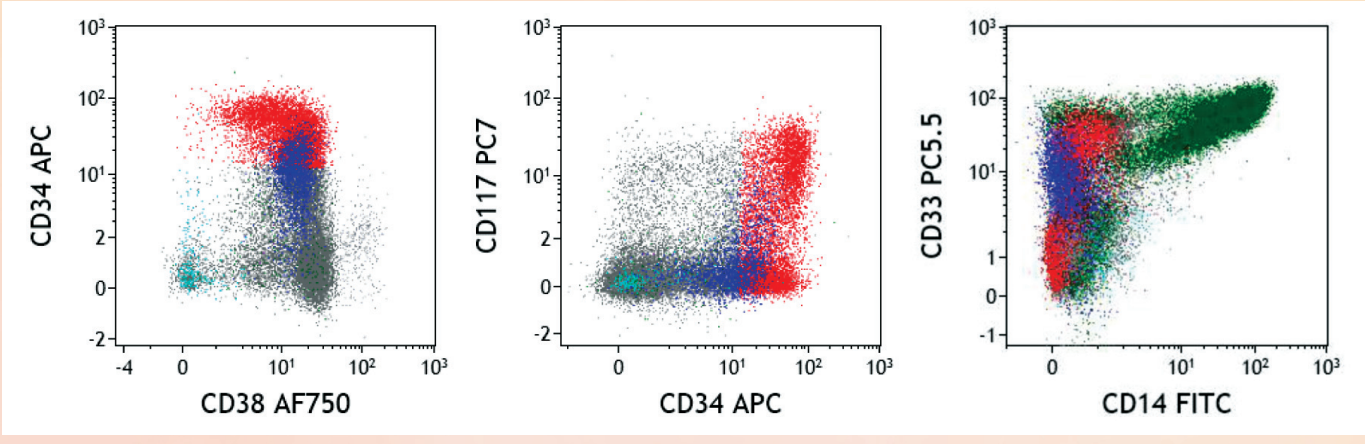
# STANDARD OPERATING PROCEDURES FOR IMMUNOPHENOTYPING OF HEMATOLYMPHOID NEOPLASMS




*Coordinated by*
Division of Non Communicable Diseases

Indian Council of Medical Research
2016

# STANDARD OPERATING PROCEDURES FOR IMMUNOPHENOTYPING OF HEMATOLYMPHOID NEOPLASMS

Prepared as an outcome of ICMR Expert Group on
Immunophenotyping of Hematolymphoid Neoplasms

*Coordinated by*
Division of Non Communicable Diseases

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# Foreword

I am glad to write this foreword for Standards Operating Procoedure for diagnostic work of hematolymphoid neoplasms using flow cytometry immunophenotyping. This document is result of the hard work of various experts across the country working in the area of laboratory hemato-oncology. ICMR constituted a task force to address issues related to flow cytometry including indications of immunophenotyping, sample collection, transportation, processing, acquiring, analysis and reporting. This task force also aims to provide guidelines for panel selection, choice of reagents, quality control and safety issues in a flow cytometry laboratory.

This document represents a joint effort of large number of individuals & it is my pleasure to acknowledge dedication and determination of each member involved in this task.

This document addresses on various issue related to good quality practices in laboratory work up of flow cytometric immunophenotyping and will be of use to pathologists, cytometrists, hematologists, technologists and scientists working in this field.

Dr. S Swaminathan
Secretary, Department of Health Research
and Director General, ICMR

# Preface

I take this opportunity to thank Indian Council of Medical Research and all the expert members of the subcommittees for having faith in me and considering me as Chairperson of ICMR Task Force project on constituting a task force to address issues related to flow cytometric immunophenotyping in leukemia lymphoma lab.

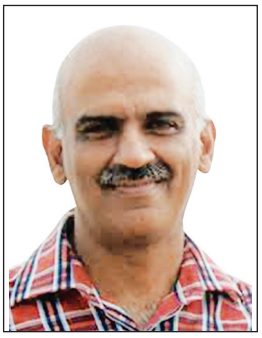
ICMR constituted a task force to address issues related to flow cytometry including indications of immunophenotyping, sample collection, transportation, processing, acquiring, analysis and reporting. Laboratory professionals from different cancer centers across country were slected to address these. The task force aims to provide guidelines for panel selection, choice of reagents, quality control and safety issues in a flow cytometry laboratory.

The taskforce committee included laboratory and medical experts from different parts of the country practicing clinical flow cytometry routinely with an emphasis on lymphoma nad leukemia immunophenotyping. These SOPs will be useful to various cancer centers doing management of leukemia and lymphomas all across the country.

Kusum Verma

(Dr. Kusum Verma)
Chairperson
ICMR Task Force Project

# Message



Flow cytometry has evolved from a niche laboratory technique to routine tool used by clinical pathologists for diagnosis and monitoring of patients with cancer and immune deficiencies. Immunophenotyping by flow cytometry has become standard practice in management of patients with hematolymphoid neoplasm.

Despite being in this field for more than 3 decades, its widespread use within India, there is a considerable variability in various issues related to diagnosing hematolymphoid neoplasms. It starts from sample collection, transportation, processing, acquiring, analysis and finally reporting of these tumors. Though there are published international guidelines and methodologies, however, none from India existed.

ICMR constituted a group of cytometrists from various centers from India doing leukemia lymphoma to devise Standard Operating Procedure (SOPs) for above issues. This group has successfully compiled various SOPs with an emphasis on lymphoma and leukemia immunophenotyping. This is our first attempt to bring some uniformity in reporting of hematolymphoid neoplasms in India.

(Dr. Sumeet Gujral)
Principal Investigator,
ICMR Task Force Project

# Message

Flow cytometric immunophenotyping remains an indispensable tool for the diagnosis, classification and monitoring of hematolymphoid neoplasms like lymphoma, chronic lymphoproliferative disorders, plasma cell neoplasm, acute leukemia, paroxysmal nocturnal hemaglobinuria, mast cell disease, myelodysplastic syndromes and myecloproliferative disorders.

We have seen advances in flow cytometry technology including instrumentation, availability of large panel of reagents and newer flurochromes. Newer WHO classfication of hematolymphoid neoplasms have made it mandatory to use this technology as a routine in management of such cases. It endorses a multiparametric approach to diagnosis and outlines the morphologic, immunophenotypic, and genotypic (cytogenetic as well as molecular based) features characteristic of each disease entity.

This document represents current thinking of National experts on this topic based on available evidence and will have to be revised as we move.

(Dr. R.S. Dhaliwal)
Head, NCD

# Acknowledgement

The ICMR constituted a team to work on various Standard Operating Procedure (SOPs) for workup of hematolymphoid neoplasms by flow cytometry. Team was from premier institutes and included cytometrists, patholgogists, scientists, hematologists and oncologists. Various centers all across India were included in this and were requested to work on various SOPs starting from indications to sample collection, sample transport, processing, acquisition, reporting to quality control, waste disposal and safety issues.

The group has come up with various SOPs, which will be useful for any laboratory dealing with hematolymphoid neoplasms.

I would like to express gratitude to Dr. Soumya Swaminathan, Secretary, Department of Health Research and Director General, Indian Council of Medical Research, for taking her special interest and understanding the need of formulating the standard operating procedures for the Immunophenotyping of Hematolymphoid Neoplasms. I would like to thank Dr. R.S. Dhaliwal for his support and coordination in finalizing this document.

I would like to take this opportunity to thank Dr. Kusum Verma, Chairperson, ICMR Task Force on Immunophenotyping Neoplasms. The efforts made by Dr. Sumeet Gujral, Principal investigator for taking up this excercise voluntarily are highly appreciated. The ICMR appreciately acknowledge the valuable contribution of members in extending their support in formulating Standard Operating Procedures.

Tanvir Kaur

(Dr. Tanvir Kaur)
Programme Officer

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# Contents

# 1. INTRODUCTION

WHO 2008 has classified hematolymphoid neoplasms based on clinical, morphological, immunological (immunohistochemistry and flow cytometric immunophenotyping), cytogenetic and molecular evaluation of various tissues including peripheral blood, bone marrow, lymph nodes, spleen and extranodal lymphoid tissues. These ancillary techniques are mandatory for the diagnosis, prognosis and management of hematolymphoid neoplasms that constitute approximately 8% of all tumors.

Immunophenotyping by flow cytometry is standard practice in the diagnosis, classification and monitoring of patients with hematolymphoid neoplasm. Lack of uniformity and considerable variability continues to exist in various methodologies used for sample evaluation and the reporting format. The ICMR constituted a task force to address issues related to flow cytometry including indications of immunophenotyping, sample collection, transportation, processing, acquiring, analysis and reporting. Also the task force aims to provide guidelines for panel selection, choice of reagents, quality control and safety issues in a flow cytometry laboratory.

The taskforce committee included laboratory and medical experts from different parts of the country practicing clinical flow cytometry routinely with an emphasis on lymphoma and leukemia immunophenotyping.

These documents have been written in collaboration with various cytometrists (pathologists and oncologists) from across India, and are based primarily on their expert opinion and published literature. It should be recognized that these documents/SOPs/guidelines are based on information that is available at the present time, and will change with advances in the technology and knowledge. All findings should be interpreted in the context of clinical, morphological and various ancillary techniques, whenever possible.

This is first successful attempt to define broad guidelines suitable for the initial evaluation of hematolymphoid neoplasm. With goal to provide good laboratory practices, ICMR proposes to have similar documents for cytogenetics and molecular diagnostics in the hematolymphoid neoplasms.

# 2. INDICATIONS FOR FCM IN A SUSPECTED CASE OF HEMATOLYMPHOID NEOPLASM

FCM analysis is used as a sensitive screening technique to document the presence of hematologic malignancy and assist in demonstrating the absence of disease. The aim of this document is to guide laboratories in the standardization of assay for flow cytometric immunophenotypic analysis. Immunophenotyping plays crucial part in the accurate diagnosis and classification of hematolymphoid malignancies as per recent WHO classification. The usual indications for immunophenotyping are as follows:

1. Cytopenias, especially bicytopenia and pancytopenia (since isolated cytopenias, particularly anemia, commonly occur in non-neoplastic diseases, these alone should not automatically trigger flow cytometric analysis).
2. Elevated leukocyte count, including lymphocytosis, monocytosis, and eosinophilia.
3. Presence of atypical cells or blasts in the peripheral blood, bone marrow, or body fluids.
4. Plasmacytosis or monoclonal gammopathy.
5. Organomegaly and tissue masses. Flow cytometry is extremely useful in the diagnosis and subclassification of tissue-based lymphoid neoplasia with the exception of Hodgkin's lymphoma. This testing is indicated in lymphadenopathy, organomegaly (including but not limited to splenomegaly and hepatomegaly), and tissue infiltrates (including but not limited to skin, mucosal sites, and bone)
6. Mast cell neoplasms[2,3].

In contrast, **flow cytometry is generally not indicated** in the following situations:

1. Mature neutrophilia
2. Polyclonal hypergammaglobulinemia
3. Polycythemia
4. Thrombocytosis
5. Basophilia

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# 3. ISOP IMMUNOPHENOTYPING: SPECIMEN COLLECTION, TRANSPORTATION TO THE TESTING FACILITY (LABORATORY)

**Contents:**



The detailed guidelines for standard precautions, specimen collection, labeling and transportfor variety of laboratory tests including FCM are published by the clinical laboratory standards institute (CLSI, formerly NCCLS). Clinical laboratories may refer to these guidelines for detailed information.

### 3.1 Standard Precautions

All human specimens should be treated as potentially infectious and handled according to *"standard precautions"* given by Center for Disease Control and Prevention (CDC). Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of blood-borne pathogens.(Refer for standard precautions: http://www.cdc.gov/HAI/settings/outpatient/outpatient-care-gl-standared-precautions.html)

### 3.2 Specimen collection:

Correct sample collection is important for reliable and reproducible results. Incorrect specimen collection can necessitate re-sampling, cause delay in diagnosis and treatment, increase patient suffering and increased costs.FCM immunophenotyping can be done in a variety of specimens for the diagnosis, staging, prognosis and monitoring of the diseases, especially in hematolymphoid neoplasms. The specimens in which FCM is easily done include:

- Peripheral blood (PB),
- Bone marrow (BM),
- Body fluids (pleural fluid, ascitic fluid, pericardial fluid vitreous fluid and cerebrospinal fluid),
- Fine needle aspirates (FNA) from lymph nodes (LN) and other lesions.
- Fresh tissue samples

**3.2.1 Peripheral blood:** It should be collected by standard venipuncture into an appropriate anticoagulant (discussed in following section 3). Small gauge needle or pulling hard may damage erythrocytes and probably also damage leukocytes. Sample can be collected directly into syringe or evacuated tube containing anticoagulant. Cases where collection of specimen is done in a syringe without anticoagulant, one should immediately transfer it into a tube containing anticoagulant and gently mix it by inverting 8-10 times. For CD4 testing, the specimens shall be kept at room temperature (between 18-22 C) after the collection, and transferred to the laboratory at the earliest [Reference: CDC guidelines for CD4 Cytometry 14:702–714 (1993)]. The collected specimens should not be refrigerated or frozen before testing.

**3.2.2 Bone Marrow aspirate or core biopsy:** It is advised to collect first pull of bone marrow aspirate, approximately 0.5 to 2 ml, in order to get representative specimen and avoid dilution with peripheral blood.In case of "dry tap", a core biopsy can be submitted without addition to fixatives in RPMI media or normal saline or else biopsy can be vortexed in RPMI media, till it becomes cloudy. The cloudy media containing disaggregated cells shall be sent for FCM and biopsy for routine histopathology evaluation (Ref 5).

**3.2.3 Body fluids:** Fluids shall be collected as per standard medical practice. For CSF, special precautions should be taken, it should be processed within an hour or two. If delay in the processing is anticipated, CSF should be collected in RPMI media with bovine serum (Ref 8 and 9).

**3.2.4 Fresh tissue biopsies:**Tissues like lymph node biopsy, splenic biopsy and other lymphoid tissue biopsies can be routinely analyzed by flow cytometry. Biopsies must be submitted fresh without fixation. It should be transported in RPMI media or sterile saline-soaked gauze at removal, and kept moist throughout transport to the laboratory (Ref – NCCLS guidelines).

**3.3 Specimen anticoagulation:**

*3.3.1 Bone marrow or Peripheral blood*

a. *Immunophenotyping*: The anticoagulants that are available include dipotassiumethylenediamine tetra-acetic acid ($K_2$EDTA; referred as EDTA in the following text), sodium or lithium heparin, and acid citrate dextrose (ACD). Any of the above three anticoagulants are acceptable for PB or BM samples for the immunophenotypic evaluation for hematolymphoid neoplasms and are known to preserve majority of the clinically relevant antigen expression up to 12 to 24 hours. Heparin is most reliable in terms of antigen preservation, especially in older specimen (> 24 hrs of sample collection) (Ref 11). ACD and heparin are shown to be superior to EDTA for maintaining viable leucocytes overnight (ref 10, 11). Moreover EDTA may influence the expression of certain antigens such as CD10, CD11b, CD16 and CD64 (Ref 12). Advantage of heparin anticoagulation for BM aspirate is enhanced for the FCM analysis of myeloid antigen expression in myelodysplastic syndromes (MDS). Granulocytes begin to show significant apoptosis after 6 hours in EDTA or ACD anticoagulation, with heparin showing the least amount of apoptosis. However, if one is processing sample on the same day of collection and also needs morphological evaluation, then EDTA is the anticoagulant of choice (NCCLS).

b. *Quantitative flow cytometric assays*: Use EDTA or heparin evacuated tubes for assays like CD4+ T-cell enumeration or CD34+ stem cell quantitation. Because acid citrate dextrose is added as a liquid to blood collection tubes, its use would make it difficult to calculate the final sample volume accurately and hence not recommended. For absolute counts, use of an accurate sample volume is critical. Although forward light scatter is maintained in EDTA for only 24 hours, the widespread adoption of CD45 versus side light scatter gating for lymphocyte quantitation has allowed the reliable results for EDTA anti-coagulated

samples upto 72 hours for quantitative assay like CD4 counts in HIV.Reject specimens that cannot be processed within 72 hours (CDC guidelines).

*3.3.2 Fresh Tissue Specimens*

In general, anticoagulant is not required for fresh tissue samples. These may be transferred to a transport media (see below) or saline soaked gauze.

*3.3.3 Body Fluids*

For body fluids like pleural fluid, pericardial fluid, and ascitic fluid anticoagulation is not required unless until the fluids are grossly hemorrhagic in nature. One can simply collect samples in a clean sterile container. However, cerebrospinal fluid (CSF) needs special attention. CSF samples should be processed within 1 hr after lumbar puncture; otherwise it should be stabilized with RPMI media or equivalent material to avoid deterioration of the cells due to the rapid *in vitro* cytotoxic effects of CSF on leukocytes.

**Note:** In the event of delayed processing, in case of reference laboratories, one can use stabilization media like Cyto-Chex® Blood Collection tube (Streck), Transfix® (Cytomark), especially for clinical trials (Ref 13).

**3.4 Specimen labeling and test ordering:**

Good clinical practice dictates that specimen containers be labeled immediately at the point of collection by the collector (e.g., phlebotomist at the bedside).The label should include at least two unique patient identifiers (e.g., full patient name, medical record number, or patient birth date). Samples should be submitted with the requisition or order. The latter (paper or electronic) should include time and date of collection and, preferably, the name of the phlebotomist or physician obtaining the samples.

**3.5 Specimen Age and Integrity (including transportation):**

Specimen age is a critical variable in FCM analysis, so all specimens must be labeled with the date and time of collection and processed as soon as possible. This is particularly important in cases of tumors with high proliferation rate (e.g. Burkitt's lymphoma, ALL) or in patients where chemotherapy and/or radiation treatments were given. A 48-hour cut-off for specimen age is appropriate; however, irreplaceable specimens should not be rejected if they exceed the 48-hours. An attempt should be made to derive useful information from such samples.A statement regarding exceeded specimen age should be noted in the final report. Each and every sample is precious and should not be discarded. Some antigens are more labile than others (e.g., CD138) and may be preferentially affected with increasing specimen age. Results of old specimen should be interpreted cautiously as it may lead to false negativity of labile antigens as well as non-specific antibody binding.

*3.5.1 Bone marrow or Peripheral blood:*

Bone marrow and peripheral blood samples for leukemia/lymphoma evaluation usually can be stored at room temperature for 24 to 36 hours without loss of significant antigenicity. This typically can be expanded to 48 to 72 hours if such samples are diluted 1:1 with cell culture media (e.g. RPMI-1640 media) and stored at 4 °C.

*3.5.2 Peripheral blood for Quantitative Blood Lymphocyte Subset Analysis:*

Blood samples for quantitative lymphocyte subset analysis can be held for up to 72 hours in EDTA-anticoagulated containers without clinically significant changes in the concentrations or percentages of lymphocytes but only if CD45 versus side light scatter gating is used. If forward light scatter is a

component of the lymphocyte gating, then EDTA anticoagulated samples typically have a 24-hour limit before significant loss of forward light scatter of the lymphocytes. Because of this and the overall superiority of CD45 versus side light scatter gating, the use of forward light scatter based lymphocyte gating is not recommended.

Quantitative lymphocyte analysis was performed initially with density gradient purified samples. The CD4 T-cell subset is vulnerable particularly to low-temperature–induced loss during density gradient purification resulting in falsely low CD4 counts (so-called 'refrigerator AIDS' or ''RAIDS''). This issue essentially has disappeared with the switch to whole blood lysis methods, which allow blood samples to be held at room or refrigerated temperatures without loss of accurate gating. Examine the tubes for sample integrity before transporting to the testing laboratory. Repeat specimens may be requested, if there is evidence of gross hemolysis or clots. Keep the specimen at room temperature (preferably below 28°C) and do not refrigerate or freeze. Avoid extreme temperatures. High temperatures (greater than 37°C) may cause cellular destruction (Ref: CDC & WHO manual).

*3.5.3 Solid Tissues/Lymphnodes:*

Ideally, tissue biopsy samples should be processed immediately into a single-cell suspension. This can be done by the classic ''mince and slice'' technique, with or without the use of a steel mesh screen. The crude suspension should be placed in a 1:1 dilution of cell media (e.g. RPMI-1640) and stored at 4 C until a decision is made whether or not to process the sample. It is possible (but not ideal) to hold suitably prepared solid tissue for several hours (up to 24 hours) and still can have a reasonable degree of confidence that clinically relevant immunophenotyping data can be obtained. For this, samples should be small (1 cm in greatest dimension) and thin (0.5 cm). Such slices should be wrapped in saline-soaked gauze, and then immersed in sterile saline in a sealed container stored at 4°C. At the earliest opportunity, samples should be processed into a crude cells suspension (discussed previously) or a decision made whether or not to perform immunophenotyping. Recently, cryopreservation is also shown to be useful in storage of fresh biopsies for later FCM immunophenotyping.

*3.5.4 Body Fluids:*

For sample viability, use tissue culture transport media (e.g. RPMI 1640). However, autofluorescence may increase with the use of tissue culture media and consideration should be given to the impact of low viability vs. autofluorescence when choosing a transport media. Cold (4°C) storage may be useful for prolonged storage of some specimens such as pleural fluids and cell suspensions prepared from tissues. Avoid extreme temperatures.

A stained smear or tissue imprint and/or tissue section should accompany specimens for reference (for referral labs). An alternative is the addition of a stabilization fluid to the sample; this may allow specimen storage up to few days for FCM analysis.

It is crucial to have written guidelines for technical staff (e.g., histo-technologists, cyto-technologists, and medical technologists) and professional medical staff (e.g., pathologists, surgeons, radiologists, and residents). In particular, there should be documentation of training of such individuals and annual competency testing. (Clinical lab Med 27 (2007) 607 – 707)}

### 3.6 Sample processing:

The two common methods used for processing of flow cytometry samples for hematolymphoid neoplasms include the Lyse-Wash - Stain method and the second one being the Stain-Lyse-Wash method. These two methods involve two different types of lysing solutions - one with fixative and another without fixative. It

is important to note that if one chooses Lyse-Stain-Wash method, where the lab makes a cell suspension before staining, the lysing solution used should be without fixative as the same can affect the antigen-antibody reaction. In the Stain-Lyse-Wash method, lysing solution containing fixative can be used as the antigen-antibody reaction has already taken place before the lysing step.

*3.6.1 Lyse stain wash Processing method*

In this method processing of hematolymphoid samples for immunophenotyping starts with lysing (red cell) of the sample, followed by wash and then staining (lyse – wash – stain method). The first step is to obtain a single cell suspension followed by antibody staining, data acquisition and data analysis.

Lysing is usually used for Peripheral blood or Bone Marrow samples or hemorrhagic body fluids / aspirate (to get rid of red cells for immunophenotyping).

### 3.6.1 Cell Suspension preparation

#### a) Peripheral Blood (PB) / Bone Marrow (BM)

A ratio of 1:10 of sample to lysing solution is recommended. (Mix 0.5 ml of the sample with 4.5 ml of lysing solution in a test tube. Incubate for 6–8 minutes at room temperature. Centrifuge for 2 minutes at 400xg at room temperature to remove red cell debris. Discard the supernatant. Gently tap the pellet to re-suspend the cells and add 2 ml of PBS-A. Wash by centrifuging for 2 minutes at 400xg at room temperature to remove the left over lysing solution. Discard the supernatant and re-suspend in PBS-A to get the final cell suspension. Target cell count – 0.5 to 2 x $10^6$ cells per 50 µl per assay tube.

#### b) Body Fluids / Fine Needle Aspirates

Centrifuge (400xg at room temperature for 2 minutes in swing rotor centrifuge) to obtain a cell pellet. Gently tap the pellet to resuspend the cells and add 2 ml of PBSA. Wash by centrifuging for 2 minutes at 400xg at room temperature. Discard the supernatant and re-suspend in PBSA to get the final cell suspension. Target cell count – 0.5 to 2 x $10^6$ cells per 50µl per assay tube.

As body fluids and aspirates may have low cell yields one may have to reduce the target cell count to as low as 25,000 to 50,000 cells per tube and select priority tubes from a given panel. Optimal acceptable percent of viable cells is about 80%.

*For hemorrhagic fluid samples, a lysing step may be required (as described above for PB/BM).

#### c) Lymph Node Biopsy / Tissue Biopsy in Normal saline

Suspend the biopsy in normal saline and transfer to a small petridish. Cut the biopsy into tiny bits and gently tease with the help of scissors and forceps. The cells can be transferred from the petridish to a test tube with micropipetters. Centrifuge the sample to obtain a cell pellet (400xg at room temperature). Gently tap the pellet to re-suspend the cells and add 2 ml of PBSA. Wash by centrifuging for 2 minutes at 400xg at room temperature. Discard the supernatant and re-suspend in PBSA to get the final cell suspension. The target cell count is around 0.5 to 2 x $10^6$ cells per 50µl per assay tube.

### 3.6.2 Staining

Antibody staining is performed according to the manufacturer's instructions. After the final cell suspension is ready and target cell concentration is obtained, antibodies can be added as per the leukemia/lymphoma panels of the laboratory (based on history, clinical details, morphology and other findings or else as per

the policy of the laboratory). As mentioned above, for low cell yield sample (e.g., cerebrospinal fluid) one may have to select priority tubes from given panel. A given panel may have four to six tubes per panel with each tube containing three to six antibodiesor more (colors) based on cytometer configuration. One can add individual antibodies or pre-prepared antibody cocktails based on the sample work-load and the laboratory policy. It is highly recommended to use per-prepared antibody cocktails to avoid the pipetting errors.

Add 50µl of cell suspension to individual antibodies/antibody cocktail (the volume of antibody added is based on the titration volume determined for each antibody). Mix gently by tapping and incubate for 15-20 minutes at room temperature in the dark. Wash with 3–5 ml of PBSA by centrifuging for 2 minutes at 400xg at room temperature to remove excess unbound antibodies.

Discard the supernatant and re-suspend in 0.5ml of 0.5% paraformaldehyde. For cytoplasmic and nuclear staining, cells are fixed and permeabilized before antibody staining and such reagents may be prepared in-house or procured commercially. After staining, the cells are acquired on the flow cytometer, data is stored and analyzed and an immunophenotyping report is generated. Turnaround time for reporting a case of leukemia/lymphoma immunophenotypingcan be from 1-2 days. A stringent internal as well as external quality control program for lab operation should be maintained.

*3.6.2 Stain-Lyse-Wash processing method*

**3.6.2.1 Staining:** This method starts with the addition of appropriate antibody cocktail into the properly labelled tubes as per the titered volume. Then 100 ul of patient's sample (peripheral blood, bone marrow or fluid or another specimen) containing approximately 0.5-1×$10^6$ cells is added to the tubes with antibody cocktail. Cells will be mixed with antibody cocktail by vortex al low speed followed by incubation for 15-20 mins at RT in the dark. This method is followed by red cell lysis as described below.

**3.6.2.2 Lysing:** 2ml of working concentration lysing solution (as per manufacturer's instructions) is added to sample incubated with the antibody cocktail, mixed by vortexing at low speed and then followed by incubation for 10 mins at RT in the dark. It is then followed by centrifugation at 400g for 5 mins (RT) and decanting the supernatant (leaving 50-100 µl of fluid in the tube).

**3.6.2.3 Washing:** 2ml of PBS- BSA or shealth fluid is added to wash the excess reagents and cell debris (repeat one more time). The supernatant is decanted leaving 50-100 µl of fluid in the tube. It is ten followed by resuspension of the cells in the PBS or sheath fluid and acquisition on flow cytometer.

Note-For Kappa and lambda light chain evaluation, two additional sample pre- washing steps are suggested, prior to the acquistion in the Stain-lyse-wash protocol.

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# 4. PANEL DESIGNING: SELECTION OF ANTIBODIES AND FLUOROCHROME CONJUGATES

## Introduction

Immunophenotyping is a method of analysis of heterogeneous populations of cells identifying different antigens expressed on the surface or inside the cells using antibodies produced against respective antigens. World Health Organization (WHO) has classified hematolymphoid neoplasms on the basis of immunophenotype of the tumor cells. Flow cytometric immunophenotyping allows rapid and simultaneous identification of multiple antigens expressed on a single cell using multiple antibodies conjugated with a variety of fluorescent dyes. To classify hematolymphoid neoplasms correctly, an optimal combination i.e. panel of antibodies conjugated with different fluorochromes is needed. There are several published guidelines for the immunophenotyping of hematolymphoid neoplasms. Notably, "Bethesda International Consensus Recommendations (2006) on the Immunophenotypic Analysis of Hematolymphoid Neoplasia by Flow Cytometry", which is followed by Indian "Guidelines for Immunophenotyping of Hematolymphoid Neoplasms by Flow Cytometry" (2008) held at Tata Memorial Hospital, Mumbai and recently, Euroflow has published detailed document on eight color panel for leukemia and lymphoma immunophenotyping. Clearly there is wide variability in the use of antibodies across the laboratories depending upon the medical indication, type of specimens, instrument configuration and analysis approach. In India, one more factor is important which adds to this variability and that is "cost of the test". Therefore, a uniform and standardized panel of antibodies is practically difficult to apply. This document highlights the principles behind selection of reagents i.e. antibodies and their fluorochrome combination, which would help in designing an antibody panel.

## 4.1 Selection of antibodies

In the last few years, the application of flow cytometry has become very popular in India and simultaneously this technology has evolved substantially. Increasingly, more colors of fluorescence are being detected by cytometers, and faster analysis is becoming possible and to meet increasing demands more reagents are becoming available for a wide variety of applications. With easy availability of multiple antibodies with variety of fluorochromes, it is possible to standardize almost all immunophenotypic assays. International consensus guidelines have been published for certain common assays. Laboratories performing such assays are advised to follow the guidelines as mentioned below:

- CD4+ T cell counts: National institute of allergy and infectious diseases (Cytometry1993;14: 702-714. )
- PNH evaluation: Michael J. Borowitz et al, Cytometry B Clin Cytom. 2010 ;78:211-30.
- CD34+ stem cell analysis: ISHAGE Sutherland DR et al, J Hematother 1996;3:213–226.

Different approaches of panel designing for immunophenotyping are being practiced by different laboratories that includes comprehensive panel, primary screening panel followed by secondary disease

specific panel, morphology or clinical information guided panel and indication based panel (reference TMH guidelines 2008). Whichever approach one opt for, three basic concerns should be addressed:

1. The panel should address medical indication as requested by the referring physician e.g. acute leukemia or chronic lymphoproliferative disorder.
2. It should study adequate markers to establish the diagnostic and prognostic classification of hematological neoplasm or document the absence of the disease.
3. It should be flexible enough to accommodate new reagent which is proven to have greater specificity for diagnosis and prognosis.

To address these concerns, panels should be designed in such a way that it should allow (see Table 1):

- identifcation of a cell lineage e.g. B cells, T cells, myeloid cells etc
- identifcation of immature and mature cells e.g. blasts, lymphocytes, granulocytes etc.
- differentiation of normal verses abnormal cells e.g. hematogones vs leukemic blasts
- identification of all major cell population in the given specimen

It is further recommended that the panel should include (see Table 1):

- CD45 in each cocktail as CD45 versus side scatter gives clear separation between different hematopoietic cells (shown in Fig 1).
- Lineage specific cocktails i.e. panel should include reagents common to a particular lineage, focusing on cells of particular group of cells like B cells, T cells, monocytes or granulocytes etc. This will allow adequate analysis of target subset of cells.
- Common backbone markers for uniform gating across all cocktails (tubes): CD45 is a good marker to use as a primary backbone marker, however, to make it more sensitive, it is preferred to use one of lineage sensitive marker as a backbone marker like CD7 or CD3 for T cell tubes, CD19 for B cell tubes, CD13 or CD33 and/or CD117 for myeloid tubes.
- Markers whose presence or loss or altered intensity indicates neoplastic nature of cells, these markers are called as leukemia associated immunophenotype (LAIP).
- Markers that are differentially expressed with maturation within a particular lineage should be combined to identify synchronous maturational patterns of antigen expression. Abnormalities in the patterns of maturation are common and are extremely useful for the recognition of hematopoietic neoplasms. For example in the B cell lineage, the combination of CD10, CD20 and CD45 is useful to follow early B cell maturation pattern (Figure 2).
- A screening tube which helps to identify different cell populations in the given specimen.
- An appropriate combination of antibody clones and fluorochrome conjugates permitting minimal interference with each other.

Constructing and validating antibody combinations is the primary responsibility of the laboratory. Recently, Euroflow has provided detailed eight color panel with complete details of antibody fluorochrome combinations, they validated for leukemia and lymphoma immunophenotyping (Refer Euroflow). Many laboratories in India use morphology or clinical information to select the panel to be tested on the submitted specimen. However, morphology is inherently subjective and can suggest diagnoses that lead to suboptimal panel selection in some cases, e.g., lymphoma mistaken for acute leukemia or vice versa.

This problem may be compounded by the often-compromised morphologic quality available for aged samples in a reference lab setting (Bethesda). FCM analysis with the comprehensive antibody panel is the most preferred approach, since it permits the detection of aberrant marker expression on neoplastic cells which in turn facilitates the follow up of these patients if post treatment minimal residual disease analysis is needed, and also allows detection of other unexpected hematological abnormalities. But because of financial concerns it may not be practical to use comprehensive antibody panel, however using a screening tube (one or two) which can primarily identify nature of tumor i.e. blastic or mature is helpful, also helps to identify other major subsets of cells present in the specimen and may identify other minor abnormalities. This can be further followed by other markers if needed.

Table 1 enlists the markers published by major consensus guidelines like Bethesda consensus 2006 and Euroflow panels.

| **Table 1. Useful markers (reagents) for acute leukemia panel** | |
|---|---|
| Backbone Marker | CD45 |
| Markers of immaturity | CD34, dim CD45, TdT |
| Myeloid lineage | Cytoplasmic MPO, CD117, CD13, CD33, CD15, CD11b |
| Monocytic lineage | CD4, CD14, CD11b, CD11c, CD36, CD64, CD65, CD66, HLADR |
| Megakaryocytic | CD41, CD61, CD36 |
| B Lymphoid | CD10, CD19, CD22, cytoCD79a, CD20, |
| T Lymphoid | Cytoplasmic CD3, surface CD3, CD1a, CD2, CD4, CD5, CD7, CD8 |
| Plasmacytoid-dendritic | CD123, CD302 |
| Erythroid | CD235a, CD71, CD36 |
| | Leukemia associated immunophenotypes (LAIPs) |
| AML | CD7, CD19, CD56 |
| B/T ALL | CD13, CD15, CD33, CD117 |
| **Markers for B cell chronic lymphoproliferative disorder (lymphoma) panel** | |
| General | CD19, CD20, CD22, cyto79a, CD79b, CD23, FMC7, Kappa, Lambda |
| Markers useful for further subclassification in B NHL | |
| CLL/MCL | CD5, CD23, CD43, CD200, CD38, Zap70 |
| FCL/BL/DLBCL | CD10 |
| HCL | CD11c, CD25, CD103, CD123 |
| **Markers for T cell chronic lymphoproliferative disorder (lymphoma) panel** | |
| General | CD2, CD3, CD4, CD5, CD7, CD8, TCR, TCR |
| | Markers useful for further subclassification in T-NHL |
| ATLL/CTCL | CD25, CD26 |
| AITL | CD10 |
| ALCL | CD30 |
| LGL | CD56, CD57 |
| EATCL | CD103 |
| NK cells | CD16, CD56, CD57, CD94, CD158 |
| **Markers for plasma cell dyscrasia panel** | |
| General | CD138, CD38, CD19 and cytoplasmic kappa & lambda |
| Abnormality associated | CD56, CD20, CD28, CD117; also loss of CD27 & CD81 |

### 4.2 Selection of fluorochromes:

Recent advances in the field of flow cytometry have made range of fluorochromes available allowing from four color to more than ten color flow cytometry. Selection of fluorochromes starts with the instrument configuration. Clinical flow cytometers come with one, two or three lasers and also different filter combinations. The type and number of lasers and detectors dictate whether the optical system can excite a given fluorochrome and properly detect a given combination of fluorochrome. One has to refer to the flow manual to decide before procuring reagent fluorochrome combinations and to minimize potential spillover of adjacent fluorochromes. Match fluorochrome choices to the platform capacity. (See table below) Older fluorochromes like Fluorescein isothiocyanate (FITC), phycoerythrin (PE), Allophycocynine (APC) are being used for decades and we have enough experience to use them without much of validation exercise. However, one has to be more cautious with newer tandem dyes as they are known to have spillover in parent channel.

#### Determine fluorochrome - conjugate strategy

In designing an antibody and its fluorochrome combination, there are basic principles that should be followed and certain issues associated with it need to be understood. The assignment of antibodies to fluorochromes is determined mainly by the background staining of, and spectral overlap between, the chosen fluorescent dyes as these two factors affect the sensitivity of resolution between positive and negative signals.

- As a general rule, strongly expressed antigens should be coupled with dim fluorochromes and weakly expressed antigens should be coupled with bright fluorochromes (values from stain index). For example CD5 expression on B cells can only be identified using bright fluorochrome.
- Avoid the antibody-fluorochrome (Ab-F) combination that gives high background in negative population as high background resulting in low resolution between positive and negative population causing difficulties in interpretation. Choose the Ab-F having wide separation between negative and positive signal peaks.
- One has to know the possible spectral overlap between the dyes. One of the methods to know it is fluorescent-minus-one (FMO) technique. Ab-F combinations causing significant spectral overlap should be avoided. To accomplish this three basic strategies are suggested.

i. To use markers that are expressed on different populations of cells, the populations being of different lineages, maturational stages, or functional subpopulations.

ii. To use reagent pairs for which the expected result is either dual positive or dual negative, avoiding situations where either reagent alone is positive.

iii. To limit the intensity of the fluorochrome that causes a compromise in sensitivity to a more suitable range, either by careful antigen selection or by dilution of labeled with unlabeled reagent.

- PE and the PE tandem fluorochromes give the brightest signals, followed by APC and APC tandem fluorochromes, and finally by the small-molecule fluorochromes such as fluorescein isothiocyanate (FITC), Pacific Blue, and the Alexa series. Brillint violet (BV) dyes also give moderate (BV510) to bright (BV421) signal.
- An unknown or new surface antigen should be studied with brighter fluorochrome (higher stain index).
- When using tandem conjugated antibody one should observe that PE-Cy5 (Cyanine 5) can non-

specifically bind to monocytes and B cells leading to high non-specific background.

- APC-Cy7 doesn't work well with fixatives and it dissociates leading to increased spillover in APC channel and needs more compensation. It is recommended to analyze fixed samples within 4 hours.
- Exposure to light and higher temperature can affect tandem dyes.
- Different lots of tandems may require significantly different amount of compensation. Fluorescence resonance excitation transfer (FRET) may also occur between two adjacently placed fluorochromes (PE & APC Cy7 - PE-Cy7).
- Recently introduced fluorochromes (BV dyes) excited with violet laser, although may give higher background, are stable and have minimum spillover with other dyes.

Fluorochrome assignment is an understanding of the impact of overlapping spectral emission on detection sensitivity and minimizing its contribution. Errors generated by particular antibody and fluorochrome combinations may promote a high degree of spreading into spillover channels, limiting the usefulness of certain reagent combinations. e.g., it is not a good idea to use CD19 PE and CD5 FITC combination in a case of CLL, wherein, because of the data spread of CD19 in CD5 channel, it becomes difficult to appreciate CD5 staining. Also, when a relatively bright antibody like CD8 is used in the APC-Cy7 channel, the resulting data spread in the APC channel can interfere with APC-positive cells. Although during fluorochrome selection, commercial availability of fluorochrome-conjugated reagents is important, but, with the advent of custom conjugation services, this consideration should not be allowed to compromise the quality of the data to be obtained.

### 4.3 Antibody Titration

Cells possess some natural autofluorescence of organic molecules (such as NADH) giving background staining which overlaps with emission spectrum of dyes such as FITC and PE. High background staining can also result from the nonspecific binding of a fluorescent conjugate to cellular elements, which occurs when the antibody concentration is too high. In either case, the problems caused by high background staining can be avoided by the titration of antibody reagents before their use. Titration is the method of determination of optimal amount of antibody concentration to obtain maximum signal (positive values) to noise (background values) ratio (SNR). Titration of each antibody is essential to determine the appropriate staining concentration in order to saturate all antigens on the cell and to avoid non-specific binding caused by excess amount of reagent. Perform titration curves for each antibody-fluorochrome conjugate with multiple concentration of antibody reagent and then determine SNR and select volume that has highest SNR. Usually, a correct titration of antibodies leads to use of low quantity of reagent than recommended by respective manufacturer leading to the cost cutting.

To minimize pipetting errors in reagent delivery and to reduce daily pipetting time, it is always good practice to make cocktails of reagent combination with shelf life of one month. Isotype controls are used to identify non-specific binding of antibody-flurochrome to vulnerable cells like degenerating cells or monocytes etc. However, the cells which are stained with isotype controls should not be used to set the thresholds. Since they are no longer considered essential, do not rely on them too heavily. The best control is to stain cells with all reagents except for the one of interest – Fluorescence minus One (FMO). FMO controls should be used whenever accurate discrimination is essential or when antigen expression is relatively low.

| Table 2. Commonly used fluorochromes for different laser combinations. | |
|---|---|
| **Laser (nm)** | **Commonly used fluorochromes** |
| **Violet 405** | Pacific blue or Horizon V450 or Brilliant violet 421 or AmCyan or VioBlueand<br>Krome Orange or Pacific orange or Horizon V500 or BV510 or VioGreen |
| **Blue 488** | FITC or Alexa 488<br>PE<br>PE-Texas Red (ECD)<br>PE-Cy5 or PerCP or PerCP-Cy5.5<br>PE-Cy7 |
| **Red 633/640** | APC or Alexa flour 647<br>Alexafluor 700<br>APC Cy7 or APC H7 |

| Table 3.Combinations of different fluorochromes for available instruments with scope of maximum colors. | | | |
|---|---|---|---|
| **4 color** | **5 color** | **6 color** | **8 color** |
| FITC<br>PE<br>PerCP/PerCP Cy5.5/ECD<br>APC | FITC<br>PE<br>ECD<br>PE-Cy5/PerCP Cy5.5/ APC<br>PE-Cy7<br>and<br>(FITC, PE, ECD, APC and PC7) | FITC<br>PE<br>PerCP Cy5.5/ PE-Cy5<br>PE-Cy7<br>APC<br>APC-H7/APC Cy7 | PB/V450/BV421/AmCyan<br>PO/V500/BV510<br>FITC<br>PE<br>PerCP Cy5.5/ PE-Cy5<br>PE-Cy7<br>APC<br>APC-H7/APC Cy7 |

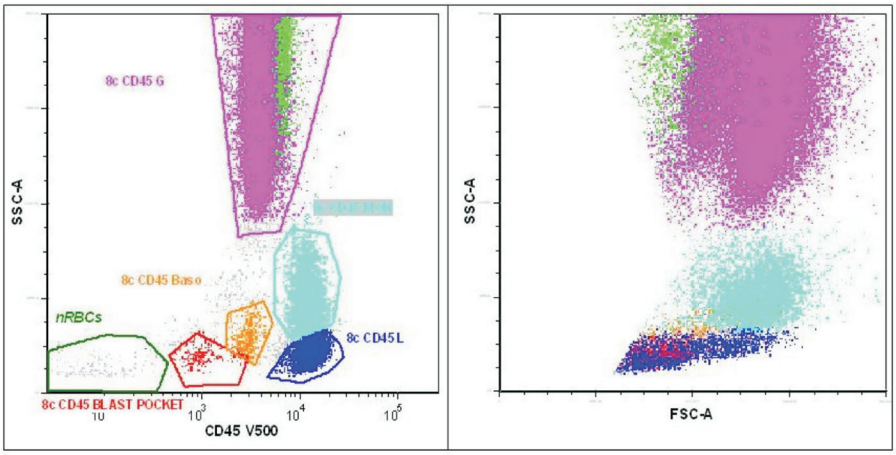


**Figure 1** shows CD45 versus side scatter dot plot (left) and forward scatter versus side scatter dot plot (right). CD45 versus side scatter dot plot shows well separation between different nucleated cells which is limitedly separated in forward versus side scatter. Here different populations are gated and different colors are given for their identification. (pink dots- granulocytes, light blue- monocytes, dark blue- lymphocytes, orange dots- basophils, red dots- precursor cells/blasts, green dots- nRBCs, light green- eosinophils).

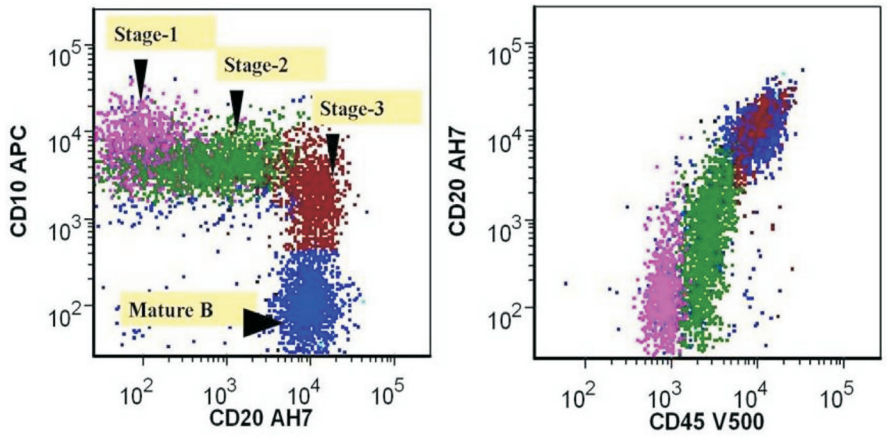


**Figure 2:** Dot plots showing normal maturation pattern of B cell precursors with synchronous expression of CD10, CD20 and CD45 at different stages of maturation.

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# 5. QUALITY CONTROL – FLOW CYTOMETRY LABORATORY

Laboratory is instructed to do the following in addition to the following quality management systems as per ISO15189:2012 (Ref) or any other such document (Reference CLSI).

The laboratory shall document various standard operating procedures. Laboratory shall take care of various pre analytical, analytical and post analytical variables.

### 5.1 Sample collection, handling, transportation and storage

**5.1.1 Sample collection and handling:** Each lab shall have a documented policy for sample acceptance and rejection criteria, anticoagulant used, age of the sample, transport temperature, processing and storage (24-72 hrs based on specimen type and anticoagulant used). Laboratory may define its own rejection criteria. However, sub-optimal samples include presence of clot, hemolysis, improper container, inappropriately labeled, samples received beyond 48 hours after collection. In case of unacceptable samples, the same should be informed to the treating physician and recorded. All samples including bone marrow are precious and efforts should be made to assay and do possible interpretations before completely rejecting a specimen.

**5.1.2 Sample transport and storage:** Blood and Bone marrow samples may be transported and stored at room temperature (i.e. 18 to 24°C) and should reach the testing laboratory within 12-24 hours. Fluid samples and aspirates and suspected cases of Burkitts lymphoma may be transported at 2-8°C. CSF, samples should be processed within 1 hour of lumbar puncture; otherwise they should be stabilized to avoid deterioration of cells due to the rapid in vitro cytotoxic effects of CSF on leucocytes. They can be stabilized using transport media like RPMI with 5% fetal bovine serum or validated transport media. The examined specimens shall be stored for re-examination and/ or additional tests for a minimum defined period. Lysed and stained samples can be re-suspended in buffered-formaldehyde solution (fixative) and stored at 2-8°C, if acquisition is going to be delayed. Samples should not be frozen. For Paroxysmal nocturnal hemoglobinuria (PNH) studies, analysis of granulocytes should be ideally carried out within the first few hours of collection (upto maximum 24 hrs) and of RBCs upto 7 days.

**5.2 Reagents:** All labs should have a policy for confirming and documenting the reactivity of the reagents used in the lab. This may be achieved by direct analysis with reference materials, verification testing of old vs. new reagents, and checking against routine controls. All reagents should be stored as per the manufacturer's guidelines.

**5.2.1 Reagent Labeling:** All reagents –antibodies, controls, calibrators, and solutions should be adequately labeled. The labels should include details regarding the date of preparation or reconstitution, volume of reagent, titers for antibodies and expiry dates.

**5.2.2 Reagent Expiry:** The laboratory must assign an expiration date to any reagents that do not have a manufacturer-provided expiration date. The assigned expiration date should be based on known stability,

frequency of use, storage conditions, and risk of deterioration. In addition, for various reagents/antibodies, laboratory shall keep the documentation of receipt, storage conditions, in-use state, antibody cocktails and assay development/optimization.

**5.3 Quality control (QC) for reagents and assays:** The frequency of running QC material depends on the type of test being performed. For measurements of CD4+ T-cells two levels of commercial controls are ideal. Internal positive controls can be used only for leukemia/lymphoma samples. Such internal control cells are the residual normal hematopoietic cells in the patient's own sample. Like external controls, there must be objective criteria for acceptable performance of internal controls. Laboratory shall document its quality control policy for each test and maintain records. It is desirable to do two levels of commercial control for CD34 + stem cell enumeration; however, participation in a proficiency testing (PT) program is mandatory.

### 5.4 Instrument

Diagnosis should be performed on flow cytometer made by standard companies that provide precise and verifiable procedures for operating and evaluating the performance of the machine. All instruments and equipment should be properly installed, operated, maintained, serviced, and monitored to ensure that malfunctions of these instruments and equipment do not adversely affect the analytical results. The maintenance procedures should be followed as per the manufacturer's guidelines. A maintenance log detailing the daily, weekly, monthly, semi-annual and annual maintenance procedures should be maintained. Any unscheduled maintenance procedures must be documented.

**5.4.1 Instrument set-up and quality control:** Using reagents and software, company technical staff shall optimize the instrument for optical alignment, electronic standardization, sensitivity/linearity and compensation. Each lab should have a documented policy defining the procedure for appropriate instrument function checks to be performed for all instruments prior to a daily run. Instrument function checks are ideally done by commercially available reference beads. These may differ depending upon type of instrument. The following parameters shall be monitored

- Laser current and laser power
- PMT Voltages
- Fluorochrome sensitivity
- Laser delay (applicable on multi laser instruments)
- Window extension (when applicable)
- Area Scaling Factors (applicable on digital flow cytometers)
- Fluidics

The values for PMT Voltages and Laser parameters shall be plotted on a LJ chart and monitored.

Though there are many protocols available in the literature to monitor, it is prudent to follow manufacturer's instructions and specifications. Vendors supply beads and respective automated software systems for monitoring the instrument. e.g., (to mention a few):

CALIBRITE beads with FACS Comp software (BD biosciences)

BDCanto Seven color set up

BD Cytometer Setup and Tracking beads with BD FACSDiva software.

Flow-Check Fluorospheres (Beckman Coulter) to verify instrument optical alignment and fluidics.

Flow-Set beads with Cyto-Comp or ADC software (Beckman Coulter) etc.

Rainbow beads (Spherotech) for alignment and voltage setup.

Frequency of performance check: Laboratory shall have a policy on its instrument performance check based on its work load like daily, or after every cold start, or once a week.

**5.4.2 Compensation Controls:** Each lab needs to document the compensation procedure. This can be done manually or by automated methods. Compensation may be done using microbeads (spherobeads) or cells containing mutually exclusive populations of the same fluorochrome. However it is important to optimize the settings given by the beads with cells to be used in the actual experiment. Frequency of doing compensation settings is decided by the individual labs. However it is essential to reestablish compensation values after any hardware change, laser realignment, change in filters, optics or any other such parameters which affect instrument performance. It is essential to note that compensation settings are stable for a given set of PMT voltages. Change in PMT voltages may lead to adverse effect on compensation values, and should be avoided.

**5.4.3 Cross-instrument performance:**

If more than one instrument is used in the lab for the same test, verify that results are concordant / equivalent and this should be done at least twice in a year.

**5.5 Assay Procedures:**

**5.5.1 Viability Testing:** Each lab should have a documented policy for evaluation of viability. Viability testing may be done using dye exclusion methods (eg. Trypan Blue) or by using DNA binding dyes (e.g., 7-AAD).

**Leukemia/Lymphoma Immunophenotyping-** Immunophenotyping is best done within 48 hours of phlebotomy. Delayed processing may lead to degeneration. Each lab should establish procedures to ensure that viable cells are analyzed. It does not imply that all specimens with low viability must be rejected. If specimen viability is below the established laboratory minimum, test results may not be reliable and this should be noted in the final report. Routine viability testing may not be necessary. However, the viability testing is recommended in specimens with a high risk of loss of viability, such as FNAC samples, stored fluid samples, disaggregated lymph node specimens.

The viability testing in lymphocyte subset enumeration may not be necessary on specimens of whole blood which are analyzed within 24 hours of drawing. ISHAGE recommends assessment of viable CD34 stem cell counts (post thaw stem cell product).

**5.5.2 Antibody Panels:** A comprehensive panel of antibodies that covers common subtypes of hematolymphoid neoplasm should be available. Clinical as well as other findings including morphology are very useful in deciding on a particular panel of antibody. Laboratory shall demonstrate documented panels and also the procedures as well as records of adding additional reagents/antibodies, if no opinion could be made based on primary panels. It is good laboratory practice to prepare antibody cocktails for panels for routine immunophenotyping of hematolymphoid neoplasms. Reduction in number of samples requiring repeat processing and also reduction in repeat procedures can be monitored as quality improvement indicators.

For CD34+ stem cell enumeration appropriately conjugated Class II or Class III anti-CD34 monoclonal antibodies are used. For performing IVD tests (using kits/materials) the manufacturer's instructions should

be followed. If procedures are altered, documentary proof should be provided that the modification has not altered the results.

**5.5.3 Cell Concentration:** It is important to define the cell concentration to be used per assay tube for a given assay. This may be based as per the manufacturer guidelines or on existing clinical practices. A recommended cell concentration to be used for immunophenotyping of hematolymphoid neoplasm is 0.1 -1 million (0.1 -1 x $10^6$) cells per assay tube. It is important to note that as antibody staining is mainly volume dependent, the sample volume in the assay remains constant. The cell number can be adjusted by spinning down the cells and re-suspending them in a desired volume. For fluids, aspirates and specimens with low counts, lower cell concentration may be used and restricted panels may be applied as per the clinical scenario (and morphology). Laboratory shall document cell concentration policy.

**5.5.4 Sample / Data Acquisition:** In the screening of peripheral blood/bone marrow samples for a new case of hematolymphoid neoplasms, at least 10,000 total events should be acquired for each tube which should contain a minimum of 500 events of tumor cells/blasts/atypical lymphoid cells. More events may be acquired if there is marked degeneration of sample or when rare populations are being evaluated.

a. PNH analysis has been divided into routine analysis (defined as identifying an abnormal population of 1% or more) and high-sensitivity analysis (in which as few as 0.01% PNH cells are detected). Laboratory should clearly mention in their scope whether it is doing routine analysis, high sensitivity analysis or both. For routine analysis, PNH testing on neutrophils with or without monocytes should be performed. PNH diagnosis only based on RBC analysis is not recommended. When standardizing the PNH testing in the laboratory, it should be performed on a known PNH positive sample with both Type I and Type III cells rather than a normal sample. Standard guidelines must be followed for use of reagents/antibody for different types of cells. MJ Borowitz et al. Cytometrry Part B, Clinical Cytometry 78B:211-230 (2010).

b. Lymphocyte subset enumeration- For single platform measurements, manufacturer guidelines are to be followed.

c. HLAB27 assay: FCM assessment of HLAB27 shall be done with at least two different antibody clones of defined specificity to avoid false positive assignment due to cross-reactivity of monoclonal antibodies (Reference: Cytometry B Clin Cytom 54(1): 28-38).

d. CD34+enumeration- A statistically valid number of CD34+ events are collected to ensure clinically relevant precision and accuracy. The allowable coefficient of variation for CD34+ cell counts should be 10%. To achieve this precision, a minimum of 100 CD34+ events should be counted, as recommended by the ISHAGE guidelines.

**5.5.5 Flow cytometry data analysis:** Although analysis of flow cytometry data can be done with acquisition software present with instrument, for immunophenotyping of leukemia/lymphoma it is better to use flow cytometry analysis softwares like Kaluza, FCS Express, Flow Jo, Winlist, Infinicyt etc. It is useful to create analysis templates for the common fixed panels of acute leukemia or Lymphoma. Final data analysis must be done by experienced person, preferably pathologist.

**5.5.6 Gating Strategies:** Each lab should have documented policies of appropriate gating strategies for different lesions. CD45 versus light scatter gating is a must while evaluating acute leukemia. For evaluation of lymphoma/myeloma, the concept is to track the abnormal population using lineage sensitive antibody/ antibodies as tracking marker/s. For CD34+ stem cell enumeration sequential (Boolean) gating systems should be followed. ISHAGE protocol is ideal for CD34 stem cell enumeration. CD45/CD3 gating is essential for CD4 subset enumeration.

**5.5.7 Quadrant markers:** Threshold determination for positive or negative population should be based on the knowledge of the cell of interest. Controls such as unstained cells/isotype controls are suggested but have limited use and therefore their use in not essential.

**5.5.8 Reporting:** The report in addition should include name/type of instrument and software used, cell preparation method, gating strategies and percentage of gated cells examined. It should also include descriptive information about the immunophenotype of the abnormal cells, if identified, and comments necessary to facilitate the interpretation. The details of the antibodies used can be given in a tabulated format along with the interpretation of positivity or negativity. Stress should be laid on interpreting the intensity of positivity and not the percentages. While interpreting the intensity of positivity as moderate, bright or dim, the abnormal population should be evaluated against known normal leukocyte populations. Final impression should be clearly stated along with a differential diagnosis if required. Comments and suggestions regarding useful follow up test or other ancillary techniques should be added. For laboratories doing other ancillary techniques like cytogenetics and molecular diagnostics, it is desirable that the final report should have a mention of these. The lab is recommended to follow the recent WHO guidelines for the classification of hematolymphoid neoplasms.

**5.5.9 Data Backup and Storage:** Analysis plots on which final diagnosis is made should be stored either in PDF format or as a hard copy. All list mode files and final reports should be stored for a minimum period of 10 years. The laboratory may consider giving the list mode files (particle data) to the patient on request for obtaining second opinion or for treatment elsewhere. The laboratory shall have a documented procedure and maintain record of the same.

**5.6 Personnel:** The technologists/scientists shall have at least a 3 month hands on experience in a busy flow cytometry laboratory. The operating technician should attend annual CMEs and workshops. Basic training in laboratory hematology in the form of lectures and also peripheral blood smear examination is desirable. The signatory authority should have at least three month experience in a clinical cytometry laboratory. However for immunophenotyping of hematolymphoid neoplasm, one year experience in a busy clinical cytometry laboratory is desirable. Exposure to training programs like CME/workshops on a yearly basis is essential.

## Reference


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2. Clinical and Laboratory Standards Institute. Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition. CLSI document H42-A2 [ISBN 1-56238-000-0]. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2006.
3. Clinical and Laboratory Standards Institute. Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition. CLSI document H43-A2 [ISBN 1-56238-000-0]. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2006.
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# 6. FLOW CYTOMETRY AND BIOSAFETY

## 6.1 General Summary

Hazards in flow cytometry laboratory may be biological, chemical, radioactive, laser, and other. Knowledge of the origin of the sample and awareness of the potential presence of pathogens are keys for safety of lab personnel. All unfixed materials (blood samples and various body fluids materials) should be considered as bio-hazardous

Biosafety considerations should be taken into account for handling of all specimens. The laboratory must develop appropriate procedures to identify the potential biological hazards of the specimens and for aerosol containment, waste management and equipment maintenance.

Laboratories are responsible for written, documented Standard Operating Procedures to implement appropriate safety precautions and practices based on local regulatory agencies.

## 6.2 To prevent exposure to bio-hazardous agents, follow these guidelines.

1. Assure that the personnel are trained in the required procedures with strict adherence to the Standard Precautions described in the institutional policy,
2. Laboratory staff must be immunized for hepatitis B.
3. Entry to the laboratory should be restricted to authorized personnel only. Laboratory door should remain closed at all times when samples are being analyzed or sorted.
4. Use of the bio-safety cabinet (BSC- typically Class II, Type A, or Type A/B3) is highly recommended.
5. All procedures must be performed carefully to minimize the creation of aerosols. Infectious or potentially infectious materials should not be sorted unless suitable containment measures are applied. A droplet containment module should be installed to reduce the risk of exposure to generated droplets and aerosols.
6. All unfixed materials (blood samples and various body fluids materials) should be considered as bio-hazardous. Known specimen should have a note divulging potentially infectious or biologically hazardous nature.
7. All spills should be reported. Work surfaces are to be decontaminated with 10% household bleach after any spillage of sample or hazardous material and after completion of experiment/test by each user.
8. Expose waste container contents to 10% bleach before disposal. Dispose of waste in accordance with local regulations.
9. Take proper precaution and wear suitable protective clothing, eye wear and gloves.

10. Prevent waste overflow by emptying the waste container daily or whenever the waste indicator is on.
11. Hands must be washed after handling any sample and also before leaving the laboratory.
12. Laboratory coats must be worn in the work area. These coats should be removed before leaving the laboratory.
13. Eating, drinking, smoking or applying cosmetics in the work area is prohibited.
14. Use only mechanical devices for pipetting. No mouth pipetting should be practiced.

### 6.3 Flow generated aerosols and droplets

Flow cytometer with cell sorter can generate microdroplets that causes aerosol production. It can be minimized using aerosol containment module.

### 6.4 In the event of a potential retrovirus exposure

Even though the risk of infection is minimal in the event of exposure of skin or mucous membranes to infectious body fluids, the following immediate steps are advised.

Contaminated skin should be meticulously cleaned for 10 minutes using an appropriate antiseptic solution and water. Contaminated eyes and mucous membranes should be irrigated for 5 minutes using water. Notify your supervisor.

### 6.5 Waste management

The procedures of waste management and equipment maintenance must be adapted to the biosafety status of the handled biological material (as per national and international recommendations).

All biohazardous material must be decontaminated and placed in sealed & leak-proof containers with proper labelling.

Effluents: Usually, the effluent is collected from the instrument in a flask containing fresh concentrated bleach to achieve a 10% final concentration. All waste is disposed according to institutional and local regulations for waste disposal.

Maintenance of fluid lines and parts of instrument: Fluid lines disinfection requires regular instrument runs with a 1:10 fresh dilution of a 5.25% sodium hypochlorite solution for at least 10 minutes, followed by distilled water.

Flow cytometer: follow the manufacturer's recommendations for appropriate disinfection.

### 6.6 Laser Safety guidelines

Access to the laboratory must be limited to authorized staff while the laser is operating in the UV mode or whenever it is necessary to operate a laser without protective shielding.

In such cases, the appropriate protective goggles or glasses must be worn while working in the laboratory.

Only qualified personnel shall operate or adjust any laser.

### Reference


1. Penn state Milton S.Hershey Medical Centre Core research facility guidelines(2004)
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# 7. TESTING FOR VIABILITY AND ADEQUACY OF CELL YIELD

## 7.1 Cell concentration

Single cell suspension of specimens is primary requirement for the flow cytometric immunophenotyping staining and analysis. Laboratories use different methods of cell preparation and staining. Assay type also determines deferent staining protocols, some requiring direct labeling, some lyse wash staining; some may even prefer cells Ficoll gradient separated.

For the purpose of proper staining and to get reliable data, cell concentration in a given volume for the antibody volume has to be optimally adjusted. Manufacturers recommended amounts of antibody to be used generally assume normal ranges for the number of target cells to be stained. However, the cell numbers vary between different specimens, and also different disease conditions. Leukemic sample (peripheral blood or bone marrow) may be hypercellular; some specimens may not have sufficient cell numbers in the peripheral blood or bone marrow.

Generally, the cell concentration as determined by different investigators, should be between 0.1 to 1 million cells per assay tube and 50-100µl as a staining volume. If the sample is a fluid or a fine needle aspirate (FNA), the lower concentration can be taken lower. For the FNA and fluids, where the cell yield tends to be low the cell concentration can be lowered to 25,000 to 50,000 cells per tube. In that case, one has to select priority tube from a given panel.

Every laboratory must decide the ideal cell concentration in a given volume and the concentration of the antibody by titration experiments.

The following guidelines may be useful for sample adjustment

Depending on the staining method, it is necessary to obtain cell counts before staining.

1. Following the red cell lysis step or ficoll gradient separation, the cell yield should be determined with an automated cell counter or by Neubauer chamber. The final concentration should be adjusted to have a target cell number between 0.1 to 1 million cells per assay tube. When processing bone marrow by direct lysis technique, it is often necessary to dilute the sample 1:10 in PBS (phosphate buffer saline) before performing either a manual count or an automated count.
2. If WBC count is low, spin the sample and readjust the counts appropriately by adding saline buffer so as to get 1 $X10^6$ cells per 100µl. Use 100µl of whole blood/BM.
3. If sample is densely populated dilute with saline buffer so as to get 1 $X10^6$ cells per 100µl.

## 7.2 Dead cell identification in flow cytometer

Dead cells can non-specifically bind antibody and thus may appear to the flow cytometer as positive events. Thus, identifying and removing data points representing dead cells is a critical step in ensuring accurate results.

There is no set rule concerning the viability level below which a specimen is unacceptable for immunophenotyping.

A lower viability, in a specimen with nearly all neoplastic cells, should not pose any problems in data analysis than those with a scanty proportion of tumor cells.

Since bone marrow and other tissues specimens are considered to be irreplaceable, such specimen even with low viability should be processed and analyzed by using an appropriate gating. Attempt should be made to generate the interpretable data. The inaccuracies of the analyzed data should be noted in the final report.

Viability may be assessed by a real-time flow cytometric analysis using propidium iodide, 7-amino-actinomycin D (7-AAD), EMA, Hoechst or DAPI dyes. Alternatively, viability can be measured, correlatively, through the use of dye exclusion methods prior to flow cytometric analysis by light microscopy such as Trypan blue. In a real-time assay dead cell events (dead cells will take up the stain, while the viable cell will remain unstrained) can be easily and accurately identified and removed electronically. For assays like CD34 stem cell, or CD4/CD8 counts follow the guideline given by the respective protocol. However, this is not much practicable in assays where incorporating such dye results in losing a fluorescence channel, expensive and also the inconvenience of requiring immediate evaluation (DNA stain will leak out of cells during fixation and spread through the whole preparation).

An alternative, but less accurate, method is to gate dead cells out by the forward scatter parameter. The dead cells have a lower refractive index than viable cells and therefore scatter less light in the forward direction and tend to have slightly higher side scatter. They can be removed by gating out the lower FSC and slightly higher SSC events, where the dead cells and the debris accumulate.