



BD™ ELISPOT

ELISPOT KIT

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.

Advantages and Relevance of the Assay

Unique strengths of the assay include the following:

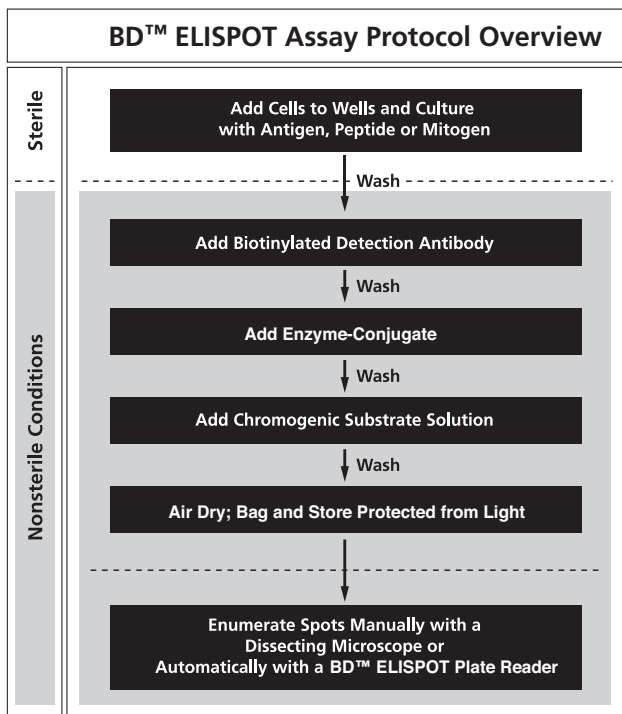
- High sensitivity
- High throughput, high content analysis
- Minimal volume of biological material required
- Applicability to frozen/thawed biological samples
- Compatibility with other assays. For example, cells analyzed by BD™ ELISPOT can be transferred for cloning, proliferation assays, flow cytometry,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

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Assay Overview



BD™ ELISPOT Kit Contents

- Plates: 2 Pre-coated BD™ ELISPOT plates
- Biotinylated Detection Antibody
- Enzyme Conjugate (Streptavidin-HRP)
- Assay Diluent
- Wash Concentrate (20×)
- PBS (10×)
- AEC Substrate Buffer
- AEC Chromogen
- Certificate of Analysis, with lot-specific optimal dilution instructions

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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. **Warning**

Wash Concentrate (component 51-9003738) contains 0.002% (w/w), Assay Diluent (component 51-2572KC) contains 0.003% (w/w) and Streptavidin-HRP (component 51-9000229) contains 0.004% of a CMIT/MIT mixture (3:1), which is a mixture of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC No 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC No 220-239-6] (3:1).

Hazard statements

May cause an allergic skin reaction.

Precautionary statements

Wear protective gloves / eye protection.

Wear protective clothing.

Avoid breathing mist/vapours/spray.

If skin irritation or rash occurs: Get medical advice/attention.

IF ON SKIN: Wash with plenty of water.

Dispose of contents/container in accordance with local/regional/national/international regulations.

3. **Warning**

AEC Substrate (component 51-2577KD) contains 7.99% methanol (w/w).

Hazard statements

Combustible liquid.

Harmful if swallowed.

May be harmful in contact with skin.

May cause damage to organs.

Precautionary statements

Wear protective gloves / eye protection.

Wear protective clothing.

Avoid breathing mist/vapours/spray.

IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.

IF SWALLOWED: Call a POISON CENTER/doctor if you feel unwell.

IF ON SKIN: Wash with plenty of water.

4. Danger

AEC Chromogen (component 51-2578KD) contains 97.8% N,N-dimethylformamide (w/w).

Hazard statements

Flammable liquid and vapour.

Harmful if inhaled.

Causes serious eye irritation.

May damage fertility or the unborn child.

Precautionary statements

Keep away from heat/sparks/open flames/hot surfaces. - No smoking

Wear protective clothing/ eye protection.

Wear protective gloves.

IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.

IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do.

Continue rinsing.

Dispose of contents/container in accordance with local/regional/national/international regulations.

5. The enzyme conjugate contains BSA. The source of all animal proteins is from USDA-inspected abattoirs located in the United States.

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Reagent Preparation

1. **Detection Antibody Solution:** According to instructions on the *Certificate of Analysis* or lot specific data sheet, add required quantity of Detection Antibody to Assay Diluent, vortex or mix. For best performance, prepare Detection Antibody Solution immediately prior to use.
2. **Streptavidin-HRP (SAv-HRP) Solution:** According to instructions on the Certificate of Analysis, add required quantity of SAv-HRP to Assay Diluent, vortex or mix. For best performance, prepare SAv-HRP Solution immediately prior to use.
3. **Wash Buffer:** Dilute required quantity of 20× Wash Concentrate immediately with deionized water, mix
Note: If the Wash Concentrate contains visible crystals, warm to room temperature and mix gently until dissolved.
4. **PBS:** Dilute required quantity of 10× PBS with deionized water, mix.
5. **AEC Substrate Solution:** No more than 15 minutes prior to use, mix 20 µL of AEC Chromogen with each 1 mL of AEC Substrate Buffer, vortex or mix. Discard any remaining prepared AEC Substrate Solution after use

Assay Protocol

Note: Use ELISPOT plates and reagents under aseptic conditions (eg, in laminar flow hood) for Steps 1 – 3. Plates are pre-coated with antibody and ready for the addition of cells. Blocking and wetting are not required.

Cell Activation (under aseptic conditions)

1. Prepare mitogen or antigen, diluted in complete medium (eg, RPMI 1640 with FBS, Pen/Strep, and L-glutamine). Add 100 µL/well to BD™ ELISPOT plate.
2. Prepare cell suspensions at different densities, (eg, 1×10^5 to 2×10^6 cells/mL). Add 100 µL/well of each cell suspension to BD ELISPOT plate microwells.

3. Replace lid and incubate at 37°C in a 5% CO₂ humidified incubator. The duration of the incubation time will vary (eg, 2hr – 24hr). Specific activation conditions will vary, depending on cell type, kinetics, and analyte of interest. Please see *Certificate of Analysis* or lot specific data sheet provided with each BD ELISPOT Kit for assay conditions including suggested cell types and incubation times of suggested positive controls. After step 3, aseptic conditions are no longer required.

Note: Cells may be diluted in a regular tissue culture plate starting at 10⁵/well in triplicate wells with 1:3 or 1:4 serial dilutions down the plate, then transferred to the BD ELISPOT plate.

Detection Antibody

1. Aspirate cell suspension. Wash wells 2× with deionized (DI) water. Allow wells to soak for 3 – 5 min at each wash step.
2. Wash wells 3× with 200 µL/well prepared Wash Buffer (see *Reagent Preparation*, step 3). Discard Wash Buffer.
3. Add prepared Detection Antibody Solution at 100 µL per well (see *Reagent Preparation*, step 1).
4. Replace lid and incubate for 2 hr at room temperature.

Enzyme

1. Discard Detection Antibody solution. Wash wells 3× with 200 µL/well prepared Wash Buffer. Allow wells to soak 1 – 2 minutes at each wash step.
2. Add prepared Streptavidin-HRP Solution at 100 µL/well (see *Reagent Preparation*, page 8, step 2).
3. Replace lid; incubate for 1 hr at room temperature.

Substrate

1. Discard Streptavidin-HRP solution. Wash wells 4× with 200 µL/well prepared Wash Buffer. Allow wells to soak 1 – 2 minutes at each wash step.
2. Wash wells 2× with 200 µL/well prepared PBS (see *Reagent Preparation*, step 4).
3. Add 100 µL of prepared AEC Substrate Solution to each well (see *Reagent Preparation* step 5). Monitor spot development from 5 – 60 minutes. Do not let color overdevelop as this will lead to high background.

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4. Stop substrate reaction by washing wells with DI water.
5. Air-dry plate at room temperature for 2 hr to overnight until it is completely dry. Removal of plastic tray supporting the 96-well plate will facilitate drying. Store plate in a sealed plastic bag, in the dark, until it is analyzed.

Analysis

1. Enumerate spots manually by inspection under a dissecting microscope (or magnifying glass), or automatically using an ELISPOT plate reader.

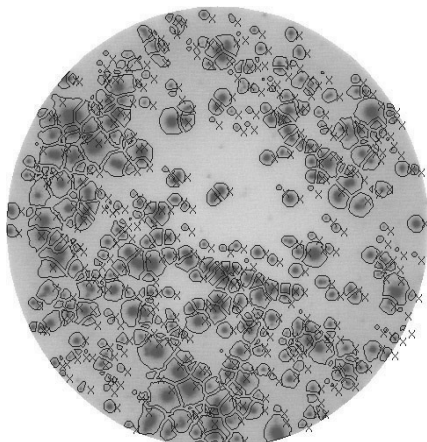
BD™ ELISPOT Technical Tips

1. To avoid damage to the PVDF membrane in the wells, do not touch membrane filters with pipette tips (during pipetting of samples/reagents) or with washing apparatus (during plate-washing).
2. To prevent edge effects, place ELISA plates in a single layer during each incubation step (avoid stacking plates).
3. To prevent cells from moving during incubation, open and close the incubator door slowly.
4. 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.
5. Store color-developed, dried plates in the dark in a sealed plastic bag, to avoid color reduction by light and air.
6. To identify the optimal cell concentration, use a wide range of cell concentrations (eg, 10^3 – 10^6 cells/well) in the first experiment.
7. When scanning a plate in an ELISPOT plate reader, make sure the plate is completely inserted into the base.
8. Monitor spot development carefully, as development time is different for different cell types, even when using the same antibody pair.

9. 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
 - 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.

Sample Data

A



B

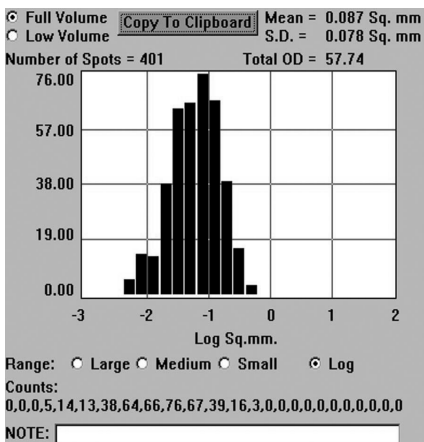


Figure 1. BD™ ELISPOT analysis of human IFN- γ -producing cells. Primed human PBMCs were stimulated (overnight) with PMA and ionomycin in the microwell of a BD ELISPOT plate that was pre coated with the NA/LE anti-human IFN- γ antibody. Biotinylated anti-human IFN- γ antibody was used to detect the captured IFN- γ . Spots were visualized using SAV-HRP 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.

References

1. Helms, T., B. Boehm, R. Asaad, R. Trezza, P. Lehmann, and M. Tary-Lehmann. 2000. Direct visualization of cytokine-producing recall antigen-specific CD4 memory T cells in healthy individuals and HIV patients. *J. Immunol.* 164: 3723.
2. Sedgwick, J., and P. Holt. 1983. A solid-phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. *J. Immunol. Meth.* 57: 301.
3. Czerkinsky, C.C., L.A. Nilsson, H. Nygren, O. Ouchterlony, and A. Tarkowski. 1983. A solidphase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody secreting cells. *J. Immunol. Meth.* 65: 109.
4. Ronnblom, L., B. Cederblad, K. Sandberg, and G. Alm. 1988. Determination of herpes simplex virus-induced alpha interferon-secreting human blood lymphocytes by a filter immuno-plaque assay. *Scand. J. Immunol.* 2: 165.
5. Czerkinsky, C., G. Andersson, H. Ekre, L. Nilsson, L. Klareskog, and O. Ouchterlony. 1988. Reverse ELISPOT assay for clonal analysis of cytokine production. *J. Immunol. Meth.* 110: 29.
6. Fujihashi, K., J. McGhee, K. Beagley, D. McPherson, S. McPherson, C.-M. Huang, and H. Kiyono. 1993. Cytokine-specific ELISPOT assay: single cell analysis of IL-2, IL-4, and IL-6 producing cells. *J. Immunol. Meth.* 160: 181.
7. Power, C., C. Grand, N. Ismail, N. Peters, D. Yurkowski, and P. Bretscher. 1999. A valid ELISPOT assay for enumeration of ex vivo antigen-specific IFN- γ producing T cells. *J. Immunol. Meth.* 227: 99.
8. Tary-Lehmann, M., D. Hricik, A. Justice, N. Potter, and P. Heeger. 1998. Enzyme-linked immunosorbent assay spot detection of interferon- γ and interleukin 5-producing cells as a predictive marker for renal allograft failure. *Transplantation.* 66: 219.
9. Yip, H., A. Karulin, M. Tary-Lehmann, M. Hesse, H. Radeke, P. Heeger, R. Trezza, F. Heinzel, T. Forsthuber, and P.V. Lehmann. 1999. Adjuvant-guided type-1 and type-2 immunity: infectious/noninfectious dichotomy defines the class of response. *J. Immunol.* 162: 3942.
10. VanCott, J., H. Staats, D. Pascual, M. Roberts, S. Chatfield, M. Yamamoto, M. Coste, P. Carter, H. Kiyono, and J. McGhee. 1996. Regulation of mucosal and systematic antibody responses by T helper cell subsets, macrophages, and derived cytokines following oral immunization with live recombinant Salmonella. *J. Immunol.* 156: 1504.

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