BD Biosciences CANTO II user procedure: 3 lasers

Blood collected in EDTA:

Normal blood	Myelaemia	MDS	PNH

Preparing reagents:

1) Washing buffer: PBS 1X + FCS 1% = 500µl FCS for 50 ml final volume

2) Lysis reagent at room temperature: Pharmlyse® or other approved reagent.
Pharmlyse®: reagent supplied **10X** it must therefore be diluted to 1/10with clean distilled water.

3) Antibodies:
CD16 PC7: Dilute 1/10 in washing buffer
CD15 Hor V450: Dilute 1/10 in washing buffer

SOP PNH WORKSHOP BD Biosciences CANTO II 3 lasers			
	CLONE	**FLUORO CHROME 6C**	**Ab VOLUMES (microL)**
CTRL iso	G155-178	FITC	20
FLAER		AF488	5
CD66b	G10F5	FITC	10
CD11b	D12	PE	2
CD55	IA10	FITC and APC	10
CD59	H19	FITC	10
CD24	ML5	PE	20
CD33	P67.6	PC5.5	20
CD16	3G8	PC7	5 (dil 1/10th)
CD14	MOP9	APC	5
CD15	MMA	V450	5 (dil 1/10th)
CD45	HI30	V500	2.5

Sample preparation:

Blood count: **ANC = _____, ____ G/L**

Volume of blood to process:**vol = 5 / _____, ____ G/L = _____ ml**

- If vol ≤ 1 ml use a 15 ml conical bottom tube
- If 1 ml < vol ≤ 2 ml fractionate the blood to perform lysis in 2 tubes, etc.

Lysis to add: _____ **ml x 10 =** _____ **ml**

Homogenize the mixture, incubate at room temperature for at least **10 min** and no more than **20 min** if the lysis is incomplete
Complete with the washing buffer, mix by inverting
Centrifuge at **200 g** for **5 min**

Aspirate the supernatant, **do not vortex**, fractionate the cell pellets by tapping the bottom of the tube, add **10 ml** of washing buffer
At this stage, if the lysis was done in several tubes, group all the cells together in a 15ml tube

Centrifuge at **200 g** for **5 min**
Aspirate the supernatant and resuspend using the same procedure

Mix by pipetting in a final volume of **180µl**. In this way, **45µl** of cell suspension contains around **10^6 ANC**

VERY IMPORTANT: SUSPENSION HOMOGENEOUS +++++

Marking:

Prepare the antibody mixture in the tube before depositing the cells.

T	V 450	V500	FITC AF488	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	Adjust the volume
0	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
1	**-**	**-**	CD55-59 **10µl-10µl**	CD11b **2µl**	**-**	**-**	**-**	**-**	**+33µl** buffer
2	**-**	**-**	CRTL ISO **20µl**	CD11b **2µl**	**-**	**-**	**-**	**-**	**+33µl** buffer
3	CD15 **5µl DIL !**	CD45 **2.5µ**l	FLAER **5µl**	CD24 **20µl**	CD33 **20µl**	**-**	**-**	CD14 **5µl**	**-**
4	CD15 **5µl DIL !**	**-**	CD66b **10µl**	**-**	CD33 **20µl**	CD16 **5µl DIL !**	CD55 **10µl**	CD14 **5µl**	**-**
	DIL: for CD15 and CD16 prepare a dilution = **2µl Ab+18µl washing buffer (1/10)**								

NB: it is advisable to use the Nakao test tube before the Nakao control tube to avoid contamination between tubes.

Vortex the antibody mixture then add, in each tube, **45µl of cell suspension**, vortex, incubate at room temperature and away from light for **30 min**
Prepare **Tube 0** with the remaining cell suspension (see §5 Panels)

Wash with **3 ml** of washing buffer per tube, centrifuge **200 g** for **5 minutes**.

Aspirate the supernatant, **do not vortex**, fractionate the cell pellets by tapping the bottom of the tube, resuspend by pipetting in **300µl of washing buffer**.
The cell suspension is vortexed before acquisition.

If the acquisition lasts for more than 10 minutes, it is advised to suspend it to resuspend the cells by vortexing.

Parameters to record:

All tubes:

FSC-A	**FSC-H**	**FSC-W**	**SSC-A**	**SSC-H**	**SSC-W**	**Time**
Tube 1:	**FL-1-A**	**FL-2-A**				
Tube 2:	**FL-1-A**	**FL-2-A**				
Tube 3:	**FL-1-A**	**FL-2-A**	**FL-3-A**		**FL-6-A**	**FL-7-A** **FL-8-A**
Tube 4:	**FL-1-A**		**FL-3-A**	**FL-4-A** **FL-5-A**	**FL-6-A**	**FL-7-A**

Adjust the FSC **area scaling factor** for the cells in the 1st tube

Adjust the **THRESHOLD** on the FSC; it should be the same for all tubes

Ensure that the **data is saved** in **FCS 3.0**

Wash the cytometer before starting the acquisition in automatic clean mode with the carousel: 1 FACSCLEAN tube **1 min**, 1 FACSRINSE tube **1 min**, 1 FACSFLOW tube **1 min**

Storage Gate: **ALL EVENTS**

Stopping Gate: **$5x10^5$ ANC**

The run speed will be adjusted to the ABORT % during the acquisition:

If >5% either reduce speed
or dilute the cell suspension with 50µl washing buffer, repeat dilution until a satisfactory abort rate is reached

Wash the cytometer between each sample in automatic clean mode with the carousel: 1 FACSCLEAN tube **1 min**, 1 FACSRINSE tube **1 min**, 1 FACSFLOW tube **1 min**

Do not forget Tube 0: acquisition of 20,000 events on the tube of unmarked cells = reference for leukocyte population distribution
If, during acquisition, the ANC % of a marking tube is not similar to tube 0, suspend the acquisition, remove the tube and resuspend by vortexing and then resume the acquisition and check for an improvement in the ANC %.

Gating strategy: see analysis sheet attached

Colour code of populations analysed (GTLLF/GEIL):

Granulocytes: Red
Monocytes: Green
Lymphocytes: Pink
PNH clone: Black

FSC/SSC: gate excluding debris

Tube 0: Record the % of granulocytes which must be the same in all of the following tubes

Tube 1 Nakao TEST:

FSC-H/ FSC-A singlet gate
SSC / CD11b Mono and Neutro gate
CD 55+ CD59 / CD11b PNH clone detection gate

See PDF files attached

Tube 2 Nakao CTRL: identical

See PDF files attached

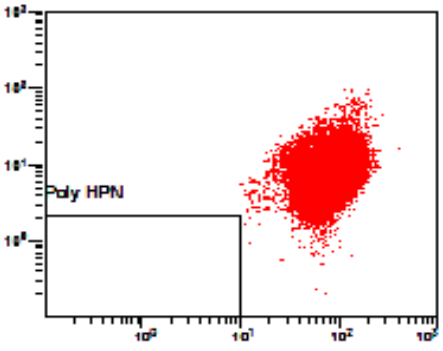
Tube 3 Guidelines:

* **FSC-H/ FSC-A** Singlet gate
* **FSC / SSC** "Morpho" living cells gate
* **SSC/CD45:** large granulocytes excluding eosinophils, and "weak CD45" cells
*__CD33/ CD15__ to specifically determine "poly" and "mono"
*__Flaer / CD24__ and __FLAER / CD14__ expression on two dot-plots
Make a "poly PNH" and "mono PNH" gate

See PDF files attached

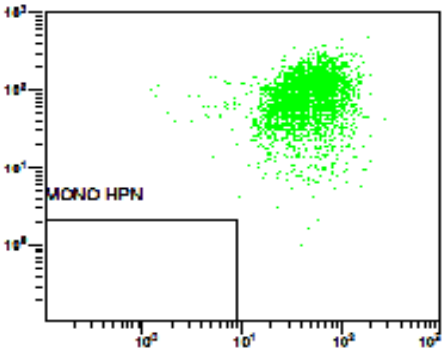
Example of normal cells

CD24



FLAER

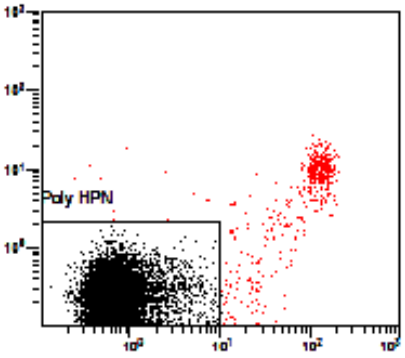
CD14



FLAER

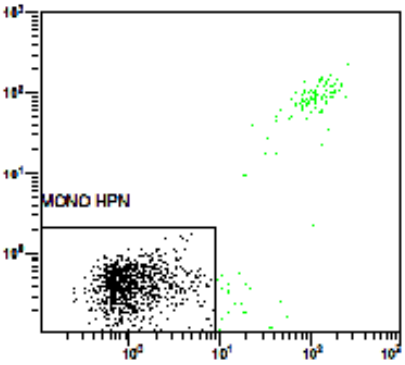
Example of PNH cells

CD24



FLAER

CD14



FLAER

Tube 4 BD Biosciences:

* **FSC-H/ FSC-A** Singlet gate
* **FSC / SSC** "Living" cell gate

* **SSC/CD33:** large granulocytes excluding eosinophils and "weak CD45" cells
*__CD33/ CD15__ to specifically determine "poly" and "mono"

See PDF files attached

Adjusting the cytometer and compensation matrix

Purpose: To obtain "comparable images" with different machines of the same type and more if possible

Rainbow beads: Vortex before preparation, dilute as directed, vortex dilution prior to acquisition +++

Analysis sheet: Position respectively the 8th peak (last peak) of FL1, FL2, FL4, FL5, FL6, FL7 and FL8 and the 7th peak (penultimate peak) of FL3 **as close as possible to the target value given below**

	FL1 (8th peak)	FL2 (8th peak)	FL3 **(7th peak)**	FL4 (8th peak)	FL5 (8th peak)	FL6 (8th peak)	FL7 (8th peak)	FL8 (8th peak)
median MFI	44537	88277	54789	4387	187549	31629	201102	166204

Target values corresponding to the average of 3 runs

See run example on PDF file attached (the target values are those in the table and not in the example)

Compensation matrix: Created following BD Biosciences guidelines using Compbeads (not provided) or fresh blood **without using too many antibodies.**

For example: the compensation matrix obtained at la Pitié, which should be similar to yours (we always have minimal differences between centres testing the strategy):

BD Guidelines tube

		FL1	FL2	FL3	FL6	FL7	FL8
FITC	FL1		1.24	0.03	0	0	2.16
PE	FL2	13.1		0	0	0	0.48
PerCP-Cy5.5	FL3	4.65	35.46		0	0.17	0.63
APC-H7	FL6	0	0	3.5		0	0
V450	FL7	0	0	0	0		10.69
V500	FL8	4	0	0	0	19.55	

BD Company tube

		FL1	FL3	FL4	FL5	FL6	FL7
FITC	FL1		0.03	0.68	0	0	0
PerCP-Cy5.5	FL3	4.65		25.97	1.95	0	0
PC7	FL4	0.05	2.37		0	0.57	0
APC	FL5	0	0	0		2.64	0
APC-H7	FL6	0	3.50	42.4	7.04		0
V450	FL7	0	0	0	0	0	

Results sheet

See Excel table containing patient data and cytometry data