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Simon Valayer, Marie Alexandre, Mélanie Prague, Abdoul Habib Beavogui, Seydou Doumbia, et al.. Evaluation of waning of IgG antibody responses after rVSV Δ G-ZEBOV-GP and Ad26.ZEBOV, MVA-BN-Filo Ebola virus disease vaccines: a modelling study from the PREVAC randomized trial. *Emerging microbes & infections*, 2024, Online ahead of print. 10.1080/22221751.2024.2432353 . hal-04817450

HAL Id: hal-04817450

<https://inria.hal.science/hal-04817450v1>

Submitted on 5 Dec 2024

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To link to this article: <https://doi.org/10.1080/22221751.2024.2432353>



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Accepted author version posted online: 19 Nov 2024.



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Publisher: Taylor & Francis & The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group, on behalf of Shanghai Shangyixun Cultural Communication Co., Ltd

Journal: *Emerging Microbes & Infections*

DOI: 10.1080/22221751.2024.2432353



Evaluation of waning of IgG antibody responses after rVSVΔG-ZEBOV-GP and Ad26.ZEBOV, MVA-BN-Filo Ebola virus disease vaccines: a modelling study from the PREVAC randomized trial

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Abstract

rVSVΔG-ZEBOV-GP and Ad26.ZEBOV, MVA-BN-Filo are WHO-prequalified vaccination regimens against Ebola virus disease (EVD). Challenges associated with measuring long-term clinical protection warrant the evaluation of immune response kinetics after vaccination.

Data from a large phase 2 randomized double-blind clinical trial (PREVAC) were used to evaluate waning of anti-Ebola virus (EBOV) glycoprotein (GP_{1,2}) antibody concentrations after rVSVΔG-ZEBOV-GP or Ad26.ZEBOV, MVA-BN-Filo vaccination with linear mixed-effect regression models.

After a post-vaccination peak, each vaccination strategy was associated with a decrease of anti-EBOV GP_{1,2} antibody concentrations with distinct kinetics, highlighting a less-rapid decline in antibody levels after vaccination by rVSVΔG-ZEBOV-GP. One year after administration of the vaccine, antibody concentrations were higher in children compared to adults for both vaccines, although with different effect sizes: 1.74-fold higher concentrations (95% confidence interval [CI] [1.48; 2.02]) for children 12–17 years old to 3.10-fold higher concentrations (95% CI [2.58; 3.69]) for those 1–4 years old compared to adults for

Ad26.ZEBOV, MVA-BN-Filo versus 1.36-fold (95% CI [1.12; 1.61]) to 1.41-fold (95% CI [1.21; 1.62]) higher at month 12 higher than these values for adults, with relatively small changes from one age category of children to another for rVSVΔG-ZEBOV-GP. Antibody concentrations also differed according to geographical location, pre-vaccination antibody concentration, and sex.

In combination with knowledge on memory response, characterization of the major determinants of immune response durability of both vaccinations may guide future EVD control protocols.

Clinical Trials Registration. ClinicalTrials.gov registration: NCT02876328.

Keywords. Ebola virus disease; vaccine; immunogenicity; antibody; Western Africa; modelling;

Introduction

Ebola virus (EBOV) has caused recurrent outbreaks of Ebola virus disease (EVD) for more than four decades. The Western African outbreak (2013–16) prompted a global research response that included the rapid acceleration of vaccine clinical trials [1,2].

Two vaccines—rVSVΔG-ZEBOV-GP (based on a recombinant vesicular stomatitis Indiana virus) and Ad26.ZEBOV, MVA-BN-Filo (dose 1 based on a recombinant human adenovirus type 26, followed by dose 2 of modified vaccinia virus Ankara)—have been prequalified by the World Health Organization (WHO) and have received marketing authorization by the European Medicines Agency (EMA) [3]. Also, rVSVΔG-ZEBOV-GP has been licensed by the U.S. Food and Drug Administration (FDA). Both vaccines have been shown immunogenic and safe in numerous clinical trials [4-11]. However, the sporadic nature of EVD outbreaks has limited the ability to conduct large phase 3 efficacy trials, with the notable exception of rVSVΔG-ZEBOV-GP having been shown to provide protection in a

immediate-versus-deferred ring vaccination trial conducted during the 2013–16 outbreak in Guinea [12]. This efficacy was recently reaffirmed by a study conducted during the 2018–20 outbreak in the Democratic Republic of the Congo [13]. Although definitive correlates of protection have not yet been demonstrated, previous studies have suggested that immunoglobulin G (IgG) antibodies targeting EBOV glycoprotein (GP_{1,2}) are associated with protection against EVD, albeit without a validated protective threshold [14]. The marketing authorization for Ad26.ZEBOV, MVA-BN-Filo has therefore been primarily based on extrapolation from experimental nonhuman primate efficacy studies and vaccine immunogenicity assessed by antibody responses in human clinical trials. However, the exact level of protection remains unknown.

Both rVSVΔG-ZEBOV-GP and Ad26.ZEBOV, MVA-BN-Filo vaccination strategies have been used widely in recent EVD outbreaks [15-17]. In the absence of trials to assess longer-term efficacy, the characterization of the durability of antibody responses provides an important, albeit partial, indicator of the durability of protection. Additionally, it is important to characterize the host factors that may be associated with the magnitude, kinetics, and durability of the antibody responses to each vaccine. The Partnership for Research on Ebola Vaccinations (PREVAC) [18] has recently published results of its randomized trial evaluating the immunogenicity and safety of antibody responses in adults and children from several Western African countries over a 12-month period following vaccination with either rVSVΔG-ZEBOV-GP or Ad26.ZEBOV, MVA-BN-Filo [19]. Here, we report the results of a modelling study, based on the PREVAC data, that evaluated the kinetics of antibody responses among participants with different demographic characteristics throughout the 12-month period after vaccination.

Materials and methods

Study design and population

The PREVAC trial (NCT02876328) was an international, randomized, double-blinded, placebo-controlled clinical trial that assessed three vaccination strategies in healthy adults and children older than 1 year in Guinea, Liberia, Mali, and Sierra Leone. Vaccine strategies included: (1) one dose of rVSVΔG-ZEBOV-GP, with a second dose at day 56; (2) one dose of rVSVΔG-ZEBOV-GP, followed by a placebo dose at day 56 (referred to as rVSVΔG-ZEBOV-GP-placebo arm); and (3) a dose of Ad26.ZEBOV, followed by a dose of MVA-FN-Filo at day 56. Because rVSVΔG-ZEBOV-GP is approved as a one-dose vaccine regimen, the experimental two-dose arm was not included in this modelling study; rather, modelling was based on data from participants of rVSVΔG-ZEBOV-GP-placebo; Ad26.ZEBOV, MVA-BN-Filo; and pooled “placebo–placebo” arms as defined by the protocol. The primary endpoint of the trial was the anti-EBOV GP_{1,2} IgG antibody concentrations measured 12 months after vaccination [18]. After the initial dose of vaccine or placebo, participants were scheduled for follow-up visits at day 7 (± 3 days), day 14 (± 3 days), and day 28 (± 7 days). All participants were administered a second dose of a vaccine or placebo on day 56 (53–66 days), followed by visits at day 63 (7 ± 3 days after the second dose), month 3 (± 14 days), month 6 (± 1 month), and month 12 (± 1 month).

Antibody assay

Serum concentrations of IgG binding antibodies against EBOV GP_{1,2} were measured before vaccination and at each follow-up visit by the Filovirus Animal Non-Clinical Group (FANG) enzyme-linked immunosorbent assay (ELISA). Details and results from the formal validation of the FANG assay were previously described [20] and discussed in supplementary section S3.6.1 of the PREVAC trial primary publication [19]. The standard operating procedure that contains the FANG assay for the PREVAC trial is in the Supplementary Material. Analyses

were performed by two separate laboratories according to the country of participant origin: the Liberian Institute for Biomedical Research (LIBR) in Liberia analyzed samples from Guinea and Sierra Leone, and the National Institutes of Health (NIH) National Institute of Allergy and Infectious Diseases (NIAID) Integrated Research Facility at Fort Detrick (IRF-Frederick) in Maryland, USA, analyzed samples from Liberia and Mali.

Statistical analysis

Linear mixed-effect regression models were used to investigate anti-EBOV GP_{1,2} IgG dynamics after the post-vaccination peak and to determine any association with participant characteristics. Two models were used to independently evaluate the two active vaccination strategies: model A for rVSVΔG-ZEBOV-GP–placebo and pooled placebo arms; model B for Ad26.ZEBOV, MVA-BN-Filo and pooled placebo arms. The dependent variable was the log₁₀-transformed anti-EBOV GP_{1,2} IgG concentrations over time. Modelling began at the peak of anti-EBOV GP_{1,2} IgG concentrations (day 28 for model A and month 3 for model B) and ended at month 12. Time was modeled as a linear trend with one slope for model A (rVSVΔG-ZEBOV-GP) and two slopes for model B (Ad26.ZEBOV, MVA-BN-Filo), with a transition at month 6 to better consider the specific dynamics. The following variables were analyzed as fixed-effect covariates (single-effect and interaction with time): vaccine arm (vaccine versus pooled placebo arms, with placebo as reference), age category (1–4 years, 5–11 years, 12–17 years, ≥18 years [reference]), sex (women, men [reference]), country (Guinea, Liberia, Sierra Leone, Mali [reference]), laboratory for FANG assay analyses, anti-EBOV GP_{1,2} IgG concentrations prior to vaccination, vaccination time (hour in the day) for dose 1 and dose 2, body mass index (BMI; for adults) or Z score (for children), malaria status (defined as a known clinical malaria case within a month prior to the boost), HIV-1 infection status, lymphopenia, neutropenia, and eosinophilia. The analyses were performed on available

data; only 5.2% of antibody response measurement data were missing (all time points considered) and considered missing at random. To build the multivariate model and to find the best trade-off between the goodness of fit and model simplicity, the final selection among the multivariable models was guided by the Akaike Information Criterion (AIC). An effect was considered significant if the p -value was less than 0.05 using a Wald test. Both models included a random effect on the intercept, and model A included a random effect on the slope. Both models were fitted using restricted maximum-likelihood estimation (REML). The fixed-effect covariates in the final model were determined to be age category, sex (only for the rVSVΔG-ZEBOV-GP–placebo arm), country, and pre-vaccination anti-EBOV GP_{1,2} IgG concentrations. The performance of the models in predicting the anti-EBOV GP_{1,2} IgG concentrations at month 12 was evaluated by a leave-one-out cross-validation.

For both models, the effect of each covariate is presented as a ratio of the GMC in a natural scale between the category of interest and its reference. In addition, tables were created for the probability of reaching a concentration threshold (200; 600; and 1,000 EU/mL, corresponding to empirical thresholds used in previous clinical trials [19,21] with unknown clinical significance) at the end of the study and the time required after the beginning of the vaccination strategy for 50% of the population to drop below the anti-EBOV GP_{1,2} IgG GMC threshold, according to the age category (and sex for the rVSVΔG-ZEBOV-GP arm).

Additional details regarding the analyses are provided in the Supplementary Material. All analyses were performed with R, version 4.1.3 (R Foundation for Statistical Computing).

Results

Study population

The PREVAC randomized trial enrolled 1,400 adults and 1,401 children. Participants with administration errors, missing covariate data, and total missing data on the antibody levels

within the model timeframe were removed from the analyses. Thus, we utilized data from 781 (out of 802) participants randomized to the single-dose rVSVΔG-ZEBOV-GP arm (administered as rVSVΔG-ZEBOV-GP–placebo), 779 (out of 799) participants randomized to the Ad26.ZEBOV, MVA-BN-Filo arm, and 791 and 786 (out of 801) participants randomized to the pooled placebo arm in models A and B, respectively. The age, sex, country, and an anti-EBOV IgG concentration of >200 EU/mL prior to vaccination were well-balanced among arms [19].

Anti-EBOV GP_{1,2} IgG dynamics

Both vaccination strategies resulted in robust peak IgG responses when measured approximately 28–34 days after completion of the respective vaccination regimen. Graphical representations of the multivariable models' outputs are presented for the overall population, by age, sex (Figure 1), country, and pre-vaccination antibody concentrations (see Supplementary Material). Very slow waning anti-EBOV GP_{1,2} IgG mean concentrations (GMCs) were observed after rVSVΔG-ZEBOV-GP vaccination from the peak (28 days) to month 12. Although the early post-vaccination peak was higher after Ad26.ZEBOV, MVA-BN-Filo vaccination, anti-EBOV GP_{1,2} IgG GMCs decreased at two different rates from the peak (month 3, after dose 1), with rapid decline to month 6 and slower decline from month 6 to month 12.

Determinants of rVSVΔG-ZEBOV-GP single-dose vaccination and antibody concentration

The anti-EBOV GP_{1,2} IgG response after a single dose of rVSVΔG-ZEBOV-GP varied according to age, sex, country, and IgG titer prior to vaccination. At peak and month 12, higher GMCs were associated with a younger age, female sex, and higher GMCs prior to vaccination. All age categories of children had higher IgG GMCs than adults: the model

outputs, expressed as GMC ratios, estimated that GMCs for children were 1.32-fold higher (95% confidence interval [CI] [1.10; 1.57]) to 1.41-fold higher (95% CI [1.22; 1.64]) at peak and 1.36-fold (95% CI [1.12; 1.61]) to 1.41-fold higher (95% CI [1.21; 1.62]) at month 12 than these values for adults, with relatively small changes from one age category of children to another. Women had estimated 1.15-fold higher (95% CI [1.01; 1.30]) GMCs than men at peak, with an increase to 1.40-fold higher (95% CI [1.22; 1.59]) at the end of the study. Compared to participants from Mali, GMCs of participants from Sierra Leone were lower with a ratio of 0.80 (95% CI [0.67; 0.95] at peak and 0.69 (95% CI [0.58; 0.81]) at month 12, and GMCs of participants from Guinea were lower with a ratio of 0.73 (95% CI [0.62; 0.85]) at peak and of 0.74 (95% CI [0.63; 0.86]) at month 12. No difference was seen in GMCs from Liberia and Mali. Antibody concentrations higher than 200 EU/mL prior to vaccination were associated with a 1.31-fold higher (95% CI [1.07; 1.57]) response at peak and up to a 1.19-fold higher (95% CI [0.99, 1.41]) response at the end of the study. All GMC ratios for rVSV Δ G-ZEBOV-GP are presented in Table 1.

The model allowed estimation of the percentage of the IgG GMCs at month 12 remaining above different empirical thresholds (>200, >600, and >1,000 EU/mL) (Table 2). For rVSV Δ G-ZEBOV-GP, there was a very high and homogenous percentage of GMCs above 200 EU/mL at the end of study for children (99% for females and 97% for males) and for adults (97% for females and 94% for males). A 600 EU/mL threshold increased the difference in percentage for adults, diminishing to 75% for females and to 61% for males at end of the study; in contrast, at the 600 EU/mL threshold, the percentage for children decreased less than adults and remained homogenous within each age category compared to adults. At the 1,000 EU/mL threshold, the percentage dropped to 54% for female adults, 38% for male adults, 66% for female children, and 53% for male children. Overall, a higher estimated percentage of females reached the designated threshold compared to males,

regardless of age category. Additional probability explorations with a continuous threshold scale and time after vaccination required for 50% of the population to drop below the same three anti-EBOV GP_{1,2} GMC thresholds are shown in the Supplementary Material.

Determinants of Ad26.ZEBOV, MVA-BN-Filo vaccination and antibody concentration

The anti-EBOV GP_{1,2} IgG GMCs after vaccination with Ad26.ZEBOV, MVA-BN-Filo differed according to age, country, and IgG titer prior to vaccination. At peak and month 12, significantly higher IgG GMCs were associated with a younger age and location in Mali. Higher IgG GMCs prior to vaccination were associated with higher antibody concentrations at month 12. In contrast to the rVSVΔG-ZEBOV-GP model, sex was not associated with the antibody response after Ad26.ZEBOV, MVA-BN-Filo vaccination.

Age had a major effect on the IgG GMCs at peak (90 days after dose 1), with 4.68-fold higher (95% CI [3.82; 5.64]) IgG GMCs for children 1–4 years old compared to adults. With increased age, the difference in peak GMCs compared to adults was diminished to 2.82-fold higher (95% CI [2.32; 3.38]) for children 5–11 years old and 1.93-fold higher (95% CI [1.57; 2.35]) for those 12–17 years old. Similar trends were found at the end of the study, with 1.74-fold higher (95% CI [1.48; 2.02]) concentrations for children 12–17 years old and 3.10-fold higher (95% CI [2.58; 3.69]) concentrations for those 1–4 years old compared to adults. Participants from Guinea and Sierra Leone had a lower peak IgG GMCs than those from Mali with ratios of 0.59 (95% CI [0.49; 0.72]) and 0.38 (95% CI [0.30; 0.47]), respectively. These differences were maintained at month 12 with ratios of 0.80 (95% CI [0.67; 0.95]) and 0.61 (95% CI [0.49; 0.74]), respectively, compared to Mali. The IgG GMCs were also lower in Liberia compared to Mali with a ratio of 0.57 (at both peak and at month 12, 95% CI [0.44; 0.73] and [0.45; 0.72], respectively). Furthermore, pre-vaccination IgG GMCs higher than

200 EU/mL were associated with 1.38-fold higher (95% CI [1.12; 1.68]) IgG GMCs at the end of the study. All GMC ratios for Ad26.ZEBOV, MVA-BN-Filo are presented in Table 3.

There was a much higher percentage of a minimum IgG GMCs of 200 EU/mL at month 12 for children compared to adults (76%), a difference which slightly decreased with increased age (from 98% for children 1–4 years old to 91% for those 12–17 years old). In addition, the percentage for adults dropped to 28% for the 600 EU/mL GMC threshold and to 12% for the 1,000 EU/mL GMC threshold. With higher thresholds, the effect of age on the estimated percentage in children was even clearer. For the threshold 600 EU/mL, the percentages were 53% (12–17 years) and 77% (1–4 years); for the 1,000 EU/mL threshold, the percentages were 30% (12–17 years) and 56% (1–4 years). The percentage table is shown in Table 4. Additional probability explorations with a continuous threshold scale and time after vaccination required for 50% of the population to drop below the same three anti-EBOV GP_{1,2} GMC thresholds are available in the Supplementary Material.

Discussion

This modelling study, based on a large international randomized clinical trial, provides new information on the longitudinal dynamics of immune responses for the two currently approved EVD vaccination strategies. A more rapid decline in antibody levels after vaccination by Ad26.ZEBOV, MVA-BN-Filo were observed. Higher IgG responses in children than in adults were observed, with a more prominent difference after Ad26.ZEBOV, MVA-BN-Filo vaccination. Higher IgG responses were seen in participants with higher pre-vaccination antibody concentrations for both vaccination strategies and in women for the rVSVΔG-ZEBOV-GP vaccine only.

The observed differences in antibody response kinetics between the two vaccines may be explained by their distinct features, including different platforms and vectors, the surface

GP_{1,2} used in the vaccine, and the dosage. Our study confirms that vaccine-specific features are not the only variables responsible for differential response profiles, but host demographic characteristics also have a major impact on the immune response after vaccination. This impact differed between the two vaccination strategies, with greater variability for the Ad26.ZEBOV, MVA-BN-Filo vaccine.

Age was particularly associated with the humoral response to Ad26.ZEBOV, MVA-BN-Filo, with IgG GMCs at 12 months three times higher in the youngest children (1–4 years old) than in adults. The overall trend of higher immune responses in younger children is consistent with similar findings reported previously [22]. It has been well-documented in the context of other vaccines that age at vaccine receipt greatly influences subsequent vaccine-specific immune responses that are generally lower with increased age and extremely low in the elderly [23]. In this study, factors contributing to IgG response differences between adults and children include the lack of dose adaptation for participant age or BMI. Additionally, pre-existing memory responses to environmental adenoviruses may impair immune responses to adenovirus-based vaccines [24,25]. However, pre-existing immunity to Ad26 did not have an effect on vaccine-induced immune response in EVD or coronavirus disease (COVID-19) studies [26].

Sex was found to influence the humoral response to the rVSVΔG-ZEBOV-GP vaccination strategy, with women exhibiting higher IgG GMCs than men across age categories, in line with what had already been described [27,28]. Although this difference was significant but relatively small at peak, it reached 40% at 1 year after vaccination, putting it at the same order of magnitude as the effect of age on this vaccination strategy.

Pre-vaccination IgG concentrations influenced responses for both vaccination strategies, with higher humoral response at month 12 among participants with higher baseline concentrations. It should be noted that a history of EVD or previous vaccination against EVD were both

exclusion criteria for the PREVAC trial but were self-reported. The unexpectedly notable baseline antibody concentrations were also found in previous clinical studies [21,29] and prompted use of centralized analysis of the samples conducted in an independent laboratory (Q2 Solutions, which is validated for regulatory purposes) [30]. Pre-vaccination IgG may derive from prior unrecognized, unrecalled, or subclinical (or asymptomatic) EBOV infection, as suggested in previous studies [31,32]. This phenomenon aligns with the trial's geographic context, conducted in the three Western African countries most severely affected by the extensive 2013–16 EVD outbreak. Notably, pre-existing antibodies were more common in participants from these countries than those from Mali and rare in the youngest children born post-epidemic. Alternatively, though scored as “positive”, detection of low concentrations of IgG in the assay may represent prior exposure to cross-reacting antigens.

Since the FANG antibody assay was performed in two different laboratories, it is difficult to discriminate between country-specific and laboratory-related variation in the PREVAC trial. The FANG assay performance is associated with some degree of variability between laboratories despite use of similar method protocols and reagents [33]. The FANG assay has been commonly used in multiple vaccine trials [4,6,8,11], and its limitations have largely been addressed in the annex of a previous PREVAC paper²¹. However, a difference was observed between Malian and Liberian participants after the Ad26.ZEBOV, MVA-BN-Filo vaccine; notably, this geographic variation was observed despite analysis in the same central laboratory and confirmed in a second centralized analysis of the same data [29]. This geographic variation, unexplained by levels of baseline antibody concentrations, might be resultant of unmeasured genetic or exposure factors.

While this modelling is drawn from a single study, one of its main strengths is the design, as it relies on data from a multi-country randomized double-blind clinical trial that incorporated two authorized vaccination strategies, was based on a large recruitment

population from several Western African countries and included rural and urban participants with balance across sex and age strata.

Our results provide important insight to the longitudinal dynamics of the anti-EBOV GP_{1,2} IgG response, which, while not a validated correlate of protection, can be considered the best immunological marker associated with vaccine efficacy against EVD [34]. The probability of having an IgG titer above the empirical threshold of 200 EU/mL IgG at 1 year remained above 90% in most cases, except in adults vaccinated with Ad26.ZEBOV, MVA-BN-Filo. However, at higher thresholds, the probability decreased, raising questions about the level of immune response necessary for optimal protection. It remains to be determined whether the same host characteristics also impact the memory immune response in previously vaccinated individuals. Current data suggest that vaccinated individuals are likely protected one-year post-vaccination, but it is still too early to recommend changes to the EVD vaccination program, including adaptations for specific populations. However, it is crucial to closely monitor the waning of immune responses over time to ensure sustained vaccine efficacy longitudinally across different populations.

Recent EVD outbreaks are sporadic and rapidly brought under control due to a well-coordinated response that includes vaccination campaigns. Thus, it is challenging to detect infectious breakthroughs, cases that could prompt the need for additional injections. Our modelling data highlight the need for research on future EVD vaccination strategies, especially evaluating the value of follow-up doses with the same or different vaccine. The ongoing PREVAC trial (PREVAC-UP) will characterize the durability of immune response up to 5 years post-vaccination and will not be published before 2025; results from this trial will be particularly useful in assessing the antibody level dynamics after 1 year, estimating the durability of humoral response over time and if, when, and for whom follow-up vaccination might be appropriate.

Acknowledgments

We are grateful to the Ministries of Health of Guinea, Liberia, Sierra Leone, and Mali, which permitted the conduct of the trial. We furthermore thank The Alliance for International Medical Action and all site collaborators for their contribution in the implementation of the trial. The authors and study team thank the participants who consented to the trial. We also thank Anya Crane (Integrated Research Facility at Fort Detrick, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Fort Detrick, Frederick, MD, USA) for critically editing the manuscript and Jiro Wada (Integrated Research Facility at Fort Detrick, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Fort Detrick, Frederick, MD, USA) for preparing figures.

Part of the experiments presented in this paper were carried out using the PlaFRIM experimental testbed, supported by Inria; Centre National de la Recherche Scientifique; Laboratoire Bordelais de Recherche en Informatique and Institut de Mathématique de Bordeaux, Université de Bordeaux; Bordeaux INP; and Conseil Régional d'Aquitaine (see <https://www.plafrim.fr>).

Declaration of interest statement

No authors have associations that might pose a conflict of interest.

Additional information

Funding

This work was supported by the Innovative Medicines Initiative 2 Joint Undertaking (IMI2JU) under grant number 115854, EBOVAC1. This project has also received support from the European Union's Horizon 2020 research and innovation programme and EFPIA.

The dissemination represents only the authors' views and IMI2JU is not responsible for any use of the information contained in the dissemination.

This work was also supported by a dedicated Institut national de la santé et de la recherche médicale (Inserm) allocation on behalf the French Research Ministry.

This work was supported in part by the National Institutes of Health (NIH), by Inserm, and by the London School of Hygiene and Tropical Medicine (LSHTM). The clinical trial was conducted with the support of Janssen, Bavarian Nordic, and Merck Sharp & Dohme Corp., which provided the vaccines.

This work was also supported in part by the National Cancer Institute contract number HHSN261201500003I through the Frederick National Laboratory for Cancer Research.

This work was supported in part through the Laulima Government Solutions, LLC, prime contract with NIH National Institute of Allergy and Infectious Diseases (NIAID) under contract number HHSN272201800013C. I.M.B. performed this work as an employee of Laulima Government Solutions, LLC. J.H.K. performed this work as an employee of Tunnell Government Services (TGS), a subcontractor of Laulima Government Solutions, LLC, under contract number HHSN272201800013C.

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PREVAC-UP trial was part of the EDCTP2 program supported by the European Union (grant number RIA2017S-2014 – PREVAC-UP) and by the US National Institute of Allergy and Infectious Diseases of the National Institutes of Health.

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Data availability statement

All data used are from the recently published PREVAC trial [19]. Data from this study can be accessed by submitting a request to Professor Yazdan Yazdanpanah (yazdan.yazdanpanah@aphp.fr). The data availability begins one year after completion of the 5-year follow-up of the PREVAC trial.

Code availability

The code to reproduce the analyses can be accessed via https://github.com/sistm/PREVAC_UP_WP_Modeling.git

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Figure 1. Anti-EBOV-GP_{1,2} IgG response (EU/mL) from the post-vaccination peak to 12 months after the first dose of rVSVΔG-ZEBOV-GP or Ad26.ZEBOV, MVA-BN-Filo, respectively. (rVSVΔG-ZEBOV-GP) and (d) (Ad26.ZEBOV, MVA-BN-Filo): Observed and modelled (multivariable models) antibody GMCs over time in vaccine arm and pooled placebo arms in overall population. (b) (rVSVΔG-ZEBOV-GP) and (e) (Ad26.ZEBOV, MVA-BN-Filo): modelled (multivariable models) IgG

GMCs over time in active vaccine arm according to age category. (c) (rVSVΔG-ZEBOV-GP): modelled (multivariable models) IgG GMCs over time in active vaccine arm according to sex. IgG GMCs over time after (Ad26.ZEBOV, MVA-BN-Filo) vaccination is not presented according to sex, as sex was not included in the model (not significantly associated). Dots represent individually observed serum samples. The dashed horizontal line in each panel indicates 200 EU/mL anti-EBOV-GP_{1,2} IgG concentration. GMC = geometric mean concentration. GP_{1,2} = glycoprotein. EBOV = Ebola virus.

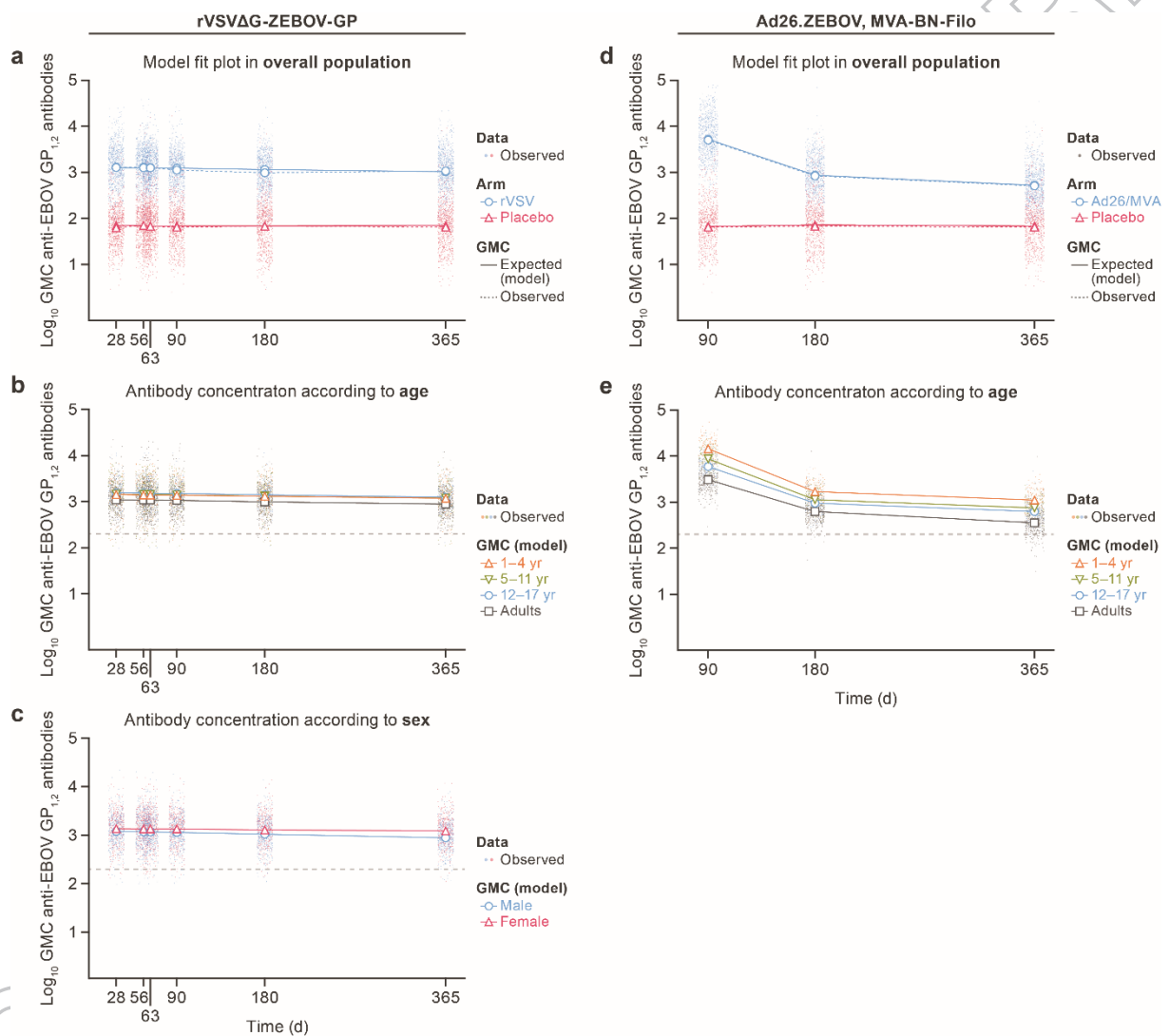


Table 1. Mixed-model analysis of variables associated with anti-EBOV GP_{1,2} IgG antibody concentrations after rVSVΔG-ZEBOV-GP vaccination.

	Anti-EBOV GP _{1,2} antibody	Anti-EBOV GP _{1,2} antibody
	Peak*	End of study*
Variable	Ratio of geometric mean concentration [95% CI]	Ratio of geometric mean concentration [95% CI]
Age category
Adults	Reference	Reference
Children 1–4 yr	1.32 [1.10; 1.57]	1.36 [1.12; 1.61]
Children 5–11 yr	1.35 [1.14; 1.57]	1.37 [1.16; 1.61]
Children 12–17 yr	1.41 [1.22; 1.64]	1.41 [1.21; 1.62]
Sex
Males	Reference	Reference
Females	1.15 [1.01; 1.30]	1.40 [1.22; 1.59]
Country (laboratory)
Mali (IRF-Frederick)	Reference	Reference
Guinea (LIBR)	0.73 [0.62; 0.85]	0.74 [0.63; 0.86]
Sierra Leone (LIBR)	0.80 [0.67; 0.95]	0.69 [0.58; 0.81]
Liberia (IRF-Frederick)	1.07 [0.87; 1.30]	1.01 [0.81; 1.24]
Pre-vaccination antibody level
< 200 EU/mL	Reference	Reference
> 200 EU/mL	1.31 [1.07; 1.57]	1.19 [0.99; 1.41]

*The peak and the end of the study were defined as 90 days and 12 months after receipt of the prime vaccination, respectively. EBOV = Ebola virus. IRF-Frederick = Integrated Research

Facility at Fort Detrick. GP_{1,2} = glycoprotein. LIBR: Liberian Institute for Biomedical Research. yr = years. EU/mL = enzyme linked immunosorbent assay units per milliliter. CI = confidence interval.

Table 2. Probability of having an antibody concentration above threshold 12 months after rVSVΔG-ZEBOV-GP vaccination (one dose).

	Probability of reaching anti-EBOV GP _{1,2} antibody threshold (EU/mL) at day 365 [95% CI]		
	> 200	> 600	> 1,000
Females
Children 1–4 yr	0.99 [0.97; 1.00]	0.84 [0.78; 0.90]	0.67 [0.59; 0.74]
Children 5–11 yr	0.99 [0.97; 1.00]	0.84 [0.78; 0.90]	0.66 [0.58; 0.74]
Children 12–17 yr	0.99 [0.98; 1.00]	0.85 [0.79; 0.90]	0.68 [0.60; 0.76]
Adults	0.97 [0.96; 0.99]	0.75 [0.71; 0.80]	0.54 [0.49; 0.60]
Males
Children 1–4 yr	0.97 [0.95; 0.99]	0.73 [0.66; 0.80]	0.51 [0.43; 0.60]
Children 5–11 yr	0.97 [0.95; 0.99]	0.74 [0.67; 0.80]	0.53 [0.45; 0.60]
Children 12–17 yr	0.97 [0.95; 0.99]	0.76 [0.69; 0.82]	0.55 [0.47; 0.63]
Adults	0.94 [0.91; 0.96]	0.61 [0.56; 0.66]	0.38 [0.33; 0.43]

EBOV = Ebola virus. GP_{1,2} = glycoprotein. yr = years. EU/mL = enzyme linked immunosorbent assay units per milliliter. CI = confidence interval.

Table 3. Mixed-model analysis of variables associated with anti-EBOV GP_{1,2} antibody concentrations after Ad26.ZEBOV, MVA-BN-Filo vaccination.

	Anti-EBOV GP _{1,2} antibody	Anti-EBOV GP _{1,2} antibody
	Peak*	End of study*
	Ratio of geometric mean concentration [95% CI]	Ratio of geometric mean concentration [95% CI]
Age category
Adults	Reference	Reference
Children 1–4 yr	4.68 [3.82; 5.64]	3.10 [2.58; 3.69]
Children 5–11 yr	2.82 [2.32; 3.38]	2.09 [1.78; 2.44]
Children 12–17 yr	1.93 [1.57; 2.35]	1.74 [1.48; 2.02]
Country (laboratory)
Mali (IRF-Frederick)	Reference	Reference
Guinea (LIBR)	0.59 [0.49; 0.72]	0.80 [0.67; 0.95]
Sierra Leone (LIBR)	0.38 [0.30; 0.47]	0.61 [0.49; 0.74]
Liberia (IRF-Frederick)	0.57 [0.44; 0.73]	0.57 [0.45; 0.72]
Pre-vaccine antibody concentration
< 200 EU/mL	Reference	Reference
> 200 EU/mL	0.93 [0.75; 1.13]	1.38 [1.12; 1.68]

*The peak and the end of the study were defined as 90 days and 12 months after receipt of the prime vaccination, respectively. EBOV = Ebola virus. IRF-Frederick = Integrated Research Facility at Fort Detrick. GP_{1,2}: glycoprotein. LIBR: Liberian Institute for Biomedical Research. yr = years. EU/mL = enzyme linked immunosorbent assay units per milliliter. CI = confidence interval.

Table 4. Probability of having an antibody concentration above threshold 12 months after Ad26.ZEBOV/MVA-BN-Filo vaccination.

	Probability of reaching anti-EBOV GP _{1,2} antibody threshold (EU/mL) at day 365 [95% CI]		
	> 200	> 600	> 1,000
Children 1–4 yr	0.98 [0.96; 0.99]	0.77 [0.70; 0.82]	0.56 [0.48; 0.63]
Children 5–11 yr	0.94 [0.91; 0.97]	0.61 [0.54; 0.68]	0.38 [0.30; 0.45]
Children 12–17 yr	0.91 [0.88; 0.94]	0.53 [0.45; 0.60]	0.30 [0.24; 0.36]
Adults	0.76 [0.72; 0.79]	0.28 [0.24; 0.32]	0.12 [0.09; 0.14]

EBOV = Ebola virus. GP_{1,2} = glycoprotein. yr = years. EU/mL = enzyme linked immunosorbent assay units per milliliter. CI = confidence interval.

Supplementary Material

Supplementary Figs. 1–3, Tables 1–2, methods, results, and appendix

Supplementary methods

rVSVΔG-ZEBOV-GP linear mixed-effects model (LMEM)

To describe the log₁₀ anti-Ebola-virus (anti-EBOV) glycoprotein (GP_{1,2}) immunoglobulin G (IgG) antibody concentrations in the rVSVΔG-ZEBOV-GP-placebo arm, an LMEM was built, in which the fixed intercept was adjusted for the following:

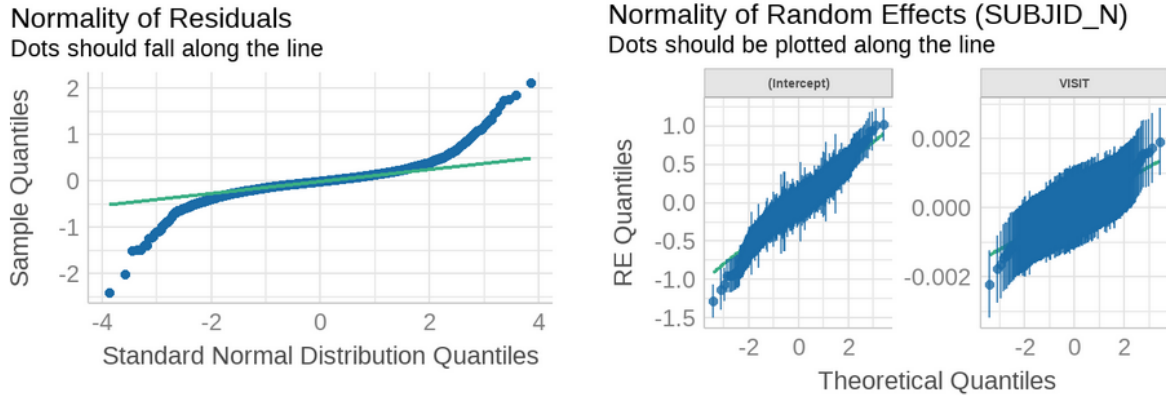
- treatment arm (vaccine [Vacc]), with placebo as reference), sex (female, with male as reference), country (Guinea, Liberia, and Sierra Leone [SL]), with Mali as reference), age category (children 1–4, 5–11, and 12–17 year, with adults as reference), and pre-vaccination antibody concentration, as single effects; and
- sex, country, age category, and pre-vaccination EBOV GP_{1,2} IgG antibody concentrations in interaction with vaccine treatment.

The fixed slope was adjusted for sex, treatment arm, and interaction between these two co-variables. In addition to covariate effects, random effects on both intercept and slope were considered. The resulting model, labelled Model A, is defined by the following equation:

$$\begin{aligned}
Y_{ij} = & \beta_0 + \beta_2 \mathbb{I}_{i \in Vacc} + \beta_3 \mathbb{I}_{i \in Female} + [\beta_4 \mathbb{I}_{i \in Guinea} + \beta_5 \mathbb{I}_{i \in Liberia} + \beta_6 \mathbb{I}_{i \in SL}] \\
& + \beta_7 Abbn_i + [\beta_8 \mathbb{I}_{i \in 1-4yo} + \beta_9 \mathbb{I}_{i \in 12-17yo} + \beta_{10} \mathbb{I}_{i \in 5-11yo}] \\
& + \mathbb{I}_{i \in Vacc} (\beta_{13} \mathbb{I}_{i \in Female} + [\beta_{14} \mathbb{I}_{i \in Guinea} + \beta_{15} \mathbb{I}_{i \in Liberia} + \beta_{16} \mathbb{I}_{i \in SL}] \\
& + [\beta_{17} \mathbb{I}_{i \in 1-4yo} + \beta_{18} \mathbb{I}_{i \in 12-17yo} + \beta_{19} \mathbb{I}_{i \in 5-11yo}] + \beta_{20} Abbn_i) \\
& + t_{ij} (\beta_1 + \beta_{12} \mathbb{I}_{i \in Female} + \beta_{11} \mathbb{I}_{i \in Vacc} + \beta_{21} \mathbb{I}_{i \in Female} \mathbb{I}_{i \in Vacc}) \\
& + \gamma_{0i} + \gamma_{1i} t_{ij} + \varepsilon_{ij}
\end{aligned}$$

Model A

Y_{ij} represents the \log_{10} IgG antibody concentration of the participant i at time point j . Thus, t_{ij} characterizes the variable of time rescaled to consider the peak at 28 days as the baseline ($t=0$). The variable $Abbn_i$ is the continuous covariate pre-vaccine IgG antibody concentration and describes the anti-EBOV GP_{1,2} IgG antibody concentration of the participant i measured at inclusion, before vaccine or placebo injection. The vector of parameters $\beta = (\beta_0, \dots, \beta_{21})$ represents the fixed effects related to the different covariates, and the parameters γ_{0i} and γ_{1i} are the random effects on the intercept and the slope, respectively. Random effects were assumed as normally distributed with mean 0, and covariance matrix Ω was assumed unstructured, with ω_0^2 and ω_1^2 the variances of γ_{0i} and γ_{1i} , respectively, and ρ their correlation. Finally, the variable ε_{ij} represents the residual error assumed to be normally distributed with mean 0 and variance σ^2 . This model was used to fit data from the rVSVΔG-ZEBOV-GP-placebo arm from the peak ($t=0$) to month 12 ($t=337$). Model A parameter estimates and the q-q plots of random effects and residuals are provided below.



Model A random effects and residuals

Shown are q-q plots of the residuals (left) and random effects (right) on the model, estimated on \log_{10} anti-EBOV GP_{1,2} IgG antibody concentrations in the rVSVΔG-ZEBOV-GP-placebo arm (1,572 participants). EBOV = Ebola virus. GP_{1,2} = glycoprotein. RE = random effects.

Model A parameter estimates

Parameter estimates of the LMEM, estimated on \log_{10} anti-EBOV GP_{1,2} IgG antibody concentrations in the rVSVΔG-ZEBOV-GP-placebo arm (1,572 participants).

Parameters	Estimate	Standard error	Pr (> t)
Fixed effects			
β_0 (intercept)	0.485	0.052	<1e-3
β_1 (slope)	-3.946e-6	5.240e-5	0.940
β_2 (vaccine arm)	2.350	0.073	<1e-3
β_3 (female)	1.932e-3	0.025	0.937
β_4 (Guinea)	0.201	0.032	<1e-3
β_5 (Liberia)	0.103	0.036	4.34e-3
β_6 (Sierra Leone)	0.275	0.036	<1e-3
β_7 (pre-vaccine antibody concentration)	0.681	0.027	<1e-3
β_8 (1–4 yr)	-0.096	0.031	1.81e-3
β_9 (12–17 yr)	-0.034	0.031	0.278
β_{10} (5–11 yr)	-0.051	0.030	0.093
β_{11} (vaccine arm, slope)	-3.952e-4	7.374e-5	<1e-3
β_{12} (female, slope)	-2.838e-5	7.697e-5	0.712
β_{13} (female, vaccine arm)	0.051	0.034	0.136
β_{14} (Guinea, vaccine arm)	-0.427	0.045	<1e-3
β_{15} (Liberia, vaccine arm)	-0.145	0.05067	4.39e-3
β_{16} (Sierra Leone, vaccine arm)	-0.518	0.05161	<1e-3
β_{17} (1–4 yr, vaccine arm)	0.254	0.04425	<1e-3
β_{18} (12–17 yr, vaccine arm)	0.195	0.04325	<1e-3
β_{19} (5–11 yr, vaccine arm)	0.201	0.04239	<1e-3
β_{20} (pre-vaccine IgG antibody concentration, vaccine arm)	-0.505	0.03873	<1e-3
β_{21} (female, vaccine arm, slope)	2.966e-4	1.082e-4	6.22e-3
Random effects and residual error			
ω_0^2 (variance γ_{0i})	9.667e-2	4.058e-3	
ω_1^2 (variance γ_{1i})	4.708e-7	4.193e-8	
ρ (correlation)	-0.44		
σ^2 (variance of residual error)	4.898e-2	9.115e-4	

EBOV = Ebola virus. GP_{1,2} = glycoprotein. β = vector of parameters. yr = years. Pr (>|t|) = *p*-value.

Model A single covariable mixed models

For defining the variables included in the multivariable rVSVΔG-ZEBOV-GP LMEM, the following variables were analyzed as fixed-effect covariates (single-effect and interaction with time): vaccine arm (vaccine versus pooled placebo arms, with placebo as reference), age category (1–4, 5–11, 12–17, or ≥18 years [reference]), sex (female, male [reference]), country (Guinea, Liberia, Sierra Leone, Mali [reference]), laboratory for Filovirus Animal Non-Clinical Group (FANG) assay analyses, anti-EBOV GP_{1,2} IgG concentrations prior to vaccination, vaccination time (hour in the day) for dose 1 and dose 2, body mass index (BMI; for adults) or Z score (for children), malaria status (defined as a known clinical malaria case within a month prior to the boost), HIV-1 infection status, lymphopenia, neutropenia, and eosinophilia. The results of the single covariable mixed models for rVSVΔG-ZEBOV-GP are presented in the following table:

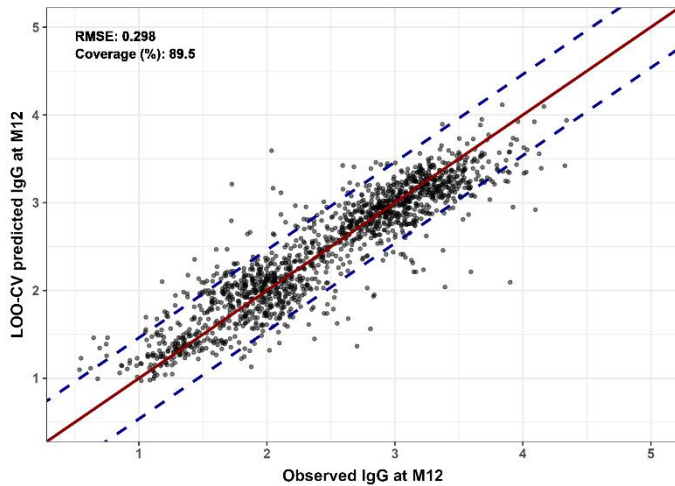
Covariate	Estimate	Estimate (log titer)	p value
None (only visit)	Peak	2.519	<1e-3
	Slope	-1.525e-04	<1e-3
Arm (ref: placebo)	Peak	1.187	<1e-3
	Slope	-2.692e-04	<1e-3
Age (ref: adults)	Peak	-	<1e-3
	Slope	-	0.565
Sex (ref: male)	Peak	-2.398e-02	0.504
	Slope	1.352e-04	0.002
Country (ref: Mali)	Peak	-	<1e-3
	Slope	-	<1e-3
Antibodies at baseline	Peak	4.074e-01	<1e-3
	Slope	-2.244e-04	<1e-3
Vaccination time (ref: 8–11 hours)	Peak	-	0.844
	Slope	-	<1e-3
BMI (adults) (ref: normal BMI)	Peak	-	0.461
	Slope	-	0.542
Z score BMI (children) (ref: -1 / +1)	Peak	-	0.111
	Slope	-	0.254
Malaria (ref: no malaria)	Peak	1.765e-01	0.013
	Slope	-1.963e-04	0.025
Lab (ref: IRF)	Peak	1.415e-01	<1e-3
	Slope	1.623e-05	0.720
HIV (adults)	Peak	2.537e-01	0.112
	Slope	2.969e-04	0.143
Syphilis (adults)	Peak	-1.241e-01	0.512
	Slope	1.968e-04	0.430
Lymphocytes (adults)	Peak	3.155e-02	0.353
	Slope	-7.504e-05	0.073
Neutrophils (adults)	Peak	1.110e-02	0.666
	Slope	-5.221e-05	0.065
Eosinophils (adults)	Peak	6.744e-02	0.553
	Slope	-2.671e-04	0.035

Model A evaluation of the predictive quality at month 12

To evaluate the performance of the rVSVΔG-ZEBOV-GP LMEM to predict antibody concentration, we performed a leave-one-out cross-validation (LOOCV).

At each iteration k of the procedure (with $k = 1, \dots, K$ where K is the number of participants), the complete dataset was split into a training and a testing dataset. The training dataset was built as the complete dataset from which the observation at month 12 from the k th participant was removed. The mixed model developed on the complete dataset was then estimated on the training dataset and used to predict the observation of the testing dataset (i.e., the observation

at month 12 of the k th participant). This procedure was repeated as much time as the number of participants in the complete dataset and two criteria were then calculated on predictions to evaluate how accurate they are: (1) the percent coverage (the higher the better) and (2) the root mean squared error (RMSE, the lower the better). Results are displayed in the following figure representing the comparison of observations at month 12 and the corresponding LOOCV predictions. The model showed good quality of predictions with a percent of coverage of 90% and an RMSE of 0.298 EU/mL.



Results of LOOCV: observation vs. predictions

plot [Model A]. The x axis represents individual observations at month 12 (successively removed from the training dataset), and the y axis represents predictions of IgG at month 12 made by models at each iteration. Each black dot represents the result of one iteration of the LOOCV (prediction made for the observation removed from the dataset).

The red solid line represents the relationship Observation = Prediction. Dashed blue lines represent the 95% confidence interval built on the distribution of the residuals of the model estimated on complete data (i.e., $\text{Observation} \pm 1.96 \cdot \text{SD}$, with SD being the standard error of the error model of the LMEM estimated on complete data). LOOCV = leave-one-out cross-validation. IgG = immunoglobulin G. SD = standard deviation. LMEM = linear mixed-effects model.

Ad26.ZEBOV, MVA-BN-Filo LMEM

To describe the \log_{10} anti-EBOV GP_{1,2} IgG antibody concentrations in the Ad26.ZEBOV, MVA-BN-Filo-placebo arm from the peak at month 3 to the end of the study, an LMEM with two slopes was built, considering a breaking point at month 6. In this model, the fixed intercept, the first and the second slopes were adjusted distinctively for:

- a) treatment arm (vaccine [Vacc], with placebo as reference), country (Guinea, Liberia, Sierra Leone [SL], with Mali as reference), age category (children 1–4, 5–11, and 12–17 years, with adults as reference), and pre-vaccination IgG antibody concentration, as single effects; and
- b) country, age category, and pre-vaccination antibody concentrations in interaction with vaccine treatment.

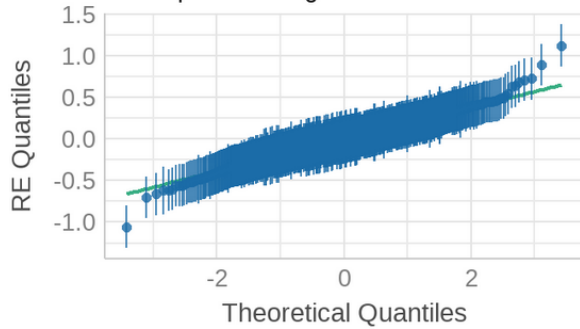
Similar to Model A, the variable of time was rescaled to consider the peak (month 3) as the baseline ($t=0$). After rescaling, the first slope is then considered from $t=0$ d (peak) to the breaking point, $\tau=90$, and the second slope from $t=\tau$ to $t=275$ (month 12). In addition to covariate effects, random effects only on intercept were considered. The resulting model, labelled Model B, is given by the following equation:

$$\begin{aligned}
Y_{ij} = & \beta_0 + \beta_1 \mathbb{I}_{i \in Vacc} + [\beta_2 \mathbb{I}_{i \in 1-4y0} + \beta_3 \mathbb{I}_{i \in 12-17y0} + \beta_4 \mathbb{I}_{i \in 5-11y0}] \\
& + [\beta_5 \mathbb{I}_{i \in Guinea} + \beta_6 \mathbb{I}_{i \in Liberia} + \beta_7 \mathbb{I}_{i \in SL}] + \beta_8 Abb n_i \\
& + \mathbb{I}_{i \in Vacc} ([\beta_9 \mathbb{I}_{i \in 1-4y0} + \beta_{10} \mathbb{I}_{i \in 12-17y0} + \beta_{11} \mathbb{I}_{i \in 5-11y0}] \\
& + [\beta_{12} \mathbb{I}_{i \in Guinea} + \beta_{13} \mathbb{I}_{i \in Liberia} + \beta_{14} \mathbb{I}_{i \in SL}] + \beta_{15} Abb n_i) \\
& + T_{ij}^a (\beta_{16} + \beta_{17} \mathbb{I}_{i \in Vacc} \\
& + [\beta_{18} \mathbb{I}_{i \in 1-4y0} + \beta_{19} \mathbb{I}_{i \in 12-17y0} + \beta_{20} \mathbb{I}_{i \in 5-11y0}] \\
& + [\beta_{21} \mathbb{I}_{i \in Guinea} + \beta_{22} \mathbb{I}_{i \in Liberia} + \beta_{23} \mathbb{I}_{i \in SL}] + \beta_{24} Abb n_i) \\
& + T_{ij}^a \mathbb{I}_{i \in Vacc} ([\beta_{25} \mathbb{I}_{i \in 1-4y0} + \beta_{26} \mathbb{I}_{i \in 12-17y0} + \beta_{27} \mathbb{I}_{i \in 5-11y0}] \\
& + [\beta_{28} \mathbb{I}_{i \in Guinea} + \beta_{29} \mathbb{I}_{i \in Liberia} + \beta_{30} \mathbb{I}_{i \in SL}] + \beta_{31} Abb n_i) \\
& + T_{ij}^b (\beta_{32} + \beta_{33} \mathbb{I}_{i \in Vacc} \\
& + [\beta_{34} \mathbb{I}_{i \in 1-4y0} + \beta_{35} \mathbb{I}_{i \in 12-17y0} + \beta_{36} \mathbb{I}_{i \in 5-11y0}] \\
& + [\beta_{37} \mathbb{I}_{i \in Guinea} + \beta_{38} \mathbb{I}_{i \in Liberia} + \beta_{39} \mathbb{I}_{i \in SL}] + \beta_{40} Abb n_i) \\
& + T_{ij}^b \mathbb{I}_{i \in Vacc} ([\beta_{41} \mathbb{I}_{i \in 1-4y0} + \beta_{42} \mathbb{I}_{i \in 12-17y0} + \beta_{43} \mathbb{I}_{i \in 5-11y0}] \\
& + [\beta_{44} \mathbb{I}_{i \in Guinea} + \beta_{45} \mathbb{I}_{i \in Liberia} + \beta_{46} \mathbb{I}_{i \in SL}] + \beta_{47} Abb n_i) + \gamma_{0i} \\
& + \varepsilon_{ij}
\end{aligned}$$

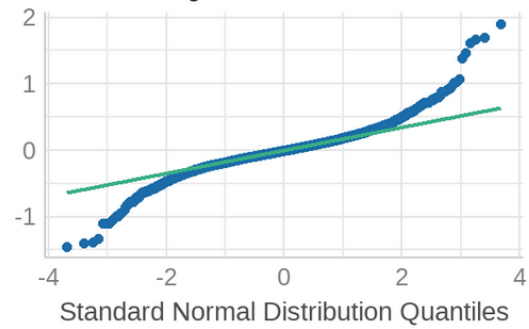
Model
B

Y_{ij} represents the \log_{10} antibody concentration of the participant i at time point j . Thus, t_{ij} characterizes the rescaled time. The variables T_{ij}^a and T_{ij}^b represent time relative to the first and the second slope, respectively, with $T_{ij}^a = \min(t_{ij}, \tau)$ and $T_{ij}^b = \max(t_{ij} - \tau, 0)$. The variable $Abbn_i$ is the continuous covariate pre-vaccine antibody concentration and describes the anti-EBOV GP_{1,2} IgG antibody concentration of the participant i measured at inclusion, before vaccine or placebo injection. The vector of parameters $\beta = (\beta_0, \dots, \beta_{47})$ represents the fixed effects related to the different covariates, and the parameter γ_{0i} is the random effect considered on the intercept. The random effect is assumed as normally distributed with mean 0 and variance ω_0^2 . Finally, the variable ε_{ij} represents the residual error assumed to be normally distributed with mean 0 and variance σ^2 . Model B parameter estimates and the q-q plots of random effects and residuals are provided above.

Normality of Random Effects (SUBJID_N)
Dots should be plotted along the line



Normality of Residuals
Dots should fall along the line



Model B random effects and residuals

Shown are q-q plots of the residuals (left) and random effects (right) on the model, estimated on \log_{10} anti-EBOV GP_{1,2} antibody concentrations in the Ad26.ZEBOV, MVA-BN-Filo-placebo arm (1,565 participants). EBOV = Ebola virus. GP_{1,2} = glycoprotein.

Model B parameter estimates

Parameter estimates of the LMEM, estimated on \log_{10} anti-EBOV GP_{1,2} IgG antibody concentrations in the Ad26.ZEBOV, MVA-BN-Filo-placebo arm (1,565 participants).

Parameters	Estimate	Standard error	Pr (> t)
Fixed effects – intercept			
β_0 (intercept)	0.451	0.060	< 1e-3
β_1 (vaccine arm)	3.150	0.086	< 1e-3
β_2 (1–4 yr)	-0.100	0.037	7.928e-3
β_3 (12–17 yr)	-0.055	0.039	0.156
β_4 (5–11 yr)	-0.069	0.037	0.065
β_5 (Guinea)	0.186	0.038	< 1e-3
β_6 (Liberia)	0.139	0.044	1.491e-3
β_7 (Sierra Leone)	0.329	0.044	< 1e-3
β_8 (pre-vaccine antibody concentration)	0.691	0.033	< 1e-3
β_9 (1–4 yr, vaccine arm)	0.742	0.053	< 1e-3
β_{10} (12–17 yr, vaccine arm)	0.360	0.053	< 1e-3
β_{11} (5–11 yr, vaccine arm)	0.522	0.053	< 1e-3
β_{12} (Guinea, vaccine arm)	-0.448	0.054	< 1e-3
β_{13} (Liberia, vaccine arm)	-0.347	0.061	< 1e-3
β_{14} (Sierra Leone, vaccine arm)	-0.701	0.063	< 1e-3
β_{15} (pre-vaccine IgG antibody concentration, vaccine arm)	-0.629	0.047	< 1e-3
Fixed effects – first slope			
β_{16} (slope)	-3.552e-5	7.222e-4	0.961
β_{17} (vaccine arm)	-9.772e-3	1.029e-3	< 1e-3
β_{18} (1–4 yr)	8.682e-4	4.569e-4	0.058
β_{19} (12–17 yr)	-1.064e-3	4.715e-4	0.024
β_{20} (5–11 yr)	-4.407e-4	4.509e-4	0.328
β_{21} (Guinea)	9.306e-4	4.676e-4	0.047

β_{22} (Liberia)	-6.582e-4	5.245e-4	0.210
β_{23} (Sierra Leone)	-1.753e-4	5.457e-4	0.748
β_{24} (pre-vaccine antibody concentration)	1.780e-4	3.993e-4	0.656
β_{25} (1–4 yr, vaccine arm)	-3.068e-3	6.440e-4	< 1e-3
β_{26} (12–17 yr, vaccine arm)	-1.285e-4	6.399e-4	0.841
β_{27} (5–11 yr, vaccine arm)	-1.632e-3	6.332e-4	0.010
β_{28} (Guinea, vaccine arm)	-6.002e-4	6.530e-4	0.358
β_{29} (Liberia, vaccine arm)	4.458e-4	7.224e-4	0.537
β_{30} (Sierra Leone, vaccine arm)	1.370e-3	7.617e-4	0.072
β_{31} (pre-vaccine antibody concentration, vaccine arm)	7.183e-4	5.684e-4	0.206
Fixed effects – second slope			
β_{32} (slope)	6.528e-4	3.534e-4	0.065
β_{33} (vaccine arm)	-2.809e-3	5.010e-4	< 1e-3
β_{34} (1–4 yr)	-7.768e-4	2.212e-4	< 1e-3
β_{35} (12–17 yr)	9.509e-4	2.271e-4	< 1e-3
β_{36} (5–11 yr)	5.373e-4	2.185e-4	0.014
β_{37} (Guinea)	-2.621e-4	2.263e-4	0.247
β_{38} (Liberia)	8.952e-5	2.556e-4	0.726
β_{39} (Sierra Leone)	-5.516e-4	2.607e-4	0.034
β_{40} (pre-vaccine antibody concentration)	-4.131e-4	1.950e-4	0.034
β_{41} (1–4 yr, vaccine arm)	1.172e-3	3.128e-4	< 1e-3
β_{42} (12–17 yr, vaccine arm)	-6.456e-4	3.077e-4	0.036
β_{43} (5–11 yr, vaccine arm)	-1.817e-4	3.075e-4	0.555
β_{44} (Guinea, vaccine arm)	4.451e-4	3.166e-4	0.160
β_{45} (Liberia, vaccine arm)	-2.005e-4	3.519e-4	0.569
β_{46} (Sierra Leone, vaccine arm)	4.444e-4	3.659e-4	0.225
β_{47} (pre-vaccine antibody concentration, vaccine arm)	8.607e-4	2.767e-4	1.884e-3
Random effects and residual error			
ω_0^2 (variance of γ_{0i})	5.550e-2	3.004e-3	
σ^2 (variance of residual error)	7.153e-2	1.908e-3	

EBOV = Ebola virus. GP_{1,2} = glycoprotein. β = vector of parameters. Pr ($>|t|$) = p -value.

Model B single covariable mixed models

For defining the variables included in the multivariable Ad26.ZEBOV, MVA-BN-Filo LMEM, the following variables were analyzed as fixed-effect covariates (single-effect and interaction with time): vaccine arm (vaccine versus pooled placebo arms, with placebo as reference), age category (1–4, 5–11, 12–17, or ≥ 18 years [reference]), sex (female, male [reference]), country (Guinea, Liberia, Sierra Leone, Mali [reference]), laboratory for FANG assay analyses, anti-EBOV GP_{1,2} IgG concentrations prior to vaccination, vaccination time (hour in the day) for dose 1 and dose 2, BMI (for adults) or Z score (for children), malaria status (defined as a known clinical malaria case within a month prior to the boost), HIV-1 infection status, lymphopenia, neutropenia, and eosinophilia. The results of the single covariable mixed models for Ad26.ZEBOV, MVA-BN-Filo are presented in the following table:

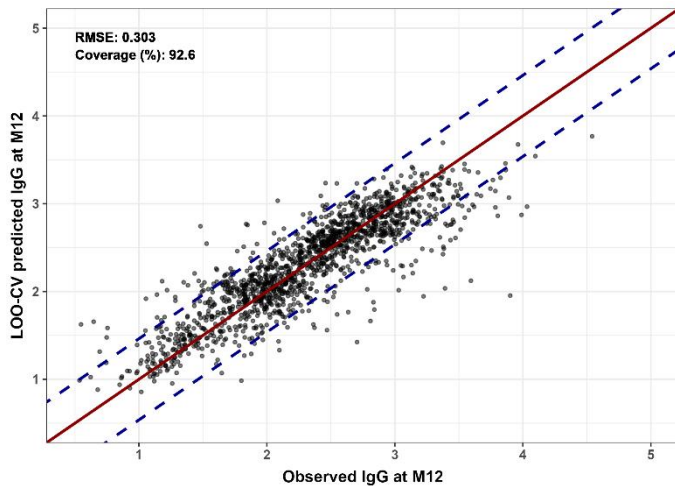
Covariate	Estimate	Estimate (log titer)	p value
None (only visit)	Peak	2.82	<1e-3
	Slope	-6.739e-04	<1e-3
Arm (ref: placebo)	Peak	1.239	<1e-3
	Slope	-8.895e-04	<1e-3
Age (ref: adults)	Peak	-	0.003
	Slope	-	0.026
Sex (ref: male)	Peak	-6.307e-02	0.097
	Slope	1.486e-04	0.334
Country (ref: Mali)	Peak		<1e-3
	Slope		0.632
Antibodies at baseline	Peak	3.175e-01	<1e-3
	Slope	1.944e-04	0.208
Vaccination time (ref: 8–11 hours)	Peak	-	0.243
	Slope	-	0.805
BMI (adults) (ref: normal BMI)	Peak	-	0.594
	Slope	-	0.464
Z score BMI (children) (ref: -1 / +1)	Peak	-	0.957
	Slope	-	0.980
Malaria (ref: no malaria)	Peak	1.180e-01	0.115
	Slope	1.971e-04	0.515
Lab (ref: IRF)	Peak	1.686e-01	<1e-3
	Slope	-1.502e-05	0.923
HIV (adults)	Peak	2.057e-02	0.919
	Slope	6.773e-05	0.944
Syphilis (adults)	Peak	-6.431e-02	0.729
	Slope	9.842e-04	0.234
Lymphocytes (adults)	Peak	1.413e-02	0.670
	Slope	-4.010e-06	0.978
Neutrophils (adults)	Peak	-3.730e-03	0.891
	Slope	2.840e-05	0.800
Eosinophils (adults)	Peak	1.889e-01	0.047
	Slope	1.971e-04	0.515

Model B evaluation of the predictive quality at month 12

To evaluate the performance of the Ad26.ZEBOV, MVA-BN-Filo LMEM to predict antibody concentration, we performed an LOOCV.

At each iteration k of the procedure (with $k = 1, \dots, K$ where K is the number of participants), the complete dataset was split into a training and a testing dataset. The training dataset was built as the complete dataset from which the observation at month 12 from the k th participant was removed. The mixed model developed on the complete dataset was then estimated on the training dataset and used to predict the observation of the testing dataset (i.e., the observation

at month 12 of the k th participant). This procedure was repeated as much time as the number of participants. In the complete dataset and two criteria were then calculated on predictions to evaluate how accurate they are: (1) the percent coverage (the higher the better) and (2) the RMSE (the lower the better). Results are displayed in the following figure representing the comparison of observations at month 12 and the corresponding LOOCV predictions. The model showed good quality of predictions with a percent of coverage of 93% and an RMSE of 0.303 EU/mL.



Results of LOOCV: observation vs. predictions

plot [Model B]. The x axis represents individual observations at month 12 (successively removed from the training dataset), and the y axis represents predictions of IgG at month 12 made by models at each iteration. Each black dot represents the result of one iteration of the LOOCV (prediction made for the observation

removed from the dataset). The red solid line represents the relationship observation = prediction. Dashed blue lines represent the 95% confidence interval built on the distribution of the residuals of the model estimated on complete data (i.e., observation $\pm 1.96 \cdot SD$, with SD being the standard error of the error model of the LMEM estimated on complete data). LOOCV = leave-one-out cross-validation. IgG = immunoglobulin G. SD = standard deviation. LMEM = linear mixed-effects model.

Antibody concentration ratios and probabilities

Calculation of geometric mean concentration (GMC) ratios

For each of the estimated LMEMs (Model A and Model B), the effect of each categorical covariate (age, sex, country, and pre-vaccination IgG antibody concentration) was evaluated as the ratio of the GMCs of anti-EBOV GP_{1,2} IgG antibody between the category of interest and its reference. Ratios of GMCs and their 95% confidence intervals at the peak and at the end of the study were estimated with a classic bootstrap procedure:

- a) We sampled with replacement 1,572 and 1,565 individuals on the original dataset for Model A and Model B, respectively.
- b) We estimated the LMEM (A or B), depending on the vaccination arm of interest, and computed prediction individual trajectories.
- c) We calculated along the predicted trajectories the ratios of GMCs, on the original scale of antibody concentration, for each category of each covariate at both the peak and the end of the study.

We repeated steps a, b, and c 2,000 times, which enabled us to derive the mean and the 95% confidence interval of each variable of interest. This method was applied successively for both vaccine arms (rVSVΔG-ZEBOV-GP and Ad26.ZEBOV, MVA-BN-Filo).

Calculation of probabilities of having antibody concentrations above a threshold and the time required from vaccination to drop below the threshold

For each studied arm (rVSVΔG-ZEBOV-GP and Ad26.ZEBOV, MVA-BN-Filo) and resulting LMEMs, simulations were performed to evaluate the probabilities of having concentrations above a given threshold at the end of the study and their 95% confidence intervals. For each simulation s , the following method was applied:

- Simulation of the population: A population of 7,500 participants with their characteristics (vaccine, age, sex, country, and pre-vaccination IgG antibody concentration), used as covariates in the models, was simulated. For categorical covariates (vaccine, age, sex, and country), we kept the same distributions as in the observed dataset. The continuous covariate “pre-vaccination IgG antibody concentration” was simulated distinctively for each participant profile (vaccine \times age - category \times sex \times country) as a normal law the mean and variance of which were estimated on the original dataset.
- Simulation of model parameters: A set of fixed parameters $\beta^s = (\beta_0^s, \dots, \beta_N^s)$, with $N=21$ for Model A and $N=47$ for Model B, was simulated considering a multinormal distribution $\beta^s \approx \mathcal{N}(\hat{\beta}, \Theta)$, where $\hat{\beta}$ is the vector of fixed parameter estimates, \mathcal{N} is the normal distribution, and Θ the extracted part of the Fisher information matrix (FIM) corresponding to fixed parameters. Standard deviations of random effects (ω_0^s for intercept and ω_1^s for slope), and of residual error (σ^s), were simulated, considering a binormal distribution (intercept and slope) for the random effects of Model A and a normal distribution for both random effect (only intercept) of Model B and residual errors. Similar to fixed parameters, mean and variance/covariance matrices of these distributions were defined by their model estimates ($\hat{\omega}_0$, $\hat{\omega}_1$, and $\hat{\sigma}$) and the FIM. Using simulated standard deviations of random effects, individual values of random effects (γ_{0i}^s and γ_{1i}^s) were derived for the 7500 participants of the simulated dataset such that in Model A: $(\gamma_{0i}^s, \gamma_{1i}^s)^T \approx \mathcal{N}\left(\begin{pmatrix} 0 \\ 0 \end{pmatrix}, \begin{pmatrix} (\omega_0^s)^2 & \rho\omega_0^s\omega_1^s \\ \rho\omega_0^s\omega_1^s & (\omega_1^s)^2 \end{pmatrix}\right)$ and in Model B: $\gamma_{0i}^s \approx \mathcal{N}(0, (\omega_0^s)^2)$. Similarly, residual errors ε_{ij}^s were simulated for each time point of each participant, considering a normal distribution $\mathcal{N}(0, (\sigma^s)^2)$.
- Calculation of probabilities: Using simulated model parameters (β^s, γ^s , and ε^s) and participant characteristics, the LMEM used to fit observed data (of either model) was used to derive the individual dynamics of \log_{10} anti-EBOV GP_{1,2} IgG antibody concentrations of the 7,500 simulated participants. Based on these dynamics, the proportion of participants reaching a designated IgG antibody threshold at the end of the study (month 12) was calculated for each profile of interest (see Table 2 and Table 4 in the main text).
- Calculation of time to threshold: Using these same simulated parameters and participants characteristics, an LMEM was used to identify individual time required for antibody concentrations to drop below the IgG antibody threshold. To identify this time, the R-function optimize was used to find the time t such as $|\text{Threshold} - Y_s(t)| = 0$, where $Y_s(t)$

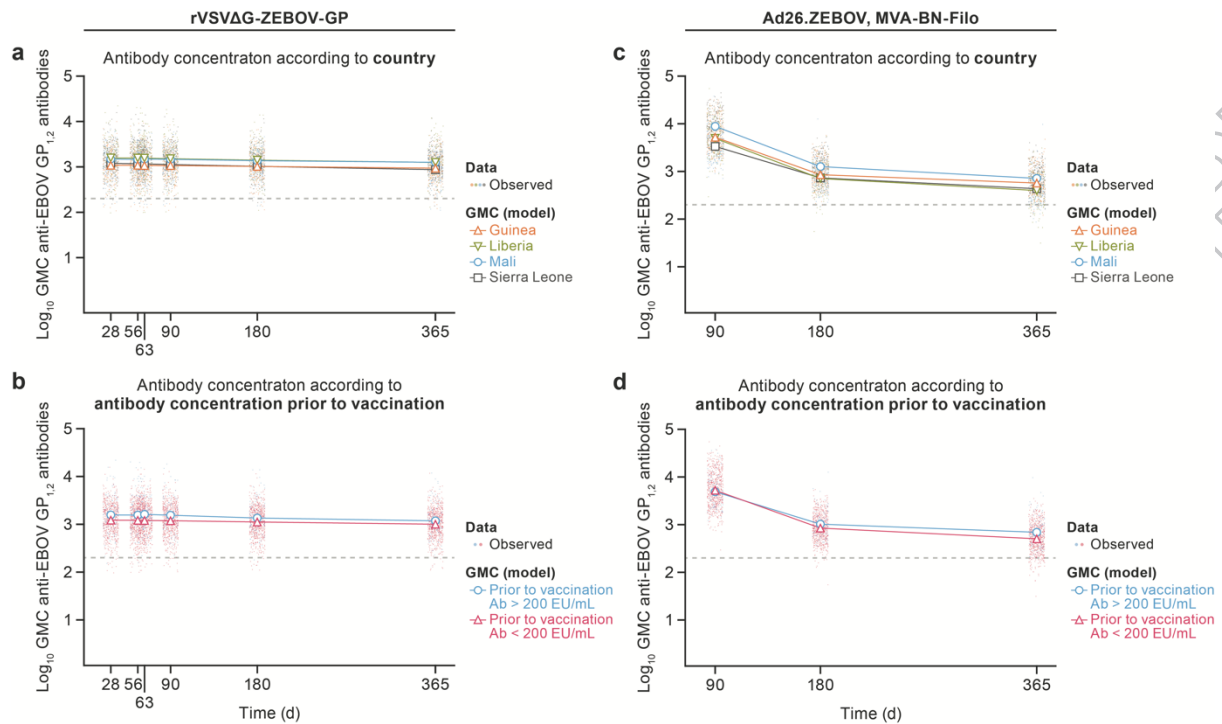
is the model output evaluated at time t . The median of all individual times was then calculated.

This method was repeated 2,000 times, which enabled us to derive the mean and the 95% confidence interval for each targeted probability and time to threshold. This process was performed for both vaccine arms (rVSVΔG-ZEBOV-GP and Ad26.ZEBOV, MVA-BN-Filo) according to their respective model.

ACCEPTED MANUSCRIPT

Supplementary results

Post-vaccination IgG antibody response dynamics



Supplementary Figure 1: Post-vaccination IgG antibody response dynamics by subgroup.

IgG antibody response dynamic post-vaccination from the peak to 12 months after the first dose of rVSVΔG-ZEBOV-GP or Ad26.ZEBOV, MVA-BN-Filo vaccine, respectively.

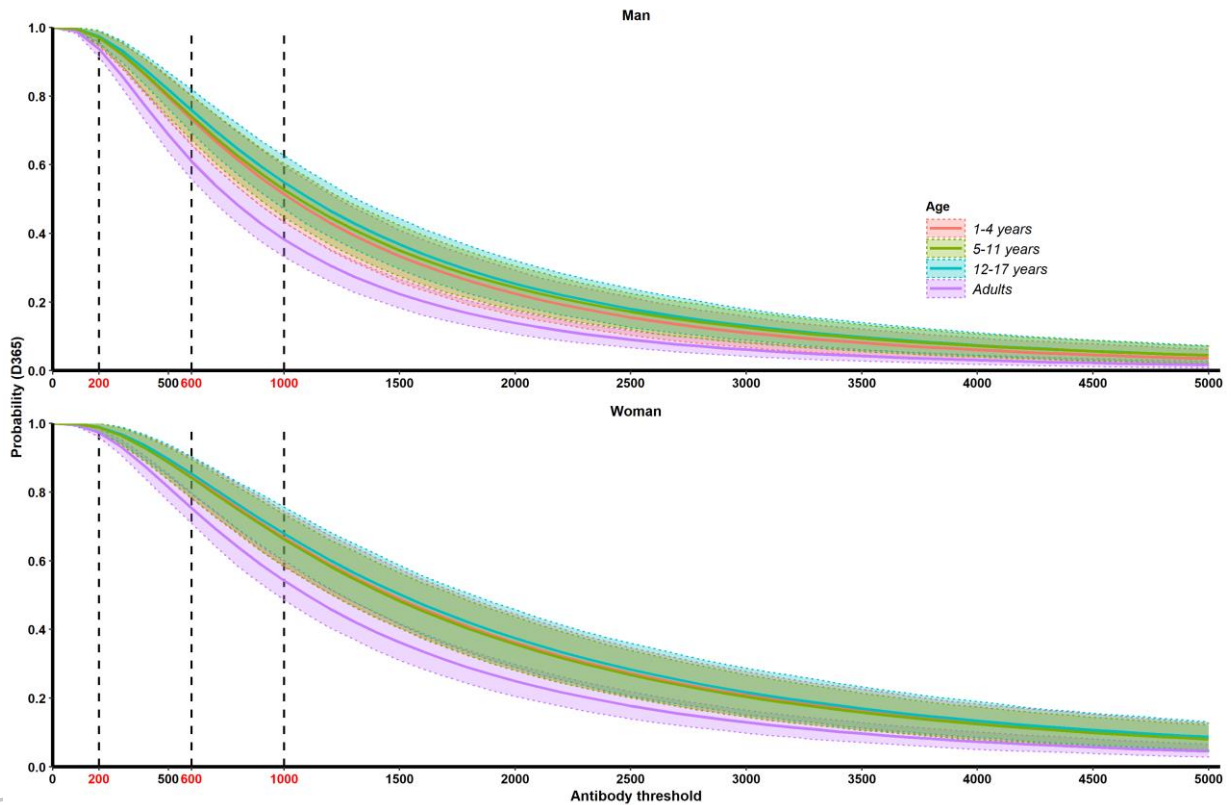
(a) (rVSVΔG-ZEBOV-GP) and (c) (Ad26.ZEBOV, MVA-BN-Filo): modelled antibody GMC over time according to country.

(b) (rVSVΔG-ZEBOV-GP) and (d) (Ad26.ZEBOV, MVA-BN-Filo): modelled antibody GMC over time according to antibody concentrations prior to vaccination. Dots represent individual observed serum samples. The dashed line in each panel indicates 200 EU/mL anti-EBOV GP_{1,2} antibody concentration.

EBOV = Ebola virus. GMC = geometric mean concentration. GP_{1,2} = glycoprotein.

rVSVΔG-ZEBOV-GP modeling

The time after vaccination required for 50% of the population to drop below the same three anti-EBOV GP_{1,2} GMC thresholds varied from 1.57 years (female children 12–17 years old) to 0.38 years (male adults) to fall below the threshold of 1,000 EU/mL, from 2.75 years (female children 12–17 years old) to 1.26 years (male adults) to fall below 600 EU/mL, and from 5.45 years (female children 12–17 years old) to 3.31 years (male adults) to fall below 200 EU/mL (Supplementary Information).



Supplementary Figure 2: Results of the rVSVΔG-ZEBOV-GP modeling.

Probability of anti-EBOV GP_{1,2} IgG antibody response at one year after vaccination with rVSVΔG-ZEBOV-GP vaccine (one dose) by sex and age.
EBOV = Ebola virus. GP_{1,2} = glycoprotein.

Supplementary Table 1: Dropping below the anti-EBOV GP_{1,2} IgG antibody threshold after rVSVΔG-ZEBOV-GP vaccination.

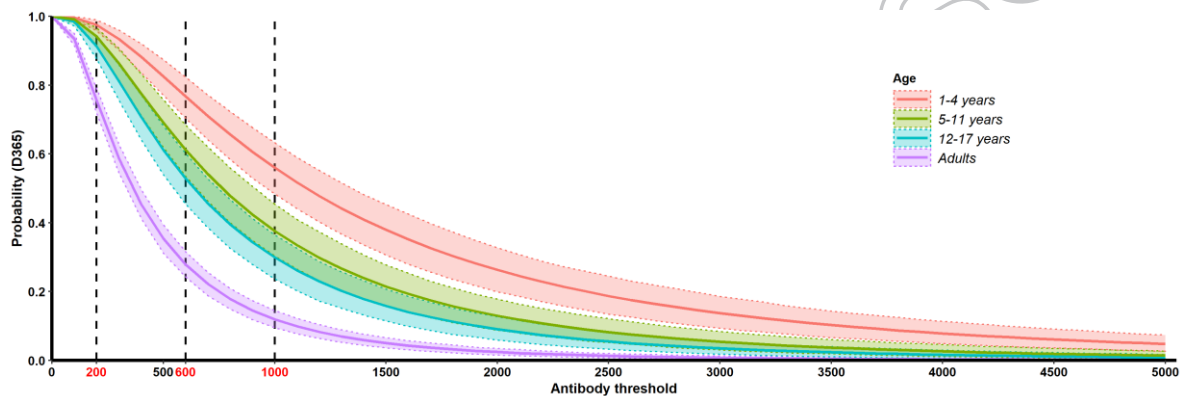
Time required after rVSVΔG-ZEBOV-GP vaccine administration for 50% of the population (by sex and age) to drop below concentration threshold for anti-EBOV GP_{1,2} IgG antibodies.

	Number of years to drop below anti-EBOV antibody threshold of 200 EU/mL	Number of years to drop below anti-GP_{1,2} EBOV antibody threshold of 600 EU/mL	Number of years to drop below anti-GP_{1,2} EBOV antibody threshold of 1000 EU/mL
Female			
1–4 yr	5.38 [4.46; 6.56]	2.69 [2.15; 3.35]	1.52 [1.09; 1.97]
5–11 yr	5.36 [4.45; 6.51]	2.66 [2.18; 3.31]	1.49 [1.10; 1.92]
12–17 yr	5.45 [4.51; 6.72]	2.75 [2.19; 3.44]	1.57 [1.15; 2.06]
Adults	4.93 [4.08; 5.32]	2.00 [1.70; 2.33]	0.89 [0.65; 1.14]
Male			
1–4 yr	3.89 [3.31; 4.59]	1.79 [1.45; 2.18]	0.89 [0.60; 1.21]
5–11 yr	3.95 [3.42; 4.63]	1.85 [1.53; 2.22]	0.94 [0.67; 1.22]
12–17 yr	4.03 [3.48; 4.67]	1.93 [1.61; 2.30]	1.02 [0.74; 1.30]
Adults	3.31 [2.95; 3.72]	1.26 [1.06; 1.47]	0.38 [0.21; 0.56]

EBOV = Ebola virus. GP_{1,2} = glycoprotein. yr = year. EU/mL = enzyme linked immunosorbent assay units per milliliter.

Ad26.ZEBOV, MVA-BN-Filo modeling

After the first injection of the Ad26.ZEBOV, MVA-BN-Filo vaccine, the projected time required for 50% of the population to drop below a threshold varied from 1.16 years (children 1–4 years) to 0.42 years (adults) for the 1,000 EU/mL threshold, from 1.82 years (children 1–4 years) to 0.54 years (adults) for the 600 EU/mL threshold, and from 3.24 years (children 1–4 years) to 1.55 years (adults) for the 200 EU/mL threshold (Supplementary Information).



Supplementary Figure 3: Results of the Ad26.ZEBOV, MVA-BN-Filo modeling.

Probability of anti-EBOV GP_{1,2} IgG antibody response at one year after vaccination with Ad26.ZEBOV, MVA-BN-Filo vaccine by age.

EBOV = Ebola virus. GP_{1,2} = glycoprotein.

Supplementary Table 2: Dropping below the anti-EBOV GP_{1,2} IgG antibody threshold after Ad26.ZEBOV, MVA-BN-Filo vaccination.

Time required after Ad26.ZEBOV, MVA-BN-Filo vaccine administration for 50% of the population (by age) dropping below concentration threshold for GP-EBOV antibodies.

	Number of years to drop below anti-EBOV GP _{1,2} antibody threshold of 200 EU/mL	Number of years to drop below anti-EBOV GP _{1,2} antibody threshold of 600 EU/mL	Number of years to drop below anti-EBOV GP _{1,2} antibody threshold of 1000 EU/mL
Children 1–4 yr	3.24 [2.48; 4.49]	1.82 [1.46; 2.39]	1.16 [0.96; 1.45]
Children 5–11 yr	2.82 [2.18; 4.07]	1.34 [1.10; 1.72]	0.66 [0.49; 0.83]
Children 12–17 yr	2.48 [1.95; 3.34]	1.09 [0.90; 1.33]	0.50 [0.47; 0.61]
Adults	1.55 [1.42; 1.72]	0.54 [0.48; 0.61]	0.42 [0.41; 0.43]

EBOV = Ebola virus. GP_{1,2} = glycoprotein. yr = year. EU/mL = enzyme linked immunosorbent assay units per milliliter.

Supplementary appendix: FANG assay



Standard Operating Procedure

FANG ASSAY	SOP_LAB_03	Version 1.0 10/08/2018
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Written by Jonathan Marchand, LIBR team	Validated by Renaud Vatrinet LWG leader	Approved by Jerome Pierson, H�el�ene Esp�erou, Deborah Watson-Jones
From NIH	From INSERM	From NIH, Inserm, LSHTM
Date 10/08/2018	Date 10/08/2018	Date 10/08/2018
Signature	Signature	Signature

Application date = Date of approval

Distribution To all members of PREVAC study

Mode of distribution PREVAC Website

Revision history (highlighted in yellow modifications)		
Version	Date	Description
1.0	10/08/2018	Creation of the SOP



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2. Definitions and abbreviations
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1. Purpose and scope

The scope of this Standard Operating Procedure (SOP) encompasses the entire process of executing an Enzyme Linked Immunosorbent Assay (ELISA) for the detection of filovirus (e.g., Marburg virus, MARV; *Sudan ebolavirus*, SUDV; *Zaire ebolavirus*, EBOV) glycoprotein (GP) specific immunoglobulin G (IgG) in sera, including coating the microplates with antigen, preparation of the reference material, controls, and test samples, execution of the assay, and analysis of the data.

The purpose of this SOP is to describe the performance of an ELISA to quantify the humoral immune response to filovirus GP in non-human primates human species that have been vaccinated with a filovirus vaccine, exposed to filovirus antigen preparations or virus, or have been passively transferred with polyclonal or monoclonal anti-filovirus GP IgG. The immune response to the filovirus is a measure of protection; therefore, measurement of anti-filovirus GP antibody is an appropriate marker of an immune response to filovirus vaccines.

This assay is designed to quantify IgG antibodies against filovirus GP using an ELISA in which purified recombinant glycoprotein (rGP) is used as the solid-phase immobilized antigen and an enzyme-conjugated anti-IgG secondary antibody is used as the reporter or signal system. The assay endpoint will be reported as the serum mean concentration of anti-filovirus GP specific IgG (ELISA Units/mL).



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2. **Definitions and abbreviations**

Limit of Detection (LOD): The lowest concentration of an analyte that a bioanalytical procedure can reliably differentiate from background noise. The LODs have been determined empirically for each antigen. The “official” LOD can be found in the appropriate qualification final report for each filovirus and species tested.

Lower Limit of Quantification (LLOQ): The LLOQ refers to the minimum concentration of analyte in the original serum sample that can be reliably quantified with a fixed degree of accuracy and precision (% Relative Standard Deviation of $\leq 20\%$). There is no maximum Limit of Quantification (max-LOQ) as there is no maximum allowable concentration of analyte in the original serum sample. Serum samples with a high anti-GP IgG concentration may be diluted so that their “in the well” readings are within the acceptable range of the assay (as shown by dilutional linearity).

Negative Control (NC): The negative control is a pool of naïve species-matched sera that have been screened for anti-filovirus GP IgG and found to be below detection. The NC is tested at 1:50 dilution in duplicate on each test plate.

Plate Acceptance Criteria: The plate acceptance criteria determine whether the **entire microplate** meets the minimum standards defined during assay development. The plate acceptance criteria includes criteria to evaluate the reference standard curve, quality control samples, and negative control. If a plate fails plate acceptance criteria then the test samples on the plate are considered failed.

Quality Control Sample (QC): The Quality Control samples are made of naïve species-matched serum mixed with anti-filovirus immune serum (from vaccinated animals) with a known relative amount of analyte. Typically, there are two levels of QCs tested on a plate (with concentrations at the High and Low ends of the assay range), and the QCs are tested once per plate.

Quantity Sufficient (Q.S.): This is the technique of “bringing the solution up to a final volume.” For example, when preparing a buffer, the solute is added



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to the diluent to make an initial, concentrated version of the solution, which is eventually diluted with the diluent to the desired final volume and end concentration.

Reference Standard (RS): The Reference Standard is made of naïve species-matched serum mixed with anti-filovirus immune serum (from vaccinated animals) with a known relative amount of analyte. Typically, the RS is diluted in duplicate across each assay plate. The first reference standard for each filovirus was assigned a concentration of 1000 ELISA Units/mL. The quality controls and subsequent reference standards will be calibrated from that lot of RS.

Replicate %CV (Coefficient of Variation): The Replicate %CV measures the precision of concentration values within a single duplicate dilution point of the reference standard dilution series. This calculation is performed by the software Softmax Pro, BioAssay database automation, or equivalent data analysis software. Replicate %CV is calculated as:

$$\left(\frac{\text{Standard Deviation}}{\text{mean}} \right) * 100$$

Replicate %RE (Relative Error): The %RE measures the accuracy (relative to the fitted 4PL curve) of concentration values within a single duplicate dilution point within the reference standard dilution series. This calculation is performed by the software Softmax Pro, BioAssay database automation, or equivalent data analysis software. %RE is calculated as:

$$\%RE = \left(\frac{\text{Observed value} - \text{Nominal}}{\text{Nominal}} \right) * 100$$

Reportable Value: The reportable value (RV) is the final value for a sample that is reported in ELISA Units/mL after the plate has passed the plate acceptance criteria and the sample has passed the test sample acceptance criteria. The RV may be derived from either the result from a single analysis or the results from multiple independent analyses (median of the passing



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results), depending on the need of the study as specified in the study protocol or at the discretion of the study director.

Test Sample (TS) Acceptance Criteria: The test sample acceptance criteria determine whether the **individual test samples** meet the minimal standards defined during assay development. If multiple test samples are tested on the same plate and one or more of the test samples fail test sample acceptance criteria, the failing sample(s) do not impact the pass or fail status of the other samples tested on that plate.

3. Procedure

3.1 Responsibility

It is the responsibility of the person or persons undertaking this assay to ensure that they are fully familiar with this SOP before commencing, that the SOP is executed accurately, and that all relevant documentation is completed. Documentation will include pertinent supportive facility forms, raw data printed upon completion of each assay, and data analysis of the raw data. All personnel must be trained in performing data analysis to ensure compliance with pertinent regulatory requirements as specified by Food and Drug Administration (FDA), or other agencies.

3.2 Specimen Handling

3.2.1 Safety

Caution must be exercised in handling all materials associated with this assay.

- Take precautions to avoid skin and mucous membrane (eye, nose, and mouth) contact with all samples, chemicals, and reagents used.
- Treat all human blood/sera as potentially contaminated with human pathogens and handle according to Biosafety Level – 2 (BSL-2) practices and procedures.
- When working with human serum, make all dilutions/serial dilutions within a Biosafety Cabinet (BSC) class II.

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- Once the plates have been washed, addition of reagents (secondary antibodies, etc.) may be added on the bench top.

3.2.2 Arrival, Tracking, and Reporting

Serum specimens to be analyzed in the anti-GP IgG ELISA that are produced by Battelle will be inventoried with respect to the specimen receipt or collection date, condition, and other information. This information must be recorded in a database or alternative inventory system, which will be kept or referenced in the appropriate study binder.

Additionally, critical reagents (e.g., reference standards, quality controls, negative controls) will be inventoried in a similar manner.

3.2.3 Sample Storage

Frozen sera will be thawed at room temperature (RT) just before use, or in a refrigerator (2-8 °C) overnight. Thawed sera will be thoroughly, but carefully, mixed just prior to testing.

For long-term storage, serum samples (test samples, QCs, NC, and RS) will be kept at $\leq -60^{\circ}\text{C}$. It is recommended that the fewest freeze thaw (F/T) cycles possible be used. It is also recommended that aliquots be made to minimize the frequency of freeze-thaw cycles, though aliquots should be no less than 30 μL in volume whenever possible.

For short-term storage, serum samples may be stored at 2-8 °C or at room temperature (RT).

For calculation of “time at 2-8 °C” and F/T cycle numbers, the following applies. If using the Sample Tracking System Database, the “time at 2-8 °C” and number of F/T cycles is automatically calculated. If using a paper form for documentation of “time at 2-8 °C” and F/T cycle numbers the following apply. See Table 1 for an illustration of “time at 2-8 °C” and F/T cycle number calculation. NOTE: this table may be re-formatted as long as the same information is captured.

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- The first day the serum sample is thawed is counted as Day 0. However, if a sample is thawed and frozen within the same day, the “time at 2-8 °C” will be advanced by 1 day.
- When the serum sample is returned to the freezer, the total time (in days) will be the number of days the sample was out of the freezer. For example, if a sample was removed from the freezer on Monday and returned on Wednesday, the total “time at 2-8 °C” will be 2 days.
- Each subsequent time a serum sample is thawed, the time (in days) at 2-8°C will be added to the previous total. There is no requirement to record cumulative “time at 2-8 °C” after each F/T cycle.
- Each time the serum sample is frozen the F/T cycle number will be advanced by one.

Date Thawed	Date Frozen	Time ¹ at 2-8°C (this Freeze/Thaw Cycle)	Time ¹ at 2-8°C (Cumulative)	Freeze/Thaw Cycle Number
08-04-14	08-06-14	2	2	1
08-17-14	08-17-14	1	3	2
09-14-14	09-15-14	1	4	3
09-24-14	09-24-14	1	5	4
10-05-14	10-07-14	2	7	5

¹Time = Days

Table 1. Time at 2-8 °C and Freeze/Thaw Cycle Example.

3.3 Reagents, Equipment, and Supplies

The following lists include, but are not limited to, the reagents, equipment, and supplies necessary to perform this SOP.

Reagents (e.g., conjugate, substrate, etc.) must be qualified prior to use in the assay. QCs and RS lots must be qualified prior to use in the assay.



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3.3.1 Reagents

- Zaire Human ELISA Reagents (BMI/JVAP) - Do not substitute manufacturer.
 - Filovirus rGP
 - Reference Standard (RS) Serum
 - Quality Control High (QCH)
 - Quality Control Low (QCL)
 - Negative Control (NC)
- Peroxidase AffiniPure Goat Anti-Human IgG, Fcy Fragment Specific (Jackson Immuno Research Labs, Cat. No. 109-035-098, Lot No. 118460) - do not substitute (other than lot number; may need to requalify optimum dilution).
- 1X PBS: (Sigma Cat. No. P3813, HyClone Cat. No. SH30256.02, or equivalent)
- Reverse osmosis deionized (RODI), reagent grade, or Millipore grade water (H₂O), or equivalent
- Polyoxyethylene Sorbitol Monolaurate: (Tween-20) (Fisher Cat BP337-500, or equivalent)
- Non-fat dry milk: (LabScientific Cat. No. M0841, or equivalent)
- Peroxidase Substrate (3,3',5,5' tetramethylbenzidine, TMB): (Thermo Scientific Cat. No. N301, or equivalent)
- Stop Solution: (Thermo Scientific Cat. No. N600, or equivalent).

3.3.2 Equipment

- Single and multi-channel pipettes of various sizes
- Microtiter plate washer (BioTek ELx405 or equivalent)
- Microtiter plate reader with 450 nm and 650 nm filter (Molecular Devices, Bio-Tek, or equivalent)
- PC compatible computer and printer



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- “Softmax Pro” data analysis software, or equivalent
- Timer or facility clock
- Freezer $\leq -20^{\circ}\text{C} \pm 4^{\circ}\text{C}$
- Freezer $\leq -60^{\circ}\text{C}$
- Refrigerator $2-8^{\circ}\text{C}$.

3.3.3 Supplies

- Barrier/Filter Pipette tips of various sizes
- Clean containers capable of holding 50 mL, 100 mL, 1000 mL, and 2000 mL
- Disinfectant: minimum ~1.0% hypochlorite solution, or equivalent
- Reagent reservoir trays
- Screw-cap 15 mL tubes (Fisher Cat No. 05-539-5, or equivalent)
- 1.5 mL microcentrifuge tubes (Fisher Cat. No. 05-408-137, or equivalent)
- 96-well plates: Immulon 2HB Microplate (Thermo Scientific Cat. No. 3455, Fisher Cat. No. 14-245-61, or equivalent)
- Titer Tubes (Fisher Cat. No. 02681381, or equivalent)
- Paper towels
- Aluminum foil
- Plastic wrap

3.4 Overall Anti-Filovirus GP IgG ELISA Assay Procedures

3.4.1 Study Binder Organization

The following is a suggestion for how to organize the filovirus GP study binders. The exact organization will depend on the particular needs of the study. The study binder will include tab designations and may be organized as follows:



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- Data Tables (Appendix 4)
- Memos and Deviations
- Critical Reagents Preparation Forms
- Reagent Preparation Forms (Appendix 1, Appendix 2)
- Plate Coating Log and Usage Form (Appendix 3)
- Sample Freeze Thaw Forms
- Equipment Forms
- Process Forms with data printouts (e.g., Softmax Files), organized by test operator, date, and plates run (Appendix 5)

3.4.2 Reagents and Preparation

A unique lot number, storage temperature, date of preparation, date of expiration, and preparer's initials must be assigned for each reagent. Additionally, the quantity of reagents prepared may be scaled up or down from that described as long as all concentrations remain as outlined below.

- **1X PBS:** Add 1 packet of PBS (pre-measured by manufacturer to make 1 L each) to 900 mL H₂O (RODI, reagent grade, or Millipore grade water). Q.S. to 1 L with H₂O. Use a magnetic stirrer or mix gently until dissolved. Alternatively, use commercially available 1X PBS or dilute 100 mL of 10X PBS solution in 900 mL H₂O. Store at 2-8 °C for up to one month.
- **ELISA Plate Coating Buffer:** (1X PBS containing filovirus rGP). Add rGP to 1X PBS to a final concentration designated. Store at 2-8 °C until used. Use by close of business and discard the excess coating buffer after coating the plates. Note that new lots of rGP sources will need to be qualified into the assay. Prepare approximately 11 mL per plate.
- **Wash Buffer:** 1X PBS + 0.1% Tween-20 - To prepare 1 L of Wash Buffer, add 1 packet of 1X PBS powder to approximately 900 mL of H₂O (RODI, reagent grade, or Millipore grade water), then add 1.0 mL of Tween-20.

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Q.S. to 1 L with H₂O. Alternatively, add 100 mL of 10X PBS and 1.0 mL Tween-20 to approximately 800 mL H₂O. Q.S. to 1 L with H₂O. Store at RT; expires after 1 month.

- ELISA Diluent:** 1X PBS + 5% non-fat dry milk (w/v) + 0.1% Tween-20 (v/v)

- To prepare 1 L of ELISA Diluent, add 50 ± 0.5 g of non-fat dry milk to approximately 800 mL of 1X PBS and mix. Add 1 mL of Tween-20 and mix. Q.S. to 1 L with 1X PBS. Alternatively, add 50 ± 0.5 g of non-fat dry milk to approximately 800 mL of H₂O and 1 packet of 1X PBS powder (or 100 mL of commercially prepared 10X PBS). Add 1 mL of Tween-20 and mix. Q.S. to 1 L with water. Store at 2-8 °C for up to two weeks.
- Water (H₂O):** Obtain from an Aqua Solutions Water Purification System; e.g., Millipore, or equivalent. Store at RT for up to two months or according to the manufacturer's instructions. This water meets the American Society for Testing and Materials (ASTM) Type II requirements for reagent grade water.

3.4.3 Plate Coating (rGP Adsorption to Microtiter Plates)

- Record the preparation of filovirus rGP-coated microplates on the FANG ELISA Plate coating Form or equivalent. Record the manufacturer's lot number for the microtiter plates being coated. Assign a unique identifier number to each newly coated plate.
- Transfer the ELISA Plate Coating Buffer to a reagent reservoir tray. Dispense 100 µL to each well of the microtiter plate.
- Cover the stacked plates (e.g., foil, plastic wrap, or similar material) and incubate at 2-8 °C for a minimum of 14 hours. Plates must be used within 7 days of preparation.
- If plates are not used within the allowable time period, then destroy the plates and record the destruction date.



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3.4.4 Overview of Assay Workflow

- A brief overview of the recommended workflow:
 - (1) Remove reagents from cold storage as necessary
 - (2) Aliquot TMB and store protected from light at RT
 - (3) Aliquot ELISA Diluent for the conjugate dilution and store at RT
 - (4) If the samples are frozen, pull the test samples from freezer to RT to thaw
 - (5) Dilute test samples, RS, and QCs in titertubes
 - (6) Wash coated assay plates
 - (7) Transfer diluted samples from titer tubes to the washed assay plates and incubate
 - (8) Return samples to the freezer or store at 2-8°C depending on the upcoming testing needs
 - (9) Wash assay plates, add goat anti-species IgG conjugated antibody (or equivalent depending on species being tested), and incubate
 - (10) Wash assay plates, add TMB substrate, protect from light, and incubate
 - (11) Add stop solution and read plate

Record sample preparation and the assay procedure

3.4.5 RS, QC, and Test Sample Dilutions in Dilution Tubes

- The ELISA Diluent should be warmed to RT prior to the addition of serum. Simply aliquoting into a titertube box or cryovials will allow the ELISA Diluent to warm quickly.
- Flick the serum tubes to mix prior to aspirating the volume (recommend 3-5 times).
- When dispensing serum into ELISA Diluent, first pre-wet the tip by



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aspirating up and down (recommend 2-3 times), dispense the volume into the ELISA Diluent, and then rinse the pipette tip by mixing up and down (recommend 3-5 times).

- Create a 1X dilution (1:50) of the NC in ELISA Diluent. If the RS is prepared in a Master Box, add ~250 µL of 1:50 diluted NC to each tube in Column 12. If the RS is prepared directly on the plate add 100 µL of the 1:50 diluted NC to wells A12 and B12.
- Create a 1X dilution of the RS in ELISA Diluent
 - (1) Human Filovirus ELISA (1:1.5 serial dilution): In a Reference Standard Master Box (S-MP), add ~1000 µL of the initial RS dilution to each titer tube in Column 1, and 300 µL ELISA Diluent to each titer tube in Columns 2-11 (Figure 1). Next, create an 11-point 1.5-fold dilution series for the RS by transferring 600 µL across the dilution series from Column 1 to Column 11 (the dilutions are referred to as S1 through S11, with S1 being the initial dilution and S11 being the last dilution). Discard 600 µL from Column 11 with the used pipette tips.

	1	2	3	4	5	6	7	8	9	10	11	12
Plate 1												
A	RS Initial											NC
Plate 2												
B	RS Initial											NC
Plate 3												
C	RS Initial											NC
Plate 4												
D	RS Initial											NC

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Plate 5											
E	RS Initial										NC
Plate 6											
F	RS Initial										NC
G											
H											

Figure 1. Preparation of the S-MP.

- Create a 1X dilution of each QC in ELISA Diluent. The QCs will be diluted two-fold down the rGP-coated plate (Rows C-H) in Columns 11 and 12.
- Create a 1X dilution of each test sample (TS) in ELISA Diluent. Generally, a starting 1X dilution is 1:50; however, other starting dilutions may be used based on the sample or study design. The TSs will be diluted two-fold down the rGP-coated plate (Rows C-H) in single columns (Columns 1-10). Note that pre-screen samples are not further diluted, instead are tested at a single 1:50 point dilution.
- For ease, a test sample master box (T-MP) may be prepared for each operator. For example, prepare the initial test sera dilution of the TSs and QCs in the T-MP as shown in Figure 2 for test samples run in the full ELISA or Figure 3 for pre-screen test samples.
- Additionally, for ease, each operator may use a titer tube box to prepare the initial and serial dilutions. 100 µL of these completed dilutions can then be transferred directly to rGP-coated plates, and no diluting directly on the plate is needed. This preparation is beneficial when running multiple plates in a single run. For example, if starting with 1:50 dilutions, prepare the initial dilutions of the TSs and the QCs by adding 245 µL of ELISA diluent to row C, columns 1-12. Then add 5 µL of TS and QC to the appropriate tubes in row C. Add 125 µL of ELISA diluent to rows D-H, columns 1-12. Two-fold dilutions are then prepared by transferring 125 µL

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down the titer tube box from row C through row H using a multi-channel pipette.

a)

	1	2	3	4	5	6	7	8	9	10	11	12
	Plate 1											
A	TS1	TS2	TS3	TS4	TS5	TS6	TS7	TS8	TS9	TS10	QCH	QCL
	Plate 2											
B	TS11	TS12	TS13	TS14	TS15	TS16	TS17	TS18	TS19	TS20	QCH	QCL
	Plate 3											
C	TS21	TS22	TS23	TS24	TS25	TS26	TS27	TS28	TS29	TS30	QCH	QCL
	Plate 4											
D	TS31	TS32	TS33	TS34	TS35	TS36	TS37	TS38	TS39	TS40	QCH	QCL
	Plate 5											
E	TS41	TS42	TS43	TS44	TS45	TS46	TS47	TS48	TS49	TS50	QCH	QCL
	Plate 6											
F	TS51	TS52	TS53	TS54	TS55	TS56	TS57	TS58	TS59	TS60	QCH	QCL
G												
H												

Figure 2. Preparation of the T-MP (Full ELISA)

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	1	2	3	4	5	6	7	8	9	10	11	12
	Plate 1											
A	TS1		TS7		TS13		TS19		TS25		QCH	QCL
B	TS2		TS8		TS14		TS20		TS26			
C	TS3		TS9		TS15		TS21		TS27			
D	TS4		TS10		TS16		TS22		TS28			
E	TS5		TS11		TS17		TS23		TS29			
F	TS6		TS12		TS18		TS24		TS30			
G												
H												

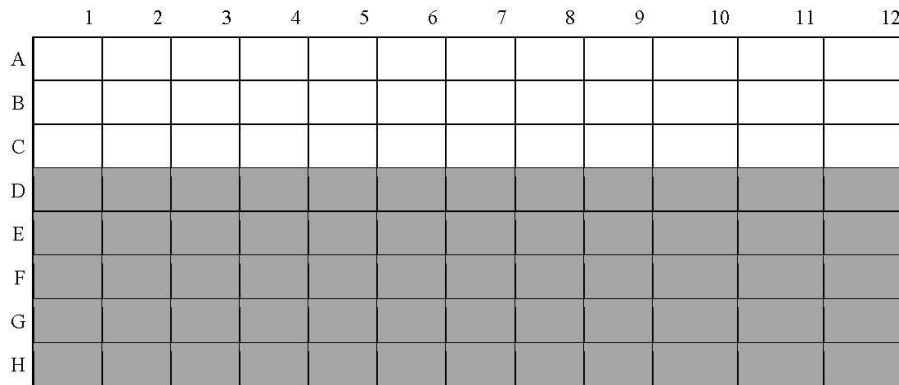
Figure 3. Preparation of the T-MP (Pre-Screen ELISA)

3.4.6 Transfer of Samples from the Dilution Tubes to the Test Plate

- Processing of plates at each step will occur one plate at a time.
- All plates will follow the steps described in this section except when a “pre-screen plate” is analyzed. A “pre-screen plate” is analyzed when a large number of samples anticipated to be negative for anti-GP IgG are analyzed. A “pre-screen plate” consists of up to 30 samples which are analyzed at a single dilution (1:50) in duplicate. In such cases, follow the alternative plate set-up option described below.
- Using a plate washer, or manually, wash the filovirus rGP-coated plate three times with ELISA Wash Buffer (300 µL per well).
- Add 100 µL of ELISA Diluent to the wells of the filovirus rGP-coated assay plate as shown in Figure 4 for test samples run in the full ELISA or Figure 5 for pre-screen test samples.

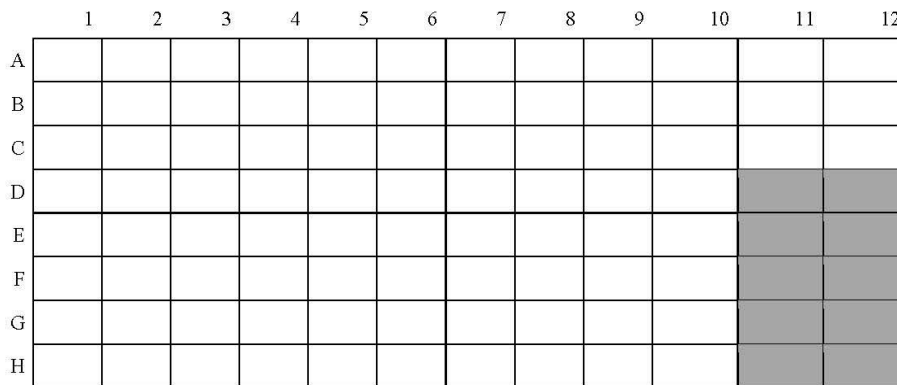
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Add 100 μ L of ELISA Diluent to the shaded wells.

Figure 4. Addition of ELISA Diluent to the Filovirus rGP-Coated Microplate



Add 100 μ L of ELISA Diluent to the shaded wells.

Figure 5. Addition of ELISA Diluent to the Filovirus rGP-Coated Microplate for Pre-Screens

- Using a multi-channel pipette, mix the RS and NC dilutions up and down (recommend 3-5 times), aspirate, and add 100 μ L of the diluted RS/NC

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from the S-MP to Rows A and B of the plate. The RS and NC will be plated without further dilution.

- For test samples run in the full ELISA, using a multi-channel pipette, mix the TS and QC dilutions up and down approximately five times, aspirate, and transfer 200 μ L of each 1X TS and QC into Row C from the appropriate row of the T-MP per the plate layout in Figure 6. Using a multi-channel pipette, serially transfer 100 μ L of TS/QC in Row C down the plate from Row C through Row H. This creates a 6-point two-fold dilution series. Discard 100 μ L from Row H with the used pipette tips. NOTE: This step is not done if the operators have already prepared their serial dilutions using a titer tube box (section 3.4.5).

	1	2	3	4	5	6	7	8	9	10	11	12
A	RS											NC
B	RS											NC
C	TS1	TS2	TS3	TS4	TS5	TS6	TS7	TS8	TS9	TS10	QC High	QC Low
D												
E												
F												
G												
H												

Figure 6. Standard Assay Plate Map Containing Diluted RS, QCs, NC, and up to 10 Test Samples (TSs)

- For pre-screen samples, using a multi-channel pipette, mix the RS and NC dilutions up and down approximately five times, aspirate, and add 100 μ L of the diluted RS/NC from the S-MP to Rows A and B of the plate. The RS and NC will be plated without further dilution. The QCs will be

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diluted down the plate. Using a single-channel pipette, add 200 µL of the diluted QCs to wells C11 and C12 according to the plate map below. Using a multi-channel pipette, create a 6-point two-fold dilution series for the QCs. Discard 100 µL from the last well in the series with the used tips. Using a multi-channel pipette, add 100 µL of the diluted TS from the T-MP in duplicate wells according to figure 7.

	1	2	3	4	5	6	7	8	9	10	11	12
A	RS											NC
B	RS											NC
C	TS1	TS1	TS7	TS7	TS13	TS13	TS19	TS19	TS25	TS25	QCH	QCL
D	TS2	TS2	TS8	TS8	TS14	TS14	TS20	TS20	TS26	TS26		
E	TS3	TS3	TS9	TS9	TS15	TS15	TS21	TS21	TS27	TS27		
F	TS4	TS4	TS10	TS10	TS16	TS16	TS22	TS22	TS28	TS28		
G	TS5	TS5	TS11	TS11	TS17	TS17	TS23	TS23	TS29	TS29		
H	TS6	TS6	TS12	TS12	TS18	TS18	TS24	TS24	TS30	TS30		

Figure 7. Pre-Screen Assay Plate Map Containing Diluted RS, QCs, NC, and up to 30 Pre-Screen Samples

- Incubate the plate(s) for 1 hour ± 5 min at 37°C ± 2°C.

3.4.7 HRP Conjugated Secondary Antibody Dilution and Incubation

- Prepare the conjugate dilution and use within 2 hours of preparation. Dilute the conjugated secondary antibody in ELISA Diluent. Prepare approximately 11 mL of diluted conjugated secondary antibody per test plate. Store at RT protected from light and mix thoroughly without

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creating bubbles prior to use by inverting the tube gently several times or by pipetting the solution up and down with a serological pipet. Any remaining diluted conjugated secondary antibody should be discarded ≤ 2 hours after preparation.

- Upon completion of the first incubation, remove the assay plate(s) from the incubator and wash the plate(s) 3 times with ELISA Wash Buffer as described in Section 3.4.6.
- Pour the diluted HRP-conjugated secondary antibody into a reagent reservoir. Using a multi-channel pipette, transfer 100 μL of the diluted conjugated secondary antibody into all wells of the assay plates.
- Incubate the plate(s) for 1 hour \pm 5 min at $37^\circ \pm 2^\circ\text{C}$.

3.4.8 TMB Substrate and Incubation

- TMB must be equilibrated to RT before use. It is considered light sensitive and should be stored in the dark until used in the assay. DO NOT pipette directly from bottle nor pour excess back into stock bottle as this may contaminate the stock bottle. It is recommended to pour out an aliquot into a clean container to allow to warm to RT instead of leaving stock bottle at RT for longer than necessary.
- Obtain approximately 11 mL of TMB substrate per assay plate. Do not remove more TMB than will be needed; once the reagent is poured into a reservoir, it may not be returned to the bottle.
- Upon completion of the secondary antibody incubation, remove the test plate(s) containing the secondary (conjugate) antibody from the incubator, and wash the plate(s) 5 times with ELISA Wash Buffer as described in Section 3.4.6.
- Pour the TMB substrate into a reagent reservoir. Using a multi-channel pipette, transfer 100 μL of the substrate into all wells of the assay plates.
- Incubate each plate 10 \pm 2 minutes at RT with TMB.



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3.4.9 Stop Solution Addition and Incubation

- Pour the TMB Stop Solution into a reagent reservoir. Using a multi-channel pipette, transfer 100 μ L of the TMB Stop Solution into all wells of the assay plates. Avoid creating bubbles, as they can interfere with the accuracy of the optical density values when reading the plates.
- Read the plates using a Bio-tek microplate reader, Molecular Devices SpectraMax plate reader, or other suitable alternative. Read at a wavelength of 450 nm with a default reference wavelength of 650 nm.
- **NOTE: If using a SpectraMax plate reader, it is very important that each user ensures that preferences are set up to automatically save the SoftMax Pro raw data file to the computer's hard drive. It is also important the print and initial/date their results immediately as this is the raw data.**
- Plates must be read within 30 minutes of addition of Stop Solution, though reading the plates as soon as possible after the addition of Stop Solution is optimal.

3.5 Calculation of the Anti-Filovirus GP IgG Concentrations and Acceptance Criteria

- There are two ways to calculate the anti-GP IgG concentrations and evaluate the acceptance criteria. The first way is to upload the OD values into the ELISA module of the BioAssay database and use the automation to perform the calculations. This process is described below.

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- If plates were not read on a SpectraMax plate reader, copy/paste the raw data (OD values) from the data acquisition software (e.g., Gen5™ Secure software) into the appropriate SoftMax Pro template for data calculation.
- Open the “pda raw data file using the appropriate SoftMax Pro template.
- SoftMax Pro will graph the reference standard curve and calculate the Mean Value, Standard Deviation, %CV, Result, Mean Result, and %RE for each dilution of the RS.
- Softmax Pro will calculate the concentration of each dilution for every QC sample and every TS via interpolation using the reference standard curve. The software will also calculate the Mean Result, the Adjusted Result, the Mean Adjusted Result, and the %CV of the Mean Adjusted Result for the TSs and QCs.
- Inspect the plate to determine if it passes plate acceptance criteria, then evaluate if the test samples pass the test sample acceptance criteria. NOTE: A flow chart depicting the plate/sample evaluation and censoring process is appended to this SOP.

3.5.1 Plate Acceptance Criteria

3.5.1.1 Negative Control Acceptance Criteria

The negative control must have an average OD value ≤ 0.20 . If the NC fails, the plate fails and need not be evaluated further.

3.5.1.2 Reference Standard Curve Evaluation

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Reference Standard Curve Censoring Criteria Based on Percent Relative Error:

- The reference standard curve will be evaluated based on the percent relative error (%RE) of the individual dilution pairs, excluding the anchor points of the standard curve as defined below.
- The anchor points of the standard curve are defined as follows:
 - The lower anchor points are defined as low concentration points where the mean OD value of the dilution pair is less than (<) the maximum allowable OD value for the negative control wells.
 - The high anchor points are defined as high concentration points where the predicted OD (that is, the OD fit to the model for the known concentration of the dilution point) is greater than (>) 0.90 x D (the upper asymptote from the 4PL model).
 - All remaining reference standard dilutions pairs are considered to be in the “linear range” of the standard curve.
- The criteria for the remaining dilution pairs (excluding the anchor points) are as follows:
 - At least 5 points must be included in the “linear range” of the reference standard curve.
 - At least (\geq) 75% of the reference standard dilutions in the “linear range” must have %RE (absolute value) \leq 20%, exclusive of censored points (see below).
- Censoring of the Reference Standard Curve
 - Censoring of the RS curve will not be allowed.

3.5.1.3 QC Acceptance Criteria

- All QCs must fall within the acceptable concentration range for that lot of QC. The QCs may be censored following the rules defined in Section 3.5.2.
- If the QC acceptance criteria are met (along with the RS and NC acceptance criteria), then the plate passes and the data will be reported as described below. However, if a test sample (analyzed at a starting



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dilution of 1:50) on a failing plate has a concentration that is less than or equal to the assay LOD (LLOQ if LOD has not been determined) then, at the discretion of the study director or designee, this sample may be considered passing and the concentration will be accepted. This only applies to plates that fails plate acceptance criteria because of not meeting the quality control criteria.

3.5.2 Test Sample and Quality Control Censoring Criteria

- Test samples and QCs will be censored following the same rules.
- Test sample and QC dilutions will be censored if:
 - (1) A dilution is outside of the RS range. The RS range is the lowest and highest dilution points on the RS curve.
 - (2) A dilution is above the upper asymptote of the RS curve or below the lower asymptote of the RS curve.
 - (3) A dilution is less than or equal to the mean OD value of the NC.
 - (4) If the OD value of the 1:50 dilution is \leq the NC cutoff for the species being evaluated, mask all points but the 1:50 dilution point and pass the sample. Flag the sample by reporting the NC calculated value.
 - (5) If a test sample with a starting dilution of 1:50 has an OD value for the 1:50 dilution \leq 0.337 (upper bound of the 95% prediction interval as calculated in the report "Prediction Intervals for Naïve Human Samples in the anti-GP IgG ELISA" performed as part of QD-438) mask all points but the 1:50 and 1:100 dilution points and pass the sample. Flag the sample by reporting " \leq 0.337".
 - (6) This same guidance applies to any species where a 95% confidence interval has been calculated. If a 95% predication interval has been

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calculated, then use the upper bound of this interval as the OD value cutoff.

(7) The within-assay %CV > 20%. Censor the dilution point furthest from the mean concentration, reevaluate the %CV and continue censoring if the %CV is still > 20%. See below for more details.

(8) For any sample that needs repeated, it may be run at the most appropriate starting dilution to maximize the number of dilution points that fall within the range of the RS.

Note: For the following guidance, the number of censored points is counted after any points are censored per the above guidance (e.g., outside the RS range, below the NC, etc.).

- For samples with 4, 5 or 6 dilutions within the range of the RS:
 - (1) At least three dilutions must be within the range of the RS after censoring for the result to be acceptable.
 - (2) If 5 or 6 dilutions fall within range, up to 2 dilutions may be censored. If the within-assay %CV > 20% after censoring 2 dilutions, then the sample fails and will be repeated. However, per Study Director input, the “only censor up to 2 points” guidance may be implemented in ongoing studies, but must be clearly documented in the Study Records (e.g., memo or other appropriate document).
 - (3) If 4 dilutions fall within the range of the RS before censoring, then 1 dilution can be censored. If the within-assay %CV > 20% after censoring one dilution, then the sample fails and will be repeated.
 - (4) If 3 dilutions fall within the range of the RS before censoring, then 1 dilution can be censored. If the within-assay %CV > 20% after censoring one dilution, then the sample fails and will be repeated.

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- For samples with 4 or more points outside the range of the RS before censoring:
 - (1) If a high-titered test sample does not have at least 3 uncensored dilution points within the range of the RS, mark the sample as failing. The test sample may be repeated at a more appropriate dilution (e.g., higher dilution) if that new dilution will increase the chances of having more dilution points fall within the range of the RS.
 - (2) If a test sample has 3 or more dilutions below the range of the RS, but was not started at 1:50, then repeat the sample at a more appropriate dilution to maximize the number of dilutions that fall on the RS curve. Mark the sample as failing.
- For samples with dilution points both above and below the range of the RS before censoring:
 - (1) If a sample has dilution points both above and below the range of the RS then at least three dilutions must be within the range of the RS after censoring for the result to be acceptable (maximum of 1 censored point).
- For low-titered samples (samples with 3 or more points below the range of the RS when started at 1:50):
 - (1) If only 1 dilution falls within the range of the RS, then the sample passes.
 - (2) If 2 dilutions fall within the range of the RS before censoring and the within-assay %CV > 20% and the 1:50 dilution point is ≤ 0.337 , mask all points but the 1:50 and 1:100 dilution point. Flag the sample by reporting " ≤ 0.337 ".
 - (3) If 2 dilutions fall within the range of the RS before censoring and the within-assay %CV > 20% and the 1:50 dilution point is > 0.337 , then mark the sample as failing and repeat the sample.



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- (4) If 3 dilutions fall within the range of the RS before censoring, then only one dilution can be censored. If the within-assay %CV > 20% after censoring one dilution, then the sample fails and will be repeated.
- (5) Occasionally, a negative or low titered test sample will have a dilution point(s) at or near the low end of the dilution series that has an OD value that is slightly above the low end of the RS range. In this situation other, more concentrated dilution points of the test sample will have OD values that are lower than this dilution point and possibly even below the RS range.



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3.5.3 Calculation of the Reportable Value

The reportable value is given as a concentration in ELISA units/mL

3.5.3.1 Reportable Value for Concentration of Test Samples Run in the Full ELISA

Concentration based on the result of at least TWO Independent Analyses:

- (1) The first and second analyses of the test samples must be performed by two independently-operating test operators on a single day, or by a single test operator on two separate days, or equivalent combination thereof. True independence is considered the independent preparation of the TSs and RS, and their dilution in the ELISA plate. However, it is preferred (but not required) that the QCs and NC also be independently prepared. All subsequent steps starting with preparation of conjugate may be performed by a single test operator and still be considered independent analyses.
- (2) Both analyses must be on plates that pass the plate acceptance criteria described in Section 3.5.1. If one or both plates do not pass the plate acceptance criteria, then the test sample(s) on the failing plates must be repeated before final results can be calculated and reported.
- (3) When analyzing a test sample at least two independent times, the final anti-filovirus GP IgG concentration is calculated as the **median** of the independent passing test sample results. The standard deviation and the %CV are also calculated for the test sample. At the study director's discretion, the mean may also be reported.
- (4) Follow the guidance in Section 35.2. for determination of each individual test sample concentration.
- (5) The two independent passing test sample results must have a %CV \leq 30%.
 - If the %CV \leq 30% then the median of the two independent test sample results is reported. Note that this is the same as the mean.

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- If the %CV > 30% then the test sample must be repeated one time (total of three independent passing results).
- If the serum concentration is low, such that the median of the sample is less than or equal to the LOD (or LLOQ if the LOD is not available) of the assay but the %CV is greater than 30%, then the test sample does not need to be repeated. Instead, report the median of the two independent test sample results and make a notation that the %CV > 30%. For samples that are below the RS range, assign the sample a concentration of 0.00 ELISA units/mL for the purposes of calculating the median.
- If the %CV of the three test sample results is $\leq 30\%$ then the median concentration of the three results is reported. If the %CV of the three results is still > 30%, then the test sample must be repeated again (total of four independent test sample results). However, if the serum concentration is low, such that the median of the sample is less than or equal to the LOD/LLOQ of the assay but the %CV > 30%, then the test sample does not need to be repeated. Instead, report the median of the three independent test sample results and make a notation that the %CV > 30%.
- If the %CV is still > 30% after the fourth analysis, then the median concentration of the four results is reported and a notation is made that the %CV is > 30%.

3.6 Documentation Process

Documentation of the data analysis process will be conducted utilizing % RE.

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- **The ELISA Analysis Automation will upload the following to the database:**
 - Optical Density file (.xls or equivalent)
 - ELISA Analysis Results file (.xml analysis logic file)
- **It is not necessary to print the files after analysis. If a hard copy is desired, open the ELISA Report file (html) in a suitable program and print.**

The BioAssay Database uses a specific number of decimal places for the following values:

- Antibody concentrations for QCs and test samples = 2 decimal places
- OD value of NC = 3 decimal places
- R² value of RS curve = 4 decimal places.





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4. References

Logue J, Tuznik K, Follmann D, Grandits G, Marchand J, Reilly C, Sarro YDS, Pettitt J, Stavale EJ, Fallah M, Olinger GG, Bolay FK, Hensley LE. Use of the Filovirus Animal Non-Clinical Group (FANG) Ebola virus immuno-assay requires fewer study participants to power a study than the Alpha Diagnostic International assay. *J Virol Methods*. 2018 Feb 23;255:84-90. doi: 10.1016/j.jviromet.2018.02.018. [Epub ahead of print]





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5. Appendices

Appendix 1 – ELISA Diluent Prep Form

FANG ELISA Diluent Reagent Prep Form					
Reagent Name:	FANG ELISA Diluent				
Lot Number Assigned:					
Expiration Date Assigned:					
Initials and Date:					
Equipment	NIH Decal / ID Number / Serial Number				
P1000 Pipette					
Dry Scale					
Refrigerator					
Reagent	Manufacturer	Catalog #	Lot #	Expiration Date	Initials and Date
1X PBS	Gibco				
Non-Fat Dry Milk	LabScientific	M0841			
TWEEN 20	Sigma-Aldrich	P2287			
Directions					
Remove 100ml from a 500ml bottle of 1xPBS to another bottle of 1xPBS					
Add 25g dry milk, shake until dissolved and wait until bubbles disappear.					
Add 500µL TWEEN 20.					
Bring volume in ELISA Diluent bottle back up to 500mL using excess 1X PBS.					
Shake until thoroughly mixed.					
STORE at 4C					
EXPIRES AFTER TWO WEEKS					





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Appendix 2 – FANG Wash Buffer Prep Form

FANG Wash Buffer Reagent Prep Form					
Reagent Name:	FANG Wash Buffer				
Lot Number Assigned:					
Expiration Date Assigned:					
Initials and Date:					
Equipment	NIH Decal / ID Number / Serial Number				
P1000 Pipette					
Reagent	Manufacturer	Catalog #	Lot #	Expiration Date	Initials and Date
PBS 1x	Gibco				
TWEEN 20	Sigma-Aldrich	P2287			
Directions					
Add 500µL TWEEN 20 to PBS and shake until dissolved.					
STORE AT ROOM TEMPERATURE					
EXPIRES AFTER ONE MONTH					





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Appendix 3 – FANG Plate Coating Form

FANG Anti-Zaire Ebola Virus GP IgG ELISA
Microplate Coating and Use Log

STUDY: _____

Equipment	Manufacturer	Model	Serial Number				
Pipette				Equipment and Reagents Verified, Plates Prepared, and Incubation Started By/Date:			
Multichannel Pipette							
Refrigerator							
Reagents/Supplies	Manufacturer	Catalog #	Lot Number	Stock Concentration	Volume	Expiration Date	
1X PBS	N/A	N/A		1X	11 ml per plate		
rGP	Virus:	ABL	N/A	17Oct13	1.53mg/mL	3.6uL per plate	N/A
	ZBOV						
Immulon 2 HB Microplate	Fisher Scientific	14-245-61		N/A	N/A	N/A	
Prepare coating buffer. Combine reagents and mix well. Add 100µL to each well. Cover the top plate with a plate sealer. Wrap stacks of plates with plastic wrap, then wrap in foil to protect from light. Incubate in refrigerator for a minimum of 14 hours. Make plates fresh the evening before testing.							
Incubation Start Time: _____							
Microplate Identifier		Date of Use / Initials		Date of Destruction / Initials			





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Appendix 4 – FANG Run Sheet

2.0 Participant sample record list: Initials and Date _____

Sample #	Sample ID	Pull Sheet Date	Plate #	Comments (i.e. color is pink/red)	Dilution	Reagent
TS1			1		50	RS
TS2			1		50	QCH
TS3			1		50	QCL
TS4			1		50	Neg Control
TS5			1		50	HRP 2*
TS6			1		50	Positive Ref
TS7			1		50	Date
TS8			1		50	
TS9			1		50	
TS10			1		50	
TS11			2		50	
TS12			2		50	
TS13			2		50	
TS14			2		50	
TS15			2		50	
TS16			2		50	
TS17			2		50	





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Appendix 5 – FANG Incubation Time Sheet

Time Sheets:

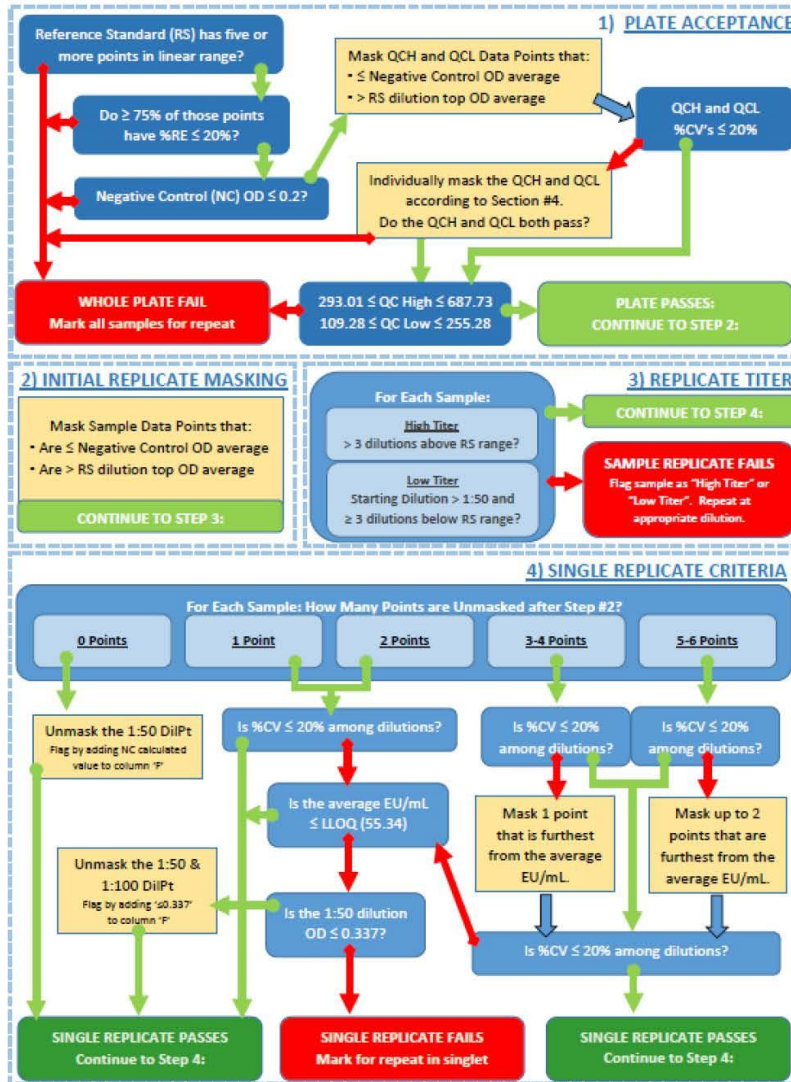
Initials and Date _____ Use these tables to indicate to record start / stop times when running multiple plates

Plate #	1 st Incubation	2 nd Incubation	Substrate	Read time
	Time on _____ Time off _____	Time on _____ Time off _____	Time on _____ Time off (STOP) _____	
	Time on _____ Time off _____	Time on _____ Time off _____	Time on _____ Time off (STOP) _____	



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Appendix 6 – Plate/Sample Evaluation and Censoring Flow Chart



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