E. coli (K-12 & Alkaline Lysis) HCP ELISA Kit User Guide

PLEASE READ THE DOCUMENT CAREFULLY BEFORE EXPERIMENT

Product No.: 1301302
Version: A/2
For Research Use Only. Not for diagnostic or therapeutic use.

Huzhou Shenke Biotechnology Co., Ltd

■ Product Name

E. coli (K-12 & Alkaline Lysis) HCP ELISA Kit

■ Package

96 tests/Kit

■ Intended Use

This kit is suitable for the quantitation of host cell proteins (HCPs) of *E. coli* K-12 strain (e.g., DH5α, Top10, JM109) in Plasmid DNA (pDNA) intermediates or bulk prepared chemically by alkaline lysis.

The kit is for Research Use Only and is not for use in diagnostic procedures or for therapeutic purposes in humans or animals.

■ Detection Principle

Different from traditional physical disintegration technique, the HCPs in the pDNA samples are exposed to high alkaline solutions and denatured during the extraction process, leading to a large difference in the detection results measured by immuno-assays.This kit is based on the solid-phase enzyme-linked immunosorbent assay (ELISA) with a double-antibody sandwich technique to detect residual host cell proteins (HCPs) from *E. coli* K-12 strains. A sheep polyclonal antibody specific to *E. coli* (K-12 strains) HCPs prepared by alkaline lysis was employed in the assay to capture any remaining HCPs in the sample. The antibody coverage is assessed by the current mainstream method. Both the Calibration Standard and test samples were simultaneously added to the microtiter plate coated with the affinity purified capture antibody and followed by incubation and washing. The biotinylated antibody was added to the microtiter plate to bind the HCPs and then reacted with streptavidin labeled with HRP (Horseradish Peroxidase). TMB (3,3',5,5' -tetramethylbenzidine) substrate was added into reaction, HRP catalyzed the oxidation of TMB by H_2O_2 to produce a blue-colored product (maximum absorption peak at 655 nm). Then the stop solution was added to terminate the enzymatic reaction, resulting in a yellow-colored product (maximum absorption peak at 450 nm). The absorbance values at 450 nm wavelength were positively correlated with the HCPs concentration in the Calibration Standard and the sample. The concentration of HCPs in the sample can be calculated using the dose-response curve.

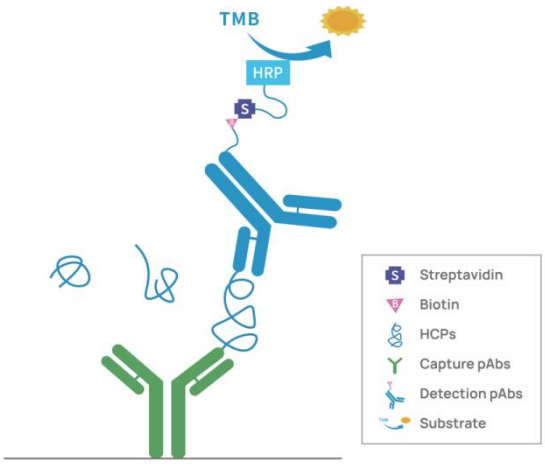


Figure 1. Schematic diagram

■ Kit Contents

Table 1. Kit Components

Reagent	Part No.	Quantity	Note
E. coli HCP-AC Calibration Standard	PNB002	3 bottles	Lyophilized powder. Dissolve it with 500 µL Reconstitution Solution (PNC002), and let it stand for about 5 minutes until transparent. Please refer to the bottle label for details.
Anti-*E. coli* HCP-AC Microtiter Strips	PNA002	8 well × 12 strips	Strips pre-coated with sheep (*Ovis aries*) anti-*E. coli* HCP-AC antibody in a vacuumed bag with desiccant. Seal and store immediately after use.
Reconstitution Solution	PNC002	2 × 1.5 mL	Only used for dissolving *E. coli* HCP-AC Calibration Standard (PNB002).
Wash Buffer Concentrate (10×)	PNF001	3 × 25 mL	For plate washing and dilution of Calibration Standard, biotinylated antibody, streptavidin-HRP and samples. Dilute 10 times with freshly prepared ultra-pure water to obtain 1×Wash Buffer solution.
Anti-*E. coli* HCP-AC: Biotinylated Conjugate (20×)	PNG002	1 × 600 µL	Biotinylated anti-*E. coli* HCP-AC antibody (sheep polyclonal) in a protein matrix with preservative. Dilute 20 times with 1×Wash Buffer before use.
Streptavidin-HRP (100×)	PNH002	1 × 140 µL	Streptavidin is labeled with HRP. Keep sealed and protected from light. Dilute 100 times with 1×Wash Buffer before use.
TMB Substrate	PND002	1 × 12 mL	Equilibrate to room temperature for 20 minutes before use. Keep sealed and protected from light.
Stop Solution	PNI002	1 × 6 mL	Avoid direct contact with eyes, skin, and clothing
Sealing Film	PNK001	3 pieces	Cover the strips with it during incubation to prevent contamination and liquid evaporation.

Note: *Room temperature refers to 25±3℃.*

■ Storage Conditions

Store the kit at 2-8℃. Please check the expiration date on the labels. Store opened or reconstituted components as shown in Table 2.

Table 2. Recommended storage conditions for opened components

Component	Stability
Anti-*E. coli* HCP-AC Microtiter Strips	Store in the bag with desiccant at 2-8℃ for up to 60 days.
Reconstituted *E. coli* HCP-AC Calibration Standard	Store at 2-8℃ for up to 30 days.

■ Materials Required (Not Provided in the Kit)

- Sterile centrifuge tubes for dilution
- Absorbent paper for plate drying
- Pipette Tips: 1000 μL, 100 μL, 10 μL
- Multi-channel reagent reservoirs (50 mL)

■ Equipment

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 620 nm to 650 nm.
- Single or multi-channel pipettes.
- Microplate thermoshaker (For sample incubation. If this equipment is not available, a standing incubation is an option. See the Assay Experiment part for details.)
- Incubator (optional)
- Plate washer (optional)

■ Workflow

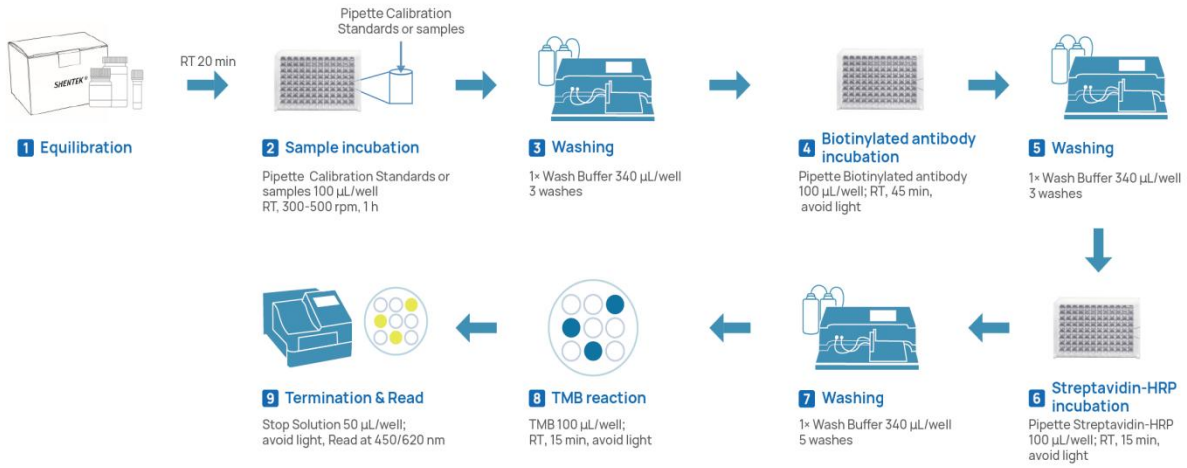


Figure 2. Procedure Flowchart

1. Preparation

(1) Equilibration

- Allow the kit to equilibrate at room temperature for 20 minutes, Return to 2-8℃ after use.
- Take appropriate amount of strips to a strip holder according to the experiment design. Immediately reseal unused strips with desiccant and store at 2-8℃.

(2) Preparation of Reagents

- *E. coli* HCP-AC Calibration Standard solution: Pipette 500 µL of Reconstitution Solution (PNC002) into the bottle containing *E. coli* HCP-AC Calibration Standard (PNB002). Gently invert 3-5 times to mix well and let it stand for 5 minutes. Save the remaining solution under the recommended condition.

  Note: *If two or more calibration-standard vials are required, reconstitute each vial as directed, combine the reconstituted solutions, and mix gently before use. Do not change the specified reconstitution volume.*
- 1×Wash Buffer: Dilute 1 volume of Wash Buffer Concentrate (10×) (PNF001) with 9 volumes of ultra-pure water. For example, add 25 mL Wash Buffer

Concentrate (10×) (PNF001) to 225 mL of ultra-pure water to make 250 mL of 1×Wash Buffer. Mix well before use.

Note: *If the Wash Buffer Concentrate (10×) (PNF001) is cloudy or contains precipitates, heat at 37℃ until it clears.*

(3) Preparation of Calibration Standard Solutions

- Prepare *E. coli* HCP-AC Calibration Standard solutions as indicated in Figure 3 and Table 3.

Figure 3. Graphic scheme of *E. coli* HCP-AC Calibration Standard solutions

Table 3. Preparation of *E. coli* HCP-AC Calibration Standard solutions

Tubes	Dilution procedure	Conc.(ng/mL)
ST1	Dilute the reconstituted *E. coli* HCP-AC Calibration Standard to ST1 with 1×Wash Buffer	729
ST2	300 µL ST1 + 600 µL 1×Wash Buffer	243
ST3	300 µL ST2 + 600 µL 1×Wash Buffert	81
ST4	300 µL ST3 + 600 µL 1×Wash Buffer	27
ST5	300 µL ST4 + 600 µL 1×Wash Buffert	9
ST6	300 µL ST5 + 600 µL 1×Wash Buffer	3
NCS	1×Wash Buffer	0

- Calculate the volume of 1×Wash Buffer to prepare the ST1:

$$\frac{(A\ \mu g \times 1000\ ng/\mu g) \times C\ \mu L}{B\ (500\ \mu L) \times (D\ ng/mL/\ 1000\ \mu L/mL)} - C\ \mu L = 1\times Wash\ Buffer\ volume\ (X)$$

- A = Content of Calibration Standard (µg)
- B = Volume of Reconstitution Solution (PNC002, 500 µL)
- C = Volume of reconstituted calibration standard solution for ST1 preparation (e.g. 729 µL)
- D = Concentration of ST1 calibration standard solution (729 ng/mL)

For example, the label indicates that each bottle of *E. coli* HCP-AC Calibration Standard (PNB002) contains 0.74 μg (A). Add (B) volume (500 μL) of Reconstitution Solution (PNC002) into the bottle for reconstitution. Then take (C) volume (recommended 729 μL) of the reconstituted solution and add 751 μL (X) of 1×Wash Buffer to prepare a ST1 calibration standard solution with a concentration of 729 ng/mL (D).

$$\frac{(0.74\ \mu g \times 1000\ ng/\mu g) \times 729\ \mu L}{500\ \mu L \times (729\ ng/mL\ /\ 1000\ \mu L/mL)} - 729\ \mu L = 751\ \mu L$$

(4) Sample Preparation

- Test samples: pDNA products (e.g., in-process samples). Make sure samples are clear and transparent, and insoluble substances need to be removed by centrifugation or filtration.
- Conduct sample stability studies to prevent degradation or denaturation during the experiment. Avoid unvalidated repeated freeze-thaw cycles. For long-term storage, -70°C is recommended to avoid degradation.
- Dilute the samples with 1×Wash Buffer to achieve a proper range of HCP concentration within the calibration curve.
- For the first use, a method validation is recommended to verify sample suitability before the subsequent routine test. This will help to set up appropriate sample dilution series.

  Note: *Please contact us for support of validation protocol.*

2. Assay Experiment

(1) Sample Incubation

- Pipette 100 μL of Calibration Standard, controls and samples into each designated well according to the experimental design. Avoid foaming bubbles during pipetting. It is recommended to prepare 2-3 replicates for each concentration.
- Seal the plate and incubate on microplate thermoshaker at 300-500 rpm for 1 hour at room temperature. It is possible to incubate the plate without shaking for 2 hours if such a device is not available.

Table 4. Example of microplate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	NCS	NCS	NCS		S1	S1	S1					
B					S2	S2	S2					
C	ST6	ST6	ST6		S3	S3	S3					
D	ST5	ST5	ST5		S1+SRC	S1+SRC	S1+SRC					
E	ST4	ST4	ST4		S2+SRC	S2+SRC	S2+SRC					
F	ST3	ST3	ST3		S3+SRC	S3+SRC	S3+SRC					
G	ST2	ST2	ST2									
H	ST1	ST1	ST1									

- ✧ "ST1-ST6" indicate 6 concentration gradients, "NCS" as negative control, "S1-S3" as test samples , and "S1 SRC-S3 SRC" as the spiked recovery controls for each sample.
- ✧ The number of replicates and the spiked samples can be determined by method validation.

(2) Biotinylated Antibody Reagent Preparation and Incubation

- 1×Anti-*E. coli* HCP-AC: Biotinylated Conjugate: Prepare the 1×Anti-*E. coli* HCP-AC: Biotinylated Conjugate by diluting the Anti-*E. coli* HCP-AC: Biotinylated Conjugate (20×) (PNG002) with 1×Wash Buffer in a sterile centrifuge tube. Prepare 1×Anti-*E. coli* HCP-AC: Biotinylated Conjugate fresh, mix gently and use it immediately.
- Wash the plate with 340 µL of 1×Wash Buffer per well. Wipe off any liquid from the bottom outside of the plate. Repeat washing for 3 times. Do not allow the wells to be completely dry before adding the substrate.
- Pipette 100 µL of 1×Anti-*E. coli* HCP-AC: Biotinylated Conjugate into the corresponding wells as indicated earlier.
- Seal the plate and incubate for 45 minutes at room temperature, and protect from light.

(3) Streptavidin-HRP Preparation and Incubation

- 1×Streptavidin-HRP: Prepare the 1×Streptavidin-HRP by diluting the Streptavidin-HRP (100×) (PNH002) with 1×Wash Buffer in a sterile centrifuge tube. Prepare 1×Streptavidin-HRP fresh, mix gently and use it immediately.

- Wash the plate with 340 μL of 1×Wash Buffer per well. Wipe off any liquid from the bottom outside of the plate. Repeat washing for 3 times. Do not allow the wells to be completely dry before adding the substrate.
- Pipette 100 μL of 1×Streptavidin-HRP into the corresponding wells.
- Seal the plate and incubate for 15 minutes at room temperature, and protect from light.

(4) Substrate Incubation

- Equilibrate the TMB Substrate (PND002) for 20 minutes at room temperature.
- Wash the plate with 340 μL of 1×Wash Buffer per well. Wipe off any liquid from the bottom outside of the plate. Repeat washing for 5 times. Do not allow the wells to be completely dry before adding the substrate.
- Add 100 μL of TMB Substrate (PND002) into wells, and incubate at room temperature for 15 minutes, and protect from light.

  Note: *Do not use Sealing Film (PNK001) during this step.*

(5) Termination

- Add 50 μL of Stop Solution (PNI002) into each well.

  Note: *The order of adding Stop Solution (PNI002) should be the same as the order of adding the TMB Substrate (PND002). While adding samples, suspend the tips above the liquid to prevent contact with the solution in the wells and minimize the risk of bubble formation.*
- Incubate at room temperature for 5 minutes, protect from light.

(6) Read

- Read absorbance at 450 nm/620-650 nm.

3. Calculation and Analysis

- The $OD_{450\ nm}$ value of each well should be calculated by subtracting their respective long wavelength, as of $OD_{620\ nm}$ in this case. If the microplate reader is not equipped with long wavelength measurement, this step can be omitted.
- The $OD_{450\text{-}620\text{-}N}$ value of calibration curve fitting points and samples should be calculated by subtracting the $OD_{450\text{-}620}$ of NCS, then take the average value of replicates.

- Perform a 4-parameter logistic regression model using the subtracted Calibration Standard concentration values to obtain a calibration curve equation. The data analysis software for calibration curve fitting could be the built-in software from microplate reader. If not, we recommend to use professional standard curve software such as Curve Expert, ELISA Calc, and so on.
- Substitute the average OD value of the samples into the equation to calculate the sample concentration.

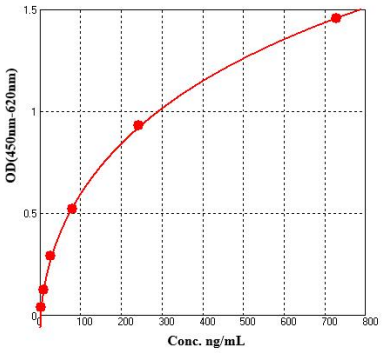
■ Limitations

- The product is only applicable to the detection of HCPs derived from the alkaline lysis method.
- The samples pH should be between 6.5 and 8.5. Beyond this range may cause abnormal results.

■ Assay Performance

- Linearity & Range: 3-729 ng/mL, $R^2 \geq 0.990$
- QL: 3 ng/mL
- Specificity: No cross-reactivity with CHO, Vero, HEK293T, and *Hansenula polymorpha*.
- Typical calibration curve and data:

Calibration Standards (ng/mL)	Abs. At (450 nm-620 nm)	AVG
729	1.5319	1.5268
	1.5628	
	1.4857	
243	1.032	1.0011
	1.0058	
	0.9656	
81	0.5986	0.5903
	0.609	
	0.5634	
27	0.3714	0.3635
	0.3614	
	0.3577	
9	0.1925	0.1974
	0.2019	
	0.1978	
3	0.1125	0.1124
	0.1095	
	0.1151	
0	0.0702	0.0701
	0.0702	
	0.0699	



4-PL: $Y = \frac{A-D}{1+(\frac{X}{C})^B} + D$

A = 3.94571

B = -0.57096

C = 1732.85755

D = -0.05957

R^2 = 0.99973

■ Additional Information

- ✧ This kit is intended for use by qualified technicians only.
- ✧ Consumables, for example sterile disposable tips, tubes and reservoirs are only allowed for single use. It is recommended to wipe with 75% ethanol before and after each use. Follow the specified pipetting procedure carefully.
- ✧ Users should validate the assay before testing their samples.
- ✧ Dilution should be gentle and thorough to avoid excessive foaming.
- ✧ Stop Solution (PNI002) contains 1 M hydrochloric acid. Avoid direct contact with eyes, skin, and clothing.
- ✧ Do not mix the kit reagents with different lot numbers.
- ✧ Use fresh sterile water or ultra-pure water, and ensure the water temperature does not exceed 37℃.
- ✧ Seal or cover the microplate immediately after sample loading to avoid liquid evaporation.
- ✧ Avoid drying the wells before substrate incubation.
- ✧ Store unused microtiter strips in a sealed bag with desiccant to prevent contamination.
- ✧ Centrifuge Anti-*E. coli* HCP-AC: Biotinylated Conjugate (20×) (PNG002) and Streptavidin-HRP (100×) (PNH002) before use to avoid any loss of the reagent.
- ✧ To avoid pipetting errors, pipette or sampling accurately for dilution of standards and samples, for example, a minimum volume of 5 μL is recommended.
- ✧ *E. coli* HCP-AC Calibration Standard solutions, Biotinylated Antibody solution and Streptavidin-HRP solution are recommended for single use due to stability issue. Prepare freshly before each experiment.
- ✧ TMB Substrate (PND002) should be colorless. If not, discard it and contact us for assistance.
- ✧ Pipette carefully to avoid any bubbles, and gently shake the plate for thorough mixing. Sometimes air, resulting in bubbles, can be drawn into the micropipette or dispensed into the wells. If this happens, bubbles can influence optical density values and detection results.
- ✧ Reading should be completed within 30 minutes after termination.
- ✧ Avoid the samples containing sodium azide (NaN_3), which will deactivate the HRP and lead to the underestimation of HCP levels.

■ Troubleshooting

Problem	Possible Cause	Solution
High background signal (OD)	Cross-contamination of reagents, including distilled water	Prepare fresh reagents before the experiment and prevent cross-contamination.
	Cross-contamination of equipment, including pipettes and centrifuge	Clean the equipment with 75% ethanol before experiment.
	Environmental contamination	Separate the working bench to avoid contamination.
	Insufficient washing	Increase the wash buffer volume or wash times, and remove any remaining liquid before proceeding to the next step.
Abnormal values	Improper washing	Swiftly and completely shake off any excess liquid, and avoid reusing paper towels to minimize contamination.
	Improper sampling	Add the samples to the bottom of the wells using micropipettes, and avoid splashing to the neighboring wells.
	Plate sealing	Promptly cover the plate with the Sealing Film (PNK001) and remove it carefully to prevent splashing.

If any other difficulties, please contact us for technical support.

■ References

- ChP<9101> Guidance for Analytical Procedures Validation
- ChP<3412>Determination of Residual *E. coli* Proteins
- USP<1103> Immunological Test Methods Enzyme-Linked Immunosorbent Assay (Elisa)
- USP<1132> Residual Host Cell Protein Measurement in Biopharmaceuticals
- Ph. Eur. <2.6.34> Host-Cell Protein Assays

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Support & Contact

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