INTENDED USE

The **Herceptin** (humanized Anti-Her2 IgG1/ trastuzumab) ELISA Kit is an immunoassay for quantifying active Herceptin in mouse or human serum/plasma, or in other appropriately qualified samples.

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. Like many humanized antibodies, Herceptin infusion may induce antibodies: human anti-human antibodies (HAHA), human anti-drug antibodies (HADA).

The ADI Herceptin ELISA is designed to measure the active drug antibody concentration in mouse or human serum/plasma. The presence of endogenous animal or human IgG1 do not interfere in the assay.

PRINCIPLE OF THE TEST

The Herceptin ELISA kit is based upon capture of active Herceptin to the HER2 antigen coated on the plate. Bound Herceptin is then detected by anti-human IgG HRP. After a washing step, chromogenic substrate is added and color is developed by the enzymatic reaction of HRP on the TMB substrate, which is directly proportional to the amount of Herceptin present in the sample. Stopping Solution is added to terminate the reaction, and absorbance at 450nm is then measured using an ELISA microtiter well reader. The concentration of Herceptin in samples and control is calculated from a curve of standards containing known concentrations of Herceptin.

STORAGE AND STABILITY

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.

KIT CONTENTS

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To Be Reconstituted: Store as indicated.

Component	Preparation Instructions
Sample Diluent Concentrate (20x) Cat.#. 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 2-8ºC until the kit lot expires or is used up.
Wash Solution Concentrate (100x) Cat. # 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.
Anti-Human IgG-HRP Conjugate Concentrate (100x) Part No. 200-514, 0.15ml	Peroxidase conjugated anti-human IgG in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10 ul of concentrate to 1 ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return 100X to 2-8ºC storage.

Ready For Use: Store as indicated on labels.

Component	Part	Amt	Contents
Antigen Coated Strip Plate	200-511	8-well strips (12)	Coated with HER2 antigen and post-coated with stabilizers.
Herceptin Standards			
0.5 ng/ml 1 ng/ml 2.5 ng/ml 5 ng/ml 10 ng/ml	200-513B 200-513C 200-513D 200-513E 200-513F	0.65 ml 0.65 ml 0.65 ml 0.65 ml 0.65 ml	Five (5) vials, each containing purified recombinant Herceptin with designated concentrations; diluted in buffer with protein, detergents and non-azide antimicrobials as stabilizers.
Positive Control [Herceptin] range on label	200-512	0.65 ml	Herceptin of stated concentration range; diluted in buffer with protein, detergents and non-azide antimicrobials 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 Antibody HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate; 0.2 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.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Standards, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains 1% 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

ASSAY DESIGN AND SET-UP

Sample Collection and Handling
Culture medium, serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference (See Limits of the Assay, page 6). For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. For all samples, clarify by centrifugation and/or filtration. If samples will not be assayed immediately, store frozen for long-term storage.

DILUTE serum samples in Working Sample Diluent. Dilutions of 1:5k–1:500k may be appropriate for standard drug treatment regimens. For accuracy, multiple dilution steps are recommended, as follows:

1) 10ul serum + 990ul diluent = [1:100],
2) 10ul [1:100] + 490ul diluent = **[1:5k].**

Diluted samples are stable for at least a year refrigerated.

Assay Validation
Validate the performance of the Herceptin sample and matrix in the assay system for recovery (see Limits of the Assay, page 6), as follows:

Recovery – a measure of the interference of the sample matrix (diluent effect) in providing accurate quantitation of Herceptin in the sample relative to the Herceptin Standards.

Prepare and run a series of dilutions of the Herceptin sample (within the Standard range) in Working Sample Diluent to determine the dilutions that give consistent and accurate quantitation. Serum and plasma require greater than 1/400 dilution to obtain consistent quantitation or complete antigen recovery.

Recovery Limits – Herceptin was spiked into dilutions of human serum & plasma, 1 pool and 9 individual samples, or Sample Diluent (Control), at a final concentration of 4.5 ng/ml.

Results: recovered values ranged from **74** to **102%** of Control with sera diluted 1/500. Recovery was **less** when serum was diluted less than 1/100. Low recovery suggests serum factors that interfere with Herceptin binding to the antigen on the plate.

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 10 Standard 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 ul 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 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.

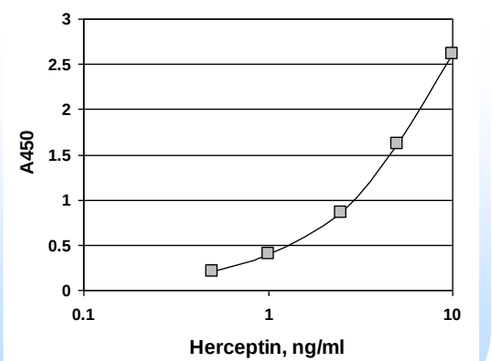
CALCULATION OF RESULTS

1. The results may be calculated using any immunoassay software package. The four-parameter curve-fit is recommended. If software is not available, Herceptin concentrations may be determined as follows:
2. Calculate the mean OD of duplicate samples.
3. On graph paper plot the mean OD of the standards (y-axis) against the concentration (ng/ml) of Herceptin (x-axis). Draw the best fit curve through these points to construct the standard curve. A point-to-point construction is most common and reliable.
4. The Herceptin concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
5. Multiply the values obtained for the samples by the dilution factor of each sample.
6. Samples producing signals higher than the 100 ng/ml standard should be further diluted and re-assayed.

Typical Results:

Wells	Calibrators		A450 nm
A1,2	**Negative Diluent Blank**		0.02
B1,2	0.5 ng/ml	**Standard**	0.20
C1,2	1 ng/ml	**Standard**	0.40
D1,2	2.5 ng/ml	**Standard**	0.86
E1,2	5 ng/ml	**Standard**	1.61
F1,2	10 ng/ml	**Standard**	2.61
G1,2	**Positive**	**Control**	1.02
H1,2	Sample	**1:500**	0.51

Sample Result: **1.26** ng/ml x 500 dilution = **630** ng/ml



PERFORMANCE CHARACTERISTICS

Specificity
The plate is coated with HER2 antigen to which Herceptin binds with high affinity. Other antibodies or binding proteins may also bind to the HER2-antigen coated plate; however the Anti-Human IgG-HRP conjugate will not bind to mouse antibodies or non-antibody human serum proteins. Therefore, the assay is highly specific for measuring Herceptin activity only.

Precision
Samples containing low, medium and high concentrations of Herceptin were assayed as duplicates in multiple assays (n=5) to obtain between-assay reproducibility. Coefficients of variation were calculated for the concentrations using a point-to-point curve-fitting program.

Herceptin concentrations were measured with good between-assay (**2.1** to **3.5** %CV) reproducibility.

Sample	Herceptin ng/ml	Inter-assay %CV
High Concn	5.66	**3.1**
Medium Concn	2.78	**3.5**
Low Concn	1.28	**2.1**

Recovery
Herceptin was spiked into human serum or plasma diluted 1/500 in Sample Diluent (1 pooled and 9 individual samples), and assayed in duplicate. **Recovery** was calculated comparing the observed (O) values to the expected (E) values for each diluted sample. All serum and plasma samples were 0 herceptin (E).

O/E values ranged from **74%** to **102%**. See Limits of the Assay.

Human Serum & Plasma Samples	Herceptin Concn (E) = 4.50 ng/ml	
	Observed (O)	O/E %
BC Pooled Serum	4.60	**102**
Serum, male A	3.71	**82**
Serum, male B	3.33	**74**
Serum, female C	3.93	**87**
Serum, female D	3.66	**81**
Serum, male E	3.82	**85**
Plasma, male F	3.76	**84**
Plasma, male G	3.39	**75**
Plasma, female H	4.13	**92**
Plasma, female I	3.83	**85**

QUALITY CONTROL

Reagents Accurate and reproducible assay results rely on proper storage, handling and control of reagent and sample temperature. Store all reagents as indicated, and warm to room temperature only those to be used in the assay. Shelf-life of the critical reagents and samples will diminish with extended exposure to non-refrigeration, resulting in inaccurate assay results. All solutions should be clear. Cloudiness or particulates are indications of reagent contamination or instability and may interfere with proper performance of the assay. Do not use.

Sample Controls A Positive Serum Control is provided with the kit, assigned with an Herceptin concentration value range. Recovery in this range is an indicator of proper assay performance. Each lab should also assay internal control samples, which represent the lab's expected sample population and that are maintained stabilized. A Sample Diluent blank should also be run; OD should be <0.3 and lower than 0.5 ng/ml Standard OD.

Standard Curve The signal generated by the standards should be continuously increasing in OD from the lowest Standard to the highest Standard, with a difference greater than 1.2 OD. Non-uniform or low signals may indicate problems with technique, protocol directions and/or reagent preparation, use or stability. Do not rely on results generated from an assay with these issues.

Technique Accurate and reproducible assay results rely on good lab technique regarding pipetting, plate washing and handling of samples and reagents.

Equipment Precision of results relies on uniform and effective washing techniques; an automatic washer may be used. ELISA reader and pipettes should be properly calibrated.

LIMITS OF THE ASSAY

1. The assay measures Herceptin activity, i.e., antibody that actually binds to the HER2-antigen coated plate, relative to Herceptin standards that are presumed to be 100% active antibody. Factors in the sample that diminish Herceptin binding, e.g., HER2 antigen or other Herceptin -binding molecules, may reduce apparent Herceptin concentration in the assay (**Recovery**).

2. Assays that measure Herceptin mass concentration may not have a tight correlation with the Herceptin activity assay, e.g., full Herceptin recovery may be determined by different factors.

3. The **recovery** (accuracy of Herceptin measurement in stored serum) may be diminished if not diluted at least 1/500 in Sample Diluent (see Recovery, above and page 6). Recovery in fresh, individual human or mouse serum or plasma samples may differ, and has not been determined.

4. Multiple-dose intravenous administration of Herceptin in humans has resulted in mean peak and trough ranges of 63 – 216 ug Herceptin /ml of serum (product data). The ELISA assay detection range is 0.5 - 10 ng IgG/ml. So Herceptin samples with the above doses will require dilutions of 6,000-500,000-fold.

Instruction Manual No. M-200-510-HLG

Herceptin
(trastuzumab/ anti-HER2 humanized IgG)

ELISA Kit # 200-510-HLG

For Quantitation of Active Herceptin in Mouse or Human Serum/Plasma

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

ELISA Kit Components		Amount	Part
HER2 Antigen Coated Microwell Plate		8-well strips (12)	200-511
Herceptin Control		0.65 ml	200-512
Herceptin Standard	0.5 ng/ml	0.65 ml	200-513B
Herceptin Standard	1 ng/ml	0.65 ml	200-513C
Herceptin Standard	2.5 ng/ml	0.65 ml	200-513D
Herceptin Standard	5 ng/ml	0.65 ml	200-513E
Herceptin Standard	10 ng/ml	0.65 ml	200-513F
Anti-Human IgG-HRP Conjugate (100X)		0.15 ml	200-514
Sample Diluent Concentrate (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-200-510-HLG