STANDARD OPERATING PROCEDURE				
Ragon Institute of MGH, MIT and Harvard	EliSpot Assay		**SOP#**	TC-SOP-009
	Originated by:	Alicja Trocha	Date:	23 November 17
	Laboratory:	Walker Laboratory	Pages:	1 of 8
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I. PURPOSE

The purpose of this procedure is to outline the ElisSpot assay performed in Ragon laboratories.

II. SAFETY

This procedure needs to be carried out in the BSL2+ laboratory following all BL2+ regulations.

III. SCOPE

This procedure applies to all employees, students, contractors and visitors that work on the CTL Feeding protocol.

IV. REQUIREMENTS

Training to be obtained by qualified trainer or Subject Matter Expertise (SME).

V. RESPONSIBILITIES

A. The Ragon Institute **qualified trainers** are responsible for the overall implementation of this procedure and ensuring compliance and for periodic review of this procedure. Updates if any may be initiated by the qualified trainers or Subject Matter Expertise (SME).
B. **All employees, students, contractors and visitors** are required to follow this procedure.

VI. REAGENTS

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	Mabtech	3310-8

Coloring Reagent Components

Item	Manufacturer	Order Number
BCIP	BioRad	170-6539
NBT (Nitroblue Tetrazolium Chloride)	BioRad	170-6532
DMF (N, N-Dimethyl-formamide)	Fisher	D119-500

Tris (Molecular Biology Grade)	Fisher	BP-152-5
$MgCl_2$:$6H_2O$	Fisher	M33-500

ELIspot Plates

Item	**Manufacturer**	**Order Number**
"White Plates" 0.45µm Hydrophobic High Protein Binding, Immobilon-P Memb.	Millipore	S2EM004M99 Current Lot F6DN58189
"Clear Plates" 0.45µm Hydrophobic High Protein Binding, Immobilon-P Memb.	Millipore	MAIPS4510 Current Lot F5HN6502

Other Supplies

Item	**Manufacturer**	**Order Number**
PBS	Sigma	D8537
TWEEN-20 (Enzyme Grade)	Fisher	BP337-100

VII. PROCEDURE

Day1: Coating the Plate

ALL TO BE DONE IN THE HOOD WITH STERILE TECHNIQUE.

1. Find ELIspot plates for coating. They are in a white and green box labeled Millipore Multiscreen Filtration Plates.
2. To coat the plate, the following items are needed:
   - Coating antibody (Anti-Human mAb 1-DK1 at 1mg/ml). All antibodies are found in a gray case in the 4^0C (coating antibody has a green cap).
   - A 15 ml conical tube with 10 ml of sterile PBS
   - Plate
   - Multichannel pipettor (manual or electronic)
3. Take 20µl of coating antibody and place in 10ml of sterile PBS (it's a 1:500 dilution). This is for one plate. If you want to coat 3, it's 60µl of antibody in 30ml of PBS. **If you are using the electronic pipettor, make a little extra as it takes a surplus of liquid. For example, for one plate, use 22 uL of coating antibody in 11 mL of sterile PBS.**
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.
   - 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.
8. Wash the plate
   - 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).

- Using the multichannel pipettor, wash the plate with PBS ~~with 1% heat inactivated FCS (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 reservoir.
- 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.
- 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 (or 20µl of the matrix pools) to the corresponding wells.
    - 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).
    - 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 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
    - 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 PHA at a 1:10 dilution.
13. Place the plate in the 37^0C incubator overnight.

DAY 3: DEVELOPING THE PLATE

ALL TO BE DONE IN THE HOOD WITH STERILE TECHNIQUE.

14. Wash the plate
    - 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.

15. Add Biotin
    - Take 5µl of Anti-Human IFN-γ mAb 7 B6-1-Biotin (commonly called Biotin) 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 Biotin 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 hour.

    NOTE: some people incubate for 1.5 hrs…I've found the same results with 1 hour.
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!!!**
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 (new adaptation since 2016)
    - **Cover a 15 mL conical in aluminum**
    - **Add 400 uL of Color Development Buffer to 10 mL of ddH2O (white tap by the sink)**
    - **Add 100 uL of color development solution A to the solution**
    - **Add 100 uL of color development solution B to the solution**
    - **NOTE: The above steps have to be done in order**
    - **Add 100 uL to each well**
    - **Cover your plate in foil to protect it from light**
    - **Wait 15-20 min or until your spots develop**

- Dump the color reagents into the soapless bucket.

22. Disinfect the plate with 0.05% Tween20 in PBS. (This is made by adding 250µl of Tween20 to a 500ml bottle of PBS.)
    - Add 100µl of the PBS 0.05% Tween to each well of the ELIspot plate.
    - Allow the plate to sit in the hood for 10 minutes.
    - Dump into soapless bucket.
23. Now stop the coloring process by washing the plate in the sink.
    - Wash 3 times with tap H_2O.
    - Leave to dry on a blue chuck.

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

24. Place Expose in the bucket used for washing and place on the autoclave cart.

INSTRUCTIONS FOR MAKING REAGENTS (if making them since this depends how you are purchasing your reagents)

Tris Buffer

1. Get a clean 1-liter beaker.
2. Place 800ml of ddH_2O in the beaker.
3. Measure 12 grams of Tris and place in the 1L beaker.
   - The Tris is located with the Walker chemicals under the bench in a large vat.
4. Add 0.12 grams of $MgCl_2$:$6H_2O$ into the beaker.
5. Stir with magnetic stirring rod on automatic heating/stirring plate (stirring rods are located in the drawer in the bench by the chemicals).
6. Once dissolved, pH the solution to 9.5.
   - ASK FOR ASSISTANCE in pHing if you're new at this.
7. Add 200ml of ddH_2O to the beaker, bringing it up to 1000ml.
8. Filter the solution with a 0.22µm CA filter.
   - Located under the bench opposite the PCR area.
   - The filters come in 250ml, 500ml, and 1000ml bottles. A 1000ml bottle would be ideal since you have 1 liter of liquid. Otherwise 2 of the 500ml bottles, etc.

NBT Color Reagent Stock: for 1ml of solution.

1. Get the NBT from the -20^0 C in Alicja's tissue culture room.
2. At the scale down by Suqin's desk, measure out 30mg of powder.
3. Place in a glass vial that has been covered with aluminum foil.
   - The vials are located above LN_2 freezer #4.
4. Add 70% N, N-Dimethyl-formamide (commonly called DMF).
   - DMF is located in the Walker chemical area.
   - For 1ml of solution 70% DMF = 0.7ml of DMF.
   - When measuring the DMF, either use glass pipets or be swift using the plastic pipets because DMF corrodes plastic.
5. Add 30% ddH2O to the vial.
   - For 1ml of solution 30% H2O = 0.3ml H2O.
6. Mix by shaking the vial.
7. Store at 4^0C.

BCIP Color Reagent Stock: for 1ml of solution.

1. Get the BCIP from the -20^0 C in Alicja's tissue culture room.
2. At the scale down by Suqin's desk, measure out 15mg of powder.
3. Place in a glass vial that has been covered with aluminum foil.
   - The vials are located above liquid nitrogen freezer #4.
4. Add 1 ml of DMF.
   1.