

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 contamination by host cell proteins from CHO. Such contamination can reduce the efficacy of the therapeutic agent and result in adverse toxic or immunological reactions and thus it is desirable to reduce HCP contamination 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 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 need of a more sensitive and specific method to demonstrate reactivity to individual HCPs in your samples Cygnus Technologies recommends a method we

find superior to 2D Western blot. We term this method 2D HPLC-ELISA. 2D HPLC-ELISA can yield much better sensitivity and specificity as compared to 2D Western blot. For more information on this 2D HPLC-ELISA analysis please contact our Technical Services department.

Special procedures were utilized in the generation of these antibodies to insure that low molecular weight and less immunogenic contaminants 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 contaminants as well as in routine final product release testing. *Cygnus* also makes another CHO HCP kit, Cat# F015. The antibodies in this kit are also affinity purified polyclonals, but were made using CHO HCPs obtained from a gentle cell lysate. Both 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 two kits. We cannot predict which kit will be most suitable for your applications. You may want to evaluate both kits simultaneously. Alternatively, if any one kit has been validated to meet your analytical needs then it is not necessary to consider the other kit. Because of the high sensitivity and broad reactivity of the antibodies, this generic kit has been successfully validated for testing of final product HCPs in many different products regardless of growth and purification process. When the kit is satisfactorily validated 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 validation 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 is of sufficient titer and broad antigen reactivity, it may be possible to substitute the standards used in this kit for ones made from the contaminants 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 validated 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 contaminants 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.

Principle of the Procedure

The CHO HCP assay is a two-site immunoenzymetric assay also termed ELISA. Samples potentially containing CHO proteins are reacted in microtiter strips coated with an affinity purified capture antibody. A second, horseradish peroxidase enzyme labeled anti-CHO antibody is reacted either simultaneously or 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 tetramethyl benzidine (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 200ng/mL. 1 mL/vial	F139
Stop Solution 0.5N 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.

* The substrate reagent should not be used if its stopped absorbance at 450nm is greater than 0.1.

* 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 (150 - 200 rpm)

Sample Diluent (recommended Cat # I028)

Distilled water

1 liter wash bottle for diluted wash solution

Precautions

* For Research or Manufacturing use only.

* Stop reagent is 0.5N H₂SO₄. Avoid contact with eyes, skin, and clothing. At the concentrations used in this kit, none of the other reagents are believed to be harmful.

* 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

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 minus a substrate blank 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. The Hook varies as a function of protocol. Using the Simultaneous Protocol, samples greater than 10 µg/mL of total HCP may give absorbances less than the 200 ng/mL standard. Using the High Sensitivity Protocol, 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/parallelism 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. 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 validation 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 1-028 its matrix begins to approach that of the standards thus reducing any inaccuracies caused by dilution 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.

Limitations

* Before relying exclusively on this assay to detect host cell proteins, each laboratory should validate that the kit antibodies and assay procedure utilized yield acceptable specificity, accuracy, and precision. A suggested protocol for this validation can be obtained by contacting our Technical Services Department or at our web site, www.cygnustechnologies.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 validated 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 offer a service for fractionation of HCPs using 2-D HPLC methods followed by detection in ELISA.

* 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 contaminants. 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 (NaN_3) which will destroy the HRP activity of the conjugate and could result in the under-estimation of HCP levels.

Assay Protocols

* Two different assay protocol options are specified below. Both methods require approximately 3.5 hours for completion. The "High Sensitivity Protocol" is a sequential method providing the highest level of sensitivity (LOQ) at approximately 700 pg/mL total HCP equivalents. The "Simultaneous Protocol" option

involves a single simultaneous incubation of sample and enzyme labeled antibody. This procedure yields an LOQ of approximately 2 ng/mL of total HCP equivalents. Because the Simultaneous Protocol provides for a dilution of a smaller sample volume with the conjugate antibody, it may yield less non-specific interference from certain sample types than the High Sensitivity Protocol. For this reason we recommend the Simultaneous Protocol in cases where additional sensitivity is not needed. When using the Simultaneous Protocol it is recommended to use the 0, 2, 8, 25, 75, and 200 ng/mL standards. When performing the High Sensitivity Protocol use the 0, 0.7, 2, 8, 25, and 75 ng/mL standards. Use of the 200 ng/mL standard in this protocol can result in absorbances off scale. These protocols will provide an adequate level of sensitivity for most applications.

* 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 use of an approved microtiter plate shaker or rotator for the immunological steps. These can be purchased from most laboratory supply companies. Alternatively you can purchase an approved, pre-calibrated shaker directly from *Cygnus Technologies*. 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. 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.

* 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 insure 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 insure 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 contaminated. 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.

High Sensitivity 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.
2. Cover & incubate on rotator at ~ 180rpm for 1 hour at room temperature, 24°C \pm 4°.
3. 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 μ L. Dump and bang 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 rotator at ~ 180rpm for 2 hours at room temperature, 24°C \pm 4°.
6. 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 μ L. 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.
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 blanking on the Zero standard.

Simultaneous Protocol

1. Pipette 50 μ L of standards, controls and samples into wells indicated on work list. Do not use standard B (0.7ng/mL) to make the standard curve. Use only Standards A, and C through G.
2. Pipette 100 μ L of anti-CHO:HRP (#F148) into each well
3. Cover & incubate on rotator at ~ 180rpm for 3 hours at room temperature, 24°C \pm 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 μ L. 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 100 μ L of TMB substrate (#F005).
6. Incubate at room temperature for 30 minutes. DO NOT SHAKE.
7. Pipette 100 μ L of Stop Solution (#F006).
8. Read absorbance at 450/650nm blanking on the Zero standard.

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, 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. CVs for samples < 4 ng/mL may be greater than 10%.

* For optimal performance the absorbance of the substrate when blanked against water should be <0.1.

* It is recommended that each laboratory assay appropriate quality control samples in each run to insure that all reagents and procedures are correct.

Example Data

High Sensitivity Protocol				
Well #	Contents	Abs. at 450nm	Mean Abs.	ng/mL HCP equivalents.
1A	Zero Std	0.145		
1B	Zero Std	0.139	0.142	
1C	0.7ng/mL	0.189		
1D	0.7ng/mL	0.178	0.184	
1E	2ng/mL	0.267		
1F	2ng/mL	0.284	0.276	
1G	8ng/mL	0.552		
1H	8ng/mL	0.567	0.560	
2A	25ng/mL	1.414		
2B	25ng/mL	1.408	1.411	
2C	75 ng/mL	2.319		
2D	75 ng/mL	2.389	2.354	
2E	sample A	2.774		
2F	sample A	2.694	2.634	>200
2G	sample B	0.264		
2H	sample B	0.270	0.267	1.9

Simultaneous Protocol				
Well #	Contents	Abs. at 450nm	Mean Abs.	ng/mL HCP equivalents.
1A	Zero Std	0.151		
1B	Zero Std	0.153	0.152	
1C	2ng/mL	0.235		
1D	2ng/mL	0.231	0.233	
1E	8ng/mL	0.480		
1F	8ng/mL	0.473	0.477	
1G	25ng/mL	0.924		
1H	25ng/mL	0.938	0.931	
2A	75ng/mL	1.610		
2B	75ng/mL	1.625	1.618	
2C	200 g/mL	2.087		
2D	200 g/mL	2.078	2.083	
2E	sample A	2.291		
2F	sample A	2.315	2.303	>200
2G	sample B	0.238		
2H	sample B	0.236	0.237	2.1

Performance Characteristics

Cygnus Technologies has validated this assay by conventional criteria as indicated below. A more detailed copy of this "Validation Summary" report can be obtained by request. This validation is generic in nature and is intended to supplement but not replace certain user and product specific qualification and validation 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 insure 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 validation 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 <100pg/mL for the High Sensitivity protocol and <600pg/mL for the Simultaneous protocol.

The lower limit of quantitation (LOQ) is defined as the lowest concentration, where concentration coefficients of variation (CVs) are <20%. The LOQ is ~0.7 ng/mL for the High Sensitivity assay and ~2ng/mL for the Simultaneous assay.

Precision

Both protocols should yield similar precision profiles in the range of the samples reported below. The table below shows both intra (n=20 replicates) and inter-assay (n=5 assays) precision determined on 3 pools using the High Sensitivity protocol. 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 indicate 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 validate 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 offer a service and/or consultation on fractionation of HCPs using 2 Dimensional HPLC methods followed by detection in the ELISA. 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 (<5.0 and >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 validate 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 > 200 ng/mL were assayed as unknowns. The hook capacity of the High Sensitivity Protocol, defined as that concentration which will give an absorbance reading less than the 75ng/mL standard was >200 µg/mL. The hook capacity for the Simultaneous Protocol defined as that concentration which will give an absorbance less than the 200ng/mL standard is ~ 10µg/mL.

Ordering Information/ Customer Service

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

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