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Flow cytometric monitoring for residual disease in B lymphoblastic leukemia post T cell engaging targeted therapies

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Abstract

The use of targeted therapy is growing in the setting of hematopoietic neoplasms. Flow cytometry is a cornerstone of residual disease monitoring post therapy in this group of malignancies. Often, there is overlap between antigens targeted by immunotherapies and gating reagents utilized for population identification by flow cytometry. Such overlap can render a previously excellent gating reagent insufficient for disease detection. Recently, several anti CD19 T cell engaging immunotherapeutic agents and an anti CD22 immunotoxin have been FDA approved for use in B lymphoblastic leukemia (B-LL), with an anti CD22 T cell engaging agent in development. In the setting of such targeted therapies, CD19 and CD22 expression may be altered, compromising the use of these reagents for identification of abnormal blasts. We describe herein a strategy for flow cytometric monitoring for residual disease in patients with B-LL post T cell engaging anti CD19 and anti CD22 therapies.

Keywords

CAR T cells; CD19; CD22; minimal residual disease; B lymphoblastic leukemia

Introduction

The traditional approach to treatment of hematopoietic neoplasms has focused on cytotoxic therapies, which non-selectively attack proliferating cells. While such approaches show efficacy in killing tumor cells, these therapies also kill normal proliferating cells in a manner that leads to undesirable side effects. Recently, investigators have identified ways to harness the power of the immune system to selectively target a variety of neoplasms. Targeted therapies are becoming more commonly and widely utilized in treating hematopoietic neoplasms (Ghosh et al., 2017; Gill, 2016; Maude, 2017; Maude et al., 2015; Tasian & Gardner, 2015).

One such class of targeted therapy functions by engaging T cells to destroy tumor cells using cell-mediated cytotoxicity. Such therapies include chimeric antigen receptor expressing T

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cells (CAR T cells) (Maude et al., 2014; Maude et al., 2015; Tasian & Gardner, 2015) and bi-specific T cell engaging small molecules (BiTEs)(Goebeler & Bargou, 2016; Zhu et al., 2016). CAR T cells are T cells, typically autologous, which are genetically engineered to express a chimeric antigen receptor (CAR) directed against a neoplastic cell. BiTEs, by contrast, are small molecules with specificity against a tumor marker and also against a T cell antigen, commonly CD3. Both CAR-T cells and small molecule BiTEs drive tumor specific T cell mediated cytotoxicity.

CAR T cells and BiTEs have been investigated in a number of therapeutic settings including precursor and mature B cell neoplasms, acute myeloid leukemia, plasma cell neoplasms, and non-hematopoietic neoplasms (Ghosh et al., 2017; Gill, 2016; Li et al., 2018; Maude, 2017; Maude et al., 2015; Tasian & Gardner, 2015). These therapies have been employed most effectively in the setting of B lymphoblastic leukemia (B-LL) and mature B cell non-Hodgkin lymphoma (Grupp et al., 2013; Maude et al., 2015; Tasian & Gardner, 2015). In fact, 2017 saw FDA approval of the first commercial anti CD19 CAR T cell for the treatment of relapsed refractory B-LL (Bach et al., 2017; Maude et al., 2018; Prasad, 2018).

CD19 has been a target in a number of CAR T cell trials and as well is the target of the BiTE, blinatumomab (Goebeler & Bargou, 2016; Zhu et al., 2016). CD19 is well suited as a target tumor antigen for B cell malignancies as it is expressed on the vast majority of mature and immature B cell neoplasms. With regard to normal populations, expression of CD19 is largely isolated to B cells and precursors. CAR T cells directed against CD19 (CAR T-19) will deplete both normal and abnormal CD19 expressing B cells. This has important implications for detection of residual disease post CAR T-19 therapy.

For the same reason that CD19 is an excellent target for immunotherapy, CD19 is an excellent gating reagent for enrichment of neoplastic B cell populations post therapy when looking for residual disease. Post CAR T-19 therapy however, CD19 is no longer reliable as a gating reagent. Not only does CAR T-19 lead to depletion of normal and abnormal B cells, but in addition, post therapy, patients may relapse with CD19 negative disease (Maude et al., 2015; Tasian & Gardner, 2015). For this reason, novel gating strategies are required to evaluate patients post treatment with T cell engaging anti CD19 targeted therapies (Cherian et al., 2018). Furthermore, CD22 expression may be compromised as patients may receive Inotuzumab, an anti-CD22 immunotoxin, and new T cell engaging therapies are under development that target CD22 (Fry et al., 2018; Haso et al., 2013) with such therapies being used in isolation or in conjunction with anti-CD19 therapies. In the era of targeted therapies, flexibility and redundancy is needed in gating strategies utilized for residual disease detection.

This monograph will describe optimal strategies for evaluating patients with B-LL post T cell engaging therapies with a focus on residual disease detection post anti-CD19 therapy. Strategies for residual disease detection in patients who have also received anti CD22 therapies will be discussed as well. Basic protocol 1 focuses on assessment of patients with B-LL for residual disease post therapy who have received anti CD19 therapy, or are post anti CD19 and CD22 therapies. The protocol relies on basic strategies for multicolor immunophenotyping outlined in Unit 6.21 (Brent L. Wood, 2005).

Basic Protocol 1. Detection of residual disease in B-LL post anti CD19 T cell engaging therapy and/or anti CD22 therapy

Post therapy with T cell engaging agents that target CD19 and/or CD22, both normal and abnormal B cells are depleted. CD19 is typically an excellent reagent to enrich for neoplastic B cells, and identifying CD19 positive cells is a frequent initial gating step in assessing patients with B cell neoplasms for residual disease post therapy; however, post CAR T-19 therapy, patients may relapse with either CD19 positive or CD19 negative disease. A typical CD19 defined B cell gate will enrich for CD19+ relapsed or residual disease, but will not include CD19 negative disease present post therapy. In the setting of anti CD19 therapies, the use of CD19 is not adequate as a gating reagent. For this reason, it is critical to adjust the gating strategy to allow for sensitive disease detection in these patients.

In our laboratories we utilize a gating strategy that employs expression of CD22 or CD24 by B cells to enrich for normal and neoplastic B cells in patients post anti CD19 therapies (gating strategy is outlined in Figure 1)(Cherian et al., 2018). We exclude CD66b positive cells to remove neutrophils, which are also highlighted by CD24. Cells expressing CD22 or CD24 without CD66b are then displayed on a variety of two-dimensional dot plots to identify residual disease using a difference from normal approach (Chen & Wood, 2017; B. L. Wood, 2016). In patients who have received anti-CD22 therapy prior to or post CD19 directed therapy, however, CD22 may not be a reliable target for gating. Furthermore, not all B-LLs are CD24 positive. In such cases multiple approaches may be necessary, including identification of cells with intracellular CD79a and aberrant patterns not found in normal immune cells.

Materials list

EDTA or heparin anticoagulated bone marrow or peripheral blood specimen at room temperature, <48 hours old.

Phosphate-Buffered Saline (PBS)

Bovine serum albumin (BSA)

RPMI Medium

Buffered NH₄Cl lyse/fixative solution with 0.25% formaldehyde

This solution is used for red cell lysis post staining. For recipe, see following reference: B. L. Wood, 2005, Current Protocols Unit 6.21

Red cell lysing solution (pre lyse solution)

This solution is used for red cell lysis prior to staining. See complete recipe under Reagents and Solutions below.

NaN₃, diluted to 0.1% in PBS

Fix and Perm cell fixation and permeabilization kit (Fix & Perm™ Cell

Permeabilization Kit, Invitrogen by Thermo Fisher Scientific, Carlsbad, CA)

Formaldehyde Ultrapure 10%

Diluted to 0.25% or 0.5% in PBS for this procedure.

Monoclonal antibodies (Table 1): It is recommended that you create and validate cocktails. Antibody combinations utilized in our laboratories are provided below:

1. For modified BD (Becton Dickinson) LSRII flow cytometer with an additional yellow laser. (The yellow laser is used to detect the fluorochrome Alexa 594): CD10 BV421/CD66b FITC/CD22 PE/CD34 PerCP-Cy5.5/CD20 PE-Cy7/CD38 Alexa594/CD24 APC/CD45 APC-H7

For BD (Becton Dickinson) FACSCanto II:

2. CD10 BV421/CD66b FITC/CD22 PE/CD34 PerCP-Cy5.5/CD20 PE-Cy7/CD38 BV510/CD24 APC/CD45 APC-H7
3. CD15 v450/CD20 FITC/CD22 PE/CD34 PerCP-Cy5.5/CD19 PE-Cy7/CD38 BV510/CD79a APC/CD45 APC-H7

Antibodies were obtained from Becton Dickinson (BD Biosciences, San Jose, CA), Beckman-Coulter (Fullerton, CA), Biolegend (San Diego, CA), eBioscience (San Diego, CA), or Dako Agilent (Santa Clara, CA). Details provided in Table 1.

Red cell lysing solution (see recipe in Reagents and Solutions)

Sheath fluid

Centrifuge

Flow cytometer equipped with lasers and filters allowing excitation and detection of all utilized fluorochromes.

In one laboratory we use a modified BD (Becton Dickinson) LSRII flow cytometer with an additional yellow laser. In the other laboratory, a BD (Becton Dickinson) FACSCanto II flow cytometer is used.

Data analysis software

Basic protocol steps

I. Specimen preparation: Staining and Red Cell Lysis—Red cell lysis can be performed either before or after staining. Protocols for both options are presented below.

A: Red cell lysis post staining

1. Whole blood or bone marrow specimens are appropriate for this procedure. Filter and dilute specimens as needed with RPMI to achieve a WBC count of $1.0\text{--}10.0 \times 10^3$ cells/ μl .
2. Incubate the sample and antibody in the dark for 15 minutes
 - a. For samples with WBC $5.0\text{--}10.0 \times 10^3$ use 100 μl of sample and 100 μl of antibody cocktail, gently vortex to mix, then incubate

- b. For samples with $\text{WBC} < 5.0 \times 10^3$ use 200 μl of sample and 100 μl of antibody cocktail, gently vortex to mix, then incubate
- 3 Subject sample to simultaneous NH_4Cl lysis and formaldehyde (0.25%) fixation (for lyse/fixative solution recipe see B. L. Wood 2015, current protocol 6.21) for 15 minutes at room temperature in the dark.
 - a. For samples with $\text{WBC} 5.0\text{--}10.0 \times 10^3$ use 1.5ml of lyse/fixative, gently vortex to mix, then incubate
 - b. For samples with $\text{WBC} < 5.0 \times 10^3$ use 3.0ml of lyse/fixative, gently vortex to mix, then incubate
- 4 Following incubation, spin down sample for 5 min at $518 \times g$ then decant supernatant.
- 5 Rack sample tubes for a few seconds to mix then add 3 ml PBS with 0.3% BSA and spin for 5 min at $518 \times g$, then decant supernatant.
- 6 Rack sample tubes for a few seconds to mix then re-suspend samples in 100 μl of PBS with 0.3% BSA. Proceed to II. Acquisition.

B. Red cell lysis prior to staining

- 7 Whole blood or bone marrow specimens are appropriate for this procedure. Subject specimen to red cell lysing solution (pre lyse solution: 150 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA) at a ratio of 1:9 (volume of sample: volume of lysing solution) for 10 minutes at room temperature.
- 8 Spin down sample for 5 min at $500 \times g$ then decant.
- 9 Wash cells with PBS, spin for 5 min at $500 \times g$ then decant. Repeat.
- 10 Dilute specimens with PBS to achieve a WBC count of 20×10^3 cells/ μl
- 11 Incubation:
 - a. For Cocktails 1 and 2 (all surface staining) incubate 100 μl of sample and antibody in the dark for 30 minutes at room temperature
 - b. For Cocktail 3 with intracellular CD79a incubate 100 μl of sample and all antibodies except CD79a for 15 minutes at room temperature, then fix cells by adding Fixation Medium A (Fix & PermTM Cell Permeabilization Kit, Invitrogen by Thermo Fisher Scientific, Carlsbad, CA) per manufacturer's recommendations and incubate with fixative for an additional 15 minutes.
- 12 Resuspend cells in 3–4 mL of PBS with 0.1% BSA and 0.1% NaN_3 .
- 13 Spin down sample for 5 min at $500 \times g$ then decant.
 For Cocktail 3 permeabilize cells by adding Fixation Medium B (Fix & PermTM Cell Permeabilization Kit, Invitrogen by Thermo Fisher Scientific, Carlsbad, CA) per manufacturer's recommendations, add anti-CD79a, incubate 20 minutes

at room temperature, add 3–4 mL of PBS with 0.1% BSA and 0.1% NaN₃, spin down sample for 5 min at 500 × g then decant

- 14 Re-suspend samples in 3–4 mL of PBS with 0.1% BSA and 0.1% NaN₃.
- 15 Spin down sample for 5 min at 500 × g then decant.
- 16 Subject sample to formaldehyde (0.5%) fixation for 15 minutes at room temperature in the dark.

II. Acquisition—Run specimens on a flow cytometer with a collection goal of >500,000 total events.

Acquisition of > 1,000,000 increases MRD detection sensitivity.

Analyze data as outlined below and demonstrated in Figure 1.

III. Gating Strategy/Data Analysis

A. Gating strategy for cocktails 1, 2, and 3 for samples collected post CD19 targeted therapy, e.g. CAR T-19 or BiTE therapies, (see Figure 1 for details)

- 17 Begin by evaluating all events versus CD45 APC-H7 to ensure stable acquisition (Figure 1A).

One can use this plot to screen for interference with data acquisition due to issues including fluidics problems, bubbles, laser delay, and others. If problems are noted, one can either analyze only events showing stable data acquisition, or if insufficient events are present, repeat the study. In some laboratories, time is used for this purpose rather than event number but both parameters will provide similar information. In the case illustrated in Figure 1, there is a minor disruption of data acquisition near the end of the data collection (see red arrow), perhaps due to the tube running dry during data collection. This segment may be excluded from analysis using a rectangular gate.

- 18 Create a singlet gate (Figure 1B) on a plot of forward scatter height (FSC-H) vs. area (FSC-A). Doublets will be excluded from analysis.

Doublet exclusion is particularly important in the setting of rare event analysis as the unusual staining pattern of doublets may skew results and mimic residual disease.

- 19 Display all singlets on a plot of FSC versus side scatter (SSC) in order to assess for debris and viability (Figure 1C).

Debris, erythroid cells, and poorly viable cells will typically have lower FSC and higher SSC. Alternatively or in addition, a plot of CD45 versus FSC can be evaluated (this strategy is especially useful for identifying red cell debris); however, if using this strategy it is important to be aware that B-LL may be CD45 negative and to not exclude neoplastic CD45 negative cells.

- 20** Assess debris/poorly viable events to ensure that this area does not contain significant populations of dead neoplastic cells (Figure 1C).

In cases of poor specimen viability, the neoplastic cells may fall into the “debris” area. Additionally, B cell precursors may have relatively low forward scatter, therefore it is critical to ensure that they are not being excluded from analysis by this gate. Note: Non-viable cells may show abundant non-specific staining patterns complicating analysis. In specimens with significant percentage of non-viable events, take care to not over interpret findings.

- 21** Exclude debris and non-viable cells

- 22** B cell identification

- a.** For cocktails 1 and 2:

Display the not debris/viable gate on a plot of CD22 versus CD66b and on a plot of CD24 versus CD66b (Figure 1D and E). Draw a gate around cells positive for CD22 or positive for CD24 without CD66b. Events expressing CD22 or CD24 without CD66b will be enriched for B cells.

The strategy of using expression of CD22 or CD24 is critical as CD22 and CD24 are variably expressed on B cells during maturation from an early hematogone to a mature B cell (Figure 1F shows differential expression of CD22 and CD24 on B cells). CD66b is utilized to exclude granulocytic cells, which express CD24.

- b.** For cocktail 3:

Viable/non debris events that are positive for intracellular CD79a are defined as B cells.

- 23** Display B cells (positive for CD22 or CD24 without CD66b using cocktails 1 or 2 and positive for cytoplasmic CD79a using cocktail 3) on a series of two dimensional dot plots and evaluate B cell maturation patterns (some examples of plots one might evaluate are shown in Figure 2). It is recommended that you evaluate all possible combinations of fluorescent parameters. These plots can then be used as a baseline for residual disease detection using a different from normal approach.

Normal B cells show conserved and predictable patterns of antigen expression during maturation while abnormal B cell precursors that one sees in the setting of leukemia show antigen expression patterns that vary from normal maturational patterns. These abnormal patterns of antigen expression allow identification of abnormal blast populations by flow cytometry. A thorough discussion of strategies for residual disease and minimal residual disease detection (MRD) is beyond the scope of this manuscript but is the subject of many excellent

reviews(Chen & Wood, 2017; B. L. Wood, 2016). Figure 3 shows an example with ~5% leukemic cells.

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It is important to note that the distribution of normal cells present may shift post anti CD19 T cell engaging therapies. The type of changes one may encounter are outlined in Figure 4 and discussed in detail below under the heading “Expected Results: Negative Samples”.

B. Gating strategy for samples post both CD22 and CD19 targeted therapy, (e.g. CAR T-19 or BiTE and CD22 CAR T cell or Inotuzumab therapies, (see figure 5): Note: If only CD22 was targeted and CD19 was not targeted, standard analysis using CD19 to enrich for B cells is appropriate.

- 24 Follow steps 17–21 above, then utilize the strategies that follow for analysis of cocktails 1 and 2 (steps 25–28) and for cocktail 3 (steps 29–30).

For cocktails 1 and 2: CD22 expression is not dependable in this situation and, since CD24 may be negative on B-LL, additional strategies must be utilized. Results from a baseline analysis can be used for residual disease detection using a different from normal approach.
- 25 Display the not debris/viable-cell gate on a plot of CD24 versus CD66b (Figure 5A) and draw a gate around cells positive for CD24 without CD66b (Gate 1).
- 26 Display the not debris/viable-cell gate on a plot of CD22 versus CD66b. If a population of cells positive for CD22 can be clearly delineated draw a gate around them. If not (Figure 5B)-examine CD22, CD10, CD24, CD34, and CD45 in a series of plots to get a better delineation of the CD22 positive population. If cells positive for CD22 are identified draw a gate around them (Figure 5C).
- 27 If a population of cells positive for CD22 cannot be clearly delineated (Figure 5G), search for B lymphoblasts with abnormal levels of CD10, CD34, CD38, or CD45 (Figure 5H) and draw a second gate that is patient specific.
- 28 Events in these gates (cells expressing CD24 without CD66b, CD66b negative cells expressing CD22, and/or cells expressing abnormal CD10, CD34, and /or CD45) are combined for the final analysis gate (Figure 5D, E, and I).

For cocktail 3: When CD22 and CD19 expression are not dependable, CD79a can be utilized to enrich for B-cells
- 29 Display the not debris/viable-cell gate on a plot with CD79a intracellular versus SSC and draw a gate around cells positive for CD79a (Figure 6A).
- 30 Display cells positive for CD79a intracellular on a series of two dimensional dot plots (Figure 6B–D) and evaluate B cell maturation patterns (some examples of plots one might evaluate are shown in Figure 6). It is recommended that you evaluate all possible combinations of fluorescent parameters.

IV Report Results

- 31** Report whether or not an abnormal population is present (i.e. “No abnormal blast population present” or “Abnormal blast population present”).

For negative samples, report the limit of quantification of your assay as established during assay validation in your laboratory.

- 32** Report the size of the abnormal population if one is detected.

It is critical that you report the denominator when providing a percentage (white blood cells, mononuclear cells, etc.). If using CD45 to define the WBC as the denominator, note that B-LL may be CD45 negative and take care to include CD45 negative blasts in the denominator.

- 33** Report the immunophenotype of the population of interest.

Reagents and Solutions

Lyse/fixative solution for post stain red cell lysis: Buffered NH_4Cl lysing solution with 0.25% formaldehyde made as outlined in protocol 6.21 (B. L. Wood, 2005).

Red cell lysing solution for red cell lysis prior to staining: 150 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA) at a ratio of 1:9 (volume of sample: volume of lysing solution)

COMMENTARY

The primary application of this protocol is to identify residual B-LL in patients post anti CD19 therapy and/or anti CD22 therapy. It may also be employed when evaluating B-LL in which expression of CD19 and/or CD22 is low and in the rare cases of B-LL which are CD19 negative. This protocol allows for detection of both overt and low-level residual disease (e.g. MRD).

Given the recent FDA approval of anti-CD19 T cell engaging therapies and an anti-CD22 immunotoxin for relapsed/refractory B-LL it is likely that patients receiving such therapies will present to the clinical flow cytometry laboratory for residual disease detection. In the era of targeted therapies, knowledge of the specific therapy a patient has received is critical in order to optimize gating strategies for disease detection post therapy. As, noted, post T cell engaging anti CD19 therapies such as CAR T-19, patients may relapse with CD19 negative disease, therefore, the traditional strategy of using CD19 for B cell enrichment is no longer adequate. Furthermore, these patients are increasingly receiving anti-CD22 therapy, either CAR T cell or immunotoxin, once they relapse with CD19 negative disease. Assays for MRD detection in the era of targeted therapies must allow for flexibility and incorporate adequate redundancy to reliably detect abnormal populations.

The importance of undertaking a thorough validation process before attempting residual disease detection [in particular MRD] cannot be over-emphasized. Any validation process when assessing for MRD must include a determination of limit of detection (LOD) and limit of quantification (LOQ) of the assay. A complete discussion of validation is beyond the

scope of this manuscript. The reader is referred to the following document for further discussion of proper assay validation (B. Wood et al., 2013). Validation, training, and experience are critical components to consider when developing any assay for MRD detection (Keeney et al., 2017).

Troubleshooting

It is critical to note that there are normal populations that may express CD22 or CD24 that are not included in a traditional CD19 defined B cell gate. These cell subsets must be recognized to avoid erroneously calling such populations residual disease. CD22 may be expressed by a number of cell types including dendritic cells (including but not limited to plasmacytoid dendritic cells) and basophils (Acon-Laws et al., 2011; Reineks et al., 2009). Expression of CD22 on these populations is antibody clone dependent, therefore it is crucial to recognize the characteristics of the anti CD22 clone you are utilizing and ideally, to avoid use of clones with broader expression profiles.

It is essential to be aware that there are some CD34+ progenitor populations that may express CD22 without CD24 (Figure 4). These populations are seen at relatively low levels in reactive marrow specimens and in marrow specimens post cytotoxic therapy; however, these populations may be relatively increased post anti CD19 therapies. Recognition and accurate identification of CD19 negative progenitor populations is critical so as not to mistakenly label such populations as residual disease.

One important population to account for is neutrophils, a non-B cell population that expresses CD24. These can generally be recognized and distinguished from putative B cell precursors using a combination of CD66b expression (see Figure 1E) and side scatter characteristics (SSC), as neutrophils typically have higher SSC than blasts; however, distinction may be more challenging in the setting of an older sample undergoing neutrophil degeneration.

Some leukemia subtypes may be challenging to detect because they express decreased CD22 or CD24. B-LL with an *MLL/KMT2A* rearrangement is often characterized by absence of CD10 and expression of CD15. This leukemia subtype may be particularly difficult to identify using the described protocol, as this subtype is often negative for CD24 (Schwartz et al., 2003). Further complicating detection of this leukemia subtype is the finding that *MLL/KMT2A* rearranged B-LL may have decreased intensity CD22 expression (Shah et al., 2015). When looking for residual disease in patients with *MLL/KMT2A* rearranged B-LL, alternative B cell markers, such as CD79a, may be considered to enrich for residual disease, and in some cases, alternative genetic based methods for MRD detection such as fluorescence in situ hybridization (FISH) or molecular sequencing based methods may be considered.

Expected results

Negative samples—In samples with no evidence of residual disease taken from patients who have not received prior anti CD19 therapy, the B cell enriching gate defined by expression of CD22 or CD24 without CD66b will show the full spectrum of B cell

maturation from early hematogones to mature B cells (Figure 2). Plasma cells are not included using this gating strategy. Post anti CD19 therapy, populations that express CD19 are depleted. CD19 negative populations positive for CD22 or CD24 without CD66b will be relatively increased in this setting (Figure 4). These populations include dendritic cells, basophils, and some CD34 positive progenitor populations. Recognition of such population is critical to avoid misidentifying normal populations as residual disease. When reporting a negative sample it is desirable to include the LOD and LOQ as determined during the assay validation process.

Positive samples—Samples positive for residual disease will contain abnormal blast populations with an immunophenotype that falls off the spectrum of normal maturation (Figures 3, 5, and 6). A thorough understanding of normal immunophenotypic patterns is critical for recognition of abnormal populations. When an abnormal population is present, the specimen should be called positive, the size of the abnormal population should be commented upon (% abnormal cells/denominator), and the immunophenotype of the abnormal cells should be described. When reporting a percentage of abnormal cells, the denominator used should be stated in the report.

Time considerations

The basic protocol will take less than 90 minutes to complete. Analysis time may vary based on the nature of the specimen.

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Significance statement

The use of targeted therapy is growing in the setting of hematopoietic neoplasms. Identification of residual disease by flow cytometry can be challenging post targeted therapy as there may be overlap between antigens targeted by specific therapies and antigens utilized to identify residual disease or characterize abnormal populations. In this manuscript, we describe a strategy for flow cytometric monitoring for residual disease in patients with B lymphoblastic leukemia post T cell engaging anti CD19 and anti CD22 therapies.

Figure 1. Gating Strategy Post CD19 Directed T cell Engaging Therapy for Cocktails 1 and 2
 A) Begin by displaying all events on a plot of CD45 APC-H7 versus event number (time may be used instead of event number) to assess for stable data acquisition. In this example there is a mild disruption near the end of acquisition (see arrow), possibly due to the tube running dry. The rectangular region may be excluded from further analysis. B) Create a plot of FSC-H versus FSC-A. Singlet events have coordinated FSC-H and FSC-A and are included in the drawn gate, while doublets fall out of this gate and are excluded from further analysis. C) Assess sample for viability/debris. As cells die, they may lose FSC and gain SSC. The drawn gate includes events with optimal viability and excludes debris and many non-viable cells as well as a subset of erythroid precursors. Cells falling out of this region should be evaluated to ensure they are not blasts. Optimal staining will be seen in more viable cells. D and E) This protocol uses expression of CD22 or CD24 without CD66b ("B cell gate") to enrich for normal B cells and for residual leukemic cells. CD22 and CD24 are both expressed on B cells with variable intensity associated with different stages of maturation. Therefore, an "or" gating strategy, including cells with expression of either CD22 or CD24 is utilized to maximize B cell identification. CD66b positive cells are excluded in order to exclude neutrophils (circle), which are CD24 positive. F) This dot plot shows the relationship of CD22 and CD24. Stage one hematogones express brighter CD24 and lower CD22 while mature B cells express brighter CD22 and lower CD24. Changes in expression of CD22 and CD24 seen with normal maturation are represented by the arrow.

Figure 2. Normal populations present in the “B cell gate”

A–F) Plots shown in Figure 2 show cells in the “B cell gate” as defined by expression of CD22 or CD24 without CD66b (as outlined in Figure 1) from a normal marrow sample with a full spectrum of B cells ranging from early hematogones to mature B cells. The arrows present highlight changes in antigen expression with maturation. It should be noted that this “B cell gate” defined by expression of CD22 or CD24 without CD66b includes cells which are not CD19 positive B cells, but that express either CD22 or CD24. Such populations may include some dendritic cell populations and basophils, and as well include CD34 positive progenitor populations (these are discussed further in Figure 6). Many non B cells/non progenitors included in this gate lack expression of CD10 and CD20 and are highlighted by the region shown in plot A. The majority of these populations express CD22 without CD24 and occupy the position highlighted by the circle in plot F.

Figure 3. Residual B-LL in the "B cell gate"

Plots shown in Figure 3 show cells in the "B cell gate" as defined by expression of CD22 or CD24 without CD66b from a sample involved by residual B-LL. The neoplastic population is highlighted in red and expresses both CD22 and CD24. By contrast with normal B cells and precursors typically seen in the marrow (see Figure 2 for comparison), the neoplastic B cells in this case are bright for CD10 and show abnormally decreased expression of CD38 and CD45.

Figure 4. Normal populations present in the “B cell gate” post anti CD19 T cell engaging therapies

Post anti CD19 T cell engaging therapies such as CAR T-19 or blinatumomab, the B cell gate is depleted of normal and abnormal CD19 positive cells. The example shown in this figure is from a patient who is 3 months post CAR T-19 therapy with no evidence of residual disease. All plots show cells in the “B cell gate” as defined by expression of CD22 or CD24 without CD66b. In this setting one will see a relative increase of CD22 positive/CD24 negative cells including both mature populations (orange) and progenitor populations (highlighted in mauve and green). Although the cells present in this case are CD20 negative, the green population may normally show low CD20 expression in some cases. These normal populations should be recognized and distinguished from residual disease.

Figure 5. Gating Strategy Post CD19 and CD22 Directed Therapy for Cocktails 1 and 2

A–E) The patient presented with relapse post CD19 CAR T cell therapy. A) Cells are gated on Singlets and Viable cells according to A–C in Figure 1. CD24 positive and CD66b negative cells are rare. B) CD22 positive and CD66b negative cells are identified but CD22 is dim. C) CD45 versus CD22 allows a cleaner CD22 positive gate. D) Abnormal cells in red are CD19 and CD45 negative. E) Abnormal cells are CD10 bright. F–I) This patient relapsed post CD19 and CD22 CAR T-cell therapy. F) Cells are gated on Singlets and Viable cells according to A–C in Figure 1. CD24 positive and CD66b negative cells are rare. G) CD45 versus CD22 shows a decrease in CD22 expression. H) CD45 versus CD10 allows a cleaner abnormal cell gate. I) Abnormal cells have a spectrum of CD22 expression from dim to negative.

Figure 6. Gating Strategy Post CD19 and CD22 Directed Therapy for Cocktail 3

Patient (same patient as Figure 5) relapsed post CD19 and CD22 CAR T-cell therapy. Cells are gated on Singlets and Viable cells according to A–C in Figure 1. A) Abnormal cell gate is drawn around CD79a positive cells. B) Abnormal cells (shown in red) have a spectrum of CD22 expression from dim to negative. C) Abnormal cells express CD38 and CD15. D) Abnormal cells are CD20 negative.

Table 1

Antibody Panels

	Antibody	Fluorochrome	Clone	Vendor
Cocktail 1	CD10	BV421	HI10a	BD
	CD66b	FITC	80H3	BC
	CD22	PE	eBio4KB128	eBiosciences
	CD34	PerCP Cy5.5	3G12	BD
	CD20	PE-Cy7	B9E9 (HRC20)	BC
	CD38	A594	HB-7	BD
	CD24	APC	ALB9	BC
	CD45	APC-H7	2D1	BD
Cocktail 2	CD10	BV421	HI10a	BD
	CD66b	FITC	80H3	BC
	CD22	PE	S-HCL-1	BD
	CD34	PerCP Cy5.5	3G12	BD
	CD20	PE-Cy7	B9E9 (HRC20)	BC
	CD38	BV510	HB-7	Biolegend
	CD24	APC	ALB9	BC
	CD45	APC-H7	2D1	BD
Cocktail 3	CD15	v450	MMA	BD
	CD20	FITC	B-Ly1	DAKO
	CD22	PE	S-HCL-1	BD
	CD34	PerCP Cy5.5	8G12	BD
	CD19	PE-Cy7	J3-119	BC
	CD38	BV510	HB-7	Biolegend
	CD79a	APC	HM47	BC
	CD45	APC-H7	2D1	BD

Abbreviations: Brilliant violet 421 (BV421), Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Peridinin chlorophyll protein cyanine 5.5 (PerCPCy5.5), Phycoerythrin cyanine-7 (PECy7), Alexa Fluor 594 (A594), Allophycocyanin (APC), Allophycocyanin H7 (APCH7), Brilliant violet 510 (BV510), BD Horizon dye V450 (v450)