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User Manual

Disclaimer: Products are intended for research use only

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E.coli (Protein Expression Strains) HCP ELISA Kit (One-step ELISA) User Guide

PLEASE READ THE DOCUMENT CAREFULLY BEFORE EXPERIMENT

Product No.: 1301301-1
Version: A/1
For Research Use Only

Biofargo, Inc.

■ Product Name

E.coli (Protein Expression Strains) HCP ELISA Kit (One-step ELISA)

■ Package

96 tests/Kit

■ Intended Use

This kit is intended for use in determining the presence of host cell proteins (HCPs) in products manufactured by expression in *E.coli* originated from BL21, such as interleukin (IL), recombinant human interferon (rhIFN), recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), recombinant human tumor necrosis factor (rhTNF), growth-promoting factor (EGF/FGF/PDGF), et.al.

The kit is for RESEARCH USE ONLY and not intended for clinical use.

■ Product Description

This kit employs a solid-phase Enzyme-linked Immunosorbent Assay (ELISA) with a double-antibody sandwich technique to detect residual *E.coli* (Protein expression strains) host cell proteins (HCPs) in the sample. Polyclonal antibody specific to *E.coli* (Protein expression strains) HCPs was employed in the assay to capture any remaining HCPs in the sample. Both the Calibration Standard (or test sample) and the HRP (Horseradish Peroxidase) labeled with anti-*E.coli* (Protein expression strains) HCP antibody were simultaneously added to the pre-coated microtiter plate, and followed by incubation and washing steps. Then TMB (3,3',5,5'-tetramethylbenzidine) substrate added for reaction, HRP catalyzed the oxidation of TMB by H_2O_2 to produce a blue product (maximum absorption peak at 655nm). Afterwards the stop solution is added to terminate the enzymatic reaction, resulting in a yellow color product (maximum absorption peak at 450nm). The absorbance values at 450nm wavelength were positively correlated with the HCPs concentration in the Calibration Standard and the sample. The concentration of *E.coli* (Protein expression strains) in the sample can be calculated using the dose-response curve.

No special treatment is required for the test sample and its suitability could be verified by the appropriate dilution ratios, using this kit.

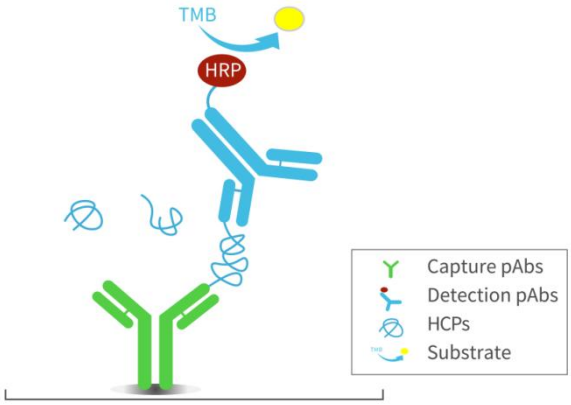


Figure 1. Schematic diagram

■ Kit Contents

Table 1. Kit Components

Reagent	Part No.	Quantity	Note
E.coli HCP-E Calibration Standard	PNB003	2 bottles	Lyophilized powder. Dissolve it with 500 µL Reconstitution Solution and let it stand for about 5 minutes until transparent. Please refer to the details on the label of the tube.
Anti-*E.coli* HCP-E Microtiter Strips	PNA003	8 well ×12 strips	Strips pre-coated with sheep anti-*E.coli* (Protein expression strains) HCP affinity antibody in a vacuumed bag with desiccant. Seal and store immediately after use.
Reconstitution Solution	PNC002	1×1.5 mL	Only used for dissolving *E.coli* HCP-E Calibration Standard.
Diluent	PNE004	2×25 mL	For dilution of Calibration Standard, Anti-*E.coli*-E:HRP (100×) and samples.
Wash Buffer Concentrate (10×)	PNF001	2×25 mL	It is easy to crystallize at low temperatures, and redissolved in 37 ℃ water bath before use. Dilute 10 times with freshly prepared ultra-pure water for plate washing.

Anti-*E.coli* HCP-E:HRP (100×)	PNN001	1×120 μL	Affinity purified sheep antibody conjugated to HRP need to be 100 times diluted in diluent (PNE004).
TMB Substrate	PND002	1×12 mL	Equilibrate to room temperature for 20 minutes before use. Sealed and keep away from light.
Stop Solution	PNI002	1×6 mL	1 M hydrochloric acid. Avoid direct contact with eyes, skin, and clothing. Wear goggles while handling.
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℃. Use within the expiration date labeled upon the kit package. The opened components should be stored as follows.

Table 2. Recommended storage conditions for opened components

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

■ Materials Required But Not Supplied in the Kit

- Sterile centrifuge tubes for dilution
- Clean tissue paper for plate drying
- Pipette Tips
- 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
- Incubator (optional)
- Plate washer (optional)

■ Workflow

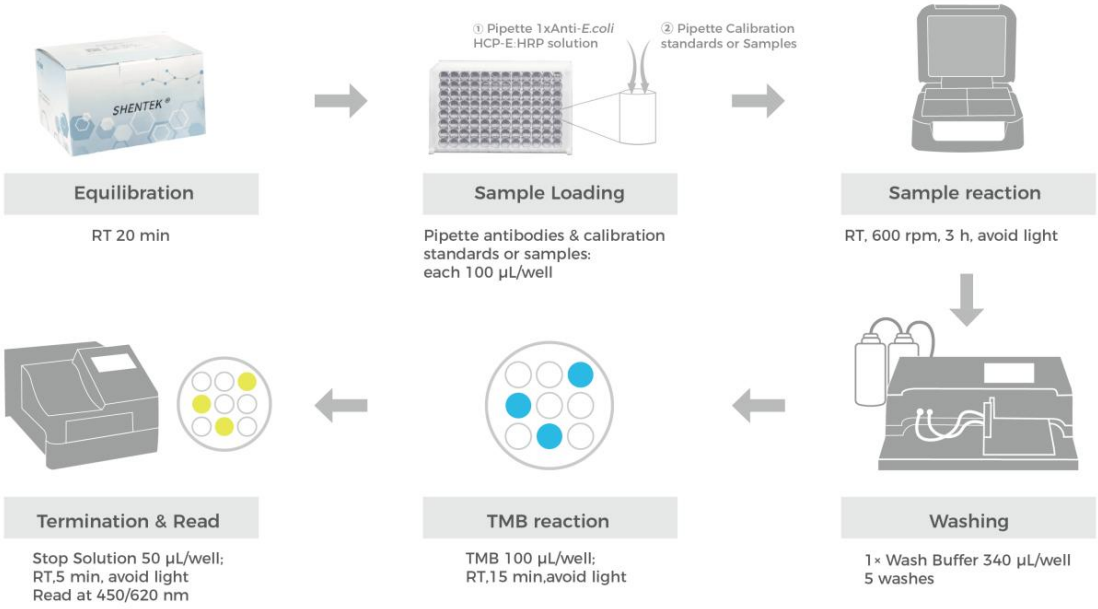


Figure 2. Procedure Flowchart

1. Preparation

(1) Equilibration

- Before use, allow the kit to equilibrate at room temperature for 20 minutes; Return to 2-8℃ after use.
- Take the appropriate amount of strips to a strip holder according to the experiment design. Please store the remaining strips in the bag with desiccant at 2-8℃.

(2) Preparation of Reagents

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

Note: Do not use any other volumes of Reconstitution Solution to dissolve the

Calibration Standard.

- 1× Wash Buffer: Dilute the Wash Buffer Concentrate (10×) at 1:10 with ultra-pure water. For example, add 25mL Wash Buffer Concentrate (10×) to 225mL of ultra-pure water to make 250mL of 1× Wash Buffer. Mix well before use.

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

- 1×Anti-*E.coli* HCP-E:HRP: Prepare the 1×Anti-*E.coli* HCP-E:HRP by diluting the Anti-*E.coli* HCP-E:HRP (100×) with Diluent in a sterile centrifuge tube. Prepare it freshly and gently mix the solution and use it immediately.

(3) Preparation of Calibration Standard Solutions

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

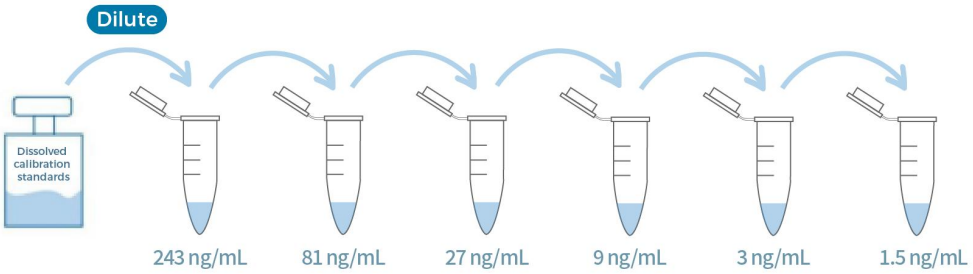


Figure 3. Graphic scheme of *E.coli* HCP-E Calibration Standard solutions

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

Serial Dilution Tube	Dilution procedure	Conc. (ng/mL)
ST1	Dilute the reconstituted *E.coli* HCP-E Calibration Standard to ST1	243
ST2	300 µL ST1 + 600 µL Diluent	81
ST3	300 µL ST2 + 600 µL Diluent	27
ST4	300 µL ST3 + 600 µL Diluent	9
ST5	300 µL ST4 + 600 µL Diluent	3
ST6	300 µL ST5 + 300 µL Diluent	1.5
NCS	Diluent	0

(4) Sample Preparation

- Test samples: In-process samples, harvested bulk, drug substance and drug

product. 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 repeated freeze-thaw cycles. Long-term storage at -70°C is recommended to avoid degradation.
- Dilute the samples with a suitable diluent to achieve a proper range of HCP concentration within the calibration curve.
- An initial test is recommend before the subsequent routine test by verifying sample suitability through setting up appropriate sample dilution series,

Note: Please contact us for support of validation protocol.

2. Assay Experiment

(1) Sample Loading

- Pipette 100 µL of 1×Anti-*E.coli* HCP-E:HRP Solution into each designated well according to the experiment design.
- Pipette 100 µL of Calibration Standard, controls and samples into the corresponding wells as indicated earlier. Avoid foaming bubbles during pipetting. It is recommended to prepare 2-3 parallels for each concentration.
- Seal the plate and incubate on microplate thermoshaker at 600 rpm for 3 hours at room temperature and protect from light.

Table 4. Example of 96-well plate 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” means 6 concentration gradients, “NCS” refer to negative control, “S1-S3” refer to test samples , and “S1 SRC-S3 SRC” refer to the spiked test samples.

✧ The number of replicates and the involvement of spiked samples can be determined by method validation.

(2) Substrate Incubation

- Equilibrate the TMB substrate for 20 min at room temperature.
- Wash the plate with 1×Wash Buffer for about 340 μL each 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 into wells, and incubate at room temperature for 15 minutes, protect from light.

  Note : Do not use sealing film during this step.

(3) Termination

- Add 50 μL of Stop Solution into each well.

  Note: The adding sequence should be the same as the adding sequence of the TMB solution. Suspend the tips while adding samples 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.

(4) Reading

- Read absorbance at 450/620-650nm.

3. Calculation and Analysis

- The OD value of each well should be calculated by the difference between OD_{450nm} and their respective long wavelength. If the microplate reader is not equipped with long wavelength measurement, this step can be omitted.
- Subtract the OD value of the NCS from each calibration point and samples, and record the mean of the duplicate wells.
- Perform a 4-parameter logistic regression model using the Calibration Standard concentration values and OD values to obtain the calibration curve equation (see Table 6). Substitute the average OD value of the sample into the equation to calculate the sample concentration, which should be multiplied by the dilution factor to obtain the actual sample concentration.
- The software for data analysis of the standard curve could be the one that comes

with the microplate reader. If not, it is recommended to use professional standard curve software such as Curve Expert, ELISA Calc, and so on.

- For samples with absorbance values above the Calibration Standard ST1, the initial test should be performed to determine an appropriate dilution before retesting. The HCP concentration in the sample is calculated from the test value multiplied by its corresponding dilution factor. If the spiked samples are simultaneously set at this dilution level and the recovery rate should meet the requirements of the corresponding regulations.

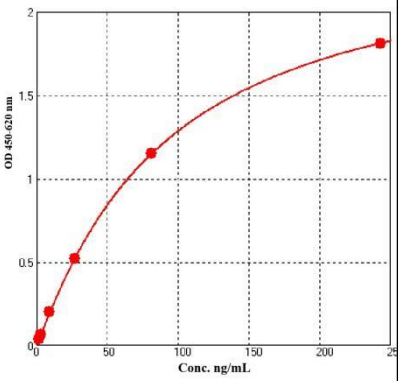
■ Limitations

- For research purposes only; not intended for clinical use.
- Specifically designed for detecting residual protein content in products manufactured by expression in BL21 of *E.coli*. A method validation is required in other types of *E.coli* protein expressing strains.
- This kit is not suitable for E.coli cloning strains or samples disintegrated by alkaline lysis. Please contact us for support of the related kits and services.
- Recommend sample pH between 6.5 and 8.5, and measurements may be compromised if the sample pH is out of range.

■ Assay Performance

- Linearity & Range: 1.5-243 ng/mL, R^2>0.990.
- LLOQ: 1.5 ng/mL.
- Specificity: No cross-reactivity with CHO, Vero, HEK293T, *Hansenula polymorpha* HCPs.
- Typical calibration curve and results:

Calibration Standards (ng/mL)	Abs. At (450 nm-620 nm)	AVG
243	1.9061	1.8938
	1.8819	
	1.8935	
81	1.2158	1.2347
	1.2672	
	1.2211	
27	0.6172	0.6007
	0.6005	
	0.5843	
9	0.3059	0.2831
	0.2767	
	0.2666	
3	0.1486	0.1469
	0.1487	
	0.1433	
1.5	0.1189	0.1199
	0.1197	
	0.1211	
0	0.0824	0.0816
	0.0838	
	0.0785	



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

A = 2.41626
B = 1.09106
C = 89.16833
D = 0.01118
R^2= 0.99995

■ Additional Information

- ✧ This kit is intended for use by qualified technicians only.
- ✧ Use sterile disposable tips, tubes and reservoirs, etc. separately. 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 is 1M HCl. Avoid direct contact with eyes, skin, and clothing.
- ✧ Do not mix the kit reagents from 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-E:HRP(100×) before use avoid any loss of the reagent.
- ✧ Accurately pipetting or sampling for dilution of standards and samples, for example, minimum volume of 5 μL is recommended.
- ✧ *E.coli* HCP-E Calibration Standard Solutions and 1×Anti-*E.coli* HCP-E:HRP are recommended for single use due to instability issue. Prepare freshly before each experiment.
- ✧ TMB Substrate should be colorless. If not, discard it and contact us for assitance.
- ✧ Pipette carefully to avoid any bubbles, and gently shake the plate for thorough mixing. Sometimes air, resulting in bubbles, can be drawn into the pipette or dispensed into the wells. If this happens, bubbles can influence optical density values and 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	Freshly prepared prior to experiment
	Cross-contamination of equipments, including pipettes and centrifuge	Clean the equipment with 75% ethanol before experiment
	Environment 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 and remove it carefully to prevent splashing.

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

■ References

- ICH. M10 Bioanalytical Method Validation And Study Sample Analysis
- FDA. Bioanalytical Method Validation
- USP<1132> Residual Host Cell Protein Measurement in Biopharmaceuticals
- EP<2.6.34> HOST-CELL PROTEIN ASSAYS
- ChP<9012> Guidance of Quantitative Method Validation for Biological Samples
- ChP<3412> Determination of Residual E.coli Proteins

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Support & Contact

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