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Universal Test Kit for Residual Protein A Detection

Product Manual

Residual Detection of Various Types of Protein A in Antibodies, Fc-fusion Proteins and other Products (Based on Detachable 8-well × 12-strip ELISA Plate)

Hyasen Biotechnology Co., Ltd.

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Statement

- Scope of Application

This kit is intended for research purposes only, and is not to be used for medical diagnosis.

Without written authorization from our company, this product or any of its components shall not be used for any commercial development or application purposes.

- Disclaimer

By purchasing this assay kit, the corresponding user agrees to assume all potential risks associated with the individual or combined use of this assay kit.

Users should make purchases from our company or through channels officially authorized by our company. Otherwise, our company will not assume any obligation for consultation or compensation. In extreme cases where the assay kit significantly deviates from the standards stated in the product instructions and fails to meet the intended purpose of the product, our company's compensation will be only limited to the replacement of the assay kit at its purchase price, free of charge.

Operators should carefully read the operating instructions before using this product.

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1. Background Information

Protein A is naturally derived from *Staphylococcus aureus*, specifically the A-type strain. It has a molecular weight of approximately 42 kDa and consists of five highly homologous IgG-binding domains, namely E, D, A, B, and C. Each domain can bind to IgG and adopts a reversely paralleled triple α-helical structure. Helices I and II bind to the Fc region, while helices II and III interact with VH3-type Fab regions. Taking advantage of the property of Protein A to bind strongly to Fc under neutral conditions and dissociate under acidic conditions, it is widely used in the industrial purification of therapeutic antibodies.

To improve the elution pH, enhance tolerance to alkaline washing, and increase dynamic binding capacity to antibodies, Protein A has undergone continuous genetic engineering modifications in both academic and industrial settings. For example, the widely used MabSelect SuRe™ developed by Cytiva consists of four Z domains in tandem, where the Z domain is a double mutant form of the B domain (A1V and G29A). This modification improves the tolerance of Protein A to sodium hydroxide regeneration and disrupts the binding of Protein A to Fab while retaining its binding capacity to Fc. Engineered Protein A ligands commonly utilize different domains from wild-type Protein A, different mutant forms within the same domain, and combinations of different domain numbers (4-6) in tandem.

When Protein A is used for antibody purification, its inevitable shedding results in protein A residues, making it a process-related impurity in antibody drugs. Studies have shown that Protein A can bind and block Fc, cross-link a portion of B-cell membrane-type antibody Fab, and interact with TNFR, Willebrand factor, C1qR, and other molecules, leading to adverse reactions in human body. The ICH Q6B guideline requires the testing of process-related impurities. The 2020 edition of the Chinese Pharmacopoeia includes requirements for residual protein A detection in the general chapter on recombinant monoclonal antibody (mAb) products, as well as in specific monographs for two mAb products, "Nivolumab Injection" and "Conbercept Ophthalmic Injection." The US FDA and the US Pharmacopoeia chapter <130> also provide requirements for protein A quantification testing.

2. Detection Principle

This assay kit is used to detect residual Protein A in antibodies and Fc-fusion proteins. The principle of this assay kit is based on a sandwich immunoassay, utilizing an acidic buffer to dilute the sample and effectively separate residual Protein A from the sample's Fc portion, and the steps involved are simple. As shown in Figure 1, the provided assay plate in the kit is pre-coated with

anti-Protein A mAb. The acid-diluted series of Protein A standards and samples are directly added to the pre-coated assay plate. After incubation at room temperature with shaking for 1 hour, unbound substances are washed away, resulting in the formation of a "coated antibody-Protein A" complex on the assay plate. Subsequently, biotinylated anti-Protein A detection antibodies are added, forming a "coated antibody-Protein A-biotinylated detection antibody" complex. After incubation at room temperature for 1 hour, unbound substances are washed away. Then, streptavidin-horseradish peroxidase (SA-HRP) complex is added and incubated at room temperature for 20 minutes, resulting in the formation of a "coated antibody-Protein A-biotinylated detection antibody-SA-HRP" complex on the assay plate. After washing away unbound substances, TMB substrate is added. The enzyme-catalyzed reaction of the substrate on the solid phase generates a color reaction. After approximately 6 minutes of color development, the reaction is terminated. The results are measured using an ELISA reader at wavelengths of 450 nm, with 650 nm as the reference wavelength. The results are calculated using the difference between OD_{450nm} and OD_{650nm}, which indirectly reflects the amount of Protein A bound to the assay plate. The residual amount of Protein A in antibodies and Fc-fusion proteins can be quantitatively analyzed based on the standard curve.

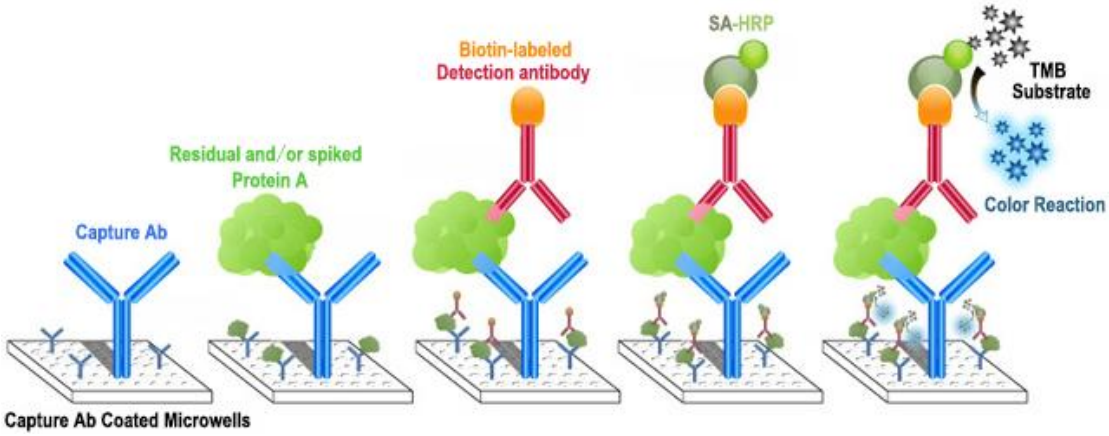


Figure 1. Principle of Sandwich ELISA for Residual Protein A Detection

3. Kit Features

(1) The coated and detection antibodies are both mAbs produced from monoclonal cell lines. They are purified using a two-step column chromatography process, ensuring batch-to-batch consistency and sustainable supply of the assay kit. The Fc regions and framework regions of both antibodies do not bind to Protein A, ensuring the scientific validity of the assay kit's design

principle.

(2) The kit is equipped with a universal Protein A ligand. When this ligand is used as a standard, it has been validated to show good recovery rates for various other ligands in actual samples, demonstrating the kit's versatility for multiple imported and domestic Protein A ligands. Additionally, the kit includes four commonly used imported and four domestic Protein A ligands commonly applied in the industry. Users can choose the corresponding ligand as a standard for testing according to their needs. If a Protein A standard not included in the kit is used, it can be diluted and prepared following the steps in section 6.2 (10) of this manual. Subsequent investigations on other Protein A ligands are ongoing and will be included in the kit if needed after evaluation.

(3) The experimental procedure is simple, requiring only sample dilution and direct addition to the plate. It does not involve complex steps such as heating, denaturation, centrifugation, or pre-incubation before sample dilution and addition to plate. The entire experimental operation takes approximately 3 hours.

(4) The kit offers high sensitivity compared to other similar products. The lowest point at the standard curve serves as the quantitation limit (10 pg/mL). The low quantitation limit ensures that users have a high degree of flexibility and selectivity in experimental design. Depending on the actual residual amount of Protein A in the test sample, the sample can be diluted to 1-0.01 mg/mL, corresponding to a final quantitation limit of 0.01-1 ppm. The ratio of OD between 10 pg/mL and 0 pg/mL on the standard curve is not less than 2 times, ensuring the stability and accuracy of experimental measurement data when the Protein A content in the sample is near the quantitation limit. It is important to note that the quantitation limit refers to the quantitation limit in a specific sample with a specific protocol. The quantitation limit of the kit is obtained through methodological validation on representative samples. When applying this kit, users should not directly adopt the quantitation limit of 10 pg/mL but should develop a methodological validation plan based on their own sample characteristics and needs of specification, and set a reasonable quantitation limit according to the results of the methodological validation.

(5) The kit has undergone comprehensive and relatively rigorous methodological validation according to the latest version of ICH-Q2 (R2). It has been validated for accuracy, precision, linearity, quantitation limit, specificity, and other parameters, meeting regulatory requirements for residual Protein A detection in antibodies and Fc-fusion protein drugs in various countries. The kit comes with a recommended validation protocol, but users can also design their own validation plan according to their specific needs.

(6) The kit demonstrates good stability. After being stored at 37℃ for 1 months, there are no

significant changes in performance. Long-term stability assessment at room temperature is currently underway. Currently, the kit supports transportation at room temperature, but it is still recommended to store the kit at 2-8℃ for long-term storage.

4. Kit Components

4.1. Components of the assay kit (as shown in Table 1)

Table 1. Components of the assay kit

	Components		Specifications	Item Number	Description
1	Anti-Protein A Antibody Pre-Coated Plate		8 wells × 12 strips * 1 plate	PA-Plate	Removable, coated with mAb whose Fc that do not bind to Protein A
2	Protein A Standard	Universal Protein A Standards	128 ng/mL 120 μL/vial	PA-STD-Univ	Select the corresponding standard based on the sample being tested; dilute with "1× Wash Buffer 1" to 640, 320, 160, 80, 40, 20, 10 pg/mL.
		Standard 1: MabSelect SuRe(Cytiva)	128 ng/mL 100 μL/vial	PA-STD-SuRe	
		Standard 2: MabSelect PrismA(Cytiva)		PA-STD-Prism	
		Standard 3: TOYOPEARL AF-rProtein A(HC)-650F(Tosoh)		PA- STD-TOYO	
		Standard 4: Eshmuno A(Merck)		PA-STD-Eshm	
		Standard 5: NMab Pro(NanoMicro)		PA-STD-NMpro	
		Standard 6: UniMab 50HC, NMab(NanoMicro)		PA-STD-50HC	
		Standard 7: MabPurix™ A/P(Sepax)		PA-STD-MPurix	
		Standard 9：MaXtar® ARPA(BioLink)		PA-STD-ARPA	
3	Wash Buffer 1		50 mL/bottle(10×)	PA-Wash-1	Dilute with deionized water as a 1× solution to be used as a dilution buffer for Protein A standards and samples, and as the wash buffer in the first step.
4	Biotin-labeled Detection Antibody		150 μL/vial(100×)	PA-Dect	mAb with Fc subtype that does not bind to Protein A; dilute with "Detection Antibody and SA-HRP Diluent" for 1× use.
5	SA-HRP		150 μL/vial(100×)	PA-SA-HRP	Dilute with "Detection Antibody and SA-HRP Diluent" for 1× use.
6	Detection Antibody and SA-HRP Diluent		30 mL/bottle(1×)	PA-Dilute	Used to dilute "Biotin-labeled Detection Antibody" and "SA-HRP".
7	Wash Buffer 2		30 mL/bottle(10×)	PA-Wash-2	Dilute with deionized water as a 1× solution to be used as the wash buffer in the second and third steps.
8	TMB Substrate Solution		12 mL/bottle(1×)	PA-TMB	Solution containing 3,3',5,5'-Tetramethylbenzidine (TMB) and H_2O_2; used directly.
9	Stop Solution		8 mL/bottle(1×)	PA-Stop	Acid solution, used directly,
10	Plate sealing film.		1 piece	/	/

Notes: 1.Standard 8 for NGL-Impact™ A ligand (No. PA-STD-NGL), is not included in this kit. Please contact us if needed.

2.To meet market demand, the company will continue to introduce more specific protein A standards, each provided with a separate product number, and these are currently not included in the kits. Please contact the company separately if needed.

3.Crystallization may occur in wash buffer 1 (10×) and wash buffer 2 (10×). It can be heated and dissolved in a water bath of 40℃-50℃, and can be stored at room temperature for three months.

4.2. Storage Conditions

The assay kit should be stored at 2-8°C, and the shelf life under this condition is 3 years.

4.3. Other Required Reagents and Materials (not provided in the kit)

Deionized water

Sample reservoir

Absorbent paper

Low-binding pipette tips with volumes ranging from 5-1000 μL

Low-binding sample dilution plate

Polypropylene centrifuge tubes (e.g., 1.5 mL, 2 mL, 5 mL, 15 mL) for sample dilution or preparation of detection antibodies

Single-channel pipette with volume adjustment range of 5-300 μL and 300-1000 μL, respectively

Multi-channel pipette with volume adjustment range of 5-300 μL

Suitable volumetric bottles for holding "1× Wash Buffer 1" and "1× Wash Buffer 2"

Microplate reader capable of reading absorbance at 450 nm (required) and 650 nm

Microplate shaker (200-500 rpm)

Software capable of four-parameter curve fitting (can also use Microsoft Excel for plotting).

5. SOP

5.1. Reagent Preparation

(1) Wash Buffer 1: Prepare a 1× solution using deionized water.

(2) Wash Buffer 2: Prepare a 1× solution using deionized water.

(3) Biotin-labeled detection antibody: Dilute with "Detection Antibody and SA-HRP Diluent" at a 1:100 ratio before use. Prepare the appropriate volume according to the experiment's needs on the day of the experiment.

(4) SA-HRP: Dilute with "Detection Antibody and SA-HRP Diluent" at a 1:100 ratio before use. Prepare the appropriate volume according to the experiment's needs on the day of the experiment.

(5) Protein A standard

The Protein A standard is prepared with "1× Wash Buffer 1" as shown in Figure 2. Prepare 9 polypropylene centrifuge tubes labeled accordingly. Add 135 μL of "1× Wash Buffer 1" to Tube 1,

900 µL of "1× Wash Buffer 1" to Tube 2, and 500 µL of "1× Wash Buffer 1" to Tubes 3 to 9. Take 15 µL of the 128 ng/mL standard solution and transfer it to Tube 1, and vortex to mix. Take 100 µL of the mixed solution from Tube 1, transfer it to Tube 2, and vortex to mix. Then, transfer 500 µL of the mixed solution to the next numbered tube, and vortex before each transfer. A series of Protein A standard solutions are prepared as above: 640 pg/mL (S1), 320 pg/mL (S2), 160 pg/mL (S3), 80 pg/mL (S4), 40 pg/mL (S5), 20 pg/mL (S6), and 10 pg/mL (S7) in concentration. Additionally, prepare a tube containing only "1x Wash Buffer 1" (S8 = 0 pg/mL).

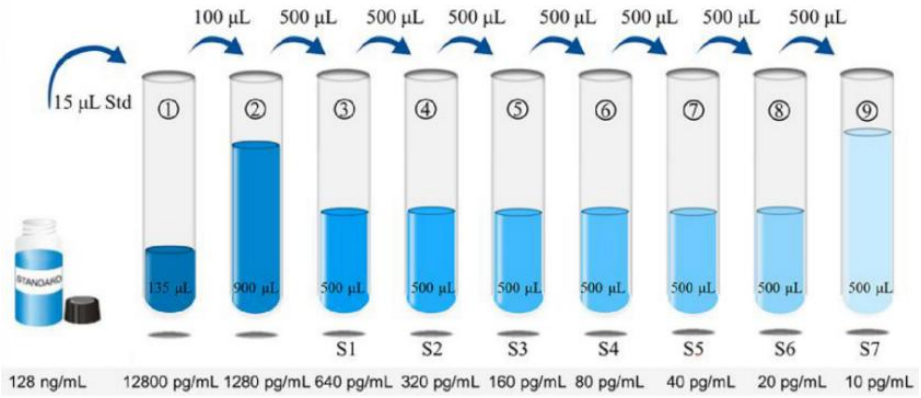


Figure 2. Preparation of Protein A Standard Series

5.2. Sample Preparation

(1) Test Sample: Dilute the sample with "1× Wash Buffer 1" and keep it ready. The sample should be diluted at least 10 times, and it is recommended to achieve a final concentration of 0.01-1 mg/mL. The volume of the diluted test sample should not be less than 200 µL.

(2) Spike Sample: The recovery rate of the spike sample is an important criterion for the applicability of the experimental system and method validation. Mix the test sample (at a concentration of twice the final concentration) with the protein A standard (at a concentration of twice the target spike concentration) in equal volumes. The volume of the prepared spike sample should not be less than 200 µL.

5.3. Experimental Procedures

The specific experimental steps are shown in Figure 3.

(1) Standard/Sample/Spiked Sample Incubation

Add 100 µL of prepared Protein A standard solutions (S1-S8), samples, and spiked samples to the wells of the pre-coated microplate. Each sample should have duplicate wells. Seal the plate with a plate seal and incubate at 500 rpm at room temperature for 1 hour.

Tap the microplate to remove the liquid, add 300 µL/well of "1× Wash Buffer 1", tap again to remove the liquid, and invert the plate on absorbent paper to allow the paper to absorb the liquid in the wells. Repeat this step two more times (a total of three times).

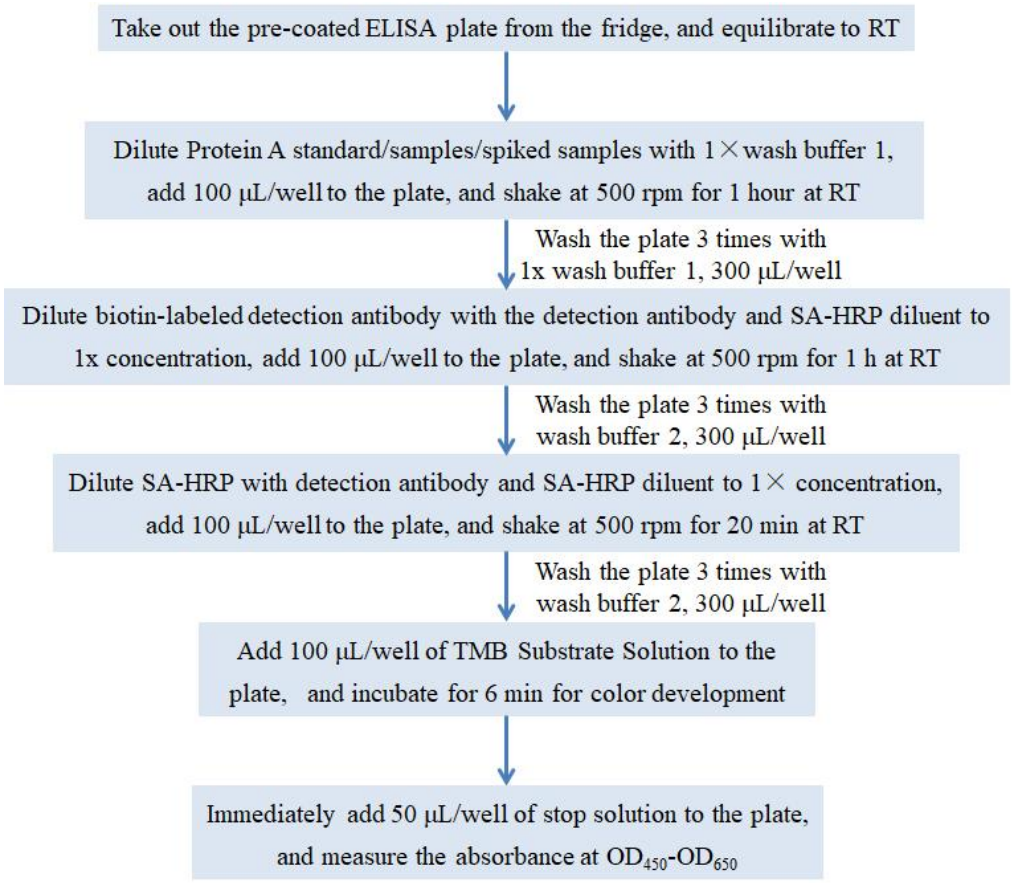


Figure 3. Workflow of ELISA Experiment for Protein A Quantification

(2) Detection Antibody Incubation

Add 100 µL/well of Biotin-labeled detection antibody (diluted) to the plate. Seal the plate with a plate seal and incubate at 500 rpm at room temperature for 1 hour.

Tap the microplate to remove the liquid, add 300 µL/well of "1× Wash Buffer 2," tap again to remove the liquid, and invert the plate on absorbent paper to allow the paper to absorb the liquid in the wells. Repeat this step two more times (a total of three times).

(3) SA-HRP Incubation

Add 100 µL/well of SA-HRP (diluted) to the plate. Seal the plate with a plate seal and incubate at 500 rpm at room temperature for 20 minutes.

Tap the microplate to remove the liquid, add 300 µL/well of "1× Wash Buffer 2," tap again to remove the liquid, and invert the plate on absorbent paper to allow the paper to absorb the liquid

in the wells. Repeat this step two more times (a total of three times).

(4) Addition of TMB Substrate and Termination:

Add 100 μL/well of TMB substrate and incubate in the dark for 6 minutes. Immediately add 50 μL/well of stop solution to terminate the reaction.

(5) Reading with a Microplate Reader

Read the absorbance at 450 nm and 650 nm using a microplate reader (should be done within 5 minutes after adding the stop solution).

(6) Result Analysis and Calculation:

Calculate OD values as OD_{450nm}-OD_{650nm}, taking the subtraction average of the duplicate wells. Perform a four-parameter logistic curve fit to the S1-S8 results using software such as the microplate reader's built-in software, Excel, or GraphPad Prism. Use the fitted standard curve to calculate the Protein A content in the samples and spiked samples.

Table 2 and Figure 4 show typical dose-response values and their corresponding fitted curve.

Table 2. Typical Dose-Response Values

Sample	Median OD	Calculated Protein A Concentration (pg/mL)
S1	2.940	640.33
S2	2.027	319.43
S3	1.244	161.61
S4	0.673	78.45
S5	0.366	40.19
S6	0.202	20.67
S7	0.114	10.12
S8	0.033	0.06
Unknown1	0.672	78.27
Unknown2	0.120	10.82

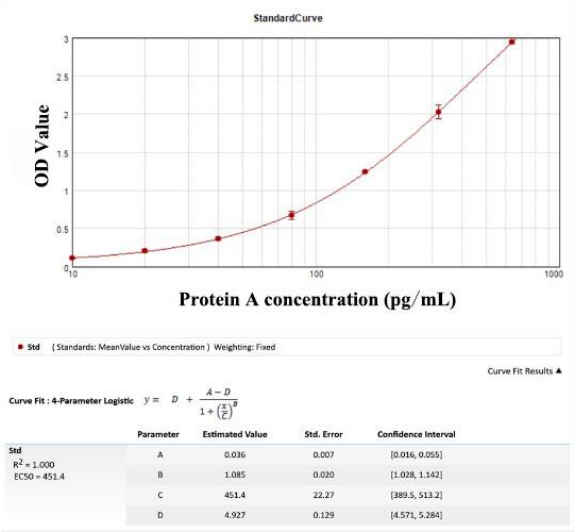


Figure 4. Typical Dose-Response Curve

6. Special Tips and Precautions

6.1. Special Tips

(1) For this kit, the only experimental condition that needs to be determined is the dilution fold of the sample. It is recommended to dilute the sample at least 10-fold to ensure the proper function of Wash Buffer 1 to detach the interaction between Fc and Protein A. In the initial experiments, prepare several samples with gradient dilutions of 10-fold and choose the appropriate dilution factor based on the preliminary detection of the actual residual concentration. This dilution factor should meet the quality standard requirements while considering the feasibility of method

validation.

(2) During the three incubation steps before adding TMB, it is recommended to shake the plate at 500 rpm. Incubation without shaking can result in an overall OD value decrease of approximately 30% (although it generally does not affect the measurement results).

(3) This kit is highly sensitive. After adding the TMB substrate, immediately start a 6-minute countdown and have the stop solution ready in the sample well to terminate the reaction promptly. The recommended OD value at the highest point of the standard curve is between 2.5～3.0. The reaction time can be extended if necessary (e.g., 1～4 minutes) depending on the experimental conditions such as room temperate.

After adding the TMB substrate, the solution turns blue (650 nm), while after adding the acid stop solution, the solution turns yellow (450 nm). Generally, as shown in Figure 5, the yellow OD_{450nm} value after the termination is approximately twice the blue OD_{650nm} value before termination. The OD_{450nm} value reaches saturation at ≥4, and to account for experimental tolerance, the OD_{450nm} value of the highest concentration (640 pg/mL) of Protein A standard curve in this kit should generally not exceed 3.5. Due to differences in operators' experimental habits, technique variations, and environmental changes, the highest OD_{450nm} value in this kit may vary, which is considered normal. Experimenters can adjust the color development time after adding TMB based on the recommendations in this manual and their own experience.

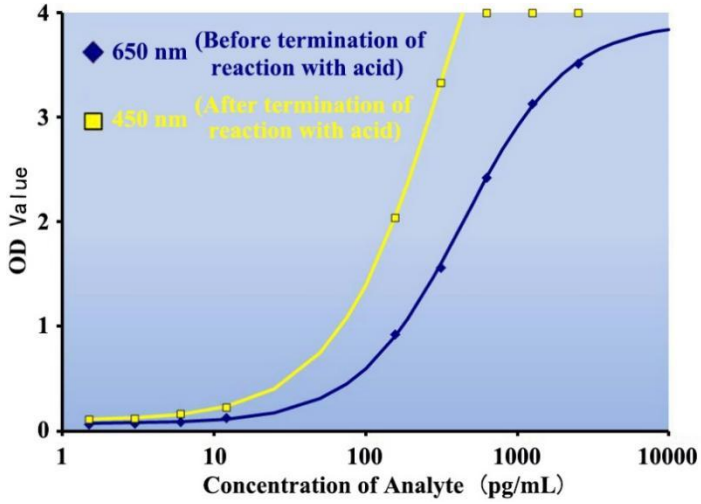


Figure 5. Relationship between OD values before and after adding the stop solution, with yellow representing 450 nm and blue representing 650 nm

(4) The stop solution is a strongly acidic solution. Caution should be taken to avoid contact with the skin and eyes during the handling process. After termination, the plate should be read as soon as possible, preferably within 5 minutes. During the plate reading, the OD_{650nm} is used as the

reference for calibration, and is preferred but not required. If there are differences in instruments, the 630 nm wavelength can be used as an alternative to 650 nm.

(5) It is recommended to include the 0-point (8 points in total) when fitting the standard curve, especially during method validation, as subtracting the protein A residual content of the sample itself is crucial for the recovery rate at the low concentration points of the standard curve.

6.2. Precautions in the Experiment

(1) Prior to starting the experiment, remove the required pre-coated plates, wash solutions 1 and 2 from the refrigerator in advance and allow them to equilibrate at room temperature for about 30 minutes. Unused strips of the plate should be returned to their original position for subsequent use. Once the experiment starts, all steps should be carried out in sequence without interruption. Ensure that the plate does not dry out between each step, as it may lead to high background values or incorrect results. Make sure that all necessary materials and reagents are prepared before starting the procedure.

(2) To avoid cross-contamination between reagents, pipette tips, and plate wells, disposable pipette tips and reagent containers should be used. Do not pour unused reagents back into the original bottles and avoid mixing up the caps of reagent bottles or tubes, as it may cause cross-contamination.

(3) During the pipetting process, pay attention to the consistency of the liquid volume aspirated by the pipette tip, especially in the case of multi-channel pipettes, and ensure the accuracy of sample addition. Try to ensure that no residual liquid remains in the pipette tips and prevent cross-contamination between wells.

(4) Different reaction times can affect the experimental readouts. The position of adding reagents to the plate wells should be consistent for each step. Each step should be performed according to the instructions provided.

(5) Proper washing of the plate is crucial, as it can affect the accuracy of the results or the level of background signals. Before using the provided wash solution, make sure that all containers used for preparing the wash solution have been rinsed with deionized water. It is recommended to add 300 μL of wash solution per well, and the plate should be quickly tapped dry. Use absorbent paper to remove the liquid from the wells as much as possible. Avoid excessive incubation time during washing and prepare the solutions to be added in advance. Do not leave the plate in a dry state for a long time during the experiment. When washing the plate, be careful not to contaminate low-concentration or blank wells with high-concentration wells. Proper plate layout should be considered during experimental design.

(6) When adding reagents to the wells, avoid damaging the pre-coated well walls. It is

recommended to gently and evenly add the samples along the same side of the well wall, preferably avoiding any residual liquid in the pipette tips.

(7) During the reaction, minimize the evaporation of the solution in the plate by covering the plate with the provided plate cover membrane.

(8) It is recommended to gently wipe the bottom of the plate with a clean tissue before reading the plate to avoid dust, fingerprints, or stains that may affect the accurate reading of OD values.

(9) The washing process may generate aerosols, which can potentially contaminate the microplate or substrates. Therefore, the washing and other experimental areas should be separated by a certain distance.

(10) Customers can use a protein A standard that is consistent with the sample being tested. If the protein A standard is not included in this kit, it can be diluted to a concentration of 128 ng/mL using the provided protein A pre-dilution solution (catalog number PA-Predilute), and distributed into low-binding polypropylene tubes at 100 µL per tube. The diluted standard can be stored at 2-8°C for up to 3 years. Table 3 provides an example of the dilution steps using a 2 mg/mL protein A solution.

Table 3. Preparation of 128 ng/mL Protein A Standard

Initial Concentration	Dilution Factor	Volume Taken from Initial Concentration	Volume of Diluent	Final Concentration
2 mg/mL	2×	15 µL	15 µL PBS	1 mg/mL
1 mg/mL	10×	20 µL	180 µLPre-dilution Solution	100 µg/mL
100 µg/mL	10×	180 µL	1.62 mLPre-dilution Solution	10 µg/mL
10 µg/mL	10×	1.6 mL	14.4 mLPre-dilution Solution	1000 ng/mL
1000 ng/mL	7.8×	15 mL	102.2 mLPre-dilution Solution	128 ng/mL

7. Validation Results (Product Performance)

To validate the performance of the kit, a representative marketed therapeutic mAb sample (with a residue protein A level of approximately 0.1 ppm) was selected, and a systematic method validation was conducted following the principles of relevant method validation guidelines. When applying this kit for methodological research, users can refer to the following validation protocol but should also develop scientifically sound validation protocols based on the characteristics of their own products and practical needs.

7.1. Experimental Protocol

(1) Protein A Standard Dilution

The 128 ng/mL protein A stock standard solution is pre-diluted to 1280 pg/mL. Each dilution should be mixed thoroughly using a vortex mixer according to the instructions provided in Table 4.

Table 4. Protein A Standard Pre-dilution

Solution Number	Diluted Protein A Concentration	Dilution Buffer (1× Wash Buffer 1) Volume	Original Protein A Volume/Concentration
1	12800 pg/mL	180 µL	20 µL 128 ng/mL
2	1280 pg/mL	1350 µL	150 µL 12800 pg/mL

Follow the instructions in Table 5 to prepare the corresponding 2× standard curve solutions. Mix each solution thoroughly using a vortex mixer.

Table 5. Preparation of 2× Standard Curve Solutions

Standard Solution Number	Diluted Protein A Concentration	Dilution Buffer (1× Wash Buffer 1) Volume	Original Protein A Solution Volume/Concentration
2×Std1	1280 pg/mL	N/A	1200 µL, 1280 pg/mL
2×Std2	640 pg/mL	600 µL	600 µL, 1280 pg/mL
2×Std3	320 pg/mL	600 µL	600 µL, 640 pg/mL
2×Std4	160 pg/mL	600 µL	600 µL, 320 pg/mL
2×Std5	80 pg/mL	600 µL	600 µL, 160 pg/mL
2×Std6	40 pg/mL	600 µL	600 µL, 80 pg/mL
2×Std7	20 pg/mL	600 µL	600 µL, 40 pg/mL
2×Std8	0	1200 µL	N/A

(2) Preparation of Standard Curve and Spike Solutions

Dilute the sample with 1× Wash Buffer 1 to a concentration of 0.2 mg/mL. Follow the instructions in Table 6 to prepare the 1× standard curve series of solutions and spike series of solutions. In a 96-well low-binding plate or low-binding tubes, add 120 µL of 1× Wash Buffer 1 or the diluted antibody solution (0.2 mg/mL). Then, add 120 µL of 2× Std1-8 (mixed in equal volumes) to each well, resulting in the preparation of the 1× standard curve solution Std1-8 or spike series of solutions. The 1× standard curve solution does not contain the antibody solution, while the spike series of solutions contain the antibody at a concentration of 0.1 mg/mL. The selection of the diluted protein concentration is crucial for method validation and actual sample testing. Users should consider factors such as the actual residual protein A level in their samples, method validation requirements, specification settings, and reporting results when determining the appropriate diluted protein concentration.

Table 6. Preparation of 1× Standard Curve and Spike Series of Solutions

Standard or Spike Solution Number	Diluted Protein A Concentration	Volume of 1× Wash Buffer 1 or Diluted Antibody	Volume/Concentration of Protein A Solution Before Dilution
Std1 or Spike1	640 pg/mL	120 µL	120 µL，1280 pg/mL
Std2 or Spike2	320 pg/mL	120 µL	120 µL，640 pg/mL
Std3 or Spike3	160 pg/mL	120 µL	120 µL，320 pg/mL
Std4 or Spike4	80 pg/mL	120 µL	120 µL，160 pg/mL
Std5 or Spike5	40 pg/mL	120 µL	120 µL，80 pg/mL

Std6 or Spike6	20 pg/mL	120 µL	120 µL，40 pg/mL
Std7 or Spike7	10 pg/mL	120 µL	120 µL, 20 pg/mL
Std8 or Spike8	0	240 µL	N/A

(3) Transfer the samples from the dilution plate (or EP tubes) to the pre-coated ELISA plate in the kit according to Table 7, with a volume of 100 µL per well. This includes the standard curve solutions and spike series of samples. Then, follow the SOP (Standard Operating Procedure) of the kit for further steps.

Table 7. ELISA Microplate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
					Standard Curve		**Sample Spikes**					
A					640 pg/mL	640 pg/mL	0.1 mg/ml+640 pg/ml	0.1 mg/ml+640 pg/ml				
B					320 pg/mL	320 pg/mL	0.1 mg/ml+320 pg/ml	0.1 mg/ml+320 pg/ml				
C					160 pg/mL	160 pg/mL	0.1 mg/ml+160 pg/ml	0.1 mg/ml+160 pg/ml				
D					80 pg/mL	80 pg/mL	0.1 mg/ml+80 pg/ml	0.1 mg/ml+80 pg/ml				
E					40 pg/mL	40 pg/mL	0.1 mg/ml+40 pg/ml	0.1 mg/ml+40 pg/ml				
F					20 pg/mL	20 pg/mL	0.1 mg/ml+20 pg/ml	0.1 mg/ml+20 pg/ml				
G					10 pg/mL	10 pg/mL	0.1 mg/ml+10 pg/ml	0.1 mg/ml+10 pg/ml				
H					0 pg/mL	0 pg/mL	0.1 mg/ml+0 pg/ml	0.1 mg/ml+0 pg/ml				

In the table, the units in pg/mL represent the final concentration of protein A in the well, while the units in mg/mL represent the final concentration of the diluted samples in the well.

7.2. Validation Design and Calculation

Two experimenters conducted independent experiments for 3 days, with 3 repetitions per day.

The serial dilutions of protein A (640~10 pg/mL and 0 pg/mL) were used as the x-axis, and the average difference in OD_{450nm} and OD_{650nm} for the duplicate wells was used as the y-axis. A four-parameter (4-PL) software was adopted for curve fitting. Based on the fitted curve and the average difference in OD values for the spike series of samples, the protein A concentration was calculated. The measured value of protein A concentration for the non-spiked samples was subtracted from this value to obtain the recovery value, and the ratio between recovery value to spiked vealue represents the recovery rate of the spiked amount.

Accuracy: The recovery rate for each sample at each spiked point represents the accuracy at that point.

Precision: For each different spiked point, the relative standard deviation (RSD) of the

recovery values from three repetitions per day by each experimenter represents the within-day precision at that point. The RSD value of the recovery values over three days by each experimenter represents the intermediate precision for that point across different days. The RSD value of the recovery values by different experimenters represents the intermediate precision for that point across different individuals.

Linearity: The actual spiked values are plotted on the x-axis, and the measured recovery values are plotted on the y-axis for linear regression, representing the linearity of the experiment.

Range: The interval that satisfies accuracy, precision, and linearity represents the range of the experiment.

Limit of Quantitation (LOQ): The lowest point within the range represents the LOQ of the experiment.

Specificity Design: The ratio of the recovery values for protein A in samples with and without the addition of host cell protein (HCP, 0.1 μg/mL) and host cell DNA (HCD, 0.1 ng/mL) exceeding the usual specification limits is used as an indicator for the specificity of the experiment. The formula for calculation is (S3-S1)/(S2-S1) × 100%. The specific design is outlined in Table 8.

Table 8. Specificity Verification Design

Sample S1	Sample S2	Sample S3
Sample (0.1 mg/mL)	+10 pg/mL protein A	+10 pg/mL protein A +0.1 μg/mL HCP +0.1 ng/mL HCD

7.3. Validation Results

The specific validation results are shown in Table 9.

Table 9. Summary of Validation Results

Validation Parameter		640 pg/mL	320 pg/mL	160 pg/mL	80 pg/mL	40 pg/mL	20 pg/mL	10 pg/mL
Accurac	Extreme (%)	83.0-114.0	85.4-106.4	87.8-107.1	85.3-102.1	83.8-100.4	90.2-108.5	86.5-120.1
	Mean (%) 95%CI(%)	96.9 (93.7,100.2)	95.0 (92.5,97.6)	94.4 (91.8,96.9)	93.4 (91.1,95.7)	93.9 (91.7-96.0)	96.8 (94.4,99.2)	99.1 (95.4,102.9)
Precision	Repeatability (%)	2.2-16.0	3.1-9.0	3.4-8.8	2.1-8.9	2.0-9.5	1.9-6.9	1.1-15.7
	Intermediate Precision (Days, %)	9.4 and 4.6	5.7and 5.8	6.6 and 5.2	5.2 and 5.7	4.0 and 6.0	6.3 and 4.6	7.5 and 9.4
	Intermediate Precision	7.2	5.8	5.8	5.3	5.0	5.4	8.2
Linearity		R2 value of 18 curves > 0.99						
LOQ		10 pg/mL						
Range		10 – 640 pg/mL						

Specificity	In the presence of 0.1 μg/mL HCP and 0.1 ng/mL HCD, recovery rate of 10 pg/mL protein A is 82.2%

The linearity of the experiment, conducted 3 times per day for 3 days by 2 experimenters (total of 18 experiments), is depicted in Figure 6. The x-axis represents the actual spiked amount of protein A, while the y-axis represents the measured spiked amount (recovery value) of protein A. Each point represents the mean and standard deviation of the 18 experiments. The correlation coefficient $R^2 \approx 1$ indicates good linearity of the experiment.

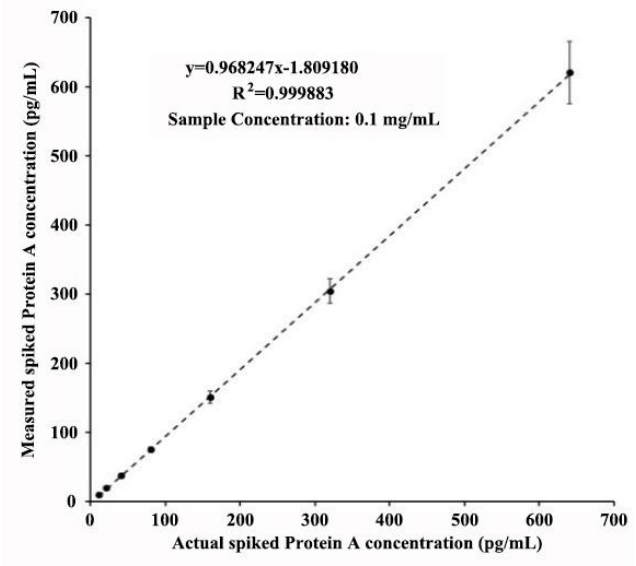


Figure 6. Linearity validation of the assay kit

In addition, a representative commercially available mAb sample (40 mg/mL) was used as a sample and diluted to concentrations of 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL according to the assay kit instructions. MabSelect SuRe (Cytiva) ligand was used as the standard curve for calculating protein A concentrations. As shown in Figure 7, the correlation coefficient $R^2 \approx 1$, indicating a linear relationship between the antibody concentration and the measured protein A concentration, demonstrating good dilution linearity.

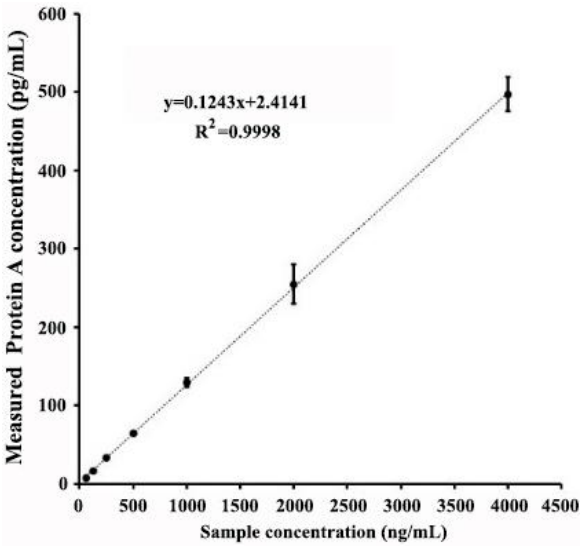


Figure 7. Linearity (dilution linearity) validation of antibody concentration and protein A content

8. Applicability of the Universal Protein A Ligand in the Assay Kit

To validate the representativeness of the universal ligand (Protein A-Universal) in the assay kit, a series of dilutions of this ligand were prepared as the standard curve following the assay kit's standard operating procedure (SOP). Other various domestically produced and imported ligands were added to the original reference Adalimumab mAb solution (final concentration 1 mg/mL) without residual protein A according to the standard curve series of concentrations. The recovery rates of the respective ligands in the mAb sample were determined, as shown in Table 10. The linear relationship between the actual and measured spiked amounts is depicted in Figure 8. The results demonstrate that the universal ligand can be used as a standard to measure the residual levels of various types of protein A ligands in antibodies and Fc-fusion proteins.

Table 10. Recovery rates of various domestically produced and imported protein A ligands using the universal protein A ligand as a standard

% / pg/mL	Ligand 1	Ligand 2	Ligand 3	Ligand 4	Ligand 5
640	106.3	99.5	77.7	102.7	109.6
320	87.1	88.4	73.9	107.0	105.6
160	94.8	88.8	79.4	104.4	109.2
80	97.9	92.5	80.9	114.0	114.2
40	102.0	99.8	92.4	118.1	120.7
20	108.5	106.8	97.2	127.6	125.8
10	97.3	89.7	88.3	117.6	116.7

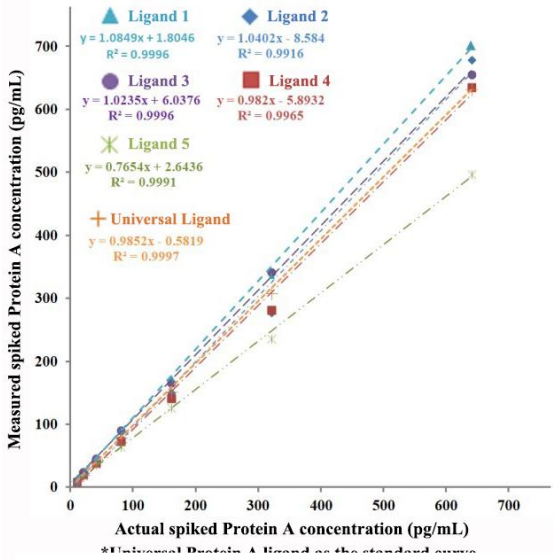


Figure 8. Fitting curve of the actual and measured spiked amounts of various protein A ligands, with universal ligand as the standard curve