SANGER INSTITUTE
STANDARD OPERATING PROCEDURE

SUBJECT: Peripheral Blood Flow Cytometry Analysis of Major Leukocyte Populations using the buffy coat

INTRODUCTION

This SOP details the characterisation of the main leukocyte populations in peripheral blood from mice by flow cytometry using the buffy coat layer after centrifugation of whole blood. This protocol uses 4-colour flow cytometry with a 4 panel staining system.

HEALTH & SAFETY

- Appropriate personal protective equipment is to be worn at all times when handling the samples
- Eye wash stations, safety showers and fire extinguishers are to be located within the laboratory.
- Appropriate training and familiarization with risk assessment must be undertaken before use of the flow cytometer
- Users should be familiar with the relevant Risk Assessments before commencing the procedure

RESOURCES

Equipment:

1. Eppendorf tubes (1.5ml & 2ml)
2. 96 well Microtiter plate, round bottom
3. Lithium Heparin-containing blood collection tubes (1ml)
4. Timer
5. P1000 and P200 single channel & 30-300 multi channel pipettes + tips
6. Multidispense pipette with 5ml and 25ml Combitips
7. Ice box filled with ice
8. Reservoirs
9. Tube rotator
10. Refrigerated microcentrifuge
11. Plate centrifuge
12. 50ml Falcon tubes + Virkon
13. Vortex

14. Lysis buffer: BD Pharm Lyse (BD 555899) - 1:10 dilution with dH_2O

15. FACS buffer
    - 1000ml 1xPBS (no Ca2+ or Mg2+) + 5g BSA, pH 7.45, filtered (store at 4°C)

16. Propidium Iodide (PI) solution (used to distinguish viable and non-viable cells):
    - 1mg/ml PI in FACS buffer (Sigma, store at 4°C). Prepare a 2x solution by diluting stock 1:200 in FACS buffer. When added to cells this will be further diluted 1:2 to give final concentration of 2.5ug/ml

17. Fc-block-solution:
    - Mouse BD FcBlock (cat no. 553142). Dilute to 10ug/ml in FACS buffer. Stock is 500ug/ml therefore dilute 1:50

18. Single Colour Controls – prepare fresh:
    - Remaining cells from all mice are pooled and used for single colour controls. Single colour controls (SCC) contain a single antibody at the dilutions described below (plus PI) and are prepared simultaneously. Fluorescence minus one (FMO) controls are also used as detailed in the tables below. Furthermore, one unstained sample and cells stained only with PI are prepared.

19. Antibodies – prepare fresh:
    - Prepare sufficient amount of antibody-mix with FACS buffer, having calculated the amount of antibodies to be used, based on titration. Tables below describe the staining panels.

ANTIBODY INFORMATION

Antibody mix

Prepare sufficient amount of antibody to stain samples using the table of dilutions below.

Tube 1	**Antigen**	**Dilution**
FITC	CD3	1 in 800
PE	CD25	1 in 640
APC/Alexa 647	CD4	1 in 320
APC-cy7	CD45	1 in 2500
Tube 2		
FITC	CD3	1 in 800
PE	NKp46	1 in 200
APC/Alexa 647	CD8	1 in 320
APC-cy7	CD45	1 in 2500
Tube 3		
FITC	-	
PE	IgD	1 in 640
APC/Alexa 647	CD19	1 in 320
APC-cy7	CD45	1 in 2500

Tube 4		
FITC	Gr1	1 in 3125
PE	NKp46	1 in 200
APC/Alexa 647	CD11b	1 in 320
APC-cy7	CD45	1 in 2500

Fluorescence minus one

There are four fluorescence minus one (FMOs) tubes used with this staining panel, the information for which can be found below.

FMO 1	Antigen	Dilution
FITC	CD3	1 in 800
PE		
APC/Alexa 647	CD4	1 in 320
APC-cy7	CD45	1 in 2500
FMO 2		
FITC	CD3	1 in 800
PE		
APC/Alexa 647	CD8	1 in 320
APC-cy7	CD45	1 in 2500
FMO 3		
FITC	-	
PE		
APC/Alexa 647	CD19	1 in 320
APC-cy7	CD45	1 in 2500
FMO 4		
FITC		
PE	NKp46	1 in 200
APC/Alexa 647	CD11b	1 in 320
APC-cy7	CD45	1 in 2500

Staining procedure

1. Collect blood vial retro-orbital bleeds within heparin coated tubes. Centrifuge samples at 5000RCF, 10 min, 8°C, remove the plasma layer and immediately proceed to next step to avoid clot formation.

2. For each sample, pipette 300 µl of blood into a 2ml Eppendorf tube, taking care to transfer leukocytes from the buffy coat (plasma/RBC interface). To this, add 1.5 ml of lysing solution (BD Pharmalyse) and place on roller for 10 minutes at room temperature (RT).

3. Centrifuge the Eppendorf tubes (400RCF, 3 min, 10°C). Discard the supernatants and re-suspend the pellet in 2 ml of lysing solution. Invert tubes 2-3 times (flick end if necessary) to resuspend pellet and place on roller for 10 minutes at room temperature (RT).

4. Centrifuge the Eppendorf tubes (400RCF, 3 min, 10°C). Discard the supernatants and re-suspend the pellet in 500 µl of FACS buffer. Vortex. (Once re-suspended filter the solution through a nylon net if clots are visible).

5. Transfer the cell suspension into a U-bottomed 96 well plate (See plate layout below) with 100 µl into Tube 1, Tube 2, Tube 3 and Tube 4 positions. Pool the remaining cell suspensions and pipette 100 µl into 16 wells for single colour controls (SCC) and fluorescence minus one (FMO).

6. After plating add 100 µl FACS buffer per well.

7. Centrifuge the plate (400RCF, 3 min, RT). Discard the supernatant.

8. Resuspend the cells in 200 µl FACS buffer per well.

9. Centrifuge the plate (400RCF, 3 min, RT). Discard the supernatant.

10. Resuspend the cells in 50 µl FcBlock per well and incubate 15 minutes at 4ºC. Switch on cytometer at this point.

11. Add 150 µl FACS buffer to each well and centrifuge the plate (400RCF, 3 min, RT). Discard the supernatant.

12. Resuspend the cells in 100 µl Antibody-Mix as seen from the tables above.
    - *In the 4 wells for each sample, use the Antibody mix for tube 1, 2, 3 and 4*
    - *In the 16 wells which contain pooled cells, use a different single antibody for each well and the 4 FMOs (see plate layout below)*

- *To SSC 1 & SSC 3 add FACS buffer.*

13. Protect from the light and incubate for 15 minutes at 4°C.

14. Add 100 µl PI solution per well (excluding the SSCs but including **SSC3**) and then incubate for a further 5 minutes on ice in the dark

15. Centrifuge the plate (400RCF, 3 min, RT). Discard the supernatant.

16. Wash the cells by adding 200 µl FACS buffer per well and centrifuge the plate (400RCF, 3 min, RT). Discard the supernatant. Repeat this wash once more.

17. Re-suspend the cells in 140 µl FACS buffer and store on ice in the dark until acquisition.

18. Define a “stopping gate” of living CD45 positive cells as 15 000 living cells. Data will be acquired until this number is reached.

19. Complete interpretation of the results, including compensation, using FlowJo software.

Plate layout

For ease, the following plate layout should be used:-

	1	2	3	4	5	6	7	8	9	10	11	12
A	SSC 1	SSC 9	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1
B	SSC 2	SSC 10	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2
C	SSC 3	SSC 11	Tube 3	Tube 3	Tube 3	Tube 3	Tube 3	Tube 3	Tube 3	Tube 3	Tube 3	Tube 3
D	SSC 4	SSC 12	Tube 4	Tube 4	Tube 4	Tube 4	Tube 4	Tube 4	Tube 4	Tube 4	Tube 4	Tube 4
E	SSC 5	FMO 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1
F	SSC 6	FMO 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2
G	SSC 7	FMO 3	Tube 3	Tube 3	Tube 3	Tube 3	Tube 3	Tube 3	Tube 3	Tube 3	Tube 3	Tube 3
H	SSC 8	FMO 4	Tube 4	Tube 4	Tube 4	Tube 4	Tube 4	Tube 4	Tube 4	Tube 4	Tube 4	Tube 4

SSC		Protocol	Channel	Volts
SSC 1	Blank	all	All	
SSC 2	CD45-APCcy7	all	FL5	
SSC 3	PI	all	FL3	
SSC 4	CD3-FITC	Tube 1	FL1	
SSC 5	CD25-PE	Tube 1/2	FL2	
SSC 6	CD4-APC	Tube 1	FL4	
SSC 7	NKp46 -PE	Tube 2	FL2	
SSC 8	CD8-APC	Tube 2	FL4	
SSC 9	IGD-PE	Tube 3	FL2	
SSC 10	CD19-APC	Tube 3	FL4	
SSC 11	Gr1-FITC	Tube 4	FL1	
SSC 12	CD11b-APC	Tube 4	FL4	

Mouse ID			
1	M000	11	M000
2	M000	12	M000
3	M000	13	M000
4	M000	14	M000
5	M000	15	M000
6	M000	16	M000
7	M000	17	M000
8	M000	18	M000
9	M000	19	M000
10	M000	20	M000