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Protein Detector™ HRP ELISpot Kit

Catalog No. 40-00-05

INTRODUCTION

The ELISpot (Enzyme Linked Immuno-Spot) assay is a powerful method for the detection and visualization of single cells secreting a protein or cytokine of interest. Derived from the widely used ELISA technique, ELISpot protocols use similar reagents and procedures. The technique is most commonly used in vaccine development studies to determine if a vaccine or other treatment has elicited the desired immune response in a cell. The use of the ELISpot application has increased in recent years because of it's ability to identify individual cells, ex-vivo, actively secreting cytokines or other proteins in the one-in-one million cell range. Additionally, antigen-specific antibodies used in ELISpot provide a detection mechanism that is highly specific for the precise cytokine of interest. The high specificity of this assay combined with its sensitivity makes ELISpot an attractive method for detection of specific protein secreting cells.

PRINCIPLE OF THE PROTEIN DETECTOR HRP ELISPOT KIT

The Protein Detector HRP ELISpot kit is designed to provide a comprehensive, flexible system for conducting ELISpot assays with any matched pair of antibodies. This kit provides optimized reagents and a flexible protocol, adaptable for many different antibody pairs. For ELISpot detection, an unlabeled antibody (ex. anti-cytokine) is coated onto a PVDF microplate. Appropriately stimulated cells are added to the wells and incubated for a period of time. During incubation, the immobilized antibody binds the cytokine secreted from the cells. Cells and unbound substances are washed away and a biotinylated antibody reactive with a different epitope of the cytokine is added. After incubation and another wash step, HRP-labeled streptavidin is added. After a final wash step, TrueBlue™ Peroxidase substrate is utilized to visualize where cytokine has been bound. Each spot represents an individual secreting cell. Spots may be read manually with a microscope or with an automated ELISpot reader.

Protein Detector ELISpot Kits provide all solutions required for blocking, washing and detection; users need to provide the protein-specific, matched antibody set (unlabeled antibody for capture and biotinylated antibody for detection). The HRP-streptavidin conjugate provided in this kit is compatible with any biotinylated antibody, therefore, the kit reagents are universal in nature and easily interchangeable with any matched antibody pair. For added-value, each kit contains five un-coated 96-well, PVDF plates that have been tested and optimized for compatibility with other kit reagents.

MATERIALS AND EQUIPMENT

Kit Component	Catalog Number	Volume
MultiScreen® Immobilon-P ELISpot (PVDF) 96-well Plate	61-00-05	5 plates
Wash Solution Concentrate (20X)	50-63-01	1 x 200 mL
10% BSA Diluent/Blocking Solution	50-61-25	1 x 25 mL
Coating Solution Concentrate	50-84-01	1 x 25 mL
Peroxidase-Labeled Streptavidin	474-3003	1 x 1 mL
TrueBlue Peroxidase Substrate	71-00-68	1 x 50 mL

Sufficient reagents are provided to test approximately 5 plates when using recommended volumes. Store the TrueBlue Peroxidase Substrate at room temperature and the remaining kit components at 2 – 8°C. Reagents are stable for a minimum of one year when stored as directed.

TrueBlue may appear clear to light blue. Product stability and performance are not affected by variations in solution color. Discard solution if it becomes turbid.

Kit solutions are not provided as sterile reagents and will require filtration to ensure sterility. See page 5 for buffer preparation and sterilization.

REQUIRED SUPPLIES AND EQUIPMENT NOT INCLUDED

- Unlabeled Capture Antibody
- Biotinylated Detection Antibody
- Ethanol or Methanol
- 0.22 µm Filter
- Microscope or ELISpot Plate Reader
- 37°C Incubator

OPTIONAL SUPPLIES

- Membrane Removal – ELI-Puncher Kit (Millipore # MELIPUNCH)

PROTEIN DETECTOR ELISPOT
AT A GLANCE

Plate Preparation (Day 1): 5.5 hours
Cell Incubation Time (Day 1) Overnight
Detection Time (Day 2): 8 – 12 hours

Prewet PVDF membrane
5 minutes
⇓
Coat with Unlabeled Capture Antibody
4 hours or overnight
⇓
Wash
5 minutes
⇓
Block
1 hour
⇓
Incubate cells
Overnight
⇓
Wash
5 minutes
⇓
Incubate Biotinylated Detection Antibody
1 hour
⇓
Wash
8 minutes
⇓
Incubate HRP-streptavidin
30 minutes
⇓
Wash
8 minutes
⇓
Develop with TrueBlue Substrate
10 minutes
⇓
Rinse
5 minutes
⇓
Air Dry Membrane
6 – 12 hours
⇓
Determine Number of Spots

NOTE ON…Warnings and Precautions

⇒ Read ALL instructions thoroughly before using the kit.

⇒ Always wear protective gloves and a lab coat for personal protection, as well as protection of the membrane and immunoassay reagents from contaminants such as skin oils or proteins.

⇒ For proper analysis of results, always include positive and negative controls, blanks and/or protein standards as appropriate.

PROTOCOL

BUFFER PREPARATION

Sufficient reagents are provided in the Protein Detector™ HRP ELISpot Kit when volumes are used as indicated. If desired, increased working volumes may be used; however, additional reagents will be necessary. Instructions for preparation of working solutions are provided below.

Working solutions can be made in bulk and stored at 2 – 8°C for up to 3 months. Kit solutions are not provided as sterile reagents. See instructions below for sterilization procedures.

1X Coating Solution

- Dilute 10X Coating Solution Concentrate 1/10 in deionized water.
- Sterilize 1X solution through filtration (0.22 µm) or autoclaving.

1X BSA Diluent/Blocking Solution

- Dilute 10X BSA Diluent/Blocking Solution 1/10 in deionized water.
- Sterilize 1X solution through filtration (0.22 µm). Do not autoclave BSA Solution.

1X Wash Solution

- Dilute 20X Wash Solution Concentrate 1/20 in deionized water.
- Sterilize 1X solution through filtration (0.22 µm) or autoclaving.

DETAILED PROCEDURES

Note: This protocol is offered by KPL as a general guideline for conducting ELISpot assays with the provided kit reagents and user-provided antibodies. Due to the nature of the assay system, each antibody pair may require a different set of conditions. It is highly recommended to optimize assay conditions based on each antibody pair.

STEPS	CRITICAL POINTS
1. Prewet PVDF membrane by adding approximately 50 µL of 70% ethanol or methanol to each well. Incubate for approximately 1 minute and wash 3 times with 100 µL/well of 1X Coating Solution.	*1. Dry PVDF membranes will not get wet unless an organic such as alcohol is used. Once wet, aqueous buffers can be used as long as the membrane is not dried again. It would take a multi-hour incubation of the membrane with no buffer in it to cause dryness.* *See Buffer Preparation above for instructions on how to prepare 1X Coating Solution.*
2. Coat wells with 100 µL of unlabeled capture antibody diluted in 1X Coating Solution for 4 hours at room temperature or overnight at 2 – 8°C.	*2. The amount of antibody needed must be determined for each antigen being tested. 5 - 10 µg/mL is a good starting range. Alternatively, use the recommended concentration from the antibody supplier.*
3. Wash wells with 100 µL of 1X Wash Solution. Empty wells and repeat 3 times.	*3. See Buffer Preparation for instructions on how to prepare the 1X Wash Solution.*
4. Block wells with 100 µL of 1X BSA Diluent/Blocking Solution. Incubate for 1 hour at room temperature.	*4. See Buffer Preparation for instructions on how to prepare the 1X BSA Diluent/ Blocking Solution. Cell media can be used as an alternative to the kit supplied BSA Diluent/Block Solution.*
5. Remove blocking buffer and add 100 µL of appropriately stimulated cells. Incubate 18 - 24 hours at 37°C under appropriate tissue culture conditions.	*5. Remove blocking media but do not wash wells. Best distribution of cells occurs when pipette tip is in the middle of the well. The time of incubation will depend on the rate of secretion of the target being measured, but overnight is a good starting point. As soon as the cells have been added, put the plate in the incubator and do not disturb it. If the plate is shifted, the spots will be disrupted. The optimal amount of cells plated will need to be determined for each individual assay through a serial dilution of cell concentrations. 100,000 cells/well is typically a good starting point. Incubation times may need to be altered up to 36 – 48 hours depending on the cell line.*

STEPS	CRITICAL POINTS
6. Wash wells with 100 µL of 1X Wash Solution. Empty wells and repeat 3 times.	
7. Add 100 µL biotinylated detection antibody diluted in 1X BSA Diluent/Blocking Solution. Incubate for approximately 1 hour at room temperature.	*7. The amount of antibody required must be determined for each antigen. 1 - 2 µg/mL is a good starting point. Alternatively, use the recommended concentration from the antibody supplier. The time and temperature of incubation can be varied and must be determined for each system. In general the higher the temperature the less time it will take to have the reaction occur.*
8. Wash wells with 100 µL of 1X Wash Solution. Empty wells and repeat 5 times.	
9. Add 100 µL HRP-streptavidin diluted approximately 1/500 in 1X BSA Diluent/Blocking Solution. Incubate 30 minutes at room temperature.	*9. The amount of HRP-streptavidin required may vary. If the membrane has an overall blue cast to it after 10 minutes of incubation with TrueBlue, the amount of HRP-streptavidin may be lowered or the number of washes in Step 8 should be increased.*
10. Wash wells with 100 µL of 1X Wash Solution. Empty wells and repeat 5 times.	
11. Add 50 µL TrueBlue Substrate and incubate for approximately 10 minutes at room temperature.	*11. The development of color can be monitored through a stereomicroscope. If background color starts to develop on the membrane, stop the reaction, as signal to noise will not improve. It is not necessary to wash with PBS after using the wash buffer as TrueBlue is not inhibited by Tween containing buffers.*
12. Wash wells with distilled water. Empty wells and repeat 5 times.	*12. The amount of water is not critical. A squirt bottle is satisfactory, but do not apply with strong force directly on the membrane.*
13. Air dry membrane 6 - 12 hours at room temperature for best viewing.	*13. After drying, membranes may be stored in the dark for several months.*
14. Count spots using a microscope or an ELISpot Plate Reader.	

TROUBLESHOOTING GUIDE

The following guide presents some of the common problems, possible causes and corrective actions associated with the ELISpot application.

PROBLEM 1: NO SIGNAL OR WEAK SIGNAL

Possible Cause	Corrective Measure
• Inadequate amount of capture or detection antibody or HRP-Streptavidin	*Test capture and detection antibodies at higher concentrations. Test HRP-Streptavidin at a lower dilution (dilution lower than 1/500 is not likely to help).*
• Over blocking	*Dilute BSA Diluent/Block to 1/15 or 1/20 instead of the recommended 1/10 dilution.*
• No cell stimulation	*Treat cells gently. Vigorous pipetting leads to cell death.* *Per reference 1, stimulate C3H/J mouse spleen cells with ConA at 2 µg/mL and culture at 1 x 10^6/mL in RPMI 1640 for 24 hours. Analyze cells using protocol described in this manual.*
• Insufficient incubation time for capture/detection antibodies or HRP-Streptavidin	*Coat with unlabeled capture antibody overnight. Incubate with biotinylated detection antibody for 2 hours. Incubate HRP-Streptavidin for 1 hour. Incubation times longer than those described here are not likely to increase the signal.*
• Wrong choice of antibody pairs	*Insure that the capture and detection antibodies react with different epitopes of the antigen being detected.*
• Missed step in procedure	*Double-check that correct ELISpot protocol was followed.*
• Reagents impacted by improper temperatures	*Insure that "room temperature" is actually 25°C and that all reagents have come to room temperature before use.*
• Excessive washing beyond recommended procedure	*Be sure the washing procedures were followed as written.*

PROBLEM 2: HIGH SIGNAL OR BACKGROUND

Possible Cause	Corrective Measure
• Excess detection antibody or HRP-Streptavidin	*Excess capture antibody is not likely to lead to background. Over abundant biotinylated detection antibody or HRP-Streptavidin is more likely to contribute to background. Reduce concentration of detection antibody and HRP-Streptavidin.* *If a generalized blue color develops over the entire membrane within 10 minutes, either the detection antibody or HRP-Streptavidin is in excess or not enough blocking has occurred. It is often very difficult to determine the background while the membrane is still wet. Dry thoroughly overnight before assessing the amount of background.*
• Non-specific spots due to protein aggregates	*Filter biotinylated antibody and/or labeled streptavidin through a 0.22 µm filter.*
• Insufficient blocking or washing, causing non-specific reaction	*The wash buffer and protocol provided should be sufficient to prevent background. The most important wash steps are after the detection antibody and after the HRP-Streptavidin.*
• Excess color development with substrate	*10 – 15 minutes should be sufficient to visualize spots with TrueBlue. Incubation past 20 minutes may result in high signal and background color. Signal development times may vary depending on assay conditions.*

PROBLEM 3: TOO MANY SPOTS OR FUZZY SPOTS

Possible Cause	Corrective Measure
• Too many cells per well	*Dilute cells before adding to well. Results of more than 100 spots per well will be difficult to quantitate or to insure distinction between spots.*
• Plates moved during incubation	*If plates are disturbed during incubation, the cells may move resulting in a large distorted spot.*

PROBLEM 4: OVERABUNDANCE OF CELLS TOWARDS PERIMETER OF WELL

Possible Cause	Corrective Measure
• Pipetting cells toward edge of well	*Pipette cells directly into center of each well.*

REFERENCES

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RELATED PRODUCTS

Product	Size	Catalog No.
TrueBlue Peroxidase Substrate	50 mL	71-00-64
HRP-labeled Streptavidin	1.0 mL	474-3000
10% BSA Diluent/Blocking Solution	2 x 100 mL	50-61-00
Coating Solution Concentrate	2 x 25 mL	50-84-00
Wash Solution Concentrate	4 x 200 mL	50-63-00

Note: The recommendations of this bulletin are provided solely for the benefit of users who need practical guidance on immunoassay procedures. Experimental conditions for the use of the suggested products are beyond the control of KPL, Inc., therefore it is impossible for KPL to implicitly guarantee the performance of the mentioned products for any and all assay procedures. Users who need additional information are encouraged to call Technical Services at 800/638-3167 or 301/948-7755. Alternatively, visit our web site at www.kpl.com for assistance.

PRODUCT SAFETY AND HANDLING

See MSDS (Material Safety Datasheet) for this product.

NOTES