Development and evaluation of a rabies enzyme-linked immunosorbent assay (ELISA) targeting IgM and IgG in human sera

by

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Abstract

Rabies is nearly 100% fatal without the pre-or post-exposure prophylaxis vaccination series. Pre-exposure Prophylaxis (PrEP) vaccination series is administered to those persons in high risk occupations due to the evidence that PrEP is the most effective method of protection from a rabies infection. Rabies virus neutralizing antibody (RVNA) is required for the protection from rabies. However, the immune mechanism and antibody kinetics of isotype switching remains unclear by current diagnostic techniques. Knowledge of these kinetics will aid in making more informed decisions on the timing and number of vaccinations needed to elicit a sufficient antibody response for protection against exposure to rabies virus. Recently, the World Health Organization (WHO) supported alternative vaccine regimens that may affect peak levels of these subclasses of RVNA. Advisory Committee on Immunization Practices (ACIP) is also currently evaluating the rationale for ideal vaccine series for amending the current rabies prevention recommendations. To date, there has not been a rapid and reliable assay to detect and quantify antibody isotype switching from the primary antibody response of IgM to the subsequent IgG antibody response that occurs during the immune response to rabies vaccination. The principal requirement of this assay is that it can reliably and reproducibly determine and correlate responses to RVNA levels and monitor IgG versus IgM response, according to current guidelines. This knowledge will aid in the understanding of the immune response as a result of rabies virus infection or immunization using a currently approved vaccine and provide additional information to guide future research.

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Dedication

Dedicated to my daughter, Alexis Kay Zajac. My constant push to achieve my dreams and goals to be a role model to pave the way for her future.

List of Abbreviations

RIG – Rabies Immune Globulin

HRIG – Human Rabies Immune Globulin

PrEP – Pre-Exposure Prophylaxis

PEP – Post Exposure Prophylaxis

WHO – World Health Organization

CDC – Center for Disease Control

ACIP - Advisory Committee on Immunization Practices

RVNA – Rabies Virus Neutralizing Antibodies

HIV – Human Immunodeficiency Virus

AIDS – Acquired Immune Deficiency Syndrome

KSU – Kansas State University

EU/mL – Equivalent Units per milliliter

IU/mL – International Units per milliliter

IgG – Immunoglobulin Gamma

IgM – Immunoglobulin Mu

CCEEV - Concentrated cell culture and embryonated egg-based rabies vaccines

APC – Antigen Presenting Cell

ELISA – Enzyme Linked Immunosorbent Assay

RFFIT – Rapid Fluorescent Focus Inhibition Test

RABV – Rabies Virus

OD – Optical Density

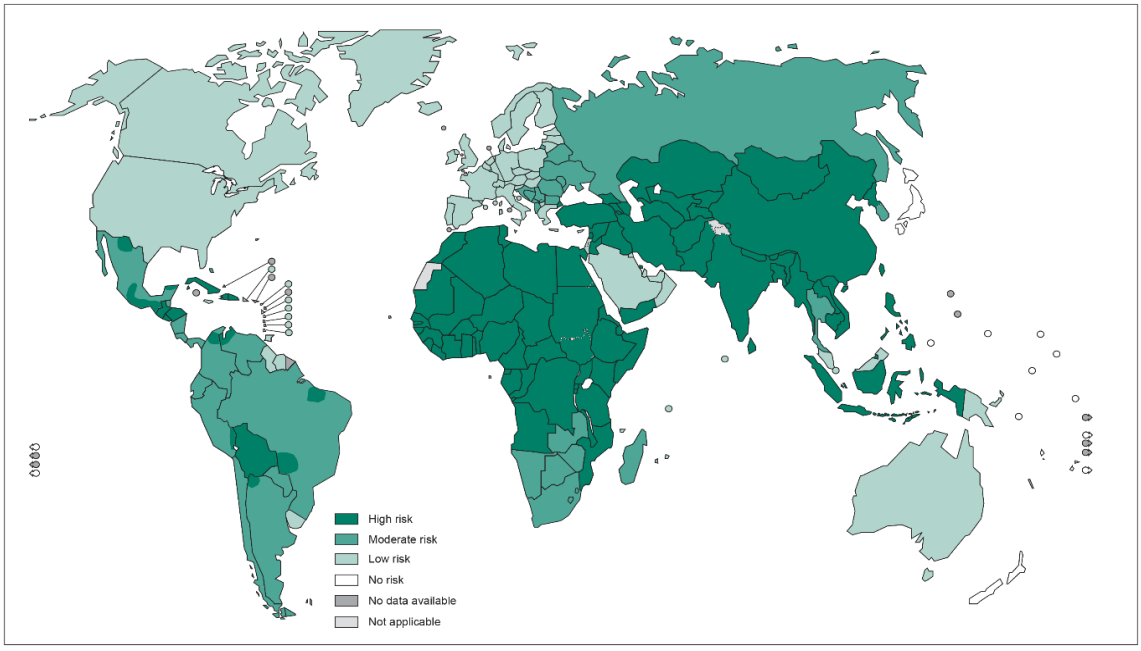
Chapter 1 - Introduction to Rabies Virus, Immune Response, and Current Diagnostics

1.1 Rabies Background

Rabies is considered one of the most lethal infectious diseases, with a case fatality rate of nearly 100% (without the receipt of pre-exposure prophylaxis consisting of the recommended vaccination series), well above other high profile infectious diseases such as Ebola (59-90%) and HIV/AIDS (80-90%) [1–4]. It remains prevalent in several regions of the world, and is endemic on all continents with a heavy burden in Africa and Asia [1,5]. Varying risk levels viewed in Figure 1. Distribution of Risk Levels for Humans contacting Rabies (2013) show moderate risk (indicated by dark green) through no risk (indicated by white) for all regions.

Figure 1. Distribution of Risk Levels for Humans Contacting Rabies (2013) [6]

Distribution of the varying risk levels for humans contacting rabies throughout the world as compiled by the World Health Organization from 2013.



Data Source: World Health Organization
Map Production: Control of Neglected Tropical Diseases (NTD)
World Health Organization

World Health Organization

Rabies is nearly 100% preventable if post exposure prophylaxis, consisting of wound cleansing (most important), administration of rabies immune globulin (RIG), and a series of four rabies vaccinations administered on days 0, 3, 7 and 14 as per the recommendation of the Advisory Committee on Immunization Practices, is received without delay [7,8]. Individuals previously vaccinated against rabies only receive the post-exposure vaccination series as a booster and not RIG [9]. Also, see Figure 2. World Health Organization (WHO) Position: Recommended Schedules for more detailed post-exposure prophylaxis guidelines [5]. When administering the vaccination series effective immunization can be achieved with reduced doses, and hence reduced cost, with a modification of the current vaccination schedule [5,10]. The World Health Organization has approved the use of a two dose vaccination series administered on day 0 and again on day 7 [5].

Figure 2. World Health Organization (WHO) Position: Recommended Schedules [5]

Recommended post-exposure prophylaxis (PEP) by the World Health Organization updated in 2018 for the varying categories of exposure for both naïve and previously vaccinated individuals.

	Category I exposure	Category II exposure	Category III exposure
Immuno-logically naive individuals of all age groups	Wash exposed skin surfaces. No PEP required.	Wound washing and immediate vaccination: - 2-sites ID on days 0, 3 and 7 - OR 1-site IM on days 0, 3, 7 and between day 14-28 - OR 2-sites IM on days 0 and 1-site IM on days 7, 21 RIG is not indicated.	Wound washing and immediate vaccination - 2-sites ID on days 0, 3 and 7[1] - OR 1-site IM on days 0, 3, 7 and between day 14-28[2] - OR 2-sites IM on days 0 and 1-site IM on days 7, 21[3] RIG administration is recommended.
Previously immunized individuals of all age groups	Wash exposed skin surfaces No PEP required.	Wound washing and immediate vaccination*: - 1-site ID on days 0 and 3; - OR at 4-sites ID on day 0; - OR at 1-site IM on days 0 and 3); RIG is not indicated.	Wound washing and immediate vaccination*: - 1-site ID on days 0 and 3; - OR at 4-sites ID on day 0; - OR at 1-site IM on days 0 and 3; RIG is not indicated.

[1] one-week, 2-site ID regimen / Institut Pasteur du Cambodge (IPC) regimen/2-2-2-0-0; Duration of entire PEP course: 7 days.
[2] two week IM PEP regimen/4-dose Essen regimen/1-1-1-1-0; Duration of entire PEP course: between 14 to 28 days.
[3] three week IM PEP regimen/Zagreb regimen/2-0-1-0-1; Duration of entire PEP course: 21 days.

* *except if complete PEP already received within <3 months*

World Health Organization

Rabies virus (RABV) is an unsegmented, single-stranded, negative-sense, enveloped RNA virus. It belongs in the order *Mononegavirales*, is in the *Rhabdoviridae* family, and belongs to the *Lyssavirus* genus of which there are fourteen species and three phylogroups [5,11]. RABV falls into the phylogroup I category and consists of five proteins that provide structural and functional support [11,12]. Viral replication and translation occur in the ribonucleoprotein complex that consists of the nucleoprotein (N), RNA-dependent RNA polymerase (L), and phosphoprotein (P) [1,13–15]. Neutralization of viral protein epitopes which bind host-cell receptors is crucial for protection against RABV via administration of rabies vaccines and immunoglobulin (RIG). This neutralization relies upon the tertiary structure formation of the matrix (M) and most importantly the glycoprotein (G). G alone is also effective for anti-rabies antibody production where monoclonal antibodies are used [12,16,17].

Clinical presentation begins similarly to most viral infections with the onset of flu-like symptoms to include, but not limited to fever, headache, anxiety, sore throat and cough [18,19]. Upon exposure, the virus travels through the outer tissues to the motor neurons where it spreads from cell to cell via the synaptic junctions and finally to the spinal cord and brain [19]. Clinically the disease manifests as either the paralytic or "furious" form that is caused by infection of the central nervous system [20–22]. More commonly, the furious form presents in approximately 80% of human cases with the typical signs of aggression; the less common paralytic form occurs in approximately 20% of cases and symptoms are lethargy and eventually paralysis that often goes misdiagnosed [19]. Ultimately it is acute encephalitis that leads to symptoms, coma, and death for either form of the disease [21,23].

1.2 Vaccines and the Immune Response

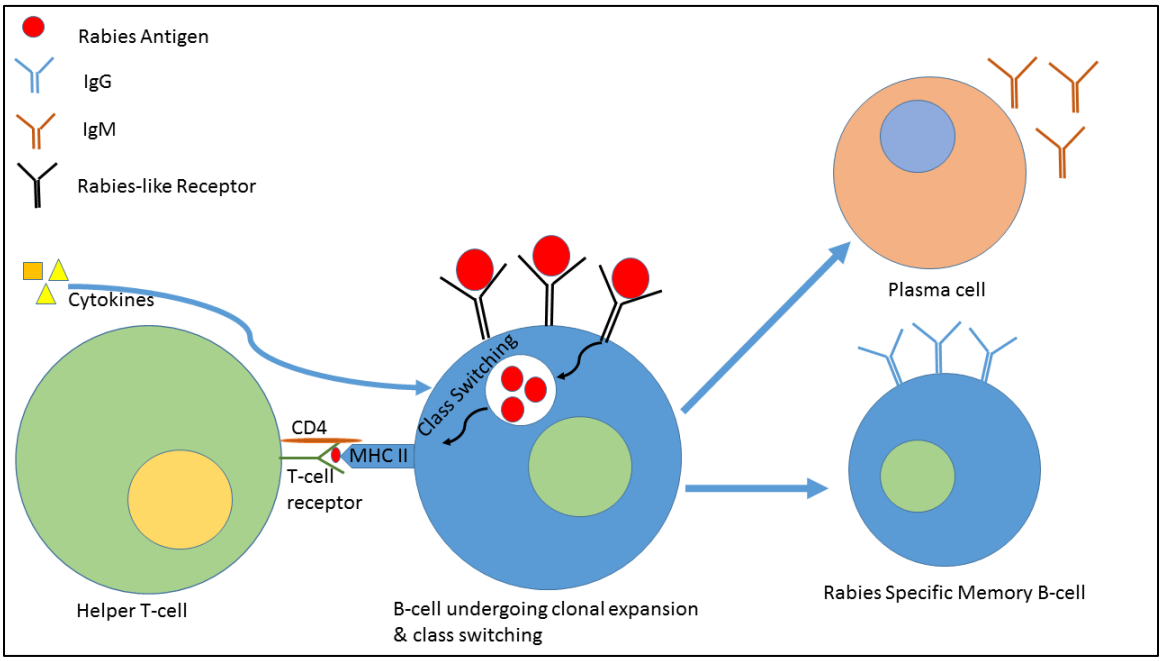
This section describes the immunological response and the cell types and other components involved in antibody production. The immunological response is important to understand the rationale for designing a separate IgM and IgG assay.

Concentrated cell culture and embryonated egg-based rabies vaccines (CCEEVs) are currently administered both via the intradermal (ID) and intramuscular (IM) routes [5,13]. ID injections allow a lower dosage than IM due to the aid of antigen-presenting

dendritic cells (APCs) that are abundantly present in the dermis [12,24]. APCs then act as a vehicle to rapidly process these antigens into peptides and present these antigen peptides via MHC II to CD4+ T-cells for activation of cytokine production that lead to class switching and elicit a strong immune response by antigen specific B-cells in a typical T cell-dependent B-cell response [24–26]. Antigen is bound to the Fab region on the B-cell receptor with secondary signaling from cytokines released by the previous CD4+ T-helper cells [27]. Somatic hypermutation occurs at the Fab region for better antigen fit and B-cells mature into plasma cells secreting IgM antibody followed by generation of memory B-cells [27]. Clones of these best antigen fit B-cells are produced that then go on to form more plasma cells and T-cell cytokines signal class switching for production of strongly neutralizing, long lasting IgG antibodies, see Figure 3 [25–27]. Upon repeat exposure (secondary immune response) to the antigen and clonal expansion of B-cells in lymphoid tissue, the response time is decreased and amount of neutralizing antibodies are increased via the process of affinity maturation during the humoral immune process [28].

Figure 3. Immune Response to Rabies Vaccination

The T-cell dependent B-cell immune response to rabies vaccination, including clonal expansion and class switching to highly neutralizing IgG antibody production.



IgM is the first immunoglobulin to respond in the presence of viral antibodies (IgM is primarily developed in plasma cells as a result of B cell activation) and IgG develops via T cell dependent immune response for lasting (humoral) immunity against an invading pathogen [25,26]. A rapid response is required by the primary immune system to be effective in recognizing and defending against pathogens, specifically rabies virus in this case [29,30]. As a result of this necessary function, the structure follows suit with IgM being a pentameric molecule capable of grabbing hold of up to ten epitopes of antigens [31].

IgG develops as a monomeric molecule against a specific antigen exposure as a result of the initial IgM primary response via class switching [28]. Affinity maturation is a result of selection of high affinity clones for expansion within the lymph nodes [28]. These responses trigger lasting immunity and a memory response via B-cells producing the immunoglobulin rapidly upon repeat exposure to the same antigen [32]. These antibodies are created in a specific manner dependent upon the type of antigen and are divided into four subclasses of IgG (IgG_1 through IgG_4) [33]. IgG antibody is the most prevalent in serum and has the ability to diffuse into tissues, unlike IgM which is short lived and cannot cross into tissues. [24–26,32,34].

1.3 Current Diagnostics

The following sections describe diagnostic detection of anti-rabies antibodies in patient sera.

1.3.1 RFFIT

Rapid Fluorescent Focus Inhibition Test is an antibody mediated virus neutralization test and is the most widely accepted assay for the quantitation of functional antibodies against rabies virus. The assay is performed in two stages (1) a virus-neutralization step, in which a standard dose of virus is mixed with the serial dilutions of a serum sample, and (2) an inoculation step, in which tissue culture cells are added to the reaction mixture. The absence or reduction of infectivity constitutes a positive neutralization reaction and indicates the presence of virus-specific antibodies in the test serum sample. The sensitivity of this neutralization test system (for measuring the growth of residual non-neutralized viruses) and the virus replication cycle time defined by the

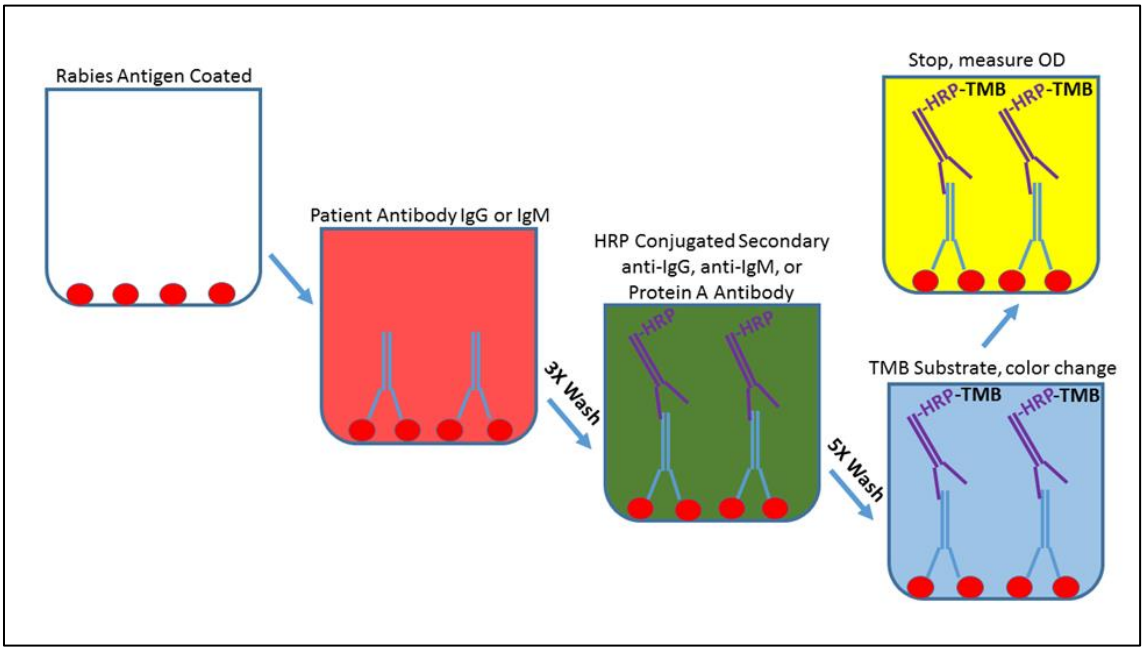
virus challenge dose to be used in the test assay. The precision with which the amounts of residual infectious virus can be measured is based on the sensitivity and specificity of the assay readout system. A fluorochrome conjugated anti-N protein rabies-specific antibody is used to measure the residual rabies virus infectivity [29,35,36]. This conjugate measures functional neutralization against the virus, not necessarily against specific Ig classes (can detect both IgM and IgG concurrently) [36,37].

1.3.2 ELISA

Indirect enzyme-linked immunosorbent assays (ELISA) are relatively inexpensive with a fast turnaround time compared to other serological testing methods. The assay uses adherence of antigens to the bottom of a micro well plate in combination with the following five principles: (1) an antigen-antibody immune reaction, (2) addition of secondary anti-rabies antibody coupled to a detector recognized by the substrate, (3) chromogenic product formed from a colorless substrate via an enzymatic chemical reaction, (4) stop solution added to cease the enzymatic reaction, (5) signal detection via a microplate reader and quantification of the optical density (OD) generated in the enzymatic reaction, see Figure 4. These in combination make this assay type one of the most specific and sensitive immunoassays to detect the biological molecule/protein of interest. By comparing the OD of the sample to the reference standard curve, the relative potency of the sample can be determined and expressed in equivalent units (EU/mL) [29,35,38,39]. Alternative secondary antibody (conjugates) can be varied for a wider range of detection utilizing the same basic assay components. EU/mL is a unit equivalent to the international units defined by seroneutralization (RFFIT).

Figure 4. Anti-Rabies Indirect ELISA Procedure

Indirect ELISA procedure utilizing rabies glycoprotein antigen coated micro titer wells to measure IgG or IgM in patient sera in response to rabies vaccination coupled with anti-IgG, anti-IgM, and Protein A horseradish peroxidase (HRP) labeled conjugate in combination with tetramethylbenzidine (TMB) substrate diluted into peroxydase substrate solution used as the detector.



Used in this assay development is the Platelia Rabies II kit which is an indirect immune-enzymatic technique allowing the detection of IgG antibodies directed against rabies virus glycoprotein ("Bio-Rad Platelia TM Rabies II Kit for in vitro Detection and Titration of IgG anti-Rabies Virus Glycoprotein in Human Serum and Plasma. Ref.:355-1180" 2015). Serum samples are distributed on a solid phase sensitized with purified rabies virus glycoprotein. The complex rabies antibodies/glycoprotein is revealed by the addition of an enzymatic conjugate (protein A derived from *Staphylococcus* aureus labeled with peroxidase allows for multi-species evaluation and binds preferentially to the Fc portion of IgG) [41–43]. The quantity of rabies antibodies in the sample is determined by comparing the OD of the sample to the R4b standard curve drawn from the quantification standards (S1 to S6) calibrated against the international WHO standard and proven to display equivalent binding compared to IgG. Validation results of the Bio-Rad Platelia Rabies Kit II against reference techniques (RFFIT) are published [43,44].

1.4 Rationale for New Diagnostics

Gold standard diagnostic tests are currently validated, accepted, and in use. However, more recently, alternative vaccine regimens have been supported by WHO that may affect the timing of seroconversion or quantity of the different classes of rabies virus neutralizing antibodies (RVNA) [5]. That difference in timing will have an effect across the board on all processes related to rabies PrEP and Post Exposure Prophylaxis (PEP). Use of measurement of rabies antibodies in human serum or donor plasma can be used to evaluate response to PrEP/PEP or to manage the production process of rabies immune globulin used in PEP in naïve individuals. This evaluation will push forward the ability to monitor and quantify rabies vaccine efficacy by monitoring isotype switching during vaccination, the specific timing of that switch, the contribution of each to RVNA, the result of affinity maturation to greater specificity and neutralizing function and better select plasma donors for manufacturing of rabies PEP immunoglobulin. The establishment and validation of this ELISA assay would be the first of its kind in distinguishing between anti-rabies IgG versus IgM and would add increased quantitation of the rabies immune response over the RFFIT and ELISA methods currently validated and utilized.

Chapter 2 - Proof of Concept and Study Design

The following sections describe previously performed proof of concept testing in the formulation of this assay development and the overall objectives in the development and evaluation of the IgM and IgG assays.

2.1 Proof of Concept

Anti-human IgG and IgM conjugate dilutions as well as a control sera for establishing an IgM standard curve for proof of concept testing were previously assessed by Amy Lyons and Dr. Susan Moore. A conjugate dilution of 1:10,000 for IgM and 1:6,400 for IgG were found to be optimal and were used as the starting reference point in this evaluation. A day 7 post vaccination serum sample was chosen to be the curve standard for establishment of the IgM quantitative assay and the kit supplied R4b standard was assessed against the IgG conjugate for establishment of the IgG quantitative assay.

2.2 Objective

The objective of the proof of concept study and the subsequent assay evaluation was to reliably detect and quantify the rabies specific IgG and IgM antibody response to vaccination and relate those antibodies to the rabies virus-neutralizing antibody (RVNA) response of the individuals who received the rabies vaccination series. Comprehensive investigation of the immune reaction of each individual's response to vaccination may contribute to our understanding of the variability of immunoglobulin class switching between individuals, potentially predict high versus low responder's effective immune defense in correlation between titer value and Ig class, and help guide future research of preventative and treatments options.

2.3 Proposed Method

Evaluation of this rabies ELISA technique targeting IgG and IgM in human sera will be assessed based upon a currently validated assay for detection of IgG in animal and human serum/plasma. This method is currently utilized for anti-rabies

glycoprotein detection in animal samples for monitoring of oral rabies vaccine baiting programs and in human plasma donors for rabies immunoglobulin production programs. This evaluation will push forward the ability to monitor and quantify rabies vaccine efficacy by monitoring isotype switching during vaccination, and better select plasma donors for the manufacturing of rabies post-exposure prophylaxis (PEP) immunoglobulin. IgM is the first immunoglobulin to respond in the presence of a viral infection and is primarily developed in plasma cells, IgG develops second. Long-lived plasma cells and memory cells combine for lasting (humoral) immunity against an invading pathogen. The establishment and validation of this assay would be the first of its kind in distinguishing between anti-rabies IgG versus IgM and would add increased quantitation of the rabies immune response over the immunofluorescent assay (IFA) method currently validated and utilized. This would provide clients with a more rapid option for evaluation of the sera samples sent in for routine rabies titer checks.

2.4 Participants

Unvaccinated Kansas State Veterinary Diagnostic Laboratory (KSVDL) employees that have given consent to have multiple blood draws for a representative sampling from Day 0 to Day 35 post vaccination with an FDA approved rabies vaccine were used in the collection of serum from each whole blood sample. This will ensure that there is adequate sera volume for both IgM and IgG quantitation and assay evaluation. Samples were de-identified as per the protocol reviewed and approved by IRB#1132.

2.5 Design

Platelia Rabies II ELISA kit (Ref: 355-1180) manufactured by Bio-Rad, which is currently validated for use to detect rabies virus glycoprotein in serum and plasma from humans was modified to evaluate human sera at varying blood draw days from 0 to 35 days post vaccination to detect IgG and IgM. Anti-human IgG (Sigma Cat# A0170) and anti-human IgM (Sigma Cat# A6907) HRP conjugates will be utilized and

evaluated in place of the kit supplied Protein A HRP conjugate. KSVDL Rabies antibody internal standards will be utilized as standards for comparison.

Table 1. Protein A ELISA Standard Curve Generation and Concentration

R4b 4 EU/mL positive control supplied by BioRad Platelia II Rabies ELISA kit. Manufacturers recommended dilution scheme and theoretical concentrations for each point on the standard curve utilized in the quantitative calculation of anti-rabies IgG in patient sera.

Quantification standards		Concentrations obtained by serial dilutions of the R4b Positive control
S6	R4b diluted to 1:100	4 EU/mL
S5	S6 diluted to 1:2	2 EU/mL
S4	S5 diluted to 1:2	1 EU/mL
S3	S4 diluted to 1:2	0.5 EU/mL
S2	S3 diluted to 1:2	0.25 EU/mL
S1	S2 diluted to 1:2	0.125 EU/mL

Table 2. Example Microplate Layout with Quantitative Method

Recommended plate set-up as per the Bio-Rad Platelia II Rabies ELISA kit manual including the kit supplied standards: R3 (negative control), R4a (0.5 EU/mL positive control), R4b (4 EU/mL positive control), and patient sera. R3, R4a, and patient samples are all diluted to the recommended 1:100 dilution prior to addition to the micro titer plate.

	1	2	3	4	5
A	R3	S4	Sample 1	Sample 9	…
B	R3	S4	Sample 2	Sample 10	
C	R4a	S3	Sample 3	Sample 11	
D	R4a	S3	Sample 4	Sample 12	
E	S6	S2	Sample 5	Sample 13	
F	S6	S2	Sample 6	Sample 14	
G	S5	S1	Sample 7	Sample 15	
H	S5	S1	Sample 8	Sample 16	

2.6 Procedure

A suitable IgM control sera will be utilized for establishing a standard curve similar to the R4b standard against Protein A conjugate (range of 0.125 to 4 EU/mL) used in the kit. Previously, the kit supplied R4b was used in establishment of the IgG standard curve. This development and evaluation will reassess that standard as the best fit for the IgG curve and delve into alternative samples for the establishment of a suitable IgG standard curve sample that provides a similar curve fit as compared to the kit supplied standard and conjugate. Further experimentation will be required to optimize and standardize the conjugate dilutions for each analyte. Incubation times and suggested dilutions will follow the manufacturer's kit instructions during establishment of appropriate conjugate dilutions and IgM and IgG standard curve creation. Based upon the proof of concept testing, those sample values (OD and EU/mL) alongside the average days post vaccination (DPV) will be the starting point for determining the optimum conjugate dilution for IgG and IgM. Followed by testing the reproducibility of the standard curve for IgM utilizing sample RAE-2 with a pre-dilution of 1:100 followed by serial two-fold dilutions for the positive control sera comparison.

Chapter 3 - Materials and Methods

The next sections describe the methodology behind all testing including RFFIT, Protein A, anti-IgM, and anti-IgG conjugated indirect ELISAs including standard curve development for the IgM and IgG assays.

3.1 RFFIT Materials & Methods

3.1.1 Challenge Virus

CVS-11 (challenge virus standard – 11) strain of rabies virus was evaluated as the challenge virus in the RFFIT assay used to quantitate the amount of RVNA present in serum samples. The CVS-11 strain was obtained from the Centers for Disease Control and Prevention (ATCC). Seed virus of the CVS-11 was grown on BHK cells to produce stock virus. Stock virus preparations were titered to obtain a working dilution of 50 $TCID_{50}$.

3.1.2 Serum samples

Serum samples used in the analysis were obtained from ten subjects who had received the same pre-exposure vaccination regimen with vaccine administered on days 0, 7, 21 or 28 as per the current ACIP guidelines. Serum samples that were collected on days 0, 7, 14, 21, 28, and 35 after initial vaccination were included in the study, in some instances a day 42 post vaccination sample was also obtained. Samples were coded using RAE- , to represent “Rabies Antibody ELISA” as is the intended purpose for the collection and storage of this sample set. All samples were heat-inactivated for 30 minutes at 56°C to remove complement factors that have been proven to interfere with neutralization. A pool of serum from unvaccinated subjects was used as the rabies antibody negative control.

3.1.3 Serological testing

RFFIT, using CVS-11 as the challenge virus strain was used to assay all serum samples for the baseline rabies virus neutralizing antibody (RVNA) titer value, as previously described (Smith, 1996). Testing was performed at the Kansas State

University Rabies Lab (Manhattan, KS). First, 100 µL of each serum sample was diluted in serial five-fold dilutions in 96-well microplates utilizing robotics for the dilution step via BioTek precision automated diluters and 100 µL if each serum dilution loaded into 8-well Lab-Tek chamber slides (Nunc™ Lab-Tek™ Chamber Slide System, catalog# 177445) after which 100 µL of the challenge virus, at a concentration of 50 $TCID_{50}$, was added (see Figure 5. RFFIT Endpoint 96-well Plate Dilutions and Figure 6 RFFIT Endpoint Transfer to 8-well Lab-Tek Slides). Viral addition results in a two-fold dilution for end titer values of 1:5, 1:25, 1:125, and 1:625. Exact endpoint titers for high-titer sera were obtained when initial titer results were above the upper limit of quantitation (> 15.0 IU/mL). High-titer sera was pre-diluted in RFFIT media to obtain a readable result within the linear range of the assay (0.1 IU/mL to 15.0 IU/mL) as defined in the current version KSU RFFIT Validation Report (2015).

Figure 5. RFFIT Endpoint 96-well Plate Dilutions

Dilution schematic for the Rapid Focus Fluorescent Inhibition Test (RFFIT). Media addition to the 96-well plate and dilutions performed via automated Bio-Tek precision automated diluters, initial samples are loaded manually by a trained technician. Serum dilutions are 1:2.5, 1:12.5, 1:62.5, and 1:312.5 after diluting.

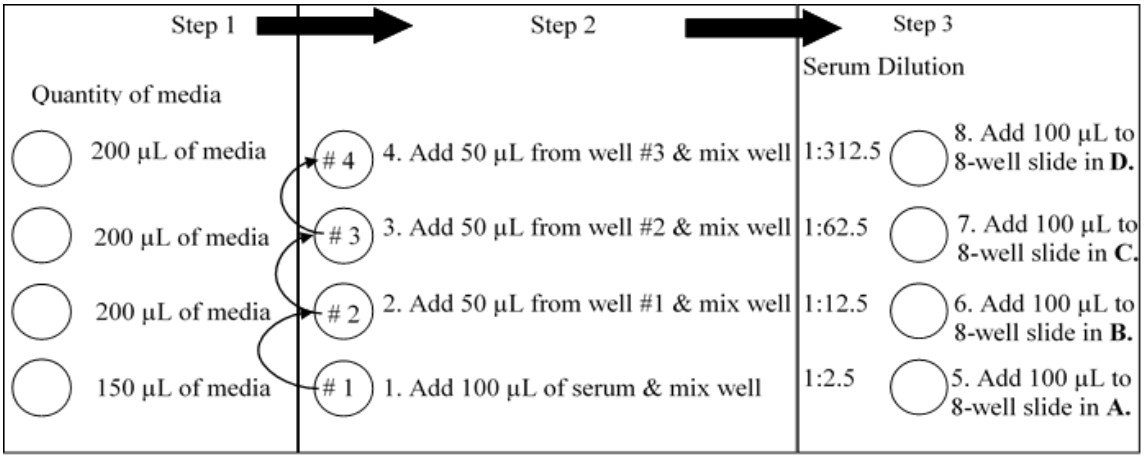


Figure 6. RFFIT Endpoint Transfer to 8-Well Lab-Tek Slides

Following serum dilutions in Figure 4. RFFIT Endpoint 96-well Plate Dilutions, 100 µL of each diluted serum is added to a separate well in an 8-well chamber slide as per the schematic below.

Serum Sample 1				Add 100 µL of each serum dilution to the well. Each endpoint will occupy 4 wells of the 8-well slide as shown. Initial serum dilutions stated. The serum samples will be diluted X2 when the virus is added.
1.A 1:2.5	**1.B** 1:12.5	**1.C** 1:62.5	**1.D** 1:312.5	
2.A 1:2.5	**2.B** 1:12.5	**2.C** 1:62.5	**2.D** 1:312.5	
Serum Sample 2				

Slides were incubated at 37ºC ± 2 ºC for 90 minutes ± 5 minutes after which 200µL of a suspension containing 5 X 10^5 BHK cells (American Tissue Cell Culture-ATCC, Catalog # CCL-10) were added to each well. Slides were placed in a 2-5% CO^2 incubator at 37ºC ± 2 ºC for 20-24 hours. After incubation, the slides were washed and fixed in 80% cold acetone, dried and stained with FITC conjugated anti-rabies antibody (anti-N, Millipore Sigma Catalog #5500). Twenty fields per well were examined under 100X magnification using a fluorescence microscope for the presence of rabies virus and RVNA titers were calculated using the Reed and Muench method (Habel, 1996). International units were calculated from the serum titer using the following formula:

Equation 1. Reed and Muench RVNA Titration Calculation

$$\frac{\text{Endpoint titer of test serum}}{\text{Endpoint titer of reference serum}} \times 2.0 \text{ IU/mL reference serum}$$

Table 3. RFFIT Endpoint Titer Results

Rapid Focus Fluorescent Inhibition Test endpoint titer results measuring functional/neutralizing antibodies expressed in IU/mL (International Units) for all Rabies Antibody ELISA (RAE) samples using CVS-11 (Challenge Virus Standard) as the neutralizing virus to determine baseline titer values. Below limit of quantitation results display as "</= 0.1" and any samples with titers > 15.0 (the assay upper limit) were pre-diluted prior to following the dilution scheme in Figure. 5. Samples RAE-1 through RAE-5, as indicated by the red box (also denoted by shading), correspond to a single patient for all draw dates (expressed in days post vaccination (DPV). RAE-35 corresponds to pooled negative samples.

Sample ID:	DPV:	Results (IU/mL):	Sample ID:	DPV:	Results (IU/mL):	Sample ID:	DPV:	Results (IU/mL):
RAE-1	0	</= 0.1	RAE-18	42	24.4	RAE-35	0	0.1
RAE-2	14	17.5	RAE-19	0	</= 0.1	RAE-36	0	</= 0.1
RAE-3	21	23.9	RAE-20	7	0.5	RAE-37	16	10.0
RAE-4	28	106.8	RAE-21	14	9.9	RAE-38	31	32.4
RAE-5	42	84.7	RAE-22	21	11.3	RAE-39	0	</= 0.1
RAE-6	0	</= 0.1	RAE-23	28	26.1	RAE-40	16	</= 0.1
RAE-7	7	2.0	RAE-24	42	18.5	RAE-41	31	5.3
RAE-8	14	12.5	RAE-25	0	</= 0.1	RAE-42	0	</= 0.1
RAE-9	21	11.3	RAE-26	7	0.9	RAE-43	7	</= 0.1
RAE-10	28	28.6	RAE-27	21	25.0	RAE-44	21	5.3
RAE-11	42	27.3	RAE-28	28	13.5	RAE-45	42	14.3
RAE-12	0	</= 0.1	RAE-29	0	</= 0.1	RAE-55	0	</= 0.1
RAE-13	7	</= 0.1	RAE-30	7	</= 0.1	RAE-56	7	</= 0.1
RAE-14	10	0.2	RAE-31	14	0.6	RAE-57	14	13.1
RAE-15	14	3.2	RAE-32	21	1.2	RAE-58	28	7.7
RAE-16	21	11.9	RAE-33	28	1.2	HRIG	HRIG	10.0
RAE-17	28	10.1	RAE-34	42	2.9			

As expected, the day 0 samples display results below the limit of quantitation (</ 0.1) and the titer result increases after each vaccination with a leveling off and stabilization of antibodies near the day 42 post vaccination draw date, see Table 3.

3.2 Protein A ELISA Materials and Methods

After the baseline gold standard RFFIT titer values were established for each sample, the next step in this evaluation was to obtain the Protein A ELISA titer values.

3.2.1 Kit

Platelia™ Rabies II Kit (Marnes-la-Coquette, France) Ref: 355-1180 for *in vitro* detection and titration of IgG anti rabies virus glycoprotein in human serum and plasma

was used for baseline IgG titer value in (equivalent units) EU/mL. The kit contains wells coated with rabies glycoprotein (G-protein) for use as the antigen in an 8-well per strip format with a secondary horseradish peroxidase (HRP) enzyme conjugated *Staphylococcus aureus* protein A (preferentially detects the Fc portion of IgG) followed by a tetramethylbenzidine (TMB) substrate that produces a colormetric reaction. Lastly, stop solution (1N sulphuric acid) is added to stop the enzymatic reaction. Version 881179 – 2015/06 kit instructions were followed to obtain the titer value in EU/mL which were calculated by comparison of the sample optical density reading against a standard curve of positive standards, calibrated to the WHO standards, supplied in the kit (R4b standard range: </= 0.125 to >/= 4 EU/mL).

3.2.2 Serum samples

Same as assayed under section 3.1.2 RFFIT Materials & Methods, section Serum Samples.

3.2.3 Serological testing

The indirect ELISA method, Bio-Rad Platelia Rabies Kit II ELISA (Marnes-la-Coquette, France) was performed at the Kansas State University Rabies Lab (Manhattan, KS) per the manufacturer's instructions. Kit supplied controls, R3, R4a, and R4b were diluted 1:100 using 10 µL sample and 990 µL of R6 diluent; when diluted 1:100, R4b = S6 in the standard curve. S6 was then serially diluted 1:2 using 500 µL of the previously made S6 and 500 µL R6 diluent to produce S5. Serial dilutions were continued using the same pipettes and volumes to produce S4, S3, S2, and S1 (S5 was diluted 1:2 to produce S4; S4 was diluted 1:2 to produce S3; S3 was diluted 1:2 to produce S2; S2 was diluted 1:2 to produce S1). Internal standards MMP-4, 0.5 Ref2017, and FBS were diluted 1:100 using 10 µL of standard and 990 µL R6 diluent. Samples RAE-4, -5, -10, -11, -16, -17, -18, -23, -24, -27, 28, 38, -45, -57, and HRIG were pre-diluted 1:10 using 20 µL sample and 180 µL R6 diluent. Pre-dilutions were initially determined on the basis of the RFFIT IU/mL results in correlation with the upper limit of the ELISA assay of 4.0 EU/mL. All other samples were tested neat on the assay. Internal standard Ref2017 was diluted 1:50 using 30 µL of standard and 1470 µL of R6 diluent used as a dilution control. See Figure

7 for plate layout. Once all pre-dilutions were performed samples and internal standards were diluted 1:100 per the Bio-Rad kit instructions using 10 µL sample and 990 µL R6 diluent. Once all 1:100 assay dilutions had been prepared, 100 µL of each sample (all controls, internal standards, and samples) were added to the micro titer plate, covered with adhesive film, and incubated at 37°C ± 2°C for 60 ±5 minutes. The plate was removed from the incubator, adhesive film removed, and prepared wash solution (10X R2) was used to perform three wash cycles with 300 µL per well each cycle. Conjugate solution (R7) was prepared at a 1:10 dilution using R2 as the diluent, 100 µL added to each well, new adhesive film added, and incubated at 37°C ± 2°C for 60 ±5 minutes. The plate was removed from the incubator, adhesive film removed, and prepared wash solution (10X R2) was used to perform five wash cycles with 300 µL per well each cycle. TMB substrate solution (R9) was prepared at a 1:11 dilution using R8 as the diluent, 100 µL added to each well away from direct light, and incubated uncovered at +18°C to +30°C for 30 ± 5 minutes in the dark. Finally, 100 µL of Stop Solution (R10) was added to each well and read immediately on a microplate reader at 450 and 630 nm to obtain the OD readings and the delta OD determined.

A Bio-Tek ELx808 microplate reader (Winooski, VT) coupled with Gen 5 (version 2.06) software set to the manufacturer's specifications for the quantitative kit parameters were used alongside the proprietary Bio-Rad Platelia II calculations in the Excel results workbook to obtain the results in EU/mL for each sample (see Figure 7. Bio-Rad Platelia II Result Excel Sheet). Samples producing results above the kit upper level of quantitation (ULOQ) were further diluted and retested to obtain an endpoint result within the quantitative range of each assay.

Figure 7. Bio-Rad Platelia II Result Excel Sheet

Indirect enzyme-linked immunosorbent assay (ELISA) Excel sheet for obtaining endpoint titer results expressed in EU/mL (Equivalent Units) for all samples using Protein A at a dilution of 1:10 as the secondary conjugate. Optical density (OD) values are input (into the yellow fields) in the top sheet along with sample IDs (in grey fields). Proprietary calculations assess criteria of the kit standards and generate a standard curve to obtain a quantitative titer result (EU/mL) for each sample.

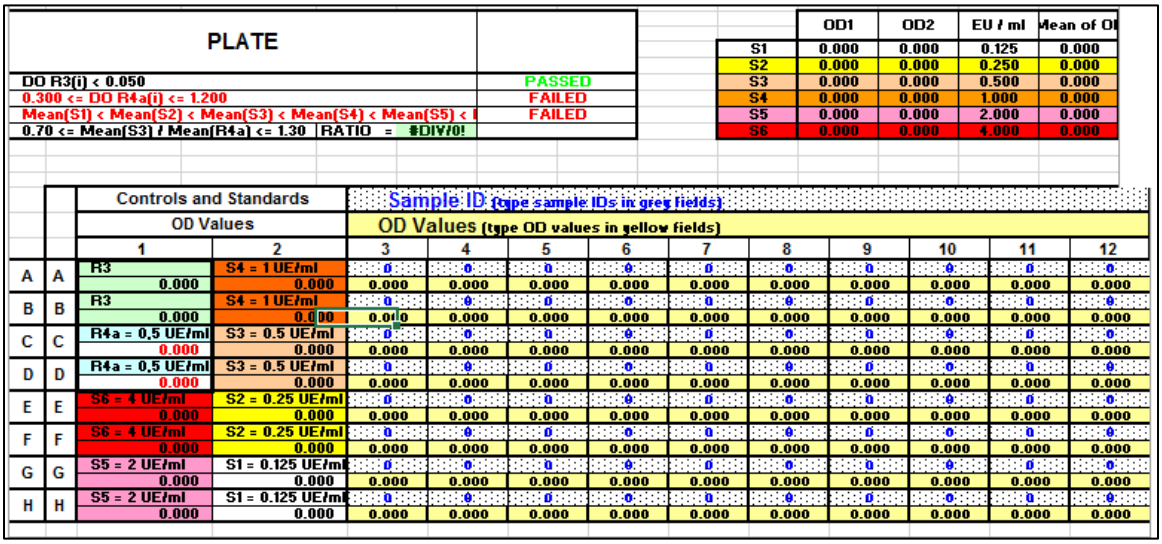


PLATE	
DO R3(i) < 0.050	PASSED
0.300 <= DO R4a(i) <= 1.200	FAILED
Mean(S1) < Mean(S2) < Mean(S3) < Mean(S4) < Mean(S5) < I	FAILED
0.70 <= Mean(S3) / Mean(R4a) <= 1.30 RATIO = #DIV/0!	

	OD1	OD2	EU / ml	Mean of OI
S1	0.000	0.000	0.125	0.000
S2	0.000	0.000	0.250	0.000
S3	0.000	0.000	0.500	0.000
S4	0.000	0.000	1.000	0.000
S5	0.000	0.000	2.000	0.000
S6	0.000	0.000	4.000	0.000

		Controls and Standards		Sample ID (type sample IDs in grey fields)									
		OD Values		OD Values (type OD values in yellow fields)									
		1	2	3	4	5	6	7	8	9	10	11	12
A	A	R3	S4 = 1 UE/ml	0	0	0	0	0	0	0	0	0	0
		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	B	R3	S4 = 1 UE/ml	0	0	0	0	0	0	0	0	0	0
		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
C	C	R4a = 0,5 UE/ml	S3 = 0.5 UE/ml	0	0	0	0	0	0	0	0	0	0
		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
D	D	R4a = 0,5 UE/ml	S3 = 0.5 UE/ml	0	0	0	0	0	0	0	0	0	0
		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
E	E	S6 = 4 UE/ml	S2 = 0.25 UE/ml	0	0	0	0	0	0	0	0	0	0
		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F	F	S6 = 4 UE/ml	S2 = 0.25 UE/ml	0	0	0	0	0	0	0	0	0	0
		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
G	G	S5 = 2 UE/ml	S1 = 0.125 UE/ml	0	0	0	0	0	0	0	0	0	0
		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
H	H	S5 = 2 UE/ml	S1 = 0.125 UE/ml	0	0	0	0	0	0	0	0	0	0
		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

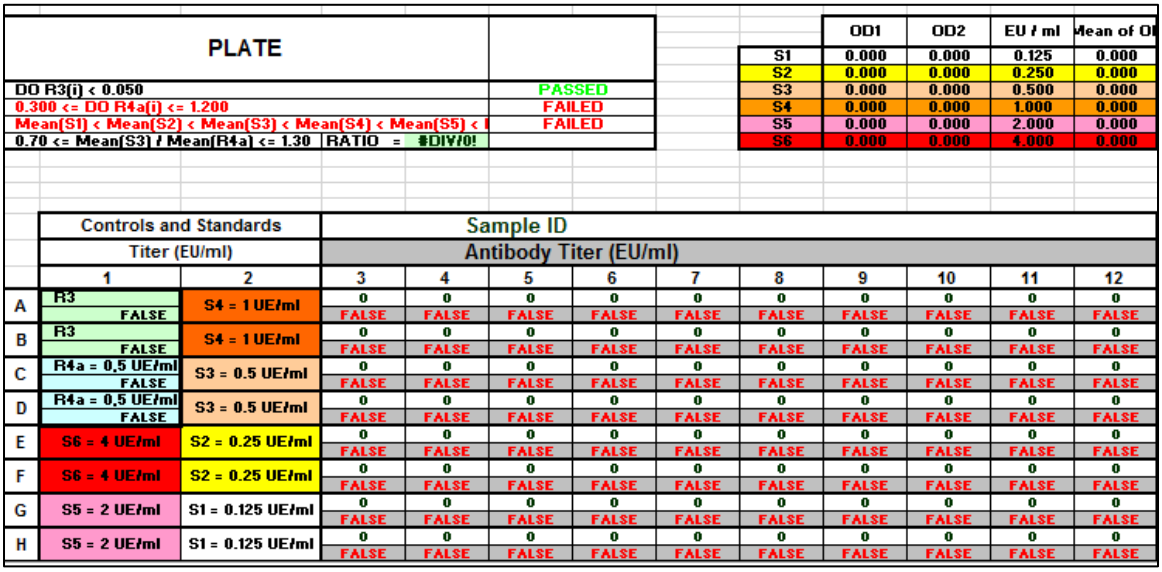


PLATE	
DO R3(i) < 0.050	PASSED
0.300 <= DO R4a(i) <= 1.200	FAILED
Mean(S1) < Mean(S2) < Mean(S3) < Mean(S4) < Mean(S5) < I	FAILED
0.70 <= Mean(S3) / Mean(R4a) <= 1.30 RATIO = #DIV/0!	

	OD1	OD2	EU / ml	Mean of OI
S1	0.000	0.000	0.125	0.000
S2	0.000	0.000	0.250	0.000
S3	0.000	0.000	0.500	0.000
S4	0.000	0.000	1.000	0.000
S5	0.000	0.000	2.000	0.000
S6	0.000	0.000	4.000	0.000

	Controls and Standards		Sample ID									
	Titer (EU/ml)		Antibody Titer (EU/ml)									
	1	2	3	4	5	6	7	8	9	10	11	12
A	R3	S4 = 1 UE/ml	0	0	0	0	0	0	0	0	0	0
	FALSE		FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
B	R3	S4 = 1 UE/ml	0	0	0	0	0	0	0	0	0	0
	FALSE		FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
C	R4a = 0,5 UE/ml	S3 = 0.5 UE/ml	0	0	0	0	0	0	0	0	0	0
	FALSE		FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
D	R4a = 0,5 UE/ml	S3 = 0.5 UE/ml	0	0	0	0	0	0	0	0	0	0
	FALSE		FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
E	S6 = 4 UE/ml	S2 = 0.25 UE/ml	0	0	0	0	0	0	0	0	0	0
			FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
F	S6 = 4 UE/ml	S2 = 0.25 UE/ml	0	0	0	0	0	0	0	0	0	0
			FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
G	S5 = 2 UE/ml	S1 = 0.125 UE/ml	0	0	0	0	0	0	0	0	0	0
			FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE
H	S5 = 2 UE/ml	S1 = 0.125 UE/ml	0	0	0	0	0	0	0	0	0	0
			FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE	FALSE

Table 4. Protein A ELISA Results

Indirect enzyme-linked immunosorbent assay (ELISA) endpoint titer results expressed in EU/mL (Equivalent Units) for all samples using Protein A at a dilution of 1:10 as the secondary conjugate. Below limit of quantitation results display as "</= 0.125" and any samples with titers > 4.0 (the assay upper limit) were pre-diluted prior to following the manufacturer 1:100 assay dilution. Samples RAE-1 through RAE-5, as indicated by the red box (also denoted by shading), correspond to a single patient for all draw dates (expressed in days post vaccination (DPV). RAE-35 corresponds to pooled negative samples. Internal standards are indicated by the purple box and kit supplied standards by the green box.

Sample ID:	DPV:	Results (EU/mL):	ELISA ID:	DPV:	Results (EU/mL):	Sample ID:	DPV:	Results (EU/mL):
RAE-1	0	</= 0.125	RAE-21	14	1.40	RAE-41	31	3.72
RAE-2	14	3.30	RAE-22	21	2.95	RAE-42	0	</= 0.125
RAE-3	21	3.86	RAE-23	28	16.62	RAE-43	7	</= 0.125
RAE-4	28	13.84	RAE-24	42	12.38	RAE-44	21	1.94
RAE-5	42	13.02	RAE-25	0	</= 0.125	RAE-45	42	9.62
RAE-6	0	</= 0.125	RAE-26	7	</= 0.125	RAE-55	0	</= 0.125
RAE-7	7	</= 0.125	RAE-27	21	7.73	RAE-56	7	</= 1.25
RAE-8	14	1.05	RAE-28	28	6.42	RAE-57	14	6.92
RAE-9	21	1.56	RAE-29	0	</= 0.125	RAE-58	28	2.19
RAE-10	28	18.25	RAE-30	7	</= 0.125	HRIG	HRIG	14.44
RAE-11	42	18.07	RAE-31	14	0.24	Ref2017	Ref2017	24.81
RAE-12	0	</= 0.125	RAE-32	21	0.46	MMP-4	MMP-4	0.14
RAE-13	7	</= 0.125	RAE-33	28	0.55	0.5Ref2017	0.5Ref2017	0.39
RAE-14	10	</= 0.125	RAE-34	42	3.92	FBS	FBS	</= 0.125
RAE-15	14	0.75	RAE-35	0	0.20	R3	R3	</= 0.125
RAE-16	21	8.97	RAE-36	0	</= 0.125	R3	R3	</= 0.125
RAE-17	28	7.47	RAE-37	16	1.46	R4a	R4a	0.46
RAE-18	42	27.57	RAE-38	31	9.23	R4a	R4a	0.45
RAE-19	0	</= 0.125	RAE-39	0	</= 0.125	R4b	R4b	4.09
RAE-20	7	</= 0.125	RAE-40	16	</= 0.125	R4b	R4b	3.93

As expected, the day 0 samples display results below the limit of quantitation (</ 0.125) and the EU/mL titer result increases after each vaccination with a leveling off and stabilization of antibodies near the day 42 post vaccination draw date, see Table 4. The internal standards (Ref2017, MMP-4, 0.5Ref2017, and FBS) all correspond to the expected values and provide the interassay validity between the initial assay and subsequent assays runs to include the pre-dilutions for endpoint titration. In addition, the kit supplied standards (R3, R4a, and R4b) produce appropriate values against the expected.

3.2.4 Standard curve determination

As neither the standard curve type nor curve fit equation is given in the Bio-Rad Platelia Rabies Kit II ELISA kit insert or via the proprietary Excel results worksheet, the concentration of the samples produced by each standard curve type (see Equation 3. Gen5 Polynomial Curve, Equation 4. Gen5 Spline Curve, and Equation 5. Gen5 Non-linear Curve; see also, Figure 8. Gen5 Generated Standard Curve for Point to Point Fit, Figure 9. Gen5 Generated Standard Curve for Polynomial Fit, Figure 10. Gen5 Generated Standard Curve for Spline Fit, and Figure 11. Gen5 Generated Standard Curve for Non-Linear Fit) offered by the Gen5 software were compared against the concentration produced by the Bio-Rad Excel results worksheet for the best fit using Equation 2. Percent Recovery where the Experimental Value is equal to the results obtained from using the anti-IgG conjugate coupled with a human IgG standard prepared internally (KAM-1) as the standard curve and the Expected Value is equal to the results obtained from using the Protein A conjugate supplied by the kit and Bio-Rad proprietary calculations.

Equation 2. Percent Recovery

$$\%R=(\text{Experimental Value}/\text{Expected Value}) \times 100$$

Figure 8. Gen5 Generated Standard Curve for Point to Point Fit

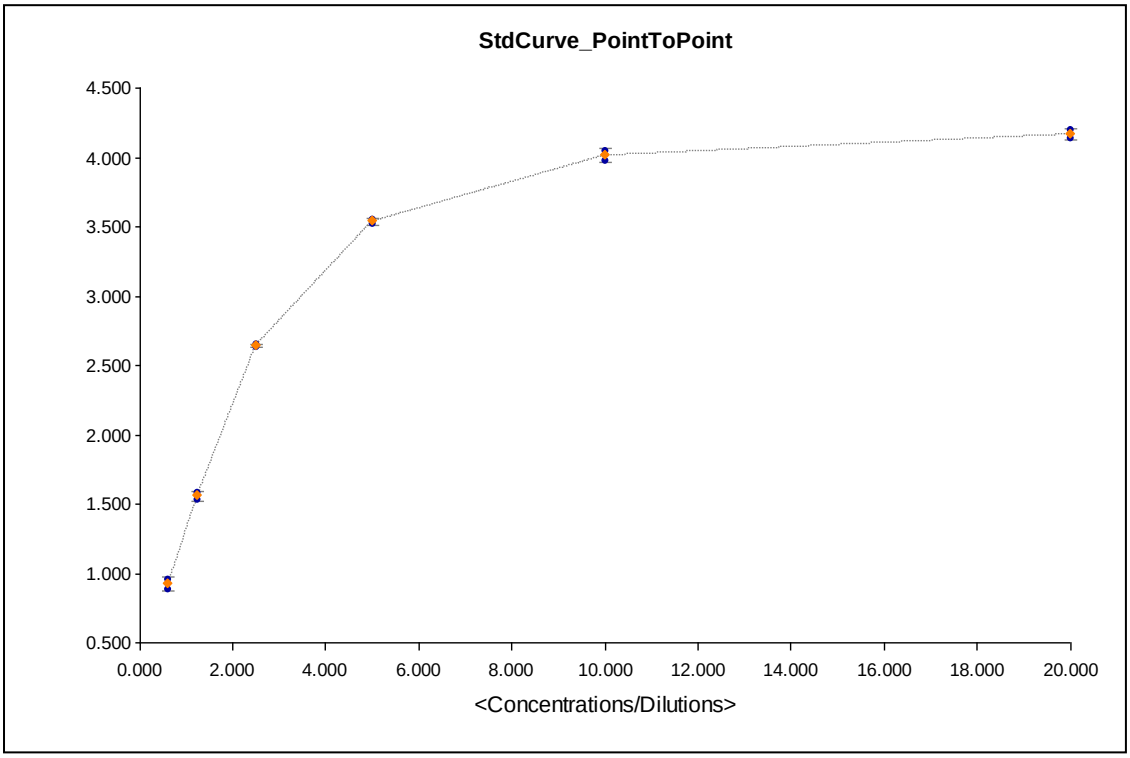


Figure 9. Gen5 Generated Standard Curve for Polynomial Fit

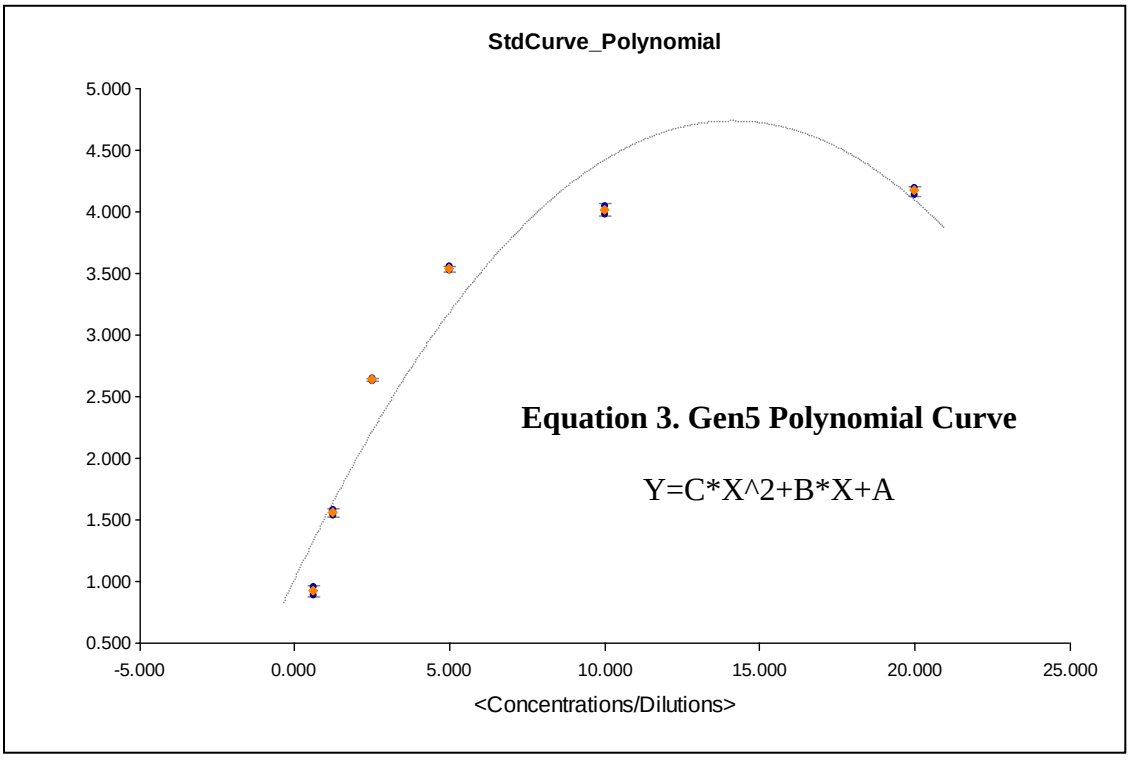


Figure 10. Gen5 Generated Standard Curve for Spline Fit

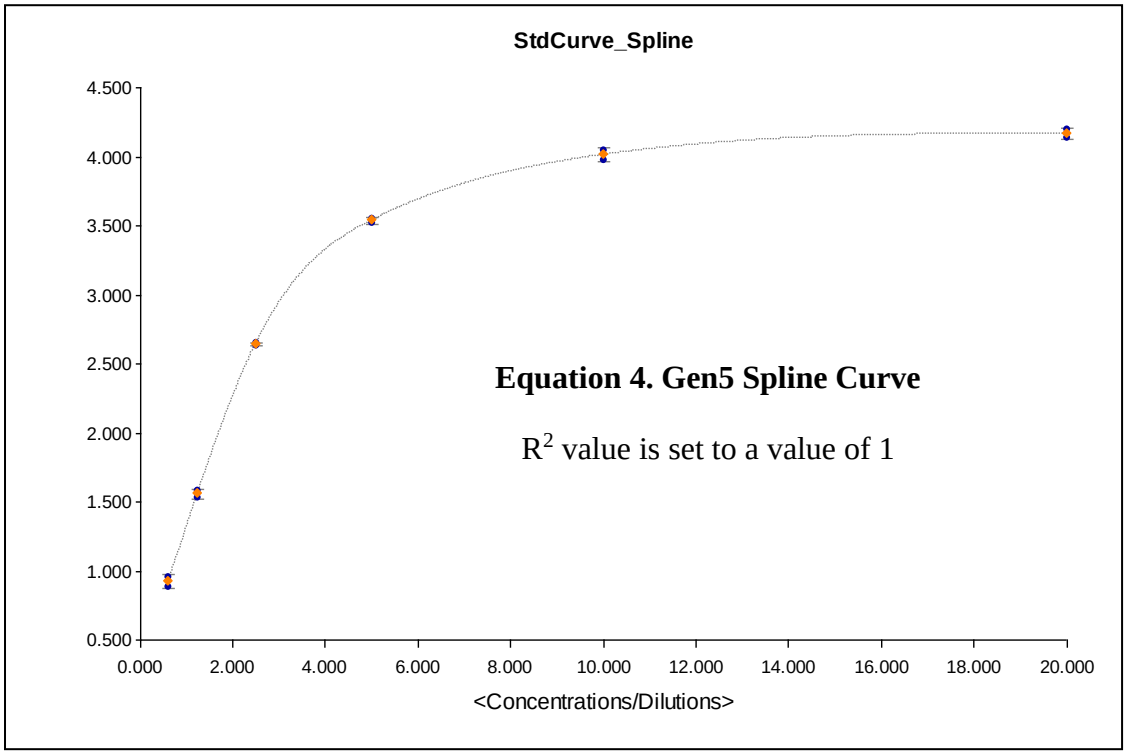
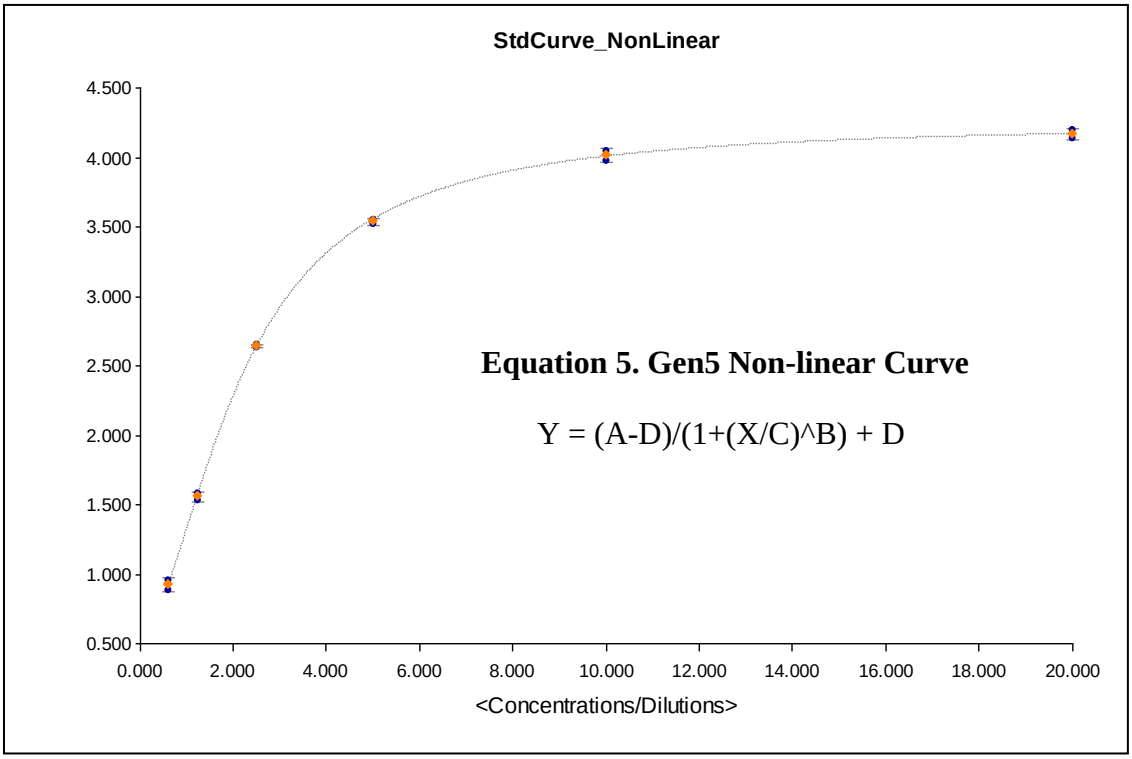


Figure 11. Gen5 Generated Standard Curve for Non-Linear Fit



Based upon the results in Table 5. IgG to Prot A %R (Based on Results in EU/mL) below, it was determined that both the Point to Point and the Non-Linear standard curves had the tightest fit upon statistical analysis of the results (EU/mL) compared to the Bio-Rad Excel results worksheet generated results (also EU/mL). All curves produced similar averages. The non-linear curve fit type produced a tighter range and had a correlation coefficient of 1 where the Polynomial had a value of .927. Non-linear was chosen for use in the IgM and IgG assays due to the above findings and the basis that there is a curve fit equation provided that can be used to check the quantification of the antibody in each sample by solving for X, X = C*([(A-D)/(y-D)]^(1/B)-1), where y equals the delta OD reading value.

Table 5. IgG to Prot A %R (Based on Results in EU/mL)

Percent Recovery of patient sample titer results using: KAM-1 human rabies immunoglobulin (HRIG) purified IgG plasma standard diluted as per the kit recommended 1:100 followed by 1:2 serial dilutions in establishment of the quantitative IgG standard curve paired with anti-IgG HRP conjugate at a dilution of 1:4800 utilizing Gen5 software standard curve types compared to R4b kit standard curve 4 EU/mL positive sample paired with Protein A HRP conjugate at a dilution of 1:10 and Bio-Rad proprietary calculations. Red boxes correspond to the Gen5 Non-linear curve as the best fit compared to the Bio-Rad results based on comparing the average value, range, and (R^2) correlation coefficient.

% R=(Experimental Value/Protein A Value)*100

*****</= results excluded due to large difference in the lower limit of quantitation of </= 0.125 for Protein A and </= 0.625 for IgG**

Sample ID:	Non-Linear	Polynomial	Point To Point	Spline	Sample ID:	Non-Linear	Polynomial	Point To Point	Spline
RAE-1		***			RAE-25		***		
RAE-2	56.12%	71.67%	58.39%	58.39%	RAE-26		***		
RAE-3	60.20%	83.73%	60.88%	60.88%	RAE-27	55.67%	73.23%	59.39%	59.39%
RAE-4	106.49%	60.71%	121.58%	121.58%	RAE-28	77.41%	95.98%	79.13%	79.13%
RAE-5	86.69%	62.12%	94.15%	94.15%	RAE-29		***		
RAE-6		***			RAE-30		***		
RAE-7		***			RAE-31		***		
RAE-8	64.33%	0.00%	63.85%	63.85%	RAE-32		***		
RAE-9	63.59%	36.54%	63.53%	63.53%	RAE-33		***		
RAE-10	54.62%	43.26%	54.26%	54.26%	RAE-34	71.79%	102.07%	76.07%	76.07%
RAE-11	69.86%	45.58%	79.77%	79.77%	RAE-35		***		
RAE-12		***			RAE-36		***		
RAE-13		***			RAE-37	81.90%	68.52%	82.38%	82.38%
RAE-14		***			RAE-38	50.42%	64.37%	52.36%	52.36%
RAE-15	82.96%	82.96%	82.96%	82.96%	RAE-39		***		
RAE-16	29.22%	41.39%	29.81%	29.81%	RAE-40		***		
RAE-17	16.65%	14.81%	16.77%	16.77%	RAE-41	52.61%	69.06%	54.55%	54.55%
RAE-18	31.67%	27.76%	33.74%	33.74%	RAE-42		***		
RAE-19		***			RAE-43		***		
RAE-20		***			RAE-44	74.43%	78.91%	76.75%	76.75%
RAE-21	82.20%	64.79%	82.55%	82.55%	RAE-45	56.15%	66.88%	61.37%	61.37%
RAE-22	65.80%	86.10%	68.27%	68.27%	RAE-55		***		
RAE-23	38.90%	41.86%	44.73%	44.73%	RAE-56	50.00%	50.00%	50.00%	50.00%
RAE-24	55.09%	57.33%	63.20%	63.20%	RAE-57	46.69%	65.91%	51.39%	51.39%
					RAE-58	82.28%	103.56%	85.71%	85.71%
R^2 Value	1	0.927	NA	1	Avg	0.62	0.61	0.65	0.65
					Min	0.17	0.00	0.17	0.17
					Max	1.06	1.04	1.22	1.22
					Median	0.60	0.65	0.63	0.63

3.3 IgM ELISA Materials and Methods

With the selection of the best curve fit alongside the previous proof of concept, the necessary groundwork is laid for moving into IgM assay development. Assessment beginning with selection of the most appropriate standard curve sample and the best fit for anti-IgM HRP working dilution for use in quantifying anti-rabies IgM in human sera is outlined below.

3.3.1 Kit

Same as assayed under section 3.2.1 Protein A ELISA Materials & Methods, with a secondary anti-human IgM HRP conjugate (Sigma Cat# A6907) used to detect IgM in place of the kit HRP Protein A. Version 881179 – 2015/06 kit instructions were followed to obtain the titer value in EU/mL which were calculated by comparison of the sample optical density reading against a standard curve of the most suitable positive sample demonstrated to have IgM antibodies.

3.3.2 Determination of IgM conjugate concentration and standard curve sample

Anti-human IgM HRP conjugate was initially tested in duplicate using serial 1:2 dilutions beginning at 1:1600 continuing until a 1:100,000 dilution was achieved. RAE-2 was selected as the sample representing IgM antibody based on a 14 DPV collection and previous results in the proof of concept study performed in 2016. RAE-2 at the assay 1:100 dilution was utilized as the primary antibody and compared to the Protein A assay and standard curve results in the presence of each IgM conjugate serial dilution. IgM conjugate was also tested in the absence of rabies antibody at a dilution of 1:12,500, comparable to that of the lot recommended 1:10,000, to assess any false reactivity or background reaction to the rabies antigen coated wells. Abnormally high coefficients of variation (%CV) are a result of probable conjugate instability or a prozone effect for the 1:6400 and 1:125,000 anti-IgM conjugate serial dilutions, see Table 6.

Table 6. IgM Conjugate Dilution Replicates

Anti-IgM conjugate titrations as the secondary against RAE-2 a day 14 post vaccination serum sample (that also had below the limit of detection result via the Protein A IgG assay) used to better establish the appropriate working dilution of anti-IgM for the purposes of detecting anti-rabies IgM in human serum. Empty wells and wells containing conjugate only were also assessed to assay potential background from the conjugate that could lead to a false positive reaction. These values were assessed based upon the kit supplied R3 negative average optical density (OD) value of 0.007. Statistical analysis including comparing the coefficient of variation contributed to the assessment of establishing the 1:1000 working dilution for the anti-IgM conjugate.

Protein A results were all </= 0.125 for sample RAE-2 and 0.007 OD for kit negative control R3

Sample ID:	Conjugate Dilution:	IgM OD:	Average:	Standard Deviation:	%CV:
RAE-2	1:1600	0.19	0.201	0.011	5.24%
RAE-2	1:1600	0.211			
RAE-2	1:3200	0.09	0.099	0.009	9.09%
RAE-2	1:3200	0.108			
RAE-2	1:6400	0.058	0.068	0.010	14.07%
RAE-2	1:6400	0.077			
RAE-2	1:12500	0.053	0.045	0.008	19.10%
RAE-2	1:12500	0.036			
RAE-2	1:25000	0.021	0.020	0.001	5.00%
RAE-2	1:25000	0.019			
RAE-2	1:50000	0.015	0.015	0.001	3.45%
RAE-2	1:50000	0.014			
RAE-2	1:100000	0.011	0.011	0.000	0.00%
RAE-2	1:100000	0.011			
Sample ID:	**Conjugate Dilution:**	**IgM OD:**	**Average:**	**Standard Deviation:**	**%CV:**
None	Empty Well	0.009	0.008	0.001	12.50%
None	1:12500	0.035	0.021	0.014	66.67%

Further analysis included comparing the Protein A results against samples tested and making the determination to test low draw date samples (less than DPV 21) to maximize the likelihood of capturing IgM alone that displayed results close to the delta 4.000 optical density value to mimic the R4b kit supplied standard curve control. The goal in standard curve determination is to ensure that the negatives are negative, positives are positive, and the curve has a clear delineation between each point. RAE-2, -8, -21, -37 were the starting samples based on the above criteria and as a comparison of the

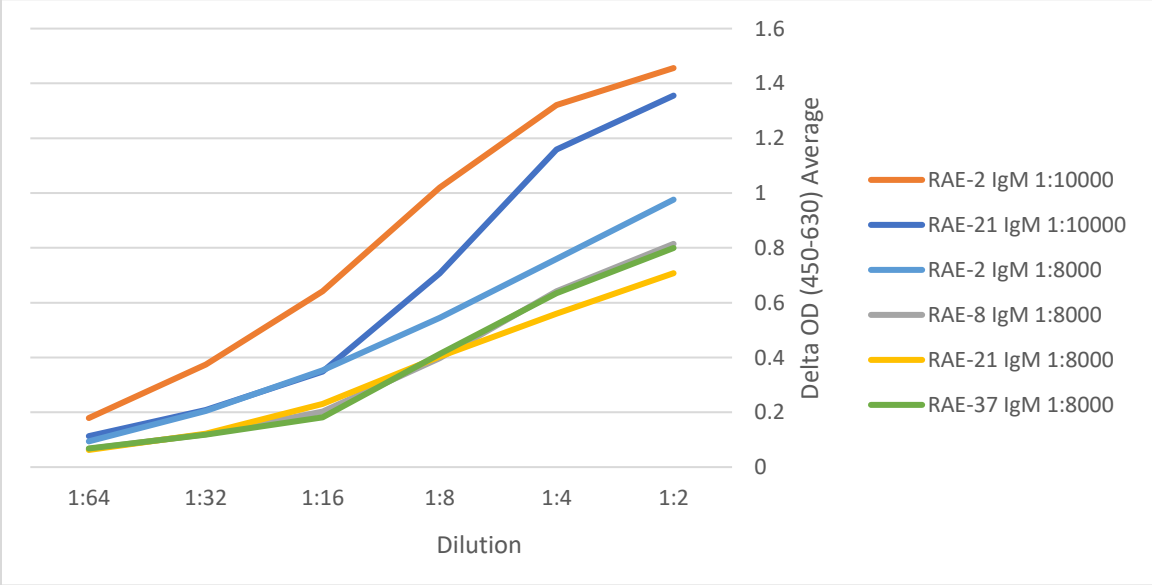
RFFIT result as compared to the previously tested IgM samples in the proof of concept testing completed in 2016.

IgM conjugate dilution of 1:8,000 was chosen as the starting point for the S1-S6 standard curve due to a low %CV (a value of 30% for percentage coefficient of variation is the upper level for acceptable precision for ELISA validation) and to keep close to the manufacturer's recommended dilution of 1:10,000. RAE-21 displayed the most appropriate curve fit under these conditions and will be tested at alternate conjugate dilutions at higher concentrations to obtain an upper limit closer to the 4.000 delta OD value (see Figure 12). IgM Standard Curve Starting Point). Dilutions for the Rb4 kit supplied 4.0 EU/mL sample were followed in generation of the standard curves for all IgM samples and provided as a reference in IgM Figure 12 and 13 below.

For all graphical representations of standard curves going forward, the X-axis (horizontal) corresponds to the dilution of the sample or the estimated concentration in EU/mL if known, and the Y-axis (vertical) correspond to the delta OD value obtained from the calibrated microplate reader.

Figure 12. IgM Standard Curve Starting Point

Representative samples utilized in the establishment of the quantitative IgM standard curve assayed against varying dilutions of anti-IgM HRP conjugate. Sample RAE-21 against anti-IgM conjugate at a working dilution of 1:10,000 displayed the most appropriate curve fit compared to the R4b kit supplied standard curve.

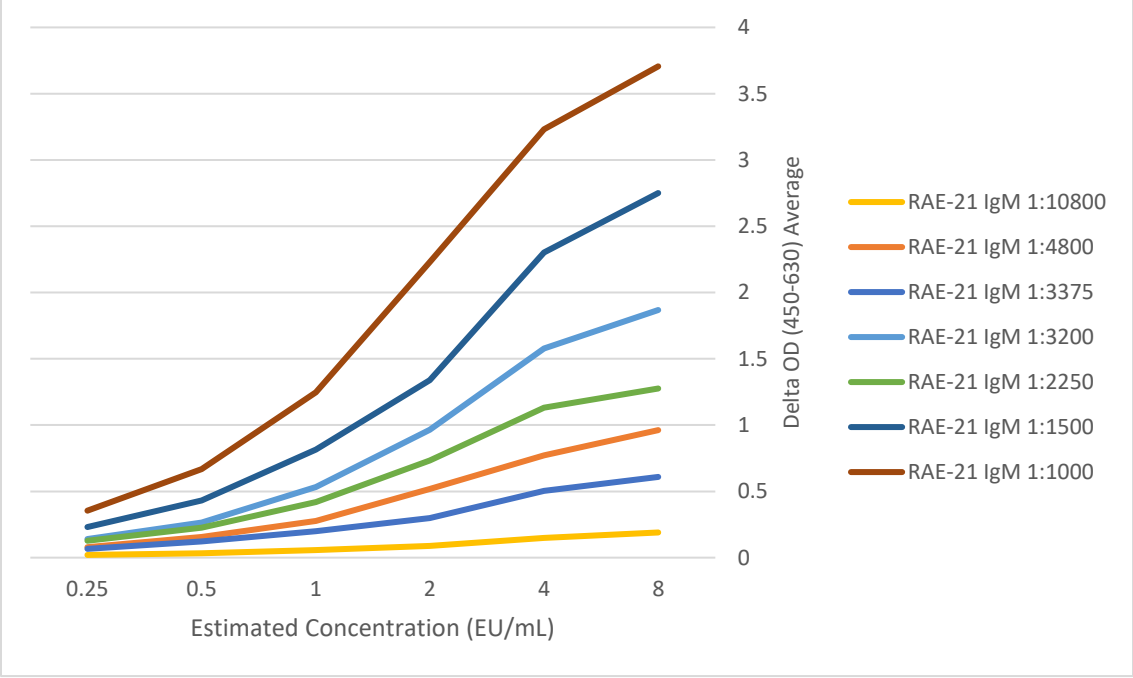


Sample RAE-21 against conjugate dilutions of 1:3200, 1:4800, 1:7200, and 1:10800 resulted in the finding that the higher concentration of conjugate did raise the upper limit to a value of approximately 1.800 delta OD while also holding the appropriate curve fit and not causing a prozone shape (see Figure 13).

IgM conjugate dilutions of 1:1000, 1:1500, 1:2250, and 1:3375 were then assayed against sample RAE-21 and found to be appropriate for standard curve fit. A dilution concentration of 1:1000 was chosen as the most appropriate for this IgM conjugate lot because of the trend of a lower %CV for higher concentrations of conjugate (from Table 6. IgM Conjugate Dilution Replicates), and the upper limit value being close to 4.000 for the delta OD value (see Figure 13). Higher concentrations of conjugate were not pursued as there is normal assay variation that can result in the shifting of the standard curve delta OD value and could cause a shift to a value much greater than the targeted upper limit of the assay.

Figure 13. RAE-21 IgM Concentration Test

Sample RAE-21 against serial 1:2 dilutions of anti-IgM conjugate at a starting dilution of 1:10,800 to establish the most appropriate IgM assay conjugate working dilution. RAE-21 paired with anti-IgM HRP conjugate at a working dilution of 1:1000 was verified to be the most appropriate fit for purpose.



3.3.3 Serum samples

Same as assayed under section 3.1.2 RFFIT Materials & Methods, section Serum Samples. The focus for detecting IgM antibody were samples drawn on days 7 and 14.

3.3.4 Serological testing

The indirect ELISA method, Bio-Rad Platelia Rabies Kit II ELISA (Marnes-la-Coquette, France) was performed using the manual method per the manufacturer's instructions. A BioTek ELx808 microplate reader coupled with Gen 5 (version 2.06) software set to the estimated IgM standard value (as estimated from comparison of the RFFIT (IgM and IgG combined effect via virus inhibition as detected by the anti-N protein conjugate) and Protein A (IgG conjugate) ELISA assays) for quantitative titer results in EU/mL for each sample (IgM range: </= 0.25 to >/= 8 EU/mL), see Table 7. Samples producing results above the IgM upper level of quantitation (ULOQ) of >/= 8 EU/mL were pre-diluted and retested to obtain an endpoint result within the quantitative range of each assay. RAE-2, -3, -8, -10, -16, -21, -22, and -37 were pre-diluted 1:10 using 20 µL sample and 180 µL R6 diluent.

Table 7. IgM ELISA Standard Curve Generation and Concentration

RAE-21 IgM standard curve sample dilution scheme. An 8 EU/mL upper limit value was estimated from the RFFIT (IgM + IgG) titer results compared to Protein A ELISA (IgG) titer results. Quantification standard concentration estimated from serial 1:2 dilutions of the 8 EU/mL upper limit value.

Quantification standards		Concentrations obtained by serial dilutions of the RAE-21 Positive control
S6	RAE-21 diluted to 1:100	8 EU/mL
S5	S6 diluted to 1:2	4 EU/mL
S4	S5 diluted to 1:2	2 EU/mL
S3	S4 diluted to 1:2	1 EU/mL
S2	S3 diluted to 1:2	0.5 EU/mL
S1	S2 diluted to 1:2	0.25 EU/mL

Table 8. IgM ELISA Titer Results

Indirect enzyme-linked immunosorbent assay (ELISA) endpoint titer results expressed in EU/mL (Equivalent Units) for all samples using anti-IgM HRP at a dilution of 1:1000 as the secondary conjugate. Below limit of quantitation results display as "</= 0.250" and any samples with titers > 8.0 (the assay upper limit) were pre-diluted prior to following the manufacturer 1:100 assay dilution. Samples RAE-1 through RAE-5, as indicated by the red box (also denoted by shading), correspond to a single patient for all draw dates (expressed in days post vaccination (DPV). RAE-35 corresponds to pooled negative samples. Internal standards are indicated by the purple box and kit supplied standards by the green box.

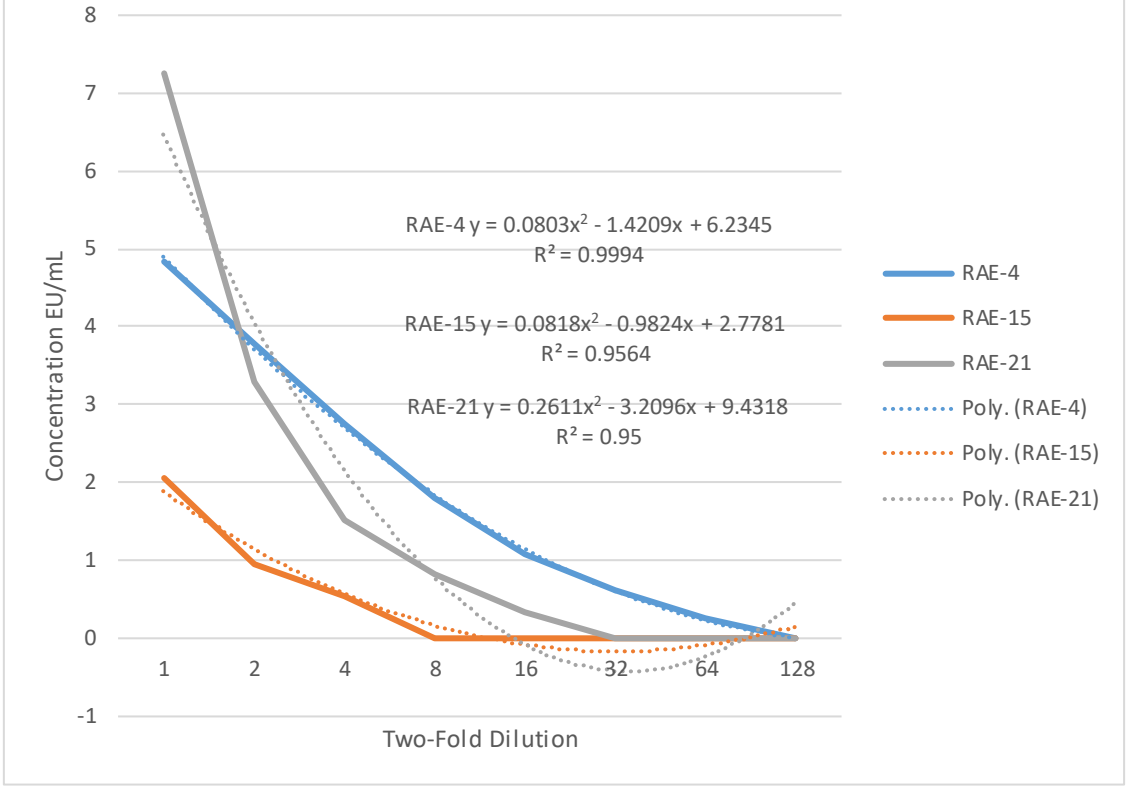
Sample ID:	DPV:	Results (EU/mL):	Sample ID:	DPV:	Results (EU/mL):	Sample ID:	DPV:	Results (EU/mL):
RAE-1	0	</= 0.250	RAE-21	14	7.04	RAE-41	31	5.02
RAE-2	14	14.39	RAE-22	21	7.96	RAE-42	0	</= 0.250
RAE-3	21	16.38	RAE-23	28	3.29	RAE-43	7	0.36
RAE-4	28	6.69	RAE-24	42	2.28	RAE-44	21	2.84
RAE-5	42	3.32	RAE-25	0	</= 0.250	RAE-45	42	1.04
RAE-6	0	</= 0.250	RAE-26	7	1.17	RAE-55	0	</= 0.250
RAE-7	7	1.64	RAE-27	21	6.23	RAE-56	7	</= 0.250
RAE-8	14	8.22	RAE-28	28	2.90	RAE-57	14	2.23
RAE-9	21	5.73	RAE-29	0	</= 0.250	RAE-58	28	1.60
RAE-10	28	13.00	RAE-30	7	</= 0.250	HRIG	HRIG	</= 0.250
RAE-11	42	4.68	RAE-31	14	1.23	Ref2017	Ref2017	</= 0.250
RAE-12	0	</= 0.250	RAE-32	21	1.74	MMP-4	MMP-4	</= 0.250
RAE-13	7	</= 0.250	RAE-33	28	1.14	0.5Ref2017	0.5Ref2017	</= 0.250
RAE-14	10	0.38	RAE-34	42	0.91	FBS	FBS	</= 0.250
RAE-15	14	2.65	RAE-35	0	</= 0.250	R3	R3	</= 0.250
RAE-16	21	5.22	RAE-36	0	</= 0.250	R3	R3	</= 0.250
RAE-17	28	2.33	RAE-37	16	8.03	R4a	R4a	</= 0.250
RAE-18	42	1.25	RAE-38	31	3.63	R4a	R4a	</= 0.250
RAE-19	0	</= 0.250	RAE-39	0	</= 0.250	R4b	R4b	</= 0.250
RAE-20	7	0.69	RAE-40	16	</= 0.250	R4b	R4b	</= 0.250

As expected, the day 0 samples display results below the limit of quantitation (</ 0.250) and the EU/mL titer result increases after each early vaccination with a decrease of antibodies near the day 28 post vaccination draw date indicating the possibility of the timing for class switching to IgG antibody production, see Table 8. The internal standards (Ref2017, MMP-4, 0.5Ref2017, and FBS) all displayed below the limit of detection values and do not provide the added interassay validity between the initial assay and subsequent assay runs to include the pre-dilutions for endpoint titration. In addition, the kit supplied standards (R3, R4a, and R4b) all produce </= 0.250 titer results as expected in quantifying IgM as these are IgG specific controls.

Dilution linearity was tested to assess the accuracy of this selection of the standard curve sample RAE-21 and the anti-IgM conjugate dilution in generating quality and reliable titer results. Low (RAE-15), medium (RAE-5), and high (RAE-21) IgM samples were chosen to represent the assay range. Trend lines accurately depict the starting value, halving of that value due to serial 1:2 dilution, and the expected shape of the curve for all three samples included in this assessment see Figure 14. Reproducibility holds within the <30% CV between the initial test results and the neat sample result for the linearity test as well. RAE-15 has a lower percent recovery due limited data points corresponding to values above the upper limit of detection (</= 0.250) for comparison to the theoretical value. This is considered acceptable with the overall actual and theoretical values being well above the 0.5 EU/mL titer value that is considered protective against rabies.

Figure 14. IgM Dilution Linearity Test

High (RAE-21), medium (RAE-4), and low (RAE-15) titer (EU/mL) value samples were serially diluted 1:2 and all dilutions were assayed via the anti-IgM ELISA using the 1:1000 conjugate working dilution to determine the dilution linearity for the assay. Actual results (all values obtained from each point on the linearity curve) were compared to the theoretical result for that sample and percent recovery (%R) calculated using %R=(Actual/Theoretical) x 100. Curve fit equations included for each sample indicated by the Poly. (Sample) curve and included equations.



Sample ID:	DPV:	Actual Results (EU/mL):	Theoretical Results (EU/mL):	%R
RAE-4	28	6.69	6.20	92.63%
RAE-15	14	2.65	2.03	76.55%
RAE-21	14	7.04	6.30	89.42%

3.4 IgG ELISA Materials and Methods

The selection of the best curve fit methodology for anti-IgM alongside the previous proof of concept testing utilizing R4b 4.0 EU/mL positive control, the necessary groundwork is laid for moving into further IgG assay development. Assessment

beginning with selection of the most appropriate standard curve sample and the best fit for anti-IgG HRP working dilution for use in quantifying anti-rabies IgG in human sera is outlined below.

3.4.1 Kit

Same as assayed under section 3.2.1 Protein A ELISA Materials & Methods, section Kit with a secondary anti-human IgG HRP conjugate (Sigma Cat# A0170) used to detect IgG in place of the kit HRP Protein A conjugate. Version 881179 – 2015/06 kit instructions were followed to obtain the titer value in EU/mL which were calculated by comparison of the sample optical density reading against a standard curve of a positive plasma sample found to be suitable for accurately detecting IgG.

3.4.2 Determination of IgG conjugate concentration and standard curve sample

Anti-human IgG HRP conjugate was tested in duplicate using serial 1:2 dilutions beginning at 1:1600 continuing until a 1:100,000 dilution was achieved. RAE-5 was selected as the primary IgG antibody due to a DPV value of 42 and the Protein A ELISA result and then used at the assay 1:100 dilution and compared to the Protein A assay and standard curve results in the presence of each anti-IgG conjugate serial dilution. Anti-IgG conjugate was also tested in the absence of rabies antibody at a dilution of 1:50,000, similar to that of the lot recommended 1:60,000, to assess any false reactivity or background reaction to the rabies antigen coated wells (see Table 9).

Table 9. IgG Conjugate Dilution Replicates

Anti-IgG conjugate titrations as the secondary against RAE-5 a day 42 post vaccination serum sample (that theoretically would contain IgG alone and had an above the limit of detection results on the Protein A ELISA) used to better establish the appropriate working dilution of anti-IgG for the purposes of detecting anti-rabies IgG in human serum. Empty wells and wells containing conjugate only were also assessed to assay potential background from the conjugate that could lead to a false positive reaction. These values were assessed based upon the kit supplied R3 negative average optical density (OD) value of 0.007. Statistical analysis including comparing the coefficient of variation contributed to the assessment of establishing the 1:4800 working dilution for the anti-IgG conjugate.

Protein A results were all 0.164 for sample RAE-5 and 0.007 OD for kit negative control R3

Sample ID:	Conjugate Dilution:	IgG OD:	Average:	Standard Deviation:	%CV:
RAE-5	1:1600	0.348	0.354	0.006	1.56%
RAE-5	1:1600	0.359			
RAE-5	1:3200	0.277	0.284	0.006	2.29%
RAE-5	1:3200	0.29			
RAE-5	1:6400	0.194	0.210	0.016	7.62%
RAE-5	1:6400	0.226			
RAE-5	1:12500	0.13	0.127	0.004	2.77%
RAE-5	1:12500	0.123			
RAE-5	1:25000	0.072	0.075	0.003	3.36%
RAE-5	1:25000	0.077			
RAE-5	1:50000	0.048	0.047	0.001	2.13%
RAE-5	1:50000	0.046			
RAE-5	1:100000	0.025	0.028	0.003	10.71%
RAE-5	1:100000	0.031			
Sample ID:	**Conjugate Dilution:**	**IgG OD:**	**Average:**	**Standard Deviation:**	**%CV:**
None	Empty Well	0.007	0.007	0.000	0.00%
None	1:50000	0.016	0.012	0.005	39.13%

R4b was initially utilized as the IgG standard curve sample as compared to the previous proof of concept testing completed in 2016. A 1:60,000 (manufacturers recommended) and a 1:10,000 dilution were chosen as the starting points for the S1-S6 standard curve due to a low %CV (a 30% %CV is the upper level for acceptable precision for ELISA validation) dilution of anti-IgG conjugate were prepared and tested against the R4b generated standard curve in duplicate.

Curve results and the upper limit of 0.700 delta OD for the 1:60,000 dilution and 1.200 delta OD for the 1:10,000 dilution in Figure 15. R4b Standard Curve + IgG Conjugate lead to the need for further analysis. Comparing the Protein A results against samples tested and making the determination to test high draw date samples (greater than or equal to DPV 21) to maximize the likelihood of capturing IgG alone that also displayed results close to the 4.000 delta OD value to mimic the R4b kit supplied standard curve control was the next step. RAE-23 was selected as the most appropriate starting sample for creation of the IgG standard curve based on the above criteria and comparison of the RFFIT and Protein A ELISA endpoint titer results.

Figure 15. R4b Standard Curve + IgG Conjugate

Kit supplied R4b 4 EU/mL positive standard utilized in the establishment of the quantitative IgG standard curve assayed against varying dilutions of anti-IgG HRP conjugate. R4b against anti-IgG conjugate at a working dilution of 1:4800 displayed the most appropriate curve fit compared to the R4b kit supplied standard curve against Protein A conjugate.

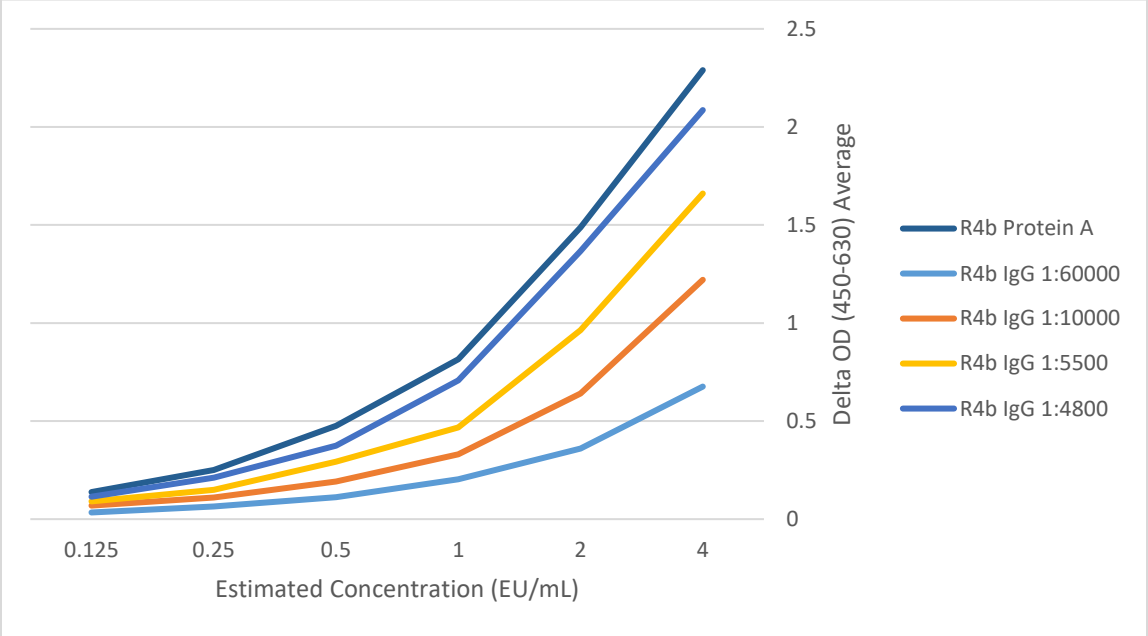
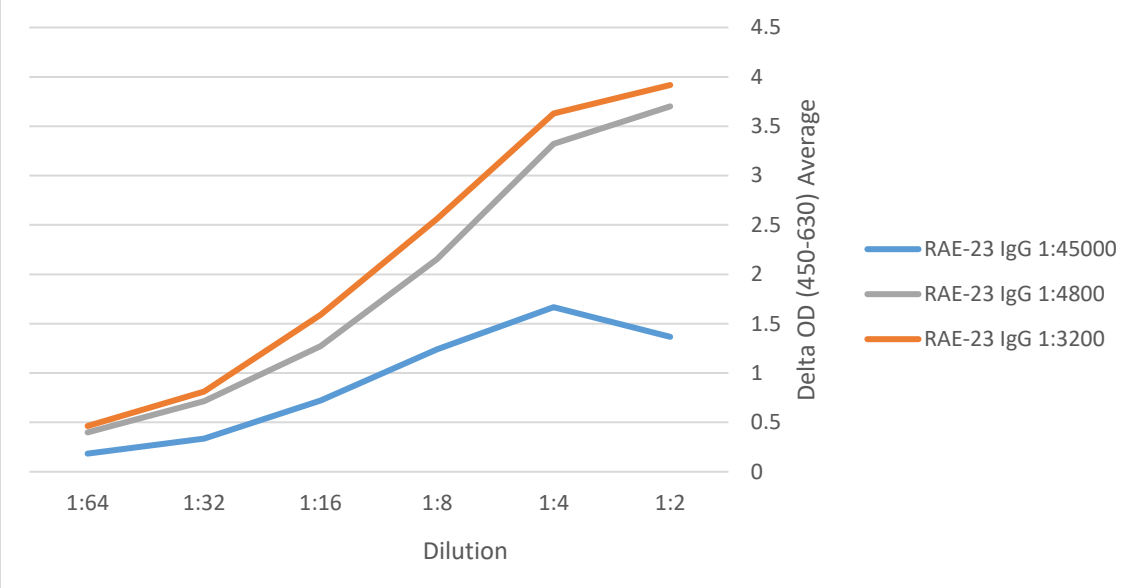


Figure 16. RAE-23 Standard Curve + IgG Conjugate shows curve results for the 1:45,000, 1:4800, and 1:3200 dilutions. Further experiments were needed to match the best fit compared to the Protein A curve results due to a prozone reaction with a 1.600

delta OD for the 1:45,000 dilution. A stronger conjugate dilution is necessary to obtain a 4.000 delta OD value for the S6 standard curve sample against IgG. RAE-23 at the 1:4800 conjugate dilution shows an ideal curve fit when compared to the Protein A R4b result and will continue to be the sample of choice for the anti-human IgG assay curve.

Figure 16. RAE-23 Standard Curve + IgG Conjugate

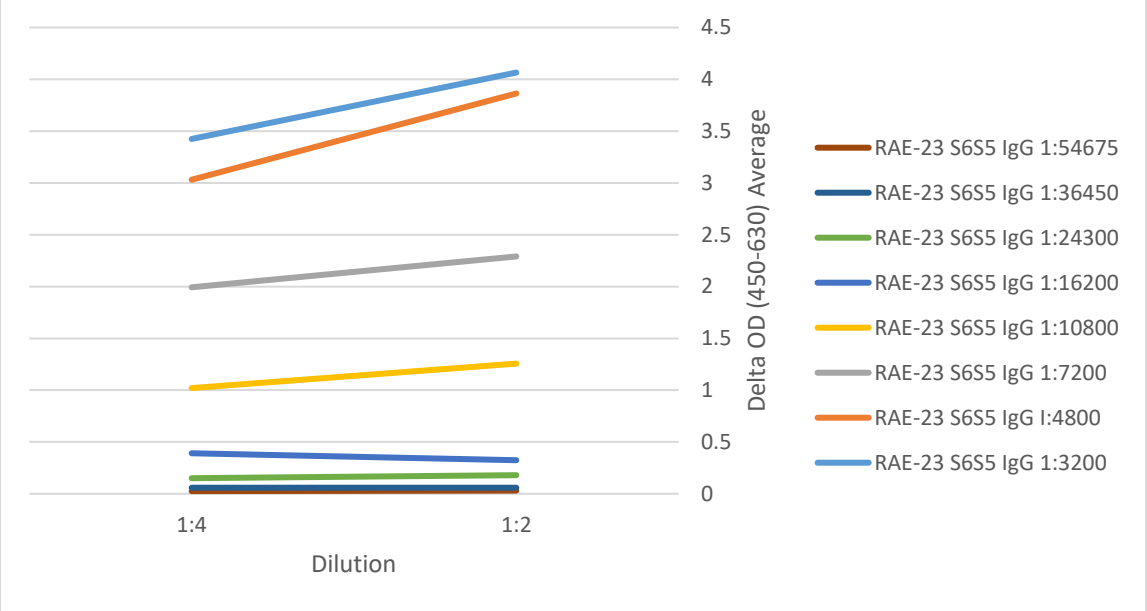
Representative samples (RAE-23 a day 28 post vaccination sample found to be the most appropriate shown in the figure) utilized in the establishment of the quantitative IgG standard curve assayed against varying dilutions of anti-IgG HRP conjugate. Sample RAE-23 against anti-IgG conjugate at a working dilution of 1:4800 displayed the most appropriate curve fit compared to the R4b kit supplied standard curve.



Samples S6 and S5 were tested to assess the presence of a similar prozone response as in Figure 17. RAE-23 S6S5 Standard Curve Prozone Test the secondary antibody were added to each S6 and S5 sample in duplicate. Conjugate dilutions of 1:3200 and 1:4800 showed an upward curve trend between those two samples closest to the target delta OD value of 4.000.

Figure 17. RAE-23 S6S5 Standard Curve Prozone Test

Quantification standards S6 and S5 for RAE-23 were assayed against varying dilutions of anti-IgG HRP conjugate to identify other dilutions producing prozone reactions such as RAE-23 against ant-IgG at a working dilution of 1:45,000 from the previous Figure 10.



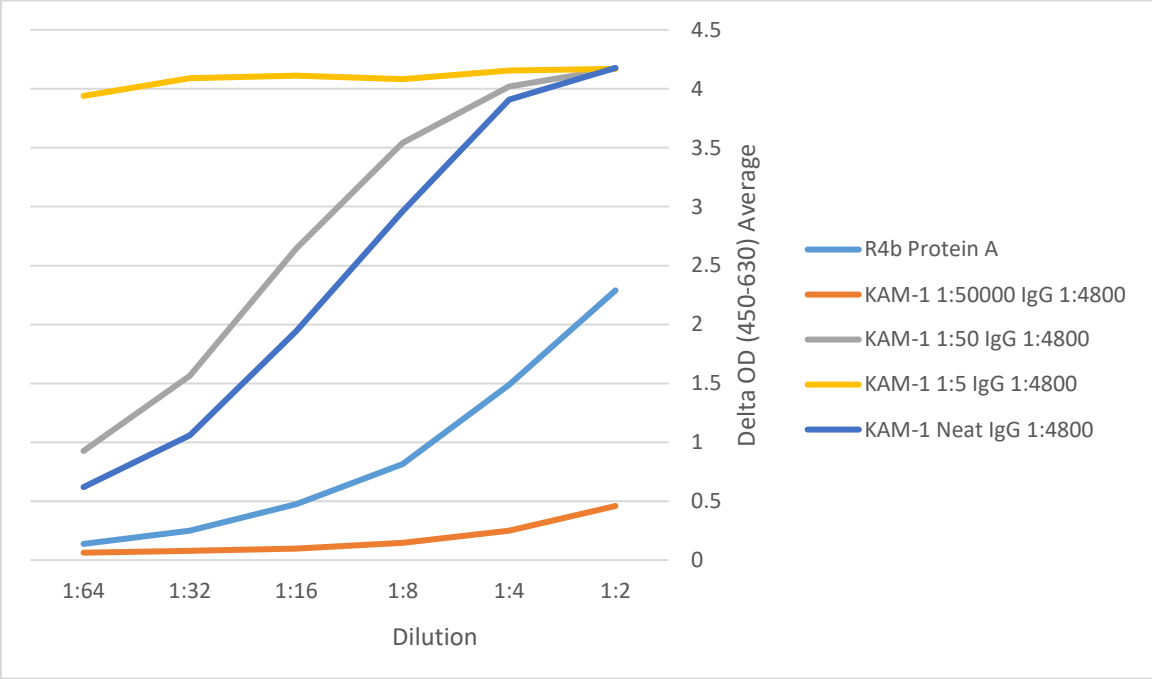
Next, all standard curve samples (S1-S6) were assayed in duplicate against the two conjugate dilutions (1:3200 and 1:4800) found to meet the criteria in the search for an ideal standard curve upper limit delta OD value. The dilution of 1:4800 was chosen as the most appropriate for this lot of anti-IgG conjugate based on the above and the %CV trend of values meeting the <30% for higher concentrations of conjugate. Higher concentrations of conjugate in the 1:3200 to 1:4800 range were not pursued as there is normal assay variation that can result in the shifting of the standard curve delta OD value and could cause a shift to a value much greater than the target.

As the day 28 post vaccination RAE-23 sample most probably contains residual IgM antibody another internal standard utilized during plasma product testing via the RFFIT method was sought out as the most appropriate fit for use in the generation of an IgG quantification standard. KAM-1 (HRIG) was next assessed as a more appropriate standard for use as the curve on the basis that the value (20.00 IU/mL) is known (defined by RFFIT) and that it is a purified IgG only containing sample. This ensured that no false reactivity against any potential remaining IgM in the sample would alter the IgM to IgG

ratio and results would be unequivocally based on IgG alone. S1-S6 was tested undiluted ("neat") and at a 1:5, 1:50, and 1:50,000 dilution (theoretical value of the 1:50 dilution upper limit is 4.0 EU/mL) to determine the best curve fit from this high titer sample. As shown in Figure 18, KAM-1 Standard Curve Comparison, the undiluted sample produced both the target delta OD value and also appropriate standard curve shape and was used as the final standard curve in the assessment of IgG titer in this study.

Figure 18. KAM-1 Standard Curve Comparison

A known purified IgG only containing human rabies immunoglobulin standard (HRIG), KAM-1, utilized at varying pre-dilutions due to the known concentration of 20 EU/mL was used in the establishment of the quantitative IgG standard curve assayed against the 1:4800 working dilution of anti-IgG HRP conjugate. KAM-1 as per the manufacturers 1:100 followed by 1:2 serial dilutions (indicated by the "Neat" designation) against anti-IgG conjugate working dilution of 1:4800 displayed the most appropriate curve fit compared to the R4b kit supplied standard curve against Protein A conjugate.



3.4.3 Serum samples

Same as assayed under section 3.1.2 RFFIT Materials & Methods, section Serum Samples. The focus for detecting IgG antibody was day 21, 28, 35, and when present 42.

3.4.4 Serological testing

The indirect ELISA method, Bio-Rad Platelia Rabies Kit II ELISA (Marnes-la-Coquette, France) was performed using the manual method per the manufacturer's instructions with the anti-human IgG conjugate substitution made. A BioTek ELx808 microplate reader coupled with Gen 5 (version 2.06) software set to the appropriate IgG standard curve values (as compared against RFFIT and Protein A ELISA) for quantitative titer results in EU/mL for each sample (IgG range: </= 0.625 to >/= 20 EU/mL), see Table 10. Samples producing results above the IgG standard upper level of quantitation (ULOQ) of >/= 20 EU/mL were pre-diluted and retested to obtain an endpoint result within the quantitative range of each assay.

Table 10. IgG ELISA Standard Curve Generation and Concentration

KAM-1 IgG standard curve sample dilution scheme. A 20 EU/mL upper limit value known from the RFFIT and Protein A ELISA titer results. Quantification standard concentration estimated from serial 1:2 dilutions of the 20 EU/mL upper limit value.

Quantification standards		Concentrations obtained by serial dilutions of the KAM-1 Positive control
S6	KAM-1 diluted to 1:100	20 EU/mL
S5	S6 diluted to 1:2	10 EU/mL
S4	S5 diluted to 1:2	5 EU/mL
S3	S4 diluted to 1:2	2.5 EU/mL
S2	S3 diluted to 1:2	1.25 EU/mL
S1	S2 diluted to 1:2	0.625 EU/mL

Table 11. IgG ELISA Results

Indirect enzyme-linked immunosorbent assay (ELISA) endpoint titer results expressed in EU/mL (Equivalent Units) for all samples using anti-IgG HRP at a dilution of 1:4800 as the secondary conjugate. Below limit of quantitation results display as "</= 0.625" and any samples with titers > 20.0 (the assay upper limit) were pre-diluted prior to following the manufacturer 1:100 assay dilution. Samples RAE-1 through RAE-5, as indicated by the red box (also denoted by shading), correspond to a single patient for all draw dates (expressed in days post vaccination (DPV). RAE-35 corresponds to pooled negative samples. Internal standards are indicated by the purple box and kit supplied standards by the green box.

Sample ID:	DPV:	Results (EU/mL):	Sample ID:	DPV:	Results (EU/mL):	Sample ID:	DPV:	Results (EU/mL):
RAE-1	0	</= 0.625	RAE-21	14	1.91	RAE-41	31	3.74
RAE-2	14	3.417	RAE-22	21	3.52	RAE-42	0	</= 0.625
RAE-3	21	4.27	RAE-23	28	15.38	RAE-43	7	</= 0.625
RAE-4	28	19.14	RAE-24	42	13.86	RAE-44	21	2.93
RAE-5	42	20.58	RAE-25	0	</= 0.625	RAE-45	42	9.46
RAE-6	0	</= 0.625	RAE-26	7	</= 0.625	RAE-55	0	</= 0.625
RAE-7	7	</= 0.625	RAE-27	21	8.70	RAE-56	7	</= 0.625
RAE-8	14	1.53	RAE-28	28	8.478	RAE-57	14	8.86
RAE-9	21	2.06	RAE-29	0	</= 0.625	RAE-58	28	3.25
RAE-10	28	15.33	RAE-30	7	</= 0.625	HRIG	HRIG	18.70
RAE-11	42	18.37	RAE-31	14	0.27	Ref2017	Ref2017	30.25
RAE-12	0	</= 0.625	RAE-32	21	1.18	MMP-4	MMP-4	</= 0.625
RAE-13	7	</= 0.625	RAE-33	28	</= 0.625	0.5Ref2017	0.5Ref2017	0.19
RAE-14	10	</= 0.625	RAE-34	42	5.85	FBS	FBS	</= 0.625
RAE-15	14	1.38	RAE-35	0	</= 0.625	R3	R3	</= 0.625
RAE-16	21	4.85	RAE-36	0	</= 0.625	R3	R3	</= 0.625
RAE-17	28	11.14	RAE-37	16	2.38	R4a	R4a	0.46
RAE-18	42	18.57	RAE-38	31	13.55	R4a	R4a	0.46
RAE-19	0	</= 0.625	RAE-39	0	</= 0.625	R4b	R4b	5.93
RAE-20	7	</= 0.625	RAE-40	16	</= 0.625	R4b	R4b	NA

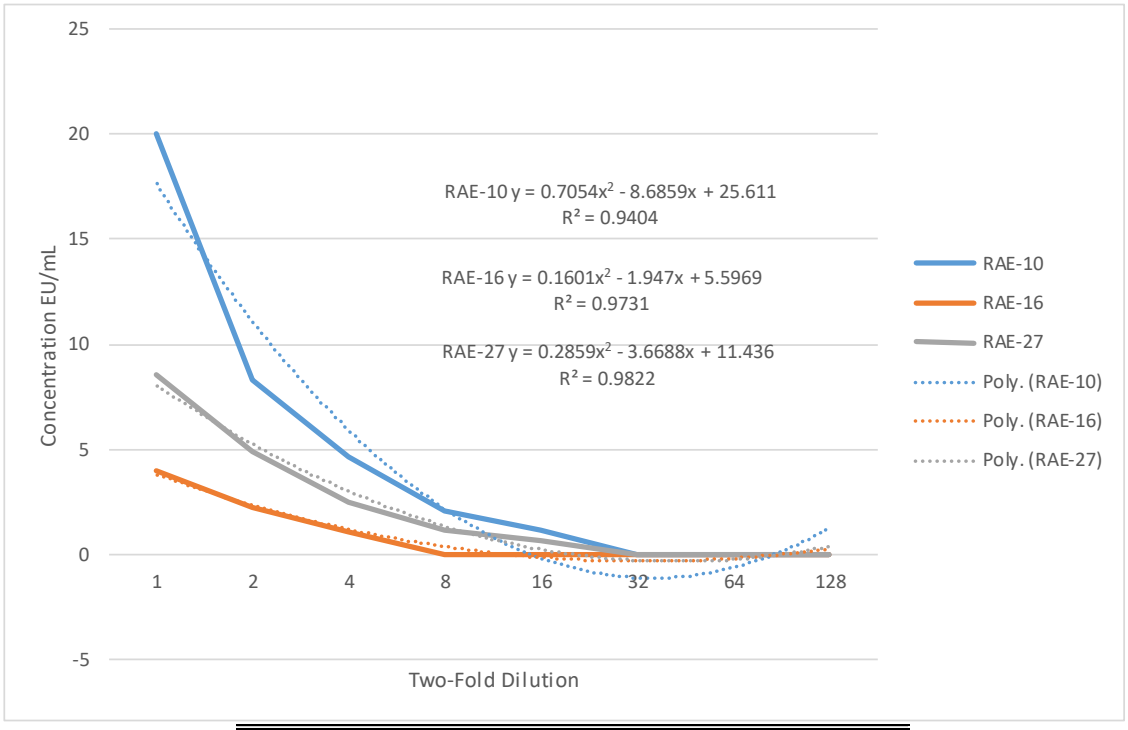
As expected, the day 0 samples display results below the limit of quantitation (</ 0.625) and the EU/mL titer result increases after each early vaccination with a slight decrease and stabilization of antibodies near the day 42 post vaccination draw date, see Table 11. A significant increase in titer occurs between day 21 to day 28 for most patients indicating the possibility of the timing for class switching from IgM to IgG antibody production. The internal standards (Ref2017, MMP-4, 0.5Ref2017, and FBS) all correspond to the expected values (except MMP-4 which has a theoretical value below the </= 0.625 lower limit of the IgG assay) to provide the interassay validity between the initial assay and subsequent assays runs to include the pre-dilutions for endpoint titration.

Also, the kit supplied standards (R3, R4a, and R4b) produce appropriate values against the expected as these are IgG specific controls.

Dilution linearity was tested to assess the accuracy of the selection of the standard curve sample and the IgG conjugate dilution (Figure 19). Low (RAE-16), medium (RAE-27), and high (RAE-10) IgG samples were chosen to represent the assay range. Trend lines accurately depict the starting value, halving of that value due to serial 1:2 dilution, and the expected shape of the curve for all three samples included in this assessment. Reproducibility holds within the <30% CV between the initial test results and the average of the 1:2 sample results for the linearity test.

Figure 19. IgG Dilution Linearity Test

High (RAE-10), medium (RAE-27), and low (RAE-16) titer (EU/mL) value samples were serially diluted 1:2 and all dilutions were assayed via the anti-IgG ELISA using the 1:4800 conjugate working dilution to determine the dilution linearity for the assay. Actual results (all values obtained from each point on the linearity curve) were compared to the theoretical result for that sample and percent recovery (%R) calculated using %R=(Actual/Theoretical) x 100. Curve fit equations included for each sample indicated by the Poly. (Sample) curve and included equations.



Sample ID:	DPV:	Actual Results (EU/mL):	Theoretical Results (EU/mL):	%R
RAE-10	28	15.33	17.91	116.83%
RAE-16	21	4.85	4.30	88.66%
RAE-27	21	8.70	9.66	111.06%

With development, evaluation, and establishment of each standard curve and conjugate working dilution, endpoint titers as a whole can be evaluated in determining the following: variability of immunoglobulin class switching between individuals, the specific timing of that switch, accurately predicting high versus low responder's effective immune defense, and discovering the time point when affinity maturation occurs.

Chapter 4 - IgM, IgG, Protein A Results and Discussion

The results of the Protein A, IgM, IgG, and RFFIT as they compare for the RAE (Rabies Antibody ELISA) sample set both in table form and graphically were evaluated to make determinations of the immunological status and Ig stage in the response both on an individual level and for the population as a whole.

4.1 Results

Referring to the results in Table 12 below, as expected there was little to no antibody response with use of anti-IgM, anti-IgG, or Protein A conjugate at day zero of the pre-exposure prophylaxis (PrEP) rabies vaccination series. Trends shown in subsequent tables and figures show that IgM is predominately the class of human immunoglobulins present in the early stages of the vaccine induced immune response, day 7-21 post vaccination. Class switching to IgG can be seen in the increase in IgG versus IgM antibodies present in the serum samples after the day 28 time point. An IgG response is detected primarily at day 21-42 post receipt of initial rabies virus vaccine.

Variation in anti-IgG and Protein A conjugate assays is due to the preferential binding of the protein A conjugate to the Fc portion of multi-species IgG and not binding all subclasses equally while anti-IgG conjugate has a higher binding affinity specifically towards human IgG . Lower and upper limits of quantitation are variable dependent upon the standard curve linear range. Of note, none of the current internal standards are appropriate for consideration in the validity of the IgM assay runs and standards will need to be reassessed to ensure interassay validity.

Table 12. Combined ELISA Results using Non-Linear Standard Curve

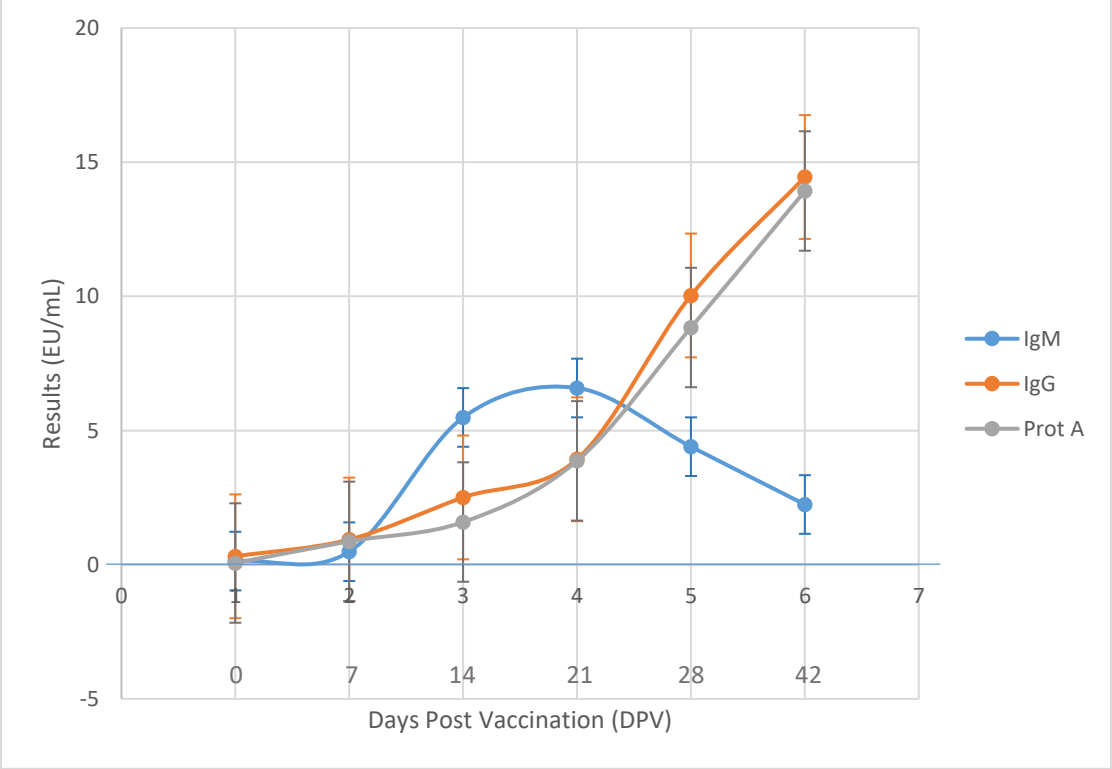
Indirect enzyme-linked immunosorbent assay (ELISA) endpoint titer results expressed in EU/mL (Equivalent Units) for all samples using anti-IgM HRP at a dilution of 1:1000 with RAE-21 as the standard curve, anti-IgG HRP at a dilution of 1:4800 with KAM-1 as the standard curve, and Protein A HRP at a dilution of 1:10 with R4b as the standard curve. Limits of quantitation range from "</= 0.250" to 8.0 for IgM, "</= 0.625" to 20 for IgG, and "</= 0.125" to 4.0 for Protein A. Any samples with titers above the assay upper limit were pre-diluted prior to following the manufacturer 1:100 assay dilution. Samples RAE-1 through RAE-5, as indicated by the red box (also denoted by shading), correspond to a single patient for all draw dates (expressed in days post vaccination (DPV). Internal standards are indicated by the purple box and kit supplied standards by the green box.

Sample ID:	DPV:	IgM EU/mL	IgG EU/mL	Prot A EU/mL	Sample ID:	DPV:	IgM EU/mL	IgG EU/mL	Prot A EU/mL
RAE-1	0	</= 0.250	</= 0.625	</= 0.125	RAE-31	14	1.23	0.27	0.24
RAE-2	14	14.39	3.42	3.30	RAE-32	21	1.74	1.18	0.46
RAE-3	21	16.38	4.27	3.86	RAE-33	28	1.14	</= 0.625	0.55
RAE-4	28	6.69	19.14	13.84	RAE-34	42	0.91	5.85	3.92
RAE-5	42	3.32	20.58	13.02	RAE-36	0	</= 0.250	</= 0.625	0.20
RAE-6	0	</= 0.250	</= 0.625	</= 0.125	RAE-37	16	</= 0.250	</= 0.625	</= 0.125
RAE-7	7	1.64	</= 0.625	</= 0.125	RAE-38	31	8.03	2.38	1.46
RAE-8	14	8.22	1.53	1.05	RAE-39	0	3.63	13.55	9.23
RAE-9	21	5.73	2.06	1.56	RAE-40	16	</= 0.250	</= 0.625	</= 0.125
RAE-10	28	13.00	15.33	18.25	RAE-41	31	5.02	3.74	3.72
RAE-11	42	4.68	18.37	18.07	RAE-42	0	</= 0.250	</= 0.625	</= 0.125
RAE-12	0	</= 0.250	</= 0.625	</= 0.125	RAE-43	7	0.36	</= 0.625	</= 0.125
RAE-13	7	</= 0.250	</= 0.625	</= 0.125	RAE-44	21	2.84	2.93	1.94
RAE-14	10	0.38	</= 0.625	</= 0.125	RAE-45	42	1.04	9.46	9.62
RAE-15	14	2.65	1.38	0.75	RAE-55	0	</= 0.250	</= 0.625	</= 0.125
RAE-16	21	5.22	4.85	8.97	RAE-56	7	</= 0.250	</= 0.625	</= 1.25
RAE-17	28	2.33	11.14	7.47	RAE-57	14	2.23	8.86	6.92
RAE-18	42	1.25	18.57	27.57	RAE-58	28	1.60	3.25	2.19
RAE-19	0	</= 0.250	</= 0.625	</= 0.125	HRIG	HRIG	</= 0.250	18.70	14.44
RAE-20	7	0.69	</= 0.625	</= 0.125	Ref2017	Ref2017	</= 0.250	30.25	24.81
RAE-21	14	7.04	1.91	1.40	MMP-4	MMP-4	</= 0.250	</= 0.625	0.14
RAE-22	21	7.96	3.52	2.95	0.5Ref2017	0.5Ref2017	</= 0.250	0.19	0.39
RAE-23	28	3.29	15.38	16.62	FBS	FBS	</= 0.250	</= 0.625	</= 0.125
RAE-24	42	2.28	13.86	12.38	R3	R3	</= 0.250	</= 0.625	</= 0.125
RAE-25	0	</= 0.250	</= 0.625	</= 0.125	R3	R3	</= 0.250	</= 0.625	</= 0.125
RAE-26	7	1.17	</= 0.625	</= 0.125	R4a	R4a	</= 0.250	0.46	0.46
RAE-27	21	6.23	8.70	7.73	R4a	R4a	</= 0.250	0.46	0.45
RAE-28	28	2.90	8.48	6.42	R4b	R4b	</= 0.250	5.93	4.09
RAE-29	0	</= 0.250	</= 0.625	</= 0.125	R4b	R4b	</= 0.250	NA	3.93
RAE-30	7	</= 0.250	</= 0.625	</= 0.125					

Anti-IgG versus Protein A conjugate results show confidence in the accuracy of the anti-IgG conjugate detecting serum IgG antibody despite the minor variability between conjugates. Curve results below show similar shape and values for both anti-rabies detecting assays as expected since Protein A and anti-IgG conjugates bind primarily to the Fc portion of IgG antibodies. However, Sigma (Cat# A0170) anti-IgG is a polyclonal antibody with specific binding for human samples making this conjugate more specific for use in human sera samples and having greater avidity towards this species versus the multi-species IgG Protein A binding ability. Utilizing a purified IgG product, KAM-1, as the IgG standard curve increases the confidence that these results are quantifying IgG alone. The IgM trend line also follows what is typically seen for the primary immune response to vaccination.

Figure 20. Average ELISA Results Compared

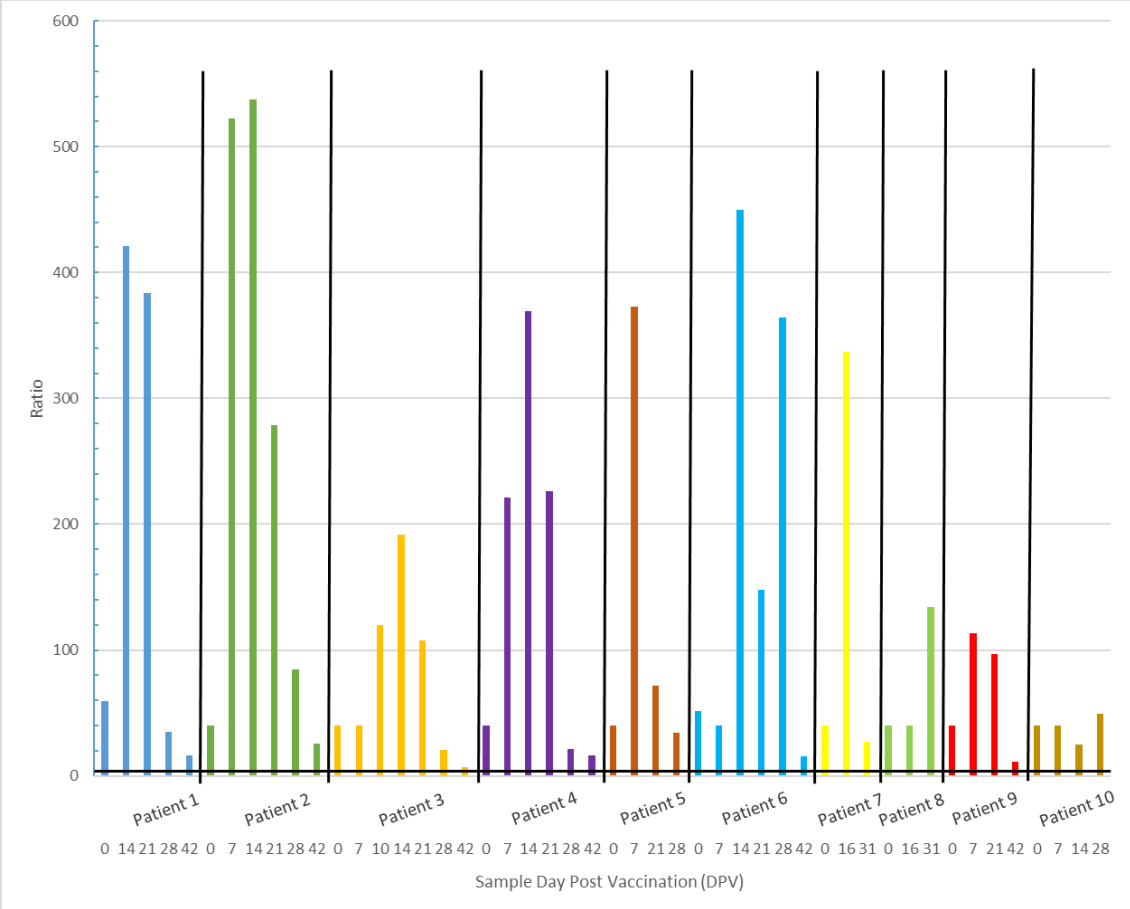
Indirect enzyme-linked immunosorbent assay (ELISA) endpoint titer results expressed in EU/mL (Equivalent Units) were averaged for each draw date comparing anti-IgM HRP, anti-IgG HRP, and Protein A HRP secondary conjugates. IgG and Protein A show comparable detection of IgG. IgM follows the estimated immune reaction in response to vaccination of an increased value from day 7 to 21 followed by the class switch to IgG between day 21 to 28 and decreasing IgM value.



The IgM to IgG ratio further solidifies the finding that IgM is predominately the primary response to vaccination or booster. Graphically, the trend of increased IgM during the median sampling of day 14 and day 21 post vaccination for most subjects is clearly observed. Variability is demonstrated between vaccine recipients regardless of the overall followed trend of IgM to IgG around the day 21 to 28 period. Grouped patient samples demonstrate the wide range in variability between an individual's response to vaccination.

Figure 21. IgM to IgG ELISA Ratio

IgM to IgG indirect enzyme-linked immunosorbent assay (ELISA) endpoint titer results expressed in EU/mL (Equivalent Units) were calculated and plotted for each draw date comparing anti-IgM HRP, and anti-IgG HRP secondary conjugates. IgM follows the estimated immune reaction in response to vaccination of an increased value from day 7 to 21 and in some instances day 28 followed by the class switch to IgG between day 21 to 28 and decreasing IgM value. Vertical black lines separate between individual patient serum sets drawn at the designated days post vaccination. The horizontal line designates the cutoff for a protective titer of 0.5 EU/mL.



Long-lasting neutralization ability being predominately IgG related is proven through correlation of the increasing IgG ELISA results alongside increasing neutralization (RFFIT) titer values. As proven for rabies virus in previous studies, it takes approximately 1 to 2 IgG molecules to neutralize three virion spikes versus only 1 IgM molecule to neutralize up to ten rabies virion spikes for neutralization of 63% of the virus present [45]. This follows the structural reasoning that IgG is monomeric (can bind

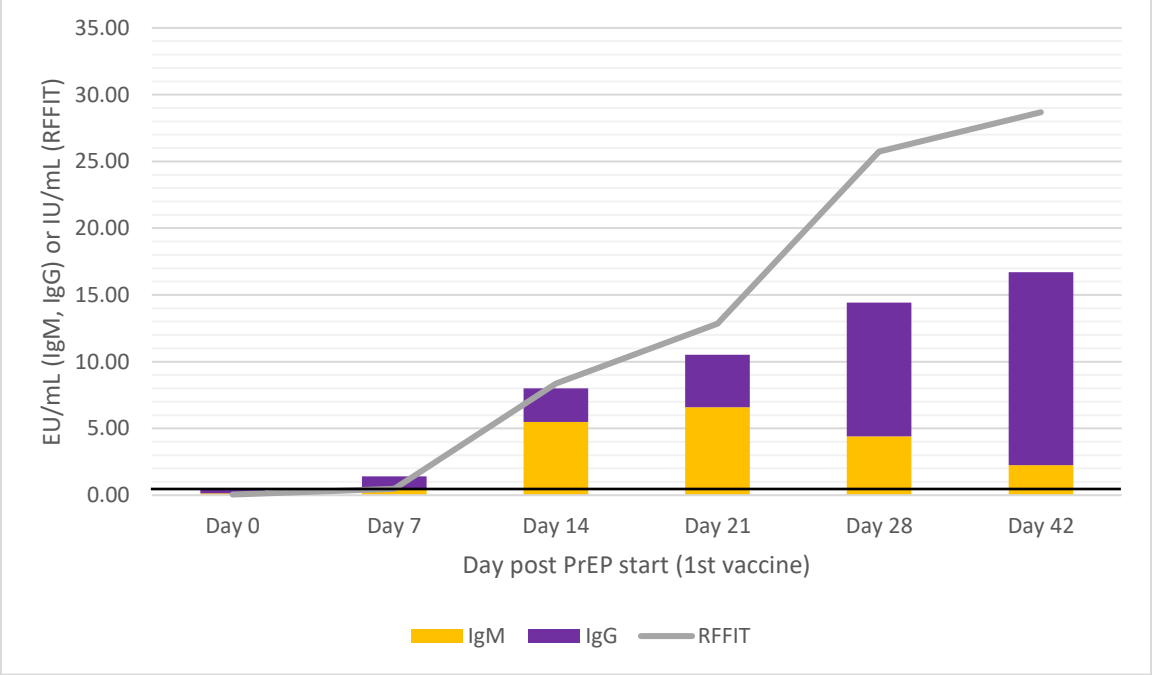
up to two molecules) and IgM is pentameric and able to bind up to ten molecules. Also of interest, during the same study, the threshold detected after the greatest neutralization effect occurred was 400 to 1000 IgG molecules per virion and approximately 100 molecules of IgM [45]. RFFIT nucleoprotein conjugate accurately detects IgM levels of antibody next to neutralizing ability even though the predominately IgG response elicits a stronger neutralization titer value compared to IgM. The smaller structural size allowing IgG to move across tissues, whereas IgM is structurally too large, is one of the reasons that IgG is present in larger quantities where long lasting neutralization is also present. In the case of vaccination and booster, IgM will be produced in response to the viral antigens present in the administered vaccine that will quickly be replaced with IgG via class switching during the humoral immune response. To more accurately quantify the IgM contribution to neutralizing ability aliquots of patient sera would need to undergo column purification with selection for the IgM-containing fraction and assayed on the Rapid Focus Fluorescent Inhibition Test (RFFIT) for endpoint titer values.

In Figure 22 below, as expected, there is no detectable antibody at day 0 for all assay methods. Day 7 post vaccination seemingly elicits an IgG response but this is explained by the difference in lower limits of detection due to the linear range differences in each assay (0.1 for RFFIT, 0.25 for IgM, and 0.625 for IgG). Assessing the need for a modified lower limit of detection for the IgG assay will need to be determined for accurately quantifying protection status at > 0.5 EU/mL as recommended by the Advisory Committee on Immunization Practices (ACIP). Class switching from IgM to IgG is observed in the IgM data (yellow) as well as the specific timing of that switch (predominately DPV 21 to 28). Rabies virus neutralizing antibodies (RVNA) correlate to long-lasting IgG evident after DPV 21 (purple coupled with linear). Affinity maturation due to selection and clonal expansion for B-cells that class switch to produce highly neutralizing IgG clones are evident in both the RFFIT results (linear) and the IgG results (purple).

Figure 22. All Results Compared (RFFIT/IgM/IgG)

Indirect enzyme-linked immunosorbent assay (ELISA) endpoint titer results expressed in EU/mL (Equivalent Units) were calculated and plotted for each draw date comparing anti-IgM HRP, and anti-IgG HRP secondary conjugates as well as comparing the neutralizing ability and contribution of each antibody to that neutralizing function via the rapid focus fluorescent inhibition test (RFFIT). The horizontal line designates the cutoff for a protective titer of 0.5 EU/mL. Class switching from IgM to IgG is visible in the IgM data (in yellow), as well as the specific timing of that switch (predominately DPV 21 to 28). Rabies virus neutralizing antibody (RVNA) correlates to long-lasting IgG evident after DPV 21 (purple IgG coupled with linear RFFIT). Affinity maturation due to clonal expansion of highly neutralizing IgG clones is displayed in both the RFFIT results (linear) and the IgG ELISA results (purple).

International units (IU/mL) were calculated from the serum titer using the following formula: (Endpoint titer of test serum/ Endpoint titer of reference serum) X 2.0 IU/mL reference serum (a WHO standard). Equivalent units (EU/mL) were calculated by comparison of the sample optical density reading against a Non-linear fit type standard curve of positive standards.



4.2 Discussion

Similar immune trends to the response to rabies vaccination are available for other human viral and bacterial infections. Rotavirus displays a similar trend with IgM present

in the early collected serum samples and lower levels in the late infection stage collected serum samples [46–49]. IgG detection via ELISA and neutralization response were the same for rotavirus as well [49] which further confirms the response in the rabies IgG and RFFIT assays. Antibody kinetics obtained via ELISA for hepatitis E virus show the typical pattern of quick increase in IgM with a seroconversion to IgG [50,51]. Enzyme immunoassays (EIA) were used to detect IgG against Epstein-Barr virus (EBV) and show the same trend of low IgG detected in newly infected patients and high in persons with reactivation of the virus or previous exposure to the virus [52]. IgM and IgG are detected at the same time in both reactivation and after initial infection [52] which follows the same immunological trend for rabies upon initial infection or vaccination followed by any booster. Despite a few mixed findings in the co-presence of IgM and IgG in early stage infection of *Treponema pallidum* leading to Syphillis, it is clear that IgM is only present in early infection with IgG being the primary immunoglobulin detected in late infection [53–55]. ELISA (or a similar immunoassay) was the chosen method for each of the examples in determining the IgM versus IgG responses. This method is quantitative, gives an objective value from a calibrated reader, is less labor intensive than similar in vitro methods, and has been shown to have similar specificity and sensitivity to the current gold standard methods of testing.

By utilizing a wide variety of validation parameters in evaluating the use of the Bio-Rad Platelia Rabies II indirect ELISA kit for use in detecting IgG and IgM in human sera, conditions are optimized to detect, with confidence, the concentrations of both IgG and IgM for each sample and accurately determine the kinetics of the immune response to rabies vaccination. Investigation of the immune reaction of each individual's response to vaccination has contributed to further our understanding of the variability of immunoglobulin class switching between individuals, the specific timing of that class switch from IgM to neutralizing IgG via clonal expansion through affinity maturation in the lymphoid tissue for these best fit immune cells. It is the starting point in being able to accurately predict high versus low responder’s effective immune defense to better classify these individuals rabies protection status. This data will also help guide future research of preventative and treatments options as well as guide further development of anti-rabies immunological assays.

Future improvements such as increased sample numbers would aid in determining trends of non-conformant patient samples such is the case with samples RAE-55 through RAE-57 collected from a single patient all showing lower titration values across sampling days. Upon collection of the data from these subjects, further testing and analysis can be conducted on other factors involved in the immune response, for example cytokines, to better expand our knowledge of these individual's atypical response to rabies vaccination. This understanding would drive further research and development of more effective vaccines or vaccine schedules. The increase in patients will allow for pooling appropriate samples for use as the IgM quantification standard for standard curve creation. After validation of the pooled IgM standard further longevity would be achieved as well as longevity in the confidence in the IgM assay.

After successful complete diagnostic validation of the Platelia Rabies II ELISA kit (parameters outlined in Chapter 5 below) with anti-IgM and anti-IgG secondary conjugates via the manual method, a partial validation can be completed utilizing the automated method by assessing precision and limits of quantification. Further evaluation and validation would branch out into human plasma samples for analysis of the immune status of plasma donors and plasma products for post-exposure prophylaxis (PEP).

As demonstrated, the modified rabies enzyme-immunosorbent assays (ELISAs) with anti-IgM or anti-IgG secondary conjugates paired with the appropriate standard curve samples are: quantitative, obtain an objective value from a microplate reader, are less labor intensive than similar methods such as the Rapid Focus Fluorescent Inhibition Test (RFFIT), and display similar specificity and sensitivity to the current gold standard methods of testing (RFFIT and Protein A ELISA). Conditions are optimized to detect, with confidence, the concentrations of both IgM and IgG for each sample and accurately determine the kinetics of the immune response to rabies vaccination.

Chapter 5 - Future Direction

A full diagnostic validation will be performed so that the IgM and IgG assays can be added to the repertoire of testing offered by the Kansas State Veterinary Diagnostic Laboratory (KSVDL) Rabies department. Appropriate IgM internal standards need to be developed in order to further quantify the validity of each assay run and be able to effectively compare interassay data. This will be achieved via the assessment of the appropriate pooling of the larger patient sampling outlined in the previous discussion section.

5.1 Assay Diagnostic Validation

Once these values are established the next phase of validation will move forward. Primary parameters to test during the validation are as follows: repeatability, robustness, intermediate precision, accuracy/linearity, specificity, dilutability, limits of quantitation. Clinical Laboratory Improvement Amendments (CLIA), GMP, General Laboratory Practices (GLP), and International Conference on Harmonisation (ICH) standards will be closely followed during this evaluation as is the requirement for use in the Kansas State University Rabies Laboratory.

5.2 Proposed Validation Design

Precision and repeatability will be measured by collecting one sample each that has known values that correspond to each dilution of the standard curve (0.25 to 8.00 EU/mL for IgM and 0.625 to 20.00 EU/mL for IgG). One sample for each EU/mL range will be selected, until each value has a representative sample and create at least 25 aliquots to store at -80ºC pending analysis. The assay will be performed on five different days with five replicates of each sample. Data from each day will appear on different rows for calculation of mean value, standard deviation (SD), confidence value (%CV) to assess both the repeatability and intermediate precision of the assay.

Robustness of temperature, time, and conjugate dilution will also be assessed. One sample for each of the EU/mL range values as per the diluted standards for quantitation will be used and run in duplicate for each parameter to test robustness.

Sample incubation temperature will be assayed at 37ºC±3ºC in six independent runs (one run for each degree ±37ºC). Conjugate incubation temperature will be assayed at 37ºC±3ºC in six independent runs (one run for each degree ±37ºC). Substrate incubation time, as per the kit instructions, already has a wide range of +18ºC to +30ºC and will not be included in this robustness assessment. Incubation time for samples will be assayed at 60 minutes ± 5 minutes in 10 independent runs (one run for each minute ± the initial 60 minute incubation time). Incubation time for conjugate will be assayed at 60 minutes ± 5 minutes in 10 independent runs (one run for each minute ± the initial 60 minute incubation time). Incubation time for substrate will be assayed at 30 minutes ± 5 minutes in 10 independent runs (one run for each minute ± the initial 30 minute incubation time). Conjugate dilution robustness will be tested by assessing the test results for the representative samples at the appropriate calculated dilution ±10µL of undiluted conjugate into the appropriate unchanged volume of diluent (one run for each 1 µL ± the appropriate calculation volume for a total of 20 replicates). Data from each day will appear on different rows for calculation of mean value, standard deviation (SD), confidence value (%CV) to assess both the robustness parameters of the assay.

Upper and lower levels of quantitation (analytical sensitivity) will be measured by the limits of quantification, dilution linearity, and parallelism. Lower limit of quantification (LLOQ) will be determined by running 16 replicates of sample diluent (R6 ready to use TRIS-EDTA buffer), calculating the mean SD, and determining the concentration at 3 SDs above the mean of the blank. Next, samples with very low and very high concentrations of IgG and IgM will be analyzed in duplicate. Calculating the average concentration and %CV will yield data points for a scatter plot of %CV as a function of concentration. From the scatter plot, determining the lowest average level above where the %CV is <20% for most of the samples for the lower level and the highest average level below where the %CV is <20% for a majority of the samples for the upper level.

Dilution linearity is measured to determine that a sample above the upper limit of quantification (ULOQ) can be diluted and yield a reliable and accurate result. This will be achieved by spiking three RFFIT negative, undiluted human sera samples with KAM-1 (IgG) and RAE-21 (IgM) at 100 to 1000 times the concentration at the ULOQ. Serial

dilutions will be made in microcentrifuge tubes until the theoretical concentration is below the LLOQ; assay these dilutions in duplicate and factor in the dilution. For the samples falling between the LLOQ and ULOQ, calculate the mean concentration, and the %Recovery for the theoretical concentrations. All concentrations falling within the previously calculated LLOQ to ULOQ range are considered to be within the acceptance criteria for the precision. Additionally, the signal versus the dilution factor will be researched to assess any suppression at concentrations that far exceed the ULOQ. Using four samples with concentrations on the high end (must be lower than the ULOQ), make six two-fold dilutions in microcentrifuge tubes and assay in duplicate next to the samples tested neat. To account for the dilution factor, calculate the %CV using readings from the neat samples and dilutions for each sample. The %CV below 20% are considered adequate for demonstrating parallelism for this ELISA method.

Human sera from subjects previously naïve will be vaccinated against rabies to be used to assess the diagnostic sensitivity and specificity of the assay. Approved samples of known status via the RFFIT method and samples of unknown status (currently undergoing RFFIT testing) will be assayed in tandem.

Lastly, sample stability has previously been investigated to assess immunoglobulin stability. See "Validation Report of the Bio-Rad Platelia Rabies II for the measurement of Anti- Rabies Glycoprotein Antibodies-Sample Stability Evaluation".

5.3 Assay Validation Results

Calculations and comparisons will be completed between the control sera and samples of known and unknown vaccination status to determine each of the following: repeatability, robustness, analytical sensitivity and specificity, thresholds, diagnostic sensitivity and specificity, reproducibility and sample stability.

5.4 Validated Assay Uses

After validation for diagnostic use, determining appropriate modifications to current rabies vaccination schedules can be achieved utilizing real world data from subjects receiving the current vaccination series. Trends can be tracked with data collection occurring over time and sampling from a large population to more accurately

assess the efficacy of protection in response to any of these modifications to the schedule as determined by the Advisory Committee on Immunization Practices (ACIP) and World Health Organization (WHO). Effective levels of protection can be determined by comparing neutralizing ability against IgM and IgG for any patients with abnormal responses to vaccination to better assess the necessity or lack thereof for subsequent boosters for appropriate stimulation of the immune response to rabies antigen. Paired with other immunological techniques such as, cytokine assays, selectively purifying and assaying sample fractions (IgM versus IgG), the wealth of rabies immunological data will be expanded and contribute to future research and development of alternative vaccinations as well as more targeted treatment options.

References

1 CDC, NCEZID, D. (2011) , CDC - The Rabies Virus. . [Online]. Available: https://www.cdc.gov/rabies/transmission/virus.html. [Accessed: 28-May-2019]

2 WHO (2018) , Ebola virus disease Fact Sheet. . [Online]. Available: https://www.who.int/en/news-room/fact-sheets/detail/ebola-virus-disease. [Accessed: 28-May-2019]

3 Fauquet, C. and International Committee on Taxonomy of Viruses. (2005) *Virus taxonomy : classification and nomenclature of viruses ; 8th report of the International Committee on Taxonomy of Viruses*, Elsevier/Academic Press.

4 Heymann, D.L. and American Public Health Association. (2008) *Control of communicable diseases manual.*, American Public Health Association.

5 WHO (2018) Rabies vaccines: WHO position paper. *Wkly. Epidemiol. Rec.* 16, 201–220

6 WHO Distribution of risk levels for humans contacting rabies, world wide 2013. . [Online]. Available: https://www.who.int/rabies/Global_distribution_risk_humans_contracting_rabies_2013.png?ua=1

7 CDC, NCEZID, D. (2016) , CDC - Rabies Serology. . [Online]. Available: https://www.cdc.gov/rabies/specific_groups/doctors/serology.html. [Accessed: 28-May-2019]

8 WHO (2019) , Rabies Fact Sheet. . [Online]. Available: https://www.who.int/en/news-room/fact-sheets/detail/rabies. [Accessed: 28-May-2019]

9 CDC, NCEZID, D. (2011) CDC - ACIP Recommendations - Rabies. at <https://www.cdc.gov/rabies/resources/acip_recommendations.html>

10 Mathias M Van Nieuwenhove, L.D. *et al.* (2019) Timing of Intradermal Rabies Pre-exposure Prophylaxis Injections: Immunological Effect on Vaccination Response. *Mil. Med.* DOI: 10.1093/milmed/usz048

11 Walker, P.J. *et al.* (2018) ICTV Virus Taxonomy Profile: Rhabdoviridae. *J. Gen. Virol.* 99, 447–448

12 WHO (2017) *The immunological basis for immunization series: module 17: rabies*

vaccines,

13 WHO (2018) WHO Expert Consultation on Rabies : third report. *WHO Tech. Rep. Ser.* 3, 183

14 Banyard, A.C. and Tordo, N. (2018) Rabies pathogenesis and immunology. *Rev. Sci. Tech. l'OIE* 37, 323–330

15 Tordo, N. (1996) Characteristics and Molecular Biology of the Virus. *Meslin FX, Kaplan MM, Koprowski H, Ed. Lab. Tech. Rabies. Geneva World Heal. Organ.* 49, 28–51

16 Wright, E. *et al.* (2008) Investigating antibody neutralization of lyssaviruses using lentiviral pseudotypes: a cross-species comparison. DOI: 10.1099/vir.0.2008/000349-0

17 Plotkin, S.A. (1980) Rabies vaccine prepared in human cell cultures: progress and perspectives. *Rev Infect Dis* 2, 433–448

18 CDC, NCEZID, D. (2011) , CDC - What are the signs and symptoms of rabies? | Symptoms | CDC. . [Online]. Available: https://www.cdc.gov/rabies/symptoms/index.html. [Accessed: 24-Sep-2019]

19 WHO (2018) Symptoms. at <https://www.who.int/rabies/home_symptoms/en/>

20 Mitrabhakdi, E; Shuangshoti, S; Wannakrairot, P.;Lewis, R.A.;Susuki, K; Laothamatias, J.. et al. (2005) Difference in neuropathogenetic mechanisms in human furious and paralytic rabies. *J. Neurol. Sci.* 238, 3–10

21 Hemachudha, T. *et al.* (2013) Human rabies: neuropathogenesis, diagnosis, and management. *Lancet Neurol.* 12, 498–513

22 Ugolini, G. (2011) Rabie virus as a transneuronal tracer of neuronal connections. *Adv. Virus Res.* 79, 165–202

23 WHO (2017) Human rabies: 2016 updates and call for data. *Wkly. Epidemiol. Rec.* DOI: 10.3389/fvets.2017.00004

24 Milligan, G.N. and Barrett, A.D.T. (Alan D.T.. (2015) *Vaccinology : an essential guide*,

25 Kindt, T.J. *et al.* (2007) *Kuby immunology.*, W.H. Freeman.

26 Tizard, I.R. (2013) *Veterinary Immunology.*, Elsevier Health Sciences.

27 Clem, A.S. (2011) Fundamentals of vaccine immunology. *J. Glob. Infect. Dis.* 3,

73–8

28 Rajewsky, K. (1998) Affinity Maturation. *Encycl. Immunol.* DOI: 10.1006/RWEI.1999.0014

29 Moore, S.M. and Hanlon, C.A. Rabies-specific antibodies: Measuring surrogates of protection against a fatal disease. , *PLoS Neglected Tropical Diseases*, 4. Mar-(2010) , e595

30 Grönwall, C. *et al.* (2012) Protective roles of natural IgM antibodies. DOI: 10.3389/fimmu.2012.00066

31 Casali, P. (1998) , Immunoglobulin M - an overview | ScienceDirect Topics. , *Encyclopedia of Immunology (Second Edition)*. [Online]. Available: https://www.sciencedirect.com/topics/immunology-and-microbiology/immunoglobulin-m. [Accessed: 15-May-2019]

32 Mak, T.W. *et al.* (2006) The Humoral Response: B Cell Development and Activation. *The Immune Response* DOI: 10.1016/B978-012088451-3.50011-9

33 Painter, R.H. (1998) IgG. *Encycl. Immunol.* DOI: 10.1006/RWEI.1999.0313

34 Isa, M.B. *et al.* (2002) Comparison of Immunoglobulin G Subclass Profiles Induced by Measles Virus in Vaccinated and Naturally Infected Individuals. *Clin. Vaccine Immunol.* 9, 693–697

35 Sekar, T. *et al.* (2018) Development of In-vitro Assays to Estimate Rabies Viral Protein in Vaccine Preparation. *J. Vaccines Vaccin.* 09, 1–6

36 Cliquet, F. and Wasniewski, M. (2015) The Fluorescent Antibody Virus Neutralization Test. In *Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention*

37 Thomas, J.B. *et al.* (1963) Evaluation of Indirect Fluorescent Antibody Technique for Detection of Rabies Antibody in Human Sera. *J. Immunol.* 91, 721–3

38 Andreasson, U. *et al.* (2015) A practical guide to immunoassay method validation. *Front. Neurol.* 6, 1–8

39 Salvi, N.C. *et al.* (2010) Validation of Indirect ELISA for Quantitative Testing of Rabies Antibodies During Production of Antirabies Serum using Equines. *Procedia Vaccinol.* 2, 3–11

40 Bio-Rad PLATELIA ™ RABIES II Kit FOR IN VITRO DETECTION AND

TITRATION OF IgG ANTI-RABIES VIRUS GLYCOPROTEIN IN HUMAN SERUM AND PLAMSA. REF.:355 1180. . (2015) , 1–16

41 Perrin, P.; Lafon, M.. S.P. (1996) Enzyme-linked immunosorbent assay (ELISA) for the determination of the glycoprotein contect of rabies vaccines. *Meslin FX, Kaplan MM, Koprowski H, Ed. Lab. Tech. Rabies. Geneva World Heal. Organ.* 49, 383–388

42 Servat, A. *et al.* (2007) A quantitative indirect ELISA to monitor the effectiveness of rabies vaccination in domestic and wild carnivores. *J. Immunol. Methods* 318, 1–10

43 Feyssaguet, M. *et al.* (2007) Multicenter comparative study of a new ELISA, PLATELIA^{TM} RABIES II, for the detection and titration of anti-rabies glycoprotein antibodies and comparison with the rapid fluorescent focus inhibition test (RFFIT) on human samples from vaccinated and non-vacc. *Vaccine* 25, 2244–2251

44 (1996) Guidance for Industry Q2B Validation of Analytical Procedures : Methodology. *U.S. Dep. Heal. Hum. Serv. Food Drug Adm. Cent. Drug Eval. Res. Cent. Drug Eval. Res.*

45 Flamand, A. *et al.* (1993) Mechanisms of rabies virus neutralization. *Virology* DOI: 10.1006/viro.1993.1261

46 Davidson, G.P. *et al.* (1983) *Serum and Intestinal Immune Response to Rotavirus Enteritis in Children*, 40

47 Grimwood,', K. *et al.* (1988) *Comparison of Serum and Mucosal Antibody Responses Following Severe Acute Rotavirus Gastroenteritis in Young Children*,

48 Riepenhoff, M. *et al.* (1981) *Development of Serum and Intestinal Antibody Response to Rotavirus After Naturally Acquired Rotavirus Infection In Man*, 8

49 Midthun, K. *et al.* (1989) *Comparison of Immunoglobulin A (IgA), IgG, and IgM Enzyme-Linked Immunosorbent Assays, Plaque Reduction Neutralization Assay, and Complement Fixation in Detecting Seroresponses to Rotavirus Vaccine Candidates*,

50 Pas, S.D. *et al.* (2013) Diagnostic performance of selected commercial HEV IgM and IgG ELISAs for immunocompromised and immunocompetent patients. *J.*

Clin. Virol. 58, 629–634

51 Huang, S. *et al.* (2010) Profile of Acute Infectious Markers in Sporadic Hepatitis E. *PLoS One* 5, 13560

52 Nystad, T.W. and Myrmel, H. (2007) Prevalence of primary versus reactivated Epstein-Barr virus infection in patients with VCA IgG-, VCA IgM- and EBNA-1-antibodies and suspected infectious mononucleosis. *J. Clin. Virol.* 38, 292–297

53 Gomez, E.; Jespersen, D.J.; Harring, J.A.; Binnicker, M.J. (2010) Evaluation of the Bio-Rad BioPlex 2200 Syphilis Multiplex Flow Immunoassay for the Detection of IgM-and IgG-Class Antitreponemal Antibodies. *Clin. Vaccine Immunol.* 17, 966–968

54 Schmidt, B.L. *et al.* (2000) *Comparative Evaluation of Nine Different Enzyme-Linked Immunosorbent Assays for Determination of Antibodies against Treponema pallidum in Patients with Primary Syphilis*, 38

55 Strandberg Pedersen, N. *et al.* (1982) *Enzyme-Linked Immunosorbent Assay for Detection of Immunoglobulin M Antibody Against the Reiter Treponeme Flagellum in Syphilis*, 16