Short EDINBURGH PROTOCOL FOR GROWTH INHIBITION ASSAY WITH CULTURED PLASMODIUM FALCIPARUM PARASITES USING MEASUREMENT OF LACTATE DEHYDROGENASE

A. Culture and Synchronization of Parasites

QUANTITIES Approx

60 wells/50μl per plate (@ 2% Hct 0.3% parasitaemia) x 15 plates = 3ml culture per plate

a) Percoll synchronisation

1. Grow parasites and monitor parasitaemia by Giemsa-stained thin smears. Parasite culture should be late trophozoite/early schizont stages just before synchronization.
2 Prepare 65% Percoll solution and sterilize it by 0.2 μm filtration. Place 5 ml of the 65% Percoll solution in a sterile 15 ml tube and place at 37ºC for at least 15 minutes prior to use.
4. Spin the culture for 5 minutes at 2000rpm in 15 ml tube.
5. Aspirate supernatant, leaving 1-1.5 ml of medium in the tube.
6. Re-suspend the pellet in leftover medium and carefully and slowly overlay it on the 65% Percoll solution. Do not mix!
7. Centrifuge at 4000rpm for 5 minutes at room temperature. The tube should have a brown layer between the medium and Percoll solution. These are late trophozoites and schizonts.
8. Carefully aspirate medium above the brown layer without disturbing it. Leave approximately 1ml of medium on top of the layer. Collect the brown layer and transfer it into a 15 ml tube.
9. Wash out the Percoll by filling tube with incomplete medium and mixing very gently by inverting the tube Centrifuge for 5 minutes at 2000rpm. Aspirate supernatant, leaving 1– 2ml of supernatant on top of the pellet, because the pellet is very fragile. Repeat the wash in complete medium and, depending on the volume of the pellet, add 200 – 600 μL of 50% washed rbcs and complete medium to bring the suspension to 2 – 3% Hct. Maintain the culture as normal and approximately 24 hours after Percoll synchronization carry out 1 or 2 sorbitol treatments

Alternative method for synchronization of trophs/schizonts

Gelofusine synchronization

1. Grow parasites and monitor parasitaemia by thin smears. Parasite culture should consist of late trophozoite and schizont stages at synchronization.
2. Spin culture at 2400rpm and remove supernatant. Re-suspend to about 40-50 % Hct with incomplete RPMI.
3. Add an equal volume of Gelofusine to the culture, mix and leave to stand at 37ºC for about 15 minutes until a clear demarcation line appears between an upper clearer layer containing the ‘floating’ trophozoites and a lower denser layer of uninfected and ring-infected rbc.. Carefully collect the upper layer to a fresh tube, fill to the top with incomplete medium to wash and spin

again as before. Remove supernatant and put pellet of trophs back into culture using approx. 3% Hct uninfected blood cells in complete medium

b) Sorbitol synchronisation

1. Determine parasitaemia of Percoll treated culture by Giemsa stained thin smear Culture should consist predominantly of ring forms.
2. Warm a tube with 5% Sorbitol solution to 37ºC. Spin culture for 5 min at 2000rpm, aspirate supernatant, removing all medium above the pellet.
3. Using a re-suspend the pellet in 10 ml of 5% sorbitol at 37ºC for every 200 µL of RBC pellet. If the pellet volume is less than 200 µL, re-suspend the pellet with 10 ml of 5% Sorbitol at 37°C. Mix, using the same pipette. Place the tube at 37ºC for 10 minutes.
4. Centrifuge at 2000rpm for 5 minutes. Aspirate supernatant. Re-suspend the pellet in complete medium and set up culture in a new flask. To obtain a very tightly synchronized culture, an additional sorbitol treatment might be applied 4 -14 hours later.

Example of Time- line for Percoll / Sorbitol Synchronization.

Percoll Treatment – Monday 5 pm,

Optional Sorbitol Treatment – between 9 pm Monday and 7 am Tuesday,

Sorbitol Treatment – Tuesday 3 pm,

Set up GIA assay – Wednesday 5 pm ± 1 hour

Harvest GIA – Friday 9am-11am

Uninfected Red Blood Cell preparation

Prepare 2% Hct suspension of uninfected human rbcs for dilutions i.e.for 10 ml of 2% Hct suspension, mix 400 µL of 50%RBC and 9.6 ml of medium

B. Preparation of test samples for GIA

Pre-adsorption

Prior to the assay, all serum samples used in the assay should be heat inactivated (56°C water bath for 20 minutes). Animal serum samples need to be pre-adsorbed with human rbcs.

1. Add 50µl of 50% Hct human O+ rbcs per 1 ml of (original serum purified) in a sterile sealed tube, mix, cover with foil and place on a rocker for 1 hour at room temperature.

2. Centrifuge 10 minutes at maximum speed in an Eppendorf 5417C to pellet rbcs then carefully transfer serum supernatant to a new tube. Place the new tube in the refrigerator at 4ºC. Discard pellet.

Sample dilutions

The following procedure should be finalised when conc. of Ab is known

Individual dilutions for test samples are done in a sterile 96-well plate or in the actual GIA assay plate (ELISA flat-bottomed) if there is shortage of sample. Pre-immune serum or normal serum from the same species should be used for Immune serum dilutions so that total serum concentration (pre-Immune, Immune, or combination of two) should be the same in all assay wells. For example:-

If highest conc. of test sample requires 20µL of immune serum in total volume of 100µL then next well in double dilution series should contain 10µL immune serum + 10µL pre-Immune or normal serum in 100µL and so on.

Control serum wells in the same assay should contain 20 µL of Pre-Immune serum in total volume of 100 µL.

C. Setting up test plate

1. Make up IgG @ desired conc. i.e. 10mg/ml
2. Double dilute IgG x 5 with pre-immune IgG as follows:

Well no.	Immune IgG	Pre-iimmune IgG
1	50	0
2	25	25
3	12.5	37.5
4	6.25	43.75
5	3.125	46.875
6	1.0625	48.975

2 Make up and add control sera in 50 µL amounts for the appropriate wells as in Fig 1. i.e. 50 µL @ 10mg/ml in both +ve and –ve controls

3. Fill outer rim of plate with 100ml incomplete medium or PBS to maintain humidity of plate

Preparation of GIA parasite suspension

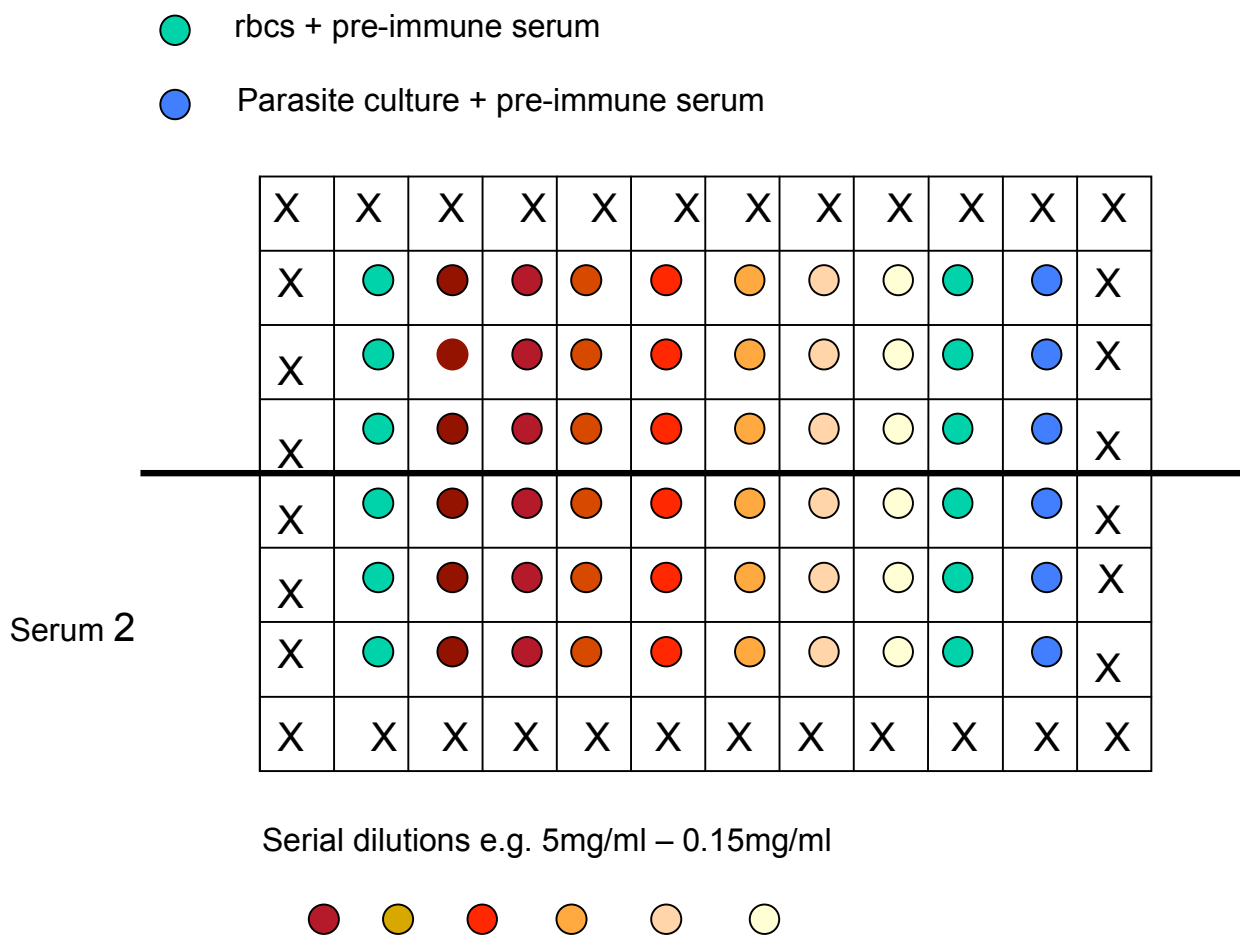
1. Determine parasitaemia of synchronized *P.falciparum* culture . Parasite culture should have mature trophozoite and schizont stages just before GIA set-up.
2. Estimate volume of the culture needed for the assay (± 3 ml of 0.3 ± 0.1% parasitaemia, 2% Hct culture is needed per plate).
3. Transfer the culture into sterile tube and spin down for 5 minutes at 2000rpm. Aspirate supernatant and re-suspend the pellet to 2% Hct with 2X complete medium
4. Dilute 2% Hct culture to a parasitaemia of 0.3 ± 0.1% using the 2% Hct suspension of uninfected human rbcs in 2X complete medium

GIA assay set-up notes

1. Final Hct in all the assay plate wells should be 2%.
2. Parasitaemia at the time of GIA set up should be 0.3 ± 0.1%.
3. Each plate should have at least 2 columns of 3 wells with RBC only ("Pre-Immune Uninfected RBC Negative Control" in Figure 1) made up in the highest concentration of Pre-Immune or normal serum.
4. Each plate should have at least 2 columns of 3 wells with Infected rbcs made up in Pre-Immune or normal serum in the highest concentration (Pre-Immune Infected RBC Positive Control in Figure 1).
5. If test samples are to be compared with the standard, use the standard in 5 serial dilutions in triplicate on each GIA assay plate

Fig 1 shows an example of an assay plate set-up for testing serum samples in triplicates in 5 dilutions (can test 2 samples per plate)

Figure 1:



6. Place assay plates in culture chamber (with moistened tissues to provide humidity), then fill chamber with mixed gas (3% CO2, 1% O2, 97% N2)* for 2 – 3 minutes, seal chamber, and place in 37ºC incubator until harvest.
7. After finishing GIA set-up, make a thin blood smear, designated “time 0 smear” from the parasite suspension used for the assay.

* or similar appropriate gas mixture e.g. 5% CO2, 3% O2, 92% N2

C. GIA Harvest

40 -42hours after GIA set-up, smear original culture, and put GIA plates in fridge to arrest growth while slide stains. If parasites are at the correct stage i.e. late trophs/schizonts then proceed with harvest. If still a bit young then leave and re-test 1-2 hours later according to original estimate of parasite stage.

* Make sure to take a smear from the pre-immune control wells to designate as ‘harvest time smear’ or ‘time 42 hour smear’ for comparison with set-up time smear.

1. When parasites in the plates are at late troph early schizont stage, fill new ELISA plate (one plate for each assay plate) with 250 µL/well ice- cold sterile PBS this time using outer three rows of each plate and place at 4ºC until needed.
2. Remove all assay plates from assay chamber and place them at 4ºC until used.
3. Label new plates with same information, written on assay plates e.g. test rabbit numbers, parasite line and date.
4. Starting with the lowest dilutions of Ab gently but thoroughly mix the contents of the wells and transfer 50 µL of culture to the ice-cold PBS in the corresponding position/row of the new plate (i.e. one row up or down respectively for the 2 assays of the original plate). Repeat step for transfer of all culture samples to the new plate.

(If carrying out FACS analysis and Giemsa staining return th eold plate to 4ºC and take 15 µL into 1ml FACS buffer and make thin smear as soon as possible thereafter)

5. While working with one plate, keep all other plates at 4ºC

6. Place the new plates in centrifuge racks into a Jouan CR412 centrifuge (or similar) fitted with 96-well plate carriers. Centrifuge for 10 minutes at 1,200rcp at 4ºC.
7. After centrifugation, carefully remove 240µL of supernatant out of all wells without disturbing the parasite/rbc pellet
8. Put plates in freezer at -20ºC or -80ºC to lyse rbcs until ready for LDH assay. Stack plates on top of each other, use an empty plate as a cover and wrap the plates with Saran wrap
9. Now is a good time to prepare the remainder of the culture left in the plates for FACS and thin smears if required.
10. Make sure to take a smear from the pre-immune control wells to designate as 'harvest time smear' or 'time 42 hour smear' for comparison with set-up time smear.

D. pLDH Assay

NB*: NBT is light-sensitive, so avoid direct light and keep solutions and plates with substrate in the dark and/or covered with aluminum foil.

1. To make up substrate, warm LDH Buffer to room temp from (frozen stock in deep-freeze) and add 1 tablet NBT per 10ml buffer and mix gently – LDH buffer contains TRITON x-100 so don't shake unless you want an NBT milkshake. Keep tube wrapped in foil.
2. Take plates from the freezer and warm to room temperature for at least 30 minutes. If plates are stacked, separate them to ensure uniform warming).
3. Prepare complete LDH substrate by adding 50 µL of 3-Acetylpyridine Adenine Dinucleotide (APAD) stock and 200 µL of Diaphorase stock to every 10 ml of NBT solution. Mix well and use IMMEDIATELY by adding 100 µL to each well.
4. Set timer to 10-20 minutes. Add 100 µL complete LDH substrate to the wells of the first. If necessary, spin the plate briefly (5 – 15 seconds) at 1,389rcp to eliminate bubbles. Cover with foil and ideally place on a flat bed shaker e.g. Titramax-100 shaker, at 400 setting at room temperature. If no shaker available, agitate gently by hand to ensure thorough mixing of substrate with cell lysate.
5. Read plates at absorbance 650 nm on ELISA Reader at 15 minutes and 30 minutes.

Calculations

NOTE*: Calculate parasitemia in the control wells before starting Percent Inhibition calculations

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Use the following formula calculate percent inhibition:

$$\% \text{ Inhibition} = 100\% - \left(\frac{\text{(A650 Immune sample - A650 RBC only)}}{\text{(A650 Pre-immune control - A650 RBC only)}} \text{ X } 100 \right)$$

Antigen Reversal Assay to estimate specificity of test sera.

1. Prepare and synchronise parasites as for GIA
2. Select desired Ab concentration – usually the serum/Ab concentration that gave 70-80% inhibition on GIA.
3. Select Ag concentrations – usually of the order of 1μg-50μg/ml
4. Incubate antibody at fixed concentration with antigen at serial concentrations for 1 hour at 37°C
5. Add parasitised cells and proceed with GIA protocol as usual.