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Product Manual

Trastuzumab Pharmacokinetic ELISA Kit

Cat. No. L00970

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

The operator should read the 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 tumour cells with high affinity. Trastuzumab can suppress the cells growth and proliferation by blocking the ability of the cancer cells to receive chemical signals.

GenScript's Trastuzumab Pharmacokinetic ELISA Kit had been comprehensively developed and validated for quantitative measurement of Trastuzumab in cynomolgus monkey serum and plasma, based on the ICH M10 and the FDA bioanalytical method validation guidance for industry. Its precision, accuracy, dilutional linearity, specificity, selectivity, stability, and hook effect were acceptable according to the guidances[1-4]. The Trastuzumab ELISA kit is a validated tool for whole Trastuzumab and its biosimilar quantification in biological matrices for drug research and development.

II. ASSAY PRINCIPLE

Trastuzumab Pharmacokinetic ELISA Kit is a sandwich ELISA assay with a pair of anti-idiotypic monoclonal capture and detection antibodies. When standards or samples are added to the capture plate, the Trastuzumab in the sample can be captured on the plate coated with the anti-Trastuzumab capture antibody. Then the Biotin Anti-Trastuzumab Antibody is added to interact with the Trastuzumab bound on the plate. Streptavidin-HRP (Streptavidin-Horseradish Peroxidase conjugate) is added to interact with the Biotin Anti-Trastuzumab Antibody. After the washing steps, TMB Solution is added, resulting in the formation of blue color. The reaction is stopped by adding Stop Solution. Adding the Stop Solution changes the color from blue to yellow. The intensity of the color can be read at 450 nm and 630 nm by a microplate reader.

The quantity of Trastuzumab in the sample is precisely quantified against a Trastuzumab standard curve.

III. ANALYTICAL CHARACTERISTICS

Features	Specifications
LLOQ	1 ng/mL
ULOQ	64 ng/mL
Intra-assay	CV≤10%
Inter-assay	CV≤15%
Minimum required dilution (MRD)	1:40, validated non-human primate plasma

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IV. KIT CONTENTS

The kit provides the following reagents and solutions for the quantitative measurement of Trastuzumab and its biosimilar in biological matrices.

Table 1. Components of the kit

Component	Quantity/Size	Part No.
Capture Plate	1 plate	Q1-80
Standard Stock	1 vial (50 µL)	Q1-10
Biotin Anti-Trastuzumab Antibody (11C4)	1 bottle (12 mL)	Q1-20
Streptavidin-HRP	1 bottle (12 mL)	Q1-30
Sample Dilution Buffer	1 bottle (60 mL)	Q1-60
20× Wash Solution	1 bottle (60 mL)	Q1-70
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 Trastuzumab.

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 with the correction wavelength set at 630 nm
- Data analysis and graphing software. It is recommended to use software which is capable of generating 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 and 37± 2 ºC incubator
- Rotary shaker
- Vortex Mixer

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 with 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 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 stability criteria that meet their needs.
2. Store specimens at -20°C or lower if not tested immediately. Avoid repeated freeze-thaw cycles.

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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 concentrations: fresh matrix (NC), 1, 2, 4, 8, 16, 32, and 64 ng/mL. Preparation of a whole set of standards is recommended in table 2. S-Int1 preparation is described below as an example.

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 (Q1-10)	5	495	500	1000
Std1	15.63	S-Int1	4	58.5	62.5	64
Std2	2	Std1	30	30	60	32
Std3	2	Std2	30	30	60	16
Std4	2	Std3	30	30	60	8
Std5	2	Std4	30	30	60	4
Std6	2	Std5	30	30	60	2
Std7	2	Std6	30	30	60	1
NC	0	/	/	30	30	/

Quality Control Preparation: QCs should be prepared with fresh matrix to generate five Trastuzumab concentrations: 1 (LLOQ), 3 (LQC), 8 (MQC), 48 (HQC) and 64 (ULOQ) ng/mL. Preparation of a whole set of standards is recommended in table 3. Q-Int1 preparation is described below as an example.

Note: QC is quality control. LQC is low quality control. MQC is medium quality control. HQC is high quality control. LLOQ is lower limit of quantification. ULOQ is upper limit of quantification.

Q-Int1 preparation: Vortex and centrifuge Standard Stock for several seconds. Dilute Standard

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Stock with a fresh matrix with a volume ratio of 1:100. For example, add 5 µL of Standard Stock to 495 µL of fresh matrix and mix it well to make 500 µL of Q-Int1.

Table 3. Recommended quality control preparation

QC ID	Dilution Factor	Source	Source Volume (µL)	Matrix Volume (µL)	Final Volume (µL)	Final Conc. (ng/mL)
Q-Int1	100	Stock (Q1-10)	5	495	500	1000
ULOQ	15.63	Q-Int1	4	58.5	62.5	64
HQC	1.33	ULOQ	30	10	40	48
MQC	6	HQC	8	40	48	8
LQC	2.67	MQC	15	25	40	3
LLOQ	3	LQC	10	20	30	1

- **Capture Plate Preparation**

It is recommended that all standards, quality controls, and samples be prepared in duplicate at least. Table 4 is an example for the setup of Trastuzumab standards and samples.

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.

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 4. Setup of standards, quality controls and samples on Capture Plate

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

S： Sample number

- **Test Procedure**

Standards and Samples Incubation

1. Dilute standards, QCs and samples with Sample Dilution Buffer with a volume ratio of 1:40.

   Note: Both standards and QCs are working solutions that have been diluted in the matrix, see PROTOCOL.Reagent Preparation for step details.

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2. Add 100 µL of the diluted standard solutions, controls and samples to the corresponding wells in the Capture Plate.
3. Cover the plate with Plate Sealer and incubate at 37°C for 60 minutes.
4. Remove the Plate Sealer and wash the plate with 260 µL of 1× Wash Solution four times.
5. Tap the inverted plate onto absorbent paper to remove residual liquid in the wells after the wash steps.

Detection Antibody Incubation

6. Add 100 µL of Biotin Anti-Trastuzumab Antibody (11C4) to all the testing wells.
7. Cover the plate with Plate Sealer and incubate at 37°C for 30 minutes.
8. Remove the Plate Sealer and wash the plate with 260 µL of 1× Wash Solution four times.
9. Tap the inverted plate onto absorbent paper to remove residual liquid in the wells after the wash steps.

Enzyme Conjugate Incubation

10. Add 100 µL of Streptavidin-HRP to all the testing wells.
11. Cover the Plate with Plate Sealer and incubate at 37°C for 10 minutes.
12. Remove the Plate Sealer and wash the plate with 260 µL of 1× Wash Solution four times.
13. Tap the inverted plate onto absorbent paper to remove residual liquid in the wells after the wash steps.

Absorbance Measurement and Calculation

14. 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.

15. Add 50 µL of Stop Solution to each well to stop the reaction.
16. Read the absorbance in the microplate reader at 450 nm against 630 nm as a reference filter.
17. Plot the standard curve with the Trastuzumab concentration (ng/mL) on the x-axis and the corresponding mean absorbance value on the y-axis.
18. Using a 4- or 5-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 and QCs
2. • Dilute the test samples and a set of standards and QCs with **Sample Dilution Buffer** with a volume ratio of 1:40.
2. • Add 100 µL of the diluted standard solutions, controls and samples to the corresponding wells. Incubate the plate at 37°C for 60 minutes.
3. • Wash the plate with 260 µL of 1× Wash Solution per well four times.
4. • Add 100 µL of Biotin Anti-Trastuzumab Antibody (11C4) to the well and incubate at 37°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 37°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 Limit of Detection**

A set of Trastuzumab calibration standards were freshly prepared and analyzed. Standard curves were constructed using a four- or five-parameter logistic curve. The typical dynamic range of the kit is 1-64 ng/mL (0.025-1.6 ng/mL diluted) and its detection limit is 1 ng/mL (Table 5 & Figure 1).

Table 5. Sample data for standard curve

Trastuzumab (ng/mL)	Absorbance (OD 450/630nm)			Measured Trastuzumab (ng/mL)	CV %	Accuracy %
	Duplicate 1	Duplicate 2	Average			
64	2.9722	2.7627	2.867	64.04	6.84	100.06
32	1.6366	1.579	1.608	32.04	2.80	100.13
16	0.8221	0.8334	0.828	15.96	0.98	99.75
8	0.4144	0.4095	0.412	7.97	0.83	99.63
4	0.2125	0.2092	0.211	4.08	1.13	102.00
2	0.1077	0.1165	0.112	2.08	6.25	104.00
1	0.0569	0.0574	0.057	0.89	0.90	89.00
N/A	0.0101	0.0172	0.014	N/A	N/A	N/A

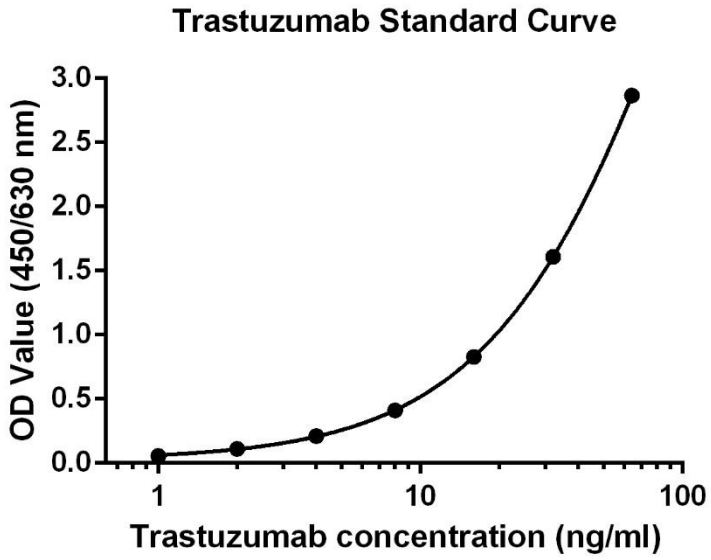

Figure 1: Trastuzumab ELISA kit standard curve.

A set of Trastuzumab calibration standards from 1 ng/mL to 64 ng/mL was then diluted with Sample Dilution Buffer with a volume ratio of 1:40.

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- **Precision and Accuracy**

Trastuzumab Quality Controls at three concentrations (P1 of 48 ng/mL, P2 of 8 ng/mL, and P3 of 3 ng/mL) were tested for precision and accuracy in three batches of the kits, with each sample tested three times.

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

QC	Trastuzumab (ng/mL)	Intra-assay (n=10)			Inter-assay (n=30)		
		Measured Trastuzumab (ng/mL)	CV %	Accuracy %	Measured Trastuzumab (ng/mL)	CV %	Accuracy %
P1	48	41.54	7.07	86.54	43.60	1.61	90.84
P2	8	7.36	7.83	92.04	7.32	2.26	91.44
P3	3	3.04	8.12	101.48	2.82	8.16	94.14

- **Selectivity**

Selectivity was tested by spiking plasma of ten different samples from non-human primate with Trastuzumab Quality Controls at two concentrations (HQC of 48 ng/mL and LLOQ of 1 ng/mL). Based on the ICH M10 guidance, the mean accuracy for LLOQ was required to be within 75%-125% of the low spiked concentration in at least 100% of the evaluated matrices. The mean accuracy for HQC was required to be within 80%-120% of the high spiked concentration in at least 100% of the evaluated matrices (Table 7).

Table 7. Selectivity analysis of the Kit

HQC-Selectivity			LLOQ-Selectivity		
Measured Trastuzumab (ng/mL)	CV%	Accuracy%	Measured Trastuzumab (ng/mL)	CV%	Accuracy%
44.51	1.29	92.72	0.83	2.68	82.70
46.88	0.67	97.67	0.80	2.07	80.10
47.26	1.37	98.45	0.92	0.30	91.50
42.27	0.15	88.06	0.96	23.74	96.00
40.92	2.04	85.26	0.79	1.40	78.70
41.23	2.54	85.90	1.19	18.41	119.40
44.03	1.33	91.73	1.06	9.75	105.80
46.77	4.30	97.43	1.02	10.24	102.00
45.08	1.60	93.91	1.11	23.08	110.70
44.29	2.15	92.26	1.20	24.25	120.10

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- **Dilutional Linearity and Hook Effect**

  Samples with high concentrations of Trastuzumab were used for the assessment of dilutional linearity (Table 9). The presence of a hook effect was investigated simultaneously. The hook effect was not observed in the assayed range (Table 8).

Table 8. Hook effect analysis of the kit

Trastuzumab (ng/mL)	Absorbance (OD 450/630nm)			CV%
	Duplicate 1	Duplicate 2	Average	
6000	5.84	5.83	5.84	0.10
600	5.87	5.87	5.87	0.10
300	5.90	5.90	5.90	0.10

Table 9. Dilutional linearity analysis of the kit

Dilution Factor	Expected Trastuzumab (ng/mL)	Measured Trastuzumab (ng/mL)	CV%	Accuracy%
1000	6	6.00	0.53	99.97
2000	3	2.86	0.97	95.30
100	6	6.34	1.26	105.60
50	6	6.36	1.02	106.00

- **Specificity**

  Trastuzumab QC samples at two concentrations (ULOQ of 64 ng/mL and LLOQ of 1 ng/mL) were spiked with different amounts of human IgG1 (64 and 640 ng/mL). The test result demonstrated that the high concentration of human IgG1 did not interfere with the detection of Trastuzumab (Table 10).

Table 10. Specificity analysis of the kit

Trastuzumab (ng/mL)	Human IgG1 (ng/mL)	Measured Trastuzumab (ng/mL)	CV%	Accuracy%
64	640	58.22	4.07	90.97
64	64	58.87	1.56	91.99
1	640	0.84	5.55	83.60
1	64	0.90	2.15	89.60

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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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