

# LEUKAEMIA AND LYMPHOMA IMMUNOPHENOTYPING

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## 1. INTRODUCTION

The aim of leukaemia and lymphoma phenotyping is to identify the cell type of the neoplastic process. This phenotypic identification should outline the cell lineage and level of maturation, as an aid to the classification of the leukaemia or lymphoma. Further, this phenotypic identification should assist in the determination as to whether the cell population is normal or abnormal and in the detection of a previously characterised population of cells in a sample for monitoring the disease remission, development or recurrence.

These assays are usually performed on blood or bone marrow specimens, fine needle aspirates, ascites, CSFs, pleural fluid etc.

## 2. SPECIMEN COLLECTION, TRANSPORT AND INTEGRITY

### 2.1. Specimen Collection

- 2.1.1. Universal precautions should be strictly observed when collecting blood samples (see 1.1 Safety Guidelines).
- 2.1.2. All samples submitted for testing must be immediately labeled with the date and time of collection and at least one unique patient identifier (complying with local regulations). When there are multiple specimens collected on the one patient then the source of the specimen must be clearly indicated on the tube (ie Blood and Bone Marrow). A request form with a unique patient identifier, presumptive diagnosis, age, sex, pertinent medication or recent treatment including dates, date and time of specimen collection, name of physician and source of sample and test required should accompany all samples.<sup>7</sup>
- 2.1.3. EDTA, ACD solution A or Sodium Heparin may be used.<sup>7</sup>
- 2.1.4. A total white cell count and differential on all Peripheral Blood Samples should be performed at the laboratory initiating the request. For distant laboratories and dispatch centres a white cell count, differential and unstained blood film should accompany each specimen.
- 2.1.5. EDTA anticoagulated blood and bone marrow specimens are suitable if the specimen will be processed within 24 hours of collection.
- 2.1.6. Sodium heparin or ACD (solution A) anticoagulated blood and bone marrow specimens may be processed within 48 hours of collection. ACD is not recommended for bone marrow aspirates because the high volume of ACD in relation to bone marrow may reduce cell viability due to changes in pH<sup>1</sup>. Note that heparin has been known to inhibit PCR reactions.
- 2.1.7. Tissue biopsies in isotonic medium (such as phosphate buffered saline, Hanks or RPMI) usually do not require anticoagulant. Note that specimens should be immersed in appropriate medium immediately on collection.
- 2.1.8. CSF should be processed as soon as possible and not more than eight hours after collection. CSF usually does not require anticoagulant

### 2.2. Transport

- 2.2.1. All specimens must be considered infectious and must be handled in a way to minimize risks for laboratory workers and third-party carriers who transport specimens.<sup>7</sup>
- 2.2.2. Packaging, labelling and transport of specimens should comply with all current local, state, national and international regulations for the regions through which the specimens will pass.
- 2.2.3. Specimens should be maintained at 18<sup>o</sup> -22<sup>o</sup> C in a leak proof container<sup>1</sup>.
- 2.2.4. Temperatures below 4 C and above 30 C must be avoided.
- 2.2.5. Except for same day deliveries it is recommended to include a minimum –maximum temperature sensor in the package to document if extremes of temperature were reached during shipping.<sup>7</sup>

## 2.3. Integrity

- 2.3.1. Visually inspect the specimen for clots, haemolysis or container defects. Where appropriate, recollect the sample if the specimen shows any visual signs of deterioration.
- 2.3.2. Specimens collected or transported outside the guidelines should be treated with caution. It is recommended to reject samples with <75% viability<sup>7</sup>. Deficiencies should be noted and the report should reflect the effect that these deficiencies may have on the results.
- 2.3.3. The viability of cells is very important as nonviable cells may nonspecifically bind to many antibodies and interfere with accurate immunophenotyping. However the sample should not be rejected solely due to low viability. Solid tumours may have lower viability than liquid samples. Cell yield is often lower in aggressive tumours like Burkitts Lymphoma. If the sample is irreplaceable and <75% viable cells are present, issue a disclaimer statement about suboptimal viability but report any abnormal populations that are identified. If no abnormal populations are identified and the viability is <75%, consider rejection and requesting a new specimen.<sup>7</sup>

## 3. SPECIMEN PROCESSING

- 3.1. The goal of handling, transporting and preparing samples with a suspected haematolymphoid malignancy is to obtain a single cell suspension while maximizing the cell yield, maintaining the viability and integrity of the cells and preventing the loss of abnormal cells.<sup>7</sup>
- 3.2. Where possible a total leucocyte count and differential should be performed before processing and the cell concentration adjusted accordingly. One should aim for a cell number of <math>1 \times 10^6</math> per test tube. Specimens which are leucocyte poor may have insufficient cells for flow cytometric analysis, thus requiring a larger collection volume of sample. Conversely standard concentrations of antibody reagents may be insufficient to saturate all binding sites in specimens with a leucocytosis, leading to possible false negative results.
- 3.3. Erythrocyte lysis methods are recommended because they are less prone to differential losses of specific subpopulations. However, using this procedure assumes that all leucocyte subsets are equally tolerant to the lysis method used. Several lysing techniques are available. Manufacturer's recommended protocol should always be followed unless data are available confirming that any modifications do not adversely affect results.
- 3.4. To obtain single cell suspensions from tissue specimens, mechanical methods should be employed. Enzyme digestion with proteolytic enzymes may destroy antigens of interest and may reduce cell viability<sup>1,4</sup>. If necessary the density gradient isolation such as Ficoll Hypaque can be used. The suspension can be filtered through nylon mesh, generally 50µm or greater in pore size. Some of these specimens required invasive procedures and every attempt should be made to obtain useful information from the samples submitted.<sup>7</sup>

## 3.4. Monoclonal Antibody Panels

- 3.4.1. Because pattern recognition plays an important role in leukaemia phenotyping, it is recommended that laboratories devise panels for acute and chronic leukaemias and lymphomas. Panels should be designed to resolve normal as well as malignant cells because normal cells act as internal reference standards<sup>1</sup>. The laboratories should then become familiar with the reactivity patterns encountered with their particular reagents. Most reagents are not absolutely specific for cells of a single lineage or there may be aberrant expression or lack of expression of reagents by neoplastic cells not seen in normal cells.<sup>7</sup>
- 3.4.2. In addition to multicolour and scatter analysis, pattern recognition may also include the fluorescence intensity of antibody-labelled cells (as a measure of the level of antigen expression). Cells should be labelled according to manufacturer's recommendations and instruments should be calibrated. Any deviation from this recommendation should be validated. In order to increase the sensitivity of the analysis, the choice of

fluorochrome may be important. It is recommended that "brighter" fluorochromes (such as fluorochromes emitting in the far red e.g. phycoerythrin) be used in cases of expected low antigen expression.

3.4.3. Surface antigen expression is generally used to assign a given case of Acute Leukaemia into one of the three broad categories: B-Lymphoid, T-Lymphoid or Myeloid.<sup>7</sup>

3.4.4. Although different antibodies can be classified by cluster of differentiation (CD) number, the different clones may show different cellular reactivity. This may be important when selecting an antibody panel. The CD number or clone name in the absence of CD number (e.g. FMC7) should be listed on the worksheet.

3.4.5. For the determination of lineage and maturation stage, the detection of cytoplasmic antigens may be of importance.

3.4.6. Any panel of antibodies must include:

- An appropriate panel of antibodies for investigating the presumptive diagnosis. The selection of antibodies used in the panel should be referenced.
- Mechanisms for internal cross-checking and lineage co-expression evaluation.
- Isotype controls matched to the test antibody may be included

Examples of screening panels are:

#### **Acute Leukaemia**

- Non lineage: CD45, CD34, HLA-DR
- T cell: CD1a<sup>7</sup>, CD2, CD3, TCR<sup>7</sup>, CD5<sup>7</sup>, CD7
- B cell: CD10, CD19, CD20
- Myeloid / Mono: CD11b<sup>7</sup>, CD13, CD14, CD15, CD33, CD64<sup>7</sup>, CDw65<sup>7</sup> CD117<sup>7</sup>
- Erythroid: Glycophorin<sup>7</sup>
- Megakaryoblastic: CD41<sup>7</sup>, CD61<sup>7</sup>

#### **Lymphoma / Chronic Leukaemia**

- Non: lineage CD38, CD45, HLA-DR,
- T cell: CD2, CD3, CD4, CD5, CD7, CD8, TCR $\alpha$  / $\beta$  <sup>7</sup>, TCR $\gamma$  / $\delta$  <sup>7</sup>
- NK: CD56, CD16 ,CD57<sup>7</sup>
- B cell: CD10, CD19, CD20, CD22, CD23, CD37<sup>7</sup>, surface membrane immunoglobulin light chains (kappa, lambda) in a single tube with a B cell marker

### **3.5. Staining Procedures**

- 3.5.1. For commercially available antibodies, follow the manufacturer's instructions. Each individual laboratory should verify any deviations from these instructions. Each individual laboratory should validate the reactivity of individual antibodies and antibody cocktails prepared in house. Antibodies should be titrated and tested on appropriate cells (up to 1x10<sup>6</sup> cells per test). All antibodies must be stored in a dark container NOTE: Insufficient antibody may result in weak staining, leading to possible false negative results. Excess antibody may cause increased non-specific staining of negatives or reduced staining of positives (prozone effect) and decreased resolution
- 3.5.2. All washes and antibody incubations should be performed in the presence of 0.1% NaN<sub>3</sub> to prevent shedding, capping or internalisation of the antigen.
- 3.5.3. Incubation should occur in the dark for 10mins at room temperature or 30mins at 4<sup>0</sup> C.

### **4. CONTROLS**

4.1. Isotype controls may be an aid in establishing levels of non-specific binding and autofluorescence. In many cases the isotype control may not be optimal for determining non-specific fluorescence because of differences in fluorochrome/protein ratio and antibody concentration between the isotype control and the test reagents. This is particularly important in certain cases of leukaemia (in particular myelomonocytic) where there is a high degree of species cross reactivity due to the presence of Fc receptors. The addition of protein (AB heat treated serum, Fetal Calf Serum, BSA) can be added to reduce non-specific staining.

4.2. Antibody panel combinations should provide clearly negative populations of cells for use in identification of thresholds for determination of levels of immunofluorescence staining to be considered positive for marker expression.

4.3. Pooled subclass controls are not recommended. It is emphasised that dim reactivity of test antibodies cannot be interpreted with certainty in the absence of appropriate negative controls.

4.4. Even when appropriate negative controls show no staining, a high level of false staining with anti-immunoglobulin (kappa/lambda) reagents may occur because these reagents bind to cytophilic serum immunoglobulin adsorbed onto Fc receptors. Standard washing procedures usually remove most of the serum and a proportion of cytophilic immunoglobulin. Individual laboratories should

validate these washing procedures. Alternatively, cells can be incubated at 37 °C for 5 minutes in a protein containing medium.

4.5. It is not practical or necessary to analyse a normal control sample on a daily or weekly basis if the laboratory is active and within-run positive and negative control results demonstrate appropriate reactivity. The most appropriate procedural control demonstrates that target cells in the sample are capable of reacting under test conditions. Each panel should include an antibody which is positive for leucocyte target cells e.g. CD45. Internal QC checks are a useful guideline in most cases. However caution should be used as malignant cells often express aberrant or inappropriate antigens.

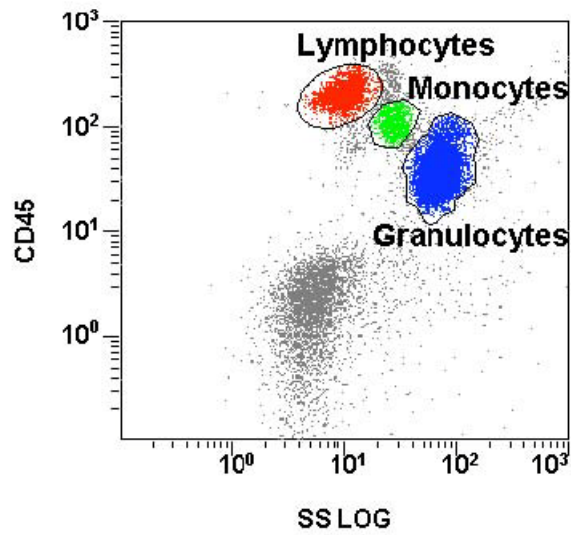
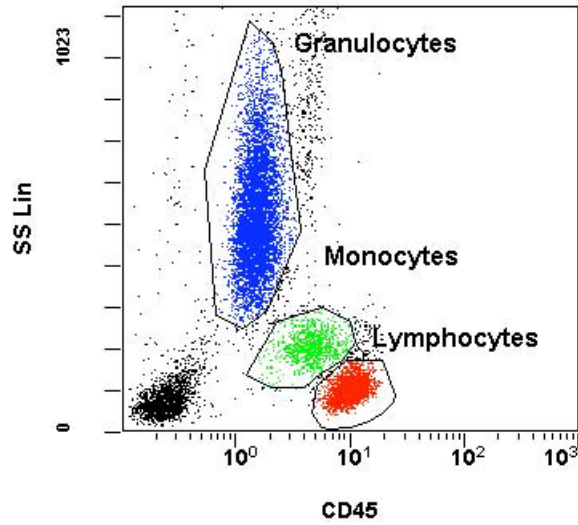
#### **4.6. Internal QC checks include**

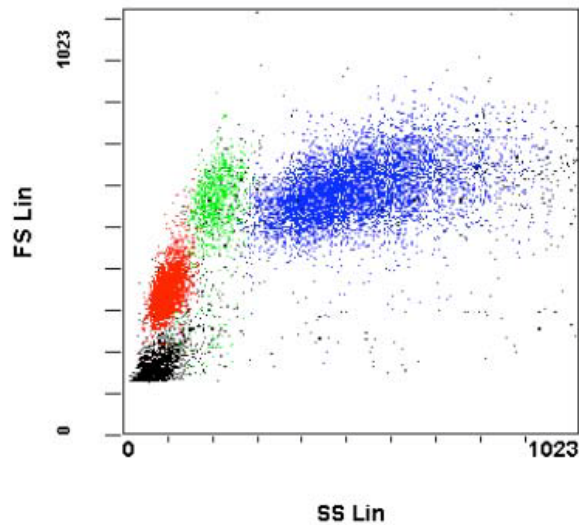
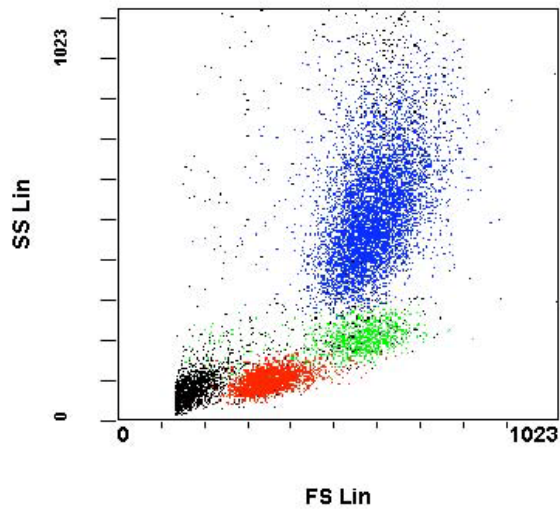
- Reproducibility of antibodies used in greater than one tube should be demonstrated
- Tcells+Bcells+Nkcells should approximately equal total lymphocytes (lymphosum)
- (CD4 +CD8) should approximately equal CD3
- Kappa+Lambda should approximately equal CD19+ B cells.

Morphologic evaluation should be performed on the same portion of specimen to be used for immunophenotyping to ensure that the specimen being analysed on the flow cytometer is representative of the putative disease process. For bone marrow aspirates and peripheral blood specimens, a smear preparation is generally sufficient. Touch preparations may be used for tissue masses and cytopsin (or similar) preparations may be preferred for fluids and disaggregated masses and biopsies<sup>1</sup>.

### **5. SAMPLE ACQUISITION AND ANALYSIS**

5.1. Test order within any panel. The gate should be set in the first tube to maximise the cells of interest and minimise contamination with the relevant particles. The discriminator / threshold is set to exclude debris, platelets etc. The appropriate isotype controls (if included) should be run next, followed by the test panel to investigate the provisional diagnosis.



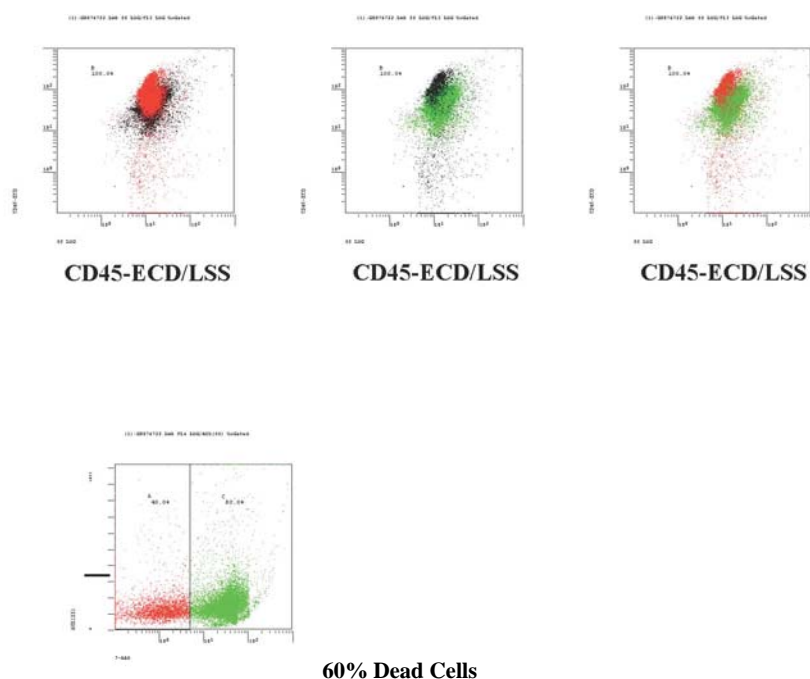


5.2. Figures 1a and 1b Common ways of displaying lysed whole blood preparations, showing lymphocyte, monocyte and granulocyte populations. Live gating should be restricted to the setting of a forward light scatter threshold. Set leucocyte gates as broadly as possible, consistent with acceptable levels of contaminating particles to avoid the exclusion of cells of interest.

5.3. Assessment of specimen viability should be done where possible, particularly on tissue specimens. Viability may be assessed by a real-time flow cytometric assay, or correlatively by manual methods. Real time assessment depends upon the use of fluorescent dyes such as propidium iodide, 7 amino actinomycin D (7-AAD) to stain nonviable cells. If cells are fixed and analysed at time points longer than 12 hours after staining, then the Ethidium MonoAzide (EMA) method of viability determination is recommended. Alternatively, a correlative measurement can be made manually by using a viability stain such as trypan blue and performing manual cell counts (100 200 cells, minimum) on a haemocytometer<sup>2</sup>. Abnormal cells which can be more fragile than normal cells may be excluded using real time viability assessment.

## 7-AAD Viability of Patient Cells

### Burkitts Lymphoma with 40% viable Cells<sup>8</sup>



### 7-AAD/SS Live Cells/Dead Cells

A statistically significant number of gated events per sample should be collected to allow for accurate assessment of minor cell populations. When the cells of interest are present in low frequencies, the total events collected should be increased proportionally.

- 5.4. It is best practice not to set any restrictive acquisition gates. It is recommended that all events be acquired and saved for re-analysis. Obvious cell populations can be discriminated by light scatter and/or antigen expression.
- 5.5. The reporting of percentages may not be possible for leukaemia samples due to the difficulties encountered with the expression of many antigens. Instead an estimate of the strength of expression of the antigens on a detected abnormal population should be made. This should be reported as “dim” or “weak” expression where there is some overlap with the appropriate negative cell population. The report can then be in the form of a qualitative description of the phenotype of the leukaemia cells.
- 5.6. It is beyond the scope of these guidelines to present a detailed analysis of different leukaemias due to the wide range of immunophenotypes that can be encountered. It is recommended to obtain familiarisation with the expression of antigens on normal cells and to use reliable reference sources

## 6. DATA REPORTING

6.1. Report all unique patient identifiers.

6.2. Report all data in terms of cluster of differentiation (CD) with a short description of the main antigen recognition characteristics.

6.3. For unclustered antibodies e.g. FMC7, report the clone name with a short description of the main antigen recognition characteristics.

6.4. Report all data indicating the phenotype of the detected abnormal population. If necessary, e.g. in the case of detection of minimal residual disease, make an estimation of the percentage of abnormal cells in the total cell population. The presence or absence of antigens together with the intensity of fluorescence when the antigen is present should be reported for the abnormal cells. If no abnormal population is identified, a statement of the relative proportions of the normal cells in the sample should be included in the report.

## **7. DATA STORAGE<sup>7</sup>**

7.1. Both the Data stored and the methods used to obtain the data must be thoroughly documented.

The variability observed in LS patterns in patient samples requires that data be stored in list-mode form because gating may not be assignable *a priori*.

7.2. All primary files, worksheets and report forms must be retained for 2 years or as required by state or local regulations which ever is longer.

7.3. After the retention period disposal is at the discretion of the Laboratory Director.

7.4. QA and QC data files should include all parameters, analysis regions and analytical results used to verify performance (instrument and method).

7.5. Data may be stored as paper hard copy and/or archival files.<sup>7</sup>

## **8. QUALITY ASSURANCE**

- 8.1. The laboratory must belong to and participate in a recognised external quality assurance program.
- 8.2. Each laboratory should determine the quality of the reagents on a regular basis and at least when changing to new lot numbers. Antibodies should be tested on appropriate cell lines and freshly prepared leucocytes to determine whether they can be used to measure the level of antigen expression.

#### **REFERENCES**

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#### **FURTHER READING**

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