Enhanced sensitivity of flow cytometry for routine assessment of minimal residual disease

In a recent paper by Béné and Kaeda,[1] technical approaches for minimal residual disease (MRD) assessment are extensively reviewed. PCR-based studies have proved to be 1-log more sensitive than flow cytometry (FC). For this reason, they are increasingly being preferred for MRD analysis, especially at the end of therapy or post hematopoietic stem cell transplantation.[1,2] It would be valuable to develop MRD flow cytometry assays with this level of sensitivity that could be applied routinely. In the present work, we analyzed MRD+ samples with a level of infiltration below the limit of detection of routine FC, which is accepted as 10^{-4} and comes from the standard acquisition of 2-5×10^5 leukocytes.[3-11] At least 10-fold more leukocytes must be acquired to increase sensitivity by 1-log; this large number of leukocytes can be acquired easily in digital cytometers by acquiring several individual tubes stained with the same combination of monoclonal antibodies, and putting them in a single file. Because the time of acquisition for each individual tube is not increased, no problems of cellular aggregation arise.

Using this approach, we acquired 6 million leukocytes from each sample using a FACSCanto flow cytometer (Becton-Dickinson). The stability of fluorescent parameters between tubes was verified (*Online Supplementary Figure S1*). The study was performed with the event rate used routinely in our laboratory, i.e. 2,000 events/sec. To explore the possibility of decreasing acquisition time, we analyzed the influence of increasing the event rate on the percentage of electronic aborts (due mainly to coincidence events), and compared MRD measurements in samples acquired at different event rates (*Online Supplementary Table S1*). We found that the acquisition event rate could be increased reliably to at least 4,000 events/sec. Using this event rate, the time of acquisition of 6 million leukocytes is suitable for routine measurements.

We assessed that this approach enhanced sensitivity by 1-log by comparing MRD analysis by FC with real-time quantitative PCR for BCR-ABL1^{p190} transcripts in serial dilutions of a Philadelphia chromosome-positive B-II acute lymphoblastic leukemia sample (Table 1A). We then selected multiple myeloma (MM) and B-cell chronic lymphocytic leukemia (BCLL) MRD+ samples with about 0.01% infiltration and diluted them 10-fold with normal leukocytes. As shown in Table 1B, MRD was detected accurately in all diluted samples (10^{-5} infiltration). Observed values were as expected ±10% in all cases.

This increase in sensitivity did not compromise the specificity of the technique. Ten million leukocytes from the bone marrow of patients (n = 3) without hematologic neoplasias and from the peripheral blood of healthy donors (n = 3) were acquired and blindly tested for the presence of events with a myelomatous or B-CLL phenotype, respectively. The results were unambiguously negative (*Online Supplementary Figure S2*). We also acquired 10 million normal leukocytes and searched for events with phenotypic characteristics of other hematologic malignancies, such as follicular lymphoma and hairy cell leukemia, with negative results.

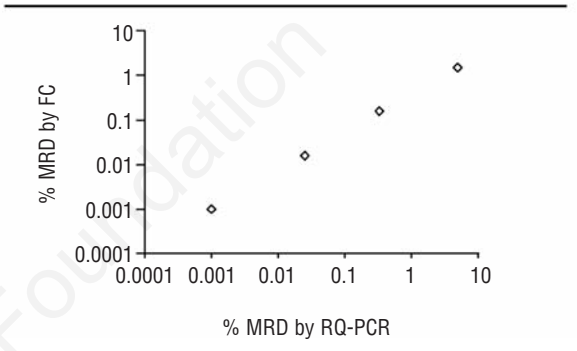
In addition to the total number of leukocytes analyzed, the sensitivity of FC depends on the number of neoplastic events that must be detected in order to obtain precise measurements.

Based on the theory that rare events follow a Poisson distribution, it is accepted that 50-100 events are needed to reliably measure the frequency of a population.[3,7,8,11,12] However, to our knowledge, no studies have really measured the influence of the number of detected tumoral cells on MRD measurement precision. Accordingly, we determined the coefficient of variation (CV) of the percentage of MRD in samples from patients with MM, B-CLL and T-cell lymphoproliferative disorders (T-CLPD) obtained

Table 1. Accuracy of minimal residual disease assessment in samples with infiltration <10^{-4}.

A

	Sample	Sample serially diluted 10-fold →		
% MRD by FC	1.5	0.16	0.016	0.001
% MRD by RQ-PCR	4.9	0.33	0.025	0.001



B

		Sample	Sample diluted 10-fold
Number of acquired leukocytes (millions)		0.5	6
MM 1	MRD (%)	0.0127	0.00127
	# Cluster events	94	54
MM 2	MRD (%)	0.017	0.0017
	# Cluster events	87	100
MM 3	MRD (%)	0.012	0.00118
	# Cluster events	82	87
MM 4	MRD (%)	0.02	0.0018
	# Cluster events	107	99
B-CLL 1	MRD (%)	0.015	0.0014
	# Cluster events	78	114
B-CLL 2	MRD (%)	0.024	0.0022
	# Cluster events	124	181
B-CLL 3	MRD (%)	0.025	0.0024
	# Cluster events	139	151
B-CLL 4	MRD (%)	0.022	0.0024
	# Cluster events	122	157

(A) Minimal residual disease (MRD) levels measured by flow cytometry (FC) or BCRABL1^{p190} transcripts quantitation by real-time quantitative PCR (RQ-PCR) in serial dilutions of a Philadelphia chromosome-positive B-II acute lymphoblastic leukemia bone marrow sample. MRD results by FC are expressed as percentage of total leukocytes. MRD results by RQ-PCR are expressed as BCR-ABL1^{p190} copy number per 100 molecules of GUS. (B) MRD levels measured by FC in samples with 10^{-5} infiltration: MRD+ samples with about 0.01% infiltration from 4 patients with multiple myeloma (MM) and from 4 with B-cell chronic lymphocytic leukemia (B-CLL) were assessed. The percentage of MRD for each sample ("Sample") represents the mean of 5 independent measurements. In each measurement, 0.5 million leukocytes were acquired. These MRD+ samples were diluted 10-fold with normal leukocytes in order to obtain tumoral infiltrations in the range of 10^{-5} ("Sample diluted 10-fold"). The percentage of MRD in diluted samples was measured by acquiring 6 million leukocytes. The number of malignant events detected in each measurement is shown.

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when detecting increasing numbers of events in the malignant cluster. As shown in Table 2, the CVs of the B-CLL and T-CLPD samples were very close to those predicted by the Poisson distribution. Fifty to 60 events were required to obtain a CV less than 15%. Strikingly, in MM samples, the CVs were around 10% regardless of the size of the cluster, even when as few as 20 malignant events were detected (possibly because it is easier to identify malignant cells from MM than from other hematologic malignancies, since myelomatous plasma cells usually occupy a space in which background events are scarce).

In summary, acquiring 6 million leukocytes is feasible with a digital cytometer on a routine basis. Because detection of 50-60 malignant cells is required to get a CV less than 15%, a sensitivity of 1×10^{-5} is achieved. Being able to routinely apply MRD FC assays with high sensitivity would be very valuable, especially in cases where molecular techniques cannot be used.

Table 2. Precision of minimal residual disease assessment according to the number of events in the malignant cluster.

	# Cluster events	MRD (%)	CV (%)
MM			
Case 1	19	0.0127	9
	43	0.013	10
	94	0.0127	10
Case 2	30	0.018	10
	44	0.017	10
	87	0.017	9
Case 3	25	0.012	10
	38	0.011	10
	82	0.012	10
Case 4	39	0.021	8
	54	0.02	10
	107	0.02	10
Case 5	22	0.0027	9
	42	0.003	10
	91	0.003	8
B-CLL			
Case 1	26	0.016	20
	36	0.014	19
	78	0.015	11
Case 2	37	0.023	18
	60	0.023	14
	124	0.024	7
Case 3	37	0.022	13
	55	0.023	9
	139	0.025	10
Case 4	36	0.021	9
	62	0.023	12
	122	0.022	10
Case 5	31	0.049	3
	55	0.051	7
	130	0.05	9
T-CLPD			
Case 1	30	0.04	12
	50	0.038	12
	272	0.04	4
Case 2	25	0.025	20
	47	0.024	13
	79	0.026	11
Case 3	48	0.031	12
	86	0.032	8
	186	0.032	8
Case 4	57	0.031	12
	106	0.031	8
	193	0.03	2

Minimal residual disease (MRD) was measured in samples from 5 patients with multiple myeloma (MM), 5 patients with B-cell chronic lymphocytic leukemia (B-CLL), and 4 patients with T-cell lymphoproliferative disorders (T-CLPD) by detecting three different levels of malignant cells (approximately 20-30, 40-60, and 80-200). Results are presented as the mean of 5 independent trials. The CV was calculated as (standard-deviation/mean) x 100.

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