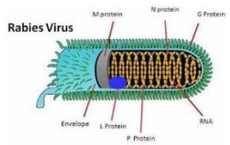
INTENDED USE

The Canine Anti-Rabies IgG ELISA Kit detects and quantifies rabies virus -specific IgG in canine serum or plasma of vaccinated, immunized and/or infected dogs, wolves or coyotes. 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.

For research use only (RUO), not for diagnosis, cure or prevention of the disease.

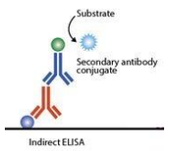
GENERAL INFORMATION



Rabies is a viral disease (Lyssavirus; ssRNA) that causes acute encephalitis (inflammation of the brain) in warm-blooded animals. It is transmitted by animals, most commonly by a bite from an infected animal, and is almost invariably fatal if post-exposure prophylaxis is not administered prior to the onset of severe symptoms. Humans and animals have been protected, and the disease eradicated in certain geographical regions, by vaccination. Most vaccines have used whole inactivated virus that has been grown in a variety of cell types; vaccines using recombinant proteins of the rabies virus are also available. For rabies control of wildlife, vaccines in bait have proven effective. Improvement of the efficacy of vaccines is an active area of investigation.

The ADI anti-Rabies ELISA is designed with high sensitivity for discriminating lower level antibodies, with specially formulated diluents to minimize interfering background signals.

PRINCIPLE OF THE TEST



The Canine Anti-Rabies IgG ELISA kit is based on the binding of antibodies in samples to rabies antigen immobilized on the microwells, and abies IgG antibody is detected by anti-Canine IgG- HRP conjugate. After a washing step, chromogenic substrate (TMB) is added and color(blue) is developed, which is directly proportional to the amount of anti-rabies 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 Canine antibody in samples is determined relative to anti-rabies calibrators.

PRODUCT SPECIFICATIONS

Specificity

Antigens prepared from whole-inactivated rabies virus subtypes 1-3 are used to coat the microwells; stabilizing postcoat contains BSA; thus, no other antibody specificity is detectable in the assay. The anti-Canine IgG HRP conjugate detects IgG from dogs, wolves and coyotes, and does not react with IgM, IgA or IgE class antibodies above background.

Assay Sensitivity

The rabies-coated plate, Low NSB Sample Diluent and anti-Canine IgG HRP concentration are optimized to differentiate anti-rabies IgG from background (non-antibody) signal with canine serum samples diluted 1:100.

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.
Sample Diluent Concentrate (20x) Cat. No. SD-20T, 10ml	Dilute 0.5ml + 9.5ml with distilled or deionized water as needed for HRP Conjugate and Sample Dilution. Label as **Working Sample/Conjugate Diluent** and store at 2-8^{o} C until the kit lot expires or is used up.
Anti-Canine IgG - HRP Conjugate Concentrate (100x) Part No. H-DgG.211, 0.15ml	Peroxidase conjugated anti-Canine IgG in buffer with protein, 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^{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 antigen, and post-coated with stabilizers.
Anti-Rabies Calibrators			
10 U/ml 25 U/ml 50 U/ml 100 U/ml	600-042B 600-042C 600-042D 600-042E	0.65 ml 0.65 ml 0.65 ml 0.65 ml	Four (4) vials, each containing anti-rabies antibody in arbitrary activity Units; diluted in buffer with protein, detergents and antimicrobial as stabilizers.
Canine Anti-Rabies IgG Positive Control	600-013	0.65 ml	Dog anti-rabies antiserum; ELISA values should be greater than **0.5** net OD
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. A multi-channel pipettor is recommended.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Anti-Canine IgG HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate; 0.2 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.

LIMITS OF THE ASSAY

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 25 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.

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 10 times the initial dilution and performed the same day as the assay.

Example: Initial (1/5): **10**ul serum + **40**ul WSD [or 0.1ml + 0.4ml]
Further (1/50): **10**ul initial (1/5) + **90**ul LNSD (1/50)

Assay Design

Review Calculation of Results (p5-7) and Limits of the Assay (above) before proceeding:

- Select the proper sample dilutions. Account for expected potency of positives and minimize non-specific binding (NSB) and other matrix effects; for example, non-immune samples should give net signal <0.5 OD. This is usually 1/100 or greater dilution for canine sera with normal levels of IgG and IgM. Dilute samples in **Working Sample Diluent (1xSD20T)** or in **Low NSB Sample Diluent (TBTm)** (see above). Note: **all samples** must be diluted in the same diluent for proper comparison – either TBTm **or** 1xSD20T.
- 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. **See Method A**.
- Run a set of Calibrators. Calibrators validate that the assay was performed to specifications, and can be used to normalize between-assay variation for enhanced precision. Reading values off a Calibrator curve has limitations, see above.
- Run the Canine Anti-Rabies IgG Positive Control; >**0.5** net OD.
- 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-Canine 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.

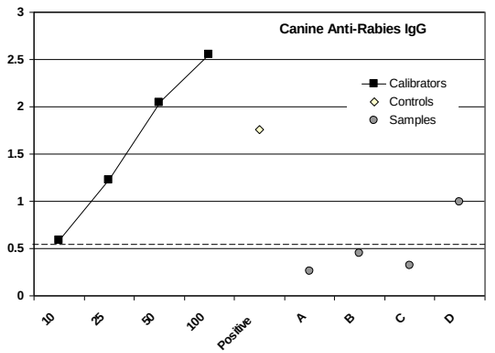
ASSAY RESULTS & PERFORMANCE

Method A. Antibody Activity Threshold Index

Compare Samples to **10 U/ml Calibrator** or **Internal Control**

= Positive/Negative Cut-off.

Example:



Results

The **sensitivity** of the assay to detect anti-rabies IgG, either natural or from immunization, is controlled so that the **10 U/ml Calibrator** represents a threshold OD for most true positives in canine serum diluted to 1:100 or greater. Visual inspection of the data in the above graph shows the following:

Calibrators – dilution curve of anti-rabies antiserum, derived from rabies viral antigen immunization, shows the OD range of the assay; high value indicates optimal sensitivity of the assay.
10 U/ml: a line has been drawn to indicate a threshold distinguishing between **Positive/Negative**. This is not a clear-cut threshold, rather a low OD area that could represent either low positives or high background negatives.

Canine Anti-Rabies IgG Positive Control – antiserum from a dog immunized with Imrab 3 (Merial), a rabies killed virus vaccine; net OD should be >**0.5.**

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 10 U/ml Calibrator can be used to calculate a **Threshold Index** that numerically discriminates Positive/Negative:

- Divide each Sample net OD by the 10 U/ml Calibrator net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

ASSAY RESULTS & PERFORMANCE (cont)

Method 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 vic

Example:

Experimental Samples are represented as follows:

C – Calibrator
P – Positive Control
E – Experimental sample

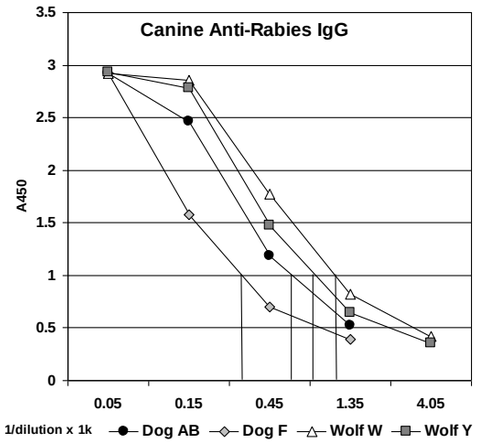
Results

Sample	Assay Net OD		Calculated Antibody Activity	
	Control	Exptl	Control	Exptl
1	0.325	2.281 **C**	0.75	**5.29**
2	0.272	1.581 **C**	0.63	**3.67**
3	0.133	0.998 **C**	0.31	**2.32**
4	0.194	0.453 **C**	0.45	**1.05**
5	0.289	0.767 **E**	0.67	**1.78**
6	0.319	0.982 **E**	0.74	**2.28**
7	0.332	1.401 **P**	0.77	**3.25**
8	0.291	0.351 **E**	0.68	0.81
9	0.402	0.325 **E**	0.93	0.75
10	0.253	0.16 **E**	0.59	0.37
Mean	0.281			
SD	0.075			
Mean +2 SD	**0.431**	= **Positive Index**		

Positive Control: Positive (>1.0) for antibody activity.
Calibrators: Ranking from 10 – 100 U/ml = 1.05 – 5.29.
Experimental: Two (2) are Positive (>1.0); 3 are Negative.

ASSAY RESULTS & PERFORMANCE (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, titers were calculated as inverse of the dilution that produced a **1.0 OD** in the assay.



Results

Dog AB: a dog immunized with Imrab 3, a rabies killed vaccine by Merial. Titer: **0.61 k**

Dog F: a dog immunized with Imrab 3, a rabies killed vaccine by Merial. Titer: **0.31 k**

Wolf W: an immunized wolf from a captive breeding program. Titer: **1.10 k**

Wolf Y: an immunized wolf from a captive breeding program. Titer: **0.85 k**

Calibrator Values

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

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

Instruction Manual No. M-600-010-DRV

Canine Anti-Rabies IgG ELISA Kit

Cat. No. 600-010-DRV, 96 tests

For Quantitation of Anti-Rabies Virus IgG in Serum, plasma or other biological fluids

For research use only (RUO), not for diagnosis, cure or prevention of the disease.

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