Behind the Scenes:

Creation of a broadly reactive *E. coli* HCP detection ELISA suitable for K Strain and B Strain Platforms

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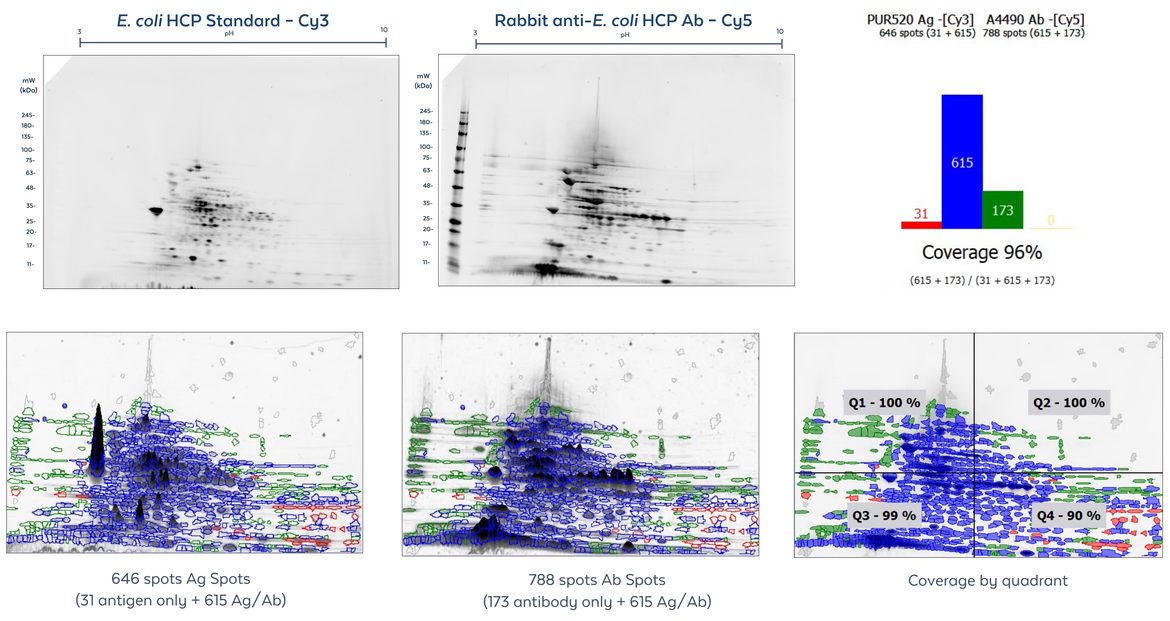
ABSTRACT

Escherichia coli **is a major expression platform to produce biopharmaceuticals. ELISAs remain the workhorse method of monitoring HCP (host cell protein) levels through the bioprocess, demonstrating HCP removal and consistency in manufacturing. Here we present the development and validation of a new E. coli HCP ELISA that has been qualified for monitoring workflows for both recombinant proteins (B-strains) and for plasmid production in GTx (K-strains).**

INTRODUCTION

Escherichia coli (*E. coli*), is a major host expression platform for production of biopharmaceuticals. It has utility for large scale production of proteins due to its high yield, rapid growth that is amenable to scale and low cost. While limited to proteins that don't require complex post-translational modifications, it is the model of choice for proteins such as cytokines and hormones, including insulin, which was first purified from *E. coli* by Eli Lily in 1978. During purification of biological drugs from the host system, the presence of host cell protein (HCP) contaminants must be assayed, both to demonstrate effective HCP clearance and manufacturing consistency. Rockland has developed an ELISA to facilitate the monitoring of *E. coli* HCP contaminants during the bioprocessing workflow. The assay has a run time of < 2 hours with a standard curve range of 1 – 250 ng/mL and an LLOQ of ≤ 3 ng/mL. The assay is compatible with both B and K *E. coli* strains and in a range of buffer matrices. Here we show how Rockland develops an HCP assay, ensuring robust performance and reproducibility in our critical reagents to effectively measure *E. coli* HCPs in bioprocess samples.

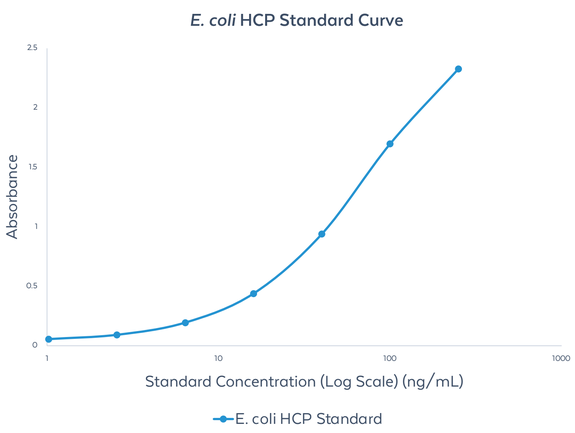
2DGE ASSESSMENT OF ANTIBODY COVERAGE



Antigen load: 100 µg. Primary Ab: anti-*E. coli* HCP (Rabbit) antibody at 5 µg/mL. Secondary Ab: Goat Anti-Rabbit IgG Cy5 Conjugated. Imaging System Used: Amersham Typhoon system. PMT: Cy3-400, Cy5-435. Resolution: 50 µm. Analysis by Melanie software.

Figure 1. The suitability of the generated antibody to the assay standard was assessed by 2D-gel electrophoresis DIBE. Cy3-conjugated antigen was separated by isoelectric point and molecular weight then the protein was transferred to nitrocellulose. The reactivity of rabbit anti-*E. coli* antibody was then resolved by Western blot. Blots were imaged in the Cy3 channel for antigen and Cy5 channel to visualize antibody binding. The DIBE images were then analyzed by Melanie software and the commonality of antigen and antibody protein spots were assessed. Coverage of the antibody to the antigen was found to be 96%.

DEVELOPMENT OF STANDARD CURVE



Example Standard Curve Performance

Assay Standard Curve [ng/mL]	Back-Calculated Interpolation [ng/mL]	% Self-recovery	% CV
250	248.4	99.4	2.71
100	101.17	101.2	2.51
40	39.41	98.5	0.97
16	16.07	100.4	3.6
6.4	6.7	104.7	3.92
2.6	2.7	103.8	3.83
1	0.94	94	6.64
0	0.19	N/A	N/A

Figure 2. In developing this assay from the Rockland generated anti-*E. coli* HCP antibody, a standard curve needed to be created that:

- Could successfully measure a broad range *E. coli* HCP concentrations
- Had a high degree of accuracy as measured by its ability to back-calculate on itself (% Recovery) using a 4-Parameter Logistic Fit (L)
- Had a high degree of precision as measured by the Percent Coefficient of Variance (% CV) of it replicates
- Could be made in a series of user-friendly dilution steps

After the rigorous testing of multiple options for standard curves, a dilution series was identified with a range of 1.0 – 250 ng/mL, with each standard curve point a 2.5x concentration dilution from the previous one. As a part of the initial assay development, optimal timing of capture, detection, and TMB development steps were identified, which demonstrated that this assay could successfully generate accurate and precise results in less than two hours.

ASSURING CONSISTENT ASSAYS

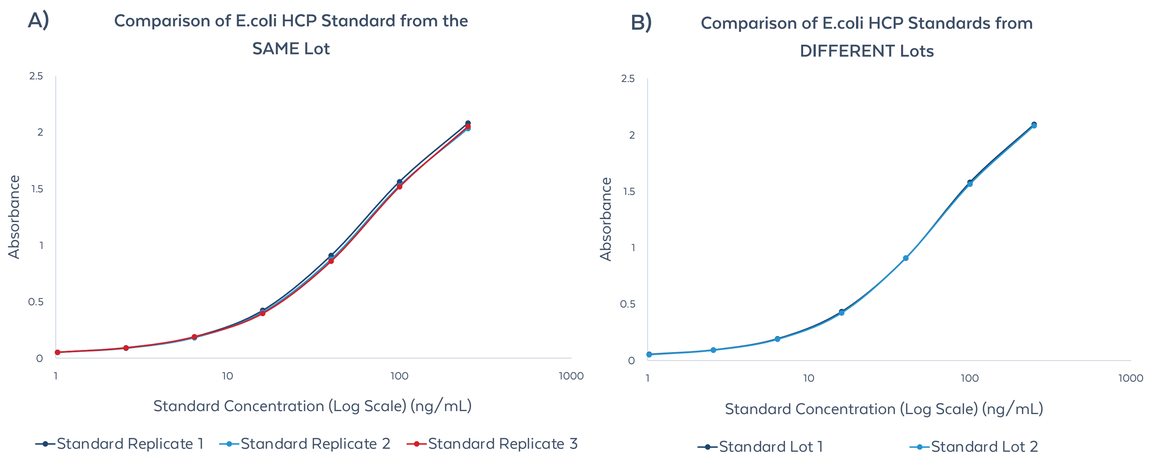


Figure 3. During the creation of the *E. coli* HCP assay, a production process was developed wherein multiple lots of lyophilized *E.coli* HCP standard would be produced over the life cycle of the assay. It was imperative that lot-to-lot consistency of *E.coli* HCP standard be maintained in order to ensure repeatable results between assays.

Rigorously tested methods were implemented to ensure lot-to-lot consistency of the standard as well as methods to confirm the consistency of the final lyophilized standard lots when they were produced.

Criteria were established that standards within the same lot, and between lots, would not be permitted to vary from each other more that 15 %, based on their 4PL %Recovery values. Lots of standard that exceeded this 15 % value would be rejected.

Figure 3A. and **3B.** demonstrate *E.coli* HCP lot-to-lot consistency within the same lot and between lots, respectively.

LOWER LIMIT OF QUANTIFICATION AND LIMIT OF DETECTION

A)

Example ELISA Assay Plate Layout and Data Set

	Standard Curve			Lower Limit of Quantification (LLOQ)								
				4 ng/mL			3 ng/mL			2 ng/mL		
	1	2	3	4	5	6	7	8	9	10	11	12
A	2.372	2.322	2.314	0.126	0.127	0.127	0.102	0.100	0.101	0.080	0.080	0.080
B	1.723	1.703	1.682	0.127	0.126	0.126	0.100	0.100	0.100	0.079	0.080	0.080
C	0.945	0.938	0.925	0.127	0.127	0.126	0.101	0.101	0.100	0.078	0.077	0.079
D	0.448	0.444	0.441	0.128	0.128	0.126	0.103	0.115	0.092	0.079	0.080	0.080
E	0.202	0.199	0.192	0.128	0.128	0.144	0.102	0.102	0.102	0.079	0.079	0.081
F	0.097	0.095	0.092	0.126	0.125	0.125	0.100	0.101	0.101	0.078	0.077	0.078
G	0.056	0.055	0.056	0.126	0.124	0.126	0.100	0.100	0.099	0.084	0.076	0.077
H	0.033	0.027	0.028	0.028	0.030	0.029	0.029	0.027	0.027	0.029	0.028	0.029
				0 ng/mL								
				Limit of Detection (LOD)								

Heat Map of Relative Absorbance Signal

Red = High

Yellow = Medium

Green = Low

B)

LLOQ Concentration (ng/mL)	Average Result (ng/mL)	Overall CV %	Overall Recovery %
4.00	3.35	15	84
3.00	2.49	17	83
2.00	1.61	17	81

C)

Average LOD (OD):	0.034
Average LOD (ng/mL):	0.22

(LOD = Blank + 3-Standard Deviations)

Figure 4. As part of the validation of the *E. coli* HCP assay, the Lower Limit of Quantification (LLOQ) and the Limit of Detection (LOD) needed to be identified. N = 4 plates were run according to the example plate layout (**Figure 4A.**), wherein spiked LLOQ samples at several concentrations were compared against the standard curve. The LLOQ was identified as the lowest concentration that had a consistent %Recovery between 70 - 130% and a %CV ≤ 25%. Although an LLOQ of 2 ng/mL could be declared (**Figure 4B.**), 3 ng/mL was declared as the LLOQ to give a margin of error for the end user, and to assure assay-to-assay consistency in results. LOD, which is defined as the absorbance of the blank plus three standard deviations, was demonstrated to be less than 1 ng/mL, and thus an LOD of ≤ 1 ng/mL was declared **(Figure 4C.).**

E. coli STRAIN SENSITIVITY

Dilution Ratio	*E. coli* Strain and Type				
	Origami2	DH5α	XL1	Rosetta	
	K			B	
	Assay Measured Concentrations (mg/mL)				
1:1,000	N/A	N/A	N/A	N/A	
1:3,000	4.3	N/A	N/A	9.6	
1:9,000	9.2	14.0	15.1	5.0	
1:27,000	11.6	10.4	10.5	5.7	
1:81,000	10.8	9.0	8.8	5.3	Dilutional Linearity Observed
1:243,000	10.1	8.5	8.7	4.8	
1:729,000	10.1	8.6	8.5	5.0	
1:2,187,000	10.1	9.3	9.3	5.2	
Concentration of Strain	10.4	8.7	8.7	5.0	
Percent Coefficient of Variation from Validation Study (N = 4-plates)	17	11	18	8	

NOTE: The *E. coli* HCP assay standard was derived from strain BL21, which is a B-strain of *E. coli.*

Table 1. Modern bioprocesses use a variety of *E. coli* strains based on their specific needs that derive from B and K strain lineages. As a part of the validation of the *E. coli* HCP assay, it needed to be demonstrated that this assay could detect antigens from several of the commonly used *E. coli* strains representing both B and K strain. Lysates from desired *E. coli* strains were prepared, serial diluted, and tested as *unknown concentrations* against a standard curve, whose 4PL characteristics were identified.

Validation protocol dictated that in order for an unknown concentration to be identified, *Dilutional Linearity* for at least three dilution steps needed to be achieved, i.e., the identified unknown concentration needed to be consistent between at least three sequential dilution steps. To identify dilutional linearity, the %CV of the identified unknown concentrations between the three dilution steps needed to be ≤ 20 %. Furthermore, to validate the assay, the %CV of the identified concentration need to be ≤ 20 % across 4-plates.

Dilutional linearity and an identification of unknown concentration was achieved against all strains tested and thus it was demonstrated that the *E. coli* HCP assay can detect multiple B and K strains of *E. coli.*

SAMPLE MATRIX BACKGROUND

Matrix Buffer	pH	Maximum % Matrix Buffer to Achieve 80 – 120 % Recovery	Dilution Factor	Successful %Recovery (YES/NO)
50 mM Tris	8.0	46.9	2.1	YES
50 mM Sodium Phosphate, 0.3 M NaCl, 0.5 M Imidazole	8.0	23.5	4.3	YES
25 mM Sodium Acetate, 0.5 M NaCl	2.5	3.1	32	YES
25 mM Citric Acid, 0.5 M NaCl	2.0	3.1	32	YES
50 mM Glycine, 50 mM Citric Acid	2.0	0.05	2048	NO

Table 2. Many bioprocesses are operated in a variety of matrix buffer backgrounds that have the potential to interfere with the *E. coli* HCP assay due to the effects of low pH and high salt content. Therefore, during validation, the *E. coli* HCP assay was challenged with several common bioprocess matrix buffers. *E. coli* HCP standard was diluted into the respective matrix buffers and then serial diluted into a non-matrix containing sample buffer until successful %Recoveries (80-120 %) could be interpolated from a standard curve. The percent of matrix buffer remaining in the solution, as well as the required dilution factor for each matrix buffer required to attain successful %Recoveries was ascertained.

Near neutral pH matrix buffers required far fewer dilutions to attain %Recovery within specification (80 – 120 %) than matrix buffers at lower pH. Only the harshest matrix buffer, 50mM Glycine, 50mM Citric Acid at pH 2.0, prevented the assay from having any meaningful %Recovery. These buffers are typically neutralized post-elution; however, the end user of the assay will be advised to test their process specific matrix buffers with this assay prior to the utilization in their process.

IN-PROCESS *E.coli* HCP IDENTIFICATION

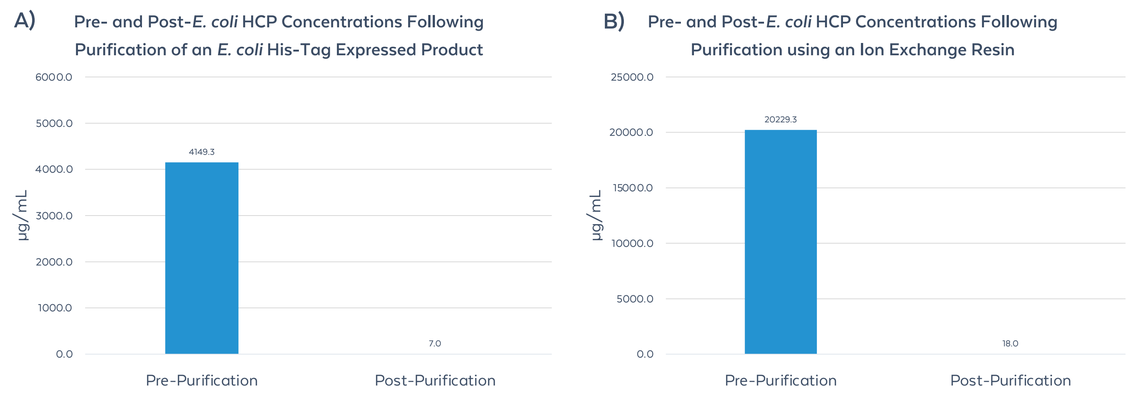


Figure 5. As the primary purpose of the *E. coli* HCP assay is to measure HCP contaminants in a bioprocess during assay validation, several in-process samples were tested in the assay both prior to and after various purification methods. As seen above, the *E. coli* HCP assay detected dramatic drops in HCP concentrations following His-Tag (**Figure 5A.**) or Ion Exchange (**Figure 5B.**) purification steps, thus demonstrating the utility of this assay in a bioprocess environment.

E. coli HCP ELISA ASSAY PERFORMANCE SPECIFICATIONS

Standard Curve	1-250 ng/mL
Assay Run Time (including wash, incubation, & TMB steps)	~2 hours
LLOQ	≤ 3 ng/mL
LOD	≤ 1 ng/mL
Compatibility with *E. coli* B and K Strains?	YES
Achievement of Dilutional Linearity for Measurements of Unknown Concentrations?	YES
Compatibility with Varying Matrix Buffers?	YES (*End user must confirm process-specific suitability*)