DETECTION OF HUMAN HLA CLASS II PROTEIN IN A VACCINE PREPARATION

By

Michelle Elizabeth Russell

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Clinical Laboratory Sciences

2011

ABSTRACT

DETECTION OF HUMAN HLA CLASS II PROTEIN IN A VACCINE PREPARATION

By

Michelle Elizabeth Russell

Proteomics has become an emerging tool in the analysis of pathological biological processes. A clinical case study in the Michigan State University Histocompatibility Laboratory presented an opportunity to become more familiar with proteomic methods to investigate the presence of a protein stimulus present in a routine vaccination. The patient had received a routine vaccination of a combined measles, mumps, rubella, and varicella vaccine and later presented with newly detected antibodies, specifically, anti-HLA-DR11. Sequence specific oligonucleotide (SSO) probe genotyping typing of the WI-38 cell line used to culture the rubella component of the vaccine has confirmed that the cell line is HLA-DR11 positive. Using immunoaffinity combined with mass spectrometry and western blot analysis, the presence of HLA-DR11 protein in the vaccine has been investigated. Following the completion of multiple assay arrangements, it was determined that HLA-DR11 protein could not be detected in the vaccine preparations tested. Though HLA-DR11 protein was not detected in any of the methods developed, it may have been due to a lack the sensitivity of the assays utilized for this project. Had the protein been positively identified, the results would have further indicated routine vaccination as an additional source of sensitization to HLA proteins.

For my husband Jeff, for giving his unconditional support and encouragement, and for Mom and Dad, for raising me to believe that it was always possible.

ACKNOWLEDGMENTS

I'd like to recognize and thank Dr. Gerlach for his guidance and financial support for this project. He answered my many questions, allowed me to work independently, supported my goals, and continues to encourage my progress as a scientist and aspiration of becoming an HLA laboratory director. I also would like to express my sincere appreciation for his help in reviewing and providing direction in the writing of my thesis. His constructive criticism and perspective helped me to sharpen my skills as a writer.

I'd like to thank John Davis, who taught me the techniques that provided the foundation of this project. His patience, technical advice, and help in troubleshooting were essential the progression of my project.

Thank you to Susan Forney, Ann Gobeski, and Arianne Mankey for teaching me the fundamentals of the HLA system and its role in solid organ transplantation.

Thank you to Dorothy Levis, Dr. A. Bradley Eisenbrey, and Dr. Jerry Rosenberg the rest of the employees of the Gift of Life Michigan HLA Laboratory. I truly appreciate their support in my academic pursuits and their encouragement in my career goal of becoming an HLA laboratory director.

Thank you to Doug Whitten of the Michigan State University Research Technology Support Facility, who ran my samples and patiently taught me the fundamentals of understanding mass spectrometry data.

I’d like to thank my committee members, Dr. David Thorne and Dr. Curtis Wilkerson, for their guidance of this project and critical assessment of my thesis. Their input on the premise of my project and critical evaluation of my writing further promote my academic and scholarly development.

Finally, I like to thank One Lambda, Inc. and Gen-Probe, Inc. for donating the protein controls used for this project.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

°C - degrees Celsius

µg - microgram

µl - microliter

% - percent

AAFP - American Academy of Family Physicians

AAP - American Academy of Pediatrics

Acc. - Accession

ACIP - Advisory Committee on Immunization Practices

AHG - antihuman globulin

AMR - antibody mediated rejection

APC - antigen presenting cell

APS – ammonium persulfate

BS3 - Bis(sulfosuccinimidyl)suberate

BSA - bovine serum albumin

CDC - Centers for Disease Control and Prevention

CID - collision induced dissociation

Da - Dalton

DNA – deoxyribonucleic acid

DPBS - Dulbecco's Phosphate Buffered Saline

DSA - donor specific antibody

DTT - dithiothreitol

EBV - espstein barr virus

ESI - electrospray ionization

FCXM - flow cytometric crossmatch

FDA - Food and Drug Administration

HBV - Hepatitis B virus

FT-MS - Fourier transform ion cyclotron

histo. - histocompatibilty

HIV-1- human immunodeficiency virus 1

HIV-2 - human immunodeficiency virus 2

HLA - human leukocyte antigen

HRP - horseradish peroxidase

kDa - kilodalton

LC-MS/MS - liquid chromatography-MS/MS

mA - milliamps

MALDI - matrix assisted laser desorption ionization

MCF - mean channel fluorescence

MFI - mean fluorescence intensity

MHC - major histocompatibility complex

ml - milliliter

mM- millimolar

MMDB – Molecular Modeling Database

Mol. - Molecular

MS - mass spectrometry

MSU HLA Laboratory - Michigan State University Histocompatibility and Immunogenetics Laboratory

NCBI - National Center for Biotechnology

ng - nanogram

NRNB - non-reduced and not-boiled

PBS - phosphate buffered saline

PDVF - polyvinylidene fluoride

pI - isoelectric point

PMSF - phenylmethanesulfonylfluoride

PRA - panel reactive antibody

RB - reduced and boiled

RCF - relative centrifugal force

RPM - rotation per minute

SDS - sodium dodecyl sulfate

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SSP - sequence specific primer

TEMED - tetramethylethylenediamine

TOF - time of flight

TST - Tuberculin Skin Test

VAST – Vector Alignment Search Tool

V - Volts

INTRODUCTION

Introduction

Proteomics has become an emerging tool in the analysis of pathological biological processes. Recently, a clinical case study in the Michigan State University Histocompatibility and Immunogenetics Laboratory (MSU HLA Laboratory) presented an opportunity to become more familiar with proteomic methods to investigate the presence of a protein stimulus in a routine vaccination. In the following case, a pediatric renal transplant candidate presented with unexpected anti-Human Leukocyte Antigen (HLA) Class II antibodies. More specifically, Anti-HLA-DR11 was the one of the strongest unexpected antibodies detected. Upon further investigation, it was determined that the patient had recently received a therapeutic injection of ProQuad®, a combined varicella, measles, mumps and rubella vaccine. It is known that the MRC-5 and WI-38 human fibroblast cell lines are used to propagate the varicella and rubella viruses for use in the vaccine preparations, respectively. Genomic molecular HLA typing of the WI-38 cell line has confirmed that the cell line is HLA-DR11 positive. In light of this connection, it is postulated that immunization to HLA proteins may also occur by routine vaccination. A variety of protein identification methods may be used to directly identify the presence of HLA Class II protein in the vaccine preparation. Such a discovery might serve as a starting point on the discussion of the role of vaccines in sensitization to other HLA proteins.

Allograft Rejection

The development and sustained presence of HLA antibodies in renal transplant candidates presents as a well-established risk to allograft transplantation.[1] When specific for renal graft HLA antigens, the presence of such alloantibodies contributes to the risk of varying

degrees of antibody mediated rejection (AMR). The range of graft rejection extends from hyperacute to chronic, depending on the specificity and titer of pre-transplant and post-transplant HLA donor specific antibody (DSA). The mechanism of AMR involves the process of donor specific antibody recognizing and binding to the non-self HLA molecules expressed on the vascular surface of the graft. Inflammatory cytokines that become elevated during initial graft rejection induce increased expression of HLA molecules on the vascular surface of the graft as well as increased HLA expression within extravascular sites such as renal tubule cells.[2] Such antibody binding leads to classical pathway of complement activation. The C4d molecule is a fragment that is produced during the classical complement activation pathway and is covalently bound to the surface of the cell. Antibody bound to the surface of endothelial cells persists for approximately three to four days, at which time bound antibodies are shed from the cellular surface. Conversely, the C4d fragment of the classical complement pathway resists shedding and becomes a permanent marker of antibody binding. Immunohistochemistry staining for C4d of renal graft biopsy is a useful indicator of humoral mediated immunologic graft rejection.[2,3]

The donor specific antibody that binds to the HLA molecules of the graft is associated with decreased graft survival. Decreased graft survival is attributed to the immune mediated damage and repair cycles that take place. Such damage results in graft vascular occlusion in the form of arteriosclerosis, and damage related to decreased renal function in the form of glomerulosclerosis and interstitial fibrosis.[2,4]

Vaccination as a Route of Sensitization

HLA antibodies develop as a result of exposure to non-self HLA molecules. Exposure may occur through by one or more mechanisms of alloimmunization. Routes of immunization include transplantation, blood products transfusion, or pregnancy.[5-8] It has been postulated that other mechanisms of immunization exist. One of these mechanisms may be through non-allogenic, or nonspecific, immunization by vaccination. For example, one study has demonstrated that a non-HLA immune stimulus resulted in the development of HLA antibodies.[9] In this study, twenty healthy subjects, ten male and ten female, received a dose of recombinant anti-Hepatitis B Virus (HBV) vaccine and a Tuberculin Skin Test (TST). Five of the subjects known to be historically TST positive were not retested. Each subject also completed a questionnaire regarding history of prior pregnancies and abortions, blood transfusions, and surgeries, indicating which subjects may have been previously exposed to HLA antigens. Serum samples were collected prior to immune stimulus and post-immune stimulus at intervals of 7, 29, and 50-56 days. HLA Class I and Class II antibody detection and analysis were completed on all subjects by Panel Reactive Antibody (PRA) evaluation using LabScreen® PRA. Eleven of the twenty were positive for either HLA Class I or Class II antibodies during the study. Nine subjects that had 0% PRA at their basal measurement presented with unique PRA percentages of greater than 0% from serum sampled at the 29 day interval. In addition, 7 of the subjects sampled at the 50 to 56 day interval were positive for antibody against HLA with PRA percentages of greater than 0%. One of these subjects only demonstrated a PRA greater than 0% at the 50-56 day sample. Most of the HLA antibodies detected in the study were low titer Class I IgG, although eight subjects did present with IgM HLA antibodies. The authors of the study postulate

that immune stimulus resulting in the detected antibody response appeared to be non-specific. The reasoning behind their hypothesis may have been due to the fact that the anti-HBV vaccine is propagated in yeast cells.

The HLA antibody response to the TST may have not been entirely non-specific. The TST is composed of a Tuberculin Purified Protein Derivative (PPD) and is isolated from culture media filtrates of a human strain of Mycobacterium Tuberculosis.[10] One such study has demonstrated homology between a 65 kDa (kilodalton) tetrapeptide of *Mycobacterium tuberculosis* and the protein structure of HLA-DR1, 3 and 4 antigens. This protein homology may represent a potential cause of sensitization.[11]

Overall, the stimulation of anti-HLA antibody study could support the premise that their administered non-HLA stimuli may have elicited the development of HLA antibodies, while the demonstrated development of antibodies against HLA-DR4 in three of the test subjects could have been due the protein homology between the 65 kDa tetrapetide of *Mycobacterium tuberculosis* and HLA-DR1, 3, and 4 antigens.

In addition to the non-specific HLA immunization through vaccination, it has been postulated that vaccination may serve as another potential source of allogenic, HLA-specific exposure, comparable to transfusion, pregnancy, and transplant. One such study has addressed this issue.[12] In this investigation, human immunodeficiency virus-1 (HIV-1) positive male subjects were administered Remune® (The Immune Response Corporation, Inc.). Remune® is an inactivated HIV-1 vaccine tested for the immunotherapeutic treatment of HIV-1 positive individuals. Remune® is propagated in the HUT-78 cell line. The HUT-78 cell line is a

cutaneous T cell lymphoma cell line of human origin.[13,14] HLA phenotyping of the HUT-78 cell line indicated it is positive for HLA- A1, -B62, -Cw3, -DR4 and -DQ8 antigens.[12,14] The HLA antibodies of individuals receiving the vaccine were assessed prior to and after initiation of Remune® therapy. The HLA types of individuals in the study were also determined in order to assess the degree of auto-reactivity. HLA antibody recognizing HLA-B62 and -DR4 were identified in individuals that received vaccine in spite of not having these antigens. These results suggest that the vaccine elicited an alloreactive response to HLA-B62 and -DR4 antigens present in the vaccine.[12] A similar mechanism of immunization may have contributed to the development of de novo or secondary response to HLA Class II antibody specificities in a pediatric renal transplant candidate at the MSU HLA Laboratory.[15]

MSU HLA Laboratory Case Study

In this case study, a pediatric renal patient presented with unexpected anti-HLA antibodies after initial crossmatch testing with his father. Patient history indicated that at the time of referral to the MSU HLA Laboratory, the 10-year-old male patient had previously received a renal graft from his mother in May of 1998. The mismatched HLA antigens of the graft were HLA-B7 and -DR8. This patient's history also included an extensive amount of blood transfusions throughout a fourteen-month period of time between June of 1997 and August of 1998. The initial testing in 2005 did not indicate HLA Class I or Class II antibodies using a LABScreen® Mixed test (One Lambda, Inc., Canoga Park, CA) and antihuman globulin (AHG) antibody methods. Concurrent with the above test, a T and B cell flow cytometric crossmatch (FCXM) between the patient and his father gave a T and B cell negative result.

After this initial evaluation, the lab did not receive additional test requests by the transplant center until May 2007, at which time the patient's father was again evaluated as a potential renal graft donor. Table 1 indicates the HLA types of the patient and his father.

Table 1. HLA Typing of Patient and Father

HLA Type Class I		
Locus	Patient	Father
A	3, 68	11, 68
B	35, 65	65, 61
HLA Type Class II		
Locus	Patient	Father
DRB1*	07, 13	11, 13
DRB3/4/5*	DRB3*, DRB4*01:03:01:02N	DRB3*
DQB1*	03:01/04/09/10/13, 0303/06/12/15	03:01/04/09/10/13, XX
DPB1*	04:01, XX	04:01, XX

Table 1. HLA Class I typing was primarily completed by a serological method. Additional testing by a molecular sequence specific primer (SSP) method was completed when the result of the serological method was unclear. HLA Class II Typing was completed by molecular methods.

An updated panel reactive antibody (PRA) test was completed at this time revealed a HLA Class I PRA of 2% and Class II PRA of 0%. The HLA Class I and Class II PRA tests were completed using the LABScreen® PRA (One Lambda, Inc.) test on a Luminex instrument (Luminex Corporation, Austin, TX). The T and B cell FCXM result between the patient and his father was negative. In September 2007, the final FCXM before the transplant surgery was T cell negative and B cell positive, revealing an unexpected positive B cell FCXM result. Table 2 is a summary of the three FCXMs between the patient and his father.

Table 2. Patient and Father FCXM Result Summary

	Negative Control MCF		Patient MCF		Channel Shift		Interpretation	
Test Date	T Cell	B Cell	T Cell	B Cell	T Cell	B Cell	T Cell	B cell
06/23/2005	334	306	325	350	-9	44	Negative	Negative
05/16/2007	301*	324*	235	340	-66*	16*	Negative	Negative
09/05/2007	318*	341*	269*	784*	-50*	435*	Negative	Positive

Table 2. Summary of the FCXMs between the patient and his father. The T and B cell cutoff values expressed as channel shifts for each of the tests are 69 and 98 on 6/23/2005, 50 and 100 on 05/16/2007, and 63* and 86* on 09/05/2007. Abbreviations: Mean Channel Fluorescence (MCF). *In cases where more than one negative control serum was run or if the patient was run in duplicate, the MCF value denotes the average of the negative control MCF, patient MCF or channel shift value.

Due to the unexpected positive B cell FCXM on 09/05/2007, an additional set of Class I and Class II PRA tests were completed on the patient's final FCXM serum; the same serum used for the FCXM run on 09/05/2007. The Class I PRA result was 0% (as expected with a negative T cell FCXM). The Class II PRA analysis revealed a 97% PRA with suspected anti-HLA-DR11 specificity. LABScreen® Single Antigen (One Lambda, Inc.) analysis confirmed antibody recognition of HLA-DR11 protein. The bead specific for HLA-DR11 resulted in mean fluorescence intensity (MFI) of 9235. MFI is the unit of fluorescence detected on each bead within the assay, where multiple beads coated with unique HLA antigens contribute to the trimmed mean in the baseline calculation of intensity. MFI may be used as a relative measure of amount of antibody detected in the sample. The Class II PRA and Single Antigen assays contain multiple HLA Class II allele specific beads. Single Antigen analysis also revealed antibody specificity to numerous HLA-DP alleles in addition to the HLA-DR11 specificity. The patient's resulting antibody specificity to HLA-DR11 aided in interpretation of the unexpected final positive B cell FCXM.

Subsequent investigation into the patient's clinical history revealed that the patient had received ProQuad®, a combined version of the routinely administered M-M-R®-II (Measles, Mumps, and Rubella Virus Vaccine Live, Merck & Co., Inc.,) and Verivax® (Varicella Virus Vaccine Live, Merck & Co., Inc.) vaccination on 05/18/2007. Vaccine administration occurred between the time of patient's 2007 initial and final T and B cell FCXMs with his father. The MSU HLA Laboratory further investigated this potential immunizing event by purchasing the cell lines used to propagate the viruses for use in the vaccination products. The cell line used to propagate the rubella component of the M-M-R®-II vaccine is the WI-38 cell line.[16] The cell lines used to propagate the Verivax® vaccine are WI-38 and MRC-5 cell lines, with final passage through the MRC-5 cell line.[17] The WI-38 and MRC-5 cell lines are available for purchase through American Type Culture Collection **(**Manassas, VA).

LITERATURE REVIEW

HLA System

The HLA system is most polymorphic antigen system within the human genome. There are two classes of antigens that are clinically relevant in reference to renal transplant. HLA Class I is a collection of loci that includes the HLA-A, -B, and -C loci. HLA Class II is a collection of loci which contains genes for the HLA -DRB1, -DQB1, and -DPB1 loci. The number of discovered alleles of the HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 loci continues to increase. In 2008, the most recently updated HLA dictionary was released. This reference lists the World Health Organization (WHO) Nomenclature Committee for the Factors of the HLA System. It defines the currently accepted HLA genotypes and their corresponding serologic equivalents.[18] Interim updates to accepted WHO Nomenclature HLA allele information can be found online at the IMGT/HLA database. The IMGT/HLA database is a collaborative database that contains new, updated, or deleted HLA alleles since the previous release.[19-21]

Genetics of the HLA System

The genes of the HLA system are located on the short arm of chromosome 6 at the 6p21.3 location and are collectively referred to as the Major Histocompatibility Complex (MHC). More than 200 genes are contained in this region of approximately 3.6 megabases.[22,23] The three major classes of the MHC are identified as Class I, Class II, and C lass III. Class I is telomeric on chromosome 6 and includes the classical HLA-A, -B, -C, genes, and non-classical HLA-E, -F, -G genes, and MICA and MICB genes. Class II is centromeric and includes the classical HLA-DR, -DQ, and -DP genes as well as the non-classical genes for transporters

associated with antigen processing, such as TAP-1 and TAP-2.[23] The Class III gene region lies between the Class I and Class II regions on the chromosome. It contains the genes for complement proteins, inflammatory cytokines, heat shock proteins, the tumor necrosis factor cluster, and other inflammatory proteins.[22,23] The Class III gene region has been characterized as the most genetically dense region of the MHC, as well as the human genome.[23] Beyond the scope of Class I and Class II antigen presentation function, role in transplantation, and immune defense mechanism, the collective set of the MHC genes have also been extensively studied for their characteristic haplotype structure and autoimmune and inflammatory disease associations.[24-26] The polygenic characteristic of the human MHC, as well as then large number of alleles at each locus, results in unique peptide presentation for different individuals. The polygeny and polymorphism of the HLA system results in an immunologic survival advantage due to its characteristic of presenting unique peptides and the ability of the immune system to react with specificity to the peptides as part of its effector response.

HLA Class I Protein Structure

The structure of the HLA Class I MHC molecule is composed of a heterodimer, consisting of the membrane spanning 43 kDa alpha chain and the non-membrane spanning 12 kDa β2-microglobulin. MHC Class I locus of contains the gene for the alpha chain. The alpha chain tertiary structure presents as three domains, α1, α2, and α3. The α1 and α2 domains are the membrane distal portions of the polypeptide, forming the antigen presentation groove. This groove consists of amino acid residues formed in the secondary structures of two opposing alpha helices anchored by a beta sheet oriented parallel to the cell surface. The primary function of

Class I is to interact with the T-cell receptor of CD8+ T-cells for antigen presentation. The antigenic peptide is held tightly within a groove by molecular interactions between both main and side chain residues of conserved and polymorphic residues. The α3 domain is proximal to the cell membrane in association with β2 microglobulin. The gene for β2 microglobulin is located on chromosome 15 of humans.[22]

Class I molecules are present on a variety of human tissue and cells. This distribution reflects the fact that the function of these proteins is to present peptides processed by the proteasome to CD8+ cytotoxic T cells. Since all nucleated cells are theoretical targets of virus infection, Class I molecules are normally expressed on all nucleated cells, though the level of expression may vary. As examples, immune cells generally express high levels of Class I proteins whereas hepatocytes express lower amounts, and erythrocytes, being non-nucleated, have little to no Class I expression.[22]

HLA Class II Protein Structure

The HLA Class II molecule is a heterodimer that is composed of an α and a β glycoprotein chain, both of which span the membrane. Both chains are encoded within the MHC Class II loci of chromosome 6. The molecular weights of the α and β chains are 34 kDa and 29 kDa, respectively. Each chain is composed of two domains. The α1 and β1 domains are in close proximity to one another, are distal to the membrane, and form the antigenic peptide binding site. The Class II structure of two opposing alpha helices anchored by a beta sheet is similar to the Class I molecule, though there is one significant difference. HLA Class II molecules have a more open antigen binding site, thus allowing for a larger peptide to bind. Peptides that have been

processed by the endosomal protein degradation pathway sit within this site. The α2 and β2 domains of the molecule are located proximal to the membrane.[22]

The distribution of Class II molecules is more restricted than Class I molecules. This may be due to a more specific role of Class II molecules in immunity. The function of Class II molecules is to present exogenous peptides that have been processed by the cellular endosomal pathway to CD4+ T cells, which subsequently activate additional effector cells. Thus, the distribution of Class II molecules is generally limited to antigen presenting cells (APCs) such as B cells, macrophages, Langerhans cells, and thymic epithelial cells. Activated T cells also demonstrate a low level of Class II expression. Class II expression on renal tubular cells in the context of transplantation has clinical relevance.[22,27]

HLA Proteins and Alloimmunization

Each molecular variant for the above mentioned HLA Class I -A, -B, and -C loci and Class II -DR, -DQ, and -DP alleles corresponds to a unique peptide sequence within the Class I or Class II molecules. In the context of solid organ transplantation, the unique peptide sequences of each HLA molecule result in cell surface antigens that may illicit an immune response. Antigenic peptide sequences of the HLA proteins have been identified in various studies.[28-32] Allelic variation at specific positions may be different enough to illicit an immune response in the form of alloantibody development and T-cell mediated alloimmunity. Consequently, the loci specific polymorphisms at the HLA- A,-B,-C, -DRB1, -DQB1, and -DPB1 loci are determined prior to organ transplantation. Combined with alloantibody detection, HLA genotyping typing allows for the HLA antigen matching between renal graft donor and recipient. It also assists in

detecting potential contraindications to transplant due to the presence of preexisting donor specific anti-HLA antibodies in recipient serum.

Routes of Sensitization

HLA antibodies often develop after exposure to non-self HLA molecules. Exposure may occur by one or more mechanisms of alloimmunization: such as, prior transplantation, blood products transfusion, or pregnancy.[5-8] Another mechanism is through non-allogenic (or non-specific) immunization by vaccination.[9] In addition, vaccination may also serve as source of allogenic, HLA specific stimulus as revealed by Page's study of anti-HLA antibody development following vaccination. This study presented patients who were treated with Remune®, an inactivated HIV-1 vaccine. These patients subsequently developed anti-HLA antibodies consistent with the HLA type of the HUT-78 cell line.[12]

Vaccine Background

The Centers for Disease Control and Prevention (CDC) releases annual vaccination schedules as part of the Morbidity and Mortality Weekly Report.[33] The guidelines are jointly approved by the Advisory Committee on Immunization Practices (ACIP), the American Academy of Pediatrics (AAP), and the American Academy of Family Physicians (AAFP). These are annual recommendations that physicians use to as a guide for administering routine vaccinations to children and adolescents. Prior to 1995, the ACIP released their childhood vaccination guidelines only every few years. The CDC recommends a variety of vaccines for children from birth until the age of eighteen. These include vaccines that induce immunity to hepatitis B, rotavirus, diphtheria, tetanus, pertussis, haemophilus type b, pneumococcal,

inactivated poliovirus, influenza, measles, mumps, rubella, varicella, hepatitis A, meningoccal, and human papilloma virus pathogens. Additional vaccinations due to incidental pathogen exposure, such as exposure to the rabies virus, may also be administered as medically indicated.

Vaccine Manufacturing

In the United States, vaccine manufacturing is monitored by the Food and Drug Administration (FDA).[34] Although highly regulated, there are variations in vaccine production. Such manufacturing variations are likely the result of specific growth, isolation, and purification processes that are dependent on pathogen characteristics. The package inserts in many vaccines contain information on the contents of growth media, strain of pathogen, and other product characteristics. The degree of disclosure in the production process varies by manufacturer.[34]

Many media and cells lines are utilized to enhance pathogen growth. They include viral propagation media such as immortalized human cell lines, yeast cells, chicken embryos, chicken fibroblasts, and vero cells (a continuous cell line from an adult African green monkey kidney).[35] examples of currently utilized human cell lines include WI-38 and MRC-5. Additionally, specialized growth media is required for propagation of bacterial pathogens.

Examples of differing pathogen isolation methods include centrifugation, cell lysis, tissue homogenation, and cellular extraction. As with the isolation methods, a variety of pathogen purification techniques are utilized. These include sterile filtration, extraction, precipitation, ultrafiltration, permeation chromatography, density gradient centrifugation, and column chromatography. The extent of disclosure of growth, isolation, and purification methods are dependent on the manufacturer.

In the cases of live attenuated viral vaccines, there may be limited purification of the viral growth media in order to preserve the efficacy of the vaccine. Vaccines manufactured from culture in human cell lines include those containing the rubella, varicella, polio and hepatitis A viruses. A model for live vaccine viral unit production has been described.[34] In this case, a vaccine for live virus is propagated intracellularly, within either a WI-38 or MRC-5 human fibroblast cell line. Once the cell culture has propagated the desired amount of virus, the cells are lysed and the virus is isolated by centrifugation and filtration.

In the previous decade, a variety of vaccines have been FDA approved and recommended for vaccination against the measles, mumps, rubella, and varicella viruses. All are manufactured by Merck & Co., Inc. (Whitehouse Station, NJ, United States). These are traditionally considered to be childhood diseases and subsequently administered to children in the early years of life as recommended by the CDC. The first described is M-M-R®-II, which is a trivalent vaccine for measles, mumps, and rubella.[16] The second is Verivax®, which is a vaccine for the varicella virus.[17] The third vaccine formulation is ProQuad®, which is a quadvalent vaccine for measles, mumps, rubella, and varicella.[36] ProQuad® appears to be the combined product of M-M-R®-II and Verivax® and was developed for the simultaneous vaccination of measles, mumps, rubella, and varicella viruses with one injection site (as opposed to two injections sites that are necessary when delivering the separate M-M-R®-II and Verivax® vaccines). The FDA indicated the availability of ProQuad® would be restricted beginning in 2007 and recommended that customers should transition to the utilization of M-M-R®-II and Verivax® for vaccination against measles, mumps, rubella, and varicella viruses.[37] ProQuad® is the vaccine which had been administered to the patient from case study described above.

Both M-M-R®-II and Verivax® vaccines contain viruses that are propagated by human diploid cell lines. The Wistar RA27/3 strain of live attenuated rubella virus is propagated inWI-38 human diploid lung fibroblasts (M-M-R®-II).[16] The Oka/Merck strain of live attenuated varicella virus is propagated in the MRC-5 human diploid cell line (Verivax®).[17] Neither of the package inserts for the above vaccines indicate the viral isolation and purification process in their product manufacturing, though the Verivax® package insert indicates that there may be residual amounts MRC-5 cellular DNA and protein present in the vaccine. A survey of the package inserts of FDA licensed vaccines currently approved for use in the United States indicates that many vaccines manufactured for use against viral infection contain residual protein from culture media. See Table 8 in Appendix A, for details on such viral vaccine residual propagation media protein contaminants.

Role of Vaccines in HLA Sensitization

The detection of WI-38 and MRC-5 derived HLA protein in vaccine preparation would indicate that these vaccines manufactured using such cell lines may be potential stimuli for immune sensitization to foreign HLA proteins. Development of anti-HLA antibodies specific to the allotypes present on the WI-38 and MRC-5 cell lines may have a clinically significant impact on histocompatibility between renal graft recipients and their potential donors. In the process of anti-HLA antibody identification, histocompatibility laboratories often rely on historical HLA antigen exposure information to appropriately identify and predict the clinical significance of anti-HLA antibodies in their relation of renal graft outcome.

Another potential source of sensitizing HLA protein in vaccines may be presented by the virus itself. One review has identified a variety of viruses that have been documented to embed

host protein molecules into their own viral envelopes.[38] HLA Class I and Class II proteins are included in this list. More specifically, host HLA- DR has been documented to be incorporated into the envelopes of the Epstein Barr virus (EBV), HIV-1, and HIV-2. Though theories exist, the mechanism for how such protein incorporation occurs in unclear. One could speculate that due to the fact that the rubella virus is an enveloped virus, it may also have the potential to incorporate host proteins into its viral envelope.[39] In addition to the more traditional routes of HLA sensitization, incorporation of host HLA Class II proteins into the rubella viral envelope could potentially serve as another source of HLA-DR sensitization.

Protein Identification

There are a variety of methods that are utilized to identify proteins in biological substances. Often, such proteins exist in cell lysates or other biological solutions in low amounts relative to other proteins. This presents a significant challenge in the identification of these low abundant proteins due to the dynamic range of mass spectrometers. Steps that can be taken to decrease the interference of the highly abundant interfering proteins include a variety of methods which may be used in combination to achieve substantial improvements in the detection of such proteins. Such approaches include immunoaffinity purification, separation by one or two dimensional polyacrylamide gel electrophoresis, or fractionation by liquid chromatography.[40,41] These isolation methods can also be used in combination with a variety of identification methods.

Immunoaffinity Purification

Immunoaffinity purification of proteins is a well-established method of protein purification and concentration. Since it is compatible with large volumes, it can be used as a preliminary step to enhance the identification of low abundance proteins in solution. The principle of this technique is that an antibody specific for the antigen is incubated with a sample containing the protein of interest. Typically, antibodies used for immunoaffinity purification are immobilized to a bead or column support to facilitate recovery of the complex. Immobilization of antibody can be by Protein A or Protein G binding or by directly crosslinking the antibody to the support structure.[42] Protein A and G are microbial proteins that have the ability to non-covalently bind antibody Fc portions with various intensities based on species and antibody subclass.[43] Following the initial Fc receptor binding step, antibodies can be covalently bound to beaded supports or columns by covalent crosslinking if desired. Direct immobilization of antibody is also an option for immobilizing the antibody to a support. There are advantages and disadvantages to each immobilization method. While Protein A or Protein G antibody immobilization results in the ideal orientation and availability of the Fab portions to react with the antigen, there tend to be problems with co-elution of antibody. These proteins will be digested with trypsin and can complicate protein identification. On the other hand, in direct covalent immobilization methods, the antibody Fab portions may not be oriented for the most efficient antigen interaction, but elution of antibody is reduced and the antibodies are relatively resistant to trypsin digestion which aids in protein identification.

Following antibody adsorption, antigen can be released from the antibody by low or high pH or by high salt concentration. For example, an immunoaffinity assay facilitates the isolation

and concentration of a protein of low abundance in a cell lysate solution. When setting up such a reaction, after an optimized incubation period, the cell lysate solution is washed away and the protein of interest is retained by the immobilized antibody. The isolated antigen can be eluted from the bead or column support into controlled volume by altering pH or salt conditions and breaking the non-covalent bonds that maintained the antibody and antigen complex.

Once the protein of interest is isolated, protein identification is more likely. This is due to the reduced complexity and dynamic range of the sample. Other methods of protein identification include Edman degradation or mass spectrometry. Another commonly utilized method is western blot, which identifies proteins through the use of antigen recognition by antibodies of known specificity. The western blot protein identification method is more appropriately utilized in highly sensitive, smaller scale protein identification experiments. The Edman degradation method is now only used for very specific applications where it is important to determine the N-terminal of the protein. Mass spectrometry is regarded as the most desirable method for large scale, high throughput protein identification and analysis.

Western Blot

The western blot method, otherwise known as protein blot or immunoblot, is a technique that is commonly used in the biological laboratory to identify proteins using antibodies of known specificity.[42,44,45] Initially, a sample is mixed with a reducing agent such as 2-mercaptoethanol or dithiothreitol (DTT), and is loaded onto a denaturing polyacrylamide gel. Sodium dodecyl sulfate (SDS) is commonly used as the denaturing agent in the sample loading buffer and in the polyacrylamide gel. SDS produces a denaturing effect of the proteins it binds.[42,44-46] This interaction confers an overall negative charge to the protein in proportion to its length. As a

result, the effect of charge on migration is eliminated, leaving the molecular weight of the protein as the primary factor in protein migration. In addition to enhancing and standardizing migration, reducing and denaturing may enhance the availability of the protein epitopes to the antibodies. Conversely, reducing and denaturing proteins in an SDS-PAGE system has the potential to destroy epitopes composed of non-linear sequences of peptide. Once the desired resolution in the first dimension has been achieved, the separated sample proteins within the gel are electrophoretically transferred to a nitrocellulose or polyvinylidene fluoride (PDVF) membrane. Alternatively, separation the 2^{nd} dimension prior to transfer to a membrane may be necessary in order to adequately resolve co-migrating proteins (of similar molecular weights) by their isoelectric point (pI).

Transfer of proteins from the polyacrylamide gel to nitrocellulose or PVDF membrane is efficiently completed by application of an electric field.[42,44,45,47] Though there are modifications in transfer apparatus and methods, the general premise is that negatively charged proteins will be transferred from a gel placed next to a membrane as they move towards the cathode in the presence of an electric field.

Upon the completion of protein transfer, the membrane is blocked to prevent non-specific background binding of antibodies. The necessity of blocking the membrane is warranted due to its high non-specific protein binding capacity, which may cause the undesirable degree of non-specific background due to antibody binding during the detection step. After blocking, the membrane is stained with primary antibody. Following the primary incubation and washing steps, the membrane is stained with labeled secondary antibody. Enzymatic and radiographic methods may be utilized for the detection of labeled antibody. Enzymatic methods using

chemiluminescent substrate are currently more favorable than radiographic detection methods. This is due to the safety and waste disposal concerns of radioactive materials. Ultimately, the protein of interest is identified and visualized based on the specificity of the primary antibody. The specificity of western blot is limited by the specificity of the primary antibody, as well as the degree of non-specific background binding by the secondary antibody. The sensitivity of this method is dependent on the detection method as well as degree of background interference. Enzymatic detection methods are known to detect up to picogram amounts of protein.[42,44,45]

Edman Degradation

The Edman degradation method of protein identification is based on N-terminal degradation.[48] A prerequisite for successful sequencing by this method is that the protein of interest cannot be N-terminally blocked. It has indicated that this may be a significant drawback in protein sequencing, due to the fact that up to 50% of eukaryotic are N-terminally blocked.[40] If this is the case, N-terminal sequencing cannot be utilized. Preparatory steps to isolate the protein of interest prior to sequencing include separation by gel electrophoresis and subsequent electrophoretic transfer to PDVF membrane. Following protein transfer to membrane, the protein of interest is be detected by staining with coomassie blue staining and visualization of banding at the estimated molecular weight. Detection of small amounts are dependent of the sensitivity of the coomassie blue stain, which is approximately 0.1 - 1 µg. This presents an obvious challenge in sequencing by the Edman degradation method, as many low abundant proteins will be present in amounts below the detectable range of the stain, limiting further analysis. If sufficient protein is present on the membrane, direct protein sequencing by Edman's method can proceed. The method is described by Edman is time consuming and usually takes

many hours. As mentioned above, the method is limited in the availability of the N-terminal peptide of the polypeptide sequence as well as the amount of protein present in the sample that can be successfully visualized by staining with coomassie blue stain.

Mass Spectrometry

Mass spectrometry (MS) is a robust method used to identify peptides and proteins. A bottom-up approach is commonly used when identifying proteins from a complex sample. The rationalization for this is due to its increased sensitivity over whole protein MS.[49] The bottom up approach relies on enzyme digestion of sample proteins prior to ionization. Trypsin is often the enzyme used for protein digestion due to its high cleavage specificity, ideal peptide length, and resulting C-terminally protonated amino acids that are amenable to ionization.[49,50]

The practical use of MS for peptide and protein identification became a commonly utilized tool with the development of soft ionization technology. Technology such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) facilitates the transfer of biomolecules from solid matrix or aqueous solution to the gas phase while maintaining structural integrity for downstream identification. The principle of MALDI is that a sample which is co-crystallized to organic matrix is ionized by the short pulse of a laser beam.[40] The effect of the pulsing laser on the matrix is that the proteins from the sample sublime into gas phase intact as they stream into the opening of the mass spectrometer as a result of high vacuum conditions.[51] Ionization by this method is appropriate for simple peptide mixtures.[49] See Figure 1 for an illustration of this concept.

Figure 1. Principle of MALDI

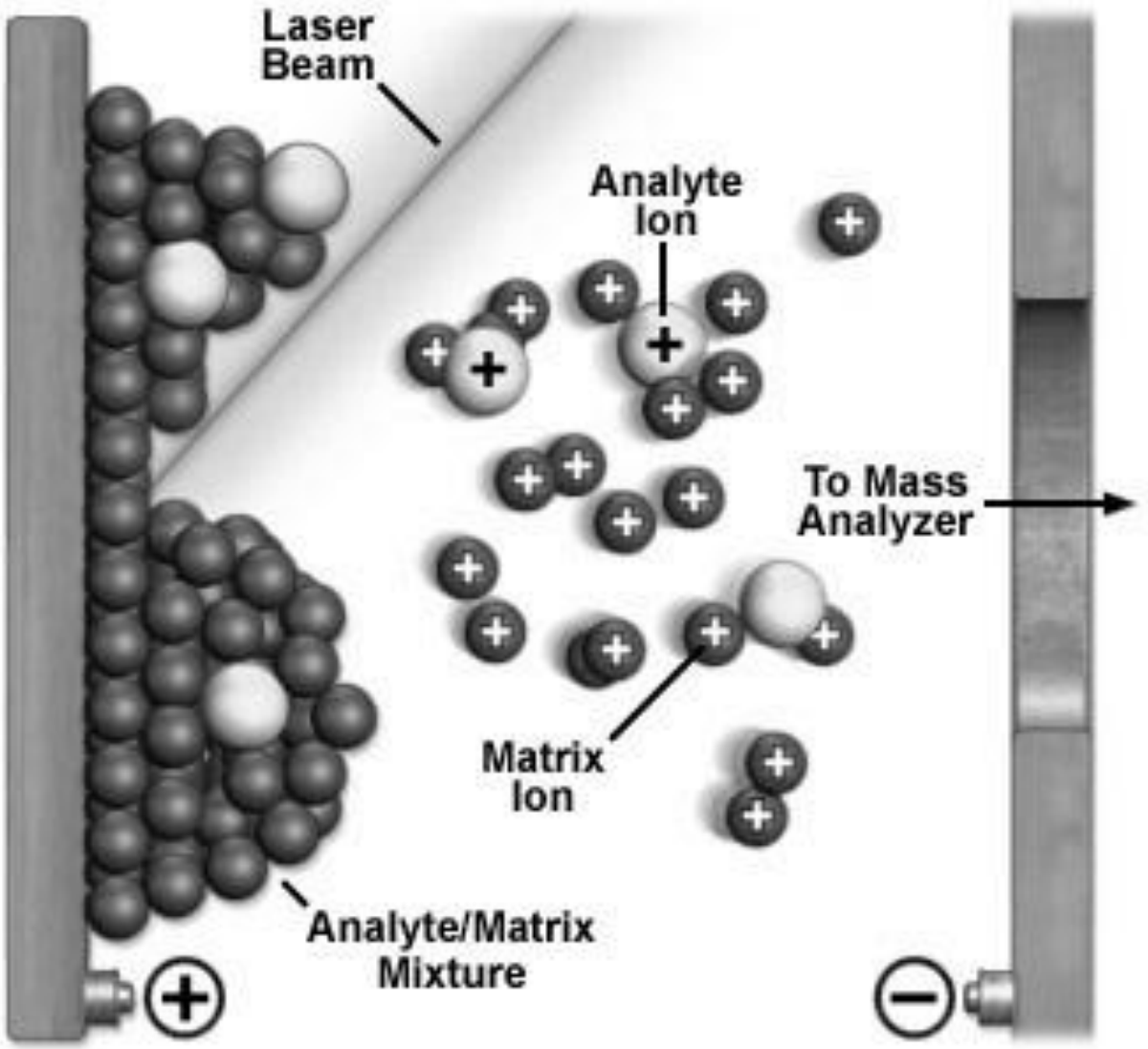

Figure 1. Principle of MALDI. The platform is coated with a mixture of the sample, depicted by the large white circles, and the matrix material, depicted by the small dark circles. As the laser pulses onto the sample matrix mixture, the sample and matrix are ionized. The matrix is formulated as a buffer between the laser and the sample so that is absorbs the energy from the laser and that the sample can be effectively ionized without being damaged. The diagram above is provided with the permission of the National High Magnetic Field Laboratory at Florida State University: http://www.magnet.fsu.edu/education/tutorials/tools/ionization_maldi.html.

ESI is the other commonly utilized soft ionization technology used for protein identification.[40,51] The principle of ESI is that the sample in the liquid state is ionized to the gas

phase by continuous low flow rate through a narrow capillary at atmospheric pressure. The tip of the capillary is held at very high potential of up to 5000 volts (V). Due to the high potential of the capillary tip, the fluid exits as a spray. Charge density accumulates on the shrinking droplet size. Due to Coulomb repulsion, the droplets sequentially decrease in size until the free analyte ions are released into the gas phase. This is referred to as a Taylor cone. Once in gas phase, ionized analytes are drawn to the interface of by the vacuum of the mass spectrometer. Ions enter the mass spectrometer via an orifice that is approximately 100 μm in diameter. See Figure 2 for an illustration of this concept.

Figure 2. Principle of ESI

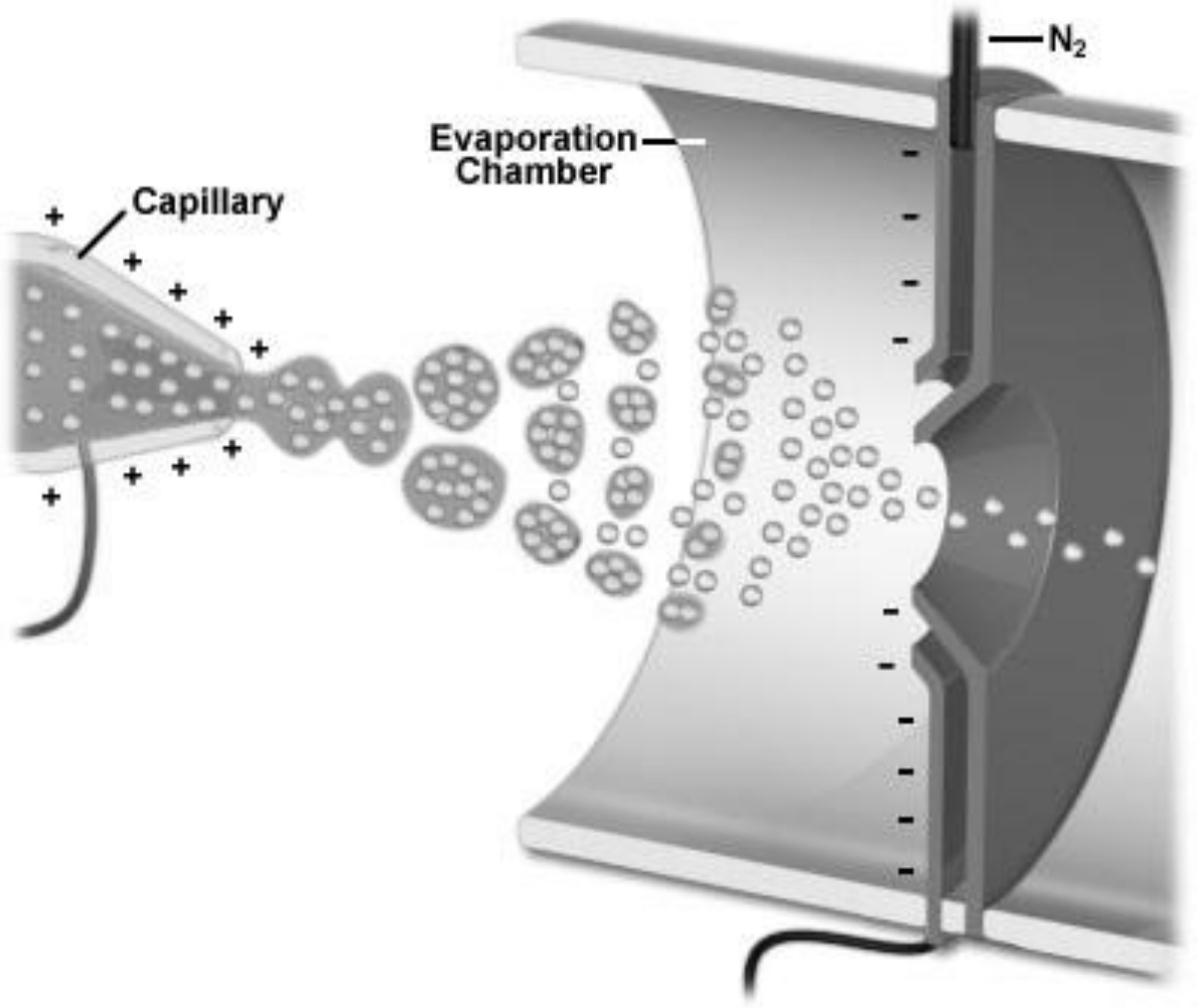

Figure 2. Principle of ESI. Liquid from the sample capillary enters the ionization chamber as a spray. As the liquid is ionized, droplets accumulate charge. Due to Coulomb repulsion, the droplets sequentially decrease in size until the free analyte ions are released into the gas phase. Once in gas phase, ionized analytes are drawn to the interface of by the vacuum of the mass spectrometer. The diagram above is provided with the permission of the National High Magnetic Field Laboratory at Florida State University: http://www.magnet.fsu.edu/education/tutorials/tools/ionization_esi.html.

Liquid chromatography is a separation technique that is especially well suited to ESI soft ionization.[49,52] This is due to of its ability to introduce highly resolved, complex samples at the site of ionization. Furthermore, when coupled to a tandem mass spectrometer platform, protein identification is possible in complex samples. This is referred to as liquid chromatography-

MS/MS (LC-MS/MS). It is the focus of this review because of its utilization as a method in this research project.

Mass analyzers play a central role in characteristics of the data obtained from the ionized sample and include sensitivity, resolution, mass accuracy, and the ability to obtain depth in peptide identification information.[49] A variety of mass analyzers exist, including ion trap, time of flight (TOF), quadrupole, and Fourier transform ion cyclotron (FT-MS). Each of these analyzers has their associated strengths and weaknesses depending on their application. Thus, the resolution, mass accuracy, sensitivity and dynamic range of the data obtained by a particular instrumentation configuration are also in part dependent on the performance of the mass analyzers utilized. In general, analyzers have analytic capabilities that are sensitive enough to detect and identify femtomole to attamole amounts of protein, with dynamic ranges of $1e^4$ to $1e^6$.[52]

When MS is utilized for protein identification by bottom-up peptide identification, mass analyzers may be configured in tandem in order to enhance identification properties. Initially, sample proteins are fractionated and concentrated by chromatography, immunoaffinity purification, or gel electrophoresis. Once the proteins of interest have been adequately isolated, the sample is digested with trypsin. The sample peptides may be further resolved by reverse phase liquid chromatography and directly introduced to the electrospray ion source. Upon ionization, the protonated peptides enter the first mass analyzer. The first mass analyzer selects a precursor ion by a computer generated peptide prioritization list. The selected precursor ion is further fragmented by collision with neutral gas atoms in a collision cell. Fragmented ions are further analyzed by the second mass spectrometer.[40,49] An alternative to this setup is separation

and fragmentation in an ion trap analyzer, where the fragment ion spectra are formed in time (not space, as described above). The resulting MS and MS/MS spectra are stored and compared to a database of theoretically obtained spectra. Theoretical candidate spectra can be limited based on user specific criteria such as mass tolerance, proteolytic enzyme constraint, and allowance for types of post translational modifications.[53]

Theoretical databases that are commonly used for identification or mass spectra are MASCOT® (Matrix Science Ltd., Boston, MA) and SEQUEST® (University of Washington, Seattle, WA), and X! Tandem© (The Global Proteome Machine Organization, available online: http://www.thegpm.org/tandem/). These programs utilize different protein identification mechanisms. The SEQUEST® database utilizes a heuristic algorithm, which correlates the acquired experimental MS/MS data with theoretical spectra and calculates a score of similarity between the two. This is referred to as the cross correlation score, or XCorr.[54] MASCOT® is a program that compares observed spectra with a database of theoretical spectra and assigns probabilities that a peptide has been observed. It is a program that is commonly used for identification of peptides and proteins by their mass spectra. Though the model is not available for peer review, MASCOT® database protein identification utilizes a MOWSE based probabilistic algorithm to identify proteins.[55,56] Proteins identified by MASCOT® have associated an Ions Score, which is based on the calculation of $-10*\log_{10}(p)$, in which the probability p is the probability that matching between the experimental and theoretical data is random. MASCOT® also reports an expectation value, the E-value, which is calculated either under the assumption that the database search follows an expected distribution or by the empirical fitting of the observed distribution of scores. The E-value is used to validate true

positive identifications versus stochastic identifications. It has been postulated that the probabilistic analysis of the protein identity scores combined from the same data using different algorithms leads to a lower rate of false positive identifications.[55,57]

Mass Spectrometry Data Validation

Theoretical database scoring programs such as SEQUEST® and MASCOT® can produce false negative and false positive assignment peptide assignments. Subsequently, peptide assignments require further validation. Validation of data by manual inspection is deemed too time consuming and requires high level of expertise when considering the large amounts of high complexity data generated in most mass spectrometry experiments. The availability of probability calculation tools serves an essential function in the protein identification process. Peptide Prophet**TM** and its incorporation into the Scaffold proteomic data validation program can be used to fulfill the peptide identification validation function.[58] In general, peptide identification by database searching is completed using a threshold model, which sorts identification results by a statistical score. The threshold for positive identification is dependent on the scoring scheme, for example, Xcorr (SEQUEST®) or P-value (MASCOT®), as well as different charge states, sample complexity, and database size. Identification based on threshold cutoffs does not take experimental variables into account. These variables include instrumentation, inter-algorithm comparison, and specificity and sensitivity tradeoffs. Peptide Prophet**TM** is an algorithm that was developed based on Bayes' law to address these issues and normalize the data. It facilitates the comparison of inter-algorithm data, such as the peptide

identity scores assigned by SEQUEST® or MASCOT®, by converting the scores into probabilities based on Bayes' Law. The equation for Bayes' Law is depicted in Figure 3.

Figure 3. Equation for Bayes' Law

$$p(+ \mid D) = \frac{p(D \mid +)\, p(+)}{p(D \mid +)\, p(+) + p(D \mid -)\, p(-)}$$

Figure 3. Equation for Bayes' Law, where Keller states, "D equals the observed data, and $p(+ \mid D)$ equals probability of correctly assigning a peptide to spectra. The factors $p(D \mid +)$ and $p(D \mid -)$ are the probabilities of an assigned peptide to an MS/MS spectrum having information D among correctly and incorrectly assigned peptides, respectively, and $p(+)$ and $p(-)$ are prior probabilities of a correct and incorrect peptide assignment, respectively".[57,58]

The algorithm utilized by Peptide Prophet is based on assessing the spectra of the entire sample, and models the results using two distributions, where the normal distribution is used for the incorrect, stochastic matches and an extreme value distribution models the correct matches. The probability of correctly assigning a peptide to a given spectra is addressed by calculating the ratio of the two distributions of a particular score. The value of normalizing the data is demonstrated by the ability to make inter-algorithm peptide assignments and protein identifications. The Scaffold program does this by comparing inter-algorithm data and utilizing Peptide Prophet**TM** to validate the data.[57]

Ultimately, the ability to identify a protein is dependent on spectral quality, which is affected by factors including ion abundance and detector sensitivity. Removing highly abundant proteins from the sample prior to digestion significantly enhances protein identification by MS because the relative amount of the protein of interest in increased. Identifying the optimal instrumentation set up for the MS application also optimizes protein identification potential.

Finally, the quality of databases used to identify peptide spectra and protein identification affect protein identification as the programs will not identify peptides not in the searched database.

Identification of HLA Class II Protein

HLA Class II proteins are heterodimers composed a 34 kDa α chain and a 29 kDa β chain. Three major isotypes of these proteins are known: namely, HLA-DR, -DQ, and -DP.

The beta chain of HLA-DR, -DQ, and -DP isotypes are known to have the majority of polymorphisms characteristic of the HLA Class II molecules. These are referred to as the HLA-DR, -DQ, and -DP antigens. The polymorphism of this antigenic system is known to be responsible for alloimmunization from blood transfusion, solid organ transplant, pregnancy, and vaccination. The polymorphisms of the HLA-DR, -DQ, and -DP molecules are routinely identified using genotyping and serologic methods. Theoretically, one could also use N-terminal sequencing, LC-MS/MS, or detection by western blot methods.

There are a variety of commercial anti-HLA Class II antibodies that available for protein detection by western blot. These include monoclonal antibodies specific for monomorphic antigenic determinants of intact or dissociated HLA Class II α and β chains.

In order to identify HLA Class II proteins by MS or N-terminal sequencing, it is necessary to know the variations in the protein sequences specific to the polymorphism of the β chains of the HLA-DR, -DQ and -DP molecules. The polypeptide sequences of the corresponding alpha chains demonstrate polymorphism to a much lesser extent. Such protein sequences can be found in a variety of databases, including the National Center for Biotechnology (NCBI), UniProt, and IMGT/HLA.[19,59-61]

When utilizing MS for protein identification, tryptic cleavage sites should also be taken into consideration. The prediction of such sites allow for pre-analytic inference on the potential diagnostic fragments created by proteolytic digestion. Peptide Cutter is a web based program that is used to determine if a suitable peptide for LC-MS/MS is available for the polymorphism to be detected. The majority of rules used by the program have been adapted from work on the specificity of proteolysis.[62] Output information includes the resulting enzyme cut sites, peptide sequences, molecular masses, amino acid lengths, and cleavage probabilities. This information may be extremely valuable when applied to the HLA Class I and II protein identification by MS. This is because prior to experimental setup, it would be worthwhile to determine whether the assay would be able to detect a unique polymorphism or not. If not, it would be more efficient to proceed with an alternative protein identification method.

Again, the Peptide Cutter program could be run prior to experimental identification of HLA proteins in order to estimate the degree of specificity that could be achieved by trypsin digestion and subsequent analysis by MS assay. Refer to Table 9 of Appendix A for an example of an in silico HLA Class II protein digestion by the Peptide Cutter program.

In addition to the specific diagnostic potential of Class II proteins based on tryptic digest, other factors that introduce variability in protein identification capacity by MS. These include relative abundance, background noise and instrument dependent factors such as dynamic range, and limit of detection. Often, the protein of interest is concentrated by immunoaffinity purification to remove inferring proteins that may be much more abundant than the protein of interest and reduces the complexity of fragment ion spectra. Instrument dependent performance characteristics also impact protein identification ability.

Specific Aim

The specific aim of this project was to confirm our hypothesis that the patient discussed in the clinical case study demonstrated unexpected sensitization to HLA-DR11 antigen due to contamination of the vaccine with HLA protein. The patient's case history revealed that the patient had received a dose of the Proquad® vaccine during this time period. Based on available vaccine product inserts, the rubella and varicella components of Proquad® are presumed to have been propagated in human cells lines. A review of vaccine product inserts has indicated that there may be carryover of proteins from the propagation media to the final vaccine product as demonstrated in Table 8 of Appendix A.

In support of the specific aim this project, an attempt was made to identify the presence of the sensitizing protein, HLA-DR11, or less specifically, HLA-DR, in vaccine preparations similar to the one administered to the patient using immunoaffinity, western blot, and mass spectrometry methods. The detection and identification of such a protein in a vaccine preparation would implicate vaccination as a potential source of clinically significant sensitization to HLA antigens.

RESEARCH OBJECTIVE

Research Objective

The research aim of this project is to detect the presence of human HLA-DR protein in the M-M-R®-II vaccine using immunoaffinity, mass spectrometry, and western blot methods to obtain support for hypothesis that the presence of HLA proteins in therapeutic vaccines may contribute to the development of anti-HLA antibodies in humans.

MATERIALS

Reagents

Unless otherwise noted, all chemicals were purchased from University Stores at Michigan State University. All water used was doubly distilled in the MSU HLA Laboratory.

Antibodies

The antibodies used for this project are described in Table 3.

Table 3. Antibody Characteristics

Antibody Specificity	Host	Clonality	Clone	Target	Intended Application	Source
HLA-DR	mouse	monoclonal	L243	human HLA-DR	immunoaffinity, western blot, etc.	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)
HLA-DR	mouse	monoclonal	B8.12.2	human HLA-DR	immunoaffinity, western blot, etc.	Santa Cruz Biotechnology, Inc.
HLA-DR	rat	monoclonal	YE2/36	human HLA-DR	immunoaffinty, western blot, etc.	Santa Cruz Biotechnology, Inc.
HLA-Class I	rat	monoclonal	YTH862.2	human HLA-Class I	immunoaffinty, western blot, etc.	Santa Cruz Biotechnology, Inc.
HLA-DRB1	rabbit	polyclonal	not applicable	human HLA-DRB1	ELISA, western blot	Abcam® (Cambridge, MA)
IgG-horseradish peroxidase (HRP)	goat	not indicated	not indicated	Mouse IgG	western blot	Santa Cruz Biotechnology, Inc.
HLA-DR11	mouse	monoclonal	not indicated	human HLA-DR11	cytotoxicity	One Lambda, Inc.

Cell Lines

The WI-38 and MRC-5 cell lines are cultured human fibroblasts purchased from American Type Culture Collection.

Protein Controls

Protein preparations containing HLA-DR 11 protein were generously donated by One Lambda, Inc. and Gen-Probe, Inc. (San Diego, CA). The protein preparation donated by One Lambda, Inc. is an antigen preparation produced from a B-cell line. Its HLA antigen profile is DR11,15, DRw51,52, DQ6,7, and DP4, X. It was documented to be composed of the entire HLA Class II protein, including the alpha and beta subunits. The transmembrane region, and cytoplasmic tail of the HLA proteins were also present in this preparation. Its estimated protein concentration is estimated to be 553 µg/ml.

The protein preparation donated by Gen-Probe, Inc. was documented to have been a cell lysate obtained from cells expressing a recombinant protein of a DRB1*11:01 allele. This recombinant HLA protein contains the alpha and beta subunits. Neither the transmembrane nor the cytoplasmic regions that would be found in a naturally occurring protein were not present as part of the protein structure in this preparation. The estimated concentration of the protein in the preparation was 10 µg/ml.

Additional positive controls were utilized for portions of this project included a Ramos whole cell immunoprecipitation (IP) control lysate (Santa Cruz Biotechnology, Inc.), a Daudi cell lysate (Santa Cruz Biotechnoloy, Inc.), and an HLA-DR positive lysate control developed and utilized in the MSU HLA Laboratory.

Documentation

Images of protein analysis results were obtained using the HP® Scanjet 5200C (Hewlett-Packard Company, Palo Alto, CA).Electronic images were edited using Paint.Net™ free software for digital photo editing (Website: http://www.getpaint.net/index.html).

METHODS

DNA Extraction

Genomic DNA was extracted from the WI-38 and MRC-5 cells lines initially using the EZ1® DNA Blood 350µl Kit on the Biorobot EZ1® Workstation (Qiagen, Valencia, CA). Due to low yield, genomic DNA was further extracted by more efficient methods, either the QIAprep® Spin Miniprep Kit (Qiagen) or by the Gentra® Purgene® Blood Kit (Qiagen). The extracted genomic DNA was quantified by measuring its absorbance at 260 nm. Its purity was evaluated by its measured absorbance at 260 nm, divided by its absorbance at 280 nm. Absorbance measurements were completed using the Genequant™ Spectrophotometer (GE Healthcare, Pittsburg, PA).

Genotyping

The WI-38 and MRC-5 cell lines were genotyped using molecular methods. Genotyping of the HLA-A, -B, -C, -DRB1, -DRB3/4/5, - DQA1, -DQB1, and -MICA loci was completed using LABType® SSO kits (One Lambda, Inc.) according to manufacturer's instructions. Hybridized products were detected using a Luminex® 100™ instrument (Luminex Corporation, Austin TX). Genotyping of the HLA-DPB1 locus of each of the WI-38 and MRC-5 cell lines was completed using the Pel-Freez® DPB1 kit (Dynal, as part of Invitrogen Corporation, Brown Deer, WI) and documented with Polaroid 667 Film (Polaroid, Minnetonka, MN).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Protein separation by molecular weight was completed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-PROTEAN® gel cassettes and

electrophoresis apparatuses (Bio-Rad Laboratories, Inc., Hercules, CA). The 12% resolving gel was prepared by combining 2.1 ml water, 4 ml of 30% acrylamide/bis 29:1 (Bio-Rad Laboratories, Inc.), and 3.75 ml 1M Tris (pH 8.8). The solution was de-gassed for approximately 10 minutes at which time .05 ml of 20% SDS, .100 ml of 10% ammonium persulfate (APS), and .004 ml of tetramethylethylenediamine (TEMED) were added, gently swirled within the container, and poured into the gel cast using a plastic transfer pipette. The polyacrylamide gel was overlaid with butanol to prevent a smiling effect.

The 3.4% stacking polyacryalamide gel was prepared by combining 11.350 ml water water, 1.75 ml of 30% acrylamide/bis 29:1 (Bio-Rad Laboratories, Inc.), and 1.9 ml 1M Tris (pH 6.8). The solution was de-gassed for approximately 10 minutes, at which time .075 ml of 20% SDS, .075 ml of 10% APS, and .015 ml of TEMED were added, gently swirled within the container and poured into the gel cast upon the polymerized resolving gel using a plastic transfer pipette.

Samples were loaded on to SDS-PAGE gel using appropriate volumes of 1X or 5X sample loading buffer. Depending on the aim of the analysis, the samples were reduced or non-reduced, and boiled or not boiled. See Appendix B for composition of the 1X and 5X SDS-PAGE sample loading buffers.

SDS-PAGE gels were run using SDS-PAGE running buffer at 60 V through the stacking gel and 120 V through the resolving gel until the Kaleidoscope Prestained Standard (Bio-Rad Laboratories, Inc.) were resolved appropriately. The Kaleidoscope Prestained Standard are referred as molecular weight marker throughout the rest of this document. See Appendix B for composition of SDS-PAGE running buffer.

Coomassie Blue R-250 Stain

Protein SDS-PAGE gels were fixed and stained using **c**oomassie blue R-250 Stain. Staining was completed with rotation overnight. See Appendix B for composition of coomassie blue R-250 Stain. Gels were destained until the background was adequately diminished and appropriate signal had been achieved. See Appendix B for composition of coomassie blue destain.

Coomassie Blue G-250 Stain

In some cases, protein SDS-PAGE gels were stained using coomassie blue G-250 Stain to enhance sensitivity. Staining was completed using EZBlue^{TM} Gel Staining Reagent (Sigma, St. Louis, MO) according to manufacturer's instructions.

Silver Stain

SDS-PAGE gels were silver stained using a modified method.[63] To prevent contamination of the samples with exogenous proteins, nitrile glove were worn while performing the protocol. Incubations were completed at room temperature with rotation on an American Rotator V R4140 (American Dade, Miami, FL) at the indicated rotations per minute (RPM). Plastic solution containers were prepared by wiping down with acetone and 70% ethanol. SDS-PAGE gels that had been previously stained with coomassie blue R-250 stain and stored in destain solution could be used in this protocol. SDS-PAGE gels that had been fixed for hour in a 45% methanol, 10% acetic acid solution could also be used. All solutions were made directly prior to use. The silver staining of an SDS-PAGE gel was initiated by incubating the gel in 30% ethanol for 30 minutes, followed by two 10 minute incubations in 20% ethanol and two 10

minute incubations in water at 40-50 RPMs. The rinsed gel was sequentially incubated at 60-80 RMPs for 1 minute each in separate containers of 0.8 millimolar (mM) sodium thiosulfate, and twice in water. Following the described steps, the gel was incubated in the dark at 60-80 RPMs for 60-75 minutes in 12 mM silver nitrate. Prior to development using a solution of 3% sodium carbonate and 0.06% formaldehyde, the gel was rapidly rinsed three times with water and three times with above development solution. Following the rinses, the gel was incubated in development solution with rotation at 60-80 RPMs until the desired intensity of the bands was reached. Development was stopped by addition of approximately 5 ml of 2.3 M citric acid.

Direct Immunoaffinity

A direct immunoaffinity reaction utilizing Dynabeads® Protein G (Invitrogen, Carlsbad, CA) was completed according to manufacturer's instructions. An exception to the manufacturer's protocol was that antibody coupling procedure was prolonged from the suggested 10 minutes to 10 to 30 minutes in some cases. In most cases, 5-10 µg of antibody were coupled to 1500 µg for each IP. This utilized 50 µl of re-suspended beads per each immunoaffinity reaction. Incubations were completed with rotation at 220 RPM on the Lab-Line® Rotator Model 2314. The antibody and antigen were co-eluted from the Dynabeads® Protein using 20 µl of 50mM glycine, pH 2.8, with rotation for 4 minutes. The eluate was immediately brought back a neutral pH using 1M Tris, pH 7.5, confirmed by spotting 0.4 µl of neutralized eluate onto pH paper.

Crosslinking

Crosslinking of the antibody to the Dynabeads® Protein G was completed according to the manufacturer's recommendations found at www.invitrogen.com/crosslinking. The

crosslinking reagent used was Bis(sulfosuccinimidyl)suberate (BS3) (Thermo Fisher Scientific Inc., Pittsburg, PA).

Indirect Immunoaffinity

An indirect immunoaffinity reaction utilizing Dynabeads® Protein G was completed by incubating the antibody and antigen overnight with tilting at approximately 4 °C. This step, known at the indirect approach, was completed prior to adsorption of antibody to the Dynabeads® Protein G because modification has been demonstrated to increase capture of low affinity antigens to the antibody as compared to traditional immunoaffinity method described above. Subsequent incubation with the Dynabeads® Protein G was completed at room temperature for approximately 75 minutes with rotation at 220 RPM on the Lab-Line® rotator model 2314. Beads were washed according to the manufacture's recommendations. The antibody and antigen were co-eluted from the Dynabeads® Protein using 20 µl of 50 mM glycine, pH 2.8, with rotation for 4 minutes. The eluate was immediately brought back a neutral pH using 1M Tris, pH 7.5, confirmed by spotting 0.4 µl of neutralized eluate onto pH paper.

Gelatin and Bovine Serum Albumin Removal

Gelatin and bovine serum albumin (BSA) removal from antibody preparations was completed using the Pierce Antibody Clean-up Kit (Thermo Fisher Scientific, Inc.). The manufacturer's instructions were followed when utilizing the Anti-HLA-DR (L243) antibody. When removing gelatin from the anti-HLA-DR (B8.12.2) antibody, addition of 0.05 M sodium chloride to the Melon^{TM} Gel Purification Buffer was used to optimize antibody elution and reduction of contaminating gelatin.

In an additional application, gelatin was removed from the M-M-R®-II vaccine preparation in three sequential incubations on the Pierce Melon Gel from the Pierce Antibody Clean-up Kit. The processed vaccine preparation was concentrated in volume using the Amicon® Ultra-0.5 Centrifugal Filter Device (VWR International, LLC, Radnor, PA). Samples were processed according to the manufacturer's instructions so that the volume of the concentrated sample would be less than 30 µl.

Antibody Immobilization Immunoaffinity

An immobilized antibody immunoaffinity reaction was completed using the Pierce® Direct IP Kit (Thermo Fisher Scientific, Inc.). Coupling the antibody to the AminoLink Plus Coupling Resin was completed following the manufacturer's protocol. The coupling incubation was completed at room temperature with rotation at 220 RPMs on the Lab-Line® Rotator Model 2314 for 120 minutes. When not directly utilized, coupled resin was stored according the manufacturer's instructions, at 4°C in 1X Coupling Buffer with 0.02% sodium azide.

Antigen immunoaffinity purification was accomplished using the manufacturer's protocol. The antigen and antibody coupled AminoLink Plus Coupling Resin was incubated overnight at 4°C. Protease inhibitors were added to each test and control reactions and included 10 µl each of phenylmethanesulfonylfluoride (PMSF), protease inhibitor cocktail, and sodium orthovanadate (Santa Cruz Biotechnology, Inc.) per each milliliter of reaction. Antigen was eluted from the AminoLink Plus Coupling Resin according to the manufacturer's instructions, with the exception that of 5µl of 1M Tris pH 8.8 instead of 1M Tris pH 9.5 was used to neutralize the elution buffer in the collection tube. The pH of the eluted solution was determined by spotting 0.4 µl of solution onto pH paper.

Eluted antigen samples were concentrated by centrifugation at ambient temperature in the SC110 Speed Vac® (Thermo Fisher Scientific, Inc.) for approximately 7.5-10 hours and refrigerated or frozen until use.

Amicon® Concentration

In some instances, samples contained protein of low abundance in a volume that exceeded that which could be loaded into a well of an SDS-PAGE gel. In these cases, the sample volume was decreased in volume using the Amicon® Ultra-0.5 Centrifugal Filter Device (VWR International, LLC.). Samples were processed according to the manufacturer's instructions so that the volume of the concentrated sample was less than 30 µl. In some cases following concentration centrifugation, a reverse spin was also carried out according to the manufacturer's instructions. This step was completed in order to reestablish the expected ionic concentration of the sample.

Dialysis

Dialysis of a solution containing a protein of interest was completed using 12-14 kDa molecular weight cutoff dialysis tubing incubated in phosphate buffered saline (PBS) with constant stirring overnight at 4 °C overnight.

Mass Spectrometry

Samples were analyzed by mass spectrometry at the Michigan State University Research Technology Support Facility. In some cases samples for mass spectrometry analysis were prepared using portions of polyacrylamide gels. Gel bands were subjected to in-gel tryptic digestion.[64] The extracted peptides were separated using a Waters® nanoAcquity using

MICHROM Bioresources (Michrom Bioresources, Inc., Auburn, CA) 0.1 x 150mm column packed with 3 µm, 200A Magic C18AQ material. Chromatography was for 35 minutes with a gradient of 2% B to 35% B in 21 min using a (Buffer A = 99.9% water/0.1% formic acid, Buffer B = 99.9% acetonitrile/0.1% formic Acid) with an initial flow rate of 1 µl /minute.

Eluted peptides were sprayed into a Thermo Fisher LTQ Linear Ion trap mass spectrometer (Thermo Fisher Scientific Inc., Pittsburg, PA) equipped with a MICHROM Bioresources ADVANCE nano-spray source (MICHROM Bioresources, Inc., Auburn CA). The top five ions in each survey scan were subjected to data-dependent zoom scans followed by low energy collision induced dissociation (CID) and the resulting MS/MS spectra were converted to peak lists using BioWorks Browser v 3.3.1 (Thermo Fisher Scientific, Inc.), using the default LTQ instrument parameters. Peak lists were searched against both human protein sequences downloaded from SwissProt and viral protein sequences downloaded from NCBI using the MASCOT® searching algorithm, v2.3. The MASCOT® output was analyzed using Scaffold v3.0.5 (www.proteomesoftware.com) to probabilistically validate protein identifications using the ProteinProphet™ computer algorithm.[58] Assignments validated above the Scaffold 95% confidence filter were considered true.

MASCOT® parameters for all databases included allowance of up to 2 missed tryptic sites, fixed modification of carbamidomethyl cysteine, variable modification of oxidation of methionine, phosphorylation of serine and threonine, a peptide tolerance of +/- 200ppm, MS/MS tolerance of 0.6 Da (Dalton), and a peptide charge state limited to +2/+3.

Samples for mass spectrometry analysis were also prepared by on-bead enzyme digestion of antibody-antigen complexes. Antibody-bound proteins were digested on-bead by washing the

beads 5 times with 50mM ammonium bicarbonate. Trypsin, in the same buffer, was added to the beads at 5ng/µl so that the beads were just submerged in digestion buffer and allowed to incubate for 6 hours. The solution was acidified by adjustment using 1% trifluoroacetic acid. The acidified solution was centrifuged briefly at 14,000 RCF to remove insoluble residue. The supernatant was removed and concentrated by solid phase extraction. Purified peptides were re-suspended in 2% acetonitrile/0.1% trifluoroacetic to 20 µl.

The extracted peptides were re-suspended in a solution of 2% acetonitrile/0.1% trifluoroacetic acid to 20 µl. From this 10 µl were automatically injected by a Waters® nanoAcquity and loaded for 5 minutes onto a Waters® Symmetry C18 peptide trap (5 µm, 180 µm x 20 mm) at 4 µl/minute in 2% acetonitrile /0.1%formic acid. The bound peptides were eluted using a Waters® nanoAcquity UPLC (Buffer A = 99.9% Water/0.1% formic acid, Buffer B = 99.9% acetonitrile/0.1% formic acid) onto a Michrom MAGIC C18AQ column (3 µm, 200A, 100 µm x 150 mm) and eluted for 35 minutes with a gradient of 2% B to 35% B in 21min at a flow rate of 1 µl/minute. Eluted peptides were sprayed into a Thermo Fisher LTQ-FT Ultra mass spectrometer (Thermo Fisher Scientific Inc., Pittsburg, PA) using a MICHROM BioresourcesADVANCE nanospray source. Survey scans were taken in the FT (25000 resolution determined at m/z 400) and the top ten ions in each survey scan were subjected to automatic low energy collision induced dissociation (CID) in the Thermo Fisher LTQ-FT Ultra mass spectrometer. The resulting MS/MS spectra were converted to peak lists using Bio Works Browser v3.3.1 using the default parameters and searched against the SwissProt human database and viral protein sequences downloaded from NCBI, using the MASCOT® searching algorithm, v 2.3. The MASCOT® output was analyzed using Scaffold (www.proteomesoftware.com) to probabilistically validate protein identifications using the ProteinProphet™ computer

algorithm.[58] Assignments validated above the Scaffold 95% confidence filter were considered true.

MASCOT® parameters for all databases included allowance of up to 2 missed tryptic sites, fixed modification of carbamidomethyl cysteine, variable modification of oxidation of methionine, a peptide tolerance of +/- 10 ppm, and a MS/MS tolerance of 0.6 Da.

Transfer of Proteins to Nitrocellulose Membrane

Proteins were transferred from SDS-PAGE gels to 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories, Inc.) at 100 milliamps (mA) for 75 minutes. See Appendix B for transfer buffer composition. Following protein transfer, the membrane was blocked in blocking buffer overnight at 4°C. See Appendix B for composition of blocking buffer.

Western Blot

The blocked nitrocellulose membrane was directly transferred to incubation buffer with primary incubation in 0.5% anti-HLA-DR (clone L243) or anti-HLA-DR (Clone B8.12.12) as indicated. See Appendix B for composition of incubation buffer. Incubation with the primary antibody was completed at room temperature, at 65 RPM on the S/P **TM** Rotator V (Baxter Scientific Products, McGaw Park, IL), for 60 minutes, with manual mixing every 10-15 minutes. Following the primary incubation, the membrane was three times for five minutes per wash in approximately 100 ml of wash buffer. See Appendix B for composition of wash buffer.

The membrane was incubated with the secondary antibody, goat anti-mouse IgG-HRP, at a dilution of 0.05% in the incubation buffer at 65 RPM on the S/P**TM** Rotator V for 60 minutes,

with manual mixing every 10-15 minutes as indicated. The membrane was washed was three times for five minutes per wash in approximately 100 ml of wash buffer. Following the final wash, excess wash buffer was drained from the membrane. The membrane was overlaid with 2 ml of working PicoMax® Sensitive Chemiluminescent HRP Substrate (Rockland Immunochemicals, Inc., Gilbertsville, PA) for one minute. The membrane was drained of substrate and exposed to Kodak BioMax® Light Autoradiography Film (Carestream Health, Rochester, NY). The film was developed and fixed in the manufacturer's recommended working dilution of Kodak GBX® Developer and Fixer (Carestream Health, Rochester, NY) in a dark room and exposed for various increments of time as applicable. Processed film was documented electronically using the HP® Scanjet 5200C.

RESULTS

Genotyping of Human Cell Lines

A variety of viruses used in therapeutic vaccination are propagated in human cell lines. For example, the rubella component of the M-M-R®-II vaccine is propagated in the human fibroblast cell line WI-38. Varicella virus utilized for therapeutic vaccination in the Varivax® vaccine is propagated in the human diploid cell line MRC-5. The HLA genotyping results of the WI-38 and MRC-5 cell lines are presented in Table 4. The HLA low resolution genotypes are reported according to the nomenclature standards of the field.[65]

Table 4. HLA Genotype of Human Cell Lines

HLA Locus	Cell Line	
	WI-38	**MRC-5**
A*	02, 68	02, 29
B*	08, 58	07, 44
C*	07, XX	05, 07
DRB1*	11, 13	04, 15
DRB3/4/5*	DRB3*02, DRB3*03	DRB4*01, DRB5*01
DQA1*	03, 04	01, 03
DQB1*	03, 06	03, 06
DPB1*	03, 04	04, XX
MICA*	008, 002 (or 020)	008, XX (or 027)

Table 4. HLA genotype of human cell lines.

Flow Chart of Assay Development

The flow chart in Figure 4 includes broad descriptions of the experimental set-up of the results depicted by the figures and tables presented in the Results section.

Figure 4. Flow Chart of Assay Development

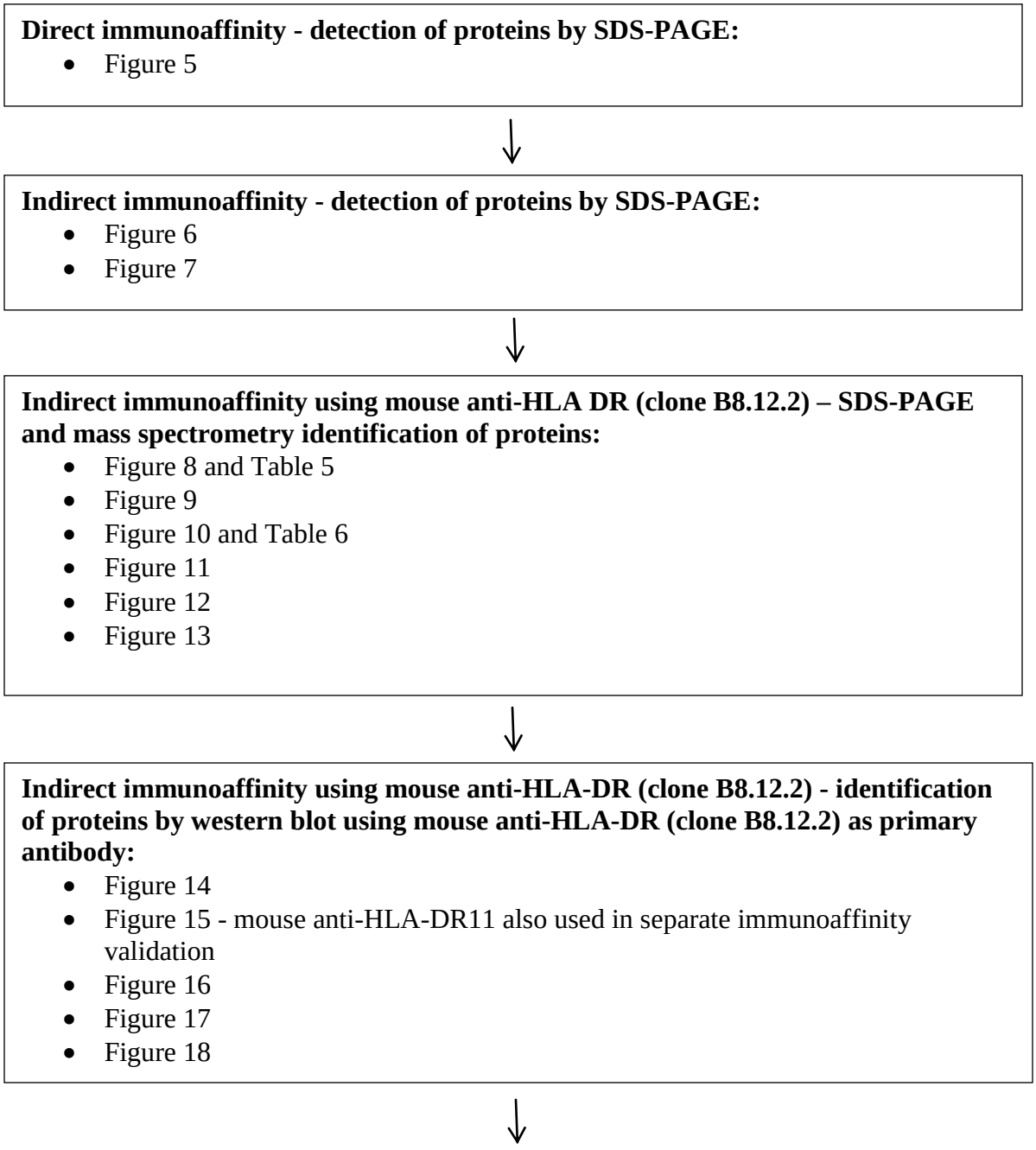

Figure 4 (cont'd)

Crosslinked immunoaffinity using mouse anti-HLA-DR (clones B8.12.2 and L243) - identification of proteins by western blot using mouse anti-HLA-DR (clone B8.12.2) as primary antibody:

- Figure 19

↓

Indirect immunoaffinity using rat anti-HLA-DR - identification of proteins by western blot using mouse anti-HLA-DR (clone B8.12.2) as primary antibody:

- Figure 20
- Figure 21
- Figure 22
- Figure 28

↓

Indirect immunoaffinity using rabbit anti-HLA-DRB1 - identification of proteins by western blot using mouse anti-HLA-DR (clone B8.12.2) as primary antibody:

- Figure 23
- Figure 27

↓

Indirect immunoaffinity using mouse anti-HLA-DR (clone B8.12.2) versus rat anti-HLA-DR versus rabbit anti-HLA-DRB1- validation of ramos whole cell lysate as positive control. Identification of proteins by western blot using mouse anti-HLA-DR (clone B8.12.2) as primary antibody:

- Figure 24

↓

Validation of western blot positive controls - effect of boiling on identification of proteins by western blot using mouse anti-HLA-DR (clone B8.12.2) versus mouse anti-HLA-DR (clone L243) as primary antibody:

- Figure 25
- Figure 26

↓

Figure 4 (cont'd)

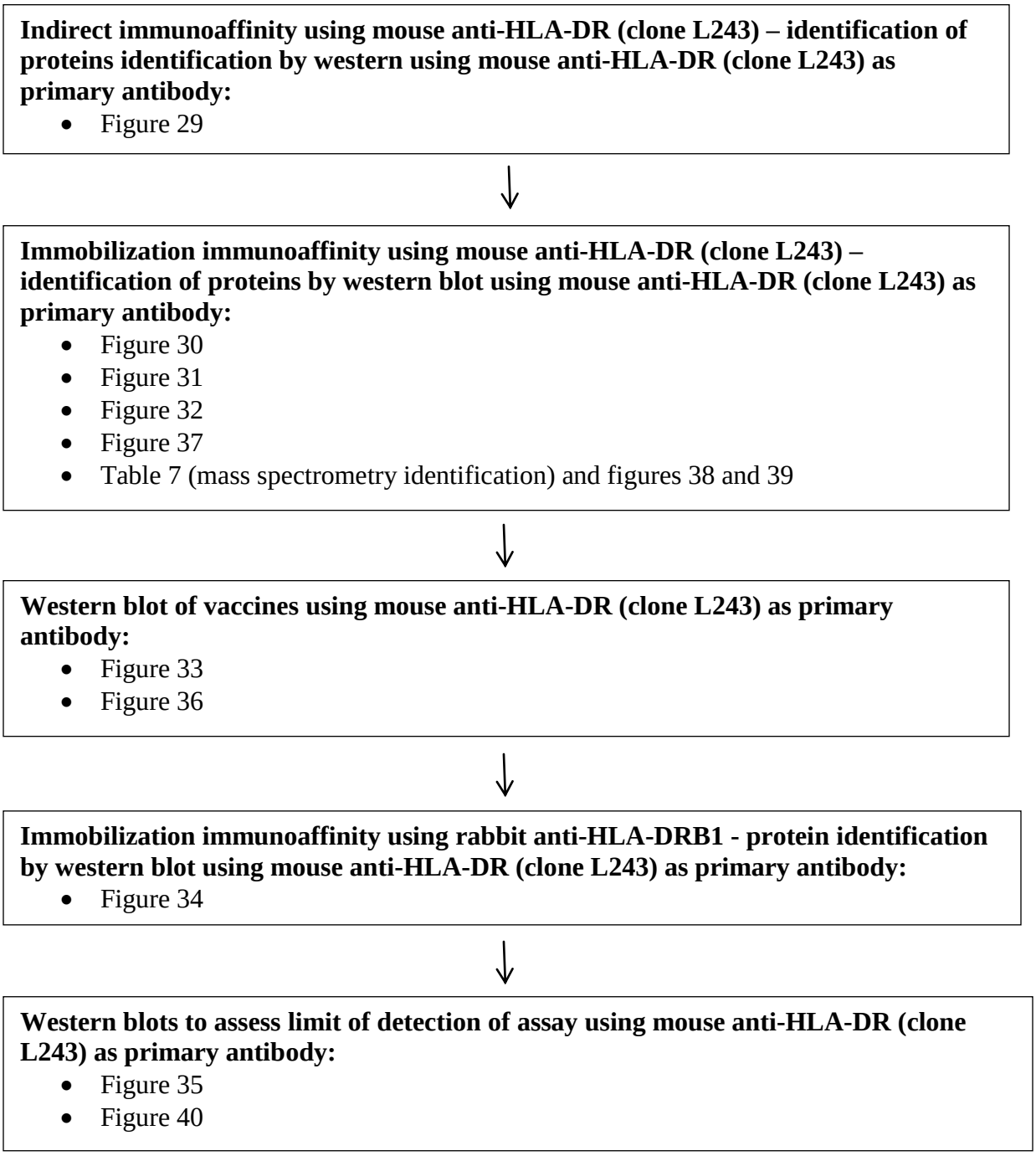

Figure 4. Flow chart of figures and tables used to demonstrate results of assay development of the Results section.

Mouse Anti-HLA-DR11 Direct Immunoaffinity Validation

Initial assay validation included using the Direct Immunoaffinity method, utilizing antibody coupled Dynabeads® Protein G with subsequent SDS-PAGE gel electrophoresis. The immunoaffinity antibody initially used in this project was a mouse monoclonal anti-HLA-DR11. The protein used to initially validate the assay was the Gen-Probe DRB1*11:01 protein preparation. The gel in the Figure 5 demonstrates the results. Samples in lanes 1 through 4 samples were loaded onto the gel reduced and boiled (RB). Samples in lanes 6-9 were loaded onto the gel non-reduced and not-boiled (NRNB). The SDS-PAGE gel was silver stained in order to visualize the proteins. Lane 1 contains molecular weight marker. Lane 2 contains the first eluate of the anti-HLA-DR11 coupled Dynabeads® Protein G incubated with Gen-Probe DRB1*11:01 protein preparation, referred to as Anti-HLA-DR11 Gen-Probe Test E1 RB. Lane 3 contains the second eluate of the anti-HLA-DR11 coupled Dynabeads® Protein G incubated with Gen-Probe DRB1*11:01 protein preparation, referred to as Anti-HLA-DR11 Gen-Probe Test E2 RB. Lane 4 contains supernatant following the initial immunoaffinity reaction (eluates observed in lanes 2 and 3), referred to as Supernatant RB. Lane 5 is a space. Lane 6 contains the first eluate of the anti-HLA- DR11 coupled Dynabeads® Protein G incubated with Gen-Probe DRB1*11:01 protein preparation, referred to as Anti-HLA-DR11 Gen-Probe Test E1 NRNB. Lane 7 contains the second eluate of the anti-HLA-DR11 coupled Dynabeads® Protein G incubated with Gen-Probe DRB1*11:01 protein preparation, referred to as Anti-HLA-DR11 Gen-Probe Test E2 NRNB. Lane 8 contains supernatant remaining from initial immunoaffinity reaction (eluates observed in lanes 6 and 7), referred to as Supernatant NRNB. Lane 9 contains molecular weight marker.

Figure 5. SDS-PAGE Gel of Mouse Anti-HLA-DR11 Direct Immunoaffinity Validation

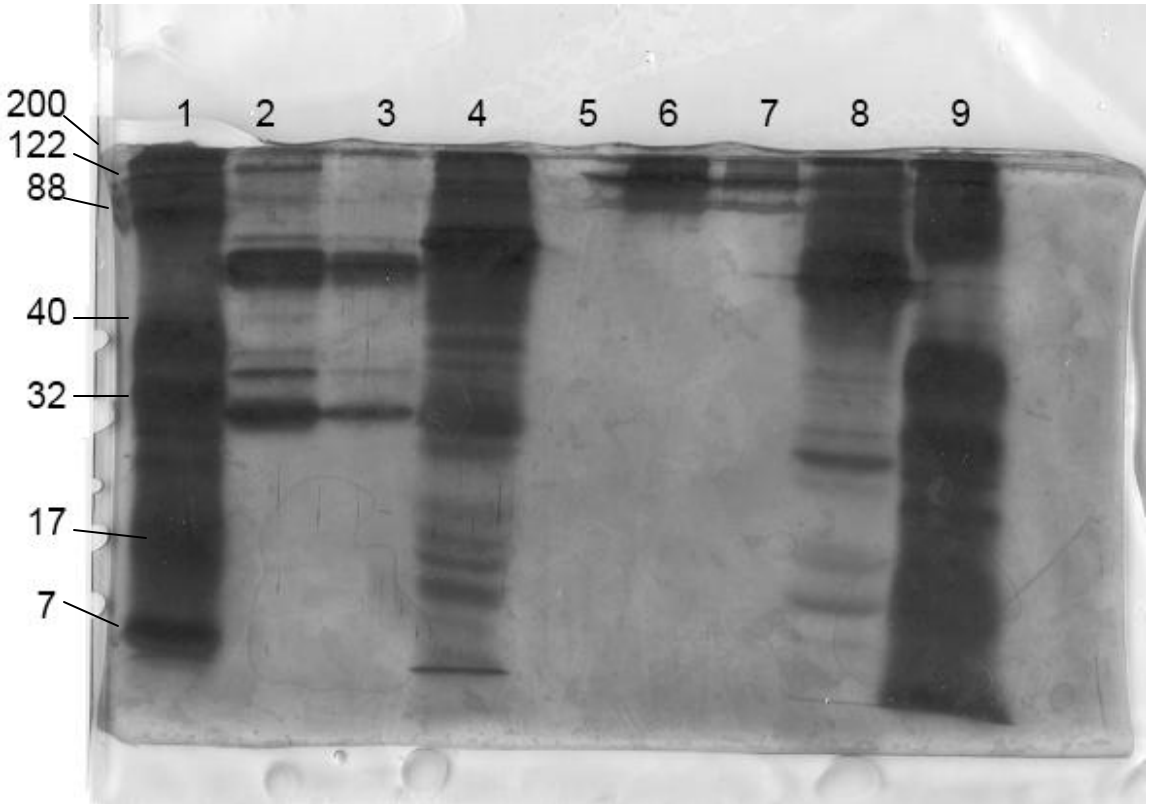

Figure 5. Silver stained SDS-PAGE gel of proteins eluted from the mouse anti-HLA-DR11 direct immunoaffinty reaction. Molecular weights of the protein standards are indicated in kDa. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker, 2. Anti-HLA-DR11 Gen-Probe Test E1 RB, 3. Anti-HLA-DR11 Gen-Probe Test E2 RB, 4. Supernatant RB, 5. Space, 6. Anti-HLA-DR11 Gen-Probe Test E1 NRNB, 7. Anti-HLA-DR11 Gen-Probe Test E2 NRNB, 8. Supernatant NRNB, 9. Molecular weight marker.

The anti-HLA DR11 antibody did not appear to capture HLA-DR11 from the Gen-Probe DRB1*11:01 protein preparation in the Direct Immunaffinity reactions. The anti-HLA-DR11 coupled to Dynabeads® Protein G were incubated with the Gen-Probe DRB1*11:01 protein preparation in two separate reactions. The antibody-antigen complexes were eluted and run on an SDS-PAGE gel either as reduced and boiled or not reduced and not boiled.

In Figure 5, the eluates in lanes 2 and 3 were reduced and boiled in an attempt to detect the disassociation of the DR11 α and β subunits. The co-elution of the anti-HLA-DR11 antibody

resulted in excessive background signal, inhibiting the detection of HLA-DR11 protein lanes 2 or 3 at the expected molecular weights of the α and β subunits of 34kDa and 29 kDa, respectively. In addition, HLA-DR11 protein was not apparent in lanes 6 or 7 at the expected molecular weight of approximately 50-55 kDa (in which the HLA Class II α and β would remain associated). It would have been appropriate to have run antibody control reactions to compare to the reduced boiled and non-reduced, not-boiled test reactions. After many attempts to troubleshoot the use of the anti-HLA-DR11 monoclonal antibody, it was decided that it was not an appropriate antibody for the assay. It was also determined that other options for the HLA-DR positive control should be explored.

Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity Validation

An indirect immunoaffinty reaction utilizing Dynabeads® Protein G was completed according to Indirect Immunoaffinity method using the anti-HLA-DR (clone B8.12.2) antibody. The modification to the procedure was that in the initial antibody antigen incubation took place at room temperature for approximately one hour. The positive controls used for the assay validation included the One Lambda DR11 protein preparation and a HLA-DR positive lysate. The eluates were visualized on a SDS-PAGE gel using the EZBlue^{TM} Gel Staining Reagent. The results of this gel are depicted in Figure 6. Lane 1 contains molecular weight marker. Lane 2 is a space. Lane 3 contains eluate of the Dynabeads® Protein G incubated with the anti-HLA-DR alone, referred to as the Antibody Control. Lane 4 contains the eluate of immunoaffinity reaction between anti-HLA-DR and the prepared human lysate, referred to as the Lysate Test. Lane 5 contains eluate from incubation of the prepared human lysate with the Dynabeads® Protein G, referred to as the Lysate Non-Specific Binding Control. Lane 6 contains eluate of immunoaffinity reaction between anti-HLA-DR and the One Lambda DR11 protein preparation,

referred to as the One Lambda DR11 Test. Lane 7 contains eluate from the One Lambda DR11 protein preparation incubated with the Dynabeads® Protein G, referred to as the One Lambda DR11 Non-Specific Binding Control. Lane 8 contains the HLA-DR positive lysate loaded directly on to the gel, referred to as HLA-DR positive lysate. All samples were reduced and boiled prior to loading as to better revolve the antibody light and heavy chains in the presence of HLA-DR protein.

Figure 6. SDS-PAGE Gel of Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity Validation

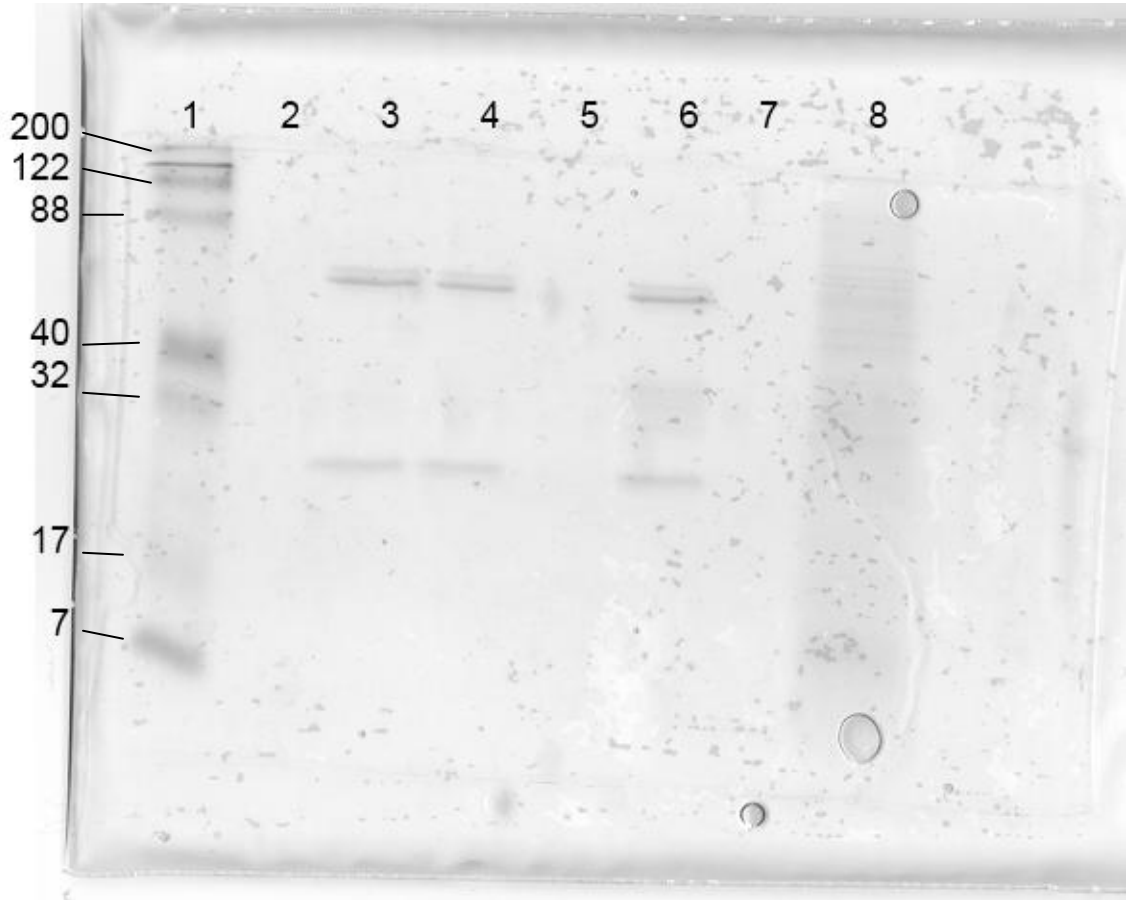

Figure 6. EZBlue™ stained SDS-PAGE gel of proteins eluted from the mouse anti-HLA-DR (clone B8.12.2) indirect immunoaffinity. Molecular weights of the protein standards are indicated in kDa. The numerical marking along the vertical axis contains approximate molecular weights of the protein molecular weight marker in kDa. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker, 2. Space, 3. Antibody Control, 4. Lysate Test, 5. Lysate Non-Specific Binding Control, 6. One Lambda DR11 Test, 7. One Lambda DR11 Non-Specific Binding Control, 8. HLA-DR positive lysate.

The Lysate Test in lane 4 did not appear to contain HLA-DR protein. The One Lambda DR11 test protein in lane 6 appeared to contain unique proteins running at approximately 30-40 kDa when compared to the Antibody Control in lane 3. The unique protein running at this molecular weight falls within the approximate range of the expected molecular weights of the disassociated HLA-DR α and β subunits, at 34 kDa and 29 kDa, respectively.

Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity Validation of Lysate

Subsequently, a repeat of the above assay was completed to address the failure of the Lysate Test. This gel was inconclusive, as can be seen in Figure 7. Lane 1 contains the molecular weight marker. Lane 2 is a space. Lane 3 contains eluate of the immunoaffinity reaction between the anti-HLA-DR (clone B8.12.2) and the HLA-DR positive lysate preparation, referred to as the Lysate Test. Lane 4 contains eluate of the HLA-DR positive lysate incubated with the Dynabeads® Protein G, referred to as the Lysate Non-Specific Binding Control. Lane 5 contains the Dynabeads® Protein G incubated with the anti-HLA-DR (clone B8.12.2), referred to as the Antibody Control. Lane 6 contains HLA-DR positive lysate loaded directly onto the gel, referred to as HLA-DR positive lysate. All samples were loaded onto the gel not reduced and not boiled.

Figure 7. Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity Validation of Lysate

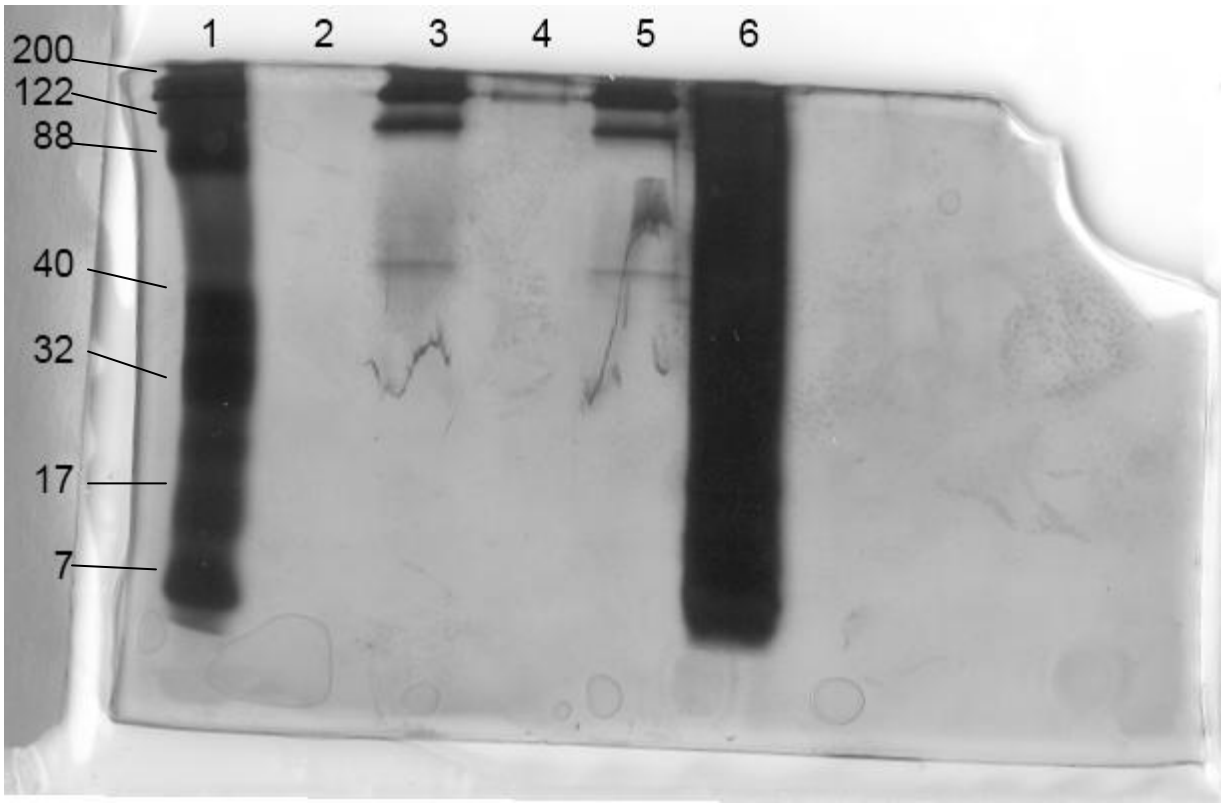

Figure 7. Silver stained SDS-PAGE gel of proteins eluted from the mouse anti-HLA-DR (clone L243) indirect immunoaffinity validation of lysate reaction. Molecular weights of the protein standards are indicated in kDa. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker, 2. Space, 3. Lysate Test, 4. Lysate Non-Specific Binding Control, 5. Antibody Control, 6. HLA-DR positive lysate.

The results from the gel in Figure 7 indicated that there may have been the presence of HLA-DR protein from the lysate eluate in lane 3 as indicated by diffuse smear between 88 and 32 kDa when compared to the Antibody Control in lane 5. Overall, it was inconclusive whether the HLA-DR positive lysate was strong enough in the gel to be validated as HLA-DR positive control.

Mouse Anti-HLA-DR (clone L243) Direct Immunoaffinity and Mass Spectrometery

Immunoaffinitity purification of a HLA-DR positive lysate was completed using the anti-HLA-DR (clone L243) antibody by a variation of the Direct Immunoaffinity method. The variation mentioned was addition of a crosslinker used to covalently bind the antibody to the Dynabeads® Protein G according to the Crosslinking method. When antibody is coupled to the Dynabeads® Protein G, they become oriented with their Fab regions facing outward towards the antigen containing solution. Addition of the BS3 crosslinker creates stable amide bonds between the lysine side chains of the protein G coating of the Dynabeads® and the lysine side chains and N-termini of the coupled antibodies. This was to serve as validation of the method prior to using the M-M-R®-II and Verivax® vaccines as test samples.

The reaction was completed and the beads were washed 8 times in PBS and submitted for on-bead digestion and mass spectrometry analysis. Using a threshold of at least 95% and a minimum of 2 unique peptides identified in Scaffold proteomic data validation program, the results were negative for any HLA proteins. Crosslinking of the antibody to the beads may have destroyed the immunoaffinity binding site on the antibody.

Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity and Mass Spectrometry I

Immunoaffinitity purification of a HLA-DR positive lysate was completed using the anti-HLA-DR (clone B8.12.2) antibody in the Indirect Immunoaffinity method. This was to serve as validation of the method prior to using the M-M-R®-II and Verivax® vaccines as test samples.

A SDS-PAGE gel was run and stained to verify the presence of potential HLA-DR protein in the preparation. Figure 8 below demonstrates the presence of potential HLADR

protein in lane 5. Lane 1 contains the molecular weight marker. Lane 2 is a space. Lane 3 contains eluate from the human lysate preclearing control, where the Dynabeads® Protein G were uncoupled and incubated with HLA-DR positive lysate to detect non-specific or human derived immunoglobulin. This is referred to as the Preclearing Control. Lane 4 contains the eluate of the anti-HLA-DR control, where the Dynabeads® Protein G were coupled with the antibody, but incubated with PBS + 0.02% Tween 20 blank as a control. This is referred to as the Antibody Control. Lane 5 contains the eluate from the immunoaffinity reaction between anti-HLA-DR (clone B8.12.2) coupled Dynabeads® Protein G which and the HLA-DR positive lysate. This is referred to as the HLA-DR Positive Lysate Test.

Figure 8. SDS-PAGE Gel of Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity I

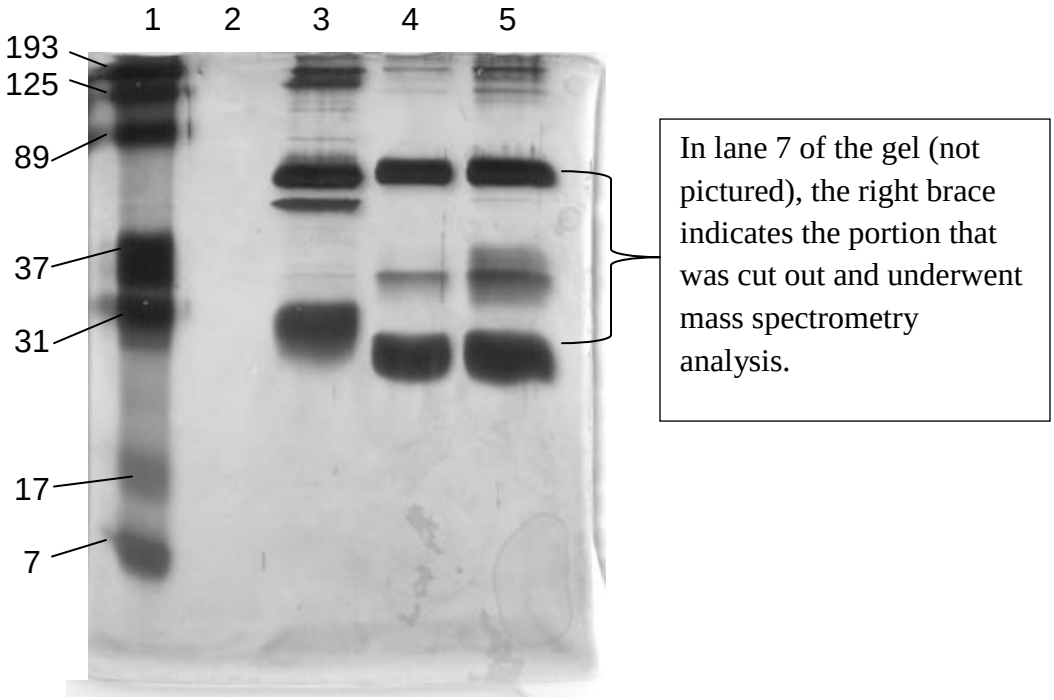

Figure 8. Silver stained SDS-PAGE gel of proteins eluted from the mouse anti-HLA-DR (clone B8.12.2) indirect immunoaffinity I reaction. Molecular weights of the protein standards are indicated in kDa. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker, 2. Space, 3. Preclearing Control, 4. Antibody Control, 5. HLA-DR Positive Lysate Test. Not pictured is gel is lane 7 and contains the eluate of reaction which replicated that of lane 5. The gel portion containing lane 7 was stained with coomassie blue R-250 stain and destained. The analogous portion of the protein marked by the right brace in lane 5 of the figure was cutout underwent mass spectrometry analysis.

The gel in Figure 8 had been cut down the middle, and the same reaction that was run in lane 5 was also run in what was lane 7. This portion of the gel was stained using coomassie blue R-250 stain and a gel cutout was made was indicated in the figure and underwent mass spectrometry analysis. An additional immunoaffinity reaction between anti-HLA-DR (clone B8.12.2) coupled Dynabeads® Protein G which and the HLA-DR positive lysate was completed and did not undergo elution. Instead, the beads were washed in buffer and underwent mass spectrometry analysis.

Table 5 demonstrates the HLA proteins identified using a threshold of at least 95% and a minimum of 2 unique peptides identified by the Scaffold proteomic data validation program. These included HLA class II histocompatibility antigens gamma chain, alpha chain, -DRB1-1 beta chains. These results demonstrated the method was sound, although not apparently highly sensitive.

Many other proteins were identified in the test and control samples. They mainly consisted of keratin, actin, collagen, tubulin and a variety of other proteins. Their presence in the test and control samples can likely be explained as common contaminant proteins.[66] A complete list of protein identifications is available upon request.

Table 5. Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity and Mass Spectrometry Results

Indentified proteins	Swiss Prot Acc. number	Mol. weight (kDa)	Number of unique spectra		Number of unique peptides		Protein identification probability	
			Bead	Band	Bead	Band	Bead	Band
HLA class II histo. antigen, gamma chain	P04233	34	3	0	3	0	100%	N/A
HLA class II histo. antigen, alpha chain	P01903	29	0	2	0	2	N/A	100%
HLA class II histo. antigen, DRB1-1 beta chain	P04229	30	0	6	0	6	N/A	100%

Table 5. Mass spectrometry results of the on-bead digest immunoaffinity reaction the gel band cutout of lane 7 described above. Abbreviations: Accession (Acc.), Molecular (Mol.), and histocompatibility (histo.).

The variation between the proteins identified in the bead and band identifications may have been due to the efficiency of trypsin digestion in each application. The proteins from the SDS-PAGE bands may have had more cut sites exposed due to SDS denaturation. This may have allowed for an increase in peptides which were better suited to MS anaylsis when compared to trypsin digestion of proteins in native conformation on the surface of the beads.

Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity and Mass Spectrometry II

Immunoaffinitity purification of 1 reconstituted vial of the M-M-R®-II vaccine was completed using the anti-HLA-DR (clone B8.12.2) antibody in the Indirect Immunoaffinity

method. See Figure 9 for results of the SDS-PAGE gel below. Lane 1 contains the molecular weight marker. Lane 2 contains the eluate from uncoupled Dynabeads® Protein G incubated with the M-M-R®-II vaccine and demonstrates non-specific binding to the Dynabeads® Protein G solid support. This is referred to as the Preclearing Control. Lane 3 is the anti-HLA-DR control, where the Dynabeads® Protein G were coupled with the antibody, but incubated with PBS + 0.02% Tween 20 blank as a control. This is referred to as the Antibody Control. Lane 4 contains the eluate from the immunoaffinity reaction between anti-HLA-DR (clone B8.12.2) coupled Dynabeads® Protein G and the M-M-R®-II vaccine. This is referred to as the MMR Test.

Figure 9. SDS-PAGE Gel of Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity II

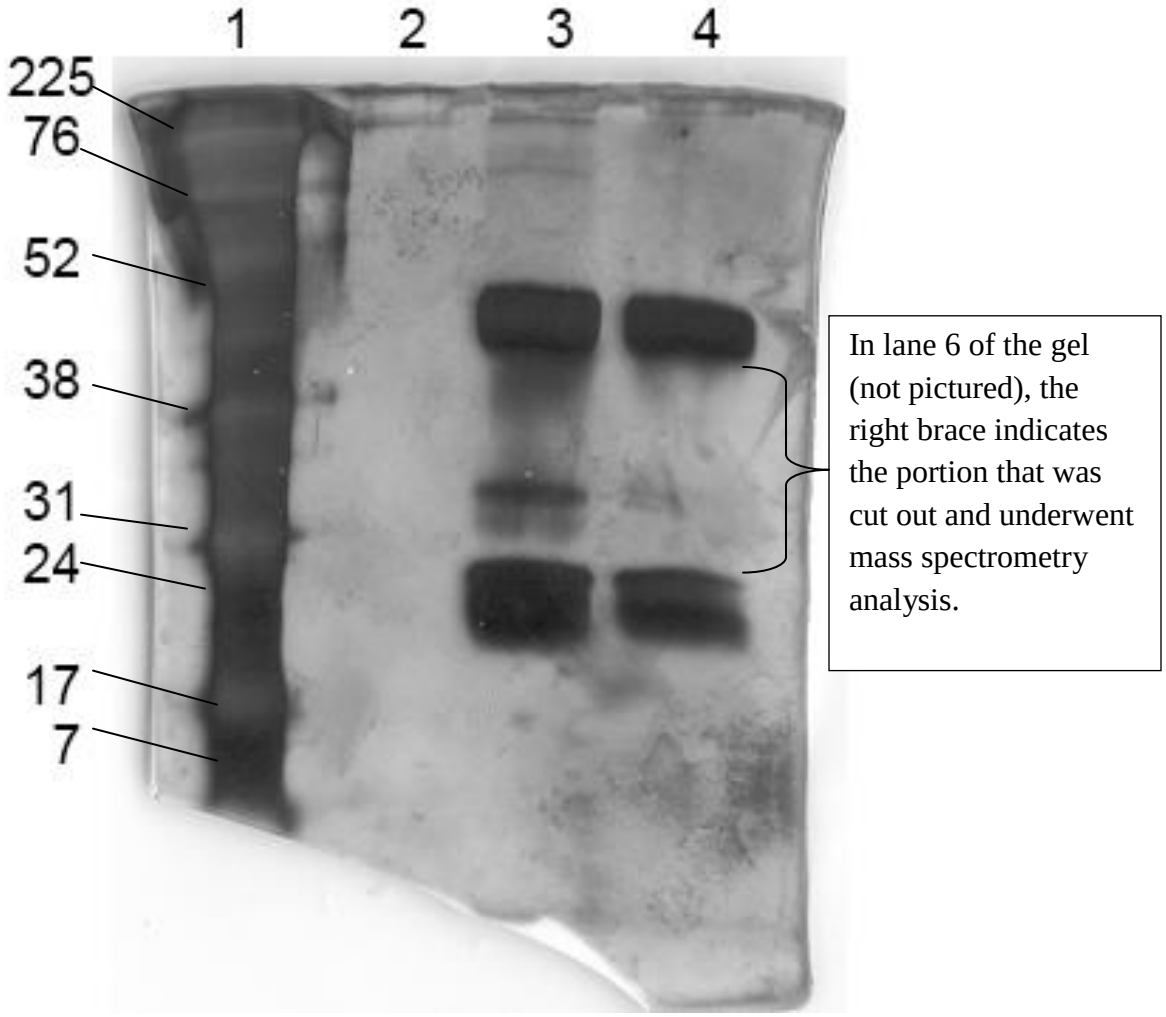

Figure 9. Silver stained SDS-PAGE gel of proteins eluted from the mouse anti-HLA-DR (clone B8.12.2) indirect immunoaffinity II reaction. Molecular weights of the protein standards are indicated in kDa.Lanes are labeled along the horizontal axis. Lane key: 1. Molecular weight marker, 2. Preclearing Control, 3. Antibody Control, 4. MMR Test. Not pictured is lane 6. It contains the eluate of reaction which replicated that of lane 4. The gel portion containing lane 6 was stained with coomassie blue R-250 stain and destained. An analogous portion of the protein marked by the right brace in lane 4 of the figure was cutout underwent mass spectrometry analysis.

The gel in Figure 9 had been cut down the middle, and the same reaction that was run in lane 4 was also run in what was lane 6 of the other half of the gel. This portion of the gel was stained using coomassie blue R-250 stain and a gel cutout was made as indicated in the figure and underwent mass spectrometry analysis. In addition, lanes replicating the Preclearing Control and Antibody Control were also run on the coomassie Blue R-250 stained portion of the gel, but were not submitted for mass spectrometry analysis. Using a threshold of at least 95% and a minimum of 2 unique peptides identified by the Scaffold proteomic data validation program, the result for the MMR test was negative for any HLA proteins.

Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity and Mass Spectrometry III

Immunoaffinitity purification of the One Lambda DR11 protein preparation was completed using the anti-HLA-DR (clone B8.12.2) antibody in the Direct Immunoaffinity method. This was to serve as additional validation of the method.

An SDS-PAGE gel was run and stained to verify the presence of potential HLA-DR protein in the preparation. Figure 10 below demonstrates the presence of potential DR protein. Lane 1 contains the molecular weight marker. Lane 2 contains the eluate from uncoupled Dynabeads® Protein G incubated with the One Lambda DR11 protein preparation, referred to as the Preclearing Control. Lane 3 contains eluate of the Dynabeads® Protein G were coupled with the antibody incubated with PBS + 0.02% Tween 20, referred to as the Antibody Control. Lane 4 contains the eluate from the immunoaffinity reaction between anti-HLA-DR (clone B8.12.2) coupled Dynabeads® Protein G and the One Lambda DR11 protein preparation, referred to as the One Lambda DR 11 Test.

Figure 10. SDS-PAGE Gel of Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity III

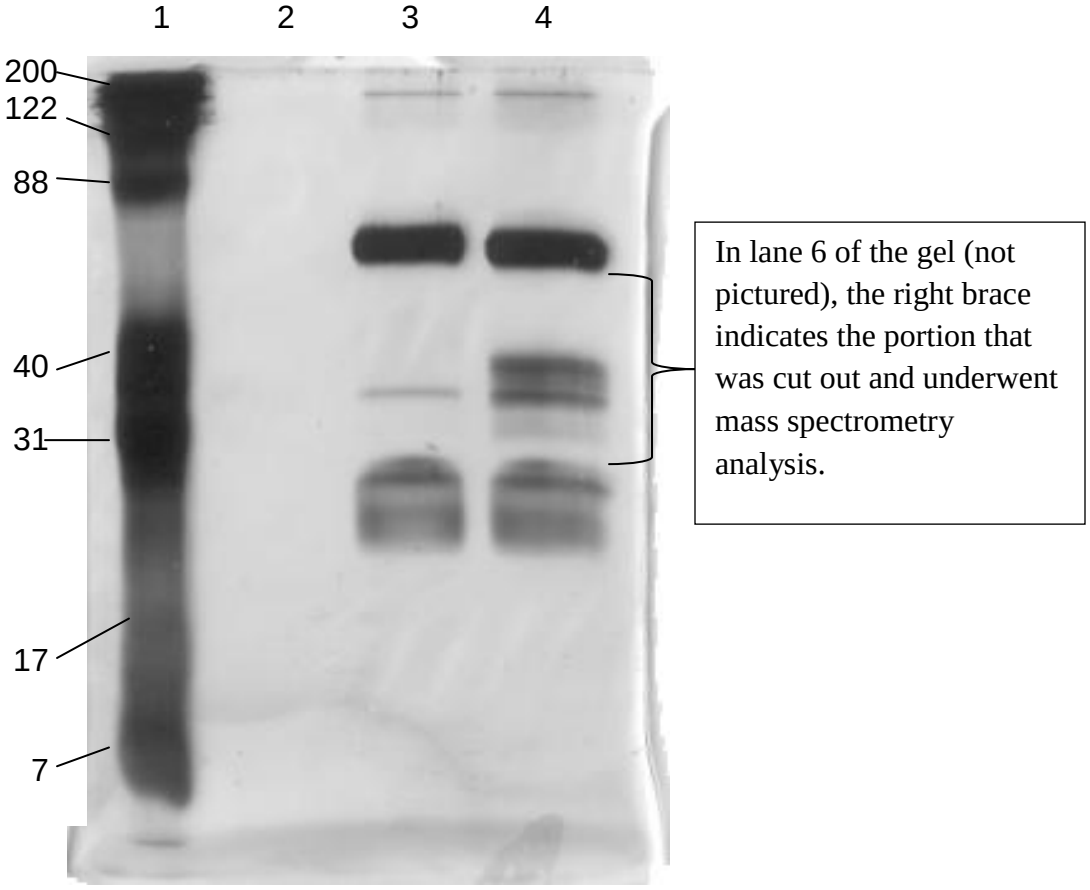

Figure 10. Silver stained SDS-PAGE gel of proteins eluted from the mouse anti-HLA-DR (clone B8.12.2) indirect immunoaffinity III reaction. Molecular weights of the protein standards are indicated in kDa. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker, 2. Preclearing Control, 3. Antibody Control 4. One Lambda DR 11 Test. Not pictured is lane 6. It contains the eluate of reaction which replicated that of lane 4. The gel portion containing lane 6 was stained with coomassie blue R-250 stain and destained. An analogous portion of the protein marked by the right brace in lane 4 of the figure was cutout underwent mass spectrometry analysis.

The unique banding in lane 4 of Figure 10 indicates the suspected presence of HLA-DR protein present and detected by the method used.

The gel in Figure 10 had been cut down the middle, and the same reaction that was run in lane 4 was also run in what was lane 6. This portion of the gel was stained using coomassie blue

R-250 stain and a gel cutout was made was indicated in the figure and underwent mass spectrometry analysis.

Analysis of the indicated cutout in lane 6 revealed the presence of a variety of HLA Class II proteins, indicating validation of the method. Table 6 demonstrates proteins identified in the gel cutout a using a threshold of at least 95% and a minimum of 2 unique peptides identified by the Scaffold proteomic data validation program.

Table 6. Mass Spectrometry III

Indentified proteins	Swiss Prot Accession number	Molecular weight (kDa)	Number of unique spectra	Number of unique peptides
HLA class II histocompatibility antigen gamma chain	P04233	34	2	2
Keratin, type I cytoskeletal 9	P35527	62	2	2
Keratin, type I cytoskeletal 10	P13645	59	2	2
HLA class II histocompatibility antigen, DQ(W3) beta chain	P05537	30	2	2
HLA class II histocompatibility antigen, DQ(5) alpha chain	P01907	28	3	2
HLA class II histocompatibility antigen, DP alpha chain	P20036	29	3	2
HLA class II histocompatibility antigen, DQ(3) alpha chain	P01909	28	3	3
HLA class II histocompatibility antigen, DRB3-2 beta chain	P01913	30	3	3
HLA class II histocompatibility antigen, DQ*0602 beta chain	P03992	30	5	4
HLA class II histocompatibility antigen, DP(W2) beta chain	P13763	29	6	4
Keratin, type II cytoskeletal 1	P04264	66	7	5
HLA class II histocompatibility antigen, DRB1-15 beta chain	P01911	30	7	6
HLA class II histocompatibility antigen, DRB1-11 beta chain	P20039	30	8	7
HLA class II histocompatibility antigen, DR alpha chain	P01903	29	9	7

Table 6. This table demonstrates proteins identified in the gel cutout.

The number of unique spectra identified for each peptide may be representative of the same amino acid sequence but have different charge states, each of which contributes to the identification of a unique peptide of an identified protein. Each protein identified in Table 6 had a corresponding protein identification probability of 100% as determined by the Scaffold proteomic data validation program.

Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity and Mass Spectrometry IV

Immunoaffinitity purification of the Gen-Probe DRB1*11:01 recombinant protein preparation was completed using the anti-HLA-DR (clone B8.12.2) antibody in the Indirect Immunoaffinity method. This purpose of this test was to further asses the validation of the method.

An SDS-PAGE gel was run and stained to verify the presence of potential HLA-DR protein in the preparation. The results are demonstrated in Figure 11. Lane 1 contains the molecular weight marker. Lane 2 contains eluate from Gen-Probe DRB1*11:01 recombinant protein preparation, where the Dynabeads® Protein G were uncoupled and incubated with the Gen-Probe DRB1*11:01 recombinant protein preparation to detect non-specific or human derived immunoglobulin. This is referred to as the Preclearing Control. Lane 3 contains the eluate of the anti-HLA-DR control, where the Dynabeads® Protein G were coupled with the antibody, but incubated with PBS + 0.02% Tween 20 blank as a control. This is referred to as the Antibody Control. Lane 4 contains the eluate from the immunoaffinity reaction between anti-HLA-DR (clone B8.12.2) coupled Dynabeads® Protein G which and the Gen-Probe DRB1*11:01 recombinant protein preparation. This is referred to as the Gen-Probe

DRB1*11:01 Test. Figure 11 below does not appear to demonstrate the presence of potential DR protein in the Gen-Probe DRB1*11:01 recombinant protein preparation.

Figure 11. SDS-PAGE Gel of Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity IV

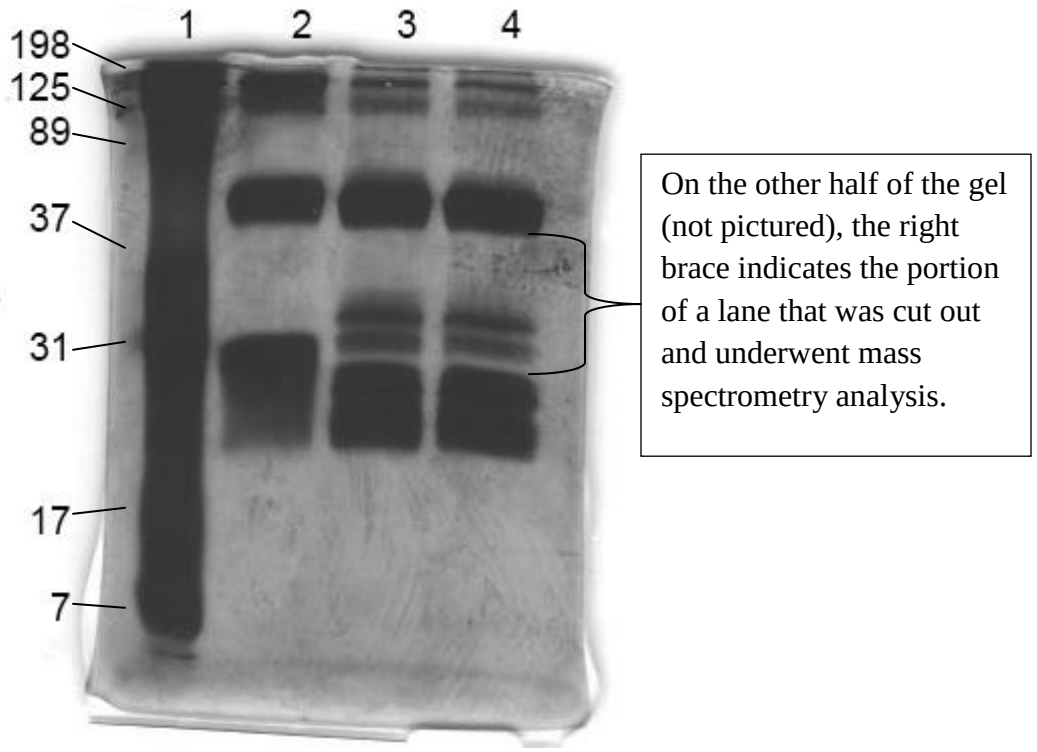

Figure 11. Silver stained SDS-PAGE gel of proteins eluted from the mouse anti-HLA-DR (clone B8.12.2) indirect immunoaffinity IV reaction. Molecular weights of the protein standards are indicated in kDa. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker, 2. Preclearing Control, 3. Antibody Control, 4. Gen-Probe DRB1*11:01 Test. Not pictured is the other half of the gel. This half of the gel was stained with coomassie blue R-250 stain and destained. The same reaction that was run in lane 4 was also run in a lane on the other half of the gel. A gel cutout was made analogous to the area indicated in the figure and underwent mass spectrometry analysis.

The gel in Figure 11 had been cut down the middle, and the same reaction that was run in lane 4 was also run in a lane on the other half of the gel. The half of the gel not pictured was stained using coomassie blue R-250 stain and a gel cutout was made analogous to the region gel

area indicated in Figure 11 and underwent mass spectrometry analysis. Using a threshold of at least 95% and a minimum of 2 unique peptides identified by the Scaffold proteomic data validation program, the result for the Gen-Probe Test was negative for any HLA proteins.

Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity and Mass Spectrometry V

Immunoaffinitity purification of the Verivax® vaccine was completed using the anti-HLA-DR (clone B8.12.2) antibody in the Indirect Immunoaffinity method. For this test, Verivax® vaccine vials were combined and dialyzed according to the Dialysis method to enhance detection of HLA-DR protein. One of the combinations utilized 2 vials of Verivax® combined for subsequent SDS-PAGE and silver stain analysis, referred to as 2X Verivax®. The other combination was 4 vials of Verivax® vaccine combined for subsequent SDS-PAGE and mass spectrometry analsysis, referred to as 4X Verivax®. It was assumed that the vaccine reconstituted as directed by the package insert would be isotonic. Combining vials of vaccine into a concentrated solution was assumed to result in the vaccine solution becoming hypteronic, potentially reducing the effectiveness of the antigen and antibody interaction of the immunoaffinity reaction. Thus, the combined vials were dialyzed against PBS overnight so that the salt concentration would be isotonic in order to optimize the antigen and antibody interaction of the immunoaffinity reaction.

An SDS-PAGE gel was run and stained to verify the presence of potential HLA-DR protein in the preparation. Figure 12 below did not appear to demonstrate the presence of potential DR protein in the 2X Verivax® preparation. Lane 1 contains the molecular weight marker. Lane 2 contains eluate from 2X Verivax®, where the Dynabeads® Protein G were uncoupled and incubated with 2X Verivax® to detect non-specific or human derived immunoglobulin. This is referred to as the 2X Verivax® Preclearing Control. Lane 3 contains

the eluate of the anti-HLA-DR control, where the Dynabeads® Protein G were coupled with the antibody, but incubated with PBS + 0.02% Tween 20 blank as a control. This is referred to as the Antibody Control. Lane 4 contains the eluate from the immunoaffinity reaction between anti-HLA-DR (clone B8.12.2) coupled Dynabeads® Protein G which and the 2X Verivax®. This is referred to as the 2X Verivax® Test. The gel in Figure 12 had been cut down the middle, and the same reaction that was run in lanes 2 and 4 were also run using 4X Verivax® in lanes on the other half of the gel. The half of the gel not pictured was stained using coomassie blue R-250 stain and gel cutouts were made analogous to the region gel area indicated in Figure 12. These gel cutouts underwent mass spectrometry analysis. Using a threshold of at least 95% and a minimum of 2 unique peptides identified by the Scaffold proteomic data validation program, the results for the 4X Verivax® Preclearing Control and 4X Verivax® Test were negative for any HLA proteins.

Figure 12. SDS-PAGE Gel of Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity V

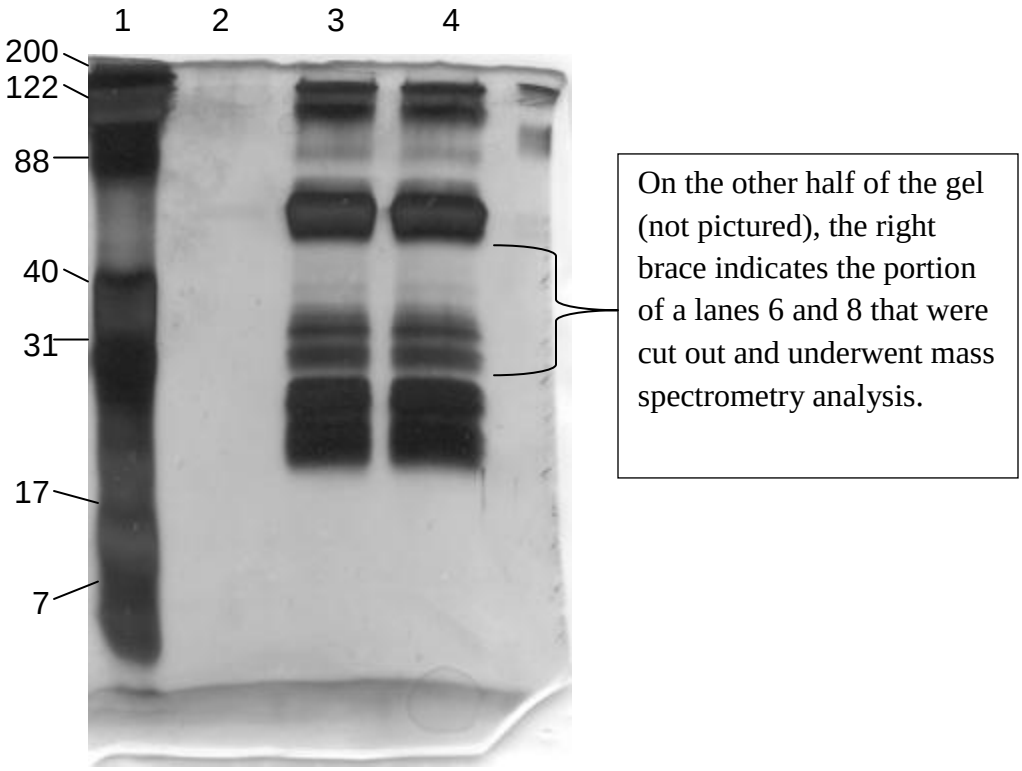

Figure 12. Silver stained SDS-PAGE gel of proteins eluted from the mouse anti-HLA-DR (clone B8.12.2) indirect immunoaffinity V reaction. Molecular weights of the protein standards are indicated in kDa. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker, 2. 2X Verivax® Preclearing Control, 3. Antibody Control, 4. 2X Verivax® Test. Not pictured is the other half of the gel. This half of the gel was stained with Coomassie blue R-250 stain and destained. The same reaction that was run in lanes 2 and 4 were also run in lanes on the other half of the gel. Gel cutouts of the 4X Verivax® Preclearing Controls were made from lane 8 and 4X Verivax® Test were made from lane 6 and analogous to the area indicated in the figure. Theses cutouts underwent mass spectrometry analysis.

Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity and Mass Spectrometry VI

Immunoaffinitity purification of the Verivax® vaccine was completed using the anti-HLA-DR (clone B8.12.2) antibody in the Protein G Indirect Immunoaffinity method. For this test, 3 vials of Verivax® vaccine were reconstituted according to the manufacturer's instructions and each vial underwent the Indirect Immunoaffinity method using the anti-HLA-DR (clone

B8.12.2) antibody (without dialysis). Following overnight incubation, the tubes of vaccine and antibody were combined and underwent binding to the Dynabeads® Protein G. An eluate was obtained and run on an SDS-PAGE gel. The SDS-PAGE gel was stained with Coomassie Blue R-250 Stain. A gel cutout was made in lane area of HLA-DR protein expected molecular weight, between the eluted antibody heavy and light chains as demonstrated in Figure 13. The gel cutout underwent mass spectrometry analysis. Using a threshold of at least 95% and a minimum of 2 unique peptides identified by the Scaffold proteomic data validation program, the results were negative for any HLA proteins.

Figure 13. SDS-PAGE Gel Cutout of Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity VI

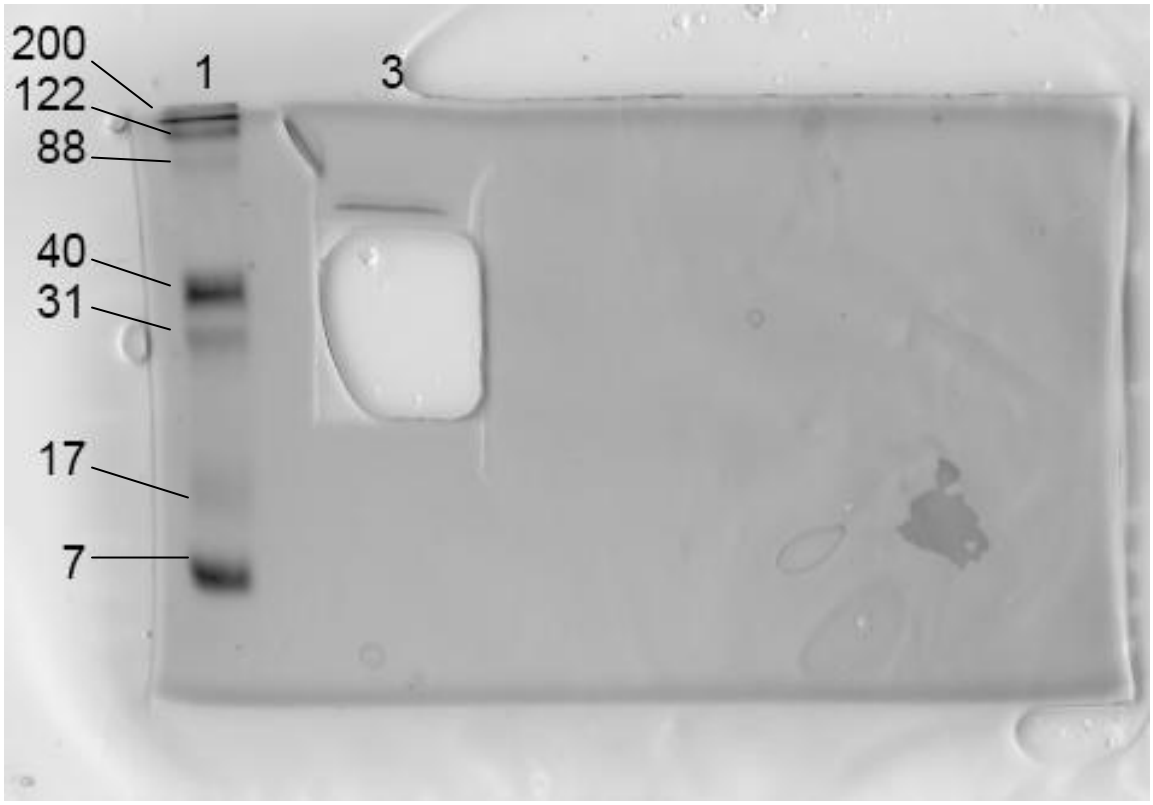

Figure 13. Coomassie blue R-250 stained SDS-PAGE gel of proteins eluted from the mouse anti-HLA-DR (clone B8.12.2) indirect immunoaffinity VI reaction. Molecular weights of the protein standards are indicated in kDa. Lanes are labeled along the horizontal axis. Lane key: 1. Molecular weight marker, Lane 3. A gel cutout of the 3 vial combined Verivax® vaccine immunoaffinity eluate was made in lane 3 in the area of HLA-DR protein expected molecular weight of 29-34 kDa (between the eluted antibody heavy and light chains). The gel cutout underwent mass spectrometry analysis.

Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity and Western Blot I

An indirect immunoaffinity reaction was completed against a HLA-DR positive lysate. The antibody used for the reaction was the anti-HLA-DR (clone B8.12.2). The eluate was reduced and boiled, run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and

detected by western blot. The primary antibody used for the blot the anti-HLA-DR (clone B8.12.2) and the secondary antibody used was goat anti-mouse IgG HRP. Figure 14 depicts the blot. The bands in Figure 14 are annotated by their interpreted identifications. The highest and lowest running bands were interpreted as the eluted mouse IgG heavy and light chains and the middle running bands were interpreted as lysate HLA Class II α and β chains.

Figure 14. Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity and Western Blot I

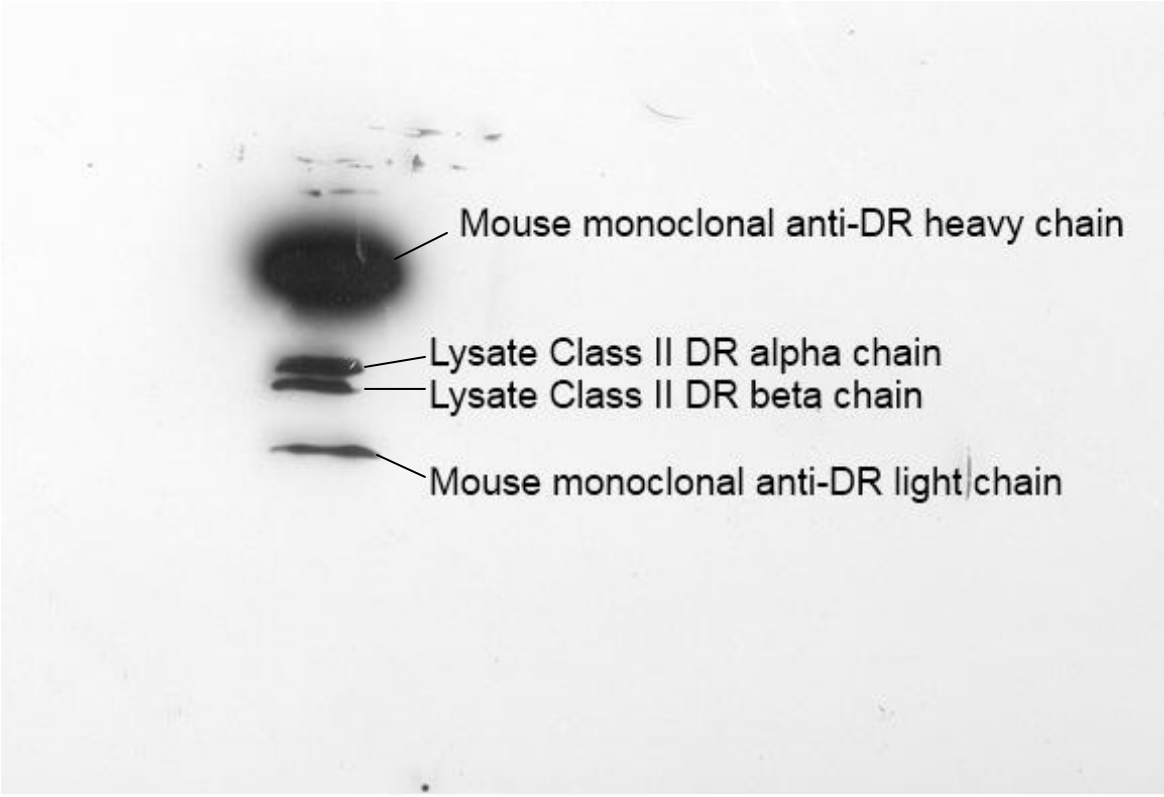

Figure 14. Western blot of mouse anti-HLA-DR (clone B8.12.2) indirect immunoaffinity I. It was interpreted that the eluate contained mouse monoclonal heavy and light chains as well as lysate HLA Class II α and β chains.

Mouse Anti-HLA-DR (clone B8.12.2) and Mouse Anti-HLA-DR11 Indirect Immunoaffinity and Western Blot II

Two indirect immunoaffinity reactions were completed against a HLA-DR positive lysate expected to be positive for HLA-DR11. In one of the indirect immunoaffinity reactions, the anti-

HLA-DR11 antibody was used. In the other indirect immunoaffinity reaction, the anti-HLA-DR (clone B8.12.2) antibody was used. The eluate was reduced and boiled, run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the lanes 1- 3 of the blot was anti-HLA-DR (clone B8.12.2) and the secondary antibody used was goat anti-mouse IgG HRP. The primary antibody used for the lanes 4 and 5 of the blot was anti-HLA-DR11 and the secondary antibody used was goat anti-mouse IgG HRP. Figure 15 depicts the image of the western blot of the immunoaffinity eluates. Lane 1 contains molecular weight marker (undetected in the blot). Lane 2 contains eluate of the Dynabeads® Protein G incubated with anti-HLA-DR (clone B8.12.2), referred to as the Anti-HLA-DR Antibody Control. Lane 3 contains eluate of the immunoaffiniy reaction between anti-HLA-DR (clone B8.12.2) and HLA-DR positive lysate, referred to as the Anti-HLA-DR Lysate Test. Lane 4 contains eluate from the Dynabeads® Protein G incubated with anti-HLA-DR11, referred to as Anti-HLA-DR11 Antibody Control. Lane 5 contains eluate of the immunoaffinity reaction between the anti-HLA-DR11 and HLA-DR positive lysate, referred to as the Anti-HLA-DR11 Lysate Test.

Figure 15. Mouse Anti-HLA-DR (clone B8.12.2) and Mouse Anti-HLA-DR11 Indirect Immunoaffinity and Western Blot II

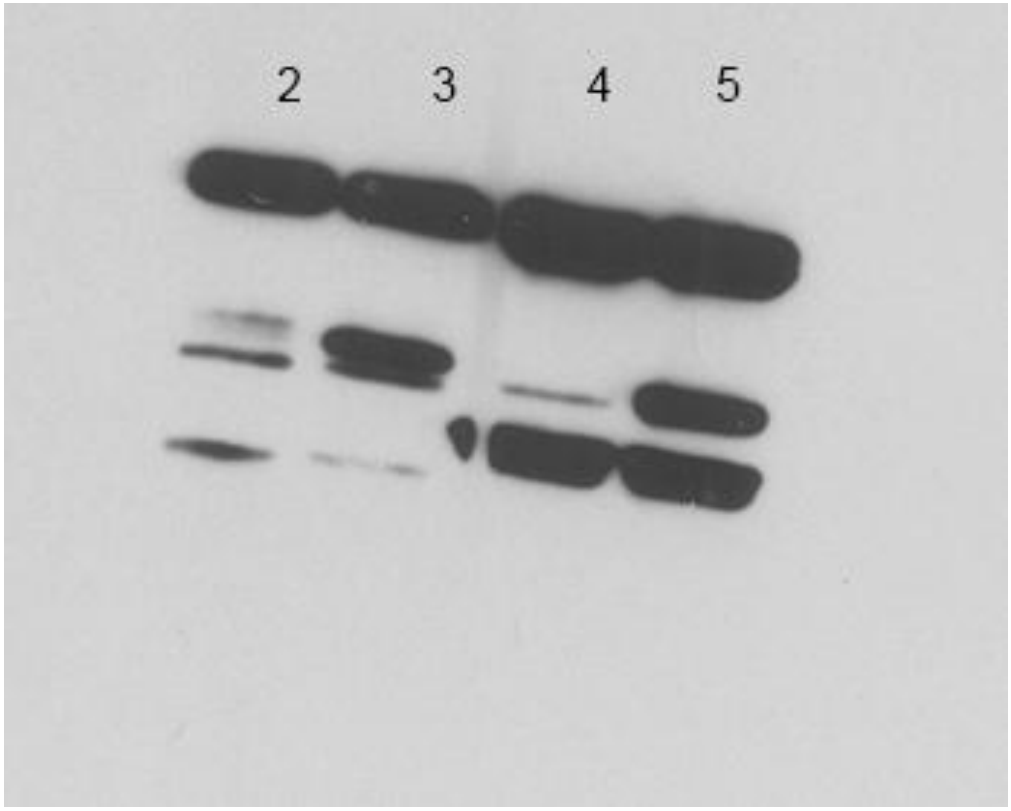

Figure 15. Western blot of mouse anti-HLA-DR (clone B8.12.2) and mouse anti-HLA-DR11 indirect immunoaffinity II. Lane key: 1. Molecular weight marker (undetected), 2. Anti-HLA-DR Antibody Control, 3. Anti-HLA-DR Lysate Test, 4. Anti-HLA-DR11 Antibody Control, and 5. Anti-HLA-DR11 Lysate Test.

It appears that unique proteins were detected eluates of the Anti-HLA-DR Lysate Test and the Anti-HLA-DR11 Lysate test whan compared the their antibody controls in lanes 2 and 4. Follow up assays revealed that using the anti-HLA-DR11 antibody as the immunoaffinity antibody or as the primary antibody for the western blot did not enhance specificity of the detection of HLA-DR11 protein. Therefore, it was not used in further testing.

Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity and Western Blot III

Indirect immunoaffinity reactions were completed against 1 vial of reconstituted Verivax® vaccine, the Gen-Probe DRB1*11:01 protein preparation, and the One Lambda DR11

protein preparation using anti-HLA-DR (clone B8.12.2). The eluates were reduced and boiled, run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot the anti-HLA-DR (clone B8.12.2) and the secondary antibody used was goat anti-mouse IgG HRP. Figure 16 depicts the image of the western blot of the immunoaffinity eluates. Lane 1 contains molecular weight marker (undetected in the blot). Lane 2 contains eluate of the Dynabeads® Protein G incubated with anti-HLA-DR (clone B8.12.2), referred to as the Antibody Control. Lane 3 contains eluate from the reaction between the uncoupled Dynabeads® Protein G and the Verivax® vaccine, and is referred to as the Verivax® Preclearing Control. Lane 4 contains eluate of the immunoaffinity reaction between anti-HLA-DR and the Verivax® vaccine, referred to as the Verivax® Test. Lane 5 is a space. Lane 6 contains eluate from the reaction between uncoupled Dynabeads® Protein G and the Gen-Probe DRB1*11:01 protein preparation, and is referred to as the Gen-Probe DRB1*11:01 Preclearing Control. Lane 7 contains eluate of the immunoaffinity reaction between anti-HLA-DR and the Gen-Probe DRB1*11:01 protein preparation, referred to as the Gen-Probe DRB1*11:01 Test. Lane 8 is a space. Lane 9 contains eluate from the reaction between uncoupled Dynabeads® Protein G and the One Lambda DR11 protein preparation, and is referred to as the One Lambda DR11 Preclearing Control. Lane 10 contains eluate of the immunoaffinity reaction between anti-HLA-DR and the One Lambda DR11 protein preparation, referred to as the One Lambda DR11 Test.

Figure 16. Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity and Western Blot III

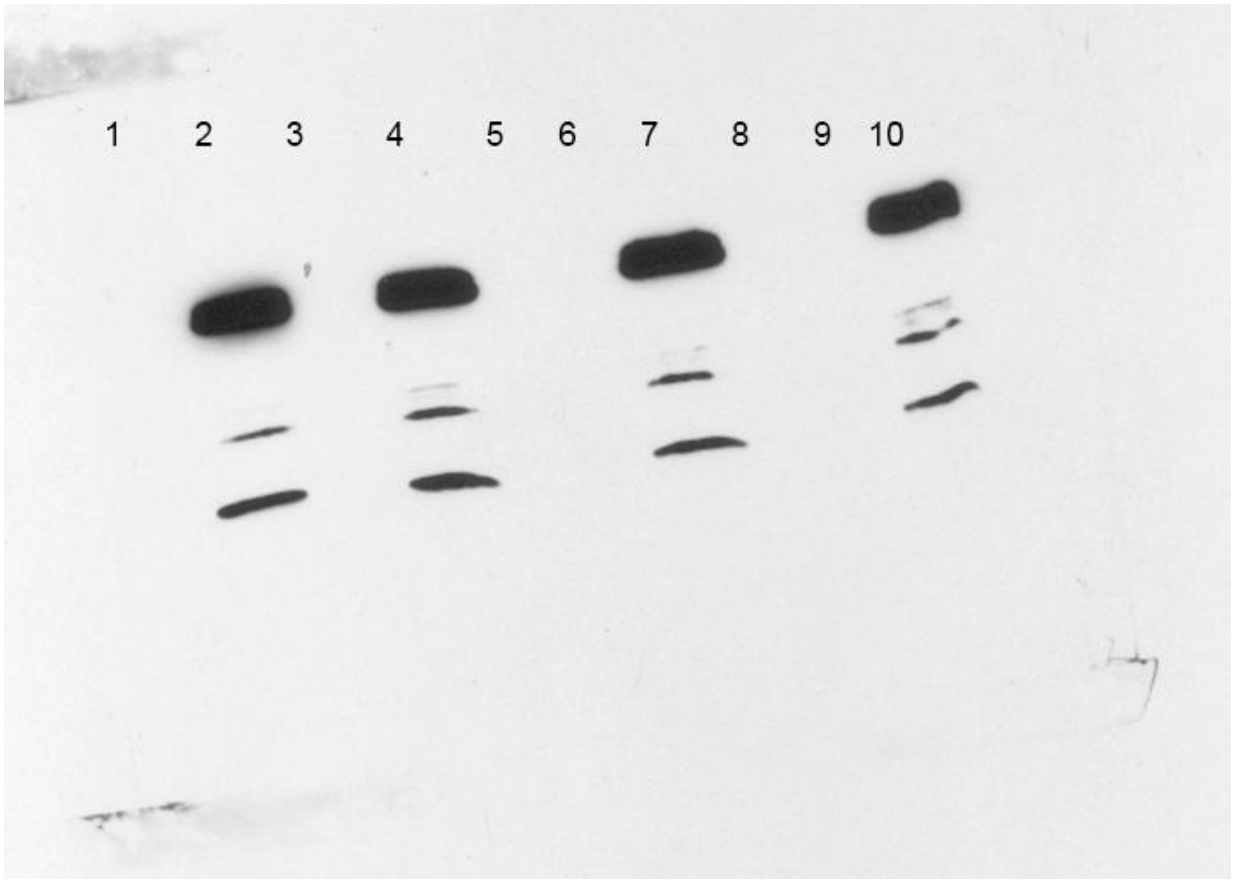

Figure 16. Western blot of mouse anti-HLA-DR (clone B8.12.2) indirect immunoaffinity III. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. Antibody Control, 3. Verivax® Preclearing Control, 4. Verivax® Test, 5. Space, 6. Gen-Probe DRB1*11:01 Preclearing Control, 7. Gen-Probe DRB1*11:01 Test, 8. Space, 9. One Lambda DR11 Preclearing Control, 10. One Lambda DR11 Test.

The results of this blot indicated that there may have been slight detection of HLA-DR protein in the Verivax® Test, Gen-Probe DRB1*11:01 Test, and the One Lambda DR11 Test when compared to the Antibody Control in lane 2. The signal was determined to be too weak to definitely confirm the presence of HLA-DR protein in the Verivax® Test.

Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity and Western Blot IV

Indirect immunoaffinity reactions were completed against 2 vials of reconstituted M-M-R®-II vaccine, the One Lamda DR11 protein preparation, and a HLA-DR positive lysate using

anti-HLA-DR (clone B8.12.2). The only variation from the procedure was in the in western blot, where the initial incubation was extended from 60 to 90 minutes. The eluate was reduced and boiled, run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot the anti-HLA-DR (clone B8.12.2) and the secondary antibody used was goat anti-mouse IgG HRP. Figure 17 depicts the image of the western blot of the immunoaffinity eluates. Lane 1 contains molecular weight marker (undetected in the blot). Lane 2 contains eluate of the Dynabeads® Protein G incubated with anti-HLA-DR (clone B8.12.2), referred to as the Antibody Control. Lane 3 contains eluate from the reaction between the uncoupled Dynabeads® Protein G and the M-M-R®-II vaccine, and is referred to as the M-M-R®-II Preclearing Control. Lane 4 contains eluate of the immunoaffinity reaction between anti-HLA-DR and the M-M-R®-II vaccine, referred to as the M-M-R®-II Test. Lane 5 contains eluate from the reaction between uncoupled Dynabeads® Protein G and the One Lambda DR11 protein preparation, and is referred to as the One Lambda DR11 Preclearing Control. Lane 6 contains eluate of the immunoaffinity reaction between anti-HLA-DR and the One Lambda DR11 protein preparation, referred to as the One Lambda DR11 Test. Lane 7 contains eluate from the reaction between uncoupled Dynabeads® Protein G and the HLA-DR positive lysate, and is referred to as the Lysate Preclearing Control. Lane 8 contains eluate of the immunoaffinity reaction between anti-HLA-DR and the HLA-DR positive lysate, referred to as the Lysate Test. Lanes 9 and 10 are spaces.

Figure 17. Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity and Western Blot IV

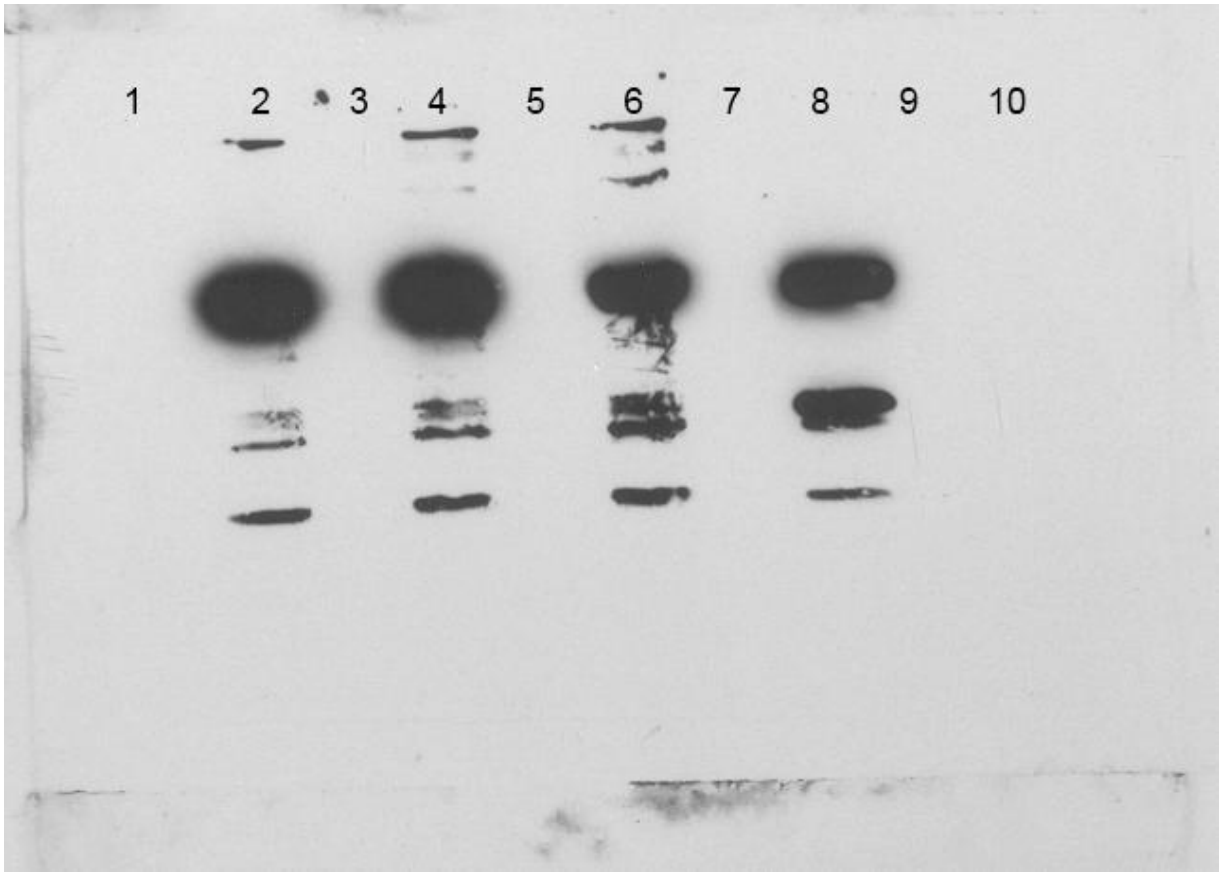

Figure 17. Western blot of mouse anti-HLA-DR (clone B8.12.2) indirect immunoaffinity IV. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. Antibody Control, 3. M-M-R®-II Preclearing Control, 4. M-M-R®-II Test, 5. One Lambda DR11 Preclearing Control. 6. One Lambda DR11 Test, 7. Lysate Preclearing Control, 8. Lysate Test, 9. Space, 10. Space.

The result of the M-M-R®-II Test was difficult to interpret due to the increased strength of banding of the Antibody Control. It was determined that because of the high level of background in the blot due to co-elution of antibody light and heavy chains, that this blot did not definitively indicate the presence of HLA-DR protein in the M-M-R®-II vaccine.

Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity and Western Blot V

A repeat of the initial indirect immunoaffinity reactions was completed against 1 vials of reconstituted M-M-R®-II vaccine, the One Lamda DR11 protein preparation, and a HLA-DR positive lysate using anti-HLA-DR (clone B8.12.2) with strict adherence to the Western Blot method. The eluate was reduced and boiled, run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot the anti-HLA-DR (clone B8.12.2) and the secondary antibody used was goat anti-mouse IgG HRP. Figure 18 depicts the image of the western blot of the immunoaffinity eluates. Lane 1 contains molecular weight marker. Lane 2 contains eluate of the Dynabeads® Protein G incubated with anti-HLA-DR (clone B8.12.2), referred to as the Antibody Control. Lane 3 contains eluate from the reaction between the uncoupled Dynabeads® Protein G and the M-M-R®-II vaccine, and is referred to as the M-M-R®-II Preclearing Control. Lane 4 contains eluate of the immunoaffinity reaction between anti-HLA-DR and the M-M-R®-II vaccine, referred to as the M-M-R®-II Test. Lane 5 contains eluate from the reaction between uncoupled Dynabeads® Protein G and the One Lambda DR11 protein preparation, and is referred to as the One Lambda DR11 Preclearing Control. Lane 6 contains eluate of the immunoaffinity reaction between anti-HLA-DR and the One Lambda DR11 protein preparation, referred to as the One Lambda DR11 Test. Lane 7 contains eluate from the reaction between uncoupled Dynabeads® Protein G and the HLA-DR positive lysate, and is referred to as the Lysate Preclearing Control. Lane 8 contains eluate of the immunoaffinity reaction between anti-HLA-DR and the HLA-DR positive lysate, referred to as the Lysate Test. Lanes 9 and 10 are spaces.

Figure 18. Mouse Anti-HLA-DR (clone B8.12.2) Indirect Immunoaffinity and Western Blot V

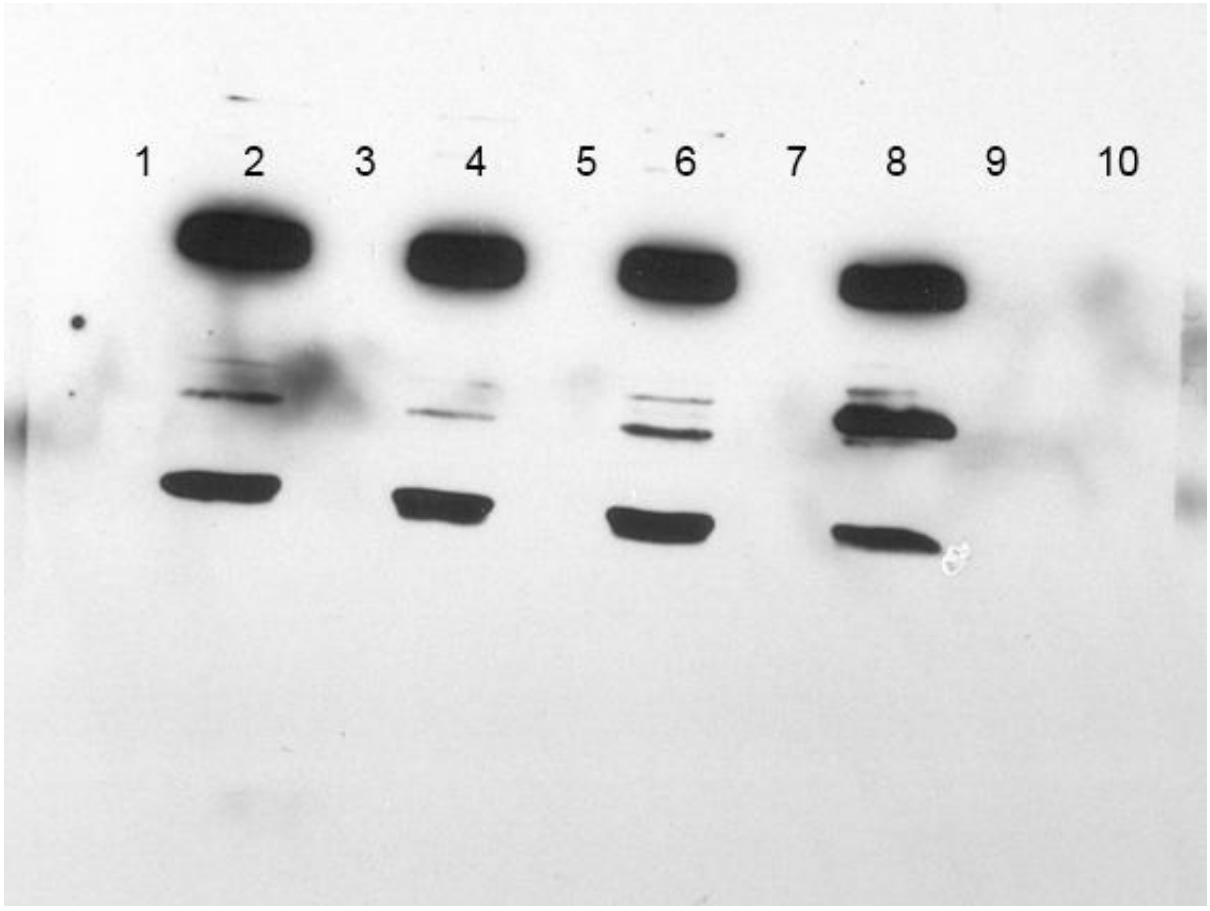

Figure 18. Western blot of mouse anti-HLA-DR (clone B8.12.2) indirect immunoaffinity V. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. Antibody Control, 3. M-M-R®-II Preclearing Control, 4. M-M-R®-II Test, 5. One Lambda DR11 Preclearing Control. 6. One Lambda DR11 Test, 7. Lysate Preclearing Control, 8. Lysate Test, 9. Space, 10. Space.

The results of the western blot in Figure 18 did not indicate the presence of HLA-DR protein in the M-M-R®-II Test. The specific banding in the Lysate Test (lane 8) indicates valid immunoaffinity and western blot assays.

Crosslinked Mouse Anti-HLA-DR (clone B8.12.2) and Mouse Anti-HLA-DR (clone L243) Direct Immunoaffinity with Western Blot

Dynabeads® Protein G were separately coupled with either anti-HLA-DR (clone L243) or anti-HLA-DR (clone B8.12.2) followed by crosslinking according to the Crosslinking method.

Following the crosslinking procedure, a direct immunoaffinity reaction was completed against each clone using a HLA-DR positive lysate. The purpose of this test was to re-evaluate the antigen binding capacity of the anti-HLA-DR, both clone L243 and clone B8.12.2. The eluates were reduced and boiled, run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot was anti-HLA-DR (clone B8.12.2) and the secondary antibody used was goat anti-mouse IgG HRP. The image of this blot is depicted in Figure 19. Lane 1 contains molecular weight marker. Lane 2 contains eluate of the Dynabeads® Protein G incubated with anti-HLA-DR (clone L243) not crosslinked to the beads, referred to as the L243 No Crosslinking Antibody Control. Lane 3 contains eluate of the Dynabeads® Protein G incubated with anti-HLA-DR (clone L243) crosslinked to the beads, referred to as the L243 Crosslinking Antibody Control. Lane 4 contains eluate from the reaction between uncoupled Dynabeads® Protein G and the HLA-DR positive lysate, and is referred to as a Lysate Preclearing Control. Lane 5 contains the immunoaffinity reaction between the anti-HLA-DR (clone L243) crosslinked beads and the HLA-DR positive lysate, referred to as the Crosslinked L243 Lysate Test. Lane 6 is a space. Lane 7 contains contains eluate of the Dynabeads® Protein G incubated with anti-HLA-DR (clone B8.12.2) not crosslinked to the beads, referred to as the B8.12.2 No Crosslinking Antibody Control. Lane 8 contains eluate of the Dynabeads® Protein G incubated with anti-HLA-DR (clone B8.12.2) crosslinked to the beads, referred to as the B8.12.2 Crosslinking Antibody Control. Lane 9 contains eluate from the reaction between uncoupled Dynabeads® Protein G and the HLA-DR positive lysate, and is referred to as a Lysate Preclearing Control. Lane 10 contains the immunoaffinity reaction between the anti-HLA-DR (clone B8.12.2) crosslinked beads and the prepared HLA-DR positive lysate, referred to as the Crosslinked B8.12.2 Lysate Test.

Figure 19. Crosslinked Mouse Anti-HLA-DR (clone B8.12.2) and Mouse Anti-HLA-DR (clone L243) Direct Immunoaffinity with Western Blot

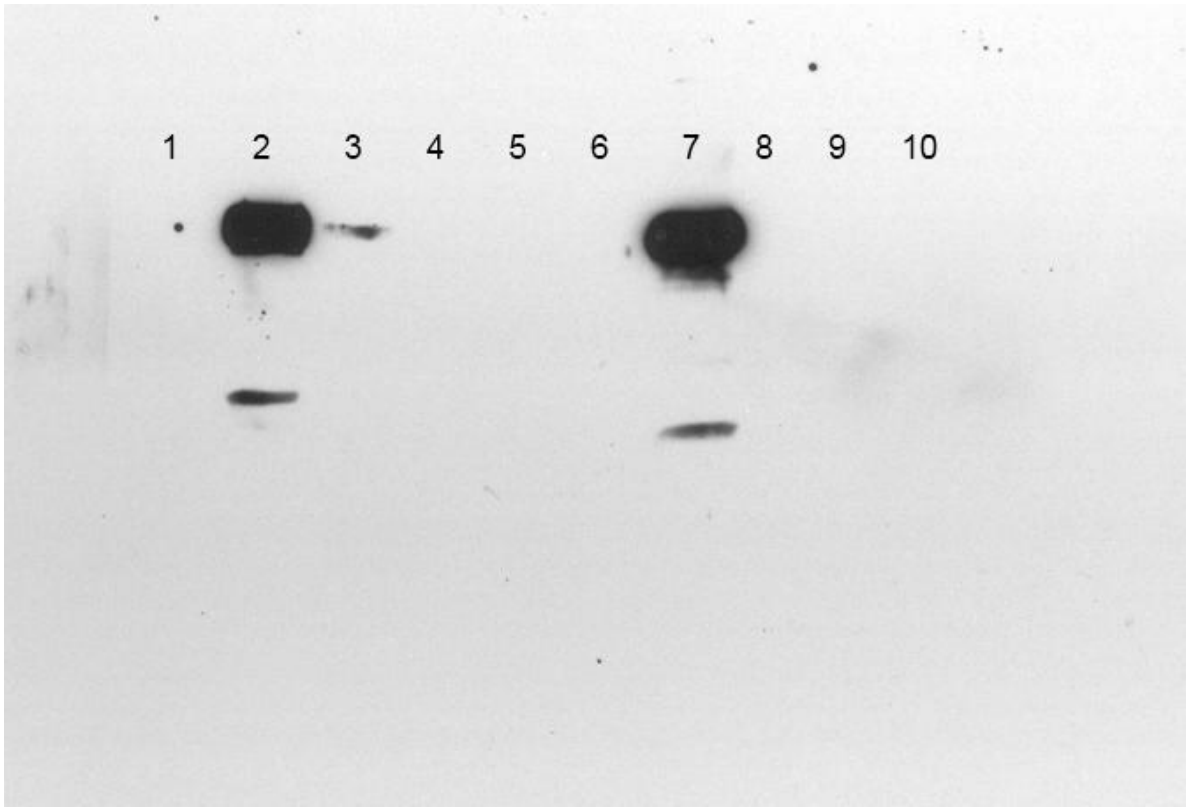

Figure 19. Western blot of crosslinked mouse anti-HLA-DR (clone B8.12.2) and mouse anti-HLA-DR (clone L243) direct immunoaffinity. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. L243 No Crosslinking Antibody Control, 3. L243 Crosslinking Antibody Control, 4. Lysate Preclearing Control, 5. Crosslinked L243 Lysate Test, 6. Space, 7. B8.12.2 No Crosslinking Antibody Control, 8. B8.12.2 Crosslinking Antibody Control, 9. Lysate Preclearing Control, 10. Crosslinked B8.12.2 Lysate Test.

The absence of bands in the Crosslinked L243 Lysate Test and Crosslinked B8.12.2 Lysate Test lanes indicated that the immunoaffinity reaction between the crosslinked beads and the HLA-DR positive lysate did not result in a positive reaction. The results further indicated that crosslinking either the anti-HLA-DR (clone L243) or the anti-HLA-DR (clone B8.12.2) interferes with its ability to bind antigen in the HLA-DR positive lysate control. The assay could

have been improved by running positive controls for the non-crosslinked immunoaffinity reactions HLA-DR positive lysate.

Rat Anti-HLA-DR Indirect Immunoaffinity and Western Blot I

Alternative antibodies were utilized in an attempt to optimize the immunoaffinity reaction that would capture HLA-DR and HLA Class I proteins. Separate indirect immunoaffinity reactions were completed against a HLA-DR positive lysate using rat anti-HLA Class I and rat anti-HLA-DR antibodies. The primary antibody used for the western blot in lanes 1 through 4 was the mouse anti-HLA Class I. A separate western blot was completed using the mouse anti-HLA-DR (clone B8.12.2) primary antibody for lanes 6 through 10. The two different antibodies were used in order to validate their use for the detection of proteins recognized by their specificity. The secondary antibody used for all lanes was goat anti-mouse IgG HRP. The image of this blot is depicted in Figure 20. Lanes 1, 6, and 7 contain molecular weight marker. Lane 2 contains eluate of the Dynabeads® Protein G incubated with rat anti-HLA Class I, referred to as the Rat HLA Class I Antibody Control. Lane 3 contains eluate from the reaction between uncoupled Dynabeads® Protein G and the HLA-DR positive lysate, and is referred to as a Lysate Preclearing Control. Lane 4 contains the immunoaffinity reaction between the rat anti-HLA Class I and the HLA-DR positive lysate, referred to as the Rat HLA Class I Lysate Test. Lane 5 is a space. Lane 8 contains eluate of the Dynabeads® Protein G incubated with rat anti-HLA-DR, referred to as the Rat HLA-DR Antibody Control. Lane 9 contains eluate from the reaction between uncoupled Dynabeads® Protein G and the HLA-DR positive lysate, and is referred to as a Lysate Preclearing Control. Lane 10 contains the immunoaffinity reaction between the rat anti-HLA-DR and the prepared HLA-DR positive lysate, referred to as the Rat HLA-DR Lysate Test.

Figure 20. Rat Anti-HLA-DR Indirect Immunoaffinity and Western Blot I

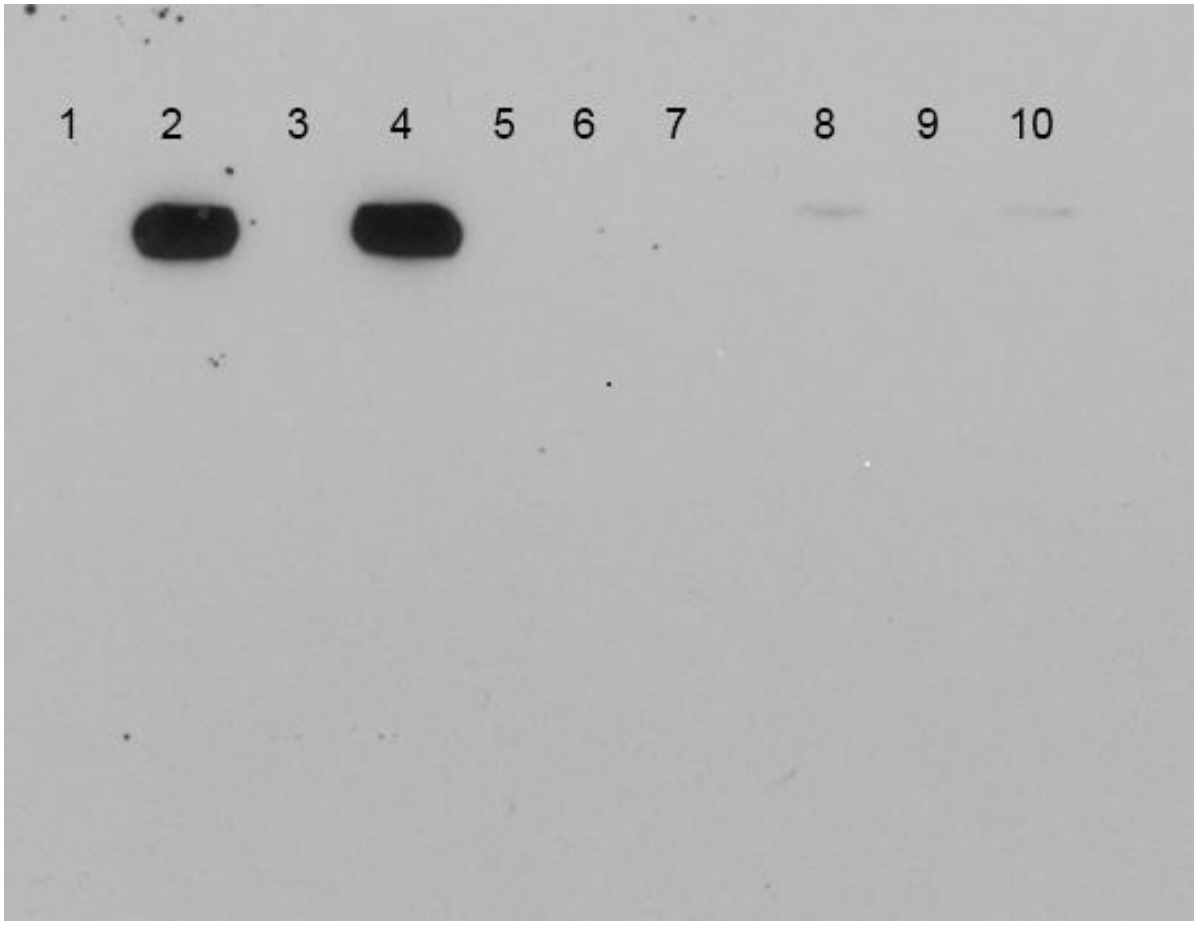

Figure 20. Western blot of rat anti-HLA-DR indirect immunoaffinity I. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. Rat HLA Class I Antibody Control, 3. Lysate Preclearing Control, 4. Rat HLA Class I Lysate Test, 5. Space, 6. Molecular weight marker (undetected), 7. Molecular weight marker (undetected), 8. Rat HLA-DR Antibody Control. 9. Lysate Preclearing Control, 10. Rat HLA-DR Lysate Test.

The results of the test indicated that neither the rat ant-HLA Class I nor rat anti-HLA-DR immunoaffinity antibodies resulted in a positive reaction with HLA Class I or HLA-DR proteins within the prepared human lysate.

Rat Anti-HLA-DR Indirect Immunoaffinity and Western Blot II

Additional indirect immunoaffinity reactions were completed to troubleshoot the rat anti-HLA-DR reaction above. The primary antibodies utilized were the rat ant-HLA-DR and the

mouse anti-HLA-DR (clone B8.12.2) against a HLA-DR positive lysate. The eluates were reduced and boiled, run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot the anti-HLA-DR (clone B8.12.2) and the secondary antibody used was goat anti-mouse IgG HRP. The image of this blot is depicted in Figure 21. Lane 1 contains molecular weight marker. Lane 2 contains eluate of the Dynabeads® Protein G incubated with rat anti-HLA-DR, referred to as the Rat HLA-DR Antibody Control. Lane 3 contains eluate from the reaction between uncoupled Dynabeads® Protein G and the HLA-DR positive lysate, and is referred to as a Lysate Preclearing Control. Lane 4 contains the immunoaffinity reaction between the rat anti-HLA-DR and the HLA-DR positive lysate, referred to as the Rat HLA-DR Lysate Test. Lane 5 is space. Lane 6 contains eluate of the Dynabeads® Protein G incubated with mouse anti-HLA-DR (clone B8.12.2), referred to as the Mouse HLA-DR Antibody Control. Lane 7 contains eluate from the reaction between uncoupled Dynabeads® Protein G and the HLA-DR positive lysate, and is referred to as a Lysate Preclearing Control. Lane 8 contains the immunoaffinity reaction between the mouse anti-HLA-DR (clone B8.12.2) and the HLA-DR positive lysate, referred to as the Mouse HLA-DR Lysate Test. Lanes 9 and 10 are spaces.

Figure 21. Rat Anti-HLA-DR Indirect Immunoaffinity and Western Blot II

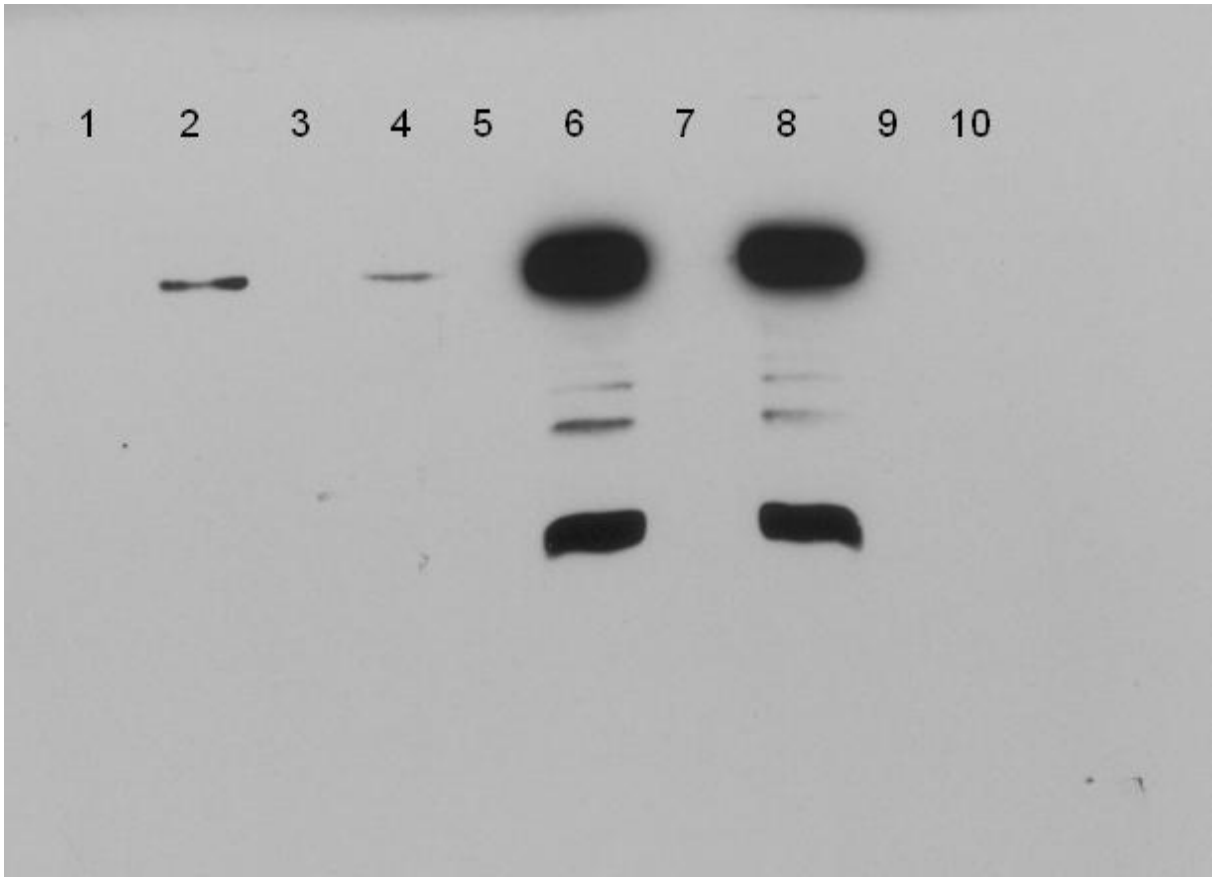

Figure 21. Western blot of rat anti-HLA-DR indirect immunoaffinity II. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. Rat HLA-DR Antibody Control, 3. Lysate Preclearing Control, 4. Rat HLA-DR Lysate Test, 5. Space, 6. Mouse HLA-DR Antibody Control, 7. Lysate Preclearing Control, 8. Mouse HLA-DR Lysate Test, 9. Space, 10. Space.

The results of this blot indicate that there was little to no HLA-DR protein detected in the rat anti-HLA-DR or mouse anti-HLA-DR reactions. This indicated that the HLA-DR11 positive lysate was not suitable as a positive control for this assay.

Rat Anti-HLA-DR Indirect Immunoaffinity and Western Blot III

The above assay was repeated using a new HLA-DR positive lysate. The image of this blot is depicted in Figure 22.

Figure 22. Rat Anti-HLA-DR Indirect Immunoaffinity and Western Blot III

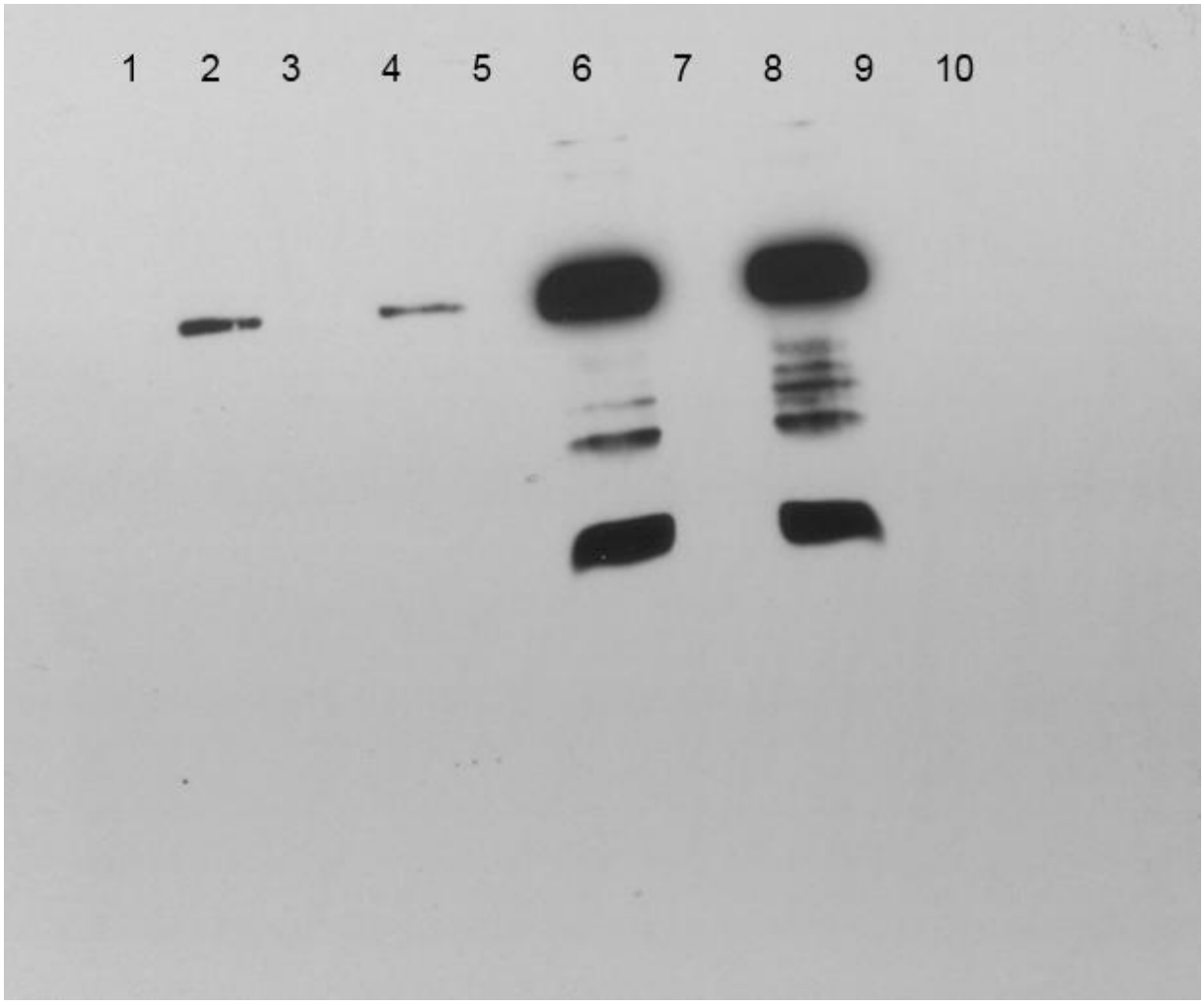

Figure 22. Western blot of rat anti-HLA-DR indirect immunoaffinity III. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. Rat HLA-DR Antibody Control, 3. Lysate Preclearing Control, 4. Rat HLA-DR Lysate Test, 5. Space, 6. Mouse HLA-DR Antibody Control, 7. Lysate Preclearing Control, 8. Mouse HLA-DR Lysate Test, 9. Space, 10. Space.

The results represented in Figure 22 demonstrated that there may have been inconsistency in the quality of the HLA-DR positive lysate, which had been used as a positive control. The HLA-DR positive lysate used in this assay appears have been positive in the Mouse HLA-DR Lysate Test but not in the Rat HLA-DR Test, indicating that the rat anti-HLA-DR might not be

suitable for an immunoaffinity reaction directed towards the M-M-R®-II vaccine preparation using this method.

Rabbit Anti-HLA-DRB1 Indirect Immunoaffinity and Western Blot I

A rabbit polyclonal anti-HLA-DRB1 antibody was utilized to further reduce background in the western blot due to co-elution of antibody with antigen from the Dynabeads® Protein G immunoaffinity reaction. Separate immunoaffinity reactions were completed against a HLA-DR positive lysate using the rabbit polyclonal anti-HLA-DRB1 either crosslinked or not crosslinked to the Dynabeads® Protein G. Another immunoaffinty reaction utilizing the mouse anti-HLA-DR (clone B8.12.2) was used as a positive control for the assay. The eluates were reduced and boiled, run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot the anti-HLA-DR (clone B8.12.2) and the secondary antibody used was goat anti-mouse IgG HRP. The image of this blot is depicted in Figure 23. Lane 1 contains molecular weight marker. Lane 2 contains eluate of the rabbit anti-HLA-DRB1 crosslinked to the Dynabeads® Protein G, referred to as Rabbit DRB1 Crosslinked Antibody Control. Lane 3 contains the immunaffinity reaction between the HLA-DR positive lysate and the rabbit DRB1 crosslinked to the Dynabeads® Protein G, referred to Rabbit Anti-HLA-DRB1 Crosslinked Test. Lane 4 contains the eluate from the reaction between uncoupled Dynabeads® Protein G and the HLA-DR positive lysate, and is referred to as a Lysate Preclearing Control. Lane 5 contains eluate of the rabbit anti-HLA-DRB1 coupled (not crosslinked) to the Dynabeads® Protein G, referred to as Rabbit DRB1 Not Crosslinked Antibody Control. Lane 6 contains the immunaffinity reaction between the HLA-DR positive lysate and the rabbit anti-HLA-DRB1 coupled to the Dynabeads® Protein G, referred to Rabbit DRB1 Not Crosslinked Test. Lane 7 contains the eluate from the reaction between uncoupled

Dynabeads® Protein G and the HLA-DR positive lysate, and is referred to as a Lysate Preclearing Control. Lane 8 contains eluate of the Dynabeads® Protein G incubated with mouse anti-HLA-DR (clone B8.12.2), referred to as the Mouse HLA-DR Antibody Control. Lane 9 contains the immunoaffinity reaction between the mouse anti-HLA-DR (clone B8.12.2) and the HLA-DR positive lysate, referred to as the Mouse HLA-DR Lysate Test. Lane 10 contains the eluate from the reaction between uncoupled Dynabeads® Protein G and the HLA-DR positive lysate, and is referred to as a Lysate Preclearing Control.

Figure 23. Rabbit Anti-HLA-DRB1 Indirect Immunoaffinity and Western Blot I

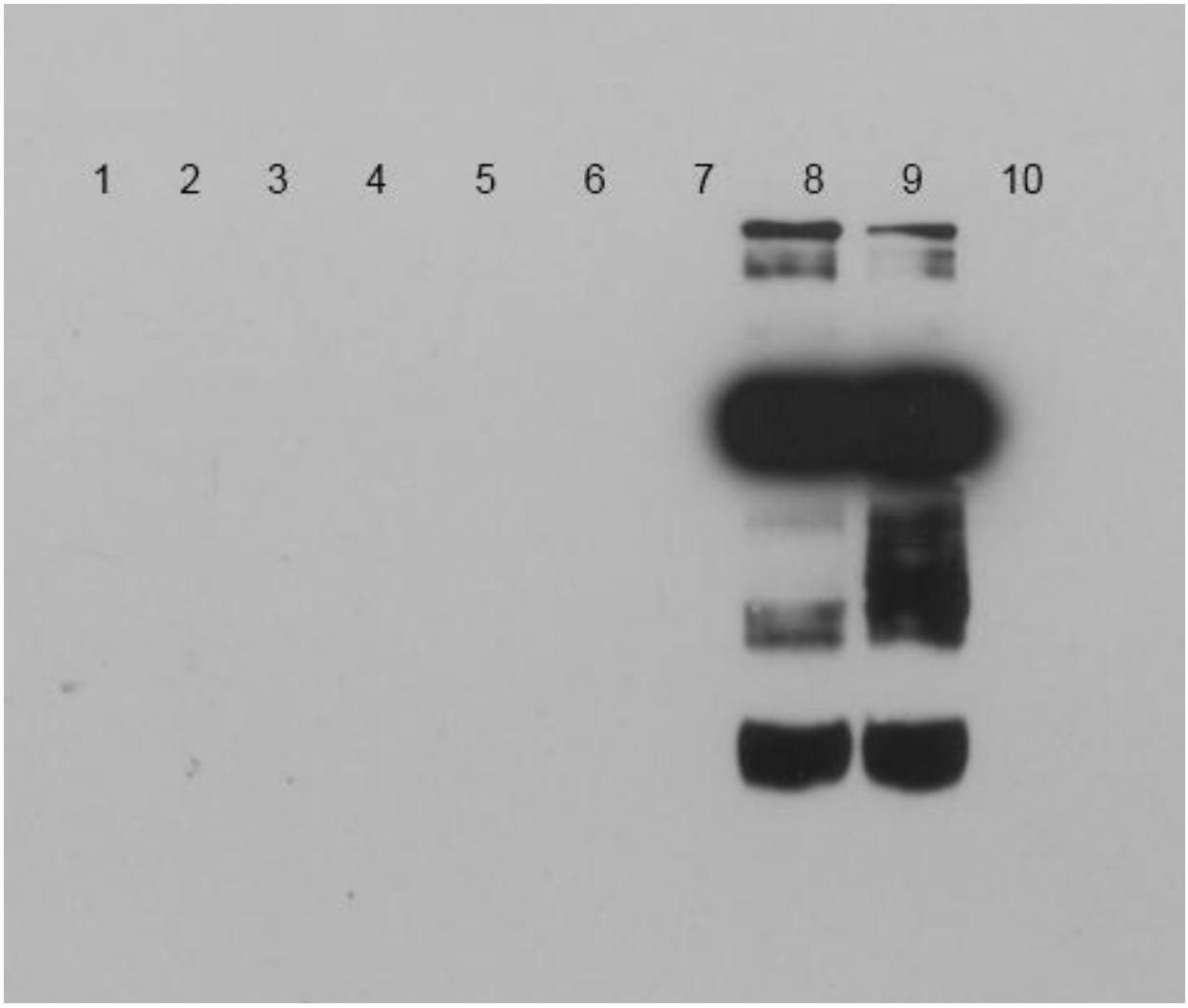

Figure 23. Western blot of rabbit anti-HLA-DRB1 indirect immunoaffinity I. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. Rabbit DRB1 Crosslinked Antibody Control, 3. Rabbit DRB1 Crosslinked Test, 4. Lysate Preclearing Control, 5. Rabbit DRB1 Not Crosslinked Antibody Control, 6. Rabbit DRB1 Not Crosslinked Test, 7. Lysate Preclearing Control, 8. Mouse HLA-DR Antibody Control, 9. Mouse HLA-DR Lysate Test, 10. Lysate Preclearing Control.

The results of this blot indicated that the rabbit polyclonal anti-HLA-DRB1 used in the crosslinked or non-crosslinked immunoaffinity reactions did not result in elution of HLA-DR protein when compared to the mouse anti-HLA-DR antibody.

Mouse Anti-HLA-DR (clone B8.12.2), Rat Anti-HLA-DR, and Rabbit Anti-HLA-DRB1 Indirect Immunoaffinity Validation of Ramos Whole Cell Lysate and Western Blot

It was determined that an additional positive control, the Ramos whole cell lysate, should be used in order supplement the development of the immunoaffinity purification assay and detection by western blot. This lysate was tested in separate indirect immunoaffinty reactions against the mouse monoclonal anti-HLA-DR (clone B8.12.2) antibody, the rat monoclonal anti-HLA-DR antibody, and the rabbit polyclonal anti-HLA-DRB1 antibody. The eluates were reduced and boiled, run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot the anti-HLA-DR (clone B8.12.2) and the secondary antibody used was goat anti-mouse IgG HRP. The results from this assay are demonstrated in Figure 24. Lane 1 contains molecular weight marker. Lane 2 contains eluate from the reaction between uncoupled Dynabeads® Protein G and the Ramos whole cell lysate and is referred to as a Ramos Lysate Preclearing Control. Lane 3 contains eluate of the Dynabeads® Protein G incubated with rat anti-HLA-DR, referred to as the Rat HLA-DR Antibody Control. Lane 4 contains the immunoaffinity reaction between the rat anti-HLA-DR and the Ramos whole cell lysate, referred to as the Rat HLA-DR Ramos Lysate Test. Lane 5 contains eluate of the Dynabeads® Protein G incubated with rabbit anti-HLA-DRB1 antibody, referred to as the Rabbit DRB1 Antibody Control. Lane 6 contains the immunoaffinity reaction between the rabbit anti-HLA-DRB1 and the Ramos whole cell lysate, referred to as the Rabbit DRB1 Ramos Lysate Test. Lane 7 contains eluate of the Dynabeads® Protein G incubated with mouse anti-HLA-DR, referred to as the Mouse HLA-DR Antibody Control. Lane 8 contains the immunoaffinity reaction between the mouse anti-HLA-DR and the Ramos whole cell lysate, referred to as the Mouse HLA-DR Ramos Lysate Test. Lane 9 contains the Ramos whole cell lysate loaded directly onto the gel as a western blot positive control, referred to as the Ramos

Western Blot Control. Lane 10 contains a Daudi cell lysate loaded directly onto the gel as an additional western blot positive control, referred to as the Daudi Western Blot Control.

Figure 24. Mouse Anti-HLA-DR (clone B8.12.2), Rat Anti-HLA-DR, and Rabbit Anti-HLA-DRB1 Indirect Immunoaffinity Validation of Ramos Whole Cell Lysate and Western Blot

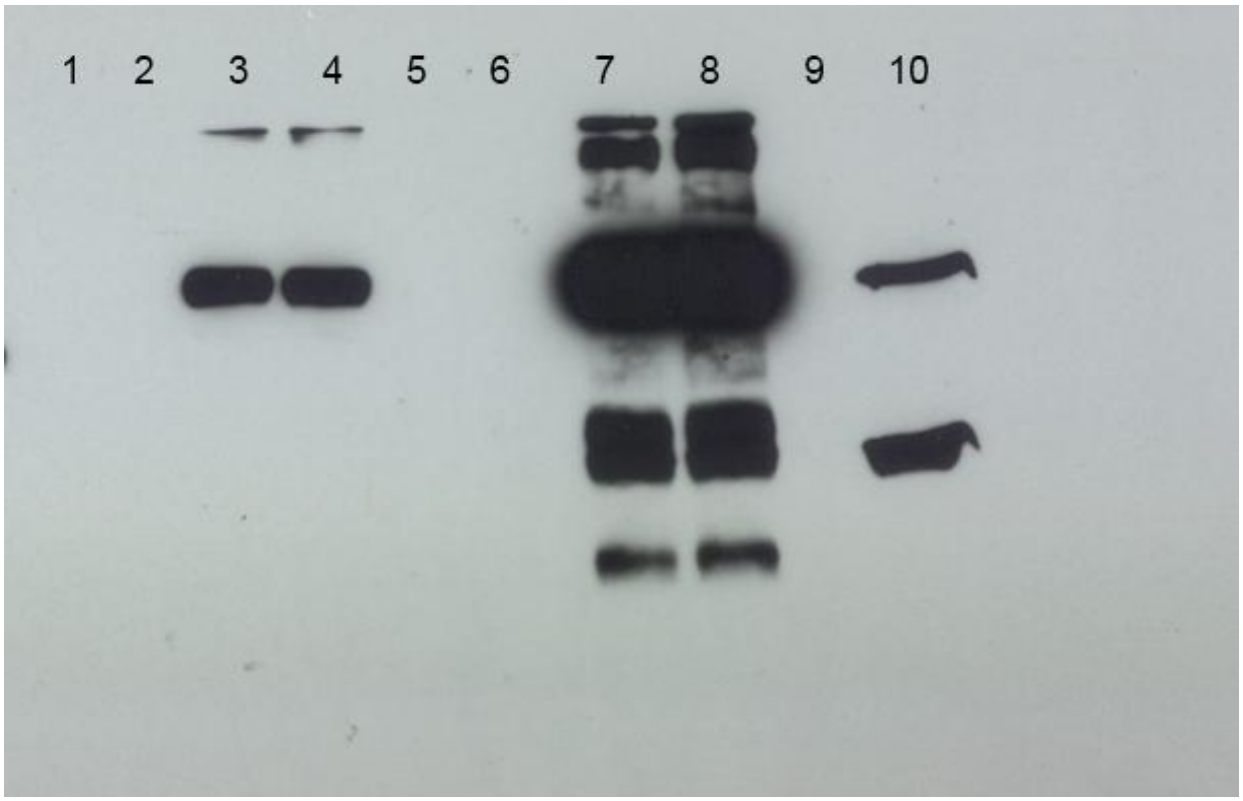

Figure 24. Western blot of mouse anti-HLA-DR (clone B8.12.2), rat Anti-HLA-DR, and rabbit anti-HLA-DRB1 indirect immunoaffinity validation of Ramos Whole Cell Lysate. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. Ramos Lysate Preclearing Control, 3. Rat HLA-DR Antibody Control, 4. Rat HLA-DR Ramos Lysate Test, 5. Rabbit DRB1 Antibody Control, 6. Rabbit DRB1 Ramos Lysate Test, 7. Mouse HLA-DR Antibody Control, 8. Mouse HLA-DR Ramos Lysate Test, 9. Ramos Western Blot Control, 10. Daudi Western Blot Control.

The results of this western blot demonstrated that immunoaffinity reactions between the Ramos whole cell lysate and the neither rat anti-HLA-DR, the rabbit anti-HLA-DRB1, nor the mouse anti-HLA-DR antibodies resulted in positive detection of unique bands on the blot when compared to their antibody controls. There were also no bands detected in the Ramos whole cell

lysate lane 9. The specificity of the bands in the Daudi cell lysate lane 9 is nuclear, as there are two unique bands, running at molecular weights of approximately 55 and 20 kDa. Because all of the eluates and the western blot controls were boiled for 5 minutes before loading them onto the SDS-PAGE gel, the HLA Class II protein was expected to be fully dissociated into their separate α and β subunits. The expected molecular weights of the HLA Class II α and β subunits are 34kDa and 29kDa, respectively. This western blot does not appear to detect unique bands running at either 34kDa or 29kDa.

Western Blot Evaluation of Positive Controls

In order to trouble shoot the apparent failure of the previous blot, another assay was set up to investigate the effect of HLA Class II protein subunit dissociation (induced by 5 minutes of boiling) and its detection in the western blot. Three western blots were set up. The samples loaded directly onto the SDS-PAGE gel were a HLA-DR positive lysate, the Ramos whole cell lysate, and Daudi cell lysate. Referred to as HLA-DR Positive Lysate, Ramos Lysate and Daudi Lysate in the Figures 25 and 26 below. They were all reduced by β-mercaptoethanol and either boiled for 5 minutes or not boiled at all. The primary antibody used lanes 1-7 of the Figure 25 blot were mouse anti-HLA-DR (clone B8.12.2). The primary antibody used for lanes 8-10 of the Figure 25 blot was mouse anti-HLA Class I. The primary antibody used in the Figure 26 blot was mouse anti-HLA-DR (clone L243). The secondary antibody used for all three blots was goat anti-mouse IgG HRP.

Figure 25. Western Blot Evaluation of Positive Controls I

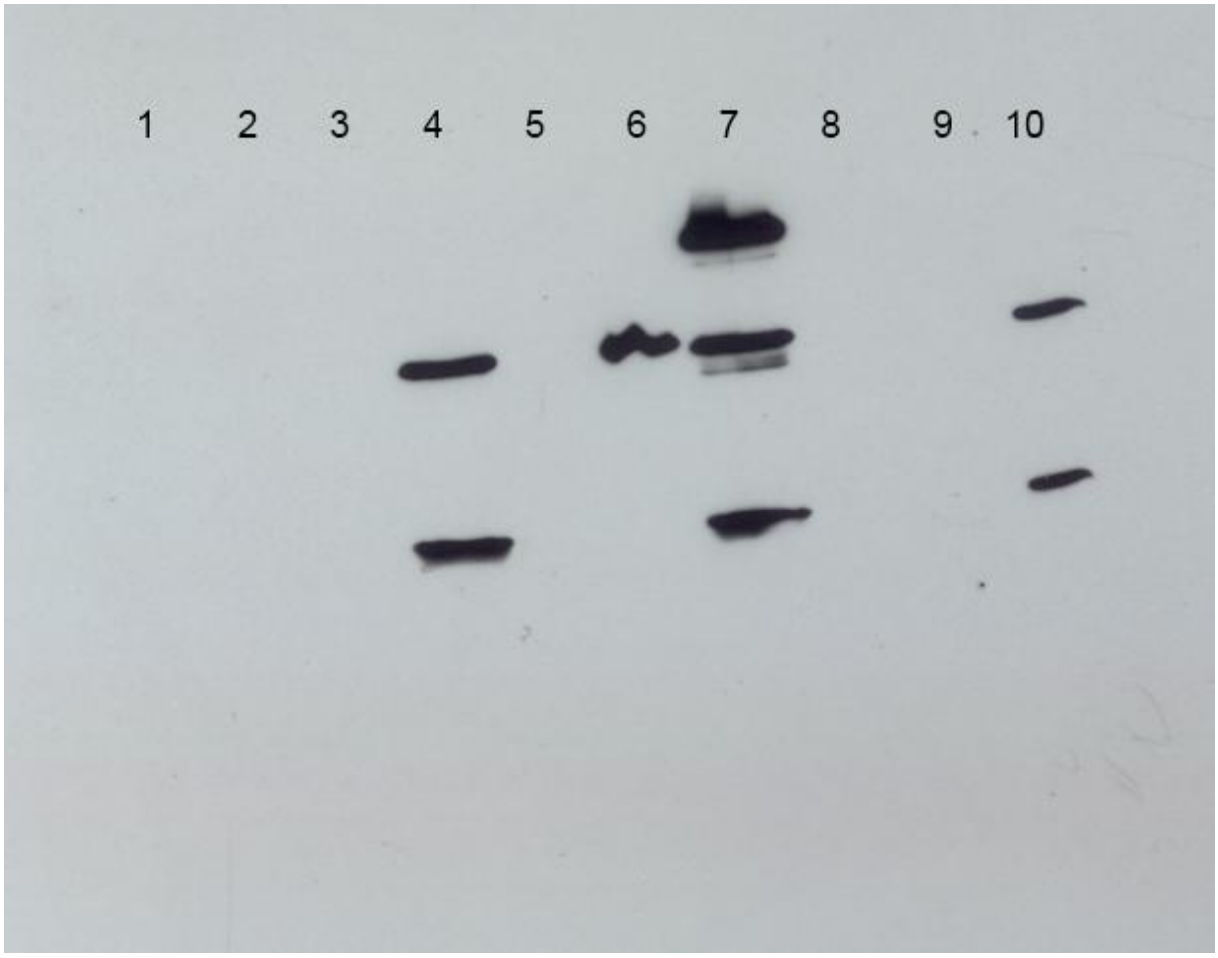

Figure 25. Western blot of evaluation of positive controls I. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. HLA-DR Positive Lysate (boiled), 3. Ramos Lysate (boiled), 4. Daudi Lysate (boiled), 5. HLA-DR Positive Lysate (not boiled), 6. Ramos Lysate (not boiled), 7. Daudi Lysate (not boiled), 8. HLA-DR Positive Lysate (boiled), 9. Ramos Lysate (boiled), 10. Daudi Lysate (boiled).

Figure 26. Western Blot Evaluation of Positive Controls II

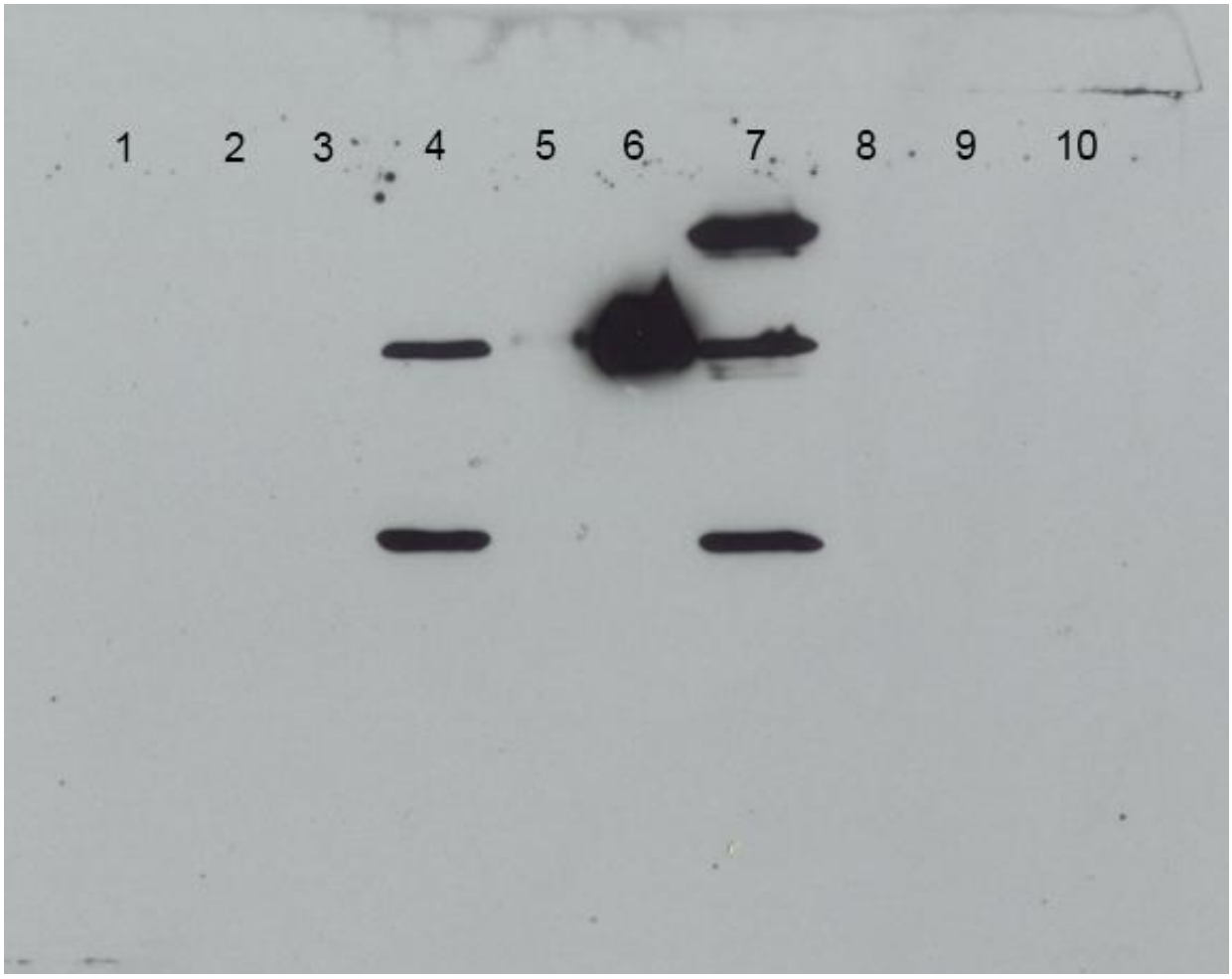

Figure 26. Western blot of evaluation of positive controls II. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. HLA-DR Positive Lysate (boiled), 3. Ramos Lysate (boiled), 4. Daudi Lysate (boiled), 5. HLA-DR Positive Lysate (not bolied), 6. Ramos Lysate (not boiled), 7. Daudi Lysate (not boiled), 8. Molecular weight marker (undetected), 9. Space, 10. Space.

The lack of detection of the Ramos Lysate in lanes 3 compared to lanes 6 within both Figures 25 and 26 demonstrated that Ramos whole cell lysate was an appropriate positive control only when not boiled prior to loading the sample onto the gel. As a result, all remaining samples loaded onto an SDS-PAGE gel for the remained of the project were not boiled.

As previously suggested, boiling HLA Class II protein dissociates the α and β subunits. This may limit detection by antibody if the epitope recognized by the detection antibody utilizes the structure of both α and β subunits. The blot utilizing mouse anti-HLA Class I was run for additional information and not as the focus of this assay. Therefore, it was not interpreted in the manner that the other lanes were. The bands detected in the lanes containing the Daudi cell lysate were of unknown specificity, as there molecular weights did not appear to coincide with the molecular weights of HLA Class II or Class I proteins. The lack of detection of the HLA-DR positive lysate further demonstrated its inconsistency as a positive control.

Rabbit Anti-HLA-DRB1 Indirect Immunoaffinity and Western Blot II

An indirect immunoaffinity reaction was completed against the Ramos whole cell lysate and the HLA-DR positive lysate. The eluates were reduced, run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot the anti-HLA-DR (clone L243) and the secondary antibody used was goat anti-mouse IgG HRP. Figure 27 depicts the western blot results. Lane 1 contains molecular weight makers loaded with .5 µg of mouse antibody in order to detect the heavy and light chains as marker on the blot. Lane 2 contains eluate of the Dynabeads® Protein G incubated with rabbit anti-HLA-DRB1 antibody, referred to as the Rabbit DRB1 Antibody Control. Lane 3 contains eluate from the reaction between uncoupled Dynabeads® Protein G and the Ramos whole cell lysate and is referred to as a Ramos Lysate Preclearing Control. Lane 4 contains the immunoaffinity reaction between the rabbit anti-HLA-DRB1 and the Ramos whole cell lysate, referred to as the Rabbit DRB1 Ramos Lysate Test. Lane 5 contains eluate from the reaction between uncoupled Dynabeads® Protein G and the HLA-DR positive lysate and is referred to as a HLA-DR Positive Lysate Preclearing Control. Lane 6 contains the immunoaffinity reaction

between the rabbit anti-HLA-DRB1 and the HLA-DR positive lysate, referred to as the Rabbit DRB1 HLA-DR Positive Lysate Test. Lane 7 is a space. Lane 8 contains Ramos whole cell lysate loaded directly onto the the SDS-PAGE gel, referred to as the Ramos Western Blot Control. Lane 9 is a space. Lane 10 contains the HLA-DR positive lysate loaded directly onto the gel, referred to as the HLA-DR Positive Lysate Western Blot Control.

Figure 27. Rabbit Anti-HLA-DRB1 Indirect Immunoaffinity and Western Blot II

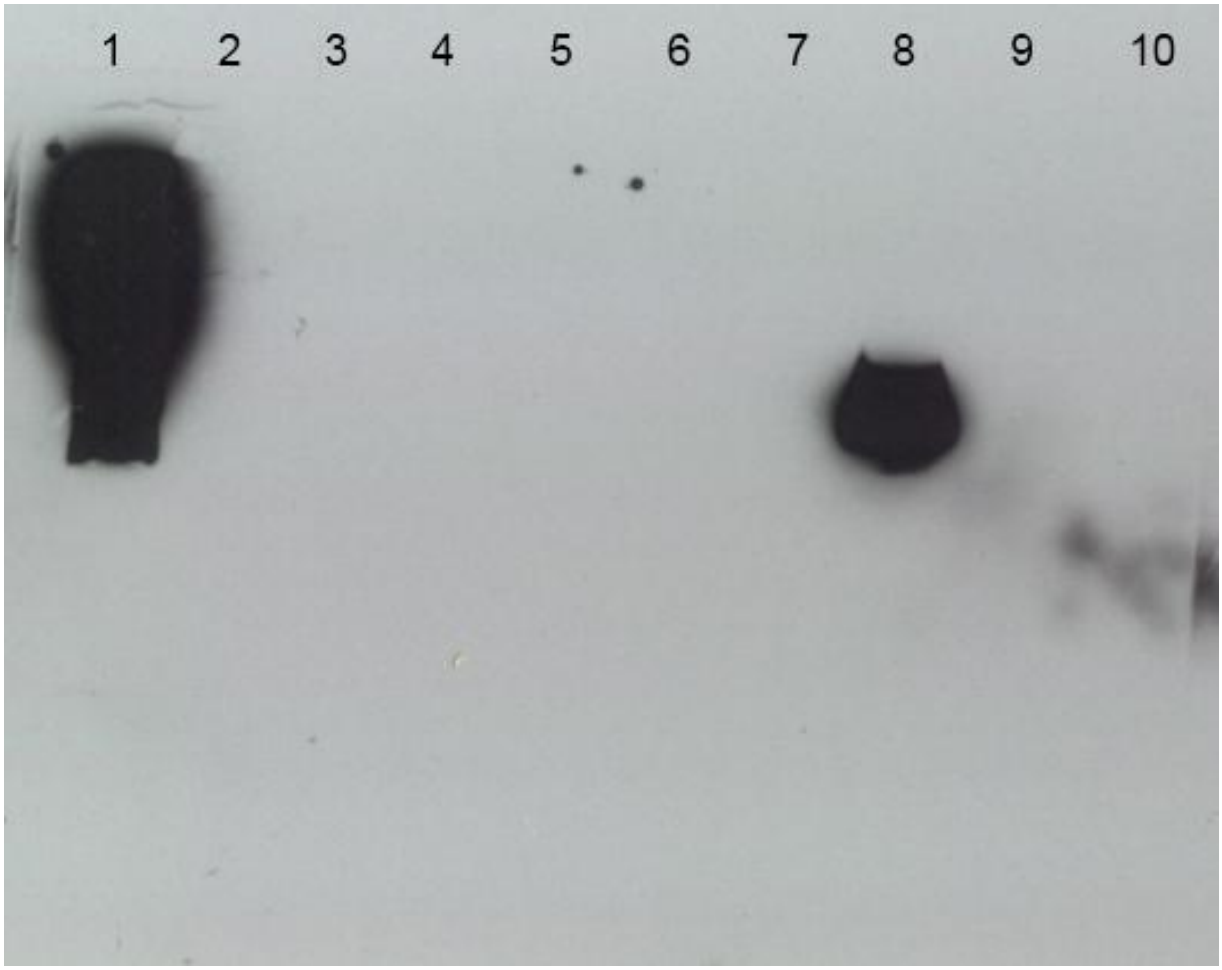

Figure 27. Western blot of rabbit anti-HLA-DRB1 indirect immunoaffinity. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker + mouse antibody, 2. Rabbit DRB1 Antibody Control, 3. Ramos Lysate Preclearing Control, 4. Rabbit DRB1 Ramos Lysate Test, 5. HLA-DR Positive Lysate Preclearing Control, 6. Rabbit DRB1 HLA-DR Positive Lysate, 7. Space, 8. Ramos Western Blot Control, 9. Space, 10. HLA-DR Positive Lysate Western Blot Control.

The results of this blot demonstrated that the immunoaffinity reaction between the rabbit anti-HLA-DRB1 and the Ramos whole cell lysate and the HLA-DR positive lysate failed to produce positive results. The detection of the band in lane 8 containing the Ramos whole cell lysate demonstrates that the western blot was a working assay. Again, the HLA-DR positive lysate was determined to be a failed, inappropriate positive control. The rabbit anti-HLA-DRB1 again failed to pull down HLA-DR protein from the Ramos whole cell lysate, indicating its failure as an immunoaffinity antibody for the assay.

Rat Anti-HLA-DR Indirect Immunoaffinity and Western Blot IV

Additional separate indirect immunoaffinity reactions were completed using the rat anti-HLA-DR and the mouse anti-HLA-DR (clone L243). The eluted proteins were run on the gel non-reduced. Detection was completed by three separate western blots. In Figure 28 in lanes 1-6 the primary antibody used was the mouse anti-HLA-DR (clone L243). In lanes 7-10 of the same figure, the primary antibody used was mouse anti-human IgG1 isotype control. The secondary antibody used for the western blot was goat anti-mouse IgG HRP. In Figure 28 lane 1 is loaded with a molecular weight marker. Lane 2 contains the eluate of the Dynabeads® Protein G incubated with rat anti-HLA-DR antibody, referred to as the Rat HLA-DR Antibody Control. Lane 3 contains eluate of the reaction between uncoupled Dynabeads® Protein G and the Ramos whole cell lysate and is referred to as a Ramos Lysate Preclearing Control. Lane 4 contains the eluate of the immunoaffinity reaction between the rat anti-HLA-DR and the Ramos whole cell lysate, referred to as the Rat HLA-DR Ramos Lysate Test. Lane 5 contains eluate of the reaction between uncoupled Dynabeads® Protein G and a HLA-DR positive lysate and is referred to as a HLA-DR Preclearing Control. Lane 6 contains the eluate of the immunoaffinity reaction between the rat anti-HLA-DR and the HLA-DR positive lysate, referred to as the Rat HLA-DR

Lysate Test. Lane 7 contains a molecular weight marker. Lane 8 contains Ramos whole cell lysate loaded directly onto the gel, referred to as Ramos Lysate Control. Lane 9 is an empty space. Lane 10 contains HLA-DR positive lysate loaded directly onto the gel, referred to as HLA-DR Lysate Control.

Figure 28. Rat Anti-HLA-DR Indirect Immunoaffinity and Western Blot IV

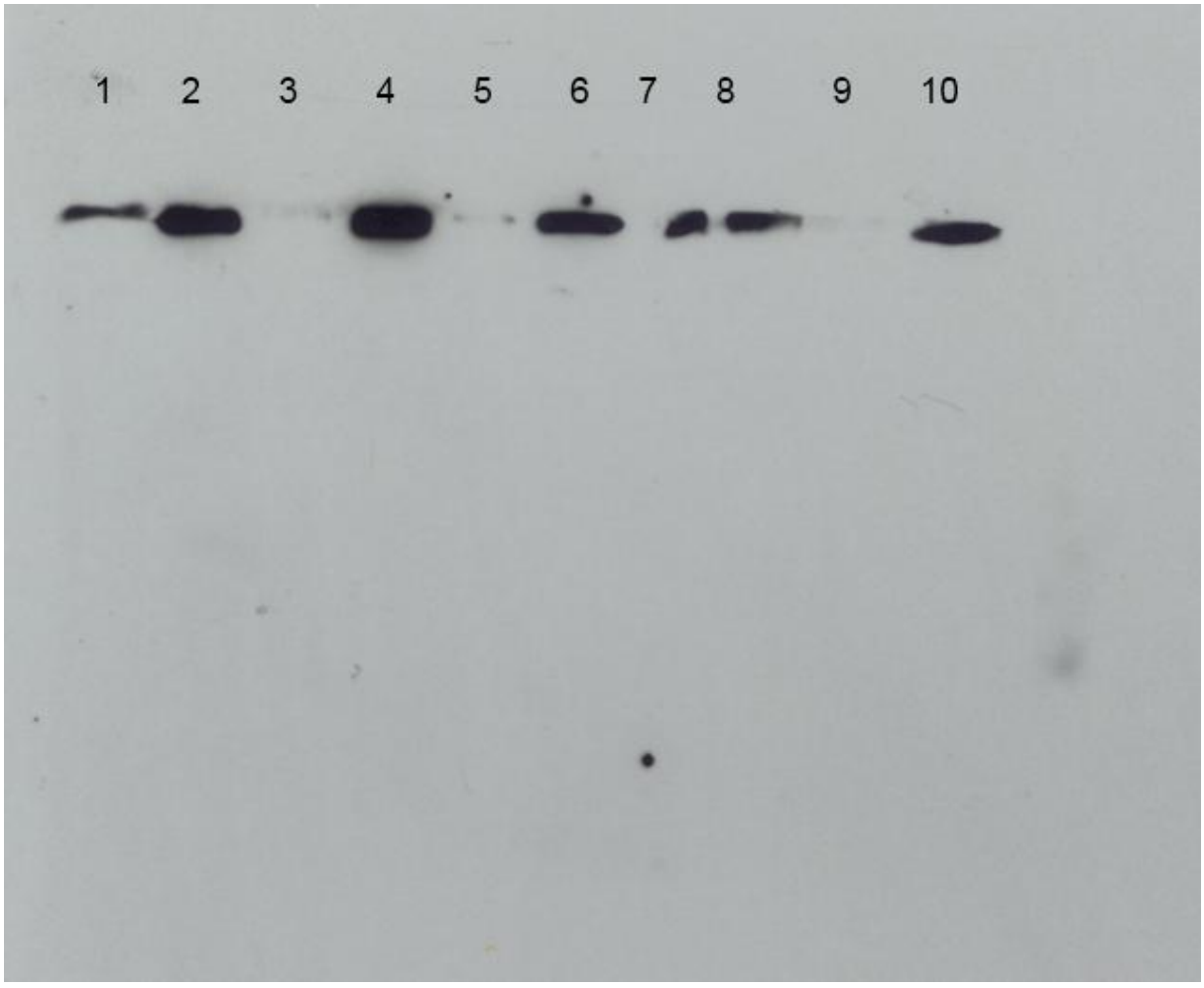

Figure 28. Western blot of rat anti-HLA-DR indirect immunoaffinity IV. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected) , 2. Rat HLA-DR Antibody Control, 3. Ramos Lysate Preclearing Control, 4. Rat HLA-DR Ramos Lysate Test, 5. HLA-DR Lysate Preclearing Control, 6. Rat HLA-DR Lysate Test, 7. Molecular weight marker (undetected), 8. Ramos Lysate Control, 9. Space, 10. HLA-DR Lysate Control.

The blot in Figure 28 demonstrated that the immunoaffinity reaction between the rat anti-HLA-DR did not result in positive detection of HLA-DR protein, as the tests lanes 4 and 6 appear to be no different from the antibody control lane 2. The band in lane 1 appears to have been spill over from lane 2.

The bands detected in the isotype control blot, lanes 7-10, were assumed to be the secondary antibody reacting with the mouse anti-human IgG1 that has bound to the lysates. The bands across the top of both blots in Figure 28 were assumed to be antibody specific bands with an expected running molecular weight of 150kDa when loaded non-reduced and not-boiled. Below these bands there was no evidence of HLA-DR protein bands running at an expected molecular weight on approximately 55kDa.

The lack of detection of the Ramos Lysate control in lane 8 demonstrates a lack of non-specific detection of HLA-DR protein by the primary antibody. This further indicated that the HLA-DR protein detected by the mouse anti-HLA-DR antibodies, clones L243 and B8.12.2, in the western blot assays was specific.

Mouse Anti-HLA-DR (clone L243) Indirect Immunoaffinity and Western Blot

In Figure 29, the primary antibody used for the entire blot was mouse anti-HLA-DR (clone L243). The secondary antibody used for the blot was the goat anti-mouse IgG HRP. The eluted proteins were run on the gel non-reduced. Lane 1 contains molecular weight marker. Lane 2 contains the eluate of the Dynabeads® Protein G incubated with mouse anti-HLA-DR (L243) antibody, referred to as the Mouse HLA-DR Antibody Control. Lane 3 contains eluate of the reaction between uncoupled Dynabeads® Protein G and the Ramos whole cell lysate and is referred to as a Ramos Lysate Preclearing Control. Lane 4 contains the eluate of the immunoaffinity reaction between the mouse anti-HLA-DR and the Ramos whole cell lysate,

referred to as the Mouse HLA-DR Ramos Lysate Test. Lane 5 contains eluate of the reaction between uncoupled Dynabeads® Protein G and a HLA-DR positive lysate and is referred to as a HLA-DR Lysate Preclearing Control. Lane 6 contains the eluate of the immunoaffinity reaction between the mouse anti-HLA-DR and the HLA-DR positive lysate, referred to as the Mouse HLA-DR Lysate Test. Lane 7 is an empty space. Lane 8 contains Ramos whole cell lysate loaded directly onto the gel, referred to as the Ramos Lysate Western Blot Control. Lane 9 is an empty space. Lane 10 contains HLA-DR positive lysate loaded directly onto the gel, referred to as the HLA-DR Positive Lysate Western Blot Control.

Figure 29. Mouse Anti-HLA-DR (clone L243) Indirect Immunoaffinity and Western Blot

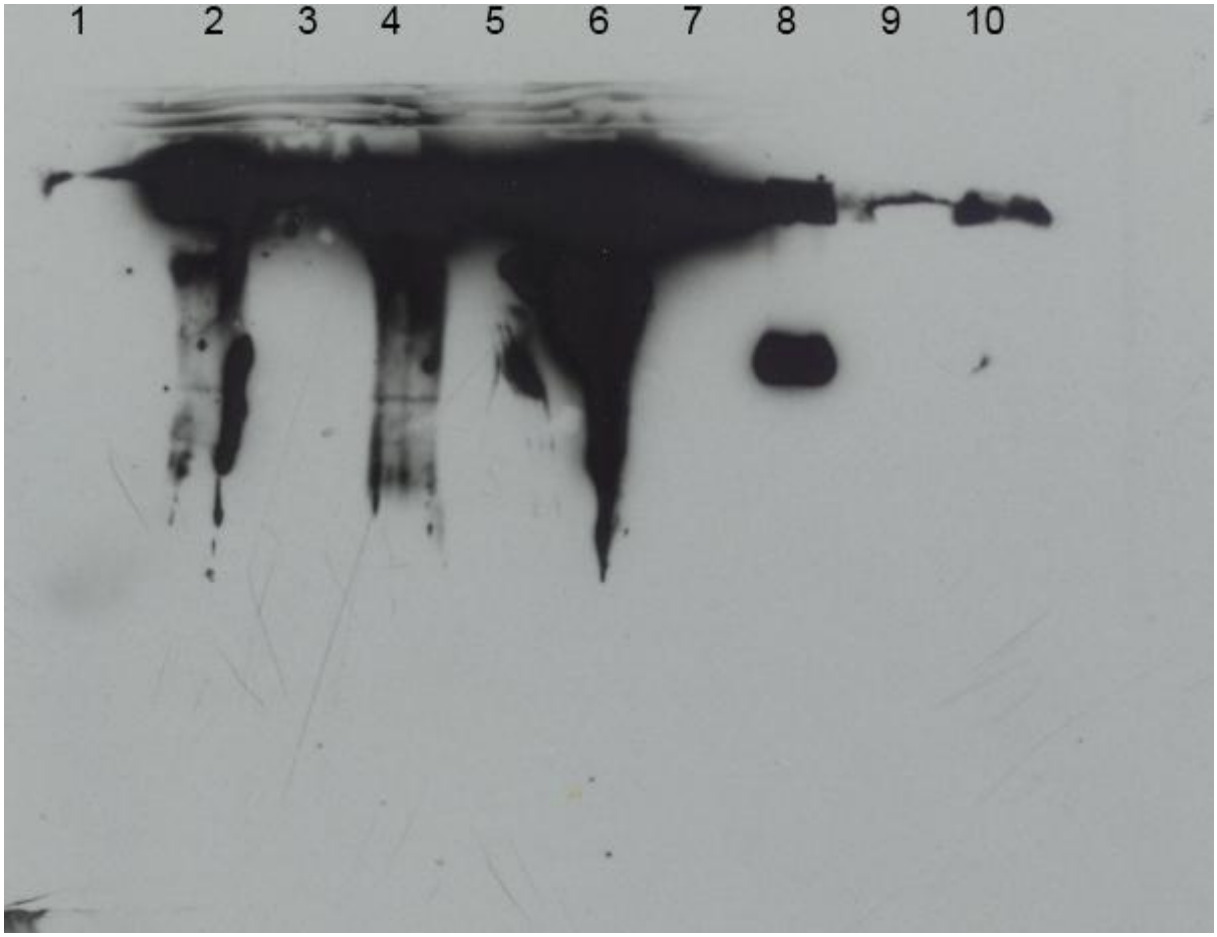

Figure 29. Western blot of mouse anti-HLA-DR (clone L243) indirect immunoaffinity. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected) , 2. Mouse HLA-DR Antibody Control, 3. Ramos Lysate Preclearing Control, 4. Mouse HLA-DR Ramos Lysate Test, 5. HLA-DR Lysate Preclearing Control, 6. Mouse HLA-DR Lysate Test, 7. Space, 8. Ramos Lysate Western Blot Control, 9. Space, 10. HLA-DR Positive Lysate Western Blot Control.

The blot in Figure 29 demonstrates the degree to which the co-eluting mouse anti-HLA-DR of the immunoaffinity reaction interfered with detection of potential HLA-DR specific protein bands. There was not a detectable difference between the antibody control in lane 2 and the lysate tests in lanes 4 and 6. The band in lane 8 appears to be specific to HLA-DR, demonstrating the utility of the Ramos whole lysate as an appropriate control for the blot. Again,

the HLA-DR positive lysate does not appear to demonstrate a band specific to HLA-DR in lane 10, indicating it was an inappropriate positive control for the western blot and the immunoaffinity reaction test.

Anti-HLA-DR (clone L243) Antibody Immobilization Immunoaffinity Western Blot I

In order to reduce excess background on the western blot, immunoaffinity reactions using anti-HLA-DR (clone L243) were completed according to the Antibody Immobilization Immunoaffinity method. Elution of the protein from the resin was completed twice. The first and seconds sets of eluates were reduced and run on separate SDS-PAGE gels, transferred to nitrocellulose membranes, and detected by western blot. The primary antibody used for the blots was the anti-HLA-DR (clone L243) and the secondary antibody used was goat anti-mouse IgG HRP. The results of the first set of elutions are demonstrated in the blot in Figure 30. The results of the second set of elutions are demonstrated in the blot in Figure 31. Lane 1 contains molecular weight marker. Lane 2 contains eluate from the mouse anti-HLA-DR (clone L243) coupled AminoLink® Plus Coupling Resin, referred to as the HLA-DR Antibody Control. Lane 3 contains eluate from the Pierce Control Agarose Resin (crosslinked 4% beaded agarose) incubated with the Ramos whole cell lysate, referred to as the Ramos Lysate Control. Lane 4 contains eluate of the immunoaffinity reaction between the anti-HLA-DR (clone L243) coupled AminoLink® Plus Coupling Resin and the Ramos whole cell lysate, referred to as the Ramos Lysate Test. Lane 5 contains eluate from the Pierce Control Agarose Resin (crosslinked 4% beaded agarose) incubated with a new HLA-DR positive lysate, referred to as the New HLA-DR Positive Lysate Control. Lane 6 contains eluate of the immunoaffinity reaction between the anti-HLA-DR (clone L243) coupled AminoLink® Plus Coupling Resin and the new HLA-DR positive lysate, referred to as the New HLA-DR Positive Lysate Test. Lane 7 contains eluate

from the Pierce Control Agarose Resin (crosslinked 4% beaded agarose) incubated with an older HLA-DR positive lysate, referred to as the Old HLA-DR Positive Lysate Control. Lane 8 contains eluate of the immunoaffinity reaction between the anti-HLA-DR (clone L243) coupled AminoLink® Plus Coupling Resin and older HLA-DR positive lysate, referred to as the Old HLA-DR Positive Lysate Test. Lane 9 contains Ramos whole cell lysate loaded directly onto the SDS-PAGE gel, referred to as the Ramos Western Blot Control. Lane 10 contains the new HLA-DR positive lysate loaded directly onto the gel, referred to as the New HLA-DR Positive Western Blot Control.

Figure 30. Anti-HLA-DR (clone L243) Antibody Immobilization Immunoaffinity Western Blot I - Elution 1

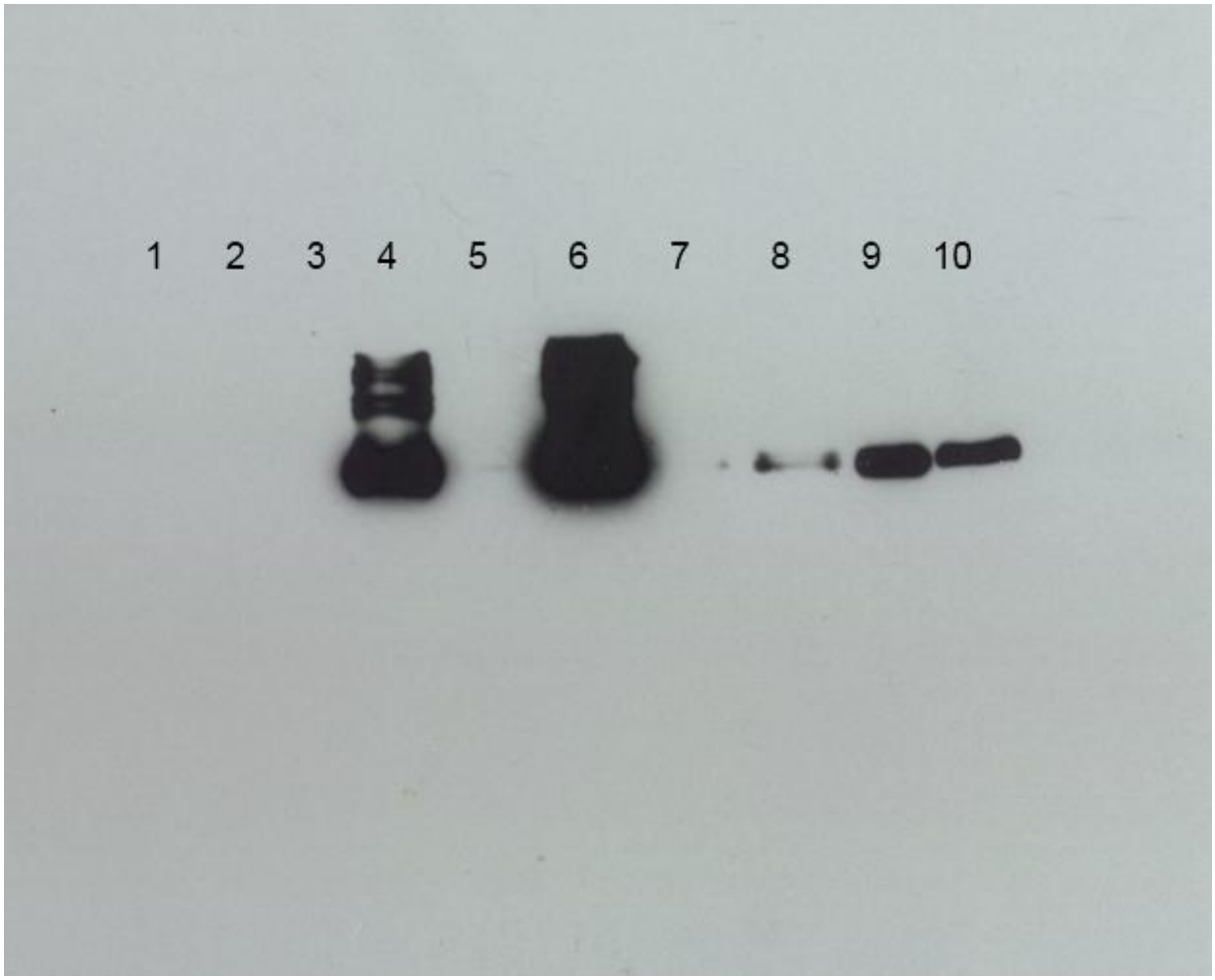

Figure 30. Western blot of anti-HLA-DR (clone L243) antibody immobilization immunoaffinity I (first elution). Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. HLA-DR Antibody Control, 3. Ramos Lysate Control, 4. Ramos Lysate Test, 5. New HLA-DR Positive Lysate Control, 6. New HLA-DR Positive Lysate Test 7. Old HLA-DR Positive Lysate Control, 8. Old HLA-DR Positive Lysate Test, 9. Ramos Western Blot Control, 10. New HLA-DR Positive Western Blot Control.

Figure 31. Anti-HLA-DR (clone L243) Antibody Immobilization Immunoaffinity Western Blot I - Elution 2

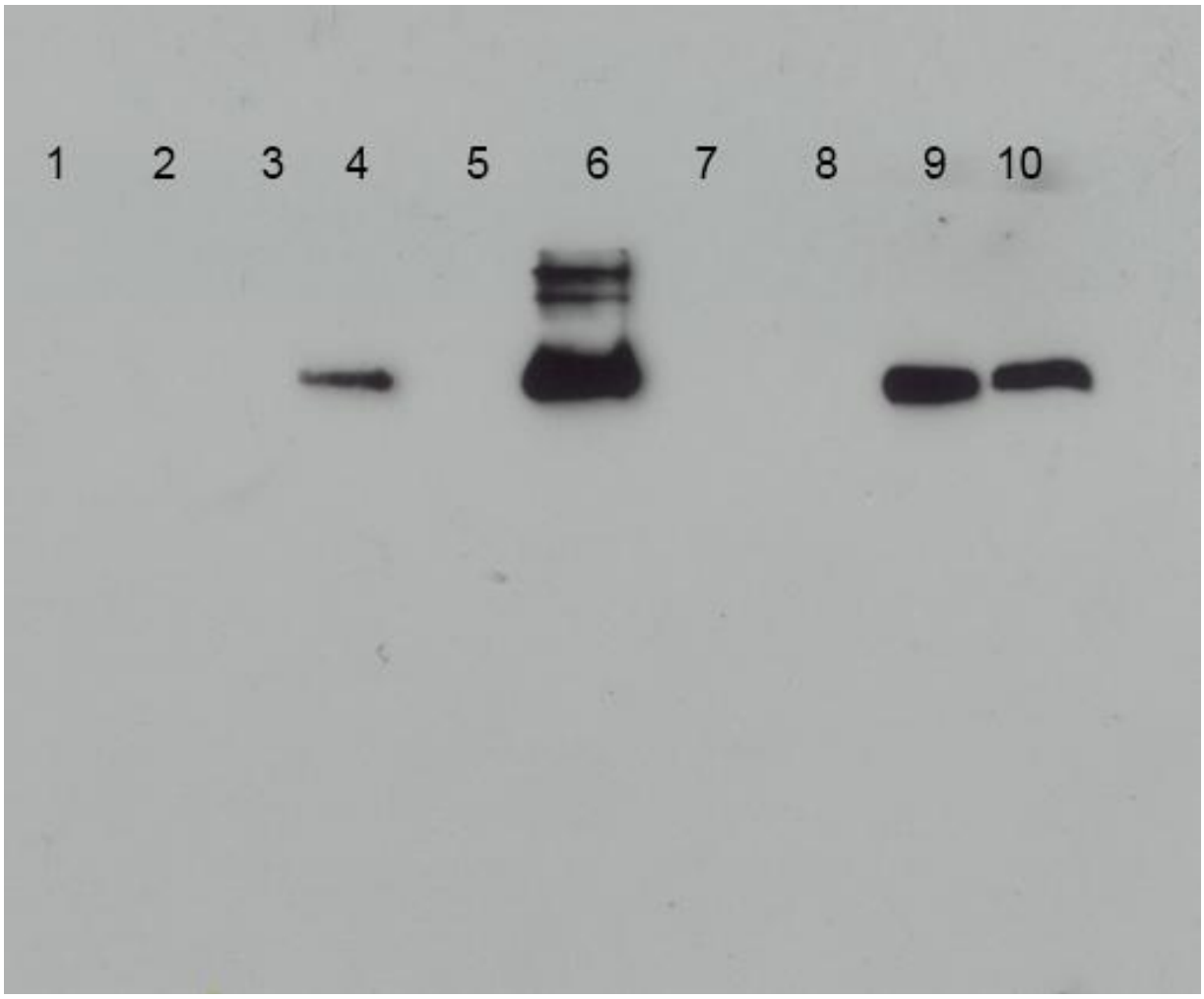

Figure 31. Western blot of anti-HLA-DR (clone L243) antibody immobilization immunoaffinity I (second elution). Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. HLA-DR Antibody Control, 3. Ramos Lysate Control, 4. Ramos Lysate Test, 5. New HLA-DR Positive Lysate Control, 6. New HLA-DR Positive Lysate Test 7. Old HLA-DR Positive Lysate Control, 8. Old HLA-DR Positive Lysate Test, 9. Ramos Western Blot Control, 10. New HLA-DR Positive Western Blot Control.

The results of the above immunoaffinity reactions indicated that there was significantly less background detected in the western blot when compared with the Direct and Indirect immunoaffinity reactions. This allowed for more precise visualization of the proteins isolated from the immunoaffinity reactions using the anti-HLA DR (clone L243). In Figure 30, the

Ramos whole cell lysate, New HLA-DR positive lysate, and Old HLA-DR positive lysate test lanes demonstrated some degree of banding. This was assumed to be specific to HLA-DR proteins. The excess signal in lanes 4 and 6 may have been due to HLA-DR protein aggregates. In Figure 31, the second elutions from the resin also demonstrated the same results, though at decreased intensities. In both Figures 30 and 31, the Ramos Western Blot Control and HLA-DR Positive Western Blot Control demonstrate distinct banding, indicating the validity of western blot assay to detect HLA-DR protein.

Anti-HLA-DR (clone L243) Antibody Immobilization Immunoaffinity Western Blot II

Immunoaffinity reactions using anti-HLA-DR (clone L243) were completed according to the Antibody Immobilization Immunoaffinity method. Two vials of Verivax® vaccine were combined in 400 µl of sterile diluent and assayed for the presence of HLA-DR protein. The eluates were reduced and run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot was the anti-HLA-DR (clone L243) and the secondary antibody used was goat anti-mouse IgG HRP. The results are demonstrated in the blot in Figure 32. Lane 1 contains molecular weight marker. Lane 2 contains eluate from the anti-HLA-DR (clone L243) coupled AminoLink® Plus Coupling Resin, referred to as the HLA-DR Antibody Control. Lane 3 contains eluate from the Pierce Control Agarose Resin (crosslinked 4% beaded agarose) incubated with the Verivax® vaccine preparation, referred to as the Verivax® Preclearing Control. Lane 4 contains eluate of the immunoaffinity reaction between the anti-HLA-DR (clone L243) coupled AminoLink® Plus Coupling Resin and the Verivax® vaccine preparation, referred to as the Verivax® Test. Lane 5 contains eluate from the Pierce Control Agarose Resin (crosslinked 4% beaded agarose) incubated with the 500 µg of Ramos whole cell lysate protein, referred to as the Ramos 500

Preclearing Control. Lane 6 contains eluate of the immunoaffinity reaction between the anti-HLA-DR (clone L243) coupled AminoLink® Plus Coupling Resin and 500 µg of Ramos whole cell lysate, referred to as the Ramos 500 Test. Lane 7 contains elaute from the Pierce Control Agarose Resin (crosslinked 4% beaded agarose) incubated with the 25 µg of Ramos whole cell lysate protein, referred to as the Ramos 25 Preclearing Control. Lane 8 contains eluate of the immunoaffinity reaction between the anti-HLA-DR (clone L243) coupled AminoLink® Plus Coupling Resin and 25 µg of Ramos whole cell lysate, referred to as the Ramos 25 Test. Lane 9 contains 25 µg of Ramos whole cell lysate protein loaded directly onto the SDS-PAGE gel, referred to as the Ramos 25 Western Blot Control. Lane 10 is an empty space.

Figure 32. Anti-HLA-DR (clone L243) Antibody Immobilization Immunoaffinity Western Blot II

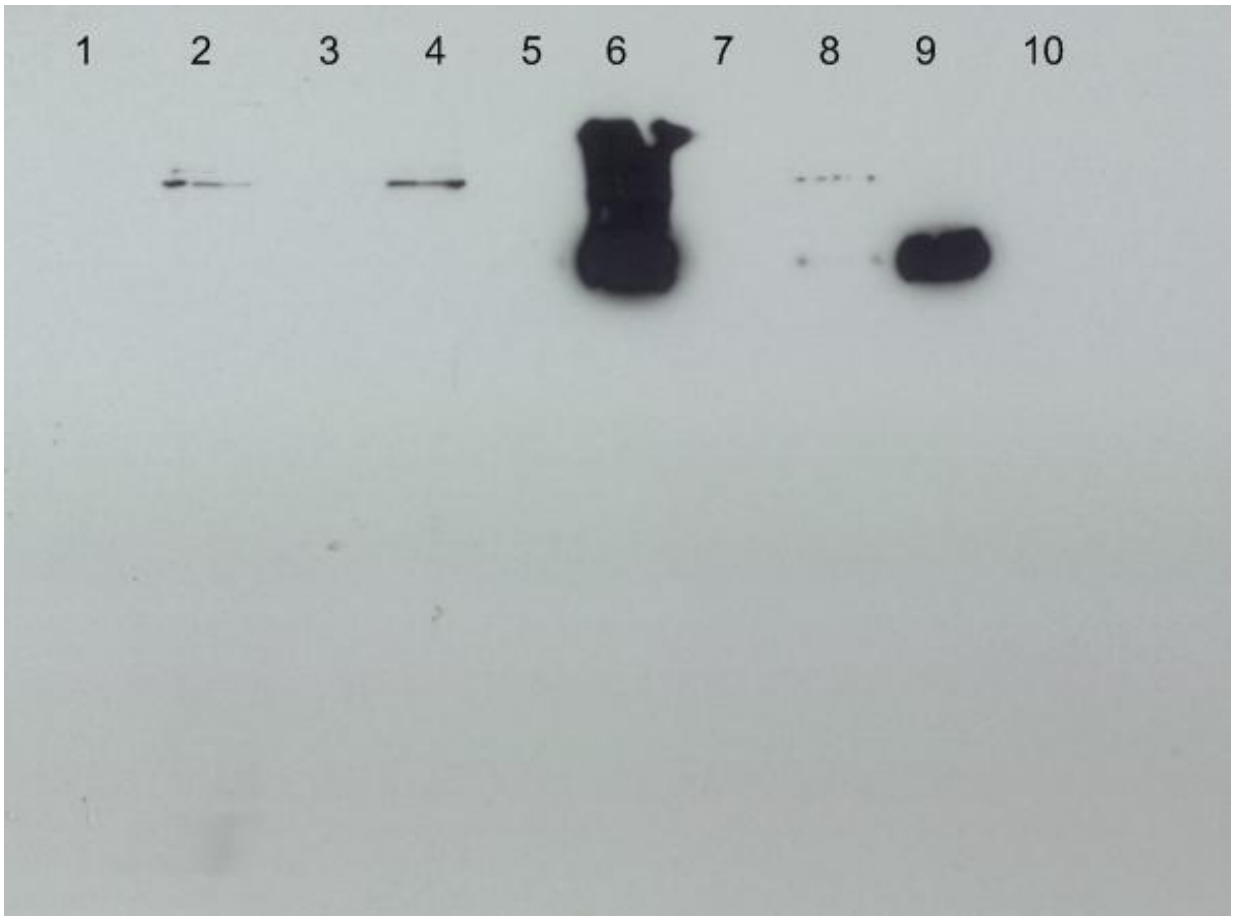

Figure 32. Western blot of anti-HLA-DR (clone L243) antibody immobilization immunoaffinity II. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. HLA-DR Antibody Control, 3. Verivax® Preclearing Control, 4. Verivax® Test, 5. Ramos 500 Preclearing Control, 6. Ramos 500 Test, 7. Ramos 25 Preclearing Control, 8. Ramos 25 Test, 9. Ramos 25 Western Blot Control. 10. Space.

The western blot result of Figure 32 indicated that the Verivax® vaccine preparation was negative for HLA-DR protein. Although there is slight banding in the Verivax® Test lane (lane 4), it was comparable to the band present in the HLA-DR Antibody Control lane (lane 2), indicating that the band was likely specific to the anti-HLA-DR antibody that has been detected as co-eluate from the resin. The Ramos 500 Test appeared as strong banding in lane 6. The Ramos 25 Test appeared as a very slight, distinct band when compared to the band of the Ramos

25 Western Blot Control. The strong difference in intensities between the Ramos 25 Test and Ramos 25 Western Blot control bands indicated the decreased sensitivity of the immunoaffinity reaction to concentrate the HLA-DR protein when compared to loading the protein directly onto the gel.

Western Blot of Verivax® Vaccine Preparation

An attempt was made to directly detect HLA-DR protein in the Verivax® vaccine by the Western Blot method. Lane 1 contains molecular weight marker. Lane 2 contains Ramos whole cell lysate was loaded onto the gel as a western blot control, referred to Ramos Western Blot Control. Lane 3 is a space. Lane 4 contains three vials of Verivax® vaccine which were reconstituted and combined in a total of 500 ul sterile diluent. The resulting preparation was concentrated according to the Amicon® Concentration method to a volume of less than 25 ul and loaded directly onto and SDS-PAGE gel. This is referred to the Verivax® Test. Lanes 5 through 10 were empty spaces. The samples were run reduced on an SDS-PAGE gel, transferred to the nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot was the anti-HLA-DR (clone L243) and the secondary antibody used was goat anti-mouse IgG HRP. The results are demonstrated in the blot in Figure 33.

Figure 33. Western Blot of Verivax® Vaccine Preparation

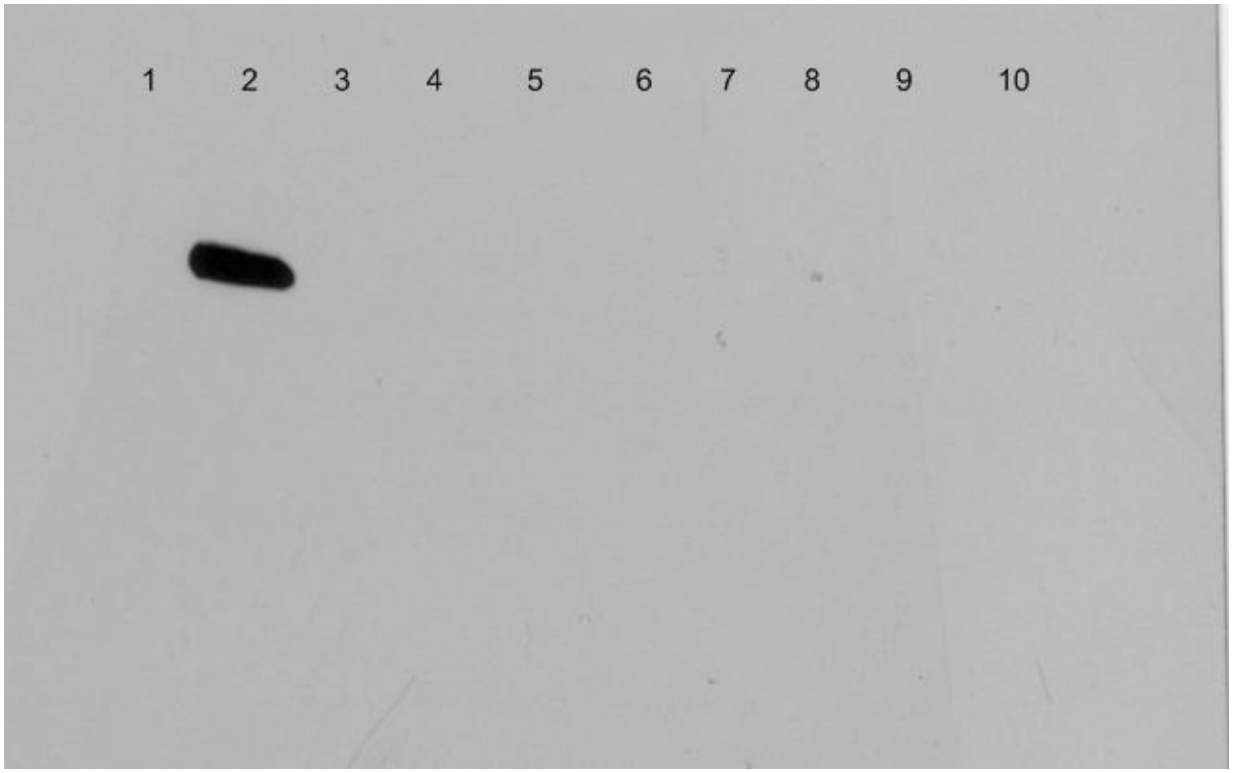

Figure 33. Western blot of Verivax® vaccine preparation. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. Ramos Western Blot Control, 3. Space, 4. Verivax® Test, 5-10. Spaces.

The result of the western blot in Figure 33 did not demonstrate the detection of HLA-DR protein in the combined, concentrated vials of the Verivax® preparation in lane 4. The band in lane 2 indicated the validity of the western blot by the detection HLA-DR in the Ramos whole cell lysate.

Rabbit Anti-HLA-DRB1 Antibody Immobilization Immunoaffinity Western Blot

Immunoaffinity reactions using the rabbit polyclonal anti-HLA-DRB1antibody were completed according to the Antibody Immobilization Immunoaffinity method. The eluates were reduced and run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot was the anti-HLA-DR (clone

L243) and the secondary antibody used was goat anti-mouse IgG HRP. The results are demonstrated in the blot in Figure 34. Lane 1 contains molecular weight marker. Lane 2 contains eluate from the Pierce Control Agarose Resin (crosslinked 4% beaded agarose) incubated with the Pierce® IPLysis/Wash Buffer buffer, referred to as Blank Preclearing Control. Lane 3 contains eluate from the rabbit polyconal anti-HLA-DRB1 antibody coupled AminoLink® Plus Coupling Resin incubated with Pierce® IPLysis/Wash Buffer buffer, referred to as the DRB1 Antibody Control. Lane 4 contains eluate from the Pierce Control Agarose Resin (crosslinked 4% beaded agarose) incubated with the 500 µg of Ramos whole cell lysate protein, referred to as the Ramos 500 Preclearing Control. Lane 5 contains eluate from the immunoaffinity reaction between the rabbit polyconal anti-HLA-DRB1 antibody coupled AminoLink® Plus Coupling Resin and 500 µg of Ramos whole cell lysate, referred to as the Ramos 500 Test. Lane 6 contains eluate from the Pierce Control Agarose Resin (crosslinked 4% beaded agarose) incubated with the 25 µg of Ramos whole cell lysate protein, referred to as the Ramos 25 Preclearing Control. Lane 7 contains eluate from the immunoaffinity reaction between the rabbit polyconal anti-HLA-DRB1 antibody coupled AminoLink® Plus Coupling Resin and 25 µg of Ramos whole cell lysate, referred to as the Ramos 25 Test. Lane 8 is a space. Lane 9 contains 25 µg of Ramos whole cell lysate protein loaded directly onto the SDS-PAGE gel, referred to as the Ramos 25 Western Blot Control. Lane 10 is a space.

Figure 34. Rabbit Anti-HLA-DRB1 Antibody Immobilization Immunoaffinity Western Blot

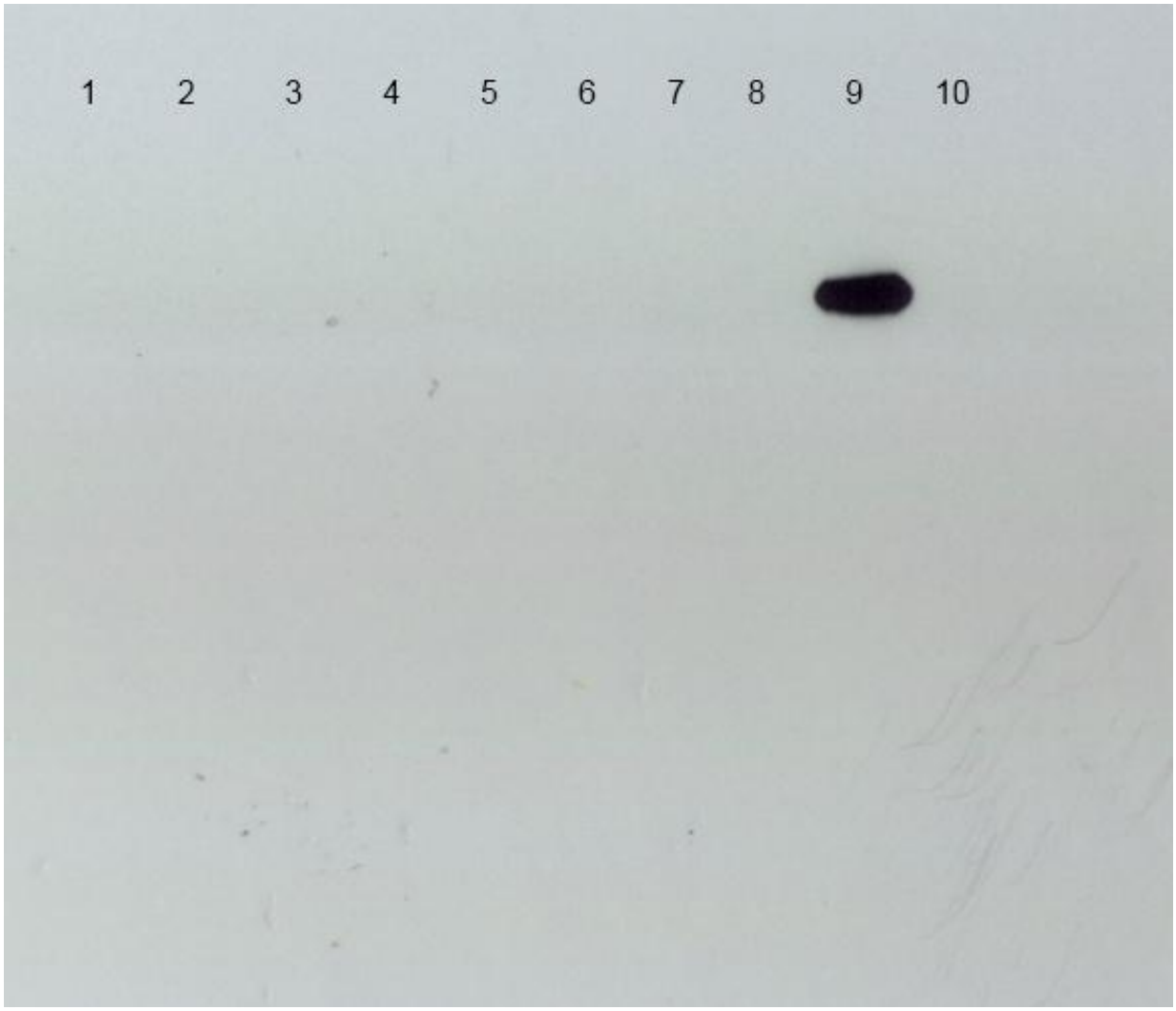

Figure 34. Western blot of rabbit anti-HLA-DRB1 antibody immobilization immunoaffinity. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. Blank Preclearing Control, 3. DRB1 Antibody Control, 4. Ramos 500 Preclearing Control, 5. Ramos 500 Test, 6. Ramos 25 Preclearing Control, 7. Ramos 25 Test, 8. Space, 9. Ramos 25 Western Blot Control, 10. Space.

The results of the western blot in Figure 34 indicated that the rabbit polyclonal anti-HLA-DRB1 antibody immunoaffinity reactions did not capture detectable HLA-DR protein. The band in the Ramos 25 Western Blot Control (lane 9) indicated the presence of HLA-DR protein and that the western blot was valid.

Western Blot to Assess the Limit of Detection of HLA-DR Protein Present in Ramos Whole Cell Lysate

An additional western blot assay was completed to estimate the limit of detection of the Ramos whole cell lysate in the assay. A serial dilution of the Ramos whole cell lysate was completed at a dilution factor of 1/2, from 25 µg to 0.98 µg. The samples were reduced, not-boiled, run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot was the anti-HLA-DR (clone L243) and the secondary antibody used was goat anti-mouse IgG HRP. The results of the western blot are depicted the in Figure 35.

Figure 35. Western Blot to Assess the Limit of Detection of HLA-DR Protein Present in Ramos Whole Cell Lysate

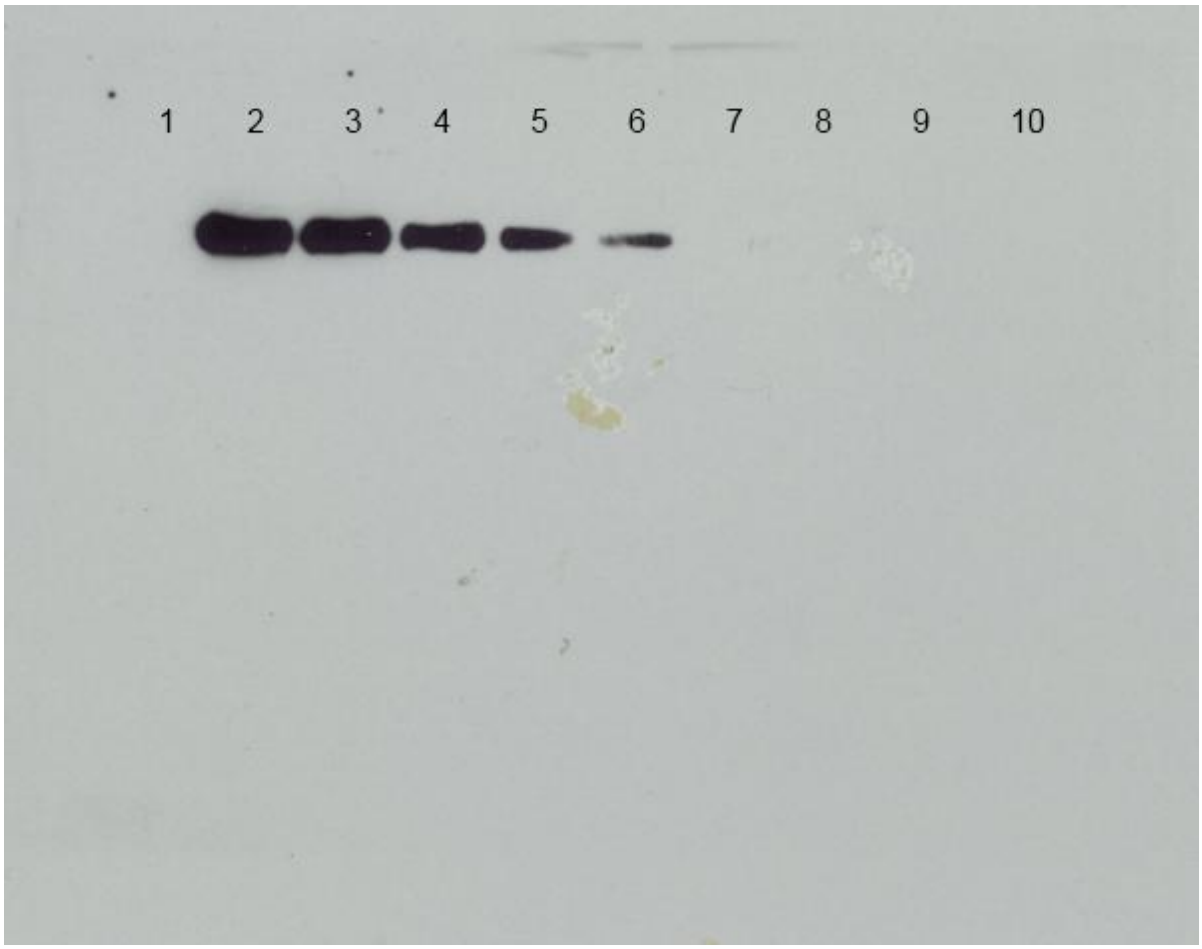

Figure 35. Western blot to assess the limit of detection of HLA-DR protein present in ramos whole cell lysate. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. 25 µg Ramos whole cell lysate, 3. 12.5 µg Ramos whole cell lysate, 4. 6.25 µg Ramos whole cell lysate, 5. 3.125 µg Ramos whole cell lysate, 6. 1.56 µg Ramos whole cell lysate, 7. 0.78µg Ramos whole cell lysate, 8. 0.39 µg Ramos whole cell lysate, 9. 0.19 µg Ramos whole cell lysate, 10. 0.095 µg Ramos whole cell lysate.

The results of the western blot in Figure 35 indicated that the limit of detection of HLA-DR protein in the western blot assay was approximately 1.56 µg of Ramos whole cell lysate.

Western Blot of M-M-R®-II Vaccine Preparation

An additional attempt was made to detect HLA-DR protein in the vaccine by omitting the immunoaffinity step. Ramos whole lysate in the amount of 6.25 µg was loaded directly into lane

2 of the SDS-PAGE gel, referred to as Unprocessed Ramos Western Blot Control. Another 6.25 µg of the Ramos whole cell lysate was processed according to Gelatin Removal Method and loaded into lane 3 of the SDS-PAGE gel and is referred to as Processed Ramos Western Blot Control. As test samples, two separate vials of M-M-R®-II preparation were reconstituted in 500ul of sterile diluent and each spiked with 6.25 µg of Ramos whole cell lysate. One of the two vials was processed according to the Gelatin Removal method and loaded into lane 5 of the SDS-PAGE gel. This sample is referred to as Processed Spiked MMR. The other spiked M-M-R®-II sample was loaded directly into lane 9 the SDS-PAGE gel, and referred to as Unprocessed Spiked MMR.

Prior to gel loading, all samples were concentrated to a volume of less than 30 µl using the Amicon® Concentration method. Following concentration to a workable sample volume, the samples were reduced, and run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot was the anti-HLA-DR (clone L243) and the secondary antibody used was goat anti-mouse IgG HRP. The results of this assay are depicted in Figure 36.

Figure 36. Western Blot of M-M-R®-II Vaccine Preparation

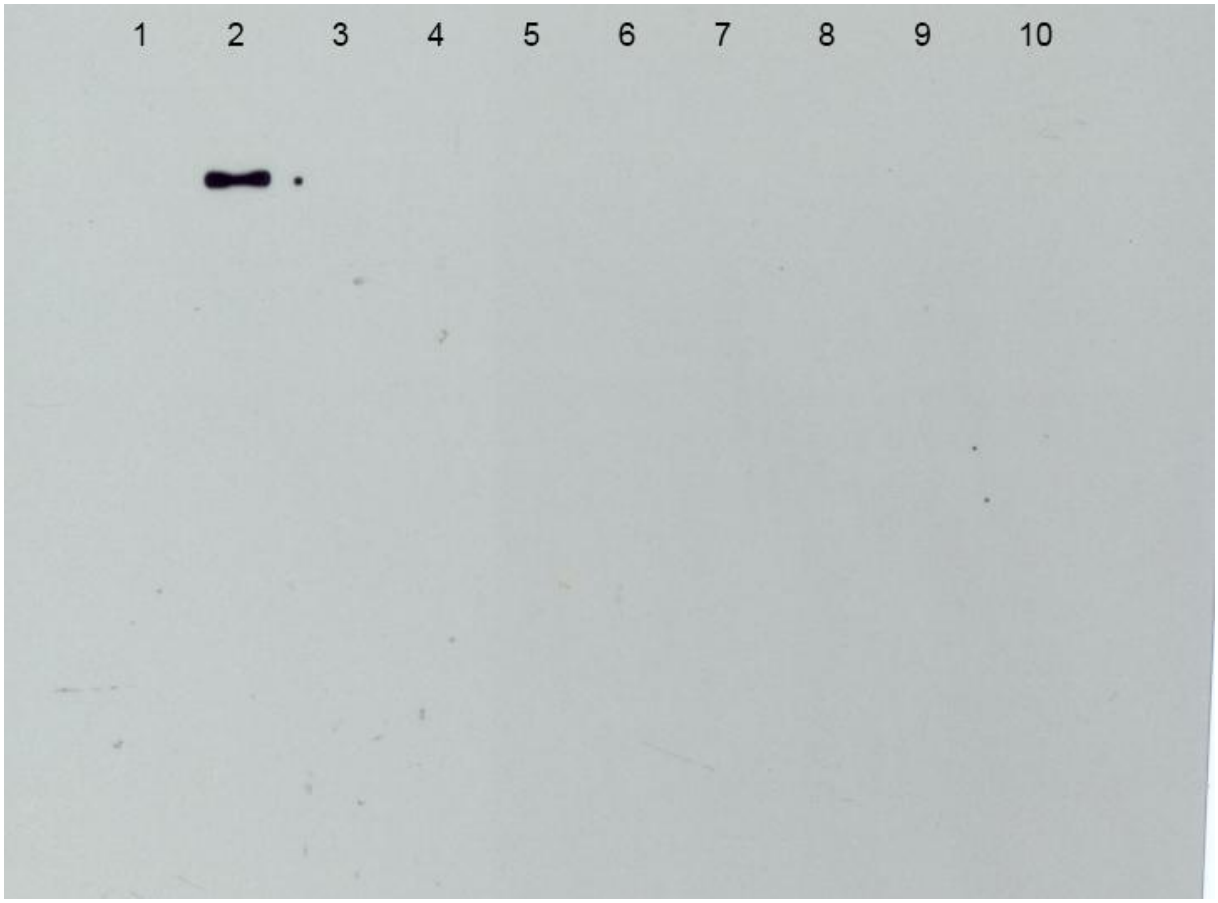

Figure 36. Western blot of M-M-R®-II vaccine preparation. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. Unprocessed Ramos Western Blot Control, 3. Processed Ramos Western Blot Control, 4. Space, 5. Processed Spiked MMR, 6. Space, 7. Space 8. Space, 9. Unprocessed Spiked MMR.

The results of the western blot assay in Figure 36 indicated that HLA-DR protein present in the Ramos whole cell lysate could not be detected after processing by the Gelatin Removal Method; nor could it be detected in the unprocessed spiked vaccine preparation. This indicates that there may have been HLA-DR protein loss due to the Gelatin Removal Method. Lack of detection of HLA-DR protein in the Unprocessed Spiked MMR (lane 9) may have been due to masking of the protein by the excess of gelatin in the unprocessed spiked vaccine sample. The

detection of HLD DR protein in the Unprocessed Ramos Western Blot Control indicates that the assay was valid.

Mouse Anti-HLA-DR (clone L243) and Mouse Anti-HLA-DR (clone B8.12.2) Antibody Immobilization Immunoaffinity Validation and Western Blot

Immunoaffinity reactions were completed in order to determine the relative efficiencies of the mouse anti-HLA-DR (clone L243) and mouse anti-HLA-DR (clone B8.12.2) in capturing HLA-DR protein from the Ramos whole cell lysate positive control. The immunoaffinity reactions were completed according to the Antibody Immobilization Immunoaffinity method. All samples were reduced and run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot was the anti-HLA-DR (clone L243) and the secondary antibody used was goat anti-mouse IgG HRP. The results are depicted in Figure 37. Lane 1 contains molecular weight marker. Lane 2 contains eluate from the mouse anti-HLA-DR (clone L243) antibody coupled AminoLink® Plus Coupling Resin incubated with Pierce® IPLysis/Wash Buffer, referred to as the Anti-HLA-DR L243 Antibody Control. Lane 3 contains eluate from the immunoaffinity reaction between the mouse anti-HLA-DR (clone L243) antibody coupled AminoLink® Plus Coupling Resin and 500 µg of Ramos whole cell lysate, referred to as the Anti-HLA-DR L243 Ramos 500 Test. Lane 4 contains eluate from the immunoaffinity reaction between the mouse anti-HLA-DR (clone L243) antibody coupled AminoLink® Plus Coupling Resin and 25 µg of Ramos whole cell lysate, referred to as the Anti-HLA-DR L243 Ramos 25 Test. Lane 5 contains eluate from the mouse anti-HLA-DR (clone B8.12.2) antibody coupled AminoLink® Plus Coupling Resin incubated with Pierce® IPLysis/Wash Buffer, referred to as the Anti-HLA-DR B8.12.2 Antibody Control. Lane 6 contains eluate from the immunoaffinity reaction between the mouse anti-HLA-DR

(clone B8.12.2) antibody coupled AminoLink® Plus Coupling Resin and 500 µg of Ramos whole cell lysate, referred to as the Anti-HLA-DR B8.12.2 Ramos 500 Test. Lane 7 contains eluate from the immunoaffinity reaction between the mouse anti-HLA-DR (clone B8.12.2) antibody coupled AminoLink® Plus Coupling Resin and 25 µg of Ramos whole cell lysate, referred to as the Anti-HLA-DR B8.12.2 Ramos 25 Test. Lane 8 contains 25 µg of Ramos whole cell lysate loaded directly onto the SDS-PAGE gel, referred to as the Ramos Western Blot Control. Lanes 9 and 10 are spaces.

Figure 37. Mouse Anti-HLA-DR (clone L243) and Mouse Anti-HLA-DR (clone B8.12.2) Antibody Immobilization Immunoaffinity Validation and Western Blot

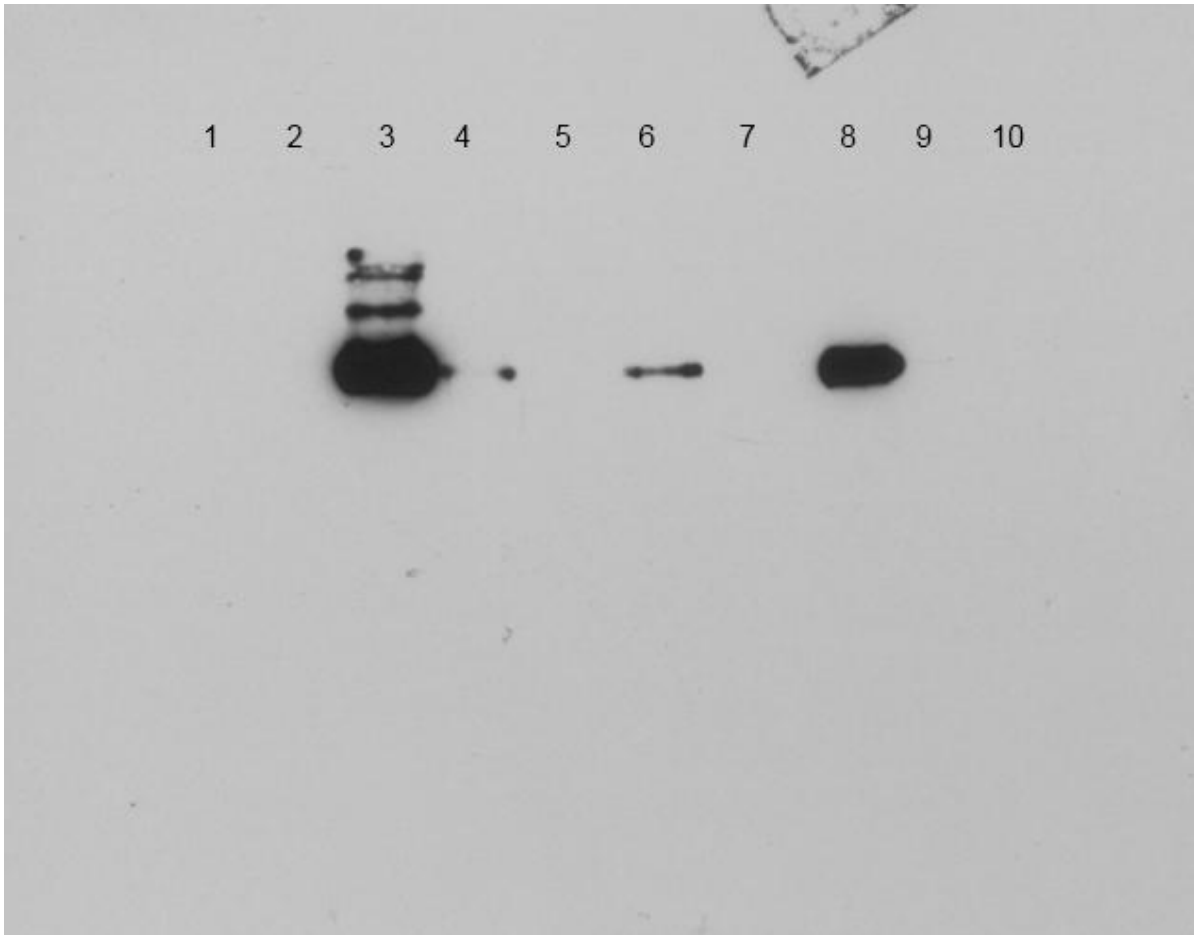

Figure 37. Western blot of mouse anti-HLA-DR (clone L243) and mouse anti-HLA-DR (clone B8.12.2) antibody immobilization immunoaffinity validation. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. Anti-HLA-DR L243 Antibody Control, 3. Anti-HLA-DR L243 Ramos 500 Test, 4. Anti-HLA-DR L243 Ramos 25 Test, 5. HLA-DR B8.12.2 Antibody Control, 6. Anti-HLA-DR B8.12.2 Ramos 500 Test, 7. Anti-HLA-DR B8.12.2 Ramos 25 Test, 8. Ramos Western Blot Control, 9. Space, 10. Space.

The relative intensities of the bands of the western blot in Figure 37 demonstrated that the mouse anti-HLA-DR (clone L243) appeared to be more efficient in the pull down of HLA-DR protein from the Ramos whole cell lysate than the mouse anti-HLA-DR (clone B8.12.2).

Anti-HLA-DR (clone L243) Antibody Immobilization Immunoaffinity with Mass Spectrometry and Western Blot III

Immunoaffinitity purifications were completed using the anti-HLA-DR (clone L243) antibody in a variation of the Antibody Immobilization Immunoaffinity method followed by mass spectrometry analysis. The variation of the method was that immunoaffinity incubation conditions were adjusted to a three hour incubation at room temperature (instead of the previously indicated overnight incubation at 4°C. Following the immunoaffinity reaction the beads were washed 8 times with PBS submitted for mass spectrometry analysis according to the on-bead enzyme digestion of antibody-antigen complexes method.

The M-M-R®-II vaccine preparation was reconstituted according to the manufacturer's instructions with the addition of protease inhibitors. Two vials of the reconstituted M-M-R®-II vaccine preparation plus of protease inhibitors were incubated sequentially with the antibody coupled beads, referred to as M-M-R®-II Test.

All controls underwent the immunoaffinity incubation in the conditions described above. One control used was the antibody coupled beads incubated with RIPA buffer (Santa Cruz Biotechnology, Inc), thus serving as an antibody control, referred to as Antibody Control. Another control used consisted of one vial of M-M-R®-II vaccine preparation reconstituted according to the manufacturer's instructions spiked with 100 µg of the Ramos IP lysate, referred to as M-M-R®-II + Ramos Control. A third control used consisted of 100 µg of Ramos IP lysate in RIPA buffer, referred to as Ramos Control. A fourth control consisted of 4µl of the One Lambda DR11 protein preparation in RIPA buffer, referred to as One Lambda Control. The incubation volumes of the test and controls were adjusted to approximately 500ul. Table 7

demonstrates the HLA proteins identified using a threshold of at least 95% and a minimum of 2 unique peptides identified by the Scaffold proteomic data validation program. Many other proteins were identified in the test and control samples.

Table 7. Anti-HLA-DR (clone L243) Antibody Immobilization Immunoaffinity with Mass Spectrometry

Indentified proteins	Swiss Prot Acc. number	Mol. weight (kDa)	Number of unique spectra					Number of unique peptides					Protein identification probability				
			N	O	P	R	S	N	O	P	R	S	N	O	P	R	S
HLA class II hist. antigen, DR alpha chain	P01903	29	0	0	2	3	9	0	0	2	3	7	N/A	N/A	100%	100%	100%
HLA class II histo. antigen, DRB1-7 beta chain	P13761	30	0	0	8	6	2	0	0	8	6	2	N/A	N/A	100%	100%	100%
HLA class II histo. antigen, gamma chain	P04233	34	0	0	2	2	3	0	0	0	2	3	N/A	N/A	100%	100%	100%
HLA class II histo. antigen, DRB1-15 beta chain	P01911	30	0	0	0	0	6	0	0	0	0	5	N/A	N/A	N/A	N/A	100%
HLA class II histo. antigen, DP beta 1 chain	P04440	29	0	0	0	0	2	0	0	0	0	2	N/A	N/A	N/A	N/A	100%

Table 7. Results of anti-HLA-DR (L243) coupled beads with on-bead trypsin digestion mass spectrometry results. Abbreviations: Accession (Acc.), Molecular (Mol.), and histo. (histocompatibility). Control and immunoaffinity assay sample key (N-S): N= Antibody Control, O= M-M-R®-II Test, P= M-M-R®-II + Ramos Control, R= Ramos Control, S= One Lambda Control.

The results of the mass spectrometry identifications are described in Table 7 by the identified HLA proteins and their associated number of unique spectra, number of unique peptides, and protein identification probability. Overall, HLA-DR protein was not detected in M-M-R®-II vaccine preparation test. HLA-DR protein was detected to varying degrees in the M-M-R®-II + Ramos Control, Ramos Control, and the One Lambda Control.

The results of the mass spectrometry analysis also indicated the identification of keratin, actin, collagen, tubulin and a variety of other proteins. Their presence in the test and control samples can likely be explained as common contaminant proteins.[66] A complete list of protein identifications from the completed assay is available upon request.

Immunoaffinty reactions were completed using mouse anti-HLA-DR (clone L243) in order to test the M-M-R®-II vaccine preparation for the presence of HLA-DR protein. The immunoaffinity reactions were completed according to the Antibody Immobilization Immunoaffinity method followed detection by western blot. The variation in the Antibody Immobilization Immunoaffinity method was the incubation conditions. Instead of incubation overnight at 4°C, the immunoaffinity incubations were completed for 3 hours at room temperature. The 3 vials of reconstituted M-M-R®-II vaccine that were tested in the immunoaffinity reaction were incubated sequentially for 3 hours with the antibody coupled beads. All samples were reduced and run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot was the anti-HLA-DR (clone L243) and the secondary antibody used was goat anti-mouse IgG HRP. The results are depicted in Figure 38. Lane 1 contains molecular weight marker. Lane 2 contains eluate from the mouse anti-HLA-DR (clone L243) antibody coupled AminoLink® Plus

Coupling Resin incubated with Pierce® IPLysis/Wash Buffer, referred to as the HLA-DR L243 Antibody Control. Lane 3 contains the One Lambda DR11 protein preparation, loaded directly onto the SDS-PAGE gel, referred to as the One Lambda DR Protein Western Blot Control. Lane 4 is an empty space. Lane 5 contains eluate from the immunoaffinity reaction between the mouse anti-HLA-DR (clone L243) antibody coupled AminoLink® Plus Coupling Resin and 3 vials of M-M-R®-II vaccine preparation, referred to as the M-M-R®-II Test. Lane 6 is an empty space. Lane 7 contains eluate from the immunoaffinity reaction between the mouse anti-HLA-DR (clone L243) antibody coupled AminoLink® Plus Coupling Resin and 1 vial of the M-M-R®-II vaccine preparation spiked with 100 µg of Ramos whole cell lyate, referred to as M-M-R®-II + Ramos Immunoaffinity Control. Lane 8 is an empty space. Lane 9 contains eluate from the immunoaffinity reaction between the mouse anti-HLA-DR (clone L243) antibody coupled AminoLink® Plus Coupling Resin and 100 µg of Ramos whole cell lysate, referred to as Ramos Immunoaffinity Control. Lane 10 contains eluate from the immunoaffinity reaction between the mouse anti-HLA-DR (clone L243) antibody coupled AminoLink® Plus Coupling Resin and the One Lambda DR11 protein preparation, referred to as the One Lambda DR11 Immunoaffinity Control.

Figure 38. Anti-HLA-DR (clone L243) Antibody Immobilization Immunoaffinity Western Blot III

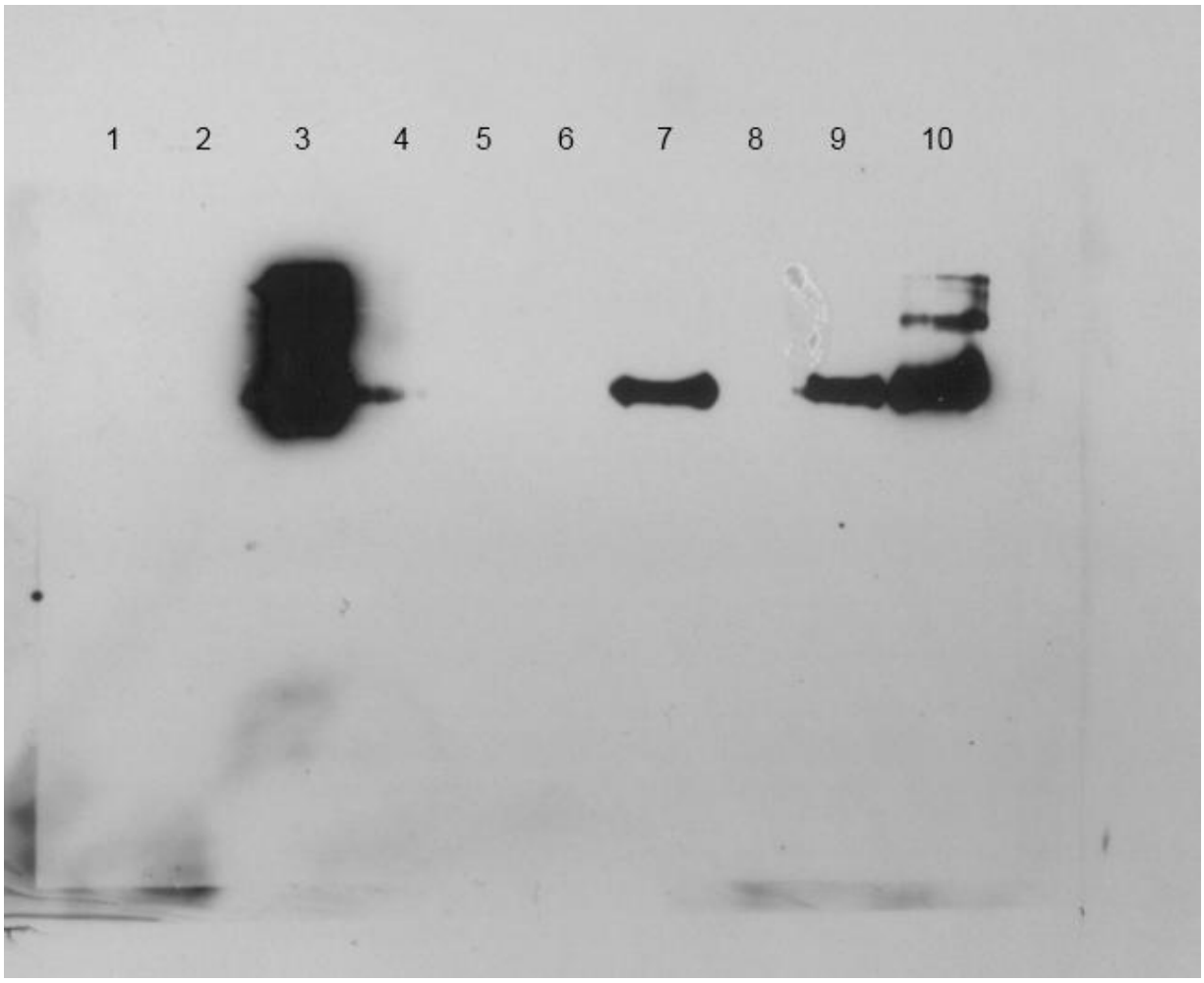

Figure 38. Western blot of anti-HLA-DR (clone L243) antibody immobilization immunoaffinity III. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. HLA-DR L243 Antibody Control, 3. One Lambda DR Protein Western Blot Control, 4. Space, 5. M-M-R®-II Test, 6. Space, 7. M-M-R®-II + Ramos Immunoaffinity Control, 8. Space, 9. Ramos Immunoaffinity Control, 10. One Lambda DR11 Immunoaffinity Control.

The results of the western blot in Figure 38 demonstrated that although HLA-DR protein was detected in the M-M-R®-II + Ramos Immunoaffinity Control, Ramos Immunoaffinity Control, and One Lambda DR11 Immunoaffinity Control, it was not detected in the M-M-R®-II Test.

Prior to their utilization in the Antibody Immobilization Immunoaffinity method, the M-M-R®-II test and controls underwent preclearing whereby they incubated for 1 hour with Pierce Control Agarose Resin (4% crosslinked agarose). Following the 1 hour incubation, elution and processing proceeded as above. The results are demonstrated in Figure 39. Lane 1 contains molecular weight marker. Lane 2 contains Ramos whole cell lysate loaded directly onto the SDS-Page Gel, referred to as the Ramos Western Blot Control. Lane 3 is an empty space. Lane 4 contains eluate from the preclearing reaction of the first M-M-R®-II vaccine preparation vial, referred to as M-M-R®-II First Vial Prelcearing Control. Lane 5 contains eluate from the preclearing reaction of the second M-M-R®-II vaccine preparation vial, referred to as M-M-R®-II Second Vial Prelcearing Control. Lane 6 contains eluate from the preclearing reaction of the third M-M-R®-II vaccine preparation vial, referred to as M-M-R®-II Third Vial Prelcearing Control. Lane 7 contains eluate from the preclearing reaction of the M-M-R®-II vaccine preparation spiked with 100 µg of Ramos whole cell lysate, referred to as M-M-R®-II + Ramos Preclearing Control. Lane 8 contains eluate from the preclearing reaction of 100 µg of Ramos whole cell lysate, referred to as the Ramos Preclearing Control. Lane 9 contains eluate from the preclearing reaction of the One Lambda DR11 protein preparation, referred to as the One Lambda DR11 Preclearing Control. Lane 10 contains molecular weight marker.

Figure 39. Anti-HLA-DR (clone L243) Antibody Immobilization Immunoaffinity Western Blot III – Preclearing Controls

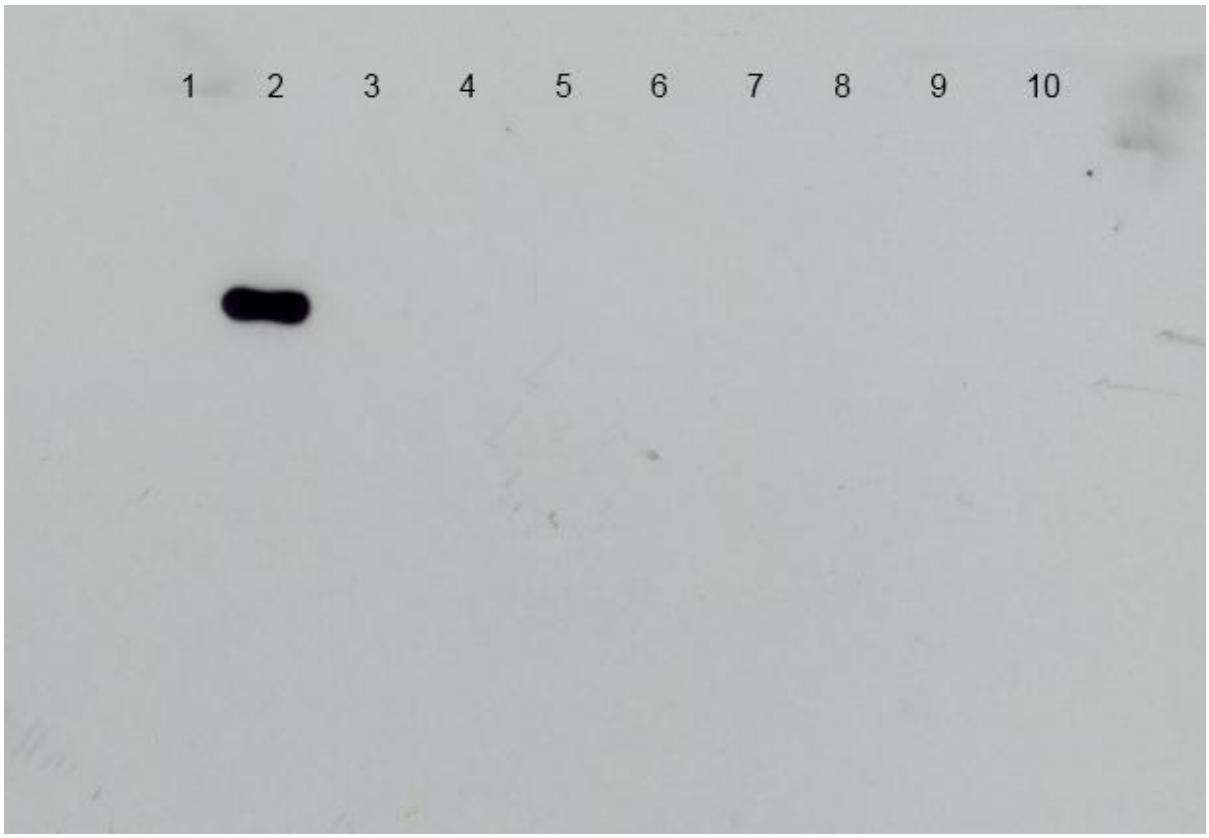

Figure 39. Western blot of preclearing controls for the anti-HLA-DR (clone L243) antibody immobilization immunoaffinity III. Lane key: 1. Molecular weight marker (undetected), 2. Ramos Western Blot Control, 3. Space, 4. M-M-R®-II First Vial Prelcearing Control, 5. M-M-R®-II Second Vial Prelcearing Control, 6. M-M-R®-II Third Vial Prelcearing Control, 7. M-M-R®-II + Ramos Preclearing Control, 8. Ramos Preclearing Control, 9. One Lambda DR11 Preclearing Control, 10. Molecular weight marker (undetected).

The results of the western blot in Figure 39 demonstrated that there was not non-specific binding of the proteins in the test and control reaction to the Pierce Control Agarose Resin (4% crosslinked agarose).

Assessment of the Limit of Detection of HLA-DR Protein in the Western Blot Assay

An additional western blot assay was completed to estimate the limit of detection of the Gen-Probe DRB1*11:01 protein preparation in the western blot assay. Although the amount on

HLA-DR protein in the Gen-Probe DR11 protein preparation could not be determined by direct measurement, the concentration of HLA-DRB1*11:01 protein in the preparation was estimated by the source be 10 µg/ml. It was from this estimation that the amount of HLA-DRB1*11:01 protein was calculated. All samples were reduced and run on an SDS-PAGE gel, transferred to a nitrocellulose membrane, and detected by western blot. The primary antibody used for the blot was the anti-HLA-DR (clone L243) and the secondary antibody used was goat anti-mouse IgG HRP. The results are depicted in Figure 40. Lane 1 contains molecular weight marker. Lane 2 contains 20 ng of Gen-Probe DRB1*11:01 protein referred to as DRB1*11:01 20 ng. Lanes 3-10 contain a serial dilution of the HLA-DRB1*11:01 protein, from 5 ng to 0.039 ng. They are referred to as DRB1*11:01 5 ng through 0.039 ng.

Figure 40. Assessment of the Limit of Detection of HLA-DR Protein in the Western Blot Assay

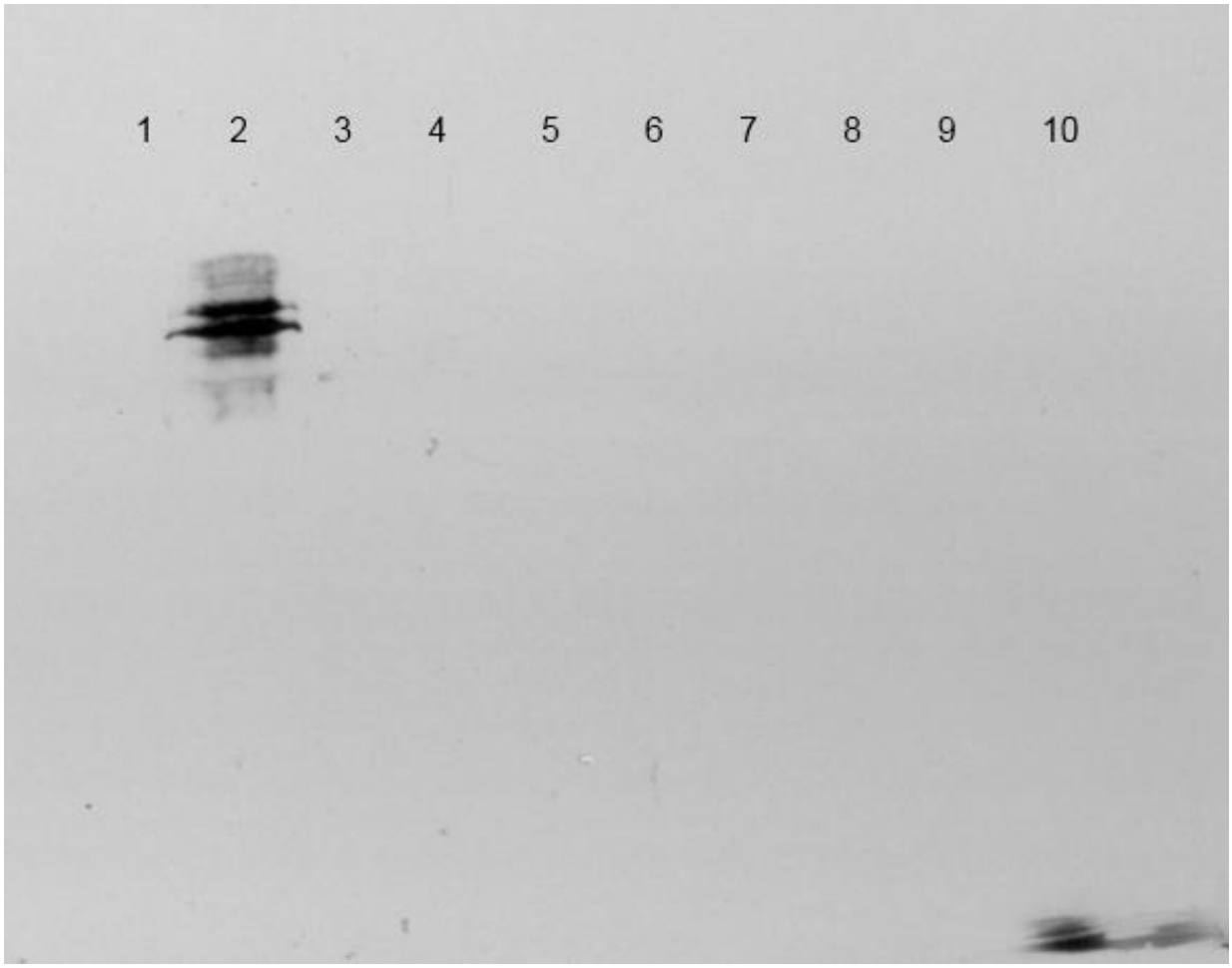

Figure 40. Assessment of the limit of detection of HLA-DR Protein in the western blot assay. Lanes are labeled along the horizontal axis. Lane Key: 1. Molecular weight marker (undetected), 2. DRB1*11:01 20 ng, 3. DRB1*11:01 5 ng, 4. DRB1*11:01 2.5 ng, 5. DRB1*11:01 1.25 ng, 6. DRB1*11:01 0.625 ng, 7. DRB1*11:01 0.313 ng, 8. DRB1*11:01 0.156 ng, 9. DRB1*11:01 0.078 ng, 10. DRB1*11:01 0.039 ng.

The results from the western blot in Figure 40 indicated the limit of detection of the western blot method was approximately 20 ng of DRB1*11:01 protein. The serial dilution in lanes 3 through 10 failed to produce any signal. This may have been true negative signal or may have been due to technical error, as there was very limited DRB1*11:01 protein to use for a serial dilution at this point in the project.

DISCUSSION

Basis for Investigation

A variety of FDA approved vaccines utilize human cell lines to propagate live attenuated pathogens for the manufacture therapeutic vaccines. A brief survey of these vaccines is described in Table 8 of Appendix A. In the instances where human cell lines are lines are utilized for pathogen propagation in these vaccines, the indicated cell lines used are the MRC-5 and WI-38 human fibroblast cell lines. Whether the propagation cell lines are of human origin or not, a variety of the package inserts also indicate that it is not rare to have cell line protein carryover into the vaccine by the end of the production process.

Traditional sources of anti-HLA antibody sensitization include transplantation, transfusion and pregnancy. Based on the information present in the vaccine product inserts, therapeutic vaccinations may also serve as a source of HLA protein and contribute to anti-HLA antibody sensitization.

The basis for this project was the development of an unexpected anti-HLA-DR11 antibody in a pediatric renal candidate. Following the detection of the novel anti-HLA antibody, investigation into the patient's history revealed recent administration of the ProQuad® vaccine. Though the patient had an earlier history of transplantation and therapeutic red cell transfusions, the antibody development coincided with the vaccine administration. Due to such a history, it was postulated that the spike of the novel anti-HLA-DR may have been due to either a primary or secondary amnestic response to HLA-DR11 antigen that may have been present in the vaccine preparation. The objective of this project was to detect HLA-DR11 protein in a vaccine preparation in order to identify it as a source of clinically significant anti-HLA antibody sensitization. If proven, vaccination history could also be utilized in the detection and

identification of clinically significant anti-HLA antibodies in renal transplant candidates and potentially lead to more accurate prediction of renal graft transplant outcomes.

Due to the discontinuation of Proquad®, it was decided to run protein detection assays on the M-M-R®-II and Verivax® vaccines, which are separate formulations of the same live attenuated pathogens present in the Proquad® vaccine preparation. In the M-M-R®-II vaccine, only the rubella component is propagated in a human fibroblast cell line. The cell line used for the propagation of the live attenuated rubella virus is the WI-38 cell line. In the Verivax® vaccine, the cell lines which are used to propagate the live attenuated varicella virus is the WI-38 cell line with final passage through the MRC-5 human fibroblast cell line.

Assay Development

Prior to developing assays that would serve to detect HLA protein in the M-M-R®-II or Verivax® vaccines, genomic HLA typing was completed on the WI-38 and MRC-5 human fibroblast cells lines used in the propagation of the rubella and varicella components of the M-M-R®-II or Verivax® vaccines, respectively . This step was completed in order to assess their potential of expressing HLA proteins, primarily HLA-DR11, serving as sensitizing agents to the pediatric renal recipient candidate in the case study. Such genomic HLA typing confirmed that the WI-38 cell line is positive for HLA-DRB1*11. This provided the foundation to move forward with assay development to detect HLA protein, more specifically HLA-DR11 protein, in the M-M-R®-II vaccine.

A variety of protein concentration and identification methods were utilized for the detection of HLA-DR11 protein present in the M-M-R®-II vaccine. It was hypothesized that

immunoaffinity purification and concentration of HLA protein would be necessary before identification by mass spectrometry or western blot methods.

Immunoaffinity purification was initially set up by coupling antibody specific for HLA-DR11 to Dynabeads® Protein G. These are commercially produced magnetic beads coated with recombinant Protein G. Protein G has been documented to have strong affinity for the Fc portion of a variety of subtypes of antibodies from a variety of different species. During the initial assay development, attempts to non-covalently couple the mouse anti-human HLA-DR11 antibody to the beads with documentation of successful antigen capture and release by SDS-PAGE electrophoresis and gel staining did not result in the detection of HLA-DR11. There was no detection of HLA-DR11protein running at its expected molecular weight of 55 kDa or as separate α and β subunits at 34 kDa and 29 kDa, respectively. Attempts to covalently bind the anti-HLA-DR11 antibody to the beads also did not appear to result in the detection of HLA-DR11 protein by SDS-PAGE analysis. It was determined that the anti-HLA-DR11 antibody was inappropriate for the assay.

Moving forward, it was determined that a mouse anti-human HLA-DR antibody would still serve the same purification and concentration function as the anti-human HLA-DR11 antibody. It also became apparent that there may be more choices of commercial anti-HLA-DR antibodies than anti-HLA antibody directed at a specific antigen, as in this case, HLA-DR11. Mouse monoclonal anti-HLA-DR (clone B8.12.2) was obtained for this purpose. When utilizing this antibody coupled to the Dynabeads® Protein G, the immunoaffinity reaction appeared to result in the detection of HLA Class II α and β subunits at 34 kDa and 29 kDa of the One Lambda DR11 protein preparation positive control as supported by Figures 6 and 10. When this method was coupled to mass spectrometry for protein identification, this method resulted in the

positive identification of HLA-DR11 protein and a variety of other HLA Class II proteins as indicated in Table 6. When the M-M-R®-II and Verivax® vaccine preparations were subjected to the same method, the results for the identification of HLA Class II protein in the Verivax® and M-M-R®-II vaccine preparation were negative. Therefore, it was determined that a more sensitive method for HLA Class II protein detection be pursued. Had the amounts of HLA protein in the One Lambda DR11 protein preparation been known, the level of detection could have set a lower limit for the amount of antigen in the vaccine.

Protein identification is a by western blot is a well-established method for protein detection and is well known for its high sensitivity. It was postulated that Class II HLA-DR immunoaffinity coupled with detection by western blot, with its enhanced sensitivity, would increase the potential for detecting HLA-DR protein in the M-M-R®-II and Verivax® vaccine preparations. The Indirect Immunoaffinity method was used for this purpose prior to detection by western blot. As the assay was developed, it became apparent that the co-elution of anti-HLA-DR light chains and heavy chains interfered with potential HLA Class II signal in the western blot, resulting in inconclusive results for detection of HLA Class II protein in the M-M-R®-II and Verivax® vaccine preparations. It was assumed that the strong, interfering background signal was a result of the of western blot secondary antibody, goat anti-mouse IgG HRP reacting with the co-eluting heavy and light chains of the immunoaffinity antibody, mouse anti-HLA-DR. This is apparent in both Figures 17 and 18. Covalently linking the mouse anti-HLA-DR antibody (both clone B8.12.2 and clone L243) to the Dynabeads® Protein G to in order to reduce background signal of co-eluting immunoaffinity antibody failed to result in the pull down of HLA-DR protein in the Direct Immunoaffinity method. This is apparent in Figure 19.

In attempt to work around the interfering background signal, rat monoclonal anti-HLA-DR and rabbit polyclonal anti-HLA-DRB1 were substituted as the immunoaffinity antibodies for the assay. Unfortunately, they failed to produce signal when compared to the mouse anti-HLA-DR as demonstrated in Figures 22 and 23.

The Antibody Immobilization Immunoaffinity method was an alternative immunoaffinity platform was pursued in order to reduce excess background on the western blot. The western blot in Figure 30 demonstrated the validation of this assay using the anti-HLA-DR (clone L243) as the immunoaffinity antibody and the Ramos whole cell and HLA-DR positive lysates as positive controls. Unfortunately, a lack of sensitivity was apparent in this western blot due to the fact that the immunoaffinity eluate did not contain as much detectable HLA-DR protein when compared to the same amount of Ramos whole cell lysate loaded directly onto the SDS-PAGE gel. Although the immunoaffinity assay did concentrate some of the HLA-DR protein from the Ramos whole cell lysate, is resulted in a band of decreased intensity when compared to direct detection on the SDS-PAGE gel.

The Verivax® vaccine preparation was later subjected to this method and the detection of HLA-DR protein in the vaccine preparation was negative as demonstrated in Figure 32. It was postulated that the negative result had been due to lack of sensitivity of the Antibody Immobilization Immunoaffinity method. An additional western blot was completed to test this hypothesis. The result of the western blot in Figure 33 demonstrated that the Verivax® is negative for HLA-DR protein when applied directly to the SDS-PAGE followed by detection via the Western Blot method.

The analysis that followed suggested that the large amount of gelatin present in the Verivax® and M-M-R®-II vaccine preparation (12.5mg/dose and 14.5mg/dose, respectively) may have inhibited binding of the primary antibody in the Western Blot method. Inhibition of western blot primary antibody binding to HLA DR may have been the result of interference of gelatin in the vaccine by running as a smear in the SDS-PAGE gels and literally blocking the interaction of the primary antibody with HLA DR that might have been present. To circumvent this issue, an attempt to remove excess gelatin by the Gelatin Removal method was completed. The result of this assay was negative for the detection of HLA-DR protein in the M-M-R®-II vaccine preparation as demonstrated in Figure 36. By using Ramos whole cell lysate both processed and unprocessed using the Gelatin Removal method, it was apparent that use of this method lead to loss of HLA-DR protein through the processing procedure.

Use of rabbit polyconal anti-HLA-DRB1 was attempted once again, as it was thought that covalent binding the antibody resin in the Antibody Immobilization Immunoaffinity method might be enhance when compared to the Dynabeads® Protein G in the Indirect Immunoaffinity method. This was not the case. As demonstrated in Figure 34, the assay could not be validated using the Ramos whole cell lysate as a positive control result and was not pursued further.

As indicated above, there was concern over the lack of sensitivity of using the immunoaffinity anti-HLA-DR (clone L243) antibody in the Antibody Immobilization Immunoaffinity method. Before moving on with the final assay of the M-M-R®-II vaccine preparation, it was decided to compare the immunoaffinity efficiency of the anti-HLA-DR (clone L243) antibody to that of the anti-HLA-DR (clone B8.12.2) antibody in it Antibody Immobilization Immunoaffinity application followed by detection by the Western Blot method. The relative intensities of the bands of the western blot in Figure 37 demonstrate that the mouse

anti-HLA-DR (clone L243) appeared to be more efficient in the pull down of HLA-DR protein from the Ramos whole cell lysate than the mouse anti-HLA-DR (clone B8.12.2). It was decided that the anti-HLA-DR (clone L243) would be the immunoaffinity antibody utilized for final assay of the M-M-R®-II vaccine preparation.

The final assay of the M-M-R®-II vaccine preparation was completed by the Antibody Immobilization Immunoaffinity followed by detection by both the MS and Western Blot methods. The MS on-bead digest method detection results failed to identify the HLA-DR protein in the M-M-R®-II vaccine preparation as described in Table 7. HLA-DR proteins were detected to varying degrees in the positive controls used in this assay.

When comparing Tables 6 and 7, the Antibody Immobilization Immunoaffinity followed by Mass Spectrometry on-bead digestion of proteins appeared to be less sensitive than the Indirect Immunoaffinity method followed by MS gel cutout digest method. This may have been due to two different factors, the first being the optimal antibody orientation on the Dynabeads® Protein G of the Direct and Indirect Immunoaffinity methods when compared to antibody immobilization on resin of the Antibody Immobilization Immunoaffinity method. The second factor may have been due to increased efficiency of the trypic digest of HLA-DR proteins in the MS gel cutout digest method versus the MS on-bead digest method.

The final assay of the M-M-R®-II vaccine preparation was completed by the Antibody Immobilization Immunoaffinity followed by detection using the Western Blot methods demonstrated a negative result in the detection of HLA-DR protein in the M-M-R®-II vaccine preparation as presented in Figure 38. The detection of HLA-DR protein in the positive controls of this assay demonstrated that the assay was valid.

Retrospective Analysis of Assay Development

There were a variety of factors that may have detracted from the progress of this project. One such factor was the choice of a positive control. At the beginning of this project, the Gen-Probe DRB1*11:01 protein preparation was assumed to be a reliable positive control and was initially the only control used for assay development and validation. It became apparent though, as the assay was being developed, that its use was not contributing to assay development as expected. This can be observed in Figure 5. Though it may have been an appropriate control at a later stage in assay validation, it should have not been the only positive control used in the initial stages of assay development.

In order to reserve the decreasing supply of the Gen-Probe DRB1*11:01 and One Lambda DR11 protein preparations as positive controls, HLA-DR positive lysates were used as positive controls as well. In fact, the weak and inconsistent activity of the HLA-DR positive lysates reduced their reliability in assay development. Ultimately, this had a significant limiting effect in the progression of assay development. An example of such an inconsistency is demonstrated between Figures 21 and 22.

The later use of the Ramos whole cell lysate was essential to the forward progress of assay development as it was an easily accessible, cost-effective, and consistent positive control. It was with the One Lambda DR11 protein preparation and the Ramos whole cell lysate that enabled the validation of the immunoaffinity, western blot, and mass spectrometry techniques.

Another factor that impacted assay development was the choice of antibody used in the immunoaffinity portions of the project. A variety of immunoaffinity antibodies used in this project were proven unsuccessful in in pulling down their target antigen. Initially, a mouse

monoclonal anti-HLA-DR11 antibody was used in this project. Repeated attempts at the validation of the antibody failed to produce positive or DR11 protein specific results. These issues are demonstrated, respectively, in Figures 5 and 15.

Additional failed antibodies included the rat monoclonal anti-HLA-DR and the rabbit polyclonal anti-HLA-DRB1 antibodies. Repeated attempts at their validation failed and are demonstrated in Figures 20, 22, 23, 27, 28, and 34. This also contributed to lack of progress in assay development.

An additional issue that hindered assay development was the reactivity of the primary western blot antibody. As attempts were made in validation of the immunoaffinity and western blot assays, it was suspected that there was inconsistency in the reactivity of the western blot assay. Although this may have been due to the inconsistency in the positive controls, it was suspected that boiling samples prior to loading them on the SDS-PAGE gels may have affected the antibody binding capacity of the western blot primary antibody to the proteins in the nitrocellulose membrane. In fact, boiling HLA Class II proteins permanently dissociates the α and β subunits of the heterodimer as reported by Springer. Additional reference demonstrated that various anti-HLA-DR antibodies have reactivity to only the associated α and β subunits of the heterodimer and negative reactivity to boiled heterodimers, which results in the dissociation of the α and β subunits.[67] This effect was demonstrated in this project by Figures 25 and 26 supported by the use of the Ramos whole cell lysate as a valid positive control. Although some reactivity after boiling was apparent prior to the assays of Figures 25 and 26, the boiling of the samples prior to loading them onto the SDS-PAGE gels was discontinued as to ensure the reactivity of the primary western blot antibody with HLA-DR proteins in the nitrocellulose membrane.

A further, unforeseen issue in assay development was the lack of the sensitivity of the Antibody Immobilization Immunoaffinity reactions when compared to direct detection by western blot. This demonstrated in Figures 30, 32 and 37, where the immunoaffinity eluates of the Ramos whole cell lysates or the HLA-DR positive lysates loaded directly onto the SDS-PAGE gel would vary in band intensities and bandwidths when compared to their counterparts that had been directly loaded onto the SDS-PAGE gels. The estimated limit of detection of the western blot assay when loading the Gen-Probe DRB1*11:01 preparation was estimated to be approximately 20 ng as demonstrated in Figure 40.

A final issue which limited the forward progress of this project was technical inconsistencies of the experimental setup and design. The author admits to occasional lapses in assay execution, most notably in the assay presented in Figure 17. Intermittently, the design of the assays from one to another was not logically thought out and was considered to be hastily pursued. By the end of the project, logical steps were taken to investigate, one by one, the variables that impacted assay development.

Conclusion

The objective of this project was to detect HLA-DR protein in the M-M-R® vaccine preparation. Although a variety of methods were utilized to achieve this objective, HLA-DR protein was not detected in the M-M-R® vaccine preparation. It is suspected that the protein may in fact be present, albeit below the limit of detection that of the assays used in this project.

The amount of protein antigen required to stimulate an immune response varies and may be dependent on factors such as primary or secondary exposure, dose and location of antigen administration, and variance in the accessibility of antigen presenting cells (APCs) to the antigen

and their corresponding interaction with B and T lymphocytes to facilitate an immune response. One recent study has validated this principle by describing the variability in titer of antibody production in mice following three two week intervals of parenteral administrations of recombinant Hepatitis B Surface Antigen (S antigen) at amounts of 5, 50, or 500 ng of antigen. It was demonstrated that low amounts of antigen, i.e. 5 ng, was sufficient to produce antibody titers comparable to those the results following 50 and 500 ng of antigens delivered at the same time intervals.[68]

Based on this study, the estimated limit of detection 20 ng of the western blot assay may have not been sensitive enough to detect HLA Class II DR protein present in an M-M-R®-II vaccine preparation. Ultimately, if present below the limit of detection, the amount of HLA-DR protein present in an M-M-R®-II vaccine preparation could have been sufficient to stimulate a primary or secondary immune response in the patient of the described case study.

SUMMARY

Summary

The DRB1*11 genotype of the cell line used to propagate the rubella viral component of the M-M-R®-II vaccine corresponded to the clinically significant anti-HLA-DR11 antibody detected in the pediatric renal recipient candidate following administration of the vaccine. It was reasonable to hypothesize that the WI-38 cell line was capable of expressing HLA-DR protein on its cellular surface and may have also produced soluble HLA- DR.[69] Such protein may have been present in the ProQuad® vaccine product. As additional evidence, multiple vaccine product inserts have indicated that there is residual protein carryover from the vaccine production process to the end product.

It is well known that many variables are involved in the production and strength of an immune response against a non-self-antigenic stimulus. These factors include amount of antigen, previous exposure to antigen, accessibility of antigen to APCs in the tissues, interaction of the APCs with lymphocytes in the lymph nodes, and co-stimulation between T and B cells in mounting an adaptive immune response. The patient in the case study was likely previously exposed to a variety of HLA antigens, including HLA-DR11, through their history of a multiple blood transfusions. If this was the case, it's possible that a very slight amount of HLA- DR11 antigen theoretically present in the ProQuad® vaccine preparation may have been sufficient to initiate a robust, secondary anamnestic immune response.

Immunoaffinity, mass spectrometry, and western blot assays were completed in order to detect HLA-DR protein in the M-M-R®-II vaccine preparation. The M-M-R®-II vaccine preparation was used as a substitute for the ProQuad® because at the time of this project

ProQuad® was no longer commercially available. The M-M-R®-II is used as a therapeutic vaccination against the rubella virus. The western blot assay was assumed to be the most sensitive of the protein isolation and detection methods utilized for this project. It was determined that the estimated limit of detection of the western blot assay was approximately 20 ng. Though HLA-DR protein was not detected in any of the methods used, it may have been due the limit of detection of the western blot assay. Theoretically, HLA-DR protein may have been present at an amount of less than 20 ng in the vaccine and still capable of producing an immune response as demonstrated in the pediatric renal recipient candidate following administration of the vaccine.

Protein homology between HLA-DR11 protein and vaccine viral proteins also may have also contributed to sensitization of the renal recipient candidate. For example, one study has demonstrated homology between a 65 kDa tetrapeptide of *Mycobacterium tuberculosis* (of the TST) and the protein structure of HLA-DR1, 3 and 4 antigens. This protein homology may represent a potential cause of sensitization directed towards HLA-DR1, 3 and 4 antigens.[11] Homology between epitopes of the HLA-DR11 protein and measles, mumps, rubella, and varicella proteins may have also been responsible for stimulating the strong immune response demonstrated in the case study. A specific epitiope shared between the HLA-DR11 and DP specificities is demonstrated in the antibody profile of the pediatric renal recipient candidate is the DPB1* 55DE and DRRB1*11:01 57DE.[32] A comparison of the three dimensional structure of the DE epitope in the HLA Class II molecules and the vaccine viral proteins mentioned above is warranted to further investigate the source of immune stimulus.

RECOMMENDATIONS

Recommendations

Further investigation into the detection of HLA proteins present in vaccine preparation may require enhanced sensitivity. This could include the use of radioactive detection in the western blot versus the chemiluminescent detection method used in this project. An additional mechanism used to enhance the sensitivity of the immunoaffinity assays would be to use multiple monoclonal or polyclonal antibodies to HLA-DR in immunoaffinity reaction, which would take advantage of binding multiple epitopes of HLA-DR proteins, thus theoretically enhancing the recovery of an antigen of low abundance.[42] Recirculating immunoaffinity columns could also be utilized to enhance the sensitivity of detecting HLA-DR proteins in larger volumes (and potentially larger amounts of HLA-DR antigen) from combined reconstituted vaccines preparations.[70,71]

A variety of FDA approved vaccines are produced by the utilization of human cell lines MRC-5 and WI-38. These include the Verivax®, M-M-R®-II, VAQTA® and TWINRIX® vaccines. Though entirely appropriate and recommended for preventing communicable disease, vaccination may lead to inadvertent sensitization of to HLA antigens due to residual contamination from the cell lines used to propagate live, attenuated pathogens.

A comparison of the three dimensional structure of the DE epitope in the HLA Class II molecules and the vaccine viral proteins mentioned above is warranted to further investigate the source of immune stimulus. Such an investigation could be initiated by identifying protein sequences and three dimension structures containing the DE epitope using available protein

databases. Sequence homology can be investigated using available Basic Local Alignment Search Tool (BLAST) available through online database and tools, such as NCBI and UniProt databases. Structural homology should be investigated using three dimensional protein structure programs. Databases and programs for this use include the IMGT/3Dstructure-DB and the Cn3D, Molecular Modeling Database (MMDB), and Vector Alignment Search Tool (VAST) three dimensional structure databases and comparison tools available through NCBI.[72-75]

Transplantation, transfusion, and pregnancy are well recognized sources of HLA antigen sensitization. In instances where histocompatibility laboratories detect and identify anti-HLA antibodies that cannot be attributed to these sources of sensitization, it is recommended that the patient's vaccination history be examined. HLA antigen exposure through therapeutic vaccination may be a source of potentially clinically significant anti-HLA antibodies. Identification of such antibodies could further aid in prediction of renal transplant outcome in newly grafted recipients.

APPENDICES

APPENDIX A

Table 8. Information on Viral Vaccine Culture Protein Contaminants

Trade Name	**Manufacturer**	**Immunizing Agent**	**Propagation Medium**	**Indicated culture protein carryover**
Pentacel®	Sanofi Pasteur Limited	Poliovirus Type 1, Type 2, and Type 3	MRC-5 cell line of human diploid cells	Not indicated
COMVAX®	Merck & Co., Inc.	Hepatitis B	*Saccharomyces cerevisiae*	1% or less of total vaccine protein is of yeast origin
VAQTA®	Merck & Co., Inc.	Hepatitis A	MRC-5 cell line of human diploid fibroblasts	Less than 1 microgram of non-viral protein
TWINRIX®	GlaxoSmithKline Biologicals	Hepatitis A	MRC-5 cells	Not more than 2.5 micrograms of MRC-5 residual proteins
TWINRIX®	GlaxoSmithKline Biologicals	Hepatitis B	*Saccharomyces cerevisiae*	No more than 5% of yeast protein
RECOMBIVAX®	Merck & Co., Inc.	Hepatitis B	*Saccharomyces cerevisiae*	Not more that 1% of yeast protein
GARDASIL®	Merck & Co., Inc.	Human papillomavirus-Types 6, 11, 16, and 18	*Saccharomyces cerevisiae*	Less than 7 micrograms of yeast protein

Table 8 (cont'd)

Trade Name	**Manufacturer**	**Immunizing Agent**	**Propagation Medium**	**Indicated culture protein carryover**
CERVARIX®	GlaxoSmithKline Biologicals	Human papillomavirus-Types 16 and 18	Baculovirus expression vector system and *Trichoplusia ni* insect cells	Less than 40 nanograms of insect cell and viral protein and less than 150 nanograms of bacterial cell protein
Influenza A (H1N1) 2009 Monovalent Vaccine	CSL Limited	Influenza A (H1N1) 2009	Allanotic fluid of embryonated chicken eggs	Less than 1 microgram of ovalbumin
Influenza Virus Vaccine (H5N1) 2009	Sanofi Pasteur	Influenza Virus H5N1	Embryonated chicken eggs	Not indicated
AGRIFLU	Novartis Vaccines and Diagnostics Inc.	Influenza virus subtypes A and type B present	Allantoic cavity of embryonated hens' eggs	Less than 0.4 micograms of egg proteins
IXIARO	Intercell Biomedical	Japanese encephalitis virus (JEV)	Vero cells	Not more than 300 nanograms of host cell protein

Table 8 (cont'd)

Trade Name	**Manufacturer**	**Immunizing Agent**	**Propagation Medium**	**Indicated culture protein carryover**
JE-VAX®	Research Foundation for Microbial Diseases of Osaka University	Japanese encephalitis virus (JEV)	Mouse intracerebral inoculation	Less than 50 nanogram of mouse serum protein
M-M-R®-II	Merck & Co., Inc.	Measles	Chicken embryo culture	Not indicated
M-M-R®-II	Merck & Co., Inc.	Mumps	Chick embryo cell culture	Not indicated
M-M-R®-II	Merck & Co., Inc.	Rubella	WI-38 human diploid lung fibroblasts	Not indicated
IPOL®	Sanofi Pasteur SA	Poliovirus Type 1, Type 2, and Type 3	Vero cells (a continuous line of monkey kidney cells)	Not indicated
IMOVAX®	Sanofi Pasteur SA	Rabies Virus	MRC-5 human diploid cells	Not indicated
RotaTeq	Merck & Co., Inc.	Rotavirus gastroenteritis	Vero cells	Cell culture media (specific amount not indicated)

Table 8 (cont'd)

Trade Name	**Manufacturer**	**Immunizing Agent**	**Propagation Medium**	**Indicated culture protein carryover**
VARIVAX®	Merck & Co., Inc.	Varicella	WI-38 human diploid lung fibroblasts, final passage through MRC-5 cells cultures	Residual components of MRC-5 cell DNA and Protein

Table 8. This table contains a survey of the package inserts of FDA licensed vaccines currently approved for use in the United States.[16,17,76-90] This survey revealed that many vaccines manufactured for use against viral infection contain residual protein from culture media. These vaccines, their manufacturer, the immunizing agent, their propagation media, and indicated culture protein carryover are listed above.

Table 9. Peptide Cutter Prediction Results

Cell line: WI-38					
Protein: DRB1*11 beta chain					
Enzyme: Trypsin					
Position of cleavage site	Resulting peptide sequence	Peptide length	Peptide mass (D)	Mass to charge ratio*	Cleavage probability^
5	MVCLR	5	621	311	100%
35	LPGGSCMAVLTVTLMVLSSPLALAGDTRPR	30	3028	1514	100%
54	FLEYSTSECHFFNGTERVR	19	2323	1162	100%
58	FLDR	4	550	275	100%
68	YFYNQEEYVR	10	1411	706	100%
77	FDSDVGEFR	9	1071	536	100%
94	AVTELGRPDEEYWNSQK	17	2022	1011	100%
109	DFLEDRRAAVDTYCR	15	1830	915	100%
122	HNYGVGESFTVQR	13	1494	747	100%
127	RVHPK	5	636	318	100%
134	VTVYPSK	7	793	397	100%
159	TQPLQHHNLLVCSVSGFYPGSIEVR	25	2782	1391	100%
162	WFR	3	508	254	100%
168	NGQEEK	6	704	352	100%
195	TGVVSTGLIHNGDWTFQTLVMLETVPR	27	2972	1486	100%
218	SGEVYTCQVEHPSVTSPLTVEWR	23	2605	1303	100%
220	AR	2	245	123	100%
227	SESAQSK	7	736	368	100%
251	MLSGVGGFVLGLLFLGAGLFIYFR	24	2548	1274	100%
254	NQK	3	388	194	100%
262	GHSGLQPR	8	851	426	100%
266	GFLS	4	422	211	100%

Table 9. Presentation of Peptide Cutter prediction of cleavage sites in Class II HLA proteins that correspond to the Class II HLA type of the WI-38 and MRC-5 cell lines. Peptide having a resulting mass to charge ratio of 400 – 2000 are within the detectable limits of the mass spectrometer instrumentation utilized for this project.[91] Polypeptide cleavage by trypic digest typically results in a +2 charge on the peptide as denoted by (*). Peptide Cutter cleavage probability is user defined as denoted by (^).

APPENDIX B

Reagents

1) 1X sample loading buffer
    a. 2.5 ml water
    b. .5 ml 1 M Tris (pH 6.8)
    c. 0.8 ml 20% SDS
    d. 0.8 ml glycerol
    e. 0.2 ml β-mercaptoethanol (reducing) or 0.2 ml water (non-reducing)
    f. trace amount of bromophenol blue
2) 5X sample loading buffer
    a. 20% (at the least) of Pierce Lane Marker Sample Buffer (Pierce Biotechnology, Thermo Scientific)
    b. 4% β-mercaptoethanol
    c. SDS-PAGE Running Buffer[92,93]
    d. 25 mM Tris Base
    e. 250 mM Glycine
    f. 0.1% SDS
3) transfer buffer[47]
    a. 25 mM Tris Base
    b. 192 mM
    c. 20% methanol
4) coomassie blue R-250 stain

a. 45% Methanol

b. 10% Acetic Acid

c. .25% Coomassie Blue R-250 Stain

5) coomassie blue destain

a. 45% Methanol

b. 10% Acetic Acid

6) blocking buffer (pH 7.4)

a. 100 ml PBS

b. 0.025% sodium azide

c. 5% Non-fat dry milk (NFDM)

d. 1% normal goat serum

e. 0.1% Tween 20

7) incubation buffer

a. 10 ml PBS

b. 5% Non-fat dry milk (NFDM)

c. 0.25% Tween 20

d. pH to 7.4

8) wash buffer

a. 100 ml PBS

b. 0.05% Tween 20

BIBLIOGRAPHY

Bibliography

1. Gebel, H., Bray, R., and Nickerson, P. (2003). Pre-Transplant Assessment of Donor-Reactive, HLA-Specific Antibodies in Renal Transplantation: Contraindication vs. Risk. American Journal of Transplantation. 3:1488-1500.

2. Feucht, H.E. and Opelz, G. (1996). The humoral immune response towards HLA class II determinants in renal transplantation. Kidney International. 50:1464-1475.

3. Feucht, H.E., Schneeberger, H., Hillerbrand, G., et al. (1993). Capillary Deposition of C4d complement fragment and early renal graft loss. Kidney International. 43(6):1333-1338.

4. Mizutani, K., Terasaki, P., Rosen, A., et al. (2005). Serial Ten Year Follow-up of HLA and MICA Antibody Production Prior to Graft Failure. American Journal of Transplanation. 5(9):2265-2272.

5. Masson, D., Bayle, F., Vichier, C., et al. (1998). Anti-HLA class I reimmunization after one HLA semi-identical blood transfusion in non naïve patients on a waiting list for a first renal allograft. Transplantation Proceedings. 30(6):2854.

6. Regan, L., Braude, P.R., and Hill, D.P. (1991). A prospective study of the incidence, time of appearance, and significance of anti-paternal lymphocytotoxic antibodies in human pregnancy. Human Reproduction. 6(2):294-298.

7. Bouma, G.J., van Caubergh, P., van Bree, S.P., et al. (1996). Pregnancy can induce priming of cytotoxic T lymphocytes specific for paternal HLA antigens that is associated with HLA antibody formation. 62(5):672-678.

8. Scornik, J.C., Ireland, J.E., Howard, R.J., et al. (1984). Assessment of the Risk for Broad Sensitization by Blood Transfusions. Transplantation. 37(3): 249:253.

9. Alberu, J., Morales-Buenrostro, L.E., de Leo, C., et al. (2007). A non-allogenic stimulus triggers the production of *de novo* HLA antibodies in healthy adults. Transplant Immunology. 18(2):166-171.

10. Seibert, F.B., and Glenn, J.T. (1941). Tuberculin purified protein derivative. Preparation and analyses if a large quantity for standard. Am Rev Tuberc. 44:9-25.

11. Aguas, A., Esaguy, N., and Sunkel, C., et al. (1990). Cross-reactivity and Sequence Homology between the 65 Kilodalton Mycobacterial Heat Shock Protein and Human Lactoferrin, Transferrin, and DRβ Subsets of Major Histocompatibility Complex Class II Molecules. Infection and Immunity. 58(5):1461-1470.

12. Page, M., Ojugo, A., Imami, N., et al. (2007). Specificity of anti-human leukocyte antigen antibody responses after immunization with Remune, an inactivated HIV-1 vaccine. AIDS. 21(3):375-377.

13. Gootenberg, J., Ruscetti, F., Mier, J., et al. (1981). Human Cutaneous T Cell Lymphoma and Leukemia Cell Lines Produce and Respond to Growth Factor. Journal of Experimental Medicine. 154:1403-1418.

14. Mann, D. and O'Brien, S. (1989). Letter to the Editor: Origin of HIV-Susceptible Human CD4+ Cell Line H9. AIDS Research and Human Retroviruses. 5(3):253-255.

15. Forney, S., Gobeski, A, and Mankey, A., et al. (2008). 42-W: Unexpected vaccination outcome. Human Immunology. 69 S1:S119.

16. M-M-R®-II Measles, Mumps, and Rubella Virus Vaccine Live. Merck & Co., Inc. Whitehouse Station, NJ. Website: http://www.merck.com/product/usa/pi_circulars/m/mmr_ii/mmr_ii_pi.pdf. Accessed 06/29/2010.

17. VERIVAX® Varicella Virus Vaccine Live. Merck & Co., Inc. Whitehouse Station, NJ. Website: http://www.merck.com/product/usa/pi_circulars/v/varivax/varivax_pi.pdf. Accessed 06/29/2010.

18. Holdsworth, R., Hurley, C.K., Marsh, S.G.E., et al. (2009). The HLA dictionary 2008: a summary of HLA-A, -B, -C, DRB1/3/4/5-, DQB1 alleles and their association with serologically defined HLA-A, B, C, DR, and DQ antigens. Tissue Antigens. 73:95-170.

19. Robinson, J., Waller, M.J., Parham, P. et al. (2003). IMGT/HLA and IMGT/MHC : sequence database for the study of the major histocompatibility complex. Nucleic Acids Research 31: 311-314.

20. Robinson, J., Waller, M.J., Parham, P. et al. (2001). IMGT/HLA - a sequence database for the human major histocompatibilty complex. Nucleic Acids Research. 29:210-213.

21. Robinson, J., Malik, A. Parham, p. et al. (2000). IMGT/HLA - a sequence database for the human major histocompatibilty complex. Tissue Antigens. 55: 280-287.

22. Janeway, C. Travers, P., Walport, M, et al. (2005) Immunobiology; The Immune System in Health and Disease, 6th Ed. New York: Garland Science Publishing.

23. Horton, R., Wilming, L., Rand, V., et al. (2004). Gene Map of the Extended Human MHC. Nature Reveiws: Genetics. 5:889-899.

24. Kelley, J., Walter, L., and Trowsdale, J. (2005). Comparative genomics of major histocompatibility complexes. Immunogenetics. 56(10);683-695.

25. Fernando, M.M., Stevens, C.R., Walsh, E.C, et al. (2008). Defining the role of MHC in autoimmunity: a review and pooled analysis. PLoS Genetics. 4(4):e1000024.

26. Stenzel, A., Lu, T., Koch, W.A., et al. (2004). Patterns of linkage disequilibrium in the MHC region on human chromosome 6p. Human Genetics. 114(4):377-385.

27. Hall, B.M., Bishop, G.A., Duggin, G.G., et al. (1984). Increased expression of HLA-DR antigens in renal tranplants: relevance to rejection response. Lancet. 2:247-51.

28. Cai, J., Hohanof, S., and Terasaki, P.I. (2006). HLA-DR antibody epitopes. Clinical Transplants. 103-114.

29. El-Awar, N., Terasaki, P.I., Cai, J., et al. (2007). Epitopes of the HLA-A, B, C, DR, DQ, and MICA antigens. Clinical transplants. 175-194.

30. Marrari, M. and Duquesnoy, R.J. (2009). Correlations between Terasaki's HLA Class II epitopes and HLAMatchmaker-defined eplets on HLA-DR and –DQ antigens. Tissue Antigens. 74(2):134-146.

31. Duquesnoy, R.J., and Marrari, M. (2009). Correlations between Terasaki's HLA Class I epitopes and HLAMatchmaker-defined eplets on HLA-A, -B and –C antigens. Tissue Antigens. 74(2):117-133.

32. Duquesnoy, R.J., Awadalla, Y., Lomago, J., et al. (2008) Retransplant candidates have donor specific antibodies that react with structurally defined HLA-DR,DQ,DP epitopes. Transplant Immunology. 18:352-360.

33. CDC. (2010). Recommended Immunization Schedules for Persons Aged 0 Through 18 Years --- United States 2010. Morbidity and Mortality Weekly Report. 58:1-4.

34. Plotkin, S., Orenstein, W., and Offit, P. (2008). Vaccines: 5th ed. Philidelphia:Saunders/Elsevier.

35. Liebhaber, H., Riordan, J., and Horstmann, D. (1967) Replication of Rubella Virus in a Continuous Line of African Green Money Kidney Cells (Vero). Proc Soc Exp Biol Med. 125(2):636-643.

36. Proquad® Meseales, Mumps, Rubella, and Varicella Virus Vaccine Live. Merck & Co., Inc. Whitehouse Station, NJ. Website: http://www.merck.com/product/usa/pi_circulars/p/proquad/proquad_pi.pdf. Accessed 06/30/2010.

37. FDA. (2010). Biologic Product Shortages. Website: http://www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/Shortages/default.htm. Accessed 06/30/2010.

38. Cantin, R., Methot, S., and Tremblay, M. (2005). Plunder and Stowaways: Incorporation of Cellular Proteins by Enveloped Viruses. Journal of Virology. 79(11):6577-6587.

39. Beatch, M., Everitt, J., and Law, LM., et al. (2005) Interactions between Rubella Virus Capsid and Host Protein p32 are Important for Virus Replication. J. Virol. 79(16):10807-10820.

40. Deutzmann, R. (2004). Structural characterization of proteins and peptides. Methods in Molecular Medicine. 94:269-297.

41. Larive, C., Lunte, S., Zhong, M., et al. (1999) Separation and Analysis of Peptides and Proteins. 71:389R-423R.

42. Harlow, E. and Lane, D. (1999). Using Antibodies. New York: Cold Spring Harbor.

43. Page, M., and Thrope, R. (2002). The Protein Protocols Handbook: Purification of IgG Using Protein A or Protein G. Totowa, NJ: Humana Press Inc.

44. Hames, B.D. and Rickwood, D. (1998). Gel Electrophoresis of Proteins: A Practical Approach: 3rd Ed. The Practical Approach Series. Oxford: Oxford University Press.

45. Bolt, M.W. and Mahoney, P.A. (1997). High-efficiency blotting of proteins of diverse sizes following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Analytical Biochemistry. 247(2): 185-92.

46. Laemmli, U.K. (1970). Cleavage of Structural Proteins during the assembly of the Head of Bacteriophage T4. Nature. 227:680-685.

47. Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. 76:4350-4354.

48. Edman, P. (1950). Method for Determination of Amino Acid Sequence in Peptides. Acta Chemica Scandinavica. 4:283-293.

49. Aebersold, R. and Mann, M. (2003). Mass spectrometry-based proteomics. Nature. 13:198-207.

50. Olsen, J.V. (2004). Trypsin cleaves exclusively C-terminal to arginine and lysine residues. Molecular & cellular proteomics. 3(6):608-614.

51. Ahmed, F. (2008). Utility of mass spectrometry for proteome analysis: part I. Conceptual and experimental approaches. Expert Rev. Proteomics. 5(6):841-864.

52. Yates, J. Ruse, C. and Nakorchevsky. (2009). Proteomics by Mass Spectrometry: Approaches, Advances, and Applications. Annu. Rev. Biomed. 11:49-79.

53. Nesvizhskii, A., Vitek, O., and Aebersold, R. (2007). Analysis of proteomic data generated by tandem mass spectrometry. Nature Methods. 4(10):787-797.

54. Sadygov, R., Cociorva, D., and Yates, J. (2004). Large-scale database searching using tandem mass spectra: Looking up the answer in the back of a book. Nature Methods. 1(3):195-202.

55. McHugh, L. and Arthur, J.W. (2008). Computational Methods for Protein Identification from Mass Spectrometry Data. PLoS Comput Biol 4(2):e12. doi:10.1371/journal.pcbi.0040012.

56. Pappin, D.J., Hojrup, P., and Bleasby, A.J. (1993). Rapid identification of proteins by peptide-mass fingerprinting. Curr Biol. 3:327–32.

57. Searle, B.C., Brundege, J.M., and Turner, M. (2007). Improving sensitivity by combining results from multiple MS/MS search methodologies with the scaffold computer algorithm. J Biomol Tech 18:6–7.

58. Keller, A., Nasvizhskii, A.I., Kolker, E., et al. (2003). A statistical model for identifying proteins by tandem mass spectrometery. Anal Chem. 75(17):4646-4658.

59. Pruitt,K.D., Tatusova, T., and Maglott, D.R. (2007). NCBI Reference Sequence (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. Nucleic Acids Res. 35(Database issue):D61-5.

60. Jain E., Bairoch A., Duvaud S., et al. (2009). Infrastructure for the life sciences: design and implementation of the UniProt website. BMC Bioinformatics. 10:136.

61. The UniProt Consortium. (2010). The Universal Protein Resource (UniProt) in 2010. Nucleic Acids Res. D142-D148.

62. Keil, B. (1992). Specificity of proteolysis. Springer-Verlag Berlin-Heidelberg-NewYork.

63. Chevallet, M., Luche, S., and Rabilloud, T. (2006). Silver staining of proteins in polyacrylamide gels. Nature Protocols. 1(4):1852-1858.

64. Shevchenko, A., Wilm, M., Vorm, O., et al. (1996) Mass Spectrometric Sequencing of Proteins from Silver Stained Polyacrylamide Gels. Anal Chem. 68(5):850-858.

65. Marsh, S., Albert, E., Bodmer, W., et al. (2010). Nomenclature for factors of the HLA system, 2010. Tissue Antigens. 75:291-455.

66. Trinkle-Mulcahy, L., Boulon, S., Wah Lam, Y., et al. (2008). Identifying specific protein interaction partners using quantitative mass spectrometry and bead proteomes. J Cell Biol. 183(2):223-239.

67. Horejsi, V, Nemec, M, Angelisova, P., et al. (1986) Characterization of seven new monoclonal antibodies angains human DR, DR+DP, and DQ + DQ3 antigens. Tissue Antigens. 28:288-291.

68. Kapusta, J, Pniewski, T., Wojciehowicz, J., et al. (2010). Nanogram Doses of Alum-Adjuvanted HBs Antigen Indduce Humor Immune Response in Mice When Orally Administered. Arch. Immunol. Ther. Exp. 58:143-151.

69. McDonald, J.C., Adamashivili, I., Hayes, J.M, et al. (1994). Soluble HLA Class II concentrations in normal individuals and transplant recipients. Comparison with soluble HLA Class I concentrations. Transplantation. 58:1268-1272.

70. Brewster, J. (2003). Isolation and concentration of Salmonellae with an immunoaffinity column. Journal of Microbiological Methods. 55:287-293.

71. Gallant, S.R. (2004). Immunoaffinity Chromatography of Proteins. Methods in Molecular Biology. 251:103-109.

72. Ehrenmann, F., Kaas, Q., and Lefranc, M.P. (2010). IMGT/3Dstructure-DB and IMGT/DomainGapAlign: a database and a tool for immunoglobins or antibodies, T cell receptors, MHC, IgSF and MhcSF. Nucl. Acids. Res. 38(S1):D301-307.

73. Wang, Y., Geer, L.Y., Chappey C., et al. (2000). Cn3D: sequence and structure views for Entrez. Trenads Biochem Sci. 25(6):300-302.

74. Wang, Y. Addess, K.J., Chen, J., et al. (2007). MMDB: annotating protein sequences with Entrez's 3D structure database. Nucleic Acids Res. 35(Database issue):D298-300.

75. Gilbrat, J.F., Madej, T., Bryant, S.H. (1996). Surprising similarities in structure comparision. Curr Opin Struct Biol. 6(3):377-385.

76. Pentacel® Diphtheria and Tetanus Toxoids and Acellular Pertussis Absorbed, Inactivated Poliovirus and Haemophilus b Conjugate (Tetanus Toxoid Conjugate) Vaccine. Sanofi Pasteur Limited. Swiftwater, PA. Website: http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM109810.pdf. Accessed 09/04/2010.

77. COMVAX® [Haemophilus b Conjugate (Meningococcal Protein Conjugate) and Hepatitis B (Recombinant) Vaccine]. Merck & Co., Inc. Whitehouse Station, NJ. Website:

http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM109869.pdf. Accessed 09/03/2010.

78. VAQTA® (Hepatitis A Vaccine, Inactivated). Merck & Co., Inc. Whitehouse Station, NJ. Website: http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM110049.pdf. Accessed 09/04/2010.

79. TWINRIX® [Hepatitis A Inactivated & Hepatitis B (Recombinant) Vaccine]. GlaxoSmithKline. Research Triangle Park, NC. Website: http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM110079.pdf. Accessed 09/04/2010.

80. RECOMBIVAX® Hepatitis B Vaccine (Recombinant). Merck & Co., Inc. Whitehouse Station, NJ. Website: http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM110114.pdf. Accessed 09/04/10.

81. GARDASIL® [Human Papillomavirus Quadrivalent (Types 6, 11, 16, and 18) Vaccine, Recombinant]. Merck & Co., Inc. Whitehouse Station, NJ. Website: http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM111263.pdf. Accessed 09/04/2010.

82. CERVARIX® [Human Papillomavirus Bivalent (Types 16 and 18) Vaccine, Recombinant]. GlaxoSmithKline. Research Triangle Park, NC. Website: http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM186981.pdf. Accessed 09/04/2010.

83. Influenza A (H1N1) 2009 Monovalent Vaccine. CSL Limited. King of Prussia, PA. Website: http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM182401.pdf. Accessed 09/04/2010.

84. Influenza Virus Vaccine (H1N1) 2009. Sanofi Paseur, Inc. Swiftwater, PA. Website: http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM112836.pdf. Accessed 09/04/2010.

85. AGRIFLU®, Influenza Virus Vaccine. Novartis Vaccines and Diagnostics, Inc. Cambridge, MA. Website: http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM192127.pdf. Accessed 09/04/2010.

86. IXIARO (Japanese Encephalitis Vaccine, Inactivated, Absorbed). Manufactured by Intercell Biomedical. Livingston,UK. Distributed by Novartis Vaccines and Diagnostics, Inc. Cambridge, MA. Website: http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM142570.pdf. Accessed 09/04/2010.

87. JE-VAX® Japanese Encephalitis Vaccine Inactivated. Manufactured by The Research Foundation for Microbial Disease of Osaka University. Suita, Osaka, Japan. Distributed by Sanofi Pasteur Inc. Swiftwater, PA. Website: http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM123761.pdf. Accessed 09/04/2010.

88. IPOL® Poliovirus Vaccine Inactivated. Sanofi Pasteur. Swiftwater, PA. Website: http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM133479.pdf. Accessed 09/04/2010.

89. IMOVAX® Rabies Vaccine. Sanofi Pasteur. Swiftwater, PA. Website: http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM133484.pdf. Accessed 09/04/2010.

90. RotaTeq (Rotavirus Vaccine, Live, Oral, Pentavalent). Merck & Co., Inc. Whitehouse Station, NJ. Website: http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM142288.pdf. Accessed 09/04/2010.

91. Wilkerson, Curtis. Personal communication. July 22, 2010.

92. Davis, B.J. (1964). Disc electrophoresis II. Method and application to human serum proteins. Ann. NY Acad. Sci. 121:404

93. Ornstein, L. (1964). Disc electrophoresis, I. Method and application to human serum proteins. Ann. NY Acad. Sci. 121:321.