Protein A ELISA Kit

96 Tests Kit
Enzyme Immunoassay for the Quantitative Detection of Protein A
For Research and Manufacturing only
Catalogue No. JTC234-Protein A

Introduction

Protein A is a 42 KDa surface protein originally found in the cell wall of Staphylococcus aureus. It is composed of five homologous immunoglobulin-binding domains that fold into a three-helix bundle. Each domain binds to immunoglobulins (Igs) of many mammalian species. It binds the heavy chain within the Fc region of IgG subclasses. The Protein A affinity chromatography is the golden standard for the capture and purification of therapeutic IgG (monoclonal antibodies) and Fc-fusion proteins in the pharmaceutical industry. Protein A leakage (free Protein A and/or Protein A-Igs complex), even though covalently conjugated to the chromatography support, may occur during the chromatographic process and could contaminate the final product. This contamination could reduce the therapeutic bio-drugs' effectiveness and cause immunological reactions. For this reason, quantifying the Protein A impurity level presents in the purified therapeutic antibodies and proteins is of extreme importance in pharmaceutical industry.

JTC Protein A ELISA kit has been developed based on enzyme-linked immunosorbent assay (ELISA) to detect and quantify of residual Protein A in order to assist manufacturers in quality control of in process streams and also in controlling the amount of Protein A in final bioproducts as much as possible.

Test principle

JTC Protein A ELISA Kit is a two-step immunoassay system based on the principle of sample treatment followed by a sandwich solid phase ELISA. In the first step, sample containing Protein A is diluted in the sample diluent provided with the kit (if necessary). The denaturing buffer is then added and mixed to dissociate the Protein A from the antibody. The sandwich solid phase ELISA utilizes an anti-Protein A polyclonal antibody for solid phase immobilization (on the wells) and an anti-Protein A polyclonal antibody for the antibody-enzyme (horseradish peroxidase) conjugate solution. In the second step, the treated (denatured) sample and standards are mixed with assay buffer and allowed to react with the solid phase antibodies. After incubation and washing, the enzyme conjugate will be added, resulting in the sandwich formation of Protein A between solid phase and conjugated antibodies. After second wash step a solution of 3,3′,5,5′-Tetramethylbenzidine (TMB) is added and incubated for 15 minutes, resulting in the development of a blue color. The color development is stopped after adding of stop solution, and the color is changed to yellow and measured spectrophotometrically at 450 nm. A 4-parameter logistic (4-PL) fit standard curve is used to calculate the concentration of Protein A in the sample. The concentration of Protein A is proportional to the color intensity of the test sample in comparison with the standards.

Materials provided with the kit

1. Sample treatment plate (STP, 1×V-shaped 96-well plate)
2. Sample diluent (1 vial, 25 mL), protein free matrix with a low-concentration detergent and preservative, ready to use
3. Denaturing buffer (1 vial, 12 mL), protein free matrix (low pH) with preservative, ready to use
4. Antibody coated wells (1×96-well ELISA plate), microtiter wells coated with polyclonal anti-Protein A antibody
5. Assay buffer (1 vial, 6 mL), containing protein matrix, and preservatives, blue color, ready to use
6. Standards set (1 mL/vial), containing 0, 0.16, 0.31, 0.63, 1.25, 2.5, 5 and 10 ng/mL of Protein A, ready to use
7. Enzyme conjugate (1 vial, 12 mL), polyclonal anti-Protein A antibody labeled with HRP in buffer, ready to use
8. Chromogen substrate reagent (1 vial, 12 mL), consisting of benzidine and hydrogen peroxide, ready to use
9. Wash solution (1 vial, 50 mL), contain Phosphate Buffered Saline solution with 0.05 % Tween 20 as detergent, concentrated (20x)
10. Stop solution (1 vial, 12 mL), 1 molar hydrochloric acid solution
11. Cardboard sealer (2 pcs)
12. Brochure barcode

Materials required but not provided

- ELISA reader with 450 nm filter (if possible 630 nm as reference filter)
- Orbital rotator with a minimum speed of 200 rpm
- Precision pipettes: 50 µL and 100 µL
- Multi-channel pipettes
- Disposable pipette tips
- Distilled water
- Absorbent paper

Notes for consumers

1. The contents of the kit should be used only for the current kit.
2. Kit should be used only by qualified technicians.
3. Kit is designed and manufactured only for the measurement of Protein A in biopharmaceutical products.
4. Do not mix kit reagents from different batch/lot numbers.
5. Kit is for Research or Manufacture Use Only.

Storage and stabilities

1. Kit should be stored at 2-8 °C upon receipt and when it is not in use.
2. Keep un-used wells in their sealed bag with desiccants.
3. Do not use expired date reagents.
4. Do not freeze.
5. For long term storage, in concentrated washing solution, crystals may form. Before preparing the work wash solution, place the vial at 37 °C to dissolve the crystals. Dilute the concentrated washing solution with distilled water, 1/20.
6. For long term storage, in denaturing buffer, crystals may also form. Before using, place the vial at 37 °C to dissolve the crystals.

General information

1. Be sure to bring all reagents at room temperature 30 minutes before starting the test.
2. All steps should be performed non-stop from the start of the test. Do not allow the wells to dry between incubation stages.
3. Use a disposable pipette tip for each sample.
4. Strips should be read within 30 minutes after adding stop solution since color will fade over time.
5. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.
6. Precision on duplicate samples should yield coefficients of variation (CV) of less than 10% for samples. In cases, where the CV of two replicates of each sample is high, the plate washing process should be evaluated.
7. One of the most important factors in achieving the accurate result is the appropriate incubation time. Before starting the test, prepare the required materials and solutions, this will be improved the accuracy by decreasing the time interval between the sampling steps.
8. In samples containing a high product antibody concentration, the linearity is not observed. It is mandatory to dilute your sample. Dilution to the range of ≤1.0 mg/mL is usually sufficient to obtain acceptable recovery.

Reagents preparation

1. Bring all reagents at room temperature (20-25 °C) before use.
2. Working wash solution: Dilute concentrated wash solution 1/20 with distilled water before use. Store working wash solution at 2-8 °C after use.

Test procedure

a) Sample treatment

1. Prepare appropriate dilutions of each sample using sample diluent.
2. Add 100 µL of standards and diluted samples into the wells of STP.
3. Add 50 µL of denaturing buffer into each well. Mix well by pipetting up and down ~15 times.
4. Incubate for 10 minutes at RT.

Note: Add denaturing buffer and pipetting as quickly and accurately as possible to avoid "end-of-run" sequential process time difference that can cause systematic inaccuracy. Actually, maintain a well-to-well or strip-to-strip time sequence for this step to ensure that all incubation times are the same for each well/ strip.

b) ELISA assay

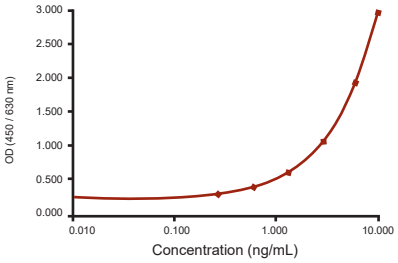
1. Before the end of 10 minutes incubation of the previous step **(a)**, add 50 µL of assay buffer into each well of the antibody coated plate.
2. Then, pipette 50 µL of the denatured standards and samples into wells containing assay buffer. As stated above, maintain a well-to-well or strip-to-strip time sequence for this step, too.
3. Cover the microtiter wells with cardboard sealer firmly. Incubate plate on orbital shaker at 200-600 rpm for 60 minutes at room temperature (20-25 °C).
4. Remove the sealer and take out wells contents by flicking the microplate into a waste container.
Rinse and flick the microtiter wells 5 times (each time with 300 µL of working wash solution). Strike the wells gently onto absorbent paper or paper towels to remove all residual droplets.
5. Add 100 µL of anti-Protein A conjugate (enzyme conjugate) into the wells.
6. Seal the plate with cardboard sealer again and incubate plate on orbital shaker at 200-600 rpm for 30 minutes at room temperature (20-25 °C).
7. Repeat step 4.
8. Dispense 100 µL of chromogen/substrate (TMB) into the microplate wells.
9. Incubate the microplate wells at room temperature in a dark place for 15 minutes to develop color.
10. Stop the reaction by adding 100 µL of stop solution to the microplate wells.
11. Measure absorbance at 450 nm by ELISA reader (use 630 nm filter as reference filter if it is available).

Result calculation

1. Calculate mean absorbance value of standards and samples at 450 nm (use 630 nm filter as reference filter if it is available).
2. Construct a 4-PL fit standard curve by plotting the mean absorbance obtained for each reference standard against its concentration in ng/mL using curve fitting software, with absorbance on the vertical (y) axis and concentration on the horizontal (x) axis.
3. Calculate the corresponding concentration of Protein A in the sample (ng/mL) from the standard curve using the mean absorbance value for each sample.

Example of Protein A standard curve

Standards (ng/mL)	OD (450/630 nm)	Mean OD
0	0.018	0.018
	0.018	
0.16	0.056	0.055
	0.054	
0.31	0.089	0.090
	0.092	
0.63	0.181	0.180
	0.179	
1.25	0.359	0.355
	0.352	
2.5	0.738	0.726
	0.714	
5	1.422	1.445
	1.468	
10	2.611	2.622
	2.634	



Note: All absorbances shown in above curve and table are for the purpose of illustration only, and should not be used to calculate unknowns. Each user should obtain his or her own data and standard curve.

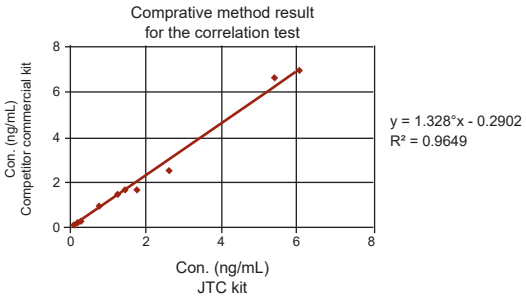
Limitations of the measurement method

Despite designing the kit to avoid interfering with different reagents, very excessive concentrations of drug samples can also additionally interfere with the accurate measurement of Protein A. It is mandatory to dilute your sample prior to assay using sample diluent to the range of ≤ 1.0 mg/mL.

Performance characteristics

1. Correlation test

JTC Protein A ELISA kit was compared with the relevant commercial kit. Eleven drug samples from different stages of production and purification were used for comparative tests. The results of comparative analysis on the samples showed a 98% correlation between JTC kit and a valid commercial kit.



2. Sensitivity

The lower limit of detection (LOD) is defined as that concentration corresponding to a signal three standard deviations above the mean of the zero standard. LOD is ~0.08 ng/mL.
The limit of quantitation (LOQ), is the lowest concentration which coefficients of variation (CV) is less than 10% with acceptable accuracy. The LOQ is ~0.16 ng/mL.

3. Test precision

Both intra- (24 replicates in one run) and inter- (30 replicates in 10 runs) assays precision was determined on three drug substance samples with different concentrations of Protein A. Results are shown in Tables 1 and 2:

Table 1. Intra-assay

Sample	No. of tests* performed	Means (ng/mL)	SD (ng/mL)	CV (%)
1	24	0.77	0.02	3.15
2	24	3.00	0.08	2.53
3	24	6.80	0.13	1.95

Table 2. Inter-assay

Sample	No. of tests* performed	Means (ng/mL)	SD (ng/mL)	CV (%)
1	30	0.78	0.05	6.24
2	30	3.35	0.16	4.73
3	30	8.45	0.39	4.63

4. Spike and recovery/interference

Despite the design of the kit to prevent interference with various buffer matrices, the buffer of your product should be evaluated for any negative inhibition on the assay before reporting the results. Samples containing a high product antibody concentration may also cause a negative interference (see the "limitations of the measurement method"). Therefore, user should validate that their sample matrices have the accurate recovery. This experiment can be performed by spiking the standard provided with this kit, into the appropriate dilution of the sample. For example, dilute 10 ng/mL standard at 1:3 ratio in the diluted sample. This yields an added spike of 2.5 ng/mL. It must be noted that the concentration of endogenous Protein A in the diluted sample should be corrected for the 25% dilution of the spike experiment. The added spike and recovery should be within acceptable range of 80% to 120%.

5. Linearity

To assess the linearity, it is mandatory to dilute sample to ≤ 1.0 mg/mL and titrated serially in the sample diluent. The recovery should be between 80%-120%. The results of linearity for three different drug samples at starting concentration (1 mg/mL) are presented as Table 3:

Table 3: Linearity of assay

Sample	Dilution Factor	Protein A (ng/mL)	Recovery (%)
1	Direct	4.38	100
	1/2	2.37	108
	1/4	1.18	107
	1/8	0.59	108
	1/16	0.24	86
2	Direct	3.08	100
	1/2	1.68	109
	1/4	0.85	111
	1/8	0.38	99
	1/16	0.18	94
3	Direct	2.41	100
	1/2	1.20	100
	1/4	0.66	110
	1/8	0.26	86
	1/16	0.12	82

6. Hook effect

To rule out possible hook effect occurrence, the Protein A assay was done on samples with high concentration of Protein A (up to 100.000 ng/mL) and no "hook effect" was observed.

Schematic procedure of Protein A test		
a) Sample treatment in STP		
Reagent	**Standard well**	**Sample well**
Sample	-	100 µL
Standard	100 µL	-
Denaturing buffer	50 µL	50 µL
Mix well by pipetting up and down ~15 times.		
Incubate for **10 minutes** at RT.		
Maintain a well-to-well or strip-to-strip time sequence for all steps to ensure that all incubation times are the same for each well/ strip.		
b) Assay in ELISA plate		
Assay buffer	50 µL	50 µL
Then, pipette 50 µL of the denatured samples and standards from STP into 96-well ELISA plate containing assay buffer. Use fresh tips for each addition.		
Cover the wells with cardboard sealer. Incubate for **60 minutes** on the rotator at 200 rpm at RT. Then, remove the cardboard sealer of the plate and empty the contents of the wells. Wash the wells 5 times according to the washing instructions.		
Anti-Protein A-HRP conjugate	100 µL	100 µL
Cover the wells with the cardboard sealer. Incubate for **30 minutes** on a rotator at 200-600 rpm at RT. Then, remove the cardboard sealer from the plate and empty the contents of the wells. Wash the wells 5 times according to the washing instructions.		
TMB	100 µL	100 µL
Incubate wells for **15 minutes** at RT in dark.		
Stop	100 µL	100 µL
Read absorbance at 450 nm (use 630 nm as reference filter if it is available).		

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