

CHO Host Cell Proteins from Culture Media

Immunoenzymetric Assay for the Measurement of CHO Host Cell Proteins commonly found in conditioned media Catalog # CM015

Intended Use

This kit is intended for use in determining the presence of CHO Host Cell Proteins (HCPs) in products manufactured by recombinant expression in CHO cells. The antibodies used in this kit were generated from HCPs typically found in protein-free conditioned growth media. 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 CHO cells is a relatively simple and cost effective method for production of proteins. Many of these products 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 from CHO. 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 ELISA are conventionally accepted. 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 the majority of the HCPs that could contaminate the product independent of the purification process. The antibodies have been generated against and affinity purified using CHO HCPs found in protein free conditioned media. 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. *Cygnus* also makes two other CHO HCP kits, Cat# F015 and Cat# F550, our most current, 3rd generation kit. The antibodies in the F015 kit were made using CHO HCPs obtained from a gentle cell lysate and should be used when the product is obtained through lysed cells such as virus production. The antibodies in the F550 kit were made from HCPs derived from conditioned media. The array and relative abundance of the antigens and antibodies are different between the CM015 and F550 kits. All 3 kits qualitatively recognize the vast majority of the same HCPs although the potential exists that the quantitative value reported for a given sample type may differ between the three kits. 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 is 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 or consider use of the F550 kit. 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 custom basis.

Principle of the Procedure

The CHO HCP assay is a sequential, two-site immunoenzymetric assay also termed ELISA. Samples potentially containing CHO proteins are reacted in microtiter strips coated with an affinity purified capture antibody. After a wash step, a horseradish peroxidase enzyme labeled anti-CHO antibody is reacted sequentially forming a sandwich complex of solid phase antibody-CHO protein-enzyme labeled antibody. The microtiter strips are then 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 CHO proteins present.

Reagents & Materials Provided

Component	Product #
Anti-CHO:HRP Affinity purified goat antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	F148
Anti- CHO coated microtiter strips 12x8 well strips in a bag with desiccant	F149*
CHO HCP Standards Solubilized CHO HCPs obtained from media with preservative. Standards at 0, 0.7, 2, 8, 25, 75, and 200 ng/mL. 1 mL/vial	F139
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 # F149.

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 - 50µL 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.

Procedural Notes

- 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 than automated plate washers. 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.
- 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 200µg/mL may give absorbances less than the 75 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 as evidenced by an apparent increase in 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. 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# I-028 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 200ng/mL standard, as described in the "Limitations" section below. If you cannot obtain a MRD within the analytical range of this assay, contact our Technical Services department for free consultation.

Quality Control

- Precision on duplicate samples should yield average % coefficients of variations of less than 10% for samples in the range of 2-200ng/mL. CVs for samples less than 2ng/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

- The protocol specifies use of an approved orbital microtiter plate shaker for the immunological steps. 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 immunological incubation steps in the plate by about one hour in order to achieve comparable results to the routine protocols. **Do not shake during the 30-minute substrate incubation step as this may result in higher backgrounds and worse precision.**
- 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 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.
- 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.**

- 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 100 μ L of standards, controls and samples into wells indicated on work list. Use only standards A – F for the standard curve. The 200ng/mL standard can be used for spike recovery and making assay controls.
2. Cover & incubate on orbital shaker at 400 – 600 rpm for 1 hour at room temperature, 24°C \pm 4°C.
3. 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 ~350 μ L. Dump and tap again. Repeat for a total of 2 washes.
4. Pipette 100 μ L of anti-CHO:HRP (#F148) into each well.
5. Cover & incubate on orbital shaker at 400 – 600 rpm for 2 hours at room temperature, 24°C \pm 4°C.
6. 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 ~350 μ L. 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.
7. Pipette 100 μ L of TMB substrate (#F005).
8. Incubate at room temperature for 30 minutes. **DO NOT SHAKE.**
9. Pipette 100 μ L of Stop Solution (#F006).
10. Read absorbance at 450/650nm.

Limitations

- Before relying exclusively on this assay to detect host cell proteins, each laboratory should qualify that the kit antibodies and assay procedure utilized 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, www.cygnustechologies.com.
- The standards used in this assay are comprised of CHO HCPs obtained from conditioned growth media. 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 CHO, this kit should be adequately reactive to HCPs from your strain. Several clients have successfully qualified this kit for their individual CHO 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. The standards used in this kit attempt to simulate typical sample protein and matrices. However, the potential exists that the product protein 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 CHO HCP impurities. This diluted standard when assayed as an unknown should give a value of 30 to 50 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 (Na₃N) which will destroy the HRP activity of the conjugate and could result in the under-estimation of HCP levels.

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL “total immuno-reactive 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. **Curve fit methods other than those recommended above may yield systematic inaccuracies as evidenced by poor “back-fit” values for the standards. 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.

Example Data

Well #	Contents	Abs. at 450-650 nm	Mean Abs.
A1	Zero Std	0.146	0.143
A1	Zero Std	0.139	
B1	0.7ng/mL	0.218	0.222
B2	0.7ng/mL	0.226	
C1	2ng/mL	0.378	0.380
C2	2ng/mL	0.382	
D1	8ng/mL	0.967	0.972
D2	8ng/mL	0.976	
E1	25ng/mL	1.894	1.915
E2	25ng/mL	1.935	
F1	75ng/mL	2.794	2.801
F2	75ng/mL	2.807	

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 that 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 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 on-line at our web site www.cygnustechnologies.com.

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 was less than 100pg/mL.

The lower limit of quantitation (**LOQ**) is defined as the lowest concentration, where concentration coefficients of variation (CVs) are less than 20%. The LOQ is ~0.7 ng/mL.

Precision

The table shows both intra (n=20 replicates) and inter-assay (n=5 assays) precision determined on 3 pools. The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
7.6ng/mL	6.3%	8.2%
24.3ng/mL	4.4%	7.0%
50.5ng/mL	5.4%	7.2%

Specificity/Cross-Reactivity

1D Western blot analysis against other cell lines of CHO HCPs indicates that most of the proteins are conserved among all strains. Thus, this assay should be useful for detecting HCP's from other CHO HCP cell lines. 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.

Human and mouse IgG at 10mg/mL were tested and shown to be un-reactive in this assay. Bovine albumin, bovine transferrin and insulin were also shown to be un-reactive.

Recovery/ Interference Studies

Various buffer matrices have been evaluated by spiking known amounts of HCPs 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) or salt concentration as well as certain detergents can cause under-recovery. In some cases, very high concentrations of the product protein may also cause a negative interference in this assay. Each user should qualify that their sample matrices and product itself yield accurate recovery in the protocol of their choice. This experiment can be performed by spiking the 200ng/mL standard provided with this kit into the sample in question. For example, we suggest adding 1 part of the 200 ng/mL standard to 4 parts of the test sample. This yields an added spike of 40ng/mL. Any endogenous HCPs from the sample itself determined prior to spiking and corrected for by the 20% dilution of that sample should be subtracted from the value determined for the spiked sample. The added spike and recovery should be within allowable limits e.g. 80% to 120%. Should you have any problems achieving adequate spike and recovery data you are strongly urged to contact our Technical Services Department for recommendations on how to overcome sample matrix interference.

Hook Capacity

Increasing concentrations of HCPs greater than 200 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration which will give an absorbance reading less than the 75ng/mL standard was greater than 200 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*:

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