A Comparison of Deep Sequencing of TCRG Rearrangements vs Traditional Capillary Electrophoresis for Assessment of Clonality in T-Cell Lymphoproliferative Disorders

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Key Words: Next generation sequencing; TCRG; Clonality; Lymphoma; Leukemia

ABSTRACT

Objectives: To design and evaluate a next-generation sequencing (NGS)–based method for T-cell receptor γ (TCRG) gene-based T-cell clonality testing on the Ion Torrent Personal Genome Machine (Life Technologies, Carlsbad, CA) platform.

Methods: We analyzed a series of peripheral blood, bone marrow, and formalin-fixed paraffin-embedded tissue specimens with NGS vs traditional capillary electrophoresis methods.

Results: Using a custom analysis algorithm that we developed, our NGS assay identified between 2,215 and 48,222 unique TCRG rearrangements in a series of 48 samples. We established criteria for assigning clonality based on parameters derived from both the relative and absolute frequencies of reads. In a comparison with standard capillary electrophoresis, 19 of 19 polyclonal samples and 24 of 27 samples that appeared clonal were in agreement. The three discrepant samples demonstrated some of the pitfalls of amplicon length–based testing. Dilution studies with T-lymphoid cell lines demonstrated that a known clonal sequence could be routinely identified when present in as few as 0.1% of total cells demonstrating suitability in residual disease testing. A series of samples was also analyzed on a second NGS platform and yielded very similar results with respect to the frequency and sequence of the clonal rearrangement.

Conclusions: In this proof-of-concept study, we describe an NGS-based T-cell clonality assay that is suitable for routine clinical testing either alone or as an adjunct to traditional methods.

Upon completion of this activity you will be able to:

- list the various strategies commonly employed for evaluation of T-cell clonality.
- describe the major differences between capillary electrophoresis (CE)–based T-cell clonality analysis and next-generation sequencing (NGS)–based T-cell clonality analysis.
- define the general criteria used for assessment of T-cell clonality by CE and NGS.

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Polymerase chain reaction (PCR)–based clonality testing is an essential component of the workup of lymphoproliferative disorders, particularly those of T-cell origin, which may lack immunophenotypic markers of clonality. Clinical laboratory testing strategies have evolved from primarily Southern blot–based assays to primarily PCR-based assays using a mixture of consensus primers designed to amplify the majority of possible unique V(D)J rearrangements. Assays targeted to the T-cell receptor γ (TCRG) gene, which rearranges early in T-cell development¹ and is thus present in a clonally rearranged state in most T-cell neoplasms,² and the T-cell receptor β (TCRB) gene, which has increased complexity because of the presence of a D segment, are the primary targets for clinical clonality assays.³,⁴ Combined use of TCRG and TCRB assays may further enhance diagnostic usefulness in suspected T-cell lymphoproliferative disorders.⁵
Currently, most T-cell clonality assays are relatively simple processes that use multiplex PCR followed by capillary electrophoresis (CE)-based resolution of the resulting amplicons. The overall spectrum of rearrangements, resolved by size, is then assessed for the presence of a dominant clonal peak by comparing it with the polyclonal background. However, these methods are subject to inherent problems and remain somewhat subjective in their interpretation. With the decreased cost and widespread adoption of massively parallel sequencing technologies (so-called next-generation sequencing [NGS]), it has become more commonplace to perform NGS in the clinical hematology laboratory, including for purposes of clonality assessment. Because NGS can provide quantitative assessment of sequence data, conversion of clonality assays to NGS is likely to provide a useful adjunct for both (1) the initial evaluation and diagnosis of a T-cell lymphoproliferative disorder and (2) the monitoring of a previously identified neoplastic population over time during treatment. Importantly, the ability to identify clonal populations at very low levels, theoretically down to single copy numbers, provides a marked advantage over current amplicon length–based methods for the detection of residual disease.

In an effort to reevaluate T-cell clonality testing in the context of NGS technology, we designed and tested an NGS-based method that combines multiplex PCR amplification of the TCRG repertoire with sequencing. Our principle goals were to establish a technique that reduces interpretive subjectivity and is effective for detecting residual disease. Furthermore, we sought to develop an analysis algorithm suitable for use with data derived from the Ion Torrent (IT) platform (Life Technologies, Carlsbad, CA), which generally demonstrates a superior performance to 50 ng/µL in TE buffer (Integrated DNA Technologies, Coralville, IA).

Patient Samples and Cell Lines

The study procedures were approved by the University of Utah Institutional Review Board. Twenty-seven samples that were previously positive for T-cell clonality in electrophoresis-based clinical testing (14 peripheral blood [PB], eight bone marrow [BM], and five formalin-fixed paraffin-embedded tissue [FFPE] samples) were obtained from our archives. PB samples from ten normal donors and nine previously analyzed polyclonal clinical samples (five BM and four FFPE samples) were obtained from our archives for a total of 19 polyclonal samples. Two cases that previously demonstrated oligoclonality (one PB and one BM sample) were also analyzed. Three human T-lymphoid cell lines (JURKAT, H-SB2, and CCRF-CEM) were obtained from ATCC (Manassas, VA) and cultured in RPMI 1640 (ATCC) supplemented with 10% fetal calf serum (HyClone, Logan, UT) and penicillin/streptomycin solution (Life Technologies) at 37°C and 5% carbon dioxide.

Dilutions of T-cell lines into polyclonal T cells were prepared using peripheral blood mononuclear cells (PBMCs) isolated from 120 mL of PB from a normal donor using Ficoll density centrifugation (GE Healthcare, Piscataway, NJ) following the manufacturer’s recommendations. T cells were quantified in the PBMC samples using flow cytometry by staining with anti-CD3 (BD Biosciences, Franklin Lakes, NJ) following the manufacturer’s recommendations. T cells were subsequently diluted in polyclonal T cells to 20%, 10%, 2.5%, 0.5%, 0.1%, 0.02%, 0.004%, and 0.0008%.

Genomic DNA was isolated using the Puregene DNA extraction kit (Qiagen, Valencia, CA) per the manufacturer’s recommendations. Following purification, DNA was analyzed for purity and concentration using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) and diluted to 50 ng/µL in TE buffer (Integrated DNA Technologies, Coralville, IA).

TCRG Repertoire PCR

Each sample was subjected to PCR amplification of the TCRG repertoire using a modified version of the assay described by Greiner et al. The assay uses PCR primers that target consensus regions of Vγ2-Vγ11 and Jγ1, Jγ2, and Jγ2 gene segments in a multiplex reaction that only amplifies TCRG loci that have undergone rearrangement, resulting in products that range in size from 160 to 215 base pairs (bp). The J primers were labeled at the 5’ end with FAM (Integrated DNA Technologies). All samples were amplified in two separate reactions and then resolved using CE as follows. Twenty-microliter PCR reactions containing 50 ng of genomic DNA, 1× HF buffer (New England Biolabs, Ipswich, MA), 0.2 mmol/L dNTPs (Idaho Technology, Salt Lake City, UT), 0.6 µmol/L of each primer (Integrated DNA Technologies), and 2 U of Phusion HS DNA polymerase (New England Biolabs) were amplified on an Applied Biosystems 9700 thermocycler (Foster City, CA) using the following cycling conditions: 95°C (30 seconds) followed by 30 cycles of 95°C.
(30 seconds), 60°C (30 seconds), and 72°C (10 seconds plus 1 additional second per cycle). Following amplification, 1 µL of the labeled PCR products was mixed with 9 µL of HiDi formamide (Applied Biosystems) and 0.5 µL of GeneScan ROX 350 internal-size standards (Applied Biosystems) and heated to 95°C for 2 minutes. The samples were run on an ABI 3130xl Genetic Analyzer (Applied Biosystems) using 36-cm capillaries and a POP-7 polymer. The samples were injected at 2 kV for 5 seconds and run at 15 kV for 950 seconds at 60°C. PCR products and internal standards were detected using filter set D. Raw data were analyzed with GeneMapper v4.0 software (Applied Biosystems). The second reaction was designed to amplify the TCRG repertoire for subsequent NGS analysis using the same conditions as described earlier, with unlabeled primers and quadruplicate reactions (totaling 200 ng of genomic DNA/specimen). Reaction products were then pooled and purified using the MiniElute PCR Purification Kit (Qiagen) per the manufacturer’s instructions, with a final elution volume of 30 µL. Concentrations were determined using a NanoDrop spectrophotometer.

Construction of IT Sequencing Libraries

Five hundred ng of amplicon was used for IT adapter ligation and PCR amplification using the NEBNext Fast DNA Library Prep Set for Ion Torrent (New England Bio-labs) following the manufacturer’s instructions. The universal library adaptors provided in the kit were substituted with 1 µL of IonXpress barcode adapter plus 1 µL of P1 adapter (Life Technologies). The adaptor-ligated library was fractionated by size according to the protocol for 200-bp sequencing reads using AMPure XP Beads (Beckman Coulter, Indianapolis, IN) and was PCR amplified for four cycles. A 1,000-fold dilution of the final library was quantified using the Library Quantification Kit–Ion Torrent/LightCycler 480 (Kapa Biosystems, Woburn, MA).

Emulsion PCR and IT Sequencing

Equimolar quantities of up to 12 sequencing libraries with different barcoded adapters were mixed, and an absolute amount of 2.8 × 10^8 molecules was used for emulsion PCR using the Ion OneTouch 200 Template Kit v2DL on the Ion Personal Genome Machine (PGM) OneTouch System (Life Technologies). The resulting template-positive ion spheres were then sequenced using an Ion 316 chip (5 × 10^6 possible reads; Life Technologies) and the Ion PGM 200 Sequencing Kit with 500 nucleotide flows on the IT PGM (Life Technologies).

Library Preparation and Sequencing on the MiSeq Platform

Approximately 350 ng of PCR product from the TCRG repertoire PCR outlined before (as measured by Qubit, Life Technologies) averaging 180 to 200 bp in size was first A-tailed with Klenow DNA polymerase at the 3’ end, followed by ligation of Illumina sequencing adapters (San Diego, CA). Library fragments were then purified (AMPureXP beads, Beckman Coulter) and analyzed for adequate ligation on an Agilent 2100 bioanalyzer (Santa Clara, CA). Limited-cycle PCR (10 cycles) with sample-specific, 7-bp index-tagged primers was then performed to enrich for ligation products. Ten indexed libraries were then pooled in equimolar amounts and diluted according to the manufacturer’s recommendations to ensure adequate cluster density. Library pools were then sequenced on an Illumina MiSeq with 2 × 150 bp paired end reads. Base calls were generated using Casava software (v1.7; Illumina) and the resulting FASTQ files analyzed as described below.

Analysis of TCRG Repertoire Data

Following sequencing, FASTQ files from each barcoded library were analyzed using a custom software package that we named “TCRDriver” (ARUP Laboratories, Salt Lake City, UT) Figure 1. TCRDriver first assigned a V and J gene segment to each read by aligning the reads to the International

Cys, cysteine; IMGT, International ImMunoGeneTics; Phe, phenylalanine.
ImMunoGeneTics information system (IMGT) reference database (http://www.imgt.org)\textsuperscript{12} using a seed-and-extend–style algorithm.\textsuperscript{13} First, matches were initiated between the references and read using 10-bp subsequences or “seeds.” Next, the matches were extended using a dynamic programming step that allowed small gaps and mismatches. The reference with the highest score was assigned to the read. The seed-and-extend approach was chosen for speed and its ability to identify the \( V \) and \( J \) gene segment terminus within the read. Once the \( V \) and \( J \) gene segments were assigned, the junction sequence was extracted from the read, defined by IMGT as the region between the conserved cysteine in the \( V \) gene and the conserved phenylalanine in the \( J \) gene.

Once the junction sequence was identified, the reads were clustered into groups. Reads were first segregated by their \( V \) and \( J \) gene segment assignments. Identical junction sequences were collapsed and sorted by the number of observations. The most commonly observed junction sequence was then compared with all other junction sequences using the Needleman-Wunsch algorithm.\textsuperscript{14} If the identity score of the alignment was 95 or greater, the junction sequences were merged. Homopolymer mismatch runs were weighted down when calculating identity scores. This process was repeated with the next most commonly observed junction sequence until all junction sequences were clustered. The analysis flowchart is shown in Figure 1.

Results

Comparison of Sequencing Data Derived From the IT With Clonality Analysis Using CE

Multiplex TCR\( \gamma \) PCR products were generated and subjected to clonality analysis via IT-NGS, followed by data processing using TCRDriver, and compared with CE. We considered cases positive for clonality using CE when a dominant peak was at least 2 times the height of the highest background peak as previously described.\textsuperscript{10} In total, 27 cases that were clonal and 19 cases that were polyclonal using CE were analyzed. We also included two cases that appeared oligoclonal on CE. Representative clonal and polyclonal cases are shown in Figure 2A and Figure 3A. Data sets for all cases demonstrated between 2,215 and 48,222 unique sequence clusters (see the “Materials and Methods” section), with a high percentage of clusters (35.55%-54.52%) containing only a single read (data not shown). Figures 2 and 3 show only the ten most abundant sequence clusters detected with NGS for each case.

It is evident that clonality can be determined from NGS data in a fashion similar to that used for CE data by a comparison with the polyclonal background. In 24 of 27 cases that were clonal on CE, NGS data showed that the presumed clonal rearrangement(s) ranged from 4.6- to 225-fold above background. The largest background cluster was defined as the third most abundant rearrangement to account for the possibility of either monoallelic or biallelic clonal rearrangements (Figure 2). The \( V \) segment identity of clonal peaks determined with NGS always corresponded to that inferred with CE when amplified by separate \( V \) primers instead of multiplexing (data not shown). Based on these results, a cutoff value of approximately fourfold higher than the background for NGS data would yield concordant results between the two methods in 43 of 46 cases interpreted as clonal or polyclonal with CE. The most abundant cluster in the 19 cases that were polyclonal was no more than 2.69-fold higher than the background (or third) cluster. Two cases that were interpreted as oligoclonal on CE (Figure 4, indeterminate [ind] samples 1 and 2) showed a clear difference compared with the polyclonal cases, as the top reads in the oligoclonal cases make up a much higher percentage of the total NGS reads (7.11%-8.95% of total reads) compared with the polyclonal cases (0.47%-2.04% of total reads). However, there was no clear distinction between the top one or two clusters and the background to confirm clonality. In the cases that were clonal on NGS using the criteria that a clonal cluster(s) must be at least fourfold more abundant than the background, the most abundant read(s) ranged from 5.89% to 94.88% of total reads. We therefore interpreted ind 1 and ind 2 as “indeterminate” with NGS. We suggest that for a case to be interpreted as clonal, the top one or two clusters must meet the criterion of being fourfold higher than the background and must comprise more than 4.5% of the total reads. The indeterminate category is reserved for those cases that meet only one of the two criteria and, as we have shown, are clearly different from the truly polyclonal cases when looking at the NGS data. However, the clinical significance of the “indeterminate” cases (or those that would have been interpreted as oligoclonal on CE) is unknown.

Based on these criteria, the three discrepant cases that were clonal on CE but not NGS were categorized as indeterminate on NGS (Figure 4, ind 3, ind 4, and ind 5). At least two of these three cases illustrate an important drawback of fragment size–based clonality testing that can be resolved with NGS. In ind 3, the rearrangements in clusters 1, 3, and 8 all have the same length (193 nucleotides [nt] on NGS, 191 nt on CE) and, when combined (as in CE analysis), form a single, apparently clonal peak. In ind 4, the rearrangements in the two most abundant clusters have the same length (203 nt on NGS, 198 nt on CE) and also result in a false clonal peak on CE (Figure 4).

Of the 24 concordant clonal cases, five of six cases that appeared to harbor monoallelic rearrangements on CE demonstrated one dominant unique sequence on IT-NGS. In one case that appeared monoallelic on CE, NGS demonstrated
that there were actually two rearrangements differing by 1 nt in length, which were poorly resolved with CE. Similarly, 17 of 18 cases that appeared clearly biallelic on CE demonstrated two dominant unique sequences on IT-NGS. One case appeared biallelic on CE because of an apparently dominant peak near the center of the polyclonal background. However, NGS demonstrated only a single dominant rearrangement. In occasional biallelic cases, the second peak on NGS only met one of the two criteria for clonality; therefore, the criteria for calling a second clonal peak of a case with biallelic rearrangements were not defined.

As outlined, NGS- and CE-based analyses were both assessed for clonality in terms of fold increase of the dominant rearrangement(s) over background. This value was higher on IT-NGS analysis in 23 of 24 cases. In the single case in which the CE data apparently indicated a greater fold increase, the NGS analysis showed a lower fold increase.

Figure 2 Comparison of capillary electrophoresis (CE) data and next-generation sequencing (NGS) data for three representative clonal samples. The relative frequency, in terms of fold-over background, is indicated for clonal peaks in the CE data and clonal sequence clusters in the NGS data. Only the top 10 sequence clusters, with percentage of total reads, is shown for the NGS data. Sample types are shown at left: FFPE, formalin-fixed, paraffin-embedded; nt, nucleotides.
frequency of the clonal sequence than the NGS data, the clonal CE peak height is overestimated because of the presence of multiple small rearrangements of identical length (188 and 193 nt on NGS).

Both NGS and CE had consistent findings on follow-up testing. Clonal 5 (PB) and clonal 21 (BM) were from the same patient, separated by more than 60 days, and each showed the same biallelic rearrangements. Clonal 7 (PB) and clonal 18 (FFPE) were also from the same patient, separated by 81 days, and also showed the same rearrangement. The remaining cases were all from different patients (data not shown).

**Comparison of Sequence Based Clonality Using the IT With Results Derived Using the Illumina MiSeq Platform**

We sought to determine if the assay results obtained with IT-NGS were comparable with results obtained on another sequencing platform, the Illumina MiSeq. We compared both the raw results and the results after clustering using...
TCRDriver. In total, 14 cases that were clonal on CE and that were analyzed on the IT platform were also analyzed on the MiSeq platform. Two cases that were polyclonal on CE were also compared. The data from a representative clonal and polyclonal case analyzed on both platforms are shown in Figure 5. We noted a much higher error rate in the IT than in the MiSeq data, as observed previously in other studies, which mostly appears to be a consequence of errors in the sequencing of homopolymers. In the IT data, the higher error rate manifests as multiple, highly similar subclusters of sequences containing one or two deleted bases compared with the most common sequence (Figure 5). Because these sequences were not present in the MiSeq data, they were considered “error” reads, validating our criteria for collapsing highly similar IT reads into a single final cluster with the TCRDriver algorithm. The frequency of the total reads present in the final clusters, containing all of the collapsed subclusters, was very similar between the two platforms Table 1. In every clonal case, the most common one or two clusters were the same on the two platforms. However, in the case of biallelic rearrangements,
they were not always in the same one to two order. In the two cases that were polyclonal on CE, the most common cluster was the same in one case (Figure 5) but differed in the other.

Effect of PCR on the Representation of the TCRG Repertoire in Sequencing Data

In this study, sequencing libraries were constructed from amplicons derived from multiplex PCR reactions. However, the number of PCR cycles may theoretically change the relative abundance of the rearrangements present in the mixture. To explore this possibility, we varied the PCR cycle number and then performed IT-NGS on two cases positive for clonal rearrangements.16 The lower dilutions (20% and 10%) mimic a clinical sample that overtly demonstrates clonality, whereas the higher dilutions mimic a sample with low-level residual disease. The lower dilutions show an approximate limit of detection of clonality at 10% clonal T cells following the criteria we set of at least 4.5% of total reads (Table 2) and at least fourfold over background (data not shown). In the higher dilutions, we found that we could reproducibly detect the presence of the clonal TCRG rearrangements in all of the 0.1% dilutions and in dilutions as low as 0.004%. Therefore, we conservatively assigned a sensitivity of 0.1% to this assay for detecting a previously sequenced clone.

Sensitivity for Detection of Minimal Residual Disease

To evaluate assay sensitivity, we created a series of dilutions of cell lines into normal polyclonal T cells (Table 2l). The T-lymphoid cell lines chosen, JURKAT, H-SB2, and CCRF-CEM, have well-characterized T-cell receptor gene rearrangements. The lower dilutions (20% and 10%) mimic a clinical sample that overtly demonstrates clonality, whereas the higher dilutions mimic a sample with low-level residual disease. The lower dilutions show an approximate limit of detection of clonality at 10% clonal T cells following the criteria we set of at least 4.5% of total reads (Table 2) and at least fourfold over background (data not shown). In the higher dilutions, we found that we could reproducibly detect the presence of the clonal TCRG rearrangements in all of the 0.1% dilutions and in dilutions as low as 0.004%. Therefore, we conservatively assigned a sensitivity of 0.1% to this assay for detecting a previously sequenced clone.

Discussion

Standard methods for assessing T-cell clonality use electrophoresis to resolve amplicons resulting from multiplex TCRG or TCRB PCR reactions. Subsequently, clonal peaks are identified by a comparison with the polyclonal sample background. In these designs, the generation of PCR
products across the range of possible V-J combinations in a single relatively short and narrow distribution may be advantageous.\(^\text{10}\) This decreases the influence of differing PCR efficiencies resulting from large variations in amplicon size and facilitates performing the assay on FFPE tissue samples in which DNA degradation may be an issue. In addition, such highly multiplexed designs may be more specific because of PCR competition among all possible amplicons.\(^\text{17}\) However, a short and narrow distribution may itself cause problems. A distinct peak observed by CE may unknowingly be composed of multiple rearrangements that create an additive signal and may thus lead to false-positive results. Another problem with all of these strategies lies in the identification of low-level residual disease in post-therapy samples. CE-based clonality assays are typically too insensitive for this purpose because of dilution of the clonal rearrangement in the sample background.\(^\text{4}\) Furthermore, despite rigorous efforts at standardization, such as those of the European BIOMED-2 group,\(^\text{3,18,19}\) a significant degree of interpretive subjectivity remains, which further complicates the use of such assays. The analysis of

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**Figure 5** Postanalysis data from TCRDriver illustrating the top cluster of a clonal (A) and polyclonal (B) patient sample sequenced on both the Ion Torrent and MiSeq platforms. The top line represents the true junction sequence, which also corresponded to the most frequent subcluster. Each line below the top subcluster indicates a subcluster that is pooled into the final cluster shown at right. Compared with the top subcluster, \(X\) indicates that the nucleotide is deleted and \(\hat{A}\) indicates an A insertion; substitutions are also indicated. The total number of collapsed subclusters used to form the top cluster is shown at the bottom. The top five subclusters are shown per platform.
cases based on quantitative sequence data using NGS may overcome these issues.

We believe that the primary advantages of NGS-based clonality testing are decreased subjectivity and the ability to detect low-level residual disease in follow-up samples if and only if the clonal sequence(s) has been previously identified in a diagnostic sample. Another advantage is the elimination of false-positive results because of the presence of multiple distinct rearrangements of the same size and their additive effect in CE-based testing. However, in terms of an initial assessment of clonality, we purposefully aligned our criteria closely with those of CE tests because, in the absence of clinical trials, the clinical rationale to argue for an enhancement is insufficient. Thus, for an initial assignment of clonality, the sensitivity of this assay is not substantially different from CE-based designs. Despite the inherent technical differences in what constitutes “background” in CE- and NGS-based assays, both methods show a similar calculated abundance, in terms of fold over background, of the clonal rearrangement. However, we did note that NGS had a slight and consistent edge in this regard. Based on dilutions of cell lines, these conservative criteria would allow for an initial assignment of clonality at the level of approximately 10% clonal T cells, very similar to the sensitivity of our CE-based method but with the advantages highlighted earlier.

In CE analysis, the polyclonal background consists of a spectrum of fragment lengths. A polyclonal T-cell population, based on the statistical distribution of V-J junction size, takes on a Gaussian distribution that does not necessarily reflect the abundance of identical rearrangements. On the other hand, a clonal peak is assumed to reflect the abundance of a particular distinct rearrangement. However, a single peak that meets the criteria for clonality may actually be composed of multiple distinct TCRG rearrangements, as demonstrated by the cases highlighted by our sequencing data. Such rearrangements are additive in the context of CE-based analyses and thus can lead to false-positive interpretations when they are assumed, in the absence of the sequencing data, to be a single rearrangement. This is illustrated by three cases (ind 3, ind 4, and ind 5) that were clonal on CE but did not meet NGS criteria (Figure 4). As stated before, this phenomenon is likely a consequence of a primer set designed to amplify rearrangements that overlap a single length distribution. An additional factor is that the length distribution of TCRG junctions is much narrower than that of TCRB junctions because of the absence of a D element and also involves a fairly small number of possible V-J combinations. Thus, NGS likely enhances the usefulness of TCRG-targeted tests by overcoming its lack of overall complexity.

Although the current study represents a significant amount of data collected using, in many cases, two NGS platforms, it should be considered as more of a proof of principle. The derived criteria for defining clonality, polyclonality, and indeterminate results may need to be adjusted, particularly in the context of clinical data. The values may be too stringent, as, for example, ind 5 (Figure 4), which was clonal on CE but did not meet NGS clonality criteria. Nevertheless, a strong correlation was observed between the vastly different methods. A somewhat more pronounced difference in peak height between pairs of presumed biallelic rearrangements in NGS data may be seen because of sequence-specific effects on the efficiency of adapter ligation during NGS library construction.

Using dilutions of T-lymphoid cell lines into normal polyclonal T cells to mimic post-treatment residual disease, we found that we were able to reproducibly detect rearrangements of known sequence in dilutions as low as 0.1%.

### Table 1

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<th>Case</th>
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sensitivity could be improved much further by maximizing total reads to better capture very rare sequences, but such strategies need to be assessed in the context of prospective clinical trials to determine if detection of very low-level residual disease with these methods is of clinical significance.

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taking into account the higher error rate for the IT, the final processed results from both platforms were very similar.

Finally, we analyzed the effect of PCR cycle numbers on the final sequencing data. We found that at cycle numbers higher than 30, often a favoring of shorter amplicons over longer amplicons was seen, likely because of magnification at higher cycles of the slightly lower PCR efficiencies of the longer amplicons. Thus, for this primer set, the cycle number should be kept to 30 or less. For NGS-based clonality assays relying on PCR primer sets that generate products with multiple, variable size distributions, PCR cycle number likely has a much greater effect on relative sequence quantitation, but this remains to be investigated.

The overall cost of an assay is another important consideration when comparing one method with another. The direct reagent costs of the assay we describe are three times greater for the IT platform and four times greater for the MiSeq platform in comparison with CE. These costs may be reduced by increasing the degree of multiplexing of samples during sequencing.

In summary, we believe that NGS-based clonality tests can be a useful primary method for assessing clonality or an adjunct to CE-based assays in difficult cases. The assay we have designed is less subjective and allows us to detect a previously identified neoplastic population down to levels of 0.1% or less. The sensitivities for both the initial assignment of clonality and the detection of low-level residual disease could easily be improved further if shown to be clinically useful. Our study also serves to highlight some of the pitfalls of traditional size-based analyses that are overcome by sequencing. Furthermore, we have demonstrated an algorithm for processing IT data that ultimately produces quantitative results that are very similar to those derived from the MiSeq platform.

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References


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