## Granzyme B ELISPOT Influenza (U01) Protocol

## 06/10/2011

Iana Haralambieva (based on the BDTM Human Granzyme B ELISPOT protocol)

**Kit**: BDTM Human Granzyme B ELISPOT set, Cat. No. 552572, Lot 02514, Exp. 2012-11-30

**\*ALL SUBJECTS WITH 1 vial of cells (=1mL or 0.5 mL) will be used. If there aren’t enough cells, drop PHA then one unstimulated then one stimulated well!**

## Preparation of Media needed for thawing and culturing PBMC:

**RPMI Thawing Media with DNase and 10%FCS:**

* Prepare in RPMI media plastic bottle and then filter with Corning filter system

**439.5 ml of RPMI with Glutamine** (**Gibco catalog number 11875**) (remove 60.5 ml of media from the original bottle and discard in a beaker)

**50 ml of FCS** (Hyclone catalog number SH30071.03, **lot number ASD29480.** Heat-inactivated aliquots stored in -20°C freezer in tissue culture room.

**5 ml of Penicillin-Streptomycin** (Sigma catalog number P7539) Aliquots stored in

-20°C freezer in tissue culture room.

**5 ml of sodium pyruvate** (Cellgro catalog number 25-000-Cl)

**0.5 ml of DNAse** (Sigma catalog number D4513). 1 mg/ml aliquots stored in -20°C freezer in tissue culture room.

* Re-cap bottle and invert to MIX WELL.
* Sterile filter using a Corning filter system (catalog number 431097) and label with name and date
  + IMPORTANT: Do not use glass bottles to filter in or store media due to risk of LPS contamination

**Pre-warm in a 37°C water bath before use to increase yield of thawed cells after cryopreservation**

**RPMI Blocking Media with 10%FCS (for blocking the Granzyme B Elispot plate):**

* + - Prepare this media following the protocol for RPMI Thawing Media with DNase and 10%FCS, just omitting the DNase!

## RPMI Culturing Media with 5% FCS:

* Prepare in RPMI media plastic bottle and then filter with Corning filter system.

**465 ml of RPMI** **with Glutamine** (**Gibco catalog number 11875**) (remove 35 ml of media from the original bottle and discard in a beaker)

**25 ml of FCS** (Hyclone catalog number SH30071.03**, lot number ASD29480.** Heat-inactivated aliquots stored in -20°C freezer in tissue culture room.

**5 ml of Penicillin-Streptomycin** (Sigma catalog number P7539) Aliquots stored in

-20°C freezer in tissue culture room.

**5 ml of Sodium Pyruvate** (Cellgro catalog number 25-000-Cl)

* Re-cap bottle and invert to MIX WELL.
* Sterile filter using a Corning filter system (catalog number 431097) and label with name and date
  + IMPORTANT: Do not use glass bottles to filter in or store media due to risk of LPS contamination

## Other reagents needed for thawing/culturing PBMCs and for Granzyme B ELISPOT

1. H1N1 Influenza A/California/07/2009 (stock of allantoic fluid 3/18/2010) -In -80°C Thermo Scientific new freezer, room 6-05, 3rd shelf from top.

Thaw right before use, keep on ice all the time and mix before use! USE Granzyme B ELISPOT stock!

1. PHA (Sigma catalog number L9132)- Stocks of 200 µg/ml are in -20°C freezer in tissue culture room. Can be kept at 4°C for 1 week after thawing.
2. Coating Buffer: PBS, pH 7.2
3. Blocking Solution/Blocking medium: RPMI with 10%FCS (for preparation see above)
4. Wash Buffer I: PBS containing 0.05% Tween-20 (0.5mL Tween-20 per 1L PBS)
5. Wash Buffer II: PBS, pH 7.2
6. Dilution Buffer: PBS, containing 10% FBS.
7. Substrate solution: TMB-H Peroxidase Substrate (Moss, Inc., 410-768-34420)

## 1. Preparation of “Supplement” Stocks for Human Granzyme B ELISPOT

**Mix H1N1 Influenza virus stock by pipetting!**

1. Influenza virus MOI=0.5 (for one plate, 12 subjects, stimulation in triplicate)

0.8 mL of Influenza virus stock (stock of allantoic fluid 3/18/2010) + 3.2 mL RPMI with 5% FCS.

Keep on ice and mix well by vortexing before using!

2. PHA (for one plate, for 12 subjects 1 stimulated PHA well per subject)

100 µL of PHA stock (200 µg/ml stock )+1.9 ml RPMI with 5% FCS

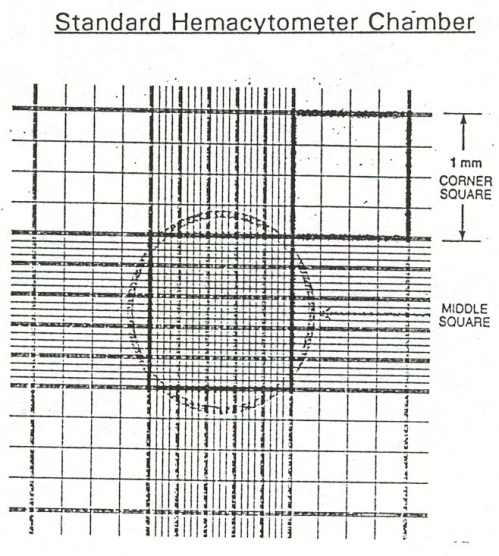
Keep on ice and mix well by vortexing before using!

## 2. Thawing of cryopreserved PBMCs

1. Checkout 1 cryovials of PBMC (1mL or 0.5mL) for each of 12 subjects (for one plate) from VATS according to VATS checkout protocol.

Ensure that the PBMC do not thaw during the checkout process (i.e. keep them on dry ice)

1. Place PBMC cryovials in -80°C freezer until you are ready to begin the thawing process.
2. Place RPMI thawing media containing DNase in 37°C water bath for 15 minutes to warm up (this is essential to recover maximal cells). Wipe the bottle with 70% ethanol before placing in tissue culture hood.
3. Label one 15 ml conical tube with the subject ID on both the side and the cap.
   1. NOTE: Ensure that the caps are kept in the same order as the tubes
4. Add 100 µl of RPMI thawing media containing DNase/10% FCS to each 15 ml conical tube using a p200 pipette.
5. Remove PBMC cryovials from -80°C freezer and place cryovials in styrofoam rack. Place sytrofoam rack containing PBMC in 37°C water bath in tissue culture room. Rapidly shake the rack (manually) so thawing can occur. Do this until a small chunk of ice remains.
6. Quickly wipe the PBMC vial with 70% ethanol and place the thawed PBMC in a blue cryotube rack (in the SAME order as the 15 ml conical tubes) and place in tissue culture hood.
7. Uncap the cryovials for each subject. Using a 2 ml pipette, remove the cells from the cryovial. Be careful not to exceed the volume of the pipette when removing the cells. Transfer the PBMC with the 2 ml pipette to the 15 ml centrifuge tube slowly. **Do not pipette the cells up and down.**
8. Mix the cells with the media in the conical tube by shaking the tube gently.
9. **Set your timer for 5 minutes.** Add 200 µl of RPMI thawing media with DNase/10%FCS to the cells SLOWLY, swirl the tube gently to mix the cells/media, wait one minute, then double the amount of media (0.5 ml) and add slowly to cells, swirl the tube gently, wait one minute, add 1 ml of media as described. Do this until cells are in a final volume of 10 ml. You should be done when your timer goes off.
10. Cap each 15 ml conical tube with the appropriately labeled cap. Invert the conical tube 5 times to mix the cells. Do not vortex the cells!
11. Centrifuge at 1200 rpm at room temperature for 7 minutes using a Beckmann bench top centrifuge.
12. While cells are centrifuging, turn on the refrigerated centrifuge. Set temperature at 4°C so it is cool when you need to use it.
13. Remove the supernatant by vacuum aspiration, leaving only few hundred μl in the tube. DO NOT ASPIRATE CELL PELLET. Re-suspend cells in remaining media by swirling gently. DO NOT VORTEX CELLS.
14. Bring volume to 10 ml with RPMI thawing media containing DNase/10% FCS.
15. Cap each 15 ml conical tube with the appropriately labeled cap. Invert the conical tube 5 times to mix the cells. Do not vortex the cells!
16. Place conical in rack and incubate in 37°C water bath for 20 minutes with intermittent inverting of tubes (every 10 minutes).
    1. NOTE: Ensure that water in the water bath does NOT exceed the top of the tubes as contamination will occur. Cover the tubes with a red weight if necessary to prevent tipping.
17. During incubation prepare FACS tubes for counting. Label FACS tubes with subject ID and add 200 µl of 1X PBS and 37.5 µl of trypan blue
18. Cool cells on ice for 7 minutes.
19. Centrifuge at 1200 rpm at 4°C for 7 minutes using one of the refrigerated bench top centrifuges.
20. Carefully remove the supernatant by vacuum aspiration without disturbing cell pellet. Resuspend cells in each conical tube in 2 ml RPMI with 5% FCS by swirling gently (MAKE SURE CELLS ARE RESUSPENDED WELL). Note: There may be a lot of dead cells and these will be filtered out in the next steps.
21. Label one 50 ml conical for each subject with the subject ID.
22. Using a 2 or 5 ml pipette, filter the cells from each subject through one BD Falcon cell strainer (catalog number 352350) into the pre-labeled conical tube for each subject.
23. Place the cells on ice.
24. Mix the conical tube containing the PBMC by inverting 3 times. Using a p20 pipette add 12.5 µl of PBMC to the appropriate FACS tube immediately before counting. Only prepare cells for 1 subject at a time.
25. Mix the contents of the FACS tube by vortexing. Using a p10 pipette, add 10 µl of the cell suspension to the counting chamber of a hemacytometer. Do not flood the hemacytometer. Make sure there are no bubbles under the coverslip. If the hemacytometer is flooded, or if there are bubbles, clean the hemacytometer with 70% ethanol and again add 10 µl of the sample.
26. Place hemacytometer under microscope and count cells (live= clear, unstained) and dead (blue) in the outer 4 quadrants.

**

Count cells in the quadrants outlined in red

1. **Open the “Influenza Granzyme B Counting Template” located in the Influenza UO1 2010\_Lab folder in the Granzyme B subfolder. Copy the blank template into a new worksheet. Label the worksheet with the date and your initials. Record both live and dead cell count on the template. The spreadsheet automatically calculates cell concentration.**

For your information, the spreadsheet calculates cells as follows:

[number of live cells/4]\*10,000\* dilution factor of 20 \*total volume (2 ml) =total cells

1. Add the indicated amount of RPMI with 5% FCS (column L of template) to the appropriate conical tube containing PBMC to adjust cell concentration to 2 x106 cells/ml. If the volume in column L is negative, spin sample down at 1200 rpm for 7 minutes at 4 degree, aspirate/discard the media and add the total amount of RPMI in column K.
   1. Drop PHA, then one unstimulated, then one stimulated if you don’t have enough cells. (see Iana if you have questions)
2. Keep cells on ice until you begin plating for the ELISPOT set-up. It is imperative that you begin plating within 30 minutes after cell counting to minimize cell death.
3. **Human Granzyme B ELISPOT -Plating and Culturing PBMCs with Influenza virus in ELISPOT plates**

**NOTE 1: Do not touch the bottom of the wells (the membrane) with the pipette tips as this will damage the membrane.**

**NOTE 2: Carry out steps 1-8 in the tissue culture hood.**

1. Two hours before plating cells, remove one **pre-coated** Granzyme B ELISPOT plate (for 12subjects) from the cold room in order to perform the blocking step.

a. Coating: (1:200 dilution, 5µg/mL final capture antibody concentration) Dilute 50µl of capture antibody in 10mL Coating buffer (sterile PBS, pH 7.2). Add 100µl of diluted antibody solution to each well of the ELISPOT plate in the tissue culture hood and seal the plate with Mylar plate sealer. Store plates at 4°C (walk in cooler) overnight! Coating should be done the day before assay setup!

1. Label the lid of the ELISPOT plate with the following information
   1. The subject ID number in each column.
   2. Unstimulated in rows H, G and F.
   3. Influenza virus MOI 0.5 in rows E, D and C.
   4. PHA in row B.
   5. Your initials, date, VATS run number, and Grz B on BOTH the lid and on the ELISPOT plate
2. Blocking: Discard coating antibody. Using a 50-300 µl multichannel pipette (AND STERILE TIPS), wash wells 1X with 200 µl/well Blocking Solution (RPMI with 10% FCS). For Blocking add 200 µl/well Blocking Solution (RPMI with 10% FCS) and incubate for 2 hours at room temperature (in the tissue culture hood).

## Prepare the Influenza virus and PHA as described under preparation of “Supplement” Stocks. Keep on ice until ready to use.

* 1. PHA (final concentration 5 µg/ml)
  2. Influenza virus MOI 0.5

1. Remove the blocking media from each well by “flicking” the plate into a glass pyrex tray (do this in the hood). Pat the plate dry on paper towels until there is no media remaining in the ELISPOT plate.
2. Using a p100 or p200, add 100 µL of cells (200,000 cells/well) for each subject (for unstimulated - triplicate, for stimulated - triplicate and one well for PHA, one column with 7 wells per subject). Mix the tube well while pipetting. DO NOT pour the cells in a reagent boat as there isn’t enough volume.
3. Add 100 µL of the appropriate supplement to the appropriate wells using a 50-300 µL multichannel pipette and STERILE tips (see template below):
   1. Add 100 µL of RPMI with 5% FCS in triplicate in H, G and F. (unstimulated)
   2. Add 100 µL of Influenza virus in triplicate in E, D and C. (stimulated)
   3. Add 100 µL of PHA to B.
4. Cover the plate with aluminum foil and place in the ELISPOT incubator (at 37°C/5% CO2 for EXACTLY 24 hours. Do not disturb the cells during the incubation period.
5. Detection antibody (from this point aseptic conditions are no longer needed!): After the incubation, begin with step 8 (Detection Antibody, page 6) of the BD Protocol (refer to their protocol for detailed instructions). Briefly, after 24 hours, remove the media from the ELISPOT plate by ‘flicking’ into the sink. Using a 50-300 µl multichannel pipette, wash the plate 2X with 250µL/well of deionized (DI) water (allow the wells to soak for 3-5 min at each wash step). Wash wells 3X with 200µL/well Wash Buffer I (PBS containing 0.05% Tween-20) and discard wash buffer. Remove wash buffer by ‘flicking’ into the sink. Pat the plate dry on paper towels between washes. Dilute detection antibody in Dilution Buffer (PBS containing 10% FBS) and add 100µL/well. Incubate the plate for 2 hours at room temperature.

a. Immediately before using, prepare detection antibody: (antibody dilution 1:250, 2µg/mL final detection antibody concentration) Dilute 40µl of biotinylated detection antibody in 10mL Dilution buffer (PBS, containing 10% FBS) and mix well. Add 100µl of diluted detection antibody solution to each well of the ELISPOT plate and incubate the plate for 2 hours at room temperature (as noted above).

1. Remove the detection antibody by ‘flicking’ the plate in the sink. Pat the plate dry on paper towels.
2. Wash wells 3X with 200µL/well Wash Buffer I (PBS containing 0.05% Tween-20) and discard wash buffer (allow the wells to soak for 1-2 min at each wash step).
3. Immediately before using, prepare the Streptavidin-HRP enzyme conjugate by adding 100 µl of Streptavidin-HRP concentrate into 10 mL of Dilution Buffer (PBS, containing 10% FBS). Mix well! Add 100 µL of DILUTED Streptavidin-HRP into each well using a multichannel pipette.
4. Incubate for 1 hour at room temperature. During the incubation, warm the Chromogen to room temperature.
5. Final washing: Wash wells 4X with 200µL/well Wash Buffer I (PBS containing 0.05% Tween-20), allowing the wells to soak for 1-2 min at each wash step. Discard the wash buffer. Wash wells 2X with 200µL/well Wash Buffer II (PBS) and discard the wash buffer.
6. Add 100 µL of Substrate solution (TMB-H Peroxidase Substrate, Moss, Inc.) to each well using a multichannel pipette.
7. Incubate the plate in the dark for 10 min at room temperature.
8. Remove the chromogen by ‘flicking’ the plate in the sink. Pat the plate dry on paper towels.
9. Rinse the ELISPOT plate 3 times with deionized water. Remove the plastic drain and rinse the bottom of the plate.
10. Pat the plate dry on paper towels. Wipe the bottom of the plate dry with paper towels.
11. Invert the plate and let dry overnight. The next day, cover the plate with the original lid.
12. **Scan** the Influenza Granzyme B ELISPOT plate after drying and make sure that the assay looks good. Save the results.
13. **Count a batch of** Influenza Granzyme B ELISPOT assays using the optimal pre-defined parameters and save the results in the specific folder.

**Influenza UO1 GrzB ELISPOT Template:**

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 Sub.ID | 2  Sub.ID | 3  Sub.ID | 4  Sub.ID | 5  Sub.ID | 6  Sub.ID | 7  Sub.ID | 8  Sub.ID | 9  Sub.ID | 10  Sub.ID | 11  Sub.ID | 12  Sub.ID |
| A | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| B | PHA | PHA | PHA | PHA | PHA | PHA | PHA | PHA | PHA | PHA | PHA | PHA |
| C | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 |
| D | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 |
| E | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 | H1N1 |
| F | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim |
| G | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim |
| H | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim | Unstim |