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SZABO-SCANDIC HandelsgmbH

Quellenstraße 110, A-1100 Wien

T. +43(0)1 489 3961-0

F. +43(0)1 489 3961-7

mail@szabo-scandic.com

www.szabo-scandic.com

[linkedin.com/company/szaboscandic](https://www.linkedin.com/company/szaboscandic) 

Chinese Hamster Ovary Host Cell Proteins

Immunoenzymetric Assay for the Measurement of Chinese Hamster Ovary Host Cell Proteins Catalog # F015

Intended Use

This kit is intended for use in determining the presence of Chinese Hamster Ovary (CHO) protein contamination in products manufactured by recombinant expression in CHO 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 CHO is a widely used procedure to obtain large, cost-effective quantities of a desired protein. 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 result in adverse toxic or immunological reactions and thus it is desirable to reduce host cell 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 contaminate the product independent of the purification process. The

antibodies have been generated against and affinity purified using a gentle mechanical and detergent lysate of washed CHO cells. 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 impurities as well as high molecular weight components would be represented in the polyclonal mixture. The kit is specific to CHO HCPs and does not react with other impurities such as growth media components. 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 another CHO HCP kit, Cat# CM015. The antibodies in this kit are also affinity purified polyclonals but were made using CHO HCPs obtained from protein free conditioned media rather than 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 qualified 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 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 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 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.

Principle of the Procedure

The CHO Host Cell Protein assay is a two-site immunoenzymetric assay. Samples which may contain CHO proteins are reacted with an affinity purified alkaline phosphatase labeled antibody. This reaction either takes place in a test tube in a sequential assay mode or simultaneously in microtiter wells coated with an anti-CHO HCP capture antibody. The two-step sequential protocol or the one step simultaneous protocol result in the formation of a sandwich complex of solid phase (capture) antibody-CHO protein-enzyme labeled antibody. The microtiter strips are then washed to remove any unbound reactants. The substrate p-nitrophenyl phosphate (PNPP) is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and will be directly proportional to the concentration of CHO proteins present.

Reagents & Materials Provided

Component	Product #
Anti-CHO:Alkaline Phosphatase Affinity purified goat antibody conjugated to alkaline phosphatase in a protein matrix with preservative. 1x22mL	F017
Anti-CHO coated microtiter strips 12x8 well strips in a bag with desiccant	F019*
CHO Standards Detergent solubilized CHO proteins in a bovine serum albumin matrix with preservative. Standards at 0, 1, 4, 20, 75 and 250ng/mL 1.5 mL/vial	F018
PNPP Substrate p-nitrophenyl phosphate in a Diethanolamine buffer with preservative. 1x22mL	F008
Wash Concentrate (20X) Tris buffered saline with preservative. 1x50mL	F004

*All components can be purchased separately except # F019.

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.
- The substrate reagent should not be used if its stopped absorbance at 405nm is greater than 0.4.
- 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 405 & 492nm. (*If your plate reader does not provide dual wavelength analysis you may read at just the 405nm wavelength.*)
- Pipettors - 50µL and 200µL
- Repeating or multichannel pipettor - 200µL
- Microtiter plate rotator (400 - 600 rpm)
- Sample Diluent (recommended Cat # I028)
- Distilled water
- 1 liter wash bottle for diluted wash solution

Precautions

- For Research or Manufacturing use only.
- 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 is greater than 0.300, 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 125 µg/mL of total HCP 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# 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 I-028, 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.

3. If the substrate has a distinct yellow color prior to performing the assay it may have been contaminated. If this appears to be the case read 200µL of substrate

against a water blank for checking substrate impurities. If the absorbance is greater than 0.4 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised. The PNPP substrate is very sensitive to environmental impurities. Do not leave bottle open or at room temperature for longer than is needed. Only remove as much reagent as is needed for your assay run and do not return any unused substrate back into the substrate bottle. Additional substrate can be purchased separately as Cat # F008.

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.
- The standards used in this assay are comprised of CHO 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 bands also 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 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 the sample matrix for interference by diluting the 250ng/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 40 to 60 ng/mL.

Consult *Cygnus Technologies* Technical Service Department for advice on how to quantitate the assay in problematic matrices.

Assay Protocols

- Two assay protocol options, offered for user convenience, are specified below. The "High Sensitivity Protocol" is termed a reverse sequential method and can provide sensitivity of <500pg/mL total HCP equivalents. In this protocol the samples, standards, and enzyme labeled antibody are first incubated in uncoated test tubes or small microfuge vials for 2 hours. After this first incubation the reactant mixture is then added to the coated microtiter wells and incubated for another 2 hours followed by washing and a substrate incubation step. This method requires approximately 6 hours for completion. When performing the "High Sensitivity Protocol" use the 0, 1, 4, 20, and 75ng/mL standards. Use of the 250ng/mL standard in this protocol may result in absorbances off scale. The "Rapid Protocol" option involves a single simultaneous 2-hour incubation of sample and enzyme labeled antibody. When using the "Rapid Protocol" it is recommended to use the 0, 4, 20, 75 and 250ng/mL standards. The 1 ng/mL standard is not recommended for use in this protocol because it may not be well discriminated from the zero standard. This procedure takes approximately 3.5 hours and yields a sensitivity of approximately 2ng/mL of total HCP equivalents.
- 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 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 90-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 405nm for the test wavelength and ~492nm 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 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.
- The conjugate will have a cloudy appearance. This is normal and does not indicate contamination. Overtime, you may observe a slight precipitate. This precipitate is inconsequential to assay results. We suggest a simple inversion of the bottle to re-suspend it.

High Sensitivity Protocol

- Use clean polypropylene test tubes or micro-centrifuge vials with caps.
- Pipette 200 μ L* of standards, (0-75ng/mL), controls, and samples into labeled tubes or vials as indicated on work list.
- Pipette 400 μ L* of anti-CHO:Alkaline Phosphatase (#F017) into each tube or vial.
*These volumes of 200 μ L for the sample and 400 μ L for the conjugate are recommended assuming the assay is performed in duplicate. If assaying in triplicate or more the relative volumes should be adjusted appropriately.
- Cap, vortex, and allow to incubate for 2 hours at room temperature, 24 $^{\circ}$ C \pm 4 $^{\circ}$ C.
- Transfer 200 μ L of the reaction mixture to duplicate coated wells in the anti-CHO coated microtiter strips.
- Cover & incubate on rotator at 400-600 rpm for 2 hours at room temperature, 24 $^{\circ}$ C \pm 4 $^{\circ}$ C.
- 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 PNPP substrate.
- Pipette 200 μ L of PNPP substrate (#F008).
- Cover & incubate at room temperature for 90 minutes. **DO NOT SHAKE.**
- Read absorbance at 405/492nm blanking on the Zero standard.

Rapid Protocol

1. Pipette 50 μ L of standards, controls and samples into wells indicated on work list.
2. Pipette 200 μ L of anti-CHO:Alkaline Phosphatase (#F017) into each well.
3. Cover & incubate on rotator at 400-600 rpm for 2 hours at room temperature, 24°C \pm 4°C.
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 PNPP substrate.
5. Pipette 200 μ L of PNPP substrate (#F008).
6. Cover & incubate at room temperature for 90 minutes. DO NOT SHAKE.
7. Read absorbance at 405/492nm 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 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, 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 < 0.4.
- 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.

Example Data

High Sensitivity Protocol			
Well #	Contents	Abs. at 405-490nm	Mean Abs.
A1	Zero Std	0.000	0.001
B1	Zero Std	0.002	
C1	1ng/mL	0.040	0.043
D1	1ng/mL	0.046	
E1	4ng/mL	0.153	0.147
F1	4ng/mL	0.141	
G1	20ng/mL	0.625	0.616
H1	20ng/mL	0.607	
A2	75ng/mL	1.367	1.378
B2	75ng/mL	1.389	

Rapid Protocol			
Well #	Contents	Abs. at 405-490nm	Mean Abs.
A1	Zero Std	0.000	0.002
B1	Zero Std	0.004	
C1	4ng/mL	0.051	0.049
D1	4ng/mL	0.047	
E1	20ng/mL	0.158	0.164
F1	20ng/mL	0.170	
G1	75ng/mL	0.535	0.539
H1	75ng/mL	0.544	
A2	250ng/mL	1.270	1.288
B2	250ng/mL	1.306	

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 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 qualification protocols can be obtained by contacting our Technical Services Department or on-line 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. The LOD is 400pg/mL for the High Sensitivity Protocol and 1.7ng/mL for the Rapid Protocol. The lower limit of quantitation (LOQ) is ~1ng/mL for the High Sensitivity Protocol and ~4ng/mL for the Rapid Protocol.

Precision

Both protocols have been shown to give very similar precision profiles in the range the samples reported below. The data below was taken from the rapid protocol and shows both intra (n=10 replicates) and inter-assay (n=5 assays) coefficients of variation (%CVs). Each laboratory is encouraged to establish precision with its protocol using a similar study.

Pool	Intra assay CV	Inter assay CV
8.5ng/mL	6.2%	8.0%
24.2ng/mL	3.6%	5.2%
72.3ng/mL	4.4%	5.3%

Hook Capacity

Increasing concentrations of CHO HCPs > 250ng/mL were assayed as unknowns. The hook capacity, defined as that concentration yielding an absorbance reading less than the absorbance of the highest standard used in the assay was ~125µg/mL.

Specificity/Cross-Reactivity

Extensive cross reactivity studies have not been performed but cumulative results from many laboratories suggest this kit is specific for CHO HCP. 1D Western blot analysis against other strains of CHO cells indicates that most of the proteins are conserved among all strains. Thus the assay should be useful for detecting the majority of HCPs from all CHO 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 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.

Each laboratory is encouraged to test cross reactivity to its strain and any other proteins and reagents that comprise their sample types such as the product itself or any excipients and stabilizers. Typical culture media additives like fetal calf serum and the purified bovine proteins BSA, Transferrin, and IgG were tested and found not to cross-react with the antibodies used in this kit.

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 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 250ng/mL standard provided with this kit, into the sample in question. For example, we suggest adding 1 part of the 250 ng/mL standard to 4 parts of the test sample. This yields an added spike of 50ng/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.

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

Cygnus Technologies, LLC
4332 Southport Supply Rd. SE
Southport, NC 28461 USA
Tel: 910-454-9442
Fax: 910-454-9443
Email: techsupport@cygnustechnologies.com

