

ImmunoGuide®

Instructions for Use

Trastuzumab ELISA (mAb-based)

Enzyme immunoassay for the specific and quantitative determination of free Trastuzumab in serum and plasma

REF: IG-AB105



12X8



2-8°C



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1. INTENDED USE

Enzyme immunoassay for the specific and quantitative determination of free Trastuzumab in serum and plasma.

2. SUMMARY AND EXPLANATION

The drug Trastuzumab (trade names Herceptin[®], Herclone[®]) 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 blood concentrations throughout the dosing interval expected to be remaining above those considered necessary for anticancer activity. Furthermore, in a separate analysis, patients with the lowest Trastuzumab serum trough concentrations had the highest rate of disease progression and shortest overall survival.

According to the prescribing information, the steady-state mean trough (C_{min}) and maximum serum concentrations of Trastuzumab were reported to be 47.4 (5-115) $\mu\text{g/mL}$ and 179 (107-309) $\mu\text{g/mL}$ for the patients with primary tumor type of either breast cancer or metastatic breast cancer dosing 8mg/kg +6mg/kg q3w, 66.1 (14.9-142) $\mu\text{g/mL}$ and 109 (51.0-209) $\mu\text{g/mL}$ for the patients with breast cancer dosing 4mg/kg +2mg/kg qw,

In this context, identification of biomarkers for (non-)response and risk factors for adverse drug reactions that might be related to serum concentrations and maintaining the effective concentration of Trastuzumab in order to potentially avoid some side effects with a reliable method might be beneficial.

3. PRINCIPLE OF THE TEST

This ELISA assay is based on Trastuzumab-specific mouse monoclonal antibody (catcher Ab, *ImmunoGuide* clone VME-5A6b). Diluted standards and samples are incubated in the microtiter plate coated with IG-VME-5A6b mAb. After incubation, the wells are washed. A horseradish peroxidase (HRP)-conjugated anti-human IgG monoclonal antibody is added and binds to the Fc part of Trastuzumab. Following incubation, wells are washed, and the bound enzymatic activity is detected by addition of chromogen-substrate. The color developed is proportional to the amount of Trastuzumab in the sample or standard. Results of samples can be determined by using the standard curve. Binding of Trastuzumab to the solid phase, pre-coated with VME-5A6b, is inhibited by recombinant human HER2 protein in a concentration dependent manner. Therefore, the *ImmunoGuide* Trastuzumab ELISA (mAb-Based) measures the free form of Trastuzumab.

4. WARNINGS AND PRECAUTIONS

1. 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.
2. In case of severe damage of the kit package, please contact **AybayTech** or your supplier in writing, latest one week after receiving the kit. Do not use damaged components in test runs but keep safe for complaint related issues.
3. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
4. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.
5. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details.
6. Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guidelines or regulations.
7. Avoid contact with Stop solution. It may cause skin irritations and burns.
8. If any component of this kit contains human serum or plasma it is indicated and if so, it has been tested and were found to be negative for HIV I/II, HBsAg and HCV. However, the 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.
9. Some reagents contain preservatives. In case of contact with eyes or skin, flush immediately with water.

5. STORAGE AND STABILITY OF THE KIT

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

6. SPECIMEN COLLECTION, HANDLING 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 (Aliquots)	Keep away from heat or direct sun light Avoid repeated freeze-thaw cycles
Stability:	3 d	6 mon	

7. CONTENTS OF THE KIT

QUANTITY	COMPONENT
1 x 12 x 8	Microtiter ELISA Plate Break apart strips coated with anti-Trastuzumab monoclonal antibody.
5 x 0.5 mL	Trastuzumab Standards A-E, Concentrate (10X) 2000; 600; 200; 60; and 0 ng/mL Used for construction of the standard curve. Contains Trastuzumab, proteins, preservative and stabilizer.
1 x 12 mL	Assay Buffer Blue colored. Ready to use. Contains proteins and preservative..
1 x 60 mL	Dilution Buffer, Concentrate (5X) Contains orange dye, proteins and preservative.
1 x 12 mL	Enzyme Conjugate Red colored. Ready to use. Contains horseradish peroxidase(HRP)-conjugated anti-human IgG mouse monoclonal antibody, Proclin [®] and stabilizers.
1 x 12 mL	TMB Substrate Solution Ready to use. Contains 3,3',5,5'-Tetramethylbenzidine (TMB).
1 x 12 mL	Stop Solution Ready to use. 1 N Hydrochloric acid (HCl).
1 x 50 mL	Wash Buffer, Concentrate (20x) Contains buffer, Tween [®] 20 and Kathon [™] .
2 x 1	Adhesive Seal For sealing microtiter plate during incubation.

8. MATERIALS REQUIRED BUT NOT SUPPLIED

1. Micropipettes (< 3% CV) and tips to deliver 5-1000 μ L.
2. Bidistilled or deionised water and calibrated glasswares (e.g. flasks or cylinders).
3. Wash bottle, automated or semi-automated microtiter plate washing system.
4. Microtiter plate reader capable of reading absorbance at 450 nm (reference wavelength at 600-650 nm is optional).
5. Absorbent paper towels, standard laboratory glass or plastic vials, and a timer.

9. 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 pre-treatment 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 readily at the appropriate time. Allow all reagents and specimens to reach room temperature (20-25 °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 the caps of vials. Always cap not used vials. Do not reuse wells 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. Do not open the pouch until it reaches room temperature. Unused wells should be returned immediately to the resealed pouch including the desiccant.

10. PRE-TEST SETUP INSTRUCTIONS

10.1. Preparation of Components*

Dilute/ dissolve	Component		Diluent	Relation	Remarks	Storage	Stability
10 mL	Wash Buffer	up to 200 mL	Distilled Water	1:20	Warm up at 37°C to dissolve crystals. Mix vigorously.	2-8 °C	4 w
10 mL	Dilution Buffer	up to 50 mL	Distilled Water	1:5		2-8 °C	4 w

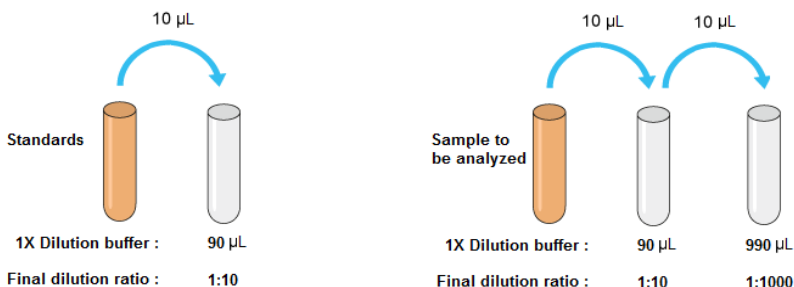
* Prepare Wash and Dilution Buffers before starting the assay procedure.

10.2. Dilution of Standards and Samples

The dilutions depicted below are examples of how to obtain final dilutions for standards and samples. Standards and samples should be properly diluted as homogenous mixture before starting the assay procedure. It is recommended mixing the standards and samples well to homogenous solution at each dilution step. We are recommending that each laboratory determines the best initial dilution for their samples in order to minimize retesting.

1. 10 μL of standard added to 90 μL of 1X dilution buffer, giving the total volume of 100 μL and a dilution of 1:10.
2. 10 μL of sample added to 90 μL of 1X dilution buffer, giving the total volume of 100 μL and a dilution of 1:10.
3. 10 μL of 1:10 diluted sample added to 990 μL of 1X dilution buffer, giving the total volume of 1000 μL and a dilution of 1:1000. This 2nd dilution should NOT be done with the standards.
4. Samples with a drug concentration above the measuring range should be rated as ">highest standard". The result should not be extrapolated. The sample in question should be further diluted with 1X Dilution Buffer and then retested.

Standard/Sample Dilution



11. TEST PROCEDURE

11.1. GENERAL REMARKS

- 11.1.1. Before performing the assay, samples and assay kit should be brought to room temperature (about 30 minutes beforehand) and ensure the homogeneity of the solution.
- 11.1.2. All Standards should be run with each series of unknown samples.
- 11.1.3. Standards should be subject to the same manipulations and incubation times as the samples being tested.
- 11.1.4. All steps of the test should be completed without interruption.
- 11.1.5. Use new disposable plastic pipette tips for each reagent, standard or specimen in order to avoid cross contamination.

11.1.6. The total pipetting time needed for dispensing all samples into the wells should not exceed 5 minutes. If this is difficult to achieve the samples should be pre-dispensed in a separate neutral polypropylene microplate and then transferred into the reaction ELISA plate by a multi channel pipette.

11.2. ASSAY PROCEDURE

1.	Pipette 100 μL of Assay Buffer into each of the wells to be used.
2.	Pipette 75 μL of each 1:10 Diluted Standard, and 1:1000 Diluted Samples into the respective wells of the microtiter plate. Bubble formation during the pipetting of standards and samples must be avoided. <u>Wells</u> A1: Standard A B1: Standard B C1: Standard C D1: Standard D E1: Standard E F1 and so on: Samples (Serum/Plasma)
3.	Cover the plate with adhesive seal. Shake plate carefully by tapping several times. Incubate the plate on a bench top for 60 min at room temperature (RT, 20-25°C).
4.	Remove adhesive seal. Aspirate or decant the incubation solution. Wash the plate 3 X 350 μL of Diluted Wash Buffer per well. Remove excess solution by tapping the inverted plate on a paper towel.
5.	Pipette 100 μL of Enzyme Conjugate (HRP-anti human IgG mAb) into each well.
6.	Cover plate with adhesive seal. Shake plate carefully by tapping several times. Incubate the plate on a bench top for 30 min at RT.
7.	Remove adhesive seal. Aspirate or decant the incubation solution. Wash the plate 3 X 350 μL of Diluted Wash Buffer per well. Remove excess solution by tapping the inverted plate on a paper towel.
8.	Pipette 100 μL of Ready-to-Use TMB Substrate Solution into each well.
9.	Incubate 10 min at RT. Avoid exposure to direct sunlight.
10.	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.
11.	Measure optical density (OD) with a photometer at 450 nm (Reference at OD620 nm is optional) within 15 min after pipetting the Stop Solution.

11. 3. QUALITY CONTROL

The test results are only valid 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/controls must be found within the acceptable ranges as stated above and/or label. 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 reviewed: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.

11. 4. CALCULATION OF RESULTS

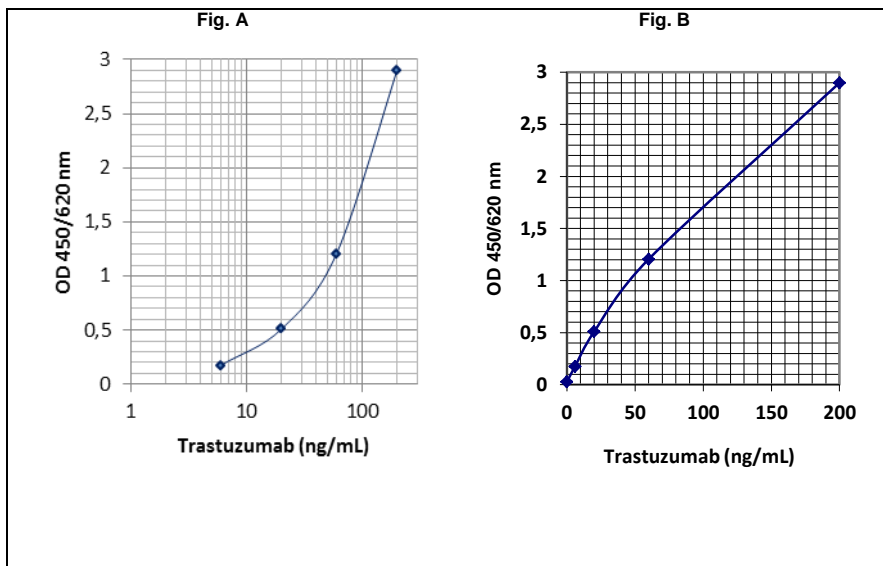
A standard curve should be constructed using the standard concentration (X-axis) versus the OD450 (or OD450/620) values (Y-axis). This can be done manually using graph paper or with a computer program. Concerning the data regression by computer, it is recommended to primarily use the "4 Parameter Logistic (4PL)" or alternatively the "point-to-point calculation". In case of manual plot there are 2 options: Semilog graph (see Fig. A) or linear graph (see Fig. B). Semilog graph paper is available at <http://www.papersnake.com/logarithmic/semilogarithmic/>.

The concentration of the samples can be read from this standard curve as follows. Using the absorbance value for each sample, determine the corresponding concentration of the drug from the standard curve. This value always has to be multiplied by the individual dilution factor (usually 1000). If any diluted sample is reading greater than the highest standard, it should be further diluted appropriately with 1X Dilution Buffer and retested. Also this second dilution has to be used for calculation of the final result. We are recommending that each laboratory determines the best initial dilution for their samples in order to minimize retesting.

Typical Calibration Curve

(All steps were performed at 23°C. Just an example. Do not use it for calculation!)

1:10 Diluted Standard	A	B	C	D	E
Concentration (ng/mL)	200	60	20	6	0
Mean OD _{450/620 nm}	2.899	1.205	0.508	0.175	0.026



12. ASSAY CHARACTERISTICS

12.1. SPECIFICITY

There is no cross reaction with any other proteins present in native human serum. A screening test was performed with 36 different native human sera. All produced OD_{450/620 nm} values less than the mean OD of standard D (6 ng/mL). No cross reaction was observed with sera spiked with the other therapeutic antibodies including Infliximab, Adalimumab, Etanercept, Rituximab, Tocilizumab, Omalizumab and Bevacizumab at concentrations up to 2mg/mL. All produced mean OD_{450/620 nm} values less than the mean OD of standard D (6 ng/mL). In addition, binding of Trastuzumab is inhibited by recombinant human HER2 in a concentration dependent manner. Therefore, the *ImmunoGuide* Trastuzumab ELISA (mAb-based) measures the biologically active free form of Trastuzumab, i.e. not pre-occupied by HER2.

12.2. SENSITIVITY

The lowest detectable level that can be clearly distinguished from the zero standard is 2 ng/mL (zero standard +2SD read from the curve) under the above-described conditions. Analytical sensitivity is 2 ng/mL, and corresponding to the detection limit (limit of quantification) of 2 µg/mL for undiluted clinical samples because the serum or plasma samples are instructed to be diluted at 1:1000 before starting the assay.

12.3. PRECISION

Intra-assay CV: <10%.

Inter-assay CV: <10%.

12.4. RECOVERY

Recovery rate was found to be >95% with native human serum and plasma samples when spiked with exogenous Trastuzumab.

13. AUTOMATION

The *ImmunoGuide* Trastuzumab ELISA (mAb-based) is suitable also for being used by an automated ELISA processor.

14. REFERENCES

1. Kang YK, Rha SY, Tassone P, Barriuso J, Yu R, Szado T, Garg A, Bang YJ. A phase IIa dose-finding and safety study of first-line pertuzumab in combination with trastuzumab, capecitabine and cisplatin in patients with HER2-positive advanced gastric cancer. *Br J Cancer*. 2014;111(4):660-6.
2. Sanford M. Subcutaneous trastuzumab: a review of its use in HER2-positive breast cancer. *Target Oncol*. 2014 Mar;9(1):85-94.
3. Ismael G, Hegg R, Muehlbauer S, Heinzmann D, Lum B, Kim SB, Pienkowski T, Lichinitser M, Semiglazov V, Melichar B, Jackisch C. Subcutaneous versus intravenous administration of (neo)adjuvant trastuzumab in patients with HER2-positive, clinical stage I-III breast cancer (HannaH study): a phase 3, open-label, multicentre, randomised trial. *Lancet Oncol*. 2012;13(9):869-78.
4. Leyland-Jones B, Colomer R, Trudeau ME, Wardley A, Latreille J, Cameron D, Cubedo R, Al-Sakaff N, Feyereislova A, Catalani O, Fukushima Y, Brewster M, Cortés J. Intensive loading dose of trastuzumab achieves higher-than-steady-state serum concentrations and is well tolerated. *J Clin Oncol*. 2010;28(6):960-6.
5. Baselga J, Carbonell X, Castañeda-Soto NJ, Clemens M, Green M, Harvey V, Morales S, Barton C, Ghahramani P. Phase II study of efficacy, safety, and pharmacokinetics of trastuzumab monotherapy administered on a 3-weekly schedule. *J Clin Oncol*. 2005;23(10):2162-71.
6. Hourcade-Potelleret F, Lemenuel-Diot A, McIntyre C, Brewster M, Lum B, Bittner B. Use of a population pharmacokinetic approach for the clinical development of a fixed-dose subcutaneous formulation of trastuzumab. *CPT Pharmacometrics Syst Pharmacol*. 2014 Jan 2;3:e87. doi: 10.1038/psp.2013.63.
7. Tokuda Y, Watanabe T, Omuro Y, Ando M, Katsumata N, Okumura A, Ohta M, Fujii H, Sasaki Y, Niwa T, Tajima T. Dose escalation and pharmacokinetic study of a humanized anti-HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *Br J Cancer*. 1999;81(8):1419-25.

8. Goldenberg MM. Trastuzumab, a recombinant DNA-derived humanized monoclonal antibody, a novel agent for the treatment of metastatic breast cancer. *Clin Ther.* 1999;21(2):309-18.
9. Arpino G, Michelotti A, Truini M, Montemurro F, Russo S, Palumbo R, Zamagni C, Latorre A, Bruzzese D, Riccardi F, De Laurentiis M, Beano A, Biganzoli L, Zaniboni A, Laudadio L, Malagoli M, Bilancia D, Schettini F, Giuliano M, Cazzaniga ME, De Placido S. Demographic, tumor and clinical features of clinical trials versus clinical practice patients with HER2-positive early breast cancer: results of a prospective study. *J Cancer Res Clin Oncol.* 2015 Aug 25. [Epub ahead of print]
10. Laboissiere RS, Buzelin MA, Balabram D, De Brot M, Nunes CB, Rocha RM, Cabral MM, Gobbi H. Association between HER2 status in gastric cancer and clinicopathological features: a retrospective study using whole-tissue sections. *BMC Gastroenterol.* 2015;15(1):157.
11. Kim YS, Sym SJ, Baek MY, Park I, Hong J, Ahn HK, Park J, Cho EK, Lee WK, Chung M, Kim HS, Lee JH, Shin DB. Low-dose capecitabine plus trastuzumab as first-line treatment in patients 75 years of age or older with HER2-positive advanced gastric cancer: a pilot study. *Cancer Chemother Pharmacol.* 2015 Oct 19. [Epub ahead of print]
12. Martin-Castillo B, Lopez-Bonet E, Cuyàs E, Viñas G, Pernas S, Dorca J, Menendez JA. Cancer stem cell-driven efficacy of trastuzumab (Herceptin): towards a reclassification of clinically HER2-positive breast carcinomas. *Oncotarget.* 2015;6(32):32317-38.
13. Xu W, Bi Y, Zhang J, Kong J, Jiang H, Tian M, Li K, Wang B, Chen C, Song F, Pan X, Shi B, Kong X, Gu J, Cai X, Li Z. Synergistic antitumor efficacy against the EGFRvIII+HER2+ breast cancers by combining trastuzumab with anti-EGFRvIII antibody CH12. *Oncotarget.* 2015 Oct 14. doi: 10.18632/oncotarget.6111. [Epub ahead of print]
14. Yeo B, Kotsori K, Mohammed K, Walsh G, Smith IE. Long-term outcome of HER2 positive metastatic breast cancer patients treated with first-line trastuzumab. *Breast.* 2015 Oct 8. pii: S0960-9776(15)00209-X. doi: 10.1016/j.breast.2015.09.008.
15. D'Avino C, Paciello R, Riccio G, Coppola C, Laccetti P, Maurea N, Raines RT, De Lorenzo C. Effects of a second-generation human anti-ErbB2 ImmunoRNase on trastuzumab-resistant tumors and cardiac cells. *Protein Eng Des Sel.* 2014;27(3):83-8.
16. Clay TM, Osada T, Hartman ZC, Hobeika A, Devi G, Morse MA, Lyerly HK. Polyclonal immune responses to antigens associated with cancer signaling pathways and new strategies to enhance cancer vaccines. *Immunol Res.* 2011;49(1-3):235-47
17. Wong JY, Raubitschek A, Yamauchi D, Williams LE, Wu AM, Yazaki P, Shively JE, Colcher D, Somlo G. A pretherapy biodistribution and dosimetry study of indium-111-radiolabeled trastuzumab in patients with human epidermal growth factor receptor 2-overexpressing breast cancer. *Cancer Biother Radiopharm.* 2010;25(4):387-94.
18. Disis ML, Wallace DR, Gooley TA, Dang Y, Slota M, Lu H, Coveler AL, Childs JS, Higgins DM, Fintak PA, dela Rosa C, Tietje K, Link J, Waisman J, Salazar LG. Concurrent trastuzumab and HER2/neu-specific vaccination in patients with metastatic breast cancer. *J Clin Oncol.* 2009;27(28):4685-92.
19. Alvarez-Rueda N, Ladjemi MZ, Béhar G, Corgnac S, Pugnère M, Roquet F, Bascoul-Mollevi C, Baty D, Pèlerin A, Navarro-Teulon I. A llama single domain anti-idiotypic antibody mimicking HER2 as a vaccine: Immunogenicity and efficacy. *Vaccine.* 2009;27(35):4826-33.
20. Morse MA, Hobeika A, Osada T, Niedzwiecki D, Marcom PK, Blackwell KL, Anders C, Devi GR, Lyerly HK, Clay TM. Long term disease-free survival and T cell and antibody responses in women with high-risk Her2+ breast cancer following vaccination against Her2. *J Transl Med.* 2007 Sep 6;5:42.