Practical Laboratory Medicine 22 (2020) e00191

Contents lists available at ScienceDirect

Practical Laboratory Medicine

journal homepage: www.elsevier.com/locate/plabm

Validation and interpretation of IGH and TCR clonality testing by Ion Torrent S5 NGS for diagnosis and disease monitoring in B and T cell cancers

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ARTICLE INFO

Keywords:
Leukemia
TCR
Clonality
Next-generation sequencing
Lymphoma
NGS
IGH
Validation

ABSTRACT

Cancers of B and T lymphocytes are the most common hematologic malignancies in the US. Molecular assays for assessing clonal rearrangements of the immunoglobulin receptor (IGH) and T-cell receptor (TCR), commonly referred to as B- and T-cell clonality, as well as determination of IGH somatic mutation status, enables improved diagnostic accuracy and disease monitoring. Here we describe validation of NGS LymphoTrack (IGH, TCRG, Invivoscribe, Inc) with Ion Torrent S5 sequencing, which employs a different sequencing chemistry and has not been previously reported for NGS clonality to our knowledge. We also demonstrate the concordance of clonality by LymphoTrack with S5 sequencing with other molecular methodologies and with clinical measurements of disease. We show that LymphoTrack with S5 sequencing identifies previously detected IGH and TCRG clonal sequences across matched biopsy specimens and clinical timepoints, enabling more precise and sensitive disease monitoring for B- and T-cell cancers compared to PCR fragment or capillary sequencing. In sum, our study demonstrates that the LymphoTrack assays with Ion Torrent S5 sequencing 1) can be used successfully for IGH and TCR clonality with reproducible results; 2) generates and quantifies clonal sequences to enable highly precise comparison of samples; 3) are substantially more sensitive than PCR fragment and return clonality results in specimens that failed PCR fragment assays; and 4) the TCRG assays are highly concordant with clinical and histopathologic diagnoses. Taken together, the LymphoTrack with Ion S5 NGS clonality assays offer a sensitive and precise method for diagnostic testing and disease monitoring in B- and T-cell cancers.

1. Introduction

Cancers of B and T lymphocytes are the most common hematologic malignancies in the US (https://seer.cancer.gov/). The increased sensitivity of molecular assays for assessing clonal rearrangements of the immunoglobulin receptor (IGH/K) and T cell receptor (TCR), commonly referred to as B and T cell clonality, enables improved diagnostic accuracy and disease monitoring [1–4]. Compared to earlier "PCR fragment" assays, in which PCR products are separated by size using slab or capillary gel electrophoresis, newer methodologies that use massively parallel ("next-generation sequencing", NGS) are more sensitive and specific for detecting clonal rearrangements of IGH and TCR [5–14]. The increase in sensitivity and specificity is due in part to their return of unique nucleic acid sequences. In contrast,

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https://doi.org/10.1016/j.plabm.2020.e00191
Received 29 August 2019; Accepted 17 November 2020

PCR fragment assays only return the size in base pairs of the PCR amplicon as determined by gel band or capillary electrophoresis peaks, which is less specific than the nucleic acid sequence of the amplicon and also the band or peak may contain multiple clonal PCR fragments of the same size. NGS-derived sequence data can be used to more accurately compare multiple specimens from the same patient separated by body site or collection time, which enhances diagnostic and staging accuracy and enables more precise disease monitoring [5,8,9,9,10,15–19]. As with any new methodology, routine clinical use of NGS methods to detect IGH/TCR clonality requires comparison to alternative and established (validated) methods as well as evaluation and implementation of new interpretive limits.

NGS clonality methods vary widely. Most assay genomic DNA, but some evaluate expressed clonal rearrangements by isolating RNA and converting to cDNA for input to the clonality assay. The primers used for amplification of IGH or TCR rearrangements vary widely, and often are not reported by manufacturers or laboratories. The genes involved in rearrangements are many and diverse, and generally speaking, assays do not exhaustively test for all genes or all possible rearrangements [20]. Finally, while the sequencing technologies used for clinical testing are similar, there are nevertheless technical differences in the methods as well as differences in user interface and software implementation. For example, most published evaluations of NGS clonality have used Illumina-based sequencing (for example [5,7,9]), but there are several Illumina platforms and there are other sequencing methods as well (e.g., Ion Torrent). To achieve consistent and reliable routine clinical testing, all of these factors must be evaluated during the validation of a new NGS clonality assay.

Washington University School of Medicine (WUSM) and Barnes-Jewish Hospital (BJH) in St. Louis have large clinical programs that treat patients with the entire spectrum of B and T cell cancers, including B and T cell lymphomas and leukemias and multiple myeloma. Testing for B and T cell clonality and, in Chronic Lymphocytic Leukemia (CLL) testing for IGH somatic hypermutation (SHM), is a critical component of diagnosis, staging, and monitoring for these diseases and the results contribute to therapeutic decision-making [7,8,15,21]. Given the reported higher sensitivity and specificity of NGS methods for IGH and TCR clonality testing, we decided to move from a method that used PCR fragment analysis by capillary electrophoresis (Invivoscribe, Inc) to an NGS-based method. Here we describe how we validated the NGS LymphoTrack assays [9,10,22] (Invivoscribe, Inc) with the Ion Torrent S5 sequencing instrument, which employs a different sequencing chemistry than the Illumina platforms [23,24] and has not been previously reported for use with NGS clonality studies to our knowledge. We also discuss the pitfalls in comparing to other molecular methodologies and to clinical measurements of disease and in interpreting results. These issues have implications for comparison of clonality results from disparate NGS methods or from different laboratories and for education and guidance offered to ordering physicians.

2. Materials & methods

2.1. Clinical cases and tissue processing

Clinical specimens received for routine assessment of IGH or TCR clonality or IGH somatic hypermutation were identified for the validation study. Cases were selected to represent all types of tissue submitted for clonality testing (fresh bone marrow, fresh lymph node biopsies, fresh vitreous fluid, fresh or fixed formalin paraffin-embedded (FFPE) skin and lymph node biopsies, and fresh peripheral blood), the most common disease classifications and other entities (e.g., atypical T cell infiltrate), and PCR clonality results (e.g., clonal, polyclonal, no rearrangement, inconclusive, see below for definitions). Disease classifications based on Pathology reports and patients' disease status were recorded. Genomic DNA was extracted from >200 blood, bone marrow, and fresh or formalin-fixed, paraffin-embedded (FFPE) tissue specimens (Table 1).

2.2. Clonality testing by PCR fragment capillary electrophoresis analysis

These samples had been previously subjected to IGH or TCR clonality or IGH somatic hypermutation testing using size analysis of fluorescently labeled PCR fragments separated by capillary electrophoresis or Sanger sequencing by capillary electrophoresis using TCRG or IGH FR1/2/3 or IGH Somatic Hypermutation Assay for ABI fluorescence detection kits (Invivoscribe) and ABI 3130 or 3500 instruments, (Thermo Fisher Scientific, Life Technologies Corporation, Carlsbad, CA), all based on manufacturers' protocols (Invivoscribe Technologies: TCRG Gene Clonality Assay 1207002Xv7.40, IGH Gene Clonality Assay, 1101002Xv7.50), BIOMED-2 guidelines [1–4], and laboratory validation. Fragment analysis was conducted with GeneMapper software on the ABI instrument using the Sizing Analysis Method and the Profiler_Plus_v2 panel with size standard GS500ROX. For IGH clonality analysis, multiplex primer mixes target the framework 1, 2 or 3 (FR1, FR2 or FR3) regions within the variable region and the joining (J) region of the lg heavy chain locus. For TCRG clonality analysis, two multiplex primer mixes target conserved regions within the variable (V) and the joining (J) regions that

Table 1
Total sample numbers by assay and specimen type.

	TCRG	IGH	IGH/SHM	Total
BM	20	5	20	45
PB	21	12	39	72
Fresh Tissue	14	14	0	28
FFPE Tissue	17	7	4	28
Vitreous Fluid	0	22	0	22
Total	72	60	63	195

flank the hypervariable antigen-binding region 3 (CDR3) in 2 separate reactions. Several manufacturer-supplied controls were included with each run. For IGH, each reaction included both a clonal and a polyclonal positive control with expected size ranges: for tube B reaction mix 285, 250–295 base pairs (bp), respectively, and for tube C reaction mix 145, 100–170 bp, respectively. For TCRG, each reaction included both a clonal and a polyclonal positive control with expected size ranges: for tube A reaction mix 211, 145–255 base pairs (bp), respectively, and for tube B reaction mix 80–220, 167 bp, respectively. Both IGH and TCRG also included a "size ladder" amplification control with primers for several genomic regions that each produced a different amplicon size, thereby acting as a control for DNA degradation (i.e., size of fragments, full amplicon ladder present indicates the DNA fragments in the specimen are long enough for amplification and detection of any IGH/TCRG amplicons if they are present) and success of PCR in general. Water served as the negative control. Results were interpreted according to latest EuroClonality/BIOMED-2 guidelines for interpretation and reporting of Ig/T-cell receptor clonality testing in suspected lymphoproliferations [25]. Specifically, the following interpretive guidelines were followed for consistent reporting: Clonal rearrangement = one peak detected in any of the reactions within the expected size range for each reaction mix, if in a polyclonal background, must be $\geq$ 50% higher than the highest polyclonal peak, full size range of amplification control detected and positive controls detected; Bi-clonal rearrangement = as for Clonal but two peaks; Oligoclonal rearrangement = as for Clonal but 3–4 peaks; Polyclonal rearrangement = several peaks of different sizes within the expected size range with no predominate single/dual peak(s), full size range of amplification control detected and positive controls detected; No rearrangement = full size range of amplification control detected and positive controls detected but no IGH/TCR amplicons detected; Inconclusive - positive controls detected, but IGH or TCR amplicons from the specimen were not detected and amplification control was not detected or was incompletely detected. Fragment sizes in base pairs for each clonal peak were reported by reaction mix/tube. Duplicates were run for each assay and reproducible results were required for reporting, in other words, only clonal peaks of the same size in both replicates were reported. For IGH somatic hypermutation analysis, a multiplex primer mix was used to target the framework 1 region (FR1) and the resulting amplicon was subjected to capillary sequencing on the ABI 3130XL or 3500 instrument either directly or after gel extraction of the clonal band. Somatic Hypermutation (SHM) analysis was performed with IMGT (http://www.imgt.org/) and IgBlast (https://www.ncbi.nlm.nih.gov/igblast/). The presence or absence of somatic hypermutation was determined by aligning the V region sequence of the sample to the germline V region sequence and calculating the (number of mismatched bases/total number of bases compared) X 100 and subtracting from 100%. If >2%, the sample was reported to have somatic hypermutation.

2.3. Performance of clonality assays by next-generation sequencing (NGS)

The LymphoTrack *IGH* FR1 (Cat #: 71210007), *IGH* FR2 (Cat #: 71210037), *IGH* FR3 (Cat #: 71210047), and *TRG* assays (Cat #: 72270007) (Invivoscribe, Inc) for NGS and were used. Libraries were prepared from DNA extracted from patient specimens described above. Patient sample DNA was diluted to 10 ng/μL, or if the sample was less than 10 ng/μL, it was used undiluted; 5 μL of input was used for each assay. For clonal and polyclonal controls, 50 ng total DNA was used. For library preparation, emulsion PCR was performed on the Ion OneTouch 2 instrument (Thermo Fisher Scientific) using the Ion 520 & 530 Kit – OT2 protocol [26], with the exception of the library pooling and dilution step. Enrichment on the Ion OneTouch ES instrument (Thermo Fisher Scientific) and sequencing on the Ion S5 (Thermo Fisher Scientific) followed the manufacturer's protocols [26–28]. The Ion S5 Ion 520 chip capacity is 3–6 million reads and the 530 chip capacity is 15–20 million reads total. Up to five different samples and/or gene targets were included per run using the included indexing oligos to identify different sample input libraries. IGH or TCR clonal and polyclonal controls (Invivoscribe, Inc) and no template controls (molecular grade water) were included with each run. Purified amplicons were quantified on both the Agilent 2200 Tapestation with High Sensitivity D1000 Screentape and the Agilent 2100 Bioanalyzer [29] using the High Sensitivity DNA Analysis kit and protocol [29] (Agilent Technologies). To assess between-run, within-run, inter-operator, reagent lot and instrument effects, the IGH and TCR clonal and polyclonal controls and clinical samples were assayed in triplicate on the same run, on different dates over a period of 6 months, on both Ion S5 instruments, by two technologists, on two chip types (520 or 530), across at least three kit lots, using $\geq$3 different indexes, and using two library quantitation methods (Agilent 2100 or 2200). Samples yielding inconclusive results were repeated. The results of these assays were quite consistent within samples, with average standard deviations of <1% across different test conditions. Further validation of these assays, including comparison to the previous results from PCR fragment by capillary electrophoresis and establishing clinical interpretation guidelines, is described in the Results section of this manuscript.

2.4. NGS clonality testing data analysis

Sequencing.fastq files were downloaded and analyzed with the LymphoTrack PGM or LymphoTrack IGH SHM PGM Software (Invivoscribe, Inc.) was used to align and merge sequences with $\leq$1 base pair mismatches; merged sequences were ranked by percentage of total sequencing reads. Per manufacturer's recommendations, a minimum of 20,000 sequencing reads was required for interpretation of clonal and polyclonal controls and patient samples; samples below this cutoff were considered inconclusive. Both software packages identify V-gene and J-gene usage, calculate percentage of total reads for a given sequence read, rank the sequence reads by percentage of total reads, and calculate the cumulative percentage of total reads at each rank. The IGH SHM software package also calculates for each sequence read, the mutation rate of the V sequence (percentage of mismatched bases of the total number of bases compared), whether the sequence is in frame, presence of a stop codon, and the percentage of the V region covered by the sequence read. IMGT and IgBlast were used to compare clonal sequences between samples and between LymphoTrack and clonoSeq results.

3. Results

3.1. Validation study plan

We chose the LymphoTrack assays (Invivoscribe, Inc) due to their similarity to the PCR fragment assays (Invivoscribe, Inc) that were previously used in the Barnes-Jewish Hospital Molecular Diagnostics laboratory (BJH MDL), which would provide clinical continuity for patients who had been previously tested and smooth the transition for ordering physicians and clinical staff. In addition, the LymphoTrack assays are compatible with the Ion Torrent S5 system, which was the only type of NGS instrument available for use in the BJH MDL.

The LymphoTrack assays are sold as Research Use Only (RUO) and therefore require a validation study for use and reporting in a clinical laboratory. Thus, we planned a validation study to include a set of samples that were representative of those routinely submitted for IGH and TCR clonality and/or IGH somatic hypermutation testing, including those from patients with known or suspected B or T cell lymphomas or leukemias, and including all specimen types submitted for clonality testing: fresh bone marrow, fresh lymph node biopsies, fresh vitreous fluid, fresh or fixed formalin paraffin-embedded (FFPE) skin and lymph node biopsies, and fresh peripheral blood. Greater than 200 patient samples were included in the validation study (Table 1). Of these, 181 had been previously tested and results reported using the BJH MDL validated PCR fragment size IGH or TCRG clonality and/or IGH somatic hypermutation testing using Invivoscribe reagents and ABI fluorescence capillary electrophoresis detection. Details on the methods and results interpretation (clonal, bi-clonal, oligoclonal, polyclonal, no rearrangement, and inconclusive), which were based on the latest EuroClonality/BIOMED-2 guidelines for interpretation and reporting of Ig/T-cell receptor clonality testing in suspected lymphoproliferations [25], are in the Methods. For IGH somatic hypermutation, the results included hypermutation, no hypermutation, and inconclusive. Samples with all possible PCR results were included in the validation set (Table 2). The remaining 20 samples were serial peripheral blood specimens from multiple myeloma patients that had been subjected to clonoSeq testing (Adaptive Biotechnologies) for minimal residual disease (MRD) monitoring and were compared to LymphoTrack IGH SHM results. These ~200 samples comprised the set used for comparison to the NGS clonality assays being validated.

3.2. Assay optimization and data analysis

To determine the acceptable amount of diluted amplicon to add to the library pool for the NGS assays, library inputs of 100, 200, 300, and 400pM from IGH/TCR clonal, IGH/TCR polyclonal, and no template controls, and five patient samples previously tested for clonality by PCR fragment analysis were assayed. Sequencing. fastq files were analyzed with the LymphoTrack PGM Software (Invivoscribe, Inc.) to align and merge sequences with $\leq$1 base pair mismatch; merged sequences were ranked by percentage of total sequencing reads. Per manufacturer's recommendations, a minimum of 20,000 sequencing reads was required for a positive control sample or patient sample to be considered valid for interpretation. In contrast, No template control samples were required to be $<$ 20,000 reads to be considered valid. Data analysis with the LymphoTrack software also identified V-gene and J-gene usage, calculated the percentage of total reads for a given sequence read, ranked the sequence reads by percentage of total reads, and calculated the cumulative percentage of total reads at each rank.

Analysis of control data and manufacturer recommendations were incorporated to develop criteria for a valid run:

1. IGH/TCR clonal control: top ranked merged rearrangement sequences $\geq$2.5% of total reads matching the known control gene rearrangements and total read counts >20,000, and
2. IGH/TCR polyclonal control: top ranked merged rearrangement sequences <1% of total reads and total read counts >20,000, and
3. No template control total read counts <20,000.

Using these criteria, 19 of 21 IGH runs and 15 of 20 TCR runs were considered valid. The two failed IGH runs and 1/5 failed TCR runs were due total read counts for polyclonal controls <20,000 due to insufficient template loaded during library preparation, which was addressed by using a minimum concentration of 10 ng/μL for clonal and polyclonal controls. Four failed TCR runs were due to total read counts >20,000 and detection of TCR clonal control rearrangement sequences in the No template controls due to contamination by

Table 2
Results of PCR fragment and IGH SHM capillary electrophoresis sequencing.

	TCRG	IGH	IGH/SHM[a]
Clonal	16	13	N/A
Bi-clonal	18	9	N/A
Oligoclonal	7	1	N/A
Polyclonal	30	26	5
Hypermutation	N/A	N/A	9
No hypermutation	N/A	N/A	19
No rearrangement	0	8	N/A
Inconclusive	1	3	10
Total	72	60	43

[a] Does not include 20 clonoSeq samples.

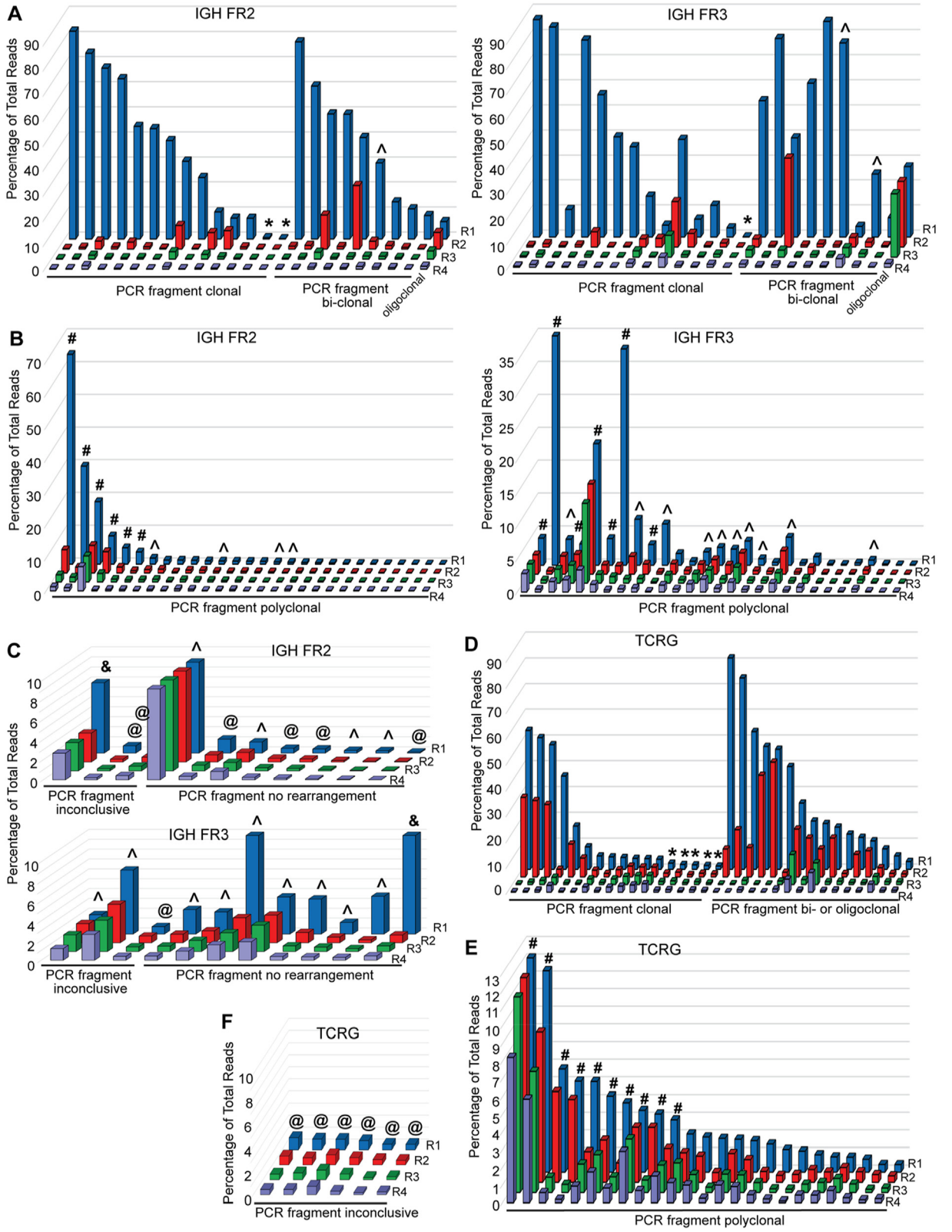

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Fig. 1. Accuracy of IGH FR2 and FR3 and TCRG NGS clonality testing compared to PCR fragment analysis. A-C) Bar graphs show the percentage of total reads for the four top-ranked clonal sequences for NGS IGH FR2 and FR3, grouped by their PCR fragment analysis results. FR2 and FR3 results are plotted in the same specimen order. (A - mono-/bi-/oligoclonal, B - polyclonal, C - inconclusive or no rearrangement). D-F) Bar graphs show the percentage of total reads for the four top-ranked clonal sequences for NGS TCRG, grouped by their PCR fragment analysis results. (D - mono-/bi-/oligoclonal, E – polyclonal, F - inconclusive). (R1 – rank 1, R2 – rank 2, R3 – rank 3, R4 – rank 4. * PCR fragment clonal but NGS polyclonal; ^ <20,000 total reads; # PCR fragment polyclonal but NGS clonal; & PCR fragment inconclusive/no rearrangement but NGS clonal; @ PCR fragment inconclusive/no rearrangement but NGS polyclonal.)

multiple TCR clonal controls being included on the same run. This problem was avoided in subsequent runs by including only one clonal control per run. Excluding these failed runs, all clonal controls identified the known gene rearrangements as the top two sequences at ≥ 2.5% of total reads; all polyclonal controls identified greater than 10 gene rearrangements, none of which comprised >1% of total reads. Total reads for valid samples ranged from 21,143 to 2,610,644. Total average reads varied somewhat by specimen type, with vitreous fluid having the lowest average total, likely due to paucity of B cells, and TCR libraries had the highest average totals (Fig. S1). Input library concentrations of 100, 200, 300, or 400pM for controls and patient samples consistently yielded total read counts >20,000. To reduce potential contamination from high input libraries, 100pM concentration was used for further studies. Samples yielded one to greater than 50 sequences, with clonal samples yielding a median of 5.6 sequences and polyclonal samples yielding a median of 23.1 sequences.

3.3. Linearity and limit of detection

Two dilution series of clonal controls were used to establish linearity and the limit of detection for clonal populations for IGH and TCR NGS assays. Clonal control DNA (IVS-0019, IGH or IVS-0021, TCR, Invivoscribe, Inc) was diluted into polyclonal control DNA (IVS-0000, Invivoscribe, Inc) at 100%, 30%, 20%, 10%, 5%, 2%, and 1%. Linearity of the dilution series was measured by calculating the correlation coefficient (R^2) of expected versus observed percentage of total reads of the top-ranked merged sequences (Fig. S2). The IGH clonal control contains one gene rearrangement (IGHV1-69_13) and thus the top-ranked sequence comprises a much larger percentage of reads at all dilutions compared to the 2nd rank sequence, which is derived from the polyclonal control diluent. The TCR clonal control contains two rearrangements (Vg11_Jg1/2 and Vg8_Jg1/2) and thus the *sum* of the two top-ranked sequences are nearly equal to the expected percentage, and each comprise a much larger percentage of reads at all dilutions compared to the 3rd rank sequence, which is derived from the polyclonal control diluent. The correlation coefficients were high for the top-ranked sequences for all dilution series (≥0.89). Even at the lowest clonal DNA input (1%, 0.5 ng), the known clonal sequence was consistently detected as the top-ranked sequence for all series, though it was, as expected, below the cutoff of 2.5% of total reads that is recommended by the manufacturer for clonality determination.

3.4. Accuracy of NGS clonality testing compared to PCR fragment analysis

To establish accuracy, TCRG and IGH FR2 and FR3 clonality cases previously assayed using the PCR-based fragment size analysis by capillary electrophoresis were tested with the LymphoTrack NGS assays (IGH FR1 is described in section 3.8). Overall, concordance was high. As shown in Fig. 1A, in specimens with IGH FR2 and FR3 clonal (mono-, bi-, oligoclonal) peaks detected by PCR fragment analysis, LymphoTrack NGS almost always detected dominant clonal sequences, often with greater than 10% of total reads for the top ranked sequence. There were two discordant specimens. In one case, the top-ranked sequences in both FR2 and FR3 comprised less than 2.5% of the total reads, which was below the manufacturer's recommended cutoff for clonality; and in the other case, the total reads were less than 20,000 in both FR2 and FR3. For PCR fragment-determined clonal specimens, the concordance was 22/24 (91.7%). In specimens with IGH FR2 and FR3 detection of polyclonal but not clonal peaks detected by PCR fragment analysis (Fig. 1B), LymphoTrack NGS detected dominant clonal sequences (>2.5% of total reads) in 8/28 specimens. In another four specimens, both FR2 and FR3 had <20,000 total reads, and thus were not interpretable (inconclusive). Thus, for PCR fragment determined IGH FR2 and FR3 polyclonal specimens, the concordance was 16/28 (57.1%). As shown in Fig. 1C, in specimens in which PCR fragment analysis was inconclusive (PCR size control ladder failed) or showed no rearrangement (PCR size control ladder amplified but no IGH rearrangements detected), two cases showed a dominant clonal sequence (>2.5% of total reads) in IGH FR2 or FR3. In five specimens, the FR2 and FR3 NGS assays detected polyclonal sequences (>0 and < 2.5% of total reads), and in four specimens, neither FR2 nor FR3 had >20,000 total reads, and thus were not interpretable. For PCR fragment determined IGH FR2 and FR3 inconclusive/no rearrangement specimens, the concordance was 4/11 (36.4%). Overall, for specimens tested with IGH FR2 and FR3, concordance was 42/67 (62.7%), with discordance in 15 of the 25 discordant specimens (60%) due to detection of clonal or polyclonal sequences that were not detected by PCR fragment analysis.

TCRG concordance was also high. Fig. 1D shows that, in specimens with TCRG (mono-, bi-, oligoclonal) peaks detected by PCR fragment analysis, LymphoTrack NGS almost always detected dominant clonal sequences, often with greater than 10% of total reads for the top ranked sequence. There were five discordant specimens in which the top-ranked sequences comprised less than 2.5% of the total reads, yielding a concordance of 28/33 (84.5%). In specimens with TCRG detection of polyclonal but not clonal peaks detected by PCR fragment analysis (Fig. 1E), LymphoTrack NGS detected dominant clonal sequences (>2.5% of total reads) in 10 specimens, yielding a concordance in 14/24 specimens (58.3%). In specimens in which PCR fragment analysis of TCRG was inconclusive (PCR size control ladder failed), NGS LymphoTrack detected polyclonal TCRG sequences (>0 and < 2.5% of total reads) and >20,000 reads in all cases, yielding 0% (0/6) concordance (Fig. 1F). Overall, for specimens tested with TCRG, concordance was 42/63 (66.7%), with discordance

in all 16/16 discordant specimens (100%) due to detection of clonal or polyclonal sequences by NGS that were not detected by PCR TCRG fragment analysis.

Taken together, for 130 specimens tested by PCR fragment analysis for IGH FR2 and FR3 or TCRG, NGS LymphoTrack clonality showed concordant interpretation in 84 specimens (84/130, 64.6%), with the majority of discordant specimens (31/46, 67.4%) due to detection of clonal or polyclonal sequences by NGS that were not detected by PCR TCRG fragment analysis.

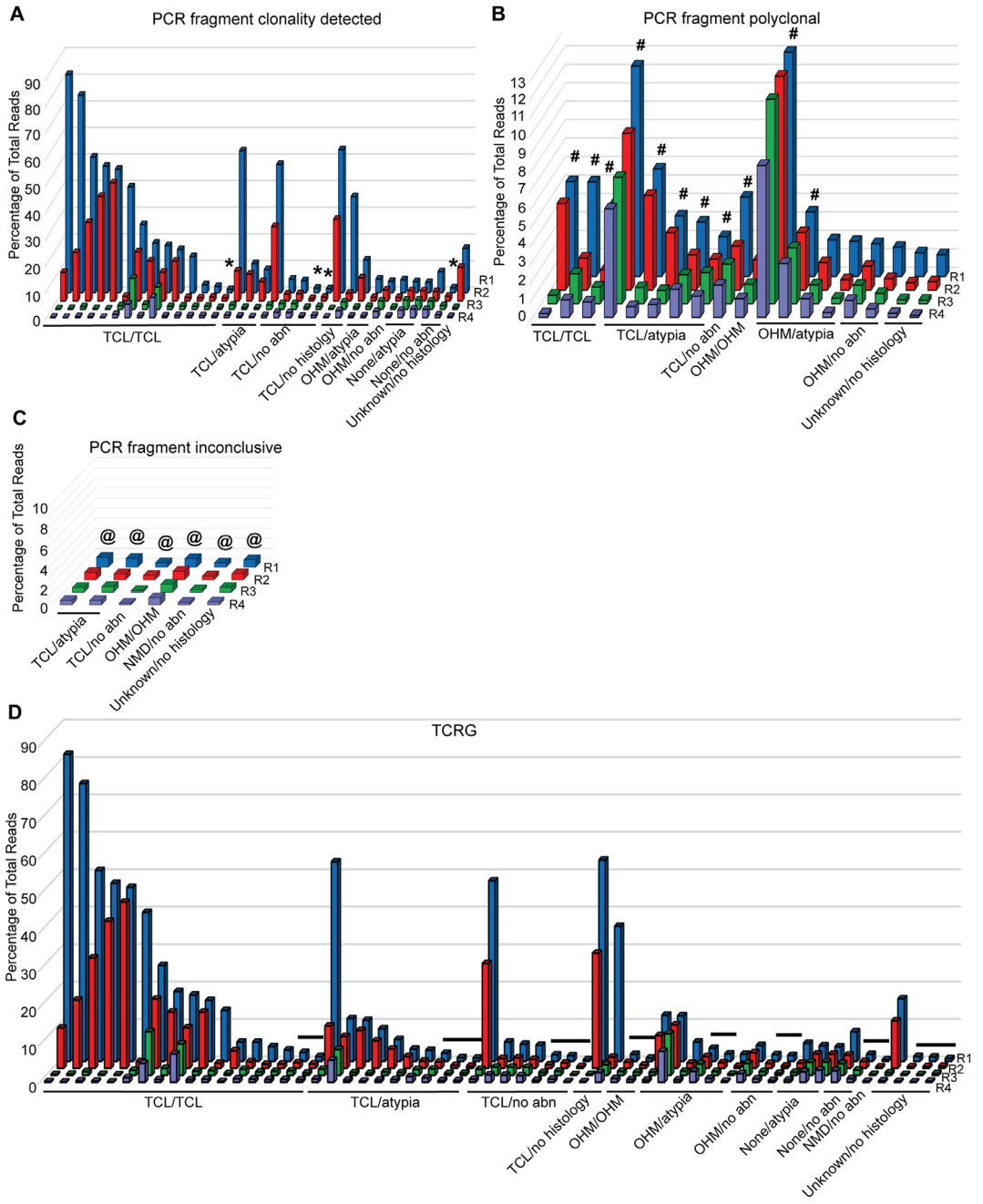


Fig. 2. NGS TCRG clonality results are consistent with clinical and histopathological diagnoses. A-C) Bar graphs show the percentage of total reads for the four top-ranked clonal sequences for NGS TCRG, grouped by their PCR fragment analysis results and sorted by clinical and histopathological diagnoses. (A - mono-/bi-/oligoclonal, B - polyclonal, C - inconclusive or no rearrangement. * PCR fragment clonal but NGS polyclonal; ^ <20,000 total reads; # PCR fragment polyclonal but NGS clonal; & PCR fragment inconclusive/no rearrangement but NGS clonal; @ PCR fragment inconclusive/no rearrangement but NGS polyclonal.) D) Bar graph shows the percentage of total reads for the four top-ranked clonal sequences for NGS TCRG, grouped by their PCR fragment analysis results. (R1 – rank 1, R2 – rank 2, R3 – rank 3, R4 – rank 4; horizontal lines indicate polyclonal NGS results).

3.5. NGS TCRG clonality testing results are consistent with clinical and histopathological diagnoses

We next sought to assess whether clonality testing by LymphoTrack NGS more closely matched the clinical or histopathological diagnoses. Clinical data was missing for a substantial number of IGH cases, so we focused on TCRG testing for which complete clinical and histopathological data was available. Specifically, we considered a relevant clinical diagnosis to include T-cell leukemia or lymphoma, other hematologic malignancy (e.g., AML, MDS), or non-malignant inflammatory disorder (e.g., psoriasis, atopic dermatitis) as determined from the electronic medical record. Histopathological diagnoses were extracted from pathology reports from the same specimen when available. We first evaluated NGS clonality results (≥2.5% of total reads) grouped by PCR fragment assay results (clonality detected, polyclonal, inconclusive/No rearrangement). As shown in Fig. 2A–C, NGS detected clonality in specimens with clinical and histopathology diagnoses of T-cell leukemia/lymphoma (13/14 PCR fragment clonal), even when PCR fragment analysis identified only a polyclonal profile (3/3). In specimens with clinical diagnoses of T-cell leukemia/lymphoma and histopathology showing atypia, NGS detected clonality in 3/3 PCR fragment clonal and 4/4 PCR fragment polyclonal. In specimens with clinical diagnoses of T-cell leukemia/lymphoma and histopathology showing no abnormalities, NGS detected clonality in 3/5 PCR fragment clonal and 1/1 PCR fragment polyclonal. Detection of clonality by NGS was less frequent for specimens without clinical or histopathological diagnoses of T-cell leukemia/lymphoma was mostly in agreement with PCR fragment results. For PCR fragment inconclusive, NGS TCRG detected a polyclonal profile in all specimens. Overall, regardless of grouping by PCR fragment results, NGS TCRG results, using a 2.5% cutoff, were generally consistent with clinical and/or histopathological diagnoses, with clonality detected in the majority of patients or specimens with T-cell leukemia/lymphoma (28/36, 77.8%) and half of those without these diagnoses (11/21, 52.4%) (Fig. 2D). Notably, Fig. 2D also shows that the dominance of the clonal sequences as measured by percentage of total reads is considerably higher in the specimens with clinical and/or histopathological diagnoses of T-cell leukemia/lymphoma (average 19.7%, standard deviation 23.0%) compared to those without either of these diagnoses (average 4.3%, standard deviation 4.4%). Taken together, these results demonstrate that NGS clonality, like other clonality methods, can detect clonal gene rearrangements in specimens with and without malignancy as determined by other diagnostic methods. An advantage of NGS clonality is that individual clonal sequences are quantified and our results using LymphoTrack with Ion S5 sequencing demonstrate that specimens with T-cell leukemia/lymphoma diagnoses generally have substantially higher levels of TCR clonal sequences relative to the polyclonal background, which is consistent with other reports using other NGS methods [7,12,30].

3.6. Evaluating alternative interpretive guidelines for IGH and TCR clonality by NGS

The results of the PCR fragment size assays for IGH and TCR clonality typically are not reported in a binary (i.e., positive/negative) manner because their output is a complex pattern that often includes IGH/TCR rearrangements from normal polyclonal and from clonal populations of lymphocytes [20,25]. The interpretations used in reporting from the BJH MDL, which were based on BIOMED-2 guidelines [25], included clonal, bi-clonal, oligoclonal, polyclonal, no rearrangement, and inconclusive. Clinicians expressed frustration that the results of PCR fragment clonality assays did not provide sufficient information to determine whether a sample contained malignant cells or a recurrence of a previously diagnosed malignancy, or rather an oligoclonal population of non-malignant cells.

While clonality testing alone is not sufficient to diagnose malignancy, implementation of NGS clonality testing offered the opportunity to evaluate new interpretation cutoffs for clonality. We sought to determine whether it was possible to simplify the interpretations and offer more precise reporting of TCR and IGH clonality using the new NGS clonality assays. Per the manufacturer, greater than 2.5% was the cutoff for a “clonal sequence”. However, this simple cutoff did not compare the relative proportions of sequences, which was conceptually a part of the BJH MDL interpretive guidelines for the PCR fragment assay as recommended by the BIOMED-2 guidelines [25]. In addition, recent studies of the LymphoTrack NGS IGH assays with Illumina sequencing demonstrated that addition fold-over-background detection cutoffs could improve specificity, though at the cost of reduced sensitivity [5]. Therefore, we evaluated two possible interpretive guidelines for the NGS clonality assays: Guideline A used a simple cutoff of 2.5% and Guideline B required that a sequence have greater than or equal to three times the number of reads as the second, third, or fourth most prevalent sequence, in order to include mono-, bi-, and oligoclonal patterns in the category of “clonal”. Both guidelines included two interpretations: clonal or polyclonal (Table 3). We compared the conclusions of each guideline to the interpretive results of the previously used IGH FR2 and FR3 and TCRG PCR fragment assays.

As shown in Fig. 3A, the concordance of both Guideline A and B to the PCR fragment assay results was very high for IGH clonal (96% for each, 27/28). For IGH polyclonal, concordance was lower (68% 19/28 for A, 75% 21/28 for B). This was due to a greater number of clonal sequences detected by NGS compared to the PCR fragment assay (32 and 25% of 28 samples reported as polyclonal by PCR fragment assay were clonal by Guidelines A and B, respectively). For TCRG clonal, the concordance for Guideline A was high (85% 28/33) but for Guideline B was 57% (19/33); for polyclonal 58% and 79% (14/24 and 19/24) were concordant for Guidelines A and B, respectively. Similar to the IGH comparison, the difference in concordance for TCRG polyclonal was due to clonal sequences detected in samples reported as polyclonal by PCR fragment.

Table 3
Interpretive guidelines tested for IGH and TCR clonality by NGS.

	Interpretive Guideline A	Interpretive Guideline B
Clonal	≥1 clonal sequence with ≥2.5% of total reads	≥1 clonal sequence with ≥2.5% of total reads AND ≥3 × 2nd/3rd/4th sequence
Polyclonal	No clonal sequences with ≥2.5% of total reads	Does not meet criteria for Clonal

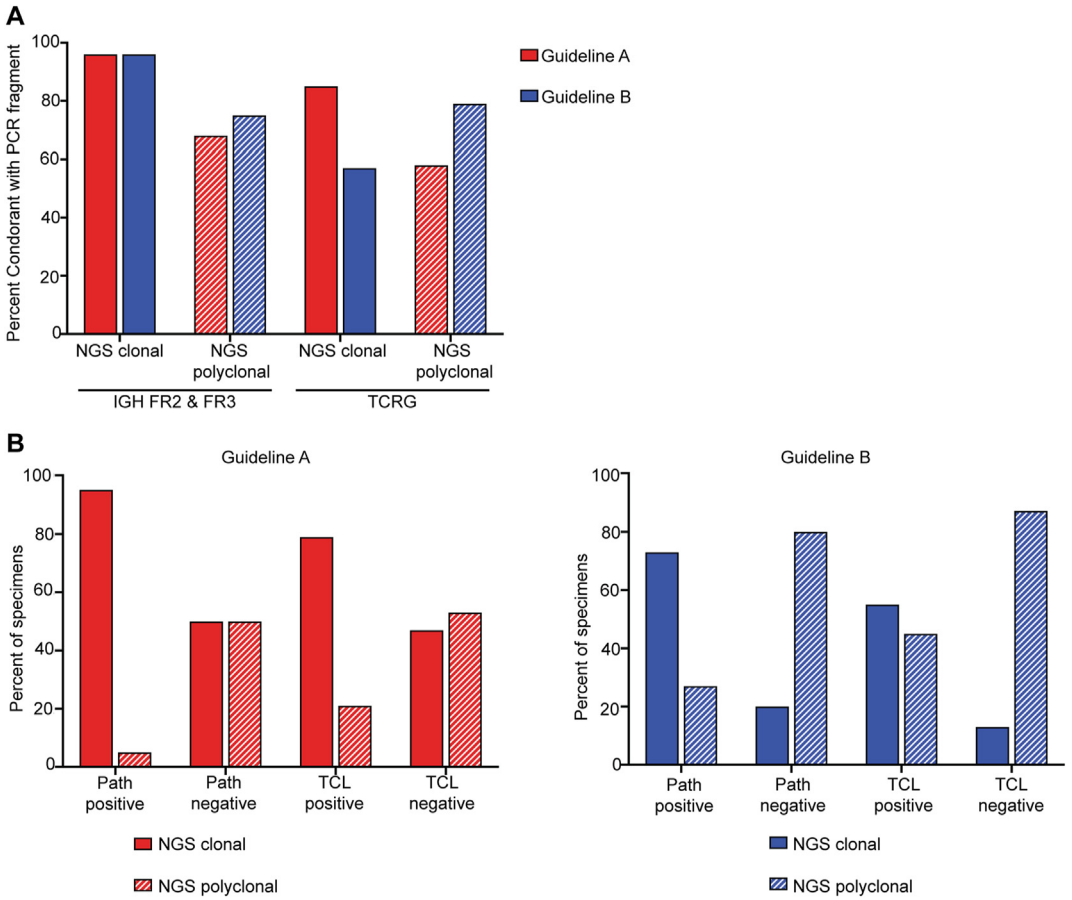
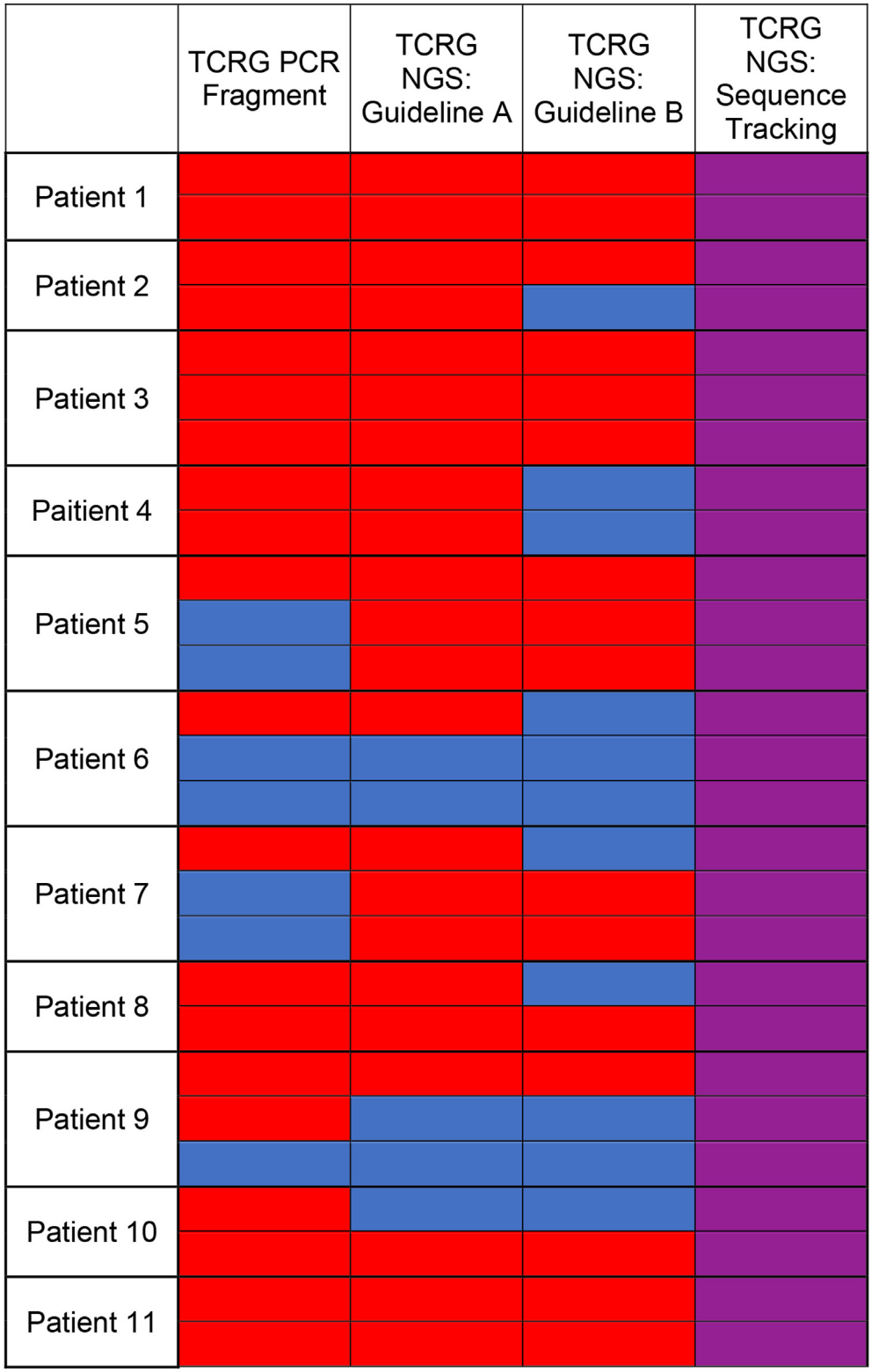


Fig. 3. Comparison of interpretive guidelines for NGS clonality assays. A) Bar graph shows percent concordance with PCR fragment analysis for NGS clonal and polyclonal interpretations for IGH FR2 and FR3 and TCRG using Guideline A or B (Table 3). B) Bar graphs show percent of specimens with NGS clonal or polyclonal interpretations using Guideline A (left panel) or B (right panel) for specimens with histopathological (Path) or clinical (TCL) diagnoses of T-cell leukemia/lymphoma.

Both guidelines demonstrated relatively consistent performance for IGH. However, concordance was markedly lower for TCR. This difference could be due to the fact that the PCR fragment assay is not a perfect gold standard. Alternatively, the two methods may have differential detection sensitivity in different disease states. The Cutaneous T Cell Lymphoma (CTCL) Clinic at WUSM provides the vast majority of specimens for TCR clonality testing and this patient population requires frequent monitoring for disease burden. Moreover, specimens from these patients have a wide range histopathological diagnoses, which, combined with clonality results, contribute to therapeutic decision-making. Ideally, clonality results should be relatively consistent with histopathological diagnoses [7,12]. Therefore, we next compared these two interpretive guidelines to the clinical and histopathological diagnoses of the tested specimens.

We evaluated whether NGS results corroborated the pathology reports (when available) and/or were consistent with disease status. To accomplish this, we extracted the diagnostic lines from pathology reports from validation specimens and determined clinical diagnoses for each patient from the electronic medical record. For simplicity, we converted these to binary categorical variables. Diagnostic lines from matched pathology reports reporting any T-cell leukemia/lymphoma were coded “Path positive”; non-malignant diagnoses (e.g., atypical T-cell infiltrate, dermatitis) were coded “Path negative”. Similarly, any patient diagnosed with a T-cell leukemia/lymphoma was coded “TCL positive”; non-T cell malignancies (e.g., B cell or myeloid malignancies or non-malignant disease) were coded “TCL negative”.

As shown in Fig. 3B, TCRG NGS interpretations using Guideline A were more often clonal when T cell malignancy was identified in matched histopathology reports (21/22, 95%) or the patient had known T-cell leukemia/lymphoma (30/38, 79%). When pathology was negative or T-cell leukemia/lymphoma was not diagnosed in the patient, using Guideline A resulted in a polyclonal interpretation in about half of cases (15/30, 50%; 8/15, 53%). The total numbers of specimens for each comparison are different because matched pathology reports were not available for all those subjected to NGS clonality. In contrast, interpretations following Guideline B were less often clonal when T-cell leukemia/lymphoma was identified in matched histopathology reports (16/22, 73%) or the patient had known T-cell leukemia/lymphoma (21/38, 55%). When pathology was negative or T-cell leukemia/lymphoma was not diagnosed in the patient, Guideline B reported polyclonal results in the majority of cases (24/30, 80%; 13/15, 87%). In sum, use of a fold-over-background detection cutoff for TCRG improved specificity, though at the cost of reduced sensitivity, when compared to clinical or histopathologic



Red: clonal
Blue: polyclonal
Purple: ≥ 1 common sequence

(caption on next page)

Fig. 4. NGS clonality detects clonal sequences in matched patient samples. Eleven patients had two or more specimens with NGS results. All patients had at least one common sequence detected in every specimen.

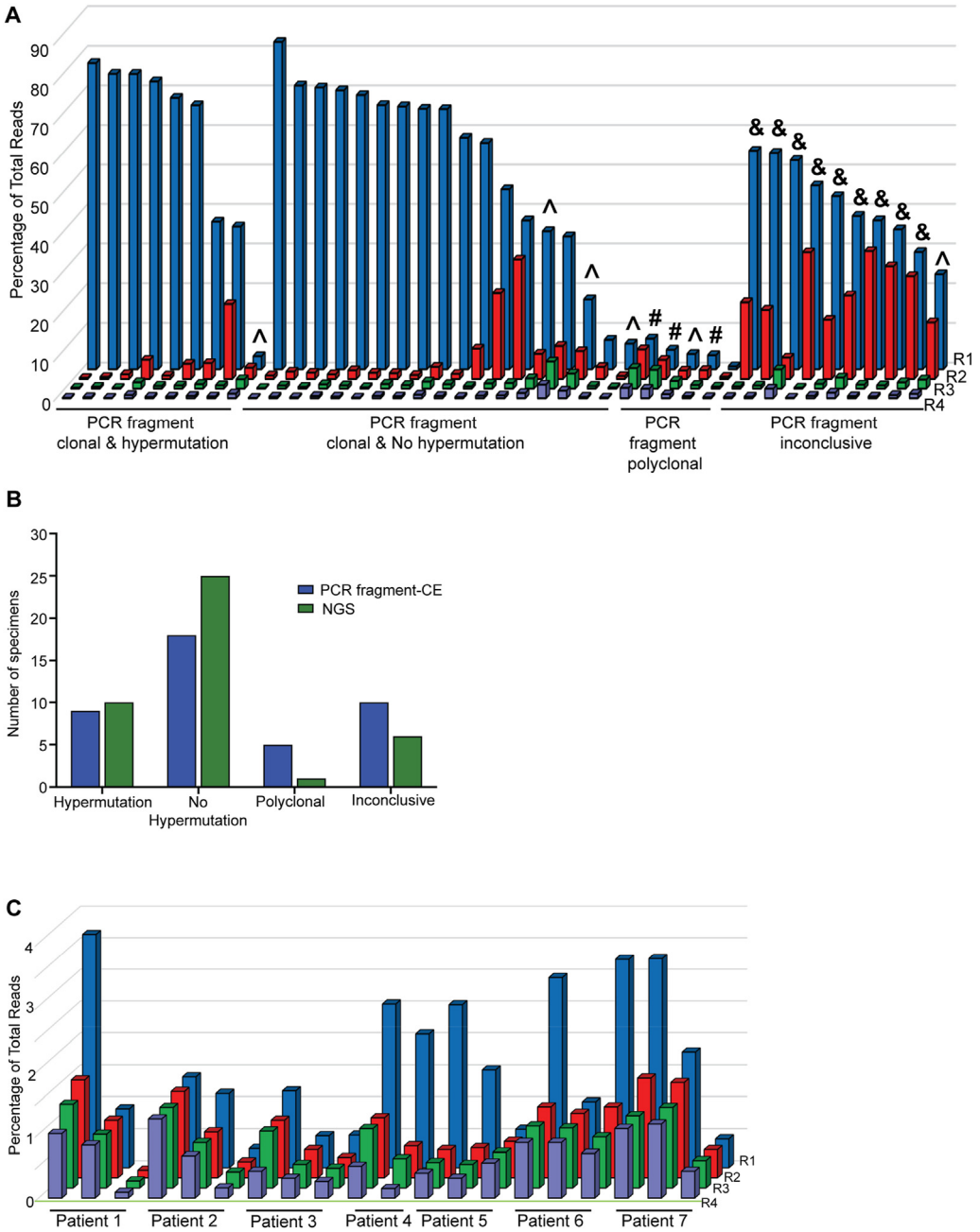


Fig. 5. NGS improves determination of somatic hypermutation status. A) Bar graph shows the percentage of total reads for the four top-ranked clonal sequences for NGS IGH FR1, grouped by their PCR fragment and capillary electrophoresis sequencing hypermutation analysis results. (R1 – rank 1, R2 – rank 2, R3 – rank 3, R4 – rank 4. ^ <20,000 total reads; # PCR fragment polyclonal but NGS clonal; & PCR fragment inconclusive/no rearrangement but NGS clonal.) B) Bar graph shows the number of specimens assayed by PCR fragment capillary electrophoresis sequencing or NGS separated by result category for IGH somatic hypermutation (SHM). C) Bar graph shows the percentage of total reads for the four top-ranked clonal sequences for NGS IGH FR1 for the 7 patients with serial samples tested by NGS IGH FR1 and SHM.

diagnosis.

3.7. NGS TCRG sequences are consistently detected in multiple samples from the same patient

Another important advantage of NGS clonality assays is that the sequences of gene rearrangements are detected and quantified, which is more specific than PCR fragment size and enables more precise tracking of previously identified clones. For CTCL, comparison of previously identified clones to those identified in subsequent biopsies is frequently requested and informs clinical decision-making. In our validation study, there were 11 patients with two or more specimens with TCRG NGS clonality results. Three of these patients had concordant interpretations for all specimens and all testing methods. However, for all 11 patients, there was at least one common TCR sequence detected in every specimen. While the common sequence for each patient sometimes fell below interpretive cutoffs for clonality, its identity and percentage of total reads could be determined in every specimen (Fig. 4). Thus, a clonal TCRG sequence can be tracked and compared across different biopsy sites and timepoints.

3.8. NGS method is more robust for detection of IGH somatic hypermutation than PCR fragment analysis

We used the LymphoTrack IGH FR1 assay and analysis with the LymphoTrack IGH SHM PGM software to determine the somatic hypermutation status in chronic lymphocytic leukemia (CLL) samples (hypermutation = greater than 2% of nucleotides mutated). We tested 42 clinical specimens and compared to results of the previously reported IGH FR1 PCR fragment-capillary electrophoresis sequencing (CE) and analysis through IMGT and IgBlast to determine the mutation rate. The results are shown in Fig. 5. Similar to NGS FR2 and FR3, half of samples with PCR fragment polyclonal results (3/6) had detectable clonality and hypermutation status by NGS (Fig. 5A). In addition, 10 samples did not have interpretable sequence from the PCR fragment capillary electrophoresis sequencing, usually due to the presence of two PCR fragments of similar size that could not be effectively separated by gel electrophoresis and caused overlapping sequencing electropherograms. In 9/10 (90%), NGS returned interpretable sequence and hypermutation status was determined (3/9 hypermutation positive). In the remaining 26 that were PCR fragment clonal with or without hypermutation by capillary electrophoresis sequencing, 22 were concordant by NGS, one sample with PCR fragment capillary electrophoresis sequencing hypermutation was below the cutoff by NGS (1.76%), and three were inconclusive due to insufficient total reads (Fig. 5A–B). Taken together, the NGS assay was generally concordant with the PCR fragment capillary electrophoresis sequencing assay, but importantly it enabled assignment of hypermutation status in an additional 12 samples.

3.9. Clonal sequences identified by NGS may not be consistent between testing platforms

For acute lymphocytic leukemia and multiple myeloma, disease burden monitoring via quantitation of Ig clonal sequences has become increasingly important for prognostic assessment and clinical decision-making [17,18,31,32]. To evaluate LymphoTrack for monitoring of clonal sequences, we subjected 2–3 matched samples from each of 7 multiple myeloma patients (20 total samples) to the LymphoTrack FR1 assay and MRD software analysis (Fig. 5). In 6/7 patients, matched clonal sequences were detected in all samples (Table S1). The samples had also been tested by the clonoSeq assay from Adaptive Biotechnologies. However, LymphoTrack sequences from only 1/7 patients matched sequences from clonoSeq assays (Tables S2–3). This disparity may have been due to technical differences in the methods, further addressed in the Discussion.

4. Discussion

Here we have shown that the results of NGS LymphoTrack (Invivoscribe) assays sequenced with the Ion Torrent S5 instrument compare favorably with PCR fragment analysis, demonstrating high concordance with the PCR fragment results, histopathology diagnoses of specimens, and disease diagnoses of the patients tested. Successful detection of clonal sequences at 1% (0.5 ng) of input DNA indicates that LymphoTrack NGS clonality testing on the Ion Torrent S5 is more sensitive than PCR fragment analysis, consistent with other reports using Illumina sequencing platforms [5,7,12–14]. Because of this sensitivity and the inherent ability of NGS to return and quantitate clonal sequences, NGS clonality testing may be used for detection of a known clonal sequence that is present in a very small percentage of cells, such as when multiple specimens from the same patient are taken simultaneously or when a follow-up specimen is tested in a patient being evaluated for relapse. Indeed, this study demonstrates that LymphoTrack with Ion S5 sequencing can be used to identify and quantify specific clonal sequences in matched and/or follow-up specimens from the same patient as with other NGS platforms [5,7,10,19].

In cases in which NGS and PCR fragment assays were not concordant, there were several patterns. The majority of discordance was in PCR fragment polyclonal or inconclusive cases in which the NGS assay identified clonal or polyclonal patterns, respectively, consistent with previous reports of NGS being more sensitive than PCR [7,12,13,19,30]. In a small number of PCR fragment clonal cases, LymphoTrack IGH or TCRG identified only a polyclonal background. These cases were reproducible as they also showed polyclonal patterns upon repeating the assay. One possible reason for the discrepancy is that multiple rearrangements with the same amplicon size could yield the same peak size and give the appearance of a clonal peak by PCR fragment analysis while NGS would return individual sequences for each rearrangement. Given that the amplified regions for the NGS and PCR assays are very similar, it is less likely that PCR detected a rearrangement that NGS could not. With any of the samples tested, it is possible that assaying for IGK or TCRB rearrangements would have identified additional clones. Studies have shown some increase in clonality detection by adding these assays [2,3,30,33]. Unfortunately, they were not available from Invivoscribe for the S5 platform at the time of this study.

IGH and TCR clonality assays are challenging to report in a binary (i.e., positive/negative) manner because their output is a complex pattern composed of rearrangements from normal polyclonal and clonal populations of lymphocytes [20,25]. Consortia such as BIOMED-2 [25] and EuroClonality-NGS working group [13,14,19] have established guidelines to standardize reporting, but these are still complex. To determine if a simplified reporting strategy could address these issues, we tested two interpretive guidelines in which only clonal or polyclonal interpretations were reported, with either the manufacturer-recommended 2.5% of total reads cutoff (A) or the 2.5% cutoff plus a fold-over-background cutoff (B). We found that addition of the fold-over-background cutoff increased specificity but at a cost of decreased sensitivity, which has been reported by other groups using other NGS platforms [10], suggesting that this effect is not limited to the Ion S5 sequencing platform. The reduction in sensitivity for fold-over-background cutoffs raises concern for missed diagnoses, which is concerning especially given recent reports of the importance of early detection of TCR clonality in cutaneous T cell lymphoma [7,12,34]. Moreover, NGS testing returns the clonal sequences themselves, which can be used to compare multiple biopsy sites. Detection of the same clonal sequence in multiple biopsy sites simultaneously and/or over time may be more clinically important than its dominance in the polyclonal background and increases the likelihood that it is a malignant clone. Such precision is a major advantage of NGS clonality testing. Thus, we would argue that tracking of clonal sequences should be incorporated into routine reporting.

NGS also provides a more robust assay methodology for measuring somatic hypermutation of IGH in CLL. The previous PCR fragment plus capillary sequencing assay failed to provide interpretable sequence in nearly 40% of validation samples, 75% of which were successfully sequenced and hypermutation status determined by the NGS assay. In contrast, it was not possible to effectively assess a comparison of the LymphoTrack assay to the clonoSeq (Adaptive) assay for evaluation of clonal sequences in multiple myeloma samples. While matching clonal sequences were detected by the LymphoTrack assay in nearly all matched patient samples, comparison to clonoSeq sequences showed concordant sequences in only 1/7 patients. This disparity could be due to technical differences, including different primers, which are proprietary for clonoSeq. The lack of concordance could also be due to differences in the length of the reported sequences. The clonoSeq reports listed 12–44 nucleotide fragment sequences while the LymphoTrack sequences were >100 nucleotides. Unfortunately, there was insufficient material for additional testing with a third sequencing platform, such as Illumina.

In sum, our study demonstrates that the LymphoTrack assays with Ion Torrent S5 sequencing 1) can be used successfully for IGH and TCR clonality with reproducible results; 2) generates and quantifies clonal sequences to enable highly precise comparison of samples; 3) are substantially more sensitive than PCR fragment and return clonality results in specimens that failed PCR fragment assays; and 4) the TCRG assays are highly concordant with clinical and histopathologic diagnoses. Taken together, the LymphoTrack with Ion S5 NGS clonality assays offer a sensitive and precise method for diagnostic testing and disease monitoring in B and T cell cancers.

Acknowledgements

We would like to thank Daniel Weber for pathology and clinical records searches and Rodney Kowalewski for excellent technical work on the validation assays.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2020.e00191.

Funding

This research received support from the Department of Pathology at Washington University School of Medicine in St. Louis.

Conflict of interests

The authors state that they have no competing interests to declare.

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