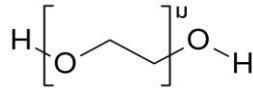## INTENDED USE

The **Rat Anti-PEG [polyethylene glycol] IgM** ELISA Kit is an immunoassay suitable for detecting and quantifying antibody activity specific for PEG in serum or plasma. For research use only (RUO), not for diagnosis, cure or prevention of the disease.

GENERAL INFORMATION

PEGylation is a process of covalent and non-covalent attachment of **polyethylene glycol (PEG)** polymer chains to molecules such as drugs or therapeutic proteins. The covalent attachment of PEG to a drug or protein can reduce the immunogenicity, antigenicity, and increase the hydrodynamic size which prolongs its circulatory time. PEGylation imparts several pharmacological advantages such as improved drug solubility, reduced dosage frequency, extended circulating life, increased drug stability, and enhanced protection from proteolytic degradation. Currently 15 PEGylated pharmaceuticals are on the market; Adynovate, Plegridy, Peginseatide, Pegloticase, Certolizumab pegol, Methoxy polyethylene glycol-epoetin beta, Pegaptanib, Pegvisomant, Peginterferon alfa-2a, Doxorubicin HCL liposome, Peginterferon alfa-2b, Pegaspargase, and Pegademase bovine. An emerging body of literature has shown the presence of anti-PEG antibodies being induced by treatment with the PEGylated therapeutic drugs. The existence of anti-PEG antibodies has been correlated with the loss of therapeutic efficacy and an increase in adverse effects.

PRINCIPLE OF THE TEST
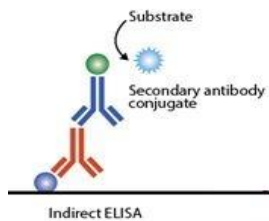
The Anti-PEG ELISA kits are based on the binding of antibodies in samples to the PEG antigen immobilized on the microwells. Bound antibody is detected by an anti-species antibody-HRP conjugate. After a washing step, chromogenic substrate (TMB) is added and color is developed by the HRP substrate, which is directly proportional to the amount of anti-PEG antibodies present in the sample. Stop solution is added to terminate the reaction, and absorbance is then measured using an ELISA reader at 450nm. The presence of antibody in samples is determined relative to anti-PEG 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. WG-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 (10x) Cat. No. SD10G, 10ml	Dilute the entire volume, 10ml + 90ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample/Conjugate Diluent** and store at 2-8ºC until the kit lot expires or is used up.
Anti-Rat IgM HRP Conjugate Concentrate (100x) Part: H-RtM.2a11, 0.15ml	Peroxidase conjugated anti-species antibody in a buffer with 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 2-8º C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
BSA-PEG Microwell Strip Plate	PEG-011	8-well strips (12)	96 microwell coated with PEG-BSA and post-coated with stabilizers.
Anti-PEG Calibrators			
1.25 U/ml 2.5 U/ml 5 U/ml 10 U/ml	PEG-092B PEG-092C PEG-092D PEG-092E	0.65 ml 0.65 ml 0.65 ml 0.65 ml	Four (4) vials, each containing anti-PEG antibodies; in buffer with protein, detergents and antimicrobial as stabilizers.
Anti-PEG Positive Control	PEG-092PC	0.65 ml	Anti-PEG in buffer with stabilizers and antimicrobial.
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-species Ig HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate; 0.2 to 1L.
- Stock bottle to store diluted Wash Solution; 0.2 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-PEG activity, a combination of antibody concentration and avidity for the PEG antigen. Antibodies with substantially different total Ig concentrations may display similar anti-PEG 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).

ASSAY DESIGN AND SET-UP

Sample Collection and Handling

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. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage.

Review Interpretation of Results (p5-7) 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 lower than the **1.25 U/ml Calibrator**. This is usually 1:10 or greater dilution for rat serum 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. Blank OD should be <0.3.

- Run the **Anti-PEG Positive Control**; value range is on the vial label.

- Run a set of **Calibrators**, which validate that the assay was performed to specifications: **10 U/ml** should give a high signal (>1.5 OD); **1.25 U/ml** should give a low signal which can be used to discriminate at the Positive/Negative threshold (see Interpretation of Results, p. 5).

Plate Set-up

Bring all reagents to room temperature (18-30º 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 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.
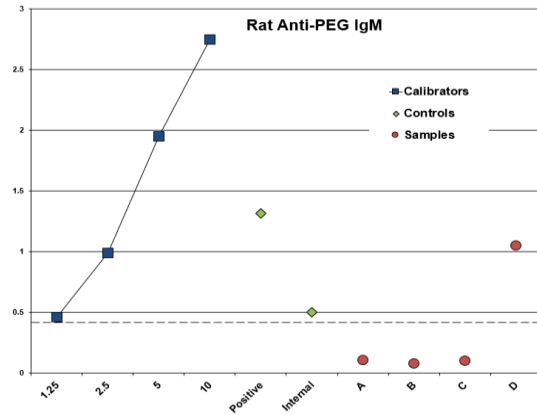
Method A. Antibody Activity Threshold Index

Compare Samples to **1.25 U/ml Calibrator** or **Internal Control**

=Positive/Negative Cut-off.

Example:

Results
The **sensitivity** of the assay to detect anti-PEG IgM, from either natural immune response or immunization, is controlled so that the **1.25 U/ml Calibrator** represents a threshold OD for most true positives in rat serum diluted 1:10 to 1:25. Visual inspection of the data in the above graph shows the following:

Calibrators – dilution curve of an anti-PEG antibody shows the OD range of the assay; high value indicates optimal sensitivity of the assay.

1.25 U/ml: a 'Cut-off' 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.

Positive Control – antiserum reactive to PEG; value range is on the vial label. This Control can be used to assess reproducibility and to normalize between-assay variation.

Internal Control – a true positive from an immune individual that represents the investigator's experience in distinguishing low positive from negative samples (not in kit). This should be run in each assay to supplement the 1.25 U/ml Calibrator for Positive/Negative discrimination purposes.

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 **1.25 U/ml** Calibrator can be used to calculate a **Threshold Index** that numerically discriminates Positive/Negative (see p6):
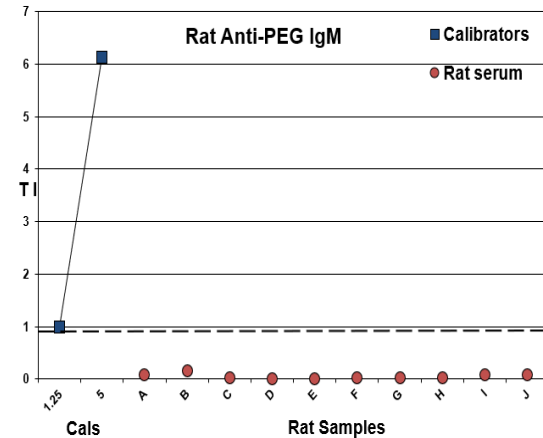
❖ Divide each Sample net OD by the 1.25 U/ml Calibrator net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 is **Negative** for antibody.

Example:

Rat Serum/Plasma IgM
A panel of non-immunized laboratory rats was tested for anti-PEG IgM (1:50 dilution). **Threshold Index** was calculated using the **1.25 U/ml Cal**.

Results

Anti-PEG IgM: all sera were negative (below the 1.0 threshold) at 1:50 dilution.

Notes:
1. **Positives** may be due to prior encounter with PEG or may be an aspect of the innate immune repertoire.

2. When the **Positive Index** is **above 5.0**, using a dilution curve to calculate titer is a more accurate quantitation method (see Method C).

3. The **sensitivity** of the assay may be adjusted by changing the sample dilutions: a) increase dilution (e.g., 1:100) to lower the signals of borderline positives to negative; b) decrease dilution (e.g., 1:25) to convert borderline samples to positive. With the latter, the values of negatives may increase, so an alternative threshold should be considered using known negatives to develop a **Positive Index** (see below) or use an **Internal Control** (Page 5).

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 sera dilution.

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

Method C. Titers from Sample Dilution Curves

The titer of elevated 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. The Positive and Sensitivity Control values 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
= **IgM Antibody Activity Units**

Specificity

Purified PEG(40k)-BSA is used to coat the microwells; thus, the assay is specific for antibodies directed to PEG or BSA. The anti-species HRP conjugate reacts with divalent or multivalent antibodies of the specified isotype (IgG, IgM, IgA, IgE) that are specific to PEG, and have bound to the PEG on the plate.

Calibrators, Controls, 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:

http://4adi.com/commerce/info/showpage.jsp?page_id=1060&category_id=2430&visit=10

Instruction Manual No. PEG-080

Rat Anti-PEG IgM ELISA Kit

Cat. No. PEG-080

For quantitation of anti-PEG antibodies 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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ELISA Kit Components	Amount	Part
PEG-BSA Coated Microwell Strip Plate	8-well strips (12)	PEG-011
Anti- PEG Positive Control	0.65 ml	PEG-092PC
Anti- PEG Calibrator 1.25 U/ml	0.65 ml	PEG-092B
Anti- PEG Calibrator 2.5 U/ml	0.65 ml	PEG-092C
Anti- PEG Calibrator 5 U/ml	0.65 ml	PEG-092D
Anti- PEG Calibrator 10 U/ml	0.65 ml	PEG-092E
Anti-Rat IgM HRP Conjugate (100X)	0.15 ml	H-RtM.2a11
Sample Diluent (10X)	10 ml	SD10G
Wash Solution Concentrate (100X)	10 ml	WG-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	M-PEG-080