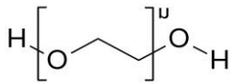
INTENDED USE

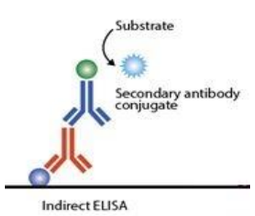
The **Human Anti-PEG [polyethylene glycol] IgG** ELISA Kit is an immunoassay suitable for detecting and quantifying antibody activity specific for PEG in serum or plasma. For research use only (RUO), not for diagnosis, cure or prevention of the disease.

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, Naloxegol, 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^{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. 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 2-8^{o}C until the kit lot expires or is used up.
Anti-Human IgG HRP Conjugate Concentrate (100x) Part: H-HuG.112, 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 2-8^{o} 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 U/ml 2.5 U/ml 5 U/ml 10 U/ml	PEG-012B PEG-012C PEG-012D PEG-012E	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-012PC	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-Herceptin activity, a combination of antibody concentration and avidity for the Herceptin antigen. Antibodies with substantially different total Ig concentrations may display similar anti-Herceptin 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 U/ml Calibrator**. This is usually 1:100 or greater dilution for human 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 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 (18-30^{o} C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 8Calibrator 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.

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-Human 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.

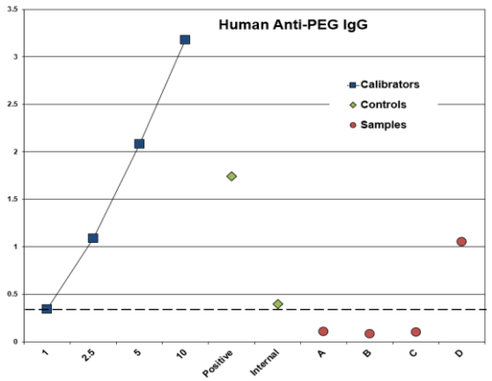
ASSAY PERFORMANCE

Method A. Antibody Activity Threshold Index

Compare Samples to **1 U/ml Calibrator** or **Internal Control**

=Positive/Negative Cut-off.

Example:



Results

The **sensitivity** of the assay to detect anti-PEG IgG, from either natural immune response or immunization, is controlled so that the **1 U/ml Calibrator** represents a threshold OD for most true positives in human serum diluted 1:50 to 1:100. 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 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 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 U/ml** Calibrator can be used to calculate a **Threshold Index** that numerically discriminates Positive/Negative (see p6):

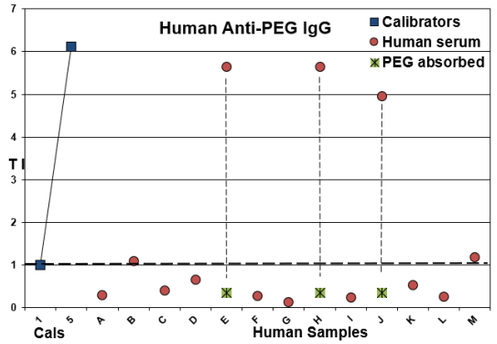
- Divide each Sample net OD by the 1 U/ml Calibrator net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 is **Negative** for antibody.

INTERPRETATION OF RESULTS

Example:

Human Serum/Plasma IgG

A panel of human sera of unknown history was tested for anti-PEG IgG (1:100 dilution). **Threshold Index** was calculated using the **1** U/ml **Cal**.



Results

Anti-PEG IgG: three samples (E,H,J) were positive (above the 1.0 threshold) at 1:100 dilution; 2 samples (B,M) were borderline; the remaining samples were negative.

The positive samples were converted to negative when pre-absorbed with PEG.

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., 1:200) to lower the signals of borderline positives to negative; b) decrease dilution (e.g., 1:50) 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.

INTERPRETATION OF RESULTS (cont)

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
   = **IgG Antibody Activity Units**

PRODUCT SPECIFICATIONS

Specificity

Purified PEG-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.

PRECAUTIONS AND SAFETY INSTRUCTIONS

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

Instruction Manual No. PEG-010

Human Anti-PEG IgG ELISA Kit

Cat. No. PEG-010

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.

ALPHA DIAGNOSTIC INTERNATIONAL

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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-012PC
Anti- PEG Calibrator 1 U/ml	0.65 ml	PEG-012B
Anti- PEG Calibrator 2.5 U/ml	0.65 ml	PEG-012C
Anti- PEG Calibrator 5 U/ml	0.65 ml	PEG-012D
Anti- PEG Calibrator 10 U/ml	0.65 ml	PEG-012E
Anti-Human IgG HRP Conjugate (100X)	0.15 ml	H-HuG.112
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-010