INTENDED USE

The **Human Anti-Herceptin/trastuzumab** or anti-drug antibodies (HADA) ELISA Kit is an immunoassay suitable for detecting and quantifying human antibody activity against **Herceptin/Trastuzumab** of any isotype, in human serum or plasma. It is validated for human sample but it should work in other species as well. This kit is for research use only.

GENERAL INFORMATION

HER2 (Human Epidermal Growth Factor Receptor 2/New/Erbb2) is over-expressed in ~30% of breast cancers. Herceptin, a fully humanized monoclonal antibody (IgG1k) that neutralized Her2 and used to treat some breast cancer patients. Humanized antibodies are 'Animal Antibodies' that have been engineered by recombinant DNA-technology to reduce the overall content of the animal-portion of IgG so as to increase acceptance by humans or minimize 'rejection'. The portion of the mouse IgG that remains in the 'humanized IgG' or even the human portion may be recognized as foreign by humans and may result into the generation of "**Human Anti-Drug Antibodies (HADA or Human Anti-Mouse Antibodies or HAMA)**. Some patients receiving Herceptin developed some form of anti-Herceptin (HADA) response. The presence of anti-Drug antibody (e.g., Human Anti-Herceptin IgG) may limit the long-term usage the humanized antibody (Herceptin). The prevalence of anti-drug antibodies are highly dependent upon the nature of sample, duration of therapy, and sensitivity of the assay. Therefore it is necessary to monitor the presence of anti-Herceptin antibody levels in patients receiving long-term Herceptin immunotherapy.

PRINCIPLE OF THE TEST

The Human Anti-Herceptin IgG ELISA kit is a double antigen sandwich ELISA based on the binding of anti-Herceptin antibodies (any isotype) in samples to Herceptin immobilized on the microwells; bound anti-Herceptin Ig's are detected by simultaneously binding to Herceptin-HRP (horseradish peroxidase) enzyme, forming a sandwich. After a washing step, chromogenic substrate (TMB) is added and color (blue) is developed, which is directly proportional to the amount of antibody present in the sample. Stopping Solution is added to terminate the reaction (converts blue color to yellow), and A450nm (yellow color) is measured using an ELISA reader. The presence of anti-Herceptin antibody in samples is determined relative to controls.

PRODUCT SPECIFICATIONS

Specificity
Purified Herceptin is used to coat the microwells and detected by Herceptin-HRP; thus the assay is specific for antibodies directed to Herceptin and not simply detect common human-anti-human Ig's (HAHA). It is possible that some samples may have common HAHA but not antibodies specific to Herceptin. The Herceptin-HRP conjugate reacts with divalent or multivalent antibodies of either isotype (IgG, IgM, IgA, IgE). The assay, however, will not distinguish between antibodies made to the mouse or human portions of Herceptin. This kit should detect antibodies to Herceptin in any species.

Assay Sensitivity
The Herceptin antigen coating level and Herceptin-HRP conjugate concentration are optimized to differentiate anti-Herceptin IgG from background (non-antibody) signal with human serum samples diluted 1:20 or higher.

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 the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample Diluent** and store at 2-8^{o}C until the kit lot expires or is used up.
Herceptin - HRP Conjugate Concentrate (100x) Part: 200-323, 0.15ml	Peroxidase conjugated mouse IgG in buffer with 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^{o} C storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
Herceptin IgG Microwell Strip Plate	200-521	8-well strips (12)	Coated with mouse IgG antigen, and post-coated with stabilizers.
Anti-Heceptin IgG Calibrators			
5 U/ml 10 U/ml 20 U/ml 40 U/ml	200-522B 200-522C 200-522D 200-522E	0.65 ml 0.65 ml 0.65 ml 0.65 ml	Four (4) vials, each containing human anti-rituximan IgG; in buffer with protein, detergents and antimicrobial as stabilizers.
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 IgG 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 IgG activity, a combination of antibody concentration and avidity for the Herceptin antigens. Antibodies with substantially different total Ig's concentrations may display similar anti-Herceptin IgG 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. For other samples, 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. We recommend an initial testing of samples at 1:20 dilutions and if the background remains <0.300 then samples can be tested at 1:10-1:20 dilution.

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.

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 lower than the **5 U/ml Calibrator**. This is usually 1/50 dilution or greater dilution for human 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. 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 A**, has limitations. See Limits of the Assay (above).

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 internal 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.

ASSAY DESIGN AND SET-UP (continued)

- 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 Rituximab-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.

INTERPRETATION OF RESULTS

Calculation of Results

Consider several data reduction methods to best represent the relationships among experimental and control groups, to determine **Positive Immune** and **Negative Non-immune** or **Pre-immune**, and to **Quantitate** positive antibody levels.

Method A. Use of a Calibrator Curve

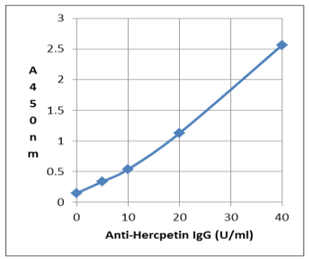
When the dilution curves of samples are parallel to the Calibrator curve (see Limits of the Assay, page 3, and Results, page 5), the anti-mouse IgG activity units may be determined by interpolation from the Calibrator curve.

Sample values = curve value, U/ml x 1/sample dilution

Method B. Antibody Activity Threshold Index

Compare Samples to **10 U/ml Calibrator** or **Internal Control = Positive/Negative Cut-off**.

Example:



Anti-Herceptin IgGs Standard Curve (do not use this for sample calculations) /4_ADI_ELISA

Results

The **sensitivity** of the assay to detect anti-Herceptin IgG, from either natural exposure or drug administration, is controlled so that the **5 U/ml Calibrator** represents a threshold OD for most true positives in human serum diluted in the Sample Diluent at 1:50 or greater.

1 unit of anti-Herceptin standards=1 ng/ml of the polyclonal used in this kit. Other anti-Herceptin or anti-human IgG antibodies may react differently and produce different activity (binding to human IgG) and concentration curves.

The **5 U/ml Calibrator** can be used to calculate a **Threshold Index** that numerically discriminates Positive/Negative, as follows:

- Divide each Sample net OD by the **5 U/ml Calibrator** net OD. Values above 1.0 are a measure of **Positive** Antibody Activity; below 1.0 are **Negative** for antibody.

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

ASSAY PERFORMANCE

Detection Range and Specificity

The Antigen Sandwich ELISA format allows for the detection and quantitation of ñbridgingò bivalent and/or multi-valent antibodies of any species or of any immunoglobulin isotype and/or subclass ï IgG, IgM, IgA or IgE.

This assay, as with all other assays that measure antibody activity, produces:

a) different signal levels with equivalent amounts of each antibody, or

b) the same signal level with different amounts of each antibody. This means that an individual antibody, calibrated in mass units (e.g., ng/ml) cannot serve as a standard curve to quantify other antibodies in mass units.

c) The value for the anti-Herceptin IgGs in samples are different from different regions of the standards curve ï a measure of non-parallelism due to differences in avidity of the antibodies for the human IgG antigen. When this occurs, use a different method for quantitation (e.g., Method B or C).

INTERPRETATION OF RESULTS

C. 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 vice versa.

Species Reactivity

ELISA kit #200-520-AHG is designed to detect anti-Herceptin antibodies in human samples. If antibodies to Herceptin are induced in animals (mouse or rat) then those antibodies will also be detected since the detection reagent is Herceptin-HRP. This kit has been tested with human samples. However, it has not been tested for anti-Herceptin/IgG in mouse, rat or other species.

Expected Results

An in-house study of normal human serum samples at 1:25 or 1:100 dilutions did not yield values greater than the lowest standard (5 U/ml).

According to information from Herceptin (Genentech).

Immunogenicity:

As with all therapeutic proteins, there is a potential for immunogenicity. Among 903 women with metastatic breast cancer, human anti-human antibody (HAHA) to Herceptin was detected in one patient using an enzyme-linked immunosorbent assay (ELISA). This patient did not experience an allergic reaction. Samples for assessment of HAHA were not collected in studies of adjuvant breast cancer. The incidence of antibody formation is highly dependent on the sensitivity and the specificity of the assay. Additionally, the observed incidence of antibody (including neutralizing antibody) positivity in an assay may be influenced by several factors including assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies to Herceptin with the incidence of antibodies to other products may be misleading.

PRECAUTIONS AND SAFETY INSTRUCTIONS

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

General References

Hudis CA (2007) N. Engl. J. Med. 357, 39-51; Yu D (200) Oncogen 19, 6115-6121; Kute T (2004) Cytometry 57a, 86-93; Santin AD (2008) Int. J. Gynecol. Obstet. 102, 128-131; http://www.herceptin.com/herceptin/patient/index.jsp

Instruction Manual No. M-200-520-AHG

Anti-Herceptin/Trastuzumab IgGs (HADA or Human Anti-Drug Antibodies)

ELISA Kit # 200-520-AHG

For Quantitation of Anti-Herceptin/ Trastuzumab IgGs in Serum or Plasma in Human or other species.

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ELISA Kit Components	Amount	Part
Herceptin Coated Microwell Strip Plate	8-well strips (12)	200-521
Anti- Heceptin IgG Calibrator	5 U/ml 0.65 ml	200-522B
Anti- Heceptin IgG Calibrator	10 U/ml 0.65 ml	200-522C
Anti- Heceptin IgG Calibrator	20 U/ml 0.65 ml	200-522D
Anti- Heceptin IgG Calibrator	40 U/ml 0.65 ml	200-522D
Herceptin HRP Conjugate (100X)	0.15 ml	200-523
Sample Diluent (20X)	10 ml	SD20T
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	M-200-520-AHG

DRAFT Version-Use the manual supplied with the kit.