Acute Myeloid Leukemia Minimal Residual Disease Detection: The Difference from Normal Approach

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The identification of residual leukemia following therapy, termed minimal or measurable residual disease (MRD), has emerged as one of the most important prognostic factors for patients with acute leukemia, including acute myeloid leukemia (AML). Flow cytometry is a preferred method for MRD detection due to its general applicability and the rapid results that it makes available. In this article, the basic protocol outlines a simple and efficient method for the labeling of hematopoietic cells from bone marrow or peripheral blood with a panel of monoclonal antibodies designed both to highlight patterns of normal maturation and allow identification of neoplastic hematopoietic progenitor populations with a high degree of sensitivity and specificity. The method was developed in a clinical laboratory setting for the diagnosis of myeloid stem cell disorders and neoplasms, and has been extensively validated both technically and clinically for the detection of MRD in AML. © 2020 The Authors.

Basic Protocol: Staining and flow cytometry for AML minimal residual disease detection
Support Protocol: Analysis and interpretation of data for AML minimal residual disease detection

Keywords: acute myeloid leukemia • AML • flow cytometry • minimal residual disease • MRD

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INTRODUCTION

The detection of residual acute myeloid leukemia (AML) after therapy by flow cytometry is dependent on identifying immunophenotypic differences between leukemic cells and discrete stages of normal hematopoietic maturation. This implies the ability to observe normal stages of hematopoietic maturation in order to make this assessment. It also requires that immunophenotypic abnormalities that are different from normal maturation be present on the leukemic cells. Such differences may take the form of alterations in antigen intensity for antigens normally expressed by cells of a particular lineage and maturational stage, abnormalities in the timing of expression relative to a maturational sequence, or expression of antigens not normally expressed by a particular maturational stage or lineage of cells, e.g., lymphoid antigens expressed on myeloid progenitors. The assay described in this protocol is designed to allow the observation of maturation from hematopoietic...
stem cells along each of the major hematopoietic cell lineages (neutrophilic, monocytic, erythroid, B cell, plasmacytoid dendritic cell, and basophil) as a reference point for the identification and enumeration of discrete populations of leukemic cells that differ from normal maturation.

The Basic Protocol describes the procedure for labeling bone marrow or peripheral blood cells with a combination of antibodies that allow simultaneous dissection of normal hematopoietic maturation and identification of residual AML. Also included is a Support Protocol discussing the approaches used for interpretation of the resulting flow cytometric data. Reagents and Solutions outlines the preparation of solutions required for the Basic Protocol to be successfully executed. The Commentary discusses critical features of the assay, including troubleshooting, comparison with the leukemia-associated immunophenotype (LAIP) approach, and time requirements.

### BASIC PROTOCOL

**STAINING AND FLOW CYTOMETRY FOR AML MINIMAL RESIDUAL DISEASE DETECTION**

The following method was devised to minimize sample manipulation and maximize recovery for all white blood cell populations while providing low fluorescence background and preserving the spectral characteristics of tandem fluorochromes. The addition of a low concentration of fixative to the lysing solution allows for the simultaneous lysing and fixing of the sample and preservation of the light scatter properties of the sample, and provides improved separation of compromised from viable cells. Addition of a subsequent wash step reduces background fluorescence due to unbound fluorochrome and provides more controlled timing of fixation, which enhances the stability of certain tandem fluorochromes. The procedure outlined is generic and can be used for any surface immunophenotyping assay.

#### Antibody panel

The AML MRD assay consists of three antibody combinations that have been highly optimized to minimize antibody interactions and provide optimal antigen intensities with low background. The antibody combinations may be acquired on any flow cytometer capable of detecting the fluorochromes indicated—in our laboratory a BD LSRII equipped with blue, red, violet, and yellow lasers—or with substitution of the CD38 conjugate, other suitable 10+ color cytometers lacking a yellow laser, e.g., BC Navios. Each lot of antibody must be individually titered for optimal signal-to-noise and binding saturation prior to being put into use. The antibody combinations may be pipetted as individual antibodies, but creation of antibody cocktails is advised to minimize pipetting errors and provide consistent reagent performance.

The M1 reagent combination (Table 1) is designed to observe the early maturation of hematopoietic stem cells (CD34+, CD38low) into each of the major cell lineages. The M2 reagent combination (Table 2) is designed to observe the maturation of early myelomonocytic precursors to later-stage mature forms, and includes identification of plasmacytoid dendritic cells and basophil cell lineages. The M3 reagent combination (Table 3) is designed to identify the abnormal expression of T and NK cell–associated lymphoid antigens on progenitor populations, a common type of abnormality in AML. In particular, the inclusion of CD34 and CD38 in each combination allows the assessment of all antigens on hematopoietic stem cells—a small normal subpopulation commonly showing immunophenotypic abnormality in AML that is of potential therapeutic and prognostic significance. Together, these three tubes form a comprehensive strategy for the identification of residual AML.

If antibody cocktails are created, a procedure should be used to verify that each antibody has been added to the cocktail and that the performance of each antibody is as expected.
### Table 1  M1 Myeloid Panel Surface Antibody Combinations

| Fluorochrome | Clone | Vendor | Catalog no. |
|--------------|-------|--------|-------------|
| CD13         | PC7   | My7    | BC          | Custom     |
| CD15         | FITC  | MMA    | BD          | 340703     |
| CD19         | PE-CF594 | HIB19 | BD          | 562294     |
| CD33         | PE    | P67.6  | BD          | 340679     |
| CD34         | APC   | 8G12   | BD          | 340667     |
| CD38<sup>a</sup> | A594 | HB-7   | BD          | Custom     |
| CD45         | APC-H7 | 2D1   | BD          | 641408     |
| CD71         | APC-A700 | YDJ1.2.2 | BC      | Custom     |
| CD117        | PC5   | 104D2D1| BC          | IM2733U    |
| HLA-DR       | PB    | Immu-357| BC       | A74781     |

<sup>a</sup>Substitute CD38 BV510 (Clone HB-7; BioLegend, #356612) for instruments lacking a yellow (594 nm) laser.

### Table 2  M2 Myeloid Panel Surface Antibody Combinations

| Fluorochrome | Clone | Vendor | Catalog no. |
|--------------|-------|--------|-------------|
| CD4          | ECD   | SFC112T4D11 | BC    | 6604727     |
| CD13         | PC7   | My7    | BC          | Custom     |
| CD14<sup>a</sup> | PURE | RMO52 | BC          | IM0643     |
| CD14         | PC5.5 | RMO52 | BC          | A70204     |
| CD16         | APC-A700 | 3G8   | BC          | B20023     |
| CD34         | APC   | 8G12   | BD          | 340667     |
| CD38<sup>b</sup> | A594 | HB-7   | BD          | Custom     |
| CD45         | APC-H7 | 2D1   | BD          | 641408     |
| CD64         | FITC  | 22     | BC          | IM1604U    |
| CD123        | PE    | 7G3    | BD          | 554529     |
| HLA-DR       | PB    | Immu-357| BC       | A74781     |

<sup>a</sup>PURE antibody is used to reduce the intensity of CD14 while maintaining antibody saturation. The desired level of CD14 intensity is about 1 log lower than maximum intensity on the instrument used.

<sup>b</sup>Substitute CD38 BV510 (Clone HB-7; BioLegend, #356612) for instruments lacking a yellow (594 nm) laser.

### Table 3  M3 Myeloid Panel Surface Antibody Combinations

| Fluorochrome | Clone | Vendor | Catalog no. |
|--------------|-------|--------|-------------|
| CD5          | PC5   | BL1a   | BC          | IM2637U    |
| CD7          | PE    | M-T701 | BD          | 340656     |
| CD33         | PC7   | P67.6  | BD          | 333949     |
| CD34         | APC   | 8G12   | BD          | 340667     |
| CD38<sup>a</sup> | A594 | HB-7   | BD          | Custom     |
| CD45         | APC-H7 | 2D1   | BD          | 641408     |
| CD56         | A488  | B159   | BD          | 557699     |
| HLA-DR       | PB    | Immu-357| BC       | A74781     |

<sup>a</sup>Substitute CD38 BV510 (Clone HB-7; BioLegend, #356612) for instruments lacking a yellow (594 nm) laser.
### Table 4  Sample Preparation

| WBC count ($\times 10^6$/ml) | Specimen (μl) |
|------------------------------|---------------|
| $<$5                         | 200 $\times$ 2 tubes (set up each tube in duplicate) |
| 5-10                         | 200           |
| 10-20                        | 100           |
| $>$20                        | Dilute to $20 \times 10^6$ cells/ml, then use 100 |

This may be easily done by running the cocktail on a normal bone marrow sample and comparing the results to results from a prior sample prepared using another lot of cocktail, or to results obtained with individually pipetted reagents. The detailed procedure for performing the verification is outside the scope of this protocol.

### Materials

- Bone marrow aspirate
- RPMI 1640 medium (see recipe)
- Antibody cocktail (see discussion above and Tables 1 to 3)
- Lyse-fix reagent (see recipe)
- PBS/0.3% BSA (see recipe)
- 5-ml polystyrene tubes with 35-μm filter caps (Fisher Scientific, #08-771-23)
- Hematology analyzer, e.g., Sysmex XP-300
- 5-ml (12 $\times$ 75−mm) polystyrene tubes
- Flow cytometer

1. Label a 5-ml polystyrene tube that has a 35-μm filter cap with the sample accession number.
2. Filter approximately 0.5 ml of bone marrow aspirate through the filter cap.
   
   *If the bone marrow is difficult to filter, switch to a CellTrics 50-μm filter (Sysmex, #04-0042-2317).*
3. Obtain a cell count using a hematology analyzer.
4. Dilute the specimen with RPMI, if necessary, based on the cell count.
   
   *For MRD, if the cell count is $>$20 $\times 10^6$ cells/ml, dilute to $20 \times 10^6$ cells/ml.*
5. Determine the amount of specimen to label according to Table 4.
6. Label the appropriate number of 12 $\times$ 75−mm, 5-ml polystyrene tubes with the sample accession number and antibody combination label.
7. Pipette specimen into each labeled tube according to Table 4.
8. Pipette 100 μl of the appropriate antibody cocktail into each corresponding labeled tubes.
9. Vortex the tube briefly to mix.
10. Incubate in the dark for 15 min at room temperature.

### Lysis and fixation

11. Add 1.5 ml of Lyse-fix reagent. Add an additional 1.5 ml if the sample volume added was 200 μl (total 3 ml Lyse-fix reagent).
12. Vortex the tube briefly to mix.
13. Incubate in the dark for 15 min at room temperature.
14. Centrifuge for 5 min at 530 × g, room temperature.
15. Decant and vortex to resuspend the cell pellet.
16. Add 3 ml of PBS/0.3% BSA to the tube.
17. Centrifuge for 5 min at 530 × g, room temperature.
18. Decant and vortex to resuspend the cell pellet.
19. Resuspend the cells with a minimum of 100 μl of PBS/0.3% BSA.
20. Store in the dark at room temperature.

**Flow cytometry**
21. Acquire the entire sample on the cytometer as soon as possible.

> When the sample has a cell count of >20 × 10^6/ml, this should result in roughly 1 × 10^6 nucleated cells acquired.

> The same procedure described here may be used for peripheral blood, provided the sample is checked for clots prior to preparation.

**ANALYSIS AND INTERPRETATION OF DATA FOR AML MINIMAL RESIDUAL DISEASE DETECTION**

The analysis of data produced by this assay is complex and requires an experienced interpreter familiar with normal immunophenotypic patterns of hematopoietic maturation as well as common immunophenotypic abnormalities seen in AML. A complete discussion of both topics is beyond the scope of this article, and the reader is referred to published literature (Cherian & Wood, 2012; Cherian, Wood, & Borowitz, 2016; Wood, 2004, 2007). However, there are a few common analytical tasks that can be performed on any sample to provide an improved starting point for interpretation (Wood, 2006). Assessment of the quality of the acquisition and preparation of the sample are important initial tasks.

**Acquisition stability**

The data from each tube should be displayed versus time or event number to confirm stability of the acquisition. A parameter should be chosen that is sensitive to alterations in fluidic stability, typically a parameter that has a signal of moderate to high intensity and that is on a laser different from that used to trigger data acquisition, e.g., not the 488-nm blue laser. In this assay CD45 APC-H7 is a good choice; see Figure 1.

**Doublet discrimination**

Doublet discrimination should then be performed to exclude coincident events or aggregates of cells and ensure as much as possible that events interpreted are individual cells or singlets. This is typically achieved by plotting forward scatter in a two-parameter dot plot as a combination of area, height, and/or width, followed by gating of the singlet population; see Figure 2.

**Viability**

Viability and overall quality of the sample are assessed by examining the light scatter properties of the sample, generally by plotting forward scatter (FS) versus side scatter (SS); see Figure 3. Apoptotic cells will show an initial loss of FS and gain of SS, followed by loss of both FS and SS as the cells further degenerate. The ability to see these changes is highly dependent on the way in which the sample is processed; the method of choice must be optimized to provide consistent light-scatter properties that reflect cell viability. Of note, nucleated erythroid cells show a loss of FS using this method due to the lysing reagent, but are retained, and the FS instrument settings (threshold and FS
Acquisition stability. Stable sample acquisition will show consistent parameter intensities for each population over time, as pictured for CD45 APC-H7 versus event number. Fluidic instability or sample exhaustion during acquisition will show intensity changes that can be excluded by appropriate gating.

Doublet discrimination. Single cells have a defined relationship between area, height, and width, while cell aggregates or doublets deviate from this relationship. Gating on single cells or singlets, pictured using area versus height, allows for minimization of artifactual doublets that may result in composite immunophenotypes and can confuse residual disease assessment.

PMT voltage) must be properly set to ensure inclusion of this population during acquisition. A viability gate is used to reduce background nonspecific antibody binding to compromised cells and stromal material, the intention being to include viable white cells without regard to nucleated erythroid cells. While the viability gate improves the quality and interpretability of the data, it assumes proportional loss of white cells with reduced cell viability, which is generally but not always correct. Relaxation of the gate to include
Figure 3  Viability gating. Light scatter properties alone can be used to assess sample quality and exclude non-viable cells and debris, pictured here using a forward scatter (FSC) versus side scatter (SSC) gate, the latter displayed on a log scale so populations have more similar distribution regardless of SSC intensity.

lower-FS events is possible and perhaps desirable under some circumstances, provided that data quality is not significantly impaired. In principle, a viability dye that emits at a long wavelength following excitation from the violet laser could be incorporated, provided that the instrument configuration is suitable, but this would require independent validation.

Progenitor population identification

The initial identification of progenitors is readily accomplished using CD45 and SS gating in the progenitor or “blast” area; see Figure 4. Mature lymphocytes are always present in marrow and blood samples, and serve as a reference point for identification of progenitors, having uniform bright CD45 and low SS. The progenitor gate should include all events having CD45 less intense than the mature lymphocyte population. Due to the large number of maturing neutrophilic progenitors usually present, the progenitor gate should exclude as much as possible the high-SS maturing myeloid populations. However, some samples may show variable hypogranularity of the maturing myeloid cells, recognized by a level of SS similar to that of monocytes and approaching that of lymphocytes, in which case it is more important to include all CD34+ progenitors in the progenitor gate at the expense of contamination by maturing myeloid cells. This recognition is facilitated by gating and coloring the CD34+ progenitors so they can be directly visualized on CD45 versus SS plots. CD45 very low to negative events largely consist of nucleated erythroid cells, plasma cells, aggregated platelets, and debris, and thus can be safely excluded, as AML only very rarely shows loss of CD45 expression to that degree.

Leukemic population identification

The identification of residual leukemia requires a detailed knowledge of normal maturation patterns of antigen expression that is beyond the scope of this procedure, and also requires an experienced interpreter. The general strategy is to identify a subset of events that is different from any normal stage of hematopoietic maturation, with a distribution of events distinct from artifact and discrete enough to allow sequential gating of the
Figure 4  Progenitor gating. Hematopoietic progenitors may be identified and gated using CD45 and side scatter (SSC) as the populations having CD45 slightly lower than that of mature lymphocytes (blue) and SSC lower than maturing neutrophils (green). Normal CD34⁺ progenitors (red) and B cell progenitors (cyan) are included in the progenitor gate, and mature lymphocytes (blue), monocytes (magenta), and maturing neutrophils (green) are excluded.

A population in multidimensional space in a manner that excludes artifactual events and normal hematopoiesis and includes all leukemic events. See Figure 5.

Recognition of normal populations can be facilitated by coloring the progenitors (CD34⁺ and/or CD117⁺) and each of the major cell lineages a different color. The interpreter should review all combinations of dot plots that are likely to be informative for this purpose, which need not be all possible dot plots but will generally be a major subset of these. The subset of dot plots that we have found to be most informative with this reagent panel is illustrated in Figure 6. Of particular importance is evaluation of the hematopoietic stem cell population (CD34++/CD38low), which is readily performed by displaying CD34⁺ events in a series of two-parameter dot plots containing CD38 versus each of the other parameters in the reagent combinations. It is also important to review the full range of maturing monocyte and myeloid populations in order to recognize abnormal immature populations of these lineages—as may be seen in AML having monocytic or myeloid differentiation—as well as to recognize potential aberrant maturational changes in these lineages that might suggest a preexisting myeloid stem cell disorder.

Immunophenotypic abnormalities in AML can be grouped into classes having recurring features that often reflect the predominant maturational stage of the leukemia.

Abnormalities in subsets of leukemic cells having immunophenotypes similar to those of normal hematopoietic stem cells are common in AML and may be associated with properties such as self-renewal and resistance to chemotherapy, and thus are important to evaluate (van Rhenen et al., 2007; Zeijlemaker et al., 2019). It is important to keep in mind that while this component of the leukemia may be relatively easy to recognize, there may be more mature components (CD34⁺ or CD117⁺) derived from these more primitive cells that are more challenging or perhaps impossible to define with a particular reagent combination. A judgment must then be made about the remaining CD34⁺ progenitors as to whether they are of sufficient concern, by also being potentially aberrant, to be included in the MRD enumeration, or whether only the stem cells that can be cleanly
Figure 5  Sequential gating. Progenitors (blasts) are gated using CD45 versus SSC, and residual leukemia identified using CD34 versus CD117 (top left). The gated leukemic population (MRD1) is then further gated (MRD2) to exclude early promyelocytes having increased CD13 (top right), then B cells (CD19+) and erythroid cells (CD71^bright) are excluded (MRD3; bottom left), and finally outliers for CD45 and CD33 are excluded, resulting in a relatively homogeneous population that includes all leukemic cells and excludes normal cells to the extent possible with this reagent combination.

A subset of AML expresses CD34 without a well-defined hematopoietic stem cell—like subpopulation. In these cases, knowledge of normal patterns of early antigen expression with commitment to each of the major lineages (B cell, monocytic, neutrophilic, erythroid, basophilic, plasmacytoid dendritic cell) is critical for MRD recognition. A relatively clear example of this principle is seen in t(8;21) AML due to a characteristic immunophenotype that includes varied aberrant expression of increased CD34, B cell antigens such as CD19, and CD56 (Khoury et al., 2003). An example of MRD having an immunophenotype similar to CD34^+ myeloid committed progenitors is provided in Figure 8.

Roughly one-third of normal-karyotype AML contain a mutation in the NPM1 gene, and these cases have a relatively distinctive immunophenotype that is important to recognize for MRD detection (Zhou et al., 2019). Characteristic of this type of AML is a more
Figure 6  Dot plots to review. Progenitors gated using CD45 versus SSC are displayed for reagent combinations M1 and M2 as an example of the number of dot plots that are advisable to review when making an assessment for MRD. Note that the gated CD34^+ progenitors are specifically displayed (bottom right) as CD38 versus each of the other parameters to assess for immunophenotypic abnormalities on the hematopoietic stem cells. Populations are colored as follows: CD34^+ progenitors (red), monocytes (magenta), myeloid (green), plasma cells (yellow), basophils (purple), plasmacytoid dendritic cells (cyan), MRD (blue).
AML MRD, hematopoietic stem cell abnormalities. Progenitors gated using CD45 versus SSC identify an expanded hematopoietic stem cell population (blue) that differs from normal CD34+ progenitors (red) by having abnormal expression of CD38 (decreased), CD34 (slightly decreased), CD123 (increased), CD56 (increased), and CD7 (variably increased). Note the variability in expression of some antigens that may be seen, CD7 in this example.

A mature immunophenotype with little to no expression of CD34, decreased to absent HLA-DR, and increased CD33, often at a level higher that monocytes, monocytes being the normal population with the highest level of CD33 expression and thus a useful reference point. As a result, a dot plot of CD33 versus HLA-DR gated on progenitors by CD45 versus SS is a useful starting point for evaluation. An example of NPM1+ AML MRD is provided in Figure 9. A similar gating strategy can be used for acute promyelocytic leukemia.

AML with monocytic differentiation can be a particularly difficult subset of AML on which to perform MRD detection using this and other reagent panels. Often the key is to identify a population of immature monocytes (usually CD14 low to negative) having immunophenotypic abnormalities in the expression of CD4, CD14, CD15, CD64, and/or HLA-DR with aberrant CD56 expression at a moderate to high level seen in a significant subset of cases. Relying on expression of high CD64 or HLA-DR to identify monocytes is
AML MRD, t(8;21). Progenitors gated using CD45 versus SSC identify an abnormal population of CD34+ progenitors (orange) that differs from normal CD34+ progenitors (red) by having abnormal CD34 (increased), CD19 (bright), CD15 (variable with uniform CD34 intensity), and CD56 (subset). Note the absence of a clear hematopoietic stem cell subpopulation.

Figure 8  AML MRD, t(8;21). Progenitors gated using CD45 versus SSC identify an abnormal population of CD34+ progenitors (orange) that differs from normal CD34+ progenitors (red) by having abnormal CD34 (increased), CD19 (bright), CD15 (variable with uniform CD34 intensity), and CD56 (subset). Note the absence of a clear hematopoietic stem cell subpopulation.

risky, as these antigens are fairly commonly decreased in this subtype of AML. Rather, a comprehensive evaluation of antigen expression with cross-correlation between reagent combinations is required for confident identification. An example of monocytic AML MRD is provided in Figure 10.

Enumeration
Relatively commonly, the leukemic population is best recognized in one of the three reagent combinations acquired. Every effort should be made to identify a corresponding population in each of the two other reagent combinations, relying on the common reagents to extrapolate between tubes. Finding a similar population at a similar frequency in more than one combination provides assurance that the finding is real and less likely to be due to a technical artifact. Rarely, significant differences in enumeration may be seen between combinations, in which case the interpreter must make a judgement as to which combination is likely most informative and most complete for identifying the leukemic population. That combination should be used for enumeration, and is not always the one...
AML MRD, NPM1-mutated. Progenitors gated using CD45 versus SSC identify an abnormal population of progenitors (blue) that differs from normal CD34+ progenitors (red) and monocytes (magenta) by having abnormal HLA-DR (variably decreased), CD33 (brighter than monocytes), CD13 (increased), CD34 (mostly negative), CD38 (slightly decreased), and CD71 (increased). Note the absence of a clear hematopoietic stem cell subpopulation.

with the highest enumeration, due to specificity issues with a particular combination. The denominator for enumeration historically is total CD45+ events, although total nucleated cells may ultimately be preferable, but would require use of a nucleic acid–binding dye and CD45 in a fourth reagent combination.

REAGENTS AND SOLUTIONS

**Lyse-fix reagent**

Add 80.2 g ammonium chloride (NH₄Cl), 8.4 g sodium bicarbonate (NaHCO₃), and 3.7 g disodium EDTA to a 1-L volumetric flask. Fill flask to 1 L with reagent-grade deionized water. Mix thoroughly and filter through a 45-μm Millipore filter. This results in a 10× stock that can be stored at 4°C for 3 months. Add 50 ml of 10× stock and 11.25 ml of 10% formaldehyde (methanol-free, Polysciences, #04018) to a 500-ml volumetric flask. Fill flask to 500 ml and mix thoroughly. Prepare lysing solution fresh every day of use.

**PBS/0.3% BSA**

Add one packet of phosphate-buffered saline (PBS) mix (Gibco, #21600-010) and 10 ml of 30% bovine serum albumin (BSA; Sigma-Aldrich, #A7284) to a 1-L volumetric
flask. Fill flask to 1 L with reagent-grade deionized water. Mix thoroughly and filter through a 45-μm Millipore filter.

**RPMI 1640 medium**

Add 1 packet of RPMI 1640 medium (Gibco, #31800-002) and 2 g NaHCO₃ to a 1-L volumetric flask. Add about 800 ml of reagent-grade deionized water and mix well, at least 15 min. While mixing, add just enough 1 N HCl to cause the magenta color to turn burnt orange. Fill to 1 L with deionized water and discard 120 ml of the RPMI. Add 10 ml of 100× pen-strep (Invitrogen, #15140-122) and 10 ml of 100× minimal essential medium (MEM; Invitrogen, #1120-052) to the RPMI and adjust pH to 7.1 using 1 N HCl and 1 N NaOH. After mixing 10 min, filter through 45-μm Millipore filter, and then add 100 ml of newborn calf serum (Invitrogen, #20610-074).

**COMMENTARY**

**Background Information**

The method outlined above was developed for the definition of normal pathways of hematopoietic maturation and the efficient identification of neoplastic hematopoietic cell populations in a clinical laboratory setting. The assay relies on both the ability of the reagent panel to subdivide hematopoietic progenitors into many discrete and defined sub-populations of cells and the specificity of the reagents to identify immunophenotypic abnormalities on leukemic cells that lie outside the range of normal patterns of expression seen on similar normal precursors. Consequently, the same assay can be used for the diagnosis of a variety of myeloid neoplasms including AML, myelodysplastic syndromes, and myeloproliferative neoplasms.
Extension of the diagnostic assay to the identification of smaller residual populations of leukemic progenitor characteristic of AML largely requires the labeling and acquisition of a larger number of cells to achieve a desired statistical target for sensitivity and reproducibility. Experience with this assay indicates that immunophenotypic abnormalities suitable for detection of residual disease to the level of 0.1% of white cells are seen on at least 90% of AML. A substantial number of AML (~40%) exhibit more significant immunophenotypic abnormalities that allow detection of residual disease to a level of 0.01% of total white cells. If the desired reproducibility for enumeration is taken to be a CV of 10%, a total of 1 million cells need to be acquired to reach that statistical target for reproducibility at a sensitivity of 0.01%. The assay has been designed to reach that target for marrow samples having relatively normal cellularity, which are most post-treatment samples from end of induction onward. If the sample is less cellular, so that fewer total cells are acquired, the sensitivity for detection is unlikely to be impaired, but the enumeration will exhibit a greater degree of variability while remaining acceptable for most applications.

Critical Parameters

Leukemia-associated immunophenotype versus difference from normal

Leukemic blasts in AML invariably demonstrate immunophenotypic differences from normal hematopoietic precursors, the number and magnitude being dependent on the reagents used and the manner in which the panel is constructed. Immunophenotypic differences that lie outside of normal maturation at diagnosis have been termed the leukemia-associated immunophenotype (LAIP), and can be represented by one or more sequential gates defining a multiparametric space that contains few to no cells in normal bone marrow or peripheral blood.

Once defined at diagnosis, a LAIP may be used to interrogate samples after therapy for the presence of events that meet the definition and hence may represent residual leukemia. While attractive as an objective and relatively simple way to define residual disease, this has a few limitations. (1) It requires a pre-treatment sample to allow definition of the LAIP, and therefore is difficult to apply in environments where only post-therapy samples are obtained, e.g., tertiary referral centers or reference labs. (2) It is dependent on the reference samples used to represent normal hematopoiesis. It is important to use reference samples from a similar time point post-therapy as the time point that will be tested for residual disease, since the background and immunophenotypes of normal hematopoietic cells in regenerating post-therapy samples may be different from those of resting bone marrow. (3) If any event that appears in the regions defined as the LAIP are taken to be residual disease without regard to distribution or shape, nonspecific binding events or noise will impair the specificity of the assay and lead to potential false positive results. (4) It assumes stability of the immunophenotype post-therapy, which is known to be not entirely true for AML, and so may give rise to under-enumeration of residual disease including false negative results. The use of more than one LAIP per leukemia is advocated to minimize this risk. (5) It may be difficult to reconcile the enumeration obtained from multiple LAIPs on the same sample, especially when incomplete or partial populations define the LAIP. Typically, it is advocated that the highest value of all the LAIPs assessed be used for enumeration.

A more general approach for the detection of residual disease in post-therapy samples is to apply a similar process for defining LAIPs as that used for pretreatment samples, but incorporating the shape and distribution of events in multiparametric space to identify discrete abnormal populations that differ from normal hematopoiesis, and to exclude noise. This analytical approach has been termed Difference-from-Normal (DfN), and is a superset of the LAIP approach. Pretreatment LAIPs may be used as the starting point for DfN analysis, if available, but are not required; therefore, the approach may be used without a pre-treatment immunophenotype. Rather than assuming stability of immunophenotypes post-therapy, the analysis may be extended to encompass any abnormal population observed, and thus can take into account even major shifts in immunophenotype. While the DfN approach effectively improves on most of the limitations of a strict LAIP approach, it requires a more detailed knowledge of normal and regenerating hematopoiesis by the interpreter and introduces some subjectivity in the definition of populations and in the recognition and exclusion of noise. Consequently, a higher degree of experience and training is needed for implementation, and standardization between interpreters is more difficult to achieve.
Troubleshooting

A subset of samples may show evidence of nonspecific binding with one or more reagents, often as diagonal or highly correlated linear relationships between parameters. Some antibody conjugates are more prone to this phenomenon than others. While the reagent panel described has been optimized to reduce the likelihood of this problem, it may still occur in a small subset of samples. Generally, this problem is mediated by plasma components such as complement (Wood & Levin, 2006), antibodies, or other plasma proteins. Washing the sample twice with RPMI or PBS/BSA will eliminate the artifact at the expense of increased cell loss and activation of some cellular subpopulations.

Anticipated Results

Samples without identifiable residual disease represent roughly 75% of post-therapy or pre-transplant samples using this assay.

Time Considerations

Using the Basic Protocol, it takes roughly 45 min to process a single sample.

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