

PER.C6[®] cells Host Cell Proteins

Immunoenzymetric Assay for the Measurement of PER.C6[®] cell line Host Cell Proteins Catalog # F530

Intended Use

This kit is intended for use in determining the presence of host cell protein contamination in products manufactured by expression in the PER.C6[®] 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 PER.C6[®] 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 contamination by host cell proteins (HCPs) from PER.C6[®] cells. 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 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 PER.C6[®] cell line HCPs found in protein free conditioned media. They have then been characterized against HCP found in samples from 3 commercial products expressed in PER.C6[®] cells. 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 a method we term 2D HPLC. We find this method far superior to 2D

Western blot in sensitivity and specificity. The 2D HPLC fractionation of the conditioned media derived HCPs used in the ELISA kit standards showed reactivity to ~530 fractions presumed to be individual HCPs. For more information on this 2D HPLC 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.

This highly sensitive ELISA kit has been validated for testing of final product HCPs using actual in-process and final drug substance samples from 3 different drug products. Based on this experience this assay should have application as a multi-use assay for other products expressed in PER.C6[®] cells. Each user of this kit is encouraged to perform a similar validation study to demonstrate it meets their analytical needs. Provided this kit can be satisfactorily validated 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 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 provides sufficient sensitivity 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 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 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.

Principle of the Procedure

The PER.C6[®] cell line assay is a two-site immunoenzymetric assay. Samples containing PER.C6[®] cell HCPs are reacted simultaneously with a horseradish peroxidase (HRP) enzyme labeled anti-PER.C6[®] cell antibody (goat polyclonal) in microtiter strips coated with an affinity purified capture anti-PER.C6[®] 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, 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 PER.C6® cell line HCPs present.

Reagents & Materials Provided

Component	Product #
Anti-PER.C6®cell:HRP Affinity purified goat antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	F531
Anti- PER.C6®cell coated microtiter strips 12x8 well strips in a bag with desiccant	F532*
PER.C6®cell HCP Standards PER.C6® cell HCPs in bovine albumin with preservative. Standards at 0, 2, 8, 25, 75, and 200ng/mL. 1 mL/vial	F533
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 # F532.

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.

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.

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 # 1028)

Distilled water

1 liter wash bottle for diluted wash solution

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. PER.C6® 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 contamination 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 contamination. 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 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. 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# 1028 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 I028, 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 validate that the kit antibodies and assay procedure yield acceptable specificity, accuracy, and precision. A suggested protocol for this validation can be obtained from our Technical Services Department or our web site.

* The standards used in this assay are comprised of PER.C6® cell line HCPs obtained after the culture of null PER.C6® cells in protein free culture 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 sensitive protein staining methods like silver stain or colloidal gold. 2D HPLC fractionation of this same conditioned media showed 530 protein fractions reactive in this ELISA. Because the majority of HCPs will show antigenic conservation among all strains of PER.C6® cells this kit should be adequately reactive to the vast majority of HCPs from your transfected cell line. 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 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 contaminants. This diluted standard when assayed as an unknown, should give an added HCP value in the range 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 (NaN₃) which will destroy the HRP activity of the conjugate and could result in the under-estimation of HCP levels.

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. 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 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.

* 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.**

* If the substrate has a distinct blue color prior to assay it may have been contaminated. If the absorbance of 100µL of substrate plus 100µL of stop against a water blank is greater than 0.1 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.

* Strips should be read within 30 minutes after adding stop solution since color will fade over time.

Assay Protocol

1. Pipette 50 μ L of standards, controls and samples into wells indicated on work list.
2. Pipette 100 μ L of anti-PER.C6[®] cell:HRP (#F531) into each well.
3. Cover & incubate on rotator at ~ 180rpm for 2 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.

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 <8 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.

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.	ng/mL HCP equivs.
1A	Zero Std	0.109		
1B	Zero Std	0.108	0.109	
1C	2ng/mL	0.139		
1D	2ng/mL	0.138	0.139	
1E	8ng/mL	0.240		
1F	8ng/mL	0.245	0.243	
1G	25ng/mL	0.429		
1H	25ng/mL	0.433	0.431	
2A	75ng/mL	1.042		
2B	75ng/mL	1.047	1.045	
2C	200ng/mL	2.358		
2D	200ng/mL	2.281	2.320	
2E	sample A	0.450		
2F	sample A	0.438	0.444	25.5
2G	sample B	1.060		
2H	sample B	1.053	1.057	76.4

Performance Characteristics

Cygnus Technologies has validated this assay by conventional criteria as indicated below. A copy of this validation report can be obtained on our web site or 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 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 ~0.5 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 ng/mL.

Precision

Both intra (n=20 replicates) and inter-assay (n=10 assays) precision were determined on 3 pools with low (~8ng/mL), medium (~25ng/mL), and high concentrations (~75ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	6.2%	5.2%
Medium	5.0%	5.6%
High	2.4%	6.5%

Specificity/Cross-Reactivity

Western blot and ELISA analysis against 3 commercial PER.C6® cell lines indicate that most of the proteins are conserved among all lines. Therefore this assay should be useful for detecting HCPs from other PER.C6® cell line derived products. Western blot, both 1 & 2 dimensional, 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 more sensitive and specific 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.

Cross reactivity to non-HCP components has not been extensively investigated with this kit. You should evaluate components in your samples for positive interferences such as cross reactivity and non-specific binding. Negative interference studies are described below.

Recovery/ Interference Studies

Various buffer matrices commonly used in purification as well as in-process and final formulation drug substances expressed in PER.C6® cells were evaluated by adding known amounts of PER.C6® cell HCP preparation used to make the standards in this kit. All of these samples yielded acceptable recovery defined as between 80-120%. The standards used in this kit contain 8mg/mL of bovine serum albumin intended to simulate non-specific protein affects of most sample proteins or virus products. However very high concentrations of some products may interfere in the accurate measurement of HCPs. In general, extremes in pH (<5.0 and >8.5), high salt concentration, high polysaccharide concentrations, urea, organic solvents, and most detergents can cause under-recovery. Each user should validate that their sample matrices yield accurate recovery. Such an experiment can be performed, by diluting the 200ng/mL standard provided with this kit, into the sample matrix in question as described in the "Limitations" section. *Cygnus* offers a more concentrated form of the HCP used to prepare the kits standards for your spike recovery and preparation of analyte controls.

Hook Capacity

Increasing concentrations of HCPs > 200 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration yielding an absorbance reading less than the 200 ng/mL standard was ~1 mg/mL.