

ImmunoGuide®

Instructions for Use

Antibody to Trastuzumab ELISA

Enzyme immunoassay for the semi-quantitative determination
of free antibodies to Trastuzumab in serum and plasma

REF: IG-BB105



12X8



2-8°C



IVD

TANI Medikal Ltd. Sti.
Turan Gunes Bulvari Korman Sitesi 51/J 06550 Ankara
Tel : +90 312 440 90 20
Fax : +90 312 440 35 15
e-mail: info@immunoguide.com
www.immunoguide.com

<u>Contents</u>	<u>Page</u>
1. Intended Use	2
2. Summary and Explanation	2
3. Principle of the Test	2
4. Warnings and Precautions	2
5. Storage and Stability of the Kit	3
6. Specimen Collection, Handling and Storage	3
7. Contents of the Kit	4
8. Materials Required but not Supplied	4
9. Procedure Notes	4
10. Pre-Test Setup Instructions	5
10.1. Preparation of Components	5
10.2. Dilution of Samples	5
11. Test Procedure	5
11.1. General Remarks	5
11.2. Assay Procedure	6
11.3. Quality Control	6
11.4. Calculation of Results	7
12. Assay characteristics	8
12.1. Specificity	8
12.2. Sensitivity	9
12.3. Precision	10
13. Automation	10
14. References	11

1. INTENDED USE

Enzyme immunoassay for the semi-quantitative determination of free antibodies to 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.

According to the prescribing information, the use of Trastuzumab might be associated to the development of anti-Trastuzumab antibodies in various percentages of patients during therapy with the drug. The ImmunoGuide 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.

3. PRINCIPLE OF THE TEST

This *ImmunoGuide* anti-drug antibody(ies) (ADA) kit is a bridging type ELISA for the determination of free antibodies against the drug Trastuzumab in serum and plasma samples. During the first incubation period, ADA in serum or plasma samples are captured by the drug coated on the microtiter wells. After washing away the unbound components from samples, a peroxidase-labelled drug conjugate is added and then incubated. ADA, if present in sample, will make a bridge, with its identical Fab arms, between the drug coated on the well and the other drug molecule labelled with peroxidase. After a second washing step, the bound enzymatic activity is detected by addition of tetramethylbenzidine (TMB) chromogen-substrate. Finally, the reaction is terminated with stop solution. The positive reaction is expected to be related to the presence of ADA in the sample.

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 *Tani Medikal* 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 have 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 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

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	

* Drug administration/infusion may camouflage/mask the presence of anti-drug antibodies (ADA) in serum/plasma samples. Therefore, blood sampling time is also critical for detection of ADA. It is proposed to obtain blood sample just before administration of the drug.

7. CONTENTS OF THE KIT

QUANTITY	COMPONENT
1 x 12 x 8	Microtiter Plate Break apart strips pre-coated with the drug Trastuzumab.
1 x 1 mL	Negative Control Ready to use. Contains serum and <15 mM NaN ₃ .
1 x 1 mL	Positive Control Ready to use. Contains Trastuzumab-specific antibody and <15 mM NaN ₃ .
1 x 15 mL	Dilution Buffer Ready to use. Contains proteins and <15 mM NaN ₃ .
1 x 12 mL	Assay Buffer Blue colored. Ready to use. Contains proteins and <15 mM NaN ₃ .
1 x 12 mL	Enzyme Conjugate Red colored. Ready to use. Contains horseradish peroxidase(HRP)-conjugated Trastuzumab, 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 µL.
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

* Prepare Wash Buffer before starting the assay procedure.

10.2. Dilution of Samples^{a,b}

Sample	To be diluted	With	Remarks
Serum/ Plasma	1:10	Dilution Buffer	For dilution at 1:10; 10 µL Sample + 90 µL Dilution Buffer

^a Negative and Positive Controls are ready-to-use and should NOT be diluted with the dilution buffer.

^b Incubate 1:10 diluted serum/plasma samples for 15 min at room temperature (20-25°C) before pipetting 50 µL of each 1:10 diluted sample per well for analysis.

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.

11.2. ASSAY PROCEDURE

1.	Pipette 100 µL of Assay Buffer into each of the wells to be used.
2.	<p>Pipette 50 µL of each Ready-to-Use Negative Control, Ready-to-Use Positive Control, and 1:10 Diluted Samples (as described in section 10.2) into the respective wells of the microtiter plate. Bubble formation during the pipetting of standards and samples must be avoided.</p> <p style="margin-left: 40px;"><u>Wells</u></p> <p style="margin-left: 40px;">A1: Negative Control</p> <p style="margin-left: 40px;">B1: Negative Control</p> <p style="margin-left: 40px;">C1: Positive Control</p> <p style="margin-left: 40px;">D1 and so on: Diluted samples (Serum/Plasma)</p>
3.	Cover the plate with adhesive seal. Shake plate carefully by tapping several times. Incubate the plate on 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 300 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-drug) into each well.
6.	Cover the plate with adhesive seal. Shake plate carefully by tapping several times. Incubate the plate on bench top for 60 min at room temperature (RT, 20-25°C).
7.	Remove adhesive seal. Aspirate or decant the incubation solution. Wash the plate 3 X 300 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 15 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 below 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

For the run to be valid, the OD_{450 nm} of the Positive Control should be ≥ 0.500 and the OD_{450 nm} of each Negative Control should be ≤ 0.150 . If not, improper technique or reagent deterioration may be suspected and the run should be repeated.

The results are evaluated by dividing each individual OD results by the Cut-off OD value. The results are expressed in arbitrary units (AU/mL).

Cut-off value = 2 x the mean OD_{450 nm} of Negative Control = 3 AU/mL

80 different naive samples have been measured for estimating the cut-off value. In order to avoid a sample from being reported as false positive the cut-off value was determined by 2 times of the mean of Negative Control. All 80 screened naive samples show ODs lower than the cut-off value.

Samples which have an equal and higher OD than the cut-off value are considered to be positive.

Range	Interpretation
\geq Cut-off (3 AU/mL)	POSITIVE
$<$ Cut-off (3 AU/mL)	NEGATIVE

An example for semi-quantitative calculation for a positive sample

OD of patient's sample = 0.402

The mean OD of Negative Control = 0.067

Cut-off value (3 AU/mL) = $2 \times 0.067 = 0.134$

Result for the patient's sample = $0.402/0.134 \times 3 \text{ AU/mL} = 9 \text{ AU/mL}$

The results themselves should not be the only reason for any therapeutical consequences. They have to be correlated to other clinical observations. In addition, the positive reaction should be clarified whether it is true or false positive following the confirmatory assay as mentioned in section "12.1. Specificity" below.

12. ASSAY CHARACTERISTICS

12.1. SPECIFICITY

The detection of ADA formation is highly dependent on the sensitivity and specificity of the assay used.

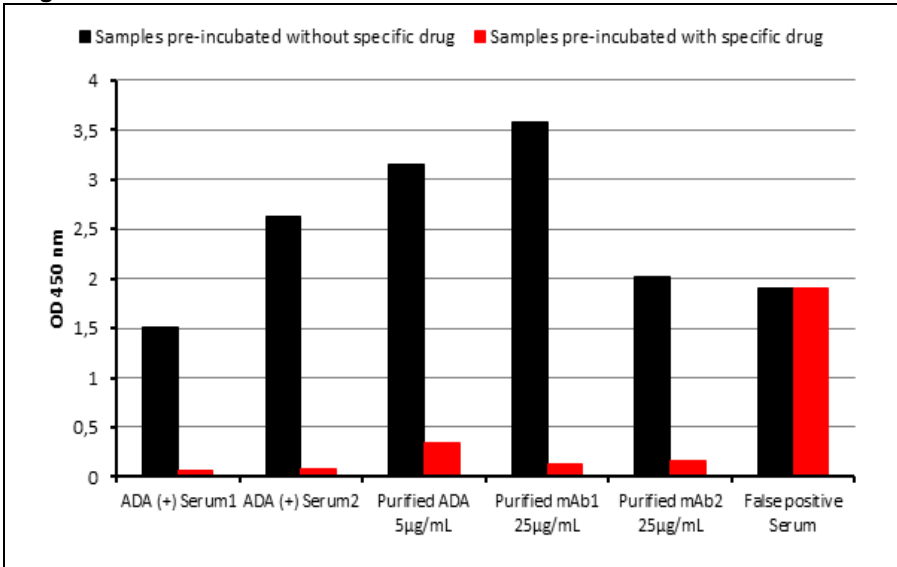
The initial screening assay should be sensitive to low and high-affinity ADA. Endogenous and exogenous components in serum or plasma may influence assay results. Measuring immune responses to therapeutic protein products that possess Ig tails, such as mAb and Fc-fusion proteins, may be particularly difficult when RF is present in serum or plasma. RF is generally an IgM antibody that recognizes IgG, although other Ig specificities have been noted. Therefore, there is frequently a need to dilute patient samples and to make approach for minimizing interference from RF to maintain a reasonable ability to detect ADA. However, dilution and/or addition of RF-blocking reagents may not solve all potential interference related with matrix components contributing to non-specific signal in samples.

If the serum/plasma sample of a patient, a candidate for a specific monoclonal drug treatment, is negative at the time before starting drug therapy and become positive during treatment, it could be concluded that the induction of the specific ADA is suspected in this individual. As shown in Figure 1, the true positive reaction of the sample is inhibited by pre-incubation of the sample with the specific drug itself (samples are spiked with certain amount of drug). However, inhibition was not observed in false positive reaction related with other components in sample (Figure 1).

Based on the approach used in calculating the assay cut-off value of anti-drug antibody (ADA) ELISAs, approximately 1-5% of tested samples are expected to generate false-positive ADA response during initial screening analysis. Therefore, further confirmation of the specificity of the ADA activity in the samples, identified as positive, is also recommended by recent publications. In addition, the FDA released a guidance paper on immunogenicity testing of therapeutic proteins: (<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM192750.pdf>)

In order to confirm the specificity of the ADA signal, a Confirmatory Assay, (competitive drug inhibition test), is proposed by ImmunoGuide. The Confirmation Test is performed with the Confirmatory Reagent. (coded IG-CR105) This Confirmatory Reagent is available as a separate product and can be obtained from ImmunoGuide upon request

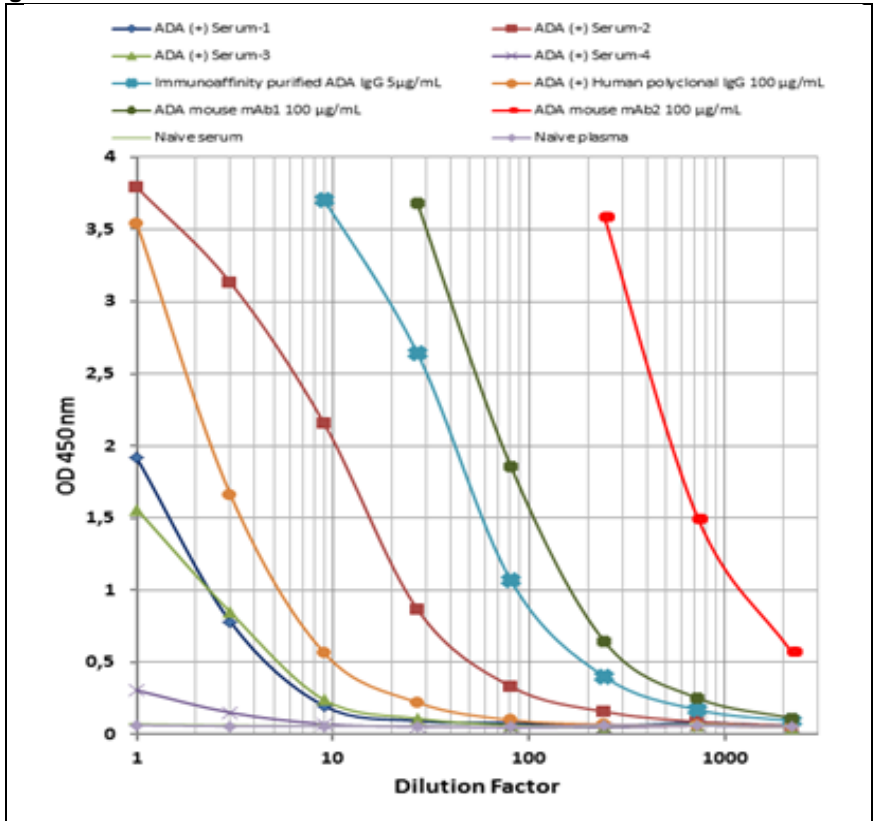
Figure 1.



12.2. SENSITIVITY

Generally, the sensitivity of the ADA assay is calculated as the lowest concentration of the positive control that can consistently generate a positive signal. However, as shown in Figure 2 below, data obtained at the *ImmunoGuide* Laboratory, the assay sensitivity also differs significantly depending on the high vs low affinity antibody used for the construction of the standard curve in the assay system. For example, when the immunoaffinity-purified ADA was exogenously added into naive serum, it was observed that the lowest detectable level that can be clearly distinguished from the negative control value is 7ng/mL by the *ImmunoGuide* bridging ELISA. It is much lower when mAb2 is used instead. Therefore, it is clear that the assay sensitivity will be highly dependent on the properties of a particular positive control to be used in the assay (Figure 2).

Figure 2.



2.3. PRECISION OF THE KIT

Intra-assay CV: <10%.

Inter-assay CV: <10%

13. AUTOMATION

The *ImmunoGuide* Anti Drug Antibody (ADA) ELISA kits are 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, Cognac 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.