In vitro culturing of *Plasmodium falciparum* parasites

I – Principle

This SOP describes the process of the continuous *in vitro* culturing of *Plasmodium falciparum* erythrocytic stages in human red blood cells (RBCs).

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 OHS 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.
- Perspex box (Microisolator)
- CO2 incubator (at 37C)
- Mr. Frosty containers.
- Neubauer Haemocytometer Cell Counting Chamber
- Water Bath (at 37C)
- MACS Magnet – Miltenyi Biotec. Vario Macs magnetic cell separator
- Bottle of gas (to gas Microisolator that contains cell culture) (1%O_2, 5%CO_2 and 94%N_2)
- Liquid Nitrogen tank
- Esky – for transport of samples between facilities

- Fridge 4C
- Freezer -20C
- Freezer -80C
- vacuum aspirator (Suction Vacuum System installed in BSCII)

Reagents/Chemicals

- Albumax-II – (Thermo Fisher - Cat.no #C36950)
- Casein Sodium Salt, 500g (Thermo Fisher - Cat.no # J65590.36)
- cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail – (Sigma Cat.no #4693159001)
- Count Bright™ Absolute Counting Beads – (Life Tech Cat.no #C36950)
- Dihydroethidium (DHE) – (Thermo Fisher Cat.no # D11347)
- Dry Ice
- EDTA-disodium salt- Thermo Fisher Cat.no # 17892
- E-64 (trans-Epoxysuccinyl-L-leucylamido(4-guanidino) butane). Sigma Cat.no # E3132-10MG; 357.4 g/mol
- HEPES (Thermo Fisher - Cat.no #15630130)
- Hypoxanthine (Sigma - Cat.no #H9636)
- Isopropanol 2.5L – Thermo Fisher
- MycoAlert – Lonza kit 100 tests
- Percoll- GE Healthcare; (Cat.no # 17-0891-01) (density 1.130 g/mL)
- PBS- 1X Filtered Phosphate Buffer Saline
- RPMI 1640 POWDER (Thermo Fisher - Cat.no #31800022)
- Sodium Azide (powder) – Thermo Fisher
- Sodium bicarbonate (Sigma - Cat.no #S5761)
- Sodium Chloride (Sigma - Cat.no # 1064045000)
- Sodium hydroxide (CHEM-SUPPLY - Cat.no #SA178)
- Sorbitol (D-sorbitol) (Sigma - Cat.no# S1876-1KG)
- SYBR™ Green I Nucleic Acid Gel Stain. (Invitrogen - Cat.no #S7563)
- Trypan Blue Solution, 0.4% (Thermo Fisher - Cat.no #15250061)
- Human sera (pool 10 donors)

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)
- Vented flasks (Various manufacturers)
- 1ml disposable transfer pipette (Various manufacturers)
- Cryovials (Corning)
- 500 mL disposable bottles (Corning)
- Parafilm Laboratory Cling film
- Bluey
- Scissors
- FACS tubes (Various manufacturers)

- Microplate 96-well plates sterile (U-well and V-well)
- syringe filters – Acrodisc syringe filters, Supor Membrane, Pall Life Science 1.2um 32 mm cat#4656
- Corning microscope slides, frosted one side, Merch Cat# CLS294875X25

Recipes used in SOPs.

FACS Buffer

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

Complete culture media (CCM) –

For human cells and malaria parasite culturing (it may contain any of the following)

- RPMI 1640 media
- HEPES buffer
- 3.6% sodium bicarbonate
- 5% AlbuMAX II (for culture requiring appropriate PfEMP1 expression, 2.5% AlbuMAXII, 2.5% human sera)
- Gentamicin
- Hypoxanthine
- Heat inactivated human sera.

Wash Buffer

- RPMI 1640 media (only)

Hypoxanthine

To prepare 200 mM hypoxanthine aliquots to be used in RPMI media for malaria parasite culturing.

- 10 mL 5 M NaOH
- 40 mL dH_2O
- 1.36 g (200 mM) hypoxanthine to bottle.

EDTA, Disodium Salt

Solution for preventing RBC clumping in blood smears.

- RPMI 1640 media (only) prepared in distilled water.

Freezing solution

- 10% DMSO in FBS.

Malaria Thawing solution (MTS)

- Add 3.5 g NaCl to 100 mL dH2O.

Mr. Frosty container

- Filled with Isopropanol

Sodium hydroxide (NaOH) – Stock at 5M

For adjusting PH in solutions

- 20 g of sodium hydroxide pellets to 100 mL dH2O

Glossary:

- **CCM:** complete culture media
- **HCT**: haematocrit
- **IEs:** parasitized red blood cells (RBCs infected with *Plasmodium falciparum*)
- **RBCs:** red blood cells
- **pRBC :** parasitized red blood cells (RBCs infected with *Plasmodium falciparum*)
    i. **Note: Trophozoites (aka trophs), schizonts, rings, Merozoites (aka meros)** refer to different stages of the life cycle of Plasmodium parasites within the RBC
- **TC**: Tissue culture
- **uRBCs:** red blood cells

IV – SOPS

Continuous asexual parasite culture

P. falciparum lab strains can be cultured continuously in vitro. Below is an overview of the culturing process. Specific steps are outlined in detail below.

Protocol Overview
Culture can be in wells (for example, 96 well plates), flat dishes (2-30ml), or flasks Prepare cells in a microfuge tube at 1/1 000 dilution. Hematocrit 3-6% (this is the proportion of packed RBC in the total media) Gas Mixture – 1% oxygen, 3% carbon dioxide, 96% nitrogen. Gas the culture chambers prior to incubation at 37C Culture media – changed daily, or at lower/higher frequency depending on the parasitemia. Old culture media can be aspirated directly from the dish, or culture can be spun (1500rpm, 5min) and spent media aspirated prior to culture being resuspended. Culture parasitemia can be measured using Giemsa Stain thin smear. Cultures need to be diluted as necessary using uninfected RBCs.

Count Red Blood Cells

Estimate parasitemia by counting red blood cells infected with *P. falciparum*.

Steps
Prepare cells in a microfuge tube at 1/1 000 dilution. ▪ Add 45 uL PBS ea. to 3x microfuge tubes. ▪ Resuspend pRBCs (or other cells) ▪ Add 5 uL sample to tube 1 1/10 ▪ Transfer 5 uL from tube 1 to tube 2 1/100 ▪ Transfer 5 uL from tube 2 to tube 3 1/1 000 Take 10 uL from tube 3 and load it into the counting chamber (Neubauer) Check 15-50 cells per big corner square. Count all cells in big 4 corner squares. Alternative method via Giemsa staining 1. Take 2-7 ul of parasite culture. 2. Drop onto glass slide

3. Use another glass slide to smear the culture – apply only LIGHT pressure, hold second slide on an angle, touch blood drop and move smoothly along the slide.
4. Dry slide at room temperature
5. Fix slide in 100% methanol for 10seconds
6. Stain slide with 10% Giemsa Stain (made up in MilliQ water) for 1-5 min.
7. Rinse slide with tap water, dry (blot or airdry)
8. Count parasite infected RBCs and RBCs

Serum Processing

To process human serum (or plasma) to be used as a supplement in the culture of malaria parasite-infected RBCs.

Steps

Serum thawing requires min. 24 h

1. Remove fresh frozen serum bags from -20 °C storage in the morning.
2. Place bags in a cold room o/n
3. Transfer bags to a 37 °C incubator on the morning of the following day. Place bags in a bunding tray to prevent spills.
    - Discard any bags that have leaked.
4. Once thawed, place plasma bags in the hood
5. Label 500 mL disposable bottles with plasma reference/batch number
6. Ethanol spray and wipe the middle tube of the plasma bag.
7. Cut the tube and pour plasma into disposable bottles.
    - Keep bags separate.
    - Do not add more than 300 mL/bottle.
8. Heat inactivates at 56 °C
    - 1 h in a water bath, or
9. Transfer heat-inactivated plasma into 50 mL tubes.
10. Aliquot-filtered plasma into 45 mL per 50 mL tube
    - Label tubes with “PL” and the plasma batch for the year
    - Place any remaining plasma in 50 mL tube in the incubator.
        - Incubate for 1 week and check for contamination.
11. Freeze plasma at -20 °C upright.

Red Blood Cells processing

To process/wash Red Cell units into packed RBCs for use in malaria culture.

Steps

<u>Prepare materials.</u>

- Wash buffer (RPMI 1640 alone)

- Red Cells delivered from Red Cross

SUMMARY METHOD

1. Remove Red Cross “Red Cells” from the fridge.
2. Invert to mix.
3. Aliquot 30 mL of blood across 50 mL tubes
4. Keep 2 tubes: place others in the fridge.
5. Add 15 mL of Wash Buffer to the remaining 2 x 50 mL tubes.
    - Note: exact ratio is not important
6. Spin
7. Remove supernatant.
8. The pellet is packed with RBCs at 100% haematocrit.

DETAILED METHOD

1. Obtain Red Cell bag from the culture room fridge.
2. Check the volume of blood in the bag.
3. Label 50 mL falcon tubes with “Red cells”, the date, and the sample code
    - Will be pouring 30 mL aliquots.
    - So, if you have 300 mLs of blood, you need 10 x 50 mL tubes.
4. Place bluey, scissors and 50 mL tubes and Red Cell bag into the biosafety hood
    - Ensure ethanol all items, including the Red Cell bag.
5. Invert Red Cell bag several times to mix.
6. Ethanol spray and wipe the middle tube protruding from the top of the plasma bag.
7. Cut the tube to leave a ~6 cm long spout.
    - Try and choose a section of the tube that contains little/no blood.
    - Fold the tube and cut along the fold.
    - DO NOT TOUCH NEAR CUT END OF SPOUT – you might contaminate the blood.
8. Pour 30 mLs of Red Cells into each 50 mL tube.
    - Aim spout into 50 mL tube but avoid touching inside.
    - Hold the blood bag via the far bottom corner.
    - Pour quickly and smoothly to avoid bubbles and splashing.
9. Add 15 mL of wash buffer to each tube you wish to wash.
    - Place other “unwashed” Red Cells into the fridge
10. Centrifuge on “blood wash” program
11. Remove supernatant.
    - The pellet contains packed RBCs ~ 100% haematocrit.
    - Expect about 15 mLs (I think!)
12. Write “packed RBC’s” on the lids of the washed/packed RBCs, to differentiate them from unwashed tubes.

Parasite Synchronization

Synchronize parasites in the ring state.

Steps

1. 5% (w/v) sorbitol solution:
2. Warm complete culture media to 37 °C in a water bath
3. Place 5% sorbitol on bench to equilibrate to room temperature.
4. Resuspend the entire culture and transfer it into 50 mL tube.
5. Centrifuge at 1,500 rpm, 5 mins, ramp 6, break 0-1
6. Remove supernatant and resuspend in 5% sorbitol to achieve 5% hematocrit (HCT). i.e If you had a 10 mL culture at 5% HCT, resuspend in 10 mL of 5% sorbitol.
7. Incubate for 5 minutes at room temperature.
8. Centrifuge at 1,500 rpm, 5 mins, ramp 6, break 0-1
9. Remove supernatant.
10. Resuspend the RBC pellet in pre-warmed complete culture media to achieve 5% HCT.
11. Transfer to the appropriate dish or flask
12. Place culture into culture chamber, re-gas, and incubate at 37 °C.

Percoll Purification

To purify late-stage *Plasmodium falciparum* parasite-infected RBCs from uninfected RBCs

Steps

1. Make 65% Percoll
2. Collect each culture and centrifuge at 1,500 rpm 5 mins.
3. Remove supernatant and resuspend with media in total vol. 4 mL.
4. Tilt 65% Percoll at a 45° angle and gently overlay 2 mL resuspended blood culture.
5. Repeat for the second tube, then repeat for other cultures.
6. Centrifuge tubes at 1,820 x g (3,300 rpm), 10 mins, low acceleration, no brake
7. Remove the top supernatant.
8. Collect purified parasites at the interface with minimal Percoll.
    - Use P200 or transfer pipette.
    - Uninfected RBCs will pellet.
9. Place purified parasites in a new 50 mL tube
    - Check purity via Giemsa staining.
10. Wash with 10 volumes 1x PBS
    - Keep in pre-warmed media if not used immediately.
    - Will keep parasites healthy.
11. Discard the supernatant and use purified parasites as needed.

Freezing parasites

Freeze cultures of parasite-infected RBCs for long-term storage.

Steps

1. Ensure the freezing container (Mr. Frosty) is topped up with isopropanol and place at 4 °C
2. Culture parasites in a petri dish at 5% HCT
3. Check via microscopy and ensure parasitemia is approx. 3% parasitemia. Parasites need to be frozen at ring stage if planning to use for continuous culture. If other stages, use for short term stimulations and protein assays only.
4. Label cryovial with Initials, date, strain, para stage: GMO (if needed)
5. Collect 4 mL parasite culture from petri dish.
6. Centrifuge at 1500 rpm, 5 mins
7. Remove supernatant: Approx. 200 uL pellet.
8. Gently agitate the pellet.
9. Slowly add 400 uL Glycerolyte (1:2 ratio) drop by drop to pellet. Gently agitate the tube after each addition. Incubate for 5 mins after 100 uL has been added. Gently mix via pipette: Allows Glycerolyte to penetrate cells.
10. Place cryovials in freezing container and transfer to a -80 C freezer overnight
11. Transfer cryovials to dry ice in esky and transport to liquid N_2 room
12. Place cryovials in liquid N_2 storage
13. Record storage location in Lab Archives
14. Mark off number of Mr. Frosty uses (max. 5)

Magnet Purification of trophozoites

Isolate trophozoites from 200 mLs culture at >5% parasitaemia

Steps

Activity occurring: Within BSCII

PART 1: Magnet Purification

PREPARATION

1. Obtain all materials.
   - Magnet, columns, stopcocks, drawing up needles (blunt needle), wire cutter, rack, magnet "stand".
   - Columns can be "C" size or "D" size columns. Note – C is a discontinued product.
   - Media (CCM or wash buffer), ethanol, MQ water
   - 50 mL tubes and 1.5 mL cryovials
   - Frosty Boy and freezing solution
   - Waste bottle – for cleaning column at the end

2. Place frosty boys into the fridge if freezing cells

3. Check stage and parasitaemia of culture.
   - Want 5-15% late trophs, tightly synchronised.

4. Collect culture from T175 flask into 50 mL tube at 10% HCT.
   - i.e. If you have 200 mL of culture at 5% HCT, transfer into 50 mL tubes, spin (1500 rpm, acc 6, break 1), resuspend in total of 100 mL CCM.

- Should now have 2 x 50 mL tubes, place one into the incubator.

5. Label 50 mL tubes
   - For each column, need 3 filter tubes, 1 elution tube, and 1 waste tube.
     - Waste 1, Filter 1A, Filter 1B, Filter 1C, and Elute 1
     - Waste 2, Filter 2A, Filter 2B, Filter 1C, and Elute 2

6. Set up magnet.
   - Use correct column and stopcock for parasite strain.
     - Eg. Use column TS 1 with stopcock TS if isolating 3D_TS strain.
   - Using a syringe, wash column by flushing 5-10 ml media through side port of stopcock up into column, then allow to run out needle and into a waste 50 mL tube.
   - Practice setting drip rate of 1 drip per second.
     - Keep column wet by adding media to top of column as needed.
   - Close stopcock and ensure media level is just above column matrix.
     - Alternately, if ready to magnet, start adding parasite culture immediately

MAGNET

1. Load 5 mL of culture onto each column, **stopcock closed.**

2. Place corresponding collection tube under each filter
   - i.e, Tube "Filter 1A" under column 1, tube "Filter 2A" under column 2….
3. Open stopcock to allow culture to run into collection tube.
4. Adjust drop rate to 1 drop per second of flowthrough.
   - Can go slower if needed, but not faster!
5. Run culture through columns into filter tubes.
   - Top up volume in column as required with transfer pipette.
6. It is important that filters do not run dry during filtration.
7. Once filter tube A contains ~25 mL of flowthrough, **without closing stopcock**, quickly remove tube A and replace with tube B.
8. Now re-filter the flowthrough you have in tube A into tube B.
9. Once filter tube B contains ~25 mL of flowthrough, **without closing stopcock**, quickly remove tube B and replace with tube C.
10. Now re-filter the flowthrough you have in tube B into tube C.
11. Once tube C contains ~25 mL of flowthrough, you can either proceed to wash step, OR refilter tube C into tube D.

WASH – this helps to remove uRBC from column.

12. Whilst the column is still in the magnet, run ≥**30 mL** of media through the column.
    - 30 mLs minimum – want flow through to have no blood tinge.
    - Can also VERY GENTLY syringe 1-2 mLs media from side port up into column to help dislodge uRBC that are stuck in matrix.

ELUTION

13. Run the column dry (otherwise you can't disconnect the column without making mess)
14. Close stopcock
15. Remove column from magnet and place into corresponding 50 mL elution tube.
16. Run up to 45 mL of wash buffer through column until it runs clear.
    - Half volume from top, other half injected from side port up into column.

17. Centrifuge eluent at 1500 rpm, ramp 6, break 1, 5 minutes at RT.

Part 2: Counting and storing.

PREPARE uRBC

1. Add 5 mL packed uRBCs into a 50 mL tube.
2. Top up to 45 mL with CCM, and place into **37°C** water bath until warm.

PURITY CHECK

3. Remove supernatant from culture.
4. Using 1 uL of pellet, prepare a blood smear and determine purity.
    - Aiming for >90% parasitaemia
5. Resuspend pellets 1-2 mL media each.

CALCULATE CELL CONCENTRATION

1. Add 90 uL Trypan Blue to 2x separate wells (A & B) in 96W_VB counting plate.
2. Transfer 10 uL pRBC suspension to Trypan Blue well A (1/10)
3. Transfer 10 uL from well A to Trypan Blue well B (1/100)
4. Load counting chamber with 10 uL from each well.
5. Select dilution containing approx. 15-50 cells per big corner square.
6. Count 4x corner squares and calculate total cell number.
7. Determine how many vials of 1E8 cells to store/usage.
   e.g. If 1.62E9 pRBCs total = 16.2x vials @ 1E8 cells/vial
8. If storing, following steps below section.

Part 3: Clean up.

Clean the column.

1. Disconnect stopcock from column, to remove column from the magnet.
2. Reconnect stopcock to column and place over 50 mL waste tube.
3. Wash each column with ~50 mL of MQ water.
    - Inject most of this volume through side port of stopcock, then empty by tipping into a waste bottle.
    - Some water should also run from top of column out bottom of stop cock.
    - Increase volume if needed - until water runs clear.
4. Repeat above step using 80% v/v ethanol.
5. Use vacuum aspirator to dry column and stopcock.

Storage of purified parasite-infected RBCs

To store purified parasite-infected RBCs (pRBCs) for future use in stimulation assays.

Steps
Activity occurring: Within BSCII PREPARATION 1. Ensure 4x Mr Frosty is available and isopropanol is topped up. 2. Place Mr Frosty in a cold room 3. Add 5 mL packed uRBCs to 50 mL tube (for a stored uRBC control)

4. Top up to 45 mL with CCM and place in 37 °C water bath.
5. Print 30x cryovial labels each with.

    i. *Parasite strain* pRBCs
    ii. 1EX cells in Glyc.
    iii. Date
    iv. uRBCs in Glyc.
    v. 300 uL
    vi. Date

PROTOCOL

8. Magnet purify pRBCs as previously described
9. Check elutions via microscopy.
    - Take photo and print for lab book.
10. Combine all elutions >90% pRBC purity into 1x 50 mL tubes.
11. Count as outline above

CELL HARVEST

12. Centrifuge pRBC and uRBC tubes at 1 500 rpm, 5 min, accel 6, decel 0
13. Discard supernatant as close to pellet as possible
14. Add 500 uL CCM to pRBC tube and resuspend pellet.
15. Back calculate pRBC volume.

e.g. If 700 uL total volume after adding 500 uL CCM = 200 ul pRBCs

16. Adjust pRBC volume to 20 mL with CCM.
17. Centrifuge pRBC tube at 1 500 rpm, 5 min, accel 6, decel 0

18. Place labels on cryovials during spin step
19. Collect freezing containers from cold room and transfer to TC fridge.

CALCULATE GLYCEROLYTE VOLUME

20. Determine volume of Glycerolyte to add to pRBCs.
    - Need to add at 2:1 ratio.

e.g., If 200 uL packed cells, add 400 uL Glycerolyte
= 600 uL total vol.

21. Determine volume of resuspended cells to add to respective vials.

e.g. If 600 uL total vol. to be added to 16.2x vials = 37 uL/vial

FREEZING CELLS

22. Discard pRBC supernatant as close to pellet as possible

23. Slowly add Glycerolyte drop by drop to packed pRBCs.
24. Gently agitate tube after each addition
25. Gently mix via pipette and ensure established volume is correct.
    - If volume is under, add more Glycerolyte to adjust.
    - If volume is over, adjust calculations to add increased volume to each vial.

26. After resuspending, transfer Glycerolyte suspended pRBCs into dedicated cryovials.
    - Place any remaining pRBC material into leftover cryovial for batch testing.
27. Place all pRBC cryovials into 1x freezing container and place at -80 °C.

28. Remove supernatant from uRBC tube.
29. Transfer 2 mL packed uRBCs to new 50 mL tube.
30. Add 4 mL Glycerolyte as described above.
    - Sufficient for 20x cryovials
    - Adjust volumes if > 20x pRBC vials.

31. Transfer 300 uL Glycerolyte suspended uRBCs into dedicated cryovials.
32. Place all uRBC vials into freezing container and transfer to -80 °C storage.

33. Move cryovials the following day into a dedicated -80 °C box.

NOTES

- Increasing Glycerolyte will reduce pRBC survival during thaw.
- Using less Glycerolyte will make the solution too small for even aliquoting.
- Excess uRBCs are stored as previous thaws have yielded less cells compared to pRBCs.
- Do not store pRBCs long-term at -80 °C.
- Mid-late trophs (32-38 h) are preferred for freezing pRBCs.
- Schizonts may rupture during thawing process.

Thaw parasites

Resuscitate malaria parasite-infected RBCs after long-term frozen storage.

Steps

Activity occurring: Within BSCII

*Add all thaw solutions via drop method i.e. 1 drop/sec
*Agitate tube between drops

1. Transfer MTS and PBS to the biosafety hood
2. Prepare MTS: PBS solution in 50 mL tube.
    - Add 5 mL MTS.
    - Add 5 mL 1x PBS.
3. Pre-warm thaw solutions and media in the water bath
4. Remove the frozen cryovial from -80 °C or liquid nitrogen storage.
5. Transfer cryovial to biosafety hood and thaw quickly
    - Should contain 600 uL total vol. (200 uL iRBCs)
6. Gently add 500 uL MTS to cryovial

7. Transfer cryovial contents to labelled 50 mL tube.
8. Incubate for 5 min.
9. Add an additional 4 mL of MTS.
10. Centrifuge at 1,500 rpm, 5 mins
11. Remove supernatant and resuspend pellet via agitation.
12. Add 5 mL MTS: PBS
13. Centrifuge at 1,500 rpm, 5 mins
14. Remove the supernatant and resuspend pellet via agitation.
15. Add 5 mL PBS.
16. Centrifuge at 1,500 rpm, 5 mins
17. Remove supernatant and resuspend pellet via agitation.
18. Resuspend pellet in 10 mL media.
19. Add blood to 5% haematocrit.
    - Usually require additional 300 uL fresh blood
20. Transfer to 10 mL dish
21. Incubate at 37 °C in malaria gas mix (1% O_2 , 5% CO_2 and 94% N_2).

Merozoite Harvest and Count

To isolate and count purified parasite merozoites.

Steps

Activity occurring: Within BSCII

- Culture *Plasmodium falciparum* parasites at 5-10% trophs and magnet purify as above protocol.
- Return to culture at 12 PM in 30 mL dish with excess media (45 mL)
- Harvest at 11 PM-12AM and add 1:1 000 E-64 to the schizont stage. If E64 is added prior to segmentation of parasites, culture will not mature.
    - Final conc. 10 uM.
- Incubate for 6-8 h, smear and filter as below when fully developed merozoites.
- Note – timings can be modified depending on use of merozoites, and parasite cell cycle.

<u>HARVEST MEROZOITES</u>

1. *Ensure 0.1% casein, wash buffer, PBS are prepared
2. Harvest culture and wash plate
3. Remove supernatant and check parasitemia.
4. Block 1.2 um filters by injecting 5 mL 0.1% casein into *mero* tube.
    - Keep syringes for aspirating parasites.
    - Use 1 filter per 1x T175 flask used.
5. Rinse tube with casein and remove.
6. Resuspend schizonts well with wash buffer.
    - Use 5 mL wash buffer per 1x T175 flask used.
7. Aspirate schizonts into 5 mL syringe
8. Rupture schizonts by passing through filter into *merozoites* tube.

9. Rinse syringe with wash buffer and filter again to remove excess meros/hemozoin. Note at this step, merozoites can be passed over magnet filter again to remove all hemozoin if needed.
10. Pellet merozoites by centrifuging at 2 200 x g (3 300 rpm), 15 mins, accel 6, decel 6
11. Remove supernatant and check merozoites via Giemsa staining.
    - May not be able to see pellet.
12. Resuspend in PBS and transfer to microfuge tubes.
    - Add 1mL PBS per 1x T175 flask used.
    - PBS used if using merozoites for ELISA (do not use wash buffer)

STAIN MEROZOITES

13. Remove SYBR dye from -20 °C and thaw.
    - Use DHE (at 1:100 dilution) instead if using GFP parasites.
14. Prepare SYBR at 1:5 000 dilution.
    - Add 1 uL SYBR to 5 mL PBS.
15. Setup microfuge tubes with different merozoites dilutions
    - Will be stained at 1:10 000 SYBR dilution.
    - Or 1:200 DHE dilution

16.

Name	Dye	PBS	Meros	Total Vol.
1/5	50 uL SYBR	30 uL	20 uL	100 uL
1/10	50 uL SYBR	40 uL	10 uL	100 uL
1/20	50 uL SYBR	45 uL	5 uL	100 uL

17. Incubate samples for 20 min, RT in the dark.
    - DHE is done at 37 °C
18. Prepare FACS tubes with 400 uL FACS buffer.
19. Add to each tube.
    - 20 uL beads
    - 2 uL stained merozoites
20. Include the following controls.
    - FACS buffer
    - Unstained meros.
    - Stained meros only
    - Beads only (Count Bright™ Absolute Counting Beads)
21. Transport to FACS facility
22. Run “mero count” program.
    - Count 1 000 beads/tube.
    - Do not need to run controls unless issue arises.

Schizont Lysate

To prepare schizont lysate from purified parasites using

Steps

Activity occurring: Within BSCII

PREPARATION

1. Prepare 50 mL filtered 1x PBS.
    a. Include Protease Inhibitor (PBS-PI) if using lysate for ELISAs.
        - Add 2x tablets to 20 mL sterile 1x PBS. cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail
        - Vortex until dissolved
    b. Do not use PBS-PI if using for PMBC stimulation.

2. Magnets purify schizonts (42 h p.i.) as previously described.
3. Check elution's via microscopy.
    a. Take photo and print for lab book.
4. Combine all elution's >90% pRBC purity into 1x 50 mL tubes.

CALCULATE CELL CONCENTRATION

5. Add 90 uL Trypan Blue to 2x separate wells (A & B) in 96W_VB counting plate.
6. Transfer 10 uL pRBC suspension to Trypan Blue well A (1/10)
7. Transfer 10 uL from well A to Trypan Blue well B (1/100)
8. Load counting chamber with 10 uL from each well.

9. Select dilution containing approx. 15-50 cells per big corner square.
10. Count 4x corner squares and calculate total cell number.

CELL HARVEST

11. Centrifuge pRBC and uRBC tubes at 1 500 rpm, 5 min, accel 6, decel 0

12. Add 500 uL PBS to pRBC tube and resuspend the pellet.
13. Back calculate pRBC volume.

e.g. If 700 uL total volume after adding 500 uL CCM = 200 ul pRBCs

14. Add equivalent cell volume from uRBC tube to new 50 mL tube.
15. Adjust volume in both tubes to 20 mL with PBS.
16. Centrifuge tubes at 1 500 rpm, 5 min, accel 6, decel 0
17. Discard supernatant as close to pellet as possible
18. Resuspend pRBCs and uRBCs at 20% HCT in PBS in 50 mL tube.
    a. e.g. If 200 uL pRBCs, add 800 ul PBS-PI (1 mL total)

19. Aliquot 250 uL resuspended cells into labelled 1.5 mL tubes
    a. e.g. If vol. is 1 mL, aliquot 4x 250 uL into 1.5 mL tubes

20. Print microfuge tube labels during next step.

Parasite strain
Schizont Lysate
1.62E9 cells/mL equivalent.
Date

uRBC Lysate
Date

FREEZE/THAW LYSIS

21. Place dry ice in 500 mL plastic beaker or blood pot
22. Slowly add 3x volumes (80-100% v/v) ethanol directly to dry ice
    a. Perform in fume hood to avoid spitting from dry ice/ethanol.

23. Place 1.5 mL tubes in float
24. Place float in dry ice/ethanol beaker using tweezers.
    a. For 3 min until frozen
25. Remove float and place in water bath.
    a. For 3 min until thawed
26. Repeat for total 5x cycles.
27. Place tubes in esky with wet ice and transfer to sonicator room

SONICATION

Location of sonicator:

Sonicator is in Equipment Room 2. Procedure to be carried out in the Equipment Room 2. Cover the sonicator with a polystyrene foam box to reduce the vibration.

28. Place 1.5 mL tubes in the sonicator tube rack
    a. All 6x tube spots must be occupied to ensure consistent sonication.
29. Sonicate tubes.
    a. High power, 5x cycles
    b. 30 sec on, 30 sec off
30. Place tubes on wet ice immediately after sonication
31. Centrifuge tubes in a microfuge (in cold room) at 12,000 rpm, 10 min
32. Collect supernatant and pool.
33. Aliquot supernatant at 50 uL/tube into labelled 1.5 mL tubes
34. Store pRBC and uRBC lysate at -80 °C

Mycoplasma testing

To test parasite-infected RBC lines for Mycoplasma contamination. All cultures MUST be tested for mycoplasma prior for use in cell stimulation assays.

Steps
Activity occurring: Within BSCII

Culture parasite-infected RBCs in 10 mL dishes described.
2. Prepare 200 mL CCM (Complete Culture Media) without Gentamicin
3. Replace spent media with antibiotic-free (AF) media.
4. Passage parasites 3x in AF-media
5. Split parasites to 0.5% parasitemia in AF-media
6. Incubate for 4 days in conditioned media.
    - i.e. No media change
7. Harvest parasite-infected RBCs
8. Collect 1 mL supernatant and transfer to labelled 1.5 mL microfuge tubes
9. Samples will be tested by using the Lonza MycoAlert mycoplasma detection kit