Serological Array for the Diagnosis of Viral Infection of the Central Nervous System

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List of Contents

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List of Tables

List of Figures

Abstract

Encephalitis caused by the alphaherpes viruses HSV 1, HSV 2 and VZV can be devastating and rapid, accurate diagnosis is required. Whilst existing molecular techniques are invaluable in diagnosing acute disease, detection of antibody is needed to confirm infection and to make a diagnosis after the acute stage or during post-infectious encephalitis. Current immunoassays are limited by the volume of sample required. The aim of this project was to develop a rapid, accurate, low sample volume assay to improve diagnosis using Luminex technology.

The immunodominant proteins of HSV and VZV, glycoprotein D (gD) and glycoprotein E (gE), were expressed in insect cells using a baculovirus expression vector. Expressed proteins were purified, characterised and used to develop in-house enzyme-linked immunosorbent assays (ELISA) to detect HSV and VZV type-specific antibodies. The performance of each newly developed in-house ELISA was compared with commercial ELISA assays using well characterised serum panels. An excellent correlation between the in-house ELISAs and the commercial ELISA assays (100% for HSV gD and 99% for VZV gE) was observed. To differentiate between HSV-1 and HSV-2 a new commercial ELISA assay (Omega) utilising a branched chain peptide (peptide 55 which provides immune selection of HSV-2 specific antibody) was evaluated against two commercially available HSV-2 ELISA assays. The Omega assay showed an overall agreement of 97.6% with Western blot and other ELISA assays.

The two expressed proteins, together with peptide 55, were used to develop a triplex fluorescent microbead immunoassay for the simultaneous detection and quantitation of anti-viral antibody in human sera. Initially a monoplex assay for each analyte was developed and optimised individually and then the three assays were mixed together in a triplex assay. Results for HSV-1 gD and VZV gE obtained from the triplex assay showed a 100% agreement with HSV-1 and VZV in-house ELISA results. In the case of peptide 55, the triplex assay results showed better sensitivity than the Omega ELISA assay with an overall agreement with Western blot and other assays of 98.4%. In addition, in order to facilitate the diagnosis of alphaherpesviruses CNS infections the triplex assay was joined

together with a biplex fluorescent microbead immunoassay designed for detecting and measuring human IgG and albumin in CSF and serum samples. The sensitivity and reproducibility of the resultant five-analyte multiplex immunoassay and the previous triplex assays were compared and found to have equivalent sensitivity and specificity. The sensitivity and minimal sample requirements of the new assay suggests that it will be a powerful tool for the diagnosis and study of both acute and post-infectious viral encephalitis.

Declaration

No portion of the work referred to in this thesis has been submitted in support an application for another degree or qualification of this or any other university or other institute of learning.

Abdulrahman M. AL-Sulaiman

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I would further like to honor the memory of my dear uncle Sulaiman A. Al Yosef whom I lost during my PhD, he was like a father for me. I miss you dearly.

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Lastly and most important I wish to express my thanks to my parents for their patience, faith and unconditional love.

Dedication

I dedicate this PhD to my parents who were my first teachers and have always supported me and taught me though their example to always put God first and to my lovely wife, brothers, sisters and friends who have steadfastly supported me during the period of my studies.

Preface

The author holds a BSc in Medical Microbiology from the Faculty of Science, King Saud University, Kingdom of Saudi Arabia (1990) and MSc in Molecular and Medical Microbiology (2000) from School of Medicine, University of Manchester in the UK. The author joined the School of Medicine at University of Manchester as a postgraduate (PhD) student in September 2006.

Publications Arising From This Work

1. Use of Molecular Array Technology for Diagnosis and Study of Viral Infection of Central Nervous System. **Al-Sulaiman A. M**, P.J Vallely, P. E Klapper. Postgraduate Day in University of Manchester, 22nd July 2008. Poster presentation

2. Comparative Performance of a Immunosorbent Assay Using a Novel HSV-2 Type Specific Enzyme-Linked Targeted Chain Oligopeptide (peptide 55). **Al-Sulaiman A. M**, P.J Vallely , P. E Klapper. European Society for Clinical Virology (ESCV) Winter Meeting in Amsterdam, the Netherlands, January (2009). Poster presentation.

3. Expression of the Major Capsid Protein of JC and BK Polyomavirus and Herpes Simplex and Varicella Zoster Virus Glycoproteins Using a Plasmid Based System in Insect Cells. B. Abedi Kiasari, **Al-Sulaiman A. M** P.J. Vallely, P.E. Klapper. European Society for Clinical Virology (ESCV) Winter Meeting in Amsterdam, the Netherlands, January (2009). Poster presentation.

4. Comparative Performance of a Immunosorbent Assay Using a Novel HSV-2 Type Specific Enzyme-Linked Targeted Chain Oligopeptide (peptide 55). **Al-Sulaiman A. M**, P.J Vallely, P. E Klapper. Journal of Clinical and vaccine immunology. 2009 Jun; 16(6):931-4. Paper

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6. Development of a Microbead Fluorescent Immunoassay for the Determination and Quantitation of Antibody Response to Herpes Simplex and Varicella Zoster Viruses **Al-Sulaiman A. M**, P.J Vallely, P. E Klapper. The 4th Saudi International Conference in Manchester, UK, July (2010). Oral presentation.

List of Abbreviations

^{0}C	Degrees Celsius
µg	Microgram
µL	Microlitre
aa	Amino acid
ABTS	2,2'-Azinobis [3-ethylbenzthiazoline-6-sulfonic acid]-diammonium salt) substrate
ACNPV	*Autographa californica* nuclear polyhedrosis virus
AIDS	Acquired immune deficiency syndrome
B	Blank
BBB	Blood brain barrier
BM	Bone marrow
BMT	Bone marrow transplantation
bp	Base pair
CASG	Collaborative Antiviral Study Group
CDC	Centers for Disease Control
CFT	Complement fixation test
CIE	Counter immune electrophoresis
CJD	Creutzfeld-Jakob disease
CMV	Cytomegalovirus
CNS	Central nervous system
CNSV	Central nervous system vasculitis
CPE	Cytopathic effect
Cryo-EM	Cryo-Electron microscope
CSF	Cerebrospinal fluid
CT	Computed tomographic scan
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbidiimide hydrochloride
EEG	Electroencephalogram

ELISA	Enzyme-linked Immunosorbent Assay
EM	Electron microscopy
F	Forward
FDA	American **f**ood and drug administration
g/L	Gram per litre
g/ml	Gram per millilitre
HIV	Human immunodeficiency virus
hr5	Transcriptional enhancer, AcNPV homologous region 5
HRP	Horse radish peroxidase
HSE	Herpes simplex encephalitis
HSV	Herpes simplex virus
HSV-1	Herpes simplex type one
HSV-2	Herpes simplex type two
ICD	International Classification of Disease
ie1	AcNPV immediate early promoter
IEF	Isoelectric focusing
IgG	Immunoglobulins G
IgM	Immunoglobulins M
Kb	Kilo base
KCL	Potassium chloride
kDa	Kilo Dalton
LIC	ligation-independent cloning
LOD	Limit of detection
MFI	Median fluorescent intensity
mg/dL	Milligrams/decilitre
$MgCl_2$	Magnesium chloride
min	Minute
mM	Micromolar
MOI	Multiplicity of infection
MRI	Magnetic resonance imaging
NAT	Nucleic acid Amplification Technology
NCBI	National Centre for Biotechnology Information
ng	Nanogram
NHANES	National Health and Nutritional Examination

NIAID	National Institute of Allergy and Infectious Diseases
nm	Nanometre
nt	Nucleotide
ORF	Open reading frames
P	Prototype
P10	an AcNPV very late promoter
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PML	progressive multifocal leukoencephalopathy
pmol	Picomole
PNS	Peripheral nervous system
polh	polyhedron
R	Reverse
s	Second
SDW	Sterile distilled water
Sf9	*Spodoptera frugiperda*
SSPE	Subacute Sclerosing Panencephalitis
STDs	Sexually transmitted disease
TEM	Transmission electron microscopy
U	Unit
UK	United Kingdom
UL	Unique long region
US	Unique short region
USA	United States of America
UV	Ultra-violet
V	Volt
v/v	Volume/volume
VHS	Virion host shut-off
VP	Viral Protein
VZV	Varicella Zoster Virus
w/v	Weight per volume
WNV	West Nile Virus

Chapter 1

Introduction

1. The Human Central Nervous System

1.1 Central Nervous System Infection

Infection of the CNS can be caused by any of four main pathogen groups: bacterial, viral, fungal and protozoal (Stone and Hawkins 2007; Granerod *et al.,* 2010). Bacterial infections can produce pyogenic infections (e.g., meningitis; brain abscess; subdural and epidural abscesses), or be caused by *Mycobacterium tuberculosis, Treponema pallidum*, or *M. Leprae* (Whitley and Gnann, 2002). Fungal infections may cause meningitis or meningoencephalitis, brain abscess, or spinal epidural infection. Protozoal infections include toxoplasmosis, malaria and amoebic infection. Viral infections may cause meningitis, encephalitis, myelitis, acquired immune deficiency syndrome (AIDS), and post-infectious syndromes (Solomon *et al.,* 2007). Experimental studies have improved the understanding of how viruses enter the host, invade host defence mechanisms and infect the CNS. Viruses have developed many mechanisms for invading the nervous system and can enter the CNS through two distinct routes: neuronal or haematogenous.

1.2 Encephalitis

Encephalitis is a particularly life threatening inflammation of the brain parenchyma which occurs with various clinical presentations (Stone and Hawkins 2007; Solomon *et al.,* 2007). From an epidemiological and pathophysiological perspective, encephalitis is distinct from meningitis, although on clinical evaluation, both often co-exist with signs and symptoms of meningeal inflammation, such as photophobia, headache or a stiff neck. Encephalitis may be due to an acute infection; a post-infectious immune-mediated process; or a paraneoplastic syndrome. In addition there are a number of non-infectious causes including autoimmune diseases and cardiovascular conditions, which can cause illnesses indistinguishable from infectious encephalitis (De Marcaida and Reik., 1999; Reznicek *et al.,* 2010a).

1.2.1 Viral Encephalitis

Encephalitis is an uncommon manifestation of human viral infection; it may develop during or after infection with any of more than 100 different viruses, producing infection which varies in epidemiology, modes of transmission and clinical features (Debiasi and Tyler 2004; Philip 2009; Reznicek *et al.,* 2010a). The disease can be either classified either according to the causative agent or the clinical presentation. Based on the clinical features encephalitis can be divided into three distinctive categories: acute viral encephalitis, postinfectious encephalomyelitis and chronic encephalitis (Domingues 2009).

1.2.2 Clinical Classification of Encephalitis

1.2.2.1 Acute Viral Encephalitis

Acute encephalitis is a severe neurological syndrome usually associated with significant morbidity and mortality. There are two form of acute encephalitis: epidemic (affecting a significant number of people in a community examples being encephalitis caused by arboviruses and enteroviruses) or sporadic (affecting non-related individuals) such as encephalitis caused by herpes simplex type 1 (HSV-1).

1.2.2.1.1 Epidemic Acute Viral Encephalitis

Arboviruses are common causes of epidemic encephalitis. The term *arbovirus* refers to Arthropod-borne viruses that include viruses passed to humans by members of the phylum Arthropoda (which includes insects and spiders). The major causes of arbovirus encephalitis include the members of the viral families *Togaviridae* (such as Eastern equine encephalitis, Western equine encephalitis, and Venezuelan equine encephalitis), *Flaviviridae* (St.Louis encephalitis, Japanese encephalitis, Tick-borne encephalitis, Murray Valley encephalitis, Russian spring-summer encephalitis, and Powassan), and *Bunyaviridae* (California encephalitis) (Reznicek *et al.,* 2010b). The most common times of year for these illnesses are summer and autumn when mosquitoes and ticks are most

prevalent. Damp environments favour large populations of mosquitoes thus increasing the risk of arbovirus infections.

1.2.2.1.2 Sporadic Acute Viral Encephalitis

Herpes simplex type 1 (HSV-1) is one of the most important causes of sporadic fatal encephalitis, with a 95% mortality rate if not treated, and significant morbidity in survivors such that only about 2.5% of those affected regain complete normal function (Tyler, 2004; Wilhelimina *et al*., 2006 ; Reznicek *et al.,* 2010a). Herpes simplex type 1 is responsible for 90-95% of cases while herpes simplex type 2 (HSV-2) contributes the remaining 5-10% of herpes simplex encephalitis (HSE) cases (Stone and Hawkins 2007). The incidence of HSE is estimated to be approximately 1 in 250,000 to 1 in 500,000 individuals per year (Whitley, 2006). The classical clinical presentation of HSE is of subacute progression of fever, hemicranial or generalized headache, behavioural abnormalities, focal seizure activity and focal neurological deficits, most often dysphasia or hemiparesis. Clinical recognition and laboratory investigation can aid early specific antiviral therapy which can significantly reduce the morbidity and mortality of cases.

1.2.2.2 Sub-Acute Viral Encephalitis

Post or para-infectious encephalitis appears to be an acute autoimmune monophasic disorder of the CNS triggered by either vaccination or a variety of common viral infections such as the childhood exanthems measles or varicella. Symptoms usually begin five to 10 days after the onset of the disease. The most common subtype is acute disseminated encephalomyelitis (ADEM) characterised neuropathologically by perivenular inflammation and demyelination and typically seen in children and adolescents (Reznicek *et al.,* 2010a). It is very difficult to distinguish ADEM from encephalitis clinically, however it can be distinguished pathologically from acute encephalitis. Infection with measles virus can lead to subacute sclerosing panencephalitis (SSPE). This develops some years after infection with the virus (range from 2 to 12 or more years). It may be related to virus reactivation within infected neuronal cells and the subsequent immune response to the infection. It is characterised by intractable seizures and progressive neurologic deterioration. Postinfectious encephalomyelitis is a disease of white matter, while acute viral encephalitis

is predominantly a disease of grey matter, although white matter may also be infected; especially basal ganglia, thalami and in some cases brainstem (Heininger and Seward 2006).

1.2.2.3 Chronic Viral Encephalitis

Chronic encephalitis is a rare progressive neurological disorder. Paraneoplastic limbic encephalitis is an example of a chronic autoimmune condition that produces depression, memory loss, remote malignancy and seizures. While symptoms progress with time, the diagnosis is usually based on finding of a malignancy (lung cancer) in 80% of cases (Stone and Hawkins 2007). In immunosuppressed patients (HIV, organ transplantation, severe combined immunodeficiency (SCID), hyperimmunoglobulinemia M, Multiple Sclerosis (MS) and Crohn's) reactivation of human polyomavirus JC enhance viral replication resulting in cytolytic destruction of oligodendroglia, leading to a clinical manifestation known as Progressive Multifocal Leukoencephalopathy (PML) (Khalili and White 2006; Maginnis and Atwood 2009). In such case the disease is characterised by multiple foci of demyelination of cerebral white matter. Symptoms include ataxia, hemiplegia, paralysis, vision loss and speech impairment. Diagnosis of PML is based on viral DNA detection in CSF and/or detecting white matter lesions by magnetic resolution imaging (MRI) (Maginnis and Atwood 2009).

1.3 Herpes Viruses

As mentioned above a number of different viruses can cause encephalitis, however the *Herpesviridae* family contains the most important causes of viral encephalitis. Herpes simplex encephalitis (HSE) and acute disseminated encephalomyelitis (ADEM) are the most important neurological disorders caused by herpes simplex (HSV) and varicella zoster viruses (VZV) respectively. These viruses are closely related, however they can cause different forms of encephalitis, this project therefore focuses on these viruses and their neurological complications.

1.3.1 Introduction and History

The name herpes comes from the ancient Greek ' □ρπης' meaning to creep or crawl, and refers to the spreading nature of the infectious lesions typically caused by this virus. A medical historian (Celsus) was the first to describe an actual herpetic lesion: he stated that they were initially rounded but with time became diffused "as a serpent" to form a belt (Roizman *et al.,* 2007). The association between continuous eruptions and fever was first noted by the Roman scholar Herodotus; he noted a relationship between lip vesicles, mouth ulcers and fever, and defined the above association as “herpes febrilis”. This was later elaborated on by Galen who found that HSV recurrences developed at the same original anatomical site. From these early descriptions and up until the 17th Century, the term herpes was used to describe many skin lesions. In the 18th Century smallpox and varicella were distinguished as two individual diseases by Heberden (1767), and in 1892 it was suggested that varicella and herpes zoster had an infectious aetiology and were closely related (Cohen *et al.,* 2007b). In 1893 Vidal recognized the route of transmission of HSV from one individual to another and by the 19th Century the vesicular nature of lesions associated with herpetic infections was well recognised (Roizman *et al.,* 2007). As the 20th century progressed and electron microscopy become available, Almeida *et al.* (1962) demonstrated that the virions found in varicella and herpes zoster vesicle fluid were identical. In 1984, Straus *et al*. compared VZV DNA from an immunocompromised patient with varicella to DNA taken from the same patient during a later episode of herpes zoster, using restriction endonucleases, and showed that herpes zoster resulted from reactivation of the varicella virus (Straus *et al*., 1984).

By the beginning of the 20th Century the infectious nature of HSV was unequivocally recognised by Lowenstein (Roizaman et al., 2007). He inoculated a rabbit cornea with fluids obtained from lesions of HSV keratitis and from vesicles of HSV labialis. He found that by using these fluids he was able to produce lesions similar in nature to those found in the human. Furthermore, vesicle fluid from patients with herpes zoster failed to produce similar lesions in the rabbit eye model. Advances in viral culture allowed the virus to be propagated in a variety of laboratory animals, and led to the understanding that HSV could infect not only the eye, but other sites in the human body such as the skin and the CNS (Whitley and Roiza man, 2001). In the 1930s, Andrews and Carmichael detected

neutralizing antibody to HSV in serum of patients suffering from herpetic lesions (Roizman *et al.,* 2007). They noted that some of these patients later developed fresh labial lesions; however these lesions were typically less severe than the earlier ones. It was believed that HSV could apparently occur in the presence of existing humoral immunity resulting in the recognition of an important biologic property of HSV: the ability to establish a latent infection and subsequently reactivate from latent infection. In 1968 antigenic and biologic differences between HSV types were demonstrated by Nahmias and Dowdle who detected the antigenic differences between herpes simplex type 1 (HSV-1) and herpes simplex type 2 (HSV-2) (Nahmias and Dowdle 1968). In addition they also established the principle that HSV-1 is more frequently associated with non-genital infection, "above the belt", whilst HSV-2 is predominantly responsible for genital infection “below the belt".

1.3.2 Classification

The International Committee for the Taxonomy of Viruses (ICTV) classifies members of the family *Herpesviridae* in the recently established order *Herpesvirales*, and into three subfamilies *Alphaherpesvirinae* (α), *Betaherpesvirinae* (β), and *Gammaherpesvirinae* (γ), mainly on the basis of their biologic and molecular properties (Davison *et al.,* 2009). Members of each of the subfamilies are further classified into different genera on the basis of their genome organisation and deoxyribonucleic acid (DNA) sequence homology. To date, over 100 different herpes viruses have been recognised infecting a diverse range of both warm and cold-blooded species, however, only eight are known to infect humans (Table 1.1). Of these eight, 3 are members of the alpha herpesviruses (HSV-1, HSV-2 and VZV) and have a relatively broad host range, a short replication cycle (18-24 hours) which is cytolytic, producing a cytopathic effect (CPE) in cell cultures characterised by eosinophilic intranuclear inclusion bodies and multinucleated giant cells with subsequent cell death. The alphaherpes viruses establish latent infections primarily in sensory neurons. In contrast, the beta herpesviruses cytomegalovirus (CMV) and human herpes virus types 6 and 7 (HHV-6 and HHV-7) have a longer reproductive cycle with restricted host range. Infected cells typically become enlarged and latency is maintained within lymphocytes and cells of the reticulo-endothelial system, kidney, secretory glands and other tissues. The

gamma herpesviruses; Epstein-Barr virus (EBV) and human herpes virus type 8 (HHV-8) also have a restricted host range (T or B lymphocytes) and latency is most commonly established in lymphoid tissue (Roizman *et al.,* 2007).

Table 1.1 The Classification of Human Herpesviruses

Virus	Abbreviation	Genus	Designation	G + C content (mol %)	Genome (bp)
Herpes simplex virus type 1	HSV-1	α 1	Human herpesvirus 1	67	152,261
Herpes simplex virus type 2	HSV-2	α 1	Human herpesvirus 2	69	154,746
Varicella- zoster virus	VZV	α 2	Human herpesvirus 3	46	124,884
Epstein–Barr virus	EBV	γ 1	Human herpesvirus 4	60	171,823
Human cytomegalovirus	HCMV	β 1	Human herpesvirus 5	57	230,283
Human herpesvirus 6	HHV-6	β 2	Human herpesvirus 6	43	159,321 161,573 162,114
Human herpesvirus 7	HHV-7	β 2	Human herpesvirus 7	40	144,861 153,080
Kaposi's sarcoma-associated herpesvirus (KSHV)	HHV-8	γ 2	Human herpesvirus 8	54	~140,500

Adapted from Davison and Clements 2005.

1.3.3 Viral Structure

1.3.3.1 Herpesvirion

The herpesvirus virion has characteristic architecture, and is composed of four distinct structural elements; a core containing a linear double-stranded DNA, an icosadeltahedral capsid surrounding the core, an amorphous asymmetric material surrounding the capsid called the tegument and an outer lipid bilayer envelope with viral glycoprotein spikes on its surface (Figure 1.1). Herpesviruses are relatively large in size, ranging from 120-300nm, due to the varying thickness of the tegument, and contain more than 30 distinct

proteins designated as virion polypeptides (VP). These proteins include 8 capsid constituents, at least 20 tegument components and more than 11 proteins; mostly glycosylated, in the envelope (Roizman *et al.,* 2007).

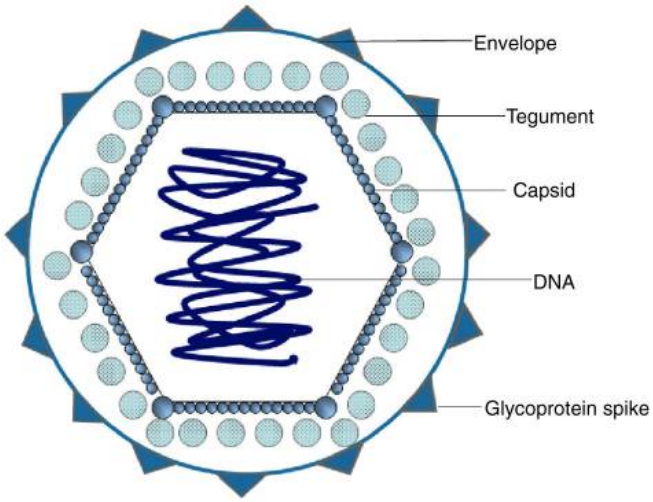

Figure 1.1 Schematic representation of herpesvirus structure (Adapted from Novak and Peng 2005)

1.3.3.2 Envelope

The sensitivity of virions to lipid solvent and detergents indicates that the envelope of the herpesviruses contains lipids, presumably derived from patches of altered cellular membranes (Spear and Roizman 1972; Roizman *et al.,* 2007). Electron microscopic analysis of virus particles has shown that the envelope has the typical trilaminar appearance of the host cell membrane, and it appears that herpesvirus acquires its envelope lipids from its host nuclear membrane, modified by the insertion of virus glycoprotein spikes (Cleator and Klapper 1995). Transmission electron microscopy (TEM) and monoclonal antibody studies, particularly immuno-gold labelling techniques, have been used to shows that herpesviruses have numerous protrusions of glycoprotein spikes, each

constructed from only one type of virus glycoprotein (Stannard *et al.,* 1987). In a recent detailed study by Grunewald *et al.,* 2003 using Electron cryomicroscopy (Cryo-EM), the number of spikes in the envelope of HSV-1 was shown to range from 595 to 758 with a mean of 659 per virion, with the average centre–to-centre spacing between 9-13 nm; however the glycoproteins were not evenly dispersed on the surface and some regions of the envelope were quite sparsely populated. The same study found that the length of spikes ranged from 10 to 25 nm, they were around 4 nm wide, and most were terminated in a globule approximately 6 nm across.

1.3.3.2.1 Glycoproteins

The envelope of herpes viruses encodes several types of glycoproteins and the glycoprotein composition of the spikes varies between species. Some are unique to a particular subfamily or virus; whilst others are conserved throughout the herpes virus family suggesting that these glycoproteins play an essential role in the infection cycle (Shin *et al*., 2003). HSV and VZV contain more than 30 distinct proteins; of these 11 (HSV) and 7 (VZV) glycosylated glycoproteins are found on the virion envelope and are also exposed on the surface of the infected cells. These glycoproteins are first synthesised as non-glycosylated nascent polypeptides and during transit through the Golgi apparatus undergo extensive post-translational modification. After trimming of some mannose residues and addition of high mannose oligosaccharides in the Golgi apparatus they form complex type oligosaccharides. Finally the processed, mature glycoproteins will be inserted in the nuclear or cellular membranes. The functions of these glycoproteins have been shown to include virus attachment, penetration, envelopment, egress and membrane fusion. They also act as major antigenic determinants for the cellular and humoral immune responses of the host (Avitabile *et al.,* 1994; Roizman *et al.,* 2007).

1.3.3.2.2 HSV Glycoproteins

The envelope of HSV contains 11 glycoproteins designated as; (glycoprotein B (gB), glycoprotein C (gC), glycoprotein D (gD), glycoprotein E (gE), glycoprotein G (gG), glycoprotein H (gH), glycoprotein I (gI), glycoprotein K (gK), glycoprotein L (gL).

1.3.3.2.2.1 Glycoprotein B (gB)

The gene encoding glycoprotein B is found within the unique sequences of the long component of UL27, it is known as VP7, and consists of a 904 amino acids (aa) sequence, with an molecular weight of 110 kd. Glycoprotein B is an essential protein containing two or three transmembrane segments, and is required for viral entry, cell-cell fusion and viral growth in cell culture (Roizman *et al.,* 2007). Glycoprotein B is conserved in all the human herpesviruses, and it is an important target for both humoral and cell-mediated immune responses (Manservigi *el al*. 1990). Studies have shown that mutant viruses containing a temperature sensitive (ts) mutation in the gB gene fail to synthesise mature gB and cannot produce infectious virions at non-permissive temperatures (Little *et al* 1981). Ali and Forghani (1990) studied the immune response to gB and found that nearly all human sera reacted with gB meaning that HSV infection in humans induces gB-reactive antibodies that are directed against the total molecule. Animal studies by Ghiasi *et al.,* (1994) found also that mice inoculated with recombinant gB expressed in a baculovirus system were protected from lethal challenge with HSV-1.

1.3.3.2.2.2 Glycoprotein C (gC)

The gene encoding glycoprotein C is found within the unique sequences of the long component UL44, it is known as VP7.5, and consists of 511aa sequences. This glycoprotein is a non-essential protein and is heavily N- and O-glycosylated (Roizman *et al.,* 2007). It plays a role in the interaction with complement component C3b to stop complement activation, and has also been reported to play a role in the adsorption of the virus to the cell surface by binding to either glycosaminoglycans of the heparan sulphate or chondroitin sulphate (Lubinski *et al.,* 1999; Roizman *et al.,* 2007). Experimental studies found that gC is non essential because viruses with mutations in this gene can grow normally in tissue culture. *In vitro* experiments using monoclonal antibodies raised against gC show that it can neutralise HSV-1 and protect animals against lethal HSV-1 challenge (Balachandran *et al*., 1982). Animal studies also showed that antibodies raised in mice vaccinated with a recombinant gC are protective against lethal challenge with HSV-1 (Ghiasi *et al.,* 1992).

1.3.3.2.2.3 Glycoprotein D (gD)

The gene encoding glycoprotein D is found within the unique sequences of the short component US6, it is known as VP17/18 and consists of a 394 aa residue. This glycoprotein is the strongest inducer of neutralizing antibodies. gD is essential for infectivity and for penetration of the virus into the cell. Glycoprotein D interacts with different cellular receptors such as HveA, nectin 1, and a modified heparan sulphate (Ankel *et al* 1998; Roizman *et al.,* 2007). Glycoprotein D in HSV-1 and HSV-2 is essentially identical making it a type common antigen for herpes simplex viruses. Serological studies have shown gD is an important target for both humoral and cell-mediated immune responses to HSV infection, and antibody against gD appears earlier than those directed against other glycoproteins (Ikoma *et al*., 2002). Ankel *et al.,* 1998 also found that in insect cells (Sf9) expressing individual HSV-1 glycoproteins (gB, gC, gD, gE, gG) on their surface, gD was the most potent IFN-α inducer in the viral envelope. Furthermore, animal studies showed that mice vaccinated with recombinant gD were protected against lethal challenge, and that gD induced some killer cell activity (Ghiasi *et al.,* 1999).

1.3.3.2.2.4 Glycoprotein e (gE) and Glycoprotein I (gI)

The genes encoding glycoprotein E and Glycoprotein I are found within the unique sequences of the short component of the herpesvirus genome. The protein encoded by US8 and US7 are of 550 and 390 aa sequences respectively. Glycoprotein E and gI form a ***heterodimer and*** can bind to the Fc portion of immunoglobulin (Bell *et al*., 1990). This binding may help HSV-1 to escape from immune cytolysis by blocking or altering the function of Fc. Animals studies have shown that mice vaccinated with recombinant gE produce low levels of complement-dependent neutralizing antibody. However Ghiasi *et al.,* (1992) showed that mice vaccinated with gE developed a delayed type hypersensitivity response to HSV-1 and that mice systemically vaccinated with gE were protected from lethal intraperitoneal and lethal ocular HSV-1 challenge.

1.3.3.2.2.5 Glycoprotein G (gG)

The gene encoding glycoprotein G is found within the unique sequences of the short component US4, gG consists of a 238 aa residue in HSV-1, and 699 aa in HSV-2 (Ashley *et al.,* 1999). gG-1 and gG-2 have similar sequences at their amino terminal, especially a segment of 153 amino acids on their carboxyl termini. However, the N-terminal part of the cell-associated gG-2 is unique for HSV-2. Therefore, it was suggested as a prototype antigen for detection of type-specific antibodies against HSV-2 (Ashley *et al.,* 1998). Sequencing studies have determined the size of gG in HSV-1 as being approximately 25 kd, and during infection the polypeptide is partially glycosylated to give an apparent molecular weight of 60 kd, whereas for HSV-2 the fully glycosylated form appears to be 92kd (Parkes *et al.,* 1991). This glycoprotein has been widely used in serological assays as a type-specific antigen for serological studies in individuals infected with HSV-1 and/or HSV-2 (Ashley *et al.,* 1998).

1.3.3.2.2.6 Glycoprotein H (gH) and Glycoprotein L (gL)

The genes encoding glycoprotein H and glycoprotein L are found within the unique sequences of the long component UL22 and UL1 of the herpes simplex virus genome. The proteins are 535 aa and 224 aa respectively. Both glycoproteins are conserved in all human herpesviruses: gH is an essential protein and it seems to play a role in infectivity of the virus and cell to cell fusion (Roizman *et al.,* 2007). Studies have shown that viruses which lack gH can egress from the cell but are not infectious. Antibody to gH can neutralize virus infection and prevent cell-cell spread (Gompels and Minson 1989; Blacklaws *et al.,* 1990). On the other hand, inoculation of experimental animals with recombinant gH does not protect against subsequent viral infection (Gompels *et al.,* 1991). Out of the 11 glycoproteins in the HSV-1 envelope, gH and gL alone are present as a hetero-dimer in the viral envelope and plasma membranes of the infected cells. The complex has an essential function in viral fusion and in the cell-to-cell spread of virions (Forrester *et al*., 1992). The formation of a hetero-dimer is essential for correct folding and processing of gH.

1.3.3.2.2.7 Glycoprotein K (gK) and Glycoprotein M (gM)

The genes encoding glycoprotein K and glycoprotein M are found within the unique sequences of the long component UL53 and UL10. Glycoprotein K and glycoprotein M consist of 338 and 473aa sequences respectively. Sequence studies suggested that glycoprotein K contains three or four transmembrane segments and is not an essential protein but is required to prevent an infected cell fusing with adjacent cells (Avitabile *et al.,* 2003). In contrast gM contains six to eight transmembrane segments, and whilst it is not a protein essential for the replication of virus in cell culture it is required for efficient capsid envelopment and exocytosis.

1.3.3.2.2.8 Glycoprotein J (gJ)

Glycoprotein J gene is found within the unique sequences of the short component US5, and consists of 92 aa protein. Glycoprotein J is a minor non essential glycoprotein and is reported to block apoptosis during infection of cells with gD (Zhou *et al.,* 2000).

Table 1.2 Characteristics of Individual HSV Glycoproteins

Antigen	Gene Encoding	Molecular Weight	Function	Required for Infectivity
Glycoprotein B	UL 27	110 Kd	Required for viral entry, cell fusion, and needed for viral growth in cell culture	Essential
Glycoprotein C	UL 44	130 Kd	Binds to the C3b component of complement, may play a role in blocking host response to infection. If deleted it appears to enhance viral pathogenicity	Non essential
Glycoprotein D	US 6	60 Kd	Related to viral infectivity The most potent inducer of neutralizing antibodies	Essential
Glycoprotein E	US 8	80 Kd	Forms complex with gI binds to the Fc portion of IgG thus it may help virus infected cells to escape from immune cytolysis	Non essential
Glycoprotein G	US 4	HSV-1 25 Kd HSV-2 60 Kd	Antigen specific to herpes simplex virus type eg. HSV-1 gG-1, HSV-2 – gG-2.	Non essential
Glycoprotein I	US 7	70 Kd	Thought to be involved with gE at the Fc receptor	Non essential
Glycoprotein H	UL 22	110 Kd	Forms a complex with gL, required for transport of both proteins to plasma membrane, attachment, penetration, egress envelopment, membrane fusion and cell-to-cell spread.	Essential
Glycoprotein L	UL 1		Forms a complex with gH (see above) required for entry mediated by gH and fusion.	Essential
Glycoprotein J	US 5		Minor glycoprotein reported to block apoptosis.	Non essential
Glycoprotein K	UL 53	40 Kd	Required for efficient viral exocytosis; contains s*yn*-locus but not found in plasma membrane.	Non essential
Glycoprotein M	UL 10	53 to 63 Kd	Myristoylated protein; required for efficient capsid envelopment and exocytosis.	Non essential

1.3.3.2.3 VZV Glycoproteins

As for HSV, the VZV genome encodes 7 glycoproteins termed as gB, gC, gE, gH, gI, gL and gK.

1.3.3.2.3.1 Glycoprotein B (gB)

Glycoprotein B is the second most abundant glycoprotein in the VZV envelope; it is encoded by a unique sequence of the short component ORF 31. VZV gB shares 49% amino acid similarity with HSV-1 gB (Cohen *et al.,* 2007). Therefore, antibodies (either polyclonal or monoclonal) to HSV-1 gB can detect both HSV-1 and VZV gB. Because of its homology with HSV-1, gB is suggested to be important for VZV entry into the host cell. gB appears in infected cells as two polypeptides bound by disulfide residues. Under non-reducing conditions gB appears to have a molecular weight of a 140 Kd. In contrast, under reducing conditions it resolves as two proteins with a molecular weight of 60 and 70 Kd. Two tyrosine-based motifs can be found in its cytoplasmic domain these are needed for endocytosis in the Golgi network. It also contains N and O-linked sugars and is sialylated, sulphated and palmitylated (Cohen *et al.,* 2007).

1.3.3.2.3.2 Glycoprotein C (gC)

As for VZV gB, VZV gC has 34% homology with HSV-1 gC, and is encoded by the unique sequences of the short component ORF 14. Due to its similarity with HSV-1 gC it is believed that gC has a role in virus entry. Glycoprotein C contains N-linked sugars, and because there are several strains of VZV, gC can range in size from 80 to 170 kd (Cohen *et al.,* 2007). Due to this variation the amount of gC found on the surface of the infected cells varies depending on the virus strain. gC is nonessential for viral growth in cell culture, because mutant strains lacking gC can replicate efficiently in cell culture. However animal studies show that gC deficient strain cannot replicate in foetal human skin implanted in Severe Combined Immunodeficiency Disease (SCID) mice (Moffat *et al.,* 1998).

1.3.3.2.3.3 Glycoprotein E (gE)

Glycoprotein E is a typical type I transmembrane glycoprotein and it is known as the most abundant VZV glycoprotein found on the surface of infected cells, with an apparent molecular weight of 85 to 100 kd (Grose 1990; Cohen *et al.,* 2007). The gene encoding gE is found within the unique sequences of the short component ORF68. Several studies have showed that gE has an essential role in VZV infectivity, is required for virus replication, syncytium formation, and is an integral part of VZV fusion machinery (Mo *et al.,* 2002; Cole *et al*., 2003; Berarducci *et al.,* 2006). Glycoprotein E is highly modified by N-linked and O-linked glycosylation, sialylation and sulphation. Antibodies to VZV gE can neutralize virus infectivity. During viral infection, gE forms a protein complex with gI (Mo *et al*., 2002). Studies have shown that on the surface of infected cells the gE-gI complex can function as a virus-encoded Fc receptor for nonimmune IgG (Litwin *et al.,* 1992).

1.3.3.2.3.4 Glycoprotein I (gI)

The gene encoding glycoprotein I is found within the unique sequences of the short component ORF67. Glycoprotein I is translated as a 58 to 62 kd, protein, contains N- and O-linked sugars and is part of the heterodimeric gE-gI Fc receptor (Roizman *et al.,* 2007). Glycoprotein I is not essential for virus replication in human cultured cells. However it is required for replication in Vero cells, human skin and T-cell xenografts in SCID mice. It is required for normal maturation of gE and suitable distribution of gE on the cell surface. The gE and gI complex can be found in virus-infected cultures and in vesicular lesions in patients suffering from varicella and zoster. Both gE and gI undergo endocytosis from the cell membrane however the gE-gI complex undergoes endocytosis more efficiently than either protein alone (Olson and Grose 1998).

1.3.3.2.3.5 Glycoprotein H (gH)

Glycoprotein H is a product of the unique sequences of the short component ORF68. It appears as a 118 kd protein and contains N-linked but not O-linked sugars (Roizman *et al.,* 2007). gH is highly conserved among herpes viruses and is known to be the third most abundant glycoprotein among VZV glycoproteins. It requires gL to be processed in the Golgi network in order to be transported to the cell surface (Maresova *et al.,* 2000). It contains a tyrosine-based motif (YNKI) within the short cytoplasmic tail that mediates clathrin-dependent endocytosis in the infected cells (Cohen *et al.,* 2007). This results in reduced cell surface expression and decreased cell-to-cell fusion. Monoclonal antibody studies found that gH plays a role in virus entry, cell-to-cell spread and egress of the virus from infected cells (Rodriquez *et al*., 1993).

1.3.3.2.3.6 Glycoprotein L (gL)

The gene encoding glycoprotein L is found within the unique sequences of the long component ORF60. gL appears as a 20 kd and is thought to contain N-linked sugars. It forms a complex with gH. In contrast with gH, gL does not require gH for maturation, therefore it is fully processed in the Golgi network in the absence of gH (Cohen *et al.,* 2007). Glycoprotein L accumulates in the cytoplasm but not on the surface of the transfected cells when it is expressed alone or with gH (Duus and Grose, 1996). Expression of both gL and gH together leads to syncytia formation, which does not happen if each protein is expressed alone.

1.3.3.2.3.7 Glycoprotein K (gK)

The gene encoding glycoprotein K is found within the unique sequences of the short component ORF5. Glycoprotein K appears as a 40 kd protein glycosylated with N-linked sugars and appear to be required for viral replication (Mo *et al.,* 1999). In wild type VZV virus, overexpression of gK leads to inhibition of syncytia formation.

Table 1.3 Characteristics of Individual VZV Glycoproteins

Antigen	Gene Encoding	Molecular Weight	Function	Required for Infectivity
Glycoprotein B	ORF 31	140 Kd	The second most abundant glycoprotein, required for virus entry	Essential
Glycoprotein C	ORF14	80-170 Kd	virus entry	Non essential
Glycoprotein E	ORF 68	85-100 Kd	The most abundant VZV glycoprotein, it is required for virus infectivity, replication and syncytium formation.	Essential
Glycoprotein H	ORF 37	110 Kd	The third most abundant glycoprotein, required for virus entry, cell-to-cell spread and egress.	Essential
Glycoprotein I	ORF 67	58-62 Kd	Required for normal maturation of gE	Non essential
Glycoprotein L	ORF 60	20 Kd	Require for gH maturation	Essential
Glycoprotein K	ORF 5	40 Kd	Required for viral replication	Non essential

1.3.3.3 Tegument

The tegument is the electron dense material located between the capsid and the envelope; it occupies about two-thirds of the volume enclosed within the virion membrane. It lacks distinctive features when viewed in thin section, however it appears to be fibrous on negative staining (Roizman *et al.,* 2007). The thickness of the tegument is variable depending on the location of the virion within the infected cell. Thus, viral particles found within the cytoplasmic vacuoles seem to have thicker tegument than those found within the perinuclear space. However it appears that the virus, rather than the infected cell, controls this variation (Roizman *et al.,* 2007). The tegument serves as a delivery compartment for proteins that are needed early in infection. In HSV it contains at least 20 viral proteins, the function of only some of which are known e.g. VP16 virion transactivator protein, virion host shut-off (VHS) protein, VP 1-2 (may be responsible for

releasing DNA at the nuclear pore during viral entry), VP22 (confers the ability of spread from cell to cell) (Vittone *et al.,* 2005; Roizman *et al.,* 2007).

1.3.3.4 Capsid

All members of the *Herpesviridae* family have an icosahedral capsid, and alphaherpesviruses have a diameter ranging from 125 to 130 nm and are composed of 162 capsomers. Cryo-EM Studies found that the outer shell of the capsid is composed of five proteins, VP5 (149 kd), VP26 (12kd), VP23 (34 kd), VP19C (50kd) and UL6. The VP5 protein is the major capsid protein and forms the structural subunits of the capsomers (Roizman *et al.,* 2007). For herpes simplex viruses Cryo-EM studies found that the HSV capsid is composed of an outer and intermediate layer arranged in a T= 16 symmetry and T= 4 lattice respectively. The symmetric unit consists of 1 penton subunit, 15 hexon subunits and 51/3 triplexes.

1.3.3.5 Genome

The genomes of herpesviruses are single linear double stranded DNA genomes (ranging from 124-230 kb in length) which encodes at least 70 to 74 genes respectively (Kemble *et al*., 2000; Zerboni *et al.,* 2005; Cohen *et al.,* 2007). There are two covalently linked components, designated as long (L) and short (S) sequences. Each component consists of unique sequences bracketed by inverted repeats (Sheldrick and Berthelot, 1975; Wadsworth *et al.,* 1975). The genome organisation is similar among all herpesviruses, however the genome sequence varies in the specific proteins that are encoded and the percentage of G+C; HSV-1 has 67%, HSV-2 69% and VZV 46% (Roizman and Whitley 1993). In general the sequence arrangement of herpesviruses DNA is characterised by the presence of a reiterated terminal sequence of greater than 100 base pairs in length. Based on the format of this sequence the herpesviruses were placed into six groups from A to F; HSV-1 and HSV-2 are in group E whilst VZV is in group D (Pellett and Roizman 2007).

1.3.3.6 Replication

The replication of herpesviruses is initiated by the interaction between viral glycoproteins and cell surface receptors. Replication of *Herpesviridae* has been most intensively studied with HSV-1, however it is basically the same for all herpesviruses. Entry of HSV into cells depends upon interaction of gB, gC, gD and gH/gL glycoproteins with multiple cellular receptors, resulting in fusion of the envelope with the cell membrane. It has been reported that initial attachment of HSV with host cell is mediated by association with gC and/or gB, by binding to glycosaminoglycans (GAG) of heparan sulphate. While this interaction significantly enhances the efficiency of HSV infection it is not essential, at least not for the infection of tissue culture, since gC^{-} mutant virus can grow normally (Herold *et al.,* 1991; Spear 2004). Glycoprotein D together with other glycoproteins (gB, gH and gL) enables the fusion of the envelope with the plasma membrane (Bender *et al.,* 2005). Upon attachment, the HSV envelope fuses with the plasma membrane and the nucleocapsid and the tegument proteins are released into the cytoplasm.

Entry of the nucleocapsid and the tegument protein into the cytoplasm results in absolute cessation of cellular metabolism and division. Most of the tegument proteins contribute to the early shut-off of cell function, however two of these are particularly important; *α-trans*-induction factor (*α-TIF*) and virion host shut-off protein (VHS) (Roizman, 2007). The α-*TIF* acts to initiate viral DNA transcription, whilst VHS destabilises and degrades host cell mRNA and subsequently causes early shut-off of cellular metabolism and protein synthesis. Soon after the VHS destabilises and degrades viral immediate-early (α-), early beta (β-) and late gamma (γ-) mRNA, leading to the sequential regulation of viral gene expression. The nucleocapsid is transported to the nuclear pores, the capsid starts to degrade, and the viral DNA is released into the host nucleus, where it is immediately circularised (Whitley *et al.,* 1998). In order to start transcription the circularised viral DNA binds to a cellular protein known as OCT-1 via a *cis*-acting site. α-*TIF* binds a cellular factor C1 and this in turn binds to the OCT-1-DNA complex which acts in trans to induce transcription of the α or early, gene products. Initially six *α*-proteins are synthesised; five are regulatory proteins whereas the sixth hinders the presentation of antigenic peptides on the infected cell surface. These proteins regulate viral genes and their expression is essential for the expression of early β and late γ genes. After production of alpha proteins,

mRNA encoding beta gene products appear 5-7 hours post-infection. The early β gene products are composed of several enzymes and DNA-binding proteins involved in DNA replication and nucleic acid metabolism (e.g. ribonucleotide reductase, thymidine kinase, thymidylate synthase, alkaline DNase, DNA polymerase, helicase).

Viral DNA is replicated by a rolling circle mechanism yielding head-to-tail concatemers. During virion assembly these concatemers are cleaved into monomers. The production of β proteins induces transcription of γ genes and the resulting late γ mRNA is translated into γ or structural proteins involved in the assembly of the progeny virion. After synthesis of capsid proteins, capsid assembly occurs in the cell nucleus in several stages. Viral DNA is cleaved and packaged into nucleocapsids and then associates with patches of nuclear membrane which have been modified by insertion of viral glycoproteins, on the inside of which tegument proteins have aggregated. Mature nucleocapsids bud through the nuclear membrane and acquire envelope and tegument. The enveloped virion is then processed by transit through the Golgi, de-enveloped and then re-enveloped in the transGolgi network. Finally, the re-enveloped virions are transported in vesicles to the plasma membrane where they are released into the extracellular space (Roizman 2007). The entire replication cycle takes about 18 to 24 hours and the process leads to cytolysis.

1.3.3.7 Latency

Most humans become infected with one or more of the herpesviruses during childhood, and after clearance of acute infection, herpesviruses enter a dormant state known as latency. Each virus has developed a different mechanism by which it can remain latent within the infected cell, thus evading host cell immune defences. Herpes simplex types 1 and 2 and VZV establish latency in the dorsal root ganglia. HSV Infection starts by intimate contact with an individual who is shedding the virus. Virus enters the host via a mucosal surface (oral or genital) or breaks within the skin. Following entry, the virus replicates in the epithelial cells and establishes a productive infection that spreads to the surrounding tissue, leading to the virus entering the nerve endings. It then travels to the nuclei of sensory ganglia by retrograde movement where it establishes latency (Figure 1.2). During latency, virus replication occurs only in a minute number of sensory neurons with the virus remaining in an episomal form. Reactivation of the virus occurs in some of the

infected neurons due to a number of stimuli such as stress, fever, exposure to ultraviolet light and immunosuppression (Roizman *et al.,* 2007).

Viral gene expression is essential for reactivation; during latency productive viral genes are transcriptionally inactive, the cytopathic effects of productive infection are therefore absent and only the latency associated transcripts (LAT) are expressed (Kang *et al.,* 2003). Once reactivation occurs, the virus travels back along the nerve (antegrade transport) to the nerve endings, at the initial site of infection, and infects the mucosal surface and the surrounding tissue. Reactivation, when it occurs, can be asymptomatic in some patients, whereas in others the characteristic blisters of HSV infection can be seen. By contrast VZV latency is less well characterised: following primary infection (chickenpox), virus becomes latent in sensory nerve ganglia, the virus may then reach the ganglia either through haematogenous spread or by centripetal neural transport. Unlike HSV, VZV has a unique mode of latency where it has been demonstrated within the ganglia in both neural and non-neural cells, and multiple genes are transcribed during latency. Furthermore, reactivation of VZV leads to zoster (shingles) where the herpetic lesions are limited to the path of the infected nerve.

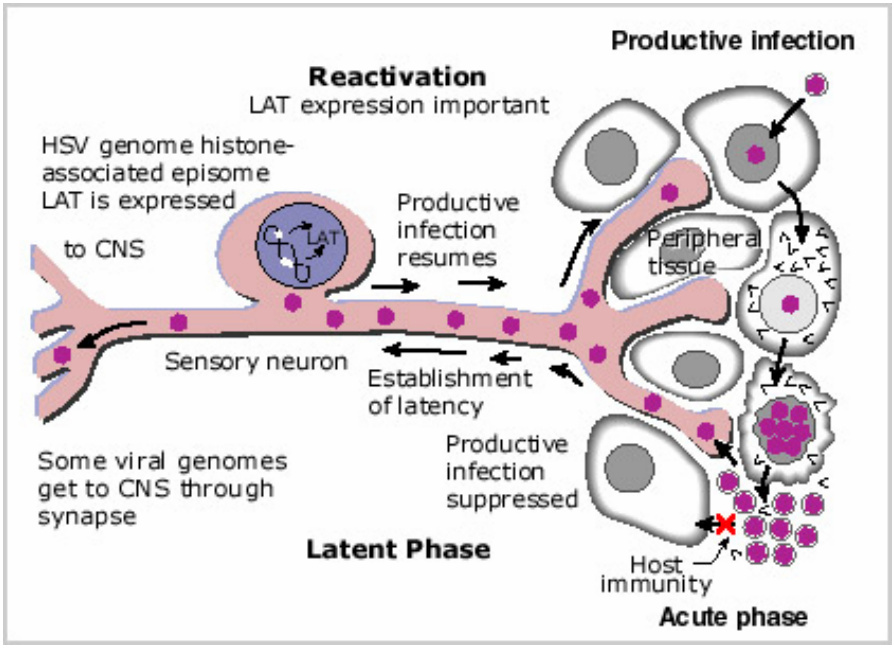

Figure 1.2 The mechanism by which HSV infects epidermal tissue innervated by sensory neurons. Productive infection follows infection of the peripheral tissue, however entry of the virus into neurons leads to latent infection in a significant proportion of individuals. Taken from http://darwin.bio.uci.edu/~faculty/wagner/hsv7f.html (Accessed 16/7/2010).

1.3.4 Herpesvirus Infection

HSV has been associated with infection of oral mucous membranes (gingivostomatitis, herpes labialis, and pharyngitis), genital membranes (genital herpes), neonatal and congenital infection, eczema herpeticum (in patients with underlying atopic dermatitis, Darier's disease or Sezary syndrome), visceral HSV infection (in immunocompromised hosts), encephalitis, myelitis, meningitis, and ocular complications (Remeijer *et al.,* 2004; Whitley 2004; Kimberlin 2005; Kimberlin and Whitley 2005). VZV has been associated with a number of clinical manifestations ranging from a mild illness such as chickenpox to potentially significant complications such as those involving the central nervous system (Koskiniemi *et al.,* 2002; Frenos *et al.,* 2007).

1.3.4.1 HSV Infection

1.3.4.1.1 Epidemiology

Herpes simplex viruses are found throughout the world, and have been reported in developed and developing countries. Humans are the only reservoir, and no seasonal or gender variation has been observed. Both viruses are capable of causing a variety of clinical illnesses range from mild illness to sporadic, severe and life-threatening illness in all age groups. Transmission usually occurs from infected people, who excrete the virus symptomatically or asymptomatically, to a susceptible person during close personal contact. The mode of transmission differs between the viral types, and whilst different parts of the body are usually affected, there is considerable overlap between the epidemiology and clinical manifestations of the two viruses (Roizman *et al.,* 2007). Infection with HSV-1 is usually acquired during early childhood and adolescence. It is estimated that 90% of the population become HSV-1 seropositive by the fourth decade of their lives (Smith and Robinson 2002; Weiss, 2004; Roizman *et al*., 2007). The transmission route of HSV-1 in younger age groups (< 5 years) appears to be from their mothers through kissing, whereas in older age (>15 years) the main route is from their infected partners. The prevalence of HSV-1 infection correlates with age, socioeconomic statues, race and geographical location (Nahmias *et al*., 1990; Rosenthal *et al*., 1997; Whitley *et al*., 1998). In less developed countries, approximately 33% of children (< 5

years) have serological evidence of HSV-1 infection, and this goes up to 70-80% by early adolescence (Whitley and Roizman 2001). In comparison, in the middle and upper classes of more developed countries, individuals become infected later; seroconversion appears in only about 20% of children by the age of 5 years, rising to 40-60% by the age of 24-40 years (Whitley and Roizman 2001). In the United States, race affects acquisition of HSV-1 the National Health and Nutritional Examination (NHANES) survey showed that more than 35% of African-American children and 18% of white children are HSV seropositive by age 5 (Roizman *et al.,* 2007).

The prevalence of HSV-1 between countries varies. In the UK, Vyse *et al.,* (2000) found that the prevalence of HSV-1 antibodies in cohorts born in 1986-1987 increased from 34% to 41% compared to those born 1994-1995. In other countries such as Brazil, Estonia, India, Morocco and Sri Lanka, the prevalence of HSV-1 was found to vary by geographical site ranging from 78.5%-93.6% in adult males, to 75.5%-97.8% in adult females. In addition, in a recent study of the prevalence of HSV-1 and HSV-2 in European countries, Bulgaria and Czech Republic had the highest rate for HSV-1 83.9% and 80.6% respectively, whereas the lowest was found to be in Finland 52.4% (Pebody *et al.,* 2004). Although various studies reported that HSV-1 seroprevalence increases significantly with age, in both sexes, in all countries, it is suggested that due to the improvement in socioeconomic status the epidemiology of HSV-1 has changed (Cowan *et al.,* 2003). Nahmias *et al* (1990) reported that the prevalence of HSV-1 antibodies in Iceland has decreased from 80% to 74% between 1979 and 1985. More recently, in the UK, the prevalence of HSV-1 antibodies increased from 34% in those born in 1986-1987 to 41% in those born 1994-1995. Above the age of 15 years, the prevalence continued to increase (Vyse *et al*., 2000). In addition, the prevalence of HSV-1 antibodies in 10-14 years olds declined from 34% in 1986-1987 to 24% in 1994-1995 (Vyse *et al*., 2000). In the UK, during the early 1990s, 44% of blood donors and 59.5% of STD clinic attendees were HSV-1 seropositive (Cowan *et al*., 1996). In a large recent study by Pebody *et al* (2004), it was found that the median age of HSV-1 acquisition between European countries varied, ranging from 5-9 years in Bulgaria and Czech Republic in contrast to > 25 years in Finland, Netherlands and England and Wales.

Historically HSV-2 is associated with genital infection, however recent studies have reported an increased number of genital and neonatal cases caused by HSV-1 (Looker and

Garnett 2005; Xu *et al.,* 2006). In a study by Xu *et al.,* (2006) to assess secular trends of HSV-1 and HSV-2 in the USA population using the 1988-1994 and 1999-2004 National Health and Nutritional Examination (NHANES) data, an increasing number of genital herpes cases caused by HSV-1 (1.8%) was found in the 1999-2004 data, in comparison with the 1988-1994 data (0.4%). Despite recent declines in HSV-1 genital infection, HSV-2 was responsible for most genital lesions worldwide. A number of risk factors influence the acquisition of HSV-2 genital infection such as age, sex, tobacco use, education level, socioeconomic status, recreational drug use and number of lifetime sexual partners (Wald 2004; Beydoun *et al.,* 2010). Pebody *et al.,* (2004), found that HSV-2 was widely distributed in the general population (>12 years) of European countries. The prevalence of HSV-2 was found to be higher in women than men and it was higher in Bulgaria (23.9%) in comparison with 4.2%, 13.9%, 13.4%, 8.8% and 6% in England and Wales, Germany, Finland, Belgium and Netherlands respectively (Pebody *et al.,* 2004). In USA the seroprevalence of HSV-2 was found to be 21.9% in age groups 12 years and older and it is higher in women (25.6%) than men (17.8%), especially among blacks (45.9%) rather than whites (Malkin 2004). In addition it is estimated that 30% to 65% of pregnant women in the USA are suffering from genital herpes (Corey *et al.,* 2009).

1.3.4.1.2 Pathogenesis

Infection with HSV begins when the virus comes into contact with mucosal epithelium or damaged cutaneous epithelium and invades cells. HSV infection can occur at a variety of sites, however it is most commonly found in oropharyngeal and genital mucosa (Miller and Dummer, 2007). The first time an individual become infected with HSV of either type this infection is called a *primary infection*. Such primary infection occurs in a completely non-immune person. However if an individual already exposed to HSV-1 becomes infected with HSV-2, or vice versa, then the infection is described as an *initial infection*. Following virus replication at the initial site of infection the virus is retrogradally transported via neurons to dorsal ganglia where it become latent. Since oral and genital infections are by far the most common sites of infection, the trigeminal (HSV-1) and sacral (HSV-2) ganglia are the usual sites of latent infection. Subsequently reactivation of the latent virus will lead to another round of infection and this is called *recurrent infection* (Roizman et *al.,* 2007). It is also possible for an individual to be re-infected with his own strain of virus but at

another site, which is described as an *endogenous re-infection*, or if it is a different strain as an *exogenous re-infection*. Primary or recurrent HSV infection will lead to cellular death due to the cytolytic activity of viral replication and as a result of the inflammatory response. During HSV infection the immune system will eliminate viral replication by both specific and non-specific host defence mechanisms. Non-specific responses are usually related to the production of α- and β interferons, leading to the stimulation of macrophages and natural killer cells (NK), followed by cytotoxic T lymphocyte response (Avgil and Ornoy 2006). Later in the course of infection the CD8+ T lymphocyte will be present and 2 to 6 weeks later neutralising and antibody-dependent cytotoxic T-cells will be present, and will persist for life. However it does not prevent infection with HSV during a recurrent or re-infection stage. This explains why neonatal infection cannot be entirely avoided in the presence of maternal transplacental antibodies (Dwyer and Cunningham 1993).

1.3.4.1.3 Clinical Manifestations

1.3.4.1.3.1 Oropharyngeal Infection

Primary oral infection with HSV-1 occurs early in life and can be asymptomatic, or present with a number of symptoms such as fever, sore throat, oedema, anorexia and malaise. Classically it presents as multiple painful sores on the tongue, nose, lips and on the skin around the mouth. Symptomatic infection in younger age groups can be painful the child usually feels sick and has a fever, headache and the intraoral lesions and associated swelling makes eating and drinking liquids extremely difficult (Roizman *et al.,* 2007). Therefore, children may require hospitalisation for dehydration. In younger adults infection is usually associated with pharyngitis and mononucleosis like syndrome (Annunziato and Gershon 1996). The duration of the incubation period ranges from 2 to 12 days whilst the duration of the illness may take 2 to 3 weeks. During that time virus can be isolated from the mouth and saliva of asymptomatic children for as long as 23 days with an average of 7 to 10 days (Amir *et al.,* 1997). Recurrent infection with HSV-1 appears as a cluster of vesicles (3-5), which are milder, localized and/or as recurrent keratoconjunctivitis, and cause illness that is often of shorter duration than primary or initial infection. Recurrence may occur in the absence of any clinical symptoms (Collier *et al.,* 2000). Recurrence is usually triggered by a variety of stimuli, including sunlight, physical trauma, stress, respiratory infections and hormonal changes, however it is not known whether these

stimuli act by a common pathway. Recurrent infection starts with pain, burning, tingling and itching at the site of the primary infection and/or in adjacent areas, due to either centrifugal spread via peripheral nerves or contiguous spread to new epithelial cells (Steiner 1996). This is followed by the appearance of blisters or vesicles approximately 24h later at the mucocutaneous junction of the lip or inside the mouth. However, it can also be found in other sites innervated by the affected neurone such as the chin and inside the nose (Figure 1.3). Vesicles usually crust over within 72 h, although complete healing takes from 8 to 10 days. During this time the virus is shed from the vesicles and the amount of shedding decreases as the lesion crusts over (Liljeqvist *et al.,* 2009). The frequency of recurrence varies between different individuals, however it may occur from 1 to 6 times per year and 5% to 10% of affected people will have more frequent recurrences (Miller *et al.,* 2004).

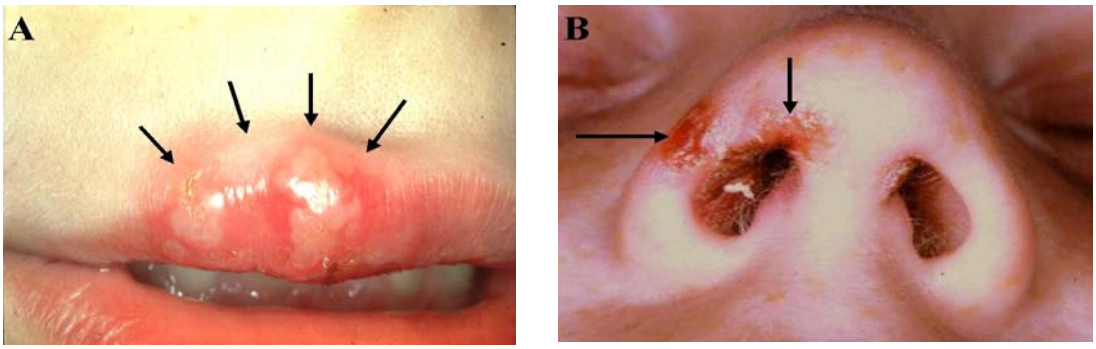

Figure 1.3 shows a characteristic appearance ***of*** a cold sore on the mucocutaneous junction of the upper lip in combination with considerable tissue oedema (arrows) and vesicle formation (A). In the other picture (B), a cold sore involving the mucocutaneous junction of the nostrils appears in its later stage where the ulcerated areas (arrows) have crusted over. Taken from http://www.healthcliniconline.co.uk/genitalherpesinfo.htm. (Accessed in 16/7/2010).

1.3.4.1.3.2 Genital Infection

Genital herpes is one of the most common sexually transmitted diseases (STDs). Historically, HSV-2 has been associated with genital infection, however recent reports suggest that a considerable and increasing number of genital isolates are of HSV-1, especially in developed countries (Lafferty *et al.,* 2000; Scoular *et al*., 2002; Anzivino *et al.,* 2009). Primary infection with HSV-2 may be asymptomatic or sub-clinical, however primary infection is usually more severe than recurrent infection (Whitley *et al*., 1998). Infection starts with the appearance of small raised macules and papules in the infected area, and then develops into diverse vesicles and papules that progress later to develop ulcers. Symptoms usually last for approximately 3 weeks and differ between the sexes; in females there is the appearance of painful lesions located in the vulva and cervix, 10-15% of patients may suffer from urinary retention syndrome, and up to 25% will develop aseptic meningitis, whereas in males lesions occur on the glans penis or penile shaft, however other parts of the body such as buttocks, thighs and perineum may also be affected (Roizman *et al.,* 2007). In severe primary infection sacral radiculomyelitis can occur leading to urinary retention and meningoencephalitis (Roizman *et al.,* 2007). The clinical course of primary genital herpes infections among patients with HSV-1 and HSV-2 are similar, however there are differences in the epidemiology and natural history of the disease caused by the two viral subtypes (Kinghorn, 1994). Primary genital HSV-1 infection is less likely than HSV-2 infection to result in recurrent disease (Benedetti *et al*., 1994; Engelberg *et al*., 2003). Genital HSV-1 infections are also characterised by lower levels of asymptomatic shedding, lower transmission frequency to new partner, longer intervals between recurrence and lower clinical recurrence rates (Mindel *et al.,* 1986; Ghani *et al.,* 1996). Infection with HSV-1 in childhood may protect adults from most genital HSV-1 infections. The clinical course of HSV recurrent genital infection is usually mild with a duration from 7 to 10 days: in females it causes minor vulvar irritation, whilst in males it is characterised by the appearance of a limited number of vesicles (3-5) on the penis shaft (Roizman *et al.,* 2007). However, although recurrent infection is mild it is problematic as it can occur as a frequent event.

1.3.4.1.3.3 Dermal Infection

Skin infection with HSV can occur at any part of the body especially if there is disruption of the skin integrity. Such infections include herpes gladiatorium, herpetic whitlow, and *eczema herpeticum.* The latter is the most common skin infection in patients suffering from skin disorders such as atopic dermatitis. The most frequently affected part of the body is the head and neck (Wollenberg *et al.,* 2003). Patients with *eczema herpeticum* present with dome shaped vesicles along with fever, malaise and lymphadenopathy. The herpetic lesions can be either localised like those of VZV with a dermatomal distribution or disseminated like those in Kaposi's varicella-like eruption (Roizman *et al.,* 2007). Herpes gladiatorium is a type of HSV infection mainly found in wrestlers, judo players or other athletes who are in very close skin contact with each other. Transmission occurs through abraded skin, and the most infected area is the upper parts of the body such as head, neck and face (Larkin 2004).

Herpetic whitlow is a painful infection of the digits recognised since 1909 and caused by either HSV-1 or HSV-2. It is mostly seen among health care professionals especially doctors and dentists who are accidently inoculated from an infected patient or contaminated instrument (Walker 2004; Bowling *et al.,* 2005; Wu and Schwartz 2007). In such cases infected doctors or dentists who do not use medical gloves can cause HSV oral infection in their patients: it is therefore an occupational hazard among doctors and dentists (Cleator and Klapper, 1995). Gill *et al.,* (1988) estimated the incidence of herpetic whitlow among health care professionals to be 2.4 cases per 100000 individuals per year. However this figure was decreased dramatically after implementation of universal precautions (Wu and Schwartz 2007). A similar infection can also be found in children who suck their thumbs. Walker *et al.,* (1990) reported that 80% of children with digital HSV-1 infection had oral infection.

1.3.4.1.3.4 Ophthalmic Infection

HSV infection of the eye is less common than oral and genital infection, although it remains a predominant cause of unilateral infectious corneal blindness worldwide (Liesegang 2001). HSV-1 and HSV-2 can both cause ocular disease but HSV-1 is

responsible for more than 95% of herpes ocular infections especially beyond the newborn age. However recent studies have reported that HSV-2 ocular infection may be increasing (Pepose *et al.,* 2006). Ocular infection can occur as result of primary infection or as endogenous re-infection caused by auto-inoculation from either primary or recurrent infection sites (Roizman *et al.,* 2007). Recurrent ocular infection by HSV can cause several ocular pathologies such as blepharitis, conjunctivitis, epithelial keratitis and stromal keratitis. In neonates the most common manifestations of ocular infection are conjunctivitis and keratitis, however other infections such as neonatal herpes, fatal meningoencephalitis or disseminated disease (immunocompromised patient) have been reported (Leflore *et al.,* 2000; Whitley and Roizman 2001; Klemann 2003).

1.3.4.1.3.5 Neonatal Infection

Neonatal herpes is a devastating consequence of vertical transmission of HSV to neonates (Brady and Bernstein 2004). Infection occurs as a result of primary or recurrent genital infection with either HSV-1 or HSV-2, although the incidence of infection by each type varies with geographical region (Gaytant *et al.,* 2002). Neonatal infection can occur transplacentally or during birth when the baby is exposed to maternal lesions or secretions during vaginal birth. Prenatally infection is thought to be responsible for 75 – 80% of cases (Brady and Bernstein 2004; Roizman *et al.,* 2007). In contrast 10% of neonatal infection occurs after birth, through contact with an infected medical staff or family member who is shedding HSV-1 (Kimberlin 2005; Roizman *et al.,* 2007). The clinical presentation of neonatal herpes is variable, but it is usually symptomatic and whilst the initial signs and symptoms are often nonspecific, it may be fatal. In such cases mortality and morbidity rates are high even with therapy (Rudnick *et al.,* 2002). Based on the clinical presentation, neonatal herpes is divided into three categories: disseminated disease with visceral organ involvement, CNS disease and infection limited to skin, eyes and mucosa (SEM). SEM and CNS associated infection comprise about 45% and 30% respectively of most cases (Parker and Montrowl 2004; Corey and Wald 2009).

In CNS infection, morbidity varies depending on the HSV subtype; neonates with CNS HSV-2 infection have higher morbidity rates than those infected with HSV-1. Even after treatment with Aciclovir, 50% of neonates will have developed moderate to severe

neurological abnormalities by one year of age (Corey and Wald 2009). Morbidity plus mortality rates with disseminated infection are higher than CNS infection, even with Aciclovir treatment the mortality rate is up to 30% (Kimberlin *et al.,* 2001). Transmissions of HSV from mother to baby are affected by a number of factors including type of maternal infection (primary, recurrent), maternal antibody levels and type of delivery (vaginal or caesarean). Mothers suffering from primary HSV genital lesion near or at the time of delivery are at greater risk of transmitting HSV to their babies than those having recurrent infection, due to either the low level or the absence of protective maternal antibodies during delivery. The infant infection rates where the mother has a primary infection range from 30% to 50%, in comparison to less than 2% in those with recurrent infection. It is therefore recommended for pregnant women with acute genital herpes, near or at the time of the delivery, to have a Caesarean rather than vaginal delivery. Furthermore it is believed that infant infection rates are reduced in recurrent infection when the genital lesions are absent, due to the presence of protective, maternal transplacental antibodies. The incidence of neonatal herpes infection is estimated to be 1 in 1,700 to 1 in 8,200 in USA, whereas it is 1.65 in 100,000 per annum in UK (Kimberlin and Whitley 2005; Corey and Wald 2009).

1.3.4.1.3.6 Neurological Complications

Various neurological complications have been associated with primary or recurrent HSV infection. These complications include meningitis, Bell's palsy, myelitis, Alzheimer's disease, intractable focal epilepsy, multiple sclerosis myelitis, radiculitis and encephalitis (Craig *et al.,* 1973; Gantz *et al.,* 1999; Furuta *et al.,* 1998; Steiner *et al.,* 2001; Mori *et al.,* 2004). Of these, encephalitis is the most serious and is usually caused by HSV-1. It is believed to be the most commonly diagnosed sporadic fatal encephalitis in developed and developing countries. A number of factors influence the incidence of HSV neurological infections including age and host immune status, however, generally such infection leads to significant morbidity and mortality even after treatment. Meningitis can be caused by HSV and it is seen most commonly in patients suffering from primary genital infection, with a percentage incidence ranging from 30% in women to 13% in men (Philip, 2009). However it is an unusual manifestation during recurrent infection, as the patient should have presenting protective antibody (of either type). In addition as the prevalence of oral

HSV-1 in developed countries turns out to be decreasing in younger age groups, the incidence of meningitis is increasing (Philip 2009).

1.3.4.1.3.7 Herpes Simplex Encephalitis (HSE)

1.3.4.1.3.7.1 Pathogenesis

Epidemiological studies show that 90% of the population have antibodies to HSV-1 by their 4th decade of life, and recurrent infection (usually cold sores) occurs in 38% of individuals. This indicates that HSV-1 is present in the population through recurrent infection (Roizman *et al.,* 2007). Primary infections occur when the virus comes in contact with mucosal surfaces. Following binding of viral glycoproteins to cellular receptors, initial viral replication occurs at the site of infection. The virus is transported via neurons to the dorsal root ganglia (HSV-1) or sacral dorsal ganglia (HSV-2) where latency is established. Whether HSE is cause by primary infection or viral reactivation or both is uncertain, and the exact mechanism by which HSV enters the brain and causes disease is still undetermined. However several studies have suggested that the virus can reach the brain during (1) primary infection of the CNS; (2) viral reactivation in the trigeminal ganglion with subsequent virus spread via the trigeminal nerve to the temporal lobes; (3) *in-situ* reactivation of latent virus from CNS tissue (Steiner *et al.,* 2007; Solomon *et al.,* 2007). There has also been suggestion that the virus can enter the brain via the olfactory route (Mori *et al.,* 2004). This is supported by the observation of HSV virus particles in the olfactory tract of patients suffering from HSE. Animal studies showed that when the olfactory tract was infected with the virus, the virus was able to reach to the CNS (Steiner *et al.,* 2007).

A number of studies have set out to determine whether HSE is caused by primary infection or reactivation. Using restriction endonuclease typing methods on HSV isolates from HSE patients, it was found that in one-third of HSE cases the virus apparently enters the CNS via primary infection, whereas in two-thirds of cases the infection results from viral reactivation (Whitley and Gnann, 2002). Whatever the cause of infection whether primary or reactivated, infection is usually subacute with a progressively deteriorating course with commonly recognised symptoms such as high fever, severe headache, nausea, personality changes, motor seizures and dysphasia. Raschilas *et al.,* (2002) reported that the most common feature in 85 out of 93 HSE patents was disorientation (76%), speech

disturbances (59%) and behavioural changes (41%). Furthermore, seizures were noted in one third of the patients.

1.3.4.1.3.7.2 Epidemiology

As stated previously HSV-1 is principally associated with oral mucocutaneous lesions and HSV-2 with genital lesion. Similarly, HSV-1 is most commonly associated with HSE beyond the neonatal period, whereas HSV-2 is associated with neonatal meningitis and encephalitis. Over 90% of HSE cases in adults and children is caused by HSV-1, whereas the remaining cases (10%) are due to HSV-2 (Mori *et al.,* 2004; Whitley, 2006; Granerod and Crowcroft 2007). In a population-based study in the UK, Davison *et al.,* (2003), analysed hospital admissions reports for all adults and children less than 17 years of age from 1/4/1989 to 31/3/1998 using the World Health Organisation International Classification of Disease (ICD) codes, and found that 6414 of adults and children were hospitalised with a diagnosis of viral encephalitis. Out of these cases, 2574 were specifically diagnosed, 52% of cases had HSV infection. A similar result was found in USA, Khetsuriani *et al.,* (2002) reported that HSV was found to be the causative agent of 74% of all hospitalised viral encephalitis cases. Moreover in multicenter study by Lee *et al.,* (2003) in Taiwan to determine the causative agent in patients suffering from acute encephalitis using PCR, the most frequently identified virus was found to be HSV (52%), followed by VZV (18.6%). Furthermore in a study by Glaser *et al.,* (2003) HSV-1 was also found to be the most frequently identified viral agent in the first 2.5 years of the California Encephalitis Project, followed by enterovirus and VZV virus.

The incidence of acute encephalitis varies between different countries. In the UK Davison *et al.,* (2003) estimated an annual rate of 1.5/100,000 in the general population and 2.8/100,000 in children, with the highest incidence in infants under the age of one (8.7/100000). In the USA the incidence was found to be between 1 in 250,000 to 1 in 500,000 individuals per year (Whitley 2006). One-third of cases are less than 20 years of age and on-half older than 50 years (Whitley & Gnann 2002). Rantalaiho *et al.,* (2001) also studied the incidence of acute encephalitis in Finland and found that 3231 cases were reported during the years 1967 to 1991, with an average incidence of 1.4/100000 adults/year, and the percentage of cases increased from 36% during 1967-1971 to 59% during 1987-1991. In the study, HSV was identified most often (16%), followed by VZV

(5%), although the leading cause of encephalitis in patients aged 65 years or older was VZV. Moreover, in a study using PCR to detect various viruses in the CSF of over 3000 patients with encephalitis, VZV was the most frequently detected virus found in 29 % of patients this was followed by HSV (11%) (Koskiniemi 2001).

1.3.4.2 VZV Infection

1.3.4.2.1 Epidemiology

Varicella–zoster virus has a worldwide geographic distribution and is the casual agent of two distinct viral syndromes; in unvaccinated populations, primary VZV infection presents as varicella (chickenpox), a contagious disease that occurs usually in early childhood, and can cause epidemics in non-vaccinated children or young adults in temperate and tropical climates (Katz *et al.,* 2004; Liesegang 2008). Reactivation of the virus leads to another form of infection known as zoster (shingles) which occurs mainly in older adults. VZV is transmitted from an infected person to a susceptible person via inhalation of aerosol droplets from the infected person. Such transmission has been reported in susceptible family members, military institutions, hospitals and day care centres (Kelly *et al.,* 1991; Sawyer *et al.,* 1994; Wharton 1996; Liesegang 2008; Weaver 2009). The peak age for infection varies between 1-4 and 5-9 years in different countries. Climate, and social factors can affect the acquisition of varicella; in most temperate climates the incidence of acquisition is more than 90% before adolescence, whereas in tropical climates the disease is acquired later in life and children are less susceptible than adults, due to the reduction of VZV transmission (Koskiniemi *et al*., 1997; Mandal *et al.,* 1998; Lolekha *et al.,* 2001). The seroepidemiology of varicella has changed dramatically since the introduction of vaccination, however in the UK where there is no routine population-wide vaccination schedule, 80% of the population has been infected by the age of 10 years. In a study by Brisson (2003), it was reported that average incidence rates of varicella and zoster between 1991 and 2000 were 1.921 and 3.73 per 100,000 populations respectively with an equivalent hospitalisation rates. In other European countries, a larger study by Nardone *et al.,* (2007) to determine the sero-epidemiology of VZV within 11 countries in the European region reported that 50% of children in all 11 countries become seropositive by the age of 5, whereas it is 86.0% to 97% in children older than 5 years of age and this

increases to 90% in adolescents. In the Netherlands, the seropositivity increases sharply with age, from 18.4% in younger age groups (0-1 years) to 48.9%, 59.0%, 75.7% and 93.0% for 2, 3, 4 and 5 year olds respectively, reaching a rate that varies between 97.5% and 100% for older age groups (de Melker *et al.,* 2006). In contrast, in the USA where the vaccine was introduced in 1995 the incidence has diminished and the average rate of mortality from varicella infection has reduced from 0.37 to less than 0.15 per 1 million of population (Nguyen *et al.,* 2005; Liesegang 2008).

A number of factors influence the reactivation of latent VZV including age, sex, ethnicity, genetic susceptibility, exogenous boosting of immunity from varicella contacts, underlying cell-mediated immune disorders, mechanical trauma, psychological stress, and immunotoxin exposure (Thomas and Hall 2004). Age plays an important role in the incidence and severity of herpes zoster (HZ); in those older than 60 years, 20 to 30% will develop HZ at some stage of their lives and this increases to 50% in those aged 85 years or more with an approximately 1% chance of more than one attack of zoster (Cooper 1987; Chapman *et al.,* 2003; Oxman *et al.,* 2005; Weaver 2009). In addition to age, the immune status also plays an important role in development of HZ, 15% of bone marrow transplant patients experience herpes zoster after transplantation. In addition the incidence in HIV patients is estimated to be 15 to 25 times higher than it is in the general population (Buchbinder *et al*., 1992).

1.3.4.2.2 Pathogenesis

Primary infection with VZV occurs as results of direct contact with vesicular fluid or through inhalation of respiratory droplets from an infected person. After adhering to the mucosal surface the virus may replicate in the epidermal cells or may also replicate in the reticulo-endothelial organs such as liver and spleen where it infects T cells. Although the exact mechanism is still unknown, animal experiments suggest that soon after entering the circulation, the virus is transported to skin by infected T cells (Arvin 2000; Cohen *et al.,* 2007). The process of spread to the skin is countered by a pronounced innate immune response mediated by epidermal cells. The process of spread form the initial site of infection takes 10 to 21 days, and is followed by the appearance of skin lesions throughout the body (Amlie-Lefond and Jubelt 2009). During this time viraemia continues as a result

of T cell trafficking along infected skin and visceral tissues and may continue even after the appearance of lesions. It is believed that infected T cells play an important role in primary infection, particularly tonsillar T cells that express skin homing markers (Cohen *et al.,* 2007b). After primary infection, the virus becomes latent at the sensory ganglia and thoracic ganglia. Later in life and influenced by various factors may reactivate, and infect the skin in the dermatome innervated by the nerve, causing the manifestation known as shingles. In addition, in some cases reactivation may again lead to generalised infection especially in immunocompromised patients, resulting in granulomatous arteritis (Amlie-Lefond and Jubelt 2009). In such cases there may be a number of complications including stroke, myelitis, meningitis and encephalitis. These complications may occur without the appearance of skin lesions.

1.3.4.2.3 Clinical Manifestations

1.3.4.2.3.1 Varicella (chickenpox)

Varicella or chicken pox is the outcome of VZV primary infection. It generally develops in healthy children within 15 days (range 8-21 days) after exposure to the virus and is characterised by fever, headache; malaise or pharyngitis, followed by the development of generalised vesicular rash within the first 48 hours (Thomas and Liesegang 2008). Lesions are usually pruritic and appear as successive crops of vesicles within a 3 to 4 day period, usually appearing first on the face and scalp and then spreading to the trunk and later to the extremities (Figure 1.4). Lesions may also be found in the mucous membranes of the oropharynx and conjunctivae. New lesion formation continues for 3 to 6 days reaching a total number ranging from 10 to 2000, however in most children the number of lesions is less than 300 (Cohen *et al.,* 2007). After the onset of the first cutaneous lesions, fever, irritability, listlessness and anorexia are prominent for 24 to 72 hours. Lesions are formed from clear fluid-filled vesicle surrounded by an irregular erythematous margin and become turbid and start to crust within 24 to 48 hours after their appearance. The course of varicella is usually self limited in immunocompetent individuals, however in some cases it may lead to several complications including secondary bacterial infection, CNS involvement, and pneumonia. In very rare cases complications may lead to death (Steiner *et al.,* 2007; Rack *et al.,* 2009). The most frequent bacterial complication is with group A β- haemolytic streptococci such as *Staphylococcus aureus* and *Streptococcus pyogenes*.

These organisms can cause secondary bacterial skin infection, cellulitis, bacterial lymphadenitis or subcutaneous abscesses (Cohen *et al.,* 2007).

Varicella pneumonia is often transient, but interstitial pneumonitis with severe hypoxemia that progresses rapidly to cause respiratory failure may occur in severe cases. During pregnancy, primary varicella places the foetus at risk from two routes: infection *in utero* and neonatal infection. Congenital varicella syndrome may occur in 0.4-2% of children born to mothers with primary varicella zoster infection during the first 20 weeks of gestation. This results in disabling abnormalities, including large area of scarring on the skin, hypoplastic limbs, chorioretinitis, cataracts and brain abnormalities (Harger *et al*., 2002; Thomas and Liesegang 2008).Varicella hepatitis may occur and may be associated with severe vomiting. Aspirin is contraindicated in children with varicella because it predisposes to liver damage (Reyes syndrome). Varicella infection is more severe in immunocompromised hosts including those with malignancy, HIV infection, or undergoing immunosuppressive therapy or solid organ transplantation. In these patients with varicella infection, skin lesions will continue to appear for several weeks, vesicles will become large and hemorrhagic, pneumonia will develop and the patient will suffer from disseminated intravascular coagulation (Feldman *et al*., 1975).

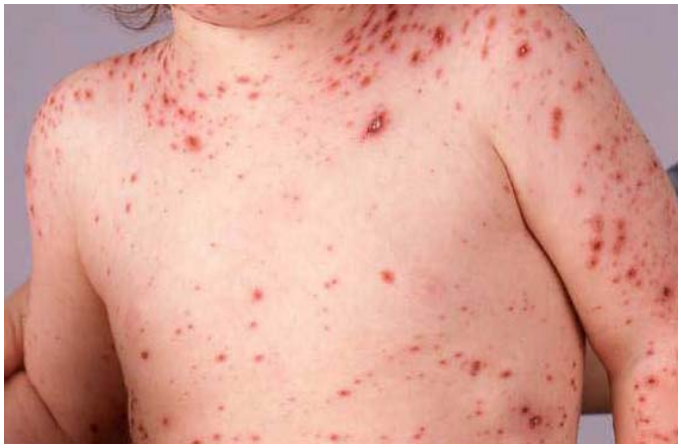

Figure 1.4 Vesicles of chickenpox appear on the face, trunk and the extremities. Taken from http://howtodealwithstuff.com/how-to-deal-with-chicken-pox/ (accessed on 17/7/2010).

1.3.4.2.3.2 Zoster (shingles)

Herpes zoster is the other form of clinical manifestation caused by VZV and occurs usually when the virus reactivates from the sensory nerve ganglia resulting in a unilateral vesicular rash adjacent to sensory dermatomes. The zoster rash is characterised by clusters of vesicular lesions on an erythematous base, founded on the skin along the path of the infected nerve or nerves (Cohen *et al.,* 2007). Individual lesions start to appear at one site or different sites along the affected dermatome, and are initially similar to those of the varicella rash. Later, as the disease progresses, the lesions become large and fluid-filled and may involve the entire dermatome. The appearance of new lesions lasts for 3 to 7 days they then crust and heal within about 14 days in most cases, although healing may be extended to 4 to 6 weeks in some cases. The disease is usually associated with a number of symptoms including a prodrome fever, malaise, headache and pain in the dermatome. In addition the patient may suffer from prolonged disorders such as anorexia, mood changes, post herpetic neuralgia (PHN), insomnia and severe depression (Thomas and Liesegang 2008). The intractable pain associated with the latter is believed to be the leading cause of suicide in the elderly.

1.3.4.2.3.3 Neurological Complications

Several recent reports confirm the association of VZV infection with a number of neurological complications. These complications can be divided into three categories, those associated with primary infection, those associated with recurrent infection and those that result from administration of varicella vaccine (Levin *et al.,* 2008; Persson *et al.,* 2009; Chouliaras *et al.,* 2010). In primary infection, the most common complications associated with the CNS are cerebellar ataxia, meningitis, Reye's syndrome, Guillain–Barré syndrome, and encephalitis (Liptai *et al.,* 2009). Acute cerebellar ataxia develops a week to 3 weeks following the appearance of the rash in children below 15 years of age and presents as gait ataxia, tremor, vomiting and headache. The exact mechanism by which the disease occurs is still unknown, and it has never been reported in association with zoster (Steiner *et al.,* 2007). Guillain–Barré syndrome (GBS), a rare complication accounts for about 7% of all primary infection complications, and it has also been associated with zoster. There are no characteristic differences between these complications and similar

ones caused by other infections. As GBS can occur as a result of primary or reactivated infection by several viruses, the exact incidence due to VZV is unknown (Steiner *et al.,* 2007).

Reye's syndrome is a non-inflammatory encephalopathy reported to occur in children aged 5 to 14 years (Gilden 2004)). The disease is characterised by intractable vomiting, lethargy, and coma and in some cases leads to permanent neurological deficit or death. Acute encephalitis is a rare disorder of varicella infection, however it is a serious complication with mortality rates reaching approximately 10% and it occurs most often in children aged 5 to 14 years (Fairley and Miller 1996). Other neurological disorders such as transient focal deficits, aseptic meningitis, transverse myelitis and hemiplegia have been reported only infrequently. Reactivation of the virus from ganglia may lead to other neurological complications. The most usual and serious complication of zoster is post herpetic neuralgia (PHN). The disease is characterised by hypersensitivity of the skin to touch or temperature changes persisting for several months or years (Whitley *et al*., 1998). Pain is a feature of disease, however in some patients, a severe debilitating and chronic pain after the clearance of the lesion can be seen (Whitley *et al*., 1998). Age is the most important predictor of PHN, in patients who develop zoster after age 60, the risk for PHN is as high as 45% (Klinschmidt-DeMasters and Gilden, 2001). In immunocompromised patients zoster develops after radiation therapy; bone marrow transplantation; or in association with lymphoma, leukaemia, cancer, AIDS, or even prolonged steroid use. In addition, in these patients VZV is considerably more likely to disseminate after zoster to multiple cutaneous sites and internal organs and to spread to the CNS. The other cause of VZV neurological complication is related to VZV vaccination: although introduction of VZV vaccine reduces the fatal complications, hospital admissions, and socioeconomic consequences of the disease, a number of adverse events have been reported, and it is estimated these occur in 52.7 per 100,000 doses, of which 2.6 per 100,000 are serious (Chaves *et al.,* 2008). Acute disseminated encephalomyelitis (ADEM) is an uncommon disorder of the central nervous system that occurs after viral illness or vaccination. ADEM resulting from VZV vaccination is estimated to occur in 1 in every 1000 cases of ADEM (Idrissova *et al.,* 2003). Symptoms usually occur after days (2 to 30 days) of neurological illness and are characterised by an acute onset of focal neurological signs and encephalopathy (Sonneville *et al.,* 2009). Symptoms include; neck stiffness, seizures and fever, and although the latter is not a constant feature, it is present in 75% of severe cases (Sonneville *et al.,* 2009).

1.4 Diagnosis of Encephalitis

The diagnosis of primary or recurrent HSV and VZV infection with neurological involvement depends mainly on laboratory investigation (Linde *et al*. 1997). These laboratory investigations include detecting the viral antigens or nucleic acids in the brain, cerebrospinal fluid examination (CSF), imaging studies and/or the demonstration of the intrathecal production of specific antibodies. Because of the high morbidity and mortality of the disease, an accurate diagnosis is important particularly for HSV and VZV encephalitis since several effective antiviral drugs are widely available for their treatment (Najioullah *et al*. 2000).

1.4.1 Clinical Evaluation

Clinical evaluation of a patient with encephalitis is usually not diagnostic, because a number of infective agents and non-infective causes must be considered (Sauerbrei and Wutzler 2002; Kennedy 2004). However, knowing the full history of vaccination, viral disease and observation of skin or mucous membrane lesions present, tonsil hypertrophy, lymphadenopathy, splenic enlargement etc, will help to narrow the list of causes and may give clues to the presence of particular viral infections, leading to determine the best diagnostic test to be used (Reznicek *et al.,* 2010a). Evidence of a systematic infection such as gastrointestinal, upper respiratory and pulmonary diseases also limits the spectrum of any viral agent. However, in some cases evidence of infection may not help. For example in HSE patients, it has been noted that when signs and symptoms of patients with biopsy-proven HSE were compared with those who did not have HSV CNS infection, no distinguishing clinical characteristic was found (Whitley and Gnann 2002). In addition, in several cases of VZV infection of the CNS, no classical skin manifestations were reported (Koskiniemi *et al*., 2002).

1.4.2 Brain Biopsy

Since the early 1990s, the gold standard diagnostic test for HSE encephalitis by direct detection of the virus in the brain was brain biopsy (Debiasi *et al*. 2002). Whilst this is an invasive technique, it provides a definitive diagnosis of HSE but it is now only applied in very rare cases. Through a burr-hole craniotomy or trephine flap surgery a brain biopsy is performed by taking a wedge or needle aspirate of brain tissue. The tissue sample is then examined using immunofluorescence or immunocytochemistry staining techniques or is viewed under an electron microscope. Even with enhanced neuroimaging techniques this procedure carries its own morbidity and mortality through surgical complications, and at the same time it suffers from the disadvantage that as the sample is very small, not all parts of the brain can be sampled (Granerod *et al.,* 2010). Nowadays, with the advent of molecular diagnostic methods the need for brain biopsy has been reduced and it is only used when a specific diagnosis cannot be established by another procedure or when the information obtained from brain biopsy is considered of sufficient value to justify the risks of the surgery undertaken (Kennedy and Chaudhuri 2002).

1.4.3 Neurodiagnostic Test

Many neurodiagnostic tests including computed tomographic (CT) scan, electroencephalogram (EEG), and magnetic resonance imaging (MRI) scan can be used to identify abnormalities that are indicative of HSV or VZV encephalitis. The sensitivity of EEG in such cases is about 84%, whereas the specifity is only 32 %, (Whitley and Kimberlin 2005; Whitley 2006). Studies have reported a lateralised EEG abnormality in a significant percentage of cases that are not proven to be caused by HSV (Booss and Esiri 2003). CT scans can suggests a diagnosis of HSE, however they are not sensitive in the early stage of the illness and only show abnormality in 30% of patients suffering from ADEM (Whitley 2006; Sonneville *et al.,* 2009). Approximately 40% of patients with HSV encephalitis have normal CT scans. In contrast MRI is a more sensitive diagnostic tool than EEG and CT and it allows the revelation of damage to the blood-brain barrier, together with oedema and necrosis in the temporal lobe (Tyler 2004). MRI can be used for diagnosis of early stages of HSE infection, however in approximately 10% of HSE cases MRI scans appear normal (Sener 2002; Whitley *et al*., 2004). In general although

neurodiagnostic methods can yield diagnostic information, the do not replace CSF examination and should only be employed as a supportive method in diagnosis of HSE or ADEM.

1.4.4 Cerebrospinal Fluid Examination

Cerebrospinal fluid (CSF) examination can be helpful in diagnosis and can help to exclude bacterial cases. However, its collection is contraindicated in the presence of high intracranial pressure, severe cerebral oedema, and in those with brain shift. In more than 90% of viral infections of the CNS the CSF cell count is abnormal (Debiasi and Tyler 2004). It should be noted however that in 5% to 10% of paediatric HSE cases CSF findings are not diagnostic because similar results are found in confirmed cases and in those with diseases that mimic HSE (Whitley 2006). Moreover, in 19 to 33% of ADEM patients CSF findings can be normal (Lin *et al.,* 2007). White blood cell count and protein in CSF will become elevated as the disease progresses. Increases in protein level are due to the production of local immunoglobulin, and to the effect of cerebral vascular permeability as a result of inflammation. Glucose concentration in viral infection is normal, but on rare occasions, the CSF glucose will be reduced in the presence of viral meningoencephalitis. As for neurodiagnostic methods, CSF examination alone is not sufficient for a definitive diagnosis of HSE and ADEM.

1.4.5 Polymerase Chain Reaction Assay (PCR)

PCR is a powerful tool; it can be used in diagnosis of many diseases where nucleic acid (DNA or RNA) can be detected (DeBiasi *et al*., 2002). Since the last decade, the advent of PCR techniques has improved the diagnosis of central nervous system infections especially those caused by viruses (Fredrick and Relman 1999). PCR on CSF was first used in the early ‘90s to diagnose HSE and enteroviral meningitis; lumbar puncture for collection of CSF is substantially less invasive than brain biopsy, and it has now replaced this as the gold standard for the diagnosis of HSE (Puchhammer-Stockl *et al*., 1990; Debiasi *et al.,* 2002). The success of PCR in neurovirological diagnosis is due to the sensitivity and rapidity of the procedure. Therefore it has been applied in many studies to investigate encephalitis caused by HSV-1 and VZV and other viruses associated with neurological

disease (Klapper *et al.,* 1990; Cassinotti *et al*., 1996; Casas *et al*., 1997). Although sensitivity and specificity of PCR in the diagnosis of neurological disease, particularly in the case of HSE, was found to be 98% and 94% respectively in comparison to brain biopsy (Kennedy and Chaudhuri 2002). However PCR results are most likely to be positive during acute infection, when the amount of replicating virus is maximal, and virus often disappears within 7 to 14 days of Aciclovir treatment (Klapper and Cleator 1998; Sauerbrei *et al.,* 2000; Tyler 2004). In a study to diagnose different causes of neurological infectious by Sindic *et al.,* (2003), it was reported that HSV DNA was detectable during the first 2 to 12 days of onset of neurological illness, but the CSF became negative by the late phase of the disease. PCR is a sensitive and specific test for detecting HSV DNA in CSF, however it is less helpful in detecting VZV DNA in CSF samples (Nau *et al.,* 1998; Gilden *et al.,* 2002; Nagel and Gilden 2007). VZV infection can involve different parts of the central and peripheral nervous system and, unlike HSV, which follows an acute course it can be subacute or chronic. Gilden *et al.,* (1998) studied four patients with subacute to chronic VZV infection of the CNS; he found that PCR was positive only when frank neurologic deficit and multiple lesions on MRI scanning were evident. The study concluded that PCR could not always provide such exquisite sensitivity for the diagnosis of VZV-mediated neurological disease. A similar result was found by Nagel *et al.,* (2007), who reported that VZV DNA was only detected in 4 (28%) out of 14 samples of patients proven to have VZV vasculopathy based on clinical and laboratory investigation (imaging, angiographic and CSF abnormalities). This demonstrated that using PCR exclusively for diagnosis of VZV vasculopathy resulted in misdiagnosis of 10 (70%) samples. Thus, a positive PCR for VZV DNA in CSF is helpful, but a negative PCR does not exclude the diagnosis.

In general it is important that a PCR result is interpreted cautiously and is considered in relation to the clinical presentation and the duration of antiviral therapy. False-negative test results have also been reported due to limited virus replication and to the presence of PCR inhibitors in CSF (Puchhammer-Stöckl *et al*. 1990; Tyler 2004). On the other hand, the likelihood of finding a positive PCR result is reduced following a few days of acyclovir treatment and in untreated patients from whom CSF samples are obtained late after the onset of neurological symptoms (Lakeman and Whitley 1995; Chataway *et al.,* 2004). Finally, because PCR is so sensitive, any contamination of the sample either from the environment or during sample handling will affect the result and may give a false positive result. Thus, whilst PCR can be a highly sensitive and specific technique, it does have

limitation, particularly in viral neurological applications. For this reason an ideal diagnostic route would include serological methods for detection of intrathecal antibody, interpreted in relation to clinical and imaging information.

1.4.6 Serological Tests

Serologic methods have been used to provide indirect evidence of CNS viral infection by detection of IgM or IgG in serum and CSF samples. In HSV primary infection antibody is produced 4-10 days after the initial infection and more than 90% of the population becomes seropositive by their 4th decade of life. Because HSE occurs as a consequence of primary, reinfection, or reactivated latent infection, interpretation of serum antibody alone in the diagnosis of HSE must be exercised with caution. Capillary circulation within the CNS has unusually non-leaky vessel walls with tight endothelial intercellular junctions forming the so-called blood brain barrier (BBB). The BBB is a specific system of capillary endothelial cells that protect the brain from harmful substances in the blood stream, while supplying the brain with the required nutrients for proper function. The peripheral capillaries allow relatively free exchange of substances across and between cells. However the BBB strictly limits transport into the brain through both physical and metabolic barriers. The BBB blocks all molecules except those that cross cell membranes by means of lipid solubility (such as oxygen, carbon dioxide, ethanol, and steroid hormones) and those that are allowed in by specific transport systems (such as sugars and some amino acids). The structure of the blood brain barrier is illustrated in Figure 1.5.

The BBB acts to prevent the free passage of antibody present in peripheral blood from the brain. In normal circumstances only about 1/500th of the concentration of antibody found in peripheral blood will traverse the BBB and be found in CSF. When a CNS infection occurs, the amount of antibody found in the CSF will increase as a result of localized, intra-brain, synthesis of antibody to the invading pathogen. To demonstrate this intrathecal production of antibody, investigation of the CSF to detect specific oligoclonal IgG bands in CSF can be performed or a decrease in the ratio of antibody in CSF compared to blood can be determined. The presence of intrathecal antibody synthesis provides evidence of recent neurologic infection (Linde *et al*., 1997). The IgG index is the ratio of IgG to albumin in the CSF and serum (Cinque and Linde 2003(B)), and the albumin CSF: serum quotient

(Q_{alb}) is an indicator of blood-CSF barrier function. Albumin (produced by the liver) is a natural marker for BBB function, and is neither synthesised nor metabolised intrathecally; therefore its presence in CSF provides an indicator of BBB function. IgG can be produced intrathecally by perivascular infiltrates of B lymphocytes residing in the CNS but can also appear as a result of transfer from peripheral blood through the BBB as a result of inflammation or trauma to the BBB. Thus increasing levels of IgG found within the CNS, can be explained either by increasing production of IgG as a result of infection or as a result of BBB dysfunction. In such cases comparing Q_{alb} to Q_{IgG} will help in discrimination between the two cases. Several indices have been devised including Delpech and Lichtblau's IgG index, Tourtellottes IgG index and Reiber hyperbolic discrimination function (Delpech and Lichtblau 1972(b); Tibbling *et al.,* 1977; Link and Tibbling 1977(b); Reiber and Peter 2001).

Klapper *et al.,* (1981) proposed a modification of the formula to calculate an antibody specific index (IA) to relate the serum:CSF HSV antibody ratio with the serum:CSF albumin ratio. In the study antibody to HSV was measured by radioimmunoassay. The study showed a raise in antibody index (AI) in 24 (92.31%) out of 26 patients with proven herpes encephalitis. Moreover, a raised AI was observed by the 3rd day of two cases with neurological disease, however most of the patients, had had the disease for at least 10 days before HSV specific antibody could be detected. Thus while the technique was useful for the diagnosis of infection in those presenting in the later stages of infection it was not useful for the diagnosis of acute infection. As for HSE, serological methods have an important role in the diagnosis of VZV CNS infections (Nagel *et al.,* 2007; Nigel *et al.,* 2008; Amlie-Lefond and Jubelt 2009). In a study by Nagel *et al.,* (2007) to evaluate the detection of VZV IgG in comparison to detection of VZV DNA in CSF for the diagnosis of VZV vasculopathy; it was reported that anti-VZV IgG was detected in 14 (100%) patients with vasculopathy and in a later study in 28 (93%) of 30 patients with VZV vasculopathy compared with only 9 who had VZV DNA in the CSF (Nagel *et al.,* 2008).

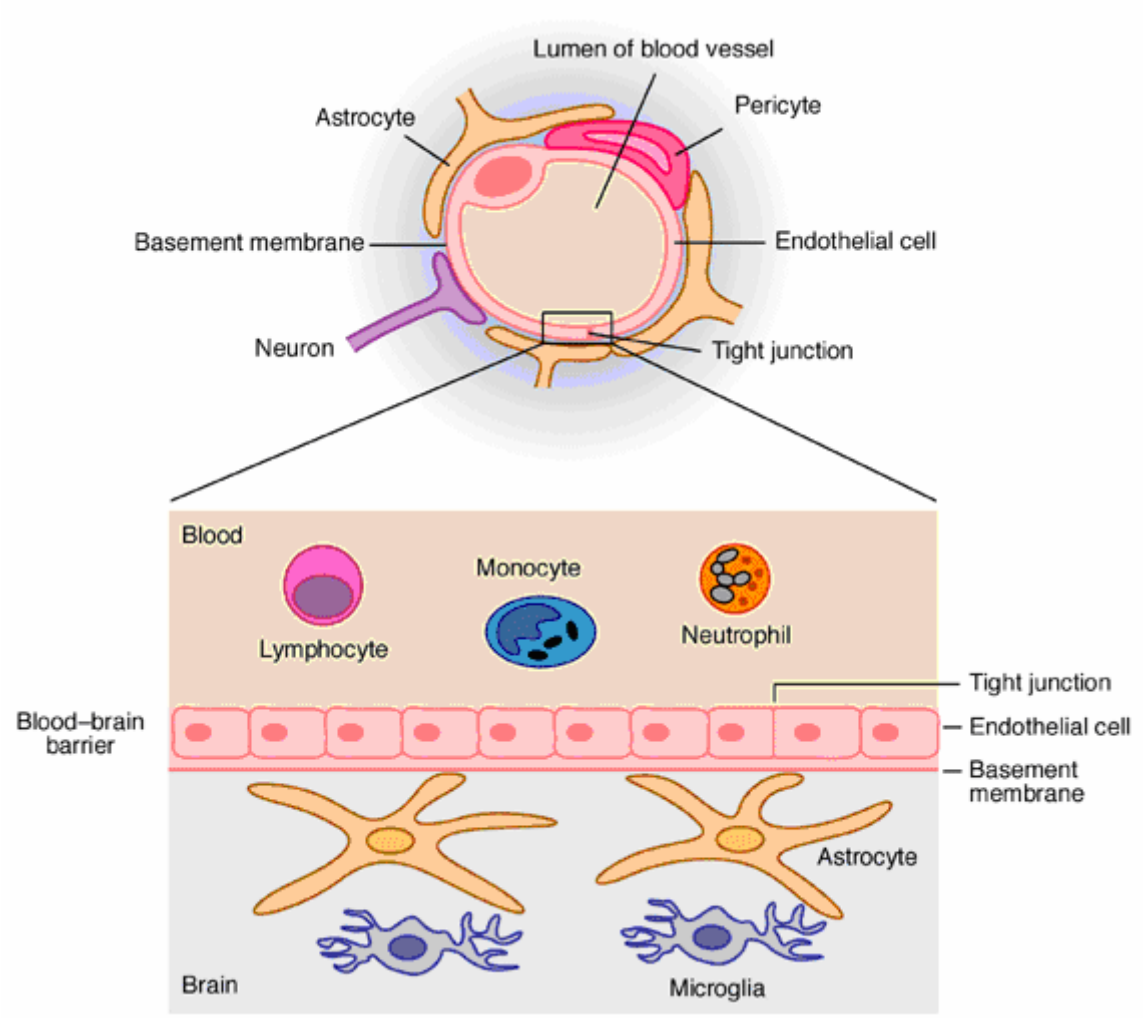

Figure 1.5 The structure of the Blood-Brain Barrier. Taken from www.stanford.edu/.../mechanism%20of%20action.htm (acceded on 16/7/ 2010)

1.4.7 The need for a Simple and Sensitive Assay for Diagnosis of Encephalitis

It is important to differentiate between viral encephalitis and post-infectious encephalomyelitis, since the management and prognosis are often quite different. As stated earlier, the diagnosis of acute HSV and VZV infections of the CNS is usually based upon the detection of viral DNA in CSF. Patients presenting late in infection or in convalescence post-treatment can be shown to have specific intrathecal IgG production within the CSF. In contrast in post-infectious encephalitis viral DNA has rarely if ever been demonstrated within the CSF of patients with this form of encephalitis. Detection of specific intrathecal IgG synthesis in post-infectious encephalitis can provide the aetiology of the infection.

Two methods are currently used for the measurement of antigen specific IgG in CSF: calculation of specific-antibody indexes and antigen-specific immunoblotting of oligoclonal IgG (Linde *et al*. 1997). Both methods involve comparing viral specific antibodies in CSF and serum samples taken on the same day and they have similar sensitivity and specifity for the diagnosis of HSV and VZV encephalitis (Monteyne *et al.,* 1997; Chataway *et al.,* 2004). Whilst these assays are useful in confirming antigen-specific antibody, within CSF they are labour intensive, require replication with multiple antigens, are time consuming and may require relatively large sample volumes for the test. Since encephalitis is a serious disease with a high morbidity and mortality rate, a simple and sensitive assay with a rapid turnaround time would be helpful to screen for the presence of multiple antigen-specific antibodies within the CSF. In recent years, it has been of increasing interest to find an assay for simultaneous measurement of many proteins within the same sample. The recent advent of particle-based flow cytometric assays such as the Luminex-Based technology (Figures 1.6 and 1.7) has increased hopes that many of these limitations can be overcome. Such assay techniques allow the detection of multiple analytes within the sample with high sensitivity using very low sample volumes.

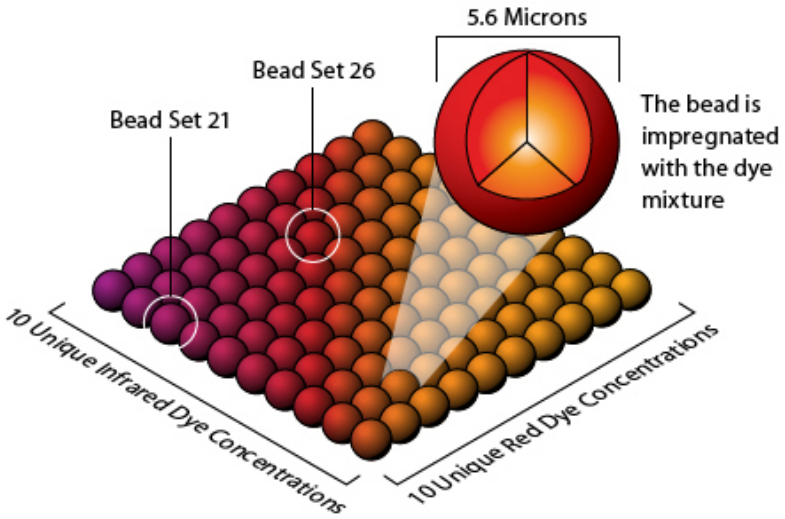

Figure 1.6 Luminex technology, colour coding of microspheres allows discrimination of different sets of beads taken from http://www.panomics.com/product/96/ (accessed 29/4/2010).

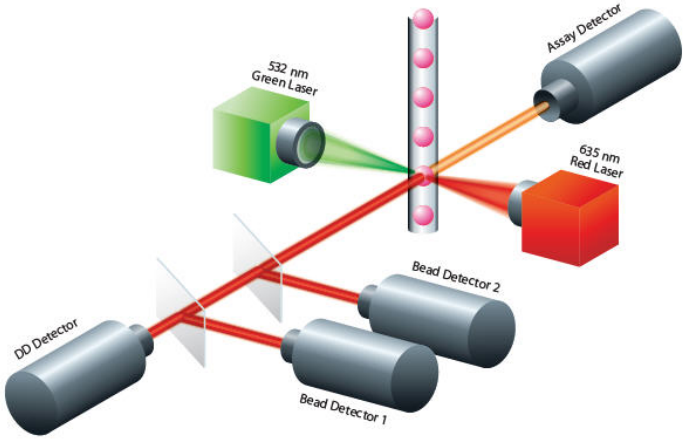

Figure 1.7 Luminex technology, immunoassays are performed (one target per bead set) and the reaction read in a flow through cell (analogous to a flow cytometer) using 2 excitation lasers. One laser identifies the bead set (thereby identifying the immunoassay analyte) and the second laser is used to excite the fluorescently labelled reporter molecule present in the immunoassay. Taken from http://www.panomics.com/product/96/ (accessed 29/4/2010).

1.5 Study Aim

The aim of this study is to investigate the feasibility of using Luminex-based technology to improve the diagnosis of CNS infections especially those caused by HSV and VZV. A microsphere-based multiplexed serological array will be developed to allow the simultaneous detection and measurement of the total IgG specific IgG to HSV-1, HSV-2 and VZV and serum albumin within the same sample (CSF and serum). To allow maximum sensitivity of detection and ensure specificity in detection of viral-specific IgG in CSF highly purified antigens of HSV and VZV are needed. This will be achieved by expression of highly immunogenic viral glycoproteins. As discussed above HSV and VZV express several antigenically distinct glycoproteins on their envelope. Many of these glycoproteins are specific to genera or subfamilies. For example, three of the HSV glycoproteins (gD, gG, gJ) lack counterparts in VZV, and only gB, gH, gL and gM are found in all the α-, β- and γ- herpesviruses. In HSV several immunological studies have found that the major structural glycoproteins are gB, gC and gD. These glycoproteins are inducers of cellular and humoral immune responses within the infected host. Of these gD an HSV type common antigen (found in HSV-1 and HSV-2) is the most potent inducer and antibodies to gD appear early in the course of infection. The most abundant glycoproteins within the VZV envelope are gE, gB and gH respectively. However, many immunological studies have reported that gE is the most abundant glycoprotein within the infected host.

In order to provide suitable antigens to be used in the assay HSV gD and VZV gE will be expressed in insect cells using a baculovirus expression system. Expressed proteins will be then purified and characterised. The proteins will be evaluated in standard ELISA procedures using well characterised serum panels. For differentiation between HSV-1 and HSV-2 antibodies a novel immunodominant epitope of glycoprotein G2 presented in a branched chain format (peptide 55) will be utilised. The peptide together with the recombinant proteins will be used to develop a triplex Luminex immunoassay. Finally another multiplex assay will be developed, in this assay a modified assay for the detection and measurement of human IgG and albumin in CSF and serum developed in this department by Al-Jinden (2009) will be included with the triplex assay. Once the multiplex assay has been established, the assay will be applied to samples from patients with proven herpes encephalitides caused by HSV-1, HSV-2 and VZV. In addition to improving

diagnosis, development of such an assay promises to lead to an improvement in the understanding of the pathogenesis of these infections and thereby contribute to the design of new treatment strategies.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

Guanidine thiocyanate (GuSCN), Tri-sodium citrate di-hydrate, Sodium dodecyl sulphate (SDS), N-lauroyl-sarcosine (sodium salt), ethylenediaminetetraacetic acid (EDTA), Trizma base, Ethidium bromide solution, Dithiothreitol (DTT), Carbenicillin, Dimethyl sulfoxide (DMSO), Potassium Chloride (KCl), Potassium Phosphate, Sodium Phosphate ($Na_2HPO_4.7H_2O$, dibasic, heptahydrate), Sodium Chloride (NaCl), sodium hydroxide (NaOH), Glycerol, Tween 20 (polyoxyethylenesorbitan-monolaurate), Bicinchoninic acid (BCA) protein assay, sucrose, Trypan blue, FAST™ BCIP/NBT Tablet (4-Chloro-2-methylbenzenediazonium/3-Hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate), Anti-Human IgG peroxidase (γ-chain) specific conjugate, Anti-mouse IgG (whole molecule-peroxidase antibody), Mouse monoclonal anti-polyhistidine-peroxidase antibody and Kanamycin (50mg/ml) were purchased from Sigma Aldrich Ltd., Poole, UK.

Laboratory disinfectants TriGene, was purchased from Scientific Laboratory Supplies Ltd., (SLS), Nottingham UK.

Propanol-2-ol (Isopropanol), Ethanol, Hydrochloric acid and Acetone were purchased from BDH Chemicals Ltd., Poole, UK.

Deoxynucleoside triphosphates (dNTPs) , HyperLadder™ I and HyperLadder™ II were purchased from Bioline Ltd., London, UK.

AmpliTaq Gold™ DNA polymerase (5U/µL), GeneAmp 10X PCR Buffer (contains 15 mM $MgCl_2$), were purchased from Applied Biosystems, Warrington, UK.

E-Gel® Low Range Quantitative DNA Ladder, E-Gel 2% Agarose, TlowE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0), NuPAGE® Novex 4-12% Bis-Tris Gels 1.0 mm, Novex® Sharp Pre-Stained Protein Standard, Invitrolon™ PVDF Filter Paper Sandwich (0.45µm), NuPAGE LDS Sample buffer (4x), NuPAGE® MES SDS Running Buffer (20x), NuPAGE® Transfer Buffer (20x), NuPAGE® sample reducing agent (10x), NuPAGE® Antioxidant, SimplyBlue™ SafeStain, T10X Blue Juice™ gel loading buffer, and 10X TBE buffer, Ultra pure water (UPW), XCELL SureLock™ Mini-Cell

electrophoresis running devise, XCELL II™ blotting module, filter papers and blotting pads were purchased from Invitrogen Life Technologies, Paisley, UK.

T4 DNA Polymerase (LIC-qualified), 10X T4 DNA Polymerase Buffer, 100 mM DTT, 25 mM dATP, 25 mM EDTA, Nuclease-free sterile distilled water, SOC Medium, Mobius 200 Columns, Mobius 200 Filters, Mobius Buffer Kit, RNase A Solution, Bacterial Resuspension Buffer, Bacterial Lysis Buffer, Mobius Neutralization Buffer, Detox Agent, Mobius Equilibration Buffer, Mobius Wash Buffer, Mobius Elution Buffer, BacVector® insect cell medium, Insect CytoBuster Reagent, His-Binding Purification Kit, and N-a-Tosyl-L-Lysine Chloromethyl Ketone Hydrochloride (TLCK) , were purchased from Merck, Novagen, Nottingham, UK.

Magnesium chloride, Glycogen, Agarose, bovine serum albumin (BSA), ABTS (2,2'-Azinobis [3-ethylbenzthiazoline-6-sulfonic acid]-diammonium salt) tablets and substrate buffer (Sodium perborate, citric acid and disodium hydrogen phosphate) were purchased from Roche diagnostics Gmbh, Mannheim, Germany.

Mouse monoclonal antibody to human IgG heavy Chain (Cat: 05-4500), was purchased from Zymed laboratory, South San Francisco, California, USA.

PhycoLink Streptavidin R-Phycoerythrin (Cat: PZPJ31S), was purchased from Europa Bioproducts Ltd., Cambridge UK.

R-phycoerythrin-conjugated goat anti-human IgG (FC(Y) chain specific) (Cat:109-116-098), was purchased from Stratech Scientific Newmarket, Suffolk, UK.

Mouse anti-human albumin (Cat: RDI-TRK4T24-15C7), was purchased from Fitzgerald Industries International, Inc., Concord, USA.

Biotin conjugated with goat (Fab-segment) anti-human albumin (Cat: BI-3825) was purchased from Cambridge Biosciences Ltd., Cambridge, UK.

SeroMAP Carboxylated microspheres regions 10, 15, 20, 25, and 30 were purchased from Luminex Corporation, Austin, Texas, USA.

N-Hydroxy-sulphosuccinimide (Sulpho-NHS), (Cat: 24510) and 1-ethly-3-(3-dimethylaminopropyl)-carbidiimide hydrochloride (EDC), (Cat: 22980) were purchased from Perbio, North Nelson Industrial Estate Cramlington, Northumberland, UK.

Newborn bovine serum (NBBS) was purchased from MP Biomedicals, Ohayo, UAS.

Pyrogen free water, injectable water, was purchased from Phoenix Pharmaceuticals Ltd, Gloucester, UK.

Absolute ethanol, methanol, propanol were purchased from Fisher Scientific, Leicestershire, UK.

Rabbit polyclonal anti-human IgG-H&L covalently liked to Horseradish peroxidase (ab6759), Rabbit anti-mouse IgG-H&L–F2 fragment and anti-human IgG-H&L covalently liked to alkaline phosphatase, mouse monoclonal to HSV-1 and HSV-2 gD clone (2C10)) and VZV gE clone (9C9), Rabbit polyclonal anti-mouse IgG-H&L F (ab) 2 fragment (ab5763) and Rabbit polyclonal anti-human IgG-H&L covalently liked to Alkaline Phosphatase (ab6760) were purchased from Abcam, Cambridge, UK.

Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse polyclonal antibodies was purchased from DAKO, Glostrup, Denmark.

Bactotryptone was purchased from DIFCO laboratories, Detroit, USA.

Bacto-yeast extract was purchased from Beta Lab., Surrey, UK.

LB agar was purchased from Oxoid, Hampshire, UK.

Micron YM-100 Centrifugal F Micron YM-100 Kit, was purchased from Millipore Corporation Bedford, USA.

Potassium Chloride (200mM) was purchased from Clear Diagnostic, LLC., Alamed, USA.

2.1.2 Equipment and Instruments:

Omnifuge 2.0 centrfuge, Megafuge 1.0 centrifuge, 27 ^{0}C and 37 ^{0}C incubator from Heraeus equipment Ltd., Essex, UK.

Shaker incubator model G25, New Brunswick Scientific Co. Inc., New Jersey USA.

Laminar flow cabinet from Envair Ltd., Lancashire, UK.

Ultraviolet illumination Herolab E.A.S. UV-T-28 MP system, from Anachem Ltd., Luton, UK).

Ultraviolet absorbance spectrophotometry (GeneQuant II) from Pharmacia Biotech, UK).

BIOMAT class II microbiological safety cabinet from Medical Air Technology Ltd., Manchester, UK.

Weighing balances (Sartorius type 1518 MP) from Data Weighing systems, Chicago, USA.

Water bath (Grant instruments Ltd., Cambridge, UK).

Sharp microwave from Sharp Electronics, LTD, UK.

Labpette Discovery pipette {AK-00134 (range from 5 µl–10 µl), AK-11879 (range from 50 µl – 200 µl), and AK-32058 (range from 200 µl-1000 µl)} from Accutek Laboratories, San Diego, USA.

Beckman ultracentrifuge and centrifugation tubes were purchased from Beckman, California, USA.

BioPlex 100 workstation from Bio-Rad, USA.

pH meter PHM 92 from Lab Radiometer, Copenhagen, Denmark.

Immunofluorescent microscope type BH2 RFCA from Optivision Ltd., Olympus, Japan.

Vacuum filtration manifold from Millipore Corporation, Bedford Massachusetts, MA, USA.

Dot Blot manifold I from Schleicher&Schuell, Germany.

Tissue culture plate and flasks, 15ml and 50ml plastic tubes, sterile screw capped cryovials, Scotlab cryofreezing container were purchased from Greiner bio-one (Cellstar), Gloucestershire, UK.

Glass culture bottles 50ml, 250ml 500ml and 1 L from Schott Duran, Germany.

ELISA plates Nunc “high binding” (Life Technologies Ltd, Paisley, UK), Costar “medium binding” (Corning, Costar Incorporated, USA), Falcon (Becton Dickinson Lab ware, California, USA).

96-well filter bottom microtitre plate was purchased from Fisher Scientific, Loughborough, UK.

Automated ELISA plate washer (ELx 50) and Reader (ELx808) from Biotek Northstar Scientific Ltd., Biotek instruments, USA.

Glass bottles automated washing machine LANCE 910 from Lancer UK Ltd., Cambridge, UK.

Agarose gel carrier (gel electrophoresis apparatus GNA-100), Hoefer SE 250 Mighty small II apparatus, Power supply ESP 500/400, ultra-violet spectrophotometer from Pharmacia Biotec, Amersham, UK.

Thermocycler machine (GeneAmp®PCR System 9700) from applied Biosystems, Warrington, UK.

Nikon digital camera coolpix 995 type, Japan.

2.1.3 DNA Controls and Plasmids

Varicella Zoster virus DNA (1 X 10^4 DNA copies/µl), was purchased from Autogen Bioclear, Ltd., Wiltshire, UK. Herpes simplex virus type 1 DNA, was provided by the Department of Virology, University of Manchester. The DNA was extracted from HSV-1 Macintyre strain (VR3) grown in Vero cell containing 10^4 TCID50/ml. Bacterial Plasmids (pIEx/Bac-3 3C/LIC), 3C/LIC β-gal Control Insert and test plasmid (ampicillin resistant) was purchased from Merck, Novagen, Nottingham, UK.

2.1.4 Oligonucleotides

1. HSV-1 gD and VZV gE primers were purchased from Invitrogen, Life Technologies, UK.

Virus	Primers	**Sequence**	Length
HSV-1	Sense	5'-CAGGGACCCGGTATGGGGGGGGCT-3'	24 bp
	Anti-sense	5'GGCACCAGAGCGTTCTAGTAAAACAAGGGCTGG-3'	33 bp
VZV gE	Sense	5'-CAGGGACCCGGTATGGGGACAGTTAATAAA-3'	30 bp
	Anti-sense	5'-GGCACCAGAGCGTTTCACCGGGTCTTATCTAT-3'	32 bp

2. Vector Upstream and Downstream primers were purchased from Merck, Novagen, Nottingham, UK.

Genes	Primers	Sequence
IE1 Promoter	Sense	5′- TGGATATTGTTTCAGTTGCAAG -3′
IE1Terminator	Anti-sense	5′- CAACAACGGCCCCTCGATA -3′

2.1.5 Insect and Competent Cells

1. NovaBlue GigaSingles™ Competent Cells and Sf9 insect cells were purchased from Merck, Novagen, Nottingham, UK.

Strain	Genotype
Sf9 Insect cells	Sf9 cell line was derived from pupal ovarian tissue of the Fall army worm *Spodoptera frugiperda*.
NovaBlue GigaSingles™ Competent Cells	

2.1.6 Enzyme-linked Immunosorbent assay (ELISA)

BioKit Bioelisa HSV-2 IgG was purchased from Biokit, Barcelona, Spain. Focus HerpeSelect HSV-2 IgG was purchased from Focus Diagnostic, Cypress, California, USA. PATHOZYME™ Viro HSV-2 ELISA IgG was purchased from Omega Diagnostics Ltd., Scotland, UK.Virotech HSV-1 IgG ELISA kit was purchased from Genzyme Virotech GmbH, Germany.

ELISA Coating buffer

1- 1x Phosphate buffered saline (PBS) pH 7.3.

2- 0.1 Carbonate/bicarbonate buffer pH 9.6.

Wash buffer: 1x PBS, 0.05 % v/v Teen 20.

Blocking buffer: 2% w/v skimmed milk in 50ml 1x PBS pH 7.3, 0.1% v/v Tween 20.

Sample diluent buffer: 1x PBS pH 7.30, 0.1% v/v Tween 20.

2.1.7 Protein Expression Systems

InsectDirect™ System and BacMagic™ Transfection kits were purchased from Merck, Novagen, Nottingham, UK.

2.1.8 Software and Databases

1- BioMath-Tm Calculation for Oligos (http://www.promega.com/biomath/calc11.htm).

2.The National Center for Biotechnology Information (GenBank) (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide&itool=toolbar).

3.Basic Local Alignment Search Tool (BLAST) databases (http://www.ncbi.nlm.nih.gov/BLAST/).

5. BioEdit Biological Sequence Alignment Editor (Version 7.0.5.3), a free programme (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) Copyright 1997-2005 by Tom Hall.

2.2 Methods

2.2.1 Clinical Specimens

2.2.1.1 Control Serum Samples

1- A clinical standard positive serum (highly positive for HSV-1, HSV-2 and VZV) and 8 known negative samples (negative for HSV-1, HSV-2 and VZV) were provided by Clinical Virology, Manchester Medical Microbiology Partnership, Manchester Royal Infirmary.

2- IgG deficient serum (Cat no: S5143) and normal human standard serum (Cat no: H4522) was purchased from Sigma-Aldrich , Dorset, UK.

2.2.1.2 HSV and VZV Serum Samples

A total of 6 panels of serum were used during this project, all specimens were re-used for this study in accordance with current Royal College of Pathologists Guideline (Guidance on the use of clinical samples retained in the pathology laboratory, 2007 http://www.rcpath.org/resources/pdf/G035GuidanceUseofClinicalSamplesSept07.pdf). Specimens and associated clinical data were collected; the specimens were anonymised by renumbering and removal of all patient identifiers from the data before use in this study. Serum panels are as follows:

1- Panel 1: consisted of 254 human sera, samples were collected from the serum bank of Clinical Virology Manchester Royal Infirmary between January 2006 and June 2007 and stored in 100 µl aliquots at -70^{0}C. All samples had been routinely tested for the presence of HSV IgG antibody using a HSV type common and type specific assays. Out of the 254 samples 90 samples were previously tested for the presence of VZV IgG antibody using Human VZV IgG assay.

2- Panel 2: consisted of 93 human sera from 57 patients with proven HSV infection. Samples were collected from the serum bank of Clinical Virology Manchester Royal Infirmary between January 1994 and April 1998 and stored in 100 µl aliquots at -70^{0}C. All samples had been tested for the presence of HSV antibody using indirect

immunofluorescence test. Samples were also tested and subtyped for HSV-1 and HSV-2 IgG antibody using a number of type specific assays.

3- Panel 3: consisted of 400 human sera from female commercial sex workers in Dacca, Bangladesh. Samples were collected from collected from female prostitutes in Bangladesh and stored in 100 µl aliquots at -70 ^{0}C. All samples had been tested and subtype for the presence of HSV-1 and HSV-2 IgG antibody using a number of type specific assays.

4- Panel 4: consisted of 200 human sera selected from among 8,000 samples taken from pregnant woman attending Antenatal Clinic at Manchester Royal Infirmary for serological screening for antibodies to rubella, syphilis and other sexually transmitted diseases (STDs). Samples were collected between January 1994 and April 1998 and stored in 100 µl aliquots at -70^{0}C. All samples had been tested and subtyped for the presence of HSV-1 and HSV-2 IgG antibody using Bioelisa HSV-1 and HSV-2 IgG type specific assays.

5- Panel 5: consisted of 60 human sera originally obtained from patients with proven HSV infection, attending the Genito-Urinary Medicine Clinic (GUM) at Manchester Royal Infirmary. Samples were collected between January 1994 and April 1998 and stored in 100 µl aliquots at -70^{0}C. All samples had been tested and subtype for the presence of HSV-1 and HSV-2 IgG antibody using Bioelisa HSV-1 and HSV-2 IgG type specific assays. Patient age ranged from 13 to 67 years and the group comprised of 28 males and 32 females.

6- Panel 6: consisted of 100 human sera, samples were collected from the serum bank of Clinical Virology Manchester Royal Infirmary between January and June 2008 and stored in 100 µl aliquots at -70^{0}C. All samples had been tested for the presence of VZV IgG antibody using the Liaison VZV IgG assay, and 37 samples out of 100 samples were tested by the AtheNA Multi-Lyte® MMRV IgG Plus assay for the detection of Measles, Mumps, Rubella and Varicella antibody.

2.2.2 DNA Extraction using Guanidinium Thiocyanate (GUSCN)

VZV DNA was extracted from clinical samples using the GuSCN method as described by Casas *et al*. (1995). Briefly, a volume of 200 µL freshly prepared lysis buffer (4M GuSCN, 0.5% N-lauroyl sarcosine, 1mM dithiothreitol, 25mM sodium citrate and 20µg/ml glycogen) was placed into a sterile 1.5 ml Eppendorf tube then mixed with 50 µL of specimen (or sterile distilled water for extraction negative control) and incubated at room temperature for 10 minutes. To precipitate the DNA 25µL of 3M sodium acetate was added to each tube followed by 275 µL of ice-cold isopropanol. Tubes were incubated at room temperature for 5 minutes. DNA pellets were then collected by centrifugation for 10 minutes at 12000 g. The supernatant was discarded and the DNA pellet was washed with 500 µL of 70% ice-cold ethanol followed by centrifugation as above, to collect the DNA. The supernatant was then gently removed and the pellet left to air dry at room temperature for 10 min before being re-suspended in 50 µL sterile deionised water. The samples were stored at -20^{0}C in single-usage aliquots (10 µL).

2.2.3 Construction of Recombinant Plasmid Containing the Full open Reading Frame of HSV-1 gD and VZV gE genes.

2.2.4.1 Determining the Sequence of HSV-1 gD and VZV gE genes

The sequence of HSV-1 gD and VZV gE were obtained by examining all published genome sequences in Genbank, 10 VZV and 8 HSV sequences were taken and compared by analysing all sequences from the 5' to 3' ends using BioEdit Sequence Alignment Editor Programme.

2.2.3.2 Primer Design and Preparation

The published HSV-1 genome sequence (accession NC-001806) and VZV gE genome sequence (accession NC-001348) found in Genbank were used to design primers for amplification the entire open reading frame containing gD, from 138419bp to 139419bp and gE, from 115808 to 117679. To generate a specific vector-compatible overhang, primers incorporated a tail at the 5'-end. The G + C content and

melting temperature were calculated using BioMath Oligo calculator. Blast alignment of the primers was also performed. All primers were diluted in Tris acetate buffer (10mM Tris and 1mM EDTA) to give a primer stock concentration of 100µM and then divided into 100µl aliquots and kept at -20^{0}C. The 100µl stock aliquot was further diluted in 400µl ultra pure water to give a working concentration of 20µM of each primer. The working stock was aliquoted into 100 µl volumes and further aliquoted into 10 µl volumes and stored at -20^{0}C for later use.

2.2.3.3 Polymerase Chain Reaction (PCR)

2.2.3.3.1 Contamination Control

PCR assay contamination was prevented by carrying out standard precautions as described by Kwok and Higuchi (1989). Briefly all pre and post PCR reagents and specimens were stored in separate areas. Separate sets of supplies (pipettes, racks, test tubes, lab coats, gloves and plugged tips) were used for setting up PCR mixtures and for sample preparation. PCR were carried out using three different rooms. PCR master mix and all reagents were contained within the first room ("DNA free" room) which was controlled strictly so that no contaminating DNA source was brought in to this room. DNA extraction and addition of the DNA samples to the master mix was performed in a separate "DNA preparation" room. The DNA amplification took place in a third room “PCR product detection”. Nothing was transferred from the DNA preparation room to the DNA free room, and both rooms were periodically subjected to ultra-violet (UV) irradiation to destroy any extraneous DNA before and after every operation. In addition, work surfaces were cleansed with 1N HCL, negative controls consisting of ultra pure water were included for each DNA extraction and PCR run. Moreover, all reagents that were need for preparation of the PCR master mix were kept in the DNA free room and aliquoted into single-usage volumes for storage.

2.2.3.3.2 PCR Amplification of HSV-1 gD and VZV gE

For amplification of HSV-1 gD a 45 µl master mix reaction containing 5 µl of 10×buffer (50mM KCl, 1.5mM $MgCl_2$ and10mM Tris-HCl; pH 8.3), 1 µl (10mM) of dNTPs, 1 µl of each oligonucleotide primer, 36.5 µl SDW and 0.5 U of AmpliTaq Gold polymerase were

prepared in the DNA free room. In the DNA preparation room a 5 µl volume of each sample, including a negative control, was added (in a final volume of 50 µl). The cycling comprised a single initial denaturation at 94^0C for 6 min followed by 40 cycles of denaturation at 94^0C for 1 min, annealing at 58^0C for 1 min, extension at 72 ^{0}C for 1 min using the PCR thermocycler**.** Amplification of VZV gE was carried out using the same master mix reaction volumes and cycling conditions except that the extension time was extended to 1 min 30 second rather than 1 min at 72 ^{o}C.

2.2.3.3.3 Detection of PCR Products by E-Gel Electrophoresis

For detection of the PCR product a volume of 9µl PCR product was mixed with 1 µl of 10x loading buffer (65% (w/v) sucrose, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.3% (w/v) bromophenol blue), plus 10 µl of deionised water. Thereafter 20µl of each sample was analysed using 2% w/v Agarose E-Gel electrophoresis at 12 volts for 15 min. Ten microliters of ladder (Hyper Ladder™ II) was added to 10 µl of deionised water and loaded into the first well. This ladder contains 15 regularly spaced bands ranging from 50bp – 2000bp. Any empty wells were loaded with 20 µl of deionised distilled water.

2.2.3.3.4 Purification of PCR Product

For purification of PCR products a modified Microcon centrifuge method (YM-100 Centrifugal F Micron YM-100 Kit) was used. Briefly: a microconcentrator was assembled (1.5 ml collection tube and spin column), 300 µl of KCL (200 mM) was then added, and a 50µl PCR product was added to the spin column. The microconcentrator was centrifuged at 500 x g for 15 minutes, and then 300 µl of sterile deionised water was added to the reservoir of the microconcentrator. After another round of centrifugation for 15 minutes, 35 µl of TlowE Buffer (10 mM Tris HCL, 0.1mM EDTA, pH 8.0) was added. The microconcentrator was removed and inverted into a new 1.5 ml tube, and centrifuged at 500 x g for 5 minutes. The microconcentrator was then discarded and 10 µl of the purified product was analysed using a 2% E gel electrophoresed at 12 volts for 15 minutes. The remainder of the purified product was stored at -20^0C for the next stage.

2.2.3.4 Preparation of the 3C/LIC insert for HSV-1 gD and VZV gE

The 3C/LIC insert for each PCR product of each virus was prepared as follows: 10 µl of each purified PCR product was mixed with 2 µl 10X T4 DNA polymerase buffer, 2 µl 25 mM dATP, 1 µl 100 mM DTT, 5.6 µl Nuclease-free water, and 0.4 µl 2.5 U/µl T4 DNA Polymerase (final volume 20 µl) in a sterile 1.5 ml tube kept on ice. Four microliters of the 3C/LIC ß-gal control insert (3081bp with a concentration of 2µg/pmol) and negative control (4µl of deionised water) was added. The reaction was started by adding the T4 DNA Polymerase enzyme. The mixture was incubated in a water bath at 22^0C for 30 minutes. The enzyme was then inactivated by re-incubating the tubes in a water bath at 75^0C for 20 minutes. The treated PCR product was then stored at -20^0C.

2.2.3.5 Annealing the Vector and 3C/LIC Insert

Annealing of the 3C/LIC Insert for each virus into the pIEx/Bac-3 3C/LIC plasmid vector was formed by assembling an annealing reaction in a sterile 1.5 ml tube, containing 1 µl of 3C/LIC vector, 2 µl T4 DNA polymerase treated 3C/LIC insert (0.02 pmol). The annealing reaction was started by adding 1 µl of (25mM) EDTA with stirring by a pipette tip; the tubes were then incubated in a water bath at 22^0C for 5 min.

2.2.3.6 Transformation

2.2.3.6.1 Preparation of Luria-Bertani (LB) Broth Medium

Luria Bertani (LB) medium was prepared by dissolving 5g Bactotryptone, 2.5g yeast extract and 5g NaCl in 450ml distilled water, the volume was then made up to 500ml and pH was adjusted to 7.5 with sodium hydroxide. The solution was then autoclaved for 15 minutes at 120^0C, and after autoclaving the LB media was allowed to cool to room temperature. Five hundred microliters Carbenicillin was then added (to reach a final concentration of 50µg/ml). The media was then stored in the cold room (4^0C).

2.2.3.6.2 Preparation of 1.5% w/v LB Agar Media

LB agar media was prepared by adding 6 grams of LB agar to 500 ml of LB broth medium in a 1L bottle and mixed well. The mixture was autoclaved for 15 minutes at 120^0C. After autoclaving the LB agar was left to cool to approximately 40^0C and then 500 µl of Carbenicillin was added (to reach a final concentration of 50µg/ml). The media was then poured into plates (approximately 25ml/plate) and left at room temperature for 15 minutes before storage at 4^0C.

2.2.3.6.3 Transformation of Competent Cells

Two ampoules containing 0.2ml of competent cells (NovaBlue GigaSingles™ were taken from the freezer (-70^0C) and placed on ice and left to thaw for 5 min. The tubes were then flicked gently 4 times to mix the contents and aliquoted into 20µl in labelled 1.5 ml tubes and left on ice. To each tube 1 µl of the annealing reaction (samples, negative and positive control) was added. To determine the transformation efficiency 1 µl (0.2 ng) of test plasmid was added to a separate tube, and incubated for 5 min on ice. The tubes were then heated in a 42^0C water bath for 30 seconds and then placed on ice for 2 minutes. Two hundred and fifty microliters of SOC medium (at room temperature) was then added to each tube on ice. Tubes were then incubated on an orbital shaker at 37^0C with shaking at 250 rpm for 60 minutes. After incubation 25 µl, 50 µl, and 70 µl from each tube was plated on LB agar medium (50µg/ml Carbenicillin). The plates were left on the bench for 10 minutes to allow excess liquid to be absorbed and then inverted and incubated overnight at 37^0C.

2.2.3.6.4 Colony Screening for Recombinant Plasmid

After overnight incubation at 37^0C 5 colonies (approximately 1mm in diameter) from each plate were taken with a sterile 200 µl pipette tip (a copy of each colony was touched in another plate) in a sterile 0.5 ml tube containing 50 µl sterile water. The tube was then vortexed to disperse the cells, and placed in a heating block at 99^0C for 5 minutes to lyse the cells. The tubes then centrifuged at 12,000 g for 1 minute to remove the cell debris. Ten µl of the supernatant was then transferred into another sterile tube placed on ice. For each

reaction 10 µl of each sample was added to 40 µl master mix reaction containing 5 µl of 10×buffer (50mM KCl, 1.5mM $MgCl_2$ and10mM Tris-HCl; pH 8.3), 1 µl (10mM) of each dNTP, 1 µM of each oligonucleotide primer (5 pmol/ µl), 31.5 µl SDW and 0.25 µl of AmpliTaq Gold polymerase (1.25 U). The cycling parameters for 35 identical cycles of; a denaturation step at 94^0C for 1 minute, annealing step at 55^0C for 1 minute and extension step at 72 ^{o}C for 2 minutes and the last step was extended for 5 minutes. PCR was performed using vector-specific primer alone, or in combination with HSV-1 gD or VZV gE gene specific primers. The PCR product was then analysed using 2% w/v Agarose E-Gel.

2.2.3.6.5 Preparation of Plasmid DNA

A single pure bacterial colony from a freshly streaked plate containing either HSV-1 gD or VZV gE recombinant plasmid was inoculated into 5 ml LB broth containing Carbenicillin (50µg/ml) and incubated at 37^0C with vigorous shaking (300 rpm) for 8 h. A few drops of the broth culture was then transferred into a further bottle containing 35 ml LB broth (50µg/ml Carbenicillin) and incubated at 37^0C with vigorous shaking (300 rpm) for 18 h. Cells were harvested at 5000x g for 10 min and supernatant was discarded, tubes were inverted for several seconds to drain any remaining medium.

2.2.3.6.6 Purification of Plasmid DNA

Extraction of the recombinant plasmid was performed using the Ultra Mobius 200 Plasmid Purification kit. Briefly: the bacterial pellet was resuspended in 2.1 ml of bacterial resuspension buffer, and gently vortexed for 1 minute. A 2.1 ml volume of bacterial lysis buffer was then added to the mixture and tubes were incubated at room temperature for 5 minutes, then 2.1 ml of Mobius™ neutralizing buffer was added. The tube was mixed gently and incubated for a further 5 minutes. After incubation the tube was centrifuged at 10,000 X g for 2 minutes to remove the bulk of insoluble materials. During centrifugation 5 ml of Mobius equilibration buffer was added to the Mobius 200 column reservoir. The column was then placed into a 15 ml conical tube and the entire clarified lysate was added to the column reservoir. Five ml of washing buffer was added and the entire volume was left to flow through column by gravity. The column was placed in a new 15 ml conical

tube and 2 ml of Mobius elution buffer was added. The eluted plasmid DNA was then collected and concentrated and desalted by adding 1.4 ml of isopropanol and centrifuged at 15000 x g for 20 minutes. The pellet was then washed by adding 2 ml of 70 % ethanol (room temperature) and centrifuged at 15000 x g for 10 minutes. Finally the supernatant was discarded and the pellet was resuspended in 150µl nuclease-free water.

2.2.3.6.7 Determining the purity and concentration of the plasmid DNA

The concentration and purity of the plasmid DNA was measured using ultraviolet absorbance spectrophotometry at a wavelength of 260nm. Quantitation of the DNA samples was estimated from the equivalence between an OD_{260} reading of 1.0 and a concentration of double-stranded DNA of 50 µg/ml. In addition, the purity of the DNA preparation was evaluated from the ratio of the OD_{260} and OD_{280} reading with an OD_{260}/OD_{280} ratio of 1.8 being characteristic of protein-free DNA. The DNA was then stored in single aliquots (20µl) at -20 ^{0}C.

2.2.4 Insect Cell Culture

2.2.4.1 Recovery of Frozen cells

A frozen vial (5x10^6 cells/ml) of *Spodoptera frugiperda* 9 **(**Sf9) insect cells was retrieved from liquid nitrogen storage and immediately immersed in a 28^{0}C water bath until cells were fully thawed (approximately 2-3 min). Under a laminar flow hood cleaned with 70% ethanol, the outside of the vial was sterilised with 70% ethanol and cells were transferred to a sterile 50ml polypropylene centrifuge tube. A 10ml amount of pre-warmed (28^{0}C) media (BacVector® Insect Cell Medium) was added drop wise to the cells. The cells suspension was gently pipetted (3-5 times) and the entire contents were then transferred to a 75cm^2 cell culture flask. The flask was gently rocked by hand to evenly disperse the cells and placed in a humidified incubator at 28^{0}C for 30-60 min. Cells were then examined under an inverted microscope to verify attachment to the bottom of the flask, the medium (containing residual DMSO from frozen cells) was then removed and replaced with a 20ml fresh pre-warmed media containing 5% (v/v) foetal calf serum, the flask was then incubated at 28^{0}C. Cells were examined daily until the monolayer become 85-95% confluent (3-4 days).

2.2.4.2 Insect Cells Count

Cells were counted using the trypan blue exclusion method. Briefly: a 100 µl volume of Sf9 cells was added to 100 µl of trypan blue solution (0.4% Trypan blue in 0.85% saline) and was pipetted 3-5 times. Twenty microliters of the mixture was immediately added to the haemocytometer (Fast Read Cell counter 102, ISL Paignton, Devon, UK). Live cells (unstained by trypan blue) were counted in 4 large squares. The total number of cells/ml was calculated as follows:

Number of cells in squares counted/ Number of squares counted x (10^4 x dilution factor).
Cells viability was determined as follows:
Total live cells counted / total cell count (live cells + dead cells).

2.2.4.3 Monolayer Sf9 Culture

The media from a confluent monolayer in a 75-cm^2 flask (85-95%) was removed and replaced by 5ml fresh pre-warmed media. Flasks were then tapped by hand (approximately 3-5 times) to dislodge cells, the suspension was transferred to a sterile 50 ml tube. Cells were examined, counted (as previously described) and seeded in new 75-cm^2 flasks containing 15 ml of antibiotic free BacVector® Insect Cell Medium at a density of 2-5 x10^5 viable cells/ml. Flasks were then incubated at 28^0C and examined on a daily basis under an inverted microscope and sub-cultured when it formed an 85-95% confluent monolayer (3-4 days).

2.2.4.4 Suspension Sf9 Culture

Sf9 cells for suspension culture were prepared as described above, except that cells were seeded in a 250 ml culture bottles containing pre-warmed BacVector® Insect Cell Medium at 5-6 x10^5 viable cells/ml, in 20 or 30 ml culture volumes. The cultures were incubated at 150rpm on orbital shakers at 28^0C. The caps contained a 0.2 µm filter (Sarstedt, Aktiengesellschaft & Co, Germany), and no antibiotics were used. For larger experiments, 500 ml or 1000 ml culture bottles, with up to 100 ml or 300 ml respectively volumes were used. Cells were examined daily and counted every 48 hours as previously described using

the trypan blue method and were subcultured when the cells density reached 4-5 x10^6 viable cells/ml. Culture bottles were routinely washed before and after any experiment using an automated glass bottle washing machine and then autoclaved for 40 min at 121^{0}C.

2.2.4.5 Cells Storage

A suspension culture of Sf9 cells with a density of 4-5 x10^6 cells/ml and more than 90% viability was transferred to a 50 ml tube, cells were harvested by centrifugation at 1500g for 10 min. Cell were then re-suspended in freezing media containing an equal volume of BacVector® Insect Cell Medium containing 20% (v/v) DMSO and 5%(v/v) fetal calf serum. Cells were gently pipetted 2-3 times to ensure complete mixing, and an aliquot of 1 ml of cells suspension was transferred to a sterile cryogenic vial. Vials were then placed in Scotlab polystyrene box (freezing box) containing isopropanol and stored at -20^{0}C for 2h, then transferred to - 70^{0}C for 12-18 h, and finally placed in a liquid nitrogen tank for longer storage.

2.2.5 Protein Expression

2.2.5.1 Insect Direct Expression System

2.2.5.1.1 Monolayer Culture (6-Well Plate)

Protein expression for each virus was carried out using a 6-well culture plate. Briefly: 12 ml freshly diluted of Sf9 cells (5 x10^5 viable cells/ml) was prepared from an exponentially growing shaker culture. Two ml of diluted cell suspension was added to each well. The plate was gently rocked by hand to evenly disperse the cells and incubated at 28^{0}C for 60 min. For each well (except the negative control well) 2 µl of plasmid DNA was mixed with 100 µl of antibiotic free BacVector® Insect Cell Medium in 0.5 ml tube, 10 µl of Insect GeneJuice Transfection reagent was then mixed with 100 µl in a 0.5 ml tube. Diluted plasmid DNA was then added dropwise to the diluted GeneJuice Transfection reagent and mixed gently to avoid precipitation, and incubated at room temperature for 15 min. Media in each well was then aspirated, and an 800 µl volume of media was mixed with each tube and the entire volume added to each well, plates were incubated for 48 to 72 h at 28^{0}C.

2.2.5.1.2 Suspension Culture

For protein expression 10 ml of suspension culture was used. Eight ml of a fresh dilution of Sf9 cells (1 x10^7 viable cells/ml) was prepared from an exponentially growing shaker culture. In a 1.5 ml tube, 20 µl of plasmid DNA was diluted in 1 ml of BacVector® Insect Cell Medium, at the same time 100 µl of GeneJuice Transfection reagent was diluted in 1ml of the same media in a 1.5 ml tube. The diluted plasmid DNA was then added dropwise to the diluted GeneJuice Transfection reagent and was mixed gently to avoid precipitation, and left at room temperature for 15 min. Finally the entire volume was added to the cells and incubated for 48 hours at 150rpm on orbital shakers at 28^0C with caps containing a 0.2 µm filter.

2.2.5.2 BacMagic Expression System

2.2.5.2.1 Construction of Recombinant Baculovirus

2.2.5.2.1.1 Preparation of Cell Culture for Transfection

Cell culture for transfection was prepared using a 25-cm^2 flask. Briefly: 1 h before starting, three 25-cm^2 flasks (sample, positive and negative control) were seeded with a 2 ml dilution of Sf9 cells (1 x10^6 viable cells/ml) from an exponentially growing shaker culture. Flasks were then gently rocked to evenly disperse the cells and incubated at 28^0C for 30 min to allow the cells to attach to the bottom of the flask.

2.2.5.2.1.2 Preparation of Transfection Mixture

The transfection mixture was prepared using a sterile 1.5 ml tube, briefly a 1.5 ml tube containing 1ml BacVector® Insect Cell Medium, 5 µl Insect GeneJuice, 5 µl BacMagic DNA (100 ng total), and 5µl recombinant plasmid were prepared for each transfection. For the negative control 5 µl of the same media was added instead of the recombinant transfer plasmid and for the positive control a transfection control plasmid was used (500 ng). Tubes were then mixed with gentle agitation and incubated at room temperature for 30 min.

2.2.5.2.1.3 Co-Transfection

Co-transfection was carried out by removing the culture media from each flask using a sterile pipette, 1 ml of the transfection mixture was added dropwise to the centre of the flask. Flasks were incubated overnight at 28^0C and 1 ml of BacVector® Insect Cell Medium was added to each flask after the initial incubation period was finished. Flasks were re-incubated for 5 days; and examined daily under the inverted microscope for signs of infection. When cells demonstrated late signs of infection, cells were harvested by low speed centrifugation 1500 x g for 10 min. The supernatant (containing the recombinant virus) was transferred to a new sterile tube and stored at 4^0C for the next stage.

2.2.5.2.1.4 Recombinant Virus Amplification

Virus amplification was carried out using a 50 ml suspension culture. A 50 ml dilution of Sf9 cells (1 $x10^6$ viable cells/ml) from an exponentially growing shaker culture was transfected at low multiplicity of infection (MOI) of < 1 pfu/cell. The MOI was calculated based on the expected virus titer (1 $x10^7$ pfu/ml) using the following formula: required inoculum (ml) = multiplicity of infection (pfu/cell) x total number of cells / titre of viral stock (pfu/ml). The required volume of recombinant virus was added to the bottle and incubated at 150rpm on an orbital shaker at 28°C with caps containing a 0.2 μm filter for 4-5 days. Cells were then harvested by low speed centrifugation at 1000 x g for 10 min, and the supernatant was transferred to another sterile bottle containing 5% (v/v) foetal calf serum and stored in the dark at 4^0C.

2.2.5.2.1.5 Recombinant Virus Titration ($TCID_{50}$)

Recombinant virus titration was carried out using the tissue culture infectious dose 50% ($TCID_{50}$) method. Briefly, a 96 well plate was seeded with Sf9 cells (2 $x10^6$ viable cells/ml) from an exponentially growing shaker culture. One hundred μl added to each well (2 $x10^4$ viable cells/100μl) and the plate was incubated for 2h at 28^0C to allow the cells to attach to the bottom of the plate. During that time a 10 fold serial dilution (10^{-1} - 10^{-10}) of the virus was prepared in 1.5 ml tubes using BacVector® Insect Cell Medium. After incubation the media was removed and 100 μl of the diluted virus was added (8 duplicates

for each dilution), the last column was left as a negative control (containing 100 µl of non infected cells). Plates were then incubated for 1 h at 28^0C to allow the virus to be adsorbed, later the medium was removed and replaced by 200 µl of fresh media and the plate was incubated at 28^0C in a humidified incubator for 4-5 days. Wells containing signs of infection were counted and the virus titration end-point was calculated using the Spearman-Karber formula. An example of the calculation is shown below in Table 2.1.

According to the Spearman-Karber formula:

Log10 Median Dose = $X_0 - (d/2) = d\ (\sum r_i/n_i)$

Where :

$X_0 =$ $\log_{10}$ of the reciprocal of the highest dilution at which all test inocula are positive.

D = $\log_{10}$ of the dilution factor (the difference between the log dilution intervals).

n_i = number of test inocula used at each individual dilution.

r_i = number of positive test inocula (out of n_i).

$\sum(r_i/n_i) = \sum$ (P) = sum of the proportion of positive tests beginning at the highest dilution showing 100% positive results (this summation starting at dilution X_0).

Table 2.1 Example Results Obtained Using 8 Wells per Dilution and 0.1 cm^3 Inoculum

Virus Dilution	N_i	r_i	Proportion Positive (P)	1-P
10^{-1}	8	8	1	0.00
10^{-2}	8	8	1	0.00
10^{-3}	8	8	1	0.00
10^{-4}	8	8	1	0.00
10^{-5}	8	8	1	0.00
10^{-6}	8	8	1	0.00
10^{-7}	8	5	0.625	0.375
10^{-8}	8	2	0.250	0.750
10^{-9}	8	1	0.125	0.875
10^{-10}	8	0	0.000	0.1

X_0 6.0; d = 1.0

Log 10 50% end-point dilution = (6-0.5) + (1x (8/8 + 5/8 + 2/8 +1/8))

= (6-0.5) + 1 x (1+ 0.625 + 0.25 + 0.125)

= 5.5 + 2

= 7.5

Infection dose = Log_{10} $TCDI_{50}$ inoculated ($0.1cm^3$) = 7.5 = $10^{7.5}$ $TCID_{50}$
Titre = $TCID_{50}$/unit volume, usually $1cm^3$.
Therefore the titre = $10^{8.5}$ $TCID_{50}/cm^3$

2.2.6 Protein Expression

2.2.6.1 Protein Expression Optimisation

Protein expression optimisation was carried out using different multiplicities of infection (MOI) and time duration in small scale protein expression experiments. Glass culture bottles (9x50ml) were seeded with 10 ml dilutions of Sf9 cells ($2x10^6$ viable cells/ml) from an exponentially growing shaker culture. Bottles were divided into three groups (3bottles for each group), each was inoculated with different MOI (5, 7, 10) and incubated at 28^0C. A bottle from each group was harvested either at 48, 72 or 96 h post-infection (POI) and analysed for protein expression. The optimum MOI and time of harvest was used for large scale protein expression.

2.2.6.2 Large Scale Protein Expression

Large scale protein expression was carried out using 100–300 ml volumes of suspension culture. Based on the protein optimisation results 500 or 1000 ml culture bottles were seeded with 100 or 300 ml of Sf9 cells ($2x10^6$ viable cells/ml) from an exponentially growing shaker culture. Each bottle was transfected at an MOI of 10 and incubated on an orbital shaker at 28^0C with caps containing a 0.2 μm filter for 72 h. Transfected cells were examined before harvesting for the appearance of late signs of infection using an inverted microscope. The cell pellet were either used immediately for protein purification or stored at -20^0C after performing 3 washes with ice-cold PBS (pH 7.3).

2.2.6.3 Protein Purification

2.2.6.3.1 Protein Solubilisation

Protein solubilisation was carried out using a suspension culture; Sf9 cells ($2.5x10^8$) were transfected at a MOI of 10 and then incubated for 3 days on an orbital shaker at 28°C. After late signs of infection were observed, cells were harvested by low speed centrifugation (2000g x 10 min), and washed 3 times with ice-cold PBS (pH 7.3). Cell pellets ($5x10^7$/ml) were then resuspended in ice-cold solubilising buffer (20m*M* Tris-HC., pH 7.8, 2 m*M* Phenylmethylsulfonyl fluoride (PMSF), 1 mM Tosyl-Lys-chloromethylketone (TLCK)) and then the same volume of buffer containing 2% (v/v) of Pentaethyleneglycolmonodecylether ($C_{10}E_5$) was added (final detergent concentration 1%). Cell suspensions were then incubated on ice for 2 h. After incubation cell debris was removed by low speed centrifugation (2000g x 10 min) and the supernatant was ultracentrifuged (70000g x 1h, 4^0C). Fifty µl of the final supernatant (containing solubilised protein) was stored at $+4^0$C for protein analysis and the remainder was used in the purification step.

2.2.6.3.2 Protein Purification

The purification was carried out using the his-bind purification kit. Briefly: in a 20 ml tube 500 µl of Ni-NTA his-binding resin was mixed with the remainder of the solubilised protein (~ 10 ml). The tube was then placed on an orbital shaker (150 rpm) at room temperature for 2h. The purification column was assembled (chromatography column + 15 ml collecting tube), and then the entire volume was added into the reservoir of the column. The column was left to flow by gravity (~ 5-10 min) at room temperature and then transferred to a new collecting tube and 4 ml of washing buffer was added to the column and left to flow through. The washing step was repeated once and the column was transferred to a new collecting tube and 1 ml of 1x elution buffer (4 M imidazole, 2 M NaCl, 80 mM Tris-HCl, pH 7.9) was added. The elution step was repeated using 500 µl in a new collecting tube to ensure no protein was left over in the column. All tubes were stored at $+4^0$C and 15 µl from each tube was used for protein analysis.

2.2.6.3.3 Protein Quantification

The purified recombinant protein concentrations were determined using Bicinchoninic acid (BCA) protein assay. The assay was carried out according to the manufacturer's instructions, briefly; serial dilutions of bovine serum albumin (BSA) ranging from 0 to 100µg/ml were prepared in sterile water from a 1 mg/ml stock. Twenty five µl of each sample (BSA standards and protein samples) were added in triplicate into the 96-well assay plate, followed by the addition of 200 µl of working solution. The plate was incubated at 37^0C for 30 min and was then cooled to room temperature. The concentrations of BSA standard and purified proteins were then determined by measuring absorbance at 562 nm using an ELx808 Universal Microplate Reader. Using Microsoft Windows Excel 2003, BSA standards results were used to create a standard curve which was used to determine the concentration of each purified protein in each sample.

2.2.7 Protein Characterisation

2.2.7.1 Indirect Immunofluorescence (IF) Assay

Indirect immunofluorescence (IF) analysis was performed using fluorescein-conjugated rabbit anti mouse IgG (cell surface, cytoplasmic). Briefly, Sf9 cells were infected with recombinant baculoviruses expressing either HSV-1 gD or VZV gE at an MOI of 10 and incubated at 28^0C for 72 h. To examine cell surface immunofluorescence, 100 µl of cells were incubated with 100 µl of 1/100 dilution of either mouse anti gD or gE monoclonal antibody for 1 h at 37^0C with gentle agitation. Cells were then washed with PBS and a 20 µl of cells were applied to the appropriate spot of the Lab-Tek chamber slides and placed on heating block to dry at 40^0C for 15 min. Slides were then fixed with 99% v/v acetone for 10 min at room temperature, washed 2 times for 5 min on an orbital shaker with sterile distilled water, washed again and incubated with fluorescein-conjugated rabbit anti-mouse IgG antibody for 1 h at 37^0C. After incubation slides were washed again and left to air dry at room temperature for 10 min. To examine intracellular (total cell) immunofluorescence, cells were washed with PBS, and 20 µl of cells were applied to the appropriate spot on the slide and placed on a heating block to dry at 40^0C for 15 min. Slides were fixed with 99% v/v acetone for 20 min, washed and dried as above and incubated with 1/200 dilution of either mouse anti gD or gE monoclonal antibody for 1 h at 37^0C. Cells were then washed

and dried and incubated with fluorescein-conjugated rabbit anti-mouse IgG antibody for 1 hr at 37^0C. Slides were washed again and left to air dry for 10 min at room temperature. Finally slides were mounted with 50 µl volume of mounting medium (glycerine buffer, PBS, pH 7.3) examined with an incident light fluorescence microscope. A negative control (non infected cells) was included in each slide for comparison.

2.2.7.2 Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis (SDS-PAGE)

To determine the size of the expressed proteins, proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing and reducing conditions. Fourteen µl of sample (transfected cells ($5x10^3$/ml), solubilised protein, purification flow, first wash, second wash, first elute and second elute) were mixed with sample loading buffer (4µl of NuPAGE LDS sample buffer, 6µl deionised water and 1µl reducing agent). Samples were heated at 70^0C for 10 min and 16µl of each sample was analysed using NuPAGE 4–12% Bis-Tris gel for 45 min at 200V with running buffer (50ml running buffer, 950ml deionised water). A Novex® Sharp Pre-Stained Protein Standard containing 12 pre-stained protein bands in the range of 3.5 - 260 kDa was electrophoresed in parallel with the test samples. The gel was removed from the tank and placed in a plastic container, rinsed three times for 5 minutes with 100 ml deionised water to remove SDS and buffer salts. Excess water was drained and 20ml of Coomassie Blue stain was added and kept on a shaker for 1 h at room temperature. For maximum staining sensitivity 20 ml of 20% NaCL was added to the same water and left on the shaker for another 2 h.

2.2.7.3 Immunoblot

2.2.7.3.1 Western Blot

Western blot analyses were carried out under denaturing reducing conditions. After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, and further developed by immunoblotting with the appropriate antibody and the antigen/antibody complex visualised with alkaline phosphatase- labelled antibody. In brief, a piece of PVDF membrane was soaked in transblotting buffer (50ml transfer buffer, 200 ml Methanol, 750ml deionised water) for 20 min. A sandwich was made as in Figure

2.1, with a rolling motion to avoid trapping air bubbles. The sandwich cassette was lowered into the tank with the membrane towards the anode (+) electrode. The electrophoretic transblot was performed at 100 V for 50 minutes. The transblotted PVDF membrane was then left in blocking buffer (2.5 mg skimmed milk, 50ml PBS pH 7.3, 0.1% Tween 20) overnight at 4^0C. The PVDF membrane was incubated with gentle agitation either anti-HSV gD or anti-VZV gE monoclonal antibody IgG (1:2000), diluted in blocking buffer for 1 h at 37^0C. The membrane was then washed 3 times with washing buffer (15ml PBS, 0.05% Tween 20) at room temperature for 5 min and incubated with anti mouse IgG alkaline phosphatase conjugate (1:2000) for 1h at 37^0C. Both primary and secondary antibodies were prepared in diluent buffer (10mM PBS, 0.1% Tween 20). After washing the membrane was incubated with one tablet of FAST^{TM} BCIP/NBT dissolved in 10 ml of deionised water on a rotor at room temperature until the bands appeared (10-15 min). The membrane was rinsed with distilled water and left to dry on filter paper.

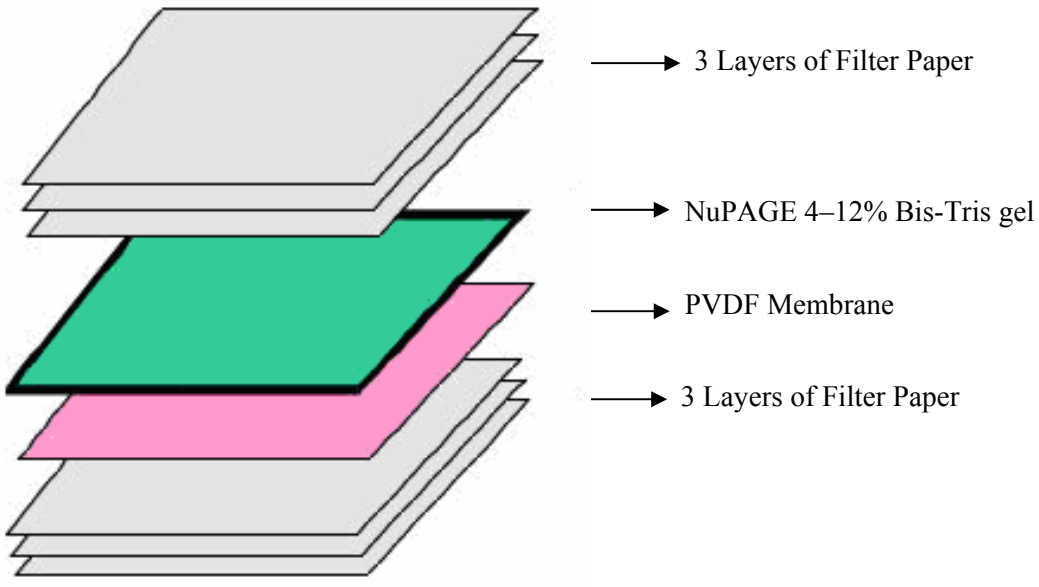

Figure 2.1 Figure shows the different layers of the sandwich assembly of the western blotting apparatus. It consisted of 3 layers of filter paper, NuPAGE 4–12% Bis-Tris gel, PVDF membrane and another 3 layers of filter paper.

2.2.7.3.2 Dot Blot

All samples i.e. transfected cells ($5x10^3$/ml), solubilised protein, purification flow, first wash, second wash and eluted protein were used in dot blot analysis. Samples (35µl) were transferred to dot blot manifold and vacuum-absorbed onto a 0.45µ nitrocellulose membrane. Each sample was washed three times with 100 µl of washing buffer (10mM PBS, 0.05% Tween 20) air-dried and then blocked in blocking buffer (2.5 mg skimmed milk, 50ml PBS pH 7.3, 0.1% Tween 20) overnight at 4°C. The membrane was then incubated with anti-HSV gD or anti-VZV gE monoclonal antibody IgG (1:2000), diluted in blocking buffer for 1 h at 37^0C with gentle agitation. Membranes were then washed 3 times with washing buffer (15ml PBS, 0.05% Tween 20) at room temperature for 5 min and incubated with anti mouse IgG alkaline phosphatase conjugate (1:2000), diluted in diluent buffer (10mM PBS + 0.1% Tween) for 1 h at 37^0C. After further washing at room temperature, membrane was immersed in a solution of one tablet of SIGMA FAST^{TM} dissolved in 10 ml of deionised water on a rotor at room temperature until the bands appeared (10-15 min). The membrane was rinsed with distilled water and left to dry on filter paper.

2.2.7.4 Enzyme-linked Immunosorbent Assay (ELISA)

All samples i.e. transfected cells ($5x10^3$/ml), purification flow, first wash, second wash and 1 and 2 eluted protein for each virus was used in ELISA analysis. A two-fold dilution of each sample from 1:100 to 1:800 of HSV-1 gD and VZV gE was prepared in coating buffer (PBS pH 7.3), and 100 µl of each diluted protein was added to selected wells and incubated overnight at 4^0C. The plate was then washed 5 times with washing buffer (10mM PBS pH 7.3, 0.05% Tween 20) using an automated universal microplate washer. one hundred µl of diluted (1/2000) mouse monoclonal antibody against HSV gD or VZV gE in diluent buffer (10mM PBS pH 7.3, 0.1% Tween 20) was added to the appropriate well and incubated at 37^0C for 1 h in humid box. The plate was washed and dried, and 100 µl of anti-mouse IgG horseradish peroxidase (HRP) conjugate (1:2000) was added to all wells and incubated for 30 min at 37^0C. Plates were then washed again and colour reaction was developed by adding 100 µl of ABTS substrate to each well. After incubation at room

temperature for 10 to 15 min, absorbances were read at 405 nm using an ELx808 Universal microplate reader.

2.2.8 Evaluation of the Performance of Peptide 55 Assay for Detecting HSV-2 IgG Antibody in Human Serum

To evaluate the performance of the PATHOZYME™ VIRO HSV-2 ELISA IgG assay based on peptide 55 for the detection of HSV-2 IgG antibodies, the assay was compared with 2 other commercially available ELISA kits; HerpeSelect HSV-2 (Focus Diagnostics, Cypress, CA, USA), based on purified recombinant type-specific gG-2 antigen and Bioelisa HSV-2 IgG type specific assay (Biokit, S.A, Barcelona-Spain) based on purified gG2 protein. The three assays are referred to in this study as the HerpeSelect, Bioelisa, and Omega assays, respectively. A well characterised serum panel consisting of 254 serum samples were used for the evaluation of the Omega assay (Panel 1)

2.2.8.1 Omega Assay

According to the manufacturer's instructions, kit components and test serum were brought to room temperature (20^0C to 25^0C) before starting the assay. Coated plates were soaked with 300 µl of diluted washing buffer and incubated for 5 min at room temperature. The buffer was removed by striking the wells (upside down) against absorbent paper. A 50 µl volume of assay controls (ready-to-use high-positive, low-positive and negative) or diluted test sera (1:10 in serum diluent reagent) were added to the appropriate well in the plate. Assay controls were tested in duplicate and 1 well was left empty to serve as a substrate "blank". The plate was covered with a lid and incubated for 1 h at 37^0C and then washed 3 times with an automated ELISA plate washer. Fifty microliters of anti-human IgG HRP conjugate was added to each well and covered with a lid and incubated for 30 min at 37^0C. The plate was washed as above and a 100 µl of substrate was added per well including the blank well. After 15 min incubation at room temperature, the enzymatic reaction was stopped by addition of 100 µl of stop solution. Absorbances were read on an automated ELx808 Universal Microplate Reader at 450 nm immediately after stopping the reaction.

2.2.8.2 HerpeSelect Assay

Assay components were brought to room temperature prior to starting the assay and the coated plate was soaked with 300 µl of diluted washing buffer which was incubated for 5 min at room temperature and buffer removed by striking the wells against absorbent paper. A 100 µl volume of the sample diluent for the "blank" well and 100 µl of each specimen; assay control (negative and positive) calibrators were tested in duplicate and test sera (diluted 1:101 in serum diluent reagent) added to the appropriate wells in the plate. The plate was covered with a lid and incubated for 1 h at room temperature (20^0C to 25^0C), before being washed 3 times with washing buffer. One hundred microliters of anti-human IgG HRP conjugate was added to each well, the plate was covered with a lid and incubated for 30 min at room temperature. The plate was washed as above and a 100 µl of substrate was added per well including the blank well. After 10 min incubation at room temperature, the enzymatic reaction was stopped by addition of 100 µl of stop solution and absorbances were read at 450 nm within 1 hour of stopping the assay.

2.2.8.3 Bioelisa Assay

Assay components were brought to room temperature and gently mixed prior to use, then 100 µl of each diluted specimen (1/100); test sera or controls were added to the corresponding wells, the blank well left empty. The plate was covered with lid, incubated for 1 h at 37^0C, washed 3 times with washing buffer and then left on absorbent tissue to remove any excess liquid from the wells. One hundred microliters of anti-human IgG HRP conjugate was added to each well except the blank. The plate was covered with a lid and incubated for 30 min at 37^0C. The plate was then washed as above and a 100 µl of substrate-TMB solution was added to each well including the blank. After 30 min incubation at room temperature, the enzymatic reaction was stopped by addition of 100 µl of stop solution and absorbances were read at 450 nm.

2.2.8.4 Virotech HSV-1 Assay

All samples (254) were retested using Virotech HSV-1 type specific gG-1 IgG assay according to the manufacturer's instructions. Assay components were first brought to room temperature and gently mixed prior to use, 100 µl of the ready to use sample diluent for the "blank" well and 100 µl of the assay negative control and cut-off control were tested in duplicate. Test sera (diluted 1:100 in serum diluent reagent) were added to the appropriate well in the plate. The plate was covered with a lid, incubated for 30 min at 37^0C, washed 4 times with washing buffer and then left on absorbent tissue to remove any excess liquid from the wells. One hundred microliters of anti-human IgG HRP conjugate was added to each well which was covered with a lid and incubated for 30 min at 37^0C. The plate was washed as above and 100 µl of substrate solution was added to each well. After 30 min incubation at 37^0C, the enzymatic reaction was stopped by addition of 50 µl of stop solution and absorbances were read at 450/650 nm.

2.2.8.5 Western blot Assay

All samples that gave discordant results with the three assays where sent to Alva Diagnostics Laboratories (Omega, Scotland, UK) for Western blot analysis. Briefly; assay was performed using a Mini-Protean 3 Electrophoresis Cell (Bio-Rad Laboratories, Hemel Hempstead, UK) according to the manufacturers instructions. HSV antigens were prepared from baby hamster kidney cells infected with either HSV-1 strain 17 syn^+ (Brown *et al*., 1973) or HSV-2 strain HG52 (Timbury, 1971). Proteins were separated by SDS polyacrylamide gel electrophoresis using concentrations of acrylamide in the stacking and resolving gels of 5% and 7% respectively. Gels were made with acrylamide cross linked with bis-acrylamide (19 to 1). After transfer of the proteins to a nitrocellulose membrane, the membrane was blocked with blocking buffer (4% newborn calf serum and 3% bovine serum albumin in Tris-buffered saline) then the membrane was cut into strips. The strips were incubated overnight in blocking buffer with either human serum diluted 100-fold, or gG1 or gG2-specific monoclonal antibodies diluted 10,000-fold (East Coast Bio Inc., Miami, USA, cat. no. H1A020 and H2A023 respectively). Strips were washed and bound human antibodies were detected with peroxidise-conjugated goat anti-human antibodies while bound gG1- and gG2-specific mouse monoclonal antibodies were detected with

peroxidise-conjugated goat anti-mouse antibodies. Following visualisation of bands with a fluorometric substrate the sera were scored as positive or negative for HSV-2 antibodies according to whether a typical gG2 band appeared.

2.2.8.6 Index Value Calculation

The Index Value of each assay was calculated according to the manufacturer's instructions. Briefly: In HerpeSelect an index values was obtained for each sample run, based on the absorbance of the patient sample divided by the mean absorbance of the cut-off calibrator; an index value of <0.9 and > 1.1 were defined as negative and positive respectively and a value of ≥ 0.9 but ≤ 1.1 was considered equivocal. In the Omega and Bioelisa ELISA kits index values were obtained for each sample run, based on the absorbance of the patient sample divided by the mean absorbance of the cut-off (low-positive control). A ratio of <0.9 and > 1.1 was considered negative and positive respectively and a ratio of ≥ 0.9 but ≤ 1.1 was considered equivocal. In the Virotech assay results were expressed as Virotech units, where < 9.0 was considered negative and from ≥ 9.0 but ≤11.0 was considered equivocal and > 11.0 was considered positive.

2.2.8.7 Statistical analysis

The correlation (r) between Omega assay and other ELISA assays was calculated in Microsoft® Excel 2007 using the Pearson's product-moment coefficient function. The probability (P) of observing each r value (if the null hypothesis that there is no relation between the two measurements was true) was calculated using correlation P calculator in Microsoft® Excel 2007. Assay sensitivity, specificity, positive and negative productive value was calculated using the Focus assay as a reference test according to the following method:

		Test Results (ELISA Assay)	
		Positive	Negative
Reference Test	Positive	d	c
	Negative	b	a

Where: d: True Positive c: False Negative
b: False Positive a: True Negative

Therefore:

Sensitivity = d / (c+d) x 100

Specificity = a / (a+b) x 100

Positive productive value = d / (b+d) x 100

Negative productive value = a / (a+c) x 100

2.2.9 Development of an In-house ELISA Using HSV-1 gD and VZV gE

The HSV-1 gD and VZV gE recombinant proteins were used to develop an in-house ELISA to detect IgG antibody against each virus in human sera. Optimisation of the in-house ELISA assay was carried out by checkerboard titration using different ELISA plate and coating buffers.

2.2.9.1 Determining the Optimal Dilution of Antigen and Human Serum

A two-fold dilution series from 1:25 to 1:3200 of HSV-1 gD and VZV gE was prepared in coating buffer (PBS pH 7.3). A 100 µl volume of each diluted antigen was added to all wells in each column on the 96 microtitre plate from column 1-11 and incubated overnight at 4^0C. Plates were then washed 5 times with washing buffer (10mM PBS pH 7.3, 0.05% Tween 20) using an automated universal microplate washer. For determining the optimal

dilution of human serum a two-fold dilution of a clinical standard reference serum (high positive for HSV-1 and HSV-2) starting from 1:25 to 1:3200 was prepared in serum diluent buffer (10mM PBS pH 7.3, 0.1% Tween 20) and was added to all rows from A1-A10. Column 11 was used as a serum control (no human serum was added) and column 12 was used as blank. The plates were then incubated at 37°C for 1 h in a humid box before washing as above. A 100 µl volume of anti-human IgG HRP conjugate (1:2000) was added to all wells and plates were incubated for 30 min at 37^0C and then washed as above. Later plates were washed again and colour reaction was developed by adding 100 µl of substrate ABTS to each well and incubating at room temperature for 10 to 15 min. Absorbances were read at 405 nm using an ELx808 Universal microplate reader. Based on the optimisation results, human sera from each individual panel were tested using the standard ELISA method.

2.2.9.2 ELISA Standard Method

Costar microtitre ELISA "medium binding" plates were coated with 100µl of recombinant HSV gD or VZV gE protein diluted to a final optimized concentration of 0.50µg/ml in coating buffer (PBS, pH 7.3), and incubated overnight at 4^0C. The plates were washed 5 times with washing buffer (PBS, 0.05% Tween 20, pH 7.3) and tapped dry on paper towels then blocked with blocking buffer (2% BSA in wash buffer) for 1h at 37^0C, and then washed and dried. A standard curve was generated by making two-fold dilutions (1/10 to 1/10240) using a clinical standard reference serum (strongly positive for HSV-1, HSV-2 and VZV antibody) tested in duplicate, other samples, patient serum, and controls were diluted 1:100 in sample diluent buffer (PBS, 0.1% Tween 20, pH 7.3). One hundred microliters of each diluted sample, standard, controls (positive and negative sample) and patient serum were added to the coated plates and incubated at 37 ^{0}C for 1h, then washed 5 times with wash buffer. After washing 100 µl of diluted mouse anti-human IgG (1/2000) conjugated with horseradish peroxidase in sample diluent buffer was added to the plates and incubated for 30 min at 37^0C. The plate was washed again and colour reaction was developed by adding a 100 µl of substrate ABTS to each well and incubating at room temperature for 10 to 15 min. Absorbances were read at 405 nm using an ELx808 Universal Microplate Reader. A diagram of the ELISA experiment procedure is shown in Figure 2.2.

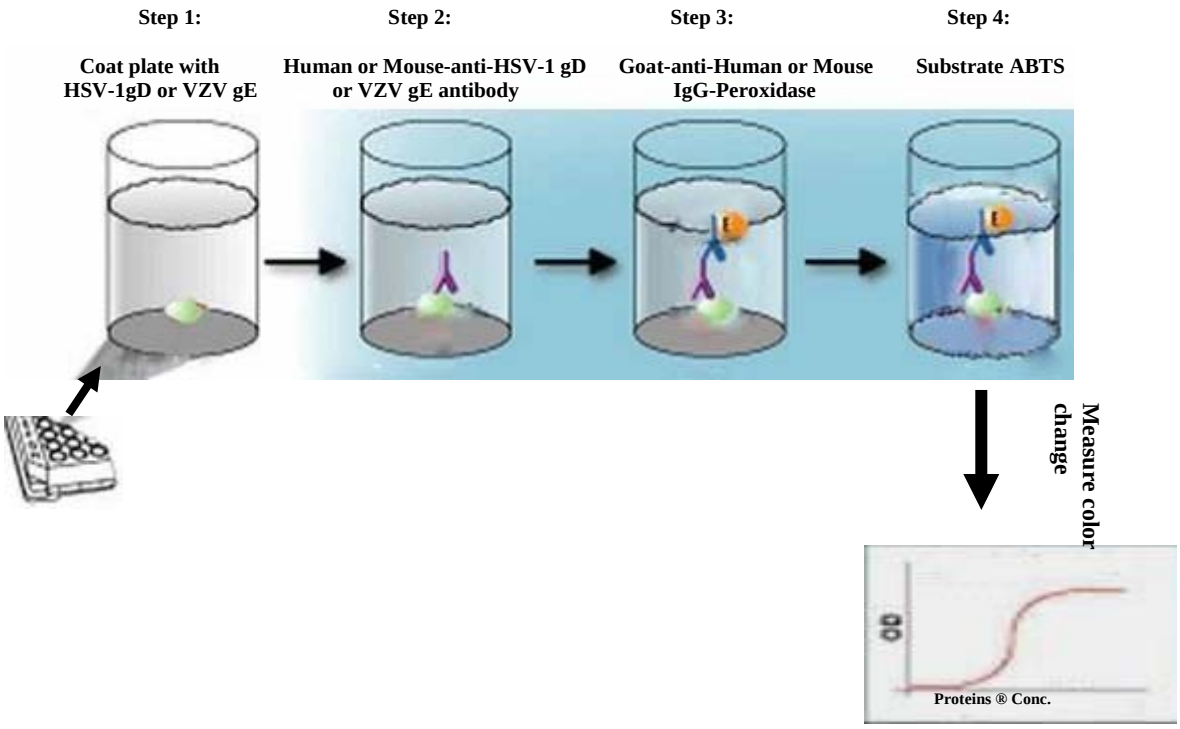

Figure 2.2 ELISA plate was coated with the recombinant protein overnight at 4^0C, washed and then incubated at 37^0C with the 100 µl of selected sample. Plates were washed again and re-incubated with antibody conjugated to horseradish peroxidase. Finally 100 µl of anti-human IgG conjugated with substrate was added and left until the colour developed at room temperature and the absorbance was read at 405nm.

2.2.9.3 Statistical Analysis of ELISA Results

The cut-off point of each assay was determined by calculating the mean absorbance of 8 negative control samples plus 3 standard deviations. Intraplate, interplate, and day-to-day variation of the ELISA was evaluated by repeated analysis of 10 serum samples (negative and positive). Sensitivity, specificity, positive and negative predictive values were determined as described above (section 2.2.11.5) using Western blot and Liaison assays as a reference test for HSV-1 gD and VZV gE respectively.

2.2.10 Development of a Fluorescence Microbead Immunoassay (FMIA) for the Detection of IgG Antibody to HSV-1, HSV-2 and VZV in Human Sera

2.2.10.1 Acceptance Criteria for HSV-1 gD, Peptide 55 and VZV gE Microbead Immunoassay

To ensure accurate measurement and maximum reproducibility of the assay the acceptable criteria for evaluation of assay results were defined as follows:-

1- Fluorescence analysis data for each well should be obtained from 100 beads/region/well.
2- Beads aggregation percentage for each replicate well should be ≤ 50%.
3- Background readings for each antigen (Blank well) should be ≤ 100 mean fluorescence intensity (MFI).
4- The standard deviation between each duplicate reading should be ≤ 30% between all standard replicates.
5- When the plate met the acceptance criteria, each antigen was assessed individually.

2.2.10.2 Standard Curve Generation

To generate a standard curve for each analyte the same clinical standard positive serum sample used in the ELISA assay was included in each plate. In order to quantitate the concentration of any analyte, the response that was produced by the tested sample was compared with the standard curve. The unknown sample concentration was determined by interpolation from the standard curve. Since the amounts of the antibody for each virus in the clinical positive standard serum were unknown the standard was defined to contain an arbitrary number of units of specific antibodies AB units (ABU), the standard serum contained 3200 ABU for VZV and 4800 ABU for HSV-1 and 4800 ABU for HSV-2. Results for each tested sample were reported as ABU/mL of specific antibody.

2.2.10.3 Microsphere Coupling Method

The purified recombinant proteins HSV-1 gD, VZV gE and peptide 55 were coupled to different SeroMAP carboxylated microspheres, using a standard modified two step carbidiimide reaction (Staros *et al.,* 1986). The coupling procedure was carried out using 6.25x 10^6 (500 µl) of the original microspheres (region 10, 15, or 20). Beads were collected by vacuum filtration and washed twice with freshly prepared PBS (0.14M sodium chloride, 0.021M potassium phosphate, 0.013M sodium phosphate, and 0.0021M potassium chloride pH 7.3) by centrifugation at 13,000g for 2 min, aspiration of the supernatant, addition of 200 µl of PBS, sonification for 30 seconds, and resuspension of the pellet in 500 µl of PBS. Five hundred µl of freshly prepared activation buffer (PBS, 5 mg/ml of EDC, 5mg/ml Sulpho-NHS, pH 7.3) was added to each set of beads, the suspension was then incubated for 30 min at room temperature on an orbital shaker in the dark. The activated beads were washed twice as described above and resuspended in 100 µl of gD (50µg/ml), gE (50µg/ml) and peptide 55 (25µg/ml) diluted in pyrogen free water and incubated for 1.5 h at room temperature on an orbital shaker in the dark. Microspheres were then pelleted by centrifugation, washed again, and resuspended in 1 ml of PBS and stored at 4^0C in the dark until use.

2.2.10.4 Beads Calculation

Bead suspensions were counted by adding 9 µl of bead suspension to a fast read counting chamber under a light microscope, the number of beads in the large squares were counted (contains 16 small squares). The total number of beads in each 1 ml was calculated as follows:

Number of beads in 1 ml (n) = (Total number of beads ÷ no of grids counted) 10^6
15000 / region/ 25 µl to be added
Bead dilution = (beads count / 40) ÷ 15000
Total PBS volume required = ((no of wells x 2) + 2) 25
Beads volume required = total volume required ÷ beads dilution
PBS volume required = total volume required – bead volume

Example

If the total beads count was = 5.5×10^6

Beads dilution = $(5.5 \times 10^6 \div 40) / 15000 = 9.17$

Total volume required = (96 wells x2) +2) 25 = 4850 µl

Beads volume required = 4850 ÷ 9.17 = 525.9 µl

PBS volume required = 4850 – 525.9 = 4324.1 µl

2.2.10.5 Triplex Microbead Immune Assay for the Quantitation of HSV-1, HSV-2 and VZV IgG in Human Sera

A standard curve was generated by making two-fold dilutions (1/10 to 1/10240) using the standard clinical serum sample tested in duplicate. Other patient serum and controls (known positive and negative samples) were diluted 1:100 (2 µl sample + 198 µl of serum diluent) in sample diluent buffer (1% NBBS, PBS pH 7.3, 0.05% Tween 20). Twenty five µl of each sample (diluted standard, assay controls, patient sera) was added in duplicate to each well of a 96-well filter bottom microtitre plate. In each plate 25 µl of sample diluent was added to the first 2 wells of the plate as a blank. Then 25 µl of premixed coupled microsphere bead (5000/region/well) was added to each well and the plate was protected from exposure to light and incubated at room temperature on an orbital shaker for 30 min. Plates were then washed 3 times with 100 µl of wash buffer (PBS, 0.05 % Teen 20) using a vacuum filtration manifold, 100 µl per well of 2.5 µg/ml of R-Phycoerythrin-conjugated goat anti-human IgG was added. Following 30 min incubation at room temperature and washing, the microspheres were resuspended in 125 µl of washing buffer and read using a BioPlex 200 workstation. Sample data were produced from a 5-parameter logistic curve (5PL).

2.2.10.6 Beads Interference Testing

The interference between all bead sets were carried out by comparing the results of the standard curves obtained from each assay for each single analyte and the triplex analyte assays.

2.2.10.7 Statistical Analysis

The cut-off point of each assay was determined by calculating the mean MFI of 10 negative samples plus 3 standard deviations and the minimal levels of detection were determined for each assay. Assay reproducibility was determined by measuring intra and inter assay variation, for intra-assay variation the mean percentage coefficient of variation (CV) of 10 samples tested in duplicate within the same plate were calculated. Inter-assay variation was determined by testing 10 serum samples in duplicate on two different days, and the CV was calculated. Sensitivity and specificity were determined for all assays by comparing the results from each assay with its related ELISA assay.

2.2.11 Development of Bi-plex Assay for the Quantitation of Albumin and IgG in Human Serum and CSF samples

2.11.1 Microsphere Coupling Method

The development of the bio-plex assay for the quantitation of albumin and IgG was modified from the method described by Al Jindan (2009). Briefly, five hundred µl (6.25x 10^6) of SeroMAP carboxylated microspheres beads (region 25 and 30), were washed and activated as described before (section 2.10.5). Activated beads were then resuspended in 100 µl of monoclonal anti-human IgG, or monoclonal anti-human albumin diluted in pyrogen free water to a final optimised concentration of 2.5 µg/ml. Beads were then incubated for 1.5 h at room temperature on an orbital shaker in the dark, pelleted by centrifugation, resuspended in 125 µl of washing buffer and read using a BioPlex 200 workstation. The standard carve was a 5-parameter logistic curve (5PL).

2.2.11.2 Biplex Assay for the Quantitation of Albumin and IgG in Human Serum and CSF samples

A standard curve was generated by making 10 two-fold dilutions (1/10 to 1/10240) of the human serum standard tested in duplicate starting from (3.9µg/dl for IgG and 5µg/dl for albumin), other samples and controls were diluted 1/10000. Beads were then collected by vacuum filtration and washed twice with freshly prepared PBS, and a 25 µl of premixed

coupled microspheres for both anti-human IgG and anti-human albumin (5000/regen/well) was added to each well of the 96-well filter bottom microtitre plate. One hundred µl per well 2.5 µg/ml of R-Phycoerythrin-conjugated goat anti-human IgG and 100 µl of 2.0 µg/ml of biotin conjugated with goat anti-human albumin was added to each well. Following 30 min incubation at room temperature and 2 washing steps, 100 µl of streptavidin R-Phycoerythrin was added to each well. After another 30 min incubation and washing, the microspheres were resuspended in 125 µl of washing buffer and read using a BioPlex 200 workstation. The standard curve was a 5-parameter logistic curve (5PL).

2.11.3 Statistical Analysis

The cut-off point of the assay was determined by calculating the mean MFI of 10 blank samples plus 3 standard deviations and the minimal levels of detection were determined for each assay. Assay reproducibility was determined by measuring intra and inter assay variation, for intra-assay variation the mean percentage coefficient of variation (CV) of 10 samples tested in duplicate within the same plate were calculated. Inter-assay variation was determined by testing 10 serum samples in duplicate on two different days, and the percentage CV was calculated.

2.2.12 Development of Multiplex Microbead Immunoassay for Detection and Quantitation of IgG Antibody Response to HSV-1, HSV-2, VZV, Albumin and IgG in Human Serum and CSF

A standard curve was generated by making 12, 4-fold dilutions (1/10 to 1/10485760), of the clinical standard serum sample (starting from 0.3g/l for albumin and 0.05g/l for IgG) tested in duplicate, other samples and controls were diluted to 1/500 and 1/5000 for serum and CSF respectively. Beads were then collected by vacuum filtration and washed twice with freshly prepared PBS, and 25 µl of premixed coupled microspheres (25000/well) was added to each well of the 96-well filter bottom microtitre plate. One hundred µl per well of diluted R-Phycoerythrin-conjugated goat anti-human IgG (1/200) and 100 µl of diluted biotin conjugated with goat anti-human albumin (1/999) was added to each well. Following 30 min incubation at room temperature and 2 washing steps, 100 µl of streptavidin R-Phycoerythrin (1/400) was added to each well. After 30 min incubation and

a second washing step the microspheres were resuspended in 125 µl of washing buffer and read using a BioPlex 200 workstation. The standard carve was a 5-parameter logistic curve (5PL).

2.2.12.1 Beads Interference and Statistical Analysis

Bead interference was tested as described before, the results obtained from the standard curves of each assay for each single analyte were compared with its relevant curve in the multiplex assays. The cut-off point of the assay was determined by calculating the mean MFI of 10 blank samples for human IgG and albumin and 10 negative samples for HSV-1, HSV-2 and VZV plus 3 standard deviations. The high and minimal levels of detection were also determined. Assay reproducibility was determined by measuring intra and inter assay variation.

Chapter 3

Results

3. Results

3.1 Determining the Sequence of gD of HSV-1 and VSV gE

Nine complete sequences for the full HSV-1 gD gene and 12 sequences for the VZV gE gene obtained from Gene Bank were analyzed and compared with each other. Alignment analyses used the BioEdit Sequence Alignment Editor Programme. Both HSV-1 gD and VZV gE sequences were found to be identical for all isolates. The full length of HSV-1 was 1185 bp spanning the region 138419 to 139603bp of HSV-1 genome, and the full length of VZV gE was 1872bp spanning the region 115808 to 117679 of the VZV genome. The sequence of HSV-1 gD (accession NC001348) and VZV gE (accession NC001348) gene was selected for this study. Both selected sequences are shown in Tables 3.1 and 3.2.

Table 3.1 The Full Open Reading Frame Sequence of HSV-1 gD (Accession NC001348)

	Sequence
1	atgggggggg ctgccgccag gttgggggcc gtgattttgt ttgtcgtcat agtgggcctc
61	catggggtcc gcggcaaata tgccttggcg gatgcctctc tcaagatggc cgaccccaat
121	cgctttcgcg gcaaagacct tccggtcctg gaccagctga ccgaccctcc gggggtccgg
181	cgcgtgtacc acatccaggc gggcctaccg gacccgttcc agccccccag cctcccgatc
241	acggtttact acgccgtgtt ggagcgcgcc tgccgcagcg tgctcctaaa cgcaccgtcg
301	gaggcccccc agattgtccg cggggcctcc gaagacgtcc ggaaacaacc ctacaacctg
361	accatcgctt ggtttcggat gggaggcaac tgtgctatcc ccatcacggt catggagtac
421	accgaatgct cctacaacaa gtctctgggg gcctgtccca tccgaacgca gccccgctgg
481	aactactatg acagcttcag cgccgtcagc gaggataacc tggggttcct gatgcacgcc
541	cccgcgtttg agaccgccgg cacgtacctg cggctcgtga agataaacga ctggacggag
481	attacacagt ttatcctgga gcaccgagcc aagggctcct gtaagtacgc cctcccgctg
541	cgcatccccc cgtcagcctg cctctccccc caggcctacc agcagggggt gacggtggac
601	agcatcggga tgctgccccg cttcatcccc gagaaccagc gcaccgtcgc cgtatacagc
661	ttgaagatcg ccgggtggca cgggcccaag gccccataca cgagcaccct gctgcccccg
841	gagctgtccg agacccccaa cgccacgcag ccagaactcg ccccggaaga ccccgaggat
901	tcggccctct tggaggaccc cgtggggacg gtggcgctgc aaatcccacc aaactggcac
961	atcccgtcga tccaggacgc cgcgacgcct taccatcccc cggccacccc gaacaacatg
1021	ggcctgatcg ccggcgcggt gggcggcagt ctcctggcag ccctggtcat ttgcggaatt
1081	gtgtactgga tgcaccgccg cactcggaaa gccccaaagc gcatacgcct cccccacatc
1141	cgggaagacg accagccgtc ctcgcaccag cccttgtttt actag

Table 3.2 The Full Open Reading Frame Sequence of VZV gE (accession NC001348)

No	Sequence
1	atggggacag ttaataaacc tgtggtgggg gtattgatgg ggttcggaat tatcacggga
61	acgttgcgta taacgaatcc ggtcagagca tccgtcttgc gatacgatga ttttcacatc
121	gatgaagaca aactggatac aaactccgta tatgagcctt actaccattc agatcatgcg
181	gagtcttcat gggtaaatcg gggagagtct tcgcgaaaag cgtacgatca taactcacct
241	tatatatggc cacgtaatga ttatgatgga tttttagaga acgcacacga acaccatggg
301	gtgtataatc agggccgtgg tatcgatagc ggggaacggt taatgcaacc cacacaaatg
361	tctgcacagg aggatcttgg ggacgatacg ggcatccacg ttatccctac gttaaacggc
421	gatgacagac ataaaattgt aaatgtggac caacgtcaat acggtgacgt gtttaaagga
481	gatcttaatc caaaacccca aggccaaaga ctcattgagg tgtcagtgga agaaaatcac
541	ccgtttactt tacgcgcacc gattcagcgg atttatggag tccggtacac cgagacttgg
481	agctttttgc cgtcattaac ctgtacggga gacgcagcgc ccgccatcca gcatatatgc
541	ttaaaacata caacatgctt tcaagacgtg gtggtggatg tggattgcgc ggaaaatact
601	aaagaggatc agttggccga aatcagttac cgttttcaag gtaagaagga agcggaccaa
661	ccgtggattg ttgtaaacac gagcacactg tttgatgaac tcgaattaga ccccccccgag
841	attgaaccgg gtgtcttgaa agtacttcgg acagaaaaac aatacttggg tgtgtacatt
901	tggaacatgc gcggctccga tggtacgtct acctacgcca cgtttttggt cacctggaaa
961	ggggatgaaa aaacaagaaa ccctacgccc gcagtaactc ctcaaccaag aggggctgag
1021	tttcatatgt ggaattacca ctcgcatgta ttttcagttg gtgatacgtt tagcttggca
1081	atgcatcttc agtataagat acatgaagcg ccatttgatt tgctgttaga gtggttgtat
1141	gtccccatcg atcctacatg tcaaccaatg cggttatatt ctacgtgttt gtatcatccc
1201	aacgcacccc aatgcctctc tcatatgaat tccggttgta catttacctc gccacattta
1261	gcccagcgtg ttgcaagcac agtgtatcaa aattgtgaac atgcagataa ctacaccgca
1321	tattgtctgg gaatatctca tatggagcct agctttggtc taatcttaca cgacgggggc
1381	accacgttaa agtttgtaga tacacccgag agtttgtcgg gattatacgt ttttgtggtg
1441	tattttaacg ggcatgttga agccgtagca tacactgttg tatccacagt agatcatttt
1501	gtaaacgcaa ttgaagagcg tggatttccg ccaacggccg gtcagccacc ggcgactact
1561	aaacccaagg aaattacccc cgtaaacccc ggaacgtcac cacttatacg atatgccgca
1621	tggaccggag ggcttgcagc agtagtactt ttatgtctcg taatattttt aatctgtacg
1681	gctaaacgaa tgagggttaa agcctatagg gtagacaagt ccccgtataa ccaaagcatg
1741	tattacgctg gccttccagt ggacgatttc gaggactcgg aatctacgga tacggaagaa
1801	gagtttggta acgcgattgg agggagtcac gggggttcga gttacacggt gtatatagat
1861	aagacccggt ga

3.2 Primer Design

Since there was no variation between the sequences obtained from Genbank for HSV-1 and VZV, the full published genome sequence for both viruses was used to design primers for amplification. The forward and reverse primers for each virus were designed by adding an extra sequence from the 5' end (underlined) to develop an overhang for the directional cloning target of the PCR product in the 3C/LIC vector. There were no virus encoding sequences present after the termination codon (TAG) at the 3' end. Blast alignment results showed 100% homology with all published isolates for both viruses, primers were then synthesised commercially (Invitrogen). Primers used for amplification of each gene are detailed in Table 3.3 and 3.4.

Table 3.3 Primers Used for Amplification of the Full Length of HSV-1 gD

Primer name (gene)	Primer sequence	Length	Product size (bp)
HSVgDF	5'-CAGGGACCCGGTATGGGGGGGGCT-3'	24 bases	1211 bp
HSVgDR	5'GGCACCAGAGCGTTCTAGTAAAACAAGGGCTGG3'	33 bases	

Table 3.4 Primers Used for Amplification of the Full Length of VZV-1 gE

Primer name (gene)	Primer sequence	Length	Product size (bp)
VZVgEF	5'-CAGGGACCCGGTATGGGGACAGTTAATAAA-3'	30 bases	1898 bp
VZVgER	5'-GGCACCAGAGCGTTTCACCGGGTCTTATCTAT-3'	32 bases	

3.3 Amplification and Thermo-cycling Profile

3.3.1 Amplification of HSV-1 gD

Amplification of HSV-1 gD was carried out using the GeneAmp^{R} PCR system 9700 thermal cycler. The first attempt was carried out using 5 µl of the positive controls added to a 45 µl master mix reaction (in a final volume of 50 µl), containing 5 µl of 10×buffer (50mM KCl, 1.5mM $MgCl_2$ and10mM Tris-HCl; pH 8.3), 1 µl (10mM) of dNTP, 0.5 (50mM) of $MgCl_2$, 0.25 µM of each oligonucleotide primer, 38 µl SDW and 0.25 U of AmpliTaq DNA polymerase. The cycling program used included a single initial denaturation at 94 ^{o}C for 12 min followed by 40 cycles of denaturation at 94 ^{o}C for 30 seconds, annealing at 50^{0}C for 30 seconds and extension at 72 ^{0}C for 30 seconds. Results showed inadequate amplification was observed when using AmpliTaq DNA polymerase (Figure 3.1). AmpliTaq Gold was therefore used together with some modification in cycling parameters and reagent concentration. The reaction mixture comprised: 5 µl of 10×buffer (50mM KCl, 1.5mM $MgCl_2$ and10mM Tris-HCl; pH 8.3), 1µl (10mM) of each dNTP, 1µM of each oligonucleotide primer, 36.5 µl SDW and 0.5 U of AmpliTaq gold

DNA polymerase. The cycling program used included a single initial denaturation at 94^0C for 6 min followed by 40 cycles of denaturation at 94^0C for 1 minute, annealing at 58^0C for 1 min and extension at 72^0C for 1 min. Results showed a clear band with the expected size (1210 bp). The PCR product was then purified using a modified Microcon centrifuge method and stored at -20^0C (Figure 3.2).

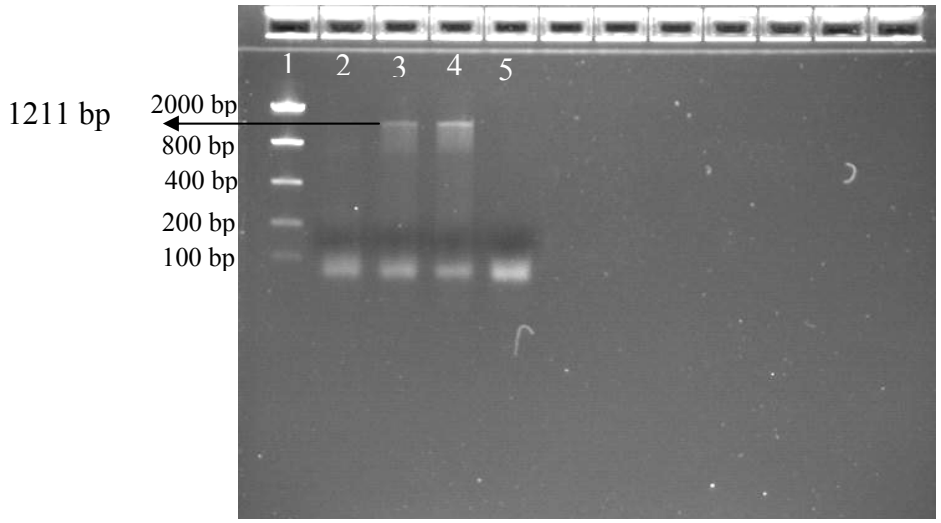

Figure 3.1 2% E-Gel® agarose gel electrophoresis illustrating the first attempts at amplifying HSV-1 gD. Lane 1 contains E-Gel® Low Range Quantitative DNA Ladder. Lane 2, 3 and 4 PCR products. Negative controls Lane 5.

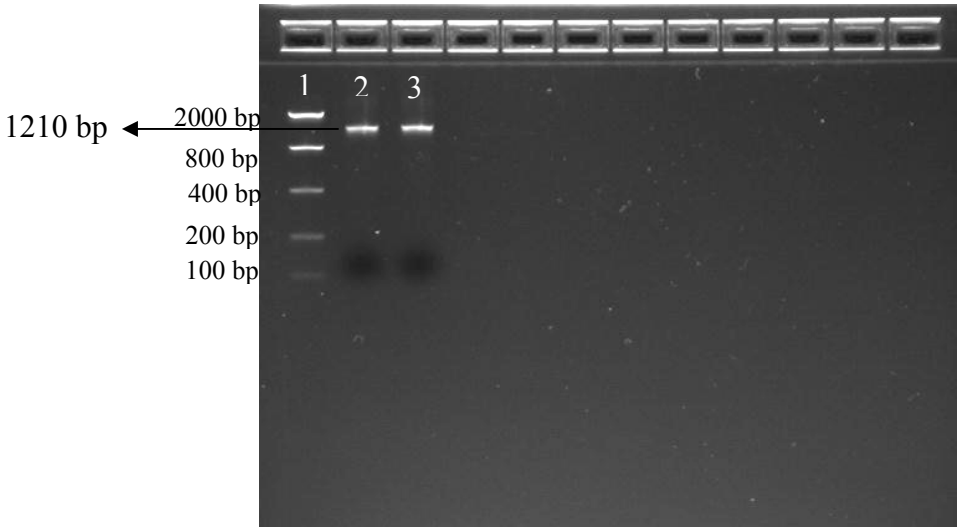

Figure 3.2 2% E-Gel® agarose gel electrophoresis shows a clear HSV-1 gD PCR product with the expected size (1210pb) in lane 2 and 3. Negative controls lane 4.

3.3.2 Amplification of VZV gE

Difficulty was encountered in amplifying the full open reading frame containing VZV gE. In initial experiments 5 µl of the positive control was added to 45 µl of master mix reaction (giving a final volume of 50 µl), containing 5 µl of 10×buffer (50mM KCl, 1.5mM $MgCl_2$ and10mM Tris-HCl; pH 8.3), 1 µl (10mM) of dNTP's, 1.5 µl (50mM) of $MgCl_2$, 0.75 µM of each oligonucleotide primer, 36.5 µl SDW and 0.25 U of AmpliTaq Gold DNA polymerase. The cycling program used included a single initial denaturation at 94^0C for 6 min followed by 40 cycles of denaturation at 94^0C for 1 min, annealing at 58^0C for 1 min and extension at 72^0C for 1 min. However, no product was detected. Modification of $MgCl_2$ 2.25 (50mM), concentration and cycling parameters was then attempted. The cycling parameters included, a single initial denaturation at 94^0C for 6 min followed by 40 cycles of denaturation at 94^0C for 1 min, annealing at 55^0C for 1.30 min and extension at 72^0C for 1 min. However still, no band could be detected. To confirm that the starting

material (positive control) used was not the problem, another PCR was performed using primers amplify a 275 bp target in the thymidine kinase gene of VZV (Table 3.5). The amplification conditions were as follows: 5 µl of 10×buffer (50mM KCl, 1.5mM $MgCl_2$ and10mM Tris-HCl; pH 8.3), 1 µl (10mM) of each dNTP, 1.5 (50mM) of $MgCl_2$, 0.75 µM of each oligonucleotides primer, 35.8 µl SDW and 0.2 U of AmpliTaq Gold DNA polymerase. The cycling program used included a single initial denaturation at 94^0C for 7 min followed by 45 cycles of 94^0C for 1 min, 72^0C for 1 min. The PCR product was then analyzed on 2% E-Gel. Results showed that a PCR product with the expected size was detected (Figure 3.3).

At this stage the experiment was repeated using the same reagent concentrations and cycling parameters used for amplifying HSV-1 gD with the slight modification that the annealing step was extended for 1.30 min rather than 1 min at 58^0C. Results showed that a PCR product with the expected size was detected (Figure 3.4). The PCR product was then purified using a modified Microcon centrifuge method and stored at -20^0C (Figure 3.5).

3.5 Primers Used to Amplify the 275 bp Target in the Thymidine Kinase gene of VZV gene.

Primer name (gene)	Primer sequence	Length	Product size (bp)
VZV 1	5'-AGTACCTGGGATTGAAGACACGTTATTCG-3'	29 bases	275bp
VZV 2	5'-AAGTGTTGTCCTGAACGGCATTAACAAGC-3'	29 bases	

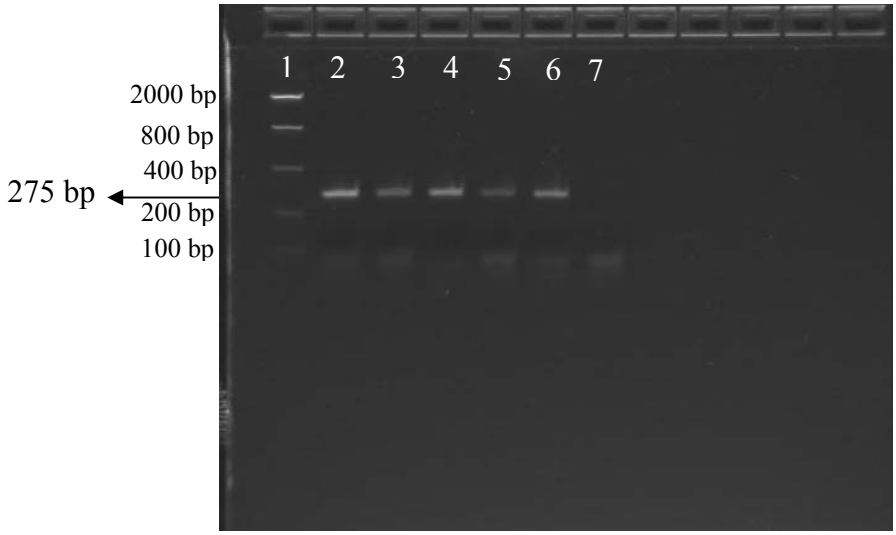

Figure 3.3 2% E-Gel® agarose gel electrophoresis illustrating the result of amplification of the 275bp product of the thymidine kinase gene of VZV. Lane 1 contains E-Gel® Low Range Quantitative DNA Ladder, Lanes 2, 3, 4, 5 and 6 show a PCR product with the expected size. Negative control lane 7.

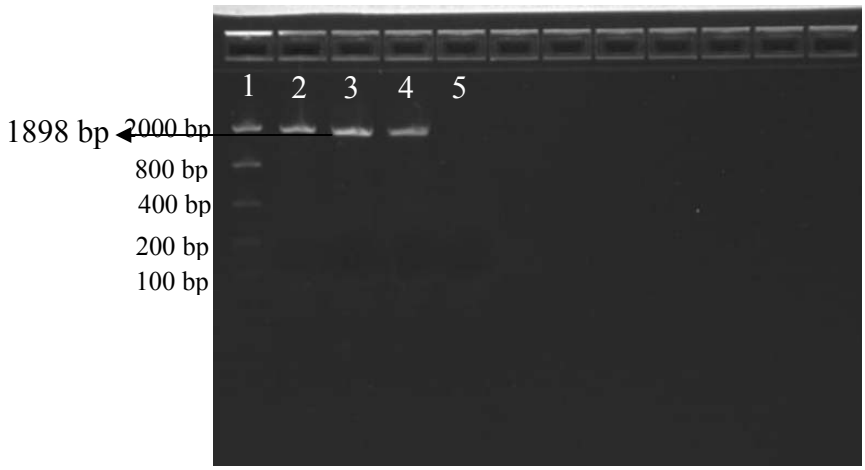

Figure 3.4 2% E-Gel® agarose gel electrophoresis illustrating the result of amplification of VZV gE using the same reagent concentrations as for HSV-1 gD amplification with slight modification of the annealing step. Lane 1 contains E-Gel® Low Range Quantitative DNA Ladder, Lane 2, 3 and 4 shows a PCR product with the expected size (1898bp). Negative control lane 5.

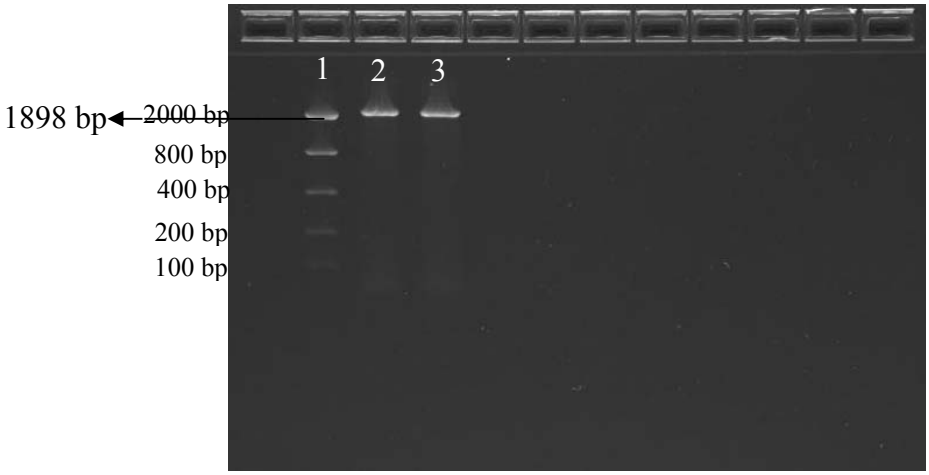

Figure 3.5 2% E-Gel® agarose gel electrophoresis illustrating the result of purification of VZV gE PCR product. Lane 1 contains E-Gel® Low Range Quantitative DNA Ladder, Lanes 2 and 3 show the PCR product after purification.

3.4 Gene Cloning

3.4.1 Cloning Strategy

The cloning procedure for HSV-1 gD and VZV gE was based on ligation-independent cloning (LIC); this include the preparation of the 3C/LIC insert and insertion of this into the pIEx/Bac-3 3C/LIC plasmid vector. Finally, recombinant plasmids were then transformed into competent cells (InsectDirect system) or used to generate the recombinant baculovirus (BacMagic system). The plasmid pIEx/Bac-3 3C/LIC full circular map is shown in Figure 3.6.

3.4.2 Construction of the Recombinant Plasmids

The strategy for the construction of the recombinant plasmids containing the complete open reading frame of HSV-1 gD or VZV gE is shown in Figure 3.7. The 3C/LIC insert of each gene was created by treating the PCR product with T4 DNA polymerase in the presence of dATB to generate vector compatible overhangs. The 3C/LIC inserts were annealed into the pIEx/Bac-3 3C/LIC and the resultant plasmids was transformed into NovaBlue GigaSingle competent cells. The annealing and the efficiency of transformation were tested by including a test plasmid as a positive control. LB agar medium was used for plating of competent cells and colonies were observed after overnight incubation. Colonies containing the recombinant plasmids was detected by PCR using vector specific primers (Table 3.6) and a combination of vector sense primer with antisense primer of each virus gene to confirm that each colony contained the correct insert and reading frame. PCR screening results using vector specific or the combination primer showed that a plasmid containing HSV-1 gD or VZV gE with the right size was generated (Figure 3.8 and 3.9 a and b).

Table 3.6 Vector Specific Primers Used for Screening of Recombinant Colonies

Primer name (gene)	Primer sequence	Length	Product size (bp)
IE1 Promoter Primer	5′- TGGATATTGTTTCAGTTGCAAG-3′	22 bases	435 bp + Interest gene
IE1 Terminator Primer	5′- CAACAACGGCCCCTCGATA -3′	19 bases	

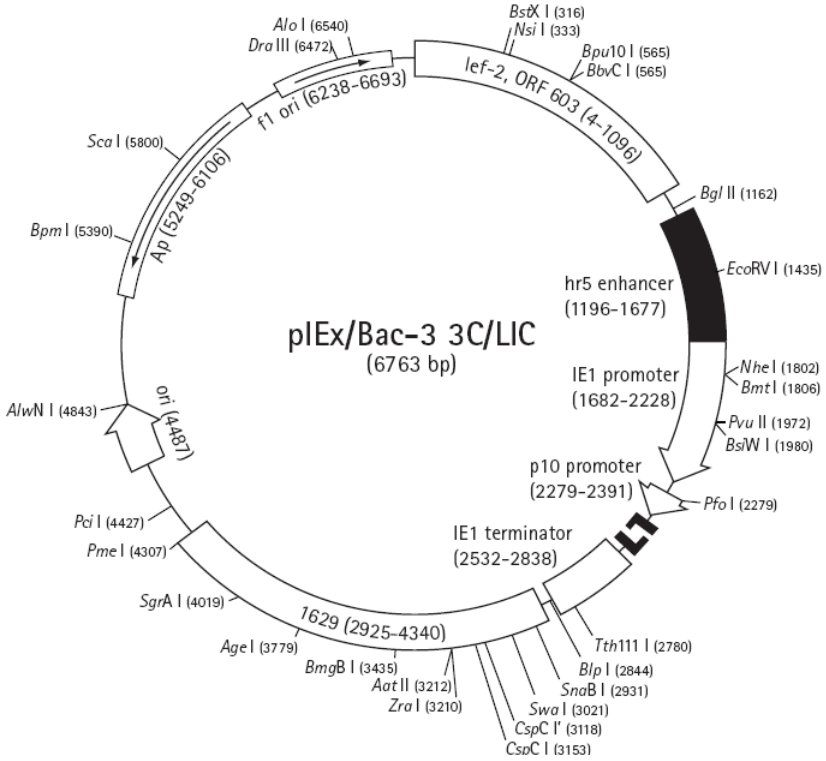

Figure 3.6 Vector pIE/Bac-3 3C/LIC circular map (6802bp) containing the baculovirus hr5 enhancer, IE1 immediate early promoter to drive transcription in the transient transfection mode and the p10 promoters to allow protein expression in the early and late stage of baculovirus infection. Taken from: http:// www.emdbiosciences.com/html/NVG/pIExBacTable.html (accessed26 July2010).

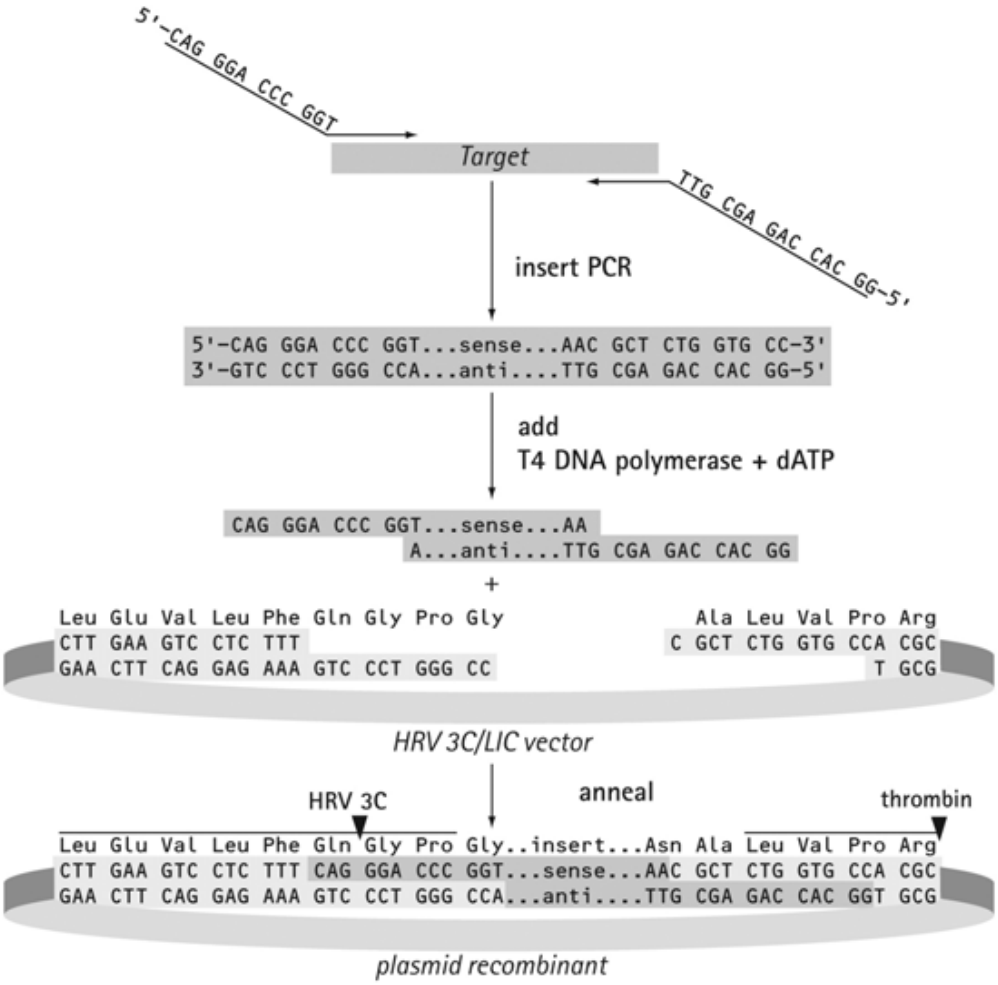

Figure 3.7 Details of the 3C/LIC cloning strategy, PCR product containing the 3LIC extension was treated with LIC-qualified T4 DNA polymerase in the presence of dATB, and then annealed to the pIEx/Bac-3 3C/LIC plasmid vector. Taken from: http:/ / www.emdbiosciences.com/html/NVG/pIExBacTable.html (accessed 26 July 2010)

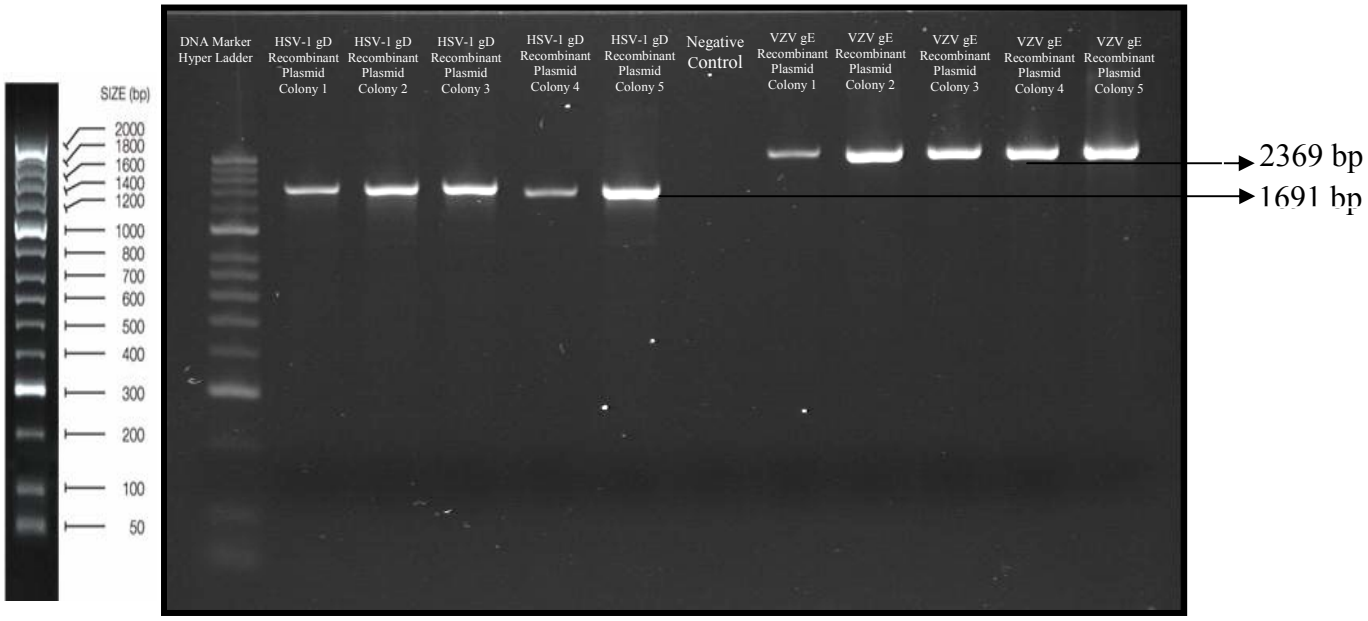

Figure 3.8 PCR colony screening results of the recombinant plasmids containing the full open reading frame of HSV-1 gD or VZV gE using vector specific primers. Lane 1 DNA marker (Hyper Ladder II), Lanes 2-5: HSV-1gD colony screening result (5 colonies), Lane 7 negative control, Lanes 8-12: VZV gE colony screening result (5 colonies).

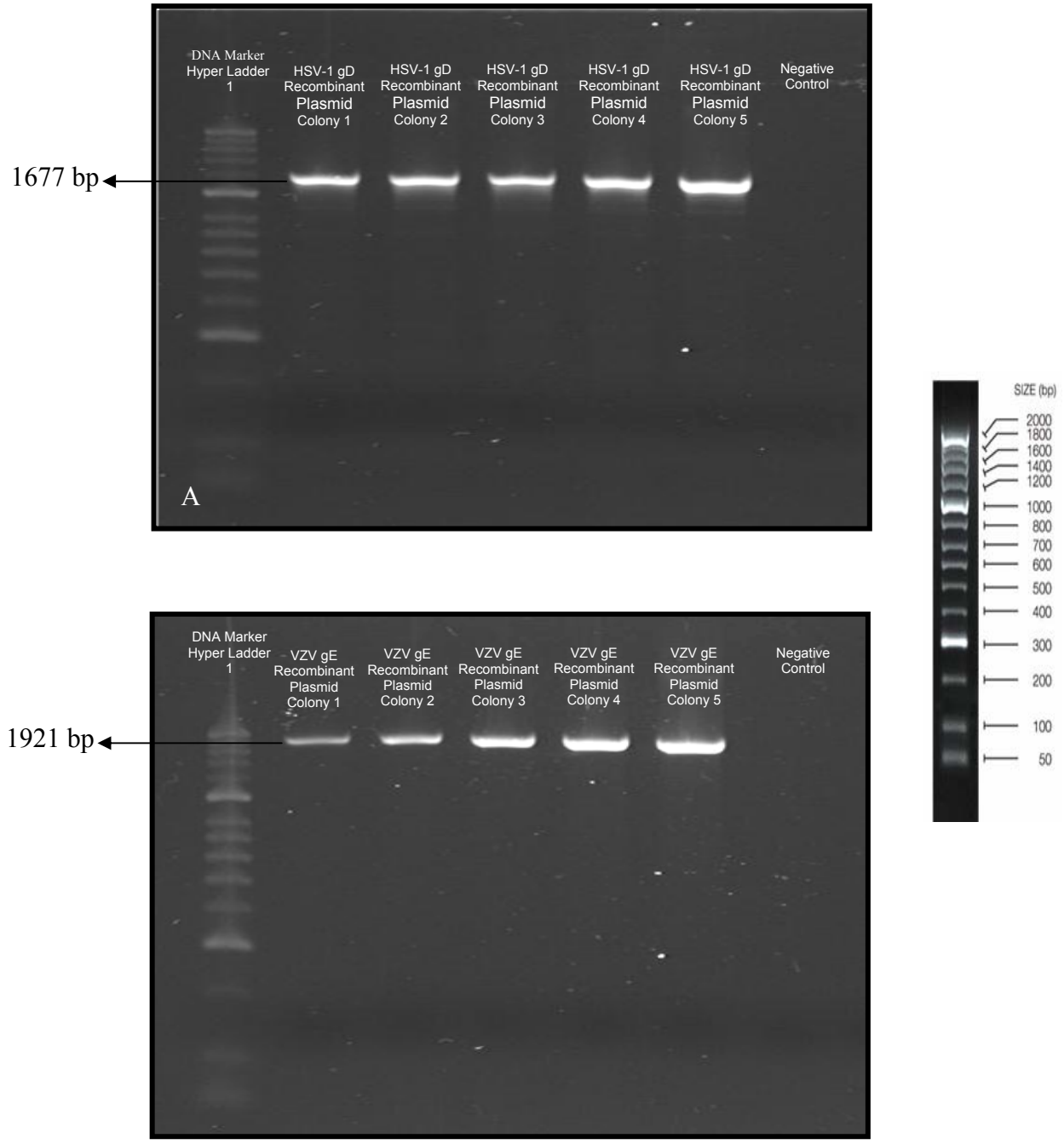

Figure 3.9 Screening of the recombinant plasmid containing the full open reading frame of HSV-1 gD (A) and VZV gE (B) using sense primer of vector and antisense primer of each virus gene.

3.4.3 Plasmid DNA Purity and Concentration

Plasmid concentration was determined using UV spectrophotometry. The plasmid DNA yield with A260/A280 absorbance > 1.7 was found to be 25µg/ml and 30µg/ml for HSV-1 gD and VZV gE respectively. The plasmid copy number for each virus insert was calculated using the formula:

Plasmid molecular weight = Number of base pairs x molecular mass of deoxynucleoside base pair

For HSV-1 gD plasmid the molecular weight of the pIEx/Bac-3 3C/LIC (6763bp) plus HSV-1 gD (1242) = (6.763+1.242) x 10^3 x 660 = 5.28 x 10^6 Dalton
Each 1 Mole of plasmid contains 5.28 x 10^6 g and with Avogadro's number = 6.023 x 10^{23} then each plasmid contains 6.023 x 10^{23} molecules
Using the UV spectrophotometer the concentration of HSV-1 gD plasmid was found to be 25µg/ml, therefore:
25µg of recombinant plasmid contains: 6.023 x 10^{23} x 20 / 5.28 x 10^6 x 10^6
= 22.81 x 10^{11} copy/ml (11.4 x 10^9 copy/5µL).

For VZV gE plasmid the molecular weight the pIEx/Bac-3 3C/LIC (6763bp) plus VZV gE (1934) = (6.763+1.934) x 10^3 x 660 = 5.74 x 10^6 Dalton
30 µg of recombinant plasmid contains: 6.023 x 10^{23} x 20 / 5.74 x 10^6 x 10^6
= 20.99 x 10^{11} copy/ml (10.5 x 10^9 copy/5µL).

3.5 Insect Cell Culture

Insect cells (Sf9) were grown in BacVector® Insect Cell medium (serum free) either as a monolayer or suspension culture with variable densities. Cells were used for transfection with recombinant plasmid or baculovirus, virus amplification and for protein expression. Stock cells (for long term storage) were prepared first from an original vial of Sf9 cells by preparing an exponentially growing suspension culture supplemented with 5% v/v foetal calf serum. Cell viability was determined by examining and counting cells at intervals using a Trypan blue exclusion method. Healthy cells were not stained with Trypan blue and appeared rounded with distinct cell boundaries when compared to unhealthy stained granular cells (Figure 3.10). Cells at low passage number were adjusted to cell density of $4\text{-}5x10^6$ cells/ml and frozen and stored in liquid nitrogen as described before (section 2.2.6.5). A number of experiments were carried out to optimize cell growth conditions and protein expression. The cell doubling time was found to be 72 h and 24 h for monolayer and suspension cultures respectively.

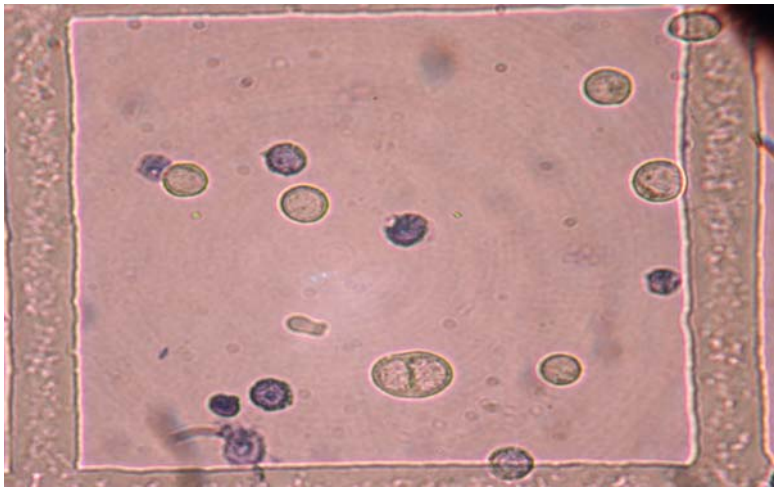

Figure 3.10 A 100 µl suspension of Sf9 cells was mixed with 100 µl of trypan blue solution, 20 µl of the mixture was immediately added to the haemocytometer chamber, the figure shows the appearance of live cells and some dead cells which appeared blue under the inverted microscope.

3.6 Protein Expression

3.6.1 InsectDirect™ System

In the InsectDirect™ expression system HSV-1 gD and VZV gE were expressed using 6 well plates and 10 ml suspension cultures. Cells were examined daily (24, 48 and 72 h p.i) no clear signs of infection were observed in comparison with uninfected cells. Transfected cells continued to grow normally and beyond confluency, as they were not subjected to contact inhibition. However after 72 h p.i signs resembling those of an unhealthy culture, such as floating cells, appearance of granules and cells lysis were observed in both transfected and non transfected cells. Protein expression was observed by 48 h (50-65% of cells) and maximum protein expression was found at 72 h p.i (95% of cells) as assessed by immunofluorescence assay.

3.6.2 BacMagic Expression System

Recombinant baculovirus for both genes of interest was prepared using a 25-cm^2 flask seeded with a 2 ml dilution of Sf9. A mixture of Insect GeneJuice, BacMagic DNA and recombinant plasmid was prepared and incubated at room temperature for 30 min and then added dropwise to the centre of the flask. The transfected cells were then incubated at 28^0C and examined on a daily basis for the appearances of signs of infection. After incubation (3-5 days), signs of infection were observed and classified as early, late and very late based on the time of appearance. Early signs of infection were observed after 48 h p.i, cells increased in diameter (30-50%) and nuclei in some cells appeared to fill the whole of the cell (Figure 3.11b). Late signs of infection appeared between 48 and 72 h p.i, cells stopped growing, and showed granular and vesicular appearance when compared to the uninfected cells (Figure 3.11c). Finally the very late signs appear after 72 h p.i when cells started to detach from the plate and the remaining attached cells started to lyse and show signs of clearing (Figure 3.11d).

Infected cells medium was then collected by low speed centrifugation and subjected to DNA purification. PCR analysis of the recombinant baculovirus using vector specific primer or combination primer confirmed the presence of each insert. Recombinant baculovirus was then amplified from the first virus stock (expected virus titre 1 $x10^7$

pfu/ml) using 50 ml suspension and the tissue culture infectious dose 50% ($TCID_{50}$) was calculated for each virus stock. Results showed that the 50% ($TCID_{50}$) was 10^9 $TCID_{50}$/ml for HSV-1 gD and 10^{10} $TDCI_{50}$/ml for VZV gE. Optimisation was then carried out using 10 ml of suspension culture, in which different MOI and harvesting times were investigated. Based on the IF test result an MOI of 10 and harvesting time of 72-96 h were found to be the optimum conditions for protein expression, therefore these condition were used in the large scale expression using a 100ml suspension culture.

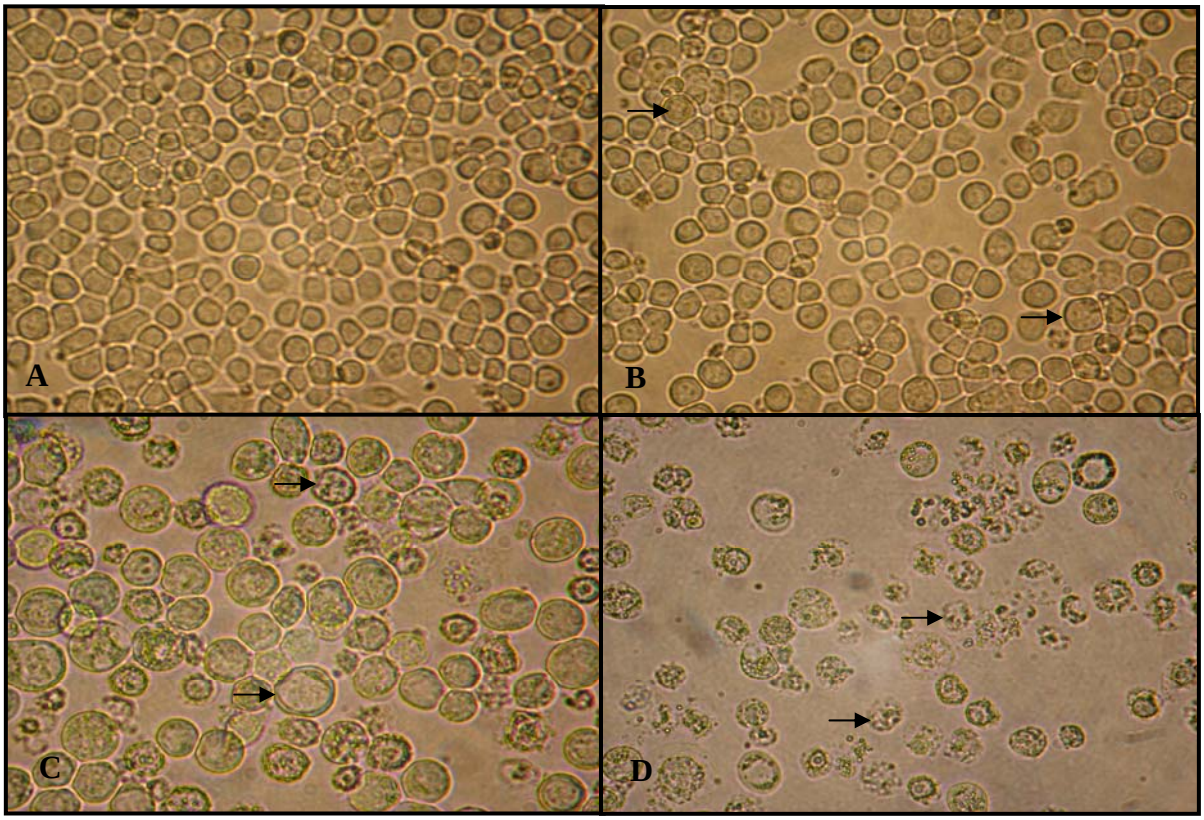

Figure 3.11 Appearance of the non-infected sf9 cells grown in 75-cm^2 flask at a density of 2-3x10^6 cells/ml in serum free medium (A) and viral infected cells with recombinant baculovirus at multiplicity of infection of 10. Transfected Sf9 cells with recombinant baculovirus began to stop dividing and showed increasing cell diameter 48 h p.i (B). At 72 h p.i cells started to vesiculate and showed a granular appearance (C), and nuclear enlargement (C), with cell lysis at 96 h p.i (D).

3.7 Protein Purification and Quantification

Since the large amount of the fully glycosylated form of HSV-1 gD and VZV gE was found to be integrated into the outer membrane of Sf9 cells, extraction of the proteins from the cell membrane was considered an essential step in protein purification. A number of methods were evaluated such as disruption of cell membrane with glass beads or solubilising membrane protein with detergents. Use of a non-ionic detergent ($C_{10}E_5$) was found to be the most suitable method. Protein quantification results using the BCA assay showed that yields of purified protein from the small scale suspension cultures of the InsectDirect^{TM} expression system ranged from 25 to 60µg/10ml, whereas 130 to 210 µg/10ml was obtained with the BacMagic expression system. For large scale protein expression; the purified protein yield of the BacMagic expression system ranged from 0.5 to 1.5 mg /100 ml. An example of the protein quantification method and the mechanism of action of detergent on integral and peripheral membrane protein are illustrated in Figure 3.12-14.

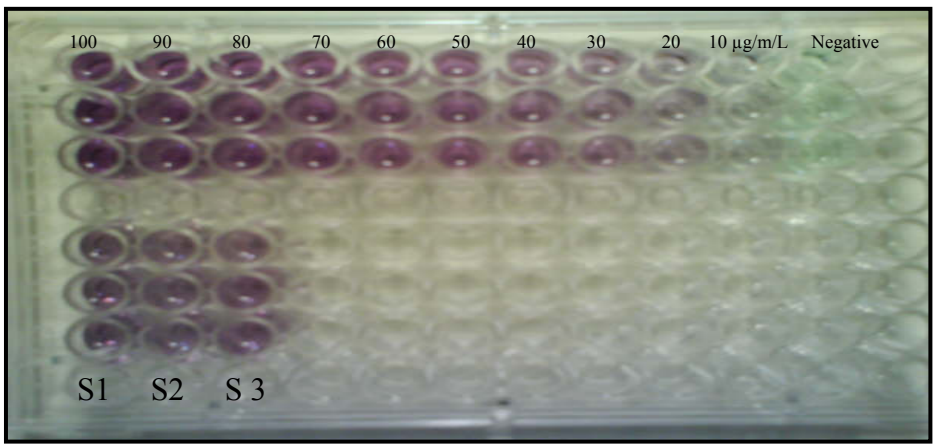

Figure 3.12 Absorbance of each well of the standard and 3 samples (triplicate) were read at 562 nm using a microtitre plate reader. BSA standards were used to create a standard curve using Microsoft Windows Excel (2003), which was then used to determine the unknown protein concentration in each sample.

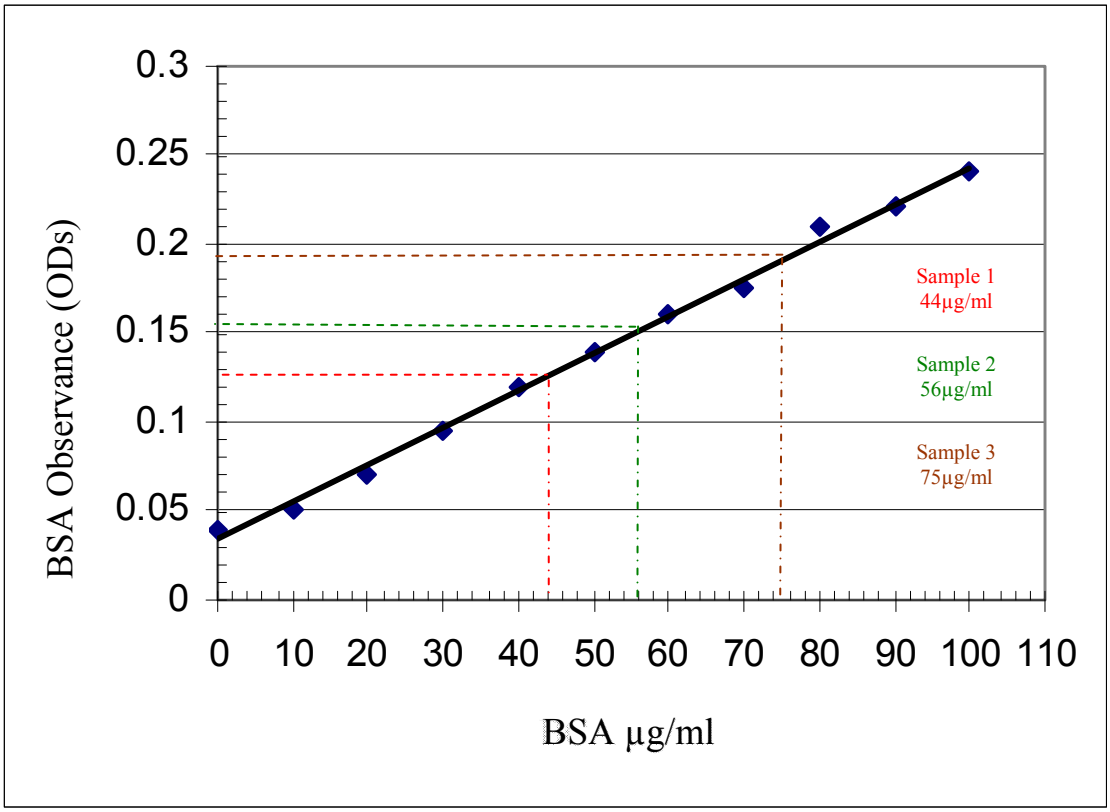

Figure 3.13 Purified proteins of HSV-1 gD and VZV gE concentrations were measured using BSA protein assay, with BSA as a standard. A standard curve was used to determine each protein concentration in each sample.

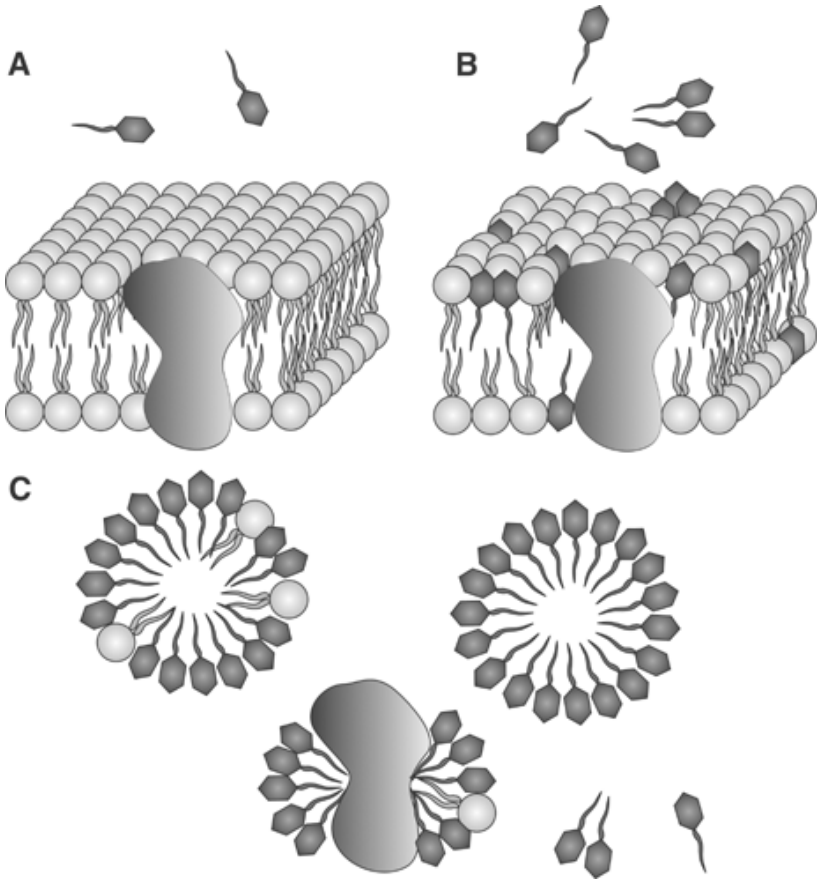

Figure 3.14 Solubilisation of membrane proteins. Cell membranes are bilayers of lipid molecules, detergents will insert into the bilayer (A and B) and at high concentrations will form mixed micelles with lipids and membrane proteins, rendering the proteins soluble (C). Taken from www.currentprotocols.com/protocol/ps0408 (accessed 22/6/2010).

3.8 Protein Characterisation

Protein characterisation is an important step in protein expression as it allows determination of the size, purity and immune reactivity of the expressed protein. A number of methods were employed including Indirect Immunofluorescence (IF) assay, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Immunoblot (Western blot and dot blot) and Enzyme-Linked Immunosorbent Assay (ELISA).

3.8.1 Indirect Immunofluorescence (IF) assay

To determine the efficiency of protein expression and the localisation of the expressed proteins, indirect immunofluorescence staining was carried out using anti HSV gD and VZV gE monoclonal antibodies. Both expressed glycoproteins were detected in the cytoplasm of acetone-fixed transfected cells (Figure 3.15 A and C) and at the cell membrane of the unfixed cells (Figures 3.15 B and D). To determine the specificity of the staining in the assay, transfected cells with recombinant plasmid containing HSV gD were reacted with VZV gE monoclonal antibody and vice versa. No staining was observed (Figure 3.15 E) in comparison with the negative control (Figure 3.15 F). Expressed proteins were observed at 48h p.i in both the InsectDirect and BacMagic expression systems. The percentage of positive cells ranged from 50% to 75%. Optimum expression of the protein was observed at 72h (95%).

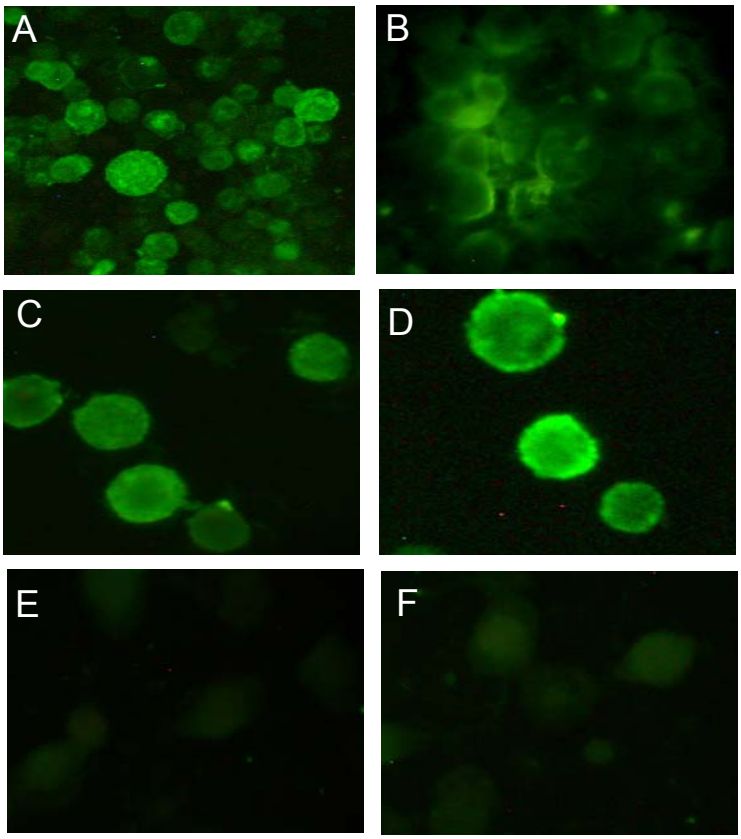

Figure 3.15 Expression of HSV-1 gD and VZV gE in insect cells. Cells were infected with recombinant plasmid or baculovirus. At 72h p.i., transfected cells were analysed for synthesis and localisation of the viral glycoproteins within the cell and at the cell surface. Expressed glycoproteins gD and gE were detected in the cytoplasm of the acetone-fixed transfected cells (A and C) and on cell membranes of the unfixed cells (B and D). Neither antibody reacted with the negative control (E).

3.8.2 SDS-PAGE and Immunoblot Analysis

The effects of various MOI and expression times of infected cells on the production of recombinant proteins were examined by SDS-PAGE analysis. In the InsectDirect expression system Sf9 cells were transfected with each recombinant plasmid and the synthesis of each protein was analysed using SDS-PAGE at different harvesting time points (48, 72 and 96h). Recombinant proteins were first solubilised using a 1% non-ionic detergent ($C_{10}E_5$), purified and then subjected to 4-12 % SDS-PAGE under denaturing

reducing conditions. Results showed that both recombinant proteins were expressed in Sf9 cells at 48h; however the peak of expression was obtained after 72 h p.i (Figure 3.16). Coomassie blue-stained SDS–PAGE showed that recombinant HSV-1gD and VZV gE appeared as single bands with a molecular weight of 64 kda and 98 kda respectively (Figure 3.17). Similar results were obtained using the BacMagic expression system, Sf9 cells were transfected with each recombinant baculovirus with different MOI (5, 7, and 10) and harvested at different time points (48, 72 and 96h) and then subjected to 4-12 % SDS-PAGE under denaturing reducing condition. Results showed that maximal protein expression levels were obtained at 72h p.i with an MOI of 10 (Figure 3.18). Western blot and dot blot analysis of the expressed proteins using monoclonal anti HSV-1 gD and VZV gE also showed that each protein was expressed in Sf9 cells (Figure 3.19 and 3.20). In comparison with the molecular weight markers, the full glycosylated form of HSV-1gD and VZV gE appeared as 64 kd and 98 kd proteins respectively. SDS-PAGE and immune blot analysis of recombinant infected cell medium did not detect any of the expressed proteins.

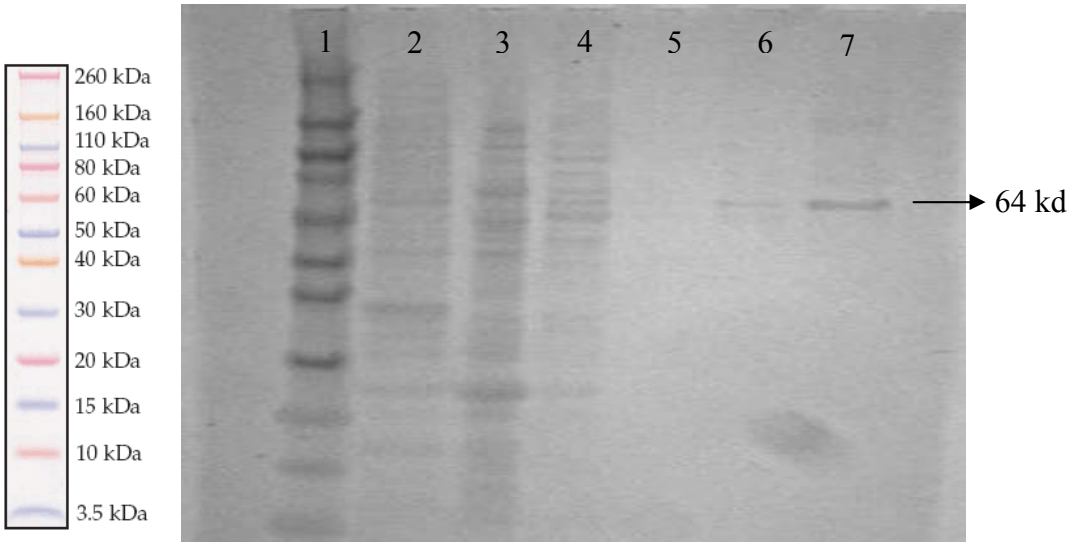

Figure 3.16 HSV-1 gD time course Coomassie blue staining of recombinant plasmid infected Sf9 cell extract following SDS-PAGE. Sf9 cells were infected with the HSV-1 gD recombinant plasmid and at 72 h p.i cells were harvested and the expressed proteins were solubilised, purified and then lysed directly in sample loading buffer. Gels were then run on 4-12 % SDS-PAGE under denaturing reducing conditions. Lane 1 molecular weight marker; Lane 2 transfected cells; Lane 3 solubilised protein; Lane 4 purification flow; Lane 5 first wash; Lane 6: 48 h eluted protein; Lane 7: 72 h eluted protein.

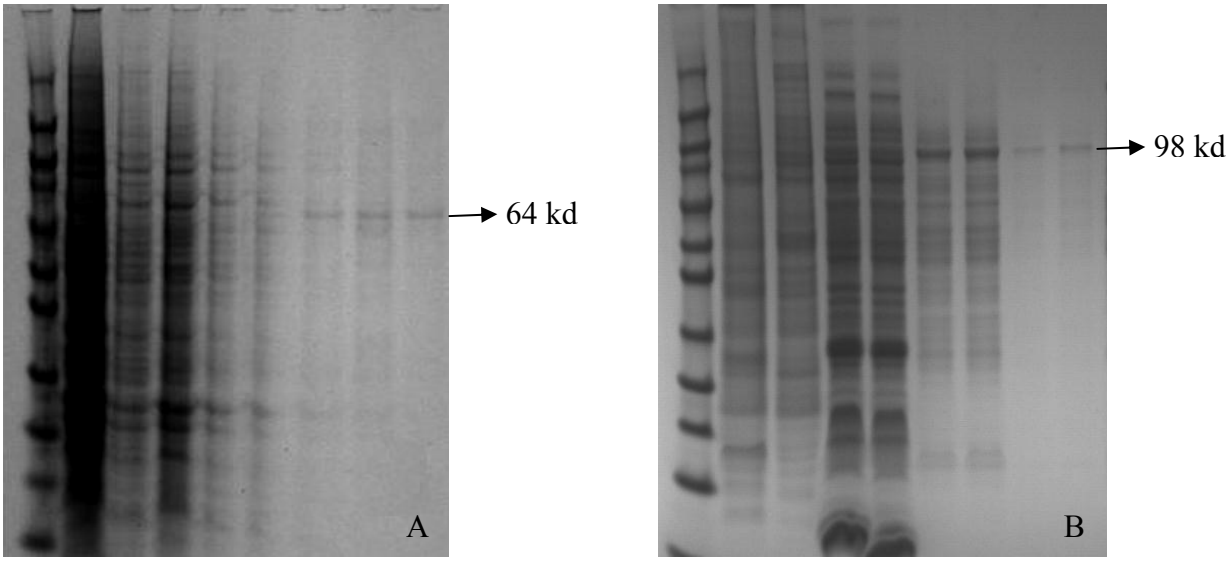

Figure 3.17 Coomassie blue staining of recombinant proteins following SDS-PAGE under denaturing reducing conditions. HSV-1gD (A) and VZV gE (B) recombinant proteins appeared as single bands with a molecular weight 64 kda and 98 kda respectively. Lane 1 molecular weight marker; Lane 2 transfected cells; Lane 3 solubilised protein; Lane 4 purification flow; Lane 5 first wash; Lane 6: 48 h eluted protein; Lane 7: 72 h eluted protein.

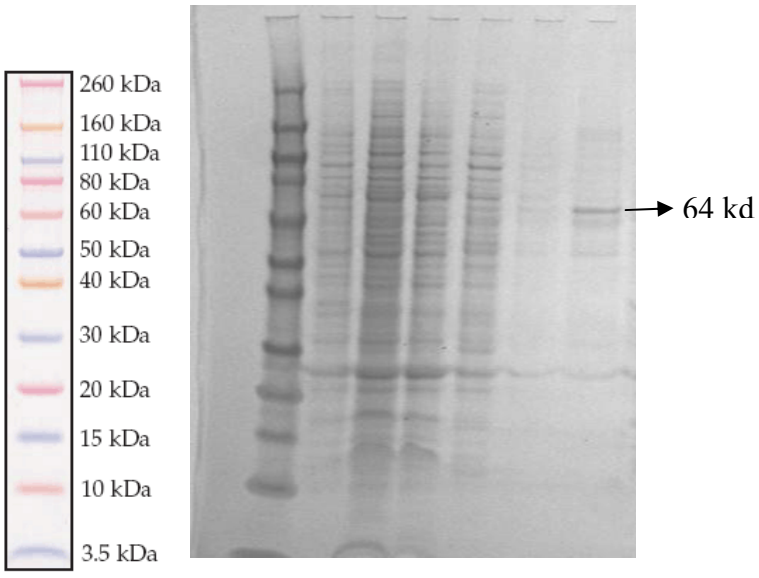

Figure 3.18 Coomassie blue staining of infected Sf9 cells transfected with HSV-1 gD recombinant baculovirus at an MOI of 10 and harvested after 72 h p.i. Lane 1 molecular weight marker; Lane 2 transfected cells; Lane 3 solubilised protein; Lane 4 purification flow ; Lane 5 first wash; Lane 6: 48 h eluted protein; Lane 7: 72 h eluted protein.

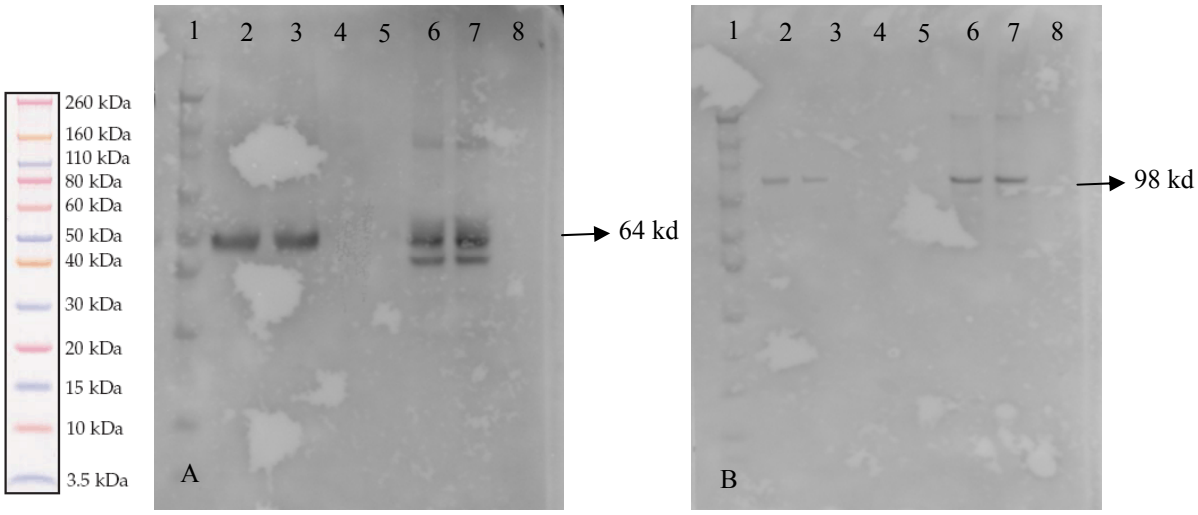

Figure 3.19 Western blot analyses of the expressed proteins using monoclonal anti HSV gD (A) and monoclonal anti VZV gE (B). Sf9 cells were transfected with the selected recombinant baculovirus and cells extract were subjected to 4-12% SDS-PAGE under denaturing reducing conditions. Proteins were then transferred to PVDF membrane and incubated with the selected antibody, washed, then incubated with peroxidase-labelled antibody. The membrane was then incubated with fast red substrate until the bands appeared. Lane 1 molecular weight marker; lane 2 second eluted recombinant protein; lane 3 first eluted recombinant protein; lane 4 purification wash; lane 5 purification flow; lane 6 solubilised protein; lane 7 transfected cells; Lane 8 transfected cell media.

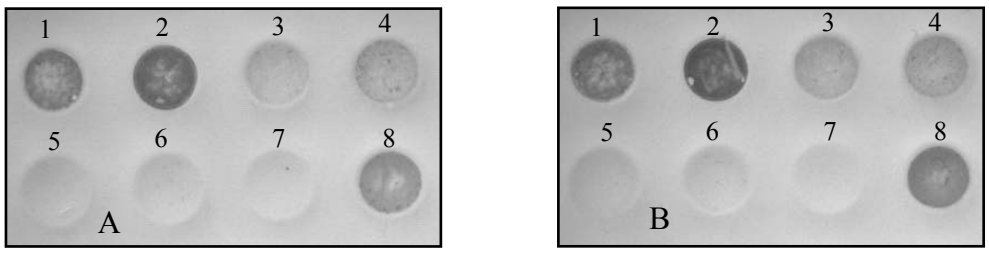

Figure 3.20 Dot blot analyses of HSV-1 gD (A) and VZV gE (B) recombinant protein. Samples were transferred to dot blot manifold and vacuum-absorbed onto a 0.45 μm nitrocellulose membrane, incubated with the selected antibody and washed, then incubated with peroxidase-labelled antibody. The membrane was then incubated with fast red substrate until the bands appeared. Lane 1 transfected cells; lane 2 solubilised protein; lane 3 purification flow; lane 4 first wash; lane 5 second wash; lane 6 negative control (non infected cells were subjected to the same purification process); lane 7 infected cell media; lane 8 eluted recombinant protein.

3.8.3 Enzyme-Linked Immunosorbent Assay (ELISA)

The immune reactivity of each expressed protein was evaluated using an ELISA assay with mouse monoclonal antibody against each protein. The ELISA plate was coated either with cells transfected with the recombinant baculovirus or diluted purified proteins (Figure 3.21).

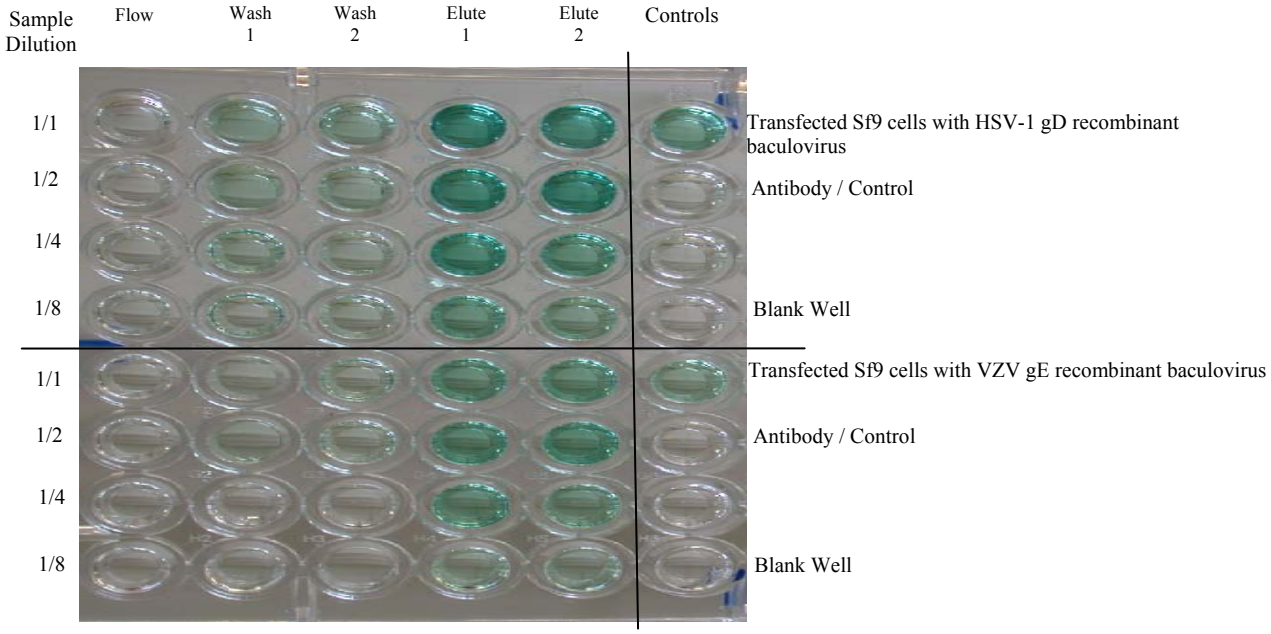

Figure 3.21 ELISA analyses of HSV-1 gD and VZV gE recombinant proteins. ELISA plate were coated with each sample dilution, incubated overnight at 4^0C, washed and incubated with the selected monoclonal antibody, washed again and then incubated with HRP antibody. Colour reaction was developed by ABTS substrate.

3.9 Evaluation of the Performance of Truncated Peptide (Peptide 55) for Detecting HSV-2 IgG Antibody in Human Serum

The performance of the Omega assay to detect and serologically differentiate HSV-2 antibody was evaluated using 254 serum samples (Panel 1). Samples had been tested by the clinical laboratory (Clinical Virology Manchester Royal Infirmary) using an HSV type common IgG assay DIAMEDIX Herpes 1+2 IgG (IVAX Diagnostics, Inc., Miami, USA). HSV positive samples were further tested by Biokit HSV-2 type specific gG-2 IgG (Biokit, S.A, Barcelona-Spain), and Virotech HSV-1 type specific gG-1 assay (Appendix 1 Table 1). Based on these assays results sera were grouped into one of four groups: group 1 sera were HSV-1 positive and HSV-2 negative (n= 143), group 2 sera were HSV-1 negative and HSV-2 positive (n= 39), group 3 were HSV-1 and HSV-2 positive (n= 37), and group 4 were negative for both HSV-1 and HSV-2 (n= 35).

All samples were tested with each of the three HSV-2 type specific assays (Omega, HerpeSelect and Bioelisa) according to the manufacturer's instructions. Absorbances were read at 450 nm for all assays. The details of these assays were as follows: HerpeSelect and Bioelisa required a 1:101 dilution of serum; high-positive, low-positive, and negative controls. Omega assay required a 1:10 dilution of serum HerpeSelect required a 1 h serum incubation, a 30 min conjugated incubation and a 10 min substrate incubation at room temperature (20 to 25^{0}C), whereas Omega and Bioelisa ELISA kits required a 1 h serum incubation, a 30-min conjugate incubation and 15 and 30 minutes respectively for substrate incubation at 37^{0}C. Samples with discordant results were retested in duplicate using the three ELISA assays and further tested using Western blot as a gold standard assay. Analysis of the results obtained from all 3 ELISA assays showed that 55 (22.65%) samples were positive for HSV-2 antibody and 176 (69.29%) samples were negative in all three assays (Appendix 1 Table 2). The remaining 23 (9.1%) samples that gave discordant results between the three kits were retested by western blot assay. Western blot analysis showed that 21 samples were positive for HSV-2 and only one sample was negative for HSV-2; the remaining sample showed only a very faint band typical of an HSV infection but it was not possible to detect a band corresponding to either gG1 or gG2, therefore this sample was classified as Western blot equivocal. Of the 21 positive results, 3 (14.3%) were detected using Bioelisa, 14 (66.7%) were detected using HerpeSelect and 16 (76.2%) were detected using

Omega. Western blot results showed that of the 22 discordant samples, 17 (77.3%) were in agreement with Omega, 15 (68%) were in agreement with HerpeSelect and 3 (13.6%) were in agreement with Bioelisa (Table 3.7). The concordance between the tests was: Omega / HerpeSelect 94.9%, Omega / Bioelisa 89.8% and HerpeSelect / Bioelisa 89.8%. Sensitivity, specificity, positive predictive value and the negative predictive value was calculated for each kit (as described in section 2.2.11.7) using the HerpeSelect assay as a reference standard (Table 3.8). The correlation (r) between the Omega assay and HerpeSelect and Bioelisa was 0.96 and 0.86 respectively. Assay results were also plotted to generate a scattergram and a regression line of the results obtained with 95% confidence intervals. Regression analysis (r^2) results are shown in Figures 3.22-24.

Table. 3.7 Discordant Test Results Obtained by the Three ELISA Tests Retested by Western Blot

Western Blot	Omega Assay			HerpeSelect Assay			Bioelisa Assay		
	Positive	Equivocal	Negative	Positive	Equivocal	Negative	Positive	Equivocal	Negative
Positive	16	0	4	14	2	5	3	0	18
Equivocal	1	0	0	0	0	0	0	0	0
Negative	0	0	1	0	0	1	0	1	0
Total	22			22			22		

Table 3.8 Omega and Bioelisa Assay Results in Comparison to HerpeSelect Assay

HerpeSelect Assay	Omega Assay			Bioelisa Assay		
	Positive	Negative	Equivocal	Positive	Negative	Equivocal
Positive	73	5	0	58	23	0
Negative	8	167	1	3	170	0
Equivocal	0	0	0	0	0	0
Total	254			254		

Sensitivity: 93.6 % and 71.6 % respectively, for Omega and Bioelisa.

Specificity: 96.6 % and 98.3 % respectively, for Omega and Bioelisa.

Positive Predictive Value: 90.1% and 95.1 % respectively, for Omega and Bioelisa.

Negative Predictive Value: 97.1% and 88.1 % respectively, for Omega and Bioelisa.

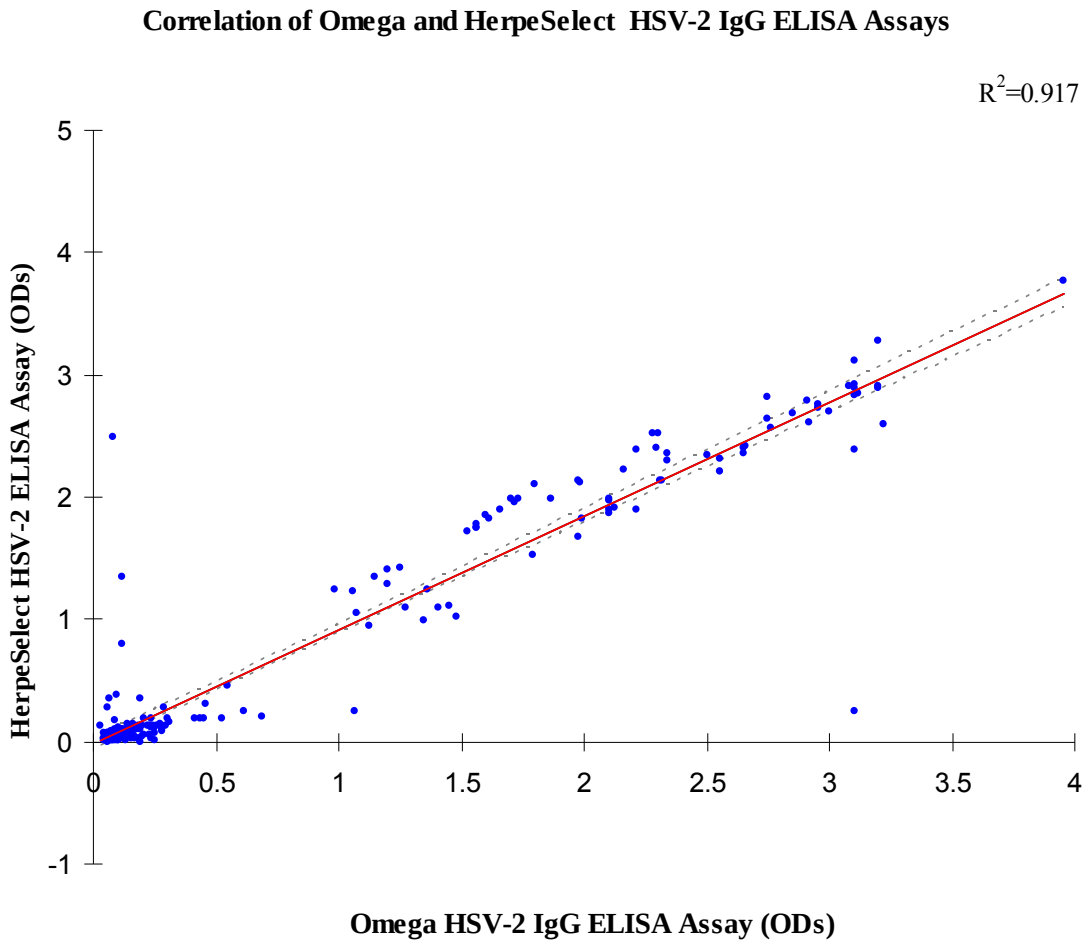

Figure 3.22 Regression analysis on 254 sera where HSV-2 antibody concentration was determined by the Omega assay (X axis) and the HerpeSelect assay (y axis).

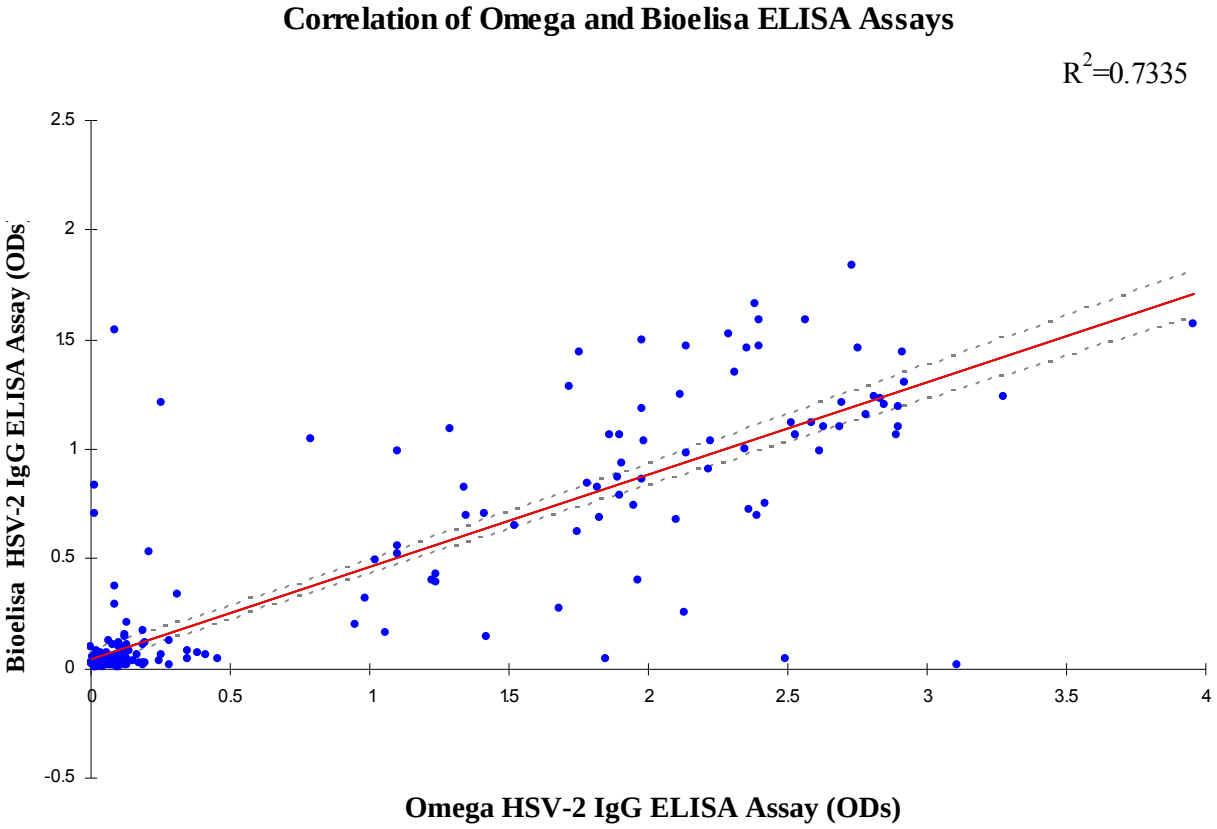

Figure 3.23 Regression analysis on 254 sera where HSV-2 antibody concentration was determined by the Omega assay (X axis) and the Bioelisa assay (y axis).

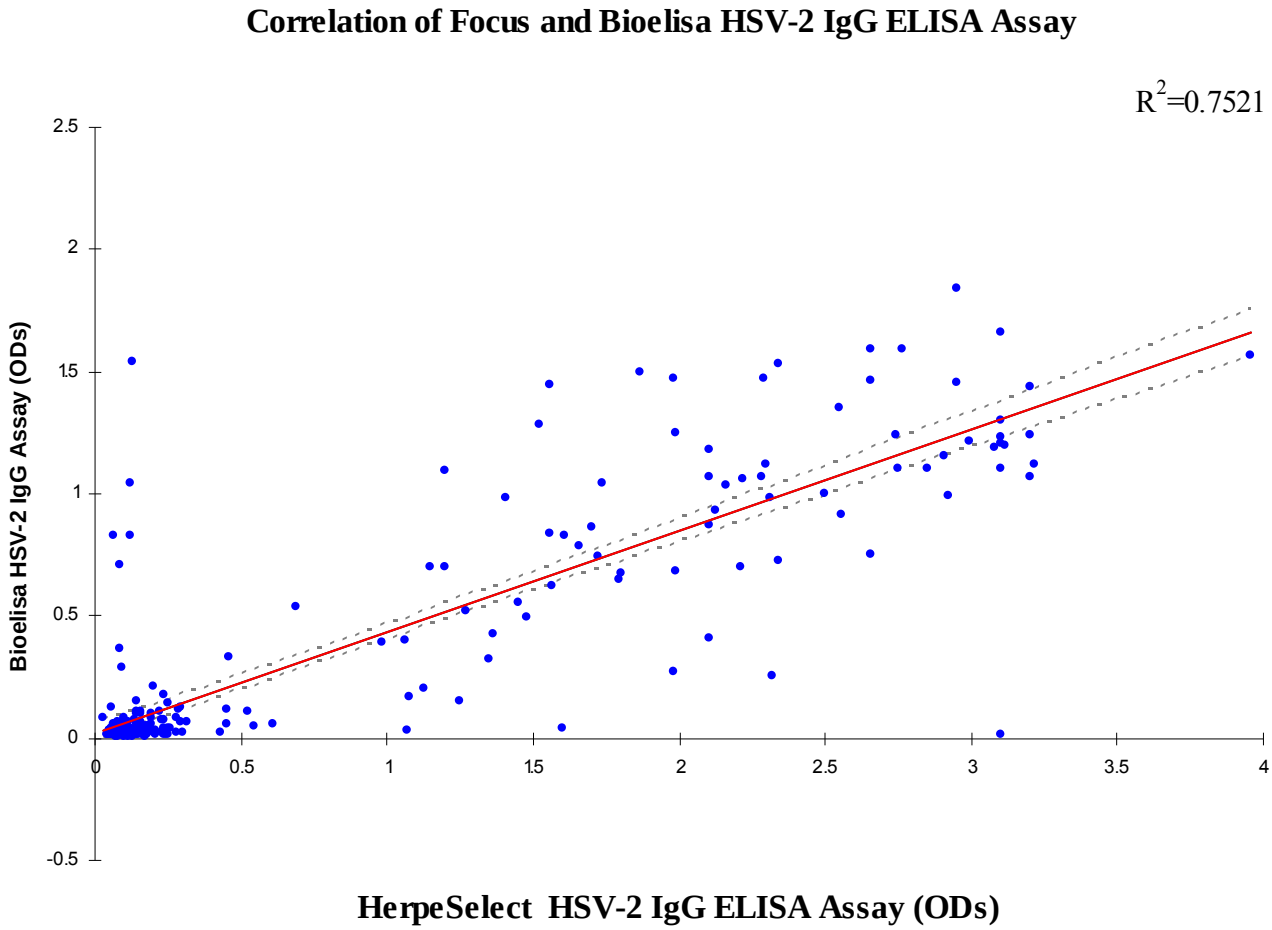

Figure 3.24 Regression analysis on 254 sera where HSV-2 antibody concentration was determined by the HerpeSelect assay (X axis) and the Bioelisa assay (y axis).

3.10 Development of the In-house ELISA Assay

To develop an in-house ELISA for detecting antibody to HSV and VZV using the expressed proteins, the optimal working dilutions of each antigen, antibody conjugate, and serum dilution were determined by checkerboard titration using different ELISA plates, coating buffers and blocking buffers.

3.10.1 Optimal Dilution of Antigen and Human Serum

To determine the optimal dilution of antigen that gave the highest OD reading a two-fold dilution of each protein was titrated against different dilutions of the clinical standard reference serum. A positive control serum with a dilution of 1/100 was tested with each protein dilution in each plate. Based on the results a sample dilution of 1/100 with a protein concentration of 50µg/ml, appeared to be the optimum working dilution for both HSV-1 gD and VZV gE. Therefore this sample dilution and protein concentration was selected for the standard assay (Table 3.9 and 3.10).

Table 3.9 Titration of HSV-1 gD Recombinant Protein Against the Reference Clinical Standard Serum

Standard Serum Dilution	OD Reading at 405nm						
	HSV-1 gD Protein Dilution						
	1/25	1/50	1/100	1/200	1/400	1/1600	1/3200
1/25	4*	4*	3.125	1.812	0.821	0.575	0.328
1/50	4*	4*	2.225	1.328	0.668	0.497	0.194
1/100	4*	**3.951**	1.594	0.985	0.539	0.454	0.110
1/200	2.281	2.109	0.817	0.682	0.413	0.334	0.082
1/400	1.554	1.218	0.628	0.426	0.297	0.233	0.080
1/800	0.895	0.692	0.351	0.298	0.231	0.176	0.083
1/1600	0.487	0.386	0.243	0.179	0.120	0.098	0.080
1/3200	0.231	0.165	0.176	0.120	0.079	0.080	0.080
Neg Serum Control 1/100	0.121	0.121	0.115	0.112	0.101	0.095	0.099
Blank	0.065	0.065	0.057	0.055	0.058	0.052	0.051

* Represents the maximum OD reading of the plate reader

Table 3.10 Titration of VZV gE Recombinant Protein Against the Reference Clinical Standard Serum

Standard Serum Dilution	OD Reading at 405nm						
	VZV gE Protein Dilution						
	1/25	1/50	1/100	1/200	1/400	1/1600	1/3200
1/25	4*	4*	3.325	1.913	0.923	0.675	0.328
1/50	4*	4*	2.289	1.298	0.696	0.456	0.214
1/100	4*	**3.921**	1.574	0.955	0.598	0.398	0.150
1/200	2.186	2.199	0.837	0.674	0.448	0.311	0.082
1/400	1.754	1.278	0.528	0.399	0.259	0.258	0.083
1/800	0.899	0.599	0.381	0.220	0.199	0.196	0.079
1/1600	0.521	0.352	0.211	0.169	0.137	0.088	0.072
1/3200	0.301	0.265	0.155	0.110	0.089	0.080	0.070
Neg Serum Control 1/100	0.129	0.125	0.123	0.119	0.117	0.111	0.099
Blank	0.066	0.062	0.064	0.055	0.054	0.062	0.051

* Represents the maximum OD reading of the plate reader

3.10.2 Optimal Dilution of the Anti-Human Conjugate (HRP)

To determine the optimal dilution of the anti-human conjugate a serial dilution of the conjugate were titrated against a 1/100 dilution of the reference serum. A negative control serum was included in each plate. Results showed that whenever the conjugate concentration was increased an increase in OD was observed. Since there was a little difference between the OD values at 1/1500 and 1/2000 dilution the 1/2000 dilution was selected for the standard assay in order to minimise reagent use (Table 3.11 and 3.12).

Table 3.11 Optimisation of the Anti-Human Conjugate with HSV-1 gD

Conjugate Dilution	HSV-1 gD Protein OD Reading at 405nm using PBS as a coating buffer						
	Conjugate Dilution						
	1/500	1/1000	1/1500	1/2000	1/2500	1/3000	1/3500
Reference Serum 1/100	4*	4*	3.902	**3.550**	3.612	3.385	2.985
Neg Serum Control 1/100	0.124	0.119	0.120	0.122	0.119	0.085	0.075
Blank	0.059	0.073	0.058	0.067	0.060	0.065	0.068

* Represents the maximum OD reading of the plate reader

Table 3.12 Optimisation of Anti-Human Conjugate with VZV gE

Sample	VZV gE Protein OD Reading at 405nm using PBS as a coating buffer						
	Conjugate Dilution						
	1/500	1/1000	1/1500	1/2000	1/2500	1/3000	1/3500
Reference Serum 1/100	4*	4*	3.815	**3.621**	3.426	3.112	2.832
Neg Serum Control 1/100	0.115	0.101	0.099	0.087	0.089	0.092	0.085
Blank	0.069	0.063	0.075	0.075	0.067	0.071	0.059

* Represents the maximum OD reading of the plate reader

3.10.3 Effect of Coating Buffer type on the In-House ELISA

To determine the optimum coating buffer to be used for the assay, 2 coating buffers were evaluated; 0.1 M carbonate/bicarbonate (pH 9.6) and PBS (pH 7.3). Recombinant proteins were diluted in each coating buffer and then the standard ELISA method was carried out. Results showed a slight reduction in the OD reading when the carbonate buffer was used in comparison to the PBS (Table 3.13 and 3.14). Therefore the PBS was selected as a coating buffer for the standard assay.

Table 3.13 HSV-1 gD OD Reading Using Carbonate as a Coating Buffer

Sample	HSV-1 gD Protein OD Reading at 405nm						
	Conjugate Dilution						
	1/500	1/1000	1/1500	1/2000	1/2500	1/3000	1/3500
Reference Serum 1/100	4*	4*	3.615	3.210	3.126	2.912	2.512
Neg Serum Control 1/100	0.110	0.111	0.089	0.077	0.081	0.112	0.111
Blank	0.057	0.063	0.065	0.052	0.061	0.062	0.055

* Represents the maximum OD reading of the plate reader

Table 3.14 VZV gE OD Reading Using Carbonate as a Coating Buffer

Sample	VZV gE Protein OD Reading at 405nm						
	Conjugate Dilution						
	1/500	1/1000	1/1500	1/2000	1/2500	1/3000	1/3500
Reference Serum 1/100	4*	4*	3.621	3.210	3.022	2.989	2.732
Neg Serum Control 1/100	0.115	0.111	0.081	0.083	0.082	0.113	0.101
Blank	0.065	0.053	0.065	0.072	0.075	0.066	0.098

* Represents the maximum OD reading of the plate reader

3.10.4 Effect of Microtitre Plate type on the In-House ELISA

The type of ELISA plate is an important factor in developing an in-house ELISA. Different types of ELISA plate are found commercially, each made from different materials. Three ELISA plates were tested to determine which one could be used in the standard ELISA. Recombinant proteins were diluted in PBS (50µg/l) and each plate was coated overnight at 4ºC, and then tested with the standard method. Results showed that different OD readings were obtained from each plate. Although the Nunc plate "high binding" give a better OD reading with the positive control, a high OD reading was observed in the serum control well (no antigen added). The Costar "medium binding" plate was therefore selected for use as it gave a good signal to noise ratio to serum control well (Table 3.15 and 3.16).

Table 3.15 OD reading of HSV-1 gD from Different ELISA Plates

Sample	HSV-1 gD Protein OD Reading at 405nm						
	Conjugate Dilution						
	1/500	1/1000	1/1500	1/2000	1/2500	1/3000	1/3500
	Costar "medium binding" Polystyrene						
Reference Serum 1/100	4*	4*	4*	3.950	3.612	3.285	2.985
Neg Serum Control 1/100	0.111	0.119	0.110	0.109	0.102	0.085	0.075
Blank	0.059	0.073	0.058	0.067	0.060	0.065	0.068
OD Ratio	40.54	37.82	38.01	38.1	35.41	39.82	39.8
	Nunc "high binding" Polystyrene						
Reference Serum 1/100	4*	4*	4*	4*	3.812	3.585	2.995
Neg Serum Control 1/100	0.254	0.210	0.190	0.151	0.129	0.119	0.109
Blank	0.089	0.083	0.078	0.077	0.069	0.065	0.068
OD Ratio	17.72	21.43	23.68	29.47	29.55	30.12	27.48
	Falcon (Nylon)						
Reference Serum 1/100	3.924	3.752	3.210	2.814	2.542	2.210	1.985
Neg Serum Control 1/100	0.105	0.119	0.115	0.110	0.105	0.093	0.076
Blank	0.045	0.049	0.050	0.043	0.048	0.042	0.040
OD Ratio	37.37	31.53	27.9	28.42	24.21	23.76	26.12

* Represents the maximum OD reading of the plate reader

Table 3.16 OD Reading of VZV gE from Different ELISA Plates

Sample	VZV gE Protein OD Reading at 405nm						
	Conjugate Dilution						
	1/500	1/1000	1/1500	1/2000	1/2500	1/3000	1/3500
	Costar "medium binding" Polystyrene						
Reference Serum 1/100	4*	4*	4*	3.810	3.626	3.212	2.832
Neg Serum Control 1/100	0.115	0.101	0.099	0.087	0.089	0.092	0.085
Blank	0.069	0.063	0.075	0.075	0.067	0.071	0.059
OD Ratio	39.13	44.55	56.2	47.24	38.5	33.83	33.32
	Nunc "high binding" Polystyrene						
Reference Serum 1/100	4*	4*	4*	4*	3.902	3.611	2.864
Neg Serum Control 1/100	0.194	0.198	0.201	0.182	0.152	0.135	0.118
Blank	0.059	0.073	0.058	0.067	0.060	0.065	0.068
OD Ratio	23.21	22.73	24.29	23.13	25.67	26.75	24.27
	Falcon (Nylon)						
Reference Serum 1/100	3.851	3.721	3.125	3.091	2.984	2.612	2.210
Neg Serum Control 1/100	0.102	0.089	0.091	0.079	0.068	0.077	0.067
Blank	0.056	0.051	0.059	0.048	0.052	0.049	0.057
OD Ratio	37.75	40.89	39.56	39.13	43.88	33.92	32.99

* Represents the maximum OD reading of the plate reader

3.10.5 ELISA Assay Reproducibility

Assay reproducibility was determined by measuring the intra and inter assay variation of the mean percentage of coefficient of variation (CV %) for 10 samples tested in duplicate using the same plate on the same day or on two different days. Results showed that assays reproducibility (intraplate and interplate) CVs was found to vary by less than 10% (Table 3.17).

Table 3.17 ELISA Intra and Inter Assay Reproducibility Results

Sample Number*	Assays Reproducibility			
	Intra assay variation		Inter assay variation	
	HSV-1 gD ELISA % CV	VZV gE ELISA % CV	HSV-1 gD ELISA % CV	VZV gE ELISA % CV
1	3.21	5.23	3.32	5.14
2	1.23	2.63	1.79	3.23
3	3.12	2.11	2.15	3.26
4	2.63	6.81	3.81	5.28
5	1.98	2.25	2.23	3.45
6	2.91	6.82	3.91	3.11
7	5.21	6.29	3.58	5.12
8	1.21	6.11	7.21	6.21
9	3.51	2.81	5.25	4.01
10	6.28	4.56	2.87	6.02

*Samples were selected from serum panel 1

3.11 Evaluation of In-house ELISAs for the Determination of Serological Status of HSV and VZV in Human Sera

All serum panels (section 2.2.1.2) were tested using the developed in-house ELISA assay with the standard method. The cut-off point for each assay was calculated using 8 negative sera as described before (section 2.2.10.2.5). An optical density (O.D) value of less than 0.177 for HSV-1 gD or 0.215 for VZV gE was considered negative, a sample with an O.D equal to or greater than 0.177 for HSV-1 gD and 0.215 for VZV gE was considered positive. Samples were tested at a dilution of 1/100 and any samples with an OD value equal to the cut-off point values were re-tested in duplicate. Assay controls (positive and negative sample) and a blank well were included in each assay run. The highest and lowest assay detection levels were calculated based on the checkerboard titration result. The highest levels of detection were found to be 4800 ABU and 3200 ABU for HSV-1 gD and VZV gE respectively, the lowest level was 4.8 ABU and 3.2 ABU respectively.

3.11.1 HSV Serum Panels

3.11.1.1 Panel 1 Sera

The HSV-1 gD in-house ELISA was used to test serum panel 1, these samples were previously tested to determine the serological status of HSV using an HSV type common and a type specific assay, based on these assay results they were grouped into four groups (section 3.10). Additionally this panel was further used to evaluate the Omega HSV-2 assay. The discordant samples between all ELISA kits were retested by Western blot assay as the gold standard assay (section 3.10). Samples were tested by the in-house ELISA and results showed that 86.22% (219/254) of samples were positive for gD and 13.78% (35/254) were negative for gD (Appendix 1 Table 3). On the basis of these results and the previous results obtained from different ELISAs the in-house was found to have good correlation with the other assays (Table 3.18 and 3.19). Assay sensitivity, specificity, negative predictive value and positive predictive value were calculated using Western blot as a reference test. Results showed that the in-house ELISA has a 100% agreement with the Western blot results.

Table 3.18 Comparison of Panel 1 Test Results with HSV-1 gD In-House ELISA Results

HSV-1 gD ELISA	DIAMEDIX Herpes 1+2 IgG Assay			Biokit HSV-2 IgG Assay			Biokit HSV-1 IgG Assay			Virotech HSV-1 IgG Assay		
	Positive	Negative	Equivocal	Positive	Negative	Equivocal	Positive	Negative	Equivocal	Positive	Negative	Equivocal
Positive	103	15	0	60	47	0	122	21	0	110	3	0
Negative	0	32	0	1	0	0	0	29	0	3	31	0
Equivocal	0	0	0	0	0	0	0	0	0	0	0	0
Total	150			108			172			147		

Table 3.19 Comparison of Panel 1 Test Results for the Omega Assay Evaluation and the HSV-1 gD In-House ELISA

HSV-1 gD ELISA	Omega HSV-2 Assay			Biokit HSV-2 IgG Assay			Focus HSV-2 IgG Assay			Western Blot Assay		
	Positive	Negative	Equivocal	Positive	Negative	Equivocal	Positive	Negative	Equivocal	Positive	Negative	Equivocal
Positive	79	140	0	61	158	0	81	138	0	23	0	0
Negative	0	35	0	0	35	0	0	35	0	0	0	0
Equivocal	0	0	0	0	0	0	0	0	0	0	0	0
Total	254			254			254			23		

3.11.1.2 Panel 2 Sera

Samples were previously tested to determine the sub-type of HSV using complement fixation test (CFT), Immunofluorescence assay (IF), an in-house ELISA, Bioelisa HSV-1 and HSV-2 IgG ELISA (BioKit S.,A., Barcelona. Spain), Gull HSV Type 1 and Type 2 Specific IgG (Gull Laboratories, Salt Lake City, Utah, USA), CAPTIA Select HSV2-G test (Centocor, Malvern, USA), Biotest Herpes Simplex 1 and 2 IgG, Biotest Herpes Simplex IgG Index (Biotest Srl, Trezzano sul Naviglio, Italy), Clark HSV-1 and HSV-2 assays (Clark Laboratories, Jamestown. USA) and Chiron Riba HSV Type 1 and 2 Strip Immunoblot Assay (Chiron, Cergny Pontoise, France). Tests results are detailed in Appendix 2 Table 1. Serum samples were tested randomly by the in-house ELISA and they were all found to be positive for HSV-1 gD antibody. Comparison of panel 2 in-house ELISA results with the results obtained from the Chiron assay (gold standard assay) showed that test results agreed in 95.7% (89/93) samples. The remaining 4 sample were positive by the in-house ELISA and negative by the Chiron assay and other ELISA assays (Table 3.20).

Table 3.20 Comparison of Panel 2 Test Results with HSV-1 gD In-House ELISA Results

Sample No	CFT	FAT	HSV In-house ELISA	HSV Immunoblot assay	HSV type by Bioelisa test	HSV type by Gull test	HSV type by CAPTIA Select HSV-2	HSV-1 In-house gD ELISA	*Anticipated
9	<1:10	1	-	-	-	-	-	Positive	Primary HSV-1
39	<1:10	-	-	-	-	-	-	Positive	Primary HSV-1
82	<1:10	1	-	-	-	-	-	Positive	Primary HSV-2
92	1:10	1&2	-	-	-	-	-	Positive	Primary HSV-2

* Anticipated results based on available laboratory and clinical information provided

3.11.1.3 Panel 3 Sera

Samples were previously tested to determine the serological status of HSV using Chiron RIBA HSV Type 1 and 2 Strip Immunoblot Assay (Chiron, Cergny Pontoise, France). A selection of 90 samples from this panel was retested with Bioelisa, Gull, CAPTIA Select HSV-2-G, Biotest and Clark assays. Tests results are detailed in Appendix 2, Table 2. Samples of this panel were tested randomly by the in-house ELISA and results showed that all tested sera were positive for HSV-1gD antibody. Comparing panel 3 in-house ELISA results with the results obtained from the Chiron assay revealed 100% agreement.

3.11.1.4 Panel 4 Sera

Panel 4 consisted of 200 human sera from among 8,000 samples from pregnant woman attending an Antenatal Clinic. Samples were previously tested to determine the serological status to rubella, syphilis and other sexually transmitted diseases (STDs). To determine the serological status of HSV, samples had been tested using Bioelisa HSV-1 and HSV-2 type specific assay. Samples were tested randomly by the in-house ELISA and results showed that all samples were positive for HSV-1 gD antibody. Comparing panel 4 in-house ELISA results with the results obtained from the Bioelisa showed 100% agreement. The remaining 53 samples were positive by the in-house ELISA and negative by the HSV-1 and HSV-2 Bioelisa (Appendix 2, Table 3).

3.11.1.5 Panel 5 Sera

Panel 5 consisted of 60 human sera originally obtained from patients with proven HSV infection, attending the Genito-Urinary Medicine Clinic (GUM). Samples were previously tested to determine the serological status of HSV using Bioelisa HSV-1 and HSV-2 type specific assay (Appendix 2 Table 4). For 45 out of 60 samples data on virus isolation and subtype by PCR was available. Samples were tested randomly by the in-house ELISA and results showed that all samples were positive for HSV-1 gD antibody. Comparing panel 5 in-house ELISA results with the results obtained from the Bioelisa showed that test results agreed in 66.67% (40/60) and the remaining 20 samples were positive by the in-house ELISA and negative by the HSV-1 and HSV-2 Bioelisa.

3.11.2 VZV Serum Panels

To evaluate the VZV gE in-house ELISA assay, two serum panels were used. Panel 1 comprised; 90 samples out of the 254 originally selected for the evaluation of the Omega assay. These were tested for the presence of VZV IgG using Human VZV IgG assay (HUMAN Diagnostics, Germany), and the Liaison VZV IgG assay (Appendix1, Table 3). The other panel was used panel 6 (100 samples) these were also tested for the presence of VZV IgG antibody using the Liaison VZV IgG assay. Thirty seven samples out of the 100 sample were also tested for presence of VZV IgG with the AtheNA Multi-Lyte® MMRV IgG Plus assay (Zeus Scientific, Inc., New Jersey, USA). The two serum panels were tested in the in-house ELISA, panel 1 results showed that 92.9% (236/254) of samples were positive and 5.91% (15/254) were negative for VZV gE, whereas panel 6 showed that 93% (93/100) of samples were positive and 7% (7/100) were negative for VZV gE (Appendix 2 Table 1).

Comparing panel 1 in-house ELISA results with the results obtained from the Liaison VZV and Human VZV IgG assay revealed that the test results agreed in 98.8% of samples (251/254) for the Liaison and 100% (100/100) of Human test (Table 3.21). Three samples were positive by the in-house ELISA and equivocal by the Liaison assay (even after retesting the sample in duplicate) were positive by the Humane assay. Comparing panel 6 in-house ELISA assay results with the results obtained from the Liaison VZV IgG assay and AtheNA Multi-Lyte® MMRV IgG Plus assay revealed that test results agreed in 99% (99/100) for the Liaison and 100% (37/37) for the AtheNA Multi-Lyte® MMRV IgG Plus assays (Table 3.22). The equivocal sample with the Liaison assay was positive by the AtheNA Multi-Lyte® MMRV IgG Plus assay. Using the Liaison VZV IgG as a reference test the sensitivity, specificity, positive and negative predictive values for the in-house ELISA in each panel were 98.75%, 100%, 100%, 100% for panel 1 and 98.93 %, 100%, 100% and 100% respectively for panel 6 (Table 3.25). Assay results were also plotted to generate a scattergram and a regression line of the results obtained with 95% confidence intervals. Regression analysis (r^2) results are shown in Figure 3.25.

Table 3.21 Comparison between VZV gE In-House ELISA and LIAISON VZV IgG and HUMAN VZV IgG Assay for their Ability to Detect VZV Antibody in Panel 1 Sera.

VZV gE In-House ELISA	LIAISON VZV IgG Assay			HUMAN VZV IgG Assay		
	Positive	Negative	Equivocal	Positive	Negative	Equivocal
Positive	236	0	3	81	0	0
Negative	0	15	0	0	9	0
Equivocal	0	0	0	0	0	0
Total	254			90		

Table 3.22 Comparison between VZV gE In-House ELISA and LIAISON VZV IgG and AtheNA Multi-Lyte® MMRV IgG Plus Assay for Their Ability to Detect VZV Antibody in Panel 6 Sera.

VZV gE In-House ELISA	LIAISON VZV IgG Assay (100) Samples			AtheNA Multi-Lyte Assay (37) Samples		
	Positive	Negative	Equivocal	Positive	Negative	Equivocal
Positive	92	0	1	30	0	0
Negative	0	7	0	0	7	0
Equivocal	0	0	0	0	0	0

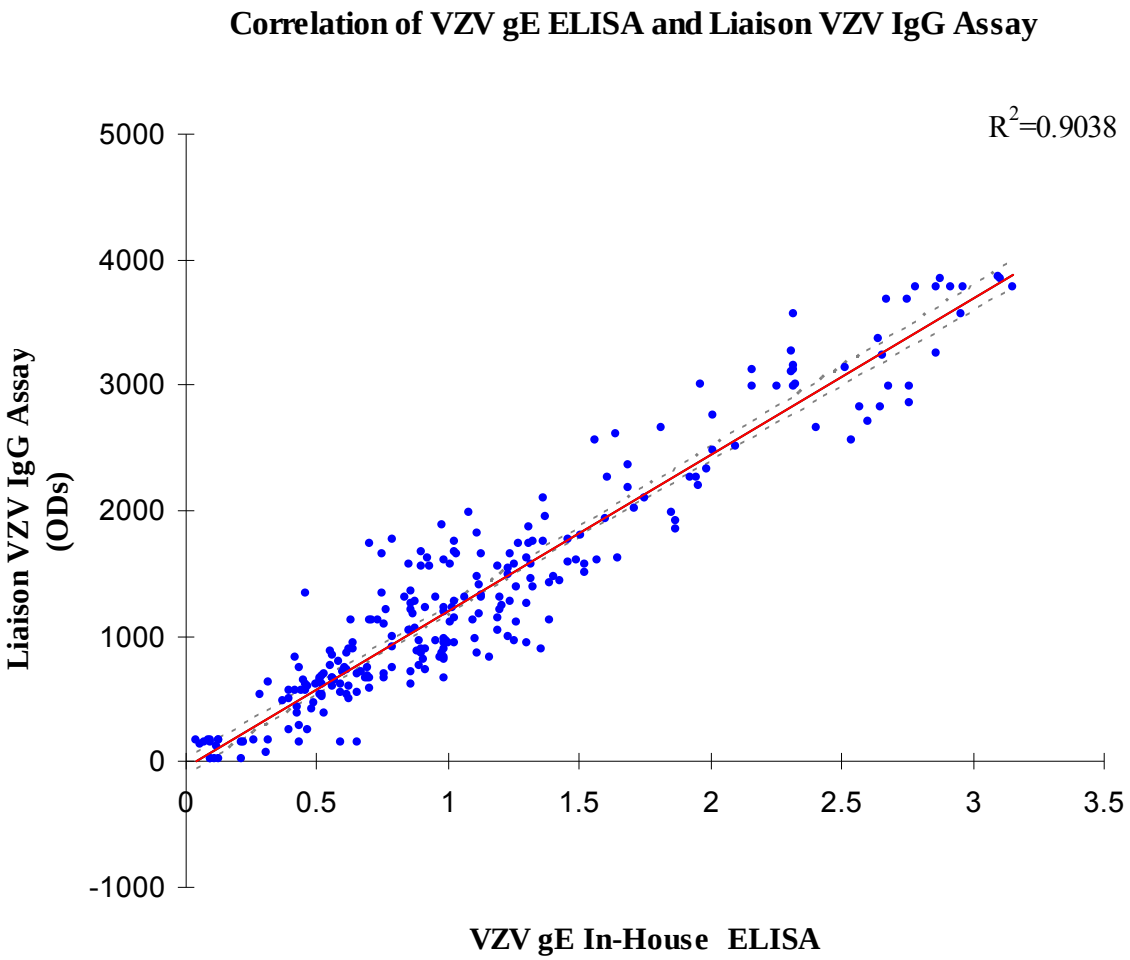

Figure 3.25 Regression analysis on 254 sera with VZV antibody concentration determined by VZV gE In-house ELISA assay (X axis) and the Liaison VZV IgG assay (y axis).

3.12 Development of a Triplex Fluorescence Microbead Immunoassay (FMIA) for the Detection of IgG Antibody to HSV-1, HSV-2 and VZV in Human Sera

A fluorescence microbead immunoassay to detect and measure HSV-1, HSV-2 and VZV antibody levels in human serum was developed. Using a standard modified two step carbidiimide reaction (Figure 3.26); each antigen (HSV-1 gD, VZV gE and peptide 55) was coupled directly to the different SeroMAP carboxylated microspheres. A monoplex assay for each antigen was developed individually and then the three assays were mixed in a triplex assay for simultaneous detection of these viruses. A standard curve was then generated using the same clinical standard serum sample used to develop the in-house ELISA for HSV-1 gD and VZV gE.

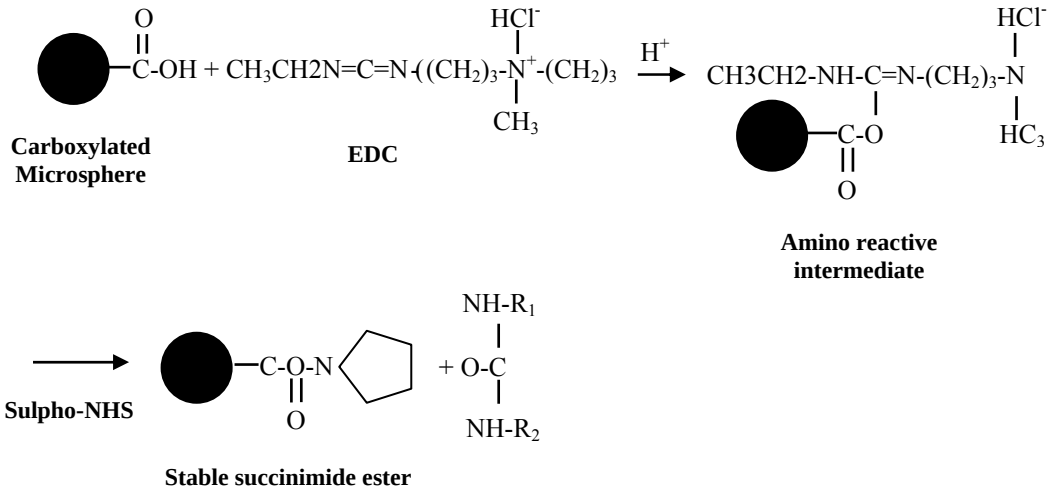

Figure 3.26 The figure shows the two step carbidiimide reactions EDC and Sulpho-NHS for conversion of the carboxyl groups on the microsphere surface into stable amine reaction groups.

3.12.1 Assays Optimisation

To determine the optimal assay condition for each antigen to be used a number of factors were investigated such as serum dilution, bead and antigen concentration, conjugate dilution and sample diluent buffers. Each assay was tested individually using three different diluted antigen (100µl/ml, 50µl/ml and 25µl/ml), sample dilution (1/50, 1/100 and 1/500) prepared in different sample diluent buffer (PBS+0.05% Tween, PBS + 0.05% Tween +1% NBBS and PBS + 0.05% Tween +2% NBBS), anti-human phycoerythrin-streptavidin conjugate (1/100, 1/200, 1/300) and bead concentration (2500, 5000). Beads were coupled as described in section 2.2.10.3 and then the standard curve for each antigen was generated using a two fold dilution of the standard clinical reference serum starting from 3200 ABU/mL for VZV and 4800 ABU/mL for HSV (1/10 to 1/10240). Results obtained from each assay showed that the optimal standard curve with maximum Fluorescent intensity were obtained from beads (5000/well) conjugated with 50µl/ml, 50µl/ml and 25µl/ml of HSV-1 gD, VZV gE and peptide 55 prepared in 1% NBBS, PBS pH 7.3, 0.05% Tween 20 of sample diluent buffer respectively, tested with 1/100 dilution of human serum dilution and detected with 1/200 dilution of conjugate (Tables 3.23-25 and Figures 3.27-29). Other serum and conjugate dilutions, antigens and bead concentrations failed to generate a good working standard curve as the observed concentration of some of the standards were not detected or below the expected concentration in some assays or the florescence intensity obtained was very low in others (examples are shown in Figures 3.30-32). Based on these results a bead concentration of (5000/well) with a 1/100 serum dilution and detected with a 1/200 dilution of conjugate, were selected for use in further assays. Using the optimal standard curve for each assay, the bead sets were mixed together in a single assay. Conjugated beads (5000/region/well) were used to generate a standard curve using a two fold dilution of the standard serum as mentioned above and the assay was performed as described in section 2.13.6.

Table 3.23 Standard Curve Values for Peptide 55 Monoplex assay using Microspheres (5000/well) Conjugated with 25µg/mL of the Peptide

Sample	Fluorescent Intensity	Fluorescent Background	Standard Deviation	CV %	Observed Concentration	Expected Concentration	Recovery Rate %
B	27	27	2.12	7.86			
S1	6919	6892	121	1.75	4676.63	4800	97
S2	5624	5597	469	8.38	2507.55	2400	104
S3	3587	3560	67	1.88	1155.64	1200	96
S4	2192	2165	39	1.78	616.37	600	103
S5	1137	1110	39	3.47	293.21	300	98
S6	637	610	13	2.09	156.15	150	104
S7	316	289	18	6.24	72.67	75	97
S8	177	150	6	3.78	37.81	37.5	101
S9	99	71	5	6.45	18.77	18.75	100

B: blank S: standard,

Florescence intensity: the median fluorescence intensity of the selected analyte

CV %: Percentage coefficient of variation

Recovery rate (Observed concentration /Expected concentration) x 100%

Table 3.24 Standard Curve Values for HSV-1 gD Monoplex Assay Using Microspheres (5000/well) Conjugated with 50µg/mL of Recombinant Protein

Sample	Fluorescence Intensity	Fluorescence Background	Standard Deviation	% CV	Observed Concentration	Expected Concentration	Recovery Rate %
B	26.3	26	1.06	4.04			
S1	17372	17346	335.88	1.94	4741.98	4800	99
S2	14466	14440	39.24	0.27	2424.5	2400	101
S3	10495	10466	399.87	3.82	1214.45	1200	101
S4	6459	6432	301.93	4.69	576.24	600	96
S5	4081	4055	102.18	2.52	316.89	300	105
S6	2179	2153	54.09	2.51	147.37	150	98
S7	1210	1184	64.7	5.46	73.11	75	98
S8	682	656	51.97	7.93	37.72	37	100
S9	376	349	0.35	0.1	18.52	18.5	101
S10	213	186	38.18	2.5	9.31	9.75	104
S11	110	84	1.06	1.26	4.48	4.88	97

B: blank S: standard

Fluorescence intensity: the median fluorescence intensity of the selected analyte

CV %: Percentage coefficient of variation

Recovery rate (Observed concentration /Expected concentration) x 100%

Table 3.25 The Standard Curve Values for VZV gE Monoplex Assay Using Microspheres (5000/well) Conjugated with 50µg/mL of Recombinant Protein

Sample	Fluorescence Intensity	Fluorescence Background	Standard Deviation	%CV	Observed Concentration	Expected Concentration	Recovery Rate %
B	34	33.5	2.19	6.54			
S1	24528	24495	73.54	0.3	432.96	3200	14
S2	24570	24537	9.19	0.04	470.88	1600	30
S3	24671	24637	176.78	0.72	755.14	800	95
S4	24574	24540	116.32	0.47	421.31	400	108
S5	23686	23652	259.15	1.1	215.45	200	107
S6	17761	17727	79.9	0.45	94.05	100	94
S7	11684	11650	109.25	0.94	53.99	50	108
S8	5197	5163	14.14	0.27	23.11	25	92
S9	3113	3078	221.68	7.2	13.45	12.5	108
S10	1605	1571	123.39	7.85	6.11	6.25	98

B: blank S: standard

Fluorescence intensity: the median fluorescence intensity of the selected analyte

CV %: Percentage coefficient of variation

Recovery rate (Observed concentration /Expected concentration) x 100%.

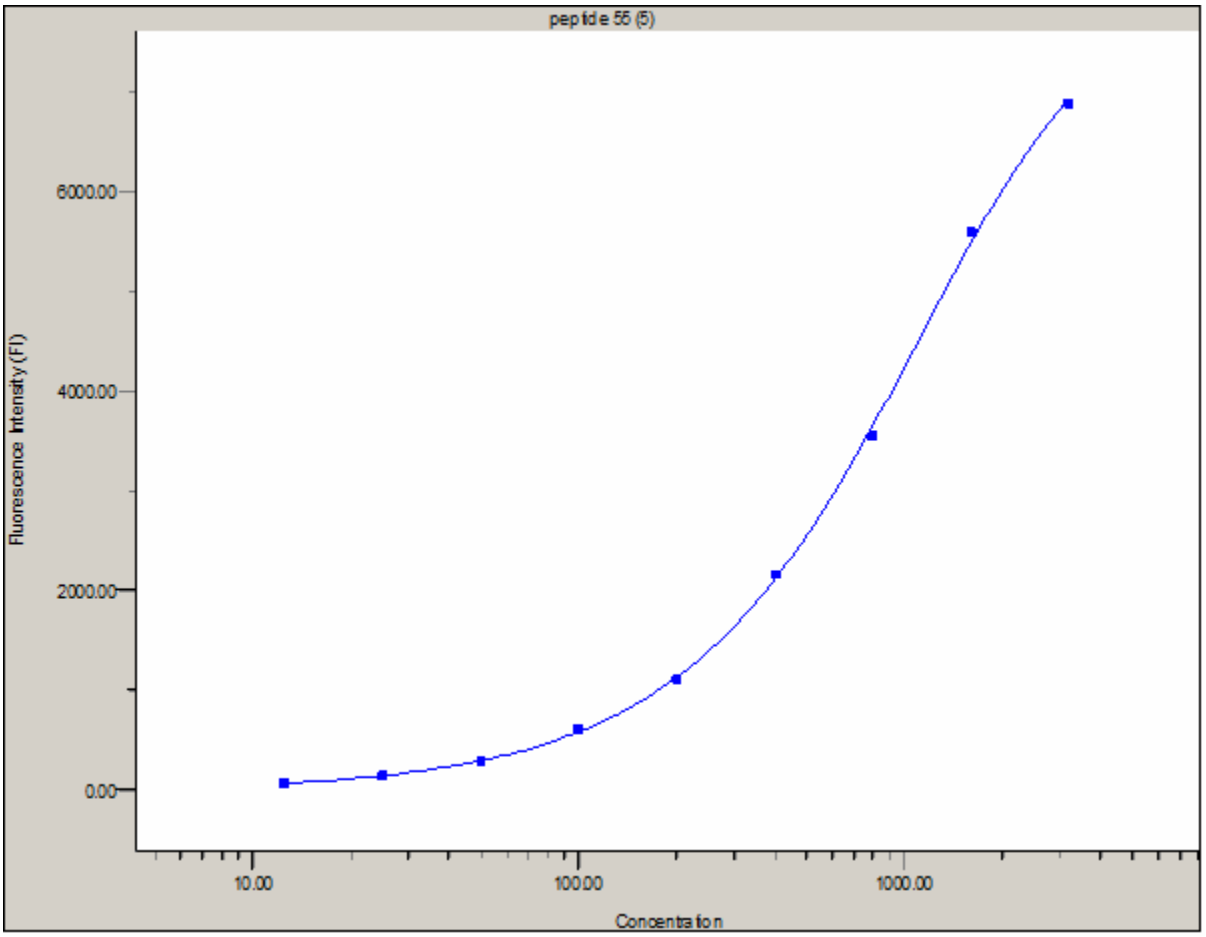

Figure 3.27 The standard curve generated from Peptide 55 monoplex assay using microsphere beads (5000/well) conjugated with 25µg/mL of the peptide prepared in (PBS + 0.05% Tween +1% NBBS) of serum diluent buffer and detected with 1/200 of the conjugate.

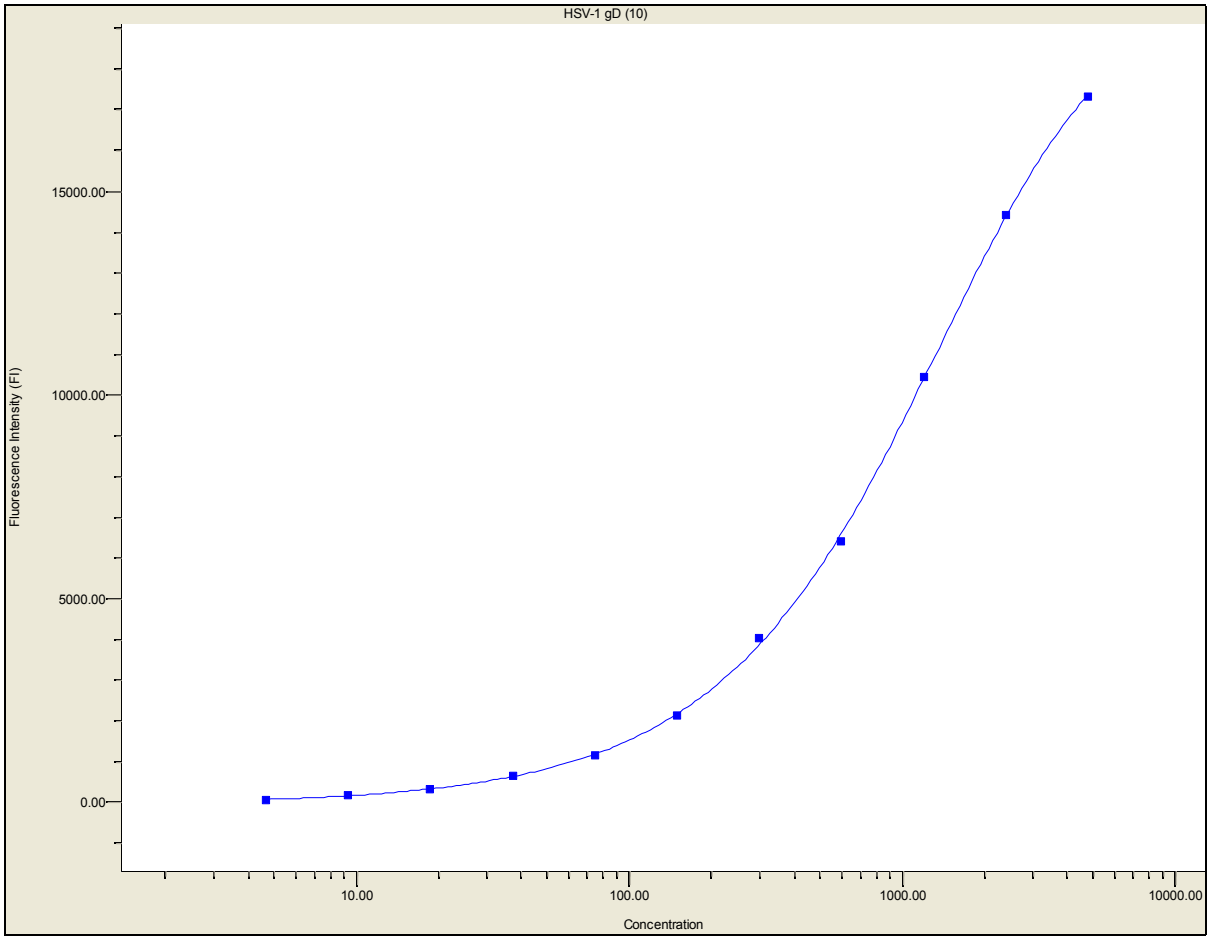

Figure 3.28 The standard curve generated from HSV-1 gD monoplex assay using microsphere beads (5000/well) conjugated with 50µg/mL of the gD antigen prepared in (PBS + 0.05% Tween +1% NBBS) of serum diluent buffer and detected with 1/200 of the conjugate.

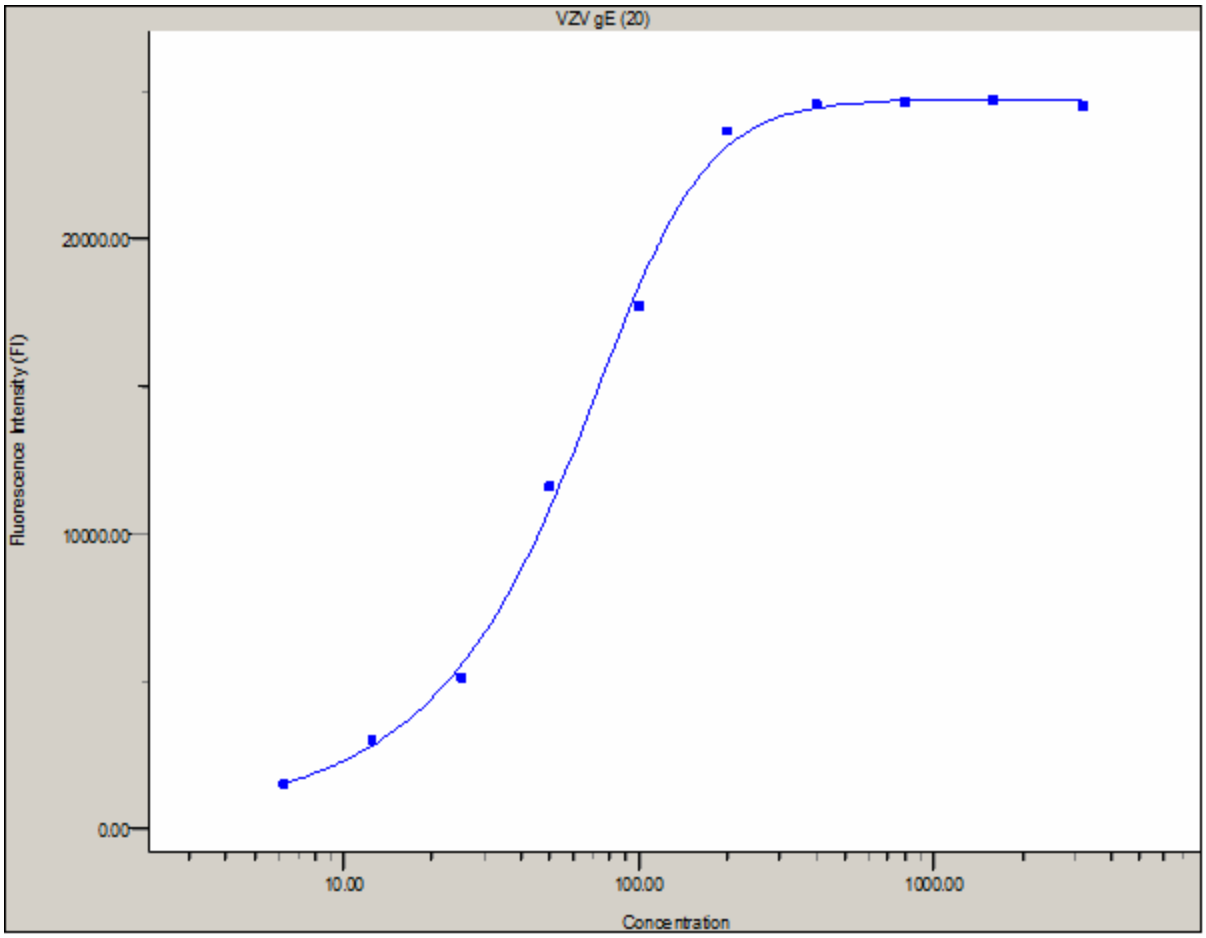

Figure 3.29 The standard curve generated from VZV gE monoplex assay using a microsphere beads (5000/well) conjugated with 50µg/mL of the gE antigen prepared in (PBS + 0.05% Tween +1% NBBS) serum diluent buffer and detected with 1/200 of the conjugate.

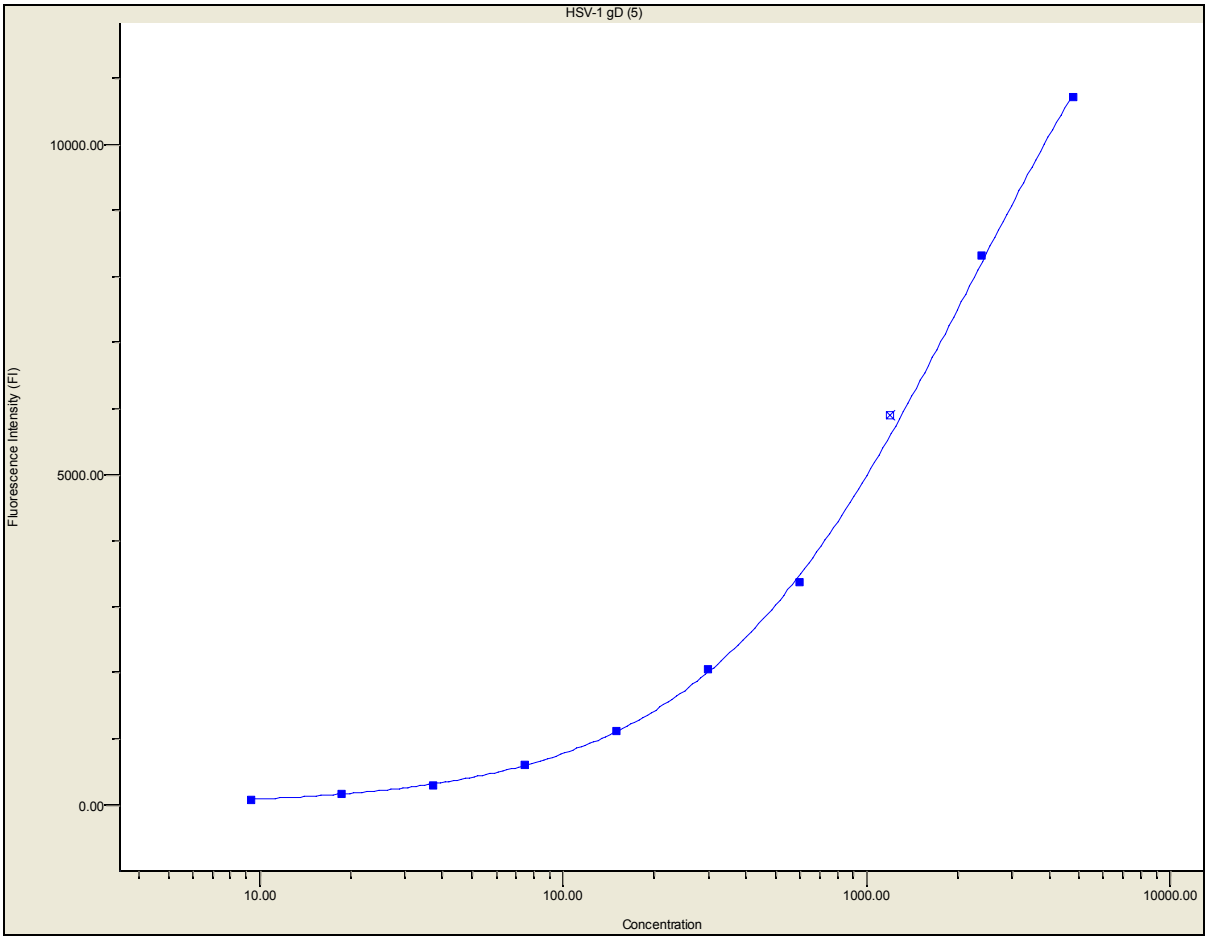

Figure 3.30 Standard curve generated from HSV-1 gD monoplex assay using microsphere beads (5000/well) conjugated with 100µg/mL of the recombinant protein prepared in (PBS + 0.05% Tween + 2% NBBS) serum diluent buffer and detected with 1/100 dilution of the conjugate.

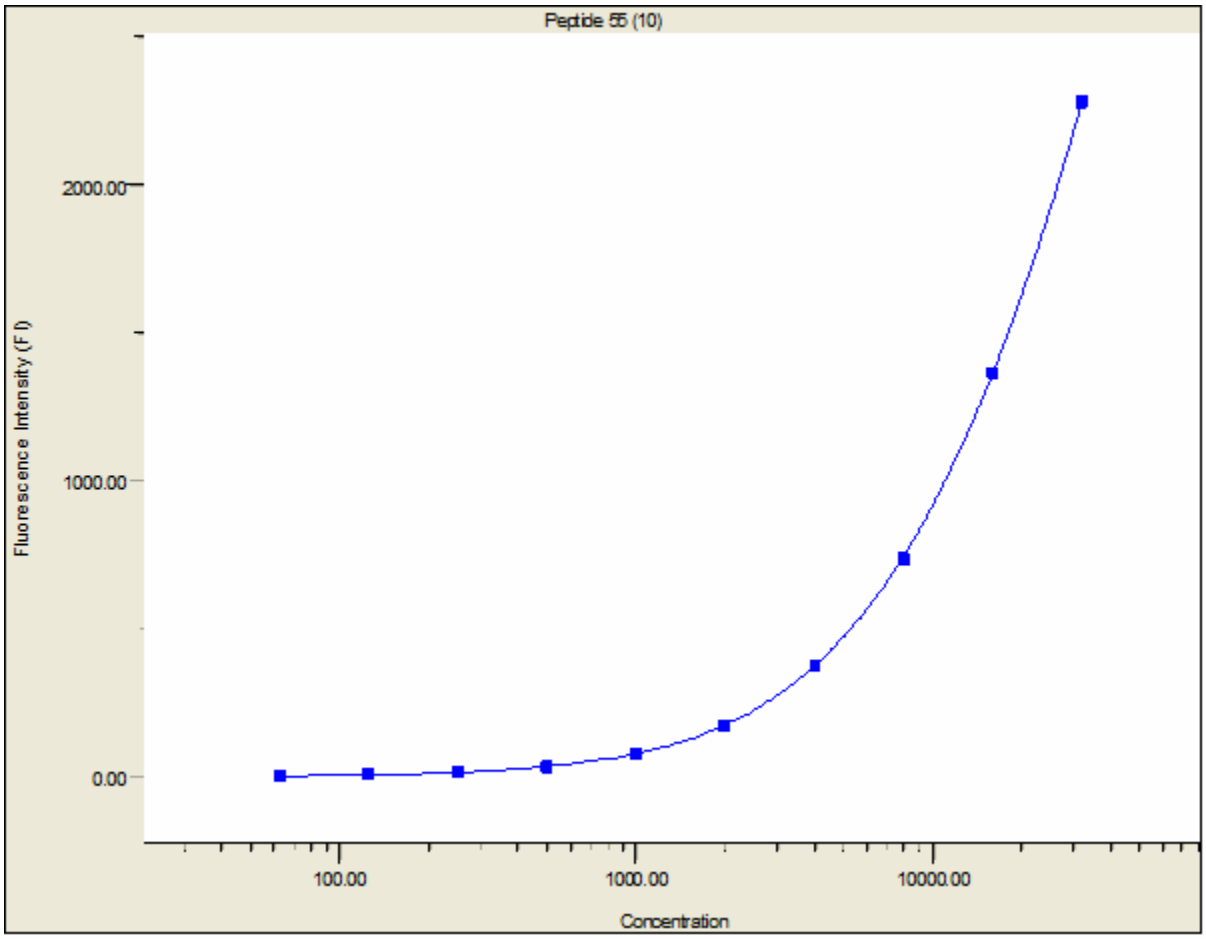

Figure 3.31 Standard curve generated from Peptide 55 monoplex assay using microsphere beads (5000/well) conjugated with 100µg/mL of the peptide prepared in (PBS + 0.05% Tween +2% NBBS) serum diluent buffer and detected with 1/200 dilution of the conjugate.

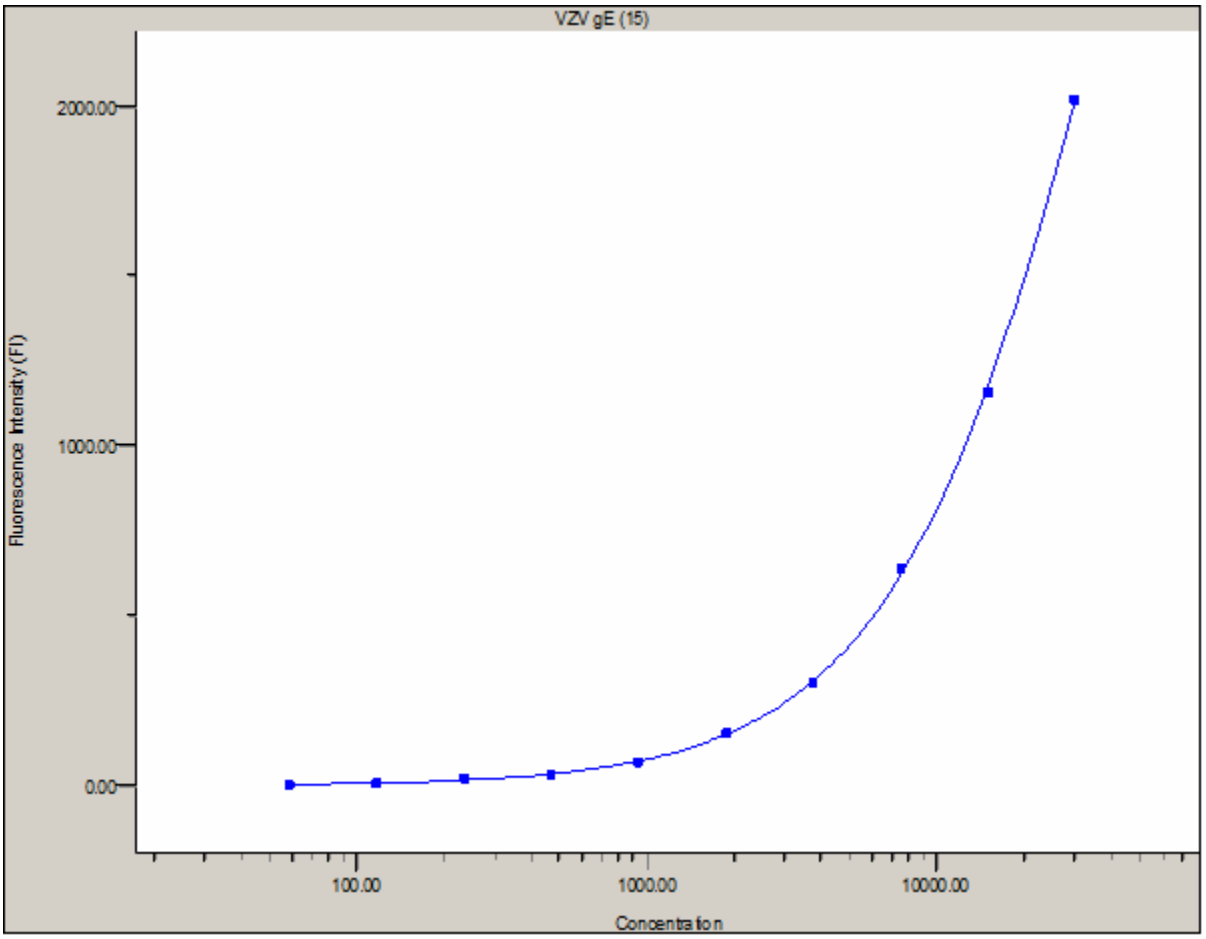

Figure 3.32 Standard curve generated from VZV gE monoplex assay using a microsphere beads (5000/well) conjugated with 100µg/mL of the recombinant protein prepared in (PBS + 0.05% Tween +2% NBBS) serum diluent buffer and detected with 1/100 dilution of the conjugate.

3.8.2 Triplex Assay Validation

3.8.2.1 Bead Interference

Bead interference was tested by comparing the results obtained from the standard curve generated from each antigen as a monoplex assay with the standard curve results generated as a triplex assay. A similar result was obtained from assays for each antigen with slight reduction in the fluorescence intensities between HSV-1 gD assay as a monoplex and triplex (Figures 3.33-35). There was no difference in the median fluorescence intensity (MFI) of the blank well in each assay (Tables 3.26-28).

3.8.2.2 Assay Sensitivity

Assay sensitivity was determined by calculating the mean values of MFIs generated from 10 blank wells plus 3 standard deviations the assay detection limit was determined in ABU/mL by interpolation from the standard curve. Based on the results the highest detection level was 3200 ABU/mL and 4800ABU/mL for VZV and HSV antibody respectively, and lowest assay detected levels were found to be 1.2 ABU/mL and 1.5ABU/mL for VZV and HSV respectively.

3.8.2.3 Assay Reproducibility

Assay reproducibility was determined by measuring intra and inter assay variation, the mean percentage of coefficient of variation (CV %) of 10 samples tested in duplicate within the same plate on the same day or on two different days was calculated. Assay reproducibility (intra and inter assay) was found to be less than 10% (Table 3.29 and 3.30).

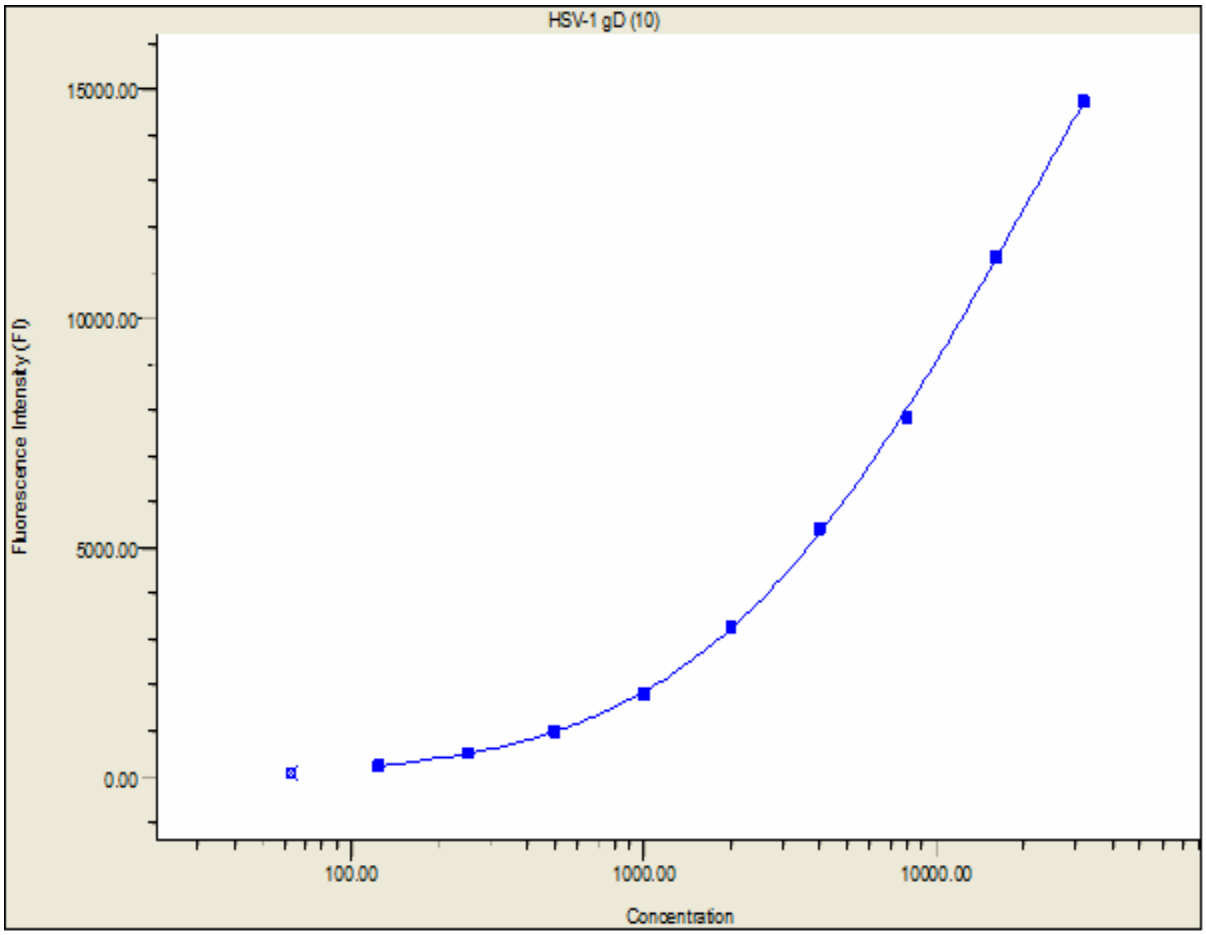

Figure 3.33 Standard curve generated from HSV-1 gD triplex assay using microsphere beads (5000/well) conjugated with 50µg/mL of the recombinant protein prepared in (PBS + 0.05% Tween +1% NBBS) serum diluent buffer and detected with 1/200 dilution of the conjugate.

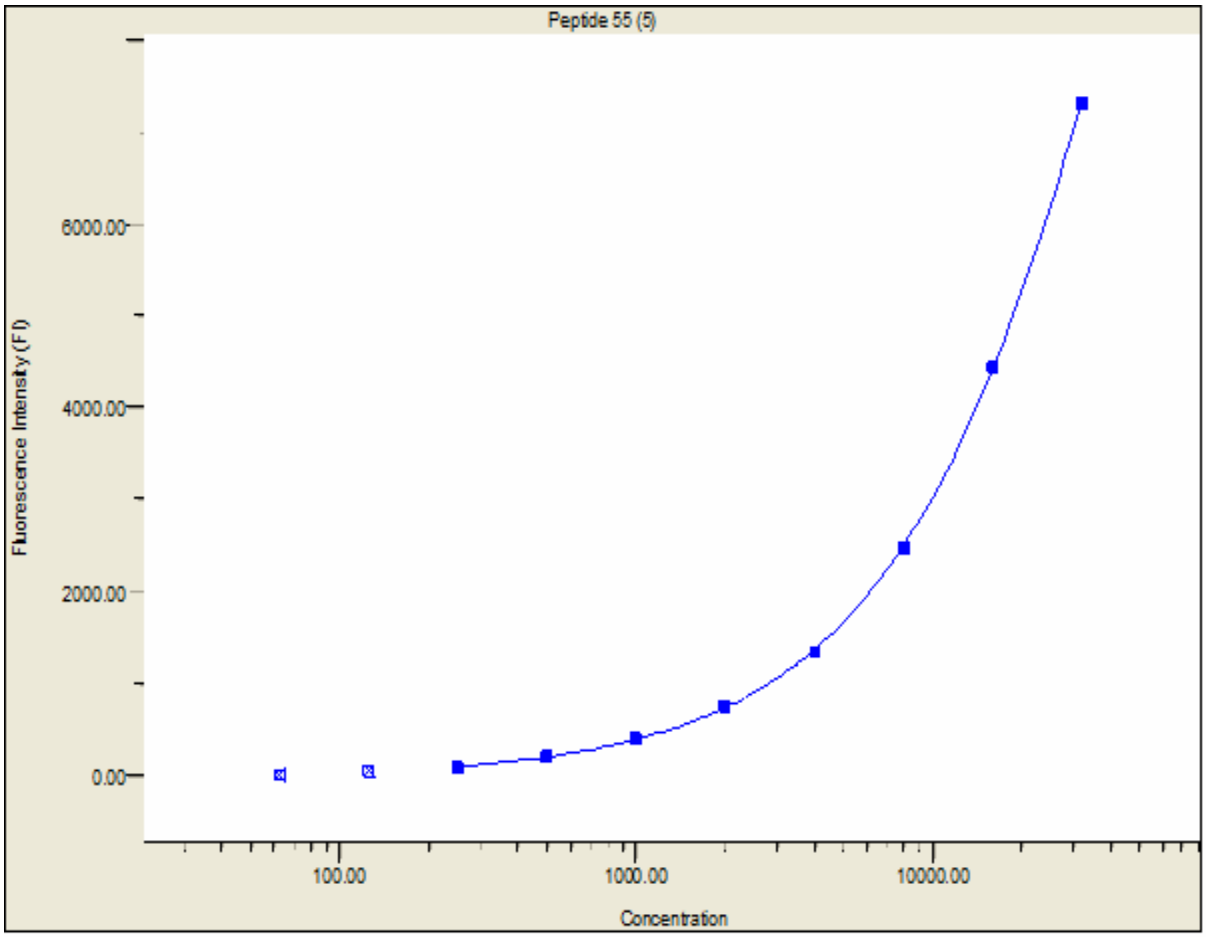

Figure 3.34 Standard curve generated from peptide 55 triplex assay using microsphere beads (5000/well) conjugated with 25µg/mL of the peptide prepared in (PBS + 0.05% Tween +1% NBBS) serum diluent buffer and detected with 1/200 dilution of the conjugate.

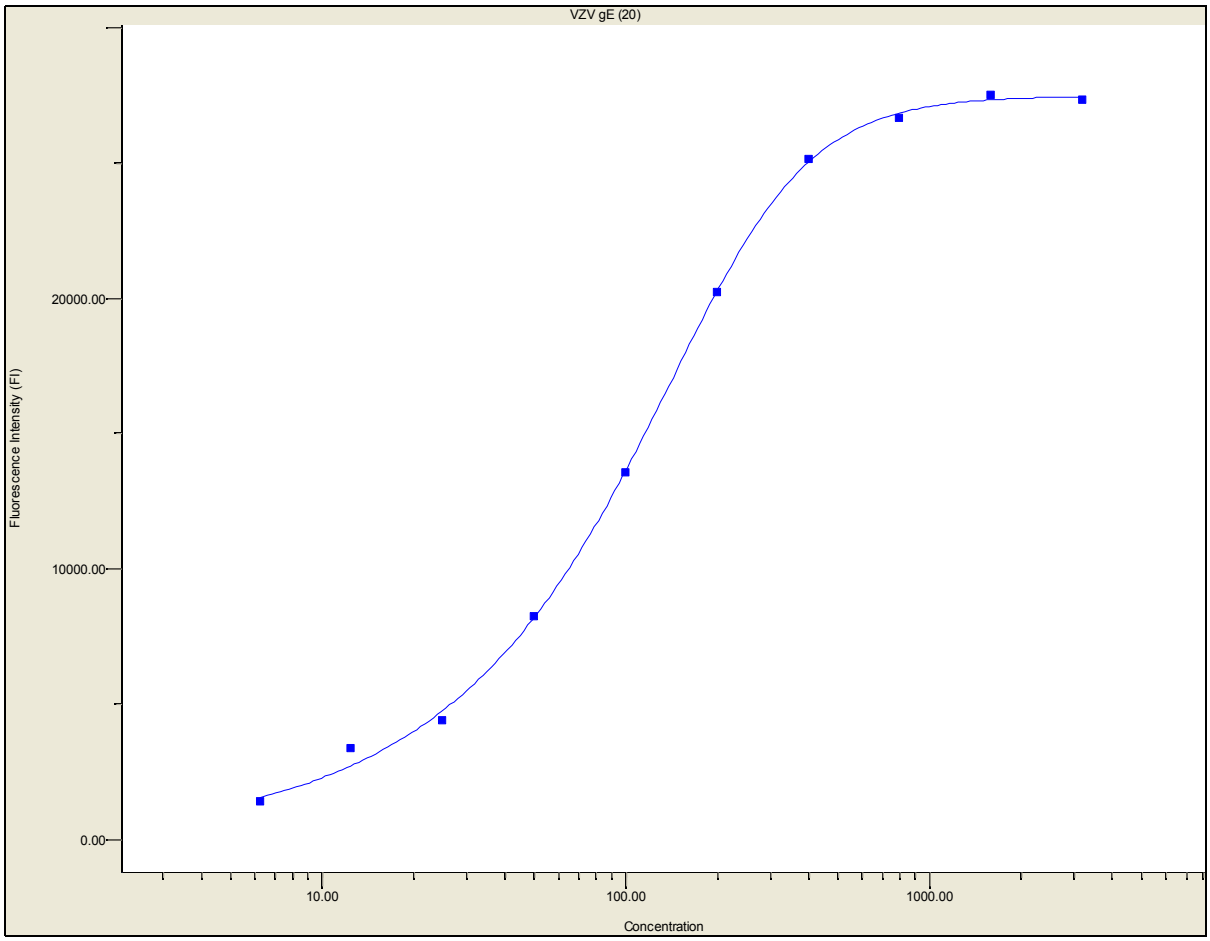

Figure 3.35 Standard curve generated from VZV gE triplex assay using microsphere beads (5000/well) conjugated with 50µg/mL of the peptide prepared in (PBS + 0.05% Tween +1% NBBS) serum diluent buffer and detected with 1/200 dilution of the conjugate.

Table 3.26 Standard Curve Values Obtained from the VZV gE Triplex Assay

Sample	Fluorescence Intensity	Fluorescence Background	Standard Deviation	%CV	Observed Concentration	Expected Concentration	Recovery Rate %
B	15	15	39.95	9.76			
S1	27424	27359	687.66	2.51	1632	3200	51
S2	27591	27526	82.38	0.3	832	1600	52
S3	26778	26712	16.26	0.06	720	800	90
S4	25225	25159	43.13	0.17	412	400	103
S5	20306	20240	96.87	0.48	200	200	100
S6	13662	13596	14.5	0.11	100	100	100
S7	8316	8251	330.57	4.01	50.5	50	101
S8	4480	4414	936.92	8.22	22.75	25	91
S9	3435	3369	585.84	6.39	12.75	12.5	102
S10	1500	1435	42.07	2.93	5.69	6.25	91

B: blank S: tandard,

Fluorescence intensity: the median fluorescence intensity of the selected analyte

CV %: Coefficient of variation (Standard Deviation / Fluorescent Background) x 100%

Recovery Rate: (Observed concentration /Expected concentration) x 100%

Table 3.27 Standard Curve Values from the HSV-1 gD Triplex Assay

Sample	Fluorescence Intensity	Fluorescence Background	Standard Deviation	%CV	Observed Concentration	Expected Concentration	Recovery Rate %
B	18	18	0.71	3.93	0	0	0
S1	14780	14762	82.38	0.56	4741.98	4800	99
S2	11401	11383	42.43	0.37	2423.5	2400	101
S3	7891	7873	53.74	0.68	1215.45	1200	101
S4	5478	5460	224.15	4.1	578.24	600	96
S5	3335	3317	70.36	2.12	317.89	300	105
S6	1867	1849	52.33	2.83	145.37	150	98
S7	1023	1005	10.96	1.09	73.51	75	98
S8	565	547	25.1	4.58	36.92	37	100
S9	293	275	5.66	2.06	18.22	18.5	101
S10					9.51	9.75	104

B: blank S: standard,

Fluorescence intensity: the median fluorescence intensity of the selected analyte

CV %: Coefficient of variation (Standard Deviation / Fluorescent Background) x 100%

Recovery Rate: (Observed concentration /Expected concentration) x 100%

Table 3.28 Standard Curve Values from Peptide 55 Triplex Assay

Sample	Fluorescence Intensity	Fluorescence Background	Standard Deviation	%CV	Observed Concentration	Expected Concentration	Recovery Rate %
B	17	17	1.47	8.62	0	0	
S1	7350	7332	107.13	1.46	4741.87	4800	99
S2	4480	4463	155.21	3.48	2413.49	2400	101
S3	2515	2497	108.19	4.33	1214.25	1200	101
S4	1381	1364	79.55	5.83	588.24	600	98
S5	784	766	38.18	4.98	314.09	300	105
S6	426	408	5.66	1.38	147.37	150	98
S7	235	217	13.44	6.17	74.51	75	99
S8	125	107	7.78	7.25	36.98	37	100
S9					18.32	18.5	99
S10					9.51	9.75	104

B: blank S: standard,

Fluorescence intensity: the median fluorescence intensity of the selected analyte

CV %: Coefficient of variation (Standard Deviation / Fluorescent Background) x 100%

Recovery Rate: (Observed concentration /Expected concentration) x 100%

Table 3.29 Triplex Intra Assay Reproducibility Results

Sample Number	Intra assay variation								
	HSV-1 gD			VZV gE			Peptide 55		
	Fluorescence MFI	Standard Deviation	CV %	Fluorescence MFI	Standard Deviation	CV %	Fluorescence MFI	Standard Deviation	CV %
1	205	8.49	4.14	510	21.92	4.3	228	19.04	8.32
2	7700	56.21	0.73	176	2.12	1.21	4267	10.25	0.24
3	5515	62.23	1.13	2188	32.53	1.49	647	16.26	2.51
4	5641	34.29	0.61	302	4.95	1.64	1240	47.73	3.85
5	3105	159.81	5.15	202	5.66	2.8	299	12.73	4.26
6	245	2.47	1.01	143	3.54	2.46	496	4.24	0.86
7	153	7.07	4.62	503	9.9	1.97	312	4.98	1.68
8	8566	457.14	5.34	239	16.97	7.08	42	0.71	1.68
9	1962	21.92	1.12	80	3.54	4.42	206	9.55	4.63
10	8927	331.63	3.71	256	1.06	0.41	100	3.18	3.16

CV %: Percentage coefficient of variation recovery rate (Observed concentration /Expected concentration) x 100%

Table 3.30 Triplex Assay Inter Assay Reproducibility Results

Sample Number	Inter assay variation								
	HSV-1 gD			VZV gE			Peptide 55		
	Fluorescence MFI	Standard Deviation	CV %	Fluorescence MFI	Standard Deviation	CV %	Fluorescence MFI	Standard Deviation	CV %
1	215	8.65	4.21	509	21.82	4.2	208	20.24	9.69
2	7720	55.21	0.71	169	2.02	1.11	4297	10.55	0.22
3	5495	61.23	1.10	2190	32.63	1.39	647	16.26	2.51
4	5641	34.29	0.61	302	4.95	1.64	1239	47.63	3.82
5	3105	159.81	5.15	202	5.66	2.8	299	12.73	4.26
6	255.3	2.37	1.11	149	3.54	2.35	489	4.14	0.84
7	149	7.17	4.60	513	9.5	1.95	322	4.88	1.59
8	8576	457.84	5.21	229	16.87	7.10	41	0.71	1.66
9	1966	21.98	1.15	85	3.59	4.45	216	9.85	4.42
10	8917	335.23	3.61	259	1.02	0.42	110	3.28	3.19

CV %: Percentage coefficient of variation recovery rate (Observed concentration /Expected concentration) x 100%

3.8.3 Evaluation of the Triplex Fluorescence Microbead Immunoassay for the Determination of Serological Status of HSV and VZV in Human Sera

The triplex assay was evaluated by testing panel 1 sera. The serological status of HSV-1, HSV-2 and VZV in this panel had been previously determined using the developed in-house ELISAs and other HSV specific assays (Omega, Focus, Biokit and Virotech). Samples were tested as described in section 2.13.6, each assay was first evaluated based on the acceptance criteria (section 2.13.2), and then the results from each individual assay were compared with the relevant ELISA assay. Results showed a 100% agreement between both the In-house ELISA and the fluorescence microbead assay for HSV-1 gD and VZV gE (Tables 3.31 and 3.32). For Peptide 55 the two assays agreed in 99.2 % (252/254). The remaining two samples were positive by the fluorescence microbead assay and negative by the Omega assay (Table 3.33). The correlation (r) between each ELISA assay with its fluorescence microbead assay was 0.988, 0.956 and 0.968 for peptide 55, HSV-1 gD and VZV gE respectively. Furthermore, in comparison to Liaison VZV IgG

Assay, the VZV fluorescence microbead assay gave similar results to VZV gE in-house ELISA assay and HUMAN VZV IgG Assay (Table 3.23). Assay results were plotted to generate a scattergram and regression analysis (r^2) results are shown in Figures 3.36-39.

Table 3.31 Comparison of Results Obtained from HSV-1 gD In-House ELISA and gD Fluorescence Microbead Immunoassays

VZV gE In-House ELISA	VZV gE Fluorescence Microbead Immunoassay		
	Positive	Negative	Equivocal
Positive	239	0	0
Negative	0	15	0
Equivocal	0	0	0
Total	254		

Table 3.32 Comparison of Results Obtained from VZV gE In-House ELISA and gE Fluorescence Microbead Immunoassays

HSV-1 gD In-House ELISA	HSV-1 gD Fluorescence Microbead Immunoassay		
	Positive	Negative	Equivocal
Positive	219	0	0
Negative	0	35	0
Equivocal	0	0	0
Total	254		

Table 3.33 Comparison of Results Obtained from Peptide 55 ELISA and Fluorescence Microbead Immunoassays

Omega Assay	Peptide 55 Fluorescence Microbead Immunoassay		
	Positive	Negative	Equivocal
Positive	77	0	0
Negative	2	175	0
Equivocal	0	0	0
Total	254		

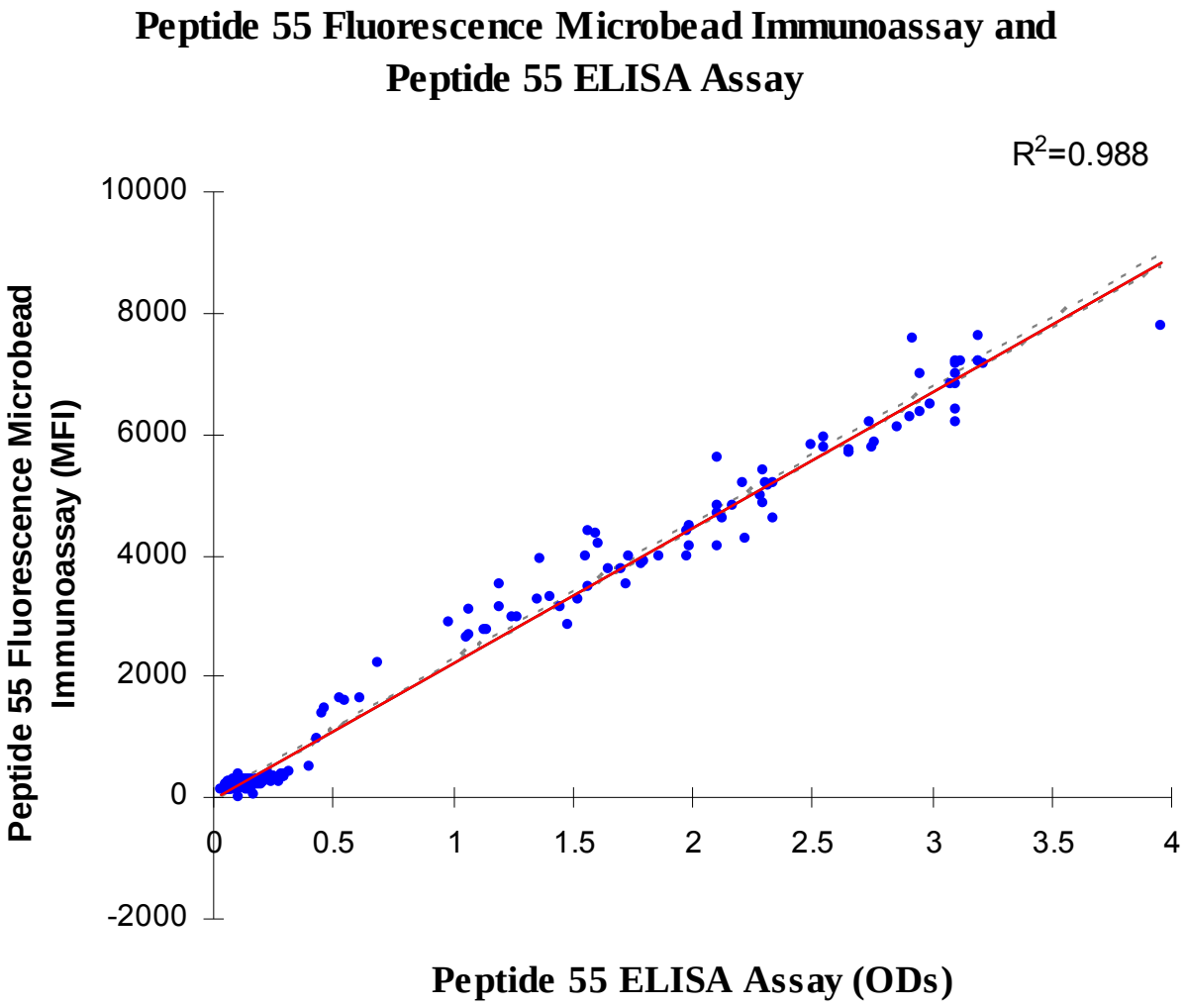

Figure 3.36 Regression analysis on measured serum HSV-2 antibody concentration comparing Omega ELISA (χ axis) and the Peptide 55 fluorescence microbead immunoassay (y axis). The measurements were performed on 254 sera; regression was 98.8 % (R^2 regression coefficient).

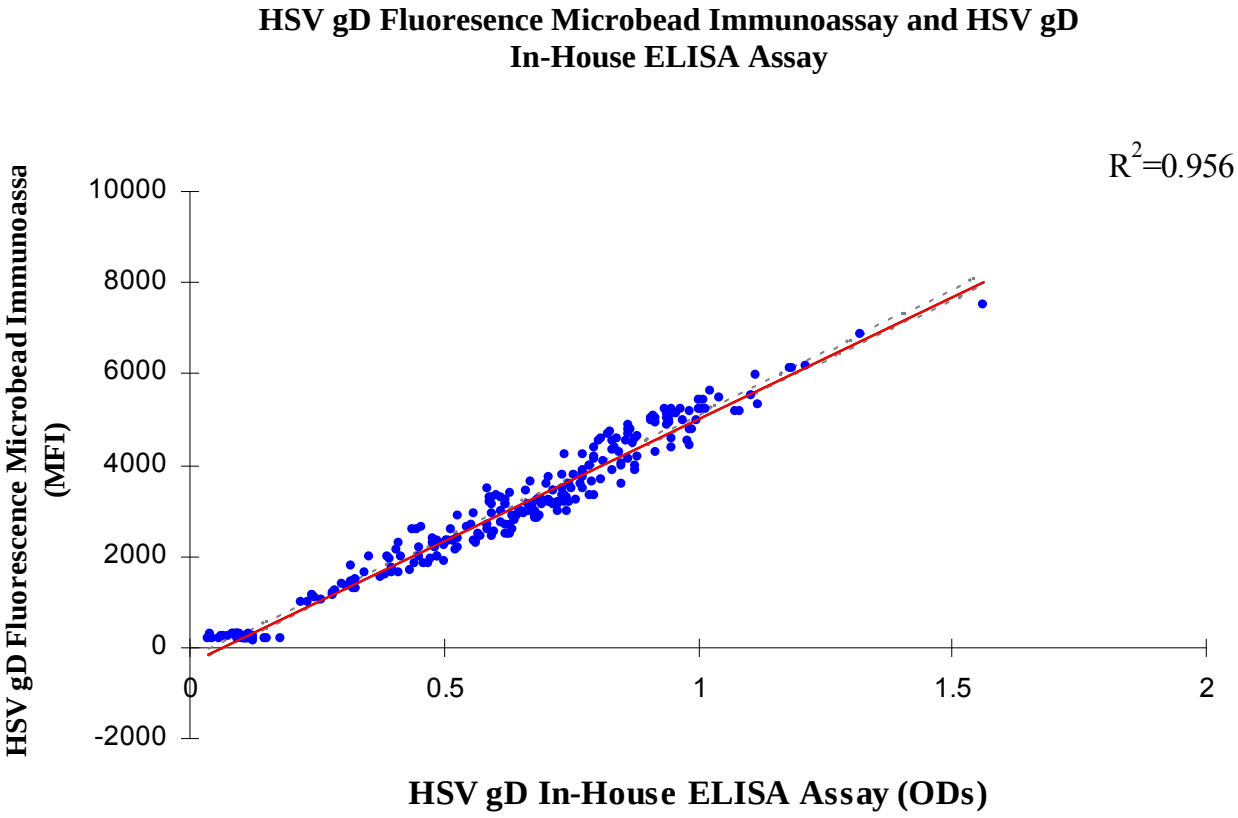

Figure 3.37 Regression analysis on measured serum HSV antibody concentration comparing HSV-1 gD in-house ELISA (X axis) and the HSV-1 gD fluorescence microbead immunoassay (y axis). The measurements were performed on 254 sera; regression was 95.6 % (R^2 regression coefficient).

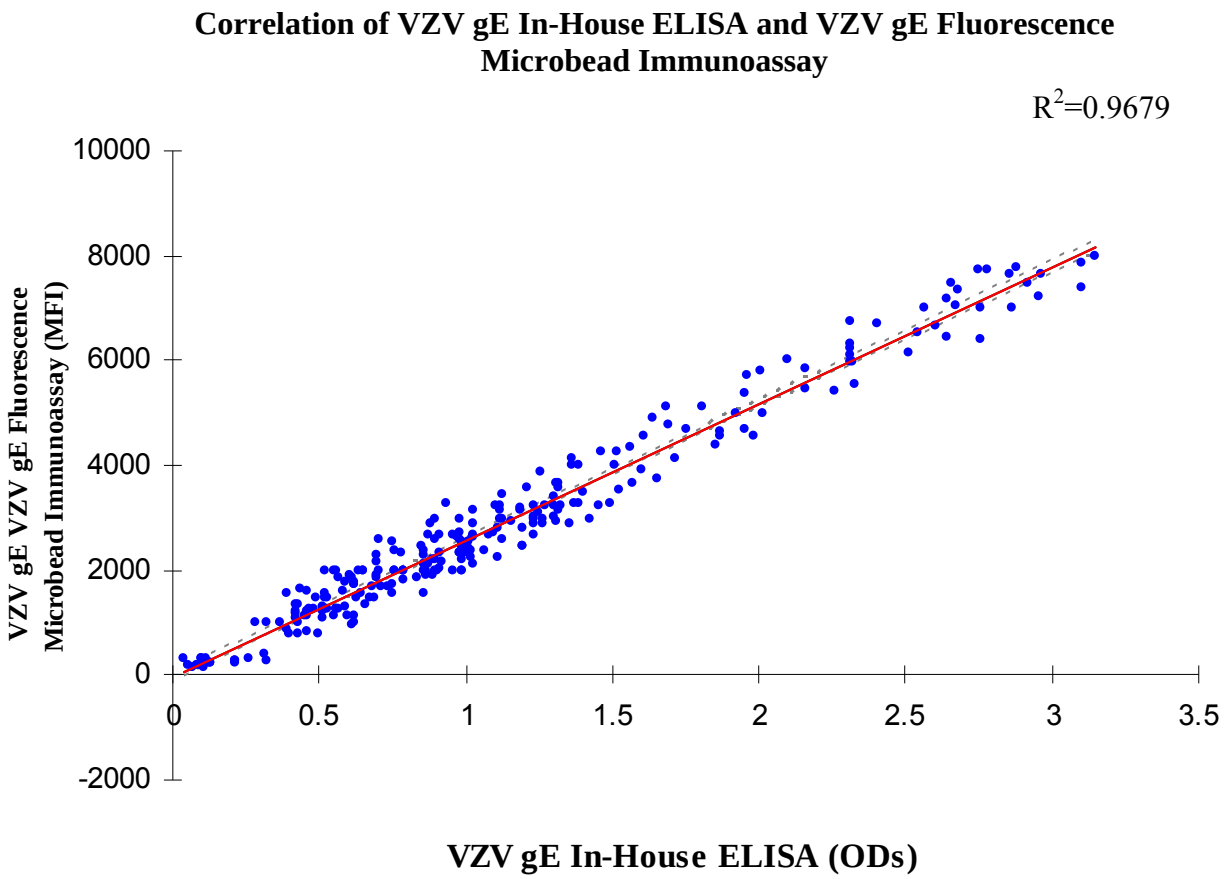

Figure 3.38 The figure shows the results of regression analysis on measured serum comparing VZV antibody concentration between VZV gE in-house ELISA (X axis) and the VZV gE fluorescence microbead immunoassay (y axis). The measurements were performed on 254 sera; regression was 96.79 % (R^2 regression coefficient).

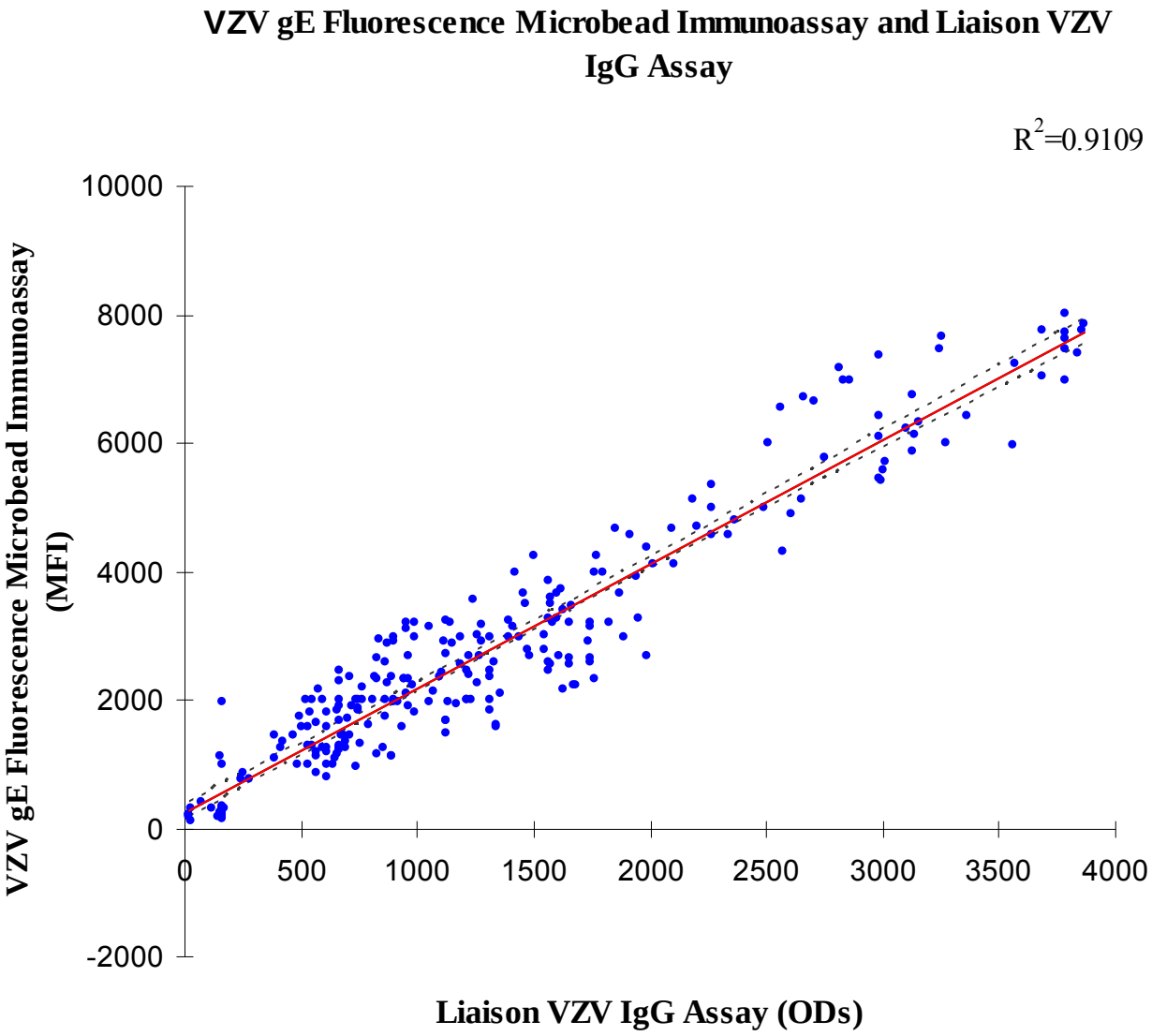

Figure 3.39 Regression analysis on measured serum VZV antibody concentration comparing Liaison VZV IgG Assay (X axis) and the VZV gE fluorescence microbead immunoassay (y axis). The measurements were performed on 254 sera; regression was 91.09 % (R^2 regression coefficient).

3.9 Development of a Biplex Assay for the Quantitation of Albumin and IgG in Human Serum and CSF samples

In order to generate a suitable standard curve that could be used to detect and quantitate both human IgG and albumin in serum and CSF samples a series of 8 two fold dilution of the human standard serum starting from 3.9µg/dl for IgG and 0.5µg/dl for albumin were used. the assay was evaluated based on the acceptance criteria (section 2.13.2). Results showed that the assay was able to detect both analytes in the same sample (Figures 3.40-41). In addition using the triplex condition (bead type, serum diluent, washing buffer and incubation times) the median Fluorescent intensity (MFI) of the blank well in each assay was less than 350 (Tables 3.34-35). Assay reproducibility was also determined by measuring intra and inter assay variation of 10 samples tested in duplicate. Assay reproducibility was found to be less than 10% (Table 3.36 and 3.37). the limit of the detection of the assay was found to be 0.03 µg/dl and 0.3 µg/dl for IgG and Albumin.

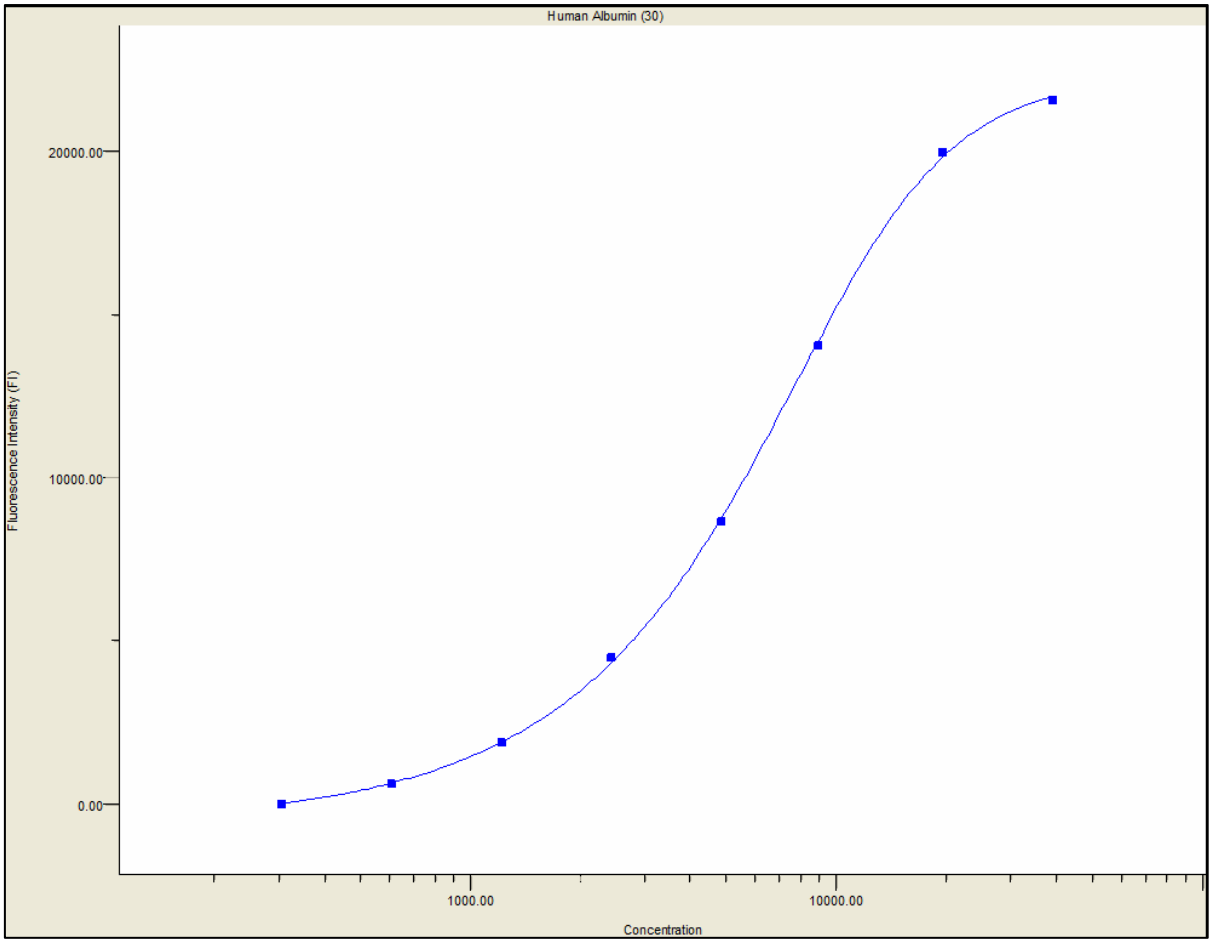

Figure 3.40 Standard curve generated from Albumin biplex assay using microsphere beads (5000/well) conjugated with 100µg/mL of the anti-human albumin prepared in (PBS + 0.05% Tween +1% NBBS) serum diluent buffer.

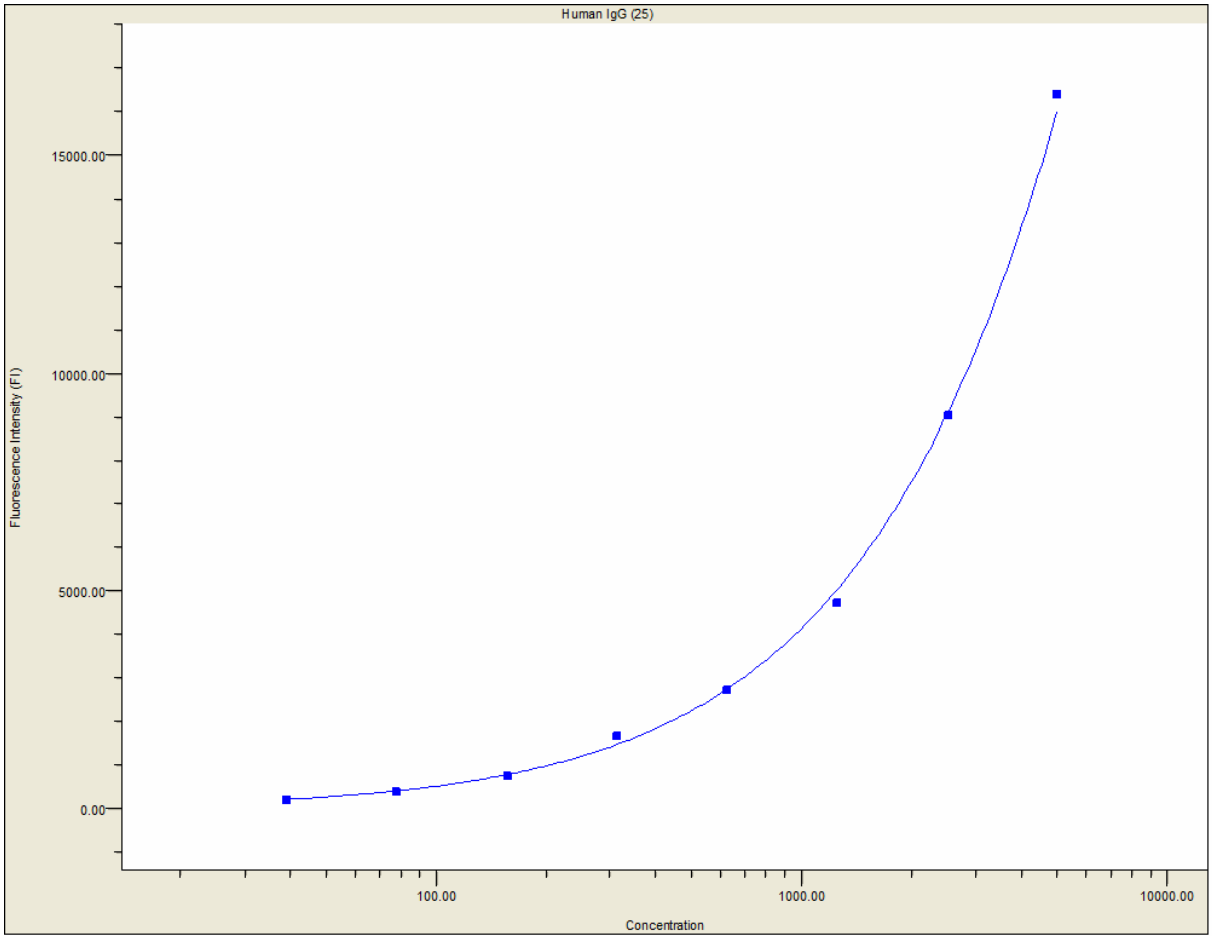

Figure 3.41 Standard curve generated from IgG biplex assay using microsphere beads (5000/well) conjugated with 100µg/mL of the anti-human albumin prepared in (PBS + 0.05% Tween +1% NBBS) serum diluent buffer .

Table 3.34 Standard Curve Values Obtained from the Albumin Biplex Assay

Sample	Fluorescence Intensity	Fluorescence Background	Standard Deviation	%CV	Observed Concentration	Expected Concentration	Recovery Rate %
B	246	249	25	9.65			
S1	22590	21578	551.65	2.56	35942.58	39000	92%
S2	21016	20005	946.46	4.73	20265.63	19500	104%
S3	9984	9795	324.5	3.31	9580	9750	102%
S4	9671	8659	791.25	9.14	4781.61	4875	98
S5	5526	4514	408.43	9.05	2507.79	2437.5	103
S6	2921	2310	180.48	4.1	1209.5	1218.75	99
S7	1667	655	15.91	2.43	601.11	609.38	99
S8	1056	944	33.23	3.52	307.63	304.69	101

B: blank S: standard,

Fluorescence intensity: the median fluorescence intensity of the selected analyte

CV %: Coefficient of variation (Standard Deviation / Fluorescent Background) x 100%

Recovery Rate: (Observed concentration /Expected concentration) x 100%

Table 3.35 Standard Curve Values Obtained from the IgG Biplex Assay

Sample	Fluorescence Intensity	Fluorescence Background	Standard Deviation	%CV	Observed Concentration	Expected Concentration	Recovery Rate %
B	310	315					
S1	16964	16419	904.5	5.51	5142.81	5000	103
S2	9564	9390	209	2.13	2470	2500	102%
S3	5282	4937	455.94	9.23	1158.48	1250	93
S4	3301	2756	62.58	2.27	621.11	625	99
S5	2248	1703	39.24	2.3	360.55	312.5	115
S6	1317	772	50.56	6.54	149.35	156.25	96
S7	962	867	84.15	9.7	75.25	78.13	96
S8	781	656	63.99	5.64	39.38	39.06	101

B: blank S: standard,

Fluorescence intensity: the median fluorescence intensity of the selected analyte

CV %: Coefficient of variation (Standard Deviation / Fluorescent Background) x 100%

Recovery Rate: (Observed concentration /Expected concentration) x 100%

Table 3.36 Bioplex Intra Assay Reproducibility Results

Sample Number	Intra assay variation					
	IgG			Albumin		
	Fluorescence Intensity	Standard Deviation	CV %	Fluorescence Intensity	Standard Deviation	CV %
1	4027	48.79	1.21	20101	202.37	1.01
2	10390	253.85	2.44	22301	2.47	0.11
3	8641	1025.3	11.87	15173	1794.64	11.83
4	9754	374.06	3.83	4060	83.09	2.05
5	11364	21.92	0.19	6517	136.47	2.09
6	5508	51.62	0.94	18384	552.25	3
7	1711	41.01	2.4	21089	117.73	0.56
8	11214	863.73	7.7	16977	724.08	4.27
9	6278	184.91	2.95	1628	152.74	9.38
10	3176	124.8	3.93	12110	158.75	1.31

Table 3.37 Biplex Inter Assay Reproducibility Results

Sample Number	Inter assay variation					
	IgG			Albumin		
	Fluorescence Intensity	Standard Deviation	CV %	Fluorescence Intensity	Standard Deviation	CV %
1	3988	38.12	0.96	20290	101.5	0.5
2	10119	190.92	1.89	21931	28.21	0.13
3	9012	860.9	9.55	16011	1661.53	11.83
4	9233	360.1	3.90	4120	73.11	1.77
5	11174	31.82	0.28	6822	141.27	2.07
6	5913	61.34	1.04	19324	572.55	2.96
7	1689	39.1	2.31	22014	217.03	0.99
8	10909	790.23	7.24	16169	741.28	4.58
9	6199	154.72	2.5	1920	139.94	7.29
10	3250	128.9	3.97	13090	176.55	1.35

3.10 Development of a Multiplex Microbeads Immunoassay for Detection and Quantitation of IgG Antibody Response to HSV-1, HSV-2, VZV, Albumin and IgG in Human Serum and CSF

In order to generate a suitable standard curve for the multiplex assay and due to the difficulty of obtaining a commercial standard serum for the 5 analytes, the previous standard serum was used. The concentration of IgG and albumin in the clinical standard serum was determined by testing serial dilution of the clinical standard using the biplex assay. Based on the biplex assay results the IgG and albumin concentration was found to be 3g/l and 0.5g/l respectively. A standard curve for the 5 analytes was then generated by 12 4-fold dilution of the clinical standard serum. Results have showed that the generated curves were suitable for determining the concentration of each analyte in the same sample (Figures 3.42-46). MFI background of the multiplex assay was generally found to be les than 350 MFI. Assay sensitivity and reproducibility was tested and the CVs values for each analyte were found to be les than 10% (Tables 38-39).

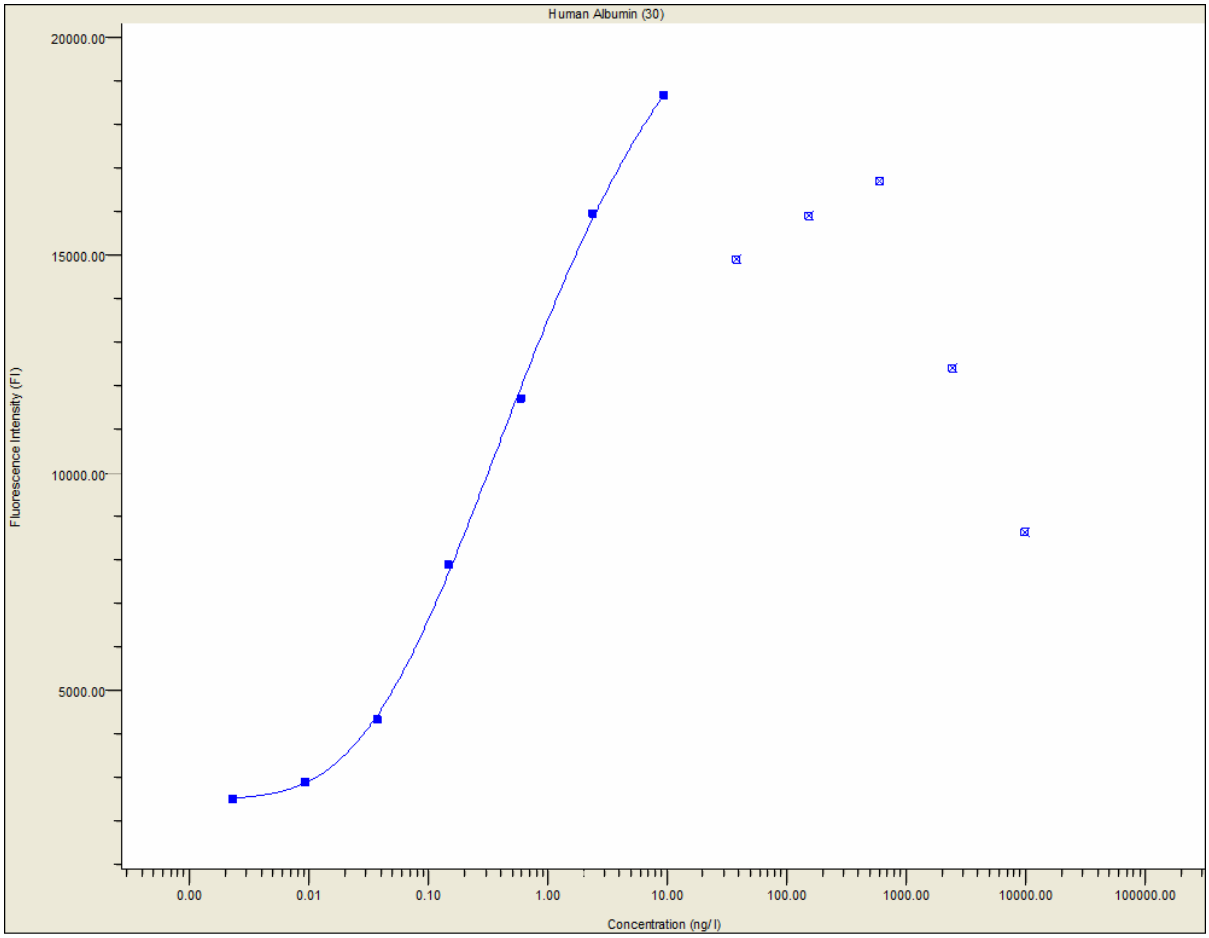

Figure 3.42 Standard curve generated from albumin multiplex assay using microsphere beads (5000/well) conjugated with 100µg/mL of the anti-human albumin prepared in (PBS + 0.05% Tween +1% NBBS) serum diluent buffer.

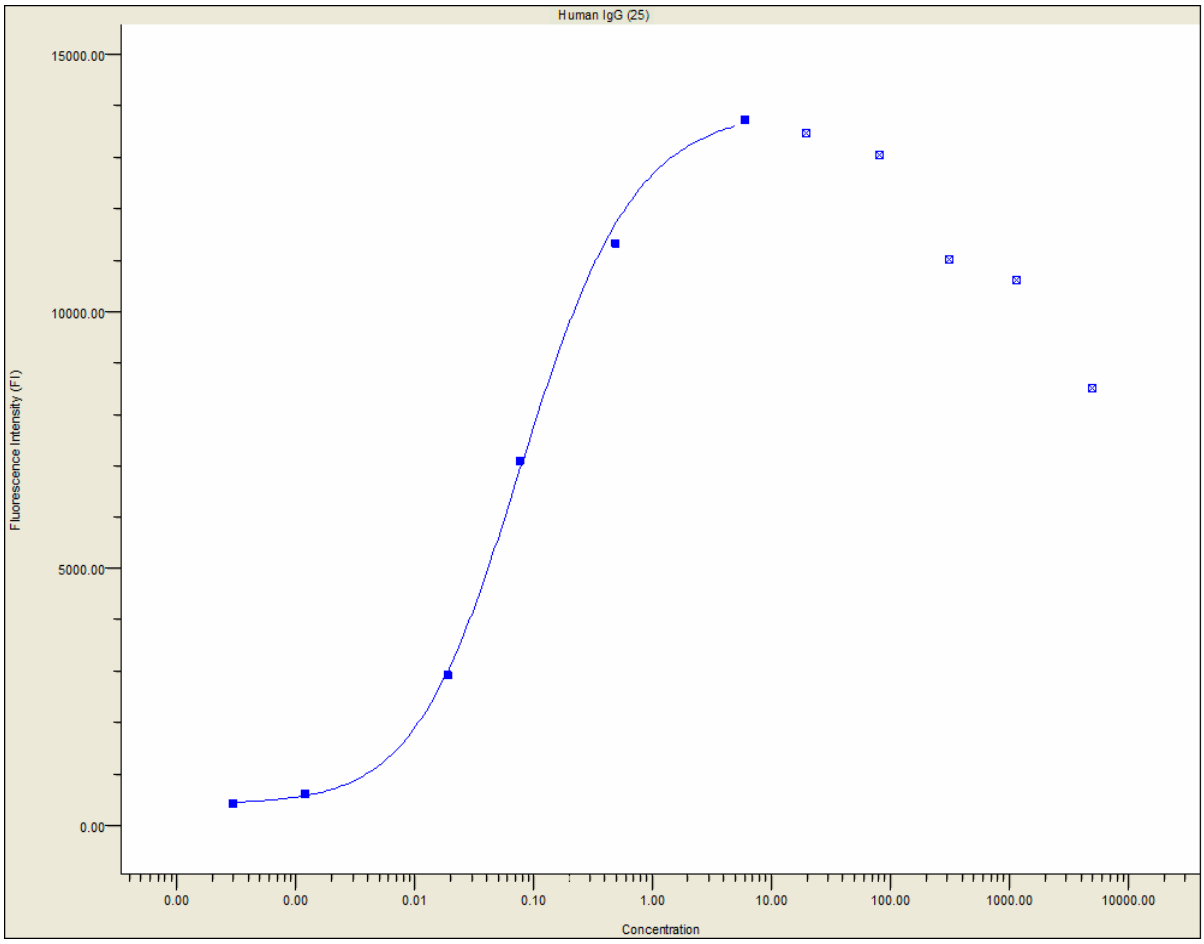

Figure 3.43 Standard curve generated from IgG multiplex assay using microsphere beads (5000/well) conjugated with 100µg/mL of the anti-human albumin prepared in (PBS + 0.05% Tween +1% NBBS) serum diluent buffer.

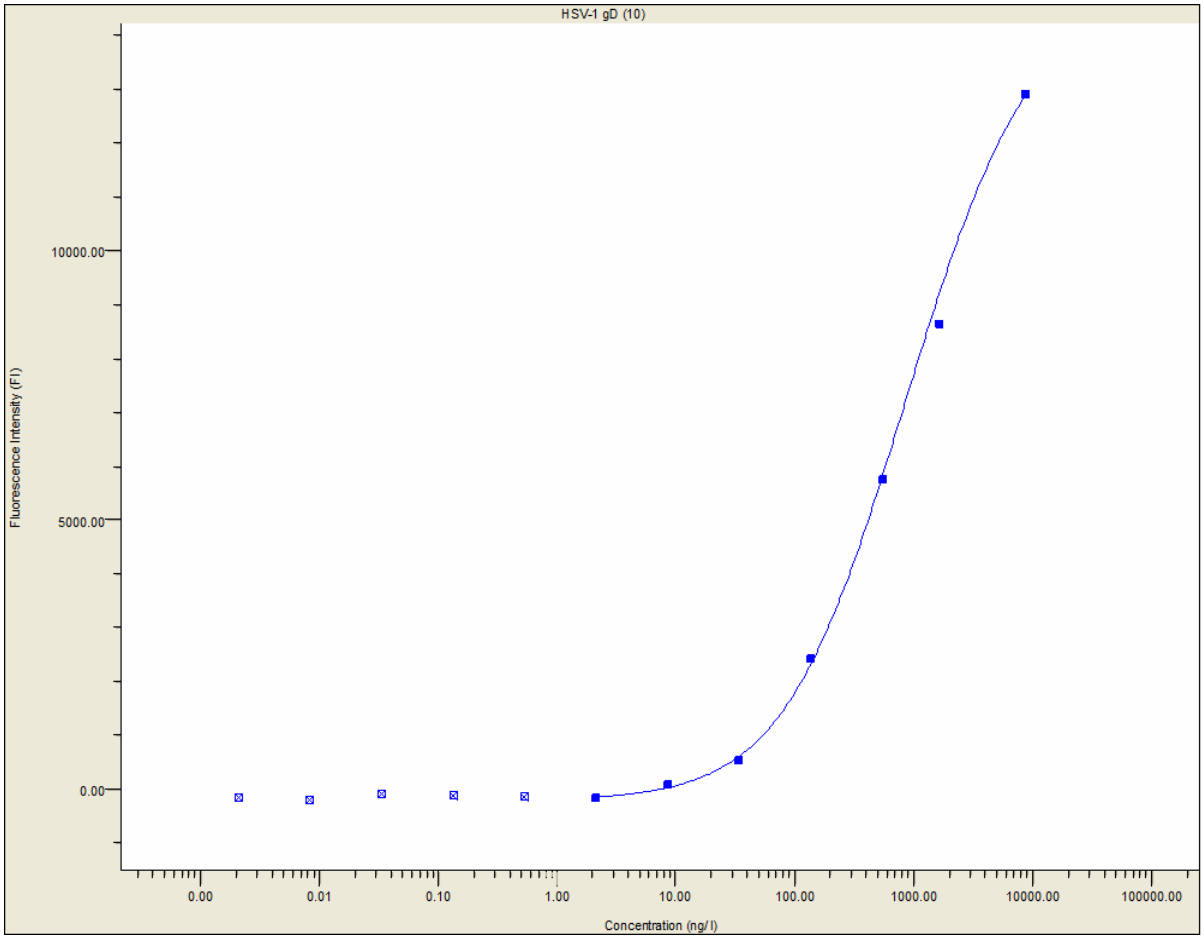

Figure 3.44 Standard curve generated from HSV-1 gD multiplex assay using microsphere beads (5000/well) conjugated with 100µg/mL of the anti-human albumin prepared in (PBS + 0.05% Tween +1% NBBS) serum diluent buffer.

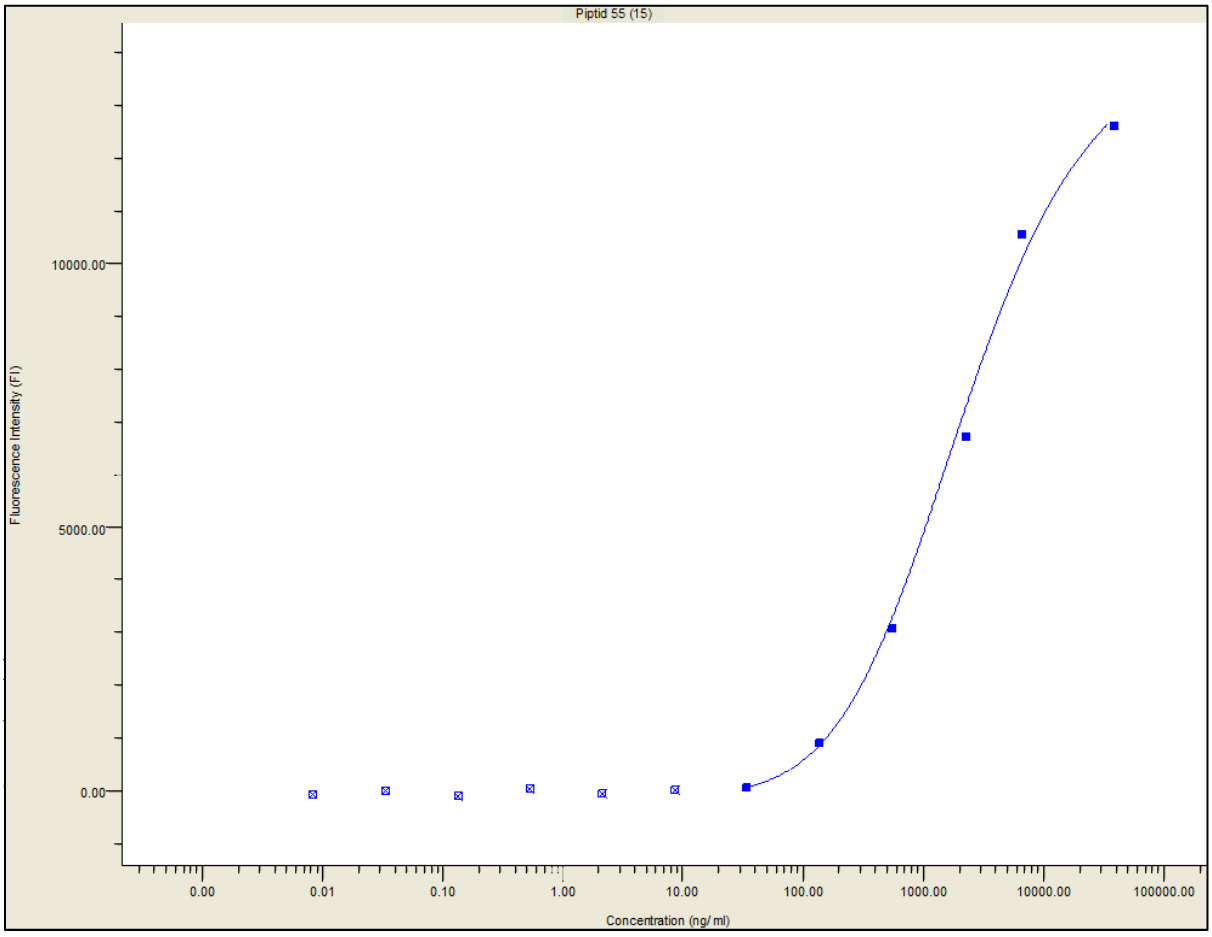

Figure 3.45 Standard curve generated from peptide 55 multiplex assay using microsphere beads (5000/well) conjugated with 100µg/mL of the anti-human albumin prepared in (PBS + 0.05% Tween +1% NBBS) serum diluent buffer.

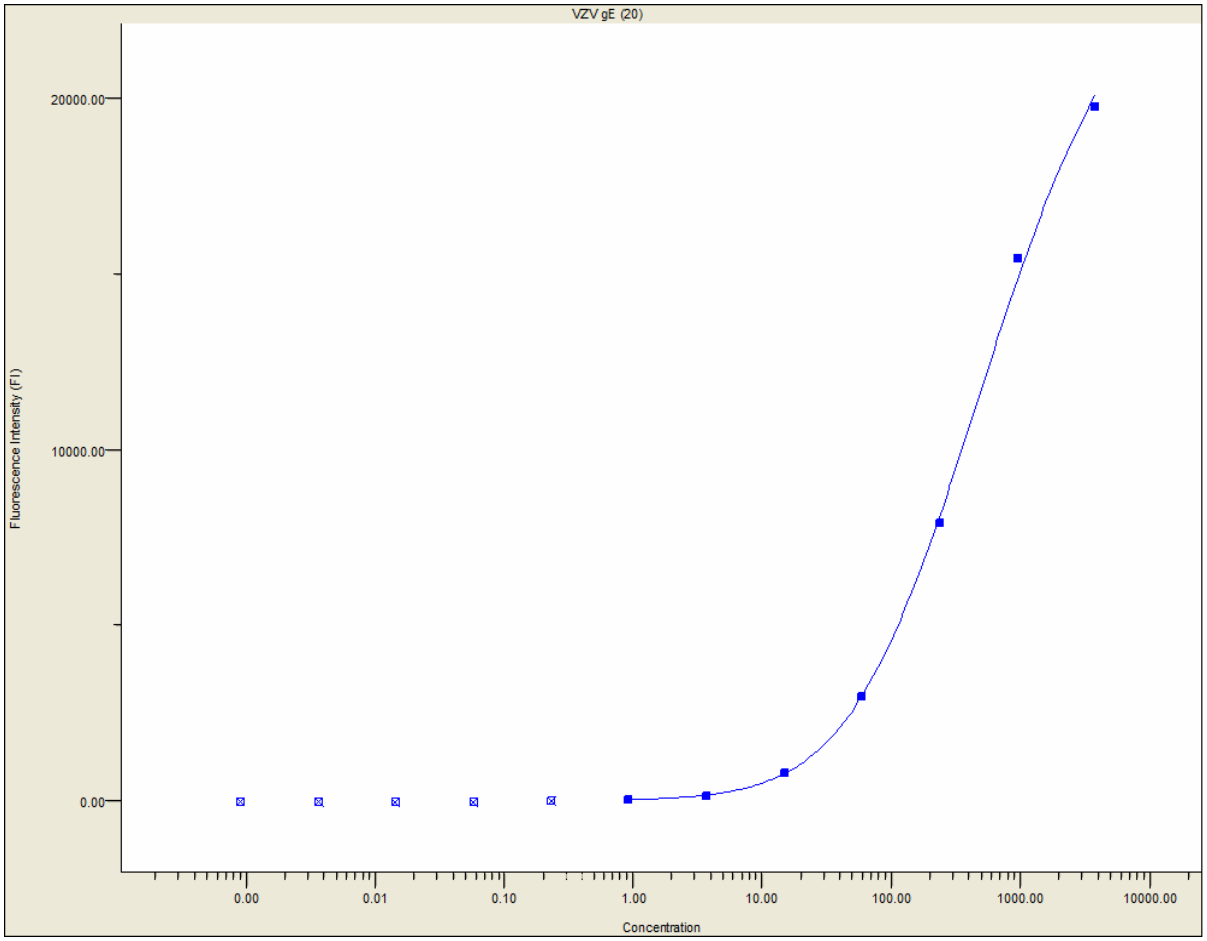

Figure 3.46 Standard curve generated from peptide VZV gE multiplex assay using microsphere beads (5000/well) conjugated with 100µg/mL of the anti-human albumin prepared in (PBS + 0.05% Tween +1% NBBS) serum diluent buffer.

Table 3.38 Multiplex Intra Assay Reproducibility Results

Sample Number	Intra assay variation														
	Albumin			IgG			HSV-1 gD			Peptide 55			VZV gE		
	Fluorescence MFI	Standard Deviation	CV %	Fluorescence MFI	Standard Deviation	CV %	Fluorescence MFI	Standard Deviation	CV %	Fluorescence MFI	Standard Deviation	CV %	Fluorescence MFI	Standard Deviation	CV %
1	19367	852.77	4.4	11085	305.82	2.76	3860	267.29	6.92	1009	69.3	6.86	15769	1092.83	6.93
2	14016	111.02	0.79	6869	189.5	2.76	2907	139.3	4.79	8934	795.5	8.9	19783	43.84	0.22
3	7706	492.15	6.39	2460	24.75	1.01	1144	63.29	5.53	5973	211.07	3.53	15454	208.95	1.35
4	3822	250.32	6.55	1254	108.19	8.62	363	12.02	3.31	2334	170.41	7.3	7906	85.21	1.08
5	528	7.42	1.4	16366	602.81	3.28	6701	540.94	8.07	521	43.84	8.41	2149	31.82	1.48
6	11640	818.48	7.03	11364	938.33	8.26	3589	93.69	2.61	243	12.37	5.09	2149	31.82	1.48
7	8568	356.03	4.16	15395	985.71	5.36	923	56.21	6.09	1552	21.92	1.41	1493	19.8	1.33
8	11327	756.6	6.68	10687	388.2	3.63	21087	419.21	2	1465	28.64	1.95	763	57.63	7.55
9	8697	672.1	7.73	17154	1653.92	9.64	10953	56.57	0.52	4340	255.97	5.9	20086	387.49	1.93
10	5396	358.5	6.64	16570	560.74	3.38	4499	130.11	2.89	12084	422.85	3.5	6701	540.94	8.07

Table 3.39 Multiplex Inter Assay Reproducibility Results

Sample Number	Inter assay variation														
	Albumin			IgG			HSV-1 gD			Peptide 55			VZV gE		
	Fluorescence MFI	Standard Deviation	CV %	Fluorescence MFI	Standard Deviation	CV %	Fluorescence MFI	Standard Deviation	CV %	Fluorescence MFI	Standard Deviation	CV %	Fluorescence MFI	Standard Deviation	CV %
1	18537	711	3.84	10095	335.5	3.3	4160	292.9	7.04	1108	85.01	7.67	16319	995.53	6.1
2	13516	211.82	1.67	6266	212.5	3.4	3401	199.8	5.87	9135	775.52	8.45	18353	63.8	0.35
3	6909	442.66	6.41	2950	54.15	1.84	2114	73.39	3.47	5853	221.33	3.78	16424	198.94	1.21
4	4212	320.11	7.6	1198	112.15	9.35	459.	11.82	2.57	3139	140.5	4.48	8497	92.31	1.09
5	628	40.56	6.5	15396	592.41	3.84	6199	590.84	9.5	629	53.85	8.55	1978	49.12	2.48
6	12820	718.40	0.06	13129	831.13	6.33	4099	103.5	2.52	360	18.09	5.02	2620	41.5	1.58
7	7967	276.8	3.5	16325	991.21	6.07	1013	85.8	8.5	2259	29.08	1.29	1877	23.5	1.25
8	12129	696.5	5.74	11686	710.2	6.01	22004	329.5	1.5	1962	30.55	1.56	813.5	67.11	8.25
9	6999	492.6	7.04	16112	1463.12	9.08	11551	86.47	0.75	5310	512.87	9.66	19855	415.49	2.09
10	6281	395.5	6.3	15980	492.34	3.08	4097	130.11	3.18	11055	352.85	3.19	6090	520.5	8.55

Chapter 4

Discussion

4. Discussion

Conventional methods for detecting CNS infection with alphaherpesviruses (HSV-1, HSV-2, VZV) and determining intrathecal IgG antibody production are time and sample consuming. They can exhaust up to 1 ml of sample and the assays may take more than 24 h to produce results. A more rapid assay requiring minimal sample volume and time to produce results would therefore be an important diagnostic tool for use in patients with CNS infection. The overarching aim of the present study was to improve the diagnosis of viral CNS infections. In the present study an immunoassay capable of detecting HSV-1, HSV-2 and VZV IgG and human IgG antibody and albumin within serum and CSF samples was developed. A number of factors were taken into consideration in the development of such an assay including assay sensitivity, specificity, simplicity, reproducibility, reliability, multiplicity, cost per test and time to result. The developed assay requires approximately 2.5 hours for full analysis of results and uses as little as 2 µl of sample.

In recent years the need for high-throughput screening and rapid progress in serological assays has stimulated the development of a number of new serological array methods such as the Luminex test (Hsu *et al.,* 2009). The Luminex technology was developed based on an earlier technology used for the enumeration and characterisation of suspensions of mammalian cells called flow-cytometry. The assay uses up to 100 different microsphere sets dyed by internal red and infrared fluorochromes with different intensities (Figure 1.6). Each microsphere set can be coupled individually with a different analyte (antigen, peptide, antibody) through a simple chemical conjugation. The captured analyte can then be revealed through the attachment of a second ligand labelled with a fluorophore (phycoerythrin). The conjugated microspheres are then lined up in a single file to enter the detection chamber, where two lasers are used for detection. A red laser (635nm) is used to excite the internal red and infrared dyes of the microsphere which allows the classification of each microsphere (by determination of the region number), and a green laser (532nm) detects the second labelled antibody associated with the binding of the analyte (Figure 1.7). Data gathered from the detection chamber can then be analysed via software that controls the instrument and results are displayed as a histogram and bead map which is continuously updated during the reading process (Giavedoni 2005).

The Luminex system was first used by Lisi *et al*., (1982) to develop an assay for detecting soluble antigens (IgG), and since that time a number of assays have been established to detect and quantify antibodies against several targets (Mc Hugh *et al*., 1986; Mc Hugh *et al*., 1989; Scillian *et al*., 1989; Syrjala *et al*., 1991; Best *et al*., 1992; Stewart *et al*., 1993; Laakel *et al*., 1996; Drouvalakis *et al.,* 1999). Results of these studies have proven the speed, efficiency and utility of the Luminex technology for simultaneous, rapid, sensitive and specific antibody detection. It has been suggested that it could be an alternative method to ELISA, since it reduces labour, sample volume, and reagent costs, as well as assay time. Nowadays, with the advances in instrumentation, software, microsphere technology and assay reagents, Luminex based assays have been widely used to detect and quantify antibodies against several types of viruses (Dias *et al*., 2005; Waterboer *et al*., 2005; Johnson *et al*., 2005; Lukacs *et al*., 2005; Dias *et al.,* 2005; Martins *et al.,* 2008; Binnicker *et al.,* 2008; Watson *et al.,* 2009; Martins *et al.,* 2009a; Xia *et al.,* 2010).

Although the Luminex technology seemed to be a promising approach in this project for the diagnosis of herpes infections, a major challenge in developing such an assay was obtaining high quality, fully functional proteins for each virus that displayed conformation-dependent epitopes capable of detecting type specific/common antibody in serum and CSF samples. As stated earlier, herpesvirus glycoproteins are highly immunogenic. Among these glycoproteins HSV gD and VZV gE are the most abundant of the glycoproteins found on the virion surface and they elicit both humoral and cell mediated immunity. A number of prokaryotic and eukaryotic expression systems have been used for *in vitro* production of HSV gD and VZV gE including bacteria (Watson *et al.,* 1982), yeast (Valenzuela *et al.,* 1983; Stanberry *et al.,* 1987; Van Kooij *et al.,* 2002), Vaccinia (Paoletti *et al.,* 1984), mammalian cells (Stanberry *et al.,* 1988; Haumont *et al.,* 1996) and baculovirus (Krishna *et al.,* 1989; Ghiasi *et al.,* 1991;Landolfi *et al.,* 1993; Sisk *et al.,* 1994; Kimura *et al.,* 1997; Ikoma *et al.,* 2002). Although *E.coli* and yeast expression systems have been used for the last 20 years as an invaluable resource for production of large quantities of proteins, proteins expressed by these systems lack many of the post translational modifications associated with proteins produced by eukaryotic cells (glycosylation and cleavage). Other systems are also limited in terms of safety and the quantity of recombinant protein produced. However, baculovirus expression systems have been found to be the most useful system to produce fully functional glycosylated

glycoproteins similar to their authentic counterparts from mammalian cells (Fotouhi *et al.,* 2008).

Several important features that make baculovirus a successful expression vector include; (1) its large genome (134 kb) which contains a number of non essential genes that can be replaced by an exogenous gene; (2) many of these genes, particularly the very late ones, are under the control of a powerful promoter (polyhedron and P10) that allow abundant expression of the recombinant gene (Ooi *et al*., 1989). Finally baculoviruses are safer expression systems as compared to other mammalian viruses due to their limited insect host range. The baculovirus expression system is based on the introduction of a foreign gene into a non-essential region of the viral genome via homologous recombination with a transfer vector containing the cloned gene. The production of foreign protein is then achieved by infection of insect cell cultures with the resultant recombinant virus. The system was first used to express HSV-1 gD by Krishna *et al.,* (1989), aiming to study the antigenic regions within HSV-1 gD that are recognised by the immune system. In the study, recombinant baculovirus was generated using HSV-1 strain SC16 DNA as template to clone the full ORF of gD gene. SF21 cells were transfected with the recombinant virus, and the expressed gD was purified and characterised using SDS-PAGE and immunoblotting. The study showed that HSV-1 gD was expressed in insect cells, and appeared as 48kd, however it was slightly smaller than virion HSV-1 gD made in baby hamster kidney (58kd). The study found that antibody taken from mice infected with HSV-1 reacted with both proteins. Immunisation of mice with an extract of the expressed gD induced a high titre of complement and independent neutralising antibody (Krishna *et al.,* 1989). In a further study to investigate the possibility of using baculovirus to express a large quantity of HSV-1 gD, Ghiasi *et al.,* (1991), constructed a recombinant baculovirus containing the full-length of HSV-1 gD using HSV-1 strain KOS as the template. In the study, SF9 cells were transfected and the recombinant protein was purified using immunoaffinity selection. Characterisation of the recombinant protein was carried out using SDS, immunofluorescence, western blot and ELISA. The study showed that recombinant protein was expressed, transported to the membrane of Sf9 cells and reacted with gD specific antibody. Although the expressed protein was slightly bigger than that reported previously (52kd), it still appeared to be slightly smaller than HSV-1 gD made in Vero cells. Landofi *et al.,* (1993) also expressed the full-length of HSV-2 gD, aiming to study the viability of using gD2 as a vaccine subunit. In the study, recombinant

baculovirus was generated using HSV-2 strain 12 DNA as the template to clone the full-length gD gene. Sf9 cells were transfected and the recombinant protein was solubilised using CHAPS detergent and purified. Characterisation of the recombinant protein was carried out using SDS denaturation, Western blot and ELISA. The study showed that HSV-2 gD was expressed in insect cells and appeared as 57.5kd. In addition the expressed protein reacted with the gD specific monoclonal and polyclonal antibody. Serum obtained from mice immunised with the recombinant gD2 reacted with both native gD1 and gD2 proteins. Similar results were also obtained by Fotouhi *et al.,* (2008), when expressing gD2 in Sf9 cells. Recombinant baculovirus containing the full-length HSV-1 gD was used to transfect Sf9 cells and the expressed protein was characterised using SDS, Western blot and ELISA. Western blot results showed two bands with approximately molecular weights of 41-43 kd and 52-55 kd. The latter corresponding to the glycosylated form of gD2, in addition, immunisation of guinea pigs with the recombinant protein elicited humoral responses. Other truncated soluble forms of HSV-1 gD have also been expressed in baculovirus for various reasons. Sisk *et al.,* (1994) expressed two forms of gD, a truncated form gD-1(306t) that has three signals for addition of N-Linked oligosaccharides (N-CHO) and a full-length gD (QAA) that contain 3 mutations to eliminate the addition of N-CHO. Both proteins were expressed in insect cells, however the latter was retained in the cell, rather than being transported to the cell membrane. The other protein was secreted in the media. Western blot analysis showed that both proteins reacted with specific monoclonal antibody. Protein yield obtained from each form was different, in the case of the gD (306t) the protein yield was 20mg/l whereas it was 1 -5 mg/l from the gD (QAA).

In the case of VZV, Wu *et al.,* (1997), expressed the full-length and truncated forms of VZV gE (the amino-terminal (N) region and the carboxy-terminal (C) region), aiming to determine the neutralising epitopes found within gE using a library of monoclonal antibodies (MAbs). Three recombinant baculoviruses were generated using VZV Boston strain as a template to clone the different forms of gE. Sf9 cells were transfected with the recombinant viruses, and the expressed gD was characterised using SDS-PAGE, immunofluorescence and Western blot. The study showed that all forms of gE were expressed 72 h p.i in insect cells and, based on the immunofluorescence results, the localisation of the expressed protein was found inside the cell and in the cell membrane. In addition Western blot results showed that all MAbs reacted strongly with 4 protein bands with the apparent molecular weight ranging between 70 to 98kd which resembles the full-

length glycosylated form of gE. Furthermore, dot blot analysis of the cell media also reacted with the antibodies, indicating that another soluble form of gE was secreted into the media. This soluble form resembled the N region of the gE since it did not contain a TMR core and was therefore secreted in the cell media. The study concluded that VZV gE contains 3 distinct antigenic domains which reacted with all the MAbs.

Although the baculovirus system has been widely used for the production and study of structural and non-structural proteins of a number of viruses including, human polyomavirus, poliovirus, influenza virus, human papillomavirus and herpesvirus (Possee, 1986; Neufeld *et al.,* 1991; Ghiasi *et al*. 1991b; Sisk *et al.,* 1993; Landolfi *et al.,* 1993; Ghiasi *et al*., 1994; Kimura *et al*., 1997; Kimura *et al.,* 1998; Ikoma *et al.,* 2002;) traditional baculovirus expression methods have several disadvantages in terms of low frequency of recombination, protein production and the requirement for several rounds of plaque purification (Fotouhi *et al.,* 2008). Nowadays, most of these problems have been overcome by improved cloning methods, transfer vector generation, recombinant virus isolation, cell culture technology and simplification of recombinant protein purification.

In order to determine a suitable strategy for protein expression and purification using baculovirus, a number of commercially available expression systems (Bac-N-Blue, Bac-to-Bac, BaculoDirect (Invitrogen), InsectDirect system, BacMagic system, BacVector system (Merck Bio Sciences)), and purification methods (Immunoaffinity chromatography and His-Tag purification strategy using NI-NTA resin) were critically evaluated. Based on the evaluation results two systems were found to be suitable to be used in the present study, the InsectDirect and BacMagic expression systems. These systems employ a combination of expression vector, high efficiency transfection reagent, lysis reagents and Ni- NTA His-Bind Resin for affinity purification of his-tag fusion proteins (Loomis *et al*., 2005). Selection of these systems was based on a number of factors including; (1) simplicity and efficiency of vector plasmid construction; (2) no requirement for restriction enzyme digestion; (3) plaque assay purification was not required; (4) plasmid vectors contained a 10 his-tag on the N-terminal to simplify purification of the expressed proteins; (5) both systems could use the same plasmid construct and cell type. To date, this is the first time that these systems have been used for expressing HSV and VZV glycoproteins.

The InsectDirect System is a plasmid-based expression system that enables rapid small-scale expression in insect cells. It can be scaled up to isolate milligram quantities of target protein without the need to generate a recombinant baculovirus. However, as expression of the target protein in large-scale was required the BacMagic expression system was also selected. The BacMagic system is an improved baculovirus expression system that consists of an *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome that lacks a 1629 bp region of the ORF of the polyhedron (*polh*) coding region. This deletion in the *polh* coding region was replaced by bacterial artificial chromosome (BAC). During reconstruction of the recombinant virus in insect cells, only homologous recombination within the cells can restore the function of the viral ORF 1629 and replace the BAC sequence in the BacMagic DNA with a promoter and the target coding sequence. This unique combination has two important features:- it prevents non-recombinant baculovirus from replicating in insect cells, and it allows the viral DNA to be propagated as circular DNA in bacterial cells (competent cells). Because only recombinant baculovirus can replicate in insect cells, a plaque assay step is not required. In traditional baculovirus expression systems plaque assay is required in order to isolate and purify the recombinant virus and this process may take from 7 to 14 days.

In order to get the full benefit of these two expression systems, the pIEx/Bac-3 3C/LIC plasmid vector was chosen. This vector is a dual-purpose vector that offers unique flexibility for cloning and expression of proteins in insect cells. It can be used directly for transfection of insect cells to produce the recombinant protein or as a donor plasmid that can be used to generate a recombinant baculovirus in the BacMagic system. The pIEx/Bac-3 3C/LIC plasmid vector has a unique 3C/LIC site which is designed for directional cloning of the gene of interest efficiently and without the need for restriction enzyme digestion (Loomis *et al*., 2005). Moreover, it contains the baculovirus enhancer/immediate early promoter combination (hr5/ie1) for plasmid-mediated expression and early baculovirus expression (see Figure 3.9 for plasmid circular map). Furthermore it contains the baculovirus p10 very late promoter for robust baculovirus-mediated expression. This vector also encodes a 10 his-tag sequence at the N-terminal to simplify purification of expressed protein.

In order to clone HSV-1 gD and VZV gE genes into the LIC site of the plasmid vector the appropriate gene sequences that are capable of producing fully functional gD and gE proteins in insect cells had to be identified. Identification of the sequences was carried out by comparing several published sequences for each virus obtained from GenBank. Alignment results of these sequences revealed that both genes are highly conserved among HSV and VZV isolates and no variation was observed. Therefore, a single sequence for each virus was selected to design a primer that amplifies the full ORF of each gene. Primers were designed manually using the selected sequences. The primers start with sequences at the 5'-ends to create a specific vector-compatible overhang, followed by the first ATG in the coding region sequence. A stop codon (TAG) was also included at the 3' ends and no HSV or VZV noncoding sequences were present after the stop codon. Blast alignment of both primers sequence showed homology of approximately 100%.

For amplifying HSV-1 gD a number of cycling parameters and conditions were evaluated. Since the final aim of the amplification was to produce product of the correct size rather than to develop a sensitive PCR assay for diagnostic purposes, the sensitivity of the PCR was not tested. Initial experiments using routine cycling parameters and conditions routinely used in the department for HSV-1 amplification were used. However this approach produced a poor yield of product, inadequate for the purposes required. Therefore, these parameters were optimised by alteration of magnesium chloride ($MgCl_2$) concentration, increase of the annealing temperature to 58°C and extension of each step in the cycling by 30 seconds. These optimising conditions significantly improved the amplification of HSV-1 gD and PCR results showed an adequate yield of PCR product of the correct size.

A similar difficulty was encountered in amplification of VZV gE, cycling parameters, and conditions were adapted from an existing PCR used in the department, however in this instance no PCR product was detected at all. Alteration of magnesium chloride concentration and cycling parameters did not improve the PCR results. At first it was suspected that the starting material (VZV DNA) used as a template was not viable. Therefore a PCR using a different primer that amplifies a 275pb product in the thymidine kinase gene of VZV DNA was used. Results showed a distinct band with the expected size. This suggested that the starting material was viable and the PCR protocol needed to be further optimised. Therefore a further round of optimisation step was carried out, and the

best results were obtained when the annealing step was increased from 1 min to 1.5 min at 58 ºC and a PCR product with the correct size was produced.

The cloning procedure for HSV-1 gD and VZV gE was carried out as described by the manufacturer. PCR products for each virus were purified to remove any remaining primers, dNTPs, or short PCR products and the concentration of the purified PCR product was calculated by comparing the appearance of the PCR product band on 2% E gel with a low range quantitative ladder. Ten µl of the PCR product was estimated to contain approximately 0.2 pmol of DNA. As mentionend previously the pIEx/Bac-3 3C/LIC plasmid vector contains the LIC site, this method takes advantage of the 3′ → 5′ exonuclease activity of T4 DNA Polymerase to create very specific 11 or 12 base single-stranded overhangs in the presence of dATP. Therefore, the PCR product was treated first with LIC-qualified T4 DNA Polymerase to generate the specific vector-compatible overhangs. Constructed plasmid containing either gD or gE and the necessary expression cassettes was generated efficiently using a ligation-independent cloning strategy. Results were confirmed by PCR using vector specific primers in one experiment, and a combination of vector specific sense primer with HSV-1 gD or VZV gE antisense primers in another (Figure 3.11 and 3.12). Such results suggest that both genes have been efficiently cloned in the right orientation within the LIC site of the plasmid and downstream of the P10 promoter. Additionally, it demonstrates the simplicity of generation of the pIEx/Bac-3 3C/LIC plasmid vector and efficiency of the cloning strategy. Recombinant plasmid was scaled up and purified using the UltraMobius plasmid kit, which provides a low endotoxin transfection-quality plasmid for transfection of insect cells. At this stage the recombinant plasmid required for the InsectDirect expression system (plasmid based expression system) and for generating recombinant baculovirus was ready to use. In order to construct the recombinant baculovirus containing either HSV gD or VZV gE the desired plasmid was mixed with BacMagic DNA in the presence of Sf9 cells. Generation of recombinant baculovirus was confirmed by the appearance of signs of infection in Sf9 cells and by PCR results obtained from amplification of the target gene and the surrounding sequences. Recombinant baculoviruses were then amplified and virus titer was determined (10^9 to 10^{10} $TCID_{50}$/ml).

For protein expression, several types of insect cells have been used previously including Sf9, Sf21 and High Five cell lines. These cells are suitable for InsectDirect and baculovirus

systems and can produce a fully functional protein similar to the native form. Although High Five cells allow better protein secretion and post-translational processing of recombinant proteins, compared to Sf9 and Sf21, they are difficult to grow and maintain in routine lab work. Therefore Sf9 and Sf21 are used for protein expression more frequently. Sf21 cells are somewhat more disparate in size and form producing rather more irregular monolayers and plaques than Sf9 cells. Therefore Sf9 was selected for protein expression in this study. Protein expression was first carried out using the InsectDirect system on a small scale using 6 well plates and then scaled up to 10ml suspension culture. In order to transfect Sf9 cells with the recombinant plasmid an Insect GeneJuice Transfection Reagent (Merck property) was used. This Reagent is a liposome-based transfection reagent designed for transfection of insect cells (Loomis *et al.,* 2005). During protein expression using this system no signs of infection were observed in the transfected cells even 96 h postinfection. Cells continued to grow normally until they started to detach from the plate and the remaining cells, attached to the plate, started to lyse. This observation was in accordance with previous reports, confirming that recombinant plasmids use endogenous insect cell transcriptional machinery for production of the recombinant protein (Loomis *et al.,* 2005). The traditional cytopathic effect caused by baculovirus infection during protein expression is absent in this system. In this case protein expression can only be monitored by obtaining samples of transfected cells every day for SDS and IF analysis. In the BacMagic system optimisation of protein expression was carried out using 25 cm^2 flasks, this was then scaled up to 10ml and 300 ml of suspension culture. To determine the optimal expression conditions the effects of different MOI and harvesting times, were investigated. As for the InsectDirect system, samples from each step were analysed using SDS and IF.

Production of each protein in insect cells was confirmed and monitored using IF and SDS. Immunofluorescence is an important method that can provide valuable information on the production and localisation of the expressed proteins. Acetone fixed and unfixed transfected Sf9 cells with recombinant plasmid or baculovirus reacted with the appropriate monoclonal antibody. In addition there was no cross reactivity between each protein when it was treated with the opposite monoclonal antibody. Such results indicate that recombinant proteins were expressed in both systems and each expressed protein contained the epitopes that were recognised by the monoclonal antibody. The IF results also show the localisation of the expressed protein. In acetone-fixed cells the expressed protein was

found to be localised within the cytoplasm and the nucleus of the infected cells. However, in unfixed cells, the expressed protein was found in the cell membrane. These localisation results are in accordance with previous studies and confirm that recombinant proteins were synthesised and, because they have membrane anchored sequences, they were correctly transported to the cell membrane resembling the formation of glycoproteins (as when HSV or VZV infects cells) (Ghiasi *et al.,* 1991; *Wu et al.,* 1997; Olson *et al.,* 1997). Moreover SDS and IF results showed that HSV-1 gD and VZV gE were expressed 48 h post infection in both systems. However the highest level (95% by IF) of expression of protein was observed between 72h to 96h post infection. In addition the peak time for protein expression in the BacMagic system was obtained at an MOI of 10 combined with a harvesting time between 72 to 96 h post infection. During optimisation of protein expression of the BacMagic system it was noticed that harvesting the cells after 96h did not increase the production of the protein. This suggests that a shut down in cell protein production occurred during baculovirus protein expression (O'Reilly *et al.,* 1992). Thus, an MOI of 10 with cell harvesting time between 72 to 96 h were considered to be the optimal conditions for production of both proteins in the BacMagic system and these were used in large scale protein expression.

Immunoreactivity of the recombinant proteins was also studied by Immunoblot (western blot and dot blot) using monoclonal antibody targeted against each protein. In immunoblot, samples (cell lysate, solubilised protein, purification flow and wash, transfection media and negative control (solubilised protein from uninfected cells) were analysed using the appropriate monoclonal antibody. Results showed that cell lysate, solubilised proteins and purified proteins samples reacted with the monoclonal antibody, whereas the negative control as well as the media of the transfected cells did not. This confirmed that both proteins were expressed and either retained in the cell or cell membrane and not secreted into the medium. Such results are in accordance with previous studies (Ghiasi *et al.,* 1991; Wu *et al.,* 1997; Fotouhi *et al.,* 2008). SDS and western blot results showed that gD and gE were expressed and present as proteins of relative molecular weight of 64 kd and 98kd respectively. In the case of HSV-1 gD the observed size (64kd) was larger than any previously reported recombinant protein expressed in insect cells using other baculovirus expression systems (Krishna *et al.,* 1989; Ghiasi *et al.,* 1991; Fotouhi et al., 2008). Such results suggest that in this project the baculovirus expressed gD underwent more extensive post-translation addition of carbohydrate than other reported proteins using this system. In

the case of VZV gE the observed size (98 kd) was similar to that reported by Wu and Forghani (1997) and bigger than that reported by Olson *et al.,* (1997). In both studies VZV gE was expressed using traditional baculovirus systems under the control of the polyhedron promoter. The results obtained from SDS, immunoblot and IF assays confirmed that these recombinant proteins were antigenically and functionally similar to their authentic counterparts, therefore production and purification of these proteins was carried out.

As stated previously, one of the unique features of using this expression system is the simplicity of purification of the recombinant protein. The plasmid contains a 10 polyhistidine tag sequence upstream of the cloning site (3C/LIC). In this case the fused protein will be expressed with an N-terminal his-tag, which can be used later to purify the expressed protein using the nickel-nitrilotriacetic acid (Ni-NTA) system. In addition, expression of the target protein immediately downstream of the HRV 3C protease cleavage site helps the removal of the encoded fused sequences after purification of the recombinant protein if it is needed. Other purification methods have been also reported in similar experiments such as immunoaffinity chromatography, immunoselection using magnetic beads, high-performance liquid chromatography (HPIEC) and gel filtration (Ghiasi *et al.,* 1991a; Sisk *et al.,* 1994; Damhof *et al.,* 1994; Welling-Wester *et al.,* 1998). However, the Ni-NTA method has proven to be a powerful tool for purification of a number of his-tag recombinant proteins (Westar *et al.,* 2001; Dobrovetsky *et al.,* 2007). The Ni-NAT is an immobilised metal affinity chromatography (IMAC), which works on the interaction between the Ni^{2+} ions immobilised on the NTA matrix and specific amino acid chains like histidine. Peptides that contain the polyhistidine tag can be retained by IMAC and eluted using imidazole or by altering of the pH concentration. Based on the immunofluorescence results, the localisation of the recombinant protein was found within the cell and cell membrane. Proteins found within the cell resemble the non or partially glycosylated form, whereas the full glycosylated form is found on the surface of the infected cells. The latter proteins are similar to those found on the native virus and contain all epitopes that are recognised by human antibody. Therefore the next focus of the work was to purify these proteins.

Purification of integral membrane proteins is always challenging since the aim is to isolate these proteins in their native form in a highly purified state. In general integral membrane

proteins consist of two parts; a hydrophilic part exposed to the outer environment and a hydrophobic part bound to the hydrocarbon chains of the lipids in the cell membrane. Thus, in order to isolate these proteins the cell wall of the transfected cells must be disrupted first. For disruption of cell walls several methods are used; mechanical (glass beads, sonication, high-pressure homogenizer) and non mechanical (enzymatic methods). However, based on critical review of previous studies similar to this work we decided to use detergents for isolation of these proteins (Landolfi *et al.,* 1993; Damhof *et al.,* 1994; Welling-Wester *et al.,* 1998). Solubilising proteins using detergent is based on mimicking the lipid bilayer found in the insect cell wall. Detergents contain a polar group at one end and a long hydrophobic carbon chain on the other. During the solubilising process the polar group of the detergent binds with water molecules via hydrogen bounds, while the hydrocarbon chains aggregate via hydrophobic interaction. This aggregation process leads to the formation of micelles. In such cases membrane proteins can incorporate these micelles via hydrophobic interaction. The hydrophobic part of the protein will be surrounded by several layers of detergent molecules and the hydrophilic part will be exposed to the aqueous medium. The resulting protein-detergent complex allows such proteins to stay in solution.

Several types of detergents are available for solubilising membrane proteins and selecting the most suitable detergent is important since some proteins are susceptible to denaturation during isolation. Previous studies have used different types of detergents and buffers for solubilising proteins such as 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), N-dodecyl-N,N-dimethylammonio)undecanoate (DDMAU), N-dodecyl-N,N-dimethylammonio)butyrate (DDMAB), SDS, pentaethyleneglycol monodecyl ether ($C_{10}E_5$) and radioimmunoprecipitation assay buffer (RIPA) (Krishna *et al.,* 1989; Landolfi *et al.,* 1993; Damhof *et al.,* 1994; Welling-Wester *et al.,* 1998).

During protein solubilisation several attempts were carried out to optimise solubilisation of HSV-1 gD and VZV gE using RIPA buffer and two types of detergents (CHAPS, $C_{10}E_5$). However the best result was obtained using a method described previously by Welling-Wester *et al.,* (1998) using a non ionic detergent ($C_{10}E_5$) with some modification. Welling-Wester *et al.,* (1998) studied the viability of using DDMAU, DDMAB, $C_{10}E_5$, Octylglucoside, Dodecyl-b-D-maltoside, and Hecameg detergents for extraction and purification of HSV-1 gD recombinant protein expressed in insect cells. In their study, gD

recombinant protein was solubilised with 1% to 2% final concentration of each detergent, and the recombinant protein was purified using High-Performance Liquid Chromatography (HPLC). Protein purification results showed that the optimum protein yields were obtained using 1% DDMAU, DDMAB and $C_{10}E_5$ (24.3-37.8 mg/L). Although the DDMAU and DDMAB gave similar and slightly higher protein yields (37.8 mg/l) than $C_{10}E_5$ (24.3 mg/l), these detergents could not be obtained commercially. Therefore $C_{10}E_5$ was used in this study. Recombinant protein solubilisation was carried out as described in the previous study but extending the incubation step from 1 h to 2 h on ice. Solubilisation was assessed by SDS and immunoblot assay. Results showed that recombinant proteins could be solubilised from the insect cell membrane.

The supernatant of the solubilising mixture after ultracentrifugation was used for protein purification. The first attempts to purify gD and gE were unsuccessful. Based on the purification kit instructions a 1x dilution of the 4x elution buffer (4 M imidazole, 2 M NaCl, 80 mM Tris-HCl, pH 7.9) provided within the kit can be used for eluting most of the his-tag proteins. However SDS and dot blot results showed that after elution the protein was observed in the solubilised sample, but not in other samples taken from the purification process (column flow, 1^{st} wash, 2^{nd} wash and eluted sample). These results suggests that the recombinant protein was bound to the resin, and was not eluted using 1x elution buffer (1M imidazole, 0.5 mM NaCl, 20mM Tris-HCl, pH 7.9). Therefore, in an attempt to optimise the elution step a serial concentration of the elution buffer was used. The best result was obtained by increasing the concentration of the elution buffer to 3x (3M imidazole, 0.70 mM NaCl, 26.7mM Tris-HCl, pH 7.9). The need for this high concentration of imidazole suggests that the recombinant proteins were bound strongly to the Ni^{2+} resin. This strong binding of the recombinant protein with the resin may be related to the length of the histidine tag. Indeed such results were reported previously by Mohanty *et al.,* (2004). In their study the effect of the polyhistidine tag length (6 his or 10 his) and position (N or C terminal) during expression of membrane proteins in *E.Coli* was investigated. The study concluded that both the length and position significantly affected protein yield, purification, and solution properties. Expression of proteins with a 10 histidine tag increases the binding affinity of the protein to the resin, but neither affect detergent extraction nor change the secondary structure of the protein (Mohanty *et al.,* 2004). The protein concentration obtained by InsectDirect and BacMagic expression systems as determined by BCA protein assay was higher than previously reported for the

full-length of HSV-1 gD (6 mg/L using InsectDirect and 15mg/L using BacMagic) (Sisk *et al.,* 1994). As for HSV-1 gD, similar VZV gE protein yields were obtained using both systems.

In general, HSV-1 gD and VZV gE glycoproteins were expressed in both expression system as early as 2 days p.i, and yielded the same proteins with no differences in size or immunoreactivity. The only differences found between the two systems were the protein yields. Such results were expected because each system was originally designed for different purposes. Although the InsectDirect system protein yield can be scaled up by using several culture bottles, it is not cost effective for production of recombinant protein in large quantities, since reagents such as plasmid purification kits and insect GeneJuice are required constantly for protein expression. However the system is ideal for small-scale screening and production of recombinant protein. In contrast the BacMagic system is a virus based system, therefore it is suitable for large production of recombinant protein. Virus titre can be scaled up and stored at +4 ºC with 5% foetal calf serum for as long as 6 months or can be stored at -70 ^{0}C for longer periods.

At this stage the immunoreactivity of the expressed type common antigen HSV (gD) and VZV gE proteins were tested by Western blot and dot blot methods using the appropriate monoclonal antibody. However in order to differentiate between HSV-1 and HSV-2 antibody in the proposed assay, a novel immunodominant epitope of glycoprotein G2 presented in a branched chain format (peptide 55) was utilised. HSV-1 and HSV-2 have approximately 83% nucleotide sequence similarity, and for some proteins they share more than 85% identity (Dolan *et al.,* 1998). Both serotypes therefore show extensive serological cross-reactivity. However, a variety of studies have concluded that only glycoprotein G (gG2) expresses epitopes specific for HSV-2 (Marsden *et al.,* 1984; Roizman *et al.,* 1984). Consequently, this glycoprotein has been used as the basis of type-specific serological assays for the diagnosis of HSV-2 infection (Ashley *et al.,* 1988; Ashley *et al.,* 1998; Hashido *et al.,* 1997; Parkes *et al.,* 1991; Ribes *et al.,* 2002). Epitope mapping of gG2 by Levi *et al.,* (1996), suggested that some epitopes may cross-react with HSV-1-specific antibody. Therefore in an effort to develop even greater selectivity for HSV-2, several research groups have studied HSV-2 type-specific epitopes within gG2. Regions comprising amino acids 350 to 427 and 525 to 587 and also regions comprising amino acids 625 to 641 and 676 to 699 were identified by two independent groups (Grabowska *et*

al., 1999; Levi *et al.,* 1996), and a secreted protein of gG2 comprising amino acids 23 to 340 was described by Liljeqvist *et al.,* (2002) and Gorander *et al.* (2003). Marsden *et al.* (1998) also developed multiple antigenic peptides corresponding to residues 561 to 578 of gG2, designated peptide 55. This peptide comprises four peptide copies attached to a branched lysine core and separated from the lysine core by four glycine residues. The sensitivity of the peptide for the detection of HSV-2 antibody was evaluated by screening a panel of HSV-2-positive human serum samples previously characterised by virus isolation and typed by indirect immunofluorescence with a type-specific monoclonal antibody. Peptide 55 was found to be sensitive and specific and no false-positive results were detected with HSV-1 antibody positive or HSV-1 or HSV-2 antibody-negative samples. In addition Oladepo *et al.,* (2000) compared the performance of an assay based on peptide 55 with that of a commercially available HSV-2 specific IgG ELISA assay kit based on affinity-purified gG2 (Gull Laboratories) and found that peptide 55 had the same sensitivity. However, the assay using peptide 55 was found to have higher specificity than that using the complete protein. Nilsen *et al.,* (2003) tested the performance of an ELISA based on peptide 55 and compared it with the performance of two different assays; an ELISA developed by Ho *et al.,* (1992) based upon native gG2 selected by affinity to *Helix pomatia* lectin, and an ELISA based on affinity-purified gG2 (Gull Laboratories), and found that the performance of the assay with peptide 55 was better than that of the other ELISA methods.

Based on the results of these previous studies, peptide 55 seemed to be a good candidate for the proposed assay. However, in order to confirm previous reports, and to test the sensitivity and specifity of this peptide, a comparative study between an ELISA assay based on peptide 55 (Omega) with another type specific HSV-2 ELISA assay (Bioelisa and HerpeSelect) was carried out. In the study the sensitivity and specificity for detection of HSV-1 IgG antibody with each assay was tested by screening a well characterised serum panel (panel 1). This panel consisted of 254 samples which had been previously tested for the presence of HSV-1 and HSV-2 antibody using type common and type specific assays. Based on these earlier assay results the panel was subdivided into 4 different groups (section 3.10). In order to evaluate the performance of peptide 55 and the other ELISA kits for detecting HSV-1 IgG antibody in this panel, all samples were tested with all three assays. Comparative results showed that the overall agreement between the three kits was 90.94% (231/254), and no false-positive test results were detected by any of the three

assays. The 23remaining discordant samples (9.06%) were further tested using the Western blot assay. Results showed that 21 samples were positive for HSV-2 and 1 was negative. The remaining sample was counted as Western blot equivocal since no gG1 or gG2 band could be detected. Using the HerpeSelect assay as a reference test, the sensitivity and specifity of the Omega and Bioelisa assays were calculated. The Omega assay showed higher sensitivity in detecting HSV-2 antibody (93.6%) when compared to the Bioelisa (71.6%). This may indicate that the antigen used by the Omega assay (peptide 55) is more sensitive than the one used by the Bioelisa. On the other hand, the specificity of the Bioelisa assay was slightly higher (98.3%) when compared with the Omega assay (96.6%). Furthermore, comparison of the results obtained from the Omega assay for the 4 designated groups, showed that the total number of samples correctly identified by the Omega assay was 249 (98.0%) out of 254 samples tested. Such results reinforce the view that the region comprising peptide 55 is clearly an immunodominant epitope and that the design of anchoring multiple copies of peptide 55 within a branched lysine core allows contact with both arms of an antibody having the cognate paratope and permits the highly sensitive detection of antibodies of even low affinities. In addition, although the concordance between the individual assays for HSV-2 was relatively poor and was poor in comparison to the "gold standard," the Western blot immunoassay, of the three assays evaluated, the results of the Omega assay showed the greatest concordance with those of Western blotting. Such results were not unexpected. Western blotting identifies antibody reactivity with separated bands of protein. It is therefore of inherently greater specificity than an assay reliant upon the detection of antibody to a single protein antigen or peptide.

The comparative study results showed the superior performance of the Omega assay in comparison to the performance of the Bioelisa and the HerpeSelect assays, in accordance with data provided previously (Oladepo *et al.,* 2000*;* Nilsen *et al.,* 2003). On the basis of these results peptide 55 was found to be suitable to be used in the proposed assay for differentiation between HSV-1 and HSV-2 antibody. In order to evaluate the sensitivity and specificity of the baculovirus expressed proteins of HSV gD and VZV gE for the detection of human antibody, in-house ELISAs for each protein were developed.

Initially an in-house ELISA assay for each antigen was developed and optimised by checkerboard titration using the recombinant HSV-1 gD and VZV gE proteins. During this stage a number of factors were investigated including antigen concentration, ELISA plate

type, antigen diluent buffers, number of washing steps required, conjugate concentration and the incubation time required between each step. A constant amount of each antigen (50µg/well) diluted in 1 X PBS (pH 7.3), incubated overnight at 4 ºC was found to be the optimum protein concentration for coating each of the Costar ELISA plates (medium binding). In addition, ELISA results showed that the maximum OD reading was obtained using this antigen concentration in combination with 1/100 dilution of the standard positive human serum, detected with 1/2000 of anti-human IgG conjugate. ELISA assay reproducibility was tested by measuring intra and inter assay variation using 10 human samples. Results showed that the reproducibility of the developed ELISA assays using these conditions was less than 10%. Therefore these conditions were selected for the standard ELISA procedure, in which all serum panels were tested.

The performance of the developed in-house ELISA assays in the present study were evaluated by screening 6 well characterised serum panels using the ELISA standard method. For evaluating HSV-1 gD ELISA, 5 serum panels were used. Using panel 1 sera (previously used to evaluate peptide 55), gD ELISA assay showed that 86.2% (219/254) of the samples were positive and 13.8 % (35/254) were negative for HSV. Of these 254 samples 150/254 were previously tested with a type common HSV assay (DiaMedix Herpes 1+2 ELISA). In the DiaMedix assay, a mixture of purified and inactivated herpes simplex virus 1 and 2 were used to coat the polystyrene microwells. Comparison of gD ELISA assay results with the DiaMedix results showed that both assays agreed in 90% (135/150) of the samples (Table 3.18). The remaining 15 samples were found to be positive by the gD ELISA and negative by the DiaMedix assay. Interestingly, 12 of these samples were positive by the Virotech HSV-1 IgG assay and 2 were positive by the Bioelisa HSV-2 IgG assay. Thus, it appears that the gD ELISA detected HSV positive samples that the DiaMedix assay missed, suggesting a higher sensitivity. The higher level of detection may be due to the type of antigen used in the assay. In addition, of the false negative results obtained by the DiaMedix assay 10% (15/150) may be due to the fact the antibody level in these samples was below the detection level of the assay. Comparison of the gD ELISA results with the remaining assays used for characterising the panel or used to test the performance of peptide 55 showed 100% agreement with gD ELISA (Table 3.20). Such results indicate the sensitivity of the developed assay for detecting HSV type common antibody.

Using the serum samples from panel 2, the HSV-1 gD ELISA showed 94.3% (66/70) agreement with the gold standard assay (Chiron assay). The remaining 4 samples were identified as negative by the Chiron assay but found to be positive by the gD ELISA. Originally, panel 2 sera were characterised using a number of commercially available assays, each assay used a different format of gG2 antigen (section 3.12.1.2). However, analysis of the results for these 4 samples obtained from the previous assays together with their clinical data showed that 3 samples were found to be positive by the CFT and IF assays and the remaining sample was positive by CFT assay. In addition the clinical data for all these samples indicate an HSV-1 or HSV-2 infection (Table 3.21). The most likely explanation for these results is that the type common antigen utilised in the Chiron immunoassay lacks specificity. The Chiron RIBA HSV-1 and HSV-2 Strip Immunoblot Assay utilised recombinant HSV-1 gG1, gG2 together with gD2 equivalents and a synthetic peptide for HSV-1 gB-1 immobilised on nitrocellulose strips. Therefore, it may be that the gD2 used in the assay lacks some of the epitopes needed for the recognition by the antibody. On the other hand, using the full-length gD in the in-house ELISA assay increases the possibility of recognition by the different types of antibody produced in the human sera against the several epitopes found within the gD antigen. An alternative explanation may be that the gD antibody titre found in this sample was not sufficient to allow binding to the gD2 found in the Chiron assay.

In order to evaluate the performance of the gD ELISA in a high HSV-2 prevalence population, serum panel 3 was used. Serum panel 3 consisted of 90 samples obtained from a population with a very high level of sexually transmitted disease (including large numbers with genital herpes). Originally samples had been tested to determine the HSV serostatus and HSV subtype using a number of ELISA assays including; HSV-1 and HSV-2 Bioelisa, Gull, CAPTIA Select HSV-2-G, Biotest, Clark assays and the Chiron RIBA HSV Type 1 and 2 Strip Immunoblot Assay (the gold standard assay). In this panel, the gD ELISA assay showed 100% agreement with the Chiron assay. Such agreement in this panel was not unexpected, because in such a group the production of antibody against type specific antigens (gG) usually occurs later after the appearance of the type common antigen (gD) antibody.

In order to further evaluate the performance of the gD assay in a different population 200 samples selected from among 8000 pregnant women of different age groups attending an

antenatal clinic was tested (panel 4). These samples were previously tested for HSV IgG antibody using the Bioelisa HSV-1 IgG and HSV-2 type specific assay (Appendix B Table 3). The gD assay results showed a 73.5% (147/200) agreement with the previous results. The remaining 53 samples were positive by the gD ELISA and negative by both of the Bioelisa assays. In this panel no type common assay had been used to determine the serostatus of these samples, however, looking to the age of these patients reveals they were all over 20 years of age. Based on seroprevalence studies in developed countries, 85% of the population become seropositive by early adolescence (Whitley *et al.,* 2001; Pebody *et al.,* 2004). Therefore, in this case it is likely that all these patients have been exposed to HSV-1 infection. Such results indicate that in a clinical setting screening samples for HSV serostatus should be performed using a type common assay rather than type specific assays.

Similar findings were obtained following screening of serum panel 5. This panel consisted of 60 samples obtained from 75 patients attending a GUM clinic. The samples had been previously tested for HSV IgG antibody using Bioelisa HSV-1 and HSV-2 IgG assay. The patient ages ranged from 13 to 67 years and virus isolation results for 45 out of 60 samples were available. The gD ELISA assay showed 66.67% (40/60) agreement with the Bioelisa HSV-1 and HSV-2 IgG assy. The remaining 20 samples were positive by the gD ELISA and negative by both Bioelisa assays. Analysis of the virus isolation results of theses samples showed that in 13 samples, the virus was isolated, whereas it was not in the other 7 samples. In addition patient age ranged from 15 to 53 years. It is know that the antibody response could be detected in the absence of the virus. Indeed, in 7 samples from this panel, although no virus was isolated, IgG antibody for HSV was detected. In addition, the age of the patients suggests that they would be likely to have had HSV-1 infection.

For evaluation of the VZV gE ELISA assay two serum panels were used; panel 1 and panel 6. As stated earlier, panel 1 serum consisted of 254 samples, 150 of these samples were tested previously for VZV IgG antibody by Human VZV IgG assay, whereas the remaining samples were not tested. Therefore in order to provide a baseline test for comparison all sera were retested for VZV IgG using the Liaison VZV IgG assay. The Liaison VZV IgG assay is an automated quantitative assay that uses magnetic particles coated with VZV antigens (partially purified extract of infected cell cultures, ROD strain). Using this panel, gE ELISA assay showed 98.8 % (251/254) agreement with the Liaison VZV IgG assay.

The remaining 3 samples were positive by gE ELISA and equivocal by the Liaison assay even after retesting. Interestingly these 3 samples were positive by the Humana assay. In addition, comparing the 150 sample results obtained from the Humana assay with the gE ELISA results showed 100% agreement. The other panel used for evaluating VZV gE ELISA was panel 6. Panel 6 consisted of 100 samples previously tested by the Liaison VZV IgG assay. Furthermore of these 100 samples 37/100 had also been tested by the new AtheNA Multi-Lyte® MMRV IgG Plus assay. This new assay was developed by Zeus Scientific and is based on Luminex technology for detecting and determining IgG antibody response to Measles, Mumps, Rubella and Varicella Zoster virus in the same sample. The assay uses a mixture of beads conjugated with VZV antigen (the manufacturer's leaflets does not define which type of antigen was used) or other virus antigens. Using this panel, the VZV gE ELISA showed 99% (99/100) agreement with the Liaison and 100% (37/37) with the AtheNA Multi-Lyte® MMRV IgG Plus assays (Table 3.23). The equivocal sample in the Liaison assay was positive by the AtheNA Multi-Lyte® MMRV IgG Plus assay. When the Liaison assay is considered as the reference assay, the sensitivity, specificity, positive and negative predictive value for the in-house ELISA were 98.75%, 100%, 100%, 100% in panel 1 and 98.93 %, 100%, 100%, 100% in panel 6 respectively. The high correlation between the two assays and the gE ELISA indicates that the developed ELISA assay is suitable for detection of VZV IgG antibody in human serum.

In general the results obtained from all serum panels showed good correlation between the developed ELISAs and the commercial ELISA assays. Such high correlation indicates that the expressed proteins used in this assays are highly purified and sensitive. In addition the epitopes found in these proteins were correctly displayed. Therefore, based on all the above results it was concluded that both proteins and peptide 55 were suitable to be used in the triplex assay.

For the purpose of developing the triplex assay peptide 55 was kindly provided by Dr Howard Marsden, Institute of Virology, Glasgow. Initially an assay for each analyte was developed individually as a monoplex assay by direct conjugation of each antigen (gD, gE, peptide 55) to the carboxylated microsphere. Previous studies have reported the instability of some monoclonal antibodies during the conjugation procedure, because some antibodies cannot tolerate adherence to a solid support (Hermanson 1996; Peppard 2000; Al-Jindan 2009). In addition, other studies have reported that in some cases exposure of the protein

(monoclonal antibody) to the chemical conjugation procedure may lead to changes in the protein structure which leads to the instability of the proteins and denaturation (Hermanson 1996; Peppard 2000). The conjugation procedure was carried out using a two step carbodiimide chemical reaction (Staros *et al.,* 1986). The principle of this reaction is based on a reaction between the carboxyl group on the surface of each microsphere with a water soluble EDC, resulting in production of an unstable amine reacting intermediate. This unstable molecule was stabilised by the addition of Sulpho-NHS, allowing cross-linking from substitution of succinimide moieties for lysine E-amino groups of the protein (Grabarek and Gergely 1990). The mechanism of the two step reaction is summarised in Figure 4.1.

The most important step in assay development was found to be optimisation of the conjugation procedure. During experimental work this step was found to be difficult, due to the fact that any small changes in the optimum conditions are likely to affect the conjugation of the microspheres to the antigens. A number of factors were investigated including activation buffer preparation; washing buffer content; number of washing steps; beads concentration; nonspecific binding; peptide or baculovirus expressed protein concentration; incubation time required between each step; and finally the buffer used for storage of the activated beads.

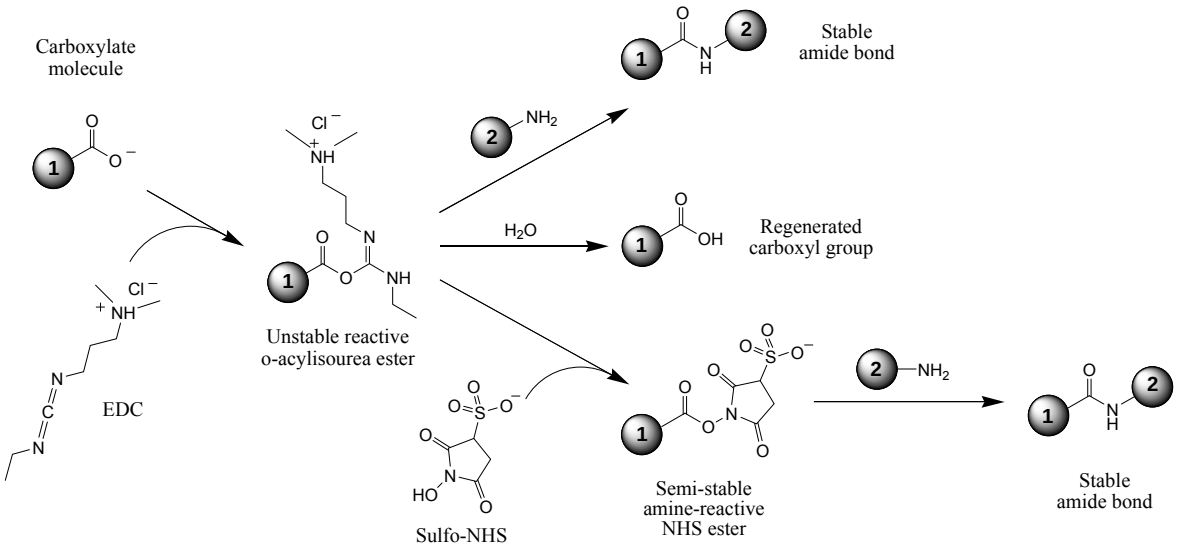

Figure 4.1 Two step carbodiimide reaction in which antigens are cross linked with the carboxyl group at the surface of the microsphere. Taken from www.piercenet.com/.../browse.cfm?fldID=02030312 (accessed on 10/8/2010).

During this stage of assay development a number of factors were found to have a major impact on the conjugation reaction. For example, reagent stability was found to be an important factor in the conjugation reaction especially EDC and sulpho-NHS. Both reagents are very sensitive to light and humidity and they need to be stored properly. It was noted during different optimisation experiments that using these reagents straight from the freezer had an effect on the conjugation process. This can be explained by the increasing humidity of reagents which occurred when the reagent bottle is opened and exposed to a warmer atmosphere within the laboratory. In such a case reagent performance will be affected and this will become apparent with time. To ensure reproducibility of experiments a number of standard procedures were always followed, these included:

1- All reagents needed were labelled with the day and time on which they were first used and they were not used beyond the expiry date.
2- Reagent bottles were stored in accordance with manufacturer's instructions in a sealed container to ensure a humidity free atmosphere.

3- Before opening any containers all reagents were placed at room temperature for 1 h before use.
4- PBS required for conjugation and washing buffers was always freshly prepared as a 500 ml volume, stored at 4ºC and used for only 5 days.
5- Microsphere bead sets were always stored in the fridge (4ºC), removed only when needed and placed on an orbital shaker in the dark at room temperature for 1 h before use.
6- Conjugation buffers were always freshly prepared in 10 ml volumes and used on the same day.

Following standardised procedures for conjugation and handling of reagents during the process of assay optimisation and development improved the performance, stability and reproducibility of each assay. The Phycoerthyrin Streptavidin R-Phycoerythrin purchased from Europa was used in the assay based on the recommendation of Luminex Ltd and was found out to give good results. Antigen concentration required for the assay was optimised by coupling each bead set with different concentration of each antigen (25 µg/ml, 50 µg/ml, 100 µg/ml). However the best results were obtained with 50µg/ml and 25 µg/ml for gD/gE and peptide 55 respectively.

Another factor that needed to be taken into account to produce the Luminex assay was reducing the non-specific binding. One of the major problems with Luminex assays is that human sera may contain antibody (heterophile antibodies) that can bind directly to the beads (Martins *et al.,* 2004; Martins *et al.,* 2006). Several studies have reported the presence of these antibodies in different serum panels and their frequency was found to vary between 59 to 80% (Kricka 1999; Waterbore *et al.,* 2005). Such binding may result in strong background reactivity independent of the antigen coupled to the beads. High backgrounds can also result from interaction between different reagents. This may be through the adherence of reagents to microsphere beads. In the present study the choice of microsphere bead type in combination with adequate blocking buffer was found to be important. Although several microsphere bead types are available commercially the SeroMap microsphere beads were selected. These microspheres were developed by Luminex to reduce the non-specific binding found within human serum samples. Waterbore *et al.,* (2006) studied the suppression of non-specific binding in serological Luminex assays using two types of Luminex beads (MultiAnalyte, SeroMap). In the study,

human sera was first pre-incubated with 0.5% polyvinylacohol (PVA), 0.8% polyvinylpyrrolidone (PVP) or 0.5% Super ChemiBlock (CBS-K) and then added to each of the bead types coupled with E6 protein of the human papillomavirus (HPV) type 16. Results showed that when the MultiAnalyte beads (not derivatised with glutathione) were used, the MFI value exceeded 5000 in some sera. In contrast using the MultiAnalyte beads together with a PVP, PVA, CBS-K or with a combination of PVP and PVA (PVX) the non-specific binding was slightly reduced (MFI 250-700). However, optimum results were obtained when using the beads with a mixture of PVX and CBS-K (MFI 70). When SeroMap beads were used in the absence of additives to the pre-incubation buffer the non-specific binding was reduced by 69%. The study concluded that non-specific binding can be eliminated by SeroMap beads and/or pre-incubated human sera in PVX and CBS-K buffer. Similar results were also reported by Martin *et al.,* (2006) who compared the MFI generated from three types of Luminex beads (Lot A, Lot B (Multi-Analyte), SeroMap) after diluting the human sera in diluent buffer containing 10% fetal bovine serum (FBS). Background binding was reduced by up to 83%, 47.6% and 90.2% in Lot A, Lot B and in SeroMap beads respectively.

In the present study the effect of using the SeroMap beads together with 0.05 -0.1% of Tween and 1-2% of NBBS for preventing the non-specific binding was investigated. Compared to all of the blocking reagents used, a sample diluent buffer containing 1% NBBS, PBS pH 7.3, and 0.05% Tween 20 was found to be the best choice due to its high blocking of the non-specific background. In addition, use of such buffer resulted in a high signal to noise ratio (a measure of signal strength relatively to the background). The MFI value obtained from all monoplex assays was generally less than 25. Such results indicate that a combination of highly purified antigen together with an efficient diluent buffer and 2 washing steps between each assay step limited the background in each monoplex assy. Similar results was obtained from each bead set in the triplex assay. The MFI value obtained for each antigen in the triplex assay was less than 18.

Bead interference is important to consider during development of multiplex assays. In the triplex assay this was evaluated by comparing the MFIs obtained from each monoplex assay with its corresponding assay in the triplex assay format. Results showed a lower background MFI obtained in the triplex compared to the monoplex assay (MFI 13.5-18). This indicates that the recovery rate and the stability of the assay was not affected by

mixing three beads sets together. The optimised triplex assay reproducibility and sensitivity was calculated. The reproducibility of the triplex assay was tested by calculating the percentage coefficient of variation (%CV) of 10 samples tested in duplicate within the same day and on different days. In general, the mean of the CVs of all tested samples was less than 10%. Similar CV value results in newly developed assays were reported in previous studies (Martins *et al.,* 2009a; Xia *et al.,* 2010). The sensitivity of the assay was also determined by testing 10 samples and the limit of detection of each was determined. Due to the difficulty of obtaining a standard serum sample with known antibody concentration for each antigen, a clinical reference serum sample known to have high levels of antibodies to each of the antibodies to be detected was used to generate a standard curve in which the concentration of antibody found within the sample could be calculated. The concentration of antibody for each analyte within unknown samples was reported as arbitrary units (ABU/ml). Based on the triplex assay results the limit of detection (LOD) for the assay was found to be 1.2 ABU/ml and 1.5 ABU/ml for VZV and HSV respectively. In addition there were no significant differences between the LOD of the monoplex and the triplex assays for each viral antibody.

The performance of the triplex assay in detection and quantitation of antibody response to HSV-1, HSV-2 and VZV viruses was evaluated by screening panel 1 serum. As stated above, the panel was tested for these viruses using a number of assays including the developed in–house ELISA for HSV gD and VZV gE. Results showed a 100% agreement with the in-house ELISA for both HSV-1 gD and VZV gE. For peptide 55 the two assays (Omega, peptide 55 microbeads) agreed in 99.2 % (252/254) of the samples. The remaining two samples were determined negative by the Omega assay and positive by peptide 55 microbead assay. Reanalysis of the results of these two samples from the Omega assay revealed that these two samples gave OD values (0.451 and 0.462) near the cut-off point (0.470). In addition they were determined as HSV-2 positive by the Western blot assay. Such results indicate that the peptide 55 microbead assay has a higher sensitivity than the Omega ELISA assay. Furthermore a linear regression analysis between the triplex assay results and results obtained from each relevant antigen ELISA assay showed a correlation of 0.988, 0.956 and 0.968 for peptide 55, HSV-1 gD and VZV gE respectively. Similar correlation has been reported in some studies (Van Gageldonk *et al.,* 2008; Al-Jindan 2009) and a poorer correlation found in others (Pickering *et al.,* 2002; De Voer *et al.,* 2008).

At the start of this study a Luminex assay for the detection of HSV-1, HSV-2 and VZV in the same sample was not available and the initial aim was to develop such an assay. In the last 3 years, 3 multiplex flow immunoassays for identifying HSV-1 and HSV-2 type specific IgG (AtheNA Multi-Lyte HSV type specific test system, BioPlex 2200 and Plexus HerpesSelect) were approved by the American Food and Drug Administration (FDA) (Martins *et al.,* 2009a; Binnicker *et al.,* 2010). The AtheNA Multi-Lyte HSV type specific assay (Zeus Scientific) employs recombinant gG1 and gG2 antigen coupled microspheres. The assay was evaluated by screening 2 well characterised serum panels (317 and 150 samples obtained from sexually active adults and expectant mothers). Assay sensitivity and specificity for HSV-1 was 98.4%, and 92.5% respectively in sexually active adults and 100.0% and 96.2% expectant mothers respectively. In the case of HSV-2 sensitivity and specificity was 97.6% and 96.2% in sexually active adults and 100% and 97.8% in expectant mothers, respectively. The BioPlex 2200 HSV-1 and HSV-2 assay (Bio-Rad Laboratories) employs an HSV-1 gG1 specific recombinant antigen and HSV-2 gG2 specific synthetic peptide. This assay was evaluated by screening 289 and 286 samples from sexually active individuals for HSV-1 and HSV-2 respectively. In addition, 399 samples from expectant mothers were tested. Assay sensitivity and specificity for HSV-1 was 97.6% and 90.1% in sexually active individuals and 96.3% and 99.0% in expectant mothers. In the case of the HSV-2 assay sensitivity and specificity were 90.6% and 98.2 in sexually active adults and 96.9% and 100% in expectant mothers. The Plexus HerpesSelect HSV-1 and HSV-2 (Focus Diagnostic) assay utilises a recombinant HSV-1 gG1 and HSV-2 gG2. Samples obtained from sexually active adults (300) and expectant mothers (300) were used to evaluate this assay and showed respective sensitivities and specificities of; 96.5%, 92.2% for HSV-1; 94.3%, 95.5% for HSV-2 in expectant mothers; and 91.0%; 96.5% for HSV-1; 96.3%, 97.4% for HSV-2 in sexually active adults.

The performance of the AtheNA assay was also evaluated in comparison with immunoblot (HerpeSelect 1 and 2 immunoblot), Western blot and ELISA (HerpeSelect HSV-1 and HSV-2 IgG) assays by Martins *et al.,* (2009a). In their study 332 well characterised serum samples categorised into 5 groups were tested by the multiplex and the HerpeSelect HSV 1 and 2 ELISA assays. In the case of HSV-1 results showed an agreement of 94.8% and the sensitivity and specifity of the assay was 95.8% and 93.4% respectively. For HSV-2 the two assays agreed on 96.9% of samples and the sensitivity and specifity of the multiplex

assay was 92.6% and 98.7% respectively. In comparison for the immunoblot assay (the assay was composed of 4 bands; recombinant gG1and gG2, antihuman serum control band and a HSV-1 and 2 whole-virus lysate band) results of the two assays agreed in 95.8% of samples and showed a higher sensitivity (97.9%) but lower specificity (92.9%) for HSV-1. In the case of HSV-2 the assay agreed in 97.9% and the sensitivity and specificity was better than previously reported by the ELISA assay; 96.7% and 98.3% respectively. The remaining discrepant samples (n=50) were further tested by Western blot and the results were compared to those obtained with the multiplex assay. Analysis of the results showed that the overall agreement between the two assays were 95.8% and 98.8% for HSV-1 and HSV-2 respectively, and the sensitivity and specificity of the multiplex assay was 94.9% and 97.0% for HSV-1 and 98.9% and 98.7% for HSV-2. Of the 50 discrepant samples 11 were found to be positive by the immunoblot assay (using the type common band), however they were not typed. Three out of these 11 samples reacted in the multiplex assay, 2 were gG1 positive and the remaining sample was gG2 positive. In addition, 3 samples of the 50 discrepant samples showed positive results by the immunoblot assay for gG1 or gG2 but were negative for the type common band, therefore they were classified as atypical samples. Three samples were reported positive for either HSV-1 or HSV-2 by the multiplex, ELISA and Western blot assays. The study reported that these samples should be reported as negative. However, based on the results of other assays they are most likely to be false negative results. The study concluded that all of the three assays preformed equally well, however, it was noted that although both the ELISA and the immunoblot employed the same recombinant antigen (gG1 and gG2); there were no great concordances between the two assays. For example in the group of samples that contained either HSV-1 or HSV-2 antibodies the immunoblot assay gave a better performance compared to Western blot assay. In contrast the ELISA assay performs well in dual infected and seronegative samples compared to the western blot assay and the immunoblot assay. In addition the study reported that including a type common antigen band in the immunoblot assay was an advantage over the ELISA and the multiplex assays.

Binnicker *et al.,* (2010) evaluated the performance of the three multiplex flow immunoassays for the simultaneous detection of HSV-1 and HSV-2 type specific antibody by screening 505 serum samples. In the study, all samples (505) were tested for the presence of HSV-1 and HSV-2 type specific antibody using HerpeSelect 1 and 2 ELISA and then tested by the three assays. Discordant samples between all the assays were further

tested by HSV type specific Western blot assay. After resolving all discordant samples (16 samples) the total agreement of the three assays with the HerpeSelect ELISA assay for HSV-1 was 96.8%, 98.8% and 97.6% for the AtheNA, Bio-Plex and Plexus respectively. In the case of HSV-2 the total agreement was 88.5%, 97.8% and 97.2 for each assay respectively. In addition, qualitative intra and inter assays reproducibility when testing 20 samples (positive and negative result) was 100%. Furthermore the timing required for full analysis of 90 samples by the AtheNA, Bio-Plex and Plexus assays were 6.1, 2.6 and 4.7 hours respectively, versus 5.2 h for the HerpeSelect ELISA assay. The study concluded that the three assays performed well in detecting HSV-1 and HSV-2. However, the clinical data and previous laboratory results for the tested samples used in the study were not available, so the results could not be correlated with the clinical picture. Therefore the performance of the tested assays was only based on its correlation with the HerpeSelect ELISA assay.

Taking into account the findings of the present study comparing the individual developed ELISA assays and the triplex assay with the commercially available assays, clearly shows that the developed triplex assay performed well in comparison to the relevant ELISAs and Western blots. In addition the developed assay has the advantage of using an HSV type common antigen that showed 100% agreement with all ELISA assays used and with Western blot. The employment of peptide 55 for detecting HSV-2 type specific antibody gave results that showed better agreement with Western blot than the other evaluated HSV-2 type specific ELISA assays. The ability of the assay to detect antibody responses to HSV-1, HSV-2 and VZV viruses in 90 samples within approximately 2.5 h using only 2µl of sample volume in comparison to 5-10 µl by the previous multiplex and 10-50 µl by the other ELISA assays is a distinctive advantage .

As stated earlier detection of viral specific antibody within the CNS compartment (intrathecal antibody) is known to be a reliable method for diagnosis of viral encephalitis. However, as a consequence of such infection the permeability of the BBB may be affected. Therefore in order to confirm the production of intrathecal antibody an antibody index has to be calculated. Traditional methods used nowadays for calculating antibody index are time, labour and sample consuming. In the present study a biplex assay based on Luminex technology developed in this department by Al-Jindan (2009) was utilised. The assay is based on 2 microsphere beads sets each coupled with either anti-human IgG or anti-human

albumin. Because phycoerythrin conjugated anti-human albumin antibody was not available commercially, an anti-human albumin monoclonal antibody labelled with biotin was used with streptavidin-coupled phycoerythrin. The assay was previously optimised, validated and evaluated by testing CSF quality control samples obtained from the United Kingdom National External Quality Assessment Service (NEQAS). The Biplex assay results were found to be highly correlated with the NEQAS samples results and the correlation coefficient of albumin and IgG was 0.9629 and 0.9921 respectively. The assay was also used to test 63 paired samples (CSF and serum) obtained from Malawian children with pneumococcal meningitis in order to assess the relationship between infection and hearing loss (Al-Jindan 2009).

The biplex assay protocol was adapted with some modifications (the microsphere beads types, sample diluent buffer, washing buffer and incubation times used were different). Although the original biplex assay showed a good correlation with the NEQAS sample results, it also showed a high MFI background. In the previous study several attempts were made to reduce the background to an acceptable reading these measures included pre-incubating the plates with blocking agent (Tween-20, 0.02%) and increasing the washing steps and the washing intervals. However even after introducing these modifications the MFI was only reduced to 452 and 820 for IgG and albumin respectively. These MFIs are higher than that obtained for the triplex assay (13.5-18). Therefore, a new attempt was to reduce the background levels in this assay before including it with the triplex assay. The assay was repeated using the same conditions determined as optimal for the triplex assay. Interestingly using these conditions reduced the MFI of the assay to 280 and 350 for IgG and albumin respectively. Such results confirm the previous findings that using the SeroMAP and the diluent buffer has a positive effect on blocking of the nonspecific binding to the beads. Other attempts to reduce the background even further by increasing numbers of washing steps or altering NBBS and Tween-20 concentration in sample diluent buffer were not successful. Therefore, the obtained background was considered as an optimal background results for the albumin/IgG biplex assay.

After obtaining an acceptable background for the biplex assay, the main challenge in developing the multiplex was to obtain a standard serum sample capable of generating a good standard curve for all 5 analytes in order to determine their concentration in the same sample. Since such a standard was not available commercially, the previous clinical

standard serum used in the ELISA and the triplex assay was used. To determine the IgG and albumin concentration in the standard sample, a serial dilution of the clinical standard serum was tested 3 times in duplicate using the biplex assay and then the mean value for each dilution was calculated. Using the biplex results, different concentrations of the clinical standard sample were used to generate a suitable standard curve for the 5 analytes. However during this step it was not possible to generate a good standard curve for all 5 analytes together using any of the two assays conditions (serum dilution). In the triplex assay, the best standard curve was generated by making two-fold dilutions of the standard serum starting from 1/10 to 1/10240. In contrast the best standard curve for the biplex assay was generated by making two-fold dilutions of the standard sample starting from 1/10000 to 1/5120000. When the triplex assay starting dilution was used for the multiplex assay the MFI reading obtained for the IgG and albumin was higher than the capacity of the machine to read (>24000 MFI). In contrast when the starting dilution of the biplex was used it was not possible to obtain good MIF reading (< 400). Therefore, In order to generate a standard curve for HSV-1 gD, VZV gE and peptide 55 4-fold dilutions of the standard serum starting from 1/10 to 1/2621440 at an IgG and albumin concentration of 0.3g/l and 0.05g/l respectively were made. At this starting point the standard sample contained 4800 and 3200 ABU of HSV-1, HSV-2 and VZV respectively. The standard thus consisted of 12 points. The first 6 points (1/10 to 1/10240) were used to generate a standard curve for HSV-1, HSV-2 and VZV, whereas the remaining 6 points (1/40960 to 1/2621440) were used for IgG and albumin. In order to obtain a good standard curve for HSV-1, HSV-2 and VZV the results of the last 6 points in the standard curve (S7 to S12) had to be masked. The reading obtained from S7 point in the standard curve for HSV-1, HSV-2 and VZV is equal to the blank well. In contrast the first 6 points in the standard curve (S1 to S6) had to be masked in order to generate a good standard curve for IgG and albumin. The reading obtained for IgG and albumin in these points was over 24000 MFI. Such standard dilution factor used in the multiplex assay (4 fold) is widely used in similar multiplex assays (van Gageldonk *et al.,* 2008; Martins *et al.,* 2009b).

Once a good standard curve had been obtained for each analyte the dilution factor for serum and CSF samples was investigated. In the triplex assay a dilution factor of 1/100 was found to be an optimal dilution for serum samples. In contrast the optimal dilution factor used previously in the biplex was 1/10000 and 1/1000000 for IgG and albumin respectively. Therefore in order to determine the best dilution factor for each sample (CSF

and serum) for the multiplex assay, a series of dilution factors were tested (1/100, 1/500, 1/1000, 1/5000, 1/10000). Based on the results 1/5000 and 1/500 was used as the optimal dilution factors for serum and CSF respectively. These dilutions were selected in order to allow the normal antibodies for HSV-1, HSV-2 and VZV to fall in the median range of the first 6 points (S3-S4) and in the median of the last 6 points for the IgG and albumin (S9-S10). After determining the sample dilution factor, the sensitivity and reproducibility of the multiplex assay was then evaluated by calculating the intra and inter assay variation, the percentage CV was found to be less than 10%. In addition combination of the two assays in a multiplex assay did not affect the sensitivity or limit of detection of the two assays. The multiplex assay results showed good correlation with previous results obtained from each individual assay. However it was noted that the background MFIs obtained from the triplex analytes (gD, gE, peptide 55) were increased to approximately 220 MFI. This increase in the MFI was due to the combination of the 5 analytes in a single assay. Since using the same reagents and experiment conditions in the triplex assay alone did not affect the assay background levels (13.5-18). Generally the background of the 5 analytes in the multiplex assay ranged from 250 to 350 MFI. Such background levels are better than reported in the biplex assay and is still within the acceptable ranges in comparison to similar assays. For example, based on the Plexus HerpeSelect HSV-1 and HSV-2 assay, a test run is acceptable with a blank well background reading of $\leq$ 800 MFI. The obtained background reading from the multiplex was well below this value and was therefore determined to be the optimal background reading for the multiplex assay. Following optimisation of the multiplex assay the next step was assay validation. However in the present study it was not possible to validate the assay due to the difficulty of obtaining paired samples from cases with and without neurological symptoms.

In summary, although the alphaherpesviruses are known to cause local cutaneous infections, however each virus is associated with a number of neurological complications including encephalitis. The CNS is well protected against such infections being protected in the bony compartment and surrounded by the CSF. Therefore in normal conditions the entry of peripheral immune cells to the CNS is limited. However when the infection occurs the CNS is capable of initiating an immune response. In clinical practice diagnosis requires the use of different immuno assays in order to detect each virus specific antibodies in human sera and CSF samples. Different assays settings may increase the possibility of

aliquot errors, testing time as will as sample volume required for each assay. Therefore in the present study the visibility of using Luminex technology to improve the diagnosis of these viruses and their complications were investigated.

In the present study two immuno dominant proteins of HSV-1, HSV-2 (gD) and VZV (gE) were expressed in insect cells using baculovirus expression systems. Expressed proteins were purified, characterised and used to develop two in-house ELISAs. The performances of the developed in-house ELISAs were tested by screening with characterise serum panels. Results have showed the superior performance of each in-house ELISAs in detecting HSV and VZV antibody in comparison to the commercially available ELISA assays. These results indicate that each expressed protein displays conformation-dependent epitopes capable of detecting antibodies for each virus. Therefore the developed ELISAs which are proven to be sensitive and specific can be utilised in central or local laboratories for detecting HSV and VZV antibodies in human sera. In addition, in order to differentiate between HSV-1 and HSV-2 antibody and due to the high risk of neonatal herpes infection when primary maternal genital HSV infection occurs, a comparative study was carried out to evaluate a newly developed ELISA assay based on peptide 55 for the detection of HSV-2 type specific antibody. Assay performance was compared with two other HSV-2 type specific assays by screening a will characterised serum panel. Comparative results have showed that the new assay had better performance than other evaluated assay with high level of agreement with Western blot assay results. Therefore the new assay can be a sensitive serological test for diagnosis of HSV-2 genital infection, especially during pregnancy. Moreover with the increasing test volumes required every year for diagnosis of these viruses and there complications the two expressed proteins together with peptide 55 were used to develop a triplex fluorescent microbead immunoassay for the simultaneous detection and quantitation of anti-viral antibody in the same human sera (Binnicker 2010). Assay sensitivity was tested by screening a well characterised serum panel and the assay was found to be sensitive, specific, reducible and showed high level of agreement with other commercially approved immuno assays used to evaluate the serum panel. The advantage of the developed triplex assay is its ability to measure antibody to the three viruses. The developed Luminex assay by contrast, is sensitive, rapid and requires very low test sample volumes; as little as 2µl can be used to detect antibody to the three viruses in comparison to other immuno assays which requires 5-30µl (5-10µl for each virus). The latter finding is particularly important when attempting to detect antibody in a sample such

as CSF where only low test volumes are commonly available. Therefore the triplex assay can be a valuable serological method for diagnosis of alphaherpesviruses infections in laboratories in where it can save labour, reagent cost, and time required for diagnosis of infection with these viruses. In order to improve the diagnosis of alphaherpesviruses CNS infection, a multiplex fluorescent microbead immunoassay was developed by combining the triplex assay with a biplex assay designed to detect and quantitate both human IgG and albumin in serum and CSF samples. The multiplex assay developed in this study was optimised, tested using standard samples and has proved its reproducibility and ability to detect and quantitate each virus antibody as well as human IgG and albumin in the same sample.

In order to validate the multiplex assay a series of paired samples from proven cases of HSE and ADEM should be tested as well as paired samples from normal patients (control groups). The latter samples will be required to determine the normal ranges of values of IgG index and albumin ratios Serum:CSF using this novel assay. Previous reported values for these parameters may or may not be valid for this new assay due to its higher sensitivity and lower detection limits. It is of course difficult to obtain samples of CSF from normal i.e. non-diseased individuals due to the invasiveness of the lumbar puncture procedure. However it may be possible to obtain CSF samples either from patients without neurological disease who are undergoing procedures leading to the removal of CSF such as pregnant women undergoing any epidural injections or patients undergoing spinal anastasia. A series of paired serum and CSF pairs are available to validate the novel assay through the UK National encephalitis study. Unfortunately these were not available at the time of this project. In order to properly calculate the antibody index to confirm the production of intrathecal antibody paired samples (i.e. serum and CSF taken at the same time) from each patient are necessary. There are many factors can contribute the variation in antibody index level including the timing in which the two samples were taken. The immune response of each patient may influence the degree of BBB damage and intrathecal IgG production. Selection of the timing in which the paired samples were taken is also important. As stated before although the production of intrathecal antibody may be detected in the early stage of CNS infection (Klapper *et al.,* 1981). Reliable detection of intrathecal antibody synthesis is not achieved until 14 or more days after the onset of the neurological infection. Therefore sample from the convalescent stage are required. This does not affect the important value of detecting of intrathecal antibody as a diagnostic

method. Once the multiplex assay has been validated it can be utilised for the diagnosis of alphaherpesviruses CNS infections. In addition it can be also used for diagnosing other encephalitis cases with unknown aetiology. It is reported that 85% of encephalitis cases reported world wide are of unknown aetiology (Davison *et al.,* 2003; Granerod and Crowcroft 2007). In a recent multi-centre study in the UK by Granerod *et al,.*(2010) the infectious cause in 203 cases of encephalitis was found only in 63% of cases, in which the specific aetiologies included HSV (19%), ADEM (11%), autoantibody-associated (8%), VZV (5%) and *Mycobacterium tuberculosis* (5%). However in 37% of these cases no aetiology was reported. The developed multiplex assay can be combined with baculovirus based expression system developed in this study to allow the immune response to a much wider rang of pathogens to be identified. The application of this technique, particularly in the study of most forms of encephalitis not associated with active CNS infection (e.g. ADEM) promises to dramatically improve the diagnostic yield in encephalitis.

The advantage of the Luminex technology is apparent, particularly in its ability of expansion to include 100 targets of interest in a single assay which can be individually discriminated. The simultaneous detection and quantitation of specific antibody response to individual targets in a small volume of a single sample can be achieved. In addition savings of reagent cost and assay time can be acquired from using such an assay. In comparison to conventional methods these advantages are clearly a strong impetus for the routine use of this assay in the clinical laboratory and for research purposes.

Chapter 5

References

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Appendices

Appendix 1

Table 1 Summary of the Results Obtained from the Different ELISA Assays for Panel 1 Sera.

Sample NO	Age	DIAMEDIX HSV 1+2 T/C	Assay Results	Bioelisa HSV 1 IgG T/C	Assay Results	Bioelisa HSV 2 IgG T/C	Assay Results	Virotech HSV-1	Assay Result	Human VZ IgG T/C	Assay Results
1	52	1.01	POS	-	-	-	-	0.532	POS	-	-
2	56	4.97	POS	-	-	-	-	1.691	POS	11.8	POS
3	48	5	POS	-	-	-	-	1.414	POS	5.3	POS
4	34	4.51	POS	-	-	-	-	1.311	POS	-	-
5	58	3.66	POS	-	-	-		1.498	POS	5.4	POS
6	38	-	-	3.41	POS	7.94	POS	0.921	POS	9.6	POS
7	95	30	POS	7.5	POS	13.7	POS	1.982	POS	-	-
8	34	4.15	POS	-	-	-	-	1.516	POS	7.2	POS
9	36	1.69	POS	-	-	-	-	0.64	POS	8.4	POS
10	69	4.06	POS	-	-	-	-	1.492	POS	9	POS
11	51	4.53	POS	-	-	-	-	1.732	POS	6.3	POS
12	46	1.23	POS	-	-	-	-	0.693	POS	-	-
13	25	4.61	POS	-	-	-	-	1.55	POS	-	-
14	31	-	-	2.87	POS	0.16	NEG	0.893	POS	-	-
15	54	2.56	POS	-	-	-	-	0.937	POS	8.2	POS
16	50	-	-	5.55	POS	3.07	POS	1.01	POS	-	-
17	23	-	-	0.27	NEG	0.17	NEG	0.915	POS	-	-
18	28	3.77	POS	-	-	-	-	1.343	POS	5.2	POS
19	88	4.71	POS	-	-	-		1.514	POS	-	-
20	47	4.57	POS	-	-	-	-	1.443	POS	-	-
21	42	-	-	3.59	POS	0.32	NEG	0.941	POS	6.2	POS
22	64	4.53	POS	-	-	-	-	1.352	POS	3.9	POS

Sample NO	Age	DIAMEDIX HSV 1+2 T/C	Assay Results	Bioelisa HSV 1 IgG T/C	Assay Results	Bioelisa HSV 2 IgG T/C	Assay Results	Virotech HSV-1	Assay Result	Human VZ IgG T/C	Assay Results
23	50	5.34	POS	-	-	-	-	1.615	POS	1.5	POS
24	28	4.8	POS	-	-	-	-	1.671	POS	-	-
25	37	5.03	POS	-	-	-	-	1.484	POS	6.6	POS
26	75	4.78	POS	-	-	-	-	1.533	POS	-	-
27	63	5.48	POS	-	-	-	-	2.175	POS	9.7	POS
28	26	5.38	POS	-	-	-	-	1.69	POS	3.6	POS
29	42	4.93	POS	-	-	-	-	1.553	POS	-	-
30	47	4.31	POS	-	-	-	-	1.387	POS	6.5	POS
31	42	4.16	POS	-	-	-	-	1.374	POS	11.2	POS
32	84	5.11	POS	-	-	-	-	1.799	POS	11.4	POS
33	35	1.2	POS	-	-	-	-	0.489	POS	5.2	POS
34	35	3.37	POS	-	-	-	-	0.56	POS	3.4	POS
35	61	5.43	POS	-	-	-	-	0.56	POS	4.1	POS
36	74	2.48	POS	-	-	-	-	1.151	POS	-	-
37	7	4.62	POS	-	-	-	-	1.74	POS	2.5	POS
38	52	4.62	POS	-	-	-	-	1.764	POS	4.6	POS
39	5	2.61	POS	-	-	-	-	1.228	POS	3	POS
40	29	-	-	2.01	POS	0.59	NEG	0.761	POS	-	-
41	72	5.1	POS	-	-	-	-	1.754	POS	0.8	NEG
42	62	4.94	POS	-	-	-	-	1.835	POS	5.1	POS
43	62	4.58	POS	-	-	-	-	1.526	POS	3.8	POS
44	72	4.67	POS	-	-	-	-	1.644	POS	0.4	NEG
45	53	4.46	POS	-	-	-	-	1.747	POS	3.2	POS
46	11	3.59	POS	-	-	-	-	1.525	POS	1.9	POS

Sample NO	Age	DIAMEDIX HSV 1+2 T/C	Assay Results	Bioelisa HSV 1 IgG T/C	Assay Results	Bioelisa HSV 2 IgG T/C	Assay Results	Virotech HSV-1	Assay Result	Human VZ IgG T/C	Assay Results
47	55	1.03	POS	-	-	-	-	0.636	POS	7.8	POS
48	67	4.5	POS	-	-	-	-	1.567	POS	-	-
49	52	3.66	POS	-	-	-	-	1.387	POS	7.6	POS
50	36	4.41	POS	-	-	-	-	1.642	POS	6.5	POS
51	56	2.98	POS	-	-	-	-	1.255	POS	4.6	POS
52	45	-	-	3.99	POS	0.14	NEG	1.20	POS	-	-
53	37	-	-	4.35	POS	2.97	POS	1.765	POS	7.5	POS
54	58	4.45	POS	-	-	-	-	1.653	POS	-	-
55	28	-	-	2.56	POS	0.13	NEG	1.32	POS	-	-
56	42	-	-	4.4	POS	0.17	NEG	0.982	POS	-	-
57	58	2.51	POS	-	-	-	-	0.749	POS	6.2	POS
58	21	-	-	1.21	POS	0.22	NEG	0.992	POS	-	-
59	4	3.92	POS	-	-	-	-	1.273	POS	5.8	POS
60	29	-	-	1.27	POS	0.1	NEG	1.230	POS	-	-
61	32	-	-	3.64	POS	0.43	NEG	1.341	POS	-	-
62	47	1.94	POS	-	-	-	-	0.652	POS	0.4	NEG
63	37	2.11	POS	-	-	-	-	0.789	POS	-	-
64	67	3.97	POS	-	-	-	-	1.384	POS	0.3	NEG
65	52	4.69	POS	-	-	-	-	1.812	POS	-	-
66	62	3.9	POS	-	-	-	-	1.441	POS	3.5	POS
67	24	-		1.35	POS	0.49	NEG	1.563	POS	-	-
68	48	4.08	POS	-	-	-	-	1.491	POS	-	-
69	30	4.99	POS	-	-	-	-	1.678	POS	-	-
70	74	4.56	POS	-	-	-	-	1.503	POS	6.7	POS

Sample NO	Age	DIAMEDIX HSV 1+2 T/C	Assay Results	Bioelisa HSV 1 IgG T/C	Assay Results	Bioelisa HSV 2 IgG T/C	Assay Results	Virotech HSV-1	Assay Result	Human VZ IgG T/C	Assay Results
71	49	4.11	POS	-	-	-	-	1.291	POS	6.1	POS
72	58	5.38	POS	-	-	-	-	1.548	POS	-	-
73	37	-	-	5.75	POS	0.11	NEG	1.342	POS	-	-
74	57	-	-	6.02	POS	0.55	NEG	0.875	POS	-	-
75	50	-	-	5.68	POS	4.56	POS	0.991	POS	-	-
76	38	-	-	3.7	POS	4.45	POS	1.231	POS	-	-
77	47	-	-	3.82	POS	0.2	NEG	0.897	POS	-	-
78	38	-	-	6.6	POS	0.09	NEG	1.021	POS	6.3	POS
79	21	-	-	4.81	POS	0.18	NEG	1.231	POS	-	-
80	20	-	-	0.3	NEG	6.57	POS	0.761	POS	-	-
81	48	-	-	1.47	POS	0.16	NEG	0.879	POS	-	-
82	32	-	-	0.07	NEG	1.7	POS	0.997	POS	-	-
83	49	-	-	5.51	POS	0.5	NEG	1.230	POS	-	-
84	60	4.83	POS	-	-	-	-	1.862	POS	-	-
85	55	4.79	POS	-	-	-	-	1.825	POS	-	-
86	25	4.88	POS	-	-	-	-	1.63	POS	8.1	POS
87	66	3.47	POS	-	-	-	-	1.324	POS	2.1	POS
88	100	20.3	POS	-	-	-	-	1.964	POS	2187	POS
89	26	4.26	POS	-	-	-	-	1.618	POS	-	-
90	48	4.7	POS	-	-	-	-	1.514	POS	5.4	POS
91	38	23	POS	-	-	-	-	1.934	POS	-	-
92	37	12.4	POS	-	-	-	-	1.444	POS	1934	POS
93	51	-	-	0.09	NEG	2.73	POS	1.320	POS	-	-
94	44	-	-	3.57	POS	3.82	POS	0.998	POS	4.7	POS

Sample NO	Age	DIAMEDIX HSV 1+2 T/C	Assay Results	Bioelisa HSV 1 IgG T/C	Assay Results	Bioelisa HSV 2 IgG T/C	Assay Results	Virotech HSV-1	Assay Result	Human VZ IgG T/C	Assay Results
95	46	-	-	0.18	NEG	0.77	NEG	0.124	NEG	-	-
96	68	-	-	-0.01	NEG	0.09	NEG	1.937	POS	-	-
97	45	-	-	-0.01	NEG	0.21	NEG	1.695	POS	7	POS
98	20	-	-	0.87	NEG	1.04	E	1.317	POS	-	-
99	16	-	-	2.18	POS	0.1	NEG	1.202	POS	-	-
100	30	-	-	-0.01	NEG	3.33	POS	1.346	POS	-	-
101	32	-	-	4.54	POS	1.54	POS	0.981	POS	-	-
102	62	-	-	5.85	POS	0.08	NEG	1.431	POS	8.7	POS
103	53	-	-	5.71	POS	0.12	NEG	0.963	POS	-	-
104	31	-	-	0.46	NEG	5.73	POS	0.875	POS	-	-
105	49	-	-	7.07	POS	3.19	POS	1.475	POS	-	-
106	24	-	-	11.35	POS	0.33	NEG	1.561	POS	-	-
107	41	-	-	5.71	POS	0.3	NEG	0.921	POS	-	-
108	47	-	-	8.85	POS	0.5	NEG	1.321	POS	-	-
109	49	-	-	6.98	POS	5.94	POS	0.992	POS	-	-
110	25	-	-	7.35	POS	-	-	0.998	POS	2.4	POS
111	35	-	-	3.78	POS	0.05	NEG	0.768	POS	-	-
112	40	-	-	0.05	NEG	2.18	POS	0.643	POS	-	-
113	42	-	-	3.56	POS	0.49	NEG	0.797	POS	-	-
114	23	-	-	3.64	POS	0.23	NEG	0.832	POS	-	-
115	28	-	-	2.83	POS	0.57	NEG	0.786	POS	-	-
116	63	4.55	POS	-	-	-	-	1.626	POS	4.6	POS
117	68	4.21	POS	-	-	-	-	1.675	POS	4.3	POS
118	86	4.45	POS	-	-	-	-	1.634	POS	-	-

Sample NO	Age	DIAMEDIX HSV 1+2 T/C	Assay Results	Bioelisa HSV 1 IgG T/C	Assay Results	Bioelisa HSV 2 IgG T/C	Assay Results	Virotech HSV-1	Assay Result	Human VZ IgG T/C	Assay Results
119	67	4.32	POS	-	-	-	-	1.635	POS	5.1	POS
120	47	1.96	POS	-	-	-	-	0.867	POS	6.4	POS
121	13	-	-	0.31	NEG	0.2	NEG	0.076	NEG	-	-
122	34	-	-	0.41	NEG	0.18	NEG	0.109	NEG	-	-
123	41	-	-	0.25	NEG	4.27	POS	1.512	POS	-	-
124	21	-	-	0.35	NEG	0.23	NEG	0.137	NEG	-	-
125	23	-	-	0.37	NEG	0.27	NEG	0.111	NEG	-	-
126	29	-	-	0.45	NEG	0.36	NEG	1.201	POS	-	-
127	37	-	-	0.74	NEG	0.64	NEG	0.088	NEG	-	-
128	26	-	-	-	-	-	-	0.128	NEG	-	-
129	49	-	-	0.1	NEG	2.2	POS	1.148	POS	-	-
130	29			2.01	POS	0.13	NEG	0.856	POS	-	-
131	50	4.3	POS	-	-	-	-	1.845	POS	-	-
132	45	-	-	0.13	NEG	0.15	NEG	0.171	NEG	-	-
133	35	29.8	POS	-	-	-	-	1.688	POS	960.6	POS
134	31	0.57	NEG	-	-	-	-	0.255	NEG	-	-
135	9	-	-	0.2	NEG	0.5	NEG	0.094	NEG	4000	POS
136	20	0.5	NEG	-	-	-	-	1.134	POS	477	POS
137	40	-	-	3.7	POS	0.5	NEG	0.764	POS	-	-
138	50	-	-	1.5	POS	0.5	NEG	0.683	POS	-	-
139	45	-	-	3.3	POS	14.8	POS	0.675	POS	-	-
140	47	-	-	2.7	POS	4.8	POS	0.821	POS	-	-
141	33	-	-	3.5	POS	0.5	NEG	0.634	POS	-	-
142	51	30	POS	-	-	-	-	1.665	POS	2672	POS

Sample NO	Age	DIAMEDIX HSV 1+2 T/C	Assay Results	Bioelisa HSV 1 IgG T/C	Assay Results	Bioelisa HSV 2 IgG T/C	Assay Results	Virotech HSV-1	Assay Result	Human VZ IgG T/C	Assay Results
143	34	-	-	3.8	POS	3.4	POS	0.677	POS	-	-
144	45	-	-	3.1	POS	7.8	POS	0.853	POS	-	-
145	45	-	-	3.7	POS	0.5	NEG	0.712	POS	-	-
146	44	-	-	4.4	POS	0.5	NEG	0.873	POS	-	-
147	33	30	POS	-	-	-	-	1.575	POS	-	-
148	105	-	-	4.3	POS	8.8	POS	0.990	POS	-	-
149	67	-	-	4.2	POS	0.8	NEG	0.985	POS	-	-
150	105	22.7	POS	-	-	-	-	1.639	POS	1503	POS
151	38	9.1	POS	-	-	-	-	0.838	POS	1715	POS
152	105	24.2	POS	-	-	-	-	1.527	POS	1395	POS
153	54	30	POS	-	-	-	-	1.497	POS	-	-
154	64	21.2	POS	-	-	-	-	1.477	POS	3276	POS
155	59	30	POS	-	-	-	-	1.713	POS	1130	POS
156	68	30	POS	-	-	-	-	1.52	POS	1470	POS
157	47	30	POS	-	-	-	-	1.729	POS	-	-
158	24	1.13	POS	-	-	-	-	0.852	POS	-	-
159	39	25.1	POS	-	-	-	-	1.374	POS	723.4	POS
160	69	26.5	POS	-	-	-	-	1.639	POS	-	-
161	27	20.5	POS	-	-	-	-	1.602	POS	1490	POS
162	89	17.7	POS	-	-	-	-	1.695	POS	468.2	POS
163	47	21.8	POS	-	-	-	-	1.746	POS	2766	POS
164	22	15.3	POS	-	-	-	-	1.032	POS	-	-
165	30	-	-	3.9	POS	0.8	NEG	0.932	POS	-	-
166	21	-	-	4.6	POS	0.5	NEG	1.201	POS	-	-

Sample NO	Age	DIAMEDIX HSV 1+2 T/C	Assay Results	Bioelisa HSV 1 IgG T/C	Assay Results	Bioelisa HSV 2 IgG T/C	Assay Results	Virotech HSV-1	Assay Result	Human VZ IgG T/C	Assay Results
167	52	-	-	4.2	POS	0.5	NEG	1.101	POS	-	-
168	44	-	-	3.2	POS	0.5	NEG	0.987	POS	-	-
169	81	-	-	4.9	POS	0.5	NEG	0.991	POS	-	-
170	19	0.5	NEG	-	-	-	-	0.078	NEG	1158	POS
171	37	30	POS	-	-	-	-	0.882	POS	-	-
172	49	-	-	7.4	POS	0.5	NEG	0.765	POS	-	-
173	47	2.3	POS	-	-	-	-	1.505	POS	-	-
174	46	23.5	POS	-	-	-	-	1.543	POS	797.5	POS
175	71	24.1	POS	-	-	-	-	1.766	POS	4000	POS
176	53	28.6	POS	-	-	-	-	1.751	POS	441.7	POS
177	24	18.9	POS	-	-	-	-	1.846	POS	2759	POS
178	36	30	POS	-	-	-	-	1.668	POS	530.5	POS
179	62	20.6	POS	-	-	-	-	1.644	POS	-	-
180	48	0.5	NEG	-	-	-	-	0.091	NEG	10.59	NEG
181	25	0.5	NEG	-	-	-	-	0.057	NEG	-	-
182	46	20.1	POS	-	-	-	-	1.878	POS	471.4	POS
183	5	0.5	NEG	-	-	-	-	0.077	NEG	1120	POS
184	21	-	-	0.1	NEG	0.5	NEG	0.064	NEG	-	-
185	27	-	-	0.2	NEG	0.5	NEG	0.048	NEG	-	-
186	38	0.5	NEG	-	-	-	-	0.082	NEG	2271	POS
187	39	0.5	NEG	-	-	-	-	0.072	NEG	-	-
188	6	0.5	NEG	-	-	-	-	0.083	NEG	-	-
189	27	0.5	NEG	-	-	-	-	1.986	POS	-	-
190	53	0.5	NEG	0.5	NEG	0.5	NEG	0.095	NEG	-	-

Sample NO	Age	DIAMEDIX HSV 1+2 T/C	Assay Results	Bioelisa HSV 1 IgG T/C	Assay Results	Bioelisa HSV 2 IgG T/C	Assay Results	Virotech HSV-1	Assay Result	Human VZ IgG T/C	Assay Results
191	4	-		0.1	NEG	0.5	NEG	0.076	NEG	-	-
192	7	0.5	NEG	-	-	-	-	0.114	NEG	23.84	NEG
193	64	0.5	NEG	-	-	-	-	0.705	POS	-	-
194	38	0.5	NEG	-	-	-	-	0.33	NEG	-	-
195	28	0.64	NEG	-	-	-	-	0.085	NEG	-	-
196	15	0.5	NEG	-	-	-	-	0.036	NEG	273.3	POS
197	42	-	-	5	POS	0.5	NEG	1.153	POS	-	-
198	84	-	-	4.7	POS	0.5	NEG	1.342	POS	-	-
199	32	-	-	4.3	POS	1.3	POS	0.998	POS	-	-
200	14	0.69	NEG	-	-	-	-	0.048	NEG	-	-
201	27	-	-	2.9	POS	0.5	NEG	0.886	POS	-	-
202	42	0.5	NEG	-	-	-	-	0.077	NEG	-	-
203	38	-	-	4.4	POS	0.5	NEG	0.994	POS	28.65	POS
204	3	0.5	NEG	-	-	-	-	1.43	POS	11.44	NEG
205	27	0.5	NEG	-	-	-	-	0.074	NEG	-	-
206	37	-	-	4.5	POS	0.5	NEG	1.211	POS	-	-
207	66	-	-	4.2	POS	6.7	POS	0.931	POS	-	-
208	4	0.5	NEG	-	-	-	-	0.089	NEG	14.86	NEG
209	5	0.5	NEG	-	-	-	-	0.098	NEG	12.68	NEG
210	21	0.5	NEG	-	-	-	-	0.116	NEG	-	-
211	51	-	-	4.4	POS	0.5	NEG	1.531	POS	1266	POS
212	26	-	-	4.9	POS	0.5	NEG	1.223	POS	-	-
213	60	0.5	NEG	-	-	-	-	0.064	NEG	-	-
214	25	0.5	NEG	-	-	-	-	0.05	NEG	185.7	POS

Sample NO	Age	DIAMEDIX HSV 1+2 T/C	Assay Results	Bioelisa HSV 1 IgG T/C	Assay Results	Bioelisa HSV 2 IgG T/C	Assay Results	Virotech HSV-1	Assay Result	Human VZ IgG T/C	Assay Results
215	60	-	-	5.2	POS	0.5	NEG	0.994	POS	-	-
216	3	0.5	NEG	-	-	-	-	1.82	POS	-	-
217	52	-	-	2.9	POS	1	E	0.763	POS	1837	POS
218	53	-	-	4	POS	5.7	POS	1.210	POS	-	-
219	34	-	-	1.2	POS	3.3	POS	0.651	POS	-	-
220	28	-	-	1.1	POS	11.6	POS	0.723	POS	-	-
221	26	29.9	POS	1.1	POS	4.5	POS	0.665	POS	-	-
222	32	-	NT	0.8	NEG	4.6	POS	0.531	POS	-	-
223	30	-	NT	0.8	NEG	1.3	POS	0.123	NEG	-	-
224	31	-	NT	1.3	POS	6.1	POS	0.735	POS	-	-
225	28	-	NT	4.6	POS	2.6	POS	1.320	POS	-	-
226	35	-	POS	2.4	POS	5.5	POS	1.21	POS	-	-
227	35	-	NT	3.8	POS	1.9	POS	0.892	POS	-	-
228	57	-	NT	4.3	POS	1.9	POS	1.112	POS	-	-
229	37	-	NT	4.4	POS	8.3	POS	1.221	POS	-	-
230	39	-	NT	3.1	POS	1.1	POS	0.988	POS	-	-
231	45	-	NT	5.8	POS	3.5	POS	1.341	POS	-	-
232	49	-	NT	8.1	POS	2.2	POS	1.632	POS	-	-
233	35	-	NT	4.0	POS	9.0	POS	1.221	POS	-	-
234	12	-	NT	3.2	POS	6.0	POS	1.132	POS	-	-
235	30	-	NT	4	POS	4.2	POS	1.213	POS	-	-
236	34	-	NT	5.1	POS	5.2	POS	1.231	POS	-	-
237	46	-	NT	5.1	POS	2.0	POS	1.301	POS	-	-
238	29	-	-	4.4	POS	2.9	POS	1.251	POS	-	-

Sample NO	Age	DIAMEDIX HSV 1+2 T/C	Assay Results	Bioelisa HSV 1 IgG T/C	Assay Results	Bioelisa HSV 2 IgG T/C	Assay Results	Virotech HSV-1	Assay Result	Human VZ IgG T/C	Assay Results
239	60	-	-	3.2	POS	7.3	POS	1.021	POS	-	-
240	43	-	-	4.6	POS	2.4	POS	1.321	POS	-	-
241	42	-	-	4.3	POS	2.2	POS	1.215	POS	-	-
242	38	-	-	4.3	POS	1.2	POS	1.431	POS	-	-
243	23	-	-	3.7	POS	1.4	POS	1.324	POS	-	-
244	36	-	-	0.69	NEG	4.25	POS	0.210	NEG	-	-
245	22	-	-	0.78	NEG	1.98	POS	0.130	NEG	9.9	POS
246	55	-	-	0.19	NEG	1.52	POS	0.218	NEG	-	-
247	47	-	-	0.09	NEG	7.54	POS	0.232	NEG	-	-
248	43	-	-	0.1	NEG	1.7	POS	0.112	NEG	-	-
249	46	-	-	0.19	NEG	4.11	POS	0.242	NEG	-	-
250	39	-	-	0.04	NEG	5.43	POS	0.045	NEG	-	-
251	4	-	-	0.05	NEG	2.02	POS	0.032	NEG	-	-
252	44	-	-	0.08	NEG	1.55	POS	0.045	NEG	-	-
253	54	-	-	0.14	NEG	5.87	POS	0.012	NEG	-	-
254	24	-	-	0.29	NEG	5.09	POS	0.120	NEG	-	-

Table 2 Summary of the Results Obtained from Omega, Focus, Biokit and Western blot Assays for Panel 1 Sera.

Sample No	Omega OD Reading	Omega Result	BioKit OD Reading	BioKit Result	Focus OD Reading	Focus Result	Virotech OD Reading	Virotech Result	Western blot result
1	0.899	POS	0.074	NEG	0.728	POS	0.532	POS	POS
2	0.059	NEG	0.022	NEG	0.018	NEG	1.691	POS	-
3	0.067	NEG	0.017	NEG	0.02	NEG	1.414	POS	-
4	0.163	NEG	0.033	NEG	0.054	NEG	1.311	POS	-
5	0.692	POS	0.535	POS	0.025	NEG	1.498	POS	POS
6	1.2	POS	1.094	POS	2.345	POS	-	-	-
7	2.745	POS	1.236	POS	3.615	POS	1.982	POS	-
8	0.057	NEG	0.032	NEG	0.063	NEG	1.516	POS	-
9	0.097	NEG	0.075	NEG	0.386	POS	0.64	POS	-
10	0.109	NEG	0.04	NEG	0.036	NEG	1.492	POS	-
11	0.094	NEG	0.033	NEG	0.033	NEG	1.732	POS	POS
12	0.132	NEG	0.006	NEG	0.011	NEG	0.693	POS	-
13	0.069	NEG	0.021	NEG	0.073	NEG	1.55	POS	-
14	0.301	NEG	0.02	NEG	0.195	NEG	-	-	-
15	1.56	POS	0.841	POS	2.065	POS	0.937	POS	-
16	1.72	POS	0.739	POS	2.262	POS	0	NT	-
17	0.067	NEG	0.016	NEG	0.039	NEG	0.915	POS	-
18	0.2	NEG	0.025	NEG	0.046	NEG	1.343	POS	-
19	0.097	NEG	0.028	NEG	0.044	NEG	1.514	POS	-
20	0.0278	NEG	0.083	NEG	0.134	NEG	1.443	POS	-
21	0.068	NEG	0.017	NEG	0.03	NEG	-	-	-

Sample No	Omega OD Reading	Omega Result	BioKit OD Reading	BioKit Result	Focus OD Reading	Focus Result	Virotech OD Reading	Virotech Result	Western blot result
22	0.061	NEG	0.013	NEG	0.013	NEG	1.352	POS	-
23	0.103	NEG	0.023	NEG	0.019	NEG	1.615	POS	-
24	0.048	NEG	0.015	NEG	0.031	NEG	1.671	POS	-
25	0.085	NEG	0.37	NEG	0.177	NEG	1.484	POS	-
26	0.073	NEG	0.061	NEG	0.022	NEG	1.533	POS	-
27	0.104	NEG	0.065	NEG	0.029	NEG	2.175	POS	-
28	0.048	NEG	0.03	NEG	0.063	NEG	1.69	POS	-
29	0.064	NEG	0.015	NEG	0.016	NEG	1.553	POS	-
30	0.046	NEG	0.011	NEG	0.009	NEG	1.387	POS	-
31	0.057	NEG	0.13	NEG	0.066	NEG	1.374	POS	-
32	0.249	NEG	0.14	NEG	0.07	NEG	1.799	POS	-
33	0.157	NEG	0.101	NEG	0.035	NEG	0.489	POS	-
34	0.108	NEG	0.032	NEG	0.02	NEG	0.56	POS	-
35	0.147	NEG	0.153	NEG	0.022	NEG	0.56	POS	-
36	0.156	NEG	0.025	NEG	0.022	NEG	1.151	POS	-
37	0.14	NEG	0.078	NEG	0.138	NEG	1.74	POS	-
38	0.069	NEG	0.041	NEG	0.033	NEG	1.764	POS	-
39	0.067	NEG	0.017	NEG	0.012	NEG	1.228	POS	-
40	0.399	NEG	0.057	NEG	0.024	NEG	-	-	NEG
41	2.3	POS	1.123	POS	2.516	POS	1.754	POS	-
42	1.98	POS	0.273	NEG	0.683	POS	1.835	POS	POS
43	0.104	NEG	0.022	NEG	0.047	NEG	1.526	POS	-
44	2.921	POS	0.89	POS	2.614	POS	1.644	POS	-

Sample No	Omega OD Reading	Omega Result	BioKit OD Reading	BioKit Result	Focus OD Reading	Focus Result	Virotech OD Reading	Virotech Result	Western blot result
45	0.074	NEG	0.019	NEG	0.049	NEG	1.747	POS	-
46	0.05	NEG	0.011	NEG	0.013	NEG	1.525	POS	-
47	0.076	NEG	0.022	NEG	0.056	NEG	0.636	POS	-
48	0.087	NEG	0.023	NEG	0.035	NEG	1.567	POS	-
49	0.985	POS	0.393	NEG	1.913	POS	1.387	POS	POS
50	2.101	POS	1.183	NEG	0.621	POS	1.642	POS	POS
51	0.07	NEG	0.008	NEG	0.015	NEG	1.255	POS	
52	0.074	NEG	0.018	NEG	0.087	NEG	-	-	-
53	0.052	NEG	0.012	NEG	0.018	NEG	1.765	POS	-
54	0.082	NEG	0.029	NEG	0.066	NEG	1.653	POS	-
55	0.067	NEG	0.02	NEG	0.04	NEG	-	-	-
56	0.137	NEG	0.031	NEG	0.032	NEG	-	-	-
57	0.078	NEG	0.007	NEG	0.042	NEG	0.749	POS	-
58	0.167	NEG	0.017	NEG	0.021	NEG	-	-	-
59	0.21	NEG	0.018	NEG	0.066	NEG	1.273	POS	-
60	0.169	NEG	0.009	NEG	0.026	NEG	-	-	-
61	0.293	NEG	0.062	NEG	0.123	NEG	-	-	-
62	0.099	NEG	0.062	NEG	0.019	NEG	0.652	POS	-
63	0.114	NEG	0.007	NEG	0.028	NEG	0.789	POS	-
64	0.045	NEG	0.017	NEG	0.014	NEG	1.384	POS	-
65	0.057	NEG	0.038	NEG	0.046	NEG	1.812	POS	-
66	0.157	NEG	0.064	NEG	0.031	NEG	1.441	POS	-
67	0.526	POS	0.105	NEG	0.065	NEG	-	-	E
68	0.121	NEG	0.014	NEG	0.065	NEG	1.491	POS	-

Sample No	Omega OD Reading	Omega Result	BioKit OD Reading	BioKit Result	Focus OD Reading	Focus Result	Virotech OD Reading	Virotech Result	Western blot result
69	3.2	POS	1.066	POS	2.266	POS	1.678	POS	-
70	0.077	NEG	0.021	NEG	0.04	NEG	1.503	POS	-
71	0.067	NEG	0.013	NEG	0.022	NEG	1.291	POS	-
72	0.17	NEG	0.046	NEG	0.133	NEG	1.548	POS	-
73	0.088	NEG	0.024	NEG	0.023	NEG	-	-	-
74	0.125	NEG	0.021	NEG	0.019	NEG	-	-	-
75	1.561	POS	1.442	POS	3.625	POS	-	-	-
76	2.5	POS	1.002	POS	2.348	POS	-	-	-
77	0.11	NEG	0.021	NEG	0.022	NEG	-	-	-
78	0.434	NEG	0.02	NEG	0.04	NEG	-	-	-
79	0.086	NEG	0.019	NEG	0.018	NEG	-	-	-
80	3.955	POS	1.452	POS	3.764	POS	-	-	-
81	0.12	NEG	0.02	NEG	0.026	NEG	-	-	-
82	2.102	POS	0.406	NEG	1.471	POS	-	-	POS
83	0.055	NEG	0.022	NEG	0.046	NEG	-	-	
84	0.549	POS	0.045	NEG	0.374	POS	1.862	POS	POS
85	0.082	NEG	0.028	NEG	0.076	NEG	1.825	POS	-
86	0.095	NEG	0.027	NEG	0.022	NEG	1.63	POS	-
87	0.24	NEG	0.07	NEG	0.116	NEG	1.324	POS	-
88	1.8	NEG	0.675	POS	2.414	POS	1.964	POS	POS
89	0.123	NEG	0.038	NEG	0.044	NEG	1.618	POS	-
90	0.207	NEG	0.028	NEG	0.058	NEG	1.514	POS	-
91	0.062	NEG	0.83	NEG	0.018	NEG	1.934	POS	-
92	0.139	NEG	0.024	NEG	0.035	NEG	1.444	POS	-

Sample No	Omega OD Reading	Omega Result	BioKit OD Reading	BioKit Result	Focus OD Reading	Focus Result	Virotech OD Reading	Virotech Result	Western blot result
93	1.7	POS	0.863	POS	2.22	POS	-	-	-
94	1.792	POS	0.467	POS	1.519	POS	-	-	-
95	0.176	NEG	0.015	NEG	0.042	NEG	0.124	NEG	-
96	0.065	NEG	0.013	NEG	0.028	NEG	1.937	POS	-
97	0.152	NEG	0.012	NEG	0.036	NEG	1.695	POS	-
98	0.107	NEG	0.055	NEG	0.027	NEG	1.317	POS	-
99	0.079	NEG	0.026	NEG	0.045	NEG	-	-	-
100	3.1	POS	0.887	POS	2.395	POS	-	-	
101	1.062	POS	0.401	NEG	1.223	POS	-	-	POS
102	0.058	NEG	0.015	NEG	0.007	NEG	-	-	-
103	0.107	NEG	0.022	NEG	0.013	NEG	-	-	-
104	2.294	POS	1.469	POS	3.497	POS	-	-	-
105	1.612	POS	0.829	POS	2.481	POS	-	-	-
106	0.05	NEG	0.029	NEG	0.028	NEG	-	-	-
107	0.127	NEG	0.03	NEG	0.024	NEG	-	-	-
108	0.068	NEG	0.026	NEG	0.045	NEG	-	-	-
109	1.656	POS	0.787	POS	2.297	POS	-	-	-
110	0.182	NEG	0.051	NEG	0.02	NEG	-	-	-
111	0.123	NEG	0.03	NEG	0.032	NEG	-	-	-
112	1.601	POS	0.0433	NEG	2.662	POS	-	-	POS
113	0.107	NEG	0.053	NEG	0.01	NEG	-	-	-
114	0.167	NEG	0.016	NEG	0.034	NEG	-	-	-
115	0.234	NEG	0.174	NEG	0.088	NEG	-	-	-

Sample No	Omega OD Reading	Omega Result	BioKit OD Reading	BioKit Result	Focus OD Reading	Focus Result	Virotech OD Reading	Virotech Result	Western blot result
116	0.084	NEG	0.02	NEG	0.003	NEG	1.626	POS	-
117	0.089	NEG	0.017	NEG	0.028	NEG	1.675	POS	-
118	0.451	NEG	0.119	NEG	0.054	NEG	1.634	POS	POS
119	0.089	NEG	0.029	NEG	0.176	NEG	1.635	POS	-
120	0.103	NEG	0.009	NEG	0.021	NEG	0.867	POS	-
121	0.122	NEG	0.017	NEG	0.029	NEG	0.076	NEG	-
122	0.121	NEG	0.022	NEG	0.022	NEG	0.109	NEG	-
123	2.554	POS	0.91	POS	2.215	POS	1.512	POS	-
124	0.111	NEG	0.018	NEG	0.031	NEG	0.137	NEG	-
125	0.249	NEG	0.018	NEG	0.072	NEG	0.111	NEG	-
126	1.074	POS	0.165	NEG	1.056	POS	1.201	POS	POS
127	0.247	NEG	0.011	NEG	0.033	NEG	0.088	NEG	-
128	0.236	NEG	0.013	NEG	0.056	NEG	0.128	NEG	-
129	2.165	POS	1.038	POS	2.732	POS	1.148	POS	-
130	0.064	NEG	0.017	NEG	0.022	NEG	-	-	-
131	0.066	NEG	0.02	NEG	0.043	NEG	1.845	POS	-
132	0.113	NEG	0.011	NEG	0.009	NEG	0.171	NEG	-
133	1.735	POS	1.041	POS	3.748	POS	1.688	POS	-
134	0.1	NEG	0.023	NEG	0.049	NEG	0.255	NEG	-
135	0.169	NEG	0.034	NEG	0.036	NEG	0.094	NEG	-
136	0.198	NEG	0.21	NEG	0.06	NEG	1.134	NEG	-
137	0.104	NEG	0.029	NEG	0.034	NEG	-	-	-
138	0.092	NEG	0.29	NEG	0.11	NEG	0.683	POS	-
139	2.34	POS	1.528	POS	3.671	POS	-	-	-

Sample No	Omega OD Reading	Omega Result	BioKit OD Reading	BioKit Result	Focus OD Reading	Focus Result	Virotech OD Reading	Virotech Result	Western blot result
140	1.562	POS	0.623	POS	1.747	POS	-	-	-
141	0.146	NEG	0.054	NEG	0.064	NEG	-	-	-
142	1.365	POS	0.43	NEG	1.237	POS	1.665	POS	POS
143	1.2	POS	0.703	POS	1.821	POS	-	-	-
144	3.1	POS	1.303	POS	2.921	POS	-	-	-
145	0.187	NEG	0.042	NEG	0.022	NEG	-	-	-
146	0.054	NEG	0.034	NEG	0.013	NEG	-	-	-
147	0.167	NEG	0.03	NEG	0.035	NEG	1.575	POS	-
148	1.523	POS	1.283	POS	2.612	POS	-	-	-
149	0.094	NEG	0.021	NEG	0.034	NEG	-	-	-
150	0.126	NEG	0.035	NEG	0.025	NEG	1.639	POS	-
151	0.152	NEG	0.039	NEG	0.104	NEG	0.838	POS	-
152	0.063	NEG	0.036	NEG	0.011	NEG	1.527	POS	-
153	0.112	NEG	0.017	NEG	0.022	NEG	1.497	POS	-
154	0.614	POS	0.057	NEG	0.14	NEG	1.477	POS	POS
155	2.658	POS	0.511	POS	0.971	POS	1.713	POS	-
156	0.073	NEG	0.019	NEG	0.011	NEG	1.52	POS	-
157	0.063	NEG	0.043	NEG	0.35	NEG	1.729	POS	-
158	1.25	POS	0.148	NEG	1.784	POS	0.852	POS	
159	0.077	NEG	0.021	NEG	0.014	NEG	1.374	POS	-
160	0.06	NEG	0.028	NEG	0.01	NEG	1.639	POS	-
161	1.987	POS	1.246	POS	2.236	POS	1.602	POS	-
162	0.082	NEG	0.033	NEG	0.024	NEG	1.695	POS	-
163	0.119	NEG	0.828	POS	1.341	POS	1.746	POS	POS

Sample No	Omega OD Reading	Omega Result	BioKit OD Reading	BioKit Result	Focus OD Reading	Focus Result	Virotech OD Reading	Virotech Result	Western blot result
164	0.083	NEG	0.019	NEG	0.025	NEG	1.45	POS	-
165	0.165	NEG	0.024	NEG	0.053	NEG	-	-	-
166	0.144	NEG	0.016	NEG	0.085	NEG	-	-	-
167	0.086	NEG	0.021	NEG	0.026	NEG	-	-	-
168	0.111	NEG	0.027	NEG	0.037	NEG	-	-	-
169	0.076	NEG	0.02	NEG	0.032	NEG	-	-	-
170	0.13	NEG	0.021	NEG	0.007	NEG	0.078	NEG	-
171	1.07	POS	0.032	NEG	0.243	NEG	-	-	POS
172	0.28	NEG	0.022	NEG	0.016	NEG	-	-	-
173	0.098	NEG	0.014	NEG	0.008	NEG	1.505	POS	-
174	0.061	NEG	0.027	NEG	0.002	NEG	1.543	POS	-
175	0.072	NEG	0.055	NEG	0.02	NEG	1.766	POS	-
176	0.23	NEG	0.019	NEG	0.048	NEG	1.751	POS	-
177	0.06	NEG	0.018	NEG	0.007	NEG	1.846	POS	-
178	0.127	NEG	1.539	POS	0.036	NEG	1.668	POS	
179	3.1	POS	0.016	NEG	3.111	POS	1.644	POS	-
180	0.146	NEG	0.017	NEG	0.074	NEG	0.091	NEG	-
181	0.183	NEG	0.05	NEG	0.048	NEG	0.057	NEG	-
182	1.128	POS	0.203	NEG	0.465	POS	1.878	POS	POS
183	0.284	NEG	0.119	NEG	0.019	NEG	0.077	NEG	-
184	0.314	NEG	0.064	NEG	0.022	NEG	0.064	NEG	-
185	0.083	NEG	0.051	NEG	0.029	NEG	0.048	NEG	-
186	0.196	NEG	0.056	NEG	0.028	NEG	0.082	NEG	-

Sample No	Omega OD Reading	Omega Result	BioKit OD Reading	BioKit Result	Focus OD Reading	Focus Result	Virotech OD Reading	Virotech Result	Western blot result
187	0.064	NEG	0.046	NEG	0.016	NEG	0.072	NEG	-
188	0.258	NEG	0.039	NEG	0.028	NEG	0.083	NEG	-
189	0.145	NEG	0.102	NEG	0.028	NEG	1.986	POS	-
190	0.277	NEG	0.083	NEG	0.037	NEG	0.095	NEG	-
191	0.158	NEG	0.106	NEG	0.032	NEG	0.076	NEG	-
192	0.145	NEG	0.108	NEG	0.057	NEG	0.114	NEG	-
193	0.079	NEG	0.044	NEG	0.016	NEG	0.705	POS	-
194	0.089	NEG	0.71	NEG	0.012	NEG	0.33	NEG	-
195	0.09	NEG	0.027	NEG	0.049	NEG	0.085	NEG	-
196	0.086	NEG	0.03	NEG	0.015	NEG	0.036	NEG	-
197	0.222	NEG	0.111	NEG	0.019	NEG	-	-	-
198	0.13	NEG	0.032	NEG	0.018	NEG	-	-	-
199	2.312	POS	0.38	POS	1.074	POS	-	-	-
200	0.083	NEG	0.044	NEG	0.024	NEG	0.048	NEG	-
201	0.097	NEG	0.08	NEG	0.025	NEG	-	-	-
202	0.097	NEG	0.034	NEG	0.016	NEG	0.077	NEG	-
203	0.085	NEG	0.047	NEG	0.015	NEG	-	-	-
204	0.055	NEG	0.021	NEG	0.004	NEG	1.43	POS	-
205	0.234	NEG	0.039	NEG	0.018	NEG	0.074	NEG	-
206	3.2	POS	1.236	POS	3.275	POS	-	-	-
207	2.281	POS	1.067	POS	2.527	POS	-	-	-
208	0.194	NEG	0.102	NEG	0.002	NEG	0.089	NEG	-
209	0.112	NEG	0.066	NEG	0.017	NEG	0.098	NEG	-

Sample No	Omega OD Reading	Omega Result	BioKit OD Reading	BioKit Result	Focus OD Reading	Focus Result	Virotech OD Reading	Virotech Result	Western blot result
210	0.116	NEG	0.044	NEG	0.073	NEG	0.116	NEG	-
211	0.063	NEG	0.054	NEG	0.016	NEG	-	-	-
212	0.292	NEG	0.127	NEG	0.283	POS	-	-	-
213	0.067	NEG	0.038	NEG	0.018	NEG	0.064	NEG	-
214	0.228	NEG	0.076	NEG	0.028	NEG	0.05	NEG	-
215	0.194	NEG	0.08	NEG	0.35	NEG	-	-	-
216	0.078	NEG	0.062	NEG	0.054	NEG	1.82	POS	-
217	2.321	POS	0.257	NEG	0.78	POS	-	-	POS
218	3.08	POS	0.862	POS	1.893	POS	-	-	-
219	0.122	NEG	1.047	POS	0.793	POS	-	-	-
220	1.865	POS	1.493	POS	2.681	POS	-	-	-
221	1.99	POS	0.684	POS	1.342	POS	-	-	-
222	2.912	POS	0.731	POS	0.944	POS	-	-	-
223	1.35	POS	0.32	NEG	0.582	POS	-	-	-
224	1.146	POS	0.698	POS	1.645	POS	-	-	-
225	1.482	POS	0.493	NEG	1.024	POS	-	-	-
226	3.22	POS	1.121	POS	1.389	POS	-	-	-
227	1.274	POS	0.517	NEG	0.744	POS	-	-	-
228	3.12	POS	0.453	NEG	1.079	POS	-	-	-
229	2.998	POS	1.215	POS	1.665	POS	-	-	-
230	0.462	NEG	0.335	NEG	0.314	POS	-	-	-
231	3.1	POS	0.511	NEG	1.205	POS	-	-	-
232	2.218	POS	1.06	POS	1.703	POS	-	-	-

Sample No	Omega OD Reading	Omega Result	BioKit OD Reading	BioKit Result	Focus OD Reading	Focus Result	Virotech OD Reading	Virotech Result	Western blot result
233	2.125	POS	0.934	POS	1.904	POS	-	-	-
234	2.952	POS	1.456	POS	2.27	POS	-	-	-
235	2.75	POS	0.832	POS	1.634	POS	-	-	-
236	2.552	POS	1.354	POS	2.045	POS	-	-	-
237	2.854	POS	0.746	POS	1.756	POS	-	-	-
238	2.213	POS	0.696	POS	2.389	POS	-	-	-
239	2.654	POS	1.592	POS	1.421	POS	-	-	-
240	1.404	POS	0.987	POS	0.71	POS	-	-	-
241	3.1	POS	0.59	POS	0.053	NEG	-	-	-
242	0.081	NEG	0.046	NEG	2.49	POS	-	-	-
243	3.1	POS	1.662	POS	2.382	POS	-	-	-
244	2.954	POS	1.839	POS	2.735	POS	-	-	-
245	0.103	NEG	0.067	NEG	0.037	NEG	-	-	-
246	0.253	NEG	0.044	NEG	0.054	NEG	-	-	-
247	1.98	POS	1.472	POS	2.136	POS	-	-	-
248	2.101	POS	0.875	POS	1.28	POS	-	-	-
249	2.654	POS	1.46	POS	1.189	POS	-	-	-
250	2.765	POS	1.59	POS	2.3	POS	-	-	-
251	2.34	POS	0.726	POS	1.225	POS	-	-	-
252	1.454	POS	0.558	POS	0.942	POS	-	-	-
253	3.2	POS	1.438	POS	2.171	POS	-	-	-
254	2.102	POS	1.066	POS	1.725	POS	?	POS	-

Table 3 Summaries of the Results Obtained from HSV-1 gD, Peptide 55, VZV gE, VZV LIAISON ELISAs and HSV-1 gD, Peptide 55 and VZV gE Triplex Immunoassay.

Sample No	HSV-1 gD ELISA OD Reading	Result	HSV-1 gD Luminex MFI Reading	Result	Peptide 55 ELISA OD Reading	Result	Peptide 55 Luminex MFI Reading	Result	VZV gE ELISA OD Reading	Results	VZV gE Luminex MFI Reading	Results	VZV LIAISON Assay	Results
1	0.51	POS	2200	POS	0.899	POS	2164	POS	2.46	POS	5760	POS	3258	POS
2	0.673	POS	3650	POS	0.059	NEG	220	NEG	0.75	POS	1568	POS	1342	POS
3	0.808	POS	4589	POS	0.067	NEG	230	NEG	0.531	POS	1236	POS	696	POS
4	0.786	POS	3350	POS	0.163	NEG	231	NEG	0.786	POS	1810	POS	987	POS
5	1.003	POS	5430	POS	0.692	POS	2230	POS	0.618	POS	1750	POS	865	POS
6	1.01	POS	5429.5	POS	1.2	POS	3500	POS	1.012	POS	2560	POS	1569	POS
7	0.414	POS	1989	POS	2.745	POS	6200	POS	0.861	POS	2112	POS	1360	POS
8	0.834	POS	3887	POS	0.057	NEG	199	NEG	0.712	POS	1680	POS	1120	POS
9	0.319	POS	1800	POS	0.097	NEG	210	NEG	1.236	POS	3210	POS	1650	POS
10	1.561	POS	7510	POS	0.109	NEG	234	NEG	1.3	POS	3399	POS	1625	POS
11	0.863	POS	4859.8	POS	0.094	NEG	250	NEG	0.863	POS	1998	POS	1210	POS
12	0.319	POS	1450	POS	0.132	NEG	265	NEG	0.319	POS	983	POS	640	POS
13	0.739	POS	3158	POS	0.069	NEG	256	NEG	0.521	POS	1561	POS	527	POS
14	0.877	POS	4596	POS	0.301	NEG	354	NEG	0.701	POS	1967	POS	1133	POS
15	0.486	POS	2351	POS	1.56	POS	3980	POS	0.98	POS	2987	POS	1892	POS
16	1.32	POS	6883	POS	1.72	POS	3500	POS	0.368	POS	1003	POS	486	POS
17	0.091	NEG	279.3	NEG	0.067	NEG	264	NEG	0.982	POS	2697	POS	1610	POS
18	0.652	POS	2980	POS	0.2	NEG	238	NEG	0.554	POS	1987	POS	762	POS
19	0.865	POS	4120	POS	0.097	NEG	197	NEG	1.81	POS	5120	POS	2654	POS
20	0.517	POS	2310	POS	0.0278	NEG	125	NEG	0.521	POS	1456	POS	676	POS
21	0.66	POS	3450	POS	0.068	NEG	129	NEG	0.432	POS	980	POS	161	EQV
22	0.882	POS	4190	POS	0.061	NEG	189	NEG	0.425	POS	1098	POS	391	POS

Sample No	HSV-1 gD ELISA OD Reading	Result	HSV-1 gD Luminex MFI Reading	Result	Peptide 55 ELISA OD Reading	Result	Peptide 55 Luminex MFI Reading	Result	VZV gE ELISA OD Reading	Results	VZV gE Luminex MFI Reading	Results	VZV LIAISON Assay	Results
23	1.025	POS	5610	POS	0.103	NEG	164	NEG	0.391	POS	867	POS	563	POS
24	0.809	POS	3665	POS	0.048	NEG	189	NEG	1.75	POS	4675	POS	2098	POS
25	0.86	POS	4535.5	POS	0.085	NEG	285	NEG	0.86	POS	2269	POS	1260	POS
26	0.56	POS	2350	POS	0.073	NEG	168	NEG	0.42	POS	1165	POS	825	POS
27	1.081	POS	5150	POS	0.104	NEG	365	NEG	1.081	POS	2680	POS	1983	POS
28	0.94	POS	4890	POS	0.048	NEG	156	NEG	0.425	POS	1350	POS	426	POS
29	0.851	POS	3576.5	POS	0.064	NEG	186	NEG	0.761	POS	2001	POS	1210	POS
30	0.461	POS	1850	POS	0.046	NEG	146	NEG	0.628	POS	1490	POS	1120	POS
31	0.714	POS	3450	POS	0.057	NEG	167	NEG	1.029	POS	2643	POS	1654	POS
32	0.984	POS	4766.8	POS	0.249	NEG	354	NEG	1.608	POS	4562	POS	2261	POS
33	0.325	POS	1268	POS	0.157	NEG	129	NEG	0.515	POS	1235	POS	663	POS
34	0.565	POS	2260	POS	0.108	NEG	123	NEG	0.462	POS	850	POS	249	POS
35	0.95	POS	4370.8	POS	0.147	NEG	146	NEG	0.673	POS	1456	POS	711	POS
36	0.454	POS	2200	POS	0.156	NEG	185	NEG	0.421	POS	1203	POS	570	POS
37	0.74	POS	4210	POS	0.14	NEG	136	NEG	0.398	POS	780	POS	246	POS
38	0.941	POS	5010	POS	0.069	NEG	133	NEG	1.31	POS	3651	POS	1867	POS
39	0.286	POS	1230	POS	0.067	NEG	136	NEG	0.286	POS	995	POS	526	POS
40	0.757	POS	3800	POS	0.399	NEG	520	NEG	0.757	POS	2361	POS	1101	POS
41	0.988	POS	4761	POS	2.3	POS	5400	POS	0.213	NEG	210	NEG	15	NEG
42	0.972	POS	4980	POS	1.98	POS	3980	POS	0.972	POS	2645	POS	828	POS
43	1.12	POS	5304	POS	0.104	NEG	165	NEG	1.12	POS	3125	POS	1410	POS
44	0.959	POS	5120	POS	2.921	POS	7560	POS	0.215	NEG	254	NEG	158	NEG
45	0.946	POS	4943.3	POS	0.074	NEG	129	NEG	0.561	POS	1986	POS	593	POS
46	0.394	POS	1955.5	POS	0.05	NEG	128	NEG	0.394	POS	1563	POS	502	POS
47	1.104	POS	5520	POS	0.076	NEG	210	NEG	2.011	POS	4982	POS	2486	POS
48	0.831	POS	4300	POS	0.087	NEG	205	NEG	0.986	POS	2563	POS	1189	POS

Sample No	HSV-1 gD ELISA OD Reading	Result	HSV-1 gD Luminex MFI Reading	Result	Peptide 55 ELISA OD Reading	Result	Peptide 55 Luminex MFI Reading	Result	VZV gE ELISA OD Reading	Results	VZV gE Luminex MFI Reading	Results	VZV LIAISON Assay	Results
49	0.588	POS	3480	POS	0.985	POS	2900	POS	0.785	POS	2310	POS	1765	POS
50	0.59	POS	3290	POS	2.101	POS	4800	POS	0.705	POS	2596	POS	1741	POS
51	0.482	POS	2190	POS	0.07	NEG	198	NEG	0.482	POS	1236	POS	411	POS
52	0.876	POS	3860	POS	0.074	NEG	198	NEG	0.851	POS	2456	POS	1564	POS
53	0.546	POS	2614	POS	0.052	NEG	179	NEG	1.32	POS	3654	POS	1456	POS
54	0.814	POS	4050	POS	0.082	NEG	209	NEG	0.697	POS	2289	POS	661	POS
55	0.448	POS	2600	POS	0.067	NEG	201	NEG	1.689	POS	4789	POS	2364	POS
56	0.642	POS	2756	POS	0.137	NEG	297	NEG	0.642	POS	1569	POS	938	POS
57	0.247	POS	1090	POS	0.078	NEG	198	NEG	0.468	POS	1256	POS	592	POS
58	0.284	POS	1210.9	POS	0.167	NEG	256	NEG	0.59	POS	1299	POS	544	POS
59	0.56	POS	2925.5	POS	0.21	NEG	325	NEG	0.895	POS	2216	POS	1669	POS
60	0.476	POS	1950	POS	0.169	NEG	260	NEG	0.957	POS	1987	POS	1314	POS
61	0.851	POS	4024	POS	0.293	NEG	380	NEG	0.439	POS	1654	POS	568	POS
62	0.111	NEG	187.8	NEG	0.099	NEG	168	NEG	0.111	NEG	125	NEG	23	NEG
63	0.519	POS	2350	POS	0.114	NEG	245	NEG	0.616	POS	958	POS	734	POS
64	0.707	POS	3735.3	POS	0.045	NEG	135	NEG	0.098	NEG	325	NEG	30	NEG
65	1.18	POS	6120	POS	0.057	NEG	156	NEG	1.95	POS	5361	POS	2265	POS
66	0.603	POS	3329	POS	0.157	NEG	314	NEG	0.603	POS	1896	POS	722	POS
67	0.397	POS	1750	POS	0.526	POS	1650	POS	1.32	POS	3589	POS	1569	POS
68	0.846	POS	4281	POS	0.121	NEG	246	NEG	0.75	POS	2563	POS	1653	POS
69	0.978	POS	4500	POS	3.2	POS	7214	POS	0.523	POS	1254	POS	614	POS
70	0.985	POS	4422.3	POS	0.077	NEG	168	NEG	1.52	POS	4258	POS	1498	POS
71	0.625	POS	2498	POS	0.067	NEG	186	NEG	0.753	POS	1711	POS	698	POS
72	0.881	POS	4620	POS	0.17	NEG	224	NEG	0.881	POS	2890	POS	871	POS
73	0.686	POS	3250	POS	0.088	NEG	198	NEG	2.009	POS	5780	POS	2753	POS
74	0.852	POS	3990	POS	0.125	NEG	213	NEG	0.431	POS	780	POS	282	POS

Sample No	HSV-1 gD ELISA OD Reading	Result	HSV-1 gD Luminex MFI Reading	Result	Peptide 55 ELISA OD Reading	Result	Peptide 55 Luminex MFI Reading	Result	VZV gE ELISA OD Reading	Results	VZV gE Luminex MFI Reading	Results	VZV LIAISON Assay	Results
75	1.212	POS	6150	POS	1.561	POS	3459	POS	1.369	POS	3256	POS	1953	POS
76	1.043	POS	5480	POS	2.5	POS	5800	POS	0.638	POS	1982	POS	897	POS
77	0.839	POS	4590	POS	0.11	NEG	199	NEG	0.618	POS	1792	POS	536	POS
78	1.015	POS	5210	POS	0.434	NEG	985	NEG	2.514	POS	6125	POS	3139	POS
79	0.678	POS	2987	POS	0.086	NEG	189	NEG	1.64	POS	4899	POS	2610	POS
80	0.624	POS	2469	POS	3.955	POS	7800	POS	0.981	POS	2598	POS	860	POS
81	0.53	POS	2896	POS	0.12	NEG	192	NEG	1.963	POS	5698	POS	3012	POS
82	0.526	POS	2399	POS	2.102	POS	5600	POS	1.39	POS	3990	POS	1420	POS
83	0.612	POS	2730	POS	0.055	NEG	178	NEG	2.315	POS	6752	POS	3129	POS
84	1.074	POS	5190	POS	0.549	POS	1590	POS	0.129	NEG	213	NEG	15	NEG
85	0.933	POS	5230	POS	0.082	NEG	188	NEG	0.933	POS	3259	POS	1560	POS
86	0.999	POS	4990	POS	0.095	NEG	196	NEG	2.31	POS	6213	POS	3102	POS
87	0.376	POS	1541	POS	0.24	NEG	267	NEG	0.311	NEG	398	NEG	69	NEG
88	0.511	POS	2343	POS	1.8	NEG	3900	NEG	1.461	POS	4259	POS	1770	POS
89	0.62	POS	2680	POS	0.123	NEG	211	NEG	0.531	POS	1459	POS	390	POS
90	0.912	POS	5090	POS	0.207	NEG	243	NEG	0.912	POS	2350	POS	890	POS
91	0.686	POS	2850	POS	0.062	NEG	139	NEG	0.789	POS	1986	POS	748	POS
92	0.776	POS	3820	POS	0.139	NEG	229	NEG	1.231	POS	3012	POS	1542	POS
93	0.516	POS	2597.9	POS	1.7	POS	3789	POS	0.516	POS	1280	POS	526	POS
94	0.725	POS	2988	POS	1.792	POS	3860	POS	0.985	POS	2367	POS	817	POS
95	0.103	NEG	260	NEG	0.176	NEG	298	NEG	1.125	POS	3450	POS	1659	POS
96	0.878	POS	3960	POS	0.065	NEG	198	NEG	1.023	POS	3159	POS	1275	POS
97	0.77	POS	3598	POS	0.152	NEG	201	NEG	1.21	POS	3569	POS	1236	POS
98	0.284	POS	1130	POS	0.107	NEG	189	NEG	1.254	POS	3856	POS	1568	POS
99	0.681	POS	2865	POS	0.079	NEG	188	NEG	0.998	POS	2310	POS	940	POS
100	0.986	POS	5160	POS	3.1	POS	6200	POS	0.986	POS	2459	POS	666	POS

Sample No	HSV-1 gD ELISA OD Reading	Result	HSV-1 gD Luminex MFI Reading	Result	Peptide 55 ELISA OD Reading	Result	Peptide 55 Luminex MFI Reading	Result	VZV gE ELISA OD Reading	Results	VZV gE Luminex MFI Reading	Results	VZV LIAISON Assay	Results
101	0.761	POS	3250	POS	1.062	POS	2640	POS	0.957	POS	2685	POS	963	POS
102	0.823	POS	4650	POS	0.058	NEG	167	NEG	2.541	POS	6541	POS	2559	POS
103	0.726	POS	3123	POS	0.107	NEG	270	NEG	1.953	POS	4685	POS	2198	POS
104	0.743	POS	3254	POS	2.294	POS	4860	POS	1.854	POS	4390	POS	1985	POS
105	0.938	POS	5110	POS	1.612	POS	4209	POS	0.621	POS	980	POS	607	POS
106	0.786	POS	3984	POS	0.05	NEG	199	NEG	0.621	POS	1120	POS	894	POS
107	0.586	POS	2689	POS	0.127	NEG	290	NEG	0.498	POS	789	POS	610	POS
108	1.112	POS	5980	POS	0.068	NEG	165	NEG	0.874	POS	2689	POS	1269	POS
109	0.874	POS	4461	POS	1.656	POS	3756	POS	0.897	POS	2597	POS	1563	POS
110	0.594	POS	3265	POS	0.182	NEG	266	NEG	0.594	POS	1128	POS	154	**EQV**
111	0.915	POS	4250	POS	0.123	NEG	237	NEG	0.915	POS	2687	POS	1221	POS
112	0.943	POS	4985	POS	1.601	POS	4340	POS	1.402	POS	3490	POS	1466	POS
113	0.826	POS	4710	POS	0.107	NEG	310	NEG	1.23	POS	2690	POS	1485	POS
114	0.479	POS	2380	POS	0.167	NEG	259	NEG	1.115	POS	3210	POS	1821	POS
115	0.907	POS	5012	POS	0.234	NEG	340	NEG	1.019	POS	2389	POS	1226	POS
116	1.187	POS	6120	POS	0.084	NEG	157	NEG	1.112	POS	2260	POS	870	POS
117	0.62	POS	3125	POS	0.089	NEG	169	NEG	0.691	POS	1456	POS	680	POS
118	0.562	POS	2350	POS	0.451	NEG	**1390**	**POS**	0.562	POS	1256	POS	662	POS
119	0.735	POS	3490	POS	0.089	NEG	193	NEG	0.735	POS	1689	POS	1120	POS
120	0.285	POS	1219	POS	0.103	NEG	223	NEG	1.712	POS	4126	POS	2015	POS
121	0.092	NEG	271.9	NEG	0.122	NEG	235	NEG	0.092	NEG	169	NEG	16	NEG
122	0.231	POS	1012	POS	0.121	NEG	229	NEG	1.428	POS	2987	POS	1438	POS
123	0.26	POS	1050	POS	2.554	POS	5789	POS	1.567	POS	3658	POS	1600	POS
124	0.109	NEG	190	NEG	0.111	NEG	216	NEG	2.601	POS	6650	POS	2706	POS
125	0.097	NEG	290	NEG	0.249	NEG	311	NEG	0.989	POS	1987	POS	1227	POS
126	0.24	POS	1125	POS	1.074	POS	3120	POS	2.256	POS	5421	POS	2994	POS

Sample No	HSV-1 gD ELISA OD Reading	Result	HSV-1 gD Luminex MFI Reading	Result	Peptide 55 ELISA OD Reading	Result	Peptide 55 Luminex MFI Reading	Result	VZV gE ELISA OD Reading	Results	VZV gE Luminex MFI Reading	Results	VZV LIAISON Assay	Results
127	0.126	NEG	216	NEG	0.247	NEG	325	NEG	2.1	POS	5998	POS	2504	POS
128	0.108	NEG	199	NEG	0.236	NEG	328	NEG	1.023	POS	2108	POS	950	POS
129	0.505	POS	2310	POS	2.165	POS	4798	POS	2.641	POS	6423	POS	3365	POS
130	0.87	POS	4790	POS	0.064	NEG	196	NEG	1.26	POS	2965	POS	1393	POS
131	0.657	POS	2943	POS	0.066	NEG	240	NEG	0.858	POS	1568	POS	610	POS
132	0.096	NEG	210	NEG	0.113	NEG	206	NEG	1.365	POS	4000	POS	1760	POS
133	0.595	POS	2456	POS	1.735	POS	3960	POS	0.987	POS	2219	POS	981	POS
134	0.124	NEG	168	NEG	0.1	NEG	225	NEG	0.854	POS	1983	POS	1048	POS
135	0.103	NEG	209	NEG	0.169	NEG	268	NEG	2.315	POS	6315	POS	3159	POS
136	0.118	NEG	275	NEG	0.198	NEG	310	NEG	0.489	POS	1456	POS	470	POS
137	0.592	POS	3200	POS	0.104	NEG	199	NEG	1.491	POS	3259	POS	1600	POS
138	0.11	POS	198	NEG	0.092	NEG	160	NEG	1.198	POS	2458	POS	1315	POS
139	0.624	POS	3125	POS	2.34	POS	5210	POS	1.198	POS	2459	POS	1213	POS
140	0.526	POS	2202	POS	1.562	POS	4380	POS	1.301	POS	2999	POS	1259	POS
141	0.681	POS	2816	POS	0.146	NEG	240	NEG	1.109	POS	2789	POS	1475	POS
142	0.707	POS	3210	POS	1.365	POS	3950	POS	2.159	POS	5462	POS	2986	POS
143	0.63	POS	2465	POS	1.2	POS	3150	POS	0.864	POS	1921	POS	1172	POS
144	0.629	POS	3402	POS	3.1	POS	7210	POS	2.31	POS	5987	POS	3269	POS
145	0.597	POS	2950	POS	0.187	NEG	290	NEG	2.917	POS	7460	POS	3782	POS
146	1.008	POS	5210	POS	0.054	NEG	169	NEG	0.621	POS	1730	POS	496	POS
147	0.864	POS	4680	POS	0.167	NEG	29	NEG	0.891	POS	1890	POS	964	POS
148	0.948	POS	5213	POS	1.523	POS	3250	POS	0.912	POS	2010	POS	735	POS
149	0.689	POS	2856	POS	0.094	NEG	164	NEG	1.128	POS	2589	POS	1330	POS
150	0.584	POS	2578	POS	0.126	NEG	219	NEG	0.835	POS	1838	POS	1314	POS
151	0.243	POS	1120	POS	0.152	NEG	248	NEG	1.312	POS	2921	POS	1736	POS
152	0.572	POS	2420	POS	0.063	NEG	149	NEG	0.989	POS	1987	POS	901	POS

Sample No	HSV-1 gD ELISA OD Reading	Result	HSV-1 gD Luminex MFI Reading	Result	Peptide 55 ELISA OD Reading	Result	Peptide 55 Luminex MFI Reading	Result	VZV gE ELISA OD Reading	Results	VZV gE Luminex MFI Reading	Results	VZV LIAISON Assay	Results
153	0.644	POS	2856	POS	0.112	NEG	227	NEG	0.589	POS	1789	POS	615	POS
154	0.569	POS	2498	POS	0.614	POS	1650	POS	2.758	POS	6415	POS	2987	POS
155	0.745	POS	3589	POS	2.658	POS	5741	POS	0.581	POS	1598	POS	794	POS
156	0.807	POS	4500	POS	0.073	NEG	146	NEG	0.459	POS	1601	POS	1342	POS
157	0.554	POS	2689	POS	0.063	NEG	260	NEG	2.159	POS	5862	POS	3129	POS
158	0.325	POS	1489	POS	1.25	POS	2982	POS	2.321	POS	5983	POS	3561	POS
159	0.436	POS	1691	POS	0.077	NEG	136	NEG	0.432	POS	1328	POS	751	POS
160	0.795	POS	4150	POS	0.06	NEG	166	NEG	2.328	POS	5561	POS	2998	POS
161	0.668	POS	2987	POS	1.987	POS	4150	POS	1.236	POS	2896	POS	1278	POS
162	0.634	POS	2589	POS	0.082	NEG	197	NEG	0.261	NEG	321	NEG	169	NEG
163	0.737	POS	3350	POS	0.119	NEG	250	NEG	3.102	POS	7391	POS	3841	POS
164	0.907	POS	4980	POS	0.083	NEG	210	NEG	1.128	POS	2987	POS	1311	POS
165	0.41	POS	1653	POS	0.165	NEG	209	NEG	1.39	POS	3251	POS	1125	POS
166	0.864	POS	4760	POS	0.144	NEG	211	NEG	0.569	POS	1852	POS	656	POS
167	0.797	POS	3350	POS	0.086	NEG	195	NEG	1.025	POS	2890	POS	1148	POS
168	0.353	POS	1987	POS	0.111	NEG	180	NEG	2.314	POS	6103	POS	2987	POS
169	0.965	POS	5220	POS	0.076	NEG	173	NEG	0.51	POS	1102	POS	648	POS
170	0.076	NEG	250	NEG	0.13	NEG	167	NEG	1.065	POS	2351	POS	1309	POS
171	0.488	POS	1989	POS	1.07	POS	2685	POS	0.524	POS	1985	POS	522	POS
172	0.95	POS	4590	POS	0.28	NEG	260	NEG	0.861	POS	2361	POS	712	POS
173	0.714	POS	3125	POS	0.098	NEG	187	NEG	1.19	POS	3150	POS	1052	POS
174	0.647	POS	2975	POS	0.061	NEG	129	NEG	0.897	POS	2982	POS	897	POS
175	0.831	POS	4520	POS	0.072	NEG	161	NEG	2.961	POS	7640	POS	3785	POS
176	0.918	POS	4910	POS	0.23	NEG	295	NEG	0.983	POS	2321	POS	942	POS
177	0.742	POS	2968	POS	0.06	NEG	186	NEG	2.678	POS	7356	POS	2987	POS
178	0.44	POS	2560	POS	0.127	NEG	235	NEG	1.651	POS	3724	POS	1620	POS

Sample No	HSV-1 gD ELISA OD Reading	Result	HSV-1 gD Luminex MFI Reading	Result	Peptide 55 ELISA OD Reading	Result	Peptide 55 Luminex MFI Reading	Result	VZV gE ELISA OD Reading	Results	VZV gE Luminex MFI Reading	Results	VZV LIAISON Assay	Results
179	0.695	POS	3210	POS	3.1	POS	6987	POS	2.674	POS	7025	POS	3681	POS
180	0.153	NEG	189	NEG	0.146	NEG	297	NEG	0.126	NEG	215	NEG	165	NEG
181	0.093	NEG	280	NEG	0.183	NEG	230	NEG	0.921	POS	2150	POS	1624	POS
182	0.915	POS	5010	POS	1.128	POS	2753	POS	0.652	POS	1985	POS	552	POS
183	0.177	NEG	198	NEG	0.284	NEG	365	NEG	1.159	POS	2931	POS	835	POS
184	0.126	NEG	125	NEG	0.314	NEG	420	NEG	2.402	POS	6710	POS	2658	POS
185	0.083	NEG	278	NEG	0.083	NEG	195	NEG	1.865	POS	4653	POS	1855	POS
186	0.092	NEG	289	NEG	0.196	NEG	228	NEG	1.987	POS	4569	POS	2337	POS
187	0.123	NEG	167	NEG	0.064	NEG	146	NEG	1.116	POS	2986	POS	1183	POS
188	0.063	NEG	260	NEG	0.258	NEG	359	NEG	0.128	NEG	215	NEG	164	NEG
189	1.003	POS	5210	POS	0.145	NEG	167	NEG	2.782	POS	7715	POS	3780	POS
190	0.118	NEG	191	NEG	0.277	NEG	294	NEG	1.231	POS	2980	POS	989	POS
191	0.058	NEG	210	NEG	0.158	NEG	193	NEG	0.318	NEG	280	NEG	165	NEG
192	0.116	NEG	167	NEG	0.145	NEG	236	NEG	0.115	NEG	325	NEG	120	NEG
193	0.298	POS	1395	POS	0.079	NEG	188	NEG	0.892	POS	2210	POS	768	POS
194	0.085	NEG	266	NEG	0.089	NEG	123	NEG	1.925	POS	4982	POS	2269	POS
195	0.149	NEG	199	NEG	0.09	NEG	199	NEG	3.1	POS	7853	POS	3864	POS
196	0.067	NEG	254	NEG	0.086	NEG	180	NEG	0.459	POS	1201	POS	615	POS
197	0.677	POS	3150	POS	0.222	NEG	311	NEG	1.026	POS	2651	POS	1745	POS
198	0.668	POS	3128	POS	0.13	NEG	195	NEG	0.561	POS	1250	POS	854	POS
199	0.612	POS	3298	POS	2.312	POS	5190	POS	0.876	POS	2130	POS	1068	POS
200	0.108	NEG	185	NEG	0.083	NEG	203	NEG	1.506	POS	3989	POS	1796	POS
201	0.456	POS	2651	POS	0.097	NEG	210	NEG	0.684	POS	1689	POS	668	POS
202	0.111	NEG	196	NEG	0.097	NEG	199	NEG	1.865	POS	4563	POS	1917	POS
203	0.795	POS	4380	POS	0.085	NEG	187	NEG	1.598	POS	3912	POS	1941	POS
204	0.321	POS	1280	POS	0.055	NEG	169	NEG	0.098	NEG	316	NEG	159	NEG

Sample No	HSV-1 gD ELISA OD Reading	Result	HSV-1 gD Luminex MFI Reading	Result	Peptide 55 ELISA OD Reading	Result	Peptide 55 Luminex MFI Reading	Result	VZV gE ELISA OD Reading	Results	VZV gE Luminex MFI Reading	Results	VZV LIAISON Assay	Results
205	0.11	NEG	195	NEG	0.234	NEG	365	NEG	0.982	POS	2310	POS	829	POS
206	0.407	POS	2130	POS	3.2	POS	7190	POS	1.011	POS	2410	POS	1109	POS
207	0.694	POS	3125	POS	2.281	POS	4990	POS	0.695	POS	1850	POS	749	POS
208	0.038	NEG	210	NEG	0.194	NEG	198	NEG	0.086	NEG	192	NEG	164	NEG
209	0.04	NEG	297.6	NEG	0.112	NEG	225	NEG	0.057	NEG	184	NEG	142	NEG
210	0.218	POS	1012	POS	0.116	NEG	231	NEG	1.19	POS	3201	POS	1138	POS
211	0.753	POS	3456	POS	0.063	NEG	169	NEG	1.021	POS	2231	POS	1678	POS
212	0.836	POS	4390	POS	0.292	NEG	345	NEG	0.069	NEG	159	NEG	161	NEG
213	0.045	NEG	198	NEG	0.067	NEG	182	NEG	0.452	POS	1150	POS	653	POS
214	0.344	POS	1650	POS	0.228	NEG	312	NEG	0.652	POS	1967	POS	162	**EQV**
215	0.703	POS	3560	POS	0.194	NEG	259	NEG	0.788	POS	1980	POS	913	POS
216	0.499	POS	1897	POS	0.078	NEG	195	NEG	1.192	POS	2789	POS	1547	POS
217	0.398	POS	1611	POS	2.321	POS	5160	POS	1.458	POS	3215	POS	1585	POS
218	0.611	POS	2986	POS	3.08	POS	6841	POS	1.325	POS	3250	POS	1396	POS
219	0.947	POS	5140	POS	0.122	NEG	260	NEG	2.568	POS	6983	POS	2827	POS
220	0.734	POS	3310	POS	1.865	POS	3960	POS	1.36	POS	4120	POS	2105	POS
221	0.748	POS	3179	POS	1.99	POS	4489	POS	0.756	POS	1986	POS	661	POS
222	0.413	POS	2259	POS	2.912	POS	6290	POS	0.897	POS	1985	POS	863	POS
223	0.453	POS	1987	POS	1.35	POS	3260	POS	1.269	POS	3216	POS	1742	POS
224	0.791	POS	3643	POS	1.146	POS	2786	POS	0.459	POS	1120	POS	569	POS
225	0.666	POS	2981	POS	1.482	POS	2864	POS	0.563	POS	1269	POS	664	POS
226	0.387	POS	1598	POS	3.22	POS	7150	POS	1.098	POS	2715	POS	1128	POS
227	0.776	POS	3750	POS	1.274	POS	2987	POS	2.861	POS	6980	POS	3785	POS
228	0.623	POS	3210	POS	3.12	POS	7190	POS	1.25	POS	3120	POS	955	POS
229	0.723	POS	3158	POS	2.998	POS	6487	POS	0.217	POS	253	POS	150	POS
230	0.325	POS	1298	POS	0.462	NEG	**1453**	**POS**	1.321	POS	3145	POS	1747	POS

Sample No	HSV-1 gD ELISA OD Reading	Result	HSV-1 gD Luminex MFI Reading	Result	Peptide 55 ELISA OD Reading	Result	Peptide 55 Luminex MFI Reading	Result	VZV gE ELISA OD Reading	Results	VZV gE Luminex MFI Reading	Results	VZV LIAISON Assay	Results
231	0.772	POS	3854	POS	3.1	POS	7150	POS	1.357	POS	2897	POS	898	POS
232	0.595	POS	3125	POS	2.218	POS	4280	POS	2.645	POS	7160	POS	2816	POS
233	0.499	POS	2219	POS	2.125	POS	4590	POS	1.3	POS	3210	POS	950	POS
234	0.443	POS	1820	POS	2.952	POS	6380	POS	2.956	POS	7230	POS	3569	POS
235	0.599	POS	2546	POS	2.75	POS	5780	POS	0.549	POS	1120	POS	887	POS
236	0.788	POS	3978	POS	2.552	POS	5940	POS	0.036	NEG	328	NEG	165	NEG
237	0.773	POS	3468	POS	2.854	POS	6125	POS	2.876	POS	7761	POS	3852	POS
238	0.693	POS	3210	POS	2.213	POS	5210	POS	0.996	POS	2315	POS	960	POS
239	0.471	POS	1856	POS	2.654	POS	5689	POS	1.106	POS	3210	POS	985	POS
240	0.711	POS	3190	POS	1.404	POS	3290	POS	0.698	POS	1894	POS	662	POS
241	0.733	POS	3800	POS	3.1	POS	6420	POS	2.657	POS	7469	POS	3241	POS
242	0.706	POS	3165	POS	0.081	NEG	179	NEG	1.524	POS	3510	POS	1569	POS
243	0.522	POS	2156	POS	3.1	POS	6811	POS	3.15	POS	8001	POS	3785	POS
244	0.635	POS	2874	POS	2.954	POS	6998	POS	0.658	POS	1359	POS	693	POS
245	0.31	POS	1349	POS	0.103	NEG	2.9	NEG	2.86	POS	7645	POS	3258	POS
246	0.108	NEG	222	NEG	0.253	NEG	324	NEG	1.265	POS	2897	POS	1116	POS
247	0.479	POS	2290	POS	1.98	POS	4397	POS	0.904	POS	1987	POS	808	POS
248	0.488	POS	1987	POS	2.101	POS	4130	POS	2.75	POS	7750	POS	3681	POS
249	0.632	POS	2678	POS	2.654	POS	5711	POS	0.699	POS	2158	POS	576	POS
250	0.507	POS	2341	POS	2.765	POS	5860	POS	2.759	POS	6980	POS	2856	POS
251	0.588	POS	2680	POS	2.34	POS	4620	POS	0.61	POS	1860	POS	750	POS
252	0.388	POS	1965	POS	1.454	POS	3145	POS	0.096	NEG	167	NEG	163	NEG
253	0.797	POS	4123	POS	3.2	POS	7612	POS	1.687	POS	5120	POS	2180	POS
254	0.776	POS	4210	POS	2.102	POS	4710	POS	1.56	POS	4325	POS	2567	POS

Appendix 2

Table 1 Summary of the Results Obtained from the Different Assays for Panel 2 Sera.

Sample No.	**CFT*	***FAT*	*'In-house' ELISA*	*Chiron Test*	*HSV type by Bioelisa Test*	*HSV Type by Gull test*	*HSV Type by CAPTIA Select HSV2-G*
1	<1:10	1&2	1&2	-	-	-	2
2	<1:10	1&2	1&2	1	1	-	2
3	1:40	1&2	1&2	1&2	1&2	1&2	2
4	< 1:10	1	1&2	-	1&2	1&2	2
5	1:10	1&2	1&2	1	1	1	2
6	1:80	1&2	1&2	1	1	1	-
7	<1:10	1&2	1&2	-	-	-	-
8	1:40	1&2	1&2	1	1	1	-
9	<1:10	1	-	-	-	-	-
10	<1:10	1	1	-	-	-	-
11	<1:10	1&2	1&2	1	1	1	-
12	1:10	1&2	1&2	1	-	2	2
13	1:80	1&2	1&2	1&2	1&2	1&2	2
14	1:20	1&2	1&2	1	1	1	-
15	1:20	1&2	1&2	1	1	1	-
16	1:20	1&2	1&2	1	1	1	-
17	1:20	1&2	1&2	1	1	1	-
18	1:10	1&2	1&2	Positive	1	1	2
19	<1:10	1	1&2	Positive	1&2	1&2	2
20	<1:10	1	1&2	Positive	1&2	1&2	2
21	1:20	1&2	1&2	1	1	1	-
22	1:160	1&2	1&2	1	1	1	-
23	1:160	1&2	1&2	1	1	1&2	-
24	1:320	1&2	1&2	1	1	1&2	-
25	1:40	1&2	1&2	1	1	1&2	-
26	1:10	1&2	1&2	1	1	1	-
27	1:10	1&2	1&2	Positive	1	1	2
28	1:160	1&2	1&2	1&2	1&2	1&2	2
29	1:20	1&2	1&2	1&2	1&2	1&2	2
30	1:20	1&2	1&2	1&2	1&2	1&2	2
31	1:20	1&2	1&2	1&2	1&2	1&2	2
32	1:80	1	1	1	-	-	-
33	1:20	1&2	1&2	1	-	1	-
34	1:20	1&2	1&2	1	1&2	1&2	2
35	1:80	1&2	1&2	1	1	1	-
36	1:80	1&2	1&2	1	1	1	-
37	1:80	1&2	1&2	1	1	1	-
38	1:80	1&2	1&2	1&2	1&2	1&2	2
39	<1:10	-	-	-	-	-	-
40	1:80	1&2	1&2	1	1	1	-
41	1:320	1&2	1&2	1	1	1	Equivocal
42	1:320	1&2	1&2	1	1	1	2
43	1:40	1&2	1&2	1	1	1	-
44	1:160	1&2	1&2	1	1	1	-
45	1:40	1&2	1&2	1	1	1	-
46	1:160	1&2	1&2	1	1	1	2
47	1:80	1&2	1&2	1&2	1&2	1&2	2
48	1:80	1&2	1&2	1	1	1	-
49	1:80	1&2	1&2	1	1	1	-
50	1:160	1&2	1&2	1	1	1&2	2
51	1:40	1&2	1&2	1	1	1	-
52	1:40	1&2	1&2	1	1	1	-
53	1:80	1&2	1&2	1	1	1	-
54	1:80	1&2	1&2	1	1	1	-
55	1:80	1&2	1&2	1	1	1&2	-
56	1:40	1&2	1&2	1	1	1&2	-
57	1:160	1&2	1&2	1	1	1&2	-

Sample No.	*CFT	**FAT	'In-house' ELISA	Chiron Test	HSV type by Bioelisa Test	HSV Type by Gull test	HSV Type by CAPTIA Select HSV2-G
58	1:80	1&2	1&2	1&2	1&2	1&2	2
59	1:160	1	1&2	1	-	1	-
60	<1:10	1&2	1&2	-	-	-	-
61	1:20	1&2	1&2	-	-	-	-
62	1:40	1&2	1&2	1	1	-	-
63	1:20	1&2	1&2	1	1	1	-
64	1:40	1&2	1&2	1	1	1	-
65	1:20	1&2	1&2	1	1&2	1&2	2
66	1:20	1&2	1&2	1	1&2	1&2	2
67	1:40	1&2	1&2	1	1&2	1&2	2
68	1:40	1&2	1&2	1	1&2	1&2	2
69	1:80	1&2	1&2	1	1	1	-
70	1:160	1&2	1&2	1	1	1	-
71	1:20	1&2	1&2	2	2	2	2
72	1:80	1&2	1&2	2	2	2	2
73	1:40	1&2	1&2	1	1	1	2
74	1:80	1&2	1&2	1	1&2	1	-
75	1:40	1&2	1&2	1	1&2	1&2	2
76	1:80	1&2	1&2	1&2	1&2	1&2	2
77	1:80	1&2	1&2	1&2	1&2	1&2	2
78	1:80	1&2	1&2	1	1	-	-
79	1:80	-	-	-	-	-	-
80	1:80	1	1&2	1	1	1	-
81	<1:10	1&2	1&2	-	-	-	-
82	<1:10	1	-	-	-	-	-
83	1:160	1&2	1&2	2	-	2	2
84	1:160	1&2	1&2	1	1	1	-
85	1:160	1&2	1&2	1&2	1&2	1&2	2
86	1:160	1&2	1&2	1	1	1	-
87	1:80	1&2	1&2	Positive	-	-	2
88	1:320	1&2	1&2	1	1	1	-
89	1:320	1&2	1&2	2	2	2	2
90	1:40	1&2	1&2	1&2	1&2	1&2	2
91	1:160	1&2	1&2	1	1	1&2	-
92	1:10	1&2	-	-	-	-	-
93	1:80	1&2	1	1	1	1	-

* CFT: Complement fixation test **FAT: Indirect immunofluorescence test

Sample No.	Chiron Test	HSV Type by Bioelisa Test	HSV Type by Gull Test	HSV Type by CAPTIA Select HSV2-G	HSV Type by Biotest Test	HSV Type by Clark Test
1	1	1&2	1&2	2	1	1 & 2
2	1	1	1	-	1	1 & 2
3	1	1	1	-	1	1 & 2
4	1	1	1&2	2	1	1 & 2
5	1	1	1	-	1	1
6	1	1	1	-	1	1 & 2
7	1	1	1	-	1	1 & 2
8	1	1	1	-	1	1 & 2
9	1	1	1	-	1	1 & 2
10	1	1	1	Equivocal	1	1 & 2
11	1	1	1	-	1	1 & 2
12	1	1	1	-	1	1 & 2
13	1	1	1	-	1	1
14	1	1	1	-	1	1 & 2
15	1	1	1	-	1	1 & 2
16	1	1	1	-	1	1 & 2
17	1	1	1	-	1	1 & 2
18	1	1	1	-	1	1 & 2
19	1	1	1	-	1	1
20	1	1	1	-	1	1
21	1	1	1	-	1	1 & 2
22	1	1	1	-	1	1 & 2
23	1	1	1	-	1	1 & 2
24	2	2	1&2	2	1	1 & 2
25	2	2	2	2	1	1 & 2
26	2	2	2	2	1	1 & 2
27	2	1&2	1&2	2	1 & 2	1 & 2
28	2	1&2	1&2	2	1	1 & 2
29	2	1&2	1&2	2	1 & 2	1 & 2
30	2	1&2	1	-	1	1 & 2
31	2	2	2	2	1	1 & 2
32	2	2	2	2	1	1 & 2
33	2	2	2	2	1	1 & 2
34	2	2	2	2	1 & 2	1 & 2
35	2	1&2	1&2	2	1	1 & 2
36	2	2	1&2	2	1 & 2	1 & 2
37	2	2	2	2	1	1 & 2
38	2	2	-	2	1	1 & 2
39	2	2	2	2	1	1 & 2
40	2	1&2	1	2	1 & 2	1 & 2
41	2	2	2	2	1	1 & 2
42	2	2	1&2	2	1 & 2	1 & 2
43	2	2	1&2	2	1	1 & 2
44	2	2	1&2	2	1	1 & 2
45	2	1&2	1&2	2	1	1 & 2
46	2	1&2	1&2	2	1	1 & 2
47	2	1&2	-	-	1	1 & 2
48	2	2	2	2	1	1 & 2
49	2	2	2	2	1	1 & 2
50	2	1&2	1&2	2	1	1 & 2
51	2	1&2	2	2	1	1 & 2
52	2	2	1	2	1	1 & 2
53	2	2	2	2	1	1 & 2
54	2	2	1	2	1	1 & 2
55	2	2	-	2	-	1 & 2
56	1&2	1&2	1&2	2	1	1 & 2
57	1&2	1&2	1&2	2	1	1 & 2

Sample No.	Chiron Test	HSV Type by Bioelisa Test	HSV Type by Gull Test	HSV Type by CAPTIA Select HSV2-G	HSV Type by Biotest Test	HSV Type by Clark Test
58	1&2	1&2	1&2	2	1	1 & 2
59	1&2	1&2	1&2	2	1 & 2	1 & 2
60	1&2	1&2	1&2	2	1	1 & 2
61	1&2	1&2	1&2	2	1	1 & 2
62	1&2	1&2	1&2	2	1	1 & 2
63	1&2	1&2	1&2	2	1	1 & 2
64	1&2	1&2	1&2	2	1	1 & 2
65	1&2	1&2	1&2	2	1	1 & 2
66	1&2	1&2	1&2	2	1	1 & 2
67	1&2	1&2	1&2	2	1	1 & 2
68	1&2	1&2	1&2	2	1	1 & 2
69	1&2	1&2	1&2	2	1	1 & 2
70	1&2	1&2	1	2	1	1 & 2
71	1&2	1&2	1&2	2	1	1 & 2
72	1&2	1&2	1&2	2	1	1 & 2
73	1&2	1&2	1&2	2	1	1 & 2
74	1&2	1&2	1&2	2	1	1 & 2
75	1&2	1&2	1&2	2	1	1 & 2
76	1&2	1&2	1&2	2	1	1 & 2
77	1&2	1&2	1&2	2	1	1 & 2
78	1&2	1&2	1&2	2	1	1 & 2
79	1&2	1&2	1&2	2	1 & 2	1 & 2
80	1&2	1&2	1&2	2	1	1 & 2
81	1&2	1&2	1&2	2	1 & 2	1 & 2
82	1&2	1&2	1&2	2	1	1 & 2
83	1&2	1&2	1&2	2	1 & 2	1 & 2
84	1&2	1&2	1&2	2	1	1 & 2
85	1&2	1&2	1&2	2	1	1 & 2
86	1&2	1&2	1&2	2	1	1 & 2
87	1&2	1&2	1&2	2	1	1 & 2
88	1&2	1&2	1&2	2	1	1 & 2
89	1&2	1&2	1&2	2	1	1 & 2
90	-	-	-	-	-	-

Sample No.	Age	HSV-1 (O.D. 450)	HSV-1 Result	HSV-2 (O.D. 450)	HSV-2 Result	HSV Type
1	15	0.879	+	0.097	-	1
2	15	0.090	-	0.055	-	-
3	15	0.125	+	0.066	-	1
4	15	1.640	+	0.096	-	1
5	15	0.672	+	0.085	-	1
6	15	0.941	+	0.059	-	1
7	15	0.901	+	0.129	-	1
8	15	2.063	+	0.131	-	1
9	15	0.206	+	0.086	-	1
10	15	1.030	+	0.129	-	1
11	15	0.002	-	0.002	-	-
12	15	0.039	-	0.034	-	-
13	16	1.016	+	0.079	-	1
14	17	0.844	+	0.056	-	1
15	17	1.793	+	0.121	-	1
16	17	1.007	+	0.088	-	1
17	17	0.766	+	0.116	-	1
18	17	1.709	+	0.085	-	1
19	17	0.939	+	0.103	-	1
20	17	0.018	-	0.014	-	-
21	18	2.324	+	0.145	-	1
22	18	0.532	+	0.054	-	1
23	18	1.808	+	0.124	-	1
24	19	0.005	-	0.002	-	-
25	19	0.030	-	0.024	-	-
26	19	1.059	+	0.114	-	1
27	19	1.957	+	0.100	-	1
28	20	0.313	+	0.070	-	1
29	20	0.278	+	0.078	-	1
30	20	0.343	+	0.035	-	1
31	20	0.665	+	0.073	-	1
32	20	0.320	+	0.024	-	1
33	20	1.891	+	0.088	-	1
34	20	0.038	-	0.033	-	-
35	20	1.033	+	0.067	-	1
36	20	0.347	+	0.061	-	1
37	20	1.568	+	0.135	-	1
38	20	1.656	+	0.092	-	1
39	20	0.890	+	0.103	-	1
40	20	1.877	+	0.697	+	1 & 2
41	20	0.807	+	0.086	-	1
42	20	0.714	+	0.065	-	1
43	21	1.137	+	0.088	-	1
44	21	0.555	+	0.048	-	1
45	22	0.088	-	0.035	-	-
46	22	1.947	+	0.062	-	1
47	22	1.068	+	0.085	-	1
48	22	0.016	-	0.003	-	-
49	22	0.005	-	0.012	-	-
50	23	0.011	-	0.004	-	-
51	23	0.762	+	0.091	-	1
52	23	0.939	+	0.100	-	1
53	23	0.452	+	0.127	-	1
54	23	1.589	+	0.078	-	1

Positive : Negative 'cut-off' O.D. values for HSV-1 were 0.117, 0.134, 0.227 in different assays.
Positive : Negative 'cut-off' O.D. values for HSV-2 were 0.200, 0.211, 0.475 in different assays.

Sample No.	Age	HSV-1 (O.D. 450)	HSV-1 Result	HSV-2 (O.D. 450)	HSV-2 Result	HSV Type
55	23	1.00	+	0.044	-	1
56	24	0.056	-	0.012	-	-
57	24	0.198	+	0.074	-	1
58	24	0.020	-	1.521	+	2
59	24	0.237	+	0.065	-	1
60	24	1.315	+	0.089	-	1
61	24	0.665	+	0.244	+	1 & 2
62	24	2.133	+	0.158	-	1
63	24	0.081	-	0.615	+	2
64	25	1.440	+	0.737	+	1 & 2
65	25	0.027	-	0.017	-	-
66	25	1.203	+	0.077	-	1
67	25	2.090	+	0.901	+	1 & 2
68	25	1.933	+	0.064	-	1
69	25	1.00	+	0.112	-	1
70	25	0.022	-	0.001	-	-
71	25	0.110	-	0.047	-	-
72	25	0.676	+	0.416	+	1 & 2
73	25	0.024	-	0.013	-	-
74	26	0.034	-	0.747	+	2
75	26	0.881	+	0.337	+	1 & 2
76	26	0.369	+	0.110	-	1
77	26	1.781	+	0.088	-	1
78	26	1.374	+	0.090	-	1
79	26	1.306	+	0.238	-	1
80	26	1.671	+	0.281	-	1
81	26	1.416	+	0.240	-	1
82	27	1.298	+	0.105	-	1
83	27	1.707	+	0.235	-	1
84	27	1.898	+	0.259	-	1
85	27	0.009	-	0.014	-	-
86	27	0.015	-	0.024	-	-
87	28	0.525	+	0.072	-	1
88	28	1.927	+	0.096	-	1
89	28	0.035	-	0.027	-	-
90	29	1.473	+	0.142	-	1
91	29	0.113	-	0.302	+	2
92	29	1.362	+	0.143	-	1
93	29	1.404	+	0.135	-	1
94	29	0.036	-	0.010	-	-
95	29	0.031	-	0.773	+	2
96	29	0.466	+	0.561	+	1 & 2
97	29	1.886	+	0.099	-	1
98	30	0.033	-	0.108	-	-
99	30	0.022	-	0.158	-	-
100	30	0.055	-	0.034	-	-
101	30	1.022	+	0.094	-	1
102	30	1.676	+	0.278	-	1
103	31	2.069	+	0.177	-	1
104	31	2.397	+	0.251	-	1
105	31	1.630	+	0.222	-	1
106	31	0.035	-	0.026	-	-
107	31	0.042	-	0.036	-	-
108	31	2.305	+	0.182	-	1
109	31	0.274	+	0.126	-	1
110	31	1.589	+	0.32	-	1
111	31	2.586	+	0.289	-	1
112	32	0.123	-	0.038	-	-

Positive : Negative 'cut-off' O.D. values for HSV-1 were 0.117, 0.134, 0.227 in different assays.
Positive : Negative 'cut-off' O.D. values for HSV-2 were 0.200, 0.211, 0.475 in different assays.

Sample No.	Age	HSV-1 (O.D. 450)	HSV-1 Result	HSV-2 (O.D. 450)	HSV-2 Result	HSV Type
113	32	0.535	+	0.246	-	1
114	32	0.022	-	0.015	-	-
115	32	1.753	+	0.222	-	1
116	32	1.956	+	0.264	-	1
117	32	1.690	+	0.245	-	1
118	32	2.029	+	0.164	-	1
119	32	1.283	+	0.587	+	1 & 2
120	32	0.032	-	0.010	-	-
121	32	1.348	+	0.147	-	1
122	33	0.055	-	0.252	-	-
123	33	0.061	-	0.086	-	-
124	33	0.114	-	0.008	-	-
125	34	1.268	+	0.596	+	1 & 2
126	34	2.138	+	0.293	-	1
127	34	0.810	+	1.690	+	1 & 2
128	35	1.586	+	0.158	-	1
129	35	1.712	+	0.168	-	1
130	35	1.994	+	1.019	+	1 & 2
131	35	0.025	-	0.026	-	-
132	35	0.028	-	0.042	-	-
133	35	1.072	+	0.137	-	1
134	35	0.941	+	0.199	-	1
135	35	0.009	-	0.019	-	-
136	35	1.050	+	0.179	-	1
137	35	0.023	-	0.014	-	-
138	35	0.979	+	0.492	+	1 & 2
139	35	0.021	-	0.016	-	-
140	36	2.259	+	0.251	-	1
141	36	0.002	-	0.017	-	-
142	36	0.199	-	1.143	+	2
143	36	1.822	+	0.263	-	1
144	36	1.525	+	0.268	-	1
145	37	1.002	+	1.043	+	1 & 2
146	37	0.686	+	0.061	-	1
147	37	2.353	+	0.564	+	1 & 2
148	37	0.021	-	0.023	-	-
149	37	1.156	+	0.146	-	1
150	38	1.825	+	0.156	-	1
151	38	0.025	-	0.067	-	-
152	38	0.020	-	1.229	+	2
153	39	1.311	+	0.225	-	1
154	39	2.073	+	0.166	-	1
155	39	1.936	+	1.124	+	1 & 2
156	39	1.739	+	0.376	-	1
157	40	0.059	-	1.072	+	2
158	40	0.027	-	0.020	-	-
159	40	1.063	+	1.221	+	1 & 2
160	40	0.855	+	0.162	-	1
161	40	0.014	-	0.017	-	-
162	40	0.054	-	0.068	-	-
163	40	0.039	-	0.036	-	-
164	40	0.023	-	1.337	+	2
165	40	0.030	-	0.003	-	-
166	40	1.806	+	1.753	+	1 & 2
167	41	0.818	+	0.162	-	1
168	41	1.545	+	0.179	-	1
169	41	1.148	+	0.524	+	1 & 2
170	41	1.188	+	0.160	-	1

Positive : Negative 'cut-off' O.D. values for HSV-1 were 0.117, 0.134, 0.227 in different assays.
Positive : Negative 'cut-off' O.D. values for HSV-2 were 0.200, 0.211, 0.475 in different assays.

Sample No.	Age	HSV-1 (O.D. 450)	HSV-1 Result	HSV-2 (O.D. 450)	HSV-2 Result	HSV Type
171	41	0.138	-	0.091	-	-
172	41	1.766	+	0.146	-	1
173	41	0.789	+	0.674	+	1 & 2
174	41	0.022	-	0.013	-	-
175	41	0.359	+	0.125	-	1
176	42	0.588	+	0.106	-	1
177	42	1.973	+	0.108	-	1
178	42	0.022	-	0.026	-	-
179	42	1.932	+	0.188	-	1
180	42	1.323	+	0.161	-	1
181	42	0.596	+	0.260	-	1
182	42	0.060	-	0.084	-	-
183	42	1.485	+	0.268	-	1
184	43	1.087	+	0.731	+	1 & 2
185	43	1.778	+	0.602	+	1 & 2
186	43	2.177	+	0.19	-	1
187	43	2.219	+	1.704	+	1 & 2
188	44	2.888	+	0.304	-	1
189	44	0.224	-	0.118	-	-
190	44	0.875	+	0.257	+	1 & 2
191	44	1.910	+	0.400	+	1 & 2
192	45	0.269	+	0.040	-	1
193	45	1.420	+	0.085	-	1
194	45	2.209	+	0.172	-	1
195	45	0.015	-	0.009	-	-
196	46	0.041	-	0.025	-	-
197	46	1.173	+	0.027	-	1
198	46	0.044	-	0.018	-	-
199	47	2.181	+	0.066	-	1
200	50	0.117	-	0.039	-	-

Positive : Negative 'cut-off' O.D. values for HSV-1 were 0.117, 0.134, 0.227 in different assays.
Positive : Negative 'cut-off' O.D. values for HSV-2 were 0.200, 0.211, 0.475 in different assays.

Table 4 Results Bioelisa HSV-1 and HSV-2 IgG assays in Patients Attending the Genito-Urinary Medicine Clinic (Panel 5).

Case No.	*Sex*	*Age*	*Swab No*	*Date Received*	***Virus Isolated*	*Serum No.*	*Date of Collection*	*O.D. 450 HSV-1*	*HSV-1 Result*	*O.D. 450 HSV-2*	*HSV-2 Result*	*HSV Type*
1	m	30	**1**	19/12/97	HSV-2	**1**	13/05/96	2.029	+	0.371	+	1&2
2	m	36	**2**	08/01/97	HSV-1	**4**	20/01/97	0.552	+	0.068	-	1
3	m	35	**3**	04/06/97	HSV-2	**6**	04/06/97	0.187	+	0.059	-	1
4	m	45	**4**	04/06/97	HSV-1	**7**	04/06/97	0.061	-	0.031	-	-
5	m	67	**5**	26/08/97	+	**8**	10/06/97	1.770	+	0.227	-	1
6	m	33	**6**	25/06/97	HSV-2	**9**	25/06/97	0.097	-	0.140	-	-
7	f	16	**7**	09/07/97	HSV-1	**10**	09/07/97	0.028	-	0.025	-	-
8	m	32	**8**	22/09/97	HSV-2	**11**	22/07/97	1.124	+	0.095	-	1
9	f	30	**9**	28/07/97	HSV-1	**12**	28/07/97	0.071	-	0.060	-	-
10	f	22	**10**	11/08/97	HSV-2	**13**	11/08/97	0.034	-	0.058	-	-
11	m	35	**11**	19/08/97	HSV-2	**14**	15/08/97	0.575	+	0.237	-	1
12	f	22	**12**	20/08/97	HSV-2	**15**	20/08/97	1.662	+	0.098	-	1
13	m	48	**13**	21/08/97	HSV-2	**16**	21/08/97	0.889	+	0.143	-	1
14	f	26	**14**	22/08/97	HSV-1	**17**	22/08/97	0.043	-	0.068	-	-
15	f	31	**15**	26/08/97	HSV-1	**18**	26/08/97	0.040	-	0.104	-	-
16	f	23	**16**	27/08/97	-	**19**	27/08/97	0.111	-	1.00	+	2
17	f	36	**17**	29/08/97	HSV-2	**20**	29/08/97	0.005	-	0.006	-	-
18	f	21	**18**	10/09/97	-	**21**	10/09/97	1.229	+	0.055	-	1
19	f	26	**19**	11/09/97	-	**22**	11/09/97	0.028	-	0.024	-	-
20	m	25	**20**	15/09/97	HSV-2	**24**	15/09/97	0.416	+	0.301	+	1&2
21	f	20	**21**	16/09/97	-	**25**	16/09/97	0.028	-	0.031	-	-
22	f	18	**22**	17/09/97	HSV-2	**26**	17/09/97	1.563	+	0.132	-	1
23	f	23	**23**	26/09/97	HSV-2	**27**	26/09/97	0.677	+	0.101	-	1
24	f	39	**24**	02/10/97	HSV-2	**28**	02/10/97	0.951	+	0.576	+	1&2
25	f	17	**25**	14/10/97	HSV-1	**29**	14/10/97	0.236	+	0.02	-	1
26	m	22	**26**	16/10/97	HSV-2	**30**	16/10/97	0.030	-	0.033	-	-
27	m	20	**27**	16/10/97	HSV-2	**31**	16/10/97	0.058	-	0.537	+	2
28	m	21	**28**	22/10/97	HSV-2	**32**	22/10/97	0.206	+	0.451	+	1&2

Positive : Negative 'cut-off' O.D. values for HSV-1 were 0.102, 0.132, 0.134, 0.136, 0.165, 0.287, 0.403, 0.676 in different assays.
Positive : Negative 'cut-off' O.D. values for HSV-2 were 0.169, 0.211, 0.268, 0.293, 0.367, 0.488, 0.753, 0.825 in different assays.

Case No.	Sex	Age	Swab No	Date Received	**Virus Isolated	Serum No.	Date of Collection	O.D. 450 HSV-1	HSV-1 Result	O.D. 450 HSV-2	HSV-2 Result	HSV Type
29	f	18	29	22/10/97	HSV-2	33	22/10/97	0.355	+	0.245	-	1
30	m	23	30	25/10/97	HSV-1	34	24/10/97	0.178	+	0.039	-	1
31	m	44	31	10/12/97	HSV-1	35	10/12/97	1.233	+	0.062	-	1
32	m	31	32	29/10/97	HSV-1	36	29/10/97	0.622	+	0.179	-	1
33	f	24	33	05/11/97	-	37	05/11/97	0.037	-	0.031	-	-
34	f	17	34	17/11/97	HSV-1	39	17/11/97	0.857	+	0.108	-	1
35	f	28	35	11/11/97	HSV-2	40	18/11/97	0.949	+	0.266	-	1
36	f	22	36	24/11/97	HSV-1	41	24/11/97	0.054	-	0.047	-	-
37	m	20	37	26/11/97	HSV-2	42	26/11/97	0.071	-	0.081	-	-
38	f	33	38	28/11/97	HSV-1	43	28/11/97	0.619	+	0.109	-	1
39	m	27	39	02/12/97	HSV-2	44	02/12/97	1.221	+	1.436	+	1&2
40	f	21	40	09/12/97	HSV-2	46	09/12/97	0.233	+	0.072	-	1
41	m	26	41	19/12/97	HSV-2	47	19/12/97	1.343	+	0.336	+	1&2
42	f	35	42	22/12/97	HSV-2	48	22/12/97	0.816	+	0.424	+	1&2
43	m	21	43	24/07/97	HSV-2	49	25/05/96	0.065	-	0.013	-	-
44	f	21	44	15/01/98	HSV-2	51	15/01/98	0.055	-	0.805	+	2
45	m	25	45	16/01/98	HSV-2	52	16/01/98	0.022	-	1.569	+	2
46	f	16	46	14/11/97	NT	38	14/11/97	0.057	-	0.713	+	2
47	f	36	-	No Iso.	No Iso.	53	20/01/98	0.144	-	1.319	+	2
48	m	13	-	No Iso.	No Iso.	55	30/01/98	2.282	+	0.425	-	1
49	m	32	-	No Iso.	No Iso.	57	02/02/98	2.174	+	0.105	-	1
50	m	26	-	No Iso.	No Iso.	58	04/02/98	0.310	-	0.105	-	-
51	f	29	-	No Iso.	No Iso.	59	03/02/98	0.062	-	0.027	-	-
52	f	33	47	11/09/97	NT	60	11/09/97	0.511	+	0.201	-	1
53	m	33	-	No Iso.	No Iso.	62	10/02/98	1.975	+	0.747	-	1
54	m	19	-	No Iso.	No Iso.	64	26/03/98	0.296	+	0.048	-	1
55	f	32	-	No Iso.	No Iso.	66	20/02/98	1.739	+	0.136	-	1
56	m	53	-	No Iso.	No Iso.	67	12/02/98	0.077	-	0.052	-	-

Positive : Negative 'cut-off' O.D. values for HSV-1 were 0.102, 0.132, 0.134, 0.136, 0.165, 0.287, 0.403, 0.676 in different assays.
Positive : Negative 'cut-off' O.D. values for HSV-2 were 0.169, 0.211, 0.268, 0.293, 0.367, 0.488, 0.753, 0.825 in different assays.

Case No.	*Sex*	*Age*	*Swab No.*	*Date received*	***Virus Isolated*	*Serum No.*	*Date of Collection*	*O.D. 450 HSV-1*	*HSV-1 Result*	*O.D. 450 HSV-2*	*HSV-2 Result*	*HSV Type*
57	f	60	**50**	19/05/98	HSV-2*	**68**	19/05/98	0.041	-	0.052	-	-
58	f	29	-	No Iso.	No Iso.	**71**	07/05/98	0.006	-	0.431	+	2
59	m	15	-	No Iso.	No Iso.	**74**	16/06/98	0.070	-	0.066	-	-
60	f	24	-	No Iso.	No Iso.	**75**	25/06/98	2.193	+	0.109	-	1

No Iso. : No virus isolated * Isolation typed by Immunofluorescence test ** Virus isolate was subtyped by PCR NT: Not typed

Positive : Negative 'cut-off' O.D. values for HSV-1 were 0.102, 0.132, 0.134, 0.136, 0.165, 0.287, 0.403, 0.676 in different assays.

Positive : Negative 'cut-off' O.D. values for HSV-2 were 0.169, 0.211, 0.268, 0.293, 0.367, 0.488, 0.753, 0.825 in different assays.