BD™ ELISPOT

ELISPOT Set

Instruction Manual

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Introduction

The enzyme-linked immunospot (ELISPOT) assay is a powerful tool for detecting and enumerating individual cells that secrete a particular protein *in vitro*.[1] Based on the sandwich enzyme-linked immunosorbent assay (ELISA), the ELISPOT assay derives its specificity and sensitivity by employing high affinity capture and detection antibodies and enzyme-amplification. Although originally developed for analyzing specific antibody-secreting cells,[2,3] the assay has been adapted for measuring the frequencies of cells that produce and secrete other effector molecules, such as cytokines.[4,5,6] The sensitivity of the assay lends itself to measurement of even very low frequencies of analyte-producing cells (eg, 1/300,000).[1] Recent developments in assay plate design and in ELISPOT plate-reader instrumentation have significantly improved the utility of the ELISPOT method for objective and rapid analysis of analyte-producing cells.[1]

Advantages and Relevance of the Assay

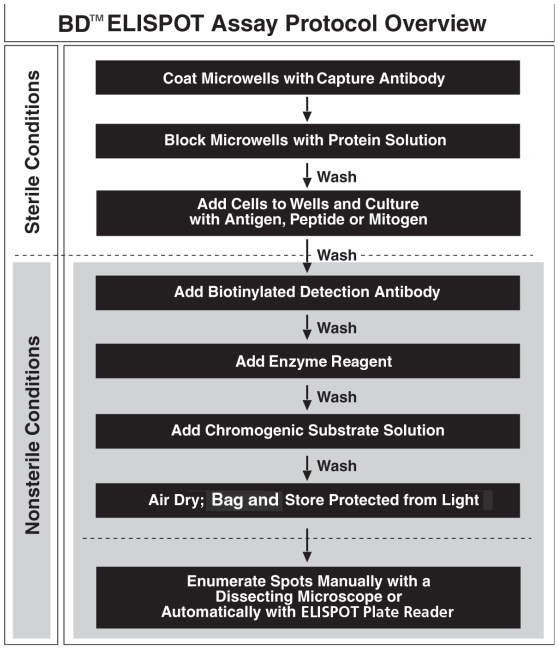
Unique strengths of the assay include the following:

- High sensitivity
- High throughput, high content analysis
- Minimal volume of biological material required
- Applicability to frozen/thawed biological samples
- Compatibility with other assays. For example, cells analyzed by BD™ ELISPOT can be transferred for cloning, proliferation assays, flow cytometry, or other methods of analysis.

The BD™ ELISPOT assay may be applied within many areas of biological research, including the following:

- Transplantation
- Vaccine development
- Th1/Th2 analysis
- Autoimmunity
- Cancer
- Allergy
- Infectious disease
- Epitope mapping
- Humoral immunity

Assay Overview



BD™ ELISPOT Set Contents

- 10 ELISPOT plates
- Unlabeled Capture Antibody (no azide/low endotoxin format); sufficient reagent for coating 10 plates
- Biotinylated Detection Antibody; sufficient reagent for 10 plates
- Enzyme Conjugate (Streptavidin-HRP); sufficient for 10 plates
- Certificate of Analysis, providing lot-specific optimal reagent concentrations

Warnings and Precautions

1. The Detection Antibody contains less than 0.1% sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.
2. The enzyme conjugate contains 2.5% BSA. All serum proteins are from USDA-inspected abattoirs located in the United States.

Assay Protocol

Note: Use ELISPOT plates and reagents under aseptic conditions (eg, in laminar flow hood) for Steps 1 – 7. Solutions noted with an asterisk (*) are described in the ***Buffers and Reagents*** section on page 7.

Coating Antibody

1. Dilute capture antibody in Coating Buffer* (see ***Certificate of Analysis*** provided with each BD™ ELISPOT Set for antibody dilution information). Add 100 µl of diluted antibody solution to each well of an ELISPOT plate.
2. Store plates at 4ºC overnight.

Blocking

3. Discard Coating Antibody. Wash wells 1× with 200 µl/well Blocking Solution*.
4. Add 200 µl/well Blocking Solution and incubate for 2 hr at room temperature.

Cell Activation

5. Discard Blocking Solution. Prepare mitogen or antigen, diluted in complete medium (eg, RPMI 1640 with FBS, Pen/Strep, and L-glutamine). Add 100 µl/well to ELISPOT plate.
6. Prepare cell suspensions at different densities, (eg, 1×10^5cells/ml - 2×10^6 cells/ml). Add 100 µl/well of each cell suspension to ELISPOT plate microwells.
7. Replace lid. Incubate ELISPOT plate at 37ºC, in a 5% CO_2 and humidified incubator. The duration of the incubation time will vary (eg, 2 hr – 24 hr). Specific activation conditions will vary, depending on cell type, kinetics, and analyte of interest. Please see ***Certificate of Analysis*** provided with each BD™ ELISPOT Set for assay conditions, suggested cell types, and incubation times of suggested positive controls. After step 7, aseptic conditions are no longer needed.

   Note: Cells may be diluted in a regular tissue culture plate starting at 10^5/well in triplicate wells with 1:3 or 1:4 serial dilutions down the plate, then transferred to the ELISPOT plate.

Detection Antibody

8. Aspirate cell suspension. Wash wells 2× with deionized (DI) water. Allow wells to soak for 3 – 5 min at each wash step.
9. Wash wells 3× with 200 µl/well Wash Buffer I*. Discard Wash Buffer.
10. Dilute Detection Antibody in Dilution Buffer* (see ***Certificate of Analysis*** for antibody dilution information). Add 100 µl per well.
11. Replace lid and incubate for 2 hr at room temperature.

Enzyme Conjugate

12. Discard Detection Antibody solution. Wash wells 3× with 200 µl/well Wash Buffer I. Allow wells to soak for 1 – 2 minutes at each wash step.
13. Dilute Enzyme Conjugate (Streptavidin-HRP) in Dilution Buffer*. (see ***Certificate of Analysis*** for dilution information). Add 100 µl/well diluted enzyme reagent.
14. Replace lid; incubate for 1 hr at room temperature.

Substrate

15. Discard enzyme conjugate solution. Wash wells 4× with 200 µl/well Wash Buffer I. Allow wells to soak for 1 – 2 minutes at each wash step.
16. Wash wells 2× with 200 µl/well Wash Buffer II*.
17. Add 100 µl of Final Substrate Solution* to each well. Monitor spot development from 5 – 60 min. Do not allow spots to overdevelop, as this will lead to high background.
18. Stop substrate reaction by washing wells with DI water.
19. Air-dry plate at room temperature for 2 hr or overnight until it is completely dry. Removal of plastic tray under plate will facilitate drying. Store plate in a sealed plastic bag in the dark, until it is analyzed.
20. Enumerate spots manually by inspection under a dissecting microscope or automatically using an ELISPOT plate reader.

Buffers and Reagents

1. Coating Buffer (1× Phosphate Buffered Saline [PBS]): 8 g NaCl; 0.2 g KCl; 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4; H_20 to 1 liter. Adjust pH to 7.2, autoclave or sterile-filter and store at 4°C.
2. Blocking Solution: Cell culture medium (ie, RPMI 1640) containing 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin–L-Glutamine (Gibco-BRL No. 10378-016).
3. Wash Buffer I: 1× PBS containing 0.05% Tween-20 (0.5 ml Tween-20 per 1 L PBS).
4. Wash Buffer II: 1× PBS
5. Dilution Buffer: 1× PBS containing 10% FBS.
6. Substrate Solution: BD™ AEC Substrate Reagent Set (Cat. No. 551951) is recommended. Alternatively, substrate solution may be prepared as follows:
   a. Prepare AEC (3-amino-9-ethyl-carbazole; Sigma A-5754) stock solution: 100 mg AEC in 10 ml DMF (N,N-Dimethylformamide; Sigma D-4551).

   Caution: Dispense DMF in fume hood. Store solution in glassware.

   b. Prepare 0.1 M Acetate Solution: add 148 ml of 0.2 M acetic acid/glacial acidic acid to 352 ml of 0.2 M sodium acetate. Adjust volume to 1 L with water; adjust pH to 5.0.
   c. For Final Substrate Solution, add 333.3 µl of AEC stock solution to 10 ml 0.1 M Acetate Solution. Filter through 0.45 µm filter. Add 5 µl of H_20_2 (30%) and use immediately.

BD™ ELISPOT Technical Tips

1. Take care not to puncture the membrane on the bottom of the ELISPOT plate wells. The membranes in the ELISPOT microwell plates are fragile; do not touch the bottom of the wells with the ends of the pipet tips when adding cells or reagents and when washing plates.
2. To identify the optimal cell concentrations for ELISPOT analysis, use a wide range of cell concentrations (eg, 10^3 – 10^6 cells per microwell) in the first experiment.
3. Do not disturb the incubator or ELISPOT plate during the cell culture process to avoid streaks and ambiguous spots.
4. Do not stack the plates in the incubator. Place each ELISPOT plate individually on the shelf to allow an even distribution of heat to each microwell and to avoid edge effects.
5. High background in blank wells (ie, strong red color) can sometimes be overcome by performing the following steps properly:
   - Stringency of washes with PBS-Tween—follow washing instructions carefully. One or more additional washes may be necessary.
   - Soaking and washing the plate with PBS prior to adding substrate. Tween-20 from the wash buffer can interfere with the substrate development, causing high background.
   - If using a substrate other than the one recommended and optimized for BD™ ELISPOT reagents, the detection antibody and the enzyme conjugate concentrations must be optimized by the researcher for best results.
   - Dry the plate longer if necessary. The speed at which the plate completely dries depends on the relative humidity in the environment.
   - Wash cells thoroughly prior to the experiment to avoid the carryover of natural cytokines made by the cells in a preliminary culture or of recombinant cytokines that have been added exogenously.
   - Monitor the substrate development carefully. Do not overdevelop.
6. After completion of the experiment, do not dry the microplate at a temperature higher than 37°C; this may cause cracking of the membrane filters.
7. Store color-developed, dried plates in a sealed plastic bag protected from light to avoid color reduction that can be caused by air or light.
8. When scanning a plate in an ELISPOT plate reader, make sure the plate is completely inserted into the base.

Sample Data

A.

B.

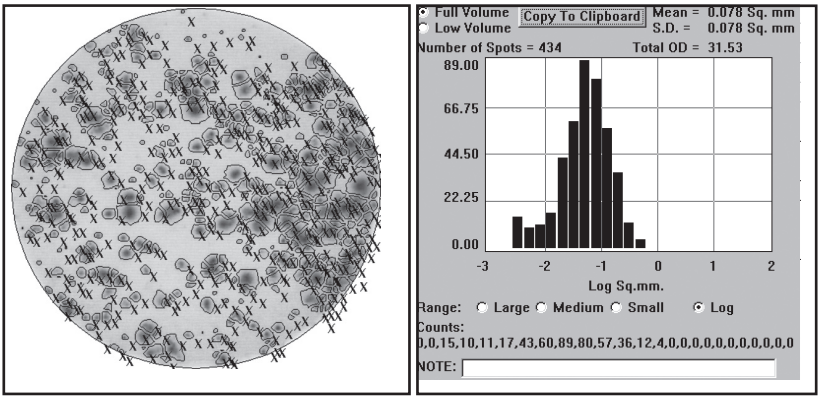


Figure 1. BD™ ELISPOT analysis of human IL-5-producing cells. Primed human PBMCs were restimulated (4 hr) with PMA and ionomycin in the microwell of an ELISPOT plate that was precoated with the NA/LE anti-human IL-5 (5 µg/ml). Biotinylated anti-human IL-5 (2 µg/ml) was used to detect the captured IL-5. Spots were visualized using SAv-HRP enzyme and AEC substrate, followed by image analysis and spot enumeration, as shown in Panel A. The spot size distribution of the PMA and ionomycin-induced response, as measured by image analysis, is shown in Panel B.

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