# STATISTICAL ANALYSIS PLAN FOR HVTN IMMUNOGENICITY

## Protocol HVTN 135 (v3.0)

*A phase 1 clinical trial to evaluate the safety and immunogenicity of the HIV-1 CH505 transmitted/founder gp120 adjuvanted with GLA-SE in healthy, HIV-exposed uninfected infants*

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# Statistical Analysis Plan for Immunogenicity

## Protocol: HVTN 135 (v3.0)

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# SAP Modification History

The version history of, and modifications to, this statistical analysis plan are described below.

| SAP Version | Modification |
|---|---|
| 1.0 | Initial |
| 2.0 | Add ADCC |
| 3.0 | Add ICABA, BAMA, ADCP, BCP, and NAb assay |
| 4.0 | Modify ICABA, BAMA and ADCP assay |
| 5.0 | Modify ADCP assay |
| 6.0 | Modify BAMA assay |
| 7.0 | Modify NAb Assay |
| 8.0 | Add PVMA and modify NAb Assay |

# Table of Contents

# 1 OVERVIEW

The following describes the Statistical Analysis Plan (SAP) for the analysis of immunogenicity data from HVTN **135** for Protocol Team (PT) reports for immunogenicity data, and the FSR for Immunogenicity.

# 2 PROTOCOL SUMMARY

## 2.1 Title

HVTN 135 is a phase 1 clinical trial to evaluate the safety and immunogenicity of the HIV-1 CH505 transmitted/founder gp120 adjuvanted with GLA-SE in healthy, HIV-exposed uninfected infants

## 2.2 Study products and routes of administration

- CH505TF gp120: CH505TF gp120 with the adjuvant GLA-SE. GLA-SE is an oil-in-water stable emulsion (SE) containing the immunological adjuvant Glucopyranosyl Lipid A (GLA). The vaccine will be administered by intramuscular (IM) injection in the thigh at 2 doses of gp120 (5 or 20 mcg) and 2 doses of adjuvant (2.5 mcg or 5 mcg).
- Placebo: Sodium Chloride for Injection, 0.9% USP, administered IM in the thigh at volumes to match the active product.

## 2.3 Schema

| | | Injection schedule in weeks | | | | |
|---|---|---|---|---|---|---|
| **Group** | **N** | **Week 0** | **Week 8** | **Week 16** | **Week 32** | **Week 54** |
| **Part A** | | **Initial Safety** | | | | |
| 1 | 5 | 20 mcg CH505TF gp120 +2.5 mcg GLA-SE | 20 mcg CH505TF gp120 +2.5 mcg GLA-SE | 20 mcg CH505TF gp120 +2.5 mcg GLA-SE | 20 mcg CH505TF gp120 +2.5 mcg GLA-SE | 20 mcg CH505TF gp120 +2.5 mcg GLA-SE |
| 2 | 2 | Placebo | Placebo | Placebo | Placebo | Placebo |
| **Part B** | | **Safety Ramp-Up** | | | | |
| 3 | 2 | 20 mcg CH505TF gp120 +5 mcg GLA-SE | 20 mcg CH505TF gp120 +5 mcg GLA-SE | 20 mcg CH505TF gp120 +5 mcg GLA-SE | 20 mcg CH505TF gp120 +5 mcg GLA-SE | 20 mcg CH505TF gp120 +5 mcg GLA-SE |
| 4 | 2 | Placebo | Placebo | Placebo | Placebo | Placebo |
| **Part C** | | **Immunogenicity of CH505TF Recombinant Protein** | | | | |
| 5 | 16 | 20 mcg CH505TF gp120 +5 mcg GLA-SE | 20 mcg CH505TF gp120 +5 mcg GLA-SE | 20 mcg CH505TF gp120 +5 mcg GLA-SE | 20 mcg CH505TF gp120 +5 mcg GLA-SE | 20 mcg CH505TF gp120 +5 mcg GLA-SE |
| 6 | 3 | Placebo | Placebo | Placebo | Placebo | Placebo |
| 7 | 5 | 5 mcg CH505TF gp120 +5 mcg GLA-SE | 5 mcg CH505TF gp120 +5 mcg GLA-SE | 5 mcg CH505TF gp120 +5 mcg GLA-SE | 5 mcg CH505TF gp120 +5 mcg GLA-SE | 5 mcg CH505TF gp120 +5 mcg GLA-SE |
| 8 | 3 | Placebo | Placebo | Placebo | Placebo | Placebo |
| **Total** | **38** | **28 vaccinees, 10 placebos** | | | | |

Notes: To ensure safety, enrollment will proceed in stages.
Groups 3 and 5 (20 mcg CH505TF and 5 mcg GLA-SE) and Groups 2, 4, 6, and 8 (placebo) will be pooled in the ultimate analyses.

# 3 OBJECTIVES AND ENDPOINTS

## 3.1 Primary objectives and endpoints

*Primary objective 1:*

To evaluate the safety and tolerability of HIV-1 CH505 transmitted/founder virus Env gp120 immunogen (CH505TF gp120) adjuvanted with Glucopyranosyl Lipid A - stable emulsion (GLA-SE) in healthy HIV-1 exposed uninfected (HEU) infants

*Primary endpoint 1:*

Local and systemic reactogenicity signs and symptoms, laboratory measures of safety, weight gain, and AEs and SAEs

*Primary objective 2:*

To determine whether vaccination with CH505TF gp120 adjuvanted with GLA-SE initiates B-cell lineages potentially capable of generating a broadly neutralizing antibody response

*Primary endpoints 2:*

Magnitude of HIV-1 Env gp120, CD4 binding site and V1V2-specific serum IgG binding antibodies, as assessed by BAMA two weeks after the 5th vaccination

Quantification and phenotypic characterization of peripheral B cells capable of binding HIV-1 Env gp120, the CD4 binding site (including differential binding to the IΔ371 mutant) and the V1V2 binding site, as assessed by flow cytometry two weeks after the 3rd and 5th vaccinations

## 3.2 Secondary objectives and endpoints

*Secondary objective 1:*

To evaluate the effect of vaccination with CH505TF gp120 adjuvanted with GLA-SE on antibody responses to the South African Expanded Program on Immunization (EPI) schedule vaccinations

*Secondary endpoint 1:*

EPI vaccine-specific antibody responses, as assessed by Pediatric Vaccine Multiplex Assay (PVMA) 2 weeks after the 5th vaccination

*Secondary objective 2:*

To evaluate the ability of the vaccine regimen to elicit HIV-specific nAbs.

*Secondary endpoint 2:*

Magnitude and breadth of serum neutralization of vaccine-matched viral isolates, and viruses engineered to detect precursors of CD4 binding site and V1V2 antibodies 2 weeks after the 5th vaccination.

*Secondary objective 3:*

To measure Fc-mediated antibody effector functions

*Secondary endpoints 3:*

Response rate and magnitude of vaccine-elicited serum binding antibodies to FcR proteins, as assessed by BAMA 2 weeks after the 5th vaccination

Response rate and magnitude of serum Antibody-dependent cellular cytotoxicity (ADCC), as assessed by flow cytometry and/or luciferase assays 2 weeks after the 5th vaccination

Response rate and magnitude of serum Antibody-dependent cellular phagocytosis (ADCP), as assessed by flow cytometry 2 weeks after the 5th vaccination

## 3.3 Exploratory objectives

*Exploratory objective 1:*

To compare antibody and B cell responses generated in infants in response to CH505TF gp120 adjuvanted with GLA-SE with adults vaccinated with the same immunogen and same adjuvant on the same schedule (HVTN 115)

*Exploratory objective 2:*

To characterize B cell lineages and evaluate the amount of B-cell receptor somatic hypermutation

*Exploratory objective 3:*

To evaluate effects of maternal antibody in cord blood, serum/plasma, and breast milk on the humoral response of infants

*Exploratory objective 4:*

To evaluate additional vaccine-induced antibody effector functions such as Antibody-dependent neutrophil phagocytosis (ADNP) and/or Antibody dependent complement deposition (ADCD)

*Exploratory objective 5:*

To measure the kinetics and maturation of the polyclonal antibody response with biophysical measurements (eg, BioLayer Interferometry, Surface Plasmon Resonance)

*Exploratory objective 6:*

Evaluation of breast milk and fecal microbiome at birth and over time to explore the effect of the microbiome on the development of the immune system and on vaccine immunogenicity

*Exploratory objective 7:*

To use systems biology approaches (eg, high resolution flow cytometry. transcriptomics, proteomics, metabolomics and/or plasma cytokine/chemokine assessment) to determine whether the pre- and/or postvaccination systemic immune milieu is associated with vaccine immunogenicity.

*Exploratory objective 8:*

To further evaluate immunogenicity, additional assays may be performed based on the HVTN Lab Assay Algorithm

*Exploratory objective 9:*

To conduct analyses related to furthering the understanding of HIV, immunology, vaccines, and clinical trial conduct

## 4 COHORT DEFINITION

Healthy HIV-1–uninfected infants born to HIV-1–infected mothers through caesarian delivery in South Africa. To quantify maternal HIV antibody response, mothers will also be enrolled in the study but will not receive study product.

## 5 POTENTIAL CONFOUNDERS

Characterization of the safety of the vaccine is susceptible to confounding by adverse events not related to the vaccine that by chance occur more often in one arm of the trial than another. Therefore, analyses involving adverse events will incorporate the reported relationship to product as assessed by HVTN staff.

## 6 RANDOMIZATION

A participant's randomization assignment will be computer generated and provided to the HVTN clinical research site (CRS) pharmacist through a Web-based randomization system. At the institution, the pharmacist with primary responsibility for dispensing study products is charged with maintaining security of the treatment assignments (except in emergency situations as specified in the HVTN Manual of operations [MOP]).

## 7 BLINDING

Participants and site staff (except for site pharmacists) will be blinded as to participant treatment arm assignments (eg, vaccine or placebo). Study product assignments are accessible to those HVTN CRS pharmacists, DAIDS protocol pharmacists and contract monitors, and SDMC staff who are required to know this information to ensure proper trial conduct. Any discussion of study product assignment between pharmacy staff and any other HVTN CRS staff is prohibited. The HVTN SMB members also are unblinded to treatment assignment to conduct review of trial safety.

When a participant leaves the trial prior to study completion, the participant will be told he or she must wait until all participants are unblinded to learn his or her treatment assignment.

In some cases, the CRS, PSRT, or study sponsor may believe unblinding of the site PI and participant would be appropriate to facilitate the clinical management of an AE or SAE. The HVTN Unblinding MOP specifies procedures for emergency unblinding, and for early unblinding for medical reasons.

# 8 STATISTICAL ANALYSIS

This section describes the final study analyses, unblinded as to treatment arm assignment. All data from enrolled participants will be analyzed according to the initial randomization assignment regardless of how many vaccinations they received. Analyses are modified intent-to-treat in that individuals who are randomized but not enrolled do not contribute data and hence are excluded. Because of blinding and the brief length of time between randomization and enrollment—typically no more than 4 working days—very few such individuals are expected.

Analyses for primary endpoints will be performed using SAS and R. All other descriptive and inferential statistical analyses will be performed using SAS, StatXact, or R statistical software.

No formal multiple comparison adjustments will be employed for multiple safety endpoints, multiple primary immunogenicity endpoints, or secondary endpoints. However, multiplicity adjustments will be made for certain immunogenicity assays, as discussed below, when the assay endpoint is viewed as a collection of hypotheses (eg, testing multiple peptide pools to determine a positive response).

## 8.1 Analysis variables

The analysis variables consist of baseline participant characteristics, safety, and immunogenicity for primary- and secondary-objective analyses.

## 8.2 Baseline comparability

Treatment arms will be compared for baseline participant characteristics using descriptive statistics.

## 8.3 Safety/tolerability analysis

Since enrollment of mother-infant pairs is concurrent with the infant receiving the first vaccination, all infants will have received at least 1 vaccination and therefore will provide some safety data.

### 8.3.1 Reactogenicity

The number and percentage of participants experiencing each type of reactogenicity sign or symptom will be tabulated by severity and treatment arm and the percentages displayed graphically by arm. For a given sign or symptom, each participant's reactogenicity will be counted once under the maximum severity for all injection visits. In addition, to the individual types of events, the maximum severity of local pain or tenderness, induration or erythema, and of systemic symptoms will be calculated. Kruskal-Wallis and Wilcoxon rank sum tests will be used to test for differences in severity between arms.

### 8.3.2 AEs and SAEs

AEs will be summarized using MedDRA System Organ Class and preferred terms. Tables will show by treatment arm the number and percentage of participants experiencing an AE within a System Organ Class or within preferred term category by severity or by relationship to study

product. For the calculations in these tables, a participant with multiple AEs within a category will be counted once under the maximum severity or the strongest recorded causal relationship to study product. Formal statistical testing comparing arms is not planned since interpretation of differences must rely heavily upon clinical judgment.

A listing of SAEs reported to the DAIDS Regulatory Support Center (RSC) Safety Office will provide details of the events including severity, relationship to study product, time between onset and last vaccination, and number of vaccinations received.

### 8.3.3 Local laboratory values

Box plots of local laboratory values will be generated for baseline values and for values measured during the study by treatment arm and visit. Each box plot will show the first quartile, the median, and the third quartile. Outliers (values outside the box plot) will also be plotted. If appropriate, horizontal lines representing boundaries for abnormal values will be plotted.

For each local laboratory measure, summary statistics will be presented by treatment arm and timepoint, as well as changes from baseline for post enrollment values. In addition, the number (percentage) of participants with local laboratory values recorded as meeting Grade 1 AE criteria or above as specified in the Division of AIDS Table for Grading the Severity of Adult and Pediatric Adverse Events will be tabulated by treatment arm for each postvaccination timepoint. Reportable clinical laboratory abnormalities without an associated clinical diagnosis will also be included in the tabulation of AEs described above.

### 8.3.4 Reasons for vaccination discontinuation and early study termination

The number and percentage of participants who discontinue vaccination and who terminate the study early will be tabulated by reason and treatment arm.

## 8.4 Immunogenicity analysis

### 8.4.1 General Approach

For the statistical analysis of immunogenicity endpoints, data from enrolled participants will be used according to the initial randomization assignment regardless of how many injections they received. Additional analyses may be performed, limited to participants who received all scheduled injections per protocol. Assay results that are unreliable, from specimens collected outside of the visit window, or from HIV-infected participants post-infection are excluded.

Since the exact date of HIV infection is unknown, any assay data from blood draws 4 weeks prior to an infected participant's last seronegative sample and thereafter may be excluded. If an HIV-infected participant does not have a seronegative sample post enrollment, then all data from that participant may be excluded from the analysis.

Discrete categorical assay endpoints (eg, response rates) will be analyzed by tabulating the frequency of positive response for each assay by antigen and treatment arm at each timepoint for which an assessment is performed. Crude response rates will be presented with their corresponding 95% confidence interval estimates calculated using the score test method [1]. Because of the small numbers of control participants in each group, no adjustment will be made to the vaccine arm estimates for the false positive rates in the control arms. Barnard or Fisher's exact tests will be used to compare the response rates across treatment groups, with a significant difference declared if the 2-sided p-value is ≤ 0.05; however the primary analysis will prioritize the one-sample comparison of those response rates to zero due to the power considerations

discussed above. In general Barnard's is preferred since under most circumstances it is more powerful than Fisher's [2].

In addition to response rate estimates for each timepoint, the probability of observing at least 1 positive response by a given timepoint and the probability of observing more than 1 positive response by a given timepoint will be estimated, with corresponding confidence intervals, for each vaccine arm using maximum likelihood-based methods [3].

For quantitative assay data (eg, magnitudes and magnitude-breadth AUCs from the neutralizing antibody multiplex assay), graphical and tabular summaries of the distributions by antigen, treatment arm, and timepoint will be made. For all primary and secondary immunogenicity endpoints, box plots and plots of estimated reverse cumulative distribution curves will be used for graphical display of all of the study arms. Typically, the results will be shown for each vaccine arm and for the set of control arms pooled into one group.

Some immunologic assays have underlying continuous or count-type readout that are dichotomized into responder/nonresponder categories (eg, nAb multiplex assay positivity). If treatment arm differences for these assays are best summarized by a mixture model, then Lachenbruch's test statistic [4] will be used to evaluate the composite null hypothesis of equal response rates in the 2 arms and equal response distributions among responders in the 2 such arms. This test statistic equals the square of a binomial Z-statistic for comparing the response rates plus the square of a Wilcoxon statistic for comparing the response distributions in the subgroup of responders. A permutation procedure is used to obtain a 2-sided p-value. For estimation, differences in response rates between arms will be estimated using the methods described above, and in the subgroup of positive responders, differences in location parameters between arms will be estimated using the methods described above.

Based upon previous HVTN trials as well as experience conducting infant clinical trials in the study population, missing up to 25% of immunogenicity results for a specific assay is anticipated due to study participants terminating from the study early, problems in shipping specimens, or low cell viability of processed peripheral blood mononuclear cells (PBMCs). To achieve unbiased statistical estimation and inferences with standard methods applied in a complete-case manner (only including participants with observed data in the analysis), missing data need to be missing completely at random (MCAR). Following the most commonly used definition, MCAR assumes that the probability of an observation being missing does not depend on any participant characteristics (observed or unobserved). When missing data are minimal (specifically if no more than 20% of participants are missing any values), then standard complete-case methods will be used, because violations of the MCAR assumption will have little impact on the estimates and hypothesis tests.

If a substantial amount of immunogenicity data is missing for an endpoint (at least 1 value missing from more than 20% of participants), then using the methods that require the MCAR assumption may give misleading results. In this situation, analyses of the immunogenicity endpoints at a specific timepoint will be performed using parametric generalized linear models fit by maximum likelihood. These methods provide unbiased estimation and inferences under the parametric modeling assumptions and the assumption that the missing data are missing at random (MAR). MAR assumes that the probability of an observation being missing may depend upon the observed responses and upon observed covariates, but not upon any unobserved factors. Generalized linear models for response rates will use a binomial error distribution and for quantitative endpoints, a normal error distribution. For assessing repeated immunogenicity measurement, linear mixed effects models will be used. If the immunological outcomes are left- and/or right- censored, then the linear mixed effects models of Hughes [5] will be used, because they accommodate the censoring. In addition, secondary analyses of repeated immunogenicity measurements may be done using weighted GEE [6] methods, which are valid under MAR. All

the models described above in this paragraph will include as covariates all available baseline predictors of the missing outcomes.

Some “resource-intensive” immunogenicity endpoints are only measured in subset of participants, eg, immunogenicity endpoints based on mucosal samples. For such endpoints, exploratory analyses will be conducted to assess the correlation of participant characteristics measured in (nearly) all participants with the resource-intensive endpoints. For example, if the same assay is performed on blood and mucosal samples, then a scatterplot and Spearman rank correlation coefficient (r) will be used to assess the correlation of responses. If at least moderate correlations exist (eg, $r \geq 0.3$), then the semiparametric efficient analysis method of Rotnitzky and Robins [7] will be used (described in Gilbert, Sato et al. for application to vaccine studies (83)) to estimate the mean of the resource-intensive endpoint for each group and to compare means between groups.

### 8.4.2 Multivariate Display of Immunogenicity Endpoints

Data visualization techniques may be used to explore the relationship among immunogenicity readouts. The set of readouts may be based on one of the primary endpoints on the set of primary endpoints, or on immunogenicity endpoints that also include secondary or exploratory endpoints. To understand the relationship between pairs of readouts, scatter plots may be used when the number of readouts is small or for a larger number of readouts, a heatmap showing the degree of correlation between any two pairs. Principal component analysis (PCA) and associated ‘biplots’ of the scores and loadings are particularly useful to understand associations between readouts, especially when readouts are correlated (84). PCA is a method to reduce the dimensionality of the number of readouts to a smaller set of values (principal components) that are normalized linear combinations of the readouts in such a way that the first principal component accounts for the most variability in the data and subsequent components, while maximizing variability, are uncorrelated with each other. A ‘biplot’ displays the first and second principal component scores and principal component loadings. The x-axis is the value from the first principal component and the y-axis is the second principal component, where each axis label includes the percentage of variation in the total set of readouts captured by the principal component. The top axis is the first principal component loadings and the right axis is the second principal component loadings. An arrow is drawn for each immunogenicity readout (eg, antibody binding magnitudes) from the origin to the point defined by its first two principal component loadings. The length of the arrow represents the amount of total variation of the set of readouts captured by the given readout. The direction of an arrow conveys the extent to which the variation of a readout is in the direction of the first or second principal component. The angle between two arrows conveys information about the correlation of the two readouts, with a zero-degree angle denoting perfect correlation and a 90-degree angle denoting no correlation. Each arrow on the biplot is labeled by the immunogenicity readout it represents. A biplot is annotated with key meta-information such as the treatment arm (most common application) or a demographic category. Depending on the application, K-means clustering and hierarchical clustering may also be applied for multivariate graphical display of immunogenicity readouts.

### 8.4.3 Analysis of Multiplexed Immunoassay Data

When a small panel of analytes (eg, ≤ 5) is being assessed in a multiplexed immunoassay, the analysis of response rates and response magnitudes will be evaluated and compared as described under the general approach. When a larger panel is being assessed, two approaches may be considered to evaluate the magnitude and breadth of these responses. First, Magnitude–Breadth (M-B) curves maybe employed to display individual- and group-level response breadth as a function of magnitude. Response breadth of neutralizing antibodies is defined as the average of the log10 nAb titer over the panel of isolates, where titers that are below the limit-of-

detection are set to half of that limit. Response breadth of binding antibodies is similarly defined as the average of the response over the panel of antigens.

## 8.5 Analyses and data sharing prior to end of scheduled follow-up visits

Any analyses conducted prior to the end of the scheduled follow-up visits should not compromise the integrity of the trial in terms of participant retention or safety or immunogenicity endpoint assessments. In particular, early unblinded analyses by treatment assignment require careful consideration and should be made available on a need to know basis. Interim blinded safety and immunogenicity data should not be shared outside of the SMB, PSRT, the protocol team leadership, the HVTN Executive Management Team, the study product developer, and the study sponsor and/or its designee(s) for their regulatory reporting unless approved by the protocol leadership and the HVTN leadership.

### 8.5.1 Safety analyses

During the course of the trial, unblinded analyses of safety data will be prepared approximately every 4 months for review by the SMB. Ad hoc safety reports may also be prepared for SMB review at the request of the PSRT. Refer to the process described in the HVTN Unblinding MOP for any requests for unblinded safety data prior to the end of the scheduled follow-up visits.

### 8.5.2 Immunogenicity analyses

An unblinded statistical analysis by treatment assignment of a primary immunogenicity endpoint may be performed when all participants within any study Part (A, B, or C) have completed the corresponding primary immunogenicity visits and data are available for analysis from at least 80% of these participants. Similarly, an unblinded statistical analysis by treatment assignment of a secondary or exploratory immunogenicity endpoint may be performed when all participants within any study Part have completed the corresponding immunogenicity visit and data are available for analysis from at least 80% of these participants. The Laboratory Program will review the analysis report prior to distribution to the protocol chairs, DAIDS, study product developer, and other key HVTN members and investigators. Reports for distribution or presentation should use PubIDs and not PTIDs for individual responses. Distribution of reports will be limited to those with a need to know for informing future trial-related decisions. The HVTN leadership must approve any other requests for HVTN immunogenicity analyses prior to the end of the scheduled follow-up visits.

# 9 IMMUNOGENICITY TABLES AND FIGURES, BY ASSAY

## 9.1 Antibody Dependent Cellular Cytotoxicity (ADCC)

ADCC assay is conducted to address protocol (v3.0) secondary objective 3, that is, to measure Fc mediated antibody effector functions. A subset of serum specimens collected from participants enrolled in HVTN135 are tested to assess response rates and magnitude of serum Antibody Dependent Cellular Cytotoxicity (ADCC). Antibody responses to cells expressing HIV Env is measured with established Luciferase and GranToxiLux (GTL) assays using infant (participant) sera. To quantify maternal immune responses, paired mothers' sera from corresponding visits also tested, even though the mothers do not receive any study product.

ADCC assays are performed on infant participant serum samples collected at visit 1 (cord blood), visit 2 (whole blood), and visit 12 (whole blood). For the analysis, visit 2 is considered as "primary baseline", and visit 1 as an "exploratory baseline". The primary plots and tables will not include visit 1, only visit 2 and 12. Paired maternal serum samples at visits 2 and 12 are also assayed to assess maternal immune response.

As the study participants (infants) are HIV-exposed, the infant baseline responses are expected to be higher than usual due the influence of the maternal immune system. For this reason, visit 2 primary baseline values are not used in response calls, and an assay specific fixed positivity threshold (provided by the lab) will be used instead.

### 9.1.1 Antibody Dependent Cellular Cytotoxicity (Luciferase)

The antigen analyzed in ADCC Luciferase assay is HIV-1 CH505 IMC. CEM.CCR5.NKR cells infected with HIV-1 CH505 IMC are tested in a 96-well plate for the Luciferase ADCC assay. Participant sera in addition to controls are incubated with the IMC-infected cells and ADCC is detected through the use of Viviren luminescence.

The data from Luciferase assay is semiquantitative. Two definitions of the magnitude of the response are considered: peak of specific killing (definition 1), and area under the curve (AUC) (definition 2). The paired maternal immunes responses are also plotted for comparison, but not included in primary analysis. There is no baseline subtraction applied to the responses, and a fixed positivity threshold is applied to background (plate-level assay background) subtracted percent killing or loss of luciferase activity. A positive response is defined as >=10 after background subtraction of baseline sample and positivity at one of the first dilutions.

#### 9.1.1.1 List of Tables

- Infant ADCC response rate of peak background subtracted percent killing or loss luciferase activity by treatment group, study visit (v2 and v12), and antigen
- Distribution of peak background subtracted percent killing or loss luciferase activity among all infant participants by treatment group, study visit (v2 and v12), and antigen
- Distribution of peak background subtracted percent killing or loss luciferase activity among infant positive responders only by treatment group, study visit (v2 and v12), and antigen
- Distribution of pAUC (peak AUC) among all infant participants by treatment group, study visit (v2 and v12), and antigen
- Distribution of pAUC (peak AUC) among infant positive responders only by treatment group, study visit (v2 and v12), and antigen

#### 9.1.1.2 List of Graphs

- Barcharts of ADCC Luciferase response rates and boxplots of ADCC response magnitudes by treatment group, study visit (v2 and v12), and antigen
- *Exploratory figures [all figures include both positive and negative responders and are implemented for both magnitude definitions. All figures are implemented for all vaccine groups combined, and for all placebo groups combined]*
    - Scatterplot of infant response magnitude at v1 vs v2 by antigen [this plot helps to justify and inform the use of v1 responses as a substitute for missing v2 responses]
    - Scatterplot of infant response magnitude vs maternal response magnitude at each study visit (v2 and v12) by antigen [this plot informs on whether maternal immune responses are associated with infant immune responses, and potentially informative for isolating vaccine-induced immune responses]
    - Side by side boxplot of infant response magnitude and maternal response magnitude at each visit (v2 and v12) by antigen, with mother-infant pairs connected across the side-by-side boxplot [this plot is an alternative way of visualizing the mother-infant association shown in the last plot]
    - Side by side boxplot of maternal minus infant response magnitude by visit (v2 and v12) and antigen [this plot shows the distribution of the difference in maternal and infant immune responses, i.e., a specific contrast of the two immune responses]

- Side by side boxplot of infant v12 minus v2 response magnitude and maternal v12 minus v2 response magnitude by antigen, with mother-infant pairs connected across the side-by-side boxplot [this plot examines whether infant change in immune response is associated with maternal immune response change]

### 9.1.2 Antibody Dependent Cellular Cytotoxicity (GranToxiLux or GTL)

The antigen analyzed in ADCC Luciferase assay is Clade B CH505 gp120. CEM.CCR5.NKR cells coated with Clade B CH505 gp120 are tested in a 96-well plate for the GTL assay. Participant sera in addition to controls are incubated with the HIV protein and ADCC detected using granzyme B substrate (GTL).

The data from GTL is quantitative, measuring the percent of Granzyme B (GzB) activity or the percent of antigen-coated target cells positive for GzB. Two definitions of magnitude of response are considered: the peak background subtracted % GzB activity (definition 1), and area under the curve (AUC) (definition 2). The paired maternal immune responses are also tabulated and plotted for comparison, but not included in primary analysis. There is no baseline subtraction applied to the responses, and a fixed positivity threshold is applied to background (plate-level assay background) subtracted %GzB activity. A positive response is defined as >= 8% GzB activity.

#### 9.1.2.1 List of Tables

- Infant response rate of peak activity by treatment group, study visit (v2 and v12), and antigen
- Distribution of peak activity among all infant participants by treatment group, study visit (v2 and v12), and antigen
- Distribution of peak activity among infant positive responders only by treatment group, study visit (v2 and v12), and antigen
- Distribution of AUC among all infant participants by treatment group, study visit (v2 and v12), and antigen
- Distribution of AUC among infant positive responders only by treatment group, study visit (v2 and v12), and antigen

#### 9.1.2.2 List of Graphs

- Barcharts of infant peak activity response rates and boxplots of peak activity response magnitudes by treatment group, study visit (v2 and v12), and antigen
- *Exploratory figures [all figures include both positive and negative responders and are implemented for both magnitude definitions. All figures are implemented for all vaccine groups combined, and for all placebo groups combined]*
  - Scatterplot of infant response magnitude at v1 vs v2 by antigen [this plot helps to justify and inform the use of v1 responses as a substitute for missing v2 responses]
  - Scatterplot of infant response magnitude vs maternal response magnitude at each study visit (v2 and v12) by antigen [this plot informs on whether maternal immune responses are associated with infant immune responses, and potentially informative for isolating vaccine-induced immune responses]
  - Side by side boxplot of infant response magnitude and maternal response magnitude at each visit (v2 and v12) by antigen, with mother-infant pairs connected across the side-by-side boxplot [this plot is an alternative way of visualizing the mother-infant association shown in the last plot]
  - Side by side boxplot of maternal minus infant response magnitude by visit (v2 and v12) and antigen [this plot shows the distribution of the difference in maternal and infant immune responses, i.e. a specific contrast of the two immune responses]

- Side by side boxplot of infant v12 minus v2 response magnitude and maternal v12 minus v2 response magnitude by antigen, with mother-infant pairs connected across the side-by-side boxplot [this plot examines whether infant change in immune response is associated with maternal immune response change]

## 9.2 Infected Cells Antibody Binding Assay (ICABA)

The ICABA assay is conducted to address the exploratory objective to evaluate immunogenicity of the vaccine regimens. ICABA will test for the presence of antibodies binding to the surface of HIV-1 infected cells in the serum of infant participants using Infectious Molecular Clone (IMC)-infected target cells. To quantify maternal immune responses, paired mothers' sera from corresponding visits also tested, even though the mothers do not receive any study product.

CEM.CCR5.NKR cells infected with HIV-1 IMC CH0505, and mock-infected cells are tested in a 96-well plate for the ICABA. Participant sera in addition to controls are incubated with the IMC-infected cells and the presence of bound antibodies is detected on the surface of the cells using flow cytometry.

ICABA assays are performed on infant participant serum samples collected at visit 1 (cord blood), visit 2 (whole blood), and visit 12 (whole blood). For the analysis, visit 2 is considered as "primary baseline", and visit 1 as an "exploratory baseline". The primary plots and tables will not include visit 1, only visit 2 and 12. Paired maternal serum samples at visits 2 and 12 are also assayed to assess maternal immune response.

As the study participants (infants) are HIV-exposed, the infant baseline responses are expected to be higher than usual due the influence of the maternal immune system. For this reason, visit 2 primary baseline values are not used in response calls, and an assay specific fixed positivity threshold (provided by the lab) will be used instead.

The data from ICABA are quantitative, measuring the Mock Background Subtracted %IgG. Responses are measured for IgG. The paired maternal immune responses are also tabulated and plotted for comparison, but not included in primary analysis. There is no baseline subtraction applied to the responses, and to make response calls a fixed positivity threshold is applied to background (plate-level assay background) subtracted response magnitudes. A positive response is defined as Mock Background Subtracted %IgG >= 5%.

### 9.2.1 List of Tables

- Infant ICABA response rate by treatment group, study visit (v2 and v12)
- Distribution of ICABA response magnitude among all infant participants by treatment group, study visit (v2 and v12)
- Distribution of ICABA response magnitude among infant positive responders only by treatment group, study visit (v2 and v12)
- Distribution of AUC among all infant participants by treatment group, study visit (v2 and v12),
- Distribution of AUC among infant positive responders only by treatment group, study visit (v2 and v12)

### 9.2.2 List of Graphs

- Barcharts of infant response rates and boxplots of response magnitudes by treatment group, study visit (v2 and v12), and antigen
- *Exploratory figures [all figures include both positive and negative responders. All figures are implemented for all vaccine groups combined, and for all placebo groups combined]*
  - Scatterplot of infant response magnitude at v1 vs v2 [this plot helps to justify and inform the use of v1 responses as a substitute for missing v2 responses]

- Scatterplot of infant response magnitude vs maternal response magnitude at each study visit (v2 and v12) [this plot informs on whether maternal immune responses are associated with infant immune responses, and potentially informative for isolating vaccine-induced immune responses]
- Side by side boxplot of infant response magnitude and maternal response magnitude at each visit (v2 and v12), with mother-infant pairs connected across the side-by-side boxplot [this plot is an alternative way of visualizing the mother-infant association shown in the last plot]
- Side by side boxplot of maternal minus infant response magnitude by visit (v2 and v12) [this plot shows the distribution of the difference in maternal and infant immune responses, i.e. a specific contrast of the two immune responses]
- Side by side boxplot of infant v12 minus v2 response magnitude and maternal v12 minus v2 response magnitude, with mother-infant pairs connected across the side-by-side boxplot [this plot examines whether infant change in immune response is associated with maternal immune response change]

## 9.3 Binding Antibody Multiplex Assay (BAMA)

The BAMA analysis corresponds to the primary endpoint to assess magnitude of HIV-1 Env gp120, CD4 binding site and V1V2-specifc serum IgG binding antibodies, as assessed by BAMA 2-weeks post 5th vaccination. The following protocol objectives and endpoints will be assessed using customized BAMA:

Primary endpoints 2: Magnitude of HIV-1 Env gp120, CD4 binding site and V1V2-specific serum IgG binding antibodies, as assessed by BAMA two weeks after the 5th vaccination

Secondary Objective 3: To measure Fc-mediated antibody effector functions

Secondary Endpoint 3: Response rate and magnitude of vaccine elicited serum binding antibodies to FcR proteins, as assessed by BAMA 2 weeks post 5th vaccination

Exploratory Objective 3: To evaluate effects of maternal antibody in cord blood, serum/plasma, and breast milk on the humoral response of infants.

The customized HIV-1 BAMA employs flow cytometry-based technology that utilizes antibody and antigen interactions to test for the presence of specific antibodies in an unknown sample with the added advantage of multiplexing the antigens of interest [6,7,8]. The partially purified HIV antigens are covalently coupled to specific fluorescent bead sets. Bead sets coupled to antigens of interest are mixed to allow for multiplexing and samples added to the beads. This step is followed by the addition of a secondary anti-human antibody conjugated to biotin and/or phycoerythrin (PE) to allow for a fluorescent readout of secondary antibody binding. Microspheres are read on either a Bioplex 200 or Luminex Flexmap 3D instrument to measure Fluorescence Intensity (FI).

FcR BAMA: FcR BAMA utilized Fc Receptor (FcR) proteins tetramerized with phycoerythrin (PE) as a detection reagent to determine antigen specific binding antibody interactions with FcRs. Microspheres are read on a Flexmap 3D instrument.

The following immune response magnitude definitions are considered for this assay:

i. Net binding Mean Fluorescence Intensity (Net MFI) Mean fluorescence intensity for the antigen wells minus the MFI for the blank well on the assay plate (Definition 1)
ii. Titration (EC50/Area Under the Curve, AUC): If high binding is observed and it's deemed scientifically important, samples may be titrated to calculate antibody titers (Definition 2)

Both response magnitudes will be plotted and analyzed on a log scale. Both Definition 1 and Definition 2 are measured post-fifth vaccination. Definition 1 will be measured at baseline, while Definition 2 will also be measured at baseline for most IgG antigens except CH505TF.6R.SOSIP.664.v4.1_avi.2-Bio/293F, gp70_CH505TF_V1V2_avi/293F, and gp70_CH505TF_V1V2_N156KN160K_avi/293F. FcR data will be run at a single dilution (1:50) and therefore Definition 1 will be employed.

Primary antigens for analysis, with 'match' or 'mismatch' status with respect to the study vaccine, are as shown. Antigens considered 'wild type' vs. 'mutant type' are designated as such:

| | | |
|---|---|---|
| CH0505_CON D7gp120_avi/293/Mon | Matched | Wild type |
| CH0505_CON D7gp120_D368R_avi/293/Mon | Matched | Mutant type |
| CH505TF D7gp120d371_avi/293/Mon | Matched | Mutant type |
| CH505TFD8N156KN160K_avi/293i Mon | Matched | |
| CH505TF.6R.SOSIP.664.v4.1_avi.2-Bio/293F | Matched | |
| Con 6 gp120/B | Mismatched | |
| Con S gp140 CFI | Mismatched | |
| 1086C_D7gp120.avi/293F | Mismatched | |
| gp41 | Mismatched | |
| gp70_CH505TF_V1V2_avi/293F | Matched | |
| gp70_CH505TF_V1V2_N156KN160K_avi/293F | Mismatched | |

Infant participant serum samples are assayed at visit 1 (cord blood), visit 2 (whole blood), and visit 12 (whole blood). For the analysis, visit 2 is considered as the preferred baseline, and when not available visit 1 will be used; we refer to this as 'v1/2'. Paired maternal serum samples at visits 2 and 12 are also assayed to assess maternal immune response.

As the study participants (infants) are HIV-exposed, the infant baseline responses are expected to be higher than usual due the influence of the maternal immune system. For this reason, visit 1/2 baseline values are not used in response calls. HVTN 115 Part A baseline data will be used as statistical baseline to determine the antigen specific positivity thresholds for HVTN 135, for the antigen(s) have the same detection and conjugate (dilution 1:50 and Isotype-Conjugate (ex. IgG-PE)) in the two studies.

Infant samples are declared to have a positive response if they meet the following three conditions:

1. net MFI values >= antigen specific cutoff values (max(95th percentile of statistical baseline, 100 net MFI))
2. net MFI values > 3x antigen specific median HVTN 115 baseline net MFI values
3. MFI values > 3x antigen specific median HVTN 115 baseline MFI values

The differential binding response is measured by the ratio of magnitude of response (net MFI) to wild type vs mutant type antigens. A differential binding response is considered to be positive when it meets the three criteria above, plus three additional criteria at screening dilution or selected dilution (when samples are titrated):

4. MFI ratio (wild type/mutant type) ≥ 2.5
5. net MFI ratio (wild type/mutant type) ≥ 2.5
6. wild type antigens' MFI and net MFI ≥ 250

Epitope binding percentages for the set of CD4-bs analytes are calculated when wild type's differential binding response is positive as (Wildtype net MFI - Mutant net MFI)/Wildtype net MFI × 100 at the selected dilution where most of both Wild Type and Mutant Type analytes' net MFI fall in the assay linear range [100, 22000] net MFI at a time point.

### 9.3.1 List of Tables

- Summary table of BAMA response rate for infant participants by treatment group, study visit (v1/2 and v12), and antigen
- Summary statistics of Net MFI among all infant participants by treatment group, study visit (v1/2 and v12) and antigen
- Summary statistics of Net MFI among infant positive responders only by treatment group, study visit (v1/2 and v12), and antigen
- Summary statistics of BAMA AUC among all infant participants by treatment group, study visit (v12), and antigen
- Summary statistics of BAMA AUC among infant positive responders only by treatment group, study visit (v12), and antigen
- Summary statistics of differential binding among all infant participants by treatment group, study visit (v1/2 and v12) and antigen

### 9.3.2 List of Figures

- Bar plots of infant BAMA response rates and boxplots of Net MFI and AUC response magnitudes by treatment group, study visit (v1/2 and v12), and antigen [When both net MFI and AUC data are available, first show AUC data plots then net MFI plots]
- Bar and box plots show infant differential binding response rates and the distribution of differential binding response rates by antigen, visit, and treatment group

- Scatter plot of infant wild type vs. mutant binding response by antigen, visit, and treatment group
- *Exploratory figures: [all figures include both positive and negative responders and are implemented for both magnitude definitions, when possible. All figures are implemented for all vaccine groups combined, and for all placebo groups combined]*
    - Scatterplot of infant response magnitude at v1 vs. v2 by antigen [this plot helps to justify and inform the use of v1 responses as a substitute for missing v2 responses]
    - Scatterplot of infant response magnitude vs. maternal response magnitude at v1/2 by antigen [this plot informs on whether maternal immune responses are associated with infant immune responses, and potentially informative for isolating vaccine-induced immune responses]
    - Scatterplot of infant response magnitude at v1/2 vs v12 by antigen [this plot informs on whether passively-acquired immune responses are associated with active immune responses, and potentially informative for isolating vaccine-induced immune responses]
    - Scatterplot of infant vs. maternal differential binding at v1/2 by antigen
    - Side by side boxplot of infant response magnitude and maternal response magnitude at each visit (v1/2 and v12) by antigen, with mother-infant pairs connected across the side-by-side boxplot [this plot is an alternative way of visualizing the mother-infant association]
    - Side by side boxplot of infant differential binding response and maternal differential binding response at each visit (v1/2 and v12) by antigen, with mother-infant pairs connected across the side-by-side boxplot
    - Boxplot of infant v12 minus v1/2 response magnitude (fold change) by antigen [this plots a change in antibody post-baseline]
    - Scatterplot of infant response magnitude vs. infant gp41 response magnitude by visit (v1/2 and v12) and antigen [this plot explores the utility of gp41 responses as a marker of maternally-acquired antibody]
    - Boxplot of infant response magnitude to a given antigen minus infant response magnitude to gp41, by visit (v1/2 and v12) and antigen [this plots a specific contrast in gp41 and vaccine-induced antibody]

## 9.4 Antibody Dependent Cellular Phagocytosis (ADCP)

ADCP assay is conducted to assess the ability of polyclonal IgG, from participant serum samples, to mediate phagocytosis. The ADCP analysis corresponds to the secondary endpoint to assess the response rate and magnitude of serum antibody-dependent cellular phagocytosis (ADCP), as assessed by flow cytometry 2 weeks post 5$^{th}$ vaccination.

ADCP assay is a qualified assay employing flow cytrometric-based technology that measures the ability of antibodies to mediate phagocytosis. Biotinylated antigen conjugated to neutravidin beads are incubated with patient serum or plasma, purified IgG, or monoclonal antibodies. A monocyte cell line (THP-1) is added to the immune complexes and spinoculated at 4$^{o}$C followed by incubation at 37$^{o}$C. Cells are then analyzed for bead internalization by flow cytometry (bead positive versus bead negative detection). The primary antigen for analysis is CH505TF_D7gp120.avi/293F. Additional antigens may be assessed on an exploratory level.

A phagocytic score is determined based on the ratio of experimental sample to PBS control. Mean phagocytosis score is defined as: (% bead positive for participant x MFI bead positive for participant) / (% bead positive for PBS only control x MFI bead positive for PBS only control).

38 infant participant serum samples are assayed at visit 1 (cord blood), visit 2 (whole blood), and visit 12 (whole blood). For the analysis, visit 2 is considered as the preferred baseline, and when not available visit 1 will be used; we refer to this as 'v1/2'. Paired maternal serum samples at timepoint(s) visit 2 and visit 12 may also be assayed.

As the study participants (infants) are HIV-exposed, the infant baseline responses are expected to be higher than usual due the influence of the maternal immune system. For this reason, primary baseline values are not used in response calls. HVTN 115 baseline data will be used as statistical baseline to determine the antigen specific positivity thresholds for HVTN 135.

Positivity calls will be based on two criteria:

1. Average ADCP score >= antigen specific cutoff values (max(95th percentile of statistical baseline, 1))
2. Average ADCP score > 3x antigen specific median 115 baseline average ADCP score

### 9.4.1 List of Tables

- Summary table of ADCP response rates for infants by treatment group, study visit (v1/2 and v12), and antigen
- Summary statistics of average phagocytosis score among all infant participants by treatment group, study visit (v1/2 and v12), and antigen
- Summary statistics of average phagocytosis score among infant positive responders only by treatment group, study visit (v1/2 and v12), and antigen

### 9.4.2 List of Figures

- Bar plots of infant ADCP response rates and boxplots of average phagocytosis score by treatment group, study visit (v1/2 and v12), and antigen
- *Exploratory figures [all figures include both positive and negative responders. All figures are implemented for all vaccine groups combined, and for all placebo groups combined]*
    - Scatterplot of infant response magnitude vs. maternal response magnitude at each study visit (v1/2 and v12) by antigen [this plot informs on whether maternal immune responses are associated with infant immune responses, and potentially informative for isolating vaccine-induced immune responses]
    - Side by side boxplot of infant response magnitude and maternal response magnitude at each visit (v1/2 and v12) by antigen, with mother-infant pairs connected across the side-by-side boxplot [this plot is an alternative way of visualizing the mother-infant association shown in the last plot]
    - Side by side boxplot of maternal minus infant response magnitude by visit (v1/2 and v12) and antigen [this plot shows the distribution of the difference in maternal and infant immune responses, i.e., a specific contrast of the two immune responses]
    - Side by side boxplot of infant v12 minus v1/2 response magnitude and maternal v12 minus v2 response magnitude by antigen, with mother-infant pairs connected across the side-by-side boxplot [this plot examines whether infant change in immune response is associated with maternal immune response change]

## 9.5 Env-specific B-cell Phenotyping (BCP)

The Env-Specific B cell Phenotyping (BCP) assay will be used to identify and characterize HIV-1 Env-specific B cells and plasmablasts induced by vaccination. The BCP analysis corresponds to

the primary objective to determine whether vaccination with CH505TF gp120 adjuvanted with GLA-SE initiates B-cell lineages potentially capable of generating a broadly neutralizing antibody response. HVTN135 study design will also allow for the comparison infant and adult immune responses to CH505TF gp120 as it mimics the design of HVTN115 that evaluated CH505TF gp120 in adult humans. The study will evaluate two dose levels of the vaccine (20 and 5 mcg) and two dose levels of the adjuvant (2.5 and 5 mcg) given at five time points over 54 weeks. The study consists of 38 total participants: 28 vaccinees and 10 placebo recipients. All vaccines as well as the placebo will be delivered via intramuscular injection (IM).

Samples from baseline (Obtained from cord blood), visit 8 and visit 12 from the maximum of 38 infants in the protocol will be assayed as part of this study.

HIV-1 Env-specific B cells induced by vaccination were identified and characterized using fluorescently-labeled recombinant Env proteins in the context of a flow cytometry panel to identify and characterize those B cells.

Total B cells are identified using doublet exclusion, lymphocyte scatter profile, a viability dye to exclude dead cells, and are negative for lineage markers: CD3, CD56 and CD14; and positive for CD19 and CD20. B cells are further gated as IgD-, then IgG+, and IgA+.

Data from Low IgD- B cells (<5000) and assay results deemed unreliable for analysis by the lab were excluded from the analysis.

To assess positivity for the detection of IgD-, IgG+, and IgA+ B cells, the 85th percentile of the proportion of cells in the subset was calculated, among all placebo recipients (C2+C4+C6+C8) for a given cell subset and visit (visits 8 and 12) and used as the threshold for defining a positive response. Positivity was not assessed for visit 1.

### 9.5.1 List of Tables

- BCP response rates for infants by treatment group, study visit (v8 and v12), and cell subset
- Distribution of frequency of B cell among all infant participants by treatment group, study visit (v8 and v12), and cell subset
- Distribution of frequency of B cell among infant positive responders only by treatment group, study visit (v8 and v12), and cell subset

### 9.5.2 List of Figures

- Barcharts of infant BCP response rates and boxplots of frequency of B cell by treatment group, study visit (v8 and v12), and antigen
- Boxplots of frequency of B cell by treatment group, study visit (v1, v8, and v12), and antigen for placebo groups only without assessing positivity

## 9.6 Neutralizing Antibody (NAb)

Serum samples from all 38 infants in this trial against 6 viruses will be assessed in the TZM-bl neutralizing antibody assay to determine the kinetics, magnitude, and breadth of neutralizing activity.

Cell-free virus is pre-incubated with multiple dilutions of test sample prior to adding infection-susceptible target cells. Infection is recorded at each dilution of sample after a single round of infection. Titers of neutralizing antibodies are defined as the sample dilution that reduces the infection by 50% or 80%; the cut-off used may differ depending on assay conditions and the nonspecific activity of pre-immune samples and other normal test samples.

Neutralizing antibodies against tier 1 and tier 2 strains of HIV-1 will be measured in TZM-bl cells as a function of a reduction in Tat-induced luciferase reporter gene expression after a single round of infection with molecularly cloned, Env-pseudotyped viruses. TZM-bl (also called JC57BL-13) is a HeLa cell clone that was engineered to express CD4 and CCR5 and to contain integrated reporter genes for firefly luciferase and E. coli ß-galactosidase under control of an HIV-1 long terminal repeat (LTR). The cells are highly permissive to infection by most strains of HIV, including primary HIV-1 isolates and molecularly cloned Env-pseudotyped viruses. DEAE-dextran (Diethylaminoethyl-Dextran) is used in the medium during neutralization assays to enhance infectivity. Expression of the reporter genes is induced in trans by viral Tat protein soon after infection. Luciferase activity is quantified by luminescence and is directly proportional to the number of infectious virus particles present in the initial inoculum. The assay is performed in 96-well culture plates for high throughput capacity. Use of a clonal cell population provides enhanced precision and uniformity. The assay has been formally optimized and validated for single-round infection with either uncloned or molecularly cloned Env-pseudotyped viruses produced by transfection in 293T cells.

All designated serum samples will be assayed against Tier 1 and Tier 2 strains of virus by starting with a 1:10 dilution of serum to obtain a neutralizing antibody titer. Titers will be defined as the serum dilution that reduces RLUs by 50% (or 80%) relative to the RLUs in virus control wells after subtraction of background.

Mandatory assays with visit 12 samples (2 weeks post final vaccination) will measure neutralization titers in TZM-bl cells against the Env-pseudotyped vaccine strain (CH0505TF) and a vaccine strain lineage virus that exhibits a tier 1A neutralization phenotype (CH0505.w4.3). Additional assays with the above mentioned viruses and vaccine strain lineage viruses that exhibit a tier 2 neutralization phenotype (CH0505s.G458Y.N279K.2/GnT1-, CH0505TF.gly4/GnT1-, CH0505s.G458Y.N279K.N280D.6/GnT1-, and CH505TF.gly4.S365P/GnT1-) will be performed in TZM-bl cells with visit 12 samples. Two indicator viruses, CH0505s.G458Y.N279K.2/GnT1- and CH0505TF.gly4/GnT1-, were developed by Dr. David Montefiori to detect precursors from the CH235 and CH103 bNAb lineages respectively, and are paired with mutant viruses CH0505s.G458Y.N279K.N280D.6/GnT1- and CH505TF.gly4.S365P/GnT1- to confirm specificity of the nAb response. If a sample was positive for an indicator virus it wastested against the paired mutant virus; otherwise, the mutant virus was not tested. The magnitude of the precursor response is the fold difference in ID50 (or ID80) response, indicator vs. mutant virus.

| Evaluated bNAb Lineages Binding Specificity | Indicator Virus | Knock out (mutant) Virus |
|---|---|---|
| CH235 | CH0505s.G458Y.N279K.2/GnT1 | CH0505s.G458Y.N279K.N280D.6 /GnT1 |
| CH103 | CH0505TF.gly4/GnT1- | CH505TF.gly4.S365P/GnT1- |

The magnitude of the nAb response will be measured by the ID50 (ID80) neutralization titer which is defined is the serum dilution that reduced relative luminescence units (RLUs) by 50% and 80% relative to the RLUs in virus control wells (cells + virus only) after subtraction of background RLU (cells only). The response to a given virus/isolate is considered positive if the neutralization titer is above a pre-specified cutoff. The prespecified positivity cutoff for ID50 and ID80 is 10. The ID50 (ID80) titers for negative responders are censored at 5 (half of the positivity cutoff).

The magnitude of the precursor response is defined as the fold difference in ID50 (ID80) titer between the indicator virus and mutant virus. When the titer for the mutant virus is below the positivity cutoff, the precursor magnitude is right-censored and computed as the ID50 (ID80) titer for the indicator virus relative to the positivity cutoff. When the titer for the indicator virus is below the positivity cutoff, the sample is excluded from the analysis.

A positive precursor response is defined by a more than 3-fold difference in neutralizing antibody titer, indicator virus vs. mutant virus. This same threshold is applied to the fold-difference in ID50 titer and to the fold-difference in ID80 titer.

Given the right-censoring of precursor response magnitudes, we will use the Kaplan-Meier (KM) method to estimate the distribution of the precursor response magnitude and the proportion of participants with positive precursor response. A 95% confidence interval for this proportion will be calculated using the Greenwood formula. The KM-based estimated proportion is interpreted as the precursor response rate among participants with a positive response to the indicator virus. The KM-based reverse cumulative distribution describes the distribution of the magnitude of the precursor response among participants with a positive response to the indicator virus.

Groups 3 and 5 (20 mcg CH505TF and 5 mcg GLA-SE) and Groups 2, 4, 6, and 8 (placebo) were combined for analyses.

Tables show the response rates to each virus/isolate and corresponding 95% confidence intervals calculated by the Wilson score test method [1], as well as summary statistics among positive responders and both responders and non-responders. Precursor response rates estimated using the KM method and corresponding 95% confidence intervals calculated by Greenwood formula will also be presented. Comparisons of response rates between treatment arms (T1 vs. T3+ T5 vs. T7) for each virus will be summarized. Also, comparisons of ID50 and ID80 titers for each virus between treatment arms (T1 vs. T3+ T5 vs. T7) among all participants and among positive responders will also be presented.

Plots of neutralizing antibody titers show both response rates or precursor response and the distribution of magnitude of response to an individual virus or magnitude of precursor response. Positive responses are indicated by dots color-coded by treatment group, and negative responses by gray triangles. Data points for each participant (if any) are connected by a grey line. Box plots based upon data from only responders are superimposed on the distributions. The mid-line of the box denotes the median and the ends of the box denote the 25th and 75th percentiles. Whiskers extend to the extreme data points that are no more than 1.5 times the interquartile range or if no value meets this criterion, to the data extremes. Scatter plots of neutralizing antibody titers will show paired indicator and mutant virus titers within for each participant. Reverse CDF using Kaplan-Meier-based reverse cumulative distribution functions estimation by cell lineage will show the proportion of samples with fold change >= 3 (threshold for positivity) distribution of precursor response by cell lineage and treatment group over time for each lineage.

### 9.6.1 List of Tables

- Response rate and corresponding 95% confidence intervals using the Wilson score method by isolate, inhibitory dilution, visit and treatment group (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7).
- Precursor response rate and corresponding 95% confidence intervals using Greenwood formula by indicator/mutant pair, inhibitory dilution, visit and treatment group (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7).

- Summary statistics for NAb titers (minimum, 25th percentile, median, 75th percentile, maximum, mean, standard deviation) among all participants, by isolate, inhibitory dilution, visit, and treatment group (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7).
- Summary statistics for NAb titers (minimum, 25th percentile, median, 75th percentile, maximum, mean, standard deviation) among positive responders, by isolate, inhibitory dilution, visit, and treatment group (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7).
- Summary statistics for nAb precursor response (fold difference, indicator vs. mutant virus) (minimum, 25th percentile, median, 75th percentile, maximum, mean, standard deviation) among all participants, by indicator/mutant pair, inhibitory dilution, visit, and treatment group (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7).
- Summary statistics for nAb precursor response (fold difference, indicator vs. mutant virus) (minimum, 25th percentile, median, 75th percentile, maximum, mean, standard deviation) among all precursor positive participants, by indicator/mutant pair, inhibitory dilution, visit, and treatment group (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7).
- Response rate comparison of treatment arms (T1 vs. T3+ T5 vs. T7).
- Precursor response rate comparison of treatment arms (T1 vs. T3+ T5 vs. T7).
- Response magnitude comparison of treatment arms among all participants (T1 vs. T3+ T5 vs. T7).
- Response magnitude comparison of treatment arms among positive responders (T1 vs. T3+ T5 vs. T7).

### 9.6.2 List of Figures

- Boxplots on positive responders of neutralizing antibody titers by virus, visit, and treatment arm (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7).
- Boxplots on all participants of neutralizing antibody titers by virus, visit, and treatment arm (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7).
- Boxplots of positive responders of neutralizing antibody titers to indicator viruses, next to positive responders of neutralizing antibody titers to mutant viruses, by indicator/mutant virus pair, visit, and treatment arm (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7)
- Boxplot on positive precursor responses for fold-difference in neutralizing antibody titer to indicator vs. mutant virus, by indicator/mutant virus pair, visit, and treatment arm (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7)
- Scatterplot of indicator virus neutralizing antibody titer vs. mutant virus neutralizing antibody titer, by indicator/mutant virus pair and visit, coloring points for different treatment arms (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7)
- Kaplan-Meier-based reverse cumulative distribution plot show the distribution of precursor response by cell lineage and treatment arm (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7)

## 9.7 Pediatric Vaccine Multiplex Assay (PVMA)

The Pediatric Vaccine Multiplex Assay (PVMA) will be used to assess EPI vaccine-specific antibody responses in infant participants, while ELISA will be used to measure recombinant hepatitis B surface antigen levels in infant participants two weeks after the fifth vaccination (Month 13, Visit 12). The following protocol objectives and endpoints will be evaluated:

*Secondary objective 1:*

To evaluate the effect of vaccination with CH505TF gp120 adjuvanted with GLA-SE on antibody responses to the South African Expanded Program on Immunization (EPI) schedule vaccinations

*Secondary endpoint 1:*

EPI vaccine-specific antibody responses, as assessed by PVMA or ELISA 2 weeks after the 5th vaccination

The PVMA simultaneously measures antibodies against Haemophilus influenzae type B (HiB), diphtheria, tetanus, pertussis, rubella and respiratory syncytial virus (RSV). The assay developed by Itell, Permar and Fouda et al. is a reliable, high-throughput technique that requires minimal sample volume and measures multiple antibody concentrations [9]. Hepatitis B-specific antibodies are measured using a 384-well ELISA format. A hepB standard is used in each assay for quality control.

The following IgG concentrations will be considered to be "protective", according to WHO recommendations: 10 mIU/mL anti-hepB IgG, 0.15 µg/mL anti-HiB IgG, 0.1 IU/mL anti-tetanus IgG, 0.1 IU/mL anti-diphtheria IgG, and 10 IU/mL anti-rubella IgG [9]. As WHO-recommended protective IgG thresholds are not available for pertussis and respiratory syncytial virus (RSV), the lower limit of quantification (LLOQ) will be used as the response threshold. For results below the LLOQ, values are truncated at LLOQ/2. For results above the upper limit of quantification (ULOQ), values are truncated at ULOQ.

The LLOQ and ULOQ of the responses are as follows:

| **Antigen** | **LLOQ** | **ULOQ** |
|---|---|---|
| pertussis | 0.686 IU/mL | 55.6 IU/mL |
| RSV | 0.0762 ug/mL | 6.17 ug/mL |

Analyses will restrict attention to participants who received all relevant recommended EPI vaccine doses prior to Month 13/Visit 12. For example, anti-HiB IgG antibodies will be evaluated in infants who received all 3 doses of DTaP-IPV-HepB-Hib. The following is the RSA EPI vaccine schedule through 12 months:

| **Age** | **Vaccines** |
|---|---|
| Birth | Bacille Calmette-Guérin (BCG)<br><br>Oral Polio Vaccine (OPV) -0 |
| 6 weeks | Oral Polio Vaccine (OPV) -1<br><br>Rotavirus (RV) -1<br><br>Pneumococcal conjugate (PCV) -1 |

| | |
|---|---|
| | Hexavalent (DTaP-IPV-HepB-Hib) -1 |
| 10 weeks | Hexavalent (DTaP-IPV-HepB-Hib) -2 |
| 14 weeks | Rotavirus (RV) -2<br>Pneumococcal conjugate (PCV) -2<br>Hexavalent (DTaP-IPV-HepB-Hib) -3 |
| 6 months | Measles/Rubella (MR) -1 |
| 9 months | Pneumococcal conjugate (PCV) -3 |
| 12 months | Measles/Rubella (MR) -2 |

Tables will show the percent of infants with protective antibody levels or those with a detectable response and corresponding 95% confidence intervals calculated by the Wilson score test method [1], as well as summary statistics among participants with protective levels of antibodies or those with a detectable response and among all participants. Differences in the percent of infants with protective antibody levels/detectable response between vaccine and placebo groups (T1 + T3+ T5 + T7 vs. pooled placebo) will be reported alongside 95% confidence intervals for each antigen. Also, geometric mean ratios of IgG concentrations against each antigen between vaccine and placebo groups (T1 + T3+ T5 + T7 vs. pooled placebo) will be evaluated among all participants and reported alongside 95% confidence intervals.

Boxplots of concentrations of antibody will show both percent of infants with protective antibody levels/detectable responses and the distribution of IgG concentration. Participants with protective levels of antibodies/detectable responses will be indicated by dots color-coded by treatment group, and negative responses by gray triangles. Data points for each participant (if any) are connected by a grey line. Box plots based upon data from only participants with protective levels of antibodies/detectable responses are superimposed on the distributions. Alternatively, for plots including all participants, box plots based upon data from all participants are superimposed on the distributions. The mid-line of the box denotes the median and the ends of the box denote the 25th and 75th percentiles. Whiskers extend to the extreme data points that are no more than 1.5 times the interquartile range or if no value meets this criterion, to the data extremes. Scatterplots will show infant PVMA response vs. NAb response and infant PVMA response vs. BAMA IgG AUC response.

### 9.7.1 List of Tables

- Percent of infants with protective antibody levels or those with a detectable response and corresponding 95% confidence intervals using the Wilson score method by antigen, visit and treatment group (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7).
- Summary statistics for IgG concentrations (minimum, 25th percentile, median, 75th percentile, maximum, mean, standard deviation) among all participants, by antigen, visit, and treatment group (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7).

- Summary statistics for IgG concentrations (minimum, 25th percentile, median, 75th percentile, maximum, mean, standard deviation) among participants with protective levels of antibodies or those with a detectable response, by antigen, visit, and treatment group (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7).
- Differences in the percent of infants with protective antibody levels/detectable response between vaccine and placebo groups (T1 + T3+ T5 + T7 vs. pooled placebo) will be reported alongside 95% confidence intervals using the Wilson score method for each antigen.
- Geometric mean ratios of IgG concentrations against each antigen between vaccine and placebo groups (T1 + T3+ T5 + T7 vs. pooled placebo) will be evaluated among all participants and reported alongside 95% confidence intervals.

### 9.7.2 List of Figures

- Boxplots on participants with protective levels of antibodies of IgG concentrations or those with a detectable response by antigen, visit, and treatment arm (Placebo (C2+C4+C6+C8), T1, T3+ T5, T7).
- *Exploratory figures [all figures include both positive and negative responders. All figures are implemented for all vaccine groups combined, and for all placebo groups combined]*
    - Scatterplot of infant PVMA response vs. NAb response
    - Scatterplot of infant PVMA response vs. BAMA IgG AUC response

## 10 REFERENCES


1. Agresti A, Coull BA. Approximate is better than "exact" for interval estimation of binomial proportions. Am Stat. 1998;52(2):119-26.
2. Lydersen S, Farmland MW, Laake P. Recommended tests for association in 2 x 2 tables. Stat Med. 2009;28(7):1159-75.
3. Hudgens MG. Estimating cumulative probabilities from incomplete longitudinal binary responses with application to HIV vaccine trials. Stat Med. 2003;22(3):463-79.
4. Lachenbruch PA. Comparisons of two-part models with competitors. Stat Med. 2001;20(8):1215-34.
5. Rotnitzky A, Robins JM. Semiparametric regression estimation in the presence of dependent censoring. Biometrika. 1995;82(4):805-20.
6. Tomaras GD, Yates NL, Liu P, Qin L, et al. 2008. "Initial B-cell responses to transmitted human immunodeficiency virus type 1: Virion Binding Immunoglobulin M (IgM) and IgG Antibodies Followed by Plasma Anto-gp41 Antibodies with Ineffective Control of Initial Viremia." J Virol. Dec; 82(24):12449-63.
7. Yates NL, Liao H, Fong Y, Camp A et al. 2014. "Vaccine-Induced EnV V1/V2 IgG3 Correlates with lower HIV-1 Infection Risk and Declines Soon After Vaccination". Sci Transl Med. Mar 19;6(228):228ra39.PMCID:PMC4116665.
8. Yates NL, decamp AC, Korber BT, Liao HX, et al. 2018. "HIV-1 Envelope Glycoproteins from Diverse Clades Differentiate Antibody Responses and Durability among Vaccinees." J Virol. Jan 31. Pii:JVI.01843-'7.Doi:10.1128/JVI.01843-17.
9. Itell HL, McGuire EP, Muresan P, Cunningham CK, McFarland EJ, Borkowsky W, Permar SR, Fouda GG. 2018. Development and application of a multiplex assay for the simultaneous measurement of antibody responses elicited by common childhood vaccines. Vaccine. 2018 Sep 5;36(37):5600-5608.