ASSAY SPECIFICATIONS (continued)

Sensitivity

Highest sensitivity (lowest LOD) can be achieved with 1) optimal assay precision (especially well-washed plates to achieve lowest blank values), 2) low sample dilution (i.e., highest HCP concentration), and 3) extended sample time of incubation. For example, overnight incubation at room temperature (plate covered to avoid evaporation and/or contamination) can increase the low end sensitivity to under 5 U/ml. Generated signal will increase such that the high end standards may be off-scale. The HRP Conjugate and TMB Substrate incubation times may also be increased to further lower detection limits, with proper controls.

Standards: Assay Interpretation and Limits

Standards are composed of combined HCPs from the above 5 E. coli strains. The Standard Curve is an average of a family of dilution curves representing each antibody specificity contributed by the capture and detection components. Dilution curves of any subset of HCPs in the lab's particular recombinant protein processing step may not be parallel with the Standard Curve, leading to possible disparate quantitation between samples read from the upper and lower regions of the curve. Therefore, the lab should construct and use instead a dilution curve composed of the particular HCP subset(s) derived from the in-house samples.

The Standards, a complex mixture of proteins, glycoproteins and carbohydrates from *E. coli* cultures, are value-assigned in units per ml. The Calibration Standard units are based on BCA assays using BSA as standard:

1 U/ml (E. coli) = 1 ng/ml (BSA).

Measurement of protein by wavelength absorption or colorimetric assays relies on standard curves of a specified protein, such as BSA, that will have different potency (e.g. extinction coefficient) in the assay than any other protein or protein mixture. Therefore, assignment of ng/ml would be inaccurate for the E. coli protein mixture.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Calibrators, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains dilute sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water. MSDS for TMB, sulfuric acid and BND can be requested or obtained from the ADI website:

STORAGE AND STABILITY

The microwell plate and all other reagents, if unopened, are stable at 2-8^{O}C until the expiration date printed on the kit box label. Stabilities of the working solutions are indicated under Reagent Preparation.

Instruction Manual No. M-800-130-ECP

E. coli HCP (Host Cell Proteins)

ELISA Kit Cat. No. 800-130-ECP

For Quantitation of E. coli HCP in biological Solutions

For research use only (RUO), not for diagnosis, cure or prevention of the disease.

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INTENDED USE

The Alpha Diagnostics Int'l E. coli HCP ELISA Kit is an in vitro immunoassay for the quantitation of Host Cell Proteins (HCP) from E. coli cultures used for production of recombinant proteins. The assay is also suitable for other samples such as extracts of foods, vaccines, or other products or processes with proper control for assay compatibility.

INTRODUCTION

A large number of genes have been cloned and expressed in various host cells (E. coli, yeast, baculovirus, NSO, Sp2/0, HEK, CHO cells). The translated recombinant proteins may remain within the cell, requiring host cell disruption for release, and/or may be secreted into the culture medium. The target recombinant proteins would then be purified from unwanted host cell protein (HCP), often with the aid of a tag (e.g., His, GST, MBP). While traces of HCP (which are often present in the purified material) may not represent a major problem for recombinants that are used for in vitro or research use applications, an increasing number of recombinant proteins are developed for therapeutic purposes (insulin, erythropoietin, GM-CSF or humanized antibodies such Rituximab & Xolair), where the presence of HCP is potentially toxic or allergic, may create other health hazards, or otherwise affect the efficacy of the drug. In these cases, detecting residual HCP and establishing minimum acceptable levels is required. Of two typical and powerful methods used for HCP characterization, Western Blot can reveal the number, size and relative concentrations of HCPs, while ELISA can provide ultra-sensitive detection and quantification using an easy, rapid assay that accommodates large numbers of samples and replicates.

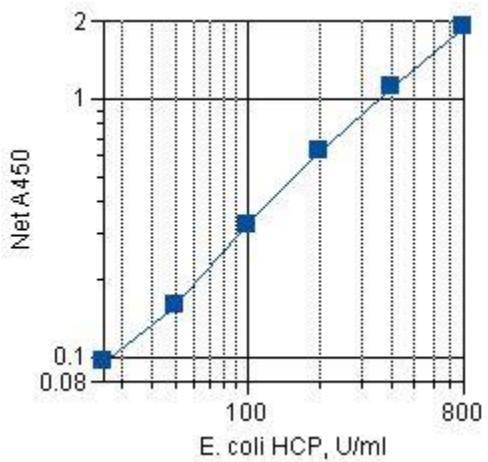
During the production of recombinant proteins, host cells die and decompose; thus, regardless of whether the recombinant product is obtained from extracellular medium or after disrupting the host cell, the entire repertoire of host cell proteins present as potential contaminants in downstream purification and processing of the recombinant protein product. The ADI E. coli HCP ELISA relies on polyclonal antibodies from multiple hosts immunized with lysates of 6 E. coli strains commonly used in recombinant technology -- antibodies with Western Blot-demonstrated multivalent specificities for the wide array of E. coli HCPs. The E. coli HCP ELISA, then, provides a broad-range, sensitive tool to conveniently and efficiently screen for the several potential contaminants that may accompany the recombinant protein during processing.

PRINCIPLE OF THE TEST

The E. coli HCP ELISA kit is based on the binding of *E. coli* HCP proteins in samples to two antibodies, one immobilized on the microwells, and the other conjugated to horseradish peroxidase (HRP) in solution. After a washing step, chromogenic substrate (TMB) is added and color is developed by the enzymatic reaction of HRP on the substrate, which is directly proportional to the amount of antigen present in the sample. Stopping Solution is added to terminate the reaction, and absorbance at 450nm is then measured using an ELISA microwell reader. The level of *E. coli* proteins in samples is determined relative to *E. coli* HCP Standards.

CALCULATION OF RESULTS (continued)

Method 2: Standard Curve



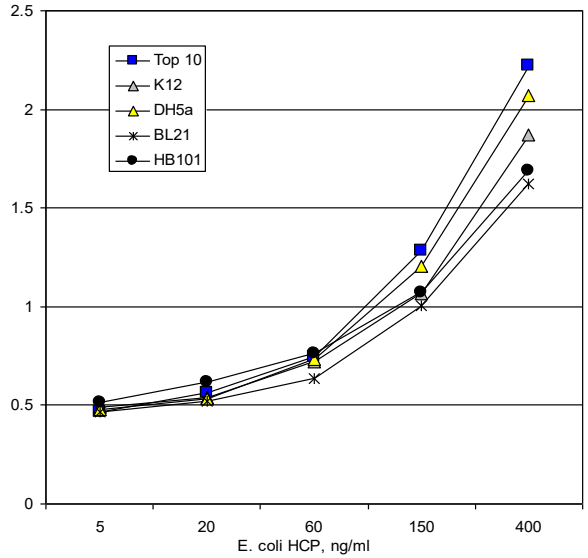
E. coli HCP concentrations in unknown samples and controls may be determined by interpolating from a standard curve, and then multiplying the values by the dilution factor to obtain HCP concentration in the original solution (see Assay Limits, page 7).

A typical dilution curve of the **Calibration Standard** (Part 800-133) is shown.

ASSAY SPECIFICATIONS

Specificity

Antibodies used for capture and detection are a blend of separate and over-lapping specificities to a wide range of E. coli HCPs, produced in multiple host species immunized with preparations containing full HCP repertoires from 5 commonly used E. coli strains. Western blots show antibody reactivities toward a broad array of proteins, and dilution curves in ELISA show similar cross-reactivity and sensitivity of the assay to each of the 5 E. coli strains.



CALCULATION OF RESULTS

Method 1: Sensitivity Threshold

Use the **Sensitivity Standard** [25 U/ml] as a precise Positive Control to represent a Limit of Detection (LOD) threshold for an assay run. Samples having an OD above the threshold would be considered as Positive for HCP.

[LOD = Mean OD of diluent only + 2 SD].

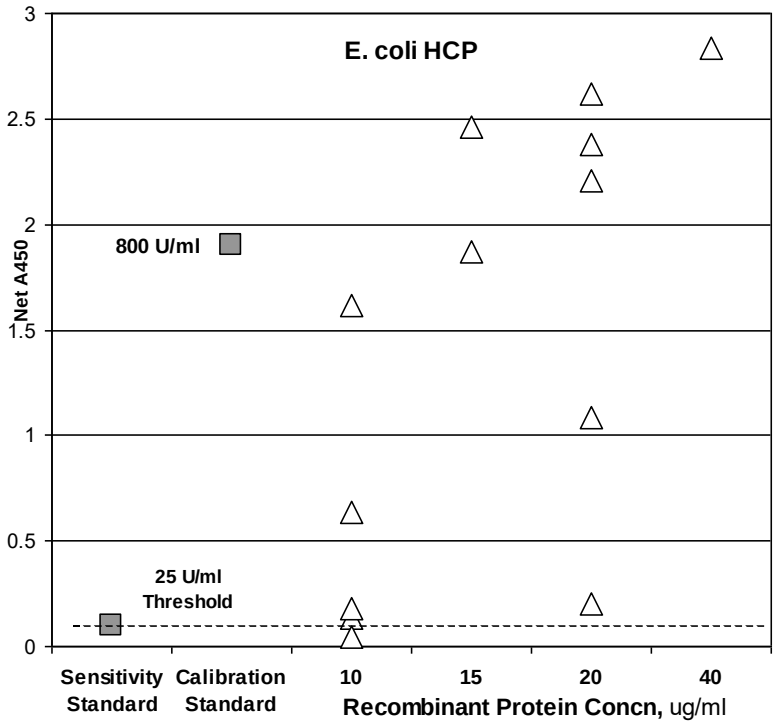
Use the **Calibration Standard** [800 U/ml], as a midrange positive control, to normalize between-assay variation of sample values.

[Sample, net OD ÷ Calibration Std, net OD]

TYPICAL RESULTS

Sensitivity Threshold

Thirteen (13) commercial recombinant proteins (his-tag) supplied for in vitro research use only were assayed at dilutions of 1:5 to 1:50. Using the 25 U/ml Sensitivity Standard OD as the threshold cut-off for positive, the assay detected clear positive HCP in 8 of the 12 proteins, with 3 proteins very near the sensitivity threshold.



KIT CONTENTS

To Be Reconstituted: Store as indicated.

Component	Instructions for Use
Sample Diluent Concentrate (20x) Cat. No. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample Diluent** and store at 2-8ºC until the kit lot expires or is used up.
Wash Solution Concentrate (100x) Cat. No. WB-100, 10ml	Dilute the entire volume 10ml + 990ml with distilled or deionized water into a clean stock bottle. Label as **Working Wash Solution** and store at RT until kit is used entirely.
E. coli HCP Standards [lyophilized]	E. coli HCP in buffer with stabilizers. Dilute in **Working Sample Diluent** according to vial labels. Use within a day or store frozen. **Calibration Standard** 800-133 2 vials [800 U/ml] **Sensitivity Standard** 800-132 2 vials [25 U/ml] The reconstituted **Calibration Standard** [800 U/ml] and **Sensitivity Standard** [25 U/ml] are positive controls demonstrating the range and reproducibility of the assay. They may be diluted to produce a Standard curve (See Assay Limits, page 6); also, reconstituting the Calibration Standard in one-half the volume indicated on the label yields a higher level standard (1600 U/ml) for increased signal. For most accurate quantitation, a calibration curve should be constructed using dilutions of the lab's identified HCP contaminated preparations, assigning concentration values in arbitrary percentage units, or another basis as desired. The Standards may be reconstituted and/or diluted in the diluent of the lab's samples in order to determine diluent effect on HCP recovery in the samples.
Anti- E. coli HCP HRP Conjugate Concentrate (100x) Part No. 800-134, 0.15ml	Anti-E. coli HCP-HRP conj. in buffer with protein, detergents and antimicrobial as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return concentrate to 2-8ºC storage.

Ready For Use: Store as indicated on labels.

Component	Part No.	Amt	Contents
Anti-E. coli HCP Microwell Strip Plate	800-131	8-well strips (12)	Coated with purified anti-E. coli HCP antibodies from multiple host species.
TMB Substrate	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	Dilute sulfuric acid.

Materials Required But Not Provided:

- Pipettors and pipettes that deliver 100ul and 1-10ml. A multi-channel pipettor is recommended.; Disposable glass or plastic 5-15ml tubes for diluting samples, and Antibody-HRP Concentrate; Grad. cylinder to dilute Wash Concentrate and Sample Diluent Concn; 200ml to 1L; Stock bottle to store diluted Wash Solution; 200ml to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength.

SAMPLE PREPARATION AND HANDLING

❑ **Caution!** The presence in the lab of preparations containing high levels of E. coli HCP may produce contamination of diluents, samples, etc., without stringent handling to avoid this issue. High blank values (A450=>0.400), poor precision, and other unexpected results may indicate HCP contamination problems. This is not a problem with the kit, and requires that the operator take extra steps to eliminate HCP contamination from the testing environment.

❑ HCPs may adsorb to glass or plastic containers/vials, especially at low concentrations. To minimize loss of this type, the WB100 Wash Solution Concentrate may be spiked into samples at 100-fold dilution. For larger volumes, addition of Tween 20 to 0.1% would be suitable.

❑ Certain constituents of a sample, e.g., high or low pH, denaturants, high salts, may alter full recovery of HCPs in the assay. These possibilities should be determined by spiking/recovery studies. Dilutions of the high standard of the kit into prospective sample matrix may be used for limited determinations of interference with HCP recovery.

❑ Perform solution-only negative control testing to ensure the compatibility of the sample solution in the assay.

QUALITY CONTROL

The Standards provided with the kit represent controls demonstrating the range for *E. coli* HCP-positive samples. Recovery as follows indicates proper assay performance:

Sensitivity Standard [800-132]: OD value above diluent only blank.
Calibration Standard [800-133]: net OD > 1.0 above Sensitivity Std.

Each lab should also assay internal control samples, which represent the lab's expected sample population and that are maintained stabilized. A Diluent only blank should also be run.

❑ The E. coli HCP ELISA is a **sequential sandwich** assay that is not susceptible to high dose hook effects (lower signal at very high HCP concentrations). Also, sample constituents that could interfere with the HRP activity, including sodium azide, are removed by washing prior to HRP Conjugate addition and are, therefore, avoided.

ASSAY PROCEDURE

Bring all reagents to room temperature (18-30° C) equilibration (at least 30 minutes).

DILUTE Samples in Working Sample Diluent according to expected HCP levels and/or trial testing.

PERFORM ALL STEPS AT ROOM TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

1. Set-up

- Determine the number of wells for the assay run. Duplicates are recommended, including 2 wells for each standard, sample and control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
- Before sample addition, add 200-300ul Working Wash Solution to each well and let stand for **15** to **30** minutes.
- Aspirate or dump the liquid and pat the plate dry on a paper towel.

2. 1st Incubation [100ul – 60 min; 5 washes]

- Add 100ul of standards, samples and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for 60 minutes.
- Wash wells 5 times and pat dry on fresh paper towels. As an alternative, an automatic plate washer may be used. Improper washes may lead to falsely elevated signals and poor reproducibility.

[Incubation may be extended for increased sensitivity; see p. 6]

3. 2nd Incubation [100ul – 60 min; 6 washes]

- Add 100ul of Working Anti-E. coli HCP-HRP Conjugate to each well.
- Incubate for 60 minutes.
- Wash wells 6 times as in step 2.

4. Substrate Incubation [100ul – 15 min]

- Add 100ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
- Incubate for 15 minutes in the dark, e.g., place in a drawer or closet.

Note: If your microplate reader does not register optical density (OD) above 2.0, incubate for less time, or read OD at 405-410 nm (results are valid).

5. Stop Step [Stop: 100ul]

- Add 100ul of Stop Solution to each well.
- Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

6. Absorbance Reading

- Use any commercially available microwell plate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.