Evaluation of Enzymatic Techniques for Screening Amphetamines and Alcohol in Oral Fluid

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by

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I would also like to dedicate this thesis to my gran, who sadly lost her battle with cancer during its completion.

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List of Abbreviations

Abbreviation	Definition
AMP	Amphetamine
ATS	Amphetamine type stimulants
CH_3CHO	Acetaldehyde
CV	Coefficient of Variation
DIP	Drug intervention programme
EI	Electron Impact
ELISA	Enzyme linked immunosorbant assay
EQC	External Quality Control
EtOH	Ethanol
GC-FID	Gas chromatography - flame ionisation detector
GC-MS	Gas chromatography mass spectrometry
HPLC	High Pressure Liquid Chromatography
IEC	International Electrotechnical Commisson
IQC	Internal Quality Control
ISO	International Organisation for Standardisation
LC-MS-MS	Liquid chromatography –mass spectrometry – mass spectrometry
LOD	Limit of Detection
Ltd	Limited
MAMP	Methamphetamine
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxy-n-ethylamphetamine

Abbreviation	Definition
MDMA	3,4-methylenedioxymethamphetamine
NAD	Nicotinamide adenine dinucleotide
PFPA	Pentafluoropropionic anhydride
PIT	Preliminary impairment test
QC	Quality Control
RIA	Radioimmunoassay
SD	Standard Deviation
SIM	Selected Ion Monitoring
SPE	Solid phase extraction
THC	Tetrahydrocannabinol
THC-COOH	11-*nor*-9-carboxy-delta-9-tetrahydrocannabinol
TMB	3, 3', 5, 5' tetramethylbenzidine
UK	United Kingdom
UKAS	United Kingdom Accreditation Service
UKNEQAS	United Kingdom National External Quality Assessment Service

Abstract

Evaluations of the Immunalysis enzyme linked immunosorbent assay (ELISA) and Immunalysis enzymatic assay for ethyl alcohol were undertaken to evaluate their suitability for screening drugs of abuse (namely amphetamine and methamphetamine) and alcohol in oral fluid samples collected with the Quantisal Collection Device. Multi-analyte controls were prepared for the drugs of abuse screen and diluted with Quantisal buffer prior to analysis to match the dilution in the Quantisal Oral Fluid Collection Device that was used to collect the samples. These samples were analysed over time to evaluate stability and case samples were analysed to evaluate sensitivity and specificity.

Alcohol calibrators and controls were evaluated for linearity and stability before being applied to case samples to evaluate the sensitivity and specificity of the method.

The amphetamine assay was found to be highly sensitive and specific. The methamphetamine assay was found to be highly specific but no positive samples were analysed so the sensitivity could not be evaluated. The multi-analyte controls were found to be stable over a fourteen month period. The Immunalysis ELISA assays were found to be suitable for screening oral fluid samples.

The alcohol assay was found to be linear over the 0 – 300mg/dL range and the calibrators and controls were found to be stable over time. The assay was found to be highly sensitive and specific and best suited to high throughput laboratories expecting mainly negative samples. However, it would not be cost effective for smaller laboratories or those expecting a high number of positives, where going straight to confirmation by head-space gas chromatography with flame ionisation detection would be recommended.

1 Introduction

1.1 Background

Toxicology is the study of poisons and their effects, and has a wide range of applications in medicine, law and sport. A poison is defined as any substance, which either formed in the body or taken into the body can cause an impairment of health (1, 2). This allows for the fact that almost all substances (including everyday essentials such as water and oxygen) can act as poisons and impair health if a high enough dose is taken (2). Other substances such as cyanide can be fatal even if only a small dose is received. Paracelsus reported this fact in the early 16th century noting that even medicinal substances could be poisonous if a large quantity was consumed.

"Alle Dinge sind ein Gift und nichts ist ohne Gift,
nur die Dosis bewirkt, daß ein Ding kein Gift ist."

"Poison is in everything, and nothing is without poison.

The dosage makes a thing not a poison."

Paracelsus, 1493-1541

Quotation courtesy of The Handbook of Pesticide Toxicology: Principles (3)

The dose required for toxic effects to be produced is known as the toxic dose but the actual quantity involved varies between individuals according to a number of factors. These include height, weight, sex, age, body water content, health, previous exposure to the drug, tolerance, mood at time the drugs were taken, effects of other drugs and the route of administration.

Over the years, many matrices have been used to analyse for drugs and poisons in humans with tissues such as the liver, which have higher concentrations than the matrices commonly used today, being used for post mortem toxicology. Blood and urine are presently the most common matrices used in forensic toxicology for drugs of abuse testing. This is because these matrices are readily available and there is a lot of published information about typical concentrations of drugs found, thus aiding interpretation. Other "alternative" matrices which can be used in post mortem

toxicology include the brain, liver, lung, muscle, stomach contents, bile, vitreous humour, hair, oral fluid, sweat and nail clippings. Over the last few decades, advances in technology has seen better sensitivity of instrumentation and this has brought hair and oral fluid testing, which typically have lower concentrations of drugs than the traditional matrices, to the front of the field.

This project will look at the suitability of oral fluid as a testing matrix for the screening and subsequent confirmation of specifically alcohol and amphetamines.

1.2 Oral Fluid

1.2.1 Anatomy of Saliva Glands

The secretion product of the head and mouth salivary glands is commonly known as saliva. Between half a litre and one and a half litres of saliva is produced daily from these glands (4-7). Saliva collected from the mouth also contains small amounts of gingival crevicular fluid, cellular debris and blood. Saliva is composed of 99% water, 0.3% protein (largely amylase) and 0.3% mucins (5, 8). Saliva glands comprise two regions, the acinar region which contains the cells capable of secretion and the ductal region lined with water impermeable cells that carry the secretions to the outlets in the mouth (8, 9).

Saliva is produced from three main glands and many minor glands (7). Under resting conditions 70% is produced from the submandibular glands, 25% is produced by the parotid glands and the other 5% is produced from the sublingual glands and the other minor glands that produce saliva. When stimulated about 50% comes from the parotid glands (4, 7, 8). The main salivary glands are shown in Figure 1 courtesy of The Free Dictionary.

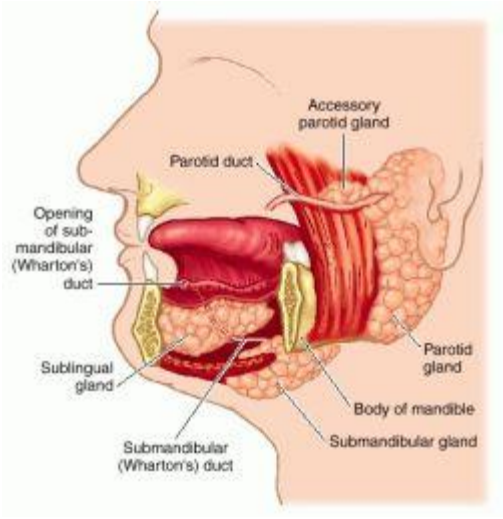


Figure 1: The Saliva Glands courtesy of The Free Dictionary (10)

Under resting conditions, saliva has a typical pH of 6.8 (typical range pH 5.6 – 7.9) (8, 11). When stimulated, saliva is excreted faster and becomes more basic and approaches the pH of plasma (4, 8, 11).

In 1993, the New York Academy of Sciences meeting on saliva testing decided to differentiate between saliva taken from the mouth and that taken directly from the saliva glands. As the fluid in the mouth is a mixture of the excretion products from the various glands in the mouth and cellular debris it was decided that saliva should be used to describe the glandular secretions taken directly from the saliva glands and that oral fluid should be used to describe the fluid taken from the mouth by either expectoration or by placing absorbents in the mouth (5, 8, 11, 12).

1.2.2 Advantages of Oral Fluid Testing

The main advantage of oral fluid as a matrix for drug testing is that collection is simple and non-invasive and samples can be collected under observation. The collection of an oral fluid sample can be carried out by the individual themselves by swabbing the inside of their mouth with a cotton swab or by expectorating (spitting) into a sample vial. Suction and draining of oral fluid from the mouth have also been used to collect samples (8, 11, 12). This can be done quickly and on site, which is beneficial for both the individual concerned and the individual collecting the sample (13). In general, people do not like providing blood, urine or hair samples for analysis

as they feel this is an invasion of their privacy (8). Urine samples can be easily adulterated or switched to avoid the detection of drugs, as the sample collection cannot be easily witnessed. This is not the case with oral fluid as there is a waiting time before sample collection in which any adulterants in the mouth will have been swallowed, diluted or expectorated (8). Also, the fact that sample collection is witnessed means the possibility of switching the sample to a sample known to be free from drugs is greatly reduced.

Oral fluid provides information about recent drug use (i.e. drugs taken within a few hours of the sample being collected and up to 48 hours after use for some drugs) due to the short window of detection. As a consequence, oral fluid provides a good indication of the drugs that were present in the blood stream at the time of collection and therefore has the potential to provide information relating to the effects the individual was experiencing at the time of collection (8).

Another advantage is that the concentration of drugs in oral fluid can be related to the concentration of drugs in plasma. Drugs found in oral fluid are typically the non-ionised, unbound parent drug. Since it is the free lipophilic drug and drug metabolites that can cross cell membranes, such as the blood – brain barrier, and cause physiological effects, free drug concentrations in plasma and in oral fluid can potentially be correlated with drug effects (5). Cone *et al* (14) found that for cocaine, the saliva concentrations correlated well with effects. In a separate study, Cone *et al* (15) stated that amphetamine in oral fluid parallels the plasma drug concentration. In contrast, many papers state that there is no correlation between oral fluid and plasma drug concentrations. Schepers *et al* (16) found a poor correlation between the oral fluid and plasma concentrations for amphetamine and methamphetamine. The results supported those from an earlier study by Cook *et al* (17). In 2007, Willie *et al* (18) carried out a multi – drug study on oral fluid: blood ratios from drivers suspected of driving under the influence of drugs and found the ratios to be highly variable.

1.2.3 Disadvantages of Oral Fluid Testing

As oral fluid is a biological fluid, it has the potential to transmit infectious diseases. Thus samples need to be handled with care like other biological fluids such as blood

and urine. Saliva production is reduced by some drugs, both prescription drugs (such as amitryptaline and paracetamol) and illicit drugs (such as amphetamine), drugs which block the central nervous system, and also by some medical conditions, including stress and diabetes (5, 7, 8, 11).This can make collection of oral fluid samples from individuals who fall into these categories difficult. There are also some individuals who are repulsed by spitting and thus rules out the use of expectoration methods for the collection of oral fluid.

Although this is also classed as an advantage, another drawback with oral fluid testing is the short window of detection (8). Drugs with short half lives or those which are rapidly metabolised will not be detectable in oral fluid for a long period of time, as it is generally the parent drug that is found in oral fluid. As a result, in cases of those suspected of being under the influence of drugs it is vital that the oral fluid sample is collected as soon as possible to maximise the chance of detection. The generally accepted window of detection of oral fluid is from the time of administration to approximately four half lives after it enters the body (5). The half-life of a drug is the time taken for its concentration to decrease by a half.

One of the biggest drawbacks with oral fluid testing is the small volume of sample collected (8). Many collection devices will only allow approximately 1mL of sample to be collected which, if many analyses are required, can present a problem. Many collection devices also dilute this 1mL of sample with buffer which can present a problem if the drugs are present at a low concentration, as it may be approaching the limit of detection of the analytical instrumentation. This problem was recently highlighted in a study by Gjerde *et al*, who carried out a large scale drug and alcohol study in Norway using the Statsure oral fluid collection device (19). This device used a collection pad to collect (up to) 1mL of oral fluid and diluted it with 1mL buffer. The authors reported that they were unable to recover 1mL of the oral fluid/buffer mixture meaning in most cases they had less than 1mL of sample to analyse (19).

Another disadvantage relates to recent administration of a drug in oral form as residue from smoking or small fragments of the drug may remain in the mouth and as such will contaminate the oral fluid sample and give a much higher concentration of the drug than is actually present (11).

1.2.4 Applications of Oral Fluid Testing

With drug testing becoming more widespread, companies such as those involved in drug maintenance programs, many private companies who offer drug testing for employers and the police (roadside and as part of the drug interventions program (DIP)), have been looking to move towards oral fluid testing as a method for drug testing as it is easier to collect than other matrices. The police for example can collect an oral fluid sample at the roadside rather than having to take the suspect to the station to provide a blood or urine sample which wastes time and money. The DIP programme tests those arrested for certain offences and aims to identify those taking drugs and giving them the option of deferring a prison sentence by enrolling them in drug treatment programmes. The test results from the DIP programme are not used in court against the suspect. As part of health and safety regulations employers are able to test employees for the presence of alcohol or drugs only if consent is given and there is a genuine reason for the test (20). Employers are required to have a policy on drug and alcohol testing if they wish to carry out such tests. Not all companies have such a policy in place, but areas such as the transport and manufacturing sectors, where intoxication could endanger the lives of others, are most likely to have one in place.

While a policy on workplace drug testing or consent is required for drug testing in most cases, it is a legal requirement in certain areas, such as the public transport industry. The Transport and Works Act 1992 (21) states that it is an offence for anyone working on public transport systems to be intoxicated while at work.

As a result of the current recession, many companies which have not had a legal requirement to carry out workplace testing have started to test employees in an effort to dismiss them from their job without redundancy pay in a bid to save costs (22). Many people argue that an employee's human rights to privacy may be breached if testing is carried out using urine and hair, as they have longer windows of detection and as such do not reflect what, if any, effects the person is currently experiencing whilst at work. The longer windows of detection from these matrices could detect any recreational use of drugs out-with working hours that can be argued, are not affecting the person during work hours.

Drug maintenance programmes are used to monitor a person's abstinence from drugs. This may be for many reasons with child custody cases and conditions of bail being amongst the most common reasons. Drug maintenance programmes are also used to monitor those on the methadone programme to ensure that they are not continuing to abuse heroin while on methadone and to ensure that they are indeed taking their methadone and not selling it to provide money for other drug habits.

The police in England and Wales also use oral fluid drug testing as a way of getting drug users who have been arrested for trigger crimes into treatment. Tests are routinely carried out on those who are arrested for petty crimes such as burglary or theft as part of the drug interventions programme (DIP) (23). This initiative aims to reduce crime carried out by drug abusers by getting them into treatment and the indications are that it has been working with a report published in November 2007 indicating a 26% reduction in crime by those entering DIP (24).

To test for alcohol intoxication at the roadside, the police use a hand-held breathalyzer, however there is currently no equivalent hand-held device approved to test for drug use at the roadside. Several collection devices have been tested over the years but as yet none has been deemed acceptable for roadside testing (11). The preliminary impairment test (PIT) is used to determine if a person is unfit to drive through drug intoxication and involves a series of simple tests and the measurement of pupil size. Technology is constantly evolving and in 2008 a British-based company announced they would be releasing a new handheld oral fluid drug testing device (25). The device will be unveiled in November 2010 and will undergo performance evaluation tests before being made commercially available but the manufacturers hope that the device will meet any criteria set out by the British government for roadside drug testing devices (26). The North Review, published by the Department of Transport in June 2010, states that the government is looking to implement a device that can detect drugs in oral fluid (27). British police were given the power to carry out preliminary impairment tests on drivers suspected of using drugs in 2003, but as yet no suitable device for such a test exists. Section 6C of the Railways and Transport Safety Act 2003 (28) states that:

"A preliminary drug test is a procedure by which a specimen of sweat or saliva is—

(a) obtained, and

(b) used for the purpose of obtaining, by means of a device of a type approved by the Secretary of State, an indication whether the person to whom the test is administered has a drug in his body."

The Home Office Scientific Development Branch stated that "*there is currently no type approval specification for roadside screening devices to detect drugs and so they cannot be used for enforcement purposes*" and that they were working with external agencies on the specification and design of a suitable device (29). They went on to state that "*it will be a couple of years before our multi-drug device is available and type-approved for use as the scientific development work behind it is highly complex*" (29).

1.3 Abuse of Drugs and Alcohol

Drug and alcohol abuse is a growing problem in the United Kingdom (30, 31). Alcohol is the most commonly abused substance in the United Kingdom although drugs such as cannabis are also widely abused. According to the world drug report 2009, Scotland was the amphetamine abuse capital of Europe with 2.2% of the population (2006 data) abusing amphetamines and amphetamine type stimulants (ATS) (excluding ecstasy) (32). Recently published data in the world drug report 2010 indicates that the Czech Republic has now overtaken Scotland as the amphetamine and amphetamine type stimulants (ATS) (excluding ecstasy) capital of Europe, as use in Scotland has dropped to 1.4% (2009 data) (33). Scotland also has the second highest rate of ecstasy abuse in Europe at 2.5% of the population (2009 data), down from 3.2% in 2006, with only the Czech Republic having a higher rate at 3.6% (2008 data) (3.5% in 2004) (32, 33). Scotland has a higher rate of amphetamine and ATS abuse than England and Wales (1.4% in Scotland compared to 1.0% in England and Wales (2009 data)) (33). Scotland also has a higher rate of ecstasy abuse than England and Wales (2.5% in Scotland compared to 1.8% in England and Wales (2009 data)) (33).

Alcohol related deaths in the United Kingdom have doubled between 1991 and 2007 according to the office for national statistics (31) as illustrated in Figure 2.

In 2007 there were 1,399 deaths which listed alcohol as the underlying cause of death in Scotland with a further 966 listing alcohol as a contributory cause of death

(34). 68.5% (959 of the 1,399 deaths) (34) of those who died as a result of an alcohol related illness were male. The Glasgow area had the highest death rate due to alcohol for both sexes (34) with the death rate for Scottish males double that of the rest of the United Kingdom (35).

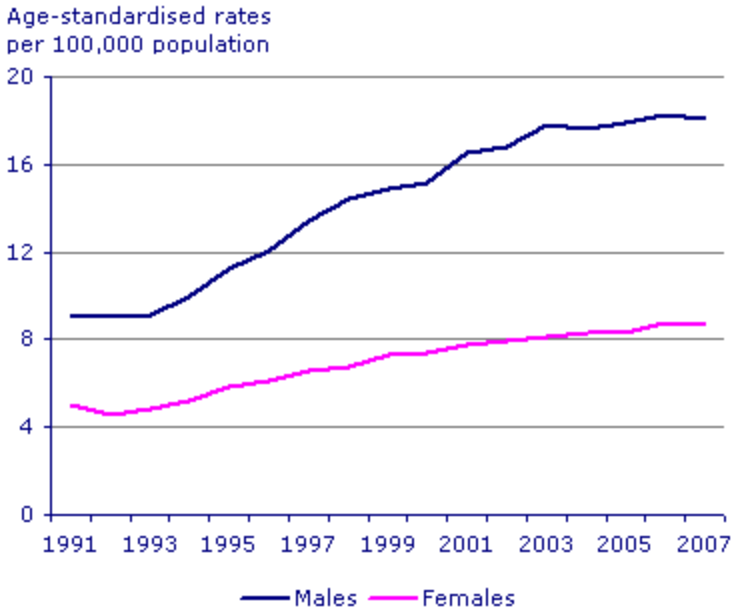


Figure 2: Alcohol Related Death Rates by sex in the United Kingdom 1991 – 2007 (31)

Drug related deaths in Scotland have doubled since 1996 according to the General Register Office for Scotland (30) as illustrated in Figure 3.

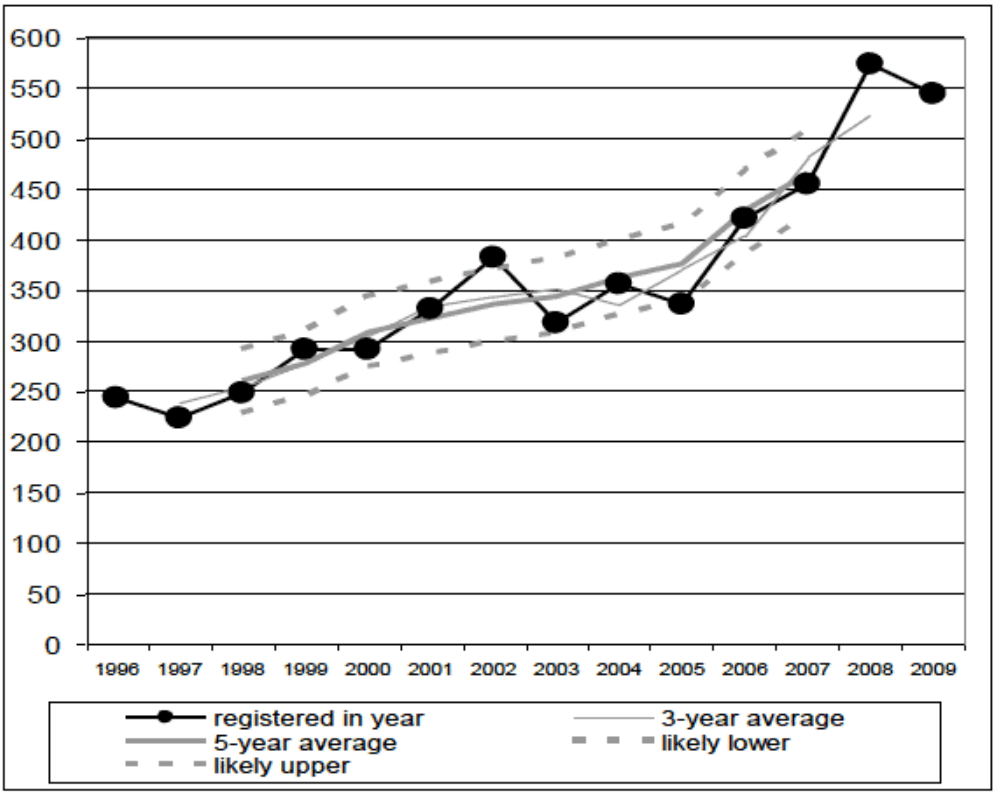


Figure 3: Drug Related Deaths in Scotland 1996 – 2009 (30)

Figure 3 shows a fairly steady increase in drug related deaths since 1996.

1.4 Screening Methods

1.4.1 Drugs of Abuse

Enzyme Linked ImmunoSorbent Assay (ELISA) is one of the most common screening techniques for drugs of abuse and is the screening method chosen for this project. Using an enzymatic method, such as ELISA to screen samples for drugs of abuse is a quick and relatively cheap method to eliminate negative samples from the more expensive and time consuming confirmatory tests (30, 31).

ELISA works by having microplate wells coated with an antibody specific to the drug that is being looked for. The sample is added along with an enzyme labelled drug. The samples are incubated in the dark for a set period of time. The drug and enzyme labelled drug compete for binding sites on the antibody during the incubation period. The enzyme conjugates typically contain azide free preservatives as the presence of azides may interfere with the antigen – antibody interactions and produce erroneous results. The plates are washed several times with water to remove any unbound materials and a substrate is added which allows a colour to develop in proportion to

the amount of enzyme present (and inversely proportional to the concentration of drug in the sample well). The samples are incubated for a set period of time in the dark. After this incubation period, a stop solution (typically an acid) is added to destroy any unbound substrate and prevent further reaction. The colour of the solution is changed by the addition of the stop solution. The absorbance of the plates is then read. The process taking place in the wells is shown in Figure 4.

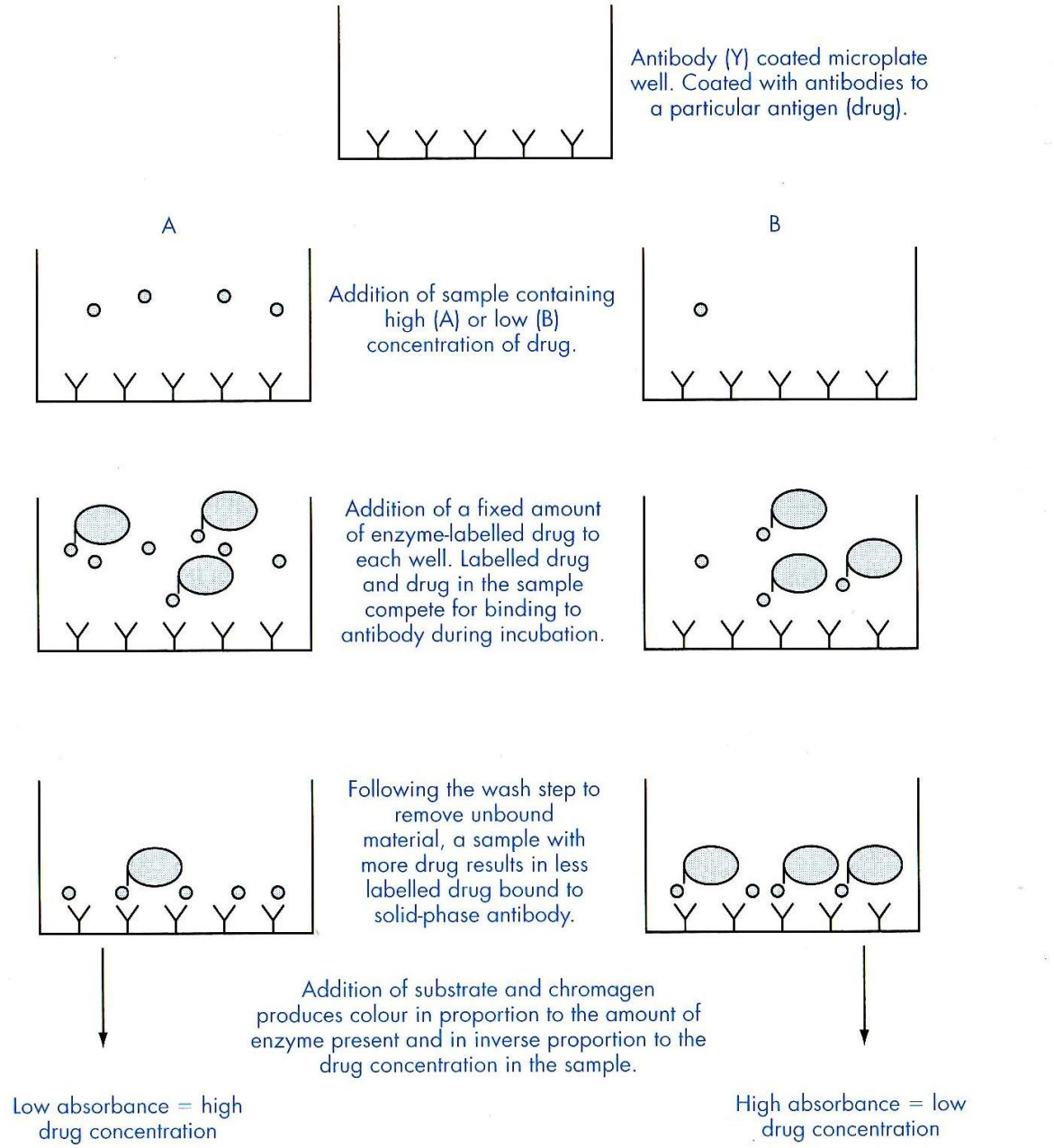
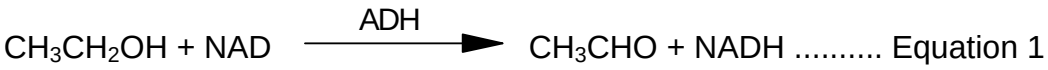


Figure 4: Principle of ELISA Courtesy of Pharmaceutical Press (36)

1.4.2 Alcohol

The Immunalysis Corporation (Pomona, CA) oral fluid alcohol assay works on the basis of alcohol dehydrogenase's (ADH) high affinity for reaction with ethanol in the presence of nicotinamide adenine dinucleotide (NAD) as shown in the equation below**.**



This simple method works by adding buffer and an enzyme which contains NAD and ADH to the sample and incubating in the dark for a set period of time. During this time, a colour develops in the wells. The absorbance of the plates is read following the incubation.

1.4.3 Other Screening Methods

A number of other screening methods are available with hand-held screening devices becoming popular with the police. Laboratory based techniques such as radioimmunoassay (RIA) (which has now been replaced by ELISA) (36) and liquid chromatography – mass spectrometry – mass spectrometry (LC-MS-MS) can also be used. LC-MS-MS appears to be the laboratory based screening method of the future as it offers the advantage over current screening methods by being able to identify specific drugs rather than just the drug group (37). It can also analyse for several hundred drugs in a single analysis (38). This technique can also be used semi-quantitatively which is advantageous if only a limited sample is available for analysis. It would also allow for any sample which may require a dilution prior to analysis to be identified at the screening stage, thus speeds up the confirmation step and saves wasting sample by extracting an undiluted sample when the drug will be off scale. The disadvantage of this technique is the cost and run time as multiple drug standards would be needed and a lot of validation work to set up the method for all of the analytes each particular laboratory is interested in.

1.5 Confirmatory Methods

1.5.1 Drugs of Abuse (Amphetamines)

For this project, the presence of amphetamines in oral fluid was confirmed by gas chromatography mass spectrometry (GC-MS) following solid phase extraction (SPE) to remove the bulk of the impurities in the sample. Liquid chromatography mass spectrometry (LC-MS) is also frequently used to confirm the presence of amphetamines. SPE is an extraction technique used to remove impurities from

samples to stop them interfering with the chromatography on the instrument. Cartridges have a silica based packing material and the analytes of interest bind to it allowing the impurities to be washed out. The analytes of interest are then selectively eluted without the impurities. In the case of amphetamines the samples were derivatised (in this instance, with pentafluoropropionic anhydride (PFPA)) to improve the chromatography. The internal standards, used to quantify the drug concentrations, were deuterated analogues of each of the analytes of interest. Deuterated standards will have similar retention times as the standards themselves and allows for easier identification.

1.5.2 Alcohol

The presence of alcohol in oral fluid samples was confirmed by using headspace gas chromatography with a flame ionisation detector (GC-FID). For this project, the standards and controls were diluted with Quantisal buffer (one part sample, three parts buffer) to match the dilution in the oral fluid collection device. A semi-automatic diluter was used to dilute the standards, controls and case samples with internal standard in clearly labelled headspace vials. All samples were analysed in duplicate. The vials were capped and placed in the carousel for analysis by headspace GC-FID.

1.6 Quality Control

Although there is no formal requirement for drug testing laboratories in the United Kingdom to have accreditation to the international testing standard, ISO/IEC 17025, many laboratories that carry out the testing do have this accreditation, however, only a limited number have accreditation for oral fluid testing. As part of quality control measures, there are various proficiency testing schemes in operation in the UK which accredited laboratories must participate in. However, these generally focus on the traditional matrices used in forensic toxicology such as blood and urine. As part of the proficiency testing scheme, spiked samples are sent to the accredited laboratories to ensure that they are correctly identifying the drugs present and accurately quantifying the concentrations in the sample. The concentration of the drugs in the sample is not given to the testing laboratories until after they have

submitted their results for the samples. Samples similar to this are commercially available as external quality controls (EQC's) but these samples detail the concentrations of the drugs present. At present, the EQC's are widely available for traditional matrices such as blood and urine with only a limited number being available for other matrices, such as oral fluid, and thus making internal quality controls (IQC's) very important in oral fluid testing. IQC's are control samples for the desired analytes that are prepared in-house to a known concentration and serve the same purpose as the EQC's. In order to gain accreditation to ISO/IEC 17025, laboratories must meet certain requirements. These include the validation of methods to ensure they are robust and fit for purpose, in addition to having standard operating procedures for all aspects of the testing process. A quality control system should be implemented and as there are currently no external quality control schemes available for oral fluid, the quality control samples must therefore be prepared in-house. The United Kingdom National External Quality Assessment Service (UKNEQAS) has a proficiency testing scheme that is currently being piloted for oral fluid testing but this is only at the developmental stage and has not been fully rolled out as yet. Schewart style quality control charts will be used to monitor the results of the IQC's and EQC's.

1.7 Quantisal Oral Fluid Collection Device

The Quantisal oral fluid collection device was used to collect oral fluid samples for this project. The device has a pad with a volume adequacy indicator that turns blue when 1mL of oral fluid has been collected. The pad is then stored in 3mL of Quantisal buffer to give a total volume of 4mL. Filters can be used to squeeze the fluid out of the pad and allow the oral fluid/buffer mixture to be transferred to labelled vials prior to analysis. A study by Langel *et al* showed the Quantisal device had recoveries in excess of 80% for amphetamine, 3,4-methylenedioxymethamphetamine (MDMA) and ethanol (39).

1.8 Aims and Objectives

The aims of this project were to evaluate a commercially available ELISA assay for drugs of abuse, in particular, amphetamine and methamphetamine in oral fluid and to evaluate a commercially available enzymatic assay for ethanol in oral fluid.

To evaluate the ELISA assay, multi-analyte controls were prepared and analysed over time to evaluate the stability of the controls and assess the suitability of the assays. To make the method more time and cost effective, the oral fluid samples were evaluated using the same ELISA method that is presently used in-house for other matrices (e.g. blood and urine) to allow oral fluid samples to be screened within the same batch as other matrices. The method was then applied to case samples collected with a commercially available oral fluid collection device and any amphetamine or methamphetamine positives will be confirmed using the in-house confirmation method of analysis by gas chromatography mass spectrometry (GC-MS). Some negative samples were also confirmed to allow the sensitivity and specificity of the assay to be evaluated. Confirmations for other drug groups were not carried out during this project as there is not a validated in-house method for these drugs in oral fluid.

To evaluate the enzymatic assay for ethanol in oral fluid, ethanol controls were prepared and analysed over time to evaluate the stability of the controls and assess the suitability of the assay. The method was then applied to case samples collected with a commercially available oral fluid collection device and any positives confirmed for alcohol using the in-house confirmation method of analysis by headspace gas chromatography flame ionisation detector (headspace GC-FID). Some negative samples were also confirmed to allow the sensitivity and specificity of the assay to be evaluated.

2 Evaluation of Multi-Analyte Oral Fluid Controls Using Immunalysis ELISA

2.1 Materials and Reagents

The following materials and reagents were used in this project.

2.1.1 Enzyme Linked Immunosorbent Assay (ELISA) Test Kits

Drugs of abuse testing kits for each of the drugs of interest were manufactured by Immunalysis Corporation (Pomona, CA) and purchased from their UK distribution company, Agriyork 400 Ltd (Pocklington, UK). The product code for each kit is detailed in Table 1. Each kit contained all the necessary reagents for the analysis, including an enzyme conjugate, a substrate solution and a stop solution. The substrate solution for all of the assays was 3, 3', 5, 5' tetramethylbenzidine (TMB) and urea peroxidase in buffer. The stop solution (1M hydrochloric acid) was the same for all assays. The enzyme conjugate for each assay is different and detailed in Table 1. The manufacturer's specification for each assay is given in Appendix 2 – ELISA Assay Specification.

The calibrators were diluted with 100mM phosphate buffer solution (product code: PBS-1000) and Quantisal dilution buffer (product code: EXTBUF-1000) was used to dilute oral fluid controls. These buffers were also manufactured by Immunalysis Corporation (Pomona, CA) and purchased from their UK distribution company, Agriyork 400 Ltd (Pocklington, UK). The assays and buffers were stored at or below 8°C in the refrigerator.

Table 1: Product Codes and Enzyme Conjugates for ELISA Kits

Drug Group	Product Code	Enzyme Conjugate*
Amphetamine	209-0480	S-(+)-amphetamine
Benzodiazepines	214-0480	Benzodiazepine derivative
Buprenorphine	236-0480	Buprenorphine derivative
Cannabinoids	205-0480	THC-COOH derivative**
Cocaine	206-0480	Benzoylecgonine derivative
Methadone	232-0480	Methadone derivative
Methamphetamine	211-0480	S-(+)-methamphetamine
Opiates	207-0480	Morphine derivative

* Enzyme conjugates are labelled with horseradish peroxidase in a buffered, protein solution with stabilizers at pH 7.6 and contain azide free preservatives. The solutions are dyed pink for clarity.

** The cannabinoid enzyme conjugate (11-*nor*-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH)) is buffered at pH 8.5.

2.1.2 Drug Standards

2.1.2.1 **Drug Standards for ELISA**

The drug standards used for this project were manufactured by Cerilliant (Round Rock, TX) and purchased from LGC Standards (Teddington, UK). The details of the drug standard including the concentration and product code are found in Table 2. Certificates of analysis were provided with each drug standard.

Table 2: Drug Standards for ELISA

Drug Standard	Concentration*	Product Code
S-(+)-Amphetamine	1mg/mL	A-008
Oxazepam	1mg/mL	O-902
Buprenorphine	0.1mg/mL	B-902
(-)-11-nor-9-carboxy-delta9-THC	0.1mg/mL	T-018
Benzoylecgonine	0.1mg/mL	B-007
(+/-)-Methadone	0.1mg/mL	M-019
S-(+)-Methamphetamine	1mg/mL	M-020
Morphine	0.1mg/mL	M-030

* All drugs come in sealed vials at the stated concentration in 1mL of methanol.

2.1.2.2 **Drug Standards for GC-MS**

The drug standards used for this project were manufactured by Cerilliant (Round Rock, TX) and purchased from LGC Standards (Teddington, UK). The details of the drug standard including the concentration and product code are found in Table 3. Certificates of analysis were provided with each drug standard.

Table 3: Drug Standards for Amphetamine Confirmations

Drug Standard	Concentration*	Product Code
(±)-Amphetamine	1mg/mL	A-007
(±)-Methamphetamine	1mg/mL	M-009
(±)-MDA	1mg/mL	M-012
(±)-MDMA	1mg/mL	M-013
(±)-MDEA	1mg/mL	M-065

* All drugs come in sealed vials at the stated concentration in 1mL of methanol.

2.1.2.3 **Internal Standards for Amphetamine Confirmations**

The deuterated drug standards used for this project were manufactured by Cerilliant (Round Rock, TX) and purchased from LGC Standards (Teddington, UK). The details of the deuterated drug standard including the concentration and product code are found in Table 4. Certificates of analysis were provided with each drug standard.

Table 4: Internal Standards for Amphetamine Confirmations

Drug Standard	Concentration*	Product Code
(±)-Amphetamine-d11	100µg/mL	A-016
(±)-Methamphetamine-d14	100µg/mL	M-092
(±)-MDA-d5	100µg/mL	M-010
(±)-MDMA-d5	100µg/mL	M-011
(±)-MDEA-d6	100µg/mL	M-081

* All drugs come in sealed vials at the stated concentration in 1mL of methanol.

2.1.3 Collection of Blank Oral Fluid

As commercially available collection devices dilute the sample in differing volumes of buffer, it was decided to collect blank oral fluid by expectoration, as neat oral fluid would allow the flexibility of diluting samples by an appropriate factor at a later stage

to match the dilution factor in the collection device of the sample in question should the sample be collected with a different collection device. Neat oral fluid was collected from a single donor who had not taken any of the drugs included in the analysis or those closely related to them prior to sample collection. Collection of oral fluid was carried out in one day by expectorating approximately 300mL into a beaker. The oral fluid was transferred to a large storage bottle and stored in the freezer until required.

2.1.4 Preparation of Calibrators

2.1.4.1 Preparation of Calibrators for ELISA

As the in-house calibrators for this assay were also used for the routine blood and urine analysis which was accredited to ISO/IEC 17025, the calibrators were prepared by a member of technical staff to comply with the accreditation. The calibrators were prepared in the following way: The levels were prepared from stock solutions of the drugs at a higher concentration than required and diluted to the required concentration with 25mL of water. The concentration of each drug in the four levels used for the calibration is shown in Table 5. The calibrators were stored in amber bottles at or below 8°C in the refrigerator. The calibrators were diluted with buffer prior to use.

Table 5: Preparation of ELISA Calibrators

Drug	Calibrators (ng/mL)				Cut-off (ng/mL)
	Lv1	Lv2	Lv3	Lv4	
Amphetamine	0	25	100	500	25
Benzodiazepines	0	10	60	300	10
Buprenorphine	0	5	20	100	5
Cannabinoids	0	2	10	50	2
Cocaine	0	10	60	300	10
Methadone	0	5	20	100	5
Methamphetamine	0	25	100	500	25
Opiates	0	10	60	300	10

2.1.4.2 **Preparation of Standards for Amphetamine Confirmations**

The mixed amphetamine standard was prepared by adding 1mL of each of the drug solutions detailed in Table 3 to a single 100mL volumetric flask and diluting to the mark with methanol. The solution was inverted several times to ensure the solution was thoroughly mixed and transferred to a labelled storage bottle. This is the stock solution and was stored in the freezer at or below -18^{o}C. To make the working solution, 1mL of the stock solution was added to a 10mL volumetric flask and it was made up to the mark with methanol. The solution was inverted several times to ensure the solution was thoroughly mixed and transferred to a labelled storage bottle. This is the working solution and was stored in the fridge between 3-8^{o}C.

2.1.4.3 **Preparation of Internal Standard for Amphetamine Confirmations**

To prepare the amphetamine internal standard, 1mL of each of the solutions detailed in Table 4 was added to a single 10mL volumetric flask and diluted to the mark with methanol. The solution was inverted several times to ensure the solution was thoroughly mixed and transferred to a labelled storage bottle. This is the internal standard stock solution and was stored in the freezer at or below -18^{o}C. To make the

working solution, 1mL of the stock solution was added to a 10mL volumetric flask and it was made up to the mark with methanol. The solution was inverted several times to ensure the solution was thoroughly mixed and transferred to a labelled storage bottle. This is the working internal standard solution and was stored in the fridge between 3-8^{o}C.

2.1.5 Preparation of Controls

2.1.5.1 **Preparation of Controls for ELISA**

A number of solutions were prepared containing the drugs of abuse at appropriate concentrations. 1mL of each drug solution was pipetted from the vial into the appropriate volumetric flask (in the case of oxazepam this was after a 1 in 10 dilution) and made up to the mark with blank oral fluid. Some solutions had more than one drug present as the controls were to be prepared with the drugs at the same concentration. The concentration of the solutions prepared along with the volume of the spiked solution added to the controls is indicated in Table 6. The controls were then pipetted into clearly labelled small vials and stored in the freezer until required.

Table 6: Preparation of in-house ELISA Controls

Drug Standard (Concentration)	Working Solution (µg/mL)	Cut-off (ng/mL)	Control (ng/mL)		Spike Volume (µL)	
			- 50%	+ 50%	- 50%	+ 50%
S-(+)-Amphetamine (1mg/mL)	100	25	13	38	13	38
S-(+)-Methamphetamine (1mg/mL)						
Oxazepam (1mg/mL)*	10	10	5	15	50	150
Benzoylecgonine (100µg/mL)						
Morphine (100µg/mL)						
+/- Methadone (100µg/mL)	10	5	3	8	30	80
Buprenorphine (100µg/mL)						
THC-COOH (100µg/mL)	10	2	1	3	10	30

* A one in ten dilution was required for oxazepam prior to making the mixed solution with benzoylecgonine and morphine.

Amphetamine and methamphetamine formed one of the drug mixtures, oxazepam, morphine and benzoylecgonine formed another and methadone and buprenorphine formed the third. The cannabis solution was not part of a mixed drug solution.

2.1.5.2 **Preparation of Controls for Amphetamine Confirmations**

A separate bottle of amphetamine stock solution (10µg/mL) was prepared as described above and marked as for controls only. From this solution, 0.45mL is added to a 100mL volumetric flask and made up to the mark with blank oral fluid. This gives a control spiked at 45ng/mL. The solution was inverted several times to ensure it was thoroughly mixed and 1.2mL aliquots were transferred to screw cap vials which were labelled as amphetamine oral fluid controls and stored in the freezer until required.

2.1.6 Equipment

Samples were washed using an MRX plate washer and analysed using an MRX microplate reader using a 450nm filter, all of which were purchased from Dynex Technologies (Chantilly, VA). Revelation software version 4.25 was used to process the results.

The pipettes used in this project were calibrated by the United Kingdom Accreditation Service (UKAS) to ISO/IEC 17025 standard. Standards were prepared in volumetric flasks provided by Fisherbrand (Leicestershire, UK).

The samples were analysed by GC-MS using an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass spectrometer. The system was automated using an Agilent 7683B series auto-sampler and processed using MSD Chemstation software (version G1701EA E.02.00.493). The gas chromatograph was fitted with a DB-5MA + DG column (30m x 0.25mm x 0.25µm). All GC-MS related components were purchased from Agilent Technologies (Berkshire, UK).

2.1.7 Case Samples

Case samples for this project were provided from three sources. Quantum Diagnostics (Essex, UK) provided approximately 100 samples for this project. The Centre for Drug Misuse Research based at the University of Glasgow (Glasgow, UK) provided approximately 210 samples for this project. Nine further samples were analysed as part of the United Kingdom National External Quality Assessment Service (UKNEQAS) proficiency testing scheme for oral fluid.

2.2 Methods

2.2.1 Method of Analysis (ELISA)

The flowchart below shows the method for the diluted oral fluid controls which were generally used. An initial comparison of neat controls was done and the oral fluid QC's were not diluted with 750µL Quantisal buffer. The method used for ELISA is detailed in Figure 5.

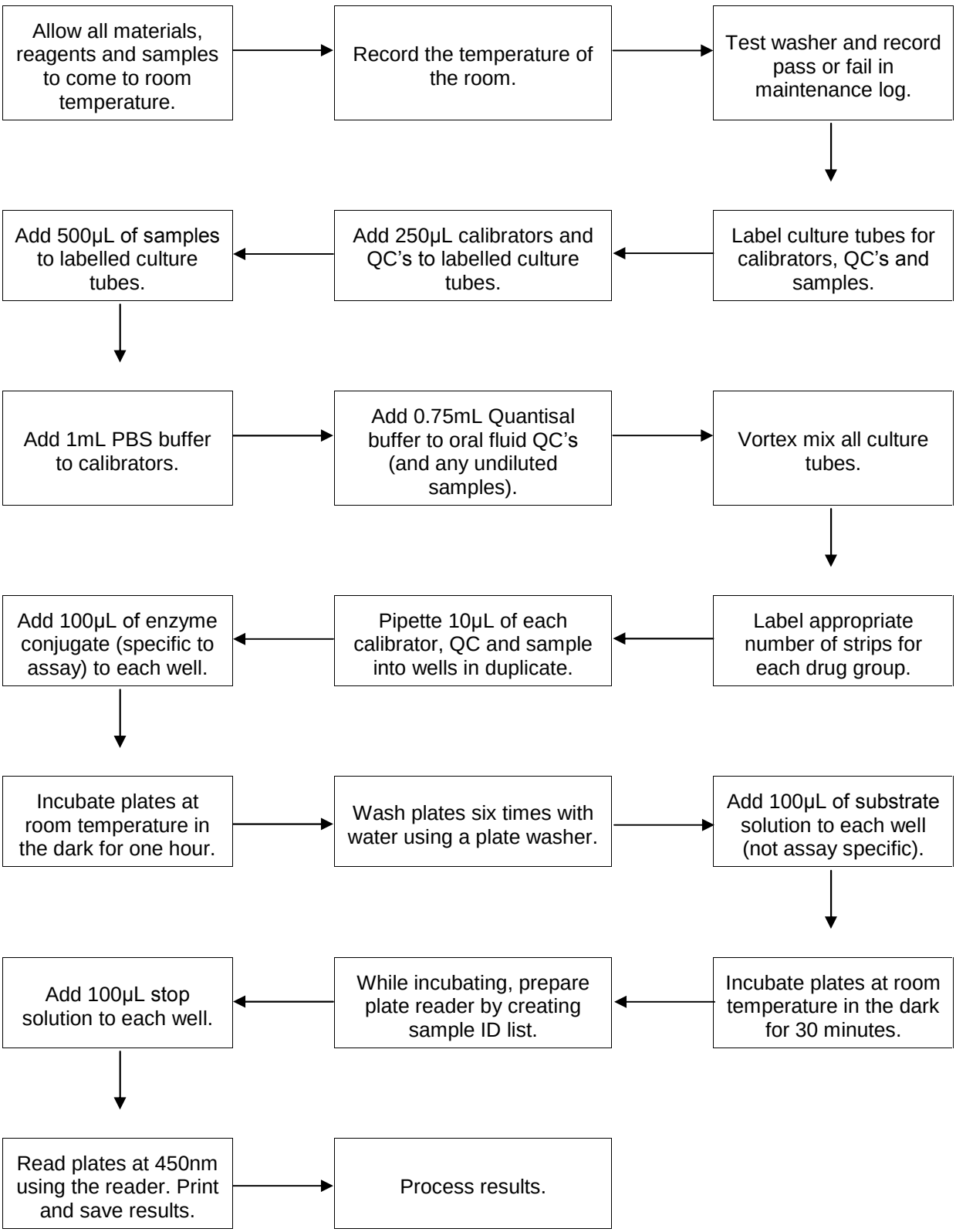


Figure 5: Flowchart Showing the ELISA Method

2.2.2 Data Processing

To process the results, the percentage binding of the calibrators and controls were calculated. The absorbance for the first positive calibrator was chosen as the cut-off value for the assay. The binding of the blank calibrator and the blank control were assumed to have 100% binding. The ratio of the absorbance of the other calibrators to the absorbance of the blank calibrator allows the percentage binding to be calculated for the calibrators and in a similar fashion the ratio of the positive controls absorbance to the blank control allows the percentage binding for the controls to be calculated. The formula used to calculate the percentage binding is given below:

$$\text{Percentage binding} = (B/B_0) \times 100 \quad \text{..... Equation 2}$$

Where B is the mean absorbance of calibrator or control and B_0 is the mean absorbance of the blank calibrator or blank control.

Using a different blank for the controls and calibrators allows any matrix effects on the absorbance to be considered. Using the percentage binding is a good way to normalize the results as the absorbance recorded will vary from day to day due to different assays and length of time incubated. The ratio between the levels should remain relatively constant and this will be monitored using a QC chart for each of the assays investigated. The results were used to construct Schewart style control charts as detailed in section 2.3.3.

As the samples are analysed in duplicate, the mean absorbance value for the sample is used for the calculation outlined above. The variation between these duplicate results is also monitored and if it is out with an acceptable level then the outlier can be discarded and the absorbance value from the other well used for the percentage binding calculation. The acceptable level of variation between the duplicate calibrators is 15% and between duplicate samples is 20%.

2.2.3 Method of Analysis (Confirmations)

Amphetamines are extracted by a solid phase extraction method. The extraction procedure for amphetamines in oral fluid is shown in Figure 6. A worksheet for the extraction procedure utilised was required to be filled in as part of the accreditation and is shown in Appendix 1.

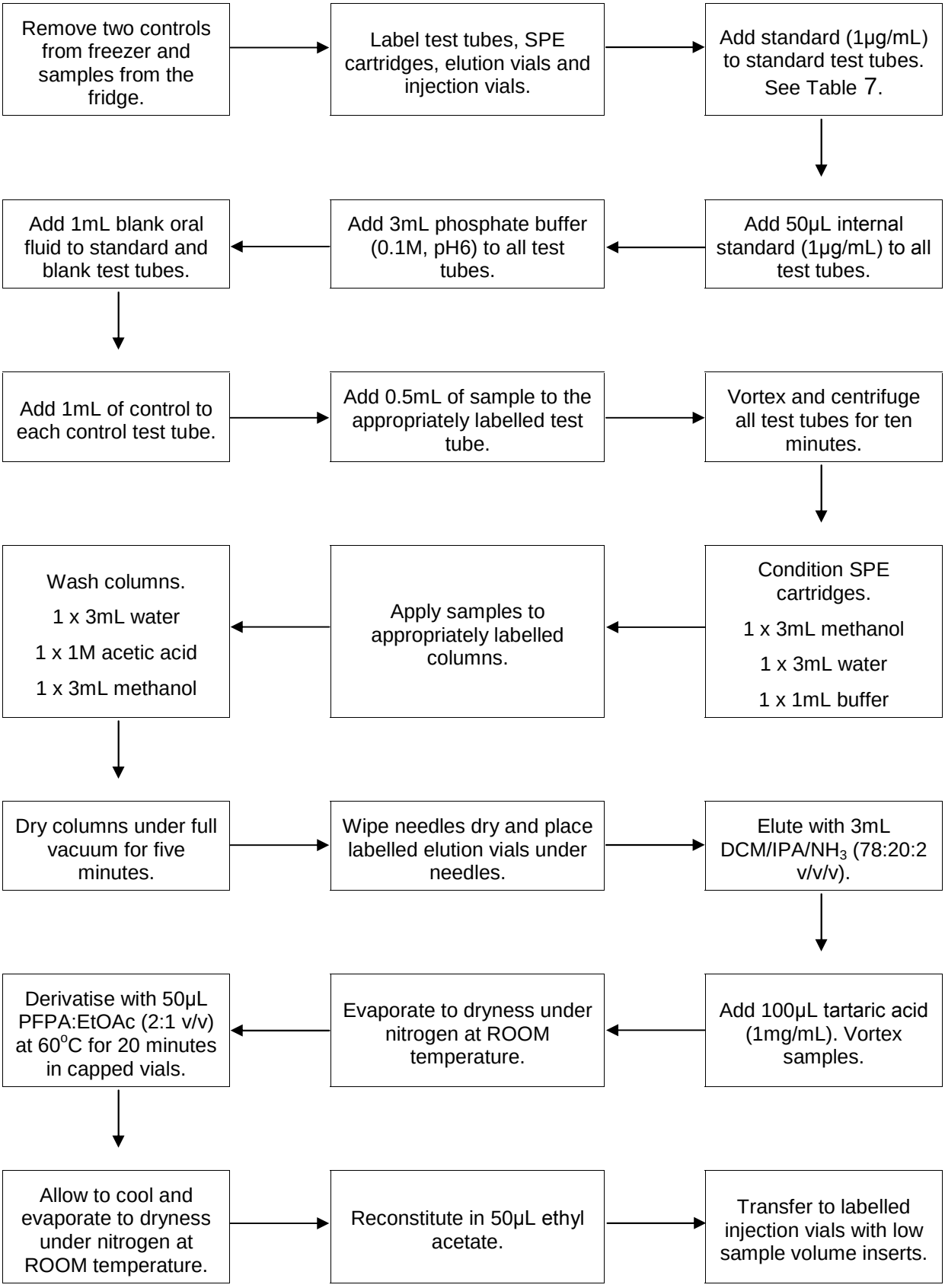


Figure 6: Flowchart for Confirmation of Amphetamines in Oral Fluid

The standard concentrations used are shown in Table 7.

Table 7: Volume of Mixed Amphetamine Standard for Amphetamines Extraction

Standard Concentration (ng/mL)	Volume of mixed amphetamines standard (1µg/mL)	Volume of mixed amphetamines internal standard (1µg/mL)
0	0µL	50µL
5	5µL	50µL
10	10µL	50µL
25	25µL	50µL
50	50µL	50µL
100	100µL	50µL
200	200µL	50µL

2.2.4 GC-MS Conditions

The GC was operated in splitless mode, with 1µL of sample being injected by the auto-sampler. The injection port was heated to 225^{o}C. The oven temperature was initially set at 55^{o}C and held for two minutes. The temperature was then ramped at 20^{o}C/min to 200^{o}C and then at 10^{o}C/min to 250^{o}C and on to 300^{o}C at 25^{o}C/min. The final temperature of 300^{o}C was held for two minutes. Helium (99.99% purity) was used as the carrier gas at a flow rate of 1mL/min. The sample was transferred via a transfer line heated to 250^{o}C to a 70eV electron impact (EI) ionisation source heated to 230^{o}C. The mass spectrometer was operated in the selected ion monitoring (SIM) mode. The ions monitored are shown in Table 8.

Table 8: Ions Monitored in SIM Mode

Drug	IS Ion	Quantifying Ion	Qualifying Ion	Qualifying Ion
Amphetamine	194	190	118	91
Methamphetamine	211	204	160	118
MDA	330	325	190	162
MDMA	344	339	204	162
MDEA	359	353	218	162

2.2.5 Processing of Results

MSD Chemstation software was used to process the data and calculated the ratio of the quantifier ion from the desired analytes to the quantifier ion from corresponding deuterated internal standard. The resulting calibration graphs gave linear responses for all analytes of interest over the calibration range of 5 – 200ng/mL. The results from the controls were used to construct Schewart style control charts as detailed in section 2.3.3.

2.3 Experimental Section

2.3.1 Response of Calibrators

To determine the suitability of the calibrators, a blank and three positive calibrators for each drug was evaluated as part of the ISO/IEC 17025 accreditation and was not repeated for this project. From this work, calibrators were prepared at the concentrations detailed in Table 5 above and a set of calibrators were run with each plate to ensure that each assay was acceptable and to determine the cut-off value for the assay on each separate run, as this was susceptible to change between lot numbers of assays and slightly different incubation times in each analysis. The

responses for the assays of interest are detailed in the results section. The mixed drug calibrators were used for each drug group.

2.3.2 Limit of Detection

To determine the limit of detection of the assays, twelve replicates of the blank control were run in duplicate on one plate. The mean and standard deviation for these samples were calculated and the mean minus two times the standard deviation was calculated (as the blank for the ELISA assays is the highest value) to determine the limit of detection. This work was only carried out for the amphetamine and methamphetamine plates.

2.3.3 Preparation of Shewart-Style Quality Control Charts

The mean value for the control chart was determined by averaging the percentage binding values from the first positive calibrator over a period of ten runs. The data from these runs was also used to calculate the mean ±2SD and the mean ±3SD and these values are indicated on the appropriate charts. Control charts for both the 50% above and 50% below the cut-off controls were constructed in the same way to that of the cut-off control charts. The same procedure was followed for the preparation of control charts for the amphetamine confirmation method.

Any subsequent sets of controls which were prepared were evaluated and if the results fell within the limits of the previous control, they were plotted on the previous control chart. If the values were out with the limits, a new control chart was constructed in the same way as the original charts. The results for the amphetamine and methamphetamine assays can be found in section 2.4.4. The charts for the other drugs can be found in Appendix 3 – ELISA QC Charts.

2.3.4 Criteria for Acceptability

All results should fall within three standard deviations of the mean and ideally within two standard deviations of the mean. One control outwith the mean ±2SD is acceptable provided the other is within that range. Trends of controls being higher or lower than “normal” may be observed due to small differences in the preperation of

calibrators. When the controls are repeatedly falling out with the acceptable range it indicates that the controls are no longer stable for the drug group in question.

2.3.5 Stability of Drugs in Oral Fluid

To evaluate the stability of drugs in oral fluid the percentage binding of the controls were calculated as described above (in section 2.2.2) and plotted on a QC chart. The stability of the controls is monitored over time by monitoring any significant change in the percentage binding, as indicated on the QC chart by the points being out with the acceptable range of the mean ±2SD. Over time, drugs will become unstable and start to break down resulting in an upward trend being observed in the QC charts. As multi-analyte controls were prepared, trends for other drugs groups were also monitored. The controls were stored in neat oral fluid, while case samples were diluted with buffer.

2.3.6 Sensitivity and Specificity

The sensitivity of the assay is defined as the efficiency of the assay in detecting positive samples and was calculated as follows and expressed as a percentage:

$$\text{Sensitivity} = (TP/ (TP + FN)) *100 \quad \text{..... Equation 3}$$

Where TP = True positives and FN = False negatives.

The specificity of the assay is defined as the efficiency of the assay in detecting negative samples and was calculated as follows and expressed as a percentage:

$$\text{Specificity} = (TN/ (TN + FP)) *100 \quad \text{..... Equation 4}$$

Where TN = True negatives and FP = False positives.

2.4 Results and Discussion

2.4.1 Calibration

The calibration for the assays used for oral fluid analysis was calculated using revelation software. The software displayed an error message if the calibration was not acceptable.

2.4.2 Limit of Detection

The limit of detection for amphetamine and methamphetamine in diluted oral fluid using Immunalysis ELISA assays is shown in Table 9.

The absorbance value for the limit of detection for amphetamine within this batch is 1.706 which is much higher than the absorbance value of the cut off concentration from this batch (0.594), thus there is no problem with sensitivity for this assay. Similarly methamphetamine, which has an absorbance value of 1.264 for the limit of detection in this batch, is sufficiently sensitive as the LOD is much higher than the absorbance value at the cut-off concentration (0.557) from this batch.

Table 9: Limit of Detection Results

Sample No:	Amphetamine	Methamphetamine
	Mean of duplicate absorbance values	Mean of duplicate absorbance values
1	1.896	1.347
2	1.853	1.347
3	1.933	1.295
4	1.957	1.378
5	1.912	1.440
6	1.844	1.390
7	1.917	1.317
8	2.011	1.437
9	1.792	1.485
10	1.700	1.453
11	1.791	1.448
12	1.896	1.526
Mean	1.875	1.405
Standard Deviation (SD)	0.085	0.071
2 x SD	0.169	0.141
Mean – 2SD	1.706	1.264
%CV	4.5	5.0

2.4.3 Stability of Drugs in Neat Oral Fluid

As the controls are being evaluated for a number of different drugs, the results for each drug will be shown in a table with a set of charts for one drug shown as an example.

The mean value and the values for the mean ±2SD and the mean ±3SD for each drug are given in the table below. The figures shown were calculated from the results obtained from the first six runs using the controls.

Table 10: Percentage Binding of Drugs of Abuse in Neat Oral Fluid at the Cut-off Concentration

Drug	Mean-3SD	Mean-2SD	Cut-off Mean	Mean+2SD	Mean+3SD
AMP	22.76	24.89	29.15	33.40	35.53
BEN	49.96	54.06	62.26	70.47	74.57
THC	89.29	90.92	94.19	97.46	99.10
COC	64.03	67.09	73.19	79.30	82.35
METH	63.89	67.18	73.75	80.33	83.62
MAMP	35.86	37.22	39.94	42.65	44.01
OP	7.21	19.84	45.11	70.38	83.01

Table 11: Percentage Binding of Drugs of Abuse in Neat Oral Fluid at 50% Below the Cut-off Concentration

Drug	Mean-3SD	Mean-2SD	-50% Mean	Mean+2SD	Mean+3SD
AMP	8.26	9.43	11.78	14.13	15.31
BEN	28.47	33.22	42.73	52.23	56.99
THC	49.37	54.11	63.58	73.05	77.79
COC	29.01	39.47	60.41	81.34	91.81
METH	15.25	27.24	51.21	75.18	87.16
MAMP	16.13	19.74	26.95	34.17	37.78
OP	8.18	11.19	17.22	23.24	26.25

Table 12: Percentage Binding of Drugs of Abuse in Neat Oral Fluid at 50% Above the Cut-off Concentration

Drug	Mean-3SD	Mean-2SD	+50% Mean	Mean+2SD	Mean+3SD
AMP	5.50	5.91	6.73	7.54	7.95
BEN	17.25	21.17	29.00	36.83	40.75
THC	41.41	45.81	54.59	63.38	67.77
COC	25.20	31.42	43.86	56.31	62.53
METH	8.32	17.26	35.14	53.01	61.95
MAMP	11.39	14.25	19.96	25.68	28.54
OP	7.18	8.37	10.75	13.12	14.31

As can be seen from the tables above, some drugs have a very narrow range of acceptability, from the mean minus three times the standard deviation to the mean plus three times the standard deviation, while others have a much larger range of acceptability. The percentage binding for the controls spiked at 50% below the cut-off concentration are lower than the percentage binding at the cut-off concentration as the controls were undiluted whereas the calibrators are diluted in 1mL of buffer which results in the controls appearing to be at a higher concentration than they actually are.

A sample set of charts at the cut-off value, 50% above and 50% below this value are shown below for amphetamine. The results are plotted against run number to allow any trends to be observed. The time period between the first and last run is approximately four months.

The control chart for amphetamine at the cut-off concentration in neat oral fluid is shown in Figure 7.

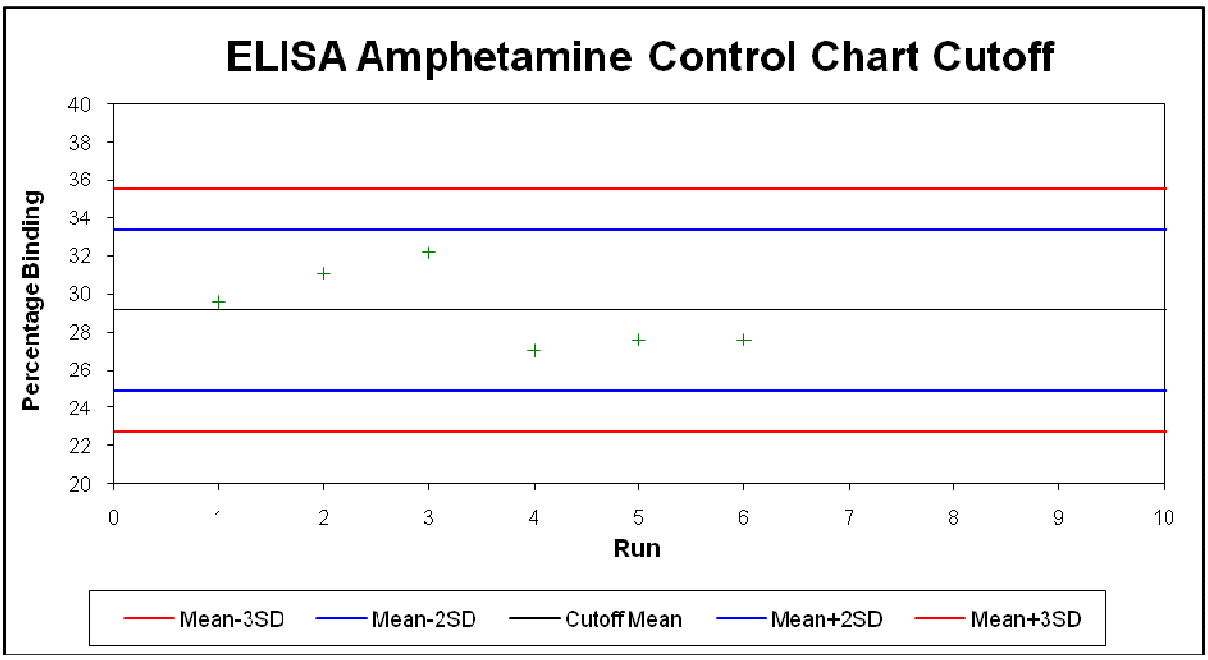


Figure 7: ELISA Control Chart for Amphetamines in Neat Oral Fluid at the Cut-off Concentration

As can be expected, the results all fall within two standard deviations of the mean as the six results were used to calculate the limits of the chart.

The control chart for amphetamine at 50% below the cut-off concentration in neat oral fluid is shown in Figure 8.

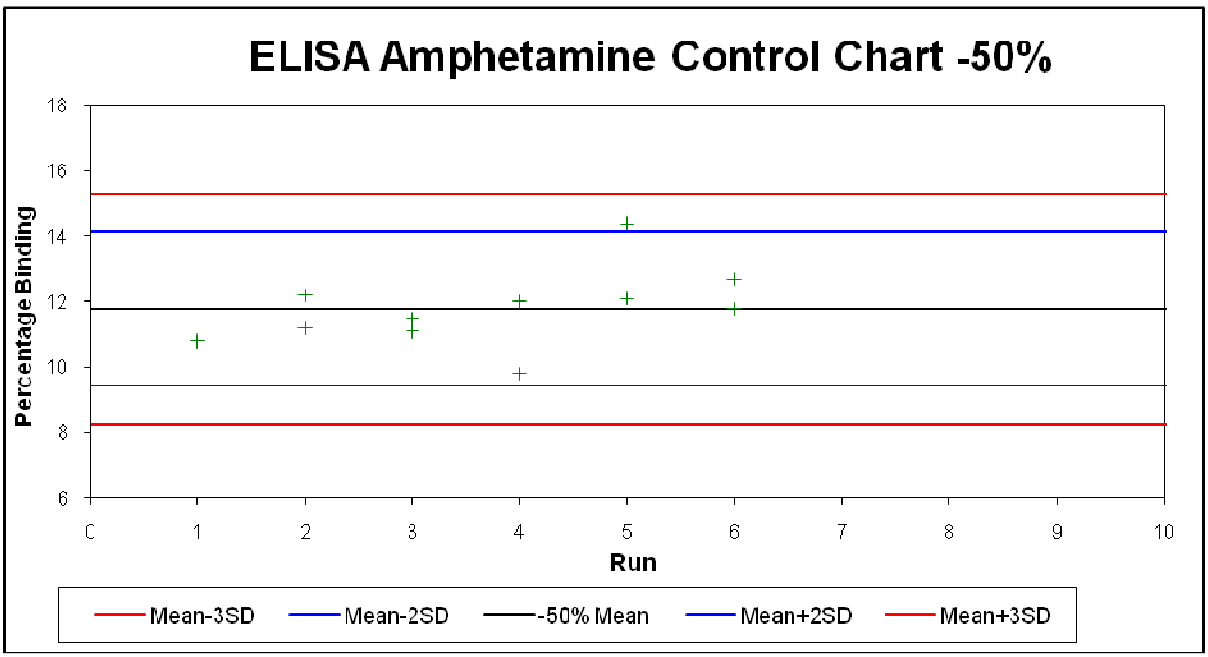


Figure 8: ELISA Control Chart for Amphetamines in Neat Oral Fluid at 50% Below the Cut-off Concentration

As can be seen from the chart above, all but one sample fell within the mean ±2SD range. All data points on the chart were used to calculate the limits. It is worth noting that for this particular control the range from minus three times the standard deviation to plus three times the standard deviation is rather small, only around eight percent, which means that a slight pipetting error or an innaccuracy in incubation time will make the control likely to fall out with the acceptable range.

The control chart for amphetamine at 50% above the cut-off concentration in neat oral fluid is shown in Figure 9.

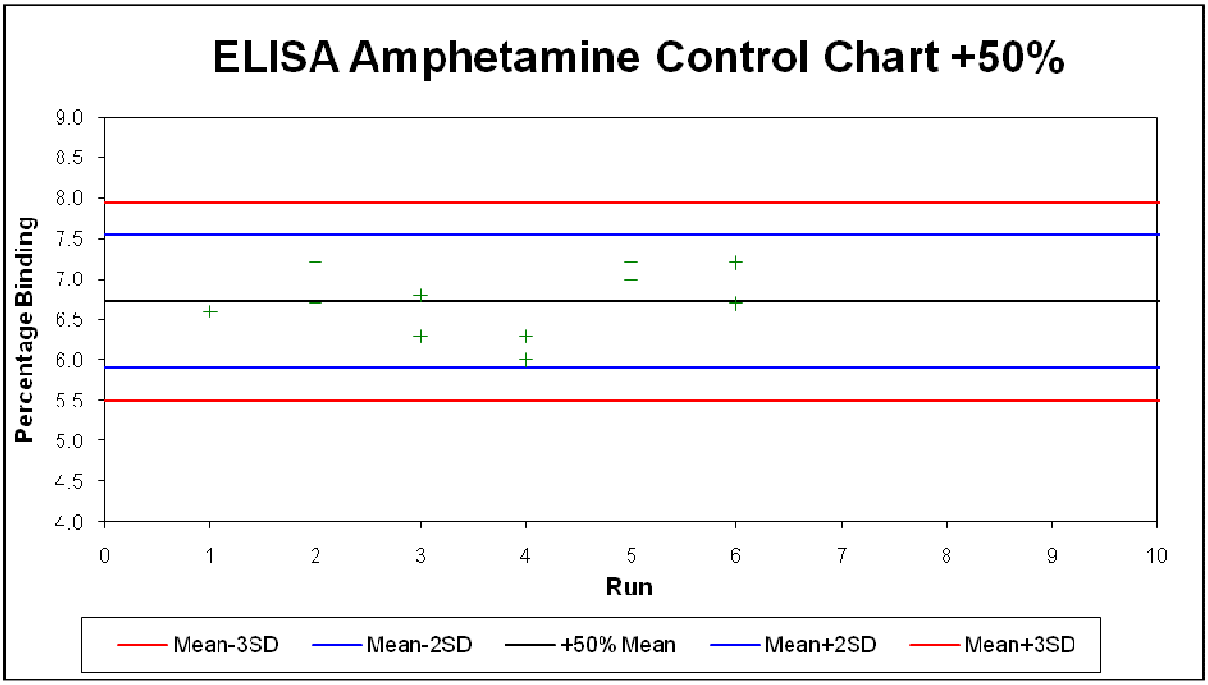


Figure 9: ELISA Control Chart for Amphetamines in Neat Oral Fluid at 50% Above the Cut-off Concentration

As can be seen from the chart above, all the results fall within the mean ±2SD range. All data points on the chart were used to calculate the limits. It is worth noting that for this particular control the range from minus three times the standard deviation to plus three times the standard deviation is incredibly small, only around three percent, which means that a slight pipetting error or an innaccuracy in incubation time will make the control likely to fall out with the acceptable range.

As the range for the neat oral fluid controls was so small it was decided that diluted controls would be used for the remainder of the project and thus no further data was collected for the neat oral fluid controls.

2.4.4 Stability of Drugs in Diluted Oral Fluid

As the controls are being evaluated for a number of different drugs, the results for each drug will be shown in a table with a set of charts for the main analytes of interest in this project, amphetamine and methamphetamine, being shown as an example. The charts for the other drugs can be found in Appendix 3 – ELISA QC Charts.

The mean value and the values for the mean plus and minus two and three times the standard deviation for each drug are given in the table below. The figures shown were calculated from the results obtained from the first ten runs using the controls.

Table 13: Cut-off Concentration for Drugs of Abuse in Diluted Oral Fluid

Drug	Mean-3SD	Mean-2SD	Cut-off Mean	Mean+2SD	Mean+3SD
AMP	23.2	25.1	29.0	33.0	34.9
BEN	49.1	54.0	63.8	73.7	78.6
THC	87.6	89.8	94.3	98.7	100.9
COC	60.0	63.9	71.5	79.2	83.0
METH	54.0	59.9	71.8	83.6	89.5
MAMP	36.0	37.2	39.8	42.3	43.6
OP	11.3	23.2	47.0	70.8	82.7

Table 14: 50% Below Cut-off Concentration for Drugs of Abuse in Diluted Oral Fluid

Drug	Mean-3SD	Mean-2SD	-50% Mean	Mean+2SD	Mean+3SD
AMP	18.1	22.7	31.9	41.1	45.7
BEN	56.2	60.3	68.6	76.8	81.0
THC	58.1	65.1	79.1	93.0	100.0
COC	47.2	59.1	83.0	106.8	118.8
METH	33.8	51.7	87.5	123.4	141.3
MAMP	20.8	27.8	41.7	55.6	62.6
OP	25.5	29.4	37.2	45.1	49.0

Table 15: 50% Above Cut-off Concentration for Drugs of Abuse in Diluted Oral Fluid

Drug	Mean-3SD	Mean-2SD	+50% Mean	Mean+2SD	Mean+3SD
AMP	11.0	13.5	18.3	23.2	25.7
BEN	42.9	47.0	55.3	63.6	67.8
THC	41.8	51.7	71.4	91.2	101.0
COC	33.1	44.4	67.1	89.8	101.2
METH	34.7	47.3	72.4	97.6	110.2
MAMP	18.7	23.2	32.3	41.4	45.9
OP	15.1	17.5	22.4	27.3	29.7

The results above show that there is a larger range of acceptable values for the controls when diluted with Quantisal buffer. This improves the range for amphetamine from around 3% to around 12% for the 50% above the cut-off concentration which gives more flexibility for any marginal error during the analysis. However, for the same control, the range of acceptability for methadone has increased from around 35% to around 50%. This wide range means that if there is a problem with the stability of methadone then it may be more difficult to see as it may not be apparent as the results may still be within the acceptable range. The percentage binding for the controls spiked at 50% below the cut-off concentration are lower than the percentage binding at the cut-off concentration as the controls are diluted in 0.75mL of buffer whereas the calibrators are diluted in 1mL of buffer which results in the controls appearing to be at a higher concentration than they actually are.

The data presented in the charts below spans a period of approximately 14 months, although the gap between analyses is not consistent as oral fluid samples were not arriving on a regular basis at the start of the project.

2.4.4.1 **Amphetamine**

The control chart for amphetamine at the cut-off concentration in diluted oral fluid is shown in Figure 10.

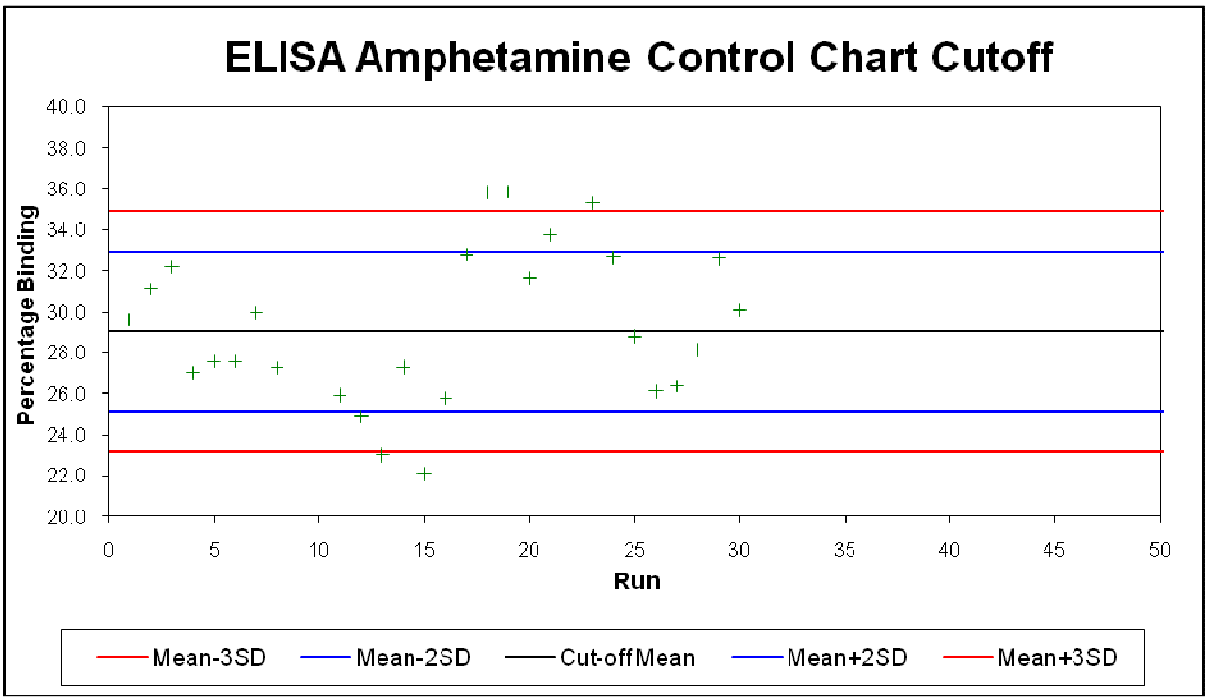


Figure 10: Cut-off Concentration for Amphetamine in Diluted Oral Fluid

The cut-off control chart for amphetamines in diluted oral fluid shows that for multiple different preparations of the calibrators, the percentage binding between the first positive calibrator and the negative calibrator remains fairly constant, with the majority of points falling within the acceptable range. A number of sets of calibrators (six) have been used while this QC chart has been in operation and none of them has deviated significantly from the mean. The cumulative mean (30 runs) for this chart (29.3%) is slightly higher than the mean (10 runs) displayed on the chart (29.0%) and represents a change of 0.9% from the originally calculated mean and confirms the consistency in the preparation of multiple calibrators used in this study.

The control chart for amphetamine at 50% below the cut-off concentration in diluted oral fluid is shown in Figure 11.

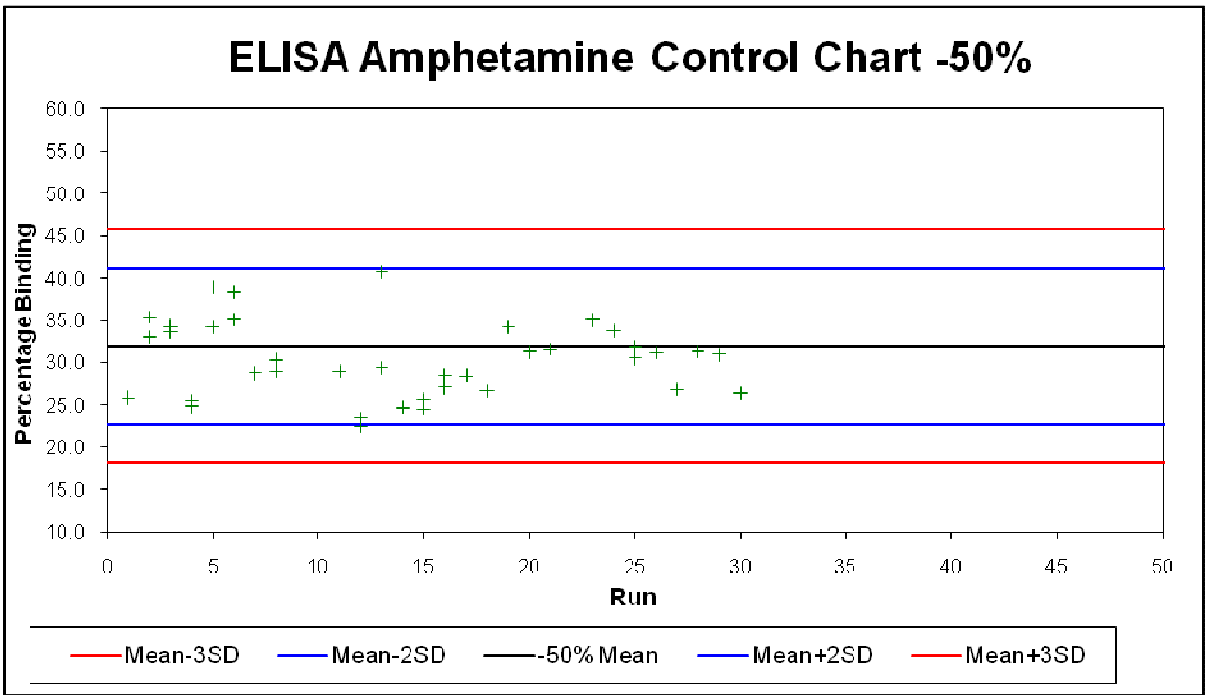


Figure 11: 50% Below Cut-off Concentration for Amphetamine in Diluted Oral Fluid

The control chart for the control spiked at 50% below the cut-off level shows good stability for amphetamine over the course of the charts use, which was approximately fourteen months, with all but one value falling within the acceptable range. The cumulative mean (30 runs) for this chart (30.2%) is slightly lower than the mean (10 runs) displayed on the chart (31.9%) and represents a change of -5.5% from the originally calculated mean and provides evidence that there is no significant loss of amphetamine from the controls.

The control chart for amphetamine at 50% above the cut-off concentration in diluted oral fluid is shown in Figure 12.

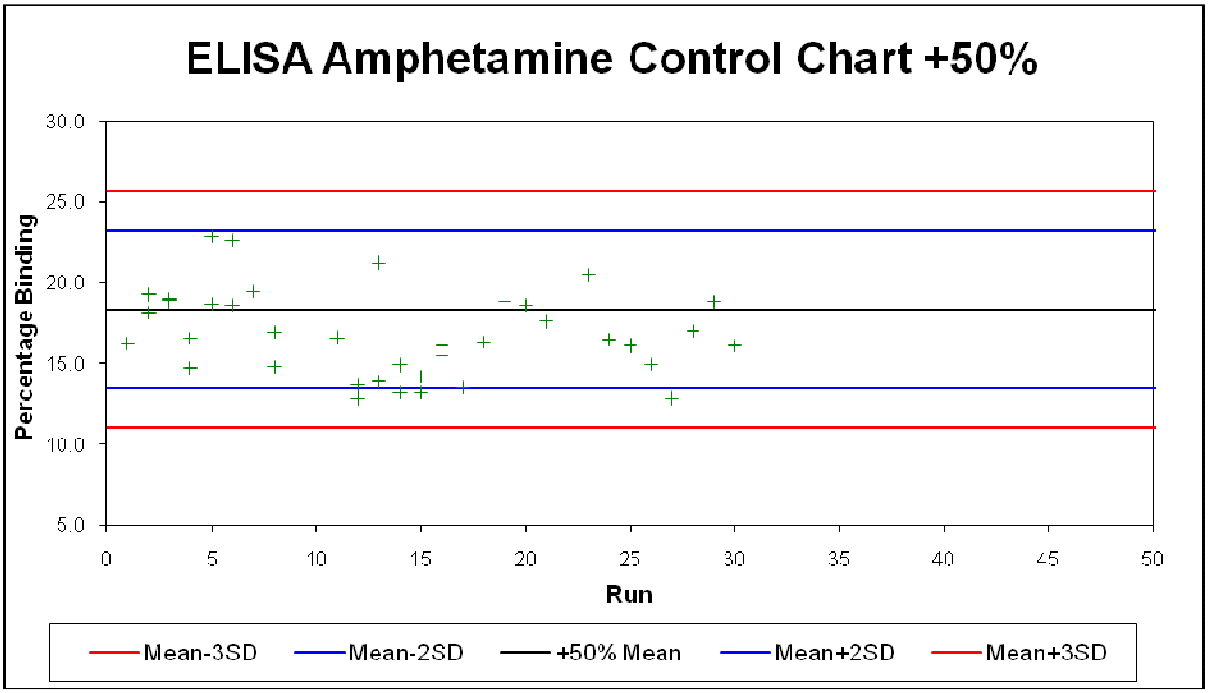


Figure 12: 50% Above Cut-off Concentration for Amphetamine in Diluted Oral Fluid

The control chart for the 50% above the cut-off value for amphetamine also shows good stability over time with almost all samples falling within the acceptable range. The cumulative mean (30 runs) for this chart (16.8%) is slightly lower than the mean (10 runs) displayed on the chart (18.3%) and represents a change of -8.3% from the originally calculated mean. While an 8.3% change in the mean value over the period of use appears to be large, it is only 1.5% lower than the original value but the percentage binding for this control was low and as such will give a higher percentage change in value. The change is small enough to show that the control is stable over time.

The amphetamine cut-off and control charts all show good consistency over the fourteen month period that they have been in operation as evidenced by almost all of the points being within the acceptable range. The cut-off chart shows the most disagreement and this is likely to be due to experimental error in the multiple preparations of the calibrators that were in use throughout this project. This provides evidence that amphetamine controls are stable in neat oral fluid stored in a freezer and diluted prior to analysis for a period in excess of twelve months.

2.4.4.2 **Methamphetamine**

The control chart for methamphetamine at the cut-off concentration in diluted oral fluid is shown in Figure 13.

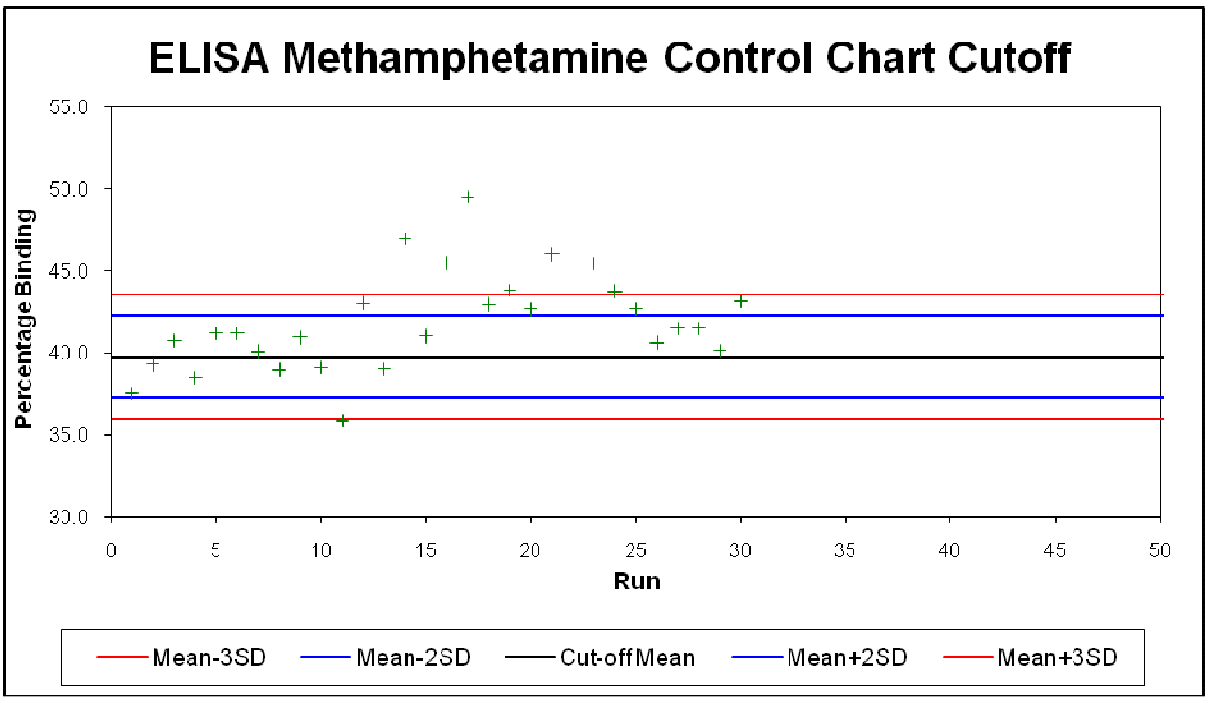


Figure 13: Cut-off Concentration for Methamphetamine in Diluted Oral Fluid

For a period of time, the cut-off concentration has failed for methamphetamine. This is likely to be down to two contributing factors. The first could result from an error in the preparation of the calibrator as the values during this period were noticeably higher than those typically seen in this chart. This is highly likely as the calibrators used were those used for the routine ELISA screen and the calibrators were prepared by several different people during this period. The second reason is the very low range of acceptability for this chart. Acceptable values must fall within a 5.1% range, which contrasts sharply with the cut-off chart for some other drug groups where the acceptable range is greater than 20%. An upwards trend is observed at this point and that could indicate that the methamphetamine calibrator was starting to break down, although a new preparation of the calibrator was also giving high results. The cumulative mean (30 runs) for this chart (41.8%) is slightly higher than the mean (10 runs) displayed on the chart (39.8%) and represents a change of 5.2% from the originally calculated mean and shows that there is no substantial variation when preparing new calibrators.

The control chart for amphetamine at 50% below the cut-off concentration in diluted oral fluid is shown in Figure 14.

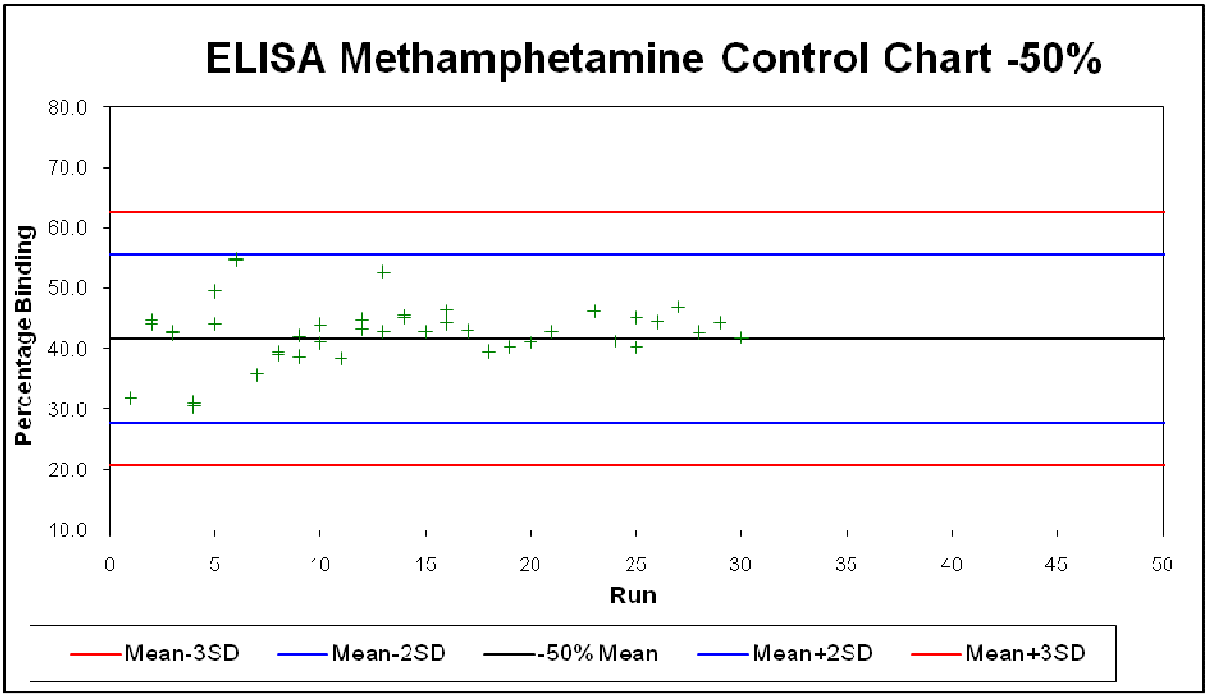


Figure 14: 50% Below Cut-off Concentration for Methamphetamine in Diluted Oral Fluid

The control chart for the control spiked at 50% below the cut-off level shows good stability for methamphetamine over a period of fourteen months with all values falling within the acceptable range. The cumulative mean (30 runs) for this chart (42.8%) is slightly higher than the mean (10 runs) displayed on the chart (41.7%) and represents a change of 2.6% from the originally calculated mean and provides evidence that there is no loss of methamphetamine from the controls whatsoever.

The control chart for amphetamine at 50% above the cut-off concentration in diluted oral fluid is shown in Figure 15.

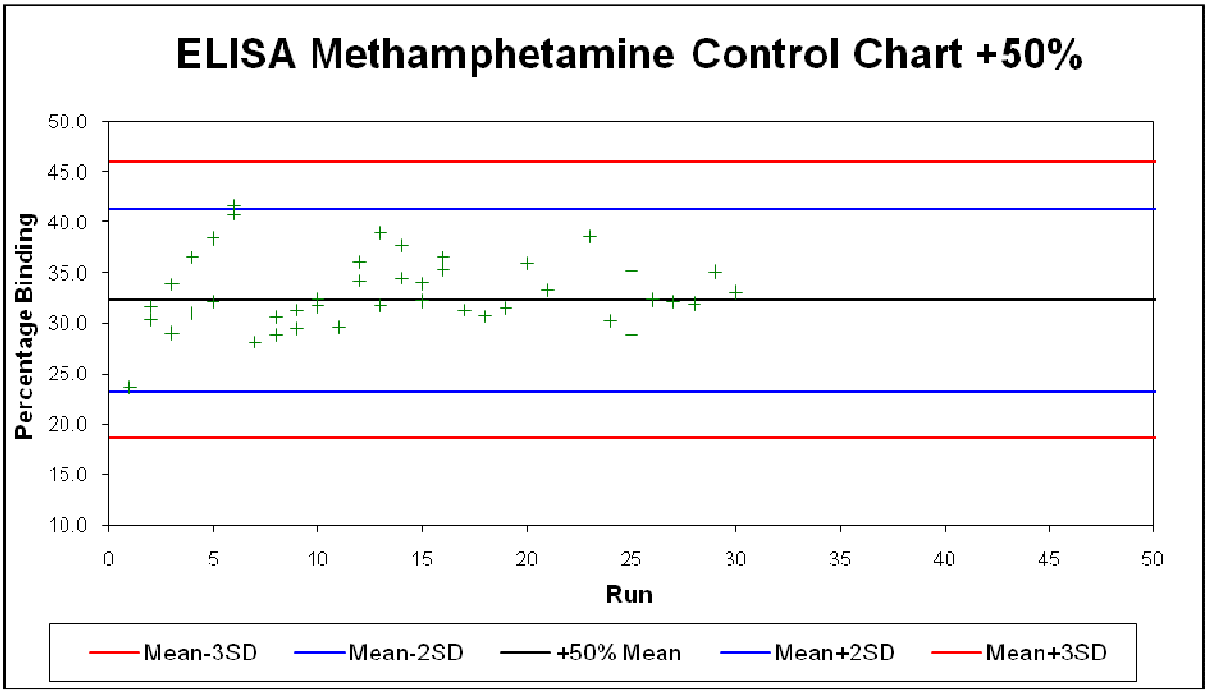


Figure 15: 50% Above Cut-off Concentration for Methamphetamine in Diluted Oral Fluid

The control chart for the control spiked at 50% above the cut-off level shows good stability for methamphetamine over a period of fourteen months with almost all values falling within the acceptable range. The cumulative mean (30 runs) for this chart (33.1%) is slightly higher than the mean (10 runs) displayed on the chart (32.3%) and represents a change of 2.4% from the originally calculated mean and provides evidence that there is no loss of methamphetamine from the controls over the time period studied.

The methamphetamine cut-off and control charts all show good consistency over the fourteen month period that they have been in operation as evidenced by almost all of the points being within the acceptable range. This provides evidence that amphetamine controls are stable in neat oral fluid stored in a freezer and diluted prior to analysis for a period in excess of twelve months.

2.4.5 Amphetamine and Methamphetamine ELISA Control Chart Conclusions

The results from the control charts for amphetamine and methamphetamine indicate that controls prepared and stored in the manor used in this project would be suitable for use for a period in excess of one year after preparation, however, a longer study

would be required to determine exactly how long the controls are stable for. Some other drugs are exhibiting an upward or downward trend in their control chart suggesting that they may no longer be stable, however, they are still within the acceptable range at present and the trend is mirrored in the cut-off chart which suggests that it may be more to do with a change in calibrator rather than a stability issue. The control charts for the other drugs are included for reference in Appendix 3 – ELISA QC Charts. The cut-off charts however, show a different picture with the amphetamine control chart which has a wide range of acceptable values being perfectly acceptable but the methamphetamine chart which had a narrow range of acceptable values was out when there was a slight error in the preparation of the calibrators. Interestingly, if the cumulative data was used for methamphetamine then almost all results would be acceptable as the standard deviation is larger due to a higher number of different sets of calibrators being prepared. If the controls are found to be stable over a longer period of time then it would make sense to make a larger quantity of controls and collect more data for the construction of the control charts to give a more representative mean and standard deviation for the long term.

The percentage change from the mean after ten runs to the mean after thirty is deceptively high for amphetamine and methamphetamine as they have low percentage binding values in comparison to the other drug groups in the controls. Increasing the number of runs to collate data for the cut-off charts would be an improvement to the current method as the calibrators are prepared far more frequently and by numerous different people, thus resulting in greater variation than the controls which are prepared far less frequently. Keeping the number of runs for the 50% above and below controls would be the best way forward as there should not be as much variation in these values.

2.4.6 Amphetamine QC Charts for GC-MS

Control charts were created for each of the five analytes in the amphetamine analysis. Only the control chart for amphetamine will be shown. The control charts for the other analytes can be found in Appendix 4 – Confirmation QC Charts. The control chart for amphetamine is shown in Figure 16.

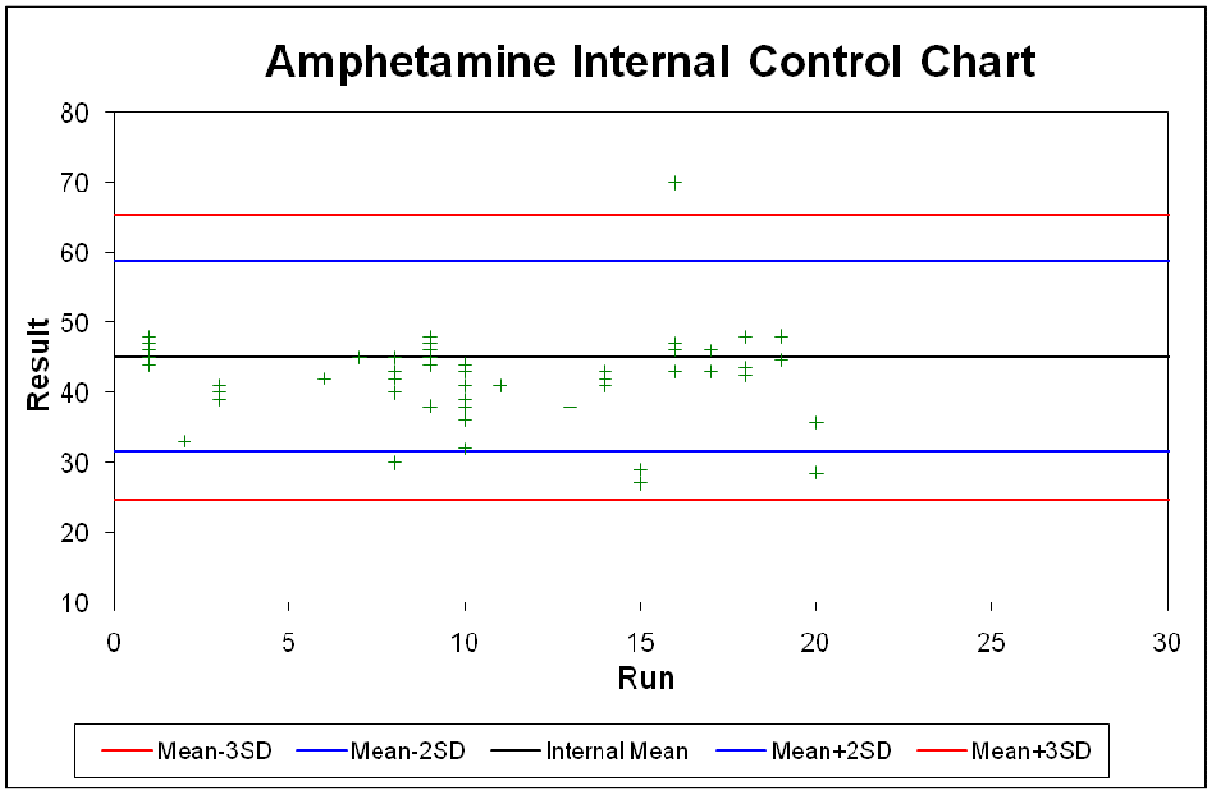


Figure 16: Amphetamine Control Chart

The control chart for amphetamine shows that the majority of samples fall within the acceptable range and that there is no downward trend indicating that there is no loss of analyte over time. None of the other control charts exhibited any loss of analyte over time.

2.4.7 Case Samples

2.4.7.1 Confirmed Samples

A number of case samples (both positives and negatives) were confirmed for the presence of amphetamines by GC-MS. The results of the case samples are given in Table 16. The confirmation results in the table are split as the amphetamine ELISA plate screened for amphetamine and MDA while the methamphetamine ELISA plate screened for methamphetamine, MDMA and MDEA.

Table 16: Results from Amphetamine Confirmations

Sample	Screening		Confirmations (results in ng/mL)				
Sample ID	AMP	MAMP	AMP	MDA	MAMP	MDMA	MDEA
015070	N/A	N/A	Neg	Neg	Neg	Neg	Neg
027660	Neg	Neg	Neg	Neg	Neg	Neg	Neg
027713	Neg	Neg	Neg	Neg	Neg	Neg	Neg
041616	Positive	Neg	58	Neg	Neg	Neg	Neg
041669	Neg	Neg	Neg	Neg	Neg	Neg	Neg
041670	Neg	Neg	Neg	Neg	Neg	Neg	Neg
041676	Neg	Neg	Neg	Neg	Neg	Neg	Neg
042532	Positive	Neg	Neg	Neg	Neg	Neg	Neg
043746	Neg	Neg	Neg	Neg	Neg	Neg	Neg
043839	Neg	Neg	<5	Neg	Neg	Neg	Neg
043881	Neg	Neg	Neg	Neg	Neg	Neg	Neg
043914	Neg	Neg	<5	Neg	Neg	Neg	Neg
044102	Neg	Neg	15	Neg	Neg	Neg	Neg
044116	Neg	Neg	Neg	Neg	Neg	Neg	Neg
057429	Positive	Neg	142	Neg	Neg	Neg	Neg
092020	Positive	Neg	281	Neg	Neg	Neg	Neg
092045	Positive	Neg	30	Neg	Neg	Neg	Neg
092073	Positive	Neg	192	Neg	Neg	Neg	Neg
092089	Positive	Neg	79	Neg	Neg	Neg	Neg
092109	Positive	Neg	316	Neg	Neg	Neg	Neg
092110	Positive	Neg	>800	Neg	Neg	Neg	Neg
092119	Positive	Neg	651	Neg	Neg	Neg	Neg
092123	Positive	Neg	110	Neg	Neg	Neg	Neg
092264	Positive	Neg	54	Neg	Neg	Neg	Neg
092292	Positive	Neg	>800	Neg	Neg	Neg	Neg
092325	Positive	Neg	>200	Neg	Neg	Neg	Neg
092328	Positive	Neg	160	Neg	Neg	Neg	Neg
092336	Positive	Neg	473	Neg	Neg	Neg	Neg

Sample	Screening		Confirmations (results in ng/mL)				
Sample ID	AMP	MAMP	AMP	MDA	MAMP	MDMA	MDEA
092339	Positive	Neg	548	Neg	Neg	Neg	Neg
092350	Positive	Neg	38	Neg	Neg	Neg	Neg
096563	Positive	Neg	779	Neg	Neg	Neg	Neg
097494	Positive	Neg	250	Neg	Neg	Neg	Neg
097573	Positive	Neg	>200	Neg	Neg	Neg	Neg
098102	Positive	Neg	62	Neg	Neg	Neg	Neg
098695	Positive	Neg	78	Neg	Neg	Neg	Neg
099260	Positive	Neg	78	Neg	Neg	Neg	Neg
099376	Positive	Neg	>200	Neg	Neg	Neg	Neg
099382	Positive	Neg	>200	Neg	Neg	Neg	Neg
CW028	Positive	Neg	Positive	Neg	Inconclusive	Neg	Neg
CW078	Positive	Neg	Neg	Neg	Neg	Neg	Neg
CW090	Positive	Neg	Inconclusive	Inconclusive	Inconclusive	Inconclusive	Inconclusive
CW144	Positive	Neg	Neg	Neg	Neg	Neg	Neg
CW147	Positive	Positive	>200	Neg	Neg	Neg	Neg
CW157	Positive	Neg	Neg	Neg	Neg	Neg	Neg
CW176	Positive	Positive	>200	Neg	Neg	Neg	Neg
CW179	Positive	Neg	73	Neg	Neg	Neg	Neg
CW184	Positive	Neg	Neg	Neg	Neg	Neg	Neg
CW221	Positive	Neg	Neg	Neg	Neg	Neg	Neg

2.4.7.2 **Unconfirmed Samples**

Approximately 250 other oral fluid samples were screened for amphetamines during this project but due to sample volume limitations and instrumentation problems there was not enough sample left to confirm. Some problems with the instrument meant that some batches of samples failed due to poor chromatography and low sample areas. Consequently, there was insufficient sample left for a repeat analysis. There was only a limited sample provided as many of the samples had previously been analysed by another laboratory. Samples collected for analysis in Forensic Medicine

and Science were part of a drug treatment program and were frequently analysed for other drug groups, thus being insufficient in volume for amphetamine analysis.

2.4.8 Sensitivity and Specificity

2.4.8.1 **Amphetamine and MDA**

This assay screened for amphetamine and MDA so the following calculations apply to these drugs as a group and not individually. There were 29 true positives, 11* true negatives, 6 false positives and no* false negatives for the amphetamine assay.

*Three samples confirmed positive but screened negative as they were below the screening method cut-off and were thus deemed to be true negatives and not false negatives.

Following the calculation given in section 2.3.6 the sensitivity of the amphetamine assay was calculated as 100% and the specificity was calculated as 65%.

2.4.8.2 **Methamphetamine, MDMA and MDEA**

This assay screened for methamphetamine, MDMA and MDEA so the following calculations would normally apply to these drugs as a group and not individually. However, in this case, as there were no positives for any of the drugs, the calculation does hold true for each drug individually. There were no true positives, 44 true negatives, 2 false positives and no false negatives for the methamphetamine assay.

Following the calculation given in section 2.3.6 the sensitivity of the methamphetamine assay was unable to be calculated as there were no positive confirmations and the specificity was calculated as 96%.

2.4.9 Case Sample Conclusions

Most of the confirmation results matched up with the screening results for the 48 confirmation tests carried out. However, there were a few discrepancies. Inevitably, some of these are false positives which are to be expected, but there were a few “false negatives” which is worrying. Admittedly, the “false negatives”, when confirmed gave results below the lowest standard and after taking the dilution factor

into account (only 0.25mL of sample was available for analysis), the results were below the in-house ELISA cut-off concentration and were therefore correctly marked as negative by ELISA. The amphetamine assay was found to be very sensitive and correctly detected 100% of the positive samples. Both assays of interest had high specificity of 65% and 96% for amphetamines and methamphetamines respectively, which shows both assays were good at eliminating negative results from confirmation tests.

Overall the amphetamine and methamphetamine ELISA assays work well and the controls prepared in-house are stable over time. This method can be used as an effective screening tool in a routine toxicology laboratory as only samples which confirmed as below the screening cut-off concentration were falsely screened as negative. However, some further work on the sensitivity of the assay at low concentrations would be recommended to ensure complete confidence in the assays.

3 Alcohol Enzymatic Analysis

3.1 Materials and Reagents

3.1.1 Immunalysis Corporation (Pomona, CA) Oral Fluid Alcohol Kit

An alcohol oral fluid kit (502-0500, Lot No: EK4354) containing reagent A (Tris buffer with 0.1% sodium azide as a preservative), reagent E (containing ADH and NAD in Tris buffer with stabilizers and 0.1% sodium azide as a preservative) and Quantisal extraction buffer (product code: EXTBUF-1000) along with blank 96 well plates were manufactured by Immunalysis Corporation (Pomona, CA) and purchased from Agriyork 400 Ltd (Pocklington, UK), their UK based distributor. The kits were stored at or below 8°C in the refrigerator. The specification for the assay is given in Appendix 5 – Alcohol Assay Specification.

3.1.2 Ethanol Standards

3.1.2.1 **Calibrators**

Ethanol certified reference standards in 1.2mL of water at the concentrations detailed in Table 17, were manufactured by Cerilliant (Round Rock, TX), and purchased from their UK based distributor, LGC Standards (Teddington, UK). The standards were stored at or below 8°C in the refrigerator. The same calibrators were used for the screening and confirmation methods.

Table 17: Product Codes for Ethanol Calibrators

Calibrator Concentration (mg/dL)	Product Code
10	E-040
25	E-035
50	E-029
80	E-030
100	E-031
200	E-032
300	E-033
400	E-036

3.1.2.2 **Controls**

Ethanol controls in 1.1mL of water at the concentrations detailed in Table 18, were manufactured by Medidrug (Kent, UK) and purchased from LGC Standards (Teddington, UK), their UK based distributor. The controls were stored at or below 8°C in the refrigerator. The same controls were used for both the screening and confirmations.

Table 18: Product Codes for Alcohol Controls

Control Concentration (mg/dL)	Product Code
30	20030
80	20080
300	20300

3.1.3 Reagents

1-propanol (HPLC grade, Part No: 29328-8) was purchased Sigma Aldrich (Dorset, UK).

3.1.4 Materials

Samples were diluted in 20mL headspace vials (part number 20CV-125) purchased from Kinesis (Cambridgeshire, UK) and capped using crimp caps (part number CRC20-04) that were also purchased from Kinesis (Cambridgeshire, UK).

3.1.5 Collection of Neat Oral Fluid

Neat oral fluid was collected from a single donor (who had not consumed alcohol within 24 hours of sample collection) by expectoration to allow greater flexibility at a later stage to dilute the samples to match the dilution factor of the collection device. Oral fluid was expectorated into a beaker and transferred to a storage bottle at the end of the collection period. This bottle was then stored in the freezer until required.

3.1.6 Preparation of Calibrators

The blank or alcohol free calibrator was prepared by adding 1.2mL of deionised water to a small vial and adding 3.6mL of the Quantisal extraction buffer. The positive calibrators were prepared by adding 1.2mL of the respective controls supplied by LGC Standards (Teddington, UK) (10, 25, 50, 80, 100, 200, 300 and 400mg/dL) to the appropriately labelled vials and adding 3.6mL of Quantisal extraction buffer to each vial. The calibrators were then vortex mixed to ensure they were thoroughly mixed. The calibrators were stored in a refrigerator at or below 8°C until required.

3.1.7 Preparation of Controls

The cut-off value for the oral fluid alcohol assay was set at 25mg/dL. A blank control was prepared using neat oral fluid that was collected from a donor who had not consumed alcohol within 24 hours of sample collection. Positive controls were

prepared at 50% below and 50% above the cut-off value (i.e. 12.5 and 37.5mg/dL respectively). Two vials (1.1mL each) of 300mg/dL alcohol control (Medidrug) were added to a small vial. This was followed by 4.4mL of water to dilute the solution to 100mg/dL. The solution was vortex mixed. To prepare the 50% below and 50% above controls, 1.25mL and 3.75mL of the 100mg/dL alcohol solution was added to the appropriately labelled 10mL volumetric flasks and made up to the mark with blank oral fluid. The solutions were inverted several times to ensure the solution was thoroughly mixed then transferred to a storage bottle and 30mL of Quantisal buffer was added to each control to match the dilution of the calibrators and samples. The controls were stored in a refrigerator at or below 8°C until required.

3.1.8 Preparation of Alcohol Internal Standard (1-propanol)

The alcohol internal standard was prepared by adding 1-propanol (0.33mL) to a 1L volumetric flask and making up to the mark with de-ionised water to give a concentration of 150mg/dL. The solution was inverted several times to ensure it was thoroughly mixed and transferred to a labelled bottle and stored at room temperature.

3.1.9 Equipment

Plates were read using a MRX microplate reader using a 340nm filter which was purchased from Dynex Technologies. Revelation software (version 4.25) was used to control the MRX microplate reader and read the absorbance of the samples.

The pipettes used in this project were calibrated by the United Kingdom Accreditation Service (UKAS) to ISO/IEC 17025 standard. Standards were prepared in volumetric flasks provided by Fisherbrand (Leicestershire, UK).

The dilutions for the alcohol confirmations were carried out using a Compudil 300 dilutor which was purchased from Hood & Tucker (Surrey, UK).

Alcohol confirmations were carried out on two GC-FIDs. Both instruments were fitted with ThermoQuest Trace GC 2000 series gas chromatographs purchased from Thermo Scientific (Hertfordshire, UK). The instruments were fitted with different auto-samplers, one used a ThermoQuest HS 2000 auto-sampler and the other used a

ThermoQuest HS 850 auto-sampler. Both auto-samplers were purchased from Thermo Scientific (Hertfordshire, UK). ChromQuest software (version 2.53) was used on both instruments and was purchased from Thermo Scientific (Hertfordshire, UK). The gas chromatographs were fitted with a RXT-1 (30m x 0.25mm x 0.25µm) and a RXT-2 (30m x 0.25mm x 0.25µm) GC column purchased from Thames Restek (Buckinghamshire, UK).

3.1.10 Case Samples

Case samples for this project were provided from a single source. Quantum Diagnostics (Essex, UK) provided approximately 100 samples for this project.

3.2 Methods

3.2.1 Manufacturer's Instructions for Alcohol Enzymatic Assay

The procedure used in this project was an adapted version of the method recommended by the manufacturer (Immunalysis Corporation (Pomona, CA) Corporation Catalog Number 502-0500, Version 6/2006), who recommend the use of a five point calibration at 0, 20, 40, 80 and 160mg/dL. To allow this method to follow the in-house ELISA method for drugs of abuse, it was decided to use a four point calibration including a blank. A flowchart detailing the steps in this procedure is shown in Figure 17.

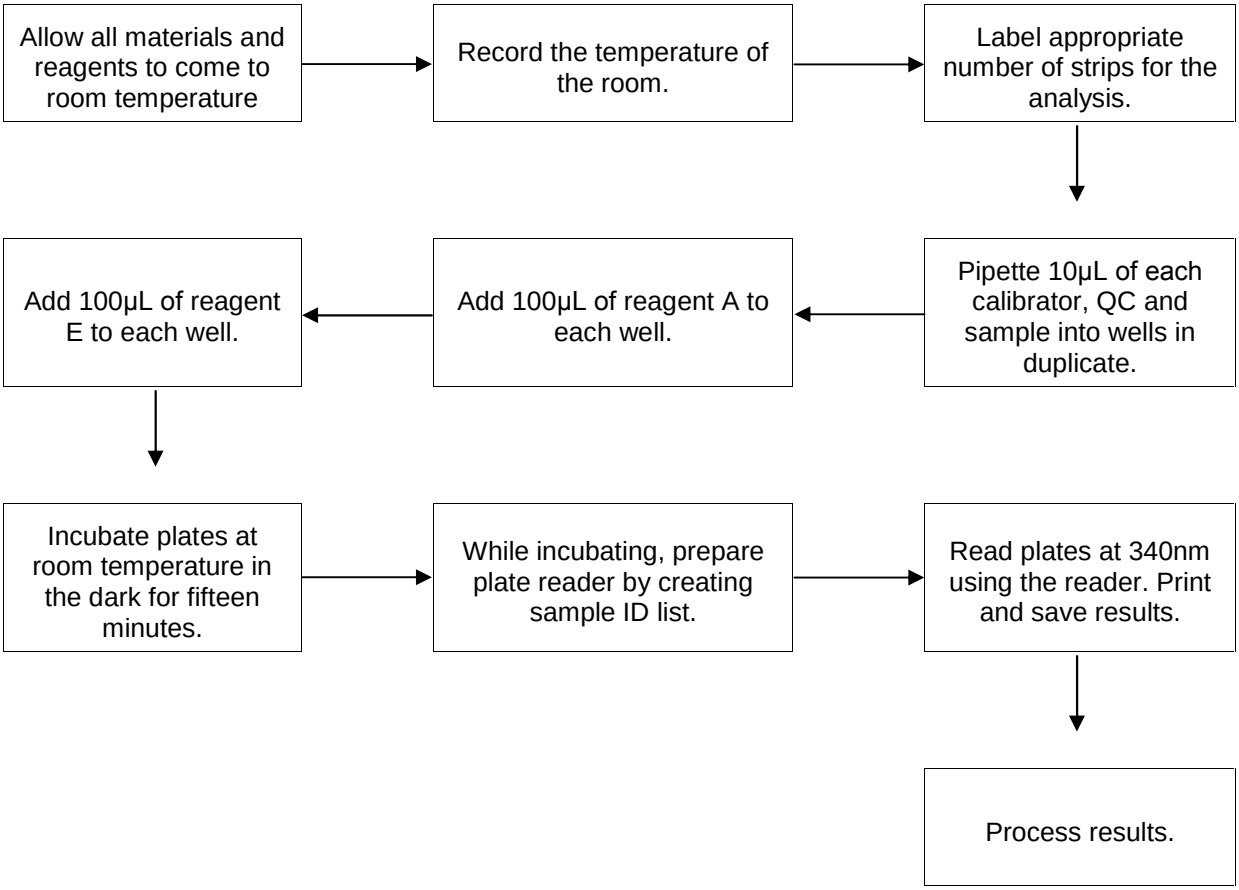


Figure 17: Method for Alcohol Screening

3.2.2 Method for Alcohol Confirmations

The Compudil dilutor was used to pipette 200µL of calibrator, QC or sample into labelled vials with 500µL of internal standard. Each sample was analysed in duplicate. The vials were then crimped and loaded into the auto-sampler tray for analysis.

3.2.3 GC-FID Conditions

Both instruments were programmed with the same method. The auto-sampler placed the sample in the incubation block to heat the sample vial to 60^{o}C for four minutes. The syringe, heated to 60^{o}C, injected 1mL of the headspace into the GC. The GC was maintained at 60^{o}C throughout the run.

3.3 Experimental Section

3.3.1 Response of Calibrators (Linearity)

To evaluate the linearity of the alcohol assay, an initial calibration was prepared (i.e. diluted one in four with Quantisal buffer to match sample dilution) and processed at concentrations of 25, 80, 100, 200, 300 and 400md/dL. Subsequent analyses made use of a four point calibration at concentrations of 0, 25, 80 and 300mg/dL. A four point calibration was chosen over the full calibration to keep running costs down and because the method was only being used semi-quantitatively to eliminate negative samples from further analysis. The linearity evaluation was repeated using a full calibration (0, 10, 25, 50, 80, 100, 200, 300 and 400mg/dL).

3.3.2 Limit of Detection

To determine the limit of detection of the assay, 12 replicates of the blank control were run in duplicate on one plate. The mean and standard deviation for these samples were calculated and the mean plus two times the standard deviation was determined to be the limit of detection.

3.3.3 Precision

Two sets of plates were run, each with two different sets of calibrators and controls, on the same day. The seven data points gathered were used to calculate the intra-day precision. Twenty-one controls were analysed over twelve runs, each with a set of calibrators and controls, on different days and this data was used to calculate the inter-day precision.

3.3.4 Preparation of QC Charts

To allow the preparation of a Shewart style QC chart, six batches over six different days were run with a set of calibrators and a set of controls. A set of controls for the oral fluid alcohol enzymatic assay is defined as a blank control, a control at 50% below the cut-off level and a control at 50% above the cut-off level. The result for the first positive calibrator (in this instance 25mg/dL) was chosen as the cut-off value for

the assay. A QC chart was constructed after six replicates of the controls had been run. Subsequent data points were plotted on this chart to monitor the stability of the controls over time.

QC charts were constructed for the cut-off value and the controls at 50% above and 50% below the cut-off value. As all samples are analysed in duplicate, the variation between these results was monitored and any outlying points could be disregarded. The acceptable level of variation between the calibrators was 15% and between samples was 20%.

3.3.5 Stability of Calibrators

As ethanol is a volatile substance, it was decided to evaluate the stability of the controls over a two week period to determine if any ethanol was lost when the calibrators were stored in screw cap reagent bottles in the refrigerator. The absorbance of each of the calibrators was recorded and plotted on one graph to show any changes. Analyses were carried out on the day of preparation and on seven other occasions over the fourteen day period.

3.3.6 Stability of Alcohol Controls in Oral Fluid

The stability of oral fluid samples spiked with alcohol at 40mg/dL was examined over a fourteen day period. Spiked oral fluid was prepared by adding 0.5mL of 400mg/dL alcohol standard to a 5mL volumetric flask and making up to the mark with neat oral fluid. This solution was transferred to a 20mL volumetric flask and made up to the mark with Quantisal buffer. 1mL aliquots of this solution were pipetted into small storage vials with half being stored in the refrigerator below 8°C and the other half stored in a cupboard at room temperature (typically 16 - 21°C). Two vials from each set of storage conditions were analysed on six separate days. Samples were analysed on the day of preparation and on five further occasions over the two week period.

3.3.7 Sensitivity and Specificity

The sensitivity and specificity of the Immunalysis ethyl alcohol assay was determined by calculating the sensitivity and specificity as described in section 2.3.6.

3.4 Results and Discussion

Some of the work for this project was done using reagents that had past their expiry date. This meant that reagent E was darker than normal. It was, however, not a significant problem for the assay as a calibration was run with each batch and therefore any effect was consistent across the batch.

3.4.1 Linearity

Figure 18 illustrates the linearity of the alcohol calibration in the 0 – 400mg/dL range.

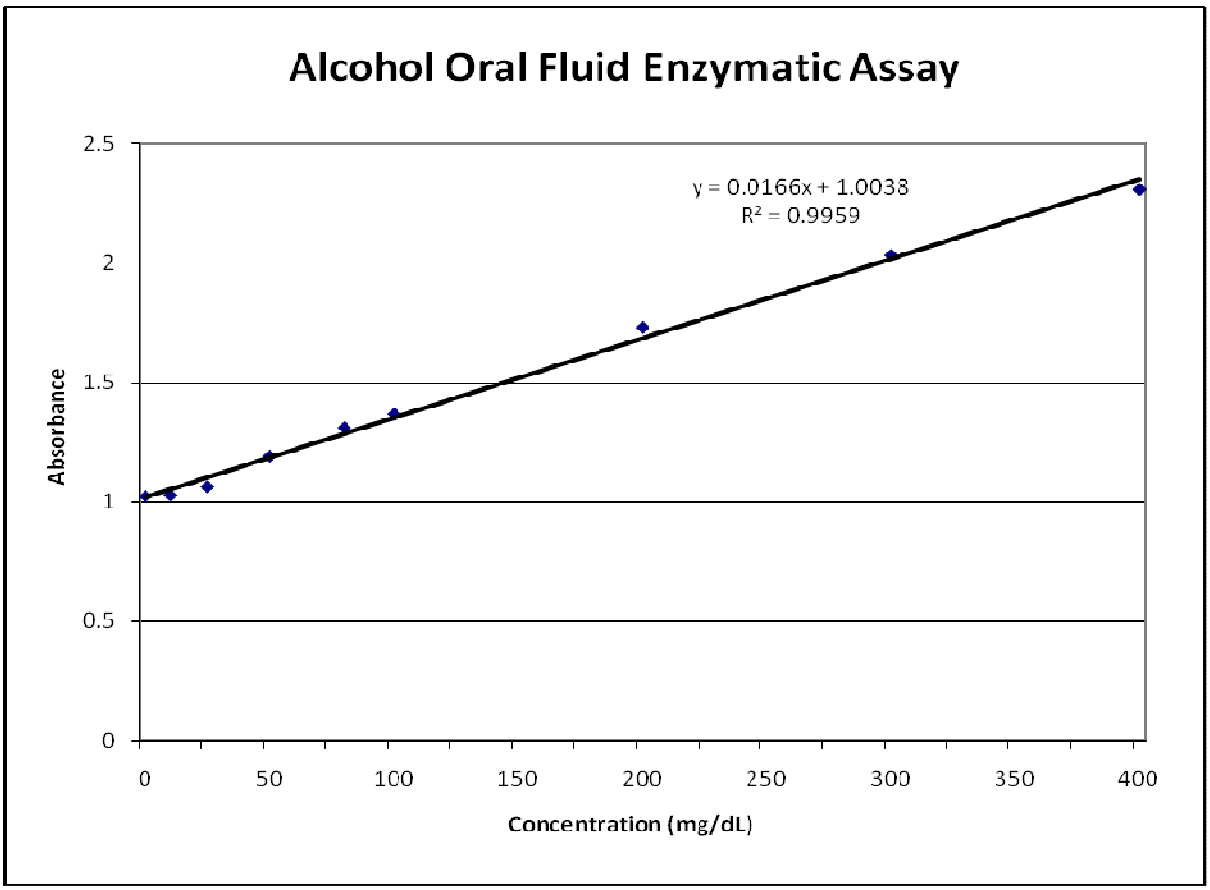


Figure 18: Example of Linearity of Full Calibration

As can be seen above, the Immunalysis Corporation (Pomona, CA) alcohol oral fluid assay demonstrates good linearity over the 0 – 400mg/dL range as evidenced by the R^2 value, which was above 0.99. As this method was being evaluated as a screening method, a full calibration was not necessary, and four points from the calibration above were run with all other batches.

An example of this four point calibration is shown in Figure 19.

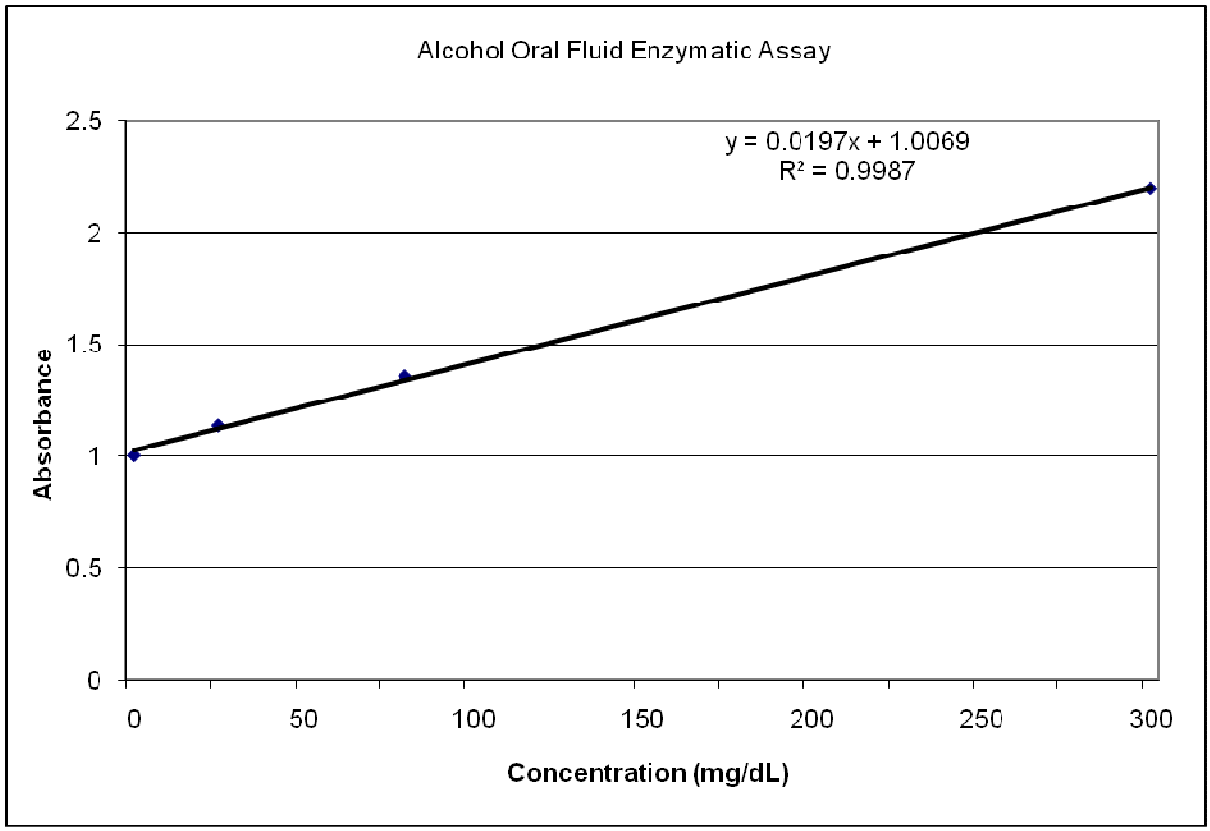


Figure 19: Example of Alcohol Oral Fluid Linearity

The Immunalysis Corporation (Pomona, CA) alcohol oral fluid assay demonstrates good linearity when used with a four point calibration over the 0 – 300mg/dL range as evidenced by the R^2 value, which was above 0.99.

3.4.2 Limit of Detection

Table 19 summarises the statistical variation of twelve replicates of the blank control that were analysed in duplicate in a single batch.

Table 19: LOD Results

	Mean Values
Mean	**0.972**
STD DEV	0.0146
2 X STD DEV	0.0291
MEAN + 2SD	**1.001**
%CV	1.50

The results above show that the blank control gives reproducible results when run on one plate as evidenced by the coefficient of variation of 1.5%. The absorbance of the cut-off concentration (25mg/dL) within this batch was 1.049. The absorbance of the blank calibrator within this batch was 0.954. The 50% below the cut-off control, spiked at 12.5mg/dL, had an absorbance value of 1.035 within this batch and is also higher than the limit of detection thus indicating that the assay is fit for purpose.

3.4.3 Precision

Table 20 summarises the statistical data for inter and intra-day precision of the assay by looking at the mean absorbance values.

Table 20: Intra- and Inter Day Precision

Intra-Day Precision (n=7)			**Inter-Day Precision (n=21)**		
	50% Below	**50% Above**		**50% Below**	**50% Above**
Mean	1.025	1.059	**Mean**	1.022	1.122
Std Dev	0.021	0.018	**Std Dev**	0.027	0.039
%CV	2.1	1.7	**%CV**	2.7	3.5

The precision data indicates that there is less variation between results from runs on the same date than there is between runs from different days. While the coefficient of variation for the intra-day precision is lower (<2.5%), the inter-day precision is still acceptable (<4%). This shows that there is good reproducibility of results on the same day and on different days. The 50% below the cut-off value control is quite close to the limit of detection for the assay.

3.4.4 Stability of Calibrators

The stability of the alcohol calibrators was monitored by plotting the absorbance reading versus time over a two week period. The calibrators were stored in screw cap bottles in a fridge between analyses. The results are shown Figure 20 .

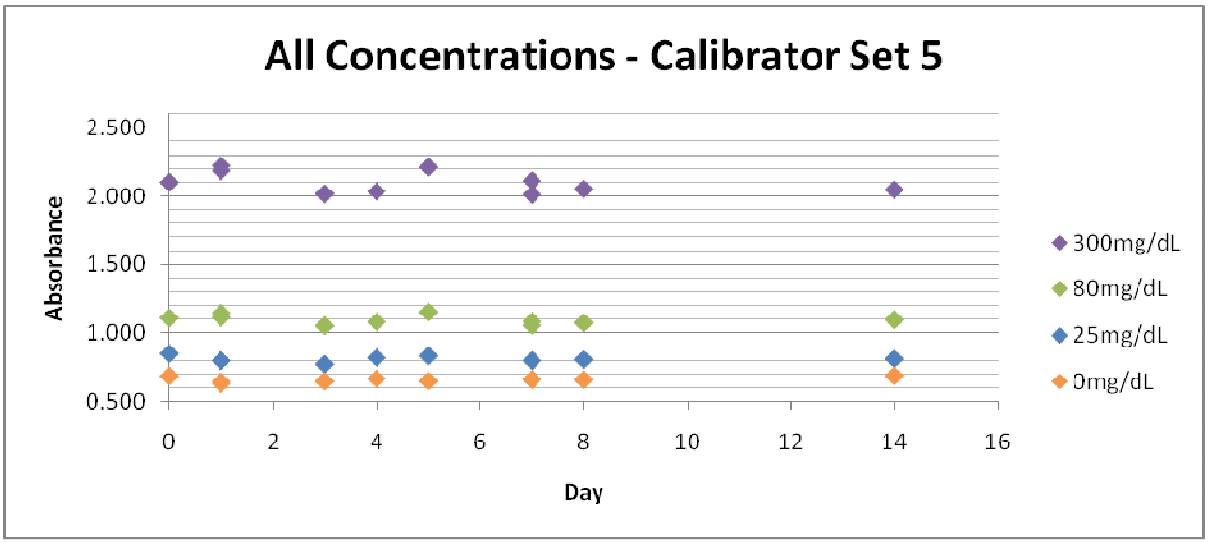


Figure 20: Stability of Alcohol Calibrators over a Two Week Period

Figure 20 demonstrates that there is no substantial loss of alcohol from the calibrators over a two week period. The absorbance values seen on this chart are lower than the LOD for the lowest standard as a new set of reagents were used that were lighter in colour to a previous set that had been used to calculate the LOD data.

While the calibrators are stable over time when refrigerated, the highest calibrator exhibited a loss of absorbance depending on how long it had been since the

calibrator had been pipetted into the wells and reagent E was added to the wells. The results of a short study to investigate this effect are shown in Figure 21.

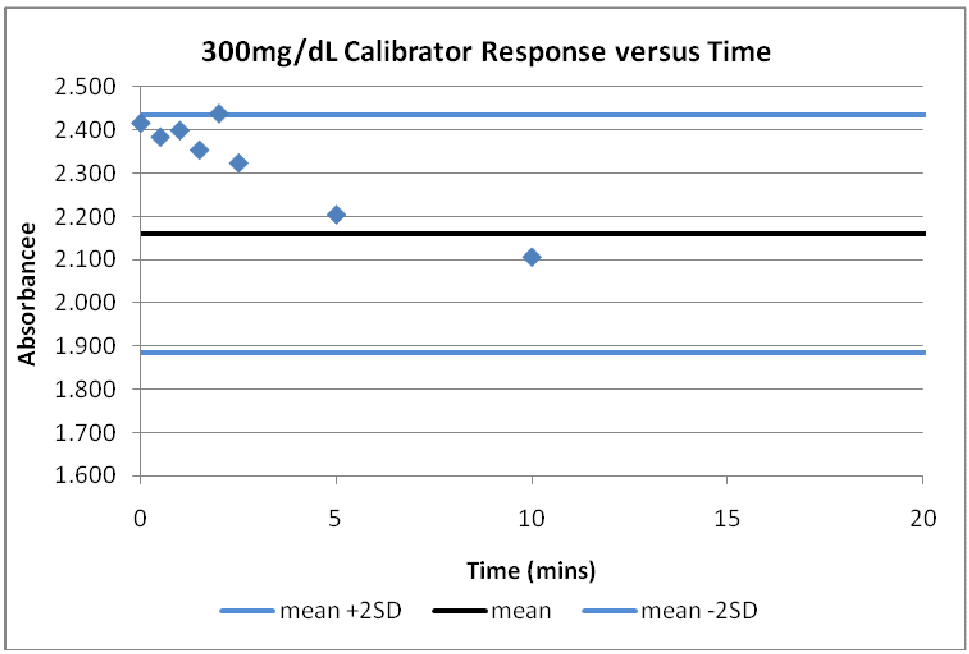


Figure 21: Calibrator Response versus Time

Figure 21 shows that although all results fall within two standard deviations of the mean, there is a considerable loss in absorbance reading with increasing time before adding the reagent. This becomes a problem if a large number of samples are being analysed at the same time, as the later samples will appear to have higher concentrations than they actually have due to this apparent loss. The problem however, seems to be limited to the higher concentrations of alcohol as no significant loss was noticed with the lower calibrators. Although a slight loss was observed for the 80mg/dL calibrator, (data shown in Appendix 6 – Alcohol Stability Charts) in terms of absolute absorbance, it is not too significant and could be attributed to experimental uncertainty.

In addition, the first samples to be pipetted are the calibrators and thus if the absorbance of the highest calibrators falls over time then the slope of the chart will be lowered and consequently this method is not suitable for quantitative analysis. Four strips from the plate were typically used for a batch, but for the four batches that used the full twelve strips on the plate, the mean absorbance value for the

300mg/dL calibrator from those batches was 1.438, which if plotted on the QC chart, Figure 21, would be well outside the acceptable range. This could represent a problem for any routine laboratory that was analysing large numbers of samples.

3.4.5 Stability of Alcohol Controls in Oral Fluid

The stability of oral fluid controls was evaluated for different storage conditions. Eppendorf vials and screw cap vials were used for the comparison with a set of each being evaluated while at room temperature and one set stored in a refrigerator.

3.4.5.1 **Eppendorf Vials**

The stability of the oral fluid samples spiked with alcohol and refrigerated in eppendorf vials are illustrated in Figure 22. Set 1 was only run for seven days.

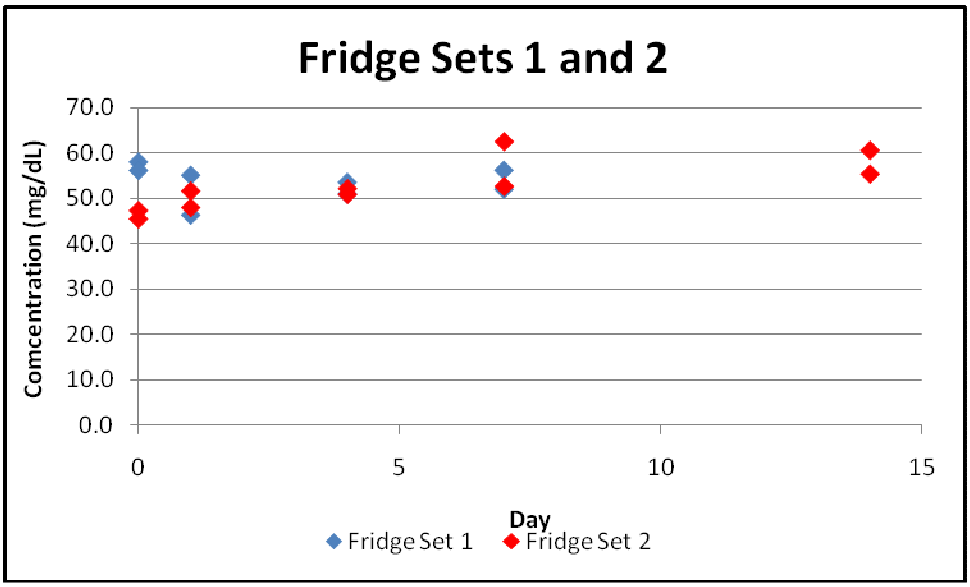


Figure 22: Stability of Spiked Oral Fluid Samples Stored in a Refrigerator Over Fourteen Days

Figure 22 demonstrates that oral fluid samples spiked with alcohol are stable over a fourteen day period if stored in eppendorf vials in a refrigerator at or below 8°C. The variation between the results is due to experimental error and not due to a loss of any sample thus it can be deduced that storing samples in eppendorf vials in a

refrigerator is a suitable way to store alcohol positive oral fluid samples in the short-term.

The stability of the oral fluid samples spiked with alcohol and stored at room temperature in eppendorf vials are illustrated in Figure 23. Set 1 was only run for seven days.

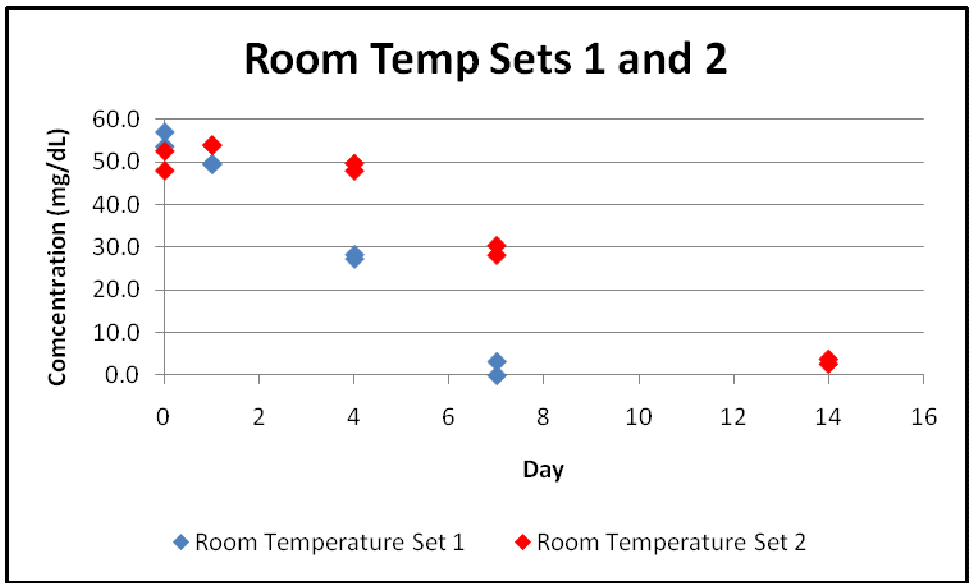


Figure 23: Stability of Spiked Oral Fluid Samples Stored at Room Temperature Over Fourteen Days

Figure 23 highlights that alcohol is not stable in oral fluid samples stored in eppendorf vials over a fourteen-day period if stored in the dark at room temperature. Given that these samples came from the same solution as those that were stored in the refrigerator over the same fourteen-day period and exhibited no loss of alcohol, it can be assumed that the most likely reason for the loss of alcohol from these samples is due to the storage conditions, in particular the temperature at which they were stored. The rate of decrease in alcohol concentration is high enough to warrant concern for samples that are transported overnight or perhaps for a longer period of time in non-refrigerated conditions. This is however, only a screening method and these findings would need to be confirmed by headspace gas chromatography – flame ionisation detector (GC-FID).

3.4.5.2 **Screw Cap Vials**

Figure 24 and Figure 25 below summarise the data generated for alcohol spiked oral fluid samples stored in screw cap vials over a fourteen day period when refrigerated and at room temperature respectively.

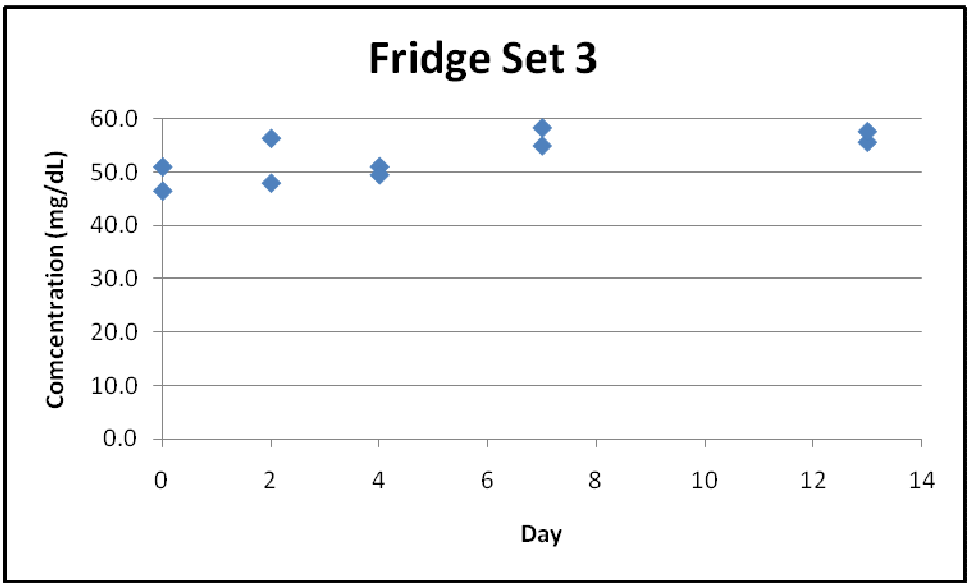


Figure 24: Stability of Spiked Oral Fluid Samples Stored in the Fridge Over Fourteen Days

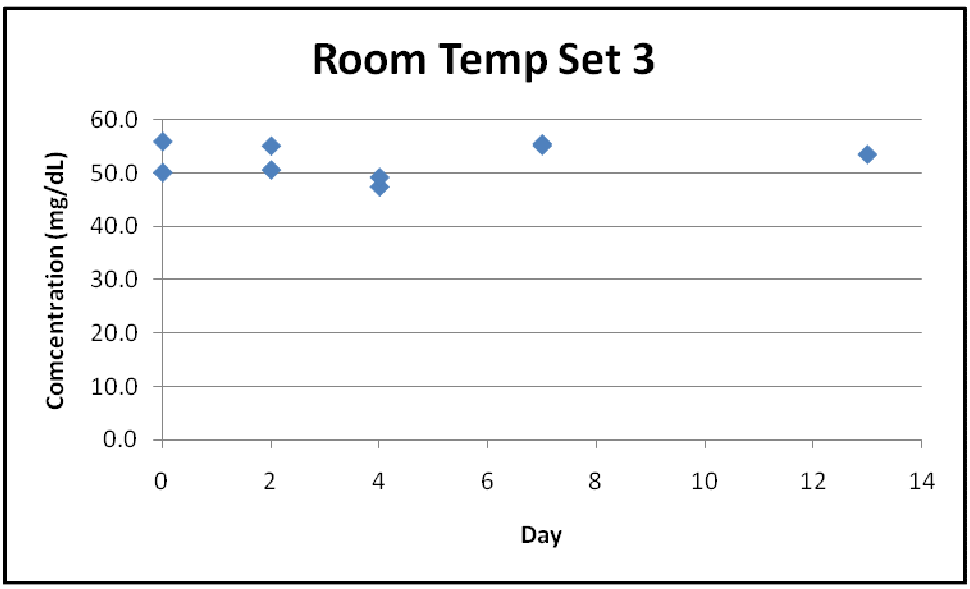


Figure 25: Stability of Spiked Oral Fluid Samples Stored at Room Temperature Over Fourteen Days

Figure 24 andFigure 25 demonstrate that there is no loss of alcohol from screw cap vials when either stored at room temperature or refrigerated. This indicates that screw cap vials are a more suitable storage medium for alcohol containing oral fluid

samples than eppendorf vials and that they should be used for the storage of controls.

3.4.6 QC Charts

QC charts were constructed for the cut-off value (Figure 26), the 50% below the cut-off control (Figure 27) and the 50% above the cut-off control (Figure 28).

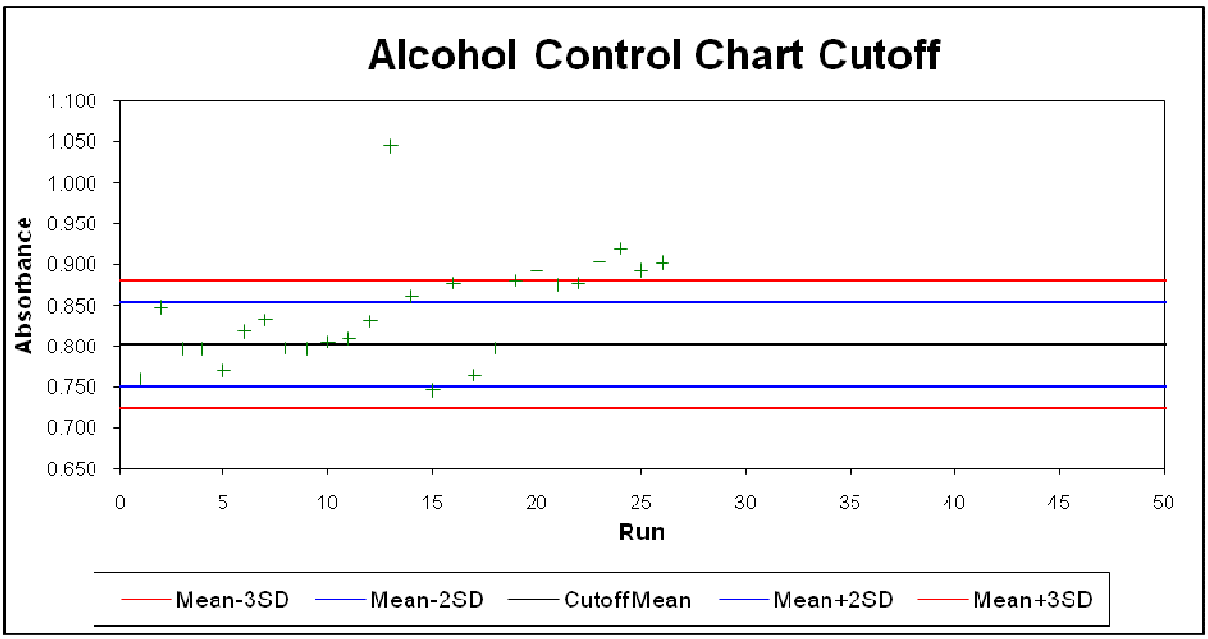


Figure 26: Alcohol Control Chart Cut-off Value

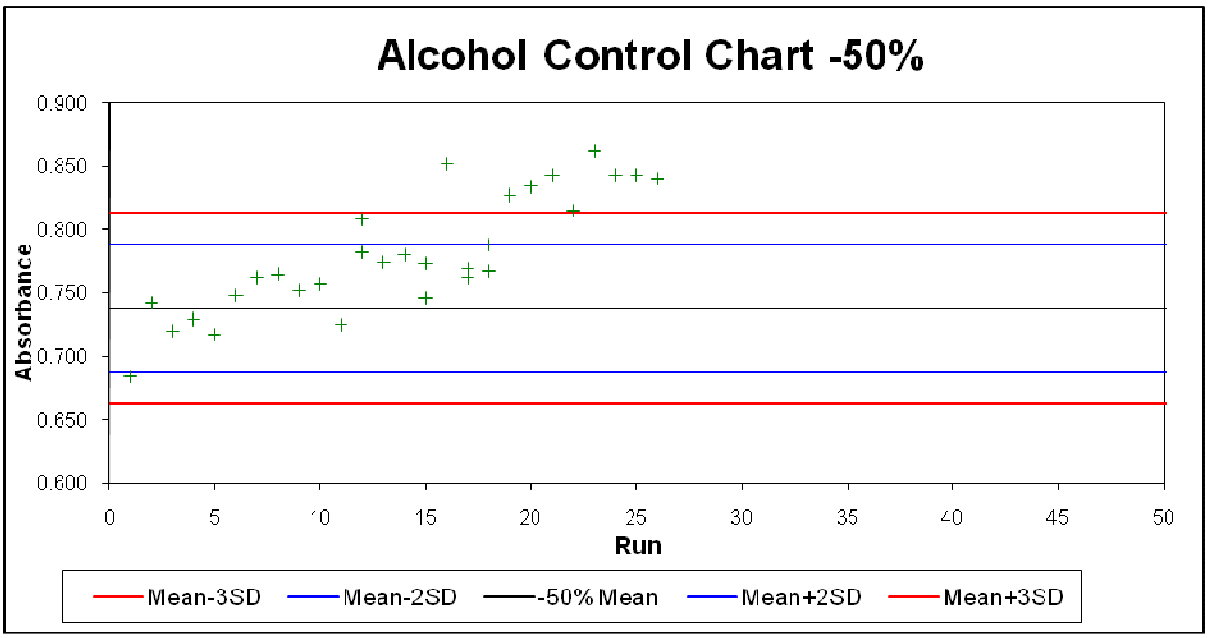


Figure 27: Alcohol Control Chart 50% Below Cut-off

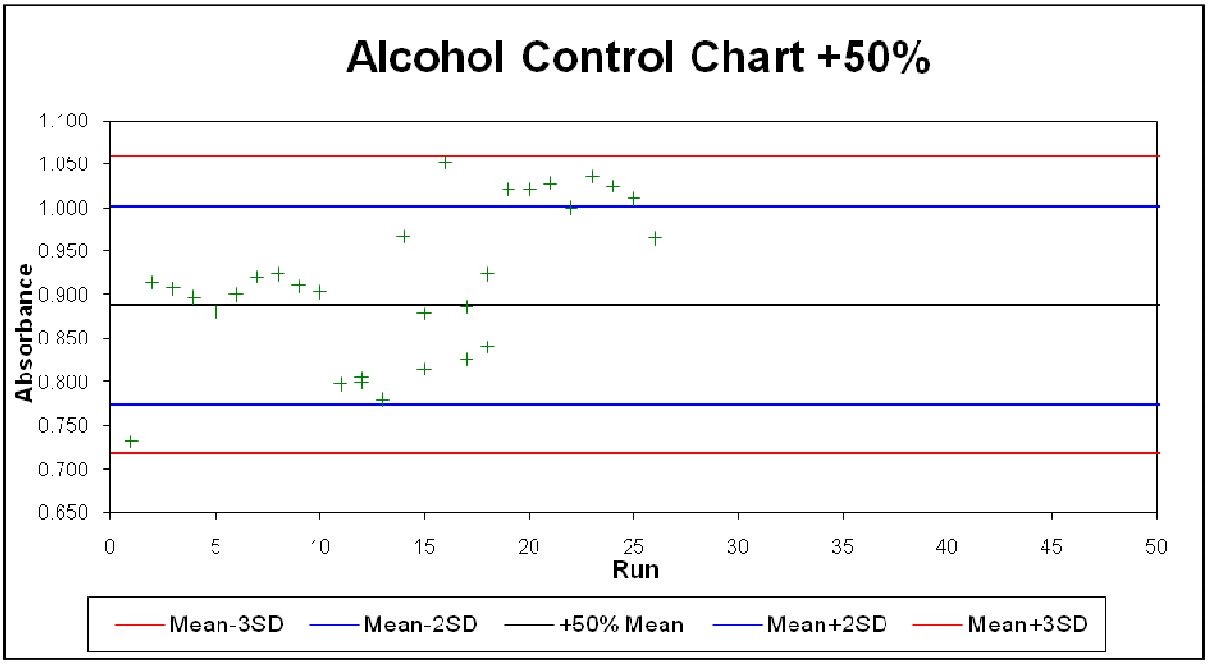


Figure 28: Alcohol Control Chart 50% Above Cut-off

Figure 26 – Figure 28 above highlight the problem with Shewart-style QC charts for this analysis. They demonstrate that the controls give fairly sporadic results as there are a number of factors that influence the absorbance. There is a general upwards trend in each of the above charts as reagent E gets closer to its expiry date. The azide preservative in the solution gives the solution its colour and gets darker as the solution approaches its expiry date. The reagent has a short shelf life, only a couple of months, and slight changes can be observed in the QC charts over time

3.5 Case Samples

3.5.1 Confirmed Samples

A number of samples (both positive and negative screening results) were confirmed by headspace GC-FID for the presence of alcohol. The results are detailed in the Table 21.

Table 21: Confirmation Results for Alcohol Samples

Sample Details	Alcohol Results	
Sample ID	**Screening**	**Confirmation**
015070	Neg	Neg
027660	Neg	Neg
027713	Neg	Neg
041616	Positive	Neg
041669	Neg	Neg
041676	Neg	Neg
042532	Neg	Neg
043746	Neg	Neg
043839	Neg	Neg
043881	Neg	Neg
043914	Neg	Neg
044102	Positive	23
044116	Neg	Neg
044194	Positive	314
055625	Positive	180
057429	Borderline	Neg
089931	Neg	Neg
092020	Neg	Neg
092045	Neg	Neg
092055	Positive	12
092073	Neg	Neg
092089	Positive	20
092108	Positive	Neg

Sample Details	Alcohol Results	
Sample ID	**Screening**	**Confirmation**
092109	Neg	Neg
092110	Neg	Neg
092113	Neg	Neg
092119	Positive	Neg
092123	Borderline	Neg
092264	Neg	Neg
092290	Positive	22
092292	Neg	Neg
092302	Borderline	11
092325	Neg	Neg
092328	Neg	Neg
092336	Neg	Neg
092339	Neg	Neg
092350	Neg	Neg
096563	Borderline	Neg
097494	Neg	Neg
097573	Neg	Neg
098102	Neg	Neg
099249	Positive	31
099260	Neg	Neg
099376	Neg	Neg
099382	Neg	Neg

The confirmation results generally match the screening results and there were no false negatives from the samples that were confirmed. This is somewhat surprising as there is not a large difference between the blank and cut-off calibrators absorbance values so more false negatives would have been expected. These results indicate that the assay would be useful as a screening technique for alcohol screening.

3.5.2 Unconfirmed Samples

Approximately fifty other oral fluid samples were screened as part of this project but due to the limited sample volumes obtained by oral fluid collection devices there was not enough left for confirmations as the samples had previously been analysed by another laboratory.

3.5.3 Sensitivity and Specificity

There were 8 true positives, 31 true negatives, 6 false positives and no false negatives for the alcohol assay.

NB: For the purposes of this calculation, borderline cases were treated as being positives.

Following the calculation given in section 2.3.6 the sensitivity of the alcohol assay was calculated as 100% and the specificity was calculated as 84%.

3.6 Conclusions

The Immunalysis Ethyl Alcohol Screening Kit has shown potential as a screening method but would require further work and validation before it demonstrated as fit for use in a routine testing laboratory. The calibrators and controls have shown short term stability over a two week period, which is the likely time frame for sample analysis in a high throughput routine testing laboratory.

A more suitable method of evaluating the performance of the control samples would need to be devised and evaluated as the current QC charts are unsuitable for a

small laboratory who would not use all of the reagent in one or two days and would therefore limit the assay's use to high throughput laboratories.

The assay consistently produced a linear response for the calibration range tested and as such has potential as a semi-quantitative test, which would be useful for highlighting any samples that may require a dilution prior to analysis. However, the issue of the highest calibrator "losing" absorbance over time would need to be thoroughly investigated as this would lower the gradient of the calibration and samples pipetted later in the plate would appear to be of a higher concentration than they actually are, while earlier low concentration samples could appear negative. This would forfeit the point of the screening test as false negatives would be produced and samples requiring dilution would not be properly identified.

If the issues outlined are successfully addressed and the analysis of test samples proves successful then this method could be used as a screening procedure for alcohol in oral fluid. The main benefits of the assay are that it is simple to use and provides a fast screening result. In addition, it has the potential to be used semi-quantitatively if the issues mentioned above are resolved. However, the assay does have its limitations in that the sample preparation step for analysis is to pipette the sample into wells, which is the same as the confirmation method. So in this respect, the sample preparation is as time-consuming as the confirmation method and it would make more sense to go straight to the confirmation method even though the results for the screening test would be obtained within 15 minutes whereas the confirmation method would take several hours. If the assay was being used in a setting where most samples were expected to be negative, for example in the workplace, then it would be a good choice.

In a laboratory that gets many positives, such as a forensic laboratory that carries out alcohol testing in road traffic cases then this method is only likely to add to the workload and therefore could not be justified.

Overall, the assay would not be recommend for a laboratory that is only analysing a small number of alcohol analyses a day as it would offer no significant advantage due to the issues highlighted above. In addition, it would not be recommend the for a high throughput laboratory analysing several hundred samples daily if they are expecting many positives as it would be an added expense and waste of time. It

would however, be very useful if utilized in a laboratory analysing samples for drug abstinence in the workplace.

Initial results from real samples gave promising results, but further real samples should be tested before this method is put to use routinely in a laboratory.

4 Conclusions

The Immunalysis ELISA assays worked well with oral fluid that had been diluted one in four with Quantisal buffer. The Quantisal oral fluid collection device dilutes 1mL of sample with 3mL of buffer giving one of the largest dilutions of all commercially available oral fluid collectors. This raised the possibility of screening results giving false negatives due to the buffer diluting the sample below the cut-off concentration. This did not appear to be the case and means that the Quantisal oral fluid collection device offers the distinct advantage of having more oral fluid/buffer mixture to analyse than other commercially available devices.

The Immunalysis ELISA assays for drugs of abuse that were used for this project proved sufficiently sensitive to detect the low drug concentrations found in the diluted oral fluid/buffer mixture. The assays did raise a couple of questions with very low amphetamine concentrations that were below the lowest calibrator. The confirmation results for the samples that “falsely” screened negative showed that they were below the ELISA assay’s in-house cut-off limit and were therefore accurately marked as negative by ELISA. The Immunalysis ELISA assays are therefore suitable for use as a laboratory based screening technique for oral fluid samples.

The alcohol enzymatic assay showed some potential as a screening technique for oral fluid. The assay showed good linearity and of the confirmed samples, there were no false negatives identified. The study did highlight some issues that would require further investigation before being used routinely in a toxicology laboratory. This study showed a worrying trend of the absorbance of the highest calibrator falling with time between addition to the well and addition of the other reagents. From a single analysis, this relationship appeared to be linear but further investigation would be required to confirm this relationship.

One of the reagents appeared to get darker in colour, and thus gave different values for its absorbance once opened and this meant that the preferred in-house method of using Shewart-style QC charts was unsuitable for this analysis. No obvious alternative method of monitoring quality control was available and this would need to be rectified if the assay was to be used in a laboratory accredited to ISO/IEC 17025 where the monitoring of quality control samples is so important. The reagent

becoming darker was observed after one day and this could present a problem to small scale laboratories that are not carrying out many analyses.

Overall, this method has the potential to be valuable to laboratories testing oral fluid samples where sample volume is limited and also to high throughput laboratories where most of the samples are expected to be negative, such as laboratories that carry out workplace testing.

5 Further Work

Further work to evaluate the stability of the controls would be to prepare several litres of the controls and analyse weekly for up to five years to assess the long term stability of multi-analyte controls. Several batches of controls should be prepared simultaneously and analysed at the same time to ensure the results are reproducible.

Concurrently, controls could be prepared and stored, ready diluted in Quantisal buffer, to allow a comparison between the stability of diluted and neat controls over time. Both the neat and diluted controls should be prepared on the same day with the same blank oral fluid and drug stock solutions and stored in the same freezer to minimise the differences between the controls, thus reducing the number of variable factors which may affect the stability.

Another step would be to examine why some drug groups have such a wide range of acceptable values and others have a narrow range. Examining whether each drug's assay works better at a specific temperature and whether that is a reason for the more sporadic results for some drugs, could improve the reproducibility of the results for some assays.

As the calibrators for ELISA used a different buffer to the oral fluid controls (to keep the method in line with the in-house blood and urine method), the effect of this could be investigated to see if there is any matrix effects from the different buffers.

Further samples should be collected and analysed purely for amphetamines if possible to allow sufficient sample to be analysed and any repeats carried out. This would allow the questionable false negatives to be clarified and further data to be collected to allow for a more accurate confirmation of the borderline positive confirmations.

Spiking samples at and below the lowest calibrator from the confirmation method would provide information about the assays suitability at low concentrations.

The first step would be to devise a more suitable method of quality control. Once this is in place, the next step would be to evaluate the long term stability of the calibrators and controls. Longer stability would reduce the running costs of the screening test as certified alcohol standards are expensive. While the stability study is underway, an

evaluation of the effect of reagent E could be undertaken by analysing two batches of calibrators and controls, one with a new reagent E each time and the other using a previously opened reagent E. My results indicated that there was a slight increase in the absorbance after just one day, however, that could have been due to slight differences in the analysis such as a slightly longer incubation time or a change in laboratory temperature. The effect of these should also be investigated.

The apparent loss of alcohol from the highest calibration over time is a serious issue that would also need to be fully investigated prior to being implemented in a laboratory. This could be done by analysing controls on one plate and leaving a set period of time between the additions of controls to each strip and adding reagents A and E once all strips have had sample added. Also, a strip of calibrators could be added to a plate every two minutes to see any change in absorbance when reagents A and E are added to all strips at the same time.

6 References

1. Duffus J, Worth H. Fundamental Toxicology: RSC; 2006.

2. Anderson RA. Forensic Toxicology. In: White PC, editor. Crime Scene to Court. 2nd ed. Cornwall: RSC; 2004. p. 318-49.

3. Krieger R, editor. Handbook of Pesticide Toxicology: Principles. 2nd ed. California, USA: Academic Press; 2001.

4. Diem K, Lentner C, editors. Scientific Tables. 7th ed. Macclesfield: Geigy Pharmaceuticals; 1973.

5. Spiehler V. Drugs in Saliva. In: Moffat AC, Osselton MD, Widdop B, editors. Clarke's Analysis of Drugs and Poisons: Pharmaceutical Press; 2004. p. 109-23.

6. Ritschel WA, Tompson GA. Monitoring of Drug Concentrations in Saliva: A Non-invasive Pharmacokinetic Procedure. Methods and Findings in Experimental and Clinical Pharmacology. [Article]. 1983;5(8):511-25.

7. Aps JK, Martens LC. Review: The physiology of saliva and transfer of drugs into saliva. Forensic Science International2005 Jun 10;150(2-3):119-31.

8. Spiehler V, Cooper G. Drugs-of-Abuse Testing in Saliva or Oral Fluid. In: Jenkins AJ, editor. Drug Testing in Alternate Biological Specimins: Humana Press Inc; 2008. p. 83-99.

9. Turner R. Mechanisms of Fluid Secretion by Salivary Glands. Annals of the New York Academy of Sciences1993;694(Saliva as a Diagnostic Fluid):24-35.

10. Gland Salivary. [cited 05/01/2009]; Available from: **http://img.tfd.com/dorland/thumbs/gland_salivary.jpg**.

11. Gallardo E, Queiroz JA. The Role of Alternative Specimens in Toxicological Analysis. Biomedical Chromatography2008;22(8):795-821.

12. Malamud D. Guidelines for Saliva Nomenclature and Collection. Annals of the New York Academy of Sciences1993;694(Saliva as a Diagnostic Fluid):xi-xii.

13. Cone EJ, Oyler J, Darwin WD. Cocaine Disposition in Saliva Following Intravenous, Intranasal, and Smoked Administration. J Anal Toxicol. [Article]. 1997 Oct;21(6):465-75.

14. Cone EJ, Kumor K, Thompson LK, Sherer M. Correlation of Saliva Cocaine Levels with Plasma-Levels and with Pharmacologic Effects After Intravenous Cocaine Administration in Human-Subjects. J Anal Toxicol. [Article]. 1988 Jul-Aug;12(4):200-6.

15. Cone E, Huestis M. Interpretation of Oral Fluid Tests for Drugs of Abuse. Annals of the New York Academy of Sciences2007;1098(1):51-103.

16. Schepers RJF, Oyler JM, Joseph RE, Cone EJ, Moolchan ET, Huestis MA. Methamphetamine and amphetamine pharmacokinetics in oral fluid and plasma after controlled oral methamphetamine administration to human volunteers. Clinical Chemistry2003 Jan;49(1):121-32.

17. Cook CE, Jeffcoat AR, Sadler BM, Hill JM, Voyksner RD, Pugh DE, et al. Pharmacokinetics of Oral Methamphetamine and Effects of Repeated Daily Dosing in Humans. Drug Metab Dispos. [Article]. 1992 Nov-Dec;20(6):856-62.

18. Wille SMR, Raes E, Lillsunde P, Gunnar T, Laloup M, Samyn N, et al. Relationship Between Oral Fluid and Blood Concentrations of Drugs of Abuse in Drivers Suspected of Driving Under the Influence of Drugs. Ther Drug Monit. 2009 Aug;31(4):511-9.

19. Gjerde H, Normann P, Christopherson A. The Prevalence of Alcohol and Drugs in Sampled Oral Fluid is Related to Sample Volume. J Anal Toxicol2010;34:416 - 9.

20. Directgov. Drug Testing and Employee Monitoring. 2009 [12/07/2009]; Available from: **http://www.direct.gov.uk/en/Employment/HealthAndSafetyAtWork/DG_10026594**.

21. Office of Public Sector Information. Transport and Works Act 1992. 1992 [14/07/2009]; Available from: **http://www.opsi.gov.uk/acts/acts1992/ukpga_19920042_en_4#pt2-ch1**.

22. Taylor D. Rise in use of Drug Testing to Sack Employee's Without Redundancy Pay. The Gaurdian. 2009 18/05/2009.

23. Home Office. Drug Interventions Programme (DIP). 2003 [12/07/2009]; Available from: **http://drugs.homeoffice.gov.uk/drug-interventions-programme/strategy/**.

24. Home Office. The Drug Interventions Programme (DIP): Addressing Drug use and Offending Through 'Tough Choices'. 2007 [12/07/2009]; Available from: **http://www.homeoffice.gov.uk/rds/pdfs07/horr02c.pdf**.

25. Concateno. Philips and Concateno Announce Revolutionary Drugs-of-Abuse Testing System Concateno PLC; 2008 [12/07/2009]; Available from: **http://www.concateno.com/index.php?cPath=78_193&news_id=123**.

26. Concateno. Oslo: Concateno to preview new portable drug testing device at ICADTS. Concateno PLC; 2010 [15/09/2010]; Available from: **http://www.concateno.com/news-events-publications/press-releases/16171/**.

27. Jackson P, Hilditch C. A Review of Evidence Related to Drug Driving in the UK: A Report Submitted to the North Review Team. London, UK: Clockwork Research LTD, Transport Df;2010.

28. Crown Office. Railways and Transport Safety Act 2003. 2003 [15/09/2010]; Available from: **http://www.legislation.gov.uk/ukpga/2003/20/schedule/7**.

29. Home Office Scientific Developement Branch. Drug Driver Testing. 2010 [15/09/2010]; Available from: **http://tna.europarchive.org/20100413151426/http://scienceandresearch.homeoffice.gov.uk/hosdb/drug-detection/drug-driver-testing/index.html**.

30. General Register Office for Scotland. Drug-Related Deaths in Scotland in 2009. In: Scotland GROf, editor. Edinburgh: General Register Office for Scotland; 2009.

31. Office for National Statistics. Alcohol Deaths. 2009 [updated 13/07/200921/07/2009]; Available from: **http://www.statistics.gov.uk/CCI/nugget.asp?ID=1091**.

32. United Nations Office on Drugs and Crime. World Drug Report 2009. Vienna: United Nations; 2009.

33. United Nations. World Drug Report 2010. Vienna: United Nations; 2010.

34. NHS Scotland. Alcohol Statistics Scotland 2009. 2009 [21/07/2009]; Available from: **http://www.alcoholinformation.isdscotland.org/alcohol_misuse/files/alcohol_stats_bul_09.pdf**.

35. Audit Scotland. Drug and Alcohol Services in Scotland. Audit Scotland; 2009 [14/07/2009]; Available from: **http://www.audit-scotland.gov.uk/docs/health/2009/nr_090326_drugs_alcohol.pdf**.

36. Hand C, Baldwin D. Immunoassays. In: Jickells S, Negrusz A, editors. Clarke's Analytical Toxicology. Cambridge: Pharmaceutical Press; 2008. p. 375 - 91.

37. Yong CY, Chua WT, Moy HY, Yao YJ, Lui CP. Screening of drug of abuse in urine by LC/MS/MS. Clinica Chimica Acta;411(11-12):910-1.

38. Dresen S, Ferreiros N, Gnann H, Zimmermann R, Weinmann W. Detection and identification of 700 drugs by multi-target screening with a 3200 Q TRAP(A (R)) LC-MS/MS system and library searching. Anal Bioanal Chem. [Proceedings Paper]. Apr;396(7):2425-34.

39. Langel K, Engblom C, Pehrsson A, Gunnar T, Arinieme K, Lillsunde P. Drug Testing in Oral Fluid— Evaluation of Sample Collection Devices. J Anal Toxicol2008;32:9.

7 Appendices

7.1 Appendix 1 – Amphetamine Worksheet

Worksheet for the Extraction of Amphetamines from Oral Fluid

***IMPORTANT – A Batch Record Form (FMS2005/F001) must be completed before starting the extraction process ***	Analyst: _____ Date: _____

Follow the steps outlined below, noting when asked the solution or reagent numbers. Initial and date each step as completed and ask the witness to initial and date when prompted.

==========

STEP ONE: Preparation of Standard Calibrators, Samples and QC's (WITNESSED)

The preparation of the following standards, samples and controls must be carried out in a fume hood or safety cabinet using labelled glass test-tubes. Tubes should be labelled for the standards, blank, spike and samples.

- To each standard test tube add the following volumes of Amphetamines working standard (1µg/mL) **(SOL No.:_____).**
- 50µL of 1µg/mL internal standard (AMP-d11, MAMP-d14, MDA-d5, MDMA-d5 and MDEA-d6) **(SOL No.:_____)** should be added to all test tubes.
- A blank with just internal standard should also be prepared.
- Two spikes should be prepared by adding 1mL of the positive amphetamine oral fluid QC (X2) **(CON No.:_____) (CON No.:_____)**.
- A total of 9 blanks and standards.

Once all standards have been added, 3mL of 0.1M phosphate buffer pH 6.0 **(SOL No.:_____)** should be added, followed by 1mL of blank oral fluid **(SOL No.:_____)**.

- Pipette 0.5mL of each sample into the appropriately labelled test-tube and note any changes to the sample volume on form FMS2005/F001.

Standard (concentration)	Volume of Working Solution (1µg/mL)	Volume of Internal Standard (1µg/mL)
Std 1 (5ng/mL)	5µL	50µL
Std 2 (10ng/mL)	10µL	50µL
Std 3 (25ng/mL)	25µL	50µL
Std 4 (50ng/mL)	50µL	50µL
Std 5 (100ng/mL)	100µL	50µL
Std 6 (200ng/mL)	200µL	50µL
Blank (0ng/mL)	0µL	50µL
Spike (45ng/mL)	1mL of QC	50µL

Note: Date of calibration used: _____

- Vortex mix all standards, samples and spike then centrifuge at 2500rpm for 10 minutes.

Analyst/Date: _____	**Witness/Date:** _____

STEP TWO: **Sample Extraction**

- Place labelled CleanScreen columns on the vacuum manifold.
- Add 3mL of methanol **(CHEM No.:_____).**
- Add 3mL of deionised water **(CHEM No.:_____)**.
- Add 1mL of 0.1M phosphate buffer pH 6.0 **(SOL No.:_____)**.
- Transfer the buffered sample to the column and allow to pass through completely **(WITNESSED)**.

Analyst/Date: _____ **Witness/Date:** _____

- Add 3mL of deionised water **(CHEM No.:_____)**.
- Add 1mL of 1M acetic acid **(SOL No.:_____)**.
- Add 3mL of methanol **(CHEM No.:_____)**.
- Dry under full vacuum for 5 minutes.
- Place labelled 4mL glass vials in a rack within the vacuum manifold **(WITNESSED)**.

Analyst/Date: _____ **Witness/Date:** _____

- Elute with 2mL of DCM/IPA/NH_3 (78:20:2 v/v/v) **(SOL No.:_____)**.
- Add 100µL of tartaric acid **(SOL No.:_____)**.
- Evaporate to dryness under N_2 at **ROOM** temperature.
- Add 50µL of PFPA:EtOAc (2:1 v/v) **(SOL No.:_____).**
- Cap the vials and derivatise at 60ºC for 20 minutes.
- Evaporate to dryness under N_2 at **ROOM** temperature.
- Reconstitute in 50µL of ethyl acetate **(SOL No.:_____)** and transfer to labelled injection vials with inserts.

Analyst/Date: _____ **Witness/Date:** _____

7.2 Appendix 2 – ELISA Assay Specification

7.2.1 Immunalysis Amphetamine ELISA Specification

Specificity
The specificity of the Immunalysis ELISA for Amphetamine was determined by generating inhibition curves for each of the compounds listed below The antisera cross-reactivities are listed in Table 2.

Table 2

Compound	Approx. ng/ml equivalent to 25ng amphetamine	Cross-reactivities percentage
l-Amphetamine	865	2.9
Hydroxyamphetamine HCl	57	44
l-Methamphetamine HCl	1250	2
dl-MDA (methylenedioxyamphetamine)	10	250
d-Methamphetamine HCl	417	6.5
dl- HMA (hydroxy methoxyamphetamine)	100	25
Phentermine	28	89
Fenfluramine	>2500	<1
d-Ephedrine	>2500	<1
l-Ephedrine	>2500	<1
d-Phenylpropanolamine	>2500	<1
l-Phenylpropanolamine	>2500	<1
dl-MDMA (methylene dioxymethamphetamine)	>2500	<1
dl-MDEA (methylene dioxyethylamphetamine)	>2500	<1
d-Pseudoephedrine	>2500	<1
l-Pseudoephedrine	>2500	<1
dl-MBDB (N-methyl-3,4methylene dioxyphenyl-2-butanamine)	>2500	<1
Tyramine	>2500	<1
Methylphenidate	>2500	<1

Cross-Reactivity with Unrelated Drugs

Aliquots of a human urine matrix were spiked with the following compounds at a concentration of 5,000 ng/ml. None of these compounds gave values in the assay that were equal to or greater than the assay sensitivity level (1 ng/ml).
Acetaminophen, Acetylsalicylic acid, Aminopyrine, Ampicillin, Amobarbital, Ascorbic acid, Atropine, Barbital, Benzoylecgonine, Butabarbital , Caffeine , Cocaine, Carbamazepine, Codeine , Chloroquine , Chlorpromazine, Carbromal , Desipramine, Dextromethorphan, Dextropropoxyphene , 5,5-Diphenylhydantoin, 10-11-Dihydrocarbamazepine , Diazepam, Ethosuximide, Estriol, Estrone, Estradiol, Ethotoin, Glutethimide , Hexobarbital, Ibuprofen, Imipramine, Lidocaine, LSD, Methadone , Methadone-primary metabolite, Methaqualone, Methbarbital , Mephenytoin, *-Methyl-*-propylsuccinimide, Mephobarbital, Methyl PEMA, Methsuximide , 4-Methylprimidone, Morphine , Meperidine , Niacinamide, Norethindrone, N-Normethsuximide, Phenobarbital , Phensuximide, PEMA, Primidone, Phencyclidine, Pentobarbitol, Phenothiazine, Phenylpropanolamine, Procaine, Quinine, Secobarbital, Tetracycline, Tetrahydrozoline, THCCOOH

REFERENCES
1. Urine Testing for Drugs of Abuse, National Institute on Drug Abuse Research Monograph. 73: 95-97 (1986).
2. N.Weiner. Norepinephrine, epinephrine and the sympathomimetic amines. In: The Pharmacological Basis of Therapeutics. 7th ed. p.145-180 (New York: MacMillan 1985).
3. J. Caldwell and P.S. Sever. The Biochemical Pharmacology of Abused Drugs. Clinical Pharmacology and Therapeutics. 16: 625- 638 (1974).
4. R. C. Baselt. In: Advances in Analytical Toxicology, Vol.1. p.87 - 93. Ed. R. C. Baselt, Biomedical Publications, Foster City, CA (1984).

IMMUNALYSIS CORPORATION
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IMMUNALYSIS

AMPHETAMINE DIRECT ELISA KIT

Version: 05/2008

Immunalysis Corporation :
Catalog Number 209-0192 2 x 96 well plates
Catalog Number 209-0480 5 x 96 well plates
Catalog Number 209-4800 50 x 96 well plates

THE IMMUNALYSIS AMPHETAMINE DIRECT ELISA KIT IS INTENDED FOR FORENSIC USE ONLY.

The Immunalysis Amphetamine Direct ELISA Kit provides only a preliminary analytical test result. A more specific alternate chemical method must be used in order to obtain a confirmed analytical result. Gas chromatography/ mass spectrometry (GS-MS) is the preferred confirmatory method (1). Professional judgement should be applied to any drug of abuse test result, particularly when preliminary positive results are used.

EXPLANATION OF THE TEST

The Immunalysis Amphetamine Direct ELISA Kit is a specific and sensitive in-vitro test to detect the presence of d-amphetamine in forensic samples such as whole blood, oral fluids, serum, plasma and urine. While the assay will detect amphetamine use, interference by l-amphetamine and pseudo-ephedrine is virtually nonexistent.

Amphetamine is a potent central nervous system stimulant. The (+)-isomer also referred to as d-amphetamine is three to four times more potent than the (-)-isomer, l-amphetamine (2). Amphetamine may be metabolized and excreted as the p-hydroxy isomer. Amphetamines act by inducing euphoria, irritability, anxiety and paranoia. Urinary excretion rates are influenced by the urinary pH with acidic urine favoring the excretion of unchanged drug(2). Up to 80% of a given dose may be excreted unchanged, especially in acid urine. Alkaline urine reduces the excretion of unchanged amphetamine to less than 5% of the dose.

PRINCIPLES OF THE PROCEDURE

The Immunalysis Amphetamine Direct ELISA Kit (for d-amphetamine measurement) is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture.

A 10 µl. aliquot of a diluted unknown specimen is incubated with a 100 µl. dilution of enzyme (Horseradish peroxidase) labeled d-amphetamine derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 1 ng/ml.

The Immunalysis Amphetamine Direct ELISA Kit avoids extraction of urine sample for measurement. It employs a d-amphetamine directed antiserum. Due to the proprietary method of orienting the antibody on the polystyrene micro-plate much higher sensitivity is achieved compared to passive edsorption. This allows an extremely small sample size reducing matrix effects and interference with binding protein(s) or other macromolecules.

MATERIALS AND EQUIPMENT

Materials and equipment required but not supplied with the Immunalysis Amphetamine Direct ELISA Kit are itemized below:
12x75 mm Disposable Glass or Plastic Culture Tubes to pre-dilute samples (if required).
Manual or electronic micropipets (single channel or multi channel) or automated pipetting stations.
Refrigerator (for kit storage).
Interval Timer.
Wash bottle or Plate Washer.
Micro-plate reader capable of reading at 450 nm and 650 nm.

REAGENTS

Immunalysis Amphetamine Direct ELISA Kit Contents

Component	192 Test Kit Cat# 209-0192	480 Test Kit Cat# 209-0480	4800 Test Kit Cat# 209-4800
96 well Micro-plate	2	5	50
d-Amph-Conjugate	25 ml	60 ml	750 ml
Neg Std	2 ml	5 ml	3 x 5 ml
TMB Substrate	30 ml	2 x 30 ml	750 ml
Stop Reagent	25 ml	55 ml	750 ml

96 well micro-plate. The micro-plate is coated with polyclonal anti-d-amphetamine via a spacer chain to provide optimally oriented binding sites. The plates are sealed in a moisture and air barrier pouch with a desiccant.

Enzyme Conjugate. The conjugate solution contains d-amphetamine labeled with horseradish peroxidase in a buffered, protein solution with stabilizers, pH 7.6 containing azide free preservatives. (Colored Red)

Negative Control. This bottle contains drug free synthetic urine containing azide free preservatives.

TMB chromogenic substrate. The color reagent contains 3,3',5,5' tetramethylbenzidine and peroxide in buffer.

Stop solution. This contains 1 N hydrochloric acid.

Precautions
1. Not for Internal or External Use in Humans or Animals.
2. There should be no eating or drinking within work area.
3. Always wear gloves and a protective lab coat.
4. No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.
5. **Do not add sodium azide to samples as preservative.**
6. **Do not use external controls containing sodium azide.**
7. Use disposable pipet tips to avoid contaminating chromogenic substrate reagent. Discard reagent if it turns blue.
8. Do not pour chromogenic substrate back into container after use.
9. Do not freeze reagents.
10. Do not mix reagents from different kit lot numbers.
11. Keep reagents out of direct sunlight.
12. Handle Acid stop reagent with care, since it is corrosive.
13. Bring all reagents to room temperature.
14. Viscous forensic samples should always be diluted in phosphate buffered saline or distilled water prior to pippetting.
15. Ensure the bag containing the micro-plate strips and dessicant is sealed well, if only a few strips are used.

General. Precise pipetting is the essence of successful radio immunoassay. Micropipets supplied by "Eppendorf" or "SMI" with disposable tips are excellent when used carefully according to instructions to insure the necessary accuracy. New automatic dispensers improve reliable delivery.

Storage. The expiration date of the kit is stated on the label. The kit can be expected to perform satisfactorily until the expiration date if stored in the refrigerator at 2 – 4° C.

Indications of Deterioration. A drop of greater than 50% in the A_0 value (zero standard absorbance reading) for a constant incubation time indicates deterioration of the antibody plate, enzyme conjugate or chromogenic substrate. A significant shift of the standard curve to the right would result from deterioration of the standards. Development of blue color in the chromogenic substrate without the addition of enzyme conjugate indicates contamination of the substrate.

SPECIMEN COLLECTION

Precautions.
The Immunalysis Amphetamine Direct ELISA Kit is to be used with human forensic samples, such as whole blood, oral fluids, serum, urine and plasma. Immunalysis has not tested all possible applications of this assay. Cutoff criteria are important in deciding the sample dilution.

Additives.
Specimens to which sodium azide has been added affect the assay.

Storage and Handling Instructions.
Urine samples should be stored at 2 - 4° C until use. Samples should be well mixed before assay. Repeated freezing and thawing should be avoided. Urine samples should be shipped refrigerated with Blue Ice or equivalent.

DETAILS OF THE PROCEDURE

All reagents must be brought to room temperature (20-25° C) before use.

The procedure as described below may be followed in sequence, using manual pipettes. Alternatively all reagents may be added using an automated pipettor.

1. Dilute forensic specimens, to the necessary range with Phosphate Buffer Saline pH 7.0. (Urine samples are normally diluted 1:20 for a Amphetamine cutoff of 500 ng/ml.) The dilution factor and volume added can be adjusted based on the laboratory's cutoff.

2. Add 10 µl. of appropriately diluted calibrators and standards to each well in duplicate.
3. Add 10 µl. of the diluted specimens in duplicate (recommended) to each well.
4. Add 100 µl. of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.
5. Incubate for 60 minutes at room temperature (20-25° C) preferably in the dark, after addition of enzyme conjugate to the last well.
6. Wash the wells 6 times with 350 µl. distilled water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells. If testing samples containing abnormally high amounts of hemoglobin (some Postmortem samples), use 10 mM Phosphate buffered saline pH 7.0-7.4. This will lower potential nonspecific binding of hemoglobin to the well, thus lowering background color.
7. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.
8. Add 100 µl. of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
9. Incubate for 30 minutes at room temperature, preferably in the dark.
10. Add 100 µl. of Stop Solution to each well, to change the blue color to yellow.
11. Measure the absorbance at a dual wavelength of 450 nm and 650 nm.
12. Wells should be read within 1 hour of yellow color development.

The following data represent a typical dose/response curve.

d-amphetamine ng/ml	Absorbance
0	2.459
10	0.891
25	0.431
50	0.255

The dose/response curve shown above should not be used in assay calculations. It is recommended that at least one in-house positive quality control be included with every assay run. A dose response curve or a cutoff calibrator should be run with every plate.

RESULTS

If the average sample absorbance is equal to or less than the average absorbance of the laboratory positive reference standard the sample is POSITIVE for amphetamine. If the average sample absorbance is greater than the average absorbance of the laboratory positive reference standard the sample is called NEGATIVE for amphetamine.

Alternatively a dose response curve can be established by plotting standard concentration (abscissa) against corresponding absorbance (ordinate). Values for unknown samples are obtained by interpolation from the curve.

SPECIFIC PERFORMANCE CHARACTERISTICS

Accuracy
Forty whole blood samples and 40 urine samples collected from presumed non-users were tested in the Immunalysis Amphetamine Direct ELISA Kit. One hundred percent of these normal samples measured negative at 50 ng/ml for whole blood and 500 ng/ml for urine. Thirty five whole blood samples which were previously confirmed positive for amphetamine by GC-MS employing a cut-off of 50 ng/ml, were tested in the Immunalysis Amphetamine Direct ELISA Kit. All of the samples were found to be positive i.e. above the cut-off of 50 ng/ml.

Precision
The precision of the Immunalysis Amphetamine Direct ELISA Kit has been verified by assessment of the mean, standard deviation (SD) and coefficients of variation (CV) in data resulting from repetitive assays.

Intra-assay Precision
Intra-assay precision was determined with reference controls.
A 0, 10, 25 and 50 ng/ml standard was assayed five times in the same assay. The results are tabulated in Table 1.

Table 1

Amphetamine (ng/ml)	Mean Abs.	S.D.	C.V.%
0	2.399	0.115	4.8
10	0.897	0.095	10.6
25	0.458	0.061	13.32
50	0.271	0.022	8.12

Sensitivity
Assay sensitivity based on the minimum amphetamine concentration required to produce a four standard deviation from assay A_0 is 1 ng/ml.

7.2.2 Immunalysis Benzodiazepine ELISA Specification

Specificity

The specificity of the Immunalysis Benzodiazepines ELISA for was determined by generating inhibition curves for each of the compounds listed below The antisera cross-reactivities are listed in Table 2A and Table 2B.

TABLE 2A

Analyte	Conc pg/well	Oxazepam pg/well	Cross Reactivity
Oxazepam	250	250	100%
Alprazolam	50	90	180%
	100	240	240%
OH-Alprazolam	100	70	70%
	250	300	120%
Bromazepam	500	350	70%
	1000	700	70%
Chlordiazepoxide	500	150	30%
	1000	180	18%
Clonazepam	50	35	70%
	100	70	70%
7-amino-Clonazepam	500	200	40%
	1000	250	25%
Diazepam	100	70	70%
	250	300	120%
Nordiazepam	100	50	50%
	250	200	80%
Estazolam	100	70	70.00%
	250	300	120%
Flurazepam	100	100	100%
	250	375	150%
2-OH--Ethyl-Flurazepam	500	>1200	>240
N-desmethyiflunitrazepam	500	450	90%
	1000	1000	100

TABLE 2B

Analyte	Conc pg/well	Oxazepam pg/well	Cross Reactivity
Halazepam	500	500	100%
	1000	950	95
Lorazepam	50	45	90%
	100	85	85%
Lorazepam -Gluc.	1000	ND	ND
Lormetrazepam	500	600	120%
Medazepam	500	200	40%
	1000	400	40%
Midazolam	100	60	60%
	250	110	44%
Nitrazepam	500	350	70%
	1000	700	70%
Prazepam	500	225	45%
	1000	500	50%
Temazepam	500	1000	200%
Triazolam	50	37.5	75%
	100	80	80%
OH-triazolam	50	40	80%
	100	80	80%
Oxazepam Gluc.	1000	ND	ND
Clorazepate	100	50	50%
	250	150	60%
Flunitrazepam	100	30	30%
	250	150	60%

Cross-Reactivity with Unrelated Drugs

Aliquots of a human urine matrix were spiked with the following compounds at a concentration of 10,000 ng/mL. None of these compounds gave values in the assay that were equal to or greater than the assay sensitivity level (2 ng/mL).

Acetaminophen, Acetylsalicylic acid, Amphetamine, Aminopyrine, Ampicillin, Ascorbic acid, Atropine, Benzoylecgonine, Caffeine, Cocaine, Carbamazepine, Codeine, Chloroquine, Chloropromazine, Carbromal, Desipramine, Dextromethorphan, Dextropropoxyphene, 5,5-Diphenylhydantoin, 10-11-Dihydro-carbamazepine, Ethosuximide, Estriol, Estrone, Estradiol, Ethotoin, Glutethimide, Ibuprofen, Imipramine, Lidocaine, LSD, Methadone, Methadone-primary metabolite, Methaqualone, Methamphetamine, Mephenytoin, 5-Methyl-5-propylsuccinimide, Methyl PEMA, Methsuximide, 4-Methylprimidone, Morphine, Meperidine, Niacinamide, Norethindrone, N-Normethsuximide, Phensuximide, PEMA, Primidone, Phencyclidine, Phenothiazine, Phenylpropanolamine, Procaine, Quinine, THC-COOH

REFERENCES

1. Urine Testing for Drugs of Abuse, National Institute on Drug Abuse Research Monograph, 73,1986.
2. S.C. Harvey,:"Hypnotics and Sedatives" in The Pharmacological Basis of Therapeutics 7th Ed.,1985 L.S. Goodman and A. Gilman, T.W. Rall and F. Murad, edd. (New York, Macmillan, (pp339-51)
3. Greenblatt, D.J., Lacasse Y., and Shader, R.I.: "Acute Overdosage with Benzodiazepine Derivatives." Clin. Pharmacol. Ther. 21:4976, 1977.
4. Blum, K.: Handbook of Abusable Drugs, Gardner Press, p. 371, 1984.
5. R.C. Kelley et al, "Association of Benzodiazepines with death in a major metropolitan area" Journal of Analytical Toxicology 6, 1982 p. 91-96.
6. Kaplan, S.A. and Jack, M.L.: "Metabolism of the Benzodiazepines: Pharmacokinetic and Pharmacodynamic Considerations" In: The Benzodiazepines: From Molecular Biology to Clinical Practice. E. Costa, Ed. Raven Press, New York p. 173, 1983.

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IMMUNALYSIS

BENZODIAZEPINES ELISA KIT

Ver 05/2008

Immunalysis Corporation:
Catalog Number : 214-0192 2 x 96 well plates
214-0480 5 x 96 well plates
214-4800 50 x 96 well plates

THE IMMUNALYSIS BENZODIAZEPINES DIRECT ELISA KIT IS INTENDED FOR FORENSIC USE ONLY.

The Immunalysis Benzodiazepines Direct ELISA Kit provides only a preliminary analytical test result. A more specific alternate chemical method must be used in order to obtain a confirmed analytical result. Gas chromatography/ mass spectrometry (GC-MS) is the preferred confirmatory method (1). Professional judgement should be applied to any drug of abuse test result, particularly when preliminary positive results are used.

EXPLANATION OF THE TEST

The Immunalysis Benzodiazepines Direct ELISA Kit is a sensitive in-vitro test to detect the presence of Benzodiazepines in forensic samples such as whole blood, serum, plasma and urine.

Benzodiazepines - are a class of widely prescribed central nervous system depressant drugs with sedative, muscle relaxant and anti-convulsant activities. Chronic use does result in moderate dependence and tolerance to the drug. The use of alcohol in conjunction with the benzodiazepines has been shown to have a greater suppressive effect to the central nervous system than that attributable to either chemical alone. Benzodiazepines are usually administered orally and are absorbed rapidly. The metabolism of Benzodiazepines is mainly in the liver and excreted in the urine as a variety of structurally related metabolites. Metabolic similarities include removal of substituents from the B ring of the 1,4 benzodiazepines and alpha hydroxylation of the triazolobenzodiazepines, hydroxylation of the 3 position carbon of the B ring and conjugation of hydroxylated metabolites followed by urinary excretion as glucuronides.(8)

PRINCIPLES OF THE PROCEDURE

The Immunalysis Benzodiazepines Direct ELISA Kit is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture.

A 10 µl. aliquot of a diluted unknown specimen is incubated with a 100 µl. dilution of enzyme (Horseradish peroxidase) labeled Benzodiazepine derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 2 ng/mL.

The Immunalysis Benzodiazepines Direct ELISA Kit avoids extraction of urine or blood sample for measurement. It employs an Oxazepam directed antiserum. Due to the proprietary method of orienting the antibody on the polystyrene micro-plate much

higher sensitivity is achieved compared to passive adsorption. This results in extremely small sample size reducing matrix effects and interference with binding proteins(s) or other macromolecules.

MATERIALS AND EQUIPMENT

Materials and equipment required but not supplied with the Immunalysis BENZODIAZEPINES Direct ELISA Kit are itemized below:

12x75 mm Disposable Glass or Plastic Culture Tubes to predilute samples (if required).
Manual or electronic micropipets (single channel or multichannel) or automated pipetting stations.
Refrigerator (for kit storage).
Interval Timer.
Wash bottle or Plate Washer.
Microplate reader capable of reading at 450 nm. And 650 nm.

REAGENTS

Immunalysis Benzodiazepines Direct ELISA Kit Contents.

Component	192 Test Kit Cat# 214-0192	480 Test Kit Cat# 214-0480	4800Test Kit Cat# 214-4800
96 well Micro-plate	2	5	50
Benzo- Conjugate	25 mL	60 mL	750 mL
Neg Std	2 mL	5 mL	3 x 5 mL
TMB Substrate	30 mL	2 x 30 mL	750 mL
Stop Reagent	25 mL	55 mL	750 mL

96 well micro-plate. The micro-plate is coated with polyclonal anti-Oxazepam via a spacer chain to provide optimally oriented binding sites. The plates are sealed in a moisture and air barrier pouch with a desiccant.

Benzodiazepine-Enzyme Conjugate. The conjugate solution contains a Benzodiazepine derivative labeled with horseradish peroxidase in a buffered, protein solution with stabilizers, pH 7.8 containing non azide preservatives. (Colored Red)

Negative Standard. This bottle contains drug free synthetic urine containing azide free preservatives.

TMB chromogenic substrate. The color reagent contains 3,3',5,5' tetramethylbenzidine and urea peroxidase in buffer.

Stop Reagent. This contains 1 N hydrochloric acid.

Precautions

1. Not for Internal or External Use in Humans or Animals.
2. There should be no eating or drinking within work area.
3. Always wear gloves and a protective lab coat.
4. No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.
5. Do not add sodium azide to samples as preservative.
6. Do not use external controls containing sodium azide.
7. Use disposable pipet tips to avoid contaminating chromogenic substrate reagent. Discard reagent if it turns blue.
8. Do not pour chromogenic substrate back into container after use.
9. Do not freeze reagents.
10. Do not mix reagents from different kit lot numbers.
11. Keep reagents out of direct sunlight.
12. Handle stop reagent with care, since it is corrosive.
13. Bring all reagents to room temperature.
14. Viscous forensic samples should always be diluted in phosphate buffered saline or distilled water prior to pipetting.
15. Ensure the bag containing the micro-plate strips and dessicant is well sealed if only a partial plate is used.

General. Precise pipetting is the essence of successful immunoassay. It is critical to pipet right at the center and bottom of each well to ensure good replicates and coefficients of variation Micropipets supplied by "Eppendorf" or "SMI" with disposable tips are excellent when used carefully according to instructions to insure the necessary accuracy. New automatic dispensers improve reliable delivery.

Storage. The expiration date of the kit is stated on the label. The kit can be expected to perform satisfactorily until the expiration date if stored in the refrigerator at 2 – 4° C.

Indications of Deterioration. A drop of greater than 50% in the A_0 (zero-standard absorbance reading) for a constant incubation time indicates deterioration of the antibody plate, enzyme conjugate or chromogenic substrate. A significant shift of the standard curve to the right would result from deterioration of the standards. Development of blue color in the chromogenic substrate without the addition of enzyme conjugate indicates contamination of the substrate.

SPECIMEN COLLECTION

Precautions.
The Immunalysis Benzodiazepines Direct ELISA Kit is to be used with human forensic samples, such as urine, whole blood, serum and plasma. Immunalysis has not tested all possible applications of this assay. Cutoff criteria are important in deciding the sample dilution.

Additives.
Specimens to which sodium azide has been added affect the assay.

Storage and Handling Instructions.
Urine samples should be stored at 2 - 4° C until use. Samples should be well mixed before assay. Repeated freezing and thawing should be avoided. Urine samples should be shipped refrigerated with Blue Ice or equivalent.

DETAILS OF THE PROCEDURE

All reagents must be brought to room temperature (20-25° C) before use.

The procedure as described below may be followed in sequence using manual pipettes. Alternatively all reagents may be added using an automated pipettor.

1. Dilute forensic specimens, to the necessary range with Phosphate Buffer Saline pH 7.0. (Urine samples are normally diluted 1:10 for a Oxazepam cutoff of 200 ng/mL.) The dilution factor and volume added can be adjusted based on the laboratory's cutoff.
2. Add 10 µl. of appropriately diluted calibrators and standards to each well in duplicate.
3. Add 10 µl. of the diluted specimens in duplicate (recommended) to each well.
4. Add 100 µl. of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.
5. Incubate for 60 minutes at room temperature (20-25° C) preferably in the dark, after addition of enzyme conjugate to the last well.
6. Wash the wells 6 times with 350 µl. distilled water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells. If testing samples containing abnormally high amounts of hemoglobin (some Postmortem samples), use 10 mM Phosphate buffered saline pH 7.0-7.4. This will lower potential nonspecific binding of hemoglobin to the well, thus lowering background color.
7. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.
8. Add 100 µl. of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
9. Incubate for 30 minutes at room temperature, preferably in the dark.
10. Add 100 µl. of Stop Solution to each well, to change the blue color to yellow.
11. Measure the absorbance at a dual wavelength of 450 nm and 650 nm.
12. Wells should be read within 1 hour of yellow color development.

The following data represent a typical dose/response curve.

Oxazepam pg/well	Absorbance
0	2.839
20	2.12
50	1.438
75	1.265
100	0.901
250	0.438
Clonazepam pg/well	Absorbance
0	2.839
50	1.705
100	1.158
Lorazepam pg/well	Absorbance
0	2.639
50	1.585
100	1.106

The dose/response curve shown above should not be used in assay calculations. It is recommended that at least one in-house positive quality control sample be included with every assay run.
A dose response curve or a cutoff calibrator should be run with every plate.

RESULTS

If the average sample absorbance is equal to or less than the average absorbance of the laboratory positive reference standard the sample is **POSITIVE** for Benzodiazepines. If the average sample absorbance is greater than the average absorbance of the laboratory positive reference standard the sample is called **NEGATIVE** for Benzodiazepines .

Alternatively a dose response curve can be established by plotting standard concentration (abscissa) against corresponding absorbance (ordinate). Values for unknown samples are obtained by interpolation from the curve.

SPECIFIC PERFORMANCE CHARACTERISTICS

Precision
The precision of the Immunalysis BENZODIAZEPINES Direct ELISA Kit has been verified by assessment of the mean, standard deviation (SD) and coefficients of variation (CV) in data resulting from repetitive assays.

Intra-assay Precision
Intra-assay precision was determined with reference controls. A 0,25,50 and 100 ng/mL Oxazepam standard was assayed five times in the same assay. The results are tabulated in Table 1.

TABLE 1

Oxazepam (pg/well)	Mean Abs.	S.D.	C.V.%
0	2.651	0.124	4.7
50	1.456	0.087	6.0
75	1.239	0.054	4.4
100	0.891	0.047	5.3
250	0.462	0.035	7.6

Sensitivity
Assay sensitivity based on the minimum oxazepam concentration required to produce a four standard deviation from assay Ao is 0.5 ng/mL.

7.2.3 Immunalysis Cannabinoid ELISA Specification

TABLE 1

carboxy THC (ng/ml)	Mean Abs.	S.D.	C.V.%
0	1.905	0.139	7.3
2	1.114	0.103	9.4
5	0.752	0.066	8.8
10	0.549	0.042	7.7

Sensitivity
Assay sensitivity based on the minimum THCA concentration required to produce a four standard deviation from assay zero dose response (Ao) is 1 ng/ml.

Specificity
The specificity of the Immunalysis Cannabinoids ELISA for was determined by generating inhibition curves for each of the compounds listed below The antisera cross-reactivities are listed in Table 2.

TABLE 2

Compound	Approx. ng/ml equivalent to ng THCA/ml	Cross-reactivities
11-nor-9-carboxy-Δ^9-THC	11	110
Δ^8-THC	48	21
Δ^9-THC	22	45
11-hydroxy-Δ^9-THC	>1000	<5
8-11-Dihydroxy Δ^9-THC	>1000	<5
Cannabinol	>1000	<5
Cannabidiol	>1000	<5

Cross-Reactivity with Unrelated Drugs
Aliquots of a human urine matrix were spiked with the following compounds at a concentration of 2,000 ng/ml. None of these compounds gave values in the assay that were equal to or greater than the assay sensitivity level.

Acetaminophen, Acetylsalicylic acid, Amphetamine, Aminopyrine, Ampicillin, Amobarbital, Ascorbic acid, Atropine , Barbital, Butabarbital, Caffeine , Cocaine, Carbamazepine, Codeine , Chloroquine, Chloropromazine, Carbromal , Desipramine, Dextromethorphan, Dextropropoxyphene , 5,5-Diphenylhydantoin, 10-11-Dihydrocarbamazepine, Diazepam, Ethosuximide, Estriol, Estrone, Estradiol, Ethotoin, Glutethimide , Hexobarbital, Ibuprofen, Imipramine, Lidocaine, LSD, Methadone , Methadone-primary metabolite, Methaqualone, Methamphetamine, Metharbital, Mephenytoin, α-Methyl-α-propylsuccinimide, Mephobarbital, Methyl PEMA, Methsuximide , 4-Methylprimidone, Morphine , Meperidine , Niacinamide, Norethindrone, N-Normethsuximide, Phenobarbital , Phensuximide, PEMA, Primidone, Phencyclidine, Pentobarbital, Phenothiazine, Phenylpropanolamine, Procaine, Quinine, Secobarbital, Tetracycline,Tetrahydrozoline

REFERENCES

1. Urine Testing for Drugs of Abuse, National Institute on Drug Abuse Research Monograph . 73, 1986.
2. Rodgers. R., Crowl C.P., Eimstad W.M., et al.: Homogeneous enzyme immunoassay for cannabinoids in urine. Clin. Chem. 24: 95 (1978).
3. Teale, J.D., Foxman, E.J., King, L.S., Piel, E.M. and Marks, V.: The development of a radioimmunoassay for cannabinoids in blood and urine. J. Pharm. Pharmacol. 27: 485 (1975).
4. Mule, S.J., Lomax, P. and Gross, S.J.: Active and passive marijuana exposure tested by three immunoassays and GC/MS in urine. J. Anal. Toxicol. 12: 113 (1988).
5. Cone, E.J. and Johnson, R.E.: Contact highs and urinary cannabinoid excretion after passive exposure to marijuana smoke. Clin. Pharmacol. Ther. 40: 247 (1986).

IMMUNALYSIS CORPORATION
Pomona, Ca. 91767
(909) 394-2203.

IMMUNALYSIS CANNABINOIDS (THCA/CTHC) DIRECT ELISA KIT

Ver: 01/2010

Immunalysis Corporation:
Catalog Number 205-0192 2 x 96 well plates
Catalog Number 205-0480 5 x 96 well plates
Catalog Number 205-4800 50 x 96 well plates

THE IMMUNALYSIS THC DIRECT ELISA KIT IS INTENDED FOR FORENSIC USE ONLY.

The Immunalysis Cannabinoids Direct ELISA Kit provides only a preliminary analytical test result. A more specific alternate chemical method must be used in order to obtain a confirmed analytical result. Gas chromatography/ mass spectrometry (GC-MS) is the preferred confirmatory method (1). Professional judgement should be applied to any drug of abuse test result, particularly when preliminary positive results are used.

EXPLANATION OF THE TEST

The Immunalysis Cannabinoids Direct ELISA Kit is a specific and sensitive in-vitro test to detect the presence of cannabinoids in forensic samples such as whole blood, serum, plasma and urine.

Δ^9-THC (a member of the cannabinoid family) is the primary psychoactive ingredient of marijuana (1). Cannabinoid metabolites appear in urine two to four hours after a marijuana smoke and may persist for days (up to thirty) (1- 3). Thus a urine assay reasonably serves to detect cannabis use even though a considerable period may have elapsed since smoking or ingestion of marijuana.

PRINCIPLES OF THE PROCEDURE

The Immunalysis Cannabinoids Direct ELISA Kit is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture.

A 10 µl aliquot of a diluted unknown specimen is incubated with a 100 µl dilution of enzyme (Horseradish peroxidase) labeled carboxy THC (THCA) derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 1 ng/ml.

The Immunalysis THC Direct ELISA Kit avoids extraction of urine or blood sample for measurement. It employs a polyclonal high affinity, purified carboxy THC antibody. Due to the proprietary method of orienting the antibody on the polystyrene micro-plate much higher sensitivity is achieved compared to passive adsorption. This results in extremely small sample size reducing matrix effects and interference with binding proteins(s) or other macromolecules.

MATERIALS AND EQUIPMENT

Materials and equipment required but not supplied with the Immunalysis THC Direct ELISA Kit are itemized below:
12x75 mm Disposable Glass to pre/dilute samples (if required).
Manual or electronic micropipets (single channel or multichannel) or automated pipetting stations.
Refrigerator (for kit storage).
Interval Timer.
Wash bottle or Plate Washer.
Microplate reader capable of reading at 450 nm. and 650 nm.

REAGENTS

Immunalysis Cannabinoids Direct ELISA Kit Contents.

Component	192 Test Kit Cat# 205-0192	480 Test Kit Cat# 205-0480	4800 Test Kit Cat# 205-4800
96 well Micro-plate	2	5	50
THC-Conjugate	25 ml	60 ml	750 ml
Neg Std.	2 ml	5 ml	3 x 5 ml
TMB Substrate	30 ml	2 x 30 ml	750 ml
Stop Reagent	25 ml	55 ml	750 ml

96 well micro-plate. The micro-plate is coated with polyclonal anti-carboxy THC via a spacer chain to provide optimally oriented binding sites. The plates are sealed in a moisture and air barrier pouch with a dessicant.

THC-Enzyme Conjugate. The conjugate solution contains a THCA derivative labeled with horseradish peroxidase in a stabilized protein solution, pH 8.5 containing a preservative. (Colored Red).

Negative Control. This bottle contains drug free synthetic urine containing azide free preservatives.

TMB chromogenic substrate. The color reagent contains 3,3',5,5' tetramethylbenzidine and urea peroxidase in buffer.

Stop Reagent. This contains 1 N hydrochloric acid.

Precautions
1. Not for Internal or External Use in Humans or Animals.
2. There should be no eating or drinking within work area.
3. Always wear gloves and a protective lab coat.
4. No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.
5. Do not add sodium azide to samples as preservative.
6. Do not use external controls containing sodium azide.
7. Use disposable pipet tips to avoid contaminating chromogenic substrate reagent. Discard reagent if it turns blue.
8. Do not pour chromogenic substrate back into container after use.
9. Do not freeze reagents.
10. Do not mix reagents from different kit lot numbers.
11. Keep reagents out of direct sunlight.
12. Handle stop reagent with care, since it is corrosive.
13. Bring all reagents to room temperature.
14. Viscous forensic samples should always be diluted in phosphate buffered saline or distilled water prior to pippetting.
15. Ensure the bag containing the micro-plate strips and dessicant is sealed well, if a few strips are used.

General. Precise pipetting is the essence of successful immunoassay. It is critical to pipet right at the center and bottom of each well to ensure good replicates and coefficents of variation Micropipets supplied by "Eppendorf" or "SMI" with disposable tips are excellent when used carefully according to instructions to insure the necessary accuracy. New automatic dispensers improve reliable delivery.

Storage. The expiration date of the kit is stated on the label. The kit can be expected to perform satisfactorily until the expiration date if stored in the refrigerator at 2 -4^0C.

Indications of Deterioration. A drop of greater than 50% in the A_0 (zero-standard absorbance reading) for a constant incubation time indicates deterioration of the antibody plate, enzyme conjugate or chromogenic substrate. A significant shift of the standard curve to the right would result from deterioration of the standards. Development of blue color in the chromogenic substrate without the addition of enzyme conjugate indicates contamination of the substrate.

SPECIMEN COLLECTION

Precautions.
The Immunalysis Cannabinoids Direct ELISA Kit is to be used with human forensic samples such as whole blood, serum, urine and plasma. Immunalysis has not tested all possible applications of this assay. **The Cutoff criteria are important in deciding the sample dilution. It is recommended to dilute most blood samples either 1:5 or 1:10 depending on the cutoff used by the laboratory.**

Additives.
Specimens to which sodium azide has been added affect the assay.

Storage and Handling Instructions.
Urine samples should be stored at 2 – 4^0C until use. Samples should be well mixed before assay. Repeated freezing and thawing should be avoided. Urine samples should be shipped refrigerated with Blue Ice or equivalent.

DETAILS OF THE PROCEDURE.

All reagents must be brought to room temperature (20-25^0 C) before use.
The procedure as described below may be followed in sequence using manual pipettes. Alternatively all reagents may be added using an automated pipettor.

1. Dilute forensic specimens, to the necessary range with Phosphate Buffer Saline pH 7.0. (Urine samples are normally diluted 1:10 for a THCA cutoff of 50 ng/ml.) The dilution factor and volume added can be adjusted based on the laboratory's cutoff.
2. Add 10 µl. of appropriately diluted calibrators and standards to each well in duplicate.
3. Add 10 µl. of the diluted specimens in duplicate (recommended) to each well.
4. Add 100 µl. of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.
5. Incubate for 60 minutes at room temperature (20-25^0 C) preferably in the dark, after addition of enzyme conjugate to the last well.
6. Wash the wells 6 times with 350 µl. distilled water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells. If testing samples, containing abnormally high amounts of hemoglobin (some Postmortem samples), use 10 mM Phosphate buffered saline pH 7.0-7.4. This will lower potential nonspecific binding of hemoglobin to the well, thus lowering background color.
7. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.
8. Add 100 µl. of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
9. Incubate for 30 minutes at room temperature, preferably in the dark.
10. Add 100 µl. of Stop Solution to each well, to change the blue color to yellow.
11. Measure the absorbance at a dual wavelength of 450 nm and 650 nm.
12. Wells should be read within 1 hour of yellow color development.

The following data represent a typical dose/response curve.

CTHC ng/ml	Absorbance
0	1.905
2	1.413
5	0.955
10	0.751

The dose/response curve shown above should not be used in assay calculations. It is recommended that at least one in-house positive quality control sample be included with every assay run.
A dose response curve or a cutoff calibrator should be run with every plate.

RESULTS

If the average sample absorbance is equal to or less than the average absorbance of the laboratory THCA/CTHC positive reference standard the sample is POSITIVE for cannabinoids. If the average sample absorbance is greater than the average absorbance of the laboratory THCA/CTHC positive reference standard the sample is called NEGATIVE for cannabinoids.

Alternatively a dose response curve can be established by plotting standard concentration (abscissa) against corresponding absorbance (ordinate). Values for unknown samples are obtained by interpolation from the curve.

SPECIFIC PERFORMANCE CHARACTERISTICS

Accuracy
35 whole blood samples and 60 urine samples collected from presumed non-users were tested in the Immunalysis Cannabinoids Direct ELISA Kit. One hundred percent of these normal samples measured negative at 20 ng/ml of THCA equivalents for whole blood and 50 ng/ml of THCA equivalents for urine. Forty whole blood samples which were previously confirmed positive for cannabinoids by GC-MS employing a cut-off of 10 ng/ml THCA were tested in the Immunalysis Cannabinoids Direct ELISA Kit . All the samples were found to be positive i.e. above the cut-off of 20 ng/ml.

Precision
The precision of the Immunalysis Cannabinoids Direct ELISA Kit has been verified by assessment of the mean, standard deviation (SD) and coefficients of variation (CV) in data resulting from repetitive assays.

Intra-assay Precision
Intra-assay precision was determined with reference controls.
A 0,2,5 and 10 ng/ml standard was assayed eight times in the same assay. The results are tabulated in Table 1.

7.2.4 Immunalysis Cocaine ELISA Specification

Precision
The precision of the Immunalysis Cocaine Metabolite Direct ELISA Kit has been verified by assessment of the mean, standard deviation (SD) and coefficients of variation (CV) in data resulting from repetitive assays.

Intra-assay Precision
Intra-assay precision was determined with reference controls.
A 0,10,25 and 50 ng/ml standard was assayed five times in the same assay. The results are tabulated in Table 1.

TABLE 1

Benzoylecgonine (ng/ml)	Mean Abs.	S.D.	C.V.%
0	2.187	0.173	8.0
10	1.117	0.129	11.55
25	0.605	0.045	7.4
50	0.418	0.033	7.8

Sensitivity
Assay sensitivity based on the minimum Benzoylecgonine concentration required to produce a four standard deviation from assay Ao is 1 ng/ml.

Specificity
The specificity of the Immunalysis Cocaine Metabolite ELISA for was determined by generating inhibition curves for each of the compounds listed below The antisera cross-reactivities are listed in Table 2.

TABLE 2
Cross-reactivities related drugs

Compound	Approx. ng/ml equivalent to 50ng BE	Cross-reactivity
Benzoylecgonine	50	100
Cocaine	750	6.66
Cocaethylene	>5000	<1
Norcocaine	>5000	<1
Norcocaethylene	>5000	<1
Ecgonine	>10000	<0.5
Ecgonine Methyl Ester	>10000	<0.5

Cross-Reactivity with Unrelated Drugs
Aliquots of a human urine matrix were spiked with the following compounds at a concentration of 2000 ng/ml. None of these compounds gave values in the assay that were equal to or greater than the assay sensitivity level (<1 ng/ml).

Acetaminophen, Acetylsalicylic acid, Amphetamine, Aminopyrine, Ampicillin, Amobarbital, Ascorbic acid, Atropine, Barbital, Butabarbital, Caffeine , Carbamazepine ,Codeine , Chloroquine , Chlorpromazine, Cerbromal ,Desipramine, Dextromethorphan, Dextropropoxyphene, 5,5- Diphenylhydantoin, 10-11-Dihydrocarbamazepine, Diazepam, Ethosuximide , Estriol, Estrone, Estradiol, Ethotoin, Glutethimide, Hexobarbital , Ibuprofen, Imipramine, Lidocaine, LSD, Methadone, Methadone - primary metabolite, Methaqualone, Methamphetamine, Methorbital , Mephenytoin, a-Methyl-a-propylsuccinimide , Mephobarbital, Methyl PEMA, Methsuximide , 4-Methylprimidone, Morphine , Meperidine, Niacinamide, Norethindrone, N-Normethsuximide, Phenobarbital , Phensuximide, PEMA, Primidone, Phencyclidine, Pentobarbital, Phenothiazine, Phenylpropanolamine, Procaine, Quinine, Secobarbital, Tetracycline, Tetrahydrozoline, THCCOOH

REFERENCES

1. Urine Testing for Drugs of Abuse, National Institute on Drug Abuse Research Monograph. 73: 95-97 (1986).
2. R.K. Siegel. In: National Institute on Drug Abuse. Research Monograph Series 50. pp. 92-110 (1986).
3. M.J. Kogan, K.G. Vereby, A.C. De Paca, R.B. Resnik and S.J. Mule. Anal. Chem. 49:1985 (1977).
4. Diagnostic Products Corp.- Double Antibody COCAINE/BENZOYLECGONINE Assay.
5. Roche Diagnostics, Abuscreen COCAINE/BENZOYLECGONINE Radio- Immunoassay.
6. Syva Corp. EMIT COCAINE/BENZOYLECGONINE Assay.
7. J. Ambre. J. Anal. Toxicol. 9:241 (1985).

IMMUNALYSIS CORPORATION
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IMMUNALYSIS COCAINE METABOLITE DIRECT ELISA KIT
(Benzoylecgonine Specific)

Ver: 05/2008

Immunalysis Corporation:
Catalog Number 206-0192 2 x 96 well plates
Catalog Number 206-0480 5 x 96 well plates
Catalog Number 206-4800 50 x 96 well plates

THE IMMUNALYSIS COCAINE METABOLITE DIRECT ELISA KIT IS INTENDED FOR FORENSIC USE ONLY.

The Immunalysis Cocaine Metabolite Direct ELISA Kit provides only a preliminary analytical test result. A more specific alternate chemical method must be used in order to obtain a confirmed analytical result. Gas chromatography/ mass spectrometry (GS-MS) is the preferred confirmatory method (1). Professional judgement should be applied to any drug of abuse test result, particularly when preliminary positive results are used.

EXPLANATION OF THE TEST

The Immunalysis Cocaine Metabolite Direct ELISA Kit is a specific and sensitive in-vitro test to detect the presence of Benzoylecgonine (BE) in forensic samples such as whole blood, serum, plasma and urine.

Cocaine abuse is widespread and its prevalence may be increasing in all social and age strata (1). The drug is generally inhaled or smoked (1,2). Several methods for measurement of Cocaine Metabolite in urine exist (3-6). Benzoylecgonine, a major metabolite appears within minutes in urine (3). Since the number and proportion of metabolites vary in subjects, results are expressed in benzoylecgonine equivalents per ml. The Immunalysis Cocaine Metabolite Direct ELISA Kit is a single incubation assay providing results similar to those obtained by existing methods (4-6). Native (unaltered) cocaine urine concentration is far lower than that of its major metabolite benzoylecgonine. After intra- venous administration of l00mg cocaine urine concentrations ranged from 1.2 - 2.4 ug/ml compared with concentrations ranging from 5 - 55 ug/ml for benzoylecgonine (3). Cocaine was undetectable (at a 50 ng/ml cut-off) 12 hours after administration in comparison with benzoylecgonine which persists hours to days after administration (7). It has been suggested that a benzoylecgonine/cocaine ratio of less than 100 is indicative of use within the past 10 hours (7).

PRINCIPLES OF THE PROCEDURE

The Immunalysis Cocaine Metabolite Direct ELISA Kit is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture.

A 10 µl. aliquot of a diluted unknown specimen is incubated with a 100 µl. dilution of enzyme (Horseradish peroxidase) labeled Benzoylecgonine derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added.

The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 1 ng/ml.

The Immunalysis Cocaine Metabolite Direct ELISA Kit avoids extraction of urine or blood sample for measurement. It employs an Benzoylecgonine directed antiserum. Due to the proprietary method of orienting the antibody on the polystyrene micro-plate much higher sensitivity is achieved compared to passive adsorption. This allows an extremely small sample size, reducing matrix effects and interference with binding proteins(s) or other macromolecules.

MATERIALS AND EQUIPMENT

Materials and equipment required but not supplied with the Immunalysis Cocaine Metabolite Direct ELISA Kit are itemized below:

12x75 mm Disposable Glass or Plastic Culture Tubes to predilute samples (if required).
Manual or electronic micropipets (single channel or multichannel) or automated pipetting stations.
Refrigerator (for kit storage).
Interval Timer.
Wash bottle or Plate Washer.
Microplate reader capable of reading at 450 nm. and 650 nm.

REAGENTS

Immunalysis Cocaine Metabolite Direct ELISA Kit Contents.

Component	192 Test Kit Cat# 206-0192	480 Test Kit Cat#206-0480	4800 Test Kit Cat#206-4800
96 well Micro-plate	2	5	50
BE- Conjugate	25 ml	60 ml	750 ml
Neg Std	2 ml	5 ml	3 x 5 ml
TMB Substrate	30 ml	2 x 30 ml	750 ml
Stop Reagent	25 ml	55 ml	750 ml

96 well micro-plate. The micro-plate is coated with polyclonal anti-benzoylecgonine via a spacer chain to provide optimally oriented binding sites. The plates are sealed in a moisture and air barrier pouch with a dessicant.

Benzoylecgonine-Enzyme Conjugate The conjugate solution contains a Benzoylecgonine derivative labeled with horseradish peroxidase in a buffered, protein solution with stabilizers, pH 7.6 containing azide free preservatives. (Colored Red)

Negative Standard. This bottle contains drug free synthetic urine containing azide free preservatives.

TMB chromogenic substrate. The color reagent contains 3,3',5,5' tetramethylbenzidine and urea peroxidase in buffer.

Stop Reagent. This contains 1 N hydrochloric acid.

Precautions
1. Not for Internal or External Use in Humans or Animals.
2. There should be no eating or drinking within work area.
3. Always wear gloves and a protective lab coat.
4. No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.
5. **Do not add sodium azide to samples as preservative.**
6. **Do not use external controls containing sodium azide.**
7. Use disposable pipet tips to avoid contaminating chromogenic substrate reagent. Discard reagent if it turns blue.
8. Do not pour chromogenic substrate back into container after use.
9. Do not freeze reagents.
10. Do not mix reagents from different kit lot numbers.
11. Keep reagents out of direct sunlight.
12. Handle stop reagent with care, since it is corrosive.
13. Bring all reagents to room temperature.
14. Viscous forensic samples should always be diluted in phosphate buffered saline or distilled water prior to pippetting.
15. Ensure the bag containing the micro-plate strips and dessicant is well sealed if only a few strips are used.

General. Precise pipetting is the essence of successful immunoassay. It is critical to pipet right at the center and bottom of each well to ensure good replicates and coefficients of variation Micropipets supplied by "Eppendorf" or "SMI" with disposable tips are excellent when used carefully according to instructions to insure the necessary accuracy. New automatic dispensers improve reliable delivery.

Storage. The expiration date of the kit is stated on the label. The kit can be expected to perform satisfactorily until the expiration date if stored in the refrigerator at 2 – 4° C.

Indications of Deterioration. A drop of greater than 50% in the A_0 (zero-standard absorbance reading) for a constant incubation time indicates deterioration of the antibody plate, enzyme conjugate or chromogenic substrate. A significant shift of the standard curve to the right would result from deterioration of the standards. Development of blue color in the chromogenic substrate without the addition of enzyme conjugate indicates contamination of the substrate.

SPECIMEN COLLECTION

Precautions.
The Immunalysis Benzoylecgonine Direct ELISA Kit is to be used with human forensic samples, like whole blood, serum and plasma. Immunalysis has not tested all possible applications of this assay. Cutoff criteria are important in deciding the sample dilution.

Additives.
Specimens to which sodium azide has been added affect the assay.

Storage and Handling Instructions
Urine samples should be stored at 2 – 4° C until use. Samples should be well mixed or vortexed before assay. Repeated freezing and thawing should be avoided. Urine samples should be shipped refrigerated with Blue Ice or equivalent.

DETAILS OF THE PROCEDURE.

All reagents must be brought to room temperature (20-25° C) before use.

The procedure as described below may be followed in sequence using manual pipettes. Alternatively all reagents may be added using an automated pipettor .

1. Dilute forensic specimens, to the necessary range with Phosphate Buffer Saline pH 7.0. (Urine samples are normally diluted 1:10 for a Benzoylecgonine cutoff of 300 ng/ml.) The dilution factor and volume added can be adjusted based on the laboratory's cutoff.
2. Add 10 µl. of appropriately diluted calibrators and standards to each well in duplicate.
3. Add 10 µl. of the diluted specimens in duplicate (recommended) to each well.
4. Add 100 µl. of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.
5. Incubate for 60 minutes at room temperature (20-25° C) preferably in the dark, after addition of enzyme conjugate to the last well.
6. Wash the wells 6 times with 350 µl. distilled water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells. If testing samples containing abnormally high amounts of hemoglobin (some Postmortem samples), use 10 mM Phosphate buffered saline pH 7.0-7.4. This will lower potential nonspecific binding of hemoglobin to the well, thus lowering background color.
7. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.
8. Add 100 µl. of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
9. Incubate for 30 minutes at room temperature, preferably in the dark.
10. Add 100 µl. of Stop Solution to each well, to change the blue color to yellow.
11. Measure the absorbance at a dual wavelength of 450 nm and 650 nm.
12. Wells should be read within 1 hour of yellow color development.

The following data represent a typical dose/response curve.

Benzoylecgonine ng/ml	Absorbance
0	2.045
10	1.455
25	0.896
50	0.584

The dose/response curve shown above should not be used in assay calculations. It is recommended that at least one in-house positive quality control sample be included with every assay run. A dose response curve or a cutoff calibrator should be run with every plate.

RESULTS

If the average sample absorbance is equal to or less than the average absorbance of the laboratory positive reference standard the sample is POSITIVE for Benzoylecgonine. If the average sample absorbance is greater than the average absorbance of the laboratory positive reference standard the sample is called NEGATIVE for Benzoylecgonine.

Alternatively a dose response curve can be established by plotting standard concentration (abscissa) against corresponding absorbance (ordinate). Values for unknown samples are obtained by interpolation from the curve.

SPECIFIC PERFORMANCE CHARACTERISTICS

Accuracy
60 whole blood samples and 35 urine samples collected from presumed non-users were tested in the Immunalysis Cocaine Metabolite Direct ELISA Kit . One hundred percent of these normal samples measured negative at 50 ng/ml of Benzoylecgonine for whole blood and 300 ng/ml of Benzoylecgonine for urine. Thirty five whole blood samples which were previously confirmed positive for Benzoylecgonine by GC-MS employing a cut-off of 50 ng/ml, were tested in the Immunalysis Cocaine Metabolite Direct ELISA Kit. All the samples were found to be positive i.e. above the cut-off of 50 ng/ml.

7.2.5 Immunalysis Methadone ELISA Specification

Intra-assay Precision
Intra-assay precision was determined with reference controls.
A 0, 125, 250 and 500 pg/well standard was assayed eight times in the same assay. The results are tabulated in Table 1.

Table 1

Methadone (pg/well)	Mean Abs.	S.D.	C.V.%
0	2.595	0.061	2.36
125	1.408	0.043	3.04
250	0.744	0.045	6.03
500	0.239	0.016	6.6

Specificity
The specificity of the Immunalysis Methadone Direct ELISA for was determined by generating inhibition curves for each of the compounds listed below The antisera cross-reactivities are listed in Table 2.

TABLE 2
Cross-reactivities related drugs

Compound	Approx. ng/ml equivalent to 300ng Methadone	Cross-reactivities
Methadone	300	100
Methadol	600	50
EDDP	>6000	< 5
LAAM	2000	15
Nor-LAAM	>30000	<1

Cross-Reactivity with Unrelated Drugs

Aliquots of a human urine matrix were spiked with the following compounds at a concentration of 50000 ng/ml. None of these compounds gave values in the assay that were equal to or greater than the assay sensitivity level (<5 ng/ml).

Acetaminophen, Acetylsalicylic acid, Amphetamine, Aminopyrine, Ampicillin, Amobarbital, Ascorbic acid, Atropine, Barbital, Benzoylecgonine, Butabarbital, Caffeine , Carbamazepine , Cocaine, Codeine , Chloroquine , Carbromal ,Desipramine, Dextromethorphan, Dextropropoxyphene, 5,5- Diphenylhydantoin, 10-11-Dihydrocarbamazepine, Diazepam, Ethosuximide , Estriol, Estrone, Estradiol, Ethotoin, Glutethimide, Hexobarbital , Ibuprofen, Imipramine, Lidocaine, LSD, Methaqualone, Methamphetamine, Metharbital , Mephenytoin, a-Methyl-a-propylsuccinimide , Mephobarbital, Methyl PEMA, Methasuximide , 4-Methylprimidone, Morphine , Meperidine, Niacinamide, Norethindrone, N-Normethasuximide, Phenobarbital , Phensuximide, PEMA, Primidone, Phencyclidine, Pentobarbital, Phenylpropanolamine, Procaine, Quinine, Secobarbital, Tetracycline, Tetrahydrozoline, THCCOOH

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IMMUNALYSIS

METHADONE DIRECT ELISA KIT

Ver: 05/2006

Immunalysis Corporation:
Catalog Number 232-0096 1 x 96 well plates
Catalog Number 232-0480 5 x 96 well plates
Catalog Number 232-4800 50 96 well plates

THE IMMUNALYSIS METHADONE DIRECT ELISA KIT IS INTENDED FOR FORENSIC USE ONLY.

The Immunalysis METHADONE Direct ELISA Kit provides only a preliminary analytical test result. A more specific alternate chemical method must be used in order to obtain a confirmed analytical result. Gas chromatography/ mass spectrometry (GS-MS) is the preferred confirmatory method (1). Professional judgement should be applied to any drug of abuse test result, particularly when preliminary positive results are used.

EXPLANATION OF THE TEST

The Immunalysis METHADONE Direct ELISA Kit is a specific and sensitive in-vitro test to detect the presence of Methadone and in forensic samples such as whole blood, oral fluids, serum and urine.

PRINCIPLES OF THE PROCEDURE

The Immunalysis Methadone Direct ELISA Kit is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture.

A 10 µl. aliquot of a diluted unknown specimen is incubated with a 100 µl. dilution of enzyme (Horseradish peroxidase) labeled Methadone derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 25 ng/ml.
The Immunalysis METHADONE Direct ELISA Kit avoids extraction of urine or blood sample for measurement. It employs a Methadone directed antiserum. Due to the proprietary method of orienting the antibody on the polystyrene micro-plate much higher sensitivity is achieved compared to passive adsorption. This allows an extremely small sample size, reducing matrix effects and interference with binding proteins(s) or other macromolecules.

MATERIALS AND EQUIPMENT

Materials and equipment required but not supplied with the Immunalysis Methadone Direct ELISA Kit are itemized below:
12x75 mm Disposable Glass or Plastic Culture Tubes to predilute samples (if required).
Manual or electronic micropipets (single channel or multichannel) or automated pipetting stations.
Refrigerator (for kit storage).
Interval Timer.
Wash bottle or Plate Washer.
Microplate reader capable of reading at 450 nm. And 650 nm.

REAGENTS

Immunalysis METHADONE Direct ELISA Kit Contents.

Component	96 Test Kit Cat# 232-0096	480 Test Kit Cat# 232-0480	4800 Test Kit Cat# 232-4800
96 well Micro-plate	1	5	50
Methadone- Conjugate	15 ml	60 ml	750 ml
Neg Std	2 ml	5 ml	3 x 5 ml
TMB Substrate	30 ml	2 x 30 ml	750 ml
Stop Reagent	25 ml	55 ml	750 ml

96 well micro-plate. The micro-plate is coated with polyclonal anti-Methadone via a spacer chain to provide optimally oriented binding sites. The plates are sealed in a moisture and air barrier pouch with a dessicant.

Methadone-Enzyme Conjugate. The conjugate solution contains a Methadone derivative labeled with horseradish peroxidase in a buffered, protein solution with stabilizers, pH 7.6 containing non azide preservatives. (Colored Red)

Negative Standard. This bottle contains drug free synthetic urine containing azide free preservatives.

TMB chromogenic substrate. The color reagent contains 3,3',5,5' tetramethylbenzidine and urea peroxidase in buffer.

Stop Reagent. This contains 1 N hydrochloric acid.

Precautions
1. Not for Internal or External Use in Humans or Animals.
2. There should be no eating or drinking within work area.
3. Always wear gloves and a protective lab coat.
4. No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.
5. **Do not add sodium azide to samples as preservative.**
6. **Do not use external controls containing sodium azide.**
7. Use disposable pipet tips to avoid contaminating chromogenic substrate reagent. Discard reagent if it turns blue.
8. Do not pour chromogenic substrate back into container after use.
9. Do not freeze reagents.
10. Do not mix reagents from different kit lot numbers.
11. Keep reagents out of direct sunlight.
12. Handle stop reagent with care, since it is corrosive.
13. Bring all reagents to room temperature.
14. Viscous forensic samples should always be diluted in phosphate buffered saline or distilled water prior to pippetting.
15. Ensure the bag containing the micro-plate strips and dessicant is well sealed if only a few strips are used.

General. Precise pipetting is the essence of successful immunoassay. It is critical to pipet right at the center and bottom of each well to ensure good replicates and coefficents of variation Micropipets supplied by "Eppendorf" or "SMI" with disposable tips are excellent when used carefully according to instructions to insure the necessary accuracy. New automatic dispensers improve reliable delivery.

Storage. The expiration date of the kit is stated on the label. The kit can be expected to perform satisfactorily until the expiration date if stored in the refrigerator at 2 – 4° C.

Indications of Deterioration. A drop of greater than 50% in the A_0 (zero-standard absorbance reading) for a constant incubation time indicates deterioration of the antibody plate, enzyme conjugate or chromogenic substrate. A significant shift of the standard curve to the right would result from deterioration of the standards. Development of blue color in the chromogenic substrate without the addition of enzyme conjugate indicates contamination of the substrate.

SPECIMEN COLLECTION

Precautions.
The Immunalysis Methadone specific Direct ELISA Kit is to be used with human forensic samples, such as whole blood, oral fluids, serum, plasma and urine. Immunalysis has not tested all possible applications of this assay. **Cutoff criteria are important in deciding the sample dilution.**

Additives.
Specimens to which sodium azide has been added affect the assay.

Storage and Handling Instructions.
Urine samples should be stored at 2 - 4°C until use. Samples should be well mixed before assay. Repeated freezing and thawing should be avoided. Urine samples should be shipped refrigerated with Blue Ice or equivalent.

DETAILS OF THE PROCEDURE.

All reagents must be brought to room temperature (20-25° C) before use.

The procedure as described below may be followed in sequence using manual pipettes. Alternatively all reagents may be added using an automated pipettor.

BLOOD
For a blood cutoff of 100 ng/ml - 200 ng/ml dilute all controls and samples 1:10 in Phosphate Buffer Saline and use 10 ul of the diluted sample in each well.

ORAL FLUIDS/ SALIVA
For a saliva cutoff of 25 ng/ml - 50 ng/ml dilute all controls and samples 1:10 in Phosphate Buffer Saline and use 25 ul of the diluted sample in each well

URINE
For a urine cutoff of 300 ng/ml dilute all controls and samples 1:40 in Phosphate Buffer Saline and use 10 ul of the diluted sample in each well.

1. Dilute forensic specimens, to the necessary range with Phosphate Buffer Saline pH 7.0. The dilution factor and volume added can be adjusted based on the laboratory's cutoff.
2. Add 10 µl. of appropriately diluted calibrators and standards to each well in duplicate.
3. Add 10 µl. of the diluted specimens in duplicate (recommended) to each well.
4. Add 100 µl. of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.
5. Incubate for 60 minutes at room temperature (20-25° C) preferably in the dark, after addition of enzyme conjugate to the last well.
6. Wash the wells 5 times with 350 µl. distilled water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells. If testing samples containing abnormally high amounts of hemoglobin (some Postmortem samples), use 10 mM Phosphate buffered saline pH 7.0-7.4. This will lower potential nonspecific binding of hemoglobin to the well, thus lowering background color.
7. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.
8. Add 100 µl. of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
9. Incubate for 30 minutes at room temperature, preferably in the dark.
10. Add 100 µl. of Stop Solution to each well, to change the blue color to yellow.
11. Measure the absorbance at a dual wavelength of 450 nm and 650 nm.
12. Wells should be read within 1 hour of yellow color development.

The following data represent a typical dose/response curve.

Methadone Pg/well	Absorbance
0	2.587
125	1.417
250	0.752
500	0.222

The dose/response curve shown above should not be used in assay calculations. It is recommended that at least one in-house positive quality control sample be included with every assay run.
A dose response curve or a cutoff calibrator should be run with every plate.

RESULTS

If the average sample absorbance is equal to or less than the average absorbance of the laboratory positive reference standard the sample is POSITIVE for Methadone. If the average sample absorbance is greater than the average absorbance of the laboratory positive reference standard the sample is called NEGATIVE for Methadone.

Alternatively a dose response curve can be established by plotting standard concentration (abscissa) against corresponding absorbance (ordinate). Values for unknown samples are obtained by interpolation from the curve.

SPECIFIC PERFORMANCE CHARACTERISTICS

Accuracy
50 known negative urines were screened using a cutoff of 300 ng/ml of Methadone with the Immunalysis Methadone Direct ELISA kit. All 50 samples screened negative. 25 urine samples containing amounts of Methadone greater than 300 ng/ml, confirmed by GC/MS were screened using a cutoff of 300 ng/ml of Methadone with the Immunalysis Direct ELISA Kit. All 25 samples screened positive at the 300 ng/ml cutoff

Precision
The precision of the Immunalysis Methadone Direct ELISA Kit has been verified by assessment of the mean, standard deviation (SD) and coefficients of variation (CV) in data resulting from repetitive assays.

7.2.6 Immunalysis Methamphetamine ELISA Specification

Specificity
The specificity of the Immunalysis ELISA Methamphetamine was determined by generating inhibition curves for each of the compounds listed below The antisera cross-reactivities are listed in Table 2.

Table 2
Methamphetamine Antiserum Cross-reactivities

Compound	Approx. ng/ml equivalent to 50ng/ml d-methamphetamine	Cross-reactivities
d,l-methamphetamine	77	65
l-methamphetamine	625	8
d,l-MDMA	37	135
d-amphetamine	2500	2
l-amphetamine	1500	3.4
p-hydroxyamphetamine	>5000	<1.0
d,l-MDA	>5000	<1.0
d,l-MDEA	500	10
d,l- MBDB	1000	5
d,l-HMA	>5000	<1.0
fenfluramine	1000	5
d-ephedrine	4300	1.2
l-ephedrine	>5000	<1.0
d,l-ephedrine	>5000	<1.0
beta - phenethylamine	>5000	<1.0
phentermine	>5000	<1.0
d-phenylpropanolamine	>5000	<1.0
d,l-phenylpropolamine	>5000	<1.0
d-pseudoephedrine	>5000	<1.0
l-pseudoephedrine	>5000	<1.0
tyramine	>5000	<1.0

Cross-Reactivity with Unrelated Drugs
Aliquots of a human urine matrix were spiked with the following compounds at a concentration of 10,000 ng/ml. None of these compounds gave values in the assay that were equal to or greater than the assay sensitivity level (1 ng/ml).

Acetaminophen, Acetylsalicylic acid, Aminopyrine, Ampicillin Amobarbital, Ascorbic acid, Atropine, Barbital, Benzoylecgonine, Butabarbital, Caffeine , Cocaine, Carbamazepine, Codeine, Chloroquine, Chlorpromazine, Carbromal, Desipramine, Dextromethorphan, Dextropropoxyphene, 5,5-Diphenylhydantoin, 10-11-Dihydro carbamazepine, Diazepam, Ethosuximide, Estriol, Estrone, Estradiol, Ethotoin, Glutethimide, Hexobarbital, Ibuprofen, Imipramine, Lidocaine, LSD, Methadone, Methadone-primary metabolite, Methaqualone, Metharbital , Mephenytoin, 3-Methyl-3-propylsuccinimide, Mephobarbital, Methyl PEMA, Methsuximide , 4-Methylprimidone, Morphine, Meperidine, Nitrazinamide, Norethindrone, N-Normethsuximide, Phenobarbital, Phensuximide,PEMA, Primidone, Phencyclidine, Pentobarbital, Phenothiazine, Procaine, Quinine, Secobarbital, Tetracycline, Tetrahydrozoline, THCCOOH.

REFERENCES

1. Urine Testing for Drugs of Abuse, National Institute on Drug Abuse Research Monograph, 73,1988.
2. R.C. Baselt. In : Advances in Analytical Technology, Vol.1. Randall C. Baselt edd. (Biomedical Publications, Foster City, CA. 87-93).
3. Driscoll, R.C., Barr, F.S., Gragg, B.J. and G.W. Moore. Determination of Therapeutic Blood Levels of Methamphetamine by GC. J.Pharm. Sci. 60:1492.1971.

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METHAMPHETAMINE DIRECT ELISA KIT

Version: 05/2008

Immunalysis Corporation :
Catalog Number 211-0192 2 x 96 well plates
Catalog Number 211-0480 5 x 96 well plates
Catalog Number 211-4800 50 x 96 well plates

THE IMMUNALYSIS METHAMPHETAMINE DIRECT ELISA KIT IS INTENDED FOR FORENSIC USE ONLY.

The Immunalysis Methamphetamine Direct ELISA Kit provides only a preliminary analytical test result. A more specific alternate chemical method must be used in order to obtain a confirmed analytical result. Gas chromatography/ mass spectrometry (GS-MS) is the preferred confirmatory method (1). Professional judgement should be applied to any drug of abuse test result, particularly when preliminary positive results are used.

EXPLANATION OF THE TEST

The Immunalysis Methamphetamine Direct ELISA Kit is a specific and sensitive in-vitro test to detect the presence of d-methamphetamine in forensic samples such as whole blood, oral fluids, serum, plasma and urine. While the assay will detect amphetamine use, interference by l-methamphetamine and pseudo-ephedrine is virtually nonexistent.

Methamphetamine is a potent central nervous system stimulant(1) with less peripheral actions than amphetamine. The (+)-isomer also referred to as d-methamphetamine is ten times more potent than the (-)-isomer, l-methamphetamine. Amphetamines act by inducing euphoria, irritability, anxiety and paranoia Methamphetamine is metabolized to its active metabolite amphetamine (via N-demethylation) and is further metabolized by hydroxylation and deamination of amphetamine. Urinary excretion rates are influenced by the urinary pH with acidic urine favoring the excretion of unchanged drug(2). Alkaline urine reduces the excretion of unchanged methamphetamine to less than 5% of the dose.

PRINCIPLES OF THE PROCEDURE

The Immunalysis Methamphetamine Direct ELISA Kit (for d-methamphetamine measurement) is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture. A 10 µL aliquot of a diluted unknown specimen is incubated with a 100 µL dilution of enzyme (Horseradish peroxidase) labeled d-methamphetamine derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 1 ng/ml.

The Immunalysis Methamphetamine Direct ELISA Kit avoids extraction of urine sample for measurement. It employs a d-methamphetamine directed antiserum. Due to the proprietary method of orienting the antibody on the polystyrene micro-plate much higher sensitivity is achieved compared to passive adsorption. This allows an extremely small sample size, reducing matrix effects and interference with binding proteins(s) or other macromolecules.

MATERIALS AND EQUIPMENT

Materials and equipment required but not supplied with the Immunalysis Methamphetamine Direct ELISA Kit are itemized below.
12x75 mm Disposable Glass or Plastic Culture Tubes to pre-dilute samples (if required).
Manual or electronic micropipets (single channel or multi channel) or automated pipetting stations.
Refrigerator (for kit storage).
Interval Timer.
Wash bottle or Plate Washer.
Micro-plate reader capable of reading at 450 nm and 650 nm.

REAGENTS

Immunalysis Methamphetamine Direct ELISA Kit Contents.

Component	Test Kit Cat#211-0192	480 Test Kit Cat#211-0480	4800 Test Kit Cat#211-4800
96 well Micro-plate	2	5	50
d-Meth-Conjugate	25 ml	60 ml	750 ml
Neg Std	2 ml	5 ml	3 x 5 ml
TMB Substrate	30 ml	2 x 30 ml	750 ml
Stop Reagent	25 ml	55 ml	750 ml

96 well micro-plate. The micro-plate is coated with polyclonal anti-d-methamphetamine via a spacer chain to provide optimally oriented binding sites. The plates are sealed in a moisture and air barrier pouch with a desiccant.

Enzyme Conjugate The conjugate solution contains d-methamphetamine labeled with horseradish peroxidase in a buffered protein solution with stabilizers, pH 7.6 containing azide free preservatives. (Colored Red)

Negative Standard. This bottle contains drug free synthetic urine containing azide free preservatives.

TMB chromogenic substrate. The color reagent contains 3,3',5,5' tetramethylbenzidine and peroxide in buffer.

Stop solution. This contains 1 N hydrochloric acid.

Precautions
1. Not for Internal or External Use in Humans or Animals.
2. There should be no eating or drinking within work area.
3. Always wear gloves and a protective lab coat.
4. No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.
5. Do not add sodium azide to samples as preservative.
6. Do not use external controls containing sodium azide.
7. Use disposable pipet tips to avoid contaminating chromogenic substrate reagent. Discard reagent if it turns blue.
8. Do not pour chromogenic substrate back into container after use.
9. Do not freeze reagents.
10. Do not mix reagents from different kit lot numbers.
11. Keep reagents out of direct sunlight.
12. Handle stop reagent with care, since it is corrosive.
13. Bring all reagents to room temperature.
14. Viscous forensic samples should always be diluted in phosphate buffered saline or distilled water prior to pipetting.
15. Ensure the bag containing the micro-plate and dessicant is well sealed, if only a few strips are used.

General. Precise pipetting is the essence of successful radio immunoassay. Micropipets supplied by "Eppendorf" or "SMI" with disposable tips are excellent when used carefully according to instructions to insure the necessary accuracy. New automatic dispensers improve reliable delivery.

Storage. The expiration date of the kit is stated on the label. The kit can be expected to perform satisfactorily until the expiration date if stored in the refrigerator at 2 – 4°C.

Indications of Deterioration. A drop of greater than 50% in the A_0 value (zero standard absorbance reading) for a constant incubation time indicates deterioration of the antibody plate, enzyme conjugate or chromogenic substrate. A significant shift of the standard curve to the right would result from deterioration of the standards. Development of blue color in the chromogenic substrate without the addition of enzyme conjugate indicates contamination of the substrate.

SPECIMEN COLLECTION

Precautions.
The Immunalysis Methamphetamine Direct ELISA Kit is to be used with human forensic samples, such as whole blood, oral fluids, serum, urine and plasma. Immunalysis has not tested all possible applications of this assay. **Cutoff criteria are important in deciding the sample dilution.**

Additives.
Specimens to which sodium azide has been added affect the assay.

Storage and Handling Instructions.
Urine samples should be stored at 2 - 4 degrees centigrade until use. Samples should be well mixed before assay. Repeated freezing and thawing should be avoided. Urine samples should be shipped refrigerated with Blue Ice or equivalent.

DETAILS OF THE PROCEDURE.

All reagents must be brought to room temperature (20-25° C) before use.

The procedure as described below may be followed in sequence using manual pipettes. Alternatively all reagents may be added using an automated pipettor .

1. Dilute forensic specimens, to the necessary range with Phosphate Buffer Saline pH 7.0. (Urine samples are normally diluted 1:20 for a methamphetamine cutoff of 500 ng/ml.) The dilution factor and volume added can be adjusted based on the laboratory's cutoff.
2. Add 10 µL of appropriately diluted calibrators and standards to each well in duplicate.
3. Add 10 µL of the diluted specimens in duplicate (recommended) to each well.

4. Add 100 µl of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.
5. Incubate for 60 minutes at room temperature (20-25° C) preferably in the dark, after addition of enzyme conjugate to the last well.
6. Wash the wells 6 times with 350 µL distilled water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells. If testing samples containing abnormally high amounts of hemoglobin (some Postmortem samples), use 10 mM Phosphate buffered saline pH 7.0-7.4. This will lower potential nonspecific binding of hemoglobin to the well, thus lowering background color.
7. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.
8. Add 100 µL of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
9. Incubate for 30 minutes at room temperature, preferably in the dark.
10. Add 100 µL of Stop Solution to each well, to change the blue color to yellow.
11. Measure the absorbance at a dual wavelength of 450 nm and 650 nm.
12. Wells should be read within 1 hour of yellow color development.

The following data represent a typical dose/response curve.

d-methamphetamine ng/ml	Absorbance
0	1.519
10	0.849
25	0.471
50	0.359

The dose/response curve shown above should not be used in assay calculations. It is recommended that at least one in-house positive quality control sample be included with every assay run. A dose response curve or a cutoff calibrator should be run with every plate.

RESULTS

If the average sample absorbance is equal to or less than the average absorbance of the laboratory positive reference standard the sample is POSITIVE for methamphetamine. If the average sample absorbance is greater than the average absorbance of the laboratory positive reference standard the sample is called NEGATIVE for methamphetamine.

Alternatively a dose response curve can be established by plotting standard concentration (abscissa) against corresponding absorbance (ordinate). Values for unknown samples are obtained by interpolation from the curve.

SPECIFIC PERFORMANCE CHARACTERISTICS

Accuracy
Sixty whole blood samples and 40 urine samples collected from presumed non-users were tested in the Immunalysis Methamphetamine Direct ELISA Kit . One hundred percent of these normal samples measured negative at 50 ng/ml for whole blood and 500 ng/ml for urine. Fifty five whole blood samples which were previously confirmed positive for methamphetamine by GC-MS employing a cut-off of 50 ng/ml, were tested in the Immunalysis Methamphetamine Direct ELISA Kit . All of the samples were found to be positive i.e. above the cut-off of 50 ng/ml.

Precision
The precision of the Immunalysis Methamphetamine Direct ELISA Kit has been verified by assessment of the mean, standard deviation (SD) and coefficients of variation (CV) in data resulting from repetitive assays.

Intra-assay Precision
Intra-assay precision was determined with reference controls.
A 0,10, 25 and 50 ng/ml standard was assayed five times in the same assay. The results are tabulated in Table 1.

Table 1

Methamphetamine (ng/ml)	Mean Abs.	S.D.	C.V.%
0	1.652	0.089	5.4
10	0.697	0.055	7.9
25	0.504	0.0614	12.2
50	0.366	0.0391	10.7

Sensitivity
Assay sensitivity based on the minimum methamphetamine concentration required to produce a four standard deviation from assay Ao is 1 ng/ml.

7.2.7 Immunalysis Opiate ELISA Specification

Sensitivity
Assay sensitivity based on the minimum morphine concentration required to produce a four standard deviation from assay Ao is 0.25 ng/mL.

Specificity
The specificity of the Immunalysis ELISA for Opiates was determined by generating inhibition curves for each of the compounds listed below The antisera cross-reactivities are listed in Table 2.

TABLE 2
Cross Reactivities with Related Drugs

Compound	Approx. ng/mL equivalent to 25 ng/mL morphine	Cross-reactivities
Morphine	25	100
Dihydrocodeine	29.5	85
Codeine	12.5	200
Morphine 3-glucuronide	40	62.5
Morphine-6-glucoronide	>1000	<4
Hydrocodone	27	93
Hydromorphone	31	81
6-acetyl-morphine	30	83
6-acetyl-codeine	42	41.7
Oxycodone	120	21
Oxymorphone	125	20
Normorphine	800	3
Norcodeine	300	8.3
Noroxycodone	>10000	<0.25
Noroxymorphone	>10000	<0.25
Naloxone	250	10
Meperidine	>5000	<0.5
Normeperidine	>5000	<0.5
Fentanyl	>5000	<0.5
Norfentanyl	>5000	<0.5
Buprenorphine	>10000	<0.25
Norbuprenorphine	>10000	<0.25

Cross-Reactivities with Unrelated Drugs

Aliquots of a human urine matrix were spiked with the following compounds at a concentration of 10,000 ng/mL. None of these compounds gave values in the assay that were equal to or greater than the assay sensitivity level (0.25 ng/mL).

Acetaminophen, Acetylsalicylic acid, Amphetamine, Aminopyrine, Ampicillin, Amobarbital, Ascorbic acid Atropine , Barbital, Benzoylecgonine, Butabarbital, Caffeine, Cocaine, Carbamazepine, Chloroquine , Chlorpromazine, Carbromal, Desipramine, Dextromethorphan, Dextropropoxyphene , 5,5-Diphenylhydantoin, 10-11-Dihydrocarbamazepine, Diazepam, Doxepin, EMDP,Ethosuximide, Estriol, Estrone, Estradiol, Ethotoin, Glutethimide, Hexobarbital, Ibuprofen, Imipramine, LAAM, Lidocaine, LSD, MDA, MDMA, Methadone, Methadone-primary metabolite, Methaqualone, Methamphetamine, Methartbital , Mephenytoin, a-Methyl-a-propylsuccinimide, Mephobarbital, Methyl PEMA, Methsuximide , 4-Methylprimidone, Meperidine , Niacinamide, Norethindrone, N-Normethsuximide, Nor LAAM, Nortriptyline, PCP, Phenobarbital , Phensuximide, PEMA, Primidone, Phencyclidine, Pentobarbital, Phenothiazine, Phenylpropanolamine, Procaine, Propoxyphene, Quinine, Secobarbital, Tetracycline, Tetrahydrozoline, THCCOOH , Tramadol, O-desmethyl Tramadol, N-Desmethyl Tramadol

REFERENCES

1. Urine Testing for Drugs of Abuse, National Institute on Drug Abuse Research Monograph, 73,1956.
2. Drugs on the Job. Time Magazine, March 17, 1986
3. E.L.Wey and T.K.Adler. Bull. Wld. Hlth. Org. 27:359 (1962)
4. R.C. Baselt. In : Advances in Analytical Technology, Vol.1. Randall C. Baselt edd. (Biomedical Publications, Foster City, CA. 112- 116).

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IMMUNALYSIS OPIATES DIRECT ELISA KIT

Ver: 05/2009

Immunalysis Corporation:
Catalog Number 207-0192 2 x 96 well plates
Catalog Number 207-0480 5 x 96 well plates
Catalog Number 207-4800 50 x 96 well plates

THE IMMUNALYSIS OPIATES DIRECT ELISA KIT IS INTENDED FOR FORENSIC USE ONLY.

The Immunalysis Opiates Direct ELISA Kit provides only a preliminary analytical test result. A more specific alternate chemical method must be used in order to obtain a confirmed analytical result. Gas chromatographyl mass spectrometry (GC-MS) is the preferred confirmatory method (1). Clinical consideration and professional judgement should be applied to any drug of abuse test result, particularly when preliminary positive results are used.

EXPLANATION OF THE TEST

The Immunalysis Opiates Direct ELISA Kit is a specific and sensitive in-vitro test to detect the presence of Opiates in forensic samples such as whole blood, oral fluids, serum, plasma and urine.

Heroin/morphine abuse is a major problem in society (2). In the body, both heroin (diacetylmorphine) and morphine are largely converted to morphine-3-glucuronide (MG)(3). The Immunalysis Opiates ELISA Kit measures heroin, morphine, codeine, hydrocodone and their metabolites.

PRINCIPLES OF THE PROCEDURE

The Immunalysis Opiates Direct ELISA Kit (for morphine equivalents measurement) is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture.

A 10 µl aliquot of a diluted unknown specimen is incubated with a 100 µl dilution of enzyme (Horseradish peroxidase) labeled morphine derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 0.25 ng/mL.

The Immunalysis Opiates Direct ELISA Kit avoids extraction of urine sample for measurement. It employs an Opiates directed antiserum. Due to the proprietary method of orienting the antibody on the polystyrene micro-plate much higher sensitivity is achieved compared to passive adsorption. This allows an extremely small sample size reducing matrix effects and interference with binding proteins(s) or other macro-molecules.

MATERIALS AND EQUIPMENT

Materials and equipment required but not supplied with the Immunalysis Opiates Direct ELISA Kit are itemized below:
12x75 mm Disposable Glass or Plastic Culture Tubes to prediute samples (if required).
Manual or electronic micropipets (single channel or multichannel) or automated pipetting stations.
Refrigerator (for kit storage).
Interval Timer.
Wash bottle or Plate Washer.
Microplate reader capable of reading at 450 nm. and 650 nm.

REAGENTS

Immunalysis Opiates Direct ELISA Kit Contents.

Component	192 Test Kit Cat# 207-0192	480 Test Kit Cat# 207-0480	4800 Test Kit Cat# 207-4800
96 well Micro-plate	2	5	50
Morphine-Conjugate	25 mL	60 mL	750 mL
Neg Std	2 mL	5 mL	3 x 5 mL
TMB Substrate	30 mL	2 x 30 mL	750 mL
Stop Reagent	25 mL	55 mL	750 mL

96 well micro-plate. The micro-plate is coated with polyclonal anti-morphine via a spacer chain to provide optimally oriented binding sites. The plates are sealed in a moisture and air barrier pouch with a dessicant.

Morphine Conjugate. The conjugate solution contains a morphine derivative labeled with horseradish peroxidase in a buffered, protein solition with stabilizers, pH 7.6 containing non azide preservatives. (Colored Red)

Negative Standard. This bottle contains drug free synthetic urine containing non azide preservatives.

TMB chromogenic substrate. The color reagent contains 3,3',5,5' tetramethylbenzidine and urea peroxidase in buffer.

Stop solution. This contains 1 N hydrochloric acid.

Precautions
1. Not for Internal or External Use in Humans or Animals.
2. There should be no eating or drinking within work area.
3. Always wear gloves and a protective lab coat.
4. No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.
5. **Do not add sodium azide to samples as preservative.**
6. **Do not use external controls containing sodium azide.**
7. Use disposable pipet tips to avoid contaminating chromogenic substrate reagent. Discard reagent if it turns blue.
8. Do not pour chromogenic substrate back into container after use.
9. Do not freeze reagents.
10. Do not mix reagents from different kit lot numbers.
11. Keep reagents out of direct sunlight.
12. Handle stop reagent with care, since it is corrosive.
13. Bring all reagents to room temperature.
14. Viscous forensic samples should always be diluted in phosphate buffered saline or distilled water prior to pippetting.
15. Ensure the bag containing the micro-plate strips and dessicant is well sealed if only a few strips are used.

General. Precise pipetting is the essence of successful immunoassay. It is critical to pipet right at the center and bottom of each well to ensure good replicates and coefficents of variation Micropipets supplied by "Eppendorf" or "SMI" with disposable tips are excellent when used carefully according to instructions to insure the necessary accuracy. New automatic dispensers improve reliable delivery.

Storage. The expiration date of the kit is stated on the label. The kit can be expected to perform satisfactorily until the expiration date if stored in the refrigerator at 2 – 4° C.

Indications of Deterioration. A drop of greater than 50% in the A_0 (zero-standard absorbance reading) for a constant incubation time indicates deterioration of the antibody plate, enzyme conjugate or chromogenic substrate. A significant shift of the standard curve to the right would result from deterioration of the standards. Development of blue color in the chromogenic substrate without the addition of enzyme conjugate indicates contamination of the substrate.

SPECIMEN COLLECTION

Precautions.
The Immunalysis Opiates Direct ELISA Kit is to be used with human forensic samples, such as urine, whole blood, oral fluids, serum and plasma. Immunalysis has not tested all possible applications of this assay. Cutoff criteria are important in deciding the sample dilution.

Additives.
Specimens to which sodium azide has been added affect the assay.

Storage and Handling Instructions.
Urine samples should be stored at 2 -4° C until use. Samples should be well mixed before assay. Repeated freezing and thawing should be avoided. Urine samples should be shipped refrigerated with Blue Ice or equivalent.

DETAILS OF THE PROCEDURE.

All reagents must be brought to room temperature (20-25° C) before use.

The procedure as described below may be followed in sequence using manual pipettes. Alternatively all reagents may be added using an automated pipettor.

1) Dilute forensic specimens, to the necessary range with Phosphate Buffer Saline pH 7.0. (urine samples are normally diluted 1:20 for a cutoff level of 300 ng/mL of morphine.) The dilution factor can be adjusted based on the laboratory's cutoff.

2) Add 10 µl. of appropriately diluted calibrators and standards to each well in duplicate.
3) Add 10 µl. of the diluted specimens in duplicate (recommended) to each well.
4) Add 100 µl. of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.
5) Incubate for 60 minutes at room temperature (20-25° C) preferably in the dark, after addition of enzyme conjugate to the last well.
6) Wash the wells 6 times with 350 µl. distilled water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells. If testing samples containing abnormally high amounts of hemoglobin (some Postmortem samples), use 10 mM Phosphate buffered saline pH 7.0-7.4. This will lower potential nonspecific binding of hemoglobin to the well, thus lowering background color.
7) Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate, does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.
8) Add 100 µl. of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
9) Incubate for 30 minutes at room temperature, preferably in the dark.
10) Add 100 µl. of Stop Solution to each well, to change the blue color to yellow.
11) Measure the absorbance at a dual wavelength of 450 nm and 650 nm.
12) Wells should be read within 1 hour of yellow color development

The following data represent a typical dose/response curve.

Morphine ng/mL	Absorbance
0	2.669
5	1.238
10	0.794
25	0.133

The dose/response curve shown above should not be used in assay calculations. It is recommended that at least one in-house positive quality control sample be included with every assay run.
A dose response curve or a cutoff calibrator should be run with every plate.

RESULTS

If the average sample absorbance is equal to or less than the average absorbance of the laboratory morphine positive reference standard the sample is POSITIVE for Opiates. If the average sample absorbance is greater than the average absorbance of the laboratory morphine positive reference standard the sample is called NEGATIVE for Opiates.

Alternatively a dose response curve can be established by plotting standard concentration (abscissa) against corresponding absorbance (ordinate). Values for unknown samples are obtained by interpolation from the curve.

SPECIFIC PERFORMANCE CHARACTERISTICS

Accuracy
40 whole blood samples and 40 urine samples collected from presumed non-users were tested in the Immunalysis Opiates Direct ELISA Kit . One hundred percent of these normal samples measured negative at 25 ng/mL for whole blood and 300 ng/mL for urine. Fifty whole blood samples which were previously confirmed positive for Opiates by GC-MS employing a cut-off of 25 ng/mL, were tested in the Immunalysis Opiates Direct ELISA Kit . All of the samples were found to be positive i.e. above the cut-off of 25 ng/mL.

Precision
The precision of the Immunalysis Opiates Direct ELISA Kit has been verified by assessment of the mean, standard deviation (SD) and coefficients of variation (CV) in data resulting from repetitive assays.

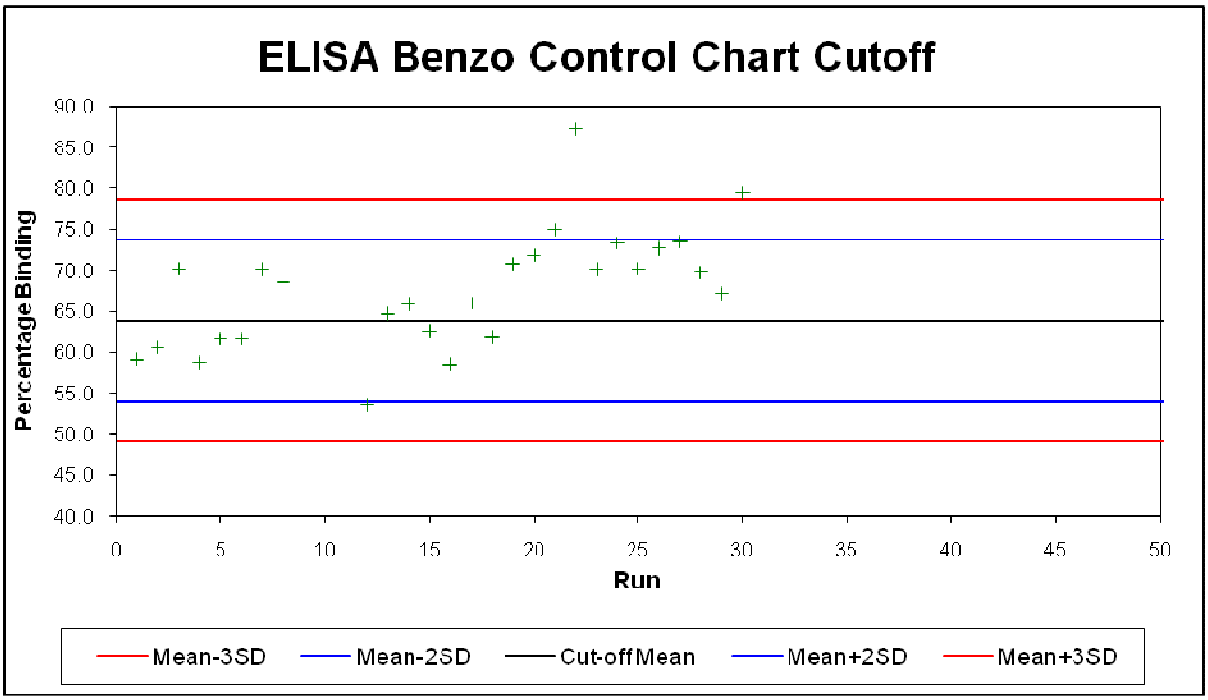
Intra-assay Precision
Intra-assay precision was determined with reference controls. A 0,5, 10 and 25 ng/mL standard was assayed five times in the same assay. The results are tabulated in Table 1.

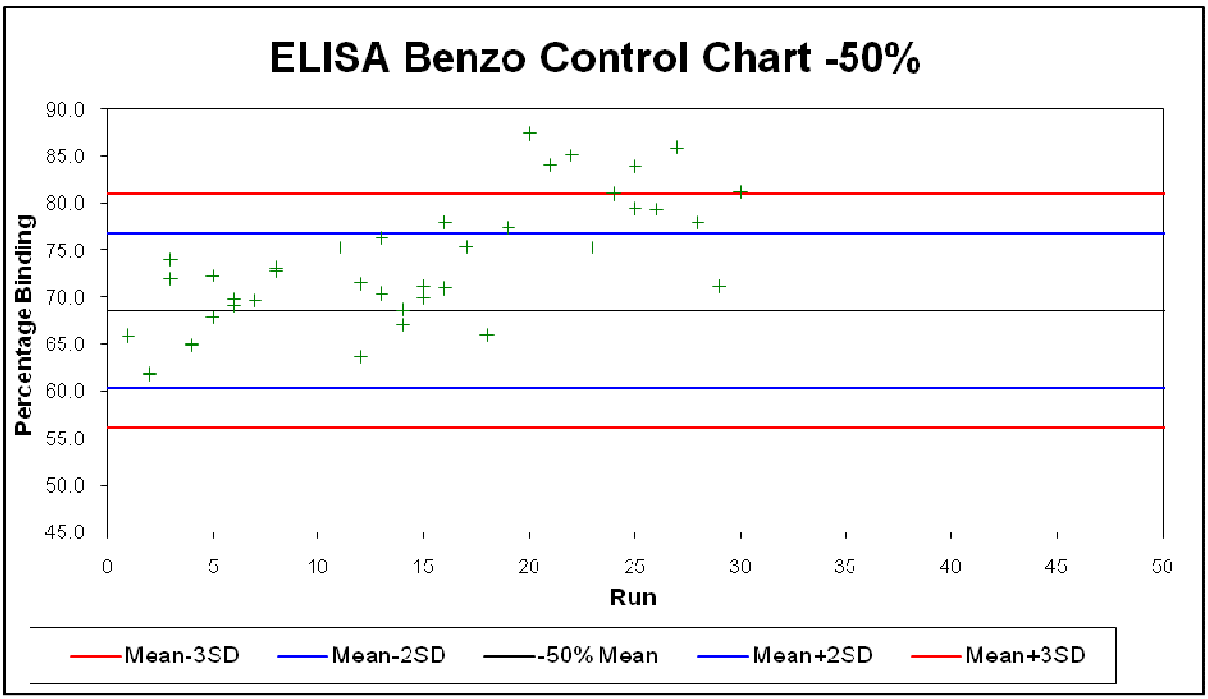
TABLE 1

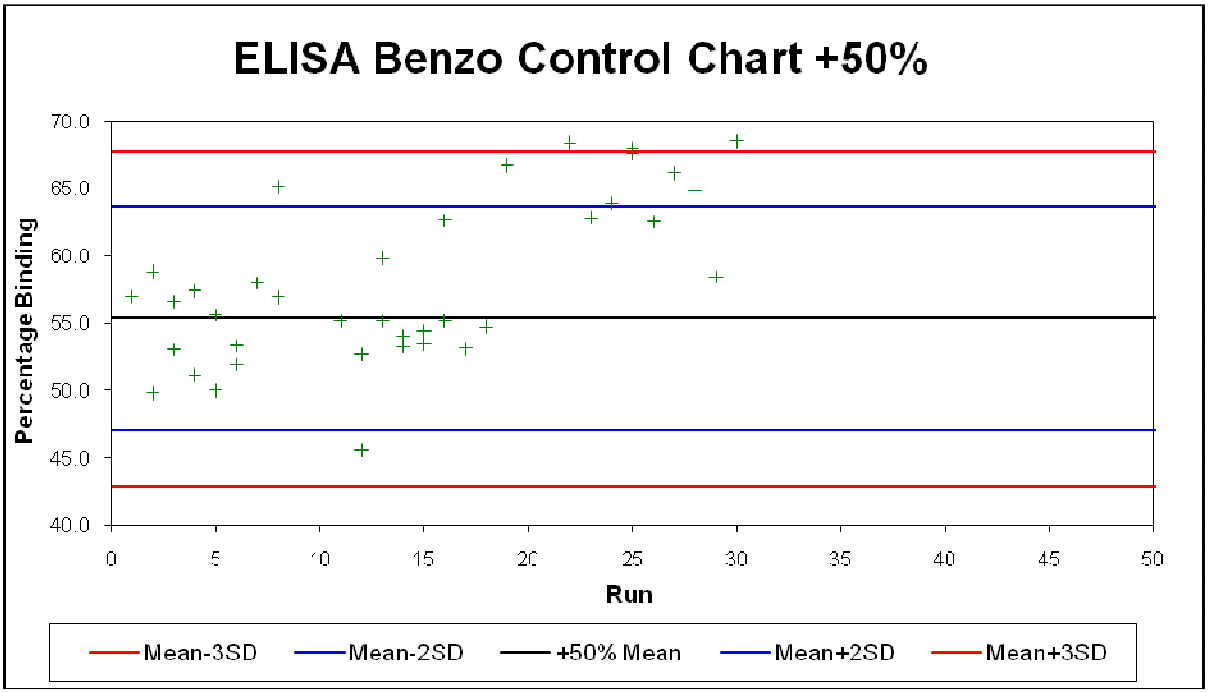
Morphine (ng/mL)	Mean Abs.	S.D.	C.V.%
0	2.654	0.21	7.9
5	1.198	0.145	12.1
10	0.806	0.093	11.54
25	0.099	0.011	11.1

7.3 Appendix 3 – ELISA QC Charts

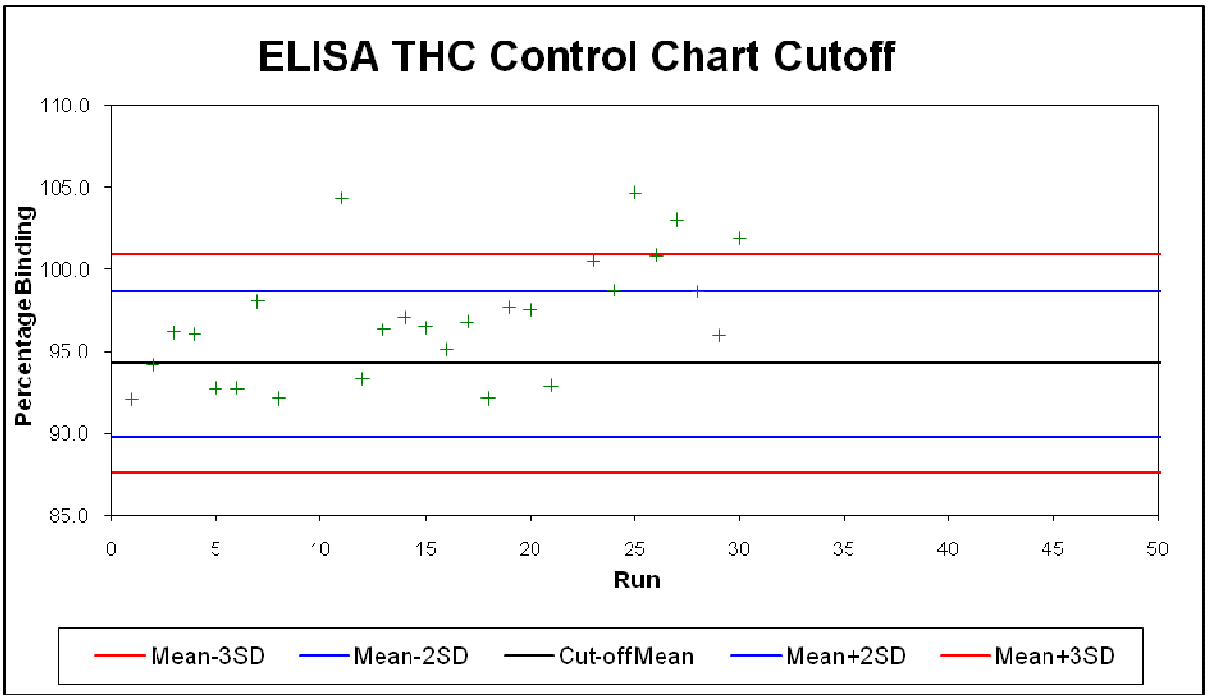
7.3.1 Benzodiazepine Control Charts

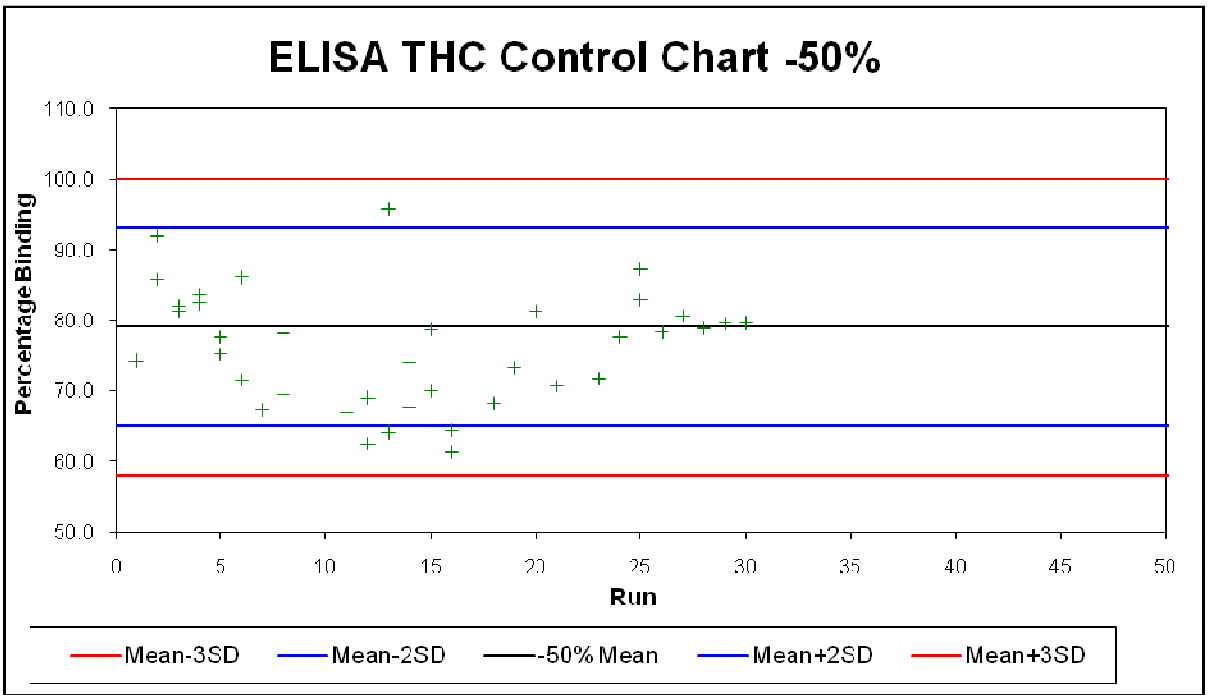


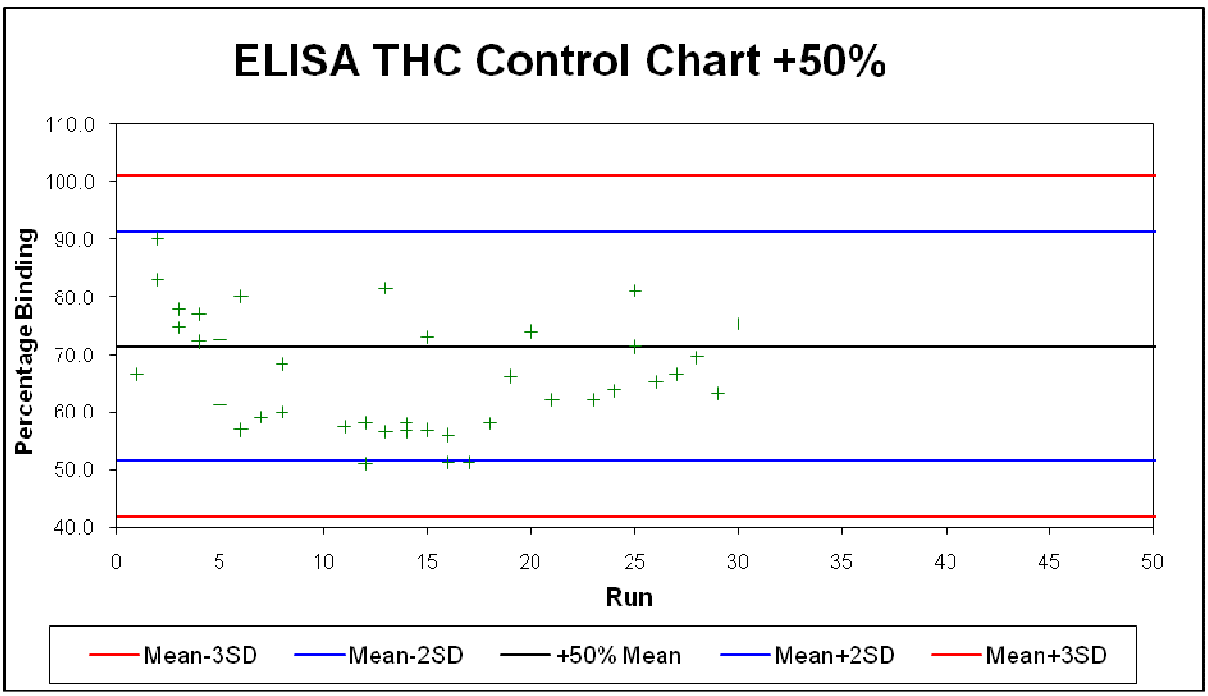




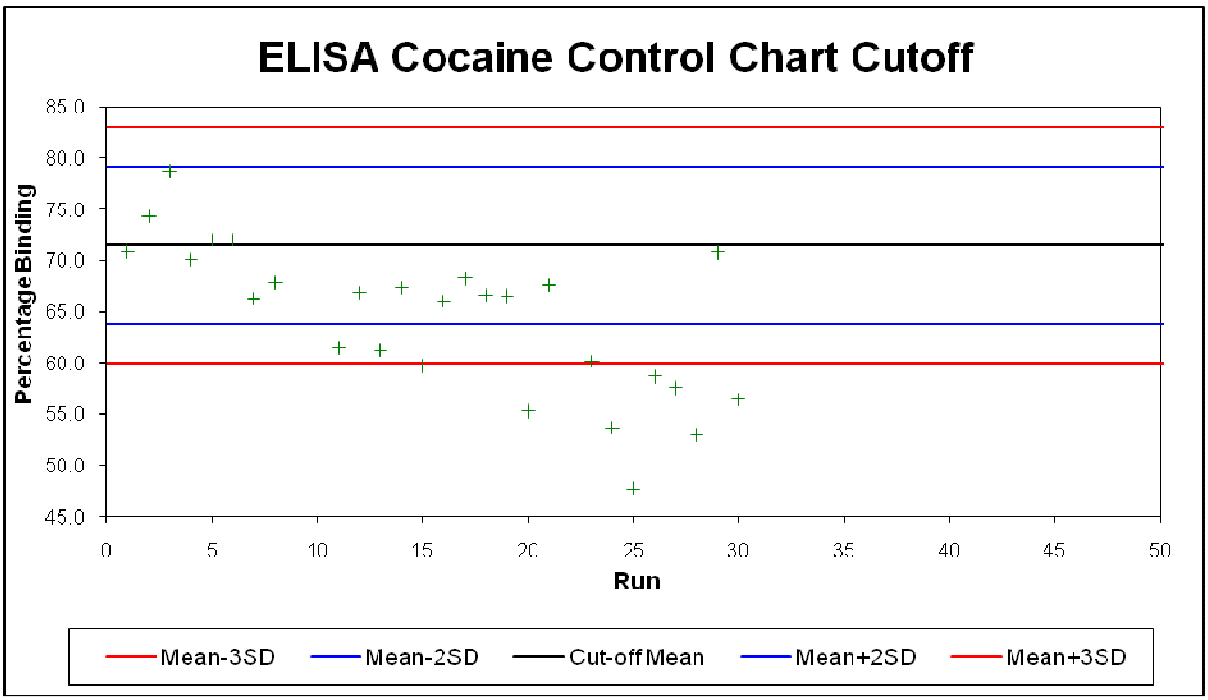
7.3.2 Cannabinoid Control Charts

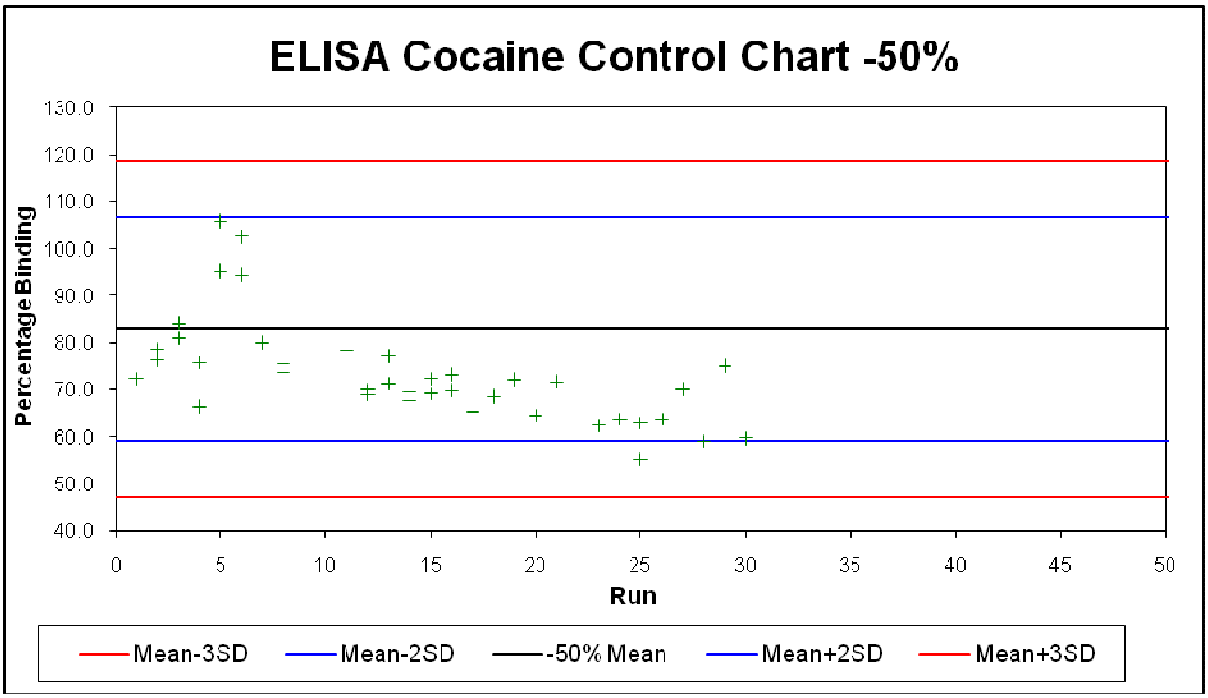


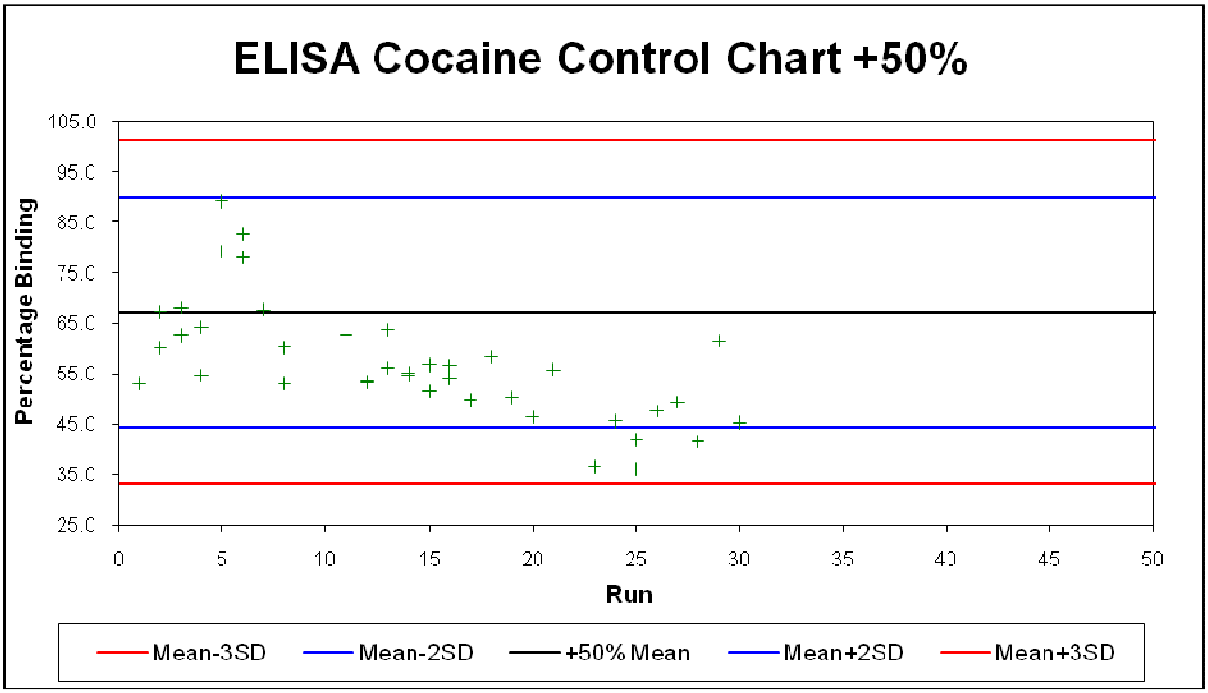

ELISA THC Control Chart -50%
Percentage Binding
110.0
100.0
90.0
80.0
70.0
60.0
50.0
0
5
10
15
20
25
30
35
40
45
50
Run
Mean-3SD
Mean-2SD
-50% Mean
Mean+2SD
Mean+3SD


ELISA THC Control Chart +50%
Percentage Binding
110.0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
0
5
10
15
20
25
30
35
40
45
50
Run
Mean-3SD
Mean-2SD
+50% Mean
Mean+2SD
Mean+3SD

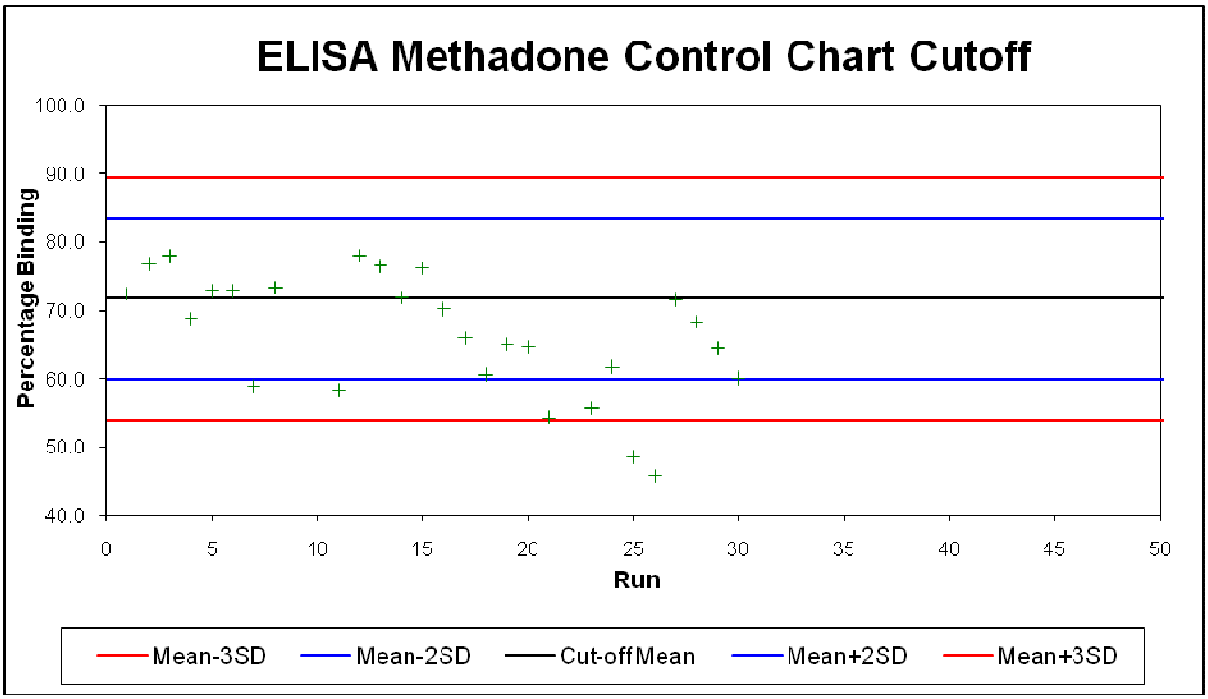
7.3.3 Cocaine Control Charts

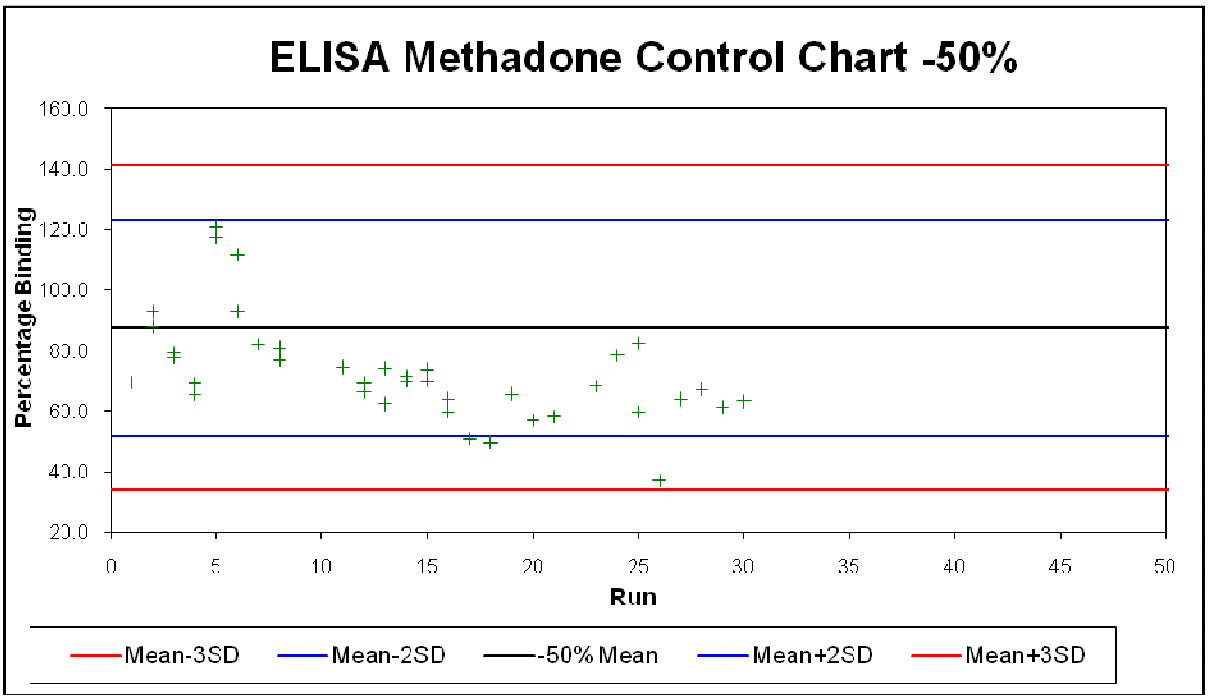


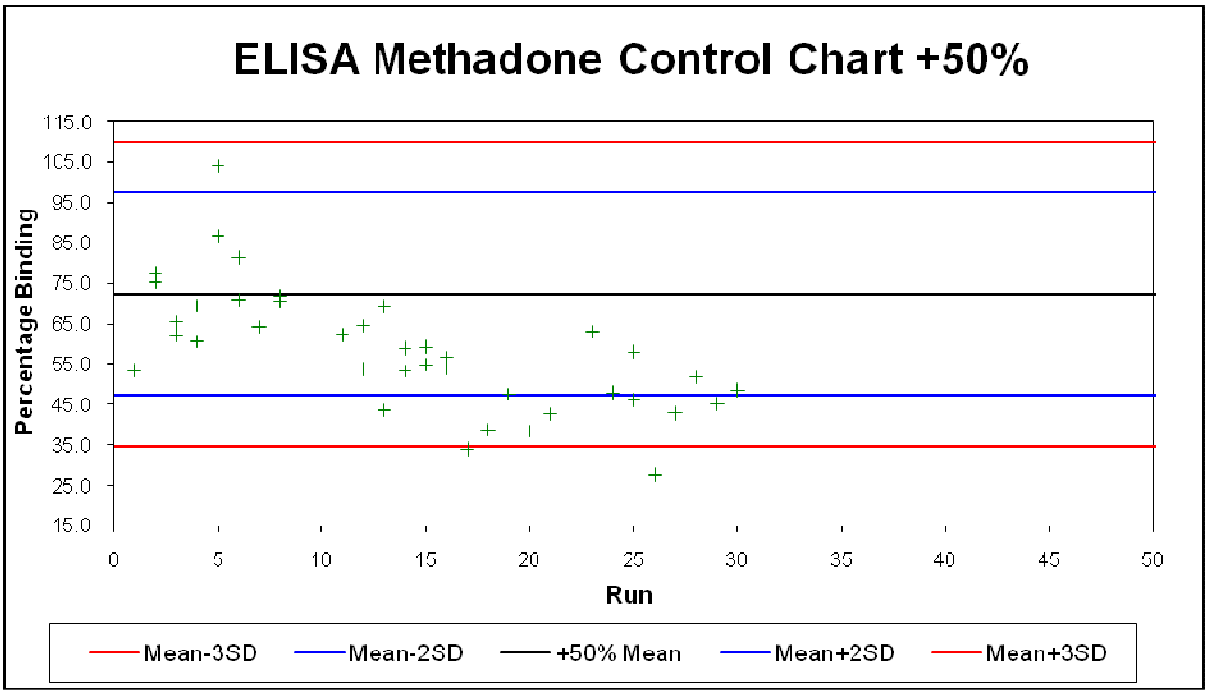




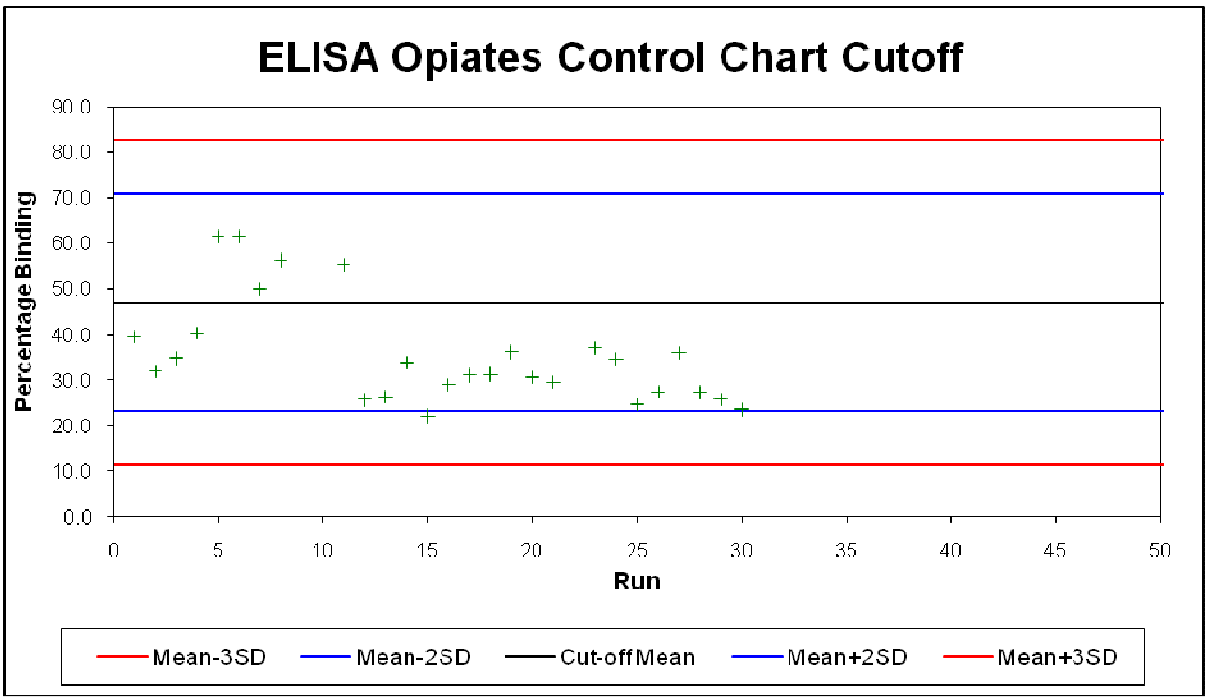
7.3.4 Methadone Control Charts

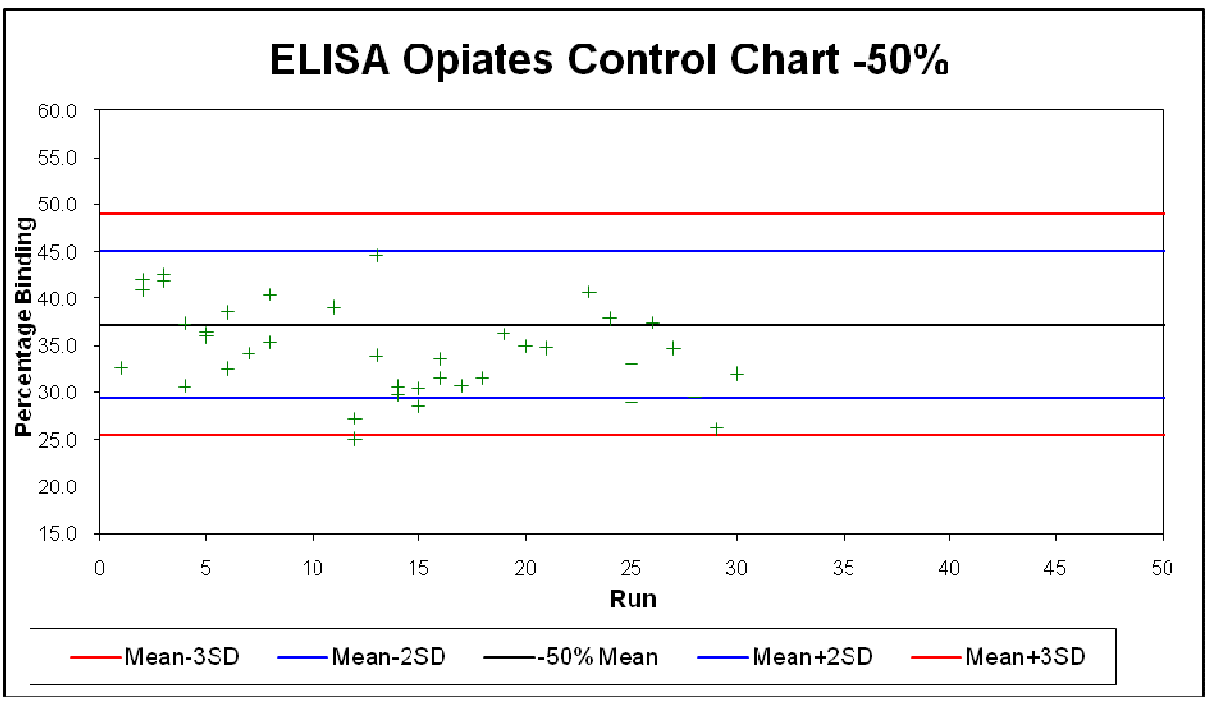


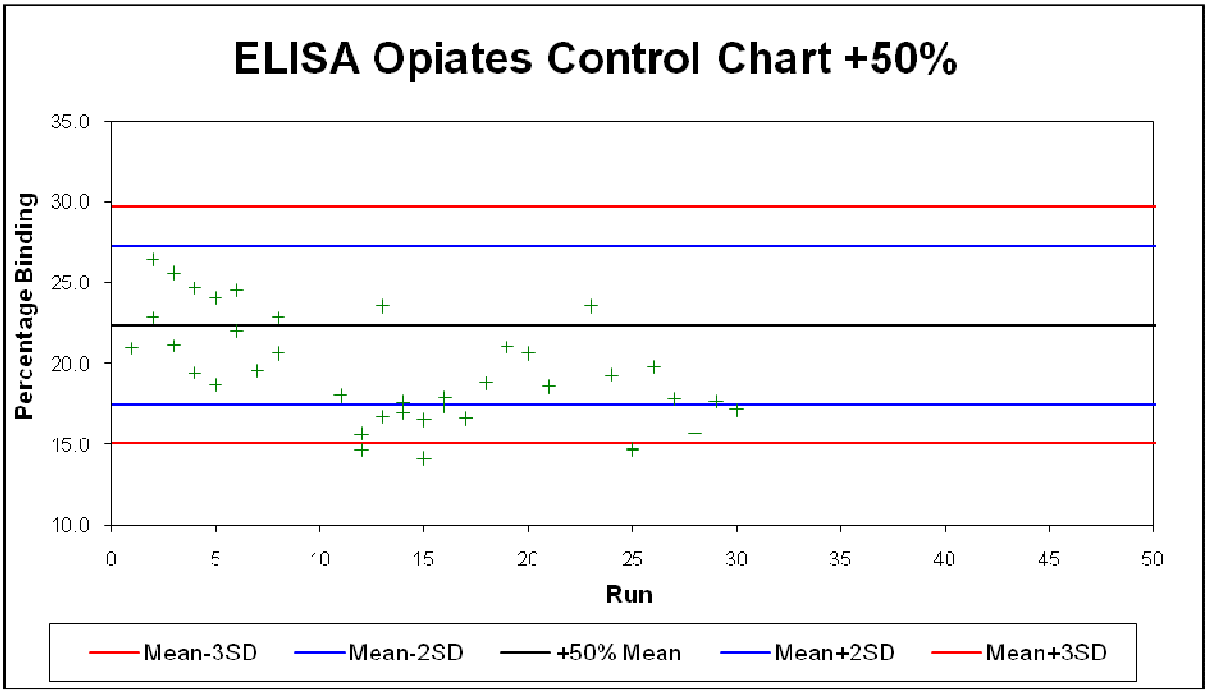

ELISA Methadone Control Chart -50%
Percentage Binding
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0
5
10
15
20
25
30
35
40
45
50
Run
Mean-3SD
Mean-2SD
-50% Mean
Mean+2SD
Mean+3SD


ELISA Methadone Control Chart +50%
Percentage Binding
115.0
105.0
95.0
85.0
75.0
65.0
55.0
45.0
35.0
25.0
15.0
0
5
10
15
20
25
30
35
40
45
50
Run
Mean-3SD
Mean-2SD
+50% Mean
Mean+2SD
Mean+3SD

7.3.5 Opiate Control Charts

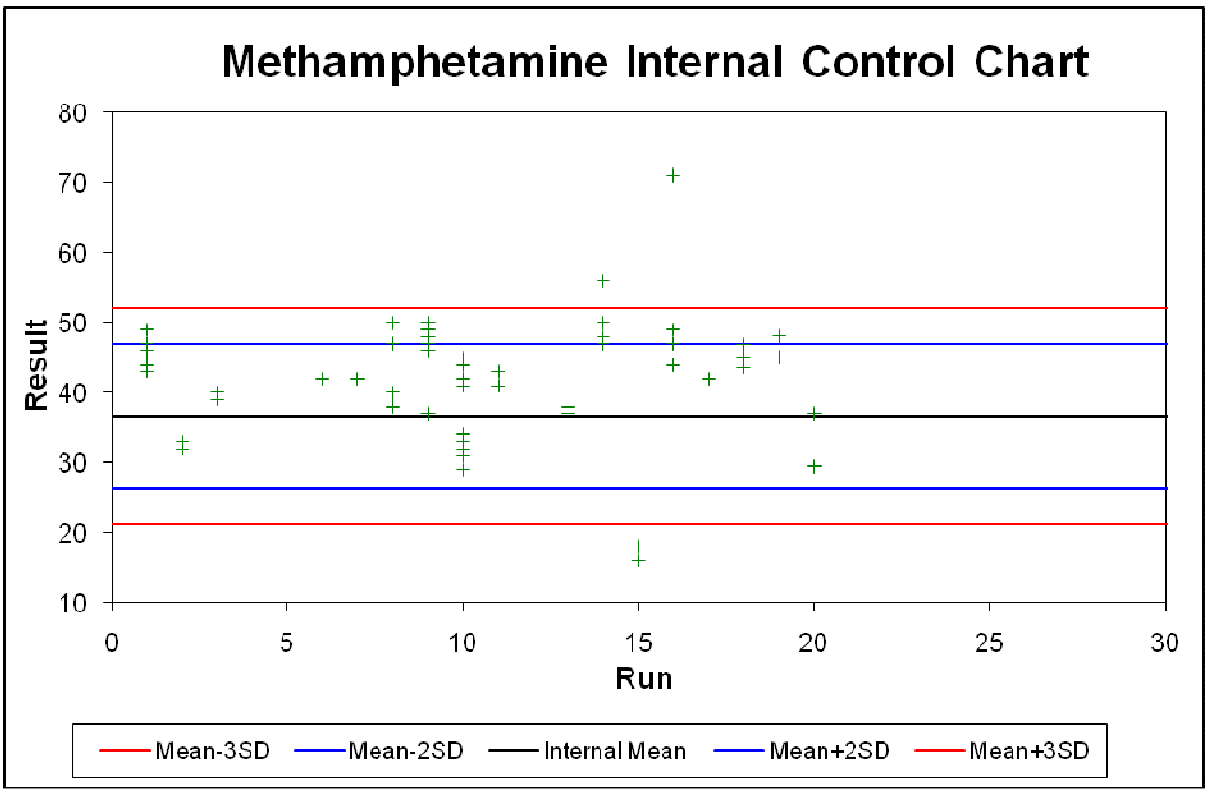




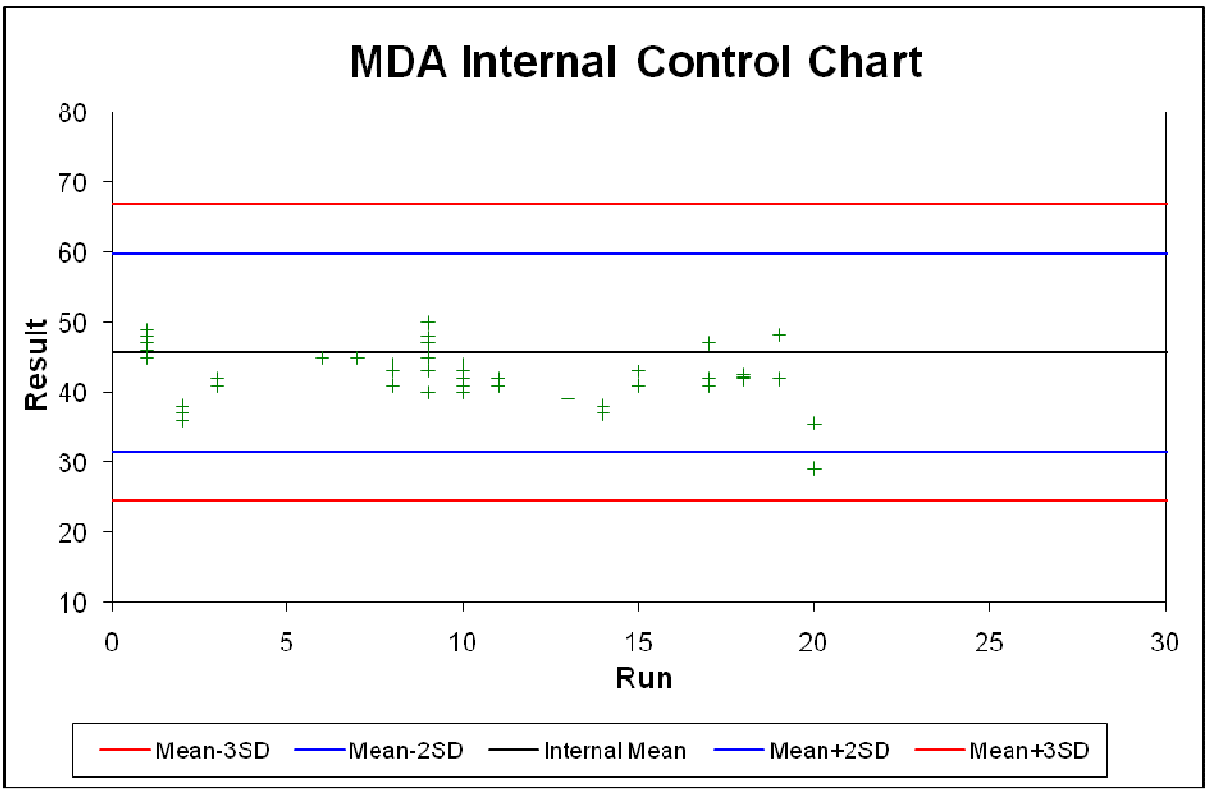


7.4 Appendix 4 – Confirmation QC Charts

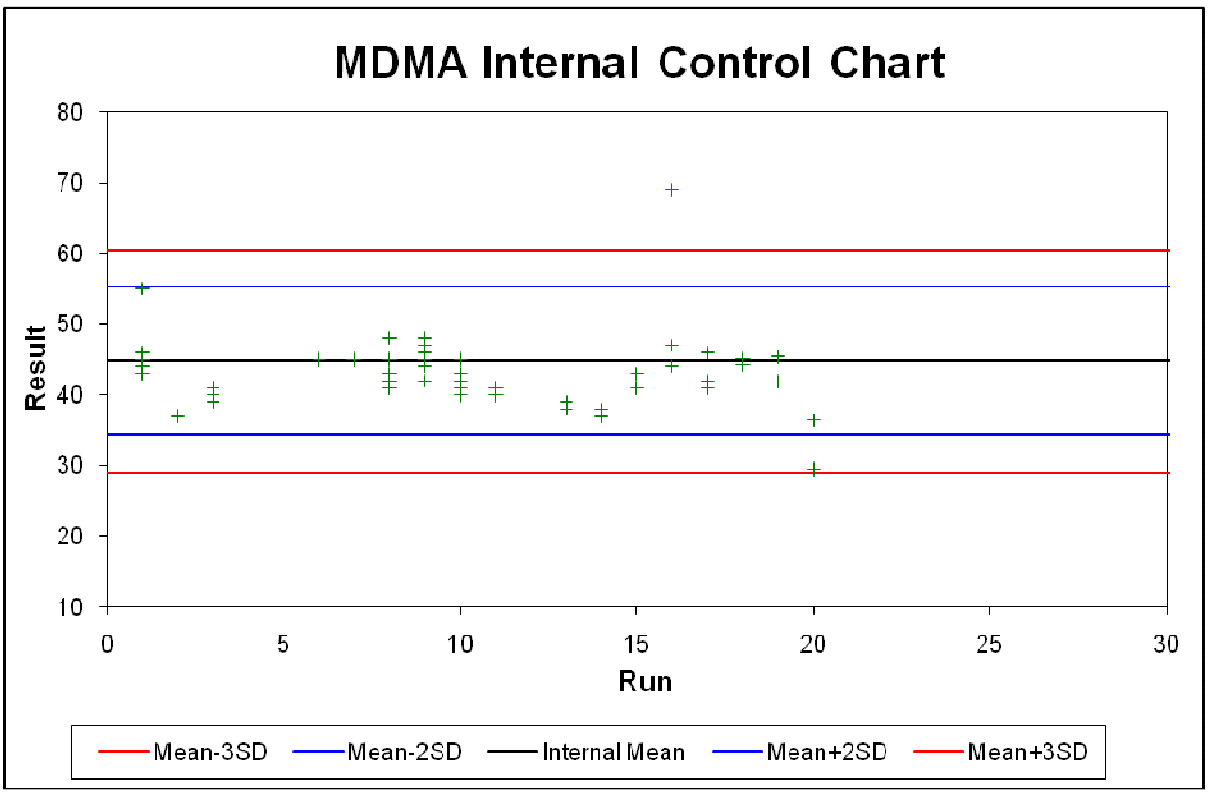
7.4.1 Methamphetamine Control Chart



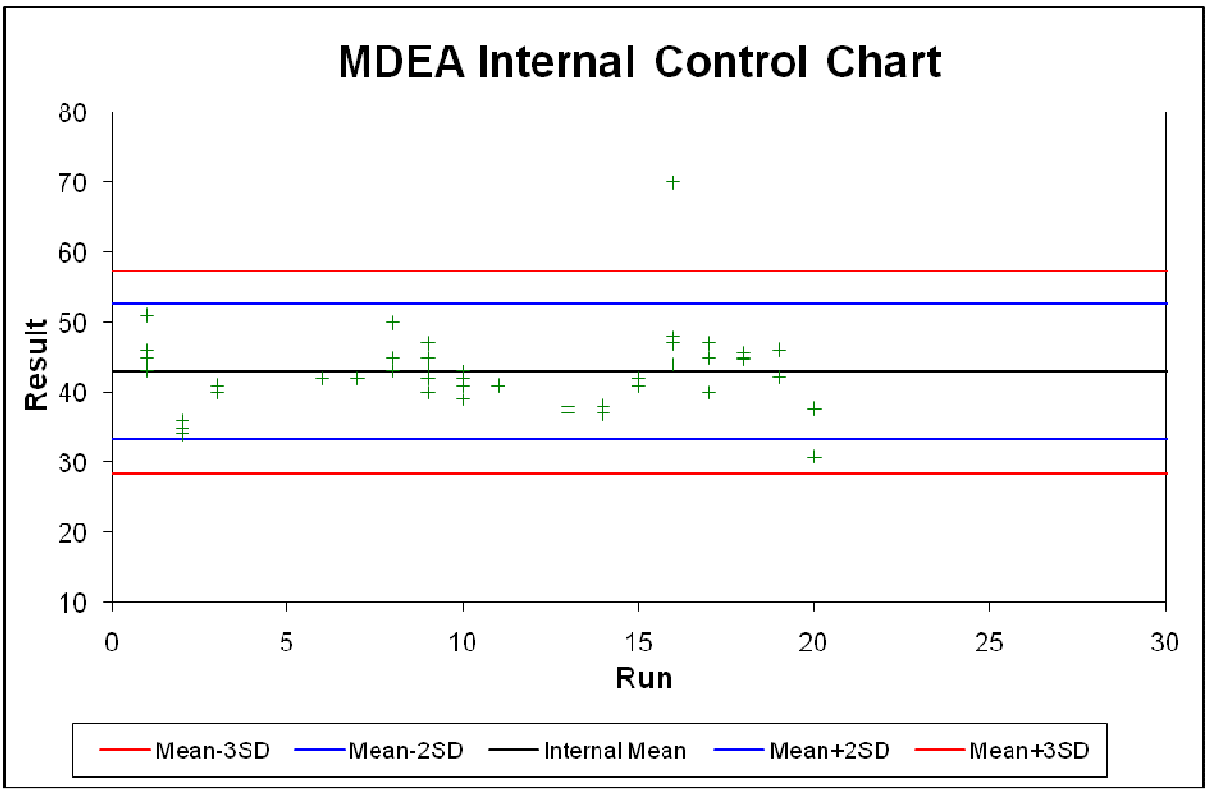
7.4.2 MDA Control Chart



7.4.3 MDMA Control Chart



7.4.4 MDEA Control Chart



7.5 Appendix 5 – Alcohol Assay Specification

IMMUNALYSIS

ORAL FLUID ETHYL ALCOHOL ASSAY

Version: 6/2006

Immunalysis Corporation:
Catalog Number: 502-0500

THE IMMUNALYSIS ORAL FLUID ETHYL ALCOHOL ASSAY IS INTENDED FOR FORENSIC USE ONLY.

The Immunalysis Ethyl Alcohol Assay is intended for the detection of ethyl alcohol in human oral fluid collected with the Quantisal™ Oral Fluid collector. Specimens containing alcohol should be confirmed with an alternate, non-enzymatic technology such as headspace gas chromatography.

EXPLANATION OF THE TEST

Alcohol intoxication can lead to loss of alertness, coma, death and as well as birth defects. Determination of ethyl alcohol in blood and saliva is commonly used for measuring legal impairment, alcohol poisoning, etc. Gas chromatography and enzymatic methods are commercially available for the determination of ethyl alcohol in biological fluids. (1,2) The Immunalysis Ethyl Alcohol Assay is an enzymatic method intended for the quantitation of ethyl alcohol in human oral fluid collected with the Quantisal™ oral Fluid collector.

PRINCIPLE OF THE PROCEDURE

Immunalysis Ethyl Alcohol Assay is based on the high specifity of alcohol dehydrogenase (ADH) for ethyl alcohol in the presence of nicotinamide adenine dinucleotide (NAD) as shown in the following:

$$EtOH + NAD \xrightarrow{\text{alcohol dehydrogenase(ADH)}} CH3CHO + NADH$$

An aliquot of the diluted oral fluid solution collected with the Quantisal collector is then added to each microplate well in duplicates. The assay buffer (RA) and enzyme (RE)are added to each well.
The plates are then covered and left at RT for 15 minutes. The plates are then read at 340nm.

The Quantisal™ oral fluid collection system collects 1 mL of neat oral fluid and dilutes it with 3 mL of preservative buffer. This results in a 1 to 4 dilution factor. However since both controls and samples are diluted the same way all concentrations in this insert refer to neat oral fluid equivalents.

MATERIALS PROVIDED:

- 1 x 55 mL bottle of Reagent A (RA) containing Tris buffer with 0.1% sodium azide as preservative.
- 1 x 55 mL bottle of Reagent E (RE) containing ADH and NAD in Tris buffer with stabilizers and 0.1% sodium azide as a preservative.
- 1 x 20 mL of the Quantisal extraction buffer.

MATERIALS REQUIRED BUT NOT PROVIDED

- 4 ethyl alcohol calibrators - negative (0.0g/dL), 0.02g/dL, 0.04g/dL, 0.08 g/dL and 0.16 g/dL are needed. Any controls employed should have external GC confirmation. These solutions should be diluted 1 to 4 in the Quantisal™ extraction buffer prior to use.
- 96 well flat bottom polystyrene micro-plates (**available from Immunalysis**)
- pipetors capable of pipeting 10uL and 100uL
- disposable pipette tips.
- refrigerator for kit storage
- interval timer
- microplate reader capable of measuring absorbance at 340 nm (**available from Immunalysis**)

PRECAUTIONS

1. The test is for forensic use only.
2. Don't use the reagents beyond the expiration dates.
3. Ethyl alcohol is very volatile and samples and controls should be stored tightly capped in a refrigerator
4. Handle all the human fluids as if they are potentially infectious
5. Not for internal or external use in humans or animals
6. There should be no eating or drinking in the work area
7. Always wear gloves and a protective lab coat
8. No pipetting should be done by mouth
9. Do not freeze reagents
10. Keep reagents out of direct sunlight
11. Bring all reagents to room temperature before proceeding

REAGENT PREPARATION

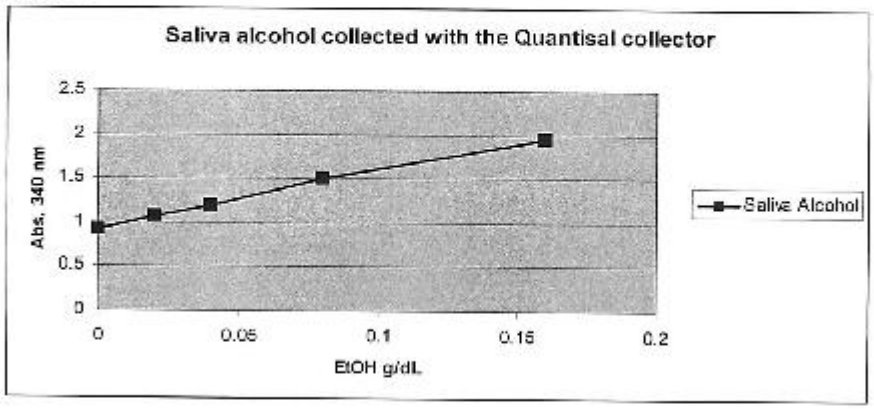
The reagents are ready to use, requiring no reconstitution or dilutions. All assay reagents and samples should be brought to room temperature before use.

PROCEDURE

1. Dilute the 0.0, 0.02, 0.04, 0.8 and 0.16 g/dL calibrators, 1 to 4 in the Quantisal™ extraction buffer solution.
2. Add 10 uL of the diluted calibrators to the appropriate positions on the micro-plate in duplicate.
2. Add 10 uL of the sample solution obtained from the Quantisal™ transport tube to the appropriate positions in duplicate.
6. Add 100 uL of RA assay buffer and 100uL of enzyme RE to each well.
7. Cover the plates and incubate in the dark at RT for 15 minutes.
8. Read the plate at 340nm
9. Calculate results

The following data were obtained based using 10 uL of sample volume from calibrators spiked with ethanol collected with the Quantisal™ oral fluid collector

FIGURE 1



LIMIT OF DETECTION

10 replicates of the negative oral fluid collected with the Quantisal™ oral fluid collection device were assayed. The mean and standard deviation were computed. The statistical LOD of this method is computed by extrapolating the value of the mean plus two standard deviations of the replicates of the negative. This vale was computed to be 0.007 g/dL or 7 mg/dl.

PRECISION

The following data were obtained based on 10 uL of treated samples (1 to 4 dilution) with ethanol spiked a 0.02, 0.04, 0.08 and 0.16 g/dL. (20,40,80 and 160 mg/dL)

Intra assay precision was determined by running 10 replicates of the above levels of alcohol in saliva diluted with the Quantisal™ extraction buffer within the same run.

EtOH	N=10 (within run)		
g/dL	Avg.	Std.	CV%
0	0.922	0.034	3.71
0.02	1.02	0.027	2.62
0.04	1.21	0.021	1.75
0.08	1.56	0.054	3.47
0.16	2.09	0.065	3.09

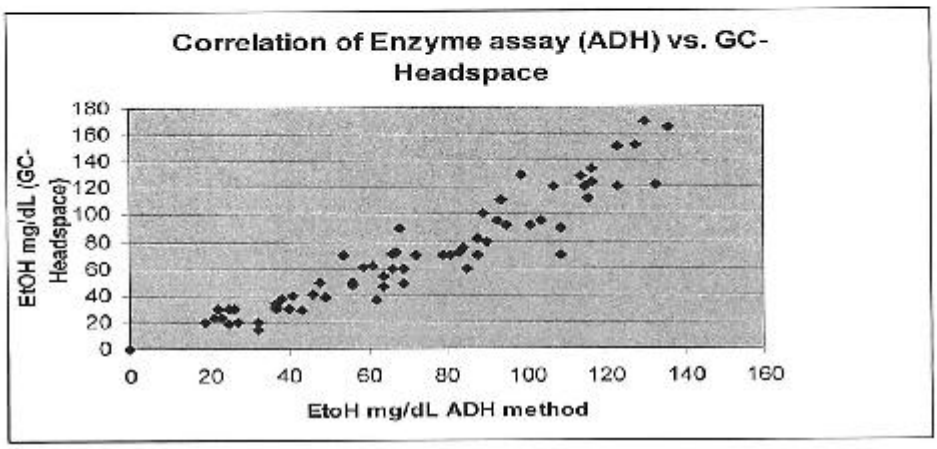
Inter assay precision was determined by running 5 replicates of the above levels of alcohol in saliva diluted with the Quantisal™ extraction buffer per run over 10 independent runs over 10 days.

EtOH g/dL	Mean	S.D.	C.V.%
0.00	0.924	0.026	2.80
0.02	1.077	0.057	5..31
0.04	1.194	0.045	3.73
0.08	1.503	0.098	6.52
0.16	1.951	0.140	7.19

ACCURACY

73 oral fluid samples collected with the Quantisal™ oral fluid collector were assayed for the presence of Ethanol by a GC-Headspace method were compared with the corresponding values obtained using the Immunalysis Oral Fluid Ethyl alcohol assay. **The correlation coefficient between the two methods was 0.951.** The results are summarized below

FIGURE 2



SPECIFICITY

Cross reactivity with various organic compounds were tested in the assay.

Compound	Level Tested mg/dL	% Cross Reactivity
Acetaldehyde	2000	0
Acetone	2000	0
Albumin	500	0
Ascorbic Acid	500	0
Bilirubin	30	
n-Butanol	2000	9
Creatinine	500	0
Ethylene Glycol	2000	2.5
D-Galactose	10	0
Glucose	3000	0
Hemoglobin	800	0
Isopropanol	2000	6.5
Methanol	2000	0
n-Propanol	2000	11.5
Riboflavin	7.5	0
Sodium Chloride	6000	0
Urea	6000	0

EXTRACTION EFFICIENCY

Negative oral fluid was spiked with ethanol at the following concentrations 40, 80 and 120 mg/dL. The concentrations were diluted 1 to 4 with the Quantisal™ extraction buffer and the concentrations verified in the assay. An aliquot of each concentration of the neat oral fluid spike was poured into test tubes and a Quantisal™ collection pad was placed in each aliquot until the volume adequacy indicator turned blue. The pad was then introduced into the transport tube containing the extraction buffer and capped. The tubes containing the pads were stored in the dark overnight at room temperature and assayed the next day to evaluate extraction efficiency.

Initial Spike EtOH mg/dL	Recovery of pad EtOH mg/dL	Extraction Efficiency
40.64	39.52	97.24
78.16	77.99	99.78
117	118.27	101.09

TRANSPORTATION STABILITY

Negative oral fluid was spiked with ethanol at the following concentrations 40,80 and 120 mg/dL. Three aliquots of each concentration of the neat oral fluid ethanol spike were poured into test tubes and a Quantisal™ collection pad was placed in each aliquot until the volume adequacy indicator turned blue. The pad was then introduced into the transport tube containing the extraction buffer and capped. One tube of each concentration was stored at 2-8° C with the pads on while the other two tubes of each concentration containing the pads were shipped via commercial courier in a cardboard shipping box. The shipment contained no blue ice or dry ice. A temperature recorder was included with the shipment. The next day upon receipt of the shipment, one tube was cooled for 30 minutes in a refrigerator (4° C) while the second tube was kept on the lab bench for a corresponding 30 minutes. After 30 minutes the tubes were uncapped and analyzed along with the reference tube stored in house at 2-8° C overnight.

Reference Tube = Tube not shipped out and stored at 2-8° C overnight
Transport Tube I = Tube shipped overnight via common carrier and stored on the lab bench for 30 minutes prior to opening the tube
Transport Tube II = Tube shipped overnight via common carrier and stored in the refrigerator at 4° C for 30 minutes prior to opening the tube.

Reference Tube EtOH mg/dL	Transport Tube I EtOH mg/dL	% of Reference tube	Transport tube II EtOH mg/dL	% of Reference tube
39.44	42.05	106.62	41.16	104.36
76.13	75.59	99.29	80.27	105.44
111.98	117.9	105.29	116.8	104.30

The chart recorder included with the shipment recorded a minimum temperature of 25.5° C / 78° F with a max of 46.6° C / 116° F. The temperature inside the cardboard shipping box was greater than 37.8° C/100° F from 2.00PM through 7.00 PM

RESULTS

The rate of alcohol metabolism is dependent upon factors such as gender, age, body weight, use of medication and general health condition.

LIMITATIONS OF PROCEDURE

Caution: Do not use volatile solvents in the area when performing the assay.

Ethyl Alcohol is volatile and precautions should be taken to prevent evaporation of alcohol from samples, calibrators and controls.

Legal alcohol intoxication levels vary. The test result should be interpreted with clinical signs, symptoms and field sobriety tests.

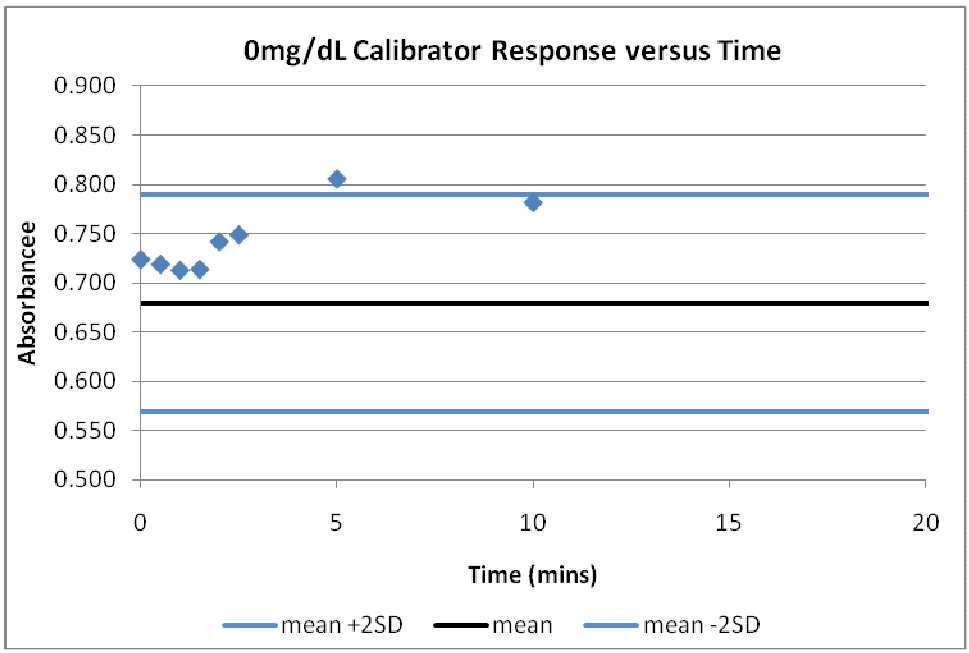
REFERENCES

1. Baselt R.C., Cravey R.H.: Disposition of Toxic Drugs and Chemicals in Man, 4th Edition, 1995 pp 293-296.
2. Beutler H.O.: Ethanol, Methods of Enzymatic Analysis, Vol VI. 3rd edition 1984, pp 598-605.

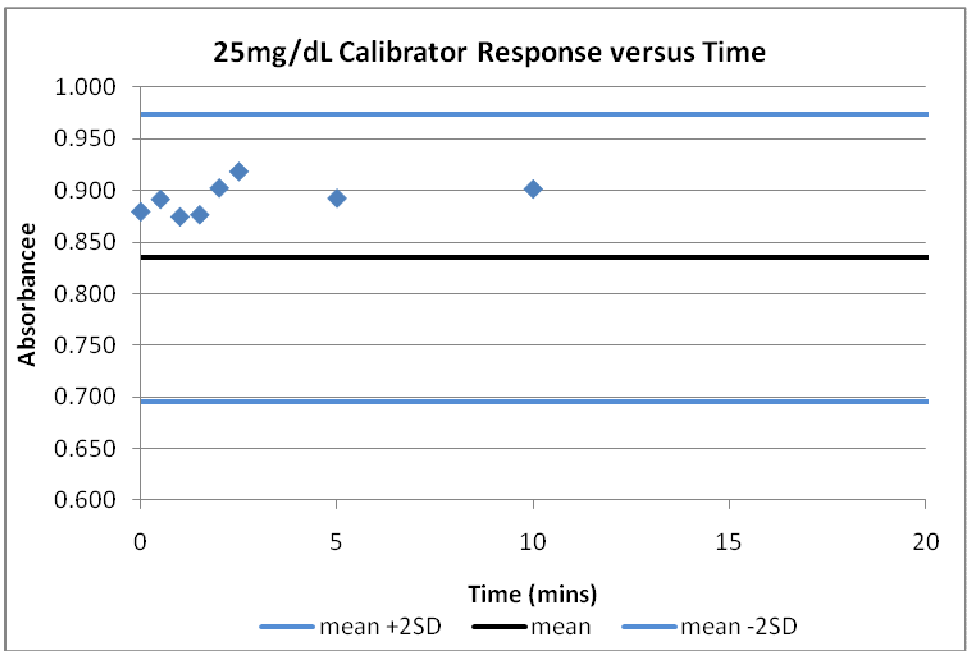
Immunalysis Corporation
Pomona, CA 91767

7.6 Appendix 6 – Alcohol Stability Charts

7.6.1 0mg/dL Calibrator



7.6.2 25mg/dL Calibrator



7.6.3 80mg/dL Calibrator