Marked Variability in Reported Minimal Residual Disease Lower Level of Detection of 4 Hematolymphoid Neoplasms

A Survey of Participants in the College of American Pathologists Flow Cytometry Proficiency Testing Program

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• ***Context.***—**Flow cytometry is often applied to minimal residual disease (MRD) testing in hematolymphoid neoplasia. Because flow-based MRD tests are developed in the laboratory, testing methodologies and lower levels of detection (LODs) are laboratory dependent.**

Objectives.—**To broadly survey flow cytometry laboratories about MRD testing in laboratories, if performed, including indications and reported LODs.**

Design.—**Voluntary supplemental questions were sent to the 549 laboratories participating in the College of American Pathologists (CAP) FL3-A Survey (Flow Cytometry—Immunophenotypic Characterization of Leukemia/Lymphoma) in the spring of 2014.**

Results.—**A total of 500 laboratories (91%) responded to the supplemental questions as part of the FL3-A Survey by April 2014; of those 500 laboratories, 167 (33%) currently perform MRD for lymphoblastic leukemia, 118 (24%) for myeloid leukemia, 99 (20%) for chronic lymphocytic leukemia, and 91 (18%) for plasma cell myeloma. Other indications include non-Hodgkin lymphoma, hairy cell leukemia, neuroblastoma, and myelodysplastic syndrome. Most responding laboratories that perform MRD for lymphoblastic leukemia reported an LOD of 0.01%. For myeloid leukemia, chronic lymphocytic leukemia, and plasma cell myeloma, most laboratories indicated an LOD of 0.1%. Less than 3% (15 of 500) of laboratories reported LODs of 0.001% for one or more MRD assays performed.**

Conclusions.—**There is major heterogeneity in the reported LODs of MRD testing performed by laboratories subscribing to the CAP FL3-A Survey. To address that heterogeneity, changes to the Flow Cytometry Checklist for the CAP Laboratory Accreditation Program are suggested that will include new requirements that each laboratory (1) document how an MRD assay's LOD is measured, and (2) include the LOD or lower limit of enumeration for flow-based MRD assays in the final diagnostic report.**

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Although multicolor flow cytometry was originally introduced into the field of diagnostic hematopathology as an ancillary or companion diagnostic test, this powerful tool is becoming a primary diagnostic modality, independent of morphologic or even conventional cytogenetic findings.[1] This has been particularly true when flow cytometry is used for rare-event analysis, such as minimal residual disease (MRD) testing.[2–4] Although some may consider MRD testing by flow cytometry an evolving field, its use is widely prevalent. There are numerous publications to support its use in specific disease indications, including acute lymphoblastic leukemia,[5–9] acute myeloid leukemia,[10–14] chronic lymphocytic leukemia,[15–19] and plasma cell myeloma.[20–24]

For a clinician, it is not inherently clear which methodology is used or what the lower level of detection (LOD) is when an "MRD test" is ordered. Depending on the

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indication and practice setting, some laboratory MRD testing is performed using nucleic acid assays (eg, *BCR-ABL* testing), and other MRD testing is performed using flow cytometry; sometimes, both are performed. Of late, the molecular pathology community has been working to standardize the reporting of molecular MRD testing to enhance patient care.[25]

Because flow cytometry technology and methodology are evolving and are compared with potentially more-sensitive molecular techniques, the LOD of flow-based MRD testing continues to improve. Thus, the term *minimal* may be a moving target, which has led some to suggest that we describe MRD as *measurable residual disease*.[26] Too often, clinicians and pathologists lump "MRD" into one category, although there are clear examples indicating major heterogeneity in testing for specific diseases, as seen in a recent publication on a small survey of MRD testing in plasma cell myeloma.[27]

The Diagnostic Immunology Resource Committee acts as the expert scientific and educational resource for the College of American Pathologists (CAP) in diagnostic immunology and flow cytometry. Volunteer members from 4 academic and 2 private, medium-to-large, flow cytometry laboratories oversee the proficiency testing (PT) for hundreds of flow cytometry laboratories enrolled in our surveys. Challenges include both "wet" and "dry" specimens. Because products from patients with acute leukemia, chronic lymphocytic leukemia, and plasma cell myeloma are not readily available through CAP or other commercial sources, the CAP does not provide an MRD PT product. Regardless, the pathologists and technologists comprising the committee recognize the increasing role of MRD in the field of diagnostic hematopathology.

The primary purpose of this study was to examine the prevalence of MRD testing by flow cytometry among the participants of our largest leukemia and lymphoma immunophenotyping survey, which includes sending out wet specimens to hundreds of flow cytometry laboratories 2 times annually. In addition to enumerating the number of flow laboratories performing MRD, we sought to identify the most common indications for MRD testing, as well as the laboratories' reported LODs.

MATERIALS AND METHODS

A supplemental questionnaire was sent out to 549 flow cytometry laboratories participating in the CAP FL3 (Flow Cytometry) Survey. With the FL3 Survey, subscribing flow cytometric laboratories receive "wet" challenges of tumor cells to be processed, stained, and analyzed, and finally, an interpretation is generated. The brief questionnaire (Figure 1) was developed by members of the Diagnostic Immunology Resource Committee, and the questions were included as part of the FL3-A (Immunophenotypic Characterization of Leukemia/Lymphoma) survey mailing in the first part of 2014.

RESULTS

Of the 549 laboratories participating in the FL3-A Survey, 500 laboratories voluntarily responded to the supplemental questions in the survey by April 2014, an overall response rate of 91%. The first question addressed whether the laboratory performed MRD testing or planned to do so in the next 12 months. As shown in Table 1, 164 laboratories (32.8% of respondents) currently perform MRD testing for any indication, presumably on bone marrow samples because marrow is the typical specimen source for this analysis. Twenty-eight laboratories (5.6% of respondents) plan to begin MRD testing in the next 6 months and most laboratories (67.2%; 336 of 500) do not perform MRD testing.

Our questions specifically asked about the most common indications for MRD testing that we see in our own laboratories, including lymphoblastic leukemia, myeloid leukemia, chronic lymphocytic leukemia, and plasma cell myeloma. Possibly because of the extensive study by the Children's Oncology Group,[5] the most commonly performed MRD assay was for lymphoblastic leukemia, which 167 of the 500 laboratories (33.4%) perform (this number may also include laboratories that intend to perform MRD in the next six months) (Table 2). For the other 3 most common indications, there were slightly fewer laboratories, with 118 (23.6%) performing MRD for myeloid leukemia, 99 (19.8%) for chronic lymphocytic leukemia, and 91 (18.2%) for plasma cell myeloma (Table 2). Ten laboratories (0.2%) reported "other," and provided textual responses to those categories, including B-cell and T-cell non-Hodgkin lymphomas (other than chronic lymphocytic leukemia) (4 laboratories; <0.1% of all respondents), hairy cell leukemia (3 laboratories; <0.1%), neuroblastoma (2 laboratories; <0.1%), and myelodysplastic syndrome (1 laboratory; <0.1%).

Because each laboratory uses a laboratory-developed test to detect MRD by flow cytometry, we expected some heterogeneity in the LODs of the MRD assays. Therefore, as part of our survey, we asked each laboratory to approximate what its LOD was for each MRD assay, independent of the number of events acquired, the minimum number of events necessary to interpret an abnormal population compatible with MRD, and the number of antigens studied. Obviously, the number of events collected, the number of "colors" used in the assay, and other technical variables are important in attaining the maximal possible assay LOD. In addition, best practices would suggest that the LOD be measured by dilutional/recovery experiments, rather than being estimated.[28] For the purposes of this study, however, we asked each laboratory to provide its approximate LOD only, without additional data to substantiate the response. Our primary intent was to document each laboratory's interpretation of the definition of *minimal*.

For each of the indications, including lymphoblastic leukemia, myeloid leukemia, chronic lymphocytic leukemia, and plasma cell myeloma, we asked each laboratory to approximate its LOD for each assay as 0.1%, 0.01%, 0.001%, or other. As shown in Figure 2, the most commonly reported LOD was lower, overall, for lymphoblastic leukemia, with 54.4% (87 of 160) of laboratories reporting an LOD of 0.01%. The other 3 MRD assays were more likely to be reported with a higher LOD, and nearly 50% of laboratories selected 0.1% as the LOD for those indications; 15 laboratories or fewer selected 0.001% for one or more of the assays, which represents less than 3% of all laboratories participating in the survey.

Some laboratories reported "other" for the LOD for each category (Figure 2) and responded with a textual comment or number. For lymphoblastic leukemia, the LOD ranged from 0.0002% to 1%—a 5000-fold difference in reported LOD among those laboratories. For myeloid leukemia, chronic lymphocytic leukemia, and plasma cell myeloma, no laboratories reported LODs below 0.001%, but a few laboratories enumerated the LOD as 1%. Again, in all 3 indications, that represents a 1000-fold difference in MRD

1. Does your laboratory perform minimal residual disease (MRD) testing by flow cytometry?
   - ○ 151 Yes
   - ○ 469 We will begin testing within the next 12 months
   - ○ 152 No

   If you answered NO to the above question, STOP here.

2. Which disease(s) do you evaluate for MRD? (Fill all that apply.)
   - ○ 364 Lymphoblastic leukemia
   - ○ 360 Myeloid leukemia
   - ○ 263 Chronic lymphocytic leukemia
   - ○ 426 Plasma cell myeloma
   - ○ 010 Other, specify:_____

3. What is the approximate lower level of detection of your assay?

	(399)	(398)	(401)	(010)
Lymphoblastic leukemia	○ 0.1%	○ 0.01%	○ 0.001%	○ Other, specify: _____
Myeloid leukemia	○ 0.1%	○ 0.01%	○ 0.001%	○ Other, specify: _____
Chronic lymphocytic leukemia	○ 0.1%	○ 0.01%	○ 0.001%	○ Other, specify: _____
Plasma cell myeloma	○ 0.1%	○ 0.01%	○ 0.001%	○ Other, specify: _____
Other:_____	○ 0.1%	○ 0.01%	○ 0.001%	○ Other, specify: _____
Other:_____	○ 0.1%	○ 0.01%	○ 0.001%	○ Other, specify: _____
Other:_____	○ 0.1%	○ 0.01%	○ 0.001%	○ Other, specify: _____

4. If a proficiency testing (PT) program was available for MRD, would your laboratory participate in this Survey?
   - ○ 151 Yes
   - ○ 152 No

Figure 1. *Copy of supplemental questions and answer choices, as listed in the paperwork accompanying the FL3-A Survey (Flow Cytometry—Immunophenotypic Characterization of Leukemia/Lymphoma).*

Table 1. Laboratories Currently Performing Minimal Residual Disease (MRD) Testing

	Total Respondents, No.	Currently Test, No (%)	Will Begin Within 12 Mo, No (%)	Do Not Test, No (%)
MRD	500	164 (32.8)	28 (5.6)	308 (61.6)

LODs among laboratories with the highest and lowest LODs. The results of this survey demonstrate that the definition of *minimal* varies greatly among flow cytometry laboratories.

COMMENT

Although previous, smaller surveys have shown major heterogeneity in flow cytometry–based MRD testing of patients with plasma cell myeloma,[27] this is the first report, to our knowledge, that summarizes the major variability in

Table 2. Assay Type and Number of Laboratories Currently Performing Minimal Residual Disease Analysis, n = 500

Disease (Assay Type)	Laboratories, No. (%)
Lymphoblastic leukemia	167 (33.4)
Myeloid leukemia	118 (23.6)
Chronic lymphocytic leukemia	99 (19.8)
Plasma cell myeloma	91 (18.2)
Other[a]	10 (0.2)

[a] Other includes textual answers including all lymphomas, B cell lymphoma, T cell lymphoma, non-Hodgkin lymphoma, hairy cell leukemia, Sézary syndrome, myelodyplastic syndrome, and acute leukemias.

how an individual flow cytometry laboratory defines MRD analysis in 4 relatively common hematolymphoid neoplasms. Clearly, laboratories are reporting what is thought to be measurable within its own laboratory-developed test; however, it is not clear whether those LODs are correctly interpreted by, or even relayed to, the ordering provider. For example, laboratories reporting a result of less than 0.001% would need to collect a minimum of 5 million events, assuming 50 target events were collected, to allow enumeration of the leukemic population with an acceptable coefficient of variation.[29] The clinician, pathologist, and/or patient may perceive all MRD test results as equivalent, even though some laboratories measure MRD much differently than other laboratories do. As shown in this report, 10-fold to 100-fold differences in LODs among laboratories are common. These differences have the potential to affect a patient's clinical management, so it is necessary to clearly communicate the LOD of the assay to patients and clinicians. Clearly, this will be important in precursor B-lymphoblastic leukemia because studies in children have demonstrated that patients with MRD values greater than 0.01% in bone marrow at the end of induction therapy need to be assigned to high-risk protocols when compared with those patients who have MRD values of less than 0.01%.[30]

In an effort to improve the transparency of an individual laboratory's MRD assay, the CAP Diagnostic Immunology Resource Committee will be working with the Laboratory Accreditation Program to create 2 new checklist items for the 2015 edition of the Flow Cytometry Checklist. Both will be introduced as phase II deficiencies, and both will fall under the category "Rare Event Flow Cytometric Assays." Such assays could include MRD assays, as well as paroxysmal nocturnal hemoglobinuria testing to look for small paroxysmal nocturnal hemoglobinuria–type clones. The first checklist item will be related to flow cytometry methodology and test development. We acknowledge that the number of events collected, the number of "colors," the individual's leukemia/lymphoma/neoplasm–associated immunophenotype, and other variables will ultimately affect the LOD for each laboratory's MRD assay. Moreover, our study did not address variability in processing methods (eg, hypotonic red cell lysis or prestaining or poststaining staining versus enriching for mononuclear cells with centrifugation over a Ficoll gradient). In addition, we note that laboratories use different "denominators," which we did not specifically address—total events versus $CD45^+$ leukocytes versus mononuclear cells (of non-Ficolled samples). However, the CAP will now require that each laboratory have documentation of method validation that demonstrates the LOD or lower limit of enumeration for each of its MRD assays. For an assay such as flow cytometry, where the analyte is cell based, this could be accomplished by performing dilutional studies using known patient samples or other suitable material to find the lowest possible percentage of cells that can be definitively distinguished as an abnormal population from a background of normal cell types comprising blood and bone marrow.[13] The second key component of the new checklist items is to not only to ensure that each laboratory has measured its MRD assays' LODs but also to ensure that the LOD is transparent to the ordering provider. Thus, the second new checklist item will require that, for rare event flow cytometric assays, the LOD or lower limit of enumeration be included in the diagnostic report.

Although these 2 changes to the checklist will improve laboratory-specific and test-specific definitions of MRD, a logical next step to improve testing will be to create PT material to evaluate a laboratory's ability to detect rare events. Indeed, because 73.7% (157 of 213) of respondents in our survey indicated interest in an MRD PT product, if available, there is apparently broad interest in MRD PT. Although there is one pilot program for acute lymphoblastic leukemia MRD testing through the United Kingdom National External Quality Assessment Service, MRD PT products for myeloid leukemia, chronic lymphocytic leukemia, and plasma cell myeloma are not readily available through CAP or other commercial sources. Certainly, development of these products will require significant time and commitment. Undoubtedly, implementation of an MRD PT program will only improve the harmonization of MRD testing by flow cytometry, particularly if the product is based on accuracy and graded. Moreover, the promulgation of consensus recommendations for antibody panels for flow

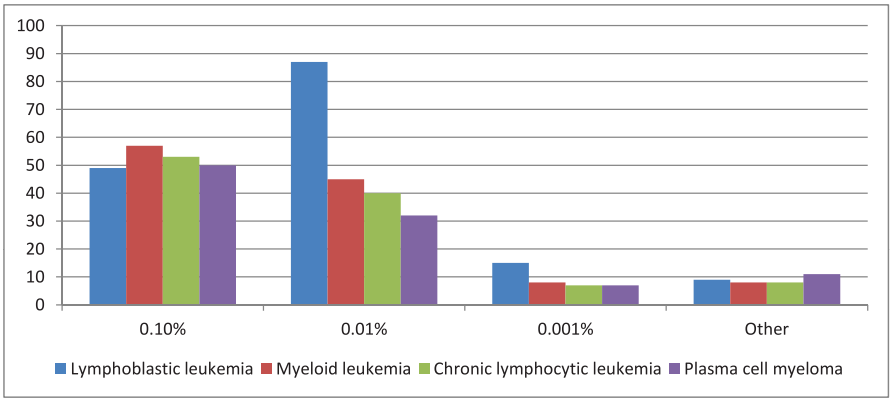

Figure 2. *Reported lower levels of detection by disease type: lymphoblastic leukemia (blue), myeloid leukemia (red), chronic lymphocytic leukemia (green), and plasma cell myeloma (purple). X-axis denotes category response by disease and reported lower level of detection. Y-axis denotes number of laboratories responding in each category.*

cytometry MRD testing should help harmonize both the methodology for MRD testing across laboratories and, simultaneously, help reduce the huge variation in how different flow cytometry laboratories define LODs for MRD by improving the sensitivity of the less-proficient laboratories. In this regard, an initiative is currently under way through the Foundation for the National Institutes of Health to standardize MRD testing in precursor B-cell acute lymphocytic leukemia. Finally, as clinicians, clinical trials, professional societies, and patients set expectations for MRD testing and necessary LODs, clinical laboratories will need to respond to market demands by delivering a product that meets expectations.

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