CAP® cell Host Cell Proteins

Immunoenzymetric Assay for the Measurement of
CAP® cell line Host Cell Proteins
Catalog # F820

Intended Use

This kit is intended for use in determining the presence of host cell protein impurities in products manufactured by expression in the CAP® human cell line. The kit is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals.

Summary and Explanation

Expression of therapeutic proteins and vaccines in the CAP® human cell line is a cost effective method for production of commercial quantities of a drug substance. The manufacturing and purification process of these products leaves the potential for impurities by host cell proteins (HCPs) from CAP® cells. 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 pollutants 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 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, 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 in goats and affinity purified using CAP® cell line HCPs found in protein free conditioned media. Western blot, 1 dimensional, was used as a preliminary method and established that the antibodies reacted to the majority of HCP bands resolved by the PAGE separation. Further characterization to more highly resolved proteins was accomplished by 2D PAGE silver stain compared to 2D Western blot showing reactivity to the majority of HCPs in all quadrants for the gel. 2D Western blot lacks the sensitivity and specificity for a meaningful assessment of the percentage coverage to individual HCPs. To give a better measure of the coverage to individual HCPs we use a method called Antibody Affinity Extraction (AAE). In this method HCPs are bound to an affinity column of anti-HCP antibody, eluted, concentrated and then fractionated by 2D PAGE. The silver stain spots from the starting sample are compared to the silver stain spots from the 2D fractionated AAE sample. Because much more sample can be loaded on to the AAE column, and multiple extracts can be pooled and concentrated, AAE is much more sensitive than 2D WB. The comparison of a silver stain to silver stain makes spot matching much easier and the removal of most of the product protein by AAE further improves the specificity over 2D WB. A total of 760 HCPs were seen in the starting sample. The AAE sliver stain was able to match 559 of the 760 spots for coverage of 74%.

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. 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.

This highly sensitive ELISA kit has been qualified for testing of final product HCPs using actual in-process and final drug substance samples. Each user of this kit is encouraged to perform a similar qualification study to demonstrate it meets their analytical needs. Provided this kit can be satisfactorily qualified for your samples, the application of a more process specific assay may not be necessary, in that such an assay would only provide information redundant to this generic assay. However, if your qualification studies indicate the antibodies in this kit are not sufficiently reactive with your process specific HCPs it may be desirable to also develop a more

process specific ELISA. This later generation assay may require the use of a more specific and defined antisera. Alternatively, if the polyclonal antibody used in this kit provides sufficient sensitivity and broad antigen reactivity, it may be possible to substitute the standards used in this kit for ones made from the impurities that typically co-purify through your purification process and thus achieve better accuracy for process specific HCPs. The use of a process specific assay with more defined antigens and antibodies in theory may yield better specificity, 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.

Principle of the Procedure

The CAP® cell line assay is a two-site immunoenzymetric assay. Samples containing CAP® cell HCPs are reacted simultaneously with a horseradish peroxidase (HRP) enzyme labeled anti-CAP® cell antibody (goat polyclonal) in microtiter strips coated with an affinity purified capture anti-CAP® cell antibody. The immunological reactions result 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 CAP® cell line HCPs present.

Reagents & Materials Provided

Component	Product #
Anti-CAP® cell:HRP Affinity purified goat antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	**F821**
Anti-CAP® cell coated microtiter strips 12x8 well strips in a bag with desiccant	**F822***
CAP® cell HCP Standards CAP® cell HCPs in I094 with preservative. 8 standards at 0, 3, 6, 12, 25, 50, 100, and 200ng/mL. 1 mL/vial.	**F823**
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 # F822.

Storage & Stability

- All reagents should be stored at 2°C to 8°C for stability until the expiration date printed on the kit.
- Reconstituted wash solution is stable until the expiration date of 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.

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 # I094)
- Distilled water
- 1 liter wash bottle for diluted wash solution

Precautions

- For Research or Manufacturing use only.
- Stop reagent is 0.5M H_2SO_4. 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 manually 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. CAP® cells are a human cell line. As such they contain many proteins with antigenic homology to other human cell lines and proteins. For this reason

precautions should be taken to avoid pollutants of these kit reagents with other human source materials. Mucosal aerosols, human dander etc. can contaminate the microtiter wells, HRP conjugate or the standards vials resulting in high and/or variable absorbances. Technicians performing this assay should avoid breathing or talking over the open wells or bottles. Gloves and a mask will prevent most pollutants. If available, a laminar flow bio-safety cabinet is a good place to perform the pipetting steps. Keep the microtiter plate covered during all incubation steps.

3. 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 samples upstream in the purification process. Samples with HCP greater than 1mg/mL may give absorbances less than the 200ng/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 sample at all dilutions 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. If a hook effect is possible, samples should also be assayed diluted. If the HCP concentration of the undiluted or less diluted sample is less than a more 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# I094 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 I094, its matrix begins to approach that of the standards, thus reducing any inaccuracies caused by dilutional artifacts. Other prospective diluents must be tested for non-specific binding and 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 perform a qualification study to demonstrate that the kit antibodies and assay procedure are fit for the intended purpose and will yield acceptable specificity, accuracy, and precision. A suggested protocol for this qualification can be obtained from our Technical Services Department or our web site. Cygnus offers a qualification/testing service using various orthogonal methods for determination of antibody coverage and identification of individual HCPs in downstream samples. These methods include 2D PAGE, Antibody Affinity Extraction (AAE), 2D HPLC, 2D Western blot, and Mass Spectrometry.
- The standards used in this assay are comprised of CAP® cell line HCPs obtained after the culture of null CAP® cells in protein free media. 2D PAGE & AAE analysis of the antibodies used in this kit demonstrate that they recognize the majority of individual HCPs.
- 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 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. This diluted standard when assayed as an unknown, should give an added HCP value in the range of 30 to 50ng/mL. Consult *Cygnus Technologies* Technical Service Department for advice on how to quantitate the assay in problematic matrices.
- Avoid the assay of samples containing sodium azide (NaN3) which will destroy the HRP activity of the conjugate and could result in the under-estimation of HCP levels.

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples in the range of 8-200ng/mL. CVs for samples <8ng/mL may be greater than 10%.
- It is recommended that each laboratory assay appropriate quality control samples in each run to insure that all reagents and procedures are correct.

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, you are advised to contact Technical Services for input on the best way to achieve your desired goals.
- The protocol specifies use of an approved microtiter plate shaker or rotator 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 step in the plate by about one hour in order 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.**
- Bring all reagents to room temperature. Set-up plate spectrophotometer to read dual wavelength at 450nm for the test wavelength and ~650nm for the reference.
- Thorough washing is essential to proper performance of this assay. Automated plate washing systems or other vacuum aspiration devices are not recommended. The manual method described in the assay protocol is preferred for best precision, sensitivity and accuracy. A more detailed discussion of this procedure can be obtained from our Technical Services Department or on our web site. In addition, a video demonstration of proper plate washing technique is available in the 'Technical Help' section of our web site.
- All standards, controls, and samples should be assayed at least in duplicate.
- Maintain a repetitive timing sequence from well to well for all assay steps to 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 your 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.**
- Strips should be read within 30 minutes after adding stop solution since color will fade over time.

Assay Protocol

1. Pipette 50µL of standards (#F823), controls and samples into wells indicated on work list.

2. Pipette 100µL of anti-CAP® cell:HRP (#F821) into each well.

3. Cover & incubate on rotator at 400-600rpm for 2 hours 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.

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!** 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 450nm	Mean Abs.
1A	0ng/mL	0.112	0.113
1B	0ng/mL	0.113	
1C	3ng/mL	0.136	0.135
1D	3ng/mL	0.133	
1E	6ng/mL	0.159	0.156
1F	6ng/mL	0.153	
1G	12ng/mL	0.207	0.206
1H	12ng/mL	0.205	
2A	25ng/mL	0.319	0.324
2B	25ng/mL	0.329	
2C	50ng/mL	0.566	0.570
2D	50ng/mL	0.574	
2E	100ng/mL	0.990	0.995
2F	100ng/mL	1.000	
2G	200ng/mL	1.881	1.818
2H	200ng/mL	1.754	

Performance Characteristics

Cygnus Technologies has qualified this assay by conventional criteria as indicated below. A copy of this qualification report can be obtained on our web site or by request. This qualification 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 qualification protocols can be obtained by contacting our Technical Services Department or at our web site.

Sensitivity

The lower limit of detection (**LOD**) is defined as that concentration corresponding to a signal two standard deviations above the mean of the zero standard. LOD is ~0.9 ng/mL.

The lower limit of quantitation (**LOQ**) is defined as the lowest concentration, where concentration coefficients of variation (CVs) are <20%. The LOQ is ~1.5 ng/mL.

Precision

Both intra (n=20 replicates) and inter-assay (n=10 assays) precision were determined on 3 pools with low (~12ng/mL), medium (~25ng/mL), and high concentrations (~50ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	8.2%	6.3%
Medium	3.4%	6.6%
High	3.6%	3.5%

Accuracy and Specificity

Accuracy and specificity were determined by dilutional linearity and spike recovery analysis of 5 samples; a harvest sample, 3 in-process intermediates, and the final drug substance. All samples gave dilutional linearity and spike recovery data within the 80% to 120% acceptable range. (See the Catalog F820 ELISA Qualification Summary data available on the Cygnus Technologies web site for the raw data). Dilutional linearity and spike recovery analysis are the most critical qualification experiments and should be performed by all labs for each type of sample they will be testing. These experiments assure the kit has excess antibody for the HCPs in a given sample and that the product protein itself and nothing in the sample matrix interfere in the accurate and specific detection of HCP.

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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