CYGNUS TECHNOLOGIES
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Pichia pastoris 3G Host Cell Proteins

Immunoenzymetric Assay for the Measurement of *Pichia pastoris* Host Cell Proteins Catalog # F1015

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* GlycoSwitch® host cells. The *Pichia* GlycoSwtich® strains allow for the engineering of human-like glycosylation structures on the protein of interest. Patents, strains, vectors and other materials related to the *Pichia* GlycoSwitch® Expression System and licenses for its use as to express recombinant products are owned by Research Corporation Technologies, Inc. (RCT), Tucson, Arizona. 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. 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 co-purify with the product independent of the purification process. The antibodies have been generated against and affinity purified using *Pichia* GlycoSwitch® HCPs recovered from conditioned media. The antibodies used in this kit were characterized by Antibody Affinity Extraction (AAE™) and Mass Spectrometry, demonstrating reactivity to 88 -100% of HCPs.

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. The suitability of this kit for a given sample type and product must be determined and qualified experimentally by each laboratory. 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 a custom basis.

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.

Reagents & Materials Provided

Component	Product #
Anti-*P. pastoris*, 3G:HRP Affinity purified goat antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	**F1016**
Anti-*P. pastoris*, 3G coated microtiter strips 12x8 well strips in a bag with desiccant	**F1017***
***P. pastoris*, 3G Standards** Solubilized P. pastoris HCPs in a bovine serum albumin matrix with preservative. Standards at 0, 3, 6, 12, 25, 50, 100, and 200ng/mL. 1 mL/vial	**F1018**
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**

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 # I028)
- Distilled water
- 1 liter wash bottle for diluted wash solution

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.

Precautions

- For Research or Manufacturing use only.
- Stop reagent is 0.5M H_2SO_4. 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 20x wash concentrate to 1x in 1 liter of distilled water, label with kit lot and expiration date, and store at 4°C.

Procedural Notes

1. Complete washing of the plates to remove excess unreacted reagents is essential to good assay reproducibility and sensitivity. 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 0.300, evaluate plate washing procedure for proper performance.

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. AAE and Mass Spectrometry analysis of the antibodies used in this kit demonstrates that they recognize the HCPs in the standards preparation.
- Certain sample matrices may interfere in this assay. The standards used in this kit attempt to simulate typical sample protein and matrices. However, the potential exists that the product protein itself or other components in the sample matrix may result in either positive or negative interference in this assay. High or low pH, detergents, urea, high salt concentrations, and organic solvents are some of the known interference factors. It is advised to test all sample matrices for interference by diluting the 200ng/mL standard 1 part to 4 parts of the matrix containing no or very low *HCP* impurities. When assayed as an unknown, this diluted standard should give an added HCP value in the 32 to 48ng/mL range. 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 (NaN_3) which will destroy the HRP activity of the conjugate and could result in the under-estimation of HCP levels.

Assay Protocol

- 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 protocol specifies 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 about one hour to achieve comparable results to the 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. 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 Resources' 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.
- Strips should be read within 30 minutes after adding stop solution since color will fade over time.

Assay Protocol

1. Pipette 100µL of anti-*P. pastoris*, 3G:HRP (#F1016) into each well.

2. Pipette 25µL of standards, controls and samples into wells indicated on work list.

3. Cover & incubate on orbital shaker at 400-600rpm for 1 hour at room temperature, 24°C ± 4°C.

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

Example Data

Well #	Contents	Abs. at 450-650nm	Mean Abs.
H2	Zero Std	0.011	0.012
H1	Zero Std	0.012	
G2	3ng/mL	0.077	0.078
G1	3ng/mL	0.078	
F2	6ng/mL	0.146	0.148
F1	6ng/mL	0.150	
E2	12ng/mL	0.267	0.275
E1	12ng/mL	0.284	
D2	25ng/mL	0.586	0.595
D1	25ng/mL	0.604	
C2	50ng/mL	1.085	1.114
C1	50ng/mL	1.143	
B2	100ng/mL	1.935	1.948
B1	100ng/mL	1.962	
A2	200ng/mL	2.934	2.977
A1	200ng/mL	3.020	

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL "total immuno-reactive HCP equivalents". 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!**

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples in the range of 6-200ng/mL. CVs for samples less than 6 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.

Performance Characteristics

Cygnus Technologies has qualified this assay by conventional criteria as indicated below. A copy of this qualification report can be requested on our web site by clicking "Request a Qualification Summary" on the product page. 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 sample types containing process derived HCPs within or above the analytical range of this assay should be evaluated for dilutional 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 three standard deviations above the mean of the zero standard. LOD is ~0.2 ng/mL.

The lower limit of quantitation (**LLOQ**) is defined as the lowest concentration where concentration coefficients of variation (CVs) are less than 20%. The LLOQ is ~3ng/mL.

Specificity/Cross-Reactivity

Cross reactivity to non-HCP components has not been extensively investigated with this kit. You should evaluate components in your samples for positive interferences such as cross reactivity and non-specific binding. Negative interference studies are described below.

Precision

Both intra (n=20 replicates) and inter-assay (n=10 assays) precision were determined on 4 controls with low (~3.75 ng/mL), low-middle (~20 ng/mL), high-middle (~75 ng/mL) and high (~150 ng/mL) concentrations. The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra-assay CV	Inter-assay CV
Low	6.1%	4.3%
Low-middle	6.0%	0.0%
High-middle	7.1%	2.7%
High	7.3%	2.8%

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. The standards used in this kit contain 8mg/mL of bovine serum albumin intended to simulate non-specific protein effects of most sample proteins. However, very high concentration of some products may interfere in the accurate measurement of HCPs. In general, extremes in pH (less than 5.0 and greater than 8.5), high salt concentration, high polysaccharide concentrations, urea, organic solvents, and most 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 200ng/mL standard provided with this kit into the sample matrix in question as described in the "Limitations" section.

Ordering Information/ Customer Service

Cygnus Technologies also offers kits for the extraction of 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

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

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