Activation Induced Marker Assay (AIM)

I – Principle

Full title: Stimulation of PBMC/Spleen/Tonsil cells using Activation Induced Marker (AIM) assay.

This SOP provides a guide for stimulating PBMC/Spleen/Tonsil cells and measuring activation-induced markers (AIMs) to determine antigen-specific response. PBMC/Spleen/Tonsil samples are stimulated with *Plasmodium*-parasitised red blood cells (pRBCs) to investigate malaria-specific cellular response. Upon T cell receptor (TCR) engagement to parasite peptide-MHC complex, antigen-experienced T cells are activated and induce the expression of surface protein markers designated as AIMs. AIMs serve as surrogate markers to identify antigen-specific T cells.

Positive stimulation control can be obtained by incubating cells with Staphylococcal enterotoxin B (SEB) or anti-CD3/CD28/CD49d. These stimulants activate T cells by binding to TCR in a non-antigen-specific manner. Responses can be compared to other antigens, such as Cytomegalovirus (CMV) peptide 55.

II – Safety Overview

- It is the responsibility of all personnel handling human blood samples to ensure they are vaccinated against Hepatitis B and are trained to treat all biological samples as potentially infectious.
- The Safety and Ethics Committee must approve all work before commencement.
- Standard personal protective equipment (PPE) must be worn when performing laboratory work unless otherwise stated. Standard PPE includes.
    - Long-sleeved lab gown, enclosed footwear, safety glasses and gloves.
- Please be aware of the risks involved in working with human samples.
    - Care must be taken when handling blood and purified cell populations obtained from clinical samples.
- Class II Biosafety Cabinet must be used when handling unfixed blood.
    - Cabinet must be sealed and exposed to UV light for 20 min prior to use.
    - Cabinet surface must then be wiped down with 80% (v/v) ethanol.

Any items for use in the cabinet (including pipettes) must be decontaminated with 80% (v/v) ethanol.

III – Equipment, Reagents and Consumables

Equipment

- Finnpipette™ F2: P2, P20, P200, and P1000
- Finnpipette™ F2: Multichannel pipette 30-300uL
- Eppendorf Centrifuge 5910
- Class 2 Biosafety Cabinet
- CellDrop DeNovix (Automatic Cell counter)
- Neubauer Haemocytometer Cell Counting Chamber
- CO2 incubator (at 37C)
- Water Bath (at 37C)
- Fridge 4C
- Freezer -20C
- Freezer -80C
- Liquid Nitrogen tank
- Esky – for transport of samples between facilities
- DynaMag™-2 Magnet (Thermo Cat no#: 12321D)

Reagents/Chemicals

- Acridine Orange / Propidium Iodide Assay Protocol (DeNovix, Cat no# CD-AO-PI1.5)
- Benzonase Nuclease (Sigma Aldrich - Cat no# E1014-25KU)
- CMV peptide – JPT PepMix HCMVA (pp65) PM-PP65-2
- DynaBeads Human T-Activator CD3/CD28 (Gibco - Cat no#11161D)
- Foetal bovine serum (FBS) Heat-inactivated (Heat inactivated 56°C, 45 min) (GIBCO -Thermo Fisher)
- PBS- 1X Filtered Phosphate Buffer Saline
- Protein Transport Inhibitor containing Brefeldin A (BFA) (BD Biosciences - Cat no# 555029)
- RPMI 1640 Medium, powder (Thermo Fisher - Cat no# 31800089)
- Sodium Chloride (Sigma Cat no# 1064045000)
- Staphylococcal enterotoxin B from Staphylococcus aureus (Merck Cat no# S4881- 1mg Lyophilized Powder
- Trypan blue 0.4% (Thermo Fisher - Cat no# 15250061)
- UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen - Cat no# 10977015)

Consumables

- Tips (various manufacturers) 10, 20, 200 and 1000 µL for use with Finnpipette™ F2 pipettes (listed above)
- 1.5 ml polypropylene tubes (Various manufacturers)
- 10 ml polypropylene tubes (Various manufacturers)
- 50 ml polypropylene tubes (Various manufacturers)
- Petri Dishes (Various manufacturers)
- 1ml disposable transfer pipette (Various manufacturers)
- Cryovials (Corning)
- FACS tubes (Various manufacturers)
- 96-well Clear TC-treated Microplate, Individually Wrapped, with Lid, Sterile (U- and V- bottom)

Recipes used in SOPs.

FACS Buffer

- 100 mL 1x PBS
- 1 mL Heat Inactivated-FBS
- 20 mg sodium azide

Malaria Thawing solution (MTS)

- Add 3.5 g NaCl to 100 mL dH2O.

Glossary:

- **R10:** RPMI 1640 supplemented with 10%FBS.
- **CCM:** complete culture media
- **RBCs:** red blood cells
- **pRBC:** parasitized red blood cells (RBCs infected with *Plasmodium falciparum*)
- **SEB** Staphylococcal enterotoxin B from Staphylococcus aureus SEB

IV – SOPS

PBMC/SPLEEN/TONSIL CELLS Thawing.

Procedure to be carried out in BSCII – Lab#8

Process	Steps
Thaw PBMC/ Spleen/Tonsil cells	1. Thaw PBMC/Spleen/Tonsil cells according to the relevant SOP.
Determine which stims can be performed	2. After calculating the total PBMC/Spleen/Tonsil cells, determine which stimulation conditions can be performed for each sample. 3. Record on plate template. 4. Stimulation priority is listed in the Comments section.
Resuspend PBMC/Spleen/Tonsil cells	5. Resuspend PBMC/Spleen/Tonsil cells in sterile R10 (WITHOUT Benzonase Nuclease) at $1x10^6$ cells in 100 uL ($10x10^6$ cells/mL).
Rest PBMC/Spleen/Tonsil cells	6. Transfer the appropriate volume of PBMC/Spleen/Tonsil cells into a 96-well U-bottom plate. 7. Top up each well to a total vol. of 200 uL R10 per well. a. Example: Ideally, 100 uL of each PBMC/Spleen/Tonsil Cells sample should be transferred to a well for each stimulation condition. Then, top up with an additional 100 uL R10. If <100

	uL of PBMC/Spleen/Tonsil Cells sample is added, be sure to account for this volume by adding >100 uL R10. 8. Rest the plate in the incubator at 37 °C, 5% CO2 for 1-2 h, or as required for specific assay setup.

Parasite thawing (if stimulating with alternative antigens skip this step)

Procedure to be carried out in BSCII – Lab#8

Process	Steps
Prepare Media	1. Make up Malaria Thaw Solution (MTS) – Filtered 3.5% 2. NaCl (in sterile MilliQ water). 3. Prepare MTS: PBS (1:1).
Thaw red blood cells (RBCs)	4. Transfer parasitised RBC (pRBC) and uninfected RBC (uRBC) vials to a biohazard hood and thaw vials by warming in hand. 5. Once thawed, add 20 uL MTS dropwise while flicking. Rest 5 min at room temperature. 6. Add 1 mL MTS dropwise while flicking. Gently transfer the cryovial to 1.5 mL microfuge tube. Centrifuge at 2,000 rpm, 2 min in microcentrifuge. Discard supernatant using a P1000 pipette. 7. Add 1 mL of MTS/PBS dropwise while flicking. Centrifuge at 2,000 rpm, 2 min in microcentrifuge. Discard supernatant using a P1000 pipette. 8. Add 1 mL PBS dropwise while flicking. Centrifuge at 2,000rpm, 2 min in microcentrifuge. Remove supernatant using a P1000 pipette. 9. Gently resuspend the pellet in 1 mL of warmed R10.
Count RBCs	10. Take 10 mL of cell suspension and mix it with 10 mL of Trypan blue (may want to dilute further prior to counting). Load 10 uL stained RBCs into a haemocytometer and observe under the microscope. 11. If many dead cells are observed, repeat wash and cell count. 12. Count live RBCs within the top left 16 squares and bottom right 16 squares. 13. Determine cell concentration using calculation in the Comments section. 14. Record RBC counts in the lab book.
Resuspend RBCs	15. Centrifuge at 2,000rpm, 2 min in microcentrifuge. Discard supernatant using a P1000 pipette. 16. Resuspend RBCs in appropriate volume of R10 to make $2x10^6$ cells in 100 uL (~$20x10^6$ cells/mL)

PBMC/Spleen/Tonsil Cells Stimulation

Procedure to be carried out in BSCII – Lab#8

To stimulate PBMC/Spleen/Tonsil cells, that have been rested for 24 h, with various antigens to compare different activation markers.

Process	Steps
Stimulation of PBMC/Spleen/Tonsil cells with RBCs	Add 50 uL of R10 containing pRBCs or uRBCs (1:1 PBMC/Spleen/Tonsil Cells: RBC; 1x 10^6 PBMC/Spleen/Tonsil Cells: 1x 106 PBMC/Spleen/Tonsil Cells) to each sample, according to their designated stimulation condition.
Stimulation of PBMC/Spleen/Tonsil cells with SEB	1. SEB stock is stored as 1 mg/mL (in sterile water) in 50 uL aliquots. 2. Dilute SEB in R10 (See Table 1). 3. For SEB-stimulated samples, add 50 uL of diluted SEB to each sample. 4. For media control wells, add 50 uL of R10 to each sample. 5. All wells should now have a total volume of 250 uL.
Stimulation of PBMC/Spleen/Tonsil cells with anti-CD3/CD49b beads	1. Pre-warm R10 media in 37 °C water bath 2. Remove DynaBeads from fridge. 3. Setup biohazard hood 4. Transfer 1 mL R10 media into sterile 1.5 mL tube 5. Vortex DynaBeads for 1 min 6. Add vortexed DynaBeads immediately to R10 media in 1.5 mL tube. § Use 2.5 uL beads/well. 7. Vortex 1.5 mL tube for 5 sec 8. Place tube into magnetic device (DynaL MPC-E-1) for 1 min 9. Aspirate and discard supernatant from tube whilst in magnetic device 10. Remove tube from device. 11. Add required R10 media to tube. o Use 50 uL DynaBeads solution/well. 12. Remove 96W_RB cell plate from incubator. 13. Thoroughly resuspend DynaBeads in tube and transfer 50 uL/well o Add 50 uL/well R10 media for any unstimulated wells. 14. Return 96W_RB cell plate to the incubator for 20 h
Stimulation of PBMC/Spleen/Tonsil cells with protein/peptide (eg CMV p65)	Use 1X10^6 cellsIncubation time is 6-16 h 1. BFA is added; incubation is done in tubes. o Wait 1 h for BFA if also stimming with protein. o Unlike peptides, antigen preparations may require processing by APCs (antigen-presenting cells). ▪ This would be disrupted if BFA is immediately added. 2. PepMix has enough for 25x tests o Seems that 1 ug/well would be sufficient. o That is approx. 1.7 uL if resuspended in 42 uL ▪ 2 uL if it had been resuspended as recommended.

	▪ Protocol says to add 2 uL to 98 uL media (1:50) o Need to use DMSO as NEGATIVE control. o Activated T cells will downregulate TCR receptors to varying degrees.
	17. Gently resuspend cells using a multichannel pipette. Incubate plate in an incubator at 37 °C, 5% CO2 for 17-18 h.

PBMC/Spleen/Tonsil Cells Flow Cytometry Staining

Procedure to be carried out in BSCII – Lab#8

Staining panel can vary – record specifics for each assay. The panel must be optimised prior to assay. Protocol below may need modifications (for example, staining times/temperatures) depending on specific panel set up.

Process	Steps
Preparation	1. Make up 2% FBS/PBS. Place in fridge.
Storing cell culture supernatant	2. Following 17-18 h stimulation, centrifuge plate at 1,500 rpm, 5 min. 3. Transfer supernatant using a multichannel pipette in duplicates to labelled 96-well U-bottom plate (ie, ~100 uL supernatant per sample into 2 plates). Seal the plate using Parafilm sealing film and place plate in an appropriate storage box. Store in a -80 °C freezer directly. Record box position in lab book. 4. Add 200 uL of 2% FBS/PBS to each well of cell plate and resuspend using a multichannel pipette. 5. Centrifuge plate at 1,500rpm, 5 min. Aspirate supernatant
Perform first surface stain. at 37 °C	6. Prepare first “chemokine” surface stain mix (including Fc Block) 7. Add 50 uL/well of the first stain mix). 8. Resuspend wells using a multichannel pipette. Incubate plate in the incubator at 37 °C, 5% CO2 for 45 min. 9. Top up each well with 150 uL of 2% FBS/PBS. Centrifuge plate at 1500rpm, 5 mins. Aspirate supernatant.
Perform Live/Dead Staining	10. Wash each well with 200 uL of PBS (WITHOUT FBS). 11. Centrifuge plate at 1,500rpm, 5 min. Aspirate supernatant. 12. Add 50 uL live/dead stain (made in PBS only – see Table 3). 13. Resuspend using a multichannel pipette. Incubate the plate in the dark at room temperature for 15 min. 14. Add 150 uL/well 2% FBS/PBS. 15. Centrifuge plate at 1,500 rpm, 5 mins. Aspirate supernatant.
Perform second surface stain at room temperature.	16. Prepare the second surface stain mix. Add 50 µL/well of second stain mix. 17. Resuspend using a multichannel pipette. Rest the plate in the dark at room temperature for 30 min. 18. Add 150uL/well 2% FBS/PBS.

	19. Centrifuge plate at 1,500rpm, 5 mins. Aspirate supernatant. 20. Repeat wash by adding 200 uL of 2% FBS/PBS. 21. Centrifuge plate at 1,500 rpm, 5 mins. Aspirate supernatant.
Fixing cells with BD stabilising fixative	22. Dilute 3X BD Stabilizing Fixative (in MilliQ water so it becomes 1x BD stabilizing fixative). This is to be made FRESH each time. 23. Resuspend cells in 200 uL of 1X BD Stabilizing Fixative. 24. Incubate plate in the dark at room temperature for 20 min. 25. Centrifuge plate at 1,500rpm, 5 min. Aspirate supernatant. 26. Resuspend in 150 uL of 2%FBS/PBS (FACS Buffer) and transfer to labelled FACS tubes 27. Acquire events on flow cytometer.

Staphylococcal enterotoxin B

Process	Steps
Prepare aliquots.	SEB stock is prepared as 1 mg/mL (in sterile water) in 50 uL aliquots (50 ug of SEB per aliquots)

Vial- pack size 1 mg

Preparation Instructions

1. Open the vial inside the fume hood.
2. Add 1ml of MilliQ Sterile water into the vial and pipette up and down until the powder is completely dissolved
3. Aliquot the dissolved SEB in PCR tubes: add 50 ul per tube and store at 4 degrees in the "Schedule Posions Box"
4. Lock the box and record usage in Schedule Posions Logbox

Storage/Stability The freeze-dried toxin stored at 4 °C for over 1 year showed no loss in biological activity or changes in its solubility in water.

https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/126/857/s4881pis.pdf

Stimulation priority list example : uRBC, pRBC | SEB |Media

Cell Concentration Calculation

- Total cell amount = Cell count x 10,000 x cell volume
- E.g. If cells counted is 200 cells;

- 200 x 10,000 x 5 = $10E^6$ cells total in 5 m