INTENDED USE

The Mouse Anti-Rabies IgG ELISA Kit detects and quantifies rabies virus-specific IgG in mouse serum or plasma of vaccinated, immunized and/or infected animals. 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/or standardizing vaccine batches and protocols.

GENERAL INFORMATION

Rabies is a disease that causes acute encephalitis (inflammation of the brain) in warm-blooded animals. It is zoonotic (i.e., transmitted by animals), most commonly by a bite from an infected animal but occasionally by other forms of contact. Rabies is almost invariably fatal if post-exposure prophylaxis is not administered prior to the onset of severe symptoms. Early-stage symptoms of rabies are malaise, headache and fever, progressing to acute pain, violent movements, uncontrolled excitement, depression, and hydrophobia. Finally, the patient may experience periods of mania and lethargy, eventually leading to coma. The primary cause of death is usually respiratory insufficiency. Worldwide, the vast majority of human rabies cases (approximately 97%) come from dog bites. In the United States, however, animal control and vaccination programs have effectively eliminated domestic dogs as reservoirs of rabies. In several countries, including the United Kingdom, Australia and Japan, the virus has been eradicated entirely.

Rapid and accurate laboratory diagnosis of rabies in humans and other animals are essential for timely administration of post exposure prophylaxis. Within a few hours, a diagnostic laboratory can determine whether or not an animal is rabid and inform the responsible medical personnel. The laboratory results may save a patient from unnecessary physical and psychological trauma, and financial burdens, if the animal is not rabid. The nature of rabies disease dictates that laboratory tests be standardized, rapid, sensitive, specific, economical, and reliable. The standard test for rabies testing is dFA. All rabies laboratories in the United States perform this test (post-mortem) on animals suspected of having rabies.

PRINCIPLE OF THE TEST

The Mouse Anti-Rabies IgG ELISA kit is based on the binding of mouse anti- rabies virus in samples to rabies virus antigen immobilized on the microwells, and anti- rabies virus IgG antibody is detected by anti-mouse IgG-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-rabies virus IgG 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 activity of mouse antibody in samples is determined relative to mouse anti-rabies virus calibrators.

PRODUCT SPECIFICATIONS

Specificity
Antigens prepared from whole-inactivated rabies virus subtypes 1-3 is used to coat the microwells; stabilizing postcoat contains BSA; thus, no other antibody specificity is detectable in the assay. The anti-mouse IgG HRP conjugate specifically detects IgG, including the IgG1,2a,2b subclasses, and will not react with IgM, IgA or IgE class antibodies.

Assay Sensitivity
The rabies virus-coated plated and the anti-mouse IgG HRP concentration are optimized to differentiate anti-rabies virus IgG from background (non-antibody) signal with mouse serum samples diluted 1:100.

Calibrator Values
The Calibrators are composed of dilutions of mouse IgG antibody to rabies virus. Values are assigned as arbitrary anti-rabies virus activity units (see Limits of the Assay).

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
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-Gc, 0.15ml	Peroxidase conjugated anti-Mouse IgG in buffer with protein, detergents and antimicrobial 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 100X to 2-8^{o}C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
Rabies Microwell Strip Plate	600-051	8-well strips (12)	Coated with rabies viral antigens, and post-coated with stabilizers.
Mouse Anti-Rabies IgG Calibrators			
10 U/ml 25 U/ml 50 U/ml 100 U/ml	600-032B 600-032C 600-032D 600-032E	0.65 ml 0.65 ml 0.65 ml 0.65 ml	Four (4) vials, each containing mouse antibodies with anti-rabies IgG levels in arbitrary activity Units; diluted in buffer with protein, detergents and antimicrobial as stabilizers.
HRP Conjugate Diluent	TBT	12 ml	Buffer with protein, detergents and antimicrobial as stabilizers. Use as is for HRP dilution
Low NSB Sample Diluent	TBTm	60 ml	Buffer with protein, detergents and antimicrobial as stabilizers. Use as is for sample dilution
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 IgG HRP Concentrate.
- Graduated cylinder to dilute Wash 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.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Calibrators, Sample Diluent, and HRP Antibody 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, if not already on file, can be requested or obtained from the ADI website.

LIMITATIONS OF THE ASSAY

Quantitation of Antibody in a Sample
The ELISA measures anti-Rabies activity, a combination of antibody concentration and avidity for the rabies antigens. Antibodies with substantially different total Ig concentrations may display similar anti-Rabies activities, due to differences in avidity. The quantitation or activity of the samples is, therefore, appropriately expressed in activity Units (titer), rather than mass units of Ig (e.g., ug/ml).

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 Rabies avidity will often have non-parallel dilution curves. In these cases, antibody activity is best expressed as a titer relative to a reference positive such as the 100 U/ml Calibrator, or another Calibrator in the kit (see Calculation of Results).

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 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.

Assay Design
Review Calculation of Results (p5-7) and Limits of the Assay (above) 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 <0.5 OD. This is usually 1/100 or greater dilution for mouse sera with normal levels of IgG and IgM.
- 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 (**See Method A**). Blank OD should be <0.3.
- Run a set of Calibrators. Calibrators validate that the assay was performed to specifications; results can be used to normalize between-assay variation for enhanced precision. Reading values off a Calibrator curve, **Method B**, has limitations. See Limits of the Assay (above).
- Run a range of sample dilutions for expected higher positives that allows calculation of antibody **Titer** (when specific titer is at least 4-fold higher than non-immune)**. See Method C.**
- Run samples in duplicate if used for quantitation; non-immunes that are significantly lower than immunes may be run in singlicate. The Calibrators that are used for quantitation, e.g., for between-assay normalization, should be run in duplicate. When determining titer from a dilution curve, singlicates can be run if more than two dilution points are used for titer calculations.

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 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.
- 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-Mouse 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.

CALCULATION OF RESULTS

Several data reduction methods may be considered to best represent the relationships among experimental and control groups, to determine **Positive Immune** and **Negative Non-immune**, and to **Quantitate** positive antibody levels.

Method A. 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 determined as **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 **quantifies** the positive Antibody Activity level.

Example:

	Net OD		Antibody Activity	
Sample	Control	Exptl	Control	Exptl
1	0.243	2.358	0.49	**4.79**
2	0.351	0.597	0.71	**1.21**
3	0.286	1.421	0.58	**2.89**
4	0.357	1.268	0.73	**2.58**
5	0.512	0.857	**1.04**	**1.74**
6	0.342	1.296	0.70	**2.63**
7	0.298	0.608	0.61	**1.24**
8	0.285	0.369	0.58	0.75
9	0.157	0.864	0.32	**1.76**
10	0.187	0.543	0.38	**1.10**
Mean	0.302			
SD	0.095			
Mean +2 SD	**0.492**	= **Positive Index**		

CALCULATION OF RESULTS (continued)

Method B. Use of a Calibrator Curve

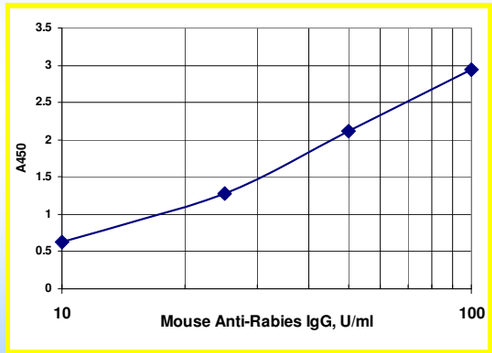
When the dilution curves of samples are parallel to the Calibrator curve (see Limits of the Assay), the anti-Rabies activity units may be determined by interpolation from the Calibrator curve, as follows:

1. The results may be calculated using any immunoassay software package. If software is not available, anti-Rabies activity concentrations may be determined as follows:
2. Calculate the mean OD of duplicate samples.
3. On graph paper plot the mean OD of the calibrators (y-axis) against the concentration (U/ml) of anti-Rabies (x-axis). Draw the best fit curve through these points to construct the calibrator curve. A point-to-point construction is most common and reliable.
4. The anti-Rabies activity concentrations in unknown samples and controls can be determined by interpolation from the calibrator curve.
5. Multiply the values obtained for the samples by the dilution factor of each sample.
6. Samples producing signals higher than the 100 U/ml calibrator should be further diluted and re-assayed.

Typical Results:

Wells	Calibrators & Samples		A450 nm	U/ml
A1, A2	**Negative Diluent Control**		0.10	0
B1, B2	10 U/ml	**Calibrator**	0.57	10
C1, C2	25 U/ml	**Calibrator**	1.22	25
D1, D2	50 U/ml	**Calibrator**	2.09	50
E1, E2	100 U/ml	**Calibrator**	2.96	100
F1, F2	**Sample** [Diluted 1:100]		1.46	31

Calculated: 100-fold dilution x 31 U/ml = **3.1 kU**/ml in serum



CALCULATION OF RESULTS (continued)

Method C. Titers from Sample Dilution Curves

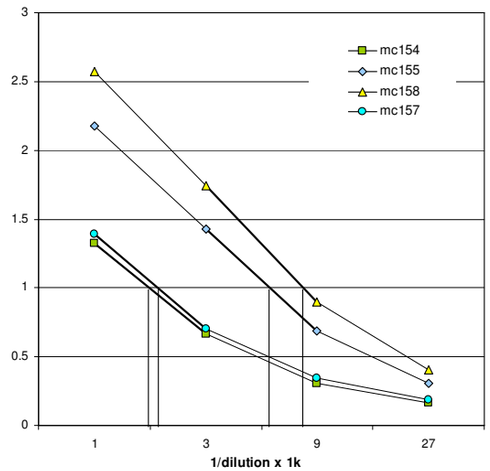
The titer of antibody activity calculated from a dilution curve of each sample is recommended as the most accurate quantitative method. Best precision can be obtained using the following guidelines:

1. Use an OD value Index in the mid-range of the assay (2.0 – 0.5 OD); this provides the best sensitivity and reproducibility for comparing experimental groups and replicates. An arbitrary 1.0 OD is commonly used.
2. Prepare serial dilutions of each sample to provide a series that will produce signals higher and lower than the selected index. With accurate diluting, duplicates may not be required if at least 4 dilutions are run per sample.
3. A 5-fold dilution scheme is useful to efficiently cover a wide range which produces ODs both above and below 1.0 OD. The dilution scheme can be tightened to 3-fold or 2-fold for more precise comparative data.
4. A Calibrator value in the mid-OD range (e.g., 100 U/ml) can be used to normalize inter-assay values.

Calculations
1. On a log scale of inverse of Sample Dilution as the x-axis, plot the OD values of the two dilutions of each positive sample having ODs above and below the OD value of the Index (arbitrary or selected Calibrator).
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 IgG Antibody Activity Units**

Example:

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



Titer Values

mc154 = 1.72 kU
mc155 = 5.70 kU
mc157 = 1.85 kU
mc158 = 7.90 kU

Instruction Manual No. M-600-030-MRG

Mouse Anti-Rabies IgG

ELISA Kit Cat. No. 600-030-MRG

For Quantitative Determination of Anti-Rabies IgG in Serum

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ELISA Kit Components		Amount	Part
Rabies Coated Microwell Strip Plate		8-well strips (12)	600-051
Mouse Anti-Rabies IgG Calibrator	10 U/ml	0.65 ml	600-032B
Mouse Anti-Rabies IgG Calibrator	25 U/ml	0.65 ml	600-032C
Mouse Anti-Rabies IgG Calibrator	50 U/ml	0.65 ml	600-032D
Mouse Anti-Rabies IgG Calibrator	100 U/ml	0.65 ml	600-032E
Anti-Mouse IgG HRP Conjugate (100X)		0.15 ml	MsH-Fc
HRP Conjugate Diluent		12 ml	TBT
Low NSB Sample Diluent		60 ml	TBTm
Wash Solution Concentrate (100X)		10 ml	WB-100
TMB Substrate		12 ml	80091
Stop Solution		12 ml	80101
Product Manual		1 ea	M-600-030