

CRANFIELD UNIVERSITY

CATHERINE RIX

DETECTING LIFE ON MARS AND THE LIFE MARKER CHIP:
ANTIBODY ASSAYS FOR DETECTING ORGANIC MOLECULES IN
LIQUID EXTRACTS OF MARTIAN SAMPLES

CRANFIELD HEALTH

PhD THESIS

Academic Year: 2008 - 2011

Supervisor: Professor David C. Cullen

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Doctor of Philosophy

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ABSTRACT

The Life Marker Chip instrument, which has been selected to fly as part of the 2018 ExoMars rover mission payload, aims to detect up to 25 organic molecules in martian rocks and regolith, as markers of extant life, extinct life, meteoritic in-fall and spacecraft contamination. Martian samples will be extracted with a solvent and the resulting liquid extracts will be analysed using multiplexed microarray-format immunoassays. The LMC is under development by an international consortium led by the University of Leicester and the work described within this thesis was carried out at Cranfield University as part of the consortium's broader program of work preparing the LMC instrument for flight in 2018. Within this thesis four specific areas of LMC instrument development are addressed: the investigation of immunoassay compatible liquid extraction solvents, the study of likely interactions of martian sample matrix with immunoassays, the development of antibodies for the detection of markers of extinct life and demonstration of solvent extraction and immunoassay detection in a flight representative format.

The purpose of the first area of work was to confirm whether the proposed extraction solvent for the LMC, a water/water miscible organic solvent/surfactant capable of extracting polar and apolar target molecules, is compatible with LMC format immunoassays. In order to achieve this, and given the context that the final suite of assays that will fly on the LMC instrument have not yet been developed, a set of model immunoassays were tested for compatibility with the proposed extraction solvent, and also for compatibility with several alternative extraction solvents, in LMC relevant ELISA and microarray formats. The results indicate that LMC format immunoassays are compatible with the proposed extraction solvent, allowing instrument development to progress with this solvent, whilst also indentifying a number of potential alternatives.

In the second area of work the likely impact of the martian sample matrix on LMC format immunoassays was investigated. This was achieved by testing performance of a set of model assays, in LMC relevant ELISA and microarray formats, with both individual Mars relevant sample matrix components that have been reported on Mars and with liquid extracts from Mars analogue samples collected on Earth. The results of these experiments indicate that LMC format immunoassays are expected to function effectively with samples similar to those analysed by the NASA Phoenix lander, however a number of sample matrix components/sample matrix types were identified

that may have a detrimental impact on the ability of LMC immunoassays to detect target molecules on Mars. These results enabled recommendations to be made for future work both to improve assay performance and also to better understand how data from other ExoMars rover instruments can be used to identify samples that are not suitable for analysis by the LMC.

The third area of work investigates the use of phage display immunised recombinant antibody libraries for the rapid production of antibodies against small, apolar and chemically inert extinct life target molecules. Two strategies, optimised for the selection of antibodies which bind to small apolar molecules were used to screen an immunised library, and the selected populations were analysed but no appropriate clones were isolated. The implication is that this type of library is unlikely to be suitable for the generation of antibodies against markers of extinct life, within the timescales of the LMC instrument development. As a result of this study an ongoing program of work has been implemented within the LMC project to investigate alternative strategies for the production of suitable antibodies, whose success is more likely as a result of the work within this section.

The final area of work within this thesis brings together elements of the other three areas in order to perform an end to end demonstration of extraction and detection by the LMC instrument. This was achieved by spiking a Mars analogue sample with a representative target molecule, extracting the spiked sample with the proposed extraction solvent and analysing the liquid extract with a flight representative breadboard. This work confirmed the compatibility of a flight representative assay with a liquid extract from a Mars analogue sample. In addition completion of this end to end demonstration contributed to attainment of an appropriate technology readiness level for the LMC instrument, allowing progression to the next stage of instrument development.

In summary significant progress has been made and improved understanding gained in four interrelated areas of LMC instrument development, and this has contributed towards the ongoing preparation of the LMC instrument for flight in 2018, as well as advancing scientific knowledge in these areas.

Keywords: Astrobiology, ExoMars, extraction solvents, sample matrix effects, recombinant antibodies, phage display, microfluidics.

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LIST OF ABBREVIATIONS

2 x TY	tryptone yeast extract liquid medium
ABB	assay breadboard
AF633	Alexa Fluor® 633
ATP	adenosine tri-phosphate
B[a]P	benzo[a]pyrene
BSA	bovine serum albumin
CAB	Centro de Astrobiologica
cAMP	cyclic adenosine monophosphate
CCD	charge-coupled device
CDR	complementarity determining region
CMC	critical micelle concentration
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
E. Coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ESA	European Space Agency
GC-MS	gas chromatography-mass spectrometry
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HRP	horse radish peroxidase
HPLC	high performance liquid chromatography
IgG	immunoglobulin G
KLH	keyhole limpet hemocyanin
LB	Lysogeny Broth
LMC	Life Marker Chip

LPS	lipopolysaccharide
LTA	lipoteichoic acid
MeCN	acetonitrile
MeOH	methanol
MeOH P80	20/80 % methanol/water with 1.5 g/L P80
MER	Mars Exploration Rover
MILDI	Mars Immunoassay Life Detection Instrument
MOMA	Mars Organics Molecules Analyser
MPBS	Marvel phosphate buffered saline
NASA	National Aeronautics and Space Administration
P20	polysorbate 20
P80	polysorbate 80
PAH	polyaromatic hydrocarbons
PBS	phosphate buffered saline
PBST	phosphate buffered saline Tween® 20 buffer
PEG	polyethylene glycol
PTFE	polytetrafluoroethylene
RNA	Ribonucleic acid
rpm	rotations per minute
SAM	Sample Analysis at Mars
SAP	shrimp alkaline phosphatase
scAb	single chain antibody fragment
scFv	single chain variable antibody fragment
SOLID	Signs of Life Detector
SPDS	Sample Processing and Distribution System
TAE	tris-acetate ethylenediaminetetraacetic acid
TBC	to be confirmed

TEA	triethylamine
TEGA	Thermal and Gas Analyser
TMB	tetramethylbenzidine dihydrochloride
TRL	technology readiness level
TYE _{AG}	tryptone yeast extract agar solid media with glucose
TYE _{AKG}	tryptone yeast extract agar solid media with Ampicillin, Kannamycin and glucose
TYE _{KG}	tryptone yeast extract agar solid media with Kannamycin and glucose
V _L	gene encoding variable region of antibody on light chain
V _H	gene encoding variable region of antibody on heavy chain
WCL	Wet Chemistry Laboratory

1 Introduction and Context, Thesis Objectives and Structure

1.1 Introduction

The work described within this thesis is related to the development of the Life Marker Chip (LMC) instrument for the detection of evidence of past and/or present life on Mars. This introduction gives background information relating to the LMC instrument, including an overview of the principles of operation of the LMC, and also explains how the work within this thesis relates to the broader program of work developing the LMC instrument for flight.

1.1.1 Mars, the ExoMars mission and the LMC instrument

Although the current environment and climate of Mars are likely to be inhospitable to Earth-like life, due to factors such as the extreme cold, lack of liquid water and high levels of surface radiation (Dartnell *et al*, 2007), there is evidence that Mars may once have been habitable (Schwartz and Mancinelli 1989; McKay and Davis 1991; Paige 2005; Bibring *et al*, 2006). From a practical point of view Mars is close to Earth, which has allowed its exploration in more detail than any other planet (Jakosky *et al*, 2007). Therefore Mars is considered to be an appropriate initial target for extraterrestrial life detection. If life did originate on Mars during early warmer and wetter periods, but has since died out, preserved evidence of extinct life may persist. In addition the recent discovery of methane in the martian atmosphere (Formisano *et al*, 2004) has promoted speculation that there could be methane producing microbes surviving in protected environments beneath the martian surface today.

The proposed 2018 joint European Space Agency (ESA) – National Aeronautics and Space Administration (NASA) ExoMars rover mission (ESA 2011b) will carry a payload of instruments dedicated to characterising the martian environment and detecting evidence of past or present life. In contrast to all previous explorations of Mars, which have examined samples collected from the martian surface or near sub-surface, the ExoMars rover will be equipped with a drill capable of collecting samples from a maximum depth of two meters below the martian surface. At these depths the preservation of evidence of extinct life and the presence of extant life is considered more likely (Pavlov *et al*, 2002; Stalport *et al*, 2008). Included in the ExoMars rover

payload is the LMC instrument, the first immunoassay based instrument selected to fly on a planetary exploration mission. The LMC, which is under development by an international consortium led by the University of Leicester, will analyse samples of martian rock and regolith for sub-parts per million concentrations of a pre-defined set of up to 25 organic molecules using multiplexed microarray immunoassays.

Target molecular species for the LMC are drawn from five categories: markers of extant life, markers of extinct life, abiotically produced organic molecules as markers of meteoritic in-fall, as well as markers of ground based contamination and a number of controls (Table 1-1). In spite of the highly diverse nature of life on Earth, there are particular biomolecules, membrane components and energy production and storage mechanisms that are ubiquitous (Parnell *et al*, 2007). Therefore the detection of these families of molecules on Mars, or the detection of the stable breakdown products of these types of molecules would provide good evidence for the presence of extant or extinct Earth-like life on Mars. In order to be able to detect molecular species, rather than specific molecules, which would restrict the effectiveness of the LMC since there are many variations possible in these types of molecules, it is important that the antibodies selected for the LMC have broad specificity. For example an anti-hopane antibody should be selected that is cross reactive with hopanes, rather than an antibody that is specific for a single hopane.

Table 1-1: LMC target molecule categories (for a full discussion of target molecule types for the LMC please see Parnell *et al* (2007))

Category	Description	Examples
Extant life	Biologically produced organic molecules which are rapidly broken down outside of the cell.	Informational macromolecules – e.g. DNA/RNA Energy storage/transfer – e.g. adenosine tri-phosphate Cell membrane components – e.g. lipoteichoic acid
Extinct life	Biologically produced organic molecules that are stable over geological timescales and diagenesis (alteration products) of biologically produced molecules over geological timescales.	Hopanes (derived from cell membranes of prokaryotes) Steranes (derived from cell membranes of eukaryotes) Isoprenoids (from chlorophyll and archeal membranes)
Meteoritic in-fall	Abiotically produced organic molecules known to be contained within meteorites which are expected to be present on Mars due to evidence of meteorite bombardment.	Amino acids - glycine, amino isobutyric acid, alanine Polyaromatic hydrocarbons (PAHs) – e.g. pyrene, phenanthrene
Contamination	Organisms commonly found to inhabit space craft assembly cleanroom facilities	Staphylococcus Micrococcus Bacillus
Controls	Synthetically produced organic molecules, not expected to be present on Mars	Atrazine Fluorescein

1.1.2 Immunoassays for life detection in planetary exploration

The exploitation of immunoassays for *in-situ* planetary exploration applications is a relatively recent development but immunoassay based detection of organic molecules, such as pesticides, in environmental samples on Earth is well established (Krotzky and Zeeh 1995; Mouvet *et al*, 1997; Knopp *et al*, 2000; Gauger *et al*, 2001; Mallet *et al*, 2001; Rigou *et al*, 2004). Antibody based technologies can be packaged into small, lightweight devices for *in-situ* use, for example in lateral flow tests, and offer the potential for high sensitivity detection of specific organic molecules in a low volume and light weight format with low power requirements; all desirable attributes for a space mission. In addition to the LMC there are a small number of other planetary exploration immunoassay based instruments under development including the Signs of Life Detector (SOLID) (Parro *et al*, 2011), the Biochip for Organic Matter Analysis in Space (BiOMAS) (Baqué *et al*, 2011) and the Mars Immunoassay Life Detection Instrument (MILDI) (McKay *et al*, 2000).

Previous attempts to detect complex organic molecules on Mars, have focused on the use of gas chromatography-mass spectrometry (GC-MS) based instruments (Anderson *et al*, 1972; Hoffman *et al*, 2008) such as those carried by NASAs 1976 Viking landers and the more recent NASA Phoenix lander. To date no complex organics have been detected by GC-MS and the recent discovery of perchlorate salts on Mars has led to the conclusion that this failure may be due to perchlorate mediated degradation of organics at the high temperatures required for sample volatilisation prior to injection onto a GC column (Hecht *et al*, 2009; Navarro-González *et al*, 2010a). Therefore an additional advantage of an immunoassay based life detection approach, compared to better established (in terms of space flights) GC-MS type instruments, is that sample volatilisation is unnecessary. This aspect is discussed in more detail in Chapter 4 of this thesis.

Since proposal of the LMC instrument concept in 2003 immediate concerns regarding the stability of antibodies and other immunoassay reagents to the operational conditions of a Mars mission, including the radiation environment and fluctuating temperature over mission durations, have been addressed in work carried out both within the LMC consortium, and by other groups developing immunoassay based instruments for planetary exploration (Thompson *et al*, 2006; Wilson 2007; Postollec *et al*, 2009; Derveni 2010; de-Diego-Castilla *et al*, 2011). However there are still challenges/issues to be overcome, a number of which are addressed in this thesis and are listed in Section 1.2.

1.1.3 LMC instrument design and principles of operation

The current (correct as of November 2011) design of the LMC flight instrument consists of four “single use” modules that can each extract and analyse a single, approximately one gram, sample of martian regolith or crushed rock (Figure 1-1). On Mars, the drill and associated sample processing and distribution system (SPDS) on the ExoMars rover will deliver a one gram sample of regolith or crushed rock to one sample processing module of the LMC. The sample will be subject to liquid extraction together with the application of ultrasonic energy to extract and solubilise target molecules.

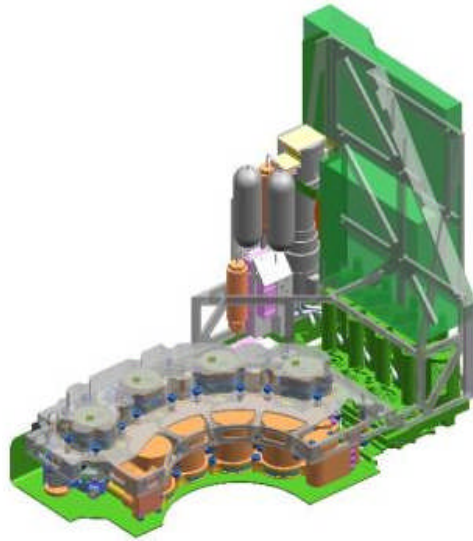


Figure 1-1: Current (2011) design of the LMC flight instrument

The liquid extract will be pumped into a single analysis module of the LMC, which consists of microfluidic channels that allow the implementation of a multiplexed inhibition immunoassay (up to 25 assays in a single multiplex) in a format similar to a lateral flow immunoassay (Posthuma-Trumpie *et al*, 2009). Note that the LMC instrument utilises an inhibition format assay because many of target molecules for the LMC (see Table 1-1 for examples) are small molecules. The key aspects of the immunoassay within the microfluidic channel are two chambers: chamber one, referred to as the pad chamber, contains a “freeze-dried cocktail” of immunoassay reagents including fluorescently labelled antibodies and chamber two, referred to as the array chamber, contains a microarray of immobilised versions of the target molecules on an optical waveguide chip. Evanescent field excitation of fluorophores on antibodies bound to the microarray is achieved by coupling laser light (635 nm) into the optical waveguides and final readout of the immunoassay signal is achieved via imaging of the presence of the fluorescent labels on the microarray with a CCD based light detection system.

On Mars, following extraction of the martian sample, the liquid extract will be pumped through the microfluidic channel into the first chamber and incubated for approximately 1 hour. During this hour the freeze dried fluorescently labelled antibodies and other components of the “freeze dried cocktail” dissolve into the liquid extract. If there are target molecules present in the liquid extract their corresponding antibodies will bind to them (Figure 1-2).

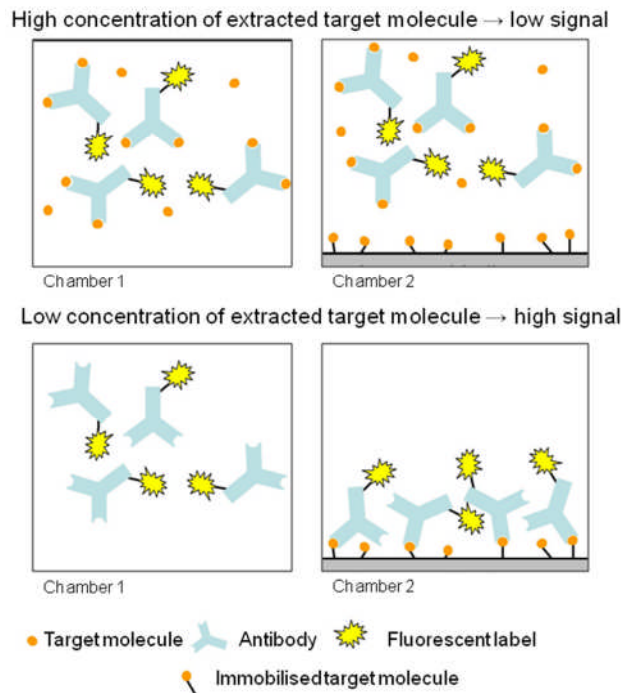


Figure 1-2: Schematic of LMC inhibition assay showing effect of extracted target molecule concentration on signal.

An inhibition assay format was selected for the LMC because many of the LMC targets are small molecules and are therefore unsuitable for sandwich assay formats and it is desirable for all assays in the multiplex to be performed using the same format. In addition inhibition assays require only one antibody per target, rather than a pair of antibodies as required for a sandwich assay reducing the number of antibodies that must be sourced/raised for the instrument.

Following this period of incubation in the pad chamber the liquid extract and dissolved antibodies are pumped into the array chamber where they come into contact with a microarray consisting of immobilised versions of the target molecules. Antibodies whose binding sites are not occupied by extracted target molecules will bind to their corresponding immobilised target molecule on the microarray (Figure 1-2), whereas those antibodies with occupied binding sites will be unable to bind to the microarray. Following a period of incubation unbound antibody will be washed away by pumping more liquid extract through the microfluidic channel and fluorophores on bound antibodies will be evanescently excited and imaged. The end result is a fluorescent signal that decreases with increasing target molecule concentration.

1.2 Thesis aim and objectives

The aim of the work reported within this thesis was to advance the understanding of the use of immunoassays for life detection experiments on Mars, specifically within the context of development of the LMC instrument for ExoMars. The objectives of this thesis were focused on four interrelated areas of LMC development relevant to immunoassays and which are stated in the following sections.

1.2.1 Objective 1: To determine compatibility of the proposed extraction solvent with LMC format immunoassays

One of the key early challenges for the LMC was the development of an extraction methodology that efficiently extracts a diverse range of target molecules, from polar extant life markers to apolar extinct life markers, and critically is also compatible with immunoassays. Work within the LMC consortium led to the proposal of a water miscible organic solvent/water/surfactant based extraction solvent. The first aim of this thesis was therefore to confirm whether the proposed extraction solvent for the LMC, is compatible with immunoassays performed in LMC relevant formats.

1.2.2 Objective 2: To investigate the impact of liquid extracts from martian samples on LMC format immunoassays

Sample matrix can have a significant effect on immunoassay performance and immunoassays have not previously been performed with martian samples. The second aim of this thesis was to investigate the likely impact of the Mars sample matrix on immunoassays, in order to determine whether LMC format immunoassays are likely to function with martian samples and to identify any risks relating to specific sample types or components.

1.2.3 Objective 3: Generation of antibodies for the detection of extinct life biomarker targets

There are few antibodies available either commercially, or from other research groups that can detect markers of extinct life. Therefore in order for the LMC to detect this scientifically important category of target molecules it is necessary to produce antibodies within relatively short timescales and within limited resources. Previous work

carried out within the LMC consortium (Derveni 2010) has identified that immunised recombinant antibody libraries are a potential route to the rapid production of such antibodies. This aim was therefore to establish whether an existing immunised recombinant antibody library could be used, within the context of short timescales and limited resources, to produce antibodies for detection of extinct life target molecules for the LMC.

1.2.4 Objective 4: Demonstration of LMC format extraction and detection in flight representative hardware

This objective was to bring together elements of the other objectives of the work within this thesis by performing an end to end demonstration of extraction of a representative target molecule from a spiked sample followed by immunoassay detection using flight representative hardware and thereby to confirm that the overall LMC concept is valid.

1.3 Thesis structure

Each results chapter within this thesis (Chapters 3 to 6 inclusive) corresponds to addressing a single thesis objective as described in Section 1.2, and contains an introduction, detailed aims and objectives and, where relevant, a literature review in addition to presenting and discussing results. These areas of work, although reported as distinct chapters, were carried out in overlapping timescales.

Much of the work contained within this thesis adopted a common approach to experimental design; therefore to avoid unnecessary repetition of information Chapter 2 describes this common experimental design and the corresponding materials and methods which were used in several of the four areas of work. Experimental design and materials and methods specific to single areas of work are included within the appropriate chapter.

The final chapter of this thesis comprises a brief summary of findings for each section of work together with a discussion/overview of implications for the ongoing development of the LMC and any broader implications for the planetary exploration and immunoassay communities and details of planned future work.

2 Common Experimental Design and Materials and Methods

2.1 Common experimental design

The flight format LMC will include a maximum of 25 antibodies to detect up to 25 molecular species as markers of extant life, extinct life, meteoritic in-fall, contamination and controls. At the current time antibodies for many of the high priority science targets for the LMC have not yet been sourced/produced and therefore are not available for use. However it is desirable to be able to characterise immunoassay performance under various LMC relevant conditions, e.g. in the proposed extraction solvent, in liquid extracts from Mars analogue samples and in flight representative hardware, at an early stage in instrument development in order to mitigate risks and justify continuing funding of development activities.

The methodology adopted within this thesis, and elsewhere within LMC development work, was to use a set of antibodies available from commercial and academic sources to model the likely performance of the 25 flight immunoassays under a variety of conditions. The suite of antibodies that will fly on the LMC are likely to be derived from a number of sources which are likely to include polyclonal, monoclonal and recombinant antibody production techniques. Thus the set of model antibodies used in this work were selected to include these antibody types in addition to including antibodies that recognise target molecules drawn from each of the five categories of target molecule for the LMC, with the exception of the extinct life category¹. The model antibodies used within this thesis are listed in Table 2-1.

¹ At the time of this work there were no antibodies available, either commercially or from other academic groups, that recognised any extinct life target molecules. The extinct marker class of targets are viewed as the most challenging to raise antibodies against because they are small, hydrophobic and chemically inert. Chapter 5 of this thesis reports work carried out using a recombinant antibody library to select antibodies that bind to extinct life target molecules, but for the purposes of the work reported within chapters 3,4 and 6 of this thesis no immunoassays from the extinct life marker category were tested.

Table 2-1: LMC model antibodies used in this study

Target molecule	Target category	Target function	Target molecule characteristics	Molecular weight	Antibody type	Source
Atrazine	Control	Synthetically produced organic molecule	Small, limited water solubility	215.7 Da	Humanised scAb	Immunised sheep recombinant antibody library
Benzo[a]pyrene /pyrene ²	Meteoritic in-fall	Abiotically produced molecule(s) found in meteorites	Small, apolar, poor water solubility	252.3 Da / 202.3 Da	Monoclonal	Mouse
cAMP	Extant	Intra cellular signalling molecule	Small polar	329.2 Da	Monoclonal	Mouse
Fluorescein	Control	Synthetically produced organic molecule	Small polar	332.3 Da	Polyclonal	Rabbit
GroEL	Extant	Heat shock protein	Protein, polar	~ 800 KDa	Polyclonal	Rabbit
LPS	Extant /contamination	Outer cell membrane of gram negative bacteria	Glycolipid	> 1000 kDa	Monoclonal	Mouse
LTA	Extant /contamination	Outer cell membrane of gram positive bacteria	Glycolipid	Not determined	Monoclonal	Mouse

cAMP = cyclic adenosine monophosphate, LPS = lipopolysaccharide, LTA = lipoteichoic acid, scAb = single chain antibody fragment

² This antibody was raised against benzo[a]pyrene but also cross reacts with a number of polycyclic aromatic hydrocarbons more commonly detected in meteorites including pyrene (Matschulat, 2005).

Many of these model antibodies are unlikely to fly on the LMC instrument since either they are not raised against high priority science targets, or are not sensitive enough to achieve the desired sub-parts per million detection levels for the LMC instrument. For example GroEL, a molecular chaperone found in a large number of bacteria (Zeilstra-Ryalls *et al*, 1991), is a high level biomarker of terrestrial life and therefore unlikely to a good marker for martian life. Structures of the target molecules for six of the seven antibodies are shown in Figure 2-1. GroEL, which is a 60kDa molecular chaperone is not shown.

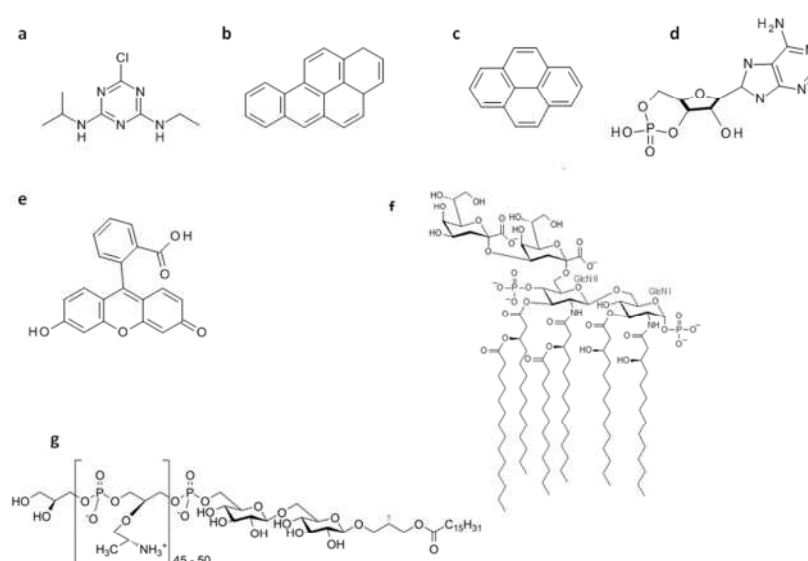


Figure 2-1: Structures of target molecules for model LMC antibodies (a) Atrazine, (b) benzo[a]pyrene, (c) pyrene, (d), cyclic adenosine monophosphate (cAMP), (e) fluorescein, (f) Lipopolysaccharide (g) Lipoteichoic acid.

Note that as described in Section 1.1 in the flight instrument antibodies are fluorescently labelled in order to generate a signal. Rather than individually fluorescently labelling 25 antibodies the LMC utilises indirect labelling via biotinylated anti-species secondary antibodies and fluorescently labelled streptavidin. Therefore appropriate biotinylated secondary antibodies were also sourced and used together with the set of model primary antibodies and labelled streptavidin to perform LMC format immunoassays.

2.1.1 LMC relevant immunoassay formats used to model LMC performance

The flight format LMC immunoassay (multiplexed microarray inhibition immunoassays implemented within a microfluidic channel with signal generation by evanescent excitation of fluorophores on antibodies bound to the microarray) is not compatible with the testing necessary to characterise antibody performance under multiple conditions due to low throughput and the limited availability of custom made components e.g. the optical waveguide chips for microarray printing and evanescent excitation. In order to overcome this, two LMC representative immunoassay formats, with higher throughput compared to the flight format assay, were developed using the model antibodies listed in Table 2-1. These two formats were:

- (i) an ELISA, which allows high throughput testing of antibody function
- (ii) a pooled microarray format assay which has lower throughput compared to the ELISA but more closely resembles the flight format assay

As explained in Section 1.1, many target molecules for the LMC instrument are small molecules and therefore assays are performed using an inhibition format. This assay format requires that versions of each target molecule be immobilised onto a surface. For small molecule targets such as cAMP and benzo[*a*]pyrene direct physical adsorption to hydrophobic³ optical waveguide chips and other hydrophobic surfaces is not appropriate, therefore carrier protein-target molecule conjugates were prepared or sourced for use in these assays.

The key differences between the ELISA, the pooled microarray format assay and the flight format assay are listed in Table 2-2

³ Physical adsorption to hydrophobic surfaces was used throughout this thesis because this was the implemented immobilisation method for the LMC at the time that this work was commenced. However covalent immobilisation is likely to offer benefits in terms of assay performance and current work has moved to the use of covalent immobilisation.

Table 2-2: Key differences between ELISA, pooled microarray and flight format assays

	Flight format	ELISA format	Pooled microarray format
Immobilisation surface and method	Microarray of target molecules (or target molecule-protein conjugates) immobilised on hydrophobic slide/chip via physical absorption	Bulk immobilisation of target molecules on polystyrene 96-well plate via physical absorption	Microarray of target molecules immobilised on hydrophobic slide/chip via physical absorption
Volume of antibody/liquid extract	6µl (volume of array chamber in microfluidic channel)	100 µl/well of 96-well plate	6 µl pooled over microarray on slide/chip
No. of assays	Multiplexed assays – up to 25 assays on a single microarray	Single assay performed in single well of 96-well plate	Multiplexed assays possible
Liquid handling	Antibodies diluted in freeze drying cocktail and freeze dried into glass fibre pads. Liquid extract pumped through microfluidic channel into pad chamber, incubated for one hour, antibodies etc. dissolve in liquid extract. Following incubation liquid extract and dissolved antibodies pumped into array chamber containing microarray.	Antibodies and freeze drying cocktail components diluted in liquid extract and pre-incubated for an hour prior to transfer into 96-well plate using a pipettor	Antibodies and freeze drying cocktail components diluted in liquid extract and pre-incubated for an hour prior to pooling over microarray area on slide/chip using a pipettor
Removal of unbound antibody	Additional volume of liquid extract pumped through microfluidic channel	96-well plate washed with buffer using commercial plate washer	Slides/chips rinsed either with flow of buffer using wash bottle or by immersion in buffer
Assay readout	Evanescence excitation of AF633 fluorophore labels by coupling laser light into optical waveguides and imaging with CCD based system	Reaction of substrate with HRP enzyme label to produce a coloured product whose absorbance is measured using a commercial plate reader	Direct excitation of AF633 fluorophore labels and imaging using commercial microarray scanner/ breadboard system

2.1.1.1 LMC format ELISA

ELISAs are a rapid and well characterised laboratory technique, which allow multiple conditions to be tested with relatively low resource requirements (*i.e.* personnel and cost of reagents). The LMC format ELISA enables characterisation of the function of LMC relevant antibodies under a wide variety of conditions and is designed to mimic, as far as possible, the flight format assay (Section 1.1). The ELISA uses the same primary antibodies, and secondary biotinylated antibodies as the microarray format assay but the fluorescent label on the streptavidin is replaced with a HRP enzyme label for the purposes of the ELISA. The antibodies and streptavidin-HRP are diluted in “freeze drying cocktail” and pre-incubated with the sample or standard under test, mimicking incubation in the pad chamber of the LMC. Following this incubation period the solution is transferred to the wells of a 96-well plate with immobilised target molecules mimicking incubation in the array chamber of the LMC. After a period of incubation, unbound antibody is washed away and the amount of antibody bound to the immobilised target molecule is measured by means of an enzyme substrate reaction, which produces a coloured product. Materials and methods for the LMC format ELISA are given in Section 2.2.2. The general approach adopted throughout this thesis, when testing the performance of model immunoassays in ELISA format was first to test binding signal (signal generated in the presence of no inhibiting antigen) as an initial screen for performance under a wide range of conditions and then to select appropriate conditions for further study in more involved inhibition ELISAs where immunoassay sensitivity under various conditions could be assessed.

2.1.1.2 LMC format pooled microarray format

The pooled microarray format assay more closely resembles the LMC flight format assay, but without any of the microfluidic aspects or evanescent excitation. Microarrays of immobilised target molecules are printed on hydrophobic surfaces (both commercially available hydrophobic slides and in-house treated silicon nitride chips were used in this work). The antibodies and fluorescently labelled streptavidin are diluted in “freeze drying cocktail” and pre-incubated with the sample or standard under test, mimicking incubation in the pad chamber of the LMC. This pre-incubated solution is then “pooled” directly over the microarray area on the hydrophobic surface, incubated for a period and unbound antibody is then washed away. Fluorophores on antibodies bound to the array are non-evanescently excited and imaged using either a

CCD based breadboard system built specifically for the LMC, or a commercial microarray scanner.

The materials and methods for running immunoassays in pooled microarray format are given in Section 2.2.3.

2.2 Common materials and methods

The following sections contain protocols for the LMC format ELISA and LMC format pooled microarray immunoassays. The materials used in each immunoassay and each assay format are defined and details of materials suppliers and product codes are given in *parenthesis*.

2.2.1 General chemicals/biochemicals

All solvents were purchased from Fisher Scientific (Loughborough, UK) and were HPLC grade unless otherwise stated.

All water used was 18 M Ω .cm grade and was produced with a Direct Q UV-3 water purification system (Millipore, Massachusetts, USA).

2.2.2 LMC Format ELISAs in 96-Well Plates

Target molecules/target molecule-protein conjugates were immobilised to the wells of Nunc MaxiSorb™ F96 MicroWell™ plates (Thermo Scientific, Roskilde, Denmark, cat. no. 442404) by incubation of 100 μ l/well target molecule in 0.05 M carbonate-bicarbonate buffer pH 9.6 (Sigma Aldrich, Poole, UK, cat. no. C3041) at a temperature of 37°C for one hour, or at a temperature of 2 – 8°C overnight. The target molecules immobilised for each immunoassay are described and the concentration at which they were used are given in Table 2-4. A set of control wells were also prepared by incubation with carbonate buffer only.

After the immobilisation step excess target molecule or target molecule-protein conjugate was removed by washing wells with 3 x 300 μ l/well 0.05 M phosphate buffered saline (Sigma Aldrich, Poole, UK, cat. no. P4417) containing 0.05 % (w/v) Polysorbate 20 (BDH/VWR, Lutterworth, UK, cat. no. 663684B) (PBST buffer) using a BW50 plate washer (Biohit, Helsinki, Finland). Residual liquid from the washing

procedure was removed from the wells by inverting the 96-well plate and tapping out on absorbent paper.

Wells were blocked to reduce non-specific interactions of antibodies with any uncoated areas of the hydrophobic plastic surfaces by incubation with 200 µl/well BSA blocking solution (0.5 % (w/v) Cohn fraction V BSA (Sigma Aldrich, Poole, UK, cat. no. A4503) in 100 mM N[2-hydroxyethyl]piperazine-N[2-ethanesulfonic acid] (HEPES buffer) pH 7.4 (Sigma Aldrich, Poole, UK, cat. no. H-7523) at a temperature of 37°C for one hour and the wells were then washed as described previously to remove excess BSA.

In the flight format LMC instrument reagents, including fluorescently labelled antibodies will be dissolved directly in the liquid extract (Table 2-3). For the purposes of LMC format ELISAs, reagents are not freeze dried. Therefore in order to approximate the expected concentrations of all components in the flight format assay, antibodies were prepared in a concentrated solution of the freeze drying cocktail and then diluted with the sample or standard under test. In addition the fluorescent label was replaced with an enzyme label for the purposes of the ELISA.

Whilst the 96-well plate was being blocked, primary antibody, biotinylated secondary antibody and streptavidin-HRP (Zymed, San Francisco, USA, used at 0.1 µg/ml, cat. no. 43-4323) were diluted in 2 x strength freeze drying cocktail, at 2 x the desired final concentration. The details and concentrations of primary and biotinylated secondary antibodies used for each of the seven model immunoassays are given in Table 2-4 (page 19). This solution was then diluted 1:1 with the standard under test, also prepared at 2 x the desired concentration in 2 x concentrated extraction solvent.

In cases where the extraction solvent was not at 2 x concentration (e.g. where liquid extracts from analogue samples were used) antibodies were prepared at 2 x concentration in a solution composed of 2 x concentration freeze drying cocktail with 1 x concentration extraction solvent. This solution was then diluted 1:1 with the liquid extract under test to maintain the final extraction solvent concentration, although still resulting in 50 % dilution of the liquid extract.

Table 2-3: Formulation of Freeze Drying Cocktail (1 x concentration)

Component	Concentration	Function	Supplier	Cat. No.
Sucrose	100 mg/ml	Protect antibodies during freeze drying	Sigma Aldrich, Poole, UK	S7903
Cohn fraction V BSA	0.5 mg/ml	Protect antibodies during freeze drying		A4503
Thimerosal	0.1 mg/ml	To prevent microbial growth		71230
Allura red AC	0.05 mg/ml	Dye to aid visualisation of reagent wash out from glass fibre pads in fluidic assays		458847
HEPES	100 mM	To control pH		H-7523

The resulting mixtures, which contained 1 x concentration antibodies and 1 x concentration freeze drying cocktail components in 1 x concentration extraction solvent, were incubated for one hour at room temperature in 1.5 ml plastic tubes. Prior to use 1.5 ml tubes were blocked with BSA blocking solution for a minimum of one hour at a temperature of 37°C to minimise non-specific binding of antibodies or free target molecule to hydrophobic plastic surfaces. This incubation mimics the incubation in the pad chamber for the flight format immunoassay.

After 1 hour incubation in BSA blocked 1.5 ml plastic tubes, antibody/sample/standard mixtures were transferred to the target molecule coated and BSA blocked wells (as well as into the blocked but uncoated control wells), 100 µl/well, and incubated for a further hour at room temperature. This step mimics the incubation step in the array chamber of the flight format assay. After one hour the 96-well plate was washed as described previously.

Tetramethylbenzidine.dihydrochloride (TMB) substrate (Sigma Aldrich, Poole, UK, cat. no. T3405) in 0.05 M phosphate citrate buffer containing 0.03 % sodium perborate pH 5.0 (Sigma Aldrich, Poole, UK, cat. no. P4992) was added to the 96-well plate 100 µl/well; reaction with HRP labelled antibodies, bound to target molecule immobilised on the surface of the wells, produces a coloured product in quantities proportional to the amount of immobilised antibody.

After approximately 10 minutes the enzyme-substrate reaction was stopped by the addition of 50 µl/well of 1 M sulphuric acid. The optical density (OD) of each well was read at a wavelength of 450 nm using a Varioskan Flash plate reader (Thermo Scientific, Leicestershire, UK). Signals were background corrected by subtraction of signal produced in control wells.

For inhibition assays, where appropriate, IC_{50} (the concentration of inhibiting antigen that produces a 50 % drop in assay signal intensity) was calculated by fitting mean signal to a four parameter binding equation (Equation 1) (Findlay and Dillard 2007).

$$y = \frac{A_1 - A_2}{1 + (x / x_0)^p} + A_2$$

<p>A_1 = maximum absorbance at zero inhibitor concentration A_2 = residual absorbance at infinite inhibitor concentration p = slope of curve at midpoint x_0 = inhibitor concentration at midpoint (IC_{50}) x = inhibitor concentration</p>
--

Equation 1: Four parameter binding equation

Table 2-4: Model Immunoassay components and concentrations

Immunoassay	Component		Supplier	Cat. No.	Concentration µg/ml	
					ELISA	Microarray
Atrazine	Immobilised target	Atrazine-BSA conjugate	Dr. M. Sathe, Cranfield University, UK	N/A	1	N/A
	Primary Antibody	Anti atrazine humanised single chain antibody fragments clone 4D8	Dr. K. Charlton, ImmunoSolv, Aberdeen, UK	N/A	1/10000 diln of stock ⁴	N/A
	Secondary Antibody	Goat anti human kappa light chain biotin (bound and free)	AbCam, Cambridge, UK	Ab46807	0.5	N/A
	Free target	Atrazine	Sigma Aldrich, Poole, UK	45330		
Benzo[a]pyrene	Immobilised target	Benzo[a]pyren-6-yl)butanoic acid -BSA	Dr. M. Sathe, Cranfield University, UK	N/A	1	N/A
	Primary Antibody	mouse anti benzo[a]pyrene clone 22F12	Prof. Dr. D Knopp, Technical University of Munich, Germany	N/A	0.25	N/A
	Secondary Antibody	Goat anti mouse IgG Fc biotin	QED Biosciences, San Diego, USA	83400	0.09	N/A
	Free target	Benzo[a]pyrene	Sigma Aldrich, Poole, UK	4-0071		
Pyrene		Sigma Aldrich, Poole, UK	18551-5			
c-AMP	Immobilised target	2'-O-monosuccinyl adenosine 3', 5' - cyclic monophosphate - BSA	Dr. M. Sathe, Cranfield University, UK	N/A	1	100
	Primary Antibody	Mouse anti Adenosine 3',5'-cyclic monophosphate clone 250532	R&D Systems, Minneapolis, USA	MAB2146	0.02	0.3
	Secondary Antibody	Goat anti mouse IgG Fc biotin	QED Biosciences, San Diego, USA	83400	0.09	20
	Free target	Adenosine 3',5'-cyclic monophosphate	Sigma Aldrich, Poole, UK	A9501		
Immunoassay	Component		Supplier	Cat. No.	Concentration µg/ml	
					ELISA	Microarray

⁴ Stock concentration of anti atrazine scAb 4D8 as supplied by ImmunoSolv is unknown

Fluorescein	Immobilised target	BSA-Fluorescein Isothiocyanate	Sigma Aldrich, Poole, UK	A9771	0.5	100
	Primary Antibody	Rabbit anti fluorescein	AbCam, Cambridge, UK	ab19491	0.1	1
	Secondary Antibody	Goat anti rabbit IgG Fc biotin	QED Biosciences, San Diego, USA	84400	0.09	24
	Free target	Fluorescein sodium salt	Sigma Aldrich, Poole, UK	46969		
GroEL	Immobilised target	GroEL	Sigma Aldrich, Poole, UK	C7688	10	100
	Primary Antibody	Rabbit anti GroEL	Sigma Aldrich, Poole, UK	G6532	10	20
	Secondary Antibody	Goat anti rabbit IgG Fc biotin	QED Biosciences, San Diego, USA	84400	0.09	24
	Free target	GroEL	Sigma Aldrich, Poole, UK	C7688		
LTA	Immobilised target	Lipoteichoic Acid	Sigma Aldrich, Poole, UK	L2515	2	N/A
	Primary Antibody	Mouse anti Lipoteichoic acid	Hycult Biotechnology, Uden, Netherlands	HM2048	10	N/A
	Secondary Antibody	Goat anti mouse IgG Fc biotin	QED Biosciences, San Diego, USA	83400	0.09	N/A
	Free target	Lipoteichoic Acid	Sigma Aldrich, Poole, UK	L2515		
LPS	Immobilised target	lipopolysaccharide from salmonella enterica serotype enteritidas	Sigma Aldrich, Poole, UK	L6011	5	N/A
	Primary Antibody	Mouse anti Lipopolysaccharide core	Hycult Biotechnology, Uden, Netherlands	HM6001	0.01	N/A
	Secondary Antibody	Goat anti mouse IgG Fc biotin	QED Biosciences, San Diego, USA	83400	0.09	N/A
	Free target	lipopolysaccharide from salmonella enterica serotype enteritidas	Sigma Aldrich, Poole, UK	L6011		

2.2.2.1 Procedure for determining impact of assay conditions on HRP enzyme

The LMC format ELISA uses an enzyme label, whereas the flight format immunoassay uses a fluorescent label. Therefore it is desirable, for tests carried out in ELISA format, to be able to discriminate between changes in immunoassay performance caused by the enzyme label (which are not relevant to the flight format) and changes resulting from altered antibody performance. In order to achieve this, the response of HRP enzyme label was tested independently of the immunoassay.

Streptavidin-HRP was immobilised to the wells of Nunc MaxiSorb™ F96 MicroWell™ plates by incubation of 100 µl/well 12 ng/ml streptavidin-HRP in 0.05 M carbonate-bicarbonate buffer pH 9.6 at a temperature of 37°C for one hour. Excess streptavidin-HRP was removed by washing using a plate washer as described in Section 2.2.2. The component/solvent under test was dissolved in 2 x concentration extraction solvent and diluted 1:1 with 2 x concentration freeze drying cocktail (Table 2-3) and incubated with the immobilised streptavidin-HRP 100 µl/well, for one hour at room temperature. Following incubation the plate was washed as described previously and TMB substrate in 0.05 M phosphate citrate buffer containing 0.03 % sodium perborate pH 5.0 was added to the 96-well plate, 100 µl/well. After approximately 10 minutes the enzyme-substrate reaction was stopped by the addition of 50 µl/well of 1 M sulphuric acid. The optical density of each well was read at a wavelength of 450 nm using a Varioskan Flash plate reader (Thermo Scientific, Leicestershire, UK).

2.2.3 LMC format pooled microarray assays

This section describes the procedure for performing pooled format microarray assays. Initial work was carried out with microarrays printed using an adapted XYZ deposition robot and imaged with a breadboard built for the LMC project (Wilson 2007). Part-way through the period of time in which the work in this thesis was performed, funding was released for the purchase of a dedicated spotting robot and commercial imaging system which allowed for higher sample throughput. Both methods of microarray printing and microarray imaging are described in the following sections along with details of reagent preparation and the protocol for performing pooled microarray format assays.

2.2.3.1 Functionalisation of silicon nitride surfaces in preparation for physical adsorption of target molecule-protein conjugates

The optical waveguide chips that will be used for the flight format LMC are fabricated from silicon nitride and in order to immobilise target molecules via physical adsorption silicon nitride surfaces must first be treated with a hydrophobic silane reagent. Pooled microarray assays were carried out using silane functionalised chips fabricated from the same materials as the optical waveguide chips, but without the optical waveguides. Physical adsorption was used as this was the established protocol for LMC format immunoassays when this thesis was commenced.

Silicon nitride chips (32 mm x 4 mm) Lionix b.v. (Twente, Netherlands) were placed in a polytetrafluoroethylene (PTFE) chip holder inside a beaker. Chips were immersed in a 0.1 % solution of Dri-Decon® (Decon Laboratories Ltd., Sussex, UK) in 18 MΩ.cm water for ten minutes to remove residual organics. After ten minutes the Dri-Decon® solution was discarded and the chips were rinsed three times with 18 MΩ.cm water to remove residual detergent then dried with a flow of filtered air.

The chips were removed from the PTFE holder, placed flat in the bottom of a glass beaker and immersed in a 1M solution of sodium hydroxide (BDH/VWR, Leicestershire, UK, cat. no. 28244.262) for 30 minutes to activate the hydroxide groups on the chip surface. After 30 minutes the sodium hydroxide solution was discarded and the chips were returned to the PTFE chip holder, rinsed three times with 18 MΩ.cm water to remove residual sodium hydroxide and dried with a flow of filtered air.

The chips, still held in the PTFE holder were then immersed in HPLC grade methanol (Fisher Scientific, Loughborough, UK, cat. no. T/2306/15) for ten minutes, rinsed three times with methanol and dried with a flow of filtered air.

The PTFE chip holder, containing clean, dry chips was placed inside a glass container with a lid fitted with two threaded screw caps. In order to minimise hydrolysis of silanisation reagents this container was dried for a minimum of four hours in an oven set at a temperature of 200°C, to ensure no water was present prior to use. The container was purged with argon gas to exclude water. 60 ml of anhydrous toluene (Sigma Aldrich, Poole, UK, cat. no. 24 451-1) was injected in to the vessel through the screw cap port, via a glass syringe that had been dried at 200°C and been purged with argon gas. A volume of 1.2 ml of dichlorodimethylsilane (Fluka, part of Thermo Fisher

Scientific, Loughborough, UK, cat. no. 40150) was then injected into the vessel in the same way, to give a 0.4 M solution of dichlorodimethylsilane in toluene. The vessel was sealed and left to stand at room temperature overnight.

After approximately 16 hours the vessel was opened and the silane coating solution discarded. The chip holder containing the silane functionalised chips was placed inside a clean, dry glass beaker and the chips were immersed in HPLC grade toluene (Fisher Scientific, Loughborough, UK, cat. no. T/2306/15) for ten minutes, rinsed three times with toluene to remove any remaining dichlorodimethylsilane and dried with a flow of filtered air. The chips were then immersed in HPLC grade methanol for ten minutes, rinsed three times with methanol to remove residual toluene, and dried with a flow of filtered air. The dry silane functionalised chips were stored in a clean polystyrene *Petri*-dish until use.

Successful silane functionalisation was confirmed by measuring the contact angle of the silane functionalised chip surfaces with water. Silane functionalised surfaces are more hydrophobic than bare silicon nitride and consequently have a higher contact angle with water (90 – 100° compared to 45° for a bare silicon nitride chip surface). For each batch of silane functionalised chips three contact angle measurements were made using three different areas on the surface of a single chip using a KVS CAM 100 contact angle system (KSV Instruments, Helsinki, Finland).

2.2.3.2 Labelling BSA with Alexa Fluor® 633 for control spots on microarrays

Microarrays in the flight format assay will include immobilised spots of target molecules/target molecule-protein conjugates and also immobilised spots of fluorescently labelled BSA as calibration controls to confirm appropriate functioning of the on-board laser excitation system. Therefore spots of fluorescently labelled BSA were also included in all microarrays printed for pooled microarray assays within this work. BSA was labelled with Alexa Fluor® 633 fluorescent dye (AF633) at two molar ratios of dye to protein, 5:1 (high ratio) and 1:1 (low ratio), via reaction of amine groups on BSA protein with an amine reactive Alexa Fluor® 633 succinimidyl ester.

Cohn Fraction V BSA (Sigma Aldrich, Poole, UK, cat. no. A4503) was dissolved in 0.05 M carbonate-bicarbonate buffer pH 9.6 (Sigma Aldrich, Poole, UK, cat. no. C3041) at a concentration of 5 mg/ml. A stock solution of Alexa Fluor® 633 succinimidyl ester (Invitrogen, Paisley, UK, cat. no. A20005) was prepared by dissolving 1 mg in DMSO

and was used immediately following dilution, unused stock solution was discarded. For 5:1 ratio labelling 25 μ l of the stock solution of Alexa Fluor® 633 succinimidyl ester was added drop wise to 500 μ l of 5 mg/ml BSA with stirring. For 1:1 ratio labelling 5 μ l of the stock solution of Alexa Fluor® 633 succinimidyl ester was added drop wise to 500 μ l of 5 mg/ml BSA with stirring. Reaction mixtures were protected from light and stirred at room temperature for 1 hour. Unreacted AF633 ester was removed by desalting using a PD10 column (GE Healthcare, Buckinghamshire, UK, cat. no. 17-0851-01). Protein concentration of the purified conjugate was determined by Micro BCA™ Protein Assay Kit (Pierce, part of Thermo Fisher Scientific, Loughborough, UK, cat. no. 23225) and the conjugates were diluted to a concentration of 500 μ g/ml in PBS containing 0.01 % (w/v) thimerosal (Sigma Aldrich, Poole, UK, cat no, 71230) and stored at 2 – 8 °C until use.

2.2.3.3 Preparation of printing solutions for deposition of target molecule/target molecule protein-conjugates onto hydrophobic surfaces by contact printing

Printing solutions were prepared for each target molecule/target molecule-protein conjugate to be printed in the microarray (see Table 2-4 for details), and also for high and low ratio AF633 labelled BSA. Target molecules/target molecule-protein conjugates/AF633 labelled BSA were diluted in PBS with 2 % glycerol (Sigma Aldrich, Poole, UK, cat. no. G7757) and were stored at 2-8 °C, for a maximum of one week, until use.

2.2.3.4 Microarray printing with SPI spotting robot for hydrophobic immobilisation of target molecules/target molecule-protein conjugates

When the work reported in this thesis commenced the microarraying equipment available within the laboratories at Cranfield Health and in routine use for LMC related work, was an SPI GmbH XYZ robot (SPI GmbH, Oppenheim, Germany) fitted with an ArrayIT™ Stealth™ 32 pin print head and Stealth™ SMP3 Micro-spotting Pin (Telechem International, Sunnyvale, USA) (Figure 2-2) and two camera microscopes. The SMP3 spotting pin contains a capillary which draws liquid up inside it. Bringing the pin into contact with a suitable surface produces a spot with a diameter of 110 μ m. The camera microscopes allow the printing procedure to be observed in detail.

Prior to printing, silane functionalised silicon nitride chips were loaded into in a custom manufactured aluminium holder that is mounted onto the robot base plate and connected to a Laboport diaphragm vacuum pump (knf Lab, Trenton, USA) to hold the chip in place. One hundred microlitres of each printing solution was pipetted into the wells of an F8 strip well (Fisher Scientific, Loughborough, UK, cat. no. 95029350) and the F8 strip well was loaded into the 96-well plate holder mounted on the base plate of the spotting robot (Figure 2-2). The ultrasonic bath, also fitted onto the base plate, was filled with 18 M Ω .cm water.

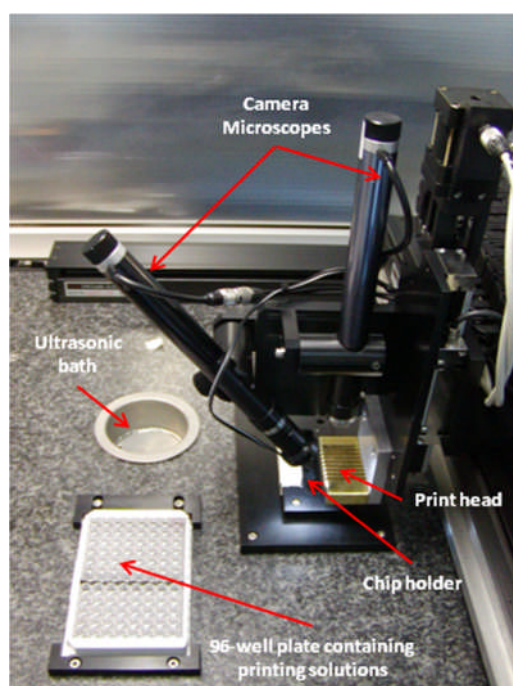


Figure 2-2: SPI GmbH XYZ robot for microarray printing

Movement of the robot was controlled by Advanced Robotic Control software (version 3.2), which was developed by SPI GmbH and supplied with the robot, using a printing program written by Dr P.K. Wilson as part of early LMC development work (Wilson 2007).

Running the printing programme resulted in the following actions

- 1) Pin lowered into the ultrasonic bath for two minutes for cleaning
- 2) Pin lifted out of the ultrasonic bath and moved over the first well of the F8 strip contained in the 96-well plate holder
- 3) Pin primed with printing solution by dipping the pin tip into the first well of the F8 strip containing the target molecule/target molecule protein conjugate

- 4) Pin moved over the array area on the chip
- 5) A column of ten spots printed, 200 μm apart by bringing the tip of the pin in contact with the chip surface to print one spot, moving the pin away from the surface by a small distance, adjusting its position in the x axis by 200 μm and repeating
- 6) Pin returned to a position over the ultrasonic bath

The cycle was then repeated, loading the pin from the second well of the F8 strip and printing a second column of spots 200 μm to the right of the first column. The maximum size of a single array was 10 x 10 spots and up to three arrays, spaced 6 mm apart, were printed onto a single chip in a period of approximately 1 hour. After printing, the edges of each microarray were marked with a diamond scribe (to facilitate location of the microarrays for pooling of reagents and imaging) and chips were incubated for one hour in a humidity chamber. After incubation chips were blocked, to minimise non-specific binding, by immersion in a solution of 1 % (w/v) Cohn fraction V BSA (Sigma Aldrich, Poole, UK, cat. no. A4503) in 100 mM HEPES pH 7.4. After two hours of immersion the chips were rinsed with water and dried with a flow of filtered air. Chips were stored at room temperature in the dark until use to avoid photobleaching of the AF633 spots.

2.2.3.5 Microarray printing with SpotBot® 3 Microarrayer for physical adsorption of target molecules/target molecule-protein conjugates

Part way through the period over which the work reported in this thesis was carried out a purpose built commercial microarraying system, the SpotBot® 3 Microarrayer (Arrayit Corporation, Sunnyvale, USA) (Figure 2-3), was purchased. The SpotBot® has a significantly smaller footprint than the SPI XYZ robot, thus is more compatible with operation inside a glove box within a cleanroom, which will be required for production of the flight model LMC instrument to minimise potential for contamination by microbes and organic molecules. The SpotBot® is designed to print microarrays onto commercially available microarraying slides, but the hardware and software can be modified in order to print microarrays onto custom produced surfaces such as the LMC optical waveguide chips. For the purposes of the work reported in this thesis the SpotBot® was used to print microarrays onto commercially available Superprotein substrate slides (Arrayit Corporation, Sunnyvale, USA, cat no. SUP) to maximise experimental throughput.

The Spotbot® (Figure 2-3) consists of a print head whose position can be altered in the x, y and z axes, a washing and drying station where the printing pin is washed by ultrasonication in a flow of PBST buffer and then dried by a flow of filtered air, a holder for a 384 well plate to contain printing solutions and a base-plate for the positioning of surfaces for printing.

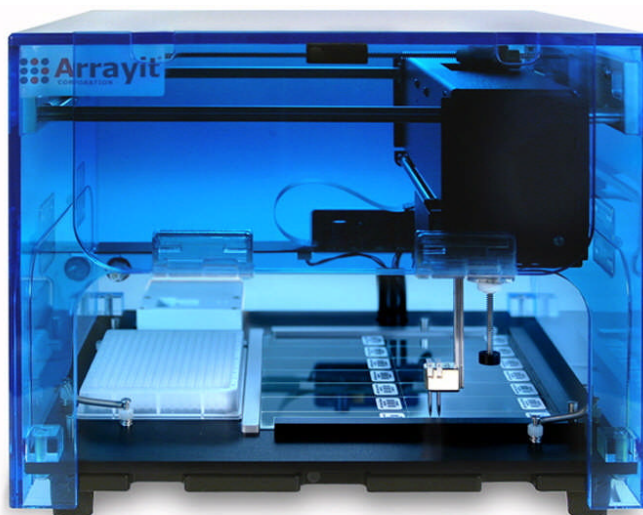


Figure 2-3: Photograph of Arrayit Spotbot® 3 Microarrayer

Prior to printing the Spotbot® print head was loaded with a single SMP3 spotting pin, printing solutions were loaded (20 μ l/well) into a 384 well plate, the washing station buffer reservoir was filled with PBST buffer and commercial microarray slides were positioned on the base-plate ready for printing.

Movement of the print head was controlled by Spotbot® Spotsuite software (version 3.6.0) which allows control of limited parameters such as the number of spots per row to be printed, the number of microarrays per slide to be printed and the timings of wash cycles. The Spotbot® was used to print up to 12 microarrays on a single slide with microarrays spaced approximately 6 mm apart. Each array consisted of rows of five replicate spots for each printing solution with a spacing of 400 μ m between each spot. Up to six slides of 12 arrays could be printed in a single print run in a period of 1hr 30 minutes, significantly increasing throughput compared to the SPI spotting robot.

After the printing program had been completed, the corners of each microarray were marked using a pencil, to enable pooling of reagents over the correct area of the slide for pooled assays, and the slides were then incubated in a humidity chamber for one

hour. After one hour, slides were blocked by immersion in a solution of 1% BSA in PBS for 2 hours in order to reduce non-specific binding to the slide surface. Slides were then washed by 5 minutes immersion in PBST followed by 5 minutes immersion in water. Finally slides were rinsed with water and dried with a flow of filtered air. Dried slides were stored at room temperature, in the dark, until use.

2.2.3.6 Pooled microarray format assays

Primary antibodies for each of the assays to be performed in the multiplex and their corresponding secondary antibodies (Concentrations are given in Table 2-4 – note that the concentrations differ from those used in ELISA format) and streptavidin AF633 (Invitrogen, Paisley, UK, used at a concentration of 10 µg/ml, cat. no. S21375) were diluted at 2 x the desired final concentration in 2 x concentrated freeze drying cocktail (Table 2-3) and then added 1:1 to the standard under test in 2 x concentrated extraction solvent.

In cases where the extraction solvent was not at 2 x concentration (e.g. where liquid extracts from analogue samples were used) antibodies were prepared at 2 x concentration in a solution composed of 2 x concentration freeze drying cocktail with 1 x concentration extraction solvent. This solution was then diluted 1:1 with the liquid extract under test to maintain the final extraction solvent concentration, although still resulting in 50 % dilution of the liquid extract. Antibody mixtures were then incubated for 1 hour in BSA blocked 1.5 ml plastic tubes to mimic incubation in the pad chamber of the flight format LMC.

Following incubation, a volume of 6 µl of antibody/liquid extract/standard solution was pooled directly over a microarray on a chip or a commercial microarraying slide using a pipette (Figure 2-4) to mimic incubation in the 6 µl volume array chamber of the LMC. Microarrays were located using either the diamond scribe marks on silicon nitride chips, or the pencil marks on commercial hydrophobic slides (Please refer to Sections 2.2.3.4 and 2.2.3.5). The slide/chip, with pooled antibody/liquid extract/standard, was incubated for one hour in a humidity chamber to minimise loss of liquid by evaporation.

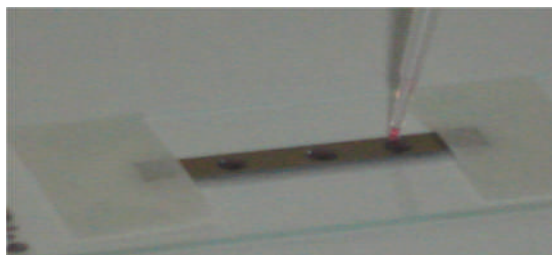


Figure 2-4: Photograph of pooled microarray immunoassay showing deposition (by pipette) of antibody/extraction solvent/liquid extract mixtures over microarrays printed onto an in-house hydrophobic silane functionalised silicon nitride chip which has been secured onto a microscope slide in this image for ease of handling

After one hour unbound antibodies were removed by washing using one of two methods

- (i) For silicon nitride chips, unbound antibodies were removed by rinsing the surface of chips with PBST using a wash bottle. The chip was then rinsed with water and dried with a flow of filtered air
- (ii) For commercial slides, which required a more rigorous wash procedure due to higher levels of background signal, unbound antibodies were removed by immersion in PBST for 5 minutes with gentle agitation, followed by immersion in water for 5 minutes with gentle agitation. Finally the slides were rinsed with a flow of water and dried with a flow of filtered air

2.2.3.7 Excitation and imaging of microarrays on in-house functionalised silicon nitride chips using in-house developed imaging system

At the beginning of the period over which the work reported in this thesis was carried out the established method for imaging microarrays within the laboratories at Cranfield Health was to use an imaging breadboard developed for the LMC project (Wilson 2007). Microarrays were imaged both before and after immunoassays had been performed to allow comparison of background signal intensity and intensity of high and low ratio AF633 labelled BSA control spots.

The Cranfield Imaging System is an optical breadboard developed and assembled for optical imaging of microarrays at a wavelength of 633 nm (Wilson 2007). The layout of the imaging breadboard is shown in Figure 2-5.

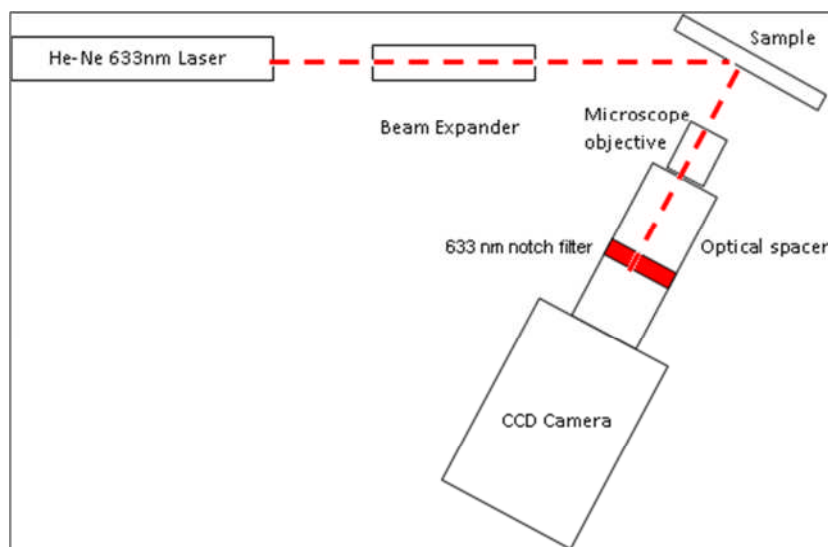


Figure 2-5: Schematic layout of the imaging breadboard for non-evanescent excitation and imaging of microarrays (Wilson 2007)

The chip being imaged was attached to a microscope slide and mounted on a positioning system (OptoSigma, Santa Ana, California, USA) consisting of X-Y-Z linear translation stages and a rotation stage. Fluorophores were excited by a 10 mW Helium-Neon 633 nm laser (Polytec, Waldbrom, Germany). The laser beam was passed through a 10 x beam expander (Intelite Inc., Genoa, USA) which spread the point source of the laser over the area of a 1 cm diameter circle, in an attempt to provide uniform illumination of the array area. Each microarray had to be aligned with the illumination area and imaged individually.

Images were captured by a Peltier-cooled SXV-H9 CCD camera (Starlight Express, Holyport, UK) mounted on an X-Y linear translation stage (Optosigma, Santa Ana, USA) with a 4 x microscope objective. A Stoptline™ 633 notch filter with an optical density of 6 at a wavelength of 633nm (Semrock, Rochester, USA) was mounted within an optical spacer in front of the CCD camera. This filter was designed to block out reflected and scattered 633nm light. Images were captured using exposure times varying from 1 to 5 minutes depending on the intensity of the signal generated by the spots.

2.2.3.8 Excitation and imaging of slides using microarray scanner

Commercial hydrophobic slides were imaged using an InnoScan 700 microarray scanner (Innopsys, Carbonne, France). The InnoScan is a two colour fluorescence scanner and uses a stabilised diode laser to excite microarrays at 635 and 532 nm and

a photomultiplier tube (PMT) to amplify the fluorescent signal. Slides were imaged, before and after running pooled format microarray assays, using a laser power of 5.0 mW at a single wavelength (635 nm) with a PMT gain of 1%.

2.2.3.9 Analysis of microarray images using ImageJ software

Images of microarrays taken before and after running pooled microarray assays were analysed using ImageJ software, a public domain Java image processing program (Rasband 2010).

The ImageJ software was used to measure the mean pixel intensity of each microarray spot (by positioning a box over each spot and then using the software to calculate the total pixel intensity within the box divided by the number of pixels within the box). Mean pixel intensities for each given immunoassay/AF633 labelled BSA spot type were then averaged. Background signal was estimated by performing the same procedure on areas of the microarray where no spots had been printed. A background correction was applied by subtracting mean background signal from mean signal for each immunoassay spot type.

2.2.3.10 Procedure for determining impact of components on AF633 fluorophore

Like antibodies, fluorescent dyes can also be affected by the conditions under which they are used. For this reason it was desirable to be able to test the performance of AF633 fluorophore under a range of conditions, independently of the immunoassay.

This was achieved by incubating the component under test with 10 µg/ml of streptavidin-AF633 and freeze drying cocktail components (Table 2-3) diluted in extraction solvent for 1 hour at room temperature in a total volume of 100 µl in the wells of a black micro titre plate (Fisher scientific, Loughborough, UK, cat no. DIS-210-190W). After one hour the fluorescence of the AF633 fluorophore was measured using a Varioskan Flash plate reader (Thermo Scientific, Leicestershire, UK). Excitation wavelength = 633 nm, emission wavelength = 660 nm).

3 Compatibility of the Proposed Extraction Solvent for the LMC with LMC Format Immunoassays

3.1 Introduction and context

The LMC will receive up to four one-gram samples of crushed martian rock or regolith from the drill on the ExoMars rover and the crushed sample will be extracted by liquid extraction with the application of ultrasonic energy. The liquid extract from the martian rock or regolith sample will then be analysed for the presence of up to 25 pre-selected molecular species using a multiplexed microarray-based immunoassay in a lateral flow type format.

Target molecules for the LMC fall into 5 categories, with varying physiochemical properties, comprising polar water soluble markers of extant life (e.g. informational macromolecules like DNA, energy transport/storage molecules such as cAMP/ATP and apolar and chemically inert markers of extinct life with limited/no solubility in aqueous solvents including hopanes and straight chain alkanes. Therefore the LMC extraction solvent must efficiently extract molecules with varying physiochemical properties and must also be compatible with antibodies and other immunoassay reagents. Given the intended multiplexed format for the LMC, where up to 25 assays may be run in a single microarray, it is essential that the LMC extraction solvent is compatible with a wide range of immunoassays, in order to have a realistic expectation of successful operation.

LMC consortium members based at Imperial College have proposed the use of 20/80 % (v/v) methanol/water with 1.5 g/L polysorbate 80 (MeOH P80), together with the application of ultrasonic energy, as a suitable extraction solvent for the LMC and have demonstrated extraction of polar and apolar LMC target molecules from spiked samples, with reasonable efficiency using this solvent (Court *et al*, 2010). In aqueous solution, at concentrations of surfactant greater than the critical micelle concentration (CMC), surfactant molecules form micelles which provide an apolar environment for hydrophobic organic molecules with poor water solubility to be extracted into, whilst more polar hydrophilic molecules are extracted directly into the bulk organic solvent water solution.

The fundamental goal of this work was therefore to confirm that immunoassays function in the proposed extraction solvent for the LMC. The LMC flight instrument design (circa 2009) incorporated an evaporation chamber to facilitate up to 30 fold concentration of liquid extracts by evaporation under reduced pressure. This feature was included to maximise the probability of detecting low concentrations of extracted target molecules. Therefore there was also a need to establish whether immunoassays are capable of functioning in the proposed LMC extraction solvent when concentrated 30 fold by evaporation.

A secondary goal of this work was to identify alternative extraction solvents, compatible with LMC format immunoassays, in-case unforeseen circumstances should arise during instrument development, resulting in a need to change the composition of the extraction solvent.

3.2 Chapter objectives

The goal of the work reported in this chapter was to confirm the compatibility of the proposed LMC extraction solvent with LMC format immunoassays. This was addressed by setting the following specific objectives:

- 1) To test functionality of LMC format immunoassays in the proposed extraction solvent, MeOH P80
- 2) To establish functionality of LMC format immunoassays in the proposed extraction solvent concentrated 30 fold by evaporation
- 3) To explore alternative extraction solvents for compatibility with LMC format immunoassays, should some aspect of the LMC instrument prove to be incompatible with the proposed extraction solvent in the future

3.3 Literature review

This literature review is divided into three sections. The first section describes methods for organic molecule extraction used previously in planetary exploration experiments and planned for future missions, including the aqueous based low temperature extraction technique planned for the LMC on ExoMars. The subsequent two sections go on to review the use of organic solvents and surfactants in immunoassays.

3.3.1 Extraction of organic molecules in the context of planetary exploration

Simple organic molecules, like methane, have been detected in the atmosphere of several other planetary bodies, including on Mars, by remote sensing using ground based infrared spectroscopy and by other instruments onboard orbiting and fly-past spacecraft (Encrenaz 1990; Formisano *et al*, 2004; Schaller and Brown 2007). There have only been two previous attempts to perform *in-situ* extraction and detection of organic molecules on other planetary bodies, both attempts were on Mars and both involved pyrolysis based extraction techniques. The GC-MS on the 1970s NASA Viking Landers (Anderson *et al*, 1972) and NASA Phoenix's GC-MS based Thermal and Gas Analyser (TEGA) (Boynton *et al*, 2001; Sutter *et al*, 2009) both failed to detect any evidence of complex organics in samples collected from the martian surface and near-subsurface. This is despite the known delivery of significant quantities of organic molecules, such as polyaromatic hydrocarbons (PAH), to the martian surface by meteorites, irrespective of any biological source of organics on Mars (Biemann *et al*, 1976).

Many theories were proposed to account for the failure to detect complex organics including inadequate sensitivity of the Viking GC-MS instrument (Levin and Straat 1977) and the degradation of organics by an oxidising agent (Levin and Straat 1981; Zent and McKay 1994; Quinn and Zent 1996; Tsapin *et al*, 2000). In 2008, oxidising perchlorate salts were detected in martian regolith by the Wet Chemistry Laboratory on Phoenix⁵ at a concentration of 0.4-0.6 weight percent (Hecht *et al*, 2009; Kounaves *et al*, 2010) and Navarro-González *et al* (2010) demonstrated that when organic carbon containing Mars like soils from the Atacama desert are mixed with one weight percent

⁵ The implications of the presence of perchlorate salts for the LMC immunoassays are addressed in more detail in Chapter 4 of this thesis

magnesium perchlorate and heated, the organics present are decomposed. Therefore the failure to detect complex organics by both the Viking GC-MS and Phoenix's TEGA may have been due to perchlorate mediated degradation of organic molecules promoted by the high temperatures experienced during pyrolysis.

The future NASA Mars Science Laboratory mission will carry a pyrolysis GC-MS instrument, Sample Analysis at Mars (SAM), for analysis of the martian atmosphere and volatiles from martian regolith samples (NASA 2010) and the ExoMars rover payload includes the Mars Organics Molecule Analyser (MOMA) a compact, low power ion trap mass spectrometer with the capability to extract organics from martian rock and regolith samples using two different methods: pyrolysis, and laser desorption (Evans-Nguyen *et al*, 2008). There is therefore a risk therefore that if perchlorates are widespread over the martian surface SAM and MOMA may also fail to detect organics, although the impact of perchlorate salts on organic compounds extracted by laser desorption is unknown.

There are numerous examples of non-pyrolysis based extraction methodologies for trace analysis of organic molecules e.g. environmental pollutants (Lopez-Avila 1999), but the majority are not compatible with the stringent mass, power and volume requirements of an instrument designed for a space mission and are therefore not considered here. Other non-pyrolysis based extraction techniques that have been proposed for use in instruments for Mars exploration missions include high temperature and pressure sub-critical water extraction, proposed for use in the Urey instrument for the detection of amino acids (Aubrey *et al*, 2008) and recently it has been demonstrated that solid phase microextraction can be used to extract the organic content of martian analogues for analysis by GC-MS (Orzechowska *et al*, 2011).

Selection of the LMC extraction methodology was driven by three requirements, (i) efficient extraction of a range of polar and apolar target molecules, (ii) compatibility with immunoassays and (iii) the need to comply with mass, power and volume constraints. In order to meet these requirements an aqueous based solvent, with a low percentage of an organic solvent and a surfactant was developed for use in a liquid extraction with the application of ultrasonic energy to break up the sample matrix and increase extraction efficiency (Court *et al*, 2010). This proposed extraction technique has the additional benefit that it is unlikely to promote perchlorate mediated degradation of organic molecules, although this had not been determined experimentally when this

thesis was commenced. The LMC therefore potentially offers a complementary extraction strategy to previous and planned future instrumentation for the detection of organic molecules in martian samples. In addition to the LMC there are also a number of other immunoassay based sensors under development such as the Signs of Life Detector (SOLID) (Parro *et al*, 2011) and the Mars Immunoassay Life Detection Instrument (MILDI) (McKay *et al*, 2000) which also propose to use organic solvent/aqueous solution based extractions.

3.3.2 Use of organic solvents in immunoassays

Immunoassays are traditionally performed in aqueous solutions, buffered to physiological pH, thus restricting immunoassay detection to target molecules with appropriate water-solubility. However interest in rapid *in-situ* immunoassay detection of poorly water soluble compounds such as pesticides, and other environmental contaminants such as polyaromatic hydrocarbons, has led to study of the performance of immunoassays in the presence of a range of organic solvents. There are numerous examples in the literature of detection of apolar target molecules, including agrochemicals and other environmental pollutants, by inhibition or competition format immunoassays in low concentrations (10-20 % (v/v)) of water miscible organic solvents. These solvents include methanol, dimethyl sulphoxide (DMSO), acetonitrile and ethanol, (Goh *et al*, 1990; Matsuura *et al*, 1993; Stöcklein *et al*, 1995; Kim *et al*, 2004; Sun *et al*, 2009).

Antibodies interact with target molecules through relatively weak non-covalent hydrogen bonding, electrostatic, Van Der Waals, and hydrophobic forces, which are dependent on the three dimensional structure and charge distribution of the antibody and the target molecule. Antibody binding sites are formed by three dimensional folding of the protein chain to create binding pockets and the way that the protein chain folds in aqueous solution is determined by the interaction of amino acids in the protein chain with water *i.e.* proteins fold in order to minimise interactions between hydrophobic amino acid side chains and polar water molecules (Petsko and Ringe 2004).

Addition of low concentrations of organic solvents can affect protein folding and therefore change the characteristics of the antibody binding site and impact on the antibody-antigen binding interaction. The physio-chemical environment in which the antibody-target molecule binding interaction takes place can also have an effect on the antibody-antigen interaction irrespective of any changes in the antibody binding site.

For example, interactions between hydrophobic target molecules and antibodies are driven by hydrophobic binding and reducing the polarity of the solution, for example by adding an organic solvent, can reduce the magnitude of hydrophobic binding forces (Setford 2000).

Antibodies have been shown to exhibit increased affinity, decreased affinity and altered specificity in low percentages of organic solvent compared to in buffer (Stöcklein *et al*, 1990; Kröger *et al*, 1998; Horáček and Skládal 2000) and it is not possible to predict how a particular antibody will behave. A study which compared the performance of 20 monoclonal antibodies, raised against the same target molecule, showed that the tolerance of clones to low concentrations of water miscible organic solvent varied widely (Matsuura *et al*, 1993).

As solvent concentration is increased above 10 – 20 % (v/v), antibody activity and sensitivity is generally reduced, and at high solvent concentrations antibodies are no longer soluble and may precipitate out of solution. However some immobilised antibodies have shown activity, all be it much reduced, in up to 100 % water miscible organic solvent including methanol and acetonitrile (Matsuura *et al*, 1993; Lu *et al*, 1997). Activity has also been demonstrated in 100% water immiscible solvents such as hexane and toluene, where a layer of residual water molecules is thought to be responsible for maintained activity; however under these conditions reaction kinetics are limited by the rate of target molecule phase transfer between the organic and aqueous phases and are generally very slow (Stöcklein *et al*, 1990; Weetall 1991).

In addition to considering the result of adding organic solvents to antibodies and target molecules it is also important to take into account the effect on other components of the immunoassay such as enzyme labels which can demonstrate altered activity in organic solvents (Lu *et al*, 1997), fluorescent labels which can show reduced fluorescent signal, immobilised antibodies and immobilised target molecules which may be desorbed from solid substrates (Stöcklein *et al*, 1995; Stöcklein and Scheller 1997), and solid substrates themselves which may be damaged by the solvents.

In summary the proposed extraction solvent for the LMC contains 20 % methanol and a survey of the literature reveals that 20 % methanol is widely used in immunoassays for the detection of targets with limited water solubility and is therefore broadly expected to be compatible with LMC format immunoassays. There are also a number of other organic solvents that are commonly used at 10 – 20 % (v/v) in immunoassays which

could be considered for ‘back-up’ extraction solvents. The flight format LMC will utilise a multiplexed format where up to 25 immunoassays will be performed using a single microarray. Given the large number of antibodies that may be included in the flight format LMC, and the potential for variation of performance between different antibodies in organic solvents, it is unlikely that any organic solvent containing extraction solvent will be optimum for all assays included on the LMC flight instrument and inevitably some level of compromise in performance is likely to be necessary.

3.3.3 Use of surfactants in immunoassays

The proposed extraction solvent for the LMC contains 1.5 g/L polysorbate 80 (P80). This review, to assess the prior use of surfactants in immunoassays will therefore focus mainly on the literature concerning the use of polysorbate in immunoassays, although other surfactants will also be considered. Polysorbates (Figure 3-1), known commercially as Tweens®, are amphipathic, non-ionic surfactants composed of fatty acid esters of polyoxyethylene sorbitan (Kerwin 2008).

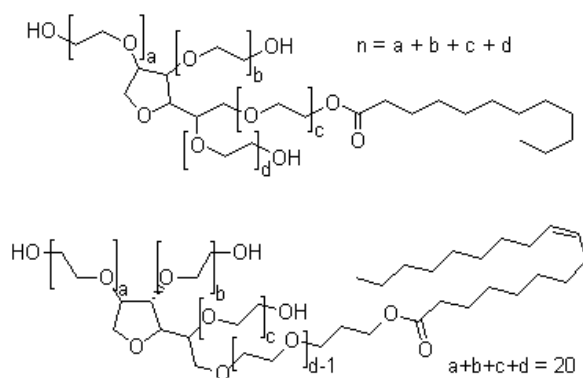


Figure 3-1: Structure of polysorbate 20 (top) and polysorbate 80 (bottom) (image taken from www.chemblink.com)

Although there is little published data regarding the influence of P80 on immunoassays, polysorbate 20 (P20) is routinely added to ELISA washing and diluting buffers to reduce background signal (Kim *et al*, 2004; Traunmuller *et al*, 2005), by removing non-specifically adsorbed biomolecules from solid substrates, and by blocking hydrophobic patches created by protein removal during wash steps (Matson 2000). Non-ionic surfactants, like P20, are also commonly added to antibody and other protein solutions to prevent physical damage during freeze drying and spray drying, for example in

commercial lateral flow tests (Kerwin 2008). P20 has been shown to increase the stability of some antibodies to organic solvents (Stöcklein *et al*, 2000) and in some cases to increase antibody binding affinity (Stöcklein and Scheller 1997). However excessive concentrations of surfactants can denature proteins, desorb proteins from substrates (Selby 1999), and inhibit enzyme-substrate reactions (Matson 2000), so their use is generally restricted to low concentrations (0.01 - 0.1 % (w/v) or 0.1 – 1 g/L) and some immunoassays have shown improved performance in the complete absence of P20 (Abuknesha and Griffith 2004) which may have resulted from interference with the hydrophobic binding interaction between the antibody and the apolar target molecule.

P20 is routinely used in immunoassays at a concentration similar to that proposed for the LMC extraction solvent, and is therefore likely to be compatible with LMC format immunoassays, although there may be an impact on hydrophobic binding interactions, which is especially relevant for LMC targets of extinct life. The surfactant selected for the proposed LMC extraction solvent is P80, which is not commonly used in immunoassays. P20 and P80 were both considered by the team at Imperial College responsible for development of the LMC extraction solvent, but P80 containing extraction solvents were found to be better at extracting a wide range of target molecules (Baki and Sephton 2007). P80 differs from P20 in that it has a lower CMC (0.012 mM compared to 0.06mM for P20) and a higher molecular weight (1310 g/L compared to 1228 g/L for P20) due to its extended hydrophobic chain (Figure 3-1). It is not anticipated that these differences will have a significant impact on immunoassay function, but there is a need to determine this experimentally.

If the LMC solvent is concentrated by up to 30 fold following extraction, the proportion of P80 in the immunoassay will be increased to a maximum of 45 g/L which is significantly higher than the concentration of P20 that is typically used in immunoassays. Higher concentrations of P20 have been shown to result in desorption of immobilised antigens and a reduction in antibody activity, which may prove to be problematic for LMC format immunoassays.

In summary due to the routine use of P20 in immunoassays it is assumed likely that similar concentrations of P80 will also be compatible with immunoassays, although it may also have an impact on hydrophobic binding interactions, but there is a need to confirm this experimentally. However 30 x concentration of the proposed extraction

solvent will result in a considerably higher concentration of polysorbate, which is expected to be problematic for the LMC.

3.4 Results and discussion

3.4.1 Overview and structure of work

The work within this chapter had three specific objectives:

- 1) To test functionality of LMC format immunoassays in the proposed extraction solvent, MeOH P80
- 2) To establish functionality of LMC format immunoassays in the proposed extraction solvent concentrated 30 fold by evaporation
- 3) To explore alternative extraction solvents for compatibility with LMC format immunoassays should some aspect of the LMC instrument prove to be incompatible with the proposed extraction solvent in the future

In order to achieve these three objectives, the functionality of seven model immunoassays in LMC representative formats (please refer to Chapter 2 for details of model immunoassays) were tested in the proposed LMC extraction solvent (MeOH P80); in 30 fold concentrated extraction solvent, which was modelled as 45 g/L P80, making the assumption that all of the methanol in the extraction solvent would be evaporated; and in a range of alternatives to the proposed extraction solvent.

The results and discussion are presented in two sections; the first section describes and discusses immunoassay performance in MeOH P80 and in 45 g/L P80. Although these results cover two of the objectives of this thesis they are grouped together to prevent unnecessary duplication of information, since the same controls were used for both sets of data. The second section describes and discusses immunoassay performance in a selection of potential alternative extraction solvents for the LMC.

Immunoassay performance was initially assessed in binding ELISA to rapidly screen assays for functionality in the tested extraction solvents. Where possible further studies were then carried out in pooled microarray binding assays to confirm performance in a more flight representative format. Finally extended studies were carried out, with a subset of conditions, to test the sensitivity of the immunoassays in the extraction solvents.

3.4.2 Immunoassay performance in the proposed LMC extraction solvent and 30 x concentrated extraction solvent

3.4.2.1 Initial assessment of immunoassay performance in MeOH P80 and 45 g/L P80 using ELISA binding signal as a performance indicator

Mean ELISA binding signals and non-specific background signal for each immunoassay, and for HRP enzyme label independently of the immunoassays (please see Section 2.2.2.1 for methodology), in MeOH P80 and in 45 g/L P80, are summarised in Table 3-1.

Mean background corrected binding signals, in MeOH P80 and 45 g/L P80, were normalised against mean background corrected binding signal in water (for ease of comparison across all immunoassays) and plotted in Figure 3-2 (all immunoassays except benzo[a]pyrene) and Figure 3-3 (benzo[a]pyrene). Data for the benzo[a]pyrene immunoassay was plotted separately because it performed significantly differently from the other immunoassays.

Table 3-1: Mean Binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs in MeOH P80 and 45g/L P80 and water as a control. Also showing binding signal generated by HRP enzyme independently of the immunoassays as a control. All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm, n = 2 or 3

	Water		MeOH P80		45 g/L P80	
	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
HRP Enzyme	0.78	0.05	0.72	0.04	0.78	0.05
Atrazine	0.59	0.12	0.62	0.07	0.29	0.07
Benzo[a]pyrene	0.18	0.11	0.82	0.07	0.48	0.07
cAMP	1.31	0.13	1.14	0.07	1.19	0.06
Fluorescein	0.69	0.21	0.60	0.08	0.53	0.20
GroEL	0.66	0.10	0.60	0.05	0.67	0.06
LPS	0.64	0.17	0.09	0.05	0.13	0.05
LTA	0.60	0.12	0.39	0.05	0.40	0.05

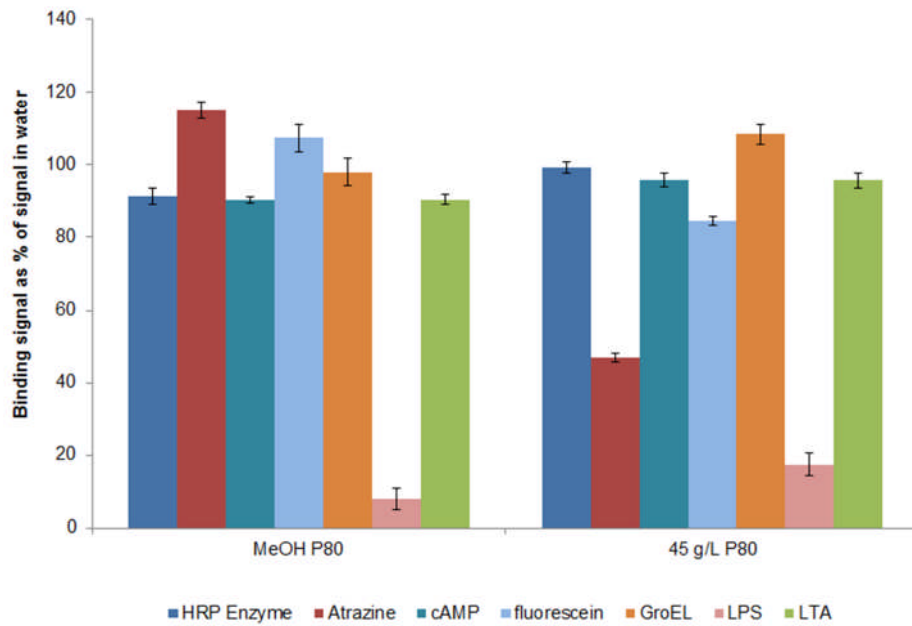


Figure 3-2: Normalised background corrected ELISA binding signal for atrazine, cAMP, fluorescein, GroEL, LPS and LTA immunoassays in MeOH P80 and 45 g/L P80. Binding signals normalised against binding signal in water. Also showing normalised binding signal produced by HRP enzyme label independently of immunoassays as a control. Error bars = +/- standard error, n=2 or 3

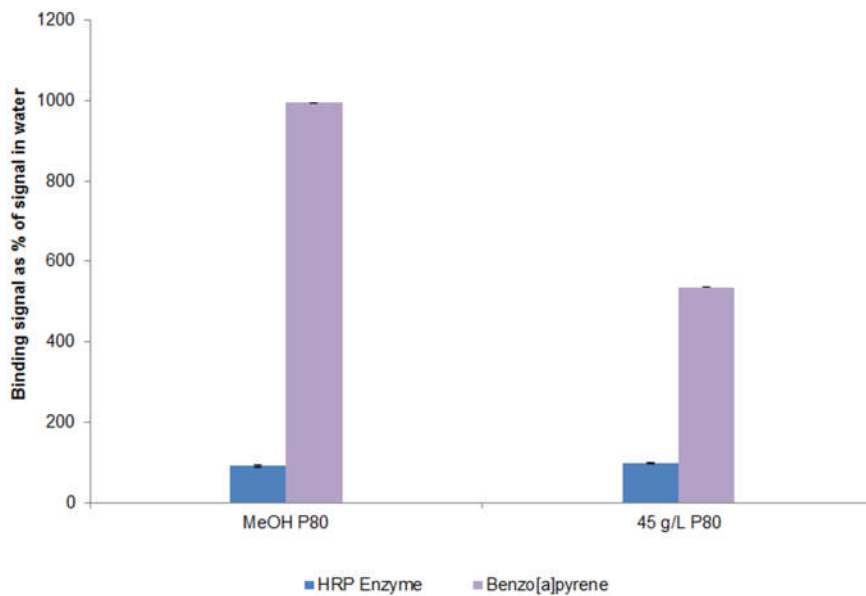


Figure 3-3: Normalised background corrected ELISA binding signal for benzo[a]pyrene immunoassay in MeOH P80 and 45 g/L P80. Binding signals normalised against binding signal in water. Also showing normalised binding signal produced by HRP enzyme label independently of immunoassays as a control. Error bars = +/- standard error, n=2 or 3

Effect of MeOH P80 on immunoassay binding signal

The data in Table 3-1, Figure 3-2 and Figure 3-3 show that binding signal was affected by MeOH P80; however the majority of the seven model immunoassays tested exhibited only small changes in binding signal (subjectively assigned as $\pm 20\%$), which are unlikely to have a significant impact on performance of the LMC flight instrument. In addition non-specific binding (signal generated by incubation of antibodies in wells that were not coated with target molecule) was reduced in MeOH P80, compared to in water (Table 3-1) for the all of the immunoassays tested. This is not unexpected given that P20 is routinely added to ELISA washing and dilution buffers to reduce background signal (Kim *et al*, 2004; Traunmuller *et al*, 2005) and indicates that the use of MeOH P80 as the LMC extraction solvent may be beneficial in terms of minimising non-specific binding.

Background corrected binding signal in the cAMP, GroEL and LTA immunoassays fell in MeOH P80 compared to water (Figure 3-2), but the level of signal reduction was similar to, or less than, the signal reduction of 10 % observed when HRP enzyme label was tested independently of the immunoassays. Therefore it is not possible to determine whether these small changes in immunoassay binding signal were attributable to changes in immunoassay performance (*i.e.* a reduction in the quantity of labelled antibody that bound to the immobilised antigen), or due to altered activity of the enzyme label after exposure to MeOH P80 which is not relevant for the flight format LMC which uses fluorescent labels. Binding signal in the fluorescein assay was unaffected. Overall this indicates that there are no fundamental issues with the use of MeOH P80 as extraction solvent for immunoassays that use polyclonal and monoclonal antibodies, raised against small polar targets, protein targets and glycolipid target molecules in terms of binding to directly immobilised proteins, small molecule protein conjugates and directly immobilised glycolipid immobilised target molecules.

Binding signal in the LPS immunoassay in MeOH P80 (Figure 3-2) fell to a level that was not distinguishable above background signal, therefore the LPS immunoassay, in its current format, could not be used with MeOH P80 as the extraction solvent. Since the anti-mouse secondary antibody used in the LPS assay (Table 2-4) is also used in the benzo[a]pyrene, LTA and cAMP immunoassays which did not exhibit large signal losses in MeOH P80, signal loss in the LPS assay is most likely due to altered interaction between the anti-LPS antibody and the immobilised LPS molecule, or due to

loss of immobilised LPS from the plate surface. Published data indicates that immobilised mycobacterial glycolipids can be stripped from 96-well plates by polysorbate 20 (Traunmuller *et al*, 2005) therefore it seems reasonable to speculate that polysorbate 80 could have a similar effect, displacing physically adsorbed LPS from the 96-well plate surface. If signal loss is due to instable immobilisation of the LPS target molecule, stability might be improved by covalent immobilisation of LPS or by conjugation of LPS to a carrier protein; there are several examples in the literature of methods developed for the conjugation of carbohydrate type molecules to proteins (Boratyn and Roy 1998; Zhang *et al*, 1998; Saksena *et al*, 2003; Gildersleeve *et al*, 2008). If signal loss is due to altered interactions between immobilised LPS and the anti-LPS antibody, it may be possible to source an anti-LPS antibody which is less sensitive to the solvent, but if binding forces are largely hydrophobic this may offer little improvement. This result is not of great concern for the LMC since there are unlikely to be a large number of glycolipid target molecules in the final set of 25 targets for flight.

Binding signal for the atrazine immunoassay, was increased by approximately 15 % in MeOH P80 compared to water (Table 3-1 and Figure 3-2). The atrazine immunoassay uses a single chain antibody fragments (scAb) primary antibody and scAbs have been shown to be less stable than complete IgGs (Fodey *et al*, 2011) having a tendency to aggregate. However in this study performance of the tested scAb (in terms of binding to immobilised conjugate) was not detrimentally changed by MeOH P80 suggesting that there are no fundamental issues with the stability of scAbs in the proposed LMC extraction solvent.

The benzo[a]pyrene ELISA (Figure 3-3) performed significantly differently from the other immunoassays tested in this study in that binding signal was ten times higher in MeOH P80 compared to water. However it should be noted that benzo[a]pyrene absolute immunoassay binding signal in water was low compared to the other immunoassays tested and absolute binding signal in MeOH P80 was comparable to the other immunoassays (Table 3-1). The observed increase in signal intensity in MeOH P80 compared to water indicates that MeOH P80 results in increased antibody affinity for the immobilised benzo[a]pyrene – BSA conjugate. A possible explanation for this could be that MeOH P80 affects how the benzo[a]pyrene-BSA protein conjugate folds, resulting in better accessibility by the antibody to the conjugated benzo[a]pyrene molecules. Benzo[a]pyrene is a small hydrophobic molecule, with low water solubility (0.0038 mg/L (Lu *et al*, 2007)) and benzo[a]pyrene and other PAH immunoassays

reported in the literature generally contain 5- 10 % methanol to solubilise standards and to extract the target molecules from environmental samples (Knopp *et al*, 2000; Matschulat *et al*, 2005). This data suggests that the use of MeOH P80 does have a significant affect on immunoassays for the detection of hydrophobic molecules, but would not prevent these types of assays from functioning in an LMC context.

Effect of 30 x concentrated proposed extraction solvent on immunoassay binding signal

Signal generated by HRP enzyme label after exposure to 45 g/L P80 was unchanged compared to the signal generated after exposure to water (Figure 3-2), therefore changes in immunoassay binding signal in 45 g/L P80 are likely to reflect real changes in antibody performance, rather than changes in the activity of the HRP enzyme label. The fact that HRP enzyme label is unaffected by 45 g/L P80 is perhaps surprising as a concentration of 45g/L P80 is considerably higher than surfactant concentrations typically included in immunoassays and surfactants have been shown to inhibit enzyme substrate reactions (Matson 2000). However in the LMC format ELISA the extraction solvent, including the P80 surfactant, is removed prior to addition of the substrate (for full details of method please see Chapter 2), therefore it is possible that addition of 45g/L P80 does affect the enzyme label on the immobilised protein, but that the changes are reversed when the surfactant containing extraction solvent is washed away, resulting in no observed change in signal generated by the enzyme.

Immunoassay binding signal was affected by 45 g/L P80 (Table 3-1, Figure 3-2, Figure 3-3); however the majority of the seven model immunoassays tested exhibited only small changes in binding signal (subjectively assigned as $\pm 20\%$) which are unlikely to have a significant impact on performance of the LMC flight instrument. Non specific binding (signal generated by incubation of antibodies in wells that were not coated with target molecule) was reduced in 45 g/L P80, compared to in water (Table 3-1) for all of the immunoassays tested. As discussed for MeOH P80, P80 is routinely added to ELISA buffers to reduce non-specific binding so this result is not unexpected.

Binding signal was unaffected by 45 g/L P80 in the cAMP and LTA immunoassays, binding signal fell by $\sim 20\%$ in the fluorescein immunoassay, and binding signal was enhanced by approximately 10 % for the GroEL immunoassay. The observed changes in binding signal could be related to changes in one/many of the interactions required to generate signal in the LMC format immunoassay including the immobilisation of the target molecules to the 96-well plate and the binding interactions of the primary

antibodies, secondary antibodies and the streptavidin-biotin. This suggests that there are no fundamental problems with concentrating the LMC extraction solvent, in terms of binding signal for immunoassays using monoclonal and polyclonal antibodies, small molecule-protein conjugates and directly immobilised glycolipids.

Binding signal in the atrazine immunoassay (Figure 3-2), fell by approximately 50 % in 45 g/L P80 compared to water, indicating that the amount of labelled antibody bound to the 96-well plate was decreased. A reduction in signal of this magnitude is not ideal but binding signal is still easily distinguishable from non-specific background signal therefore the assay is still expected to be functional. The drop in binding signal in 45 g/L P80 could indicate that scAbs format antibodies, of which the anti-atrazine antibody is an example, are generally less stable in high concentrations of P80 than whole IgG format antibodies, or the signal loss could be specific to this individual clone, or related to the anti-human kappa light chain biotin secondary antibody used in the atrazine assay (Table 2-4), or due to altered interactions between the immobilised target and the anti-atrazine antibody binding site. Testing further scAb format antibodies in 45 g/L P80 and also a range of different secondary antibodies is required to clarify this. If scAb format assays are not stable in 45 g/L P80 this may prevent their use in the LMC flight instrument, assuming that the liquid extract from the martian sample is subject to a concentration step.

The greatest reduction in binding signal in 45 g/L P80 was observed for the LPS immunoassay which was rendered non-functional. The drop in signal for the LPS immunoassay in 45 g/L P80 may be related to instability of immobilisation of the glycolipid antigen in the presence of P80, as discussed previously for MeOH P80, or may be related to changes in the interaction between immobilised LPS and the anti-LPS antibody binding site.

The benzo[a]pyrene immunoassay (Figure 3-3) again performed differently to the other immunoassays in that binding signal was enhanced in 45 g/L P80 compared to binding signal in water (approximately x 6). However it should once again be noted that binding signal in water for this immunoassay was low and that binding signal in 45 g/L P80 was not significantly higher than for the other immunoassays (Table 3-1). Increased signal may be a result of the mechanisms described previously for the benzo[a]pyrene assay in MeOH P80.

3.4.2.2 Further assessment of immunoassay performance in MeOH P80 and 45 g/L P80 using microarray assay binding signal as an indicator of performance

In addition to the testing carried out in ELISA format, two immunoassays, GroEL and fluorescein, were tested for binding signal in MeOH P80 and 45 g/L P80 in multiplexed pooled microarray format. Note that at the time of this work GroEL and fluorescein were the only two immunoassays that had been transferred successfully into microarray format, therefore they were the only assays available for use in this work. Images of microarrays taken before and after running pooled microarray immunoassays are presented in Figure 3-4.

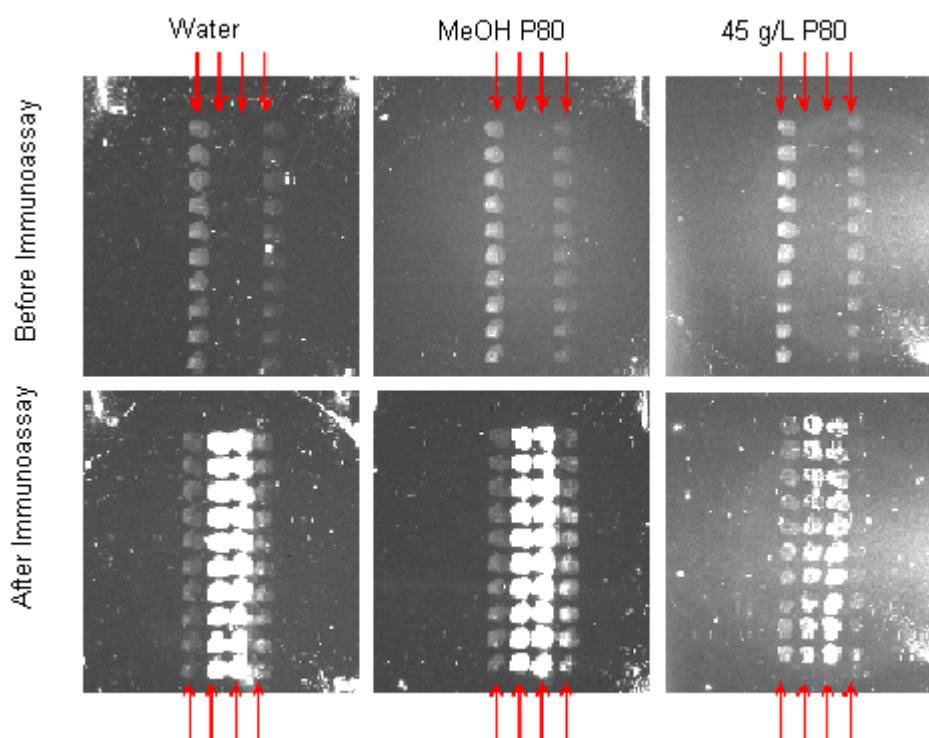


Figure 3-4: Images of microarrays of physically adsorbed proteins/protein-small molecule conjugates before and after running pooled format immunoassays in water, MeOH P80 and in 45 g/L P80

Microarrays with 4 columns of 10 replicate spots (from left to right on each array, position indicated by red arrows) high ratio AF633-labelled BSA, GroEL (not visible on pre-immunoassay images), BSA-FITC (not visible on pre-immunoassay images) and low ratio AF633- labelled BSA, printed on hydrophobic silane functionalised silicon nitride chips using SPI spotting robot. Images recorded using imaging breadboard. Image brightness and contrast has been adjusted to aid visualisation of spots.

In the images taken before running immunoassays (top of Figure 3-4) only the AF633 labelled BSA spots are visible, whereas in images taken after multiplexed pooled microarray format immunoassays (bottom of Figure 3-4) the GroEL and BSA-fluorescein spots become visible. Closer examination of the images shows that the brightness of microarray spots appears more variable on microarray assays run with MeOH P80 and with 45 g/L P80 compared to assays run in water. This is particularly obvious in the images of microarrays run in 45 g/L P80. One interpretation is that immobilised target molecule has been stripped from the chip surface, resulting in the highly variable spot structures.

The mean pixel intensity of individual spots on the microarray images shown in Figure 3-4 were measured using ImageJ software (please see Section 2.2.3.9) and the mean signal intensity calculated for each assay (typically ten replicates) to give the binding signal for each immunoassay and the mean signal intensity for the AF633 labelled BSA spots (Figure 3-5).

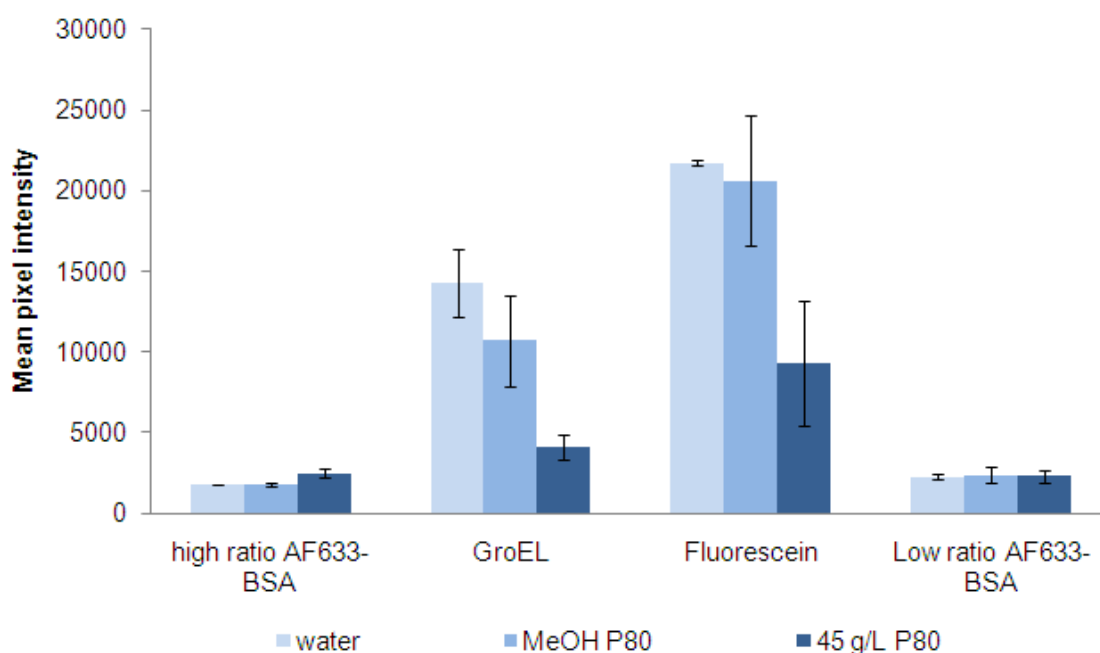


Figure 3-5: Binding signal for pooled format microarray immunoassays run in water, MeOH P80 and 45 g/L P80. Binding signal reported as mean pixel intensity of microarray spots. Microarrays printed using SPI spotting robot and imaged using imaging breadboard. Error bars = ± 1 standard deviation of the mean, n = 10

The use of MeOH P80 and 45 g/L P80 in pooled microarray immunoassays produced reductions in immunoassay binding signal compared to binding signal in water. Signal

reduction was greater in 45 g/L P80 than in MeOH P80 and variability in the signal intensity was increased (as assessed by comparison of standard deviation from the mean) with 45 g/L P80 compared to water and MeOH P80.

Comparison of the mean pixel intensity of the AF633 labelled BSA spots in images taken of the same array before and after running pooled format assays, reveals that signal was reduced after running pooled assays with water, MeOH P80 and 45 g/L P80 (Table 3-2). The highest loss of signal was observed after pooled format immunoassays in MeOH P80 (50 % reduction in signal) but signal was reduced by 40 % after running assays with water. This indicates that the immobilisation of the AF633-BSA conjugates, by physical adsorption, is not stable to the incubation and washing procedures used to run a pooled assay, even when water is used as the solvent. From this it can be inferred that immobilisation of all proteins/protein conjugates onto LMC chips by physical adsorption is also likely to be unstable. It is possible that immobilisation stability and consequently microarray assay performance in any extraction solvent could be significantly improved by the use of covalent immobilisation. Covalent immobilisation would also enable the regeneration of chip surfaces, allowing more development work to be carried out with a limited number of optical waveguide chips.

Table 3-2: Mean pixel intensity (\bar{x}) of high ratio AF633-BSA spots measured on images taken before and after pooled microarray immunoassays in MeOH P80 and 45 g/L P80 and water as a control. n=10

	<i>Before Immunoassay</i>		<i>After Immunoassay</i>		<i>Change in signal</i>
	\bar{X}	<i>% CV</i>	\bar{X}	<i>% CV</i>	
<i>Water</i>	663	10.7	408	22.8	- 39 %
<i>MeOH P80</i>	974	21.7	476	24.4	- 51 %
<i>45 g/L P80</i>	1006	32.5	691	61.5	- 31 %

The result reported in this section demonstrate that binding signal in microarray format can be generated in MeOH P80 and 45 g/L P80 for the limited set of immunoassays that were tested, although signals were affected. The data has also highlighted that there is scope to further optimise the microarray format in order to improve immunoassay performance.

3.4.2.3 Extended assessment of immunoassay performance in MeOH P80 and 45 g/L P80 using ELISA sensitivity (IC_{50}) as an indicator of performance

The effect of MeOH P80 and 45 g/L P80 on immunoassay sensitivity was assessed by running inhibition ELISAs in MeOH P80, 45 g/L P80, and with water as a control solvent. Since benzo[a]pyrene and atrazine antigens have poor water solubility 10/90 % (v/v) MeOH water was used as a control solvent in place of water for these assays.

Two examples of inhibition immunoassay curves for ELISAs run in MeOH P80 and 45 g/L P80 are plotted in Figure 3-6 (the other graphs are available in Appendix A.1), and the IC_{50} values for all immunoassays, calculated by fitting data to a four parameter binding equation (Findlay and Dillard 2007), are presented in Table 3-3.

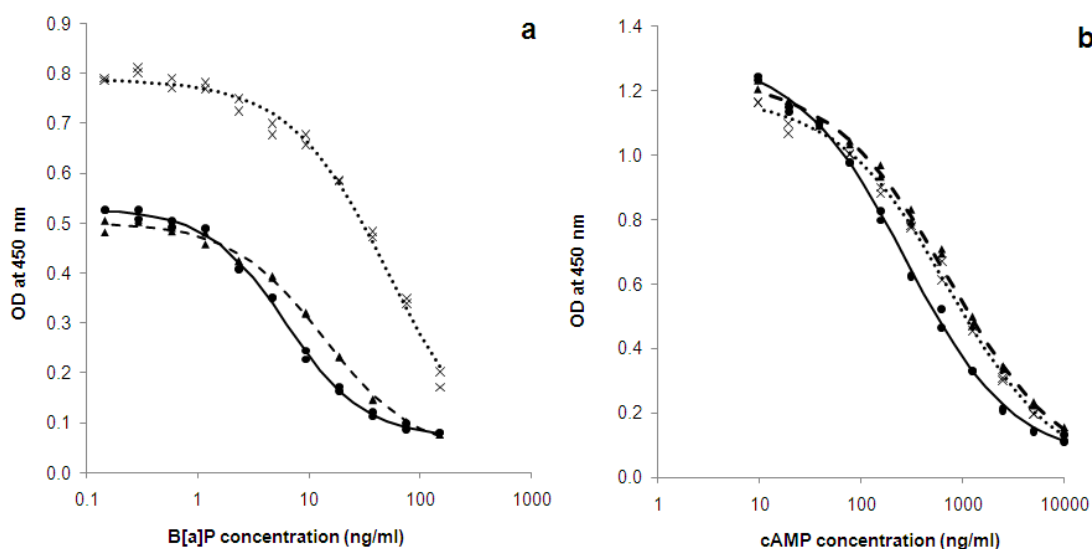


Figure 3-6: (a) B[a]P and (b) cAMP inhibition ELISAs in (●) control solvent, (X) MeOH P80, (▲) 4.5 g/L P80.

Each data point represents a single replicate. Immunoassay curves were plotted by fitting mean signal to a four parameter binding equation for assays run with (solid line) control solvent (dotted line) MeOH P80, (dashed line) 45g/L P80. The control solvent was 10 % methanol for the B[a]P assay and water for the cAMP assay

Table 3-3: IC₅₀ values for inhibition ELISAs run in MeOH P80 and 45 g/L P80
 IC₅₀ values were calculated by fitting the data for each immunoassay to a four parameter binding equation. Sum of squares due to regression was less than 0.02 for all data fitting

	IC ₅₀ (ng/ml)		
	water (*10 % MeOH)	MeOH P80	45 g/L P80
Atrazine	*1.4	24.2	24.2
B[a]P	*6.2	53.8	13.4
cAMP	274.8	717.0	717.0
Fluorescein	300.0	375.0	219.2
GroEL	2447.0	2096.5	1500.1
LPS	190.5	Not calculated ⁶	Not calculated ⁵
LTA	2.0	1.0	2.1

Immunoassay sensitivity in MeOH P80

Target molecules for six of the seven model immunoassays were successfully detected in MeOH P80, detection was not achieved for the LPS immunoassay because binding signal was too low. This reveals that immunoassays encompassing a broad range of antibody types can be used for the detection of a variety of target molecule types in MeOH P80, validating its selection as extraction solvent for the LMC. Immunoassay sensitivity (estimated as IC₅₀) in MeOH P80 (Table 3-3) was poorer (*i.e.* IC₅₀ was increased) for 4 out of the 7 immunoassays tested (atrazine, benzo[a]pyrene, cAMP and fluorescein) and sensitivity was improved (*i.e.* IC₅₀ was reduced) in two immunoassays (GroEL and LTA) in MeOH P80 compared to in a control solvent. These findings broadly agree with the findings of published studies which have determined that IC₅₀ is generally increased in methanol and other organic solvent containing solutions compared to aqueous buffers (Penalva *et al*, 2000; González-Martínez *et al*, 2003; Sun *et al*, 2009). Changes in immunoassay sensitivity are a result of altered affinity of the antibody for the free target molecule in MeOH P80 compared to the control solvent.

In the case of more traditional immunoassays, *e.g.* for environmental monitoring, or medical diagnostics, variations in sensitivity (and binding signal) of the magnitude

⁶ IC₅₀ was not calculated because the data did not fit a four parameter binding equation. In most cases this was because binding signal was not distinguishable from background signal in the solvent under test

observed in this study would be problematic due to the level of precision required (Krotzky and Zeeh 1995) and would need to be well characterised and controlled. The LMC instrument is intended to be qualitative rather than quantitative – *i.e.* to indicate whether or not a particular molecule is present above a given detection threshold, not to measure precisely how much of that molecule is there. Therefore these types of changes in immunoassay sensitivity and binding signal observed in this study are unlikely to be highly significant, particularly if the antibodies used in the flight model have high affinity and sensitivity.

The assays which demonstrated the greatest reduction in sensitivity were benzo[a]pyrene and atrazine, both molecules with limited water solubility. Stocklein *et al* (2000) proposed that binding of antigens to antibodies is more favourable in solvents in which the antigen is poorly soluble and data generated by LMC consortium members at Imperial College has shown that the solubility of pyrene, a PAH with a structure related to benzo[a]pyrene, is increased by more than tenfold in MeOH P80 compared to 20/80 % (v/v) methanol water (Court 2011). The implication is that addition of P80 to the extraction solvent increases the solubility of benzo[a]pyrene but also reduces the favourability of the antibody – benzo[a]pyrene binding interaction. This view is supported by data from a previous study in which Matschulat *et al* (2005) found that sensitivity of an immunoassay using the same anti-benzo[a]pyrene clone was unaffected by methanol at concentrations of up to 40 % (v/v). Therefore the reduction in sensitivity observed in this study is likely to be related to the presence of P80. This has implications for other immunoassays for target molecules with poor water solubility, particularly extinct life marker target molecules, where immunoassay sensitivity is likely to be poorer in P80 containing solutions. However it is also important that the extraction solvent is able to extract apolar molecules. Stocklein *et al* (2000) sought to determine the optimum solvent for both the extraction of pesticide molecules from soil samples and for the detection of extracted pesticide molecules in immunoassays and concluded that both conditions cannot be met optimally by the same solvent. For the LMC where up to 25 immunoassays will be multiplexed, the selection of an appropriate solvent becomes even more challenging and some level of compromise, in terms of immunoassay performance and target extraction efficiency, is inevitable.

Immunoassay sensitivity in 45 g/L P80

Six of the seven model immunoassays successfully detected target molecule in 45 g/L P80, detection was not achieved for the LPS immunoassay because binding signal was too low. This reveals that immunoassays encompassing a broad range of antibody types can be used for the detection of a variety of target molecule types in 45 g/L P80, therefore concentration of the proposed extraction solvent by evaporation is likely to be compatible with immunoassays. The functionality of a high number of the tested immunoassays in 45g/L P80 is perhaps surprising given that the use of P20 in immunoassay buffers is generally restricted to much lower concentrations (0.1-1 g/L) and higher concentrations of surfactants have been shown to denature proteins and to desorb proteins from substrates (Selby 1999).

IC₅₀ varied in 45 g/L P80 compared to the control solvent for the seven immunoassays tested with some immunoassays showing improved sensitivity (GroEL and fluorescein), others worsened sensitivity (atrazine, benzo[*a*]pyrene and cAMP) and some immunoassays being unaffected (LTA) (Table 3-3). IC₅₀ was not calculated for the LPS immunoassay because the binding signal in 45 g/L P80 was too low to fit the data to a four parameter binding equation. Once again many of the observed changes in IC₅₀ were of a magnitude unlikely to significantly impact on instrument performance.

In summary immunoassay performance, in terms of sensitivity was, for the majority of immunoassays tested, similar or better than in MeOH P80. This is surprising given that 45 g/L P80 is more different to typical immunoassay buffers than the proposed LMC extraction solvent. The maximum increase in IC₅₀ in 45 g/L P80 compared to water/10 % methanol was 20 fold, indicating that a 30 fold concentration of the liquid extract is unlikely to be detrimental to instrument detection limits.

3.4.2.4 Summary and future Work

This section of work had two specific objectives:

Objective 1

The first objective of this study was to confirm functionality of LMC format immunoassays in the proposed LMC extraction solvent, by extending the range of relevant immunoassays tested under these conditions. This objective was addressed by testing the performance of seven LMC relevant immunoassays in the proposed LMC extraction solvent, compared to water. As anticipated from information published in the literature (reviewed in section 3.3), the majority of the assays tested in this study functioned effectively in MeOH P80. These assays encompassed a broad range of antibody types, target molecule types and immobilised target types. The results of the study indicate that MeOH P80 should be broadly compatible with LMC format immunoassays.

Some changes in immunoassay binding signal and immunoassay IC_{50} were observed with MeOH P80 compared to in water, but the vast majority of these changes were not of significant concern for the LMC due to the analytical requirements of the instrument and the acceptance that some level of compromise in terms of immunoassay performance is inevitable given the requirement for the solvent to both extract target molecules efficiently and be compatible with the multiplexed immunoassays.

In conclusion this data confirms that the selected baseline LMC extraction solvent is likely to be compatible with the multiplexed LMC format immunoassay. Future immunoassays should be developed and optimised directly in the proposed LMC extraction solvent and further work should be carried out to extend the number of immunoassays tested in microarray format.

Objective 2

The second objective of this work was to establish whether 30 x concentration of the proposed LMC extraction solvent is compatible with immunoassays. This objective was achieved by modelling 30x concentrated LMC extraction solvent as 45 g/L P80 and testing seven LMC format immunoassays for functionality in 45 g/L P80. Some changes in immunoassay binding signal and immunoassay IC_{50} were observed with 45 g/L P80 compared to water, but the vast majority of these changes were not of

significant concern for the LMC due to the analytical requirements of the instrument and the acceptance that some level of compromise in terms of immunoassay performance is inevitable in order to meet the dual requirements of efficient extraction and immunoassay compatibility.

In conclusion, the data established the functionality of a small number of LMC format assays in the 30 x concentrated extraction solvent, demonstrating that 30-fold concentration of the liquid extract from the Martian sample is likely to be compatible with the LMC format multiplexed immunoassay. Since the completion of this work LMC flight instrument design has evolved and the facility to concentrate the LMC solvent has been removed in order to reduce mass and power consumption requirements. If a concentration step was still being considered for the LMC further work would be required to assess the performance of more immunoassays in 45 g/L P80 and also to expand the range of immunoassays tested in microarray format.

3.4.3 Immunoassay performance in alternative extraction solvents

The work in Section 3.4.2 has shown that the proposed LMC extraction solvent, MeOH P80, is likely to be compatible with LMC format immunoassays. However there is a risk that the proposed LMC solvent may prove to be unsuitable for use in the flight instrument, due to as yet unidentified factors such as incompatibility with space radiation and/or sterilisation procedures. Therefore it is necessary to identify a number of potential 'back-up' extraction solvents as possible alternatives to MeOH P80.

A range of alternative solvents was selected in collaboration with LMC consortium members based at Imperial College. Selection was based on anticipated extraction efficiency for a range of polar and apolar organic molecules and expected compatibility with immunoassays, based on literature information. The solvents and concentrations that were tested are shown in Table 3-4. Within this work no investigation of alternative surfactants was made, but this has been identified as a future piece of work for the LMC consortium⁷.

Table 3-4: Formulation of alternative LMC extraction solvents tested for compatibility with LMC format immunoassays

Extraction Solvent	Solvent concentrations tested (v/v)
Dimethylsulfoxide (DMSO) P80	2 % DMSO, 98 % water, 2.5 g/L P80 5 % DMSO, 95 % water, 2.5 g/L P80
DMSO MeOH P80	2 % DMSO, 18 % MeOH, 80 % water, 2.5 g/L P80 5 % DMSO, 15 % MeOH, 80 % water, 2.5 g/L P80
Acetone P80	10 % acetone, 90 % water, 2.5 g/L P80 20 % acetone, 80 % water, 2.5 g/L P80 30 % acetone, 70 % water, 2.5 g/L P80
Acetonitrile (MeCN) P80	10 % MeCN, 70 % water, 2.5 g/L P80 20 % MeCN, 80 % water, 2.5 g/L P80 30 % MeCN, 90 % water, 2.5 g/L P80

Determination of the extraction efficiency of the alternative solvents was carried out elsewhere within the LMC project team (Gomes 2009) and results are not reported in this thesis.

⁷ Note - in studies in late 2011, alternative surfactants of Zonyl FS-300 and Poly(dimethylsiloxane-co-[3-(2-(2-hydroxyethoxy)ethoxy)propyl]methoxysiloxane have been shown in LMC studies to be compatible with both target molecule extraction and immunoassay detection

Immunoassay performance was initially assessed by testing the impact of the alternative extraction solvents on immunoassay binding signal in ELISA format. No further studies with pooled microarray format immunoassays were carried out as part of this work due to resource limitations. Extended studies with a limited set of alternative extraction solvents were then performed by assessing immunoassay sensitivity in ELISA format.

It should be noted that when this piece of work was carried out the concentration of P80 in the proposed LMC extraction solvent had not been finalised and varied between 1.5 and 2.5 g/L. The impact of increasing the P80 concentration from 1.5 g/L to 2.5 g/L on ELISA binding signal was minimal (data not shown) therefore for the purposes of this study P80 was included in the alternative extraction solvents at a concentration of 2.5 g/L.

3.4.3.1 Initial assessment of immunoassay performance in potential alternative extraction solvents using ELISA binding signal as performance indicator

Mean ELISA binding signal for each immunoassay, and for HRP enzyme label tested independently of the immunoassays (please see Section 2.2.2.1), in a range of alternative extraction solvents, are summarised in Table 3-5 along with non-specific background binding signal for each immunoassay in each solvent. The data reported in the table is restricted to the highest concentration tested for each alternative extraction solvent. The complete data set for all tested concentrations is available in Appendix A2

Mean background corrected binding signals were normalised against mean background corrected binding signal in water (for ease of comparison across all immunoassays) and plotted in Figure 3-7 (all immunoassays except benzo[a]pyrene) and Figure 3-8 (benzo[a]pyrene). Data for the benzo[a]pyrene immunoassay was plotted separately because it performed significantly differently from the other immunoassays.

Table 3-5: Mean binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in selected concentrations of proposed alternative LMC extraction solvents and water as a control.

Also showing binding signal generated by HRP enzyme independently of the immunoassays as a control. All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm and n = 2 or 3.

	<u>Water</u>		<u>5 % DMSO 2.5 g/L P80</u>		<u>5 % DMSO 15 % MeOH 2.5 g/L P80</u>		<u>30 % acetone 2.5 g/L P80</u>		<u>30 % acetonitrile 2.5 g/L P80</u>	
	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.78	0.05	0.82	0.04	0.82	0.04	0.81	0.04	0.66	0.04
<i>Atrazine</i>	0.57	0.14	0.15	0.05	0.25	0.05	0.06	0.05	0.06	0.05
<i>B[a]P</i>	0.27	0.12	0.66	0.20	1.08	0.04	0.96	0.05	0.45	0.05
<i>cAMP</i>	0.71	0.05	0.65	0.04	0.63	0.04	0.55	0.05	0.05	0.05
<i>Fluorescein</i>	0.86	0.05	0.73	0.04	0.70	0.04	0.58	0.05	0.48	0.05
GroEL	0.68	0.05	0.61	0.04	0.62	0.04	0.59	0.04	0.37	0.04
LPS	0.54	0.13	0.22	0.08	0.15	0.08	0.09	0.05	0.06	0.05
LTA	0.97	0.16	0.13	0.05	0.11	0.05	0.07	0.05	0.06	0.06

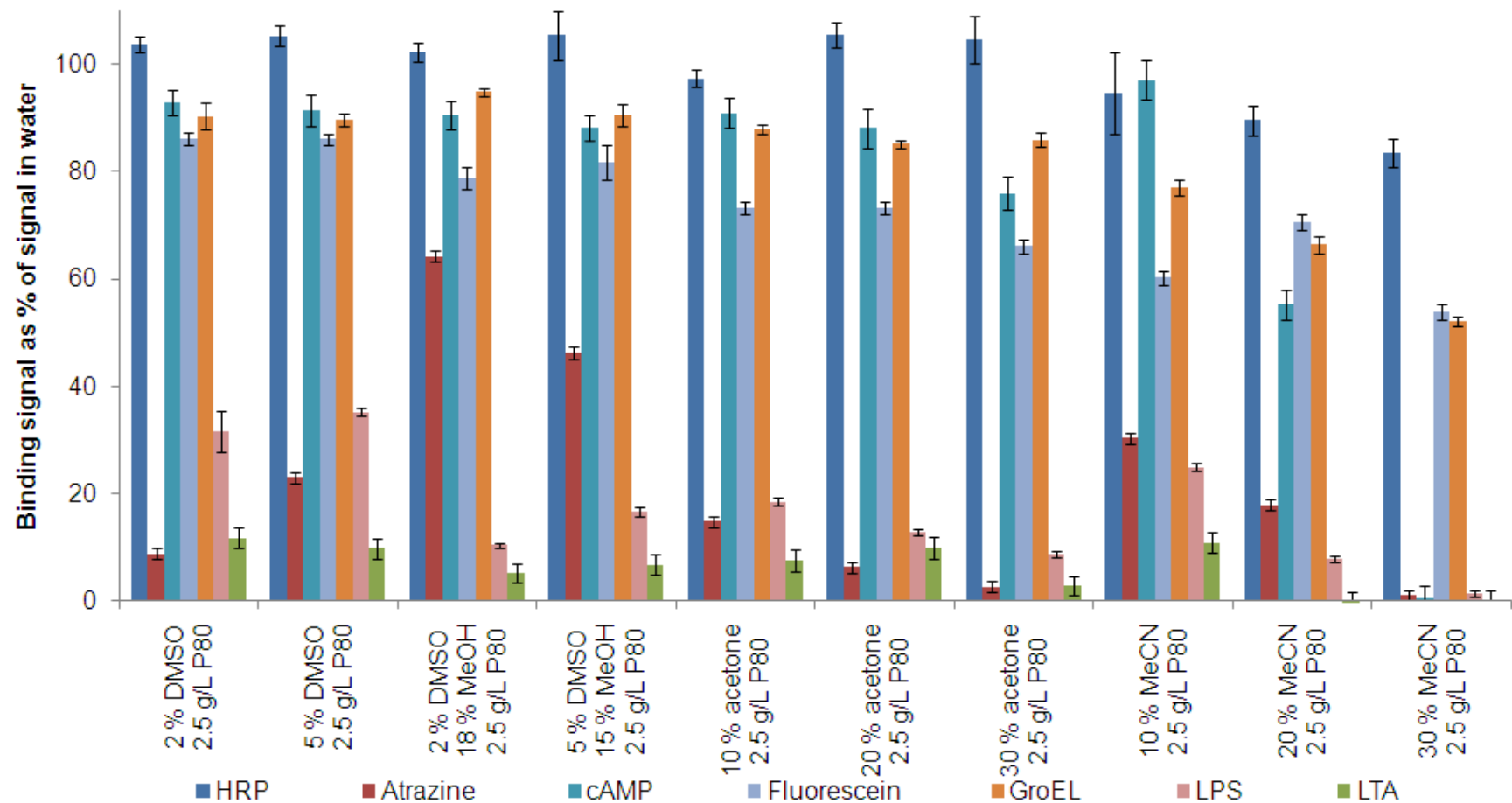


Figure 3-7: Normalised background corrected ELISA binding signal for atrazine, cAMP, fluorescein, GroEL, LPS and LTA in proposed alternative extraction solvents.

Binding signals normalised against binding signal in water. Also showing normalised binding signal produced by HRP enzyme label independently of immunoassays as a control. Error bars = +/- standard error, n=2 or 3.

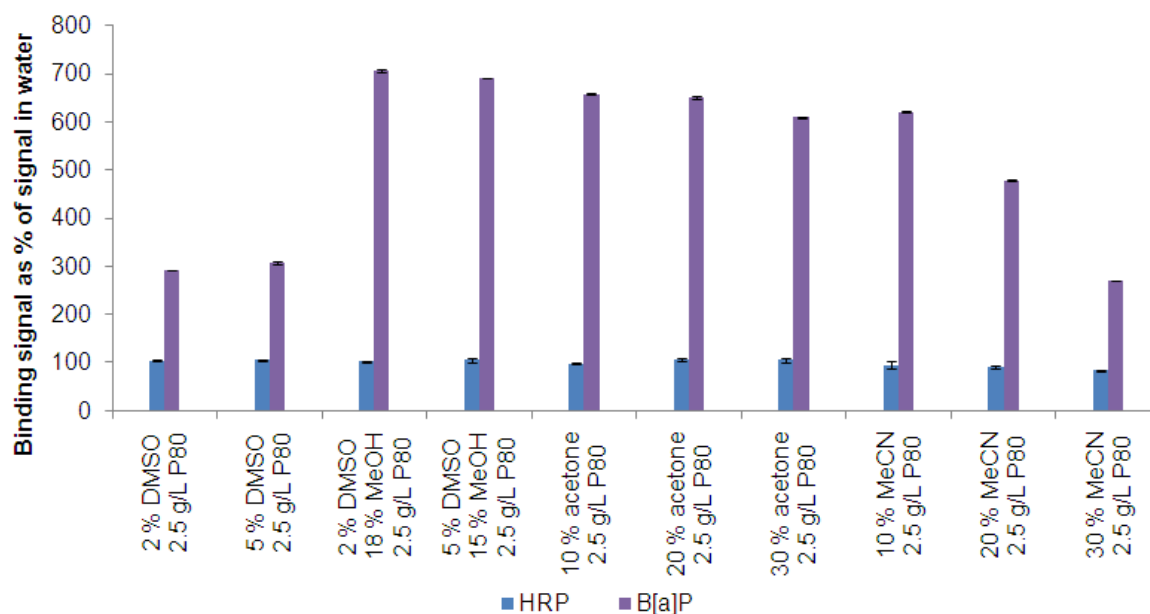


Figure 3-8: Normalised background corrected ELISA binding signal for benzo[a]pyrene in proposed alternative extraction solvents. Binding signals normalised against binding signal in water. Also showing normalised binding signal produced by HRP enzyme label independently of immunoassays as a control. Error bars = +/- standard error, n=2 or 3

The data presented in Table 3-5, Figure 3-7 and Figure 3-8 show that background corrected binding signal for each immunoassay varied across the range of alternative extraction solvents tested, with the majority of immunoassays showing lower binding signal in the alternative extraction solvents compared to in water.

Organic solvents and surfactants are known to affect enzyme labels (Lu *et al*, 1997; Matson 2000) and changes in HRP enzyme label activity were observed across the range of alternative extraction solvents tested, although generally these changes were small (<10 %). The greatest reduction in activity of the HRP label was evident in acetonitrile containing extraction solvents. Acetonitrile has previously been shown to cause irreversible changes in HRP enzyme (Lu *et al*, 1997) and hydrophilic solvents, such as acetonitrile, have been observed to be more detrimental to enzymes than less polar organic solvents like methanol (Stöcklein and Scheller 1997).

The seven immunoassays tested can be broadly be separated into two sub-groups, those that were generally robust to the addition of a proposed alternative extraction solvents (*i.e.* maintained binding signal, showing either no change, or only small reductions or enhancements in binding signal), and those that were not robust (*i.e.* those that showed large reductions in binding signal).

Four of the seven immunoassays (cAMP, fluorescein, GroEL and benzo[a]pyrene) had high tolerance to the range of extraction solvents tested, retaining binding signal that was significantly above background noise in varying concentrations of DMSO, DMSO/MeOH, acetone and MeCN with 2.5 g/L P80. Immunoassays have previously been successfully demonstrated in a range of water miscible organic solvents at low (10 – 20 % (v/v)) concentrations including acetone (Kim *et al*, 2004), MeCN (González-Martínez *et al*, 2003), DMSO (Rehan and Younus 2006) and MeOH (Rigou *et al*, 2004), therefore the finding that LMC format immunoassays function in low concentrations of acetone, MeCN, DMSO and MeOH containing extraction solvents is not surprising. The cAMP, fluorescein, GroEL and benzo[a]pyrene immunoassays are diverse, encompassing a broad range of immobilised target molecule types, target molecule types and primary and secondary antibody types. Therefore, assuming that the performance of these immunoassays is representative, it should be possible to move to an alternative extraction solvent if the MeOH P80 solvent proves unsuitable for any reason. Some previous studies have shown that monoclonal antibodies are more resistant to organic media than polyclonals (Penalva *et al*, 1999), but in the case of the immunoassays tested here there are examples of immunoassays with both monoclonal (cAMP, benzo[a]pyrene) and polyclonal (GroEL, fluorescein) primary antibodies that show good tolerance for a range of organic solvents.

The LPS, LTA and atrazine immunoassays were functional (*i.e.* binding signal was high enough to be readily distinguishable from background noise) in a very limited number of the alternative extraction solvents tested. For LTA and LPS this suggests that the primary antibodies, or the direct immobilisation of glycolipid target molecules is sensitive to the addition of the proposed alternative extraction solvents (since the anti-mouse secondary antibody used for these assays is also used for the cAMP assay which showed better functionality in the alternative extraction solvents). The poor performance of the atrazine immunoassay, which in contrast to other tested immunoassays utilises a single chain antibody fragment primary antibody, in the proposed alternative extraction solvents, is of potential concern for the LMC because it may indicate that single chain antibody fragments are less stable to organic solvents and surfactants than IgGs, equally it may indicate that this particular scAb clone is sensitive to organic solvents, whereas other scAbs may not be. The reduction in signal intensity could also be related to the anti-human kappa light chain secondary antibody, which is not used in any of the other immunoassays tested. Further testing with additional scAbs is required to confirm their performance with the alternative extraction solvents. The implication of this is that if a need arose to move to an alternative extraction solvent, it is possible that some immunoassays, which function in MeOH P80, would not function in an alternative solvent.

The benzo[a]pyrene immunoassay exhibited enhanced binding signal in the proposed alternative extraction solvents compared to water but as discussed previously (Section 3.4.2.1) absolute binding signal in water was low compared to the other immunoassays tested (Table 3-5) and therefore it would appear that the benzo[a]pyrene immunoassay functions better (in terms of binding to immobilised target molecule) in the presence of P80 and organic solvent than in water. It is possible that the immobilised benzo[a]pyrene target molecules become more accessible to the primary antibody in the presence of organic solvents and/or P80 and/or adopt a conformation that is better recognised by the antibody, resulting in higher binding signal in the alternative extraction solvents compared to water.

For the majority of tested immunoassays increasing the proportion of organic solvent included in the extraction solvent under test resulted in lower binding signal (or a smaller enhancement in signal in the case of the benzo[a]pyrene immunoassay), this was particularly evident in acetonitrile and acetone containing extraction solvents where three concentrations were tested (10, 20 and 30 % (v/v)). Some additional testing, which is not reported in this thesis, was carried out with additional alternative extraction solvents which included ethanol and propan-2-ol at concentrations varying from 10 – 30 % and in general immunoassays that were robust to the solvents tested here were also robust to ethanol and propan-2-ol.

Previous studies have shown that polar organic solvents are more detrimental to proteins than less polar organic solvents because they are more water soluble and therefore displace more of the structural water molecules from the protein (Horáček and Skládal 2000). In this study acetonitrile was more disruptive to antibody function, and also to enzyme activity, than the less polar acetone and methanol (Table 3-6) in agreement with the published data.

Table 3-6: Dielectric constant (as a measure of polarity), boiling point and molecular mass of organic solvents tested for compatibility with LMC format immunoassays

<i>Solvent</i>	<i>Dielectric Constant</i>	<i>Boiling point (°C)</i>	<i>Molecular mass (g/mol)</i>
Acetone	20.7	65	58.08
Acetonitrile	36.6	56	41.05
Dimethyl sulphoxide	47.2	82	78.13
Methanol	33.0	189	32.04
Water	80.0	100	18.01

3.4.3.2 Extended assessment of immunoassay performance in selection of proposed alternative extraction solvents using ELISA sensitivity as a performance indicator

Immunoassay sensitivity was assessed by running inhibition ELISAs in the highest concentration of each proposed alternative extraction solvent and with a control solvent, water or 10/90 % methanol water for immunoassays where target molecules were not water soluble. Two examples of typical immunoassay inhibition curves are shown in Figure 3-9, the remaining inhibition assay plots are contained within Appendix A3. Immunoassay IC_{50} values were calculated by fitting data to a four parameter IC_{50} equation and are reported in Table 3-7.

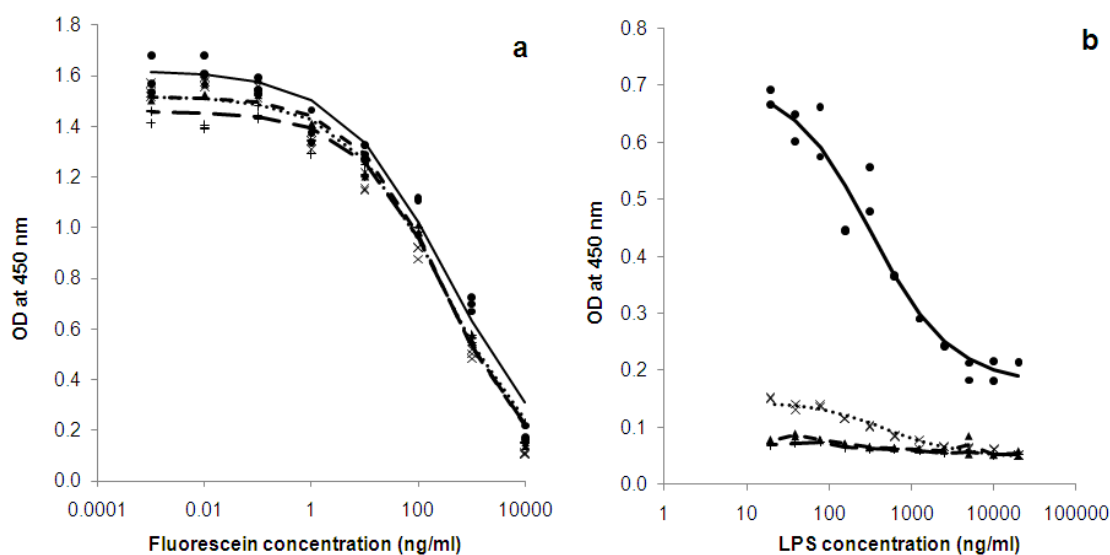


Figure 3-9: (a) fluorescein and (b) LPS LMC format inhibition ELISAs in water and alternative LMC extraction solvents

(●) water, (X) 5 % DMSO 2.5 g/L P80, (▲) 30 % acetone 2.5 g/L P80 and (+) 30 % acetonitrile 2.5 g/L P80. Each data point represents a single replicate. Immunoassay curves were plotted by fitting mean signal to a four parameter binding equation, or by linear interpolation of the mean OD where the data did not fit a four parameter binding equation (solid line) 10 % methanol, (dotted line) 5 % DMSO 2.5 g/L P80, (short dashed line) 30 % acetone 2.5g/L P80, (long dashed line) 30 % acetonitrile 2.5 g/L P80.

Immunoassay sensitivity was affected by the alternative extraction solvents in the majority of cases tested, but for several immunoassays it was not possible to calculate IC_{50} values because binding signal was reduced to a level where it was not distinguishable from background noise in the extraction solvent under test.

Table 3-7: IC₅₀ values for Inhibition ELISA assays run in alternative LMC extraction solvents. IC₅₀ values were calculated by fitting data to a four parameter IC₅₀ equation. Sum of squares due to regression were less than 0.05 for all data fitting

	<i>IC₅₀ (ng/ml)</i>			
	<i>Water (* 10 % methanol)</i>	<i>5 % DMSO 2.5 g/L P80</i>	<i>30 % acetone 2.5 g/L P80</i>	<i>30 % MeCN 2.5 g/L P80</i>
Atrazine	*5.6	1.32	106.8	Not calculated
B[a]P	*3.2	1.17	50.8	Not calculated
cAMP	331.4	229.3	2000	Not calculated
Fluorescein	352.5	300.0	300.0	347.9
GroEL	601.9	295.1	178.2	1400.0
LPS	319.7	499.9	Not calculated	Not calculated
LTA	2.8	2.5	Not calculated	Not calculated

Target molecules for each immunoassay were successfully detected in the proposed alternative extraction solvents, with the exception of instances where the immunoassays were rendered non-functional due to large reductions in immunoassay binding signal. Immunoassay sensitivity varied with the alternative extraction solvent. Since the LMC instrument aims to determine whether classes of organic molecules are present above a given detection threshold, rather than to quantify specific molecules small changes in immunoassay sensitivity are unlikely to have a significant impact on the function of the LMC instrument. However orders of magnitude changes in sensitivity are likely to be more significant.

The only orders of magnitude changes in sensitivity induced in the alternative extraction solvents were observed for the atrazine, benzo[a]pyrene and cAMP immunoassays in 30/70 % (v/v) acetone water with 2.5 g/L P80 where sensitivity was reduced. However it is also important to consider that instrument detection limit is also dependent on target molecule extraction efficiency, which has not been tested within this work. The ideal extraction solvent for the LMC will efficiently extract target molecules and also give high immunoassay sensitivity, however antibody-target molecule binding affinity is improved in solvents in which the target molecule is less soluble (Penalva *et al*, 1999; Horáček and Skládal 2000; Stöcklein *et al*, 2000), therefore a highly efficient extraction solvent is unlikely to produce the optimum immunoassay sensitivity.

3.4.3.3 Summary and future work

The specific objective of this section of work was to explore alternative extraction solvents for compatibility with LMC format immunoassays, in-case some aspect of the LMC instrument proves to be incompatible with the baseline LMC extraction solvent in the future. This objective was addressed by testing four alternative water miscible organic solvent P80 solutions, whose formulations were suggested by the team at Imperial College developing

the LMC extraction solvent, for compatibility with LMC format immunoassays. Changes in binding signal and sensitivity were observed across the range of solvents tested, but a diverse range of immunoassays did function in the alternative extraction solvents. The suitability of these solvents in terms of extraction efficiency of relevant targets was not assessed in the work reported here. A number of back-up solvents have therefore been identified that could be explored further should the need arise. Once again no single solvent was identified that is optimum for immunoassay function and some degree of compromise of immunoassay function would be required. Should the baseline LMC extraction solvent prove unsuitable for use in the flight instrument, a significant amount of work would be required to characterise the performance of each immunoassay in the LMC extraction solvent and also to assess extraction efficiency for a range of target molecules.

3.5 Conclusions

The LMC instrument will directly analyse extracts from martian samples for the presence of a wide range of target molecules including markers of extant and extinct life, meteoritic in-fall and terrestrial contamination. The need to extract a wide range of organic molecules with varying polarities and critically the requirement that the solvent used for the extraction be compatible with multiplexed immunoassays led to the proposal of MeOH P80 as a suitable extraction solvent for the LMC. Built in to the LMC was the option to concentrate the extract up to thirty fold by evaporation under reduced pressure. The objective of the work reported within this chapter was to determine whether immunoassays are compatible with extraction solvents suitable for use in the LMC instrument.

Given that the LMC immunoassay aims to be capable of extracting and detecting up to 25 target molecules with differing properties, it is highly unlikely that an extraction solvent exists that would be optimal in terms of extraction and detection of every target molecule. However this study concludes that the proposed LMC extraction solvent, MeOH P80, is an acceptable compromise in terms of performance of a wide range of LMC relevant immunoassays and its efficiency as an extraction solvent has been confirmed elsewhere and is beyond the scope of this thesis. In addition to this, extract that has been concentrated up to 30 fold by evaporation is also acceptable in terms of immunoassay performance and concentration by evaporation could be used to increase instrument sensitivity, although this feature has subsequently been removed from the flight instrument design. Finally there are a number of other organic solvent/surfactant based extraction solvents that have the potential to be compatible with LMC format immunoassays should they be required in the future.

This study therefore supports the use of MeOH P80 as the extraction solvent for the LMC. In future work new assays should be optimised directly in MeOH P80. This will ensure optimum assay performance in the extraction solvent and allow early identification of any antibodies/assays which are unsuitable for inclusion in the flight instrument due to poor compatibility with the extraction solvent.

Although there are many examples in the literature where small numbers of immunoassays against one, or several closely related target molecules have been tested for performance in multiple organic solvents *e.g.* (Melnikova *et al*, 2000; Matschulat *et al*, 2005; Rehan and Younus 2006; Zeng *et al*, 2007) as far as the author is aware there are no examples in the literature of studies where the performance of a diverse range of immunoassays, against different target molecule types have been examined in this way. This work appears to represent the best example to date of a systematic study of the effect of solvents on a diverse range of immunoassays

4 Influence of Liquid Extracts from Martian Samples on LMC Format Immunoassays

4.1 Introduction and context

In the LMC instrument samples of martian rock or regolith will be extracted via liquid extraction with ultrasonication and the resulting liquid extract, after particle filtration, will be analysed *via* a multiplexed microarray immunoassay. The consequence of using liquid extraction is that in addition to target molecules, other soluble components of the sample matrix may also be extracted and carried through into the LMC immunoassay. Hence there is a risk that extracted components of the sample matrix may compromise immunoassay performance. Since the LMC immunoassay is in an inhibition format there is a risk that soluble components of the sample matrix might produce non-specific inhibition of signal which could incorrectly be interpreted as positive detection of a target molecule. Therefore there is a need to investigate the likely interaction of the martian sample matrix with LMC format immunoassays.

One of the difficulties associated with attempting to investigate the impact of the martian sample matrix on the LMC immunoassay is that the only martian samples on Earth are meteorites, which are available in small quantities and are unlikely to well represent the current martian environment (McSween *et al*, 2009). However there is a large body of data regarding the likely composition of the martian surface which can be used to identify the types of sample matrix likely to be encountered by the LMC. Some preliminary work, carried out early in the development of the LMC, tested the performance of a single ELISA format immunoassay in the presence of a limited selection of Mars relevant sample matrix components (Wilson 2007). In the period since this work was carried out the LMC instrument design has evolved, more LMC relevant immunoassays have been developed and additional data has been reported from the 2008 Phoenix Lander mission, including the detection of perchlorate salts at high latitudes on Mars (Hecht *et al*, 2009; Kounaves *et al*, 2010). The discovery of perchlorate salts on Mars has implications for the detection of organic molecules on Mars by the LMC as well as by other non-immunoassay based instruments due to the ability of perchlorates to degrade organic molecules under high temperature conditions; this is discussed further in the literature review contained within this chapter.

Therefore the objective of the work in this chapter was to investigate the likely impact of the martian sample matrix on the LMC format immunoassay in the context of currently available data regarding Mars and current LMC instrument design, hence identifying potential risks for

the operation of the LMC instrument and facilitating future investigation of mitigating strategies for any problematic sample matrix components/types. More broadly this chapter also has relevance to the development of other immunoassay based instruments for Mars missions and for environmental analysis in extreme 'Mars-like' environments on Earth.

4.2 Chapter objectives

The objective of the work reported in this chapter was to investigate the potential interaction of martian sample matrix with LMC immunoassays, in order to identify risks for operation of the LMC instrument with martian samples and to enable the development of mitigation strategies. In order to meet this goal the following specific objectives were set

- 1) To test performance of a number of LMC relevant immunoassays in the presence of likely individual Mars sample matrix extractable components identified from known Mars geology/geochemistry.
- 2) To test performance of a number of LMC relevant immunoassays with liquid extracts from Mars analogue samples, in order to mimic the complex 'real' samples that may be encountered on Mars
- 3) To demonstrate extraction and detection of organic molecules in the presence of perchlorate salts using an LMC relevant immunoassay to address immediate concerns raised by recent Mars findings

It is important to note that in addition to influencing the LMC immunoassay the martian sample matrix may also have an impact on other elements of the LMC, for example the extraction efficiency of the MeOH P80 extraction solution and the potential for the evolution of heat during addition of the extraction solvent to the martian sample. These factors are not considered within the context of the work reported in this chapter but are part of ongoing work elsewhere within the LMC consortium. Additionally in Chapter 6 of this thesis the interactions of martian sample matrix components with the optical and microfluidic aspects of the flight format LMC are considered.

4.3 Literature review

4.3.1 The effect of sample matrix on immunoassays

Since immunoassays have not previously been performed with martian samples, there is no information in the literature specifically regarding the impact of martian sample matrix components on immunoassays, although there are examples of immunoassay analyses of martian analogue samples such as the iron rich acidic waters of the Rio Tinto in Spain (Fernandez-Calvo *et al*, 2006; Rivas *et al*, 2008). Aside from these limited examples, the most relevant information contained within the literature concerns the impact of terrestrial soil matrixes on immunoassays for analysis of environmental contaminants such as pesticides, and while a martian sample is likely to differ significantly from a terrestrial sample, this body of data provides a useful basis for identifying sample matrix components/types that affect immunoassays.

It is common to find that an immunoassay which functions well with analytical standards will function less well when tested with the more complex matrixes found in real samples, and this is one factor which has limited the more widespread use of immunoassays for environmental monitoring applications despite advantages such as low cost and the potential for rapid *in-situ* testing of samples (Glass *et al*, 2004; González-Martínez *et al*, 2007). Matrix effects in immunoassays have been shown to result in signal loss (Kröger *et al*, 1998), underestimation of target molecule concentrations (Székács and Hammock 1995), overestimation of target molecule concentration due to cross reactivity with structurally related molecules (Kröger *et al*, 1998), reduced sensitivity (Bjarnason *et al*, 2001; Fernandez-Calvo *et al*, 2006), and variation in measured target molecule concentration compared to other analysis methods such as HPLC (Mouvet *et al*, 1997; Gauger *et al*, 2001). These types of effects have been attributed to factors such as pH, ionic strength, the absorption of antibodies to insoluble material from the sample matrix, the presence of chemical compounds in the sample matrix which adversely and non-specifically affect the affinity of an antibody for an antigen (Krotzky and Zeeh 1995), the organic content of the sample (*e.g.* interference by humic acids) (Kröger *et al*, 1998), and sample interference with immunoassay detection mechanisms (*e.g.* fluorescent labels, enzyme substrate reaction etc.) (Bjarnason *et al*, 2001).

Therefore, considering the known effects of terrestrial soil matrixes on immunoassays, the properties of a martian sample that are most likely to affect immunoassay performance when analysing a liquid extract include soluble salts leading to altered ionic strength, the presence

of insoluble sub-0.45 μm particulates - *i.e.* those that will not be removed by commonly available sample extraction filters, the presence of components which form acidic or basic solutions, and the presence of components that interfere with the assay detection system *e.g.* fluorescence quenchers. The organic content of the martian sample is unlikely to be a significant concern given that the concentration of organics on Mars is anticipated to be low. Cross reactivity with related molecules is desirable for the LMC since the instrument aims to detect related classes of molecules, rather than being limited to the detection of specific molecules in order to account for likely variations between Earth life and any martian life. However cross reactivity with unrelated molecules, or molecules which are not markers of past or present biology is undesirable.

A number of strategies have been developed in order to avoid or reduce the difficulties associated with sample matrix effects in analysis of environmental samples by immunoassay. These strategies include sample dilution with water or buffer prior to analysis and solid phase extraction (Mouvet *et al*, 1997; Hennion and Barcelo 1998; Kröger *et al*, 1998; Rigou *et al*, 2004). If target molecules are present on Mars, they may only be present at very low levels therefore dilution of the sample is undesirable and solid phase extraction is not suitable because of the diverse range of targets that the LMC aims to detect, *i.e.* multiple solid phases would be required for extraction of the range of chemically diverse LMC target molecules. Due to the mass and size constraints of the LMC and the need for autonomous operation, sample processing must be kept to a minimum: However limited measures to control/reduce the impact of sample matrix mediated effects have been incorporated into the current (2011) design of the LMC *e.g.* to remove particulates liquid extracts will be filtered through a 0.45 μm filter prior to analysis and buffer salts are included in the freeze drying cocktail that is freeze dried with the antibodies (Section 2.2.2), offering some level of control over pH; but other issues relating to the Mars sample matrix have not yet been considered in detail. For example the LMC instrument uses a fluorescent readout therefore the presence of fluorescence quenchers in the martian sample may be detrimental to the immunoassay.

In the field of environmental monitoring, changes in immunoassay performance such as those described here are considered significant due to the level of precision required in order to meet with legislative requirements (Krotzky and Zeeh 1995). However the LMC is intended to give an indication of whether classes of target molecules are present on Mars above a certain detection threshold and some level of compromise regarding the performance of the instrument in the martian sample matrix is acceptable and inevitable, given the constraints on sample processing. It is essential that any ambiguity over immunoassay results which appear to indicate the presence of a target molecule, but could

also be due to non-specific interference by components of the sample matrix, is avoided. Therefore it is important that the potential effects of sample matrix are well characterised. One of the advantages of the multiplexed microarray format of the LMC immunoassay is that control assays can be run directly alongside target molecule detection assays in the same sample matrix (Fernandez-Calvo *et al*, 2006) hence reducing the risk of “false positive” results.

4.3.2 Mars sample matrix and likely interactions with the LMC immunoassay

Within this section of the literature review, general information regarding the likely geology of martian samples is considered and Mars relevant analogue sites on Earth are identified as potential sources for analogue samples within this study (Section 4.3.2.1). The review then goes on to specifically consider individual components/properties of Mars samples that are likely to interact with immunoassays (Sections 4.3.2.2- 4.3.2.7).

4.3.2.1 Mars geology/geochemistry

Since 1965 more than fifteen spacecraft have operated successfully on or near Mars (Jakosky *et al*, 2007) gathering data regarding the geology of Mars via remote sensing and *in-situ* measurements made at a number of locations on the martian surface. However it should be noted that all *in-situ* analyses of martian rocks and regolith to date have been restricted to samples collected from the martian surface and immediate subsurface (maximum depth of a few centimeters), whereas the ExoMars rover will collect samples from a maximum depth of 2 meters below the martian surface.

Data from instrumentation onboard orbitors, landers and rovers has shown that the martian crust is dominated by basaltic rock (McSween *et al*, 2009; Rossi and Gasselt 2010) with localised concentrations of phyllosilicates, hydrated sulphates (including gypsum, jarosite and kieserite), carbonates and ferric oxides including haematite (Gendrin *et al*, 2005; Langevin *et al*, 2005; Poulet *et al*, 2005; Bibring *et al*, 2006; Boynton *et al*, 2009; Palomba *et al*, 2009). Above the martian crust is a layer of regolith – dust and soils formed by physical weathering of martian rocks. The composition of martian regolith at several different sites and has been shown to be chemically similar, indicating that martian regolith is homogeneous on a global scale and is likely mixed and distributed by impact events and storms (Rieder *et al*, 1997; Wanke *et al*, 2001; Yen *et al*, 2005).

Data from the OMEGA imaging spectrometer on Mars Express has suggested that Mars's geological and climatic history can be split into three distinct eras: (Bibring *et al*, 2006). During Mars's early 'phyllocial' era Mars had an active hydrological system where rocks such as phyllosilicates were formed in water rich alkaline environments (Poulet *et al*, 2005), this was followed by the 'theikian' era of extensive volcanism leading to the release of sulphur dioxide creating an acidic environment in which sulphates such as jarosite and kieserite were formed and finally the 'siderikian' era dominated by the non-aqueous oxidation of iron bearing rocks and minerals.

The landing site for the 2018 NASA/ESA ExoMars Rover mission has not yet been selected, but the criteria used for selection will be similar to those for NASA's 2011 Mars Science Laboratory Mission which were based on a combination of engineering requirements, *e.g.* landing site slope, elevation, roughness *etc.*, and science requirements, *e.g.* indication of past/present habitability and potential for preservation of markers of extinct life (Grant *et al*, 2011). Areas on Mars with phyllosilicates are likely to be high priority targets due to the evidence of long standing bodies of water and the potential preservation properties of clay minerals (Orofino *et al*, 2010), although recent analysis has suggested that these clays may have been formed by hydrothermal ground water circulation and that water was only ever transient on the martian surface (Ehlmann *et al*, 2011b). Data from OMEGA has revealed a large diversity in the composition of the martian surface at small spatial scales (< 1km) (Bibring *et al*, 2005) therefore there are likely to be a number of different geologies within reach of a single rover landing site.

There are a number of sites on Earth with geological or environmental conditions similar to those on Mars which have been used as Mars analogues for scientific studies and for field testing of planetary exploration instruments. These environments include (but are not limited to) Iceland with its active volcanism, poorly formed soils, basaltic bedrock and sparse surface water, the cold and arid Antarctic dry valleys (Léveillé 2009), the acidic iron and sulphate rich Rio Tinto basin in Spain (González-Toril *et al*, 2003), the 24 km Haughton Impact Crater on Devon Island, Northern Canada, with its impact induced hydrothermal deposits (Lindgren *et al*, 2009) and the Atacama desert in Chile with its extreme aridity and oxidising soils (for a comprehensive review please see Léviellé (2009)). Access to samples collected from these analogue sites will allow assessment of LMC immunoassay performance using complex real samples with Mars relevant geologies.

4.3.2.2 Ionic strength of the martian sample matrix

The salt content of martian samples is relevant for the LMC because immunoassays are sensitive to ionic strength, although only salts that are soluble in the LMC extraction solvent will be carried through into the immunoassay. Martian regolith has relatively high salt content (8-25 wt %) thought to be composed primarily of a mixture of sulphates (magnesium, calcium and iron), chlorides (magnesium or sodium chlorides) and carbonates (Toulmin *et al*, 1977; Clark and Hart 1981; Wanke *et al*, 2001; Wang *et al*, 2006; Yen *et al*, 2006) although the solution chemistry of these salts in martian samples has not been extensively studied. Experiments carried out at high latitudes by the Wet Chemistry Laboratory (WCL) on the 2008 NASA Phoenix Lander, to investigate the solution chemistry of martian regolith, showed that 1:25 (v/v) extraction of regolith samples with water produced solutions which contained approximately 10 mM dissolved salts dominated by Na^+ , Mg^{2+} and ClO_4^- , with smaller contributions from Ca^{2+} , K^+ , Cl^- , CO_3H^- and possibly SO_4^{2-} (Hecht *et al*, 2009).

4.3.2.3 pH of the martian sample matrix

In addition to measuring the concentration of soluble ions in martian regolith, WCL on Phoenix also performed measurements of the pH of water soluble components of martian regolith. The pH of a 1:25 (v/v) mixture of martian regolith and water was 7.7 ± 0.5 (Hecht *et al*, 2009) which is within the range at which immunoassays normally function. However some of the salts that have been observed on Mars, such as iron (III) sulphates, form acidic solutions and it is likely that the results from the WCL may not be representative of all locations on the martian surface. The pH of the LMC immunoassay is controlled through the inclusion of buffer salts in the freeze drying cocktail that is freeze dried into the glass fibre pads (please see Section 2.2.2), therefore the addition of small quantities of acidic or basic components will not result in large changes in pH. However highly acidic or basic liquid extracts are likely to have an impact on immunoassay performance. In addition to this the emission spectra of fluorophores can be affected by changes in pH, although the Alexa Fluor® dyes, including the selected dye for the LMC, do show better tolerance to changes in pH than many other fluorophores (Johnson and Spence 2011).

4.3.2.4 Fluorescence quenchers in the martian sample matrix

Fluorescence quenchers are species that are capable of accepting energy from excited state fluorophores, reducing the emission of photons from excited state fluorophores hence reducing fluorescence (Atkins and de-Paula 2002). Quenching of fluorescence can occur via three main mechanisms, collisional deactivation; energy transfer; and electron transfer. Any

soluble components of the martian sample capable of accepting energy from excited state AF633 molecules by any of the three mechanisms described above may reduce the fluorescent signal produced by the LMC instruments. Obvious candidates for fluorescent quenching in the martian sample include iron (III) ions, which are known to act as fluorescence quenchers (Lytton *et al*, 1992).

4.3.2.5 Oxidising agents in the martian sample matrix

The 1970s Viking Landers carried a set of life detection experiments designed to look for evidence of metabolism in martian samples but results of the experiments were inconsistent with a biological basis and instead indicated that the martian regolith was chemically reactive, containing one or more oxidising agents capable of degrading organic molecules (Klein 1978; Zent and McKay 1994; Quinn and Zent 1996; Tsapin *et al*, 2000; Hurowitz *et al*, 2007; Davila *et al*, 2008). This conclusion was supported by the failure of the Viking GC-MS, and the more recent Thermal and Evolved Gas Analyser (TEGA) instrument onboard the Phoenix Lander, to detect any organic molecules in martian samples, despite their known continuing delivery to the martian surface by meteorites independently of any past or present biology on Mars (ten-Kate 2010). Many species were proposed as candidates for the martian oxidant including hydrogen peroxide (Levin and Straat 1977), hydrogen peroxide complexed with surface minerals (Quinn and Zent 1996) and iron (VI) (Tsapin *et al*, 2000). The presence of oxidising agents on Mars is of significance for the LMC, not only because they may interact with immunoassay reagents, but also because the presence of oxidising agents has implications for the survival of organic molecules, both on the martian surface, and during any attempts to extract target molecules from martian rocks/regolith.

In 2008 The Wet Chemistry Laboratory (WCL) on NASA's Phoenix Lander detected the presence of 0.4-0.6 wt % perchlorate in samples of martian regolith (Hecht *et al*, 2009). Perchlorate salts are oxidizing, but metastable, unless initiated by mechanical action, heating, or electric shock (Shriver and Atkins 1999), and therefore they are not expected to destroy organic molecules under martian conditions. Navarro-González *et al* (2010), however, demonstrated that when organic carbon containing Mars like soils from the Atacama Desert were mixed with one weight percent magnesium perchlorate and heated, the organics present were decomposed. All previous attempts to detect organic molecules on Mars, for example by the GC-MS instrument on the Viking Landers and by Phoenix's Thermal and Evolved Gas Analyser (TEGA), have relied on pyrolysis to volatilize organics present in collected samples. In the Viking experiments, samples were heated to 200, 350, and 500°C (Anderson *et al*, 1972), and TEGA progressively heated samples up to a

temperature of 1000°C (Hoffman *et al*, 2008). Heating to these temperatures in the presence of perchlorates may have destroyed the very molecules that the Viking GC-MS and TEGA were trying to detect.

The LMC's use of a low temperature (>0°C, <<+100°C, nominally +20°C) liquid extraction may offer an advantage over other instruments in the analysis of perchlorate containing samples because perchlorate salts are not expected to act as oxidants under these conditions. MOMA, the other instrument on the ExoMars rover payload capable of detection of trace organics, is a compact, low power ion trap mass spectrometer with the capability to extract organics from martian rock and regolith samples by two different methods: pyrolysis and laser desorption (Evans-Nguyen *et al*, 2008). Extraction by pyrolysis will involve evaporation of volatile organics in the martian sample by heating to high temperatures followed by injection into a GC column with an electron ionization source. Exposure of the martian sample to high temperatures could result in perchlorate mediated degradation, although recently it has been proposed to integrate the use of derivitisation reagents to increase the volatility of some organics in order to reduce the required temperature (Eigenbrode *et al*, 2011). In extraction by laser desorption, the regolith sample will be pulsed with a high power UV laser to liberate ions (ESA 2011a); it is unknown whether this technique will be affected by the presence of perchlorates.

The LMC low temperature extraction method may be beneficial in terms of preventing perchlorate mediated degradation of organic molecules, however, perchlorates are highly water soluble and therefore expected to dissolve in the LMC extraction solvent and be carried through into the LMC analysis system where the perchlorates could interact with immunoassay reagents via changes in parameters such as pH and ionic strength. Within the immunoassay literature, there appear to be no examples that demonstrate detection of small organic molecules by immunoassay in the presence of perchlorate salts, although there is one recently published study that describes no significant effect of 50 mM perchlorate on the antibody-antigen reaction by sandwich-type antibody microarray fluorescent immunoassays (Parro *et al*, 2011).

4.3.2.6 Particle size of the martian sample matrix

The immunoassay literature has demonstrated that the presence of particulates in sample matrixes can affect immunoassay performance due to absorption of antibodies. The drill on the ExoMars rover will collect a core of rock or regolith (approx 1 cm diameter x 3 cm length) which will then be crushed by a crushing station and distributed to the individual instruments onboard the rover (ESA 2011c). The size distribution of the crushed sample is likely to

depend heavily on the sample matrix type. In the LMC instrument the sample will be subject to liquid extraction and then filtered through a 0.45 µm filter. Therefore sub-0.45 µm particulates may pass into the microfluidic channel where they could cause blockages or interfere with the immunoassay. In addition to this there is also a risk that precipitation of solutes may occur within the microfluidic channel, post filtration. For example work carried out early in LMC development showed that a red precipitate formed when iron (II) sulphate was dissolved in HEPES buffer and allowed to incubate for an hour (Wilson 2007).

4.3.2.7 Other components/properties of the martian sample matrix that may interact with immunoassays

In addition to the components whose presence on Mars can be anticipated due to data from previous missions, there is an unavoidable risk that martian samples collected by the ExoMars rover may contain unexpected/unknown components which may interact with immunoassays, particularly given the lack of analysis performed previously with samples collected from below the martian surface. However there is little that can be done in advance of the mission to prepare for this eventuality, apart from the inclusion of positive control immunoassays to enable discrimination between non-specific matrix mediated signal reduction and specific inhibition due to the presence of target molecules.

4.3.3 Literature review conclusions

Previous studies have demonstrated that immunoassay performance is affected when immunoassays are performed with real samples compared to with buffers and that changes are mediated by factors such as the pH and ionic strength of the sample matrix. Data regarding the geology and geochemistry of Mars indicates that there are a wide range of geochemistries/geochemical environments which may be encountered by the LMC, depending on the selected landing site. Review of the available data regarding Mars and regarding sample matrix effects in immunoassays used for terrestrial environmental analysis allows the prediction of Mars sample components that are likely to interact with immunoassays *i.e.* soluble salts leading to altered ionic strength, the presence of species that form acidic or basic solutions, and oxidising agents. Therefore this allows the proposal of experiments to investigate the influence of the Mars sample matrix on immunoassays in order to determine which sample types might be problematic for the LMC and is the subject of the following section.

4.4 Structure of the work

The work in this chapter has three specific objectives; these three objectives have been addressed by three discrete pieces of work which are reported as three separate studies within this chapter (Sections 4.5, 4.6 and 4.7). The first study describes work carried out to investigate the impact of likely components of the Mars sample matrix on immunoassays. As a fundamental part of this study the effects of sample matrix components on the HRP enzyme label used in ELISA format, and the fluorescent AF633 label used in microarray format are also investigated independently of the immunoassays. The second study investigates the impact of liquid extracts from Mars analogue samples on immunoassays to mimic the complex nature of real martian samples. The third study extends work carried out to investigate the impact of perchlorate salts on immunoassays by addressing the combined impact of perchlorate salts on LMC relevant extraction and detection. Within the following three sections (Sections 4.5 – 4.7), the experimental design for each study and materials and methods supplementary to those in Chapter 2 are described, and results of each study are reported and discussed. The final section of this chapter (Section 4.8) summarises the work and makes recommendations based on the findings of the three studies.

4.5 Study 1: Testing the impact of individual likely Mars sample matrix components on LMC relevant immunoassays

Sample matrix components present in martian rocks and regolith and that are soluble in the LMC extraction solvent may interact with immunoassays, influencing instrument performance. The literature review in Section 4.3 of this thesis, revealed that martian regolith has relatively high (8-25 %) salt content (Toulmin *et al*, 1977; Clark and Hart 1981; Wanke *et al*, 2001; Wang *et al*, 2006; Yen *et al*, 2006) and contains one or more oxidising agents. This objective of this study is to test the compatibility of likely Mars sample matrix components, such as salts and oxidising agents, with immunoassays in LMC relevant formats in order to:

- (i) Determine whether the LMC is likely to function with martian samples
- (ii) Identify Mars relevant sample matrix components that are incompatible with immunoassays
- (iii) Identify development opportunities to improve LMC performance in the presence of likely Mars sample matrix components

4.5.1 Supplementary experimental design

The design adopted for this study aligns with the common experimental design described in Chapter 2 of this thesis: The set of model immunoassays described in Chapter 2, were used to assess the likely performance of LMC immunoassays with Mars relevant sample components. Note that within this study only four of the seven model immunoassays described in Chapter 2 were used (atrazine; benzo[a]pyrene, cAMP and fluorescein). The GroEL, LTA and LPS model immunoassays were excluded from this study, mainly due to time constraints, but their exclusion was also influenced by performance of the model immunoassays in the proposed LMC extraction solvent and also due to some of the antibodies being unavailable from the manufacturer at the time of the work.

Initial assessment of immunoassay performance was carried out using the LMC representative ELISA format (please see Section 2.2.2) to assess the impact of a diverse range of Mars relevant sample matrix components on immunoassay binding signal, at a variety of concentrations. Further studies were then carried out in pooled microarray format (please see Section 2.2.3) to confirm whether behaviour in the more flight representative microarray format was similar to that observed in ELISA format. Finally an extended study of

immunoassay sensitivity was carried out, on a limited set of sample matrix components in ELISA format.

4.5.1.1 Selection of likely Mars sample matrix components

There are many potential geologies/geochemistries, and hence sample matrix components, that may be encountered on Mars, depending on the landing site selected for the ExoMars rover. Within this study a broad range of soluble Mars relevant sample matrix components, including acidic, basic, neutral and iron (II) and (III) containing Mars relevant salts as well as perchlorate and hydrogen peroxide oxidising agents (see Table 4-1 for complete list) were tested. Components such as iron oxides, which have been reported on Mars (Bibring *et al*, 2006) but are insoluble in aqueous solutions, were not tested as individual sample matrix components, but were tested indirectly as components of some of the analogue samples which were extracted to produce liquid extracts for the second study in this chapter of work (please see Section 4.6).

Table 4-1: Mars relevant sample matrix components tested for impact on LMC relevant immunoassays

Sample matrix component	Molecular weight (g mol ⁻¹)	Ionic strength of 100 mM solution (mol dm ⁻³)
Magnesium sulphate	120.4	0.4
Sodium sulphate	142.0	0.3
Sodium chloride	58.4	0.1
Sodium carbonate	105.9	0.3
Iron (III) chloride	162.2	0.6
Magnesium perchlorate	223.2	0.3
Calcium perchlorate	237.9	0.3
Iron (II) perchlorate	254.8	0.3
Iron (III) perchlorate	354.2	0.6
Potassium chloride	74.5	0.1
Iron (II) sulphate	151.9	0.4
Iron (III) sulphate	399.9	1.5
Hydrogen peroxide	34.0	N/A

4.5.1.2 Concentration of sample matrix components tested

Although there is data concerning the abundances of various components, including salts, in martian regolith, there is limited information regarding the solution chemistry of the martian regolith. The approach adopted for this study was to test sample matrix components at a range of concentrations to an upper limit in excess of the highest reported concentration on Mars.

Phoenix's WCL made the first measurements of the solution chemistry of martian regolith and found that when 1 cm³ of regolith sampled from Phoenix's landing site was mixed 1:25 with water the resulting solution contained approximately 10mM of dissolved salt, which,

assuming a regolith density of 1g/cm^3 equates to 0.25 mmol of soluble salt in the sample (Hecht *et al*, 2009). Taking the same sample and performing an LMC relevant extraction with 3 ml of solvent (assuming a regolith density of 1g/cm^3) would produce a solution with a maximum salt concentration of 83 mM. Mars Exploration Rover (MER) AXPS analyses have been carried out at a number of sites within the Gusev Crater and the wall of the Burrough's trench was found to contain approximately 16 weight percent sulphates, which were mainly magnesium sulphate (Yen *et al*, 2005) but the solution chemistry of these samples was not determined.

Within this study the selected sample matrix components were tested at concentrations of 10 mM, 100 mM and 500 mM. The upper limit for this study (500 mM) was selected by calculation of the maximum salt concentration if 1 g of sample with 25 weight percent kieserite (in excess of the highest salt concentrations detected by MER) was subject to an LMC extraction with 3 ml of solvent, and all of the kieserite was soluble.

4.5.2 Supplementary materials and methods

Note that the majority of materials and methods for the work within this study are the same as those used throughout the other chapters of this thesis and are described within Chapter 2. This section contains supplementary materials and methods specific to the work carried out in this study and where appropriate, cross references the relevant sections of Chapter 2.

4.5.2.1 Preparation of stock solutions of sample matrix components

A 1M stock solution of each sample matrix component was prepared in MeOH P80. Details of each of the sample matrix components used are given in Table 4-2. In the case of components that did not dissolve readily at 1M concentration ultrasonic energy was applied to aid dissolution. Components that would not dissolve at 1M concentration, post ultrasonication, were used as a saturated solution and are indicated with "*" in Table 4-2. Where the hydration state of a sample matrix component was unknown, for example for iron (III) sulphate, calculations were based on a hexahydrated complex.

4.5.2.2 ELISA and microarray immunoassays

Stock solutions of sample matrix components were added into ELISA and pooled microarray format assays, maintaining a final concentration of 20/80 % (v/v) methanol water 1.5 g/L P80 following the procedures described in Section 2.2.2 and Section 2.2.3. Iron (II) sulphate stock solutions were freshly prepared for each immunoassay since oxidation to iron (III)

sulphate was observed (as evidenced by colour change from green to red) upon prolonged storage.

Table 4-2: Hydration state, supplier and product codes of sample matrix components used in ELISA and microarray experiments

Sample matrix component	Hydration state	Supplier	Product code
Magnesium sulphate	.7H ₂ O	VWR, Lutterworth, UK	101514Y
Sodium sulphate*	anhydrous	Sigma-Aldrich, Poole, UK	S67795
Sodium chloride	anhydrous	Fisher Scientific, Loughborough, UK	BP358-1
Sodium carbonate*	anhydrous	Sigma-Aldrich, Poole, UK	S7795
Iron (III) chloride	.6H ₂ O	Sigma-Aldrich, Poole, UK	F-2877
Magnesium perchlorate	.6H ₂ O	Sigma-Aldrich, Poole, UK	309303
Calcium perchlorate	.4H ₂ O	Sigma-Aldrich, Poole, UK	401420
Iron (II) perchlorate	.xH ₂ O	Sigma-Aldrich, Poole, UK	334081
Iron (III) perchlorate	.xH ₂ O	Sigma-Aldrich, Poole, UK	326348
Potassium chloride	anhydrous	Sigma-Aldrich, Poole, UK	P4504
Iron (II) sulphate	.7H ₂ O	Sigma-Aldrich, Poole, UK	21, 542-2
Iron (III) sulphate	.xH ₂ O	Riedel-de-Haën (Sigma-Aldrich), Poole, UK	12357
Hydrogen peroxide	N/A	Sigma-Aldrich, Poole, UK	216763

4.5.3 Results and discussion

4.5.3.1 Initial studies of immunoassay performance in all sample matrix components using ELISA binding signal as performance indicator

Confirmation of impact of Mars relevant sample matrix components on enzyme label

The LMC format ELISA uses enzyme labels to measure the quantity of antibody bound to immobilised target molecule via an enzyme-substrate reaction to generate a coloured product, whereas the flight format microarray immunoassay uses fluorescent labels. Therefore changes in ELISA signal due to altered activity of the enzyme label are not directly relevant to the flight format assay. In order to enable discrimination between changes in ELISA signal due to the enzyme and changes due to altered antibody performance, the signal produced by HRP enzyme after exposure to each sample matrix component was assessed independently of the immunoassays following the method described in Chapter 2 (Section 2.2.2.1). Figure 4-1 shows the normalised background corrected signal generated by immobilised HRP label, upon addition of TMB substrate, following exposure to the full range of Mars relevant sample matrix components.

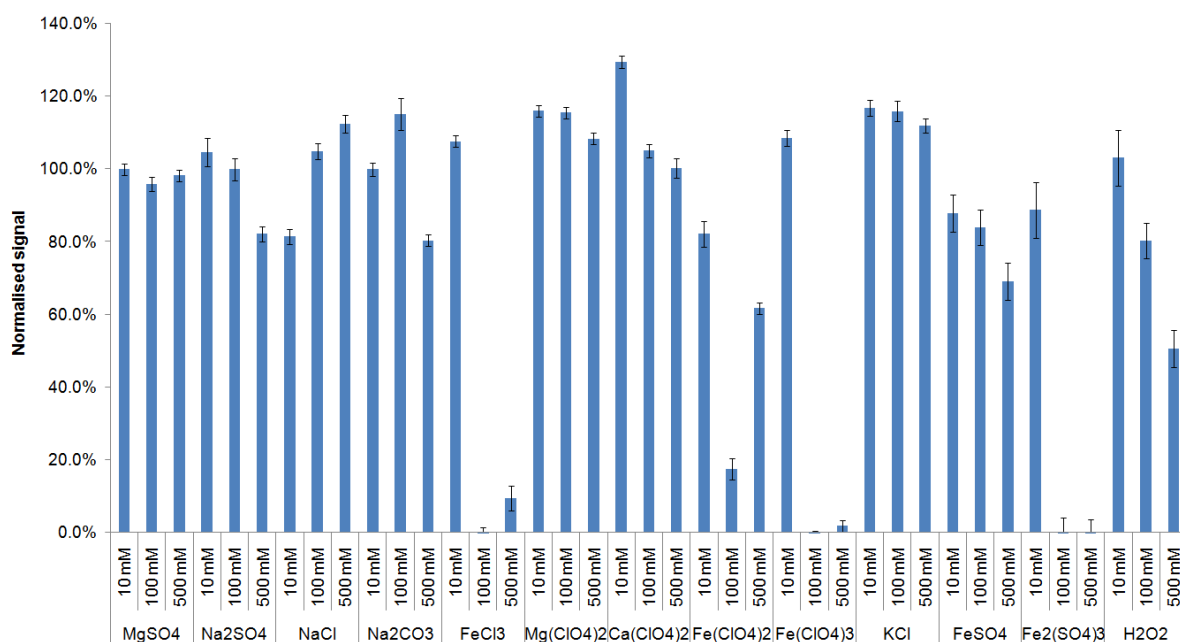


Figure 4-1: Normalised background corrected ELISA signal for immobilised HRP enzyme after incubation in MeOH P80 and freeze drying cocktail containing martian relevant salts at 10 mM, 100 mM and 500 mM concentration.

Background corrected signals normalised against background corrected signal after incubation with MeOH P80 and freeze drying cocktail only. Error bars = \pm standard error, n=2

Although signal produced by immobilised HRP enzyme did vary after exposure to Mars relevant sample matrix components, for the majority of the components tested the variations were small (subjectively assumed as $< \pm 20\%$) (Figure 4-1). Changes in enzyme activity of this magnitude are unlikely to mask any significant changes in the performance of antibodies after exposure to sample matrix components, thus validating the use of the ELISA format to investigate the effect of sample matrix components on LMC immunoassays. However iron ion containing components and hydrogen peroxide, which has previously been shown to deactivate HRP enzyme at high concentrations (Weinryb 1966), did have a significant impact on signal generated by the HRP enzyme particularly at concentrations of 100 mM and above. The consequence of this observation is that in ELISA format changes in binding signal due to altered antibody performance may be difficult to discern from changes in binding signal resulting from altered enzyme activity following exposure to hydrogen peroxide and iron ion containing sample matrix components. Therefore results must be interpreted with care for these components.

Impact of Mars relevant sample matrix components on binding signal in LMC relevant ELISA format

LMC format ELISAs for atrazine, benzo[a]pyrene, cAMP and fluorescein were performed in the presence of 10 mM, 100 mM and 500 mM of each Mars relevant sample matrix component. Binding signals were background corrected and normalised against binding signal with 0 mM sample matrix component to facilitate comparison of the data for multiple immunoassays and plotted in Figure 4-2, Figure 4-3, Figure 4-4 and Figure 4-7. The absolute values of binding signal, and non-specific background signal, are contained within Appendix B1.

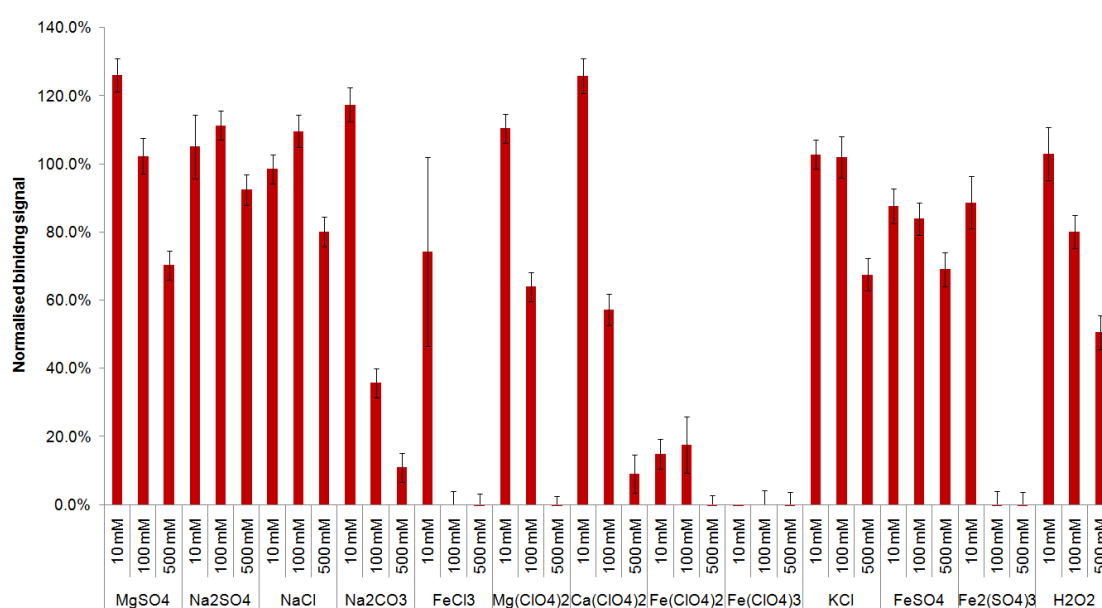


Figure 4-2: Normalised background corrected ELISA binding signal for atrazine immunoassay run in MeOH P80 with martian relevant sample matrix components at concentrations of 10, 100 and 500 mM. Binding signals normalised against binding signal with no sample matrix component. Error bars = \pm standard error, n = 2

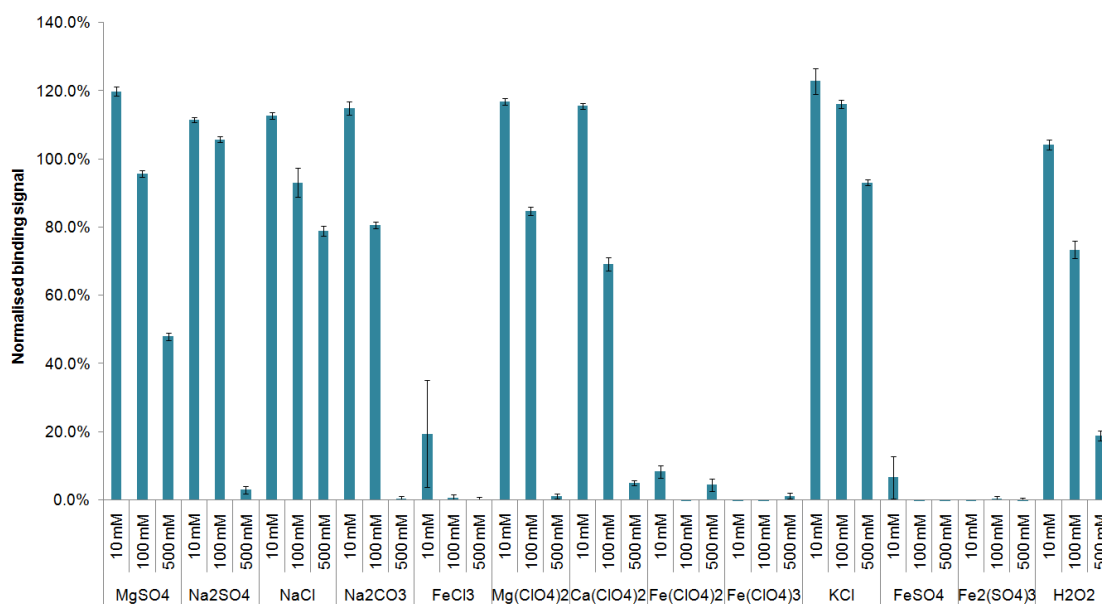


Figure 4-3: Normalised background corrected ELISA binding signal for cAMP immunoassay run in MeOH P80 with martian relevant sample matrix components at concentrations of 10, 100 and 500 mM. Binding signals normalised against binding signal with no sample matrix component. Error bars = \pm standard error, n = 2

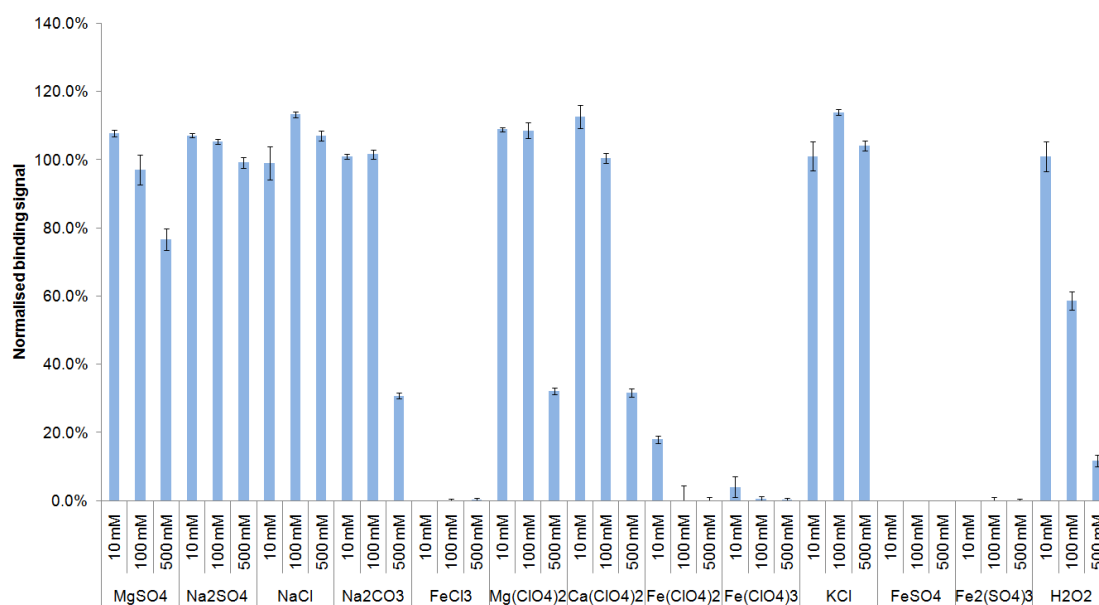


Figure 4-4: Normalised background corrected ELISA binding signal for fluorescein immunoassay run in MeOH P80 with martian relevant sample matrix components at concentrations of 10, 100 and 500 mM. Binding signals normalised against binding signal with no sample matrix component. Error bars = \pm standard error, n = 2

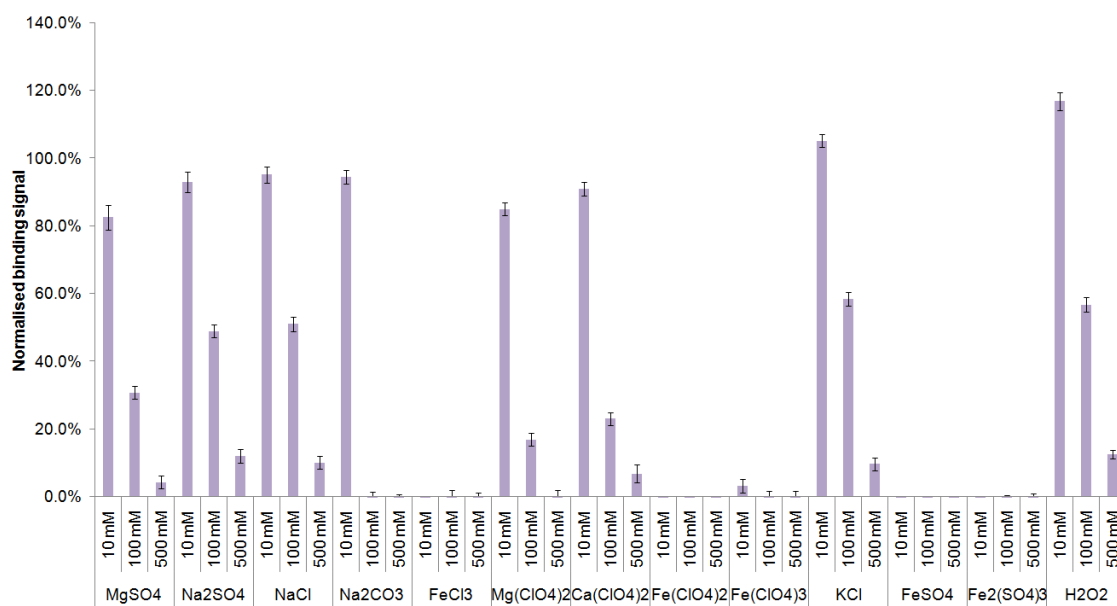


Figure 4-5: Normalised background corrected ELISA binding signal for benzo[a]pyrene immunoassay run in MeOH P80 with martian relevant sample matrix components at concentrations of 10, 100 and 500 mM. Binding signals normalised against binding signal with no sample matrix component. Error bars = \pm standard error, n = 2

ELISA binding signal for the four tested immunoassays varied across the range of sample matrix components tested and in general higher concentrations of sample matrix components had greater impact on ELISA binding signal. The atrazine, cAMP and fluorescein immunoassays showed similar performance to one another with the range of tested sample matrix components, although there were some subtle differences – for example the atrazine immunoassay was less sensitive to the addition of iron (II) sulphate than the cAMP and fluorescein immunoassays. The benzo[a]pyrene immunoassay demonstrated greater loss of binding signal upon addition of salts compared to the other three immunoassays tested, although the general trends observed were the same for all four immunoassays. The implication is that the 22F12 mouse anti benzo[a]pyrene clone is particularly sensitive to the addition of salts (note that the benzo[a]pyrene immunoassay uses the same anti-mouse secondary antibody as the cAMP assay, which was much less sensitive to the addition of salts and therefore the signal loss in the benzo[a]pyrene assay can be attributed to the 22F12 primary antibody). Antibodies, like the 22F12 clone, with this level of sensitivity to the addition of Mars relevant sample matrix components are not suitable for inclusion in the LMC flight instrument.

The sample matrix components tested can be grouped according to the lowest concentration at which they had a significant effect on ELISA binding signal (Table 4-3). Note that most sample matrix components tested had a significant effect on the benzo[a]pyrene assay at

lower concentrations than those stated in the table, but that this assay was felt to be an outlier in terms of its performance.

Table 4-3: Table grouping tested sample matrix components according to the lowest concentration for a given matrix component where significant signal loss (more than 20%) was observed

Concentration of sample matrix component at which ELISA binding signal was reduced by > 20 %			
10 mM	100 mM	500 mM	> 500 mM
Fe(III)Cl ₃ Fe(II)(ClO ₄) ₂ Fe(III) ₂ (ClO ₄) ₆ Fe(II)SO ₄ Fe(III) ₂ (SO ₄) ₃	Ca(ClO ₄) ₂ Mg(ClO ₄) ₂ Na ₂ CO ₃	MgSO ₄ Na ₂ SO ₄ NaCl H ₂ O ₂	KCl

The Mars relevant sample matrix components that reduced signal by a significant amount at 10 mM concentration were all iron ion containing salts. Iron ion containing components also had a significant impact on HRP enzyme activity; therefore care is required in the interpretation of these results. However when the HRP enzyme label was tested independently of the immunoassays (Figure 4-1) iron ion containing salts significantly reduced signal produced by the enzyme at concentrations of 100 mM and above, whereas concentrations of 10 mM significantly reduced ELISA binding signal. In most cases 10 mM iron ions reduced ELISA binding signal to a level where it was not distinguishable above background. This indicates that iron ion containing salts have a significant impact on antibody function, as well as on the enzyme label in LMC format ELISAs. Therefore martian samples with low levels of extractable iron salts may be problematic for the antibody based assays in the LMC.

The mechanism for the loss of binding signal at low concentrations of iron ion containing solutions cannot be wholly attributed to increased ionic strength, because a 10 mM solution of iron (II) sulphate, which produces a significant reduction in binding signal, has the same ionic strength (assuming an ideal solution) as a 10 mM solution of magnesium sulphate (Table 4-1), which does not produce a significant reduction in binding signal. Therefore it is likely that some of the signal loss is caused by a specific property of iron ion containing solutions, one possibility is that the buffering capacity of the LMC freeze drying buffer is insufficient to control the pH of the solution upon addition of iron ions.

Iron (II) ion containing sample matrix components produced an increase in non-specific binding (Appendix B Table B4 and Table B11Table) and the formation of precipitates were observed in ELISA wells containing iron (II) ions. These precipitates may account for the observed high background signals, resulting from non-specific adsorption of labelled antibodies to the particulates, which were then not all removed from the wells by washing.

Perchlorates and sodium carbonate at concentrations of 100 mM and greater had a significant impact on ELISA binding signal and at 500 mM the majority of the immunoassays tested were rendered non-functional. The effect of these components is unlikely to be purely related to ionic strength, for example solutions of magnesium and calcium perchlorate have lower ionic strength than equimolar solutions of magnesium sulphate (Table 4-1) (assuming ideal solutions) but magnesium sulphate did not produce similar reductions in signal. Perchlorate salts and sodium carbonate had little impact on the enzyme label (Figure 4-1); therefore it is likely that the observed signal loss is entirely due to changes in the immunological interactions. If an LMC extraction was performed on samples similar to those detected by Phoenix, which contained 0.4 – 0.6 wt % perchlorates (Hecht *et al*, 2009) the maximum extracted perchlorate concentration fed through into the immunoassay would be 17 mM (assuming extraction of 1 g regolith containing 1 % wt perchlorate with 3 ml of extraction solvent) and the data from this experiment indicates that immunoassays do function in a concentration of up to 100 mM perchlorate salts. It should be noted that calcium perchlorate increased non-specific background signal (Appendix B Table B2) whereas magnesium perchlorate did not (Appendix B Table B1), suggesting that these two compounds, which differ only by cation, are interacting differently with the immunoassays.

The components that had the least effect on the immunoassays were sodium chloride, sodium sulphate, magnesium sulphate, potassium chloride and hydrogen peroxide. For hydrogen peroxide, the changes observed in immunoassay signal were similar in magnitude to the changes observed when the HRP enzyme was tested independently of the immunoassay, suggesting that the majority of the observed signal losses were attributable to exposure of the enzyme to hydrogen peroxide.

In summary, immunoassay binding signal can successfully be generated after exposure to Mars relevant sample matrix components at concentrations of 100 mM, and in some cases after exposure to concentrations of up to 500 mM. However concentrations of 10 mM and above iron ion containing sample matrix components have a particularly detrimental impact on binding signal. Signal loss may be occurring due to changes in antibody performance, caused by increased ionic strength and altered pH, and also due to loss of immobilised target molecules from 96-well plate surfaces. Two aspects, control of pH; and stability of conjugate immobilisation, are further investigated in the following two sections.

Further experiment to investigate buffering capacity of freeze drying buffer

In order to determine whether the buffering capacity in the LMC immunoassay, which is provided by the buffer salts contained within the freeze drying buffer (Section 2.2.2) is sufficient to control immunoassay pH upon addition of Mars relevant sample matrix components, the pH of solutions of freeze drying buffer made up in MeOH P80 was measured after the addition of a range of sample matrix components.

The pH of the solution was unaffected for the majority of sample matrix components tested (Figure 4-6). However a sub-set of the components, including all of the iron-ion containing salts, did produce changes in pH. Solutions of sodium carbonate at concentrations above 10 mM in the LMC solvent and freeze drying buffer were basic, with a 500 mM solution giving a pH of approximately 10, whereas solutions of sample matrix components containing iron ions were acidic.

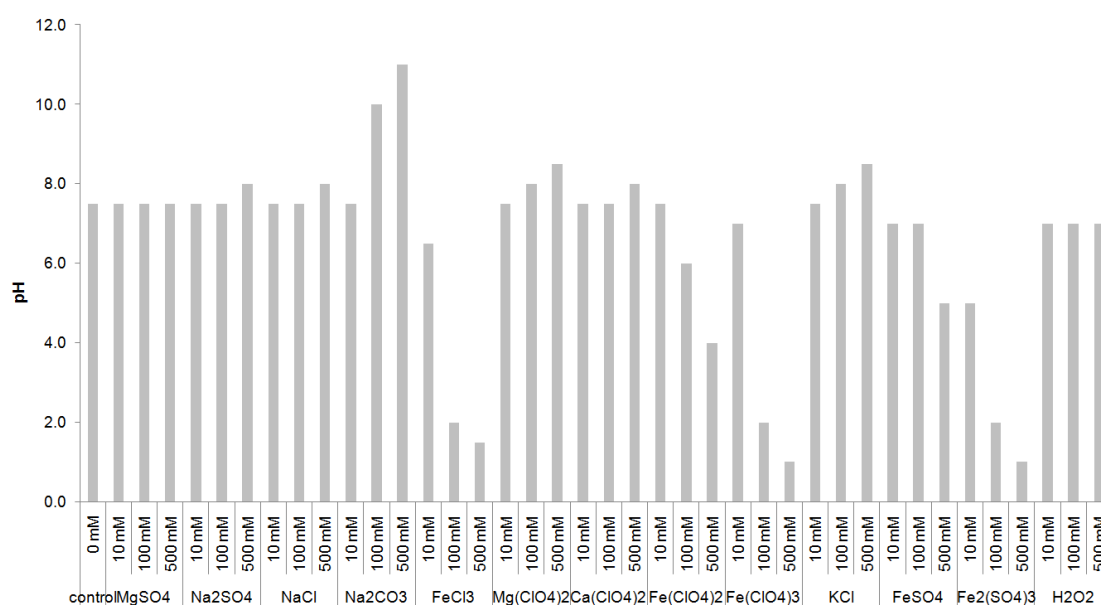


Figure 4-6: pH of sample matrix components diluted in LMC extraction solvent and freeze drying buffer

Iron (III) sulphate produced the largest change in pH with a 10 mM solution giving a pH of 5.0 and a 500 mM solution giving a pH of 1.0. These changes in pH may account for the changes in both enzyme activity and antibody performance in the presence of these sample matrix components and it is possible that immunoassay performance could be improved by increasing the buffering capacity of the freeze drying cocktail.

Further experiment to determine whether sample matrix components effect immobilisation stability or antibody function

In an attempt to better understand the interaction of Mars relevant sample matrix components with immunoassays, specifically to separately test (i) interaction of Mars relevant sample matrix with immobilised target molecules and (ii) interaction of Mars relevant sample matrix with antibodies in solution, an experiment was performed where conjugates and antibodies for two immunoassays (benzo[a]pyrene and cAMP) were separately incubated with a sub-set of Mars relevant salts (sodium chloride, iron (III) chloride, magnesium perchlorate and iron (III) perchlorate). For both immunoassays 96-well plates were coated with target-molecule protein conjugate and then blocked with BSA. Half of the coated and blocked wells were then incubated with buffer and the other half were incubated with 100 mM salt in buffer for one hour. Antibodies were diluted in LMC extraction solvent and freeze drying buffer. Half of the antibody solution was incubated with 100 mM salt, the other half with 0 mM salt. The pre-incubated antibody solutions were then transferred to the coated and blocked ELISA plates such that there were four separate sets of conditions as illustrated in Table 4-4.

Table 4-4: Details of experiment to determine impact of salt on immobilised conjugates and on antibodies in solution

Condition	Conjugate incubation	Antibody incubation
1	0 mM salt	0 mM salt
2	100 mM salt	0 mM salt
3	0 mM salt	100 mM salt
4	100 mM salt	100 mM salt

The plotted background corrected binding signals for the cAMP and the benzo[a]pyrene immunoassays are shown in Figure 4-7. Binding signal in the cAMP immunoassay was unaffected by incubation of sodium chloride, a salt that produces a pH neutral solution in the LMC extraction solvent and freeze drying buffer, with the immobilised cAMP-BSA conjugate or with the cAMP assay antibodies, and with combinations of both (Graph A in Figure 4-7). This implies that immobilisation of the cAMP-BSA conjugate is stable in the presence of 100 mM NaCl and that the antibodies (and the HRP enzyme label) for the cAMP immunoassay are also unaffected by incubation in the presence of 100 mM sodium chloride. Binding signal in the benzo[a]pyrene immunoassay was unaffected by incubation of the immobilised B[a]P-BSA conjugate, implying that immobilisation of the benzo[a]pyrene conjugate is stable in the presence of 100 mM sodium chloride (incubation condition 2), but binding signal was

reduced where 100 mM sodium chloride was incubated with the antibodies (condition 3). This observation is in agreement with previous results where the 22F12 clone was shown to be more sensitive to changes in ionic strength than other tested antibodies.

Binding signal in the cAMP and B[a]P immunoassays was also unaffected by the incubation of 100 mM magnesium perchlorate with immobilised conjugates (condition 2) (Graph C in Figure 4-7), indicating that immobilised conjugates were stable to the presence of 100 mM magnesium perchlorate. However addition of 100 mM magnesium perchlorate to the antibodies (condition 3) and to both the antibodies and the immobilised conjugate (condition 4) did produce a reduction in binding signal, which was greater for the benzo[a]pyrene immunoassay than for the cAMP immunoassay. This indicates that the loss in binding signal observed for the cAMP and B[a]P immunoassays in the presence of 100 mM magnesium perchlorate is due to changes in antibody performance and also supports the previous observation that the B[a]P immunoassay is more sensitive to the addition of salts, than the cAMP immunoassay.

For both the cAMP and B[a]P immunoassays incubation of immobilised conjugate with 100 mM iron (III) chloride, a salt which produces acidic solutions in the LMC solvent and freeze drying buffer, (condition 2) produced a significant reduction in binding signal (Graph B in Figure 4-7). This indicates that immobilisation of both cAMP-BSA and B[a]P-BSA conjugates is unstable to 100 mM iron (III) chloride, assuming that the washing procedure was sufficient to remove all traces of iron (III) chloride prior to addition of the antibodies to the 96-well plate. Incubation of iron (III) chloride with the antibodies (condition 3) and with both the antibodies and the conjugate (condition 4) produced a further reduction in binding signal. Note that as shown by the data in Figure 4-1, 100 mM iron (III) chloride has a significant impact on the HRP enzyme label independently of the immunoassay therefore it is not possible to determine whether the enzyme or the antibodies are responsible for the loss of signal from this experiment, but it seems likely that it is a combination of both since antibodies are unlikely to perform well at pH 2.0 (Figure 4-6). Incubation with iron (III) perchlorate, which also forms acidic solutions (Graph D in Figure 4-7) had similar consequences for immunoassay binding signal.

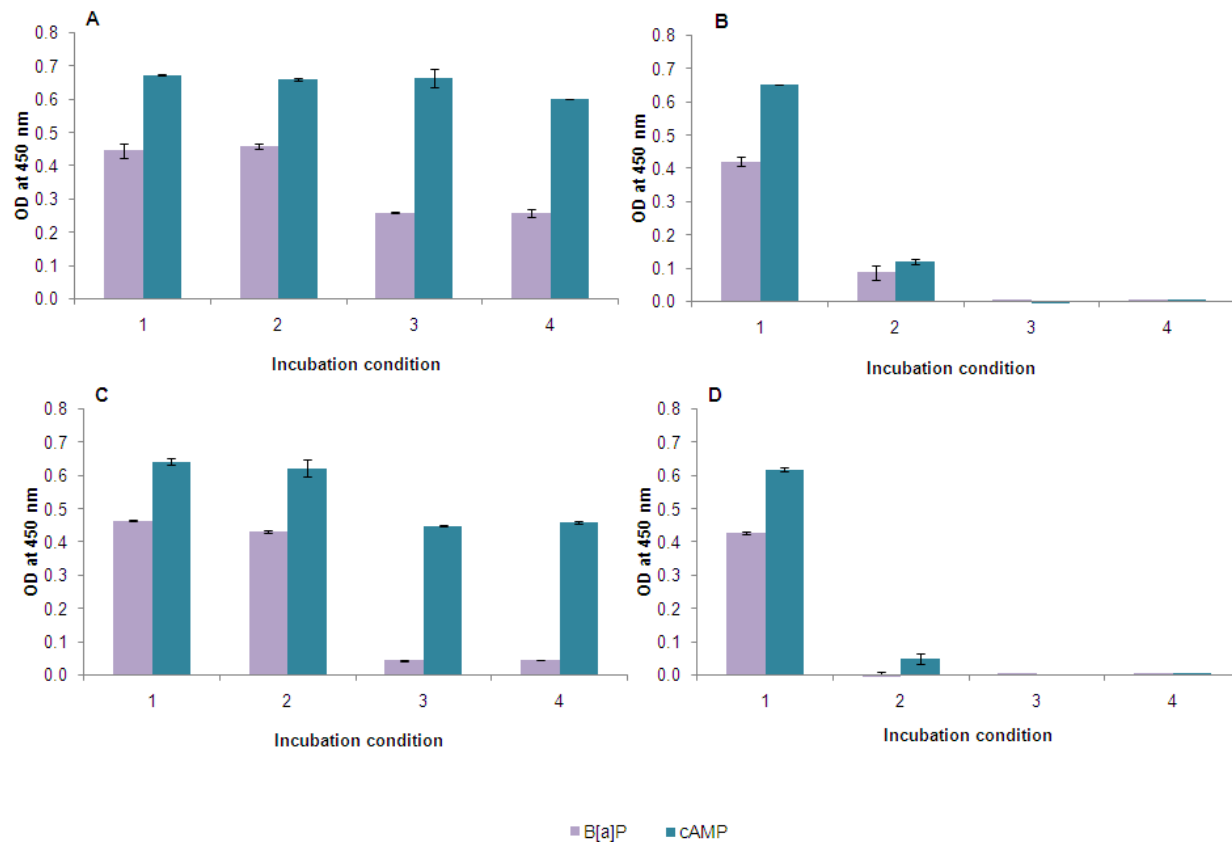


Figure 4-7: ELISA binding signal for B[a]P and cAMP immunoassays run in 20/80 % (v/v) methanol/water with 1.5 g/L P80 after incubation with 100 mM Martian relevant sample matrix components
 A NaCl, B FeCl₃, C Mg(ClO₄)₂ and D Fe(III)(ClO₄)₃. **Incubation condition 1** no sample matrix component included **incubation condition 2** sample matrix component incubated with immobilised conjugate **incubation condition 3** sample matrix component added during incubation of antibodies **incubation condition 4** sample matrix component incubated with immobilised conjugate and added during incubation of antibodies. Binding signals were background corrected by subtracting signal generated in uncoated wells. Error bars are ± standard error, n = 2.

In summary this data indicates that acidic Mars relevant sample matrix components such as iron (III) chloride and iron (III) perchlorate reduce binding signal in ELISA format by more than one mechanism, *i.e.* by stripping immobilised conjugate from the 96-well plate and by interaction with the antibodies and/or enzyme label, whereas sample matrix components which do not produce changes in pH have no impact on the immobilised conjugate therefore changes in binding signal are caused only by interaction with the antibodies and enzyme label. It may therefore be possible to reduce the impact of acidic sample matrix components by covalently linking target molecule-protein conjugates to the plate surface, or to the chip surface for microarray format assays. This is being implemented in current LMC development work.

Summary

In summary ELISA binding signal is affected by the addition of sample matrix components with the greatest changes in signal resulting from the addition of iron ion containing salts. The buffering capacity in the freeze drying buffer is insufficient to control pH in the presence of high concentrations of iron ions and these components interfere with the immunoassay by changing antibody/enzyme performance and stripping immobilised target molecule from the surface of 96-well plates. Therefore implementation of covalent immobilisation and improved pH control may be beneficial in reducing some of the sample matrix mediated signal changes. The implications of these findings are further discussed in Section 4.5.3.4.

4.5.3.2 Further studies to confirm immunoassay performance in pooled microarray format

Effect of sample matrix component on fluorescent labels

In the flight format LMC microarray immunoassays will be performed directly in a liquid extract from a martian sample. After incubation within the array chamber, antibodies that have not bound to the microarray will be washed away using several chamber volumes of liquid extract. Fluorophores on bound antibodies will then be evanescently excited whilst the liquid extract is still present in the chamber, therefore sample matrix components that interfere with the fluorescence of the fluorophores on the bound antibodies may be detrimental to the function of the flight instrument.

In order to assess the potential impact of sample matrix components on fluorescence of AF633 fluorescent label, independently of the impact on immunological interactions, streptavidin-AF633 was incubated with Mars relevant sample matrix components in the LMC

solvent and freeze drying buffer and the fluorescence of the fluorophore was then measured (please see Section 2.2.3.10 for detailed methodology).

The data in Figure 4-8 show that the majority of Mars relevant sample matrix components tested had little impact on the fluorescent signal generated by AF633 label in solution at concentrations of 10 mM, 100 mM and 500 mM. The only components that produced a significant reduction (subjectively assigned as > 20 %) in the fluorescent signal generated were iron ion containing and 500 mM sodium carbonate. At 10 mM concentration of iron (III) chloride, iron (II) perchlorate, iron (III) perchlorate, iron (II) sulphate and iron (III) sulphate the fluorescent signal generated by the AF633 label in solution was reduced to a level where it was not distinguishable above background noise.

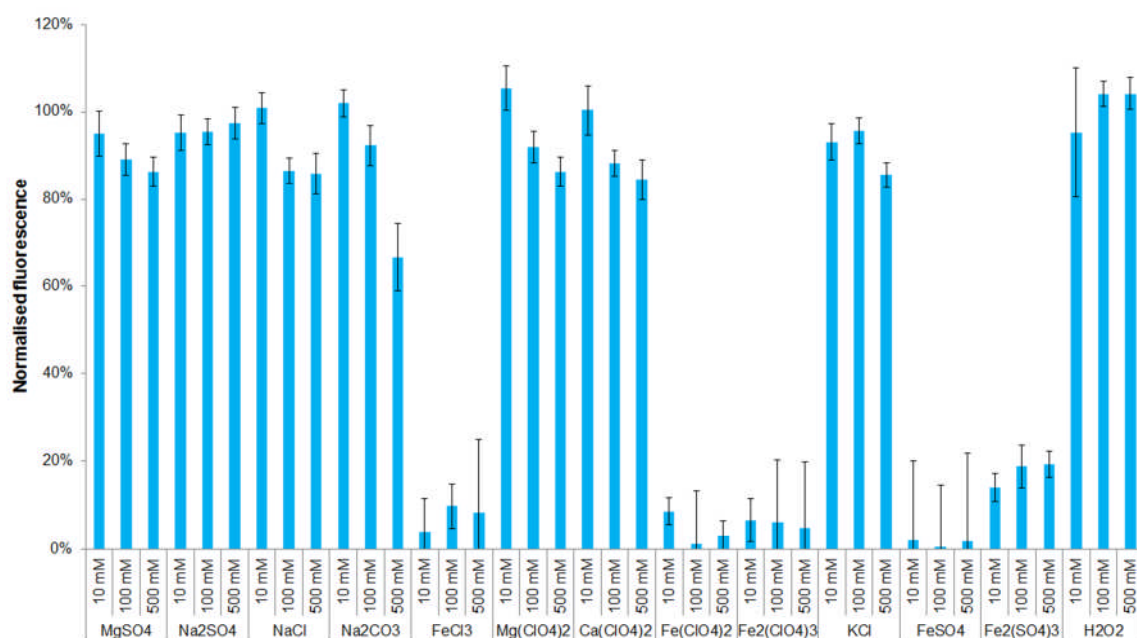


Figure 4-8: Impact of sample matrix components on fluorescence of AF633 in solution. Fluorescent signal generated in the presence of each sample matrix component was normalised by dividing by fluorescent signal produced with no sample matrix component. Error bars = \pm standard error n=2.

Invitrogen/Molecular Probes, the manufacturer of the Alexa Fluor® series of dyes claims that the dyes are insensitive to pH in the range 4-10 (Panchuk-Voloshina *et al*, 1999). A 500 mM solution of sodium carbonate has a pH of 11.0 and therefore would be expected to interfere with fluorescence of AF633 whereas 10 mM solutions of iron chloride, iron (II) and iron (III) perchlorate and iron (II) and iron (III) sulphate had a pH of greater than 4.0 and therefore would not be expected to induce large reductions in fluorescence. This implies that there may be another quenching mechanism in addition to changes in pH. Experiments performed

to establish the lowest iron ion containing concentration that produced signal reduction (data not shown) demonstrated that concentrations of iron ions of as low as 0.1 mM still affect the fluorescent signal generated by AF633.

This result is potentially of concern for the LMC since Mars samples are likely to contain iron salts, although the likely solubility of iron in martian rock/regolith samples has not been determined. Therefore it may be necessary to replace the liquid extract present in the sample chamber with fresh extraction solvent, prior to optically exciting the fluorophores to prevent reduced fluorescent signal. Technically this should be possible with the current (2011) design of the flight model; however work would be required with flight representative hardware to confirm this. In the pooled microarray format sample matrix components are washed away prior to fluorescent imaging of the microarray, therefore data from experiments performed in pooled microarray format can be used to confirm if removing the sample matrix components prior to imaging is effective at minimising loss of fluorescent signal.

Effect of sample matrix components on microarray assay binding signal

Since the microarray format assay is less well established than the ELISA format, and because this work was carried out at an early stage in the use of the newly purchased commercial microarraying system (Section 2.2.3.5), the reproducibility of the printing and assaying process was first assessed by running 12 identical pooled assays on 12 microarrays printed onto a single commercial hydrophobic slide. The mean pixel intensity of microarray spots, and AF633 labelled BSA spots are plotted in Figure 4-9. Observe that signals (pixel intensities) for each set of microarray spots vary across the slide by 5000-10000. The source of these variations in signal intensity is undetermined but could be due to many factors including, but not limited to, printing reproducibility, humidity and temperature during printing, reproducibility of pooling reagents over an array area for the microarray assay and consistency of washing following a microarray assay.

This intrinsic variation within arrays produced by the current printing and assaying protocol makes interpretation of data within this study more problematic and may mask underlying effects, since it is not possible to be certain whether changes in immunoassay and labelled BSA binding signal are due to the sample matrix component under test, or due to the intrinsic variability across the 12 arrays on each slide. Work is ongoing to optimise microarray printing for the LMC to reduce variability. However within this study there were some sample matrix components where microarray binding signal was reduced to an extent where, despite the variability in the data, it was possible to be confident that changes in signal were directly attributable to the sample matrix components.

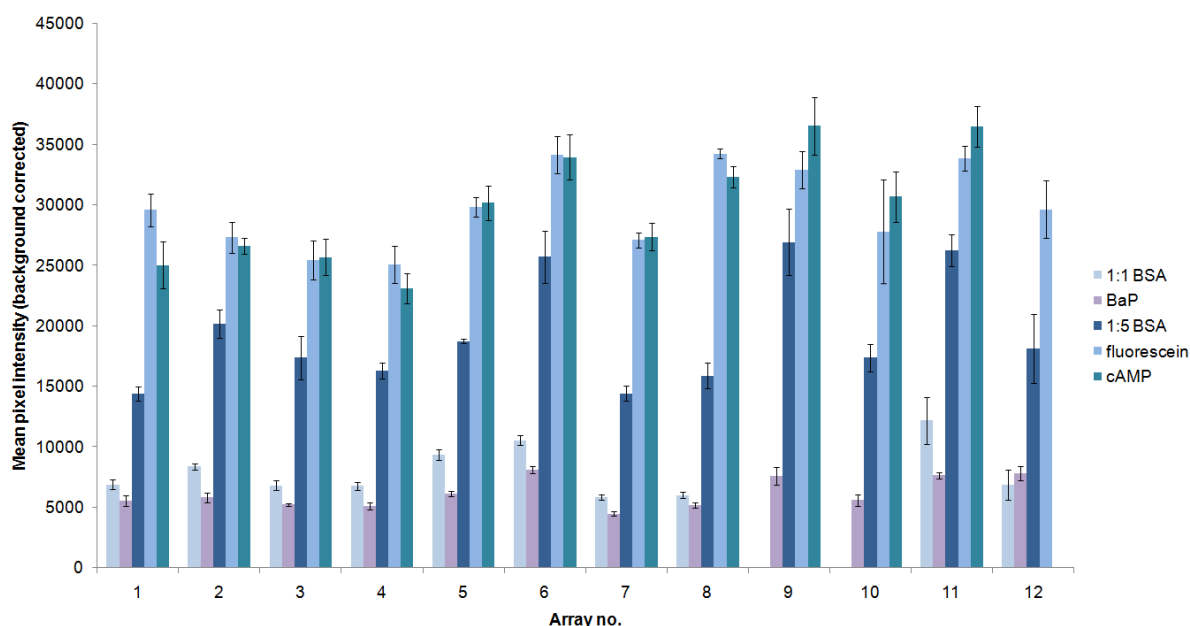


Figure 4-9: Mean pixel intensity of low ratio (1:1) AF633 labelled BSA, benzo[a]pyrene, high ratio (1:5) AF633 labelled BSA, fluorescein and cAMP microarray spots after running pooled multiplexed microarray immunoassays on all 12 arrays printed onto a single commercial hydrophobic microarraying slide. Error bars = ± 1 std error, $n = 5$. Note that cAMP assay spots were missing on array 12 and low ratio AF633 labelled BSA spots were missing on arrays 9 and 10 (not printed, due to the microarraying pin sticking in the print head).

Maximum microarray binding signal (binding signal with zero inhibiting target molecule) for the benzo[a]pyrene, cAMP and fluorescein immunoassays was assessed in the presence of a selection of Mars relevant sample matrix components, at a range of concentrations, by running pooled multiplexed microarray format immunoassays using microarrays printed onto commercially available hydrophobic microarraying slides. Slides were imaged using the Innopsys microarray scanner (representative fluorescent image shown in Figure 4-10). Mean pixel intensities were measured from the captured images and are plotted in Figure 4-11, Figure 4-12 and Figure 4-13.

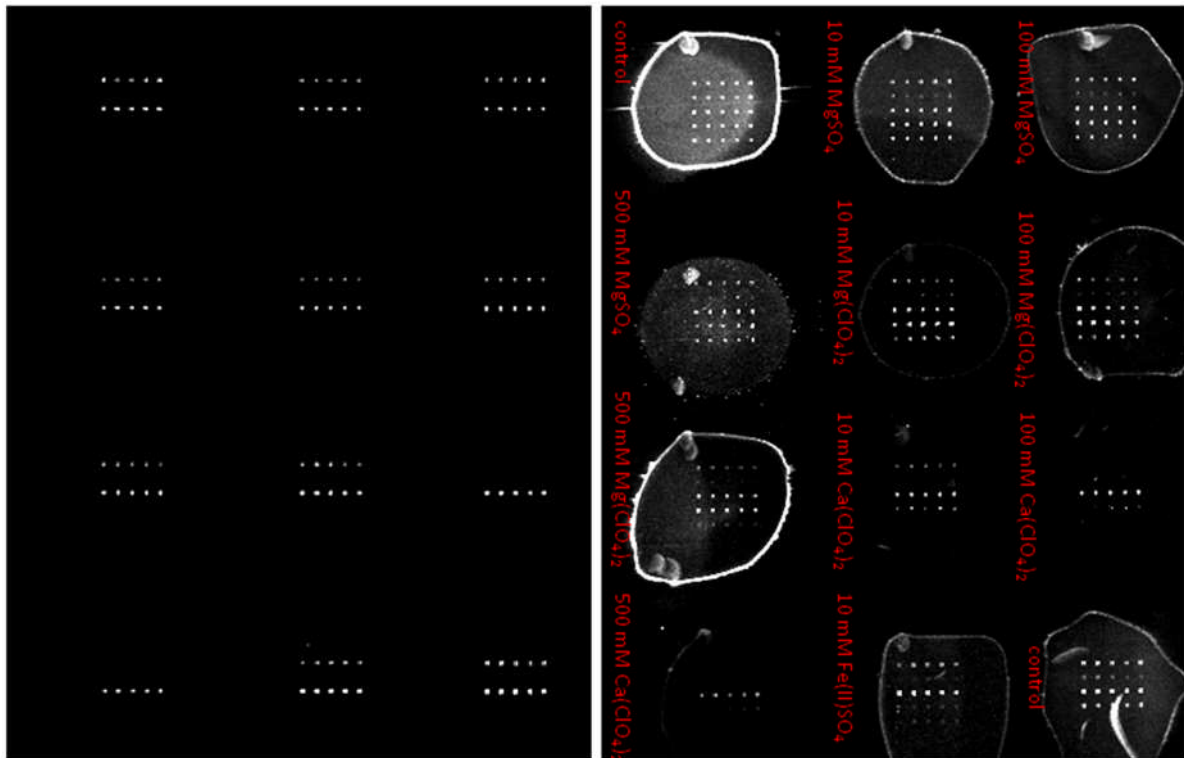


Figure 4-10: Representative fluorescent images of 12 microarrays printed and physically adsorbed onto commercial hydrophobic slide using ArrayIt® SpotBot. Images taken using Innopsys Microarray scanner before (left) and after (right) running pooled multiplexed microarray format immunoassays in the presence of Mars relevant sample matrix components.

In images taken before running the pooled immunoassays two rows of five spots are visible in each array comprising from top to bottom low ratio labelled AF633 labelled BSA and high ratio AF633 labelled BSA. In images taken after running pooled microarray assays (right) five rows of five spots are visible in each array comprising from top to bottom low ratio AF633 labelled BSA, benzo[a]pyrene immunoassay spots, high ratio AF633 labelled BSA, fluorescein immunoassay spots and cAMP immunoassay spots. Image brightness and contrast have been optimised to aid visualisation of spots in both images. Note that for two of the arrays the low ratio AF633 labelled BSA spots are missing from the image because they did not print. Microarray spots are 110 µm in diameter and are spaced 400 µm apart (centre to centre).

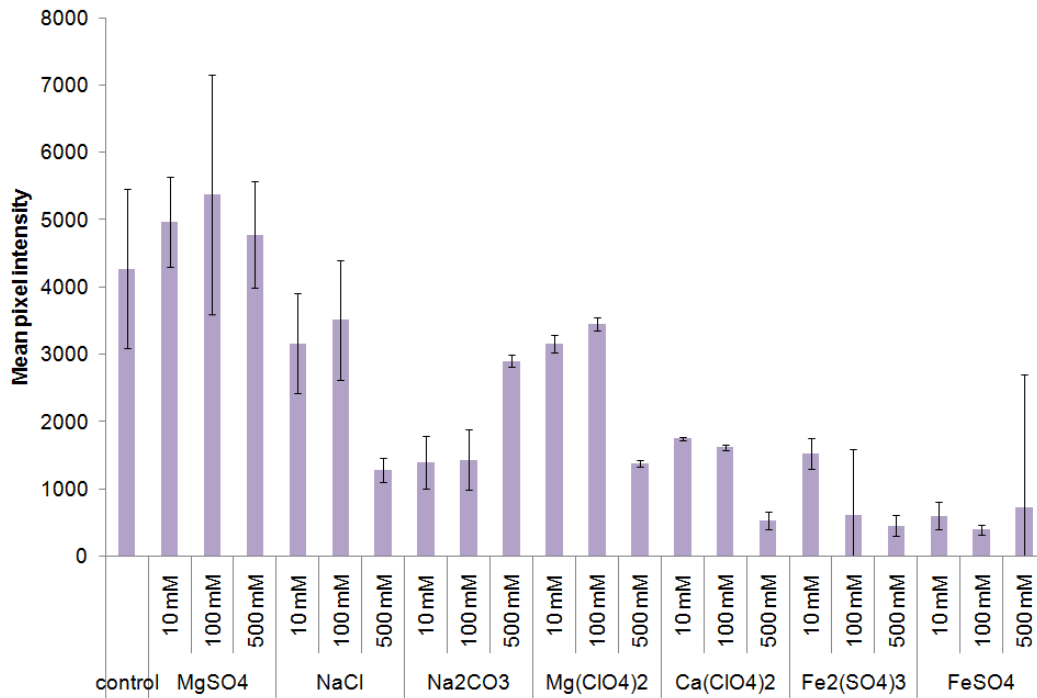


Figure 4-11: Mean pixel intensity of benzo[a]pyrene assay microarray spots after multiplexed pooled microarray immunoassay with Mars relevant sample matrix components. Error bars = ± 1 std error, n = 2-5

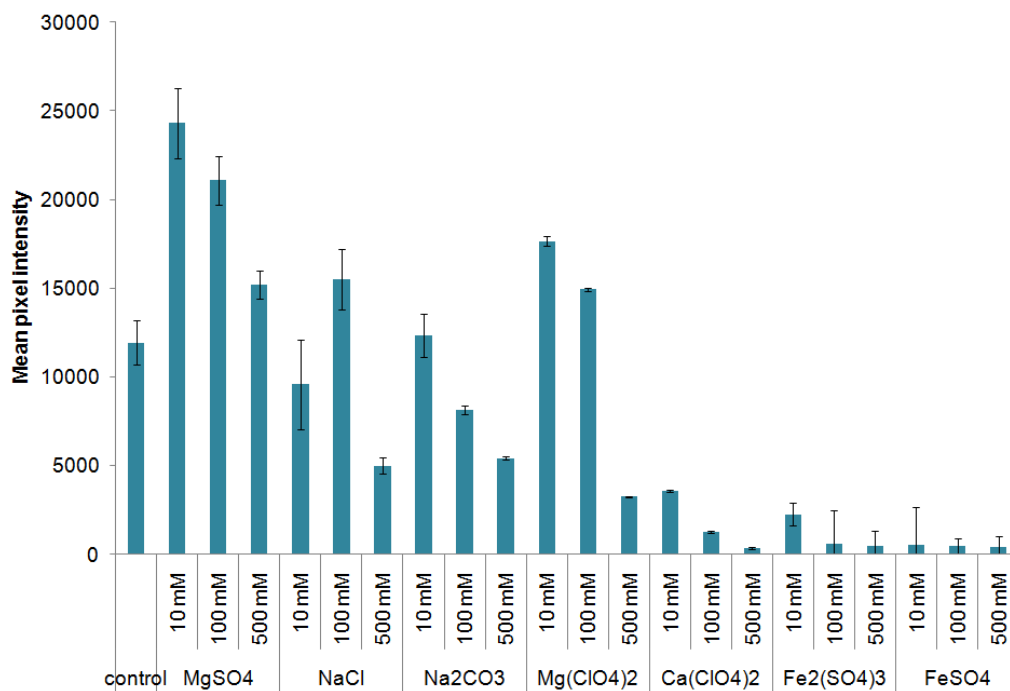


Figure 4-12: Mean pixel intensity of cAMP assay microarray spots after multiplexed pooled microarray immunoassay in the presence of Mars relevant sample matrix components. Error bars = ± 1 std error, n = 2-5

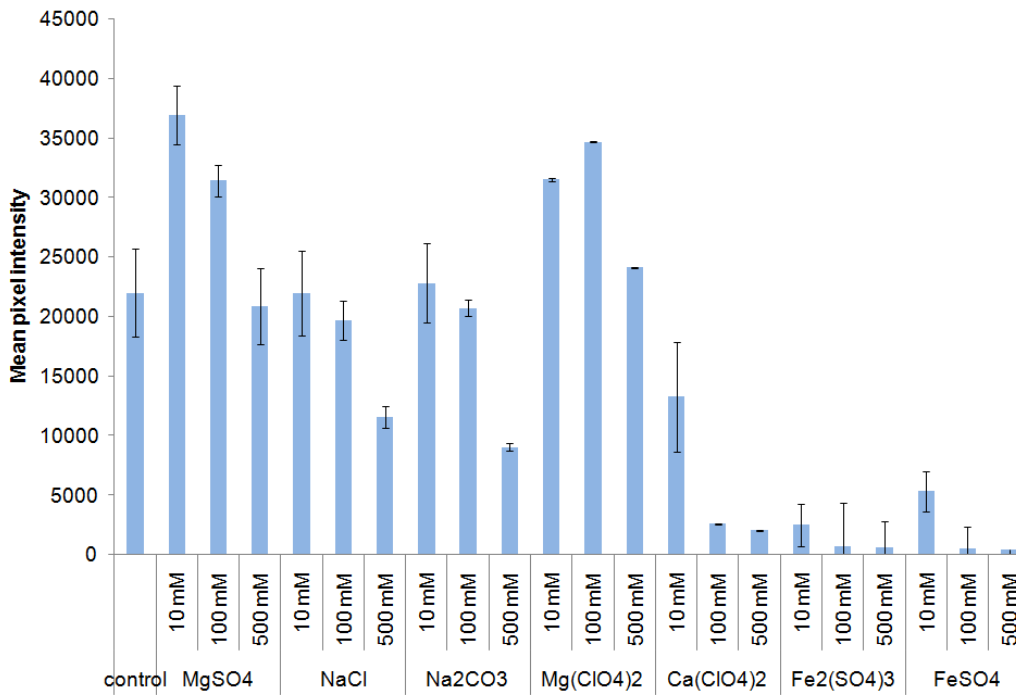


Figure 4-13: Mean pixel intensity of fluorescein assay microarray spots after multiplexed pooled microarray immunoassay with Mars relevant sample matrix components. Error bars = ± 1 std error, $n = 2-5$

Given the demonstrated variability within the microarray data, using the current printing and assay protocols, results must be interpreted with care. However the general trends in the microarray data, are in agreement with those observed for the experiments carried out in ELISA format *i.e.* iron ion containing components reduce binding signal to levels which render the immunoassay non-functional at concentrations of 10 mM and above and immunoassays function (in terms of generating binding signal) in concentrations of up to 100 mM of the non iron-ion containing sample matrix components.

None of the sample matrix components produced a significant increase in non-specific background signal in microarray format, compared to a control assay run with no sample matrix component, indicating that the increases in background signal observed with some sample matrix components in ELISA format were specifically related to the enzyme-substrate reaction and are therefore irrelevant to the flight format immunoassay. Note however that some scatter was observed in microarray images, around the edges of the arrays. This is thought to be a result of evaporation of pooled solutions, resulting in the deposition of particles on the slide surfaces (Figure 4-10).

Further analysis of impact of sample matrix components on fluorescent dyes by analysis of AF633-BSA spots on microarrays

In addition to the immunoassay spots, each printed microarray included two sets of control spots consisting of high ratio AF633 labelled BSA (1:5 dye: protein labelling ratio) and low ratio AF633 labelled BSA (1:1 dye: protein labelling ratio). The signal intensity of these control spots was measured both before and after running pooled microarray immunoassays. This was carried out to (i) investigate the impact of sample matrix components on fluorescence of immobilised AF633 BSA and (ii) to further investigate whether removal of sample matrix components from the array chamber in the flight instrument, prior to imaging the microarray, will mitigate the loss of fluorescent signal observed in the presence of iron ions.

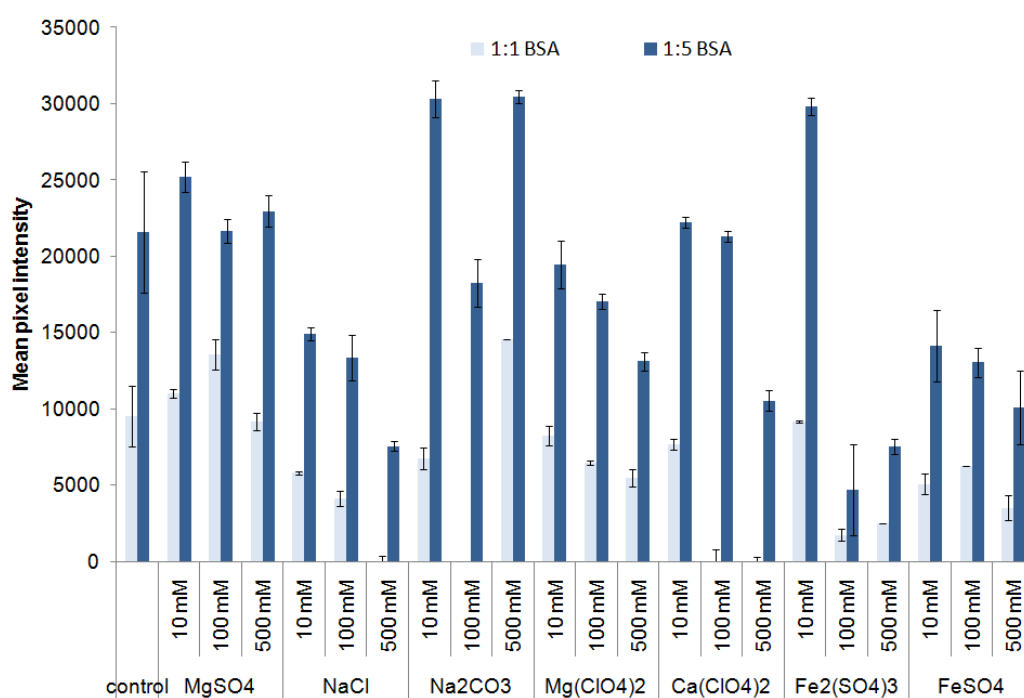


Figure 4-14: Mean pixel intensity of low ratio (1:1 ratio) AF633 labelled BSA and high ratio (1:5 ratio) AF633 labelled BSA microarray spots after multiplexed pooled microarray assay with Mars relevant sample matrix components. Error bars = ± 1 std error, n = 2-5

For the three control arrays (microarrays incubated with no sample matrix component, but subjected to the same washing conditions) signal intensity of the AF633 labelled BSA spots was reduced by between 0 and 20 % after running a pooled microarray immunoassay indicating that a quantity of immobilised AF633 labelled BSA may have been lost from the chip surface during the incubation and/or washing procedures (Figure 4-14).

Some sample matrix components produced significantly greater signal loss compared to the control arrays, even taking into account the variability observed between microarrays. *i.e.* 100 – 500 mM sodium chloride, 100 – 500 mM calcium and magnesium perchlorate and 10 – 500 mM iron (II) and iron (III) sulphate. Sodium chloride, magnesium perchlorate and calcium perchlorate at 100 – 500 mM concentration reduced fluorescent signal produced by the labelled BSA spots but did not reduce the fluorescence of AF633 when tested in solution (Figure 4-8), therefore AF633 labelled BSA spot signal loss is unlikely to be due to reduced fluorescence and more likely a result of loss of fluorescently labelled BSA from the slide surface. Conversely Iron (II) and iron (III) sulphate did reduce fluorescence of AF633 labelled BSA in solution with 10 mM concentrations reducing signal to a level that was not distinguishable from background. In the case of immobilised AF633 labelled BSA spots although incubation with iron sulphate salts did reduce the fluorescent signal, signal was still discernable above background noise, even at 500 mM concentration. This is significant because it indicates that removing the iron salts prior to imaging microarrays at least partially recovers the fluorescent signal. Therefore this may be a viable route to mitigate the impact of iron ions on fluorescent signal in the flight format instrument. Additionally it may be possible to improve immobilisation stability to the presence of sample matrix components by investigating the use of covalent immobilisation of target molecule-protein conjugates to the microarraying slide and by improving the pH control of the LMC freeze drying cocktail.

In the flight format immunoassay liquid extract will be pumped over the surface of the microarray, whereas in the pooled microarray immunoassay liquid extract was pooled on-top of the microarray and rinsed away by immersion in buffer. Therefore future work should also include assessment of sample matrix components in a microfluidic format microarray assay to determine whether the physical flow of the liquid extract over the surface has an influence on the severity of sample matrix mediated effects.

Summary

In summary binding signal in microarray immunoassays is affected by the addition of sample matrix components with the greatest changes in signal resulting from the addition of non-pH neutral salts. The mechanism for this signal loss is likely to be a combination of changes in antibody performance and loss of immobilised target molecule-protein conjugate from the surface of the microarraying substrate, therefore implementation of covalent immobilisation and improved pH control may be beneficial in reducing some of the sample matrix mediated signal changes. In solution iron ions significantly reduce fluorescent signal, probably due to fluorescence quenching, however when immobilised fluorophores are exposed to iron ions and the iron ions are then removed, signal loss is reduced, implying that washing away iron

ions prior to imaging microarrays may minimise the loss of fluorescent signal which results from the presence of these components. The implications of these findings are further discussed in Section 4.5.3.4.

4.5.3.3 Extended study with limited range of sample matrix components using ELISA sensitivity as performance indicator

In order to determine the impact of sample matrix components on the sensitivity of LMC relevant immunoassays, inhibition ELISAs were performed in the presence of a selection of martian relevant sample matrix components at concentrations of 10 mM, 100 mM and 500 mM. Studies were performed with a sub-set of sample matrix components excluding any components which prevented immunoassays from functioning. Representative inhibition assay data for the cAMP immunoassay are plotted in Figure 4-15 (other inhibition assay data plots are included in Appendix B Figure B1, Figure B2 and Figure B3). IC_{50} values (Table 4-5) for each immunoassay were calculated by fitting data to a four parameter binding equation (Section 2.1.1.1).

The four target molecules atrazine, pyrene, cAMP and fluorescein were successfully detected in the tested sample matrix components, except at high concentrations of sample matrix components where binding signal was reduced to a level where it was not distinguishable from background signal. Immunoassay IC_{50} varied in the presence of the sample matrix components, however for the LMC which aims to perform a threshold detection, variations in IC_{50} , such as those observed within this study, are unlikely to be significant.

The literature contains many examples of individual immunoassays tested for performance at a range of ionic strengths. In some examples ionic strength has strongly influenced immunoassay performance with high ionic strength producing increased IC_{50} (Liang *et al*, 2008; Hao *et al*, 2009), whereas in other examples ionic strength had had little effect of immunoassay IC_{50} (Liu *et al*, 2009), implying that the effect of increased ionic strength is highly antibody/target specific. The 22F12 anti-benzo[a]pyrene clone used within this study has previously been reported to show reduced immunoassay IC_{50} in higher ionic strength solutions (Matschulat *et al*, 2005) in agreement with the observed performance in this study.

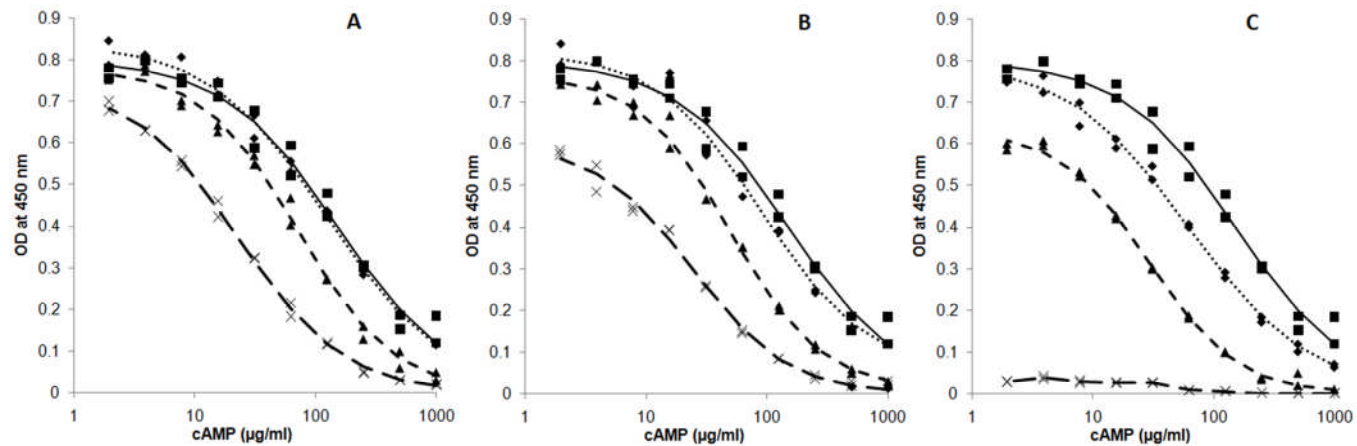


Figure 4-15: cAMP inhibition ELISA in the presence of sample matrix components **A** sodium chloride, **B** magnesium sulphate and **C** magnesium perchlorate at ■ 0 mM, ◆ 10 mM, ▲ 100 mM and X 500 mM concentration.

Each data point represents a single replicate. Immunoassay curves for each concentration of each sample matrix component were plotted by fitting mean signal to a four parameter binding equation, or by linear regression of the mean signal where the data did not fit a four parameter binding equation (solid line) 0 mM, (dotted line) 10 mM, (short dashed line) 100 mM and (long dashed line) 500 mM

Table 4-5: IC₅₀ values (ng/ml) for inhibition ELISAs run in the presence of Mars relevant sample matrix components at 10, 100 and 500 mM concentration.

	Control	NaCl			MgSO ₄			Mg (ClO ₄) ₂		
		10 mM	100 mM	500 mM	10 mM	100 mM	500 mM	10 mM	100 mM	500 mM
Atrazine	6.9	6.9	5.6	3.3	7.4	5.8	3.1	7.9	406	N/A
B[a]P	118.5	94.5	76.4	N/A	101.3	40.0	N/A	94.0	57.9	N/A
cAMP (µg/ml)	150.30	126.5	71.9	23.3	90.7	50.0	24.0	62.3	29.5	N/A
Fluorescein	444	380	470	250	470	150	100	440	410	N/A

4.5.3.4 Study 1 General Discussion

The objective of the work reported in this section was to test performance of a number of LMC relevant immunoassays in the presence of likely individual Mars sample matrix extractable components identified from known Mars geology/geochemistry. In order to achieve this immunoassays, in both ELISA and microarray format, were performed in the presence of a selection of Mars relevant salts and oxidising agents.

LMC relevant Immunoassay performance in the presence of sample matrix components

Data from ELISA and pooled microarray format assays indicate that LMC immunoassays are likely to function with martian samples with similar levels of extractable salts to those tested by Phoenix (Hecht *et al*, 2009). However samples with salt levels similar to some of the samples analysed by MER (Yen *et al*, 2005), assuming that all of the salt is soluble, are likely to compromise immunoassay performance and may even prevent assays from functioning entirely. Therefore it may be necessary to avoid analysis of samples with high salt content using the LMC. Early in LMC development work the use of ion exchange resins to remove ions from liquid extracts was considered (Wilson 2007), however this has since been rejected as an option due to the high likelihood that hydrophobic target molecules would be absorbed by the high surface area de-salting resins that were proposed.

One key limitation of this study is that the likely solubility of Mars relevant sample matrix components, once incorporated into a real matrix, may differ significantly from those tested here as pure compounds. Future work should therefore be carried out to characterise the soluble components of the types of rocks that may be encountered on Mars in order to better understand the likely solution chemistry of martian samples and the resulting implications for the LMC.

Of the four immunoassays tested the benzo[a]pyrene assay showed lower tolerance to the addition of sample matrix components and as such the 22F12 antibody would probably be considered unsuitable for inclusion in the flight suite of immunoassays. This highlights the need to assess the tolerance of antibodies to sample matrix components at an early stage in assay development, prior to investing significant effort transferring assays into flight representative formats. Screening recombinant antibody libraries has been identified as a potential route to the production of antibodies against LMC relevant target molecules and recombinant libraries have previously been used to generate receptors that function in high ionic strength solutions such as sea water (Goldman *et al*, 2002). Therefore it may be possible to design the screening procedure to favour the selection of antibodies that tolerate

high ionic strength and other conditions such as extremes of pH, although this may be unrealistic within the schedule for development of the LMC instrument.

Data from immunoassays performed in both ELISA and microarray format suggests that the Mars relevant components that have the most detrimental impact on immunoassays are those that form acidic/basic solutions in the LMC solvent and freeze drying buffer, and those that contain iron ions. It may be possible to reduce these effects by altering the formulation of the LMC freeze drying buffer to increase buffering capacity, however this may have other implications for the LMC such as changing assay performance, altering solubility of extracted target molecules and leading to the risk of precipitation of buffer salts in the microfluidic channels of the flight format immunoassay. For some Mars relevant sample matrix components it may not be possible to build in sufficient buffering capacity, therefore it may be necessary for the LMC to reject any samples that are likely to contain iron sulphates and other similar salts by using data available from the other ExoMars rover contextual instruments.

One of the major concerns for the LMC is the importance of being able to distinguish between specific inhibition of signal due to the presence of target molecule, from signal losses mediated by sample matrix effects. The primary strategy adopted to address this issue is the inclusion of control immunoassays in the flight instrument, for targets that are not expected to be present on Mars. Therefore if signal loss is observed in the control immunoassays, the implication is that the signal loss is sample matrix mediated. The data generated in this study, which used LMC relevant immunoassays with a number of differing primary and secondary antibodies, is that there were differences in the degree of signal loss between the immunoassays tested with the same sample matrix component. This result highlights the need to include a number of well characterised control assays in the flight format assay with a range of tolerances to changes in pH and ionic strength.

Impact of sample matrix components on fluorescence of AF633

Since the flight format instrument has a fluorescent readout, the impact of Mars relevant sample matrix components on fluorescence is of high significance. For the majority of Mars relevant sample matrix components tested, fluorescent signal was unaffected; however iron ion containing sample matrix components had a detrimental impact on fluorescence in solution, even when tested at low concentration. However analysis of immobilised AF633 labelled BSA microarray spots following pooled microarray assays revealed that removal of the iron ion containing sample matrix components prior to fluorescent imaging, resulted in visible fluorescent spots, which had similar intensities to control microarrays which had not

been exposed to iron salts. Therefore removal of sample matrix components prior to imaging microarrays in the flight instrument should reduce the loss of fluorescent signal resulting from the presence of iron ions. Therefore in the flight format instrument liquid extract containing soluble components of the Mars sample matrix should be washed away from the assay chamber using fresh extraction solvent, prior to imaging the arrays. Given the current design of the flight instrument this should be possible, but testing needs to be performed to confirm this.

Comparison to literature data

There are a number of immunoassay based instruments under development for life detection experiments on Mars, and for planetary exploration more generally, *e.g.* Steele *et al* (2001), Parro *et al* (2011), however there are no published studies where immunoassay performance has been systematically tested in a range of Mars relevant sample matrix components. The group developing the SOLID instrument, based at Centro de Astrobiologica in Spain, describe an experiment to determine the impact of perchlorate salts, at up to 50 mM concentration on sandwich type microarray assays and report that no substantial differences were observed compared to a control (Parro *et al*, 2011), which is in agreement with data reported within this study. The group at CAB have also published data relating to the use of their SOLID instrument prototype instrument with a range of Mars analogue samples. These results are discussed in the context of results from Study 2 (Section 4.6) where LMC format immunoassays were tested with liquid extracts from Mars analogue samples.

4.6 Study 2: Testing the impact of liquid extracts from Mars analogue samples on LMC relevant immunoassays

The work in Study 1 has shown that Mars relevant sample matrix components have a significant impact on the function of LMC format immunoassays, particularly at the highest tested concentration (500 mM). However when incorporated into complex real samples sample matrix components may behave differently, especially in terms of their solubility, and there is limited information available regarding the solution chemistry of martian rocks and regolith. In addition sub 0.45 μm particulates generated from the processing/crushing of real samples by the Sample Processing and Distribution System (SPDS) on the ExoMars rover may pass through the filter and into microfluidic channels where they may interact with the immunoassays. Therefore the objective of the work reported in Study 2 was to investigate the performance of LMC format immunoassays with liquid extracts from a range of Mars analogue samples, in an attempt to better understand likely interactions with complex 'real' samples.

4.6.1 Supplementary experimental design

The design adopted for this study aligns with the common experimental design described in Chapter 2 of this thesis: The set of model immunoassays described in Chapter 2, were used to assess the likely performance of LMC immunoassays with liquid extracts from Mars analogue samples. Note that within this study a sub-set of the immunoassays (B[a]P, cAMP and fluorescein) were used due to resource constraints.

Initial assessment of immunoassay performance, was carried out using the LMC representative binding ELISA format (Section 2.1.1.1). Further studies, with a sub-set of Mars analogue samples, were then carried out in pooled microarray binding format (Section 2.1.1.2) to confirm whether behaviour in the more flight representative microarray format was similar to that observed in ELISA format and finally an extended study of immunoassay sensitivity was carried out, on a selected set of Mars analogues in pooled microarray format. Pooled microarray format was used here in preference to inhibition ELISA format due to the lower volumes of liquid extract required for pooled microarray assays.

4.6.1.1 Selection of Mars analogues for use in study

The martian crust is dominated by basaltic rock (McSween *et al*, 2009; Rossi and Gasselt 2010) with localised concentrations of phyllosilicates (clays), hydrated sulphates (including gypsum, jarosite and kieserite), carbonates and ferric oxides including haematite. Since the

landing site for the ExoMars Rover has not yet been selected, and is unlikely to be selected prior to instrument delivery in early 2015 (correct as of late 2011), it is important to test analogue samples from as wide a range of Mars relevant geologies as possible, with the potential for more targeted testing once the landing site has been selected. Analogue samples used within this study were either selected as examples of specific mineralogies relevant to Mars e.g. phyllosilicates (clays) and gypsum, or were selected due to their origin from a relevant Mars analogue site on Earth offering a more diverse / complex sample set e.g. Iceland, which is predominantly a volcanic island, has poorly formed soils, basaltic bedrock and sparse surface water and has therefore been considered to be an analogue site for early volcanic environments on Mars (Cousins *et al*, 2010; Ehlmann *et al*, 2011a). In addition a martian simulant regolith, JSC Mars-1, developed by NASA's Johnson Space Centre, and commonly used in studies where a Mars analogue is required, was also tested. Analogue samples were not specifically collected for this work and their selection was influenced by their availability within the LMC project team and from other related academic groups. Details of all of the analogue samples used are given in Table 4-6.

Table 4-6: Analogue samples used to test performance of immunoassays in liquid extracts

Sample	Description/relevance to Mars	Source
JSC Mars-1	martian regolith simulant palagonitic tephra (< 1mm size fraction). Contains haematite	Pu'u Nene cinder cone on the Island of Hawaii (Allen <i>et al</i> , 1997)
Gypsum	hydrated calcium sulphates	Haughton impact crater, Northern Canada
Marl	a calcium carbonate rich mud or mudstone, deposited in marginal marine setting	Vigo Meano, Northern Italy (Watson <i>et al</i> , 2005)
Kimmeridge Clay	sedimentary deposit of fossiliferous marine clay	Kimmeridge Bay, Dorset, UK (Farrimond <i>et al</i> , 1984)
Rheolite	igneous volcanic extrusive rock	Iceland
Basalt rock	igneous volcanic extrusive rock	Iceland
Edfell ash	pulverised rocks and glass	Iceland
Obsidian glass	igneous volcanic extrusive rock	Iceland

4.6.2 Supplementary materials and methods

The majority of materials and methods used within this study are the same as those used throughout the other chapters of this thesis and are described in Chapter 2. This section contains supplementary materials and methods specific to the work carried out in this study and where appropriate cross references the relevant sections of Chapter 2.

4.6.2.1 Sourcing and processing of Mars analogue samples

Analogue samples collected from Iceland's volcanic fields (basalt rock, rheolite, edfell Ash and obsidian glass) and gypsum from the Haughton Crater in northern Canada were supplied by Prof. Charles Cockell (Open University, Milton Keynes, UK) as rocks and ash

composed of particles of varying size (varying from a few mm to tens of cm). These samples were crushed prior to extraction as described in Section 4.6.2.2.

JSC Mars-1 regolith simulant, the < 1 mm fraction from palagonitic tephra, was developed by NASA's Johnson Space Centre (Allen *et al*, 1997) and was used as supplied.

Kimmeridge Clay and Marl were supplied by Dr Alex Baki, Dr Rachel Gomes and Dr Richard Court (Imperial College, London, UK) in the form of fine powders prepared by manual grinding in a pestle and mortar. The powders were used as supplied.

4.6.2.2 Preparation of Mars analogue samples for extraction

In order to mimic the action of the rock crusher in the ExoMars rover SPDS, samples that were not supplied ready processed were crushed using a Clarke strong arm 10 tonne hydraulic press. Note that at the time of this work no detailed specifications were available regarding the size distribution of crushed samples produced by the SPDS and no attempt was made to characterise the size distribution of samples crushed using the hydraulic press.

Analogue samples consisting of large pieces of rock were first broken into smaller fragments using the hydraulic press. Approximately 4 g of each analogue sample, broken into small (approx. 1 cm pieces), was placed between two stainless steel plates (approx 10 cm x 10 cm x 1 cm) together with two stainless steel spacers (10 cm x 1cm x 1 mm) (shown in bottom right hand image in Figure 4-16) and the analogue sample was crushed by using the hydraulic press to bring the two plates together until they met the spacers.



Figure 4-16: Photographs illustrating processing of analogue samples (left) unprocessed analogue samples supplied by Prof. Charles Cockell, (top right) 10 tonne hydraulic press used for crushing samples in preparation for liquid extraction (bottom right) analogue sample after crushing on stainless steel plate with 1 mm spacers.

4.6.2.3 LMC representative extraction of analogue samples

Note that analogue samples were tested in two separate batches, with approximately 6 months in-between the two sets of testing. During the intervening six months work carried out at Imperial College identified that extracted target molecules were being retained by the cellulose acetate filters used to clarify liquid extracts as part of the LMC extraction process (Court and Sephton 2011). In order to avoid the loss of target molecules, and until identification of suitable alternative membrane filters was possible, it was decided to replace the filtration step with a centrifugation step. Therefore the extraction procedure used for the two batches of testing varied in terms of the method used to remove large particulates from the extract. Both protocols are described in the following two sections.

Protocol 1 – used for extraction of JSC Mars-1, Marl and Kimmeridge Clay

Three ml of MeOH P80 was added to 1g analogue sample in a glass test tube and sonicated for 20 minutes with a Sonotrode Ultrasonic UP100H probe and UP50H ultrasonic processor set at 40 % amplitude (Hielscher Ultrasonics, Teltow, Germany). The mixture was allowed to stand for 10 minutes, and the cloudy supernatant that had formed above the sedimented

analogue sample was filtered through a 0.45 µm cellulose acetate filter and stored at 2 – 8°C in glass vials until tested in the immunoassay.

Protocol 2 – modified extraction protocol used for extraction of gypsum, rheolite, basalt, edfell ash and obsidian glass

Three ml MeOH P80 was added to 1g analogue sample in a glass test tube and sonicated for 20 minutes with a Sonotrode Ultrasonic UP100H probe and UP50H ultrasonic processor set at 40 % amplitude (Hielscher Ultrasonics, Teltow, Germany). The mixture was allowed to stand for 10 minutes, and the cloudy supernatant that had formed above the sedimented analogue sample was decanted into 1.5 ml centrifuge tubes. To minimize loss of extracted components by adsorption to surfaces of the hydrophobic polymer centrifuge tubes, the tubes were first blocked with 0.5 % (w/v) BSA in 100 mM HEPES pH 7.4. The supernatants were clarified by centrifugation at 17000 G for 10 minutes and stored at 2 – 8°C in glass vials until tested in the immunoassay.

4.6.2.4 LMC format immunoassays

ELISA and microarray immunoassays were performed following the methods described in Section 2.2.2 and Section 2.2.3 with liquid extracts from analogue samples.

4.6.3 Results and discussion

4.6.3.1 Initial studies of immunoassay performance in liquid extracts from a range of analogue samples using ELISA binding signal as a performance indicator

The LMC flight format immunoassay will be performed directly in the liquid extract from a martian sample with freeze dried antibodies and other immunoassay reagents dissolved directly into the liquid extract. For ELISA format assays the antibodies and other immunoassay reagents are not freeze dried and therefore have to be added to the extract in liquid form, resulting in dilution of the extract. The maximum concentration of liquid extract that could be tested in ELISA format, whilst maintaining flight representative concentrations of solvent and buffer salts etc, was 50 % (v/v) liquid extract, diluted with 25 % (v/v) 2 x concentration MeOH P80 and 25 % (v/v) 4 x concentration antibodies in 4 x concentration freeze drying buffer.

Since it was not possible to test higher concentrations of liquid extract it was decided to test a range of liquid extract concentrations from 0 % (v/v) up to 50 % (v/v) in order to allow extrapolation of the effect of increasing the liquid extract concentration further.

The graphs in Figure 4-17, Figure 4-18 and Figure 4-19 show how ELISA binding signal and non-specific background signal was affected by adding increasing concentrations of liquid extracts from Mars analogue samples into ELISAs (whilst maintaining the same final solvent concentration), up to a maximum of 50 % (v/v) liquid extract, for the benzo[a]pyrene, cAMP and fluorescein immunoassays. ELISA binding signal and ELISA background binding signal were plotted separately for liquid extracts from each analogue sample in order to also illustrate the impact that each sample matrix type had on non-specific binding signal.

The graphs in Figure 4-17, Figure 4-18 and Figure 4-19 show that ELISA binding signal was generated for each immunoassay in the presence of up to 50 % (v/v) liquid extracts from a range of Mars analogue samples including igneous rocks, clays and gypsum. This indicates that all of the primary and secondary antibodies used in these three immunoassays, and the streptavidin-HRP label, function in the presence of extractable components from the tested analogue samples.

For the majority of analogue samples tested, increasing the proportion of liquid extract included in the ELISA immunoassay had minimal effect on immunoassay binding signal and on non-specific background signal indicating that the immunoassays were robust to the extractable components contained within the analogue samples (note that no attempt was made to characterise the soluble components in the liquid extracts). For a number of the analogue samples tested there was some evidence of reduction in binding signal at high extract concentration and it is likely that increasing the concentration of liquid extract to 100 % (v/v), as is the case for the flight format assay, would reduce the signal further. It is unclear whether signal loss is attributable directly to dissolved components of the analogue samples, or due to feed through of sub-0.45 μm particulates which may adsorb some of the antibodies (Krotzky and Zeeh 1995), or a combination of both.

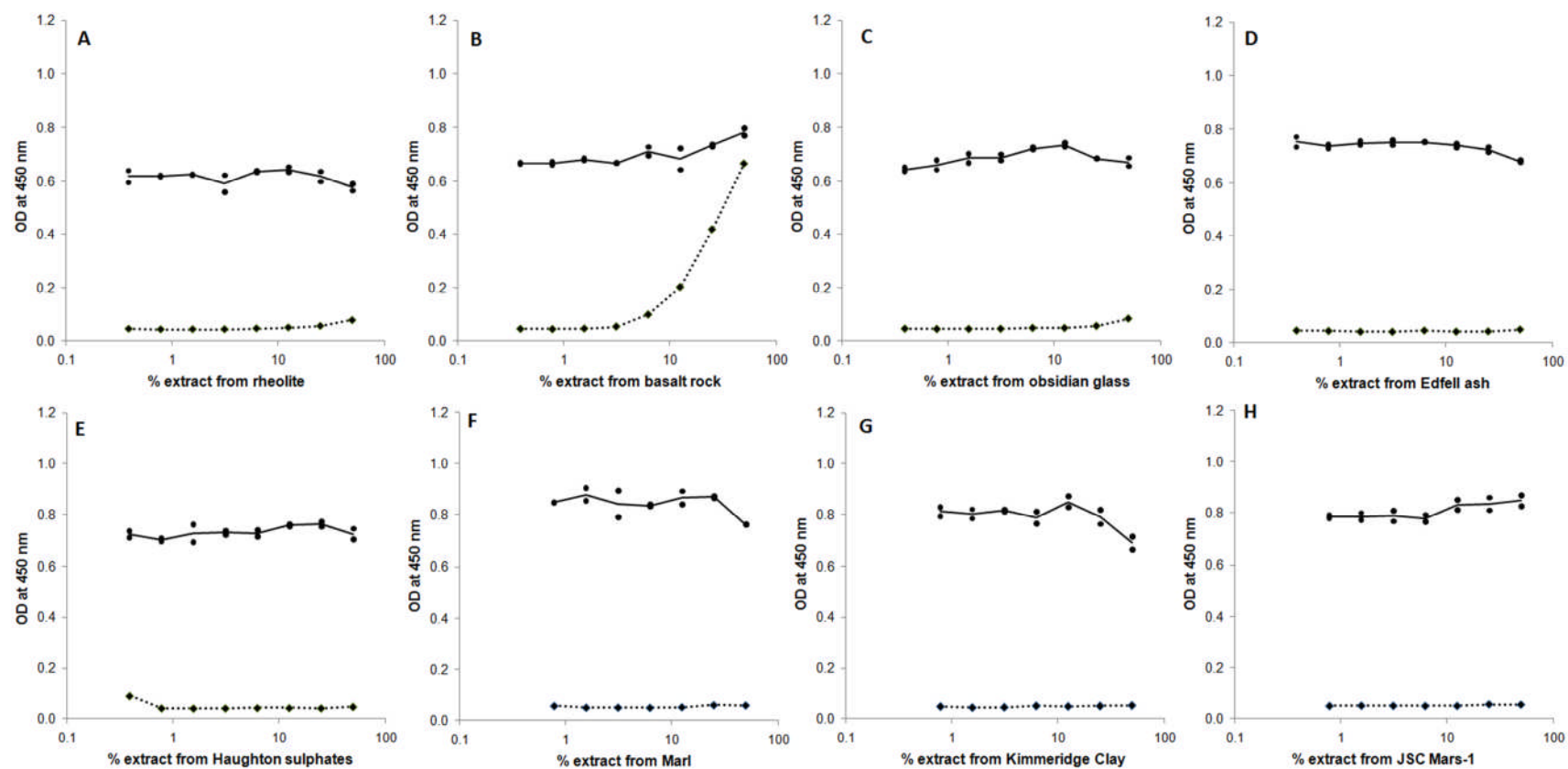


Figure 4-17: Benzo[a]pyrene binding ELISA performed in the presence of increasing concentration of liquid extract from (A) rheolite, (B) basalt rock, (C) obsidian glass, (D) Edfell ash, (E) Haughton Crater calcium sulphates, (F) Marl, (G) Kimmeridge clay and (H) JSC Mars-1. Data points are single replicates for ● binding to immobilised BSA-benzo[a]pyrene conjugate and ◆ non-specific binding to BSA. Lines are the linear interpolation of mean OD for (solid line) binding to immobilised BSA-benzo[a]pyrene conjugate and (dotted line) non-specific binding to BSA

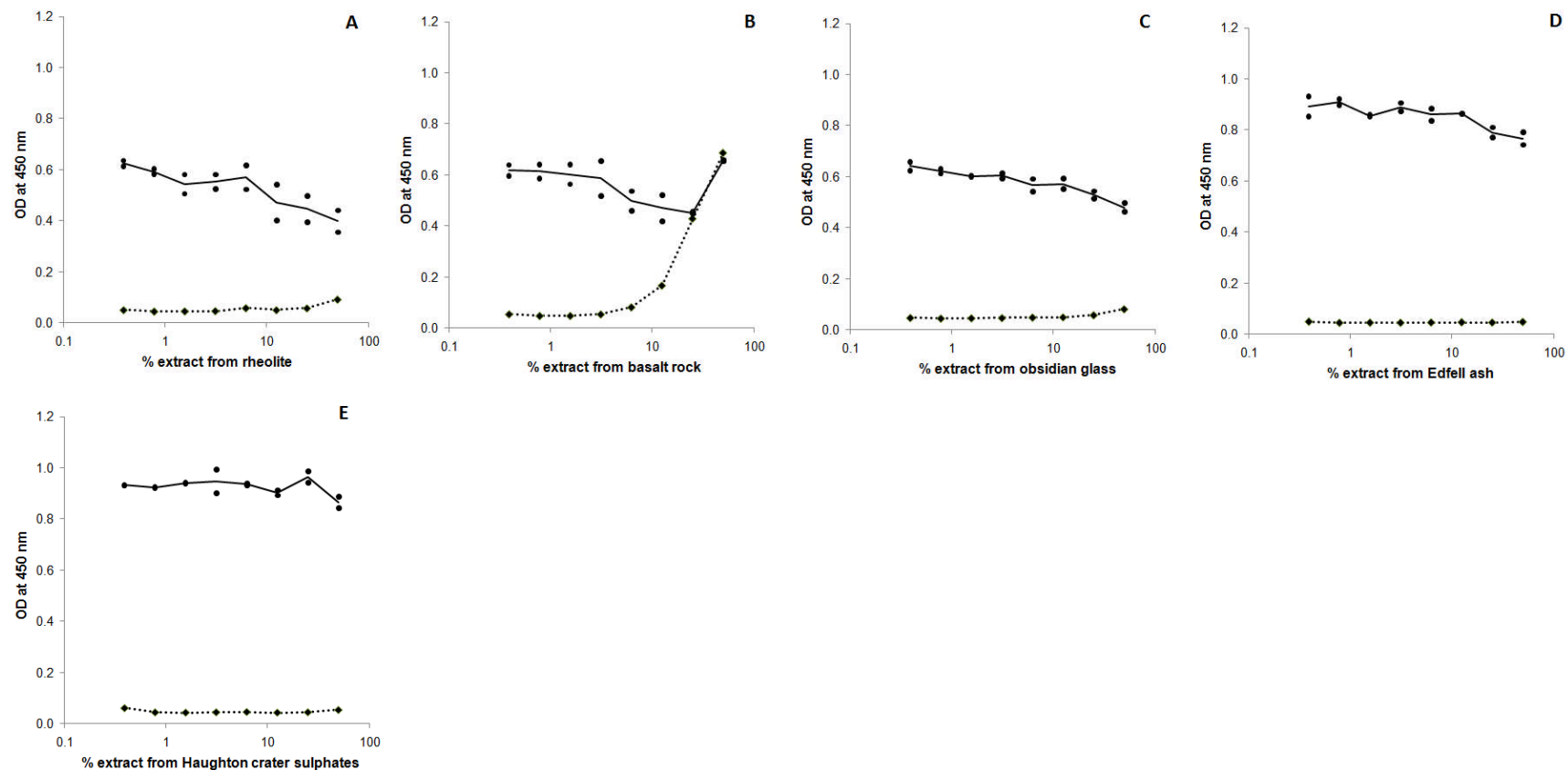


Figure 4-18: cAMP binding ELISA performed in the presence of increasing concentration of liquid extract from (A) rheolite, (B) basalt rock, (C) obsidian glass, (D) Edfell ash, (E) Haughton Crater calcium sulphates.

Data points are single replicates for ● binding to immobilised BSA-cAMP conjugate and ◆ non-specific binding to BSA. Lines are the linear interpolation of mean OD for (solid line) binding to immobilised BSA-benzo[a]pyrene conjugate and (dotted line) non-specific binding to BSA. Note that cAMP ELISAs were not performed with Marl, Kimmeridge Clay and JSC Mars-1 because this immunoassay had not yet been developed at the time of testing of these analogue samples.

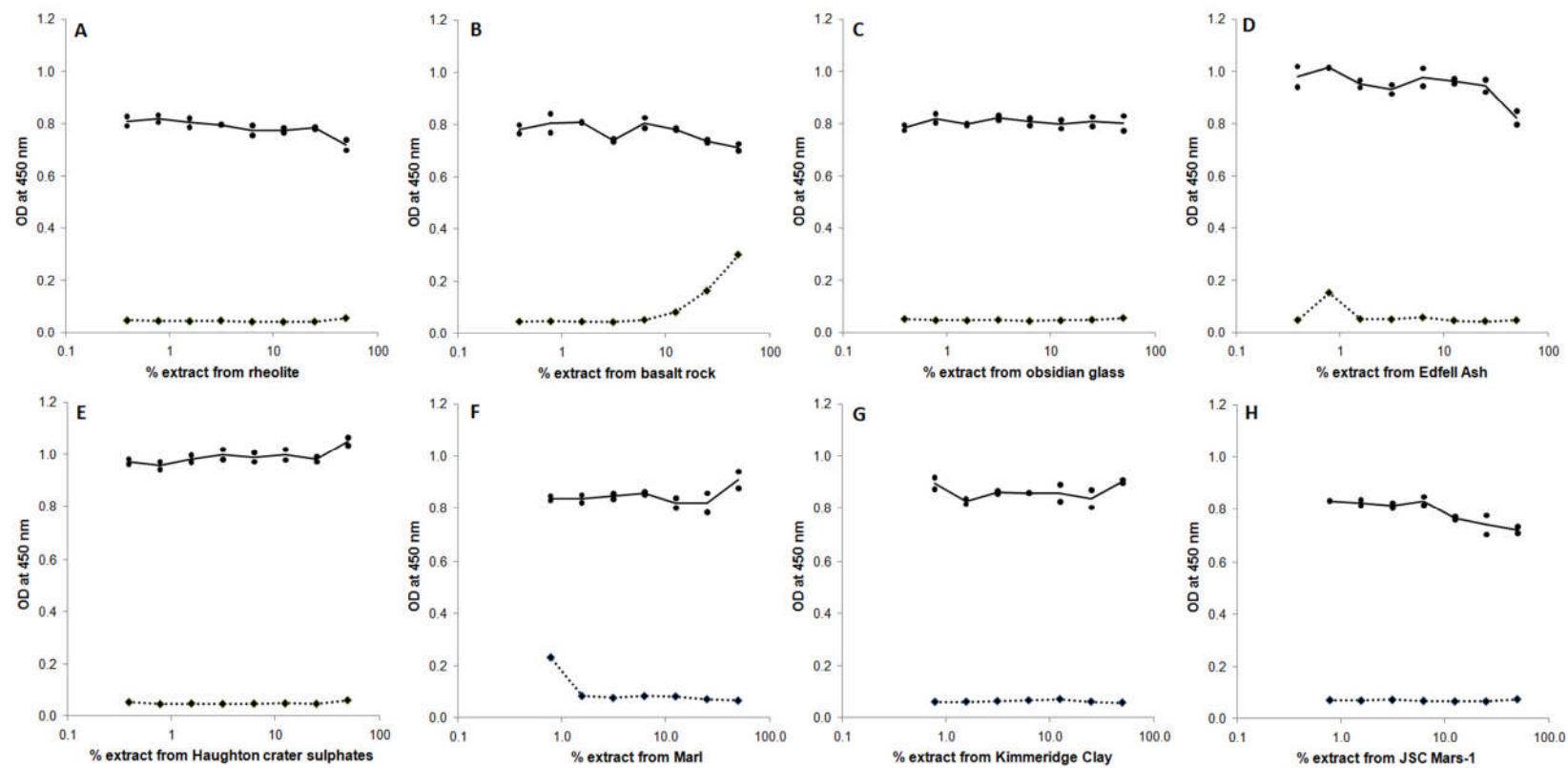


Figure 4-19: Fluorescein ELISA performed in the presence of increasing concentration of liquid extract from (A) rheolite, (B) basalt rock, (C) obsidian glass, (D) Edfell ash, (E) Houghton Crater calcium sulphates, (F) Marl, (G) Kimmeridge clay and (H) JSC Mars-1. Data points are single replicates for ● binding to immobilised BSA-FITC conjugate and ◆ non-specific binding to BSA. Lines are the linear interpolation of mean OD for (solid line) binding to immobilised BSA-benzo[a]pyrene conjugate and (dotted line) non-specific binding to BSA

For all three immunoassays liquid extract from basalt rock produced an increase in non-specific background signal at high extract concentrations (Graph B in Figure 4-17, Figure 4-18 and Figure 4-19). However when microarray format immunoassays were tested (see following section), no increase in non-specific background signal was observed, therefore increased ELISA background signal was most likely due to an interaction of extracted components, or sub 0.45 μm particulates, with the HRP enzyme rather than an interaction with the immunoassay antibodies. Since the HRP enzyme label is not used in the flight format immunoassay this is unlikely to be problematic for the LMC instrument.

4.6.3.2 Further studies to confirm impact of liquid extracts from a range of analogue samples on microarray format assays using binding signal as a performance indicator

In order to confirm whether the observations made in ELISA format were also valid in a format that more closely resembles the flight format, liquid extracts from selected Mars analogue samples was assessed in multiplexed pooled microarray format (Figure 4-20). Tests were performed using a single concentration of liquid extract, 50 % (v/v). This is the maximum concentration that can be tested using pooled microarray format since, as described previously for ELISA format assays, the antibodies and other immunoassay reagents for pooled microarray assays are not freeze dried and are in liquid format therefore resulting in dilution of the liquid extract.

The pixel intensities of microarray spots after running multiplexed pooled microarray immunoassays in the presence of 50 % (v/v) liquid extract from each analogue matrix are given in Figure 4-21, Figure 4-22 and Figure 4-23 for the benzo[a]pyrene, cAMP and fluorescein immunoassays and a representative fluorescent image is shown in Figure 4-20. As discussed in Section 4.5.3.2, there is inherent variation in microarray assay data, thought to be due to inconsistencies introduced by the current printing and pooled microarray assay protocols. Data from microarray assays must therefore be interpreted in the context of this variability.

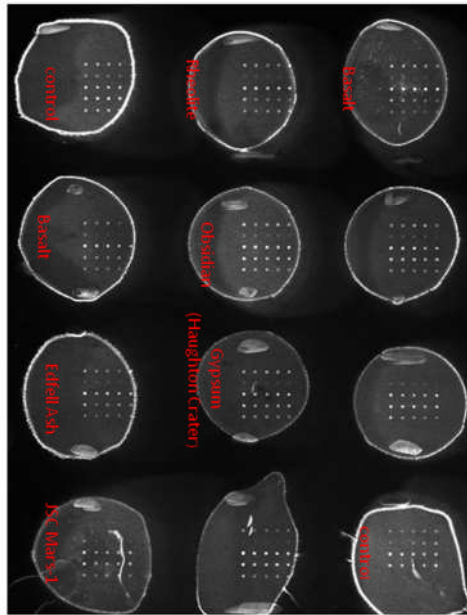


Figure 4-20: Representative fluorescent images of 12 microarrays after (right) running pooled multiplexed microarray format immunoassays in the presence of 50 % (v/v) liquid extracts from Mars analogue sample matrixes.

Microarrays printed onto commercial hydrophobic slide using ArrayIt® SpotBot, images recorded using Innopsys Microarray scanner. In images taken after running pooled microarray assays five rows of five spots are visible comprising from top to bottom low ratio AF633 labelled BSA, benzo[a]pyrene immunoassay spots, high ratio AF633 labelled BSA, fluorescein immunoassay spots and cAMP immunoassay spots. Image brightness and contrast have been optimised to aid visualisation of spots. Note that for two of the arrays the low ratio AF633 labelled BSA spots are missing from the image because they did not print. Microarray spots are 110 μm in diameter and are spaced 400 μm apart.

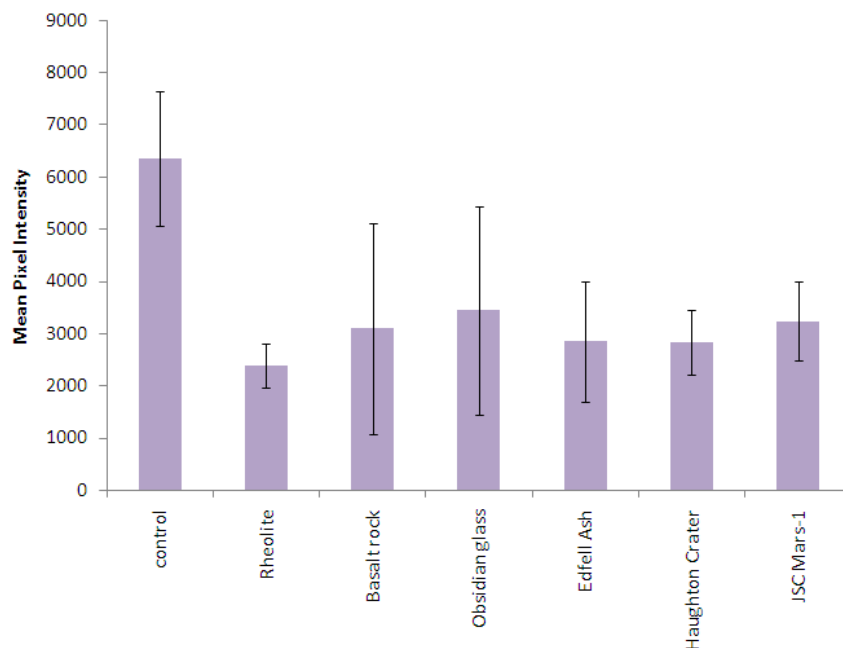


Figure 4-21: Mean pixel intensity of microarray spots for multiplexed pooled benzo[a]pyrene immunoassay run in 50 % (v/v) liquid extract from Mars analogue sample matrixes. Error bars = ± 1 standard deviation of the mean, $n = 5$

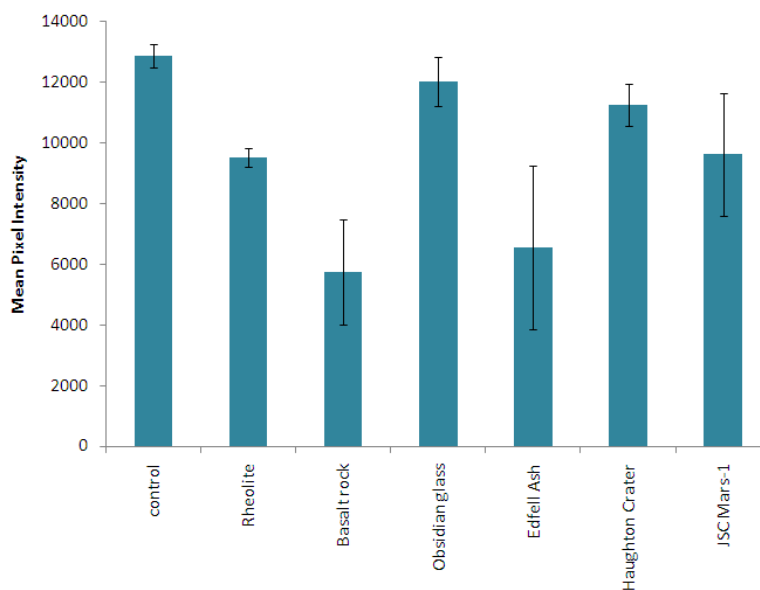


Figure 4-22: Mean pixel intensity of microarray spots for multiplexed pooled cAMP immunoassay run in 50 % (v/v) liquid extract from Mars analogue sample matrixes. Error bars = ± 1 standard deviation of the mean, n = 5

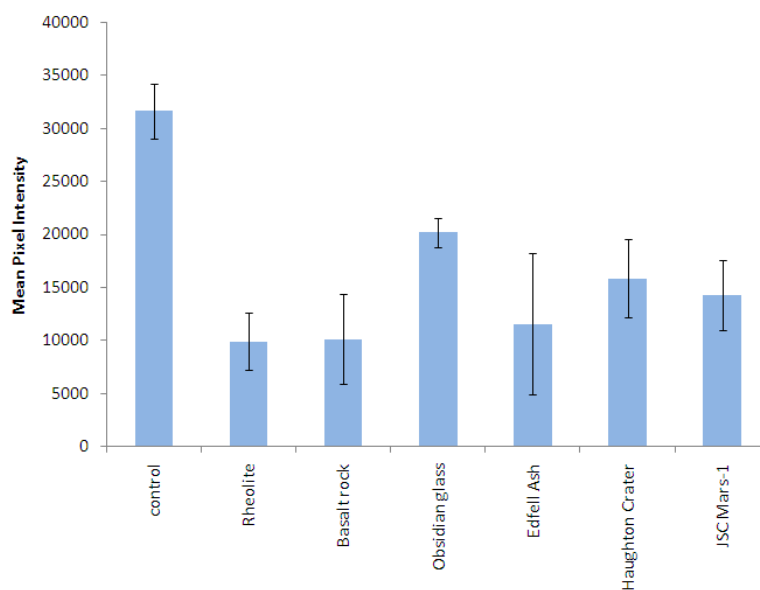


Figure 4-23: Mean pixel intensity of microarray spots for multiplexed pooled fluorescein immunoassay run in 50 % (v/v) liquid extract from Mars analogue sample matrixes. Error bars = ± 1 standard deviation of the mean, n = 5

Microarray spots for the benzo[a]pyrene, cAMP and fluorescein immunoassays all fluoresced after running multiplexed pooled microarray immunoassays in 50 % (v/v) liquid extract, indicating that all of the primary antibodies, secondary antibodies and the streptavidin-AF633 bound successfully in the presence of 50 % (v/v) liquid extracts. Note that in the flight format assay imaging is performed in a chamber containing the liquid extract, whereas in the pooled microarray format fluorescent imaging was performed after the liquid extract (and any associated particulates/precipitates) had been washed away. Therefore no comment can be made on the likely effect of the liquid extract components, including particulates, on fluorescent imaging. This aspect is further investigated in Chapter 6 of this thesis.

Binding signal was generally reduced in all of the liquid extracts tested compared to control microarrays run with extraction solvent only. Binding signal response varied across the three immunoassays tested with the cAMP immunoassay appearing to be most robust (*i.e.* exhibiting the smallest loss of binding signal) in the liquid extracts. The observed reduction in signal for all three immunoassays may be due to altered antibody performance as well as loss of immobilised target molecule-protein conjugate from the chip surface during incubation and washing procedures and the presence of particulates interfering with the immunoassay.

In microarray format the signal loss observed was generally greater than signal loss observed in ELISA format, but no issues with increased background signal were observed. Therefore for the flight format immunoassay some loss of signal intensity should be expected with real samples compared to with standards prepared in extraction solvent. The fact that immunoassays showed differing degrees of signal loss in liquid extracts from the same sample matrix further highlights the need to include a range of well characterised control assays in the flight instrument suite of assays.

4.6.3.3 Extended study with liquid extracts from a limited number of analogue samples using microarray assay sensitivity as a performance indicator

In order to investigate the impact of liquid extracts from analogue samples on immunoassay sensitivity, liquid extracts were spiked with target molecules and then run in pooled multiplexed microarray immunoassays. Pooled format microarrays were used in preference to ELISAs in this case due to the limited liquid extract volumes available. Figure 4-24 shows the mean pixel intensity for microarray spots when incubated with 50 % (v/v) liquid extracts from three Mars analogue samples spiked with varying concentrations of cAMP target molecule.

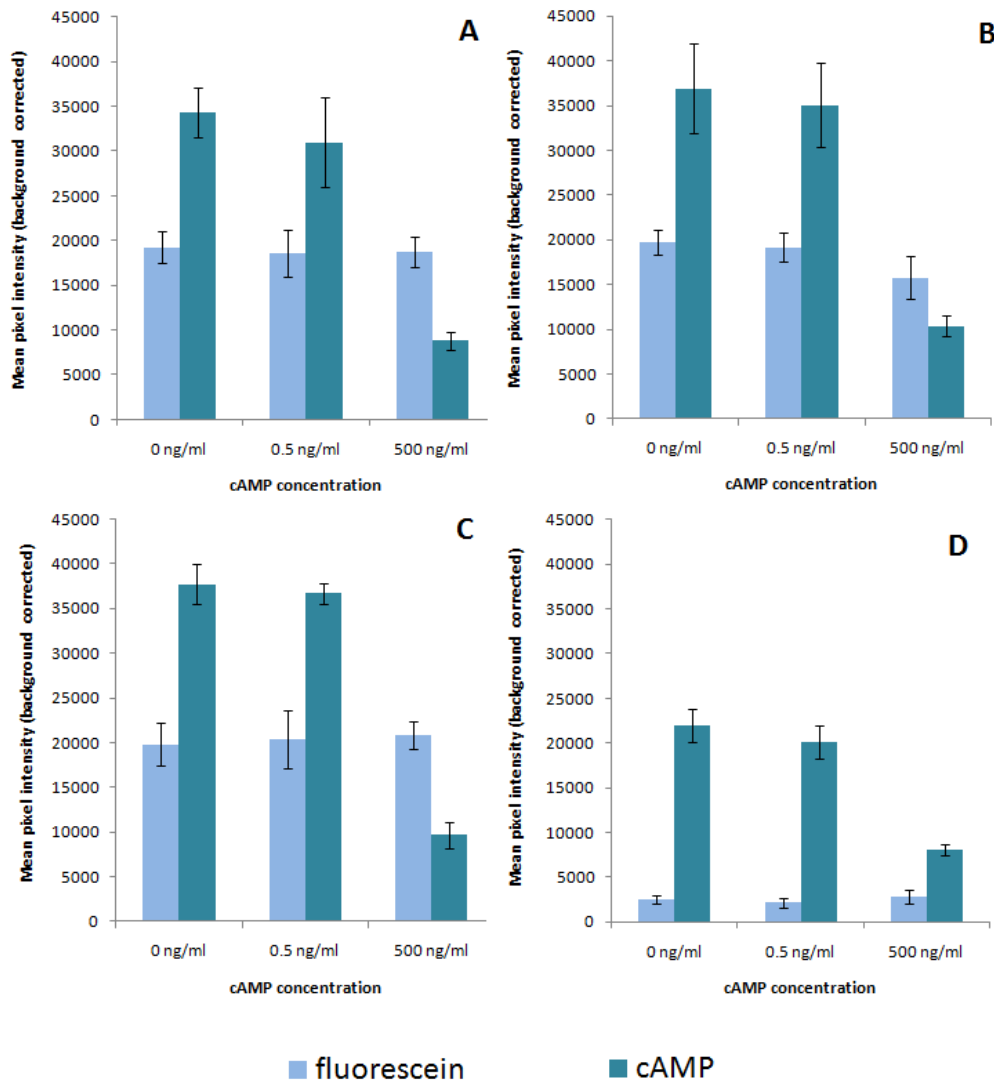


Figure 4-24: Mean background corrected pixel intensity for pooled multiplexed format fluorescein and cAMP microarray assays run with cAMP spiked liquid extracts from (A) control (extraction solvent only), (B) liquid extract from basalt rock, (C) liquid extract from gypsum (Houghton Crater), (D) liquid extract from JSC Mars-1. Error bars = ± 1 std deviation, $n = 5$

As the concentration of cAMP spiked into liquid extracts from basalt rock, gypsum and JSC Mars-1 was increased, signal for the cAMP immunoassay was inhibited confirming that the cAMP inhibition immunoassay functions in liquid extracts from these analogue samples (Figure 4-24). Signal for the fluorescein immunoassay was unaffected by increasing the concentration of cAMP spiked into the liquid extract, showing that there was no cross reactivity of the anti-fluorescein antibodies with the cAMP target molecule in any of the liquid extracts tested. (Figure 4-24). Note however that independently of the concentration of spiked cAMP, signal for the fluorescein assay was reduced by approximately 90 % in liquid

extracts from JSC Mars-1 compared to in extraction solvent and in liquid extracts from basalt rock and Haughton crater sulphates (Graph D in Figure 4-24).

In the flight format immunoassay if low signal was observed for a target molecule, but also for a control immunoassay, the likely interpretation would be that the signal loss for both assays was due to a generic matrix effect. However in the case shown here signal intensity in the fluorescein 'control' immunoassay was reduced by a matrix effect which did not affect the cAMP immunoassay to the same degree and loss of signal in the cAMP immunoassay was due to specific inhibition by the presence of cAMP target molecule spiked into the liquid extract. This result highlights the need to include a number of well characterised control immunoassays, with differing performance in sample matrixes, within the flight instrument, to reduce the risk of data misinterpretation.

Figure 4-25 shows the mean pixel intensity for microarray spots when incubated with liquid extracts from three Mars analogue samples spiked with varying concentrations of fluorescein target molecule. As the concentration of fluorescein spiked into liquid extracts from basalt rock, gypsum and JSC Mars-1 was increased, signal for the fluorescein immunoassay was inhibited demonstrating that the fluorescein inhibition immunoassay functions in liquid extracts from these analogue samples. Signal for the cAMP immunoassay was unaffected by increasing the concentration of fluorescein spiked into the liquid extract, showing that there was no cross reactivity of the anti-cAMP antibodies with the fluorescein target molecule in any of the liquid extracts tested. (Figure 4-25). Note however that independently of the concentration of spiked fluorescein, signal for the fluorescein assay was reduced by approximately 90 % in liquid extracts from JSC Mars-1 compared to in extraction solvent (Graph D in Figure 4-25).

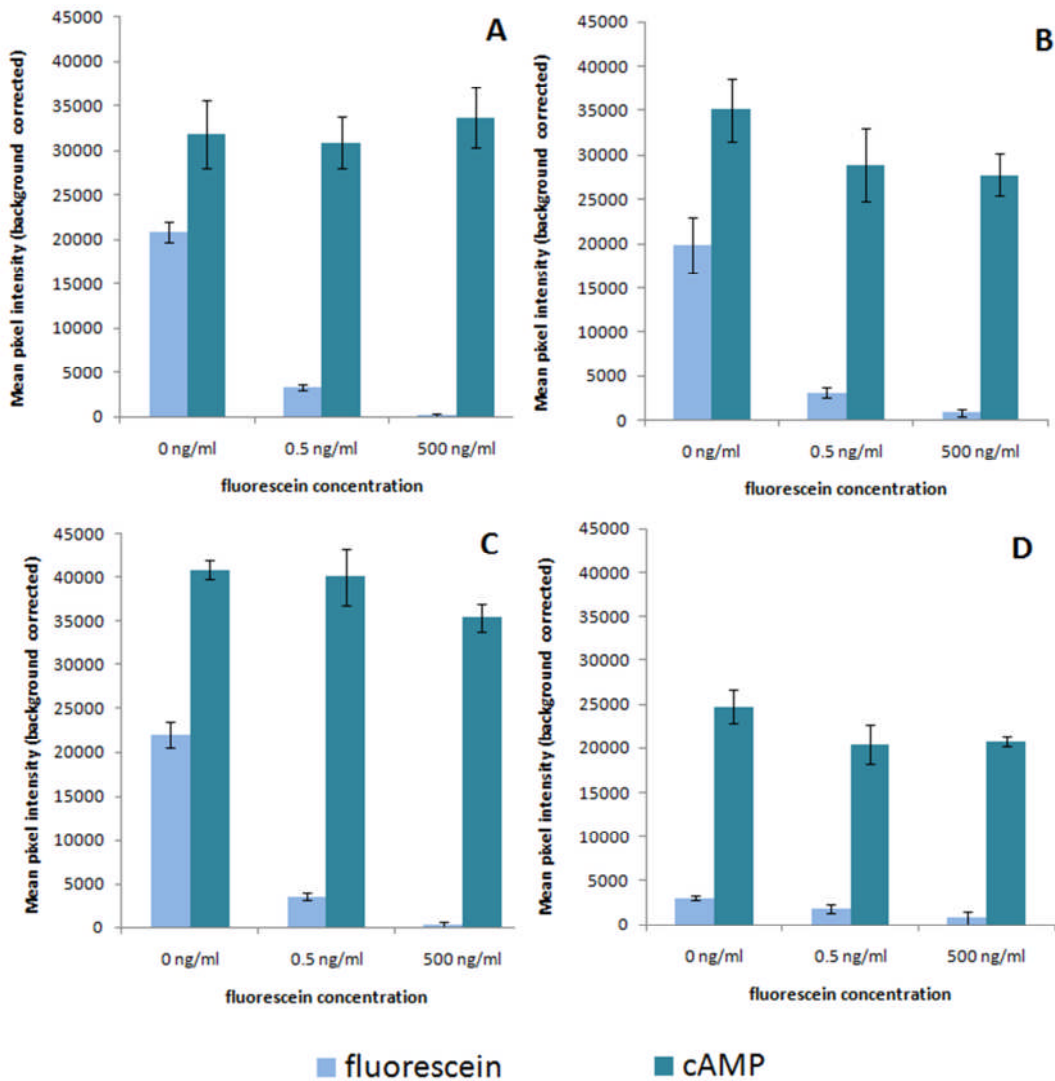


Figure 4-25: Mean background corrected pixel intensity for pooled multiplexed format fluorescein and cAMP microarray assays run with fluorescein spiked liquid extracts from (A) control (extraction solvent only), (B) liquid extract from basalt rock, (C) liquid extract from gypsum (Houghton Crater), (D) liquid extract from JSC Mars-1. Error bars = ± 1 std deviation, $n = 5$

Note that no attempt was made to calculate the IC_{50} of the fluorescein or cAMP microarray immunoassay in the liquid extracts due to the limited volume of liquid extracts available for spiking studies, therefore no specific conclusions can be drawn regarding how liquid extracts from Mars analogue samples effect immunoassay sensitivity, except to say that inhibition immunoassays do function in spiked liquid extracts and that no gross changes in sensitivity were apparent in the tested extracts from the limited concentration range tested.

4.6.3.4 Study 2 general discussion

The objective of the work reported in Study 2 was to test performance of a number of LMC relevant immunoassays with liquid extracts from Mars analogue sample matrixes in order to mimic the complex 'real' samples that may be encountered on Mars.

In order to achieve this, a selection of Mars analogue samples, selected on the basis of their relevance to Mars as well as by their availability within the time scales of this study, were extracted using an LMC representative liquid extraction with sonication and the liquid extracts were then added in to ELISA and microarray format immunoassays. In both ELISA and microarray format immunoassays some loss of binding signal was observed in all of the liquid extracts tested. Signal loss may be due to a number of factors including changes in antibody performance and adsorption of antibodies to particulate material contained within the liquid extract. Note that for all of the sample matrixes tested, although assay signal was reduced, it was not reduced to a level where it was no longer distinguishable from background, indicating that it should be possible to perform immunoassays in liquid extracts from samples such as those tested here. Different immunoassays showed varying susceptibility to signal loss in liquid extracts, which highlights the risk of misinterpreting non-specific matrix mediated signal loss as specific inhibition. The inclusion of a number of well characterised control assays in the flight instrument, with differing tolerances to sample matrix types, should help to mitigate this risk. As antibody immunoassays are developed it is important that their performance in extracts from analogue samples is characterised at an early stage as it may be necessary to (i) deselect antibodies/assays which show poor tolerance for sample matrix types likely to be encountered on Mars and (ii) to include appropriate controls for each assay.

A limitation of this study was that ELISA and pooled microarray format immunoassays were performed in a maximum concentration of 50 % (v/v) liquid extract, whereas the flight format assay will be performed in 100 % liquid extract. Additionally in the flight format immunoassay the extract will be pumped through microfluidic channels and across the surface of the microarray therefore future studies should be performed in a more flight representative format to confirm whether the use of 100 % liquid extract and the physical flow of liquid extract across the microarray surface, will have a significant impact on binding signal. This aspect is investigated further in Chapter 6 of this thesis.

There are many possible geologies that may be encountered on Mars and this study considered only a small selection of analogue samples, which by no means encompass all possible types of sample that may be encountered by the ExoMars rover. Therefore the

findings of this study are preliminary and extensive future testing must be carried out with a wider range of analogue samples. Once the landing site for ExoMars is selected, more information as regards the likely geology of samples to be encountered by the ExoMars rover will be available, but landing site selection is unlikely to occur until after the LMC instrument is integrated within the ExoMars rover (current schedule for instrument delivery is early 2015) therefore as broad a range of samples should be tested in advance of this. Potential sources of a wide variety of samples with Mars relevant geologies have been identified and include the European Space Analogue rock collection (ESAR) (Westall *et al*, 2011). No attempt was made in this study to characterise the soluble components that were released from the tested sample matrixes. In future studies it would be useful to have this information in order to be able to correlate performance in liquid extracts from analogue samples with the extractable components of that analogue sample.

The group developing the SOLID instrument have published a number of studies describing analysis of Mars analogue samples including ferric iron enriched waters and sediments from the Rio Tinto, goethite, hematite and jarosite, sands and clays. They found that these analogue samples had no effect on assay performance, with the exception of clay type samples which reduced assay signal (Parro *et al*, 2005). This contrasts with the findings of the study performed here with LMC relevant immunoassays, where all of the sample matrix types tested produced a reduction in immunoassay signal intensity. There are a number of differences between the SOLID immunoassay and the LMC immunoassay. The most significant of these differences is that SOLID immunoassays are predominantly performed in sandwich format for the detection of markers of extant life *e.g.* large biological molecules such as proteins. In the sandwich format SOLID assay liquid extracts are directly incubated over a covalently immobilised microarray of antibodies. The sample is then washed away and a second set of fluorescently labelled antibodies are incubated in buffer with the microarray. Therefore only one of the immunological interactions required for signal generation in SOLID occurs in the liquid extract, which may be beneficial in minimising sample matrix mediated effects compared to the LMC, where the entire assay is conducted directly in the liquid extract. However a sandwich assay format is unsuitable for the LMC since many of the target molecules for the LMC, including high science priority markers of extinct life, are small molecules and therefore cannot be detected using a sandwich assay. The use of covalent immobilisation, like that used in the SOLID instrument, may be beneficial for the LMC and this is being investigated as part of the package of work implemented to improve microarray reproducibility.

A further difference between SOLID and the LMC is that the extraction buffer used by SOLID is composed of 0.4 M Tris HCl pH 8 with 0.3 M NaCl and 5 % BSA and the buffering capacity is therefore likely to be superior to that of the LMC (100 mM HEPES buffer included in freeze drying cocktail). This may partly explain why SOLID format immunoassays are more robust when used to analyse real samples than LMC format immunoassays. Improvement of the buffering capacity of the LMC was identified as a possible route to improving LMC performance in Study 1 within this chapter, but buffer concentration is likely to be constrained due to the sparingly soluble nature of markers of extinct life and the risk of precipitation in solutions with high buffer salt concentrations.

Summary

In summary ELISA and pooled microarray format immunoassays function in liquid extracts from a range of Mars analogue samples including clays, basalts and gypsum. However signal intensity was generally lower compared to signal in assays run with extraction solvent only as a control. Different immunoassays showed differing degrees of signal loss in the same liquid extract further highlighting the need to include a range of well characterised control assays with differing performances in different sample matrix types. Opportunities for improving assay performance were identified, including increasing the buffering capacity in the LMC freeze drying solvent and investigating covalent immobilisation of target molecules/target molecule-protein conjugates to chip surfaces.

4.7 Study 3: the impact of perchlorate salts on LMC extraction and detection⁸

The work performed in Study 1 has shown that LMC format immunoassays can in principle function in the presence of up to 100 mM perchlorate added directly into standards. This implies that perchlorate containing samples, similar to those encountered by the Phoenix lander, are unlikely to detrimentally affect LMC format immunoassay detection of target molecules. However Study 1 made no attempt to address the effect that perchlorates salts might have on target molecules during the LMC extraction step, and the resulting impact that this might have on the immunoassay. As discussed in Section 4.3.2.5 perchlorate salts can act as oxidising agents at high temperatures, and their presence on Mars may account for the failure of the GC-MS on Viking and TEGA on Phoenix to detect any organic molecules in martian samples (Navarro-González *et al*, 2010b). Under the relatively mild conditions of an LMC liquid extraction, perchlorate is not expected to act as an oxidising agent. This study was designed to extend the work carried out in Study 1 by investigating the effect of perchlorate salts on combined LMC liquid extraction and immunoassay detection to determine whether the LMC is capable of extracting and detecting organic molecules from perchlorate containing analogue samples.

4.7.1 Supplementary experimental design

In order to confirm that organic molecules can be extracted and detected from spiked samples in the presence of perchlorate salts a martian simulant regolith, JSC Mars-1, was spiked with pyrene and perchlorate, extracted with LMC solvent and the extracts were analysed by immunoassay. Pyrene was selected as an LMC relevant model target for this study because pyrene is an abiotically produced organic molecule, which has previously been detected in meteorites (Sephton and Botta 2005) and may have been delivered to the martian surface by meteoritic in-fall. JSC Mars-1, a martian simulant regolith developed by the Johnson Space Center (Houston, USA) (Allen *et al*, 1997), was selected for use in this study because it is commonly used in studies where a martian analogue is required (Court *et al*, 2010; Gough *et al*, 2010; Kral *et al*, 2011).

⁸ Note that the work in this study forms the basis for an article which has been published in *Astrobiology* (Rix, 2011)

4.7.2 Supplementary materials and methods

The following sections describe materials and methods used for the work within this study supplementary to those described in Chapter 2 and where appropriate cross-references to the relevant sections in Chapter 2 are provided.

4.7.2.1 Spiking of Mars analogue with target molecule and perchlorate salts

The martian simulant regolith JSC Mars-1 (used as supplied) was spiked with pyrene in methanol, to a final concentration of 500 µg pyrene/g simulant regolith. This level was chosen in the combined context of the expected extraction efficiency, the known sensitivity of the model immunoassay used, and an additional factor to produce extracts that gave 100% immunoassay inhibition in the control samples. It is noted that this results in a higher level of pyrene than could be expected on Mars. To achieve this, 500 µl of 1 mg/ml pyrene in methanol was added to 1 g of JSC Mars-1 in a 7 ml glass tube, mixed by vortexing, and allowed to stand at room temperature for approximately 30 minutes. The JSC Mars-1, which consisted of a free flowing granular matrix when dry, formed into clumps when wetted with solvent. After standing, methanol was removed by evaporation; vials were loosely sealed and placed in an oven set at a temperature of +37°C overnight. After approximately 18 hours, the JSC Mars-1 had reverted to a free flowing granular matrix. Control samples were spiked with methanol containing no pyrene and mixed and dried in the same way as the pyrene spiked samples.

After spiking with pyrene, JSC Mars-1 was spiked with 1 weight percent perchlorate anion by addition of solid calcium perchlorate tetrahydrate (Sigma Aldrich, Poole, UK, cat no. 401420) or magnesium perchlorate hexahydrate (Sigma Aldrich, Poole, UK, cat no. 309303) to the dry simulant regolith. This concentration is slightly in excess of that detected by the Phoenix lander (Hecht *et al*, 2009). Fifteen and seven-tenths mg of calcium perchlorate or 16.7 mg of magnesium perchlorate (both fine crystalline powders, used as supplied) were weighed into 7 ml glass vials, and 1 g of dried pyrene-spiked JSC Mars-1 was transferred into each vial and mixed well by inversion and by vortex mixer.

4.7.2.2 Extraction and immunoassay of spiked samples

Three ml of 20/80 % (v/v) methanol/water with 1.5 g/L polysorbate 80 was added to 1g JSC Mars-1 in a glass test tube and sonicated for 20 minutes with a Sonotrode Ultrasonic UP100H probe and UP50H ultrasonic processor set at 40 % amplitude (Hielscher Ultrasonics, Teltow, Germany). Over twenty minutes of sonication the temperature of the

solution increased to above ambient temperature but less than 50°C. This extraction protocol simulates the baseline extraction protocol for the LMC. The mixture was allowed to stand for 10 minutes, and the cloudy supernatant that had formed above the sedimented analogue sample was decanted into 1.5 ml centrifuge tubes. To minimize loss of extracted components by adsorption to surfaces of the hydrophobic polymer centrifuge tubes, the tubes were first blocked with 0.5 % (w/v) BSA in 100 mM HEPES pH 7.4. The supernatants were clarified by centrifugation at 17000 G for 10 minutes and stored at 2 – 8°C in glass vials until tested in the immunoassay.

After centrifugation, it was observed that extracts from 500 µg/g and 0 µg/g pyrene spiked JSC Mars-1 samples with 1 weight percent calcium or magnesium perchlorate were colourless liquids, whereas extracts from 500 µg/g and 0 µg/g pyrene spiked JSC Mars-1 samples with no added perchlorate were yellow in colour (Figure 4-26).

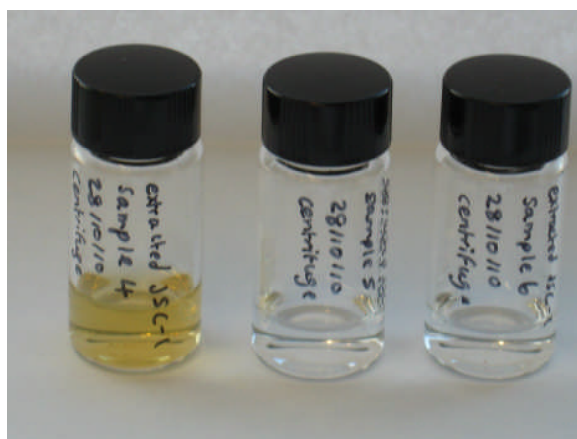


Figure 4-26: Photograph of extracts from pyrene spiked JSC Mars-1 samples. JSC Mars-1 spiked with 500 µg/g pyrene and (from left to right) spiked with no perchlorate, 1 wt % calcium perchlorate and 1 wt % magnesium perchlorate prior to extraction.

Note that this yellow coloration had not been observed with the liquid extracts from JSC Mars-1 prepared in Study 2, but that the extracts from JSC Mars-1 in Study 2 had been clarified by filtration through a cellulose acetate filter, rather than by centrifugation as was used in this study.

Liquid extracts were analysed using an LMC format ELISA as described in Chapter 2 (Section 2.1.1.1) maintaining a final concentration of 20/80 % (v/v) methanol water with 1.5 g/L P80.

4.7.3 Results and discussion

Figure 4-27 shows the immunoassay curves produced by running dilutions of extracts from spiked samples in an LMC format ELISA: Extracts from six samples comprising combinations of pyrene spike level (0 or 500 µg/g) and perchlorate spike (no perchlorate or 1 wt % calcium perchlorate or 1 wt % magnesium perchlorate).

Immunoassays with extracts from JSC Mars-1 sample spiked with 500 µg/g pyrene and 1 wt % perchlorate (Figure 4-27a) all showed decreasing immunoassay signal as extract concentration was increased, which implies that pyrene was extracted from the spiked JSC Mars-1 and detected in the immunoassay. This conclusion is further supported by examination of the immunoassay curves produced with extracts from JSC Mars-1 spiked with 0 µg/g pyrene and 1 wt % perchlorate (Figure 4-27b), where no reduction in immunoassay signal was observed with increasing extract concentration. This suggests that the reduction in immunoassay signal produced with extracts from 500 µg/g pyrene and 1 wt % perchlorate spiked JSC Mars-1 was not caused by either the perchlorate salts or by an interfering substance extracted from the JSC Mars-1 simulant regolith. Therefore, these results indicate that the presence of martian relevant levels of perchlorate salts in a well studied martian analogue sample matrix had no significant effect on LMC representative combined sample extraction and inhibition immunoassay detection of a representative small organic molecule LMC target (pyrene).

At high (> 6%) concentrations of extract from JSC Mars-1 spiked with 500 µg/g pyrene but no perchlorate however, immunoassay signal began to increase with increasing extract concentration. In addition to this, the immunoassay curve produced with the extract from the un-spiked sample (no pyrene and no perchlorate) (Figure 4-27b) showed a gradual reduction in binding signal at high extract concentrations which was not observed with extracts spiked with perchlorates (no pyrene). The extract concentration over which this reduction in signal was observed was the same as the concentration that produced anomalous results for pyrene spiked samples (no perchlorates) (Figure 4-27). This observation suggests a common underlying effect.

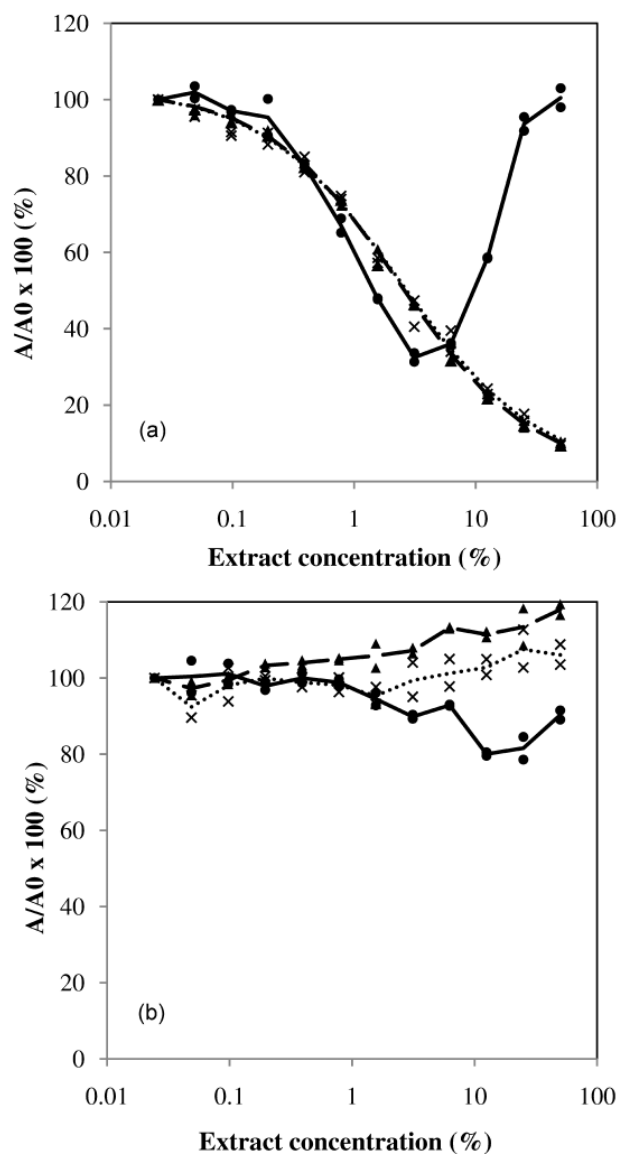


Figure 4-27: Pyrene inhibition ELISA run with extracts diluted in 20/80 % (v/v) methanol/water with 1.5 g/L polysorbate 80. Extractions performed on JSC Mars-1 spiked with (a) 500 µg/g pyrene (b) 0 µg/g pyrene and in each graph, (●) no perchlorate, (x) 1 wt % calcium perchlorate and (▲) 1 % wt magnesium perchlorate.

Each data point represents a single replicate. OD values (A) were normalised against maximum binding signal with 0 % extract (A₀). Plotted immunoassay curves are the fit to a four parameter binding equation, or where such a fit was deemed inappropriate; curves to show trends in the data were drawn by using linear interpolation between the mean normalised OD values. (solid line) no perchlorate, (dotted line) 1 wt % calcium perchlorate, and (dashed line) 1 wt % magnesium perchlorate

The immunoassay curve produced with extract from JSC Mars-1 spiked with 500 µg/g pyrene but no perchlorate (Figure 4-27a) is significantly, and unexpectedly, different from all the other relevant immunoassay curves. At low extract concentrations, the immunoassay curve is similar to that for extracts from 500 µg/g pyrene and 1 wt % perchlorate spiked JSC Mars-1, that is, immunoassay signal decreased as the extract concentration was increased.

It was noted that the anomalous behaviour correlated with the yellow coloration of the extracts that only occurred from samples where no perchlorate was added (Figure 4-26). It is therefore inferred that the substance responsible for the yellow colour of the extract, from samples that had no perchlorate, caused the anomalous changes in immunoassay signal intensity. Since the final immunoassay readout of the HRP enzyme label was carried out after the yellow coloured extract was washed away, any optical absorption due to the yellow coloration can be discounted. The conclusion is that the species producing the yellow colour interacted with the immunoassay reagents by an as yet unidentified mechanism. To better understand the absence of the yellow coloration in extracts from the perchlorate containing samples, further investigations were carried out. It was observed that when perchlorate or chloride salts were directly added to a yellow coloured extract, precipitation of a fine yellow solid occurred. This yellow solid could be removed by centrifugation to give a colourless extract. Therefore, in extracts from JSC Mars-1 spiked with 1 wt % perchlorate, the inference is that the species producing the yellow colour was precipitated due to the presence of the perchlorate salts and removed during clarification of the extracts by centrifugation, which resulted in colourless extracts.

The mechanism for this observation is unknown, but it is unlikely to be pH related given that the pH of both the yellow and colourless extracts were similar to one another. Studies have not been performed to date with other Mars relevant salts to confirm whether this observation is perchlorate salt specific or a more general effect due to presence of salts. It should be noted that this anomalous behaviour was not observed in Study 2 when ELISAs, including the benzo[a]pyrene immunoassay, were tested in liquid extracts from JSC Mars-1. This discrepancy could be a result of the different methods used to clarify the liquid extracts in the two studies *i.e.* in Study 2 liquid extracts from JSC Mars-1 were clarified by 0.45 µm cellulose acetate membrane filtration whereas in this study liquid extracts from JSC Mars-1 were clarified by centrifugation.

In addition to the anomalous changes in binding signal, the slopes of the curves produced with the three extracts from pyrene spiked JSC Mars-1 with and without perchlorate salts (Figure 4-27) also differ in that the IC₅₀ value for the extract from a sample spiked with pyrene but no perchlorate was 50 % lower than for extracts from samples spiked with pyrene and calcium perchlorate or magnesium perchlorate. The IC₅₀ values were calculated from the immunoassay data for extracts from the samples spiked with both perchlorate and pyrene, as described in Section 2.2.2, which resulted in values of 2.4 % extract concentration for the calcium perchlorate spiked sample and 2.3 % extract concentration for the magnesium perchlorate spiked sample. The immunoassay data for extracts from the

sample spiked with pyrene but with no perchlorate was not appropriate to fit to a four-parameter binding model. Nevertheless, IC_{50} was estimated by using only values of extract concentration below 6%, which resulted in an IC_{50} value of 1.0 % extract concentration.

This observation could be a result of changes in the immunoassay or changes in the extraction, or both, and it is not possible to determine which from this study. One possibility is that some pyrene may have been lost from the extract during the precipitation of the yellow solid in extracts from perchlorate spiked samples, but retained in the extracts from samples with no perchlorate, where no precipitation of the yellow solid occurred.

4.7.3.1 Study 3 General discussion

In Section 4.7 a representative immunoassay (benzo[a]pyrene) was used to demonstrate the extraction and detection of pyrene, an example of a marker of abiotic meteoritic infall, in the presence of Mars relevant concentrations of calcium and magnesium perchlorate salts. While the main objective of this study was met, that is, to demonstrate that immunoassays can be performed in the presence of martian levels of perchlorate, including an LMC representative extraction step, an unexpected sample matrix effect was observed in the absence of perchlorate. The results of this study highlight that there is a difference between performing LMC extractions and immunoassays with JSC Mars-1 spiked with and without perchlorate; the addition of perchlorate salts prevented a component of the JSC Mars-1 from interfering with the immunoassay, but also doubled immunoassay IC_{50} . This warrants further investigation, but JSC Mars-1 is only representative of one class of a diverse range of possible sample matrixes that may be encountered on Mars. Therefore, it is unlikely that this sample matrix effect will be present in all cases.

Future work will be carried out in microarray format utilizing fluorescent labels to clarify whether the observed changes in immunoassay performance are related to the HRP enzyme label, or are true changes in antibody affinity. This may result in the effects observed with the yellow coloured extracts becoming negligible. The LMC flight instrument will not incorporate a centrifugation step; rather the martian extract will be filtered through a 0.45 micrometer pore-size filter prior to introduction into the microfluidic channel. Thus, it is important to understand the implications of the findings of this study in a more flight representative format.

Recent work published by Parro *et al* (2011) describes no significant effect of 50 mM perchlorate on the liquid extraction and subsequent fluorescent microarray immunoassay detection of large biological molecules, using sandwich format assays, from spiked samples

in the presence of up to 50 mM perchlorate. The work within this study, extends the work of Parro *et al* by specifically testing the impact of perchlorates on the extraction and detection of small organic molecules in LMC relevant formats.

The LMC instrument will include up to 25 immunoassays; therefore, further work is required to determine whether the findings also apply to other LMC relevant immunoassays, and other Mars relevant sample matrixes. In addition, it should be taken into account that a spiked sample may not effectively mimic a real sample where organic molecules may be incorporated differently into the matrix; therefore, testing with natural samples spiked with martian relevant levels of perchlorate will be carried out. Ideally, trials would be performed with natural samples with naturally occurring perchlorate salts, but there are no known terrestrial samples available with martian relevant levels of perchlorates.

4.8 Chapter conclusions and recommendations for future work

The aim of the work reported in this chapter was to investigate the potential interaction of the Mars sample matrix with the LMC immunoassay in order to identify risks for operation of the LMC instrument and to enable the development of mitigation strategies. To achieve this, the impact of individual likely Mars sample matrix components and liquid extracts from a number of Mars analogue samples on LMC relevant immunoassays was tested. In addition a further study was carried out to specifically test the impact of perchlorate salts on combined LMC extraction and detection. From the work carried out in the three studies within this chapter it is possible to draw a number of conclusions and also to make recommendations relating to the future program of work for the LMC.

Work carried out in ELISA and microarray formats has demonstrated that the LMC immunoassays are likely to function with martian samples similar to those encountered by the Phoenix lander, however some of the samples encountered by MER may be more challenging for the immunoassays, particularly if all of the salt component of these samples is extractable. This highlights the need for future work to better understand the likely solution chemistry of martian samples by characterising the solution chemistry of a range of Mars analogue samples and correlating the solution chemistry data with immunoassay performance data.

A further conclusion that can be drawn from work carried out with Mars relevant sample matrix components is that the buffering capacity in the freeze drying buffer is insufficient to control pH of the immunoassay when components such as iron ions are dissolved in the LMC extraction solvent. Therefore a further piece of future work had been identified to

investigate increasing buffering capacity, however this may be limited by factors such as solubility of buffer salts and the risk of precipitation within the microfluidic channels of the LMC. Other opportunities identified to improve assay performance under high/low pH include the investigation of covalent immobilisation of immobilised target molecules to the waveguide surfaces as acidic salts were shown to remove immobilised target molecules from 96-well plates.

Immunoassays run with liquid extracts from martian samples are likely to have reduced signal intensity compared to immunoassays run with standards prepared directly in extraction solvent. In addition different immunoassays may show different levels of signal loss in liquid extracts from martian samples with specific geologies. There is therefore the need to characterise the performance of antibodies at an early stage in their development for the LMC, in order to exclude antibodies with particularly poor performance and to allow the identification of appropriate control assays, to mitigate the risk of data misinterpretation. This has been incorporated into the standard assay development procedure adopted within the LMC consortium.

Iron ions, which may be present in liquid extracts from martian samples, reduce fluorescent signal. However their effect can be reduced by washing away iron ions prior to excitation and imaging. Therefore in order to mitigate the risk of fluorescent signal loss due to iron ions, or any other species from the martian sample, the liquid extract within the array chamber of the flight instrument should be replaced with fresh extraction solvent, prior to excitation and imaging of the microarray. This is possible with the current flight model design and this capability should be retained in any future design iterations.

Both LMC immunoassay detection and the LMC low temperature aqueous extraction with ultra-sonication are not negatively influenced by the presence of perchlorate salts in martian samples at levels similar to those detected at the NASA Phoenix landing site. These results increase confidence in the LMC instruments capability to detect small organic molecules on Mars in perchlorate rich samples. The testing of further combinations of immunoassay and sample matrices will be performed to validate the assumption that the model target molecules and analogue sample used in this work are broadly representative and will include testing within a multiplexed microarray immunoassay format.

It is likely that even with improved buffering capacity and optimised immobilisation stability some samples will be unsuitable for analysis. The LMC instrument is only capable of analysing four samples therefore it is of critical importance to understand how data from the other ExoMars instruments can be used to identify samples that may be unsuitable for

analysis, due to likely sample matrix effects. Other ExoMars instruments that may provide useful information on the geological context of samples are PanCam, a multi-spectral imager, MicroOmega, a microimaging system consisting of a visible light microscope and a near IR imaging spectrometer, XRD an X-ray diffractometer and fluorescence spectrometer, and the onboard Ramen spectrometer. It is therefore recommended that prior to flight the suite of ExoMars instruments should be used to analyse multiple diverse analogue samples to enable correlation of datasets regarding the geology of the samples with LMC instrument performance.

5 Generation of Antibodies for the Detection of Extinct Life Biomarker Targets: Use of an Immunised Recombinant Antibody Library

5.1 Introduction and context

Although interest in the use of antibodies for the detection of markers of life in planetary exploration is growing (McKay *et al*, 2000; Steele *et al*, 2001; Vandenabeele-Trambouze *et al*, 2002; Parro *et al*, 2005; Schweitzer *et al*, 2005), there are few examples of antibodies developed specifically for planetary exploration applications. However there are antibodies which have been developed for use in other fields, e.g. in microbiology, and in the detection of environmental pollutants, that recognise markers of extant life, markers of meteoritic in-fall and markers of contamination and may therefore be suitable for use in the LMC instrument. Critically there are very few antibodies which recognise markers of extinct life, which are high priority science targets for the LMC because if life did exist during warmer and wetter periods on Mars it is considered unlikely that it could survive under current cold and dry martian conditions (Knoll *et al*, 2005; Bibring *et al*, 2006). Therefore there is a need to develop antibodies that recognise markers of extinct life for the LMC instrument. Extinct life markers are generally small, hydrophobic and chemically inert organic molecules therefore the production of antibodies that recognise these types of molecule is expected to be challenging.

The LMC aims to detect up to 25 target molecules or molecular species, including a number of markers of extinct life. Prior to this thesis, the LMC science team identified five extinct life markers (Figure 5-1) as preliminary targets for the production of antibodies. The selection of these targets was a pragmatic compromise based on:

- priority for the ExoMars mission as defined at a workshop held in 2006 to identify and prioritise targets for the ExoMars mission (Parnell *et al*, 2007)
- the availability of suitable materials for the production of immunogens (since extinct life markers are too small to illicit an immune response unless conjugated to an immunogenic carrier protein)
- an attempt to select a range of different types of structure to more broadly represent all molecular classes encompassed by extinct life biomarker targets for the LMC (acyclic aliphatic, cyclic aliphatic and cyclic aromatic)

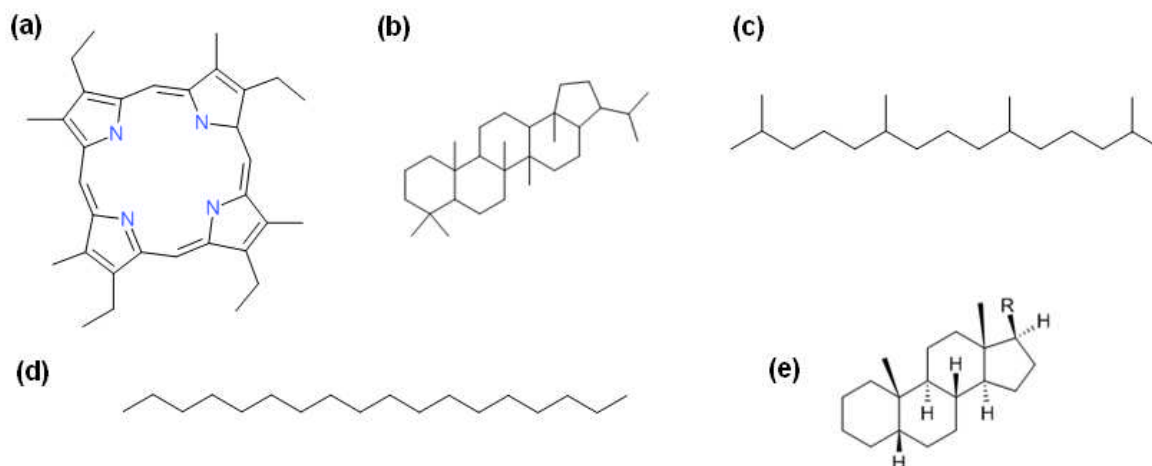


Figure 5-1: Structure of extinct life markers selected as preliminary targets for the generation of antibodies for the LMC (a) porphyrin, (b) hopane, (c) phytane, (d) a straight chain alkane (octadecane) and (e) 5 β sterane. Note that the ideal antibodies for the LMC would recognise a family of closely related molecules, rather than being highly specific for the one example of each molecular type shown here.

In 2008/2009 the LMC consortium attempted to generate antibodies to these five preliminary targets using traditional polyclonal antibody technology via immunisation of rabbits. However, the polyclonal sera showed no recognition of the relevant free target molecule, and cross reacted with irrelevant small molecule-carrier protein conjugates (Buckley 2009). At this stage in the project the production of monoclonal antibodies had already been discounted due to the long time scales and high costs involved, therefore an alternative antibody generation strategy was required and the production of recombinant antibodies, by screening phage display libraries, was identified as a promising option.

Screening recombinant antibody libraries using phage display is a powerful technique that allows the isolation of a large number of antibody clones in a short space of time. Traditionally these libraries have been used for the production of therapeutic antibodies against large target molecules, but phage display libraries are now increasingly being used for the production of antibodies against small molecule targets, primarily for use in the field of environmental analysis (Kramer and Hock 2003).

Prior to the work reported in this thesis, a naive recombinant antibody library (please see Section 5.3.3.1 for description of recombinant antibody library sources including naive libraries) was screened for antibodies that bound to phytane, hopane, 5 β sterane and porphyrin (Derveni 2009), but suitable antibodies were not isolated. It was therefore decided

to construct an immunised library with the five selected target molecules since immunisation was expected to bias the library repertoire towards clones that recognised the relevant target molecules, increasing the probability of successfully isolating appropriate antibodies. Derivatives of the five preliminary target molecules were synthesised and conjugated to keyhole limpet hemocyanin (KLH) carrier protein to produce immunogens (Figure 5-2)⁹. The immunogens were supplied to ImmunoSolv Ltd., (Aberdeen, UK), who organised immunisation of two sheep and constructed a phage display recombinant antibody library using standard methods as described in the literature (Charlton *et al*, 2001). Sheep were used for the construction of the library because anti-hapten antibodies have previously been obtained from immunised sheep libraries (Charlton *et al*, 2001) and because ImmunoSolv Ltd. has previous experience with the use of sheep for the construction of immunised libraries.

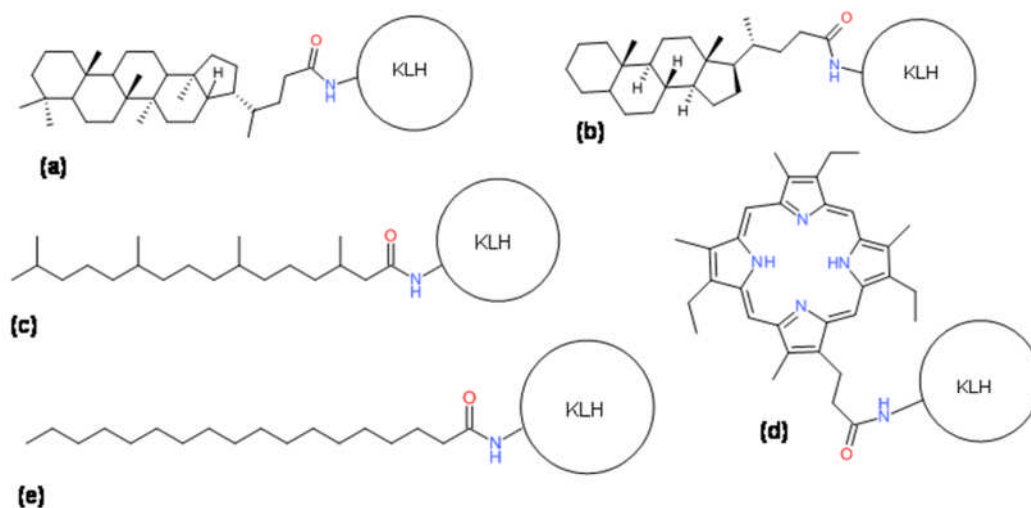


Figure 5-2: Immunogens used for production of phage display immunised recombinant antibody library

(a) hopane-KLH conjugate, (b) 5 β sterane-KLH conjugate, (c) phytane-KLH conjugate, (d) porphyrin-KLH conjugate, (e) octadecane straight chain alkane-KLH conjugate

Therefore the fundamental goal of this work was to determine whether screening the immunised recombinant antibody library, produced by ImmunoSolv, could generate antibodies capable of detecting any of the five selected markers of extinct life. Successful isolation of appropriate antibodies would not only provide antibodies that could be included on the flight instrument, but would also de-risk the future production of antibodies against a

⁹ Derivatives and conjugates were synthesised by Dr Manisha Sathe and Mrs Marjorie Allen, Cranfield University, UK

wider range of extinct life marker target molecules using recombinant antibody library technology.

5.2 Chapter objectives

The objective of the work reported in this chapter was to determine if screening the available phage display immunised recombinant antibody library could isolate antibodies that specifically recognise extinct life markers, within the context of the available resources and timescales for development of the LMC instrument.

In order to meet this goal the following specific objectives were set:

- 1) To attempt to isolate antibodies that bind to an extinct life marker molecule/molecular class by screening the immunised recombinant antibody library using a selected literature procedure for one or more targets.
- 2) To test the output of the selected literature procedure, *i.e.* both as antibody fragments expressed as fusion proteins on phage (scFvs) and, where relevant, as soluble single chain antibody fragments (scAbs), for appropriate function in an LMC relevant context.
- 3) To review the output of the selected literature procedure and if necessary to modify the protocol in order to favour the selection of clones against extinct life marker molecules.

As discussed in the introduction to this chapter the immunised library used in this work was generated using five target molecules: porphyrin, hopane, phytane, a straight chain alkane (octadecane) and 5 β sterane as examples of five molecular classes porphyrins, hopanes, isoprenoids, straight chain alkanes and steranes. Within this thesis work was carried out to isolate antibodies to three of these molecular species; hopane, octadecane and 5 β sterane. Additional screening for the remaining target molecules was carried out independently of the work reported in this thesis, but in a similar timeframe, by other members of the LMC team at Cranfield University and by ImmunoSolv Ltd in Aberdeen.

5.3 Literature review

5.3.1 Introduction

As described in the introduction to this chapter, extinct life markers are high priority targets for the LMC, but this class of target molecule is not typically detected using antibody based techniques and consequently very few antibodies are currently available to this type of molecule. Traditional antibody generation routes have been ruled out for the generation of antibodies to markers of extinct life for the LMC, for the reasons discussed in Section 5.1 and the production of recombinant antibodies by screening an immunised phage display library has been identified as a potential route forward. This review therefore aims to consider information in the literature relevant to the production of antibodies against these types of target molecule, with a specific focus on phage display and is divided into two sections. The first section considers the nature of extinct life markers and the challenges in producing antibodies to these types of molecule, with particular reference to the five targets used to construct the immunised recombinant antibody library used in this work. The second section reviews the use of phage display technology, specifically focusing on the generation of antibodies that recognise small hydrophobic target molecules.

5.3.2 Extinct life biomarkers as hydrophobic small molecule targets for the generation of antibodies

Markers of extinct life comprise biologically produced molecules that are stable over geological timescales and the geologically stable break down products of biologically produced molecules. Markers of extinct life are therefore mostly derived from lipid membranes, and from chlorophyll type pigments. Over geological timescales biologically produced molecules are altered through the reduction of double bonds and the loss of functional groups (Peters *et al*, 2005). Therefore extinct biomarkers typically comprise a saturated carbon skeleton, with few or no functional groups and consequently are hydrophobic and chemically inert in nature. Markers, such as those shown in Figure 5-1, are routinely detected in terrestrial samples using laboratory based chromatographic and spectrometric detection techniques (Fernandez-Calvo *et al*, 2006). The LMC intends to detect markers of extinct life in liquid extracts of martian samples using an antibody microarray immunoassay, therefore it is necessary to raise antibodies against these molecules.

Antibodies bind to their corresponding target molecules via relatively weak non-covalent hydrogen bonding, electrostatic, Van Der Waals, and hydrophobic forces, which are

dependent on the three dimensional structure and charge distribution of the antibody and the target molecule, however markers of extinct life are small, chemically inert and apolar and some lack a defined three dimensional structure, reducing the opportunity for binding interactions with an antibody. For this reason the production of antibodies to extinct life markers is expected to be challenging. Furthermore in order to have a realistic expectation of detecting trace levels of target molecules the LMC instrument must have a low detection limit (e.g. in the order of parts per billion or less) and therefore the antibodies selected for use in the flight instrument must have high affinities (e.g. nanomolar affinities) for their target molecules.

For the purposes of antibody generation, markers of extinct life are defined as haptens due to their low molecular weight (< 1000 Da) and it is more difficult to produce high affinity antibodies to haptens than to larger protein target molecules (Strachan *et al*, 2002). Hapten molecules are too small to illicit an immune response and must therefore be conjugated to a large immunogenic protein (termed a carrier protein) in-order to activate recognition by the immune system (Fodey *et al*, 2011). Since haptens occupy only a small fraction of the immunogen, most of the animals B cells are triggered by the conjugate and carrier protein and only a few are triggered by the hapten (Kramer 2002). Some small molecules fail to invoke an immune response even when conjugated to a carrier protein (Moghaddam *et al*, 2001). The design of conjugates used for immunisation in the production of anti-small molecule antibodies is critical to the production of antibodies with appropriate binding properties. For example the position used for conjugation of the small molecule to the carrier protein may affect the specificity of the antibodies generated (Gani *et al*, 1994), and the length of the linker used to join the small molecule and the carrier protein is also critical; if the linker is too short the determining groups of the hapten may be masked (Li *et al*, 2006). Furthermore hydrophobic molecules are viewed as particularly challenging targets for antibody generation due to difficulties associated with solubilisation for conjugation to carrier proteins. Attempts to produce antibodies to hydrophobic small molecule targets frequently result in the generation of antibodies that recognise the conjugate, or the derivative form of the small molecule but not the free target molecule (Makvandi-Nejad *et al*, 2010).

In the following sections the five markers of extinct life, all small hydrophobic molecules, used in the production of the immunised library that was screened in this work (hopane, phytane, porphyrin, 5 β sterane, and straight chain alkanes) (Figure 5-1), are described in more detail, together with examples of antibodies produced against these or related molecules where they have been documented in the literature.

5.3.2.1 Hopanes

Hopanes (Figure 5-1) are geologically stable molecules derived from hopanoids which originate in the cell membrane of prokaryotes, constituting 0.1 –5 mg/g of the dry weight of a cell (Peters *et al*, 2005). Hopanes are a high priority potential biomarker of Martian life because their bio-synthesis does not require molecular oxygen, which appears never to have been present on Mars in significant quantities (Parnell *et al*, 2007). Hopanes are viewed as challenging molecules to produce antibodies to because they consist of aliphatic carbon rings, with no polar functional groups to facilitate hydrogen bonding with an antibody binding site and they also have poor water solubility.

A conference paper given at the 2003 Lunar and Planetary Space Science Conference by the group developing the MILDI instrument briefly reported the production of polyclonal anti-hopane antibodies (Maule *et al*, 2003). This would indicate that it is possible to raise antibodies against this type of target molecule. However attempts by the LMC project team to produce polyclonal antibodies to hopane were unsuccessful (Buckley 2009).

5.3.2.2 Phytane

Phytane (Figure 5-1) is a highly stable biomarker that originates from the phytol side chain of chlorophyll and bacteriochlorophyll and also from the cell membranes of archaea (Peters *et al*, 2005). Phytane was assigned as a high priority target for the ExoMars mission because it is geologically stable and has a wide variety of biological pre-cursors (Parnell *et al*, 2007). Phytane, an acyclic aliphatic molecule, is anticipated to be a particularly challenging target to produce an antibody to, as in addition to having no functional groups, it has no conserved three dimensional structure.

There are commercially available antibodies that recognise phytanic acid (phytane with a terminal carboxylic acid group) including a rabbit polyclonal available from AbCam (Cambridge, UK, product code ab51309), but when tested by the LMC project team this antibody showed no recognition of free phytanic acid, no cross reactivity with phytane and appeared to only recognise the protein-phytanic acid conjugate (Derveni 2008). It is unknown whether it is possible to produce antibodies to this target.

5.3.2.3 Porphyrins

Porphyrins (Figure 5-1) are highly stable geological derivatives of well characterised biomolecules such as heme, chlorophyll and bacteriochlorophyll that are widely distributed among all terrestrial living organisms, from prokaryotes to mammals (Suo *et al*, 2007). In the

2006 biomarker workshop, held to define targets of relevance for the ExoMars mission, porphyrins were assigned a high priority due to their diverse sources and geological stability (Parnell *et al*, 2007).

Porphyrins have a well defined three dimensional structure as well as delocalised electron density around the pyrrole rings and nitrogen atoms which can hydrogen bond when not chelated to a metal ion. Porphyrins can chelate metal ions, which enhances their stability to diagenesis (Killops and Killops 2005) and may also influence how well they are recognised by antibodies (Schwabacher *et al*, 1989). In terrestrial living organisms porphyrin precursors contain magnesium, copper or cobalt ions, but in petroleum vanadium and nickel commonly replace these metals (Peters *et al*, 2005).

Schwabacher *et al* report the production of monoclonal antibodies, that bind to a synthetic metallo-porphyrin (Schwabacher *et al*, 1989), although the substituent groups on the pyrrole rings of the synthetic metallo-porphyrin were more polar compared to the expected hydrophobic side chains for extinct life marker porphyrin molecules. There are also commercially available antibodies to structures related to porphyrins such as haemoglobin e.g. an anti-haemoglobin antibody is available from Sigma (product code H4890) (Sigma-Aldrich).

5.3.2.4 Sterane

Steranes (Figure 5-1) are a geologically stable class of tetracyclic saturated biomarkers constructed from six isoprene sub-units and are derived from sterols which originate in the cell membranes of eukaryotes (Peters *et al*, 2005; Parnell *et al*, 2007). Unlike the structurally similar hopanoids, molecular oxygen is required for the bio-synthesis of sterols, reducing the priority of steranes as biomarker targets of ancient martian life compared to hopanes (Parnell *et al*, 2007).

There are no examples in the literature of attempts, successful or not, to raise antibodies against steranes. However there are numerous examples of monoclonal and recombinant antibodies that bind to steroids, which have a similar tetracyclic ring structure but more polar functional groups than steranes (White. *et al*, 1985; Kobayashi *et al*, 2009).

5.3.2.5 Straight chain alkanes

Lipid membranes in eubacteria and eukarya are composed of glycolipids and phospholipids; fatty acid chains (12 – 24 carbons long) attached to a polar head group (Peters *et al*, 2005). Fatty acids can be synthesised abiotically, but the probability of abiotic synthesis of long un-

branched chains is very small (Parnell *et al*, 2007). Fatty acids are stable and synthesised by two out of three of the domains for life and were therefore assigned as high priority biomarkers for the ExoMars mission (Parnell *et al*, 2007). Further discussions with various experts highlighted that on Mars, a decarboxylated straight chain alkane (*i.e.* non-branched) would be a more relevant target than a fatty acid and therefore octadecane was selected as a target for this work.

Fatty acids and straight chain alkanes are considered to be challenging targets to raise antibodies to due to their low molecular weight and conformational flexibility. There is one example in the literature of an antibody that recognises methyl palmitate, selected from a semi-synthetic human antibody library, that bound specifically to a C₁₆ chain with an IC₅₀ value of 0.2 µM (Gargir *et al*, 2002) suggesting that it is possible to raise antibodies against this type of molecule, although this sensitivity would be too low for the LMC flight instrument.

5.3.2.6 Summary

In summary markers of extinct life are hydrophobic small molecules. Their small apolar nature results in limited opportunities for interaction with an antibody binding site, therefore the production of high affinity antibodies that recognise these types of molecules is likely to be challenging. There are some examples in the literature of antibodies raised against related structures using traditional monoclonal, polyclonal and recombinant approaches; however the majority of these related structures are more polar than the targets for the LMC. Therefore part of the novelty of the work reported in this chapter is to attempt to produce antibodies against targets that are significantly more hydrophobic than the vast majority of targets against which antibodies have successfully been raised against in previous work.

5.3.3 Phage display technology for the production of antibodies that recognise small, hydrophobic and chemically inert target molecules

Phage display is the display of foreign peptides, proteins or antibody fragments on the surface of filamentous phage by fusion of the DNA coding sequence of the protein to be displayed to the gene encoding one of the phage surface proteins, most commonly the pIII coat protein (Sidhu 2001). This results in the antibody fragment or protein being displayed as a fusion protein on the surface of a phage particle which contains the genetic material that encodes the displayed protein fragment, thus creating a link between genotype and

phenotype. Phage particles displaying fusion proteins with the desired binding properties can rapidly be separated using *in-vitro* affinity selection. Because the phage particles contain the gene that encodes the displayed protein fragment it is then straightforward to isolate and clone the gene into an appropriate vector for expression, offering a rapid route to the production of the desired product.

Antibodies generated by phage display have mostly been used in therapeutic and biomedical applications, and it is only recently that interest has expanded to the development of anti-small molecule antibodies using phage display, primarily for the monitoring of pesticides, antibiotics and drug contamination in water, food and environmental samples (Brichta *et al*, 2005). This literature review focuses on applications of phage display relevant to the production of antibodies that bind to hydrophobic and small molecule targets. For more detailed information concerning the principles of phage display there are a number of comprehensive review papers available (Hoogenboom *et al*, 1998; Azzazy and Highsmith 2002; Brichta *et al*, 2005; Hoogenboom 2005).

5.3.3.1 Construction of phage display libraries for the production of antibodies that recognise small and hydrophobic targets

Phage display libraries can be produced from immunised sources (*i.e.* immunised animals) like the library used in this work, non-immunised sources (*i.e.* non-immunised animals), or can be derived synthetically/semi-synthetically. The following three sections consider each type of library in turn, discussing advantages and disadvantages and highlighting examples where specific library types have been used to generate antibodies against hydrophobic and small molecule targets.

Immunised libraries

Libraries from immunised sources are constructed by cloning genes coding the variable portions of antibody heavy and light chains directly from the B-lymphocytes of an immunised host animal. Since the immune system of the animal has been biased towards recognition of the target molecule by immunisation it is possible to select high affinity clones from a relatively small library (Brichta *et al*, 2005). The recombinant antibody library will also contain clones that are not present in the immune system of the animal due to the recombination of heavy and light chains during library construction, which increases library diversity.

Immunised libraries are limited by the immune repertoire of the animal host and it can be difficult to isolate antibodies against self antigens, non immunogenic antigens and toxic

antigens which cannot be used for immunisation (Hoogenboom 2005). This is of relevance to the LMC because many of the extinct life targets are likely to be poorly immunogenic due to their small size and lack of functional groups. For each target, or set of targets against which antibodies are to be generated, an animal must be immunised and a recombinant antibody library constructed, which takes several months. This is an important consideration for the LMC as should the screening of immunised recombinant antibody libraries by phage display prove effective in isolating antibodies that recognise selected extinct life markers, the production of further libraries using animals immunised with additional targets would be required, but this has to be carried out within the context of flight instrument delivery (currently scheduled for early 2015).

Immunised libraries have been used to successfully select antibodies, including high affinity antibodies with nanomolar sensitivities against a number of small molecule targets, some examples of which are listed in Table 5-1. Note that all of these molecules are more polar than the markers of extinct life selected as target molecules for the LMC.

Table 5-1: Examples of small molecule binding antibodies selected from immunised phage display recombinant antibody libraries

Target	Target MW (Da)	Host	IC ₅₀	Reference
Atrazine	216	Sheep	0.13 nM	(Charlton <i>et al</i> , 2001)
Mecoprop	215	Rabbit	Not given	(Li <i>et al</i> , 2000)
Simazine	202	Rabbit	Not given	(Li <i>et al</i> , 2000)
Fluorescein	332	Chicken	Not given	(Andris-Widhopf <i>et al</i> , 2000)
Monensin	671	Mouse	31 nM	(Makvandi-Nejad <i>et al</i> , 2010)
Methamidophos	141	Mouse	142 nM	(Li <i>et al</i> , 2006)

Naive/native libraries

Non immunised libraries can be created by cloning antibody diversity from the B-lymphocytes of non-immune animals. Therefore the source of genes used to construct the library is not explicitly biased to contain clones that bind to a specific target molecule (Hoogenboom 2005) and the library is expected to contain greater sequence diversity than an immunised library (Brichta *et al*, 2005). Naive libraries can be screened against any target molecule, without the need for immunisation or construction of a new library (Sheedy *et al*, 2007). In some instances antibodies against self, non-immunogenic and toxic antigens, which cannot be used for immunisation, have been isolated from naïve libraries (Brichta *et al*, 2005). Antibodies selected from non-immune libraries are often of lower affinity than those isolated from immunised libraries of a similar size, but this can be overcome by the construction of larger libraries which offer better opportunities for isolating specific and high affinity antibodies (Little *et al*, 1999; Sheedy *et al*, 2007).

Anti-small molecule antibodies have previously been isolated from naive libraries and some examples are listed in Table 5-2. Once again the target molecules against which antibodies have been selected are generally more polar than LMC biomarker molecules of extinct life.

Table 5-2: Examples of small molecule binding antibodies selected from naive phage display recombinant antibody libraries

Target	MW (Da)	Library Source	IC ₅₀	Reference
FITC	389	Salmon	Not given	(Jørgensen <i>et al</i> , 2002)
Tri nitrophenol	401	Salmon	Not given	(Jørgensen <i>et al</i> , 2002)
Phenyl-ox-azolone	161	Human	Not given	(de-Haard <i>et al</i> , 1999)
Progesterone	314	Human	Not given	(Little <i>et al</i> , 1999)
Testosterone	413	Human	Not given	(Little <i>et al</i> , 1999)

Synthetic/semi-synthetic libraries

Synthetic/semi-synthetic libraries, can be created *in-vitro* by introducing diversity into the CDRs V genes (either synthetic V genes, genes derived from a monoclonal hybridoma line, or a single phage clone (Hoogenboom 2005)). The use of synthetic/semi-synthetic libraries removes the need to use animals in the generation of recombinant antibody libraries and a single synthetic/semi-synthetic library can be screened against multiple targets. Synthetic/semi-synthetic libraries can also be used to generate antibodies against non-immunogenic and toxic target molecules. In addition, the contents, local variability and overall diversity of a synthetic/semi-synthetic library can be controlled and well defined facilitating library design (Brichta *et al*, 2005). For example a library specifically designed to produce high affinity antibodies to small molecule targets, termed a cavity library, was built using a hapten binding antibody, with a pronounced antigen binding pocket, as a structural backbone and performing site directed mutagenesis at residues commonly involved in hapten contacts (Persson *et al*, 2006).

Anti-small molecule antibodies have been isolated from both synthetic and semi-synthetic libraries, including the cavity library mentioned above, some examples of which are given in Table 5-3. Once again the majority of target molecules are more polar than the markers of extinct life selected as targets for the LMC, although it should be noted that an antibody against methyl palmitate generated from the semi-synthetic Nissim library, showed cross reactivity with hexadecane, a straight chain alkane (IC₅₀ = 0.2 μM), and straight chain alkanes are a marker of extinct life.

Table 5-3: Examples of small molecule binding antibodies selected from synthetic/semi-synthetic phage display recombinant antibody libraries

Target	MW (Da)	Library Source	IC ₅₀	Ref
Fluorescein	332	Semi-synthetic (chicken)	Not given	(van-Wyngaardt <i>et al</i> , 2004)
NIP	323	Semi-synthetic (chicken)	Not given	
Microcystin	1000	Synthetic (Tomlinson I library)	4 µM	(Strachan <i>et al</i> , 2002)
Microcystin	1000	Semi-synthetic (Griffin library)	13 nM	
Methyl palmitate	270	Semi-synthetic (Nissim library)	0.5 µM	(Gargir <i>et al</i> , 2002)
FITC	389	Semi synthetic (cavity library)	0.7 nM	(Persson <i>et al</i> , 2006)
Testosterone	413	Semi synthetic (cavity library)	0.2 µM	
NIP	323	Semi synthetic (cavity library)	2.4 µM	

4-hydroxy-3-iodo-5-nitrophenylacetic acid = NIP

Summary

Anti-small molecule antibodies have been successfully isolated from immunised, naive and synthetic/semi-synthetic phage display recombinant antibody libraries. The molecular weights of the target molecules against which antibodies have been isolated are similar to those of the five preliminary markers of extinct life identified in Section 6.1, but the target molecules to which these antibodies bind are more polar than extinct life target molecules for the LMC. Each of the three library types discussed here has its own advantages and disadvantages. The library available for screening within the work reported in this chapter was a phage display recombinant antibody library produced using an immunised sheep.

5.3.3.2 Strategies for screening phage display recombinant antibody libraries for antibodies that bind hydrophobic and hapten targets

Recombinant antibody libraries are screened for antibodies that bind to the desired target molecule using a two stage process. The first stage of screening involves the isolation of a sub-population of the library which is enriched in relevant clones using an affinity selection process commonly called panning. In the second stage of screening the enriched population is analysed, including isolation and testing of individual clones.

In a single round of panning a recombinant antibody library, displayed as fusion proteins expressed on phage particles, is exposed to an immobilised version of the desired target molecule (Figure 5-3). Phage particles expressing antibody fragments that recognise the target molecule bind to the immobilised target, whereas unbound phage, which does not recognise the target, is washed away. Bound phage is then recovered and infected into *E. coli* for amplification, to produce a population enriched in relevant binders displayed as fusion proteins on phage particles. Several rounds of panning are usually required because

non-specific binding limits the enrichment that can be achieved in a single panning round (Hoogenboom *et al*, 1998; Brichta *et al*, 2005; Hoogenboom 2005).

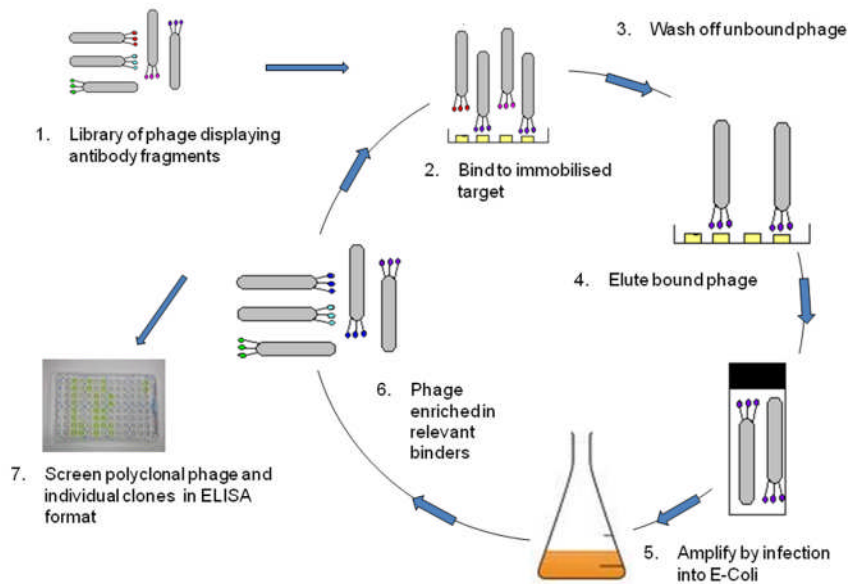


Figure 5-3: Diagram showing recombinant antibody library screening process for the isolation of relevant clones from a phage display recombinant antibody library

Panning is a versatile process and the physical parameters imposed during panning can strongly influence the nature of the antibodies that are enriched and subsequently isolated (Lou *et al*, 2001). For example using short incubation times and long wash steps can preferentially enrich clones with fast on-rates and slow off-rates, and reducing the concentrations of immobilised target molecule favours the enrichment of clones with high binding affinities (Hoogenboom 2005; Li *et al*, 2006). The ability to favour the enrichment of antibodies with specific properties, by making minor changes in panning conditions may be useful for the LMC since due to the format of the flight immunoassay it is desirable to have antibodies which have high affinities and also fast on-rates and slow off-rates. There are also examples in the literature where panning protocols have been adapted to adjust the specificity of antibodies (Zhu *et al*, 2011) which may be relevant for the LMC given the desire to produce antibodies that recognise classes of related molecules, rather than specific molecules, due to natural variation in the substituent groups on the molecules, and due to the likelihood that any life on Mars may not have evolved in an identical fashion to life on Earth.

Panning for small molecule binding antibodies

Unlike protein targets, small molecule targets are not usually directly immobilised to a substrate for panning. Within the literature there are two methods which are commonly adopted for the immobilisation of small molecule targets when panning for hapten binding antibodies. Small molecules can be conjugated to a large carrier protein (Andris-Widhopf *et al*, 2000; Li *et al*, 2000; Charlton *et al*, 2001; Li *et al*, 2006; McKenzie *et al*, 2007), often BSA or KLH, for immobilisation to solid surfaces in the panning process. In order to minimise the enrichment of carrier protein binding clones, panning is usually carried out in a background of free carrier protein, and the carrier protein used is often alternated between panning rounds (Sheedy *et al*, 2007). The second strategy that is used to immobilise small molecule target molecules for panning is to biotinylate the target molecule and then to immobilise the biotinylated target via streptavidin coated surfaces (Korpimäki *et al*, 2002; Kobayashi *et al*, 2009). In order to minimise the enrichment of streptavidin binding clones, a subtractive panning step can be used. In this step streptavidin binding clones are depleted by incubation over streptavidin coated surfaces, prior to exposure to the immobilised biotinylated target molecule.

Some previous attempts to screen phage display recombinant antibody libraries for hapten binding antibodies have resulted in the enrichment of clones that bind to the conjugated version of the target molecule, but not to the free target molecule (Moghaddam *et al*, 2001; Makvandi-Nejad *et al*, 2010). In order to avoid this, panning strategies have been adapted to incorporate the use of competitive elution, where bound phage is eluted from the target molecule-protein conjugate using the free target molecule prior to amplification in *E. coli*. This has been demonstrated to favour the enrichment and subsequent isolation of phage clones that have a higher affinity for the free target molecule, over carrier protein conjugates (Charlton *et al*, 2001; Moghaddam *et al*, 2001).

Panning for antibodies that bind to hydrophobic molecules

There are few examples in the literature of attempts to screen recombinant antibody libraries for antibodies that bind to hydrophobic targets. Antibodies against selected pesticides and herbicides with limited water solubility, such as atrazine, have been selected from recombinant antibody libraries (Li *et al*, 2000; Charlton *et al*, 2001), but such molecules are significantly more polar than LMC biomarkers of extinct life. The main issue of concern with attempting to screen phage display libraries for antibodies against hydrophobic small molecules, is that the target molecules will have poor water solubility, making it difficult to introduce a competitive elution step. However filamentous phage have been demonstrated

to retain their ability to infect *E. coli* after exposure to up to 80/20 % (v/v) methanol water, 20 %/80 % (v/v) propan-2-ol water, 99 %/1% (v/v) acetonitrile water and 60 %/40 % (v/v) dimethylformamide water mixtures (Olofsson *et al*, 1998). Therefore it is possible to perform library screening in aqueous buffers with a proportion of water miscible organic solvent to facilitate solubilisation of hydrophobic markers of extinct life.

Summary

Screening phage display recombinant antibody libraries represents a rapid and flexible means for the generation of antibodies with desired binding characteristics and also offers the potential to favour the isolation of antibodies with specific properties by making small changes in the panning protocol. There are two main panning strategies that have been used when screening for antibodies that bind small molecule targets; panning with small molecule-carrier protein conjugates and panning with small molecule-biotin conjugates.

5.3.4 Literature review conclusion

Markers of extinct life are challenging molecules to raise antibodies to due to their small size and their hydrophobic nature and there are very few examples of antibodies raised successfully against these types of molecule. Recombinant antibody technology appears to offer an efficient way to optimise the generation of antibodies against these sorts of targets. This is ideal for the LMC due to the short timescales required and the ability to optimise the properties of the antibodies selected during the panning process.

Attempting to screen phage display recombinant antibody libraries for antibodies that recognise markers of extinct life will represent one of the first attempts to (i) specifically generate antibodies for use in planetary exploration, (ii) to generate antibodies against targets that are hydrophobic and chemically inert.

5.4 Materials and methods

This section describes in detail the protocols used to screen the immunised recombinant antibody library and unlike the other chapters in this thesis is independent of the common materials and methods described in Chapter 2.

Materials used during screening are defined and details of suppliers and product codes are given in *parenthesis*. All reagents and consumables used were either supplied as sterile, or were sterilised using a benchtop autoclave (Prestige Medical, Blackburn, UK) and were subsequently handled aseptically.

5.4.1 Preparation of growth media for bacterial cultures

5.4.1.1 Liquid media

Bacterial cultures were grown in two types of commonly used liquid media, tryptone yeast extract (2xTY) and Lysogeny Broth (LB), providing the trace elements and vitamins required for growth of *Escherichia coli* (*E. Coli*) (Table 5-4). Tryptone enzymatic digest from casein (cat no. 95039), yeast extract (cat no. 70161) and sodium chloride (cat no. BP358-1) were purchased from Fisher Scientific (Loughborough, UK). The appropriate quantities of tryptone, yeast extract and sodium chloride were dissolved in 500 ml 18 MΩ.cm water in a Schott bottle and sterilised by autoclave. Liquid media was stored sealed, at room temperature, until use.

Table 5-4: Formulation of liquid media used for growth of *E. coli* cultures

Medium	Tryptone (mg/ml)	Yeast extract (mg/ml)	NaCl (mg/ml)
2xTY	16	10	5
LB	10	5	10

5.4.1.2 Agar plates

Tryptone yeast extract agar plates (TYE) with glucose and antibiotics were used for the selective growth of bacterial cultures for colony counting and colony picking (Table 5-5). Glucose, a catabolic repressor of the *lac2* promoter which is used to drive expression of the pIII-antibody fragment fusion protein (Hoogenboom *et al*, 1998), was purchased from Sigma Aldrich (Poole, UK, cat. no. G5767) and was sterilised before use by making up a 50 % (w/v) solution in water and then autoclaving. The antibiotics ampicillin (cat no. A5354) and kannamycin (cat no. K0245) were purchased from Sigma Aldrich (Poole, UK) and were used for the selective growth of library phage infected (ampicillin) and helper phage infected (kannamycin) cells.

Table 5-5: Formulation of agar plates used for growth of *E. coli* cultures

Medium	Tryptone (mg/ml)	Yeast extract (mg/ml)	NaCl (mg/ml)	Agar (mg/ml)	Glucose (w/v %)	Ampicillin (µg/ml)	Kanamycin (µg/ml)
TYE	8	5	5	15	1 %		
TYE _{AG}	8	5	5	15	1 %	100	
TYE _{KG}	8	5	5	15	1 %		50
TYE _{AKG}	8	5	5	15	1 %	100	50
Soft agar	8	5	5	7.5	1 %		

Tryptone, yeast extract, sodium chloride and agar were dissolved in water and sterilised by autoclave, then allowed to cool until comfortable to handle. The appropriate quantities of sterile 50 % (w/v) glucose solution and antibiotics were then added and the mixture was poured into Petri dishes. After the agar had solidified the agar plates were stored at 2-8°C until use. Soft agar was kept in a Schott bottle at room temperature and was warmed in a 45°C waterbath to liquefy before use.

5.4.2 General protocols for amplification and purification of phage

The following sections describe the protocols used for the amplification and purification of phage used during the screening of the immunised recombinant antibody library.

5.4.2.1 Preparation of *E. coli* cultures in logarithmic phase of growth

Phage infection of *E. Coli* is most efficient when *E. Coli* are in logarithmic phase of growth (log phase). In order to prepare log phase *E. Coli* cells for infection an autoclaved glass baffled flask containing 2xTY with 1 % (w/v) glucose was inoculated with approximately 1×10^8 TG1 *E. Coli* cells (Stratogene Stockport, UK, cat no. 200123) per ml of culture medium and the culture was grown in an KS4000i shaking incubator (IKA, Staufen, Germany) set at a temperature of 37°C and a rotation speed of 180 rpm. The increase in cell density was monitored periodically by removal of 1 ml of culture and measurement of optical density at a wavelength of 600 nm (OD₆₀₀) using a WPA cell density meter (Biochrom, Cambridge, UK). Once an OD₆₀₀ of 0.4 – 0.5 had been reached the cells were deemed to be in log phase and were used immediately.

5.4.2.2 Preparation of a high titre stock of M13K07 helper phage

The immunised recombinant antibody library was constructed using the pHEN2 phagemid vector. This vector contains the gene that encodes the p3 fusion protein, in addition to an ampicillin resistance gene, but the vector lacks the genetic material required for the generation of progeny phage. Amplification of phage is achieved by superinfection with M13K07 helper phage, which contains the necessary genes to enable assembly of progeny

phage and a kanamycin resistance gene, but also has a compromised origin of replication, ensuring that phagemid DNA is preferentially packaged. M13K07 helper phage is used in large quantities in the panning process and therefore a high titre stock was prepared from a commercially available product.

Commercial M13K07 helper phage (New England Biolabs, Hitchin, UK, cat. no. NO315S), supplied at a concentration of 10^{11} infective phage/ml, was diluted in 2xTY (10^{-2} – 10^{-12} dilution) and 50 μ l of each dilution was used to infect 500 μ l log phase TG1 *E. coli* cells (Stratagene, Stockport, UK, cat. no. 200123) grown to log phase as described in Section 5.4.2.1. Helper phage infection was carried out by incubation of phage dilutions with log phase TG1 *E. coli* in sterile 1.5 ml plastic tubes placed in a Clifton waterbath (Bennett Scientific, Newton Abbot, UK) set at a temperature of 37°C for 30 minutes. After 30 minutes the helper phage infected TG1 *E. coli* were added to 3 ml of pre-warmed soft agar (please see Section 5.4.1.2 for details of preparation) and poured over TYE_{KG} plates. The plates were incubated overnight in a Heraeus Kelvitron® 30°C static incubator (Thermo Fisher Scientific, Loughborough, UK). The following day a single colony was picked from the TYE plates and inoculated into 10 ml of 2xTY in a sterile 50 ml plastic tube and incubated for 2 hours at 37°C in a shaking incubator set at a rotation speed of 180 rpm. After two hours the contents of the 50 ml tube were transferred to a 2L baffled flask containing 500 ml of 2xTY medium and incubated for a further hour. After one hour kanamycin antibiotic was added to a final concentration of 50 μ g/ml and the contents of the flask were returned to the shaking incubator overnight.

After approximately 18 hours progeny helper phage were concentrated and purified by PEG precipitation as described in Section 5.4.2.4. The concentration of the purified helper phage was determined as described in section 5.4.2.4 and was 2.8×10^{13} infective phage/ml.

5.4.2.3 Phage rescue by helper phage infection

A small inoculum of *E. coli* cells containing the pHEN2 vector (either through transformation during library build, or through phage infection during library panning) (sufficient to give an OD₆₀₀ of approximately 0.1 = 1×10^8 cells/ml) was added to a 250 ml baffled flask containing 100 ml 2xTY with 1% (w/v) glucose and 100 μ g/ml ampicillin. The culture was grown in a shaking incubator set at a temperature of 37°C and a rotation speed of 180 rpm to log phase. A volume of 50 ml of log phase cells were superinfected with M13K07 helper phage at a ratio of 1:20 cells to helper phage. Helper phage was added to the cells followed by 30 minute infection in a waterbath set at 37°C and a further 30 minutes in a 37°C shaking incubator set at a rotation speed of 180 rpm. Ten microlitres of culture was retained for

determining the efficiency of helper phage infection and the remaining volume of culture was centrifuged at 6000 G for ten minutes. The supernatant was discarded and the resulting bacterial pellet was re-suspended in 100 ml of 2xTY (no glucose) with 100 µg/ml ampicillin in a baffled flask. The culture was incubated in a shaking incubator (30°C, 180 rpm) for 30 minutes before addition of 50 µg/ml kanamycin. The baffled flask was then returned to the shaking incubator overnight.

The efficiency of helper phage infection was determined by titration of serial dilutions (10^{-2} - 10^{-7} in 2xTY) of helper phage infected culture on TYE_{AKG} plates (50 µl/plate, in duplicate). Plates were incubated overnight at 30°C and the number of colonies that had grown were counted allowing calculation of the rate of helper phage infection. Typical infection rates were 10^8 superinfected cells/ml. Progeny phage was purified and concentrated by PEG precipitation.

5.4.2.4 Purification and concentration of rescued phage by PEG precipitation

Phage particles expressed in *E. coli* were separated from cellular debris and concentrated by PEG precipitation with polyethylene glycol 6000 (PEG 6000). PEG precipitation is an established method for the purification and concentration of phage particles expressed in bacteria (Yamamoto and Alberts 1970). PEG NaCl precipitation solution (20 % (w/v) PEG 6000, 2.5 M NaCl) was prepared by dissolving the appropriate quantities of PEG 6000 (Thermo Fisher Scientific, Loughborough, UK, cat. No. 192280051) and NaCl (Thermo Fisher Scientific, Loughborough, UK, cat. No. BP358-1) in water and then autoclaving.

Overnight liquid cultures of phage producing bacteria from phage rescue (Section 5.4.2.3) or from helper phage preparation (Section 5.4.2.2) were split into 30 ml aliquots in 50 ml sterile plastic tubes and centrifuged at 6000 G for twenty minutes using a Megafuge 11R centrifuge (Thermo Fisher Scientific, Loughborough, UK). Phage supernatants were retained and collected in clean 50 ml plastic tubes. To each tube containing 30 ml of phage supernatant was added 10 ml PEG NaCl solution and the plastic tubes were then chilled on ice for 1 hour. After one hour the tubes were centrifuged at 10700 G for 30 minutes, the supernatants were discarded and the soft white phage pellets were re-suspended in 10 ml of water and centrifuged at 6000 G for twenty minutes to remove remaining bacterial debris. Phage supernatants were retained and pooled into 30 ml lots in 50 ml plastic tubes. 10 ml PEG NaCl was added to each tube and the tubes were then chilled on ice for 20 minutes. After 20 minutes the tubes were centrifuged at 10700 G for 30 minutes and the supernatant discarded. The soft white phage pellets were re-suspended in 2 ml of PBS buffer. Finally the re-suspended phage was split between 1.5 ml plastic tubes (1 ml per tube) and centrifuged

at 16 G for ten minutes using a benchtop Heraeus Fresco minifuge (Thermo Fisher Scientific, Loughborough, UK) to remove any last traces of bacterial debris. After centrifugation the supernatants were transferred to sterile cryovials (Fisher, Loughborough, UK, cat. no. 10-500-26) and sterile glycerol (Sigma, Poole, UK, cat. no. G7893) was added to a final concentration of 20 % (v/v). PEG precipitated phage was stored at -80°C until use.

The titre of infective phage was determined by infecting log phase TG1 *E. coli* with dilutions of the PEG precipitated phage stock and titrating on TYE_{AG} plates. PEG precipitated phage was diluted in 2xTY (10⁻² – 10⁻¹²) and 50 µl of each dilution was used to infect 450 µl log phase TG1 *E. coli* cells. Infection was allowed to take place for 30 minutes whilst the dilutions of phage mixed with log phase cells were incubated in a water bath set at a temperature of 37°C. After infection, 50 µl of each dilution of phage infected into TG1 *E. coli* was spread onto TYE_{AG} plates in duplicate. Plates were incubated in a static incubator set at a temperature of 30°C overnight. The number of colonies that grew on the titration plates was counted and the concentration of the phage was calculated. Typical infective phage concentrations obtained were in the range 10¹²-10¹³ infective phage/ml

5.4.3 Protocols for panning

The following protocols describe the general panning protocols used in the screening of the immunised recombinant antibody library. Each round of panning involves affinity selection, followed by amplification of the selected pool of phage and subsequent purification. Note that these protocols describe the general panning procedures. Specific details for individual rounds of panning are described within the experimental design for each panning strategy used.

5.4.3.1 Preparation of library phage for panning

An immunised recombinant antibody library was supplied by ImmunoSolv (Aberdeen, UK) consisting of 8 sub-libraries (different VH-VL pairings), four with lambda light chains and four with kappa light chains, as glycerol stocks of XL1 Blue *E. coli* cells containing the gene encoding the variable antibody binding regions as an scFv cloned into the pHEN2 vector. Total library size was 10⁸ clones.

In preparation for panning, phage displaying single chain variable antibody fragments (scFvs) were produced from the supplied bacterial cells by performing phage rescue (Section 5.4.2.3) followed by PEG precipitation (Section 5.4.2.4) on each sub-library individually.

Following phage rescue and PEG precipitation, all PEG precipitated phage from the lambda sub-libraries were pooled together in equal volumes. This was repeated for all of the kappa sub-library phage. Lambda and kappa library phage was then pooled together in 9:1 ratio. Pooled phage was stored at -80 °C as a 20 % (v/v) glycerol stock until use.

5.4.3.2 Target molecule-carrier protein conjugates and target molecule-biotin conjugates used in panning

All of the target molecule-carrier protein conjugates and target molecule-biotin conjugates used in library panning and subsequent analysis were synthesised for the LMC project by Dr Manisha Sathe, Dr Marijan Stefinovic, Mr Luis Miguel Garcia Con and Mrs Marjorie Allen (Cranfield University, UK). Details of these conjugates are given in Sections 5.6.1 and 5.7.1. The free target molecules 17 β (H),21 α (H)-Hopane (cat. no. 07562), octadecane (cat. no. 74691) and 5 β -coprostane (cat. no. C2005) were purchased from Sigma-Aldrich (Poole, UK). Hopane was solubilised by dissolving at high concentration in dichloromethane (DCM). The DCM solution was then diluted into 20 % (v/v) methanol in PBS buffer. Octadecane and coprostane were solubilised in methanol and then diluted into 20 % (v/v) methanol in PBS buffer. Biotinylated conjugates were solubilised using DMF, diluted into methanol and then diluted into 20 % (v/v) methanol 1.5 g/L P80 in PBS buffer.

5.4.3.3 Panning with target molecule-protein conjugates (screening strategy 1)

Nunc MaxiSorpTM ImmunoTMtubes (Thermo Fisher Scientific, Roskild, Denmark, cat. no. 444202) were coated with a target molecule-carrier protein conjugate (for details of conjugates and concentrations used please see Section 5.6.1 and Section 5.7.1) by incubation with 4 ml of target molecule-protein conjugate in sterile PBS at a concentration ranging from 1 – 10 μ g/ml overnight at 2-8°C, or for 2 hours at 37°C. The coating solution was poured away and excess unbound conjugate was removed by washing the ImmunoTMtube 3 times with 4 ml of PBS buffer (Sigma Aldrich, Pool, UK, cat no. P4417). The ImmunoTMtube was then blocked to prevent non specific interactions of phage clones with uncoated areas of the hydrophobic plastic surface using 4 ml of a 2 % (w/v) solution of Marvel skimmed milk powder in PBS buffer (MPBS). Blocking was allowed to occur for two hours in an incubator set at a temperature of 37°C, or at 2-8°C overnight. Excess blocking buffer was removed by washing with 3 x 4ml of PBS buffer.

For the first round of panning 1ml of rescued PEG precipitated pooled library phage (approximately 10¹² phage particles) was added to the coated and blocked ImmunoTMtube in a buffer mix, termed in this work as the “binding buffer” (the binding buffer used was varied

depending on the panning strategy used, for details please see Section 5.6.1 and Section 5.7.1). For subsequent rounds of panning 1 ml of PEG precipitated phage recovered from the previous panning round was used (approximately 10^{12} phage). The ImmunoTMtube was sealed with Parafilm® (Thermo Fisher Scientific, Loughborough, UK, cat.no. 13-374-10) and incubated at room temperature for a total of two hours. For the first 30 minutes of the two hour incubation period the ImmunoTMtube was rotated at 20 rpm using a Stuart SB3 rotator (Bibby Scientific Ltd, Stone, Staffordshire), followed by 90 minutes static incubation. After two hours unbound phage was discarded and the ImmunoTMtube was washed with a wash buffer. The wash buffer and the number of washes used varied depending on the panning strategy (for details please see Section 5.6.1 and Section 5.7.1).

Subtractive panning

Note that in some panning rounds (please see Section 5.6.1 and Section 5.7.1 for details) phage was subject to an additional subtractive panning step, prior to application to the coated and blocked ImmunoTMtube, to deplete carrier protein binding phage. In order to achieve this 1 ml of phage in binding buffer was incubated for 1 hour at room temperature (rotating) in an ImmunoTMtube that had been coated with 1 mg/ml carrier protein. Following this hour long incubation the phage and binding buffer was transferred to the coated and blocked ImmunoTMtube as described above.

Elution of bound phage

Following incubation and washing away of unbound phage, phage bound to the immobilised conjugate were eluted either by non-specific elution or by competitive elution. Eluted phage were infected into log phase TG1 *E. coli* cells as described below.

Non-specific elution and infection

Phage bound to immobilised conjugate was eluted by the addition of 1 ml of 100mM triethylamine in water (Sigma Aldrich, Poole, UK, cat. no. T0886) to the ImmunoTMtube followed by ten minutes of rotation on a Stuart SB3 rotator. After ten minutes the triethylamine was neutralised (to prevent damage of the phage, which would result in reduced infectivity) by transferring the contents of the ImmunoTMtube into a 1.5 ml plastic tube containing 0.5 ml of 1M Tris buffer pH 7.4 (Severn Biotech Ltd., Kidderminster, UK, cat. No. 207901-10). A further 200 μ l of 1M Tris buffer was added directly to the ImmunoTMtube, so that any phage still contained within the tube could also be infected into *E. coli*. A volume of 0.75 ml of eluted phage was used to infect 9.25 ml of log phase TG1 *E. coli* cells in a 50

ml plastic tube, by static incubation in a 37°C waterbath for 30 minutes. A further 4 ml of log phage TG1 *E. coli* cells were added directly to the Immuno™ tube. The remaining unused 0.75 ml of eluted phage was stored as a 20 % (v/v) glycerol stock at a temperature of -80°C. After 30 minutes incubation in the waterbath the TG1 *E. coli* cells in the Immuno™ tube were pooled with those in the 50 ml tube.

Competitive elution and infection

4 ml of a solution of 0.1 – 10 µM of free target molecule (for details of target molecules and concentrations used please refer to Section 5.6.1 and Section 5.7.1) dissolved in a buffer termed the “elution buffer” was added to the Immuno™ tube and incubated at room temperature, rotating for 30 minutes and static for 30 minutes. A volume of 2 ml competitively eluted phage was used to infect 10 ml of log phage TG1 *E. coli* by incubation in a 50 ml tube in a 37°C waterbath for 30 minutes.

The quantity of both competitively eluted and non-specifically eluted phage was determined by titration of dilutions of eluted phage infected *E. coli* (10^{-2} – 10^{-7}) on TYE_{AG} plates (50 µl/plate in duplicate). The plates were incubated at 30°C overnight and then the concentration of eluted phage was calculated using the number of colonies that had grown. The remaining volume of phage infected bacterial culture was pelleted by centrifugation at 6000 G for 10 minutes. The supernatant was discarded and the bacterial pellet was re-suspended in 0.4 ml of 2xTY. The entire 0.4 ml of re-suspended culture was then spread onto a large TYE_{AG} plate. Plates were incubated at 30°C overnight. After approximately 18 hours of growth, cells were recovered from the agar plate by addition of 1-3 ml of 2xTY and loosening with a sterile plastic microbiological spreader (Thermo Fisher Scientific, Loughborough, UK, cat. No. LPS-141-011R). Recovered bacterial cells were stored as a 20 % (v/v) glycerol, 5 % (w/v) glucose stock at -80°C. Phage was amplified and purified in preparation for the next round of panning by phage rescue (Section 5.4.2.3) followed by PEG precipitation (Section 5.4.2.4).

5.4.3.4 Panning with target molecule-biotin conjugates (screening strategy 2)

Preparation of magnetic beads for panning

Streptavidin coated Dynabeads® (Invitrogen, Paisley, UK, cat. no. M280), supplied in PBS with 0.01 % P20 and 0.02% NaN₃, were washed to remove the P20 and azide. Washing was achieved by adding 1 ml of PBS to 200 µl of Dynabeads® in a 1.5 ml plastic tube and mixing well, the tube was then placed in a Dynal 1.5 ml tube magnetic separator rack (Invitrogen,

Paisley, UK) and allowed to stand for 2 minutes during which time the magnetic beads were drawn to the side of the tube. The rack was briefly inverted to recover any magnetic beads that had collected in the lid of the plastic tube, then the tube, still contained within the rack was allowed to stand for a further 1 minute. Supernatant was removed using a pipette, leaving the magnetic beads on the side of the plastic tube. The beads were resuspended in 1 ml of PBS and the washing procedure was repeated to a total of two washes.

After a total of two washes magnetic beads were re-suspended in 1 ml of 2% MPBS and rotated for a minimum of one hour at room temperature using a Stuart SB3 tube rotator. Just prior to use the 1.5 ml tube containing magnetic beads in MPBS was placed into a magnetic separating rack and left to stand for 2 minutes. The rack was briefly inverted to remove any magnetic beads that had collected in the lid and then stood for a further 1 minute. Two percent MPBS was removed using a pipette leaving dry magnetic beads on the side of the 1.5 ml plastic tube.

Preparation of library phage for panning

Five hundred microlitres of rescued and PEG precipitated library phage (from library rescue for pan 1 and from the previous pan for subsequent panning rounds, approx 10^{12} phage) was added to 300 μ l of 6.66% MPBS in a 1.5 ml plastic tube and rotated for 1 hour at room temperature.

Subtractive panning

For some rounds of panning a subtractive panning step was carried out in order to minimise the enrichment of clones that bound to the streptavidin coated magnetic beads. In panning rounds where subtractive panning was used 200 μ l of washed streptavidin coated magnetic beads were added to 300 μ l of library phage in 6.66% (w/v) MPBS (after an initial 1 hour incubation as described above). The beads and library phage were incubated with rotation for 1 hour at room temperature. After one hour the magnetic beads were removed using a magnetic rack and the phage solution was transferred to a fresh 1.5 ml plastic tube.

Incubation of library phage with biotinylated target molecule

Biotinylated antigen (for details please refer to Section 5.7.1) was dissolved in methanol at a concentration of 16.5mM. and then further diluted to 2.5 μ M in methanol with 7.5g/L P80. A volume of 200 μ l of this solution was added to pre-prepared 300 μ l of library phage in 6.66 % MPBS to give a final formulation of 500nmol biotinylated target molecule, 20 % (v/v)

methanol 1.5 g/L P80 and 2% (w/v) MPBS. This mixture was incubated at room temperature rotating for one hour.

After one hour the mixture of library phage and biotinylated antigen was transferred into a 1.5 ml plastic tube containing dry magnetic beads and incubated for 20 minutes rotating to allow the streptavidin on the magnetic beads to bind to the biotinylated antigen. After 20 minutes the magnetic beads were separated from the solution containing unbound phage using a magnetic rack. The beads were washed to remove non-specifically bound phage.

Bound phage were either used directly to infect log phase TG1 *E. coli* whilst still bound to beads, or were competitively eluted prior to infection using free antigen.

No elution/direct infection

Magnetic beads with associated biotinylated antigen and bound phage were re-suspended in 200 μ l of PBS and directly used to infect log phase TG1 *E. coli*. A volume of 200 μ l of re-suspended magnetic beads were added to 9.25 ml of log phase TG1 *E. coli* in a 50 ml plastic tube and incubated for 30 minutes in a 37 °C waterbath. The 50 ml plastic tubes were gently inverted periodically during the 30 minute incubation in the water bath in order to re-suspend the beads which settled at the bottom of the tube. After infection magnetic beads were removed from the culture using a Dynal 50 ml tube magnetic separator rack (Invitrogen, Paisley, UK).

Competitive elution and infection

A volume of 1 ml of 10 – 100 μ M free target molecule (please see Section 5.7.1 for details) dissolved in PBS with 20 % (v/v) methanol 1.5g/L P80 was added to the washed magnetic beads and the mixture was rotated for one hour at room temperature. After one hour the magnetic beads were separated from the competitively eluted phage solution using a magnetic separator rack and the whole 1 ml volume of supernatant was used to infect 9.25 ml of log phase TG1 *E. coli* in a plastic 50 ml tube for 30 minutes in a 37°C waterbath.

After incubation 10 μ l of eluted phage infected *E. coli* (from both direct infection and infection with competitively eluted phage) was removed and titrated on TYE_{AG} plates. Dilutions were prepared in 2xTY (10^{-2} – 10^{-7}) and 50 μ l was spread (in duplicate) onto TYE_{AG} plates. The plates were incubated at 30°C overnight and then the concentration of eluted phage was calculated by counting the number of colonies that had grown. After removal of 10 μ l for titration the remaining volume of phage infected bacterial culture was pelleted by centrifugation at 6000 G for 10 minutes. The supernatant was discarded and the bacterial

pellet was re-suspended in 0.4 ml of 2xTY. The whole 0.4 ml was then spread onto a large TYE_{AG} plate. Plates were incubated at 30°C overnight. The colonies that had grown on the large TYE_{AG} plate were recovered by addition of 1-3 ml of 2xTY and loosening with a sterile plastic microbiological spreader (Thermo Fisher Scientific, Loughborough, UK, cat no. LPS-141-011R). Recovered bacterial cells were stored as a 20 % (v/v) glycerol, 5 % (w/v) glucose stock at -80°C. Phage was amplified and purified in preparation for the next round of panning by phage rescue (section 5.4.2.3) followed by PEG precipitation (Section 5.4.2.4)

5.4.4 Protocols for analysis of enriched population of clones generated by panning (including the isolation of individual clones)

5.4.4.1 Polyclonal ELISA

Target molecule-protein conjugates and free carrier proteins were immobilised to the wells of Nunc MaxiSorb™ F96 MicroWell™ plates (Thermo Fisher Scientific, Roskilde, Denmark, cat. no. 442404) by incubation of 100 µl/well at a protein concentration of 1µg/ml overnight at 2 – 8°C or for two hours at 37°C. After the immobilisation step, excess target molecule-protein conjugate and carrier protein was removed from wells by washing with 3 x 300 µl/well Phosphate buffered saline (Sigma Aldrich, Poole, UK, cat. no. P4417) containing 0.1 % (w/v) Polysorbate 20 (BDH/VWR, Leicestershire, UK, cat. no. 663684B) (PBST buffer) and then rinsing with 3 x 300 µl/well PBS. Residual liquid was removed from the wells by inverting the 96-well plate and tapping out on absorbent paper.

Wells were blocked to reduce non-specific interactions of phage with any uncoated areas of the hydrophobic plastic surfaces by incubation with 200µl/well 2% (w/v) MPBS at a temperature of 37°C for two hours. After two hours the wells were washed as described previously. Fifty microlitres of PEG precipitated phage from each round of panning was added to duplicate wells coated with each target molecule-protein conjugate and each free carrier protein. For pan 0 material, pooled PEG precipitated phage from the library rescue (Section 5.4.2.4) was used for the ELISA. Fifty microlitres of 4% (w/v) MPBS was then added to each phage containing well and the plate was incubated at room temperature for 1 hour then washed as described previously.

After washing, anti-bacteriophage M13 antibody HRP conjugate (GE Healthcare, Chalfont St Giles, UK, cat. No. 27-9421-01) was added 100 µl/well in 2 % (w/v) MPBS buffer at 1/1000 dilution of stock and incubated for one hour at room temperature. The plate was then washed as described previously.

Tetramethylbenzidine.dihydrochloride (TMB) substrate (Sigma Aldrich, Poole, UK, cat. no. T3405) in 0.05 M phosphate citrate buffer containing 0.03 % (w/v) sodium perborate pH 5.0 (Sigma Aldrich, Poole, UK, cat. no. P4992) was added to the microtitre plate 100 µl/well; reaction with HRP labelled antibodies bound to phage immobilised on the surface of the wells produced a coloured product in quantities proportional to the amount of immobilised antibody.

After approximately 10 minutes the enzyme-substrate reaction was stopped by the addition of 50 µl/well of 1 M sulphuric acid. The absorbance of each well was read at a wavelength of 450 nm using a Varioskan Flash plate reader (Thermo Scientific, Leicestershire, UK).

5.4.4.2 Monoclonal ELISA

Preparation of phage supernatant for monoclonal ELISA

PEG precipitated phage from panning rounds was diluted 10^{-2} - 10^{-12} in 2xTY. A volume of 100 µl of each dilution was used to infect 900 µl of log phase TG1 *E. coli* in 1.5 ml plastic tubes for 30 minutes in a waterbath set at a temperature of 37°C. After 30 minutes 50 µl of cells infected with each dilution of phage were spread (in duplicate) onto TYE_{AG} plates and incubated overnight at 30°C.

The following morning individual colonies were picked from the plates using sterile pipette tips and inoculated directly into 150 µl of 2xTY with 1% (w/v) glucose and 100 µg/ml ampicillin in the wells of a Nunc 96-well tissue culture plate (Thermo Scientific, Roskilde, Denmark, cat. no. 167008). Bacterial cultures were grown overnight shaking in a 37°C incubator. The following morning small inoculums were taken from each well on the 96-well tissue culture plate using a 96 pin replicator (VWR, Lutterworth, UK, cat. No. 738-0252) and transferred into a fresh tissue culture plate containing 200 µl/well 2xTY, 1% (w/v) glucose, 100 µg/ml ampicillin. The cultures were grown at 37°C shaking for 4 hours. In order to make a stock of each clone the tissue culture plates containing overnight cultures were centrifuged at 1500 G for 10 minutes using a plate spinning rotor head (Thermo Fisher Scientific, Loughborough, UK, cat. no.11175750). The supernatants were aspirated using a multi-channel pipetter and the bacterial pellets were re-suspended in 150 µl 2xTY, 5 % (w/v) glucose, 20 % (v/v) glycerol and stored at -80°C.

After 4 hours the bacterial cultures were removed from the incubator and infected with M13KO7 helper phage by addition of 25 µl/well 2xTY containing 100 µg/ml ampicillin, 1 % (w/v) glucose and 4×10^{10} /ml M13KO7 helper phage. The tissue culture plate was incubated static for 30 minutes at 37°C to allow superinfection by helper phage and then incubated for

a period of 60 minutes shaking at 37°C. Following the 60 minute incubation the plate was spun at 1500 G for 10 minutes and supernatants were aspirated using a multi channel pipettor and discarded. Bacterial pellets were re-suspended in 200 µl/well 2xTY with 100 µg/ml ampicillin and 50 µg/ml kannamycin and grown overnight shaking at 30°C.

The following morning tissue culture plates were removed from the incubator and centrifuged at 3000 rpm for ten minutes. Phage supernatant was aspirated using a multi channel pipettor and stored in a fresh tissue culture plate at 2-8°C until used in an ELISA.

ELISA protocol

The basic protocol followed in order to run a monoclonal ELISA was the same as the protocol for running a polyclonal phage ELISA as described in Section 5.4.4.1 with some modifications which are described below. Clones were first screened for binding to appropriate conjugates in a binding ELISA. Selected clones were then screened for recognition of free target in an inhibition ELISA.

Binding ELISA

A single 96-well plate was coated with BSA conjugated to target molecule and a second 96-well plate was coated with BSA carrier protein following the method described in Section 5.4.4.1. If required, plates were also coated with KLH conjugated to target molecule and free KLH. The 96-well plates were then blocked as described in Section 5.4.4.1.

To each coated and blocked 96-well plate was added 50 µl/well 4 % (w/v) MPBS and then 50 µl/well of phage supernatant was transferred from the tissue culture plate to both 96-well plates (therefore each monoclonal phage clone was screened for binding to target molecule-protein conjugate and for binding to free carrier protein). From this point onwards the ELISA protocol was identical to that described in Section 5.4.4.1, with incubation of phage for 1 hour, followed by washing, addition of the anti-M13 secondary antibody, a further 1 hour incubation, washing and then addition of TMB substrate.

Inhibition ELISA

Two 96-well plates were coated with BSA conjugated to target molecule, and then blocked as described in Section 5.4.4.1. During the blocking step two tissue culture plates were prepared to facilitate incubation of the monoclonal phage with free target molecule prior to application to the coated and blocked 96-well plates. One tissue culture plate contained 50 µl/well 40 % (v/v) methanol (Thermo Fisher Scientific, Loughborough, UK, cat. no. M14056)

with free target molecule at a concentration of 10 μ M (for details of free target molecules used please see Section 5.4.3.2), the other contained 50 μ l/well 40 % (v/v) methanol with no target molecule as a control (to allow direct comparison of binding signal generated with and without free target molecule in the same solvent). Fifty microlitres per well of phage supernatant was transferred from the tissue culture plate containing the stock phage supernatant to each of the tissue culture plates containing methanol/water and free target molecule. Phage and methanol/water/free target molecule were incubated together for 1 hour before being transferred across to the coated and blocked 96-well plates. From this point onwards the protocol was identical to that described in Section 5.4.4.1.

5.4.4.3 Sequencing of monoclonal phage

Individual clones were grown up from the glycerol stocks stored in tissue culture plates prepared during clone picking (Section 5.4.4.2) by inoculation into 5 ml of LB containing 1% glucose and 100 μ g/ml ampicillin. Cultures were grown overnight shaking at 180 rpm in a 37°C incubator. Plasmid DNA was recovered and purified from each individual culture using a QIA Prep Spin Mini prep kit (Qiagen, Crawley, UK, cat. no.27104). Genes encoding antibody variable regions were sequenced by Beckman Coulter Genomic (Takeley, UK) using primers that were custom synthesised by Invitrogen (Paisley, UK).

AH18 Rev 5'-AAA TAC CTA TTG CCT ACG GCA GCC GCT GG-3'

GIII For 5'-GAA TTT TCT GTA TGA GGT TTT GC-3'

Sequences were compared and analysed using Lasergene® 8 software (DNASTAR, Madison, USA).

5.4.4.4 Expression of soluble scAbs

Antibody fragments were expressed as soluble single chain antibody fragments (scAbs) by cloning into the expression vector PIMS147 (Hayhurst and Harris 1999) and transforming into *E. coli*.

Preparation of PIMS147 expression vector

XL1 Blue *E. coli* cells containing PIMS147 plasmid (supplied by Ms. B Ravi, ImmunoSolv, Aberdeen, UK) were grown overnight in 20 ml of LB with 1% (w/v) glucose, 100 μ g/ml ampicillin in a shaking incubator set at 37 °C. Plasmid DNA was recovered and purified using a QIA Prep Spin Mini prep kit, and quantified using a Picodrop Microliter UV/Vis Spectrophotometer (Picodrop Ltd, Saffron Walden, UK). Plasmid DNA was then digested

using the restriction enzymes NcoI and NotI (New England Biolabs, cat. nos. R0193S and R0189L respectively). A volume of 30 μ l of plasmid DNA (approx 6 μ g) was mixed with 1 μ l (10 units) of each enzyme, 5 μ l of NE buffer and 0.5 μ l of 100xBSA buffer (buffers supplied with the enzymes) in a 1.5 ml plastic tube and made up to a total volume of 50 μ l with water. The enzymes and plasmid DNA were incubated together for 1 hour 30 minutes in a waterbath set at a temperature of 37°C. Digested DNA was run on a Tris-Acetate EDTA (TAE) 1% (w/v) agarose gel containing 1 μ g/ml ethidium bromide using TAE running buffer (Thermo Fisher Scientific, Loughborough, UK, cat. no. ELR-328-010V), at 125V for approximately 45 minutes alongside Hyperladder 1 molecular weight marker (Bioline, London, UK). Bands of DNA on the gel were visualised using a Transluminator and the band at 6kbp was excised using a clean scalpel. The excised DNA was recovered using a QIAquick Gel extraction kit (Qiagen, Crawley, UK, cat no. 28706) and purified DNA was quantified using a Picodrop spectrometer.

To prevent self ligation of the cut vector the 6kbp fragment of PIMS147 was treated with shrimp alkaline phosphatase (SAP) (Promega, Southampton, UK, cat. no. M8201) to remove the 5' terminal phosphate group. Sixty microlitres (approx 2 μ g) of purified cut PIMS147 vector was incubated with 1 μ l (1 unit) of SAP and 2 μ l SAP buffer (supplied with the enzyme) in a waterbath set at 37°C for 2 hours. SAP was deactivated by heating at 65°C for 30 minutes. SAP treated vector was stored at -20°C until use.

Digestion and purification of library DNA

Plasmid DNA isolated from library cells (prepared as described in Section 5.4.4.3) was digested using the restriction enzymes NcoI (New England Biolabs, cat. no. R0193S) and NotI (New England Biolabs, cat. no. R0189L). Thirty microlitres of plasmid DNA (approx 6 μ g) was mixed with 1 μ l (10 units) of each enzyme, 5 μ l of NE buffer and 0.5 μ l of 100xBSA buffer (supplied with enzyme) in a 1.5 ml plastic tube and made up to a total volume of 50 μ l with water. The enzymes and plasmid were incubated for 1 hour 30 minutes in a waterbath set at 37 °C. Digested DNA was run on a Tris-Acetate EDTA (TAE) 1% (w/v) agarose gel containing 1 μ g/ml ethidium bromide using TAE running buffer (Thermo Fisher Scientific, Loughborough, UK, cat. no. ELR-328-010V), at 125V for approximately 45 minutes alongside Hyperladder 1 molecular weight marker (Bioline, London, UK). Bands of DNA on the gel were visualised using a Transluminator and the band at 800bp was excised using a clean scalpel. The excised DNA was recovered using a QIAquick Gel extraction kit (Qiagen, Crawley, UK, cat no. 28706) and purified DNA was quantified using a Picodrop spectrometer.

Cloning of library insert DNA into PIMS147

Approximately 100 ng of SAP treated cut PIMS 147 vector was added to approximately 60 ng of library insert DNA (approx 1:6 molar ratio of vector to insert) together with 1 µl of T4 DNA ligase (New England Biolabs, Hitchin, UK, cat no M0202M), 2 µl of 10 x ligase buffer (supplied with T4 DNA ligase) and made up to a total volume of 20 µl with water in a 1.5 ml plastic tube. The mixture was incubated overnight at room temperature. The following morning T4 DNA ligase enzyme was inactivated by heating at 65 °C for 10 minutes. Ligated DNA was quantified using a Picodrop spectrometer and then stored at -20°C until it was transformed into *E. coli*.

Preparation of heat shock competent TG1 cells for expression of sequences containing stop codons

Several of the sequences isolated from the immunised library contained stop codons and therefore had to be expressed using an *E. coli* strain, such as TG1, that is able to read through these codons. In order to prepare heat shock competent TG1 cells an overnight culture of TG1 *E. coli* (Stratogene Stockport, UK, cat no. 200123) was grown in 5 ml of LB shaking at 37°C. The following morning 250 ml LB broth was inoculated with 5 ml overnight culture and grown shaking at 37°C to log phase of growth. The culture was then split into 8 x 50 ml sterile plastic tubes and centrifuged at 2680 G for 20 minutes, the supernatant was discarded and each bacterial pellet was re-suspended in 0.5 ml of ice cold Tris-HCl with 100 mM calcium chloride and incubated on ice for 20 minutes. After 20 minutes the tubes were centrifuged at 2680 G for 15 minutes, the supernatant was discarded and bacterial pellets were re-suspended in 3 ml of ice cold 100 mM calcium chloride (Sigma, Poole, UK, cat. no. C3881) and incubated on ice for 20 minutes. The contents of the 8 tubes were pooled into two tubes and centrifuged at 2680 G for 15 minutes. The supernatant was discarded and the bacterial pellets were re-suspended in 12.5 ml 100 mM calcium chloride with 20 % (v/v) glycerol. The suspension was aliquoted into 1.5 ml plastic tubes and stored at -80°C until use.

Transformation of PIMS147 vector with cloned library insert into *E. coli*

Plasmid DNA was transformed into *E. coli* (TG1 or XL1 Blue) using heat shock. Heat shock competent XL1 Blue *E. coli* cells (Stratogene, Stockport, UK, cat. No. 200249), or heat shock competent TG1 *E. coli* prepared as described in preceding section, were removed from storage at -80°C and allowed to thaw on ice. Thawed cells were aliquoted into pre-chilled 1.5 ml plastic tubes (100 µl/tube) and 1.7 µl of mercaptoethanol (supplied with XL1 Blue

competent cells) was added to each tube. A volume of 3 µl of plasmid DNA was added to each tube and tubes were then incubated on ice for 30 minutes. Cells were heat shocked at 42°C, by placing into a waterbath for 45 seconds. Tubes were then removed from the waterbath and returned to the ice for 2 minutes. A volume of 900 µl of pre-warmed (42°C) LB was added to each transformation mixture and incubated shaking at 37°C for 1 hour. A volume of 100 µl and 50 µl of each transformation mixture were then spread over TYE_{AG} plates (in duplicate) and incubated overnight at 37°C (XL1 Blue cells) or at 30°C (TG1 cells).

After overnight growth colonies were picked from the transformant plates and inoculated into 10 ml LB with 1% (w/v) glucose and 100 µg/ml ampicillin and grown overnight at 37°C shaking at 180 rpm. The following morning 5 ml of overnight culture was removed for plasmid DNA preparation and sequencing to confirm that the correct sequence had been cloned across. The remaining culture was centrifuged at 6000 G for 10 minutes. The supernatant was discarded and the bacterial pellet was resuspended in LB with 5 % (w/v) glucose and 20 % (v/v) glycerol for long term storage at a temperature of -80°C.

Sequencing of transformed plasmid DNA

Genes encoding the variable regions on the plasmid DNA from transformed clones were sequenced by Beckman Coulter Genomics (Takeley, UK) using primers that were custom synthesised by Invitrogen (Paisley, UK) in order to confirm that the correct sequences had been cloned across.

AH18 Rev 5'-AAA TAC CTA TTG CCT ACG GCA GCC GCT GG-3'

HuCk-FOR 5'-GAA GAT GAA GAC AGA TGG TGC-3'

Sequences were compared and analysed using Lasergene® 8 software (DNASTAR, Madison, USA).

Expression of scAb in *E. coli*

5 ml of LB with 100 µg/ml ampicillin and 1 % glucose was inoculated from glycerol stocks of each transformed clone. Cultures were grown overnight at 37°C shaking. The following morning overnight cultures were centrifuged at 6000 G for ten minutes. The supernatant was discarded and the bacterial pellet was re-suspended in 10 ml of LB with 100 µg/ml ampicillin. The culture was incubated at 25°C shaking for 1 hour and then expression of soluble scAb was induced by the addition of 50 µl 200mM Isopropyl β-D-1-thiogalactopyranoside (IPTG)

(Sigma, Poole, UK, cat. no. I1284). Cultures were then returned to the shaking incubator for a further 4 hours.

After four hours bacterial cultures were centrifuged at 6000 G for ten minutes. Bacterial pellets were re-suspended in 0.5 ml of ice cold 200 mM Tris pH 7.5 (Severn Biotech Ltd., Kidderminster, UK, cat. No. 207901-10) with 20 % (w/v) sucrose (Sigma, Poole, UK, cat. no. S0389), 10 µl 100 mM EDTA (Thermo Fisher, Loughborough, UK, cat. no. BP120-1) and 0.05 mg lysozyme (Sigma, Poole, UK, cat. no. L6876), then incubated with gentle rotation on ice for 15 minutes. After 15 minutes, 0.5 ml of ice cold 5 mM magnesium sulphate was added and the mixtures were incubated on ice, rotating for a further 15 minutes. The mixtures were then centrifuged at 6000 G for 10 minutes. The supernatants were filtered (0.2 µm) and stored at -20°C until use.

5.4.4.5 Analysis of performance of soluble scAbs by ELISA

Target molecule-protein conjugates and free carrier proteins were immobilised to the wells of Nunc MaxiSorb™ F96 MicroWell™ plates (Thermo Scientific, Roskilde, Denmark, cat. no. 442404) by incubation of 100 µl/well at a protein concentration of 1µg/ml overnight at 2 – 8°C. After the immobilisation step, excess target molecule-protein conjugate and carrier protein was removed from wells by washing with 3 x 300 µl/well PBST buffer and then rinsing with 3 x 300 µl/well PBS using a multichannel pipettor. Residual liquid was removed from the wells by inverting the 96-well plate and tapping out on absorbent paper.

Wells were blocked to reduce non-specific actions of phage antibodies with any uncoated areas of the hydrophobic plastic surfaces by incubation with 200 µl/well 2% MPBS at a temperature of 37°C for two hours. After two hours the wells were washed as described previously.

In binding ELISAs a ½ dilution series of scAb (50 µl/well) was added to wells coated with each target molecule-protein conjugate and each free carrier protein. Fifty microlitres of 4% (w/v) MPBS was then added to each well and the plate was incubated at room temperature for 1 hour and washed as described previously.

In inhibition ELISAs scAb was incubated with free target molecule dissolved in 20 % methanol (v/v) 2% (w/v) MPBS for an hour prior to addition to the coated and blocked microtitre plate, and a second sample of each scAb was incubated with 20 % (v/v) methanol 2% (w/v) MPBS with no free target molecule prior to application to the coated and blocked ELISA plates.

Anti human kappa light chain (bound and free) peroxidase conjugate (Sigma, Poole, UK) was added 100 µl/well in 2 % (w/v) MPBS buffer at a concentration of 1/1000 and incubated for one hour at room temperature. The plate was then washed as described previously.

Tetramethylbenzidine.dihydrochloride (TMB) substrate (Sigma Aldrich, Poole, UK, cat. no. T3405) in 0.05 M phosphate citrate buffer containing 0.03 % (w/v) sodium perborate pH 5.0 (Sigma Aldrich, Poole, UK, cat. no. P4992) was added to the microtitre plate 100 µl/well; reaction with HRP labelled antibodies bound to antigen immobilised on the surface of the wells produced a coloured product in quantities proportional to the amount of immobilised antibody.

After approximately 10 minutes the enzyme-substrate reaction was stopped by the addition of 50 µl/well of 1 M sulphuric acid. The absorbance of each well was read at a wavelength of 450 nm using a Varioskan Flash plate reader (Thermo Scientific, Leicestershire, UK).

5.5 Structure of work

In line with the aims and objectives of this work (Section 5.2) the immunised library was initially screened by application of a selected literature screening protocol which involved panning the library and then analysing the resulting enriched population of phage. However, the clones produced from the application of the selected literature protocol did not give appropriate performance and therefore it was necessary to modify the screening strategy used and re-screen the library.

The experimental work and results are therefore presented in two sections. The first section details the experimental design and describes and discusses the results from the first attempt to screen the immunised recombinant library using a selected literature screening protocol. The second section describes the experimental design and reports and discusses the work from a second attempt to screen the library, modified based on the outcome of the first screening strategy.

Following these two sections the outcome of both screening strategies are discussed together and conclusions/recommendations for further work are made.

5.6 Screening strategy 1: Panning using target molecule-carrier protein conjugates

5.6.1 Experimental design

The literature protocol selected for initial screening of the immunised library was that of Charlton *et al* (2001), who screened a phage display library, constructed from an immunised sheep, for antibodies that bound to atrazine using atrazine-carrier protein conjugates. The literature protocol used both non-specific and specific elution of bound phage and the alternation of carrier proteins between panning rounds. This screening protocol was selected, on the recommendation of the company who constructed the immunised library, because it had previously been used for the selection of anti-small molecule antibodies from similar libraries, including anti-atrazine and anti-homoserine lactone antibodies. The literature protocol was followed as closely as possible, although some minor modifications were necessary due to the nature of the selected target molecules.

Two extinct life marker molecules were selected for use in screening strategy 1; hopane as a representative example of a polycyclic aliphatic target molecule (Section 5.3.2), and octadecane as an example of a straight chain alkane acyclic aliphatic target molecule (Figure 5-4). The carrier protein-target molecule conjugates used in the panning of the library consisted of derivatised versions of the extinct life biomarker target molecules conjugated to carrier proteins via amide bond linkages (Figure 5-4).

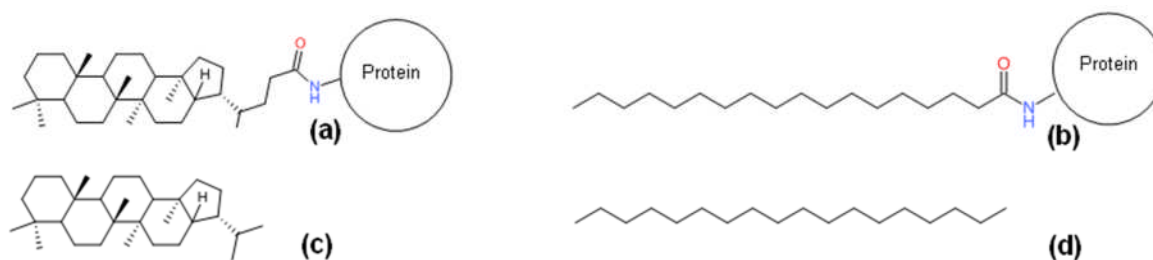


Figure 5-4: Structure of conjugates and free target molecule used in screening strategy 1 (a) hopane conjugate (b) octadecane straight chain alkane conjugate (c) free hopane and (d) free octadecane

Figure 5-5 illustrates the panning approach adopted for both targets and the details of each round of panning are summarised in Table 5-6 (hopane) and Table 5-7 (octadecane) (general procedures for panning are given in Section 5.4.3.3). Each round of panning involved exposure of phage to immobilised target molecule-protein conjugates, washing to

remove unbound/weakly bound phage followed by either TEA (non-specific) or competitive (specific) elution with free target molecule.

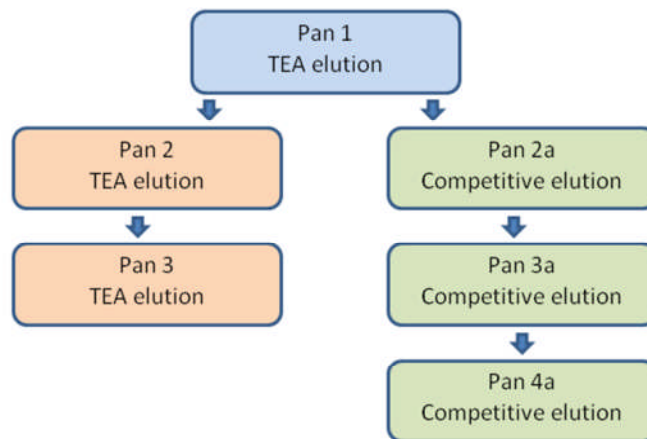


Figure 5-5: Block diagram illustrating panning protocol used for screening strategy 1

For each target the first round of panning (pan 1) used non-specific TEA elution. After amplification the material from pan 1 was split, and half was subject to two further rounds of panning with TEA elution (pan 2 and 3) whilst the other half was subject to three further rounds of panning using competitive elution with the free target molecule (pan 2a, 3a and 4a).

Table 5-6: Summary of panning protocol used in screening strategy 1 for selection of hopane binding clones

Panning Round	Subtractive pan	Coating antigen	Coating antigen concentration (µg/ml)	Binding Buffer	No. of washes	Elution reagent
1	N/A	Hop-BSA	10	2 % MPBS 0.5 mg/ml BSA	10 x PBST, 10 x PBS	TEA (100 mM)
2	1 mg/ml BSA	Hop-BSA	1	2 % MPBS 0.5 mg/ml BSA	10 x PBST, 10 x PBS	TEA (100 mM)
3	N/A	Hop-BSA	1	2 % MPBS 0.5 mg/ml BSA	10 x PBST, 10 x PBS	TEA (100 mM)
2a	1 mg/ml BSA	Hop-BSA	1	2 % MPBS 0.5 mg/ml BSA	10 x PBST, 10 x PBS	Hop (10 µM)
3a	N/A	Hop-BSA	1	2 % MPBS 0.5 mg/ml BSA	10 x PBST, 10 x PBS	Hop (1 µM)
4a	1 mg/ml BSA	Hop-BSA	1	2 % MPBS 0.5 mg/ml BSA	10 x PBST, 10 x PBS	Hop (0.1 µM)

Table 5-7: Summary of panning protocol used for screening strategy 1 for selection of octadecane binding clones

Panning Round	Subtractive pan	Coating antigen	Coating antigen concentration (µg/ml)	Binding Buffer	No. of washes	Elution reagent
1	N/A	Oct-BSA	10	2 % MPBS 0.5 mg/ml BSA	10 x PBST-X, 10 x PBS	TEA (100 mM)
2	N/A	Oct-KLH	1	2 % MPBS 0.5 mg/ml KLH	10 x PBST-X, 10 x PBS	TEA (100 mM)
3	N/A	Oct-BSA	1	2 % MPBS 0.5 mg/ml BSA	10 x PBST-X, 10 x PBS	TEA (100 mM)
2a	N/A	Oct-KLH	1	2 % MPBS 0.5 mg/ml KLH	10 x PBST-X, 10 x PBS	Oct (10 µM)
3a	N/A	Oct-BSA	1	2 % MPBS 0.5 mg/ml BSA	10 x PBST-X, 10 x PBS	Oct (1 µM)
4a	N/A	Oct-KLH	1	2 % MPBS 0.5 mg/ml KLH	10 x PBST-X, 10 x PBS	Oct (0.1 µM)

PBSTX – 0.05 % (w/v) Triton-X 100 in PBS buffer TEA – triethylamine Oct – octadecane Hop – hopane

Key adaptations vs literature protocol

- 1) For hopane only one protein conjugate was available, therefore it was not possible to alternate carrier protein between panning rounds. In order to minimise amplification of BSA binding clones a subtractive panning step was performed in alternate panning rounds. Please see Section 5.4.3.3 for details
- 2) The wash buffer used when panning for octadecane binding clones was adapted because P20 in the standard PBST wash buffer has structural similarities to octadecane. P20 was replaced with Triton-X 100 which has a polyether side chain
- 3) Free target molecules were solubilised in solutions of 20/80 % (v/v) methanol PBS, due to their poor water solubility. Please see Section 5.4.3.2 for details

5.6.2 Results and discussion

The selected literature protocol with the adaptations described in Section 5.6.1 was applied to the immunised library for the isolation of anti-hopane and anti-octadecane antibodies. Following the completion of all panning rounds the output was initially assessed by polyclonal ELISA.

5.6.2.1 Assessment of binding to target molecule-carrier protein conjugate by enriched population of phage via polyclonal ELISA

Polyclonal phage from each round of hopane panning was tested for binding to BSA, BSA-hopane and KLH (Figure 5-6) and polyclonal phage from each round of octadecane straight chain alkane panning was tested for binding to BSA, BSA-octadecane, KLH and KLH-octadecane (Figure 5-7). Pan 0 phage (phage taken from the original library population before any panning had been carried out) was also tested for binding to BSA-hopane, BSA-octadecane and KLH-octadecane conjugates, and free BSA and KLH carrier proteins to determine the inherent binding properties of the starting material.

The ELISA data for polyclonal phage selected by panning for hopane (Figure 5-6) and octadecane straight chain alkane (Figure 5-7), by both TEA elution and competitive elution show that binding to target molecule-protein conjugate increased with each panning round, with no similar increase in binding signal observed for free carrier protein. This indicates that the panning process was successful in terms of enriching the selected pool with conjugate binding phage clones.

Maximum binding signal, and therefore enrichment of conjugate binding clones was achieved by round 2 (TEA elution) and round 2a (competitive elution) for hopane and round 1 (TEA elution) and round 2a (competitive elution) for octadecane. Maximum binding signal was about 0.4 OD units lower for phage selected by competitive elution than for phage selected by non-specific elution for both hopane and octadecane, but because the ELISAs for competitively eluted and non-specifically eluted phage were not run alongside each other, these variations may be due to subtle differences in the timing of incubation steps during the ELISA, rather than a true difference in the proportion of phage that bound to the conjugate.

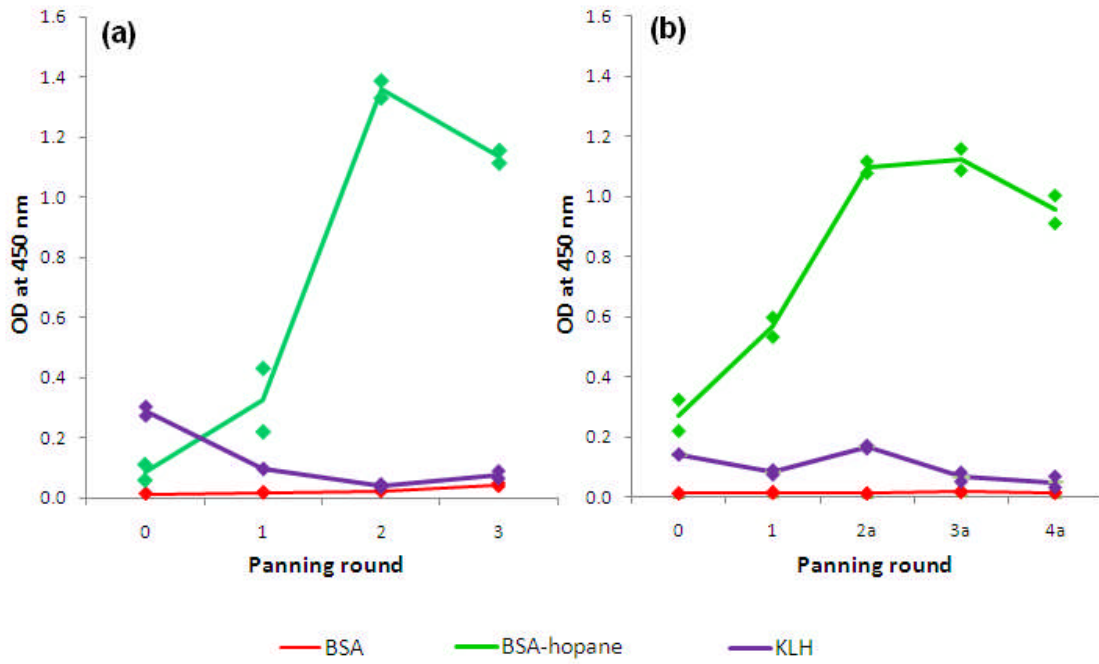


Figure 5-6: Screening strategy 1 - Hopane polyclonal phage ELISA with phage population generated by (a) non-specific TEA elution and (b) competitive elution with free hopane. Data points are individual replicates and lines are the linear interpolation of mean OD values for polyclonal phage from each panning round.

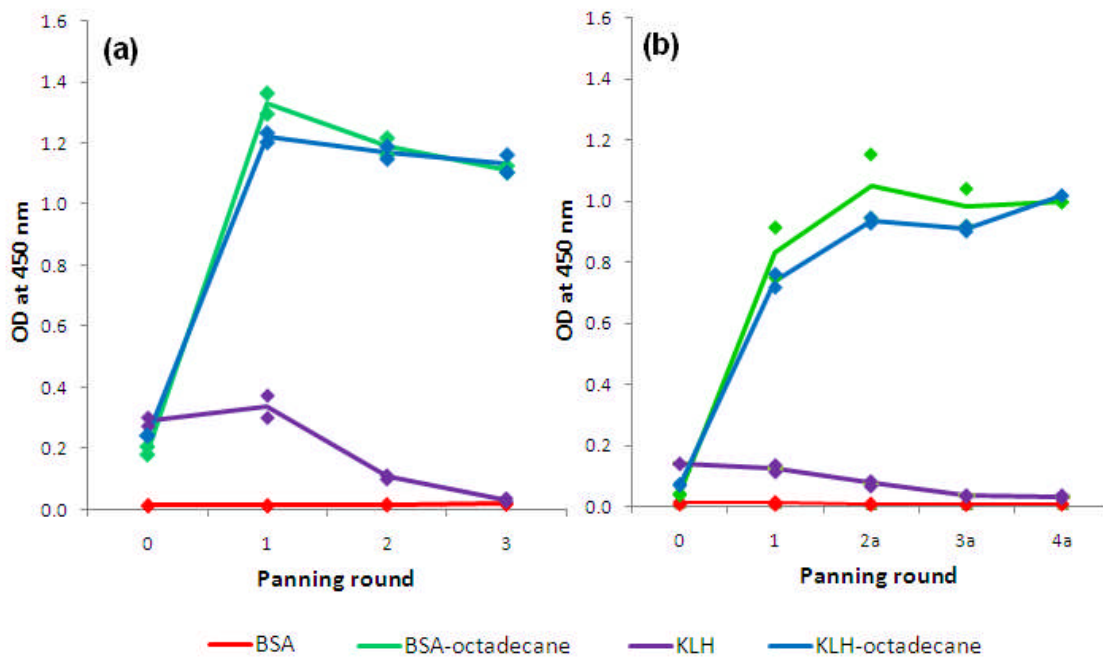


Figure 5-7: Screening strategy 1 - Octadecane polyclonal phage ELISA with phage population generated by (a) TEA elution and (b) competitive elution with free octadecane. Data points are individual replicates and lines are the linear interpolation of mean OD values for polyclonal phage from each panning round.

Polyclonal ELISA data is intended to give an indication of the effect of panning on the population of selected phage, showing trends, rather than allowing comparison of the absolute binding signal and the concentration of phage used in the ELISA from each panning round was in the order of 10^{12} infective phage particles/ml but was not identical across all pans.

For phage generated by panning for hopane binding clones, a single carrier protein (BSA) was used during panning, due to limited availability of the required starting materials. Despite this the polyclonal ELISA data shows no evidence of the presence of BSA binding clones. This demonstrates that the use of a subtractive panning step against BSA in alternate panning rounds (Table 5-6) was successful in minimising the enrichment of BSA binding clones.

The polyclonal ELISA data shows that the panning protocol successfully enriched the selected population of phage with clones that recognise the relevant carrier protein-target molecule conjugates. Comparison with previous work, including the selected literature protocol on which this protocol was based, indicates that with this level of enrichment the isolation and identification of relevant clones by monoclonal ELISA should be possible.

5.6.2.2 Assessment of binding characteristics of selected phage clones by monoclonal phage ELISA

Phage from panning rounds which had shown enrichment in binding to conjugate, but no enrichment in binding to carrier protein (hopane panning rounds 2, 3, 3a and 4a; octadecane panning rounds 1, 2, 3, 3a and 4a) were taken forward for analysis of individual clones by monoclonal ELISA. Monoclonal phage was tested for binding to conjugate and to carrier protein in a monoclonal ELISA (please see section 5.4.4.2 for detailed protocol). Table 5-8 gives an example of some typical monoclonal ELISA data and data from all monoclonal ELISAs for hopane and octadecane is summarised in Table 5-9 (hopane) and Table 5-10 (octadecane). For the purposes of this assessment clones were defined as conjugate binding clones if the binding signal produced with conjugate was greater than 5 x the intensity of the binding signal with free carrier protein. A sub-set of the clones were also tested in inhibition ELISA format to determine if they recognised the free target molecule. Clones were defined as recognising the free target if they exhibited 5 % inhibition of signal or greater with 10 μ M solutions of free target molecule.

Table 5-8 :Example of typical monoclonal binding ELISA data for 95 clones from screening strategy 1 pan 3 for hopane. Data shown as binding signal (OD at 450 nm) laid out in 96-well plate format. Clones that gave a binding signal with hopane-BSA (plate 1) of greater than 5 x binding signal with BSA (plate 2) were defined as conjugate binding clones and are highlighted in yellow (on plate 1 only).

Plate 1 -Binding of 95 phage clones to hopane-BSA conjugate (well A1 is a negative control)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.05	0.70	0.02	0.03	0.02	0.02	0.02	0.02	0.03	0.02	0.03	0.03
B	0.09	0.03	0.02	0.03	0.02	0.02	0.02	1.15	0.02	0.02	0.08	1.15
C	0.02	0.02	0.02	0.01	0.02	0.02	0.44	0.03	0.29	0.03	1.14	0.07
D	0.03	0.02	0.02	0.02	0.02	0.02	0.01	0.03	0.61	0.89	1.02	0.05
E	0.02	0.91	0.02	0.02	0.02	0.02	1.20	0.45	0.03	1.02	0.60	0.06
F	1.06	0.13	0.02	0.02	0.02	0.02	0.02	0.15	0.82	0.06	1.20	0.06
G	0.02	0.02	0.17	0.02	0.42	1.14	0.03	1.02	0.82	0.05	1.37	1.27
H	0.02	0.01	0.85	0.02	0.02	0.02	0.02	0.03	0.02	0.03	0.03	0.03

Plate 2 Binding of same 95 phage clones to BSA (well A1 is a negative control)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.01	0.01	0.02	0.02	0.02	0.02	0.03	0.01	0.02	0.02	0.02	0.02
B	0.02	0.02	0.01	0.02	0.01	0.02	0.01	0.01	0.02	0.02	0.02	0.02
C	0.02	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.02	0.02	0.03
D	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02
E	0.02	0.02	0.02	0.01	0.01	0.02	0.01	0.01	0.02	0.02	0.02	0.02
F	0.02	0.02	0.03	0.02	0.01	0.02	0.01	0.02	0.02	0.02	0.03	0.02
G	0.02	0.02	0.01	0.01	0.02	0.02	0.03	0.02	0.02	0.02	0.02	0.02
H	0.02	0.02	0.01	0.02	0.01	0.02	0.03	0.02	0.02	0.02	0.02	0.02

Table 5-9: Summary of monoclonal ELISA data for screening strategy 1 for hopane.

Panning Round	No of clones (out of 95) showing binding to	
	Conjugate	Free hopane
2	1	Not tested
3	27	Not tested
3a	21	6
4a	5	4

Table 5-10: Summary of monoclonal ELISA data for screening strategy 1 for octadecane.

Panning Round	No of clones (out of 95) showing binding to	
	Conjugate	Free octadecane
1	1	Not tested
2	51	Not tested
3	56	Not tested
3a	43	5
4a	18	4

The number of clones from each panning round that showed binding to conjugate varied, and in general the number initially increased with each panning round and then stabilised or began to fall. For example, the number of hopane-BSA conjugate binding clones selected increased from one out of 95 from panning round 2, to 27 out of 95 from panning round 3 (Table 5-9). There was also an increase in the number of selected hopane-BSA binding clones between panning rounds 1 and round 3a (competitive elution with free hopane) from 1/95 clones to 21/95 clones, but by panning round 4a the number of conjugate binding clones fell to 5/95.

Increasing the number of panning rounds in a selection generally increases the number of clones in the selected pool that bind to the conjugate, but also reduces the diversity of the selected population (Lou *et al*, 2001) and can result in an increase in the population of non-specific or non-relevant phage (O'Brien *et al*, 1999). An enrichment in non-specific phage may account for the reduction in the proportion of conjugate binding clones observed in later panning rounds. From the monoclonal ELISA data it is not possible to determine whether the conjugate binding phage clones selected are all multiple copies of the same clone, or a number of unique clones.

In order to function in the LMC inhibition immunoassay, antibodies must bind to target molecule-protein conjugate and must also bind to the free target molecule, ideally with a higher affinity than binding to conjugate. A small number of clones showed some inhibition of binding signal in the presence of 10 μ M of their respective free antigen for both hopane (Table 5-9) and octadecane (Table 5-10), but the reduction in signal was low (5 – 20 % inhibition) compared to that observed by Charlton *et al* (2001) where a sub-population of phage clones selected using competitive elution showed 90 – 40 % inhibition of binding signal in the presence of 1 μ M atrazine. This indicates that the affinity of the selected clones

for their respective free targets is likely to be poor in comparison to the affinity of the anti-atrazine clones selected by Charlton *et al.*, which had sub nanomolar IC₅₀ values.

5.6.2.3 Assessment of diversity of selected clones via amino acid sequence analysis of heavy and light chain CDRs

In order to determine the diversity of the pool of phage selected with non-specific and competitive elution using screening strategy 1, the gene coding for the expressed antibody fragment was sequenced for a number of hopane conjugate and octadecane conjugate binding clones.

A total of 20 target molecule-carrier protein conjugate binding clones were sequenced per target from pans 2 and 3 (hopane) and 1, 2 and 3 (octadecane). In addition all of the clones from pans 3a and 4a (hopane and octadecane) that had shown binding to conjugate and inhibition of binding in the presence of free target molecule were also sequenced. The amino acid sequences of complementarity determining regions (CDRs) on the heavy and light chains on the scFvs are summarised in Table 5-11 (clones selected by panning for hopane binders) and Table 5-12 (clones selected by panning for octadecane binders).

Table 5-11: CDR amino acid sequences of anti-hopane phage clones selected from screening strategy 1 with non-specific elution (TEA) and competitive elution (C). Variations in the amino acid sequences are highlighted in red. Clones with missing CDRs were truncated

Clone Identifier	Elution method	No. of copies	V _{H1}	V _{H2}	V _{H3}	V _{L1}	V _{L2}	V _{L3}
G1	TEA	8	FTSNAVG	DMSAGGRTYLNPAKLS	GTSGYFVHIDY	SGSSSNIGVFGVS	RNNKRPS	ASADNSANF
	TEA	2	LTSNAVG	DMTSSGGRTYYNPALKS	GAGAYFVHIDN	SGSSSNIGVFGVS	RNNKRPS	ASADNSANF
	TEA	2	LSSNAVG	DMSAGGRTYLNPAKLS	GTSGYFVHIDY	SGSSSNIGVFGVS	RNNKRPS	ASADNSANF
	TEA	2	FTSNAVG	DMSAGGRTYLNPAKLS	GTSGYFVHIDY	SGSSSNIGLLGVS	ASNRRPS	ASADKSAF
	TEA	1	LRSNAVG	DMSAGGRTYLNPAKLS	GTSGYFVHIDY	SGSSSNIGVFGVS	RNNKRPS	ASADNSANF
	TEA	1	LNSNAVG	DMSTGGRTYLNPAKLS	GTSGYFVHIDY	SGSSSNIGVFGVS	RNNKRPS	ASADNSANF
	TEA	1	FTSNAVG	DMSTGGRTYLNPAKLS	GTSGYFVHIDY	SGSSSNIGVFGVS	RNNKRPS	ASADNSANF
	TEA	1	LSSNAVG	DMSAGGRTYLNPAKLS	GTSGYFVHIDY	GSSSNIGVFGVS	RNNKRPS	ASADNSANF
	TEA	1	FTSNAVG	DMSAGGRTYLNPAKLS	GTSGYFVHIDY	SGSSSNIGVFGVS	RNYKRPS	ASADKSAF
	TEA	1	-	-	-	SGSSSNVGYANYVS	DATTRAS	ASYDNRDSNI
H1	C	5	LSSVGVG	NINSDGVTYYPALRS	GSALGRGFFHGGYPGNIDY	TGTSSDIGAYKYVG	NVNRKPS	GSLKSGGTAL
	C	2	-	-	-	SGSSDNVGRFPVG	GTNNRPA	TSFDSSGSIL
	C	1	-	-	-	RTNQNINGFLN	HATKLY	FQYSTLPLA
	C	2	LRSNAVG	DMSAGGRTYLNPAKLS	GTSGYFVHIDY	SGSSSNIGVFGVS	RNNKRPS	ASADNSANF

Table 5-12: CDR amino acid sequences of anti-octadecane phage clones selected from screening strategy 1 with non-specific elution (TEA) and competitive elution (C). Variations in the amino acid sequences are highlighted in red. Clones with missing CDRs were truncated

Clone Identifier	Elution Method	No. of copies	V _{H1}	V _{H2}	V _{H3}	V _{L1}	V _{L2}	V _{L3}
G1	TEA	9	FTSNAVG	DMSAGGRTYLNPAKLS	GTSGYFVHIDY	SGSSSNIGVFGVS	RNNKRPS	ASADNSANF
	TEA	2	FTSNAVG	DMSTGGRTYLNPAKLS	GTSGYFVHIDY	SGSSSNIGVFGVS	RNNKRPS	ASADNSANF
	TEA	1	LTSNAVG	DMTSSGGKTYYSALKS	DSDFGGYFVHITY	SGSSSNIGVFGVS	RNNKRPS	ASADNSANF
	TEA	1	LTSNAVG	DMTSSGGKTYYSALKS	DSGPGGYFVHPTY	SGSSSNIGVFGVS	RNYKRPS	ASADKSAF
	TEA	1	LSSNAVG	DMTSSGGKTYYSALKS	GTSGYFVHIDY	SGSSSNIGVFGVS	RNNKRPS	ASADNSANF
	TEA	1	LTSNAVG	DITSRGNTYFNPALKS	GSSTYFIHIDY	SGSSSNIGVFGVS	RNNKRPS	ASADNSANF
	TEA	1	LTSNAVG	DMTSSGGKTYYSALKS	DSGPGGYFVHPTY	SGSSSNIGVFGVS	RNYKRPS	ASADKSAF
	TEA	1	-	-	-	SGSSSNVGTYGVG	GTNSRPS	AAEDNTIFNGV
	TEA	1	-	-	-	SGNIDTWGVG	GVGNRPS	GTYTEERGN
	G1	C	8	FTSNAVG	DMSAGGRTYLNPAKLS	GTSGYFVHIDY	SGSSSNIGVFGVS	RNNKRPS
SA1	C	1	FTSNAVG	DMSAGGRTYLNPAKLS	GTSGYFVHIDY	SGSSSNIGLFGVS	DTFKRSS	ASADRSLSF

Ten complete unique sequences were identified from the 30 sequenced hopane conjugate binding clones (Table 5-11), there were also four unique truncated sequences, *i.e.* sequences missing the heavy chain region of the antibody binding site. It has been observed previously that phage with a smaller insert (usually missing the genetic material coding for the heavy chain (V_H), as was observed in this case) can dominate an enriched pool of phage after only a small number of panning rounds because they may have non selective growth advantages (Azzazy and Highsmith 2002). The majority of the sequences for hopane conjugate binding clones were highly conserved with only a few (between 1 and 5) residue variations, which were mostly located in V_{H1} and V_{H2} . One of the unique sequences, identified as H1 and selected by competitive elution, stands out from the others because it has residue variations in all six hypervariable regions and has 6 additional residues inserted into V_{H3} .

Eight complete unique sequences were identified from the 18 octadecane conjugate binding clones (Table 5-12), and a further two truncated sequences were identified. Similarly to the sequencing data from hopane panning the truncated sequences both lack V_H . The sequences from the octadecane conjugate binding clones showed most variation in the heavy chain, whereas the light chain was highly conserved and several of the sequences are identical to, or very closely resemble the sequences from the sequenced hopane conjugate binding clones. A number of the octadecane clones contained stop codons contained within the framework regions of the heavy chain. In *E. Coli* TG1 80 % of CAA and TAG stop codons are translated as glutamine, whereas the remaining 20 % go to termination reducing scFv expression. When panning for binders to monensin, an antibiotic, Makvandi-Nejad *et al* (2010) observed that by round three of panning 80 % of the selected binders contained a stop codon and they suggested that this could be a result of the toxic nature of the anti-monensin scFvs. The presence of stop codons in the selected monoclonal phage for octadecane binders may indicate that the selected conjugate binding scFvs were toxic in nature.

One clone, which is identified as clone G1 in Table 5-11 and also in Table 5-12 was present in multiple copies in the clones sequenced from hopane and octadecane panning. Assuming that the randomly selected clones that were sequenced are representative of the population of phage enriched by panning, this clone appears to dominate the enriched pool for both hopane and octadecane. Critically this clone was also isolated during parallel panning work that was carried out by ImmunoSolv, using a similar panning strategy for porphyrin and 5β sterane binding clones, in a separate facility.

The implication of the isolation of this clone in panning carried out against four separate targets (and in two different locations, which negates the possibility that the isolation of this clone from pans for multiple targets is a result of environmental contamination) is that the applied panning strategy resulted in the amplification of a non-specific phage clone, which recognises a generic feature of all of the conjugates used to pan for hopane, octadecane, porphyrin and 5 β sterane. However by monoclonal ELISA a small number of clones were identified that showed evidence of inhibition in the presence of free target molecule, suggesting that some of the selected pool of phage may be specific.

5.6.2.4 Assessment of binding characteristics of clones expressed as soluble scAb via ELISA

Three unique sequences (G1, H1 and SA1 in Table 5-11 and Table 5-12) were taken forward for expression as soluble single chain antibody fragments. G1 is the clone that dominated the output of panning for both octadecane and hopane, and was also observed by ImmunoSolv when panning for 5 β sterane and porphyrin. H1 is a unique clone selected from the enriched pool of phage generated by panning for binders to hopane using competitive elution, containing additional residues in V_{H3}, and SA1 is a unique clone selected from the enriched pool of phage generated by panning for binders to octadecane. The binding properties of the scAbs were tested by binding and inhibition ELISA Figure 5-8 (H1), Figure 5-9 (SA1), Figure 5-10 (SA1, H1 and G1).

When expressed as scAbs clone H1 bound to BSA-hopane conjugate but showed no evidence of inhibition of binding in the presence of free hopane (Figure 5-8). Similarly clone SA1 bound to octadecane-BSA conjugate, but showed no inhibition of binding in the presence of free octadecane (Figure 5-9). Both H1 and SA1 had shown a level of inhibition when expressed as fusion-scFvs displayed on phage particles (18 % and 14 % inhibition of binding signal respectively). The failure to recognise free target molecules when expressed as scAbs could be a result of different folding of antigen binding sites on the phage bound ScFv compared to the soluble scAb, (Makvandi-Nejad *et al*, 2010), or it may reflect that the level of inhibition observed in monoclonal phage ELISA was too small to be significant. Note that the concentration of phage used in inhibition ELISAs was selected using binding ELISA data to ensure a sub-saturating concentration of phage.

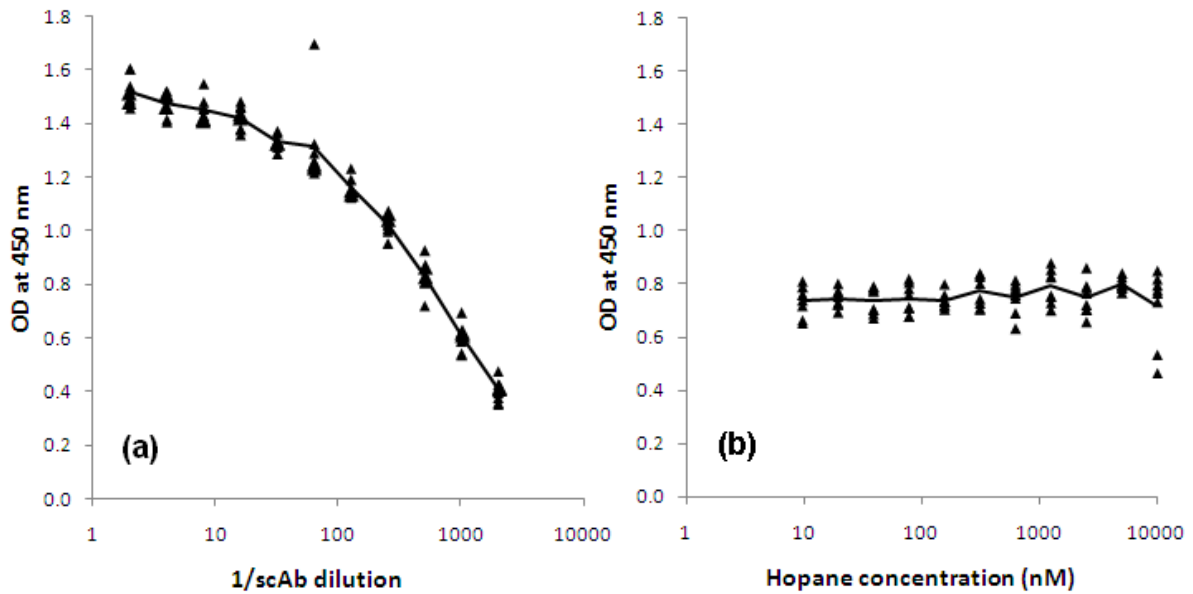


Figure 5-8: scAb ELISAs with clone H1 showing (a) Binding to hopane-BSA conjugate and (b) inhibition of binding to hopane-BSA conjugate in the presence of free hopane.

Each data point is a single replicate. Lines are linear interpolation of the mean binding signal.

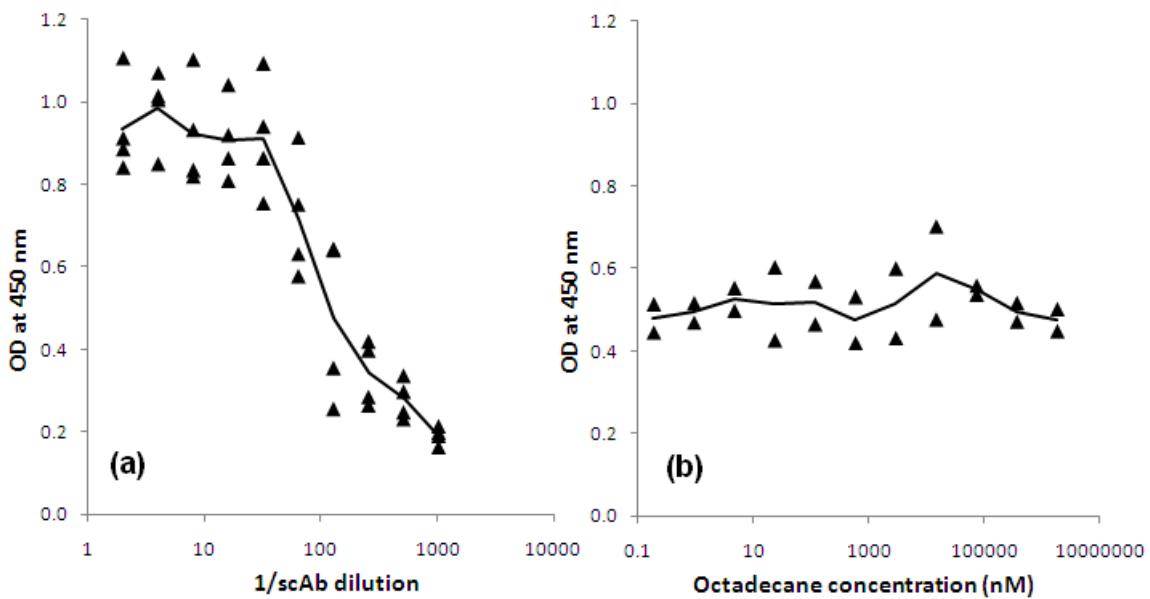


Figure 5-9: scAb ELISA with clone SA1 showing (a) binding to octadecane-BSA conjugate and (b) inhibition of binding to octadecane conjugate in the presence of free octadecane.

Each data point is a single replicate. Lines are linear interpolation of the mean binding signal.

Given the results from inhibition assays with the clones SA1 and H1 which both failed to recognise their respective free target molecules, and in particular due to the recurrence of clone G1 in panning performed for both hopane and octadecane it was decided to perform cross reactivity studies with these three clones (Figure 5-10). The three clones were tested for cross reactivity with protein conjugates of all of the targets used to generate the immunised library (hopane-BSA, octadecane-BSA, porphyrin-BSA, 5 β sterane-BSA and phytane-BSA) (Figure 5-2) in addition to testing for cross reactivity with two other hydrophobic small molecule conjugates produced for other work within the LMC project and not used for the production of the immunised library (carotane-BSA and benzo[a]pyrene-BSA).

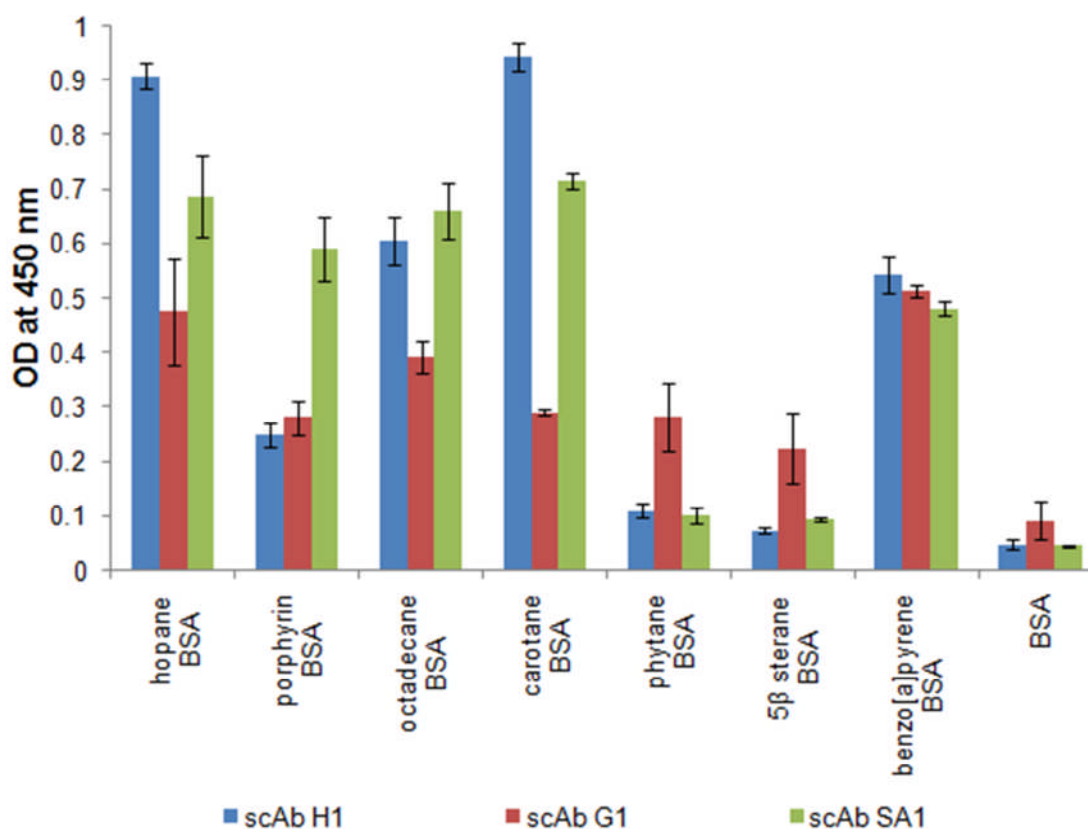


Figure 5-10: scAb binding ELISA for clones H1, G1 and SA1, with hapten-BSA conjugates. Plotted values are the mean of two replicates, error bars = +/- standard error.

Assessment of the cross reactivity of H1, SA1 and G1 by binding ELISA (Figure 5-10) showed that all three clones were cross reactive and bound to a range of hydrophobic small molecule-BSA conjugates, but not to free BSA. It has been observed previously that panning for small molecule targets can result in the selection of clones with characteristics such as

those described here (Charlton *et al*, 2001). These cross reactive conjugate binding clones have been described as interface binders, since they appear to recognise the interface between the small molecule and the carrier protein, but not the free carrier protein or the free target molecule. The use of competitive elution has previously been shown to reduce the occurrence of interface binders (Charlton *et al*, 2001), but all three of these clones were isolated by competitive elution with free target. Therefore in this case the use of competitive elution did not have the desired effect.

5.6.3 Summary and review of screening strategy 1

The immunised recombinant antibody library was screened using a literature protocol for antibodies that bound to hopane and octadecane straight chain alkane. The selected pool of clones was dominated by interface binders, which recognised the relevant target molecule-protein conjugate, but did not recognise the free target molecule and also cross reacted with other small molecule-protein conjugates. Therefore the following section constitutes a review of the outcome of screening strategy 1, leading to identification of possible adaptations to the screening protocol for application to a second attempt to screen the library.

There are two top-level scenarios that may have resulted in failure to successfully isolate appropriate antibodies using screening strategy 1. The first is that the immunised recombinant antibody does not contain any clones that recognise the free target molecules (*i.e.* the immunised sheep from which the library was constructed failed to produce an appropriate immune response). The second scenario is that target molecule binding clones were present within the library but screening strategy 1 was inappropriate, with the result that target molecule binding clones were either not enriched at all, or were enriched but not enriched sufficiently to allow isolation (given the limited number of individual clones that were isolated for characterisation). From the work reported here, it is not possible to determine which of these two scenarios is more likely, but for the purposes of the following discussion the assumption is made that the second scenario is correct and that the recombinant antibody library did contain target molecule binding clones but that screening strategy 1 was non-optimum.

Charlton *et al* (2001) successfully isolated high affinity clones that recognised atrazine (Figure 5-11), a small molecule target with limited water solubility, from an immunised recombinant antibody library using the literature protocol on which screening strategy 1 was based. However atrazine is significantly more polar than hopane and octadecane having an electronegative chlorine substituent on an aromatic ring, offering more opportunities for

binding interactions with an antibody than LMC markers of extinct life such as hopane and octadecane (Figure 5-11).

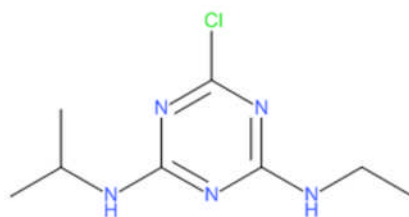


Figure 5-11: Structure of atrazine, target molecule for selected literature screening strategy

Markers of extinct life such as hopane and octadecane are expected to be poorly immunogenic. Therefore it is reasonable to speculate that clones that recognise them may only be present in the recombinant antibody library in small numbers, since a large proportion of the immune response is likely to have been generated towards the more immunogenic carrier protein (Kramer 2002). In addition it has been observed previously, when panning recombinant antibody libraries for small molecule binding clones, that clones that recognise the free target molecule with high affinity tend to have lower affinity for the target molecule-carrier protein conjugate (Charlton 2009). By panning using target molecule-carrier protein conjugates it is possible that rare target molecule binding clones may be quickly out competed by more common interface binders which have higher affinity for the conjugate. An alternative panning strategy that has been used for small molecule targets is to use biotinylated target molecules in place of protein conjugates (Korpimäki *et al*, 2002; Kobayashi *et al*, 2009). The application of biotinylated targets would remove the need for the use of a carrier protein and allow the phage antibody – target interaction to be carried out in solution, rather than with the target immobilised to a surface via a carrier protein. This may be beneficial in terms of minimising the selection of interface binding clones.

The recurrence of the same clone (G1) in phage enriched by panning with different target molecule-carrier protein conjugates (hopane, octadecane, 5 β sterane and porphyrin), both in the work reported here and in parallel work carried out by ImmunoSolv, indicated that the clones enriched by the selection strategy recognised a common element of the conjugates used for panning. The conjugates used in panning (Figure 5-2) were all small hydrophobic molecules conjugated to carrier proteins, with either no linker between the hydrophobic target molecule and the carrier protein (octadecane and phytane), or were linked to the carrier protein via a hydrocarbon chain of 3-4 carbon atoms in length. All target molecules, with or without linker, were linked to their carrier protein via an amide bond. Therefore the cross reactive clones appear to recognise one or more of these common elements *i.e.* the

amide bond linking the target molecule with the carrier protein, the hydrocarbon linker (or the hydrocarbon chain of the target where there was no linker) and generic small hydrophobic molecules presented on a protein surface. Investigation of alternative linker chemistry is impractical within the timescales of this project, but the use of alternating linkers *e.g.* hydrophobic and hydrophilic is achievable in terms of the synthetic chemistry required, and may be beneficial in minimising the selection of interface binders.

An additional concern identified when using competitive elution was that it was difficult to determine with confidence whether the molecules used for competitive elution had been successfully solubilised. This is especially relevant given the observation that competitive elution did not prevent the amplification of interface binders as expected. Extinct life target molecules such as hopane and octadecane have low solubility in aqueous solutions *e.g.* the maximum solubility of octadecane in water is 0.006 mg/L (Singer and Finnerty 1984), and the maximum solubility of hopane in water has not been determined. The successful solubilisation of free target molecule in 20/80 % (v/v) methanol PBS for competitive elution was assessed visually (by inspecting the solution for cloudiness and/or visible particulates) and it is possible that the target molecules were not well solubilised. If this was the case, phage that appeared to be competitively eluted may in-fact have been non-specifically eluted, which may account for the enrichment of interface binders rather than target molecule binding clones. Work carried out at Imperial College has identified that extinct life target molecules are significantly more soluble in MeOH P80 than in 20/80 % (v/v) methanol water, therefore the inclusion of P80 in buffers used during panning may help to solubilise hydrophobic target molecules, as well as being representative of the conditions under which the antibody must function in the flight instrument.

5.7 Screening strategy 2: panning with biotinylated target molecule with alternating linkers and streptavidin coated magnetic beads

5.7.1 Experimental design

Screening strategy 2 was designed based on the outcome of screening strategy 1 avoiding the use of a carrier protein during panning, in order to prevent enrichment of interface binding clones. To achieve this, a biotinylated target molecule was used in place of a target molecule-carrier protein conjugate, allowing the interaction between scFvs expressed on phage and the target molecule to occur in solution without the presence of a carrier protein. After incubation with phage, the biotinylated target molecule (and associated bound phage) were separated from unbound phage using streptavidin coated magnetic beads and used to directly infect *E. coli* for amplification (Hawkins *et al*, 1992), or competitively eluted with free target molecule prior to infection.

The required biotinylated target molecules had to be synthesised specifically for this work, and within the timescales of this thesis the only available biotinylated target was 5 β sterane, a cyclic aliphatic molecule, therefore screening strategy 2 used this molecule as the target. Two different 5 β sterane-biotin conjugates were used for panning, one of which had a hydrophobic pentylamine group linking the 5 β sterane molecule to the biotin and the other had a hydrophilic polyethylene glycol group (PEG3) linking the 5 β sterane molecule to the biotin(Figure 5-12). In order to prevent enrichment of clones that recognised the linker the hydrophobic and hydrophilic linkers were alternated between panning rounds.

In addition to the use of biotinylated target molecule, P80 was introduced into the buffers used during panning in order to improve solubility of hydrophobic target molecules.

Screening strategy 2 was developed based on protocols supplied by Dr. Damjana Kastelic of the Babraham Institute (Cambridge, UK) and included subtractive panning steps in order to deplete streptavidin binding clones. The overall panning procedure used is shown in Figure 5-13 and the details of each panning round are given in Table 5-13. Detailed materials and methods are described in Section 5.4.3.4.

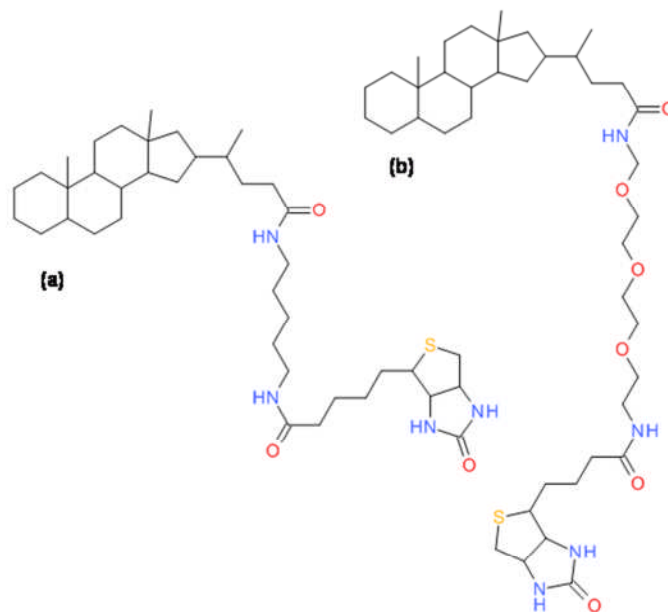


Figure 5-12: Structure of biotinylated 5 β sterane conjugates used for screening strategy 2 (a) 5 β sterane pentyl amine biotin (hydrophobic linker) and (b) 5 β sterane PEG 3 biotin (hydrophilic linker)

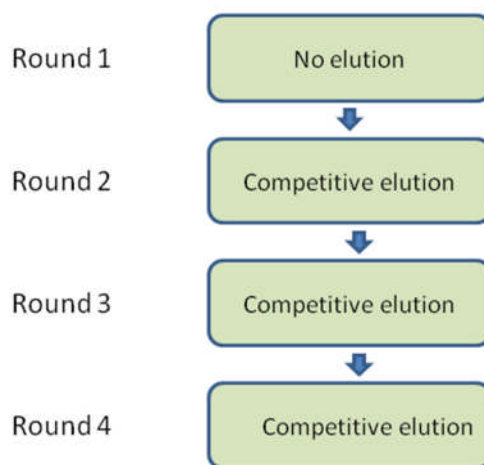


Figure 5-13 Block diagram illustrating panning protocol used for screening strategy 2

Table 5-13: Summary of panning conditions for screening strategy 2, panning using biotinylated antigen and streptavidin coated magnetic beads

Panning Round	Subtractive pan	Biotinylated antigen	Binding Buffer	No. of washes	Elution reagent
1	N/A	5 β sterane PEG 3 biotin	2 % MPBS 20 % MeOH P80	3 x PBS 20 % MeOH P80	No elution
2	Yes	5 β sterane pentyl amine biotin	2 % MPBS 20 % MeOH P80	10 x PBS 20 % MeOH P80	10 μ M coprostane in PBS 20 % MeOH P80
3	Yes	5 β sterane PEG 3 biotin	2 % MPBS 20 % MeOH P80	10 x PBS 20 % MeOH P80	10 μ M coprostane in PBS 20 % MeOH P80
4	Yes	5 β sterane pentyl amine biotin	2 % MPBS 20 % MeOH P80	10 x PBS 20 % MeOH P80	1 μ M coprostane in PBS 20 % MeOH P80

2 % MPBS MeOH P80 = 2 % milk powder in PBS buffer with 20 % (v/v) methanol and 1.5 g/L polysorbate 80

Key design features of panning strategy

- 1) Hydrophobic/hydrophilic linker alternated between panning rounds
- 2) Binding and washing performed in MeOH P80, to improve solubility of biotinylated and free target molecules
- 3) Subtractive panning used to deplete streptavidin binding clones

5.7.2 Results and discussion

Screening strategy 2 was applied to the immunised library for the enrichment of anti-5 β sterane clones. Following the completion of all four rounds of panning the enriched population of phage was analysed.

5.7.2.1 Assessment of binding to target molecule-carrier protein conjugate by enriched population of phage via polyclonal ELISA

After the completion of four rounds of panning, the output of each panning round was assessed by polyclonal phage ELISA (Figure 5-14). Polyclonal phage from each round of panning was tested for binding to 5 β sterane PEG4 BSA (hydrophilic linker), 5 β sterane pentylamine KLH (hydrophobic linker), free BSA and free KLH, in addition to BSA-phytane (no linker) as an additional control for the assessment of cross reactivity with an unrelated hydrophobic small molecule – protein conjugate. An irrelevant conjugate was introduced at polyclonal ELISA analysis stage due to the cross reactive nature of the clones that were isolated by screening strategy 1.

The polyclonal ELISA data (Figure 5-14) show that rounds of panning have enriched the selected pool with clones that bind to 5 β sterane-KLH conjugate, but have also enriched the selected pool with clones that bind to free KLH. Binding signal with KLH conjugate and with KLH is of the same magnitude, which implies that the entire binding signal observed is due to non-specific binding to KLH carrier protein, rather than recognition of the 5 β sterane-KLH conjugate. This theory is also supported by the fact that binding signal to 5 β sterane-BSA and phytane - BSA conjugates does not increase across the four rounds of panning.

Since the immunogens used for construction of the immunised recombinant antibody library were KLH conjugates (Figure 5-2) it is likely that the recombinant antibody library contains a proportion of KLH binding clones, however it is unclear how these clones were enriched in a panning protocol that did not include KLH conjugates. The use of biotinylated target molecule, with alternating hydrophobic/hydrophilic linkers was intended to minimise the selection of interface binders (clones that non-specifically recognise small hydrophobic molecules conjugated to carrier proteins) and in that respect it has been successful, because the enriched population of clones did not bind to unrelated small molecule-carrier protein conjugates. However the enriched population did not specifically bind to the relevant target molecule-carrier protein conjugate.

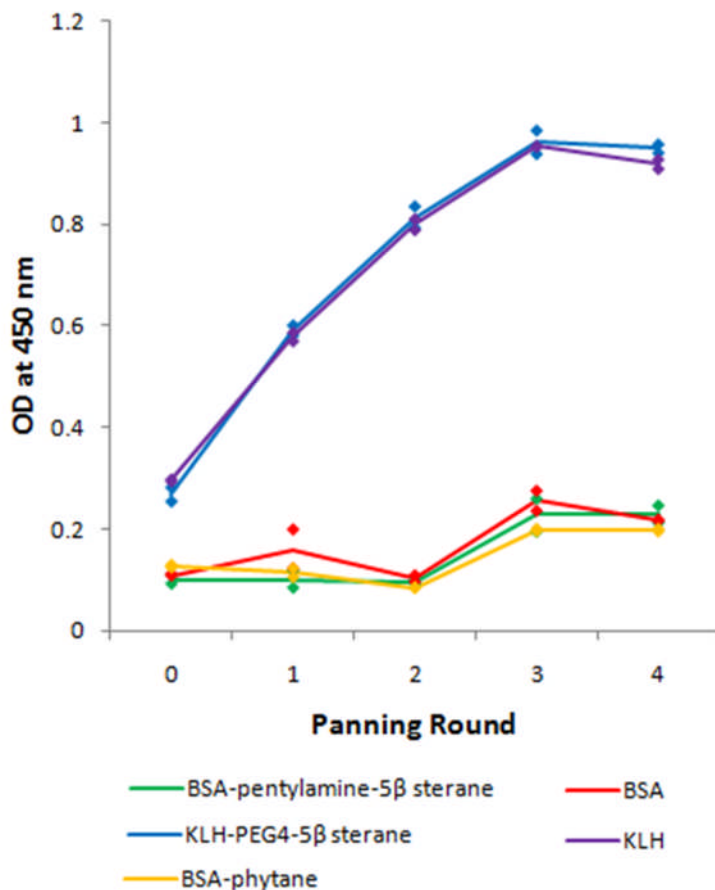


Figure 5-14: Screening strategy 2 5β sterane polyclonal phage ELISA with phage population generated by panning with biotinylated 5β sterane and streptavidin coated magnetic beads. Data points are individual replicates and lines are the linear interpolation of mean OD values for each panning round

The polyclonal ELISA data indicated that the probability of isolating target molecule binding clones from the enriched population of phage was likely to be very small and for this reason the material was not taken forward for further analysis.

5.7.3 Summary

The use of biotinylated target molecule in screening strategy 2, to replace target molecule-carrier protein conjugates, resulted in the enrichment of clones that non-specifically bound to KLH carrier protein. The enriched phage population showed no evidence of recognition of carrier protein-small hydrophobic molecule conjugates (with the exception of 5β sterane-KLH). The mechanism for the enrichment of KLH binding clones is unclear. Analysis was stopped at the polyclonal ELISA stage.

5.8 General discussion

The objective of the work reported in this chapter was to determine if screening the available immunised recombinant antibody library, by phage display, could isolate antibodies that specifically recognise extinct life markers, *i.e.* small hydrophobic molecules, within the context of the available resources and timescales for the LMC project.

In order to meet this objective, and given the context that an immunised recombinant antibody library produced using five pre-selected targets (Figure 5-1) as examples of markers of extinct life was available for use in this work, the following specific objectives were set:

- 1) To attempt to isolate antibodies that bind to extinct life marker molecule by screening the immunised recombinant antibody library using a selected literature procedure for one or more targets
- 2) To test the output of the selected literature procedure, *i.e.* both as antibody fragments expressed as fusion proteins on phage (scFvs) and, where relevant, as soluble single chain antibody fragments (scAbs), for appropriate function in an LMC relevant context
- 3) To review the output of the selected literature procedure and if necessary to modify the protocol in order to favour the selection of clones against extinct life marker molecules

The immunised recombinant antibody library was screened for antibodies that bound to hopane and for antibodies that bound to octadecane using a literature procedure that had previously been used to isolate high affinity small molecule-binding antibodies from an immunised recombinant antibody library (screening strategy 1). The analysis of the output showed, that although the polyclonal ELISA and monoclonal ELISA data appeared promising, the enriched pool of phage was dominated by interface binding clones. In order to try and address this, modifications were made to the panning strategy introducing the use of biotinylated target molecules with alternating hydrophobic and hydrophilic linkers, instead of target molecule-protein conjugates. The adapted protocol did not enrich interface binders, but instead the selected pool was dominated by non-specific KLH binding clones.

The failure to isolate appropriate clones, using first the literature protocol and second the adapted protocol may be due to a number of reasons:

1) There may be no suitable binders present in the library

The immunised recombinant antibody library used in this work may not contain any clones that bind to the free target molecules either because sheep cannot produce antibodies against these target molecules (because they are non-immunogenic or because the sheep has in-built tolerance mechanisms), or because the immunogens used in the generation of the recombinant antibody library were unsuitable due to ineffective conjugate design.

If sheep are incapable of producing appropriate antibodies against biomarker targets of extinct life, alternative animals could be used for the generation of an immunised library, or a library generated from a non-immune source could be used: For example synthetic and semi-synthetic libraries have previously been used to isolate antibodies that are difficult to produce from immunised libraries due to *in vivo* tolerance mechanisms. For example human anti-self antibodies have been isolated from a naïve library constructed from rearranged V_H and V_L genes derived from the peripheral blood lymphocytes of unimmunised health human volunteers (Griffiths *et al*, 1993). Antibodies against fatty acids, which are poorly immunogenic or non-immunogenic (Gargir *et al*, 2002), and heparan sulphate (Kuppevelt *et al*, 1998), a linear polysaccharide that is found in all animal tissues, have been isolated from semi-synthetic phage display libraries.

Structural studies carried out on hapten binding antibodies have shown that small molecules are frequently bound in a cavity or pocket formed by the heavy and light chain variable domains (Kusharyoto *et al*, 2001), and the linkers used for immunisation for this library may have been too short (linker lengths varied from 0 – 4 carbons) to allow the target molecules to access a binding pocket. In the construction of any future immunised libraries it is important that conjugate design, including the length of the linker used to attach the target molecule to the carrier protein is carefully considered.

2) Suitable binders were present in the library but were not isolated using either of the tested screening strategies

It is accepted that no single panning strategy can exploit the full diversity of a recombinant antibody library (Lou *et al*, 2001), however it is concerning that the application of two strategies did not yield any clones that showed recognition of the free target molecule. There are two principle reasons why the strategies used here may have failed; the first is that the parameters used in each of the tested panning strategies were non-optimum, in spite of the modifications made. Panning is a flexible process and making small adjustments in conditions such as the washing steps and the binding buffers can have a significant impact

on the pool of enriched phage (Makvandi-Nejad *et al*, 2010). It may be possible to further optimise the panning conditions used in order to favour the selection of appropriate clones. It is likely that a balance is required between applying enough stringency to minimise enrichment of non-specific and interface binders, but not so much stringency that target molecule binding clones, which may well have low affinities and be rare within the library, are lost. However this is likely to require considerable time to achieve and may be beyond the resources and timescales available for this project.

The other possibility is that there is a fundamental problem with the panning or analysis protocol used. Both panning protocols had common factors. One factor that may play a role in the failure of the protocols attempted was the inclusion of milk powder as a blocking agent during panning. Milk powder is a poorly defined substance and is likely to contain a range of small hydrophobic molecules, which may have interfered with the panning process. In future attempts to screen recombinant antibody libraries consideration should be given to the use of an alternative blocking protein, or to alternating the blocking protein between panning rounds. Polysorbate 20 has been demonstrated to prevent non-specific interactions of phage with plastic (Duan and Siegmund 2010) therefore it may be possible to substitute this for milk powder in future panning protocols, although this may be problematic when attempting to isolate clones that bind to straight chain alkanes due to structural similarities with polysorbate molecules.

Another concern is that LMC target molecules of extinct life may be structurally similar to those present in *E. coli* used for amplification during screening. This may have resulted in the production of phage with occupied binding sites, interfering with successful selection. Alternative non-cell base selection strategies should also be considered *e.g.* ribosome display to avoid this complication, but once again this has significant resource implications.

The analysis methods used in this thesis were low throughput (approximately 300 individual clones were analysed for each target in strategy 1). The application of high throughput selection technologies during the analysis phase of the selection would allow analysis of a much higher number of clones (in the order of tens of thousands) from the selected pool, which may facilitate the isolation of rare clones that bind to free target molecules in a pool dominated by conjugate binding and/or non-specific clones. Once again it is likely that this is beyond the resources of the project, however it may be possible to use high throughput screening in collaboration with other organisations.

In summary, although antibodies against extinct life marker targets were not generated in this work, experience has been gained regarding the challenges in producing antibodies to

small hydrophobic molecules and routes to future progression have been identified; specifically the exploitation of different library types, including immunised libraries from other sources and the use of naive/synthetic libraries in addition to the employment of high throughput screening techniques to increase the probability of isolating rare clones.

5.9 Conclusions and future work

The LMC aims to use a microarray immunoassay in order to detect the presence of markers of extant and extinct life in liquid extracts from martian samples. In order to achieve this it is first necessary to produce antibodies that recognise markers of extinct life. Markers of extinct life are small hydrophobic molecules, comprised primarily of carbon and hydrogen and are therefore perceived as challenging to raise antibodies against. The objective of the work reported in this chapter was to establish if screening an immunised phage display library would allow the isolation of antibodies that bind to a number of markers of extinct life, within the context of the limited resources available. Two attempts were made to screen the immunised recombinant antibody library and analysis of the output of the screenings determined that no suitable clones had been isolated. Therefore the conclusion is that the current immunised recombinant antibody library is unlikely to provide a rapid route to the selection of appropriate antibodies within the available timescales and resources for the LMC project.

The work here led to the identification of several potential alternative approaches to the production of appropriate antibodies, including the use of libraries from alternative sources and identification of the need for access to high throughput screening facilities. This has generated useful information that can be fed into future efforts to isolate appropriate antibodies. The rapid production of antibodies that recognise markers of extinct life is essential in order for the LMC to be ready to meet its objective of searching for extinct life on Mars. An ongoing program of work has been implemented, involving the exploitation of a number of recombinant antibody libraries from a variety of immune and non-immune sources, including synthetic/semi-synthetic libraries and the use of high throughput screening (Table 5-14)¹⁰ which it is hoped will overcome the limitations of the immunised library used within this work. Note that in late 2011 the first indications of successful isolation of clones that recognise free 5 β sterane, isolated from the CAT2 semi-synthetic recombinant antibody library using biotinylated target molecules and high throughput screening have been reported by a collaborating partner.

¹⁰ The following description of future work has been designed by the LMC project team and has included input from the author of this work

Table 5-14: Future program of work showing recombinant antibody libraries to be screened for the isolation of antibodies against small and hydrophobic target molecules of extinct life for the LMC

Library Type	Library name	Library size	Analysis method	Collaborating organisation	Rationale
Immunised camelid	N/A	1×10^8	Low throughput	Vlaams Instituut voor Biotechnologie (VIB), Gent, Belgium	Use of different animal for construction of immunised library with improved conjugate design.
Immunised sheep	N/A	circa 1×10^8	Low throughput	ImmunoSolv Ltd, Aberdeen, UK	Improved conjugate design for immunisation, further modifications to panning protocols
Semi-synthetic human	Nissim	$> 1 \times 10^8$	Low throughput	N/A	Non-immune library, may offer benefits in terms of isolating antibodies against non-immunogenic targets
Semi-Synthetic Human	CAT2.0	1.3×10^{11}	High throughput	Medimmune, Cambridge, UK	Large non-immune library, may offer benefits in terms of isolating antibodies against non-immunogenic targets, high throughput screening for isolation of rare clones
Semi-Synthetic Human	HuCal PLATINUM®	4.5×10^{10}	High throughput	AbD Serotech, Munich, Germany	Large non-immune library, may offer benefits in terms of isolating antibodies against non-immunogenic targets, high throughput screening for isolation of rare clones
Semi-synthetic	Cavity library	TBC	TBC	University of Lund, Sweden	Library design to be based around pocket binding antibodies for hapten binding

6 Demonstration of LMC Format Extraction and Detection in Flight Representative Hardware

6.1 Introduction and context

The flight format immunoassay differs from the ELISA and pooled microarray formats which have been used throughout this thesis. Successful demonstration of immunoassay function in the proposed LMC extraction solvent (Chapter 3) and with Mars relevant analogue samples/sample matrix components (Chapter 4) using ELISA and pooled microarray formats indicates that immunoassays will also function in a more flight representative format, *i.e.* in a multiplexed microarray immunoassay implemented within a microfluidic channel using evanescent excitation of fluorophores, but this must be confirmed experimentally.

As part of the ESA funded Technology Readiness Level (TRL) Upgrade Study for the LMC instrument, a flight instrument representative breadboard, which is described in detail in Section 6.3, was developed to (i) validate the implementation of multiplexed microarray immunoassays within microfluidic channels, (ii) to demonstrate the optical waveguide technology proposed for excitation of fluorophores bound to microarrays and (iii) to demonstrate read out of immunoassay signal for the LMC flight instrument. Therefore the objective of the work reported in this chapter was to perform an 'end to end' demonstration of the LMC instrument using the flight representative breadboard to analyse a liquid extract from a Mars analogue sample. The purpose of this work was to confirm that flight format immunoassays function in the proposed LMC extraction solvent and with Mars relevant analogues/sample matrix components, and to investigate the interaction of liquid extracts with the microfluidic and optical aspects of the flight format immunoassay.

6.2 Chapter objectives

The objective of the work reported in this chapter was to confirm that LMC immunoassays function with liquid extracts from Mars analogue samples in a flight representative format. In order to meet this aim the following specific objectives were set.

- 1) To confirm compatibility of liquid extracts from Mars analogue samples with the optical and microfluidic aspects of the flight format immunoassay *i.e.* fluid flow within a microfluidic channel with evanescent excitation of bound fluorophores by coupling of laser light into optical waveguides
- 2) To confirm the function of LMC relevant immunoassays in a liquid extract composed of the proposed LMC extraction solvent and sample matrix components from a Mars analogue sample in flight representative format *i.e.* immunoassays performed within a microfluidic channel with antibodies and other immunoassay reagents freeze dried in a glass fibre pad

Note that 'end to end' demonstration of the LMC instrument, from extraction to detection, using breadboards in a laboratory environment, was required in-order for the LMC to meet the required TRL for progression to the next phase of instrument development.

6.3 Description of the assay breadboard

The assay breadboard (ABB) is an optical and fluidic demonstrator and was developed by LMC Consortium members at the University of Leicester, UK; Dutch Space, The Netherlands; and LioniX b.v., The Netherlands as part of a TRL Upgrade Study funded by the European Space Agency. The ABB (Figure 6-1) consists of an optical and imaging system and a fluid control system integrated with a module composed of a stack of 'single use' immunoassay components which form the microfluidic channel where the LMC immunoassay takes place.

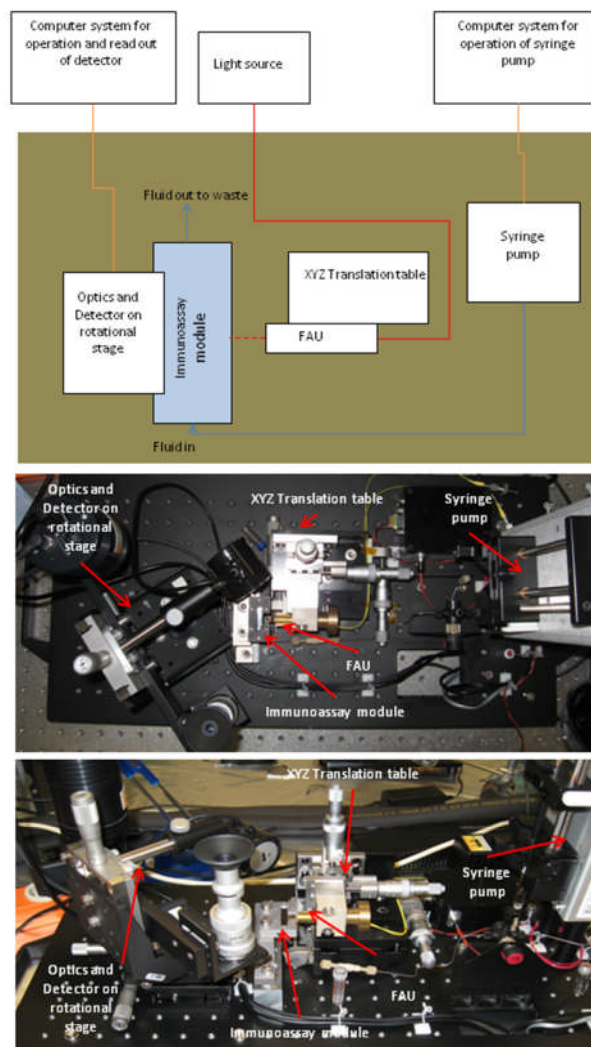


Figure 6-1: Images of the assay breadboard showing from top to bottom outline layout of the assay breadboard (not to scale), photograph of the assay breadboard top view, and photograph of assay breadboard side on view (FAU = fibre alignment unit, used to couple laser light into optical waveguide chip in immunoassay module).

In the following sections the key components of the ABB are described in more detail.

6.3.1 Assay breadboard immunoassay module

The ABB immunoassay module, which contains the microfluidic channel where the multiplexed immunoassays take place, consists of a stack of four components clamped together in an aluminium holder. The four components are (i) an optical waveguide chip, (ii) a Viton[®] gasket, (iii) a glass fibre pad containing freeze dried fluorescently labelled antibodies and other assay reagents, and (iv) a glass fluidic cover (Figure 6-2).

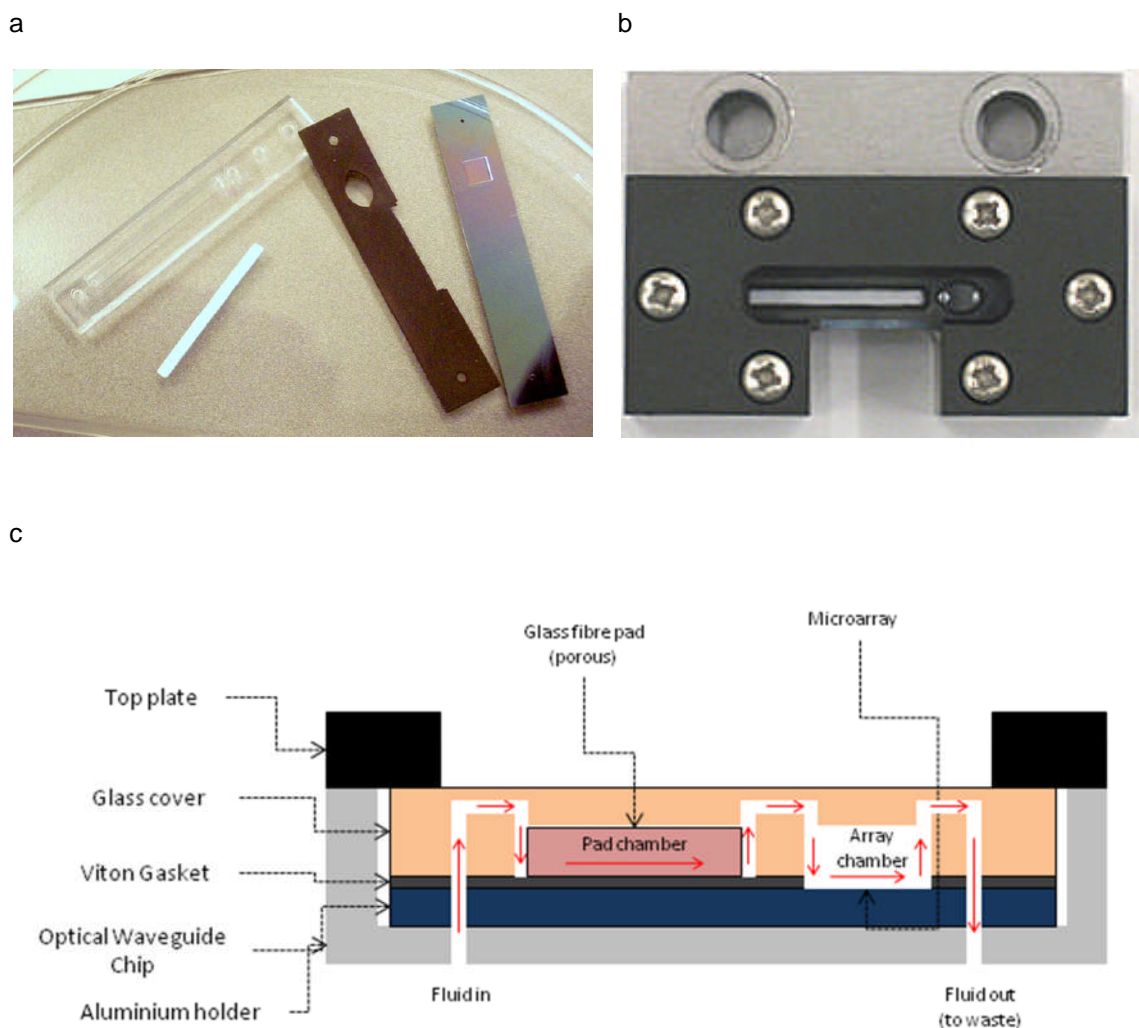


Figure 6-2: Assay breadboard immunoassay module components.

(a) (from left to right within image) glass cover, glass fibre pad, Viton[®] gasket and optical waveguide chip. (b) immunoassay module consisting of immunoassay components assembled into aluminium holder ready for integration onto ABB. (c) Cross section of assembled immunoassay module showing fluidic flow path through assembled stack (not to scale), direction of fluid flow is indicated by red arrows.

6.3.1.1 Optical waveguide chips

LMC optical waveguide chips (Figure 6-3) were custom designed and manufactured by LioniX b.v. (Twente, The Netherlands). Each chip, with dimensions of 32 x 6 mm, is fabricated using thin film deposition of silicon oxide and silicon nitride on a silicon substrate and waveguides are shaped by photolithography and plasma etching. In the array area of the chip (square area in images in Figure 6-3), the surface of the chip is etched to expose the optical waveguides to the surface allowing microarray spots to be printed directly onto the waveguides in this area. Each chip is fabricated so as to have a single hole at each end to allow fluid to enter the microfluidic channel (Figure 6-3) and to have alignment crosses at the four corners of the etched array area of the chip to aid with printing alignment (since printed microarray spots must align precisely with the waveguides in order to allow evanescent excitation of bound fluorophores). In order to immobilise target molecules in the form of a microarray the silicon nitride chip must first be surface functionalised to create a hydrophobic surface as described in Section 6.5.2.1. After running an immunoassay laser light is coupled into the waveguides at a single point and then split into 10 with a multi mode interferometer. ABB optical waveguide chips are a single use item and can only be used to run a single multiplexed microarray immunoassay on the ABB.

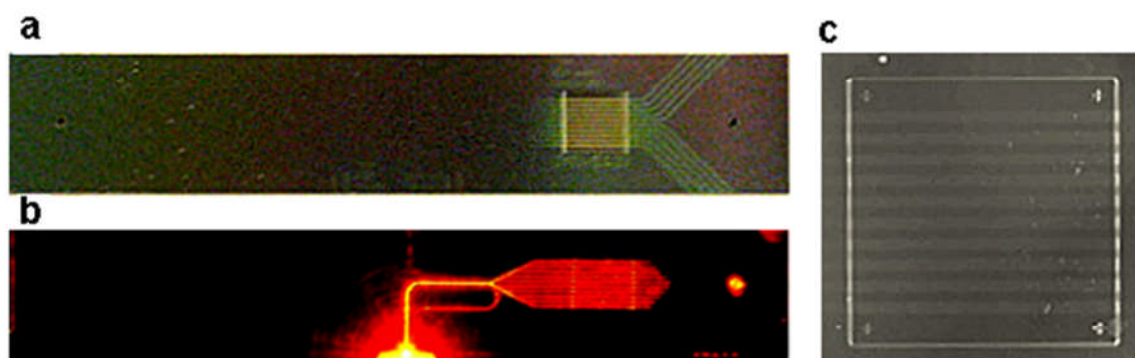


Figure 6-3: Images of optical waveguide chip

(a) photograph of optical waveguide chip, with dimensions of 32 mm x 6 mm, showing array area consisting of ten 100 µm optical waveguides spaced 200 µm apart and fluidic holes at either end of chip (b) Photograph of optical waveguide chip (32 mm x 6 mm) with laser light coupled into incoupling waveguide using FAU. (c) array area of optical waveguide chip magnified to show ten 100 µm optical waveguides, spaced 200 µm apart, and four alignment crosses, one at each corner of the array area.

6.3.1.2 Glass fluidic covers

Glass fluidic covers were designed and manufactured by LioniX b.v. The glass covers, when assembled into the immunoassay module, together with the Viton[®] gasket and the optical waveguide chip, form two chambers with connecting channels. The first is a chamber which holds the glass fibre pad with freeze dried antibodies and the second a chamber which aligns with the microarray area on the waveguide chip, allowing incubation of antibodies and liquid extracts over the microarray and allowing fluorescent and optical imaging of the array area. In the final instrument glass fluidic covers will be a single use item, but in the work reported here glass covers were cleaned and re-used for multiple assays due to their limited availability.

6.3.1.3 Viton[®] gaskets

A Viton[®] gasket (AAA Acme rubber co. inc. Tempe, Arizona, USA), cut to size using a Fenix CO₂ laser cutting /engraving system (Synrad, Mukilteo, WA, USA), is placed between the glass cover and the waveguide chip, within the immunoassay module, to provide a compliant surface against which the microfluidic channel can be sealed and to form the base of the pad chamber. The Viton[®] was cut to size so that, when the gasket was laid over the optical waveguide chip, the array area on the chip was left uncovered and the in-coupling waveguide on the right hand side of the chip was unobstructed to allow coupling of laser light into the optical waveguide chip. Two holes were cut in either end of the gasket to align with the holes in the optical waveguide chip to complete the fluid flow path (Figure 6-2). In the final instrument Viton[®] gaskets will be a single use item, for ABB assays, Viton[®] gaskets were cleaned and re-used for multiple assays.

6.3.1.4 Glass fibre pads

Glass fibre pads containing freeze dried fluorescently labelled antibodies are placed within the pad chamber formed by the glass fluidic cover and the Viton[®] gasket. The antibodies are freeze dried with a protective matrix (freeze drying cocktail) and are stable for long periods of time¹¹. As the liquid extract is pumped through the pad

¹¹ Storage studies carried out by the LMC team at Cranfield have shown no significant change in assay performance after storage of antibodies freeze dried into pads at room temperature under an inert gas for up to one year Cullen D, and Allen M (2010). Technical note LMC-CU-TN-005 One year LMC storage study outcomes. (unpublished report).

chamber, the freeze dried antibodies are re-dissolved directly into the liquid extract. Glass fibre pads are single use items. The method for preparation of glass fibre pads, including freeze drying antibodies, is described in Section 6.5.2.3.

6.3.2 Fluid control system

Fluid flow through the ABB is generated by a vertically mounted NE-501 syringe pump (Prosense BV, Oosterhout, Netherlands) which is controlled by WinPumpControl software (version 1.2.1). The syringe pump is connected to the inlet fluid port of the immunoassay module with capillary tubing, (Figure 6-1). Liquid extract is pumped from the syringe pump reservoir, through the capillary tubing and into the immunoassay module. Within the immunoassay module the liquid extract is pumped into the pad chamber where it dissolves the freeze dried antibodies and associated assay reagents contained in the glass fibre pad. Fluid from the pad chamber is then pumped into the array chamber, where it comes into contact with the printed microarray and finally fluid is pumped out through the outlet fluid port of the immunoassay module to waste, via another section of capillary tubing. The flow of fluid can be stopped as required in order to allow appropriate incubation periods in the pad chamber and in the array chamber to allow immunological reactions to take place.

6.3.3 Optics and imaging

Evanescent excitation of fluorophores on antibodies bound to the microarray is achieved by coupling laser light into the optical waveguide chips. A Fibre Alignment Unit (FAU) holds an optical fibre which feeds laser light from a 635 nm LD-635-31A laser diode (Wavelength Electronics Ltd, Kent, UK) with a power output of 1mW, to the waveguide in the immunoassay components module. The FAU is mounted on an XYZ translation table that can be moved using three orthogonally arranged precision linear translation stages allowing alignment of the optical fibre with the optical waveguide chip in the immunoassay component module, so that laser light can be coupled into the waveguides (Figure 6-4).

In order to image the excited fluorophores, an SXV-H9 thermoelectrically cooled CCD camera with a 4x microscope objective (Starlight Express, Holyport, UK) is mounted onto a rotation and translation stage above the immunoassay module. A Stoptline™ 633 notch filter with an optical density of 6 at a wavelength of 633nm (Semrock,

Rochester, USA) is mounted within an optical spacer in front of the CCD camera, to block out reflected and scattered 633nm light.

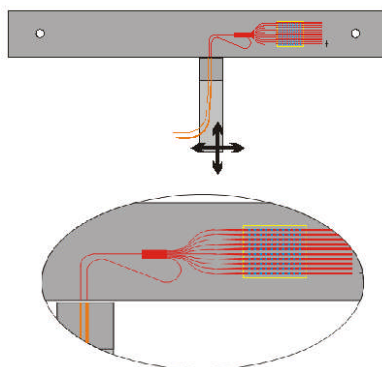


Figure 6-4: Schematic drawing of optical waveguide chip and fibre alignment unit (FAU) showing coupling of laser light into in-coupling fibre on the waveguide. Top image shows the optical waveguide chip and the FAU, the black arrows indicate two of the three axis in which the FAU can be manipulated (the third axis is into the plane of the page). The bottom image is a magnified view of the array area and the in-coupling area on the waveguide chip.

In addition to the CCD camera a 50 x magnification microscope and a Philips SPC900NC webcam are mounted onto the stage to aid with visual conformation of FAU alignment, for coupling of laser light into the optical waveguide chip, and to allow inspection of waveguides and visual recording of the fluid flow through the pad and array chambers.

6.4 Experimental design

In order to confirm functionality of LMC immunoassays in flight representative format with LMC extraction solvent and Mars relevant sample matrix components, a Mars analogue sample was spiked with a model target molecule and extracted using an LMC representative extraction protocol with MeOH P80 extraction solvent. The liquid extract was then analysed by multiplexed immunoassay using the flight representative assay breadboard. In order to discriminate between specific inhibition of signal by extracted target molecules and non-specific inhibition of signal due to sample matrix mediated effects, an un-spiked sample of a Mars analogue sample matrix was also extracted and the resulting liquid extract analysed in the same way.

Due to time constraints and the limited availability of optical waveguide chips, work was limited to spiking of a single Mars analogue with a single model target molecule and performing a single multiplexed ABB assay with each liquid extract, with no replicate runs¹².

6.4.1 Selection of immunoassays and model target molecule

Ideally the end to end demonstration would have been performed with a high science priority target for the LMC, *e.g.* a marker of extinct life, however as reported in Chapter 5 of this thesis, attempts to produce antibodies that recognise markers of extinct life have not so far been successful. Immunoassay, and therefore model target molecule selection was restricted to immunoassays that had been successfully transferred into microarray format at the time of this piece of work; GroEL and fluorescein¹³.

The fluorescein assay is intended as a positive control marker for the LMC since fluorescein, a synthetic organic molecule is not expected to be present on Mars. However for the purposes of an end to end demonstration fluorescein was considered to be the most appropriate of the two available targets since it is a small organic molecule, like many of the intended targets for the LMC. Additionally using fluorescein as the target molecule for spiking, facilitated easy confirmation of successful extraction by measuring the fluorescence of the liquid extracts and comparing to a standard curve.

Fluorescein was therefore used as the model martian target molecule for this study and the GroEL immunoassay was used as a control assay. In addition to the control assay spots of high and low AF633 labelled BSA were also printed onto the microarray. These spots had two functions, the first of which was as a positive control of fluorescence *i.e.* to confirm that the excitation and optical aspects of the ABB assay were functioning independently of the immunoassay and the second was as calibration

¹² A problem encountered during the study (described in Section 6.6.1) necessitated one of the samples to be reanalysed therefore three ABB immunoassays were performed in total for this study.

¹³ At the time of completion of this thesis, work was ongoing within the LMC project team to perform an end-to-end demonstration using the benzo[a]pyrene immunoassay, which was transferred into microarray format after the work reported in this chapter had been completed. This second end to end demonstration with involve extraction of Mars analogues spiked with pyrene (a hydrophobic marker of meteoritic in-fall) using the most recent version of a flight representative breadboard built for the LMC instrument.

spots to allow normalisation of signal intensities to correct for differing coupling efficiencies between optical waveguide chips.

6.4.2 Selection of analogue sample matrix

JSC Mars-1, a martian regolith analogue developed by the Johnson Space Centre (Houston, USA) (Allen *et al*, 1997) was selected for use in this study because it is the standard adopted by the Mars exploration community for studies where a Mars analogue is required (Court *et al*, 2010; Gough *et al*, 2010; Kral *et al*, 2011).

6.5 Supplementary materials and methods

The following sections describe the materials and methods used to perform an 'end to end' demonstration of the LMC. There is some overlap with the common materials and methods described in Chapter 2 of this thesis and where relevant this section cross references the appropriate sections in Chapter 2.

6.5.1 Preparation of liquid extracts

Samples of JSC Mars-1 were spiked and extracted by Dr Alex Baki, Imperial College London. Dr Baki's procedure for spiking JSC Mars-1 is described in the following section.

6.5.1.1 Spiking JSC Mars-1 with fluorescein model target molecule

JSC Mars-1, a commonly used martian regolith simulant (Allen *et al*, 1997), was cleaned prior to spiking to remove any residual organics, or other contamination. This was achieved by addition of 3 ml of 80/20 % (v/v) water/methanol (Fisher scientific, Loughborough, UK cat no. M14056/17) to one gram of JSC Mars-1 in a glass test tube followed by twenty minutes of sonication in an ultrasonic bath (Thermo Fisher Scientific, Loughborough, UK, cat no. FB15048). The JSC Mars-1 was then allowed to settle by standing and the supernatant was decanted. This process was repeated to a total of two extractions. Following the two pre-extractions JSC Mars-1 was dried in an oven set at a temperature of 120°C for 12 hours.

Following pre-extraction and drying JSC Mars-1 was spiked with fluorescein sodium salt (Fluka, part of Thermo Fisher Scientific, Loughborough, UK, cat. no.28803), in this context as a model martian target molecule, at two concentrations; 0 µg/g and 2.7µg/g.

These concentrations were calculated with the aim of producing liquid extracts with fluorescein concentrations that were 0 x and 30 x the IC₅₀ value of the fluorescein microarray assay (3 ng/ml) assuming a 10 % extraction efficiency. The appropriate quantity of fluorescein sodium salt was dissolved in one millilitre of 18 MΩ.cm water and mixed well with one gram of pre-extracted JSC Mars-1 by vortexing. The solvent was then evaporated over a period of several hours using a RVC 2-18 CD rotational vacuum concentrator (Christ, Osterode am Harz, Germany). Once the solvent had been removed the spiked JSC Mars-1 samples were dried further in an oven set at 40°C for 12 hours.

6.5.1.2 Extraction of spiked JSC Mars-1

Three millilitres of MeOH P80 was added to 1g JSC Mars-1 in a glass test tube and sonicated for 20 minutes with a Sonotrode Ultrasonic UP100H probe and UP50H ultrasonic processor set at 40 % amplitude (Hielscher Ultrasonics, Teltow, Germany). This extraction protocol simulates the extraction protocol for the LMC. After ultrasonication, the mixture was allowed to stand for 10 minutes, and the supernatant that had formed above the sedimented JSC Mars-1 was clarified by filtration through a 0.45 µm cellulose acetate filter and then stored at 2 - 8°C in glass vials until tested in the immunoassay¹⁴.

Estimation of extraction efficiency by fluorescence of liquid extract

In order to estimate the concentration of fluorescein extracted from spiked JSC Mars-1, 200 µl of liquid extract was placed in a white microtitre plate (Pierce, part of Thermo Scientific, Leicestershire, UK, cat. no. 15042) and the fluorescence measured using a Variskan Flash plate reader (Thermo Scientific, Leicestershire, UK) ($\lambda_{\text{ex.}} = 494\text{nm}$, $\lambda_{\text{em}} = 521\text{nm}$). A standard curve was constructed in the same microtitre plate by dissolving known concentrations of sodium fluorescein in MeOH P80 over the concentration range 0 – 5 µg/ml. The concentration of sodium fluorescein salt in the liquid extract (and hence the extraction efficiency) was calculated by linear regression and was found to be approximately 90 ng/ml, which is approximately 30x the IC₅₀ for the fluorescein microarray immunoassay.

¹⁴ Note that this work was carried out before the finding (discussed previously in Section 4.6.2.3) that filtration can result in loss of hydrophobic target molecules, by absorption to the filter membrane. This is unlikely to have an impact on the extraction efficiency of the more polar fluorescein used in this study.

6.5.2 Preparation of immunoassay components for ABB assay

6.5.2.1 Silanisation of optical waveguide chips

Optical waveguide chips were treated to produce a hydrophobic surface for immobilisation of target molecules/target molecule-protein conjugates, following the protocol described in Chapter 2 (Section 2.2.3.1) for silicon nitride chips.

6.5.2.2 Printing and immobilisation of microarrays onto optical waveguide chips via physical adsorption

Microarrays of immobilised antigen were printed onto optical waveguide chips, which had been treated as described in Section 6.5.2.1, using the SPI spotting robot following the protocol described in Chapter 2 (Section 2.2.3.4). A single microarray was printed onto each waveguide chip in the array area so that spots were printed directly on top of the ten optical waveguides. Each microarray consisted of rows of 10 spots of (i) high ratio labelled AF633 BSA, (ii) GroEL, (iii) BSA-FITC and (iv) low ratio AF633 labelled BSA. GroEL and FITC-BSA are the immobilised versions of the target molecules required for the GroEL and fluorescein immunoassays respectively. The high and low ratio AF633 labelled BSA spots were included in the array as a positive control to confirm coupling of laser light and to allow calibration/normalisation across different optical waveguide chips, which are likely to have been coupled with differing efficiency. Alignment of the printing pin with the optical waveguides was achieved using the camera microscopes on the spotting robot with the aid of the alignment crosses at the edges of the array area on the optical waveguide chips (Figure 6-3).

6.5.2.3 Freeze drying of antibodies into glass fibre pads

Glass fibre pads were prepared by cutting binder free borosilicate glass fibre sheet with a specified thickness of 215.9-292.1 μm (Pall Corporation, Port Washington, New York, USA) to dimensions of 1.6 mm x 8.65 mm with a Fenix CO₂ laser cutting /engraving system (Synrad, Mukilteo, WA, USA).

Residual organics were removed by agitating the pads in an aqueous solution of 0.1% (w/v) sodium dodecylsulphate (SDS) (Sigma Aldrich, Poole, UK, cat. no. L4390) on an orbital mixer for 1 hour. After 1 hour the SDS solution was discarded and the pads were rinsed three times by filling the beaker with 18 M Ω .cm water, swirling to ensure all

pads were immersed, and then decanting the liquid taking care that the pads remained in the beaker.

The pads were agitated for a further hour in a 1M solution of hydrochloric acid followed by three washes with water using the method described previously.

The pads were then blocked (to prevent binding of antibodies to the pad material) by agitating with BSA blocking solution (0.5 % (w/v) Cohn fraction V BSA (Sigma Aldrich, Poole, UK, cat. no. A4503) in 100 mM N[2-hydroxyethyl]piperazine-N[2-ethanesulfonic acid] (HEPES buffer) pH 7.4 (Sigma Aldrich, Poole, UK, cat. no. H7523) for one hour, followed by three water washes. The pads were dried at room temperature for a minimum of 1 hour then sealed inside clean polystyrene *Petri*-dishes with desiccant (to prevent absorbance of moisture) until use.

Anti-fluorescein and anti-GroEL primary antibodies, their associated secondary antibodies and streptavidin Alexa Fluor® 633 were diluted to the desired concentrations (See Table 2-1 in Chapter 2) in freeze drying cocktail (See Table 2-3 in Chapter 2). The required numbers of glass fibre pads were arranged on a glass microscope slide and 7.5 µl of antibodies diluted in freeze drying cocktail solution was pipetted next to each glass fibre pad. The glass fibre pads were then pushed over the solution with a pipette tip and allowed to absorb the liquid.

Glass fibre pads containing antibodies diluted in freeze drying cocktail were rapidly frozen by dropping them into a beaker of liquid nitrogen. The beaker now containing the frozen glass fibre pads was covered with aluminium foil, which was pierced several times. The beaker was then placed inside a Scanvac Coolsafe freeze drier (LaboGene APS, Lyngø, Denmark) set at a temperature of minus 60°C and a vacuum was applied for a minimum of four hours. After freeze drying, glass fibre pads were stored in a clean polystyrene *Petri*-dish with desiccant until use. Any bent or damaged pads were discarded.

6.5.3 Running microarray assays with liquid extracts using the assay breadboard

Glass fibre pads and printed optical waveguide chips were assembled into the ABB immunoassay module as shown in Figure 6-2. The fluidic path was sealed by tightening the screws on the top plate of the aluminium chip holder such that there was

a spacing of 0.3 mm between the aluminium holder and the top plate. Note that in order to reduce loss of antibodies and target molecules via adsorption to surfaces, the glass fluidic cover and Viton® gasket were blocked with BSA blocking solution (0.5 % (w/v) Cohn fraction V BSA (Sigma Aldrich, Poole, UK, cat. no. A4503) in 100 mM N[2-hydroxyethyl]piperazine-N[2-ethanesulfonic acid] (HEPES buffer) pH 7.4 (Sigma Aldrich, Poole, UK, cat. no. H7523), for a minimum of 1 hour prior to running an assay.

The liquid extract from JSC Mars-1 was loaded into the syringe, ensuring there were no trapped air bubbles and the capillary tubing was primed by pumping liquid extract through the tubing at a flow rate of 10 µl/min, until fluid exited from the end of the capillary tubing. The immunoassay module was mounted onto the ABB and the inlet port on the aluminium chip holder was connected to the vertically mounted syringe pump using capillary tubing, as shown in Figure 6-1.

The webcam, mounted on the rotation and translation stage on the ABB, was positioned above the immunoassay module so that the image produced showed the pad chamber and array chamber. The resulting video feed was used to visually inspect the progression of the liquid front through the pad and array chambers and to determine when to stop the syringe pump to allow incubation of the liquid within each chamber.

Liquid extract was pumped using the syringe pump, through the capillary tubing and into the microfluidic channel in the glass cover *via* the inlet port on the aluminium chip holder and from there into the pad chamber at a flow rate of 1 µl/min. Once the pad chamber was full and the glass fibre pad was completely wetted, as determined by observation of the video feed, the pump was stopped and the mixture was allowed to incubate for 50 minutes in the pad chamber. After 50 minutes the syringe pump was restarted at a flow rate of 1 µl/min and the video feed was used to observe the progression of the liquid front into the assay chamber. Once the assay chamber had filled with liquid, as determined by observation of the video feed, the syringe pump was stopped and the solution was incubated in the array chamber for a further 50 minutes. After this incubation period unbound antibody was washed away by pumping more liquid extract through the microfluidic path, maintaining the same flow rate. The efficiency of washing was monitored by observation of the colour of the liquid passing out of the exit port to waste, using the red dye present in the freeze dried cocktail as a tracer for all of the dissolved reagents. A flow of liquid extract was maintained until the

fluid passing out of the system was colourless, this took approximately 15 minutes. The syringe pump was then stopped and the array area on the optical waveguide chip was imaged *in-situ*, without disconnecting any of the tubing.

6.5.3.1 Evanescent excitation and fluorescent imaging of microarrays

Laser light was coupled into the incoupling waveguide of the optical waveguide chip by adjusting the position of the FAU using the XYZ translation stage. Successful coupling was confirmed by observation of laser light passing through, and scattering from, the optical waveguides on the array area of the optical waveguide chip. The microarray area of the optical waveguide chip was imaged with the CCD camera, mounted on the translation stage of the ABB, using exposure times varying from 30 seconds to 5 minutes. During imaging the ABB was isolated from external light sources by covering with a black box.

6.5.3.2 Image analysis

Images of microarrays on optical waveguide chips were analysed by measuring the mean pixel intensity of each spot using ImageJ software as described in Section 2.2.3.9. When imaging the optical waveguide chips a number of exposure times were used. Images with an exposure time such that pixel intensity was as high as possible, but where none of the spots had reached saturation (mean pixel intensity of 65000) were selected for analysis. Since the intensity of the microarray spots produced is dependent on the efficiency with which the laser light was coupled into the microarray, there is significant potential for variation between chips. This variation may result from the skill of the operator in optimising the coupling efficiency or from differences between the optical waveguide chips resulting in varying efficiency independent of the skill of the operator, e.g. quality of the optical face of the incoupling waveguide. In order to account for this variation mean pixel intensities were normalised against the mean pixel intensities of the high ratio AF633 labelled BSA spots printed onto the same optical waveguide chip.

6.6 Results and discussion

6.6.1 Fluidic operation of ABB with liquid extracts from JSC Mars-1

Liquid extracts were produced by ultrasonication of 1 g of JSC Mars-1, spiked with 0 $\mu\text{g/g}$ and 2.7 $\mu\text{g/g}$ fluorescein, with 3 ml of MeOH P80 (Figure 6-5). The liquid extract produced from the unspiked sample was colourless whereas the liquid extract produced from the spiked sample had a yellow coloration. Both liquid extracts were cloudy, after filtration through a 0.45 μm membrane filter, indicating that the extracts contained a level of fine (sub-0.45 μm) particulate material.

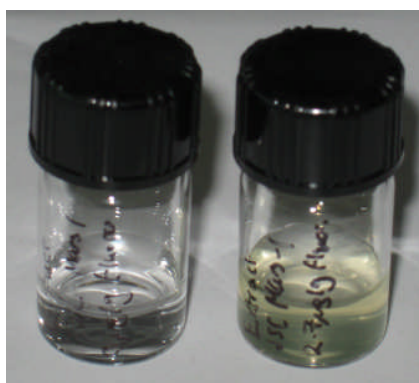


Figure 6-5: Photograph of liquid extracts produced by extracting 1 g of JSC Mars-1 spiked with (left) 0 $\mu\text{g/g}$ fluorescein and (right) 2.7 $\mu\text{g/g}$ fluorescein using 3 ml of MeOH P80 with ultrasonication

Both liquid extracts were analysed using ABB format multiplexed microarray immunoassays. No evidence of blockage of the microfluidic channels, including the capillary tubing (internal diameter of 125 μm), due to the presence of fine particulate materials was observed.

The liquid extract from JSC Mars-1 spiked with 0 $\mu\text{g/g}$ fluorescein was analysed first (run 1), followed by analysis, on the same day of the liquid extract from the 2.7 $\mu\text{g/g}$ fluorescein spiked JSC Mars-1 (run 2). However during run 2 problems were encountered with air bubbles becoming trapped in the array chamber, this issue is discussed in more detail later in this section. Therefore, although the intention had been to perform a single replicate run for each liquid extract, it was necessary to perform a second analysis of the 2.7 $\mu\text{g/g}$ fluorescein spiked JSC Mars-1 (run 3). Run 3 was carried out using a glass fibre pad from the same batch as runs 1 and 2, but an optical waveguide chip had to be freshly prepared for run 3.

Figure 6-6 shows a series of images of the microfluidic channel captured from the video footage recorded during each ABB run showing filling of the pad chamber.

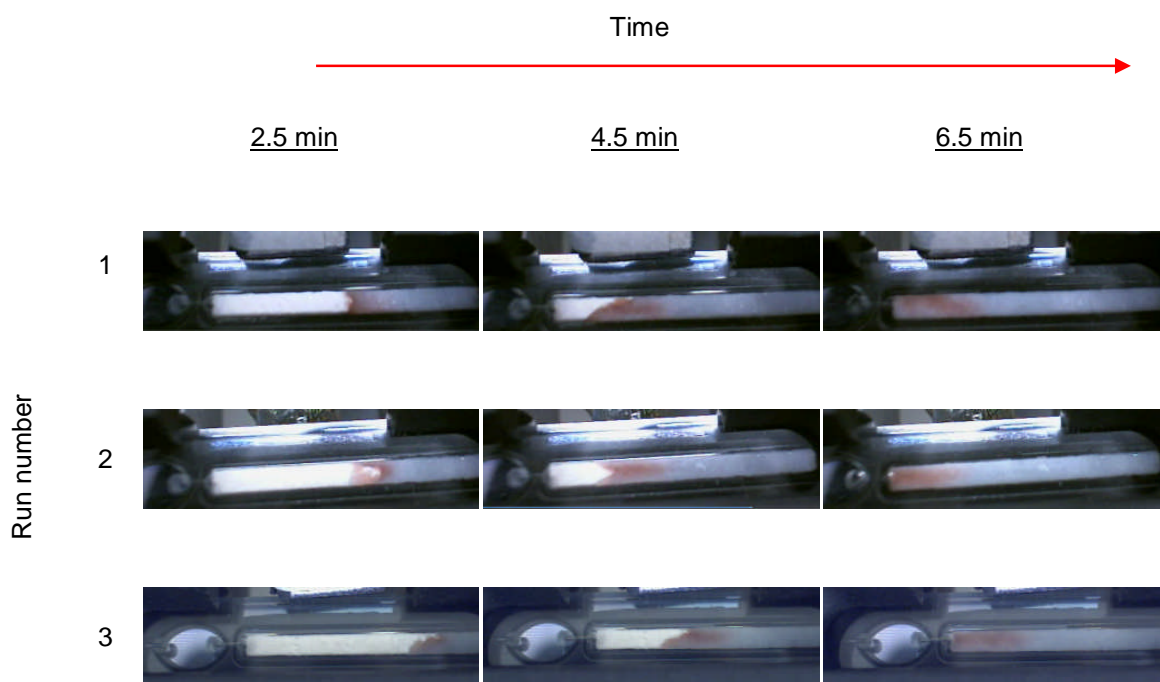


Figure 6-6: Images captured from video footage of pad chamber filling during ABB assays.

Each row shows images from a single run taken 2.5 min, 4.5 min and 6.5 min from starting the syringe pump to fill the pad chamber (fluid fill direction from right to left in each image)

In each of the three runs carried out, the pad chamber filled with liquid extract in a time of approximately 6.5 minutes from starting the syringe pump. As the liquid front moved through the glass fibre pad the red dye included in the freeze drying cocktail, which is used as a proxy for the position of all of the other solutes in the pad, migrated through the pad and was concentrated at the end of the pad closest to the exit to the array chamber. Once the pad was completely wetted the gaps around the edge of the pad then filled. There was some variation in the speed in which the pads filled and not all of the pads filled evenly. In some cases areas of the pad remained unwetted as the liquid front passed, and then were backfilled later as shown in the image for run 2 at 2.5 minutes (Figure 6-6).

After 50 minutes of incubation the syringe pump was restarted and the liquid extract was pumped from the pad chamber into the array chamber (Figure 6-7). The liquid that filled the array chamber was red in colour indicating that the dissolved solutes were

also transferred into the array chamber. Complete filling of the array chamber took approximately 3.5 minutes from starting the syringe pump. After this period of time some red coloration was still present in the pad, indicating that some of the dissolved solutes had not been transferred to the array chamber.

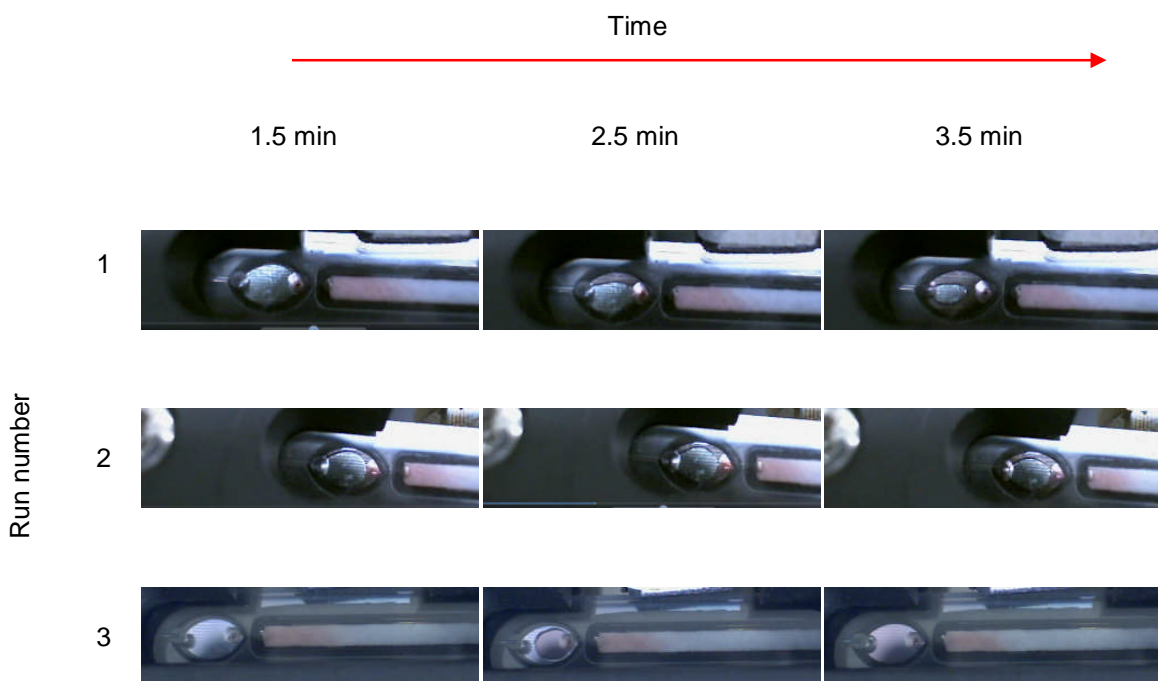


Figure 6-7: Images captured from video footage of array chamber filling during ABB assays. Each row shows images from a single run taken 1.5 min, 2.5 min and 3.5 min from starting the syringe pump to fill the assay chamber (fluid fill direction from right to left in each image)

The way in which the array chamber filled varied between the three runs and in two cases air became trapped in the array chamber. In run 1 with the liquid extract from 0 $\mu\text{g/g}$ spiked JSC Mars-1 the liquid front filled the array chamber around the edges first before the middle of the chamber then began to back fill. This resulted in an air bubble becoming trapped in the chamber that could not be dislodged by tapping. The air bubble partially overlapped with the microarray on the optical waveguide chip, but the majority of the spots on the microarray were not in contact with the air bubble. In run 2 with the extract from 2.7 $\mu\text{g/g}$ spiked JSC Mars-1 during the 50 minutes incubation in the pad chamber liquid from the pad chamber passed into the array chamber, filling it around the edges and trapping an air bubble in the array chamber. This was probably as a result of not stopping the pump early enough during pad chamber filling and allowing some liquid to pass into the channel between the pad and array chambers. In

this instance the trapped air bubble covered the whole area of the microarray and the assay had to be repeated with a new optical waveguide chip and pad. In run 3 the liquid pumped into the array chamber formed as a drop on the top of the array chamber before dropping down into the bottom of the chamber and filling the array chamber evenly with no air bubble becoming trapped.

Similar problems with trapped air bubbles have been observed previously when running ABB assays with buffered solutions, therefore it is unlikely that the problem is directly related to the use of a liquid extract with associated fine particulates. The formation of trapped air bubbles is thought to be caused by the presence of surface contaminants (e.g. particulates from the air, grease etc.) resulting from insufficient cleaning of the Viton® gasket and glass fluidic cover, which for ABB assays are reused for multiple runs. In the flight format instrument the gaskets and glass covers will be single use components and will be assembled and prepared under clean room conditions with strict quality control procedures, so this is unlikely to be an issue.

After 50 minute incubation in the array chamber, further liquid extract was pumped through the chip to remove unbound antibody. The removal of all unbound antibody was assessed by observing the colour of the liquid exiting from the waste capillary tube (Figure 6-8).

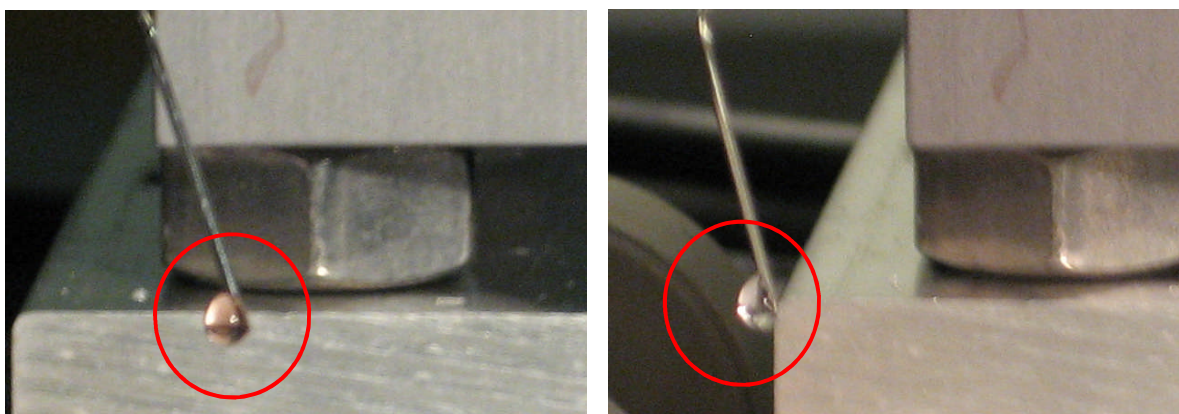


Figure 6-8: Photographs of liquid exiting waste capillary on ABB taken approx. 1 minute after starting wash (left) showing red coloration and approx. 15 minutes after starting wash (right) showing colourless liquid, indicating complete washing away of red dye and other solutes.

The first drop of liquid exiting the capillary tubing was red. After approximately 15 minutes at a flow rate of 1 $\mu\text{l}/\text{min}$ the drop of liquid exiting the chip was colourless

indicating that all of the dye and other dissolved solutes had been washed out. Observation of the pad within the pad chamber also showed that all of the residual red coloration in the pad had been washed out.

6.6.1.1 Assay readout of using ABB with liquid extracts from JSC Mars-1

The fluorescent images of the microarrays shown in Figure 6-9, captured after running ABB immunoassays with liquid extracts from 0 µg/g and 2.7 µg/g fluorescein sodium salt spiked JSC Mars-1 (from run 1 (0 µg/g), and run 3 (2.7 µg/g)), show that fluorophores on the high and low ratio AF633 labelled BSA spots fluoresce. This indicates that laser light was coupled into the optical waveguide chips generating an evanescent field which excited the fluorophores on the immobilised BSA-fluorophore conjugates. There was no evidence of significant scatter due to presence of sub 0.45 µm particulates in the liquid extract, neither was there any evidence of high background signal, indicating that the optical aspects of the ABB assay are compatible with liquid extracts from JSC Mars-1.

For the microarray run with a liquid extract from 0 µg/g spiked JSC Mars-1, the GroEL and FITC-BSA spots on the microarray fluoresce showing that both the GroEL and fluorescein immunoassays have functioned in the liquid extract. For the microarray run with a liquid extract from JSC Mars-1 spiked with 2.7 µg/g fluorescein the GroEL spots on the microarray still fluoresce brightly, whereas the BSA-FITC spots give a much fainter signal indicating that the anti-fluorescein antibody has been inhibited from binding to the microarray by the presence of extracted fluorescein.

Closer inspection of the individual microarray spots in Figure 6-9 shows that the intensity of the microarray spots is not homogeneous across the individual waveguides. This is due to two factors; striation of the evanescent field strength across the width of the waveguide and poor alignment of microarray spots with the optical waveguides. Additionally for the microarray on the left hand side of Figure 6-9, three of the GroEL microarray spots are abnormally small and one of the low ratio AF633 labelled BSA spots is missing from the image on the right hand side of Figure 6-9. This is most likely related to problems with the printing procedure *i.e.* printing pin sticking in print head as a result of residual moisture from pin washing step.

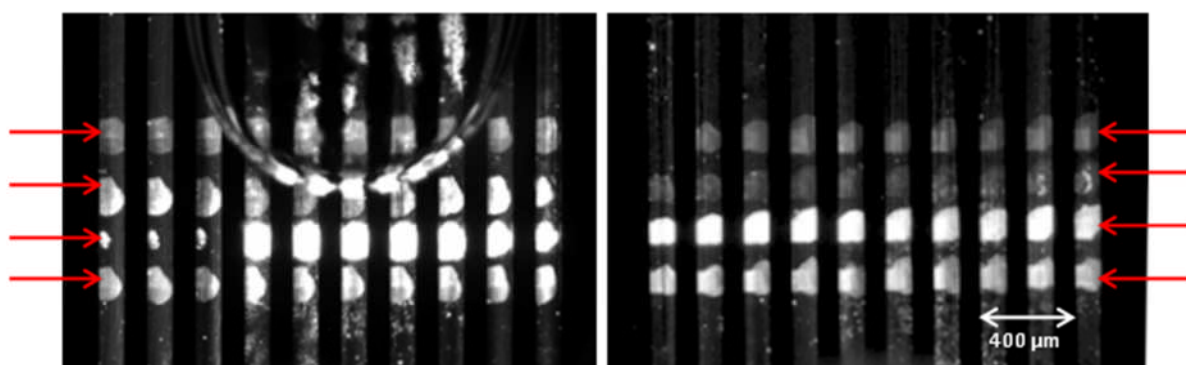


Figure 6-9: Fluorescent images of optical waveguide chips after running ABB assays with (left) extract from JSC Mars-1 spiked with 0 $\mu\text{g/g}$ sodium fluorescein and (right) extract from sample spiked with 2.7 $\mu\text{g/g}$ fluorescein.

Microarrays consists of rows of ten spots of (from top to bottom) low ratio AF633-BSA spots, BSA-fluorescein, GroEL and high ratio AF633 BSA. Position of each row of microarray spots is indicated by red arrows. Image taken using evanescent excitation on ABB with an exposure time of 2 minutes. Image brightness and contrast have been adjusted to aid visualisation of spots. Absolute mean pixel intensity of high ratio labelled BSA spots was approx 17000 in left hand image and 12000 in right hand image

6.6.1.2 Image analysis

The pixel intensity of microarray spots was measured using ImageJ software. In the images taken with a 5 minute exposure time the intensity of the GroEL microarray spots had reached saturation, therefore the images with an exposure time of 2 minutes were selected for analysis. The absolute signal intensity of the high ratio AF633 labelled BSA spots on the image with a 2 minute exposure time was higher for the microarray run with the liquid extract from 0 $\mu\text{g/ml}$ spiked JSC Mars -1 (left image in Figure 6-9) compared to the microarray run with 2.7 $\mu\text{g/ml}$ spiked JSC Mars -1 (right image in Figure 6-9) approx 17000 compared to 12000. The signal intensity generated is dependent on the coupling efficiency (*i.e.* how well laser light is coupled into the in-coupling fibre on the optical waveguide chip) which is determined by how well the operator has aligned the FAU and is also dependent on the finish of the in-coupling area on each individual optical waveguide chip. In order to compensate for this, the mean pixel intensity for each set of spots was normalised against the mean pixel intensity of the high ratio AF633 labelled BSA spots printed onto the same optical waveguide chip. In the image where an air bubble had partially overlapped with the microarray (left hand image in Figure 6-9), microarray spots which were under the

trapped air bubble, or at the interface between the air bubble and the liquid extract were excluded from the analysis.

Analysis of the intensity of the immunoassay spots (Figure 6-10) showed that the intensity of BSA-FITC spots was significantly reduced, relative to the high ratio AF633 labelled BSA spots on the same waveguide, by a liquid extract from JSC Mars-1 spiked with 2.7 $\mu\text{g/g}$ (2.7 ppm) sodium fluorescein compared to a liquid extract from JSC Mars-1 spiked with 0 $\mu\text{g/g}$ (0 ppm) sodium fluorescein.

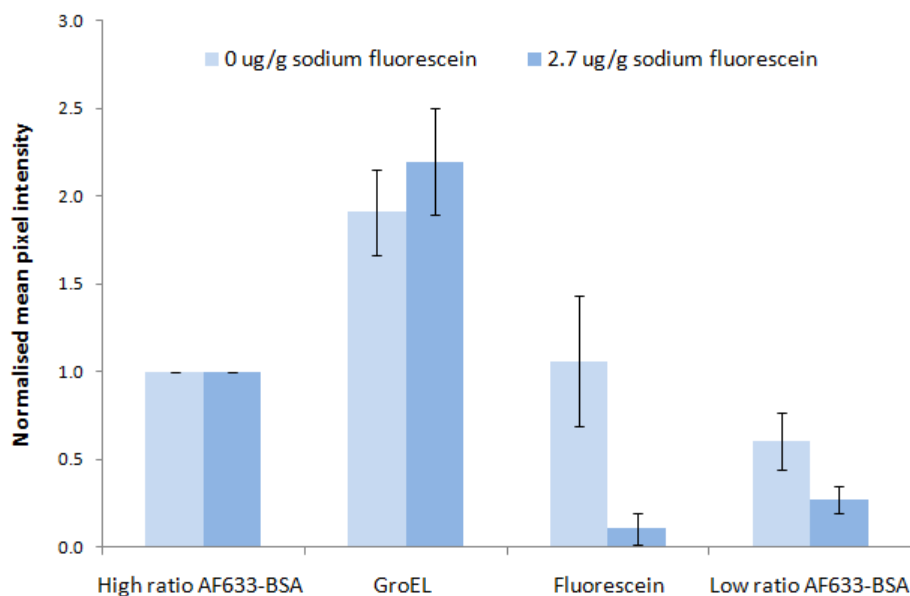


Figure 6-10: Mean pixel intensity of microarray spots after ABB immunoassay run with liquid extract from JSC Mars-1 spiked with 0 and 2.7 $\mu\text{g/g}$ sodium fluorescein salt. Mean pixel intensity normalised against mean pixel intensity for high ratio AF633 labelled BSA spot on same optical waveguide. Error bars = +/- 1 standard deviation of the mean, n = 5-10 (some spots removed from analysis due to presence of air bubble)

Conversely the intensity of GroEL spots, relative to the high ratio AF633 labelled BSA spots on the same waveguide, was unchanged by a liquid extract from JSC Mars-1 spiked with 2.7 $\mu\text{g/g}$ (2.7 ppm) sodium fluorescein compared to a liquid extract from JSC Mars-1 spiked with 0 $\mu\text{g/g}$ (0 ppm) sodium fluorescein. The implication is that the anti fluorescein antibody was inhibited from binding to the BSA-FITC microarray spots by the extracted fluorescein, whereas the anti-GroEL antibody was not. Thus GroEL was a successful positive control immunoassay. It should be noted that there was a reduction in the relative intensity of the low ratio labelled AF633-BSA spots between the immunoassays run with liquid extract from JSC Mars-1 spiked with 2.7 $\mu\text{g/g}$ (2.7 ppm) sodium fluorescein compared to a liquid extract from JSC Mars-1 spiked with 0

$\mu\text{g/g}$ (0 ppm) sodium fluorescein. This may be due to variation in printing between the two optical waveguide chips (since the optical waveguide chips used were not printed alongside each other), or it might indicate a level of cross reactivity between the anti-fluorescein antibody and the AF633 labelled BSA spots, although if that were the case the antibody would also be expected to cross react with the high ratio AF633 labelled BSA spots.

6.6.2 Summary discussion and future work

The objective of the work in this study was to demonstrate the function of the LMC in a flight representative format. The specific objectives set for this piece of work were

- 1) To confirm the compatibility of liquid extracts from Mars analogue samples with the optical and microfluidic aspects of the flight representative ABB
- 2) To confirm the function of LMC relevant immunoassays in liquid extracts from Mars analogue samples in flight representative ABB assays

The work reported in this chapter successfully met both specific objectives by demonstrating the function of the LMC (from extraction through to detection) in flight representative ABB assay format, both in terms of the compatibility of liquid extracts with the optical and microfluidic aspects of the ABB and in terms of immunoassay function. As noted in the results section some issues were encountered relating to the reproducibility of microarray printing *e.g.* missing/mis-sized microarray spots, reproducibility between chips and also relating to the formation of air bubbles in the assay array chamber. Although none of these issues were directly caused by the use of liquid extracts they clearly require resolution in order to move forward with development of the LMC flight instrument.

However despite meeting the objectives set for this work the study was limited in that it was only based on three runs on the ABB, using a single Mars analogue sample matrix. No replicates runs were carried out due to limited availability of optical waveguide chips and the time consuming nature of performing immunoassays on the ABB. Within the context of this study this is deemed to be acceptable but in order to progress LMC development it is important that reproducibility is considered. The Mars analogue JSC Mars-1 is representative of only one of many sample matrix types that

may be encountered on Mars. The highest risk aspect relating to sample matrix is likely to be precipitation of components of the sample extract when mixed with the buffer salts in the pad chamber and further work with an extended range of Mars analogues and individual sample matrix components is necessary.

The data from this study, which used GroEL and fluorescein as LMC relevant immunoassays, supports the assumption that immunoassays that have been demonstrated to function in liquid extracts (*i.e.* extraction solvent and sample matrix components) in ELISA and pooled microarray format are likely to also function in flight representative microfluidic format. However as new assays are developed and transferred into microarray format for the LMC it is essential that this finding is confirmed. Furthermore the selection of fluorescein as a model target molecule, although sufficient for the purposes of this study, was not ideal because fluorescein will not be a target molecule for the LMC, rather a positive control molecule that is not expected to be found on Mars. Fluorescein is also significantly more polar than LMC high priority markers of extinct life.

Despite these limitations this study has performed an initial 'de-risking' of the operation of the LMC flight format instrument with liquid extracts from martian samples and also satisfied the requirement to perform an end-to end demonstration of LMC extraction and detection in order to gain an acceptable TRL level to enable progression to the next phase of development.

6.7 Conclusions

The work in this chapter has successfully demonstrated extraction and detection of a small molecule from a single martian analogue sample matrix using LMC relevant multiplexed microarray assays implemented within a microfluidic channel in a flight representative format. Although this study had several limitations *i.e.* no replicates performed, small number of immunoassays tested in the microarray, it has confirmed that fundamentally the LMC immunoassay can function in a flight representative format, in addition to contributing to successful award of TRL level 4, enabling the LMC to progress to the next phase of development.

7 Thesis Summary, Conclusions and Future Work

Development of the LMC instrument continues beyond the period of this thesis, but this work has contributed significantly towards the preparation of the LMC instrument for flight in 2018. In summary, this thesis includes work that has advanced the current state of scientific knowledge in four key areas for the development of the LMC instrument: by validating the use of a water miscible organic solvent/water/surfactant extraction solvent, by demonstrating that immunoassays can function with model martian samples, by contributing to a program of work to produce antibodies against extinct life biomarker targets, and by performing an end-to end demonstration of LMC instrument function in flight representative format. This section briefly summarises the developments made within each key area of work, the status of each area of work at the conclusion of this thesis and plans for future work.

7.1 Compatibility of the proposed extraction solvent for the LMC with LMC format immunoassays

7.1.1 Summary and conclusions

The solvent used for the LMC instrument must both efficiently extract polar and apolar target molecules from martian rock and regolith, and must also be compatible with immunoassays. The objective of the work within this chapter was to confirm whether the proposed extraction solvent for the LMC (20 % methanol 1.5 g/L P80) was compatible with LMC format immunoassays. In order to investigate this, a set of model LMC immunoassays were tested for performance in MeOH P80 in ELISA and microarray format. The representative data in Table 7-1 shows the successful detection of six out of seven of the model target molecules used in this study in the LMC extraction solvent by ELISA.

This study has confirmed that the proposed extraction solvent for the LMC is generally compatible with immunoassays, although there is a risk that specific immunoassays may have poor function in the proposed extraction solvent and may therefore be unsuitable for inclusion on the flight instrument. In addition immunoassays were also shown to be compatible with 30 fold concentrated liquid extract, although the ability to concentrate the extracted sample has since been removed from the instrument design,

and a number of potential back-up solvents were identified should the current preferred solvent prove unsuitable for any reason in the future.

Table 7-1: IC₅₀ values for inhibition ELISAs run in MeOH P80 and 45 g/L P80

	IC ₅₀ (ng/ml)	
	water (*10 % MeOH)	MeOH P80
Atrazine	*1.4	24.2
B[a]P	*6.2	53.8
cAMP	274.8	717.0
Fluorescein	300.0	375.0
GroEL	2447.0	2096.5
LPS	190.5	Not calculated
LTA	2.0	1.0

For the LMC project, instrument design should therefore move forward (and has done so) based on the expectation of using MeOH P80 as extraction solvent. Beyond the scope of the LMC, the compatibility of these types of solvents with immunoassays, combined with their ability to efficiently extract apolar molecules is likely to be of interest to the planetary exploration community, and in-particular may be of benefit in the development of other immunoassay based instruments for life detection, and especially extinct life detection. In addition this type of extraction solvent may be of interest to the broader immunoassay/biosensor community due to its potential for use in *in-situ* immunoassay analysis of environmental contaminants such as pesticides.

7.1.2 Future work

It is recommended that as new antibodies become available for the LMC, they should be tested immediately for compatibility with the LMC extraction solvent, in ELISA and especially microarray format before investing significant effort transferring antibody assays into flight representative formats.

Future work is required to test how the LMC extraction solvent affects the specificity of antibodies, which was not addressed within this work. Antibodies for the LMC should ideally cross react with classes of related molecules and the solvent environment may affect this cross reactivity. This testing will be built into the routine assay development procedure for new antibodies.

Work is also required to assess the stability of the extraction solvent to space mission conditions, including the radiation environment and also to the temperature cycling that is likely to be experienced during the mission cruise phase. A number of the back-up solvents identified within this study should also be subject to radiation and temperature testing.

7.2 Impact of liquid extracts from martian samples on LMC format immunoassays

7.2.1 Summary and conclusions

Immunoassays performance can be significantly influenced by sample matrix. The objective of the work within this chapter was to determine whether immunoassays are likely to function with martian samples. In order to achieve this, the performance of a set of model immunoassays, in ELISA and microarray format, was tested with Mars relevant sample matrix components and with liquid extracts from Mars analogue samples. The typical response of ELISA signal to a range of Mars relevant sample matrix components is shown in Figure 7-1 and an example of a successful microarray format immunoassay performed in liquid extracts from four Mars analogue samples is shown in Figure 7-2.

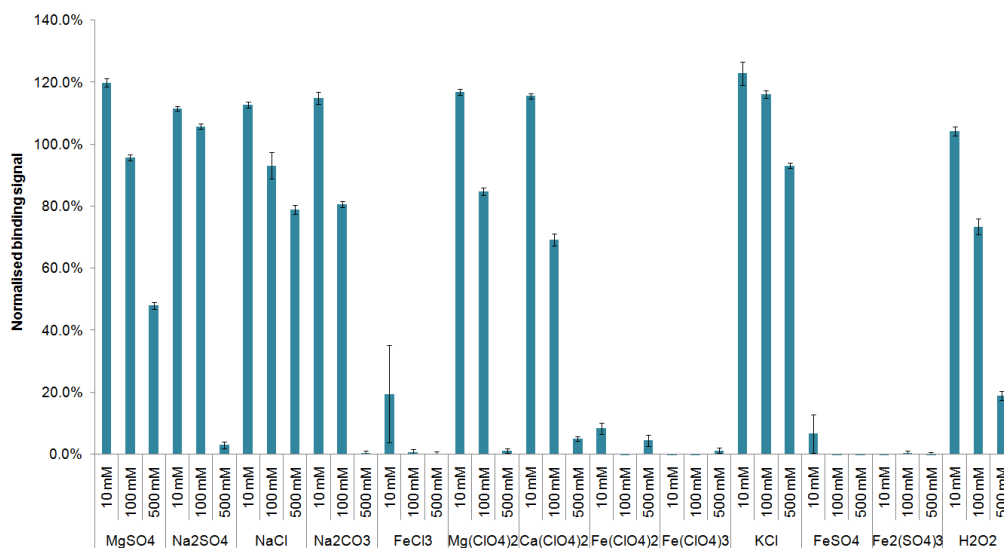


Figure 7-1: Normalised background corrected ELISA binding signal for cAMP immunoassay run in MeOH P80 with martian relevant sample matrix components at concentrations of 10, 100 and 500 mM.

Binding signals normalised against binding signal with no sample matrix component. Error bars = \pm standard error, $n = 2$

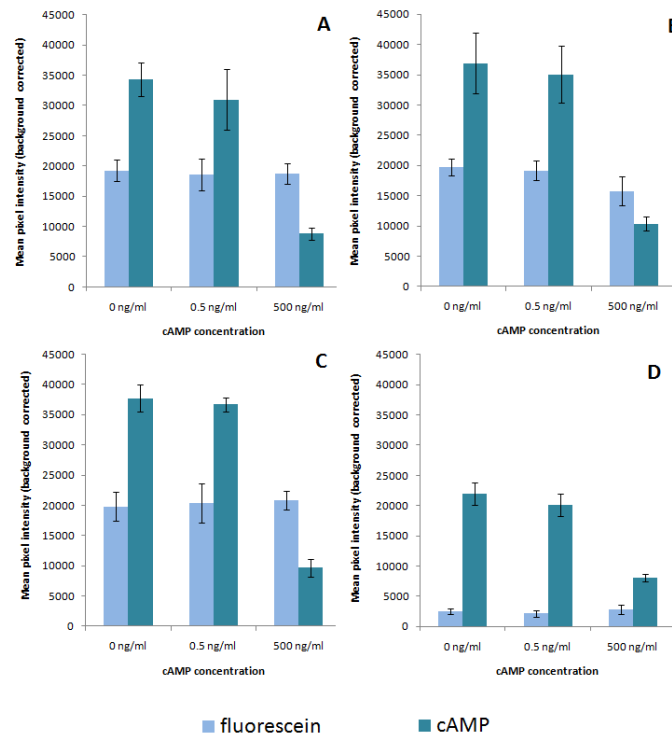


Figure 7-2: Mean background corrected pixel intensity for pooled multiplexed format fluorescein and cAMP microarray assays run with cAMP spiked liquid extracts from (A) control (extraction solvent only), (B) liquid extract from basalt rock, (C) liquid extract from gypsum (Haughton Crater), (D) liquid extract from JSC Mars-1. Error bars = ± 1 std deviation, n = 5

These studies, to investigate the likely interaction of liquid extracts from martian samples, have shown that immunoassays, in LMC format, are likely to function with martian samples similar to those encountered by the Phoenix lander. Samples with high concentrations of extractable salts, and especially iron ion containing salts may compromise assay performance and it may be necessary to avoid analysis of these types of sample. Studies also revealed that some immunoassays have poorer tolerance to the addition of sample matrix components and that signal loss with a single component/liquid extract may vary for each immunoassay tested. This has highlighted the need to screen antibodies against sample matrix components at an early stage in assay development and to include a number of well characterised control assays, to reduce the risk of data misinterpretation.

This work has significantly de-risked the operation of the LMC with martian samples, and has also identified a number of routes that can be investigated in order to improve

assay performance with martian samples. More broadly the results of this study are of interest to other immunoassay instruments for planetary exploration and for immunoassay analysis in other extreme environments on Earth.

7.2.2 Future work

It is recommended that as new antibodies for the LMC become available that they are tested, at an early stage, for their stability to a range of Mars relevant sample matrix components and liquid extracts from analogue samples. This testing should be carried out prior to investing significant effort transferring assays into microarray format and antibodies with poor stability should be deselected for further work. A number of control assays with differing tolerances to sample matrix should also be sourced for inclusion in the flight instrument to aid data interpretation.

There is a need to carry out further testing of assay performance with Mars relevant sample matrix components and liquid extracts from analogue samples in microarray format immunoassays. However before this work can be carried out effort is required to optimise the performance of LMC microarray format assays, including the investigation of covalent immobilisation of target molecule-protein conjugates to chip surfaces. The use of covalent immobilisation, rather than the current physical adsorption onto hydrophobic surfaces, may improve assay performance in the presence of sample matrix components.

In addition to altering the surface chemistry for the assay the inclusion of increased buffering capacity in the solutes freeze dried with antibodies in the glass fibre pads should be investigated in order to try and improve assay performance with Mars relevant sample matrix components that form acidic solutions.

In order to better predict the likely proportion of soluble salts in Mars samples, testing should be performed to characterise and quantify the extractable components of Mars analogue samples. This will allow the identification of sample/rock types that are likely to be unsuitable for analysis by the LMC. Testing should also be expanded to include a wider range of analogues than have been studied to date.

Finally work is required, in collaboration with other ExoMars instrument teams, to understand how data from the other instruments can be used to guide selection of suitable samples for analysis by LMC.

7.3 Generation of antibodies for the detection of markers of extinct life

7.3.1 Summary, conclusions and future work

The objective of the work within this chapter was to generate antibodies which bind to markers of extinct life using an immunised recombinant antibody library, since there are few antibodies available for this high priority class of LMC targets. Within this work two screening strategies were applied to an immunised recombinant antibody library for the production of antibodies against markers of extinct life. Despite the use of protocols specifically designed for the isolation of antibodies against small molecules, both of the screening strategies used resulted in the isolation of cross reactive and/or non-specific antibodies (Figure 7-3).

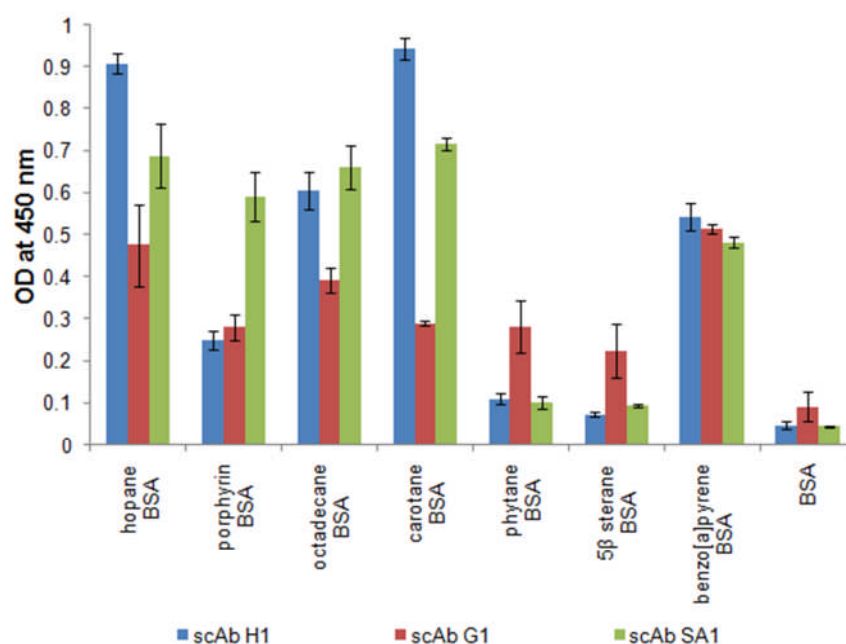


Figure 7-3: scAb binding ELISA showing cross reactive nature of selected clones with a range of hapten-BSA conjugates. Plotted values are the mean of two replicates, error bars = +/- standard error.

This outcome indicates that the screening of immunised recombinant antibody libraries is unlikely to be a suitable route for the generation of antibodies that bind to markers of extinct life for the LMC given the available resources and timescales. These results led to the conclusion that either the sheep used to construct the immunised library did not have an appropriate immune response (e.g. the targets were not recognised by the

immune system) or that there was a fundamental issue with the screening strategy (e.g. low throughput analysis did not allow the isolation of rare clones) and led to suggestions for methods to improve the probability of isolating appropriate clones.

The findings of this work may be applied beyond the LMC project for the development of antibodies against small and hydrophobic targets, for examples in the field of environmental monitoring.

Although no antibodies were produced as a direct result of the work carried out within this thesis, the findings were used to influence the implementation of a future program of work to produce antibodies (described fully in Table 5-14), which exploits immunised and non-immunised libraries from a number of sources, and also the use of high throughput screening facilities available via other organisations. The probability of producing antibodies against markers of extinct life markers in this future program of work has been significantly enhanced by the findings of the work within this thesis and in October 2011 an antibody that specifically binds to 5 β -sterane was isolated from a semi-synthetic library using high throughput screening as a direct result of the implemented program of work.

7.4 Demonstration of LMC format extraction and detection in flight representative hardware

7.4.1 Summary and conclusions

The objective of the work reported within the final chapter of this thesis was to determine whether LMC format extraction and detection function with flight representative hardware. In order to investigate this, a Mars analogue sample was spiked with a representative small molecule target and the sample was subject to LMC format extraction and detection using the flight representative assay bread board. Figure 7-4 illustrates the successful detection of fluorescein, a representative target molecule from a spiked sample of Mars analogue JSC Mars-1.

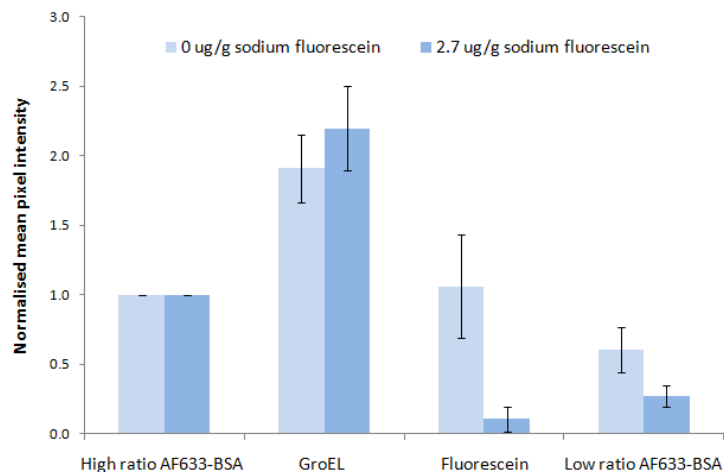


Figure 7-4: Mean pixel intensity of microarray spots after ABB immunoassay run with liquid extract from JSC Mars-1 spiked with 0 and 2.7 $\mu\text{g/g}$ sodium fluorescein salt. Decreased intensity of Fluorescein microarray assay spots with 2.7 $\mu\text{g/g}$ spiked sample demonstrates successful extraction and detection of fluorescein. Mean pixel intensity normalised against mean pixel intensity for high ratio AF633 labelled BSA spot on same optical waveguide. Error bars = ± 1 standard deviation of the mean, $n = 5-10$ (some spots removed from analysis due to presence of air bubble)

This work brought together aspects of the work carried out in Chapters 3 and 4 by confirming that LMC immunoassays function with LMC extraction solvent liquid extracts from Mars analogue samples in a flight representative format. In addition the successful completion of an end to end demonstration contributed towards award of Technology Readiness Level (TRL) 4 at the completion of the ESA funded TRL upgrade study for the LMC, enabling progression to the next stage of instrument development.

7.4.2 Future Work

Future work will involve the performance of further extractions and detections with flight representative hardware using a wider range of analogue samples and representative target molecules as the relevant antibodies become available. The eventual goal is to perform an end to end demonstration with a real sample, with target molecule incorporated within the sample, rather than with spiked samples.

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APPENDICES

Appendix A Appendix to Chapter 3

A.1 Additional inhibition immunoassay curves for ELISAs run in MeOH P80 and 45 g/L P80

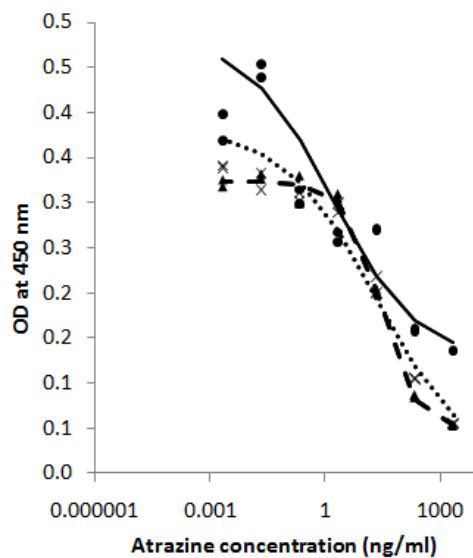


Figure A. 1: atrazine inhibition ELISA in (●) 10 % methanol, (X) MeOH P80, (▲) 4.5 g/L P80.

Each data point represents a single replicate. Immunoassay curves were plotted by fitting mean signal to a four parameter binding equation for assays run with (solid line) 10 % methanol (dotted line) MeOH P80, (dashed line) 45 g/L P80.

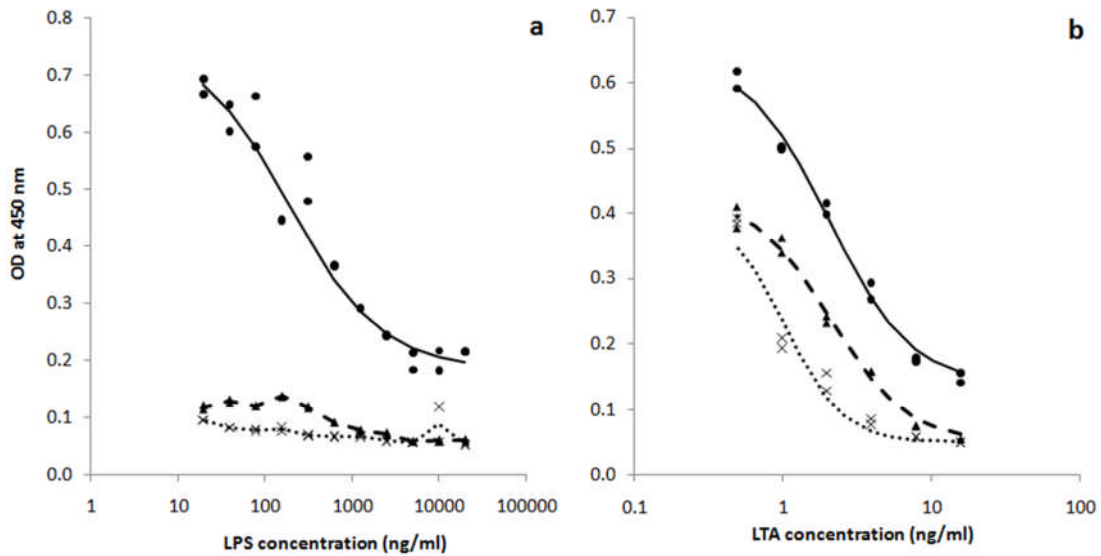


Figure A.2: (a) LPS and (b) LTA inhibition ELISA in (●) water, (X) MeOH P80, (▲) 4.5 g/L P80.

Each data point represents a single replicate. Immunoassay curves were plotted by fitting mean signal to a four parameter binding equation for assays run with (solid line) water (dotted line) MeOH P80, (dashed line) 45 g/L P80.

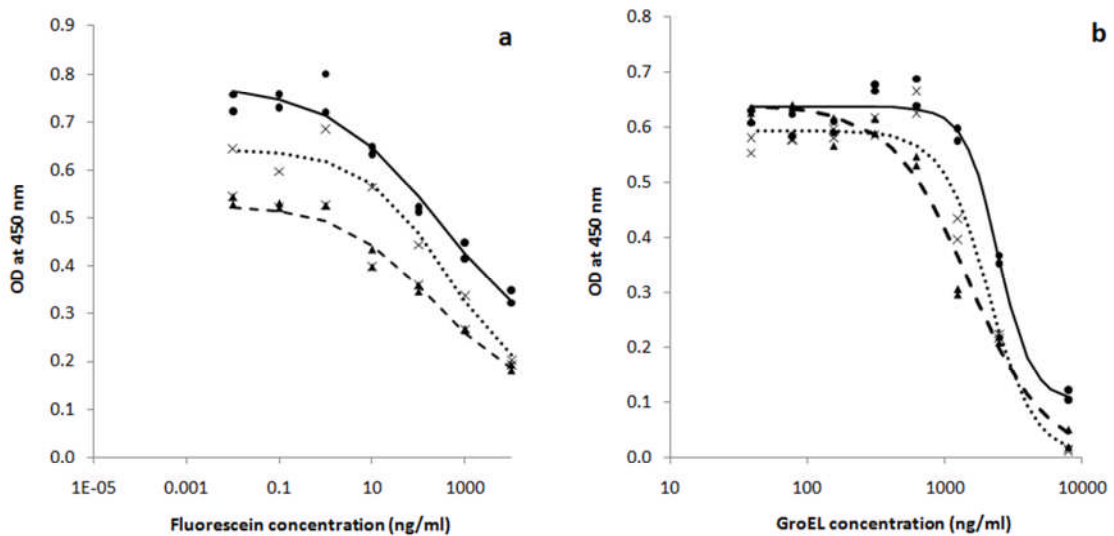


Figure A.3: (a) fluorescein and (b) GroEL inhibition ELISA in (●) water, (X) MeOH P80, (▲) 4.5 g/L P80.

Each data point represents a single replicate. Immunoassay curves were plotted by fitting mean signal to a four parameter binding equation for assays run with (solid line) water (dotted line) MeOH P80, (dashed line) 45 g/L P80.

A.2 Absolute ELISA binding data for full concentration range of alternative extraction solvents

Table A.1: Mean binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in DMSO and DMSO/methanol containing extraction solvents and water as a control.

Also showing binding signal generated by HRP enzyme independently of the immunoassays as a control. All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm, $n = 2$ or 3

	<u>Water</u>		<u>2 % DMSO 2.5 g/L P80</u>		<u>5 % DMSO 2.5 g/L P80</u>		<u>2 % DMSO 15 % MeOH 2.5 g/L P80</u>		<u>5 % DMSO 15 % MeOH 2.5 g/L P80</u>	
	\bar{X}	\bar{X}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.78	0.05	0.81	0.04	0.82	0.04	0.80	0.04	0.82	0.04
<i>Atrazine</i>	0.57	0.14	0.09	0.05	0.15	0.05	0.32	0.05	0.25	0.05
<i>B[a]P</i>	0.27	0.12	0.48	0.05	0.67	0.20	1.13	0.07	1.08	0.04
<i>cAMP</i>	0.71	0.05	0.66	0.05	0.65	0.04	0.64	0.04	0.63	0.04
<i>Fluorescein</i>	0.86	0.05	0.75	0.01	0.73	0.04	0.68	0.04	0.70	0.04
<i>GroEL</i>	0.68	0.05	0.62	0.04	0.61	0.04	0.65	0.04	0.62	0.04
<i>LPS</i>	0.54	0.13	0.20	0.07	0.22	0.08	0.12	0.08	0.15	0.08
<i>LTA</i>	0.97	0.16	0.15	0.05	0.13	0.05	0.100	0.06	0.11	0.05

Table A.2: Mean binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in acetone containing extraction solvents and water as a control.

Also showing binding signal generated by HRP enzyme independently of the immunoassays as a control. All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm, n = 2 or 3

	<u>Water</u>		<u>10 % acetone</u> <u>2.5 g/L P80</u>		<u>20 % acetone</u> <u>2.5 g/L P80</u>		<u>30 % acetone</u> <u>2.5 g/L P80</u>	
	\bar{X}	\bar{X}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.78	0.05	0.76	0.04	0.82	0.04	0.81	0.04
<i>Atrazine</i>	0.57	0.14	0.11	0.05	0.07	0.05	0.06	0.05
<i>B[a]P</i>	0.27	0.12	1.03	0.04	1.02	0.04	0.96	0.05
<i>cAMP</i>	0.71	0.05	0.64	0.04	0.62	0.04	0.55	0.05
<i>Fluorescein</i>	0.86	0.05	0.65	0.05	0.63	0.05	0.58	0.05
<i>GroEL</i>	0.68	0.05	0.60	0.04	0.59	0.04	0.59	0.04
<i>LPS</i>	0.54	0.13	0.18	0.10	0.12	0.06	0.09	0.05
<i>LTA</i>	0.97	0.16	0.12	0.06	0.13	0.05	0.07	0.05

Table A.3: Mean binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in acetonitrile containing extraction solvents and water as a control.

Also showing binding signal generated by HRP enzyme independently of the immunoassays as a control. All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm, n = 2 or 3

	<u>Water</u>		<u>10 % MeCN</u> <u>2.5 g/L P80</u>		<u>20 % MeCN</u> <u>2.5 g/L P80</u>		<u>30 % MeCN</u> <u>2.5 g/L P80</u>	
	\bar{X}	\bar{X}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.78	0.05	0.74	0.04	0.70	0.04	0.66	0.04
<i>Atrazine</i>	0.57	0.14	0.18	0.05	0.13	0.05	0.06	0.05
<i>B[a]P</i>	0.27	0.12	0.98	0.04	0.76	0.05	0.45	0.05
<i>cAMP</i>	0.71	0.05	0.68	0.04	0.41	0.04	0.05	0.05
<i>Fluorescein</i>	0.86	0.05	0.64	0.16	0.63	0.05	0.48	0.05
<i>GroEL</i>	0.68	0.05	0.53	0.04	0.48	0.05	0.37	0.04
<i>LPS</i>	0.54	0.13	0.18	0.08	0.09	0.06	0.06	0.05
<i>LTA</i>	0.97	0.16	0.15	0.06	0.06	0.06	0.06	0.06

A.3 Additional inhibition ELISA curves with alternative extraction solvents

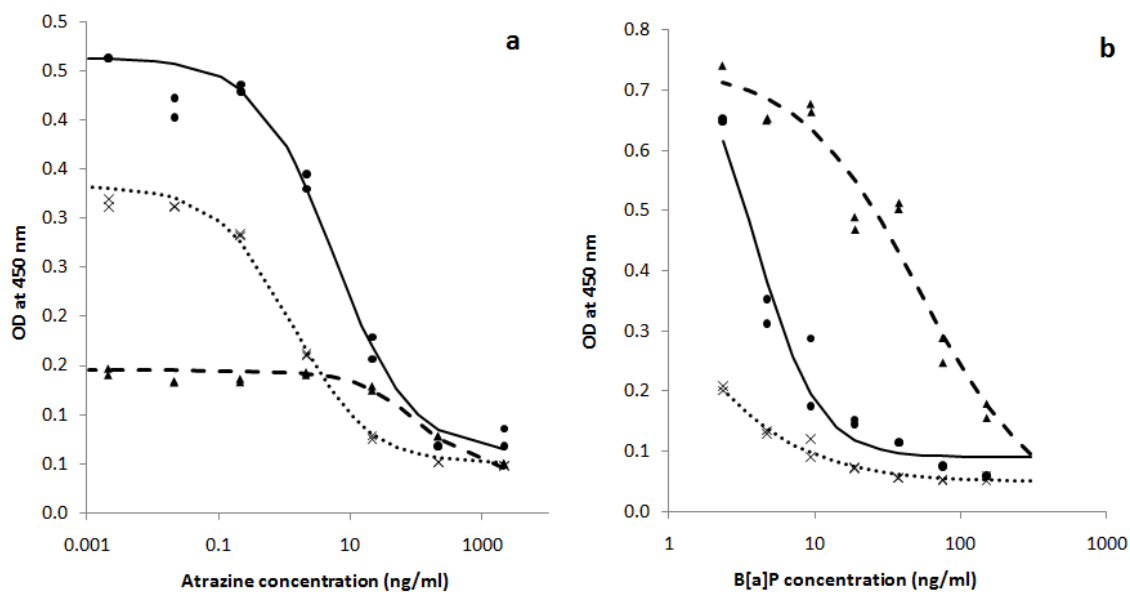


Figure A.4: (a) atrazine and (b) B[a]P LMC format inhibition ELISAs in 10 % methanol and alternative LMC extraction solvents

(●) 10 % methanol, (X) 5 % DMSO 2.5 g/L P80, (▲) 30 % acetone 2.5 g/L P80 and (+) 30 % acetonitrile 2.5 g/L P80. Each data point represents a single replicate. Immunoassay curves were plotted by fitting mean signal to a four parameter binding equation, or by linear interpolation of the mean OD where the data did not fit a four parameter binding equation (solid line) 10 % methanol, (dotted line) 5 % DMSO 2.5 g/L P80, (short dashed line) 30 % acetone 2.5 g/L P80, (long dashed line) 30 % acetonitrile 2.5 g/L P80.

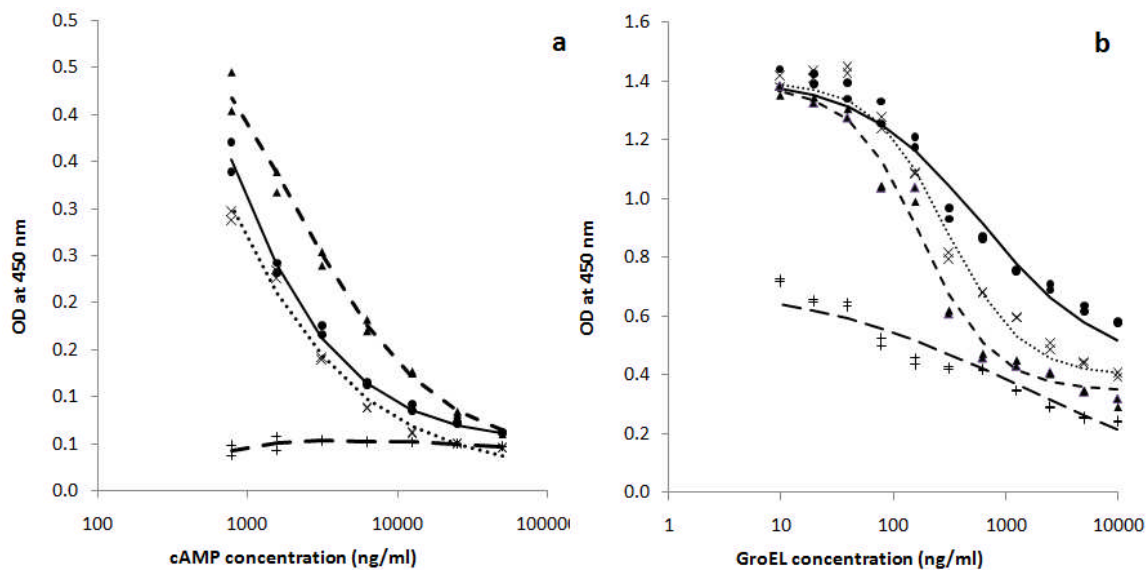


Figure A.5: (a) cAMP and (b) GroEL LMC format inhibition ELISAs in water and alternative LMC extraction solvents

(●) water, (X) 5 % DMSO 2.5 g/L P80, (▲) 30 % acetone 2.5 g/L P80 and (+) 30 % acetonitrile 2.5 g/L P80. Each data point represents a single replicate. Immunoassay curves were plotted by fitting mean signal to a four parameter binding equation, or by linear interpolation of the mean OD where the data did not fit a four parameter binding equation (solid line) 10 % methanol, (dotted line) 5 % DMSO 2.5 g/L P80, (short dashed line) 30 % acetone 2.5 g/L P80, (long dashed line) 30 % acetonitrile 2.5 g/L P80.

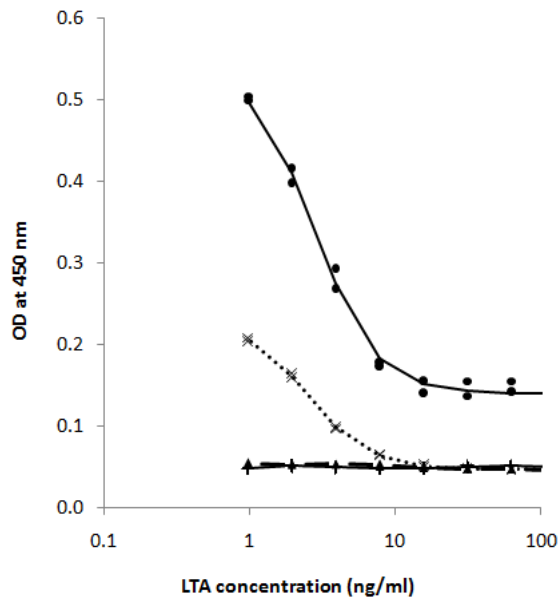


Figure A.6: LTA LMC format inhibition ELISA in water and alternative LMC extraction solvents

(●) water, (X) 5 % DMSO 2.5 g/L P80, (▲) 30 % acetone 2.5 g/L P80 and (+) 30 % acetonitrile 2.5 g/L P80. Each data point represents a single replicate. Immunoassay curves were plotted by fitting mean signal to a four parameter binding equation, or by linear interpolation of the mean OD where the data did not fit a four parameter binding equation (solid line) 10 % methanol, (dotted line) 5 % DMSO 2.5 g/L P80, (short dashed line) 30 % acetone 2.5 g/L P80, (long dashed line) 30 % acetonitrile 2.5 g/L P80.

Appendix B Appendix to Chapter 4

B.1 ELISA binding data for full range of sample matrix components

Table B1: Mean Binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in the presence of magnesium perchlorate salts. Also showing signal generated by HRP enzyme independently of immunoassay as a control.

	Control		<u>10 mM Mg(ClO₄)₂</u>		<u>100 mM Mg(ClO₄)₂</u>		<u>500 mM Mg(ClO₄)₂</u>	
	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.37	0.04	0.44	0.04	0.44	0.04	0.42	0.04
<i>Atrazine</i>	0.51	0.04	0.53	0.05	0.34	0.07	0.06	0.07
<i>B[a]P</i>	0.53	0.05	0.47	0.04	0.14	0.05	0.05	0.05
<i>cAMP</i>	0.60	0.04	0.69	0.04	0.52	0.08	0.05	0.05
<i>Fluorescein</i>	0.63	0.04	0.69	0.05	0.69	0.05	0.24	0.05

All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm (n = 2 or 3)

Table B2: Mean Binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in the presence of calcium perchlorate salts. Also showing signal generated by HRP enzyme independently of immunoassay as a control. All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm (n = 2 or 3)

	<u>Control</u>		<u>Ca(ClO₄)₂ 10 mM</u>		<u>Ca(ClO₄)₂ 100 mM</u>		<u>Ca(ClO₄)₂ 500 mM</u>	
	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.37	0.04	0.49	0.04	0.41	0.04	0.39	0.04
<i>Atrazine</i>	0.51	0.04	0.61	0.07	0.40	0.15	0.39	0.35
<i>B[a]P</i>	0.53	0.05	0.51	0.05	0.19	0.07	0.27	0.23
<i>cAMP</i>	0.60	0.04	0.70	0.06	0.46	0.08	0.14	0.11
<i>Fluorescein</i>	0.63	0.04	0.73	0.06	0.68	0.08	0.45	0.26

Table B3: Mean Binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in the presence of iron (III) perchlorate salts. Also showing signal generated by HRP enzyme independently of immunoassay as a control. All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm (n = 2 or 3)

	<u>Control</u>		<u>Fe(ClO₄)₃ 10 mM</u>		<u>Fe(ClO₄)₃ 100 mM</u>		<u>Fe(ClO₄)₃ 500 mM</u>	
	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.37	0.04	0.42	0.05	0.06	0.07	0.05	0.04
<i>Atrazine</i>	0.51	0.04	0.27	0.30	0.06	0.06	0.05	0.06
<i>B[a]P</i>	0.53	0.05	0.21	0.20	0.06	0.07	0.06	0.06
<i>cAMP</i>	0.60	0.04	0.95	1.49	0.06	0.07	0.06	0.06
<i>Fluorescein</i>	0.63	0.04	0.30	0.28	0.06	0.06	0.05	0.05

Table B4: Mean Binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in the presence of iron (II) perchlorate salts. Also showing signal generated by HRP enzyme independently of immunoassay as a control. All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm (n = 2 or 3)

	<u>Control</u>		<u>Fe(ClO₄)₂ 10 mM</u>		<u>Fe(ClO₄)₂ 100 mM</u>		<u>Fe(ClO₄)₂ 500 mM</u>	
	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.37	0.04	0.35	0.06	0.22	0.16	0.39	0.04
<i>Atrazine</i>	0.51	0.04	0.80	0.74	0.76	0.69	0.18	0.19
<i>B[a]P</i>	0.53	0.05	1.67	1.85	0.75	0.90	0.14	0.16
<i>cAMP</i>	0.60	0.04	1.26	1.21	0.68	0.78	0.23	0.20
<i>Fluorescein</i>	0.63	0.04	0.69	0.58	0.59	0.59	0.12	0.12

Table B5: Mean Binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in the presence of sodium chloride. Also showing signal generated by HRP enzyme independently of immunoassay as a control. All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm, n = 2 or 3

	<u>Control</u>		<u>NaCl 10 mM</u>		<u>NaCl 100 mM</u>		<u>NaCl 500 mM</u>	
	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.37	0.04	0.33	0.04	0.41	0.04	0.43	0.04
<i>Atrazine</i>	0.51	0.04	0.47	0.04	0.52	0.04	0.39	0.04
<i>B[a]P</i>	0.53	0.05	0.53	0.04	0.30	0.04	0.10	0.04
<i>cAMP</i>	0.60	0.04	0.67	0.04	0.56	0.04	0.52	0.08
<i>Fluorescein</i>	0.63	0.04	0.63	0.04	0.71	0.04	0.67	0.04

Table B6: Mean Binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in the presence of potassium chloride. Also showing signal generated by HRP enzyme independently of immunoassay as a control.

All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm, n = 2 or 3

	<u>Control</u>		<u>KCl 10 mM</u>		<u>KCl 100 mM</u>		<u>KCl 500 mM</u>	
	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.37	0.04	0.45	0.04	0.45	0.04	0.43	0.04
<i>Atrazine</i>	0.51	0.04	0.49	0.04	0.48	0.04	0.34	0.05
<i>B[a]P</i>	0.53	0.05	0.57	0.04	0.34	0.04	0.10	0.05
<i>cAMP</i>	0.60	0.04	0.73	0.04	0.69	0.04	0.56	0.05
<i>Fluorescein</i>	0.63	0.04	0.64	0.04	0.71	0.04	0.66	0.04

Table B7: Mean Binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in the presence of magnesium sulphate. Also showing signal generated by HRP enzyme independently of immunoassay as a control.

All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm, n = 2 or 3

	<u>Control</u>		<u>MgSO₄ 10 mM</u>		<u>MgSO₄ 100 mM</u>		<u>MgSO₄ 500 mM</u>	
	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.37	0.04	0.39	0.04	0.37	0.04	0.38	0.04
<i>Atrazine</i>	0.51	0.04	0.59	0.04	0.49	0.05	0.36	0.06
<i>B[a]P</i>	0.53	0.05	0.46	0.04	0.20	0.05	0.07	0.05
<i>cAMP</i>	0.60	0.04	0.71	0.04	0.58	0.04	0.31	0.05
<i>Fluorescein</i>	0.63	0.04	0.68	0.04	0.62	0.04	0.50	0.05

Table B8: Mean Binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in the presence of sodium sulphate. Also showing signal generated by HRP enzyme independently of immunoassay as a control. All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm, n = 2 or 3

	<u>Control</u>		<u>Na₂SO₄ 10 mM</u>		<u>Na₂SO₄ 100 mM</u>		<u>Na₂SO₄ 500 mM</u>	
	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.37	0.04	0.40	0.04	0.39	0.04	0.33	0.04
<i>Atrazine</i>	0.51	0.04	0.50	0.04	0.53	0.04	0.45	0.05
<i>B[a]P</i>	0.53	0.05	0.51	0.04	0.29	0.04	0.11	0.05
<i>cAMP</i>	0.60	0.04	0.68	0.05	0.63	0.04	0.44	0.43
<i>Fluorescein</i>	0.63	0.04	0.67	0.04	0.66	0.04	0.63	0.05

Table B9: Mean Binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in the presence of sodium carbonate. Also showing signal generated by HRP enzyme independently of immunoassay as a control. All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm, n = 2 or 3

	<u>Control</u>		<u>Na₂CO₃ 10 mM</u>		<u>Na₂CO₃ 100 mM</u>		<u>Na₂CO₃ 500 mM</u>	
	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.37	0.04	0.39	0.04	0.44	0.04	0.32	0.04
<i>Atrazine</i>	0.51	0.04	0.55	0.04	0.21	0.06	0.14	0.09
<i>B[a]P</i>	0.53	0.05	0.52	0.04	0.06	0.06	0.07	0.04
<i>cAMP</i>	0.60	0.04	0.68	0.04	0.52	0.07	0.08	0.08
<i>Fluorescein</i>	0.63	0.04	0.64	0.04	0.65	0.05	0.26	0.08

Table B10: Mean Binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in the presence of iron (III) chloride. Also showing signal generated by HRP enzyme independently of immunoassay as a control. All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm, n = 2 or 3

	<u>Control</u>		<u>FeCl₃ 10 mM</u>		<u>FeCl₃ 100 mM</u>		<u>FeCl₃ 500 mM</u>	
	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.37	0.04	0.42	0.04	0.06	0.06	0.09	0.06
<i>Atrazine</i>	0.51	0.04	0.62	0.30	0.06	0.06	0.06	0.07
<i>B[a]P</i>	0.53	0.05	1.94	2.21	0.07	0.07	0.07	0.08
<i>cAMP</i>	0.60	0.04	0.79	0.68	0.07	0.07	0.07	0.07
<i>Fluorescein</i>	0.63	0.04	0.74	0.83	0.06	0.06	0.06	0.06

Table B11: Mean Binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in the presence of iron (II) sulphate. Also showing signal generated by HRP enzyme independently of immunoassay as a control. All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm, n = 2 or 3

	<u>Control</u>		<u>Fe(II)SO₄ 10 mM</u>		<u>Fe(II)SO₄ 100 mM</u>		<u>Fe(II)SO₄ 500 mM</u>	
	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.49	0.05	0.49	0.10	0.50	0.12	0.52	0.20
<i>Atrazine</i>	0.49	0.04	0.49	0.10	0.50	0.12	0.52	0.20
<i>B[a]P</i>	0.34	0.04	0.94	0.99	0.63	0.71	0.64	0.74
<i>cAMP</i>	0.63	0.04	0.89	0.850	0.75	0.80	0.80	0.86
<i>Fluorescein</i>	0.68	0.04	0.89	0.95	0.73	0.80	0.70	0.95

Table B12: Mean Binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in the presence of iron (III) sulphate. Also showing signal generated by HRP enzyme independently of immunoassay as a control. All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm, n = 2 or 3

	<u>Control</u>		<u>Fe(III)₂(SO₄)₃ 10 mM</u>		<u>Fe(III)₂(SO₄)₃ 100 mM</u>		<u>Fe(III)₂(SO₄)₃ 500 mM</u>	
	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.49	0.05	0.45	0.04	0.05	0.05	0.05	0.05
<i>Atrazine</i>	0.49	0.04	0.45	0.04	0.05	0.05	0.05	0.05
<i>B[a]P</i>	0.34	0.04	0.28	0.30	0.05	0.05	0.05	0.05
<i>cAMP</i>	0.63	0.04	0.29	0.38	0.05	0.05	0.05	0.05
<i>Fluorescein</i>	0.68	0.04	0.23	0.34	0.05	0.05	0.05	0.06

Table B13: Mean Binding signal (\bar{X}) and mean background signal (\bar{X}_{bg}), for LMC relevant ELISAs run in the presence of hydrogen peroxide. Also showing signal generated by HRP enzyme independently of immunoassay as a control. All binding signals are given in units of optical density measured at an absorption wavelength of 450 nm, n = 2 or 3

	<u>Control</u>		<u>H₂O₂ 10 mM</u>		<u>H₂O₂ 100 mM</u>		<u>H₂O₂ 500 mM</u>	
	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}	\bar{X}	\bar{X}_{bg}
<i>HRP</i>	0.49	0.05	0.51	0.04	0.40	0.04	0.27	0.04
<i>Atrazine</i>	0.49	0.04	0.51	0.04	0.40	0.04	0.27	0.04
<i>B[a]P</i>	0.34	0.04	0.38	0.04	0.21	0.04	0.08	0.04
<i>cAMP</i>	0.63	0.04	0.66	0.04	0.47	0.04	0.15	0.04
<i>Fluorescein</i>	0.68	0.04	0.69	0.05	0.43	0.06	0.12	0.04

B.2 inhibition immunoassay curves for ELISAs run in sample matrix components

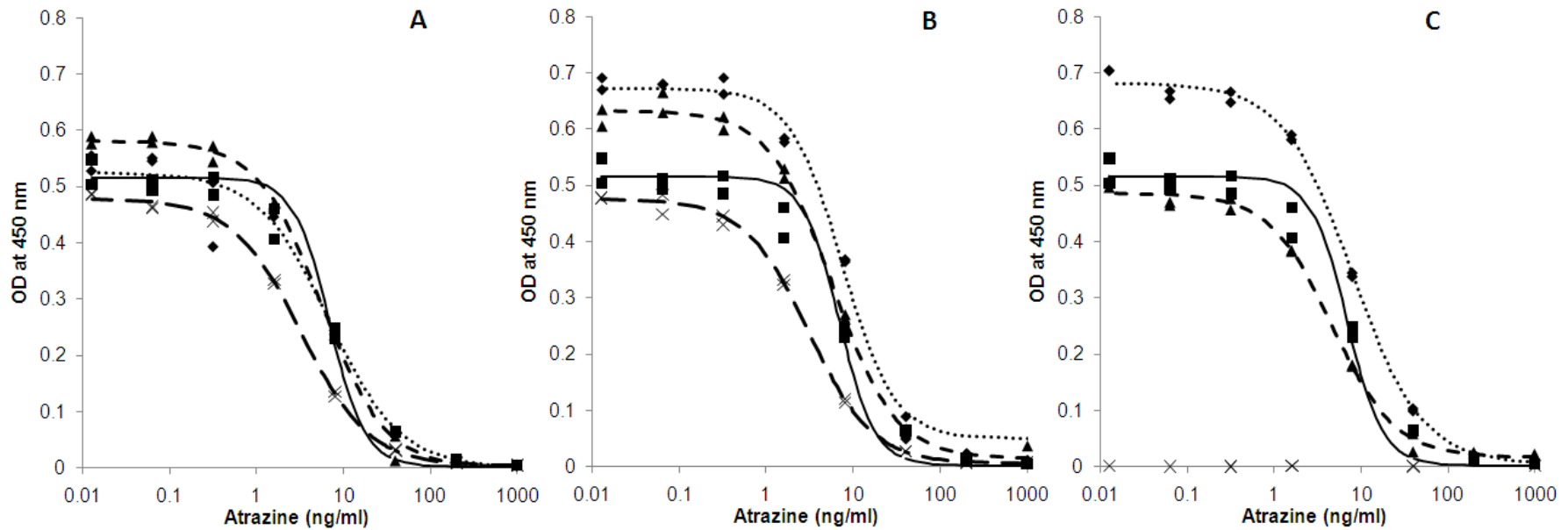


Figure B1: Atrazine inhibition ELISA in the presence of Mars relevant sample matrix components

A sodium chloride, **B** magnesium sulphate and **C** magnesium perchlorate at ■ 0 mM, ♦ 10 mM, ▲ 100 mM and X 500 mM concentration. Each data point represents a single replicate. Immunoassay curves for each concentration of each sample matrix component were plotted by fitting mean signal to a four parameter binding equation, or by linear regression of the mean signal where the data did not fit a four parameter binding equation. (solid line) 0 mM, (dotted line) 10 mM, (short dashed line) 100 mM and (long dashed line) 500 mM.

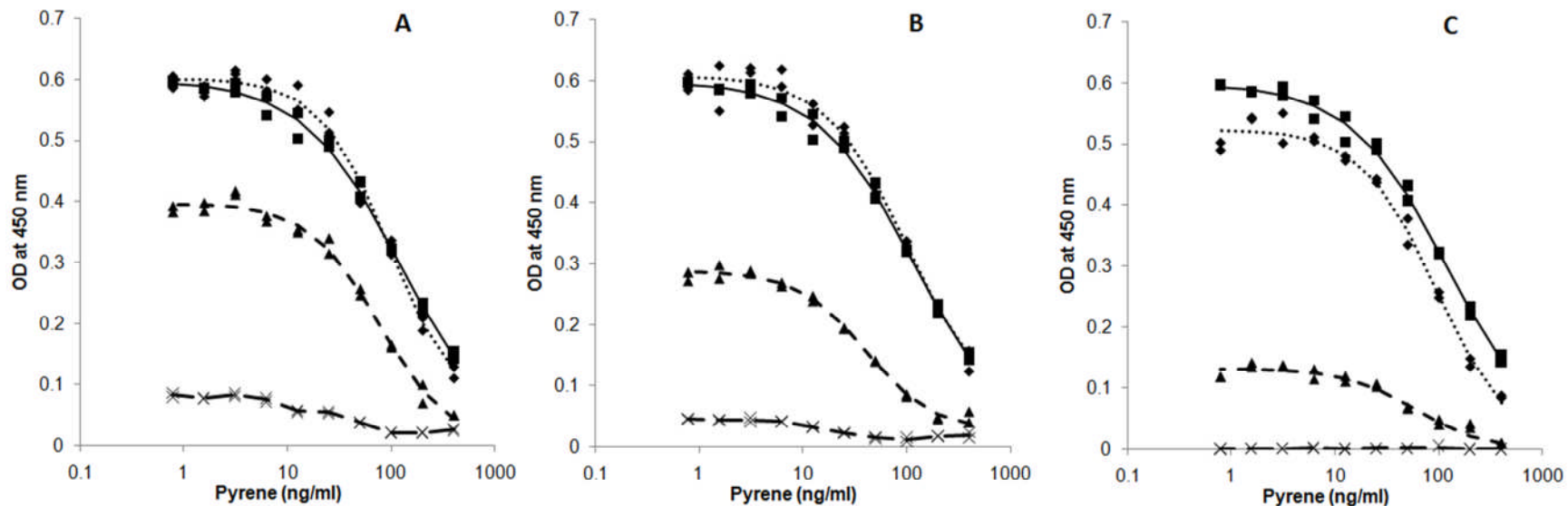


Figure B2: Benzo[a]pyrene inhibition ELISA in the presence of sample matrix components **A** sodium chloride, **B** magnesium sulphate and **C** magnesium perchlorate at ■ 0 mM, ◆ 10 mM, ▲ 100 mM and X 500 mM concentration. Each data point represents a single replicate. Immunoassay curves for each concentration of each sample matrix component were plotted by fitting mean signal to a four parameter binding equation, or by linear regression of the mean signal where the data did not fit a four parameter binding equation. (solid line) 0 mM, (dotted line) 10 mM, (short dashed line) 100 mM and (long dashed line) 500 mM. Note that pyrene (rather than benzo[a]pyrene) was used as inhibiting antigen

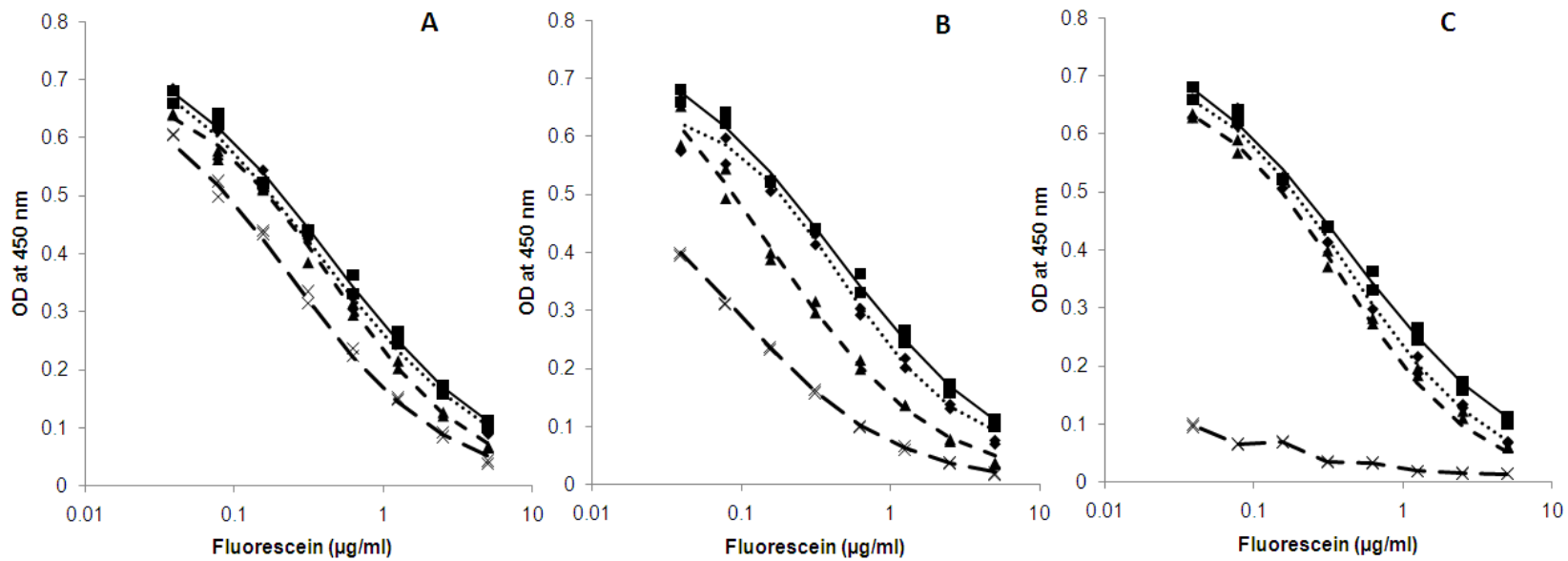


Figure B3: Fluorescein inhibition ELISA in the presence of sample matrix components **A** sodium chloride, **B** magnesium sulphate and **C** magnesium perchlorate at ■ 0 mM, ◆ 10 mM, ▲ 100 mM and X 500 mM concentration. Each data point represents a single replicate. Immunoassay curves for each concentration of each sample matrix component were plotted by fitting mean signal to a four parameter binding equation, or by linear regression of the mean signal where the data did not fit a four parameter binding equation. (solid line) 0 mM, (dotted line) 10 mM, (short dashed line) 100 mM and (long dashed line) 500 mM.

Appendix C Publications

C.1 Papers

Court R. W., Rix C. S., Sims M. R., Cullen D. C., Sephton M. A. (submitted 2011) Extraction of polar and nonpolar biomarkers from the martian soil using aqueous surfactant solutions

Rix C.S., Sims M.R., Cullen D.C., (2011). Immunological Detection of Small Organic Molecules in the Presence of Perchlorates: Relevance to the Life Marker Chip and Life Detection on Mars, *Astrobiology*, 11 (9), 839-846

Sims M.R., Cullen D.C. , Rix C.S., Buckley A., Derveni M.E., Evans D., Rhodes A., Rato C.C., Sephton M.A., Court R.W., Bulloch C., Kitchingman I., Ali Z., Pullan D., Holt J., Blake O., Sykes J., Samara-Ratna P., Canali M., Borst G., Leeuwis H., Prak A., Norfini A., Geraci E., Tavanti M., Brucato J., Holm N. (submitted 2012) Development Status of the Life Marker Chip Instrument for ExoMars

C.2 Oral presentations at conferences

Rix C., Cullen D.C., Sims M.R., Sephton M., Prak A., Borst A.G. (2010). The Life Marker Chip: A multiplexed immunoassay for the detection of life on Mars, *20th World Congress on Biosensors*, Glasgow, UK

C.3 Poster presentations at conferences

Rix C.S, Gomes R., Taylor J., Sephton M.A., Sims M.R., Cullen D.C. (2009) Performance of antibody assays in a range of solvents relevant to the Life Marker Chip multiplexed immunoassay instrument. *Applications of Micro and Nano Technology in Biosensing and Diagnostics*

Extraction of polar and nonpolar biomarkers from the martian soil using aqueous surfactant solutions

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ABSTRACT

The Life Marker Chip intends to use an aqueous surfactant solution to extract both polar AND nonpolar biomarkers from the martian soil for transport to an antibody-based detection system. Currently, a solution of 1.5 g l⁻¹ polysorbate 80 in 20:80 (vol:vol) methanol:water is being considered and appears to be suitable. However, should this solution be shown to be unsuitable for the LMC or the martian environment, it will be necessary to use a different surfactant. Here, we have investigated the ability of a range of other surfactant solutions to extract a suite of eight standards spiked on the surfaces of the martian soil simulant JSC Mars-1 and tested the compatibility of the best two surfactants with a representative antibody assay for the detection of pyrene. The results show that using 20:80 (vol:vol) methanol:water as the solvent leads to greater recoveries of standards than using water alone. The poloxamer surfactants Pluronic® F-68 and F-108 are not effective at extracting the standards from JSC Mars-1 at any of the concentrations tested here. The fluorosurfactant Zonyl® FS-300 is able to extract the standards, but not as efficiently as polysorbate 80 solutions. Most successful of the alternative surfactants was the siloxane-based surfactant poly[dimethylsiloxane-co-[3-(2-(2-hydroxyethoxy)ethoxy)propyl]methylsiloxane] (PDMSHEPMS) which is able to extract the standards from JSC Mars-1 about as efficiently as polysorbate 80 solutions. Enhanced recovery of the standards using polysorbate 80 solutions can be achieved by increasing the concentration of polysorbate 80, from 1.5 g l⁻¹ to 10 g l⁻¹, leading to an increase in the recovery of standards of about 50%; a similar increase in effectiveness is also apparent for PDMSHEPMS. Polysorbate 80 at concentrations of 1.5 g l⁻¹ and 10 g l⁻¹ and Zonyl® FS-300 and PDMSHEPMS (both at a concentration of 10 g l⁻¹) are also compatible with the representative pyrene antibody assay.

Immunological Detection of Small Organic Molecules in the Presence of Perchlorates: Relevance to the Life Marker Chip and Life Detection on Mars

Astrobiology (2011), *11* (9), 839-846

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ABSTRACT

The proposed ExoMars mission, due to launch in 2018, aims to look for evidence of extant and extinct life in martian rocks and regolith. Previous attempts to detect organic molecules of biological or abiotic origin on Mars have been unsuccessful, which may be attributable to destruction of these molecules by perchlorate salts during pyrolysis sample extraction techniques. Organic molecules can also be extracted and measured with solvent based systems. The ExoMars payload includes the Life Marker Chip (LMC) instrument, capable of detecting biomarker molecules of extant and extinct Earth-like life in liquid extracts of martian samples with an antibody microarray assay. The aim of the work reported here was to investigate whether the presence of perchlorate salts, at levels similar to those at the NASA Phoenix landing site, would compromise the LMC extraction and detection method. To test this, we implemented an LMC representative sample extraction process with an LMC representative antibody assay and used these to extract and analyze a model sample that consisted of a martian analogue sample matrix (JSC Mars-1) spiked with a representative organic molecular target (pyrene, an example of abiotic meteoritic in-fall targets) in the presence of perchlorate salts. We found no significant change in immunoassay function when using pyrene standards with added perchlorate salts. When model samples spiked with perchlorate salts were subjected to an LMC representative liquid extraction, immunoassays functioned in a liquid extract and detected extracted pyrene. For the same model sample matrix without perchlorate salts, we observed anomalous assay signals that coincided with yellow coloration of the extracts. This unexpected observation is being studied further. This initial study indicates that the presence of perchlorate salts, at levels similar to those detected at the NASA Phoenix landing site, is unlikely to prevent the LMC from extracting and detecting organic molecules from martian samples.

Development Status of the Life Marker Chip Instrument for ExoMars

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The corresponding author is Mark R. Sims

ABSTRACT

The Life Marker Chip (LMC) is one of the instruments being developed for possible flight on the 2018 ExoMars mission. The instrument uses solvents to extract organic compounds from samples of martian regolith and to transfer the extracts to dedicated detectors based around the use of antibodies. The scientific aims of the instrument are to detect organics in the form of biomarkers that might be associated with extinct life, extant life or abiotic sources of organics. The instrument relies on a novel surfactant-based solvent system and bespoke, commercial and research-developed antibodies against a number of distinct biomarkers or molecular types. The LMC comprises a number of subsystems designed to accept up to four discrete samples of martian regolith or crushed rock, implement the solvent extraction, perform microfluidic-based multiplexed antibody-assays for biomarkers and other targets, optically detect the fluorescent output of the assays, control the internal instrument pressure and temperature, in addition to the associated instrument control electronics and software. The principle of operation, the design and the instrument development status as of December 2011 are reported here.

The Life Marker Chip:

A multiplexed immunosensor experiment for the detection of life on Mars

20th World Congress on Biosensors (2009), Glasgow, UK

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ABSTRACT

The Life Marker Chip (LMC) is a multi-sample, multiplexed immunoassay microarray based instrument currently under development by an international consortium for flight on the European Space Agency's ExoMars mission to Mars. The mission is currently scheduled for launch in 2018. The LMC has been proposed as part of the mission's organics instrument suite to search for molecular organic evidence of life on Mars. This mission will include for the first time a rover equipped with a drilling system to extract samples from depths down to 2 metres below the surface where evidence of life may be preserved.

The LMC instrument comprises an optical waveguide substrate that evanescently excites fluorescent labels associated with a one hundred spot multiplexed immunoassay microarray. The array is in a micro-fluidic channel that also contains pre-dosed and dried assay reagents that dissolve into an incoming liquid sample. The LMC instrument contains multiple single-use array / micro-fluidic channels to enable multiple samples to be analysed on the Martian surface. The instrument also contains a sample processing system to automate the extraction of organics from a Martian regolith or crushed rock sample. The LMC analytical targets include organic molecules that are from the following classes: biomarkers of extinct life, biomarkers of extant life, markers of meteoritic organic material, markers of terrestrial contamination, and synthetic control markers.

Progress in LMC development will be reported and to include: (i) development of breadboard hardware demonstrators including microfluidic assay and sample extraction and processing breadboards; (ii) development of recombinant antibodies and assays to LMC targets; (iii) development of sample processing protocols and (iv) storage and stability testing of LMC assay reagents in space mission relevant scenarios.

Performance of antibody assays in a range of solvents relevant to The Life Marker Chip multiplexed immunoassay instrument

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Introduction

The Life Marker Chip (LMC) proposed for the European Space Agency's ExoMars mission (Figure 1), aims to detect evidence of extant and extinct life on Mars. The LMC will use antibodies to search for the presence of biomarker targets in samples collected from below the Martian surface.

The Life Marker Chip instrument (Figure 2) utilises microarray based inhibition assays. Antigens are immobilised on an optical waveguide chip and fluorescently labelled antibodies are mixed with an extracted Martian sample prior to exposure to the antigen array. Unbound antibodies are then washed from the surface and fluorophores are excited by coupling of laser light into the optical waveguides. This results in production of a fluorescent signal with an intensity that is related to the amount of antibody that has bound to the immobilised antigen and hence inversely to the amount of target present in the extracted sample (Figure 3).

One of the key challenges of LMC development (and the development of antibody based biosensors for other environmental targets) is the selection of a universal solvent system that will efficiently extract biomarkers – including hydrophobic targets – and is compatible with immunoassays. Antibodies generally give optimum performance at physiological pH and temperature in aqueous conditions, although a variety of antibody based assays have been shown to tolerate solvents including 10 – 20 % methanol with no detrimental effect. The approach adopted for the LMC is to use a low percentage of organic solvent, in combination with a surfactant to solubilise hydrophobic targets.

The objectives of the work reported here were to examine the performance of a range of antibody assays in solvents that may be suitable for the extraction of biomarker targets.

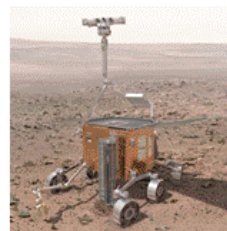


Figure 1: The ExoMars Rover concept (image courtesy of ESA)

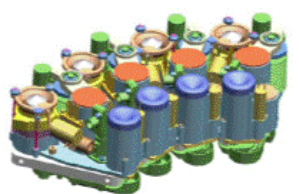


Figure 2: Design concept for the LMC Instrument (dimensions are approx. 175 x 115 x 60 mm)

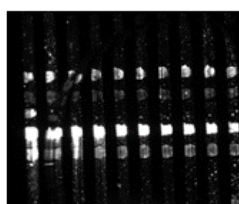


Figure 3: Example optical read out from an LMC prototype microarray (100 µm spots)

Methods/Materials

In order to assess performance in a range of solvents, assays were run in an inhibition ELISA format with antigens (or antigen protein conjugates) immobilised on polystyrene plates and HRP labelled antibodies (biotin-labelled secondary antibodies with streptavidin-HRP conjugates). The ELISA assay was designed to be representative of the microarray assay but allowed higher throughput of samples. Assays were first performed in a binding format (i.e. with no inhibiting antigen present) to assess the impact of the solvents on binding of the antibodies to the immobilised antigens. Assays were then run in a sub-set of solvents in an inhibition format to assess the impact on assay sensitivity and midpoint. Assays were selected to include a variety of antibody species and target molecules (Table 1).

Results & Discussion

Binding Assays

Figure 4 shows the varying response of binding assays to the solvents tested. Some assays e.g. fluorescein, GroEL and cyclic adenosine monophosphate (cAMP) were unaffected (< 20 % change in binding signal) by the majority of solvents, whereas the lipoteichoic acid (LTA) and atrazine assays showed high signal loss in the majority of solvents tested.

The consistently poor performance of the LTA assay may be related to unstable immobilisation of the polysaccharide antigen onto the microtitre plate.

Note that the benzo[a]pyrene (B[a]P) assay – representative of small hydrophobic biomarker targets – gave significantly higher signals in organic solvents compared to in water.

Inhibition assays

Inhibition assays were successfully demonstrated for those combinations of antibodies and solvents in Figure 4 where no significant reduction in binding signal was seen. Figure 5 contains examples of both successful (sensitivity and detection limit unaffected) and unsuccessful examples (reduced sensitivity and in some cases no evidence of inhibition).

Table 1: Assay antibody and antigen details

Assay	Antibody	Antigen
Atrazine	Recombinant scAb	Small hydrophilic
B[a]P	Mouse monoclonal	Small hydrophobic
cAMP	Mouse monoclonal	Small hydrophilic
Fluorescein	Rabbit polyclonal	Small hydrophilic
GroEL	Rabbit polyclonal	Protein
LTA	Mouse monoclonal	Polysaccharide

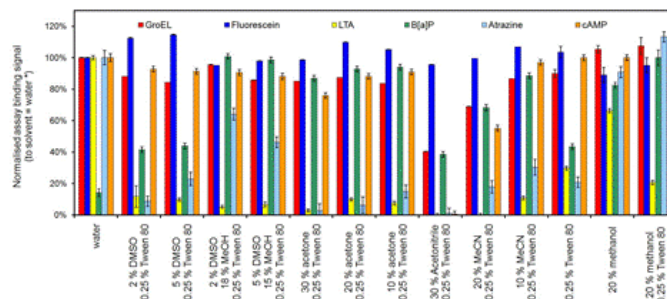


Figure 4: Impact of solvents on antibody binding assay signal intensity for selection of LMC relevant assays *Antibody binding signal normalised vs. water – exception benzo[a]pyrene assay normalised vs. 20% MeOH, 0.25% Tween 80

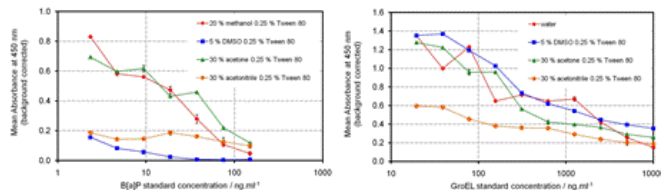


Figure 5: Benzo[a]pyrene (left) and GroEL (right) inhibition assay in a selection of solvents

Summary, Conclusions and Future Work

- Experiment implemented to assess solvent effects on a diverse range of immunoassays – i.e. for antibody variables of species / polyclonal / monoclonal / recombinant and for target variables of small hydrophobic / hydrophilic / polysaccharide / proteins
- Assays showed a diverse response to varied solvents – i.e. some assays unaffected by all/most solvents and other assays exhibited varying performance in different solvents
- Given the limited number of assays tested for each set of variables (antibody and target type) it is not possible to identify underlying trends – i.e. to have a predictive understanding

- The data reported here shows that immunoassays are possible for a wide range of targets in a variety of water / water miscible organic solvents / surfactant mixtures but for LMC selection the assays will have to be individually screened on a case-by-case basis
- Future work to involve confirming transfer of performance to microarray assays and expanding the range of assays for LMC biomarker targets
- Additionally, the intended future use of recombinant antibody techniques will allow selection of antibodies that function optimally in appropriate solvents

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