BESTCHROM
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Protein A ELISA Kit

Instruction for use

Contents

1. Background Information

Natural protein A is isolated from Staphylococcus aureus type A. Mol.Wt of natural Protein A is about 42kD. It has five IgG binding domains with high homology (E,D,A,B,C), each of which can bind IgG. Protein A enjoys high affinity with IgG, therefore it is widely used for the purification of antibodies after being covalently coupled on chromatography resins. However, when using Protein A for antibody purification, leachability of Protein A will occur inevitably and lead to Protein A residue in drug products. For antibody-based therapeutics, it is necessary to bring the Protein A impurities to the lowest level to prevent any adverse impact on patients' health.

In the *Pharmacopoeia of the People's Republic of China(2020)*, articles such as *General Discussion on Recombinant Monoclonal Antibody Products for Human Use*, as well as other two mAb-related articles concerning *Nituzumab Injection* and *Conbercept Ophthalmic Injection* all specify limit for Protein A residue. Besides, description concerning Protein A residue can also be found in FDA guidance and *United State Pharmacopoeia chapter 130*.

2. Product Application

Bestchrom Protein A residue detection kit (enzyme-linked immunosorbent assay) is A highly sensitive protein A residue detection kit. It can accurately detect ligand protein A of multiple imported and domestic resins, enabling easy and accurate detection of leached protein A ligands for Protein A resin producers.

Applicable Protein A ligands: 1#Standard, 2#Standard.

- ✧ **1#Standard: for the detection of leached ligands from AT Protein A Diamond, AT Protein A Diamond Plus, AT Protein A Diamond Ultra resins.**
- ✧ **2#Standard: for the detection of leached ligands from Novo-A Diamond, Extrem A Diamond resins.**

3. Product Characteristics

- High universality, suitable for the detection of multiple types of recombinant, alkali-stable Protein A residue.
- High detection sensitivity, standard curve OD ratio of 0ng/mL to 0.16ng/mL is approximately 2 to 5 times.
- High precision, inter-and intra-plate variability is <10%.
- Acid-treatment for Sample pre-treatment, eliminate sample boiling and the subsequent centrifugation steps. Incubation step is carried out at the same time of antibody/antigen capture and antibody detection. Eliminate inconvenience of multiple plate washing. Simplify operation.
- ➢ The Kit enjoys good stability. After being preserved at 37℃ for 1 month, kit showed no obvious change in performance. It is recommended to preserve kit at 2-8℃.

4. Detection Principle

Bestchrom Protein A ELISA kit can detect Protein A residue from samples via two-site Sandwich ELISA approach. First dilute Protein A-containing sample with Sample Diluent. Add denaturing Buffer and mix to isolate Protein A and sample antibody. Add denatured sample and let it react with the polyclonal anti-Protein A pre-coated ELISA Microplate. A second anti-Protein A antibody labeled directly with horse radish peroxidase (HRP) enzyme is simultaneously reacted forming as sandwich complex of solid phase antibody-Protein A : HRP labeled antibody. After a wash step to remove any unbound reactants, the strips are then react with tetramethyl benzidine (TMB) substrate, producing a color change(from colorless to blue). It will finally turn to yellow after using Stop Solution. Detect optical density(OD) at 450nm and 650nm. OD shall be positively related to Protein A contents in sample.

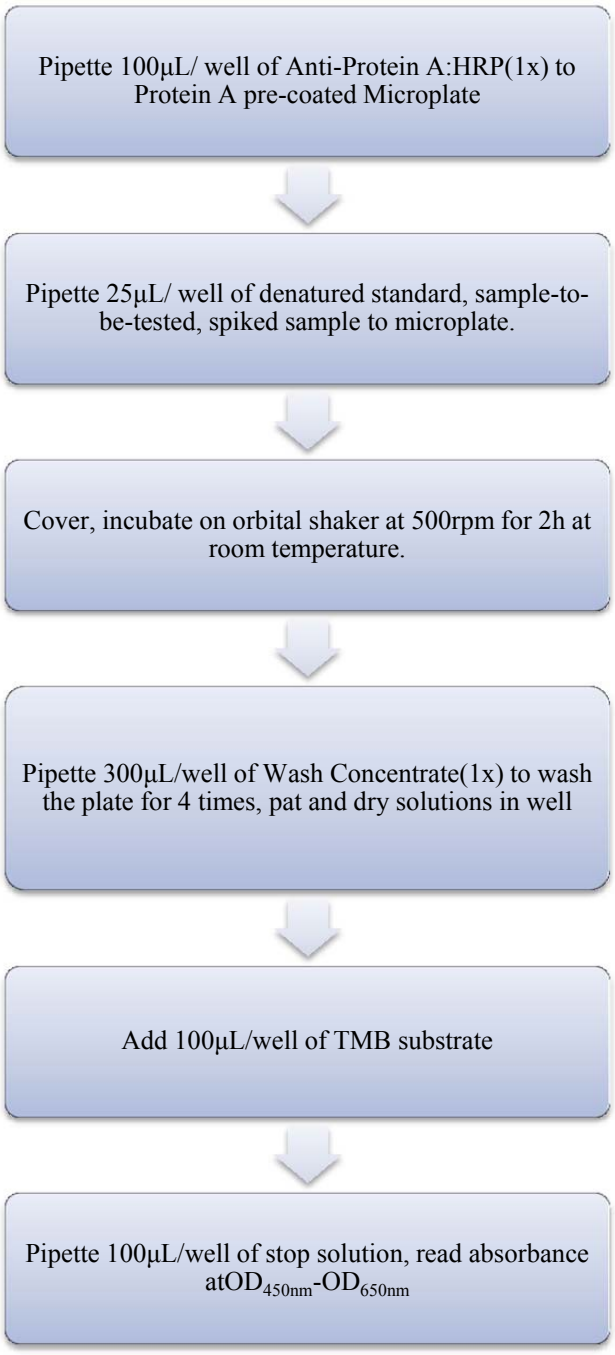
5. Kit Component

S/N	Component	Pack size	Description
1	Anti-Protein A pre-coated Microplate	8 well x12 strips	Dismantable, depend on practical requirement.
2	1#Standard	8 pcs x1mL	0,0.16,0.31,0.63,1.25,2.5,5,10ng/mL
3	2#Standard	8 pcs x1mL	0,0.16,0.31,0.63,1.25,2.5,5,10ng/mL
4	Anti-Protein A:HRP(100x)	150μL/pc	Enzyme-linked antibody, use Anti-Protein A:HRP Diluent to get 1 x dilution.
5	Anti-Protein A:HRP Diluent	12mL/bottle	Enzyme-linked antibody diluent
6	Denaturing Buffer	12mL/bottle	Citrate buffer solution, used for sample denaturation treatment
7	Sample Diluent	25mL/bottle	For sample dilution treatment
8	Stop Solution	12mL/bottle	Acid solution, corrosive
9	TMB	12mL/bottle	Solution containing 3,3',5,5' –tetramethylbenzidine and H_2O_2
10	Wash Concentrate(10x)	50mL/bottle	Use deionized water to get 1x dilution
11	Sample Treatment Plate	96 wells PCR plate	For sample denaturation treatment

6. Assay Protocol

- Kit preparation: bring all reagents to room temperature.
- Set up reagents:
  - Wash Concentrate (10x): wash with deionized water to get 1-fold dilution.
  - Anti-Protein A: HRP (100x): use Anti-Protein A: dilute HRP Diluent to 1x dilution.
- Sample preparation:
  - Sample to be tested: Dilute with Sample Diluent and set aside for use. It is recommended to get 10-fold or above dilution. Sample volume shall be no less than 100µL after dilution.
  - Spiked sample: Spiked sample recovery rate detection is an important criterion for the applicability and methodological validation of the experimental system. Volume of spiked sample shall be no less than 100µL.
- Experimental procedure:
  - Bring all reagents, components of kit to room temperature. Conduct all operation at room temperature. It is recommended to run sample in replicate for all wells (with sample).
  - Add sample: using Sample Dilute, dilute sample-to-be-tested, spiked sample to the right ratios. Pipette 50µL/well to Sample Treatment Plate. Pipette 25µL of Denaturing Buffer to each well, incubate on orbital shaker at 500rpm for 10min. Perform the same procedure to standard sample. Pipette 100µL Anti-Protein A: HRP (1x) to each well of polyclonal anti-Protein A coated microplate. Pipetter 25µL/well of denatured sample-to-be-tested, spiked sample and standard sample to microplate. Make sure run sample in replicate for each sample/standard sample. Incubate at 500rpm for 2 hours.
  - Wash: Shake the microplate to dry it. Pipette 300µL/ well of Wash Concentrate (1x), repeat the process for 4 times.
  - Coloring: pipette 100µL/ well of TMB substrate, incubate at room temperature in dark till the color of max concentration standard to dark blue.
  - **Recommended coloring time for 1#Standard and its corresponding sample-to-be-tested / spiked sample is 10 min. Coloring time for 2#Standard and its corresponding sample-to-be-tested / spiked sample is 5 min.**
  - Ending: add 100 µL/well of Stop Solution (Color turns from blue to yellow).
  - Read absorbance (OD) at 450nm and 650nm. Calculate the difference of OD between 450nm and 650 nm.

- Workflow of leached Protein A detection

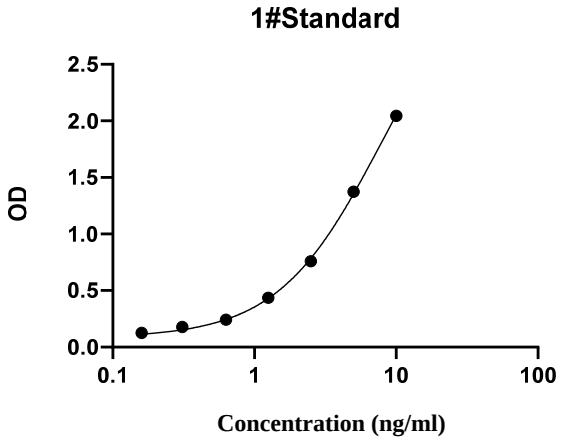


7. Data Analysis

- The calculation of OD: for each standard or sample, $OD=OD_{450nm}-OD_{650nm}$, OD should be the average value of samples run in replicate. Use microplate reader software or other softwares such as GraphPad Prism to analyze data. Using standard concentration as abscissa(X) and standard absorbance concentration as ordinate(Y), get standard curve. It is recommended to use four-parameter curve fitting equation. Calculate protein A content in sample and spiked sample using the fitted standard curve. Pay attention to the dilution factor.

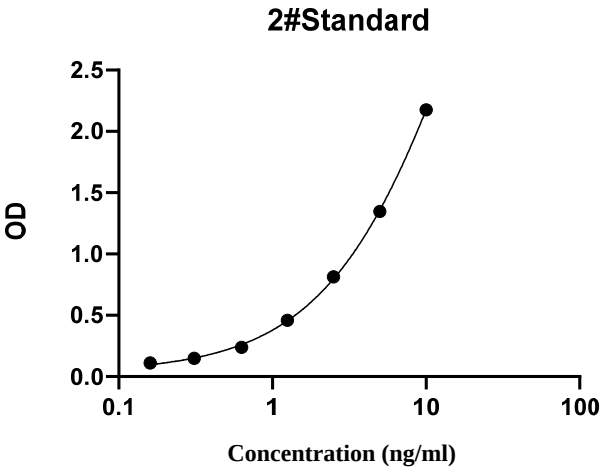
➢ For standard curve of 1#Standard

1#Standard conc. (ng/mL)	Average OD
10	2.046
5	1.373
2.5	0.759
1.25	0.436
0.63	0.242
0.31	0.178
0.16	0.125
0	0.057



➢ For standard curve of 2#Standard

2#Standard conc. (ng/mL)	Average OD
10	2.177
5	1.347
2.5	0.814
1.25	0.459
0.63	0.239
0.31	0.149
0.16	0.112
0	0.032



8. Product Performance

- Spiked experiment and recovery validation: Evaluation of 1#Standard and 2#Standard with the existence of human IgG. Since hIgG has good affinity to Protein A, mix 250ng/mL of Protein A with 25mg/mL of hIgG according to volume ratio of 1:1(ppm=10). Dilute the mixture with Sample Diluent, do denaturalizing treatment to sample. Get measured concentration of sample according to standard curve of the kit. Calculate the expected percentage value (n=9) by dividing the measured concentration by the expected concentration.
- Precision: intra and inter-assay precision.

➢ Intra-assay precision:

Conduct 16 repetitive detection to 3 Protein-A containing control samples via single experiment

#of Tests	1#Standard Concentration (ng/mL)	%CV
16	8	5.4
16	3	4.8
16	0.5	6.2

#of Tests	2#Standard Concentration (ng/mL)	%CV
16	8	6.1
16	3	5.7
16	0.5	7.3

➢ Inter-assay precision:

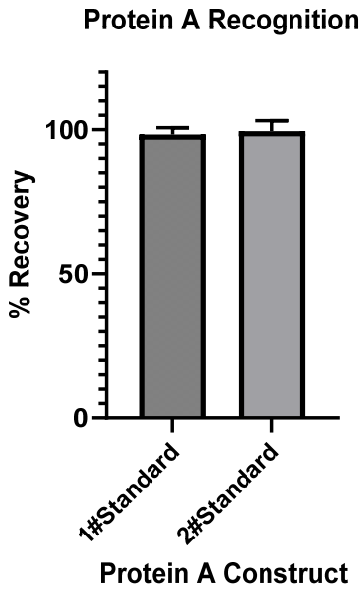
Conduct multiple detections to different Protein A-containing control samples in several days.

#of Tests	1#Standard Concentration (ng/mL)	%CV
3	8	6.4
3	3	7.2
3	0.5	5.9

#of Tests	2#Standard Concentration (ng/mL)	%CV
3	8	7.4
3	3	7.8
3	0.5	6.7

- Specificity: Protein A ELISA Kit can identify different recombinant forms of Protein A. The following picture shows evaluation of two Protein A constructs.

  Get measured concentration of sample according to standard curve of the kit. Calculate the expected percentage value (n=9) by dividing the measured concentration by the expected concentration.



9. Precautions

- Before using, all components of kit shall be recovered to room temperature.
- It is recommended to dilute sample to 1mg/mL or below, to prevent the formation of denatured precipitation.
- HOOK effect: Within certain concentration range, concentration of analyte shows positive correlation to detection signal. However, when analyte concentration reaches to a certain level, signal will decrease despite of the increase of analyte concentration. This phenomenon gets its name “hook effect” for the hook shape of concentration/signal curve. When protein A concentration is excessively high, detection level can be lower than 10ng/mL. In this case, dilution of sample (Protein A) can usually solve the issue. Therefore, when detecting Protein A leakage, it is recommended to conduct dilution linear evaluation to eliminate the impact of “Hook effect”.
- For recovery detection by adding Protein A (with known content), accepted recovery range is usually from 80%-120%. Extreme pH or salinity might cause abnormal recovery. Under some conditions, high concentrated antibody will cause negative interference. In that case, please contact Bestchrom Technical Support Team for help.
- Avoiding using pipette tip to touch the bottom of microplate, to prevent any damage to pre-coating.
- After washing the microplate, tap it to dry it. Make sure no strip is falling out.

- During the reaction, to minimize the solution evaporation from microplate, it is recommended to cover the microplate and Sample Treatment Plate using the covering membrane provided in Protein A ELISA Kit.
- Use the kit within its validity. When detecting Protein A leakage, it is necessary to use the right standard. Please avoid using reagents from different batches.
- Detection results variation might be caused by various factors, including experimental staff operation, the use of pipette.
- This kit is for in vitro research experiments only, not for clinical diagnosis.
- Other needed reagents(not provided by kit): deionized water, 1.5mL low-adsorption centrifuge tubes, high precision pipette & low adsorption pipette tip, tissue paper, microplate reader, microplate shaker (200-500rpm), software can be used for the fitting of four-parameter curve (e.g. GraphPad Prism).

10. Storage

Preserved at 2-8℃.

11. Ordering Information

Product	Cat.No.
Protein A ELISA Kit	EK001
Anti-Protein A pre-coated Microplate	EK001-01
1#Standard	EK001-02
2#Standard	EK001-03
Anti-Protein A:HRP(100x)	EK001-04
Anti-Protein A:HRP Diluent	EK001-05
Denaturing Buffer	EK001-06
Sample Diluent	EK001-07
Stop Solution	EK001-08
TMB	EK001-09
Wash Concentrate(10x)	EK001-10
Sample Treatment Plate	EK001-11