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Instructions for Use

Antibody to Trastuzumab (Herclon®, Herceptin®) ELISA

SHIKARI®
S-ATT

Enzyme immunoassay for the quantitaive determination of antibodies to Trastuzumab (Herclon®, Herceptin®) in serum and plasma

REF **TR-ATRASv2** Σ 12 x 8 2-8°C

Revision # 2.1 March 2017

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IVD

Matriks Biotek® Laboratories
www.matriksbiotek.com

Intended Use

The Matriks Biotek® Antibody to Trastuzumab (ATT) (Herclon®, Herceptin®) Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) Kit is intended for the quantitative determination of antibodies to Trastuzumab in serum and plasma. It is for professional use only.

Summary and Explanation

The HER (or ErbB) family of transmembrane tyrosine kinase receptors is composed of four members, HER1 to HER4 . HER2, a ligand-less Mr 185,000 receptor encoded by the neu proto-oncogene, is overexpressed in 25–30% of human breast cancer and has been associated with enhanced tumor aggressiveness and a high risk of relapse and death. Recent evidence indicates that HER2 amplifies the signal provided by other receptors of the ErbB family by heterodimerizing with them. The important biological role of HER2 in the signaling network that drives epithelial cell proliferation and transformation, together with its extracellular accessibility and its overexpression in some human tumors led to considering HER2 as an appropriate target for tumor-specific therapies.

The neu gene encodes a 185-kDa transmembrane glycoprotein, referred to as p185neu, HER2, or erbB-2, possessing intrinsic protein tyrosine kinase activity. The receptor consists of an extracellular domain, with four subdomains including two cysteine rich domains, a transmembrane domain, and an intracellular domain, consisting of a juxtamembrane region, a tyrosine kinase domain, and a carboxyl tail harboring autophosphorylation sites HER2 is homologous to, but distinct from, other members of the erbB family, which includes the epidermal growth factor receptor (EGFR or erbB-1), erbB-3, and erbB-4. The binding of cognate growth factors to these receptors regulates cell growth, proliferation, and diferentiation through the activation of receptor tyrosine kinases, triggering an incompletely defined signal transduction cascade. Signal transduction by these receptors is believed to involve dimerization and oligomerization, both in the form of homo-oligomers and hetero-oligomers in various erbB receptor combinations.

Trastuzumab (Herclon®, Herceptin®) is a recombinant DNA-derived humanized monoclonal antibody that selectively targets the extracellular domain of the human epidermal growth factor receptor 2 protein (HER2). The antibody is an IgG1 kappa that contains human framework regions with the complementarity-determining regions of a murine anti-p185 HER2 antibody that binds to HER2. Trastuzumab is composed of 1,328 amino acids and has a molecular weight of 148 kDa.

Trastuzumab has antitumor activity against HER2-positive human breast tumor cells in laboratory models and is active for the treatment of women with HER2-overexpressing breast cancers. On the basis of trastuzumab clinical efficacy, this antibody was approved in 1998 for clinical use for HER2 overexpressing metastatic breast cancer. Trastuzumab seems to exert its antitumor effects by several mechanisms that are not yet completely understood. In HER2 overexpressing cells, trastuzumab markedly down-regulates HER2 expression by accelerating receptor endocytosis and degradation and inhibits cell cycle progression by inducing the formation of p27Kip1/Cdk2 complexes. Trastuzumab also induces antibody-dependent cell-mediated cytotoxicity against the HER2 expressing tumor cells in animal models. This process is regulated by antibody receptors FcγRIII and FcγRIIB on myeloid cells. Other additional mechanisms that have been proposed include suppression by trastuzumab of angiogenesis and metastasis.

Accoring to the manufacturer's product insert, the use of Trastuzumab (Herclon®, Herceptin®) might be associated to the development of anti-Trastuzumab antibodies in various percentages of patients during therapy with the drug Herclon® and Herceptin®. The Matriks Biotek® Antibody to Trastuzumab ELISA Kit can be efficiently used for monitoring anti- Trastuzumab antibodies during therapy and offers the clinician a tool for decision on possible preventive measures.

Test Principle

The Matriks Biotek® Antibody to Trastuzumab (Herclon®, Herceptin®) ELISA is a sandwich assay for the determination of antibodies against Trastuzumab in serum and plasma samples. During the first incubation period, the drug Trastuzumab (Herclon®, Herceptin®) coated on the wall of the microtiter wells captures the antibodies to Trastuzumab in patient serum and plasma samples. After washing away the unbound components from samples, a Peroxidase-labelled conjugate is added to each well and then incubated. After a second washing step, the bound enzymatic activity is detected by addition of tetramethylbenzidine (TMB) chromogen-substrate. Finally, the reaction is terminated with an acidic stop solution. The intensity of the reaction color is directly proportional to the concentration of antibodies to Trastuzumab in sample.

Warnings and Precautions

1. For professional use only.

2. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood. For further information (clinical background, test performance, automation protocols, alternative applications, literature, etc.) please refer to the local distributor.

3. In case of severe damage of the kit package please contact Matriksbiotek or your supplier in written form, latest one week after receiving the kit. Do not use damaged components in test runs, but keep safe for complaint related issues.

4. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.

5. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.

6. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details.

7. Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guidelines or regulations.

8. Avoid contact with Stop solution. It may cause skin irritations and burns.

9. Some reagents contain sodium azide (NaN_3) as preservatives. In case of contact with eyes or skin, flush immediately with water. NaN_3 may react with lead and copper plumbing to form explosive metal azides. When disposing reagents, flush with large volume of water to avoid azide build-up.

10. All reagents of this test kit containing human serum or plasma have been tested and were found negative for HIV I/II, HBsAg and HCV by FDA approved procedures. However, a presence of these or other infectious agents cannot be excluded absolutely and therefore reagents should be treated as potential biohazards in use and for disposal.

Storage and Stability

The kit is shipped at ambient temperature and should be stored at 2-8°C. Keep away from heat or direct sun light. The storage and stability of specimen and prepared reagents is stated in the corresponding chapters.

The strips of microtiter plate is stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2–8°C.

Specimen Collection and Storage

Serum, Plasma (EDTA, Heparin)*

The usual precautions for venipuncture should be observed. It is important to preserve the chemical integrity of a blood specimen from the moment it is collected until it is assayed. Do not use grossly hemolytic, icteric or grossly lipemic specimens. Samples appearing turbid should be centrifuged before testing to remove any particulate material.

Storage:	2-8°C	-20°C	Keep away from heat or direct sun light Avoid repeated freeze-thaw cycles
Stability:	7 d	6 mon	

*.Trastuzumab (Herclon®, Herceptin®) infusion camouflages/masks the presence of antibody to Trastuzumab in serum/plasma samples. Therefore, blood sampling time is critical for detection of trastuzumab. Matriks Biotek® Laboratories propose to obtain blood sample just before the infusion of Trastuzumab (Herclon®, Herceptin or at least 2 weeks after the infusion of Trastuzumab (Herclon®, Herceptin).

Materials Supplied

1 x 12 x 8	MTP	**Microtiter Plate** Break apart strips. Microtiter plate with 12 rows each of 8 wells coated with trastuzumab
6 x 1 mL	STND A-F	**ATT Standards A-E** 500; 250; 125; 62; 31; 0 ng/mL Ready to use. Used for construction of the standard curve. Contains antibody to trastuzumab, human serum and <0,1% NaN_3
1 x 50 mL	ASSAY BUF	**Assay Buffer** Blue colored. Ready to use. Contains proteins, RF blockers and <0.1% NaN_3
1 x 12 mL	CONFIRMATION REAGENT	**Confirmation Reagent** Ready to use. Contains optimized concentration of the trastuzumab, proteins, stabilizer, RF blockers and <0,1% NaN_3
1 x 12 mL	POD CONJ	**Peroxidase Conjugate** Red colored. Ready to use. Contains peroxidase (POD) conjugate, RF blockers, stabilizer and preservatives.
1 x 12 mL	TMB SUBS	**TMB Substrate Solution** Ready to use. Contains TMB
1 x 12 mL	TMB STOP	**TMB Stop Solution** Ready to use. 1N HCl.
1 x 50 mL	WASHBUF CONC	**Wash Buffer, Concentrate (20x)** Contains Buffer with Tween 20.
2 x 1	ADH FILM	**Adhesive Film** For covering of Microtiter Plate during incubation.
2 x 1	SLGP	**Semi-Log Graph Paper** For constructing standard curve and calculation of results.

Materials Required but not Supplied

1. Micropipettes (< 3% CV) and tips to deliver 5-1000 µL.
2. Bidistilled or deionised water
3. Calibrated measures.
4. Absorbent paper and timer.
5. Standard laboratory glass or plastic vials, cups, etc.
6. Wash bottle, automated or semi-automated microtiter plate washing system
7. Microtiter plate reader capable of reading absorbance at 450 nm (reference wavelength 650 nm is optional).

Procedure Notes

1. Any improper handling of samples or modification of the test procedure may influence the results. The indicated pipetting volumes, incubation times, temperatures and pretreatment steps have to be performed strictly according to the instructions. Use calibrated pipettes and devices only.
2. Once the test has been started, all steps should be completed without interruption. Make sure that required reagents, materials and devices are prepared ready at the appropriate time. Allow all reagents and specimens to reach room temperature (18-25 °C) and gently swirl each vial of liquid reagent and sample before use. Mix reagents without foaming.
3. Avoid contamination of reagents, pipettes and wells/tubes. Use new disposable plastic pipette tips for each reagent, standard or specimen. Do not interchange caps. Always cap not used vials. Do not reuse wells/tubes or reagents.
4. Use a pipetting scheme to verify an appropriate plate layout.
5. Incubation time affects results. All wells should be handled in the same order and time sequences. It is recommended to use an 8-channel Micropipettor for pipetting of solutions in all wells.
6. Microplate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or an automatic microplate washing system. Do not allow the wells to dry between incubations. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled precisely with Wash Buffer, and that there are no residues in the wells.

7. Humidity affects the coated wells/tubes. Do not open the pouch until it reaches room temperature. Unused wells/tubes should be returned immediately to the resealed pouch including the desiccant.

Pre-Test Setup Instructions

1. Preparation of Components

Dilute/ disolve	Component	with	Diluent	Relation	Remarks	Storage	Stability
10 mL	Wash Buffer*	Up to 200 mL	bidist. Water	1:20	Warm up at 37°C to dissolve crystals. Mix vigorously.	2-8 °C	1 w

***.** Prepare Wash Buffer before starting assay procedure.

2. Dilution of Standards and Samples (serum/plasma)*

Sample	To be diluted	With	Relation	Remarks
Serum/Plasma	1/10	Assay Buffer	1:10 – 1:100	For dilution 1:10 10µl Sample + 90µl Assay Buffer For dilution 1:100 5µl Sample + 495µl Assay Buffer

*. Patient samples with a concentration of ATT above the measuring range are to be rated as "> highest standard". The result must not be extrapolated. The patient sample in question should be diluted with Assay Buffer and then retested.

PREPRATION OF CONFIRMATION TEST MIXTURE

Sample	To be diluted	With	Relation	Remarks
Serum/Plasma	Initially no	Confirmation Reagent*	1:10	For dilution 1:10 10µL Sample + 90µL Confirmation Reagent

Test Procedure

1	**QUANTITATIVE ELISA TEST FORMAT** Pipette 100 µL of ready-to use **Standards, Samples and Confirmation test mixture** into the respective wells of microtiter plate. Wells A1: Standard A B1: Standard B C1: Standard C D1: Standard D E1: Standard E F1: Standard F G1 and on: Sample (Serum/Plasma)
2	Cover the plate with adhesive film. Briefly mix contents by gently shaking the plate. **Incubate 60 min** at room temperature (18-25°C).
3	Remove adhesive film. Discard incubation solution. Wash plate **3 times** each with **300 µL** of diluted **Wash Buffer**. Remove excess solution by tapping the inverted plate on a paper towel.
4	Pipette 100 µL of ready-to use **Peroxidase Conjugate** into each well.
5	Cover the plate with adhesive film. **Incubate 60 min** at room temperature (18- 25°C).
6	Remove adhesive film. Discard incubation solution. Wash plate **3 times** each with **300 µL** of diluted **Wash Buffer**. Remove excess solution by tapping the inverted plate on a paper towel.
7	Pipette **100 µL** of **TMB Substrate Solution** into each well.
8	**Incubate 20 min** (without adhesive foil.) at room temperature (18-25°C) in the dark.
9	Stop the substrate reaction by adding **100 µL** of **Stop Solution** into each well. Briefly mix contents by gently shaking the plate. Color changes from blue to yellow
10	Measure optical density with a photometer at **450/650 nm** within **30 min** after pipetting of the Stop Solution.

Confirmation Test

	CONFIRMATION TEST FOR POSITIVE SAMPLES
	Incubate positive patient samples and optimized confirmation reagent for **60 minutes in a microtube**. After the incubation proceed the test procedure from step one given above.

QUALITY CONTROL

The test results are only valid only if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable standards/laws. All standards must be found within the acceptable ranges as stated above. If the criteria are not met, the run is not valid and should be repeated. In case of any deviation the following technical issues should be proven: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.

Calculation & Interpretation of Results

QUANTITATIVE INTERPRETATION

1. Using the standards (500, 250, 125, 62, 31 ng/mL) disregarding zero standard, construct a standard curve by plottingthe OD450/650 nm for each of 4 standards on the vertical (Y-axis) axis versus the corresponding ATT concentration on the horizontal (X-axis) axis, thus creating a standard curve by 4 points obtained.

2. The concentration of the samples can be read directly from this standard curve. Using the absorbance value for each sample, determine the corresponding concentration of ATT from the standard curve. Find the absorbance value on the Y-axis and extend a horizontal line to the curve. At the point of intersection, extend a vertical line to the X-axis and read the ATT concentration for the unknown sample.

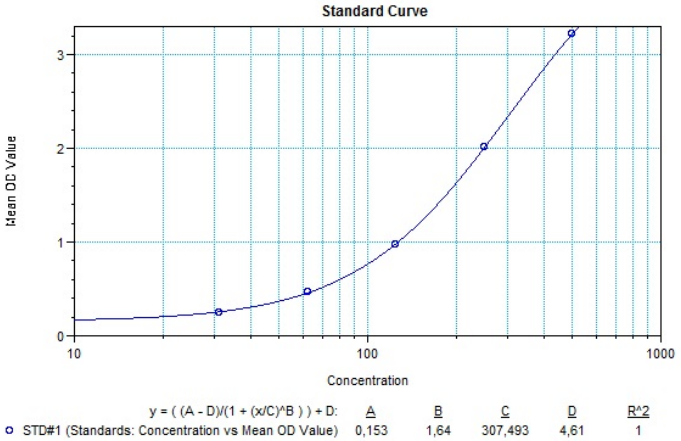
3. If computer data regation is going to be used, we recommend primarily "4 Parameter Logistic (4PL)" or secondly the "point-to-point calculation".

4. Any sample reading greater than the highest standard should be diluted appropriately with Assay Buffer and retested. Therefore, if the samples have been diluted, the concentration determined from the standard-curve must be multiplied by the dilution factor. Because the samples initially have been diluted to 1:10, the concentration determind from the standard curve must be multiplied by the dilution factor.

5. Automated method: Computer programs can also generally give a good fit.

Typical Calibration Curve

(Example. Do not use for calculation!)



Standard	Concentration (ng/mL)	Mean $OD_{450/650}$
A	500	3,225
B	250	2,010
C	125	0,976
D	62	0,467
E	31	0,251
F	0	0,045

Assay Characteristics

1. Specificity: Trastuzumab (Herclon®, Herceptin®) infusion camouflages/masks the presence of antibody to trastuzumab (ATT) in serum/plasma samples. Therefore, blood sampling time is critical for detection of ATT. It is convenient to obtain blood sample just before the infusion of trastuzumab (Herclon®, Herceptin®) or at least 2 weeks after the infusion of trastuzumab (Herclon®, Herceptin®).

2. **Sensitivity:**The lowest detectable level that can be distinguished from the zero standard is > 30 ng/mL.

3. **Precision of Kit:**
   Intra-assay CV: <20% for the ATT range of 31-500 ng/mL.
   Inter-assay CV: <20% for the ATT range of 31-500 ng/mL.

4. **Recovery:** Recovery rate was found to be higher than 96% when spiked using normal human serum samples with known concentrations.

Automation

Experiments have shown that the Matriks Biotek® S-ATT ELISA is also suitable to run on an automated ELISA processor.

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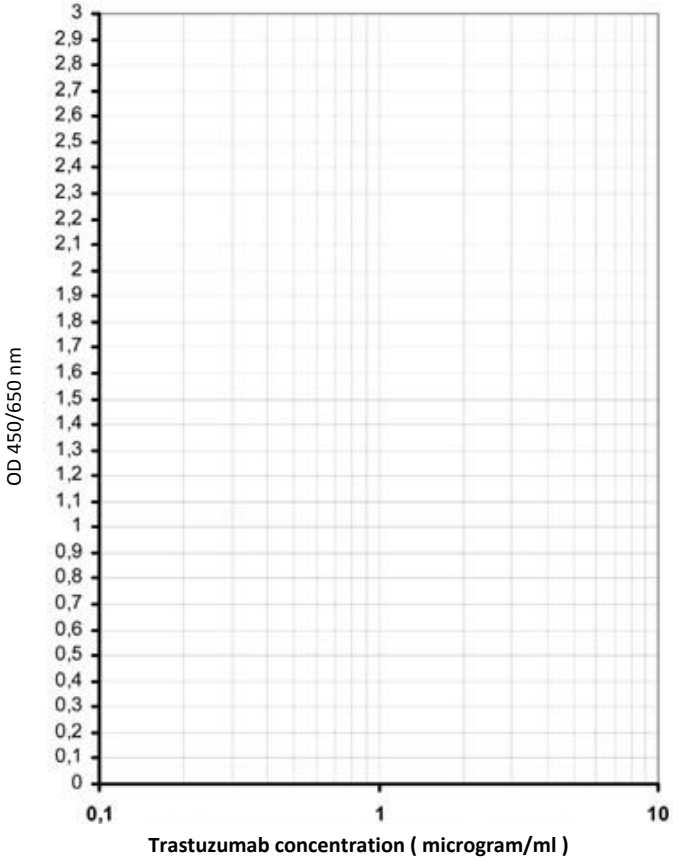
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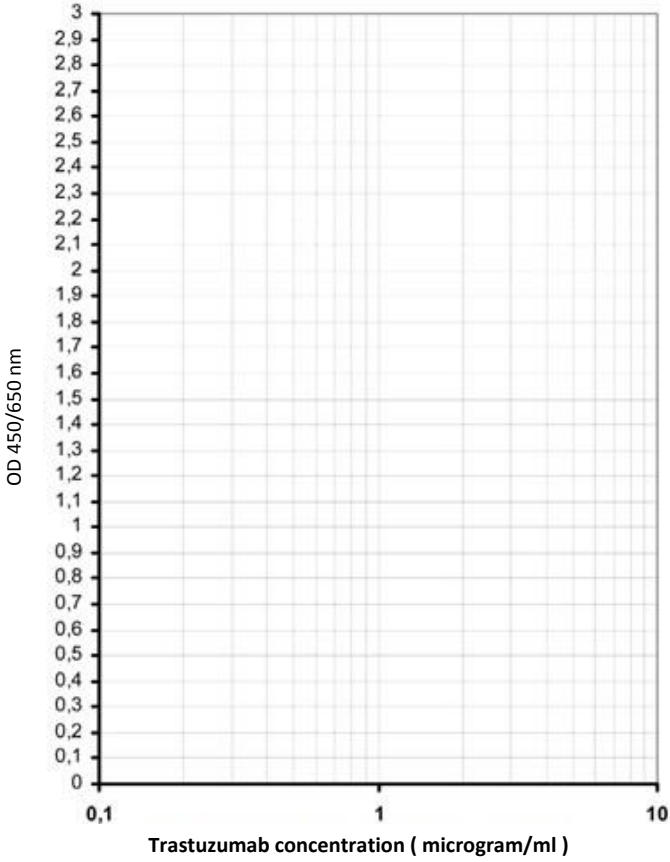
Trastuzumab concentration (microgram/ml)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Trastuzumab concentration (microgram/ml)