Human IL-2 ELISpot Assay- PROCEDURE SUMMARY

	Allow all reagents to reach room temp.; arrange and label required # of strips. 1. **Prepare all required buffers and substrates** that are not supplied in the kit (see page 2). 2. **Dilute required amount of coating capture antibody, detection antibody, and Enzyme conjugate**. that are supplied in the kit
Step 1	**Coat ELISpot** plate with diluted (1:200) capture antibody (**100 ul/well). Coat overnight at 40C**
Step 2	Aspirate and **wash 1 times** with 1x block solution**. Block with 200 ul block solution** and incubate for **2 hrs min** at room temp.
Step 3	Aspirate and **add cells (pre-activated or control) 100 ul/well.** Cover the plate and incubate for 1-24 h at 37oC in cell culture incubator.
Step 4	Aspirate cells and **wash 2 times** with water and 3 x with wash buffer-1. Add diluted (1:250x) **biotinylated detection antibody** (100 ul/well). Incubate at room temp for 2 hrs.
	Step 1-4 may should be performed under sterile conditions. However, for short incubation of a few hours, it is possible to work under non-sterile conditions if the culture media contains antibiotics.
Step 5	Aspirate and wash wells 3x with wash buffer-1. Add diluted (1:100) Streptavidin-HRP conjugate (100 ul/well). Incubate for 1 hr at room temp. .
Step 6	Aspirate and wash wells 4x with wash buffer-I and 2x with washer buffer-II. Add **100 ul/well of single solution AEC substrate** or freshly prepared AEC final substrate. Incubate for 5-60 min at room temp. . .
Step 7	**Stop reaction** by washing with water. Do not allow spots to overdevelop
Step 8	Enumerate spots manually by inspection under a dissecting microscope or automatically using an ELISPOT plate reader

CHECK LIST (Check each box after completing each of the above steps)

	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7
Start time End Time							

KIT PROFILE

Date received: _____**Cat #** IL-2-E-10 **Lot #** _____**Exp.** _____

Date kit opened _____ **Technician:** _____

Date used: **# Plates used** _ **# Remaining** _____

Instruction Manual No. 100-150-ADM

Human IL-2 ELISPOT Kit

Cat. # IL2E-1 (1 plate kit)
Cat. # IL2E-10 (10 plate kit)

For the detection and frequency of cells producing human IL-2.

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Human IL-2 ELISpot KIT Cat. # IL-2-E-10

Kit Components, 96 tests	Cat #
ELISPOT plates, 10 plates	IL2-E-10-1
Anti-human IL-2 Capture Antibody (no azide/low endotoxin format); 200X	IL2-E-10-2
Biotinylated Detection Antibody in stabilizing buffer containing 0.05% azide (250x)	IL2-E-10-3
Streptavidin-HRP Enzyme Conjugate, in a stabilizing buffer containing 2% BSA (100x)	IL2-E-10-4
Instruction Manual	IL2-E-10-M
Additional reagents and materials are required but not supplied fro the assay (see the list on	

Introduction

The enzyme-linked immunospot (ELISPOT) assay has emerged as a very powerful technique for detecting and counting of individual cells that secrete a particular protein *in vitro.* Basic principles of both ELISA and ELISpot are the same. Both assays, the sandwich enzyme-linked immunosorbent assay (ELISA) and the ELISPOT assay, derives its specificity and sensitivity by using high affinity capture and detection antibodies and enzyme-amplification. ELISpot was originally developed for analyzing specific antibody-secreting cells, the assay has been adapted for measuring the frequencies of cells that produce and secrete other effector molecules, such as cytokines. High sensitivity of the assay lends itself to measurement of even very low frequencies of analyte producing cells (eg, 1/300,000). 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.

Interleukin 2 (IL-2) is a pleiotropic cytokine produced primarily by antigen- or mitogen-activated T lymphocytes. IL-2 synthesis is induced transiently upon activation of the T cell receptor (TCR). IL-2 plays a key role in promoting the clonal expansion of antigen-activated cytolytic CD8+ T cells (CTL). IL-2 stimulates the proliferation of CD4+ T helper cells as well as natural killer (NK) cells.

The human IL-2 ELISpot assay is designed for the detection of IL-2 secreting cells at the single cell level, and it can be used to measure the frequency of human IL-2 secreting cells. ELISpot assays are well suited for monitoring immune responses to various stimuli, treatments and therapies, and they have been used for the quantitation of antigen-specific CD4 and/or CD8 T cell responses. Other methods for the assessment of antigen-specific T cell responses, such as the chromium release assay with quantitation by limiting dilution, are tedious, and require previous in vitro expansion of T cells for several days. High sensitivity of the assay lends itself to measurement of even very low frequencies of analyte producing cells (eg, 1/300,000). 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. ELISpot assays do not require prior in vitro expansion of T cells. ELISpot are suitable for high-throughput analysis using only small volumes of primary cells. As such, ELISpot assays are useful tools for research in areas as diverse as antigen recognition and vaccine development.

ADI human IL-2 ELISPOT kit is a highly sensitive assay for the detection of IL-2 producing cells.

Always include proper controls for each ELISpot experiment.

Positive Control - Use recombinant human IL-2 or cells that are known to secrete human IL-2.

Negative Control - Use the same number of unstimulated cells as stimulated cells.

Background Control - Use sterile culture media.
Detection Antibody Control - Substitute phosphate buffered saline for Detection Antibody.

CALCULATION OF RESULTS

The developed microplate can be analyzed by counting spots either manually using a dissection microscope or by using a specialized automated ELISpot reader. Specific spots are round and have a dark center with slightly fuzzy edges. Quantitation of results can be done, for example, by calculating the number of spot forming cells (SFC) per number of cells added into the well.

REPRODUCIBILITY DATA

PBMC, Peripheral blood mononuclear cells (5 x 10^5/mL) when stimulated with 50 ng/mL of phorbol 12-myristate-13-acetate and 0.5 g/mL of calcium ionomycin overnight at 37° C in 5% CO2 incubator. The sample was assayed in seven wells according to the procedure and analyzed with a dissection microscope. The #SFC were on average #310 per well.

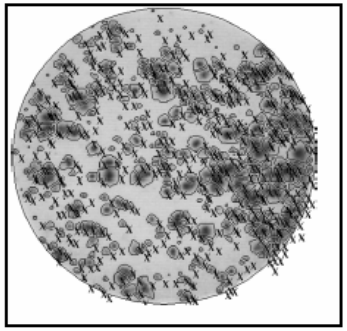

Figure 1. ELISPOT analysis of human IL-2-producing cells. Primed human PBMCs were restimulated (4 hr) with PMA and ionomycin in the microwell of an ELISPOT plate . Spots were visualized followed by image analysis and spot enumeration. The spot size distribution can be measured by image analysis, if necessary.

References

Helms, T., B. 2000, J. Immunol. 164: 3723-3732; Sedgwick, J.,1983, J. Immunol. Meth. 57: 301; Czerkinsky, C.C.,1983, J. Immunol. Meth. 65: 109; Ronnblom, L., B. 1988, Scand. J. Immunol. 2: 165-171; Czerkinsky, C., G. 1988, J. Immunol. Meth. 110: 29-36; Fujihashi, K., 1993, . J. Immunol. Meth. 160: 181-189; Power, C, 1999,J. Immunol. Meth.227: 99-107; Tary-Lehmann, M., D. 1998, Transplantation. 66: 219-224, Yip, H., A.,1999, J. Immunol. 162: 3942-3949; VanCott, J., H.,1996, J. Immunol. 156: 1504-1514

Technical help and trouble shooting

1 Take care not to puncture the membrane on the bottom of the ELISPOT plate wells. 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 (e. g, $10^3 – 10^6$ 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 (i.e, 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 in the 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.

9 The kit is designed for single use only. The layout of the assay should be carefully planned to maximize the use of the plate and reagents provided.

PRINCIPLE OF THE TEST

The enzyme-linked immunospot (ELISpot) assay was originally developed for the detection of individual B cells secreting antigen-specific antibodies. This method has since been adapted for the detection of individual cells secreting specific cytokines or other antigens. ELISpot is based upon the quantitative sandwich enzyme-linked immunosorbant assay (ELISA) technique. A capture antibody (poly or mono) specific for the protein or cytokine of interest (e.g. human IL-2) is pre-coated onto a PVDF (polyvinylidene difluoride)-backed ELISA microplate. Cultured cells (control or stimulated or treated) cells are plated into the wells and the microplate is placed into a humidified 37° C CO2 incubator for a specified period of time. During this incubation period, the immobilized antibody in the immediate vicinity of the secreting cells bind secreted IL-2. After washing away any cells and unbound substances, detection antibody (usually biotinylated polyclonal antibody specific the capture antigen, e.g. IL-2) is added to the wells. Following a wash to remove any unbound biotinylated antibody, plates are incubated with enzyme conjugate (HRP or alkaline-phosphatase conjugated to streptavidin) is added. After washing the unbound conjugate, a precipitating substrate solution (AEC or BCIP/NBT) is added. A colored precipitate forms and appears as spots at the sites of cytokine localization, with each individual spot representing an individual IL-2 secreting cell. The spots can be counted with an automated ELISpot reader system or manually using a stereomicroscope.

MATERIALS AND EQUIPMENT REQUIRED (not provided)

Adjustable micropipet (5-1000 ul) and multichannel pipet with disposable plastic tips. Reagent troughs, plate washer (recommended) and ELISA plates Reader.

Additional reagents and buffers required (not provided)

1. Prepare Coating Buffer (1× Phosphate Buffered Saline [PBS]): NaCl (8 g); KCl (0.2 g); Na2HPO4 (1.44 g), KH2PO4 (0.24 g); dissolve in 1-liter water and 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 # 10378-016). Other suitable medium may be substituted.

3. **Wash Buffer-I**: 1× PBS containing 0.05% Tween-20 (0.5 ml concn Tween-20 add to 1-L PBS).
4. **Wash Buffer-II**: 1× PBS
5. **Dilution Buffer**: 1× PBS containing 10% FBS.
6. **Prepare AEC substrate solution**. ADI has a Ready-to-use single solution substrate solution that required no weighing or buffer preparation. It is stable for several months. Cat # AEC1-100 (100 mls sufficient for 10 plates).

Alternatively AEC solution can be prepared using the following 3-solutions.

a) **Substrate Solution**: 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 glassvials.

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 ul of AEC stock solution (step 6a) to 10 ml 0.1 M Acetate Solution (step 6b). Filter through 0.45 µm filter; add 5 ul of H202 (30%, Sigma) and use immediately. Prepare substrate solution in required amounts only and do not store.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Detection antibody contains 0.05% (w/v) azide. it can be disposed of in the drains. A MSDS for azide, if not already on file, can be requested or obtained from the ADI website.

STORAGE AND STABILITY

The ELISpot well plate and all other reagents, if unopened, are stable at 2-8^{o}C until the expiration date printed on the label. The whole kit stability is at least 6 months from the date of shipping under appropriate storage conditions.

TEST PROCEDURE *(ALLOW ALL REAGENTS TO REACH ROOM TEMPERATURE BEFORE USE).* **Use ELISPOT plates and reagents under aseptic conditions** (eg, in laminar flow hood) for Steps 1 –6. Do not open ELISpot plates in not needed. Solutions noted with an asterisk (*) require reagents and preparations are described on page 2.

Coating Antibody

1. **Dilute capture antibody in Coating Buffer*** (it is typically supplied at 200x and lot specific stock dilution is also indicated on the vial). (prepare 10 ml for 1 full plate or 50 ul antibody stock in 10 ml buffer). Immediately, transfer **100 ul of diluted antibody** to each well of an ELISPOT plate. Cover the plates and **incubate plates at 4ºC** overnight.

Blocking

2. Aspirate Antibody solution and **Wash wells 1× with 200** ul/well Blocking Solution*.

3. **Add 200 ul/well Blocking Solution** and incubate for 2 hr at room temperature (25-30oC).

Cell Activation

4. Discard Blocking Solution Prepare mitogen or antigen, diluted in complete medium (eg, RPMI 1640 with FBS) Add 100 ul/well to ELISPOT plate

5. Prepare cell suspensions at different densities, (e.g, 1 × 10^5cells/ml-2 × 10^6 cells/ml). Add 100 ul/well of each cell suspension to ELISPOT plate microwells

6. Cover the plate and incubate ELISPOT plate at 37ºC, in a 5% CO2 and humidified incubator as required by the experiment (1-24 hrs).

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

The following steps may be performed in non-sterile conditions.

Detection Antibody

7. Aspirate cell suspension. **Wash wells 2× with deionized (DI)** water. Allow wells to soak for 3 – 5 min at each wash step.

8. **Wash wells 3× with 200 ul/well** Wash Buffer-I*. Discard Wash Buffer.

9. **Dilute Detection Antibody in Dilution Buffer*** (prepare 10 ml per plate; typical dilution is 1:250 or 4 ul/ml or 40 ul stock antibody in 10 ml buffer; lot sp dilution is given on the vial). **Add 100 µl per well**. Cover the plate and incubate for 2 hr at room temperature (25-30oC).

Enzyme Conjugate

10 Discard Detection Antibody solution Wash wells 3× with 200 ul/well with Wash Buffer-I. Allow wells to soak for 1 – 2 minutes at each wash step.

11. **Dilute Enzyme Conjugate (Streptavidin-HRP)** in Dilution Buffer* (dilute stock 1:100; prepare 10 ml per plate of 100 ul stock/10 ml buffer). **Add 100 ul/well** diluted enzyme reagent. Replace lid; **incubate for 1 hr at room temperature**

Substrate

12. Aspirate and discard enzyme conjugate solution **Wash wells 4×** with 200 ul/well Wash Buffer-I. Allow wells to soak for 1 – 2 minutes at each wash step.

13. **Wash wells 2× with 200 µl/well Wash Buffer II***

14. **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

15. **Stop substrate reaction** by washing wells with DI water.

16. 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.

17. Enumerate spots manually by inspection under a dissecting microscope or automatically using an ELISPOT plate reader