Pichia Pastoris Host Cell Protein ELISA Kit, G3

(Applicable to X-33 and Derivatives)

96 Wells

Catalogue Number: PH-E0021-3B1

ELISA Kit for the Quantitative Measurement of *Pichia Pastoris* HCP Residues in Protein Purification Process and End-Product

FOR RESEARCH, DEVELOPMENT AND MANUFACTURING USE ONLY!
NOT FOR THERAPEUTIC OR DIAGNOSTIC APPLICATIONS!
PLEASE READ THROUGH ENTIRE PROCEDURE BEFORE BEGINNING!

CONTENTS

1. BACKGROUND

This kit is intended for the quantitative detection of host cell protein (HCP) residues in biopharmaceuticals produced by *Pichia Pastoris* (X-33 and Derivatives) expression systems.

As a commonly used expression system, *Pichia Pastoris* produces target proteins while concomitantly triggering its own cellular apoptosis. According to data, the host proteins released into the culture medium after cell lysis number in the thousands, a large proportion of which exhibit strong immunogenicity. These proteins can induce adverse toxic or immune responses that compromise product safety and quality, thereby posing a risk of potential biological contamination. One of the core objectives of downstream processing in biopharmaceutical production is to eliminate these potential hazards.

Therefore, it is imperative to minimize the residual level of host cell proteins (HCPs). During the development of downstream purification processes, a scientifically sound method must be established for quantifying HCP concentrations in both finished and semi-finished products. Enzyme-linked immunosorbent assay (ELISA) boasts ultra-high sensitivity, and thus has been designated as the gold standard for HCP detection by domestic and international regulatory authorities including the FDA, EMA, NMPA and ICH.

2. PRINCIPLE OF THE ASSAY

This ELISA kit is applied to the solid phase sandwich Enzyme-Linked Immunosorbent Assay. The microplate has been pre-coated with a capture antibody. The antigen to be measured and the HRP-conjugated detection antibody are then added to the microplate wells sequentially to form a capture antibody-antigen-detection antibody complex. After washing, the conjugates uninvolved in the reaction are removed, and then a TMB substrate solution is added to the wells to incubate. TMB substrate solution turns blue after the oxidation of HRP and finally turns yellow immediately after adding the stop solution. The color of the TMB substrate positively correlated with the antigen bound in the initial steps. Measure the absorbance (OD value) at 450nm using a microplate reader, and create a standard curve along with the corresponding concentrations. Then, by inputting the OD values of the samples into the standard curve equation, calculate the concentration of the target

protein in the sample.

3. KITS' ADVANCEMENT

1) High Coverage: The capture and detection antibody exhibit a strong capacity for recognizing host cell proteins (HCPs), with a coverage rate of over 80% and the manufacturing process demonstrated excellent process stability across multiple batches.
2) High Antibody Titers: The antibodies used in the reagent kit are tested using the indirect method with ELISA, and the results indicate a titer of 10^6.
3) High Sensitivity: Serum antibody purification employs affinity purification to remove non-specific antibodies to the greatest extent.
4) High Stability: The production process uses a broad-spectrum protein stabilizer and microplate processing technology to enhance the stability and repeatability of the standard and microplate result.
5) Applicability: The kit was evaluated under different reaction temperatures (20-30°C) and reaction time variations (±10 minutes), demonstrating excellent reproducibility of test results
6) Optimal Diluent Buffer: The sample diluent has been optimized to accommodate the different buffer systems used at various stages of antibody purification. This significantly reduces nonspecific binding during sample analysis, improves dilution linearity and recovery rates, and maintains an extremely low background signal, facilitating accurate detection of the target sample concentration.

4. MATERIALS (Note: Store at 2-8°C)

	Reagents	Specification	Quantity
1	Pre-Coated Microplate (Detachable)	96 wells	1 plate (Keep Sealed)
2	Standard (Stock Solution) -500μg/mL	50μL	1 tube
3	Detection antibody (100×)	150μL	1 tube
4	TMB Substrates	10mL	1 vial (Avoid Light)
5	Stop Solution	10mL	1 vial

6	Wash Solution (20×)	10mL	5 vials
7	Diluent Buffer (10×)	10mL	1 vial
8	Plate Sealer		4 pieces
9	Instruction Manual		1

5. EQUIPMENT REQUIRED BUT NOT PROVIDED

1) 10-1000μL Pipettor
2) Multichannel Pipettor
3) 1L sterilized Deionized Water or Ultrapure Water
4) Sterilized EP Tubes
5) Absorbent Paper
6) Microplate Reader
7) High-speed Centrifuge
8) Mini Centrifuge
9) Microplate Washer or Washing Bottles
10) Data Analysis and Graphing Software

6. PREPARATION BEFORE ASSAY

Please read the kit instruction manual carefully before use. All reactions are performed at room temperature.

7. REAGENT PREPARATION

1) Place all kits' components at room temperature for 30 minutes before using.
2) The preparation of Wash Solution (1×): Add the contents of five 10mL of Wash Solution (20×) into 950mL of deionized water or ultrapure water to prepare 1000mL of Wash Solution (1×). If crystals have formed in the concentrate, allow it to reach room temperature and mix gently until the crystals are completely dissolved.
3) The preparation of Diluent Buffer (1×): Dilute 10mL of Diluent Buffer (10×) with 90mL of deionized water or ultrapure water to prepare 100mL of Diluent Buffer (1×). If crystals have formed in the concentrate, allow it to reach room temperature and mix gently until the crystals

are completely dissolved. The buffer is used as the Diluent Buffer for standards, samples and detection antibodies.

8. ASSAY PROCEDURE

1) Remove the microplate from the foil bag, detach the desired number of strips from the plate, immediately reseal the remaining strips and store at 4°C. Then put the ready-for-use strips on a clean microplate frame, and start the experiment after the strips returned to room temperature (**Note:** The microplate frame can be reused). The standard may stick to the tube wall due to transport turbulence. Before using, gently shake it or centrifuge for about 2 seconds.

2) Prepare Standards: When preparing the standards, label 8 tubes as IS0, S1, S2, S3, S4, S5, S6, S7 and add the following volumes of Diluent Buffer (1×) to each tube: IS0 (98μL), S1 (480μL), S2 to S7 (each 250μL).

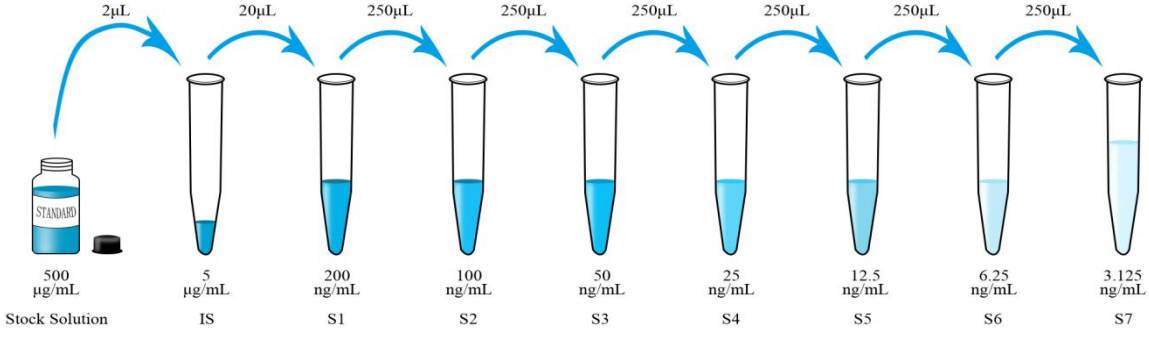
① Add 2μL of Standard (Stock Solution - 500μg/mL) into the IS0. Mix gently by pipetting up and down twice, then invert to mix thoroughly.

(**All subsequent mixing steps should be performed in the same manner—do not vortex or use any vigorous mixing method**.)

② Transfer 20μL of the resulting IS0 (5μg/mL) to the tube S1. Mix gently by pipetting up and down twice, then invert to mix thoroughly.

③ Transfer 250μL of S1 (200ng/mL) to S2, mix gently; then transfer 250μL of the resulting S2 solution (100ng/mL) to S3, mix gently. Continue this 2-fold serial dilution sequentially through S7 to achieve a final concentration of 3.125ng/mL.

NOTE: There are 7 points of diluted standards, S1: 200ng/mL, S2: 100ng/mL, S3: 50ng/mL, S4: 25ng/mL, S5: 12.5ng/mL, S6: 6.25ng/mL, S7:3.125g/mL.



3) Sample Addition: Add 100μL of standards or test samples to the microplate wells. For the Blank Control, add 100μL of Sample Diluent only.
4) Cover the plate with sealer, put it on the oscillator to mix and incubate for 90 minutes at room temperature.
5) Automated Washing:

- Put 1000mL Wash Solution (1×) into the washing bottle of the automated microplate washer for standby.
- Take out the microplate, and discard the incubation mixture. And then wash the microplate with the automated microplate washer for 5 times.
- After washing, invert plate and blot dry by hitting the plate onto absorbent paper or paper towels until there is no moisture appears.

Or Manual Washing:

- Put 1000mL Wash Solution (1×) into the washing bottle for standby.
- Take out the microplate, and discard the incubation mixture. And then invert plate and blot dry by hitting the plate onto absorbent paper or paper towels until there is no moisture appears.
- Fill each well with 300μL Wash Solution (1×) by a muti-channel pipette, let stand for 20 seconds, then discard the contents, invert plate, and blot dry by hitting the plate onto absorbent paper. Repeat this washing step for 5 times.

  Note: There should be no moisture appears in the fifth washing step.

6) Prepare Detection Antibody: Dilute 100μL of Detection antibody (100×) to its working concentration (1×) with 9.9mL Diluent Buffer and mix gently. Add 100μL of the resulting diluted detection antibody to each well, seal the plate with an adhesive plate sealer, and incubate at room temperature on a shaker for 1.5 hours.
7) Washing Step: Repeat the same procedure as step 5.
8) Add 100μL TMB substrate to each well. Cover the plate with a sealer, incubate at room temperature for about 15 minutes. If the color is light, the reaction time can be extended appropriately, but not more than 30min.

9) Add 50μL of Stop Solution to each well to stop the reaction.

10) Run the microplate reader and conduct measurement at 450nm.

11) Data analysis: Four parameter curve fitting is recommended.

9. NOTES

1) Sample Preparation

a) After collection, the samples should be aliquoted and stored at -20°C (less than 3 months) or -80°C (less than 6 months) to maintain protein activity, avoid contamination and multiple freeze-thaw cycles. If samples are to be run within 24 hours, they may be stored at 2- 8°C.

b) Ensure the frozen samples are completely thawed before use (do not thaw by heating). Mix thoroughly using a pipette or vortex mixer. If precipitates are present in the sample, they may interfere with the ELISA results; centrifugation is recommended to remove the precipitates.

c) Some chemical solvents may interfere with this experiment, such as SDS, Triton, etc.

d) It is recommended that all standards and samples be run in duplicate. Please gently mix the liquid after adding standards and samples, and confirm there's no bubble inside.

e) The process of target protein purification is often accompanied by complex buffer solutions. To exclude matrix effect, it is recommended to perform spike recovery test when using different buffers for the first time. An acceptable spike recovery range is 80-120%. High salt, low PH, polysaccharide, organic solvents, and detergents can result in lower recovery rates. The specific procedure is to add Standard S1(200ng/mL)/Diluent Buffer into a buffer solution in a 1:1 volume ratio (e.g. add 50μL of standard S1(200ng/mL)/Diluent Buffer to 50μL of the buffer solution). The recovery rate is calculated by subtracting the background concentration without the S1 from the concentration with S1, then dividing by the theoretical concentration.

2) Experimental Operation

a) Do not mix or interchange different ELISA kits or same ELISA kit but with different lot.

b) To avoid cross-contamination, use disposable sterilized tips during the experiment.

c) Total dispensing time for addition of reagents or samples to the plate should not exceed 10 minutes. If there are too many samples, a multichannel pipettor is recommended.

d) Incomplete washing will adversely affect the test outcome. All washing must be performed with Wash Solution provided. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells. Do not directly touch the bottom of the microplate. If it is covered with fingerprints or stains, it will affect the measurement.

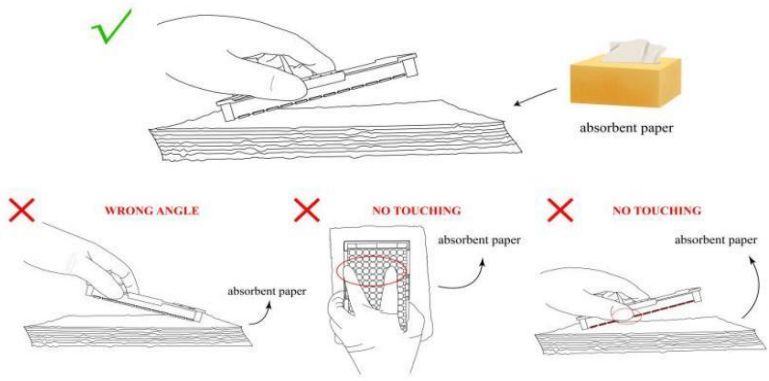
e) TMB is easily contaminated. Please protect it from light and metal.

f) Monitor the reaction time. After adding TMB substrate, observe the color change of reaction wells at regular intervals (for example, about 10 minutes). If the color turns too deep, add Stop Solution in advance to terminate the reaction.

g) The Stop Solution provided with this kit is diluted sulfuric acid. Protective gloves, clothes, and face protection should be worn.

h) The coefficient of determination of the standard curve should be $R^2 \geqslant 0.95$.

i) Schematic diagram of tapping the plate:



10. CALCULATION OF RESULTS

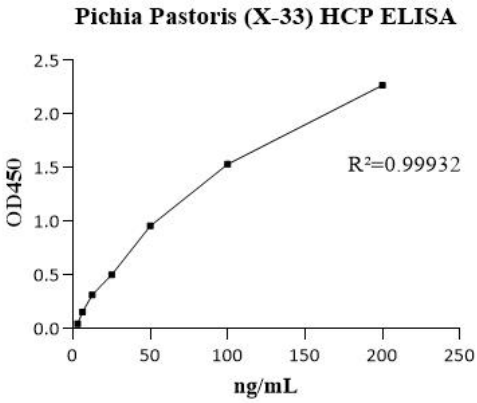
1) Average the duplicate readings for each standard and sample.

2) Construct a standard curve by plotting the average O.D. for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis, and generate a four paramater logistic (4-PL) curve-fit.

3) Calculate the concentration of samples corresponding to the mean absorbance from the standard curve.

4) Standard curve is for demonstration only.

APPENDIX 1: EXAMPLE OF STANDARD CURVE



	ng/mL	OD450
S1	200	2.270
S2	100	1.534
S3	50	0.959
S4	25	0.504
S5	12.5	0.317
S6	6.25	0.158
S7	3.125	0.046

11. QUALITY CONTROL

1) Sensitivity:

LOD: 0.39ng/mL

LOQ: 3.125ng/mL

2) Precision:

Intra Variation%: 2-5

Inter Variation%: 3-7

3) Specificity:

Sample	Concentration
Ogataea polymorpha HCP	<1.5%
Saccharomyces cerevisiae HCP	<0.2%
Pichia Pastoris HCP	>50%

12. FAQs & TROUBLESHOOTING

If the experimental results are abnormal, promptly take photos to record the color development results, fully retain the unused strips and reagents, and contact technical support. In addition, you

may refer to the troubleshooting information provided below to identify the root cause of the problem.

Problem Description	Possible Causes	Corresponding Solutions
Poor Standard Curve	Incorrect dilution of standards	Dilute the standard curve according to the specified ratio.
	Inaccurate pipetting or sample loading	Check the pipette and pipette tips.
	Incomplete washing of the microplate	Ensure the required number of washing cycles and the volume of washing solution per well.
Weak or Absent Color Development	Insufficient incubation time	Ensure adequate incubation time.
	Incorrect experimental temperature	Use the recommended incubation temperature.
	Insufficient reagent volume or missing addition	Check the pipetting and sample loading process to ensure all reagents are added in sequence with sufficient volume.
	Substrate solution not equilibrated to room temperature	Allow the TMB substrate to stand at room temperature for more than 30 minutes before color development.
Low OD Value Reading	Incorrect microplate reader settings	Verify the wavelength and filter configuration on the microplate reader. Preheat the microplate reader in advance before reading.
High Coefficient of Variation (CV)	Incorrect sample loading	Check the sample loading operation.
	Contamination on the microplate bottom	Inspect the microplate bottom for residual liquid and fingerprints.
	Foreign objects or air bubbles in wells	Confirm no foreign objects in wells before sample loading and no air bubbles after loading.
	Unsealed or incompletely sealed plate during	Seal the plate with plate sealing film.

	incubation	
High Background Value	Incomplete washing of the microplate	Wash the plate according to the method recommended in the instruction manual.
		If using an automated plate washer, check for clogs in all liquid inlets and waste outlets.
		For manual washing, appropriately increase the number of washing cycles.
		Inadequate or missed washing will result in high background.
	Incorrect incubation time or temperature	Operate strictly in accordance with the instruction manual.
	Contaminated consumables	Ensure the tubes, pipette tips and other consumables are clean.
	Contaminated washing solution	Prepare fresh washing solution.
	Contaminated substrate solution	The substrate solution is inherently colorless. Ensure the substrate is not contaminated by metal ions or oxidizing agents before use and store it away from light.
Low Sensitivity	Improper kit storage	Store all relevant reagents according to the requirements in the instruction manual.

13. CONTACT US

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