Sample Preparation for Flow Cytometry: Practical Considerations

Introduction

Flow cytometry is used to interrogate the properties of cells, including cell surface proteins, intracellular proteins, nucleic acids, cellular functions, and more[1–3]. In order to analyze these properties, single cells flow through the interrogation point in a cytometer one at a time. If they are not analyzed as single cells, incorrect data may be generated. Preparation of the specimen as a single-cell suspension is therefore essential. Fortunately, leukocytes can be easily isolated for flow cytometric examination from peripheral blood, where they exist as a cell suspension. The goal of this document is to address sample preparation of human peripheral blood and peripheral blood mononuclear cell (PBMC) samples for cell surface immunophenotyping with flow cytometry. Other specimen types and applications are not discussed.

About the author

Teri Oldaker is a certified cytometrist with more than 35 years of flow cytometry experience. She has served on the board of the International Clinical Cytometry Society (ICCS) and as a faculty member for numerous flow courses, and has authored four book chapters and more than 60 publications. Oldaker currently is a consultant in flow cytometry validation and quality and serves as vice chair for the Clinical and Laboratory Standards Institute (CLSI) H62 document *Validation of Assays Performed by Flow Cytometry*.

1 Collect peripheral blood with anticoagulant. (See Specimen Considerations section.)

2 Ship to laboratory. (See Specimen Considerations section.)

3 Receive specimen and qualify. (See Specimen Considerations section.)

4 Perform cell count and differential. (See Specimen Processing section.)

5 Choose processing method. (See Specimen Processing section.)

6 All WBC: Lyse RBC

6 PBMC: Density gradient centrifugation

7 Wash, resuspend, count cells, measure viability. (See Specimen Processing section.)

Specimen Considerations

For accurate results, flow cytometry assays require collection of the correct specimen type and volume, rapid transportation, qualification upon arrival, selection of an appropriate processing method, and staining with fluorescent antibodies. Best practices for these processes are discussed below.

Specimen Collection

All peripheral blood specimens for flow cytometry are to be handled with universal precautions, since they may transmit infectious agents[4,5]. Ideally, specimens should be processed immediately after collection. When this is not possible, the laboratory should verify that the required labeling practices, anticoagulant, holding temperature, and collection method have been followed. Peripheral blood specimens are collected into a blood collection tube (for example, BD Vacutainer® Blood Collection Tubes) or a syringe. Blood will clot in 25–60 seconds unless clotting is inhibited by an anticoagulant during collection[6,7].

Choice of Anticoagulant

The choice of anticoagulant depends on specimen type, transportation, storage requirements, and, in some cases, sample preparation method[6,8]. For peripheral blood, ethylenediaminetetraacetic acid (EDTA, a chelator), sodium heparin, or acid citrate dextrose (ACD) may be used. If a complete blood count (CBC) and white blood cell (WBC) differential are to be made from the same specimen used for flow cytometry, then EDTA is the anticoagulant of choice. Sodium heparin samples are reported to be optimally stable for 48–72 hours, EDTA samples for up to 48 hours, and ACD samples for 72 hours[8,9]. ACD anticoagulation is seldom used and is not recommended for a draw that does not fill the entire tube[8]. If the ratio of sample to ACD anticoagulant is not correct, the pH may be altered, and cell viability will be reduced. For lymphocyte assays, sodium (not lithium) heparin is the preferred anticoagulant and is required if functional testing is to be performed. EDTA is acceptable for cell surface marker testing but not for functional assays, since divalent cations are necessary for certain cell functions[10].

Table 1. Anticoagulant options for flow cytometric assays

Anticoagulant	Primary Uses	Stability	Key Considerations
EDTA	• CBC and immediate • Immunophenotyping	<48 hr	• Best morphology • Cannot be used for functional assays
Sodium heparin	• Immunophenotyping • Functional assays	<72 hr	• Less accurate CBC due to morphology changes • Lithium heparin will kill cells.
ACD	• Immunophenotyping • Functional assays	<96 hr	• Longest stability • Problems with underfilled collection vial

Shipping

The integrity of peripheral whole blood samples is well-maintained at room temperature (18–25 °C). Blood specimens should be stored and transported within this temperature range or the storage condition specified for the specific assay[6,8,11]. Fluctuation in temperature can cause changes in membrane expression of some antigens. Thus, the effect of cooling and rewarming should be validated for each assay. Transportation outside the 18–25 °C range, which may occur unintentionally in particularly hot areas in summer or particularly cold areas in winter, requires validation of at least five samples of each affected specimen type with each different anticoagulant or use of temperature monitors to enable accurate temperature exposure tracking. One approach to counteracting unintentional heating or cooling of specimens is to include one or two small sealed wet ice or cold packs in packaging in which specimens are transported to flow cytometry laboratories. The packaging should separate specimens from direct contact with any cold packs to prevent freezing[6,12]. No convincing literature exists indicating that diseased samples age significantly differently than healthy samples[11]. Therefore, tests may be validated for specimen stability using healthy specimens.

Specimen Age

Because of the perishable nature of fresh peripheral blood specimens and the potential for antigenic alterations during the aging of specimens, tracking specimen age is a critical part of the sample preparation procedure[8,9,13]. Specimens can deteriorate during transportation and when stored. Some laboratories may alter their specimen preparation depending on the reported age of the specimens at the time of receipt. In general, all perishable specimens should be processed as soon as possible after collection. The validation of any new flow cytometric assay should include documentation that the assay performs appropriately on the full range of peripheral blood specimen ages likely to be encountered by the laboratory. Contemporary airborne shipping enables virtually all flow cytometry laboratories, including reference laboratories, to receive most specimens within 48 hours post-draw[13]. However, occasionally specimens may be up to 72–96 hours old when received by the laboratory. The laboratory should verify that specimens of this age produce acceptable results.

Specimen Qualification upon Receipt

Upon receipt, the container should be examined for damage and temperature. Specimens should be visually inspected for correct specimen type, hemolysis, clots, correct labeling, correct use of collection tubes, and any other assay specific requirements, such as anonymization[10,14,15]. Hemolysis indicates that the blood has been exposed to conditions that can cause erythrocyte lysis, suggesting that leukocytes may also be affected. Severely hemolyzed specimens should be rejected. Specimen clotting, even when partial, may cause selective loss or alteration of certain cell type subpopulations. Clotted specimens should either be evaluated for effect on the assay or rejected. If a specimen is unusually warm or cold to the touch, this observation should be noted for further consideration during preparation, analysis, and interpretation even if all other evaluation criteria are satisfactory. For viability requirements, see Viability in the Specimen processing section, below.

Storage at the Testing Lab

Specimens should be stored at 18–25 °C. However, some antigens show greater variability than others with specimen age (for example, CD138 and CD16 are more labile than CD45 or CD64) and storage at 4 °C may delay degradation. The laboratory should verify the assay with different storage temperatures, time post-draw, and transportation conditions. For long-term storage, PBMC can be cryopreserved in tissue culture fluid containing protein (usually calf serum) and DMSO. Whole blood has been frozen with DMSO alone[16]. This and additional storage and thawing procedures have been described in the literature[17,18]. The quality of frozen PBMC or WBC should always be determined by measuring their viability after thawing. See below for viability requirements.

Antibody Reagents

Antibody Vial Labeling, Storage, and Inventory Management

In addition to the manufacturer labels with contents, quantity, storage requirements, and expiration date, all antibody vials should be labeled with the date of receipt, date opened, and initials of the individual first opening the vial. When a new antibody is introduced into the laboratory, it should be checked for expected labeling pattern in a flow cytometry assay. Most antibodies can be checked with normal blood or PBMC specimens to verify that they label the expected cell population and do not non-specifically bind to cells[19]. Verification of antibody reactivity with rare antigens can be performed with commercial cell lines or with specimens containing the abnormal (for example, neoplastic) cell expressing that antigen. Newly received lots of antibodies already in use should be kept separate until they are tested against previous lots of antibody and determined to be acceptable[20,21]. This applies to newly received vials of the same lot that is already in use, since shipping may have altered reactivity. For large studies, a single lot of antibody can be sequestered to reduce the need to test multiple lots.

Antibody and Cocktail Storage Considerations

Fluorescent antibodies should be stored in dark vials to prevent degradation from exposure to light. Degraded antibodies produce a lower signal and increased non-specific binding. Single antibodies can be stored at 4 °C if the solution contains sodium azide to prevent degradation due to microbial growth[22,23]. Regulatory rules prevent the use of antibodies or other reagents past their labeled expiration date in regulated laboratories[24]. For studies in research and exploratory biopharma settings, small aliquots of individual antibodies for a single experiment can be stored at –20 °C for years. Stability of frozen antibodies and use past expiration date should be verified for each antibody clone by checking their reactivity pattern with a known control specimen (for example, peripheral blood or PBMC). Multiple freeze thaw cycles should be avoided because this will cause antibody aggregation and loss of labeling activity. Therefore, frost-free freezers cannot be used because they use freeze/thaw cycles to remove frost buildup. Freezing antibodies in small aliquots (>10 µL) eliminates the need to thaw multiple times and minimizes the effects of evaporation on smaller aliquots (general laboratory practice)[22,25].

In general, antibodies conjugated with a single fluorochrome are stable for long periods with proper storage and handling[26]. However, tandem conjugated fluorochromes are less stable and prone to tandem breakdown, leading to signal artifacts. For example, PE-Cy7 can degrade over time where the Cy7 (cyanine 7) is decoupled from PE (phycoerythrin) resulting in less Cy7 signal and more PE signal[27].

Often more than one fluorescent probe is used in an assay. A collection of multiple fluorescent antibodies and any other fluorescent probes in one tube is referred to as a cocktail[28]. Cocktails make staining more efficient and reduce the possibility of pipetting error but are not as stable as individually packaged antibodies. Some laboratories report that fluorescent antibody cocktails can be stored up to four weeks when not cycled from 4 °C to room temperature more than three times. Each laboratory should determine the stability of homebrew cocktails[24,28–30]. Vendor cocktails have an expiration date.

Antibody Cocktail Design

When more than one fluorescent probe is used in an assay, this panel (or cocktail) of fluorescent antibodies must be carefully designed to reduce the possibility of erroneous results[8,15,31]. Fluorochromes emit signals in more than one collection channel of the flow cytometer, including in spectral analyzers. Therefore, a cell that should be positive for only one marker can appear to express a second marker due to incorrect compensation for fluorescence spillover or incorrect spectral unmixing[32,33]. A full description of panel design (and compensation) is beyond the scope of this document. For a good starting point, see Roederer (2001)[34] and Wood (2006)[35].

Other Reagents

Non-antibody reagents such as buffers, lysing solutions, and fixatives can be purchased from a manufacturer or developed in-house. There are advantages and disadvantages of each. Vendor-supplied reagents are quality-controlled and only need to be compared to old lots when new lots are received. In addition to the manufacturer labels listing contents, quantity, concentration, storage requirements, and expiration date, all primary reagent containers should be labeled with the date of receipt and initials of the individual receiving the reagent into the laboratory, date opened, and initials of the individual first opening the reagent.

Homebrew reagents are less expensive but require additional container labeling and testing. Homebrew reagents should have all contents recorded on labels along with date of preparation, expiration date, and preparer initials[7]. Mistakes can be made when measuring chemicals for homebrew reagents. These reagents should always be tested against previous lots to verify that they produce the same results. Reagents can also degrade or promote bacterial growth over time and should be carefully examined for any changes, such as cloudiness or change in color. In additional to lot-to-lot testing, homebrew reagents require pH checks and, in some cases, pH adjustments.

Specimen Processing

Cell Count

Different types of assays require different total numbers of either WBC or PBMC. While simple cell surface staining of a major cell population, such as that used for CD4 counts or hematopoietic neoplasia detection, can be accomplished by acquiring as few as 10,000 cells[9], detection of small cell populations, such as 0.01% residual chronic leukemia cells in a peripheral blood specimen, requires acquisition of at least 0.5–1 x 10^6 cells[36,37]. The initial specimen cell count can be performed on a variety of cell counting instruments, such as a clinical hematology instrument, particle counter, or hemacytometer[9,38]. The results of hematology instruments include both a total number of WBC and the percentage of each major cell population present (differential). These numbers will be used to calculate the volume of specimen required for the assay. The cell count performed is used to calculate the volume of specimen needed for the required number of cells. A flow cytometer can produce the same cell count and differential as a hematology instrument if absolute counting beads are included9. Any cell/particle counter is another alternative for the cell count.

For whole blood this equation is: $Volume = cell\ number\ needed\ /\ (\frac{WBC}{mL})$

For PBMC this equation is: $Volume = cell\ number\ needed\ /\ (\frac{WBC}{mL} * (\%lymphs + \%monos))$

Viability

Establishment of optimal handling and storage conditions for specimens is necessary to maintain their integrity. One way of assessing the adequacy of these procedures is to monitor the viability of specimens. The proportion of viable cells in the sample should be estimated and recorded immediately before or during the assay[39]. Specimens are usually rejected if the viability is <85%. However, exceptions are made for rare or hard-to-replace specimens[8]. Non-viable cells can be excluded from analysis by including either nucleic acid or early apoptosis stains in the staining cocktail[40,41].

Processing Method to be Used (Peripheral Blood or PBMC)

The sample processing procedure depends on the cells of interest required for the assay. In all cases red blood cells (RBC) must be removed, because RBC vastly outnumber leukocytes. RBC are removed either by lysis or density gradient centrifugation.

Whole blood lysis: If all WBC (leukocytes, comprised of lymphocytes, granulocytes, and monocytes) are required, a whole blood lysis process is used because this preserves all leukocyte populations in the sample. Several lysing techniques are available[9,42,43]. These include tris-buffered ammonium chloride and hypotonic buffer. Several proprietary lysing reagents are also available from instrument or monoclonal antibody manufacturers. When using commercial reagents, the manufacturer's recommended protocol must be followed.

The lysing solution will eventually lyse WBC in addition to RBC. Therefore, when the required lysis time has elapsed, the WBC need to be rapidly removed from the lysis solution by addition of an isotonic buffer and centrifugation. The WBC are fragile and should be washed in a balanced centrifuge at 300 x g for 5–10 minutes. The supernatant is immediately aspirated, and the cell pellet is gently resuspended in an isotonic buffer (pH 7.2–7.4). The cells are washed at least twice in isotonic buffer. Three washes are necessary when the assay is designed to detect cell surface immunoglobulin. Room temperature is ideal for most assays. Avoid wide temperature fluctuations.

PBMC: If only the peripheral blood mononuclear cells (lymphocytes and monocytes) are required, density gradient centrifugation with Ficoll-Hypaque or an equivalent is used to isolate this subset of cells[44,45]. Some vendors also provide blood collection tubes that can perform this separation. This type of separation is used primarily for functional assays of mononuclear cells[2,3], pharmacological studies, and cryopreservation[17,46]. Frozen specimens can be accumulated over time and assayed together, reducing day to day variation in an assay. The principle of density gradient centrifugation is based on the differential density of RBC and polymorphonuclear leukocytes that pass through the gradient material during centrifugation, leaving the lymphocytes and monocytes at the interface and platelets in the blood plasma above the interface, from which they can be harvested. Differential loss of some lymphocyte populations has been reported and may affect the final results of an assay[11].

Staining Process

The required numbers of cells needed for the assay are pipetted into pre-labeled tubes. Individual antibodies or a cocktail are added to the cell suspension. Cell and antibody concentrations should be optimized for saturation. Most antibodies bind to their respective antigen within seconds, but to allow staining saturation, incubation is usually allowed to proceed for at least 15 minutes. Stained cells are then washed twice in an isotonic buffer (pH 7.2–7.4). The WBC or PBMC are fragile and should be centrifuged in a balanced centrifuge at 300 x g for 5–10 minutes. Room temperature is ideal for most assays. If the antibody has a low affinity for its antigen, the stain tubes should be incubated on ice[47]. Wide temperature fluctuations should be avoided. Light exposure should be avoided, since light can reduce the fluorescence intensity of fluorochromes[48].

Cell enumeration: In some cases, absolute cell count of specific cell subsets is needed (for example, CD4 count, CD34 count, etc.). Absolute counting beads or tubes containing pre-counted beads for cell enumeration are available for determining the exact number of cells and cell concentration. In this situation, the flow cytometric assay is used to count both beads and cells. After analysis, the cell concentration can be calculated. These materials produce a known bead concentration only when they are added to the final stained, resuspended solution. If the original concentration in blood is required, the whole blood specimen should not be washed. Instead a stain, lyse, fix, acquisition protocol should be followed with no washing steps.

Cell fixation: There are two reasons to fix the final cell suspension after staining. First, any infectious agents are inactivated. Second, the stained cells are stabilized for storage[8,49,50].

Unfixed cells continue to degrade and lose fluorescent probes. Unfixed cells are only stable for a few hours. Fixed cells can be stored for several days. However, fixation can both adversely affect some fluorochromes and alter scatter properties of the cells. Each laboratory is responsible for determining the stability of their stained specimens.

Once specimens are isolated, stained, and optionally fixed, they are ready to be acquired on a flow cytometer. A complete description of flow cytometric analysis is beyond the scope of this document.

Closing Remarks

Pre-analytical handling, optimized sample preparation, and adherence to established best practices are critical to ensuring accuracy, reproducibility, and reliability of flow cytometric data. There is a rich body of literature on the topics covered in this document. For further reading see the references section.

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CORPORATE HEADQUARTERS
2 Tower Place, Suite 2000
South San Francisco, CA 94080 USA
Toll-free: 866 359 4354 in the US and Canada
Fax: 650 871 7152
fluidigm.com

SALES
North America | +1 650 266 6170 | info-us@fluidigm.com
Europe/EMEA | +33 1 60 92 42 40 | info-europe@fluidigm.com
Latin America | +1 650 266 6170 | info-latinamerica@fluidigm.com
Japan | +81 3 3662 2150 | info-japan@fluidigm.com
China (excluding Hong Kong) | +86 21 3255 8368 | info-china@fluidigm.com
All other Asian countries | +1 650 266 6170 | info-asia@fluidigm.com

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