Chemiluminescent Western Blot Kit for Detection of *E.coli* Host Cell Proteins

KCAJ07

Rockland's Chemiluminescent Western Blot Kit for Detection of *E.coli* Host Cell Proteins (HCP) combines all of the necessary reagents with a rapid proven protocol for the detection of *E.coli* HCP contamination in products manufactured by recombinant expression in *E.coli* host cells. This kit contains sufficient substrate for up to 10 mini blots at 7.5 x 8 cm^2 (1,800 cm^2) and is stable for at least 1 year when stored as indicated.

This Chemiluminescent Western Blot Kit allows for the detection of residual *E.coli* HCP protein contamination. Food and Drug Administration (FDA) specifications for large molecule biopharmaceuticals require final products to be free of host cell protein (HCP) contaminants. HCPs may be left behind during the purification process from expression hosts, such as *E.coli* cells. If HCP impurities remain in products administered to patients, contaminants can result in adverse toxic or immunological reactions. To investigate the presence of residual contamination in the final biopharmaceutical product, the usage of a polyclonal antibodies with maximum coverage against native HCP lysate provides a valuable tool to demonstrate product purity. This kit contains Rockland's proprietary anti-*E.coli* HCP antibody, validated by 2D electrophoresis workflow and shown to react with more than 300 *E.coli* proteins from SDS/DTT solubilized *E.coli* cells on a 2D Western blot assay. This kit can be used as a process development tool or routine quality control method to monitor the optimal removal of HCP contaminants.

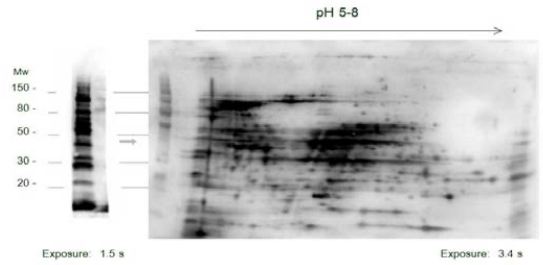


Fig 1. Western Blot. Anti-*E.coli* HCP Chemiluminescent Kit for Western blot was used to detect known amounts of bacterial HCP separated by 1D and 2D electrophoresis. After separation by SDS-PAGE proteins were transferred to a membrane which was blocked with blocking buffer (#MB-070) 1 hour at RT. The blot was probed with Anti-*E.coli* HCP antibody followed by HRP anti-Rabbit IgG (#611-1302) at 1:10,000 dilution in blocking buffer for 1 hour at RT.

I. Reagents Required

Reagent	Size	Cap Color	Notes
Anti-*E.coli* (RABBIT) Host Cell Protein Antibody (#100-401-J07)	100 µL	Red	Store at -20°C
Anti-Rabbit IgG (GOAT) Antibody Peroxidase Conjugated (#611-1302)	2 mg	Red	Store at 4°C
E.coli Host Cell Protein Control Protein (#000-001-J08)	1.5 mL	Clear	Store at -20°C
Blocking Buffer for Fluorescent Western Blotting (#MB-070-0050)	50 mL	Blue	Store at 4°C
Western Blot Incubation Box	1 Unit	N/A	N/A

Note: See Fig 2. for orientation of kit components.

II. Materials Required but Not Supplied

1. SDS-PAGE electrophoresis equipment and related materials
2. Nitrocellulose, PVDF (polyvinylidene difluoride), or other membranes for protein transfer and transfer materials
3. Microfuge tubes
4. Rocker platform for gentle mixing during incubations
5. X-ray film, cassettes, and related materials or camera-based imaging system
6. Chemiluminescent FemtoMax™ Super Sensitive HRP Substrate (#FEMTOMAX-020 or #FEMTOMAX-110)
7. Deionized water

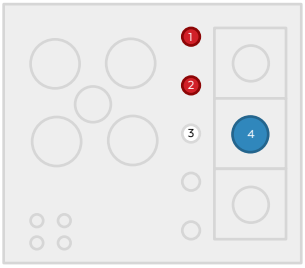


Fig 2. Component Orientation

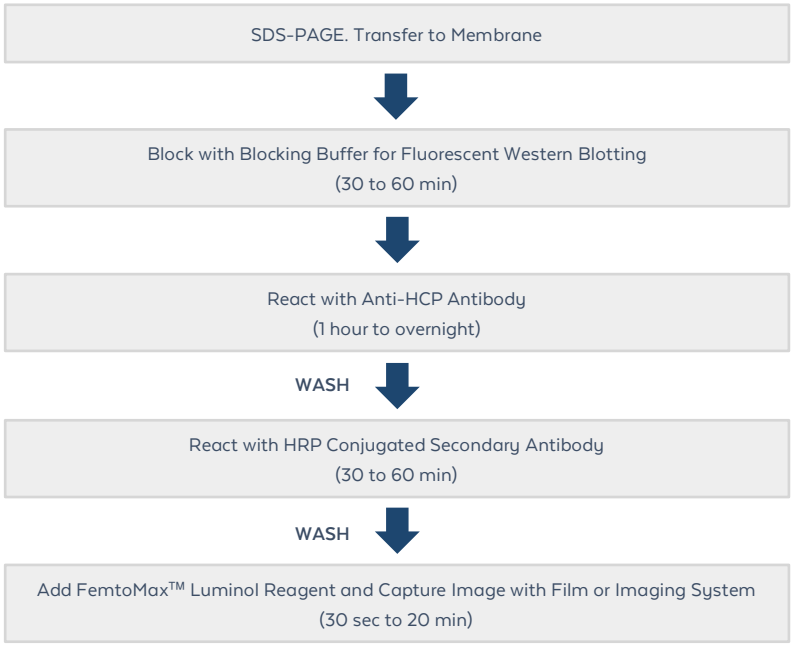
III. Storage and Stability

This kit is stable for at least one year when stored as indicated upon receipt. Individual components are stable for 3-4 weeks after dilution when stored at 4°C.

IV. Number of Assays

Components in this kit are sufficient to run approximately 10 mini blots at 7.5 x 8 cm^2 (1,800 cm^2). The amount of peroxidase conjugated secondary antibody supplied when diluted as recommended in our protocol will yield in excess of 200 mL of working solution. Adjustments in volumes for larger or smaller blots will affect the number of blots detected.

V. Flow Diagram for Chemiluminescent Western Blot Procedure



VI. Buffer Preparation

The user is to prepare the following buffers for this procedure. The exact volume of buffers required depends on the size of the membranes to be processed. We suggest preparation of 1.0 L of Tris Buffered Saline with Tween (TTBS) and 100 mL of 1x Blocking Buffer for Fluorescent Western Blotting. Prepare all solutions using ultra pure reagents and deionized (or equivalent) water. Filter the solutions and store at 4°C. Warm solutions to room temperature prior to use. Do not store solutions for more than one (1) month.

Note: Wash buffers MUST NOT contain SODIUM AZIDE or other inhibitors of peroxidase activity.

Buffer I: Tris Buffered Saline with Tween-20 (TTBS)

1. Add 800 mL of deionized water.
2. Dissolve 12.1 g of Tris base.
3. Dissolve 8.8 g Sodium Chloride (NaCl).
4. Adjust pH to 7.5 with HCl.
5. Add 1.0 mL of Tween-20 (provided).
6. Adjust volume to 1.0 L with deionized water.

VII. Preparation of Working Solutions

The Chemiluminescent Western Blot Kit for the detection of *E.coli* HCP proteins comes with a concentrated stock of anti- *E.coli* HCP antibody (primary) and peroxidase conjugated anti-Rabbit IgG antibody (secondary). Prior to use, dilute the primary antibody to a final concentration of 1µg/mL. Reconstitute and dilute the secondary antibody in a two-step process to a working solution. The working solution is ready for use. Reconstitute the peroxidase conjugated anti-Rabbit IgG antibody by adding 1.0 mL of deionized water to the RED capped vial. Mix thoroughly and maintain this stock at 4°C. For extended storage aliquot contents and freeze at -20°C or below. Avoid cycles of freezing and thawing. Prepare an intermediate dilution (1:100) by pipetting 5 µL of the stock solution to 0.5 mL of Buffer I (TTBS) in a clean microfuge tube. Prepare a second dilution (1:200) by pipetting 50 µL of the intermediate solution in a clean tube. Add 10 mL of Buffer I (TTBS). Mix thoroughly. The final dilution will contain 50 ng/mL peroxidase conjugated goat anti-Rabbit IgG antibody and will represent a 1:20,000 dilution of the stock solution.

VIII. Western Blot Method

The following method is suggested as a guideline for the use of Rockland's Chemiluminescent Western Blot Kit allows for the detection of *E.coli* HCP proteins. Each researcher must optimize Western blotting conditions for their protein of interest. Membranes composed of nitrocellulose or PVDF can be used. Nylon membranes may also be used (see "Additional Notes"). After your antigen has been immobilized onto the membrane by transfer, dotting or filtration, follow the numbered steps below to process the blot. All reactions occur at room temperature. Use a rocking platform set at low speed for gentle agitation. Always add enough solution to cover the membrane. Never let a membrane air dry during this process. Add the suggested volumes or just enough volume to cover the membrane to keep it wet. Do not touch the membrane with your skin!

Note: Some antigens in combination with the anti- *E.coli* HCP antibody may require specific conditions other than those stated below. If so, use these recommendations as a starting point for further optimization.

1. After transfer is complete, block the membrane by immersing in (1x) Blocking Buffer for Fluorescent Western Blotting (provided) and incubate at room temperature for 1 hour with gentle agitation.
   Note: See "Additional Notes" for other blocking agents that may be used.
2. Aspirate or decant the blocking solution
   Note: Aspirate using a glass pipette attached to a vacuum. Alternatively, the solution may be poured off away from the blot.
3. Immediately add 5 ml of anti-*E.coli* HCP antibody (primary) solution diluted in (1x) Blocking Buffer for Fluorescent Western Blotting to the membrane. The appropriate dilution should be determined by the end user. We would recommend a starting dilution of 1:500. Greater dilutions often result in lower backgrounds but may require longer incubation times.
4. Incubate for 1 h at room temperature with gentle agitation. If desired, the membrane can be incubated with of anti- *E.coli* HCP antibody (primary) overnight at 4° C.
5. Aspirate or decant the anti- *E.coli* HCP antibody (primary) solution.
6. Wash the blot 3x with Buffer I for 5 minutes each with gentle agitation.
   Note: Increasing the wash buffer volume or the number of washes may decrease background.
7. Aspirate or decant the wash solution and add 5 mL of the "Diluted Secondary Antibody" solution (see above for preparation).
8. Incubate for 1 hour at room temperature with gentle agitation.
9. Aspirate or decant secondary antibody solution.
10. Wash the blot as in Step 3.
11. Prepare chemiluminescent reagent just prior to use. Transfer blot to incubation box or film cassette then add 0.75 to 1.0 mL of freshly prepared chemiluminescent reagent to the membrane (7.5 x 8.0 cm). Transfer blot to incubation box then add 0.75 to 1.0 mL of freshly prepared FemtoMax reagent to the membrane (7.5 x 8.0 cm).
12. Immediately visualize the membrane by exposing X-ray film or by other imaging methods, including CCD camera-based imaging systems. For film, expose and process the film according to the manufacturer's instructions. Cover the blot with clear plastic wrap or equivalent and remove any excess liquid and any air bubbles to reduce imaging artifacts. Start with a 60 sec exposure. Exposure times may be varied for best results. For imaging systems, follow the manufacturer's instructions and vary exposure times and/or binning for best results. The length of time required to achieve optimum signal varies greatly depending on several factors. Incubation times can range from 30 sec to 20 min or more.

IX. Additional Notes

- The methods given in these instructions are to be used as a guideline. Experienced users can make deviations from the stated method. Solutions have been optimized for the stated method and any change in reagent concentration, volume, reaction time or temperature will affect the overall performance of the kit. Generally, if a variable is to be modified, only alter one condition at a time.

- Nylon membrane is more difficult to block and may result in higher levels of non-specific staining. Using 10% BLOTTO (non-fat dry milk) (#B501-0500) is suggested to block nylon membranes. Allow the blocking step to proceed for several hours to overnight at 37° C. Do not use Tween-20 when using nylon membranes.
- The blocking of membranes for western blotting can be accomplished with (1x) Blocking Buffer for Fluorescent Western Blotting only. If other Blocking buffers are used, we suggest using BSA or other blocking agent, such as 5% normal goat serum, 3% fish gelatin, or other commercially available blocking agent, depending on previous experience to lower non-specific staining.
- Always use enough solution to cover the membrane. Never let the membrane dry during the process.
- Protect the chemiluminescent reagent from light. Precise optimization is required to achieve maximum signal detection including optimizing the membrane, blocking conditions, antigen and antibodies. Detection by Rockland's FemtoMax chemiluminescent reagent requires much less sample and antibody than most commercially available ECL substrates. Use the same blotting conditions for FemtoMax chemiluminescent reagent as you would for Amersham ECL Plus™ Substrate or Pierce SuperSignal® West Femto Substrate.
- Western blots can be repeatedly exposed to X-ray film to obtain optimal results or stripped of detection reagents and reprobed.
- Use care not to touch the membrane with your skin! Wear gloves. Make certain that all equipment used in the process is free of foreign material.
- Unless specified differently, all reactions occur at room temperature.
- Use a rocking platform set at low speed for gentle agitation for all incubation steps.
- Solutions containing sodium azide or other inhibitors of peroxidase activity should not be used to dilute the secondary antibody, substrate or any other FemtoMax™ chemiluminescent reagent.
- A positive control *E.coli* protein is provided in a WHITE capped vial for use as a positive control of the primary antibody. Spot 1 to 10 µg as a control on your Western or dot blot prior to the blocking step.

X. Troubleshooting Guide

Table 3. Common Troubleshooting Techniques

Issue	Resolution	Solution
Little or no signal	**1.** Incomplete transfer of proteins	**1.** Follow all protocols included with your transfer apparatus. Check for the presence of transferred proteins using India ink stain as described in Reference 1.
	2. Poor binding of anti-*E.coli* HCP antibody (primary)	**2.** Use provided control cell lysate protein in serial dilution as a control. Decrease the dilution (increase the concentration) of anti-*E.coli* HCP antibody. Increase the incubation time of anti- anti-*E.coli* HCP antibody from 30 minutes to several hours or overnight. Increase the incubation temperature to 37°C.
	3. Inactive Peroxidase Conjugate	**3.** Be certain that all buffers are free of sodium azide, which is a strong inhibitor of peroxidase activity.
Multiple Signals	**1.** Too much protein on the blot	**1.** Verify the concentration of your protein sample, using Bradford or BCA reagent. For best results, load approximately 50-100 g of total protein (lysate) per lane.
	2. Too high concentration of anti-*E.coli* HCP antibody	**2.** Increase the dilution of anti-*E.coli* HCP antibody solution.
	3. Overexposure of signal	**3.** Decrease exposure time of film or decrease settings on camera system to decrease the signal from minor bands
High background / Poor signal-to-noise ratio	**1.** Insufficient blocking	**1.** Be certain blocking buffer has been properly prepared. Use other blocking agents. In most cases, the addition of 1.0% BSA will decrease background over the use of TTBS alone. In some cases, increased concentrations of BSA (up to 5%) are necessary.
	2. Insufficient Washing	**2.** Increase the number of wash steps and the volume of TTBS used for each wash.

XI. References

1. *Antibodies, A Laboratory Manuel.* Ed Harlow and David Lane, eds. Cold Spring Harbor Press. 1988. Chapter 12 gives an excellent overview of Western Blotting techniques, including India Ink staining.
2. *Current protocols in Molecular Biology*. J. Ausebel, et al, eds. John Wiley and Sons, New York. Gives a complete protocol of Western Blotting and Dot Blotting.
3. *Molecular Cloning: A Laboratory Manuel.* 2nd Edition. J. Sambrook, E.F. Fritsch and T. Maniatis, eds. Cold Spring Harbor Press, 1989. Chapter 18 gives detailed protocols for both the production of cell lysates and electrophoresis and blotting of proteins.
4. *Antibodies, A Practical Approach.* 2nd Edition. Catty, D., ed. IRL Press, Oxford, England. 1990. Volumes I and II represent a detailed and complete reference for most current antibody technique