Cell Stimulation Assay

I – Principle

This SOP describes the process of stimulating human whole blood ex vivo, or cryopreserved PMBC, spleen, and/or tonsil cells. Stimulations include PMA (Phorbol 12-myristate 13- acetate)/Ionomycin, lysates of or intact *Plasmodium falciparum-infected* red blood cells (Pf-lysate, pRBCs), lysate of or intact uninfected red blood cells (U-lystate, uRBC). Following stimulation, cells can be stored in PROT1, or analysed following antibody staining and acquisition via flow cytometry.

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
- Microscope – inverted.
- CO2 incubator (at 37C)
- Mr. Frosty containers
- Neubauer Haemocytometer Cell Counting Chamber
- Water Bath (at 37C)
- Liquid Nitrogen tank
- Esky – for transport of samples between facilities
- Fridge 4C

- Freezer -20C & Freezer -80C

Reagents/Chemicals

- Brilliant Stain Buffer (BD Horizon Cat no# 566385)
- Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Cat no#51-2091k7) - The kit provides two reagents, fixation/permeabilization solution and BD Perm/Wash™ Buffer.
- Ionomycin (Sigma Aldrich - Cat #I0634)
- Phorbol 12-myristate 13-acetate (Sigma Aldrich - Cat #P8139) (abbreviation: PMA)
- Protein Transport Inhibitor containing Monensin (BD Biosciences - Cat #554274) (abbreviation: Monensin)
- Protein Transport Inhibitor containing Brefeldin A (BD Biosciences - Cat #555029) (abbreviation: BFA)
- SMART Tube Proteomic Stabilizer PROT-1 (Fisher Scientific - Cat #PROT1-1L)
- Stabilizing Fixative 3X concentrate (BD – Cat no#664907)
- 1X Filtered Phosphate Buffer Saline (PBS)

Consumables

- Tips (various manufacturers) 10, 20, 200 and 1000 µL for use with Finnpipette™ F2 pipettes (listed above)
- 1.5 ml polypropylene tubes (Eppendorf)

IV – SOPS

Stimulation of whole blood ex vivo with PMA/Ionomycin and cryopreservation in Proteomic Stabiliser PROT-1

Process	Steps
Prepare cluster tubes.	1. Label empty cluster tubes a. PMA/I Stim, Nil PMA/I b. Performed in duplicate per patient/volunteer donor. 2. Obtain additional 2x cluster tubes for at least one individual to be used for unstained controls for spectral flowcytometry. a. 1x tube for PMA/I Stim b. 1x tube for Nil PMA/I
Prepare BFA and Monensin	3. Remove BFA (Golgi-Plug) and Monensin (Golgi-Stop) from 4 °C storage. Confirm that BFA contents are frozen. 4. Allow the BFA tube to thaw until contents are completely liquid (Approx. 15 min) 5. Vortex BFA and Monensin on high speed for 20 sec. 6. Prepare the BFA/Monensin master mix as described in Table 1. 7. Vortex BFA/Monensin master mix on high speed for 5 sec.

Prepare PMA and Ionomycin	8. Remove PMA and Ionomycin stocks from -20 °C storage and confirm that contents are frozen. 9. Allow tubes to thaw until contents are completely liquid. Repeatedly mix tube contents using P20 pipette. 10. Make appropriate volumes and concentrations of Intermediate PMA and Intermediate Ionomycin as described in Table 2. 11. Vortex tubes on high speed for 5 sec. Combine Intermediate PMA and Intermediate Ionomycin into a PMA/Ionomycin master mix as described in Table 2. 12. Vortex tube on high speed for 5 sec.
Add BFA and Monensin to ALL TUBES	13. Vortex BFA/Monensin master mix on high speed for 5 sec. 14. Add 5 µL of BFA/Monensin master mix to EVERY cluster tube.
Add PMA and Ionomycin to designated tubes.	15. Add 5 µL of PBS to designated cluster tubes. (Nil PMA/Ionomycin) 16. Vortex combined PMA/Ionomycin master mix on high speed for 5 sec. 17. Add 5 µL of combined PMA/Ionomycin master mix to designated cluster tubes containing the sample. (PMA/I Stim)
Add whole blood/PBMC/Tonsils or Spleen cells.	18. Obtain whole blood as described in SOP – BLOOD SAMPLE PROCESSING. 19. Add 250 µL whole blood (per cluster tube) to designated tubes. Immediately mix 6x using 1 mL pipette. 20. If components do not appear to be completely mixed, place lid on the cluster tube and vortex on high speed for 5 sec. 21. Remove the lid after this WITHOUT placing the strip lids on; incubate samples at 37 °C, 5% CO2, for 4 h at 45° angle.
Stabilise with PROT-1	22. After 4 h total stimulation time, add 364 µL (1.4x whole blood volume) PROT-1 to each sample. 23. Mix 8x, or until fully resuspended, with an electronic 1,250 uL multi-channel pipette. a. Mix speed = 6 / Mix vol. 400 uL 24. Incubate for 10 min at room temp. This is a time-sensitive step. 25. During incubation, place cluster tube lid strip onto tubes. Cut lid strip so each tube is separate from adjacent tubes. 26. Vortex cluster tubes on high speed for 5 sec or until coagulated blood on tube bottom is fully resuspended. · a. This step must be done inside the biosafety cabinet. 27. Transfer samples to -80 °C freezer immediately

Stimulation of cryopreserved PBMCs or spleen or tonsil cells with PMA/Ionomycin, followed by antibody intracellular and surface staining.

Process	Steps
All steps in the centrifuge go accelartion:6 and break 6	
PBMC or tonsils or spleen cells thaw	1. Pre-warm some R10 and RPMI in 37C water bath. 2. Thaw the cells by placing the cryovials in 37°C water bath. Swirl the vials gently with preferably keeping the cap above the water level, till a small frozen pellet remains. The entire thawing process should be done as quickly as possible. Do not leave the cryovials in the water bath unattended at any time. 3. Ethanol the cryovials and transfer the contents into a labelled 10mL falcon tube in the hood – GENTLY! 4. Rinse the cryovial with 1mL of 10% FBS+RPMI (R10) and transfer it into the falcon tube – add it dropwise! Add another 8-9 mL of warm 10% FBS+RPMI (R10) DROPWISE into the falcon using a transfer pipette. 5. Spin at 1500rpm for 5 minutes (RT) 6. Aspirate supernatant and resuspend the pellet first with 1mL RPMI. 7. After that, top tube up to 10mL of RPMI. 8. Spin the tube at 1500rpm for 5 minutes (RT). 9. After centrifugation, tip off the supernatant and resuspend the pellet in 3-5 mL of RPMI. (Depending on how concentrated – as this is to count!) 10. Take 10uL of the aliquot and mix it with 10uL of Trypan blue. Dilute further if needed. Count the cells using a hemocytometer. 11. Top the cells to 10mL with RPMI and spin at 1500rpm for 5 minutes. 12. Resuspend the cells in R10 for a concentration of: ~$1x10^6$ cells/ 100 uL ($10x10^6$ cells/mL) 13. Transfer cells to U-bottom plate: 100uL per well (media, uRBC, pRBC, PMA/Ionomycin) – place PMA/Ionomycin in a separate plate. 14. Continue with stimulation, or rest cells in a 37C incubator for ~2 hours or overnight as per specific assay protocol (resting is not necessary for PMA stim, but may be used for parasites stimulation or other conditions).
Activation with PMA/Ionomycin (For Positive Controls)	• PMA stock: 1mg/mL ($1x10^6$ ng/mL) Working concentration: 25ng/mL • Ionomycin stock: 1mg/mL Working concentration: 1ug/mL In 200uL = 5ng PMA + 0.2ug Ionomycin.
	1. Dilute PMA and Ionomycin in R10. 2. Add 50uL of PMA + Ionomycin per well. 3. Gently resuspend.

	4. Incubate cells for 6 hours at 37C. After 2 hours BFA/Monensin is added – see below.
STIMULATION with pRBCs and uRBCs lysates	5. Stimulate PBMCs/Tonsils/Spleen cells with designated stimulants for 24 hours (NOTE – stimulation time needs to be optimised for each specific assay). Top wells up to 190ul with R10. Add PBS around wells. a. 1:2 Pf-lysate/pRBC (ratio of cell:lysate/parasite is assay dependent – needs to be optimized for each specific assay) 100uL cells (1M) + 50uL pRBC lysate (2M) + 10uL IL2 (100 units/mL) + 30uL R10 b. 1:2 u-lysate/uRBC (use the same ratio as for parasites) 100uL cells (1M) + 50uL uRBC lysate (2M) + 10uL IL2 (100 units/mL) + 30uL R10
	Parasite lysates and/or intact parasites/uRBC: corresponding protocol is described in "BoyleLab_SOP_Plasmodium_culture" location: Lab Archives > SOPs and Risk Assessments.
BFA/ Monensin Plug (For PMA/Ionomycin + Media, or parasite stimulations requiring ICS)	• BFA stock: BD: 1mg/mL Working concentration: 10ug/mL. • Monensin stock: BD: 2mg/mL Working concentration: 10ug/mL. BFA/ Monensin is added during the last 4 hours of stim. Ie, 2 hours after PMA+Iono stim, add BFA/Monensin mix. NOTE – timing is dependent on specific assay – optimised as needed.
	1. For BFA+ Monensin mix: Add BFA (BD Plug; 1/12.5) + Monensin (BD Stop; 1/25) to R10. 2. Mix. 3. Add 25uL of BFA/Monensin mix to per well.
	6. Add 10 ul of BFA/Monensin master mix (10ug/mL) after 1hr stimulation, or as optimised for specific assay. 7. Incubate the plate for 23 hours at 37°C
PBMC or tonsils or spleen cell surface staining.	The staining protocol is available at: "BoyleLab_SOP_Plasmodium_culture" location: Lab Archives > SOPs and Risk Assessments. 1. Spin plates at 1500rpm for 5 minutes 2. Wash once with 200uL of PBS. 3. Spin plates at 1500rpm for 5 minutes – aspirate supernatant 4. Aspirate supernatants and add 50uL of live/dead surface stain per well and 50 uL of PBS to unstained wells. Incubate for 15 minutes. 5. After incubation, add 150 uL of 2% FBS/PBS and centrifuge at 1500 rpm for 5 mins. 6. Remove the supernatant and top it up to 200uL using 2% FBS/PBS 7. Spin the plates at 1500rpm for 5 minutes (RT).

	8. Aspirate supernatants and add 50uL of surface stain* per well and 50 uL of 2% FBS/PBS to unstained wells. Incubate for 15 minutes. 9. Spin the plates at 1500rpm for 5 minutes (RT). 10. Remove the supernatant and top it up to 200uL using 2% FBS/PBS 11. Spin the plates at 1500rpm for 5 minutes (RT).
	surface stain* refers to a panel of antibodies to cell surface markers of interest (e.g., CD4, CD8, PD-1, etc). The surface stain mix contains: 1. 15uL of R10 + 10uL of Brilliant Stain Buffer. 2. Add 25uL to all wells (pRBC/uRBC/Media only / PMA+Ionomycin).
PBMC or tonsils or spleen cells intracellular staining	1. Aspirate supernatants and add 100 uL of Cytofix/Cytoperm per well (no dilution) and incubate at 4C for 20 minutes. 2. Add 100uL of BD Perm Wash Buffer (1:10 MilliQ) and spin the plates at 1500rpm for 5 minutes (RT). 3. Remove the supernatant and top it up to 200 uL using BD Perm Wash Buffer 4. Spin the plates at 1500rpm for 5 minutes (RT). 5. Aspirate supernatants and add 50 uL of intracellular stain* per well and 50 uL of BD Perm Buffer to unstained wells. Incubate in the dark at 4C for 30 mins.
	intracellular stain* refers to a panel of anti-cytokine antibodies of interest (e.g. IL-4, IL-21, IL-10 etc)
BD Stabilizing fixative	1. Dilute 3x BD Stabilizing Fixative (in MQ H20) – made FRESH. 2. Resuspend cells in 200uL of 1x BD Stabilizing Fixative 3. Incubate for 20 minutes at RT 4. Centrifuge off. 5. Resuspend in PBS and acquire in the cytometer.

Table 1. Calculation BFA/Monensin Mastermix

	Stock Conc.	Vol. for 1x tube	Final Conc.	If 8 tubes Make for 10x	If 16 tubes Make for 18x	If 24 tubes Make for 26x	If 32 tubes Make for 34x
BFA	1 mg/mL	1.250 µL	5 µg/mL	12.50 µL	22.50 µL	32.50 µL	42.50 µL
Mon.	2 mg/mL	0.625 µL	5 µg/mL	6.25 µL	11.25 µL	16.25 µL	21.25 µL
PBS	N/A	3.125 µL	N/A	31.25 µL	56.25 µL	81.25 µL	106.25 µL
Total	N/A	5.000 µL	N/A	50.00 µL	90.00 µL	130.00 µL	170.00 µL

Table 2. PMS and Ionomycin Make-up

Reagent	**Vol. stock required for 1x cluster tube**	**Stock conc.**	***Intermediate* dilution**	**Final dilution**	**Final conc. in cluster tube**
PMA	0.00625 µL	1 mg/mL	1/400 in PBS	1/40,000	25 ng/mL
Ionomycin	0.25000 µL	1 mg/mL	1/10 in PBS	1/1,000	1 µg/mL

Calculation for PMA (Intermediate Dilution)

- Add 2 uL PMA stock into 798 µL of PBS (=1/400 dilution)

Calculation for Ionomycin (Intermediate Dilution)

	For 1x tube	?x (+ extra)	e.g. 20x samples
Ionomycin (stock)	0.25 µL	µL	5 µL
PBS	2.25 µL	µL	45 µL
Total	2.50 µL	µL	50 µL

- Make for minimum 10x samples

Calculation for Combined PMA/Ionomycin Mastermix

	For 1x tube	?x (+ extra)	e.g. 16x samples
PMA (Intermediate)	2.5 µL	µL	40 µL
Ionomycin (Intermediate)	2.5 µL	µL	40 µL
Total	5.0 µL	µL	80 µL