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## **NS/0 HCP ELISA kit Cat # F220**

### **Validation Report**

**Report dated Dec. 12, 2003**

The data reported below was generated by *Cygnus Technologies* to qualify and validate an ELISA to NS/0 Host Cell Proteins (HCPs). This ELISA kit utilizes goat and rabbit anti-NS/0 antibodies. The generation and characterization of those antibodies has been previously described. As evidenced by Western Blot staining correlated to PAGE colloidal gold staining, the anti-NS/0 antibodies react to what appears to be the majority of NS/0 HCPs. As the following data demonstrate, the NS/0 ELISA kit developed with these antibodies is an accurate, sensitive, and precise method for detection of NS/0 HCPs in typical human monoclonal antibody products and other sample matrices. The assay is capable of detecting as little as 300pg/mL of NS/0 HCP.

The data summarized below was generated by *Cygnus Technologies* to establish the performance parameters and validity of this kit to measure NS/0 HCP in a typical human antibody and buffer formulation. This data is intended to supplement and not replace user generated validation data. This data is representative of what a laboratory can expect to achieve when following the kit insert recommended protocols. Significant differences in these performance parameters may be indicative of problems with reagents, laboratory equipment, or technique and should be investigated before reporting results.

It is recommended that a user validation study include at least the following experiments to validate this kit for use with their product: (1) Each user should perform intra and inter assay precision experiments to establish their procedural proficiency. (2) Each user should perform recovery experiments using their test sample matrices. Such a study can be performed by adding known amounts of the 200ng/mL standard provided with this kit to the final product or any intermediate samples, which are to be tested. Ideally these test sample matrices should be devoid of any HCP or have very low levels (<20ng/mL) determined prior to adding the 200ng/mL standard. Such an experiment will establish the degree of sample matrix interference in the recovery of NS/0 HCPs. (3) Laboratories should also perform dilutional recovery experiments on their actual samples. This experiment assumes that at least some of the test samples from the purification process will have significant levels of HCP. Such samples will be serially diluted by some appropriate diluent previously shown to give acceptable recovery. When diluted, samples should give essentially the same value at each dilution when multiplied by the appropriate dilution factor. This experiment establishes the condition of antibody excess for accurate quantitation and determines that typical process samples do not have HCPs in the "Hook Region" of the concentration response curve.

**Materials:**

- Anti-NS/0 antibodies were generated in two goats and 6 rabbits. IgG fractions were obtained from Protein G affinity chromatography and then subjected to antigen affinity purification using lysate HCPs from an NS/0 proteins immobilized onto Sepharose 4B
- HRP conjugates to the affinity purified pooled rabbit and goat antibodies
- Coated Microtiter Plates: Blend of affinity purified antibody from both goats. Three coated plate lots were used in the validation: 57/02-1, 15082, & N102
- Standards: NS/0 HCPs obtained from lysis of cells (HCP concentrate lot # 7102). Standards made at 0, 2, 8, 25, 75, & 200ng/mL in typical antibody product formulation buffer Code # F223A
- TMB Substrate, Cat. # F005
- Wash Solution, Cat.# F004
- Stop Solution, Cat. # F006

**Data References:** Raw data for these experiments are recorded in Notebook #1-NS/0 pages 68-100 and notebook #2, pages 1-27.

**Methods:** The assay format is a 96 well microtiter strip sandwich ELISA method using HRP as the enzyme and TMB as the substrate. Two assay protocols have been validated. The so-called Standard Protocol is a “simultaneous incubation” assay procedure where sample is incubated at the same time in the coated wells with enzyme conjugated antibody. This protocol takes approximately 2.5 hours to complete and has an analytical range of 3 to 200ng/mL. The second protocol is the “High Sensitivity” assay. This is a reverse sequential method in which sample and conjugated antibody are first incubated in an uncoated tube and then the mixture transferred to the coated microtiter strip wells for a second incubation. This assay takes about 4.5 hours to complete and has an analytical range of 1 to 75ng/mL. Wells are passively coated with affinity purified goat anti-NS/0 and blocked and stabilized with a proprietary solution. The Standard Protocol uses 6 standards ranging in concentration from 0 to 200ng/mL. The High Sensitivity Protocol uses 6 standards ranging in concentration from 0 to 75ng/mL. Several other assay protocols were evaluated during the development of the ELISA. Sequential incubation of sample first with either the coated capture antibody (forward sequential) or first with the enzyme conjugated antibody (reverse sequential) was compared to the simultaneous assay in which both sample and conjugated antibody are incubated together. The effects of sample size, incubation times, and antibody conjugate concentration were also evaluated in selecting the final protocol. These variations indicated that the assay and its antibodies are robust and that minor protocol changes should not significantly affect the accuracy of the method. Thus, it is believed that the assay protocol could be modified to specifically manipulate certain other performance parameters such as more or less sensitivity, increased analytical range, or reduced assay time. Should any laboratory using this kit decide to modify the assay protocol it is recommended that they repeat at least some of the validation studies performed below. Minimally we would recommend that any modified protocol be re-validated for analytical recovery and for precision.

**ELISA Validation:** Typical absorbance data for the kits standards tested in duplicate and using the Standard Protocol and High Sensitivity Protocol are shown below.

**Typical NS/0 ELISA Standard Curve, Standard Protocol**

Standard Concentration ng/mL	Absorbance 1 <sup>st</sup> well	Absorbance 2 <sup>nd</sup> well	Mean absorbance
0	0.080	0.082	0.081
3	0.100	0.105	0.103
8	0.134	0.130	0.132
25	0.278	0.286	0.282
75	0.610	0.648	0.629
200	1.522	1.514	1.518

**Typical NS/0 ELISA Standard Curve, High Sensitivity Protocol**

Standard Concentration ng/mL	Absorbance 1 <sup>st</sup> well	Absorbance 2 <sup>nd</sup> well	Mean absorbance
0	0.065	0.067	0.066
1	0.089	0.093	0.091
3	0.143	0.137	0.140
8	0.264	0.256	0.260
25	0.586	0.596	0.591
75	1.546	1.516	1.531

**Assay Standardization/Calibration:** NS/0 cells were used to prepare standards. These cells were first washed to remove media components and then lysed by freeze thaw and high speed vortexing. The cell proteins were further solubilized in a mild detergent to further increase membrane permeability. After high speed centrifugation and filtration at 0.2 microns the resulting clarified HCPs were dialyzed against a 3500 kD cutoff membrane to remove small peptides, amino acids, and other non-HCP molecules. Total protein was then determined by BCA, using BSA as a standard. This material was assigned lot # 7102, aliquoted, and stored at -80°C for use in preparing assay standards. To minimize sample matrix interference standards were made in a “typical” formulation buffer used for the human therapeutic monoclonal antibody products except that in place of the product antibody, 2mg/mL of normal human IgG was substituted.

**Precision:** Precision is defined as the percent coefficient of variation (%CV). This is calculated by dividing the standard deviation by the mean value for a number of replicate determinations of control samples throughout the range of the assay. Both within (Intra) and between (Inter) assay precision were determined for 3 control samples. The data shown below indicate the assay can be very precise. The design goal specifications are given in the last column of each experiment. While actual precision may vary from laboratory to laboratory and technician to technician it is recommended that all operators achieve precision below these design goals before reporting results.

### Standard Protocol

Intra-assay				Inter-assay			
# of tests	Mean ng/mL	%CV	Design Goal Specification	# of assays	Mean ng/mL	%CV	Design Goal Specification
10	9.08	4.3	<15%	5	8.1	8.7	<20%
10	21.08	3.7	<15%	5	24.5	5.6	<20%
10	65.13	6.1	<15%	5	74.3	6.4	<20%

### High Sensitivity Protocol

Intra-assay			
# of tests	Mean ng/mL	%CV	Design Goal Specification
10	7.34	6.9	<15%
10	24.1	6.1	<15%
10	73.1	2.3	<15%

**Accuracy/Matrix Interference:** Defined as the ability of the assay method to correctly quantitate known concentrations of NS/0 HCP in a representative sample matrix, accuracy was evaluated by spiking NS/0 HCP into the same buffer matrices as actual final and in-process samples. The most important application of this assay is as a release test for final product. For this reason a buffer used to formulate other human antibody products was used to prepare the standards. This buffer is a Phosphate/Sucrose/Arginine/Tween 20 formulation containing 2mg/mL chromatographically purified normal human IgG to simulate the sample protein effect of typical product antibodies.

Potential interference from product antibody was first simulated by determining the recovery of 100ng/mL of HCP spiked into 10, 5, 2, and 1mg/mL of normal human IgG as shown below. Recovery of spiked HCP was very good at all hIgG concentrations however the background ODs for the 10ng/mL hIgG sample was significantly greater than the zero standard, indicating an apparent, unexpected NS/0 HCP level that is presumably due either to some cross reactivity to hIgG or some impurity in the hIgG preparation or an increase in assay non-specific binding at very high concentrations of hIgG. It is cautioned that if samples with concentrations of product antibody higher than ~2mg/mL are to be tested then, either those samples should be first diluted to 2mg/mL or assay standards at higher protein concentrations should be made to better simulate matrix effects.

### Spike and Recovery of 100 ng/mL HCPs into product simulated (hIgG) matrix

hIgG Conc. (mg/mL)	Background NS/0 activity (ng/mL) of hIgG (Non-specific binding)	NS/0 HCP Recovered (ng/mL)	% Recovery
10	2.8	120	116.7
5	0.7	111.6	110.8
2	0.5	108.2	107.7
1	0	113.6	113.6

Spike and recovery at 25, 50, & 100 ng/mL was performed on an actual final product sample submitted by a client company. That sample was in the formulation buffer with the product antibody concentration at 2.21mg/mL. The concentration of HCP measured in that sample before spiking was 9.1ng/mL. The table below shows the recovery data, ranging from 82.5% to 95%, with a mean=88%.

### Spike & Recovery into a real Final Product sample

NS/0 HCP Spike ng/mL	NS/0 Recovered ng/mL	% Recovery corrected for dilution of the sample
0	9.1	NA
25	30.3	95
50	85.6	86
100	82.5	82

Three sample buffer matrices representing in-process sample matrices were evaluated as supplied by a client. These matrices are similar to those that might be found for some in-process samples. All 3 matrices were tested unspiked and spiked at 25, 50, & 100ng/mL of HCP. The data presented below show that Matrices 2 & 3 were significantly inhibitory to spike and recovery. However, such inhibition is not surprising. The high concentration of ammonium sulfate is no doubt the problem for Matrix 2 while the acid pH and strong buffer effect of the citrate in Matrix 3 will certainly inhibit antibody binding. Because samples in these matrices will be from upstream in the purification process it is likely that the true NS/0 HCP levels will be sufficiently high as to allow for the dilution of the sample in order to overcome the matrix interference. If such in-process samples are to be routinely tested, then a more thorough analysis should be performed. Based on this experiment we recommended that samples in either matrix should be diluted approximately 20 fold to overcome the matrix interference. Alternatively, Matrix 3 could be pH neutralized or Matrix 2 & 3 could be diafiltered to get the HCPs into a more assay compatible buffer.

### Spike & Recovery into possible In-Process Buffers

Matrix	Buffer Matrix	Average % recovery
#1	50mM phosphate with 250mM NaCl	88
#2	25mM phosphate with 0.5M ammonium sulfate	30
#3	0.1M sodium citrate with 200mM NaCl ~pH4	<10

**Sensitivity:** The NS/0 concentration corresponding to a signal 2 standard deviations above the mean of the zero standard is defined as the limit of detection (LOD). This was determined from 20 replicates of the zero standard in both protocols. The mean signal of the zero standard plus 2 SD yielded an LOD of ~700pg/mL in the Standard Protocol and ~300pg/mL in the High Sensitivity Protocol. The limit of quantitation (LOQ) is defined as the lowest concentration for which the CV is typically <20%. This is determined by performing a precision profile for the assay at low concentration points and then interpolating that concentration which corresponds to a 20% CV. LOQ is stated as ~3ng/mL in the Standard Protocol and ~1ng/mL in the High Sensitivity Protocol

**Specificity:** Specificity: In sandwich ELISA, cross reactivity can manifest itself either as a false increase in HCP levels (positive cross reactivity) or as a false decrease in HCP (negative cross reactivity) when HCP present in the sample competes with the cross reactant for the kit antibodies. Other materials that may not cross react in the true immunological sense, may simulate cross reactivity due to non-specific interactions that can result in either a false increase or false decrease in the apparent NS/0 concentration. Because of the very defined nature of samples proposed for testing in this assay, an extensive study into cross reactivity was not attempted. However, it is recommended that each user evaluate known materials in their sample matrices for cross reactivity or non-specific interferences by testing those materials with and without NS/0 HCPs spiked into them.

Only bovine serum albumin (BSA), normal mouse IgG, and normal human IgG were evaluated for cross reactivity both in the absence of NS/0 HCPs and in the presence of NS/0 HCPs. BSA showed no cross reactivity at up to 8mg/mL. Normal human IgG was tested at 1, 2, 5, & 10 mg/mL with and without added NS/0. No inhibition of spiked NS/0 recovery was seen at any concentration. At 5 and 10mg/mL the unspiked hIgG preparations did show apparent NS/0 background levels of 2.8 and 8.1 mg/mL respectively. It is possible that this very low level of apparent cross reactivity, could be due to trace contaminants in the IgG preparation or to some increase in non-specific binding of the assay reagents caused by very high levels of normal hIgG. Normal mouse IgG was also tested for cross reactivity at 10, 5, 2, and 1 mg/mL and showed 0.001% cross reactivity at 1 mg/mL. Given that NS/0 cells are derived from mouse cells, it is possible that this low level of apparent cross reactivity is due to contaminating mouse serum proteins in the mIgG preparation that are conserved in NS/0 HCPs.

**Hook Capacity:** Very high concentrations of NS/0 HCP were evaluated for the hook effect. At concentrations exceeding 200 $\mu$ g/mL of a cell lysate the apparent concentration of NS/0 HCP may read less than the 200ng/mL kit standard. Samples yielding signals above the 200ng/mL standard or suspected of having HCP levels in excess of 200 $\mu$ g/mL should be assayed diluted.

**Reagent Stability Studies:** The reagents and processes used to manufacture and stabilize them are in common with many other products routinely manufactured by *Cygnus* for several years. Based on this considerable experience, the only anticipated potential stability problem is the kit standards. ELISA coated plates and enzyme antibody conjugate were stored at 4°C, room temperature, and 37°C for up to 50 days. A slight loss in activity (<20%) was seen only at 37° for both components. This small amount of deterioration under these extreme storage conditions did not have any significant impact on the accuracy of the assay since all test wells were affected proportionately. Thus, it is projected that the strips and conjugate will yield adequate kit performance for at least 1 year when stored as recommended at 4°C.

Kit standards were stored at 37°C, room temperature, 4°C, -20°C, and -80°C for 50 days to predict long term stability at recommended storage conditions and to simulate worst case shipping conditions. Only the 37°C storage condition showed any significant deterioration, with approximately a 24% loss in activity. With no loss in standards activity at room temperature or 4°C we are confident in conservatively setting a 9 month kit shelf life with 4°C storage until real time and real condition data can be collected.