Standard Operating Procedure

Title: **Flow cytometry-based invasion phenotyping assay**

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Date: 14.09.2010

All procedures should be carried out inside a class II microbiological safety cabinet, to maintain aseptic conditions and protect the operator.

All liquid and solid wastes must be treated in accordance with the local code of practice.

1. Materials

a. Reagents

Reagent name	Source	Catalog number
1% O_2, 3% CO_2, balance N_2	BOC	228447
CFDA SE	Sigma-Aldrich Co.	21888
α-chymotrypsin from bovine pancreas	Sigma-Aldrich Co.	C7762
Complete medium	See section 1, sub-section b	
DDAO SE	Invitrogen	C34553
Dimethyl sulfoxide	Sigma-Aldrich Co.	D2438
Dulbecco's phosphate buffered saline	Sigma-Aldrich Co.	D8537
Gentamicin	Invitrogen	15750037
D-(+)-glucose	Sigma-Aldrich Co.	G7021
Glutaraldehyde	Sigma-Aldrich Co.	G5882
HEPES	Sigma-Aldrich Co.	H4034
Hoechst 33342	Invitrogen	H3570
Hypoxanthine	Sigma-Aldrich Co.	H9636
Incomplete medium	See section 1, sub-section b	
Neuraminidase from *Vibrio cholerae*	Sigma-Aldrich Co.	N7885
Paraformaldehyde	Sigma-Aldrich Co.	76240
Ribonuclease A	MP Biomedicals	101076
RPMI 1640 - liquid	Invitrogen	21875091
RPMI 1640 - powder	Invitrogen	51800019
Sodium bicarbonate	Sigma-Aldrich	S5761

Reagent name	Source	Catalog number
Sodium hydroxide	VWR BDH Prolabo	191373M
SYBR Green I	Invitrogen	S7563
Triton X-100	Sigma-Aldrich Co.	T9284
Trypsin from bovine pancreas	Sigma-Aldrich Co.	T1426

b. Media

All media solution must be kept warm at +37°C throughout their use in the invasion assay.

i. Incomplete media

Reagent name	Quantity
RPMI 1640 - powder	10.43g
HEPES	7.15g
Gentamicin	0.5mL
D-(+)-glucose	2g
Hypoxanthine	1mL of a solution at 50mg/mL in 2M NaOH
MilliQ water	Enough for 1000mL

Adjust the pH of the solution to 7.2 and filter the solution through a 0.2µm membrane. Keep warm in a water bath at +37°C.

ii. Complete media

Reagent name	Quantity
Heat-inactivated human sera pool	50mL
Incomplete media	450mL
Sodium bicarbonate	16mL of a solution at 7.5% in MilliQ water

Filter the solution through a 0.2µm membrane. Keep warm in a water bath at +37°C.

c. Erythrocytes (RBC) and parasites

i. RBC

Leukocyte-depleted RBC need to be obtained in accordance with local legislation on the use of human material for research purposes. RBC need to be washed on the day the invasion assay commences, prior to their use in culture with *Plasmodium falciparum* parasites.

1. Label a 15mL tube with “washed RBC”
1. Using a disposable serological pipette, add 10mL of RPMI 1640 to the tube
2. Using a disposable serological pipette, add 4mL of leukocyte-depleted RBC to the tube

3. Centrifuge the 15mL tube at 1,800g for 5min (with brakes on)
4. Using a disposable serological pipette, aspirate and discard all of the supernatant, as well as the top layer of the cell pellet, from the centrifuged tube
5. Using a disposable serological pipette, add enough RPMI 1640 to the tube to obtain a 50% hematocrit suspension, and pipette up and down to resuspend the cell pellet

ii. Parasitized RBC (pRBC)

The culture of pRBC to be used in the invasion assay needs to be a synchronous, ring-stage culture at the time the donor pRBC are mixed with the target RBC (see section 2, sub-section c). The preparation of the donor pRBC population depends on the parasitemia of the pRBC culture to be phenotyped, as well as the donor:target ratio that will be used to setup the invasion assay (see section 2, sub-section c). If choosing a donor:target ratio of 1:1, a suspension of pRBC at a hematocrit of 2% and a parasitemia of 1-2% needs to be prepared. If, on the other hand, a ratio of 1:2 is chosen, a suspension of pRBC at a hematocrit of 2% and a parasitemia of 1.5-3% will have to be prepared. In either case, when donor pRBC and target RBC are mixed in the appropriate ratio, the parasitemia will be reduced to 0.5-1%.

d. Plasticware

Plastic	Brand and model (example)
1.5mL microfuge tubes, sterile	Eppendorf Safelock
15mL conical tubes, sterile	BD Falcon
50mL conical tubes, sterile	BD Falcon
96-well, flat-bottom plate	BD Falcon
96-well, round-bottom plate, sterile	Corning Life Sciences Costar
Filter-fitted pipette tips, sterile	Gilson Diamond Filter
Reagent reservoirs	Corning Life Sciences Costar
Serological pipettes, sterile	Corning Life Sciences Costar Stripette
Pipette tips	Starlab TipOne

e. Equipment

Equipment	Brand and model (example)
Centrifuge	Eppendorf 5810
Flow cytometer	BD LSRII
Fridge	LEC S53YC
Microbiological safety cabinet, class II	Contained Air Solutions Bio-Class 2
Microcentrifuge	VWR Galaxy MiniStar
Multichannel pipette	Gilson Pipetman Neo Multichannel P200N
Incubator	RSBiotech Galaxy R+

Equipment	Brand and model (example)
Incubation culture chamber	CBS Scientific M-312
Pipettes	Gilson Pipetman P1000, P200, P20 and P10
Pipetting controller	Brand accu-jet pro
Tube rotator	Miltenyi Biotec MACSmix
Waterbath	Grant SUB Aqua12

2. Method

a. Labeling target RBC

The choice of fluorescent label for target RBC is dependent on the choice of DNA dye, which itself depends on the flow cytometer that will be used to acquire the data at the end of the invasion assay. The fluorescence emission peak of CFDA SE is approximately 517nm, close to SYBR Green I at about 520nm. CFDA SE can thus only be used in combination with Hoechst 33342. The fluorescence emission peak of DDAO SE is close to 657nm. So, it can be used in combination with either Hoechst 33342 or SYBR Green I.

Fluorescence label/dye combination	Method sections to be completed						
CFDA SE & Hoechst 33342	a. i.	b.	c. i.	c. ii. 1./c. ii. 2.	c. iii.	d. i.	e.
DDAO SE & Hoechst 33342	a. ii.	b.	c. i.	c. ii. 1./c. ii. 2.	c. iii.	d. i.	e.
DDAO SE & SYBR Green I	a. ii.	b.	c. i.	c. ii. 1./c. ii. 2.	c. iii.	d. ii.	e.

i. CFDA SE

1. Label a 50mL tube with “RBC @ 2% hematocrit”
2. Using a disposable serological pipette, add 12mL of RPMI 1640 to the tube
3. Using a disposable serological pipette, add 0.5mL of washed RBC to the tube to obtain a 2% hematocrit
4. Mix the cell suspension gently
5. Label a 15mL tube with “stained RBC”
6. Transfer 3mL of 2% hematocrit RBC suspension from the 50mL tube to the 15mL tube
7. Centrifuge the 15mL tube at 450g for 3min (moderate brakes can be applied)
8. Using a disposable serological pipette, aspirate and discard all of the supernatant from the centrifuged tube
9. Label a 15mL tube with “CFDA”
10. Using a disposable serological pipette, add 3.1mL of RPMI 1640 to the “CFDA” tube
11. Turn off any direct light source for the reminder of the assay
12. Using a filter-fitted pipette tip, add 12.4µL of 5mM CFDA SE in DMSO to the “CFDA” tube to obtain a 20µM suspension
13. Mix the 20µM CFDA SE suspension thoroughly

14. Using a disposable serological pipette, add 3mL of 20µM CFDA SE to the "stained RBC" tube and immediately pipette the suspension up and down thoroughly to resuspend the pellet[1]
15. Place the tube on a rotator and switch on the rotation at maximum speed
16. Place the rotator inside an incubator at +37°C and incubate for 2h
17. Stop the rotation and centrifuge the "stained RBC" tube at 450g for 3min
18. Using a disposable serological pipette, aspirate and discard all of the supernatant from the centrifuged tube
19. Using a disposable serological pipette, add 3mL of complete media to the "stained RBC" tube and pipette up and to resuspend the cell pellet
20. Centrifuge the tube at 450g for 3min
21. Using a disposable serological pipette, aspirate and discard all of the supernatant from the centrifuged tube
22. Using a disposable serological pipette, add 3mL of complete media to the "stained RBC" tube and pipette up and to resuspend the cell pellet
23. Place the tube on the rotator and switch on the rotation at maximum speed
24. Place the rotator inside an incubator at +37°C and incubate for 30min
25. Stop the rotation and centrifuge the "stained RBC" tube at 450g for 3min
26. Using a disposable serological pipette, aspirate and discard all of the supernatant from the centrifuged tube
27. Using a disposable serological pipette, add 3mL of incomplete media to the "stained RBC" tube and pipette up and to resuspend the cell pellet
28. Centrifuge the tube at 450g for 3min
29. Repeat steps 26 to 28 once more
30. Using a disposable serological pipette, aspirate and discard all of the supernatant from the centrifuged tube
31. Using a disposable serological pipette, add 3mL of incomplete media to the "stained RBC" tube and pipette up and to resuspend the cell pellet
32. Set the "stained RBC" tube aside and discard all other remaining tubes

At this point, it is possible to store the cells protected from light at +4°C for up to 24h, before proceeding to treat the target RBC with enzymes.

ii. DDAO SE

1- Label a 50mL tube with "RBC @ 2% hematocrit"
2- Using a disposable serological pipette, add 12mL of RPMI 1640 to the tube
3- Using a disposable serological pipette, add 0.5mL of washed RBC to the tube to obtain a 2% hematocrit
4- Mix the cell suspension gently
5- Label a 15mL tube with "stained RBC"
6- Transfer 3mL of 2% hematocrit RBC suspension from the 50mL tube to the 15mL tube
7- Centrifuge the 15mL tube at 450g for 3min (moderate brakes can be applied)

[1] Rapidly and completely resuspending the cell pellet is important to ensure homogenous staining of the RBC population

8- Using a disposable serological pipette, aspirate and discard all of the supernatant from the centrifuged tube
9- Label a 15mL tube with “DDAO”
10- Using a disposable serological pipette, add 3.1mL of RPMI 1640 to the “DDAO” tube
11- Turn off any direct light source for the reminder of the assay
12- Using a filter-fitted pipette tip, add 6.2µL of 5mM DDAO SE in DMSO to the “DDAO” tube to obtain a 10µM suspension
13- Mix the 10µM DDAO SE suspension thoroughly
14- Using a disposable serological pipette, add 3mL of 10µM DDAO SE to the “stained RBC” tube and immediately pipette the suspension up and down thoroughly to resuspend the pellet[2]
15- Place the tube on a rotator and switch on the rotation at maximum speed
16- Place the rotator inside an incubator at +37°C and incubate for 2h
17- Stop the rotation and centrifuge the “stained RBC” tube at 450g for 3min
18- Using a disposable serological pipette, aspirate and discard all of the supernatant from the centrifuged tube
19- Using a disposable serological pipette, add 3mL of complete media to the “stained RBC” tube and pipette up and to resuspend the cell pellet
20- Centrifuge the tube at 450g for 3min
21- Using a disposable serological pipette, aspirate and discard all of the supernatant from the centrifuged tube
22- Using a disposable serological pipette, add 3mL of complete media to the “stained RBC” tube and pipette up and to resuspend the cell pellet
23- Place the tube on the rotator and switch on the rotation at maximum speed
24- Place the rotator inside an incubator at +37°C and incubate for 30min
25- Stop the rotation and centrifuge the “stained RBC” tube at 450g for 3min
26- Using a disposable serological pipette, aspirate and discard all of the supernatant from the centrifuged tube
27- Using a disposable serological pipette, add 3mL of incomplete media to the “stained RBC” tube and pipette up and to resuspend the cell pellet
28- Centrifuge the tube at 450g for 3min
29- Repeat steps 26 to 28 once more
30- Using a disposable serological pipette, aspirate and discard all of the supernatant from the centrifuged tube
31- Using a disposable serological pipette, add 3mL of incomplete media to the “stained RBC” tube and pipette up and to resuspend the cell pellet
32- Set the “stained RBC” tube aside and discard all other remaining tubes

At this point, it is possible to store the cells protected from light at +4°C for up to 24h, before proceeding to treat the target RBC with enzymes.

[2] Rapidly and completely resuspending the cell pellet is important to ensure homogenous staining of the RBC population

b. Enzymatic treatments

Regardless of the cell label used, stained target RBC are treated in the same manner throughout this section.

1- Label 6 microfuge tubes A to F
2- Using a filter-fitted pipette tip, transfer 400µL of cell suspension from the "stained RBC" tube to each of the 6 microfuge tubes
3- Using filter-fitted pipette tips, add 8µL of 1U/mL neuraminidase in RPMI 1640 to tube B and tube E
4- Place the 6 tubes on a rotator and switch on the rotation at maximum speed
5- Place the rotator inside an incubator at +37°C and incubate for 1h
6- Stop the rotation and centrifuge the microfuge tubes on a benchtop microcentrifuge for 30s
7- Using filter-fitted pipette tips, aspirate and discard the supernatant from each of the 6 tubes
8- Using a filter-fitted pipette tip, add 400µL of incomplete media to each of the 6 tubes
9- Centrifuge the tubes on a benchtop microcentrifuge for 30s
10- Using filter-fitted pipette tips, aspirate and discard the supernatant from each of the 6 tubes
11- Using filter-fitted pipette tips, add 400µL of incomplete media to each of the 6 tubes and resuspend the cell pellets by pipetting up and down
12- Using a filter-fitted pipette tip, add 2µL of 10mg/mL trypsin in RPMI 1640 to tube C
13- Using filter-fitted pipette tips, add 40µL of 10mg/mL trypsin in RPMI 1640 to tube D and tube E
14- Using a filter-fitted pipette tip, add 40µL of 10mg/mL chymotrypsin in RPMI 1640 to tube F
15- Place the 6 tubes on the rotator and switch on the rotation at maximum speed
16- Place the rotator inside an incubator at +37°C and incubate for 1h
17- Stop the rotation and centrifuge the microfuge tubes on a benchtop microcentrifuge for 30s
18- Using filter-fitted pipette tips, aspirate and discard the supernatant from each of the 6 tubes
19- Using a filter-fitted pipette tip, add 400µL of incomplete media to each of the 6 tubes
20- Centrifuge the tubes on a benchtop microcentrifuge for 30s
21- Repeat steps 18 to 20 once more
22- Using filter-fitted pipette tips, aspirate and discard the supernatant from each of the 6 tubes
23- Using a filter-fitted pipette tip, add 400µL of complete media to each of the 6 tubes resuspend the cell pellets by pipetting up and down, and discard all other remaining tubes

At this point, if sub-sections a and b have been completed on the same day, it is possible to store the cells protected from light at +4°C for up to 24h, before proceeding to incubating the target cells with the donor pRBC.

c. Plate setup and incubation

If the stained target RBC were stored at +4°C, place the tubes inside an incubator at +37°C for 5 to 10min to gently warm them up.

i. Plate layout

	PBS										
			A1	B1	C1	D1	E1	F1			
			A2	B2	C2	D2	E2	F2			
			A3	B3	C3	D3	E3	F3			
											PBS
		PBS									

ii. Plate setup

The volumes of target RBC and donor pRBC to be added to the plate depend on the parasitemia of the donor pRBC population. If the parasitemia is greater than 1% but less than 2%, 50µL of the donor pRBC population and 50µL of the target RBC population should be added to the wells, giving a ratio of 1:1. If the parasitemia is greater than 2%, it is possible to either use a donor:target ratio of 1:1 or 1:2. A ratio of 1:2 is obtained by adding 33µL of the donor pRBC population and 67µL of the target RBC population to the wells. With either ratio, the final volume will be 100µL per well.

1. 1:1 ratio

1. Label 18 wells of a 96-well, round-bottom plate, on its lid, from A to E and from 1 to 3, as indicated in sub-section c, sub-sub-section i.
2. Using a disposable serological pipette, add approximately 200µL of PBS to the 78 unlabeled wells of the plate (shaded in grey in sub-section c, sub-sub-section i)
3. Using a filter-fitted pipette tip, add 50µL of donor pRBC at 2% hematocrit, 1-2% parasitemia, to each of the 18 wells labeled A1 to F3[3]
4. Rotate the plate 180 degrees
5. Using filter-fitted pipette tips, add 50µL of target RBC at 2% hematocrit from each of the 6 microfuge tubes (see sub-section b, step 23) to the corresponding wells (e.g. 50µL from tube A to each of wells A1, A2 and A3)[4]
6. Using filter-fitted pipette tips, pipette up and down the contents of each of the 18 wells, while carefully avoiding to create bubbles (a multichannel can be used to facilitate the mixing)
7. Discard all remaining tubes

[3] Add the donor pRBC by touching the left side wall of the well with the pipette tip

[4] Add the target RBC by touching the left side wall of the well with the pipette tip. Having rotated the plate 180 degrees, this will limit the risk of carry-over between wells

2. 1:2 ratio

1. Label 18 wells of a 96-well, round-bottom plate, on its lid, from A to E and from 1 to 3, as indicated in sub-section c, sub-sub-section i.
2. Using a disposable serological pipette, add approximately 200µL of PBS to the 78 unlabeled wells of the plate (shaded in grey in sub-section c, sub-sub-section i)
3. Using a filter-fitted pipette tip, add 33µL of donor pRBC at 2% hematocrit, 1.5-3% parasitemia, to each of the 18 wells labeled A1 to F3[5]
4. Rotate the plate 180 degrees
5. Using filter-fitted pipette tips, add 67µL of target RBC at 2% hematocrit from each of the 6 microfuge tubes (see sub-section b, step 23) to the corresponding wells (e.g. 50µL from tube A to each of wells A1, A2 and A3)[6]
6. Using filter-fitted pipette tips, pipette up and down the contents of each of the 18 wells, while carefully avoiding to create bubbles (a multichannel can be used to facilitate the mixing)
7. Discard all remaining tubes

iii. Incubation

Regardless of the donor:target ratio, the plates are treated in the same manner throughout this sub-section.

1- Place the plate inside an incubator culture chamber, pre-warmed to +37°C
2- Close the chamber and gas the chamber for 3min with a mixture of 1% O_2, 3% CO_2 and a balance of N_2
3- Place the chamber inside an incubator at +37°C and incubate for 48h

d. Parasite staining

If the target RBC were stained with CFDA SE, staining of the parasites will have to be done with Hoechst 33342. If the target RBC were stained with DDAO SE, either Hoechst 33342 or SYBR Green I may be used to stain the parasites, depending on the laser lines available on the flow cytometer.

i. Hoechst 33342

1- Remove the chamber from the incubator and remove the plate from the chamber
2- Centrifuge the plate at 450g for 3min
3- Using pipette tips, draw 50µL of culture supernatant from each of the 18 wells labeled A1 to F3 (a multichannel can be used to facilitate the work throughout the whole staining procedure)
4- Using pipette tips, add 200µL of RPMI 1640 to each of the 18 wells
5- Centrifuge the plate at 450g for 3min

[5] Add the donor pRBC by touching the left side wall of the well with the pipette tip
[6] Add the target RBC by touching the left side wall of the well with the pipette tip. Having rotated the plate 180 degrees, this will limit the risk of carry-over between wells

6- Using pipette tips, draw 200µL of supernatant from each of the 18 wells
7- Using pipette tips, add 200µL of 2µM Hoechst 33342 in RPMI 1640 to each of the 18 wells and pipette up and down to resuspend the cell pellet
8- Place the plate inside an incubator at +37°C and incubate for 1h
9- Centrifuge the plate at 450g for 3min
10- Using pipette tips, draw 200µL of supernatant from each of the 18 wells
11- Using pipette tips, add 200µL of PBS to each of the 18 wells
12- Centrifuge the plate at 450g for 3min
13- Repeat steps 10 to 12 twice more
14- Using pipette tips, draw 200µL of supernatant from each of the 18 wells
15- Using pipette tips, add 200µL of 2% paraformaldehyde/0.2% glutaraldehyde in PBS to each of the 18 wells and pipette up and down to resuspend the cell pellet
16- Place the plate inside a fridge at +4°C and incubate for 1h
17- Centrifuge the plate at 450g for 3min
18- Using pipette tips, draw 200µL of supernatant from each of the 18 wells
19- Using pipette tips, add 200µL of PBS to each of the 18 wells
20- Centrifuge the plate at 450g for 3min
21- Using pipette tips, draw 200µL of supernatant from each of the 18 wells
22- Using pipette tips, add 200µL of PBS to each of the 18 wells and pipette up and down to resuspend the cell pellet

At this point, it is possible to store the plate protected from light at +4°C for later data acquisition with a flow cytometer. However, the plate has to be acquired within 7 days of the target RBC being initially stained.

ii. SYBR Green I

1. Remove the chamber from the incubator and remove the plate from the chamber
2. Centrifuge the plate at 450g for 3min
3. Using pipette tips, draw 50µL of culture supernatant from each of the 18 wells labeled A1 to F3 (a multichannel can be used to facilitate the work throughout the whole staining procedure)
4. Using pipette tips, add 200µL of PBS to each of the 18 wells
5. Centrifuge the plate at 450g for 3min
6. Using pipette tips, draw 200µL of supernatant from each of the 18 wells
7. Using pipette tips, add 200µL of 2% paraformaldehyde/0.2% glutaraldehyde in PBS to each of the 18 wells and pipette up and down to resuspend the cell pellet
8. Place the plate inside a fridge at +4°C and incubate for 1h
9. Centrifuge the plate at 450g for 3min
10. Using pipette tips, draw 200µL of supernatant from each of the 18 wells
11. Using pipette tips, add 200µL of PBS to each of the 18 wells
12. Centrifuge the plate at 450g for 3min
13. Using pipette tips, add 200µL of 0.3% Triton X-100 in PBS to each of the 18 wells and pipette up and down to resuspend the cell pellet
14. Keep the plate at room temperature and incubate for 10min
15. Centrifuge the plate at 450g for 3min

16. Using pipette tips, draw 200µL of supernatant from each of the 18 wells
17. Using pipette tips, add 200µL of PBS to each of the 18 wells
18. Centrifuge the plate at 450g for 3min
19. Using pipette tips, add 200µL of 0.5mg/mL ribonuclease A in PBS to each of the 18 wells and pipette up and down to resuspend the cell pellet
20. Place the plate inside an incubator at +37°C and incubate for 1h
21. Centrifuge the plate at 450g for 3min
22. Using pipette tips, draw 200µL of supernatant from each of the 18 wells
23. Using pipette tips, add 200µL of PBS to each of the 18 wells
24. Centrifuge the plate at 450g for 3min
25. Using pipette tips, add 200µL of 1:5,000 SYBR Green I in PBS to each of the 18 wells and pipette up and down to resuspend the cell pellet
26. Place the plate inside an incubator at +37°C and incubate for 1h
27. Centrifuge the plate at 450g for 3min
28. Using pipette tips, draw 200µL of supernatant from each of the 18 wells
29. Using pipette tips, add 200µL of PBS to each of the 18 wells
30. Centrifuge the plate at 450g for 3min
31. Repeat steps 28 to 30 twice more
32. Using pipette tips, draw 200µL of supernatant from each of the 18 wells
33. Using pipette tips, add 200µL of PBS to each of the 18 wells and pipette up and down to resuspend the cell pellet

At this point, it is possible to store the plate protected from light at +4°C for later data acquisition with a flow cytometer. However, the plate has to be acquired within 7 days of the target RBC being initially stained.

e. Acquisition on flow cytometer

Regardless of the DNA dye used to stain the parasites, the plates are treated in the same manner throughout this section.

1- Label 18 wells of a 96-well, flat-bottom plate, on its lid, from A1 to F3
2- Using pipette tips, add 250µL of PBS to each of the 18 wells (a multichannel can be used to facilitate the work)
3- Using pipette tips, draw 25µL from each of the 18 wells of the plate containing the stained cells of the invasion assay, and add this to the corresponding wells on the new plate containing only PBS
4- Pipette up and down to mix the diluted cell suspension
5- Acquire the plate containing the diluted cell suspension on a flow cytometer fitted with the appropriate laser lines for the fluorescent cell label and DNA dye chosen[7,8]

[7] The diluted suspension will yield a sample speed of approximately 2,000 events per second when using the lowest sample rate setting.
[8] Acquire 100,000 events to have a minimum of 50,000 target cells to analyse.