

# Minimal residual disease detection in Acute Myeloid Leukaemia on a Becton Dickinson flow cytometer

## Purpose

This procedure gives instruction on minimal residual disease (MRD) detection in patients with acute myeloid leukaemia (AML)

## Background

About half of the patients suffering from AML relapses within 12 months after achieving complete remission due to the presence and outgrowth of residual AML cells in bone marrow (BM), so called minimal residual disease (MRD). MRD can be measured by using immunophenotypic detection methods. Leukaemia associated phenotypes (LAP) established in newly diagnosed AML, are used in remission BM to detect the frequency of MRD cells. It has been shown by several groups that the frequency of MRD is highly predictive for the occurrence of relapse.

## Policy

All specimens for immunophenotyping should be processed preferentially within 24 hours of collection.

## Specimen

- anticoagulated (preferably with heparin) BM of AML patients in complete remission
- autologous peripheral blood stem cell transplants.

## Equipment and materials

- Flow cytometer with at least 6 parameters; 2 light scatter and at least 4 fluorescence parameters.
- Vortex mixer
- Adjustable pipettes (e.g. 10 µl, 20 µl, 100 µl, 1ml, 2 ml)
- Disposable pipette tips
- Disposable polypropylene tubes

## Abbreviations

BM	bone marrow
BSA	bovine serum albumin
LAP	leukaemia associated phenotype
Moab	monoclonal antibody

MRD	minimal residual disease
PBS	phosphate-buffered saline
PFA	paraformaldehyde
RT	room temperature
WBC	white blood cell

## Reagents

1. Ammonium Chloride Lysing Solution (10x):
  - 82.6 g (1.5 M) Ammonium Chloride (NH<sub>4</sub>CL); Sigma A-5666
  - 10 g (100 mM) Potassium Bicarbonate (KHCO<sub>3</sub>); Sigma P-4913
  - 370 mg (0.96 mM) Tetra Sodium EDTA (Na<sub>4</sub>EDTA); Sigma ED4SS
  - 1000ml Distilled H<sub>2</sub>O

*Dissolve these ingredients in 900 ml of distilled water.  
Adjust the pH by addition of either 1N HCL or 1N NaOH to a range of 7.2 – 7.4.  
Add distilled water to a volume of 1000 ml. Store ≤6 months at 2-8 °C.  
To prepare the working solution, dilute the concentrate 1:10 in distilled water.*
2. Phosphate-buffered Saline (PBS)
3. PBS-0.1% BSA; PBS containing 0.1% Bovine Serum Albumin (BSA) or Foetal Bovine Serum or Human Serum Albumin.
4. PBS-1% PFA; PBS containing 1% Paraformaldehyde (PFA).
5. Monoclonal antibodies (Moabs) in working dilution, defined by titration.

## Procedure

### 1 Bulk lysis of cells

- 1.1 Resuspend the anticoagulated BM sample to homogeneity
- 1.2 Optionally, homogenise BM by passing the sample twice through a 25 µM gauge syringe.
- 1.3 Perform a white blood cell (WBC) count.
- 1.4 Pipet the needed amount of WBC in a tube. The amount of cells is dependent on the number of needed stainings. Take for 1 staining about 2x10<sup>6</sup> cells.  
When the total available number of WBC is too low, determine only the most reliable LAP(s) with as many cells as possible. Preferably, use 2 independent LAPs to exclude false-negative MRD measurements due to phenotypic shifts.
- 1.5 Lyse red blood cells by adding ammonium chloride working solution to the cells.  
Volume of ammonium chloride working solution has to be 15 times higher at minimum than the volume of the cell suspension.
- 1.6 Mix gently.
- 1.7 Incubate for 15 min at room temperature (RT).
- 1.8 Centrifuge for 10 min 800g at RT.
- 1.9 Discard the supernatant (e.g with a vacuum pump or Pasteur pipette).
- 1.10 Resuspend the cell pellet and add PBS-0.1% BSA.
- 1.11 Centrifuge for 5-10 min 500g at RT.
- 1.12 Discard the supernatant (e.g. with a vacuum pump or Pasteur pipette).
- 1.13 Resuspend the cell pellet in PBS-0.1% BSA in the cell concentration of 20x10<sup>6</sup> WBC/ml. If not possible, use lower WBC concentrations.

## 2 Staining of cells for flow cytometry

- 2.1 Pipet the moabs that define the LAP expression as established in diagnosis material in FACS tubes. This incubation can be done with 4x10 $\mu$ l moabs, also other volumes can be used (minimum volume: 5  $\mu$ l) with adjusted sample volumes. Use the moabs distributed by the MRD project group and/or own moabs,
- 2.2 Add the lysed cells (preferably 2x10<sup>6</sup> cells, maximal end cell concentration of 20x10<sup>6</sup>/ml) to the tubes.
- 2.3 Mix gently.
- 2.4 Incubate for 15 min (or 10 min when shaking) at RT protected from light.
- 2.5 Resuspend the cell pellet and add PBS-0.1% BSA.
- 2.6 Mix gently.
- 2.7 Centrifuge for 5 min 500g at RT.
- 2.8 Discard the supernatant (e.g. with a vacuum pump or Pasteur pipette).
- 2.9 Resuspend the cell pellet in PBS-0.1% BSA.
- 2.10 If possible acquire of each tube 1x10<sup>6</sup> WBC events (characterised by CD45 expression and FSC, see appendix 1) or until tube is empty on flow cytometer.

*When flow cytometric acquisition can not be performed on the same day, then add 300-500  $\mu$ l PBS-1% PFA on the cell pellet at point 2.9. Samples can be stored at 2 - 8°C for 24 hours at maximum.*

## 4 Data analysis

Data analysis can be performed with Cellquest, Paint-a gate, FlowJo or other software..

### 4.1 Analyses of different cell populations

Analyse the percentage of CD45dim blasts, lymphocytes, monocytes and granulocytes as described in appendix 2 of the SOP "Defining Leukaemia Associated Phenotypes in newly diagnosed Acute Myeloid Leukaemia".

### 4.2 Analyses of MRD

- 4.2.1 Analyse the percentage of LAP+ cells in the WBC cell compartment, characterized by CD45-FSC, in which dead cells and cell fragments have been excluded (see appendix 1).
- 4.2.2 Gate on the LAP+ cells based on the LAP expression in newly diagnosed material (see appendix 1). Take into account that 1) the intensity of markers in the LAP might have been undergone some changes compared to diagnosis material and 2) normal cells might contaminate this LAP gate. Therefore, the gate on the LAP+ cells may have to be adjusted taking into account that most AML LAP+ cells are included but with minimal contamination of normal cells.
- 4.2.3 Verify the characteristics of the LAP+ cells by backgating the LAP+ cells in the density plots FSC/SSC and CD45/SSC. LAP+ cells in these plots should be clustered and

preferably similar to as at diagnosis. Exclude the cells with other characteristics if necessary.

4.2.4 Note the percentage of the LAP+ cells in the WBC compartment.

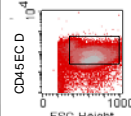
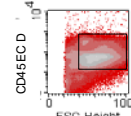
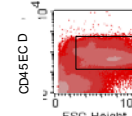
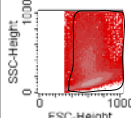
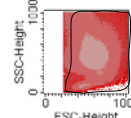
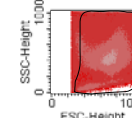
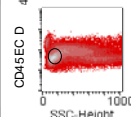
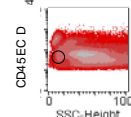
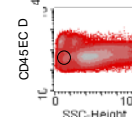
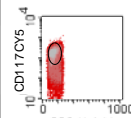
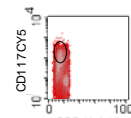
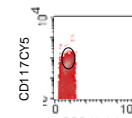
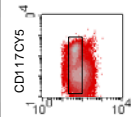
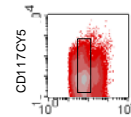
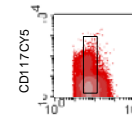
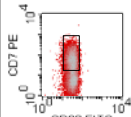
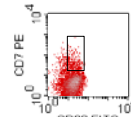
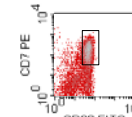
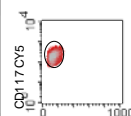
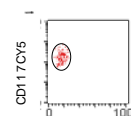
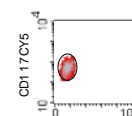
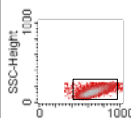
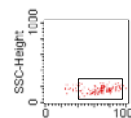
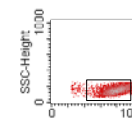
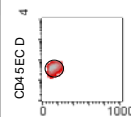
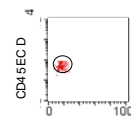
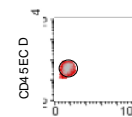
## **5 Data reporting and interpretation**

5.1 Note on the MRD REPORT form (see appendix 2):

- The percentage of blasts, lymphocytes, monocytes and granulocytes.
- The combination of markers that defines the LAP and the percentage of LAP as established in diagnosis material.
- The sensitivity of the LAPs. (The specificity and stability of the LAPs can be established only by the centre with an extensive experience in MRD measurements). The total of these parameters determines the quality of the LAP.
- The percentage of LAP and MRD in the WBC compartment.
- The conclusion concerning the presence of MRD.

On the worksheet "Commentary" (see appendix 2) a guide for this form is given.

Appendix 1: Gating strategy for MRD analyses

	De novo AML	BM after 1st cycle	BM after 2nd cycle		
Defining of CD45dim blasts in WBC compartment				No gate Characterization of WBC compartment (R1)	1
				In R1 Exclusion of cell fragments and/or dead cells (R2)	2
				In R1+R2 Identification of CD45 dim / SSC blasts (R3)	3
Defining of LAP+ cells in WBC compartment				In R1+R2+R3 Gate on CD117+ cells (R4)	4
				In R1+R2 Gate on CD117+ cells which are CD45dim (R5)	5
				In R1+R2+R3+R4+R5 Gate on CD33+CD7+ cells, which are CD117+ CD45dim : LAP+ cells (R6)	6
Backgating of LAP+ cells				In R1+R2+R3+R4+R5+R6 Backgating of LAP+ cells in a CD117/SSC plot	7
				In R1+R2+R3+R4+R5+R6 Backgating of LAP+ cells in a SSC/FSC plot	8
				In R1+R2+R3+R4+R5+R6 Backgating of LAP+ cells in a CD45/SSC plot	9

Gating strategy of MRD in BM after the 1<sup>st</sup> and 2<sup>nd</sup> cycle of chemotherapy (panels in figure columns 2 and 3) is based on the LAP expression in diagnosis AML material (panels in figure column 1). In this example the LAP is CD33+CD7+CD45dimCD117+. Frequency of LAP+ cells after the first cycle is 0.08%. No judgement can be done whether these cells are MRD cells since normal cells also may have similar LAP frequencies. After the 2<sup>nd</sup> cycle the frequency of LAP+ cells is 1.25%, which can be judged as MRD

- The analyses start with defining of CD45dim blasts in the WBC compartment. This is established by the following gating steps:

- 1 Identifying the WBC compartment by a gate on the CD45 positive cells in which events with a low FSC are excluded as being dead cells or fragments (R1).
- 2 If necessary, further exclusion of dead cells and cell fragments can be done in a SSC/FCS density plot (R2) gated in the WBC compartment (R1).
- 3 Identifying the CD45 blast gate (R3) in a CD45/SSC plot gated in the 'viable' WBC compartment (R1+R2) based on the CD45 expression at diagnosis material.

*When the CD45 expression in AML blasts at diagnosis is similar to normal blasts in control BM, the CD45 gate in follow-up material can be fine tuned by back gating normal blasts by using the primitive marker that is used in the labelling.*

- The next steps include the identification of LAP+ cells

- 4 First, in the blast region (R1+R2+R3), cells with expression of a primitive marker (in this case CD117) are gated in a CD117/SSC density plot (R4). When no primitive marker is present, use a marker which is present in a low percentage in the blast region.
- 5 Exclude those cells that do have the primitive marker (CD117 in this case) but with CD45 expression outside the gate R5 (in this case CD117+ cells with CD45 high expression are excluded).
- 6 Identify the cells which define the LAP (here CD7 and CD33) in all defined gates.

- The last steps are meant to exclude cells which are not clustered at the right place

- 7 Back gate the LAP+ cells in a density plot as used in point 4. Note that the CD117 expression in BM after 2<sup>nd</sup> cycle is lower than in diagnosis material and after the 1<sup>st</sup> cycle.
- 8 Back gate the LAP+ cells in a SSC/FSC density plot. Cells that fall outside the cluster can be excluded.
- 9 Back gate the LAP+ cells in with a CD45/SSC density plot. Again the non-clustered cells can be excluded (not present in this example).

