HeLa Host Cell Proteins

Immunoenzymetric Assay for the Measurement of
HeLa Cell Host Cell Proteins
Catalog # F810

Intended Use

This kit is intended for use in determining the presence host cell protein impurities in products manufactured by expression in HeLa host cells. The kit is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals. Users should qualify this assay for use with their product samples.

Summary and Explanation

Recombinant expression by HeLa cells is a widely-used procedure to obtain sufficient and cost effective quantities of a desired protein or virus. 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 HeLa cells. 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 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 the HCPs that could contaminate the product independent of the purification process. The antibodies have been generated against and affinity purified using a mild lysate of HeLa cells to obtain HCPs typically encountered in the initial product recovery step. Coverage of the antibody to individual HCPs was determined by a method termed Antibody Affinity Extraction (AAE). AAE is much more sensitive and specific than 2D Western blot. To determine coverage to your process specific HCPS it is recommended to use AAE. For more information on AAE analysis please 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. 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. A process specific 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 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" or "platform" 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 HeLa Host Cell Protein assay is a two-site immunoenzymetric assay. Samples containing HeLa cell proteins are reacted in microtiter strips coated with an affinity purified capture antibody. A second HRP labeled anti-HeLa antibody is reacted simultaneously, forming a sandwich complex of solid phase antibody-HeLa HCP-

enzyme labeled antibody. The microtiter strips are then washed to remove any unbound reactants. After the washes, the substrate tetramethylbenzidine is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and is directly proportional to the concentration of HeLa Host cell proteins present.

Reagents & Materials Provided

Component	Product #
Anti-HeLa:HRP Affinity purified goat antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	**F811**
Anti-HeLa coated microtiter strips 12x8 well strips in a bag with desiccant	**F812***
HeLa HCP Standards HeLa HCPs in Catalog # I094 with preservative. Standards at 0, 3, 6, 12, 25, 50, 100, and 200ng/mL. 1 mL/vial.	**F813**
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 # F812.

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 0.200, 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 samples upstream in the purification process. Samples with HCP greater than 800µg/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 dilution 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 qualify that the kit antibodies and assay procedure yield acceptable coverage, dilutional linearity, accuracy, and precision. A suggested protocol for this qualification can be obtained from our Technical Services Department or our web site.
- The standards used in this assay are comprised of HeLa HCPs solubilized by mechanical disruption and detergent. AAE analysis of the antibodies used in this kit demonstrates that they recognize the majority of distinct PAGE separated proteins 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 HeLa this kit should be adequately reactive to HCPs from your strain
- 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 3 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 40 to 60ng/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.

Assay Protocol

- The suggested assay protocol takes approximately 2.5 hours to complete and will yield a sensitivity of <2ng/mL. 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 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 step in the plate by about one hour to achieve comparable results to the shaking protocol. Do not shake during the 30-minute substrate incubation step, as this may result in higher backgrounds and worse precision.
- 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 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 your 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.**
- Strips should be read within 30 minutes after adding stop solution since color will fade over time.

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 15% for samples in the range of 6-200ng/mL. CVs for samples <6ng/mL may be greater than 15%.

- 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

1. Pipette 100µL of anti-HeLa:HRP (#F811) into each well.

2. Pipette 50µL of standards (#F813), controls and samples into wells indicated on work list.

3. Cover & incubate on orbital plate shaker at 400 - 600 rpm 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

Example Data

Well #	Contents	Abs. at 450nm-650nm	Mean Abs.
A1	0ng/mL	0.044	0.044
A2	0ng/mL	0.044	
B1	3ng/mL	0.087	0.085
B2	3ng/mL	0.083	
C1	6ng/mL	0.127	0.126
C2	6ng/mL	0.125	
D1	12ng/mL	0.229	0.223
D2	12ng/mL	0.217	
E1	25ng/mL	0.385	0.385
E2	25ng/mL	0.384	
F1	50ng/mL	0.708	0.719
F2	50ng/mL	0.730	
G1	100ng/mL	1.306	1.305
G2	100ng/mL	1.303	
H1	200ng/mL	2.636	2.580
H2	200ng/mL	2.524	

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.

Performance Characteristics

Cygnus Technologies has qualified this assay by conventional criteria as indicated below. This qualification is generic in nature and is intended to supplement but not replace certain user and product specific 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 dilution 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 website.

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 0.5 ng/mL.
The lower limit of quantitation (**LOQ**) is defined as the lowest concentration, where concentration coefficients of variation (CVs) typically are <20%. The LOQ is 2 ng/mL.

Precision

Both intra (n=20 replicates) and inter-assay (n=10 assays) precision were determined on 3 pools with low (6ng/mL), medium (25ng/mL), and high concentrations (100ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	5.5%	4.3%
Medium	3.5%	3.0%
High	4.1%	2.8%

Recovery / Interference Studies

Various buffer matrices have been evaluated by adding known amounts of HeLa 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 (<6.0 and >8.5) as well as certain detergents can cause under-recovery. Organic solvents and high salt concentration can also interfere. In some cases, very high concentrations of the product protein may also cause a negative interference in this assay. Each user should demonstrate that their sample matrices and product itself yield accurate recovery. Such an experiment can be performed by diluting 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 3 parts of the test sample. This yields an added spike of 50ng/mL. Any endogenous HeLa HCPs from the sample itself determined prior to spiking and corrected for by the 20% dilution of that sample can 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 >200ng/mL were assayed as unknowns. The hook capacity, defined as that concentration that will give an absorbance reading less than the 200 ng/mL standard was >800 µg/mL**.**

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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