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AccuSignal™ *E. coli* HCP ELISA Kit

An ELISA with broad sample compatibility and high sensitivity

Escherichia coli (*E. coli*) is a widely utilized organism in scientific research and biomanufacturing of therapeutic biologic drug substances. Monitoring the concentrations of impurities, particularly *E. coli* Host Cell Proteins (HCP), is of paramount importance in biomanufacturing processes. The primary objective of purification processes is to eliminate HCPs to minimize immunogenicity, enhance potency, and ensure the stability of a pure drug substance.

Rockland's AccuSignal™ *E. coli* HCP ELISA Kit (KJB-4003) is a robust sandwich enzyme-linked immunosorbent assay (ELISA) designed to detect contaminating *E. coli* HCPs in therapeutic products throughout their purification processes, including early, mid, late, and final stages. Additionally, it can detect HCPs from both B and K-12 *E. coli* strains, which are commonly employed in the biomanufacturing of drug substances.

E. coli HCPs from a sample are captured on the AccuSignal™ *E. coli* HCP ELISA Kit plate, which contains pre-immobilized anti-*E. coli* HCP antibodies. The kit detection antibodies (anti-*E. coli* HCP antibodies) are conjugated with Horse Radish Peroxidase (HRP). Upon exposure to 3,3',5,5'-Tetramethylbenzidine (TMB) substrate, HRP activates, resulting in a color change that can be measured with a plate reader at 450 nm. This optical density (OD) value is directly proportional to the concentration of *E. coli* HCPs, which can be calculated by interpolating against a Four-Parameter Logistic (4PL) standard calibration curve.

Key Benefits

- **Sensitive antibody** enables reliable detection of *E. coli* HCP
- **Strong *E. coli* HCP coverage** minimizes the risk of undetected HCP
- **Wide dynamic range** provides confidence in results across a large range of potential concentrations throughout the entire purification process
- **Consistently low inter- and intra-plate variation** ensures reproducible data

Table 1. General kit specifications

Specification	Parameter
Limit of Detection (LOD)	≤ 1.0 ng/mL
Lower Limit of Quantification (LLOQ)	3.0 ng/mL
Range	2.6 – 250.0 ng/mL
Precision (Intra-Assay Variability)	≤ 20%
Precision (Inter-Assay Variability)	≤ 25%
Recovery of Each Standard	80-120%

High Sensitivity

The Lower Limit of Quantitation (LLOQ) represents the lowest concentration that can be reliably measured with an acceptable level of reproducibility, while the Limit of Detection (LOD) is the lowest concentration that is distinguishable from the background.

To calculate the LLOQ and LOD, known concentrations of *E. coli* HCP were spiked into sample buffer and measured using the kit. The LLOQ was calculated as the lowest concentration for which a recovery of 70-130% was observed with a Coefficient of Variation (CV) less than or equal to 25%. The LOD was calculated as the average background result plus three standard deviations (σ) thereof. The LLOQ and LOD were verified across four independent assays.

Spike assays were conducted to validate the LLOQ of the assay. A total of 84 replicate wells were tested at each spike concentration level. At the 2 ng/mL spike concentration, the 84 replicates yielded an overall recovery of 78.9% and a CV of 18%. This value surpasses the kit's LLOQ of 3.0 ng/mL.

To determine LOD, 36 replicate wells of blank sample buffer were measured in the assay. The OD of the blank plus 3σ was 0.034. As this value falls below the OD of the 2.6 ng/mL assay calibrator at 0.102, an equivalent back-calculated value in ng/mL cannot be determined, thus these data indicated that the LOD is less than or equal to 1.0 ng/mL.

Table 2. LLOQ

E. coli HCP Concentration	4 ng/mL	3 ng/mL	2 ng/mL
Mean Interpolated Concentration (ng/mL)	3.4	2.5	1.6
Recovery (%)	83.8	82.4	78.9
CV (%)	14.9	17.4	18.0

Table 3. LOD

Parameter	Average
OD of Blank	0.029
σ of Blank	0.002
OD of Blank + 3σ (LOD)	0.034
OD at 2.6 ng/mL	0.102
Back-Calculated Value of OD + 3σ (LOD)	≤ 1.0 ng/mL*

*A precise value cannot be calculated because the OD of the Blank + 3σ falls below the 4PL calculations.

Broad Dynamic Range

The AccuSignal™ *E. coli* HCP ELISA Kit offers a wide dynamic range, thereby reducing the number of plates and time required for experiments. To demonstrate this, we utilized the protein standard provided in the kit to construct a standard curve spanning from 2.6 to 250 ng/mL (Fig. 1, Table 4). This curve was subsequently meticulously measured in triplicate across six distinct assays, resulting in a total of 18 replicates. The assay successfully validated the effectiveness of this broad range (2.6 to 250 ng/mL) and demonstrated its remarkable ability to accurately interpolate its calibrators (Recovery %) with an exceptionally strong goodness of fit ($R^2 > 0.99$).

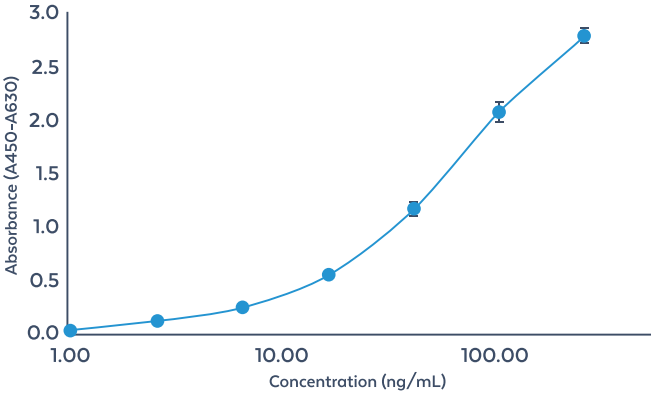

Fig. 1. Standard curve (4PL) of the AccuSignal™ *E. coli* HCP ELISA Kit.

To minimize assay repetition caused by out-of-range detection, scientists must determine the optimal dilution factors for each assay sample.

Table 4. Mean absorbance and inter-plate CV of the standard curve

Concentration (ng/mL)	Mean Absorbance (A_{450} - A_{630})	Mean Inter-plate Recovery (%)	Mean Inter-plate CV (%)
250.00	2.79	99.68	9.75
100.00	2.08	101.63	4.50
40.00	1.17	98.06	2.23
16.00	0.55	100.91	2.38
6.40	0.25	107.04	4.18
2.56	0.11	105.82	5.15

Robustness & Reproducibility

It is imperative that an assay generates data that is consistently reproducible both within the same assay and across multiple assays. Assay precision is quantified through the CV within a single experiment (intra-assay) and across multiple experiments (inter-assay).

To analyze intra-assay variation, we measured the CV% from the interpolated concentration of nine in-process samples tested in triplicate dilutions across 35 separate plates. From these results, 163 triplicate dilutions fell within the measurable range of the standard curve and were evaluated for CV%. Of these, 87% exhibited an intra-assay CV% less than 10%, with an average CV% of 5% (Fig. 2).

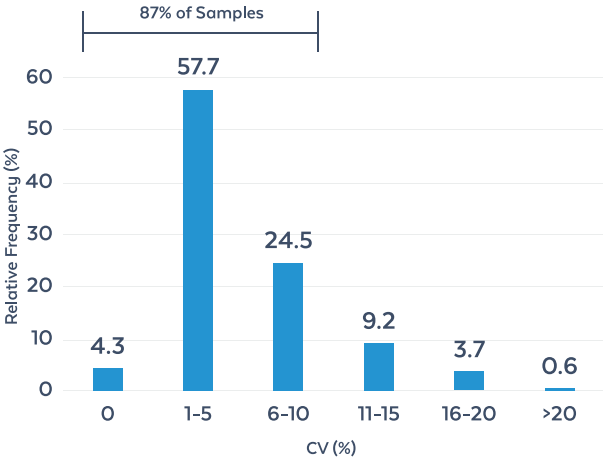

Fig. 2. Indicates the percentage of in-process measurements binned together within a given range of CV%. Approximately 87% of all samples fell between 0-10 CV% for these in-process intra-assay results.

To measure inter-assay precision, four plates were spiked with concentrations of *E. coli* HCP at 100 ng/mL, 20 ng/mL, and 5 ng/mL, each replicated four times (a total of 16 replicates). To calculate the inter-assay precision, the means of the OD were determined along with the mean recovery % and CV%. The data reveal CV% not exceeding 10% across the four plates tested (Table 5), affirming assay-to-assay reproducibility.

Table 5. Inter-assay precision

Spiked HCP Concentration (ng/mL)	Measured HCP (ng/mL)	CV (%)	Recovery (%)
100	99.2	3.1	99.3
25	24.5	4.4	97.9
5	5.5	7.3	103.4

Importance of Linearity and Sample Compatibility

Ideally, a bioprocess should demonstrate the ability to eliminate HCPs at each purification step. Therefore, it's crucial to monitor the presence of HCP impurities throughout the process. Successful monitoring of impurities is essential to optimize the various purification stages and minimize the amount of HCP in a final product.

When selecting an assay to demonstrate the removal of impurities, it's important to choose one that can demonstrate the best dilution linearity across multiple in-process samples. Dilutional linearity assesses the assay's ability to provide accurate and linear measurements of a target analyte across a range of sample dilutions. By doing this, it demonstrates sample compatibility and ensures confidence in the resulting data. As a sample is assessed across several dilutions, the same calculated stock result must be observed, which is a parameter known as parallelism. The determination of acceptable parallelism indicates that the assay is reliable and can accurately quantify the target analyte in various sample types.

As a means of demonstrating these capabilities of the AccuSignal™ *E. coli* HCP ELISA Kit, we utilized the kit to sample HCP concentrations from various stages of a purification process for a therapeutic substance. Each stage of the purification process was sampled in triplicate across four plates (12 sample replicates total) and assessed at multiple dilutions. Values were interpolated as unknown concentrations from the purification process using a 4PL fit relative to the standard curve (Fig. 3, Table 6).

The AccuSignal™ *E. coli* HCP ELISA Kit exhibits excellent parallelism (CV% <25%), ensuring consistent results across a broad range of dilutions. Moreover, it showcases the capability to monitor HCP levels throughout a purification process.

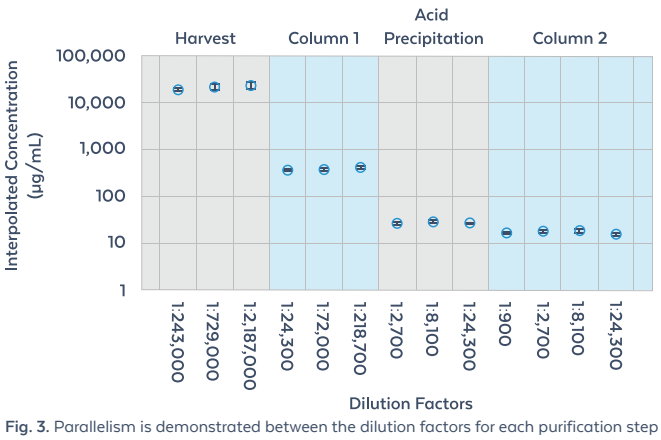

Fig. 3. Parallelism is demonstrated between the dilution factors for each purification step as indicated by the calculated *E. coli* HCP levels for each in-process step dilution value. The standard error is represented by the dark blue error bars across replicate experiments.

Table 6. Concentration of *E. coli* HCP (µg/mL) for each purification step (see Fig. 3) with the average parallelism also indicated.

Sample	HCP Concentration (mg/mL)	Average Parallelism (%)
Harvest	21684.7	24
Column 1	389.7	9
Acid Precipitation	27.5	9
Column 2 (Final Product)	18.1	12

Wide Buffer Compatibility

A biomanufacturing process to generate a purified drug substance may require a variety of buffers, known as matrix buffers, for different purification stages. Therefore, the assay must be compatible with various matrices when monitoring for HCP impurities.

To assess the potential impact of matrix effects on *E. coli* HCP purification, we conducted spike recovery assays using a diverse set of commonly employed matrix buffers. Each matrix buffer was spiked in triplicate across four plates (yielding a total of 12 replicates), with a concentration of *E. coli* HCP. Subsequently, we performed a series of dilutions in sample buffer to evaluate the compatibility of each matrix buffer. By gradually replacing matrix buffer with sample buffer, we identified the minimum dilution factor required for each matrix buffer to achieve an interpolated recovery % of 80-120% with a CV% of <20%.

The AccuSignal™ *E. coli* HCP ELISA Kit exhibits excellent compatibility with a diverse range of matrix buffers, including those with varying salt concentrations and pH levels. To ensure optimal performance, we strongly advise scientists to determine the optimal dilution factors for each assay sample. Additionally, we recommend routinely measuring the recovery of a spiked sample to identify any specific matrix effects that may arise during the process. To accomplish this, it is advisable to spike a known concentration of control protein into a control sample matrix and subsequently assess the recovery.

Table 7. Compatibility with various matrix buffers at varying pH and salt concentrations with respective minimum dilution factor required for passing Recovery (%) and CV (%).

Matrix Buffer	Minimum Matrix Dilution Factor	Recovery (%)	CV (%)
50 mM Tris, pH 8.0	2.1	112	7
50 mM Sodium Phosphate, 0.3 M NaCl, 0.5 M Imidazole, pH 8.0	4.3	80	2
25 mM Sodium Acetate, pH 2.5	32	103	6
25 mM Citric Acid, 0.5 M NaCl, pH 2.0	32	91	3

Reagent Stability

We conducted an accelerated stability study to validate a shelf life of 24 months. According to the Variable Q_{10} method, an accelerated study conducted at 25°C over 187 days is equivalent to 24 months at the intended storage condition of 2-8°C.

Therefore, the kit components were stored at 25°C and assay performance was evaluated at regular intervals over 187 days using the criteria outlined in Table 8. Notably, all criteria were met, which successfully validated the shelf life of 24 months for the AccuSignal™ *E. coli* HCP ELISA Kit.

Table 8. Test criteria and results of accelerated stability study for a 2-year shelf-life equivalent.

Parameter	Pass Criteria	Result (Day 187 at 25°C)
Absorbance of 100 ng/mL Standard	≥ 1.0	PASS
Absorbance of blank standard	≤ 0.1	PASS
Intra-Assay CV	≤ 20%	PASS
Recovery of each protein standard	80 – 120%	PASS
LOD (ng/mL)	≤ 1.0 ng/mL	PASS

DIBE Coverage

Antibody coverage is an important consideration in HCP risk mitigation. The antibody should demonstrate it can detect the majority of HCPs in a sample, yet different antibodies will have different reactivities to process-specific samples. Being able to detect the majority of proteins makes for a more reliable assessment that HCPs are being cleared during bioprocessing and if this clearance is consistent.

To demonstrate the utility of the AccuSignal™ *E. coli* HCP ELISA polyclonal antibody to a range of *E. coli* platforms, we utilized 2D Differential in Blot Electrophoresis (DIBE) technology. DIBE is an enhanced version of 2D gel electrophoresis that demonstrates how well a polyclonal antibody can interact with antigen targets in a lysate (i.e., its coverage). Through the use of DIBE, we performed coverage analysis on lysates derived from three *E. coli* platforms—DH5α, Origami2 (both K-12 strains), and Rosetta (B-strain)—against the kit antibody (see Table 9).

Table 9. *E. coli* strains tested by DIBE and associated antibody coverage

E. coli Sample	Strain	Coverage (%)
DH5α	K-12	94
Origami2	K-12	90
Rosetta	B	94

Purified supernatant proteins from the cell lines were labeled with Cy3 dye (CyDye™ reagents, DIGE assay Cy3 dye). The labeled samples were separated in the first dimension with Isoelectric Focusing (IEF) gel electrophoresis using an IPGphor™ 3 IEF system along with 7-cm, pH 3–11 NL Immobiline® DryStrips strips.

The proteins were separated in the second dimension by molecular weight using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Next the gels were transferred to a polyvinylidene difluoride membrane (PVDF) and a Western blot was performed with the AccuSignal™ *E. coli* HCP ELISA Kit antibody as the primary antibody.

To visualize this immunoreactivity, the blots were incubated with Cy5-labeled secondary antibody raised against the host species of the primary antibody (rabbit). The membranes were scanned on an Amersham™ Typhoon™ biomolecular imager, and coverage calculated using the DIBE™ coverage module in Melanie™ 9 software (see Fig. 4).

As shown in Fig. 4 and Table 9, our data demonstrates the AccuSignal™ *E. coli* HCP ELISA Kit antibody is compatible with detecting HCPs from multiple platforms and strains, with observed coverage > 90%.

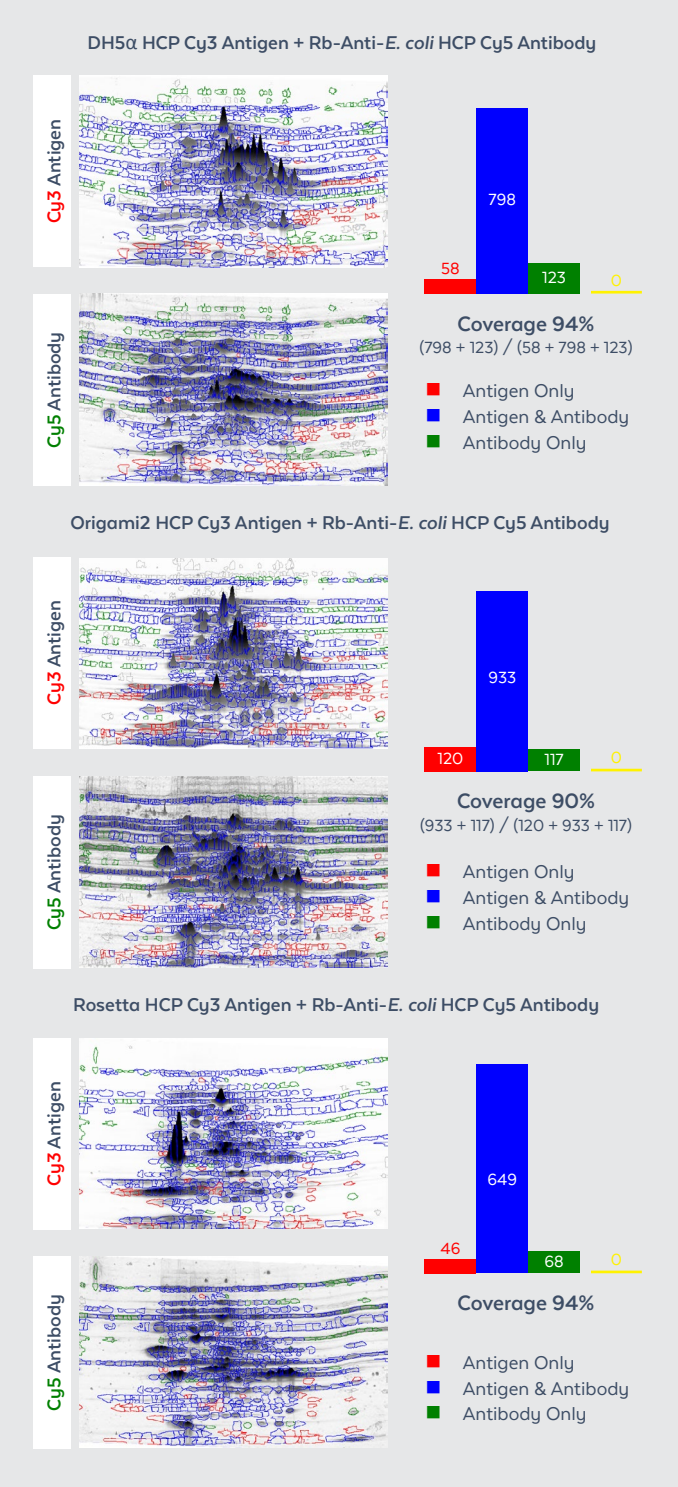

Fig. 4. Heat map and protein spot demarcation in Melanie™ 9 software, with associated coverage calculation for each strain by the anti-*E. coli* HCP antibody.

Strain Compatibility in ELISA Assay

Coverage analysis using DIBE technology showed good coverage of the detection antibody with various strains of *E. coli*. However, it was crucial to demonstrate that the kit itself could detect and quantify different B and K-12 strains of *E. coli*. To achieve this, lysates of multiple *E. coli* strains were prepared, diluted into the range of the standard curve of the assay, and their concentrations measured. The AccuSignal™ *E. coli* HCP ELISA Kit successfully detected all *E. coli* strain lysates tested (Table 10) and quantified them accurately (Fig. 5). These results confirm the ability to accurately measure *E. coli* HCP strains using the kit.

Table 10. Kit compatibility across a variety of *E. coli* strains.

B or K-12	Specific Strain	HCP Detected in Assay
B	BL21 (DE3)*	YES
B	Rosetta	YES
K-12	Origami2	YES
K-12	DH5α	YES
K-12	XL1-Blue	YES

*The Kit's standard curve was generated against *E. coli* BL21 (DE3) cells.

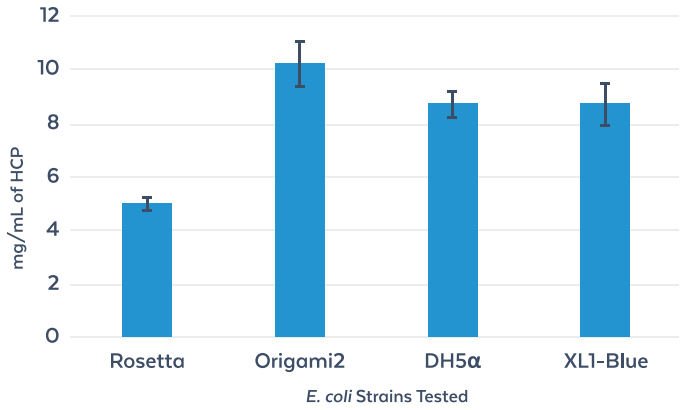

Fig. 5. Indicates the AccuSignal™ *E. coli* HCP ELISA Kit successfully quantitates HCPs from both B and K-12 strains varieties of *E. coli*.

Note: BL21 (DE3) cells are not included as it comprises the kit standard.

Kit Components

Table 11. List of AccuSignal *E. coli* HCP ELISA Kit Components

Component	Item No.	Size
E. coli HCP Antibody-coated 96-well Strip Plate	KJB-4003B	1 plate
E. coli HCP Detection Antibody	KJB-4303A	120 µL
E. coli HCP Protein Standard	KJB-0003C	1 µg
Sample Buffer	KJX-0001D	50 mL
Stop Buffer	KJX-0001G	20 mL
TMB Buffer	KJX-0001F	20 mL
Wash Buffer (10X)	KJX-0001E	60 mL
Plate Sealer	KJX-0001H	1 sheet

Ordering Information

Table 12. Ordering information for AccuSignal *E. coli* HCP ELISA Kit

Description	Item No.	Size
AccuSignal™ *E. coli* HCP ELISA Kit	KJB-4003	1 Kit

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