ROCKLAND

AccuSignal™

E. coli HCP ELISA Kit

(KJB-4003)

Contents:

Introduction

AccuSignal™ *E. coli* HCP ELISA Kit (KJB-4003) is designed to detect contaminating *E. coli* Host Cell Proteins (HCP) within therapeutics. This kit is provided as a sandwich ELISA that uses pre-immobilized anti-*E. coli* HCP detection antibodies combined with 3,3',5,5'-TMB substrate to quantitatively measure *E. coli* HCPs in a variety of therapeutic products and in-process materials from both K-12 and B-strains of *E. coli*.

Captured proteins are detected using an anti-*E. coli* HCP antibody conjugated to Horse Radish Peroxidase (HRP). The addition of TMB results in a chromogenic change that can be measured photometrically at 450 nm, with a reference wavelength at 630-650 nm. The optical density (OD) in the wells is directly proportional to the *E. coli* HCP concentration of the sample and can be interpolated to calculate the concentration of *E. coli* HCP present in the sample based on a 4PL calibration curve generated with the provided *E. coli* HCP standard.

I. Kit Components

Table 1. List of Accusignal *E. coli* HCP ELISA Kit Components

Component	Item No.	Size
E. coli HCP Antibody-coated 96-well Strip Plate[1]	KJB-4003B	1 plate
E. coli HCP Detection Antibody[2]	KJB-4303A	120 µL
E. coli HCP Protein Standard[3]	KJB-0003C	1 µg
Sample Buffer[4]	KJX-0001D	50 mL
Stop Buffer[4]	KJX-0001G	20 mL
TMB Buffer[4]	KJX-0001F	20 mL
Wash Buffer (10X)[2]	KJX-0001E	60 mL
Plate Sealer[4]	KJX-0001H	1 sheet

Note: Store kit components at 2°C to 8°C upon arrival.

[1] Antibody-coated 96-well strip plate in a 1 x 8 strip format. Each strip is packaged within a frame enclosed in a resealable foil pouch that includes a desiccant. Ready for use as supplied. Do not use wells if the foil pouch shows evidence of damage, such as tears or holes in the foil pouch.
[2] Dilute prior to use.
[3] Reconstitute and dilute prior to use.
[4] Ready to use as supplied.

II. Reagents & Materials Required but Not Provided

- Microplate shaker (up to 450 rpm)
- Interval timer
- Deionized water
- Multichannel pipettor (e.g. 50-300 µL)
- Precision single pipettes (e.g. 10 µL, 35 µL, 100 µL, 1000 µL, etc.)
- Disposable pipette tips
- Disposable microcentrifuge tube(s) or microplate

- Polypropylene centrifuge tubes (15 mL)
- Spectrophotometer microplate reader (450 nm absorbance, 630–650 nm reference filter)
- Disposable gloves
- Graduated cylinder
- Reagent reservoirs
- Vortex mixer
- Stir plate & magnetic stir bar
- Absorbent paper

Procedural Notes

1. Keep light-sensitive reagents (HRP Conjugate, TMB Substrate) in the original bottles and avoid unnecessary exposure to the light.
2. Store any unused test strips in the resealable foil pouch with desiccant to protect from moisture.
3. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
4. Incubation times or temperatures other than those stated in this insert may adversely affect the results.
5. Avoid introducing air bubbles in the test wells as this could result in lower binding efficiency.
6. All reagents should be mixed gently and thoroughly prior to use. Avoid foaming.
7. Antibody must remain at 2-8°C until immediately prior to use. Antibody may be kept on ice if necessary.

III. Assay Reagents Preparation

Preparation of Standards & Test Samples

1. Reconstitute the standard vial with 1 mL deionized water to obtain a final concentration of 1 µg/mL.
   Note: This is the "reference stock solution" that will be used below to make the standards (see Table 2).
2. Prepare dilutions of standard as indicated in Table 2.
3. Test samples should be diluted in Sample Buffer based on empirically determined criteria for each sample.

Table 2. Standards, Concentrations, and Volumes

Assay Standard	Final Conc. (ng/mL)	Initial Volume (µL)	Add Vol. to Dilution Tubes
A	250	450	150 µL of Ref stock solution (i.e. 1 mL at 1,000 ng/mL)
B	100	360	240 µL of A
C	40	360	240 µL of B
D	16	360	240 µL of C
E	6.4	360	240 µL of D
F	2.6	360	240 µL of E
G	0	360	0

Note: Dilution scheme above allows for standards to be run in triplicate. It is recommended that standards be run at least in duplicate.

Detection Antibody Working Solution Preparation

Table 3. *E. coli* HCP Detection Antibody Concentration and Volume for a Full 96-well Assay (1:100 ratio of antibody to buffer)

Sample Buffer Volume	11 mL
Anti-*E. coli* HCP Detection Antibody Volume	110 µL

1. Add 110 µL of conjugated antibody to 11 mL of Sample Buffer for use in a full 96-well assay.
2. Mix well by pipette or inversion. Do not vortex.
3. Distribute antibody working solution as described in the assay procedure.

Note: Volumes may be adjusted so long as final working concentration remains as specified.

Wash Buffer (1X) Preparation

1. Add 50 mL of Wash Buffer (10X) to 450 mL of deionized water.
2. Mix for at least 10 minutes using a magnetic stir bar.

Note: The wash buffer (1X) can be stored at room temperature (15°C to 25°C) for up to 2 weeks, after which it should be discarded.

VI. Sample Preparation & Matrix Effect

The performance of the kit was tested in various sample buffers (matrices). Some buffer systems can greatly affect the assay, causing low recovery because of suboptimal conditions (e.g. low pH).

Sample Dilution

Optimal dilution factors for each test sample must be determined by the user. It is recommended to dilute the test sample first to a minimum ratio of 1:2, or ratios as determined by the user, using the Sample Buffer and perform a serial dilution of the sample with the Sample Buffer. For low pH buffers, a dilution of 1:10 or up to 1:20 might be required.

Note: Remove insoluble protein aggregates by centrifuging.

Matrix Effect

Validate specific matrix effects if the test sample is not diluted into the Sample Buffer. Matrix effects can be quantified by spiking known concentrations of *E. coli* HCP Protein Standard into test matrix buffers. Matrix effects should be tested with different proportions of the Sample Buffer for optimal results. Recovery should be 80-120%.

V. Recommended Assay Procedure

The assay procedure takes approximately 2 hours with a detection range of 250-3.0 ng/mL.

For a reproducible assay we recommend to:

- Mix the samples and reagents before use.
- Perform the washing procedure with the recommended wash buffer volumes.
- Measure all standards and samples in duplicate or triplicate.
- Avoid the use of an automated plate washer unless validated as fit for purpose by the end user.

1. To each well add 100 µL of unknown or standard sample per well and incubate at room temperature for 30 minutes with shaking at 450 revolutions per minutes (rpm) on a shaker.
2. Wash the wells with Wash Buffer as follows:
   i. Decant the contents of the wells manually with a hard, rapid downward motion. Fluid should be captured in a receptacle designed to collect the waste. Remove all residual reagent from the microplate by tapping it on absorbent paper with the opening facing downwards.
   ii. Fill each well with 300 µL of Washing Buffer with a multichannel pipettor.
   iii. Decant the Washing Buffer from the wells with a hard, rapid downward motion. Remove all residual solution from the microplate by tapping it on absorbent paper with the opening facing downwards.
   iv. Repeat steps ii and iii three more times (total of 4 washings). Do not leave any residual moisture in the wells on each washing step.
3. To each well add 100 µL of detection antibody working solution (see Table 3).

4. Incubate at room temperature for 30 minutes, covered to protect from light, with shaking at 450 rpm.
5. Following 30 minute incubation, wash with Wash Buffer as follows:
    i. Decant the contents of the wells manually with a hard, rapid downward motion. Fluid should be captured in a receptacle designed to collect the waste. Remove all residual reagent from the microplate by tapping it on absorbent paper with the opening facing downwards.
    ii. Fill each well with 300 µL of Washing Buffer with a multichannel pipettor.
    iii. Decant the Washing Buffer from the wells with a hard, rapid downward motion. Remove all residual solution from the microplate by tapping it on absorbent paper with the opening facing downwards.
    iv. Repeat steps ii and iii three more times (total of 4 washings). Do not leave any residual moisture in the wells on each washing step.
6. To each well add 100 µL of TMB solution (see Table 3).
7. Incubate at room temperature for 30 minutes, covered to protect from light.
8. Then add 100 µL of stop solution per well. Gently tap the plate to mix, ensuring no bubbles are formed, and read plate within 5 minutes after stopping the reaction.
9. On plate reader, measure absorbance at 450 nm with the reference wavelength set at 630–650 nm.

VI. Calculations

Follow the steps below to estimate the *E. coli* HCP concentration of the test samples.

1. Calculate the relative OD 450 using the following formula: Relative OD 450 = (OD 450 of well) – (OD 630-650 nm of well).

2. Calculate the mean relative OD 450 of the replicates for each standard solution.
3. Plot the standard solutions data as mean relative OD 450 for each standard solution (Y) vs the respective concentration of the standard solutions (X).
4. Fit the standard solution data with a 4-parameter logistic (4PL) curve.
5. Estimate the *E. coli* HCP concentration of each test sample well using interpolation from the standard curve. Calculate the average of each respective sample solution concentration.

Note: If the assay samples are from dilutions, multiply the concentrations obtained from interpolations by the dilution factor.

Note: If the spectrometer used for the assay does not automatically subtract the reference wavelength, do this manually.

VII. Assay Performance Characteristics

Table 4. General kit specifications

Specification	Parameter
Goodness of fit of standard curve (4PL)	$R^2 > 0.98$
Range of the standard curve	2.6-250 ng/mL
Precision, intra-and inter-assay CV%	≤20%
Sensitivity, lower limit of quantification	3.0 ng/mL
Sensitivity, lower limit of detection	≤1.0 ng/mL

Note: Individual labs may qualify the assay at lower LLOQ values.

VIII. Troubleshooting

Table 5. Troubleshooting

Issue	Possible Solutions
Low Reproducibility	• Never mix components between kit lots. Each assay is lot-specific and designed for performance when used before the assigned expiration date. • Review the washing step procedure. Add recommended volumes of wash buffer to all wells. • Review preparation and mixing of the samples procedures. Make sure that the samples are well-mixed and not aggregated. • Make sure to have proper reagent temperatures throughout the assay. • If liquid is on underside of plate, carefully dry the underside of the plate with an absorbent towel.
High Background	• Clean all surfaces and equipment with 70% Isopropyl Alcohol before beginning assay and do not reuse pipette tips. This assay is sensitive enough to detect extraneous *E. coli* proteins on uncleaned surfaces and equipment. • Review washing step procedure. Add recommended volumes of wash buffer to all wells. • Review the assay incubation procedure. Follow recommended incubation times. • Review preparation and mixing of samples procedure. Make sure that samples are well mixed and not aggregated.
Poor Reactivity or Intra Assay Precision	• Review preparation and the mixing of samples procedure. Make sure that the samples are well mixed and not aggregated. • Review matrix effect: high concentrations of detergents, salts, pH and other matrices characteristics can influence the assay. • Use the provided sample buffer to dilute the sample to the minimum recommendations.

Table 5. Troubleshooting

Issue	Possible Solutions
Poor Fit of the Standard Curve	• Review washing step procedure. Add recommended volumes of wash buffer to all wells. • Review preparation and mixing of samples procedure. Make sure that the samples are well mixed and with no protein aggregates. • Review pipetting procedure. Make sure that the samples are pipetted into the bottom of each well. Take care not to touch the bottom of the well with the pipette tip. **Note**: This assay has been optimized to function with 4PL Curve Fits. However, additional curve fitting techniques can be attempted based on established techniques and methodologies (for example, weighting with 1/Y^2 or 5PL Curve Fits).

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