CYGNUS TECHNOLOGIES
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HEK 293 Host Cell Proteins

Immunoenzymetric Assay for the Measurement of HEK Host Cell Proteins Catalog # F650S

Intended Use

This kit is intended for use in determining the presence of Human Embryonic Kidney (HEK) host cell protein (HCP) impurities in products manufactured by transgenic expression in HEK host cells. This kit was developed and qualified for detection of HEK HCPs using various in-process and final drug substance samples from real world processes. 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 of proteins in mammalian cell culture is a cost-effective method for production of commercial quantities of drugs and other biological substances. The manufacturing and purification process of these products leaves the potential for impurities by HCPs. 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.

The antibodies used in this kit were characterized by 1D & Antibody Affinity Extraction demonstrating reactivity to the majority of HCPs as evidenced by silver and colloidal gold staining. This simple to use, objective, and semi-quantitative ELISA is a sensitive and specific method to aid in optimal purification process development, process control, and in routine quality control and product release testing. The antibodies have been generated against and affinity purified using HEK HCPs recovered from protein additive free HEK growth media. 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.

This highly sensitive ELISA kit was qualified for testing of final product HCPs by 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

Principle of the Procedure

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

Reagents & Materials Provided

Component	Product #
Anti-HEK:HRP Affinity purified antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	**F651S**
Anti-HEK coated microtiter strips 12x8 well strips in a bag with desiccant	**F652S***
HEK HCP Standards HEK HCPs in bovine serum albumin with preservative. Standards at 0, 2, 10, 25, 75, and 200ng/mL. 1 mL/vial	**F653S**
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 # F652S

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 # I700)
- Distilled water
- 1 liter wash bottle for diluted wash solution

Storage & Stability

- The kit standards must be removed and stored at -20°C.
- All other 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.

Precautions

- For Research or Manufacturing use only.
- Stop reagent is 0.5M H_2SO_4. 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.

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. 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 120µg/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 as evidenced by an apparent increase in dilution corrected HCP concentration with increasing dilution. 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# I700 available in 100mL, 500mL, or 1liter bottles. This is the same material used to prepare the kit standards. As the sample is diluted in I700, 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 specificity, 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 HCPs recovered from null cells grown. process. AAE analysis of the antibodies used in this kit demonstrates that they recognize the majority of distinct PAGE separated proteins seen using silver staining.
- 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 35 to 45ng/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 (NaN_3) which will destroy the HRP activity of the conjugate and could result in the under-estimation of HCP levels.

Assay Protocol

- Assay variables like incubation times, sample size, and other sequential incubation schemes can be altered to manipulate assay performance. 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 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 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 qualify 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.
- 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.

Assay Protocol

1. Pipette 100µL of anti-HEK:HRP (#F651S) into each well.

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

3. Cover & incubate on orbital shaker at 400 - 600rpm for 1.5 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 450-650nm	Mean Abs.
A1	Zero Std	0.163	0.163
A2	Zero Std	0.162	
B1	2ng/mL	0.219	0.223
B2	2ng/mL	0.226	
C1	10ng/mL	0.383	0.391
C2	10ng/mL	0.399	
D1	25ng/mL	0.704	0.700
D2	25ng/mL	0.695	
E1	75ng/mL	1.392	1.386
E2	75ng/mL	1.380	
F1	200ng/mL	2.653	2.633
F2	200ng/mL	2.612	

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.

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples in the range of 2-200ng/mL. CVs for samples less than 2ng/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.

Performance Characteristics

This assay was qualified using samples from various processes. Any new sample types must be qualified by your lab to determine MRD and acceptable spike & recovery as described above and in our Qualification Summary. Operators should refer to that report for specifics on methods used in qualification, expected assay performance, and approximate MRDs for typical in-process and final product samples. This qualification is generic in nature and is intended to supplement but not replace a comprehensive user and sample type qualification that should be performed by each laboratory.

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 ~0.2 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 ~1.0ng/mL.

Specificity/Cross-Reactivity

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.

Precision

Both intra (n=20 replicates) and inter-assay (n=10 assays) precision were determined on 3 pools with low (~15ng/mL), medium (~45ng/mL), and high concentrations (~135ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	7.1%	5.2%
Medium	4.2%	3.3%
High	5.4%	2.6%

Recovery/Interference Studies

Real world in-process and final formulation drug substances were evaluated by adding known amounts of the HEK 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 4mg/mL of bovine serum albumin intended to simulate non-specific protein effects of most sample proteins. 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, urea, organic solvents, 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 HCP used to prepare the kits standards for your spike recovery and preparation of analyte controls.

Hook Capacity

Increasing concentrations of HCPs greater than 200ng/mL were assayed as unknowns. The hook capacity, defined as that concentration yielding an absorbance reading less than the 200ng/mL standard was ~120μg/mL.

Ordering Information/ Customer Service

Cygnus Technologies also offers kits for the extraction and detection of HEK 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

- Residual HEK Host Cell DNA extraction and detection using PicoGreen® dye:

To place an order or to obtain additional product information contact *Cygnus Technologies*:

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