



NS/0 Host Cell Proteins

Immunoenzymetric Assay for the Measurement of NS/0 Host Cell Proteins Catalog # F220

Intended Use

This kit is intended for use in determining the presence of NS/0 host cell protein contamination in products manufactured by recombinant expression in NS/0 cells. 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 by the hybridoma cell line NS/0 is a relatively simple and cost effective method for production of monoclonal antibodies. Many of these antibodies 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 contamination by host cell proteins (HCPs) from NS/0. 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 widely accepted due to their specificity and sensitivity. While Western blot is a powerful 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 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, and in 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 against and affinity purified using a mild lysate washed of NS/0 cells to obtain HCPs typically encountered in your initial product recovery step. 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 testing.

Because of the high sensitivity and broad reactivity of the antibodies, this generic kit has been successfully validated for testing of final product HCPs in many different products regardless of growth and purification process. When the kit can be satisfactorily validated 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 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 suitability of this kit for a given sample type and product must be determined and validated 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 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 NS/0 assay is a two-site immunoenzymetric assay. Samples containing NS/0 HCPs are reacted with a horse radish peroxidase (HRP) enzyme labeled anti-NS/0 antibody in a test tube or simultaneously in microtiter strips coated with an affinity purified capture anti-NS/0 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 NS/0 HCPs present.

Reagents & Materials Provided

<u>Component</u>	<u>Product #</u>
Anti-NS/0:HRP Affinity purified antibody conjugated to HRP in a protein matrix with preservative, 1x12mL	F221
Anti-NS/0 coated microtiter strips 12x8 well strips in a bag with desiccant	F222
NS/0 HCP Standards Solubilized NS/0 HCPs in human immunoglobulin with preservative. Standards at 0, 1, 3, 8, 25, 75, & 200ng/mL, 1 mL/vial	F223
TMB Substrate 3,3',5,5' Tetramethyl benzidine, 1x12mL	F005
Wash Concentrate (20X) Tris buffered saline with preservative, 1x50mL	F004
Stop Solution 0.5N sulfuric acid, 1x12mL	F006

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.

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 # F223A)
Distilled water
1 liter wash bottle for diluted wash solution

Precautions

For research or manufacturing use only.

The standards used in this kit contain human immunoglobulin derived from human serum. The source material was tested and found negative for antibody to HIV and Hepatitis B surface antigen. No known method can offer total assurance that HIV or Hepatitis virus or other infectious agents are absent, therefore handle these reagents as if they are potentially infectious.

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.

Preparation of Reagents

- * Bring all reagents to room temperature.
- * Dilute wash concentrate to 1 liter in distilled water, and 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 manual 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 minus a substrate blank is greater than 0.300, evaluate plate washing procedure for proper performance.

2. High Dose Hook Effect 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 the sample. Samples greater than 200 µg/mL may give absorbances less than the 200 ng/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 undiluted sample may be lower than the highest standard in the kit however these samples will fail to show acceptable dilutional recovery/parallelism as evidenced by an apparent increase in HCP concentration with increasing dilution. High Dose Hook is 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 dilution (MRD) where the dilution adjusted value remains essentially constant. 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# F223A 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 F223A its matrix begins to approach that of the standards thus reducing any inaccuracies caused by dilutional artifacts. Other prospective diluents must be tested for 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 by contacting our Technical Services Department or our web site.

* The standards used in this assay are comprised of NS/0 HCPs solubilized by mechanical disruption and detergent. Western blot analysis of the antibodies used in this kit demonstrates that they recognize the majority of distinct PAGE separated bands 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 NS/0 this kit should be adequately reactive to HCPs from your strain. Several clients have successfully validated this kit for their individual NS/0 strains demonstrating acceptable specificity, accuracy, and sensitivity for process intermediate samples as well as final product. However, there can be no guarantee that this assay will detect all proteins or protein fragments from your process. In recognition of this potential limitation, we suggest reporting unknown samples in arbitrary dose units such as “ng/mL or parts per million of total immuno-reactive HCP equivalents”.

* 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 protein or other components in the sample matrix may result in either positive or negative interference in this assay. 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 a value 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 (NaN_3) which will destroy the HRP activity of the conjugate and could result in the under-estimation of HCP levels.

Assay Protocol

* Two assay protocols are offered. The Standard Assay Protocol is a simultaneous incubation of sample with HRP conjugated antibody. This yields a sensitivity of ~1ng/mL and requires 2.5 hours to complete. The Standard Protocol should provide an adequate level of sensitivity for most applications.

* The High Sensitivity Protocol uses what is called a ‘reverse sequential’ incubation scheme. In this protocol the sample and enzyme conjugated antibody are first incubated in an uncoated tube for 1 hour. After this first

incubation, the mixture is then added to the coated microtiter wells and incubated for 3 more hours. This procedure takes approximately 5 hours to complete and yields a sensitivity of less than 500pg/mL of total HCP equivalents.

*Seven standards are provided with the kit. When using the Standard Assay Protocol it is not advisable to employ the 1ng/mL standard (Standard B), since it may not be well discriminated from the Zero Standard.

Therefore, when performing the Standard Assay Protocol use the 0, 3, 8, 25, 75 and 200 ng/mL standards. When using the High Sensitivity Protocol the 1ng/mL standard should be used but it is recommended that the 200 ng/mL standard (Standard G) not be included since the OD value may be out of the linear range of most microtiter plate readers.

Therefore, when performing the High Sensitivity Protocol use the 0, 1, 3, 8, 25, and 75 ng/mL standards.

*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, users are advised to contact our technical services for input on the best way to achieve your desired goals.

* The protocols specify use of an approved microtiter plate shaker or rotator for the immunological steps. 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 routine protocols. **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.

* 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 the 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.

Standard 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-NS/0:HRP 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 pipeting 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.
5. Pipette 100 μ L of substrate.
6. Incubate at room temperature for 30 minutes. DO NOT SHAKE.
7. Pipette 100 μ L of Stop Solution.
8. Read absorbance at 450/650nm blanking on the Zero standard.

High Sensitivity Protocol

1. Use clean polypropylene test tubes or micro-centrifuge vials with caps.
2. Pipette 200 μ L of standards, controls and samples into the labeled tubes or vials.
3. Pipette 200 μ L of anti-NS/0:HRP enzyme conjugate into each tube or vial.
4. Cap, vortex and allow to react for 1 hour at room temperature, 24°C \pm 4°.
5. Transfer 150 μ L of the reaction mixture to each of duplicate coated wells in the coated microtiter strips provided.
6. Cover the strips by placing into an airtight 'zip-lock' plastic bag and place them on the microtiter plate rotator/shaker.
7. Incubate on rotator at ~ 180rpm for 3 hours at room temperature.
8. 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.
9. Pipette 100 μ L of TMB substrate.
10. Cover, place out of direct sunlight and incubate for 30 minutes at room temperature. DO NOT SHAKE.
11. Pipette 100 μ L of Stop Solution.
12. Read absorbance at 450/650nm blanking on the zero standard.

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL “total immunoreactive HCP equivalents” (See Limitations section above). 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

Standard Assay Protocol

Well #	Contents	Abs. at 450nm	Mean Abs.	ng/mL HCP equivs.
1A	Zero Std	0.000		
1B	Zero Std	0.002	0.001	
1C	3ng/mL	0.020		
1D	3ng/mL	0.025	0.023	
1E	8ng/mL	0.054		
1F	8ng/mL	0.050	0.052	
1G	25ng/mL	0.198		
1H	25ng/mL	0.206	0.202	
2A	75ng/mL	0.530		
2B	75ng/mL	0.568	0.547	
2C	200 g/mL	1.442		
2D	200 g/mL	1.434	1.438	
2E	sample A	1.755		
2F	sample A	1.715	1.735	>200
2G	sample B	0.059		
2H	sample B	0.055	0.057	8.4

Quality Control

- With the Standard Assay Protocol, 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 < 3 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.

Example Data

High Sensitivity Protocol

Well #	Contents	Abs. at 450nm	Mean Abs.	ng/mL HCP equivs.
1A	Zero Std	0.000		
1B	Zero Std	0.002	0.001	
1C	1ng/mL	0.023		
1D	1ng/mL	0.027	0.025	
1E	3ng/mL	0.077		
1F	3ng/mL	0.071	0.074	
1G	8ng/mL	0.198		
1H	8ng/mL	0.190	0.194	
2A	25ng/mL	0.520		
2B	25ng/mL	0.530	0.525	
2C	75ng/mL	1.480		
2D	75ng/mL	1.450	1.465	
2E	sample A	1.755		
2F	sample A	1.715	1.735	>75
2G	sample B	0.079		
2H	sample B	0.075	0.077	3.1

Performance Characteristics

Cygnus Technologies has validated this assay by conventional criteria as indicated below. A more detailed copy of this “Validation Summary” report can be obtained by request. This validation is generic in nature and is intended to supplement but not replace certain user and product specific 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 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 ~0.8 ng/mL in the Standard Assay Protocol and 300 pg/mL in the High Sensitivity Protocol.

The lower limit of quantitation (LOQ) is defined as the lowest concentration, where concentration coefficients of variation (CVs) are <20%. The LOQ is ~3 ng/mL in the Standard Assay Protocol and ~1ng/mL in the High Sensitivity Protocol.

Precision

Both intra (n=10 replicates) and inter-assay (n=5 assays) precision were determined on 3 pools with low (9ng/mL), medium (21ng/mL), and high concentrations (65ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	4.3	8.7
Medium	3.7	5.6
High	6.1	6.4

Specificity/Cross-Reactivity

Immunoblot analysis against other cell lines of NS/0 indicates that most of the proteins are conserved among all strains. Thus this assay should be useful for detecting HCP's from other NS/0 cell lines. Normal human IgG was shown to be nonreactive in this assay at concentrations of <5mg/mL. Above 5mg/mL there was an increase in absorbance above the Zero Standard that could be due to non-specific binding (NSB) or cross reactivity to a contaminant in the hIgG preparation. 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 were evaluated by adding known amounts of NS/0 HCP preparation 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%. While the standards used in this kit contain 2mg/mL normal human immunoglobulin, some product human IgGs particularly at very high concentrations (>5mg/mL) may interfere in the accurate measurement of HCP's. In general extremes in pH (<5.0 and >8.5), high salt concentration, and certain 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.

Hook Capacity

Increasing concentrations of HCPs > 200 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration which will give an absorbance reading less than the 200 ng/mL standard was >200 µg/mL.

Ordering Information/ Customer Service

To place an order or to obtain additional product information contact <i>Cygnus Technologies</i> Customer Support:

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