



Cygnus Technologies, Inc.

4705 Southport Supply Road SE, Suite 208
Southport, NC 28461 USA
Tel: 910-454-9442 FAX: 910-454-9443
Email: cygnustec@aol.com
Web: www.cygnustechnologies.com

***E. coli* HCP ELISA kit
Catalog # F010
Validation Summary
Report dated Feb. 20, 1999**

The data summarized below was generated by *Cygnus Technologies* to establish the performance parameters and validity of this kit to measure *E. coli* Host Cell Proteins (HCPs). This data is intended to supplement and not replace user generated validation data. The 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 a western blot using the same antibody used in this kit to demonstrate that the antibody reacts with the majority of proteins separated by SDS/PAGE. (2) Each user should perform intra and inter assay precision experiments to establish their procedural proficiency. (3) Each user should perform recovery experiments using their test sample matrices. Such a study can be performed by adding known amounts of the 250ng/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 *E. coli* proteins or have very low levels (< 4ng/mL) determined prior to adding the 250ng/mL standard. Such an experiment will establish the degree of sample matrix interference in the recovery of HCPs. (4) 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 HCPs. Such samples are to 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: Kit Lot # 17019, Exp. Date 04/30/00 used for all studies except stability
Kit Lot # 4047 was used for stability

Methods: As indicated in the data tables below both the "standard" and "rapid" protocols described in the kit insert were used in this validation.

Data References: Raw data for these experiments are recorded in Notebooks # EC-1 & EC-2.

Antibody Development and Characterization: The antibody used in this kit was generated against a number of commonly used laboratory strains and a non-pathogenic clinical isolate. These strains are as follows: K12, ATCC 37197, and from Invitrogen; strains TOP 10F, JM109, HB101, XS127/P3, and C236/P3. Western blot analysis and ELISA dilutional analysis of these and other individual strains such as BL21 and DH5 alpha, indicates that the vast majority of ECPs are antigenically conserved among all strains of *E. coli*. Thus this kit should be of utility for strains other than those specifically used above. It is recommended that each user of this kit verify by western blotting and ELISA that the kit antibody reacts with the majority of their ECPs.

Precision: Precision is defined as the percent coefficient of variation (%CV). This is calculated by dividing the mean by the standard deviation for a number of replicate determinations of three different control samples in the low, medium and high concentration range of the assay. 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
20	2.6	6.8	<10%	5	2.4	7.4	<12%
20	10.2	3.1	<8%	5	10.2	4.2	<10%
20	39.8	4.0	<10%	5	40.1	5.9	<10%

Rapid Protocol

Intra-assay				Inter-assay			
# of tests	Mean ng/mL	%CV	Design Goal Specification	# of assays	Mean ng/mL	%CV	Design Goal Specification
20	8.0	7.8	<10%	5	7.8	7.4	<12%
20	41.2	3.9	<8%	5	40.4	4.7	<10%
20	153	4.2	<10%	5	160	4.8	<10%

Recovery/Matrix Interference: The same *E. coli* HCP preparation used for the standards was spiked into various “sample buffers” to demonstrate the potential for matrix interference. HCPs were added at 4 and 40ng/mL and tested in duplicate in the standard protocol. In all cases the zero for each sample buffer was within the limit of detection for the assay and thus the buffers themselves were considered to contribute 0 ng/mL of HCPs. Acceptable recovery is specified as plus or minus 20% of the added HCP value. These data serve as examples of certain buffers or buffer components which may or may not give matrix interference. As shown below, matrix interference can be either positive (false increase in HCPs) or negative (false decrease in HCPs). Each user is encouraged to test their sample matrices for recovery in a similar experiment.

Sample Buffer Matrix	<i>E. coli</i> Added ng/mL	<i>E. coli</i> Recovered ng/mL	% Recovery (assayed/added x100)
0.05M TBS with 1 mg/mL mouse IgG, pH 7.2	4	3.8	96
“	40	38.8	97
0.05M TBS with 10mg/mL BSA, pH 7.2	4	3.8	96
“	40	41.9	105
0.05M PBS with 10mg/mL BSA, pH 7.2	4	3.7	93
“	40	41.2	103
Citrate/Phosphate with 1 mg/mL BSA, pH 6.0	4	3.5	88
“	40	37.5	94
0.05M TBS with 1% Tween 20, pH 7.2	4	2.3	58
“	40	21.0	53
0.1M Acetate, 10mg/mL BSA, 1% Triton, pH5.0	4	5.1	125
“	40	49.9	125
0.05 M Tris, 10mg/mL BSA, pH 8.5	4	4.4	110
“	40	48.2	120

Sensitivity: The *E. coli* HCP 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 10



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replicates of the zero standard. The mean signal of the zero standard plus 2 SD yielded a LOD of 250 pg/mL for the standard protocol and 880 pg/mL for the rapid protocol. The limit of quantitation (LOQ) is defined as the lowest concentration for which the CV is <20%. This is determined by performing a precision profile for the assay at several low concentration points and then interpolating that concentration which corresponds to a 20% CV. The LOQ was 330pg/mL for the standard protocol and 1.25ng/mL for the rapid protocol.

Specificity: The following strains were used to generate antibodies and have expressed a high degree of homology as determined by Western Blot analysis:

TOP 10F	XS127/P3	HB101
JM109	CJ236/P3	ATCC 37197

Several other strains of *E. coli* such as K12, BL21, and even the pathogenic strain O157-H7 have shown very similar high degrees of homology when compared to the immunization strains. Each user should evaluate the suitability of these antibodies for detection of their process HCPs by both Western Blot and this ELISA and comparing results to protein bands detected by a sensitive PAGE protein staining method such as silver staining. Other *Enterobacteriaceae* species have not been tested but may cross react with these antibodies. Certain biological reagents may have been contaminated with *E. coli* or other *Enterobacteriaceae* species during their production or storage. For example, *Cygnus Technologies* has detected “*E. coli*” HCP activity in many commercial preparations of bovine serum albumin and other protein preparations. These are presumably carryover trace contaminants of HCPs since these materials were negative by culture. Such HCP contamination of reagents can lead to apparent elevation of HCP by this assay and could thus be erroneously attributed to the user’s product and process. Therefore, it is advisable that the user test such raw materials for HCP activity.

An *E. coli* LPS (endotoxin) was tested in this assay and found not to react. Still it is not advised to use any raw materials which have endotoxin levels exceeding 1 EU/mg as this may indicate the potential for contamination with HCPs as well.

Hook Capacity: Very high concentrations of *E. coli* were evaluated for the hook effect. At concentrations exceeding 10,000 ng/mL, the apparent concentration of *E. coli* may read less than the 250ng/mL standard. Samples yielding signals above the 250ng/mL standard or suspected of having concentrations in excess of 10,000 ng/mL should be assayed diluted.

Stability: Stability of this kit and its components was established by both real time and accelerated (storage at 37°C) conditions. Average absorbance units for each standard in the rapid protocol are reported below after blanking against the zero standard. Results are compared to the assay expiration specification limits.

Standard (ng/mL)	Day 0	Day 28 at 37°C	12 months at 4°C	Expiration Specification
4	0.026	0.015	0.017	>0.013
20	0.102	0.072	0.082	>0.067
75	0.492	0.268	0.293	>0.250
250	1.739	0.795	0.880	> 0.750