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Validation of an ELISA Method for Epstein-Barr Virus Antibodies in Dried Blood Spot Specimens and Correlation of EBV Serology with DNA Detection and Cytomegalovirus Serology

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Abstract

Validation of an ELISA Method for Epstein-Barr Virus Antibodies in Dried Blood Spot Specimens and Correlation of EBV Serology with DNA Detection and Cytomegalovirus Serology

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Dried blood spot (DBS) technology offers several advantages over conventional liquid sample types in terms of cost, storage and stability especially in large population-based or field research. The Epstein-Barr virus (EBV) antibody level is widely accepted as a biomarker for psychosocial stress, based on the notion that stress triggers downregulation of cellular immune function and causes the reactivation of latent EBV and in turn, the increase of EBV IgG level as a response. Our project has two goals: First, we validated an ELISA method for measuring EBV antibody in DBS. We found a strong correlation between EBV IgG in plasma and DBS sample types (R^2 = 0.90). Second, we examined the correlations between EBV serology and EBV PCR results and cytomegalovirus (CMV) serology in DBS and serum in hopes of exploring a more direct or alternate means of measuring herpesvirus reactivation. We found that EBV IgG level poorly predicted viremia in serum (R^2 = 0.08) and viral load in DBS [F (1, 85) = 1.93, P = 0.17] and that EBV and CMV serology correlated weakly (r = 0.43).

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LIST OF ABBREVIATIONS

AIDS, acquired immune deficiency syndrome.

ANOVA, analysis of variance.

BSI, Brief Symptom Inventory.

CD, cluster of differentiation.

CDC, Centers for Disease Control and Prevention.

CMV, human cytomegalovirus.

CV, coefficient of variation.

DBS, dried blood spot.

DNA, deoxyribonucleic acid.

dNTP, deoxyribonucleotide triphosphate.

EA, early antigen.

EBV, Epstein-Barr virus.

EBNA, Epstein-Barr nuclear antigen.

EDTA, Ethylenediaminetetraacetic acid.

EIA, enzyme immunoassay.

ELISA, enzyme-linked immunosorbent assay.

gp, glycoprotein.

HLA, human leukocyte antigen.

HSV-1, Herpes simplex virus type I.

Ig, immunoglobulin.

IM, infectious mononucleosis.

LMP, latent membrane protein.

LoA, limits of agreement.

LOD, limit of detection.

MHC, major histocompatibility complex.

NK cells, natural killer cells.

OD, optical density.

PBMC, peripheral blood mononuclear cell.

PCR, polymerase chain reaction.

RBC, red blood cells.

TGF-β, transforming growth factor beta.

UW, University of Washington.

VCA, viral capsid antigen.

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Chapter 1. INTRODUCTION

1.1 BACKGROUND

The University of Washington Biomarker Lab was contracted to produce results for Epstein-Barr virus (EBV) viral capsid antigen (VCA) immunoglobulin G (IgG) level in ~2000 dried blood spot (DBS) samples for part of the Landmark Spirituality and Health Study. The data would be used to assess the relationship between psychosocial stress and immune function in healthy individuals based on the widely accepted model that a relative high titer of EBV IgG is associated with psychosocial stress. The model hypothesizes that stress downregulates the ability of cellular immunity to maintain herpesvirus latency and causes EBV to reactivate and as a response, the EBV IgG level increases.

As the ELISA kit previously employed and validated in the laboratory for testing EBV IgG on DBS had been discontinued by the manufacturer, we needed to validate a new method using another commercially available ELISA kit. We also became interested in exploring using other existing methods to assess other parameters that play roles in the relationship between stress and EBV serology, namely EBV viral load and serology and viral load of human cytomegalovirus (CMV), another latent herpesvirus that reactivates.

In this chapter, we study introductory knowledge of the different key components of this project, including the herpesviruses EBV and CMV and the sample type of DBS. We also review the literature to gain better understanding of the history and evolution of the role of EBV IgG in the

field of psychoneuroimmunology research. These learning processes help form the goals as well as hypotheses of this project.

1.1.1 *Epstein-Barr Virus*

Named after its discoverers, Epstein-Barr virus (EBV) was first observed through electron microscopy in a lymphoblast culture from Burkitt's lymphoma in 1964.[1] It was the first virus discovered to be associated with human cancer.[2] Geographic locations, socioeconomic status and living condition are all factors determining EBV seroprevalence of a population.[3] Being one of the most common human viruses in existence, it is believed that over 90% of the world's population is seropositive for EBV. The fact that it is ubiquitous makes EBV a great biomarker candidate for population based studies.

EBV is also known as human herpesvirus 4 and belongs in the gammaherpesviridae subfamily. Its double stranded, linear genome encodes nearly 100 proteins with around 172- 184 kilobase pairs and 85-100 genes.[2,4] The capsid which packs the genomes is surrounded by an envelope.[3] In between the capsid and the envelop is a structure unique to herpesviruses called the tegument. The tegument contains proteins that are important in viral replication and evasion of host immune response.[5] Glycoproteins embed the viral envelope and are vital for cell tropism and receptor recognition[6].

EBV is the major causative agent of infectious mononucleosis (IM). As it is highly transmissible via saliva, it is commonly referred to as the kissing disease. The name infectious mononucleosis describes the symptom of atypically large mononuclear lymphocytosis found in peripheral blood.[3]

These lymphocytes are activated CD8+ T cells that are thought to be responding to the B cell infection by EBV. Common signs and symptoms of patients with IM typically include pharyngitis, cervical lymphadenopathy, fever, sore throat, fatigue, headache and fever.[3] The severity of the symptoms correlates with the age of the patients. Children under 10 years of age have lower chance of developing IM symptoms when contracted with EBV and if they do, they are relatively mild. However, if primary EBV infection is acquired during adulthood the IM symptoms are generally more intense. The reason for the correlation is not known, however it has been speculated that it is due to the fact that immune response is more robust in older individuals.[3] Treatment typically involves managing severe symptoms with antipyretics and analgesics and rest. IM symptoms usually resolve in about two weeks, however, the virus is never cleared from the body as it establishes a lifelong, latent infection.[3]

In rare instances, and especially in people with compromised immune systems, reactivated EBV can cause various types of cancer and malignancies. B cell lymphomas arise from the ability of EBV to immortalize resting B cells and transform them into latently infected lymphoblastoid cell lines in the absence of effective T cell surveillance.[6] Late-stage AIDS patients and patients receiving solid organ transplant and bone marrow transplant whose immune system is being medically suppressed are at risk of developing EBV-associated B cell lymphomas. Other malignancies associated with latent EBV infections include Hodgkin's lymphoma, Burkitt's lymphoma, nasopharyngeal carcinoma and gastric carcinoma.[6]

EBV is mainly spread through saliva in the tonsillar compartment. The virus is able to cross the epithelial barrier and infect the naïve B lymphocytes through glycoprotein interactions.

Specifically, the viral gp350 glycoprotein binds to CD21 on B cells.[7] Then, the virion fuses with host cell membrane through interaction of EBV gp42 and the HLA class II molecules of the B cells.[3,6,8] Upon entry, the nucleocapsid is released into the cytoplasm and viral genome is transported to the nucleus where replication is initiated by viral DNA polymerase.[3] B cells are transformed into proliferating lymphoblast by the virus utilizing the growth program. The growth program, also known as latency 3, is the expression of nine latent proteins of transcription factor EBNA2.[3,9] Growth program can also be used to immortalize B cells *in vitro*.[10] The proliferating B cells are able to evade apoptosis by cytotoxic T cells by switching off the growth program and activating the default program with expression of latent membrane proteins LMP1 and LMP2A.[9] These survival signals allow infected B lymphoblasts to leave the germinal center as resting memory B cells. Infected memory B cells circulate in the periphery thus resulting in detectable viral level in blood. The EBV genome is circularized as a nuclear episome and replicated by host DNA polymerase during mitosis.[3,7] Latently infected resting B cells occasionally reactivate and initiate viral replication and shedding in saliva and infect new B cells and epithelial cells of the host.[11,12]

Latent EBV infection can also reactivate and become a lytic infection. Molecularly, lytic reactivation is known to be triggered by either the synthesis of the Z protein or the R protein.[11] Several factors have been known to contribute to the stimulation of reactivation *in vitro*. They include B cell receptor stimulation, hypoxia, TGF-β, DNA damage and chemical agents.[11] However, the mechanism of reactivation of latent virus *in vivo* is not well understood but it is thought to be largely triggered by B cell receptor stimulation and plasma cell differentiation.[3,11]

Cellular immune responses play roles in both controlling primary EBV infection and suppressing reactivation. EBV-specific CD4+ and CD8+ T cells and activated NK cells are primed and expand during primary infections by viral proteins and bring infection under control. Among these, the population of CD8+ T cells has the most intense response and its large expansion is thought to be responsible for the symptoms of infectious mononucleosis. CD4+ T cells expansion occurs at a smaller scale. After primary infection, a portions of the T cell pool becomes tissue-resident memory cells and circulating T cells.[13,14] They provide continuous surveillance by recognizing viral particles presented on B cell surfaces by MHC class I molecules.[15]

A variety of antibodies are produced by the host humoral immune system during primary EBV infection. IgM specific to the viral capsid antigen (VCA) is the first antibody to rise and it peaks approximately 7 days after exposure to EBV and begins to decline and does not reappear after about two months, coinciding with the rise and fall of detectable EBV viremia in peripheral blood. Anti-VCA IgG is produced alongside IgM but at a much slower rate, peaking 4 months after initial exposure and persists for life. Antibodies to Epstein-Barr virus nuclear antigen (EBNA), which is a latent antigen develops much later than antibodies to VCA. They typically appear about 60 days after exposure to virus and peak 120 days later and persist for life. Antibodies to viral early antigen (EA) can be detected in most primary infection cases in low titer and are not thought to have diagnostic value.[3,16] Neutralizing antibodies are also synthesized *in vivo* upon primary infection. Antibodies specific to gp350/220 which are glycoproteins essential for viral entry into host B lymphocytes through interaction with CD21 are considered to be the most important neutralizing antibodies as gp350 is the most abundant glycoprotein in EBV.[3,17] Differential testing for EBV

antibodies using enzyme immunoassay (EIA) is useful in distinguishing primary from past infection. However, serological pattern for EBV reactivation is ambiguous and not well defined.[18]

1.1.2 *Human Cytomegalovirus*

Like EBV, the human cytomegalovirus (CMV) is also a herpesvirus, meaning that they both have the ability to establish lifelong latent infection and periodically reactivate. In a 2001 study, the overall seroprevalence for CMV in the US was found to be 50.4 % and factors including female sex, older age and foreign birth place are associated with CMV seropositivity.[19] CMV is transmitted through the oral route and sexual activity via saliva, semen, cervical and vaginal secretions. Primary CMV infections are usually subclinical and transient and are occasionally associated with infectious mononucleosis or mononucleosis-like symptoms.[20]

CMV is described as having a broad cellular tropism which implies that it can infect a variety of cell types.[21] Glycoprotein B (gB) allows the virus to attach to host cell and another glycoprotein complex, the gH-gL dimer promotes gB fusion with host cell membrane.[21] Upon entry, lytic infection is initiated by a series of temporally regulated gene expressions.[21] Viral shedding is commonly found in urine, saliva and genital secretions and may result in transmission.[20] CMV latency is now believed to take place in CD34+ myeloid progenitor cells in the bone marrow and the CD14+ monocytes in peripheral blood that derive from them.[22] During latency, CMV limits its gene expression and is able to evade immune surveillance and persist in its host.[22]

Like EBV, CMV reactivation is usually asymptomatic in immunocompetent individuals. However, in transplant patients CMV can cause a variety of symptoms either due to reinfection under immunosuppression or serological mismatch between CMV+ donor and CMV- recipient.

Post-transplant CMV symptoms vary depending on the site of infection, but commonly patients experience a viral syndrome that includes fever, malaise, leukopenia, thrombocytopenia and elevated liver enzymes. Patients can further develop interstitial pneumonia, gastroenteritis, retinitis, hepatitis, graft failure and death.[20] If acquired congenitally, CMV can lead to severe birth defects. Congenital CMV infection is the leading cause of nonhereditary sensorineural hearing loss. It can also lead to neurodevelopment disabilities, growth failure, vision loss and in rare cases, fetal and neonatal death.[20] According to the CDC, one in five children with congenital CMV infection will develop a permanent disability.[23]

Although latent CMV reactivation poses no direct risks to immunocompetent individuals, recent studies have suggested that the repetitive CMV reemergence and persistence may cause a form of exhaustion or aging of the cellular immunity of the hosts.[24] Theories suggest that as CMV carrying individuals age, they have increased CMV specific CD8+ accumulation and decreased naïve T cells that are readily available to respond to foreign antigens other than CMV.[25] This is a possible explanation of the waning ability of elderly individuals to overcome new infections and may be an important component of predicting mortality in heathy elderly individuals.

1.1.3 *Dried Blood Spot Methodology*

Dried blood spot (DBS) is a blood sample collection technique in which drops of blood are absorbed and allowed to dry on a piece of filter paper. Dr. Robert Guthrie is often credited as being the first to utilize such technique in 1963 when he developed a bacterial inhibition assay to measure phenylalanine to screen for phenylketonuria in newborns.[26] Over the years many improvements had been made to the DBS collection technique including establishment of a quality assurance for filter paper and standardization of materials and methods by the CDC.[27] In a paper published by

the CDC in 2001, close to 180 different analytes are listed to be measureable in DBS. It also states that virtually any analyte that can be measured in whole blood, plasma or serum can be measured in DBS.[27]

There are many advantages of using DBS over conventional liquid samples. No phlebotomy training is required for the DBS sample collection which is relatively simple and less invasive than venipuncture. DBS sample needs no refrigeration and most analytes show stability when stored at room temperature. Dried blood is not considered to be biohazardous and therefor has few shipping restrictions. Furthermore, the materials required for DBS collection are inexpensive. All of the above features of DBS make it an ideal sample type for large population or remote location based studies where cost of sample collection, storage and shipping to laboratories could be dramatically lowered.[24,28–30]

However, there are also some limitations to DBS samples. One of the concerns is the small sample volume. After DBS elution, analytes become very diluted and would require a much more sensitive method for detection or quantification. Another question often raised is the variability of sample volume from one spot to another. This can be affected by collection technique, hematocrit, and location of the spot for excision (punch), which are all not easily controlled. The facts that DBS is capillary blood as opposed to venous blood, and that DBS contains red blood cell debris and intracellular matter also cast doubts in its comparability with conventional liquid samples.[28]

1.2 Literature Reviews

Since the early 1980's, there have been research interests in the relationship between stress and immune system function. There was also much speculation on stress altering and intensifying the

presentation of herpesvirus infections based on observation but without much scientific support. Psychologist Janice K. Kiecolt-Glaser and immunologist Ronald Glaser are pioneers in this area of study. Intrigued by studies that suggest positive correlations between stress and viral infections and stress and tumors in rodents as well as some prior evidence of stress-induced immunosuppression in human, Kiecolt-Glaser and Glaser's group conducted their first study in a group of 75 first-year medical students in 1983.[31] A baseline blood sample was drawn one month before and after a major exam and another sample was obtained during a period of multiple exams. In addition to blood samples, a self-report measurement of the Brief Symptom Inventory (BSI) was also documented, which provided data on stressful life events and loneliness. The natural killer (NK) cell activity was analyzed and plasma immunoglobulins were quantified. Their data showed that NK cell activities significantly decreased in samples taken during the mildly stressful event of examination compared to baseline samples ($P < 0.003$). NK cell activities were also markedly lower with samples that score high on the stressful life event and loneliness scale. Moreover, plasma immunoglobulins showed increase compared to baseline samples but only the increase of IgA was significant. They concluded that stress might have some negative effect on the antiviral activity of NK cells, which would help explain the common belief that individuals are more susceptible to viral infections while experiencing stress.

In 1984, the same group published another study using a similar experimental setup.[31] Instead of NK cell activity and plasma immunoglobulins, antibody levels to three herpesviruses EBV, CMV and herpes simplex virus type I (HSV-1) were measured. Antibody titers to poliovirus was also measured. They found EBV VCA antibody titer significantly decreased from samples taken one month before and during final exams to samples taken after students had just returned from

summer vacation ($P < 0.0001$). Similar patterns were also seen in antibody levels to CMV and HSV-1 but to a lesser extent. Interestingly, no significant change was observed in antibody level to poliovirus. The results agreed with the authors' hypothesis that there is a relationship between stress and herpesvirus latency as reflected in the change in their antibody levels. The presence of antibody to poliovirus in the subjects was due to vaccination therefore the fact that its level remained unchanged suggested that the changes in antibody levels to the herpesviruses were not due to overall changes in plasma immunoglobulin level.

Since their publication, many had used these studies as basis to explore relationships between EBV antibody levels and a variety of psychosocial stressors of interest. Stress associated with pregnancy and racial discrimination,[32] childhood advsersity,[33] attachment anxiety,[34] and perceived stress and social support[35] had all been found to correlate with EBV antibody level. However, there had been many other studies that did not find strong correlation between stress and EBV antibody level or they found the correlation was associated with another factor. Matalka et al. found significant increase in EBV VCA IgG level in female students during school exams but only in the Summer semester and not in Fall/Winter[36]. McDade et al. found that significant high EBV VCA IgG level was associated with traumatic life events in young girls but not in boys.[29] Rudzik et al. failed to find correlation between self-reported stress level and EBV antibody titer in postpartum women.[37]

1.3 Project Goals and Hypotheses

The first goal of this project is to validate an ELISA method to measure EBV VCA IgG level in DBS. From literature review, we learned that DBS is regarded as a suitable substitute for conventional serum and plasma samples. McDade et al. had published a study detailed their success in adapting a commercially available EBV ELISA kit for testing DBS samples.[29] We will

base our experiments on their work as well as UW Biomarker Lab's own experience in adapting ELISA kits for DBS testing for other analytes.

Our second goal is to study the correlations of EBV VCA IgG measurement with measurements from other existing DBS methods, namely EBV PCR, CMV IgG by ELISA and CMV PCR, with the intend of exploring the validity of EBV as a stress biomarker and the plausibility of utilizing PCR as a more direct stress biomarker. Many of the studies we reviewed used EBV IgG as an indirect biomarker for stress based on the belief that it is a response to the latent herpesvirus reactivation. If this model were true, we expect to see correlation between EBV IgG measurement and EBV PCR results. We also expect to see correlation between EBV IgG level and CMV IgG level as CMV is also a herpesvirus capable of establishing latency and reactivation.

Chapter 2. EBV DBS ELISA METHOD VALIDATION

To validate a method for measuring EBV antibodies in DBS, we took a commercially available ELISA kit that is intended for plasma and serum and adapted it for testing on DBS samples. The validation process was largely based on work previously published by McDade et al. as well as expertise from other scientists with prior experience adapting various other tests at the Biomarker Lab. Essentially, we followed the procedure per the kit's instruction manual but substitute diluted plasma or serum with DBS eluate. The method was validated by comparing measurements obtained from matched DBS and plasma samples, with the plasma measurements being the comparative method.

2.1 MATERIALS AND METHODS

2.1.1 *Matched DBS and Plasma Samples*

Mock DBS samples were made from venous whole blood patient samples collected in EDTA Vacutainer tubes. These were one to three-day-old excess samples from de-identified patients from hematology testing at Harborview Medical Center stored at 4°C. One hundred and forty-four samples were randomly selected. To prepare mock DBS samples from whole blood, samples were brought to room temperature and mixed well. One blood spot was made by pipetting 75 μL of the whole blood sample onto a sheet of Whatman 903 filter paper. The blood was allowed to be absorbed and permeate on the filter paper naturally. Multiple spots could be made on the same sheet of paper with proper spacing. The spotted papers were allowed to air-dry on bench top overnight. Prepared DBS were stored in a -80°C freezer for later use. Once the DBS had been made, whole blood samples were centrifuged for 15 minutes to allow for plasma separation. Plasma was aliquoted into separate tubes and stored in a -80°C freezer for later testing.

2.1.2 *DBS Elution*

An elution process is necessary to transform DBS into a liquid phase and be assayed as such. Essentially, a piece of DBS is placed in an eluent and through diffusion and mechanical shaking, plasma, along with cellular debris and intracellular contents are incorporated into the mixture. The elution procedure for EBV DBS ELISA is described as follows.

If taken out of the freezer, prepared blood spots are first brought to room temperature. Using either a semi-automated instrument or a manual office hole puncher, a 3.2 mm disc is cut out of each spot and into designated wells in a deep-well microtiter plate. After adding 200 μL of assay diluent as the eluent into each well, the plate is placed on a plate shaker for one hour at room temperature. The resulting eluate is assayed just as diluted plasma would according to the procedure described in the following section.

2.1.3 *EBV VCA IgG ELISA Kit*

The IBL-America EBV VCA IgG Antibodies ELISA Test Kit (No. IB79230, Minneapolis, MN) is designed to measure IgG class antibodies against EBV viral capsid antigen in serum and plasma.[38] The kit includes a 72-well plate that is coated with EBV VCA antigens, four levels of calibrators (0 U/mL, 10 U/mL, 50 U/mL and 200 U/mL) an enzyme conjugate, a substrate, a stop solution, sample diluent, and a wash buffer. The wash buffer is diluted 1:9 with distilled water while the other reagents are ready-to-use.

Plasma and serum, which are the intended sample types for this kit, are first diluted 1:100 with the sample diluent. After thorough mixing, 100 μL of the diluted sample or DBS eluent is pipetted into a coated well. The plate is sealed and incubated at room temperature for one hour to allow

binding of EBV IgG antibodies in the sample and the solid phase VCA antigens in the wells. The plate then goes through a wash step to remove any unbound material using an automated plate washer, which washes the plate three times by aspirating the contents in the wells and then adding 300 μL of diluted wash buffer. After the last wash, residual moisture is removed by inverting the plate and forcefully tapping against a paper towel.

100 μL of the enzyme conjugate is then added to the plate. The plate is sealed and incubated for 30 minutes to allow binding of enzyme conjugates with bound IgG antibodies in the wells. The plate then goes through another wash step as described previously. 100 μL of the tetramethylbenzidine substrate is then pipetted into the wells, which will allow blue color to develop in the mixture. The intensity of the color is proportional to the amount of the conjugated enzyme present in the wells. The plate is sealed and incubated for 20 minutes at room temperature.

100 μL of the stop solution is then added to the wells. The stop solution, which is an acid, denatures the enzyme and stops color from developing further and at the same time turns the mixture yellow. After thorough mixing, the plate is read at 450 nm using the BioTek microplate reader within 60 minutes. A standard curve is generated from the four calibrators of known EBV IgG concentration, from which the concentrations of unknown samples are calculated. Data analysis is done using the BioTek Gen5 software and Microsoft Excel.

2.2 DATA ANALYSES

Microsoft Excel was used to perform the majority of the statistical analyses and generation of graphs and figures. 0.05 was selected to be the alpha level, when relevant, probabilities (*P*-values) below which were considered to be statistically significant.

2.2.1 *Matched Plasma/DBS Sample Comparison*

We wanted to assess EBV VCA IgG measurements done on DBS as compared to plasma to see how concordant they are and if there is any bias. A linear regression analysis was done to examine the correlation between measurements of the 144 matched samples of DBS and plasma. Samples were tested in duplicated and samples that have measurements above 200 U/ml were excluded as they exceed assay linearity.

We would also like to perform a Bland-Altman analysis to assess the agreement of measurements obtained by both sample types. Since the plasma volume in a DBS sample is different from that of a plasma sample, we could not use the typical Bland-Altman analysis by plotting the differences between the EBV VCA IgG values obtained from the two sample types. Instead, we opted for the alternative Bland-Altman analysis of plotting the ratio of DBS values and plasma values over plasma values, the reference values. Bias was evaluated by the pattern and the number of samples that fell out of the 95% confidence interval.

After obtaining an equation for converting DBS value to plasma or serum-equivalent value as described in the next section (2.2.2), we would be able to make direct comparison of EBV VCA IgG values in plasma and serum to DBS in the same unit of measure. A population of 82 matched

DBS and serum samples independent of the validation samples that were used to create the conversion equation was used to evaluate the difference between EBV VCA IgG values in DBS and serum. A standard Bland-Altman difference plot was constructed by plotting the differences between IgG measurements obtained in serum and the serum-equivalent values calculated from IgG measurements in DBS using the equation created in 2.2.2 over the average of the two values. We examined bias and trends in the Bland-Altman plot. As a side note, this population is the same one described and explored in Chapter 3.

2.2.2 *Conversion Equation for DBS Value to Plasma/Serum-Equivalent Value*

It is important that the EBV VCA IgG measurements obtained from DBS can be compared to the ones obtained from plasma or serum, which are the conventional and intended sample types for this assay. However, it is difficult to determine the conversion factor of DBS eluate to plasma volume based on the dilution of blood. However, if matched samples of DBS versus plasma measurements have a strong linear correlation, the linear regression equation can be used as a conversion equation to determine the estimated plasma and serum equivalent value from measuring the DBS value.

2.2.3 *Linearity with Diluted Samples*

The linearity of the assay was examined by testing if it could produce accurate measurements of diluted sample according to their dilution factors. A matched plasma/DBS sample of high measurement of EBV VCA IgG was selected and diluted 1:2, 1:3 and 1:4 with assay diluent and the percent recovery was calculated using the following equation and evaluated.

$$Percent\ recovery = \frac{observed\ concentration}{expected\ concentration} \times 100\ \% \qquad (2.1)$$

2.2.4 *Inter- and Intra-Assay Coefficients of Variation (CV)*

To examine if the assay can precisely reproduce results within a run (intra-assay) and across different runs (inter-assay), a coefficient of variation (CV) analysis was done. CV was calculated using the following equation.

$$CV = \frac{\sigma}{\mu} \times 100\ \%\,, \tag{2.2}$$

where σ is the standard deviation and μ is the mean of the repeated samples being examined. Intra-assay CV was evaluated by calculating the average of the CVs of 72 of the matched mock DBS and plasma samples ran as duplicates within the same runs. Inter-assay CV was evaluated by calculated the CVs of a low and mid-level DBS control across 29 runs over five different days. These DBS control were made in-house and were run in triplicates on each plate. To eliminate variability between blood spots, eluates from DBS discs were pooled and homogenized. One measurement out of the triplicate was randomly selected for evaluation because patient samples were run in singlicates. Inter-day CV was also determined from this set of data by randomly selecting one plate out of each date and calculating the CVs from the average of the measurements.

2.2.5 *Limit of Detection*

The limit of detection (LOD) was determined by calculating the mean optical density (OD) value of 24 replicates of the zero standard, adding three standard deviations and then determining the corresponding concentration according to the standard curve equation generated by the BioTek Gen 5 software.

2.2.6 *Seropositive Cut-Off Evaluation*

A cut-off value is established by the kit manufacturer for qualitative classification of EBV seropositive and seronegative samples. The value of the cut-off is set as such that the sensitivity and specificity of the qualitative capability of the assay would be maximized. The cut-off is different from the quantitative limit of detection which distinguishes between presence and absence of detectable EBV VCA IgG. In other words, within the analytical range of the assay, a sample might have quantifiable EBV VCA IgG, but would still be classified as EBV seronegative if it falls below the cut-off value.

According to the kit instruction manual, Calibrator B is the cut-off threshold calibrator, and has a manufacturer-assigned concentration of 10 U/mL. Samples with values 20% below the cut-off (8 U/mL) were to be interpreted as being EBV VCA IgG negative. Samples with values 20% greater than the cut-off (12 U/mL) were to be interpreted as positive. Samples with values between 8 and 12 U/mL were to be interpreted as equivocal. We later determined that the threshold calibrator had a value of 18 U/mL and a ±20% range of 14-22 U/mL. This modification was made because we encountered some inconsistencies with the curve fitting using these calibrators that accompanied the kit. This process is described in detail in section 2.3.1 and further discussed in section 4.1.3.

We evaluated this new seropositive cut-off, as well as possible red cell interference by assaying 24 known seronegative DBS samples made with washed red cells and human serum albumin. We also assayed 24 replicates of the zero standard and the assay diluent for comparison. Two known positive and two known negative serum samples as determined by a qualitative EIA protocol of UW Virology were assayed as well to assess qualitative agreement. Washed cell DBS samples

were made from whole blood washed five times with phosphate-buffered saline and reconstitute with equal part 5% human serum albumin to achieve 50% hematocrit.

2.2.7 *Freeze-Thaw Stability of DBS Samples*

Since all DBS samples are stored in a freezer at -80°C if not immediately tested or for retesting at the UW Biomarker Lab, a freeze-thaw cycle study was done and not a stability study at room temperature. We defined a freeze-thaw cycle as freezing a sample at -80°C for at least 16 hours and then taking it out of the freezer and allowing it to come to room temperature at around 25°C for at least an hour. A set of 24 DBS samples were made to go through five freeze-thaw cycles before discs were punched out while a duplicate set of the same DBS samples were made to go through only one freeze-thaw cycle. The average percent recovery of samples that had gone through five freeze-thaw relative to samples that only went through one were calculated. A linear plot was also constructed for visualization

2.2.8 *Elution Time Evaluation*

The effect of the length of DBS elution time was examined to determine if it was necessary to modify the assay procedure for optimal result and convenience. We compared measuring EBV VCA IgG in an overnight eluate versus a more convenient one-hour eluate. The overnight eluate samples were prepared by eluting 72 DBS sample with 200 μL of diluent in a deep well plate. The plate was agitated on a plate-shaker for one hour before being placed into a 5°C fridge for 16 hours. The samples were assayed after another one-hour agitation on the plate shaker at room temperature. Another set of the same 72 DBS samples were eluted for one hour as described in section 2.1.2. Linear regression analysis was performed to examine correlation between different elution duration.

2.3 RESULTS

2.3.1 *Standard Curve Optimization*

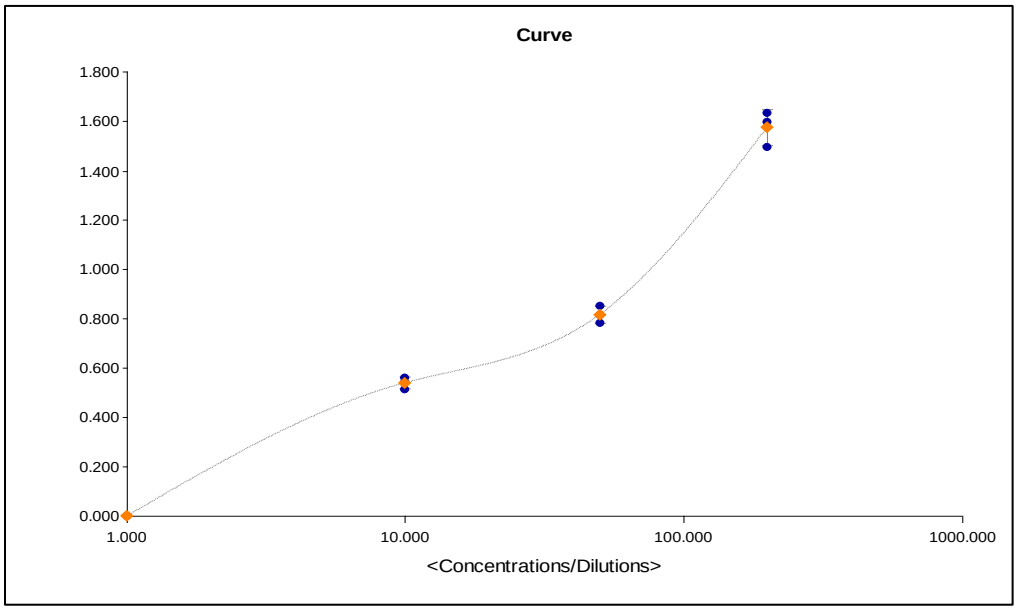
The kit provided four calibrator specimens, with assigned values (0 U/mL, 10 U/mL, 50 U/mL and 200 U/mL) for calibration. The kit instruction manual suggests a spline fit, which results in a standard curve similar to the one shown in Figure 2.1A. using the BioTek Gen5 software. We were not satisfied with this curve as there were too few points. Therefore, we decided to introduce two additional points, 25 U/mL and 100 U/mL into the standard curve. They were made by a 1:2 dilution of the 50 U/mL and 200 U/mL calibrators, respectively, using the assay diluent. As a result, we obtained a standard curve similar to the one shown in Figure 2.1B. We then realized that the new points deviated from an appropriate and expected curve, and did not calibrate well with a spline fit line. In addition, the program did not generate a line equation we could refer to for calculation for concentrations of unknown samples with spline fitting, so we sought a different method of curve fitting.

After trial and error, we opted for a 4-parameter non-linear regression fit because we had the most success of fitting points on the curve and the fact that a line equation is generated by the Gen5 program. The difficulty in fitting the standard curve also raised suspicion that the values of the standard materials might be relative rather than absolute since the kit is described as "semi-quantitative" by the manufacturer. Therefore, further investigation was performed by introducing standard points (50 U/mL, 25 U/mL, 12.5 U/mL and 6.25 U/mL) created by serial dilution from the highest standard (200 U/mL). These new points were plotted along with the original standards points to create a standard curve as seen in Figure 2.2., and the Gen5 program would generate a table similar to Table 2.1., which describes the line equation and its components. The original, kit-

provided 50 U/mL standard fell on the curve and was kept. However, the kit-provided 10 U/mL fell outside the curve and was eliminated from our procedure. We established that the new standard curve consists these points: 0 U/mL, 6.25 U/mL, 12.5 U/mL, 25 U/mL, 50 U/mL and 200 U/mL. Of those, 0 U/mL, 50 U/mL and 200 U/mL were original standards from the kit and the rest were made from serial dilution from the 200 U/mL standard.

Moreover, the 10 U/mL standard we eliminated happened to also be the cut-off standard used to determine seropositive samples. We reassigned a new value of 18 U/mL to this standard according to our new standard curve and 18 U/mL was also used as our threshold for EBV seropositivity. According to the user manual, a range of ±20% around the cut-off is reasonably defined as the grey zone. Therefore, in our assay, results of 14-22 U/mL were considered equivocal, results less than 14 U/mL were considered to be seronegative and those that were greater than 22 U/mL were considered to be seropositive.

A



B

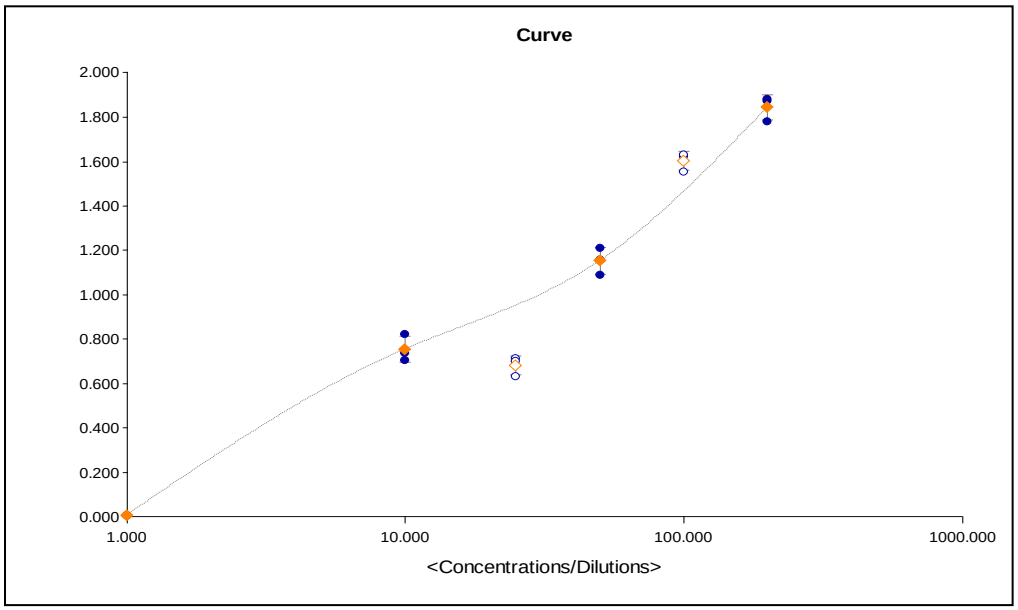


Figure 2.1. Standard curves using spline fit.
Graphical outputs of standard curves generated by the Gen5 software using spline fit. The x-axis represents the concentration (U/mL) and is in logarithmic scale as the y-axis represents the optical density. Each calibrator was run in triplicate hence the three blue dots. The orange dot are the averages of the triplicates and are used to construct the curve. **A.** shows the four original points (0 U/mL, 10 U/mL, 50 U/mL and 200 U/mL) and **B.** shows the inclusion of additional 25 U/mL and 100 U/mL made from dilution represented by the hallowed dots. They fail to fall on the curve.

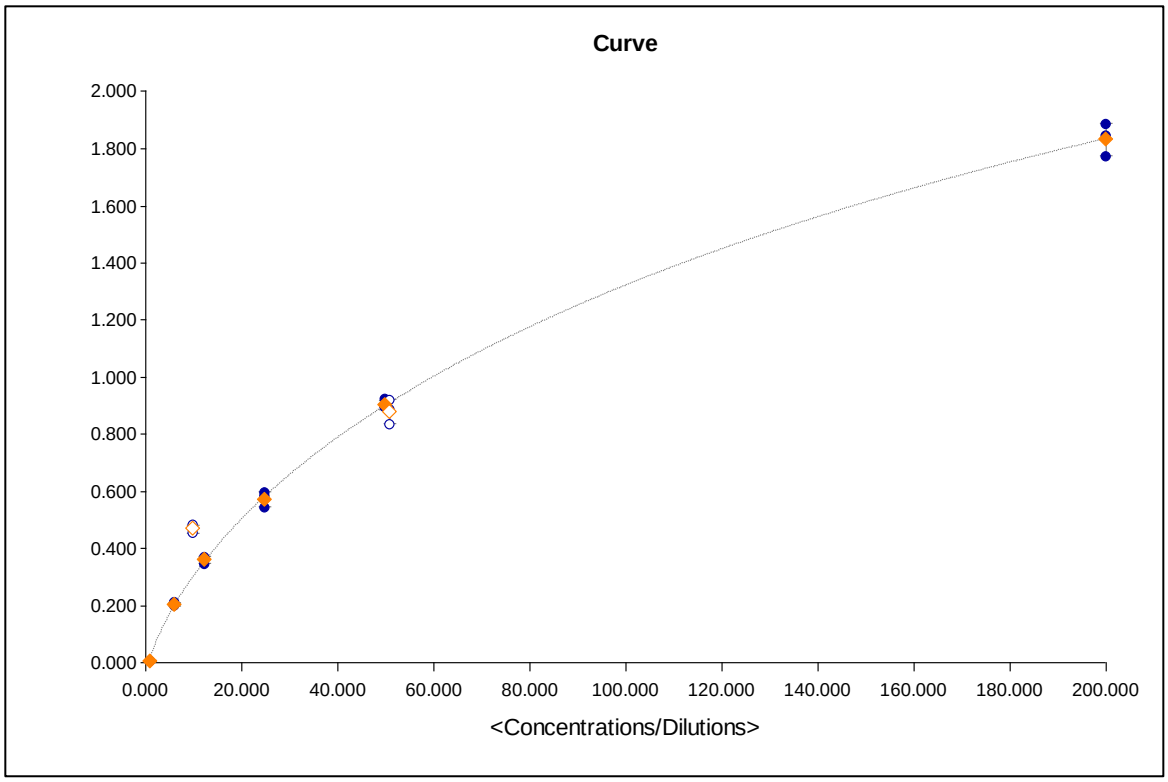


Figure 2.2. Standard curve using 4-parameter non-linear regression with additional points. Graphical output of a standard curve generated by the Gen5 software using 4-parameter non-linear regression fit with the addition of points 6.25 U/mL, 12.5 U/mL, 25 U/mL and 50 U/mL made from serial dilution from the 200 U/mL standard, and elimination of 10 U/mL. The new 50 U/mL agrees and overlaps with the original 50 U/mL and is further eliminated. The eliminated points are represented by the hallowed dots. The orange dots represent the averages of calibrators ran in triplicates. The X-axis represents the concentration of the calibrators in U/mL and the y-axis represents the optical density.

Table 2.1. Standard curve formula and its parameters.

Standard curve formula output as a table generated by the Gen5 software. The values of its parameters are given as well as the coefficient of determination (R^2).

Curve Name	Curve Formula	A	B	C	D	R^2
Curve	Y = (A-D)/(1+(X/C)^B) + D	-0.0745	0.682	381	4.8	1

2.3.2 *Matched Plasma/DBS Sample Comparison*

Eleven out of 144 samples were out-of-range high and were excluded from the analysis. As we predicted, the linear correlation between EBV VCA IgG values measured in DBS and plasma was very strong (R^2 = 0.90) with a slope of 0.73 and an intercept of 0.47 (Figure 2.3). It was also expected that DBS samples would yield weaker signals than their plasma counterparts.

Bland-Altman style ratio plot (Figure 2.4) shows good agreement with little bias between values obtained from the two different sample types, with mean ratio being 0.80, somewhat reflecting the slope of from the linear regression. Only seven out of 133 points fell out of the 95% confidence interval limit of agreement with a trend toward samples with extremely low plasma values (< 7 U/mL). However, this trend was not significant as ratios were amplified by two small values.

A more classical Bland-Altman analysis (Figure 2.5) was also done using the difference between a population independent of the one used in validation. The amount of differences scattered quite widely around the mean difference of (-4.0) with 4 points falling outside the 95% confident interval limits of agreement (LoA). The agreement was very high with samples of low values. Out of the 82 samples used in this analysis, two samples had discrepancy in EBV seropositivity between Plasma and DBS samples as determined by the cut-off range 14-22 U/mL. The two DBS samples fell in the equivocal range (both are 21 U/mL) and their plasma counterparts are in the seropositive range (> 22 U/mL).

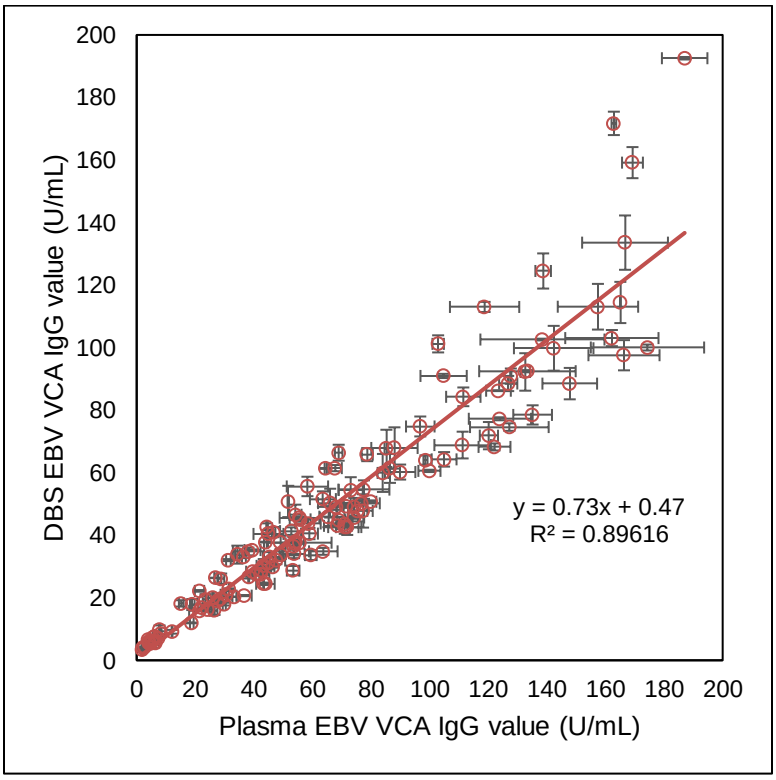


Figure 2.3. Linear regression analysis of EBV VCA IgG measured in DBS vs. plasma.
This is a scatterplot of DBS EBV VCA IgG values over plasma EBV VCA IgG values in 133 matched samples. Each point is the average of duplicate measurements and the error bars are one standard deviation away from the average value. The solid line is the linear regression trend line with its equation and R^2 displayed.

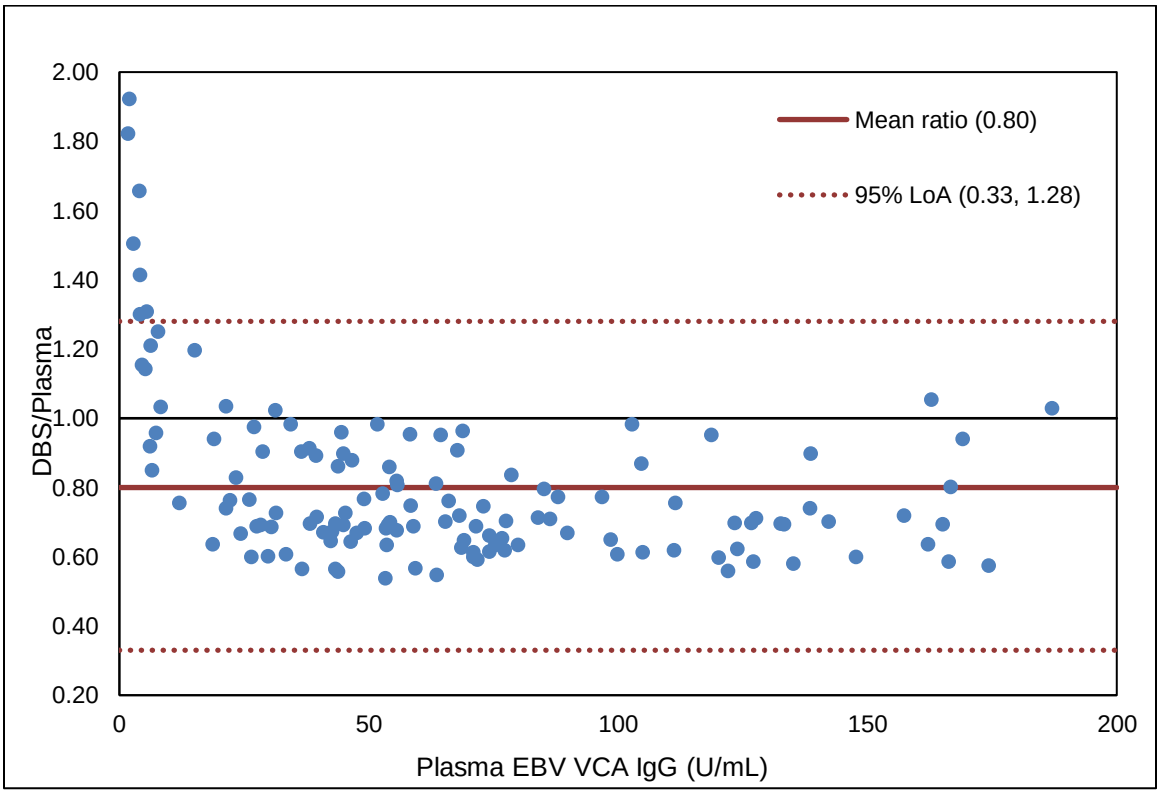


Figure 2.4. Ratio of EBV VCA IgG values in DBS and plasma over plasma values.
This is an alternative Bland-Altman style agreement plot in which the ratios of the measurements from the two methods are plotted against measurements from the comparative method. In this case, ratio of EBV VCA IgG measurements in DBS and plasma was plotted against measurements in plasma. (n = 133). The solid red line is the mean ratio (0.80). The dotted red lines are the 95% confidence interval limits of agreement (LoA).

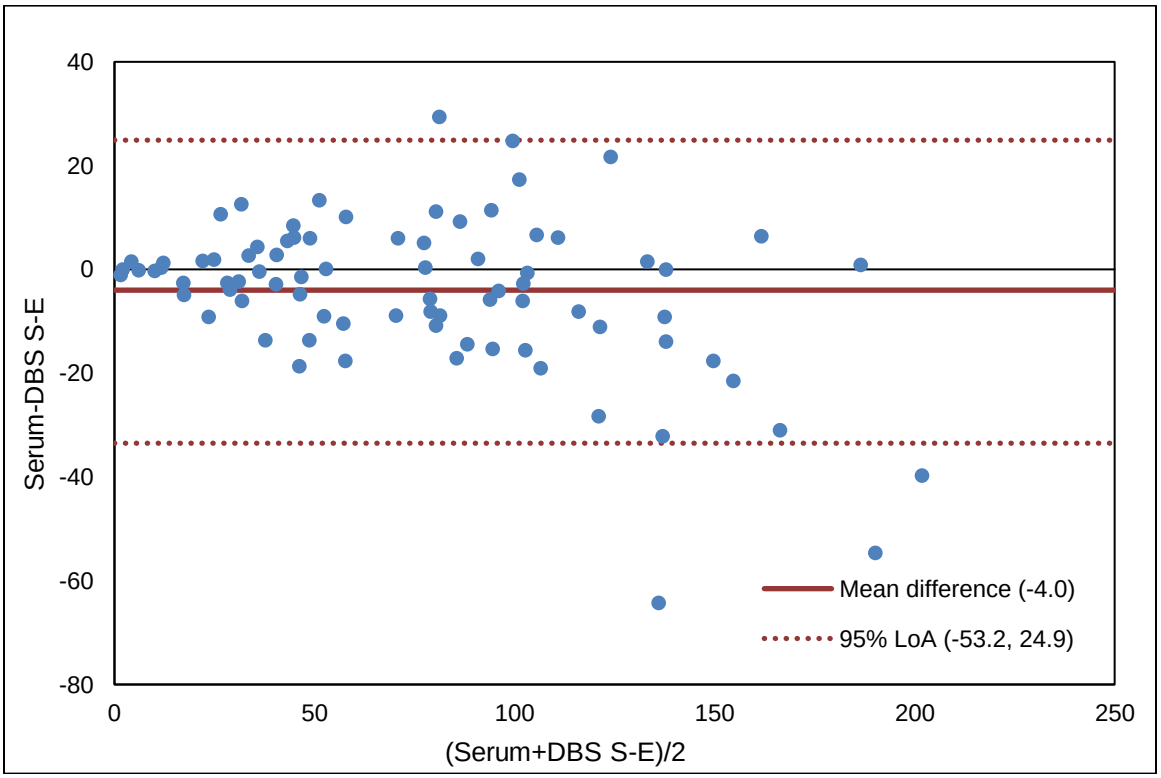


Figure 2.5. Difference of EBV VCA IgG values in DBS and serum over average values. This is a standard Bland-Altman analysis. (n = 82) EBV VCA IgG measurements were first converted to serum-equivalent values. The differences between which and the measurements in serum were plotted against the average of the two measurements. The solid red line is the mean difference (-4.0). The dotted red lines are the 95% confidence interval limits of agreement (LoA).

2.3.3 *Conversion Equation for DBS Value to Plasma-Equivalent Value*

The linear regression equation for DBS value to plasma-equivalent value according to the scatterplot (Figure 2.3) is:

$$y = 0.73x + 0.47\,, \tag{2.3}$$

where x is the plasma value and y is the DBS value. Rearranging the equation to:

$$x = 1.37y - 0.64\,, \tag{2.4}$$

in which DBS values can be input as y, and solving for x can give us an estimated plasma-equivalent value.

2.3.4 *Linearity with Diluted Samples*

The calculated percent recovery for diluted plasma and DBS samples are presented in Table 2.2

Table 2.2. EBV VCA IgG % recovery in diluted plasma and DBS samples.

Sample type	Dilution factor	Theoretical concentration	Measured concentration	% recovery
Plasma	neat		199 U/mL	
	1:2	99.5 U/mL	149 U/mL	149%
	1:3	66.3 U/mL	116 U/mL	174%
	1:4	49.8 U/mL	102 U/mL	204%
DBS	neat		197 U/mL	
	1:2	98.5 U/mL	156 U/mL	158%
	1:3	65.6 U/mL	129 U/mL	196%
	1:4	49.3 U/mL	107 U/mL	217%

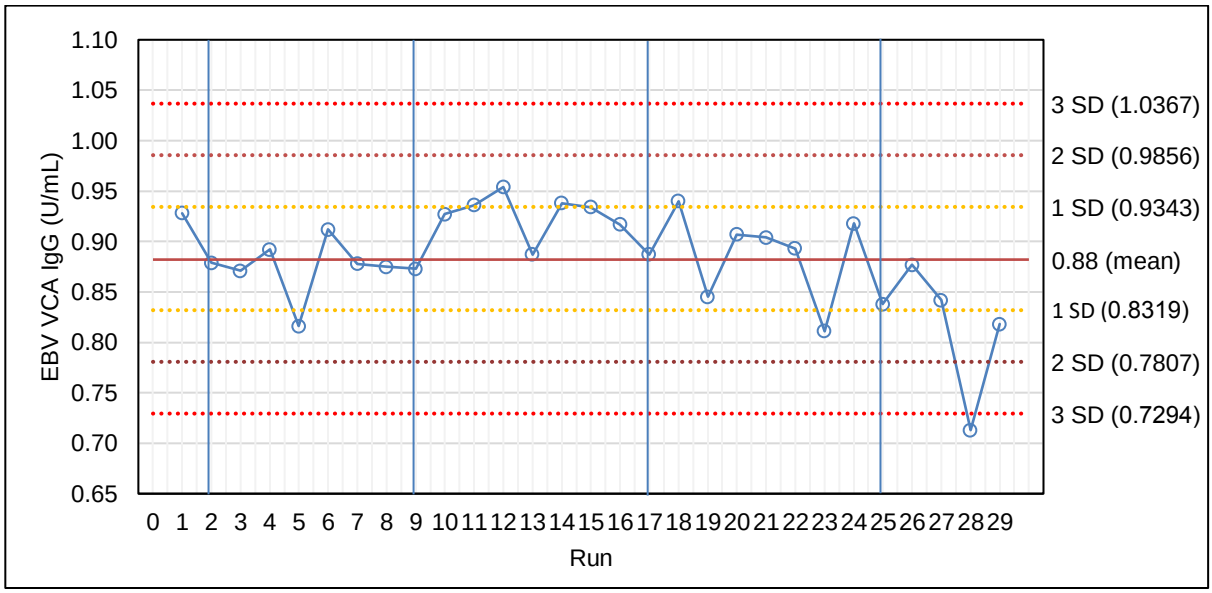
The assay overestimated the diluted samples according to their expected values in both DBS and plasma samples. However, we only tested dilution of samples with high measured values (~100-200 U/mL). The implication and possible explanation of such inconsistency will be explored further in section 4.1.4.

2.3.5 *Inter- and Intra-Assay Coefficients of Variation (CV)*

After eliminating those samples that were out-of-range high (> 200 U/mL), the intra-assay CVs were calculated for DBS and plasma samples. The intra-assay CVs were 6 % for DBS out of 132 samples and 7% for plasma out of 132 samples.

Across 29 runs over five days, the means of the in-house DBS control were 0.90 U/mL (SD = 0.08 U/mL) for the low-level control and 32.47 U/mL for the mid-level control (SD = 2.80 U/mL). The CVs between 29 runs were 6% for the low-level control and 9% for the mid-level control, yielding an average inter-assay CV of 8% for DBS samples. The CVs between different days were 6% for the low-level control and 11% for the mid-level control, yielding an average inter-day CV of 8%. The data for inter-assay variability is visualized as two separate Levey-Jennings charts for the low and mid-level in-house DBS control. (Figure 2.6)

A



B

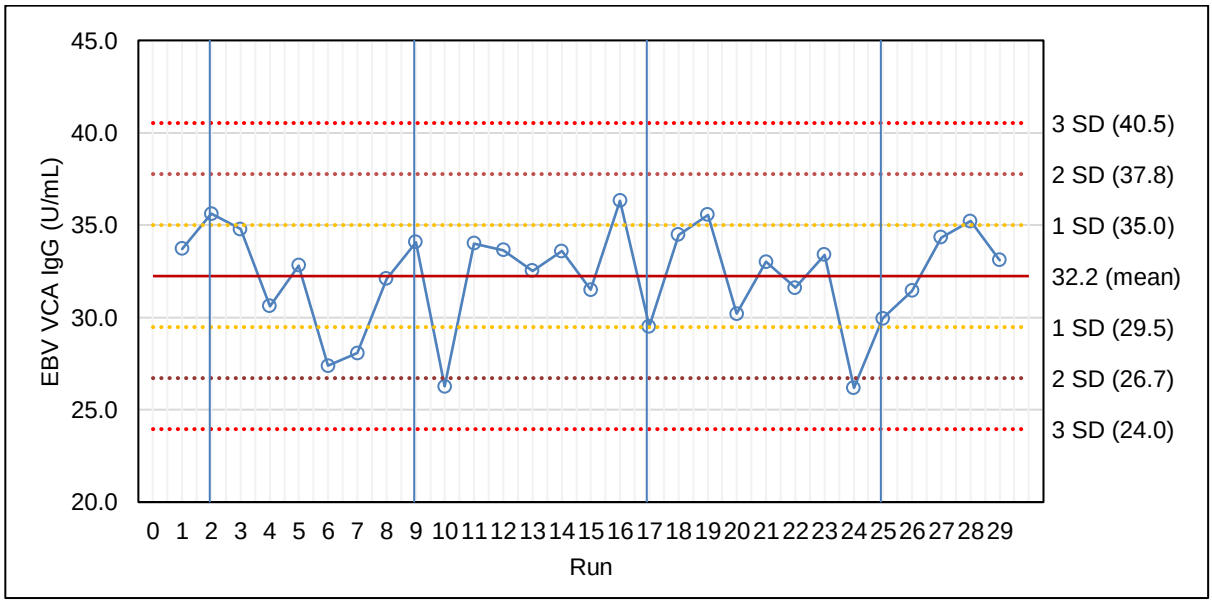


Figure 2.6. Levey-Jenning's charts for in-house DBS controls.
Each dot represents one single measurement of the in-house DBS of low-level (A) and mid-level (B). The x-axis represents the run number while the y-axis represents the EBV VCA IgG measurement in U/mL. The vertical blue lines mark assays performed on different day Each point is connected by a blue line to visualize trends. The red solid horizontal lines are the mean of the measurements and the dotted horizontal line are one, two and three standard deviations away from the mean.

2.3.6 *Limit of Detection*

The mean OD value of 20 replicates of the zero standard was calculated to be 0.005 with a standard deviation of 0.0006. The OD of the lower limit of detection was calculated to be 0.005 + 3(0.0006) = 0.0068. When we plugged this number into the standard curve equation as seen in Table 2. (y = (-0.0745-4.8)/1+(x/381)$^{0.682}$+4.8), and solved for x, we got a value of 0.97. Therefore we determined the limit of detection of this assay to be 0.97 U/mL.

2.3.7 *Seropositive Cut-Off Evaluation*

All of the measurement of replicates for the zero standard and sample diluent and most of the washed red cell DBS sample fell below the limit of detection. Only five single readings of the washed red cell DBS sample had concentration readings. The mean of which was 1.074 U/mL with a standard deviation of 0.035 and is drastically lower than the lower 20% cut-off for of 18 U/mL. (14 U/mL) Red cell debris and pigment did not seem to falsely interfere with the result of this assay by increasing OD. The mean concentration measurement of the different types of negative samples and the cut-off calibrators is visualized below. (Figure 2.7)

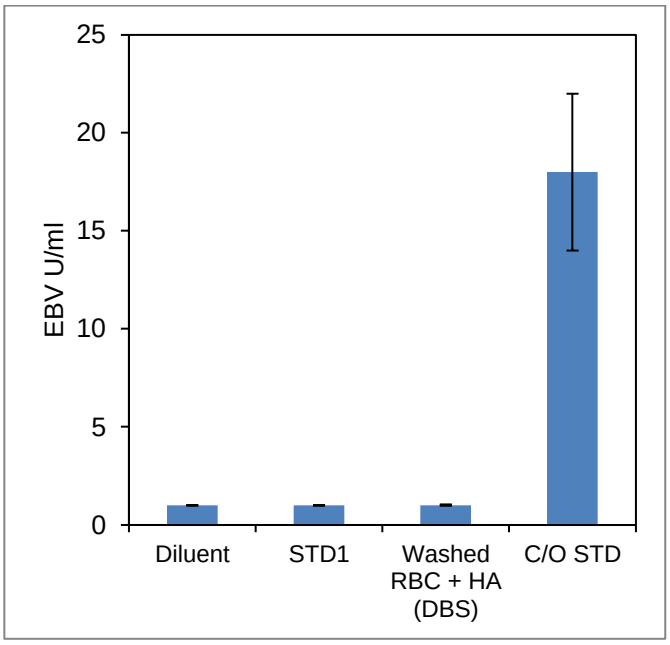


Figure 2.7. Comparison of known EBV negative samples with Cut-Off standard.
Mean EBV VCA IgG measurements of 24 replicates of the assay diluent, the zero standard (STD 1), washed RBC + HA DBS, and the Cut-Off Standard (C/O STD) (18 U/mL) are being compared. The error bars represent ± 20% around the cut-off (14-22 U/mL) in which a sample would be identified as “equivocal”.

2.3.8 *Freeze-Thaw Stability of DBS Samples*

Linear regression analysis was performed to compare the 24 DBS samples that had gone through five freeze-thaw cycles versus ones that had only gone through 1 freeze-thaw. The correlation was very strong (R^2 = 0.98) with a slope very close to one. This shows that DBS samples were extremely resistant to the deteriorating effect of repetitive freezing and thawing.

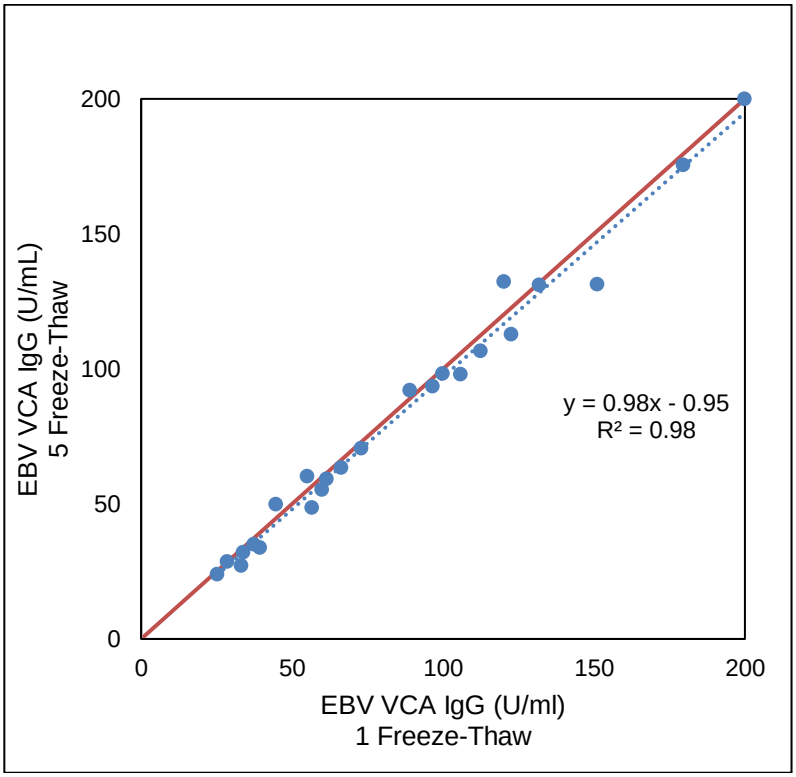


Figure 2.8. Freeze-thaw stability of EBV VCA IgG in DBS samples.
Linear regression of EBV VCA IgG measurements between one cycle of freeze-thaw and five cycles in 24 matched DBS samples. The red solid line represents equality. The blue dotted line is the linear regression trend line with line equation and R^2 value displayed.

2.3.9 *Elution Time Evaluation*

We found that the overnight eluate yielded higher EBV VCA IgG concentration results than the one-hour eluate, however, the linear correlation between the results was very strong ($R^2 = 0.98$) (Figure 2.9). DBS values are required to be converted to serum or plasma-equivalent values eventually, therefore, mathematically there would be no difference to use results from either type of eluate since their correlation was found to be so strong. One-hour elution was the obvious choice based on efficiency and convenience.

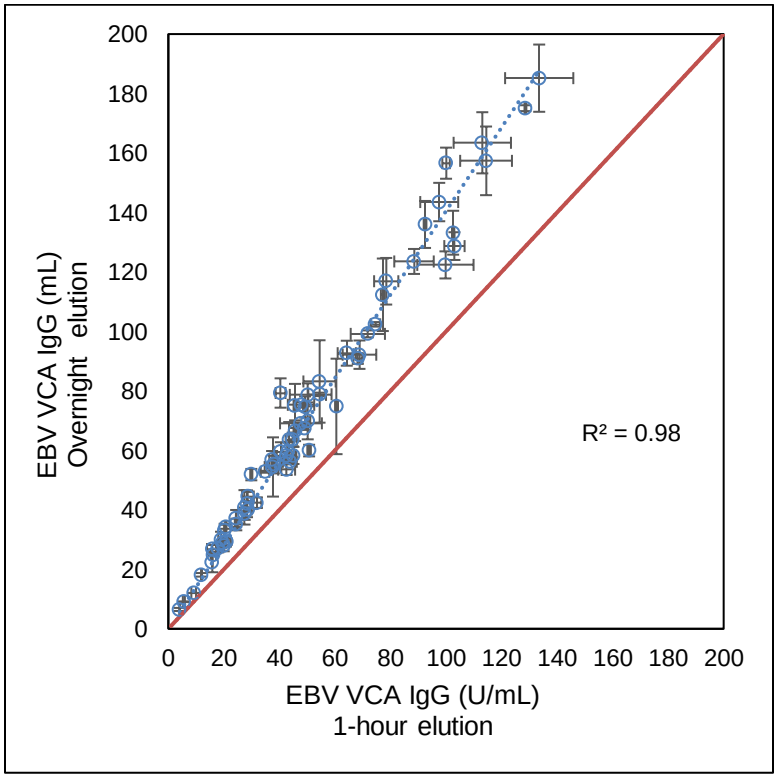


Figure 2.9. Effect of elution time on EBV VCA IgG measurement of DBS samples.
Linear regression of EBV VCA IgG measurement between overnight elution and one-hour elution between 72 matched DBS samples. The error bars are one standard deviations as each sample was ran in duplicate. The blue dotted line is the linear regression trend line with R^2 value displayed. The red line represent equality.

Chapter 3. EBV SEROLOGY AND EBV PCR, AND CMV SEROLOGY CORRELATION

One of the goals of this project is to compare and correlate serological measurement and molecular measurement of EBV in DBS samples in relation to viral reactivation. UW Virology has existing protocols for performing PCR testing for CMV and EBV on DBS and has been using them routinely on transplant patient and newborn samples. Little is known about whether viral DNA is detectable by these methods in otherwise healthy individuals with CMV and EBV reactivations. Since PCR assay is significantly more expensive than ELISA and the DBS extraction instrument at UW Virology is time consuming and can only process a small batch of samples at a time, a small pilot study on PCR positivity based on EBV VCA IgG measurement was conducted. The purpose of the pilot study was to see if there is an obvious relationship between EBV serological measurement and EBV PCR results, i.e., if PCR is only positive in samples with high IgG measurement. Eighteen DBS samples from the ELISA validation study spanning a wide range of measured EBV VCA IgG values were selected (Plasma-equivalent value 4 U/mL – 385 U/mL) and assayed using the established DBS extraction and real time PCR protocol at UW Virology. DBS extraction and real time PCR protocols as established and validated by UW Virology are described in 3.1.1-4.

Around the same time, the UW Biomarker Lab was contracted to produce results on EBV IgG, CMV IgG, EBV PCR and CMV PCR on 89 matched serum and DBS samples. This study is referred to as the USC/UCLA study and the Biomarker Lab was blinded from all information of

the population from which the samples were taken. Since this set of data pertained to areas of interest of our project, we decided to include analyses of this set of data.

The EBV IgG DBS portion was performed using the method validated as described in Chapter 2. The CMV IgG DBS portion is performed using an existing protocol described in section 3.1.5. PCR testing for EBV and CMV in both serum and DBS was sent over to UW Virology and assayed using their protocols which are described in sections 3.1.1-4.

3.1 MATERIALS AND METHODS

3.1.1 *Extraction/Digestion of DBS Samples for PCR*

The purpose of an extraction is to remove DNA from the nucleus. For DBS samples, there is an additional need to elute the sample from the filter paper. UW Virology utilizes the Casework Extraction Kit (Promega Catalog #DC6745) for extraction of DBS specimens. The kit includes 50 mL Casework Extraction Buffer, 1250 µL Nuclease Free Water, two vials of 10 mg lyophilized Proteinase K that is to be reconstituted by adding 556 µL water. An extraction buffer mix is prepared by mixing 405 µL of Casework Extraction Buffer with 45 µL of Casework Proteinase K for each DBS sample extraction. The buffer mix is also spiked with an internal control material, a plasmid called EXOBS, which is a jellyfish DNA sequence. The purpose of the internal control is to ensure that negative results are not due to inhibition of the polymerase, in which case neither the target virus DNA nor the EXOBS would amplify.

For each sample, a 6.35 mm sized disc are made from each of two blood spots for a total of two discs using a manual hole puncher. The two discs are placed into a 1.5 mL conical screw-capped

tube and 450 μL of buffer mix is added to the tube. The tube is capped and vortexed for ten seconds before being incubated on a heat block at 65°C for one hour. During the incubation, the tube is vortexed periodically at the 30 and 60-minute mark. The sample is then ready for the purification process.

3.1.2 *Purification of DBS sample for PCR*

PCR assay requires sample DNA to be extracted from the nucleus and rid of contaminants such as proteins, lipids and other cellular debris that can inhibit polymerase reactions completely. UW Virology utilizes the Promega Maxwell 16 instrument which is capable of handling 16 samples each run and the Promega Maxwell 16 Viral Total Nucleic Acid Purification Kit (Promega Catalog #AS1150) to perform the purification process of DBS samples. The kit includes 48 Maxwell 16 LEV cartridges, 20 mL lysis buffer, two vials of 1 mL Proteinase K solution, 20 mL nuclease-free Water, 50 LEV plungers and 50 0.5 mL elution tubes.

To minimize contamination, new gloves should be donned before handling the cartridges, LEV plungers and elution tubes. Cartridges are placed and centered in the Maxwell 16 LEV Cartridge Rack. Seals are peeled back with care as to not leave plastic residues on the top of the cartridges. A LEV plunger is then placed in well #8 of each cartridge and a labeled 0.5 mL elution tube is placed in front of each cartridge. 100 μL of nuclease-free water is added to the bottom of each of the elution tube with care as to not introduce bubbles into the water, which may cause suboptimal elution. The extracted DBS samples are centrifuged at 14000rmp for four minutes before 350 μL of which are pipetted to well #1 of the cartridge without disturbing the DBS discs. The Maxwell cartridge rack is then transfer to the Maxwell 16 instrument platform and ready to be run.

Once the Run/Stop button is pressed, the instrument will commence the automatic purification process. First, the sample in well #1 will be mixed by rapid up-and-down movement of the plunger. A magnetic rod will then be inserted down the middle of the plunger and capture paramagnetic particles in well #2. The plunger then travels back to well #1 to allow binding of the paramagnetic particles with the nucleic acids. After a series of capture and release washes through 7 wells, the purified DNA is finally released in the elution tubed placed in well #8. The whole process takes approximately 40 minutes. To minimize the presence of paper fiber residues in the sample, the elution tube is centrifuged again at 14000rmp for four minutes. The eluate is then carefully transfer to a clean tube without disturbing the paper fiber pellets that may have formed at the bottom.

3.1.3 *Real-Time PCR Assay*

Polymerase chain reaction (PCR) is a common technique that amplifies a specific region of a DNA molecule of interest. A typical PCR mixture includes the sample, DNA templates that contain the target DNA region, polymerase, primers that are complementary to the 3' ends of the target DNA region, deoxynucleotide triphosphates, which are the building blocks of new strands of DNA, buffer solution that provides optimal environment for the polymerase and $MgCl_2$ which provides Mg^{2+} ions that act as cofactors and catalyzes the reaction. The mixture is placed in a thermal cycler which would initially heat the mixture to a temperature of 94-96 °C. This disrupts hydrogen bonds of the DNA templates and denatures the double-stranded DNA and results in complementary single-stranded DNA molecules. Then the reaction is cooled to a temperature of 50-65 °C to allow for hybridization of the primers to the single-stranded DNA in this annealing step. Finally, the reaction enters the extension step in which the temperatures is adjusted to 75-80 °C. During this

phase, the DNA polymerase synthesize a new strand of DNA that is complementary to the template by adding on dNTPS to the primer in 5' to 3' direction which results in double the amount of the original DNA of interest, completing one cycle of PCR. The cycle then starts over and repeats for about 40-45 times, resulting in exponential amplification of PCR products at the end of the assay.

3.1.4 *TaqMan*

EBV and CMV PCR assays at UW Virology are real-time PCR assays utilizing the TaqMan technology for quantification. In a TaqMan assay, probes specific to an internal sequence are added in addition to primers in the reaction mixture. Each probe is covalently bonded to a "reporter" fluorophore molecule at the 5' end and a quencher molecule at the 3' end. The close proximity of the fluorophore and the quencher on the probe prevents the fluorophore from emitting fluorescence by way of Förster Resonance Energy Transfer. During the extension phase, the Taq polymerase, possessing the enzyme exonuclease, extends from the primers to form complementary strands of DNA along the templates and cleaves off the probes with exonuclease activity. Once the probes are cleaved, the reporter fluorophores are no longer in proximity to the quencher molecules, and thereby are able to generate measurable fluorescence light. The amount of fluorescence light emitted is proportional to the amount of DNA amplicons produced. The starting quantity of target DNA in the sample is estimated by comparing the cycle at which the PCR product accumulates in significant levels to that of a DNA standard curve samples of known quantities.

3.1.5 *CMV IgG ELISA*

Similar to the EBV ELISA method, a commercially available ELISA kit (Diamedix Corporation, No. 720-320, Miami, FL) was selected for measurement of CMV IgG concentration.[39] The package insert does not specify the type of CMV IgG the assay detects. This kit includes a 72-well

plate pre-coated with partially purified CMV antigen, three levels of calibrators, three levels of controls, sample diluent, a concentrated wash buffer that is diluted 1:19 before use, an enzyme conjugate, a substrate and a stop solution. Plasma samples are diluted 1:100 using the sample diluent. Diluted samples or DBS eluates are thoroughly mixed before being pipetted into coated wells. The plate then is incubated for 60 minutes uncovered at 37°C to allow binding of CMV IgG present in the sample with the CMV antigens in the wells. The plate then goes through a wash step using an automated plate washer, which washes the plate three times by aspirating the contents in the wells and then adding 300 μL of diluted wash buffer. After the last wash, residual moisture is removed by inverting the plate and forcefully tapping against a paper towel. 100 μL of the enzyme conjugate is then pipetted into the wells. The plate is again incubated at 37°C for 60 minutes, during which the enzyme conjugate binds with bound IgGs in the wells. The plate goes through another wash step as described previously. 100 μL of the substrate solution is then pipetted into the wells. The enzyme in the wells will continuously convert the substrate into blue products. The intensity of the color is proportional to the amount of enzyme present in the wells. The plate is incubated at 37°C for another 20 minutes to allow color development. 100 μL of the acidic stop solution is pipetted into each well to denature the enzyme, stopping further color development and turning the mixture yellow at the same time. After gentle mixing, absorbance is read at 450 nm using a BioTek plate reader. Absorbance of the kit calibrators of known IgG concentration are used to generate a standard curve, from which IgG concentrations of unknown samples are calculated. Data analysis is done using the BioTek Gen5 software and Excel.

3.2 DATA ANALYSES

Microsoft Excel was used for generation of graphs and figures. Regression analysis and one-way analysis of variance (ANOVA) were also done by Microsoft Excel. Online calculators were used for Mann-Whitney U test[40] and Fisher's exact test[41]. 0.05 was selected to be the alpha level.

3.2.1 *EBV Serology and Viral Load Correlation in DBS: Pilot Study*

Eighteen mock de-identified DBS samples with known EBV VCA IgG measurements were selected to be tested on PCR. A regression study was done to assesses the linear relationship between the two sets of data. As viral load was not detectable in all of the samples, the viral load data was transformed into PCR+ and PCR– categories. Samples that had a detectable viral load were considered PCR+ and samples that had "undetectable" PCR results were considered PCR-. The EBV VCA IgG measurements of the two categories were compared and visualized by constructing boxplots. One-way ANOVA and Mann-Whitney U tests were utilized to assess variation of the IgG measurements between PCR+ and PCR- samples.

3.2.2 *Serum and DBS Sample Type Correlation in USC/UCLA samples*

We compared the EBV VCA IgG measurements between 89 matched serum and DBS samples as well as CMV IgG measurements between 89 matched serum and DBS samples. Scatterplots were constructed and correlations were assessed by linear regression.

3.2.3 *EBV Serology and Viral Load Correlation in USC/UCLA DBS Samples*

Similar to our pilot study, we performed a regression analysis on EBV IgG and viral load measurements obtained in 86 DBS samples. Viral load data was divided into PCR + and PCR- categories. Samples that had a detectable viral load were considered PCR+ and samples that had

"undetectable" PCR results were considered PCR-. Boxplots were constructed to compare the EBV IgG measurements between the two categories. One-way ANOVA and Mann-Whitney U test were utilized to assess variation of the IgG measurements between PCR+ and PCR- samples.

3.2.4 *EBV Serology and CMV Serology Correlation in Serum*

We examined the correlation between EBV IgG and CMV IgG in matched serum samples. Only samples that were seropositive for both EBV and CMV were selected for analysis. A scatterplot was constructed to analyze the linear correlation. A regression analysis was done to determine the Pearson correlation (r) and whether or not the slope was significant. We further explored the potential of stronger correlation by dividing the data into high and low groups and examine the association of EBV and CMV levels as contingency tables. We used three different methods of categorize the IgG measurements into high and low groups. The first and second methods were dividing the data based on the means and medians respectively. The third method involved calculating the z-scores of the log transformed IgG values. Z-score for a given sample is calculated as follows:

$$z = \frac{x - \mu}{\sigma}, \tag{3.5}$$

where μ is the mean and σ is the standard deviation. Samples with values higher than the mean would have a positive z-score and would be categorized as high and vice versa.

3.3 RESULTS

3.3.1 *EBV Serology and Viral Load Correlation in DBS: Pilot Study*

In this 18-sample pilot study, regression analysis indicates that there was no linear relationship between EBV viral load and EBV VCA IgG measurement (R^2 = 0.07*)*. Boxplots showed distinct

separation and little overlaps between EBV PCR- and PCR + groups in their EBV VCA IgG measurements (Figure 3.1). The median EBV VCA IgG values of PCR- and PCR+ groups were 55 U/mL and 227 U/ml respectively. One-way ANOVA indicates there was a significant difference in EBV VCA IgG measurements between PCR- and PCR+ DBS samples [F (1, 16) = 6.71, P = 0.02]. A Mann-Whitney U test was also performed and it indicated the median EBV VCA IgG values of PCR- and PCR+ DBS samples were significantly different (U = 12, P < 0.05).

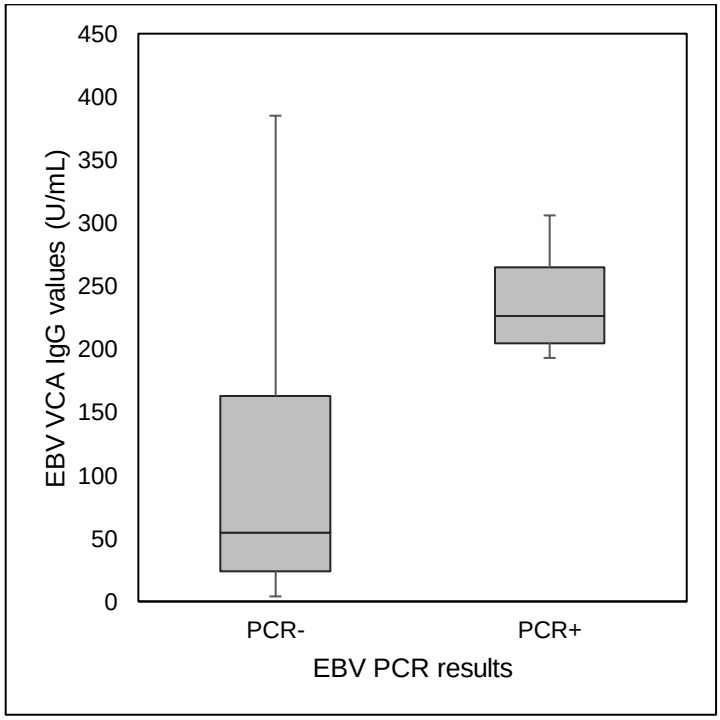


Figure 3.1. Boxplots of EBV VCA IgG measurements of PCR- and PCR+ DBS samples in pilot study.
The boxes represent the lower (Q1) and upper (Q3) quartiles. The lines within the boxes are the medians. The whiskers show the range of data. Sample size is 18 with 12 in the PCR- group and 6 in the PCR + group.

3.3.2 *USC/UCLA Study Samples*

Eighty-nine matched serum and DBS samples were assayed for EBV VCA IgG, CMV IgG, EBV PCR and CMV PCR. Certain sample types yielded no detectable analyte with certain methods in our analysis. Whether or not a method was able to detect analytes based on sample types is summarized as Table 3.3.

Table 3.3. Analyte detectability based on methods and sample types in our analysis.

Virus	Method	Sample Type	
		Serum	DBS
EBV	IgG	***Yes***	***Yes***
	PCR	***No***	***Yes***
CMV	IgG	***Yes***	***Yes***
	PCR	***No***	***No***

The inability to detect EBV and CMV DNA in serum samples and CMV DNA in DBS samples will be discussed in section 4.2. The data we were able to obtain from the other methods allowed us to conduct the analyses described in the rest of this section.

3.3.3 *Serum and DBS Sample Type Correlation*

Of the 89 matched serum and DBS samples, 82 had EBV VCA IgG data on both sample types and 77 had CMV IgG data on both sample types. The rest either had data missing on either one or both sample types due to sample insufficiency. After converting DBS values into serum-equivalent values, linear correlations of EBV VCA IgG and CMV IgG measurements are visualized as scatterplots in Figure 3.2. Regression analysis indicated strong correlation between serum and DBS samples types for both EBV VCA IgG and CMV IgG measurements. R^2 values were 0.92 and 0.85 respectively. Two samples had high CMV IgG measurements in serum but CMV seronegative in DBS samples. These discrepancies were suspected to be due to sample collection or processing errors. It was also more apparent in the CMV data that there are a few outliers whose

serum-equivalent values calculated from DBS and their matched serum values are more discrepant. This could also be due to sample collection or random assay errors.

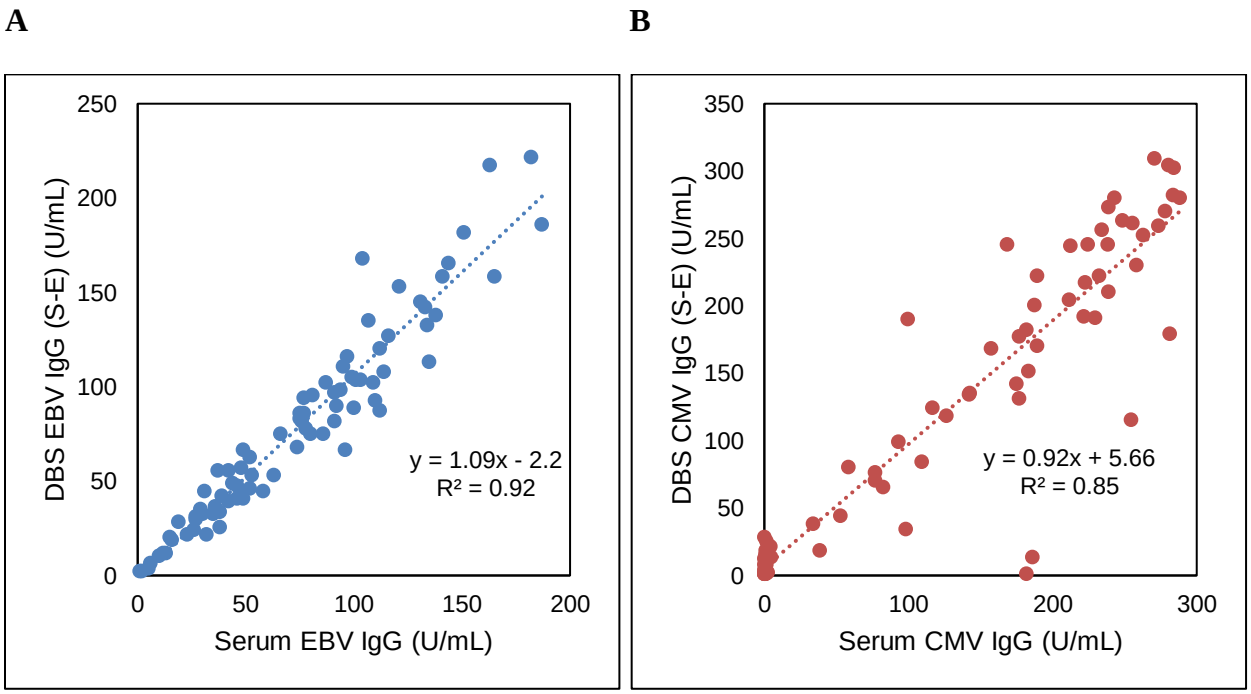


Figure 3.2. Linear regression of IgG measurements between serum and DBS sample types. A. DBS serum-equivalent values for **A.** EBV VCA IgG (n = 82) and **B.** CMV IgG (n = 77) plotted against serum values in matched serum and DBS samples. The dotted lines are the trend lines with their equations and R^2 values displayed.

3.3.4 *EBV Serology and Viral Load Correlation in USC/UCLA DBS Samples*

Data from 86 matched samples was available for this analysis. Regression analysis indicated that there was no linear relationship between EBV viral load and EBV VCA IgG measurement (R^2 = 0.08*)*. Boxplots showed overlaps and no separation between EBV PCR- and PCR + groups in their EBV VCA IgG measurements (Figure 3.3). The median EBV VCA IgG values of PCR- and PCR+ groups were 71 U/mL and 81 U/ml respectively. One-way ANOVA indicated there was no significant difference in EBV VCA IgG measurements between PCR- and PCR+ DBS samples [*F*

(1, 85) = 1.93, *P* = 0.17]. A Mann-Whitney U test was also performed and it indicated the median EBV VCA IgG values of PCR- and PCR+ DBS samples were not significantly different (*U* = 344.5, *P* = 0.17).

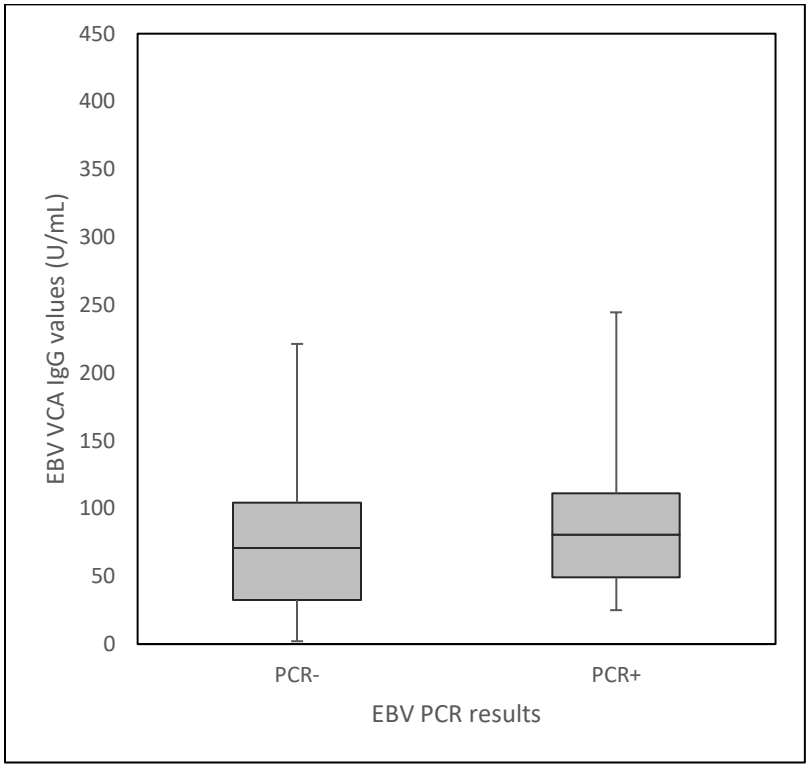


Figure 3.3. Boxplots of EBV VCA IgG measurements of PCR- and PCR+ DBS samples in USC/UCLA study samples.
The boxes represent the lower (Q1) and upper (Q3) quartiles. The lines within the boxes are the medians. The whiskers show the range of data. Sample size is 86 with 72 in the PCR- group and 14 in the PCR+ group.

3.3.5 *EBV Serology and CMV Serology Correlation in Serum*

Out of 89 serum samples, 83 had serology data for both EBV and CMV and of those, 48 were seropositive for both EBV and CMV. A detailed breakdown of the CMV and EBV serostatuses of the 83 samples is shown in Table 3.4. A regression analysis was done on the correlation of CMV and EBV IgG levels between matched serum samples and is visualized in Figure 3.4 as a scatterplot. CMV IgG measurement correlated weakly with EBV VCA IgG in serum (r = 0.43), with a significant slope (P = 0.002).

To further examined stronger correlation between EBV and CMV serology, we explored three different methods of categorizing the IgG measurements into high and low groups. Two of which were categorization based on the sample means of EBV and CMV IgG values (Figure 3.5), and categorization based on sample medians of EBV and CMV IgG values (Figure 3.6). The mean IgG values were 89 U/mL for EBV and 197 U/mL for CMV. The median IgG values were 78 U/mL for EBV and 212 U/mL for CMV. The last method of categorization was converting the log transformed IgG values in z-scores (Figure 3.7).

For each of these categorizations, a contingency table was constructed with levels of EBV and CMV IgG as variables and their association was examining by Fisher's exact test. The association between high and low EBV VCA IgG and high and low CMV IgG level was found to be significant regardless of how we categorized high and low groups. The P values determined by Fisher's exact test are 0.0005, 0.0012 and 0.0024 for categorization using sample means (Table 3.5), sample medians (Table 3.6) and z-score of log transformed IgG values (Table 3.7) respectively.

Table 3.4. EBV and CMV serostatus of USC/UCLA study samples.

EBV and CMV serostatus of 83 serum samples as determined by ELISA.

	EBV Sero +	EBV Sero -	Total
CMV Sero +	48 (58%)	6 (7%)	54 (65%)
CMV Sero -	26 (31%)	3 (4%)	29 (35%)
Total	74 (89%)	9 (11%)	83 (100%)

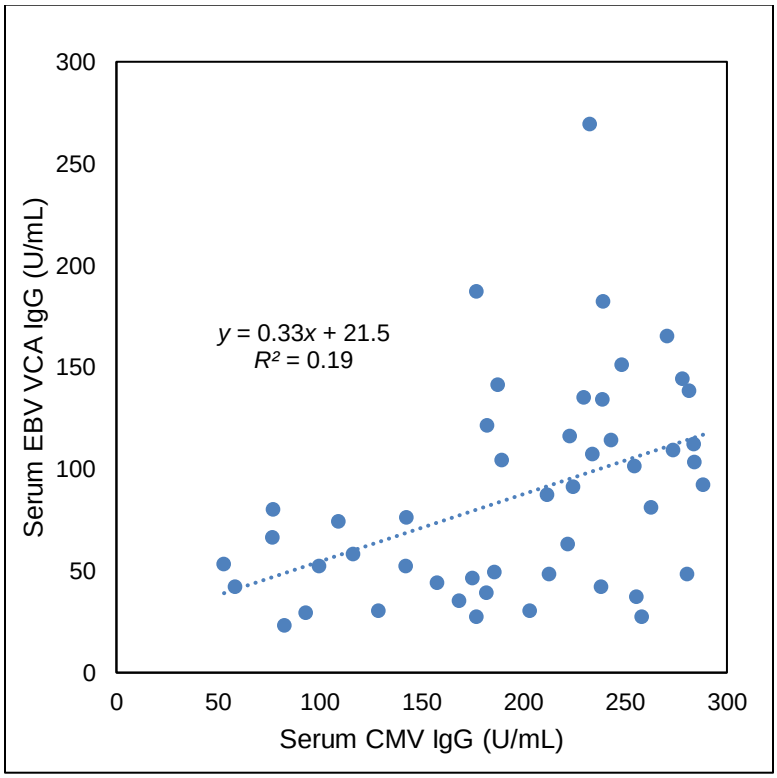


Figure 3.4. Correlation between serum CMV IgG and serum EBV VCA IgG measurements.
Scatterplot of CMV IgG vs. EBV VCA IgG values in U/mL of 48 serum samples. The blue dotted line is the trend line with its equation and R^2 value displayed.

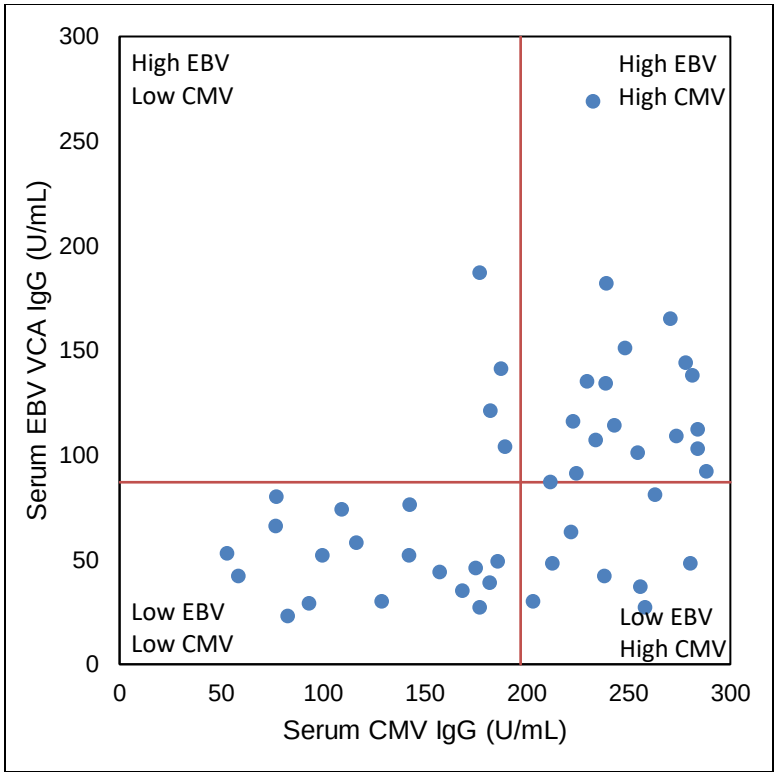


Figure 3.5. High and low EBV and CMV IgG values categorized by sample means. Scatterplot of 48 EBV and CMV seropositive samples. The quadrants are defined by mean values of EBV (87 U/mL) and CMV IgG (197 U/mL) measurements. Samples fall in quadrants corresponding to their relative EBV and CMV IgG levels. (Quadrant I: high EBV, high CMV; quadrant II: high EBV, low CMV; quadrant III: low EBV, low CMV; quadrant IV: low EBV, high CMV)

Table 3.5. Contingency table of high and low EBV and CMV IgG values categorized by sample means.

	Low CMV	High CMV	Total
High EBV	4	18	22
Low EBV	18	8	26
Total	22	26	48
Fisher's exact test P value = 0.0005			

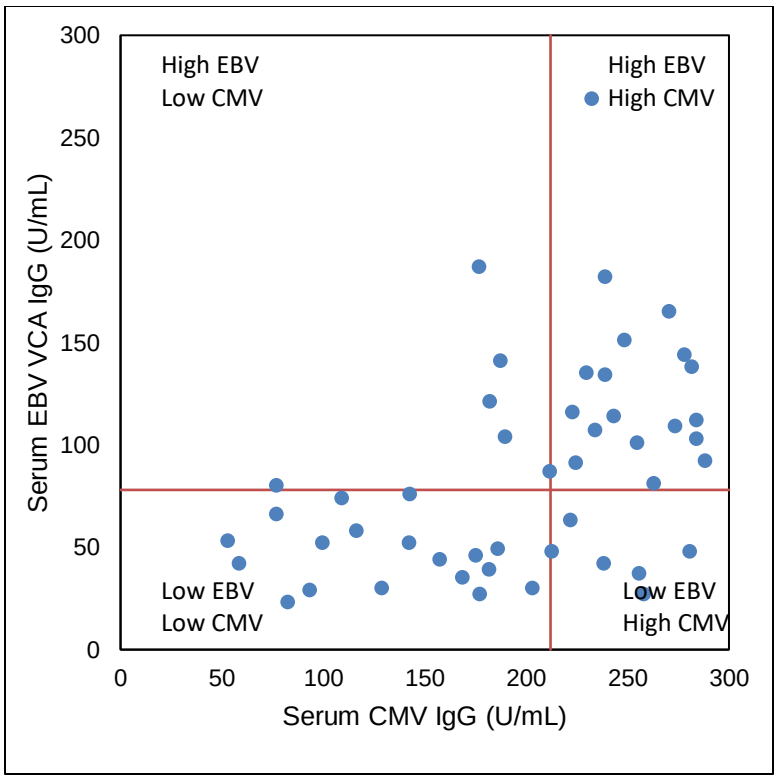


Figure 3.6. High and low EBV and CMV IgG values categorized by sample medians. Scatterplot of 48 EBV and CMV seropositive samples. The quadrants are defined by mean values of EBV (78 U/mL) and CMV IgG (212 U/mL) measurements. Samples fall in quadrants corresponding to their relative EBV and CMV IgG levels. (Quadrant I: high EBV, high CMV; quadrant II: high EBV, low CMV; quadrant III: low EBV, low CMV; quadrant IV: low EBV, high CMV)

Table 3.6. Contingency table of high and low EBV and CMV IgG values categorized by sample medians.

	Low CMV	High CMV	Total
High EBV	6	18	24
Low EBV	18	6	24
Total	24	24	48
Fisher's exact test P value = 0.0012			

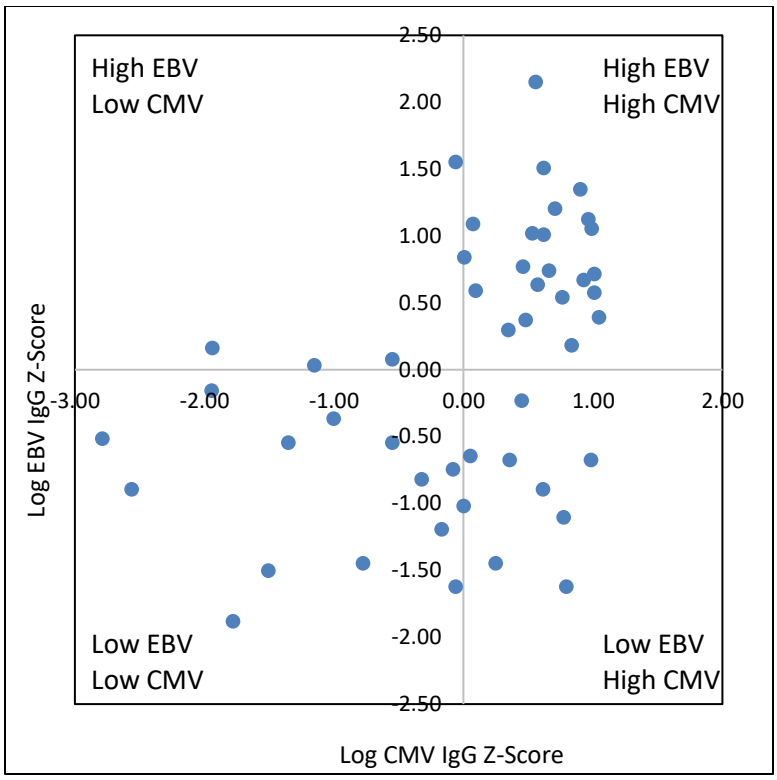


Figure 3.7. High and low EBV and CMV IgG values categorized by log transformed IgG z-scores.

Scatterplot of log transformed EBV and CMV IgG values z-scores of 48 serum samples that are seropositive for both EBV and CMV. Samples fall in quadrants corresponding to their relative EBV and CMV IgG levels. (Quadrant I: high EBV, high CMV; quadrant II: high EBV, low CMV; quadrant III: low EBV, low CMV; quadrant IV: low EBV, high CMV)

Table 3.7. Contingency table of high and low EBV and CMV IgG values categorized by log transformed IgG z-scores.

	Low CMV	High CMV	Total
High EBV	4	22	26
Low EBV	13	9	22
Total	17	31	48
Fisher's exact test P value = 0.0024			

Chapter 4. DISCUSSION

4.1 EBV DBS ELISA METHOD VALIDATION

In preparation for a large volume of study samples and as the kit for our previous validated method had been discontinued, the UW Biomarker Lab validated a method for measuring EBV antibodies in DBS. Based on previously published works[29,42] and our own experiences with adapting other ELISA assays for DBS, we took a commercially available ELISA kit that is intended for plasma and serum and adapted it for testing on DBS samples. We largely followed the procedure per the kit's instruction manual but substituted diluted plasma or serum with DBS eluate and made adjustments as needed. For instance, the standard curve needed to be optimized and is discussed further in section 4.1.2. The method was validated by comparing measurements obtained from matched DBS and plasma samples, with the plasma measurements being the comparative method.

The results of our experiments agreed with the main literature we referenced[29,42] and they showed that EBV VCA IgG measured in DBS sample type agreed and correlated well with that in plasma. An equation was established to convert EBV VCA IgG values measured in DBS to a plasma or serum-equivalent values and it is discussed in detail in section 4.1.1. The intra-assay CV and inter-assay CV in DBS replicate samples were both within acceptable range for ELISA (10% and 15% respectively). As one replicate of DBS measurement results from one elution mixture of one excised DBS disc, there may be inter-sample or inter-spot variabilities. We were not able to control and examine sample variability from two excised discs from different location of the same blood spot or two excised discs from two different blood spots. However, as our CV results suggested, these variabilities should be and negligible.

The seropositive cut-off standard was evaluated by comparing its value with values measured in known seronegative samples and DBS samples made with washed-RBC and human serum albumin. We found that the cut-off standard was conservative in calling a sample seropositive and that RBC debris and pigments did not interfere and falsely elevated the measurements. We were also able to establish the assay's lower limit of detection from the noise measured in zero standard.

A DBS freeze-thaw stability evaluation was performed as it is protocol at the UW Biomarker Lab to freeze DBS samples at -70°C. As we expected, DBS samples were inert to the deteriorating effect of freeze-thaw cycles that is usually more pronounced to wet samples. An elution time study was also done to compare EBV VCA IgG measurements between DBS eluted for one hour versus overnight (16 hours). The results showed that longer elution indeed yields stronger signals, which meaned more plasma had been incorporated into the elution mixture. However, the values from the two elution times correlated very well which justified our opting for one-hour elution time out of convenience.

This validated method was eventually put to use and EBV VCA IgG measurements were produced for ~2000 DBS samples for Landmark Spirituality and Health Study. This set of data was evaluated in the published journal article entitled, *The Mediating Role of Meaning in the Association between Stress and Health*.[43]

4.1.1 *Comparing DBS values to Plasma values*

It is necessary to convert DBS measurements to values that are comparable to plasma or serum values. In Mei et al.'s study,[27] the volume of plasma in a 3.2 mm punch of DBS was estimated to be around 1.5 μL. If we assume that all of the plasma in the DBS disc was incorporated into the

200 μL of diluent during the elution process (we know this was not the case based on our elution time study), we could calculate that there was approximately 0.75 μL of plasma in 100 μL of the eluate, which is the volume used to assay. On the other hand, to assay plasma sample, we diluted 5 μL of plasma with 495 μL of diluent, which means there was 1.01 μL of plasma in the 100 μL assay mixture. Therefore, theoretically, the ratio of plasma in DBS samples to plasma samples was 0.75 μL/1.01 μL = 0.74. As indicated by the slope of the linear regression line equation of 133 matched samples (Figure 2.3), the EBV VCA IgG value in DBS was approximately 0.73 times of its plasma counterpart.

4.1.2 *Plasma and Serum-Equivalent Conversion*

Although the closeness of the plasma ratio calculation to the slope from our linear regression was intriguing, it was based on some assumptions and overlooking factors affecting plasma volumes in DBS, such as hematocrit, blood volume, and location of punch as examined in Mei et al.'s study.[27] In addition to these factors, it is impossible to determine how much plasma had been shaken off the filter paper and how much of it remained during elution. However, we do know that elution time has a positive relationship with IgG measurement. Hence, establishing a standard elution time plus using a conversion equation to obtain a plasma or serum-equivalent value for DBS sample is a feasible and practical approach.[42] Two improvements our conversion equation can benefit from are larger sample size, which would decrease error, as well as using real DBS instead of mock DBS samples, which would take in account variables (e.g. blood volume and hematocrit) of real DBS samples that mock DBS samples were able to eliminate.

4.1.3 *Standard Curve Optimization*

We deviated from the manufacturer's instruction manual[38] and modified the way the standard curve was constructed. We initially decided to do this because we wanted to include more points in the curve and because we wanted a method of curve fitting that would generate an equation in the Gen5 software. Upon discovering that when using a 4-parameter non-linear regression curve generated from using serially dilution of the highest kit standard (200 U/mL), the 10 U/mL standard would not fall on the curve, we suspected that the concentration assigned to the kit calibrators might be relative and not absolute values. The instruction manual provides no information on how these values in arbitrary unit (U/mL) were assigned or how they are comparable to a recognized national or international standard reference materials. Therefore, it is safe to assume that the quantification of EBV VCA IgG determined by this kit is only designed to be used in this assay and not necessarily comparable to quantifications obtained by other ELISA kits or any clinical and international standard values.

4.1.4 *Linearity with Diluted Samples*

Another problem we encountered in relation to the standard curve was linearity with diluted specimens with values between measured values of 100 U/mL and 200 U/mL. We only evaluated dilution linearity with a matched DBS and plasma sample with borderline high value (~200 U/mL) because it represented the type of samples that would likely require a dilution. As shown in Table 2.1, the assay consistently overestimated the diluted samples and showed no linearity. However, as we examined our standard curve (Figure 2.2), we could see that the section of the curve that correspond to these values of interest had no calibrator points to support it; the calibrator points jumped from 50 U/mL to 200 U/mL. Therefore, we suspected that the lack of linearity with the

diluted high value samples was due to the combination of the unknown shape of the curve between those two points and the aforementioned likely randomness of the assigned calibrator value. To further investigate and possibly improve the standard curve, additional calibrator points between 50 U/mL and 200 U/mL could be incorporated to examine the linearity of the assay between those two points. Furthermore, we could also examine dilutions of samples with lower measured values and see how they would behave.

4.2 EBV SEROLOGY AND EBV PCR, AND CMV SEROLOGY CORRELATION

In this part of the project, we intended a comprehensive comparison of the results of EBV VCA IgG measurements with EBV PCR results, as well as with CMV IgG and CMV PCR measurements in matched DBS and serum/plasma samples. We formed hypotheses for these comparisons based on the well accepted theory in which EBV IgG is viewed as an indirect biomarker for stress. This theory reasons that psychosocial stress weakens cellular immunity's ability to control and maintain EBV latency and causes EBV reactivation, which in turns triggers a humoral immunity response to produce a measurable increase in EBV IgG. We hypothesized that if this model were true, EBV DNA could also be detected as a result of EBV reactivation and perhaps serve as a more direct biomarker for stress. We were also interested in EBV IgG and PCR results correlation with CMV IgG and PCR results because like EBV, CMV is also a herpesvirus that established latency and reactivates during immunosuppressed state and therefore should behave similarly.

4.2.1 *CMV PCR Detectability in DBS and Serum*

In our experiments, we were not able to detect CMV DNA in any of the 89 matched serum and DBS samples. The established protocols for EBV and CMV PCR assays in serum and DBS at UW Virology has been used for testing clinical samples. They are used primarily in monitoring viral

load in transplant patients with induced immunosuppression and screening newborns for congenital CMV infection. Correlation study of CMV viral loads between DBS and plasma had been done with transplant patient samples[44] and many studies examining using DBS with PCR to screen for neonatal CMV infection have been published.[45,46] We were not certain about whether subclinical reactivation of CMV in would manifest in detectable viremia by PCR. There had been debates in blood banking about the capability of PCR methods in detecting CMV in healthy blood donors. Conclusions varied among groups; while some were able to detect CMV DNA in peripheral blood in healthy blood donor by PCR,[47,48] others failed to produce similar results.[49,50] In one study that examined viral reactivation in older women with latent CMV infection, Thomasini et al. were able to detect viremia by PCR in 59% (n = 71) of women between 60 to 80 years of age and only 8% (n = 73) in younger women (aged 18 – 30).[14] We were blinded to the demographical information of the sample population in our experiment, but there was reason to believe that perhaps these study samples are of a younger demography. Another possibility is that our PCR method was not sensitive enough to detect CMV viral load or viremia due to reactivation in healthy individuals.

4.2.2 *EBV Serology and Viral Load Correlation in DBS*

In contrast to CMV, we were able to detect EBV DNA in EBV seropositive samples. In our pilot study (n = 18), six out of 18 samples (33%) had detectable viral load by PCR. Although the viral load quantification of these PCR + samples did not linearly correlate with their EBV VCA IgG values ($R^2 = 0.07$), PCR+ samples did show association with higher IgG values compared to PCR- samples [F (1, 16) = 6.71, p = 0.02]. The results of the pilot study could be interpreted as being in agreement with our hypothesis: If high EBV IgG values do indicate reactivation, viral load or

viremia are detectable by PCR. However, since the sample size was very small, these results might be biased.

In the USC-UCLA study samples (n = 86), we saw very different results in which 14 out of 86 (16%) DBS samples were PCR+ and 72 (84%) were PCR-. Of the PCR+ DBS samples, viral loads did not correlate with their EBV VCA IgG values (R^2 = 0.08) and there was no significant difference in EBV VCA IgG values between PCR+ and PCR- DBS samples [F (1, 85) = 1.93, P = 0.17]. Interestingly, none of the 86 matched serum samples had detectable EBV DNA by our EBV PCR method, suggesting the 14 PCR+ DBS samples might be showing EBV presence in the peripheral lymphocytes, but without detectable viremia in the serum. It also indicated that these DBS sample contained sufficient amount of peripheral blood mononuclear cells (PBMC), which is the most sensitive sample type for EBV viral detection, since EBV maintains its latent infection and proliferates primarily in B cells.

The lack of correlation between EBV IgG measurement and PCR positivity and between detectable cellular and serum/plasma viremia is supported by the literatures. Gärtner et al. found no correlation between serological parameters of EBV reactivation and viral load.[51] Luderer et al. found two out of 62 sera with EBV reactivation serological parameters with detectable EBV DNA by PCR.[52] Maurmann et al. found discrepancy between reactivation serological profile, cellular viral load and plasma viremia in 22 healthy EBV seropositive donors.[53] The findings from these studies suggest that serological profile alone is not a good indicator of EBV reactivation and the result of our experiments appears to be in agreement.

In regard to whether DBS is an accurate and sensitive enough type for detection of EBV cellular viral load in association with reactivation, a correlation study with whole blood and/or peripheral blood mononuclear cells would be appropriate. In Hakim et al.'s comparison of EBV DNA quantification between various blood components, high correlation of viral load is found between PBMC and whole blood. DBS is essentially whole blood but in very small amount. Hakim et al. used 200 μL of whole blood for DNA extraction, whereas in our DNA extraction procedure for DBS, two 6.35 mm punches of DBS discs were digested in 450 μL of buffer, which is estimated to be ~ 10.5 μL of whole blood. It is very likely sensitivity can be lost in the extreme small volume of DBS sample type.

4.2.3 *EBV Serology and CMV Serology Correlation*

We found a statistically significant ($P = 0.003$) but weak correlation ($r = 0.43$) between EBV VCA IgG values and CMV IgG values in 48 matched serum samples that were seropositive for both EBV and CMV. Additionally, we found the correlation to be stronger when we explored three ways to categorize IgG values into relatively high and relatively low groups. This finding is not dissimilar to that of Fagundes et al., where they found that the psychosocial stress of childhood adversities correlated to both EBV and CMV IgG titers, however, EBV and CMV IgG titers only moderately correlated with each other.[33] They argued that this is due to the difference in patterns and mechanisms by which EBV and CMV reactivation are influenced.

Chapter 5. CONCLUSION AND FUTURE DIRECTIONS

We were successful in taking a commercially available ELISA kit intended for detecting EBV VCA IgG in plasma or serum and adapting it for testing in DBS. The DBS sample type

demonstrates very good correlation with plasma samples, reproducibility and stability. It offers many advantages over plasma and serum in its ease and low-cost in collection and storage especially in population and field studies.[29] Optimizations could be made to improve the standard curve by introducing more calibration points. Calibrators could also be compared with a nationally or internationally recognized standard material for the quantification to be more meaningful.

We also utilized the validated DBS EBV IgG method and made a comprehensive comparison with existing IgG and PCR methods on matched DBS and serum samples with the intention of evaluating parameters of EBV and CMV reactivation. We were able to see evidence of EBV reactivation through detectable viral load in some DBS samples by PCR, however its lack of correlation with EBV VCA IgG measurement led us to conclude that EBV serological profile is not a good indicator of EBV reactivation. We also observed that detectable EBV viral load in DBS samples did not correlate with detectable viremia in serum, which is consistent with previous literature.[54] Although we were able to detect viral load in some DBS samples, correlation studies with whole blood are needed to determine validate the sensitivity of DBS samples by PCR. CMV DNA was not detectable in serum or DBS with our PCR method. This could be due to sensitivity of our assay or the demography of our sample population. Lastly, we found only a weak correlation between EBV serology and CMV serology.

There are ongoing interests in studying the relationship between stress and immunity. Evaluating psychosocial stress largely relies on self-reported questionnaires that score the subject's emotions and experiences. A measurable biomarker, may be understandably appealing to the analysis of stress as a "hard science" evidence. The notion of EBV IgG as a biomarker for stress has been

widely accepted and numerous studies had found success in associating it with psychosocial stress. However, studies with molecular profiles of EBV with PCR fail to find a strong link between EBV serology and EBV reactivation. More research is needed toward a definitive method of detecting EBV reactivation and in turn, better understanding between the connection between stress and immunity.

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