INTENDED USE

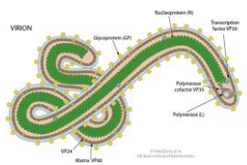
The **Humanize Ebola GP IgG (plant expressed)** ELISA Kit is an immunoassay suitable for quantifying human antibody activity to Ebola Glycoprotein (GP), including the humanized monoclonal constituting the Zmapp drug; also other human, humanized, or mouse/human chimeric antibodies from natural sources or as recombinant IgG from cell cultures, e.g., *N. benthamiana* (plantibodies), CHO, E.coli. This kit can be used in human or animal serum, plasma or other biological samples.

This immunoassay is suitable for:

- Determining biological activity of anti-Ebola GP during manufacturing process;
- Qualifying and standardizing therapeutic antibody batches & protocols;
- Assessing anti-Ebola GP activity and concentrations in human or animal serum.

The assay is for research use only (RUO), and not for therapeutic uses. 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. The mortality rate of Ebola victims is between 60-90%. Ebola virions consist several viral proteins (NP, GP, and VP40) that are highly immunogenic and could individually or together constitute effective vaccines. The FDA has allowed two drugs, **ZMapp** and an RNA interference drug called **TKM-Ebola**, to be used in people infected with Ebola under these programs during the 2014 outbreak. ZMapp is composed of three monoclonal antibodies (mAbs) that have been humanized by genetic engineering. ZMapp is being developed by Mapp Biopharmaceutical and Leaf Bio. Zmapp components are humanized monoclonal lgG1 c13C6 from MB-003 and two humanized mAbs from ZMab, c2G4 and c4G7. Like intravenous immunoglobulin therapy, ZMapp contains neutralizing antibodies that provide passive immunity to the virus by directly and specifically reacting with virus GP in a "lock and key" fashion. ZMapp is manufactured in the tobacco plant *Nicotiana benthamiana* in the bio-production process known as "pharming". Like many humanized antibodies it may also produce anti-drug antibodies (**ADA**).

This kit has been tested with the three humanized (mouse/human chimeric lgG expressed in tobacco plants) and fully human antibodies (KZ52). We recommend testing of all humanized antibodies as individual antibodies to characterize their activity potency in the assay.

PRINCIPLE OF THE TEST

This ELISA kits are based on the binding of Anti-Ebola lgGs to the recombinant, purified ZEBOV antigen (GP) immobilized on the microwells. Bound antibody is detected by antibody-HRP conjugate that recognize the GP antibody. After a washing step, substrate (TMB) is added and color (blue) is developed, which is directly proportional to the amount of anti-Ebola GP present in the sample. Stop Solution is added to terminate the reaction, and A450 is then measured using an ELISA reader. The presence of anti-Ebola GP in samples is determined relative to humanized anti-GP Calibrators and Controls.

KIT CONTENTS

The microtiter well plate and all other reagents, if unopened, are stable at 2-8^{o}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
Sample Diluent Concentrate (20x) Cat.#. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample Diluent** and store at 2-8^{o}C until the kit lot expires or is used up.
Wash Solution Concentrate (100x) Cat. # 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 ambient temperature until kit is used entirely.
Anti-Human IgG-HRP Conjugate Concentrate (100x) Part No. H-HuG-612, 0.15ml	Peroxidase conjugated anti-human IgG in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10 ul of concentrate to 1 ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 2-8^{o}C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
ZEBOV GP Coated Strip Plate	320621	8-well strips (12)	Coated with purified recombinant ZEBOV GP, and post-coated with stabilizers.
Human Anti-Ebola GP IgG Standards			
5 ng/ml 10 ng/ml 25 ng/ml 50 ng/ml 100 ng/ml	320813B 320813C 320813D 320813E 320813F	0.65 ml 0.65 ml 0.65 ml 0.65 ml 0.65 ml	Five (5) vials, each containing humanized anti-Ebola lgG (plant expressed) in buffer with protein, detergents and non-azide antimicrobials.
Positive Control [concn range on label]	320812	0.65 ml	Monoclonal c13C6 (Zmapp) of stated concentration range; in buffer with stabilizers & antimicrobials.
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. A multi-channel pipettor is recommended.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Antibody HRP Concentrate.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate washer and reader at 450 nm wavelength.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Standards, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains 1% 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

ASSAY DESIGN AND SET-UP

Sample Collection and Handling

Culture medium, serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference (See Limits of the Assay, page 6). For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. For all samples, clarify by centrifugation and/or filtration. If samples will not be assayed immediately, store frozen for long-term storage.

DILUTE samples in **Working Sample Diluent**.
Diluted samples are stable for at least a year refrigerated.

Assay Validation

Validate the performance of the Zmapp or other antibody samples and matrix in the assay system for recovery, as follows:

Recovery – a measure of the interference of the sample matrix (diluent effect) in providing accurate quantitation of Zmapp in the sample relative to the Zmapp Standards.

Prepare and run a series of dilutions of the Zmapp sample (within the Standard range) in Working Sample Diluent to determine the dilutions that give consistent and accurate quantitation. Serum and plasma require greater than 1/200 dilution to obtain consistent quantitation or complete antigen recovery.

Recovery Limits – Monoclonals c13C6, 13F6 and c6D8 were spiked into dilutions of 8 individual human sera, or Sample Diluent (Control), at a final concentration of 50 ng/ml.

Results: recovered values ranged from **68** to **82%** of Control with sera diluted 1/200. Recovery was **less** when serum was diluted less than 1/100. Low recovery suggests serum factors that interfere with antibody binding to the antigen on the plate.

Plate Set-up

Bring all reagents to room temperature (18-30^{o} C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 10 Standard wells and 2 wells for each sample and 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 ul 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 Conjugate** 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.

INTERPRETATION OF RESULTS

A variety of anti-Ebola GP antibodies can be quantified using this assay. Because each antibody will have a different, characteristic potency for binding to the GP antigen, different methods for calculating antibody concentration should be considered, as follows:

Method A. Calculation of Mass: Standard Curve.

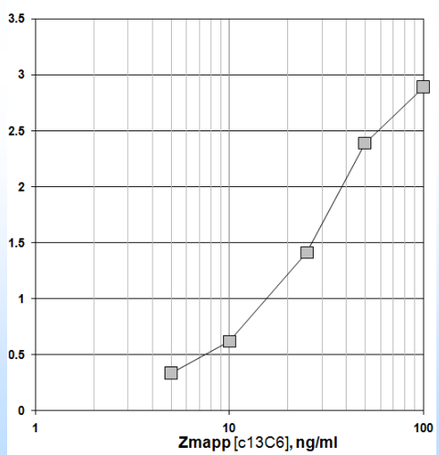
Quantitation of antibody mass concentration (e.g., ng/ml) requires a standard (dilution) curve of the actual antibody being measured. The Standards provided in the kit are dilutions of the c13C6 monoclonal, and is suitable for quantifying c13C6 in mass units.

Calculation: The results may be calculated using any immunoassay software package. The four-parameter curve-fit is recommended. Samples producing signals higher than the 100 ng/ml standard should be further diluted and re-assayed.

Typical Results:

Wells	Calibrators		A450 nm
A1,2	**Negative Diluent Blank**		0.04
B1,2	5 ng/ml	**Standard**	0.33
C1,2	10 ng/ml	**Standard**	0.62
D1,2	25 ng/ml	**Standard**	1.41
E1,2	50 ng/ml	**Standard**	2.39
F1,2	100 ng/ml	**Standard**	2.89
G1,2	**Positive**	**Control**	1.58

Positive Control [21 – 39ng/ml]: **28.0** ng/ml



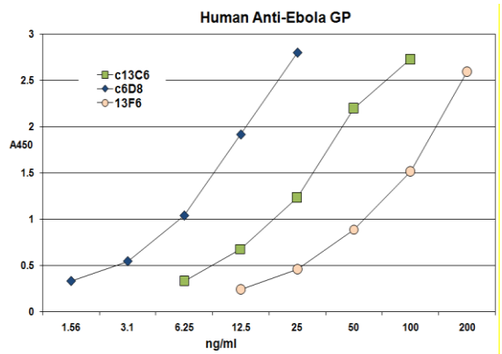
INTERPRETATION OF RESULTS (cont.)

Method B. Calculation of Mass: Antibody Dilution Curve.

Quantitation of antibody mass concentration of other human anti-Ebola GP antibodies can be performed using a dilution curve of the specific antibody, or antibody mix, being measured.
Protocol

- Prepare and run a dilution curve of each antibody; assign concentration values based on the concentration of the antibody in the stock preparation;
- Run the Ebola GP IgG Standards as Positive Controls to validate a precise and reproducible run, and to normalize between-assay variation.

Example:



Comments

- Each of the 3 humanized monoclonal antibodies shown in the above graph has a different potency for GP binding, as indicated by difference in antibody concentration required to produce a 1.0 OD signal in the assay.
- Thus, the requirement to use a dilution curve of the antibody (or combination) being tested to determine mass concentration is demonstrated.

Method C. Calculation of Activity: Standard Curve.

Quantitation of Antibody Activity for any human anti-Ebola GP antibody (or Ab mixture of constant ratio) can use the Zmapp Standard curve, as in Method A, except the units cannot be ng/ml for a non-c13C6 Ab, rather assign '**Z units**'; i.e., the Standard values would be 5 to 100 **Zu/ml**.

This method is valid if the dilution curve of the antibody (or mix) is parallel to the Zmapp Std curve. Parallelism – dilutions of the sample should read equivalent values from the top and bottom of the Standard curve to provide good assay precision. Sample readings from the upper and lower regions of the curve should differ by less than 25%.

Note: Dilutions of antibodies c6D8 and 13F6, shown in Example above, are within the parallelism criteria for reading from the Zmapp Standard curve.

INTERPRETATION OF RESULTS (cont.)

Method D. Calculation of Activity: Zmapp Potency Index
The anti-Ebola GP activity of any human IgG antibody or antibody mix may be expressed relative to the value of a Positive Control, by calculation of a **Potency Index**. Any of the Zmapp or other plant expressed Ebola GP may be use as the Positive Control. One typical method is as follows:

1. Divide each Sample net OD by the net OD of the Zmapp Positive Control = **Z units/ml.**

2. Multiply the **Z units/ml** x dilution factor = **Total Z units**

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

Note: This method works well for measuring the activity of an antibody mix such as Zmapp, especially if the ratios of the composite is altered, i.e., after injection into a host.

LIMITS OF THE ASSAY

1. The assay measures human anti-Ebola GP activity, i.e., antibody that actually binds to the GP-antigen coated plate, relative to antibody standards that are presumed to be 100% active antibody. Factors in the sample that diminish GP binding, e.g., GP antigen or other antibodies or masking molecules, may reduce apparent anti-GP IgG concentration in the assay (**Recovery**).

2. Assays that measure Ebola GP IgG mass concentration may not have a tight correlation with the antibody activity assay, e.g., full GP IgG antibody recovery may be determined by different factors.

3. The **recovery** (accuracy of humanized anti-GP IgG measurement in stored serum) may be diminished if not diluted at least 1/500 in Sample Diluent. Recovery in fresh, individual human or other animal serum or plasma, may differ and has not been determined.

PRODUCT SPECIFICATIONS

Specificity
Purified recombinant Zaire Ebola GP is used to coat the microwells; thus the assay is specific for antibodies directed to GP antigens. The humanized Ebola GP IgG conjugate used in this kit reacts with humanized IgG1 subclass and it may also detect other IgG subclasses (IgG2-4). However, human antibodies of IgA or IgM or mouse monoclonal will not be detected. We recommend that all plant expressed Ebola IgG be tested in the kit to assure reactivity.

Related Items

AE-320815-1 Anti-Humanized Ebola GP IgGs (Plant expressed) (Anti-drug antibody/ADA) ELISA kit, 96 tests, Quantitative

AE-320880-25 Humanized Anti-Ebola GP IgG (plant expressed) rapid test (test Zmapp acticty in 2-10 min), 25 cassettes/pk

AE-320870-1 Humanized Ebola IgGs-C1q activity ELISA 96 tests, Quantitative

Instruction Manual No. M-320810-1

Humanized (plant expressed) Anti-Ebola GP IgG

ELISA Kit # AE-320810-1

For Quantitation of Humanized Anti-Ebola GP IgG in Serum or Plasma or Biological Solutions

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Texas 78244 • USA.

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ELISA Kit Components	Amount	Part
ZEBOV-GP Coated Microwell Plate	8-well strips (12)	320621
Humanized anti-GP IgG Positive Control	0.65 ml	320812
Human anti-Ebola GP IgG Std. 5 ng/ml	0.65 ml	320813B
Human anti-Ebola GP IgG Std. 10 ng/ml	0.65 ml	320813C
Human anti-Ebola GP IgG Std. 25 ng/ml	0.65 ml	320813D
Human anti-Ebola GP IgG Std. 50 ng/ml	0.65 ml	320813E
Human anti-Ebola GP IgG Std.100 ng/ml	0.65 ml	320813F
Anti-Human IgG-HRP Conjugate (100X)	0.15 ml	H-HuG-612
Sample Diluent Concentrate (20x)	10 ml	SD20T
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	M-320810-1