ELISA Kit Components	Amount	Part No.
Anthrax LF-coated Microwell Strip Plate	8-well strips (12)	900-201
Anti-Anthrax LF Calibrator 125 U/ml	0.65 ml	900-202B
Anti-Anthrax LF Calibrator 250 U/ml	0.65 ml	900-202C
Anti-Anthrax LF Calibrator 500 U/ml	0.65 ml	900-202D
Anti-Anthrax LF Calibrator 1000 U/ml	0.65 ml	900-202E
Anti-Mouse Ig HRP Conjugate (100X)	0.15 ml	MsH-GAM
Sample Diluent Concentrate (10X)	10 ml	SD-20T
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	900-200-LFM

PRECAUTIONS AND SAFETY INSTRUCTIONS

Calibrators, Sample Diluent, and Anti-Mouse Ig-HRP contain ProClin 300 (0.05%, v/v). Stop Solution contains diluted 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 ProClin 300, if not already on file, can be requested or obtained from the ADI website.

LIMITS OF THE ASSAY

Quantitation of Antibody in a Sample
The ELISA measures anti-Anthrax LF activity, a combination of antibody concentration and avidity for the Anthrax LF antigen. Antibodies with substantially different specific IgG concentrations may display similar anti-Anthrax LF activities, due to differences in avidity. The quantitation or potency of the samples is, therefore, appropriately expressed in Activity Units (titer), rather than mass units of IgG (e.g., ng/ml).

Calibrators and Antibody-HRP Conjugates
The Calibrators are dilutions of mouse anti-Anthrax LF IgG. The signals of the Calibrators confirm appropriate performance of the assay and the quality of the coating antigen. The potency of the anti-mouse Ig-HRP for detecting a standardized amount of mouse IgG bound to the microwell has been confirmed prior to packaging.

Calibrator Curve Quantitation
To quantitate antibody activity from a Calibrator curve (such as provided with the kit), the dilution curve of the samples must be parallel to the Calibrator curve, to avoid different values being obtained from different regions of the curve. Antibodies that are not matched in anti-Anthrax LF avidity will often have non-parallel dilution curves. In these cases, antibody activity is best expressed as a titer; the Calibrators are internal standards that can be used to normalize between-assay signal variations to improve precision of the collected data (see page 6).

Instruction Manual No. M-900-200-LFM

Mouse Anti-Anthrax Lethal Factor (LF)

ELISA Kit # 900-200-LFM

For Semi-Quantitative Determination of Anti-Anthrax LF Ig in Biological Fluids

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INTENDED USE

The Mouse Anti-Anthrax LF ELISA Kit is an immunoassay suitable for quantifying or titering total antibody activity (IgG, IgA and IgM) specific for Anthrax LF in serum, plasma or other biological fluids.

INTRODUCTION

Anthrax, a zoonotic disease caused by the spore-forming bacterium *Bacillus anthracis*, has become a biological warfare agent of concern due to the stability and extreme lethal consequences of human inhalation of spores. *B. anthracis* evades the immune system by producing an anti-phagocytic capsule. In addition, three proteins - protective antigen (PA), lethal factor (LF), and edema factor (EF) – are produced that act in combinations to form two exotoxins known as lethal toxin and edema toxin. Development of improved vaccines for protection of livestock and for human immunization have involved preparations that include combinations of these antigens. Immunoassays that measure titer of host antibody directed against the specific *B. anthracis* antigens can be used to study the efficacy of experimental anthrax vaccines and the exposure to the bacterium and/or separate antigens.

PRINCIPLE OF THE TEST

The Mouse anti- Anthrax LF ELISA kit is based on the binding of mouse anti-Anthrax LF in samples to Anthrax LF immobilized on the microwells, and anti-Anthrax LF antibody is detected by anti-mouse IgG+IgA+IgM-specific antibody conjugated to HRP (horseradish peroxidase) enzyme. After a washing step, chromogenic substrate (TMB) is added and color is developed by the enzymatic reaction of HRP on the substrate, which is directly proportional to the amount of anti-Anthrax LF present in the sample. Stopping Solution is added to terminate the reaction, and absorbance at 450nm is then measured using an ELISA microwell reader. The titer of Anthrax LF-specific mouse Ig in samples may be calculated relative to anti-Anthrax LF reference Calibrators.

PRODUCT SPECIFICATIONS

Specificity
Purified recombinant anthrax LF is used to coat the microwells; stabilizing postcoat contains BSA. The anti-Mouse IgG+IgA+IgM (H+L) HRP conjugate reacts with mouse IgG, IgA and IgM class antibodies that bind to LF on the plate. IgE antibody would not be measured above background signals.

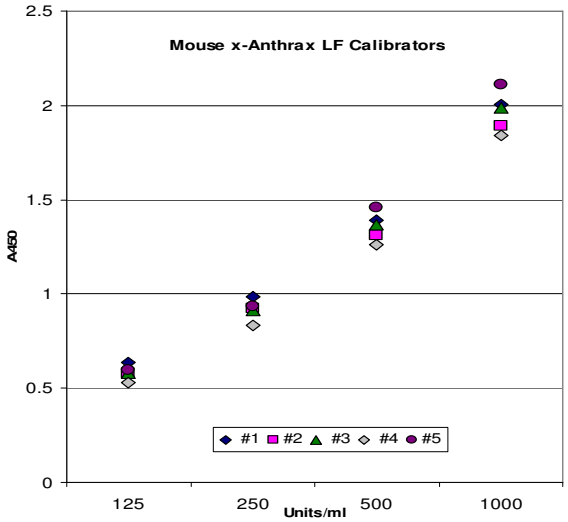
Assay Sensitivity
The diluted anti-Mouse Ig HRP produces a 1.0 OD signal (30 min anti-Mouse Ig HRP incubation) with mouse Ig coated on a microwell, separately, at 25ng IgG, 100ng IgM and 100ng IgA. The LF coating level is optimized to differentiate anti-LF Ig from background (non-antibody) signal with mouse serum samples diluted 1:100.

Calibrator Values
The Calibrators are composed of dilutions of IgG antibodies from Anthrax LF immunized mice. Values are assigned as arbitrary anti- Anthrax LF activity units (see **Limits of the Assay**, page 7).

CALCULATION OF RESULTS (continued)

Method III. Calibration of the Assay
The multi-level Calibrators are used to determine reproducibility between assays and across changes in reagent lots. Calibrators may also be used in calculations to normalize between-assay variation to enhance precision.

Calibrator Values of Multiple Runs



Normalizing Sample Values for Enhanced Precision

III. Precision of the Calibrator OD values from the 5 assays shown above ranged from **5.3** to **6.5** %CV (coefficient of variation). The 500 U/ml Calibrator was used as Index as follows:
Calibrator (or Sample) net OD ÷ 500 U/ml Calibrator net OD = **Normalized Value**.

Cal	#1	#2	#3	#4	#5	Ave	%CV
125	0.638	0.581	0.582	0.532	0.596	0.586	**6.5**
250	0.986	0.926	0.910	0.834	0.934	0.918	**6.0**
500	1.39	1.314	1.370	1.262	1.458	1.359	**5.5**
1000	2.004	1.894	1.989	1.844	2.112	1.969	**5.3**
Normalized Value using 500 U/ml Calibrator as Index						**Ave**	**%CV**
125	0.459	0.442	0.425	0.422	0.409	0.431	**4.5**
250	0.709	0.705	0.664	0.661	0.600	0.676	**4.4**
500	1.0	1.0	1.0	1.0	1.0	1.0	**na**
1000	1.44	1.44	1.45	1.46	1.45	1.449	**0.6**

Precision of the normalized Calibrator values improved to **0.6** to **4.5** %CV.

CALCULATION OF RESULTS

Several data reduction methods may be considered to optimize precision and to best represent the relationships among experimental and control groups.

Method I. Single Dilution or Low Titer Samples
With samples tested at a common dilution, report values in OD units multiplied by the inverse of the dilution. The Calibrator values are useful as internal controls to normalize between-assay signal variation (see Method III).

Typical Results:

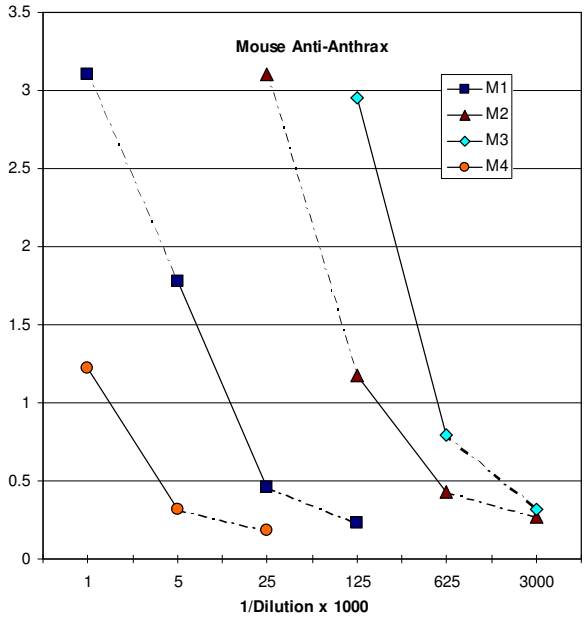
1.61 [Sample A, net OD] X **100**-fold dilution = **161** U/ml.

Method II. High Titer, Multiple Sample Dilutions
Antibody potency can be expressed in semi-quantitative activity units, using an arbitrary mid-range signal, e.g., 1.0 or 0.5 OD, as Index. The Calibrators are useful for normalizing titer values for between-assay variation (see Method III):

1. On a log scale of inverse of Sample Dilution as the x-axis, plot the OD values of the pair of dilutions of each positive sample having ODs above and below the OD value of the Index (arbitrary OD).
2. From a point-to-point line drawn between the two sample ODs, read the dilution value (x-axis) corresponding to the OD of the selected Index
   = **Total Antibody Activity Units**

Typical Results:

II. A **1.0 OD** value was used as the Index to determine titer of 4 antibodies.



Titer Values

Mouse 1 = 13k units
Mouse 2 = 180k units
Mouse 3 = 380k units
Mouse 4 = 1.5k units

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	Instructions for Use
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 Diluent** and store at 2-8°C until the kit lot expires or is used up.
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 ambient temperature until kit is used entirely.
Anti-Mouse IgG - HRP Conjugate Concentrate (100x) Part No. MsH-GAM, 0.15ml	Peroxidase conjugated anti-mouse IgG+IgA+IgM (H+L) in buffer with protein, detergents and ProClin 300 as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **HRP Conjugate Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return concentrate to 2-8°C storage.

Ready For Use: Store as indicated on labels.

Component	Part No.	Amt	Contents
Anthrax LF Microwell Strip Plate	900-201	8-well strips (12)	Coated with Anthrax LF, and post-coated with stabilizers.
Anti-Anthrax LF Calibrators			
125 U/ml 250 U/ml 500 U/ml 1000 U/ml	900-202B 900-202C 900-202D 900-202E	0.65 ml 0.65 ml 0.65 ml 0.65 ml	Four (4) vials, each containing mouse anti-anthrax LF levels in arbitrary Activity Units; diluted in buffer with protein, detergents and ProClin 300 as stabilizers.
TMB Substrate	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	1% 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 Anti-mouse Ig-HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate and Sample Diluent concentrate; 200ml to 1L.
- Stock bottle to store diluted Wash Solution; 200ml 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

Culture medium, 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. For other samples, including **tissue culture media**, clarify the sample by centrifugation and/or filtration prior to dilution in Working Sample Diluent. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage. Avoid freeze-thaw cycles.

Samples, Calibrators and Controls

Dilute **Samples** in Working Sample Diluent according to expected anti-Anthrax LF activity levels; for serum: dilute at least 100-fold (e.g., 10 ul sample + 990 ul Diluent) for reduced nonspecific signals. At least 2 dilutions of each sample are recommended in order to determine if reading values from the Calibrator curve is valid (see Limits of the Assay).

Do not dilute the **Calibrators**. Include Working Sample Diluent as a Negative Control to determine proper assay performance (signal should be < 0.3 OD) and to subtract from sample and Calibrator values to obtain net OD. Internal **Controls** that represent the lab's expected results should also be included in each assay run.

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 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.

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.

- Add 200-300ul Working Wash Solution before sample addition to each well and let stand for about 5 minutes. Aspirate or dump the liquid and pat dry on a paper towel.

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 by adding 250-300ul Working Wash Solution; aspirate or dump the liquid 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-mouse Ig-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. Positive 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.