Version 04-01/25

User's Manual

Pichia pastoris HCP ELISA kit

REF **DEIA-JY24011**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

This kit is intended for use in determining the presence of Pichia pastoris protein impurities in products manufactured by recombinant expression in Pichia pastoris host cells.

General Description

Recombinant expression by the yeast Pichia pastoris is a relatively simple and cost effective method for production of complex proteins. Many of these recombinant proteins are intended for use as therapeutic agents in humans and animals and as such must be highly purified. The manufacturing and purification process of these products leaves the potential for impurities by host cell proteins (HCPs) from Pichia pastoris. Such pollutants can reduce the efficacy of the therapeutic agent and result in adverse toxic or immunological reactions and thus it is desirable to reduce HCP impurities to the lowest levels practical. Immunological methods using antibodies to HCPs such as Western Blot and ELISA are conventionally accepted. While Western blot is a useful method aiding in the identity of HCPs, it suffers from a number of limitations. Western blot is a complex and technique dependent procedure requiring a subjective interpretation of results. Furthermore, it is essentially a qualitative method and does not lend itself to obtaining quantitative answers. The sensitivity of Western blot is severely limited by the volume of sample that can be tested and by interference from the presence of high concentrations of the intended product. While Western Blot may be able to detect HCPs in samples from upstream in the purification process it often lacks adequate sensitivity and specificity to detect HCPs in purified downstream and final product. The microtiter plate immunoenzymetric assay (ELISA) method employed in this kit overcomes the limitations of Western blots providing on the order of 100 fold better sensitivity. This simple to use, highly sensitive, objective, and semiquantitative ELISA is a powerful method to aid in optimal purification process development, process control, routine quality control, and product release testing. This kit is "generic" in the sense that it is intended to react with essentially all of the HCPs that could pollute the product independent of the purification process. The antibodies have been generated against and affinity purified using a mild lysate washed of Pichia pastoris cells to obtain HCPs typically encountered in your initial product recovery step. Western blot was used as a preliminary method and established that the antibodies reacted to the majority of HCP bands resolved by the PAGE separation.

Special procedures were utilized in the generation of these antibodies to ensure that low molecular weight and less immunogenic impurities as well as high molecular weight components would be represented. As such this kit can be used as a process development tool to monitor the optimal removal of host cell impurities as well as in routine final product release testing. Because of the high sensitivity and broad reactivity of the antibodies, this generic kit has been successfully qualified for testing of final product HCPs in many different products regardless of growth and purification process. When the kit can be satisfactorily qualified for your samples, the application of a more process specific assay is probably not necessary in that such an assay would only provide information redundant to this generic assay. However, if your qualification studies indicate the antibodies in this kit are not sufficiently reactive with your process specific HCPs it may be desirable to also develop a more process specific ELISA. This later generation assay may require the use of a more specific and defined antisera. Alternatively, if the polyclonal antibody used in this kit provides sufficient sensitivity and broad antigen reactivity, it may be possible to substitute the standards used in this kit for ones made from the pollutants that typically co-purify through your purification process and thus achieve better accuracy for process specific HCPs. The suitability of this kit for a given sample type and product must be

determined and qualified experimentally by each laboratory. The use of a process specific assay with more defined antigens and antibodies in theory may yield better sensitivity however such an assay runs the risk of being too specific in that it may fail to detect new or atypical impurities that might result from some process irregularity or change. For this reason it is recommended that a broadly reactive "generic" host cell protein assay be used as part of the final product purity analysis even when a process specific assay is available.

Principles of Testing

The Pichia pastoris assay is a two-site immunoenzymetric assay. Samples containing Pichia pastoris HCPs are reacted in microtiter strips coated with an affinity purified capture antibody. A second horseradish peroxidase (HRP) enzyme labeled anti-Pichia pastoris antibody is reacted simultaneously resulting in the formation of a sandwich complex of solid phase antibody-HCP-enzyme labeled antibody. The microtiter strips are washed to remove any unbound reactants. The substrate, tetramethylbenzidine (TMB) is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and is directly proportional to the concentration of Pichia pastoris HCPs present.

Reagents And Materials Provided

1. **Anti-P. *pastoris* coated microtiter strips**: The appropriate amount of sheep anti-P. *pastoris* HCPs polyclonal antibodies has been coated. It is sealed in an aluminum foil bag and contains a desiccant.
2. **P. *pastoris* HCPs Standard**: 2 vials. Lyophilized. Accurately measure 500 µL of the reconstitution solution, dissolve it, and let it stand for 5-10 minutes. The solution should appear clear and transparent with no visible insoluble particles. The specific concentration can be found labeled on the bottle.
3. **Standard Dilution Buffer**: 1.5 mL × 2 vials.
4. **Sample Dilution Buffer**: 25 mL × 2. Dilutions are required for test samples, and enzyme-labeled antibodies. For samples being tested for the first time, a sample suitability verification is necessary to determine the optimal dilution factor.
5. **Washing Buffer 10×**: 25 mL × 2. Crystallization may occur at low temperatures. To dissolve, the Washing Buffer can be placed in a 37°C water bath. Prior to use, dilute the Washing Buffer 10-fold with freshly prepared ddH2O.
6. **P. *pastoris* HCP-HRP Antibody 100×**: 120 µL × 1. The HRP-conjugated sheep polyclonal antibody against SF9 HCPs should be diluted 100-fold with the Sample dilution buffer before use. Avoid light.
7. **TMB Substrate**: 12 mL×1. Equilibrate for at least 20 minutes at room temperature. Store in a dark and sealed tightly.
8. **Stop Solution**: 6 mL×1. Hydrochloric acid. When handling, please wear safety goggles and avoid contact with the skin.
9. Plate sealers: 3.

Materials Required But Not Supplied

1. Microtiter plate reader spectrophotometer with dual wavelength capability at 450 & 420-650 nm. (If your plate reader does not provide dual wavelength analysis you may read at just the 450 nm wavelength.)
2. Pipettors
3. Repeating or multichannel pipettor - 100µL

4. Microtiter plate rotator (400 - 600 rpm)
5. Distilled water
6. 1 liter wash bottle for diluted wash solution
7. Absorbent pads (tissue)

Storage

2-8°C

Microtiter strips: After opening, the microtiter strips, along with the desiccant, should be sealed in a self-sealing bag for stable storage at 2-8 °C for 60 days.

Reconstituted calibrator: Short-term storage: store at 2-8 °C for 7 days; Long-term storage: store at -18 °C or below for 60 days.

Specimen Collection And Preparation

Samples: Expressing purified process samples, stock solutions and other solutions samples should be clear and transparent, with insoluble components removed through methods like centrifugation or filtration.

Prior to storing the samples, it is crucial to establish their stability and identify the optimal storage conditions. For long-term storage, it is generally recommended to keep samples at -65°C or below, while minimizing freeze-thaw cycles.

For initial use or situations where the HCPs content in the sample is unknown, it is highly advisable to conduct sample suitability verification to determine the suitable sample dilution ratio for accurate subsequent routine testing. Dilute the samples appropriately with a 1× dilution buffer based on their estimated concentration of HCPs, ensuring that the detection values fall within the quantification range of the calibration curve.

Plate Preparation

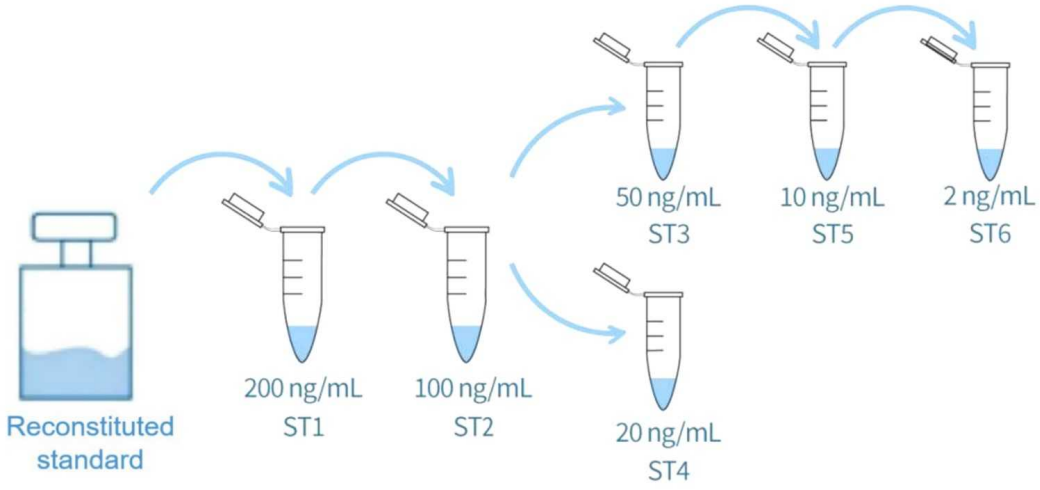
1. Remove the pre-sealed microplate from the packaging and let it equilibrate at room temperature for approximately 20 minutes. Other reagents should also be taken out in advance and allowed to equilibrate at room temperature before use. After use, promptly return them to 2-8°C storage.
2. Calculate the number of wells needed based on the number of samples to be tested. Take out the corresponding number of microplate strips, and seal the remaining strips along with the desiccant in a selfsealing bag. Place the bag back into the kit box and store it in a 2-8°C refrigerator until the expiration date.

Note: Crystallization on some well walls of the microplate is a normal phenomenon and does not require any special treatment.

Reagent Preparation

1. **P. *pastoris* HCPs Standard**: Accurately measure 500 µL of the reconstituted standard solution and transfer it into a vial. Gently invert the vial to mix, and allow it to stand for 5-10 minutes. The concentration of HCPs in the reconstituted calibration standard is equivalent to the labeled concentration. The solution can be stored at 2- 8°C after reconstitution and should be used within the expiration date.

Note: If multiple vials of calibrator are to be used simultaneously, please dissolve them separately, transfer to a 1.5 mL sterile centrifuge tube together, shake well, and then use. If the reconstitution solution of each vial of calibrator is subjected to multiple freeze-thaw cycles, it is recommended to aliquot into 1.5 mL sterile centrifuge tubes and store at ≤ -20°C.



		Concentration (ng/mL)
ST1	Dilute the standard lyophilized to ST1 with standard dilution buffer.	200
ST2	500 µL ST1 + 500 µL Dilution Buffer	100
ST3	400 µL ST2 + 400 µL Dilution Buffer	50
ST4	100 µL ST2 + 400 µL Dilution Buffer	20
ST5	100 µL ST3 + 400 µL Dilution Buffer	10
ST6	100 µL ST5 + 400 µL Dilution Buffer	2
NCS (Negative Control)	Dilution Buffer	0

2. **Washing Buffer (1× conc.)**: Dilute Washing Buffer 1 + 9; e. g. 25 mL Washing Buffer (10× conc.) + 225 mL distilled water. In case crystals appear in the concentrate, warm up the solution to 37°C e.g. in a water bath. Mix well before dilution.
3. **P. *pastoris* HCP-HRP Antibody**: The HRP-conjugated sheep polyclonal antibody against P. *pastoris* HCPs should be diluted 100-fold with Sample dilution buffer before use.

Assay Procedure

1. Add 100 µL of the P. *pastoris* HCP-HRP Antibody into the corresponding microplate wells. Ensure that the solution is added to the bottom of the wells and avoid generating bubbles during the operation.
2. Add 100 µL of the Standard Work Solution and 1× Dilution Buffer (NCS) into the corresponding microplate wells. Prepare 2-3 replicate wells for each concentration and record the location of each concentration well.
3. Add the prepared test samples into the corresponding microplate wells. After adding the samples, seal the microplate with a sealing film and place it on a microplate constant temperature shaker. Incubate at room temperature at 600 rpm for 3 hour.

Refer to the layout in Table below to arrange the 96-well plate based on the number of samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NCS	NCS	NCS									
B												
C	ST6	ST6	ST6	S1	S1	S1						
D	ST5	ST5	ST5	S2	S2	S2						
E	ST4	ST4	ST4	S3	S3	S3						
F	ST3	ST3	ST3	S1+SRC	S1+SRC	S1+SRC						
G	ST2	ST2	ST2	S2+SRC	S2+SRC	S2+SRC						
H	ST1	ST1	ST1	S3+SRC	S3+SRC	S3+SRC						

This example shows a calibration of 6 concentrations (ST1-ST6), a negative control (NCS), 3 test samples (S1-S3), and sample spike control (S1 SRC-S3 SRC) for each test. 2-3 replicate wells for each test.

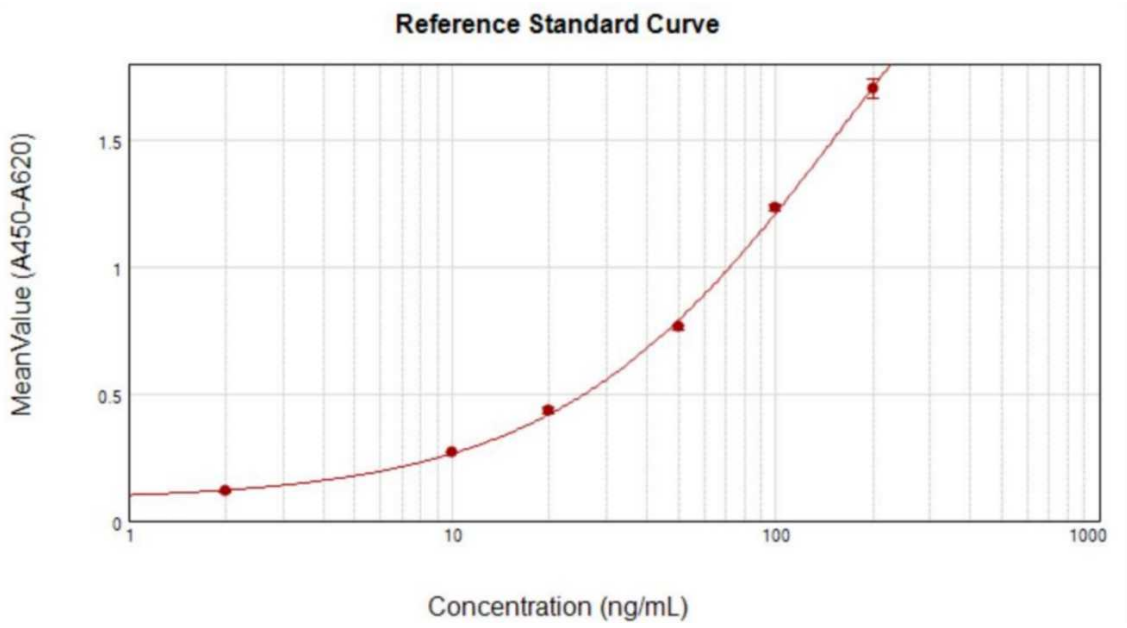
4. After incubation, remove the plate sealer, discard the contents and wash the strips 5 times with 300 µL 1× Washing Buffer, ensuring every well is filled. When washing is completed, tap the strips firmly on absorbent tissue to remove residual Washing Buffer.
5. Add 100 µl TMB substrate into each well.
6. Do not cover the strips! Incubate for 10 minutes at room temperature (15-30°C) * in the dark.
7. Add 50 µl stop solution into each well and mix well.
8. Determine absorption after 5 minutes with an ELISA reader at 450 nm and 620 nm-650 nm as a reference. If no reference wavelength is available, read only at 450 nm.

* The intensity of the color change is temperature sensitive. We recommend observing the color change and stopping the reaction upon good differentiation.

Calculation

1. The OD450 nm values of each well should be subtracted by the respective long wavelength OD value. If the plate reader is not equipped with a long wavelength filter, this step can be omitted.
2. After subtracting the OD value of the negative control from the OD values of each calibration point and sample, calculate the average of the 2 or 3 replicate wells. Perform a four-parameter fit of the concentration values and OD values of the calibration points to obtain the calibration curve equation. Substitute the average OD value of the sample into the equation to calculate the sample concentration. This concentration should be multiplied by the dilution factor to obtain the actual concentration of the sample.
3. The curve fitting software can be the one provided with the plate reader. If not available, it is recommended to use professional curve fitting software such as Curve Expert, ELISA Calc, etc.

Typical Standard Curve



Curve Fit : 4-Parameter Logistic $y = D + \frac{A-D}{1+\left(\frac{x}{C}\right)^B}$

	Parameter	Estimated Value	Std. Error	Confidence Interval
STD $R^2 = 0.999$ EC50 = 170.0	A	0.080	0.042	[-0.102, 0.262]
	B	0.965	0.150	[0.321, 1.608]
	C	170.0	77.01	[-161.3, 501.4]
	D	3.102	0.686	[0.152, 6.052]

Detection Range

2-200 ng/mL

Detection Limit

LLOQ: 2 ng/mL

LOD: 0.5 ng/mL

Specificity

It has good quantitative accuracy for common strains of Pichia pastoris (GS115, X33, etc.); no obvious cross-reaction with common expression hosts such as Sf9, E.coli, CHO, etc.; the cross-reaction rate for other yeast species (Saccharomyces cerevisiae, Hansenula, etc.) is less than 1%

Antibody coverage: 72.1%-96.0% (IMBS-2D)

Broad spectrum of quantitative calibrators: 1,139 protein spots can be identified under two-dimensional electrophoresis silver staining conditions (2D-PAGE), which is widely representative.

Precautions

1. All reagents must be prepared using sterile disposable pipettes, test tubes and sample slots, etc. Do not mix them. Avoid contamination of the connection part of the micropipette tip. It is recommended to wipe it with 75% alcohol before and after each experiment. Standardize the pipetting operation. It is strictly forbidden to suck the liquid into the pipette or place it horizontally on the table without removing the pipette tip.
2. The calibrators and samples should be gently and sufficiently diluted and mixed, avoiding excessive foam formation.
3. The stop solution is an acidic solution. Pay attention to the protection of eyes, face, hands and clothes during use.
4. Avoid mixing different batches of test kits.
5. The water used to prepare the buffer solution must be sterile water or freshly prepared ultrapure water, and the water temperature must not exceed 37°C .
6. When adding samples, carefully dispense them into the bottom of the ELISA plate without touching the well walls. Avoid bubble formation, and gently shake to mix if needed. If there are bubbles before testing on the machine, they need to be punctured with a clean 10 µL pipette or needle. Be careful not to suck away the liquid in the well, which will cause large errors in the results.
7. Cover the ELISA plate with a film during the incubation reaction to prevent sample evaporation.
8. After pouring off the buffer, the subsequent solution should be added immediately. Do not let the ELISA wells dry to prevent affecting the detection performance of the kit.
9. Unused ELISA strips need to be stored in the self-sealing aluminum foil bag attached to the kit to avoid contamination by other samples, which will cause the kit to be scrapped.
10. The preparation of calibration products and sample dilution must be accurate. The minimum sampling volume during preparation should not be less than 5 µL to prevent large errors in the results.
11. Pichia pastoris HCP enzyme-labeled antibody (100×) Please quickly centrifuge before use to shake the residual reagent in the tube cap to the bottom of the tube to prevent contamination and loss of the reagent.
12. Calibrators and 1× Pichia pastoris HCP enzyme-labeled antibodies that have been diluted to working concentrations are not recommended for reuse because their stability cannot be guaranteed.
13. The color developing solution should be a colorless and transparent liquid. Be sure to replace a clean pipette tip when aspirating to prevent HRP contamination. If a light blue color is found, please discard it.
14. Make sure to test on the machine 5-10 minutes after adding the stop solution, the result will be more stable, and the time should not exceed 30 minutes.
15. Sodium azide cannot be added to the sample because it can inhibit HRP activity and has a great impact on the test results.

Limitations

1. This product is only for research purposes and not for clinical diagnosis.
2. Too low or too high pH value of sample solution may exceed the buffer range of diluent, thus affecting the performance of the kit. It is recommended to control the pH of sample solution between 6.5-8.5.
3. The components of sample matrix and preparation should not be products expressed by Pichia pastoris, otherwise the sample quantification may be abnormal due to the residue of Pichia pastoris HCP in the components.
4. If the main product in the sample has protease activity, the sample quantification may be abnormal due to

enzyme degradation. It is recommended to inactivate the main product by appropriate means before testing.

Tel: 1-631-624-4882 (USA)
Tel: 44-161-818-6441 (Europe)
Fax: 1-631-938-8221
Email: info@creative-diagnostics.com