

INTENDED USE

The Canine Anti-KLH IgM ELISA is an indirect ELISA for the detection of and quantification of KLH (keyhole limpet hemocyanin)-specific IgM in canine serum or plasma. This immunoassay is suitable for:

- Determining **immune status** relative to non-immune controls;
 - Assessing efficacy of **vaccines**, including dosage, adjuvancy, route of immunization and timing;
 - Qualifying and/or standardizing vaccine batches and protocols.
 - Measuring natural anti-KLH antibodies of **innate immunity**.
- For research use only (RUO), not for diagnosis, cure or prevention of the disease.

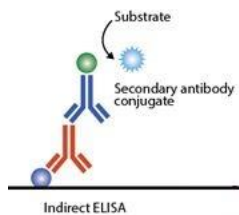
GENERAL INFORMATION



Keyhole Limpet Hemocyanin (KLH), an oxygen-transporting protein of the marine gastropod *Megathura crenulata*, is recognized as a potent immunoactivator, and therefore is widely used in research and clinical studies. Present

applications of KLH include: (a) use as a highly immunogenic antigen for assessment of immune competence of an organism, and (b) frequent use as a carrier of low molecular mass peptides and haptens, such as oligosaccharides, gangliosides or (glyco)peptides, designed to facilitate antibody production. In these cases, antibodies made to small peptides/haptens are generally raised by coupling to a large carrier protein like KLH. Antibodies are produced to both KLH and the peptide/hapten; because anti-KLH may give non-specific signals in various immunoassays, removal by solid phase immunoaffinity chromatography is common. The ELISA is useful for determining levels of anti-KLH in sera and for monitoring anti-KLH removal from purified samples after antibody production. The ADI anti-KLH ELISA is designed with high sensitivity for discriminating lower level antibodies, which may include natural anti-KLH antibodies of innate immunity, with specially formulated diluents to minimize interfering background signals.

PRINCIPLE OF THE TEST



The Canine Anti-KLH IgM ELISA kit is based on the binding of Canine anti- KLH in samples to KLH antigen immobilized on the microwells, and anti- KLH IgM antibody is detected by anti-Canine IgM-specific antibody conjugated to HRP conjugate. After a washing step, chromogenic substrate (TMB) is added and color (blue), which is

directly proportional to the amount of antibody present in the sample. Stop Solution is added (converts blue to yellow color), and A450nm is then measured using an ELISA reader. The activity of antibody in samples is calculated relative to supplied calibrators.

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 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 Diluent and store at 2-8°C until the kit lot expires or is used up.
Anti-Canine IgM - HRP Conjugate Concentrate (100x) Part No. H-DgM.211, 0.15ml	Peroxidase conjugated anti-Canine IgM in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of Working Sample Diluent is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 2-8°C.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
KLH Microwell Strip Plate	700-101	8-well strips (12)	Coated with KLH, and post-coated with stabilizers.
Anti-KLH IgM Calibrators			
10 U/ml	700-112B	0.65 ml	Four (4) vials, each containing anti-KLH levels in arbitrary activity Units; diluted in buffer with protein, detergents and antimicrobial as stabilizers.
25 U/ml	700-112C	0.65 ml	
50 U/ml	700-112D	0.65 ml	
100 U/ml	700-112E	0.65 ml	
Canine Anti-KLH IgM Positive Control	700-190PC	0.65 ml	Canine serum with anti-KLH IgM activity. Net OD > 0.5
Low NSB Sample Diluent	TBTm	30 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	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 IgM 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.

LIMITATIONS OF THE ASSAY

Quantitation of Antibody in a Sample

The ELISA measures anti-KLH activity, a combination of antibody concentration and avidity for the KLH antigens. Antibodies with substantially different total Ig concentrations may display similar anti-KLH 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 KLH 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

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 background, should be at least 1/10 of initial dilution and performed the same day as the assay.

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

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 1/200 or greater dilution for Canine 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**).
- 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 the Canine Anti-KLH IgM **Positive Control**.
- 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° 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.

- 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.
- 2nd Incubation [100ul – 30 min; 5 washes]**
 - Add 100ul of diluted Anti-Canine IgM 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.

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: Sample Diluent and anti-Protein G-HRP contain Proclin 300 (0.05%, w/v). <http://4adi.com/objects/catalog/product/extras/ELISA-Kit-SDS-MSDS-Set-1.pdf>

INTERPRETATION OF RESULTS

Quality Control & Expected Results

Sample Diluent Blank: < 0.3 OD; higher ODs suggest inadequate washing. If the OD of the Blank is greater than the lowest Calibrator, re-run the assay.

Non-immune/ Non-vaccinated Serum: Pre-immune/ non-immune sera at 1/200 dilutions should produce ODs lower than the low Calibrator. Very low antibody levels, including innate anti-KLH, may be assayed at dilutions less than 1/200; because net ODs will be elevated, compare the results with pre-immune (or KLH-adsorbed) controls at the same dilutions.

Positive Control: Activity > 0.5 net OD/ml. Significantly lower deviation indicates possible HRP deterioration; re-run the assay.

Calibrators: ODs should be in ascending order with low-to-high range > 1.0 OD. Variance of replicate values >25% indicates invalid precision for the run.

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:

Sample	Net OD		Calculated Antibody Activity		
	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

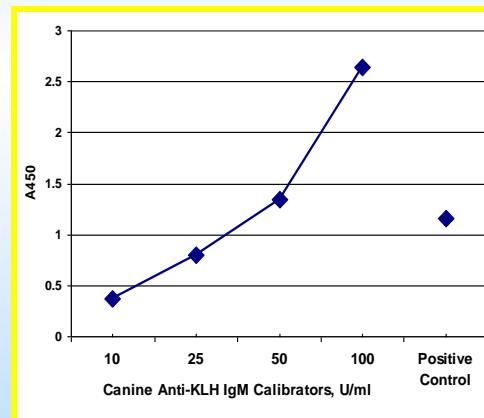
When the dilution curves of samples are parallel to the Calibrator curve (see Limits of the Assay), the anti-KLH activity units may be determined by interpolation from the Calibrator curve, as follows:

1. The results may be calculated using any immunoassay software package. If software is not available, anti-KLH 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-KLH (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-KLH 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	A450 nm
A1,2	Negative Diluent Blank	0.01
B1,2	10 U/ml Calibrator	0.38
C1,2	25 U/ml Calibrator	0.80
D1,2	50 U/ml Calibrator	1.35
E1,2	100 U/ml Calibrator	2.64
F1,2	Positive Control	1.16
G1,2	Sample 1:500	1.61

Result: 57 U/ml x 500 dilution = 28.5 kU/ml



Calibrator Values

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

CALCULATION OF RESULTS (continued)

Method C. Titters 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., 25 U/ml) can be used to normalize inter-assay values.

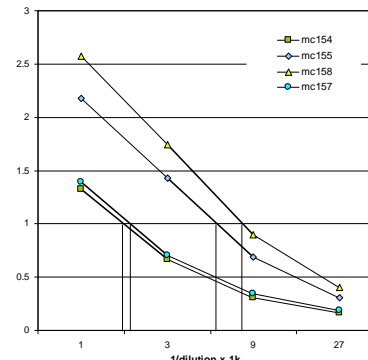
Calculations

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 IgM 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

PRODUCT SPECIFICATIONS

Specificity

Clinical grade, highly purified KLH is used to coat the microwells; stabilizing postcoat contains BSA; thus, no other antibody specificity is detectable in the assay. The anti-Canine IgM HRP conjugate specifically detects IgM, including that of dogs and wolves, and will not react with IgG, IgA or IgE class antibodies.

Assay Sensitivity

The KLH-coated plated and the anti-Canine IgM HRP concentration (which has a detection limit of 3 ng/ml) are optimized to differentiate anti-KLH IgM from background (non-immunized) signal with canine serum samples diluted 1:200.

Instruction Manual No. M-700-190-KLM

Canine Anti-KLH IgM ELISA Kit

Cat. No. 700-190-KLM, 96 Tests

For Quantitation of Anti-Keyhole Limpet Hemocyanin (KLH) IgM in Serum, plasma or other biological fluids

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



**ALPHA DIAGNOSTIC
INTERNATIONAL**

6203 Woodlake Center Drive • San Antonio • Texas 78244 • USA.

Phone (210) 561-9515 • Fax (210) 561-9544

Toll Free (800) 786-5777

Email: service@4adi.com

www.4adi.com

ELISA Kit Components	Amount	Part
KLH Coated Microwell Strip Plate	8-well strips (12)	700-101
Anti-KLH Calibrator	10 U/ml	0.65 ml 700-112B
Anti-KLH Calibrator	25 U/ml	0.65 ml 700-112C
Anti-KLH Calibrator	50 U/ml	0.65 ml 700-112D
Anti-KLH Calibrator	100 U/ml	0.65 ml 700-112E
Canine Anti-KLH IgM Positive Control	0.65 ml	700-190PC
Anti-Canine IgM HRP Conjugate (100X)	0.15 ml	H-DgM.211
Sample Diluent Concentrate (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	M-700-190