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Quantitative Serology Assays for Determination of Antibody Responses to Ebola Virus Glycoprotein and Matrix Protein in Nonhuman Primates and Humans

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Abstract

The West Africa Ebola virus disease (EVD) outbreak has reached unprecedented magnitude and caused worldwide concerns for the spread of this deadly virus. Recent findings in nonhuman primates (NHPs) demonstrate that antibodies can be protective against EVD. However, the role of antibody response in vaccine-mediated protection is not fully understood. To address these questions quantitative serology assays are needed for measurement of the antibody response to key Ebola virus (EBOV) proteins. Serology enzyme-linked immunosorbent assays (ELISA's), using a reference detection antibody, were developed in order to standardize the quantitation of antibody levels in vaccinated NHPs or in humans exposed to EBOV or immunized with an EBOV vaccine. Critical reagents were generated to support the development of the serology ELISAs. Recombinant EBOV matrix protein (VP40) was expressed in *E. coli* and purified. Two variants of the glycoprotein (GP), the ectodomain lacking the transmembrane domain (GPTM), and an engineered GP lacking the mucin-like domain (GP^{muc}) were expressed and purified from mammalian cell systems. Using these proteins, three ELISA methods were developed and optimized for reproducibility and robustness, including stability testing of critical reagents. The assay was used to determine the antibody response against VP40, GPTM, and GP^{muc} in a NHP vaccine study using EBOV virus-like particles (VLP) vaccine expressing GP, VP40 and the nucleoprotein. Additionally, these ELISAs were used to successfully detect antibody responses to VP40, GPTM and GP^{muc} in human sera from EBOV infected individuals.

1. Introduction

The re-emergence of Ebola virus (EBOV) causing death and disruption within western African nations, and the potential for spread to other nations throughout the world

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necessitates a concerted effort to develop, test, and approve efficacious vaccines to treat and prevent infection.

EBOV causes lethal hemorrhagic fever in humans and nonhuman primates (NHP) with case fatality rates of up to 90% (Feldmann and Kiley, 1999; Feldmann and Klenk, 1996). EBOV has caused the majority of Ebola virus disease (EVD) outbreaks including the 2014 outbreak in West Africa with over 27,000 cases and 11,000 deaths (Gire et al., 2014). Ebolaviruses are non-segmented, negative-strand RNA viruses belonging to the Filoviridae family, Mononegavirales order. The ebolavirus genomes consist of seven genes encoding nine major proteins in the case of EBOV. The viral proteins VP30, VP35, and nucleoprotein (NP) encapsidate the negative-stranded genome to form the nucleocapsid structure. The viral RNA dependent RNA polymerase (polymerase L) binds the viral genome and sequentially transcribes each gene. VP40 is the major matrix protein and the main protein that triggers budding of filamentous particles. The glycoprotein is expressed as a secreted form (sGP) and a trimeric glycoprotein (GP) expressed on the viral surface. The GP contains the ectodomain required for receptor binding (GP1) and fusion (GP2). GP appears to be the primary determinant for protection against lethal infection, although other proteins can also play a role (Sullivan et al., 2009). GP and VP40 can assemble into virus-like particles (VLPs) when expressed ectopically in mammalian or insect cells (Bavari et al., 2002; Noda et al., 2002; Swenson et al., 2004; Warfield et al., 2003), and other viral proteins such as NP and VP24 can also be incorporated into the particles (Bavari et al., 2002; Kallstrom et al., 2005; Swenson et al., 2004). There are multiple clinical trials evaluating the Ebola vaccines that are ongoing and using various technologies for determining immune response. A serological assay with defined antigens, controls, and other essential parameters will be of paramount importance to testing and characterizing of immune response in vaccinated subjects. Enzyme-linked immunosorbent assays (ELISAs) have been widely used for the measurement of antibodies in many different types of matrices (biological fluids, culture media) (Voller et al., 1978). Accurate measurement of antibody titers from antisera or other fluids from immunized experimental animals or human clinical trials is one of the most important read-outs in order to evaluate the immunogenicity of experimental vaccine candidates or antibody response in infected individuals. The ELISAs described here were developed to measure the binding of specific IgG antibodies in NHP and human sera to purified recombinant EBOV GP ectodomain, lacking the transmembrane domain, (GPTM), an engineered GP lacking the mucin-like domain (GP^{muc}), and the matrix protein (VP40). During the basic assay development activities, multiple parameters were tested in order to optimize these assays. Those parameters included optimization of coating antigen concentration, secondary antibody concentration, and dilution series of the standard reference detection antibody (RDA) to assure a four-parameter logistic (4PL) curve. In addition, Quality Control (QC) samples were established, the assay's limit of detection was evaluated, and the effect of multiple freeze-thaws of the RDA, QC samples, and coating antigen on assay performance was assessed. NHP serum samples from EBOV VLP vaccinated animals and serum samples from EBOV infected human individuals were then evaluated using the developed serology ELISAs.

2. Materials and Methods

2.1. Critical Reagents

EBOV GP TM and GP muc—The EBOV GP TM and EBOV GP muc antigens were generated by expression in mammalian 293T cells similar to the procedure previously reported (Lee et al., 2008). The EBOV GP TM nucleotide sequences corresponding to amino acids 1–636 was cloned into a modified pDisplay plasmid resulting in a C-terminal HA TAG (kindly provided by Dr. Erica Saphire (Scripps)). The EBOV GP muc nucleotide sequences corresponding to amino acids 1–636; delta 279–422 was cloned into a modified pDisplay plasmid resulting in a C-terminal HA TAG (kindly provided by Dr. Erica Saphire (Scripps)). Briefly, HEK293T cells were transfected with either the EBOV GP TM in the pDisplay plasmid or the EBOV GP muc in the pDisplay plasmid. Following expression, the media was collected and treated with phenylmethylsulfonyl fluoride (PMSF). Expressed proteins (either EBOV GP TM or EBOV GP muc) were purified using Q-Sepharose Fast Flow resin. Elutions were pooled and these fractions were passed over a GE S-200 HR column. S-200 fractions containing either EBOV GP TM or EBOV GP muc were pooled and dialyzed against PBS. Purified proteins were analyzed by standard BCA, SDS-PAGE, and Western Blot.

EBOV VP40—The EBOV VP40 antigen was generated by expression in *Escherichia coli* (*E. coli*) cells. To generate cDNA constructs encoding the VP40 protein with a 6xHis tag at the N-terminus, primers binding to the start and stop codons were used to reverse transcribe and PCR amplify purified viral RNA. EBOV VP40 DNA was re-cloned into a Trx (TAG) expression system containing a cleavable linker. The recombinant EBOV VP40 protein was expressed in *E. coli* and the recombinant protein was purified using affinity chromatography. The TAG was removed from the EBOV VP40 protein by protease cleavage. Purified protein was analyzed by standard BCA, SDS-PAGE, and Western Blot.

EBOV RDA standard and QCs—RDA and QCs were generated from pooled macaque plasma from macaques vaccinated with EBOV VLPs. Plasma from treated animals that showed high antibody titers to EBOV glycoprotein were chosen to generate the RDA or QC pool.

Affinity purified polyclonal antibody to Human IgG heavy and light chains (whole IgG) made in Goat and labeled with horseradish peroxidase (anti-human IgG-HRP) was purchased from KPL (Catalogue Number: 474–1006).

NHP and human sera—Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility, Covance (Denver, PA), is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Vaccinations were conducted under BSL-2 conditions. All studies were approved by the Institutional Animal Care and Use Committee at Covance. Male cynomolgus macaques of Mauritius origin of > 5 kg in weight were selected based on the size/weight requirements. Cohorts of 3–4 NHPs were vaccinated with the formulated

vaccine containing VLPs supplemented with QS-21 adjuvant (Antigenics, Lexington, MA), or QS-21 only (Warfield et al., 2015) Blood collections were performed at day -1 (pre-vaccination) and days 14, 42, 56 and 63 (post-vaccination). Blood collections for human patients were approved from the government agencies within the respective countries.

2.2. ELISA

Antigens diluted in 1× Dulbecco's Phosphate Buffered Saline (DPBS, Corning) were immobilized on 96-well MaxiSorp plates (Nunc) for 12–24 hours at 4°C. Non-specific binding sites were blocked up to 3 hours at ambient temperature using StartingBlock T20 (PBS) Blocking Buffer (Pierce/Thermo Scientific). Following automated washes using a BioTek ELx405 plate washer (1× DPBS + 0.05% Tween-20), plates were incubated with RDA, test serum or plasma dilutions for 2 hours at ambient temperature. After washing, bound antibodies were detected with a dilution of anti-human IgG-HRP (KPL) for 1 hour at ambient temperature, and 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Life Technologies) for 30 minutes at ambient temperature. Assay absorbance values were determined at 650 nm using a VersaMax plate reader (Molecular Devices).

2.3. Characterization of the RDA

The reference standard determination was performed as previously described (Miura et al., 2008). A standard plasma referred to here as RDA was selected by pooling plasma from cynomolgus macaques vaccinated with EBOV VLPs. The RDA was tested multiple times in full dilution series on separate ELISA plates coated with each of the three antigens (EBOV GP_{TM}, GP_{muc}, and VP40) and analyzed using 4PL curve fit using Softmax Pro (version 5.4, Molecular Devices). The average dilution at the inflection points of these 4PL curves was used to establish the number of arbitrary antibody units/milliliter (AU/mL) for the RDA. The RDAs used in the assays described in this paper were assigned the AU/mL concentrations of 2302 AU/ml, 1451 AU/ml, and 5195 AU/ml for GP_{TM}, GP_{muc}, and VP40, respectively.

Full dilution curve of RDA was tested as a reference standard curve on every ELISA plate and graphed with AU/ml on the X-axis and optical density at 650 nm (OD₆₅₀) on the Y-axis. The following 4PL equation was then used to quantify the AU/mL of the unknown (test) samples:

$$X = C \left(\frac{A - Y}{Y - D} \right)^{\left(\frac{1}{B} \right)}$$

In which X = AU/mL, Y = OD₆₅₀ and parameters A (lower asymptote), B (slope), C (inflection point) and D (upper asymptote) are generated from the SoftMax standard curve. The 4PL equation and the four parameters are utilized in Excel to calculate the AU/mL (X) based on OD₆₅₀ (Y) for the test samples.

2.4. Selection of coating antigen concentration and secondary antibody-HRP conjugate dilution

Multiple combinations of coating antigen amounts and anti-human IgG-HRP dilutions were tested using a single dilution (1:100) of EBOV macaque RDA. The coating antigens (EBOV GP_{TM}, GP_{muc}, or VP40) tested ranged from 800 to 6.3 ng/well in combination with anti-human IgG-HRP diluted from 1:1,000 to 1:10,000. Based on the results from the initial testing, a limited number of coating antigen concentrations and anti-human IgG-HRP were selected. These optimal coating antigen concentrations and anti-human IgG-HRP dilutions were evaluated using 2.5-fold dilutions of RDA ranging from 1:40 to 1:953,674. This RDA range generated a full RDA standard curve.

2.5. Establishing QC samples for setting the limits for RDA standard performance

Assay controls were established to determine whether the RDA curves, used to estimate the AU/mL values of unknown test samples, were performing within acceptable precision. A pool of macaque plasma from EBOV VLP vaccinated macaques distinct from the plasma used for the RDA standard was diluted in naïve macaque plasma (NMP) to establish three controls: high, medium, and low quality controls (HQC, MQC, and LQC, respectively). These three controls were diluted in StartingBlock and treated as unknown test samples. The NMP were pre-tested and shown to have low background absorbance in the assays (OD<0.15). The HQC, MQC, and LQC samples were optimized such that the OD value of the HQC targeted at the upper end of the linear range of the RDA curve, the OD value of the MQC targeted the middle of the linear range of the curve, and the OD value of the LQC targeted the lower end of the linear range of the RDA curve.

2.6. Evaluating RDA, QC samples and coating antigen performance after multiple freeze thaw cycles

The RDA, QC samples and coating antigen were tested following multiple freeze-thaw cycles. This was performed by freezing single-use aliquots at -80°C overnight and then allowing them to thaw on the bench top to ambient temperature. Individual single-use aliquots were subjected to up to six freeze-thaw cycles. All samples for each component were then evaluated in a single ELISA to reduce assay-to-assay variability.

3. Results

3.1. Selection of coating antigen concentration and secondary antibody-HRP conjugate dilution

Initial coating antigen amounts of GP_{TM}, GP_{muc}, and VP40 and anti-human IgG-HRP dilutions were tested in a checkerboard fashion using a single dilution (1:100) of EBOV macaque RDA. The optimal amount of coating antigen was determined by evaluating the signal-to-background ratios (data not shown). Once the optimal concentrations were selected, the assay was tested with limited antigen amounts of GP_{TM}, GP_{muc}, and VP40 and anti-human IgG-HRP dilutions using a full dilution curve of the EBOV macaque RDA. The OD₆₅₀ values were plotted against the dilution of RDA and the optimal concentrations were determined based on achieving the OD₆₅₀ values near 4.0 (the upper limit of the plate

reader) at the highest RDA concentration and minimal background in negative controls ($OD_{650} < 0.07$). The result of the assay for GP TM is shown in (Supplemental Figure 1). Based on these experiments, the optimal coating amount and dilution of anti-human IgG-HRP were determined for all three antigens (Table 1).

3.2. Establishing the Antibody Units (AU/mL) value for the EBOV macaque RDA standard

The EBOV macaque RDA standard was tested for each antigen at 1:2.5-fold dilutions in multiple independent experiments performed on different dates (thirteen experiments for GP TM), nine experiments for GP muc, and twenty two experiments for VP40 (Supplemental Figure 2A–C). The dilution at the inflection point (C parameter) of each 4PL curve was used to establish the number of AU/mL for the RDA in which the inflection point is automatically calculated by SoftMax during data transformation into a 4PL curve fit model. The independent experiments resulted in largely overlapping curves (Supplemental Figure 2A–C). The coefficient of variation (%CV) for the upper asymptote, slope, and inflection point were below 20% for all antigens. The higher %CV for the lower asymptote was expected as the values were extremely low and slight variations were magnified in those percentages (Table 2). Inflection point values were averaged from RDA standard curves of all the experiments for each antigen to calculate the antigen specific RDA concentration (AU/mL). As shown in Figure 1 for GP TM, out of 13 repeats, nine EC_{50} values were within the 95% confidence interval and no trending in one direction was observed. Similar results were obtained for GP muc and VP40 (data not shown).

3.3. Establishing the RDA standard curve for quantifying AU/mL values of test samples

Based on the assigned concentrations of RDA (AU/mL) for each antigen, the data from Table 2 were transformed to graph the RDA standard curve using a 4PL curve fit with AU/mL values on the x-axis and OD_{650} on the y-axis (Figure 2A–C). For example a 1:100 dilution of RDA for GP TM standard curve was transformed to 23.02 AU/ml (based on RDA concentration of 2302 AU/ml). Based on the definition of RDA concentration, the C parameter (inflection point) of the Standard Curve must be close to 1.0 AU/mL (For example, the C value is 1.04 for GP TM as shown in Figure 2A). Using OD_{650} of dilutions of unknown sample, AU/mL values for the unknown samples are calculated based on the AU/mL values of the transformed standard curve.

3.4. RDA Evaluation to determine the acceptable OD range by comparison of back-calculated AU/mL standard values to the target AU/mL values

The acceptable range within a RDA standard was evaluated by comparing the back-calculated AU/mL values to the target AU/mL values as determined in Table 3. The difference between the back-calculated and target values is considered acceptable if the difference between the two values is $\leq 20\%$. The OD values associated with the standards that met the mentioned criterion comprise the acceptable OD range. The acceptable range of OD values from a representative standard curve for GP TM, GP muc and VP40 are shown bolded in Table 3. For each assay run, the acceptable range was defined and test values falling outside this range were not quantified.

3.5. Establishing QC samples for setting the limits for RDA standard performance

To establish QC samples, dilutions of VLP-vaccinated macaque plasma pool (VMP, distinct from the RDA) were prepared in NMP to generate high (H), medium (M), and low (L) quality control (QC) samples.

For GP_{TM} a 1:5 dilution of the VMP in NMP followed by a 1:100 dilution in StartingBlock (a 1:500 dilution total) was selected to represent the HQC because the OD value was at the upper end of the RDA curve (Figure 2A). A 1:17 dilution of VMP in NMP followed by a 1:100 dilution in StartingBlock buffer (a 1:1,700 dilution total) was selected to represent the MQC because the OD value was in the middle of the RDA curve (Figure 2A). Finally, a 1:200 dilution of VMP in NMP followed by a 1:100 dilution in StartingBlock buffer (a 1:20,000 dilution total) was selected to represent the LQC because the OD value was at the lower end of the linear range of the RDA curve (Figure 2A). Similarly the QC samples were established for GP_{muc} (Figure 2B) and VP40 (Figure 2C).

QC samples were evaluated for precision by obtaining 5 – 7 independent determinations. The performance results for QC samples are shown in Table 4. All QC values had an imprecision level < 20 %.

3.6. Evaluating RDA, QC samples, and coating antigen performance after multiple freeze thaw cycles

The single-use aliquots of the RDA, QC samples, and coating antigen were subjected to up to six freeze-thaw cycles. One ELISA was performed with all RDA and all QC freeze-thaw samples and one ELISA was performed with all coating antigen freeze-thaw samples. No significant difference in signal was observed for up to six freeze-thaw cycles for the RDA or the coating antigen for GP_{TM}, GP_{muc} and VP40 (Supplemental Figure 3).

3.7. Evaluation of antibody response in vaccinated NHPs and infected humans

NHPs vaccinated with EBOV VLPs developed high IgG antibody titers against GP_{TM} (Figure 3A), GP_{muc} (Figure 3B) and VP40 (Figure 3C) after two vaccinations while NHPs vaccinated only with QS-21 adjuvant did not demonstrate appreciable antibody responses (Figure 3A–C). As a proof of concept that the assay also performs for human samples, sera from twelve humans that were infected with EBOV were tested for reactivity to GP_{TM}, GP_{muc} and VP40 using the developed serological assays. These patients were tested by qPCR immediately upon admittance to the Ebola Treatment Center. These patients were re-tested by qPCR between 10 to 14 days post admittance to the center and were qPCR negative at the time of release. Antibody responses against GP_{TM}, GP_{muc} and VP40 were observed in 11 of the 12 samples tested with the highest antibody responses from the two samples from 1977–1978 and compared to a pool of normal human sera (Figure 4).

4. Discussion

In this report we describe a set of three semi-quantitative ELISA methods for reliable measurement of antibody responses to the Ebola virus glycoproteins (GP_{TM}, GP_{muc}) and matrix protein (VP40). Using this ELISA, the quantity of antibodies in unknown (test)

samples can be determined based on a standard curve of pooled plasma derived from EBOV VLP-immunized macaques. The cornerstone of this assay is a well-characterized plasma derived from seropositive VLP-vaccinated macaques, referred to as Reference Detection Antibody (RDA). The full-dilution curve of the RDA on each antigen analyzed by a 4PL curve fit is used to determine the inflection point (EC_{50}) as a semi-quantitative measure of its reactivity to each of the antigens. The EC_{50} is then assigned as “Antibody Units per Milliliter” (AU/mL) concentration to the RDA. The assigned concentration is the basis of the standard curve used in each assay to determine the antibody titers of unknown samples in AU/mL. Using an RDA for the ELISA method allows for fewer dilutions of test samples compared with titration by full curve endpoint dilution. Also, using the RDA and QCs allows for comparison of values acquired from ELISAs performed by different analysts, on different days, or in different laboratories.

In order to identify the reagent concentrations that would provide a strong signal for the ELISA methods and optimal sensitivity, coating antigens and anti-human IgG-HRP detection antibody were titrated in checkerboard fashion. These initial studies were performed with a single dilution of the RDA and then reagent concentrations were refined using full-dilution curves for the RDA. This optimization provided an RDA curve, using a 4PL curve fit, with a well-defined upper and lower asymptote. This approach provided acceptable accuracy and precision measurements as shown in Tables 2 & 3.

It is expected that the OD_{650} values for the RDA will vary from run to run, therefore, it was important to QC the RDA standard curve. Three QC samples were identified that would target the linear portion of the RDA curve. The QC samples were tested at 1:100 dilutions for GP_{TM} and GP_{muc} serology ELISAs and 1:500 dilutions for the VP40 serology ELISA. These dilutions represent the lowest dilution of an actual test sample. This would ensure that the QC sample would mimic any potential matrix effects in the test sample. A QC range was defined based on repeated performance of the QC samples. This criterion would be used to determine an acceptable RDA standard curve.

To evaluate one parameter of assay robustness, RDA, QC samples and coating antigens were subjected to multiple rounds of freeze-thaw cycles. Based on the data presented (Supplemental Figure 3), no deficiencies in assay performance were observed with up to six freeze-thaw cycles of the RDA, QCs or coating antigens.

The glycoprotein of EBOV, considered to be the primary protective antigen (Sullivan et al., 2009), consists of the multiple domains. The receptor binding region (RBR) is deeply positioned into the core of GP1 partially concealed by the highly glycosylated regions known as glycan cap and mucin-like domain (MLD) (Kuhn et al., 2006; Lee et al., 2008). All known EBOV GP neutralizing antibodies, including components of ZMapp (Murin et al., 2014) and KZ52 (Lee et al., 2008), bind to regions outside the MLD. Our unpublished observations indicate that these neutralizing antibodies as well as a set of monoclonal antibodies to the RBR that we have recently identified bind significantly better to an MLD-deleted GP (GP_{muc}) than the full length GP ectodomain (GP_{TM}). This suggests that these epitopes are partially masked by the MLD. Therefore, we reasoned that determining the antibody responses to GP_{muc} may be valuable for future clinical use as correlates of

protective immunity. To this end, the assays described here measure the antibody responses to both GP_{TM} and GP_{muc}.

The matrix protein VP40 is highly abundant in the virus. Our observations and published literature consistently show that infected individuals mount a strong antibody response to this antigen (Becquart et al., 2014; Becquart et al., 2010). However, antibodies to VP40 are not believed to play a role in protection from EBOV disease. Nevertheless, VP40 serology can be a sensitive method of detecting exposure to the virus in humans and animals. The assay described here appears to be highly sensitive in detecting anti-VP40 antibodies in macaque plasma.

The assays described here have been extensively used in multiple studies aimed at determination of immunogenicity and efficacy of VLP vaccines (Warfield et al., 2015) as well as correlates of immunity in VLP and adenovirus vaccinated macaques (Warfield et al, Manuscript in preparation). Thus, the assay is uniquely suited for vaccine efficacy studies in NHPs. However, the secondary antibody used in our assays is an affinity purified polyclonal antibody to human IgG heavy and light chains (whole IgG). Therefore, the assay is also most likely applicable to testing human samples. Our preliminary data (Figure 4) indicate that this may be the case. However, future studies should determine if any of the assay parameters need to be further optimized for human use. Based on the expected availability of positive human sera and plasma from the current EVD outbreak in West Africa, it is expected that a reference standard can be prepared for use in this assay in place of macaque RDA for more accurate detection of human anti-EBOV antibodies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- Bavari S, Bosio CM, Wiegand E, Ruthel G, Will AB, Geisbert TW, Hevey M, Schmaljohn C, Schmaljohn A, Aman MJ. Lipid raft microdomains: A gateway for compartmentalized trafficking of Ebola and Marburg viruses. *J Exp Med*. 2002; 195:593–602. [PubMed: 11877482]
- Becquart P, Mahlakoiv T, Nkoghe D, Leroy EM. Identification of continuous human B-cell epitopes in the VP35, VP40, nucleoprotein and glycoprotein of Ebola virus. *PLoS One*. 2014; 9:e96360. [PubMed: 24914933]
- Becquart P, Wauquier N, Mahlakoiv T, Nkoghe D, Padilla C, Souris M, Ollomo B, Gonzalez JP, De Lamballerie X, Kazanji M, Leroy EM. High prevalence of both humoral and cellular immunity to Zaire ebolavirus among rural populations in Gabon. *PLoS One*. 2010; 5:e9126. [PubMed: 20161740]
- Feldmann H, Kiley MP. Classification, structure, and replication of filoviruses. *Curr Top Microbiol Immunol*. 1999; 235:1–21. [PubMed: 9893375]
- Feldmann H, Klenk HD. Marburg and Ebola viruses. *Adv Virus Res*. 1996; 47:1–52. [PubMed: 8895830]

- Gire SK, Goba A, Andersen KG, Sealfon RS, Park DJ, Kanneh L, Jalloh S, Momoh M, Fullah M, Dudas G, Wohl S, Moses LM, Yozwiak NL, Winnicki S, Matranga CB, Malboeuf CM, Qu J, Gladden AD, Schaffner SF, Yang X, Jiang PP, Nekoui M, Colubri A, Coomber MR, Fonnies M, Moigboi A, Gbakie M, Kamara FK, Tucker V, Konuwa E, Saffa S, Sellu J, Jalloh AA, Kovoma A, Koninga J, Mustapha I, Kargbo K, Foday M, Yillah M, Kanneh F, Robert W, Massally JL, Chapman SB, Bochicchio J, Murphy C, Nusbaum C, Young S, Birren BW, Grant DS, Scheffelin JS, Lander ES, Happi C, Gevaio SM, Gnirke A, Rambaut A, Garry RF, Khan SH, Sabeti PC. Genomic surveillance elucidates Ebola virus origin and transmission during the 2014 outbreak. *Science*. 2014; 345:1369–1372. [PubMed: 25214632]
- Kallstrom G, Warfield KL, Swenson DL, Mort S, Panchal RG, Ruthel G, Bavari S, Aman MJ. Analysis of Ebola virus and VLP release using an immunocapture assay. *J Virol Methods*. 2005; 127:1–9. [PubMed: 15893559]
- Kuhn JH, Radoshitzky SR, Guth AC, Warfield KL, Li W, Vincent MJ, Towner JS, Nichol ST, Bavari S, Choe H, Aman MJ, Farzan M. Conserved receptor-binding domains of Lake Victoria marburgvirus and Zaire ebolavirus bind a common receptor. *J Biol Chem*. 2006; 281:15951–15958. [PubMed: 16595665]
- Lee JE, Fusco ML, Hessel AJ, Oswald WB, Burton DR, Saphire EO. Structure of the Ebola virus glycoprotein bound to an antibody from a human survivor. *Nature*. 2008; 454:177–182. [PubMed: 18615077]
- Miura K, Orcutt AC, Muratova OV, Miller LH, Saul A, Long CA. Development and characterization of a standardized ELISA including a reference serum on each plate to detect antibodies induced by experimental malaria vaccines. *Vaccine*. 2008; 26:193–200. [PubMed: 18054414]
- Murin CD, Fusco ML, Bornholdt ZA, Qiu X, Olinger GG, Zeitlin L, Kobinger GP, Ward AB, Saphire EO. Structures of protective antibodies reveal sites of vulnerability on Ebola virus. *Proc Natl Acad Sci U S A*. 2014; 111:17182–17187. [PubMed: 25404321]
- Noda T, Sagara H, Suzuki E, Takada A, Kida H, Kawaoka Y. Ebola virus VP40 drives the formation of virus-like filamentous particles along with GP. *J Virol*. 2002; 76:4855–4865. [PubMed: 11967302]
- Sullivan NJ, Martin JE, Graham BS, Nabel GJ. Correlates of protective immunity for Ebola vaccines: implications for regulatory approval by the animal rule. *Nat Rev Microbiol*. 2009; 7:393–400. [PubMed: 19369954]
- Swenson DL, Warfield KL, Kuehl K, Larsen T, Hevey MC, Schmaljohn A, Bavari S, Aman MJ. Generation of Marburg virus-like particles by co-expression of glycoprotein and matrix protein(1). *FEMS Immunol Med Microbiol*. 2004; 40:27–31. [PubMed: 14734183]
- Voller A, Bartlett A, Bidwell DE. Enzyme immunoassays with special reference to ELISA techniques. *J Clin Pathol*. 1978; 31:507–520. [PubMed: 78929]
- Warfield KL, Bosio CM, Welcher BC, Deal EM, Mohamadzadeh M, Schmaljohn A, Aman MJ, Bavari S. Ebola virus-like particles protect from lethal Ebola virus infection. *Proc Natl Acad Sci U S A*. 2003; 100:15889–15894. [PubMed: 14673108]
- Warfield KL, Dye JM, Wells JB, Unfer RC, Holtsberg FW, Shulenin S, Vu H, Swenson DL, Bavari S, Aman MJ. Homologous and heterologous protection of nonhuman primates by ebola and Sudan virus-like particles. *PLoS One*. 2015; 10:e0118881. [PubMed: 25793502]

- We have developed and standardized three quantitative serology ELISAs to detect anti-Ebola antibodies
- The serology ELISAs detect antibodies against two forms of the Ebola glycoprotein, and Ebola matrix protein
- Each serology ELISA can detect anti-Ebola antibodies generated in non-human primates vaccinated with Ebola virus-like particles
- Each serology ELISA can detect anti-Ebola antibodies in humans previously exposed to the Ebola virus.

Figure 1. Trending C-parameter values of EBOV macaque RDA tested on 13 plates over 13 different test dates. Solid line: average C value assigned to RDA as AU/ml and dashed lines: 95% confidence interval.

Figure 2.

RDA Standard curves for EBOV (A) GP TM, (B) GP muc, (C) VP40. Closed symbols represent High (HQC), medium (MQC), and low (LQC) quality controls. Panel (A) GPdTM, a 1:5 dilution of the EBOV macaque QC pool was performed to establish the HQC, a 1:17 dilution of the EBOV macaque QC pool was performed to establish the MQC, and a 1:200 dilution of the EBOV macaque QC pool was performed to establish the LQC (closed circles).

Figure 3. Analysis of NHPs vaccinated with VLPs. NHPs receiving EBOV VLPs (Circles) and adjuvant only (squares) are shown. Antibody responses against (A) GP TM, (B) GP muc, (C) VP40 were quantitated in NHP sera from day -1 (pre-vaccination) through day 63 (post-vaccination).

Figure 4.

Analysis of humoral immune response in human infected samples. Antibody responses against GP Δ TM, GP Δ muc, or VP40 were quantitated in human sera.

Table 1

Optimal coating antigen amounts and dilutions of anti-human IgG-HRP were determined for all three antigens.

	GP TM	GP muc	VP40
Antigen (ng/well)	75	100	300
Anti-human IgG-HRP (dilution)	1:5000	1:8000	1:5000

Table 2

Four parameter values of RDA standard curves obtained from multiple experiments for each of the three antigens. A: upper asymptote, B: slope, C: inflection point, D: lower asymptote. Standard deviations (SD) and coefficient of variation (%CV) are shown for each parameter. The average C-parameters (2302 for GP_{TM}, 1451 for GP_{muc}, and 5195 for VP40) were assigned as the AU/mL value for EBOV macaque RDA for the respective antigen.

Antigen	# of repeats	4PL Curve Parameters	Mean	SD	%CV
EBOV GP _{TM}	13	A	3.85	0.08	2%
		B	0.96	0.02	2%
		C	2302	383	17%
		D	0.0458	0.0092	20%
EBOV GP _{muc}	9	A	3.59	0.14	4%
		B	0.937	0.026	3%
		C	1451	202	14%
		D	0.0353	0.0095	27%
EBOV VP40	22	A	3.83	0.11	3%
		B	0.989	0.028	3%
		C	5195	624	12%
		D	0.0493	0.0076	15%

Table 3

Analytical Recovery of the standard curves. The acceptance range (in bold) is established by selecting the values that demonstrate recovery 80–120% of the nominal concentration.

RDA Standard	EBOV GP TM			EBOV GP muc			EBOV VP40		
	Nominal Concentration (AU/mL)	Backfit Concentration (AU/mL)	Analytical Recovery (Percent)	Nominal Concentration (AU/mL)	Backfit Concentration (AU/mL)	Analytical Recovery (Percent)	Nominal Concentration (AU/mL)	Backfit Concentration (AU/mL)	Analytical Recovery (Percent)
1	23.020	31.504	137%	111.450	432.407	388%	51.950	104.374	201%
2	9.208	7.780	84%	44.580	61.166	137%	20.780	19.115	92%
3	3.683	3.661	99%	17.832	15.295	86%	8.312	7.136	86%
4	1.473	1.517	103%	7.133	6.74	94%	3.325	3.522	106%
5	0.589	0.598	101%	2.853	2.914	102%	1.330	1.339	101%
6	0.236	0.23	98%	1.141	1.179	103%	0.532	0.524	99%
7	0.094	0.09	95%	0.456	0.456	100%	0.213	0.211	99%
8	0.038	0.035	93%	0.183	0.172	94%	0.085	0.084	99%
9	0.015	0.014	93%	0.073	0.069	94%	0.034	0.034	100%
10	0.006	0.007	116%	0.029	0.03	103%	0.014	0.014	103%
11	0.002	0.004	166%	0.012	0.014	120%	0.005	0.005	92%
12	0.001	0.003	311%	0.005	0.009	193%	0.002	0.003	138%

Determination of the recommended ranges of AU/mL values of high (HQC), medium (MQC), and low (LQC) Quality Controls. A %CV was obtained for each of the HQC, MQC, and LQC over different dates to establish a recommended acceptable range for each control.

Table 4

Antigen	# of repeats	QC samples	Mean AU/ml	SD	%CV	Min= Mean - (2*SD)	Max= Mean + (2*SD)
EBOV GP TM	7	HQC	391.06	37.67	9.6%	315.71	466.40
		MQC	99.68	4.71	4.7%	90.27	109.09
		LQC	8.91	0.68	7.7%	7.54	10.27
EBOV GP muc	5	HQC	310.25	19.21	6.2%	271.83	348.68
		MQC	82.28	5.85	7.1%	70.58	93.98
		LQC	8.60	0.41	4.7%	7.78	9.41
EBOV VP40	5	HQC	2023.86	402.51	19.9%	1218.85	2828.88
		MQC	638.82	56.11	8.8%	526.60	751.04
		LQC	66.50	4.53	6.8%	57.44	75.56