Age related seroepidemiological survey of measles, mumps, rubella, varicella zoster, herpes simplex type 1 and 2 viruses

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy
In the Faculty of Medical and Human Sciences

2015

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Abstract

Age stratified seroepidemiological studies play a crucial role in the design and assessment of vaccination strategies. An existing multiplex bead immunoassay for measles, mumps, rubella and varicella zoster virus antibodies together with a newly developed multiplex bead immunoassay for herpes simplex virus type 1 and type 2 antibodies were used to investigate the age-related seroepidemiology of these viruses in England during 2012.

To develop the HSV-1 and HSV-2 antibody assay, attempts were made to produce full length of HSV-1 and HSV-2 glycoprotein G using a baculovirus vector expression system. While HSV-1 gG protein was produced, the proteins were extensively aggregated. Native glycoprotein G molecules undergo partial removal of HSV-1 signal sequence and HSV-1 short membrane anchor sequence during post translational modification. It is possible that such post translational modification is not performed when protein is processed in insect cell culture. Attempts to produce an HSV-2 glycoprotein G were not successful. It is possible that the high GC-content of HSV-2 glycoprotein G led to poor fidelity of copying the PCR amplification sequence. Commercially available truncated HSV-1 gG and HSV-2 gG were therefore used to develop a duplex microbead immunoassay for the simultaneous detection of specific HSV antibodies in human sera. The resultant assays performed with low sensitivity and

specificity (HSV-1 of 89% and 66%, respectively and for HSV-2 of 79% and 85%, respectively) compared to the reference HerpeSelect ELISA.

The MMRV multiplex bead immunoassay proved rapid, and required minimal sample volume to semi-quantify MMRV specific antibodies. The seroepidemiology of MMR results was compared with previous seroepidemiological studies performed in 1996 in England. The comparison showed an increase in the proportion of individuals who were positive for mumps and measles antibodies in the 2012 survey. The proportion of individuals positive for rubella was essentially unchanged. The increase in the proportion of individuals positive for mumps and measles antibodies in 2012 show the effectiveness of the change in MMR vaccination policy for England from 1996 onward. For VZV, the proportion of individuals who were positive for varicella antibodies between the 1996 and 2012 serological surveys were essentially unchanged. The comparison showed that most young children are susceptible to VZV. At this level of immunity, it can be expected that varicella will continue to produce epidemics of infection in the population, unless varicella vaccination is implemented as a part of routine childhood vaccination.

Declaration

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

Acknowledgement

I wish to thanks to all the people who gave me valuable advice and support during the period of my PhD study.

Firstly, I would like to thanks to my supervisors, Professor Pam Vallely, Professor Paul Klapper, Professor Ray Borrow and my adviser, Dr. Carol Yates for their patience and helpful advice. Besides this, I also wish to express my sincere gratitude to Dr Rachael Almond and all the other staff of the Department of Virology, University of Manchester and Vaccine Evaluation Unit, Public Health England, Manchester Royal Infirmary for their guidance and support in the laboratory.

Then, I would like to thank my colleagues, friends, brothers and sisters for their moral and material support throughout my PhD study.

Finally yet important, I would like to special acknowledge to my parents, for providing me support, lifelong guidance and advice which is much appreciated and invigorated me to complete this thesis.

Dedication

I dedicate this PhD to my parents who were always supported and guided me during the period of my studies.

List of Abbreviations

µL	microlitre
µM	MicroMole
^{0}C	Degree Celsius
5PL	5-parameter logistic curve
aa	Amino acid
AcNPV	*Autographa california* nuclear polyhedrosis virus
Adj	Adjusted
agg	aggregation
AU	Arbitrary number of specific antibodies
AU/mL	Arbitrary unit per milliliter
B	Blank
BCA	Bicinchoninic acid
BCIP/NBT	4-Chloro-2-methylbenzenediazonium/3-hydroxy-3 naphthoic acid 2,4-dimethylanilide phosphate
BEVS	Baculovirus expression vector system
BHI	Brain heart infusion
bp	Base pair
BSA	Bovine serum albumin
CD4+ T-cells	Mature T-helper cells
CDC	Centers for Disease Control and Prevention
CHCA	α-Cyano-4-hydroxycinnamic acid
CO_2	Carbon Dioxide
CPE	cytopathogenic effect
CV	Coefficient of variation
dATP	Deoxyadenosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDC	1-ethyl-3-(-3dimethylaminopropyl)-carbodiimide hydrochloride
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
Exp	Expected concentration of the standard point
F protein	Fusion protein
FI	Fluorescence intensity
H protein	Haemaggutinin protein
HRP	Horseradish peroxidase
HSV	Herpes Simplex Virus
HSV-1 gG	Herpes simplex virus type 1 glycoprotein G
HSV-1	Herpes simplex virus type 1
HSV-2 gG	Herpes simplex virus type 2 glycoprotein G
HSV-2	Herpes simplex virus type 2
HT	High throughput
IgG	Immunoglobulin G
IRL	Internal long repeat sequences
IRS	Internal short repeat sequences
IU/mL	International unit per millilitre
kbp	Kilo base pair
kDa	Kilo Dalton
LB	Luria broth
LIC	Ligation independent cloning
LLOQ	Lower limit of quantitation
M	Mole
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time-of-Flight
MALLS	Multiangle Laser Light Scattering

MEM	Minimum essential medium
MFI	Median fluorescence intensity
mg/mL	Milligram per millilitre
$MgCl_2$	Magnesium Chloride
MIA	Multiplex immunoassay
mL	Millilitre
mL/min	Millilitre per minutes
MMR	Measles, mumps and rubella
MMRV	Measles, mumps, rubella and varicella zoster
MS	Mass spectrometer
NaCl	Sodium chloride
NBBS	Newborn bovine serum
NCBI	National Centre for Biotechnology Information
NHS	UK National Health Service
NIBSC	National Institute for Biological Standards and Control
nm	Nanometer
Obs	Observed concentration of the standard point
obs/exp	Standard curve recovery
OOR	Out of range
ORF	Open reading frame
P	Probability
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHE	Public Health England
PHN	Post-herpetic trigeminal neuralgia
p-value	Probability of obtaining the observed sample result
QC	Quality control

Q-TOF	Quadrupole time-of-flight
r	Correlation coefficient
RIVM	Rijksinstituut voor Volksgezondheid en Milieu
ROC	Receiver operator characterisation
rpm	Revolutions per minute
RU/mL	Relative unit per millilitre
RUBI-1-94	International rubella serum sample
SAB	Sabourard's broth
SDS	Sodium dodecyl sulphate
Sf9	*Spodoptera frugiperda*
SOD	Superoxide dismutase
Sulpho-NHS	N-hydroxysulfosuccinimide
$TCID_{50}$	50% tissue culture infectious dose
TD-PCR	Touchdown polymerase chain reaction
Tm	Melting temperature
TMB	Tetramethylbenzidine
TMHMM	TransMembrane prediction using Hidden Markov Models
TRL	Terminal long repeat sequences
TRS	Terminal short repeat sequences
UK	United Kingdom
UL	Unique long region
US	Unique short region
USA	United States of America
UV	Ultra violet
V	voltage
v/v	Volume/volume
VZV	Varicella Zoster virus
w/v	Weight per volume

WHO	World Health Organization
z-value	The number of standard deviations an observation above the mean

1.0 Introduction

Age stratified seroepidemiological studies (i.e. epidemiological study through the use of serological testing) play a crucial role in the design and assessment of immunisation strategies. These include assessment of the impact of existing strategies both on the target group and the rest of the population, monitoring the effect of any change in strategy and providing data necessary for predicting the effects of policy changes. Seroepidemiological investigations survey the geographical distribution of vaccinated people in a population. Such information is of value in planning for the provision of health services and health protection interventions and for estimating rates of infection in the susceptible populations (Lobel and Kagan, 1978). In order to perform large-scale seroepidemiological studies for multiple pathogens, it is necessary to develop multiplex immunoassays. The present study concerns the development of an age-stratified seroprevalence study of measles, mumps, rubella, varicella zoster, herpes simplex virus (HSV) type-1 and type-2 in the population of England.

1.1 Measles

1.1.1 Classification and virus structure

Measles, also known as Rubeola or Morbilli, is a member of the family *Paramyxoviridae*, genus Morbillivirus (Rima and Duprex, 2006). The word ‘Measles’ is derived from middle Dutch, known as ‘blemish’ (Rima

and Duprex, 2006; De Swart, 2008). Measles, like other Paramyxoviruses, is lipid enveloped with a single stranded negative RNA genome. It contains six structural proteins, two of which span the membrane to form oligomeric spikes. These glycoproteins are Haemaggutinin (H) protein and Fusion (F) protein. The virus particles are pleiomorphic ranging in size from 100 to 300 nm in diameter.

1.1.2 Pathogenesis

Measles infection is initiated by binding of the measles haemagglutinin (H) protein to host cellular receptors (Moss and Griffin, 2012; Yanagi *et al.,* 2006). Then, the fusion (F) protein interacts with the cell membrane to allow fusion between the virus and host cell membranes (Rima and Duprex, 2005). Once inside the cell, the virus uncoats, enters the cell nucleus and undergoes transcription and translation to form new viral proteins which assemble to produce new virus particles that bud from the cell membrane (Rima and Duprex, 2006; Yanagi *et al.,* 2006).

1.1.3 Clinical Features

Measles is a highly contagious disease which is transmitted by aerosols or direct contact with respiratory secretions (Asaria and MacMahon, 2006; De Swart, 2008). The average incubation period (from exposure to onset of rash) for measles infection is 14 days. The prodrome period for measles infection is normally characterised by fever, cough,

runny nose, conjunctivitis and the production of Kopliks spots (oral mucosal membrane lesions) (Griffin *et al.,* 2012; De Swart, 2008). The prodrome for measles infection lasts for four to five days before the characteristic maculopapular rash (Figure 1.1) appears on the face and spread to the whole body (Asaria and MacMahon, 2006). The vast majority of patients will recover soon after the appearance of rash, however, in some, complications may occur. Measles virus can cause diarrhoea, pneumonia, acute encephalitis, subacute encephalitis, otitis media or corneal ulceration (Asaria and MacMahon, 2006; Rima and Duprex, 2006; De Swart, 2008). The complication rates for measles infection varies by age, geographical distribution, and level of immunity (De Swart, 2008). In industrialised countries, the most common complications of measles are otitis media, pneumonia and encephalitis (Van Den Hof *et al.,* 1999).

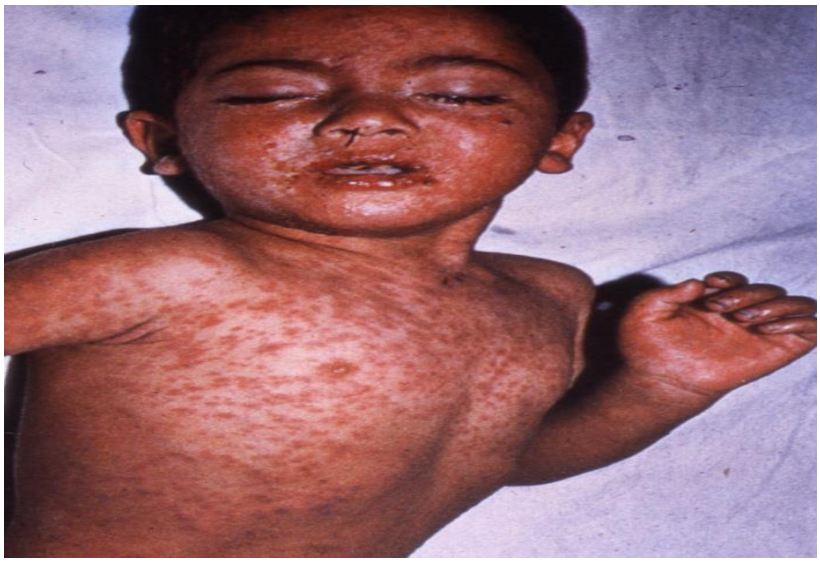


Figure 1.1: Red confluent popular rashes of measles appearing on the face, trunk and extremities. Taken from: http://dermatlas.med.jhmi.edu/derm/IndexDisplay.cfm?ImageID=598177566 (accessed on 03/10/2012)

1.2 Mumps

1.2.1 Classification and Virus structure

It is suggested that the word 'Mumps' came from a British word "to mump", which means to grimace. The first clinical description of mumps was by the ancient Greek physician, Hippocrates, in the fifth century B.C. Mumps virus belongs to the genus Rubulavirus, family *Paramyxoviridae* (Galazka *et al.,* 1999; Hviid *et al.,* 2008). Mumps virus contains a linear, single stranded negative RNA, which is enclosed within a nucleocapsid protein coat in association with an RNA polymerase complex of both large and small phosphoprotein subunits. This ribonucleoprotein complex interacts with matrix protein and the viral envelope. The envelope consists of a bilayer membrane, which contain three glycoproteins,

Haemagglutinin-neuraminidase (HN), fusion (F) and small hydrophobic (SH) protein (Conly and Johnston, 2007; Hviid *et al.,* 2008). The human is the only known host for mumps virus.

1.2.2 Pathogenesis

Mumps virus usually spreads by direct or respiratory contact with the saliva from an infected person (Conly and Johnston, 2007; Hviid *et al.,* 2008). 'Classical' mumps begins with viral replication in the upper respiratory tract and then spreads to the lymph nodes, which results in viraemia. Sometimes, the viraemia will spread the infection to multiple organs in the host and can lead to mumps complications. Mumps is an acute disease, and normally affects the salivary glands, and less often 'attacks' other organs such as pancreas, testis (Orchitis), ovary (Oophoritis), brain, mammary gland, liver, kidney, joint and heart (Hviid *et al.,* 2008). Unfortunately, the complications of mumps are not uncommon and occur more often in adults. Temporary hearing loss is one of the most common mumps complications among the mumps-infected patients. Many studies indicate that the most severe complications of mumps such as aseptic meningitis and orchitis, are more common in adults than children (Conly and Johnston, 2007; Falk *et al.,* 1989; Hviid *et al.,* 2008).

1.2.3 Clinical Features

Mumps is a highly contagious disease, however, mumps is usually more benign in comparison to the other vaccine preventable diseases such as influenza and measles (Galazka *et al.,* 1999). The incubation period for mumps infection is 14 to 21 days, and normally the infected person excretes the virus for 6 days before symptoms appear (Collier and Oxford, 2006a). The infection begins with non-specific symptoms such as fever, headache, myalgia, malaise and anorexia. After that, the disease is characterised by swelling of the parotid gland, either unilaterally (one side) or bilaterally (both sides) (Figure 1.2) (Gupta *et al.,* 2005; Hviid *et al.,* 2008). However, a UK report documented that at least 30% of infected children have asymptomatic mumps (Gupta *et al.,* 2005). In addition, mumps infection in early pregnancy will increase the rate of spontaneous abortion (Conly and Johnston, 2007; Hviid *et al.,* 2008).

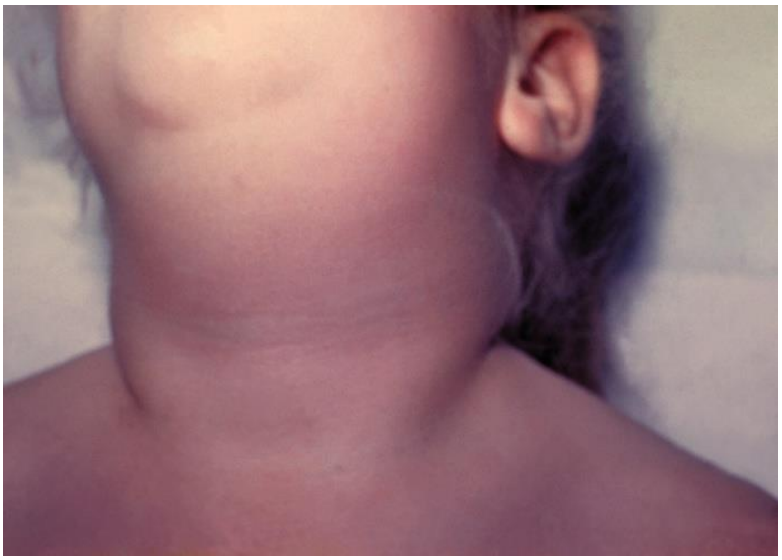


Figure 1.2: A patient with bilateral swelling on his parotid gland. Taken from: http://www.vaccineinformation.org/photos/mumpcdc003a.jpg (Accessed on: 03/10/2012)

1.3 Rubella

1.3.1 Classification and Virus Structure

Rubella, also known as German measles is the only member of the genus Rubivirus, family Togaviridae (Lee and Bowden, 2000). Humans are the only known host for rubella virus. The word ‘Rubella’ is derived from the Latin word rubeus, meaning ‘reddish’. Initially, people considered rubella as a variant of measles and named it 'third disease’. It was not until the mid-eighteenth century (1740) that a first clinical description of rubella was published by a German physician, Friedrich Hoffmann (Chen and Icenogle, 2004; De Santis *et al.,* 2006). Rubella has a single-stranded positive RNA genome, covered by a lipid membrane with a diameter of 50 to 70 nm (Collier and Oxford, 2006b). Its genome contains 9762 nucleotides, encodes 2 nonstructural proteins (p150 and p90) and 3

structural polypeptides, capsid, E1 and E2 (Bardeletti *et al.,* 1975; Chen and Icenogle, 2004; Lee and Bowden, 2000). The glycoproteins E1 and E2 are the elements that form the prominent 'spikes' on the surface of the viral envelope (Zhou *et al.,* 2007). Inside the viral envelope is an icosahedral nucleocapsid 40 nm in diameter. The viral genome is enclosed within the major nucleocapsid formed by homo-oligomerisation of the phosphoprotein (Chen and Icenogle, 2004).

1.3.2 Pathogenesis

The incubation period for rubella infection is 14 to 21 days, with the patient being infectious for up to 7 days before and 5 days after symptoms appear (Bardeletti *et al.,* 1975; Lee and Bowden, 2000). Rubella virus is transmitted by respiratory aerosol, and the major replication of the virus is usually located in the upper respiratory tract and nasopharynx (Collier and Oxford, 2006b). The virus then spreads to regional lymph nodes. Viraemia occurs 5 to 7 days after initial infection.

1.3.3 Clinical Features

The first clinical manifestation of rubella is the appearance of a maculopapular rash 14 to 17 days after exposure. The rash first appears on the face and spreads downwards from head to trunk. For adults and older children, the rash will normally last about 3 days. Lymphadenopathy persists after the rash has resolved and can last for several weeks (Lee

and Bowden, 2000). Other symptoms for rubella infection include low-grade fever, sore throat and malaise. Complications for rubella infection are uncommon, are found more frequently in adults than in children (Chen and Icenogle, 2004; Lee and Bowden, 2000) and include arthralgia, encephalitis and haemorrhagic manifestations. About 70% of mature women, who contract rubella, may suffer arthralgia or arthritis, which often affects fingers, wrists, knee and other joints (Collier and Oxford, 2006b). The major complication of rubella is congenital rubella syndrome (CRS). Congenital rubella may lead to foetal death or premature delivery; damage to all organs of the foetus and produce deafness, cataracts (Figure 1.3), heart defects, mental retardation, bone alterations and liver and spleen damage (Dontigny *et al.,* 2008; De Santis *et al.,* 2006; Tookey, 2004). The basic pathogenic mechanisms underlying congenital rubella symptoms remain unclear. Some studies have found that rubella virus has the ability to induce apoptosis in certain cell types (Bardeletti *et al.,* 1975; Dontigny *et al.,* 2008). The percentage of severe damage to the foetus can be up to 85% if the infection occurs during the first trimester (Lee and Bowden, 2000; De Santis *et al.,* 2006).

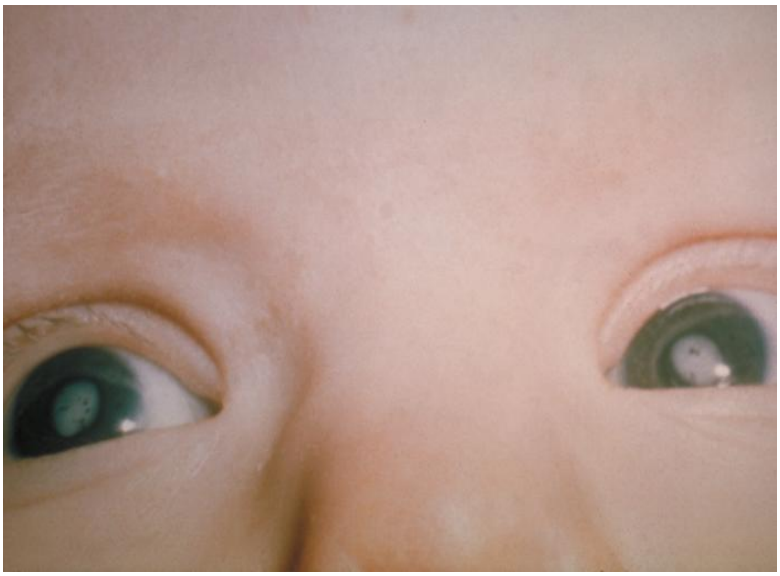


Figure 1.3: The cataracts of congenital rubella syndrome (CRS) appear in a child's eyes. Taken from http://hardinmd.lib.uiowa.edu/cdc/4284.html (accessed on 03/10/2012)

1.4 Varicella Zoster Virus

1.4.1 Classification and structure

Varicella zoster virus (VZV; human herpesvirus 3) (King *et al.,* 2012) also known as chickenpox virus or herpes zoster virus (shingles) belongs to the herpesvirus family, subfamily alphaherpesvirinae (Rockley and Tyring, 1994; Rahaus, 2006; Mueller *et al.*, 2008). The term chickenpox originated from the old English word "gican", which means to itch, while herpes is derived from a Greek word, meaning "to spread or creep" (Beswick, 1962) and zoster means "girdle or zone" (Scott-Wilson, 1978; Rockley and Tyring, 1994). Varicella zoster is an enveloped icosahedral virus with a double-stranded DNA genome and contains at least 70 genes (Rockley and Tyring, 1994). The overall size of VZV varies from 120 to 300 nm diameter. The virus is made up of four major

components, the envelope, tegument, nucleocapsid and genome (Rahaus, 2006). The outer envelope typically has a trilaminar appearance. This space between it and the nucleocapsid is interspersed by an amorphous proteinaceous material, the tegument. The VZV nucleocapsid has an icosahedral shape 100-110 nm in diameter and the size of the VZV genome is around 125 kbp (Davison and Scott, 1986; Rockley and Tyring, 1994). The VZV genome has the smallest genome of the human herpesviruses. The genome, consists of two unique regions, unique long region (UL) and unique short region (US). The UL is bounded by terminal long (TRL) and internal long repeat sequences (IRL), whereas the US is bounded by terminal short (TRS) and internal short repeat sequences (IRS). VZV genome has five repeat regions, which are located at different parts of the genome. Many studies indicate that the length of the repeat regions can be used to distinguish different VZV strains (Cohen, 2001).

1.4.2 Pathogenesis

Varicella zoster virus primary infection begins at respiratory mucosal sites, while viral replication can happen in either epidermal cells or reticuloendothelial organs such as liver and spleen. The exact mechanism for VZV pathogenesis is still unknown, but animal studies (Ku *et.al*, 2005) suggest that the virus is transported from lymph nodes to skin or ganglia by VZV-infected tonsillar CD4+ T-cells. The long incubation period (10-21 days) required for VZV infection can be explained by the

time required to overcome the innate immune response and create the typical vesicular lesions at the skin surface. The process continues until the clearance of viruses by VZV-specific T-cell immunity. After primary infection, VZV latency is established by infecting the neurons (ganglia) with cell-free VZV and infected neurons only express a limited subset of viral genes. As no infective viral particles are expressed during latency, the infected neurons survive destruction by the immune system (Abendroth and Arvin, 2001; Steiner *et al.*, 2007). The decline of VZV-specific T-cell immunity with advancing of age or immunosuppression, (such as may occur in organ transplant patients and HIV-infected patients) provokes the reactivation of VZV (Abendroth and Arvin, 2001; Steiner *et al.*, 2007). Animal model studies by Gershon and colleagues (2008) indicated that latently infected neurons expressed the non-structural VZV ORF61 (open reading frame 61) during virus reactivation, in order to stimulate lytic infection.

1.4.3 Clinical Features

Varicella zoster virus causes: chickenpox (Figure 1.4A) and shingles (Figure 1.4B). Chickenpox is a common childhood disease that produces itchy vesicular lesions (blisters). VZV spreads by the respiratory route or by direct contact with vesicular fluid from infected persons (Ku *et al.*, 2005; Collier and Oxford, 2006c). The incubation period for VZV is around 10-21 days before the development of the characteristic

chickenpox rash (Abendroth and Arvin, 2001). In 10-20% of individuals, VZV recurrent infection occurs years to several decades after the primary infection (varicella) and is most commonly manifested as shingles or herpes zoster. During reactivation, VZV reaches the skin using an axonal pathway to deliver virus to skin cells innervated by the infected nerve, causing the characteristic dermatomal rash of herpes zoster (Abendroth and Arvin, 2001; Collier and Oxford, 2006c; Steiner *et al.*, 2007). In immunocompetent individuals, recurrence of VZV is probably due to the decline of acquired immunity with increasing age. Shingles can be terribly painful and is associated with the development of post-herpetic trigeminal neuralgia (PHN). The condition may become permanent and persistent intractable pain associated with PHN has found to be leading cause of suicide in elderly patients (Schmader, 1998). If host immunity is impaired, infected T-cells can transport VZV to other susceptible sites such as liver, brain and lungs, causing severe varicella complications (Abendroth and Arvin, 2001).

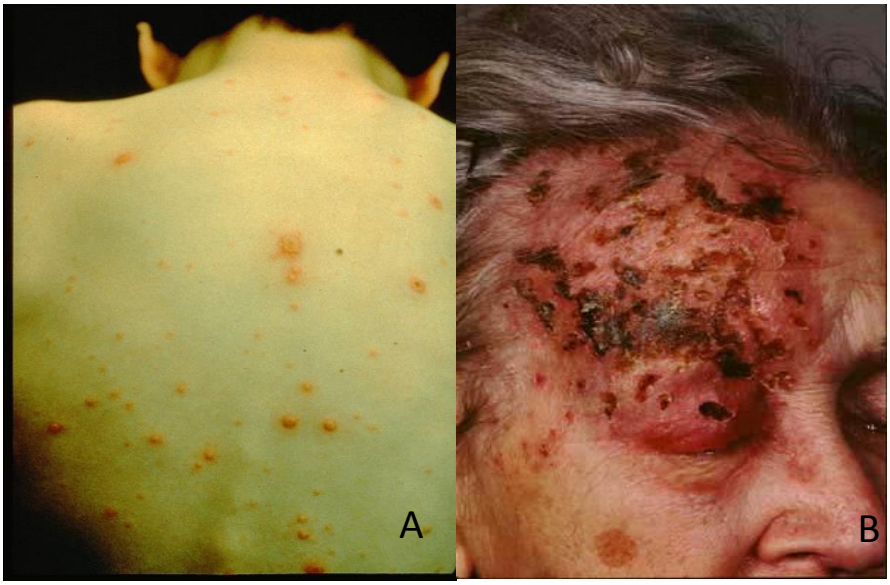


Figure 1.4: A boy with red papules and vesicles of chickenpox appearing on the back and trunk (A). Taken from: http://www.immunize.org/photos/chickenpox-photos.asp (accessed on 16/09/2014). Ophthalmic zoster (B). Taken from: http://www.patient.co.uk/doctor/shingles-and-shingles-vaccination (accessed on 16/09/2014)

1.5 Herpes Simplex Virus

1.5.1 Classification and structure

Herpes simplex virus (HSV) is a member of the Herpesviridae family, subfamily alphaherpesvirinae (Liesegang, 1992). There are two types of HSV, Herpes simplex virus type 1 (HSV-1; human herpesvirus 1) (King *et al.,* 2012) and herpes simplex virus type 2 (HSV-2) with similar characteristics, morphology and structure (Grunewald *et al.*, 2003). As with VZV, The virus consists of four major components; an electron-dense core containing the viral double-stranded DNA; an icosahedral nucleocapsid which consists of 162 capsomers surrounding the core; an amorphous

asymmetric region between nucleocapsid and envelope, termed the tegument; and the outer lipid bilayer enveloping the nucleocapsid and tegument studded with glycoprotein spikes (Figure 1.5). The average size of HSV is 120 to 300 nm in diameter. The overall diameter of virus particle depends upon the thickness of the tegument and the array of spikes. Cryo-Electron Tomography studies using spherical sections through a radius just external to the membrane permitted prediction of the number of spikes on the surface of the virus envelope to be between 595 and 758 per virion, with a 13 nm average centre-to-centre spike spacing (Grunewald *et al.*, 2003).

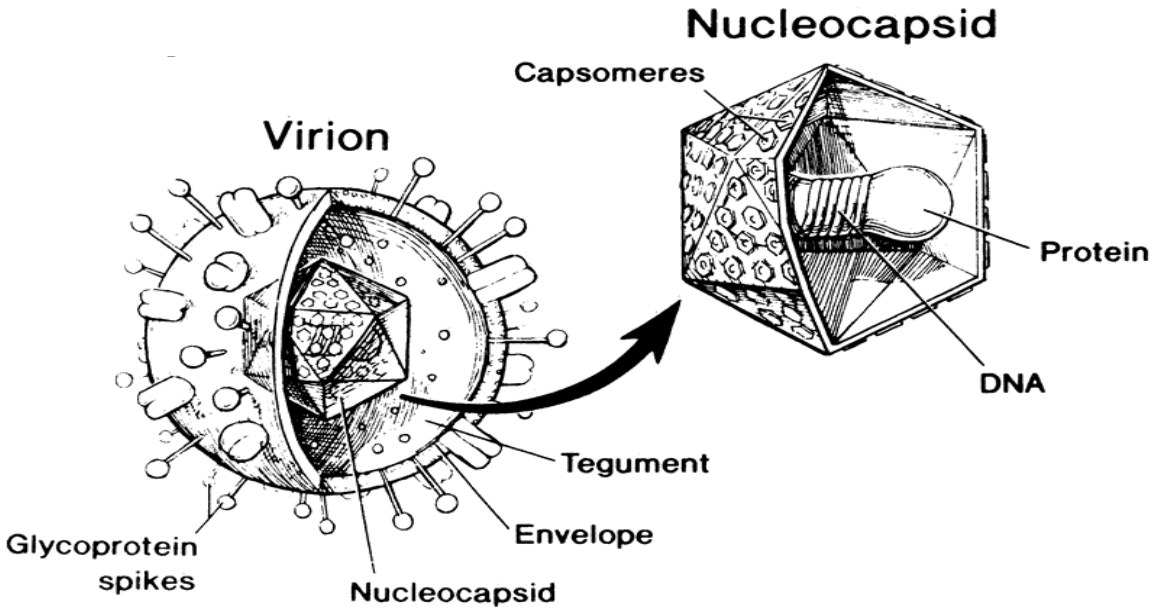


Figure 1.5: Overview of herpes simplex virus morphology. (Adapted from Liesegang, 1992)

1.5.2 Pathogenesis

Herpes simplex virus is spread by contact, the virus can be found in saliva, tears and other secretions (Arduino and Porter, 2008). Human beings are the only natural host for HSV. Herpes simplex virus type 1 is normally transmitted during childhood, whereas HSV-2 usually infects in early adulthood (Cunningham *et al.*, 2006). The usual site for the virus implantation is skin or mucous membrane. Once infection is established, infected cells undergo acantholysis, and this leads to the formation of a vesicle. In the mucous membrane, the roof of the vesicle is weak and soon collapses, forming a herpetic ulcer (Cunningham *et al.*, 2006). During first or primary infection, HSV spreads locally, and a short-lived viraemia occurs. The virus is able to escape the immune response and persist in a latent state in certain tissue. The trigeminal ganglia and sacral ganglia are the usual sites of HSV latent infection (Liesegang, 1992).

1.5.3 Clinical Features

The incubation period for HSV infection ranges from 5 to 15 days. In most cases, the primary infection is asymptomatic or mild (Arduino and Porter, 2008). As the HSV establishes latency after the primary infection, the latent virus can reactivate and produce another round of infection, known as recurrent or reactivated infection. In contrast to VZV, two types of host reinfection are also known; endogenous reinfection, in which an individual is reinfected with his/her own strain of the virus but at a different

site, and exogenous reinfection, where an individual is reinfected by a different strain of virus. Many triggers can provoke a recurrence of HSV, such as stress, pneumococcal infection, fever, irradiation and menstruation (Huang *et al.,* 2011; Padgett *et al.,* 1998). Primary infections are rare after the age of 30 years. Once an individual acquires HSV, it remains in a latent form throughout the rest of their life. Most orally infected patients and genitally infected herpes patients will experience recurrence of infection, although this recurrence is often asymptomatic. The frequency of reactivation varies with age and immunological status.

1.5.4 HSV glycoproteins

The genomes of HSV-1 and HSV-2 are complex and closely related. Herpes simplex virus type 1 and HSV-2 have two unique regions, unique long sequence (UL) and unique short sequence (US) (Steiner *et al.*, 2007). Both HSV-1 and HSV-2 contain 74 genes within their genomes and the two viruses share more than 70% genomic homology. The genes can encode up to 100 different types of proteins, involved in the cellular control, replication of virion DNA and formation of capsid, tegument and envelope of the virus. The glycoprotein molecules that will make up the spikes projecting from the virion envelope are produced in the late stages of replication. Studies have shown that 11 different types of glycoprotein are produced which have varied functions in virus replication and infection (Table 1.1). Those glycoproteins are:- 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), glycoprotein M (gM) and glycoprotein J (gJ) (Dolan *et al.,* 1998; Watson *et al.,* 2012).

1.5.4.1 Glycoprotein B

Glycoprotein B is encoded by a unique sequence within the UL region, UL27, also known as VP7. It is a class 1 transmembrane protein composed of 904 amino acids (aa) with a molecular weight of 110 kDa. The crystal-like structure of the glycoprotein B ectodomain has been resolved as a trimer, with five structural domains (Heldwein *et al.*, 2006). Glycoprotein B is the most conserved glycoprotein and serves as a fusogen, which is primarily needed for cell-cell fusion and viral entry (Hutchinson *et al.*, 1993; Heldwein *et al.*, 2006; Bender *et al.*, 2007). Hutchinson *et al.* (1993) found that mutation of gB can inhibit viral entry and inhibit production of the syncytial phenotype of the wild type HSV. Glycoprotein B is an important target of both humoral and cell-mediated immune response to HSV infection (Manservigi *et al.*, 1990; Bender *et al.*, 2007).

1.5.4.2 Glycoprotein C

Glycoprotein C is encoded within the UL sequences by gene, UL44 (VP7.5); it comprises 511 aa and has a molecular weight of 130 kDa. This

glycoprotein contains four noncontiguous regions with disulphide bond structure and two heavily glycosylated oligosaccharides (N-linked and O-linked). Although gC is a non-essential protein in viral infectivity, it is principally responsible for the attachment of the virus to cells by binding to heparan sulphate moieties (Herold *et al.*, 1991). Studies have shown that the interaction between gC and C3b fragment, which is highly dependent upon the conformation of gC, can protect the virus and virus infected cells from complement-mediated damage. Glycoprotein C is a major inducer of both humoral and cell-mediated immunity to protect against lethal HSV infection in animal experimentation (Ghiasi *et al.*, 1994; Rux *et al.*, 1996).

1.5.4.3 Glycoprotein D

Glycoprotein D is a typical type 1 transmembrane protein, and is encoded within the US. It is the gene product of US 6 (VP17/19), and consists of 319 aa with a molecular weight of 60 kDa. This glycoprotein is required for penetration of virus into the cell by interacting with the herpesvirus entry mediator (HVEM) (Inoue *et al.*, 1990; Whitbeck *et al.*, 1997). Glycoprotein D has been selected as a potential HSV vaccine candidate because; gD is a largely type common antigen of both HSV type 1 and HSV type 2, and gD is a major target of virus neutralisation (Ghiasi *et al.*, 1994).

1.5.4.4 Glycoprotein E and Glycoprotein I

Both glycoprotein E and I are encoded by the US of the HSV genome, US 8 and US 7, respectively. The molecular weight for glycoprotein E and I are 80 kDa with 550 aa and 70 kDa with 390 aa, respectively. Glycoprotein E and I together form a hetero-oligomer complex, which acts as a Fc receptor of IgG to inhibit the immune cytolysis of HSV. Studies have shown that gE/gI complex is also required in the fusion of virus and cell during virus penetration. Ghiasi and colleagues (1994) found that gE-immunised mice developed delayed-type hypersensitivity responses to protect against lethal HSV infection.

1.5.4.5 Glycoprotein G

Glycoprotein G is encoded by the unique sequences of the short component, US4. Glycoprotein G of HSV-1 and HSV-2 comprises 238 aa and 699 aa, respectively. Glycoprotein G is the only viral envelope protein that predominantly bears type-specific epitopes distinguishing HSV-1 and HSV-2. The aa similarity between gG-1 and gG-2 is only present at the short membrane anchor region (McGeoch *et al.*, 1987; Rekabdar *et al.*, 2002). In type-discriminating serology, the cell-membrane associated mature gG is used as a target for serological studies in infected patients with HSV-1 and/or HSV-2. Glycoprotein G is not essential in the virus infectivity; however, animal studies shown that vaccination with MgG-2

(mature gG-2) can protect mice from lethal HSV infection (Tunbäck *et al.*, 2000).

1.5.4.6 Glycoprotein H and Glycoprotein L

Both glycoprotein H and glycoprotein L are encoded by unique sequences of the long component, UL 22 and UL 1; they consist of 535 aa and 224 aa, respectively. Glycoprotein H is a type 1 glycoprotein with 7 N-linked glycosylation sites and 8 cysteine residues; whereas glycoprotein L is a soluble glycoprotein found in the virion and at the cell surface (Subramanian and Geraghty, 2007). Studies have shown that glycoprotein H mutation can slow down the rate of cell fusion and that glycoprotein H without the short cytoplasmic tail can reduce the infectivity of the virus (Jackson *et al.*, 2010). However, the functions of glycoprotein H in viral fusion can only be activated in the presence of glycoprotein L and as mentioned above, glycoprotein H and glycoprotein L are present as a gH/gL heterodimer complex in the envelope of the virus. Subramanian and Geraghty (2007) concluded that the gH/gL complex has an essential function in phase II of the virus-cell fusion process (formation of hemifusion).

1.5.4.7 Glycoprotein K and Glycoprotein M

Glycoprotein K and Glycoprotein M are encoded by the unique sequences of long component UL53 and UL10, with 338 aa and 473 aa, respectively. Glycoprotein K is a highly conserved glycoprotein, which consists of 2 N-linked sugar and three or four hydrophobic domains. Although glycoprotein K is not an essential glycoprotein, studies have found that gK is required in virus replication, viral assembly and plays a role in virus virulence (Zhang *et al.*, 2011). In contrast to gK, glycoprotein M is a type III integral membrane with 8 transmembrane domains (McGeoch *et al.*, 1988). Glycoprotein M is not essential for virus replication, but is required for secondary envelopment in infected cells and virus egress pathway (Baines *et al.*, 2007).

1.5.4.8 Glycoprotein J

Glycoprotein J is a type 1 membrane protein, encoded by US5 and comprising 92 aa. This glycoprotein is classed as ‘non-essential’ protein and is found to inhibit caspase and apoptosis activation during virus infection (Jerome *et al.*, 2001; Aubert *et al.*, 2008).

Table 1.1: Characteristics of HSV glycoproteins

Glycoprotein	Gene encoding	Molecular weight	Function
B	UL27	110 kDa	Essential protein. Required for cell-cell fusion, viral entry and viral growth in cell culture.
C	UL44	130 kDa	Non-essential protein. Required for virus adsorption and protection of the virus from complement-mediated damage by binding with C3b fragment.
D	US6	60 kDa	Required for virus penetration and is a potential HSV vaccine candidate. Essential for virus infectivity.
E	US8	80 kDa	Non-essential protein. Required for viral fusion and acts as a Fc receptor of gE by binding with gI.
I	US7	70 kDa	Required for gE/gI complex. Non-essential protein
G	US4	25 kDa (HSV-1) 60 kDa (HSV-2)	Non-essential protein. Required for antigen specific to HSV-1 and HSV-2.
H	UL22	110 kDa	Essential for virus infectivity. Form a complex with gL. Required for virus penetration, egress envelopment, cell fusion and cell-to-cell spread.
L	UL1	25 kDa	Essential protein. Required for gH/gL complex.
K	UL53	40 kDa	Required for virus replication, viral assembly and virus virulence. Non-essential protein
M	UL10	51 kDa	Required for secondary envelopment and virus egress pathway. Non-essential protein.
J	US5	9.6 kDa	Non-essential protein. Required for caspase and apoptosis inhibition.

1.6 Epidemiology

1.6.1 Definition

The word epidemiology is derived from the Greek, with *epi* meaning 'upon', *demos* meaning 'people' and *logos* meaning 'study'. Many definitions of epidemiology have been proposed, but commonly epidemiology is defined as the study of the distribution and determinants of disease frequency as implicated in health related problems and control of disease (Bonita and Beaglehole, 2006). As for seroepidemiology, this is one of the tools for epidemiological surveillance and is based on the testing of serum samples. Seroepidemiology uses serum antibody quantitative methods to study antibody coverage in the human population. In epidemiology, definition of the distribution and determinants of disease are necessary. The distribution of disease considers the pattern of health events in a population, which involves one broad aspect of epidemiology, called descriptive epidemiology (Grimes and Schulz, 2002). Descriptive epidemiology provides such questions as who is getting a disease, and where and when the disease is occurring. Knowledge of such distribution is essential to generate information on possible causal and preventive factors of disease in the population. In general, viral epidemiological investigations can be categorised into three terms, endemic, epidemic and pandemic (Green *et al.,* 2002). Endemic disease means multiple or continuous chains of disease transmission in the population, while epidemic disease results in peaks of disease incidence, which exceed the

base line of endemic disease. If a disease is categorised as ‘pandemic’, it means it is an epidemic worldwide, such as influenza and HIV (Human immunodeficiency virus).

1.6.2 Epidemiological studies in virus infection

Nowadays, the science of epidemiology directly affects most people. It allows them to take evidence based decisions in their lives and forms the way that government, public health agencies, and medical organisations make policy decisions that affect people. In medical virology, epidemiology plays an essential role in studies of transmission and control of virus infections (Bonita and Beaglehole, 2006). In study of infection, knowledge of epidemiology is important to finding the source of an outbreak and identification of the agent responsible. An example is smallpox, where epidemiological studies became an important tool to evaluate control measures during smallpox vaccination campaigns, which ultimately resulted in eradication of the virus and the disease it caused (Pennington, 2003). In addition, epidemiological studies also played a vital role in identifying the epidemic and spread of HIV and SARS (Severe acute respiratory syndrome), which brought a series of interventions for prevention, treatment and control of these viruses in the population (Jong-wook, 2005; Wang and Jolly, 2004). The strength of epidemiological studies sometimes allows preventive action against disease before the

complete mechanism of virus aetiology is known, such as occurred with hepatitis B virus (HBV) infection (Shepard *et.al.,* 2006).

1.6.3 Epidemiology of Measles

Measles remains as a serious problem worldwide, especially for those countries where measles is endemic and large outbreaks occur (Van Den Hof *et al.,* 1999). Africa and Asia are the major continents where most measles outbreaks have occurred in the last ten years. In 2013, the World Health Organization (WHO) stated there were 145,700 measles deaths globally, which means about 400 people died every day or 16 deaths per hour. The WHO report stated most measles deaths occur in children under the age of five and were from low-income families where health facilities are inadequate. Measles is, however, not just a problem for the developing world, in 2011, there were a large number of measles cases reported in western and central-eastern Europe (CDC, 2011, WHO, 2011) despite the wide availability of measles virus vaccine. During the outbreak in Europe, France and Ukraine reported the highest number of cases accounting for more than half of all cases reported in 2013 (CDC, 2011, Cottrell and Roberts, 2011). In the UK, since 1996, the incidence of measles cases has fallen by more than 80%, with fewer than 100 cases recorded every year (Jick and Hagberg, 2010). Unfortunately, a modest outbreak of measles occurred in 2003 dramatically increasing the number of measles cases in the UK. Such outbreaks have continued since then in

the England and Wales, about 1,564 notified cases of measles were reported between January and June 2014 (PHE, 2014a). The majority of the new measles cases in 2014 occurred in unvaccinated children and adolescents. These patients were typically infected within universities, schools, families, or by travel abroad. Measles infection is often severe in infants, elderly people, pregnant women and immunocompromised patients (Asaria and MacMahon, 2006).

1.6.4 Epidemiology of Mumps

During the pre-vaccine era, increasing urbanisation led to mumps becoming a common childhood infection with about 290 cases per annum per 100,000 populations being reported in Europe with most cases occurring in those aged of 5 to 7 years (Galazka *et al.,* 1999). Historically, mumps infection was also common within military personnel due to barrack room overcrowding. In temperate countries, mumps infections peak in winter and spring. Before the introduction of mumps vaccine, mumps used to be a major cause of aseptic meningitis and hearing loss with an excess of cases being found in males (Hviid *et al.,* 2008). In the 1980s, the introduction of routine mumps vaccination into the childhood vaccine schedule greatly reduced rates of the mumps infection in the population. In USA, there was a 99% decrease from 185,691 cases in 1968 to about 900 cases reported annually by the mid-1990s (Conly and Johnston, 2007). The epidemiology of mumps virus infection has changed

as a result of the introduction of vaccination. According to serosurveys in Western Europe, 70-90% seroprotection against mumps infection was found (Galazka *et al.,* 1999). However, mumps outbreaks continue to occur in the population. In the UK, a nationwide outbreak of mumps occurred in 2005, with 43,378 confirmed cases, 90% of which were in persons aged 19-23 years (Gupta *et al.,* 2005). The reason for mumps infection in this age group was that they were either unimmunised or they had received only one dose of vaccine (Cohen *et al.,* 2007). Recently, in England, 847 confirmed mumps cases occurred between January and March 2014, predominantly in persons aged 15-30 years of age (PHE, 2014b).

1.6.5 Epidemiology of Rubella

Rubella is a disease that occurs worldwide, the incidence tends to peak during spring in temperate countries. Before the licensing of rubella vaccine, outbreaks of rubella occurred every 3 to 5 years in Europe, mostly affecting children in the 5 to 9 year old age group (Nardone *et al.,* 2008; Tookey, 2004). According to Collier and Oxford (2006c), over 70% of women in the population were infected by rubella. In the UK, less than 2% of British born pregnant women were susceptible to rubella infection compared to 4% of women of Mediterranean origin and 8% of women originating from Asia and Africa. Since 1990, several rubella outbreaks have been reported in the UK. In 1993, cases were mostly in young men;

while in 1996, infections in pregnant women were reported. Cases were also mostly seen in immigrants and unimmunised persons (Tookey, 2004). Following an outbreak in Greece, there was an outbreak in the UK in early 1999; most of the infected people were university students from Greece. Only one infant was reported as having contracted congenital rubella six months after the outbreak (Tookey *et al.,* 2000). There were 27 European countries reported 38,847 rubella cases in 2013, with most rubella cases in young male adults in Poland (ECDC, 2014). In England, about 202 rubella cases were reported between January and June 2014, with only one laboratory confirmed case in a newborn (PHE, 2014c).

1.6.6 Epidemiology of Varicella Zoster Virus

Varicella infection is an endemic disease worldwide. However, the epidemiology of varicella differs between countries with temperate and tropical climates. In most temperate countries, more than 90% of people are infected before adolescence, with peak incidence of infection occurring during winter and early spring, whereas, in many tropical climates, disease is acquired later in life, and more adult infections occur (Choo *et al.,* 1995). Studies have shown that this variation may relate to differences in population density, environment, risk of exposure, and social factors (Weller, 1992). The highest incidence of varicella infection is in children aged 1 to 9 years (Weller, 1992). In developed countries, the average ranges of mortality rates for varicella are between 0.3 and 0.5 per million

populations (Choo *et al.,* 1995). Although varicella infection is more severe in immunocompromised people, most cases of mortality and severe morbidity infection are seen in healthy people (Weller, 1992; Heininger and Seward, 2006). In the UK, previous epidemiological studies have indicated that the majority of people acquire infection as young children aged less than 5 years old (Straus *et al.,* 1988; Brisson and Edmunds, 2003). A possible explanation for this is the increasing frequency of young children attending pre-school establishments, which facilitate exposure and transmission of viruses amongst this age group (Brisson and Edmunds, 2003).

1.6.7 Epidemiology of HSV-1 and HSV-2

HSV infection occurs worldwide without seasonal variation. Because the virus spreads by direct contact, by far the most common way to acquire HSV infection is by a kiss given from a person shedding the virus. Many people infected with HSV develop labial or lesions, but the majority of infections are asymptomatic or develop subclinical herpes. Since most infections are asymptomatic, people are unaware of their infection although they pose a risk for spreading HSV. The risk of infection to a non-immune individual through exposure to contaminated secretions can be as high as 80%. Nowadays, the development of HSV type specific antibody testing is certainly an important breakthrough in accurately diagnosing infection and the type of HSV involved, especially for those

with genital herpes. These type-specific antibody assays are based on the detection of gG-1 and gG-2 specific antibodies. The identification of herpes subtype in the patient aids guidance of the dosage and duration of the antiviral therapy (Cowan, 2000; Schmid *et al.,* 1999). Several studies have found that recurrence and viral shedding in the genital area is more for HSV-2 than for HSV-1 (Ashley and Wald, 1999; Cowan, 2000; Schmid *et al.,* 1999). In general, throughout the world, over 80% of the adult population have evidence of exposure to HSV-1; the seroprevalence of HSV-2 infection varies from country to country and within country, from 7% up to 90% or more (Cunningham *et al.,* 2006). In USA (1999-2004), 57.7% of Americans were infected with HSV-1 and 16.2% of people with HSV-2 (Xu *et al.,* 2006). HSV-1 seroprevalence has shown large differences between European countries. According to Pebody *et al.* (2004), HSV-1 seroprevalence is high in Bulgaria and the Czech Republic and lower in Belgium; whereas HSV-2 seroprevalence is high in Bulgaria. One study also found that European women are more likely to be seropositive for both types of HSV than men are. In the UK, clinical episodes of HSV infection have steadily increased over recent decades. In England, particularly, 32,279 people were newly diagnosed with genital herpes in 2013 and about 60% of those diagnosed were women (PHE, 2014d). Within the past 20 years, an increasing number of primary genital herpes infections in the UK have been found to be caused by HSV-1. Although the incidence of genital herpes is widely dependent on the age, gender

and geographical location (Morris-Cunnington *et al.,* 2004), one main factor that might contributed this change was lower seropositivity of HSV-1 in childhood. This has resulted in an increased opportunity to acquire HSV-1 infection via sexual transmission in adulthood, presenting as genital herpes infections. The significant increase in genital herpes infections in the childbearing age range has raised the incidence of cases of neonatal herpes in infants (Garland and Steben, 2014). The risk of neonatal herpes is greatest for babies born to a woman with first episode genital herpes around the time of delivery. Neonatal herpes is life-threatening, but occurs rarely in the UK, with less than 17.5 per 100,000 live births in the UK (Batra *et al.,* 2014). Another complication, Herpes simplex encephalitis (HSE) remains a serious illness with significant risk of morbidity in UK population, about 0.2 to 0.4 per 100,000 cases (Sabah *et al.,* 2012) and may occur following primary infection or in already immune individuals. Some people may suffer herpes infection of the eye during childhood or early adulthood. The recurrence rate for ocular herpes is 40% for affected individuals, and the reported case of this infection is about 50,000 per annum in the UK (Liesegang, 2001).

1.7 Vaccination

After clean water, vaccination is the second best public health intervention in the world for promoting good health (Andre *et al.,* 2008). Because of the limited opportunity to use antiviral drugs, vaccination is

one of the effective public health interventions against viral infection (Demicheli *et al.,* 2005; Galazka *et al.,* 1999; Plotkin, 2005). Vaccines elicit an immune response without causing disease and provide protective immunity against potential pathogens (Plotkin, 2005). In the UK, there are many vaccines which are given routinely to children to protect against disease such as, measles, mumps, rubella, polio, tetanus, diphtheria, meningococcus serogroup C, pneumococcus, *Haemophilus influenzae* type B and pertussis (Department of Health, 2014a). Viral vaccines are made using a small amount of inactivated virus, live attenuated virus or subunit of a virus (Lombard *et al.,* 2007). The introduction of a vaccine can have a dramatic effect upon disease incidence. For example, in 1968, there were 763,094 cases of measles with 552 deaths in USA. After a vaccine was developed and licensed, measles cases dropped to fewer than 150 per year (CDC, 2008). The major purpose of vaccine development is not only in preventing disease in individuals, but also in the community. The aim of vaccination of a community is to provide protection called ‘herd protection’. Herd protection works by reducing the number of susceptible individual in a population and interrupting transmission; for organisms that do not establish latent or persistent infections, the disease will then disappear or be eradicated from the community. However, vaccines do not guarantee complete protection to the individual from a disease, vaccine failure can occur such as when the vaccine contains insufficient antigen and the vaccine has denaturated during storage or

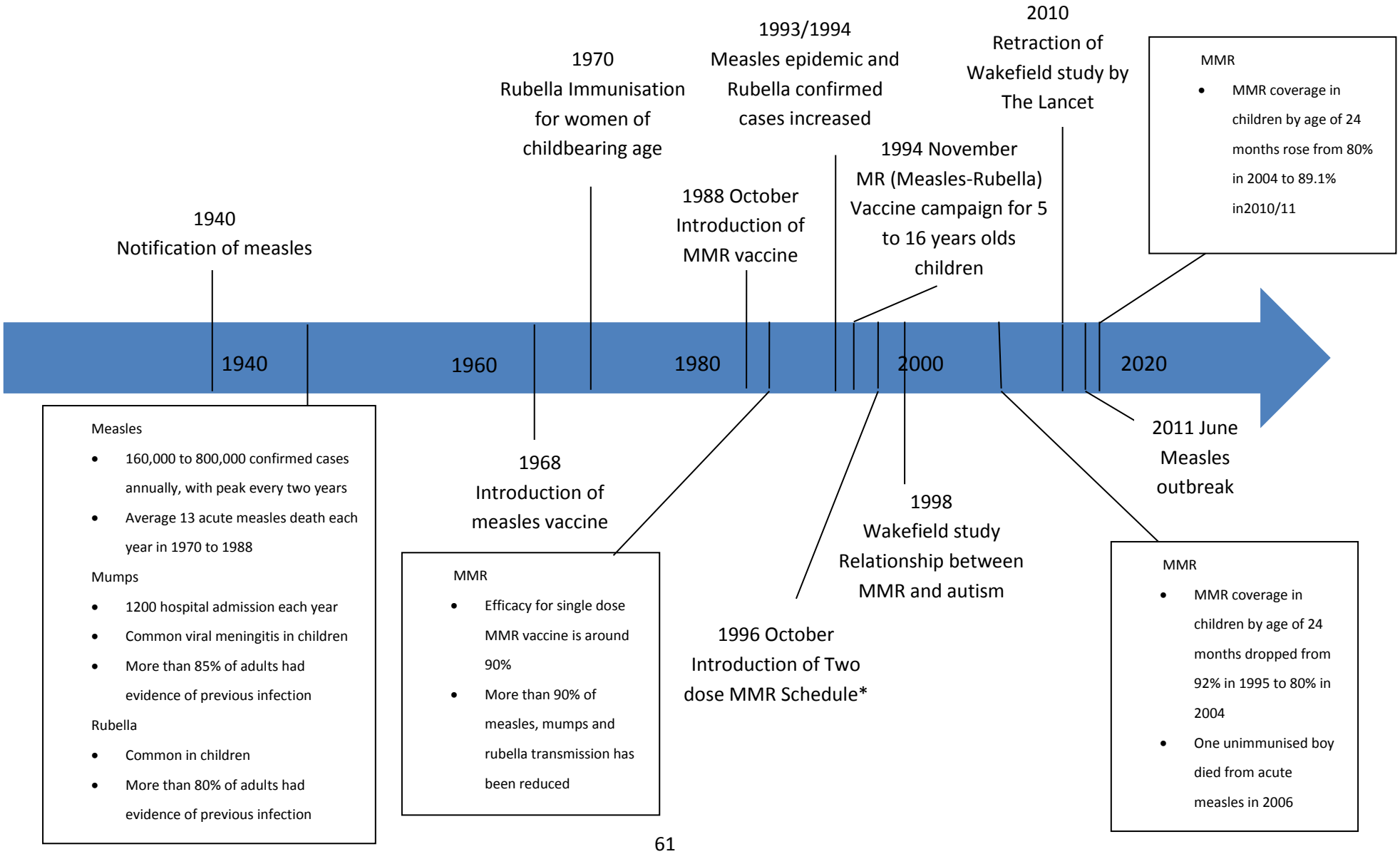
administration. Studies have shown that vaccines may cause mild reactions, such as fever, soreness at the injection or a cough, but very rarely cause serious diseases (Smeeth *et al.,* 2004). To be usable the risk caused by vaccination must be lower than the health risk associated with the disease. The efficacy or performance of a vaccine is highly dependent on individual immunity, health status and age. Sometimes, multiple doses of vaccines are required, as one dose of vaccine may not be effective in developing protective immunity.

1.7.1 MMR Vaccine

In the UK before MMR vaccine became available, infections with measles and rubella were prevented through administration of monovalent vaccines for each virus (Vyse *et al.,* 2002). Monovalent measles vaccine was introduced for infant vaccinations in 1968; while in 1970, rubella vaccine was introduced for prepubertal school girls and susceptible women (Figure 1.6) (Osborne *et al.*, 2000; Vyse *et al.*, 2006; Sonnenberg *et al.*, 2007). However, no monovalent mumps vaccine was introduced in the UK. The replacement of monovalent vaccines by a combined MMR vaccine commenced in October 1988, with the aim of eliminating all three diseases in the population (Vyse, 2002; Demicheli *et al.*, 2005). The MMR vaccine consists of three live attenuated viruses, measles, mumps and rubella. The viral strains included in the MMR vaccine are cultured in animal or human cell lines (Demicheli *et al.*, 2005). For example, mumps

and measles viruses are grown in embryonated eggs (USA) and chick embryo cell cultures; while the rubella virus is propagated using human embryo lung fibroblasts (Plotkin, 2005). MMR vaccination is usually performed in childhood and completed with two vaccinations. Two doses of MMR vaccine are typically adequate, with over 90% of children aged 12-15 months developing protective antibodies. Only 2 to 5% of children fail to develop antibodies due to the presence of passive antibody in the vaccine recipient, damaged vaccine, or other reasons (CDC, 2013). The first dose is administered between the ages of 12 and 13 months, and the second dose is given pre-school (Demicheli *et al*., 2005; Department of Health, 2014b). The MMR vaccination uptake rates reached a peak in 1995, when more than 92% of children received the first dose of vaccine in the UK (Osborne *et al.*, 2000; Vyse, 2002; Vyse *et al.*, 2006). Unfortunately, MMR vaccine uptake began to reduce and fell to only 80% or lower uptake in 2004. This was due to the controversy surrounding the use of MMR vaccine which began in 1998, when studies suggested an unfounded link between vaccination and either Crohn's disease or autism (Wakefield *et al.,* 1998). The studies were later retracted (Figure 1.6), when the UK National Health Service (NHS) and US Centers for Disease Control and Prevention (CDC) concluded that there was absolutely no evidence to show a link between use of MMR vaccine and autism or Crohn's diseases (Horton, 2004). In England and Wales, there are four types of surveillance systems to monitor the impact of MMR vaccination

programme. Those four methods are vaccine coverage data, clinical notification, laboratory confirmation and serological surveillance (Osborne *et al.*, 2000). Surveillance was introduced in 1988 and provides evidence to national policy that allows adjustment of vaccination programmes (Osborne *et al.*, 2000). In epidemiological surveillance, the present of IgG antibodies indicates an individual has had past infection or vaccination. In England, since 1986, anonymised blood specimens have been collected every year for serological testing (Vyse, 2002). Serum collected from appropriate age groups have been tested for antibody to measles, mumps and rubella-specific IgG.



Note:* MMR 1996 October – first dose at the age of 13 months and second dose at the ages of 4 and 5

Figure 1.2: MMR vaccination timeline in England and Wales

1.7.2 Varicella Zoster vaccine

Varicella zoster vaccine is made up of live attenuated virus immunisation which protects against the viral diseases caused by VZV (Javed *et al.*, 2011) of which there are two types; one to prevent varicella, and the other to prevent herpes zoster. Varicella vaccine was developed in the early 1970s, and Japan became the first country to use this live attenuated vaccine to prevent chickenpox. The United States has also performed routine vaccination against VZV since 1995 and showed it dramatically decreased the incidence of chickenpox in the population (Schmader *et al.*, 2012). In Europe, the varicella vaccine was licensed for use in mid 1980s; however, with the exception of Germany, most European countries do not currently vaccinate against varicella, the belief being that childhood vaccination against VZV could have a negative impact on the severity of herpes zoster in adults (Pinot de Moira and Nardone, 2005). Research has indicated that adults who have had chickenpox as a child have less risk of developing shingles in later life (Goldman and King, 2013). Studies also have suggested that adolescent vaccination is the safest and most cost effective use of varicella vaccination, but adolescent vaccination has less overall impact on varicella infection (Brisson et al., 2003; Goldman and King, 2013; Schmid and Jumaan, 2010). In the UK, varicella vaccination is not routine, however, in 2005, vaccination was recommended for all health care staff in the UK who were non-immune and was also recommended for siblings of children who were immunocompromised (Brisson and Edmunds, 2003). The varicella vaccine is safe, only 4% of children who receive the vaccine develop mild side effects such as rash, edema or pruritus at the site of

injection (Wise *et al.*, 2000). The duration of protection from varicella vaccine is still unknown. In general, vaccinated persons do not have evidence of waning immunity and clinical research suggests that the protective effect of varicella vaccine is at least 6 years (Hendriksz *et al.*, 2011). However, there is a risk that loss of vaccine-induced immunity to varicella increases with age. If varicella vaccine is introduced, proactive surveillance to ensure those vaccinated persons are still protected from varicella will be necessary.

Unlike varicella vaccine, only a single dose of zoster vaccine is required. The zoster vaccine was introduced in the UK in September 2013, for all those age 70 years and above (Department of Health, 2014). Zoster vaccine contains 14 times more virus than the varicella vaccine as it is intended to boost cell-mediated immunity (Javed *et al.*, 2011). Clinical trials have shown that zoster vaccine reduces the risk of herpes zoster by 61% and PHN by 67% (Oxman *et al.,* 2005). However, the zoster vaccine cannot be used as treatment for patients who have active shingles or for immunocompromised patients (Hendriksz *et al.,* 2011).

1.7.3 Development of HSV vaccine

Herpes simplex virus establishes lifelong infection and currently no method is available to eradicate the virus from the body. There are antiviral medications, such as acyclovir, valacyclovir, famciclovir and penciclovir that can reduce or inhibit virus replication, duration of illness and severity (Koelle and Corey, 2008). However, these methods are imperfect, and can only reduce approximately 50% of HSV transmission in immunocompetent persons. Ideally, a candidate vaccine might be

considered successful if the vaccine prevented the HSV primary clinical episode, reduced frequency or severity of recurrences, and reduced shedding in asymptomatic persons (Whitley and Roizman, 2002). Modern research on HSV vaccines has focussed on subunit vaccines containing viral glycoproteins and live attenuated vaccines (Stanberry *et al.*, 2002; Whitley and Roizman, 2002; Halford *et al.*, 2011). Most of the vaccines have been shown to be at least partially effective in animal studies; however, none of them are therapeutically effective and they are poorly immunogenic in clinical trials. (Hosken, 2005; Johnston *et al.,* 2013). An investigational vaccine, produced by GlaxoSmithKline, containing glycoprotein D from HSV-2 formulated in AS04 adjuvant (alum + monophosphoryl lipid A) progressed into a Phase III clinical trial. The trial showed the vaccine gave only limited protection; it was approximately 55 % effective against HSV-1, but not effective against HSV-2 even though it induced antibodies against HSV-2. The studies suggested that a possible reason was the rate of neutralisation of each of the viruses in that HSV-1 appears to be more easily neutralised by antibodies than HSV-2 (Hosken, 2005; Johnston *et al.,* 2013).

1.8 Laboratory techniques

A problem with previous seroepidemiological studies has been the use of different assays that may give rise to variation in antibody levels. Standardisation of assays against known reference standards and the use of the same assay procedure are therefore desirable when conducting age related seroprevalence studies for different target organisms. In recent years, the large cost of conducting these studies has meant that fewer

surveys have been performed and there has been a consequent decline in reporting of current seroepidemiology. Over the last several years, there has been increased demand for high-throughput, cost effective and accurate techniques for the study and diagnosis of disease in clinical and research laboratories (Sheppard *et al.,* 2011). The focus has thus moved from single analyte ELISA to multiplex analyte immunoassays.

Cloning of antigens for use in immunoassay is widely used. In molecular cloning, DNA fragments representing the gene or genes that are to be reproduced are inserted into a cloning vector. The cloning vectors contain genetic information that allows host cells to replicate them. After the insertion of the plasmid vector into a host cell, genetic materials are transformed into the host cells. (Finney *et al.,* 2001; Lessard, 2013) In medical virology, molecular cloning has been used to study viral replication pathways and has been used for individual virus protein expression for example to produce vaccine for virus infections, such as for human papillomavirus (Cutts *et al.,* 2007) or hepatitis B virus (Hilleman, 1987). One popular protein expression systems is the baculovirus expression system.

1.8.1 Baculovirus expression vector system

The baculovirus expression vector system (BEVS) allows the introduction of a foreign gene into the viral replication genome region via recombination with a transfer vector containing the target gene. The system was first introduced in 1983 and rapidly became one of the most powerful eukaryotic vector systems for recombinant protein expression (Pharmingen, 1999). The system originated from basic studies of

baculovirus use in bio-control (Hitchman *et al.*, 2009). The baculovirus family of *Baculoviridae*, is a lethal pathogen of insects. It comprises a double stranded, circular and coiled DNA molecule held within a rod shaped capsid (Isaac *et al.*, 2004; Hitchman *et al.*, 2009; Pharmingen, 1999). These viruses have a bi-phasic life cycle and are found mainly in arthropods. The most common baculovirus used for expression studies is *Autographa california* nuclear polyhedrosis virus (AcNPV). In BEVS, several baculovirus genes, which are non-essential for the baculovirus life cycle, are replaced by target genes. Due to the large baculovirus genome, it is difficult to insert the foreign genes directly; hence, they are cloned into transfer vectors (Pharmingen, 1999). The co-transfection of transfer vectors and baculovirus genome take place in the continuous cell line, Sf9, which was developed from the ovaries of the fall armyworm (*Spodoptera frugiperda*), this allows the recombination of target genes with AcNPV DNA. The baculovirus infection of Sf9 cells results in the restriction or shut-off of host cell expression (Isaac *et al*., 2004), this allows a high rate of recombinant protein production. The amount of recombinant protein production is around 50% of the total insect cell protein (Pharmingen, 1999).

There are several factors, which have contributed to this system's popularity. First, BEVS is an eukaryotic expression system and capable of producing several post-translational modifications such as N- and O-linked glycosylation, phosphorylation and proteolytic cleavage that are present in higher eukaryotic cells (Isaac *et al.,* 2004). In addition, baculoviruses are essentially non-pathogenic to mammals and have a restricted host range, mainly in invertebrate species. No helper cell lines or helper virus is

required for baculovirus because it contains all of the genetic information required for its replication. Furthermore, AcNPV DNA can accommodate large segments of foreign DNA, around 130kbp. Nowadays, BEVS are widely used in recombinant protein production, gene delivery and expression vectors in mammalian cells (Hitchman *et al.*, 2009). In medical virology, BEVS has been found useful in the studies of characterisation and type-specificity of viral antibodies in human serum samples (Sulaiman, 2010).

1.8.2 Multiplex Bead Immunoassay

There are different types of multiplex immunoassays (MIA) available and have been widely used to quantify multiple analytes from biological fluid samples, however, the multiplex bead immunoassay was used in this study. Multiplex bead immunoassay is a combination of existing techniques such as microspheres, lasers and traditional chemistry, to perform a wide variety of bioassays quickly, cost effectively and accurately (Maecker *et al.*, 2010; Varro *et al.,* 2007). Such systems use microspheres, which are coated with a reagent specific to a particular bioassay for capture and detection of the specific analytes. Microspheres are around 5 to 6 micrometer in diameter and internally dyed with 2 different flurophores (Figure 1.7). About 100 different microsphere sets are created with a specific, known, ratio of the two dyes. Because each microsphere is unique, the flow-based detector can simply identify a bead (and its associated analyte) through laser illumination of bead and detection and quantification of any attached fluorophore via illumination with a second laser (Varro *et al.,* 2007).

One of the major advantages of MIA is the ability to measure multiple analytes within a single sample at the same time. The assay is more sensitive than conventional ELISA, and uses smaller amounts of sample, about 5 µL. In medical virology, studies have used MIA in viral serological surveillance, diagnostics and vaccine development (Kellar *et al.,* 2001; Yang *et al.,* 2008; Rao *et al.,* 2004).

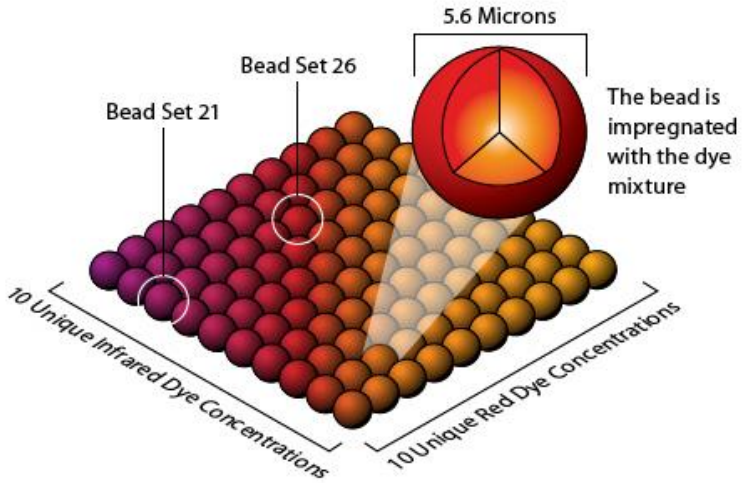


Figure 1.7: The colour coding of microspheres with a specific ratio of two dyes, red and infrared fluorophores. Taken from: http://www.panomics.com/product/96/ (Accessed on 03/10/2012)

1.9 Study aim

The aim of this study was to investigate the age-related seroepidemiology of measles, mumps, rubella and varicella zoster using a multiplex bead immunoassay and also to develop a multiplex bead immunoassay for the measurement of total specific IgG antibodies to herpes simplex type 1 and type 2 viruses to allow investigation of the age-related seroepidemiology of these viruses. A multiplex bead immunoassay for measles, mumps, rubella and varicella zoster, previously developed by the RIVM (Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven), was adapted for use in the study and was validated using a standardised sample panel. As multiplex bead immunoassay antigens for all viruses except HSV-1 and HSV-2 were already available, the study initially focused on producing HSV-1 and HSV-2 specific antigens to develop a similar multiplex bead immunoassay HSV-1 and HSV-2 antibodies. The expression of the immunodominant glycoproteins of HSV-1 and HSV-2 (gG1 and gG2) was attempted using the InsectDirect baculovirus vector expression system, the commercially available truncated glycoprotein G for HSV-1 and HSV-2 was also obtained and used to develop a duplex Luminex immunoassay.

2.0 Materials and Methods

The methods are divided into three main sections. Sections 2.1 to 2.8 incorporate the expression of HSV-1 glycoprotein G and HSV-2 glycoprotein G using the baculovirus expression system; section 2.9.1 describes the seroepidemiology of measles, mumps, rubella and varicella zoster virus in England using an MMRV multiplex bead immunoassay; section 2.9.2 describes the development of the HSV bi-plexed assay.

2.1 Reagents

2.1.1 Virus samples and Plasmids

Herpes simplex virus type 1, (strain Macintyre) and HSV-2, (clinical isolate KA01), were provided by the Department of Virology, University of Manchester. Plasmids pIEx/Bac-3 3C/LIC, 3C/LIC β-gal Control Insert, and transfection control plasmid (ampicillin resistant) were purchased from Merck, Novagen, Nottingham, UK.

2.1.2 Cell lines

Vero cells were provided by the Department of Virology, University of Manchester; Sf9 insect cells, *Spodoptera frugiperda* (Nishikawa *et. al,* 2003), were purchased from Merck, Novagen.

2.1.3 Primer

HSV-1 gG and HSV-2 gG oligonucleotide primers were synthesised by Invitrogen, Life Technologies, Paisley, UK.

Virus	Primers	Sequences	Length
HSV-1	Sense	5'-CAGGGACCCGGTATGTCGCAGGGC-3'	24bp
	Anti-sense	5'-GGCACCAGAGCGTTCCCCATACCCTACCC-3'	29bp
HSV-2	Sense	5'-CAGGGACCCGGTATGCACGCCATCGCTCCCAGGTTG-3'	36bp
	Anti-sense	5'-GGCACCAGAGCGTTCTAATCCCGCTCGGGTGGCAGA-3'	36bp

Truncated HSV-1 gG and truncated HSV-2 gG oligonucleotide primers were synthesised by Invitrogen, Life Technologies.

Virus	Primers	Sequences	Length
Truncated HSV-1	Sense	5'-CAGGGACCCGGTATGTCGCAGGGC-3'	24bp
	Anti-sense	5'-GGCACCAGAGCGTTCTACAGCGGGGTATGT-3'	30bp
Truncated HSV-2	Sense	5'- CAGGGACCCGGTATGAGCCTGCTGGTG-3'	27bp
	Anti-sense	5'-GGCACCAGAGCGTTCTATTTGTTCGGGTTCGG-3'	32bp

Vector upstream and downstream primers were purchased from Merck, Novagen.

Genes	Primers	Sequences
IE1 Promoter	Sense	5'- TGGATATTGTTTCAGTTGCAAG-3'
IE1 Terminator	Anti-sense	5-CAACAACGGCCCCTCGATA-3′

2.1.4 Protein expression system

InsectDirect system transfection kits were purchased from Merck, Novagen.

2.2 Cell culture of Vero cells and Sf9 insect cells

2.2.1 Vero cells

2.2.1.1 Recovery of Vero cells

Vero cells were recovered from liquid nitrogen using the protocol described by Ammerman *et al.* (2008). One vial of Vero cells (1 mL; $2x10^5$ cells/mL), was gently swirled in a 37^oC water bath (Grant instruments Ltd, Cambridge, UK) after being removed from liquid nitrogen. The vial was then decontaminated by spraying with 70% ethanol (BDH Chemical Ltd, Poole, UK) in a biohazard class two safety cabinet (Medical Air Technology Ltd, Manchester, UK). Following this, 1 mL of Vero cell suspension from the vial was transferred into a 15 mL centrifuge tube containing 10 mL of growth media (Eagle's Minimum Essential Medium (MEM) (Biosera Ltd, East Sussex, UK) with 10% foetal calf serum (Biosera Ltd)). The cell suspension was centrifuged using Megafuge 1.0 centrifuge (Heraeus equipment Ltd, Essex, UK) at 750 x g for 5 minutes at room temperature, then, the supernatant was gently discarded and the pelleted cells resuspended in 15 mL of growth media. The cell suspension was transferred into a 75 cm^2 cell culture flask (Greiner Bio-One Cellstar, Gloucestershire, UK) and incubated at 37^0C with 5% CO_2 (Heraeus Equipment Ltd., Essex, UK). Vero cells in the culture flask were observed daily using an inverted microscope (Wolf Laboratories Ltd, York, UK) until the cells had reached 95 to 100% confluency.

2.2.1.2 Sub culturing the Vero cells

After 48-hour incubation, when the cells had reached 95% confluency the growth medium was removed. The cells in the flask were

washed using 10 mL of 0.25% ($^{v}/_{v}$) trypsin (Sigma Aldrich Ltd, Poole, UK) phosphate buffered saline (PBS) (Thermo Scientific OXOID Ltd, Hampshire, UK). The flask was gently rocked by hand to allow the trypsin solution to make contact with the whole of the surface of the monolayer. This was repeated once, the liquid removed into a plastic beaker and the flask incubated at 37^{0}C for 3 to 5 minutes. After that, 11 mL of growth medium was pipetted several times across the side of the flask to ensure complete detachment and reduce cell clumping. The cells were then counted (see Section 2.2.1.3) and diluted to a cell density of $2x10^{5}$ cells per mL, 15 mL was transferred into a 75 cm^{2} cell culture flask and incubated in a 37^{0}C incubator with 5% of CO_2 (Heraeus) until the cells reached 95 to 100% confluence.

2.2.1.3 Cell count

Vero cells were counted using a trypan blue exclusion method (Strober, 2001). A dilution of the cell suspension was prepared by adding 0.2 mL of cell suspension to 0.1 mL of trypan blue (0.4% w/v trypan blue in 0.85% of saline) (Sigma Aldrich Ltd) in a bijou bottle. A volume of 40 to 50 µL of the mixture was added to the haemocytometer (Fast read 102™ disposable counting chamber; Immune Systems Ltd, Devon, UK). Live cells unstained by trypan blue were counted in four large squares and cell concentration was calculated as below:

Total number of cells counted in the four large squares = N

Number of cells per large square = N/4

Number of cells per cm^{3} of diluted cell suspension = $(N/4)*10^{4}$

Allowing for the dilution with trypan blue = $((N/4)*10^{4})*3/2$ Cell/mL

2.2.2 Sf9 insect cells

2.2.2.1 Recovery of Sf9 frozen insect cells

A frozen vial (5-6 x 10^5 cells/mL) of Sf9 insect cells was thawed from liquid nitrogen by immersing the vial in a 37°C water bath for 2 to 3 minutes. Then, the outside of the vial was sterilised with 70% of ethanol (BDH Chemical Ltd). The cells were then transferred into 15 mL centrifuge tube containing 10 mL of pre-warmed (28°C) media (BacVector® Insect cell medium) (Merck, Novagen). The cells were centrifuged using Megafuge 1.0 centrifuge (Heraeus equipment Ltd) with speed at 900 x g for 4 minutes at room temperature, the supernatant was discarded and the pelleted cells resuspended in 20 mL pre-warmed medium containing 5% ($^v/_v$) of foetal calf serum. The entire suspension was then seeded into a 75 cm^2 cell culture flask and incubated in a 28°C incubator (Heraeus). The cells were examined daily using an inverted microscope (Wolf Laboratories Ltd) until the cell monolayer reached confluence of 85-95%.

2.2.2.2 Sub culture of insect cells

After the cells had reached confluence of 85-95% (3 to 5 days), the cells were subcultured. Briefly: The media was removed from the flask and replaced by 5-10 mL fresh pre-warmed media with 5% ($^v/_v$) foetal calf serum. The cells were then removed from the flask using a sterile disposable cell scraper (Greiner Bio-One), and the cell suspension transferred into a sterile Universal tube. Cells were then examined, counted (as described in Section 2.2.1.3) and resuspended at a density of 3-5 x 10^5 viable cells/mL into sterile 75cm^2 flasks containing 15-18 mL of antibiotic free BacVector® Insect cell Medium. All the flasks were then

placed in an incubator at 28^0C and examined daily under an inverted microscope. The cells were cultured until the cell monolayer become 85-95% confluent.

2.2.2.3 Insect cells storage

When the insect cells had reached 90-95% viability, the cell medium from the flask was discarded and replaced with 10 mL of pre-warmed medium. The cells were then rinsed from the flask using a sterile disposable cell scraper (Greiner Bio-One) and centrifuged using Megafuge 1.0 centrifuge (Heraeus) at 1450 x g for 10 minutes. The supernatant was removed and the cell pellet resuspended gently in 1 mL of BacVector® medium containing 20% ($^v/_v$) dimethyl sulfoxide (DMSO) (Sigma Aldrich Ltd) and 5% ($^v/_v$) foetal calf serum. Following this, the cell suspension was transferred to a sterile cryogenic vial and placed in Scotlab polystyrene box (freezing container) (Nalgene Nunc International Corporation, Rochester, USA) containing isopropanol (BDH Chemical Ltd). The freezing container was stored in the -80^oC freezer for 12-18 hour before the cryogenic vials were immersed in liquid nitrogen for longer storage.

2.3 Cell infection

2.3.1 Infection of cells with HSV-1 and HSV-2

The growth medium over confluent monolayers of Vero cells was removed and replaced with 11 mL of warmed (27^0C) maintenance medium (Eagle's MEM with 0.5% ($^v/_v$) foetal calf serum). An aliquot of 1 mL of virus (10^6 $TCID_{50}$/mL) was added to the flasks. The flasks were then rocked gently to ensure good distribution of the virus across the cell layer. The

flasks were then incubated in a 37^0C incubator with 5% CO_2 (Heraeus) and the infected cells were examined daily.

2.3.2 Harvesting virus

Virus was harvested using a freeze-thaw method (Ammerman *et. al,* 2008). Once the cells inside the flask were observed to have developed cytopathogenic effect (CPE), the flask was placed in a -20^0C freezer (RLFF13246, Labcold Ltd, Hampshire, UK) for one hour. The flask was then removed from the freezer and placed at room temperature. Once defrosted, the flask was shaken by hand to detach the cells. This process was repeated once and the medium in the flask aliquoted into sterile 1.5 mL screw-cap Eppendorf tubes. All tubes were stored in the -80^0C freezer and 1 mL of each batch was used for virus titration.

2.3.3 Virus titration in cell culture

Virus titre was determined using the 50% tissue culture infectious dose ($TCID_{50}$) method (LaBarre and Lowy, 2001). A volume of 100 µL of a Vero cell suspension (10^5 $TCID_{50}$/mL) was added to each well of a 96-well microtitre plate (Greiner Bio-One) and incubated at 37^0C in 5% CO_2 in air for 24 hours. A 10-fold serial dilution of virus (10^{-1} to 10^{-11}) was prepared in bijoux bottles using maintenance medium. Using an 8-channel 25-200 µL pipette (StarLab Ltd, Milton Keynes, UK), the growth medium in each well was discarded and replaced with 100 µL of virus dilution (8 duplicates for each dilution). For the cell control, 100 µL of maintenance medium was added into each well, which was located in the last column of a 96-well plate. Plates were then incubated at 37^0C in 5% CO_2 in air for 48 hours.

Wells with signs of infection (CPE) were counted and calculated using the Spearman-Karber formula as below:

Log10 Median Dose = X_0 – (d/2) = d (Σri/ni)

Where:

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

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

ni = number of test inoculates used at each dilution.

ri = number of positive test inoculates (out of ni).

Σ(ri/ni) = Σ (P) = sum of the proportion of positive tests beginning at the highest dilution showing 100% positive results (this summation starting at dilution X_0).

2.3.4 DNA extraction using QIAamp MinElute Virus spin kit

HSV-1 and HSV-2 DNA were extracted from Vero cells using QIAamp MinElute virus spin kit (Qiagen Ltd, Manchester, UK) following the manufacturer's protocol (Qiagen, 2010a). Briefly, a volume of 200 µL of specimen or sterile distilled water (as a negative control) was pipetted into a 1.5 mL microcentrifuge tube containing 25 µL of Qiagen protease. A volume of 200 µL of lysis buffer (Qiagen AL buffer containing guanidinium chloride), including 28 µg/mL of carrier RNA was added into solution and mixed by pulse-vortexing for 15 seconds. The tube was then incubated at 56^0C in a heating block (Grant) for 15 minutes. After incubation, 250 µL of ice-cold ethanol (96-100%) (BDH Chemical Ltd) was added to the sample with further incubation for 5 minutes at room temperature (approximately

27^0C). The mixtures were then transferred into the QIAamp MinElute column and centrifuged using Biofuge pico centrifuge (Heraeus) at 6000 x g for 1 minute. The column was centrifuged 3 times at 6000 x g for 1 minute. Each time, a clean 2 mL collection tube was used and 500 µL of different buffer solutions were added. Those solutions were buffer AW1, buffer AW2, and ice-cold absolute (100%) ethanol (BDH Chemical Ltd). The column was then centrifuged at full speed (20,000 x g) for 3 minutes and incubated at 56^0C in a heating block with the lid opened for 3 minutes in order to dry the membrane or solution completely. The supernatant in the collection tube was discarded and the column placed into a sterile 1.5 mL Eppendorf tube followed by 200 µL of RNase-free water was added to the centre of the membrane. The column was then incubated at room temperature for a further 3 minutes followed by centrifugation at 20,000 x g for 1 minute. The supernatants or extracted DNAs were stored at -20^0C freezer until required.

2.4 Polymerase chain reaction for HSV-1 gG and HSV-2 gG

2.4.1 Determining the sequences of HSV-1 gG and HSV-2 gG

All published genome sequences of HSV-1 gG (4 sequences) and HSV-2 gG (2 sequences) were obtained from the US National Institutes for Health genetic sequence database (GenBank) and then aligned and compared (from 5’ to 3’ end) using BioEdit Sequence Alignment Editor Program (Version 7.0.5.3; http://www.mbio.ncsu.edu/bioedit/bioedit.html).

2.4.2 Primer design for the full length and truncated version of HSV-1 gG and HSV-2 gG

The full length and truncated version of HSV-1 gG and HSV-2 gG primer sets were designed using the entire genome sequences of HSV-1 (NC-001806) and HSV-2 (NC-001798) published in Genbank. Primer design incorporated a specific vector-compatible overhang at the 5' end. The G + C content and melting temperature for the primer sets were determined using Primer Blast (Basic Local Alignment Search Tool) from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). An NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was also performed to align primer sequences and published sequences in Genbank. In order to prepare the primers for polymerase chain reaction (PCR) use, all primers were diluted in Nuclease-free sterile distilled water (Merck, Novagen) to achieve a concentration of 100 µM and then stored at -20^{0}C after dividing into 100 µL aliquots (primer stock). The 100 µL primer stock was further diluted in 400 µL of sterile distilled water (Merck) to achieve 500 µL of working stock with a concentration of 20 µM. The working stock was then divided into 100 µL and further aliquoted into 10 µL volumes. Those working stocks were then stored at -20^{0}C freezer until required.

2.4.3 Contamination Control

PCR contamination control was carried out using standard precautions as described by Aslanzadeh, 2004. The pre and post PCR reagents and samples were stored in separate areas to avoid cross contamination. Separate sets of supplies, such as pipettes, racks, lab coats, gloves and PCR tubes required for setting up PCR mixture and for

sample preparation were prepared. The PCR procedure was performed in three different rooms: the first room was a 'DNA free' room, where the storage and preparation of PCR mixture and all reagents occurred. The DNA free room was strictly controlled so that no contamination sources could be brought into this room. PCR mixtures were then transferred to a 'DNA preparation room', for addition of DNA templates to the mixture. The DNA amplification was performed in a third room, the 'PCR product detection' room. All the PCR preparation steps were performed in a PCR UV cabinet. In addition, negative controls containing ultra-pure water were included in every PCR reaction to monitor for potential contamination.

2.4.4 PCR amplification of HSV-1 gG and HSV-2 gG

For amplification of HSV-1 gG and HSV-2 gG, AmpliTaq Gold 360 master mix (Invitrogen Life Technologies) was used. Briefly: a 45 µL of AmpliTaq Gold 360 mixture reaction containing 25 µL AmpliTaq Gold 360 master mix, 1 µL of each oligonucleotide primer (20 µM) and 18 µL of Ultra-pure PCR water (Bioline Ltd, London, UK) were prepared in a DNA free room. A volume of 5 µL of each DNA sample (HSV-1 and HSV-2) was then added to each mixture to give a final volume of 50 µL. For HSV-1 gG amplification, the cycling comprised a single initial denaturation at 95^0C for 10 minutes followed by 40 cycles of denaturation at 95^0C for 1 minute, annealing at 60^0C for 30 seconds (every 8 cycles reduce 1^0C annealing temperature) and extension at 72^0C for 2 minute; whereas for HSV-2 gG, the cycling started with a single initial denaturation at 95^0C for 10 minutes followed by 40 cycles of denaturation at 95^0C for 1 minute, annealing at 58^0C for 1 minute and extension at 72^0C for 1 minute using the PCR

thermocycler machine (GeneAmp®PCR System 9700) (Applied Biosystems, San Diego, USA).

The amplification of truncated HSV-1 gG was started with a single initial denaturation at 95^0C for 10 minutes followed by 40 cycles of denaturation 95^0C for 1 minute, annealing at 60^0C for 1 minute and extension at 72^0C for 1 minute; whereas for truncated HSV-2 gG, the cycling started with a single initial denaturation at 95^0C for 10 minutes followed by 40 cycles of denaturation 95^0C for 1 minute, annealing at 62^0C for 1 minute and extension at 72^0C for 1 minute.

2.4.5 Detection of PCR products by E-gel electrophoresis

The detection of PCR products was performed by mixing a volume of 9 µL of PCR product with 1 µL of 10x loading buffer (containing 65% (w/v) sucrose, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.3% (w/v) bromophenol blue; Bioline Ltd, London, UK), plus 10 µL of deionised water. A volume of 10 µL of ladder (HyperLadder ™ I (Bioline Ltd) for HSV-1 gG or EasyLadder IV (Bioline Ltd) for HSV-2 gG) was added to 10 µL of distilled water. Thereafter, 20 µL of each sample and ladder were analysed using 2% w/v Agarose E-gel electrophoresis (Invitrogen Life Technologies) at 12 Volts for 30 minutes. The DNA band was then visualised using Alphamager 2200 transilluminator (Bio-Techne, Minneapolis, USA) with 365 nm wavelength.

2.4.6 Detection of PCR products by 1.5% agarose gel electrophoresis

2.4.6.1 Preparation of 1.5% agarose gel

A total of 1.5 g agarose powder (Bioline Ltd) was added to a glass bottle containing 100 mL of 1X TBE buffer (89 mM Tris base, 89 mM Boric acid and 2 mM EDTA, pH 8.0; Sigma Aldrich Ltd). The mixture was then heated using a 700 watt microwave oven (Sharp Electronics, LTD, UK) for 60 seconds with intervals of shaking every 15 seconds until the agarose powder was completely dissolved. The mixture was then reheated for 3 minutes. The molten agarose was then cooled to 60^0C before pouring onto an agarose gel slab containing a 15 well comb (Scie-Plas Ltd, Cambridge, UK).

2.4.6.2 Detection of PCR products

After gel solidification, the agarose gel slab was placed in a T-V-Gel tank (Scie-Plas Ltd) and the comb gently removed. The tank was filled with 1X TBE buffer until the buffer covered the gel in the tank. Next, 20 µL of PCR products were loaded into the wells on the gel. A volume of 15 µL of DNA ladder (HyperLadder™ I (Bioline) for HSV-1 gG, truncated HSV-1 gG and truncated HSV-2 gG or EasyLadde II (Bioline) for HSV-2 gG) was added to one well as a DNA marker. Electrophoresis was performed at 90 Volts with 400 Ampere for 2 hours. After that, the gel was removed from the buffer and cut into 4 columns (DNA marker, negative control and two samples) using a sterile disposable scalpel (Swann Morton Ltd, Sheffield, UK). The DNA marker containing gel was stained using Midori Green DNA stain (NIPPON Genetics Europe GmbH, Dueren, Germany) for 15 to 30

minutes. The stained gel was then aligned adjacent with the unstained gel and the DNA bands visualised using a blue light transilluminator (Geneflow Ltd, Staffordshire, UK) at 480 nm wavelength.

2.4.6.3 Excision of DNA from the gel

The DNA bands were excised using the blue light transilluminator and a sterile disposable scalpel (Swann Morton Ltd). Before band excision, 1.5 mL Eppendorf tubes were weighed. The gel was then transferred on to an autoclaved glass sheet and placed onto the blue light transilluminator. The bands were cut from the gel and placed into the 1.5 mL Eppendorf tubes. The tubes were then reweighed to determine the weight of excised gel.

2.5 Purification of PCR products from a gel

For purification of PCR products from a gel, the QIAquick gel extraction kit (Qiagen Ltd, London, UK) was used (Qiagen, 2010b). Three volumes of buffer QG were added to 1 volume of gel (300 µL of buffer QG was added to each 100 mg of gel). The mixture was then incubated at 50^0C for 10 minutes with intervals of vortexing of the tube every 2 or 3 minutes during the incubation in order to solubilize the agarose completely. Next, 1 gel volume of ice-cold isopropanol was added to a sample and which was then placed in a QIAquick spin column with 2 mL collection tube. The column was centrifuged using Biofuge pico centrifuge (Heraeus equipment Ltd) with speed at 17,900 x g for 1 minute, and then 500 µL of buffer QG was added to the reservoir of the column. After another round of centrifugation for 1 minute, 750 µL of buffer PE (contain

96-100% ethanol) was added and the tube re-centrifuged for 1 minute. The column was inserted into a new 2 mL collection tube and centrifuged again at 17,900 x g for 1 minute to remove residual ethanol from buffer PE. The QIAquick column was placed into a clean 1.5 mL Eppendorf tube, and 50 µL of buffer EB (10 mM Tris-CI, pH 8.5) was added to the centre of the QIAquick membrane.To increase DNA concentration, the column was incubated at room temperature for 2 minutes and centrifuged at 17,900 x g for 1 minute. The QIAquick column was discarded and 9 µL of the purified product was electrophoresed using a 2% E-gel at 12 volts for 30 minutes (as described in section 2.4.5). The remainders of the purified products were stored at -20^{0}C until required.

2.6 Recombinant plasmid construction for HSV-1 gG and HSV-2 gG

2.6.1 Preparation of the 3C/LIC insert for HSV-1 gG and HSV-2 gG

For generation of a recombinant plasmid for each virus (HSV-1 gG and HSV-2 gG), the 3C/LIC insert for purified PCR product of each virus was prepared using pIEX/Bac 3C/LIC plasmid vector kit (Merck, Novagen). Briefly, 10 µL of purified PCR product was mixed with 2 µL of 10X T4 DNA polymerase buffer, 1 µL of 100 mM DTT, 2 µL of 25 mM dATP, 4.6 µL of nuclease-free water, and 0.4 µL of 2.5 U/µL T4 DNA polymerase to give a final volume of 20 µL in a sterile 1.5 mL Eppendorf tube (kept on ice). For the positive and negative control, 4 µL of 3C/LIC β-gal control insert (3081 bp with a concentration of 2 µg/pmol) (positive control) and deionised water (negative control) (Merck) was added. Thereafter, all the mixtures

were incubated at 22^0C heating block for 30 minutes and the tubes incubated at 75^0C for 20 minutes to inactivate the enzyme. The treated PCR products were then stored at -20^0C for the next step.

2.6.2 Annealing the vector and 3C/LIC insert

The annealing of the 3C/LIC insert for each treated PCR product (HSV-1 gG and HSV-2 gG) into the pIEX/Bac-3 plasmid vector was prepared as follows: 1 µL of 3C/LIC vector was mixed with 2 µL of T4 DNA polymerase treated 3C/LIC insert (0.02 pmol) and 1 µL of 25 mM EDTA (Merck, Novagen). The annealing reaction was started by adding EDTA with stirring by a pipette tip and then, incubated at 22^0C in a heating block for 5 minutes. The transfer vector were then stored at -20^0C until required.

2.6.3 Recombinant Plasmid Analysis

2.6.3.1 Transformation of competent cells

For the confirmation of correct target sequences insertion of the recombinant plasmid, transformation of competent cells was prepared. Briefly, two tubes containing 0.2 mL of GigaSingles competent cells (Merck, Novagen) were taken from the -80^0C freezer and left to thaw on ice for 5 minutes. The tubes were then flicked gently 2 times to mix the contents and 1 µL of the annealing reaction (samples and negative control) was added to each tube. To determine transformation efficiency, 1 µL of test plasmid was added to a separate tube containing cells and incubated for 5 minutes on ice. The tubes were then heated in a 42^0C water bath for 30 seconds and placed on ice for 2 minutes. The mixtures were then added to 250 µL of SOC medium (at room temperature) and

incubated at 37^0C with shaking at 250 rpm for 60 minutes. After the incubation, 50 µL from each tube was plated out on Luria Broth (LB) agar medium (Sigma Aldrich Ltd) (50 µg/mL Carbenicillin (Sigma Aldrich Ltd)). The plates were left on the bench for 15 minutes to allow excess liquid to be absorbed and then incubated overnight at 37^0C incubator (Heraeus equipment Ltd).

2.6.3.2 Colony screening for recombinant plasmid

After overnight incubation, 3 colonies (approximately 1 mm in diameter) from each plate were taken using a sterile pipette tip in a sterile 0.5 mL tube containing 50 µL of sterile water (Merck). The tube was then vortexed and placed in boiling water for 5 minutes to lyse the cells. Thereafter, the tube was then centrifuged using Biofuge pico centrifuge (Heraeus equipment Ltd) at 12,000 g for 1 minute to remove the cell debris. A volume of 10 µL of the supernatant was transferred into another sterile 0.5 mL tube and placed on ice. For each reaction, 10 µL of each sample was added to 40 µL AmpliTaq Gold 360 master mix reaction containing 25 µL AmpliTaq Gold 360 master mix (Invitrogen Life Technologies), 1 µL of each oligonucleotide primer (20 µM) and 13 µL of sterile Ultra-pure PCR water (Bioline Ltd). The PCR parameter was started with a single initial denaturation at 95^0C for 10 minutes followed by 35 cycles of denaturation at 95^0C for 1 minute, annealing at 58^0C for 1 minute and extension at 72^0C for 2 minutes and the last step was extended for 5 minutes. The PCR was performed using vector-specific or virus-specific primer alone, or in combination with vector-specific (IEI promo/IEI term) or

virus-specific primers. The PCR product was then visualised using 2 % w/v agarose E-Gel.

2.7 InsectDirect Protein Expression system

2.7.1 Preparation of cell culture for Transfection

For transfection of cell cultures, 25 cm^2 flasks were used. Briefly: Four 25 cm^2 flasks (2 samples, positive and negative control) were seeded with a 2 mL dilution of Sf9 ($2X10^5$ cells/mL) from a 75 cm^2 confluent monolayer cell culture. Flasks were then incubated at 28^0C for 24 hours to allow the cells to reach 80-90% confluence.

2.7.2 Preparation of Transfection mixture

The transfection mixture was prepared as follows: for each transfection, 15 µL of plasmid DNA (15 µg in total) was diluted with 1 mL of serum free BacVector Insect cell medium. Also, 100 µL of Insect GeneJuice transfection reagent (Merck, Novagen) was diluted with 1 mL of serum free BacVector Insect cell medium. The plasmid DNA mixture was then added dropwise to the Insect GeneJuice transfection reagent, gently vortexed to avoid precipitation and then incubated at room temperature for 15 minutes.

2.7.3 Transfection of Insect cells

After 24-hours incubation, the culture media from each 25 cm^2 flask was discarded using a sterile pipette, 1 mL of transfection mixture was added dropwise to the centre of the flask. Flasks were then incubated for overnight (10 to 14 hours) in a 28^0C incubator after which 1 mL of

BacVector® Insect cell medium was added to each flask. Thereafter, flasks were continued to be incubated for 5 days. The insect cells were examined daily under an inverted microscope (Wolf Laboratories Ltd) for signs of infection. Once insect cells had demonstrated late signs of infection, cells were harvested using Megafuge 1.0 centrifuge (Heraeus equipment Ltd.) with low speed centrifugation (1500 x g) for 10 minutes. The supernatant, which contained the recombinant virus, was transferred to a sterile tube and stored at 4^0C until required.

2.7.4 Recombinant virus analysis

The supernatant from transfected cell culture was extracted using QIAamp MinElute Virus spin kit (Qiagen) as described in section 2.3.4. A volume of 10 µL of each DNA extracted sample, including negative controls (uninfected cells), was mixed with 40 µL AmpliTaq Gold 360 (Invitrogen Life Technologies) master mix reaction containing 25 µL of AmpliTaq Gold 360 master mix , 1 µM of each oligonucleotide primer (20 µM) and 13 µL of sterile Ultra-pure PCR water (Bioline Ltd). The cycling parameter comprised a single initial denaturation at 94^0C for 10 minutes, followed by 35 cycles of denaturing step at 94^0C for 1 minute, an annealing step at 55^0C for 1 minute and an extension step at 72^oC for 2 minutes and hold at 72^0C for 5 minutes. PCR reaction was performed using virus specific primers. After amplification, the PCR product was then visualised using 2% w/v agarose E-gel (Invitrogen Life Technologies).

2.8 Protein Purification

2.8.1 Preparation of lysates using Insect PopCulture® Reagent

For each virus, 10 mL of transfected cell culture was mixed with 500 µL of Insect PopCulture (Merck, Novagen) and 4 µL of benzonase® nuclease (10 Units) (Merck, Novagen). The mixture was then mixed by gentle inversion and incubated at room temperature for 15 minutes. The supernatant, containing the solubilised protein was then transferred to a sterile universal tube and stored at -20^{0}C freezer until needed.

2.8.2 His-Select iLAP column purification method

The protein purification was carried out using His-Select iLAP 5 mL column purification method. Briefly: 5 mL of supernatant was added into the iLAP column (Sigma Aldrich Ltd) with the bottom cap firmly in place. The column was then capped and incubated at room temperature for 40 minutes. After the incubation, the column was secured on a ring stand to perform the subsequent gravity flow purification. The bottom cap was then removed and a 1.5 mL Eppendorf tube placed below the column. After this, the top cap was removed to allow the cell lysate and unbound proteins to drain completely from the column. The sample of this column flow-though was collected for SDS-PAGE analysis. The resin bed at the column was washed with 2 mL of HIS-Select wash buffer (containing 300 mM NaCI, 50 mM sodium phosphate and 10 mM imidazole, pH 8.0) (Sigma Aldrich Ltd) being careful not to disturb the bed of resin at the bottom of column. The wash solution was then completely drained from the column and saved for SDS-PAGE analysis. Thereafter, the target protein was eluted into a

sterile 2.0 mL collection tube by adding 1 mL of HIS-Select elution buffer (containing 300 mM NaCl, 50 mM sodium phosphate and 250 mM imidazole, pH 8.0) (Sigma Aldrich Ltd) onto the top of the resin bed. All the collecting tubes were stored in the -20^{0}C freezer until needed.

2.8.3 Protein concentration

The purified recombinant proteins were concentrated using a Amicon Ultra-4 centrifugal filter unit with a molecular weight cut-off of 30 kDa (Merck, Novagen). Briefly: 500 µL of purified recombinant protein was added into a Microcon device and centrifuged using a Biofuge pico centrifuge (Heraeus equipment Ltd.) at 14,000 x g for 30 minutes. The column was centrifuged 2 times at 14,000 x g for 20 minutes. Each time a clean concentrate tube was used and 500 µL of PBS (Sigma Aldrich Ltd) were added. The column was then placed into a new concentrate tube over the top of the device; the assembly was inverted and centrifuged for 3 minutes at 1,000 g for 3 minutes to transfer concentrated protein to tube. Thereafter, the filter device was removed from the tube and the purified protein stored at -20^{0}C freezer until needed.

2.8.4 Protein Quantification

The concentrations of the purified recombinant protein were determined using Bicinchoninic acid (BCA) protein assay (Thermo Scientific OXOID Ltd, Hampshire, UK) from the manufacturer's protocol (Sigma Aldrich Ltd). Briefly: serial dilutions of bovine serum albumin (BSA) ranging from 0 to 1000 µg/mL were prepared with sterile water from a 1 mg/mL stock. Then, 25 µL of each samples (BSA standard and purified

protein samples) were added in duplicate into a 96-well microtitre plate (Greiner Bio-One), followed by addition of 200 µL of working solution. The plate was then incubated at 37^0C incubator for 30 minutes and cooled to room temperature. The concentrations of BSA standard and purified protein were then determined by measuring absorbance at 562 nm using an ELx808 Universal Microplate Reader (BioTek, Bedfordshire, UK). BSA standard results were used to create a standard curve using Microsoft Excel 2007 (Microsoft, Seattle, USA), in order to determine the concentration of each purified protein.

2.8.5 Protein characterisation

2.8.5.1 Sodium Dodecyl Suphate Polyacrylamide gel Electrophoresis (SDS-PAGE)

A volume of 15 µL of sample (purification flows, wash, elute and concentrate) was mixed with 30 µL of sample loading buffer (containing 50 mM Tris-HCl pH 6.8 (Thermo Scientific OXOID Ltd), 2% SDS (Sigma Aldrich Ltd), 10% glycerol, 1% DTT (Sigma Aldrich Ltd), 0.02% bromophenolblue (Sigma Aldrich Ltd) and 8 M urea (Sigma Aldrich Ltd)) and heated in a boiling water bath (100^0C) for 5 minutes. Following this, 15 µL of each sample was loaded onto a NuPage 4-12% Bis-Tris SDS-PAGE gel (Invitrogen Life Technologies) with protein marker (High-range rainbow molecular weight marker from GE Healthcare, Life Sciences, Buckinghamshire, UK) applied to one well. The gel was electrophoresed using XCELL SureLock™ Mini-Cell electrophoresis tank (Invitrogen Life Technologies) at 200 volts for 35 minutes with 1 x NuPAGE® MES SDS running buffer (50 mL 20x NuPAGE MES SDS running buffer (Invitrogen

Life Technologies), 950 mL deionised water (Merck)). The gel cassette was removed from the tank, rinsed 2 times with 100 mL of deionised water (Merck) for 5 minutes. Thereafter, the gel was stained with 20 mL of Instant blue (Expedeon, Cambridge, UK) and kept on an orbital mixer (Denley, Oxford Labs, Oxford, UK) at 250 rpm for 1 hour at room temperature. Following this, the gel was then destained with 20 mL of deionised water (Merck) and photographed on an Alphamager 2200 transilluminator (Bio-Techne).

2.8.5.2 Western Blot

Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane and further analysed by immunoblotting (Towbin *et al.,* 1992). Briefly, a piece of nitrocellulose membrane filter paper sandwich with 0.45µm pore size (Invitrogen Life Technologies) and 4 pieces of blotting pad were soaked in transblotting buffer (50 mL 20x NuPAGE® transfer buffer (Invitrogen Life Technologies), 100 mL absolute methanol (100%) (BDH Chemical Ltd), 850 mL deionised water (Merck) for 20 minutes. A sandwich was made as in Figure 2.1 and transferred into a cassette. The cassette was positioned into the XCell SureLock™ mini cell (Invitrogen Life Technologies) and the cassette filled with transblotting buffer until the gel/membrane sandwich was covered in transfer buffer. The outer chamber was filled with 650 mL of deionised water (Merck) and a voltage of 100 V applied to electrophoretically transblot at 100 V for 40 minutes. Thereafter, the transblotted nitrocellulose membrane was then incubated with blocking buffer (2.5 g skimmed milk (Thermo Scientific OXOID Ltd), 50 mL PBS (Sigma Aldrich Ltd) pH 7.3, 0.1% Tween 20 (Sigma Aldrich

Ltd)) on an orbital shaker (Heidolph, Essex, UK) with speed at 150 rpm at room temperature for 1 hour. The membrane was washed 3 times with washing buffer (15 mL PBS, 0.05% v/v Tween 20) at room temperature for 5 minutes and incubated with either anti-HSV-1 gG (catalogue number:H1379) (Abcam, Cambridge, UK) or anti-HSV-2 gG monoclonal antibody IgG (catalogue number:H1206) (Abcam) (1:1,000) at room temperature for 1 hour. The membrane was then washed 3 times with washing buffer for 5 minutes and incubated with goat anti-mouse polyvalent immunoglobulin alkaline phosphatase conjugate (1:1,000) (Sigma Aldrich Ltd) at room temperature for 1 hour. After washing step, the membrane was treated with one tablet of FAST™BCIP/NBT [4-Chloro 2-methylbenzenediazonium/3-hydroxy-3 naphthoic acid 2,4-dimethylanilide phosphate; contained 0.15 mg/mL BCIP, 0.30 mg/mL NBT, 100 mM Tris buffer and 5 Mm $MgCl_2$] (Sigma Aldrich Ltd, Poole, UK) dissolved in 10 mL of deionised water on an orbital shaker (Heidolph) with speed at 150 rpm at room temperature until the bands appear (15-30 min). The membrane was then rinsed with water and left to dry at room temperature.

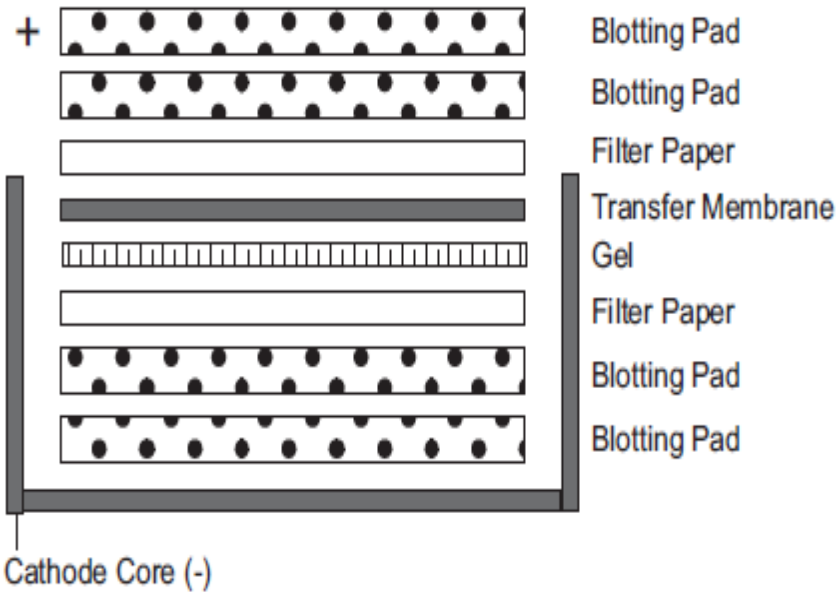


Figure 2.1: Western blotting sandwich (Taken from: http://tools.lifetechnologies.com/content/sfs/manuals/blotmod_pro.pdf) (Accessed: 03 October 2014)

2.8.5.3 Light Scattering

The purified protein was analysed using Multiangle Laser Light Scattering (MALLS) analysis by the Faculty of Life Sciences, protein facility of The University of Manchester. Briefly, 500 µL of purified recombinant protein was applied to a Superdex 200 10/300GL gel filtration column (GE Healthcare Ltd) running at a flow rate of 0.75 mL/min in PBS (Sigma Aldrich Ltd). The protein sample was then eluted from the column and passed through an in-line DAWN HELEOS-II laser photometer (laser wavelength 658 nm) (Wyatt Technology Europe GmbH, Dernbach, Germany) with a QELS dynamic light scattering attachment, and an Optilab rEX refractometer (Wyatt Technology Europe GmbH). The light scattering intensity and eluent refractive index for the purified protein were analysed using Astra (version 6.1) software (Wyatt Technology Corporation, Santa Barbara, CA, USA).

2.8.5.4 Mass spectrometer

2.8.5.4.1 BioMerieux VITEK mass spectrometer

The purified recombinant protein was analysed using a BioMerieux VITEK mass spectrometer (BioMérieux Ltd, Hampshire, UK). Briefly, 10 µL of the purified protein was added to the VITEK MS-DS target slide. The target spot was then added 1 µL of VITEK MS-α-Cyano-4-hydroxycinnamic acid (CHCA) matrix (BioMérieux Ltd) and the spot was allowed to dry at room temperature for 5 minutes. Thereafter, the yellowish crystal was formed on the spot and the target slide was then placed into the Biomerieux VITEK workstation. The spectrum of the proteins was detected using VITEX MS software (BioMérieux Ltd)**.**

2.8.5.4.2 Intact Mass spectrometer

The purified protein was further analysed using an Agilent 6510 Quadrupole time-of-flight (Q-TOF) mass spectrometer coupled to an Agilent 1200 series HPLC (Agilent technologies, Santa Clara, USA). Briefly: The Q-TOF mass spectrometer was previously set to the standard mass range mode and calibrated. A volume of 5 µL of purified protein was injected onto a de-salting guard column (Agilent 5 x 3 mm PLRP-S) in 0.3 mL/min 5% ACN/H_2O (0.1% formic) and run into the Q-TOF mass spectrometer with fragmentor set to 215 V, 35^0C source, 8 L/min drying gas and a nebuliser of 30 psig. Thereafter, sample data were deconvoluted using Agilent MassHunter (version 5.0) software.

2.9 Multiplex immunoassay

2.9.1 Development of a Bioplexed fluorescent bead assay for the quantitative detection of IgG antibodies to Measles, Mumps, Rubella and Varicella Zoster virus (MMRV) in human sera

2.9.1.1 Acceptance criteria for MMRV Bioplex assay

A series of acceptance criteria for the MMRV Bioplex assay were developed to ensure accurate measurement and maximum reproducibility when performing the assay. The criteria for evaluation of assay results were defined as follow:-

1. Initial plate acceptance criteria
    a. No errors/ incidents / Analyte Fluorescence Intensity (FI) inconsistencies affecting the background, the standard curve, a Quality Control (QC) (both dilutions) across all antigens or the whole plate.
    b. Background (defined blank wells) Median Fluorescence Intensity (MFIs) for the antigens assayed ≤ 200.
2. Initial antigen acceptance criteria
    a. No errors/ incidents that could have affected the background or standard curve of the antigen.
    b. The background MFI ≤ 200.
    c. At least one blank replicate has a bead count (total beads detected per specific analyte) of ≥ 80.

d. At least one blank replicate has a % bead aggregation (agg) of ≤ 50.

3. Standard curve acceptance criteria

a) All remaining standard curve replicates have a bead count of ≥ 80.

b) All remaining standard curve replicates have a % agg of ≤ 50.

c) The assay precision was determined using a percentage of coefficient of variation for replicate wells (%CV) (Reed *et al.*, 2002; PHE, 2013). All standard curve points have a %CV ≤ 30 (or a %CV > 30 and the difference in replicate FI ≤ 10).

d) All standard curve points have a percentage of standard curve recovery (obs/exp) ≥ 80 and ≤ 120. The obs/exp is used to determine the overall accuracy of the bioplex assay and was calculated as below:

Percentage of standard curve recovery = obs/exp*100

Where:

Obs = Observed concentration of the standard point

Exp = Expected concentration of the standard point

4. QC Acceptance criteria

a) No errors/ incidents that could have affected both dilutions of QC/ test sera.

b) QC/ test sera replicates have a bead count of ≥ 80.

c) QC/ test sera replicates have a % agg of ≤ 50.

d) QC/ test sera has a %CV ≤ 25 (excluding the result is flagged by '*' or out of range for the standard curve ('OOR'), and results that

are less than lower limit of quantitation for the standard curve (≤LL0Q) at a dilution of 1/200). Result from QC1, QC2 and QC3 must be valid and fall within the assigned ranges of the QC tracking sheet for the QC to be acceptable.

5. Test sera Acceptance criteria

a) Correct sample results for data which are out of range (>OOR or <OOR).

b) Samples >OOR are repeated in the higher dilution and samples <OOR or with MFI's below the lowest concentration of the standard curve are corrected to the established LLOQ (0.001 IU/mL for measles, 0.1 RU/mL for mumps, 0.01 IU/mL for rubella and 0.005 IU/mL for varicella) for statistical purposes.

2.9.1.2 Microsphere coupling method

Purified measles strain Edmonston (RIVM), mumps strain Jeryl Lynn (RIVM), rubella strain HPV-77 (GenWay Biotech Inc., San Diego, USA) and Varicella-Zoster virus strain VZ-10 (GenWay Biotech Inc.) antigens were coupled to different carboxylated MicroPlex Microspheres (Luminex Corporation, Austin, Texas, USA), dye regions 43, 45, 49 and 51, respectively using a modified two step carbodiimide reaction method (Staros *et al.,* 1986). Briefly, 1 mL of a homogeneous carboxylated microsphere suspension was added to a 1.5 mL reaction vial and centrifuged using MIKRO 200 Microcentrifuge (Hettich Lab Technology, Massachusetts, USA) at 13,000 x g for 4 minutes. The supernatant was then removed and re-suspended the bead in 1 mL of freshly prepared

bead activation buffer (containing prepared PBS (containing 0.14M sodium chloride (Sigma Aldrich Ltd), 0.021M potassium phosphate (Sigma Aldrich Ltd), 0.013M sodium phosphate (Sigma Aldrich Ltd), and 0.0021M potassium chloride (Sigma Aldrich Ltd), pH 7.3), 5 mg/mL 1-ethyl-3-(-3dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (Thermo Scientific OXOID Ltd) and 5 mg/mL Sulpho-NHS (N-hydroxysulfosuccinimide) (Thermo Scientific OXOID Ltd)). The tube was wrapped in aluminium foil and incubated at room temperature with shaking at 50 rpm for 20 minutes using a Polymax 1040 orbital Shaker (Heidolph, Essex, UK). After the incubation, the supernatant was removed by centrifugation at 13,000 x g for 4 minutes and washed twice with freshly prepared PBS by centrifugation at 13,000 x g for 4 minutes. The antigen solution for conjugation was prepared according to Table 2.1. The supernatant of the activated bead was aspirated and re-suspended in 500 µL of antigen solution. For the bead conjugation of measles, the antigen solution was incubated at room temperature for 30 minutes and sonicated using an XB3 ultrasonic bath (Grant Instruments Ltd) for 30 seconds before being added to activated beads. The tube was then vortexed, wrapped in aluminium foil and incubated at room temperature with shaking at 50 rpm for 2 hours (±5 minutes). The bead was washed 3 times with 1 mL of PBS, followed by re-suspension in 1 mL of freshly prepared bead storage buffer (containing PBS, 0.1% BSA; Sigma Aldrich Ltd) and 0.05% sodium azide (Severn Biotech Ltd, Worcestershire,UK)). The tube was vortexed on high speed for 30 seconds and then sonicated for 30 seconds. The activated beads were then transferred to a labelled 1.5 Eppendorf tube and stored at $4^{O}C$ in the dark until needed.

Table 2.1: MMRV bead conjugation ratios

Antigen	Strain	Concentration (µg/12.5 x 10^6 microspheres)	Amount of antigen (concentration) µL (µg/µL)	Amount of PBS (µL)
Measles	Edmonston	150	185 (0.9)	365
Mumps	Jeryl Lynn	75	92 (0.9)	458
Rubella	VZ-10	15	33 (0.5)	517
Varicella zoster	HPV-77	55	51.7 (1.17)	498.3

2.9.1.3 Bead calculation

The bead suspensions were counted by adding 20 µL of bead suspension into the sample introduction port of a Cellometer® disposable cell counting chamber (Nexcelom Bioscience, Lawrence, USA) which was then placed under a light microscope (Nikon, London, UK) with 10x magnification. The numbers of beads were counted in 4 small squares in a corner square. The total number of beads in each 1 mL was calculated as follows:

No of beads/mL = (no. of beads counted in 4 small squares x 4) x10^4

2.9.1.4 Bioplexed fluorescent bead assay for the quantitation of MMRV IgG in human sera

A standard curve was generated by making three-fold dilutions (from 1/400 to 1/7873200) using an Anti-Rubella immunoglobulin standard; RUBI-1-94 (National Institute for Biological Standards and Control (NIBSC), Hertfordshire, UK) (containing antibody level of measles; 63 IU/mL, mumps; 4385 RU/mL, rubella; 1600 IU/mL and Varicella Zoster; 22 IU/mL). Samples and standards were tested in duplicate. The test

serum samples, IgG deficient serum (Sigma Aldrich Ltd), controls (QC1 (QCRRUBQC1), QC2 (QCRRUBQ2) and QC3 (Anti-Varicella Zoster virus control serum), were all purchased from NIBSC) were diluted 1 in 200 in sample diluent buffer (containing PBS pH 7.3, 0.05% ($^{v}/_{v}$) Tween-20 and 3% BSA). A volume of 25 µL of each sample (diluted standard, assay control and patient sera) was added in duplicate to each well of a 96-well filter bottom microtitre plate (Merck, Novagen). 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 bead (4000 beads/region/well) was added to each well and the plate covered with aluminium foil was incubated at room temperature with shaking using a plate shaker (IKA®-Werke GmbH, Staufen, Germany) at 600 rpm for 45 minutes (±2 minutes). The plate was then washed 3 times with 100 µL of PBS using a vacuum filtration manifold (Merck, Novagen). The detector antibody ("conjugate") - R-Phycoerythrin conjugated Goat anti-Human IgG (Stratech Scientific Ltd, Suffolk, UK) was prepared in PBS with a dilution of 1 in 200 during the washing procedure. After the washing step, 50 µL of conjugate was added to each well; the plate was covered with aluminium foil and incubated at room temperature with shaking at 600 rpm for 30 minutes (±2 minutes). The plate was then washed 3 times with PBS as described above and 100 µL of PBS added to each well. The plate was then shaken at room temperature at 600 rpm for at least 5 minutes before analysed using a Bioplex 200 workstation (Bio-Rad Laboratories Ltd, Hertfordshire, UK). The sample data were analysed using a 5-parameter logistic curve (5PL) fitting programme using Bioplex Manager Software, Version 4.1.1 (Bio-Rad Laboratories Ltd). The evaluation and validation of this MMRV

Bioplex assay were tested using 40 RIVM serum samples with a broad range of antibody concentrations previously determined by RIVM. After the validation and evaluation, the same MMRV Bioplex conditions as above were selected for testing the MMRV serum samples.

2.9.1.5 MMRV serum Panels

The samples were previously collected from the sero-epidemiological surveillance of the National immunisation programme of England in 2012. An age-stratified random selection of 612 samples from this panel, with the age range from 0 to 60 and above years of age, was used. The test samples were anonymised with the only information provided being age, gender and laboratory of sample origin (Appendix 8). These parameters were required to allow an epidemiological profile for each virus to be established. The test samples were stored in the seroepidemiological surveillance -80^{0}C freezer bank at the Public Health England (PHE) regional laboratory in Manchester. The sample size of this study was determined using the single proportion sample size method, assuming a normal distribution with 5% significant level and margin of error, 0.05. The formula for the single proportion sample size method is shown below:

$n=Z^2P(1-P)/e^2$

Where:

n = minimum sample size

Z = the table value for standard normal distribution at 5% significance level =1.96

e = degree of precision / Margin of error

P = estimated prevalence of antibodies

2.9.1.6 Statistical analysis

For validation and evaluation of the MMRV Bioplex assay, the mean of the test panel results for three independent runs were related to the results provided by RIVM using linear regression. The values of correlation coefficient (r) were calculated to determine the variation between each antigen with its RIVM results. The cut-off level for each assay was assigned based on the RIVM cut-off level (Van der Berg *et al.,* 2014; Waaijenborg *et al.,* 2013). The assigned cut-off values for this MMRV assay were further used to interpret the specific IgG antibody status for the MMRV serum panel, with ≥ 0.2 IU/mL for measles, ≥ 45 RU/mL for mumps, ≥ 10 IU/mL for rubella and ≥ 0.26 IU/mL for varicella, respectively. The specific IgG status for each assay was categorised into two groups, positive sample (specific IgG antibody ≥ assigned cut-off) and negative sample (specific IgG antibody < assigned cut-off).

In order to examine the recent seroprevalence of MMRV specific antibodies in England, 612 anonymised serum samples collected in 2012, were tested using validated MMRV Bioplex assay as above. The generated MMRV data were grouped based on three parameters, age group, gender and sample origin, respectively. To examine the potential changes in seroepidemiology of these diseases in England, the significance of difference between the 2012 proportion of antibody and the antibody proportions generated from the past studies (De Melker *et al.,* 2003; Nardone *et al.,* 2003; Vyse *et al.,* 2004; Pebody *et al.,* 2000) were tested using chi-square test. Furthermore, the significance of difference between the gender and regions for the 2012 proportion of antibody, were also examined using a chi-square method. GraphPad Prism 6 (GraphPad software Inc., La Jolla, CA, USA) and Microsoft Excel 2007 (Microsoft, Seattle, USA) were used for data analysis in this study.

2.9.2 Development of a Bi-plexed fluorescent bead assay for the quantitative detection of IgG antibodies to HSV-1 and HSV-2 in human sera

2.9.2.1 Evaluation of the HSV-1 and HSV-2 IgG Antibody in human serum samples for validation of HSV-1 and HSV-2 Bi-plexed assay

2.9.2.1.1 DiaSorin Liaison HSV type specific IgG assay

The HSV status (HSV-1 positive, HSV-2 positive, HSV-1 and HSV-2 positive or HSV-1 and HSV-2 negative) of serum samples were evaluated using DiaSorin Liaison HSV type specific IgG (DiaSorin Ltd, Dartford, UK) assay by the clinical virology department in Manchester Royal Infirmary.

2.9.2.1.2 HerpeSelect ELISA

All samples were retested using the HerpeSelect (Focus Diagnostics Inc, Cypress, USA) assay according to the manufacturer's protocol. Briefly, all the assay components were brought to the room temperature prior to commencing the assay. The test serum samples and controls (high positive, low positive and cut-off calibrator) were diluted in 1 in 101 dilutions with sample diluent reagent. A volume of 100 µL of sample diluent for the 'blank' and 100 µL of each diluted specimen were added to the appropriate wells in the plate. The assay controls and calibrator were tested in triplicate with 100 µL of diluted mixture into each appropriate well. The coated plate was then placed in a humid chamber and incubated for 1 hour at room temperature. Thereafter, the plate was washed 3 times with washing buffer (300 µL wash buffer/well/cycle) using an ELx50 microplate

strip washer (BioTek, Bedfordshire, UK). A volume of 100 µL of peroxidase-conjugated goat anti-human IgG horseradish peroxidase (HRP) was added to all tested well including the blank well and the plate was then placed in a humid chamber and incubated for 30 minutes at room temperature. After incubation, the plate was washed as above and 100 µL of substrate (tetramethylbenzidine (TMB) and hydrogen peroxide) was added to all tested wells. After 10 minutes incubation at room temperature, the enzymatic reaction was stopped by addition of 100 µL of stop solution (1 M sulphuric acid) and absorbances for each well were read using an ELx808 absorbance microplate reader (BioTek) at a wavelength of 450 nm.

2.9.2.1.3 Index Value Calculation

The Index value of each assay was calculated according to the manufacturers' instructions. Briefly, the LIAISON analyser was automatically calculated an index value for HSV-1 or HSV-2 IgG level based on a 10-point master curve, which was adjusted according to a 2-point calibration of the assays. The results for the test sample were graded as positive (index greater than 1.10), negative (index lower than 0.9) or equivocal (between 0.90 and 1.10) based on the thresholds. For the HerpeSelect ELISA assay, the cut-off calibrator was formulated to give the optimum differentiation between negative and positive sera. The mean value for the cut-off calibration wells must be within 0.100 to 0.700 OD units and all the replicate cut-off calibrator ODs should be within 0.10 absorbance units from the mean value. The control index value was reported as index values relative to the cut-off calibrator. For the high

positive control, the index value should be greater than 3.5; low positive should be between 1.5 and 3.5; meanwhile for the negative control, the index value should be less than 0.8. The patient index value was calculated based on the absorbance of the patient's sample divided by the mean absorbance of the cut-off calibrator and the interpretation of the test samples were defined in Table 2.2.

Table 2.2 Interpretation of test sample for HerpeSelect ELISA assay

OD value	Interpretation (Manufacturer's Criteria)
Greater than 1.10	Positive. Presumptive for the present of IgG antibodies to HSV.
Between 0.90 and 1.10	Equivocal
Less than 0.90	Negative. No IgG antibodies to HSV were detected.

2.9.2.2 Development of HSV Bi-plexed assay

2.9.2.2.1 Acceptance criteria for HSV-1 and HSV-2 Bi-plexed assay

Acceptance criteria for the HSV Bi-plexed assay were developed to ensure accurate measurement and maximum reproducibility when performing the assay. The criteria for evaluation of assay results were defined as follow:-

1. Background (defined blank wells) Median Fluorescence Intensity for (MFIs) the antigens assayed ≤ 200.
2. Each analyte has a bead count of ≥ 80 beads/region/well.
3. Each analyte has a % bead aggregation of ≤ 50.
4. All standard curve points have a %CV ≤ 30.
5. All standard curve points have a percentage of standard curve recovery (obs/exp) ≥ 80 and ≤ 120.

2.9.2.2.2 Standard curve generation

The standard curves for HSV-1 and HSV-2 were generated using RUBI-1-94 (NIBSC), the HSV status of this standard was previously determined using the HerpeSelect (Focus Diagnostics Inc) assay. In order to quantitate the concentration of HSV-1 and HSV-2 antibodies, the sample curves for HSV-1 and HSV-2 were compared with the rubella and VZV standard curves by the method of cross standardisation described by Plikaytis and colleagues (1994). The first step was to determine whether the dilution curves for HSV-1 and HSV-2 antibodies against rubella and VZV were paralleled. Briefly, the calibration of the standard references and serially diluted HSV sample curves were described using a log-log model

and a fully specified logit-log model. The log-log model is a linear function relating to the log of fluorescence intensity of the series to the log of the dilution (Plikaytis *et al.,* 1991). A regression line generated for each HSV assay was compared with rubella and VZV standard curves. The values of correlation coefficients (r) were calculated to determine the variation between each HSV sample curve with rubella and VZV standard curves.

For the fully specified logit-log model, the formula is shown as below:

$\text{Logit (MFI)}_{fs} = \text{Log}[(\text{MFI} - \text{MFI}_{min})/(\text{MFI}_{max} - \text{MFI})]$

Where,

MFI_{max} and MFI_{min} = unknown quantities and correspond to the MFIs at zero and infinite concentrations, respectively. MFI_{max} and MFI_{min} are theoretically equal to the upper and lower asymptotes, respectively.

MFI = the mean florescence intensity of each serially diluted HSV sample point

To facilitate visual comparison between standard reference curve and the HSV sample curves, the data generated from the fully specified logit of the fluorescence intensity formula were plotted against the relative dilution series. The formula for relative dilution series is showed as below:

$$\text{Relative dilution} = \frac{\text{actual sample dilution}}{\text{maximum dilution in series}} \times 100$$

For assigning the antibody concentration of HSV in the RUBI-1-94 reference serum, each relative HSV dilution point was calculated based on the formula below:

$$\text{Concentration} = \frac{\text{interpolated dilution from standard curve}}{\text{actual sample dilution}} \text{ x Calibration factor}$$

Where,

Calibration factor = initial concentration of the standard reference serum

The assigned HSV antibody concentration in this study was the mean of the calculated antibody concentration of serial dilution HSV points. For comparison purposes, the coefficient of variation between the standard curve and HSV sample curve was determined using the formula below:

Coefficient of variation = (standard deviation/mean) x 100%

The standard serum was defined to contain arbitrary units of specific antibodies (AU/mL).

2.9.2.2.3 Microsphere coupling method

Purified recombinant proteins HSV-1 and HSV-2 were coupled to different carboxylated MicroPlex Microspheres (Luminex Corporation), dye regions 43 and 45, respectively. Briefly, 1 mL of a homogeneous carboxylated microsphere was added to a 1.5 mL reaction vial and centrifuged using MIKRO 200 Microcentrifuge (Hettich Lab Technology) at 13,000 x g for 4 minutes. The supernatant was then removed and re-suspended the bead in 1 mL of freshly prepared bead activation buffer (containing prepared PBS (containing 0.14 M sodium chloride (Sigma Aldrich Ltd), 0.021M potassium phosphate (Sigma Aldrich Ltd), 0.013 M sodium phosphate (Sigma Aldrich Ltd), and 0.0021 M potassium chloride (Sigma Aldrich Ltd), pH 7.3), 5 mg/mL 1-ethyl-3-(-3dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (Thermo Scientific OXOID Ltd) and 5 mg/mL Sulpho-NHS (Thermo Scientific OXOID Ltd)). The tube was wrapped using aluminium foil and incubated at room temperature with

shaking at 50 rpm for 20 minutes using a Polymax 1040 orbital Shaker (Heidolph, Essex, UK). After the incubation, the supernatant was removed by centrifugation at 13,000 x g for 4 minutes and washed twice with freshly prepared PBS by centrifugation at 13,000 x g for 4 minutes. In order to determine the optimum antigen concentration for HSV bead conjugation, 3 different antigen concentrations were tested, with 0.5 µg/mL, 1 µg/mL and 2 µg/mL, respectively. All the antigen concentrations were diluted from an antigen concentration of 3 µg/mL. The supernatant of the activated bead was aspirated and re-suspended in 500 µL of antigen solution. The tube was then vortexed, wrapped in aluminium foil and incubated at room temperature with shaking at 50 rpm. In order to determine the optimum incubation time for HSV bead conjugation, 3 different incubation times were tested, 1.5 hours, 2 hours and 3 hours, respectively. After the incubation, the bead was washed 3 times with 1 mL of PBS, followed by re-suspension in 1 mL of freshly prepared bead storage buffer (containing PBS, 0.1% BSA; Sigma Aldrich Ltd) and 0.05% sodium azide (Severn Biotech Ltd, Worcestershire,UK)). The tube was vortexed on high speed for 30 seconds and then sonicated for 30 seconds. The activated beads were then transferred to a labelled 1.5 Eppendorf tube and stored at +4°C in the dark until needed.

2.9.2.2.4 Bead calculation

The bead suspensions were counted by adding 20 µL of bead suspension into the sample introduction port of a Cellometer® disposable cell counting chamber (Nexcelom Bioscience) which was then examined under a light microscope (Nikon, London, UK) with 10x magnification. The

numbers of beads were counted in 4 small squares in a corner square. The total number of beads in each 1 mL was calculated as follows:

No of beads/mL = (no. of beads counted in 4 small squares x 4) $x10^4$

2.9.2.3 Bi-plexed fluorescent bead assay for the quantitation of HSV-1 and HSV-2 IgG in human sera

A standard curve was generated by making two-fold dilutions (from 1/10 to 1/10240) using RUBI-1-94 (NIBSC). Samples and standards were tested in duplicate. The test serum samples, IgG deficient serum (Sigma Aldrich Ltd), controls (QC1 (originated from unknown patient samples with HSV-1 positive), QC2 (originated from unknown patient samples with HSV-2 positive) and QC3 (originated from unknown patients samples with HSV-1 and HSV-2 positive)), were diluted 1 in 100 and 1 in 200 in sample diluent buffer (containing PBS pH 7.3, ($^v/_v$) 0.05% Tween-20 and 2% NBBS (Newborn bovine serum) (Biosera, UK)). As the commercially available recombinant HSV-1 and HSV-2 proteins for this HSV Bi-plexed assay were fused to human superoxide dismutases (SOD), and it might affected the sensitivity and specificity of the assay to determine the HSV subtype and antibody level of the test samples, the commercially available human SOD (Sigma-Aldrich,UK) was used in this assay. The human SOD was added to sample diluent buffer in order to neutralise the anti-SOD antibody in the human test samples to prevent the SOD cross-interferences of test samples and antigens. In order to optimise the amount of SOD used for neutralising the test samples, 5 different SOD concentrations were tested, nil, 0.0025 g/L, 0.005 g/L 0.01 g/mL and 0.1

g/mL, respectively. A volume of 25 µL of each sample (diluted standard, assay control and patient sera) was added in duplicate to each well of a 96-well filter bottom microtitre plate (Merck, Novagen). 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 beads (5000 beads/region/well) was added to each well and the plate covered with aluminium foil. This was then incubated at room temperature on a plate shaker (IKA®-Werke GmbH) at 600 rpm for 45 minutes (±2 minutes). The plate was then washed 3 times with 100 µL of PBS using a vacuum filtration manifold (Merck, Novagen). The detector antibody ("conjugate") - R-Phycoerythrin conjugated Goat anti-Human IgG (Stratech Scientific Ltd) was prepared in PBS with a dilution of 1 in 200 during the washing procedure. After the washing step, 50 µL of conjugate was added to each well; the plate was covered with aluminium foil and incubated at room temperature with shaking at 600 rpm for 30 minutes (±2 minutes). The plate was then washed 3 times with PBS as described above and 100 µL of PBS added to each well. The plate was then shaken at room temperature at 600 rpm for at least 5 minutes before analysis using a Bioplex 200 workstation (Bio-Rad Laboratories Ltd, Hertfordshire, UK). Sample data were analyzed using a 5-parameter logistic curve (5PL) fitting programme using Bioplex Manager Software, Version 4.1.1 (Bio-Rad Laboratories Ltd).

2.9.2.4 Statistical analysis

. Optimisation of the HSV Bi-plexed assay conditions, such as antigen concentration and incubation times for the bead conjugation, were determined based on the parallelism and correlation analysis of the

standard curve. A standard curve generated for each assay was separately compared with rubella and VZV standard curves. The values of correlation coefficients (r) were calculated to determine the variation between each HSV standard curve with rubella and VZV standard curves. The optimisation of serum dilutions and SOD concentration was achieved by comparing the IgG concentration of 3 serum samples for each assay condition. A total of 126 HSV serum samples, were previously tested to determine HSV status using HerpeSelect ELISA and used to determine the cut-off values of HSV Bi-plexed assay. The cut-off point, assay sensitivity and specificity of each assay were calculating using a receiver operator characterisation (ROC) curve. Microsoft Excel 2007 (Microsoft, Seattle, USA) was used for data analysis in this study.

3.0 Results

The results are divided into three main sections. Sections 3.1 to 3.6 describe the expression of HSV-1 glycoprotein G and HSV-2 glycoprotein G using the baculovirus expression system; section 3.7 the development of the HSV Bi-plexed assay; section 3.8 describes the seroepidemiology of measles, mumps, rubella and varicella zoster virus in England using an MMRV multiplex bead immunoassay.

3.1 Virus titration

The titre of the stock preparation of HSV-1, Macintyre strain and HSV-2 clinical isolate KA01, were 10^8 $TCID_{50}$/mL and $10^{6.875}$ $TCID_{50}$/mL, respectively. An example calculation is shown in Table 3.1.

Table 3.1 Titration of HSV-1, Macintyre strain

Virus dilution	ni	ri	Proportion positive (P)	1 - P
10^{-1}	8	8	1	0
10^{-2}	8	8	1	0
10^{-3}	8	8	1	0
10^{-4}	8	8	1	0
10^{-5}	8	8	1	0
10^{-6}	8	8	1	0
10^{-7}	8	4	0.5	0.5
10^{-8}	8	0	0	0
10^{-9}	8	0	0	0
10^{-10}	8	0	0	0
10^{-11}	8	0	0	0

X0 = 6.0; d = 1.0

Log_{10} 50% end-point dilution = (6-0.5) + (1x (8/8 + 4/8))

= 7.0

Infection dose = Log_{10} $TCDI_{50}$ inoculated ($0.1cm^3$) = 7.0 = 10^7 $TCID_{50}$

Titre = TCID50/unit volume, usually 1 mL.

Therefore, the titre of HSV-1, Macintyre strain = $10^8 TCID_{50}$/mL

3.2 Polymerase chain reaction (PCR) for HSV-1 gG and HSV-2 gG

3.2.1 Sequence of HSV-1 gG and HSV-2 gG

A total of 4 published sequences for HSV-1 gG and 2 published sequences for HSV-2 gG were analysed using the BioEdit sequence alignment editor programme. The full length of HSV-1 gG sequence was taken from accession NC-001806 (Table 3.2), containing 716 bp, from the position of 136,744 bp to 137,460 bp, whereas HSV-2 gG sequence was selected from NC-001798 (Table 3.3), containing 2099 bp, from the position of 137,878 bp to 139,977 bp. According to the NCBI BLAST result, both selected sequences for HSV-1 gG and HSV-2 gG were aligned to 20 and 25 published isolate sequences, respectively.

Table 3.2 The complete sequence of HSV-1 gG (NC-001806)

	Sequences
1	atgtcgcagg gcgccatgcg tgccgttgtt cccattatcc cattcgtttt ggttcttgtc
61	ggtgtatcgg gggttcccac caacgtctcc tccaccaccc aaccccaact ccagaccacc
121	ggtcgtccct cgcatgaagc ccccaacatg acccagaccg gcaccaccga ctctcccacc
181	gccatcagcc ttaccacgcc cgaccacaca ccccccatgc caagtatcgg actggaggag
241	gaggaagagg aggaggaggg ggccggggat ggcgaacatc ttgagggggg agatgggacc
301	cgtgacaccc taccccagtc cccgggtcca gccgtcccgt tggccgggga tgacgagaag
361	gacaaaccca accgtcccgt agtcccaccc cccggtccca acaactcccc cgcgcgcccc
421	gagaccagtc gaccgaagac acccccccacc agtatcgggc cgctggcaac tcgacccacg
481	acccaactcc ccccaaaggg gcgacccttg gttccgacgc ctcaacatac cccgctgttc
541	tcgttcctca ctgcctcccc cgccctggac accctcttcg tcgtcagcac cgtcatccac
601	accttatcgt ttttgtgtat tgttgcgatg gcgacacacc tgtgtggcgg ttggtccaga
661	cgcgggcgac gcacacaccc tagcgtgcgt tacgtgtgcc tgccgtccga acgcgggtag

Table 3.3 The complete sequence of HSV-2 gG (NC-001798)

	Sequences
1	atgcacgcca tcgctcccag gttgcttctt ctttttgttc tttctggtct tccggggaca
61	cgcggcgggt cgggtgtccc cggaccaatt aatccccccca acaacgatgt tgttttcccg
121	ggaggttccc ccgtggctca atattgttat gcctatcccc ggttggacga tcccgggccc
181	ttgggttccg cggacgccgg gcggcaagac ctgccccggc gcgtcgtccg tcacgagccc
241	ctgggccgct cgttcctcac ggggggggctg gttttgctgg cgccgccggt acgcggattt
301	ggcgcaccca acgcaacgta tgcggcccgt gtgacgtact accggctcac ccgcgcctgc
361	cgtcagccca tcctccttcg gcagtatgga gggtgtcgcg gcggcgagcc gccgtcccca
421	aagacgtgcg ggtcgtacac gtacacgtac cagggcggcg ggcctccgac ccggtacgct
481	ctcgtaaatg cttccctgct ggtgccgatc tgggaccgcg ccgcggagac attcgagtac
541	cagatcgaac tcggcggcga gctgcacgtg ggtctgttgt gggtagaggt gggcggggag
601	ggccccggcc ccaccgcccc cccacaggcg gcgcgtgcgg agggcggccc gtgcgtcccc
661	ccggtccccg cgggccgccc gtggcgctcg gtgcccccgg tatggtattc cgcccccaac
721	cccgggtttc gtggcctgcg tttccgggag cgctgtctgc ccccacagac gcccgccgcc
781	cccagcgacc taccacgcgt cgcttttgct ccccagagcc tgctggtggg gattacgggc
841	cgcacgttta ttcggatggc acgacccacg gaagacgtcg gggtcctgcc accccattgg
901	gccccgggg ccctagatga cggtccgtac gcccccttcc caccccgccc gcggtttcga
961	cgcgccctgc ggacagaccc cgagggggtc gaccccgacg ttcgggcccc cctaaccggg
1021	cggcgcctca tggccttgac cgaggacgcg tcctccgatt cgcctacgtc cgctccggag
1081	aagacgcccc tccctgtgtc ggccaccgcc atggcgccct cagtcgaccc aagcgcggaa
1141	ccgaccgccc ccgcaaccac tactcccccc gacgagatgg ccacacaagc cgcaacggtc
1201	gccgttacgc cggaggaaac ggcagtcgcc tccccgcccg cgactgcatc cgtggagtcg
1261	tcgccactcc ccgccgcggc ggcaacgccc ggggccgggc acacgaacac cagcagcgcc
1321	cccgcagcga aaacgccccc caccacacca gcccccacga ccccccccgcc cacgtctacc
1381	cacgcgaccc cccgccccac gactccgggg ccccaaacaa cccctcccgg acccgcaacc
1441	ccgggtccgg tgggcgcctc cgccgcaccc acggccgatt ccccccctcac cgcctcgccc
1501	cccgctaccg cgccggggcc ctcggccgcc aacgtttcgg tcgccgcgac caccgccacg
1561	cccggaaccc ggggcaccgc ccgtaccccc ccaacggacc caaagacgca cccacacgga
1621	cccgcggacg ctccccccgg ctcgccagcc cccccacccc ccgaacatcg cggcggaccc
1681	gaggagtttg agggcgccgg ggacggcgaa ccccccgatg acgacgacag cgccaccggc
1741	ctcgccttcc gaactccgaa ccccaacaaa ccacccccccg cgcgccccgg gcccatccgc
1801	cccacgctcc cgccaggaat tcttgggccg ctcgccccca acacgcctcg ccccccccgcc
1861	caagctcccg ctaaggacat gccctcgggc cccacacccc aacacatccc cctgttctgg
1921	ttcctaacgg cctcccctgc tctagatatc ctctttatca tcagcaccac catccacacg
1981	gcggcgttcg tttgtctggt cgccttggca gcacaacttt ggcgcggccg ggcggggcgc
2041	aggcgatacg cgcacccgag cgtgcgttac gtatgtctgc cacccgagcg ggattag

3.2.2 Primer design

In order to express the target proteins, primer sets were designed to amplify the complete sequences from both viral genomes. For the truncated version of both viruses primer sets were designed based on the primers published by Ikoma *et al.* (2002). The specific vector-compatible overhang sequences for both truncated primer sets were replaced by 3'C/LIC vector specific sequences. The forward primer included the start codon (ATG) at the 5', whereas the reverse primer included the stop codon (CTA) at 5' to terminate protein transcription. The specific vector-compatible overhang tails (underlined; Table 3.4, 3.5, 3.6 and 3.7) were added to both 5' ends of the forward and reverse primer, respectively, for the directional cloning of PCR products in the 3'C/LIC vector. In addition, a total of 9 extra virus non-encoding sequences (highlighted; Table 3.4) were added to HSV-1 gG reverse primer, in order to avoid primer-dimer formation (Brownie *et al.,* 1997). According to the NCBI BLAST alignment, the primer sets for both viruses showed 100% homology to all published HSV genomes (HSV-1 gG and HSV-2 gG).

Table 3.4 The primer set used to amplify full sequences of HSV-1 gG

Primer	Primer sequence	Length	Product size (bp)
HSV-1 gGF	5'-CAGGGACCCGGTATGTCGCAGGGC-3'	24bp	752
HSV-1 gGR	5'-GGCACCAGAGCGTTCCCCATACCCTACCC-3'	29bp	

Table 3.5 The primer set used to amplify full sequences of HSV-2 gG

Primer	Primer sequence	Length	Product size (bp)
HSV-2 gGF	5'-CAGGGACCCGGTATGCACGCCATCGCTCCCAGGTTG-3'	36bp	2123
HSV-2 gGR	5'-GGCACCAGAGCGTTCTAATCCCGCTCGGGTGGCAGA-3'	36bp	

Table 3.6 The primer set used to amplify truncated sequences of HSV-1 gG

Primer	Primer sequence	Length	Product size (bp)
HSV-1 tgGF	5'- CAGGGACCCGGTATGTCGCCGGGC-3'	24bp	537
HSV-1 tgGR	5'- GGCACCAGAGCGTTCTACAGCGGGGTATGT-3'	30bp	

Table 3.7 The primer set used to amplify truncated sequences of HSV-2 gG

Primer	Primer sequence	Length	Product size (bp)
HSV-1 tgGF	5'- CAGGGACCCGGTATGAGCCTGCTGGTG-3'	27bp	1278
HSV-1 tgGR	5'-GGCACCAGAGCGTTCTATTTGTTCGGGTTCGG-3'	32bp	

3.2.3 Amplification of HSV-1 gG and truncated HSV-1 gG

The amplification of HSV-1 gG and truncated HSV-1 gG were carried out using AmpliTaq Gold 360 master mix with touchdown-PCR conditions. Non-specific bands (Figures 3.1 and Figures 3.2) were observed, even with modifications in PCR reaction conditions such as, increasing the annealing time and extension time from 1 minute to 2 minutes per cycle, respectively; increasing the annealing temperature from 58^0C to 60^0C per cycle; and adding a GC enhancer in the PCR reaction mixture from 5 µL to 10 µL (Appendix 1). Although non-specific bands

appeared in the sample lanes, a clear band of the expected size was obtained for HSV-1 gG and truncated HSV-1 gG, with 752 bp and 537 bp, respectively. Following this, PCR products were purified as described in section 2.5.

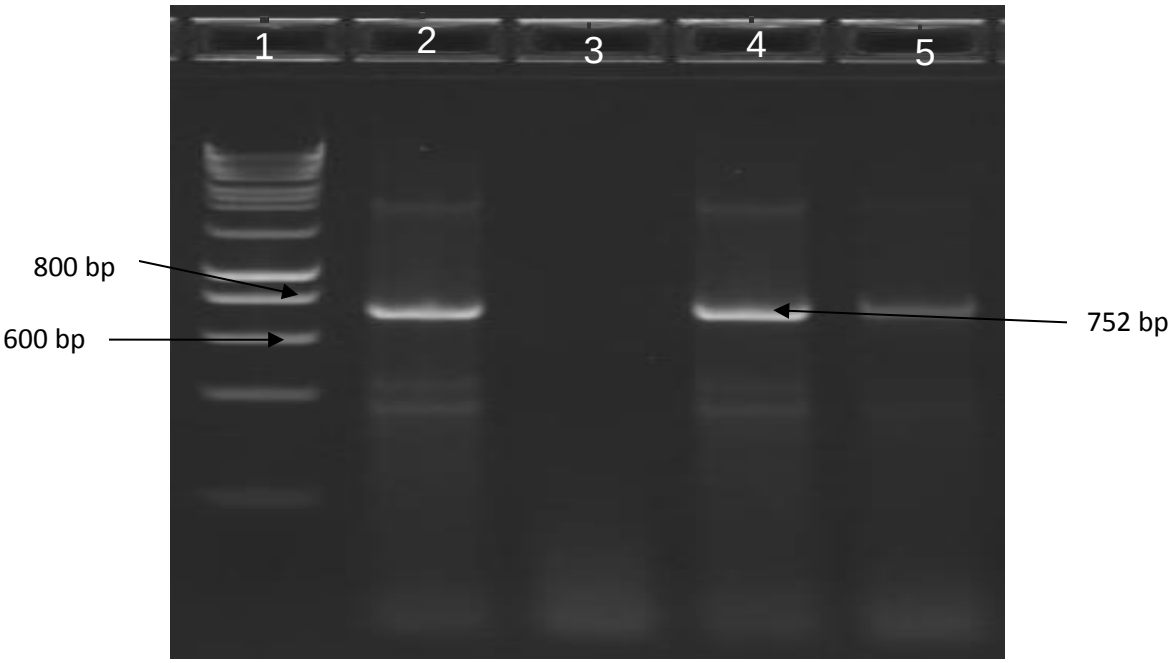


Figure 3.1: HSV-1 gG amplification using AmpliTaq Gold 360 master mix. Lane 1 contained HyperLadder™ I DNA ladder, ranging from 200 to 10,037 bp. Lane 2, 4 and 5 were PCR products with different dilutions (10^{-1} for Lanes 2 and 4; 10^{-2} dilution for Lane 5). The negative control was in Lane 3.

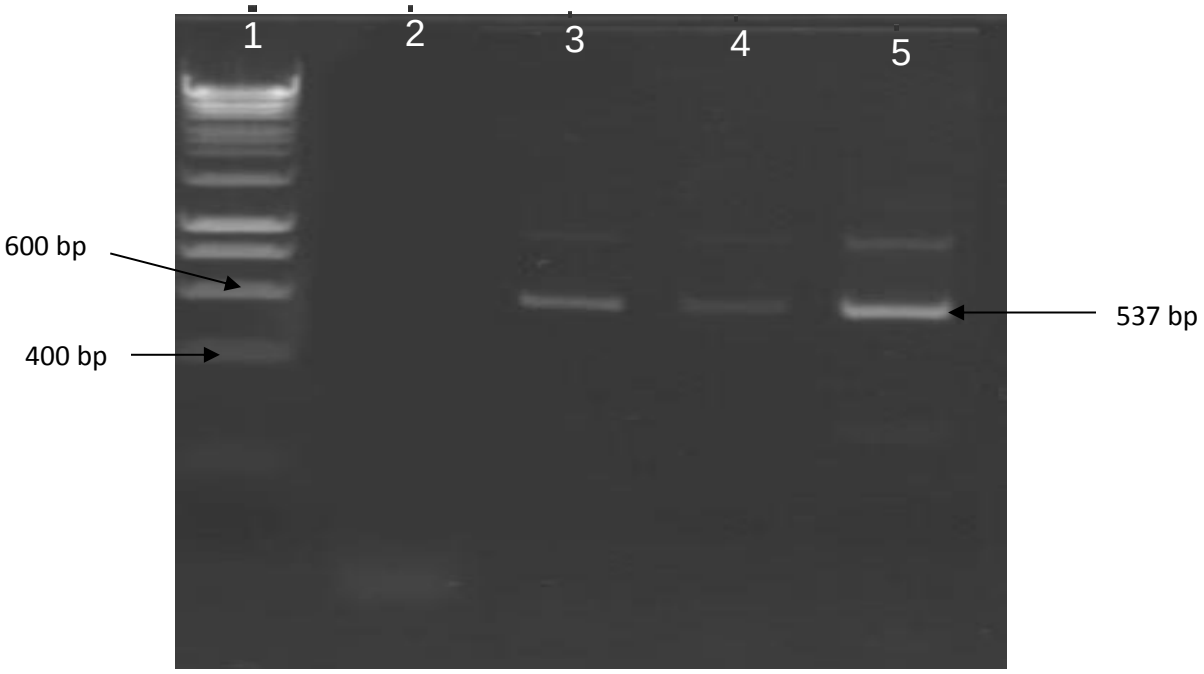


Figure 3.2: Truncated HSV-1 gG amplification using AmpliTaq Gold 360 master mix. Lane 1 contained HyperLadder™ I DNA ladder, ranging from 200 to 10,037 bp. Lanes 3, 4 and 5 were PCR products with different dilutions (10^{-1} for Lane 5; 10^{-2} dilution for Lanes 3 and 4). The negative control was in Lane 2.

3.2.4 Amplification of HSV-2 gG and truncated HSV-2 gG

Amplification of HSV-2 gG and truncated HSV-2 gG was performed using AmpliTaq gold 360 master mix and PCR parameters as described in section 2.4.4. As with HSV-1 gG amplification, a DNA amplicon together with non-specific bands were observed on the amplification of HSV-2 gG (Figure 3.3) and truncated HSV-2 gG (Figure 3.4), with the expected size, 2123 bp and 1278 bp, respectively. Modification of the PCR reaction was attempted by increasing the annealing temperature; adding the GC enhancer in the PCR reaction mixture; changing the time for annealing and extension from 1 minute to 30 seconds per cycle (Appendix 2), respectively. However, none of these modifications excluded the non-specific bands from the desired DNA amplicon. Therefore, to try to eliminate non-specific bands, the desired DNA amplicons were excised and purified as described in section 2.5.

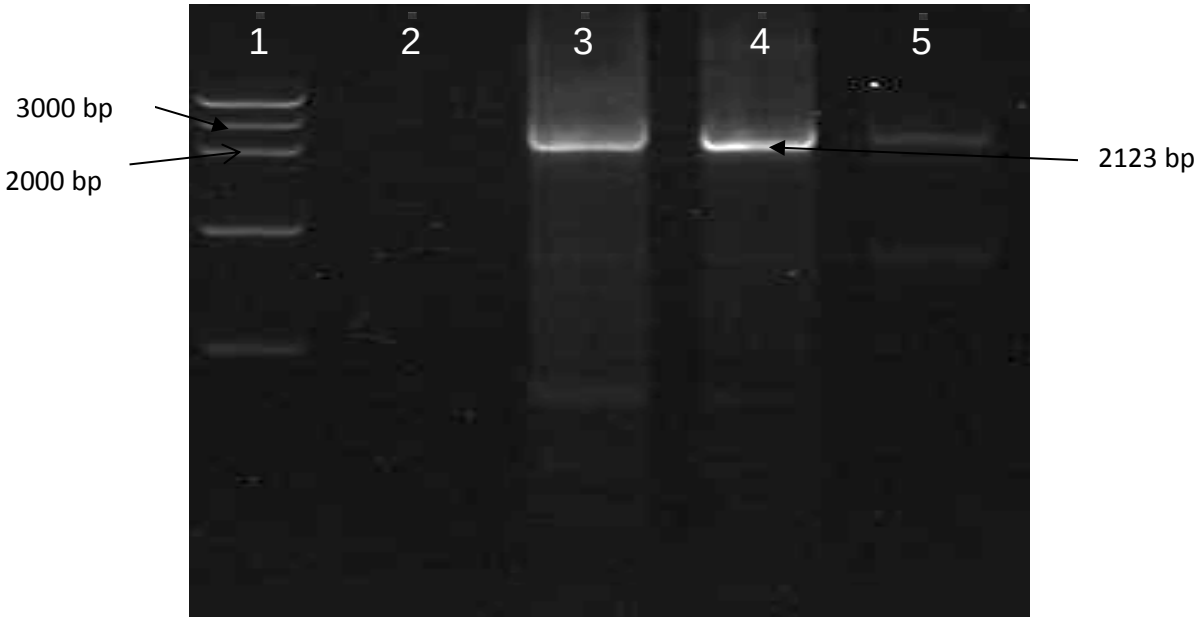


Figure 3.3: HSV-2 gG amplification using AmpliTaq Gold 360 polymerase master mix. Lane 1 contained EasyLadder II, ranging from 500 to 5000 bp. Lanes 3, 4 and 5 contained PCR products with different dilutions (Neat for Lane 3; 10^{-1} dilution for Lanes 4; 10^{-2} dilution for Lane 5). The negative control was in Lane 2.

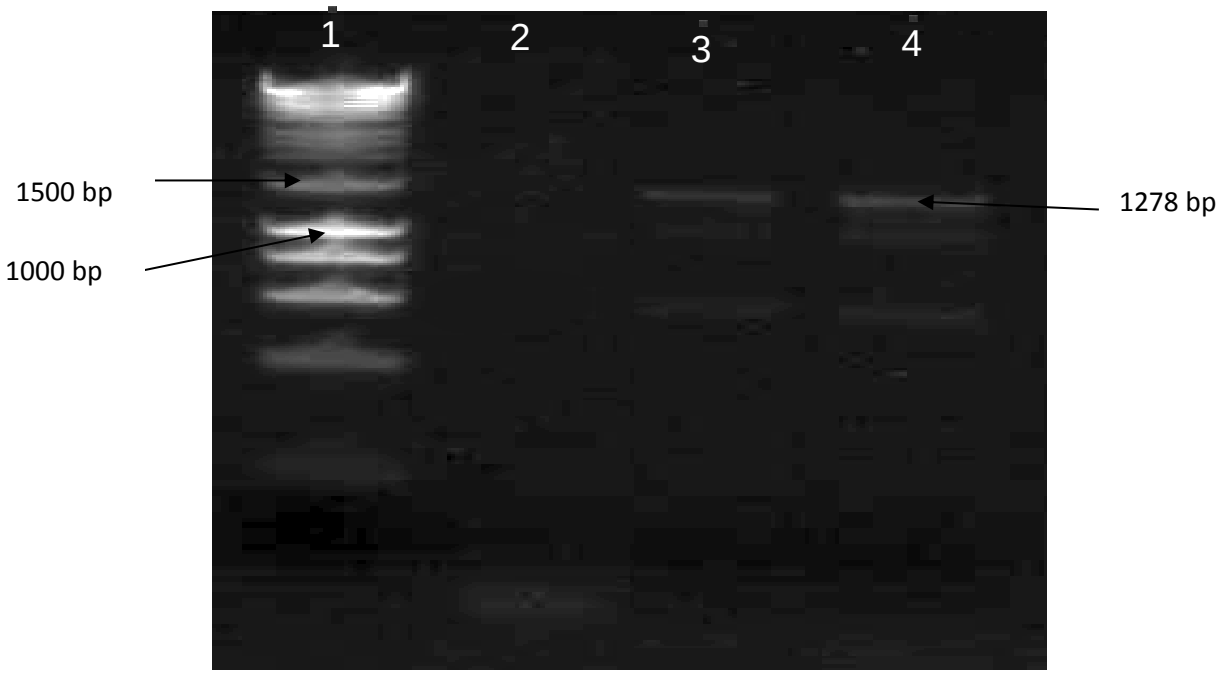


Figure 3.4: Truncated HSV-2 gG amplification using AmpliTaq Gold 360 polymerase master mix. Lane 1 contained HyperLadder™ I DNA ladder, ranging from 200 to 10,037 bp. Lanes 3 and 4 neat PCR products. The negative control was in Lane 2.

3.2.5 DNA purification

After DNA amplification, the DNA products were excised from a gel and purified using the QIAquick gel extraction kit. The purified products were then further electrophoresed using 2% E-gel. As shown in Figures 3.5 and 3.6, a single band for both HSV-1 gG and HSV-2 gG, with the expected sizes, 752 bp and 2123 bp, respectively, were obtained. For the truncated HSV-1 gG and truncated HSV-2 gG, a single band was observed, with the expected size, 537 bp (Figure 3.7) and 1278 bp (Figure 3.8), respectively. These PCR products were then used for recombinant plasmid construction.

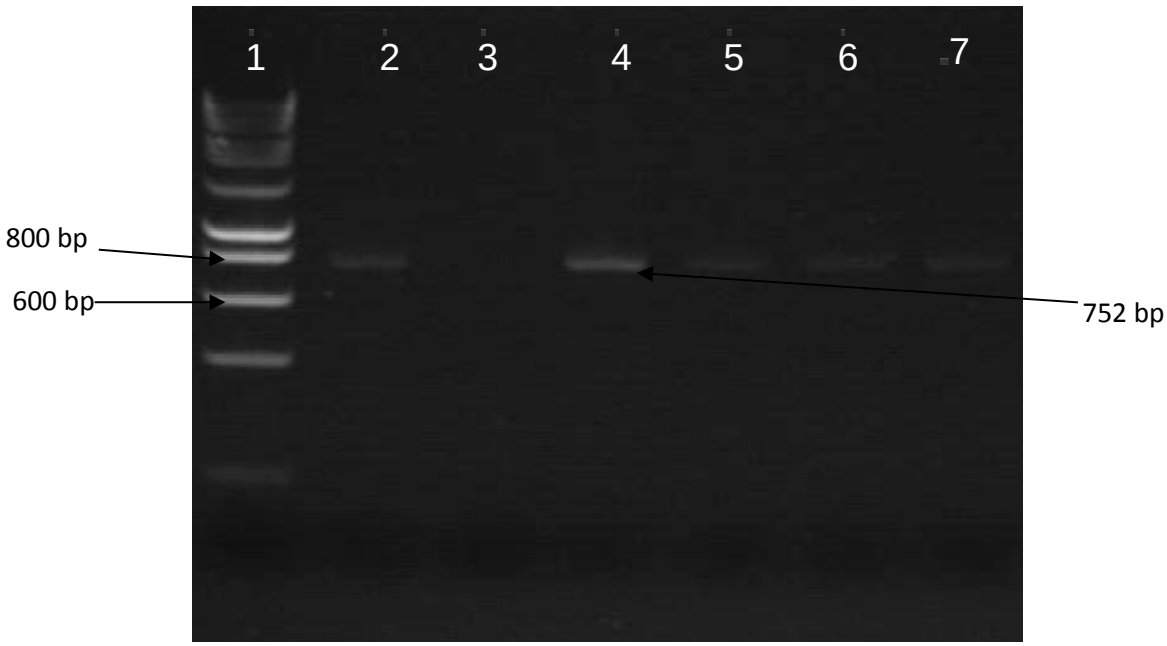


Figure 3.5: DNA purification of HSV-1 gG. A band with the expected size (2,123 bp) was visibled in Lanes 2, 4, 5, 6 and 7. Lane 1 contained HyperLadder™ I DNA ladder. Lane 3 the negative control.

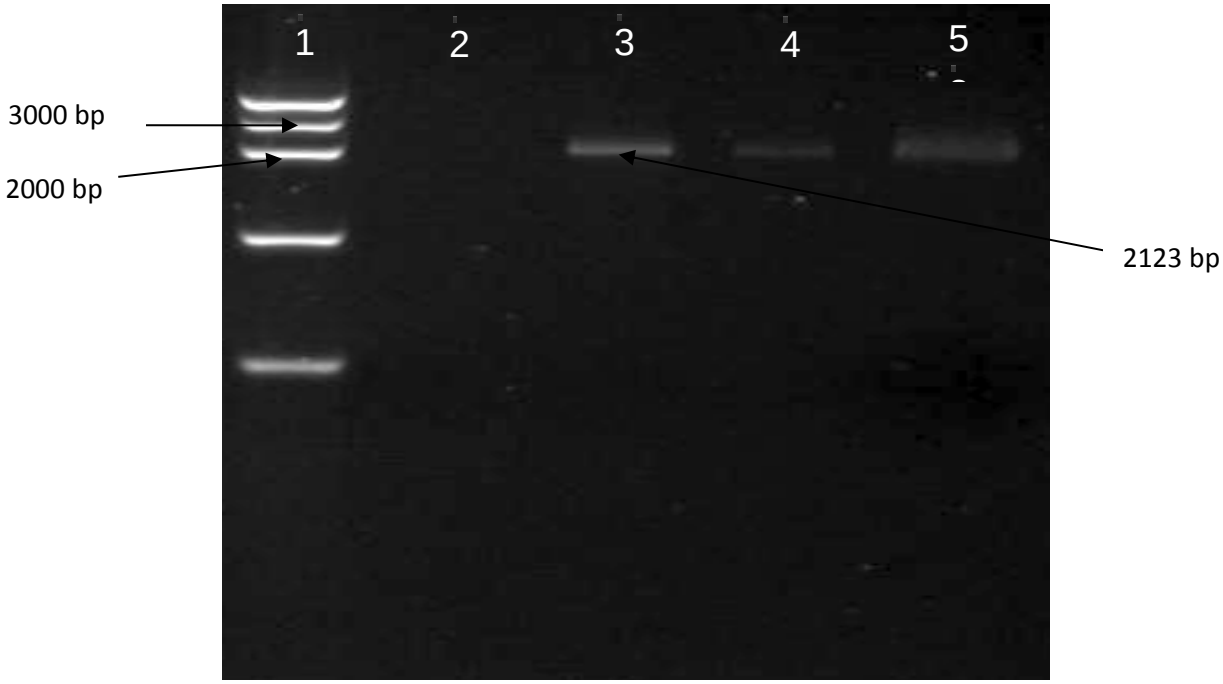


Figure 3.6: DNA purification of HSV-2 gG. Lane 1 contained EasyLadder II, Lanes 3, 4 and 5 the PCR products with the expected size (2,123 bp). Lane 2 the negative control.

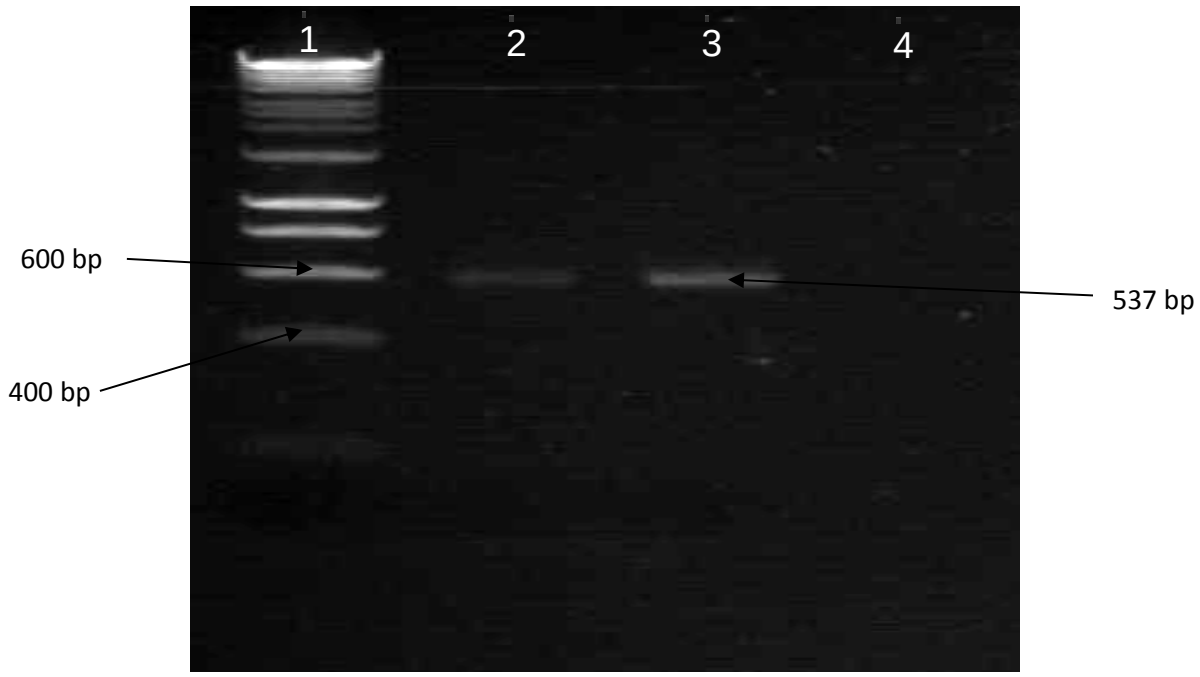


Figure 3.7: DNA purification of truncated HSV-1 gG. Lane 1 contained HyperLadder™ I DNA ladder, Lanes 2 and 3 the PCR products with the expected size (537 bp). Lane 4 the negative control.

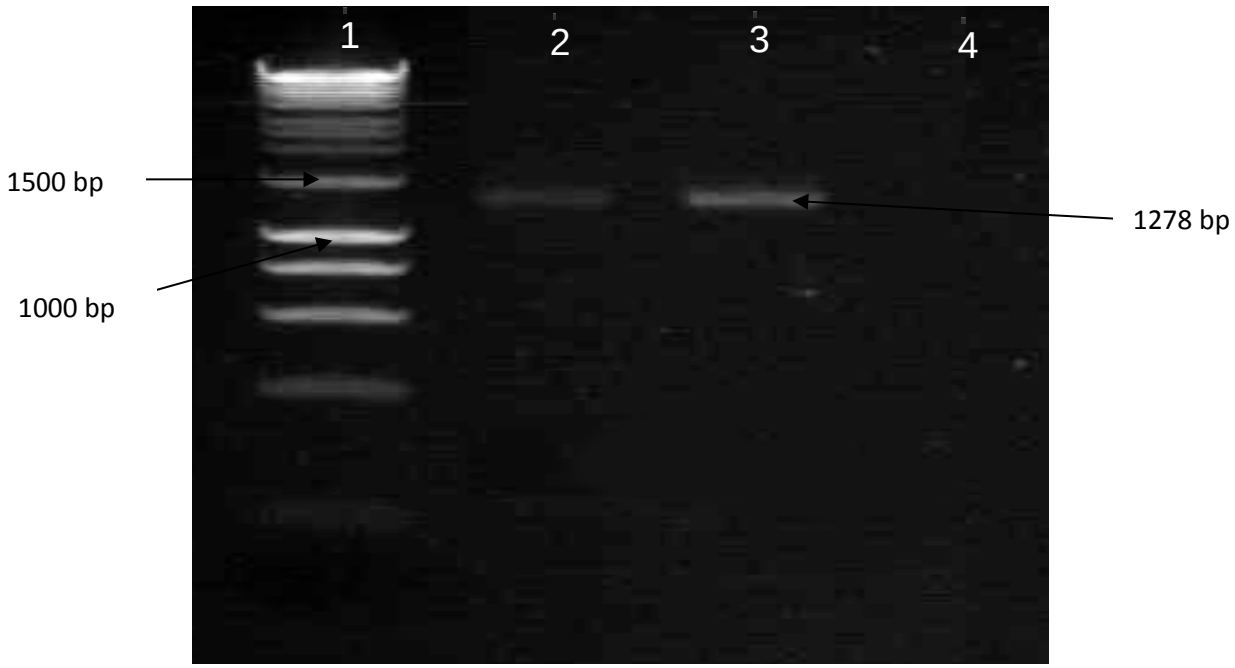


Figure 3.8: DNA purification of truncated HSV-2 gG. Lane 1 contained HyperLadder™ I DNA ladder, Lanes 2 and 3 the PCR products with the expected size (1278 bp). Lane 4 the negative control.

3.3 Recombinant Plasmid construction

3.3.1 Gene cloning strategy

Gene cloning was performed using ligation-independent cloning (LIC) that included 3C/LIC preparation and pIEx/Bac-3 3C/LIC (Figure 3.9) plasmid vector insertion, see section 2.6. Following this, the recombinant plasmids were generated using the InsectDirect vector expression system as described in section 2.7.

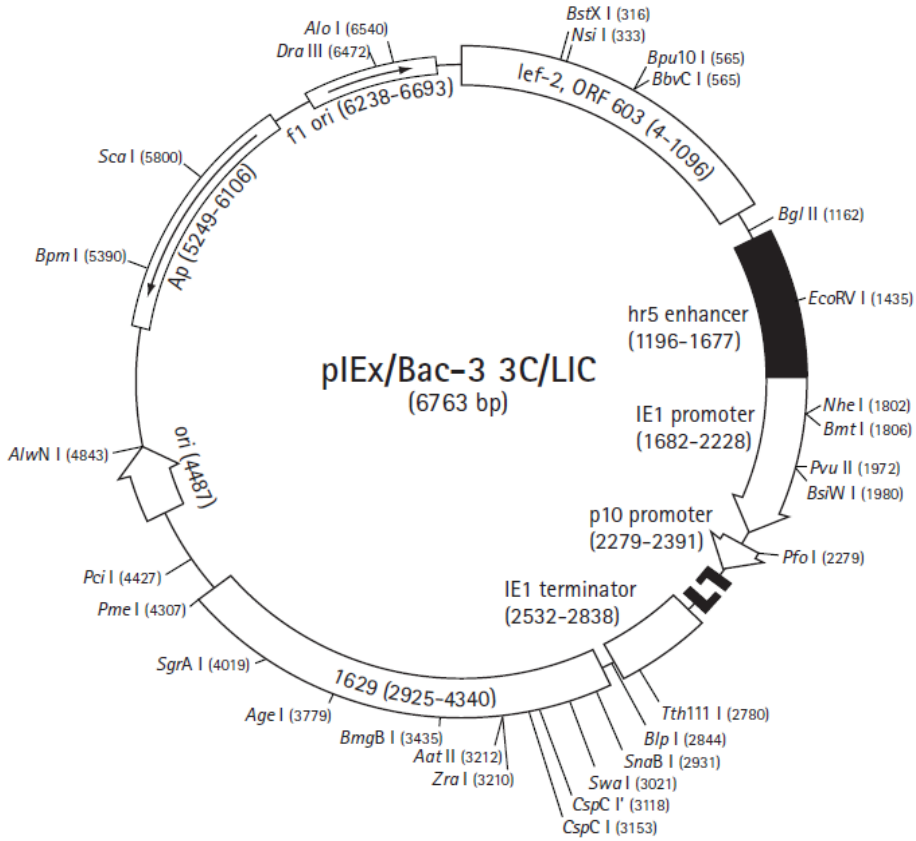


Figure 3.9: Circular map for pIEx/BAC-3 3C/LIC plasmid vector, containing 6763 bp. Taken from: http://www.merckmillipore.co.uk/life-science-research/vector-table-novagen-piex-bac vectortable/c_ru2b.s1O2BgAAAEh06cLdcae? PortalCatalogID =merck4biosciences&CountryName=United+Kingdom (Accessed 16 October 2012)

3.3.2 Recombinant Plasmid construction

The construction of recombinant plasmid for each virus was commenced by treating the purified PCR product with the 3C/LIC insect kit. The presence of LIC-qualified T4 DNA polymerase and dATP in the mixture generated the vector compatible overhangs (3C/LIC insert), which were located at both ends of the target gene (Figure 3.10). The 3C/LIC insert tails were then annealed to the 3C/LIC cloning site at the pIEx/BAC-3 3C/LIC vector to form the recombinant plasmid. The recombinant plasmids were then stored at -20^{0}C until required for the next step, colony screening.

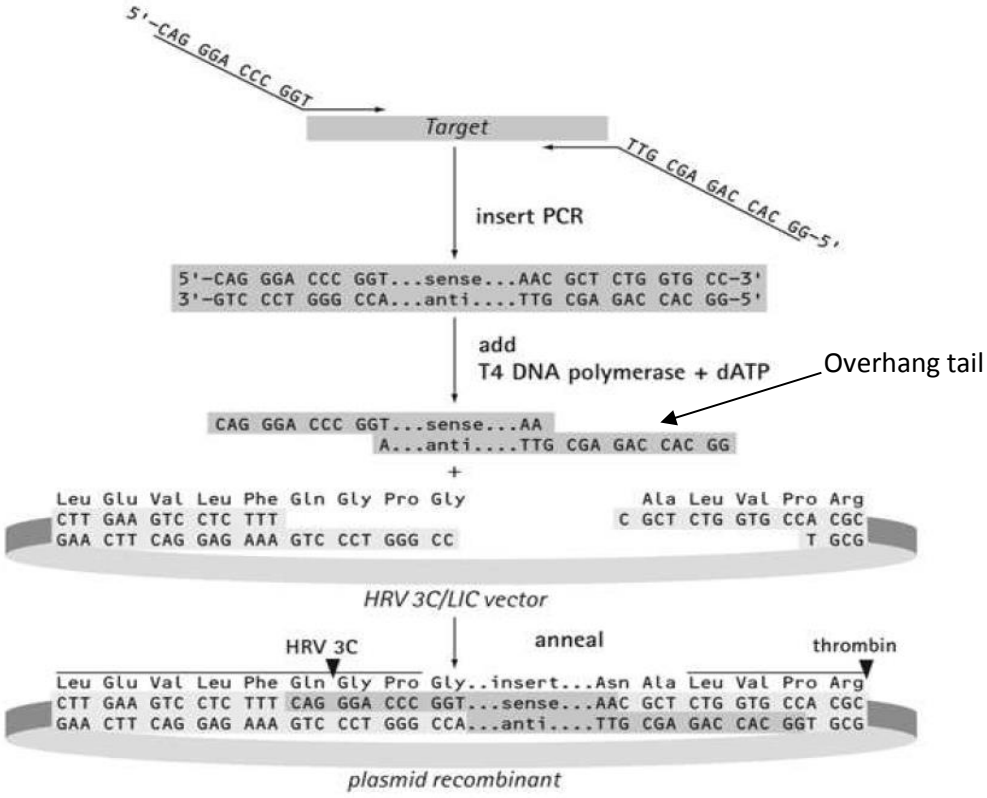


Figure 3.10: 3C/LIC cloning strategy. Taken from: http://www.merckmillipore.co.uk/life-science-research/vector-table-novagen-piex-bac-vector table/cru2b.s1O2BgAAAEh06 cLdcae?PortalCatalogID =merck4biosciences& CountryName=United+Kingdom (Accessed 16 October 2012)

3.3.3 Colony Screening

In order to reduce the presence of PCR inhibitor and to determine the correct plasmid construction, a cell transformation step was used prior to PCR screening of recombinant plasmid (Figure 3.11). During cell transformation, the recombinant plasmid acts as a 'donor plasmid' to enter a GigaSingles competent bacterial cell, *Escherichia coli* using a calcium chloride transformation method and followed by manipulation of target genes into an *E. coli* plasmid to form a DNA plasmid, as described in section 2.7.3. The DNA plasmids were then harvested and screened using PCR with virus-specific primers or vector specific primers. The virus-specific primer sets were designed to amplify the sequences of the viral glycoprotein G gene with the extra vector-compatible overhang tails added to both 5' ends of the forward and reverse primer; vector-specific primer sets were designed to amplify the sequence of viral glycoprotein G gene with the extra IEI promoter and IEI terminator sequences from the plasmid vector.

The images of PCR products showed the HSV-1 gG recombinant plasmid had been successfully constructed, with a band of 752 bp (Figure 3.12) using the virus specific primer set and a band of 1152 bp (Figure 3.13) using the vector specific primer set, respectively. As with PCR screening for truncated HSV-1 gG and truncated HSV-2 gG plasmids, the expected bands were observed, with a band of 537 bp (Figure 3.14) and 1278 bp (Figure 3.15), respectively for the virus specific primer set; a band of 937 bp (Figure 3.14) and 1678 bp (Figure 3.15), respectively for vector specific primer set. Neither the HSV-2 glycoprotein G primer set (Figure 3.12) nor the vector specific primer set (Figure 3.13) showed the expected

band on the E-gel images for the PCR screening of HSV-2 gG, suggesting that the HSV-2 recombinant plasmid was not successfully constructed. All recombinant plasmids except for the HSV-2 gG recombinant plasmid, were then used for insect cell transfection.

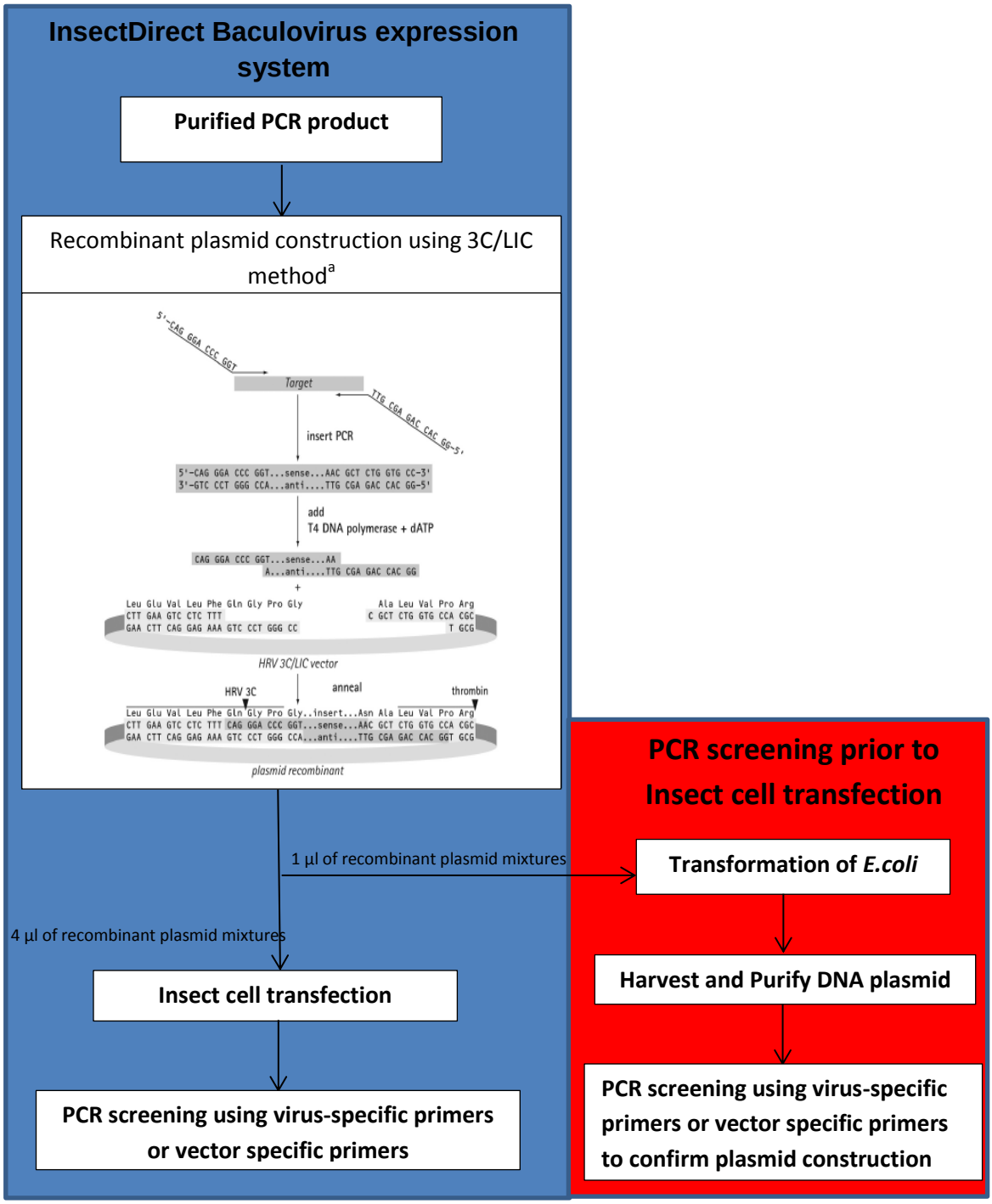


Figure 3.11: Experimental outline for verification of HSV-1 gG and HSV-2 gG recombinant plasmid construction.
[a] Diagram of the 3C/LIC strategy. After amplification with primers that included the 5'LIC extension, the PCR insert was treated with LIC-qualified T4 DNA polymerase (with the presence of dATP), annealed to the 3C/LIC vector, and the resultant nicked, circular plasmid was constructed.

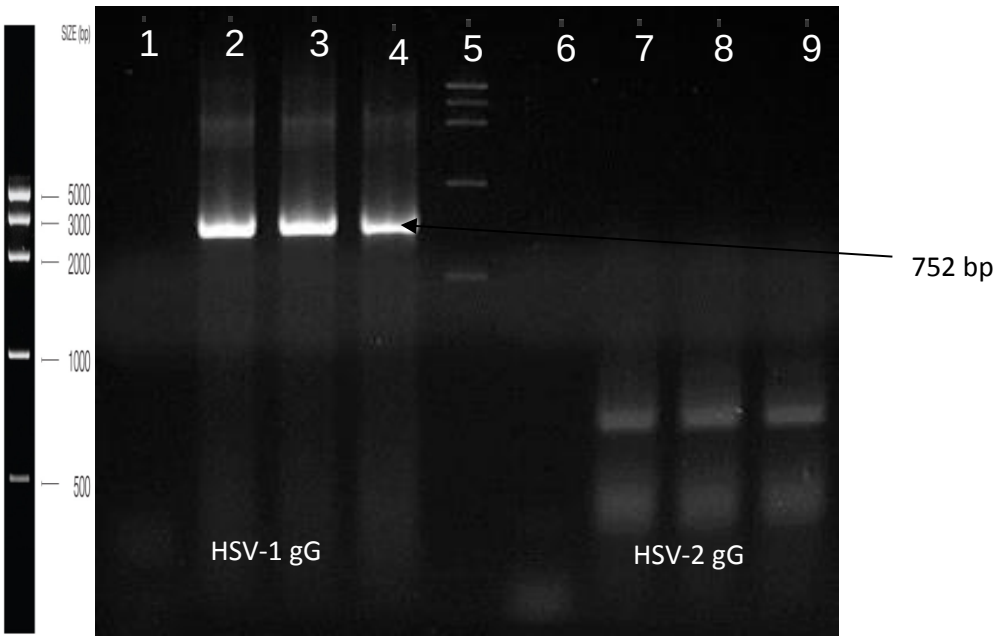


Figure 3.12: HSV-1 gG and HSV-2 gG PCR screening using HSV-1 gG specific primers or HSV-2 gG specific primers after cell transformation. Lane 5 contained EasyLadder II, Lanes 2, 3 and 4 the HSV-1 gG PCR products; Lanes 7, 8 and 9 the HSV-2 gG PCR products. Lanes 1 and 6 the negative control.

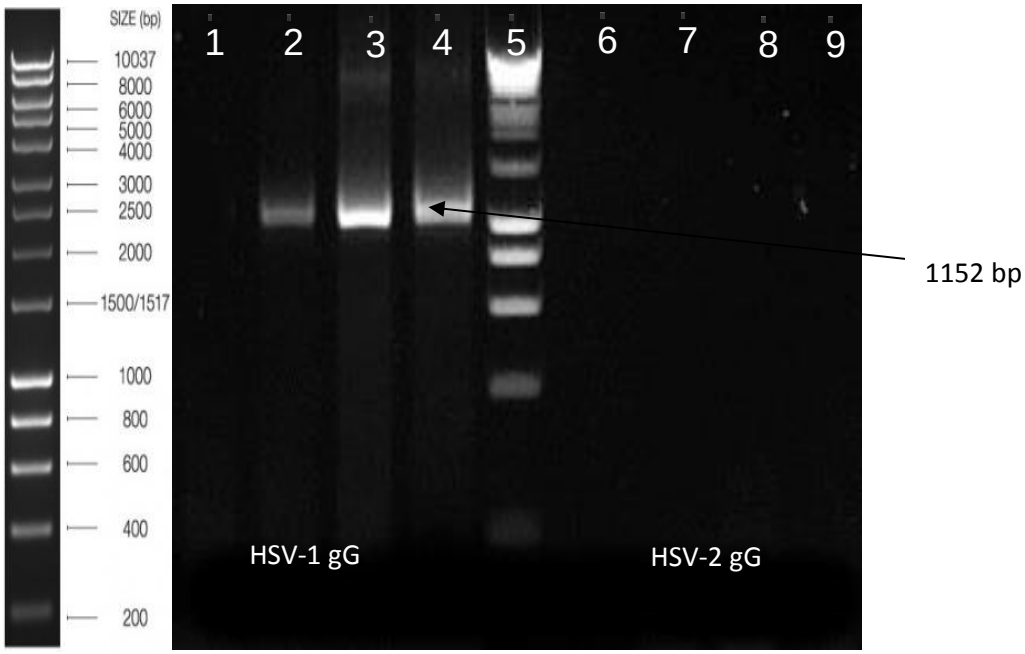


Figure 3.13: HSV-1 gG and HSV-2 gG PCR screening using vector-specific primers set after cell transformation. Lane 5 contained HyperLadder I, lanes 2, 3 and 4 showed the HSV-1 gG PCR products; Lanes 7, 8 and 9 the HSV-2 gG PCR products. Lanes 1 and 6 the negative control.

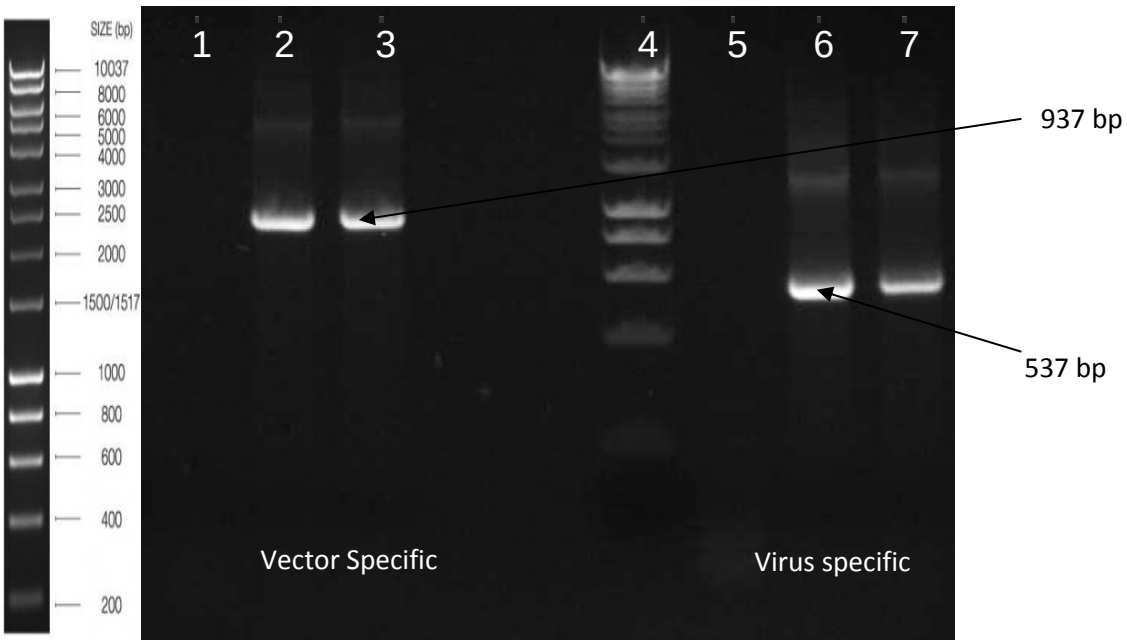


Figure 3.14: Truncated HSV-1 gG PCR screening using HSV-1 gG specific primers or vector specific primers after cell transformation. Lane 4 contained HyperLadder I, Lanes 2 and 3 the PCR products amplified using vector specific primer; Lanes 6 and 7 the PCR products amplified using virus specific primer. Lanes 1 and 5 the negative control.

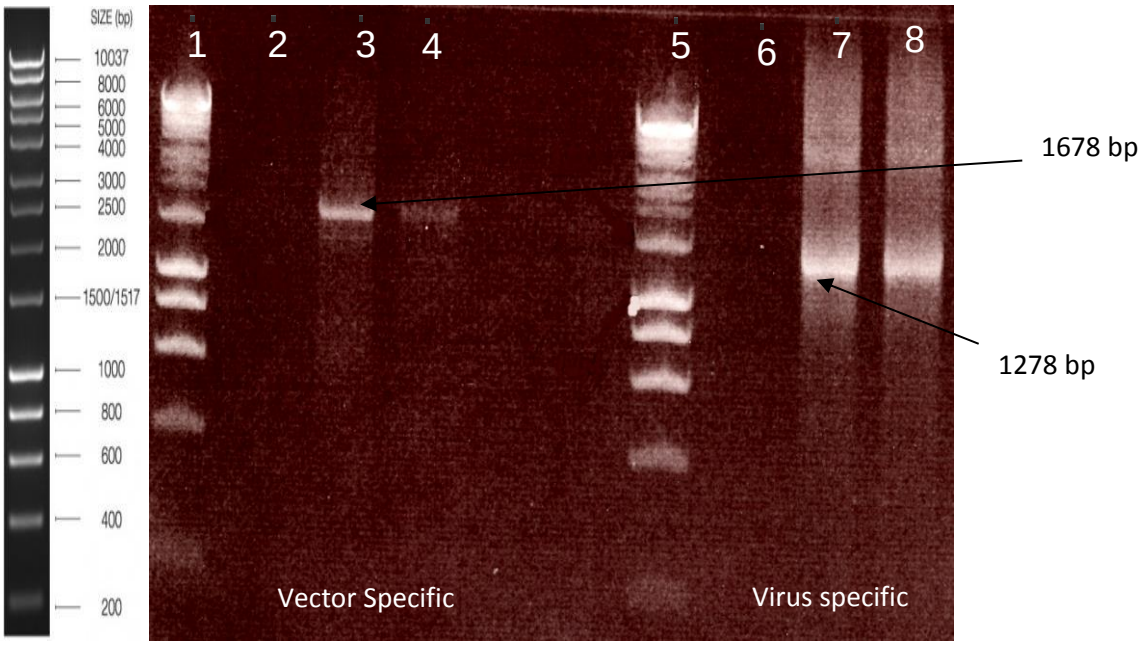


Figure 3.15: Truncated HSV-2 gG PCR screening using HSV-2 gG specific primers or vector specific primers after cell transformation. Lane 1 and 5 contained HyperLadder I, Lanes 3 and 4 the PCR products amplified using vector specific primers; Lanes 7 and 8 the PCR products amplified using virus specific primers. Lanes 2 and 6 the negative controls.

3.4 Transfection of Insect cells

The appearance of insect cells at 90 – 95% confluence is shown in Figure 3.16. The cells appeared rounded and bright with distinct cell boundaries. The recombinant plasmid with the gene of interest was added to the transfection mixture that contained Insect GeneJuice and BacVector Insect cell medium. The mixture was then added dropwise to the centre of a 25-cm^2 flask, which was seeded with 2 mL of Sf9 cells with cell density of $1x10^6$ cells/mL. All the transfected cells were then incubated at 28^0C and the signs of infection were sought daily, under an inverted microscope. Early sign of infection of Sf9 cells was observed after 48-hour incubation. Transfected cells increased their size by 40-50% as compared to non-infected cells (Figure 3.17A). Cell enlargement and vesicular appearances were frequently observed after 72-hour incubation (Figure 3.17B). The late signs of infection appeared after 5 days when transfected cells started to lyse and detach from the flask (Figure 3.17C). The transfected cells were then harvested and stored at -20^0C until required.

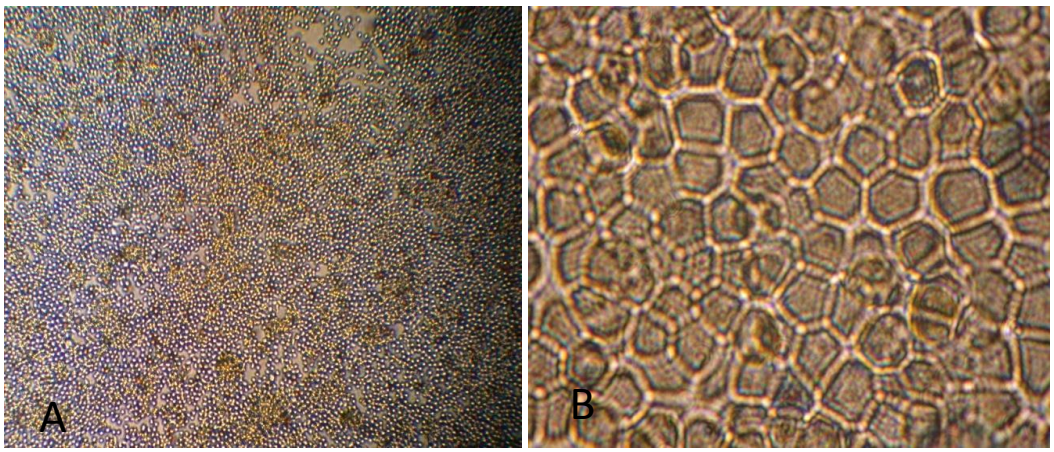


Figure 3.16: The images show 90-95% confluent monolayer Sf9 cells in a 75-cm^2 flask at a cell density of 1 x 10^6 cells/mL. The images are obtained using 10x (panel A) and 100x (panel B) objectives after 3 days incubation at 28^0C.

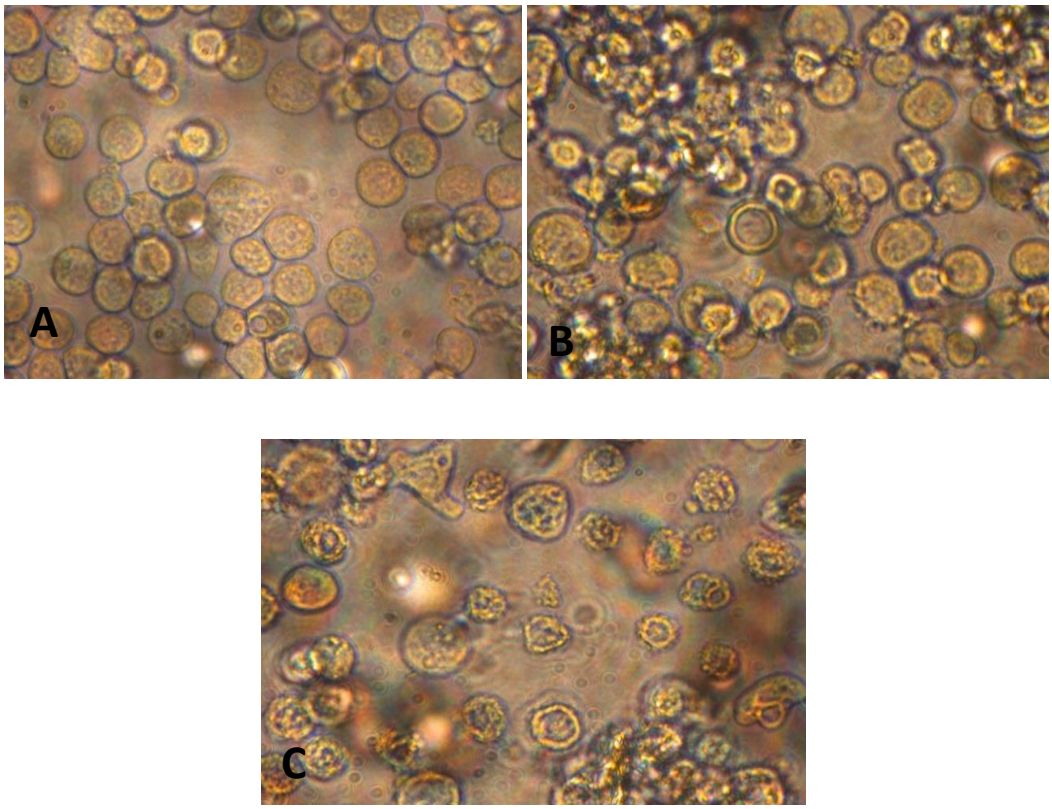


Figure 3.17: Transfection of sf9 insect cells in a 25-cm^2 flask at a cell density of 1 x 10^6 cells/mL. Panel A shows the transfected Sf9 cells are began to increase cell diameter after 48-hour incubation. Some transfected cells started to vesiculate (B) after 72 hour incubation and then cell lysis (C) after 5 days incubation.

3.5 Recombinant plasmid screening

To confirm the presence of the expressed glycoprotein after cell transfection, the DNA extracted samples were amplified using virus specific primers, as described in section 2.15. The expected band size 752 bp (Figure 3.18) was observed for HSV-1 gG extracted samples; whereas for truncated HSV-1 gG and truncated HSV-2 gG extracted samples, the expected band sizes of 537 bp (Figure 3.19) and 1278 bp (Figure 3.20) were observed, respectively.

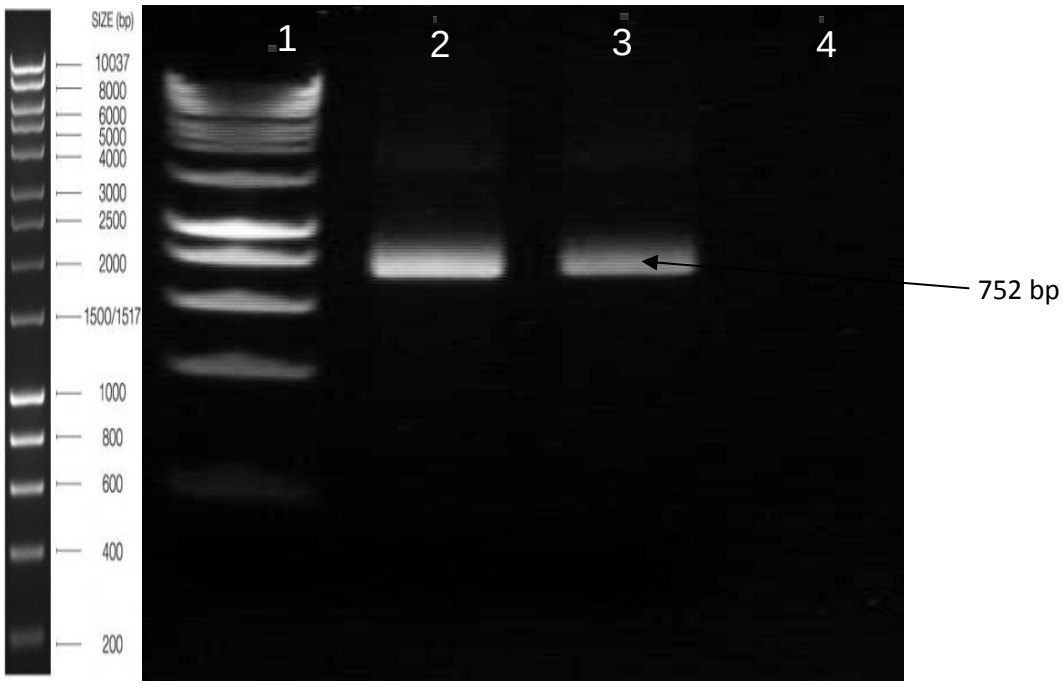


Figure 3.18: Recombinant baculovirus screening for HSV-1 gG using virus specific primer. Lane 1 DNA marker represented (HyperLadder I), Lanes 2 and 3 the HSV-1 gG PCR product with the size of 752 bp. The negative control was in Lane 4.

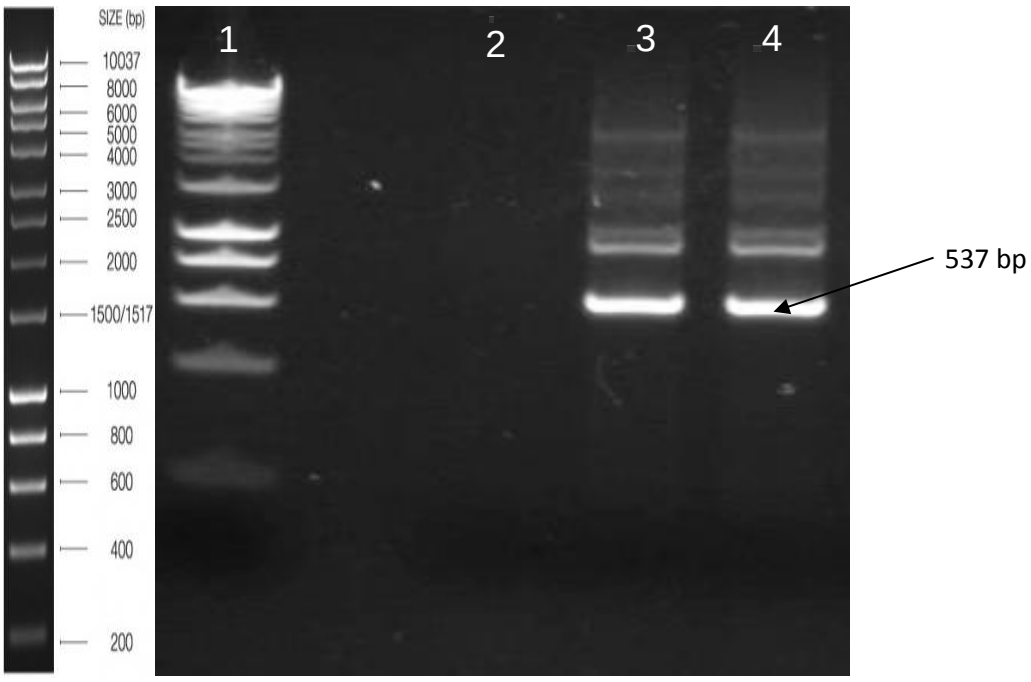


Figure 3.19: Recombinant baculovirus screening for truncated HSV-1 gG using virus specific primer. Lane 1 represented DNA marker (HyperLadder I), Lanes 3 and 4 truncated HSV-1 gG expressed protein samples with the size of 537 bp. The negative control was in Lane 2.

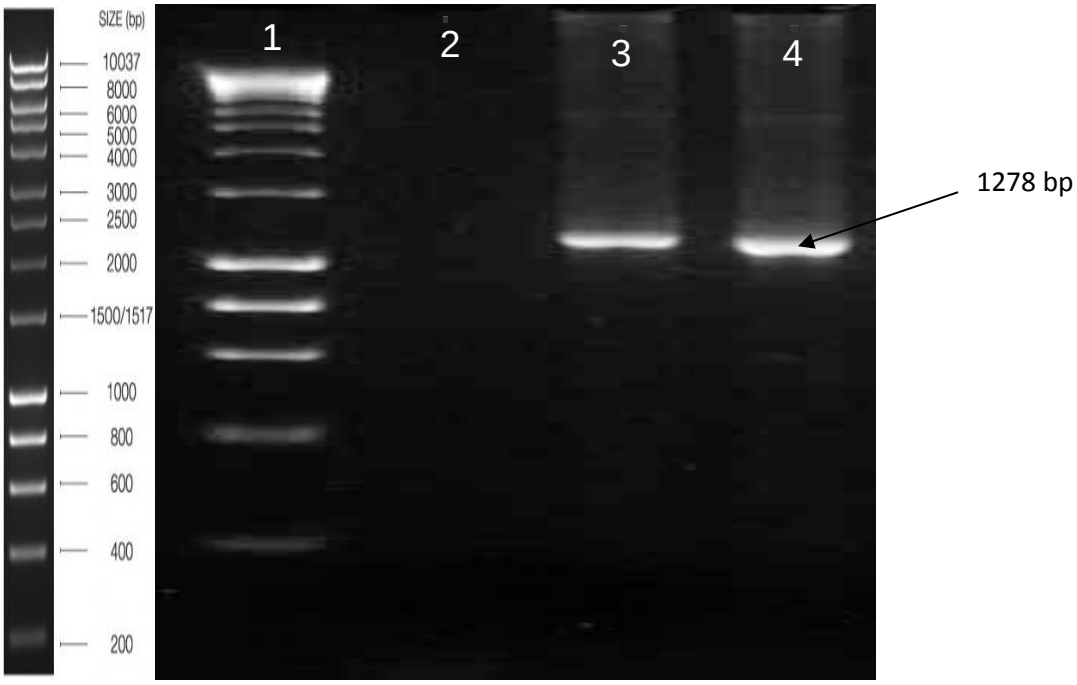


Figure 3.20: Recombinant baculovirus screening for truncated HSV-2 gG using virus specific primer. Lane 1 DNA marker (HyperLadder I), Lanes 3 and 4 truncated HSV-2 gG expressed protein samples with the size of 1278 bp. Negative control was in Lane 2.

3.6 Protein characterisation

3.6.1 Protein Quantification

The expressed proteins were purified using a nickel binding His-Select iLAP column. After purification, the concentrations of the purified recombinant proteins were determined using the BCA protein assay, as described in section 2.8.4. Based on the BSA standard curve, the concentration of the HSV-1 gG purified protein was 0.30 mg/mL, whereas for HSV-1 gG truncated protein and HSV-2 gG truncated proteins, 0.31 mg/mL and 0.34 mg/mL were quantified, respectively. The purified proteins were then used for further protein characterisation, SDS-PAGE and Western blot.

3.6.2 SDS-PAGE and Western blot

SDS-PAGE and Western blot were used to characterise recombinant proteins in order to determine the size, purity, and immune reactivity of the expressed proteins. Purified recombinant proteins were solubilised using the PAGE sample loading buffer and then loaded onto a 4-12% Bis-Tris SDS-PAGE gel under denaturing reducing condition with protein marker. When analysed by SDS-PAGE (Figure 3.21), the size of HSV-1 gG protein was difficult to distinguish among a background of multiple proteins. However, the Western blot analysis of HSV-1 gG purified protein showed the immunospecific band migrated between 76 kDa and 225 kDa (Figure 3.22), using an anti-HSV-1 gG mouse monoclonal antibody IgG as a primary antibody. This Western blot result was contradictive to the protein molecular weight calculation of 25 kDA. By using a membrane protein topology prediction method (TMHMM) based

on Markov model, there were two hydrophobic domains of the HSV-1 gG sequences (Figure 3.23), which located at residue 10-32 aa and 191-213 aa, respectively. Based on the TMHMM result, it was suspected that the purified HSV-1 gG protein typically folds into multiple conformations after protein purification leading to protein aggregation.

For truncated HSV-1 gG and HSV-2 gG, SDS-PAGE analyses revealed multiple proteins bands (Figure 3.24 and Figure 3.26). The Western blot analysis of truncated HSV-1 gG and truncated HSV-2 gG showed multiple immunoreactive bands in the sample lanes, suggesting that these purified glycoproteins formed different protein structures after the protein purification. By referring to Ikoma *et al.* (2002) and the product details of the truncated HSV-1 gG (Appendix 11) and truncated HSV-2 gG (Appendix 12) from Virusys Corporation, USA, the domain size of the immunoreactive band for truncated HSV-1 gG and truncated HSV-2 gG purified protein were 45 kDa (Figure 3.25) and 81 kDa (Figure 3.27), respectively.

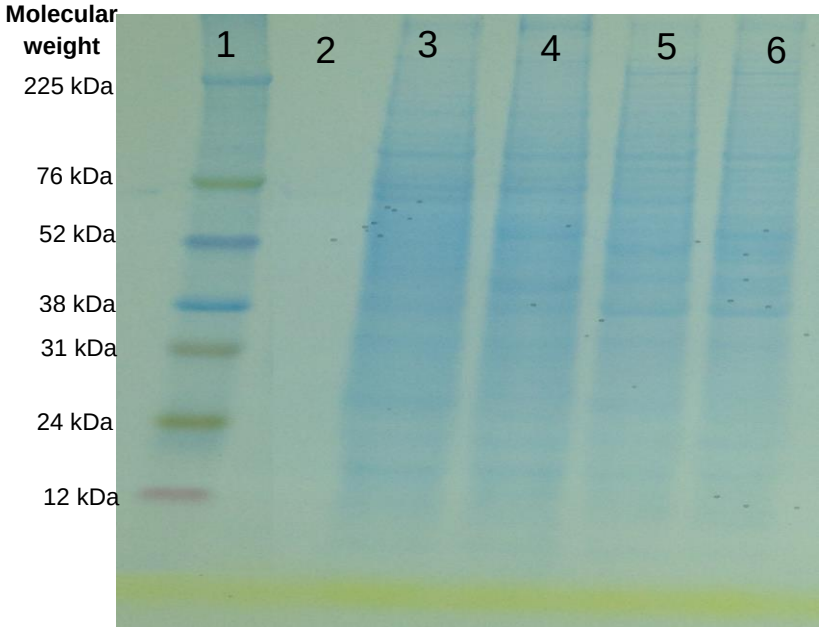


Figure 3.21: Coomassie blue staining of recombinant HSV-1 gG protein following SDS-PAGE. Lane 1 contained High-Range Rainbow; Lane 3 solubilised protein; Lane 4 contained washed protein; Lane 5 contained eluted protein; Lane 6 contained concentrated protein; Lane 2 represented negative control. The molecular weight range for the High-Range Rainbow marker is from 12 kDa to 225 kDa.

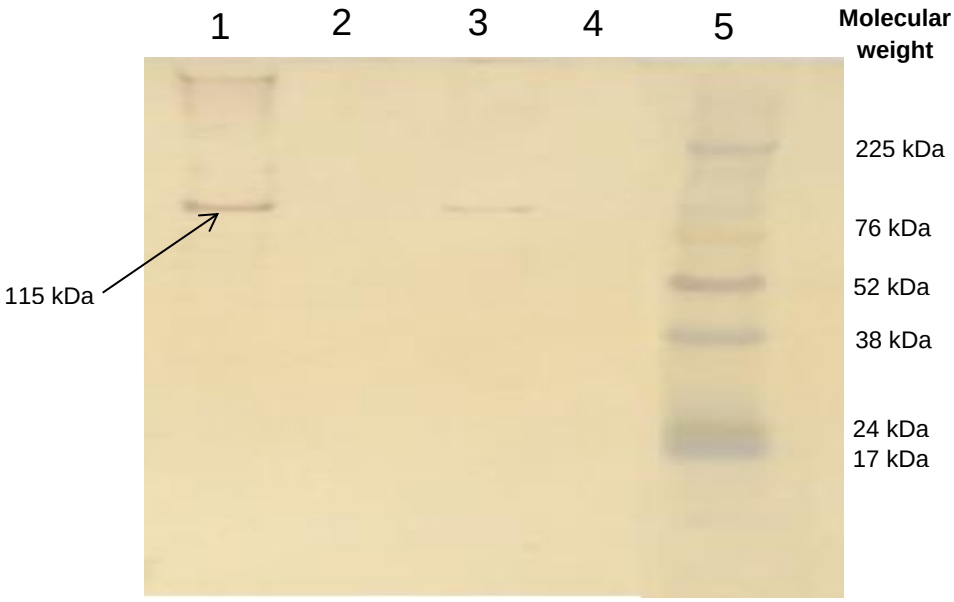


Figure 3.22: Western blot analysis of the HSV-1 gG expressed proteins using anti-HSV-1 gG mouse monoclonal antibody IgG. Lane 5 contained High-Range Rainbow molecular weight marker; Lane 1 HSV-1 gG concentrated protein; Lane 2 contained washed protein; Lane 3 contained eluted protein; Lane 4 represented negative control. The molecular weight range for the High-Range Rainbow marker is from 12 kDa to 225 kDa.

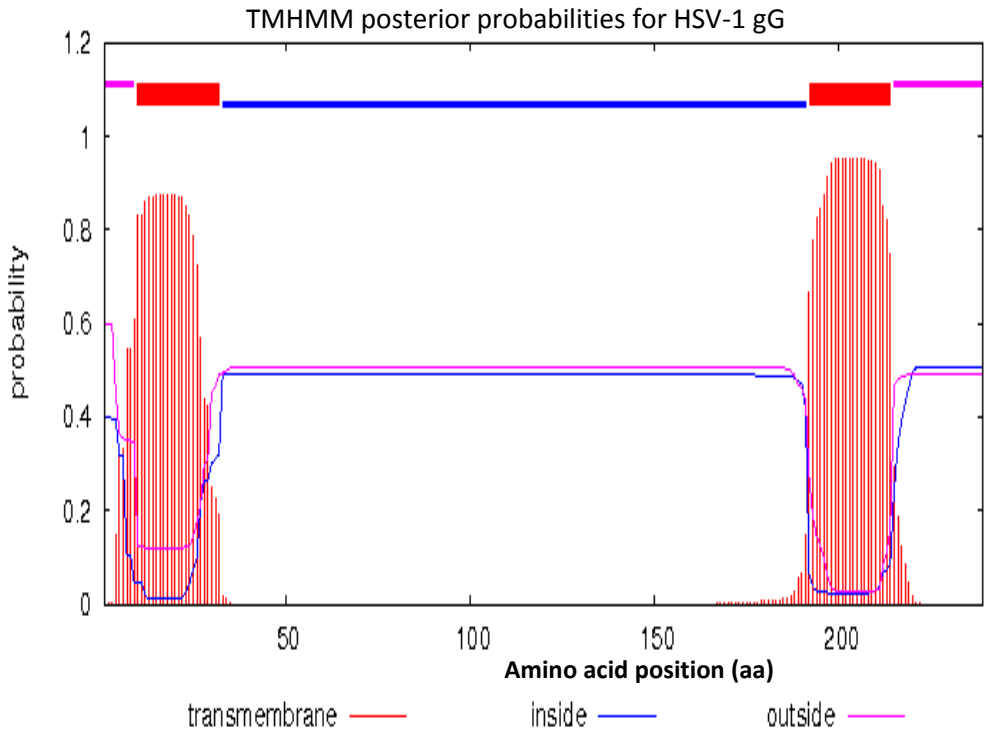


Figure 3.23: Membrane protein topology prediction analysis (TMHMM) of the HSV-1 gG expressed protein based on Markov model. Based on the graph, the HSV-1 gG expressed protein contains 2 hydrophobic sites which located at 10 to 32 aa and 191 to 213 aa, respectively. (Available at: http://www.cbs.dtu.dk/services /THMM-2.0/) (Accessed: 07 JULY 2015)

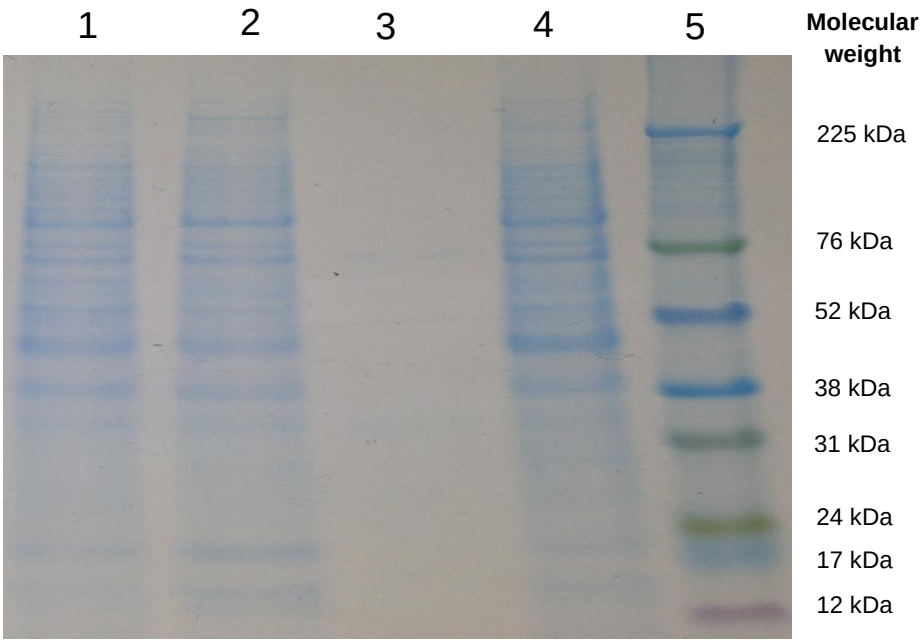


Figure 3.24: Coomassie blue staining of truncated HSV-1 gG protein following SDS-PAGE. Lane 5 contained High-Range Rainbow molecular weight marker; Lane 1 concentrated protein; Lane 2 contained eluted protein; Lane 4 contained solubilised protein; Lane 3 represented negative control. The molecular weight range for the High-Range Rainbow marker is from 12 kDa to 225 kDa.

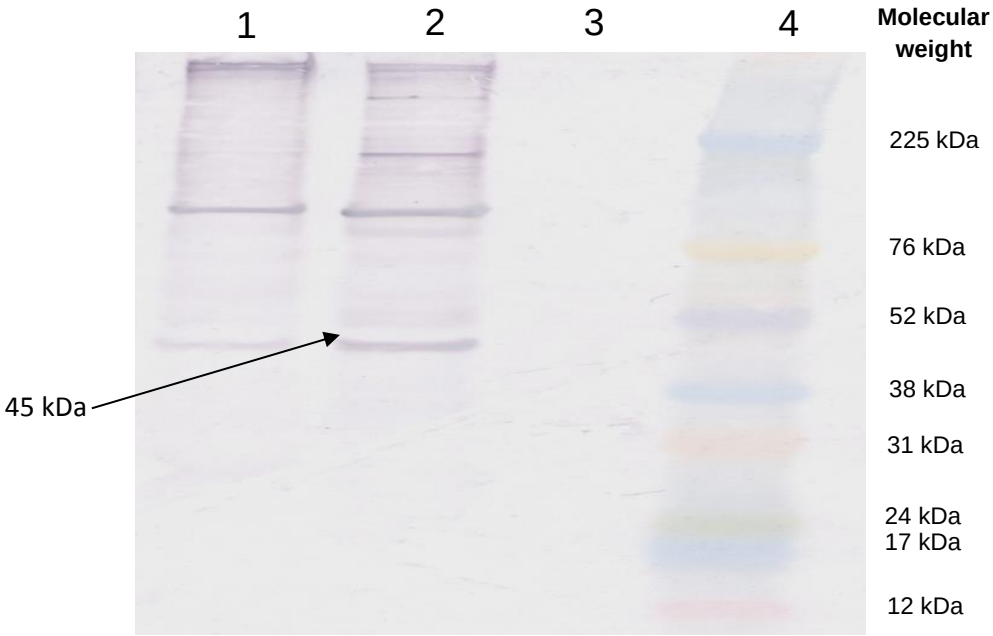


Figure 3.25: Western blot analysis of the truncated HSV-1 gG expressed proteins using anti-HSV-1 gG mouse monoclonal antibody IgG. Lane 4 contained High-Range Rainbow molecular weight marker; Lane 1 truncated HSV-1 gG concentrated protein; Lane 2 contained eluted protein; Lane 3 represented negative control. The molecular weight range for the High-Range Rainbow marker is from 12 kDa to 225 kDa.

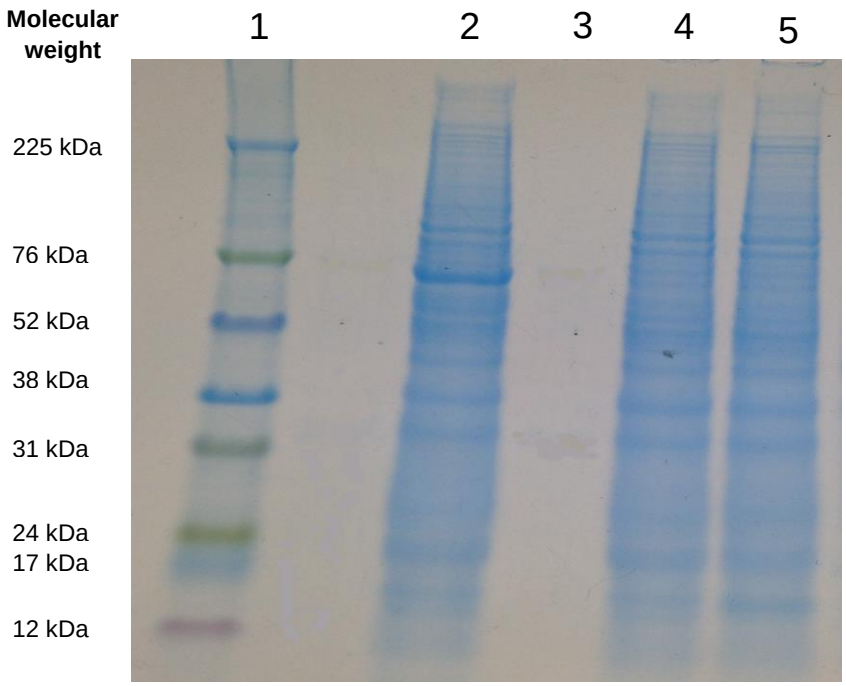


Figure 3.26: Coomassie blue staining of truncated HSV-2 gG protein following SDS-PAGE. Lane 1 contained High-Range Rainbow molecular weight marker; Lane 2 solubilised protein; Lane 4 contained eluted protein; Lane 5 contained concentrated protein. Lane 3 represented negative control (deionised water). The molecular weight range for the High-Range Rainbow marker is from 12 kDa to 225 kDa.

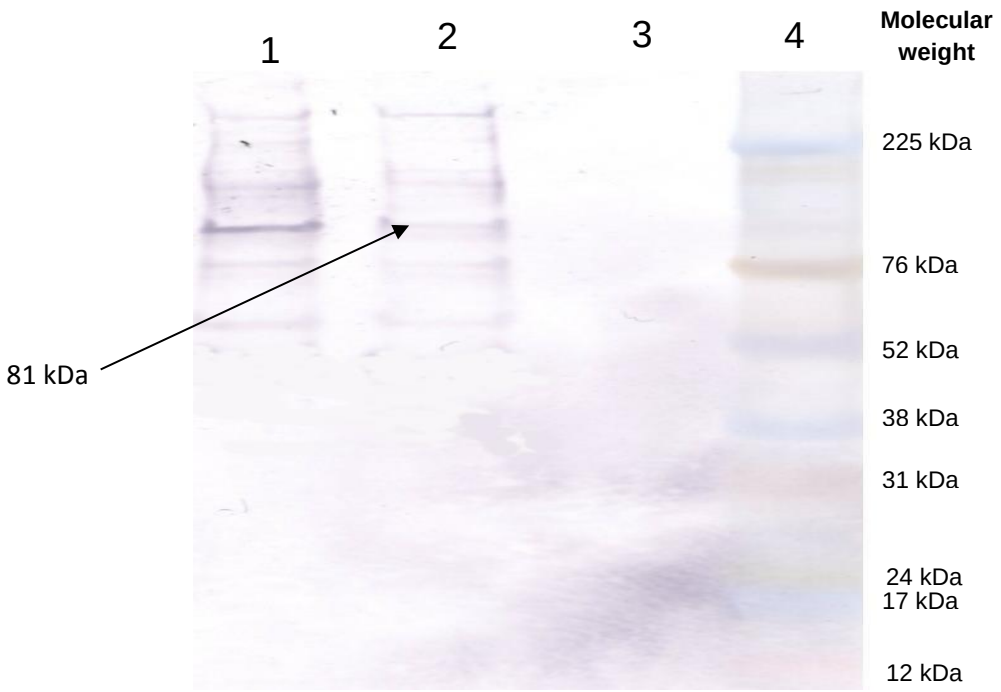


Figure 3.27: Western blot analysis of the truncated HSV-2 gG expressed proteins using anti-HSV-2 gG mouse monoclonal antibody IgG. Lane 4 contained High-Range Rainbow molecular weight marker; Lane 1 truncated HSV-2 gG concentrated protein; Lane 2 contained eluted protein. Lane 3 represented negative control (deionised water). The molecular weight range for the High-Range Rainbow marker is from 12 kDa to 225 kDa.

3.6.3 Light Scattering

The expressed HSV-1 gG purified protein was further analysed using light scattering, as described in section 2.8.5.3. Based on the light scattering analysis, multiplicity of peaks was observed for the HSV-1 gG purified protein, suggesting that the HSV-1 gG purified protein aggregated after protein purification (Appendix 7).

3.6.4 Mass spectrometer

In order to determine the actual molecular size, the expressed HSV-1 gG purified protein was analysed using a mass spectrometer, as described in section 2.8.5.4. When analysed by the BioMerieux VITEK mass spectrometer (Figure 3.28), the molecular size of HSV-1 gG protein was difficult to determine due to the formation of the multiple peak protein spectra on the VITEX MS datasheet, with a size range between 6000 Da to 7500 Da. Therefore, the HSV-1 gG protein was further analysed using Q-TOF mass spectrometer. Based on the Q-TOF mass spectrometer result (Figure 3.29), the predominant molecular size for HSV-1 gG protein was 112,965.75 Da.

As only a low yield of HSV proteins obtained when using the InsectDirect Baculovirus expression system, commercially available truncated glycoprotein G for HSV-1 and HSV-2 from Virusys Corporation, USA, were used to develop a duplex HSV Luminex immunoassay.

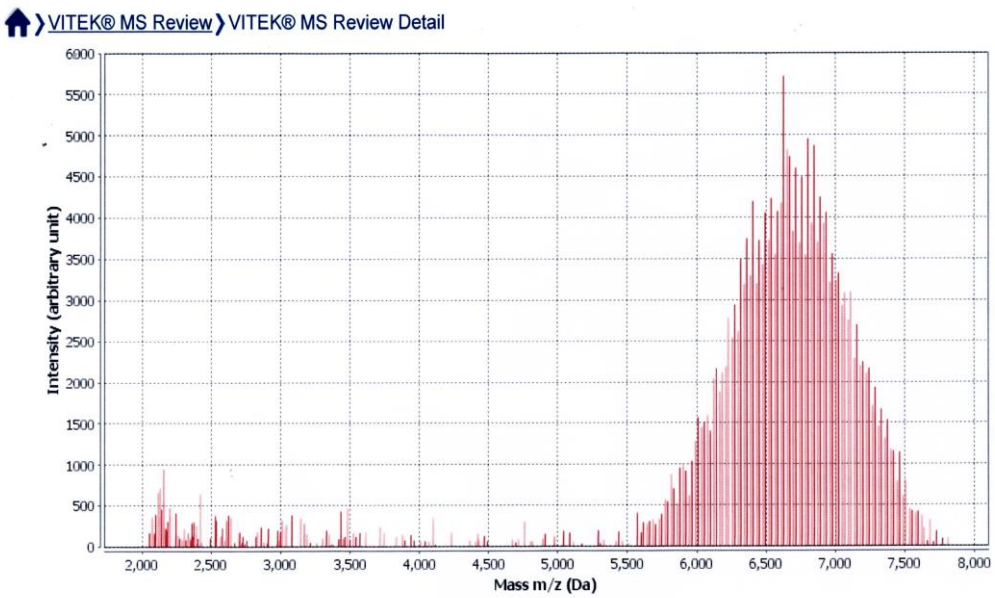


Figure 3.28: BioMerieux VITEK mass spectrometer analysis of HSV-1 gG purified protein. The graph showed the multiple peak protein spectra for the purified recombinant HSV-1 gG protein, with a size range between 6000 Da to 7500 Da.

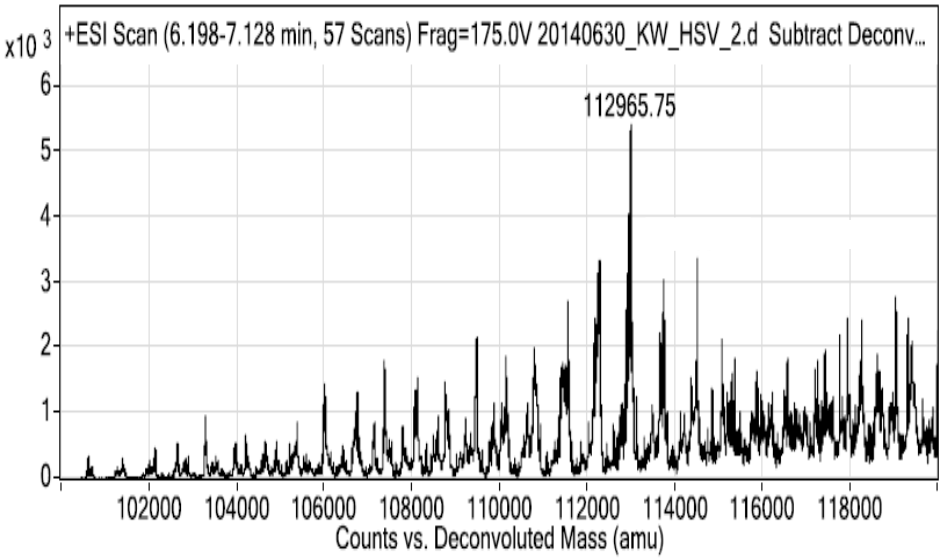


Figure 3.29: Q-TOF mass spectrometer analysis of HSV-1 gG purified protein. The graph showed the predominant molecular size for HSV-1 gG protein was 112,965.75 Da.

3.7 Development of a Bi-plexed fluorescent bead assay for the quantitative detection of IgG antibodies to HSV-1 and HSV-2 viruses in human sera

3.7.1 HSV-1 and HSV-2 serum panels

To test the HSV serum panel, the HSV-1 and HSV-2 HerpeSelect ELISA was used to verify samples which had previously been tested using the DiaSorin Liaison HSV type specific IgG assay by the Clinical Virology Department in Manchester Royal Infirmary. Based on the Diasorin assay results, the HSV serum panels were grouped into four groups, HSV-1 positive for serum panel 1, HSV-2 positive for serum panel 2, HSV-1 and HSV-2 positive for serum panel 3 and negative sera samples for serum panel 4. Overall, 50.83% (61/120) of samples were positive for HSV-1 and 49.17% (59/120) of samples were negative for HSV-1 using HSV-1 HerpeSelect ELISA; whereas for HSV-2 HerpeSelect ELISA, 52.5% (63/120) of samples were positive for HSV-2 and 47.5% (57/120) of samples were negative for HSV-2 (Appendix 9 and Appendix 10). Based on these 120 HSV test samples, the HerpeSelect ELISA data were found to have a good correlation with the results obtained from Diasorin assay. The sensitivity and specificity of the HerpeSelect assay were calculated using Diasorin assay as a reference test. This showed the sensitivity and specificity of HSV-1 HerpeSelect ELISA were 95.00% and 93.33% (Table 3.8), respectively, whereas for HSV-2 HerpeSelect ELISA, the sensitivity and specificity of the test were 96.67% and 96.49% (Table 3.9), respectively.

Table 3.8 Comparison of HSV-1 HerpeSelect ELISA test result with DiaSorin Liaison HSV-1 IgG assay

		HerpeSelect	
		Positive	Negative
Diasorin Liason	Positive	57	3
	Negative	4	56

Sensitivity of HSV-1 HerpeSelect = Number of true positive / (number of true positive + number of false negative)

= 57 / (57+3)

= 0.95 = 95.00%

Specificity of HSV-1 HerpeSelect = Number of true negative / (number of true negative + number of false positive)

= 56 / (56+4)

= 0.9333 = 93.33%

Table 3.9 Comparison of HSV-2 HerpeSelect ELISA test result with DiaSorin Liaison HSV-2 IgG assay

		HerpeSelect	
		Positive	Negative
Diasorin Liason	Positive	58	2
	Negative	5	55

Sensitivity of HSV-2 HerpeSelect = Number of true positive / (number of true positive + number of false negative)

= 58 / (58+2)

= 0.9667 = 96.67%

Specificity of HSV-2 HerpeSelect = Number of true negative / (number of true negative + number of false positive)

= 55 / (55+2)

= 0.9649 = 96.49%

3.7.2 Development of a reference standard serum

The RUBI-1-94 serum, which was previously tested to determine whether it contained anti-HSV antibodies using HerpeSelect ELISA, was used as a reference serum to develop the HSV Bi-plexed fluorescence bead assay. Each antigen (HSV-1 and HSV-2) was initially coupled to different carboxylated microspheres using a modified two step carbodiimide reaction. A standard curve for this assay was then generated using RUBI-1-94 serum with two fold-dilutions, starting from 1/10 to 1/10240. The HSV-1 and HSV-2 dilution curves were compared with rubella and VZV standard curves using a log-log model. This model was generated by plotting the logged MFI values for each bead set against log of the dilution scale. The correlation between the standard curves was determined using power regression and expressed as the correlation coefficient (r). The result showed both HSV-1 and HSV-2 dilution curves demonstrated parallelism with both the rubella and VZV standard curves, with high correlation between those antigens ($r > 0.99$) (Figure 3.30). Based on the visual comparison between the standard reference curves and the HSV sample curves, the VZV standard curve was selected as the reference standard curve for assigning the antibody concentration of HSV in the reference serum. The antibody concentration for both HSV-1 and HSV-2 assays were determined using fully specified logit-log model, as described in section 2.9.2.2.2. The model showed both HSV-1 (Figure 3.31) and HSV-2 (Figure 3.32) dilution curves parallel the VZV standard curve, with correlation coefficient of 0.9916 for HSV-1 and 0.9978 for HSV-2, respectively. In this study, the MFI corresponding to the highest concentration of both HSV-1 and HSV-2 Bi-plexed assays was used to

approximate MFI $_{max}$. The MFI $_{min}$ of HSV-1 and HSV-2 Bi-plexed assays corresponded in the MFI $_{min}$ of the VZV standard curve. For the HSV-1 sample curve (Table 3.10), two points associated with the lowest MFI of the curve, which had undefined fully specified logits due to a lower MFI compared to the MFI $_{min}$ of the VZV standard curve. Therefore, these dilution points were eliminated together with the MFI $_{max}$ point of the curve and resulted in an 8-point assay for this HSV-1 model. For the HSV-2 sample curve, two points with lowest dilution (Table 3.11), 1 in 10 and 1 in 20 dilution, respectively and two points with highest dilution of the sample curve, 1 in 5120 and 1 in 10240, respectively, were eliminated due to the high %CV (>30%) and the low MFI compared to the VZV MFI $_{min}$, which resulted in a 6-point assay for the HSV-2 model. The data showed the assigned antibody concentration for HSV-1 (Table 3.10) was 3.93 AU/mL with a standard deviation of 0.91 AU/mL and a %CV of 23.23%, whereas for HSV-2 (Table 3.11), the assigned antibody concentration was 0.30 AU/mL with a standard deviation of 0.04 AU/mL and a %CV of 14.16%.

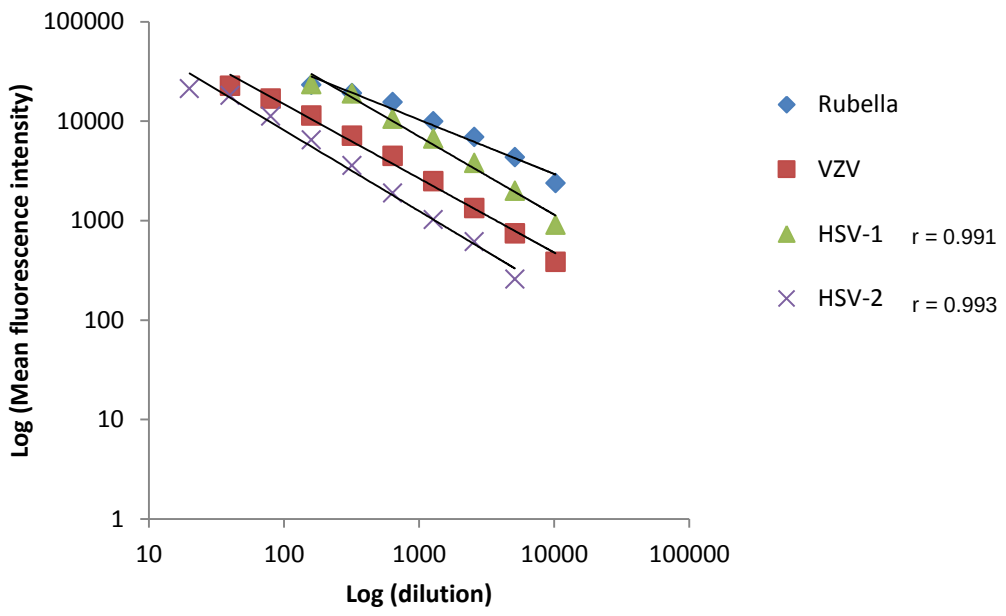


Figure 3.30: Parallelism and correlation analysis on standard curve generated from a serial dilutions of RUBI-1-94 reference serum for rubella, VZV, HSV-1 and HSV-2 antigens. The regression analysis revealed that the HSV-1 and HSV-2 dilution curves were parallel to rubella and VZV standard curve, with high correlation between the antigens, with 0.991 for HSV-1 and 0.993 for HSV-2, respectively.

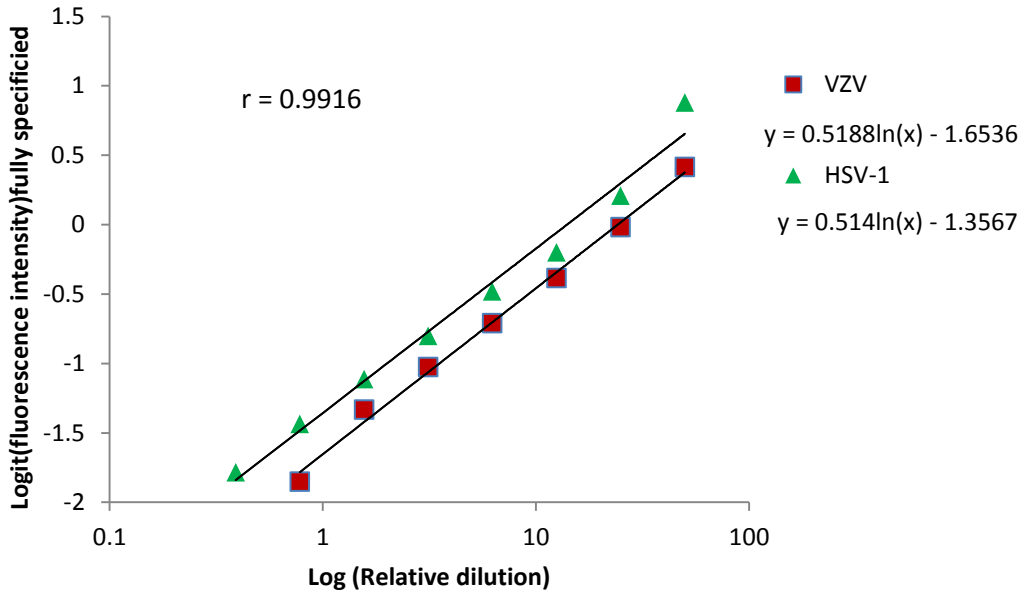


Figure 3.31: Parallelism and correlation analysis on standard curve generated from a serial dilution of RUBI-1-94 reference serum and serially diluted HSV-1 sample plotted on a fully specified logit-log scale. The correlation between the antigens for HSV-1 dilution curve was 0.9916.The slope for VZV standard curve and HSV-1 sample curve were 0.5188 and 0.5140, respectively.

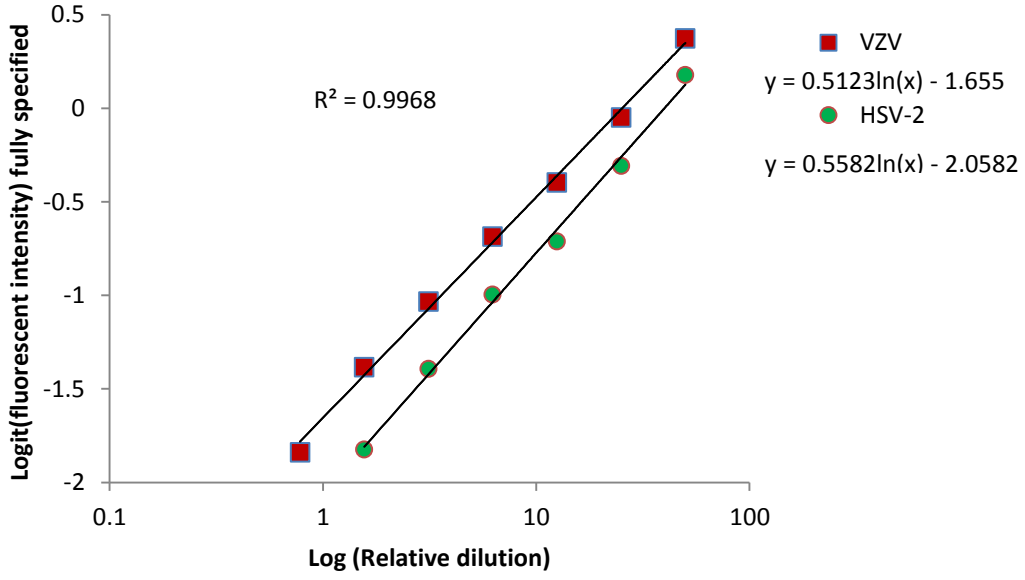


Figure 3.32: Parallelism and correlation analysis on standard curve generated from a serial dilution of RUBI-1-94 reference serum and serially diluted HSV-2 sample plotted on a fully specified logit-log scale. The correlation between the antigens for HSV-2 dilution curve was 0.9978. The slope for VZV standard curve and HSV-1 sample curve were 0.5123 and 0.5582, respectively.

Table 3.10 Antibody concentration calculation for HSV-1 Bi-plexed assay using fully specified logit-log model

Dilution	1/dilution	MFI	Logit$(MFI)_{fs}$	Relative dilution	Interpolated dilution from standard curve	Concentration in HSV-1 dilution corrected (AU/mL)
10240[a]	9.76563E-05	135	NA	0.09765625	NA	NA
5120[a]	0.000195313	265.15	NA	0.1953125	NA	NA
2560	0.000390625	513.15	-1.785686468	0.390625	3100.91	4.37
1280	0.00078125	961.25	-1.437726583	0.78125	6064.16	4.27
640	0.0015625	1805.4	-1.115513476	1.5625	11284.95	3.97
320	0.003125	3326	-0.802996572	3.125	20611.60	3.63
160	0.00625	5931.15	-0.484050233	6.25	38115.87	3.35
80	0.0125	9188.75	-0.201850552	12.5	65665.77	2.89
40	0.025	14608.4	0.206769813	25	144344.64	3.18
20	0.05	20854.3	0.877840641	50	526214.87	5.79
10[b]	0.1	23599.3	NA	NA	NA	NA
					Average concentration of HSV-1 (AU/mL)	3.93
					Standard deviation	0.91
					%CV	23.23

Note: 10240[a] and 5120[a] - the points that were undefined fully specified logits because the MFI of the points were below the MFI_{min} of VZV standard curve (MFI = 361.65), 10[b]-the point that was used to approximate MFI_{max}, MFI represents median fluorescence intensity, Logit $(MFI)_{fs}$ represents fully specified logit of the median fluorescence intensity, %CV represents the percentage of coefficient of variation, NA – not applicable.

Table 3.11 Antibody concentration calculation for HSV-2 Bi-plexed assay using fully specified logit-log model

Dilution	1/dilution	FI	Logit$(FI)_{fs}$	Relative dilution	Interpolated dilution from standard curve	Concentration in HSV-1 dilution corrected (AU/mL)
10240[a]	9.76563E-05	143.2	NA	NA	NA	NA
5120[a]	0.000195313	283.3	NA	NA	NA	NA
2560	0.000390625	597.05	-1.8226013	1.5625	0.74	0.25
1280	0.00078125	1024.8	-1.3918374	3.125	1.67	0.29
640	0.0015625	1959.4	-0.9954563	6.25	3.62	0.32
320	0.003125	3212.3	-0.7119714	12.5	6.30	0.28
160	0.00625	6180.65	-0.3071215	25	13.89	0.31
80	0.0125	10979.55	0.17844455	50	35.83	0.38
40[c]	0.025	18037.8	NA	NA	NA	NA
20[b]	0.05	NA	NA	NA	NA	NA
10[b]	0.1	NA	NA	NA	NA	NA
					Average concentration of HV-2 (AU/mL)	0.30
					Standard deviation	0.04
					%CV	14.16

Note: 10240[a] and 5120[a] - the points that were undefined fully specified logits because the MFI of the points were fell below the MFI_{min} of VZV standard curve (MFI = 361.65), 20[b] and 10[b]-the points with high %CV (CV >30%), 40[c] – the point was used to approximate MFI_{max} , MFI represents median fluorescence intensity, Logit $(MFI)_{fs}$ represents fully specified logit of the median fluorescence intensity, %CV represents the percentage of coefficient of variation, NA – not applicable.

3.7.3 Assay optimisation

In order to optimise the HSV assay, a number of factors such as, antigen concentration, incubation times for the bead conjugation, serum dilution and SOD concentration were investigated. The assay was initially tested using three different diluted antigens (0.5 µg/mL, 1.0 µg/mL and 2 µg/mL); then, three different incubation times for bead conjugation (1.5 hours, 2 hours and 3 hours) were determined. The results were determined based on the parallelism and correlation analysis on the dilution curve generated by each assay compare with rubella and VZV standard curves. The HSV Bi-plexed assay was then further analysed by testing two different sample dilutions (1 in 100 and 1 in 400) in duplicate, using five serum samples, with HSV antibody status defined by HerpeSelect assay. To determine whether human SOD affected the sensitivity and specificity of the assay, 5 different SOD concentrations (nil, 0.0025 mg/mL, 0.005 mg/mL, 0.01 mg/mL and 0.1 mg/mL) were tested.

The results showed the optimal standard curve for HSV-1 Bi-plexed assay was obtained using beads conjugated with 1.0 µg/mL of antigen with 1.5 hours coupling times (Figure 3.33 and Table 3.12). Other HSV-1 antigen dilutions and incubation times failed to generate a good parallel dilution curve with rubella and VZV standard curves when testing the standard serum, RUBI-1-94 (examples shown in Figure 3.34 – Figure 3.37). For the HSV-2 Bi-plexed assay, the conjugated bead generated high backgrounds for the blank well, with the fluorescence intensity (FI) greater than 13000 (Table 3.13). Modification of the HSV bead coupling was attempted by increasing the incubation times for the bead conjugation to 2 hours with the antigen diluted of 1.0 µg/mL. This radically reduced the

fluorescence intensity of the background (blank) of the assay to less than 2500 (Table 3.14). However, this value was still beyond the acceptance criteria, stated by the manufacturer's protocol (Bio-Rad laboratories Ltd.), which suggested an FI of less than 200 is desirable. A linear curve for the HSV-2 assay condition was generated by subtracting the assay background from the reading of the reference serum and test sera using the Bioplex Manager Software. The HSV-2 dilution curve paralleled the rubella and VZV standard curve with high correlation (Figure 3.38 and compared to other assay conditions Figure 3.39 – Figure 3.42). Therefore, the HSV-2 conjugated bead was used in the study utilised bead conjugation of 1.0 µg/mL and 2.0 hours coupling time. The antibody concentrations generated by two different serum dilutions (1 in 100 and 1 in 400) for both HSV assays paralleled the dilution curve of the standard serum, with less than 20% of coefficient of variation (CV) for each tested sample (Table 3.15 and Table 3.16). The resultant concentrations from both dilutions were almost identical, suggesting that a serum dilution of 1 in 100 was sufficient to measure a broad range of antibody concentrations for HSV-1 and HSV-2 samples. The result also revealed that there were no differences in test sensitivity when different concentrations of SOD were used in the HSV Bi-plexed assay (Table 3.17 and Table 3.18). Based on these results, the optimal conditions for the HSV-1 assay (1.0 µg/mL of diluted antigen, 1.5 hours bead coupling time, with a 1 in 100 serum dilution and nil SOD concentration) and HSV-2 assay (1.0 µg/mL of diluted antigen at 2.0 hours bead coupling time, with a 1 in 100 serum dilution and nil SOD concentration) were used to test the HSV serum samples.

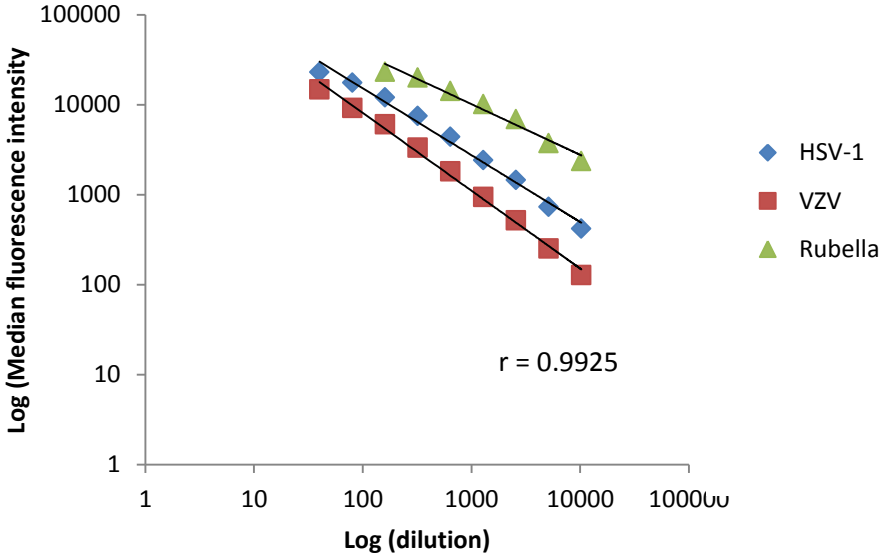


Figure 3.33: Parallelism and correlation analysis on dilution curves generated from HSV-1 with antigen diluted of 1.0 µg/mL at 1.5 hours coupling time. The regression analysis revealed that the HSV-1 dilution curves were parallel to rubella and VZV standard curve with correlation between the antigens, 0.9925.

Table 3.12 The dilution curve values for HSV-1 Bi-plexed assay using the microsphere (5000/well) conjugated with 1.0 µg/mL of HSV-1 gG recombinant antigen at 1.5 hours coupling time

Sample	Fluorescent Intensity	Flourescent Background	Standard deviation	%CV	Observed concentration	Expected Concentration	Recovery Rate %
B	62	62	0.71	1.14			
S1	262.8	200.8	8.84	4.4	0	0	95
S2	531.3	469.3	43.49	9.27	0.01	0.01	106
S3	896.8	834.8	51.27	6.14	0.01	0.02	97
S4	1693.5	1631.5	67.88	4.16	0.03	0.03	101
S5	3267.3	3205.3	108.54	3.39	0.07	0.06	109
S6	5152.5	5090.5	26.87	0.53	0.12	0.12	94
S7	9176.8	9114.8	22.98	0.25	0.24	0.25	98
S8	15000.5	14938.5	634.27	4.25	0.5	0.49	102
S9	20610.5	20548.5	3737.06	18.19	1.01	0.98	102
S10	24264	24202	746	3.08	1.96	1.97	99
S11	26019	25957	836.51	3.22	3.69	3.93	94

B: blank S: standard

%CV: percentage coefficient of variation

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

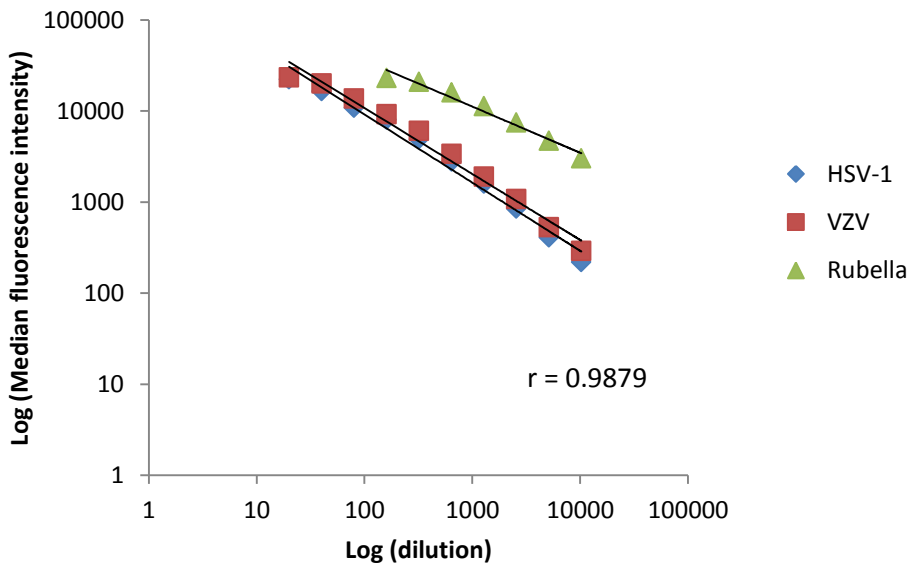


Figure 3.34: Parallelism and correlation analysis on dilution curves generated from HSV-1 with antigen diluted of 2.0 µg/mL at 1.5 hours coupling time. The correlation between the antigens for HSV-1 dilution curve was 0.9879.

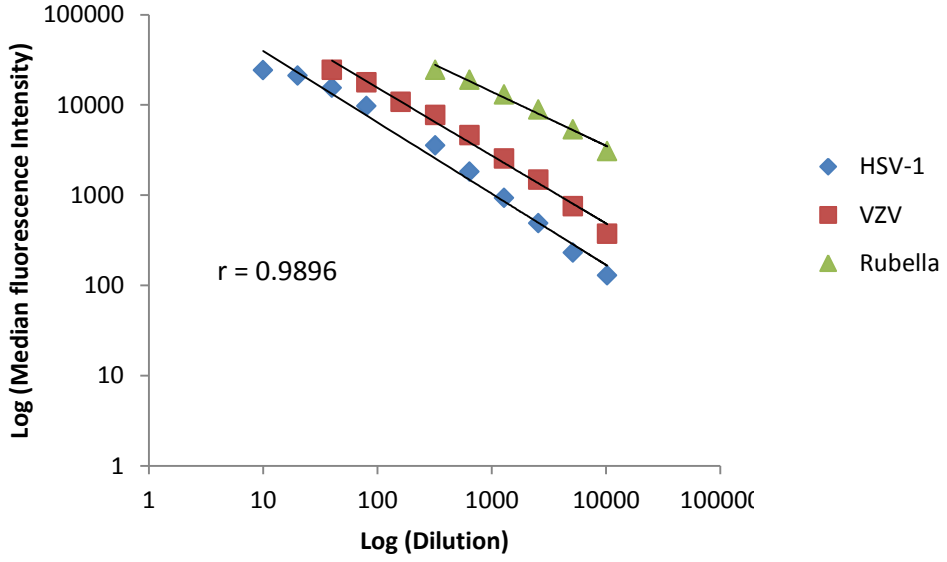


Figure 3.35: Parallelism and correlation analysis on dilution curves generated from HSV-1 with antigen diluted of 0.5 µg/mL at 1.5 hours coupling time. The correlation between the antigens for HSV-1 dilution curve was 0.9896.

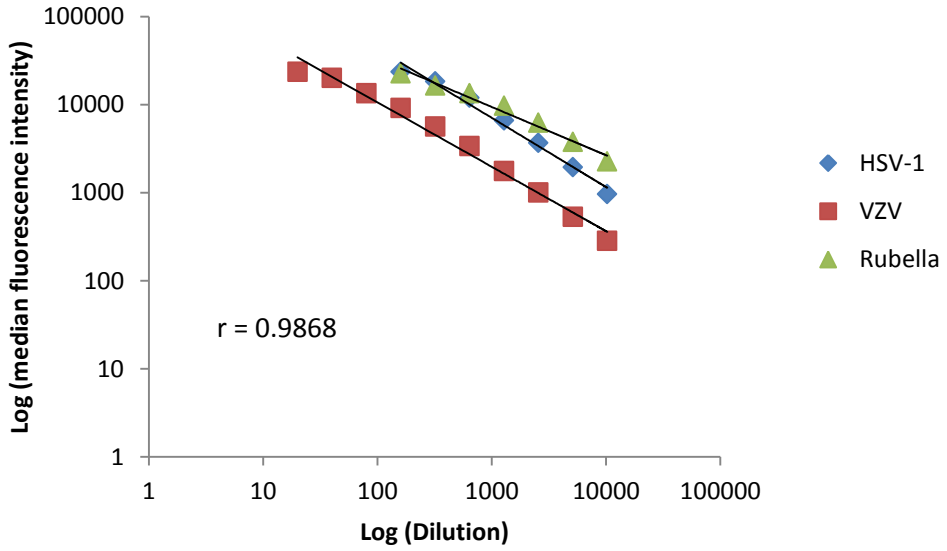


Figure 3.36: Parallelism and correlation analysis on dilution curves generated from HSV-1 with antigen diluted of 1.0 µg/mL at 2.0 hours coupling time. The correlation between the antigens for HSV-1 dilution curve was 0.9868.

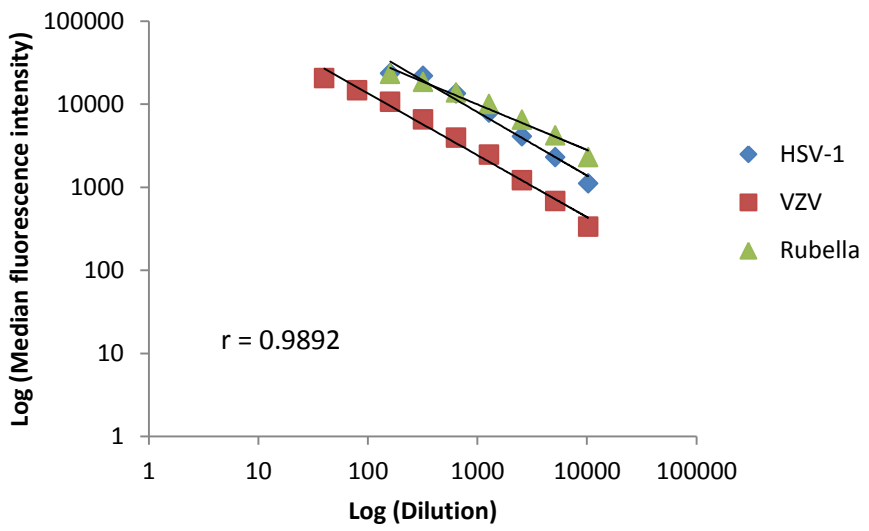


Figure 3.37: Parallelism and correlation analysis on dilution curves generated from HSV-1 with antigen diluted of 1.0 µg/mL at 3.0 hours coupling time. The correlation between the antigens for HSV-1 dilution curve was 0.9892.

Table 3.13 The dilution curves values for HSV-2 Bi-plexed assay using the microsphere (5000/well) conjugated with 2.0 µg/mL of HSV-2 gG recombinant antigen at 1.5 hours coupling time

Sample	Fluorescent Intensity	Flourescent Background	Standard deviation	%CV	Observed concentration	Expected Concentration	Recovery Rate %
B	13178.3	13178.3	642.41	4.87			
S1*	---	---				0	
S2*	---	---				0	
S3*	---	---				0	
S4	14310.3	1132	90.16	7.96	0	0	100
S5	15631.5	2453.3	772.87	31.5	0	0	96
S6	17646.3	4468	109.25	2.45	0.01	0.01	112
S7	18909.5	5731.3	1290.47	22.52	0.02	0.02	89
S8	21504	8325.8	1448.15	17.39	0.04	0.04	106
S9	23381.5	10203.3	163.34	1.6	0.08	0.07	99
S10*	---	---				0.15	
S11*	---	---				0.3	

*The standard points were masked due to the high %CV (%CV > 30).

B : blank S:standard

%CV: percentage coefficient of variation

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

Table 3.14 The Fluorescence Intensity values for the blank well of HSV-2 Bi-plexed assay with different antigen concentrations and incubation times of the bead conjugation

Antigen Concentration (µg/mL)	Incubation times	FI values*
1	1.5	7357.55
2	1.5	13178.3
1	2	2347
1	3	2337.75
0.5	1.5	14784

Note:* represented by the mean of the fluorescence intensity value for the blank well in 2 independent runs.

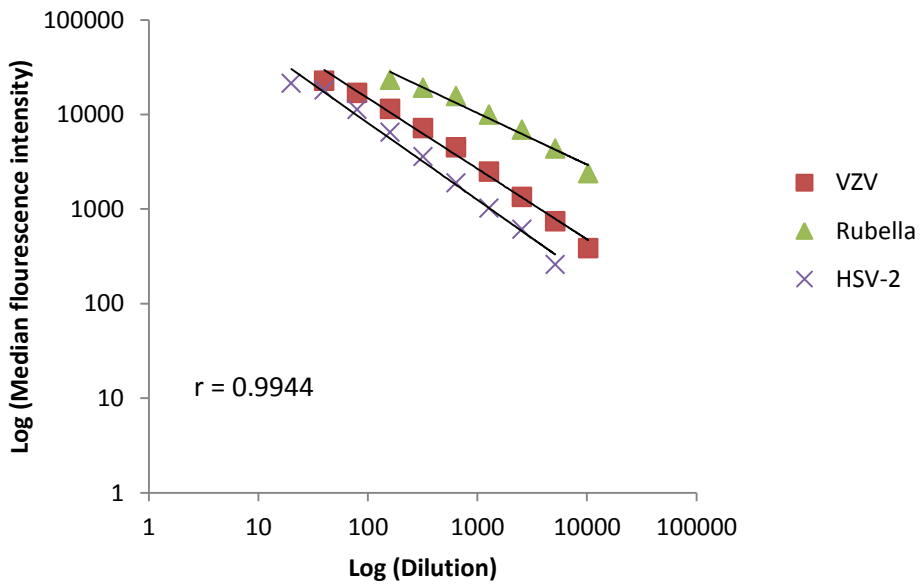


Figure 3.38: Parallelism and correlation analysis on dilution curves generated from HSV-2 with antigen diluted of 1.0 µg/mL at 2 hours coupling times. The regression analysis revealed that the HSV-2 dilution curves were parallel to rubella and VZV standard curves with correlation between the antigens, 0.9944.

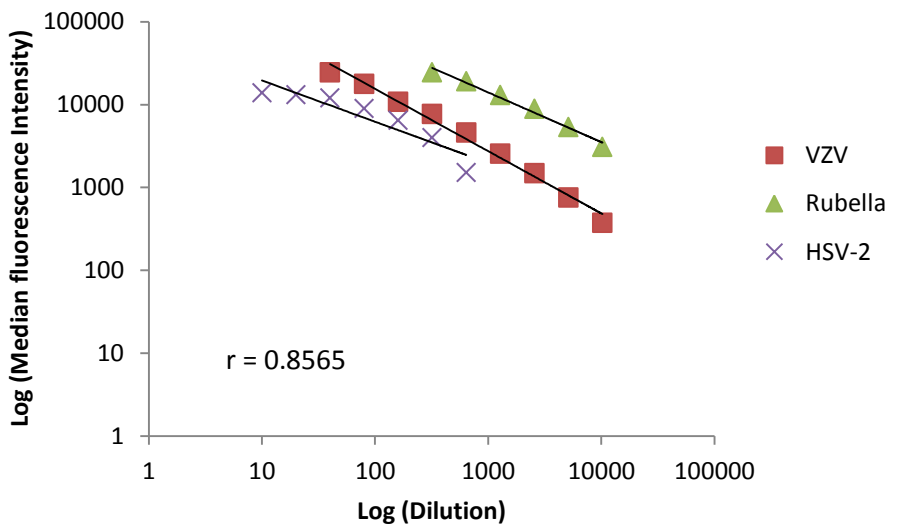


Figure 3.39: Parallelism and correlation analysis on dilution curves generated from HSV-2 with antigen diluted of 0.5 µg/mL at 1.5 hours coupling time. The correlation between the antigens for HSV-2 dilution curve was 0.8565.

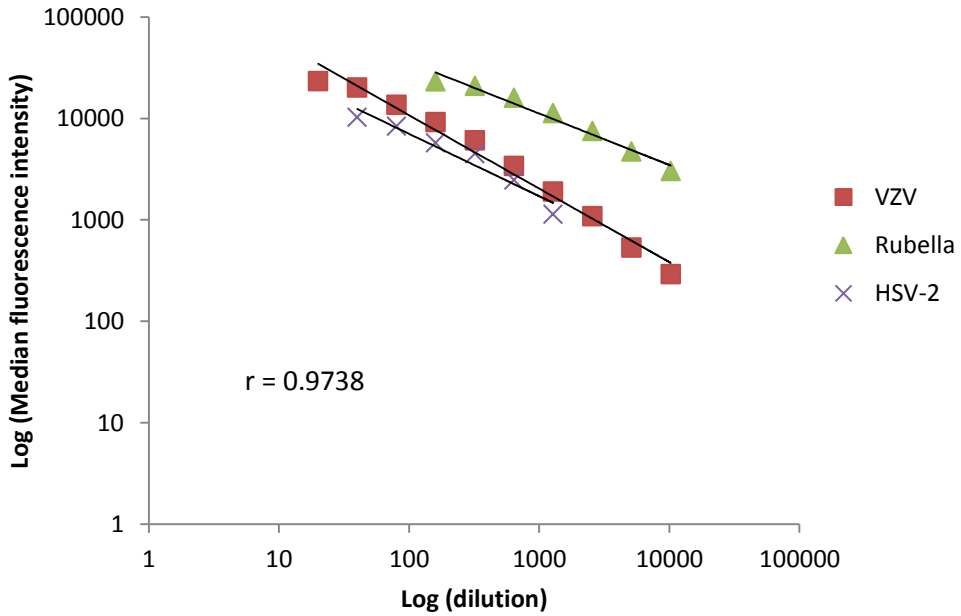


Figure 3.40: Parallelism and correlation analysis on dilution curves generated from HSV-2 with antigen diluted of 2.0 µg/mL at 1.5 hours coupling time. The correlation between the antigens for HSV-2 dilution curve was 0.9738.

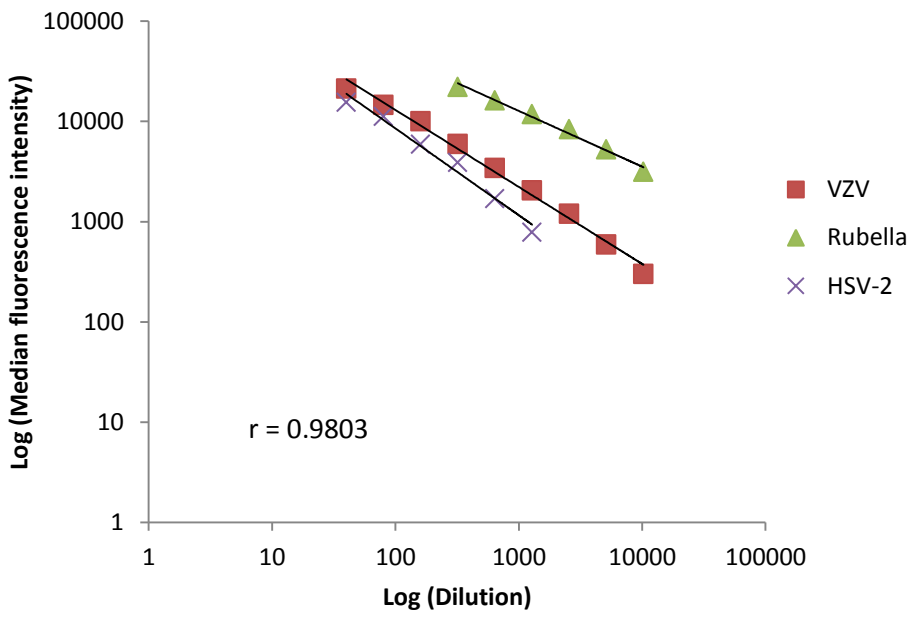


Figure 3.41: Parallelism and correlation analysis on dilution curves generated from HSV-2 with antigen diluted of 1.0 µg/mL at 1.5 hours coupling times. The correlation between the antigens for HSV-2 dilution curve was 0.9803.

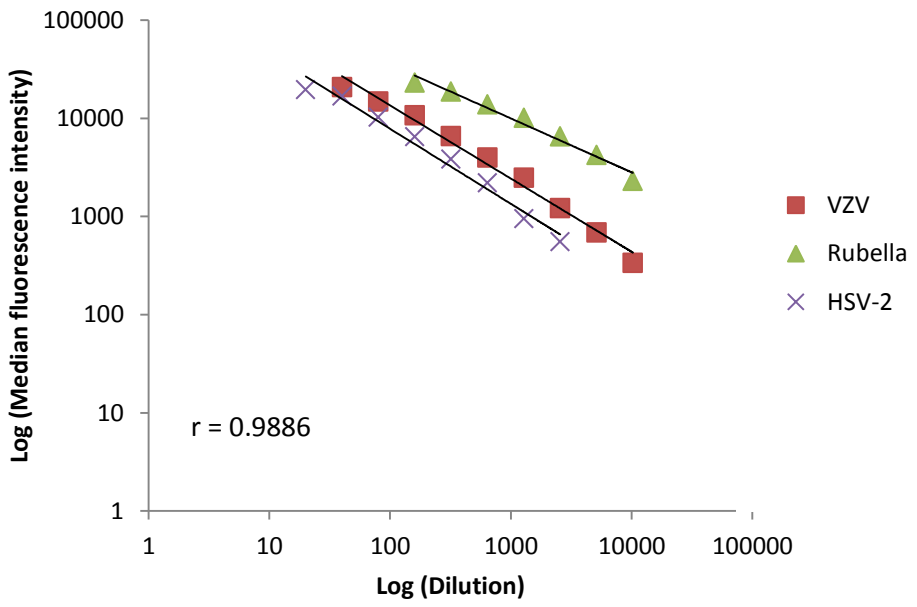


Figure 3.42: Parallelism and correlation analysis on dilution curves generated from HSV-2 with antigen diluted of 1.0 µg/mL at 3.0 hours coupling time. The correlation between the antigens for HSV-2 dilution curve was 0.9886.

Table 3.15 The IgG antibody concentration and percentage coefficient of variation (%CV) for 3 tested samples of HSV-1 Bi-plexed assay with 1 in 100 and 1 in 400 serum dilutions

Samples Number	Serum Dilutions			
	1/100		1/400	
	%CV	Antibody concentration (AU/mL)	%CV	Antibody concentration (AU/mL)
1*	0.71	4.57	6.11	5.37
2*	1.38	2.21	4.24	2.8
3*	15.18	2.28	9.47	2.72

*Sample 1 and 2 - unknown patient samples with HSV-1 positive;
Sample 3 - unknown patients samples with HSV-1 and HSV-2 positive

Table 3.16 The IgG antibody concentration and percentage coefficient of variation (%CV) for 3 tested samples of HSV-2 Bi-plexed assay with 1 in 100 and 1 in 400 serum dilutions

Samples Number	Serum Dilutions			
	1/100		1/400	
	%CV	Antibody concentration (AU/mL)	%CV	Antibody concentration (AU/mL)
1*	6.4	0.87	18.28	0.88
2*	9.43	1.33	18.33	1.43
3*	2.7	0.8	1.49	1.02

*Sample 1 and 2 - unknown patient samples with HSV-2 positive;
Sample 3 - unknown patients samples with HSV-1 and HSV-2 positive

Table 3.17 The IgG antibody concentration for the QC serum samples of HSV-1 Bi-plexed assay with different Superoxide dismutase (SOD) concentrations

SOD concentration (mg/mL)	HSV-1 IgG antibody concentration (AU/mL)		
	QC1	QC2	QC3
Nil	4.51	*0.12	2.39
	3.25	*0.17	1.08
0.0025	3.74	*0.21	1.55
	3.17	*0.17	1.57
0.005	4.71	*0.23	2.40
	3.7	OOR <	1.90
0.01	4.01	*0.15	1.96
	3.76	*0.17	1.71
0.1	4.43	*0.14	2.43
	3.99	*0.16	1.95

Note: QC1- unknown patient samples with HSV-1 positive; QC2 - unknown patient samples with HSV-2 positive; QC3 - unknown patients samples with HSV-1 and HSV-2 positive

*Star values and OOR< – observed concentration of the test samples that fall outside the standard curve range

Table 3.18 The IgG antibody concentration for the QC serum samples of HSV-2 Bi-plexed assay with different Superoxide dismutase (SOD) concentrations

SOD concentration (mg/mL)	HSV-2 IgG antibody concentration (AU/mL)		
	QC1	QC2	QC3
Nil	OOR <	0.84	1.11
	OOR <	0.87	1.11
0.0025	OOR <	0.76	0.37
	OOR <	0.65	0.97
0.005	OOR <	0.97	1.20
	OOR <	0.55	0.80
0.01	OOR <	0.75	0.56
	OOR <	0.60	0.94
0.1	OOR <	0.87	1.25
	OOR <	0.59	0.95

Note: QC1- unknown patient samples with HSV-1 positive; QC2 - unknown patient samples with HSV-2 positive; QC3 - unknown patients samples with HSV-1 and HSV-2 positive

*Star values and OOR< – observed concentration of the test samples that fall outside the standard curve range

3.7.4 Determination of the threshold IgG level for the HSV-1 and HSV-2 Bi-plexed assay

In order to determine the optimal sensitivity and specificity for HSV-1 and HSV-2 Bi-plexed assays, the cut-off values for the assays were calculated by a receiver operator characteristics (ROC) curve. The reference samples for this analysis consisted of 126 serum samples, which were previously tested to determine the HSV status using HerpeSelect ELISA. The test samples resulted was reported as AU/mL of specific HSV antibody, as described in section 2.9.2.2.2. For the HSV-1 Bi-plexed assay, the ROC analysis revealed that the optimal sensitivity of the assay was a cut-off value of 0.10 AU/mL (Figure 3.43 and Table 3.19) with 100% of sensitivity. However, with this cut-off value, the HSV-1 Bi-plexed assay only gave a specificity of 11% relative to the reference test (HerpeSelect test), with correspondingly high number of false positive test results. In order to improve the HSV-1 Bi-plexed assay, a cut-off level of 0.75 AU/mL was selected, which was produced a test sensitivity and specificity of 89% and 66%, respectively. As with the HSV-1 Bi-plexed assay, the ROC analysis indicated that the HSV-2 IgG cut-off value with optimal sensitivity and specificity (Figure 3.44 and Table 3.20) was 0.05 AU/mL, with sensitivity of 79% and specificity of 85%, respectively.

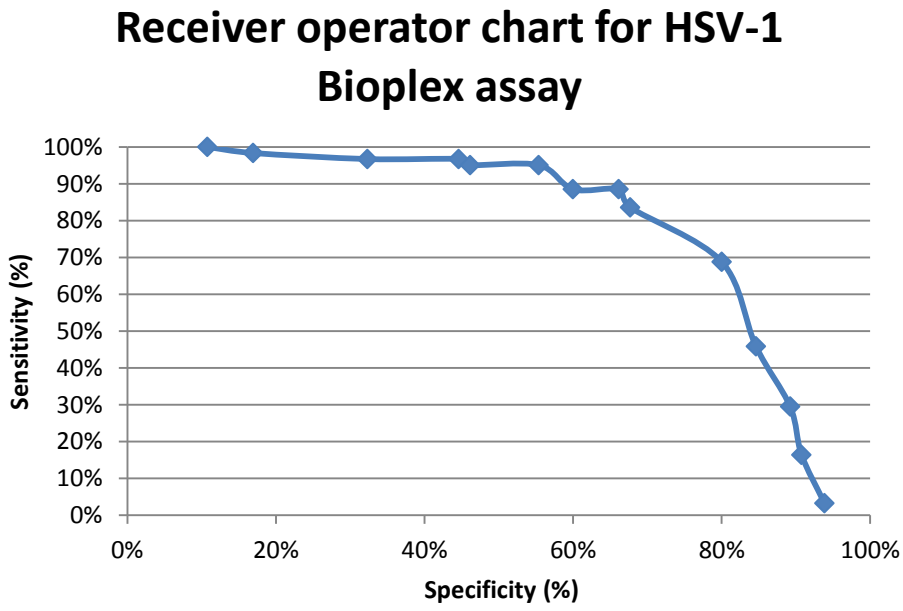


Figure 3.43: Receiver operator characteristic (ROC) curve for HSV-1 Bi-plexed assay

Table 3.19 The sensitivity and specificity of the HSV-1 Bi-plexed assay with different HSV-1 cut-off levels

HSV-1 Cut Off level (AU/mL)	Sensitivity (%)	Specificity (%)
0.10	100%	11%
0.15	98%	17%
0.20	97%	32%
0.25	97%	45%
0.30	95%	46%
0.35	95%	55%
0.50	89%	60%
0.75	89%	66%
1.00	84%	68%
2.00	69%	80%
3.00	46%	85%
4.00	30%	89%
5.00	16%	91%
10.00	3%	94%

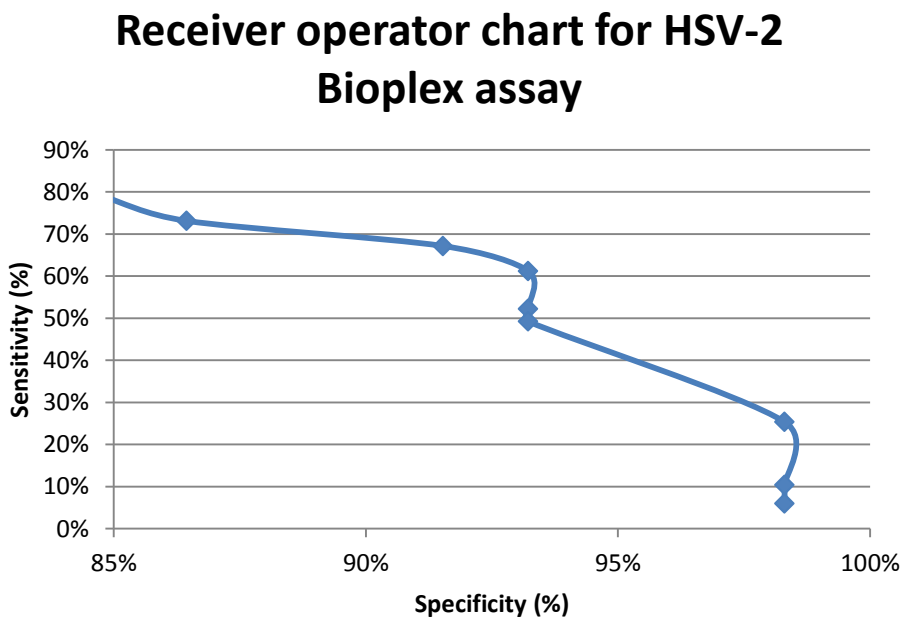


Figure 3.44: Receiver operator characteristic (ROC) curve for HSV-2 Bi-plexed assay

Table 3.20 The sensitivity and specificity of the HSV-2 Bi-plexed assay with different HSV-2 cut-off levels

HSV-2 Cut Off level (AU/mL)	Sensitivity (%)	Specificity (%)
0.05	79%	85%
0.10	73%	86%
0.20	67%	92%
0.30	61%	93%
0.40	52%	93%
0.50	49%	93%
1.00	25%	98%
2.00	10%	98%
3.00	6%	98%

3.8 MMRV Multiplex Immunoassay

3.8.1 Validation of MMRV Bioplex assay using RIVM sample panel

A multiplex fluorescent microbead immunoassay to detect and measure the measles, mumps, rubella and Varicella Zoster antibody levels in human serum, previously developed by RIVM assay, was evaluated and validated using a sample panel provided by RIVM. The panel contained 40 serum samples with a broad range of antibody concentrations. Initially, each antigen was coupled to the different carboxylated microspheres using a modified two step carbodiimide reaction. A standard curve for this Bioplex assay was generated using the rubella standard serum (RUBI-1-94) with three fold-dilutions, starting from 1/400 to 1/7873200. For validation of MMRV Bioplex assay, 40 RIVM serum samples were tested in triplicate and compared with the results obtained from the RIVM by plotting a scatterplot for each antigen. The correlation (r) between each antigen with its RIVM result was greater than 0.95, with 0.9715 for measles (Figure 3.45), 0.9806 for mumps (Figure 3.46), 0.9724 for rubella (Figure 3.47) and 0.9610 for VZV (Figure 3.48), respectively. A discrepancy between the test results and RIVM results was observed for each antigen below the RIVM assigned cut-off level. The evaluation for MMRV bioplex assay was also performed by testing 40 RIVM serum samples with two different batches of BSA, BSA from the Vaccine Evaluation Unit (VEU), Manchester Royal Infirmary and BSA from RIVM. The comparison between the VEU BSA and RIVM BSA for each antigen was high, with 0.9640 for measles (Figure 3.49), 0.9401 for mumps (Figure 3.50), 0.9896 for rubella (Figure 3.51) and 0.9255 for VZV (Figure

3.52), respectively. As with the previous comparison of VEU results and RIVM results, there was less correlation between the VEU BSA and RIVM BSA for each antigen in the lower-concentration region. The analyses indicated that there was a strong correlation and near identicality between VEU results and RIVM results for each antigen with a small discrepancy in the lower concentration region.

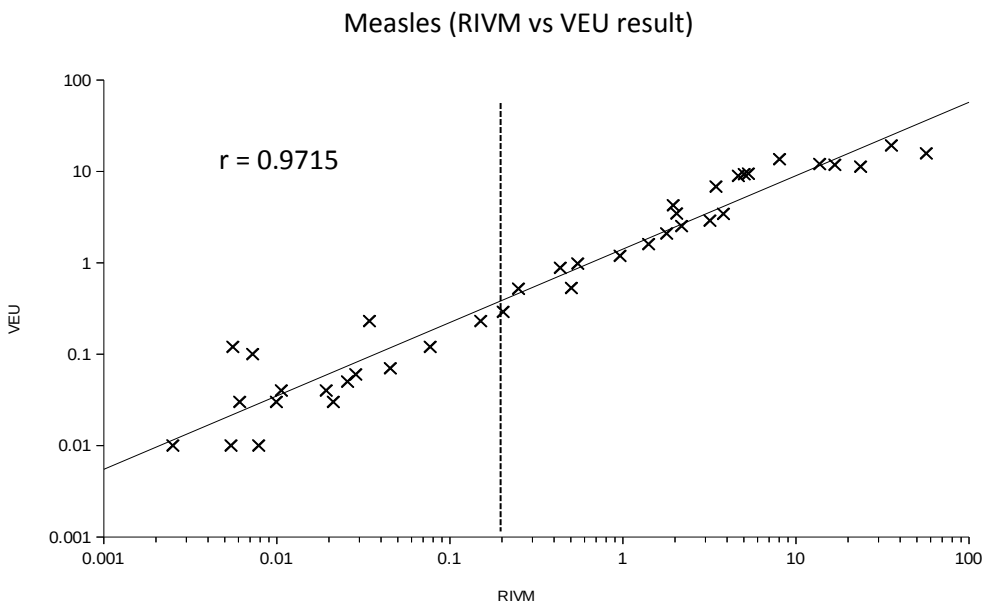


Figure 3.45: Correlation analysis on measured serum measles antibody concentration comparing RIVM result and the tested result (VEU result). The measurements were performed on 40 RIVM sera; correlation (r) was 0.9715. The cut-off level was indicated by vertical dashed line at 0.2 IU/mL**.**

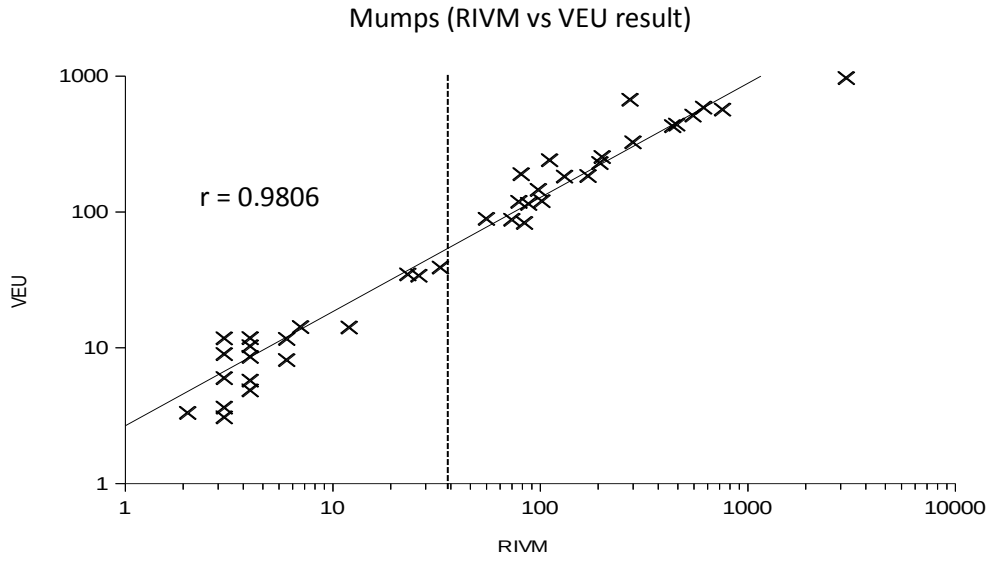


Figure 3.46: Correlation analysis on measured serum mumps antibody concentration comparing RIVM result and the tested result (VEU result). The measurements were performed on 40 RIVM sera; correlation (r) was 0.9806. The cut-off level was indicated by vertical dashed line at 45 RU/mL.

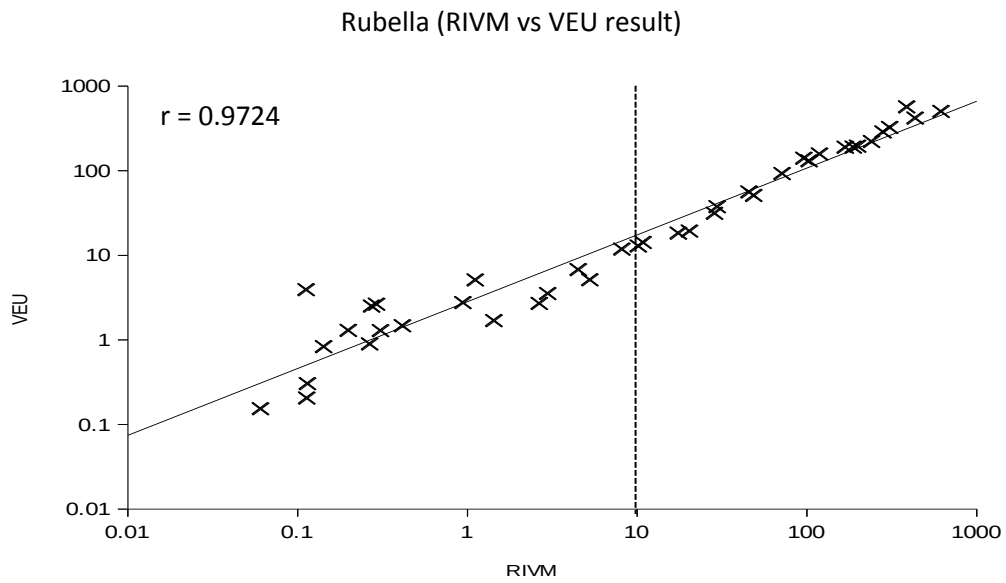


Figure 3.47: Correlation analysis on measured serum Rubella antibody concentration comparing RIVM result and tested result (VEU result). The measurements were performed on 40 RIVM sera; correlation was 0.9724. The cut-off level was indicated by vertical dashed line at 10 IU/mL.

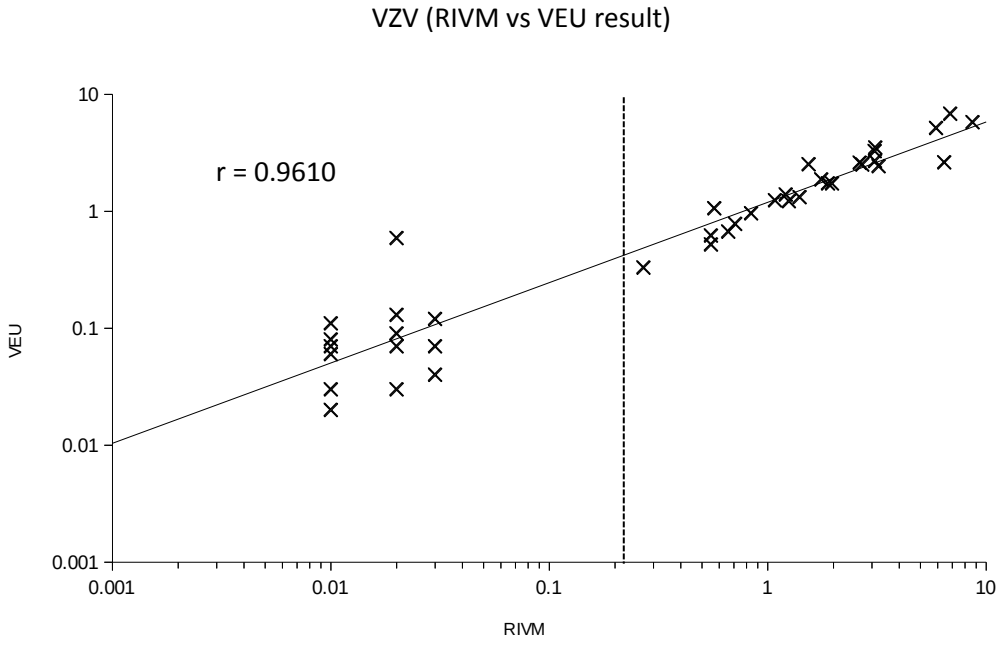


Figure 3.48: Correlation analysis on measured serum VZV antibody concentration comparing RIVM result and the tested result (VEU result). The measurements were performed on 40 RIVM sera; correlation (r) was 0.9610.The cut-off level was indicated by vertical dashed line at 0.26 IU/mL.

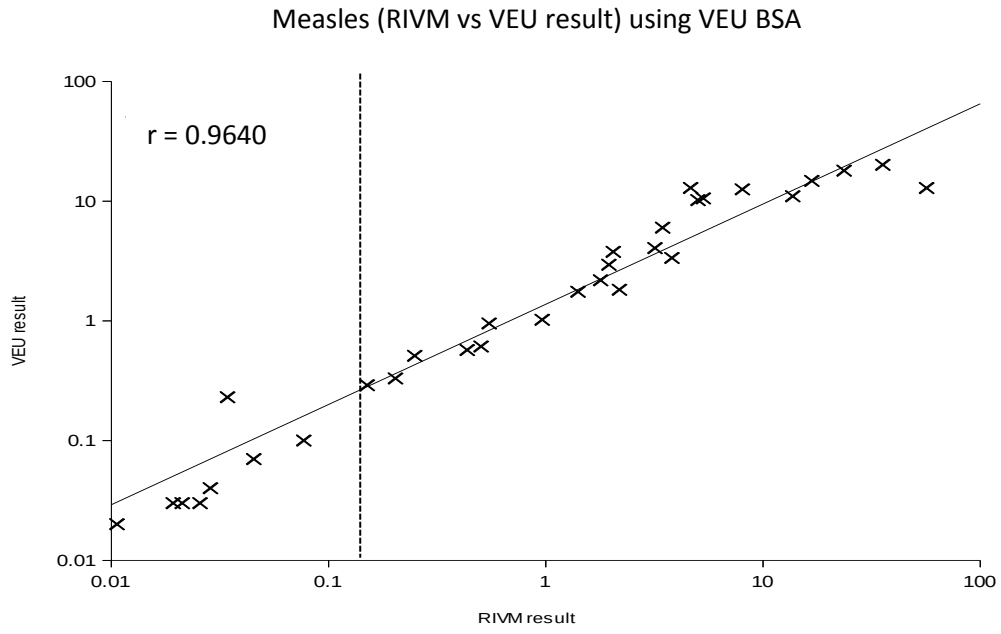


Figure 3.49: Correlation analysis on measured serum measles antibody concentration comparing RIVM result and the tested result (VEU result) using VEU BSA. The measurements were performed on 40 RIVM sera; correlation (r) was 0.9640.The cut-off level was indicated by vertical dashed line at 0.2 IU/mL.

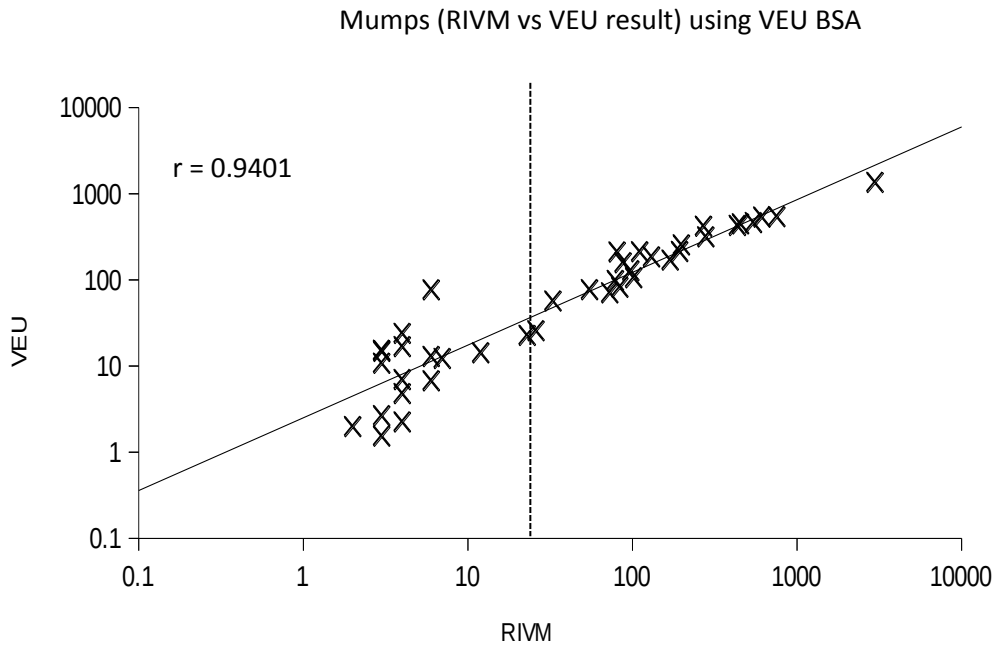


Figure 3.50: Correlation analysis on measured serum mumps antibody concentration comparing RIVM result and the tested result (VEU result) using VEU BSA. The measurements were performed on 40 RIVM sera; correlation (r) was 0.9401. The cut-off level was indicated by vertical dashed line at 45 RU/mL.

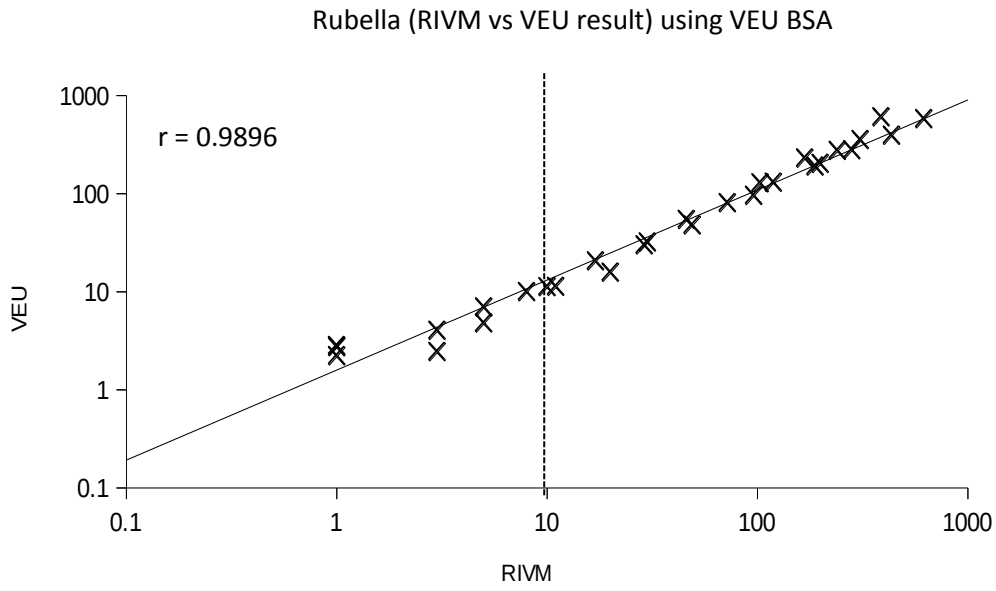


Figure 3.51: Correlation analysis on measured serum rubella antibody concentration comparing RIVM result and the tested result (VEU result) using VEU BSA. The measurements were performed on 40 RIVM sera; correlation (r) was 0.9896. The cut-off level was indicated by vertical dashed line at 10 IU/mL.

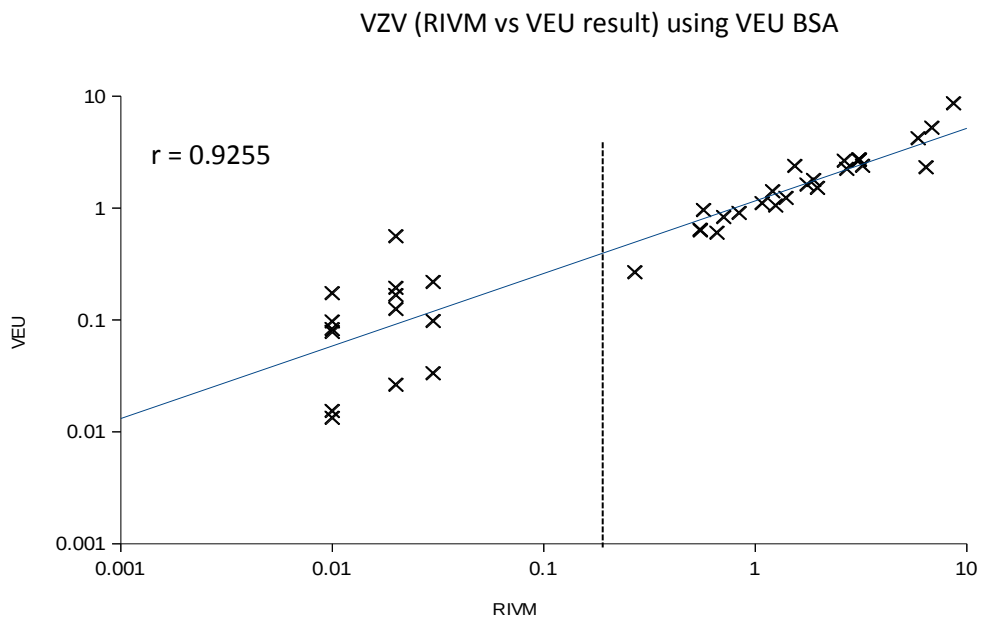


Figure 3.52: Correlation analysis on measured serum VZV antibody concentration comparing RIVM result and the tested result (VEU result) using VEU BSA. The measurements were performed on 40 RIVM sera; correlation (r) was 0.9255. The cut-off level was indicated by vertical dashed line at 0.26 IU/mL.

3.8.2 Comparison of proportion of Measles, Mumps, Rubella and Varicella Zoster antibody positive individuals in 1996 and 2012

A total of 612 MMRV test samples (Appendix 8), collected in 2012, were analysed for the presence of specific IgG antibodies against measles, mumps, rubella and varicella zoster by the MMRV multiplex assay as describe in section 2.9.1.4. The RIVM assigned protective concentrations were used to determine the proportion of MMRV seropositivity for the test samples, with ≥ 0.2 IU/mL for measles, ≥ 45 RU/mL for mumps, ≥ 10 IU/mL for rubella and ≥ 0.26 IU/mL for varicella, respectively. The antibody proportion for each assay was calculated as the

number of seropositive samples divided by the total number of samples tested.

3.8.2.1 Statistical analaysis of the proportion of Measles, Mumps, Rubella and Varicella Zoster antibody positive individuals in 1996 and 2012 using Chi-square

In order to determine whether there was a significant difference between the 1996 proportion of antibody positive individuals (De Melker et al., 2003; Nardone et al., 2003; Vyse et al., 2004; Pebody et al., 2000) and the 2012 proportion of antibody positive individuals for measles, mumps, rubella and varicella zoster viruses in England, the proportions determined in each of the two time periods were compared using Chi-square (McHugh, 2013). The results showed that there were significant differences between the proportions of antibody positive individuals in 1996 and 2012 for measles (Table 3.21) and mumps (Table 3.22), with the p-value of 0.0048 and 0.0001, respectively. For the rubella (Table 3.23) and VZV (Table 3.24) viruses, there were no significant differences between the proportion of antibody positive individuals in 1996 and 2012, with the p-value of 0.5247 and 0.9661, respectively.

Table 3.21 Proportion of measles virus antibody positive individuals in 1996 and 2012

Year	Positive samples	Negative samples	Antibody Proportion
1996	2332	434	0.8431
2012	544	68	0.8889

H_o : There were no significant differences between the proportion of measles virus antibody positive individuals in 1996 and 2012

The p-value from the chi-square was 0.0048 ($p<0.05$). Therefore, the null hypothesis (H_o) was rejected. There were significant differences between the proportion of measles virus antibody positive individuals in 1996 and 2012. In 2012, the measles antibody prevalence had increased to 0.8889 from 0.8431 in 1996 (difference between proportion = 0.04579, 95% C.I (0.01464 to 0.07695)).

Table 3.22 Proportion of mumps virus antibody positive individuals in 1996 and 2012

Year	Positive samples	Negative samples	Antibody Proportion
1996	1865	901	0.6742
2012	525	87	0.8578

H_o : There were no significant differences between the proportion of mumps virus antibody positive individuals in 1996 and 2012

The p-value from the chi-square test was 0.0001 ($p<0.05$). Therefore, the null hypothesis is rejected. There were significant differences between the proportion of mumps virus antibody positive individuals in 1996 and 2012. In 2012, the mumps antibody prevalence had increased to 0.8578 from 0.6742 in 1996 (difference between proportion = 0.1836, 95%C.I (0.1437 to 0.2234)).

Table 3.23 Proportion of rubella virus antibody positive individuals in 2012 and 1996

Year	Positive samples	Negative samples	Antibody Proportion
1996	2439	327	0.8817
2012	534	78	0.8725

H_o : There were no significant differences between the proportion of rubella virus antibody positive individuals in 1996 and 2012

The p-value from the chi-square test was 0.5247 ($p<0.05$). Therefore, the null hypothesis was accepted. There were no significant differences between the proportion of rubella virus antibody positive individuals in 1996 and 2012. In 2012, the rubella antibody prevalence had decreased to 0.8725 from 0.8817 in 1996 (difference between proportion = 0.00923, 95%C.I (-0.01922 to 0.03768)).

Table 3.24 Proportion of varicella zoster virus antibody positive individuals in 1996 and 2012

Year	Positive samples	Negative samples	Antibody proportion
1996	1677	414	0.8020
2012	491	121	0.8022

H_o : There were no significant differences between the proportion of rubella virus antibody positive individuals in 2012 and 1996

The p-value from the chi-square test was 0.9661 ($p<0.05$). Therefore, the null hypothesis was accepted. There were no significant differences between the proportion of varicella zoster virus antibody positive individuals in 1996 and 2012. In 2012, the varicella zoster virus antibody prevalence had increased to 0.8022 from 0.8020 in 1996 (difference between proportion = 0.0002, 95%C.I (-0.03562 to 0.03618)).

3.8.2.2 Comparison of the proportion of Measles, Mumps, Rubella and Varicella Zoster antibody positive individuals for gender and regions in 2012

The differences among the proportion of antibody positive individuals for gender and region in 2012 were assessed using a chi-square test. Because 4 out of 612 MMRV test samples were from individuals with unknown sex, a total of 608 test samples were analysed by gender. The results indicated that there were no significant difference between the 2012 proportion of antibody positive individuals in male and female for all the MMRV viruses, with the p-value of 0.2458 for measles (Table 3.25), 0.9998 for mumps (Table 3.26), 0.2728 for rubella (Table 3.27) and 0.6104 (Table 3.28) for VZV, respectively. For region, the 2012 MMRV seroprevalance data were categorised into three different groups: Group 1 (East, SouthWest, SouthEast and London), Group 2 (NorthEast, NorthWest and Yorkshire) and Group 3 (West Midlands and East Midlands). The proportions of antibody positive individuals in regions were almost similar for measles (Table 3.29) and mumps (Table 3.30), with no statistically significant differences, p-value of 0.3203 for measles and 0.0863 for mumps, respectively, while for the rubella (Table 3.31) and varicella zoster viruses (Table 3.32), there were significant differences between the 2012 proportion of antibody positive individuals in regions, with the p-value of 0.0310 and 0.0422, respectively.

Table 3.25 Proportion of measles antibody positive individuals by gender

Gender	positive	Negative	Antibody proportion
Male	257	37	0.8832
Female	284	30	0.9045

H_o: There were no significant differences between the 2012 proportion of measles virus antibody positive individuals by gender

The p-value from the chi-square test was 0.2458 ($p<0.05$). Therefore, the null hypothesis was accepted. There were no significant differences between the 2012 proportion of measles antibody positive individuals in male and female. The proportions of measles positive individuals in male and female were 0.8832 and 0.9045, respectively.

Table 3.26 Proportion of mumps antibody positive individuals by gender

Gender	Positive	Negative	Antibody proportion
Male	252	42	0.8571
Female	270	44	0.8598

H_o: There were no significant differences between the 2012 proportion of mumps antibody positive individuals by gender

The p-value from the chi-square test was 0.9998 ($p<0.05$). Therefore, the null hypothesis was accepted. There were no significant differences between the 2012 proportion of mumps antibody positive individuals in male and female. The proportions of mumps positive individuals in male and female were 0.8571 and 0.8598, respectively.

Table 3.27 Proportion of rubella antibody positive individuals by gender

Gender	Positive	Negative	Antibody Proportion
Male	252	42	0.8571
Female	279	35	0.8885

H_o: There were no significant differences between the 2012 proportion of rubella antibody positive individuals by gender

The p-value from the chi-square test was 0.2728 ($p<0.05$). Therefore, the null hypothesis was accepted. There were no significant differences between the 2012 proportion of rubella antibody positive individuals in male and female. The proportions of rubella positive individuals in male and female were 0.8571 and 0.8885, respectively.

Table 3.28 Proportion of varicella zoster virus antibody positive individuals by gender

Gender	Positive	Negative	Antibody Proportion
Male	233	61	0.7925
Female	255	59	0.8121

H_o: There were no significant differences between the 2012 proportion of varicella zoster virus antibody positive individuals by gender

The p-value from the chi-square test was 0.6104 ($p<0.05$). Therefore, the null hypothesis was accepted. There were no significant differences between the 2012 proportion of VZV antibody positive individuals in male and female. The proportions of VZV positive individuals in male and female were 0.7925 and 0.8121, respectively.

Table 3.29 Proportion of measles antibody positive individuals by region

Region	Positive	Negative	Antibody proportion
East, SouthEast,London and SouthWest	221	23	0.9057
NorthEast, NorthWest and Yorkshire	258	33	0.8866
Emids and Wmids	65	12	0.8442

H_o: There were no significant differences between the 2012 proportion of measles antibody positive individuals by region.

The p-value from the chi-square test was 0.3203 ($p<0.05$). Therefore, the null hypothesis was accepted. There were no significant differences between the 2012 proportion of measles antibody positive individuals by region.

Table 3.30 Proportion of mumps antibody positive individuals by region

Region	Positive	Negative	Antibody proportion
East, SouthEast,London and SouthWest	217	27	0.8893
NorthEast, NorthWest and Yorkshire	247	44	0.8488
Emids and Wmids	61	16	0.7922

H_o: There were no significant differences between the 2012 proportion of mumps antibody positive individuals in regions.

The p-value from the chi-square test was 0.0863 ($p<0.05$). Therefore, the null hypothesis was accepted. There were no significant differences between the 2012 proportion of mumps antibody positive individuals in region.

Table 3.31 Proportion of rubella antibody positive individuals by region

Region	Positive	Negative	Antibody proportion
East, SouthEast,London and SouthWest	217	27	0.8893
NorthEast, NorthWest and Yorkshire	257	34	0.8832
Emids and Wmids	60	17	0.7792

H_o: There were no significant differences between the 2012 proportion of rubella antibody positive individuals by regions.

The p-value from the chi-square test was 0.0310 ($p<0.05$). Therefore, the null hypothesis was rejected. There were significant differences between the 2012 proportion of rubella antibody positive individuals by region.

Table 3.32 Proportion of rubella antibody positive individuals by region

Data analysed for VZV	Positive	Negative	Antibody proportion
East, SouthEast,London and SouthWest	206	38	0.8443
NorthEast, NorthWest and Yorkshire	203	65	0.7575
Emids and Wmids	59	18	0.7662

H_o: There were no significant differences between the 2012 proportion of varicella zoster virus antibody positive individuals in regions.

The p-value from the chi-square test was 0.0422 ($p<0.05$). Therefore, the null hypothesis was rejected. There were significant differences between the 2012 proportion of VZV antibody positive individuals by region.

3.8.2.3 Proportion of Measles, Mumps, Rubella and Varicella Zoster antibody positive individuals by age group in 1996 and 2012

The MMRV seroprevalance data of 2012 were further analysed by age group, and were then compared with the seroprevalance studies conducted in 1996. In order to construct the comparable age groups with age groups sampled in 1996, the age groups sampled in 2012 were divided into 9 age groups for measles, mumps and rubella viruses; 0 to 1 years, 2 year, 3 to 5 years, 6 year, 7 to 8 years, 9 to 24 years, 25 to 39 years, 40 to 59 years and >60 years, respectively. For varicella zoster

virus, five age groups were assembled as follow; 0 to 4 years, 5 to 9 years, 10 to 14 years, 15 to 19 years and > 20 years. The proportion of antibody positive individuals for measles (Figure 3.53), mumps (Figure 3.54) and rubella (Figure 3.55) viruses had increased in infants aged 0 to 1 years up to children aged 2 year in both 2012 and 1996. In 2012, the proportion of measles (Figure 3.53) antibody was increased in older children and then remained constant up to the age of 60 years and above, while for 1996, the proportion of measles antibody positive decreased in children aged 7 to 8 years and increased in age 9 to 24 years to a level that remained constant through adulthood (to > 60 years). A steady increase was observed for 2012 proportion of mumps antibody positive individuals (Figure 3.54) within children aged 3 to 5 years up to children aged 7-8 years. The 2012 proportion of mumps antibody positive then decreased in the age groups from 9 to 24 years and increased in age 25 to 39 years to a level that remained constant to age of 60 years and more. There was a different profile for the 1996 proportion of mumps antibody positive. The 1996 proportion of mumps antibody positive individuals decreased in the age group of 3 to 5 years and then started to increase from 6 year old children onward. Further increases were observed for 1996 proportion of mumps antibody positive individuals in children aged 7 to 8 years to a level that remained constant in age >60 years. For the proportions of rubella antibody positive (Figure 3.55), the trends for both 1996 and 2012 were similar. The proportions were further increased in children aged 3 to 5 years to a level that remained constant in adults (>60 years). The proportions of varicella zoster virus (Figure 3.56) antibody positive individuals for both years were low in those aged 0 to 4 years and

increased in those aged 5 to 9 years and then remained constant up to > 60 years.

The overall age distribution curve revealed that the 2012 proportion of antibody positive individuals for all the viruses were higher than in 1996.

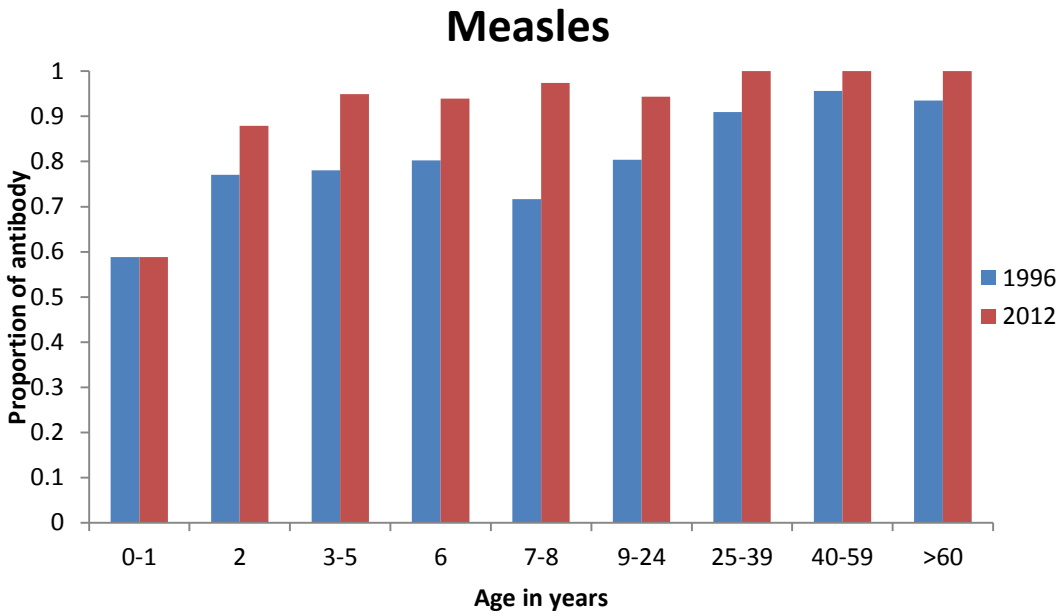


Figure 3.53: Distribution of 1996 and 2012 proportions of measles antibody positive individuals by age group

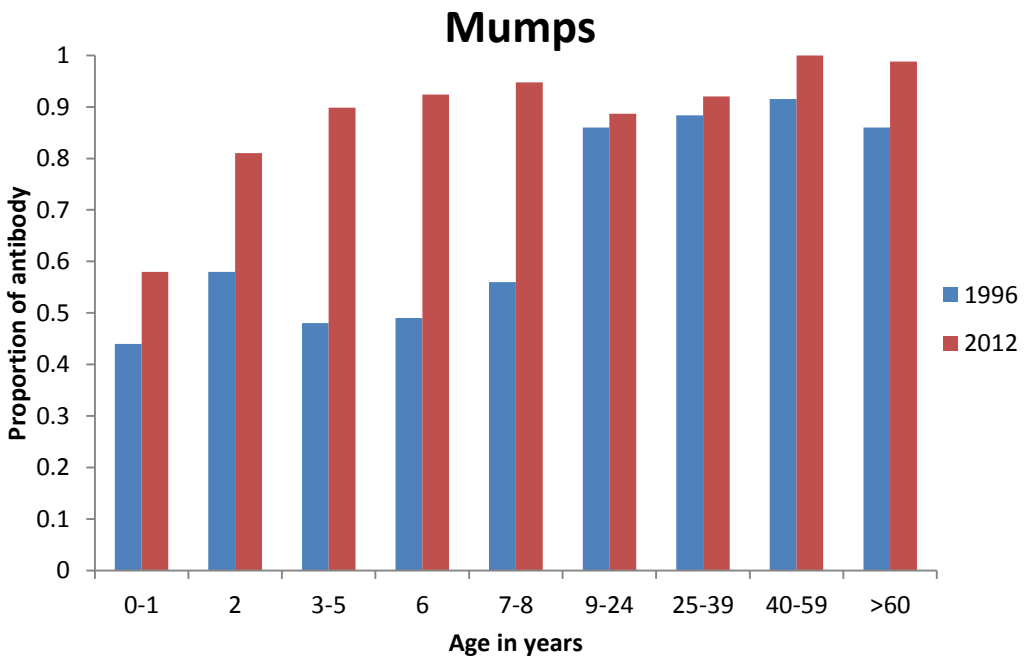


Figure 3.54: Distribution of 1996 and 2012 proportions of mumps antibody positive individuals by age group

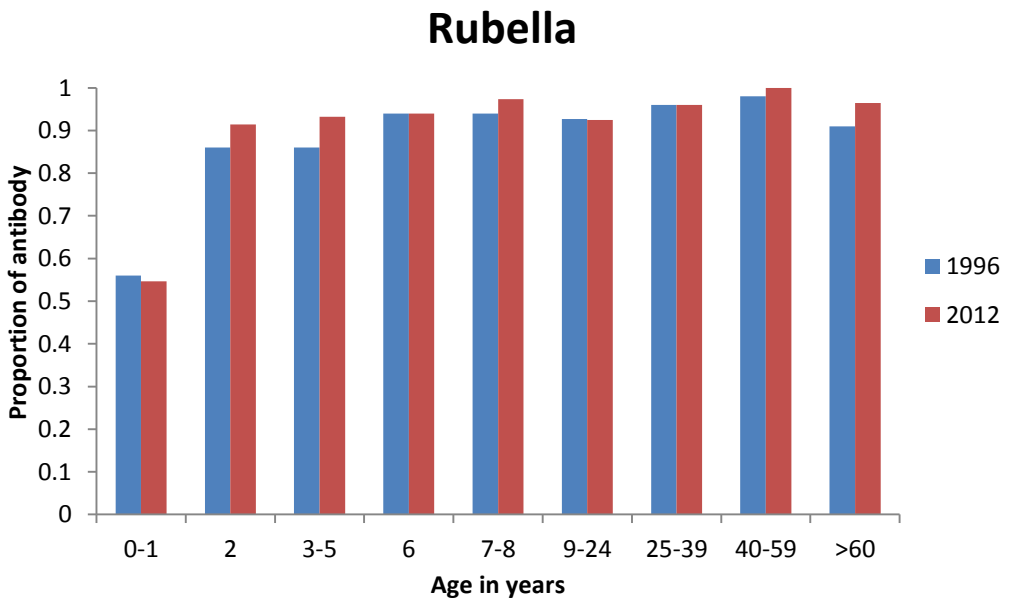


Figure 3.55: Distribution of 1996 and 2012 proportions of rubella antibody positive individuals by age group

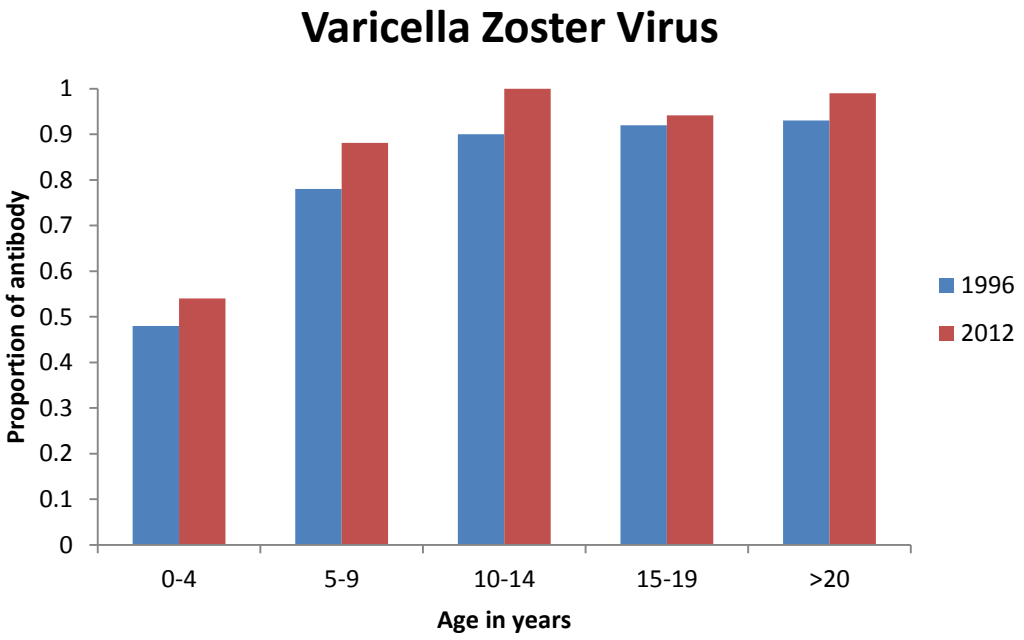


Figure 3.56: Distribution of 1996 and 2012 proportions of varicella zoster virus antibody positive individuals by age group

3.8.2.4 Multiple comparison of proportion of Measles, Mumps, Rubella and Varicella Zoster antibody positive individuals between the age group in 1996 and 2012

In order to determine the association between the age groups in 2012 and 1996 for each virus, data were further analysed using a one proportion Z-test with Bonferroni correction (Sheskin, 2004). One proportion Z-test was selected because the sample sizes of the age groups for each virus were generally small, approximately 50 samples for each age group and unequal in number. In order to avoid spurious results during the multiple-comparison between the age groups in 1996 and 2012, it was necessary to adjust the critical p-values for the assay using the Bonferroni correction. The Bonferroni correction was used to reduce the chance of false-positive results when the statistical tests were performed simultaneously. To perform a Bonferroni correction in this assay, the critical P-value (0.05) was divided by the number of age groups and compared with the p-value obtained from the one proportion Z-test for each set of the age group. The null hypothesis was rejected if p-value was less than adjusted p-value. The results indicated that there were significant differences between the 1996 and 2012 proportion of measles (Table 3.33) and mumps (Table 3.34) antibodies positive individuals for all age groups, except age 0 to 1 years, 2 year and 40 to 59 years for mumps virus and age 9 to 24 years and aged 25 to 39 years for mumps virus, respectively. For rubella virus (Table 3.35), the statistical test showed there were no significant differences between the proportion of antibody positive individuals in 2012 and 1996 for all age groups, while for varicella

zoster virus (Table 3.36), statistically significant differences were observed in the age groups 5 to 9 years, 10 to 14 years and > 20 years.

Table 3.33 Multiple comparison of 1996 and 2012 proportion of measles antibody positive individuals in age group

Age group (years)	Proportion (1996)	Proportion (2012)	z-value	p	Significant p<0.0056*
0-1	0.5882	0.5882	0.0000	1.0000	no
2	0.7702	0.8793	-2.5780	0.0099	no
3-5	0.7805	0.9491	-5.9914	p<0.0001	yes
6	0.8028	0.9393	-4.7892	p<0.0001	yes
7-8	0.7169	0.9736	-12.7762	p<0.0001	yes
9-24	0.8041	0.9433	-4.2917	p<0.0001	yes
25-39	0.9094	1.0000	-5.6854	p<0.0001	yes
40-59	0.9564	1.0000	-2.0449	0.0409	no
>60	0.9351	1.0000	-4.6330	p<0.0001	yes

* There were nine age groups with a desired critical p-value = 0.05. The Bonferroni correction tested each individual hypothesis as, critical p-value = 0.05/9 = 0.0056.

H_o: There were no significant differences between the 1996 and 2012 proportion of measles antibody positive individuals by age group.

Based on the one proportion Z-test with Bonferroni correction, there were significant differences between 1996 and 2012 proportion of measles antibody positive individuals for all the age groups, except age 0 to 1 years, 2 year and 40 to 59 years.

Table 3.34 Multiple comparison of 1996 and 2012 proportion of mumps antibody positive individuals in age group

Age group (years)	Proportion (1996)	Proportion (2012)	z-value	p	Significant p<0.0056*
0-1	0.4400	0.5798	-3.0897	0.0020	yes
2	0.5800	0.8103	-4.4650	p<0.0001	yes
3-5	0.4800	0.8983	-10.7538	p<0.0001	yes
6	0.4900	0.9242	-12.8766	p<0.0001	yes
7-8	0.5600	0.9473	-15.600	p<0.0001	yes
9-24	0.8600	0.8867	-0.6980	0.4852	no
25-39	0.8833	0.9200	-1.0426	0.2971	no
40-59	0.9150	1.0000	-4.7715	p<0.0001	yes
>60	0.8600	0.9882	-12.0458	p<0.0001	yes

* There were nine age groups with a desired critical p-value = 0.05. The Bonferroni correction tested each individual hypothesis as, critical p-value = 0.05/9 = 0.0056.

H_o: There were no significant differences between the 1996 and 2012 proportion of mumps antibody positive individuals by age group.

Based on the one proportion Z-test with Bonferroni correction, there were significant differences between 1996 and 2012 proportion of mumps antibody positive individuals for all the age groups, except age 9 to 25 years and 25 to 39 years.

Table 3.35 Multiple comparison of 1996 and 2012 proportion of rubella antibody positive individuals in age group

Age group (years)	Proportion (1996)	Proportion (2012)	Z-Score	P-value	Significant? P<0.0056*
0-1	0.5600	0.5462	0.2193	0.8264	no
2	0.8600	0.9137	-1.3306	0.1833	no
3-5	0.8600	0.9322	-2.1073	0.0351	no
6	0.9400	0.9393	0.0000	1.0000	no
7-8	0.9400	0.9736	-1.5331	0.1252	no
9-24	0.9266	0.9245	0.2683	0.7884	no
25-39	0.9600	0.9600	0.0000	1.0000	no
40-59	0.9800	1.0000	-0.6816	0.4955	no
>60	0.9100	0.9647	-2.3524	0.0187	no

* There were nine age groups with a desired critical p-value = 0.05. The Bonferroni correction tested each individual hypothesis as, critical p-value = 0.05/9 = 0.0056.

H_o: There were no significant differences between the 1996 and 2012 proportion of rubella antibody positive individuals by age group.

Based on the one proportion Z-test with Bonferroni correction, there were no significant differences between 1996 and 2012 proportion of rubella antibody positive individuals for all the age groups.

Table 3.36 Multiple comparison of 1996 and 2012 proportion of varicella zoster virus antibody positive individuals in age group

Age group (years)	Proportion (1996)	Proportion (2012)	z-value	p-value	Significant p<0.01*
0-4	0.4800	0.5399	-1.757	0.0789	no
5-9	0.7800	0.8809	-3.9886	p<0.0001	yes
10-14	0.9000	1.0000	-3.3845	0.0007	yes
15-19	0.9200	0.9411	-0.3472	0.7284	no
>20	0.9300	0.9900	-8.5280	p<0.0001	yes

* There were five age groups with a desired critical p-value = 0.05. The Bonferroni correction tested each individual hypothesis as, critical p-value = 0.05/5 = 0.01.

H_o: There were no significant differences between the 1996 and 2012 proportion of varicella zoster virus antibody positive individuals by age group.

Based on the one proportion Z-test with Bonferroni correction, there were significant differences between 1996 and 2012 proportion of VZV antibody positive individuals for age 5 to 9 years, 10 to 14 years and > 20 years.

3.8.2.5 Multiple comparison of the 2012 proportion of rubella and VZV antibody positive individuals in each region

The chi-square test described in section 3.7.3.2 indicated that only rubella and VZV proportions of antibody positive individuals showed significant differences between regions in 2012. In order to determine the region that was significantly difference from other for rubella and VZV, the test was further analysed using the Hochberg's sequential method (Hochberg, 1988). This test is a 'step-up' version of the Bonferroni test, which uses sequential methods to correct the critical p-value in order to avoid the cumulative type 1 error (i.e.: incorrect rejection of true null

hypothesis) during multiple comparisons on a single data set. The Hochberg's sequential method was initially conducted by comparing each set of the region using chi-square test and then ordered the p-values from smallest to largest in a 'step up' approach. The p-value was adjusted from the previous number of test based on the formula shown below:-

Adjusted p-value (5% of significant) = 0.05/n+1-K, where n = number of comparisons, K=1,2,3….n

The adjusted p-values were ordered from largest (unadjusted p-value) to smallest and compared with the p-value obtained from the chi-square test. The null hypothesis was rejected if a p-value was less than the adjusted p-value. The results indicated that for group 3 (West Midlands and East Midlands) region, there was a significant difference to group 1 (East, SouthWest, SouthEast and London) and group 2 (NorthEast, NorthWest and Yorkshire) regions for the proportion of rubella (Table 3.37) antibody positive individuals in 2012. For varicella zoster virus (Table 3.38), there was a significant difference between the proportion of antibody positive individuals for group 1 and group 2 regions.

Table 3.37 Multiple comparison of 2012 proportion of rubella antibody positive individuals in region

Comparison	P	Adj. P	Significant?
Group 1 vs Group 2	0.3391	0.0500	No
Group 2 vs Group 3	0.0119	0.0250	Yes
Group 1 vs Group 3	0.0099	0.0167	Yes

Note: Group 1 (East, SouthWest, SouthEast and London), Group 2 (NorthEast, NorthWest and Yorkshire) and Group 3 (West Midlands and East Midlands)

H_o: There were no significant differences between the 2012 proportion of rubella antibody positive individuals by region.

Based on the Hochberg method, the group 3 (West Midlands and East Midlands) region was significantly difference to group 1 (East, SouthWest, SouthEast and London) and group 2 (NorthEast, NorthWest and Yorkshire) regions for the proportion of rubella antibody positive individuals in 2012.

Table 3.38 Multiple comparison of 2012 proportion of varicella zoster virus antibody positive individuals in region

Comparison	P	Adj. P	Significant?
Group 2 vs group 3	0.1042	0.0500	No
Group 1 vs group 3	0.0436	0.0250	No
Group 1 vs group 2	0.0147	0.0167	Yes

Note: Group 1 (East, SouthWest, SouthEast and London), Group 2 (NorthEast, NorthWest and Yorkshire) and Group 3 (West Midlands and East Midlands)

H_o: There were no significant differences between the 2012 proportion of VZV antibody positive individuals by region.

Based on the Hochberg method, there was a significant difference between group 1 (East, SouthWest, SouthEast and London) and group 2 (NorthEast, NorthWest and Yorkshire) regions for the 2012 proportion of VZV antibody positive individuals.

4.0 Discussion

Overall immunity to measles, mumps and rubella virus (MMR) in England had increased since the introduction of MMR vaccination into the childhood immunisation programme (Vyse *et al.,* 2002). However, outbreaks of measles and mumps virus infections in England had raised concern about the level of protection to MMR in the population (Pegorie *et al.,* 2014; HPA, 2012; PHE, 2014e; Vyse *et al.,* 2000). For herpes simplex virus, changes in sexual practices and susceptibility had led to a reversal in distribution of the type of herpes simplex virus found in initial infection of the human genital tract suggesting a change in the levels of immunity to these viruses (Pereira *et al.,* 2012; Cowan *et al.,* 2002; Forward and Lee, 2003). The seroepidemiology of Varicella Zoster virus was also believed to have altered with increasing numbers of young adults suffering primary infection (chickenpox) (Fairley and Miller, 1996). This change in the age related acquisition of the virus had profound effect on levels of disease. Studies indicated that the acquisition of VZV infection during adulthood can be associated with a higher morbidity and mortality compared to a primary VZV infection in childhood, especially for the pregnant women, who may cause complicated varicella to themselves and fetus (Lamont *et al.,* 2011; Mohsen and McKendrick, 2003; Tunbridge *et al.,* 2008). Based on these assumptions, the seroepidemiological studies play an essential role in studies of transmission and control of MMRV and

HSV infections, in order to allow the breaking of the chain of infection in human population.

However, a problem with previous seroepidemiological studies has been the variation in test results when different methodologies have been used (WHO, 2013; Andrews *et al.,* 2000). In order to avoid such variability, multiplex immunoassays have been developed to standardise such assays (Binnicker *et al.,* 2011). Multiplex bead immunoassays allow concentrations of multiple antibodies to be determined within the same specimen. This technique offers substantial cost and time-saving over the ELISAs in high-throughput manner (Smits *et al.,* 2012; Binnicker *et al.,* 2010). The study was designed to investigate the age-related seroepidemiology of measles, mumps, rubella, varicella zoster, herpes simplex type 1 and type 2 viruses using multiplex bead immunoassay. As the antigens for all viruses in this study except HSV-1 and HSV-2 were commercially available, the study initially focused on producing HSV-1 and HSV-2 specific antigens to develop a similar multiplex bead immunoassay HSV-1 and HSV-2 antibodies.

4.1 HSV-1 gG and HSV-2 gG protein expression

Herpes simplex virus type 1 (HSV-1) and HSV-2 genomes are closely related and share approximately 83% of identically aligned nucleotides (McGeoch *et al.,* 1987; Dolan *et al.,* 1998). This genotypic similarity is expressed phenotypically and results in extensive antigenic

cross-reactivity between the two virus types. The description and characterisation of glycoprotein G by McGeoch and colleagues (1987) rekindled interest in distinguishing between these two HSV subtypes. Studies indicated that the severity of HSV-1 and HSV-2 infection is similar in genital herpes episodes; however, recurrences of HSV-1 are more infrequent than those that occur with HSV-2 (Ashley and Wald, 1999; Wald and Ashley-Morrow, 2002; Ikoma *et al.,* 2002). The correct diagnosis of HSV infection is crucial for counseling and treating herpes-infected patients and their partners and for seroepidemiological studies of HSV-1 and HSV-2 (Wald and Ashley-Morrow, 2002; Schmid *et al.,* 1999). HSV-1 gG and HSV-2 gG are nonessential in virus infectivity. They both have similarity between the signal sequence and in the short membrane anchor sequence (Schmid *et al.,* 1999). However, both regions are removed during posttranslational modification and in membrane integration (Rapoport, 1986). Thus, only the dissimilar epitopes of the gG molecules are exposed as antigens. Many studies have been performed using truncated glycoprotein G of both virus types as an immunogen to induce a type-specific antibody response (Schmid *et al.,* 1999; Ikoma *et al.,* 2002; Tunbäck *et al.,* 2000); however, using truncated-glycoprotein as a 'type-specific antigen' has limitations in term of specificity and sensitivity of the assay due to the mutation or deletion of the HSV gG during the expression of truncated forms (Wald and Ashley-Morrow, 2002; LeGoff *et al.,* 2014). The complete sequence of glycoprotein G was thus more likely to yield

adequate representation of native form of the glycoprotein. To achieve the aim of full mimic of ‘native’ HSV gG, the baculovirus expression system offered best mimic eukaryotic glycosylation and post-translational modification compared to other expression systems. In addition, the expression of truncated HSV-1 gG and HSV-2 gG protein were also included in this study.

The defined regions for both HSV-1 gG and HSV-2 gG DNA sequences in this study, were amplified using a PCR method. Primer design plays a crucial role in PCR as poor primer design affects the PCR efficiency through non-specific amplification of irrelevant nucleic acid sequences (Dieffenbach *et al.,* 1993). The GC-content of the targeted nucleic acid sequences, the melting point of the targeted sequences, primer length, the presence of secondary structures and self 3’ complementary sequences within the targeted nucleic acid sequences are important during primer design (Kämpke *et al.,* 2001). To amplify the complete genomes of HSV-1 gG and HSV-2 gG, the appropriate gene sequences were identified by comparing published sequences of each virus obtained from GenBank. Alignment results revealed both HSV-1 gG and HSV-2 gG genes were conserved among HSV-1 and HSV-2 isolates respectively. Therefore, a single sequence for each virus was selected to design a primer. The primer sequence for each virus showed 100% homology to published sequences using NCBI blast. For the truncated

version of HSV-1 and HSV-2 gG, both viruses primer set were designed based on the published primers of Ikoma and colleagues (2002).

For amplification of the complete genome and truncated version of HSV-1 gG, initial experiments were carried out using AmpliTaq Gold polymerase mixture with the cycling parameter published by Sulaiman (2010). The HSV-1 gG PCRs produced a poor yield of product with smearing and appearance of non-specific bands. Several modifications of the PCR reaction such as alteration of $MgCl_2$ concentration and cycling parameters did not improve the product yield (Appendix 1).

Because of inadequate amplification using AmpliTaq Gold DNA polymerase mixture, the HSV-1 gG amplification was then switched to the AmpliTaq Gold 360 master mix with touchdown-PCR cycling parameters. AmpliTaq Gold 360 master mix contains an ultrastable Taq DNA polymerase, which ensures greater productivity of PCR products by reducing the risk of pipetting errors during master mix preparation compared to AmpliTaq Gold polymerase mixture. The touchdown PCR (TD-PCR) was used in amplification of HSV-1 gG. Touchdown PCR is a modification of conventional PCR that offers a simple and rapid method to increase the efficiency of PCR without redesigning the primers (Korbie and Mattick, 2008). This protocol has proved particularly useful for amplifying templates with a high percentage of GC and for DNA templates with extensive secondary structures. The TD-PCR employs higher annealing temperatures than the projected melting temperature (T_m) of the primers in

early PCR cycles, then transitions to a lower annealing temperature over the course of the temperature cycling. This approach allows the PCR reaction to enrich the correct product over any non-specific product. By using the optimal PCR parameters as described in section 2.4, the PCR results showed adequate amplification of both the complete genome and the truncated version of HSV-1 PCR products with a reduction of non-specific amplification.

For amplification of HSV-2 gG, more problems were encountered. Similar to HSV-1 gene amplification, initial experiments were carried out using AmpliTaq Gold polymerase mixture. The percentage of GC content for HSV-2 gG genome (about 70%) is higher than HSV-1 gG genome (about 60%), therefore, a modification of cycling parameters of Sulaiman (2010) was made in which initial denaturing temperature was raised from 94^0C to 96^0C. The PCR products showed a double band on a gel with the size less than 400 bp (Appendix 2). At first, it was suspected that a primer dimer was formed (i.e. where a primer preferentially self anneals or anneals with other primer in the reaction). Thus, the experiment was repeated changing the concentration of reagents such as $MgCI_2$ and AmpliTaq Gold DNA polymerase, and cycling parameters (Appendix 2), however, the same result was observed. Ideally, the formation of primer-dimers can be avoided by optimising the $MgCI_2$ concentration in the PCR mixtures. However, the result remained the same; and suggested that the presence of a double band on the gel was not the primer dimer, but a PCR

product with incorrect size. A further suggestion to explain this result was that the template formed a hairpin loop during PCR reaction. A hairpin-loop is a pattern that occurs in a single-stranded DNA when two regions of the same strand form a double helix that ends in an unpaired loop (Singh *et al.,* 2000). The loop formation for the template could influence miss-copying of the template and form a shorter than expected PCR product. For this reason, it would have been desirable to generate different primer sets to see if the PCR could be improved to prevent the possible formation of hairpin loops. Similar to HSV-1 gene amplification, the amplification of HSV-2 gG was then attempted using the AmpliTaq Gold 360 master mix. The PCR products using the AmpliTaq Gold 360 master mix were the expected size, 2152 bp for the full genome of HSV-2 gG and 1278 bp for truncated version of HSV-2 gG, respectively. As the HSV-2 clinical isolate could be amplified, it was decided to sequence the PCR product to ensure that the complete, un-mutated gene was amplified. As only one pair of primers was used to sequence the PCR product, only partial sequences were obtained, which matched with the complete sequence of HSV-2 gG genome at 5' and 3' end, with 588 bp and 357 bp, respectively (Appendix 3 and Appendix 4). The PCR products with the expected sizes by AmpliTaq Gold 360 master mix suggested that the differing PCR mixture components of AmpliTaq Gold 360 master mix and AmpliTaq Gold polymerase mixture may be responsible for unequal DNA sequences of HSV-2 gG during PCR amplification. The AmpliTaq Gold 360 master mix

contains a PCR additive, glycerol (Appendix 5). Glycerol can stabilise the polymerase and may suppress the formation of secondary structure by lowering the melting temperature of primer-template hybridization (Chakrabarti and Schutt, 2001; Roux, 2009). However, although the presence of glycerol in the PCR mixtures may increase the yield of the PCR products, this PCR additive might also form non-specific products by impairing the amplification process. There is evidence that this did occur in that there was appearance of smearing and non-specific amplification of HSV-1 gG genes (Figure 3.1 and Figure 3.2) and HSV-2 gG genes (Figure 3.4 and Figure 3.5).

In order to remove the non-specific amplicons, the relevant size bands for HSV-1 gG and HSV-2 gG DNA amplification products were excised from a gel. The excision of bands of interest from a gel is a recognised method to permit further characterisation of PCR products in cloning (Bjourson and Cooper, 1992). However, the exposure of PCR products to UV light may result in reduced fidelity in subsequent cloning even if the DNA is exposed to UV for the minimum period of time that permits excision of a band from a gel. For this reason, the blue light transillumination was used as an alternative to UV transillumination in this study. Blue light transillumination uses a high intensity LED array. This exciting light source is a ‘safe‘ light that can avoid user exposure to UV radiation and radiation damage of DNA, and it ensures samples are free from photo nicking of the DNA. After band excision, PCR products from

each virus were purified. A distinct band formed on a gel for both HSV-1 gG and HSV-2 gG purified products with the expected size, 752 bp of complete genome HSV-1 gG, 537 bp of truncated HSV-1 gG, 2123 bp of complete genome HSV-2 gG and 1278 bp of truncated HSV-2 gG, respectively. The purified PCR products then proceeded to recombinant plasmid construction using ligation independent cloning (LIC) method.

Ligation independent cloning is a method to produce expression constructs without the use of DNA ligase and restriction endonucleases (Aslanidis and de Jong, 1990). After DNA purification, the PCR product was treated with LIC-qualified T4 DNA polymerase in the presence of dATP. The HSV-1 gG and HSV-2 gG genes with specific-compatible overhangs were generated by the 3'-5' exonuclease activity of T4 DNA polymerase. The target gene was then annealed into the pIEx/Bac-3 3C/LIC plasmid vector in the absence of ligase (Figure 3.10). The pIEx/Bac-3 3C/LIC plasmid vector was a 3C/LIC prepared version of pIEx/Bac-3 that was designed for directional cloning of gene interest. Moreover, the pIEx/Bac-3 vectors were dual-purpose vectors that can be used directly for transfection of insect cells or as a donor plasmid in the BacMagic system. The vector contains the AcNPV enhancer/immediate early promoter combination (hr5/iel) for transient transfection and early baculovirus expression, and the AcNPV p10 promoter for late/very late baculovirus expression. In addition, the pIEx/Bac-3 vector also carries a 10 His's-Tag coding sequence for purification of expressed protein. After

insertion each of HSV-1 gG and HSV-2 gG genes into the pIEx/Bac-3 3C/LIC plasmid vectors, the vectors proceeded to PCR screening of recombinant plasmid.

According to the manufacturer's (Novagen) methodology, the presence of the insertion of the target sequence verification was an essential step in recombinant baculovirus construction. If the novel gene sequence is not inserted into the plasmid, the baculovirus will not be assembled after transfection of the plasmid into permissible insect cells. To confirm the insertion of the plasmid with the HSV gG sequence, PCR screening was used prior to insect cell transfection. PCR products were analysed using 2% E-gel electrophoresis. Due to the small amount of recombinant plasmid mixture (only 0.02 pmol/µL in 5 µL was produced) and the presence of ethylene diamine tetra acetic acid (EDTA) in the recombinant plasmid mixture, the correct insertion of plasmid with HSV gG sequence was difficult to determine using PCR. EDTA is a chelating agent that can reduce the amount of free magnesium, thus influencing the efficiency and specificity of PCR reactions (Khosravinia and Ramesha, 2007; Huggett *et al.,* 2008). Although the initial experiment was started by diluting the recombinant plasmid mixture (1 in 10 or 1 in 100), neither the virus specific primer set nor vector specific primer set amplified the expected sequences. One of the possible explanations for PCR screening failure was that the concentration of recombinant plasmid was over-diluted during the dilution of the recombinant plasmid mixture, which prevents the

primer sets amplifying the target sequences. In order to reduce the presence of PCR inhibitor and to determine the correct plasmid construction, the cell transformation step as described in section 2.6.3.1 was used prior to PCR screening of recombinant plasmid. During cell transformation, the recombinant plasmid acts as a 'donor plasmid' to enter a competent bacterial cell, using a calcium chloride transformation method and followed by manipulation of target genes into an *E. coli* plasmid to form a DNA plasmid. The DNA plasmids were then harvested and screened using PCR. The recombinant plasmids were screened using virus-specific primers or vector specific primers. The PCR screening (section 3.3.3) revealed that all the HSV recombinant plasmids had been successfully constructed, except the complete sequence of HSV-2 gG recombinant plasmid. Neither the HSV-2 glycoprotein G primer set nor the vector specific primer set gave positive results, suggesting that the HSV-2 recombinant plasmid was not successfully constructed. One possible explanation for the failure to insert the HSV-2 gG sequences was that the HSV-2 gG PCR product might be in some way mutated within the vector compatible overhang tails, which prevented it annealing to the recombinant plasmid. Further work to explain the HSV-2 gG plasmid reconstruction failure is warranted. As all the HSV recombinant plasmids except complete genome of HSV-2 gG recombinant plasmid, were successfully constructed, the remaining recombinant plasmids were used to transfect insect cells using the InsectDirect protein expression system.

Insect cell cultures are now commonly used for heterologous protein expression. The most widely used insect cells for Baculovirus expression vectors system (BEVS) are the Sf21, Sf9 and High Five cell lines. The Sf21 and Sf9 cell lines are originally derived from IPLBSF-21 in the pupal ovarian tissue of *Spodoptera frugiperda* (Vaughn *et al.,* 1977) , whereas High Five cell lines, originally established from the *Trichoplusia ni* embryonic tissue (Granados *et al.,* 1994). The High Five cell lines offer better protein expression and post-translational processing of recombinant protein, compared to Sf21 and Sf9, however; they are difficult to cultivate routinely. Sf21 and Sf9 are therefore frequently used in protein expression work. The Sf9 cells are a sub-clone of the Sf21 cells and more favourably selected for their faster growth rate and high densities compared to the Sf21 cells (Pharmingen, 1999). Furthermore, Sf21 cell lines are disparate in size, producing more irregular monolayers than Sf9 cells. Thus, the Sf9 cell lines were used in this study. The Sf9 cells when healthy, appear as rounded and bright cells with distinct cell boundaries. The cells achieved confluent monolayers after 3 or 4 days incubation and cell count using trypan blue assay was regularly assessed during each subculturing to maintain optimal Sf9 cells.

The baculovirus expression system in this study was initially started using the BacMagic expression system prior to infection of Sf9 insect cells. Although the BacMagic protein expression enhanced the yield of proteins, this expression method is time-consuming; approximately 10 days are

required to express the proteins. The multiple steps of BacMagic expression method increased the risk of contamination during the process. An alternative baculovirus expression method, the InsectDirect system was therefore used. The InsectDirect System is a plasmid-based expression system that enables high throughput (HT) protein expression in insect cells without generating the recombinant baculovirus. This resulted in reduced risk of expressed proteins becoming contaminated and allowed protein expression within 48-72 hour.

During insect cell transfection with the recombinant plasmid, the maintenance media for the transfected cells started to turn cloudy after 72-hours incubation. This may suggest the appearance of biological contamination inside the flask. Therefore, the medium of transfected cells was sterility tested using SAB (Sabouraud's broth: - for detecting the presence of fungi) and BHI (*Brain Heart Infusion*: - for detecting the presence of bacteria) to determine whether contamination had occurred. Both SAB and BHI medium remained clear and yellowish after 96-hour incubation, which suggested that the medium for transfected cells was free from contamination. Based on these results (sterility testing), it was suspected that some proteins had been expressed by insect cells, which might turn the cell medium cloudy. Another possible explanation for this phenomenon was insect cell death. After 72-hour incubation, some insect cells start to die due to insufficient nutrients in the maintenance medium.

The dead cells detach from the flask and form cells clumps or precipitates that might cause the medium to become cloudy.

The early signs of infection for transfected cells were observed after 48-hours post-transfection (Figure 3.17A), with their size increased by 40-50% as compared to non-infected cells, followed by vesicular appearances after 72-hours post transfection (Figure 3.17B). The late signs of infection appeared after 5 days when most of the transfected cell lysed and detached from the flask (Figure 3.17C). In order to confirm the present of the desired expressed HSV glycoprotein G after cell transfection, the transfected medium was screened using HSV virus specific primers. Resulting fragments were analysed by 2% E-gel electrophoresis. Based on the results shown in section 3.5, it was suggested that the HSV gG proteins were successfully expressed. The expressed proteins samples were then subjected to protein purification.

The remained HSV gG expressed proteins were then harvested and purified using a His-tagged protein purification method. This method was based on the affinity between histidine residues and an immobilised metal ion (Ni_{2+}). The expressed proteins containing 10 histidine residues (introduced during plasmid construction) were bound to Ni_{2+} and recovered by elution with imidazole. Initially, 2 mL of insect cell culture was added into a single well of 96-well filter plate at one time. This method was found to be time consuming and yielded a low concentration of HSV gG production, 0.03-0.05 mg/mL, suggesting a larger scale of purification was

necessary. The protein purification was then repeated using HIS-Select iLAP 5 mL column purification method as described in section 2.8.2. The HIS-Select iLAP column is designed for one-step purification of histidine-tagged proteins directly from insect cell culture. This versatile system enabled efficient purification of expressed proteins from insect cells and avoids high ionic strength elution reagents, which can damage protein. The use of the HIS-Select iLAP column resulted in increased concentration of HSV gG proteins (Section 3.6.1), with range from 0.30 mg/mL to 0.34 mg/mL.

After purification, the size and immunoreactivity of the expressed proteins were tested by SDS-PAGE and Western blot. In SDS-PAGE, purified proteins were electrophoresed using the NuPAGE® Bis-Tris Gel. The NuPAGE® Bis-Tris Gel is a precast polyacrylamide gel designed to give optimal separation of proteins. When analysed by SDS-PAGE (Figure 3.21, Figure 3.24 and Figure 3.26), the expected size of HSV gG proteins were difficult to distinguish among a background of multiple proteins. For the protein analysis of HSV-1 gG purified protein, the Western blot result showed the immunospecific band migrated between 76 kDa and 225 kDa (Figure 3.22), using an anti-HSV-1 gG mouse monoclonal antibody IgG as a primary antibody. This HSV-1 gG immunospecific band was determined by plotting the relative migration distance (Rf) of the migrated protein with the relative molecular weight of the protein marker, with the size of 108 kDa (Appendix 6). However, this Western blot result contradicted the

protein molecular weight calculation, as the predicted molecular weight of this membrane-associated protein from DNA sequences analysis was 25,237 DA. A possible reason for this was protein aggregation of the expressed protein. Using a membrane protein topology prediction method (TMHMM), there were two hydrophobic domains in the HSV-1 gG sequence, which located at residue 1-32 aa and 191-238 aa (Figure 3.23). The TMHMM is a tool that predicts transmembrane helices in protein sequences and also discriminates between soluble and membrane protein with high degree of accuracy (Sonnhammer *et al.,* 1998). It was suspected that the purified HSV-1 gG protein typically folds into multiple conformations after protein purification due to the surface-induced aggregation. The hydrophobic portions of the HSV-1 gG protein tended to shield itself from the hydrophilic condition by aggregating together. In addition to this, it was also suspected that the HSV-1 gG proteins might contain multiple 'sticky' or complementary patches on the monomer surface, resulting in oligomer conformations of the same patterns of monomer proteins. Thus, a large micelle formation of the HSV-1 gG glycoprotein may have formed, with a hydrophilic exterior and a hydrophobic interior in the water-detergent solution. Because epitopes of the monoclonal antibody recognised the anti-HSV-1 gG mouse monoclonal antibody IgG were located in the hydrophilic domain of the protein; it was able to bind in the Western blot analysis. Another possible explanation is that the signal sequence and the short membrane anchor

sequence (which are known to be removed during post-translational modification in eukaryotic cells) were only partially removed during post translational modification processing in insect cells. These sequences might have caused or added to aggregation after protein expression due to their hydrophobic nature. The aggregation of HSV-1 gG purified protein was further supported by light scattering. This result was expected, as the previous studies by Ikoma *et al* (2002) and Liu *et al* (2015), concluded that the production of full length of HSV glycoprotein G resulted in instability and inadequate amount of proteins. As with HSV-1 gG expressed protein, the truncated version of HSV-1 gG and HSV-2 gG formed multiple conformations within the domain size of the immunoreactive band, 45 kDA and 81 kDA, respectively.

In order to determine the actual molecular weight of HSV-1 gG purified protein, purified protein was further analysed using mass spectrometry. The size of HSV-1 gG purified protein was initially determined using the BioMerieux VITEK mass spectrometer. The BioMerieux VITEK mass spectrometer is an automated system that uses the Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) technology to determine the elemental composition of a protein sample. It proved hard to determine the actual size of HSV-1 gG purified protein using this method alone, due to the formation of the multiple peak protein spectra (Figure 3.28). A right-skewed peak shape, with the highest peaks located in the range between 6000 Da and 7500 Da was observed.

A possible reason for failure to determine the protein size of HSV-1 gG using this VITEK MS was the formation of the multiple glycoforms for HSV-1 glycoprotein G. The HSV-1 gG contained the covalently attached carbohydrate chain, which was responsible in part for multiple peptides or fragments formation with almost similar positively charges during the protein ionisation and desorption steps. This glycoprotein might also form the different fragment structures and ionic charges even though cleaved at the same cleavage site due to complexity and diversity of protein-linked carbohydrates (Harvey *et al.,* 2008; Han and Costello, 2013; Azadi and Heiss, 2009). It was suspected the broadness of the protein fragment peaks on the VITEK MS datasheet was caused by carbohydrate heterogeneity. The matrix that used in this study was α-Cyano-4-hydroxycinnamic acid (CHCA). Previous studies by Stephens and colleagues (2004) have indicated that the CHCA matrix induces glyosidic cleavage of glycoproteins. During desorption and inonisation steps, more protons and internal energy were acquired from the CHCA matrix to ionise the heterogeneous protein fragments. This phenomenon caused the CHCA matrix to become unstable, thus, increasing the undesired fragmentation and chemical background. Because the VITEK MS failed to calculate the protein size of HSV-1 gG purified protein, an alternative mass spectrometer, the Quadrupole time-of-flight (Q-TOF) mass spectrometer was therefore used. The Q-TOF mass spectrometer is a mass analyzer that provides high mass accuracy and sensitivity for protein

analysis using quadrupole instruments (Chernushevich *et al.,* 2001). With the presence of the quadrupole in this mass spectrometer, the sample ions were filtered based on their mass-to-charge ratio (m/z) and the stability of trajectories through the quadrupole rods. As the product ions were initially distinguished in the quadrupole, only a low ion signal was acquired for analog detector to quantify and identify the fragment ions compared to VITEK MS, thus, improving the sensitivity and signal-to-noise ratio for this assay. The Q-TOF mass spectrometer revealed that the predominant molecular size for HSV-1 protein was 112,965.75 DA. Apart from the predominant peak, the Q-TOF MS data also showed there were a number of smaller peaks of different sizes. It was suspected that these peaks corresponded to the protein that was expressed in the host cell, which was supported by SDS-PAGE analysis. In order to gain highly pure HSV-1 gG, further downstream purification steps, such as affinity chromatography and size exclusion chromatography might be required.

In this study, the HSV-1 gG and truncated version of HSV-2 gG proteins were successfully expressed using the InsectDirect expression system. However, these expressed proteins resulted in protein aggregation and were only produced in low yield, which might interfere with the sensitivity and specificity of the assay. Time constraints prevented improvement of the HSV gG protein expression, therefore, the commercially available truncated glycoprotein G for HSV-1 and HSV-2, were used to develop a HSV Bi-plexed immunoassay in this study.

4.2 Development of a Bi-plexed fluorescent bead assay for the quantitative detection of IgG antibodies to HSV-1 and HSV-2 virus in human sera

The enzyme-linked immunosorbent assay (ELISA) is known to be a widely used method for testing the type-specific antibody to HSV-1 or HSV-2 in human samples. However, ELISA can be time and sample consuming. In addition, the results generated from different ELISA are often difficult to interpret due to the variation in sensitivity and specificity of the assays used (Morrow and Friedrich, 2003). An HSV Bi-plexed bead immunoassay based on the Luminex technology was therefore developed, in order to discriminate accurately between the specific antibodies to HSV-1 and HSV-2 and reduces the amount of sample required to test.

As discussed in section 4.1, commercially available truncated HSV-1 and HSV-2 glycoprotein G expressed using Baculovirus expression system, were used. The results obtained from ELISAs and Western blots for both truncated HSV glycoproteins G suggested the expressed proteins were immunoreactive (the product details and serological results for both expressed proteins can be found in Appendix 9 and Appendix 10). Based on these results, both commercially available truncated HSV-1 and HSV-2 glycoproteins G were thought suitable to be used in this HSV Bi-plexed assay.

One challenge for developing a multiplex assay was to obtain a standard serum sample, to generate a standard curve to allow semi-quantitation of antibody against the antigens of interest in the multiplex assay. As there was no standard serum for both HSV-1 and HSV-2, the international rubella serum sample (RUBI-1-94) was evaluated for use as a simultaneous standard for HSV-1 and HSV-2 antibody quantitation. The HSV status of RUBI-1-94 serum sample was determined using the HerpeSelect ELISA. Interestingly, this serum not only contained high IgG antibody concentrations for measles, mumps, rubella and varicella zoster virus, but also contained high HSV-1 and HSV-2 IgG antibody concentrations.

After obtaining an acceptable standard serum for the HSV Bi-plexed assay, the HSV-1 and HSV-2 truncated proteins were initially coupled to different carboxylated microsphere beads using a two-step carbodiimide reaction method. This method involves the reaction between EDC and Sulpho-NHS for conversion of the carboxyl group on the microsphere surface into a stable amine bond with the test antigen. In order to assign values for the HSV-1 and HSV-2 lgG antibodies in the HSV Bi-plexed assay, the method of cross-standardisation was used. Cross-standardisation allows the assigned antibody concentration in a reference serum against one antigen to be used to calculate the antibody concentration for additional antigens, but only if their serial dilution curves are parallel (Joseph *et al.,* 2004). Four different standard curves for HSV-

1, HSV-2, rubella and VZV respectively, were generated using RUBI-1-94 serum in two fold-dilutions, starting from 1/10 to 1/10240. The parallelisms of all these standard curves, generated from the cross-standardisation method were determined by the guideline of Plikaytis and colleagues (1994). The results showed that both HSV-1 and HSV-2 standard curves were parallel to the rubella and VZV standard curves, with a correlation coefficient greater than 0.99 (Figure 3.39). Based on this result, the antibody-binding characteristics between both HSV antigens and rubella and VZV antigens were similar and this allowed the determination of specific antibody levels in the diluted RUBI-1-94 serum samples. The parallelism between the standard curves generated from each antigen in this study also reflected a possibility of integrating the HSV beads into the MMRV multiplex immunoassay to form a hexaplex assay for simultaneous antibody detection of these viruses. Based on visual comparison, the result (Figure 3.30) showed both HSV-1 and HSV-2 sample curves were more parallel to VZV standard curve than the rubella standard curve. Therefore, the VZV standard curve was selected as the reference standard curve for assigning the antibody concentration of HSV in the reference serum. The concentration of specific HSV antibodies found in the RUBI-1-94 was reported as arbitrary units of HSV specific antibodies (AU/mL). To assign the HSV antibody in the RUBI-1-94 reference serum, a fully-specific logit-log function was used, as described in section 2.9.2.2.2. Plikaytis and colleagues (1991) had examined the performance of four

different cross-standardisation methods, log-log, logistic-log, partially specified logit-log and fully specified logit-log, respectively, for assigning the antibody concentration of *Neisseria meningitidis* group A polysaccharide by using an ELISA. Their conclusion was that the fully specified logit-log function was the most accurate model compared to the other three models for interpolating the antibody concentration of *Neisseria meningitidis* group A polysaccharide from the standard curve. The results gave an assigned antibody concentration for HSV-1 (Table 3.10) and HSV-2 (Table 3.11) of 3.93 (± 0.91) AU/mL and 0.30 (±0.04) AU/mL, respectively, with the CV below 25%. The low CV reflected the fact that both HSV-1 and HSV-2 sample curves were parallel to the VZV standard curve. Thus, if equal amounts of antibody are bound to the VZV and HSV antigens, the mean fluorescence intensity of the specific antibodies will be similar.

Several modifications such as antigen concentrations were investigated including; length of incubation for the bead conjugation, serum dilutions and SOD concentration. This optimisation was necessary as, changes in the antigen concentration for the assay and incubation time for the bead conjugation affected the parallelism and correlation of the standard curve for each antigen compared to the rubella and VZV standard curves. Following optimisation, the optimal condition for HSV-1 glycoprotein G antigen was found to be 1.0 µg/mL at 1.5 hours coupling times (Figure 3.33 and Table 3.12). However, for the HSV-2 glycoprotein

G antigen, the determination of optimal conditions was problematic. The HSV-2 gG conjugated bead was initially examined with 0.5 µg/mL at 1.5 hours coupling times, this generated a high background in a blank experimental well, with a fluorescence intensity (FI) greater than 14,000. A possible reason for the high background was the presence of the reducing agent, DTT (Dithiothreitol) and the non-ionic detergent Triton x-100, contained in the storage buffer of the native truncated HSV-2 gG protein, but not the HSV-1 gG protein. During the bead conjugation, DTT may reduce the reaction between the carboxyl groups on the surface of each microsphere activated by the EDC/Sulfo-NHS system, by cleaving the disulfide bond on the NHS ester-activated crosslinker that bind to the HSV-2 gG antigen, whereas for Triton x-100, this nonionic surfactant might compete with HSV-2 gG antigen to bind to the amide bond on the surface of microsphere. This may have caused a reduction of stable HSV-2 amide bonds on the surface of microsphere and result in high background reactivity, independent of the amount of HSV-2 gG antigen coupled. Improvement of the fluorescence intensity for the blank well was attempted, by increasing the HSV-2 gG concentration to 1.0 µg/mL at 2 hours incubation time; this yielded an FI of less than 2,500. The reason for this result may be due to the weak hydrogen bonding of Triton x-100 to the amine reacting intermediate. It was suspected that by increasing the incubation time for bead conjugation, the HSV-2 gG antigen might disrupt the weak hydrogen bond of Triton x-100 and by cross-linked to itself form

a strong covalent bonding with the amine functional group on the surface of the microsphere. Although these coupling conditions dramatically reduced the FI value for the blank well and titration of standard serum showed paralleled titration curves to rubella and VZV, this value was still beyond the acceptance criteria defined by the reader manufacturer. In order to further reduce the background, other assay conditions were attempted, such as increasing the incubation time of bead conjugation to 3 hours and increasing the concentration of HSV-2 gG antigen to 2 µg/mL. However, none of these modifications were successful. Therefore, the obtained assay condition (1.0 µg/mL at 2 hours incubation time) was considered best as could be obtained conditions for the HSV-2 Bi-plexed assay. After obtaining the conditions for both HSV glycoproteins G conjugated beads, the dilution factor for serum was investigated. This showed that both dilution factors (1 in 100 and 1 in 400) quantified almost identical antibody concentrations for the tested samples in both HSV assays (Table 3.17), with a % CV less than 25% for each tested sample. As the resultant antibody concentration obtained from both dilution factors were almost identical and parallel to the HSV standard curve, it was suggested that the serum dilution factor of 1/100 was sufficient to quantify a broad range of HSV antibody concentrations in the tested samples.

On studying the product details of both commercially available truncated recombinant HSV-1 gG and HSV-2 gG proteins, it was found that these proteins were fused with superoxide dismutase (SOD) in the N

terminal region. Superoxide dismutase (SOD) is an enzyme that catalyses the dismutation of superoxide (O2-) in order to reduce the free radical damage to protein (Liochev and Fridovich, 2007; Nozik-Grayck *et al.,* 2005; Zelko *et al.,* 2002). The presence of the SOD in the coupled antigen may lead to the binding of anti-SOD antibody in human sera. To investigate if the SOD may bind antibody, five different SOD concentrations were used to adsorb out any antibodies. However, no difference was demonstrated between the SOD concentrations when testing a human serum sample (in triplicates) for the HSV Bi-plexed assay (Table 3.19 and Table 3.20). Another 33 serum samples were subsequently tested and no differeces between SOD concentrations were determined. Therefore, SOD was not utilised to neutralise the human sera samples before testing in this HSV Bi-plexed assay.

After determining the best that could be obtained conditions for both HSV Bi-plexed assay in this study, the performance of the assay in detection and quantitation of antibody response to HSV-1 and HSV-2 viruses were evaluated. Originally, the samples had been tested to determine the HSV status using DiaSorin Liaison HSV type specific IgG and then tested by HerpeSelect 1 and 2 ELISA. The comparative study results showed that the HerpeSelect ELISA had good agreement with DiaSorin Liaison HSV type specific IgG assay on both HSV subtypes, (HSV-1, 96.67% and 96.49%, respectively, and HSV-2, 95% and 93.33%, respectively). As the HerpeSelect assay had high concordance with the

gold standard HSV diagnosis test, Western blot compared to Diasorin Liaison assay and had been extensively studied (Morrow and Friedrich, 2003; Ashley *et al.,* 1998), it was suggested that the performance of the HSV Bi-plexed assay should be based on its correlation with the HerpeSelect ELISA assay as a surrogate for the HSV western blot. The HSV Bi-plexed assay performed with low sensitivity and specificity compared to HerpeSelect ELISA assay (HSV-1 of 89% and 66%, respectively, with the cut-off level of 0.75 AU/mL and for HSV-2 of 79% and 85%, respectively, with the cut-off level of 0.05 AU/mL). A possible explanation for the result in the HSV-1 Bi-plexed assay was the low purity of the truncated HSV-1 antigens, as shown by the SDS-PAGE analysis included in the product description from the company, which showed the supplied product contained a number of irrelevant (non HSV gG) (Appendix 11). The proteins are likely to be host proteins, which also may bind antibodies in the sera. Studies showed that human sera contained antibodies, which were immunoreactived to the Insect glycoproteins (Nores *et al.,* 1991; Bencúrová *et al.,* 2004). For the HSV-2 Bi-plexed assay, the high background possibly caused by the agents in the truncated protein's diluent buffer clearly was also an issue further contributing to the low sensitivity and specificity of the assay when compared with the HerpeSelect ELISA. Another possible explanation for low agreement between both HSV Bi-plexed assay and HerpeSelect ELISA method could be that only minimal SOD concentrations up to 0.1

mg/mL were investigated. A higher concentration of SOD might be necessary to neutralise anti-SOD antibody in particular human sera. Based on these results, further improvements are required in order to include this HSV bead immunoassay into a HSV type-specific screening test.

4.3 The seroepidemiology of MMRV in England

The multiplex immunoassay assay in this study was a bead based technology that enables the determination of multiple antibodies within the same specimen. The bead was internally dyed with two or more fluorophores of differing intensities, in order to allow the flow-based detector to differentiate the individual beads. The advantages of this assay are in its ability of expansion to include up to 500 targets in a single assay. The technique therefore promises to decrease cost, minimize sample volume requirements (<5 µL) and allow testing of numerous test samples in a single assay. In comparison to conventional methods, these advantages are clearly of value in diagnostic and research uses (Kellar *et al.,* 2001; Yang *et al.,* 2008; Rao *et al.,* 2004).

In order to determine the seroprevalence of measles, mumps, rubella and varicella zoster virus in the population of England, a MMRV multiplex bead immunoassay was adapted for use in this study. The development of the MMRV multiplex bead immunoassay has been described in Smits *et al.* (2012). Smits and colleagues (2012) evaluated

the performance of the MMRV multiplex immunoassay in comparison with conventional ELISA by screening 70 serum samples. In their study, all test serum samples were initially tested for the presence of virus type specific antibodies using separate virus specific ELISAs and then tested by the MMRV multiplex immunoassay. They concluded that there was a strong agreement between the results obtained with the MMRV multiplex immunoassay and individual ELISAs, with the correlation coefficients greater than 0.97. The MMRV multiplex immunoassay provided a method with high sensitivity and specificity together with a method for the relative quantitation of antibody. Several studies have since used this procedure to determine MMRV antibodies in population based studies (Waaijenborg *et al.,* 2013; Van den Berg *et al.,* 2014). Taking into account the advantages of the multiplex immunoassay and the supporting evidence of its use in previous immunosurveillance studies, was a strong impetus for the use of the MMRV multiplex immunoassay.

In this study, the MMRV multiplex immunoassay was initially validated and evaluated using a standard panel of samples obtained from the RIVM laboratory, Bilthoven. The sample panel contained 40 serum samples, which had previously been tested by RIVM. The mean of MMRV antibody concentration in each of the samples determined in three separate runs was compared to the RIVM results by plotting a scatterplot for each virus with the RIVM assigned cut-off level (Smits *et al.,* 2012; Waaijenborg *et al.,* 2013; Van den Berg *et al.,* 2014). Regression analysis

revealed that there was a good correlation between each antigen and the RIVM value (Figure 3.45-3.48), with coefficient values of greater than 0.95. However, there was a discrepancy between the test results and RIVM results in the low antibodies concentration region. At first, it was suspected that the discrepancy between the test results and RIVM results was caused by the variation of different batches of BSA used. Bovine serum albumin is a common blocking reagent used in immunoassays to prevent non-specific binding of antibodies and to improve protein stability. The BSA acts as an 'inhibitor' to bind potential non-specific binding sites without interfering with the specific antigen/antibody binding. Although the BSA can be used successfully, in certain circumstance it can create problems in immunoassays. Several studies indicated that batch-to-batch variation of BSAs leads to assay variation (Law and Biddlecombe, 2005; Yuhong and Stuart, 2012). The possible reasons for this variation include animal component contamination, variations in the processing method and variations in different herds. Two different batches of BSAs from Sigma-Aldrich ltd, BSA purchased through RIVM and BSA purchased through VEU were therefore evaluated. However, almost similar results were observed (Figure 3.49 to 3.52). An alternate explanation for the difference observed between RIVM results and the VEU results could be attributed to differences in sample preparation during the assay. Sample preparation inevitably introduces differences, it involves sample dilution methods, bead conditions and samples storage condition differences which cannot always

be avoided. Furthermore, inter laboratory variations in instrumentation and temperature variations or other atmospheric conditions may also cause the discrepancies between the VEU results and RIVM results for each antigen in this study. Overall, the regression results revealed a strong correlation between VEU results and RIVM results for each antigen. Discrepancies between the VEU results and RIVM results for each antigen were only observed with lower antibody concentrations. To solve the problem, cut-off values were assigned for each virus antigen (Figure 3.45 to Figure 3.52). The calculation for the RIVM assigned cut-off values can be found in Smits and colleagues (2012). Briefly, samples with a concentration below the cut-off values for each antigen (≥ 0.2 IU/mL for measles, ≥ 45 RU/mL for mumps, ≥ 10 IU/mL for rubella and ≥ 0.26 IU/mL for varicella) were considered as seronegative. This MMRV immunoassay with assigned cut-off values in the lower range concentrations was then used to test the MMRV serum samples.

Following the licensing of MMR vaccine and its introduction into the childhood immunisation programme, the incidence of measles, mumps and rubella in the population of England fell dramatically (Department of Health, 2013b; Department of Health, 2013c; Department of Health, 2013d; Eliman *et al.,* 2009). However, the controversy of MMR vaccine and autism in 1998 (Wakefield *et al.,* 1998) raised public concern and led to a dramatic decline in MMR vaccination rates, with the lowest vaccine coverage of 79.9% in 2003-04 (HSCIC, 2013). This controversy had

changed the prevalence of measles, mumps and rubella antibodies and caused the incidence of these diseases to increase in the population, mainly in children and young adults, aged between 19 and 23 years (Asaria *et al.,* 2006; Gupta *et al.,* 2005). Outbreaks of measles and mumps may also result in a possible change in the prevalence of MMR antibodies in certain age groups. In the case of varicella zoster virus, an increasing numbers of young adults suffering primary infection have been found. According to Brisson and Edmunds (2003), the average incidence rate for varicella infection between 1991 and 2000 was 1,291 per 100,000 population per annum and almost 14% of the varicella cases in total occurred in young adults aged 15 to 44 years. Based on this, the seroprevalence studies of VZV were necessary to predict disease burden, as a prelude to the possible introduction of a vaccine into the UK childhood immunisation programme.

To determine the MMRV seroepidemiology in the population of England, a total of 612 anonymised MMRV test samples were analysed. Information on age, gender and laboratory of sample origin were provided for each test sample and an overview of the demographic data for the test samples can be found in Appendix 8. The proportion of MMRV antibody positive samples were determined using the RIVM assigned protective concentrations (define as RIVM assigned cut-off points) as described in section 2.9.1.6. In order to examine the potential changes in seroepidemiology of MMRV in the population of England, the proportions

of MMRV antibodies positive individuals generated in this study were then compared with the proportion of MMRV seropositive antibodies studies in 1996 (De Melker *et al.,* 2003; Nardone *et al.,* 2003; Vyse *et al.,* 2004; Pebody *et al.,* 2000).

It was shown that the proportion of MMR seropositive test samples in 2012, were generally higher than in 1996 (Section 3.8.2.1), with 84.3% and 88.9% of antibody positive individuals in the 1996 study and 2012 study for measles, respectively and antibody positive individuals of 67.4% in 1996 study and 85.8% in 2012 study for mumps, respectively. The likely reason for this was the improved effectiveness of the MMR vaccine policy in 2012 compared to 1996. According to the PHE (2014f), MMR vaccine was initially introduced as a single-dose schedule in October 1988, and then a two-dose schedule was implemented in 1996 to boost the immunological responses against MMR infections in the population of England. Several studies had previously shown that a single dose of MMR vaccine schedule provided protection from measles (Bedford, 2004; Miller, 2002). For example, Morse and colleagues (1994) showed that about 90% of the vaccine recipients were protected after receiving a single dose of MMR vaccine. Although a single dose of MMR vaccine was highly effective, 10% of the vaccine recipients failed to mount a protective response. The possible reasons for MMR vaccine failure were interference from maternal passive antibodies in the vaccine recipients (Sultana *et al.,* 2006; Nicoara, *et al.,* 1999), waning of vaccine-induced immunity (Kontio

et al., 2012) or a damaged vaccine due to temperature fluctuations during storage and transportation (WHO, 2006). Studies had revealed that mumps was the least effective vaccine among the three components of MMR vaccine (Harling *et al.,* 2004; Malaiyan and Menon, 2013; Davidkin *et al.,* 2008). The effectiveness of a single dose of mumps containing vaccine against mumps infection varied, from 64% (Harling *et al.,* 2004) to 88% (Cohen *et al.,* 2007), whereas for measles and rubella components conferred around 90% to 100% protection for a single dose of MMR vaccine (Davidkin *et al.,* 2008; Ong *et al.,* 2007; Malaiyan and Menon, 2013). The high effectiveness of a single dose MMR vaccine for measles and rubella components suggested that most MMR vaccine recipients had acquired adequate immunity to protect themselves from measles and rubella infections after vaccination. This was supported by the results in this study, which showed a significant (but small) difference in the proportion of measles positive (Table 3.21) and no significant difference in the proportion of rubella positive (Table 3.23) in both years. For mumps, the study revealed that there was a significant difference between the percentage of mumps positive antibodies in 1996 and 2012, (67.4% and 85.8%, respectively; ($p<0.05$)). The low proportion of mumps positive antibody in 1996 compared to 2012 reflected the high rate of mumps-containing vaccine failure among MMR vaccine recipients in 1996. There was a dramatic reduction in the incidence of mumps infection after the introduction of MMR vaccine, less than 250 mumps cases being reported

each year during 1990 to 1996 (Gupta *et al.,* 2005) compared to 1962 to 1981, when, 1011 cases per 100,000 populations were reported every year (Galbraith *et al.,* 1984). This meant that a person who failed to obtain protection against mumps was less likely to acquire the immunity from naturally infection because mumps was no longer circulating epidemically. However, mumps epidemics were reported in 2005, with 56,390 notified cases, with the majority of the cases occurring in inadequately MMR vaccinated persons, aged 15 to 24 years (CDC, 2006; Gupta *et al.,* 2005). In order to compensate for primary MMR vaccine failure, a two-dose MMR schedule was introduced. Although the routine second dose of MMR vaccine was given to the pre-school children (aged 3 to 5) in late 1996, the effect of the second dose MMR vaccine on herd protection in the UK was unlikely to be seen in 1996 due to the short time between the implementation of the second-dose schedule and data collection.

The differences in proportions of MMR positive antibodies in 1996 and 2012 were further investigated based on different age groups. The age groups sampled in 2012 were divided into 9 age groups in order to construct the comparable age groups of the 1996 studies. For measles and mumps, the proportions of antibody positive in 2012 were generally higher than in 1996 across the age groups. Statistical significance was reached for the proportions of measles antibody positive between 2012 and 1996 in all age groups, except in the 0 to 1 year, 2 year and 40 to 59 years groups (Table 3.33), whereas for mumps, the differences were

significant in all age groups, except for those aged 9 to 24 years and 25 to 39 years (Table 3.34). For rubella, the proportions positive in 1996 and 2012 studies were similar (Figure 3.55 and Table 3.35).

Differences in the proportion of MMR positive antibodies in 2012 compared to 1996 were mainly due to a change in UK vaccination policy. There were however, several age groups in this study that differed from this observation:

a) Proportions of measles positive antibodies in aged 7 to 8 years and 9 to 24 years.

According to the Gay and colleagues (1997), 92% of the individuals aged 5 to 16 years in 1994 were vaccinated during the national measles and rubella (MR) vaccination campaign in order to prevent predicted measles and rubella outbreaks. The effectiveness of the MR campaign was measured in terms of the proportion of rubella positive antibodies. More than 92% of the individuals aged 7 to 8 years and aged 9 to 24 years in 1996, were seropositive for rubella with no significant differences compared to the same age groups in 2012 (Table 3.35). Unlike rubella, the proportions of measles positive antibodies in 1996 were markedly lower in these age groups compared to 2012. As the measles component in the combination vaccine was highly effective and there was high vaccine coverage in these age groups, the disparity between those aged 7 to 8

years and those aged 9 to 24 years for the proportion of measles positive antibodies were unlikely to be observed.

b) The proportions of MMR positive antibodies in those aged 25 year and above.

The individuals in these age groups for both years were mainly born prior to the introduction of MMR vaccination. Because of the high incidence of measles, mumps and rubella infections in England prior to the introduction of MMR vaccination, with endemic peaks every 2 to 3 years, it was suspected that most of the individuals in this generation generally developed adequate immunity to measles and mumps through natural infection. However, a high disparity between the proportion of measles and mumps protective subjects in these age groups for both years was observed, with markedly lower proportions of positive subjects in 1996 compared to 2012.

A possible explanation for the discordance between the proportions of measles and mumps positive in these age groups was the sensitivity and specificity of the assay employed. The MMR seroepidemiological studies in 1996 used conventional ELISA (De Melker *et al.,* 2001; Nardone *et al.,* 2003; Pebody *et al.,* 2000). Until recently, enzyme immunoassay has been widely used for detection of specific antibodies to MMR in human serum. However, some studies indicated that the ELISA was insensitive in the detection of low levels of antibody, especially for mumps (Mauldin *et al.,* 2005; Ratnam *et al.,* 1995). As the titres of vaccine-

induced antibodies were generally lower than natural infection and ELISA often required a high serum dilution for antibody detection, it was suspected that the EIA might contribute to false-negative results due to the over diluted serum samples (Mauldin *et al.,* 2005; Ratnam *et al.,* 1995). This hypothesis explained why there were low proportions of mumps and measles positive individuals in 1996 compared to 2012, particularly in young children aged 2 to 8 years. It was suspected that most of the individuals aged 2 to 8 years in 1996 had a low level of vaccine-induced mumps or measles antibodies. The high serum dilution of ELISA procedures had possibly diluted those low positive antibody samples to a dilution below the minimum antibody detection of ELISA, resulting in a reducing ability to correctly classify the antibody status of the test samples. The MMRV multiplex immunoassay can detect lower level of measles and mumps antibodies in these age groups. The high proportion of measles and mumps positive individuals in 2012 compared to 1996 suggested that the MMRV multiplex immunoassay was more sensitive than ELISA. This was supported by previously published studies, which indicated that the multiplex bead immunoassay was more sensitive than the ELISA assay (Smits *et al.,* 2012; Binnicker *et al.,* 2011).

The proportion of mumps antibody positive individuals across the age groups in 1996 and 2012 differed. A possible reason for this was the lack of a mumps international standard to allow comparison of assays. Variations between mumps virus antibody assays have been reported and

are believed to relate to differences in the virus strain used and type of the antibody detected (Nardone *et al.,* 2003; Pipkin *et al.,* 1999). To compare mumps antibody levels in the year 1996 and 2012, the methodology published by Andrews *et al.,* 2000 and Smith *et al.,* 2012 was applied in this study for standardisation. The approach by Andrews *et al.* (2000) and Smits *et al.* (2012) comprised the development of a working standard for mumps by a designated reference centre, which was then used for calibration purposes. However, even with a designated standard small differences in methodology can lead to a high variability of results in mumps sero-prevalence and results need to be interpreted cautiously. Ultimately, the development of a mumps international standard and methodology is necessary, in order to directly compare mumps epidemiological results.

Although a high level of MMR immunity was found in the 2012 cohort, there were several age groups, especially in those aged 9 to 24 years who apparently remained susceptible to MMR infection.The possible reasons for this were the low level of second dose MMR vaccine coverage in the population and the impact of MMR controversy. According to HSCIC data (2013), there was less than 90% second dose MMR vaccine coverage for children reaching the age of 5 over the 5 years, from 2008 to 2013. As discussed as above, anyone who missed second dose MMR vaccine continued to be at risk of measles, mumps and rubella infections because of incomplete vaccine protection. People aged 9 to 24 years in

2012, notably the generation born during the period of MMR controversy, were generally unvaccinated with MMR vaccine due to the impact of adverse publicity (Hilton *et al.,* 2009; Anderberg *et al.,* 2011). According to PHE data (2014g), laboratory confirmed cases of measles, mumps and rubella across England and Wales in 2012, there were 2030 cases of measles, 2564 cases of mumps and 65 cases of rubella. Almost half of these infections in 2012 were in young adults who were either partially vaccinated during childhood or had not been immunised. These young adults were not just at risk themselves to infection, but they also posed an infection risk to others, especially infants, who were too young to be vaccinated.

The proportion of MMR positive samples in 2012 was further analysed by gender and regions. For gender, no significant differences were observed between the proportions positive in male and female. This result reflected the MMR vaccination was distributed equally between male and female. However, for rubella virus, the difference between the female proportion and male proportion in 2012 was slightly higher compared to measles and mumps viruses (section 3.8.2.2). Before the introduction of MMR trivalent vaccine, routine monovalent vaccination was given to pre-pubertal girls and women of childbearing age In order to reduce the risk of Congenital Rubella Syndrome (CRS) during pregnancy (Department of Health, 2013d). Supposedly, this selective rubella vaccination policy left most males susceptible to rubella infection due to

the low level of rubella specific immunity (Department of Health, 2013d). The 2012 study shows a clear increase of rubella antibody positive individuals in the male population of England over the past 20 years. For regions of England, the results indicated that the proportions of measles and mumps positive, but not rubella, were not significantly different between the three regions of England studies. As with gender, these statistical results showed that MMR vaccine was equally delivered across the population of England. Only a minor difference between the regions for the proportion of rubella positive in 2012 was observed. The possible reason for this was the imbalance in sample size between the regions. Because of limited resources, only 77 serum samples had been collected for the group 3 regions (East Midlands and West Midlands). The low sampling size in this group does not represent the actual population in the regions, and thus caused an incorrect association.

For varicella zoster virus, the overall proportion positive in the population of England were similar between 1996 and 2012, with 0.8022 and 0.8020, respectively ($P<0.05$) (Table 3.24). The high proportions of VZV positive antibody in both years were unlikely to be due to the vaccination, as the varicella vaccine has not been used routinely in England and zoster vaccine was only introduced in the elderly in September 2013 (Department of Health, 2014). The probable explanation is the similar and relatively high incidence rate of VZV infections in England in both 1996 and 2012. To examine the seroprevalance of VZV

positive antibody in 1996 and 2012 in more detail, the proportions of VZV positive in both years were further analysed by age groups. A total of 5 age groups were constructed in this study for the 2012-collected samples, in order to compare with 1996 published study (Vyse *et al.,* 2004). A significant different between the proportions of VZV positive in both 1996 and 2012 were observed in aged 5 to 9 years, aged 10 to 14 years and aged more than 20 years, respectively. The discordance between the age groups was probably due to the sensitivity and specificity of the assays employed. The overview of the assay used in 1996 can be found in Vyse and colleagues (2004).

The proportions VZV positive individuals increased rapidly with age in both years, with most of the individuals acquiring immunity by the aged of 10 year. Referring to the age distribution of VZV antibody (Figure 3.56), nearly half of the children aged 0 to 4 years did not have immunity to VZV. A large pool of susceptible children to varicella infection in those aged 0 to 4 years could be worrisome, as most of the children in this age group now attend pre-school establishments. This phenomenon produced a high risk of transmission of VZV among the susceptible children and explains outbreaks of chickenpox in this age group (Vyse *et al.,* 2004; Brisson *et al.,* 2001). To protect infants from severe or complicated varicella infections, implementation of the varicella vaccine into the routine childhood vaccination may be necessary. However, there were several issues relating to the vaccine effectiveness and the impact of the varicella

vaccine on the incidence of herpes zoster in older age (Edmunds and Brisson, 2002; Brisson *et al.,* 2003), which have interrupted the introduction of universal varicella vaccination in England. As the varicella vaccine required two doses to produce a sufficient level of protection to against the infection, there has been concern that varicella vaccination could resulted in suboptimal uptake and possibly cause an epidemiological shift of varicella infections to older age groups. However, the high incidence rates of herpes zoster in older age groups after the varicella vaccination could be overcome by vaccinating the elderly with the zoster vaccine. Although the varicella vaccine may not confer lifelong immunity, most vaccine recipients would acquire sufficient immunity to protect them from infections before they were vaccinated with the zoster vaccine. Several studies have indicated that the varicella vaccine is highly immunogenic, efficacious and safe (Bonanni *et al.,* 2009; Schmader *et al.*, 2012). Many countries around the world have performed routine varicella childhood vaccination, resulting in substantial reduction of varicella incidence in their populations (Bonanni *et al.,* 2009; Goldman and King, 2013). Recently, ECDC (European Centre for Disease Prevention and Control, 2015) published guidance for the use of varicella vaccine in order to support the introduction of routine childhood varicella vaccination in Europe. If the routine varicella vaccine schedule is introduced in England into the infant immunisation schedule, sero-surveillance of varicella will be necessary to examine the natural immunity in older age groups in the

population, who not be exposed to varicella from their children or grand children.

The proportion of VZV positive test samples in 2012 was further analysed by gender and regions. As with MMR, there was no sex-related difference in the proportion of VZV positive in 2012, which reflected a similar incidence rates for varicella infections in both males and females (Table 3.28). For region, the proportion of VZV positive antibody was higher in group 1 regions compared to other regions, but the difference was significant only between group 1 regions and group 2 regions. The possible reason for this difference was the imbalance of age distribution between the regions. The overview of the age distribution between the regions can be found in Appendix 8. The difference between group 1 regions and group 2 regions was not unexpected, as approximately two-thirds of the samples collected in group 2 regions (184/268 samples in total) were from young children aged less than 9 years. Because of the large pool of susceptible children to varicella infections in these age groups as discussed as above, it was expected that most of the individuals in group 2 regions were seronegative to VZV and this, resulted in the significant difference with group 1 regions.

Overall, the analysis revealed that the proportion of MMRV positive individuals in 2012 was higher than in 1996. The reasons for discordances between the proportions of MMR positive antibodies in both years were the effectiveness of the vaccination policy and for MMRV the sensitivity of

the assay employed. This study indicated that the MMRV multiplex bead immunoassay exhibited a better performance in the quantitation of MMRV specific antibodies compared to ELISA. In 2012, the result revealed that several age groups still remained highly susceptible to MMRV infections due to the low uptake of second dose MMR vaccine and the impact of the MMR scare. Based on this assumption, the high two doses MMR vaccine coverage was necessary, to offset the MMR transmission in the population; whereas for VZV, the implementation of the universal varicella childhood vaccination in England may be necessary in order to protect the population from the severe and complicated varicella infections. There were no sex-related differences in the seroprevalance of MMRV antibodies in 2012. For regions, the results indicated that the proportions of measles and mumps positive, but not rubella and VZV, were not significantly different between the three group regions. The significant differences between the proportion of rubella and VZV in regions were due to the imbalance of sample size and unequal age distribution between the regions.

4.4 Future Work

The PCR amplification of the complete genomes and truncated version for HSV-1 gG and HSV-2 gG using AmpliTaq Gold 360 master mix was achieved. However, the unexplained failure to construct the complete genome of HSV-2 gG recombinant plasmid is worrisome. The presence of

high GC content (about 70%) and long PCR amplified sequence (>2000bp) in complete genome of HSV-2 gG had possibly reduced the fidelity of copying of the correct target sequences. If an incorrect copy of the gene had been produced this might explain the failure to construct the complete genome of HSV-2 gG recombinant plasmid (as evidenced by PCR screening). However, there is yet no evidence of a lack of fidelity of copying. Thus, sequencing of the complete HSV-2 gG genome product will be necessary. Although the HSV-1 gG genomes was successfully expressed, the issues related to protein aggregation and low yield interrupted the further use of this expressed protein in HSV Bi-plexed assay. As discussed as above, the hydrophobic portions of the HSV-1 gG tended to misfold into multiple amyloid-like conformations in the water solution. The problem might be solved by creating an artificial environment that mimics the native membrane. As the bound hydration layer in the aqueous solution was less mobile and more ordered compared to the bulk water in the water solution (Chen *et al.,* 2008), this might result in less protein aggregation of HSV gG expressed protein and thus, the integrity and stability of this expressed protein will be preserved. To Improve yield of expressed proteins, suspension cell culture should be explored. Sf9 cells were grown as a monolayer culture in the 75-cm^2 culture flasks. Although monolayer cell cultures are easy to inspect visually under the inverted microscope, this cell culture method required periodic passaging and Sf9 cells growth was limited by surface area, which might cause a limit

production on the HSV gG expressed proteins. Cells that are cultured in suspension can be maintained in culture flask and are easier to passage compared to monolayer cell culture. In addition, the suspension culture allows high yield of expressed proteins production in a short period.

For the seroepidemiological studies of MMRV in England, the comparison in the proportions of MMRV positive antibodies between 1996 and 2012 across the age groups were well established. However, for VZV, since the age group in this study was limited to aged ≤ 20 years, these data would not detect any upward shift of varicella infections toward to the older adult. The high proportions of VZV positive antibody in young adult suggested that there are less susceptible to varicella infection in older age. The trend of MMRV seroprevalence in 2012 across the regions was difficult to determine due to an imbalance of sample size from each region which produced an unequal distribution of age related samples between the regions. The use of more samples and equal number of age related samples from each region will be needed to correct this.

For the development of the HSV Bi-plexed assay, the low agreement with HerpeSelect ELISA method suggested this assay is suboptimal. Several problems relating to the non-specific binding due to the present of the detergents in the origin protein storage buffer and the low purity of the truncated coupled antigens, possibly cause the low sensitivity and specificity of HSV Bi-plexed in this study. Exclusion of detergents in the protein storage buffer and the improvement of the purity

of the truncated HSV gG antigens are costly and time consuming, which might result in more problematic situations. Therefore, the involvements of other conventional truncated HSV gG antigens are necessary, in order to improve the sensitivity and specificity of this HSV Bi-plexed assay.

Overall, the study showed that the effectiveness of the two-dose MMR vaccination policy had increased the MMR antibody prevalence in the population. This phenomenon serves to underline the importance of two doses of MMR vaccine in order to interrupt the transmission of these diseases in the population. Therefore, to archieve measles, mumps and rubella elimination, the priority will be to maintain high MMR vaccine coverage in the population with two doses. For VZV, almost half of the children aged 0 to 4 years (48% of 1996 study and 53.99% of 2012 study) did not possess sufficient antibody against VZV. At this level of immunity, it can be expected that the outbreak will continue to occur in the population, unless the introduction of varicella vaccine into routine childhood vaccination is implemented. For HSV, a change in levels of immunity and differences in frequency of recurrence for the type of herpes simplex virus had underlined the importance of accurate diagnosis of HSV subtype, in order to ensure adequate treatment of HSV infection. However, the low agreement between the HSV bead immunoassay and HerpeSelect ELISA method (sensitivity and specificity for HSV-1 of 84% and 68%, respectively, in the cut-off level of 35 AU/mL and sensitivity and

specificity for HSV-2 of 79% and 83%, respectively, in the cut-off level of 10 AU/mL) illustrate that further investigations are required.

The development of multiplex bead immunoassay is useful for seroepidemiological studies. This assay offers opportunities to examine different analytes simultaneously, in high throughput manner. The multiplex bead immunoassay is sensitive, rapid and only required low test samples volumes compared to other conventional methods, such characteristics are particularly important when attempting to detect responses antibody in samples such as CSF, dried blood and body fluids such as urine or oral fluid. As multiplex bead immunoassay offers advantages over other conventional methods such as ELISA, it is suggested that this assay could provide an efficient approach to improve the diagnosis of viral infections in the clinical laboratory and for research purposes.

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Appendix 1

Modifications of HSV-1 gG PCR amplification

Condition	Amplitaq Gold			AmpliTaq Gold 360 master mix		
	Target band		No band	Target band		No band
	Strong	Weak		Strong	Weak	
Initial PCR condition*		X (NA)*				
Concentration of $MgCl_2$ (2.0mM)			X (NA)			
Template dilution (Nil to 10^{-3})		X (until 10^{-1})				
BioMix Red master mix					X (until 10^{-2})(NA)	
Extension time to 1:30 minutes					X (NA)	
Annealing temperature to 55^0C					X (NA)	
Extension time to 2 minutes					X	
Annealing temperature to 60^0C		X		X (NA)		
Annealing time to 1:30 minutes		X		X (NA)		
Annealing (60^0C for 1:30 minutes)			X			
With and without $MgCl_2$ (±1mM)			X			
Extension time to 1:30 minutes			X		X (NA)	
Annealing time to 30 seconds			X		X (NA less)	
Initial denaturing time to 10 minutes			X		X (NA)	
Touchdown PCR (TD)				X (S)*		
TD (annealing time to 1 minute)				X (NA)		
Nested-PCR						X
TD (Template dilution nil to 10^{-3})				X (until 10^{-2}) (NA)		
TD (32 cycling cycles)				X (NA)		

Note: Initial PCR condition* represents the PCR condition as published by Sulaiman, 2010, (NA)* represents non-specific amplification, (S)* represents smearing formation.

Appendix 2

Modifications of HSV-2 gG PCR amplification

Condition	Amplitaq Gold			AmpliTaq Gold 360 master mix		
	Target band	Band with unexpected size	No band	Target band	Band with unexpected size	No band
PCR condition*		X (DB)*				
Template dilution (Nil to 10^{-7})		X (until 10-1)				
Cycling cycles to 45		X (until 10-2)				
AmpliTaq Gold 360 master mix					X (until 10^{-2})	
Extension time to 1:30 min					X	
Annealing temperature to 55^{0}C					X	
Extension time to 2 minutes					X	
Extension time to 2:30 min					X	
Primer set (without histidine tail)			X		X	
Concentration of $MgCI_2$ (2.0mM)		X			X	
Cross mix primer (PF* with NR*)			X		X	
Cross mix primer (NF with PR*)		X		X		
Cross mix primer (NF with NR)			X	X		
Annealing (30s), extension (2 min)			X			X
Touch down - Annealing (30s), extension (2 min)		x			X	
Extracted new template (NF with NR)			X	X		
Extracted new template (NF with PR)		X		X		
Nested-PCR						X
Annealing time to 1:30 min					X	
Extension time to 1:30 min (NF with NR)				X		
Extension time to 1:30 min (NF with PR)					X	
Triplicate the MS and CI samples				X		
Template dilution (Nil to 10^{-3})				X (Until 10^{-2})		

Note: PCR condition* represents the PCR condition described on section 2.4; PF* represents previous forward primer, containing 30 bp; NR* represents new reverse primer, containing 36 bp; PR* represents previous reverse primer, containing 33 bp; NF* represents new forward primer, containing 36 bp; DB* represents double band.

Appendix 3

Alignment of PCR product (sequencing using HSV-2 gG sense primer) and HSV-2 gG complete genome

		Sequences
1	wa_hf*	------CNNN NNNNTNNTGT TAGTGC---- ATNTCGGGTC TCGCTAGTGC ATAGTCGAGT
	NC-001798*	CAGGGACCCG GTATGCACGC CATCGCTCCC AGGTTGCTTC TTCTTTTTGT TCTTTCTGGT
61	wa_hf*	GCTCAGTGCC AGTGTCCCCG GAGACGCGAG AATTAACTGC CTCCTCCAAT CGACACAGAT
	NC-001798	CTTCCGGGGA CACGCGGCGG GTCGGGTGTC CCCGGACCAA TTAATCCCCC CAACAACGAT
121	wa_hf*	GTTGTTTTCC CGGGAGGTTC CCCCGTGGCT CAATATTGTT ATGCCTATCC CCGGTTGGAC
	NC-001798	GTTGTTTTCC CGGGAGGTTC CCCCGTGGCT CAATATTGTT ATGCCTATCC CCGGTTGGAC
181	wa_hf*	GATCCCGGGC CCTTGGGTTC CGCGGACGCC GGGCGGCAAG ACCTGCCCCG GCGCGTCGTC
	NC-001798	GATCCCGGGC CCTTGGGTTC CGCGGACGCC GGGCGGCAAG ACCTGCCCCG GCGCGTCGTC
241	wa_hf*	CGTCACGAGC CCCTGGGCCG CTCGTTCCTC ACGGGGGGGC TGGTTTTGCT GGCGCCGCCG
	NC-001798	CGTCACGAGC CCCTGGGCCG CTCGTTCCTC ACGGGGGGGC TGGTTTTGCT GGCGCCGCCG
301	wa_hf*	GTACGCGGAT TTGGCGCACC CAACGCAACG TATGCGGCCC GTGTGACGTA CTACCGGCTC
	NC-001798	GTACGCGGAT TTGGCGCACC CAACGCAACG TATGCGGCCC GTGTGACGTA CTACCGGCTC
361	wa_hf*	ACCCGCGCCT GCCGTCAGCC CATCCTCCTT CGGCAGTATG GAGGGTGTCG CGGCGGCGAG
	NC-001798	ACCCGCGCCT GCCGTCAGCC CATCCTCCTT CGGCAGTATG GAGGGTGTCG CGGCGGCGAG
421	wa_hf*	CCGCCGTCCC CAAAGACGTG TGGGTCGTAC ACGTACACGT ACCAGGGCGG CGGGCCTCCG
	NC-001798	CCGCCGTCCC CAAAGACGTG CGGGTCGTAC ACGTACACGT ACCAGGGCGG CGGGCCTCCG
481	wa_hf*	ACCCGGTACG CTCTCGTAAA TGCTTCCCTG CTGGTGCCGA TCTGGGACCG CGCCGCGGAN
	NC-001798	ACCCGGTACG CTCTCGTAAA TGCTTCCCTG CTGGTGCCGA TCTGGGACCG CGCCGCGGAG
541	wa_hf*	ACTTTCGAGT ACCAGATCGA ACTCGGCGGC GAACTGCACG TGGGTCTGTT GTGGGTAN--
	NC-001798	ACATTCGAGT ACCAGATCGA ACTCGGCGGC GAGCTGCACG TGGGTCTGTT GTGGGTAGAG
601	wa_hf*	---------- ---------- ---------- ---------- ---------- ----------
	NC-001798	GTGGGCGGGG AGGGCCCCGG CCCCACCGCC CCCCCACAGG CGGCGCGTGC GGAGGGCGGC
661	wa_hf*	---------- ---------- ---------- ---------- ---------- ----------
	NC-001798	CCGTGCGTCC CCCCGGTCCC CGCGGGCCGC CCGTGGCGCT CGGTGCCCCC GGTATGGTAT
721	wa_hf*	---------- ---------- ---------- ---------- ---------- ----------
	NC-001798	TCCGCCCCCA ACCCCGGGTT TCGTGGCCTG CGTTTCCGGG AGCGCTGTCT GCCCCCACAG
781	wa_hf*	---------- ---------- ---------- ---------- ---------- ----------
	NC-001798	ACGCCCGCCG CCCCCAGCGA CCTACCACGC GTCGCTTTTG CTCCCCAGAG CCTGCTGGTG
841	wa_hf*	---------- ---------- ---------- ---------- ---------- ----------
	NC-001798	GGGATTACGG GCCGCACGTT TATTCGGATG GCACGACCCA CGGAAGACGT CGGGGTCCTG
901	wa_hf*	---------- ---------- ---------- ---------- ---------- ----------
	NC-001798	CCACCCCATT GGGCCCCCGG GGCCCTAGAT GACGGTCCGT ACGCCCCCTT CCCACCCCGC
961	wa_hf*	---------- ---------- ---------- ---------- ---------- ----------
	NC-001798	CCGCGGTTTC GACGCGCCCT GCGGACAGAC CCCGAGGGGG TCGACCCCGA CGTTCGGGCC
1021	wa_hf*	---------- ---------- ---------- ---------- ---------- ----------
	NC-001798	CCCCTAACCG GGCGGCGCCT CATGGCCTTG ACCGAGGACG CGTCCTCCGA TTCGCCTACG
1081	wa_hf*	---------- ---------- ---------- ---------- ---------- ----------
	NC-001798	TCCGCTCCGG AGAAGACGCC CCTCCCTGTG TCGGCCACCG CCATGGCGCC CTCAGTCGAC

		Sequences
1141	wa_hf* NC-001798	---------- CCAAGCGCGG AACCGACCGC CCCCGCAACC ACTACTCCCC CCGACGAGAT GGCCACACAA
1201	wa_hf* NC-001798	---------- GCCGCAACGG TCGCCGTTAC GCCGGAGGAA ACGGCAGTCG CCTCCCCGCC CGCGACTGCA
1261	wa_hf* NC-001798	---------- TCCGTGGAGT CGTCGCCACT CCCCGCCGCG GCGGCAACGC CCGGGGCCGG GCACACGAAC
1321	wa_hf* NC-001798	---------- ACCAGCAGCG CCCCCGCAGC GAAAACGCCC CCCACCACAC CAGCCCCCAC GACCCCCCCG
1381	wa_hf* NC-001798	---------- CCCACGTCTA CCCACGCGAC CCCCCGCCCC ACGACTCCGG GGCCCCAAAC AACCCCTCCC
1441	wa_hf* NC-001798	---------- GGACCCGCAA CCCCGGGTCC GGTGGGCGCC TCCGCCGCAC CCACGGCCGA TTCCCCCCTC
1501	wa_hf* NC-001798	---------- ACCGCCTCGC CCCCCGCTAC CGCGCCGGGG CCCTCGGCCG CCAACGTTTC GGTCGCCGCG
1561	wa_hf* NC-001798	---------- ACCACCGCCA CGCCCGGAAC CCGGGGCACC GCCCGTACCC CCCCAACGGA CCCAAAGACG
1621	wa_hf* NC-001798	---------- CACCCACACG GACCCGCGGA CGCTCCCCCC GGCTCGCCAG CCCCCCCACC CCCCGAACAT
1681	wa_hf* NC-001798	---------- CGCGGCGGAC CCGAGGAGTT TGAGGGCGCC GGGGACGGCG AACCCCCCGA TGACGACGAC
1741	wa_hf* NC-001798	---------- AGCGCCACCG GTCTCGCCTT CCGAACTCCG AACCCCAACA AACCACCCCC CGCGCGCCCC
1801	wa_hf* NC-001798	---------- GGGCCCATCC GCCCCACGCT CCCGCCAGGA ATTCTTGGGC CGCTCGCCCC CAACACGCCT
1861	wa_hf* NC-001798	--------- CGCCCCCCCG CCCAAGCTCC CGCTAAGGAC ATGCCCTCGG GCCCCACACC CCAACACATC
1921	wa_hf* NC-001798	---------- CCCCTGTTCT GGTTCCTAAC GGCCTCCCCT GCTCTAGATA TCCTCTTTAT CATCAGCACC
1981	wa_hf* NC-001798	---------- ACCATCCACA CGGCGGCGTT CGTTTGTCTG GTCGCCTTGG CAGCACAACT TTGGCGCGGC
2041	wa_hf* NC-001798	---------- CGGGCGGGGC GCAGGCGATA CGCGCACCCG AGCGTGCGTT ACGTATGTCT GCCACCCGAG
2101	wa_hf* NC-001798	--------- CGGGATTAG

Note: wa_hf represents PCR product, NC-001798 represents HSV-2 gG genome

Appendix 4

Alignment of PCR product (sequencing using HSV-2 gG antisense primer) and HSV-2 gG complete genome

		Sequences					
1	wa_hr* NC-001798	---------- ATGCACGCCA	---------- TCGCTCCCAG	---------- GTTGCTTCTT	---------- CTTTTTGTTC	---------- TTTCTGGTCT	---------- TCCGGGGACA
61	wa_hr* NC-001798	---------- CGCGGCGGGT	---------- CGGGTGTCCC	---------- CGGACCAATT	---------- AATCCCCCCA	---------- ACAACGATGT	---------- TGTTTTCCCG
121	wa_hr* NC-001798	---------- GGAGGTTCCC	---------- CCGTGGCTCA	---------- ATATTGTTAT	---------- GCCTATCCCC	---------- GGTTGGACGA	---------- TCCCGGGCCC
181	wa_hr* NC-001798	---------- TTGGGTTCCG	---------- CGGACGCCGG	---------- GCGGCAAGAC	---------- CTGCCCCGGC	---------- GCGTCGTCCG	---------- TCACGAGCCC
241	wa_hr* NC-001798	---------- CTGGGCCGCT	---------- CGTTCCTCAC	---------- GGGGGGGCTG	---------- GTTTTGCTGG	---------- CGCCGCCGGT	---------- ACGCGGATTT
301	wa_hr* NC-001798	---------- GGCGCACCCA	---------- ACGCAACGTA	---------- TGCGGCCCGT	---------- GTGACGTACT	---------- ACCGGCTCAC	---------- CCGCGCCTGC
361	wa_hr* NC-001798	---------- CGTCAGCCCA	---------- TCCTCCTTCG	---------- GCAGTATGGA	---------- GGGTGTCGCG	---------- GCGGCGAGCC	---------- GCCGTCCCCA
421	wa_hr* NC-001798	---------- AAGACGTGCG	---------- GGTCGTACAC	---------- GTACACGTAC	---------- CAGGGCGGCG	---------- GGCCTCCGAC	---------- CCGGTACGCT
481	wa_hr* NC-001798	---------- CTCGTAAATG	---------- CTTCCCTGCT	---------- GGTGCCGATC	---------- TGGGACCGCG	---------- CCGCGGAGAC	---------- ATTCGAGTAC
541	wa_hr* NC-001798	---------- CAGATCGAAC	---------- TCGGCGGCGA	---------- GCTGCACGTG	---------- GGTCTGTTGT	---------- GGGTAGAGGT	---------- GGGCGGGGAG
601	wa_hr* NC-001798	---------- GGCCCCGGCC	---------- CCACCGCCCC	---------- CCCACAGGCG	---------- GCGCGTGCGG	---------- AGGGCGGCCC	---------- GTGCGTCCCC
661	wa_hr* NC-001798	---------- CCGGTCCCCG	---------- CGGGCCGCCC	---------- GTGGCGCTCG	---------- GTGCCCCCGG	---------- TATGGTATTC	---------- CGCCCCCAAC
721	wa_hr* NC-001798	---------- CCCGGGTTTC	---------- GTGGCCTGCG	---------- TTTCCGGGAG	---------- CGCTGTCTGC	---------- CCCCACAGAC	---------- GCCCGCCGCC
781	wa_hr* NC-001798	---------- CCCAGCGACC	---------- TACCACGCGT	---------- CGCTTTTGCT	---------- CCCCAGAGCC	---------- TGCTGGTGGG	---------- GATTACGGGC
841	wa_hr* NC-001798	---------- CGCACGTTTA	---------- TTCGGATGGC	---------- ACGACCCACG	---------- GAAGACGTCG	---------- GGGTCCTGCC	---------- ACCCCATTGG
901	wa_hr* NC-001798	---------- GCCCCCGGGG	---------- CCCTAGATGA	---------- CGGTCCGTAC	---------- GCCCCCTTCC	---------- CACCCCGCCC	---------- GCGGTTTCGA
961	wa_hr* NC-001798	---------- CGCGCCCTGC	---------- GGACAGACCC	---------- CGAGGGGGTC	---------- GACCCCGACG	---------- TTCGGGCCCC	---------- CCTAACCGGG
1021	wa_hr* NC-001798	---------- CGGCGCCTCA	---------- TGGCCTTGAC	---------- CGAGGACGCG	---------- TCCTCCGATT	---------- CGCCTACGTC	---------- CGCTCCGGAG
1081	wa_hr* NC-001798	---------- AAGACGCCCC	---------- TCCCTGTGTC	---------- GGCCACCGCC	---------- ATGGCGCCCT	---------- CAGTCGACCC	---------- AAGCGCGGAA
1141	wa_hr* NC-001798	---------- CCGACCGCCC	---------- CCGCAACCAC	---------- TACTCCCCCC	---------- GACGAGATGG	---------- CCACACAAGC	---------- CGCAACGGTC

		Sequences					
1201	wa_hr*	----------	----------	----------	----------	----------	----------
	NC-001798	GCCGTTACGC	CGGAGGAAAC	GGCAGTCGCC	TCCCCGCCCG	CGACTGCATC	CGTGGAGTCG
1261	wa_hr*	----------	----------	----------	----------	----------	----------
	NC-001798	TCGCCACTCC	CCGCCGCGGC	GGCAACGCCC	GGGGCCGGGC	ACACGAACAC	CAGCAGCGCC
1321	wa_hr*	----------	----------	----------	----------	----------	----------
	NC-001798	CCCGCAGCGA	AAACGCCCCC	CACCACACCA	GCCCCCACGA	CCCCCCCGCC	CACGTCTACC
1381	wa_hr*	----------	----------	----------	----------	----------	----------
	NC-001798	CACGCGACCC	CCCGCCCCAC	GACTCCGGGG	CCCCAAACAA	CCCCTCCCGG	ACCCGCAACC
1441	wa_hr*	----------	----------	----------	----------	----------	----------
	NC-001798	CCGGGTCCGG	TGGGCGCCTC	CGCCGCACCC	ACGGCCGATT	CCCCCCTCAC	CGCCTCGCCC
1501	wa_hr*	----------	----------	----------	----------	----------	----------
	NC-001798	CCCGCTACCG	CGCCGGGGCC	CTCGGCCGCC	AACGTTTCGG	TCGCCGCGAC	CACCGCCACG
1561	wa_hr*	----------	----------	----------	----------	----------	----------
	NC-001798	CCCGGAACCC	GGGGCACCGC	CCGTACCCCC	CCAACGGACC	CAAAGACGCA	CCCACACGGA
1621	wa_hr*	----------	----------	----------	----------	----------	----------
	NC-001798	CCCGCGGACG	CTCCCCCCGG	CTCGCCAGCC	CCCCCACCCC	CCGAACATCG	CGGCGGACCC
1681	wa_hr*	----------	----------	----------	--------GG	GAGAGGAGGA	TGCCGGCAGG
	NC-001798	GAGGAGTTTG	AGGGCGCCGG	GGACGGCGAA	CCCCCCGATG	ACGACGACAG	CGCCACCGGT
1741	wa_hr*	CGCGGGTGAG	CCAGTTTTAC	CCCCCACGAG	CCGCCCCCCG	CGCGCCCGGG	GCCCATAAGC
	NC-001798	CTCGCCTTCC	GAACTCCGAA	CCCCAACAAA	CCACCCCCCG	CGCGCCCCGG	GCCCATCCGC
1801	wa_hr*	CCCACGCTGC	GGCCAGGAAT	TAAAGGGCCC	CTCGCCCCCA	ACACGCCTCG	CCCCCCCGCC
	NC-001798	CCCACGCTCC	CGCCAGGAAT	TCTTGGGCCG	CTCGCCCCCA	ACACGCCTCG	CCCCCCCGCC
1861	wa_hr*	CAAGCTCCCG	CTAAGGACAT	GCCCTCGGGC	CCCACACCCC	AACACATCCC	CCTGTTCTGG
	NC-001798	CAAGCTCCCG	CTAAGGACAT	GCCCTCGGGC	CCCACACCCC	AACACATCCC	CCTGTTCTGG
1921	wa_hr*	TTCCTAACGG	CCTCCCCTGC	TCTAGATATC	CTCTTTATCA	TCAGCACCAC	CATCCACACG
	NC-001798*	TTCCTAACGG	CCTCCCCTGC	TCTAGATATC	CTCTTTATCA	TCAGCACCAC	CATCCACACG
1981	wa_hr*	GCGGCGTTCG	TTTGTGCTGG	TCGCCTTGGC	AGCACAACTT	TGGCAGGGCA	GGNCAGGG-G
	NC-001798*	GCGGCGTTCG	TTTGT-CTGG	TCGCCTTGGC	AGCACAACTT	TGGCGCGGCC	GGGCGGGGCG
2041	wa_hr*	CAGCATATAC	ACGCANCCGA	GCGCNNTATN	CNNNNG----	----------	----------
	NC-001798*	CAGGCGATAC	GCGCACCCGA	GCGTGCGTTA	CGTATGTCTG	CCACCCGAGC	GGGATTAGGG
2101	wa_hr*	----------	----				
	NC-001798*	CGAGCCCACC	GTCT				

Note: wa_hr represents PCR product, NC-001798 represents HSV-2 gG genome

Appendix 5

Chemical ingredients for AmplitaqGold 360 Master Mix

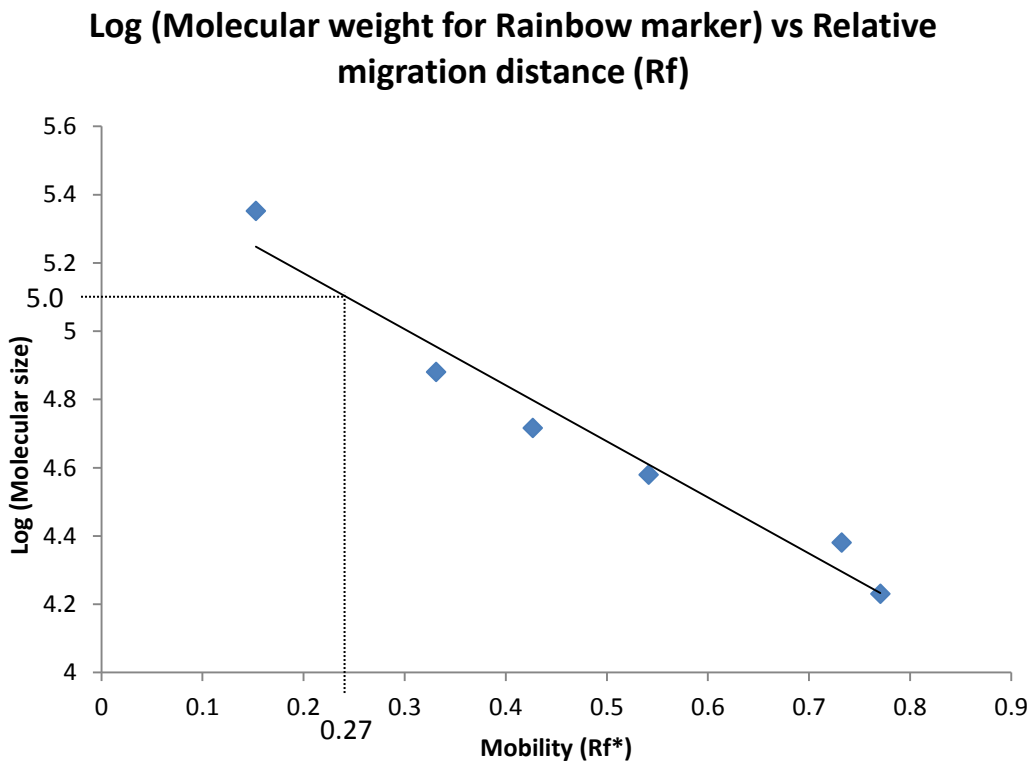
Identification of the substance/preparation: AmpliTaq Gold 360 Master Mix

Chemical Name	CAS-No.	Weight percent (%)
1,3-Propanediol,2-amino-2-(hydroxymethyl)-,hydrochloride	1185-53-1	0.5-1.5
Glycerin	56-81-5	10-30
Sodium azide	26628-22-8	<0.1

(Adapted from Material Safety Data Sheet (MSDS) for AmplitaqGold 360 Master Mix, Invitrogen, Life Technologies, Paisley, UK) Available at: https://tools.lifetechnologies.com/content/sfs/msds/2012/4398790_MTR-EULT_BE.pdf (Accessed: 19 MAY 2015)

Appendix 6

Determine molecular weight of HSV-1 gG purified protein



Note: Rf = migration distance of the protein / Migration distance of the dye front

The Rf value for HSV-1 Gg purified protein = 0.27

Log (molecular size) of HSV-1 Gg purified protein = 5.06

Molecular size of the HSV-1 Gg purified protein = $10^{5.06}$ Dalto

= 114815.36 Dalton

Appendix 7

The light scattering report for HSV-1 gG recombinant protein

ASTRA 6 Report Experiment1

File Name: Experiment1
Collection Version: 6.1.1.17

Sample:

Description:
dn/dc: 0.1800 mL/g
Concentration: 0.000 mg/mL
Injected Volume: 500.0 µL

Configuration

Description: MALLS 1 config 210113
Concentration Source: RI
Flow Rate: 0.750 mL/min

Light Scattering Instrument: DAWN HELEOS

Temperature Control: yes
Temperature: 25.0 °C
Band Broadening Correction: Yes (Instrumental: 7.926 µL, Mixing: 19.403 µL)
Cell Type: Fused Silica
Wavelength: 658.0 nm
Calibration Constant: 3.0538×10^{-5} 1/(V cm)
QELS Fiber Replaces Detector: 12 (99.9°)

Detector	Scattering angle	Gain	Normalization coefficient
1	n/a	n/a	1.000
2	13.0°	n/a	14.478
3	20.6°	n/a	2.395
4	29.6°	n/a	1.909

Detector	Scattering angle	Gain	Normalization coefficient
5	37.4°	n/a	2.569
6	44.8°	n/a	0.728
7	53.1°	n/a	0.822
8	61.1°	n/a	0.967
9	70.1°	n/a	0.930
10	80.1°	n/a	1.047
11	90.0°	n/a	1.000
12	n/a	n/a	1.000
13	109.9°	n/a	1.007
14	120.1°	n/a	0.952
15	130.4°	n/a	0.851
16	140.1°	n/a	0.727
17	149.1°	n/a	2.243
18	157.8°	n/a	2.032

RI Instrument: Optilab rEX

Band Broadening Correction: n/a
Wavelength: 633.0 nm
Calibration Constant: 3.4203×10^{-5} RIU/pixel

UV Instrument: Generic UV

Description: 280nm
Band Broadening Correction: Yes (Instrumental: 1.010 µL, Mixing: 1.004 µL)
UV Cell Length: 1.000 cm
UV Response Factor: 1.000 AU/V

Column: SX200#4

Description: PBS

Solvent: PBS, Aqueous

Refractive Index: 1.331
Viscosity: 0.894 cP

Fluid Connections

Source Instrument	Destination Instrument	Delay Volume (mL)
Generic Pump	Injector	0.000
Injector	Generic Column	0.000
Generic Column	Generic UV Instrument	0.000
Generic UV Instrument	DAWN HELEOS	0.135
DAWN HELEOS	Optilab rEX	0.135

ıx Connections

Source Instrument	Destination Instrument	Aux Channel	Calibration Constant
Generic UV Instrument	DAWN HELEOS	1	2.000×10^{-4}

Processing

Collection Time: Wednesday May 07, 2014 10:06:48 AM GMT Daylight Time
Processing time: Wednesday May 07, 2014 11:29:55.292 AM GMT Daylight Time

Basic Collection:

LS Instrument Collection Interval: 0.500 sec
QELS Instrument Collection Interval: 2.0 sec

Peak settings:

Peak Name	Peak 1	Peak 2	Peak 3
Peak Limits (mL)	13.056 - 13.969	15.340 - 16.852	8.426 - 9.099
Light Scattering Model	Zimm	Zimm	Zimm
Fit Degree	1	1	1
dn/dc (mL/g)	0.1800	0.1800	0.1800
A2 (mol mL/g²)	0.000	0.000	0.000

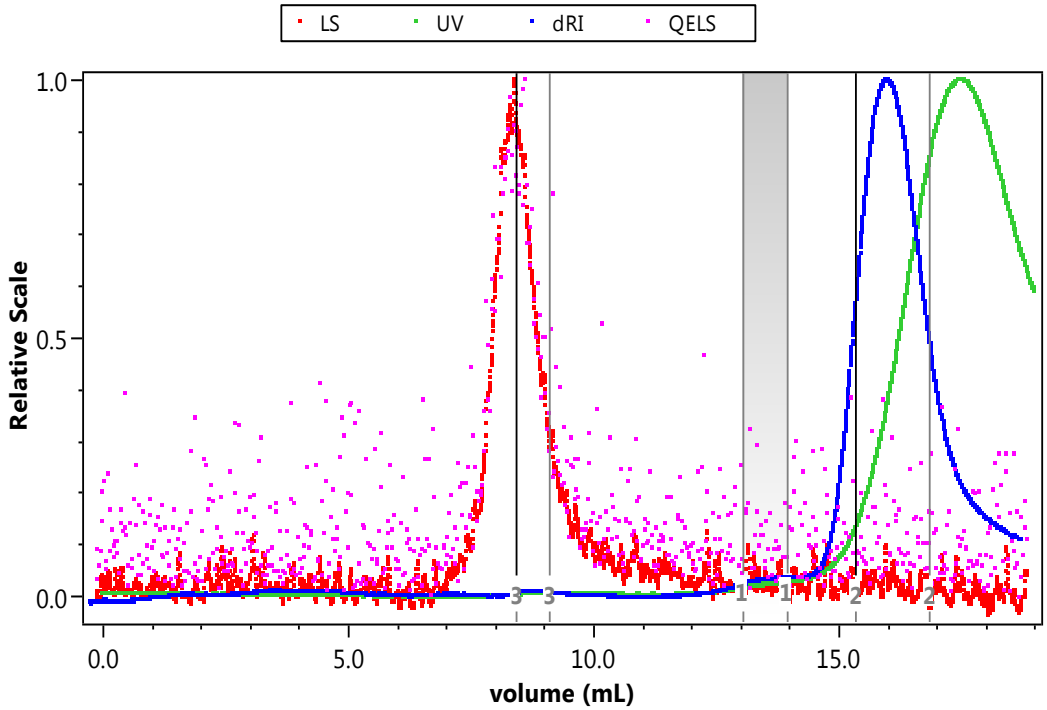
Peak Results

	Peak 1	Peak 2	Peak 3
Hydrodynamic radius (Q) moments (nm)			
Rh(Q)n	249.940 (±5.445%)	268.439 (±3.597%)	233.162 (±3.296%)
Rh(Q)w	251.738 (±5.265%)	276.309 (±3.616%)	233.672 (±3.194%)
Rh(Q)z	253.693 (±5.010%)	281.168 (±3.624%)	229.596 (±3.101%)
Rh(Q)(avg)	202.126 (±0.784%)	223.768 (±0.570%)	229.999 (±0.685%)
Masses			
Calculated Mass (µg)	0.45	20.76	0.07
Mass Fraction (%)	2.1	97.6	0.3
Molar mass moments (g/mol)			
Mn	2.908×10^{5} (±60.860%)	1.192×10^{4} (±64.879%)	3.353×10^{7} (±7.696%)
Mp	2.644×10^{5} (±48.499%)	1.121×10^{4} (±48.909%)	3.174×10^{7} (±5.540%)
Mw	3.373×10^{5} (±65.419%)	1.592×10^{4} (±75.663%)	3.816×10^{7} (±6.648%)
Mz	4.354×10^{5} (±161.246%)	2.112×10^{4} (±176.063%)	4.512×10^{7} (±13.694%)
Mz+1	6.434×10^{5} (±199.287%)	2.611×10^{4} (±236.027%)	5.416×10^{7} (±16.789%)
M(avg)	2.706×10^{5} (±9.075%)	8.723×10^{3} (±9.535%)	3.575×10^{7} (±0.633%)
Polydispersity			
Mw/Mn	1.160 (±89.351%)	1.335 (±99.670%)	1.138 (±10.170%)
Mz/Mn	1.497 (±172.349%)	1.772 (±187.637%)	1.346 (±15.709%)
rms radius moments (nm)			
Rn	n/a	n/a	66.5 (±5.9%)
Rw	n/a	n/a	66.9 (±5.5%)

Peak Results

	Peak 1	Peak 2	Peak 3
Rz	n/a	n/a	67.4 (±5.0%)
R(avg)	132.6 (±23.9%)	128.6 (±15.3%)	66.0 (±0.5%)

Define Peaks



Appendix 8

Demographic detail of MMRV test samples

Barcode	Age	Gender*	Region*
M174011	7	F	NW
M174013	7	F	NW
M174494	7	F	NW
M174495	7	F	NW
M174502	8	F	NW
M174503	8	F	NW
M174505	8	F	NW
M175136	7	F	NW
M175138	7	F	NW
M175302	61	F	NW
M175347	24	F	East
M175448	29	F	East
M175952	65	F	NE
M176144	64	F	East
M176165	45	F	East
M176194	30	F	East
M176243	0	F	NW
M176276	5	F	NW
M176277	5	F	NW
M176282	6	F	NW
M176283	6	F	NW
M176291	7	F	NW
M176292	7	F	NW
M176298	8	F	NW
M176300	8	F	NW
M176335	13	F	NW
M176355	16	F	NW
M176378	19	F	NW
M176485	81	F	NW
M176492	0	F	NW
M176519	5	F	NW
M176523	6	F	NW
M176638	22	F	NW
M176655	24	F	NW
M176661	25	F	NW
M176679	36	F	NW
M176693	46	F	NW
M176725	6	F	SW
M176871	6	F	SW
M176946	1	F	YH
M177170	38	F	SE
M177174	30	F	SE
M177218	49	F	SE
M177223	53	F	SE
M177235	57	F	SE
M177264	55	F	SE

Barcode	Age	Gender*	Region*
M177289	52	F	SE
M177369	0	F	SW
M177376	75	F	SW
M177390	1	F	NW
M177397	2	F	NW
M177398	2	F	NW
M177399	2	F	NW
M177408	3	F	NW
M177429	6	F	NW
M177430	6	F	NW
M177431	6	F	NW
M177432	6	F	NW
M177437	7	F	NW
M177439	7	F	NW
M177529	19	F	NW
M177582	34	F	NW
M177617	57	F	NW
M177664	59	F	East
M177701	32	F	East
M177780	82	F	SE
M177844	80	F	SE
M177860	64	F	SE
M177888	22	F	SE
M177913	34	F	SE
M177925	19	F	SE
M177928	18	F	SE
M177956	1	F	NW
M177957	1	F	NW
M177958	1	F	NW
M177959	1	F	NW
M177964	2	F	NW
M177965	2	F	NW
M177966	2	F	NW
M177967	2	F	NW
M177973	3	F	NW
M177975	3	F	NW
M177993	6	F	NW
M177994	6	F	NW
M177995	6	F	NW
M178001	7	F	NW
M178019	10	F	NW
M178030	13	F	NW
M178090	21	F	NW
M178116	24	F	NW
M178132	31	F	NW
M178139	38	F	NW
M178178	83	F	NW
M178179	83	F	NW
M178757	2	F	NW

Barcode	Age	Gender*	Region*
M178758	2	F	NW
M178759	2	F	NW
M178774	4	F	NW
M178783	5	F	NW
M178788	6	F	NW
M178789	6	F	NW
M178790	6	F	NW
M178791	6	F	NW
M178796	7	F	NW
M178886	19	F	NW
M178934	27	F	NW
M178973	64	F	NW
M178979	67	F	NW
M178989	78	F	NW
M179034	18	F	Wmids
M179055	15	F	YH
M179608	44	F	Emids
M179611	43	F	Emids
M179660	3	F	Emids
M179662	2	F	Emids
M179664	2	F	Emids
M179667	0	F	Emids
M179708	7	F	SW
M179711	2	F	SW
M179721	75	F	SW
M179723	49	F	SW
M179732	20	F	SW
M179768	1	F	SW
M179787	22	F	SW
M179831	4	F	LON
M179837	0	F	LON
M179853	7	F	LON
M179878	26	F	LON
M179879	27	F	LON
M179926	75	F	LON
M179966	7	F	Emids
M179975	28	F	Emids
M180027	82	F	East
M180028	81	F	East
M180035	76	F	East
M180077	22	F	East
M180108	26	F	Emids
M180153	86	F	SW
M180188	75	F	SW
M180207	6	F	YH
M180209	0	F	YH
M180210	1	F	YH
M180221	0	F	YH
M180237	0	F	YH

Barcode	Age	Gender*	Region*
M180271	5	F	YH
M180287	0	F	YH
M180307	81	F	YH
M180333	0	F	YH
M180335	4	F	SW
M180336	2	F	SW
M180346	6	F	SW
M180347	7	F	SW
M180350	0	F	SW
M180352	0	F	SW
M180354	1	F	SW
M180355	1	F	SW
M180364	7	F	SW
M180368	73	F	SE
M180371	57	F	SE
M180399	27	F	SE
M180422	83	F	SE
M180424	75	F	SE
M180455	76	F	SW
M180458	74	F	SW
M180460	41	F	SW
M180461	29	F	SW
M180466	26	F	SW
M180468	5	F	SW
M180481	6	F	SW
M180496	1	F	SW
M180507	2	F	SW
M180548	26	F	SW
M180550	65	F	SW
M180557	4	F	SW
M180562	77	F	SW
M180626	4	F	SW
M180641	2	F	NE
M180649	25	F	NE
M180717	28	F	NE
M180772	31	F	NE
M180822	75	F	Emids
M180842	84	F	SW
M180856	7	F	SW
M180858	2	F	SW
M180909	5	F	SW
M180911	7	F	SW
M180920	2	F	SW
M180923	5	F	SW
M180934	1	F	SW
M180936	1	F	SW
M180951	8	F	SW
M180957	6	F	SW
M180963	14	F	SW

Barcode	Age	Gender*	Region*
M180966	9	F	SW
M180988	26	F	LON
M181038	27	F	LON
M181533	46	F	LON
M181668	1	F	Wmids
M181669	0	F	Wmids
M181671	0	F	Wmids
M181679	2	F	Wmids
M181680	2	F	Wmids
M181683	3	F	Wmids
M181732	19	F	Wmids
M181757	21	F	Wmids
M181762	21	F	Wmids
M181811	33	F	SW
M181817	49	F	SW
M181825	32	F	SW
M181850	37	F	SW
M181874	76	F	SW
M181901	80	F	SW
M181909	48	F	SW
M181919	13	F	SW
M181921	65	F	SW
M181933	74	F	SW
M181969	1	F	SW
M181972	1	F	SW
M181979	3	F	SW
M181980	2	F	SW
M182019	8	F	SW
M182029	6	F	SW
M182030	6	F	SW
M182031	6	F	SW
M182506	6	F	YH
M182509	8	F	YH
M182518	6	F	YH
M182531	1	F	YH
M182532	15	F	YH
M182534	2	F	YH
M182560	7	F	YH
M182561	5	F	YH
M182573	6	F	YH
M182581	1	F	YH
M182583	6	F	YH
M182585	20	F	YH
M182587	44	F	YH
M182623	1	F	YH
M182630	1	F	YH
M182639	34	F	YH
M182640	1	F	YH
M182649	0	F	YH

Barcode	Age	Gender*	Region*
M182671	1	F	YH
M182675	0	F	YH
M182681	34	F	YH
M182699	1	F	YH
M182702	2	F	YH
M182704	74	F	YH
M182711	54	F	YH
M182716	0	F	YH
M182720	1	F	YH
M182729	6	F	YH
M182731	7	F	YH
M182735	4	F	YH
M182739	6	F	YH
M182746	54	F	YH
M182758	2	F	YH
M182773	19	F	YH
M182778	2	F	YH
M182791	74	F	YH
M182794	8	F	YH
M182796	6	F	YH
M182800	7	F	YH
M182801	4	F	YH
M182805	2	F	YH
M182807	5	F	YH
M182827	6	F	YH
M182828	34	F	YH
M182848	2	F	YH
M182856	34	F	YH
M183098	6	F	YH
M183134	75	F	YH
M183156	24	F	YH
M183159	6	F	YH
M183166	3	F	YH
M183194	75	F	East
M183911	3	F	SW
M183915	7	F	SW
M183924	6	F	SW
M183927	1	F	SW
M183958	67	F	SW
M183970	3	F	SW
M183975	77	F	SW
M184273	15	F	SW
M184278	1	F	SW
M184284	4	F	SW
M184288	6	F	SW
M184289	2	F	SW
M184305	76	F	SW
M184308	1	F	SW
M184871	42	F	Emids

Barcode	Age	Gender*	Region*
M184928	26	F	Emids
M184934	37	F	Emids
M184936	36	F	Emids
M184974	28	F	Emids
M185028	51	F	Emids
M185037	68	F	Emids
M185040	35	F	Emids
M185052	67	F	Emids
M185056	55	F	Emids
M185065	50	F	Emids
M185066	35	F	Emids
M185112	0	F	Emids
M185260	0	F	Emids
M185324	0	F	Emids
M185338	0	F	Emids
M185392	0	F	Emids
M185393	0	F	Emids
M185394	0	F	Emids
M185395	0	F	Emids
M185397	0	F	Emids
M187273	0	F	YH
M189721	7	F	NW
M189722	7	F	NW
M174499	7	M	NW
M174500	7	M	NW
M174501	7	M	NW
M174508	8	M	NW
M175139	7	M	NW
M175325	78	M	NW
M175937	70	M	NE
M175939	65	M	NE
M175947	67	M	NE
M176246	0	M	NW
M176247	1	M	NW
M176248	0	M	NW
M176257	2	M	NW
M176271	4	M	NW
M176279	5	M	NW
M176287	6	M	NW
M176295	7	M	NW
M176297	7	M	NW
M176301	8	M	NW
M176401	21	M	NW
M176501	2	M	NW
M176522	5	M	NW
M176526	6	M	NW
M176527	6	M	NW
M176528	6	M	NW
M176535	7	M	NW

Barcode	Age	Gender*	Region*
M176542	8	M	NW
M176619	19	M	NW
M176736	6	M	SW
M176878	8	M	SW
M177166	53	M	SE
M177173	40	M	SE
M177194	47	M	SE
M177239	57	M	SE
M177249	63	M	SE
M177293	37	M	SE
M177309	2	M	SE
M177339	68	M	SW
M177382	75	M	SW
M177393	1	M	NW
M177394	0	M	NW
M177395	0	M	NW
M177396	1	M	NW
M177401	2	M	NW
M177402	2	M	NW
M177403	2	M	NW
M177404	2	M	NW
M177409	3	M	NW
M177410	3	M	NW
M177417	4	M	NW
M177420	4	M	NW
M177426	5	M	NW
M177427	5	M	NW
M177433	6	M	NW
M177434	6	M	NW
M177435	6	M	NW
M177436	6	M	NW
M177440	7	M	NW
M177442	7	M	NW
M177443	7	M	NW
M177462	10	M	NW
M177525	18	M	NW
M177586	30	M	NW
M177636	83	M	NW
M177663	59	M	East
M177724	76	M	East
M177764	63	M	SE
M177769	82	M	SE
M177783	6	M	SE
M177785	7	M	SE
M177817	75	M	SE
M177861	71	M	SE
M177867	79	M	SE
M177883	75	M	SE
M177896	1	M	SE

Barcode	Age	Gender*	Region*
M177917	3	M	SE
M177934	8	M	SW
M177961	0	M	NW
M177962	0	M	NW
M177963	0	M	NW
M177968	2	M	NW
M177969	2	M	NW
M177971	2	M	NW
M177976	3	M	NW
M177978	3	M	NW
M177985	4	M	NW
M177997	6	M	NW
M178000	6	M	NW
M178004	7	M	NW
M178013	8	M	NW
M178024	11	M	NW
M178042	14	M	NW
M178129	27	M	NW
M178135	34	M	NW
M178713	1	M	SW
M178715	1	M	SW
M178751	1	M	SW
M178754	0	M	NW
M178755	0	M	NW
M178756	0	M	NW
M178760	2	M	NW
M178761	2	M	NW
M178762	2	M	NW
M178763	2	M	NW
M178786	5	M	NW
M178787	5	M	NW
M178792	6	M	NW
M178793	6	M	NW
M178794	6	M	NW
M178797	7	M	NW
M178799	7	M	NW
M178800	7	M	NW
M178804	8	M	NW
M178805	8	M	NW
M178813	9	M	NW
M178842	13	M	NW
M178856	15	M	NW
M178866	16	M	NW
M178896	20	M	NW
M178898	20	M	NW
M179622	38	M	Emids
M179643	14	M	Emids
M179655	7	M	Emids
M179663	2	M	Emids

Barcode	Age	Gender*	Region*
M179666	1	M	Emids
M179730	60	M	SW
M179760	68	M	SW
M179762	2	M	SW
M179772	4	M	SW
M179793	2	M	SW
M179795	58	M	SW
M179812	1	M	SW
M179838	0	M	LON
M179951	13	M	Emids
M180083	0	M	Emids
M180084	6	M	Emids
M180085	7	M	Emids
M180097	66	M	Emids
M180134	21	M	SW
M180145	44	M	SW
M180183	75	M	SW
M180187	75	M	SW
M180203	82	M	SW
M180211	0	M	YH
M180217	1	M	YH
M180222	1	M	YH
M180224	87	M	YH
M180226	5	M	YH
M180240	81	M	YH
M180242	0	M	YH
M180243	0	M	YH
M180254	0	M	YH
M180258	0	M	YH
M180281	45	M	YH
M180291	81	M	YH
M180301	0	M	YH
M180309	5	M	YH
M180311	75	M	YH
M180330	75	M	YH
M180343	5	M	SW
M180344	5	M	SW
M180353	1	M	SW
M180356	1	M	SW
M180365	7	M	SW
M180369	63	M	SE
M180394	47	M	SE
M180395	40	M	SE
M180459	2	M	SW
M180478	42	M	SW
M180491	42	M	SW
M180509	36	M	SW
M180513	49	M	SW
M180533	6	M	SW

Barcode	Age	Gender*	Region*
M180543	74	M	SW
M180551	6	M	SW
M180558	75	M	SW
M180559	8	M	SW
M180565	74	M	SW
M180588	6	M	SW
M180604	77	M	SW
M180611	74	M	SW
M180621	0	M	SW
M180632	0	M	NE
M180683	26	M	NE
M180783	41	M	NE
M180792	1	M	Emids
M180793	1	M	Emids
M180808	78	M	Emids
M180827	3	M	SW
M180833	4	M	SW
M180838	3	M	SW
M180841	1	M	SW
M180851	3	M	SW
M180855	2	M	SW
M180866	1	M	SW
M180885	7	M	SW
M180887	2	M	SW
M180893	32	M	SW
M180899	55	M	SW
M180904	7	M	SW
M180912	1	M	SW
M180913	6	M	SW
M180916	0	M	SW
M180927	1	M	SW
M180953	47	M	SW
M180960	13	M	SW
M180961	2	M	SW
M181662	6	M	Wmids
M181663	7	M	Wmids
M181670	1	M	Wmids
M181672	0	M	Wmids
M181673	0	M	Wmids
M181674	1	M	Wmids
M181675	1	M	Wmids
M181677	1	M	Wmids
M181678	2	M	Wmids
M181682	3	M	Wmids
M181709	40	M	Wmids
M181802	34	M	SW
M181814	52	M	SW
M181849	1	M	SW
M181886	40	M	SW

Barcode	Age	Gender*	Region*
M181963	7	M	SW
M181965	5	M	SW
M181968	2	M	SW
M181970	0	M	SW
M181975	1	M	SW
M181976	1	M	SW
M181977	3	M	SW
M182003	2	M	SW
M182004	2	M	SW
M182013	9	M	SW
M182017	8	M	SW
M182022	7	M	SW
M182023	7	M	SW
M182028	6	M	SW
M182033	2	M	SW
M182034	3	M	SW
M182039	4	M	SW
M182046	0	M	SW
M182047	0	M	SW
M182383	7	M	YH
M182503	39	M	YH
M182512	0	M	YH
M182538	8	M	YH
M182546	44	M	YH
M182556	44	M	YH
M182562	74	M	YH
M182572	6	M	YH
M182574	2	M	YH
M182590	24	M	YH
M182602	2	M	YH
M182659	20	M	YH
M182661	7	M	YH
M182662	1	M	YH
M182695	0	M	YH
M182722	6	M	YH
M182727	6	M	YH
M182752	5	M	YH
M182922	7	M	YH
M183081	29	M	YH
M183116	2	M	YH
M183164	5	M	YH
M183172	75	M	YH
M183576	7	M	LON
M183880	6	M	SW
M183887	7	M	SW
M183897	1	M	SW
M183898	7	M	SW
M183904	19	M	SW
M183913	31	M	SW

Barcode	Age	Gender*	Region*
M183922	12	M	SW
M183939	6	M	SW
M183943	1	M	SW
M183948	82	M	SW
M183968	2	M	SW
M183977	6	M	SW
M184281	7	M	SW
M184285	6	M	SW
M184286	6	M	SW
M184291	3	M	SW
M184293	6	M	SW
M184886	63	M	Emids
M184913	80	M	Emids
M184916	59	M	Emids
M184971	85	M	Emids
M184976	73	M	Emids
M185055	70	M	Emids
M185057	0	M	Emids
M185251	0	M	Emids
M185341	0	M	Emids
M185350	0	M	Emids
M185356	0	M	Emids
M185396	0	M	Emids
M180288	80	U	YH
M182641	10	U	YH
M182653	1	U	YH
M182682	0	U	YH

*Note: For gender, F represents female; M represents Male; U represents Unknown sexs. For regions, NW represents Northwest region; East represents East region; NE represents Northeast region; YH represents Yorkshire; SE represents Southeast region; SW represents Southwest; Emids represents East midland; Wmids represents West midland; LON represents London.

Appendix 9

Results obtained from the HSV-1 HerpeSelect ELISA for HSV serum panel

Serum panel 1 (HSV-1 positive)

No	OD value*	Index value*	HSV-1 test result
			Interpretation
1	2.950	7.74	Positive
2	OUT	OUT	Positive
3	OUT	OUT	Positive
4	2.994	7.86	Positive
5	2.902	7.62	Positive
6	OUT	OUT	Positive
7	OUT	OUT	Positive
8	2.583	6.78	Positive
9	OUT	OUT	Positive
10	0.432	1.13	Positive
11	OUT	OUT	Positive
12	OUT	OUT	Positive
13	OUT	OUT	Positive
14	OUT	OUT	Positive
15	2.261	5.93	Positive
16	2.075	5.45	Positive
17	OUT	OUT	Positive
18	0.636	1.67	Positive
19	0.323	0.85	Positive
20	OUT	OUT	Positive
21	OUT	OUT	Positive
22	OUT	OUT	Positive
23	OUT	OUT	Positive
24	OUT	OUT	Positive
25	OUT	OUT	Positive
26	2.352	6.17	Positive
27	1.404	3.69	Positive
28	1.690	4.44	Positive
29	OUT	OUT	Positive
30	OUT	OUT	Positive

Note: *OD value represents specimen optical density;
Index value = specimen optical density (OD) / mean of the Cut-off calibrator absorbance values.

Serum panel 2 (HSV-2 positive)

No	OD value*	Index value*	HSV-1 test result
			Interpretation
1	0.055	0.14	Negative
2	0.07	0.18	Negative
3	0.226	0.59	Negative
4	0.099	0.26	Negative
5	0.086	0.23	Negative
6	0.076	0.20	Negative
7	0.072	0.19	Negative
8	0.187	0.49	Negative
9	0.339	0.89	Negative
10	0.084	0.22	Negative
11	0.172	0.45	Negative
12	0.156	0.41	Negative
13	0.123	0.32	Negative
14	0.059	0.15	Negative
15	0.103	0.27	Negative
16	0.135	0.35	Negative
17	0.06	0.16	Negative
18	0.074	0.19	Negative
19	0.063	0.17	Negative
20	0.069	0.18	Negative
21	0.57	1.50	Positive
22	0.104	0.27	Negative
23	0.423	1.11	Positive
24	0.322	0.65	Negative
25	0.213	0.43	Negative
26	0.154	0.31	Negative
27	0.384	0.78	Negative
28	0.158	0.32	Negative
29	0.596	1.21	Positive
30	0.235	0.48	Negative

Note: *OD value represents specimen optical density;
Index value = specimen optical density (OD) / mean of the Cut-off calibrator absorbance values.

Serum panel 3 (HSV-1 and HSV-2 Positive)

No	OD value*	Index value*	HSV-1 test result
			Interpretation
1	OUT	OUT	Positive
2	1.469	3.86	Positive
3	2.865	7.52	Positive
4	OUT	OUT	Positive
5	OUT	OUT	Positive
6	OUT	OUT	Positive
7	2.071	5.44	Positive
8	1.526	4.01	Positive
9	OUT	OUT	Positive
10	2.992	7.85	Positive
11	2.913	7.65	Positive
12	OUT	OUT	Positive
13	2.518	6.61	Positive
14	OUT	OUT	Positive
15	OUT	OUT	Positive
16	2.747	7.21	Positive
17	0.115	0.30	Negative
18	1.108	2.91	Positive
19	2.262	5.94	Positive
20	0.103	0.27	Negative
21	2.992	7.85	Positive
22	2.881	7.56	Positive
23	2.032	5.33	Positive
24	OUT	OUT	Positive
25	1.213	3.18	Positive
26	0.213	0.56	Negative
27	OUT	OUT	Positive
28	2.976	7.81	Positive
29	0.604	1.59	Positive
30	0.851	2.23	Positive

Note: *OD value represents specimen optical density;
Index value = specimen optical density (OD) / mean of the Cut-off calibrator absorbance values;
OUT represents an out of range value was read.

Serum panel 4 (HSV-1 and HSV-2 Negative)

No	OD value*	Index value*	HSV-1 test result
			Interpretation
1	0.181	0.37	Negative
2	0.059	0.12	Negative
3	0.093	0.19	Negative
4	0.088	0.18	Negative
5	0.080	0.16	Negative
6	0.071	0.14	Negative
7	0.106	0.21	Negative
8	0.110	0.22	Negative
9	0.083	0.17	Negative
10	0.111	0.22	Negative
11	0.176	0.36	Negative
12	0.096	0.19	Negative
13	0.073	0.15	Negative
14	0.522	1.06	Positive
15	0.053	0.11	Negative
16	0.091	0.18	Negative
17	0.083	0.17	Negative
18	0.076	0.15	Negative
19	0.168	0.34	Negative
20	0.075	0.15	Negative
21	0.033	0.07	Negative
22	0.097	0.20	Negative
23	0.361	0.73	Negative
24	0.203	0.41	Negative
25	0.213	0.43	Negative
26	0.057	0.12	Negative
27	0.062	0.13	Negative
28	0.080	0.16	Negative
29	0.099	0.20	Negative
30	0.105	0.21	Negative

Note: *OD value represents specimen optical density;
Index value = specimen optical density (OD) / mean of the Cut-off calibrator absorbance values.

Appendix 10

Results obtained from the HSV-2 HerpeSelect ELISA for HSV serum panel

Serum panel 1 (HSV-1 positive)

No	OD value*	Index value*	HSV-1 test result
			Interpretation
1	0.039	0.09	Negative
2	0.105	0.24	Negative
3	0.256	0.58	Negative
4	0.071	0.16	Negative
5	0.083	0.19	Negative
6	0.119	0.27	Negative
7	0.170	0.39	Negative
8	0.081	0.18	Negative
9	0.083	0.19	Negative
10	0.078	0.18	Negative
11	0.070	0.16	Negative
12	1.406	3.20	Positive
13	0.268	0.61	Negative
14	0.211	0.48	Negative
15	0.089	0.20	Negative
16	0.081	0.18	Negative
17	0.062	0.14	Negative
18	0.065	0.15	Negative
19	0.046	0.10	Negative
20	0.098	0.22	Negative
21	0.077	0.18	Negative
22	0.104	0.24	Negative
23	0.137	0.31	Negative
24	0.063	0.14	Negative
25	1.474	3.35	Positive
26	1.282	2.91	Positive
27	0.244	0.55	Negative
28	0.102	0.23	Negative
29	1.098	2.50	Positive
30	0.125	0.28	Positive

Note: *OD value represents specimen optical density;
Index value = specimen optical density (OD) / mean of the Cut-off calibrator absorbance values.

Serum panel 2 (HSV-2 positive)

No	OD value*	Index value*	HSV-1 test result
			Interpretation
1	2.351	5.34	Positive
2	2.08	4.73	Positive
3	OUT	OUT	Positive
4	OUT	OUT	Positive
5	OUT	OUT	Positive
6	2.066	4.70	Positive
7	OUT	OUT	Positive
8	OUT	OUT	Positive
9	2.811	6.39	Positive
10	2.792	6.35	Positive
11	OUT	OUT	Positive
12	2.614	5.94	Positive
13	OUT	OUT	Positive
14	2.979	6.77	Positive
15	OUT	OUT	Positive
16	OUT	OUT	Positive
17	OUT	OUT	Positive
18	OUT	OUT	Positive
19	OUT	OUT	Positive
20	OUT	OUT	Positive
21	OUT	OUT	Positive
22	OUT	OUT	Positive
23	OUT	OUT	Positive
24	2.151	4.89	Positive
25	OUT	OUT	Positive
26	2.901	6.59	Positive
27	2.719	6.18	Positive
28	2.850	6.48	Positive
29	OUT	OUT	Positive
30	1.730	3.93	Positive

Note: *OD value represents specimen optical density;
Index value = specimen optical density (OD) / mean of the Cut-off calibrator absorbance values;
OUT represents an out of range value was read.

Serum panel 3 (HSV-1 and HSV-2 Positive)

No	OD value*	Index value*	HSV-1 test result
			Interpretation
1	2.785	6.33	Positive
2	OUT	OUT	Positive
3	OUT	OUT	Positive
4	OUT	OUT	Positive
5	OUT	OUT	Positive
6	2.298	5.22	Positive
7	2.73	6.20	Positive
8	2.224	5.05	Positive
9	0.37	0.84	Negative
10	OUT	OUT	Positive
11	2.262	5.14	Positive
12	2.418	5.50	Positive
13	OUT	OUT	Positive
14	2.928	6.65	Positive
15	2.652	6.03	Positive
16	2.465	5.60	Positive
17	0.184	0.42	Negative
18	2.179	4.95	Positive
19	2.171	4.93	Positive
20	2.376	5.40	Positive
21	OUT	OUT	Positive
22	OUT	OUT	Positive
23	OUT	OUT	Positive
24	2.416	5.49	Positive
25	OUT	OUT	Positive
26	2.887	6.56	Positive
27	OUT	OUT	Positive
28	OUT	OUT	Positive
29	2.755	6.26	Positive
30	1.508	3.43	Positive

Note: *OD value represents specimen optical density;
Index value = specimen optical density (OD) / mean of the Cut-off calibrator absorbance values;
OUT represents an out of range value was read.

Serum panel 4 (HSV-1 and HSV-2 Negative)

No	OD value*	Index value*	HSV-1 test result
			Interpretation
1	0.073	0.17	Negative
2	0.105	0.24	Negative
3	0.149	0.34	Negative
4	0.112	0.25	Negative
5	0.094	0.21	Negative
6	0.121	0.28	Negative
7	0.092	0.21	Negative
8	0.157	0.36	Negative
9	0.149	0.34	Negative
10	0.130	0.30	Negative
11	0.160	0.36	Negative
12	0.160	0.36	Negative
13	0.076	0.17	Negative
14	0.132	0.30	Negative
15	0.076	0.17	Negative
16	0.101	0.23	Negative
17	0.081	0.18	Negative
18	0.208	0.47	Negative
19	0.113	0.26	Negative
20	0.073	0.17	Negative
21	0.377	0.86	Negative
22	0.216	0.49	Negative
23	0.146	0.33	Negative
24	0.120	0.27	Negative
25	0.457	1.04	Equivocal
26	0.084	0.19	Negative
27	0.067	0.15	Negative
28	0.152	0.35	Negative
29	0.086	0.20	Negative
30	0.097	0.22	Negative

Note: *OD value represents specimen optical density;
Index value = specimen optical density (OD) / mean of the Cut-off calibrator absorbance values.

Appendix 11

The product details of HSV-1 truncated glycoprotein G (adapted from Virusys Corporation, Taneytown) Available at: https://www.virusys.com/hsv-1-antigens/hsv-1-rglycoprotein-g (Accessed: 19 MAY 2015)

Virus strain:	MacIntyre
Host Cell:	Baculovirus (Sf-9)
Cell Line Characterization:	Mycoplasma negative
Purity:	Infected cell extract enriched for viral proteins (Fused with human SOD)
Inactivation:	Greater than 90% by SDS-PAGE
Supplied in:	Phosphate Buffered Saline, 6M Urea pH 7.4
Protein concentration:	2.2 mg/mL
Fill Volume:	45 µL
ELISA (IgG) Titre:	>1:64,000
Western Blot Titre:	10 µg/cm
Reference:	J.Virol.Methods (1986), 14:111

ELISA Results

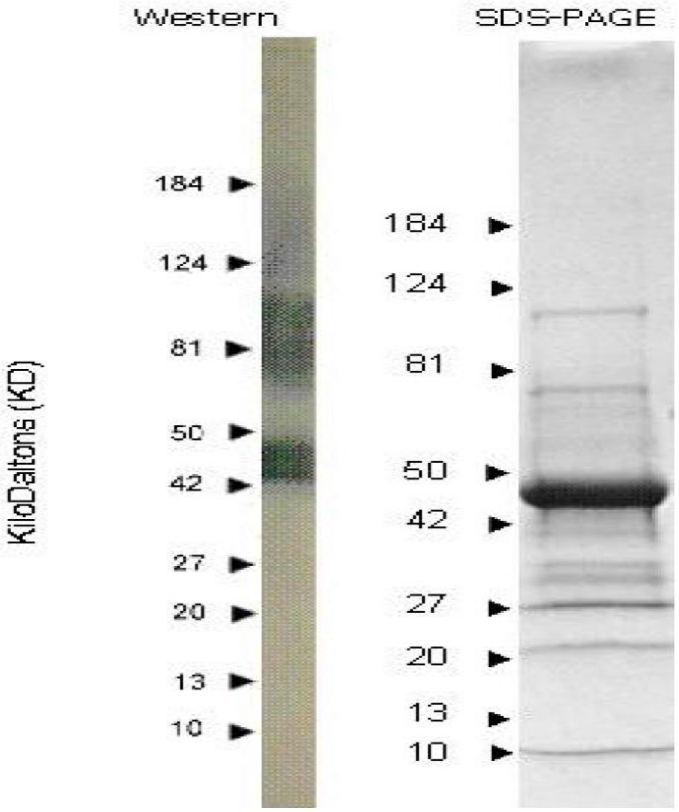
Antigen is Virusys # H1V016 HSV-1 Infected Cell Extract AND H1V133 Recombinant HSV gG-1 coated at various dilutions in PBS. Primary antibodies are human plasmas previously characterized for HSV-1 IgG serology.

HSV-1 Glycoprotein G
Cat. No. H1V133 Lot L1142044

Sample	HSV-1 Extract IgG Serology	Antigen Dilution Extract 1:4K	gG-1 1:16K	gG-1 1:64K
RN11	-	0.039	0.039	0.037
CM08	-	0.037	0.038	0.037
RN06	-	0.039	0.038	0.038
CM01	+	1.799	1.459	0.924
CM04	+/-	0.241	0.038	0.036
RN02	++	0.907	0.067	0.055
CM02	+	0.943	0.376	0.191
RN03	++	2.200	2.200	1.402
Negative Mean		0.038	0.038	0.037
Positive Mean		1.462	1.345	0.839
P/N		38.15	35.09	22.47

Western Blot and SDS-PAGE Results

Antigen is Virusys # H1V133 HSV-1 Recombinant gG at 10 µg/cm.
Primary antibody is H1A020-100 HSV-1 gG Monoclonal Antibody.



Appendix 12

The product details of HSV-2 truncated glycoprotein G (adapted from Virusys Corporation, Taneytown) Available at: https://www.virusys.com/hsv-2-antigens/hsv-2-rglycoprotein-g (Accessed: 19 MAY 2015)

Virus strain:	G
Host Cell:	Baculovirus (Sf-9)
Cell Line Characterization:	Mycoplasma negative
Purity:	Infected cell extract enriched for viral proteins (Fused with human SOD)
Inactivation:	Greater than 90% by SDS-PAGE
Supplied in:	50 mM Tris, 5 mM DTT, 1 mM EDTA, 0.1% Triton x-100 pH 8.0
Protein concentration:	1.0 mg/mL
Fill Volume:	100 µL
ELISA (IgG) Titre:	>1:64,000
Western Blot Titre:	10 µg/cm
Reference:	J.Virol.Methods (1992), 36:249

ELISA Results

Antigen is Virusys # H1V016 HSV-1 Infected Cell Extract AND H1V134 HSV Recombinant gG-2 coated at various dilutions in PBS. Primary antibodies are human plasmas previously characterized for HSV-2 IgG serology.

HSV-2 Glycoprotein G
Cat. No. H2V134 Lot I1245018

Sample	HSV-2 Extract IgG Serology	Antigen Dilution Extract 1:3200	gG-2 1:16K	gG-2 1:64K
RN11	-	0.038	0.079	0.055
CM08	-	0.039	0.061	0.040
RN06	-	0.040	0.059	0.041
CM01	+	0.475	0.055	0.043
CM04	-	0.037	0.062	0.042
RN02	++	**1.805**	**1.613**	**0.808**
CM02	+	0.198	0.064	0.055
RN03	++	0.918	0.071	0.069
Negative Mean		0.039	0.064	0.049
Positive Mean		0.849	1.613	0.808
P/N		22.05	25.04	16.39

Western Blot and SDS-PAGE Results

Antigen is Virusys # H1V134 HSV-2 Recombinant gG at 10 µg/cm.
Primary antibody is H2A023-100 HSV gG Monoclonal Antibody.