Version 01
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Product Manual

Trastuzumab Immunogenicity Kit

(Bridging ELISA)

Cat. No. L01007

For Research Use Only. Not for Use in Diagnostic Procedures.

The operator should read technical manual carefully before using this product.

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Contents

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I. DESCRIPTION

Trastuzumab, also known as Herceptin, is a human epidermal growth factor receptor 2 (HER2) inhibitor for the treatment of breast cancer. Trastuzumab is a recombinant humanized IgG1 kappa monoclonal antibody. It binds to the extracellular domain of the HER2 protein on the on the surface of HER2-positive tumor cells with high affinity. Trastuzumab can suppress the cells growth and proliferation by blocking the ability of the cancer cells to receive chemical signals.

Antibody drugs have the possibility to generate anti-drug antibodies (ADAs), which may alter drug clearance and neutralize target binding, causing reduction of drug efficacy. The immunogenicity of antibody drugs may cause anaphylaxis, infusion reactions, and immune complex disorders. Therefore, it is important to assess the presence and impact of Trastuzumab ADAs on exposure, safety, and efficacy.

GenScript Trastuzumab Immunogenicity Kit (Bridging ELISA) is designed for detection of anti-Trastuzumab antibody in serum and plasma samples. This kit utilizes a validated bridging immunoassay method based on the FDA, EMA and NMPA Immunogenicity Guidelines. Through rigorous validation studies, the kit has been demonstrated high sensitivity, specificity, and is free from matrix effects. It is an ideal tool for the analysis of ADA against Trastuzumab.

II. ASSAY PRINCIPLE

The GenScript Trastuzumab Immunogenicity ELISA Kit is a bridging immunoassay that utilizes a microplate coated with the Trastuzumab. When standards or samples are added to the capture plate, anti-Trastuzumab antibodies can be captured on the plate. The biotin-conjugated Trastuzumab is then added to interact with the anti-Trastuzumab antibodies bound on the plate. After a washing step, horseradish peroxidase conjugated streptavidin (Streptavidin-HRP) is added and to react with the 3,3',5,5'-Tetramethylbenzidine solution (TMB Solution) to develop a blue product in the solution. The reaction is stopped by adding stop solution, which turns the color yellow and the absorbance can be read at 450 nm by a microplate reader. The intensity of the reaction color is directly proportional to the concentration of antibodies to Trastuzumab in samples.

III. ANALYTICAL CHARACTERISTICS

Features	Specifications
Sensitivity	0.78 ng/mL
Detection Range	0.78-50 ng/mL
Intra-Assay	CV≤10%
Inter-Assay	CV≤15%
Minimum required dilution (MRD)	1:5, validated non-human primate plasma
Hook Effect	Not observed at 3,200 ng/mL of anti-Trastuzumab antibodies
Conveniency	All reagents and buffers for the test are provided and the test can be completed within 2 hours

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IV. KIT CONTENTS

The kit provides the following reagents and solutions for the quantitative measurement of anti-Trastuzumab antibodies in biological matrices.

Table 1. Components of the kit

Component	Quantity/Size	Part No.
Capture Plate	1 plate	R1-80
Standard Stock	1 vial (50 µL)	R1-10
Biotin Trastuzumab	1 bottle (12 mL)	R1-20
Streptavidin-HRP	1 bottle (12 mL)	R1-30
Sample Dilution Buffer	1 bottle (60 mL)	R1-60
20× Wash Solution	1 bottle (60 mL)	R1-70
Stop Solution	1 bottle (6 mL)	A1-50
TMB Solution	1 bottle (12 mL)	A1-40
Plate Sealer	2 pieces	N/A

- Capture Plate: 96 well microplates (8 wells x 12 strips); 12 strips are configured in plate; plate is sealed in a foil pouch with a desiccant.
- Standard Stock contains 100 µg/mL of anti-Trastuzumab antibody.

V. STORAGE

The unopened kit is stable for at least 12 months from the date of manufacture at 2°C to 8°C, and the opened kit is stable for up to 1 month from the date of opening at 2°C to 8°C.

VI. REAGENTS/EQUIPMENT NEEDED BUT NOT SUPPLIED

- Fresh matrix (normal serum or plasma from human or non-human primate)
- Microplate reader capable of measurement at 450 nm
- Data analysis and graphing software. It is recommended to use software which can generate a four-parameter logistic (4-PL) curve-fit
- Automated microplate washer
- Deionized or distilled water
- Graduated cylinder
- Plastic container
- Tubes to aliquot and dilute samples
- 10 µL, 200 µL, and 1000 µL precision pipettes and pipette tips
- Multichannel pipettes
- Disposable reagent reservoir

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- Absorbent paper
- Laboratory timer
- Refrigerator
- Centrifuge
- 25 ± 2 ºC incubator
- Rotary shaker
- Vortex Mixer
- Absorbent Paper

VII. PRECAUTIONS

1. All reagents containing human material should be handled as potentially infectious. Operators should wear gloves and protective clothing when handling any patient sera or serum based products.
2. Reagents that contain preservatives may be toxic if ingested, inhaled, or spilled on the skin.
3. Avoid contact of skin, eyes, or clothing with Stop Solution or TMB Substrate. Keep the container tightly closed. In case of an accident, please seek medical advice immediately.
4. Do not use the kit if there is any visible damage to the packaging or kit contents.
5. Do not mix components from different batches. Do not mix with components from other manufacturers.
6. Do not use reagents beyond the stated expiry date.
7. All reagents must be equilibrated to room temperature (20°-25°C) before running the assay. Only take an appropriate amount of reagents at once. Do not put unused reagents back into the vials as reagent contaminations may occur.
8. Before opening the Standard Stock, quickly span the vial to ensure that all the liquid has collected at the bottom and prevent the liquid from splashing when opening the lid.
9. Use only distilled or deionized water and clean glassware.
10. Do not let wells dry during the test, add reagents immediately after completing the washing steps.

VIII. SPECIMEN COLLECTION AND STORAGE

1. The handling and storage information provided here is intended to be used as a general guideline. Sample stability has not been evaluated. When samples need to be stored for a long time, users need to evaluate the stability of the samples. It is the responsibility of the individual laboratory to use all available references and/or its own studies when establishing alternate

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stability criteria that meet their needs.

2. Store specimens at -20°C or lower if not tested immediately. Avoid repeated freeze-thaw cycles.

IX. PROTOCOL

- **Reagent Preparation**

All reagents must be equilibrated to room temperature before use (20°C-25°C). All samples and reagents should be vortexed before use. Store all reagents back in the refrigerator promptly after use.

1× Wash Solution: Dilute the 20× Wash Solution with deionized or distilled water with a volume ratio of 1:19. For example, dilute 40 mL of 20× Wash Solution with 760 mL of deionized or distilled water to make 800 mL of 1× Wash Solution. Store the solution at 2°C to 8°C when not in use.

Note: If any precipitate is found in the 20× Wash Solution, incubate the bottle in a water bath (up to 50°C) with occasional mixing until all the precipitate is dissolved.

Calibration Standard Preparation: Calibration standards should be prepared with a fresh matrix (The reagents are not provided in the kit) to generate eight Trastuzumab antibody concentrations: fresh matrix (NC), 0.78, 1.56, 3.125, 6.25, 12.5, 25, and 50 ng/mL. Preparation of a whole set of standards is recommended as table 2. S-Int1 preparation is described below as an example.

Note: NC is Negative Control.

S-Int1 Preparation: Vortex and Centrifuge Standard Stock for several seconds. Dilute Standard Stock with a fresh matrix with a volume ratio of 1:100. For example, add 5 µL of Standard Stock to 495 µL of Matrix and mix it well to make 500 µL of S-Int1.

Table 2. Recommended standard preparation

Standard ID	Dilution Factor	Source	Source Volume (µL)	Matrix Volume (µL)	Final Volume (µL)	Final Conc. (ng/mL)
S-Int1	100	Standard Stock	5	495	500	1000
Std1	20	S-Int1	10	190	200	50
Std2	2	Std1	100	100	200	25
Std3	2	Std2	100	100	200	12.5
Std4	2	Std3	100	100	200	6.25
Std5	2	Std4	100	100	200	3.125
Std6	2	Std5	100	100	200	1.56
Std7	2	Std6	100	100	200	0.78
NC	0	/	/	100	100	N/A

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- **Capture Plate Preparation**

1. It is recommended that all standards and samples be prepared in duplicate at least. Table 3 is an example for setup of Trastuzumab standards and samples.
2. Count the strips according to the number of test samples and install the strips. Make sure the strips are tightly snapped into the plate frame.
3. Leave the unused strips in the foil pouch and store at 2°C to 8°C. The strips must be stored in the closed foil pouch to prevent moisture from damaging the Capture Plate.

Table 3. Setup of standards and samples on the Capture Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std1	Std1	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	Std2	Std2	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	Std3	Std3	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	Std4	Std4	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	Std5	Std5	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	Std6	Std6	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	Std7	Std7	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	NC	NC	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

S： Sample number

- **Test Procedure**

Detection Antibody Incubation

1. Dilute standards and samples with Sample Dilution Buffer with a volume ratio of 1:5.

   Note: Standards are working solutions that have been diluted in the fresh matrix, see PROTOCOL. Reagent Preparation for step details.

2. Add 100 µL of the standard solutions or samples to the corresponding wells in the Capture Plate, cover the plate with Plate Sealer and incubate at 25°C for 60 minutes.
3. Remove the Plate Sealer and wash the plate with 260 µL of 1× Wash Solution four times, tap the inverted plate onto absorbent paper to remove residual liquid in the wells after the washing steps.
4. Add 100 µL of Biotin Trastuzumab to all the testing wells, cover the plate with Plate Sealer and incubate at 25°C for 30 minutes.
5. Remove the Plate Sealer and wash the plate with 260 µL of 1× Wash Solution four times, tap the inverted plate onto absorbent paper to remove residual liquid in the wells after the washing steps.

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Enzyme Conjugate Incubation

6. Add 100 µL of Streptavidin-HRP to all the testing wells.
7. Cover the Plate with Plate Sealer and incubate at 25°C for 10 minutes.
8. Remove the Plate Sealer and wash the plate with 260 µL of 1× Wash Solution four times, tap the inverted plate onto absorbent paper to remove residual liquid in the wells after the washing steps.

Absorbance Measurement and Calculation

9. Add 100 µL of TMB Solution to each well and incubate the plate in the dark at 25°C for 15 minutes (start timing after the addition of TMB Solution to the first well).

   Note: TMB incubation time could extend to 20 minutes based on test signals.

10. Add 50 µL of Stop Solution to each well to stop the reaction.
11. Read the absorbance in the microplate reader at 450 nm as a reference filter.
12. Plot the standard curve with the anti-Trastuzumab antibody concentration (ng/mL) on the x-axis and the corresponding mean absorbance value on the y-axis.
13. Using a 4-parameter logistic curve fitting program, calculate the best-fitting linear line through the points of the standard curve.

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X. ASSAY PROCEDURE SUMMARY

1	• Prepare 1× Wash Solution and Capture Plate. • Dilute Standard Stock with **fresh matrix** to generate calibration standards.
2	• Dilute the test samples and a set of standards with **Sample Dilution Buffer** with a volume ratio of 1:5.
2	• Add 100 µL of the standard solutions or samples to the corresponding wells, Incubate at 25°C for 60 minutes .
3	• Wash the plate with 260 µL of 1× Wash Solution per well four times.
4	• Add 100 µL of the Biotin Pertuzumab to the well and mix the wells. Incubate at 25°C for 30 minutes.
5	• Wash the plate with 260 µL of 1× Wash Solution per well four times.
6	• Add 100 µL of the Streptavidin-HRP and incubate at 25°C for 10 minutes.
7	• Wash the plate with 260 µL of 1× Wash Solution per well four times.
8	• Add 100 µL of TMB Solution and incubate the plate in dark at 25°C for 15 minutes.
9	• Add 50 µL of Stop Solution to each well to stop the reaction.
10	• Read the plate immediately.

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XI. ANALYTICAL PERFORMANCE

- **Linearity and Sensitivity**

A set of anti-Trastuzumab antibody calibration standards were freshly prepared and analyzed. Standard curves were constructed using a four-parameter logistic curve. The typical dynamic range of the kit is 0.78-50 ng/mL (Table 4). The sensitivity of the kit is 0.78 ng/mL.

Table 4. Sample data for the standard curve

Standard (ng/mL)	Absorbance Value (OD 450 nm)			CV%
	Duplicate 1	Duplicate 2	Average	
50	2.40	2.34	2.37	2
25	1.41	1.36	1.39	2
12.5	0.72	0.66	0.69	6
6.25	0.37	0.37	0.37	0
3.125	0.21	0.21	0.21	1
1.56	0.12	0.13	0.12	2
0.78	0.09	0.09	0.09	0
N/A	0.05	0.05	0.05	1

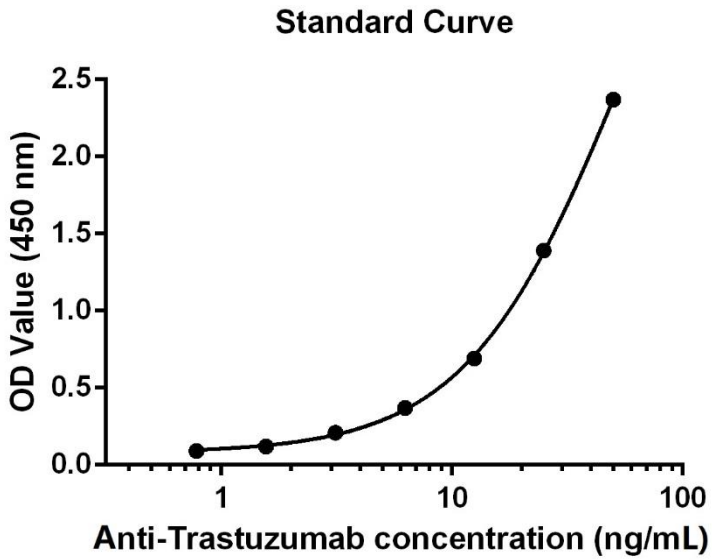

Figure 1: Trastuzumab Immunogenicity Kit (Bridging ELISA) standard curve.

- **Selectivity**

15 samples diluted with cynomolgus monkey serum from 15 different individuals were evaluated for precision and OD450 nm values to analyze the selectivity of the anti-Trastuzumab antibody assay at low concentrations of 1.5 ng/mL and at the 0 concentration (Blank) level.

The results showed that all low concentration samples diluted by serum from different individuals read close to the samples diluted by the assay matrix, and all OD450 nm values of

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low concentration samples were significantly different from those of blank samples (Table 5).

Table 5. Selectivity analysis of the kit

Dilution Buffer	Low Concentration Sample-Selectivity		Blank-Selectivity	
	Average Value (OD 450 nm)	CV%	Average Value (OD 450 nm)	CV%
Assay matrix	0.12	4.3	0.05	3.9
Individual #1	0.12	0.7	0.05	11.5
Individual #2	0.11	4.2	0.05	0.0
Individual #3	0.12	2.3	0.06	10.2
Individual #4	0.11	2.2	0.05	3.2
Individual #5	0.12	2.8	0.04	0.2
Individual #6	0.13	11.0	0.04	0.8
Individual #7	0.13	4.0	0.05	9.3
Individual #8	0.13	4.0	0.04	2.5
Individual #9	0.12	0.9	0.05	10.7
Individual #10	0.11	1.7	0.05	0.0
Individual #11	0.11	0.8	0.05	7.7
Individual #12	0.11	1.4	0.05	10.1
Individual #13	0.11	1.4	0.05	10.2
Individual #14	0.11	1.6	0.05	13.4
Individual #15	0.11	2.8	0.05	6.4

- **Precision and Accuracy**

Anti-Trastuzumab antibody at three concentrations (P1 of 50 ng/mL, P2 of 7.5 ng/mL and P3 of 2.5 ng/mL) were measured for intra-and inter-assay precision and accuracy (Table 6).

Table 6. Intra-assay and inter-assay precision and accuracy of the kit

Samples	Anti-Trastuzumab (ng/mL)	Intra-assay (n=10)			Inter-assay (n=10×3 Batches)		
		Measured Anti-Trastuzumab (ng/mL)	CV %	Accuracy %	Measured Anti-Trastuzumab (ng/mL)	CV %	Accuracy %
P1	50	45.02	6.5	90.0	44.95	4.5	89.9
P2	7.5	6.61	3.5	88.1	6.91	4.2	92.1
P3	2.5	2.27	8.0	102.9	2.38	5.1	95.2

- **MRD (minimal required dilution)**

The MRD is the minimum dilution necessary for the detection of Anti-Trastuzumab antibody in biological matrix with least interference. The MRD yields a signal close to that of the assay diluent and allows for the highest signal-to-noise ratio (S/N). Serum samples from cynomolgus monkey were serially diluted to determine the MRD of the kit, and the test result suggested that MRD was as 1:5 (Figure 2).

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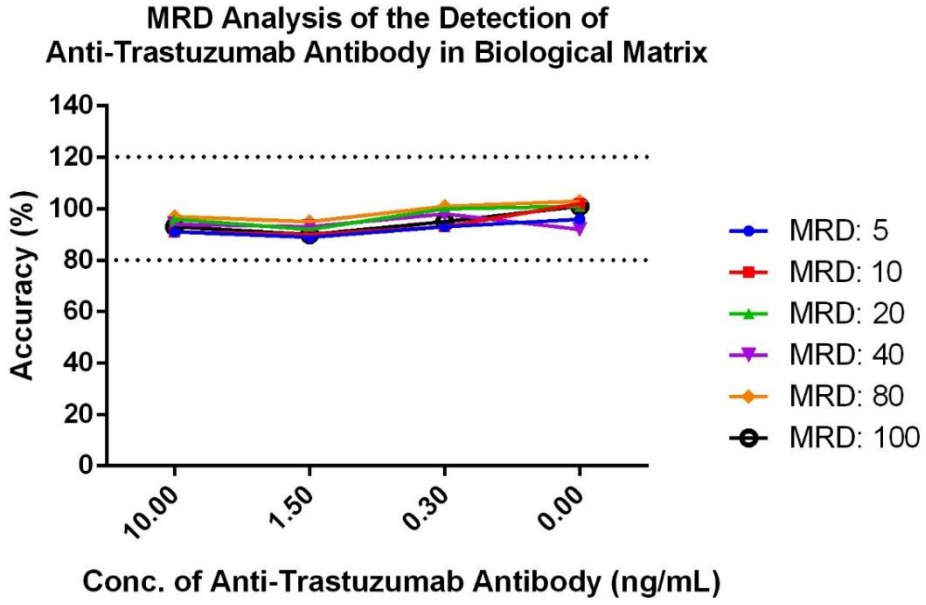

Figure 2. MRD analysis of the Kit

- **Hook Effect**

Anti-Trastuzumab antibody at different concentrations (3200, 1600, 800, 400, 200, 100, 50, 25 ng/mL) were measured for hook effect of the kit. The kit demonstrated no evidence of hook effect at 3200 ng/mL of anti-Trastuzumab antibody (Table 7).

Table 7. Hook effect analysis of the Kit

Samples	Conc.ng/mL	Average Value (OD 450 nm)	CV%
S1	3,200	5.57	7.3
S2	1,600	5.68	2.0
S3	800	5.75	5.8
S4	400	5.27	1.8
S5	200	4.80	0.6
S6	100	3.37	5.0
S7	50	2.04	3.3
S8	25	1.07	1.6

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XII. TROUBLESHOOTING

Problem	Probable Cause	Solution
Poor Precision	Wells are not washed or aspirated properly	Make sure the washing apparatus works properly and wells are dry after aspiration
	Wells are scratched with pipette tips or washing needles	Dispense and aspirate solution into and out of wells with caution
	Particulates are found in the samples	Remove any particulates by centrifugation prior to the assay
Weak/No Signal	Substrate is not added or added at the wrong time	Follow the manual to add the substrate properly
	Components are used from other lots or sources	Use only lot-specific components
	Substrate is contaminated	Use a new substrate from the same Lot
	Volumes of reagents are not correct	Repeat the assay with the required volumes as noted in the manual
	The plate is not incubated for proper time or temperature	Follow the manual to repeat the assay
	The plate is not read within the specified time range	Read the plate within 5 minutes
High Background	Plate is not washed properly	Make sure the washing apparatus works properly
	Substrate is contaminated	Use new substrate from the same Lot
	Evaporation of wells during incubations	Perform incubation steps with a plate sealer in a repeat assay
	Incorrect incubation times and/or temperatures	Follow the manual to repeat the assay

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XIII. REFERENCES

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