WELLCOME SANGER INSTITUTE

STANDARD OPERATING PROCEDURE PACKET

SANGER INSTITUTE
STANDARD OPERATING PROCEDURE

SUBJECT: Micronucleus assay

INTRODUCTION:

This document outlines the general procedures involved in the micronucleus assay. This is a mutagenic test that can identify increases in the frequency of normochromatic micronucleated erythrocytes, reticulocytes and micronucleated reticulocytes, all of which can act as markers for chromosomal damage.

ABBREVIATIONS:

EDTA = Ethylenediaminetetraacetic acid
PPE = Personal protective equipment
PPL = Project license
QC = Quality control
SD = Standard deviation
SOP = Standard operating procedure

HEALTH & SAFETY:

This procedure is covered by the following risk assessment WTSI_2097

- Appropriate personal protective equipment (PPE) is to be worn at all times when handling the samples – white coat and gloves
- New employees, or those returning from a significant period of absence, must be supervised until deemed competent at performing this procedure
- Individual risk assessments for young persons and new or expectant mothers would be performed to define any exclusions for performing this assay
- Any employees with disabilities or health issues will undertake pre-employment health questionnaires and seek advice from occupational health
- Lone working and out of hours working is permitted for those deemed competent although out of hours should be restricted to data acquisition where possible
- Ergonomic pipettes should be used to minimise the risk of repetitive strain injury as this process involves multiple pipetting steps
- Users should correctly organise their workspace to minimise stretching and other ergonomic hazards
- New users are required to be trained in the correct operation of the BD LSRII by the core flow facility staff
- The BD LSRII flow cytometer contains class III lasers but this instrument is class I by design
- Under no circumstances should the covers or interlocks on the instrument be overridden to access the lasers

RESPONSIBILITIES:

All staff performing this procedure are responsible for ensuring that this Standard Operating Procedure (SOP) and accompanying risk assessment has been read and understood. All staff should be trained and competent to perform the procedure.

RESOURCES:

Equipment and reagents

- 100 µl EDTA coated blood collection tubes without capillary (Scientific Laboratory Supplies catalogue number 078042)
- Centrifuge (for plates)
- BD Falcon 96 well Flat bottom plates with lid (VWR cat no. 353072)
- Sterilin 96 well V bottom plates with lid (ThermoFisher cat nos 611V96 & 642000)
- FACS buffer (PBS with 0.5% BSA) – see SOP0080 – Preparation of PBS.BSA
- Pipettes (single, multi and repeat) and filter tips
- Long 10 µl filter tips (SLS cat no. F171203)
- DNA dye waste container, plus funnel
- Timer
- 4 ml amber glass vials (Sigma catalogue number 27001-U)
- Antibodies (see appendix B)
- Vortex
- BD LSRII flow cytometer

Associated SOPs/Documentation:

- SOP0046 – Retro-orbital bleed
- SOP0076 – Haematology
- Micronuclei analysis template.xls

Staff: This procedure requires one member of staff to be performed optimally

METHOD – Preparing working antibody solution

1. Prepare sufficient amount of antibody-mix with FACS buffer, having calculated the amount of antibodies to be used, based on the details in appendix 2.
2. In a 4 ml amber glass vial, pipette FACS buffer, then add antibodies and Hoechst as indicated.
3. Label tubes with panel, initials of person who prepared the mix and date.
4. Mix thoroughly by vortexing and store in fridge until required.

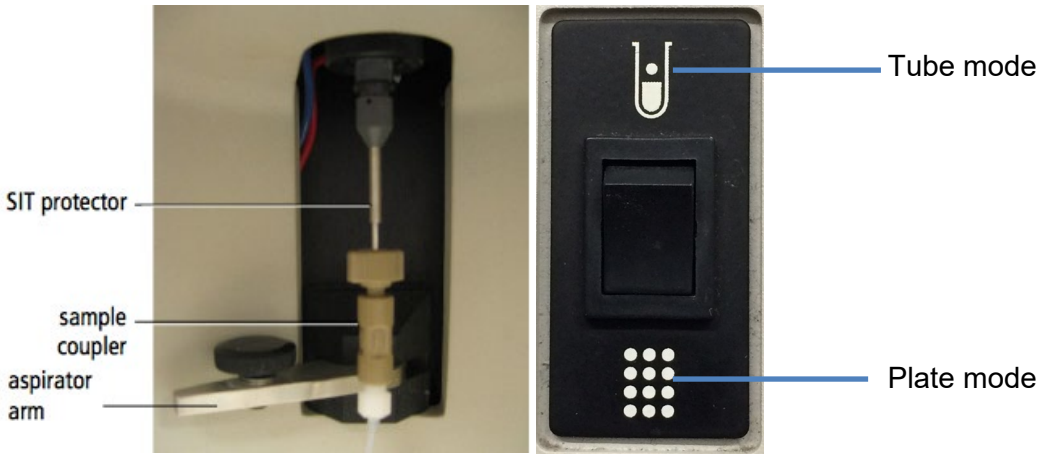
METHOD –staining whole blood

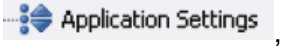
5. Blood is collected according to SOP0046 into 100 µl EDTA tubes
6. Add 49 µl FACS buffer to 96 well V bottom plate to required wells
7. Using a P2 pipette with long filter tips (DLF10ST) reverse pipette 1 µl of blood into the well
8. Note the mouse barcode number on the record sheet for entering into BD FACSDiva software and record the sample number on the EDTA tube lid
9. Repeat with both batches of blood until all samples are on the plate
10. Add 50 µl of 2x CD71/Ter119/Hoechst cocktail to each sample well using the Eppendorf multi-repeater pipette using care to ensure that there is no splashing and cross contamination of the wells.
11. Mix samples with gentle pipetting using a multichannel pipette and filter tips

12. Incubate for 20 minutes at room temperature
13. Add 150 µl of FACS buffer to each well and gently mix
14. Spin plate (400 rcf, 3 minutes) and discard supernatant into **DNA dye waste container using a funnel**
15. Resuspend in 250 µl FACS buffer
16. In 96 well flat bottom plate that will be used for acquisition, add 275 µl FACS buffer to the required wells
17. Transfer 25 µl of the stained blood sample from the 96 well V bottom plate to the acquisition flat bottom plate
18. Place the 96 well V bottom plate in the fridge as a backup in the event of an instrument failure
19. Mix samples well, avoiding generating bubbles and proceed to acquisition

METHOD – Data acquisition

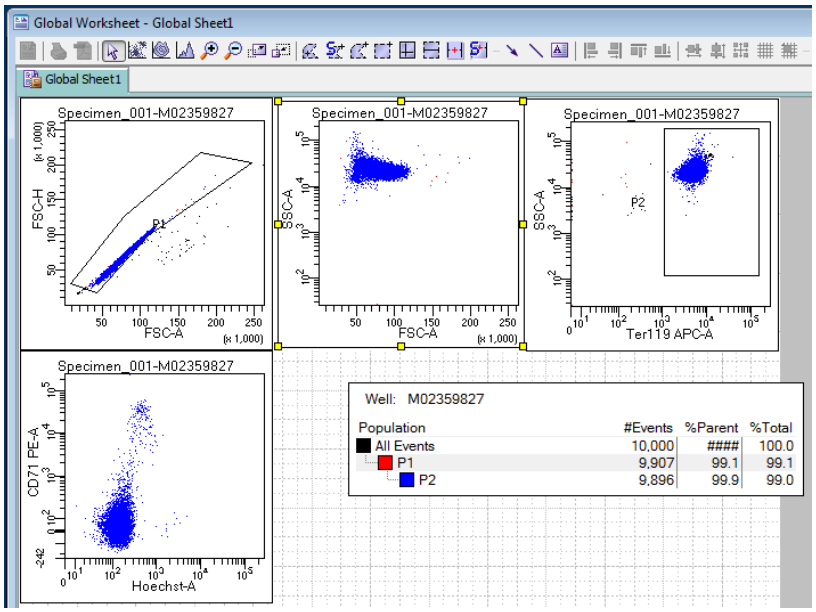
20. At the LSRII verify the instrument is in HTS mode and the HTS is connected to the sample probe – see figures below



21. Log in to BD FACSDiva.
22. Open the experiment template. Rename with today's date in the format YYYYMMDD using the inspector window.
23. Open the blood plate (double click on icon in browser window) and delete sample wells as required.
24. Label wells with the correct mouse barcode number.
25. Apply application settings by right-clicking the application settings icon in the browser window 

, select apply and choose the micronuclei application settings.
26. Place the blood plate containing samples onto HTS platform (make sure A1 is in correct position)
27. Check and/or change the FSC voltage to 450 – 470 and the SSC voltage to 250 – 270. The threshold should be 15,000. Remember, these are only a guide and may need to be adjusted on the day depending on the instrument performance

28. Open the blood samples plate and select the first well
29. Run (acquire) this sample to check the staining and that the cells are on scale, adjust FSC/SSC voltages as required
30. Verify that the FSC-H vs FSC-A singlet doublet exclusion (top left plot) and Ter119 vs SSC gates (top right plot) are set correctly (see figure below). Adjust the position of the gates as required or the voltages. You should expect >90% Ter119+ events (P2).



31. If this looks good, record this sample, a stopping gate of 250,000 Ter119+ singlet leukocyte events is set in the template.
32. Highlight the rest of the samples on the plate and click run wells

Note: The plate can be left to run but should be checked on regularly to make sure no clogs or other problems have occurred.

33. Export the data as FCS files. Go to File>Export FCS. Click OK in next pop-up window, make sure destination drive is correct (in D drive) and click OK. Transfer data to a USB stick
34. Run a cleaning plate on the LSRII. Open the experiment named ‘Cleaning 2017’, double click on the plate icon. Fill the wells with the relevant cleaning solutions as detailed on the laminated sheet and place on the HTS platform. Highlight all wells (B1-F3) and select ‘run wells’ (click OK to overwrite if prompted)
35. **When finished, put the LSRII in standby mode. The LSRII will continue to produce waste whilst on run mode and can overfill the waste container**
    - If there are no further users booked that day, the analyser and computer can be switched off
    - Otherwise, log out of FACSDiva and leave the LSRII on standby.
36. Analyse in FlowJo X by following further steps below.

Troubleshooting

- While the plate is running, check on it periodically to make sure it is progressing as expected. If the plots start looking odd, for example, all cells start to become squashed against the bottom FSC/SSC plot axis, or very few events appear, there may be a blockage. Stop the plate as soon as you notice a problem. You can try a few things to fix this:
    - Run a cleaning panel
    - Prime the sample probe
    - If in any doubt, seek advice from a member of core facility staff
- Sample mixing and correct running can be verified by checking the dot plot showing Time on the x axis and APC on the y axis and will normally be a flat tight line, large alterations to the flow rate will be indicated by broadening of the line or gaps appearing
- Make a note on the plate layout sheet about any issues that occur during the run. This will help when it comes to the data analysis and gives a valid reason if any results need to be QC failed
- If a blockage or other problem has caused data from some wells to be unusable, and if it can be resolved on the day then you can go back to the 96 well V bottom plate with the stained sample and repeat the process
- If there is a problem with just the HTS then either ask the core facility staff if one from another instrument can be used or there is the possibility to use tubes for data acquisition

Analysis of micronuclei data in FlowJo

METHOD

Arranging the Data for Analysis

1. After logging into the Mac, connect to the team drive

2. Copy the raw data from the memory stick into the folder for the correct month/day. Remove the memory stick.

3. Open the Micronuclei template in FlowJo

4. Drag and drop the raw data folder into the FlowJo workspace. All files in that folder that FlowJo recognizes as samples will be imported and should be sorted automatically into one group.
    - If it looks like this has not happened properly, double click on the name of the group and select the staining protocol to define the sample group

5. Save the FlowJo file as a workspace in your analysis folder with the date of the run (e.g. Micronuclei analysis 20171016.wsp).

Open plots

Gate hierarchy:

- FSC-H vs FSC-A
    - SSC-H vs SSC-W
        - APC-A Ter119 vs Time
            - SSC-A vs APC-A Ter119
                - PE-A CD71 vs Hoechst

Note: On any axis, click the T icon to allow adjustment of axis scaling using the slider, making sure all events can be seen and giving better visualization of the populations.

6. Open all plots as described in the following steps, but do not move any gates until they are all open and you have selected a representative sample to set gates on while synchronised.

7. Open the first sample (Figure 1)
    - X axis = FSC-A; Y axis = FSC-H
    - Set the gate on the main body of cells, as shown in Figure 1. Ignore the events below the gate, these are debris or doublets, and should not be included in the analysis.

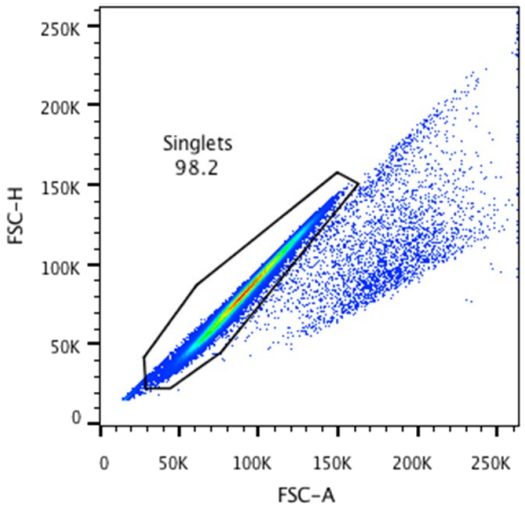


Figure 1: FSC-A vs FSC-H

8. Open ‘singlets’ to display single cells
    - X axis = SSC-W; Y axis = SSC-H (figure 2)

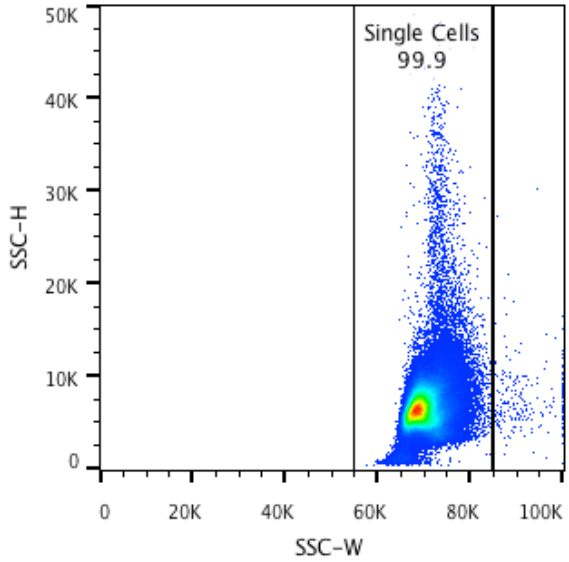


Figure 2: SSC-W vs SSC-H

9. Open Single cells to display ‘Time, Ter119 subset’
    - X axis = Time; Y axis = APC-A Ter119
    - Here you want to avoid any fluctuations in the event rate. This will often mean taking the middle section of the events (see figure 3)

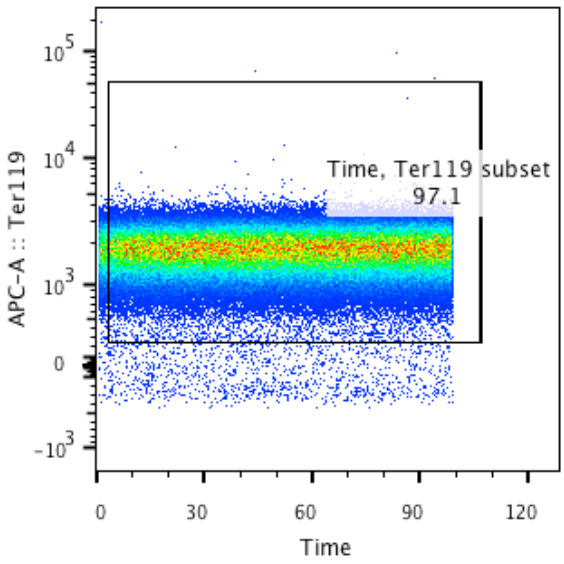


Figure 3: Time vs APC-Ter119

10. Open RBCs by double clicking ‘Time, Ter119 subset’ gate
    - X axis = APC-A Ter119; Y axis SSC-A (figure 4)

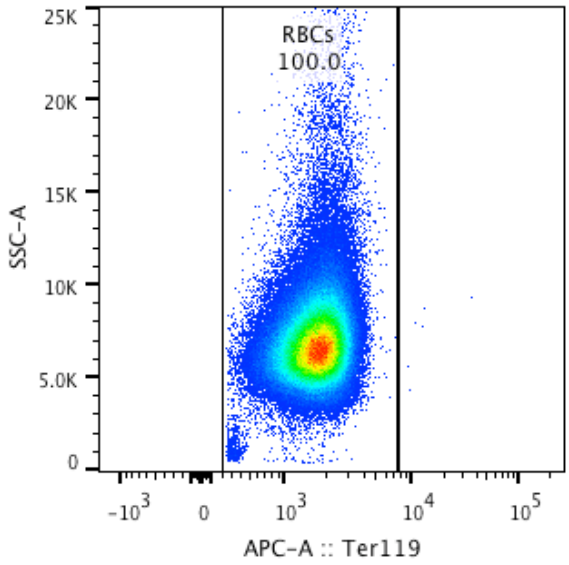


Figure 4: APC-Ter119 vs SSC-A

11. Open RBC & reticulocyte populations by double clicking ‘RBCs’ gate
    - X axis = Hoechst-A; Y Axis = PE-A CD71 (figure 5)

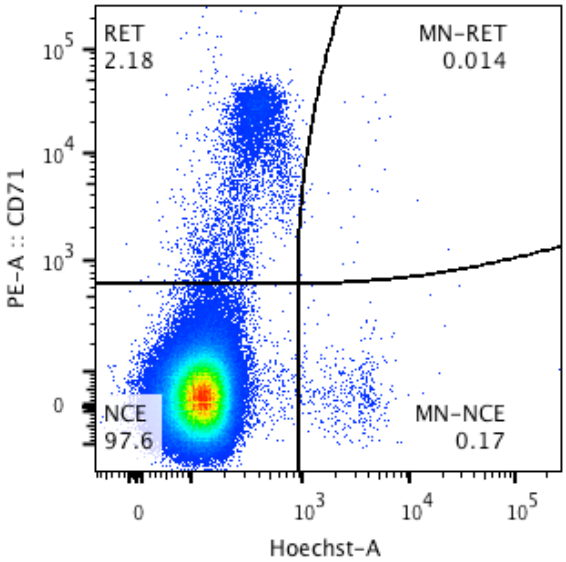


Figure 5: Hoechst vs PE-CD71

12. Synchronise the gates and move the gates of the representative sample to resemble those shown on the figures above.

13. Hold Shift and click the forward button  to cycle through all the samples, checking the populations fall into the gates correctly. If you need to adjust any individual samples, unsynchronise the gates first, then make adjustments as necessary. Once you have been through all samples, close all the plots.

14. Open the table editor  , and send the output to Excel (or copy to clipboard and paste into a new spreadsheet). Save this file in your analysis folder.

15. Save the FlowJo file and exit the program.

16. Open the Micronuclei analysis template and paste in the data under the correct headings from the raw output file. The percentages of micronucleated cells will be calculated automatically.

17. Save a .csv copy of this file and upload to the Mouse database.

Appendix A: plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33							
B	2	10	18	26	34							
C	3	11	19	27	35							
D	4	12	20	28	36							
E	5	13	21	29	37							
F	6	14	22	30	38							
G	7	15	23	31	39							
H	8	16	24	32	40							

	Samples

Appendix B: antibody panel

Reagent	Channel	Final dilution	Supplier	Catalogue #	Clone
CD71 PE	PE	2000	Biolegend	113808	RI7217
Ter119	APC	600	Biolegend	116212	TER-119
Hoechst 33452	DAPI	2000	Life technologies	H3570	N/A

Mouse barcode	Date	NCE events	RET events	MN-NCE ever	MN-RET ever	RBC events	% MN-NCE	% MN-RET	% RET
							=(E2/(C2+E2))*100	=(F2/(D2+F2))*100	=((D2+F2)/G2)*100
							#DIV/0!	#DIV/0!	#DIV/0!
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Paste in data from FlowJo export in the blank cells, values will be automatically calculated