

***Pichia pastoris* Host Cell Proteins**

Immunoenzymetric Assay for the Measurement of ***Pichia pastoris* Host Cell Proteins** Catalog # F140

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. The kit is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals.

Summary and Explanation

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 impurities 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 semi-quantitative 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. If you have a need of a more sensitive method to demonstrate coverage to HCPs in your process *Cygnus Technologies* recommends a method that is superior to Western blot called Antibody Affinity Extraction (AAE). AAE has greatly increased sensitivity and specificity to Western blot which makes it a better predictor of how the antibodies will perform in the ELISA. For additional information on AAE please visit our website and read the posted articles under Technical Documents or contact our Technical Services Department.

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 impurities 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. If you deem a more process specific assay is necessary, *Cygnus Technologies* is available to apply its proven technologies to develop such antibodies and assays on custom basis.

Reagents & Materials Provided

Component	Product #
Anti-<i>P. pastoris</i>:HRP Affinity purified goat antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	F141
Anti-<i>P. pastoris</i> coated microtiter strips 12x8 well strips in a bag with desiccant	F142*
<i>P. pastoris</i> HCP Standards Solubilized <i>P. pastoris</i> HCPs in a bovine serum albumin matrix with preservative. Standards at 0, 1, 4, 20, 75, and 250ng/mL. 1 mL/vial	F143
Stop Solution 0.5M sulfuric acid. 1x12mL	F006
TMB Substrate 3,3',5,5' Tetramethylbenzidine. 1x12mL	F005
Wash Concentrate (20X) Tris buffered saline with preservative. 1x50mL	F004

*All components can be purchased separately except # F142.

Principle of the Procedure

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.

Storage & Stability

- All reagents should be stored at 2°C to 8°C for stability until the expiration date printed on the kit.
- After prolonged storage, you may notice a salt precipitate and/or yellowing of the wash concentrate. These changes will not impact assay performance. To dissolve the precipitate, mix the

wash concentrate thoroughly and dilute as directed in the “Preparation of Reagents” section.

- Reconstituted wash solution is stable until the expiration date of the kit.

Materials & Equipment Required But Not Provided

- Microtiter plate reader spectrophotometer with dual wavelength capability at 450 & 650nm. (*If your plate reader does not provide dual wavelength analysis you may read at just the 450nm wavelength.*)
- Pipettors - 25µL and 100µL
- Repeating or multichannel pipettor - 100µL
- Microtiter plate rotator (400 - 600 rpm)
- Sample Diluent (recommended Cat # 1028)
- Distilled water
- 1 liter wash bottle for diluted wash solution

Precautions

- For Research or Manufacturing use only.
- Stop reagent is 0.5M H₂SO₄. Avoid contact with eyes, skin, and clothing
- This kit should only be used by qualified technicians.

Preparation of Reagents

- Bring all reagents to room temperature.
- Dilute wash concentrate to 1 liter in distilled water, label with kit lot and expiration date, and store at 4°C.

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL “total immunoreactive HCP equivalents” (See Limitations section above). This data reduction may be performed through computer methods using curve fitting routines such as point-to-point, cubic spline, or 4 parameter logistic fit. **Do not use linear regression analysis to interpolate values for samples as this may lead to significant inaccuracies!** Data may also be manually reduced by plotting the absorbance values of the standard on the y-axis versus concentration on the x-axis and drawing a smooth point-to-point line. Absorbances of samples are then interpolated from this standard curve.

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples greater than 4 ng/mL and less than 250 ng/mL. CVs for samples less than 4 ng/mL may be greater than 10%.
- It is recommended that each laboratory assay appropriate quality control samples in each run to ensure that all reagents and procedures are correct.

Assay Protocol

- Two protocols are offered for user convenience. The "Standard Protocol" is the highest sensitivity method (sensitivity less than 0.3ng/mL) and requires approximately 3.5 hours to complete. When using this protocol the highest standard should be the 75ng/mL standard "E". It is not advised to use the 250ng/mL standard "F" in this protocol because it may result in absorbances which are out of the linear range of most spectrophotometers. The "Rapid Protocol" requires only a 1 hour first incubation for a total assay time of ~1.5 hours. This protocol will offer an extended upper assay range such that the 250ng/mL standard may be used. The Rapid Protocol is less sensitive than the Standard Protocol and thus may not adequately discriminate the zero standard "A" from the 1ng/mL standard "B".
- The assay is very robust such that assay variables like incubation times, sample size, and other sequential incubation schemes can be altered to manipulate assay performance for more sensitivity, increased upper analytical range, or reduced sample matrix interference. Before modifying the protocol from what is recommended, users are advised to contact our technical services for input on the best way to achieve your desired goals.
- The protocols specify the use of an approved orbital microtiter plate shaker for the immunological step. These can be purchased from most laboratory supply companies. If you do not have such a device it is possible to incubate the plate without shaking however it will be necessary to extend the first immunological incubation step by one to two hours to achieve comparable results to the 3 hour shaking protocol. **Do not shake during the 30-minute substrate incubation step as this may result in higher backgrounds and worse precision.**
- It is recommended that each laboratory assay appropriate quality control samples in each run to ensure that all reagents and procedures are

correct. **You are strongly urged to make controls in your typical sample matrix using HCPs derived from your cell line. These controls can be aliquoted into single use vials and stored frozen for long-term stability.**

- Bring all reagents to room temperature. Set-up plate spectrophotometer to read dual wavelength at 450nm for the test wavelength and 650nm for the reference.
- Thorough washing is essential to proper performance of this assay. Automated plate washing systems or other vacuum aspiration devices are not recommended. The manual method described in the assay protocol is preferred for best precision, sensitivity and accuracy. A more detailed discussion of this procedure can be obtained from our Technical Services Department or on our web site. In addition, a video demonstration of proper plate washing technique is available in the 'Technical Help' section of our web site.
- All standards, controls, and samples should be assayed at least in duplicate.
- Maintain a repetitive timing sequence from well to well for all assay steps to ensure that all incubation times are the same for each well.
- Make a work list for each assay to identify the location of each standard, control, and sample.
- If the substrate has a distinct blue color prior to the assay it may have been polluted. If the absorbance of 100 μ L of substrate plus 100 μ L of stop against a water blank is greater than 0.1 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.
- Strips should be read within 30 minutes after adding stop solution since color will fade over time.

Assay Protocol

1. Pipette 25µL of standards, controls and samples into wells indicated on work list.
2. Pipette 100µL of anti-*P. pastoris*:HRP (#F141) into each well.
3. Cover & incubate on orbital plate shaker at 400-600 rpm for 3 hours at room temperature, 24°C ± 4°.
4. Dump contents of wells into waste. Blot and gently but firmly tap over absorbent paper to remove most of the residual liquid. Overly aggressive banging of the plate or use of vacuum aspiration devices in an attempt to remove all residual liquid is not necessary and may cause variable dissociation of antibody bound material resulting in lower ODs and worse precision. Fill wells generously to overflowing with diluted wash solution using a squirt bottle or by pipetting in ~350µL. Dump and tap again. Repeat for a total of 4 washes. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. Do not allow wash solution to remain in wells for longer than a few seconds. Do not allow wells to dry before adding substrate.
5. Pipette 100µL of TMB substrate (#F005).
6. Incubate at room temperature for 30 minutes. DO NOT SHAKE.
7. Pipette 100µL of Stop Solution (#F006).
8. Read absorbance at 450/650nm.

Rapid Assay Protocol

1. Pipette 25µL of standards, controls and samples into wells indicated on work list.
2. Pipette 100µL of anti-*P. pastoris*:HRP (#F141) into each well.
3. Cover & incubate on rotator at 400-600 rpm for 1 hour at room temperature, 24°C ± 4°.
4. Dump contents of wells into waste or gently aspirate with a pipettor. Blot and vigorously bang out residual liquid over absorbent paper. Fill wells generously with diluted wash solution by flooding well from a squirt bottle or by pipetting in ~350 µL. Dump and bang again. Repeat for a total of 4 washes. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. Do not allow wash solution to remain in wells for longer than a few seconds. Do not allow wells to dry before adding TMB substrate.
5. Pipette 100µL of TMB substrate (#F005).
6. Incubate at room temperature for 30 minutes. DO NOT SHAKE.
7. Pipette 100µL of Stop Solution (#F006).
8. Read absorbance at 450/650nm.

Procedural Notes

1. Complete washing of the plates to remove excess unreacted reagents is essential to good assay reproducibility and sensitivity. We advise against the use of automated or other manual operated vacuum aspiration devices for washing plates as these may result in lower specific absorbances, higher non-specific absorbance, and more variable precision. The manual wash procedure described below generally provides lower backgrounds, higher specific absorbance, and better precision. If duplicate CVs are poor or if the absorbance of the 0 standard is greater than 0.200, evaluate plate washing procedure for proper performance.

2. High Dose Hook Effect or poor dilutional linearity may be observed in samples with very high concentrations of HCP. High Dose Hook Effect is due to insufficient excess of antibody for very high concentrations of HCPs present in the samples upstream in the purification process. Samples greater than 30 µg/mL may give absorbances less than the 250 ng/mL standard. It is also possible for samples to have certain HCPs in concentrations exceeding the amount of antibody for that particular HCP. In such cases the absorbance of the undiluted sample may be lower than the highest standard in the kit however these samples will fail to show acceptable dilutional linearity/parallelism as evidenced by an apparent increase in dilution corrected HCP concentration with increasing dilution. High Dose Hook and poor dilutional linearity are most likely to be encountered from samples early in the purification process. If a hook effect is possible, samples should also be assayed diluted. If the HCP concentration of the undiluted sample is less than the diluted sample this may be indicative of the hook effect. Such samples should be diluted at least to the minimum required dilutions (MRDs) as established by your qualification studies using your actual final and in-process drug samples. The MRD is the first dilution at which all subsequent dilutions yield the same HCP value within the statistical limits of assay precision. The HCP value to be reported for such samples is the dilution corrected value at or greater than the established MRD. The diluent used should be compatible with accurate recovery. The preferred diluent is our Cat# 1028 available in 100mL, 500mL, or 1 liter bottles. This is the same material used to prepare the kit standards. As the sample is diluted in 1028 its matrix begins to approach that of the standards thus reducing any inaccuracies caused by dilutional artifacts. Other prospective diluents must be tested for recovery by using them to dilute the 250ng/mL standard, as described in the "Limitations" section below.

Limitations

- Before relying exclusively on this assay to detect host cell proteins, each laboratory should qualify that the kit antibodies and assay procedure yield acceptable specificity, accuracy, and precision. A suggested protocol for this qualification can be obtained by contacting our Technical Services Department or at our web site.
- The standards used in this assay are comprised of *Pichia pastoris* HCPs solubilized by mechanical disruption and detergent. 1D Western blot analysis of the antibodies used in this kit demonstrates that they recognize the majority of distinct PAGE separated bands seen using a sensitive protein staining method like silver stain or colloidal gold. Because the vast majority of HCPs will be conserved among all strains of *Pichia pastoris* this kit should be adequately reactive to HCPs from your strain. Other clients have successfully qualified this kit for their individual *Pichia pastoris* strains demonstrating acceptable specificity, accuracy, and sensitivity for process intermediate samples as well as final product. However, there can be no guarantee that this assay will detect all proteins or protein fragments from your process. If you desire a much more sensitive and specific method than western blot to detect the reactivity of the antibodies in this kit to your individual HCPs *Cygnus* is pleased to perform AAE as a service to provide coverage information of the antibodies to the HCPs in your process samples.
- Certain sample matrices may interfere in this assay. However the potential exists that the product protein itself, high or low pH, high salt, detergents, or other components in the sample matrix may result in either positive or negative interference in this assay. It is advised to test all sample matrices for interference by diluting the 250ng/mL standard, 1 part to 4 parts of the matrix containing no or very low HCP impurities. This diluted standard when assayed as an unknown should give a value of 40 to 60 ng/mL. Consult *Cygnus Technologies* Technical Service Department for advice on how to quantitate the assay in problematic matrices.
- Avoid the assay of samples containing sodium azide (NaN₃) which will destroy the HRP activity of the conjugate and could result in the under-estimation of HCP levels.

Example Data

Standard Protocol			
Well #	Contents	Abs. at 450-650nm	Mean Abs.
A1	Zero Std	0.058	0.059
B1	Zero Std	0.059	
C1	1ng/mL	0.093	0.092
D1	1ng/mL	0.091	
E1	4ng/mL	0.179	0.180
F1	4ng/mL	0.181	
G1	20ng/mL	0.611	0.616
H1	20ng/mL	0.621	
A2	75ng/mL	1.814	1.810
B2	75ng/mL	1.806	

Rapid Protocol			
Well #	Contents	Abs. at 450-650nm	Mean Abs.
A1	Zero Std	0.031	0.031
B1	Zero Std	0.030	
C1	4ng/mL	0.076	0.076
D1	4ng/mL	0.076	
E1	20ng/mL	0.234	0.238
F1	20ng/mL	0.242	
G1	75ng/mL	0.706	0.697
H1	75ng/mL	0.687	
A2	250ng/mL	1.804	1.776
B2	250ng/mL	1.747	

Performance Characteristics

Cygnus Technologies has qualified this assay by conventional criteria as indicated below. A more detailed copy of this "Qualification Summary" report can be obtained by request. This qualification is generic in nature and is intended to supplement but not replace certain user and product specific qualification and qualification which should be performed by each laboratory. At a minimum each laboratory is urged to perform a spike and recovery study in their sample types. In addition, any of your samples types containing process derived HCPs within or above the analytical range of this assay should be evaluated for dilution linearity to ensure that the assay is accurate and has sufficient antibody excess for your particular HCPs. Each laboratory and technician should also demonstrate competency in the assay by performing a precision study similar to that described below. A more detailed discussion of recommended user qualification protocols can be obtained by contacting our Technical Services Department or at our web site.

Sensitivity

The lower limit of detection (LOD) is defined as that concentration corresponding to a signal two standard deviations above the mean of the zero standard. LOD, Standard Protocol is less than 0.3 ng/mL. LOD, Rapid Protocol is less than 1 ng/mL.

The lower limit of quantitation (LOQ) is defined as the lowest concentration where concentration coefficients of variation (CVs) are less than 20%. LOQ, Standard Protocol is ~0.4 ng/mL. LOQ, Rapid Protocol is ~ 1.5 ng/mL.

Precision

Both intra (n=20 replicates) and inter-assay (n=5 assays) precision were determined on 3 pools with low (3.6ng/mL), medium (~19.8ng/mL), and high concentrations (~60.6ng/mL) using the Standard Protocol. The % CV is the standard deviation divided by the mean and multiplied by 100. Similar precision should be obtainable by the rapid protocol.

Pool	Intra assay CV	Inter assay CV
Low	4.3%	6.9%
Medium	3.4%	5.7%
High	6.1%	5.8%

Specificity/Cross-Reactivity

1D Western blot analysis against other strains of *Pichia pastoris* indicates that most of the proteins are conserved among all strains. Each end user must qualify that this kit is adequately reactive and specific for their samples. 1D Western blot is highly orthogonal to ELISA and to non-specific protein staining methods such as silver stain or colloidal gold. As such, the lack of identity between silver stain and western blot does not necessarily mean there is no antibody to that protein or that the ELISA will not detect that protein. If you desire a much more sensitive and specific method than Western blot to detect the reactivity of the antibodies in this kit to your individual HCPs *Cygnus* is pleased to perform AAE as a service to provide coverage information of the antibodies to the HCPs in your process samples. This method has been shown to be much at least 100 fold more sensitive than Western blots in detecting antibody reactivity to individual HCPs. The same antibody as is used for both capture and HRP label can be purchased separately.

Cross reactivity to other *Pichia* species has not been investigated.

The yeast, *Saccharomyces cerevisiae* was evaluated in both this ELISA assay and by Western Blot analysis. In the ELISA the cross reactivity on a weight-to-weight basis was ~ 1%. In the Western Blot assay *Saccharomyces cerevisiae* yielded less than 5% of the bands seen for *Pichia pastoris*.

The commercial preparation of the enzyme Alcohol Oxidase purified from *Pichia pastoris* is reactive in this assay and in Western Blot. On a weight to weight basis

the Alcohol Oxidase preparation compared to the HCPs used in the standards gave a cross reactivity of 5.1%.

Recovery/ Interference Studies

Various buffer matrices were evaluated by adding known amounts of the *Pichia pastoris* HCP preparation used to make the standards in this kit. Because this assay is designed to minimize matrix interference most of these buffers yielded acceptable recovery defined as between 80-120%. In general extremes in pH (less than 5.0 and greater than 8.5) as well as certain detergents can cause under-recovery. Each user should qualify that their sample matrices yield accurate recovery by performing a similar experiment. Such an experiment can be performed by diluting the 250ng/mL standard provided with this kit into the sample matrix in question.

Hook Capacity

Increasing concentrations of HCPs greater than 250 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration which will give an absorbance reading less than the 250 ng/mL standard was ~ 30 µg/mL.

Ordering Information/ Customer Service

Cygnus Technologies also offers kits for the extraction and detection of CHO Host Cell DNA. The following kits are available:

- Residual Host Cell DNA extraction:
Cat # D100W, DNA Extraction Kit in 96 deep well plate
Cat # D100T, DNA Extraction Kit in microfuge tubes
- Extraction and PCR amplification of CHO Host Cell DNA for use with user supplied master mix:
Cat # D555W, DNA Extraction Kit in 96 deep well plate
Cat # D555T, DNA Extraction Kit in microfuge tubes
- Residual CHO Host Cell DNA extraction and detection using PicoGreen® dye:
Cat # D550W, DNA Extraction Kit in 96 deep well plate
Cat # D550T, DNA Extraction Kit in microfuge tubes

To place an order or to obtain additional product information contact *Cygnus Technologies*:

www.cygnustechnologies.com

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