

Vero Cell 2G Host Cell Proteins

Immunoenzymetric Assay for the Measurement of Vero Cell 2G Host Cell Proteins Catalog # F975

Intended Use

This kit is intended for use in determining the presence of host cell protein impurities in products manufactured by expression in Vero cells. 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 viral vectors and vaccines in Vero cells 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 Vero 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 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 several 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 enzyme-linked immunosorbent 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 pollute the product independent of the purification process. The antibodies have been generated against and affinity purified using mild lysate of Vero cells. 1D 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. Further characterization of coverage of the antibodies to the Vero HCPs was accomplished by an immunoaffinity chromatography method termed Antibody Affinity Extraction (AAE™). This method is superior to 2D Western blot in terms of sensitivity, specificity, and predictability of how the antibodies perform in the ELISA. The AAE fractionation of Vero HCP showed reactivity to 79% of Vero cell HCPs. For additional information on AAE please visit our website and read the posted articles under Technical Resources or 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.

Each user of this kit is encouraged to perform a qualification study similar to the studies mentioned in this insert 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. 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. 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 a custom basis.

Principle of the Procedure

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

Reagents & Materials Provided

Component	Product #
Anti-Vero Cell 2G: HRP Affinity purified goat antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	F976
Anti-Vero Cell 2G microtiter strips 12x8 well strips in a bag with desiccant	F977*
Vero Cell 2G HCP Standards Solubilized Vero cell HCPs in bovine serum albumin with preservative. Standards at 0, 3, 6, 12, 25, 50, 100 and 200ng/mL. 1 mL/vial	F978
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 # F977.

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 on Reagents' section.
- Reconstituted wash solution is stable until the expiration date of the kit.

Materials & Equipment Required But Not Provided

- Microtiter plate reader spectrophotometer with dual wavelength capability at 450 & 650nm. (*If your plate reader does not provide dual wavelength analysis you may read at just the 450nm wavelength.*)
- Pipettors - 50µL and 100µL
- Repeating or multichannel pipettor - 100µL
- Microtiter plate rotator (400 - 600 rpm)
- Sample Diluent (recommended Cat # 1028)
- Distilled water
- 1 liter wash bottle for diluted wash solution

Precautions

- For Research or Manufacturing use only.
- Stop reagent is 0.5M H₂SO₄. Avoid contact with eyes, skin, and clothing.
- 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.

Assay Protocol

- 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 in order to achieve comparable results to 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.
- All standards, controls, and samples should be assayed at least in duplicate.

Limitations

- 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.**
- If the substrate has a distinct blue color prior to assay it may have been contaminated. If the absorbance of 100 μ L of substrate plus 100 μ L of stop against a water blank is greater than 0.1 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.
- Strips should be read within 30 minutes after adding stop solution since color will fade over time.

Assay Protocol

1. Pipette 100 μ L of Anti-Vero Cell 2G: HRP (#F976) into each well.
2. Pipette 50 μ L of standards, controls and samples into wells indicated on work list.
3. Cover & incubate on orbital shaker at 400-600 rpm for 2 hours at room temperature, 24°C \pm 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 ~350 μ L. 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 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.

- Before relying exclusively on this assay to detect host cell proteins, each laboratory should qualify that the kit antibodies and assay procedure yield acceptable specificity, accuracy, and precision. A suggested protocol for this qualification can be obtained from our Technical Services Department on our web site.
- The standards used in this assay are comprised of Vero cell HCPs solubilized by methods commonly used in initial harvesting steps for vaccine products. 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.
- Because the majority of HCPs will show sufficient antigenic conservation among all lines of Vero cells this kit should be adequately reactive to HCPs from your 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 method than western blot to detect the reactivity of the antibodies in this kit to your individual HCPs *Cygnus Technologies* is pleased to perform AAE as a service to provide coverage information of the antibodies to the HCPs in your process samples.
- 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 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.

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples in the range of 3-200ng/mL. CVs for samples less than 6 ng/mL may be greater than 10%.
- It is recommended that each laboratory assay appropriate quality control samples in each run to ensure 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 immunoreactive 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!**

Example Data

Well #	Contents	Abs. at 450-650nm	Mean Abs.
A1	Zero Std	0.178	0.178
A2	Zero Std	0.178	
B1	3 ng/mL	0.220	0.221
B2	3 ng/mL	0.222	
C1	6 ng/mL	0.264	0.260
C2	6 ng/mL	0.256	
D1	12 ng/mL	0.353	0.353
D2	12 ng/mL	0.353	
E1	25 ng/mL	0.541	0.533
E2	25 ng/mL	0.525	
F1	50 ng/mL	0.895	0.891
F2	50 ng/mL	0.886	
G1	100 ng/mL	1.510	1.516
G2	100 ng/mL	1.523	
H1	200 ng/mL	2.695	2.672
H2	200 ng/mL	2.648	

Performance Characteristics

Cygnus Technologies has qualified this assay by conventional criteria as indicated below. A copy of this qualification report can be requested on our web site by searching "Request Qualification Summary". 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 sample types containing process derived HCPs within or above the analytical range of this assay should be evaluated for dilutional 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 requested by contacting our Technical Services Department or on our web site.

Sensitivity

The lower limit of detection (LOD) is defined as that concentration corresponding to a signal three standard deviations above the mean of the zero standard. LOD is ~1.4 ng/mL.

The lower limit of quantitation (LLOQ) is defined as the lowest concentration, where concentration coefficients of variation (CVs) are less than 20%. The LLOQ is ~ 3 ng/mL.

Precision

Both intra (n=24 replicates) and inter-assay (n=10 assays) precision were determined on 3 pools with low (~10ng/mL), medium (~45ng/mL), and high concentrations (~125ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	8.1%	4.8%
Medium	3.8%	2.5%
High	6.1%	2.7%

Specificity/Cross-Reactivity

This assay should be useful for detecting HCPs from Vero cell lines. 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 not 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 Technologies* is pleased to perform AAE as a service to provide coverage information of the antibodies to the HCPs in your process samples.

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 capture 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

A buffer matrix commonly used in purification and final formulation of drug substances expressed in Vero cells was evaluated by adding known amounts of Vero cell HCP preparation used to make the standards in this kit. The standards used in this kit contain 8mg/mL of bovine serum albumin intended to simulate non-specific protein effects 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 (less than 5.0 and greater than 8.5), high salt concentration, high polysaccharide concentrations, and most detergents can cause under-recovery. Each user should qualify 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 Technologies* offers a more concentrated form of the HCPs used to prepare the kits standards for your spike recovery and preparation of analyte controls.

Ordering Information/ Customer Service

Cygnus Technologies also offers kits for the extraction and detection 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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