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Routine Evaluation of Minimal Residual Disease in Myeloma Using Next-Generation Sequencing Clonality Testing

Feasibility, Challenges, and Direct Comparison with High-Sensitivity Flow Cytometry

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The 2016 International Myeloma Working Group consensus recommendations emphasize high-sensitivity methods for minimal residual disease (MRD) detection, treatment response assessment, and prognostication. Next-generation sequencing (NGS) of *IGH* gene rearrangements is highly specific and sensitive, but its description in routine clinical practice and performance comparison with high-sensitivity flow cytometry (hsFC) remain limited. In this large, single-institution study including 438 samples from 251 patients, the use of NGS targeting the *IGH* and *IGK* genes for clonal characterization and monitoring, with comparison to hsFC, is described. The index clone characterization success rate was 93.6% (235/251), which depended on plasma cell (PC) cellularity, reaching 98% when PC $\geq$10% and below 80% when PC $<$5%. A total of 85% of cases were successfully characterized using leader and FR1 primer sets, and most clones showed high somatic hypermutation rates (median, 8.1%). Among monitoring samples from 124 patients, 78.6% (147/187) had detectable disease by NGS. Concordance with hsFC was 92.9% (170/183). Discordant cases encompassed 8 of 124 hsFC MRD+/NGS MRD− patients (6.5%) and 4 of 124 hsFC MRD−/NGS MRD+ patients (3.2%), all with low-level disease near detection limits for both assays. Among concordant hsFC MRD−/NGS MRD− cases, only 5 of 24 patients (20.8%) showed subsequent overt relapse at 3-year follow-up. HsFC and NGS showed similar operational sensitivity, and the choice of test may depend on practical, rather than test performance, considerations. *(J Mol Diagn 2021, 23: 181−199; https://doi.org/10.1016/j.jmoldx.2020.10.015)*

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Plasma cell myeloma (PCM) patients treated by high-dose chemotherapy and/or stem cell transplantation have historically been considered to be in complete response (CR) based on the absence of monoclonal urine and serum paraprotein, and marrow biopsy showing plasma cells (PC) comprising <5% of cellularity.[1] Subsequently, the term *stringent CR* was introduced with additional criteria requiring normalized serum free light chain ratio and lack of demonstrable PC clonality in the marrow by immunohistochemical stains or flow cytometry (FC).[2] Despite achieving CR or stringent CR, a significant proportion of PCM patients relapsed, suggesting persistent low-level disease that was not detected by the evaluation methods utilized. This observation illustrated the need for laboratory methods with high technical sensitivity for minimal residual disease (MRD) detection, for the purposes of prognostic stratification and treatment decisions.[3]

More recently, with further advances in methodology, the 2016 International Myeloma Working Group (IMWG) consensus incorporated multiparametric FC, particularly newer generations of high-sensitivity FC (hsFC), and next-generation sequencing (NGS)-based assays as disease-monitoring modalities[3] in their recommendations, emphasizing that positive MRD status has prognostic significance, even at very low level and among patients who meet criteria for CR or stringent CR.[4,5] HsFC has been demonstrated to achieve a sensitivity of 2×10^{-6} to 6×10^{-6}, depending on the methodology and number of cells analyzed.[6–8] Similarly, NGS-based assays have also shown highly encouraging results in PCM in both research[9–11] and clinical settings[12,13] using various assays. Unfortunately, due to the complexity of establishing such assays in clinical laboratories, the adoption of NGS-based clonality assays has remained limited with scarce data on their performance characteristics and, at this time, no established guidelines for broad deployment in routine clinical practice. In the current study, the authors describe their extended clinical experience using a commercially available NGS-based assay (LymphoTrack; Invivoscribe, Inc., San Diego, CA), which is performed in-house for routine clonal characterization and monitoring of patients with PCM. The authors compare its performance characteristics with their hsFC assay, which provides a technical sensitivity of 1×10^{-6}, similar to the assay recommended by the EuroFlow consortium (Leiden, the Netherlands).[6,8] In particular, the authors focus on the practicality and feasibility of establishing an NGS assay as part of routine clinical practice and describe the pitfalls and benefits of utilizing this technology.

Materials and Methods

Patient and Sample Selection

Patients treated at Memorial Sloan Kettering Cancer Center (MSKCC) with confirmed diagnoses of PCM, and whose clinical samples were submitted for immunoglobulin heavy chain (*IGH*) clonal rearrangement studies by NGS between January 1, 2016, and January 31, 2018, were identified. Cases were excluded if the initial sample received for clonal characterization showed ≤1% PC in the marrow aspirate, as this would preclude accurate clonotyping. When available, archival samples (from as early as July 2010) with higher disease involvement were also utilized to facilitate characterization of the index clones. Characterization samples with a concurrent mature or immature B- or T-cell neoplasm(s) were also excluded. Genomic DNA was extracted using standard protocols. For initial characterization, marrow aspirates in EDTA, aspirate smear slides, and formalin-fixed, paraffin-embedded tissue without decalcification were used. For monitoring samples, only marrow aspirates in EDTA were tested. Relevant patient data, including pathology report information on CD138 quantitation of PC and aspirate differential cell count, and IMWG response status[3] were also gathered. The study was approved by the local institutional review board and was performed in accordance with the Declaration of Helsinki.

IGH Clonal Rearrangement by Capillary Electrophoresis–Based Assay

Routine *IGH* clonal rearrangement studies were initially performed using commercial BIOMED-2 multiplex PCR master mixes and controls (*IGH* clonality assay tubes A, B, and C, corresponding to primers targeting the FR1, FR2, and FR3 regions; Invivoscribe, Inc.) following the manufacturer's protocols. The fluorescently labelled PCR products were separated and analyzed based on fragment length by capillary electrophoresis on an ABI 3730 DNA analyzer (Thermo Fisher Scientific, Waltham, MA). Results were interpreted according to the 2012 EuroClonality/BIOMED-2 guidelines for interpretation and reporting Ig/T-cell receptor clonality testing in suspected lymphoproliferations.[14]

Initial *IGH* Clonal Characterization by the NGS-Based Assay

Initial characterization of the disease-associated clone (index clone) was performed using commercially available primer sets targeting various regions of the *IGH* gene (LymphoTrack assay; Invivoscribe, Inc.) as previously described[15] (standard DNA input of 250 ng). Primer sets were used in sequential order: FR1, leader (conserved sequence upstream of FR1), FR2, and FR3, until at least one clonal sequence could be confidently characterized (Supplemental Figure S1). Selected cases without a detectable index clonal sequence by *IGH*-targeted testing were also tested using immunoglobulin light chain kappa (*IGK*) primers. The primer sets contain barcoded sequence adaptors, allowing demultiplexing of reads after sequencing on Illumina MiSeq instruments (Illumina, San Diego, CA). Sequences were analyzed using the LymphoTrack MiSeq software version v.2.3.1 (Invivoscribe), as well as an

analysis pipeline developed at MSKCC as described previously.[16] The criteria for clonality calling have been described in detail by the authors' group.[15] In a case with optimal total sequencing reads (>100,000), a clone was considered unequivocal if present at a frequency of 2.5% of the total *IGH* reads and at least 10 times higher than the polyclonal background. A characterization sample was considered test failure if the number of total sequencing reads was <30,000, and suboptimal if the number of reads was 30,000 to 100,000.

Detection of Index Clonal Sequences in Monitoring Samples

Assessment of monitoring samples followed similar methods as described above. However, testing was performed in duplicate reactions using a total of 1000- to 1500-ng DNA input to warrant a sensitivity of at least 1×10^{-5}. To streamline testing, cases with overt residual disease based on morphologic examination (PC ≥5% marrow cellularity) were run as singletons. Furthermore, in samples with very low-level or no residual disease by hsFC, if the index clone was not detected in the duplicates, additional replicates were run to confirm the negative status. Testing was done using only the primer sets that successfully characterized the index clone. To avoid cross-contamination during sequencing, characterization and monitoring samples from the same patient were sequenced in different runs, using different barcode indexes, on different Illumina MiSeq instruments whenever possible. If the same instrument was used, a rotating instrument schedule was instituted so samples from the same patient would not be sequenced within 3 runs of each other. To further minimize potential carryover contamination from the characterization to monitoring samples, an Illumina Template Line Wash with bleach was conducted after each MiSeq run. A no-template control and low-level positive MRD controls were also included in every run.

Quantitation of the Disease-Associated Index Clone and Normalization

Quantitation of the index clonal sequences was initially performed based on a percentage of the total *IGH* sequencing reads in the sample. Normalization using a commercially available spike-in control (LymphoQuant; Invivoscribe, Inc.) was further instituted to re-evaluate a subset of cases and estimate the proportion of the clone in the total sample. Spike-in DNA equivalent to 100 clonal cells was added to 700 ng of patient DNA (approximately 100,000 cell equivalent), before the sample underwent PCR amplification and sequencing (each case tested in duplicate). The proportion of the clone was calculated as a percent of cell equivalents for the sample and the percent of the clone in total sample using the following formulas:

Estimated # of cell equivalent for the sample = (% reads for patient's index clonal sequence/% reads for LymphoQuant) × 100 cells.

% of DNA from index clone in total DNA = (# of cell equivalent/100,000 cells) × 100%.

IGH Sequence Analysis

After demultiplexing, analysis was performed using the LymphoTrack MiSeq software version v.2.3.1 (Invivoscribe, Inc.) to determine the index clone. For monitoring samples, the LymphoTrack MRD data analysis tool version v.1.2.0 was used for analysis, querying for the previously characterized index clonal sequences as an exact match and up to 2-bp mismatches (>99% sequence homology). For cases without overt morphologic disease (<5% aspirate PC and/or lack of abnormal PC by hsFC), the results were considered positive only if the index clones were detectable in at least two separate replicates. If the index clone was found in only one replicate, the test was repeated to confirm results. Data from the run were considered invalid if either the % cluster passing filter or the % base calls above Q30 (%>Q30) were below 75% and 70% for 2×250 and 2×300 cycles, respectively. A monitoring sample was considered test failure if the number of total sequencing reads was <50,000 and suboptimal (qualified reports) if the diagnostic clone was not detected, and the number of reads was below 200,000.

Monitoring Assay Sensitivity and Reproducibility

Assay sensitivity and reproducibility were monitored through the use of a well-characterized MRD control created as a dilution of IVS-0019—positive control into IVS-0000 (Invivoscribe, Inc.) for detection of three unique clonal sequences at approximately 3×10^{-4}, 1×10^{-5}, and 1×10^{-6}. These were sequentially tracked in each run and analyzed in a similar fashion as the clinical samples.

hsFC Analysis

hsFC analysis was performed on fresh samples using the MSKCC single 10-color tube assay as previously described.[6,8] For plasma cell neoplasm MRD detection, a target acquisition of 3 to 6 million cells was used to achieve a detection sensitivity up to 1×10^{-6}.

Data Analysis

Logistic regression, test of equal or given proportions, Pearson correlation coefficient, and χ^2 goodness-of-fit test were performed using R software version v.3.4.3 (The R Foundation; *https://www.r-project.org*).

Table 1 Characteristics of All the Characterization and Monitoring Samples in This Study

		Characterization samples (n = 251)	Monitoring samples (n = 187)
Number of unique patients		251	124
Total sequencing reads	Median	503,187	520,249
	Range	521–2,294,084	613–3,086,324
Plasma cell by aspirate differential, %	Median	16	2
	Range	1–95	0–64
Plasma cell by CD138 immunostain, % cellularity	Median	30	<5
	Range	<5–95	<5–90
Abnormal plasma cell by flow cytometry, % of WBC	Median	1.60	0.022
	Range	0.0020–64.50	0.0–90.0
Abnormal plasma cell by flow cytometry, % of total plasma cells	Median	98.1	30.15
	Range	2.8–100.0	0.0–100.0

WBC, white blood cells.

Results

Clonal Characterization Success Rate by NGS Assay

In total, 251 PCM patients were included in the study with patient and sample characteristics as summarized in Table 1. Overall, index clonal sequences were successfully established in 93.6% (235/251) of all cases. The success rate correlated with the proportion of PC in the aspirate smears (Figure 1 and Supplemental Tables S1 and S2). Among cases with <5% PC, clonal characterization was successful in 79.4% of cases (27/34), compared with 95.6% (196/205) in those with ≥5% PC (P = 0.0018), and 98.1% in those with ≥10% PC. All clonality calls were concordant with the initial screening by the capillary electrophoresis–based *IGH* clonality assay. All cases without a characterizable index clone by the NGS showed polyclonal patterns by the capillary electrophoresis–based *IGH* clonality assay. These included two cases exhibiting

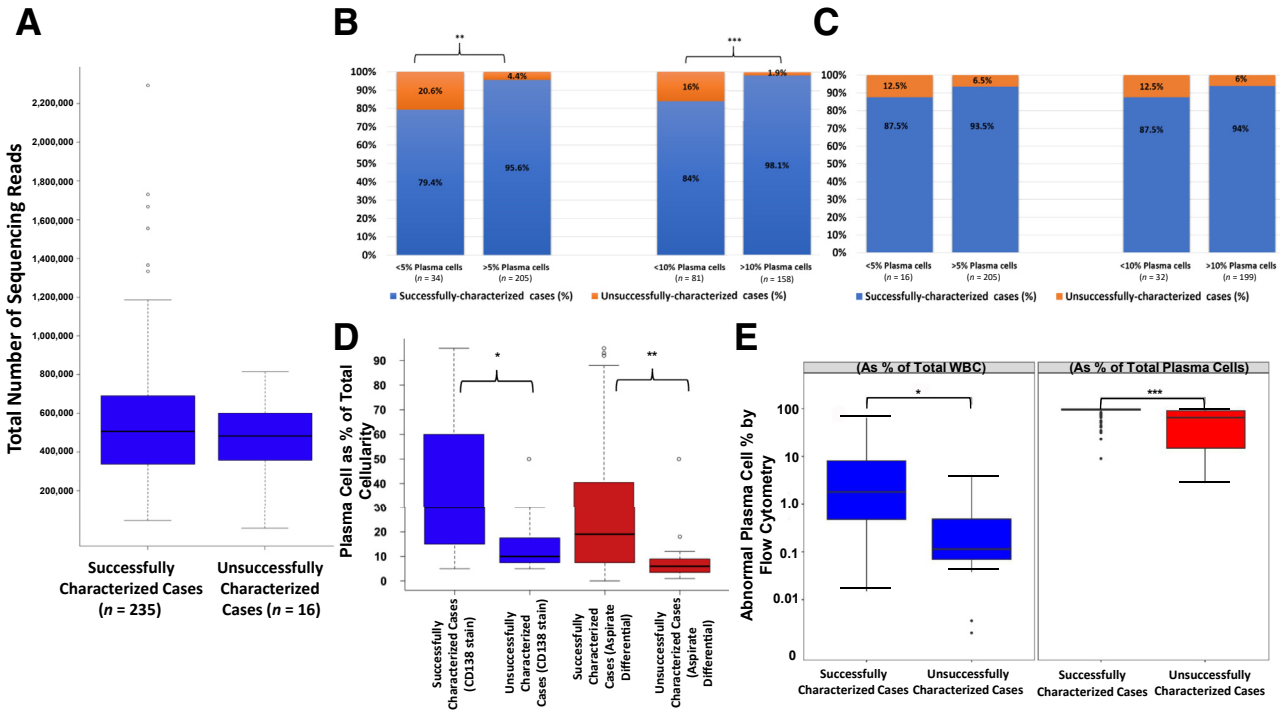


Figure 1 Comparison of characterization samples with successfully and unsuccessfully characterized index clonal sequences by the next-generation sequencing (NGS) assay: **A:** Total *IGH* sequencing reads. **B:** Comparison of success rates among cases with <5% and ≥5% plasma cells (PC), as well as <10% and ≥10% PC, by aspirate differential count. **C:** Comparison of success rates among cases with <5% and ≥5% PC, as well as <10% and ≥10% PC, by CD138 immunostain quantitation performed on core biopsies. **D:** Comparison of PC quantitation by CD138 immunostaining and by aspirate differential count. **E:** Comparison of abnormal PC quantitation by flow cytometry, as % of total white blood cells (WBC), and as % of total PC in the samples. In **A**, **D**, and **E**, the **colored boxes** represent values between the 25th and 75th percentiles, whereas the **horizontal line** within the **boxes** represents the median value. *P < 0.05, **P < 0.01, and ***P < 0.001.

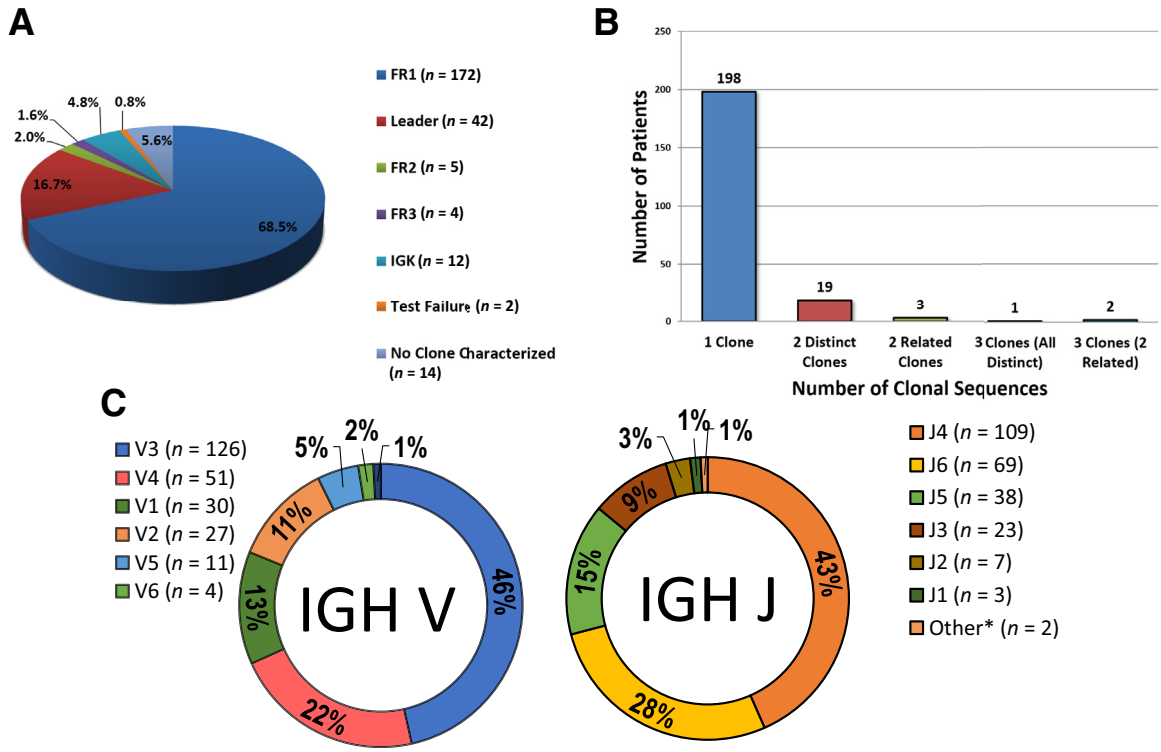


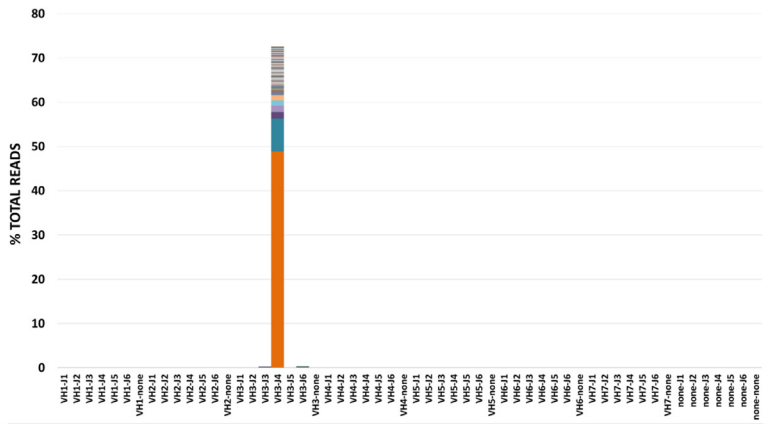
Figure 2 Characteristics of the index clonal sequences by the next-generation sequencing (NGS) assay: **A:** The proportion of total cases with clones successfully characterized by each primer sets, using a sequential testing approach as described in Supplemental Figure S1. The majority of cases can be characterized by a combination of *IGH* FR1 and leader primer sets. **B:** Number of distinct clones detected by *IGH* primers (leader, FR1, FR2, FR3 only). Note that in cases where two or three distinct clones were detected, the clones can be related and have the same IGH V-J usage. **C:** *IGH* V and J family usage among all the clones characterized by *IGH* primers. **Asterisk:** In two cases, the *IGH* J family usage cannot be reliably determined, likely due to significant mutations, insertion, or deletion of nucleotides in the rearranged clonal *IGH* sequence. $n = 251$ total cases (**A**); $n = 223$ clones (**B** and **C**). Y/N, yes/no.

prominent sequences by NGS, but below the established threshold for confident clonal calling, as well as two test failures due to low number of sequencing reads, likely related to paucity of B and plasma cells in the samples.

Most cases (85.3%, 214/251) were successfully characterized by a combination of FR1 (68.5%, 172/251) and leader primer sets (16.7%, 42/251) (Figure 2A). The median % of *IGH* sequencing reads supporting a clone was 24.40% (range, 2.50% to 90.50%) (Supplemental Table S2). Of the 223 cases characterized by *IGH* primers, 198 (88.8%) showed a single distinct clonal sequence, 19 (8.5%) showed two unrelated clonal sequences (likely biallelic) with different *IGH* V-J gene segment usages, whereas one (0.4%) showed three unrelated clonal sequences (Figure 2B). In very rare cases, significant clonal heterogeneity was detected, as evidenced by the presence of several highly related sequences with identical/very similar PCR product sizes and identical *IGH* V-J segment usage but varying in sequence by $\geq$3 bp (Figures 3 and 4, and Tables 2 and 3). Significant bias in V segment usage ($P = 2.2 \times 10^{-16}$), and V-J segment combination ($P = 2.67 \times 10^{-10}$) was noted as summarized in Figure 2C. Compared with the reference germline *IGH* sequence, most index clones showed high somatic hypermutation (SHM) rates with a median of 8.1% (range, 0.0% to 29.0%). Twelve patients (4.8%, 12/251 patients) had clonal sequences that were only detectable by *IGK* primers (Figure 2A). All clonal sequences detected were confirmed to be unique to each patient through routine quality control checks performed across all patients characterized with this assay at the authors' institution.

Disease Monitoring Samples and Concordance Rate with Flow Cytometry

A total of 187 monitoring samples (from 124 unique patients) was studied (Supplemental Figures S2 and S3), and the median interval between the characterization and monitoring samples was 9.5 months (range, 0.5 to 85.8 months). Sample characteristics are summarized in Table 1. The range of DNA input for the monitoring samples was 85.5 ng to 5000 ng, with a median of 1000 ng. The variability in total DNA input was due to two main reasons: A small proportion of samples show marked hemodilution and/or hypocellularity, limiting DNA input. At the same time, repeat replicates were done in a subset of cases, when the initial replicates showed suboptimal sequencing reads or equivocal results. The index clonal sequences were detectable in 78.6% of cases (147/187), ranging



Rank	Sequence	Length	IGH V-gene	IGH J-gene	% total reads	Mutation rate (%)	In-frame (Y/N)	No Stop codon (Y/N)
1	GCCTCTG(	281	V3-30-3_01	J4_02	60.9	5.8	Y	Y
2	GCCTCTG(	281	V3-30-3_01	J4_03	7.9	8.4	Y	Y
3	GCCTCTG(	281	V3-30-3_01	J4_04	2.7	7.6	Y	Y
4	GCCTCTG(	281	V3-30-3_01	J4_05	1.5	7.1	Y	Y
5	GCCTCTG(	281	V3-30-3_01	J4_06	1.5	9.3	Y	Y
6	GCCTCTG(	281	V3-30-3_01	J4_07	0.1	7.6	Y	Y
7	GCGTCTG(	165	V3-33_02	J4_02	0.1	5.3	N	N
8	GCCTCTG(	272	V3-30-3_01	J4_02	0.0	6.2	Y	Y
9	GCCTCTG(	284	V3-9_01	J6_02	0.0	0.0	Y	Y
10	ACCTCTG(	272	V3-7_01	J4_02	0.0	6.6	Y	Y

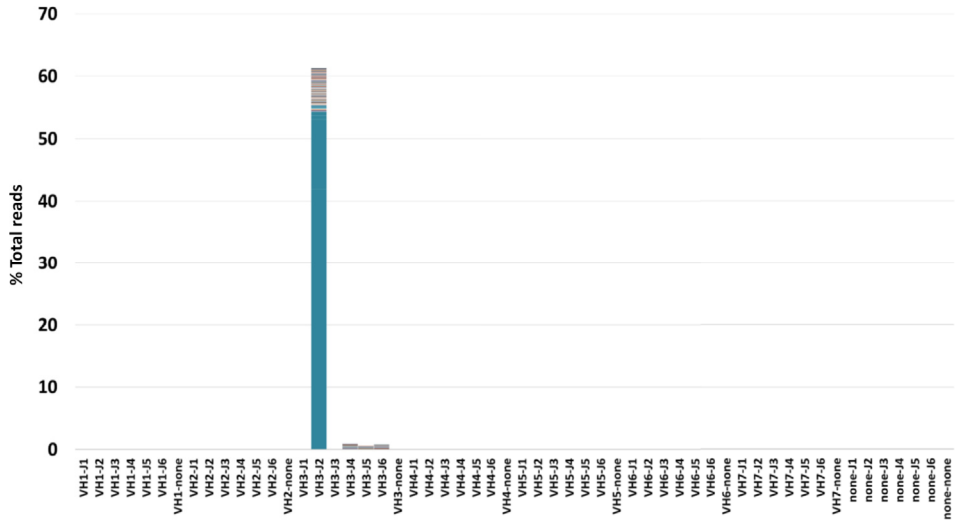
Figure 3 Examples of initial characterization of plasma cell myeloma index clonal sequence for a case with clonal heterogeneity. **Bottom panel:** After merging of sequencing reads within 2-bp differences, the sequences are ranked in descending order of % of total *IGH* sequencing reads. Sequences corresponding to index clones are highlighted in yellow. A case is considered to have clonal heterogeneity if there are multiple sequences with identical length of PCR products and *IGH* V-J gene usages, and differences of ≥3 bp from each other. **Top panel:** The sequences are grouped by *IGH* V-J gene usages, with each color representing a unique sequence after merging. **Right panel:** A comparison of the top five sequences that illustrate clonal heterogeneity is also shown. An **asterisk** represents an identical nucleotide across all five sequences, whereas a blank space or **dot** represents a difference in a nucleotide in at least one of the five sequences. Table 2 provides further information on this case. Y/N, yes/no.

from 0.00056% to 95% of the total sequencing reads (median, 3.7%). Three cases were considered test failures due to low sequencing reads (<50,000) and corresponded to cases with no or very low % of PC based on morphology and concurrent hsFC. Excluding the three failure cases, no significant differences were noted in the number of sequencing reads ($P = 0.35$) between samples with and without a detectable index clone (Figure 5A and Supplemental Tables S3 and S4), but the two groups showed significant difference in plasma cell content (Figure 5B and C). All cases retained the same index sequences compared with the diagnostic samples (>99% homology). No new productive clonal sequence was found in any of the monitoring samples.

Concurrent hsFC and NGS analyses were available for 183 monitoring samples, showing 92.9% concordance (170/183) between the two assays (Figure 6). The median number of cells collected for flow cytometry analysis was approximately 6 million cells/sample, with a range of approximately 2 to 7 million cells/sample. In all discordant cases, evidence of the index clonal sequence or an abnormal PC population was identified at very low level by the NGS assay and hsFC, respectively, near the limit of detection for both assays. In 9 of 183 cases (4.9%), a small abnormal PC population was detected by hsFC (median, 0.00095% of total WBC; range, 0.00034% to 0.052%), but the index clone was not detectable by NGS despite optimal sequencing reads (median, 823,969; range, 208,756 to 2,359,851). These included two cases with PC detected below the lower limit for accurate quantitation by hsFC (<20 events). In five of these cases, sufficient DNA was available for repeat testing with higher DNA input, which still failed to detect the index clones in any of the cases. Conversely, the NGS assay detected the index clone in 4 of 183 cases (2.2%) (range, 0.0013% to 0.26% of the *IGH* sequencing reads), but no abnormal PCs were detected by hsFC despite acquisition of over 3 million cells for analysis in each case, the minimum number of cells necessary for optimal MRD analysis. Subsequent monitoring samples from the four hsFC−/NGS+ cases confirmed the presence of abnormal PC by hsFC in one case (0.00049% of total WBC), whereas the other three cases either have no subsequent monitoring sample by hsFC, or hsFC remained negative.

Conversion of Disease Quantitation from % of Total Sequencing Reads to % of Marrow Cellularity

Based on the LymphoTrack analysis software output, the quantitation of index clonal sequences is expressed as a %



Rank	Sequence	Length	V-gene	J-gene	% total reads	Mutation rate (%)	In-frame (Y/N)	No Stop codon (Y/N)
1	GCCTCTG(	290	V3-7_01	J2_01	63.0	5.3	Y	Y
2	GCCTCTG(	275	V3-23_03	J4_02	0.0	1.8	Y	Y
3	GCCTCTG(	245	V3-20_01	J4_02	0.0	1.8	n/a	N
4	GCCTCTG(	272	V3-7_01	J5_02	0.0	0.4	Y	Y
5	GCCTCTG(	240	V3-9_01	none	0.0	0.4	n/a	N
6	GCCTCTG(	274	V3-33_06	J4_02	0.0	0.4	n/a	N
7	GCCTCTG(	263	V3-48_01	J5_02	0.0	0.0	Y	Y
8	GCCTCTG(	274	V3-33_06	J4_02	0.0	0.4	n/a	N
9	GCCTCTG(	299	V3-33_06	J6_02	0.0	0.4	Y	Y
10	GCCTCTG(	272	V3-7_01	J5_02	0.0	8.4	Y	Y

Figure 4 Examples of initial characterization of plasma cell myeloma index clonal sequence for a case without clonal heterogeneity. **Bottom panel:** After merging of sequencing reads within 2-bp differences, the sequences are ranked in descending order of % of total *IGH* sequencing reads. The sequence corresponding to an index clone is highlighted in yellow. **Top panel:** The sequences are grouped by *IGH* V-J gene usages, with each color representing a unique sequence after merging. Table 3 provides further information on this case. n/a, not available; Y/N, yes/no.

of total sequencing reads, corresponding to only those cells with rearranged *IGH* gene (B and plasma cells in the samples). Therefore, to provide more clinically meaningful assessment of the level of residual disease, retesting was performed in a subset of cases with sufficient material (n = 30) using a spike in control for normalization. Results are summarized in Supplemental Figure S4.

Monitoring Assay Sensitivity and Reproducibility

Figure 7 summarizes the findings of the limit of detection control (MRD control) sequenced in each clinical run, across 231 sequencing pools. The results show that the clones expected at 3×10^{-4} to 4×10^{-4}, 1×10^{-5}, and 1×10^{-6} are appropriately detected in 100%, 99.57% and 71.86%, respectively, confirming the assay shows highly reproducible technical sensitivity down to 1×10^{-5}. Although the index clonal sequence below 1×10^{-5} could still be detected by the assay, the result was not consistently reproducible.

MRD Negativity and Subsequent Risk of Relapse

Overall, monitoring samples from 24 of 124 unique patients (19.4%), corresponding to a total of 28 of 187 samples showed MRD negativity by both the hsFC and NGS assays (hsFC MRD−/NGS MRD−), whereas monitoring samples from an additional 12 of 124 unique patients (9.6%), corresponding to a total of 14 of 187 samples showed MRD negativity by one of the two assays (Figure 6). Of the 24 patients with concordant hsFC MRD−/NGS MRD− results, with a median follow-up time of 36.3 months, only 5 of 24 patients (20.8%) showed subsequent overt evidence of relapse, with a median progression-free survival of 8.4 months and time to next treatment of 11.6 months from the time of MRD studies. The remaining 19 patients mostly maintained the same or better IMWG response criteria. Of the 8 patients with discordant hsFC MRD+/NGS MRD− status, with a median follow-up time of 32.5 months, only 1 of 8 patients (12.5%) showed subsequent overt relapse, with a progression-free survival of 1.4 months, and time to next

Table 2 NGS Clonality Assay Data Output for Initial Clonal Characterization of a Case with Clonal Heterogeneity, after Merging of Sequencing Reads within 2-bp Differences

Total sequencing reads: 490,484

Rank	Sequence	Length, bp	Merge count	V gene	J gene	% Total reads	Cumulative %	Mutation rate to V gene, %	In-frame, Y/N	No stop codon, Y/N	V coverage
1	5′-GCCTCTGGATTCACCTT CAGTAACTATGCTATGCA GTGGGTCCGCCAGGCTCC AGGCAAGGGGCTGGAGT GGTTGGCATTTATATCA GATGATGGAAGCGTTAA ATACTACGCAGACTCCG TGAACGGCCGATTCACC ATCTCCAGAGACAATTT CAAGAATACTCTGTAT CTGCAAATGAACAGC CTGAGAGCTGAGGAGAC GGCTGTGTATTACTGTG TGAGAGATCTATTAA GGGTCTACTCGGCTCTG GACTTCGACTACTGGG GCCAGGGAACCCT-3′	**281**	**298,510**	**IGHV3-30-3_01**	**IGHJ4_02**	**60.86**	**60.86**	**5.78,**		**Y**	**100.00**
2	5′-GCCTCTGGATTCAG CTTCAAAAACTATGC AATGCACTGGGTCCG CCAGGCTCCAGGCAA GGGGCTGGAGTGGCT GACATTCATATCAGAT GATGGAAGCAGTGTAT ACTACGCAGACTCCGT GAAGGGCCGAATCACC ATCTCCAGAGACAATT CCAAGAGCACGCTGTA TCTGCACCTGAACAGC CTGAGAGCTGAGGACA CGGCTGTCTATTACTG TGTGAGAGATCTACTA AGAGTCTACTCGCCTC TGGACTTTGACTACTG GGGCCAGGGAACCCT-3′	**281**	**38,900**	**IGHV3-30-3_01**	**IGHJ4_02**	**7.93**	**68.79**	**8.44**	**Y**	**Y**	**100.00**
3	5′-GCCTCTGGATTCAG CTTCAAAAACTATGC TATGCACTGGGTCCG CCAGGCTCCAGGCAA GGGGCTGGAGTGGCT GACTTTCATATCAGA TGATGGAAGCAGTGT ATACTACGCAGACTC CGTGAAGGGCCGATT CACCATCTCCAGAGA CAATTCCAAGAGCAC GCTGTATCTGCAACT GAACAGCCTGAGAGC TGAGGACACGGCTGT CTATTACTGTGTGAG AGATCTACTAAGAGT	**281**	**13,430**	**IGHV3-30-3_01**	**IGHJ4_02**	**2.74**	**71.53**	**7.56**	**Y**	**Y**	**100.00**

(*table continues*)

Table 2 (*continued*)

Total sequencing reads: 490,484

Rank	Sequence	Length, bp	Merge count	V gene	J gene	% Total reads	Cumulative %	Mutation rate to V gene, %	In-frame, Y/N	No stop codon, Y/N	V coverage
	CTACTCGCCTCTGGA										
	CTTTGACTACTGGGG										
	CCAGGGAACCCT-3′										
4	5′-GCCTCTGGATTCAGCT	**281**	**7229**	**IGHV3-30-3_01**	**IGHJ4_02**	**1.47**	**73.00**	**7.11**	**Y**	**Y**	**97.78**
	TCAAAAACTATGCAATGC										
	ACTGGGTCCGCCAGGCT										
	CCAGGCAAGGGGCTGGAG										
	TGGCTGACATTCATATCA										
	GATGATGGAAGCAGTGTA										
	TACTACGCAGACTCCGTG										
	AAGGGCCGATTCACCATC										
	TCCAGAGACAATTCCAAG										
	AGCACGCTGTATCTGCA										
	ACTGAACAGCCTGAGAGC										
	TGAGGACACGGCTGTCTA										
	TTACTGTGTTAGAGATCT										
	ACTAAGAGTCTACTCGCC										
	TCTGGACTTTGACTACTG										
	GGGCCAGGGAACCCT-3′										
5	5′-GCCTCTGGATTTAGTT	**281**	**7173**	**IGHV3-30-3_01**	**IGHJ4_02**	**1.46**	**74.47**	**9.33**	**Y**	**Y**	**100.00**
	TCAAAAACTATGCTATG										
	CACTGGGTCCGCCAGGC										
	TCCAGGCAAGGGGCTGG										
	AGTGGCTGACTTTCATAT										
	CAGATGATGGAAGCAGTG										
	TATACTACGCAGACTC										
	CGTGAAGGGCCGATT										
	CACCATCTCCAGAGA										
	CAATTCCAAGAGCAC										
	ACTATATCTGCAACT										
	GAACAGCCTGAGAGC										
	TGAGGACACGGCTGT										
	CTATTACTGTGTGAG										
	AGATCTACTAAGAGT										
	CTACTCGCCTCTGGA										
	CTTTGACTACTGGGG										
	CCAGGGAACTCT-3′										
6	5′-GCCTCTGGATTCAG	**281**	**402**	**IGHV3-30-3_01**	**IGHJ4_02**	**0.08**	**74.55**	**7.56**	**Y**	**Y**	**97.78**
	CTTCAAAAACTATGC										
	AATGCACTGGGTCCG										
	CCAGGCTCCAGGCAA										
	GGGGCTGGAGTGGCT										
	GACATTCATATCAGA										
	TGATGGAAGCAGTGT										
	ATACTACGCAGACTC										
	CGTGAAGGGCCGATT										
	CACCATCTCCAGAGA										
	CAATTCCAAGAGCAC										
	GCTGTATCTGCAACT										
	GAACAGCCTGAGAGC										
	TGAGGACACGGCTGT										

(*table continues*)

Table 2 (*continued*)

Total sequencing reads: 490,484

Rank	Sequence	Length, bp	Merge count	V gene	J gene	% Total reads	Cumulative %	Mutation rate to V gene, %	In-frame, Y/N	No stop codon, Y/N	V coverage
	CTATTATTGTGTTCG AGATCTACTAAGACT CTACTCGCCTCTGGA CTTTGACTACTGGGG CCAGGGAACCCT-3′										
7	5′-GCGTCTGGAAGGGC CGATTCGCCATCTCC AGAGACAATTCCATG AACACGGTGTTTGTG CAAATGCGCAGCCTG AGAGCCGGGGGCACG ACTGCATTTTACTGT GCGAGAGAGGGCCCC TTCATCATGATGGTAG TGACACTGACTTCTGG GGCCAGGGAACCCT-3′	165	259	IGHV3-33_02	IGHJ4_02	0.05	74.60	5.29	N	N	46.26
8	5′-GCCTCTGGATTCAT CTTCAGTAATTATGC TATGCACTGGGTCC GCCAGGCTCCAGGCAA GGGGCTGGAGTGGGT GGCAATTGTGTCAT TTGATGGAAACAAT AAATACTACGCAG ACTCCGTGAAGGGC CGATTCACCATCTC CAGAGACAGTTCCA AGAACACAGTGTAT CTGCAGATGTACAG CCTGAGAGTTGAGG ACACGGCTGTGTAT TACTGTGCGAGAGAT CCTTCAATGAGGGTG ACTGTGGACTACTGG GGCCAGGGAACCCT-3′	272	213	IGHV3-30-3_01	IGHJ4_02	0.04	74.64	6.22	Y	Y	100.00
9	5′-GCCTCTGGATTCA CCTTTGATGATTAT GCCATGCACTGGGT CCGGCAAGCTCCA GGGAAGGGCCTGG AGTGGGTCTCAGG TATTAGTTGGAATA GTGGTAGCATAGGC TATGCGGACTCTGT GAAGGGCCGATTCA CCATCTCCAGAGACA ACGCCAAGAACTCCC TGTATCTGCAAATGA ACAGTCTGAGAGCTG AGGACACGGCCTTGT ATTACTGTGCAAAAG	284	194	IGHV3-9_01	IGHJ6_02	0.04	74.68	0.00	Y	Y	100.00

(*table continues*)

Table 2 (*continued*)

Total sequencing reads: 490,484											
Rank	Sequence	Length, bp	Merge count	V gene	J gene	% Total reads	Cumulative %	Mutation rate to V gene, %	In-frame, Y/N	No stop codon, Y/N	V coverage
	ATATGGAGTGGACC										
	CAGGACTACTACTAC										
	GGTATGGACGTCTGG										
	GGCCAAGGGACCAC-3′										
10	5′-ACCTCTGGATT	272	194	IGHV3-7_01	IGHJ4_02	0.04	74.72	6.61	Y	Y	99.56
	CACCTTTAGTTACT										
	ATTCAATGACCTGG										
	GTCCGCCAGGCTC										
	CAGGGAAGGGGCT										
	GGAGTGCGTGGCC										
	AATATAAAGCAAGA										
	TGGAAGTGGGGAAA										
	ACTATGTGGACTCT										
	GTGAAGGGCCGATT										
	CACCATCTCCAGAGA										
	CAACGCCAAGGAGTCA										
	CTGTATCTGCAAATGA										
	ACAGCCTGAGAGTCGA										
	AGACACGGCTGTATAT										
	TACTGTGCGAGAGATCG										
	CCTAGTAGCGGGGGAC										
	TTTGACTACTGGGG										
	CCAGGGAATTGT-3′										

Sequences corresponding to the index clone are in bold.
N, no; Y, yes.

treatment of 1.5 months from the time of MRD studies. The remaining 7 patients maintained the same IMWG response criteria, despite having flow cytometry MRD positivity. Finally, of the 4 patients with discordant hsFC MRD−/NGS MRD+ status, with a median follow-up time of 29.8 months, 2 of 4 patients (50.0%) showed subsequent overt relapse, with a median progression-free survival of 23.5 months and time to next treatment of 23.9 months from the time of MRD studies. The remaining 2 patients maintained the same IMWG response criteria, despite having NGS MRD positivity. The details of the follow-up on these cases are listed in Supplemental Table S5.

Discussion

NGS-based *IGH* rearrangement assays have been successfully used in clonal characterization and subsequent disease monitoring in various B-cell lineage malignancies, including PCM,[9–13] chronic lymphocytic leukemia/small lymphocytic lymphoma, mantle cell lymphoma, and B-lymphoblastic leukemias/lymphomas.[9,17–21] In all, studies offer proof-of-principle evidence that NGS is more specific and potentially more sensitive than alternative options such as quantitative PCR and multicolor FC.[9,17,19,20,22,23] At present, only one commercial strategy (ClonoSEQ; Adaptive Biotechnologies, Seattle, WA) has proven utility in clinical practice and, accordingly, has been introduced as part of the response criteria of the 2016 IMWG consensus.[3] The broad application of this testing modality in routine practice, however, relies on the ability to implement the assays across clinical laboratories where they can be performed and analyzed locally, in the context of the patient with all available clinicopathologic information. Still, there are many challenges toward routine application that need to be overcome, including the need for new standardization, guidelines for validation, and implementation processes, all of which should be performed as a multicenter and multidisciplinary collaboration effort.

Given the unique advantages that NGS clonality assays can offer over conventional low-throughput technology, the authors' group has previously validated the current NGS assay for routine clonality assessment and has established single-institution clonality calling criteria based on their experience.[15] Based on serial dilution experiments in their prior study, as well as subsequent validation studies on the assay for MRD assessment and monitoring, the authors have determined that a sensitivity of 1×10^{-6} is attainable, but difficult to establish in current routine clinical practice,

Table 3 NGS Clonality Assay Data Output for Initial Clonal Characterization of a Case without Clonal Heterogeneity, after Merging of Sequencing Reads within 2-bp Differences

Total sequencing reads: 668,530

Rank	Sequence	Length, bp	Merge count	V gene	J gene	% Total reads	Cumulative %	Mutation rate to V gene, %	In-frame, Y/N	No stop codon, Y/N	V coverage
1	5′-GCCTCTGGATTCATCTT TAGTAGTTATTGGATGA ATTGGGTCCGCCAGGCTCC AGGGAAGGGGCTGGAGTG GGTGGCCAACATAAAGCAAGA TGGAAGTGAAAAAAACCACGT GGACTCTGTGAAGGGCCGA TTCACCATCTCCAGAGACAAC GCCAAGAACTCACTGTATCT GCAAATGAACAGTCTGAGAGTC GAGGACACGGCTGTCTATTAC TGTGCCAGAGTGGATTGTAGT GGTGGTGGCTGCTACGGAGTC TGGCACTTCGATCTCTGGGGC CGTGGCACCCT-3′	**290**	**421,195**	**IGHV3-7_01**	**IGHJ2_01**	**63.00**	**63.00**	**5.29**	**Y**	**Y**	**99.56**
2	5′-GCCTCTGGATTCACCTTTA GCAGCTATGCCATGAGCTG GGTCCGCCAGGCTCCAGGGAA GGGGCTGGAGTGGGTCTCAG TTATTTATAGCGGTGGTAGCA CATACTATGCAGACTCCGTGAA GGGCCGGTTCACCATCTCCAGA GATAATTCCAAGAACACGCTG TATCTGCAAATGAACAGCCTGA GAGCTGAGGACACGGCCGTATA TTACTGTGCGAAAATCGGGACC GGTCGGAGGGTTCGGGTAATTG ACTACTGGGGCCAGGG AACCCT-3′	275	293	IGHV3-23_03	IGHJ4_02	0.04	63.05	1.78	Y	Y	98.67
3	5′-GCCTCTGGATTCACCT TTGAGGATTATGGCATGAGG TGGGTCCGCCAAGCTCCAGG GAAGGGGCTGGAATGGGTCT CTGGTCTTAATTGGAATGGTG GTAGCACAGGTTATGCAGACT CGGCAGAGACAACGCCAAGAA CTCCCTGTATCTGCAAATGAAC AGTCTGAGAGCCGAGGACACG GCCTTGAATCACTGTGCGAGAC ATGGGGCCCCGCAGCTTTGACT ACTGGGGCCGGGGAACCCT-3′	245	284	IGHV3-20_01	IGHJ4_02	0.04	63.09	1.76	n/a	N	52.42
4	5′-GCCTCTGGATTCACCT TTAGTAGCTATTGGATGAGC TGGGTCCGCCAGGCTCCAGGG AAGGGGCTGGAGTGGGTGGCC AACATAAAGCAAGATGGAAGTG AGAAATACTATGTGGACTCT GTGAAGGGCCGATTCACCAT CTCCAGAGACAACGCCAAGA ACTCACTGTATCTGCAAATG AACAGCCTGAGAGCC GAGGACACGGCTGTGTATT ACTGTGCAAGAGATTTGTGG ATCGGATGGTTCGGGGATTC CTGGGGCCAGGGAACCCT-3′	272	272	IGHV3-7_01	IGHJ5_02	0.04	63.13	0.44	Y	Y	100.00
5	5′-GCCTCTGGATTCACC TTTGATGATTATGCCATGCA	240	266	IGHV3-9_01	none	0.04	63.17	0.44	n/a	N	93.45

(*table continues*)

Table 3 (*continued*)

Total sequencing reads: 668,530

Rank	Sequence	Length, bp	Merge count	V gene	J gene	% Total reads	Cumulative %	Mutation rate to V gene, %	In-frame, Y/N	No stop codon, Y/N	V coverage
	CTGGGTCCGGCAAGCTCCAG GGAAGGGCCTGGAGTGGGTC TCAGGTATTAGTTGGAATAGT GGTAGCATAGGCTATGCGGAC TCTGTGAAGGGCCGATTCACCA TCTCCAGAGACAACGCCAAGAA CTCCCTGTATCTGCAAATGAACA GTCTGAGAGCTGAGGACATGGC CTTGTATTCTATATGGTTCGGGGC CCCTTACCCT-3′										
6	5′-GCCTCTGGATTCACCTT CAGTAGCTATGGCATGCACT GGGTCCGCCAGGCTCCAGGC AAGGGGCTGGAGTGGGTGGC AGTTATATGGTATGATGGAAG TAATAAATACTATGCAGACTCC GTGAAGGGCCGATTCACCATCT CCAGAGACAATTCCAAGAACAC GCTGTATCTGCAAATGAACAGC CTGAGAGCTGAGGACACGGCT GTGTATTACCGAATGAAAGAGA CAGCAGTGGCTGGTACTACTTT GACTACTGGGGCCAG GGAACCCT-3′	274	263	IGHV3-33_06	IGHJ4_02	0.04	63.21	0.44	n/a	N	93.83
7	5′-GCCTCTGGATTCACC TTCAGTAGCTATAGCATG AACTGGGTCCGCCAGGCTC CAGGGAAGGGGCTGGAGTG GGTTTCATACATTAGTAGTAG TAGTAGTACCATATACTACGC AGACTCTGTGAAGGGCCGATT CACCATCTCCAGAGACAATGCC AAGAACTCACTGTATCTGCAAAT GAACAGCCTGAGAGCCGAGGAC ACGGCTGTGTATTACTGTGCGAG ACTGGGGGATAGTAGTGGTATCT GGGGCCAGGGAACCCT-3′	263	262	IGHV3-48_01	IGHJ5_02	0.04	63.25	0.00	Y	Y	99.12
8	5′-GCCTCTGGATTCACC TTCAGTAGCTATGGCATG CACTGGGTCCGCCAGGCTC CAGGCAAGGGGCTGGAGTGG GTGGCAGTTATATGGTATGAT GGAAGTAATAAATACTATGCA GACTCCGTGAAGGGCCGATTC ACCATCTCCAGAGACAATTCCA AGAACACGCTGTATCTGCAAAT GAACAGCCTGAGAGCTGAGGAC ACGGCTGTGTAGCCTGTATTTTG ACTGGTTAATTGTACCCAGGGGC CCCTGACTACTGGGGCCAGGG AACCCT-3′	274	259	IGHV3-33_06	IGHJ4_02	0.04	63.29	0.44	n/a	N	92.07
9	5′-GCCTCTGGATTCACCT TCAGTAGCTATGGCATGC ACTGGGTCCGCCAGGCTCCA GGCAAGGGGCTGGAGTGGGT GGCAGTTATATGGTATGATGG	299	258	IGHV3-33_06	IGHJ6_02	0.04	63.33	0.44	Y	Y	97.36

(*table continues*)

Table 3 (*continued*)

Total sequencing reads: 668,530

Rank	Sequence	Length, bp	Merge count	V gene	J gene	% Total reads	Cumulative %	Mutation rate to V gene, %	In-frame, Y/N	No stop codon, Y/N	V coverage
	AAGTAATAAATACTATGCAGAC										
	TCCGTGAAGGGCCGATTCACCAT										
	CTCCAGAGACAATTCCAAGAACA										
	CGCTGTATCTGCAAATGAACAG										
	CCTGAGAGCTGAGGACACGGCTG										
	TGTATTACTGTGCGAAGGCACTAG										
	GATATTGTAGTGGTGGTAGCTG										
	CTACTCGAGGGTTTTCGGTAT										
	GGACGTCTGGGGCCAAGG										
	GACCAC-3′										
10	5′-GCCTCTGGATTCTCCT	272	257	IGHV3-7_01	IGHJ5_02	0.04	63.36	8.37	Y	Y	98.24
	TTAGTAATTATTGGATGAG										
	TTGGGTCCGCCAGCCTCCA										
	GGGAAGGGGCTGGAGTGGG										
	TGGCCAGTATACAGCCAGA										
	CGGAAGTGCGGAGTATTACG										
	TGGACTCTGTGAAGGGCCGAT										
	TCACCGTCTCCAGAGACAACGC										
	CAAGAACTCACTATATCTGCAG										
	ATGAACAGCCTGAGAGCCGAGG										
	ACACGGCTGTGTATCACTGTGC										
	GAAACTGAAGGATACAGCTACG										
	CGCTACGACTCCTGGGGCCAGGGA										
	ATTCT-3′										

Sequence corresponding to the index clone is bolded.
N, no; Y, yes.

given the high DNA input necessary (approximately 7 μg per sample), limitations of maximum DNA volume input by the assay, the need for multiple replicates (six to seven), and corresponding extensive sequencing capacity requirements. In the authors' cohort, many of the monitoring samples were post-treatment PCM patients with relatively low marrow cellularity, leading to extracted DNA samples with low concentrations, which in turn, limited the maximum amount of DNA that could be used in each reaction. Therefore, based on all the practical laboratory considerations, routine MRD assessment at a level of 1×10^{-5} is much more feasible and operationally easy to incorporate into clinical workflows, even in a high-volume laboratory. A technical sensitivity of 1×10^{-5} is the level that the authors' laboratory claims and can confidently and consistently achieve for clinical reporting purposes.

Based on the current study, the authors show that NGS clonality testing by this assay is a robust method for initial clonal characterization in patients with PCM. Success rates vary, however, depending on the disease burden of the diagnostic sample. To maximize success, enrichment methods may be used and are recommended if the PC content is <10% of sample cellularity. Nevertheless, the success rates in this cohort were higher than those reported in prior literature,[9,10,24] and can be attributed to the incorporation of leader primer sets and targeting of all framework regions of *IGH* (FR1, FR2, and FR3), as well as *IGK*, which might not have been used in other studies. The two major reasons for characterization failure in the authors' cohort were: low tumor burden (PC <5% cellularity), mostly in patients previously treated at other hospitals, and ineffective primer binding presumably related to SHM, which has been well-described.[9–11,15,17–19] The vast majority of cases characterized with *IGH* or leader primer sets showed one or two (biallelic or biclonal) clonal rearrangement(s). It should be noted that in very rare cases, variable degree of clonal heterogeneity was noted, as evidenced by the presence of multiple related clonal sequences with nucleotide differences greater than expected from technical artifacts such as PCR amplification errors and sequencing artifacts. In the authors' laboratory, they have observed clonal heterogeneity more frequently in certain tumor types, such as follicular lymphomas (unpublished data). Almost all characterized cases of PCM had high rates of SHM, thus this clonal heterogeneity could be inferred to be the result of ongoing SHM, leading to intraclonal diversity. It is not yet clear how this issue should be addressed in the context of disease-monitoring in such patients. Currently, the authors' strategy for monitoring is to search for the index clonal sequences using exact match and up to 2-bp mismatches. In

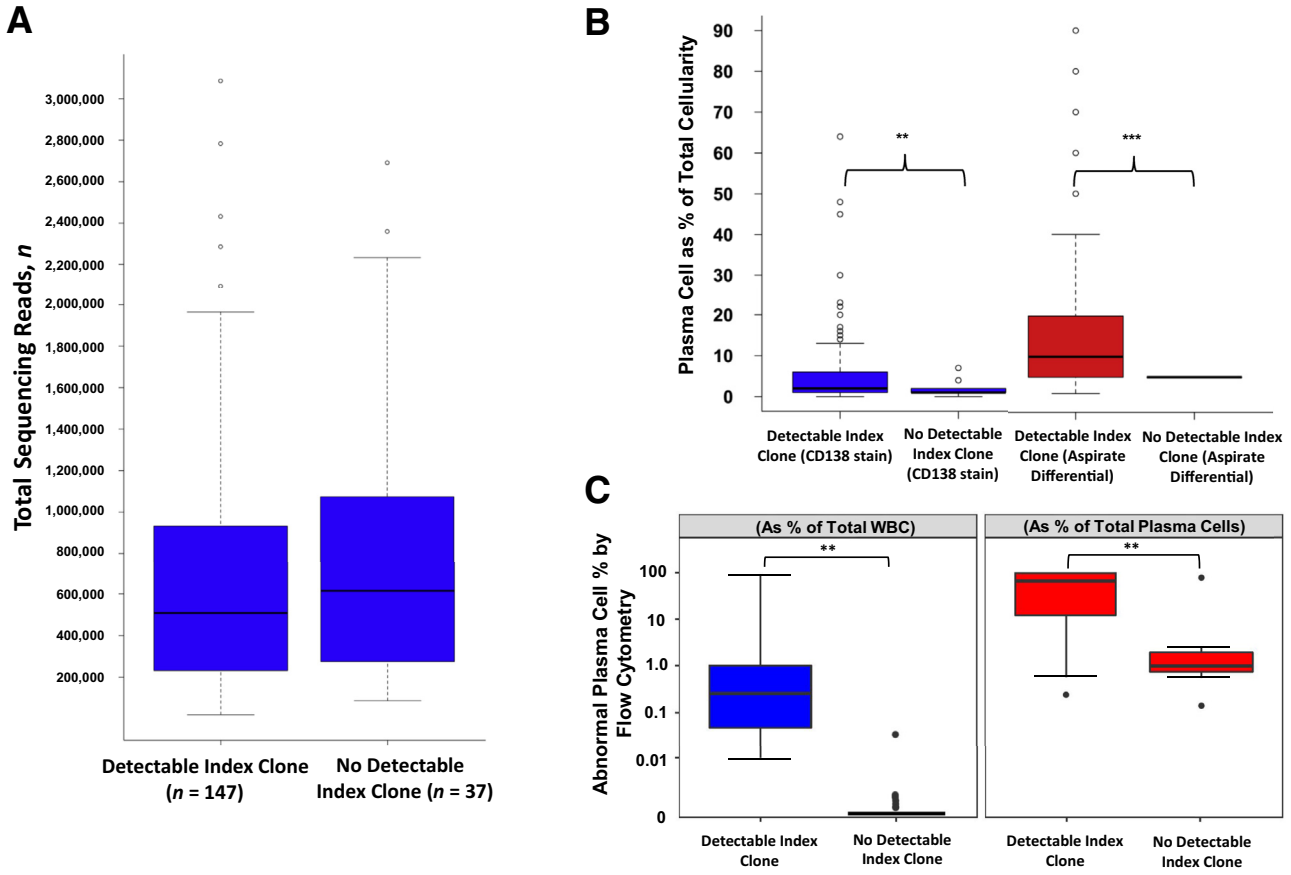


Figure 5 Comparison of monitoring samples with detectable and no detectable index clonal sequences by the next-generation sequencing (NGS) assay: **A:** Total *IGH* sequencing reads. **B:** Comparison of plasma cell (PC) quantitation by CD138 immunostains performed on the core biopsy and by aspirate differential count. **C:** Comparison of abnormal PC quantitation by flow cytometry, as % of total white blood cells (WBC), and as % of total PC in the samples. In all panels, the **colored boxes** represent values between the 25th and 75th percentiles, whereas the **horizontal line** within the **boxes** represents the median value. $^{**}P < 0.01$, $^{***}P < 0.001$.

the rare cases with clonal heterogeneity, all sequences with clonal variations are tracked independently. For these cases, during sequence searches, it might be necessary to further relax the usual criteria for the number of allowable base pair mismatches in relation to the index clonal sequence, to account for possible further clonal sequence drifts in monitoring samples. Cases with index clones characterized by *IGK* primer sets characteristically had two to four different dominant clonal sequences with at least one INTR-Kde rearrangement. The observation of multiple clonal sequences by *IGK* primers can be seen, even in cases with a single *IGK* V-J rearrangement, due to cross-annealing of V family−specific primers to other V family genes. In some specific configurations of *IGK* loci, multiple rearrangements can also be present on the same allele. The presence of up to four different *IGK* rearrangements can still be compatible with a single clonal population.[25,26]

The analysis of the monitoring data across 187 samples (124 patients) allowed the authors to make several important observations. The MSKCC hsFC assay was used for comparison,[6,8] which surpasses the expected sensitivity of the NGS assay based on the authors' selected nucleic acid input. Despite the expected differences in sensitivity, in practice, the two methods performed very similarly, showing a high concordance rate (approximately 93%) in disease detection, including those patients with stringent CR. The few observed cases with MRD status discrepancies affected both assays, and as expected, evidence of residual disease was identified at a very low level near the limit of detection for both assays. At this low level of residual disease, sampling differences might have contributed to the discrepancies. Per institutional protocol, the first bone marrow aspirate draw is generally reserved for hsFC. The subsequent aspirates are distributed for other ancillary studies with the potential to be more hemodiluted and less optimal for MRD assessment. The low number of MRD discrepant cases, however, precluded optimal assessment of their prognostic value and clinical significance as compared with MRD-negative cases by both assays, and further larger studies will be necessary. Importantly, the majority of patients with MRD-negative status by both assays remained MRD-negative in the follow-up interval, with approximately 21% of patients showing overt evidence of subsequent disease relapse, with a median follow-up time of close to 3 years.

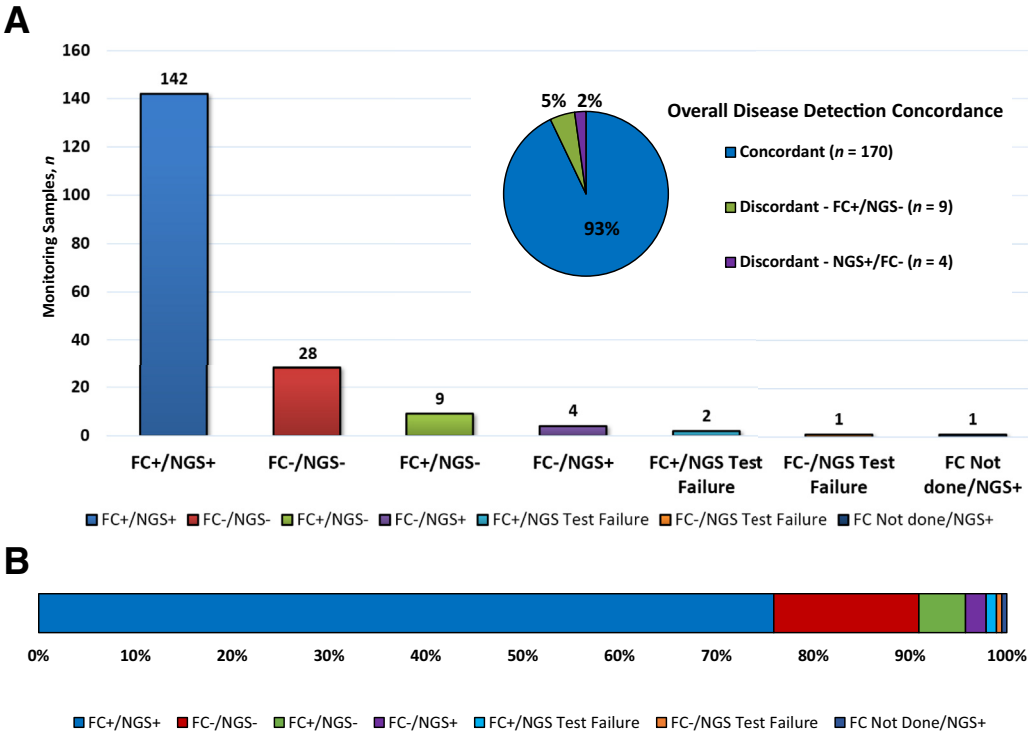


Figure 6 Comparison of residual disease detection among 187 monitoring samples by flow cytometry (FC) and next-generation sequencing (NGS) assay. Results represented by a bar graph **(A)**. Also shown is the percentage of cases with concordant and discordant residual disease status by the two assays. Results represented as cumulative percentage of total number of cases **(B)**.

Within a relatively short time between the initial characterization and subsequent monitoring samples for most of the patients in this study, the identity of the established index clonal sequences and SHM rates remained stable in the monitoring samples, suggesting that at least in most cases, using a conservative search approach for the index clonal sequence, for exact match and up to 2-bp mismatches, would allow adequate tracking of the clones. However, we acknowledge that our cohort consists of patients who have received variable treatment regimens, with variable length of follow-up time. Therefore, more comprehensive, regimen-specific studies will be necessary to evaluate any effect that treatments can have on SHM rate, clonal heterogeneity, and emergence of new index clones.[27] A notable advantage of NGS testing over hsFC, in this regard, is the ability to broaden the assessment to encompass the entire immune repertoire, the clonal architecture and clonal dynamics over time when serial samples are analyzed. This is an active area of investigation that is bound to provide valuable insight into pathogenic mechanisms, effect of immunomodulating therapies, immune surveillance, immune system constitution, and so on, and therefore argues for the added value of NGS as a more suitable or complementary approach to assessment.

While establishing the technology for routine clinical assessment, there were additional technical challenges that must be mentioned. To confidently adopt a clinical NGS assay for MRD detection with a high level of accuracy, it is crucial to take precautions to minimize cross-contamination from the characterization to subsequent monitoring samples (a source of false-positive MRD results) as well as errors in sequencing and demultiplexing resulting in incorrect assignment of sequencing reads (a source of false-negative MRD results).[28] These precautions are described in detail in *Materials and Methods*. It is worth emphasizing that, with this specific assay, the characterization and monitoring of samples from the same patient must be sequenced in separate runs, using different barcode indexes, and ideally on different MiSeq instruments, to prevent carryover contamination. Also, for monitoring samples without overt residual diseases, multiple replicates should be run to reach the target sensitivity. In this study, the best results were obtained using up to 1 μg per replicate. Depending on the volume and the general workflows in the laboratory, instrument rotation and separation of diagnostic and monitoring samples may be difficult.

Finally, an important component of MRD assessment is quantitation. Due to the methodology of NGS assays, a common and easy way to quantify the MRD level is by counting the number of detectable index clonal sequences and expressing the level as a % of total *IGH* sequencing reads. Given that these originate from amplicons of DNA from mostly B and plasma cells, but not other cells with *IGH* genes in germline configuration, this may lead to significant overestimation of MRD. Expression of residual disease as a proportion of all nucleated cells in the sample is

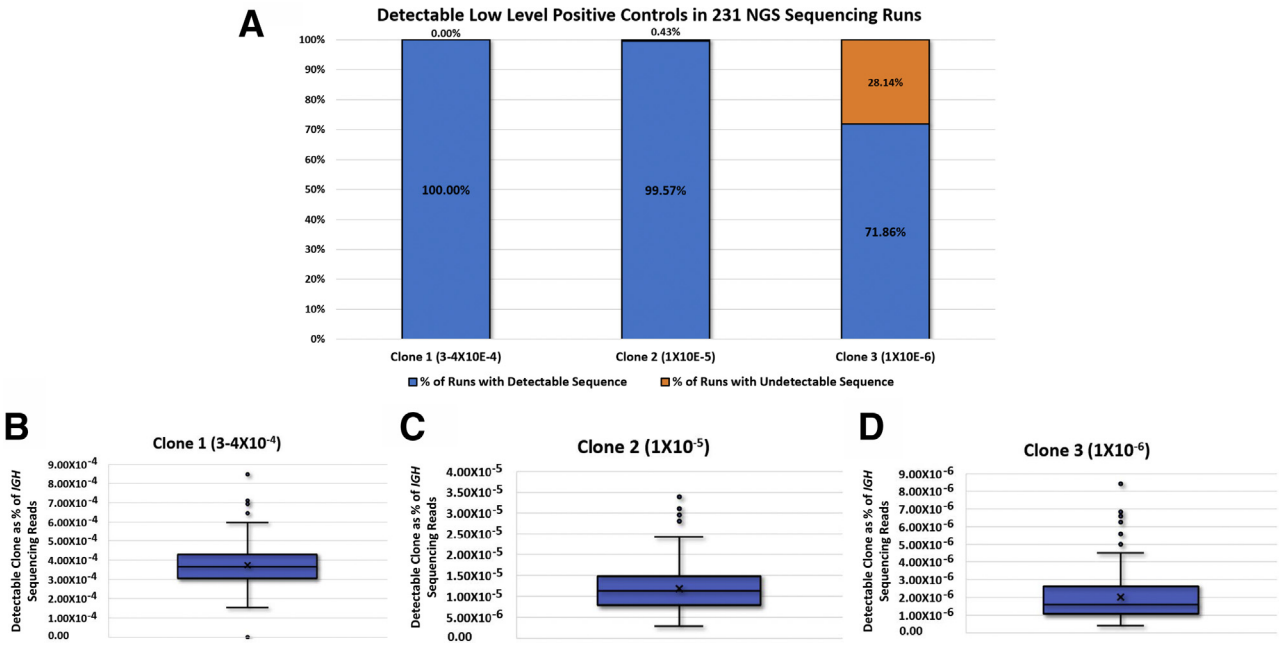


Figure 7 Detectable low-level positive controls in 231 next-generation sequencing (NGS) runs. Dilution of IVS-0019 Clonal B-cell control DNA into IVS-0000 polyclonal control DNA sample yielded three different clonal sequences at different expected concentrations, and were added to each NGS sequencing run as low-level positive controls. **A:** Overall detection of the three clones across 231 NGS runs. **B:** Detectable clonal sequence 1 (expected concentration, 3×10^{-4} to 4×10^{-4}) as % of *IGH* sequencing reads. **C:** Detectable clonal sequence 2 (expected concentration, 1×10^{-5}) as % of *IGH* sequencing reads. **D:** Detectable clonal sequence 3 (expected concentration, 1×10^{-6}) as % of *IGH* sequencing reads. In all panels, the **boxes** represent values between the 25th and 75th percentiles, whereas the **horizontal line** within the **boxes** represents the median value.

an important parameter for clinical assessment, and therefore, internal controls need to be included to allow this calculation. Different approaches have been used in the literature, including the use of plasmids containing known *IGH* gene rearrangements or spiked-in synthetic control templates into each sample to compute an average number of reads for each sequenced spike synthetic. Using a similar spike-in approach, the authors showed the utility of this conversion in a subset of cases, obtaining results that are very similar to the levels obtained by hsFC for patients with low-level residual disease. The correlation drifts in those cases with overt morphologic evidence of disease, which is likely related to the intrinsic PCR biases that are common with any amplicon-based assay. When the level of the index clone is high in the sample, we note that this sequence may be preferentially amplified over the spike-in sequence, which is added at a very low level to the sample. Further clinical validation is in progress to assess the utility of more than one spike-in control; however, the quantification of the index clones in samples with overt disease is not necessarily as clinically relevant, as historically, aspirate differential count and immunohistochemical stain quantitation of PC have been used instead for this purpose.

Based on these findings, both hsFC and NGS-based assays showed very high detection sensitivity and high concordance in residual disease detection. For individual laboratories, the choice of MRD methodology may depend on other practical factors, such as availability of instrumentation, expertise, and nature of specimens received. HsFC provides a fast turnaround time, does not require prior characterization of disease clones, but does require fresh samples for analysis. Furthermore, hsFC assays, similar to that used in this study, do require a high level of expertise for assay validation and results interpretation. NGS-based assays can be performed on DNA extracted from fresh or formalin-fixed, paraffin-embedded tissue, allowing clonal characterization using archival samples obtained several years prior. However, one potential obstacle to successful clonal characterization, particularly at a referral hospital like the authors where many patients have received treatments elsewhere, is the unavailability of DNA from samples with adequate tumor content. Although it is difficult to accurately assess the proportion of patients who fall into this category at the authors' institution, they estimate that these patients comprise up to approximately 20% of their PCM population. Nevertheless, once clonal characterization is successful, residual disease detection in subsequent samples can be automated using analysis pipelines and predefined thresholds for sequence homology, reducing subjectivity in interpretation. NGS assays also provide insights into the biology of the disease clones, whereas serial sequencing of samples from the same patient allows studying of the temporal evolution of clonal sequences. Finally, regardless of the methodology used for MRD detection, sensitivity is limited by the sample quality

and quantity. For NGS assays, high-quality DNA with high total DNA yield is crucial in generating sufficient number of sequencing reads to achieve high MRD detection sensitivity.

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Authorship Contributions

C.H. and M.E.A. designed the study and wrote the manuscript; M.S. performed statistical analyses and supported bioinformatics analysis; M.R., A.D., K.N., and O.L. designed the study; C.H., M.R., K.P.-D., C.M., J.Y., A.E.Q., J.B., C.V., Y.L., M.Z., A.D., and M.E.A. analyzed the flow cytometry and/or NGS testing data; W.Y., L.M., M.W., and Y.M. performed technical aspects of the molecular assays. Q.G. provided technical support for the flow cytometry assay. Y.H., K.H., and J.E.M. provided technical and analysis support for the NGS-based assay. E.H.R., M.H., B.T.D., B.Z.-L., and O.L. analyzed the clinical data; all authors reviewed and approved the manuscript.

Supplemental Data

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