Standard Operating Procedure
University of Edinburgh

Culturing *Plasmodium falciparum*

Safety:
Plasmodium falciparum is designated as a Category 3 pathogen (with some derogations). All culturing must be done in a Category 3 facility and the safety Code of Practice adhered to. (see separate SOP for Category 3 guidelines).

Reagents:

A CULTURE MEDIUM

RPMI 1640	Gibco cat no 318-074 (500mlsx10 = £35.10)
L-glutamine	Gibco cat no 25030-024 (100ml =£3.06)
Hepes buffer 1M solution	Gibco cat no 15630-056 (100ml = £15.57)
Gentamycin 10mg/ml solution	Gibco cat no 15710-031 (20ml =£9.18)

1M NaOH - make up in Milipore water and filter sterilise
20% Glucose solution - make up in Millipore filtered water and filter sterilise.

B. HUMAN SERUM
Obtained from the SE Scotland Blood Transfusion Service. Usually collected as blood packs. Serum/blood is removed sterilely and centrifuged at 2,500rpm to separate serum from red blood cells. Serum is carefully decanted off and stored frozen in large sterile plastic1 litre bottles. Serum from a minimum of 5 (preferably 7) donors is pooled before being aliquotted in 40 or 50ml aliquots at-20 deg C (or at -80 deg. C).

C.THAWING SLUTIONS
12% NaCl
1.6 (or1.8) % NaCl
0.9% NaCl + 0.2% glucose or dextrose

D. LYMPHOPREP
Nycomed - Axis-Shield Prod. no. 1114544 1x250 ml
Used if all white cells are to be removed.

E FREEZING SOLUTION
28% Glycerol
3% Sorbitol or Manitol
0.65% NaCl

Filter sterilise and store at -4 Deg. C

or use commercial Glycerolyte

F. PLASMAGEL orGELOFUSINE
for trophozoite floatation

G. METHANOL, GIEMSA STAIN and GIEMSA BUFFER
for staining of slides

H. SORBITOL (for parasite synchronisation)

Make up a solution of 5% sorbitol in PBS (or buffered Incomplete Medium). Filter sterilise and store at 4 deg. C

Methods:

MAKING UP COMPLETE MEDIUM

Remove 62.25ml from a 500ml bottle of RPMI1640 (with 20mM sodium bicarbonate, and with 25mM HEPES buffer [12.5ml to 500 if not already added])

Add:

5ml of 200mM glutamine* (final conc. 2mM)
5ml 20% glucose solution
1.25ml of 10mg/ml gentamicin (final conc. 25micrograms/ml)
1 ml 1M NaOH (to achieve pH 7.2-7.4)
50 (or 40)ml Human serum

*Glutamine should be stored in 5ml aliquots at -20 deg.C.
Everything else can be kept at 4 deg. C.

WASHING BLOOD

Blood should be drawn into packs/tubes containing anticoagulant ACD or CPD. Parasites generally do not appreciate heparin-based anticoagulants.

White cells/buffy coat can be removed in 2 ways:

For normal culturing

1. Spin down blood at 2000rpm for 5 mins.
2. Aspirate off serum and as much of the white-ish buffy coat layer on the surface of the red cells as possible.
3. Wash 3x in Incomplete RPMI medium aspirating , re-suspending and mixing between each wash. Use 100% or more of the volume of cells for washing each time..
4. After the third wash, re-suspend the red cells at 50% haematocrit in Complete medium.
5. This may be stored in the fridge and used up to 2 weeks from the date of drawing.

For experiments requiring all white cells to be removed

1. Layer 10ml of whole blood over 5ml Lymphoprep.
2. Spin for 15 min at top speed of the bench-top centrifuge (± 4,500rpm).
3. Aspirate supernatant and wash cells 3x with 13ml Incomplete RPMI medium
4. Re-suspend cells as above at 50% haematocrit in Complete or Incomplete according to the requirements of the experiment

ASEXUAL PARASITE CULTURING

3-6ml culture in 25cm^2 flasks (standing up)
15-25ml culture in 75cm^2 flasks (lying down)

Haematocrit - 3-6%. 5% for routine asexual cultures

Gas Mixture - 1% oxygen, 3% carbon dioxide, 96% nitrogen
or place in CO2 incubator with caps loose

Change culture medium daily.
Check parasitaemia using Giemsa-stained thin blood smear.
Smears are fixed for 30 seconds in methanol and stained with 10% Giemsa in PBS pH 7.2 for 45 minutes).

Dilute cultures as necessary using O^+ red blood cells which have been washed 3x in Incomplete medium
For dilution:

$$\frac{\text{Parasitaemia of culture}}{\text{Parasitaemia required}} \times \text{Volume required}$$

SORBITOL SYNCHRONISATION

5% sorbitol will kill all stages of the parasite except the ring forms.

1. Choose culture with with high proportion of ring forms.
2. Centrifuge culture and remove supernatant.
3. Re-suspend to original volume in 5% sorbitol (in sterile distilled water). Allow to stand at room temperature 5 minutes.
4. Centrifuge culture, remove sorbitol solution and re-suspend in Incomplete RPMI medium.
5. Wash twice by centrifuging in complete RPMI and set up the new culture.
6. Examine smear of culture after 24 hours. Culture should contain mainly large trophozoites. If parasitaemia is more than 3% trophozoityes dilute to 1%. Examine 18 hours later to identify the appearance of early rings. Depending on the 'tightness' of the synchronisation required, repeat the sorbitol treatment 3-6 hours later. This should produce a cohort of synchronous parasites. However this synchrony will only last 1-2 cycles unless sorbitol treatments are repeated periodically once or twice a wee

(ALANINE SYNCHRONISATION (as from BPRC)

Materials

Heat-inactivated Human serum A+ or AB+ (Bloedbank Leidsenhage, stored at -20°C)
Human RBC 0+ (Bloedbank Leidsenhage, stored at +4°C in RPMI)
RPMI 1640 prewarmed at 37°C
Gentamycin (stored at RT; stock 50 mg/ml) use at 15 μg/ml
0.3 M L-alanine, 10 mM Hepes pH 7.5 pre-warmed at 37°C

Method

1. Pre-warm a solution of 0.3 M alanine, 10 mM Hepes pH 7.5 at 37°C.

2. Spin down the parasite culture (5 minutes 500 g, 20°C).
3. Resuspend the pellet in 5 volumes of 0.3 M alanine, 10 mM Hepes pH 7.5.
4. Incubate for 20 to 30 minutes at 37°C .
5. Add 10 volumes of culture medium and centrifuge culture (5 min. 500 g, 20°C).
6. Resuspend the pellet in culture medium at a 5% hematocrit.

Reference
Braun Breton C. et al. 1988. Nature 332: 457-459.)

SYNCHRONISATION BY PLASMAGEL or GELOFUSINE

1. Use culture that has a reasonable percentage of trophozoite stages. Spin in a 15ml tube, remove supernatant.
2. Re-suspend pellet in Incomplete RPMI to about 40-50% haematocrit.
3. Add an equal volume of Plasmagel or Gelofusine. Mix well and leave for 10-15 minutes at 37 deg. C. or until a clear demarcation can be seen between an upper and lower level.
4. Carefully remove the upper layer to a fresh tube. Add an excess of Incomplete RPMI to wash the cells thoroughly. This upper layer should consist oof a majority (70-90% trophozoites). Rings, schizonta and uninfected red cells should be in the lower layer.
5. Depending on the requirements trophozoites may be further purified by magnet, or the trophozoites may be diluted with uninfected red cells to the required parasitaemia and placed back in culture.

DEEP-FREEZING *P. FALCIPARUM*

1. Choose cultures with a high percentage of ring forms. (All other forms will be destroyed by the deep-freezing process). 5% rings or more is ideal. Place culture in sterile tube and centrifuge at 2000rpm for 5 minutes.
2. Remove supernatant and measure volume of packed red cells. Add an equal volume of deep-freeze solution or glycerolyte slowly to the cells at room temperature and mix well to allow glycerol to penetrate cells.
3. Aliquot final mixture into small screw-topped ampoules - no more than 0,5ml per ampoule.
4. Freeze rapidy by plunging into liquid nitrogen.

THAWING OF DEEP-FROZEN *P.FALCIPARUM*

1. Make up and sterilise by filtration the 3 thawing solutions (A -12% NaCl, B - 1.6% NaCl, and C - 0.9% NaCl + 0.2% dextrose). Sterlize all solutions by filtration through 0.22micron filter.
2. Remove ampoule from liquid nitrogen, place in water bath at 37ºC until fully thawed.
3. Transfer sample to sterile tube and measure volume of blood. Using 0.2ml solution A for every 1ml thawed blood. Add solution A drop by drop, mixing constantly. Allow tube to stand for 3 minutes.
4. Add solution B, drop by drop mixing constantly, using 10ml for each 1ml of original thawed blood.
5. Add solution C also drop by drop using 10ml per ml of original sample volume.
6. Centrifuge at 2000rpm for 5 minutes.

7. Remove supernatant. Re-suspend cells slowly in complete medium to 3-5% haematocrit. Culture should then be maintained under normal conditions.
8. A drop of fresh blood should be added on the 2nd day after thawing when rings should be preparing to burst and re-invade. (If original sample was very small after thawing a drop of washed red cells may be added then to increase haematocrit and/or create a minimum culture of 3ml in which case the fresh blood can be omitted on the 2nd day).