INTENDED USE

The **Human Anti-Ebola Virus Glycoprotein IgG Combo** ELISA Kit is an immunoassay suitable for quantifying IgG antibody activity specific for glycoprotein of Ebolavirus subtypes **Zaire**, **Reston**, **Sudan** & **Bundibugyo**, in serum or plasma of vaccinated, immunized and/or infected hosts.

This immunoassay is suitable for:

- Determining **immune status** relative to non-immune controls;
- Assessing efficacy of **vaccines**, including dosage, adjuvantcy, route of immunization and timing;
- Qualifying and standardizing vaccine batches & protocols

The assay is for research use only (RUO) and is not intended nor validated for diagnosing Ebola virus disease (EVD). Reagents contain no virus or viral antigens.

GENERAL INFORMATION

Ebola virus (**EBOV**) causes severe disease in humans and in nonhuman primates in the form of viral hemorrhagic fever. Ebola Zaire attacks every organ and tissue in the human body except skeletal muscle and bone. Strains of Ebola include: **Zaire**, **Sudan**, **Reston**, **Bundibugyo** and Tai. All cause illness in sub-human primates. Ebola Reston has not caused illness in humans. The mortality rate of Ebola victims is between 60-90%.

Ebola virions consist of seven structural proteins.. At the center is the helical ribonucleocapsid, which consists of the genomic RNA wrapped around a polymer of nucleoproteins (**NP**). Associated with the ribonucleoprotein is the RNA-dependent RNA polymerase (L) with the polymerase cofactor (VP35) and a transcription activator (VP30). The ribonucleoprotein is embedded in a matrix, formed by the major (**VP40**) and minor (VP24) matrix proteins. These particles are surrounded by a lipid membrane derived from the host cell membrane. The membrane anchors a glycoprotein (**GP1,2**) that projects 7 to 10 nm spikes away from its surface. While nearly identical to Marburg virions in structure, Ebola virions are antigenically distinct. Viral proteins (NP, GP, and VP40) are highly immunogenic and could individually or together constitute effective vaccines. Promising vaccine candidates include DNA vaccines or those based on adenoviruses, vesicular stomatitis Indiana virus (VSIV) or filovirus-like particles (VLPs) as all of these candidates could protect nonhuman primates from Ebola virus-induced disease.

PRINCIPLE OF THE TEST

The Anti-Ebola IgG/IgM ELISA kits are based on the binding of antibodies (IgG/IgM) in samples to the recombinant, purified Ebola antigen (GP, NP or VP40) immobilized on the microwells. Bound antibody is detected by anti-human IgG or IgM-HRP conjugate. After a washing step, chromogenic substrate (TMB) is added and color is developed by the HRP substrate, which is directly proportional to the amount of anti-Ebola IgG or IgM present in the sample. Stop Solution is added to terminate the reaction, and Absorbance is then measured using an ELISA reader at 450nm. The presence of antibody (IgG/IgM) in samples is determined relative to anti-Ebola IgG/IgM Calibrators and Controls.

KIT CONTENTS

The microtiter well plate and all other reagents, if unopened, are stable at 2-8°C until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

To Be Reconstituted: Store as indicated.

Component	Preparation Instructions
Wash Solution Concentrate (100x) Cat. No. WB-100, 10ml	Dilute the entire volume 10ml + 990ml with distilled or deionized water into a clean stock bottle. Label as **Working Wash Solution** and store at 4°C for long term and ambient temp. for short term.
Sample Diluent Concentrate (20x) Cat. No. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample/Conjugate Diluent** and store at 2-8°C until the kit lot expires or is used up.
Anti-Human IgG-HRP Conjugate Concentrate (100x) Part: H-HuG.211, 0.15ml	Peroxidase conjugated anti-human IgG in buffer with detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample/Conjugate Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 2-8°C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
Ebola GP Mix Coated Strip Plate	325601	8-well strips (12)	Coated with a mixture of Zaire, Reston, Sudan & bundibugyo recombinant Ebola GPs, and post-coated with stabilizers.
Anti-Ebola GP Calibrators			
1 U/ml	325602B	0.65 ml	Four (4) vials, each containing anti-Ebola GP; in buffer with antimicrobial.
2.5 U/ml	325602C	0.65 ml	
5 U/ml	325602D	0.65 ml	
10 U/ml	325602E	0.65 ml	
Human x-Ebola GP IgG Positive Control	325600-PC	0.65 ml	Human serum with anti-Ebola GP reactivity; Net OD > **0.5**
Low NSB Sample Diluent Reduces non-specific binding	TBTm **Not** for HRP Conjugate dilution.	30 ml	Buffer with protein, detergents and antimicrobial as stabilizers. Use as is for sample dilution. See **Assay Design**, page 3.
TMB Substrate	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	Dilute sulfuric acid.

Materials Required But Not Provided:

- Pipettors and pipettes that deliver 100ul and 1-10ml.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Anti-Human IgG HRP Concentrate.
- Stock bottle to store diluted Wash Solution; 0.2 to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength.

ASSAY DESIGN AND SET-UP

Sample Collection and Handling

Serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference. For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage.

Caution: Human serum and other bodily fluids may contain infectious material. Always wear gloves when handling human samples, including the standards and controls (which have been tested non-reactive for HbsAg and Anti-HIV), and dispose of these samples and containers as biohazard waste.

Sample Preparation & Antibody Stability

Initial dilution of serum into **Working Sample Diluent** (WSD) is recommended to stabilize antibody activity. This enhances reproducible sampling, and stabilizes the antibody activity for years, stored refrigerated or frozen. Further dilution into **Low NSB Sample Diluent** (LNSD), which provides the lowest assay background, should be at least 5-10 times the initial dilution and performed the same day as the assay. Example:

Initial (1:10): **10** ul serum + **90** ul **WSD**
Further (1:50): **10** ul initial (1:10) + **490** ul **LNSD**
= **1:500 Final Dilution** [other dilutions may also be made].

Assay Design

Review Interpretation of Results (p5-7) before proceeding:

- Select the proper sample dilutions accounting for expected potency of positives and minimizing non-specific binding (NSB) and other matrix effects; for example, net signal for non-immune samples should be lower than the **1 U/ml Calibrator**. This is usually 1:500 or greater dilution for human serum with normal levels of IgG and IgM.
- Run the Human Anti-Ebola GP IgG Positive Control; net OD > **0.5**.
- Run a Sample Diluent **Blank**. This signal is an indicator of proper assay performance, especially of washing efficacy, and is used for net OD calculations, if required. Blank OD should be <0.3.
- Run a set of **Calibrators**, which validate that the assay was performed to specifications: **10 U/ml** should give a high signal (>1.5 OD); **1 U/ml** should give a low signal which can be used to discriminate at the Positive/Negative threshold (see Interpretation of Results, p. 5).

Plate Set-up

Bring all reagents to room temperature (18-30° C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 8 Calibrator wells and 2 wells for each sample control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
- Add 200-300ul Working Wash Solution to each well and let stand for about 5 minutes. Aspirate or dump the liquid and pat dry on a paper towel before sample addition.

Assay Procedure

ALL STEPS ARE PERFORMED AT ROOM TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

1. 1st Incubation [100ul – 60 min; 4 washes]

- Add 100ul of calibrators, samples and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for 60 minutes.
- Wash wells 4 times and pat dry on fresh paper towels. As an alternative, an automatic plate washer may be used. Improper washes may lead to falsely elevated signals and poor reproducibility.

2. 2nd Incubation [100ul – 30 min; 5 washes]

- Add 100ul of diluted Anti-Human IgG HRP to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 2.

3. Substrate Incubation [100ul – 15 min]

- Add 100ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
- Incubate for 15 minutes in the dark, e.g., place in a drawer or closet.

Note: If your microplate reader does not register optical density (OD) above 2.0, incubate for less time, or read OD at 405-410 nm (results are valid).

4. Stop Step [Stop: 100ul]

- Add 100ul of Stop Solution to each well.
- Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

5. Absorbance Reading

- Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Calibrators, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains dilute sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water. MSDS for TMB, sulfuric acid and BND can be requested or obtained from the ADI website: http://4adi.com/commerce/info/showpage.jsp?page_id=1060&category_id=2430&visit=10

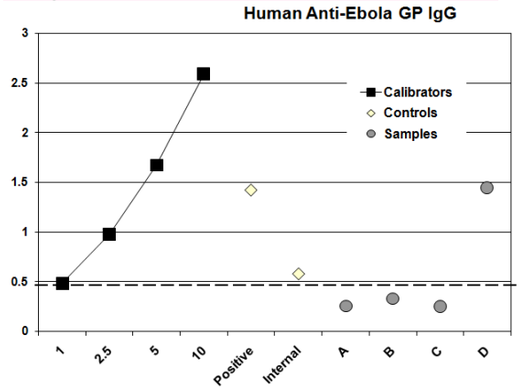
INTERPRETATION OF RESULTS

Method A. Antibody Activity Threshold Index

Compare Samples to **1 U/ml Calibrator** or **Internal Control**

= Positive/Negative Cut-off.

Example:



Results

The **sensitivity** of the assay to detect anti-GP IgG, from either natural infection or vaccination, is controlled so that the **1 U/ml Calibrator** represents a threshold OD for most true positives in human serum diluted to 1:500 or greater. Visual inspection of the data in the above graph shows the following:

Calibrators – dilution curve of an anti-Ebola GP antibody, derived from GP immunization, shows the OD range of the assay; high value indicates optimal sensitivity of the assay.
1 U/ml: a 'Cut-off' line has been drawn to indicate a threshold distinguishing between **Positive**/**Negative**. The is not a clear-cut threshold, rather a low OD area that could represent either low positives or high background negatives.

Positive Control – a humanized antibody showing reactivity to ZEBOV GP; net OD > **0.5**. This Control can be used to normalize between-assay variation.

Internal Control – a true positive from an immune human that represents the investigator's experience in distinguishing low positive from negative samples (not in kit). This should be run in each assay to supplement the 1 U/ml Calibrator for Positive/Negative discrimination purposes.

Samples A,B,C,D – 3 samples (A, B, C) are negative: below the threshold; 1 sample (D) is positive: clearly above the threshold.

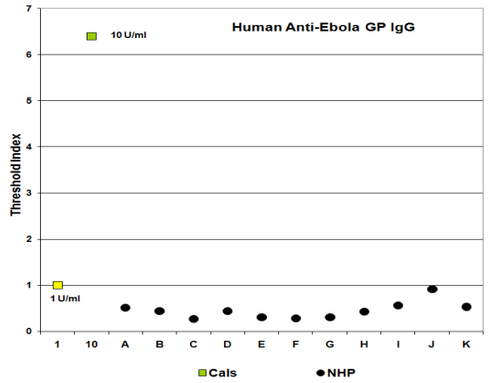
The **1 U/ml** Calibrator can be used to calculate a **Threshold Index** that numerically discriminates Positive/Negative (see p6):

- Divide each Sample net OD by the 1 U/ml Calibrator net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

ASSAY PERFORMANCE

Example:

Human Serum/Plasma IgG
A panel of stored human plasma of unknown history were tested for anti-Ebola GP IgG (**1:500 dilution**). **Threshold Index** was calculated using the **1** U/ml **Cal**.



Results

Anti-Ebola GP IgG: samples were negative (below 1.0 threshold) at **1:500 dilution**; one was borderline.

Notes:

1. **Positives** may be due to prior encounter with the virus or non-Ebola proteins with common epitopes, or may be an aspect of the innate immune repertoire.
2. The **sensitivity** of the assay may be adjusted by changing the sample dilutions: a) increase dilution (e.g., 1/1000) to lower the signals of borderline positives to negative; b) decrease dilution (e.g., 1/200) to convert borderline samples to positive. With the latter, the values of negatives may increase, so an alternative threshold should be considered using known negatives to develop a **Positive Index (**see below) or use an **Internal Control** (Page 5).

B. Positive Index
Experimental sample values may be expressed relative to the values of Control or Non-immune samples, by calculation of a **Positive Index**. One typical method is as follows:

1. Calculate the net OD mean + 2 SD of the Control/Non-immune samples = **Positive Index**.
2. Divide each sample net OD by the Positive Index. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

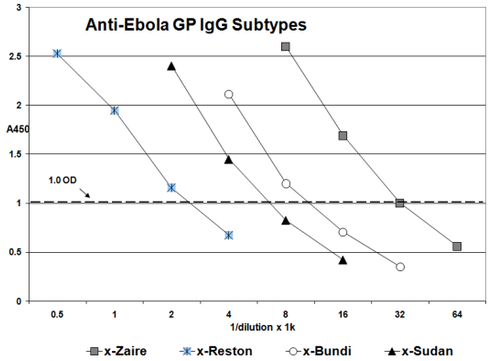
A sample value would be **Positive** if significantly above the value of the pre-immune serum sample or a suitably determined non-immune panel or pool of samples, tested at the same sample dilution.

This calculation also **quantifies** the positive Antibody Activity level, assigning a higher value to samples with higher Antibody Activity, and vice versa.

INTERPRETATION OF RESULTS (cont)

C. Antibody Titer

The most accurate method for comparing antibody potencies is by calculation of a titer, using an OD reading midrange in the dilution curves of each antibody as **Index.** In the example below, **IgG** titers were calculated as inverse of the dilution that produced a **1.0 OD** in the assay.



Results

Anti-Zaire: Rabbit antibodies specific for the Zaire glycoprotein. Titer: **32k**.

Anti-Reston: Rabbit antibodies specific for the Reston glycoprotein. Titer: **2.5k**.

Anti-Bundi: Rabbit antibodies specific for the Bundibugyo glycoprotein. Titer: **10.6k**.

Anti-Sudan: Rabbit antibodies specific for the Sudan glycoprotein. Titer: **6.5k**.

PRODUCT SPECIFICATIONS

Specificity
The following are used for coating antigens:

Ebola Nucleoprotein (NP) antigen: Zaire Ebola NP, full length, His-tag, E. coli expressed protein (>95% pure).

Ebola VP40 antigen: Zaire Ebola VP40, full length, no tag, E. coli expressed protein (>95% pure).

Ebola Glycoprotein (GP) antigen: Zaire Ebola GP, full length minus TM domain, His-tag, Sf9 expressed protein (>95% pure).

Sequence Conservation in Ebola subtypes.

	Zaire	Bundi-bugyo	Cote D'Ivo.	Res-ton	Sudan	Marb-urg
NP	100%	75%	69%	69%	67%	53%
VP40	100%	82%	69%	74%	75%	34%
GP	100%	65%	64%	57%	54%	NA

Although the 3 Ebola virus antigens are significantly conserved in various Ebola serotype and also in related Marburg viruses, it is not known if the antibodies elicited by one Ebola strain will be cross-reactive with antigens from other strains.

Instruction Manual No. M-AE-325600-XH

Recombivirus™ Human Anti- Ebola Virus Glycoprotein Combo IgG ELISA Kit

ELISA KIT # AE-325600-XH

For Quantitation of Anti-Ebola GP IgG specific for Zaire, Reston, Sudan and/or Bundibugyo Subtypes in Serum/ Plasma

For research use only, not for diagnostic or therapeutic use.

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ELISA Kit Components		Amount	Part
Ebola GP Coated Strip Plate		8-well strips (12)	325601
Human Anti- Ebola GP IgG Positive Control		0.65 ml	325600-PC
Anti- Ebola GP Calibrator	1 U/ml	0.65 ml	325602B
Anti- Ebola GP Calibrator	2.5 U/ml	0.65 ml	325602C
Anti- Ebola GP Calibrator	5 U/ml	0.65 ml	325602D
Anti- Ebola GP Calibrator	10 U/ml	0.65 ml	325602E
Anti-Human IgG HRP Conjugate (100X)		0.15 ml	H-HuG.211
Sample Diluent (20x)		10 ml	SD20T
Low NSB Sample Diluent		30 ml	TBTm
Wash Solution Concentrate (100X)		10 ml	WB-100
TMB Substrate		12 ml	80091
Stop Solution		12 ml	80101
Product Manual		1 ea	AE-325600-XH