Beckman-Coulter 6C NAVIOS 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: Versalyse®

3) Antibodies:
CD16 PC7: Dilute 1/5 in washing buffer

SOP PNH WORKSHOP Beckman Coulter 6C NAVIOS 3 lasers			
	CLONE	**FLUORO CHROME 5C**	**Ab VOLUMES (microL)**
CTRL iso		FITC	20
FLAER		AF488	5
CD11b	BEAR1	PE	10
CD55	JS11KSC2	FITC	10
CD59	P282	FITC	10
CD24	ALB9	PE	20
CD33	D3HL-60	PC5.5	2
CD16	3G8	PC7	5 (dil 1/5th)
CD45	J33	PC7	10
CD14	RMO52	APC	10
CD15	80H5	PB	10

Sample preparation:

Blood count: **ANC = _____, ____ G/L**
You are using the panels with 6 colours. You have 5 tubes.

Volume of blood to process:**vol = 6 / _____, ____ 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	PB	FITC AF488	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	Adjust the volume
0	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
1 test	**-**	CD55-59 **10µl-10µl**	CD11b **10µl**	**-**	**-**	**-**	**-**	**+25µl** buffer
2 ctrl	**-**	CRTL ISO **20µl**	CD11b **10µl**	**-**	**-**	**-**	**-**	**+25µl** buffer
3	CD15 **10µl**	FLAER **5µl**	CD24 **20µl**	CD33 **2µl**	CD45 **10µl**	CD14 **10µl**	**-**	**-**
4	CD15 **10µl**	CD55 **10µl**	CD24 **20µl**	CD33 **2µl**	CD16 **5µl DIL !**	CD14 **10µl**	-	**-**
	DIL: for CD16 prepare a dilution = **2µl Ab+ 8µl washing buffer (1/5)**							

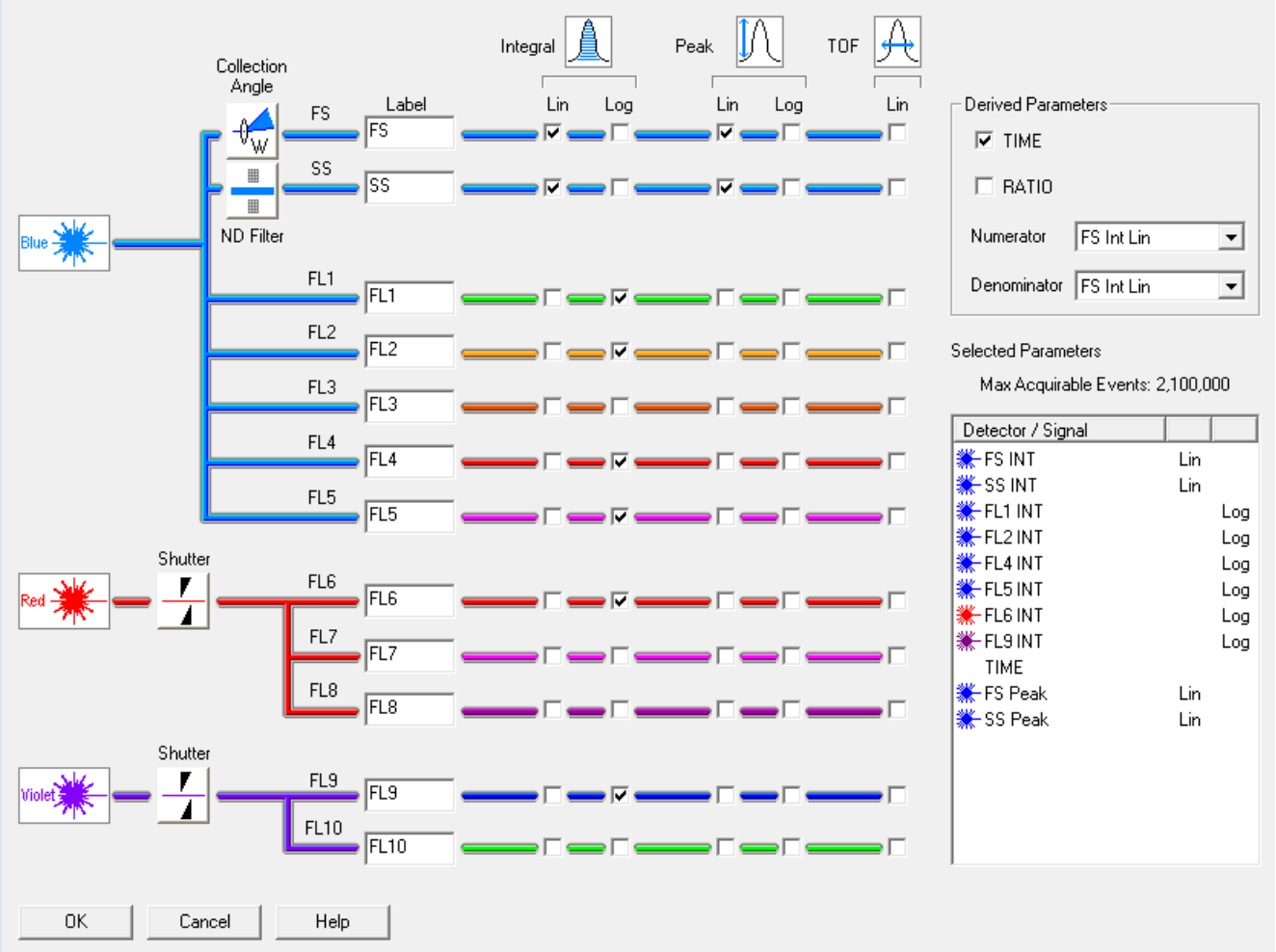
NB: it is advisable to use the Nakao test tube before the Nakao control tube to avoid contamination between tubes.

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

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 to resuspend, add**500µl of washing buffer**, **stir**
The cell suspension is vortexed before acquisition

Parameters to record:



Example for tubes 3 or 4

FSC-INT FSC-Peak SSC- INT Time

Tube 0:
Tube 1: **FL-1-LOG FL-2-LOG**
Tube 2: **FL-1-LOG FL-2-LOG**
Tube 3: **FL-1-LOG FL-2-LOG FL-4-LOG FL-5-LOG FL-6-LOG FL-9-LOG**
Tube 4: **FL-1-LOG FL-2-LOG FL-4-LOG FL-5-LOG FL-6-LOG FL-9-LOG**

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: 1 tube of CLENZ **1 min**, 1 tube of lukewarm tap water **1 min** and 1 tube of distilled water **1 min**

Storage Gate: **ALL EVENTS**

Stopping Gate: **$5x10^5$ granulocytes**
500 sec (beware of air bubbles)

NB: ensure that no air bubbles are aspirated at the end of the tube

Do not forget tube 0: acquisition of **20,000 events** in the tube of unmarked cells = reference for leukocyte population distribution, ensure that the same % of ANC is obtained in the following tubes.

Vortex the tube before starting the acquisition without exceeding 3,000 events/sec (corresponding to a theoretical abort % of less than 5%).
The TIME parameter can detect sample sedimentation (the number of events analysed/min decreases: generally after 300 sec).
In this case, use the pause rotate function, vortex the cell suspension and continue the acquisition.

These operations make it possible to acquire a maximum number of events (500,000 granulocytes are expected) while avoiding sedimentation and cell clumping

Gating strategy: see analysis sheet (flow page) attached

Colour code of populations analysed (GTLLF/GEIL):

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

FS/SS: "A" gate excluding debris

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

Tube 1 Nakao TEST:
FS INT / FS PEAK singlet gate
SS / CD11b Mono and neutro gate
CD 55+ CD59 / CD11b PNH clone gate

Tube 2 Nakao CTRL: identical

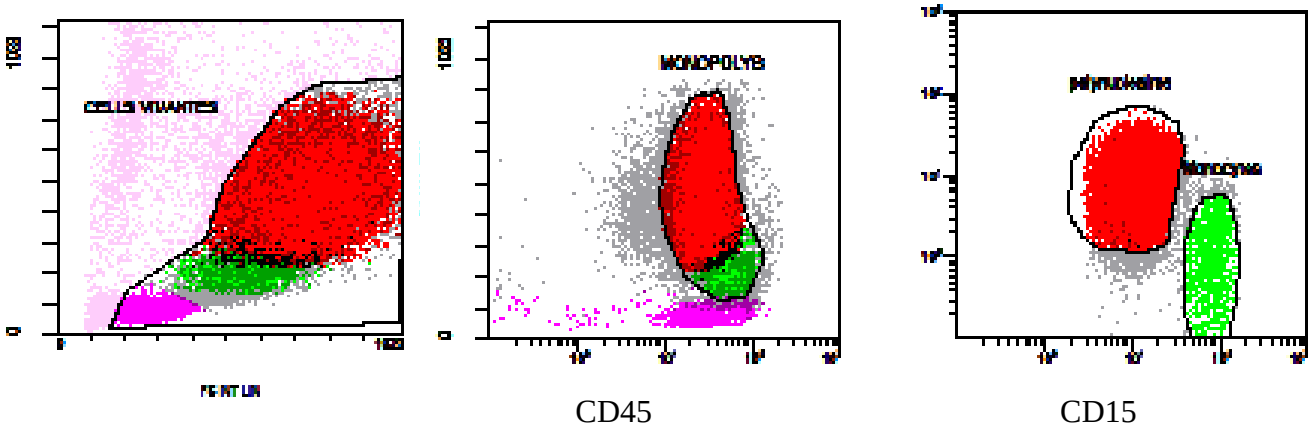
Tube 3 Guidelines: * **FS INT / FS PEAK** Singlet gate

* **FS / SS** ”Living” cell gate

* **SS/CD45:** large granulocytes excluding eosinophils, and “weak CD45” cells

*__CD33/ CD15__ to specifically determine “poly” and “mono”

Example of normal cells



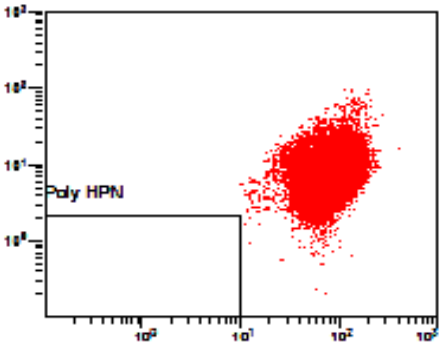
CD45 CD15

*__Flaer / CD24__ and __FLAER / CD14__ expression on two dot-plots

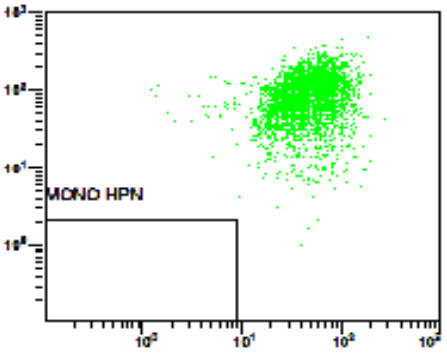
Make “poly PNH” and “mono PNH” gate

Example of normal cells

CD24



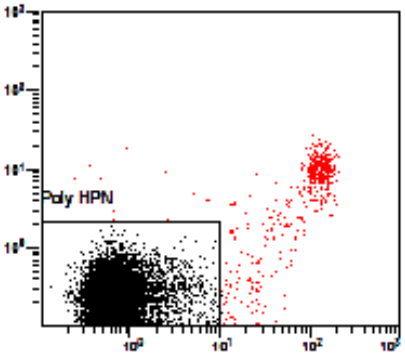
CD14



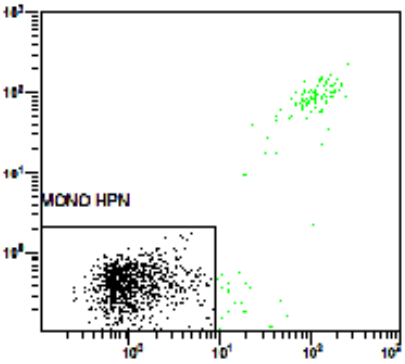
FLAER FLAER

Example of PNH cells

CD24



CD14



FLAER FLAE

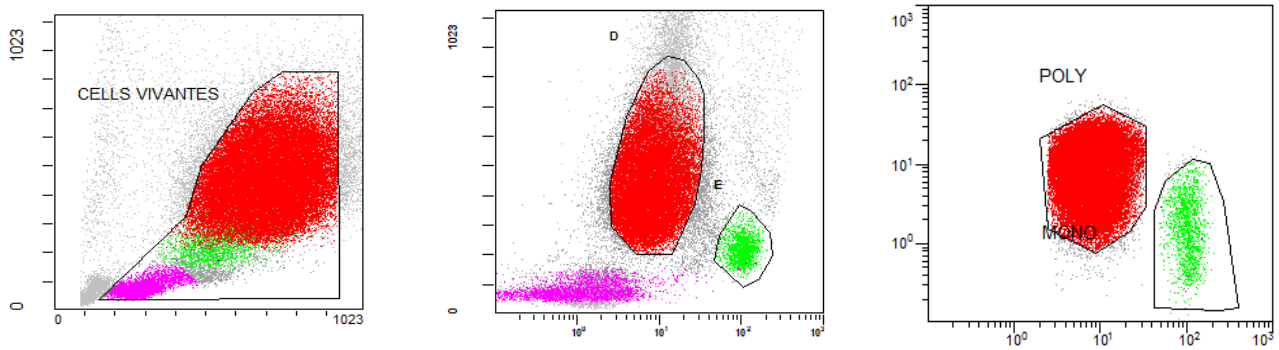
__**Tube 4 Beckman:**__ *singulet gate

* **FS / SS** ”Living” cell gate

* **SS/CD33:** large granulocytes excluding eosinophils, and "weak CD45" cells
***CD33/ CD15** to specifically determine "poly" and "mono"

Example of normal cells

CD15

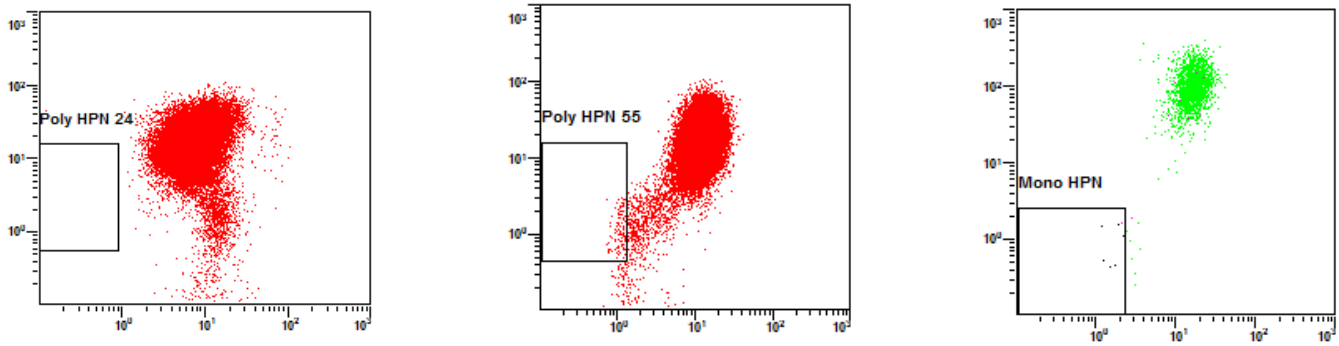


FS CD33 CD33

*CD55 CD24 CD16 expression on three dot-plots
PNH clone gate: "CD55neg AND CD24neg AND CD16low"

Example of normal cells

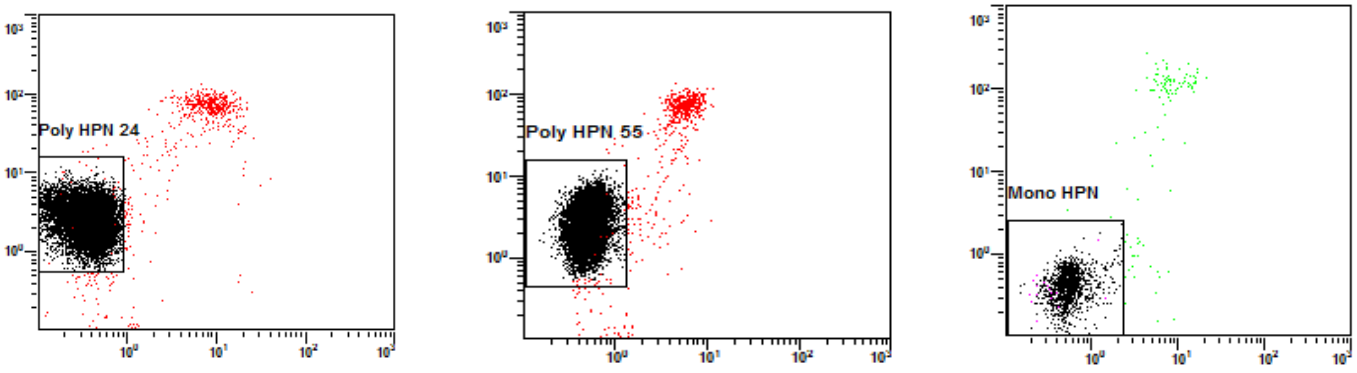
CD16 CD16 CD14



CD24 CD55 CD5

Example of PNH cells

CD16 CD16 CD14



CD24 CD55 CD55

Adjusting the cytometer and compensation matrix

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

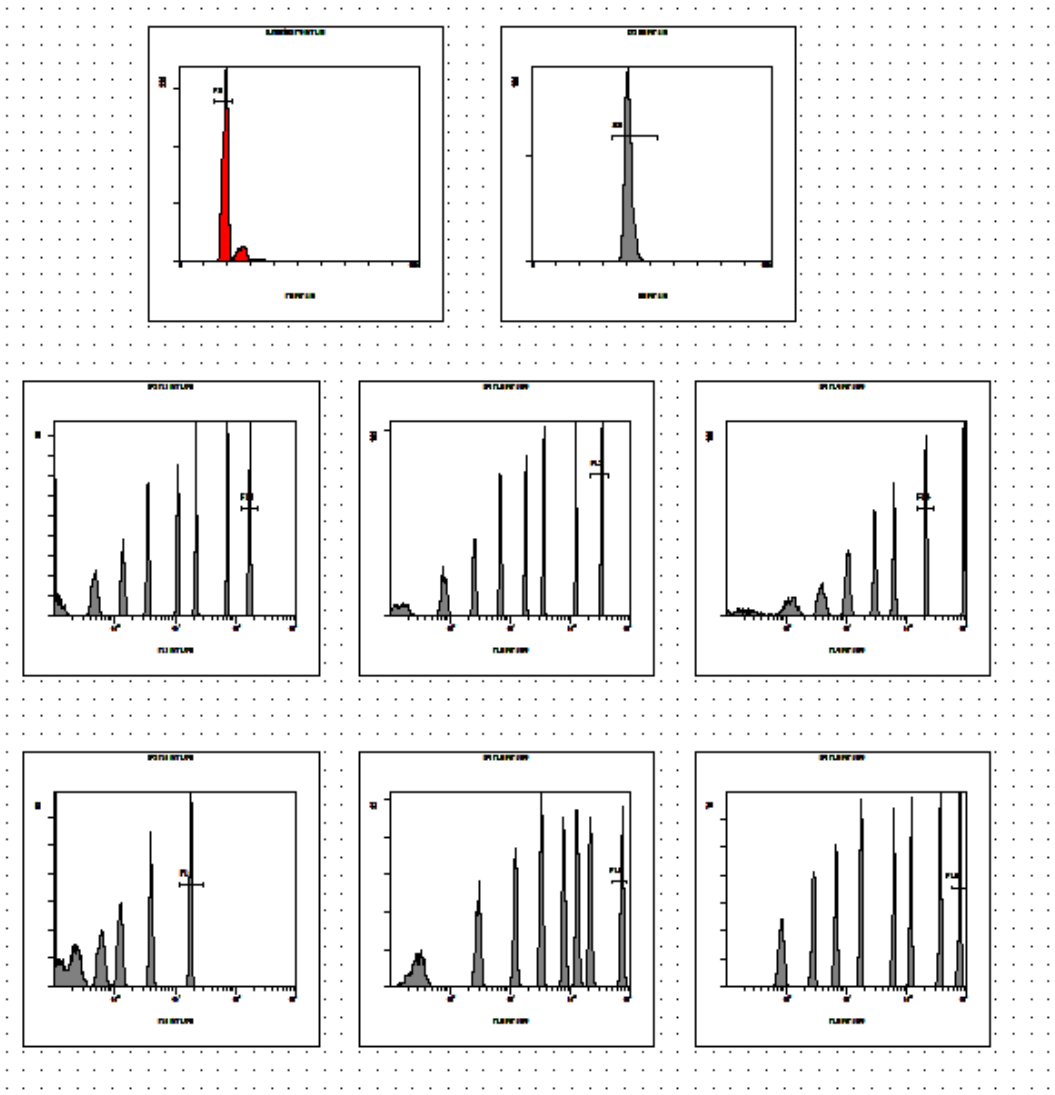
Rainbow beads: Vortex before preparation, dilute as directed, vortex dilution prior to acquisition +++++

Analysis sheet: Position respectively the 8th peak (last peak) of FL1, FL2, FL5, FL6 and FL9 and the 7th peak (penultimate peak) of FL4 as close as possible to the target value given below

NAVIOS

		FL1 (8th peak)	FL2 (8th peak)	FL4 **(7th peak)**	FL5 (8th peak)	FL6 (8th peak)	FL9 (8th peak)
	MFI	179	353	210	18	721	768

Target values corresponding to the average of 4 runs



Compensation matrix: Create following the Beckman-Coulter guidelines with Beckman Coulter CD45 or other Beckman-Coulter Abs (or workshop Abs **but be careful not to “eat up” Abs unnecessarily).** Use

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):

NB: You may call upon your local Beckman-Coulter application engineer

BC Guidelines tube	FL1	FL2	FL4	FL5	FL6	FL9
FL1		1.1	0.1	1.6	1.2	0
FL2	17.1		0.1	5.4	0.1	0
FL4	0	30.6		10.1	0	0
FL5	0.1	0.1	2.8		0	0
FL6	0	0	0.2	0.4		0
FL9	0	0	0	0	0	

BC Company tube	FL1	FL2	FL4	FL5	FL6	FL9
FL1		1.1	0.1	1.5	0.2	0
FL2	17.1		0.2	6	0.1	0
FL4	0.1	30.4		5.8	2.2	0
FL5	0.1	0.1	2.5		0	0
FL6	0	0	0.3	0		0
FL9	0	0	0	0	0	

Results sheet

See Excel table containing patient data and cytometry data