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Final Report

Study Title	Characterization of Hepatitis B Vaccine T-Cell Dependent Antibody Response in Cynomolgus Monkeys
Study Director	[redacted]
Sponsor and Testing Facility	Covance Laboratories Inc. 3301 Kinsman Boulevard Madison, Wisconsin 53704-2523 United States of America
Covance Study Number	8326556
Report Issue Date	08 December 2017
Page Number	1 of 142

TABLE OF CONTENTS

SIGNATURE PAGE

The current study was exploratory in nature and was conducted in accordance with Covance standard operating procedures and generally recognized Good Laboratory Practices.

For clarification, Hepatitis B Vaccine (Engerix B®) was considered a reagent for the purpose of use on this study. Characterization of this reagent was limited to information readily provided by manufacturer.

I, the undersigned, hereby declare the work was performed under my supervision, and the findings provide a true and accurate record of the results obtained.

[redacted]

08 DEC 2017
Date

QUALITY ASSURANCE STATEMENT

This study has been reviewed by the Quality Assurance Unit of Covance, and the report accurately reflects the raw data. The following study-specific inspections were conducted and findings reported to the Study Director (SD) and associated management.

In addition to the inspection program detailed in the following, facility and process inspection programs are also operated. Details of these programs are set out in standard operating procedures.

Inspection Dates		Phase	Date Reported to SD and SD Management
From	To		
24 Jul 2015	24 Jul 2015	Protocol Review-Madison	24 Jul 2015
28 Jul 2015	28 Jul 2015	Protocol Amendment Review-Madison	28 Jul 2015
21 Oct 2015	21 Oct 2015	Protocol Amendment Review-Madison	21 Oct 2015
28 Oct 2015	05 Nov 2015	Draft Report and Data Review	05 Nov 2015
14 Mar 2016	17 Mar 2016	Revised Draft Report Review	17 Mar 2016
15 Jun 2017	16 Jun 2017	Revised Draft Report Review	16 Jun 2017*
10 Jul 2017	10 Jul 2017	Protocol Amendment Review-Madison	10 Jul 2017*
28 Aug 2017	01 Sep 2017	Revised Draft Report and Data Review	01 Sep 2017
24 Oct 2017	27 Oct 2017	Revised Draft Report Review	27 Oct 2017
04 Dec 2017	06 Dec 2017	Final Report Review	06 Dec 2017

*Issued to SD Management on 04 Dec 2017

[redacted]

8 Dec 2017
Date

TEST SITE INFORMATION

Test Site for ELISpot Analysis

Company	Covance Laboratories Inc. 671 South Meridian Road Greenfield, Indiana 46140
Test Site Reference Number	8326556

RESPONSIBLE PERSONNEL

Study Director	[redacted]
Study Toxicologist	
Study Direction Management	
Lead Quality Assurance Contact	
Dose Formulations and Analysis	
Animal Operations	
Animal Welfare and Comparative Medicine	
Laboratory Operations	
Responsible Scientist for ELISpot Analysis	

1. SUMMARY

The purpose of this study was to characterize the Hepatitis B vaccine T-cell-dependent antibody response in cynomolgus monkeys and evaluate an assay to detect IgM and IgG antibody response and IFNγ-response to the Hepatitis B vaccine antigen (HBsAg). This study also included both a 'proof of concept' detection of anti-HBsAg in cynomolgus monkey serum samples and an assessment of analytical measures to assess the method as fit-for-purpose in GLP execution.

Female naïve cynomolgus monkeys were assigned to one group, and doses were given as indicated in the following table. Animals were dosed via intramuscular injection into the right quadriceps femoris region at a dose volume of 1 mL/animal. Animals were given one dose of the vaccine on Day 1 and a second (recall/challenge) dose on Day 29.

Group	No. of Animals Female	Dose Level (µg/dose)	Dose Concentration (µg/mL)[a]
1	4	20	20

a The HBsAg formulation was administered at a volume of 1 mL/dose.

Vaccination with HBsAg resulted in no noteworthy clinical observations or effects on body weight, food consumption or standard clinical pathology analyses, including hematology, coagulation, chemistry, and urinalysis.

For the purpose of HBsAg IgG and IgM analysis, blood (approximately 3 mL) was collected from all animals on Days 1 (prior to HBsAg injection), 5, 8, 15, 22, 29 (prior to HBsAg second injection), 36, 43, 50, and 57. Serum was prepared from all samples. Serum samples were analyzed using a validated enzyme-linked immunosorbent assay (ELISA) method for the semi-quantitative determination of IgG and IgM antibodies to HBsAg. For the ELISA-based assays, anti-HBsAg IgG serum samples were diluted 1:100 and were then serially diluted 5-fold a total of 4 times for a total of five dilutions and a final dilution of 1:62500. Anti-HBsAg IgM was diluted to 1:100 and then serially diluted 2X four times for a total of five dilutions and a final dilution of 1:1600. All dilutions were performed prior to serum being incubated on to plate-bound HBsAg (anti-HBsAg IgG and anti-HBsAg IgM Detection Kits; Alpha Diagnostic Intl Inc.). Bound antibodies were detected with horseradish peroxidase–conjugated goat anti-human IgG or IgM. After substrate addition, colorimetric analysis was used to calculate an interpolated cut point titer.

No animals exhibited endogenous cross-reactive anti-HBsAg IgM or IgG titers prior to first HBsAg injection. Vaccination with HBsAg on Day 1 produced measurable levels of IgM by Day 15 in 2 of 4 animals and measurable levels of IgG by Day 15 in 3 of 4 animals. After a second (recall/challenge) dose of HBsAg on Day 29, animals that mounted primary responses exhibited robust IgM and IgG responses. Additionally, a modest secondary IgG response was detected in one animal which did not exhibit measurable levels of IgG after primary immunization.

Analytical measures intended to assess the precision and repeatability of anti-HBsAg IgM and IgG detection were assessed retrospectively on Animals I10808 and I10811 and on serum from two known-positive animals (IM162634 and IM161303). Additionally a manufacturers validated method (kit method) was compared to the method utilized on this study for anti-HBsAg IgM and IgG detection and stability of serum samples at room temperature for up to 12 hours was assessed. The kit method and the method used on study performed similarly, with both methods producing linear absorbance and therefore robustly interpretable cutpoint titer responses across a range of IgM and IgG dilutions. Serum samples analyzed after 0, 1, 2, and 12 hours storage at room temperature performed similarly and serum samples were considered stable for up to 12 hours at room temperature. Precision and repeatability in the detection of anti-HBsAg-IgM and IgG from serum samples, kit positive control sample, and dilutions of kit cutpoint standards, as compared between analysts, between plates, and within a single known-positive sample were all found acceptable and fit for purpose.

For the purpose of HBsAg Enzyme-Linked Immunospot (ELISpot) analysis, blood (approximately 10 mL) was collected from all animals once during the predose phase and on Days 15, 29 (prior to HBsAg second injection), 43, and 57 of the dosing phase.

ELISPOT analysis performed to measure IFNγ secretion by PBMC isolated from cynomolgus monkeys immunized with [redacted]® in response to ex vivo stimulation with either HBsAg or [redacted]® antigens revealed that only one out of four study animals responded with appreciable IFNγ secretion. In this animal, maximal IFNγ secretion was noted on Day 43, and waned by Day 57.

In conclusion, intramuscular injection into the right quadriceps femoris region of naïve animals at a dose volume of 1 mL/animal with a the commercially available [redacted] HBsAg vaccine was well tolerated with no clinical observations or changes in body weights, food consumption or clinical pathology parameters. Vaccination with HBsAg on Day 1 produced measurable levels of IgM by Day 8 in 2 of 4 animals and measurable levels of IgG by Day 15 in 3 of 4 animals. After a second (recall/challenge) dose of HBsAg on Day 29, animals that mounted primary responses exhibited robust IgM and IgG responses. Additionally, a modest secondary IgG response was detected in one animal which did not exhibit measurable levels of IgG after primary immunization. Serum samples analyzed after 0, 1, 2, and 12 hours storage at room temperature performed similarly and serum samples were considered stable for up to 12 hours at room temperature. Analytical precision and repeatability in the detection of anti-HBsAg IgM and IgG from serum samples, kit positive control sample, and dilutions of kit cutpoint standards, as compared between analysts, between plates, and within a single known positive sample were all found acceptable and fit for purpose. HBsAg-specific IFNγ release was detected by ELISpot in only the animal with the greatest IgM and IgG response.

2. GENERAL STUDY INFORMATION

2.1 Objective

The objective of this study was to characterize the Hepatitis B vaccine T-cell-dependent antibody response in cynomolgus monkeys and evaluate an assay to detect IgM and IgG antibody response and IFNγ-response to the Hepatitis B vaccine antigen (HBsAg). This study also included both a 'proof of concept' detection of anti-HBsAg in cynomolgus monkey serum samples and an assessment of analytical measures to assess the method as fit-for-purpose in GLP execution.

2.2 Study Timetable

Study Initiation Date	24 July 2015
Experimental Start Date	23 July 2015
Inlife Start Date	31 July 2015
Inlife End Date	25 September 2015
Experimental Termination Date	16 August 2017
Study Completion Date	08 December 2017

2.3 Regulatory Test Guidelines

Immunophenotyping is suggested by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Draft Consensus Guideline, S8, Immunotoxicology Studies for Human Pharmaceuticals (April 2006), the Committee for Proprietary Medicinal Products Note for Guidance on Repeated Dose Toxicity, Appendix B (Committee for Proprietary Medicinal Products /SWP/1042/99; effective October 2000), and the Guidance for Industry, Immunotoxicology Evaluation of Investigational New Drugs, prepared by the Food and Drug Administration, Center for Drug Evaluation and Research (October 2002).

2.4 Protocol Adherence

The study was conducted in accordance with the Protocol and Protocol Amendments, with the exception of the Protocol Deviations. None of the deviations affected the integrity or interpretability of the results of the study.

2.5 Animal Welfare, Care, and Use Statement

Covance Laboratories is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All procedures in the protocol were in compliance with applicable animal welfare acts and were approved by the local Institutional Animal Care and Use Committee (IACUC).

2.6 Major Computer Systems

Application Name[a]	Application Function
Metasys	Monitors and controls environmental conditions and water flow within the facility (e.g., animal rooms)
REES Environmental Monitoring	Monitors and documents facility storage conditions (e.g., refrigerators, freezers, and constant temperature rooms)
Path/Tox System Pristima, supplied by Xybion Medical Systems Corporation	Captures direct online formulation data, inlife toxicology, and clinical and anatomic pathology data and study maintenance information and randomizes animals
FACS Diva version 6.1.2	Captures and analyzes flow cytometry data
Softmax Pro version 6.3	Captures and analyzes data generated by the Spectramax 340PC384 plate reader
Electronic Notes (eNotes)	Documents study-specific communications
Tox Reporting	Transfers data from Pristima for reporting purposes
Statistical Analysis Software (SAS)	Performs statistical analysis

a All version numbers of the applications are maintained by Covance.

2.7 Archive Statement

The raw data, documentation, specimens, records, protocol, and final report for this study will be stored in the Covance archives as detailed in the Protocol.

The raw data, including documentation and specimens (excluding those wet specimens obtained from blood, urine, feces, and biological fluids) generated from phases performed by Covance-Greenfield, will be archived in the storage facilities at Covance-Madison, as indicated.

3. METHODS

3.1 Test System and Study Design

3.1.1 Species Selection

Monkeys historically have been used in safety evaluation studies and are recommended by appropriate regulatory agencies.

3.1.2 Animal Specifications and Acclimation

Female naïve cynomolgus monkeys were originally received from Covance Research Products, Inc. Alice, Texas and were transferred to this study from the Covance-Madison Stock Colony. Animals were acclimated to the study room for 8 days prior to study start.

At initiation of dosing, animals were 2 to 5 years old, and their body weights ranged from 2.6 to 3.0 kg.

3.1.3 Environmental Conditions, Diet, and Water

3.1.3.1 Housing

Animals were housed in stainless steel cages. When possible, animals were socially housed. Animals were individually housed for study-related procedures.

3.1.3.2 Water

Water was provided ad libitum.

3.1.3.3 Diet

Animals were offered Certified Primate Diet #5048 (PMI Nutrition International Certified LabDiet®) twice daily unless fasted for study procedures (see Protocol Deviations).

3.1.3.4 Environment

Environmental controls were set to maintain the following animal room conditions: a temperature range of 20 to 26°C, a relative humidity range of 30 to 70%, 10 or greater air changes/hour, and a 12-hour light/12-hour dark cycle. Any variations to these conditions are maintained in the raw data and had no effect on the outcome of the study.

3.1.3.5 Dietary and Environmental Enrichment

Animals were given various cage-enrichment devices and fruit, vegetable, or dietary enrichment (that did not require analyses). Animals were commingled in accordance with Covance standard operating procedures (see Protocol Deviations).

3.1.4 Animal Identification and Assignment to the Study

Animals were identified using a tattoo and implantable microchip identification device.

Selection of animals was based on data collected during acclimation. No randomization was needed because all animals were given the same dose.

3.1.5 Study Design

Group	No. of Animals Female	Dose Level (µg/dose)	Dose Concentration (µg/mL)[a]
1	4	20	20

a The HBsAg formulation was administered at a volume of 1 mL/dose.

3.2 Test Article

Information on synthesis methods, stability, purity, composition, or other characteristics defining the test article is on file with the respective manufacturer.

3.2.1 Test Article

Test Article[a]	Storage	Lot No.	Expiration Date	Concentration
Hepatitis B Vaccine; [redacted]® (HBsAg)[b]	In a refrigerator, set to maintain 2 to 8°C	GP7E7	30 September 2017	20 µg/mL

a Provided preformulated at a concentration of 20-µg/mL hepatitis B surface antigen
b Manufactured by [redacted]

3.2.2 Test Article Formulation

Test article formulations were used as supplied by the supplier and dispensed twice according to the Covance dispensing procedure. Dose concentrations were based on the nominal concentration of the test article formulation, as provided by the manufacturer.

At least one unused vial of HBsAg from the same lot used for dosing, was transferred to the Greenfield Immunotoxicology Lab to use for coating of ELISA plates.

The HBsAg formulation was stored according to the manufacturer's recommendation, in a refrigerator, set to maintain 2 to 8°C.

3.3 Hepatitis B Vaccine (HBsAg) IgM and IgG (ELISA) and Hepatitis B Vaccine (HBsAg) Enzyme-Linked Immunospot (ELISpot) Analysis

3.3.1 Hepatitis B Vaccine (HBsAg) IgM and IgG Sample Collection and Handling

Blood samples were collected via a femoral vein from animals as follows. Animals were not fasted for sample collections unless fasted for clinical pathology sample collections.

Primary Administration

Prior to (predose) primary administration on Day 1 and on Days 5, 8, 15, and 22.

Secondary Administration

Prior to (predose) secondary administration on Day 29 and on Days 36, 43, 50, and 57 of the dosing phase.

Blood (approximately 3 mL) was collected into serum separator tubes containing no anticoagulant. After blood collection, the tubes were gently inverted to ensure adequate mixing and were transferred to the Clinical Pathology (Immunotoxicology) for processing. Samples were held on dry ice until transferred to freezer, set to maintain -60 to -80°C.

Unused samples were donated to general-purpose use at Covance.

3.3.2 Hepatitis B Vaccine (HBsAg) Enzyme-Linked Immunospot (ELISpot) Sample Collection and Handling

Blood samples were collected from all animals via a femoral vein once during the predose phase and on Days 15, 29 (predose), 43, and 57 of the dosing phase. Animals were not fasted for sample collections unless fasted for clinical pathology sample collections.

Blood (approximately 10 mL) was collected into tubes containing sodium heparin. After blood collection, the tubes were gently inverted to ensure adequate mixing and were transferred to the Clinical Pathology (Immunotoxicology) for processing. Peripheral blood mononuclear cells were isolated from blood according to a study-specific procedure. Samples were stored in a freezer, set to maintain -60 to -80°C, until packed on dry ice and shipped to Covance-Greenfield for analysis.

3.3.3 Hepatitis B Vaccine (HBsAg) IgM and IgG (ELISA) Analysis

Samples were analyzed for anti-HBsAg IgM titers and for anti-HBsAg-IgG titers. Samples were analyzed using an enzyme-linked immunosorbent assay (ELISA) method (Alpha Diagnostic International, 2015). Analytes are reported as concentrations, where possible, or as cut point titers.

Anti-HBsAg IgG and IgM were assessed as a cutpoint titration value. The cutpoint titer is defined as the reciprocal of the highest dilution of a sample that gives a reading above the cutoff. To determine the cutoff value, naïve serum from a non-vaccinated subject was analyzed repeatedly in each assay. The cutoff value was then defined as the mean of the naïve serum repetitions, plus the standard deviation, times the appropriate standard deviation multiplier. Standard deviation multipliers are calculated from the critical values for a one-tailed *t*-distribution (Frey et al., 1998). This method eliminates the establishment of arbitrary cutoff values and addresses the statistical likelihood of false positive samples.

Serial dilutions of serum samples were analyzed with an ELISA method. Samples were diluted until the response values were less than the established cutoff of the naïve serum control. The interpolated cutpoint titer was then calculated as the point at which the optical density (OD) of the sample (Y) crosses the cut point axis (X) using the slope of the line between the points above and below the cutoff.

For the purposes of assay calculation, any value reported with < was taken to represent a value below the limit of detection (quantitation) for the assay and treated as a zero (0) for assay calculations. Further, if the mean value was below the lower limit of quantitation, it was also treated as zero for any required analysis.

3.3.4 Hepatitis B Vaccine (HBsAg) Enzyme-Linked Immunospot (ELISpot) Analysis

ELISPOT assay was performed per the manufacturer's protocol and the ELISPOT analyzer was used in accordance with Covance TBS SOP. One day before the assay, ELISPOT plates were coated with 80 µL of capture antibody stock solution that was diluted 1:250 by mixing 40 µL of human IFNγ capture antibody in 10 mL of diluent A. Plates were sealed using ParafilmTM and refrigerated at 2 to 8°C overnight. The capture antibody solution was decanted and the plates were washed with DPBS once. 100 µL of CTL Test medium supplemented with 2 mM L-Glutamine or antigen/mitogen solution made in the same medium was added to the appropriate wells and incubated in a 37°C humidified incubator supplied with 5% CO_2 for 15 minutes. 100 µL of PBMC appropriately diluted in CTL Test medium supplemented with 2 mM L-Glutamine was then added and the plates were incubated for 24 hours in a 37°C humidified incubator supplied with 5% CO_2. For stimulation with PHA, 100,000 PBMC were used per well in 100 µL of CTL Test medium supplemented with 2 mM L-Glutamine). For other conditions, PBMC were used at 200,000 cells per well. Plates were washed two times with DPBS and two times with DPBS containing 0.05% Tween 20. 80 µL of a 1:250 diluted- human IFNγ detection antibody solution (prepared by mixing 40 µL of antibody stock solution in 10 mL of Diluent B) was then added to each well and the plates were incubated at room temperature for two hours. Plates were washed three times with DPBS containing 0.05% Tween 20 and then 80 µL of diluted Streptavidin-Alkaline Phosphatase conjugate solution (prepared by mixing 10 µL of Streptavidin-Alkaline Phosphatase conjugate stock solution in 10 mL of Diluent C) was added. Plates were incubated for 30 minutes at room temperature. Plates were then washed two times with DPBS containing 0.05% Tween 20 and two times with distilled water. 80 µL of developer solution (prepared by mixing 160 µL of S1, 160 µL of S2 and 92 µL of S3 sequentially with 10 mL of Diluent Blue) was then added to each well and plates were developed in the dark for 15 minutes. The protective underdrain of the plates was removed and the reaction was stopped by washing the plates in running distilled water. Plates were dried overnight and scanned/counted using a CTL-ImmunoSpot S6 Macro Analyzer.

3.3.5 Hepatitis B Surface Antigen (HBsAg) - Analytical Validation Parameters

3.3.5.1 Assumptions

It was assumed the Spectramax 340PC384 plate reader, was functioning correctly at the time of this validation.

Stability was assumed as the stability for immunoglobulin IgG and IgM (www.aruplab.com/guides/ug/tests/0050355.jsp). Serum samples are stable at ambient conditions for 8 hours; refrigerated conditions for 8 days; and frozen conditions for 1 year. Temperatures stated in GL-GEN-307 were accepted as the local tolerance (ambient conditions 15 to 20°C, refrigerated 2 to 8°C, frozen at -10 to -20°C, frozen at -60 to -80°C). This assumption was made until the completion of internal stability analysis completed in this study.

The dilution scheme and method had previously been established. If the acceptance criteria stated below was met, the analytical method was validated and considered fit for purpose.

3.3.5.2 Exclusions
The primate anti-HBsAg IgG and IgM assay utilized an antibody cut-point titer for the determination of the concentration of anti-HBsAg IgG and IgM in a sample. Precision and stability of anti-HBsAg antibody in serum was assessed. All other aspects of the performance of the assay had been [redacted]

3.3.5.3 Methodology
A 96 well strip plate was provided by Alpha Diagnostic International (catalog #4200 [IgG] and catalog #4205 [IgM]) and each microwell had HBsAg immobilized on them. Serum containing anti-HBsAg was added to the wells and bound the antigen immobilized on the surface of the well. The anti-HBsAg antibody was detected by anti-human IgG or IgM specific antibody conjugated to horse radish peroxidase (HRP) enzyme. After a washing step, chromogenic substrate 3,3', 5,5'-Tetramethylbenzidine (TMB) was added and color was developed by the enzymatic reaction of HRP on the substrate (TMB), which was directly proportional to the amount of anti-HBsAg IgG or IgM in the serum sample. Stopping solution was added to stop the reaction and the absorbance at 450 nm was then measured using the Spectramax 340PC384 plate reader (or another suitable microwell plate reader).

3.3.5.4 Method Comparison
Anti-HBsAg positive primate serum was tested using the kit method and a Covance developed method.

3.3.5.5 Precision
Primate serum samples from animals inoculated with [redacted]® (HBvx) were prepared using the previously established five-fold dilution scheme for IgG and the previously established two fold dilution scheme for IgM. The diluted samples were analyzed four times on a minimum of three different runs and at least one run was performed by a different analyst. The titer values were calculated. The mean, standard deviation, and the percent coefficient of variation (%CV) for the titer value were calculated (intra-batch precision, inter-batch precision and inter-analyst precision was calculated). The acceptance criteria were that there should be no more than a 25% CV observed.

3.3.5.6 Stability
Stability was assessed using serum from three primates (individuals or pools). Samples were separated into eight aliquots labelled A through H.

Aliquot A was analyzed after the first thaw of the sample, to provide baseline/expected values after long term storage at approximately -80°C.

Aliquots B through H were frozen at approximately -80°C. Aliquots B and C were thawed for at least 1 hour and re-frozen. Aliquot C was removed from the freezer for a second time and thawed at least 1 hour and re-frozen. Analysis of aliquots B and C occurred together on one run. Aliquot D was thawed for at least 12 hours and run. Aliquots E through H were kept at approximately -80°C until discarded prior to report finalization. The titer values were calculated and findings tabulated and compared using a Levy-Jennings chart.

The data established stability and was considered acceptable if results were between 70 and 130% of baseline for the material.

3.3.5.7 Hepatitis B Surface Antigen (HBsAg) - Analytical Validation Runs

A table of included and excluded analytical runs is presented in Table 10.8.

3.4 Method Approval

Hepatitis B vaccine T-Cell Dependent Antibody Response in cynomolgus monkeys was approved for use on 07 September 2017.

3.5 Inlife Procedures

3.5.1 Dose Administration

Dose formulations were well shaken to re-suspend the sediment of fine white particles of adjuvant (aluminum hydroxide), which settled during storage, to obtain a qualitatively slightly opaque, white suspension (see Protocol Deviations).

Primary Administration

All animals were given one dose of HBsAg at a dose volume of 1 mL/animal by intramuscular injection into the right quadriceps femoris region on Day 1 of the dosing phase.

Secondary Administration

All animals were given one dose of HBsAg at a dose volume of 1 mL/animal by intramuscular injection into the right quadriceps femoris region on Day 29 of the dosing phase.

The HBsAg injection sites were clipped free of hair (prior to dosing and as needed thereafter).

3.5.2 Clinical Observations

3.5.2.1 Health Monitoring

Animals were checked twice daily (a.m. and p.m.) for mortality, abnormalities, and signs of pain or distress (see Protocol Deviations).

3.5.2.2 Clinical Examinations

Cageside observations were conducted for each animal once daily during the predose and dosing phases, except on days when detailed observations were conducted (see Protocol Deviations). Abnormal findings were recorded (see Protocol Deviations).

Detailed observations were conducted for each animal once during the predose phase, prior to dosing on Day 1, and weekly (based on Day 1) throughout the dosing phase. Detailed observations included the evaluation of dose site. Abnormal findings or an indication of normal was recorded.

Unscheduled observations were recorded.

3.5.2.3 Postdose Observations

On each day of dosing, cageside observations were conducted for each animal approximately 1 and 4 hours postdose. Postdose observation start times for each animal were based on the dosing completion time for each animal. Postdose observations included the evaluation of the dose sites. Abnormal findings or an indication of normal was recorded.

3.5.3 Body Weights

Body weights were recorded twice during the predose phase, before dosing on Day 1, and weekly (based on Day 1) thereafter during the dosing phase.

3.5.4 Food Consumption

Qualitative food consumption was recorded daily, except on the day of animal transfer or days when animals were fasted at the time of the observation, during the predose and dosing phases (see Protocol Deviations).

3.6 Clinical Laboratory Procedures

3.6.1 Clinical Pathology

3.6.1.1 Sample Collection and Handling

Blood samples for hematology, coagulation, and clinical chemistry were collected from fasted animals via a femoral vein. Urine samples for urinalysis were collected chilled during the overnight period before blood collection from animals fasted overnight.

Blood and urine samples were collected on Day 5 of the predose phase and on Day 57 of the dosing phase.

The anticoagulants were sodium citrate for coagulation tests and potassium EDTA for hematology tests. Samples for clinical chemistry were collected without anticoagulant.

3.6.1.2 Hematology Tests

red blood cell (erythrocyte) count
hemoglobin
hematocrit
mean corpuscular volume
mean corpuscular hemoglobin
mean corpuscular hemoglobin concentration
platelet count
white blood cell (leukocyte) count
differential blood cell count
blood cell morphology
reticulocyte count

3.6.1.3 Coagulation Tests

prothrombin time
activated partial thromboplastin time

3.6.1.4 Clinical Chemistry Tests

glucose
urea nitrogen
creatinine
total protein
albumin
globulin
albumin:globulin ratio
cholesterol
triglycerides
total bilirubin
alanine aminotransferase
alkaline phosphatase
gamma glutamyltransferase
aspartate aminotransferase
creatine kinase
calcium
inorganic phosphorus
sodium
potassium
chloride

3.6.1.5 Urinalysis Tests

appearance (clarity and color)
volume
specific gravity
pH
protein
glucose
ketones
bilirubin
urobilinogen
blood
microscopic examination of sediment

3.7 Terminal Procedures

3.7.1 Final Disposition

All animals were transferred back to the stock colony after the final blood collection.

3.8 Data Evaluation and Statistical Analysis

Various models of calculators, computers, and computer programs were used to analyze data in this study. Because different models round off or truncate numbers differently, values in some tables (e.g., means, standard deviations, or individual values) may differ slightly from those in other tables, from individually calculated data, or from statistical analysis data. Neither the integrity nor the interpretation of the data was affected by these differences.

Analyses of data will include calculation of means, and standard deviations as appropriate.

4. RESULTS

4.1 Hepatitis B Vaccine (HBsAg) IgM and IgG

Hepatitis B Vaccine (HBsAg) IgM and IgG data are presented in Table 9.5. Anti-HBsAg response is presented in Figure 8.1 (IgM) and Figure 8.2 (IgG).

4.1.1 Anti-HBsAg IgM

No animals exhibited endogenous cross-reactive anti-HBsAg IgM titers prior to HBsAg injection.

One animal (Animal I10811) had a measurable IgM value of 136 at the predose measurement, the remaining three animals were below the lower limit of quantification (BLLOQ). Animal I10811 continued to have similar values from Day 8 through Day 15, at which point the cut point titer exhibited a >5-fold increase, indicating an induced IgM response to HBsAg. This was interpreted to indicate the predose and Days 1 through 8 values were a background level for this animal, and these results did not indicate presence of endogenous cross-reactive IgM antibodies to HBsAg antigen.

Upon primary immunization with HBsAg, two of four animals (Animals I10811 and I10808) exhibited a >5-fold increase in IgM antibodies from Days 8 through 15 after the initial injection, followed by a decrease from Days 15 through 29. Animal I10808 returned to nearly baseline values, while Animal I10811 remained at approximately 3-fold greater antibody presence compared to its individual baseline. Both of these animals exhibited robust antibody response to the secondary antigen challenge (vaccination) at Day 29, with Animal I10808 returning to near-peak levels within 7 days of secondary challenge and maintaining these levels until the final Day 57 collection. Animal I10811 increased circulating antibodies to 2-fold greater than the initial IgM peak response (nearly 10-fold from baseline) and then returning to approximately baseline values by its final Day 57 collection.

The remaining two animals (Animals I10809 and I10810) exhibited no measureable IgM response to either primary or secondary vaccination with HBsAg. The lack of an IgM response may be related to individual variability in response to the vaccine, and expected based on the literature (Lebrec et al., 2014). Animal I10810 had a measureable IgG response after the recall HBsAg challenge; therefore, it did appear to respond to the antigen (though less robustly) despite the fact no measureable IgM response occurred (see Section 4.1.2).

4.1.2 Anti-HBsAg IgG

No animals exhibited endogenous cross-reactive anti-HBsAg IgG titers prior to HBsAg injection.

No relevant IgG response was observed in any animal at any collection from predose through Day 15. Minimal IgG antibody production is present from Day 15 through the secondary HBsAg administration at Day 29. Noteworthy increases of at least 7-fold, and up to more than 1000-fold, in IgG titer compared to baseline in 3 of 4 animals was

observed by Day 36. One animal (Animal I10809) had a no considerable response to primary vaccination and only mild response to secondary HBsAg vaccination, with an approximately 10-fold increase in IgG present from Day 36 and sustained to the Day 57 final collection. One animal (Animal I10811) had multiple-fold (range of approximately 4 to 30-fold) higher IgG antibodies present at Day 29 (collected prior to secondary challenge) than all other animals, and subsequently exhibited the greatest IgG antibody production of all animals on study after the second administration of HBsAg. Substantial variability in the response was present, with an approximate 5-fold range observed by Day 36 between high-responder Animal I10811 and moderate responders Animals I10808 and I10810 and a 20-fold range between these moderate responders and low responder Animal I10809. An approximate 100-fold difference in values occurred between high responder Animal I10811 and low responder Animal I10809. These ranges between one low, two moderate and one high responder were present from Day 36 through the end of the study at the final Day 57 collection. Measured IgG levels peaked at Day 36 for all animals and gradually decreased through the final assessment at Day 57, but mean titer levels remained in the range of 50,000 to 100,000 as compared to a baseline 100 value.

4.2 Hepatitis B Vaccine T-Dependent Antibody Response Analytical Measures

4.2.1 Inter-Analyst Cutpoint Titer - IgM and IgG

Inter-analyst cutpoint titer - IgM and IgG data are presented in Table 10.1.

Serum samples from two known positive animals (Animals IM162634 and IM161303) and one animal (Animal I10808) collected at the terminal sacrifice from this study were run by two analysts in parallel and cutpoint titer results compared. The largest variability was in IgM response from Animal I10808 at approximately 17%; this was on a sample that was approximately 23 months old at the time of assay. All other inter-analyst variability was <10% and therefore passed analytical validation criteria for inter-analyst variability.

Additionally, a set of replicate analyses by a single analyst was generated and run in parallel to a second analyst and cutpoint titer results compared. On known positive animals (Animals IM162634 and IM161303), variability was <15% in all instances, and for IgG cutpoint was generally in the range of 5% across all analyses. Variability in the detection of IgG was >25% in Animal I10808 and therefore did not pass acceptable limits. IgM detection also varied by 16.6% in this animal. However, as the sample was past known stability, the variability in detection for this animal was not considered indicative of inter-analyst variability on a standard sample. When samples were within known stability, the assay passes acceptable inter-analyst variability.

4.2.2 Inter-Plate/Intra-Analyst - IgM and IgG

Inter-plate/intra-analyst - IgM and IgG data are presented in Table 10.2.

Serum samples from two known positive animals (Animals IM162634 and IM161303) and one animal (Animal I10808) from this study were loaded onto two identical plates each by two separate analysts. Variability of plates loaded by the same analyst was

therefore assessed, and CV's were <13% in all cases with the exception of IgG from one of two analysts in Animal I10808. When samples inside known stability were analyzed for inter-plate/intra-analyst variability, all were found to have acceptable inter-plate precision and repeatability.

4.2.3 Intra-Sample/Batch - IgM and IgG

Intra-sample/batch - IgM and IgG data are presented in Table 10.3.

To assess the precision of the repeated measure of a single sample, a sample from two known positive animals (Animals IM162634 and IM161303) and one animal from this study (Animal I10808) were loaded in quadruplicate by a single analyst, and this was performed by two analysts in parallel. Variability was less than approximately 11% in all measures, and generally <5%. Therefore the precision and repeatability of analysis measures pass analytical validation acceptance criteria.

4.2.4 Benchtop Stability - IgM and IgG

Benchtop stability - IgM and IgG data are presented in Table 10.4.

To assess the stability of serum samples at ambient temperatures, samples from two known positive animals (Animals IM162634 and IM161303), and two animals from this study (Animals I10808 and I10811) were held at room temperature for 1, 2 and 12 hours post thaw and then plated and analyzed. Change from the 0 hour time point for all samples tested was less than established acceptance criteria of 70 to 130%, and generally 5% or less, therefore indicating that samples were highly stable after thaw for up to 12 hours at room temperature/ambient conditions. Cutpoint IgM titer for stability analysis at each time point tested is presented in Figure 8.3 and IgG data is presented in Figure 8.4.

4.2.5 Method and Kit Comparison (Kit Controls and Serum Sample): Absorbance-IgM and IgG

Method and kit comparison (kit controls): absorbance - IgM and IgG data are presented in Table 10.6.

To assess the precision and repeatability of the kit and study method in detection of anti-HBsAg-IgM and -IgG, the positive control for IgM and IgG from the kit, and a serum sample from a known-positive were tested five times each with each method. IgG detection was highly repeatable within each method, and the two methods performed similarly. The kit detected slightly higher IgG absorbance in the positive control, but less in the serum sample; in total, variability was less than approximately 17% and therefore for IgG detection were considered comparable methods. IgM detection was similar but more consistent within replicates with the study method as compared with the kit method, and IgM detection was slightly higher in the positive serum sample analysis using the study method. Though variability between methods was >25%, detection of IgM was actually slightly higher with the study method, and both methods consistently detected IgM. Therefore, as the assay is always relative, and both methods do detect IgM consistently, the methods, though slightly different in quantitation of IgM, were

considered to perform acceptably and results were considered to be able to be reliably interpreted within either method.

4.2.6 Method and Kit Comparison: Absorbance - IgM and IgG

Method and kit comparison: absorbance - IgM and IgG data are presented in Table 10.5.

To assess the performance of the kit method compared with the method developed and utilized in this study, a general guideline of variability (CV 25%) was used for initial comparisons. The kit standards used to generate the standard curve for cut point titer interpretation were run according to manufacture instructions and then also run with the method utilized on this study. The standards from the kit created highly equivalent absorbance through the spectrum of dilution up to the most dilute standard (-0.005). At the highest dilution, the method used on study detected less IgG and IgM than the kit method. However, a useable standard curve is generated from either method, and in general the methods performed highly similarly. As an additional measure, the most concentrated standard was serially diluted and measured by both methods. This "High Standard Series" performed 2:1 serial dilutions, and across four sets of dilutions, both methods performed highly similarly, detecting equivalent levels of kit standard IgM and IgG and demonstrating linear response to IgM and IgG dilution.

4.2.7 Method and Kit Comparison (Study Serum Samples): Absorbance - IgM and IgG

Method and kit comparison (study serum samples): absorbance - IgM and IgG data are presented in Table 10.7.

To assess the performance, and allow comparison, of the kit and study method to detect anti-HBsAg-IgM and -IgG, samples from two animals (Animals I10808 and I10811) collected at two dates (18Sep15, and 25Sep15) on study were analyzed in parallel. A general guideline of variability (CV 25%) was used for initial comparisons. IgG detection was according to expectation and linear across the range of dilutions with either method (see Figure 8.5 and Figure 8.6). Detection at the highest dilution, for a single animal, at the first time point (18Sep15), was the single point outside established acceptance criteria (<25% CV) when the two methods were compared. This single point of curve setting was the only CV outside acceptable limits, and the IgG detection as a whole was considered highly similar between methods, highly repeatable within each method, and capable of generating robustly interpretable data. The kit method generally detected slightly higher IgG absorbance at the highest dilution, but less in the more concentrated samples (see Figure 8.5 and Figure 8.6). IgM detection was higher using the study method at most concentrations in both animals at both time points. Though variability in result was therefore higher than 25% with comparison of the two methods, the consistency of IgM detection and the linearity and fitment of the detection was highly similar between methods. Additionally, higher variability was only observed at dilutions of sample that would not be used in standard sample analysis (an ideal, not maximal, dilution is used normally). Therefore, both methods consistently and repeat-ably detected anti HBsAg-IgM. Therefore, as the assay is always relative, and both methods do detect IgM consistently, the methods, though slightly different in quantitation of IgM, were

considered to perform acceptably and results were considered to be able to be reliably interpreted within either method.

4.3 Hepatitis B Vaccine (HBsAg) Enzyme-Linked Immunospot (ELISpot)

Results of Hepatitis B Vaccine (HBsAg) ELISpot analysis are in the Hepatitis B Vaccine (HBsAg) ELISpot Report.

Using a previously validated human IFNγ ELISPOT kit, PBMC samples from four cynomolgus monkeys were analyzed.

Results showed that PBMC from all animals responded to PHA positive control by secreting IFNγ, as expected at all time points, with the notable exception of Animal I10810 at the Day 29 time point. However, three of the four animals did not respond appreciably to either HBsAg or [redacted]®. Average number of spots for IFNγ noted in these animals were mostly similar to those noted in the negative control (media). One animal (Animal I10811) responded to both HBsAg and [redacted]® in an appreciable manner. In this animal, there were no detectable levels of IFNγ on Day -5 (predose) but IFNγ secretion was noted at all time points post immunization (i.e., Days 15, 29, 43, and 57). Maximal IFNγ secretion was seen on Day 43 (2 weeks post administered dose of [redacted]®), and waned by Day 57.

4.4 Inlife Evaluations

4.4.1 Animal Fate

Animal fate data are presented in Table 9.1.

All animals survived to the scheduled end of study, when they were returned to stock.

4.4.2 Clinical Observations

Clinical observations data are summarized in Table 9.2; individual data are presented in Table 9.3.

No toxicologically relevant or HBsAg-related changes in clinical observations occurred.

4.4.3 Body Weights

Individual and summary body weight data are in Table 9.4.

No toxicologically relevant or HBsAg-related changes in body weights occurred.

4.5 Clinical Laboratory Evaluations

4.5.1 Clinical Pathology

Summary and individual hematology and coagulation, clinical chemistry, and urinalysis data are in Table 9.6, Table 9.7, and Table 9.8, respectively.

4.5.2 Hematology

No toxicologically relevant or HBsAg-related changes in hematology parameters occurred. Hematology parameters were within normal limits for all animals at the predose and dosing phase evaluations. Animal I10809 did exhibit an elevated reticulocyte count at the predose evaluation; this indicated a regenerative response was occurring during the predose phase, which was likely ongoing at study initiation, although total red blood cell (RBC) numbers are equivalent for all animals. Additionally, Animal I10809 exhibited lower total white blood cell (WBC) numbers, with corresponding decreases relative to all other animals in all subsets of WBC (neutrophil, lymphocytes, monocytes, eosinophils, basophils, and large unstained cells). Although within normal limits, these decreases were correlated with decreased immune function observed in this animal.

4.5.3 Coagulation

No toxicologically relevant or HBsAg-related changes in coagulation parameters occurred. Coagulation parameters were within normal limits for all animals at the predose and dosing phase evaluations.

4.5.4 Clinical Chemistry

No toxicologically relevant or HBsAg-related changes in chemistry parameters occurred. Chemistry parameters were within normal limits for all animals at the predose and dosing phase evaluations.

4.5.5 Urinalysis

No toxicologically relevant or HBsAg-related changes in urinalysis parameters occurred. Urinalysis parameters were within normal limits for all animals at the predose and dosing phase evaluations.

5. DISCUSSION

The T-cell-dependent antibody response (TDAR) assay has become a standard approach to assess immune function. The successful TDAR is dependent on multiple functional immune processes. Antigen uptake and presentation is followed by T cell recognition and B cell activation to produce antibodies. Alteration of any of these processes will result in abnormal antibody production and will indicate the occurrence of immunomodulation. For this reason, the TDAR assay provides a good marker of adaptive immune system function.

Hepatitis B surface antigen (HBsAg) is a clinically used human vaccine and a T-dependent antigen that, unlike sRBCs, TT, and KLH, can be used in pre-clinical and clinical safety assessment. The ability to translate an assay from animal models to a human model may enhance the predictability of immunomodulation of test articles at the clinical level.

In this study, for an ELISA-based assay for measurement of anti-HBsAg IgG- and IgM-specific antibodies, cynomolgus monkeys were immunized with HBsAg ([redacted] [HBsAg] at the 20 μg adult human dose) and were re-challenged with a second vaccination 28 days later. After the initial immunization, anti-HBsAg IgM-specific primary antibody peak responses occurred on Day 15, respectively, although two of four animals did not respond. One of these animals had a lower white blood cell and relevant subset apparent in clinical pathology examination; the decreased response may have been related. Variability in the response was expected given standard vaccine dose-responses in the literature, and it may indicated the strength of antigenic stimulation with a 20 ug HBsAg dose was not enough to consistently elicit a maximal response.

Animal I10809 appeared to have an example of the lower range of expected immunological response, but it remained within normal limits given a 20 ug dose of HBsAg antigen. Animal I10809 had immune cell (WBC and relevant subsets) decreases relative to all other animals on study. Although WBC numbers were within normal limits, the decreased response to HBsAg was well correlated to this clinical pathology finding. Based on the variability observed in this study, it was presumed a normal distribution of responses was represented here, with Animals I108011 and I10809 representing high and low responders, respectively, and Animals I10808 and I10810 representing an average response, especially with regard to IgM recall anti-HBsAg response.

The method of detection appeared to perform acceptably. After the re-challenge, anti-HBsAg IgG secondary response peaked from Days 36 to 43 and then decreased gradually through Day 57.

This study demonstrated the ability to detect and assess cynomolgus monkey antibody response to the HBsAg challenge. [redacted]

Detectable HBsAg-specific IFNγ cytokine release occurred in one of four animals on study despite all four animals having normal response to Phytohemagglutinin (PHA) mitogen and thereby demonstrating normal function of T cells and acceptable function of the assay. Though the ability to detect antigen-specific cytokine release using ELISpot was confirmed, the utility of the assay remains unclear due to detection in only one of four animals. Testing of additional animals and comparison of frozen and fresh supernatants are considered necessary to establish the utility of the assay.

6. CONCLUSION

In conclusion, intramuscular injection into the right quadriceps femoris region of naïve animals at a dose volume of 1 mL/animal with a the commercially available [redacted] HBsAg vaccine was well tolerated with no clinical observations or changes in body weights, food consumption or clinical pathology parameters. Vaccination with HBsAg on Day 1 produced measurable levels of IgM by Day 8 in 2 of 4 animals and measurable levels of IgG by Day 15 in 3 of 4 animals. After a second (recall/challenge) dose of HBsAg on Day 29, animals that mounted primary responses exhibited robust IgM and IgG responses. Additionally, a modest secondary IgG response was detected in one animal which did not exhibit measurable levels of IgG after primary immunization. Serum samples analyzed after 0, 1, 2, and 12 hours storage at room temperature performed similarly and serum samples were considered stable for up to 12 hours at room temperature. Analytical precision and repeatability in the detection of anti-HBsAg-IgM and IgG from serum samples, kit positive control sample, and dilutions of kit cutpoint standards, as compared between analysts, between plates, and within a single known positive sample were all found acceptable and fit for purpose. HBsAg-specific IFNγ release was detected by ELISpot in only the animal with the greatest IgM and IgG response.

7. ASSOCIATED STUDY INFORMATION

7.1 References

Alpha Diagnostic International, *Human Anti-HBsAg IgG: Instruction Manual No. M-4200 and No. M-4205.* San Antonio, Texas (2015).

Frey, A., Di Canzio, J., and Zurakowski, D., "A Statistically Defined Endpoint Titer Determination Method for Immunoassays," *Journal of Immunological Methods*, *221*(1):35-41. doi:10.1016/S0022-1759(98)00170-7 (1998).

Lebrec, H., Molinier, B., Boverhof, D., Collinge, M., Freebern, W., Henson, K., Mytych, D., Ochs, H., Wange, R., Yang, Y., Zhou, L., Arrington, J., Christin-Piché, M., and Shenton J., "The T-Cell-Dependent Antibody Response Assay in Nonclinical Studies of Pharmaceuticals and Chemicals: Study Design, Data Analysis, Interpretation," *Regulatory Toxicology and Pharmacology*, *69*(1):7-21. doi:10.1016/j.yrtph.2014.02.008 (2014).

7.2 Abbreviations

The following lists of abbreviations are used by Covance. Some, but not necessarily all, of this information may be needed for this report.

General Abbreviations

-	Dead animal; no value
#, N, No.	Number
% RSD	Relative standard deviation
%-Diff	Percent difference
.	No value calculated for mean and standard deviation
a.m.	Ante meridian
BID, bid	Twice a day
BODYTEMP	Body temperature
C	Comment found at the end of each group for each sex
CAM	Covariate-adjusted mean
CO	Clinical observation
CTLS, ctls	Controls
CV	Coefficient of variation
DIA	Diastolic pressure
DSNG	Dosing phase
DSNG X.X	Dosing Phase Week X. Day X
DT TY	Data type
F	Female
ID	Identification
int	Interval
IPD	Immediate postdose
LOQ	Limit of quantitation
M	Male
MAP	Mean arterial pressure
Mean; MEAN	Arithmetic mean
N	Number of measurements in a group
NA	No value; not applicable; not present
ND	None detected
NF	National Formulary
NVL	No visible lesions
Obs	Observations
OXSA	Blood oxygen saturation
P	Present
P(DR)	P value (dose response)
P(overall)	Overall P value for all groups
P(v1)	P value (verses group 1)
p.m.	Post meridian
PD	Postdose
PRED	Predose phase

General Abbreviations (Continued)

PRED X.X	Predose Phase Week X. Day X
RECO	Recovery phase
RECO X.X	Recovery Phase Week X. Day X
RESP	Respiration rate
S.E.M./SEM	Standard error mean
SD; S.D.; STAND DEV; STANDARD DEV; sd; STD.DEV	Standard deviation
SE; STDERR	Standard error
SYS	Systolic pressure
TBW	Terminal body weight
TS	Terminal sacrifice
Typ	Type
UNSCHED or SCHED	Unscheduled or scheduled
USP	United States Pharmacopeia
WK	Week
WT	Weight

General Abbreviations (Continued)

Units of Measure

amol	Attomol
BPM	Beats per minute
ºC	Degrees Celsius
cm	Centimeter
DL, dl, dL	Deciliter
EU	Ehrlich units
FL, fl	Femtoliter
fmol	Femtomol
G, g	Gram
H, h	Hours
IU	International units
KG, kg	Kilogram
L	Liter
MCG, UG, µg, ug	Microgram
MEQ	Milliequivalents
MG, mg	Milligram
MI	Million
ML, mL	Milliliter
mm	Millimeters
mmHG/mmHg	Millimeter of mercury
MMOL, mmol	Millimoles
MN, min	Minute
MOS	Milliosmoles
Msec, msec	Milliseconds
mU	Milliunits
ng	Nanogram
PG, pg	Picogram
pmol	Picomoles
PPM, ppm	Parts per million
S, s, sec	Seconds
TH	Thousand
U	Units
UL, µL, uL	Microliter
UMOL, µmol	Micromoles

Abbreviations and Units for Hematology

Abbreviation	*Units*	*Test*
ANISO		Anisocytosis
BASO	E3/uL	Absolute basophils
BASO%	%	Percent basophils
CFWB	cells/uL	White blood cell count cerebral spinal fluid
CHCMr	g/dL	Reticulocyte hemoglobin concentration mean
CHr	pg	Concentration of hemoglobin in reticulocytes
EOS	E3/uL	Absolute eosinophils
EOS%	%	Percent eosinophils
ESR	mm/hour	Erythrocyte sedimentation rate
FNBC	cells/uL	Cell poor fluid nucleated cell count
FRBC	cells/uL	Cell poor fluid red blood cell count
FWBC	cells/uL	Cell poor fluid white blood cell count
Hct	%	Hematocrit
HDW	%	Hemoglobin distribution width
HGB	g/dL	Hemoglobin
HRTC	E9/ul	High absorbance reticulocytes
HRTC%	%	Percent high absorbance reticulocytes
HYPO		Hypochromasia
LRTC	E9/ul	Low absorbance reticulocytes
LRTC%	%	Percent low absorbance reticulocytes
LUC	E3/uL	Absolute large unstained cells
LUC%	%	Percent large unstained cells
LYM	E3/uL	Absolute lymphocytes
LYM%	%	Percent lymphocytes
MCH	pg	Mean corpuscular hemoglobin
MCHC	g/dL	Mean corpuscular hemoglobin concentration
MCV	fL	Mean corpuscular volume
MCVr	fL	Reticulocyte MCV
MONO	E3/uL	Absolute monocytes
MONO%	%	Percent monocytes
MPV	fL	Mean platelet volume
MRTC	E9/ul	Mid absorbance reticulocytes
MRTC%	%	Percent mid absorbance reticulocytes
NEUT	E3/uL	Absolute neutrophils
NEUT%	%	Percent neutrophils
PCT	%	Platelet crit

Abbreviations and Units for Hematology (Continued)

Abbreviation	*Units*	*Test*
PDW	%	Platelet distribution width
PLT	E3/uL	Platelet count
POIK		Poikilocytosis
POLY		Polychromasia
RBC	E6/uL	Red blood cell count
RDW	%	Red blood cell distribution width
RDWr	%	Reticulocyte distribution width
RETIC	E3/uL	Reticulocyte count
RETIC%	%	Percent reticulocyte
TOXIC		Toxic granulation
WBC	E3/uL	White blood cell count

Abbreviations and Units for Manual Differential

Abbreviation	*Units*	*Test*
BAND	E3/uL	Absolute band neutrophils
BAND%	%	Percent band neutrophils
BANDC		Band neutrophil count
BASO	E3/uL	Absolute basophils
BASO%	%	Percent basophils
BASOC		Basophil count
BLAST	E3/uL	Absolute blast cells
BLAST%	%	Percent blasts
BLASTC		Blast cell count
EOS	E3/uL	Absolute eosinophils
EOS%	%	Percent eosinophils
EOSC		Eosinophil count
LYM	E3/uL	Absolute lymphocytes
LYM%	%	Percent lymphocytes
LYMC		Lymphocyte count
META	E3/uL	Absolute metamyelocytes
META%	%	Percent metamyelocytes
METAC		Metamyelocyte count
MONO	E3/uL	Absolute monocytes
MONO%	%	Percent monocytes
MONOC		Monocyte count
MYE,PRO	E3/uL	Absolute promyelocytes/myelocytes
MYE,PRO%	%	Percent promyelocytes/myelocytes
MYE,PROC		Promyelocytes/myelocytes count
NEUC		Neutrophil count
NEUT	E3/uL	Absolute neutrophils
NEUT%	%	Percent neutrophils
NRBC		Nucleated red blood cell count

Abbreviations and Units for Coagulation

Abbreviation	*Units*	*Test*
APTT	sec	Activated partial thromboplastin time
DDIM	ng/mL	D-dimer
FDP	µg/mL	Fibrin/fibrinogen degradation products
FIB	mg/dL	Fibrinogen
PAGA	%	Platelet aggregation - adenosine diphosphate
PAGC	%	Platelet aggregation - collagen
PAGR	%	Platelet aggregation - ristocetin
PT	sec	Prothrombin time
TT	sec	Thrombin time

Abbreviations and Units for Chemistry

Abbreviation	*Units*	*Test*
A ALB	g/dL	Absolute albumin
A Alpha1	g/dL	Absolute alpha-1 globulin
A Alpha2	g/dL	Absolute alpha-2 globulin
A BETA	g/dL	Absolute beta globulin
Adipo	ug/mL	Adiponectin
A GAMMA	g/dL	Absolute gamma globulin
A:G		Albumin:globulin ratio
ACTH	pg/mL	Adrenocorticotropic hormone
ALB	g/dL	Albumin
ALB%	%	Percent albumin
ALD	U/L	Aldolase
ALP	U/L	Alkaline phosphatase
Alpha1%	%	Percent alpha-1 globulin
Alpha2%	%	Percent alpha-2 globulin
ALT	U/L	Alanine aminotransferase
AMY	U/L	Amylase
AST	U/L	Aspartate aminotransferase
Bb	ug/mL	Complement factor Bb
BETA%	%	Percent beta globulin
C3	mg/dL	Complement 3
C4	mg/dL	Complement 4
Ca	mg/dL	Calcium
CFCK	U/L	Cerebrospinal fluid chloride creatine kinase
CFCl	mmol/L	Cerebrospinal fluid chloride
CFGL	mg/dL	Cerebrospinal fluid chloride glucose
CFK	mmol/L	Cerebrospinal fluid chloride potassium
CFNa	mmol/L	Cerebrospinal fluid chloride sodium
CFTP	mg/dL	Cerebrospinal fluid chloride total protein
CH50	units/mL	50% Complement-related hemolysis of erythrocytes
cHCO3	mmol/L	Derived bicarbonate
CHOL	mg/dL	Total cholesterol
CK	U/L	Creatine kinase
CKBB	U/L	Absolute creatine kinase BB
CKBB%	%	Percent creatine kinase BB
CKMB	U/L	Absolute creatine kinase MB
CKMB%	%	Percent creatine kinase MB
CKMM	U/L	Absolute creatine kinase MM
CKMM%	%	Percent creatine kinase MM
Cl	mmol/L	Chloride
HCO3	mmol/L	Bicarbonate
CORT	ug/dL	Cortisol

Abbreviations and Units for Chemistry (Continued)

Abbreviation	*Units*	*Test*
CORST	ng/mL	Corticosterone
CREA	mg/dL	Creatinine
CRP	mg/dL	C-reactive protein
CTLI	ng/mL	Canine trypsin-like immunoreactivity
CTX1	ng/mL	Carboxy (C)-terminal telopeptide fragment of type I collagen
DBIL	mg/dL	Direct bilirubin
DDIMM	ug/mL	D-dimer modular
ESTR	pg/mL	Estradiol
Fe	ug/dL	Iron
FERR	ng/mL	Ferritin
FeS%	%	Percent iron saturation
FFA	umol/L	Free fatty acids
FT3	pg/mL	Free T3
FT4	ng/mL	Free T4
GAMMA%	%	Percent gamma globulin
GGT	U/L	Gamma glutamyl transferase
GLDH	U/L	Glutamate Dehydrogenase
GLOB	g/dL	Globulin
GLU	mg/dL	Glucose
GGON	pg/mL	Glucagon
HAPT	mg/dL	Haptoglobin
HBA1C	%	Hemoglobin A1C
HDL	mg/dL	High density lipoprotein cholesterol
IBIL	mg/dL	Indirect bilirubin
ICA	mmol/L	Ionized calcium
IFNg	pg/mL	Interferon-gamma
IGA	mg/dL	Immunoglobulin A
IGF-1	ng/mL	Insulin-like growth factor-1
IGG	mg/dL	Immunoglobulin G
IGM	mg/dL	Immunoglobulin M
iHct	%	Ionized calcium hematocrit
IL-2	pg/mL	Interleukin-2
IL-4	pg/mL	Interleukin-4
IL-6	pg/mL	Interleukin-6
IL-10	pg/mL	Interleukin-10
IL-12p70	pg/mL	Interleukin-12p70
IL-13	pg/mL	Interleukin-13
IL-17	pg/mL	Interleukin-17
K	mmol/L	Potassium
LACT	mg/dL	Lactate

Abbreviations and Units for Chemistry (Continued)

Abbreviation	*Units*	*Test*
LDH	U/L	Lactate dehydrogenase
LDH1	U/L	Absolute Lactate Dehydrogenase 1
LDH1%	%	Percent Lactate Dehydrogenase 1
LDH2	U/L	Absolute Lactate Dehydrogenase 2
LDH2%	%	Percent Lactate Dehydrogenase 2
LDH3	U/L	Absolute Lactate Dehydrogenase 3
LDH3%	%	Percent Lactate Dehydrogenase 3
LDH4	U/L	Absolute Lactate Dehydrogenase 4
LDH4%	%	Percent Lactate Dehydrogenase 4
LDH5	U/L	Absolute Lactate Dehydrogenase 5
LDH5%	%	Percent Lactate Dehydrogenase 5
LDL	mg/dL	Low density lipoprotein cholesterol
LIP	U/L	Lipase
METHGB	%	Methemoglobin
Mg	mg/dL	Magnesium
MYO	ng/mL	Myoglobin
Na	mmol/L	Sodium
OSTEO2	ng/mL	Osteocalcin
P1NP	ng/mL	N-terminal propeptide of type 1 collagen
PAMY	U/L	P-amylase
P-CHE	umol/L	Cholinesterase - plasma
pCO2	mmHg	Partial pressure carbon dioxide
pH	pH	Ionized calcium pH
PHOS	mg/dL	Inorganic phosphorus
PINP	ng/mL	N-terminal propeptide of type 1 collagen
PLIP	mg/dL	Phospholipids
PO2	mmHg	Partial pressure oxygen
PROG	ng/mL	Progesterone
PROL	ng/mL	Prolactin
PTH	pg/mL	Parathyroid hormone
R-CHE	umol/L	Cholinesterase - red blood cells
SBA	umol/L	Total bile acids
S-CHE	umol/L	Cholinesterase - serum
SDH	U/L	Sorbitol dehydrogenase
SHGB	mAbs	Supernatant hemoglobin
SKET		Serum ketone
sO2	%	Oxygen saturation of hemoglobin
SOSMO	mosm/kg	Serum osmolality
T3	ng/dL	Triiodothyronine
T4	ug/dL	Thyroxine
T4K9	ug/dL	Canine thyroxine

Abbreviations and Units for Chemistry (Continued)

Abbreviation	*Units*	*Test*
TBIL	mg/dL	Total bilirubin
TEST	ng/dL	Testosterone
TIBC	ug/dL	Total iron binding capacity
TNF-a	pg/mL	Tumor necrosis factor-alpha
TP	g/dL	Total protein
TPI	ng/mL	Troponin I
TRAP5b	U/L	Tartrate-resistant acid phosphatase 5b
TRIG	mg/dL	Triglyceride
TSH	UU/mL	Thyroid stimulating hormone
TSHK9	UU/mL	Canine thyroid stimulating hormone
UA	mg/dL	Uric acid
UIBC	ug/dL	Unsaturated iron binding capacity
UN	mg/dL	Urea nitrogen
UN:CR		Serum UN:creatinine ratio
Vit. D	ng/mL	Vitamin D
VLDL	mmol/L	Calculated very low density lipoprotein cholesterol

Abbreviations and Units for Urine Analysis Functions

Abbreviation	*Units*	*Test*
ALBEX	mg	Urine albumin excretion
B2M:UCRE		Urine beta-2-microglobulin:urine creatinine ratio
B2MICGOS	ug/mL	Urine beta-2-microglobulin
B2MIOS	ng/mL	Urine beta-2-microglobulin
CaEX	mg	Urine calcium excretion
CaFCL	%	Calcium fractional clearance
CASTT		Cast type
ClEX	mmol	Urine chloride excretion
ClFCL	%	Chloride fractional clearance
CRCLEAR	mL/minute	Creatinine clearance
CREAX	mg	Urine creatinine excretion
FOBL		Fecal occult blood
FOBLN		Fecal occult blood negative control
FOBLP		Fecal occult blood positive control
GLUEX	mg	Urine glucose excretion
ICTO		Ictotest
KEX	mmol	Urine potassium excretion
KFCL	%	Potassium fractional clearance
KIM1	ng/mL	Urine kidney injury molecule 1
KIM:UCRE		Urine kidney injury molecule1:urine creatinine ratio
KIM:UCREA		Urine kidney injury molecule1:urine creatinine ratio
MALB	ng/mL	Urine microalbumin
MALBUCRE		Urine microalbumin:urine creatinine ratio
MALBUCREA		Urine microalbumin:urine creatinine ratio
MgEX	mg	Magnesium excretion
NaEX	mmol	Urine sodium excretion
NaFCL	%	Sodium fractional clearance
NGAL	ng/mL	Urine neutrophil gelatinase-associated lipocalin
NGAL:UC		Urine neutrophil gelatinase-associated lipocalin:urine creatinine ratio
Osteopon	ng/mL	Urine osteopontin
OST:UCREA		Urine osteopontin:urine creatinine ratio
pHMET		Urine pH by pH meter
PHOSEX	mg	Urine phosphorus excretion
PHOSFCL	%	Phosphorus fractional clearance
REDS		Reducing substances
SPGR		Urine specific gravity
TPEX	mg	Urine total protein excretion
UALB	mg/dL	Urine albumin
UALT	U/L	Urine alanine aminotransferase
UAMY	U/L	Urine amylase

Abbreviations and Units for Urine Analysis Functions (Continued)

Abbreviation	*Units*	*Test*
UAST	U/L	Urine aspartate aminotransferase
UB2:UCR		Urine beta-2-microglobulin:urine creatinine ratio
UBACT		Urine bacteria
UBIL		Urine bilirubin
Uca	mg/dL	Urine calcium
Uca:UCR		Urine calcium:urine creatinine ratio
UCAST		Casts
Ucl	mmol/L	Urine chloride
UCL:UCR		Urine chloride:urine creatinine ratio
UCLA		Urine clarity
UCLUST	ng/mL	Urine clusterin
UCLS:UCR		Urine clusterin:urine creatinine ratio
UCOL		Urine color
UCREA	mg/dL	Urine creatinine
UCRYS		Abnormal crystals
UCRYST		Abnormal crystal type
UCYC	ng/mL	Urine cystatin C
UCYC:UCR		Urine cystatin C:urine creatinine ratio
UEPI		Epithelial cells
UGGT	U/L	Urine gamma glutamyl transferase
UGGT:UCR		Urine gamma glutamyl transferase:urine creatinine ratio
UGL:UCR		Urine glucose:urine creatinine ratio
UGLU		Urine qualitative glucose
UGLUC	mg/dL	Urine qualitative glucose
UK	mmol/L	Urine potassium
UKET		Urine ketone
ULDH	U/L	Urine lactate dehydrogenase
ULEU		Urine leukocyte
UMA:UCREA		Urine microalbumin:urine creatinine ratio
UMALBOS	ug/mL	Urine microalbumin
UMg	mg/dL	Urine magnesium
UMg:UCR		Urine magnesium:urine creatinine ratio
UNa	mmol/L	Urine sodium
UNa:UCR		Urine sodium:urine creatinine ratio
UNa:UK		Urine sodium:potassium ratio
UNAG	U/L	Urine N-acetyl-B-D-glucosaminidase
UNAG:UCR		Urine N-acetyl-b-d-glucosaminidase:urine creatinine ratio
UNEX	mg	Urine urea nitrogen excretion
UNIT		Urine nitrite

Abbreviations and Units for Urine Analysis Functions (Continued)

Abbreviation	*Units*	*Test*
UOBL		Urine blood/occult
UOSMO	mosm/kg	Urine osmolality
UOTH		Urine other
UPAMY	U/L	Urine P-amylase
UpH		Urine pH
UPHO:UCR		Urine phosphorus:urine creatinine ratio
UPHOS	mg/dL	Urine phosphorus
UPRO		Urine qualitative protein
URBC		Urine red blood cell
UTP	mg/dL	Urine qualitative total protein
UTP:UCR		Urine protein:urine creatinine ratio
UUA	mg/dL	Urine uric acid
UUBG	mg/dL	Urine urobilinogen
UUN	mg/dL	Urine urea nitrogen
UUN:UCR		Urine urea nitrogen:urine creatinine ratio
UVOL	mL	Urine volume
UWBC		Urine white blood cell

Abbreviations and Units for Urine Analysis Functions (Continued)

MICROSCOPIC EXAMINATION OF URINE

Gradings

Casts, red and white blood cells, and epithelial cells	Crystals, bacteria
0 None seen	0 Not present
1 1 to 5	1 Occasional, not seen in every field
2 6 to 10	2 Few in all fields
3 11 to 20	3 Moderate in all fields
4 >20	4 Many in all fields, may obscure other elements

URINE ANALYSIS

Clinitek® 200+ Analyzer, Multistix® Strip, Clinitek Atlas

Urine Glucose		Urine Ketones		Urine Blood	
NEGATIVE	Negative	NEGATIVE	Negative	NEGATIVE	Negative
TRACE	100 mg/dL	TRACE	5 mg/dL	TRACE	Trace
1+	250 mg/dL	1+	15 mg/dL	1+	Small
2+	500 mg/dL	2+	40 mg/dL	2+	Moderate
3+	≥1000 mg/dL	3+	≥80 mg/dL	3+	Large

Urine Nitrite		Urine Protein		Urine Bilirubin	
NEGATIVE	Negative	NEGATIVE	Negative	NEGATIVE	Negative
POSITIVE	Positive	TRACE	Trace	1+	Small
		1+	30 mg/dL	2+	Moderate
		2+	100 mg/dL	3+	Large
		3+	≥300 mg/dL		

Urine Cast/Crystals		Leukocyte Esterase		Urine Color	
NT	No Type	NEGATIVE	Negative	DKYELLOW	Dark Yellow
		TRACE	Trace	CO	Pale/Colorless
		1+	Small		
		2+	Moderate		
		3+	Large		

Ictotest® Urine Bilirubin		Clinitest® Urine Reducing Substances	
-	Negative	NEGATIVE	Negative
+	Positive	TRACE	1/4 %
		1+	1/2 %
		2+	3/4 %
		3+	1 %
		4+	2 %

7.3 Comments on the Data

The following comments on the data are used by Covance. Some, but not necessarily all, of this information may be needed for this report.

The number of animals listed in the heading of the summary tables reflects the number of animals assigned to each group at the start of each respective phase, with the exception of the anatomic pathology tables, which indicate the number of animals assigned to each respective necropsy interval. The summary table for observations indicates the number of animals for which a condition was observed without regard to the specific nature, severity, reversibility, number of incidences/animal, or the length of time the condition persisted.

For the purpose of this report, the abbreviation for the manufacturer-supplied Hepatitis B Vaccine reagent was abbreviated HBvx. The abbreviation for the active Hepatitis-specific component (the viral protein) was HBsAg (Hepatitis B Surface Antigen). The Vaccine (HBvx) reagent was used to deliver the surface antigen (HBsAg), and the immune response (antibody titer) measured in this study was generated to the surface antigen.

7.4 Study Deviations

7.4.1 Protocol Deviations

Procedure	Protocol Deviations
Test System and Study Design	
Environmental Conditions, Diet, and Water	On Day 4 of the predose phase, it cannot be verified that animals were commingled. On Day 35 of the dosing phase, the animals were only provided feed once instead of twice as require by Protocol.
Inlife Procedures	
Dose Administration	On Day 1 of the dosing phase, it can not be determined that the dosing formulations were well shaken to resuspend the sediment of fine white particles of adjuvant (aluminum hydroxide) which settles during storage to obtain a qualitatively slightly opaque, white suspension due to lack of documentation.
Clinical Observations	On Day 8 of the predose phase, an indication of normal was recorded for cageside observations, although only abnormal findings were to be recorded. On Day 1 of the dosing phase, cageside observations were performed although not required by the Protocol. On Day 35 of the dosing phase, the p.m. general daily observations were not performed.
Food Consumption	On Day 35 of the dosing phase, qualitative assessment of food consumption was not performed.

These study deviations neither affected the overall interpretation of study findings nor compromised the integrity of the study.

8. FIGURES

Figure 8.1: Anti-HBsAg IgM Response

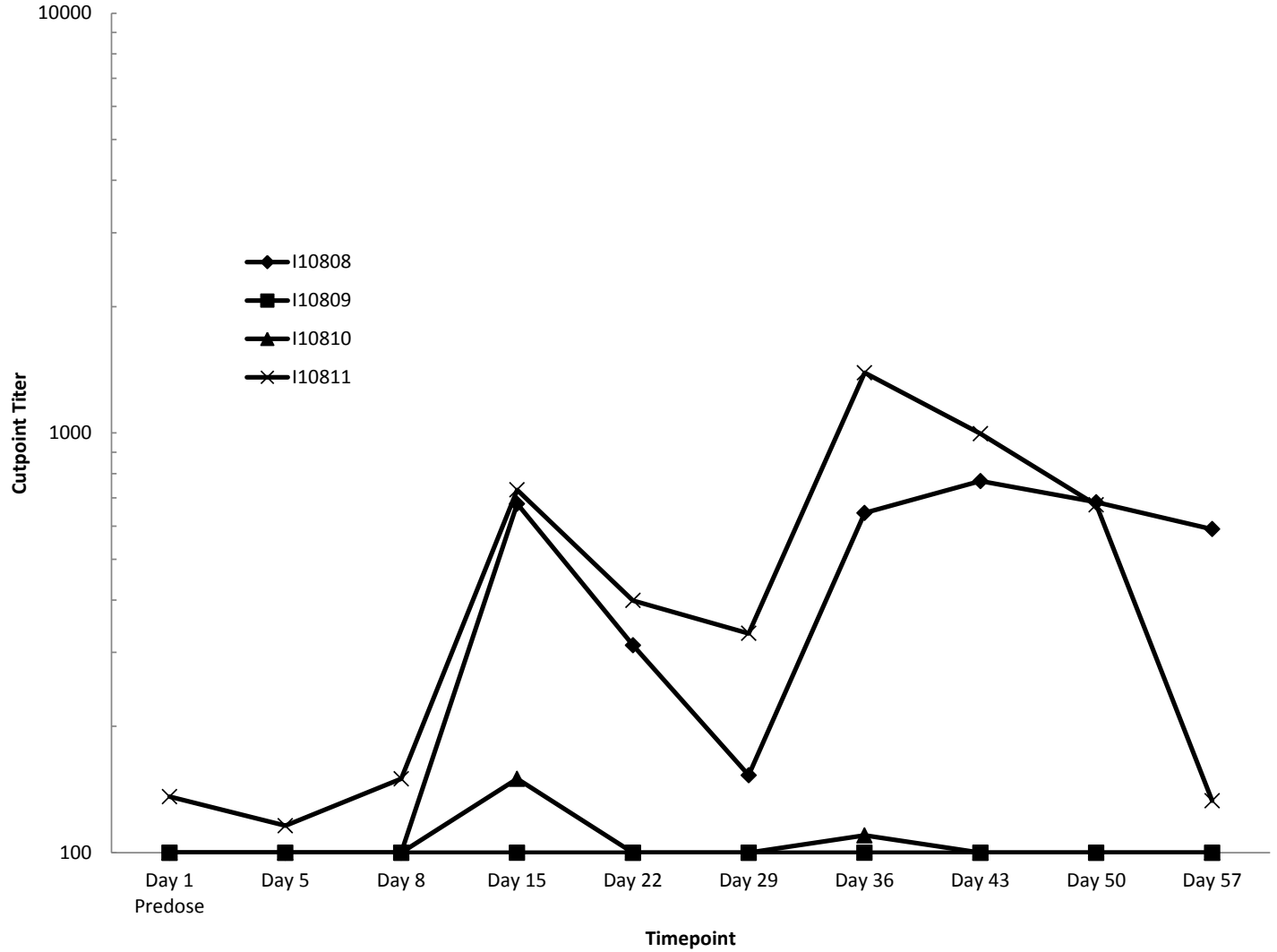


Figure 8.2: Anti-HBsAg IgG Response

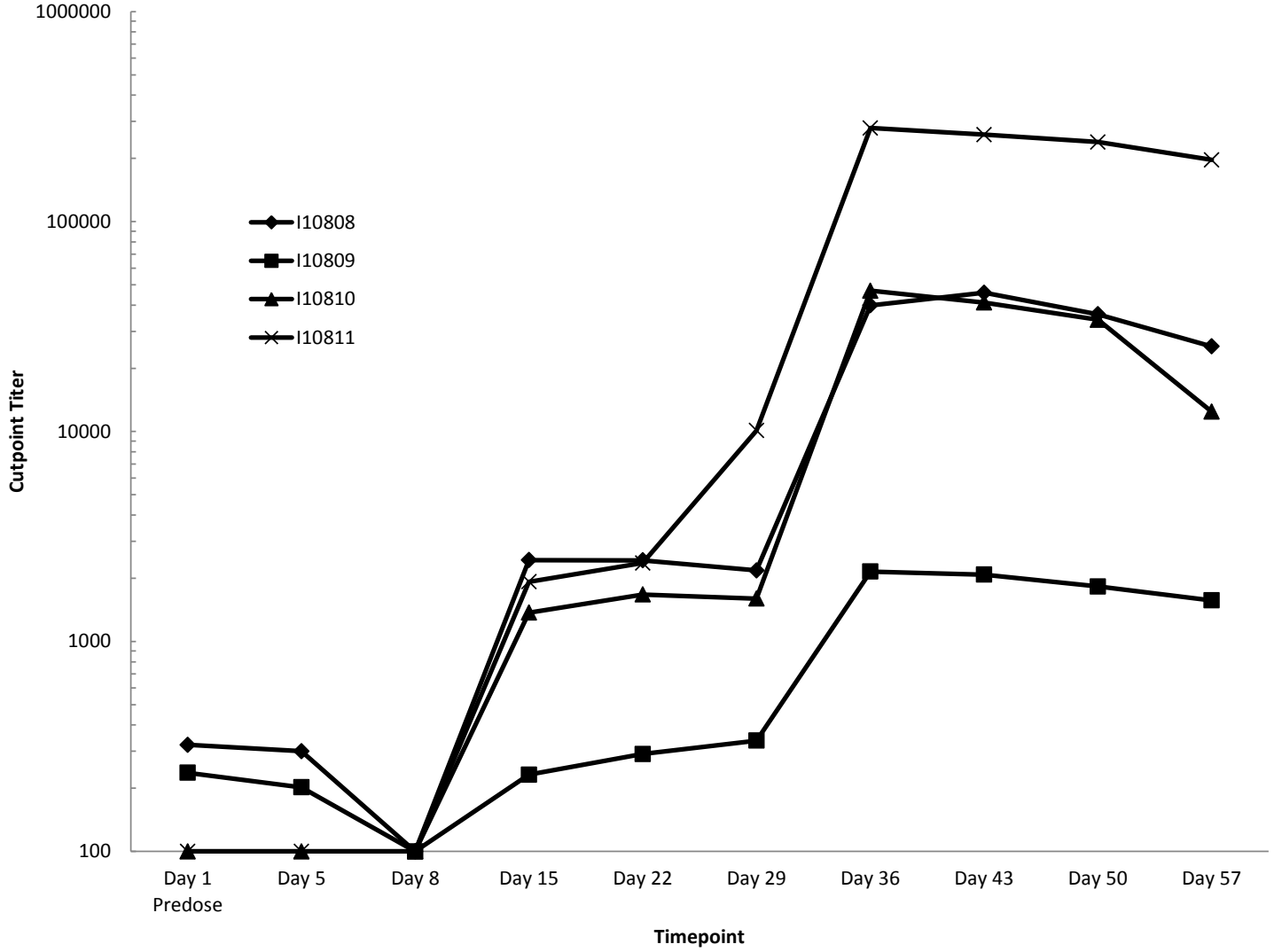


Figure 8.3: IgM Cut Point Titer - Benchtop Stability

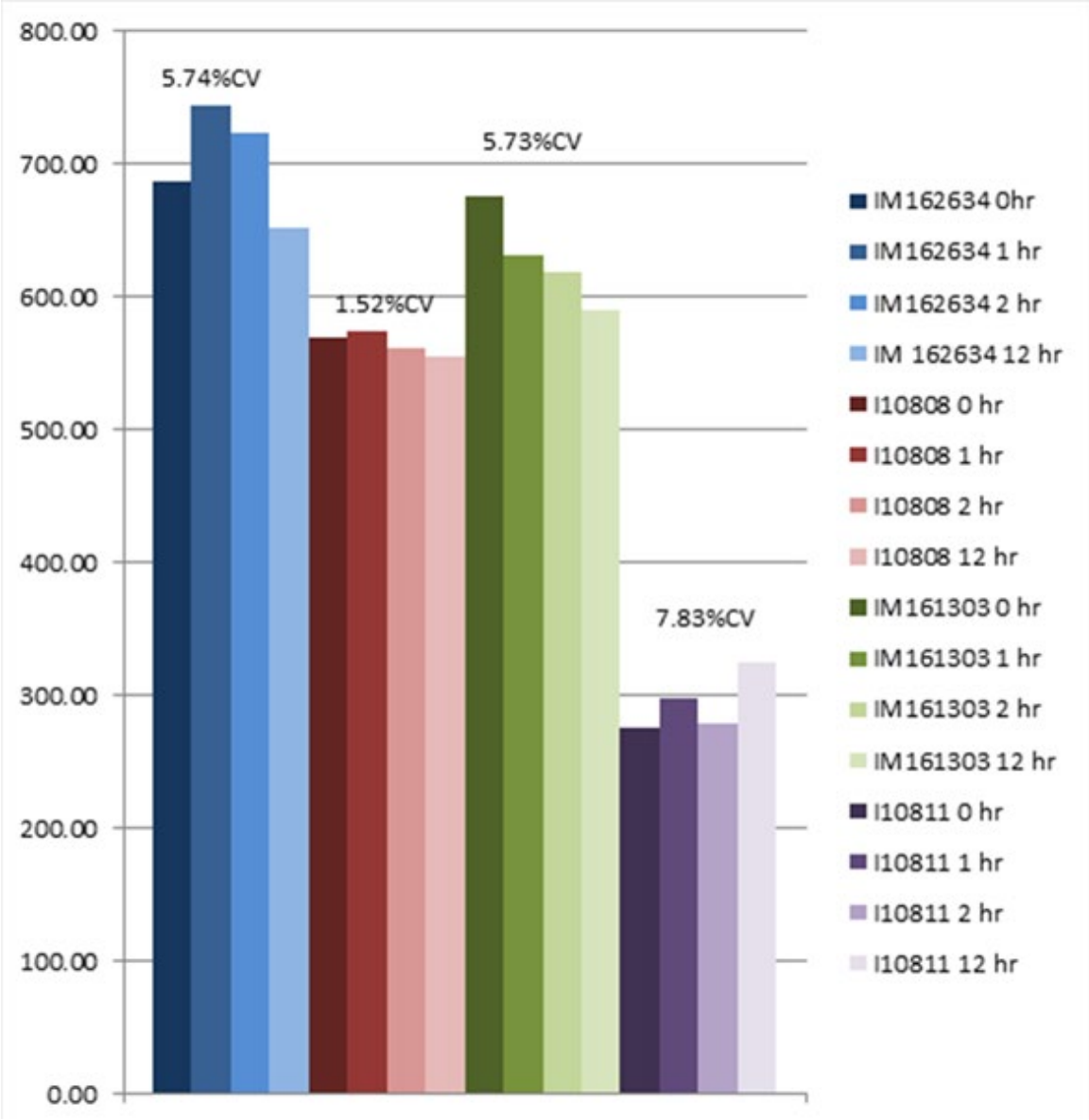


Figure 8.4: IgG Cut Point Titer - Benchtop Stability

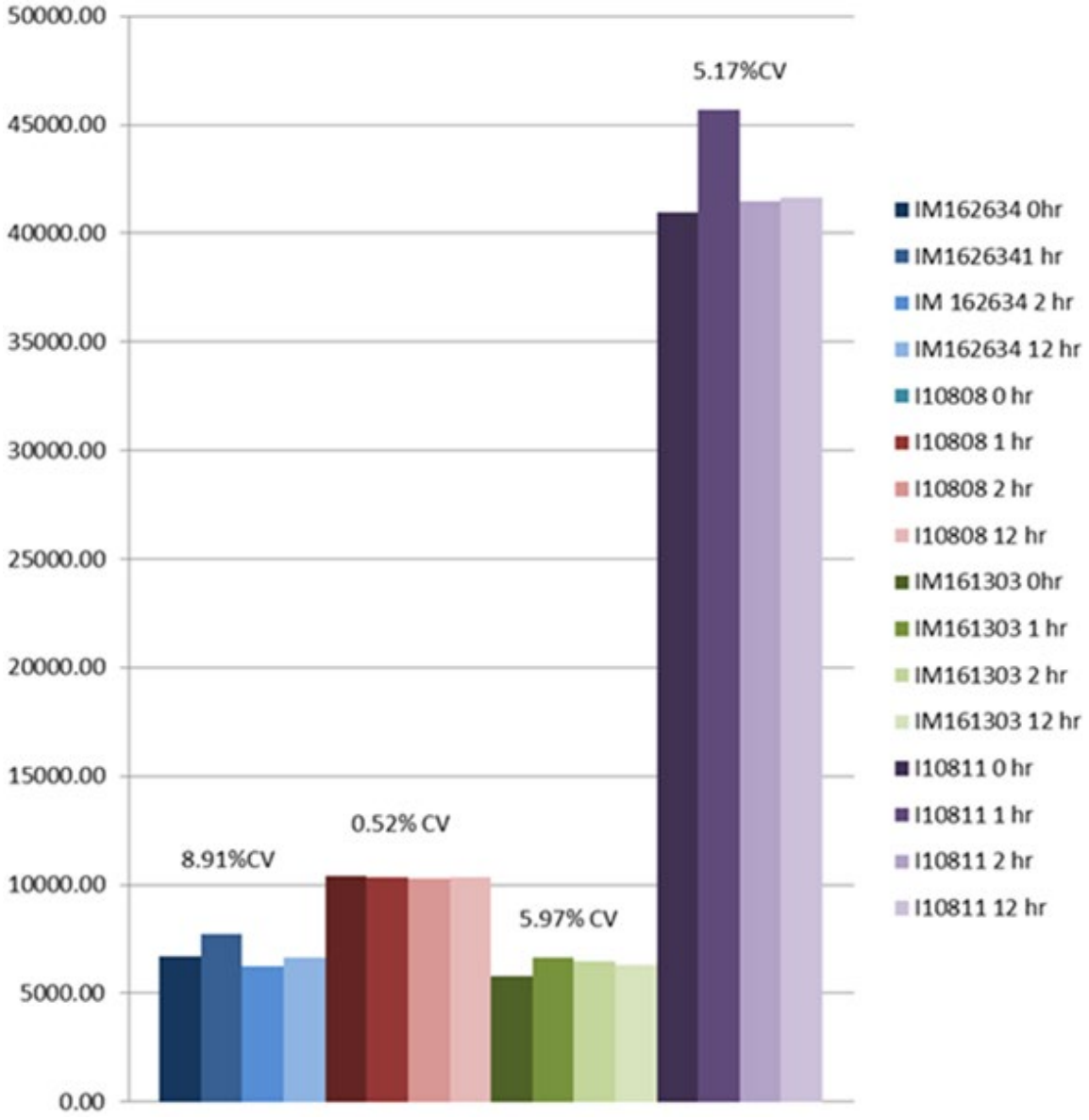


Figure 8.5: Method and Kit Comparison (Serum Samples): IgM Concentration

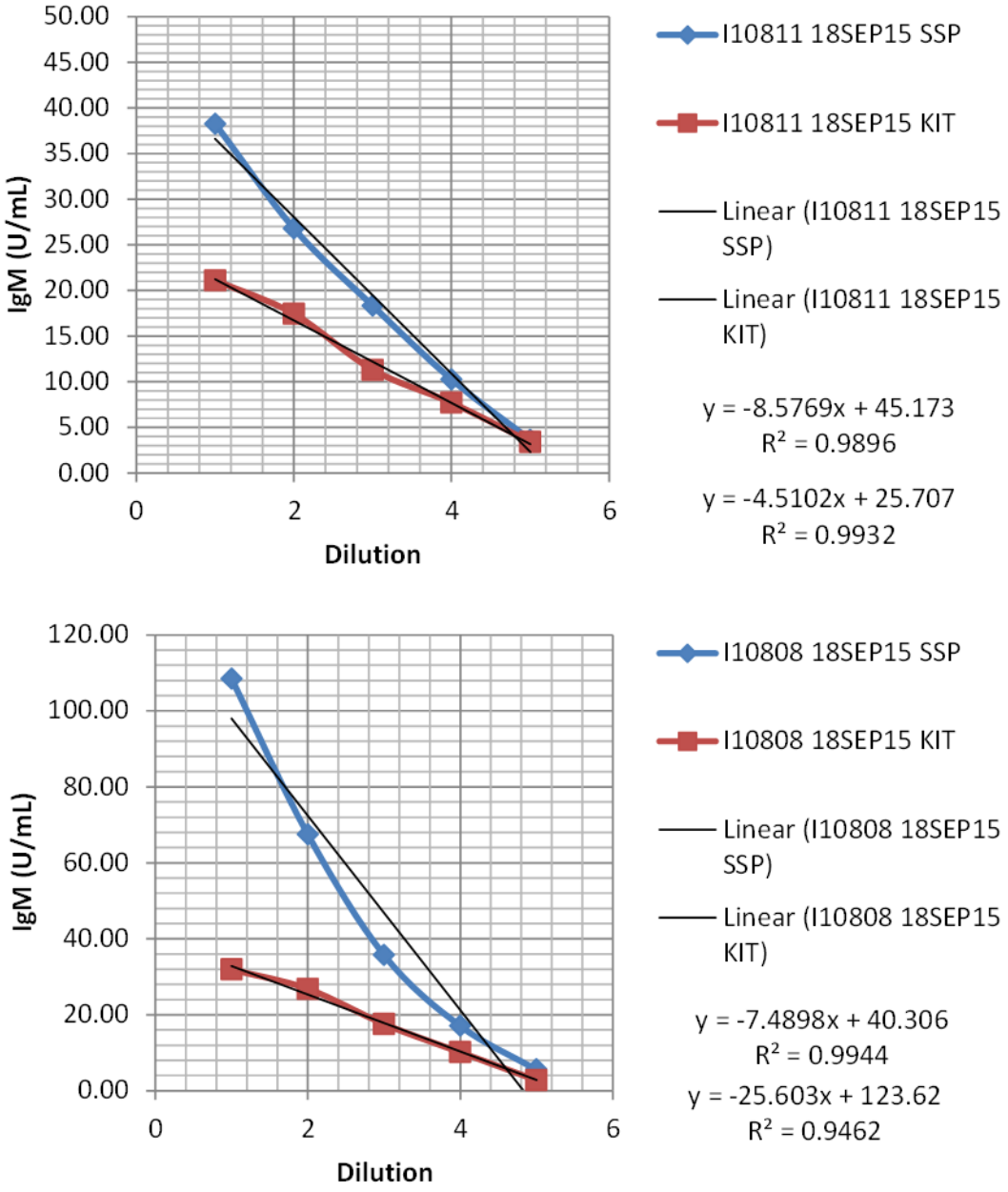
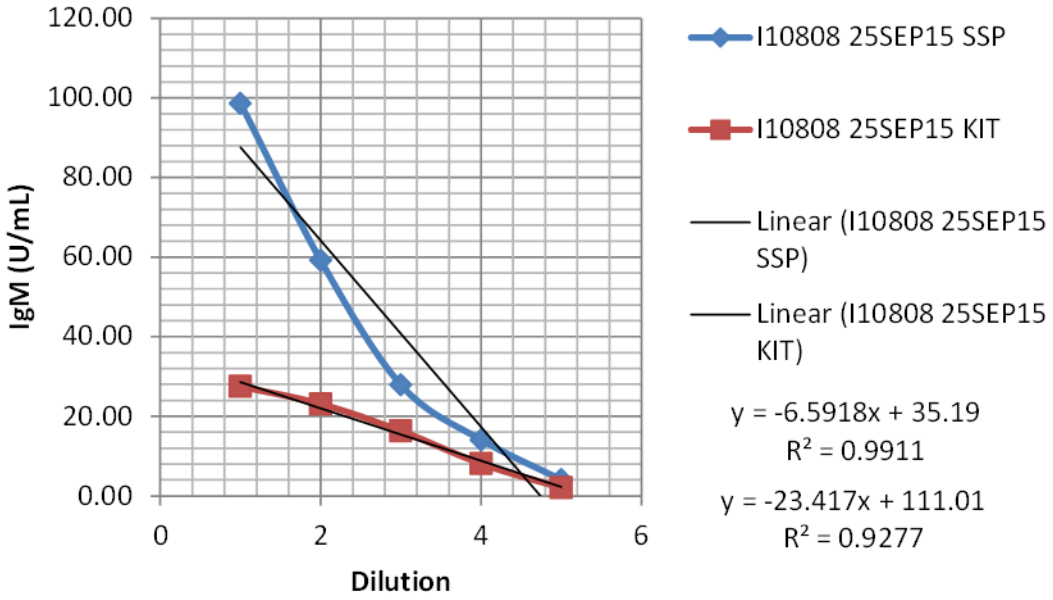


Figure 8.5 (Continued): Method and Kit Comparison (Serum Samples): IgM Concentration



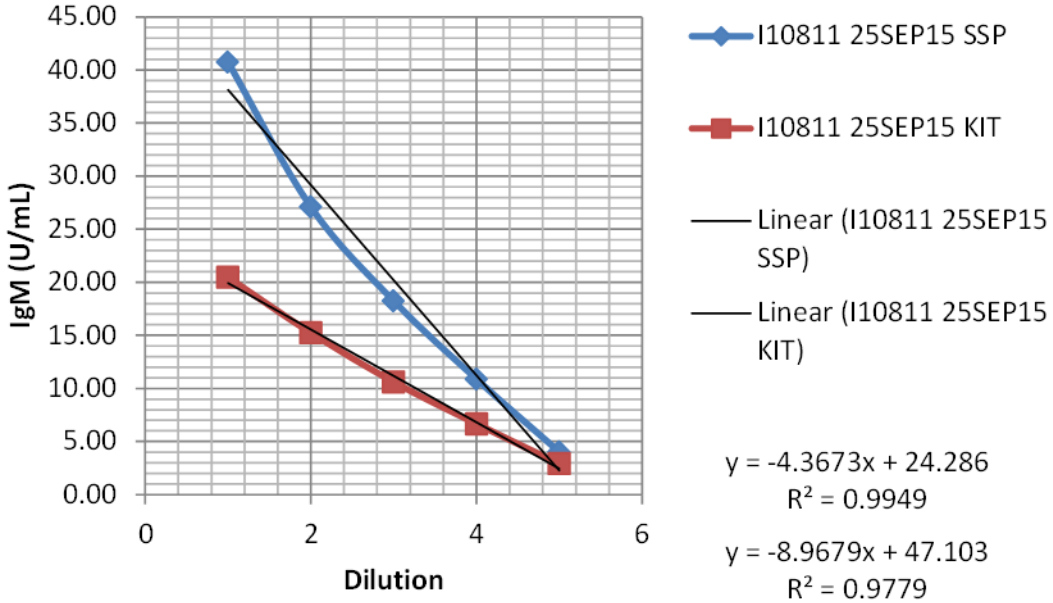


Figure 8.6: Method and Kit Comparison (Serum Samples): IgG Concentration

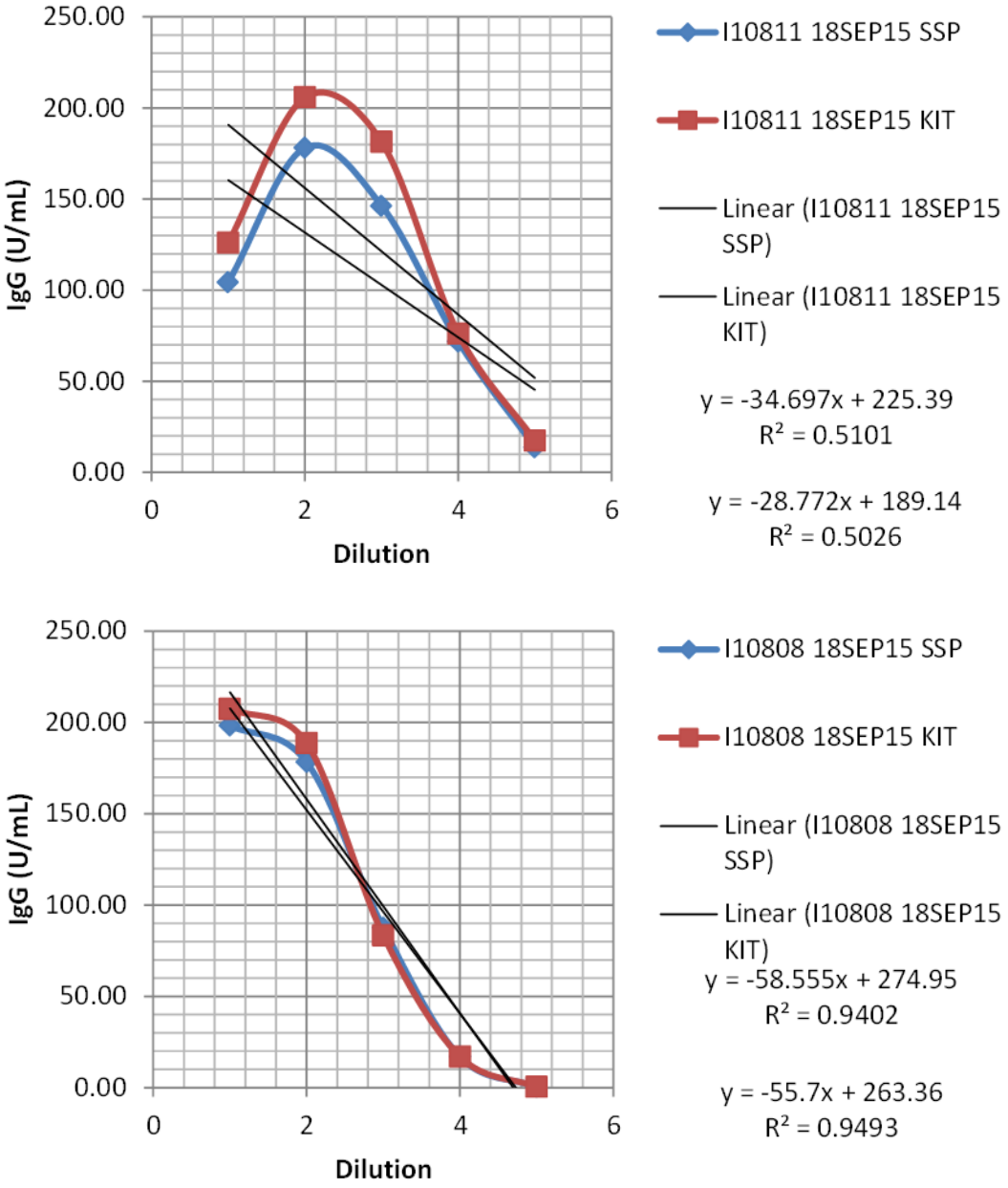
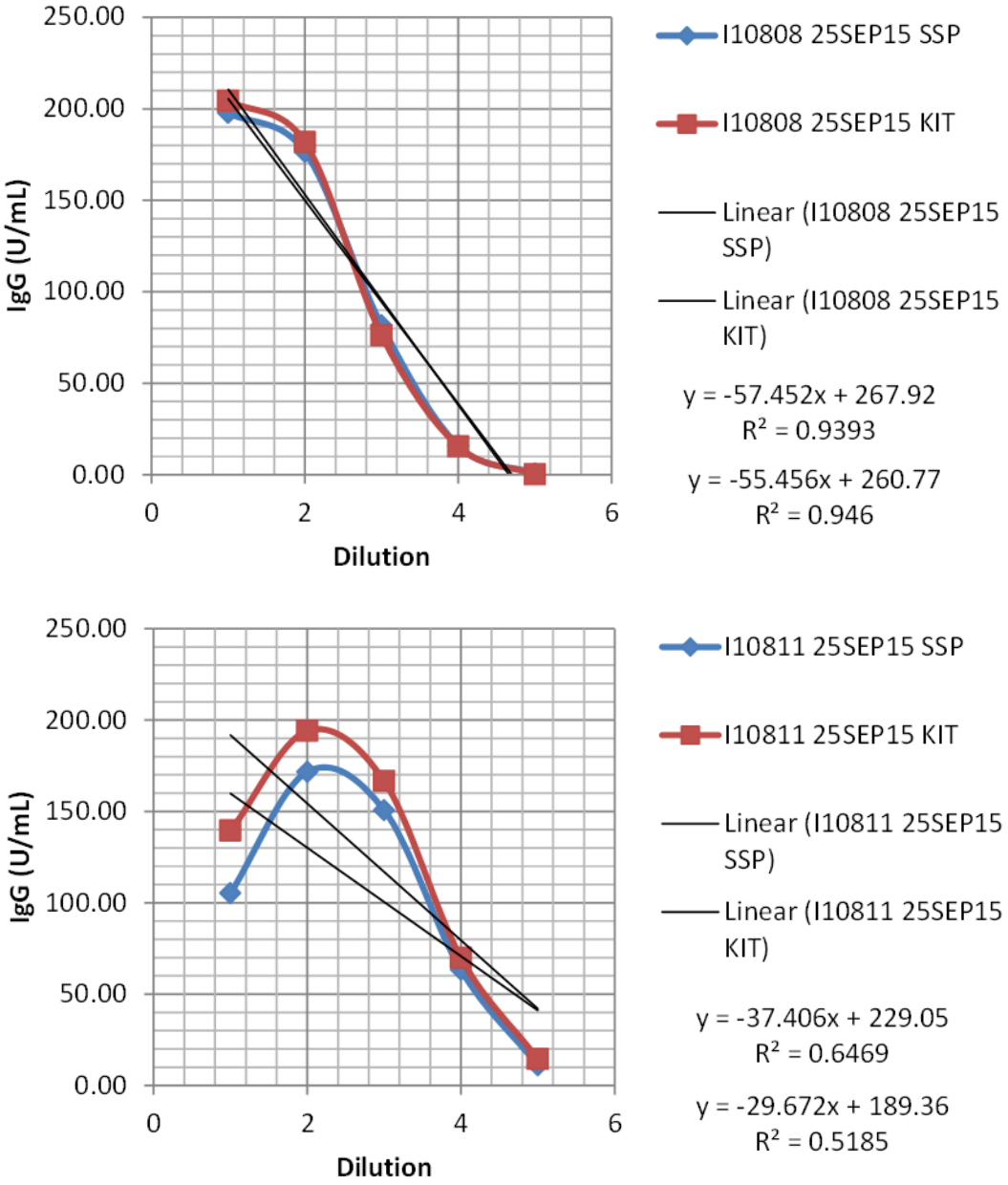


Figure 8.6 (Continued): Method and Kit Comparison (Serum Samples): IgG Concentration



9. TABLES

Table 9.1: Individual Animal Fate

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Group/ Sex	Animal Number	Date	Phase of Fate	Phase Week	Phase Day	Fate Status	Terminal Body Weight (kg)
1/F	I10808	25/SEP/15	Dosing	9	57	Marked Complete	-
	I10809	25/SEP/15	Dosing	9	57	Marked Complete	-
	I10810	25/SEP/15	Dosing	9	57	Marked Complete	-
	I10811	25/SEP/15	Dosing	9	57	Marked Complete	-

Table 9.2: Summary of Clinical Observations

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Phase: Dosing

Category / Observation	Group/Sex: 1/F Number in Group: 4
NORMAL	
No remarkable observations	4
Appearance	
prolapsed rectum	1
Discharge	
appears to be menstruating	1
red, right front foot	1
Excretion	
feces, nonformed, group observation	3
feces, nonformed, individual observation	1
Psychological assessment	
fecal painting	4
Skin and pelage	
broken skin, right front foot	1
discolored skin, dose site, brown	1
scab, dorsal thorax	1
scab, tail distal	1
scab, tail mid	1

Table 9.3: Individual Clinical Observations

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted] (HBsAg)		1.0 mL	20 µg/mL

Group/ Sex	Cage Number	Animal Number	Observation	Phase	Day(s)
1/F		I10808	NORMAL		
	1		No remarkable observations	PRED	1,8
				DSNG	1,15,29,43,57
			Excretion		
			feces, nonformed, group observation	DSNG	22,29,34,36,50
			Psychological assessment		
			fecal painting	DSNG	10,13
			Skin and pelage		
			scab, tail distal	DSNG	8
1/F		I10809	NORMAL		
	1		No remarkable observations	PRED	8
				DSNG	1,8,15,29,43,57
			Discharge		
			appears to be menstruating	PRED	1
			red, right front foot	DSNG	24
			Excretion		
			feces, nonformed, group observation	DSNG	22,29,34,36,50
			Psychological assessment		
			fecal painting	DSNG	10,13
			Skin and pelage		
			broken skin, right front foot	DSNG	24
			discolored skin, dose site, brown	DSNG	1

Table
Individual Clinical Observations

Test Article	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted] (HBsAg)	1.0 mL	20 µg/mL

Group/ Sex	Cage Number	Animal Number	Observation	Phase	Day(s)
1/F		I10810	NORMAL		
	2		No remarkable observations	PRED	1,8
	1		No remarkable observations	DSNG	1,8,15,29,43,57
			Excretion		
			feces, nonformed, group observation	DSNG	22,29,34,36,50
			Psychological assessment		
			fecal painting	DSNG	10,13
1/F		I10811	NORMAL		
	2		No remarkable observations	PRED	1
				DSNG	1,29,36,50
			Appearance		
			prolapsed rectum	DSNG	15,22
			Discharge		
			appears to be menstruating	PRED	5,8
				DSNG	43
			Excretion		
			feces, nonformed, individual observation	DSNG	57
			Psychological assessment		
			fecal painting	DSNG	10,13,15
			Skin and pelage		
			scab, dorsal thorax	DSNG	8
			scab, tail mid	DSNG	8

Table 9.4: Summary and Individual Body Weight

Test Article	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted] (HBsAg)	1.0 mL	20 µg/mL

Data Presented in "kg"

Group/ Sex	Animal Number	Phase Day	PRED 1	PRED 8	DSNG 1	DSNG 8	DSNG 15	DSNG 22
1/F	I10808		2.7	2.7	2.7	2.7	2.8	2.8
	I10809		3.0	3.0	3.0	2.9	2.9	3.0
	I10810		2.7	2.6	2.6	2.5	2.7	2.6
	I10811		2.7	2.7	2.8	2.7	2.7	2.7
	Mean		2.8	2.8	2.8	2.7	2.8	2.8
	SD		0.15	0.17	0.17	0.16	0.10	0.17
	N		4	4	4	4	4	4

Table
Summary and Individual Body Weight

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Data Presented in "kg"

Group/ Sex	Animal Number	Phase Day	DSNG 29	DSNG 36	DSNG 43	DSNG 50	DSNG 57
1/F	I10808		2.8	2.8	3.0	2.8	2.8
	I10809		3.0	3.0	3.0	3.0	3.0
	I10810		2.7	2.7	2.7	2.5	2.6
	I10811		2.8	2.9	2.9	2.9	2.9
	Mean		2.8	2.9	2.9	2.8	2.8
	SD		0.13	0.13	0.14	0.22	0.17
	N		4	4	4	4	4

Table 9.5: Summary and Individual Hepatitis B Vaccine (HBsAg) IgM and IgG

Test Article	Anti-HBsAg IgM	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Animal #	Group/Sex	Interpolated Cutpoint Titer Day 1 Predose	Interpolated Cutpoint Titer Day 5	Interpolated Cutpoint Titer Day 8	Interpolated Cutpoint Titer Day 15	Interpolated Cutpoint Titer Day 22	Interpolated Cutpoint Titer Day 29	Interpolated Cutpoint Titer Day 36
I10808	1F	<100	<100	<100	678	312	153	645
I10809	1F	<100	<100	<100	<100	<100	<100	<100
I10810	1F	<100	<100	<100	150	<100	<100	110
I10811	1F	136	116	150	732	399	333	1391
	Mean	34	29	38	390	178	122	537
	SD	68.0	58.0	75.0	369.5	208.3	158.4	635.5
	N	4	4	4	4	4	4	4

Table
Summary and Individual Hepatitis B Vaccine (HBsAg) IgM and IgG

Test Article	Anti-HBsAg IgM	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Animal #	Group/Sex	Interpolated Cutpoint Titer Day 43	Interpolated Cutpoint Titer Day 50	Interpolated Cutpoint Titer Day 57
I10808	1F	768	684	590
I10809	1F	<100	<100	<100
I10810	1F	<100	<100	<100
I10811	1F	996	674	133
	Mean	441	340	181
	SD	517.7	392.0	279.9
	N	4	4	4

Table
Summary and Individual Hepatitis B Vaccine (HBsAg) IgM and IgG
Anti-HBsAg IgG

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Animal #	Group/Sex	Interpolated Cutpoint Titer Day 1 Predose	Interpolated Cutpoint Titer Day 5	Interpolated Cutpoint Titer Day 8	Interpolated Cutpoint Titer Day 15	Interpolated Cutpoint Titer Day 22	Interpolated Cutpoint Titer Day 29	Interpolated Cutpoint Titer Day 36
I10808	1F	322	300	<100	2441	2432	2180	39874
I10809	1F	237	202	<100	232	291	337	2152
I10810	1F	<100	<100	<100	1371	1673	1600	46820
I10811	1F	<100	<100	<100	1924	2363	10107	278924
	Mean	140	126	0	1492	1690	3556	91943
	SD	165.1	150.3	0.0	946.8	993.5	4434.6	126189.8
	N	4	4	4	4	4	4	4

Table
Summary and Individual Hepatitis B Vaccine (HBsAg) IgM and IgG
Anti-HBsAg IgG

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Animal #	Group/Sex	Interpolated Cutpoint Titer Day 43	Interpolated Cutpoint Titer Day 50	Interpolated Cutpoint Titer Day 57
I10808	1F	45780	36158	25431
I10809	1F	2081	1826	1568
I10810	1F	41112	34050	12425
I10811	1F	259205	239007	196618
	Mean	87045	77760	59011
	SD	116433.9	108639.9	92255.5
	N	4	4	4

Table 9.6: Summary and Individual Hematology

Test Article	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted] (HBsAg)	1.0 mL	20 µg/mL

Group/ Sex	Animal Number	Phase Day	RBC E6/uL Predose 5	RBC E6/uL Dosing 57	HGB g/dL Predose 5	HGB g/dL Dosing 57	Hct % Predose 5	Hct % Dosing 57
1/F	I10808		6.03	5.73	13.8	13.0	45.8	44.4
	I10809		4.77	4.98	11.4	12.1	39.1	42.3
	I10810		5.84	5.61	13.6	12.7	46.8	43.1
	I10811		6.28	5.97	14.2	13.7	45.8	44.5
	Mean		5.73	5.57	13.3	12.9	44.4	43.6
	SD		0.665	0.422	1.26	0.67	3.55	1.06
	N		4	4	4	4	4	4

Group/ Sex	Animal Number	Phase Day	MCV fL Predose 5	MCV fL Dosing 57	MCH pg Predose 5	MCH pg Dosing 57	MCHC g/dL Predose 5	MCHC g/dL Dosing 57
1/F	I10808		75.9	77.5	22.8	22.7	30.1	29.3
	I10809		81.9	84.9	23.8	24.3	29.0	28.7
	I10810		80.2	76.9	23.2	22.6	29.0	29.5
	I10811		73.0	74.6	22.6	22.9	30.9	30.6
	Mean		77.8	78.5	23.1	23.1	29.8	29.5
	SD		4.05	4.46	0.53	0.79	0.93	0.79
	N		4	4	4	4	4	4

Table
Summary and Individual Hematology

Test Article	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted] (HBsAg)	1.0 mL	20 µg/mL

Group/ Sex	Animal Number	Phase	RETIC E3/uL		PLT E3/uL		WBC E3/uL	
			Predose	Dosing	Predose	Dosing	Predose	Dosing
		Day	5	57	5	57	5	57
1/F	I10808		52.4	80.9	399	363	14.31	8.53
	I10809		130.2	52.6	475	423	5.74	6.57
	I10810		61.9	71.6	504	447	15.21	10.72
	I10811		59.8	81.8	383a	341	14.43	12.47
	Mean		76.1	71.7	440	394	12.42	9.57
	SD		36.31	13.56	58.5	49.7	4.473	2.570
	N		4	4	4	4	4	4

Group/ Sex	Animal Number	Phase	NEUT E3/uL		LYM E3/uL		MONO E3/uL	
			Predose	Dosing	Predose	Dosing	Predose	Dosing
		Day	5	57	5	57	5	57
1/F	I10808		8.10	3.74	5.63	4.35	0.34	0.24
	I10809		3.01	3.55	2.51	2.69	0.18	0.27
	I10810		4.60	4.13	9.66	5.90	0.59	0.47
	I10811		5.03	5.13	8.43	6.60	0.60	0.51
	Mean		5.19	4.14	6.56	4.89	0.43	0.37
	SD		2.129	0.704	3.182	1.739	0.204	0.137
	N		4	4	4	4	4	4

a = Platelet clumps were observed

Table
Summary and Individual Hematology

Test Article	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted] (HBsAg)	1.0 mL	20 µg/mL

Group/ Sex	Animal Number	Phase	EOS E3/uL		BASO E3/uL		LUC E3/uL	
			Predose	Dosing	Predose	Dosing	Predose	Dosing
		Day	5	57	5	57	5	57
1/F	I10808		0.08	0.09	0.08	0.08	0.08	0.03
	I10809		0.01	0.02	0.01	0.03	0.01	0.01
	I10810		0.11	0.10	0.12	0.07	0.13	0.06
	I10811		0.20	0.06	0.08	0.11	0.09	0.07
	Mean		0.10	0.07	0.07	0.07	0.08	0.04
	SD		0.079	0.036	0.046	0.033	0.050	0.028
	N		4	4	4	4	4	4

Group/ Sex	Animal Number	Phase	PT sec		APTT sec	
			Predose	Dosing	Predose	Dosing
		Day	5	57	5	57
1/F	I10808		8.7	8.1	22.9	22.5
	I10809		8.4	8.0	21.2	22.1
	I10810		8.1b	8.2	18.8b	18.9
	I10811		8.4	7.7	22.8	23.9
	Mean		8.4	8.0	21.4	21.9
	SD		0.24	0.22	1.92	2.11
	N		4	4	4	4

b = Sample was slightly hemolyzed

Table
Summary and Individual Hematology

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Group/ Sex	Animal Number	Phase	ANISO		POLY		POIK	
			Predose	Dosing	Predose	Dosing	Predose	Dosing
		Day	5	57	5	57	5	57
1/F	I10808		Normal	Normal	Normal	Normal	Normal	Normal
	I10809		Normal	Normal	Normal	Normal	Normal	Normal
	I10810		Normal	Normal	Normal	Normal	Normal	Normal
	I10811		Normal	Normal	Normal	Normal	Normal	Normal

Group/ Sex	Animal Number	Phase	HYPO		TOXIC	
			Predose	Dosing	Predose	Dosing
		Day	5	57	5	57
1/F	I10808		Normal	Normal	Normal	Normal
	I10809		Normal	Normal	Normal	Normal
	I10810		Normal	Normal	Normal	Normal
	I10811		Normal	Normal	Normal	Normal

Table 9.7: Summary and Individual Clinical Chemistry

Test Article	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted] (HBsAg)	1.0 mL	20 µg/mL

Group/ Sex	Animal Number	Phase Day	GLU mg/dL Predose 5	GLU mg/dL Dosing 57	UN mg/dL Predose 5	UN mg/dL Dosing 57	CREA mg/dL Predose 5	CREA mg/dL Dosing 57
1/F	I10808		56	64	24	21	0.8	0.9
	I10809		59	62	23	20	0.8	0.9
	I10810		101	84	19	18	0.9	1.0
	I10811		81b	64	20b	18	0.6b	0.7
	Mean		74	69	22	19	0.8	0.9
	SD		21.0	10.4	2.4	1.5	0.13	0.13
	N		4	4	4	4	4	4

Group/ Sex	Animal Number	Phase Day	TP g/dL Predose 5	TP g/dL Dosing 57	ALB g/dL Predose 5	ALB g/dL Dosing 57	GLOB g/dL Predose 5	GLOB g/dL Dosing 57
1/F	I10808		7.5	7.4	4.5	4.5	3.0	2.9
	I10809		7.0	6.8	4.6	4.4	2.4	2.4
	I10810		7.3	7.1	4.5	4.4	2.8	2.7
	I10811		7.3b	6.8	4.3b	4.0	3.0b	2.8
	Mean		7.3	7.0	4.5	4.3	2.8	2.7
	SD		0.21	0.29	0.13	0.22	0.28	0.22
	N		4	4	4	4	4	4

b = Sample was slightly hemolyzed

Table
Summary and Individual Clinical Chemistry

Test Article	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted] (HBsAg)	1.0 mL	20 µg/mL

Group/ Sex	Animal Number	Phase	A:G Predose	A:G Dosing	CHOL mg/dL Predose	CHOL mg/dL Dosing	TRIG mg/dL Predose	TRIG mg/dL Dosing
		Day	5	57	5	57	5	57
1/F	I10808		1.5	1.6	129	132	46	38
	I10809		1.9	1.8	174	171	43	45
	I10810		1.6	1.6	138	118	41	54
	I10811		1.4b	1.4	174b	164	41b	45
	Mean		1.6	1.6	154	146	43	46
	SD		0.22	0.16	23.7	25.4	2.4	6.6
	N		4	4	4	4	4	4

Group/ Sex	Animal Number	Phase	TBIL mg/dL Predose	TBIL mg/dL Dosing	AST U/L Predose	AST U/L Dosing	ALT U/L Predose	ALT U/L Dosing
		Day	5	57	5	57	5	57
1/F	I10808		0.2	0.2	34	29	38	32
	I10809		0.2	0.1	31	29	41	39
	I10810		0.2	0.2	37	36	54	40
	I10811		0.3b	0.3	66b	50	44b	41
	Mean		0.2	0.2	42	36	44	38
	SD		0.05	0.08	16.2	9.9	6.9	4.1
	N		4	4	4	4	4	4

b = Sample was slightly hemolyzed

Table
Summary and Individual Clinical Chemistry

Test Article	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted] (HBsAg)	1.0 mL	20 µg/mL

Group/ Sex	Animal Number	Phase	ALP U/L		GGT U/L		CK U/L	
			Predose	Dosing	Predose	Dosing	Predose	Dosing
		Day	5	57	5	57	5	57
1/F	I10808		771	639	79	76	179	184
	I10809		297	240	48	44	207	209
	I10810		401	360	85	74	245	216
	I10811		611b	571	66b	65	369b	361
	Mean		520	453	70	65	250	243
	SD		212.3	184.9	16.4	14.6	83.8	80.2
	N		4	4	4	4	4	4

Group/ Sex	Animal Number	Phase	Ca mg/dL		PHOS mg/dL		Na mmol/L	
			Predose	Dosing	Predose	Dosing	Predose	Dosing
		Day	5	57	5	57	5	57
1/F	I10808		10.6	10.8	6.7	5.9	149	147
	I10809		10.2	10.3	5.8	5.0	145	147
	I10810		11.1	10.8	7.2	5.2	150	146
	I10811		10.5b	9.9	6.6b	5.5	143b	142
	Mean		10.6	10.5	6.6	5.4	147	146
	SD		0.37	0.44	0.58	0.39	3.3	2.4
	N		4	4	4	4	4	4

b = Sample was slightly hemolyzed

Table
Summary and Individual Clinical Chemistry

Test Article			Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted] (HBsAg)			1.0 mL	20 µg/mL

			K mmol/L		Cl mmol/L	
		Phase	Predose	Dosing	Predose	Dosing
Group/ Sex	Animal Number	Day	5	57	5	57
1/F	I10808		4.7	4.8	108	104
	I10809		5.2	4.9	107	106
	I10810		5.5	4.5	105	104
	I10811		5.4b	4.6	104b	106
	Mean		5.2	4.7	106	105
	SD		0.36	0.18	1.8	1.2
	N		4	4	4	4

b = Sample was slightly hemolyzed

Table 9.8: Summary and Individual Urinalysis

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Group/ Sex	Animal Number	Phase	UVOL mL		SPGR		UpH	
			Predose	Dosing	Predose	Dosing	Predose	Dosing
		Day	5	57	5	57	5	57
1/F	I10808		34.8	175.7	1.024	1.005	8.0	7.0
	I10809		50.6	26.8	1.017	1.017	7.5	7.5
	I10810		123.8	258.5	1.011	1.006	8.0	7.5
	I10811		63.6	193.0	1.019	1.008	8.5	7.5
	Mean		68.2	163.5	1.018	1.009	8.0	7.4
	SD		38.89	97.86	0.0054	0.0055	0.41	0.25
	N		4	4	4	4	4	4

Table
Summary and Individual Urinalysis

Test Article	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted] (HBsAg)	1.0 mL	20 µg/mL

Group/ Sex	Animal Number	Phase	UPRO		UOBL		UGLU	
			Predose	Dosing	Predose	Dosing	Predose	Dosing
		Day	5	57	5	57	5	57
1/F	I10808		NEGATIVE	NEGATIVE	NEGATIVE	1+	NEGATIVE	NEGATIVE
	I10809		NEGATIVE	NEGATIVE	NEGATIVE	TRACE	NEGATIVE	NEGATIVE
	I10810		NEGATIVE	NEGATIVE	3+	NEGATIVE	NEGATIVE	NEGATIVE
	I10811		2+	NEGATIVE	3+	NEGATIVE	NEGATIVE	NEGATIVE

Group/ Sex	Animal Number	Phase	UKET		UBIL		UUBG mg/dL	
			Predose	Dosing	Predose	Dosing	Predose	Dosing
		Day	5	57	5	57	5	57
1/F	I10808		2+	NEGATIVE	NEGATIVE	NEGATIVE	0.2	0.2
	I10809		2+	1+	NEGATIVE	NEGATIVE	0.2	0.2
	I10810		NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	0.2	0.2
	I10811		NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	0.2	0.2

Table
Summary and Individual Urinalysis

Test Article	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted] (HBsAg)	1.0 mL	20 µg/mL

Group/ Sex	Animal Number	Phase	URBC		UWBC		UEPI	
			Predose	Dosing	Predose	Dosing	Predose	Dosing
		Day	5	57	5	57	5	57
1/F	I10808		0	0	0	0	3	0
	I10809		0	0	1	0	1	0
	I10810		0	0	0	0	1	0
	I10811		4	0	2	0	1	2

Group/ Sex	Animal Number	Phase	UBACT		UCAST		CASTT	
			Predose	Dosing	Predose	Dosing	Predose	Dosing
		Day	5	57	5	57	5	57
1/F	I10808		1	3	0	0	NT	NT
	I10809		2	3	0	0	NT	NT
	I10810		2	2	0	0	NT	NT
	I10811		3	3	0	0	NT	NT

Table
Summary and Individual Urinalysis

Test Article	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted] (HBsAg)	1.0 mL	20 µg/mL

Group/ Sex	Animal Number	Phase	UCRYS		UCRYST		UCOL	
			Predose	Dosing	Predose	Dosing	Predose	Dosing
		Day	5	57	5	57	5	57
1/F	I10808		0	0	NT	NT	YELLOW	YELLOW
	I10809		0	0	NT	NT	YELLOW	YELLOW
	I10810		0	0	NT	NT	YELLOW	YELLOW
	I10811		0	0	NT	NT	RED	YELLOW

Group/ Sex	Animal Number	Phase	UCLA		UOTH	
			Predose	Dosing	Predose	Dosing
		Day	5	57	5	57
1/F	I10808		CLOUDY	CLOUDY	0	0
	I10809		CLOUDY	CLOUDY	0	0
	I10810		CLOUDY	CLOUDY	0	0
	I10811		TURBID	CLOUDY	0	0

10. ANALYTICAL VALIDATION TABLES

Table 10.1: Inter-Analyst Cutpoint Titer - IgM and IgG

Test Article	IgM Cutpoint Titer	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Run/Analyst	Anti-HBsAg	Animal IM162634 (Male) 04Nov16[a]	Animal IM161303 (Female) 04Nov16[a]	Animal I10808 (Female) Terminal Sacrifice[b]
Run 4 AMR	100.00	688.13	707.63	446.40
	100.00	742.53	645.88	397.71
	100.00	731.84	651.71	454.12
	100.00	735.74	645.99	449.13
Run 7 AC	100.00	780.73	789.86	584.88
	100.00	751.97	699.42	559.88
	100.00	767.96	709.07	584.73
	100.00	790.15	726.29	626.96
Mean	100.00	748.63	696.98	512.98
SD	0.000	32.258	49.297	85.164
N	8	8	8	8
%CV	0.00	4.31	7.07	16.60

a Sample from [redacted]
b Sample from [redacted]

Table
Inter-Analyst Cutpoint Titer - IgM and IgG
IgG Cutpoint Titer

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Run/Analyst	Anti-HBsAg	Animal IM162634 (Male) 04Nov16[a]	Animal IM161303 (Female) 04Nov16[a]	Animal I10808 (Female) Terminal Sacrifice[b]
Run 4 AMR	100.00	11102.91	11378.46	11588.59
	100.00	10967.29	10600.01	11734.63
	100.00	10955.24	10382.21	11854.92
	100.00	11138.50	10549.24	11831.91
Run 7 AC	100.00	12046.18	11462.46	12122.32
	100.00	12175.83	11468.19	12449.34
	100.00	12390.48	11179.40	14644.53
	100.00	38128.351*	11381.02	43030.354*
Mean	100.00	11539.49	11050.12	12318.03
SD	0.000	633.245	459.568	1063.905
N	8	7	8	7
%CV	0.00	5.49	4.16	8.64

* = Significant outlier p<0.05 by Grubbs Test of Significant Outliers and not included in calculations

a Sample from [redacted]

b Sample from [redacted]

Table 10.2: Inter-Plate/Intra-Analyst - IgM and IgG

Test Article	IgM	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Run/Analyst	Anti-HBsAg	Animals IM162634 (Male) 04Nov16[a]	Animal IM161303 (Female) 04Nov16[a]	Animal I10808 (Female) Terminal Sacrifice[b]
Run 4 AMR	100.00	688.13	707.63	446.40
	100.00	742.53	645.88	397.71
	100.00	731.84	651.71	454.12
	100.00	735.74	645.99	449.13
Run 9 AMR	100.00	586.93	572.96	358.91
	100.00	593.20	561.22	364.56
	100.00	629.37	482.30	357.23
	100.00	645.04	521.41	368.34
Mean	100.00	669.10	598.64	399.55
SD	0.000	64.143	76.277	43.549
N	8	8	8	8
%CV	0.00	9.59	12.74	10.90

a Sample from [redacted]
b Sample from [redacted]

Table
Inter-Plate/Intra-Analyst - IgM and IgG
IgG

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Run/Analyst	Anti-HBsAg	Animal IM162634 (Male) 04Nov16[a]	Animal IM161303 (Female) 04Nov16[a]	Animal I10808 (Female) Terminal Sacrifice[b]
Run 7 AC	100.00	12046.18	11462.46	12122.32
	100.00	12175.83	11468.19	12449.34
	100.00	12390.48	11179.40	14644.53
	100.00	38128.351*	11381.02	43030.354*
Run 12 AC	100.00	11953.15	12162.97	17999.25
	100.00	11960.88	34565.908*	23574.81
	100.00	12127.36	11855.05	22490.32
	100.00	12382.55	12312.97	20147.76
Mean	100.00	12148.06	11688.87	17632.62
SD	0.000	181.824	427.431	4681.980
N	8	7	7	7
%CV	0.00	1.50	3.66	26.55

* = Significant outlier p<0.05 by Grubbs Test of Significant Outliers and not included in calculations

a Sample from [redacted]

b Sample from [redacted]

Table 10.3: Intra-Sample/Batch - IgM and IgG

Test Article	IgM	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Run/Analyst	Anti-HBsAg	Animal IM162634 (Male) 04Nov16[a]	Animal IM161303 (Female) 04Nov16[a]	Animal I10808 (Female) Terminal Sacrifice[b]
Run 4 AMR	100.00	688.13	707.63	446.40
	100.00	742.53	645.88	397.71
	100.00	731.84	651.71	454.12
	100.00	735.74	645.99	449.13
Mean	100.00	724.56	662.80	436.84
SD	0.000	24.682	30.005	26.281
N	4	4	4	4
%CV	0.00	3.41	4.53	6.02

a Sample from [redacted]
b Sample from [redacted]

Table
Intra-Sample/Batch - IgM and IgG
IgG

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Run/Analyst	Anti-HBsAg	Animal IM162634 (Male) 04Nov16[a]	Animal IM161303 (Female) 04Nov16[a]	Animal I10808 (Female) Terminal Sacrifice[b]
Run 7 AC	100.00	780.73	789.86	584.88
	100.00	751.97	699.42	559.88
	100.00	767.96	709.07	584.73
	100.00	790.15	726.29	626.96
Mean	100.00	772.70	731.16	589.11
SD	0.000	16.544	40.681	27.836
N	4	4	4	4
%CV	0.00	2.14	5.56	4.73

a Sample from [redacted]
b Sample from [redacted]

Table
Intra-Sample/Batch - IgM and IgG
IgG

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Run/Analyst	Anti-HBsAg	Animal IM162634 (Male) 04Nov16[a]	Animal IM161303 (Female) 04Nov16[a]	Animal I10808 (Female) Terminal Sacrifice[b]
Run 4 AMR	100.00	11102.91	11378.46	11588.59
	100.00	10967.29	10600.01	11734.63
	100.00	10955.24	10382.21	11854.92
	100.00	11138.50	10549.24	11831.91
Mean	100.00	11040.99	10727.48	11752.51
SD	0.000	93.325	443.851	121.080
N	4	4	4	4
%CV	0.00	0.85	4.14	1.03

a Sample from [redacted]
b Sample from [redacted]

Table
Intra-Sample/Batch - IgM and IgG
IgG

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Run/Analyst	Anti-HBsAg	Animal IM162634 (Male) 04Nov16[a]	Animal IM161303 (Female) 04Nov16[a]	Animal I10808 (Female) Terminal Sacrifice[b]
Run 7 AC	100.00	12046.18	11462.46	12122.32
	100.00	12175.83	11468.19	12449.34
	100.00	12390.48	11179.40	14644.53
	100.00	38128.351*	11381.02	43030.354*
Mean	100.00	12204.16	11372.77	13072.06
SD	0.000	173.889	134.918	1371.578
N	4	3	4	3
%CV	0.00	1.42	1.19	10.49

* = Significant outlier p<0.05 by Grubbs Test of Significant Outliers and not included in calculations

a Sample from [redacted]

b Sample from [redacted]

Table 10.4: Benchtop Stability - IgM and IgG

Test Article	IgM Cut-Point Titer	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Run/Analyst	Timepoint	Animal IM162634 (Male)[a]	Animal I10808 (Female)[b]	Animal IM161303 (Female)[a]	Animal I10811 (Female)[b]
Run 10 AC	0 hr	686.95	569.98	676.40	275.38
	1 hr	744.08	573.91	631.88	298.32
	2 hr	722.74	561.33	619.25	278.30
	12 hr	652.68	554.79	589.59	325.36
	Mean	701.61	565.00	629.28	294.34
	SD	40.246	8.601	36.070	23.055
	N	4	4	4	4
	70%	505.92	392.93	433.47	194.81
	130%	939.56	729.73	805.02	361.79
	%CV	5.74	1.52	5.73	7.83

a Sample from [redacted]
b Sample from [redacted]

Table
Benchtop Stability - IgM and IgG
IgG Cut-Point Titer

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Run/Analyst	Timepoint	Animal IM162634 (Male)[a]	Animal I10808 (Female)[b]	Animal IM161303 (Female)[a]	Animal I10811 (Female)[b]
Run 10 AC	0 hr	6733.35	10422.00	5772.29	40962.03
	1 hr	7712.73	10333.95	6627.12	45706.58
	2 hr	6279.78	10296.84	6496.26	41483.60
	12 hr	6665.27	10375.64	6290.89	41622.66
	Mean	6847.78	10357.11	6296.64	42443.72
	SD	610.238	53.922	375.961	2193.753
	N	4	4	4	4
	70%	4395.85	7207.79	4547.38	29038.52
	130%	8163.71	13385.90	8445.14	53928.69
	%CV	8.91	0.52	5.97	5.17

a Sample from [redacted]
b Sample from [redacted]

Table 10.5: Method and Kit Comparison: Absorbance - IgM and IgG

Test Article	IgM	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Positive Kit Control		Positive Serum	
	0.004		1.257
	0.007		1.527
Method	0.003	Method	1.455
	0.005		1.451
	0.004		0.46
	0.004		1.074
	0.004		1.131
Kit	0.005	Kit	0.978
	0		1.007
	0.012		1.044
Mean	0.005	Mean	1.14
SD	0.003	SD	0.313
N	10	N	10
%CV	64.25	%CV	27.46

Table
Method and Kit Comparison: Absorbance - IgM and IgG
IgG

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

	Positive Kit Control		Positive Serum
	0.06		3.597
	0.06		3.512
Method	0.055	Method	3.537
	0.067		3.623
	0.057		3.561
	0.062		3.227
	0.088		3.293
Kit	0.072	Kit	3.203
	0.053		3.187
	0.076		3.183
Mean	0.065	Mean	3.39
SD	0.011	SD	0.188
N	10	N	10
%CV	16.77	%CV	5.54

Table 10.6: Method and Kit Comparison (Kit Controls): Absorbance - IgM and IgG

Test Article	Absorbance IgM	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Kit Standards					
Method	1.597	0.841	0.519	0.215	0
Kit	1.498	0.852	0.517	0.19	0.013
Mean	1.55	0.85	0.52	0.20	0.01
SD	0.070	0.008	0.001	0.018	0.009
N	2	2	2	2	2
%CV	4.52	0.92	0.27	8.73	141.42
High Standard Series					
Method	1.681	0.8	0.439	0.21	0.083
Kit	1.366	0.814	0.479	0.2	0.099
Mean	1.52	0.81	0.46	0.21	0.09
SD	0.223	0.010	0.028	0.007	0.011
N	2	2	2	2	2
%CV	14.62	1.23	6.16	3.45	12.43

Table
Method and Kit Comparison (Kit Controls): Absorbance - IgM and IgG

Test Article	Absorbance IgG	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Kit Standards					
Method	1.817	0.949	0.549	0.211	-0.005
Kit	1.56	0.919	0.525	0.187	0.011
Mean	1.69	0.93	0.54	0.20	0.00
SD	0.182	0.021	0.017	0.017	0.011
N	2	2	2	2	2
%CV	10.76	2.27	3.16	8.53	377.12
High Standard Series					
Method	1.623	0.824	0.395	0.196	0.098
Kit	1.641	0.948	0.455	0.196	0.096
Mean	1.63	0.89	0.43	0.20	0.10
SD	0.013	0.088	0.042	0.000	0.001
N	2	2	2	2	2
%CV	0.78	9.90	9.98	0	1.46

Table 10.7: Method and Kit Comparison (Serum Samples): Absorbance - IgM and IgG

Test Article	Absorbance IgM	Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Animal I10808 (Female) 18Sept15[a]	Dilutions				
	100	200	400	800	1600
Method	1.748	1.109	0.614	0.323	0.144
Kit	0.54	0.465	0.329	0.22	0.112
Mean	1.14	0.79	0.47	0.27	0.13
SD	0.854	0.455	0.202	0.073	0.023
N	2	2	2	2	2
%CV	74.67	57.86	42.74	26.83	17.68

Animal I10808 (Female) 25Sept15[a]	Dilutions				
	100	200	400	800	1600
Method	1.596	0.98	0.492	0.277	0.121
Kit	0.475	0.411	0.311	0.19	0.101
Mean	1.04	0.70	0.40	0.23	0.11
SD	0.793	0.402	0.128	0.062	0.014
N	2	2	2	2	2
%CV	76.55	57.85	31.88	26.35	12.74

a Sample from Covance 8326556

Table
Method and Kit Comparison (Serum Samples): Absorbance - IgM and IgG
Absorbance IgG

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Animal I10811 (Female) 18Sept15[a]	Dilutions				
	100	200	400	800	1600
Method	0.654	0.475	0.343	0.217	0.114
Kit	0.381	0.327	0.237	0.184	0.121
Mean	0.52	0.40	0.29	0.20	0.12
SD	0.193	0.105	0.075	0.023	0.005
N	2	2	2	2	2
%CV	37.30	26.10	25.85	11.64	4.21

Animal I10811 (Female) 18Sept15[a]	Dilutions				
	100	200	400	800	1600
Method	0.693	0.48	0.342	0.227	0.12
Kit	0.372	0.295	0.227	0.169	0.114
Mean	0.53	0.39	0.29	0.20	0.12
SD	0.227	0.131	0.081	0.041	0.004
N	2	2	2	2	2
%CV	42.63	33.76	28.58	20.71	3.63

a Sample from Covance 8326556

Table
Method and Kit Comparison (Serum Samples): Absorbance - IgM and IgG
Absorbance IgG

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Animal I10808 (Female) 18Sept15[a]	Dilutions				
	100	500	2500	12500	62500
Method	3.609	3.249	1.617	0.345	0.048
Kit	3.278	2.988	1.355	0.328	0.07
Mean	3.44	3.12	1.49	0.34	0.06
SD	0.234	0.185	0.185	0.012	0.016
N	2	2	2	2	2
%CV	6.80	5.92	12.47	3.57	26.37

Animal I10808 (Female) 25Sept15[a]	Dilutions				
	100	500	2500	12500	62500
Method	3.559	3.215	1.509	0.325	0.053
Kit	3.232	2.881	1.245	0.304	0.068
Mean	3.40	3.05	1.38	0.32	0.06
SD	0.231	0.236	0.187	0.015	0.011
N	2	2	2	2	2
%CV	6.81	7.75	13.56	4.72	17.53

a Sample from Covance 8326556

Table
Method and Kit Comparison (Serum Samples): Absorbance - IgM and IgG
Absorbance IgG

Test Article		Dose Volume	Dose Concentration
Hepatitis B Vaccine; [redacted]	(HBsAg)	1.0 mL	20 µg/mL

Animal I10811 (Female) 18Sept15[a]	Dilutions				
	100	500	2500	12500	62500
Method	1.919	3.248	2.673	1.331	0.288
Kit	2.018	3.254	2.875	1.244	0.334
Mean	1.97	3.25	2.77	1.29	0.31
SD	0.070	0.004	0.143	0.062	0.033
N	2	2	2	2	2
%CV	3.56	0.13	5.15	4.78	10.46

Animal I10811 (Female) 18Sept15[a]	Dilutions				
	100	500	2500	12500	62500
Method	1.935	3.13	2.752	1.179	0.24
Kit	2.225	3.072	2.646	1.146	0.289
Mean	2.08	3.10	2.70	1.16	0.27
SD	0.205	0.041	0.075	0.023	0.035
N	2	2	2	2	2
%CV	9.86	1.32	2.78	2.01	13.10

a Sample from Covance 8326556

Table 10.8: Analytical Run Table - Included and Excluded Runs

Run	Included/Excluded	Explanation for Exclusion
1, 2, 3	Excluded	Runs 1 through 3 were collected on a computer that had been inadvertently disconnected from the network and the data could not be gathered in a GLP compliant part 11 compliant and reconstructable fashion.
4	Included	NA
5,6	Excluded	The dilution series was incorrect, no serial dilution was observed, and without a serial dilution no cutpoint could be calculated and therefore the data set was not used.
7, 8, 9, 10	Included	NA
11	Excluded	The dilution series was incorrect, no serial dilution was observed, and without a serial dilution no cutpoint could be calculated and therefore the data set was not used.
12	Included	NA

NA = Not applicable

ATTACHMENTS

Protocol

COVANCE
THE DEVELOPMENT SERVICES COMPANY

Protocol

Study Title	Characterization of Hepatitis B vaccine T-Cell Dependent Antibody Response in Cynomolgus Monkeys
Study Director	[redacted]
Sponsor and Testing Facility	Covance Laboratories Inc. 3301 Kinsman Boulevard Madison, Wisconsin 53704-2523 United States of America
Covance Study Number	8326556
Version	Final
Protocol Issued	24 July 2015
Page Number	1 of 16

TEST SITE

Company	Covance Laboratories Inc. 671 South Meridian Road Greenfield, Indiana 46140
Test Site Reference Number	8326556

KEY PERSONNEL

Study Director	[redacted]
Study Toxicologist	
Lead Quality Assurance Contact	
Responsible Scientist for ELISpot Analysis	

STUDY IDENTIFICATION

Purpose
The purpose of this study is to characterize the Hepatitis B vaccine T-cell dependent antibody response assay in cynomolgus monkeys.

Proposed Study Timetable

Experimental Start Date	23 July 2015
Inlife Start Date	31 July 2015
Inlife End Date	25 September 2015
Unaudited Draft Report	30 October 2015
Experimental Termination Date	09 May 2016
Study Completion Date	09 May 2016

REGULATORY COMPLIANCE

This study is an exploratory study and will be conducted in accordance with Covance standard operating procedures (SOPs) and generally recognized good laboratory practices.

For clarification, Hepatitis B Vaccine (Engerix) is considered a reagent for the purpose of use on this study. Characterization of this reagent will be limited to information readily provided by manufacturer.

REGULATORY GUIDELINES

Immunophenotyping is suggested by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Draft Consensus Guideline, S8, Immunotoxicology Studies for Human Pharmaceuticals (April 2006), the Committee for Proprietary Medicinal Products Note for Guidance on Repeated Dose Toxicity, Appendix B (Committee for Proprietary Medicinal Products /SWP/1042/99; effective October 2000), and the Guidance for Industry, Immunotoxicology Evaluation of Investigational New Drugs, prepared by the Food and Drug Administration, Center for Drug Evaluation and Research (October 2002).

ANIMAL CARE AND USE STATEMENT

All procedures in this protocol are in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare. In the opinion of the sponsor and study director, the study does not unnecessarily duplicate any previous work, and no other model can fulfill the study requirements.

VETERINARY CARE/PALLIATIVE AND PROPHYLACTIC MEASURES

In accordance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare, medical treatment necessary to prevent unacceptable pain and suffering, including euthanasia, is the sole responsibility of the attending laboratory animal veterinarian. Palliative and prophylactic procedures may be based upon consensus agreement between the study director and attending laboratory animal veterinarian. The study director and sponsor/designee (if possible) will be included in discussions of palliative and prophylactic procedures (nonlife-threatening conditions, including suspension of dosing and removal of animals from study) recommended by the attending veterinarian. Final authority for decision making will be with the laboratory animal veterinarian.

MAJOR COMPUTER SYSTEMS

The major computer systems to be used on this study may include, but are not limited to, the following systems.

Application Name[a]	Application Function
Metasys	Monitors and controls environmental conditions and water flow within the facility (e.g., animal rooms)
REES Environmental Monitoring	Monitors and documents facility storage conditions (e.g., refrigerators, freezers, and constant temperature rooms)
Path/Tox System Pristima, supplied by Xybion Medical Systems Corporation	Captures direct online formulation data, inlife toxicology, and clinical and anatomic pathology data and study maintenance information and randomizes animals
FACS Diva version 6.1.2	Captures and analyzes flow cytometry data
Tox Reporting	Transfers data from Pristima for reporting purposes
Electronic Notes (eNotes)	Documents study-specific communications
SAS	Performs statistical analysis

a All version numbers of the applications are maintained by Covance.

QUALITY ASSURANCE

This work will be performed in compliance with Covance SOPs. Although method validations do not fall under the scope of Good Laboratory Practices regulations, they will be used as a guide for good documentation practices. The Covance Quality Assurance Unit will audit the protocol and amendments, data, and report in accordance with Covance SOPs. Quality assurance inspection reports will be provided to the study

director for review, and a Quality Assurance Statement will be provided for inclusion in the final report.

TEST ARTICLE

Identification	Hepatitis B Vaccine; [redacted]® (HBvx) The test article will be provided preformulated at a concentration of 20 µg/mL hepatitis B surface antigen and used as supplied.
Lot No(s).	Will be maintained in the raw data.
Purity	Responsibility of, and provided by, the manufacturer/supplier
Stability	Responsibility of, and provided by, the manufacturer/supplier
Test Article Storage Conditions	Store in a refrigerator set to maintain 2 to 8°C. Do not freeze; discard if frozen.
Characteristics	Information on synthesis methods, composition, or other characteristics defining the test article is on file with the respective manufacturer/supplier.
Safety	The respective manufacturer/supplier will provide occupational safety information known about the test article (e.g., Material Safety Data Sheet, safety instructions, test article identity, etc.).

TEST ANIMALS AND HUSBANDRY

Animals

Species

Naïve Cynomolgus monkeys (*Macaca fascicularis*)

Origin

Chinese

Source

Covance Madison Stock Colony

Age at Initiation of Testing
2 to 7 years

Number and Sex for Acclimation
5 females

Number and Sex Assigned to Study
4 females

Identification
An implantable microchip identification device will be used. Alternative methods of identification such as tattoo, and/or cage card may be used in the event of a microchip failure, by study design, or when animals are not removed from their home cage

Justification
Monkeys historically have been used in safety evaluation studies and are recommended by appropriate regulatory agencies. The monkey model will also be useful.

Husbandry
Housing
Animals will be housed in stainless steel cages. When possible, animals will be socially housed. Animals may be individually housed during acclimation, for study-related procedures, or for behavior or health reasons.

Diet
Certified Primate Diet #5048 (PMI Nutrition International Certified LabDiet®) will be provided twice daily unless otherwise noted. The diet is routinely analyzed by the manufacturer for nutritional components and environmental contaminants. Results of specified nutrient and contaminant analyses are on file at Covance-Madison.

Water
Water will be provided ad libitum. Water samples are routinely analyzed for specified microorganisms and environmental contaminants. Results are on file at Covance-Madison.

Contaminants
No known contaminants are present in the diet or water at levels that might interfere with this study.

Environment
Environmental controls for the animal room will be set to maintain 20 to 26°C, a relative humidity of 30 to 70%, a minimum of 10 air changes/hour, and a 12-hour light/12-hour dark cycle. The light/dark cycle may be interrupted for study-related activities.

Acclimation (Predose Phase)
For at least 1 week

Environmental and Dietary Enrichments
Animals will be given various cage-enrichment devices; fruit, vegetable, or dietary enrichment (that do not require analyses). Animals will be commingled in accordance with Covance standard operating procedures.

Randomization
Selection of animals for the study will be based on data collected during acclimation. No randomization is needed as all animals will receive the same treatment.

GROUP DESIGNATION AND DOSE LEVELS

Group	No. of Animals Female	Dose Level (µg/dose)	Dose Concentration (µg/mL)[a]
1	4	20	20

a The HBvx formulation will be administered at a volume of 1 mL/dose.

DOSING PROCEDURES

Dose Formulation (HBvx)

Procedures

Test article formulations will be used as supplied and dispensed using aseptic procedures (as needed) according to the Covance dispensing procedure. Dose concentrations are based on the nominal concentration of the test article formulation as provided by the manufacturer.

Test article will be used as supplied by the manufacturer or supplier and will be dispensed at least once.

At least 1 unused vial of HBvx, from the same lot used for dosing, will be transferred to the Immunotoxicology Lab to use for coating of ELISA plates (if needed). If unused, the vial will be returned to the Dose Formulations for storage.

Storage Conditions

The HBvx formulation will be stored according to the manufacturer's recommendation, in a refrigerator set to maintain 2 to 8°C.

Dose Administration (HBvx)

Predose Handling	Dosing formulations will be well shaken to resuspend the sediment of fine white particles of adjuvant (aluminium hydroxide) which settles during storage to obtain a qualitatively slightly opaque, white suspension.
HBvx	Primary Administration All animals will receive one dose of HBvx at a dose volume of 1 mL per animal by intramuscular injection into the right quadriceps femoris region on Day 1 of the dosing phase. Secondary Administration All animals will receive one dose of HBvx at a dose volume of 1 mL per animal by intramuscular injection into the right quadriceps femoris region on Day 29 of the dosing phase.
Special Procedures	The HBvx injection sites will be clipped free of hair (prior to dosing and as needed thereafter).

OBSERVATION OF ANIMALS

Clinical Observations

General Daily Observations

Frequency	Each animal will be observed twice daily (a.m. and p.m.).
Observations	Abnormal findings will be recorded as observed for mortality, abnormalities, and signs of pain or distress.

Unscheduled Observations

Observations	Abnormal findings will be recorded as they are observed.

Cageside Observations

Frequency	Cageside observations will be made for each animal once daily (except on the days of detailed observations and day of animal transfer) during the predose and dosing phases. Additionally, each day of dosing, postdose observations will be made for each animal approximately 1 and 4 hours postdose. Postdose observations will be based on the completion of dosing for each animal.

	Postdose observations will include the evaluation of dose site(s).
Observations	Cageside observations: Abnormal findings will be recorded. Postdose observations: Abnormal findings or an indication of normal will be recorded.
Food Consumption	Once daily (except on the day of animal transfer or days when animals are being fasted at the time of the observation), during the predose and dosing phases, food consumption will be assessed qualitatively; abnormal findings will be recorded.

Detailed Observations

Frequency	At least once during the predose phase, prior to dosing on Day 1 and weekly (based on Day 1) thereafter during the dosing phase.
Observations	Detailed observations will include the evaluation of dose site(s). Abnormal findings or an indication of normal will be recorded.

Body Weights

Frequency	At least once during the predose phase, prior to dosing on Day 1 and weekly (based on Day 1) thereafter during the dosing phase.

HEPATITIS B VACCINE (HBvx) IgM AND IgG ANALYSIS

Number of Animals and Frequency	Primary Administration (All Surviving Animals) Prior to (predose) primary administration on Day 1 and on Days 5, 8, 15, and 22. Secondary Administration (All Surviving Animals) Prior to (predose) secondary administration and on Days 36, 43, 50, and 57 of the dosing phase
Fasting Requirements	Animals will not be fasted for sample collections (unless the collection is concurrent with clinical pathology sampling).
Blood Volume to Collect	Approximately 3 mL
Collection Site	A femoral vein; an alternate vein may be used if necessary (the site of blood collection will be documented).

Anticoagulant	None; serum separator tubes
Handling after Collection and Centrifugation	Blood samples will be allowed to clot at room temperature and centrifuged within 1 hour of collection. Serum will be harvested.
Storage Conditions	Samples will be held on dry ice until transferred to freezer, set to maintain -60 to -80°C.
Analysis	Samples will be transferred to the immunotoxicology lab and analyzed for anti-HBvx IgM titers and for anti-HBvx-IgG titers. Samples will be analyzed using an enzyme-linked immunosorbent assay (ELISA) method. Analytes will be reported as concentrations where possible or as cutpoint titers.
Disposition of Samples	Unused sample will be donated to general purpose use at Covance or disposed of, at the discretion of the Study Director.

HEPATITIS B VACCINE (HBvx) ELISpot ANALYSIS

Frequency	Once during the predose phase and on Days 15, 29 (predose), 43, and 57 of the dosing phase
Number of Animals	All surviving animals
Fasting Requirements	Animals will not be fasted for sample collections (unless the collection is concurrent with clinical pathology sampling).
Blood Volume to Collect	Approximately 10 mL
Collection Site	A femoral vein; an alternate vein may be used if necessary (the site of blood collection will be documented).
Anticoagulant	Sodium Heparin tubes
Handling after Collection	After blood collection, the tubes will be gently inverted to ensure adequate mixing and transferred to the Clinical Pathology (Immunotoxicology) for processing.
Sample Processing	Peripheral blood mononuclear cells will be isolated from blood according to study-specific procedure.

Storage Conditions	Samples will be stored in a freezer, set to maintain -60 to -80°C, and subsequently packed on dry ice and shipped to Covance – Greenfield.
Shipping	After the predose, Days 15, 29, 43, and 57 collection intervals (on a Monday, Tuesday, or Wednesday), samples will be shipped by overnight carrier to Covance-Greenfield. [redacted] Covance Laboratories Inc. 671 South Meridian Road Greenfield, Indiana 46140 Telephone No.:[redacted] E-mail:[redacted] Before shipping, the recipient will be notified by e-mail as to the date and method of sample shipments.
Reporting	The responsible scientist for ELISpot analysis will be responsible for all delegated phase activities and will provide a report from the data generated to the study director for inclusion in the final report.

CLINICAL PATHOLOGY

Frequency	Blood and urine will be collected once during the predose phase and on the last day of the study.
No. of Animals	All surviving animals
Fasting Requirements	Animals will be fasted overnight for scheduled collections.
Collection Site	A femoral vein; an alternate vein may be used if necessary (the site of blood collection will be documented). Urine will be collected chilled on wet ice during the overnight period before blood collection.
Anticoagulant	The anticoagulants will be sodium citrate for coagulation tests and potassium EDTA for hematology tests. Samples for clinical chemistry will be collected without anticoagulant.
Interpretation	Clinical pathology evaluation will be used for animal selection purposes will be evaluated prior to animal randomization.

Planned Tests
In the event a planned test cannot be completed, the reason for the missing value will be recorded.

Hematology

red blood cell (erythrocyte) count
hemoglobin
hematocrit
mean corpuscular volume
mean corpuscular hemoglobin
mean corpuscular hemoglobin concentration
platelet count
white blood cell (leukocyte) count
differential blood cell count
blood cell morphology
reticulocyte count

Coagulation

prothrombin time
activated partial thromboplastin time

Clinical Chemistry

glucose	alanine aminotransferase
urea nitrogen	alkaline phosphatase
creatinine	gamma glutamyltransferase
total protein	aspartate aminotransferase
albumin	creatine kinase
globulin	calcium
albumin:globulin ratio	inorganic phosphorus
cholesterol	sodium
triglycerides	potassium
total bilirubin	chloride

Urinalysis

appearance (clarity and color)	ketones
volume	bilirubin
specific gravity	blood
pH	microscopic examination of sediment
protein	urobilinogen
glucose	

TERMINATION

Unscheduled Sacrifices and Deaths

No. of Animals	Any animals that die or are sacrificed at an unscheduled interval.
Procedures	Animals sacrificed will be sacrificed with sodium pentobarbital and discarded. Animals found dead will be discarded.

Final Disposition

Intervals	All surviving animals will be transferred back to the stock colony after the final blood collection.

REPORTS

Final Report
An electronic copy of the signed final report will be retained by Covance.

STATISTICAL EVALUATION

Analyses of immunophenotyping data will include calculation of means, and standard deviations as appropriate. Additional calculations will be included as described previously, and as deemed appropriate by the study director and statistician to assess validity of assays.

RECORD RETENTION

The raw data, documentation, specimens, records, protocol and final report generated as a result of this study will be archived in the storage facilities of Covance-Madison according to Covance SOPs.

PROTOCOL APPROVAL

[redacted]

24 JUL 2015
Date

Protocol Amendments

COVANCE
THE DEVELOPMENT SERVICES COMPANY

Protocol Amendment No. 1

Study Title	Characterization of Hepatitis B vaccine T-Cell Dependent Antibody Response in Cynomolgus Monkeys
Study Director	[redacted]
Sponsor and Testing Facility	Covance Laboratories Inc. 3301 Kinsman Boulevard Madison, Wisconsin 53704-2523 United States of America
Covance Study Number	Covance 8326556
Page Number	1 of 4

This amendment modifies the following portions of the protocol.

1. **HEPATITIS B VACCINE (HBVX) IGM AND IGG ANALYSIS**, **Number of Animals and Frequency.** Replace the text in this section with the following.

   Primary Administration (All Surviving Animals)
   Prior to (predose) primary administration on Day 1 and on Days 5, 8, 15, and 22.

   Secondary Administration (All Surviving Animals)
   Prior to (predose) secondary administration on Day 29 and on Days 36, 43, 50, and 57 of the dosing phase

Reason for Change:
To clarify that collection prior to (predose) secondary administration is on Day 29.

2. **HEPATITIS B VACCINE (HBVX) IGM AND IGG ANALYSIS**, **Handling after Collection and Centrifugation.** Replace the text in this section with the following.

Handling after Collection	After blood collection, the tubes will be gently inverted to ensure adequate mixing and transferred to the Clinical Pathology (Immunotoxicology) for processing.

Reason for Change:
To change the subsection title (no centrifugation needed) and move processing to immtox lab.

3. **HEPATITIS B VACCINE (HBVX) ELISPOT ANALYSIS, Shipping**. Replace the text in this section with the following.

After the final collection, all samples (on a Monday, Tuesday, or Wednesday), will be shipped by overnight carrier to Covance-Greenfield.

[redacted]
Covance Laboratories Inc.
671 South Meridian Road
Greenfield, Indiana 46140
Telephone No.: [redacted]
E-mail: [redacted]

Before shipping, the recipient will be notified by e-mail as to the date and method of sample shipments.

Reason for Change:

To indicate processed and cyropreserved PBMC's for ELISpot will be shipped after all collections are complete (at the end of the study).

AMENDMENT APPROVAL

The changes contained in this protocol amendment were approved by the sponsor on 28 July 2015.

[redacted]

28 JUL 15
Date

COVANCE
THE DEVELOPMENT SERVICES COMPANY

Protocol Amendment No. 2

Study Title	Characterization of Hepatitis B vaccine T-Cell Dependent Antibody Response in Cynomolgus Monkeys
Study Director	[redacted]
Sponsor and Testing Facility	Covance Laboratories Inc. 3301 Kinsman Boulevard Madison, Wisconsin 53704-2523 United States of America
Covance Study Number	Covance 8326556
Page Number	1 of 3

This amendment modifies the following portions of the protocol.

1. **QUALITY ASSURANCE.** Replace the text in this section with the following.

   This work will be performed in compliance with Covance SOPs. Although method validations do not fall under the scope of Good Laboratory Practices regulations, they will be used as a guide for good documentation practices. The Covance Quality Assurance Unit will audit the protocol and amendments, data, and report in accordance with Covance SOPs. Quality assurance inspection reports will be provided to the study director for review, and a Quality Assurance Statement will be provided for inclusion in the final report.

   Study conduct, data, and report produced by Covance-Greenfield will not be audited by a Quality assurance department.

Reason for Change:
To add that study conduct, data, and report produced by Covance-Greenfield will not be audited by a Quality assurance department because the assays being performed by Covance-Greenfield will always be conducted non-GLP.

AMENDMENT APPROVAL

The changes contained in this protocol amendment were approved by the sponsor on 20 October 2015.

[redacted]

20 Oct 15
Date

COVANCE
THE DEVELOPMENT SERVICES COMPANY

Protocol Amendment No. 3

Study Title	Characterization of Hepatitis B vaccine T-Cell Dependent Antibody Response in Cynomolgus Monkeys
Study Director	[redacted]
Sponsor and Testing Facility	Covance Laboratories Inc. 3301 Kinsman Boulevard Madison, Wisconsin 53704-2523 United States of America
Covance Study Number	Covance 8326556
Page Number	1 of 5

This amendment modifies the following portions of the protocol.

1. **HEPATITIS B VACCINE (HBvx) IgM AND IgG ANALYSIS.** Add the following as a separate section following this section.

HEPATITIS B SURFACE ANTIGEN (HBSAG) - ANALYTICAL VALIDATION PARAMETERS

Assumptions	It is assumed the Spectramax 340PC384 plate reader, is functioning correctly at the time of this validation. Stability will be assumed as the stability for Immunoglobulin IgG and IgM (www.aruplab.com/guides/ug/tests/0050355.jsp). Serum samples are stable at ambient conditions for 8 hours; refrigerated conditions for 8 days; and frozen conditions for 1 year. Temperatures stated in GL-GEN-307 will be accepted as the local tolerance (ambient conditions 15 to 20°C, refrigerated 2 to 8°C, frozen at -10 to -20°C, frozen at -60 to -80°C). This assumption will be made until the completion of internal stability analysis completed in this study. The dilution scheme and method have previously been established. If the acceptance criteria stated below is met, the analytical method will be validated and considered fit for purpose.
Exclusions	The primate anti-HBsAg IgG and IgM assay utilizes an antibody cut-point titer for the determination of the concentration of anti-HBsAg IgG and IgM in a sample. Precision and stability of anti-HBsAg antibody in serum will be assessed. All other aspects of the performance of the assay have been characterized in [redacted] and this study.

Methodology	A 96 well strip plate will be provided by Alpha Diagnostic International (catalog #4200 [IgG] and catalog #4205 [IgM]) and each microwell has HBsAg immobilized on them. Serum containing anti-HBsAg will be added to the wells and binds the antigen immobilized on the surface of the well. The anti-HBsAg antibody will be detected by anti-human IgG or IgM specific antibody conjugated to horse radish peroxidase (HRP) enzyme. After a washing step, chromogenic substrate 3,3', 5,5'-Tetramethylbenzidine (TMB) will be added and color will be developed by the enzymatic reaction of HRP on the substrate (TMB), which is directly proportional to the amount of anti-HBsAg IgG or IgM in the serum sample. Stopping solution will be added to stop the reaction and the absorbance at 450nm will then be measured using the Spectramax 340PC384 plate reader (or another suitable microwell plate reader).
Method Comparison	Anti-HBsAg positive primate serum will be tested using the kit method and a Covance developed method.
Precision	Primate serum samples from animals inoculated with [redacted]® (HBvx) will be prepared using the previously established five-fold dilution scheme for IgG and the previously established two fold dilution scheme for IgM. The diluted samples will be analyzed four times on a minimum of three different runs and at least one run will be performed by a different analyst. The titer values will be calculated. The mean, standard deviation, and the percent coefficient of variation (%CV) for the titer value will be calculated (intra batch-precision, inter batch–precision and inter-analyst precision will be calculated). **Acceptance Criteria:** There should be no more than a 25% CV observed.

Stability	Stability will be assessed using serum from three primates (individuals or pools). Samples will be separated into eight aliquots labelled A through H. Aliquot A will be analyzed after the first thaw of the sample, to provide baseline/expected values after long term storage at approximately -80°C. Aliquots B through H will be frozen at approximately -80°C. Aliquot B and C will be thawed for at least 1 hour and re-frozen. Aliquot C will be removed from the freezer for a second time and thawed at least 1 hour and re-frozen. Analysis of aliquot B and C will occur together on one run. Aliquot D will be thawed for at least 12 hours and run. Aliquots E through H will be kept at -80°C in case additional stability testing is required. The titer values will be calculated and findings tabulated and compared using a Levy-Jennings chart. **Acceptance Criteria:** The data will establish stability and will be considered acceptable if results are between 70 and 130% of baseline for the material.

Reason for Change:
To add analytical validation parameters for Hepatitis B Surface Antigen (HBsAg).

AMENDMENT APPROVAL

The changes contained in this protocol amendment were approved by the sponsor on 10 July 2017.

[redacted]

10 JUL 2017
Date

Hepatitis B Vaccine (HBsAg) ELISpot Report

COVANCE
SOLUTIONS MADE REAL®

Covance Translational Biomarker Solutions Report

Enzyme Linked ImmunoSpot (ELISPOT) Analysis of Peripheral Blood Mononuclear Cells (PBMC) from Non-Human Primates (NHP) Immunized with Hepatitis B Vaccine [redacted]®

Study: 8326556

Study Title	Characterization of Hepatitis B Vaccine T-Cell Dependent Antibody Response in Cynomolgus Monkeys
Sponsor Contact	Covance internal study
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Primary Testing Site	Covance Laboratories, Inc. Greenfield, IN 46140
Covance Project Number	8326556
Authors	[redacted]
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TABLE OF CONTENTS

SIGNATURES

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07Mar 2016
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Date

AUTHENTICATION STATEMENT

The experiments described in this report were performed by Steve Hatch, B.S and Sinnathamby Gomathinayagam, PhD. This report provides an accurate record of the results obtained. This is not a GLP Report. No audit was performed by the Quality Assurance Unit.

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ACRONYMS AND ABBREVIATIONS

CV	Coefficient of Variation
SD	Standard Deviation
PBMC	Peripheral Blood Mononuclear Cells
ELISPOT	Enzyme Linked ImmunoSpot Assay
INF-γ	Interferon-γ
PHA	Phytohemagglutinin
NHP	Non-Human Primate
DPBS	Dulbecco's Phosphate Buffered Saline
HBsAg	Hepatitis B Virus Surface Antigen
[redacted]®	Hepatitis B vaccine

1. SUMMARY

In this study, IFNγ secretion by PBMC isolated from cynomolgus monkeys immunized with [redacted]® (Hepatitis B vaccine) in response to ex vivo stimulation using hepatitis B surface antigen (HBsAg) or [redacted]® was measured using ELISPOT assay.

2. INTRODUCTION

The work described in this report is to support [redacted] PBMC isolated from 4 cynomolgus monkeys immunized with [redacted] at various time points were stimulated with HBsAg or [redacted]®, and IFNγ ELISPOT was performed. PBMC were stimulated with phytohemagglutinin (PHA) as a positive control. Results of the analysis are presented in this report.

3. MATERIALS AND METHODS

3.1 Materials

The following materials and equipment were used in the study.

Material	Vendor	Catalog number
Human IFNγ Elispot Kit	CTL	HIFNG-1
RPMI1640	Hyclone	SH30255.01
Heat inactivated FBS	Hyclone	SH30071.03
PBS with Ca++/Mg++	Hyclone	SH30264.01
L-glutamine	Gibco	25030-081
CTL Test media	CTL	CTLT-005
Anti-NHP CD45FITC	BD Biosciences	557803
Sytox Red	Molecular Probes	S34859
Flow count beads	Beckman/Coulter	7547053
PHA	Sigma	L1668-5mg
96-well deep well plate	Nunc	278743
Cell strainer	Fisher	22363548
HBsAg	Biorad	OBT0915
[redacted]® Hepatitis B vaccine	Manufactured by [redacted]e; Purchased from McKesson	Mfr # 58160082111; Product # 629870
Equipment	**Manufacturer**	
CTL-ImmunoSpot S6 Macro Analyzer	CTL ImmunoSpot	
Gallios™ flow cytometer	Beckman Coulter Inc	

3.2 Methods

3.2.1 PBMC Isolation and Cryopreservation

PBMC from 4 experimental cynomolgus monkeys immunized with [redacted]® were isolated from heparinized blood collected at various time points (Days -5 [predose] 15, 29 [predose], 43, and 57) using Lymphoprep tubes and cryopreserved in liquid nitrogen at the site of the live phase study. Samples were then shipped on dry ice to the site of ELISPOT analysis.

3.2.2 PBMC Recovery and Viability Determination

On the day of the experiment, frozen PBMC were quickly thawed in a 37°C water bath, washed with CTL test media supplemented with 2 mM L-Glutamine, pelleted down and resuspended in complete media. PBMC were then stained with FITC-conjugated anti-NHP CD45 and a viability dye (Sytox red). Viable CD45+ cell counts were determined using a flow cytometer in accordance with Covance SOP.

3.2.3 Preparation of Solutions of PHA and HBV Antigens

A stock solution of 5 mg/ml PHA was made by reconstituting 5 mg of lyophilized PHA powder in one ml of DPBS. Aliquots were made and stored frozen in ≤70°C. For PHA titration, dilutions were made in deep 96-well plates. A stock solution of HBsAg (1 mg/ml) was used to make a 2X concentrated solution at 20 µg/ml in CTL test media supplemented with 2 mM L-Glutamine. A stock solution of [redacted]® (20 µg/mL) was used to prepare a 2X concentrated solution at 2 µg/ml in CTL test media.

3.2.4 ELISPOT Assay

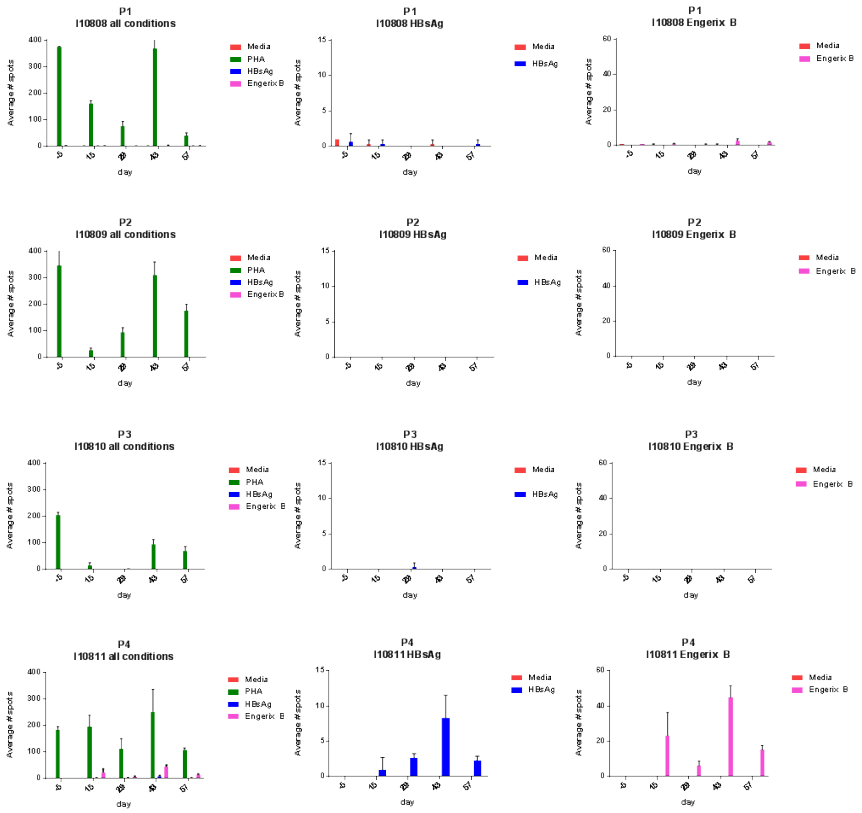
ELISPOT assay was performed per the manufacturer's protocol and the ELISPOT analyzer was used in accordance with Covance TBS SOP. One day before the assay, ELISPOT plates were coated with 80 µl of capture antibody stock solution that was diluted 1:250 by mixing 40 µl of human IFNγ capture antibody in 10 ml of Diluent A. Plates were sealed using Parafilm™ and refrigerated at 2-8ºC overnight. The capture antibody solution was decanted and the plates were washed with DPBS once. 100 µl of CTL Test medium supplemented with 2 mM L-Glutamine or antigen/mitogen solution made in the same medium was added to the appropriate wells and incubated in a 37°C humidified incubator supplied with 5% CO_2 for 15 minutes. 100 µl of PBMC appropriately diluted in CTL Test medium supplemented with 2 mM L-Glutamine was then added and the plates were incubated for 24 hours in a 37ºC humidified incubator supplied with 5% CO_2. For stimulation with PHA, 100,000 PBMC were used per well in 100 µl of CTL Test medium supplemented with 2 mM L-Glutamine). For stimulation with HBsAg and [redacted]®, PBMC were used at 200,000 cells per well. Plates were washed two times with DPBS and two times with DPBS containing 0.05% Tween 20. 80 µl of a 1:250 diluted- human IFNγ detection antibody solution (prepared by mixing 40 µl of antibody stock solution in 10 ml of Diluent B) was then added to each well and the plates were incubated at room temperature for two hours. Plates were washed three times with DPBS containing 0.05% Tween 20 and then 80 µl of diluted Streptavidin-Alkaline Phosphatase conjugate solution (prepared by mixing 10 µl of Streptavidin-Alkaline Phosphatase conjugate stock solution in 10 ml of Diluent C) was added. Plates were incubated for 30 minutes at room temperature. Plates were then washed two times with DPBS containing 0.05% Tween 20 and two times with distilled water. 80 µl of developer solution (prepared by mixing 160 µl of S1, 160 µl of S2 and 92 µl of S3 sequentially with 10 ml of Diluent Blue) was then added to each well and plates were developed in the dark for 15 minutes. The protective underdrain of the plates was removed and the reaction was stopped by washing the plates in running distilled water. Plates were dried overnight and scanned/counted using a CTL-ImmunoSpot S6 Macro Analyzer.

4. RESULTS AND DISCUSSION

Using a previously validated human IFNγ ELISPOT kit, PBMC samples from four cynomolgus monkeys were analyzed. Results are shown in Figure 1 below.

These results showed that PBMC from all animals responded to PHA positive control by secreting IFNγ, as expected at all time points, with the notable exception of Animal I10810 at the Day 29 time point. However, three of the four animals did not respond appreciably to either HBsAg or [redacted]®. The average number of spots for IFNγ noted in these animals were mostly similar to those noted in the negative control (media). One animal (I10811) responded to both HBsAg and [redacted]® in an appreciable manner. In this animal, there were no detectable levels of IFNγ on Day -5 (predose) but IFNγ secretion was noted at all time points post immunization (ie, Days 15, 29, 43, and 57). Maximal IFNγ secretion was seen on Day 43 (2 weeks post administered dose of [redacted]®), and waned by Day 57.

Figure 1



PBMC collected from 4 cynomolgus monkeys were stimulated with PHA, HBsAg and [redacted]® and IFNγ ELISPOT assay was performed.

5. CONCLUSIONS

In conclusion, ELISPOT analysis performed to measure IFNγ secretion by PBMC isolated from cynomolgus monkeys immunized with [redacted]® in response to ex vivo stimulation with either HBsAg or [redacted]® antigens revealed that only one out of four study animals responded with appreciable IFNγ secretion. In this animal, maximal IFNγ secretion was noted on Day 43, and waned by Day 57.

6. STORAGE

All the raw data in the form of Microsoft Excel sheets generated data are stored at Covance, Inc. on password protected backed-up network storage device. The raw data will be archived at the Covance Greenfield facility archives.

7. PERSONNEL INVOLVED

Work described in this report including data analysis was performed by [redacted] and [redacted] This report was written by [redacted] and QC was performed by [redacted]