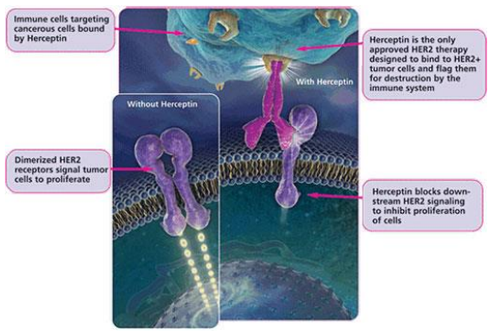
INTENDED USE

The Human Anti-Herceptin (trastuzumab) ELISA Kit is an immunoassay suitable for detecting and quantifying human antibody activity specific for Herceptin (Anti-drug Ig's), of any isotype, in human serum or plasma. The assay also detects anti-Herceptin antibodies in serum of any other species, including monkey, rat, rabbit and pig. For research use only (RUO), not for diagnosis, cure or prevention of the disease.

GENERAL INFORMATION



HER2 (Human Epidermal Growth Factor Receptor 2), also known as Neu, ErbB-2, CD340 or p185, is amplified or over-expressed in ~30% of breast cancers, and is strongly associated with increased disease recurrence and worse prognosis. Over-expression is also known to occur in ovarian, stomach, and aggressive forms of uterine cancer, such as uterine serous endometrial carcinoma. HER2 is the target of the monoclonal antibody trastuzumab (Herceptin: by Roche/Genentech). Herceptin, a fully humanized monoclonal antibody (IgG1 kappa), binds to the domain IV of the extracellular segment of the HER2/neu receptor. Herceptin has had a major impact in the treatment of HER2-positive metastatic breast cancer, being mostly effective only in cancers where HER2 is over-expressed.

Herceptin is a fully 'humanized' monoclonal antibody which does not contain 'animal' derived sequences that would be recognized by the injected patient as a foreign antigen; thus, immunological response to Herceptin is expected to be minimal. However, when large amounts of a monoclonal immunoglobulin are continually encountered in circulation, the host may mount significant 'anti-idiotypic' response = anti-Herceptin antibodies. Such antibodies might be expected to diminish the effectiveness of Herceptin as a drug, and perhaps have other metabolic consequences.

PRINCIPLE OF THE TEST

The Human Anti-Herceptin ELISA kit is a double antigen sandwich ELISA based on the binding of anti-Herceptin antibodies (any isotype or species) in samples to Herceptin antigen immobilized on the microwells; bound anti-Herceptin antibody is detected by simultaneously binding to Herceptin conjugated to HRP After a washing step, chromogenic substrate (TMB) is added and color is developed, which is directly proportional to the amount of anti-Herceptin antibody present in the sample. Stopping Solution is added to terminate the reaction, and A450nm is measured using an ELISA reader.

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/Conjugate Diluent** and store at 2-8^{o}C until the kit lot expires or is used up.
Herceptin- HRP Conjugate Concentrate (100x) Part: 200-524, 0.15ml	Peroxidase conjugated Herceptin in 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
Herceptin Microwell Strip Plate	200-521	8-well strips (12)	Coated with Herceptin, (trastuzumab) and post-coated with stabilizers.
Anti-Herceptin Calibrators			
2 U/ml 5 U/ml 10 U/ml 20 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 anti-Herceptin antibodies; 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 Herceptin 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. 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.

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 **3 U/ml Calibrator**. This is usually 1/10 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 Herceptin 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

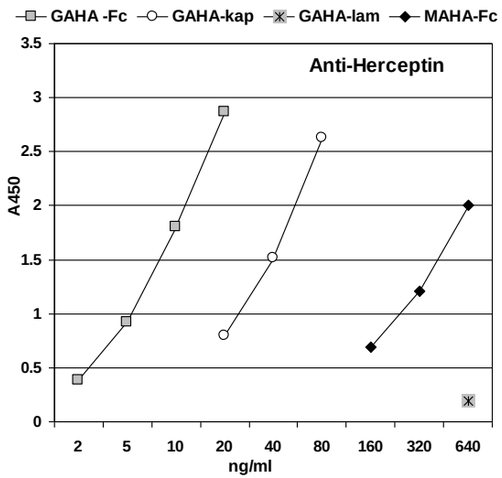
- Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.

ASSAY PERFORMANCE

Detection Range and Specificity
The Antigen Sandwich ELISA format allows for the detection and quantitation of 'bridging' bivalent and/or multi-valent antibodies of any animal species or of any immunoglobulin isotype and/or subclass – IgG, IgM, IgA or IgE.

This graph shows dilution curves of affinity-purified antibodies reactive with Herceptin as antigen, as follows:

- **GAHA-Fc** – goat polyclonal antibodies specific for the Fc region of Herceptin; affinity-purified.
- **MAHA-Fc** – mouse monoclonal antibody specific for the Fc region of Herceptin; affinity-purified.
- **GAHA-kap** – goat polyclonal antibodies specific for the kappa light chain of Herceptin; affinity-purified.
- **GAHA-lam** – goat polyclonal antibodies specific for human lambda light chain; Herceptin has no lambda light chain.



Results

- The data demonstrate measuring antibodies of different species.
- 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.
- The values for the GAHA-kap antibody were consistent when read from different regions of the GAHA curve – a measure of parallelism; values for MAHA-Fc were not. When parallelism does not occur, e.g., when antibodies differ significantly in avidity for the Herceptin as antigen, use a different method for quantitation (e.g., Method B or C, page 6,7).

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 Assay Performance, page 5), the anti-Herceptin activity units may be determined by interpolation from the Calibrator curve.

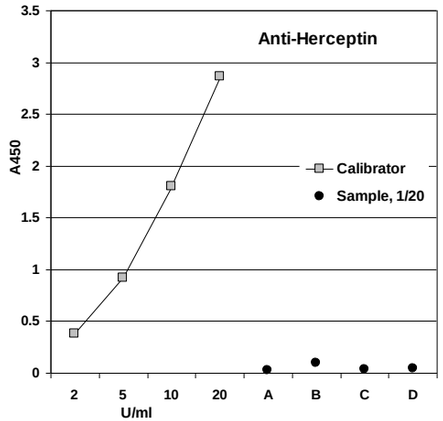
Sample values = curve value, U/ml x 1/sample dilution

Method B. Antibody Activity Threshold Index

Compare Samples to **2 U/ml Calibrator** or **Internal Control**

= Positive/Negative Cut-off.

Example:



Results
The **sensitivity** of the assay to detect anti-Herceptin, native level or from drug administration, is controlled so that the **2 U/ml Calibrator** represents a threshold OD for most true positives in human serum diluted in the Sample Diluent at 1:20 or greater.

* * * *

The **2 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 **2 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.

INTERPRETATION OF RESULTS (cont)

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.

PRODUCT SPECIFICATIONS

Specificity

Purified Herceptin (trastuzumab) is used to coat the microwells; thus the assay is specific for antibodies directed to Herceptin or other similar human IgG. The Herceptin HRP conjugate reacts with divalent or multivalent antibodies of any isotype (IgG, IgM, IgA, IgE) that are specific to Herceptin, and have bound to the Herceptin on the plate. Anti-Herceptin antibodies from any species may be detected in the assay.

Assay Sensitivity

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

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

Instruction Manual No. M-200-520-HAG

Human Anti-Herceptin (trastuzumab) antibodies (ADC) ELISA Kit

Cat. No. 200-520-HAG, 96 tests

For Quantitation of Anti-Herceptin Antibodies (ADC) in Serum, plasma or other biological fluids

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

ALPHA DIAGNOSTIC INTERNATIONAL

India Contact:

Life Technologies™
Your Molecular & Cell Technology Partner

Life Technologies (India) Pvt. Ltd.
306, Aggarwal City Mall, Opposite M2K Pitampura, Delhi – 110034 (INDIA).
Ph: +91-11-42208000, 42208111, 42208222
Mobile: +91-9810521400
Fax: +91-11-42208444
Email: customerservice@lifetechindia.com
Web: www.lifetechindia.com

ELISA Kit Components	Amount	Part
Herceptin Coated Microwell Strip Plate	8-well strips 12)	200-521
Anti- Herceptin Calibrator 2 U/ml	0.65 ml	200-522B
Anti- Herceptin Calibrator 5 U/ml	0.65 ml	200-522C
Anti- Herceptin Calibrator 10 U/ml	0.65 ml	200-522D
Anti- Herceptin Calibrator 20 U/ml	0.65 ml	200-522E
Herceptin HRP Conjugate (100X)	0.15 ml	200-524
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-HAG