Institut für HIV Forschung
SOP#09-00 **(April 2015)**

ELISpot Assay

This protocol is written for the detection of IFN-γ by antigen-specific T cells using peptide stimulation. The antibodies and stimulants used in this assay can be exchanged to detect other cytokines and proteins, but the conditions and dilutions must be optimized for each new antibody and stimulant used.

Antibodies

Item	Manufacturer	Order Number
"Coating Antibody" (Anti-Human mAb 1-DK1 at 1mg/ml)	Mabtech	3420-3
"Primary Antibody" (Anti-Human IFN-γ mAb 7 B6-1-Biotin at 1mg/ml)	Mabtech	3420-6
Streptavidin-ALP	Mabtech	3310-8

Coloring Reagent Components

Item	Manufacturer	Order Number
AP Conjugate Substrate Kit	BioRad	170-6432

ELIspot Plates

Item	Manufacturer	Order Number
"Clear Plates" 0.45µm Hydrophobic High Protein Binding, Immobilon-P Memb.	Millipore	MAIPS4510 Current Lot R3AA43964

Other Supplies

Item	Manufacturer	Order Number
PBS	Sigma	D8537
TWEEN-20 (Enzyme Grade)	Fisher	BP337-100
Fetal Calf Serum (FCS)	Sigma	F4135
Phytohaemagglutinin (PHA) at 250ug/mL	Fisher	R 30852801

Media:

R10

Item	Stock Concentration	Volume to Add	Final Concentration
RPMI 1640	-	500	-
FBS	100% (v/v)	55ml	10% (v/v)
Penicillin/Strep	Pen: 5000 IU/mL Strep: 5000ug/mL	5.5mL	Pen: 50 IU/mL Strep: 50ug/mL
L-glutamine	200mM	5.5mL	2mM
HEPES	1M	5.5ml	10mM

Protocol:

Day1: Coating the Plate

ALL TO BE DONE IN THE HOOD WITH STERILE TECHNIQUE.

1. Find ELIspot plates for coating. They are in the blue boxes labeled Millipore Multiscreen Filter Plates.
2. To coat the plate, the following items are needed:
   - Coating antibody (Anti-Human mAb IFNgamma 1-DK1 at 1mg/ml). All antibodies are found in a gray case in the 4^0C. (If performing an ELIspot for a different cytokine, such as IL21, IL4, etc. coat plate with same antibody concentration)
     - **NOTE:** IL21 Abs along with a couple of others are only at 0.5mg/ml, so to attain the same concentration, a different dilution is necessary!!
   - A 15 ml conical tube with 10 ml of sterile PBS (This can be scaled up in 50ml conicles)
   - Plate
   - Multichannel pipettor (manual or Rainin electronic repeater)
3. Take 20µl of coating antibody and place in 10ml of sterile PBS (it's a 1:500 dilution for IFNgamma). This is for one plate. If you want to coat 3, it's 60µl of antibody in 30ml of PBS, etc.
4. VORTEX THE SOLUTION WELL.
5. Place 100µl of solution into each well using the multichannel pipettor.
6. Place the plate in the 4^0C overnight. The plates are good for ~2 weeks. The rule of thumb is that if there is still liquid left in the wells, the plate can be used.

Day 2: Setting up the Plate

ALL TO BE DONE IN THE HOOD WITH STERILE TECHNIQUE.

7. First, it is best to write all the information of your experiment on the plate. For example:
   - Patient name/number.
   - Date.
   - The number of cells in each well.

- The specific peptides you are placing in each well, which can be done easily on the lid of the plate. If you have a conventional layout, such as Gag and Env consensus peptides, this is not necessary.
- Label the rows of the plate 1-12 down the left-hand side of the plate and lid (this will help you with putting peptides in the correct wells).

NOTE: it is best to write the patient info on both the lids of the plate and the plate itself, in case there is confusion with multiple lids. Be careful to keep the wells as covered as possible to avoid debris contaminating the plate.

8. Wash the plate with 1%FCS/PBS (this can be made by a placing 5ml of FCS in a new 500 ml bottle of PBS).
   - Pour the PBS with FCS into a sterile pipet basin.
   - Using the multichannel, add 100µl of the PBS to each well of the ELIspot plate.
   - Discard the PBS by flicking the plate into the soapless bucket. Often flicking into the biohazard with something to soak in it works as well, but is not completely sterile so it is better to do it in the hood.
   - WASH IN THIS MANNER 6 TIMES!

NOTE: try to do this without any splashing, because splashing may cause wells of the plate to turn blue.

9. Add R10 to each well of the ELIspot plate (30µl/well for optimals and confirms, 20µl/well for matrices).
10. Add 10µl of the appropriate peptides to the corresponding wells. We have a special 10-100ul multichannel pipet to use for this purpose.
    - The peptides are at 200 µg/ml dilution, this is a 1:10 dilution of the stock peptides at 2 mg/ml (so, 100µl of stock peptide in 900µl of RPMI with antibiotics makes the 1:10 dilution). When screening a lot of peptides, make a working plate of peptides to easily transfer with a multi-channel pipet.
    - Use a **NEW PIPET TIP EVERY TIME** you go into the 1:10 dilution of the peptide. This will help prevent contamination of the peptide.
11. Add the PBMCs to the plate:
    - Usually we add 100,000 cells per well for screening, but we can go down to 50,000 cells per well if we do not have a sufficient amount of cells. The range has been from 50,000-250,000 cells per well.
    - The cells are added in R10 at 100 µl per well:
      a. 100, 000 cells/well: 10 million cells in 10ml of R10
      b. 50,000 cells/well: 5 million cells in 10ml of R10
    - Use a P200 Multichannel pipet. Make sure to add a P200 pipet tip to the end; try to splash as little as possible while adding cell solution.
    - While adding the cells do not touch the tip of the pipet to the edge of the well of the ELIspot plate. This would lead to contamination of one well by the peptides of the previous wells.
12. Controls:
    - Negative Controls: in **AT LEAST THREE** wells we place only the R10 and the cells.
    - Positive Control: in one well we place R10, cells, and 10 µl of stock PHA at a 1:10 dilution.
13. Place the plate in the 37^0C incubator for **40 hours**.

DAY 4: DEVELOPING THE PLATE

ALL TO BE DONE IN THE HOOD WITH STERILE TECHNIQUE.

14. Wash the plate

    Manually:

    - Place a blue chuck on the back wall of the hood and under the bucket that you will be using to wash the plate. A clean bucket that does not contain any expose should be used (label the bucket explaining that there is no soap).
    - With the multichannel, add 200µl of the PBS to each well of the ELISPOT plate.
    - Discard the PBS by flicking it into the soapless bucket.
    - WASH IN THIS MANNER **6** TIMES!

    NOTE: try to do this without any splashing since splashing may cause wells of the plate to turn blue.

    Automatic Washing:

    - Use the plate washer (directions are written above it)
        - RUN daily maintenance program with water to clean the machine. (MAINT)
        - PRIME the machine with 1XPBS (Prime_200)
        - WASH using the EliSpot program (Program 07)

15. Add Biotin
    - Take 5µl of Anti-Human IFN-γ mAb 7 B6-1-Biotin (or other cytokine) and place in 10ml of sterile PBS (it's a 1:2000 dilution).
    - VORTEX SOLUTION WELL.
    - Place 100µl of solution into each well.
    - This is for 1 plate, multiply accordingly for more plates.
    - As before, IF USING THE ELECTRONIC PIPETTOR: take 5.5 µl of Biotinylated antibody and place in 11ml of PBS. The electronic pipettor uses a surplus of liquid!
16. Incubate plate in the **hood** at **room temperature** for 1.5 hours.
17. Wash plate 6 times with PBS as explained in #14.
18. Add Streptavidin.
    - Take 5µl of Streptavidin and place in 10ml of sterile PBS (it's a 1:2000 dilution).
    - VORTEX SOLUTION WELL.
    - Place 100µl of solution into each well.
    - This is for 1 plate, multiply accordingly for more plates.
    - As before, IF USING THE ELECTRONIC PIPETTOR: take 5.5 µl of streptavidin and place in 11ml of PBS. The electronic pipettor uses a surplus of liquid!
    - **Wrap plates in aluminum foil to protect from light!!! (Also turn off hood light)**
19. Incubate plate in the **hood** at **room temperature** for 1 hour.

    NOTE: some people incubate with streptavidin for only 45 minutes.
20. Wash plate 6 times with PBS as explained in #14.
21. Make color solution using BioRad AP Substrate Kit (#170-6432)
    - Dilute 25x Development Buffer 1:25 in diH2O.
    - For 1 plate: make 10ml of 1X Development Buffer by adding 400ul 25x Development Buffer to 9.6ml diH2O. (directions come with the BioRad kit; scale up as needed)
    - Add 100µl of color Reagent A (NBT) to every 10ml of 1x Development Buffer.
    - Add 100µl of color Reagent B (BCIP) to every 10ml of 1x Development Buffer.

- VORTEX SOLUTION WELL.
- Place 100µl of solution into each well of ELIspot plate using a multichannel.
- **NOTE:** Tris can be diluted ahead of time, but try not to add the color reagents until right before you add the solution to the plate.

22. Allow color reagents to remain on the plate until the spots begin to turn blue and are quite dark.
    - This usually takes place in about 5 minutes to 15 minutes depending on the level of the response. Don't leave the coloring solution on too much longer or your background will be high (wells will all turn purple)
    - Dump the color reagents into the soapless bucket.
23. Now stop the coloring process by washing the plate in the sink.
    - Wash 6 times with tap H_2O.
    - Leave to dry on ventilated portion of hood under aluminum foil. Make sure plates dry upside down at a slight angle (pin them up on their lid).

NOTE: THE LAST STEP IS THE ONLY STEP TO BE DONE OUTSIDE OF THE HOOD!!!!!!!

24. Clean/bleach the bucket, if you used one.
25. Go have lunch