Research Article

Conventional PCR-based versus next-generation sequencing-based approach for T-cell receptor γ gene clonality assessment in mature T-cell lymphomas: A phase 3 diagnostic accuracy study

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Abstract

Background: Clonality assessment is currently the major molecular analysis utilized to support the diagnosis of suspicious lymphoid malignancies. Clonal rearrangements of the V-J segments of T-cell receptor G chain locus (TCRγ or TRG) have been observed in almost all types of T neoplasms, such as T-cell-related non-Hodgkin lymphomas and leukemias. At present, the gold standard for clonality evaluation is multiplex polymerase chain reaction (PCR), plus subsequent capillary electrophoresis/heteroduplex analyses, and/or Sanger sequencing. This approach overcomes the problem with the conventional Southern blot hybridization and is more efficient, simple, fast, and reproducible. In the recent years, the new next-generation sequencing (NGS) technologies provided alternative techniques for the analysis of antigen receptors genes, which presented several advantages, such as increased efficiency, specificity (SP), sensitivity (ST), resolution, and objectivity of the results, leading to a better classification, stratification, and monitoring of lymphoid malignancies. Nonetheless, these technologies are still far from being the new gold standard since further studies are warranted to prove their utility. The present study aimed to assess the diagnostic accuracy of these two methods by comparing a commercial NGS-based assay for the evaluation of TRG locus with the gold standard PCR-based one, to fulfill the requirements of a phase 3 diagnostic accuracy study. **Methods:** We assessed the TRG gene rearrangements in 72 cases using the conventional and highly-validated PCR-based assay proposed by EuroClonality consortium, an alternative commercial PCR-based assay, namely, IdentiClone® TCR Gamma Gene Rearrangement Assay 2.0, and a commercial NGS-based assay, that is, Invivoscribe LymphoTrack® Dx MiSeq® (both by Invivoscribe Technologies Inc., San Diego, CA, USA), to determine the diagnostic accuracy of the latter, and compare them with reference diagnoses made based on observation of clinical manifestations, cytohistological, and immunohistochemical analyses. Statistical values were calculated using the Oxford CATmaker software package. **Results**: Using standardized criteria of interpretation, the obtained results showed a diagnostic accuracy of 90.3% (correspondence in 65 out of 72 cases) of the test under investigation, with a ST of 86%, a SP of 95%, a positive predicting value of 94%, and a negative predicting value of 88%, demonstrating that Invivoscribe LymphoTrack® Dx MiSeq® assay had high efficiency and reliability in detecting clonal TRG gene rearrangements in T-cell non-Hodgkin lymphomas. **Conclusions**: This diagnostic accuracy study yielded comparable results using a validated PCR-based approach and a new NGS-based one. Subsequent studies and cost-effectiveness evaluation are needed to put the NGS-based clonality assessment into routine diagnostic practice.

Keywords: Clonality, T-cell receptor gamma, BIOMED2, PTCL, Lymphoma, Leukemia, Evidence-based medicine, Diagnostic accuracy, Polymerase chain reaction, Next-generation sequencing, LymphoTrack® TRG Assay

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1. INTRODUCTION

Diagnosis of T-cell non-Hodgkin lymphomas, to date, is mostly based on clinical manifestations, cytohistomorphological, and immunohistochemical analyses. However, in 10-15% of cases, the diagnosis is still uncertain and it is necessary to perform other molecular analyses to confirm or reject the suspected diagnosis [1]. Clonality analysis is a reliable method to achieve this purpose: the evaluation of the T-cell receptor (TCR) encoding genes rearrangement can define a clonal T-cell population that supports the final diagnosis for T-cell-related lymphoid disorders. In recent years, the progresses in the technologies of establishing the clonality state have allowed researchers to better characterize, classify, and monitor hematological neoplasms [2-6].

Chromosomal aberration, fusion transcripts, and breakpoint-fusion regions can be used as markers for the assessment of clonality, providing significant clinical information, especially in monitoring the course of the disease since these types of modification are directly related to the oncogenic process and are relatively stable. However, such approaches for the clonality evaluation, due to their low frequency, can be used only in a subgroup of patients. In contrast, the analysis of antigen-receptor gene rearrangements for clonality tests could be applied to virtually all patients [2,6]. Of note, the assessment of somatic mutations is currently not recommended for the diagnosis of such types of lymphomas. In the latest edition of the World Health Organization (WHO) classification [1], the analysis of a series of somatic mutations are suggested to be potentially useful for follicular helper-related cases, but still not formally recommended as a routine diagnostic tool.

TCRs detect foreign antigens that have been processed into small peptides and have bound to major histocompatibility complex molecules on the surface of antigen-presenting cells. Each TCR consists of a dimer composed of either an alpha and a beta chain or a delta and a gamma chain. During the early T-cell development, the TCR D chain genes (TRD) first rearrange, and then the TCR G chain genes (TRG) are subjected to rearrangement, potentially resulting in the expression of a functional TCRγδ. Otherwise, after the TRG rearrangement, the TCR B chain genes (TRB) rearrange, with subsequent TCR D deletion and TCR A chain genes (TRA) rearrangement, leading to the expression of TCRαβ. Rearrangement of the TCR G and/or TCR B chains has been observed nearly in all types of T-cell lymphoproliferative disorders. Therefore, the assessment of the TRG genes can be very useful in the identification of a neoplastic population of T-cells, which generally derives from a single tumor cell and, consequently, experiences completely identical receptor genes rearrangement [7,8].

The gamma locus contains V (variable), J (joining), and C (constant) segments. During the development of T-cells, the gamma chain is synthesized through a recombination event at the DNA level, combining a V segment with a J segment. Subsequently, the C segment joins through splicing at the RNA level. The wide range of antigen recognition is achieved by the re-combination of various V segments with various J segments and, furthermore, junctional diversity adds to the rearrangement variety by incorporating random nucleotides through terminal deoxynucleotidyl transferase [9].

Different methods have been developed to detect the clonality status of the TRG gene [3,4]: The first gold standard chosen for this propose was the Southern blotting method [10,11]. Nonetheless, the strict conditions required, including the substantial amount of DNA, have led to new technologies, such as polymerase chain reaction (PCR)-based analyses (followed by heteroduplex/capillary electrophoresis (CE) and/or Sanger sequencing), emerging as the gold standards [12-24].

With the revolution ushered in by next-generation sequencing (NGS), clonality tests also benefited from the development. The application of these technologies in this context, ideally, would allow for a better characterization, with higher resolution, specificity (SP), and sensitivity (ST). Consequently, they could lead to a better and more objective classification, stratification and monitoring of lymphoid malignancies (also from the minimal residual disease [MRD] point of view) due to their higher ability to resolve a polyclonal background, distinguish pseudo-clonality, and overcome the problem of lack of materials [25-43]. Despite these, to date, NGS methods have not yet been introduced into routine clinical diagnosis and PCR-based methods are still the gold standard for this type of analysis.

In this phase 3 diagnostic accuracy study, we compared the clonality outcomes from the highly validated BIOMED-2 PCR assay [12-19], with the ones obtained from the commercial PCR-based IdentiClone® TCR Gamma Gene Rearrangement Assay 2.0 and Invivoscribe LymphoTrack® Dx TRG MiSeq® Assay (Invivoscribe Technologies Inc., San Diego, CA, USA), the latter being the NGS-based test under investigation. The goal of this study was to assess the diagnostic accuracy of this novel approach, with the aim to clinically validate it for routine diagnosis of T-lymphoproliferative disorders.

Overall, the diagnosis of lymphomas is currently based on the integration of cytohistological and immunohistochemical

analyses, genetic, and clinical tests. Whenever the diagnosis remains uncertain, the case is also subjected to molecular PCR/CE analysis (which is, in any case, part of the conventional approach). To evaluate the diagnostic accuracy of the new molecular NGS analysis, this study compared the outcomes obtained by NGS and by the standard molecular test (i.e., BIOMED-2, based on PCR/CE) separated from the other conventional procedures. In addition, to gain in-depth insights, in this study, we also calculated the correlation between the molecular NGS results and the reference diagnoses (i.e., the relationship between clonality detection and PTCL diagnosis).

2. MATERIALS AND METHODS

2.1. Study design and participants

This study was designed to evaluate the diagnostic accuracy of a new NGS technology, that is, Invivoscribe LymphoTrack® Dx TRG assay based on Illumina® MiSeq® instruments (Invivoscribe Technologies Inc., San Diego, CA, USA), and compare it with the standard BIOMED-2 assay (https://euroclonality.org/about/), a PCR-based method followed by a CE analysis.

As a preliminary investigation, this study evaluated the IdentiClone® TCR Gamma Gene Rearrangement Assay 2.0, a PCR-based method based on the same chemistry of the LymphoTrack Dx TRG, to guarantee that the only variable relied on the sequencing method.

The studied cases were enrolled over a period of 2 years, at the Hematopathology Unit, IRCCS Azienda Opedaliera-Universitaria di Bologna; Institute of Hematology and Medical Oncology "L&A Seràgnoli." Cases included 169 T-lymphoproliferative disorders, of which, unfortunately, 97 cases were not evaluable due to an insufficient amount of DNA, an excessive degradation, and/or impurity or insufficient NGS reads (<10,000). Seventy-two could be evaluated. Forty-nine T-cell non-Hodgkin lymphomas (T-NHL) and 23 reactive lymphoid hyperplasia with paracortical expansion (RLH) were collected from formalin-fixed and paraffin-embedded (FFPE) tissues ($n = 49$) or fresh/frozen tissues ($n = 23$) (Figure 1 and Table 1). All the samples were extracted from lymph node tissue. As a consequence, the potential variability and the consequent effect on the outcomes, resulting from different tissue-types, were excluded from the study. It is important to stress that the vast majority of these samples at the Hematopathology Unit derived precisely from lymph node tissues, so the considered cohort of cases also statistically represented the normal distribution of used samples.

The reference diagnoses were made according to the WHO classification of tumors of the hematopoietic and lymphoid tissues, by experienced hematopathologists. They were based on morphological, immunophenotyping, genomic, clinical, and molecular features [44]. For the PCR analyses, the positive control (for monoclonality) used was a DNA sample extracted from a FE-PD cell line, a T-NHL cell line established from a CD30+ PTCL/NOS, whereas the negative control (for polyclonality) was a DNA sample extracted from a peripheral blood sample of a healthy participant.

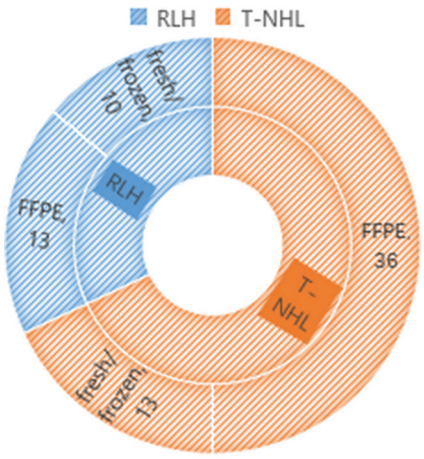

Figure 1. Distribution of the studied cases. Totally, 49 T-NHL and 23 RLH cases were recruited, including both fresh/frozen and formalin fixing and paraffin embedding samples.

All the participants gave their informed consents for molecular diagnosis. The study was designed and conducted in accordance with the Helsinki Declaration (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/). Furthermore, the study strictly followed the evidence-based medicine rules, respecting the QUADAS, REMARK, and STARD requirements [45].

In our series, samples were harvested from non-consecutive patients at the above-mentioned hospital, and they received both the reference standard test and the study test. Since the former was performed before the study initiation, we designed a retrospective study.

2.2. DNA extraction

The genomic DNA from both FFPE and fresh/frozen samples was extracted using the QIAamp® DNA mini kit (QIAGEN N.V., Hilden, Germany) according to the manufacturer's instructions (with additional procedures of de-paraffinization for the FFPE ones). The extracted DNA was then analyzed for purity and concentration on a NanoDrop® spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) by following the manufacturer's instructions, to select only the samples

Table 1. Description of studied cases. The table describes and compares the clonality assessment using BIOMED-2, Invivoscribe LymphoTrack® Dx TRG MiSeq® (Invivoscribe NGS) and IdentiClone® T-cell receptor Gamma Gene Rearrangement Assay 2.0 (Invivoscribe PCR)

Case	Sample type	Pathological diagnosis	Clonality by BIOMED-2	Clonality by Invivoscribe NGS	Clonality by Invivoscribe PCR
1	FFPE	T-NHL	Oligoclonal	Oligoclonal	Oligoclonal
3	FFPE	RLH	Oligoclonal	Oligoclonal	Clonal
4	FFPE	T-NHL	Biclonal	Biclonal	Clonal
5	FFPE	T-NHL	Monoclonal	Monoclonal	Clonal
6	FFPE	T-NHL	Biclonal	Monoclonal	Clonal
7	Fresh/frozen	RLH	Oligoclonal	Oligoclonal	Oligoclonal
8	FFPE	T-NHL	Oligoclonal	Oligoclonal	Clonal
9	FFPE	T-NHL	Polyclonal	Polyclonal	Polyclonal
10	FFPE	T-NHL	Polyclonal	Polyclonal	Polyclonal
11	FFPE	RLH	Polyclonal	Polyclonal	Polyclonal
12	FFPE	RLH	Polyclonal	Polyclonal	Polyclonal
13	FFPE	RLH	Oligoclonal	Oligoclonal	Polyclonal
14	FFPE	T-NHL	Oligoclonal	Oligoclonal	Clonal
15	FFPE	T-NHL	Oligoclonal	Monoclonal*	Clonal
16	FFPE	RLH	Polyclonal	Polyclonal	Polyclonal
17	FFPE	T-NHL	Biclonal	Biclonal	Clonal
18	FFPE	T-NHL	Biclonal	Biclonal	Clonal
19	FFPE	RLH	Polyclonal	Polyclonal	Polyclonal
20	FFPE	T-NHL	Monoclonal	Polyclonal*	Polyclonal
21	FFPE	T-NHL	Monoclonal	Monoclonal	Clonal
22	Fresh/frozen	RLH	Polyclonal	Polyclonal	Polyclonal
23	FFPE	T-NHL	Biclonal	Monoclonal	Clonal
24	Fresh/frozen	RLH	Polyclonal	Polyclonal	Polyclonal
25	Fresh/frozen	RLH	Polyclonal	Polyclonal	Polyclonal
26	FFPE	T-NHL	Monoclonal	Monoclonal	Clonal
27	FFPE	T-NHL	Monoclonal	Polyclonal*	Clonal
28	Fresh/frozen	RLH	Polyclonal	Monoclonal*	Polyclonal
29	FFPE	T-NHL	Monoclonal	Monoclonal	Clonal
30	FFPE	RLH	Monoclonal	Monoclonal	Clonal
31	Fresh/frozen	RLH	Polyclonal	Polyclonal	Polyclonal
32	Fresh/frozen	T-NHL	Oligoclonal	Oligoclonal	Not Done
33	Fresh/frozen	RLH	Polyclonal	Polyclonal	Not Done
34	FFPE	RLH	Polyclonal	Polyclonal	Not Done
35	FFPE	T-NHL	Oligoclonal	Oligoclonal	Not Done
36	FFPE	T-NHL	Monoclonal	Monoclonal	Not Done
37	FFPE	RLH	Polyclonal	Polyclonal	Not Done
38	FFPE	T-NHL	Biclonal	Biclonal	Not Done
39	Fresh/frozen	T-NHL	Biclonal	Biclonal	Not Done
40	Fresh/frozen	RLH	Polyclonal	Polyclonal	Not Done
41	FFPE	T-NHL	Monoclonal	Biclonal	Not Done
42	FFPE	RLH	Polyclonal	Polyclonal	Not Done
43	FFPE	T-NHL	Monoclonal	Monoclonal	Not Done
44	FFPE	T-NHL	Monoclonal	Biclonal	Not Done
45	Fresh/frozen	T-NHL	Biclonal	Biclonal	Not Done
46	Fresh/frozen	RLH	Polyclonal	Polyclonal	Not Done
47	FFPE	T-NHL	Monoclonal	Biclonal	Not Done
48	FFPE	T-NHL	Monoclonal	Monoclonal	Not Done
49	FFPE	T-NHL	Monoclonal	Monoclonal	Not Done
50	FFPE	T-NHL	Biclonal	Biclonal	Not Done

(*Cont'd...*)

Table 1. ***(Continued)***

Case	Sample type	Pathological diagnosis	Clonality by BIOMED-2	Clonality by Invivoscribe NGS	Clonality by Invivoscribe PCR
51	Fresh/frozen	T-NHL	Monoclonal	Monoclonal	Not Done
52	Fresh/frozen	T-NHL	Monoclonal	Biclonal	Not Done
53	FFPE	T-NHL	Monoclonal	Monoclonal	Not Done
54	FFPE	T-NHL	Biclonal	Biclonal	Not Done
55	FFPE	RLH	Polyclonal	Polyclonal	Not Done
56	FFPE	T-NHL	Biclonal	Biclonal	Not Done
57	Fresh/frozen	T-NHL	Oligoclonal	Oligoclonal	Not Done
58	FFPE	T-NHL	Monoclonal	Polyclonal*	Not Done
59	Fresh/frozen	RLH	Polyclonal	Polyclonal	Not Done
60	Fresh/frozen	T-NHL	Monoclonal	Monoclonal	Not Done
61	Fresh/frozen	T-NHL	Oligoclonal	Oligoclonal	Not Done
62	FFPE	T-NHL	Polyclonal	Polyclonal	Not Done
63	Fresh/frozen	T-NHL	Monoclonal	Polyclonal*	Not Done
64	FFPE	T-NHL	Monoclonal	Polyclonal*	Not Done
65	FFPE	RLH	Polyclonal	Polyclonal	Not Done
66	FFPE	T-NHL	Oligoclonal	Oligoclonal	Not Done
67	FFPE	T-NHL	Biclonal	Biclonal	Not Done
68	Fresh/frozen	T-NHL	Oligoclonal	Oligoclonal	Not Done
69	Fresh/frozen	T-NHL	Oligoclonal	Oligoclonal	Not Done
70	Fresh/frozen	T-NHL	Monoclonal	Biclonal	Not Done
71	FFPE	RLH	Polyclonal	Polyclonal	Not Done
72	Fresh/frozen	T-NHL	Biclonal	Biclonal	Not Done

FFPE: Formalin-fixed paraffin-embedded; T-NHL: T-cell non-Hodgkin lymphomas; RLH: Reactive lymphoid hyperplasia; PCR: Polymerase chain reaction; NGS: Next-generation sequencing.*Discordant cases.

whose 260/280 nm ratio (nucleic acids/proteins) and 260/230 nm ratio (nucleic acids/carbohydrates and others) were in the range 1.8–2.0 and 2.0–2.2, respectively, which indicate an acceptable purity. To verify the DNA integrity, a control PCR amplification was performed for all samples, identifying PLZF as the control gene. The minimum requirement to confirm a sufficient DNA integrity was a 300 bps amplification of this gene; otherwise, the sample was considered as not evaluable.

2.3. PCR analysis of TRG gene by BIOMED-2 multiplex assay and IdentiClone® TCR Gamma Gene Rearrangement Assay 2.0

For the assessment of TRG rearrangements, two multiplex PCR analyses were conducted according to the protocols of BIOMED-2 [12-19] and IdentiClone® TCR Gamma Gene Rearrangement Assay 2.0 (https://catalog.invivoscribe.com/product/identiclone-t-cell-receptor-gamma-gene-rearrangement-assay-2-0-abi-fluorescence-detection/), by at least two qualified molecular hematopathologists who were blinded to the histopathological and sequencing results. These assays differ principally in the master mixes, which include different sets of forward and reverse primers utilized with the aim of amplifying the rearranged V-J regions of the TRG locus.

An automated thermocycler (VeritiTM Dx Thermal Cycler, Applied BioSystems™, Thermo Fisher Scientific Inc., Wilmington, DE, USA) was used for this purpose. According to the assay guidelines, the preparation for the amplification was as follows: for each sample, a 50 µL PCR reaction was set up and it was composed of 100 ng of nuclear DNA. The DNA was added to the corresponding master mix (BIOMED-2 or Invivoscribe ones, depending on which of the PCR analyses was performed), which included 10 pm of each primers, 0.2 mmol/L of dNTPs, 1 U of Ampli-Taq Gold polymerase (Applied BioSystems™, Thermo Fisher Scientific Inc., Wilmington, DE, USA) for BIOMED-2 master mixes or 1.25 U of Eagle-Taq DNA polymerase (Invivoscribe Inc., San Diego, CA, USA) for Invivoscribe master mix, 5 µL of 10X Gold buffer (Applied BioSystems™, Thermo Fisher Scientific Inc., Wilmington, DE, USA), and 1.5 mmol/L of MgCl2; the samples are analyzed also with 150 µL (3 x 50 µL) of positive, negative, and no-template controls.

In accordance with the BIOMED-2 protocol, for the thermocycling profile was as follows: (1) 7 min of pre-activation at 95°C, (2) 30 s of denaturation at 95°C, (3) 30 s of primers annealing at 60°C, (4) 60 s of primers extension at 72°C, and (5) 10 min of final extension at 72°C. The steps 2–4 were repeated for 35 cycles. On the other hand, the Invivoscribe protocol required that the thermocycling

should consist of (1) 7 min of pre-activation at 95°C, (2) 45 s of denaturation at 95°C, (3) 45 s of primers annealing at 60°C, (4) 90 s of primers extension at 72°C, and (5) 10 min of final extension at 72°C. The steps 2–4 were repeated for 35 cycles. Every single amplification session on all samples were executed in duplicate, which was followed by further CE.

The PCR products for each sample were, then, evaluated by GeneScan analysis to determine the clonality state.

2.4. GeneScan analysis

To assess clonality using the GeneScan analysis, 1 µg of PCR products was mixed, for 2 min at 95°C, with 0.5 µg of standard molecular weight product (GeneScan 600 LIZTM for BIOMED-2 assay or GeneScan 400HD ROXTM for IdentiClone® assay) and with 12 µL of Hi-DiTM formamide (Applied BioSystems™, Thermo Fisher Scientific Inc., Wilmington, DE, USA), which induced the denaturation of DNA into single strands, which are necessary for a high-resolution analysis. The single-strand fragments were labeled with fluorochrome for detection in the following step [20-22].

The products, as required by the EuroClonality and Invivoscribe guidelines, were then separated through a CE system, and subsequently detected by a laser system that reads the fluorescence of the fragments, on an automatic DNA sequencer (ABI Prism® 3100, Applied BioSystems™, Thermo Fisher Scientific Inc., Wilmington, DE, USA), according to the manufacturer's instructions.

2.5. Library preparation and quantification for NGS-based analysis

The chosen assay for the NGS-based analysis of the TRG gene was the Invivoscribe LymphoTrack® Dx TRG MiSeq® (Invivoscribe Inc., San Diego, CA, USA) (https://catalog.invivoscribe.com/product/lymphotrack-trg-assay-panel-miseq/) and was conducted by at least two qualified molecular hematopathologists who were blinded to the histopathological and standard PCR results. This assay aimed to assess the gene rearrangement using consensus primers that target the V and J regions of the aforementioned gene. This method allows for the execution of a one-step PCR, and the products, also coming from several samples (up to 24 per target), were then analyzed on a single MiSeq flow cell and visualized using the associated LymphoTrack® Dx Software, allowing for a simple and reliable interpretation of the results for the determination of the TRG gene rearrangement state in support to the final diagnosis.

Different primers target different portions of the two regions (Vγ2, Vγ3, Vγ4, Vγ5, Vγ8, Vγ9, Vγ10, Vγ11 in V and JγP1, JγP2, JγP, Jγ1, Jγ2 in J), and both forward and reverse are tagged with a unique barcode, among 24 possible, like the Illumina® P5 and P7 flow cell adapters oligo sequences (Illumina® Inc., San Diego, CA, USA). For each sample, a PCR has been set up, including 5 µL of genomic DNA (Sample DNA concentrations ranged from a minimum of 12.5 to a maximum of 968.9 ng/µL, median being 207.9 ng/µL), 45 µL of master mix (including primers, dNTPs, control DNA, and reaction buffer), and 0.2 µL (0.2?) (5 U/µL) of Eagle-Taq DNA polymerase (Invivoscribe Inc., San Diego, CA, USA). The Veriti™ Dx thermocycler (Applied BioSystems™, Thermo Fisher Scientific Inc., Wilmington, DE, USA) for the amplification was then set as follows: (1) 7 min of pre-activation at 95°C, (2) 45 s of denaturation at 95°C, (3) 45 s of primers annealing at 60°C, (4) 90 s of primers extension at 72°C, and (5) 10 min of final extension at 72°C. The steps 2 to 4 were repeated for 29 cycles.

Subsequently, the PCR products were subjected to a phase of purification, to remove exceeding primers, nucleotides, salts, and enzymes. For this purpose, a solid-phase reversible immobilization (SPRI) paramagnetic bead technology was employed for high-throughput purification (Ambion Magnetic Stand 96, Thermo Fisher Scientific Inc., Wilmington, DE, USA). Briefly, using an optimized buffer, the PCR amplicons, whose size was at least 100 bps, remained bound to the paramagnetic beads while the mentioned impurities were washed away using ethanol. The amplicons were next eluted from the paramagnetic beads, thereby obtaining a purified PCR product for the following analyses.

At this point, the purified amplicons were quantified using KAPA Library quantification kit for Illumina platforms (Illumina® Inc., San Diego, CA, USA). After a dilution and the addition of 6-standard pre-diluted DNA, the products were amplified by a quantitative PCR, using the KAPA SYBR® FAST qPCR master mix and primers, which target the Illumina® P5 and P7 flow cell adapters oligo sequences from the amplicons. Using the standard curve (log10) generated from the pre-diluted standard DNA, it was therefore possible to calculate, through interpolation, the concentration (pM) of the amplicons, to obtain an equal representation of these in the final bundled library that was afterward loaded onto a flow cell in a MiSeq cartridge, so as to be sequenced on a MiSeq instrument.

2.6. NGS of TRG gene by Invivoscribe LymphoTrack® Dx TRG MiSeq® assay

In the sequencing on MiSeq instrument, based on Illumina's technologies, the obtained amplicons (minimum 50 ng) of the library were hybridized to oligonucleotides on a flow cell in a MiSeq® V2 cartridge, and, with a bridge-amplification mechanism, they were amplified to form local clonal colonies. Four types of reversible terminator bases (one for each base) were added to the flow cell and the sequencing strand of DNA

was extended one nucleotide at a time; meanwhile, a CCD camera (Charge Coupled Device camera) took an image of the of the fluorescent light emitted when the labeled nucleotides were incorporated into the sequencing strand, which was, then, cleaved to allow for the inclusion and the detection of the subsequent one. The MiSeq® Control software v2.6 (Illumina® Inc., San Diego, CA, USA), ran on a Windows PC, thus allocated raw sequences into FASTQ files using the sample-specific barcodes. These files were, then, analyzed with LymphoTrack® MiSeq® software (Invivoscribe Inc., San Diego, CA, USA) to trim the barcodes and to filter out low quality reads, off-target reads, and primer dimers. The data so obtained were, therefore, aligned to the reference TRG V and J sequences, assigning a rearrangement identity based on alignment scores.

2.7. Definition of clonality

The determination of the clonality state in the examined samples, using the two PCR-based assay, met the following criteria: Using the GeneScan analysis, after the CE and the detection on an automatic DNA sequencer (ABI Prism® 3100, Applied BioSystems™, Thermo Fisher Scientific Inc., Wilmington, DE, USA), PCR products from a T-cell monoclonal population, as result of this procedure, gave rise to 1 peak, PCR products from a biclonal (or biallelic) population yielded two peaks, PCR products from an oligoclonal population presented 3 to 5 peaks, whereas PCR products from a polyclonal population generated a Gaussian distribution pattern of the peaks [20-22].

Moreover, the determination of the clonality state, using the NGS-based Invivoscribe LymphoTrack® Dx TRG MiSeq® assay, satisfied the following criteria: after the sequencing procedure and the analysis with LymphoTrack® MiSeq® software, the samples were, thus, interpreted as clonal if there was a clonotype read with a frequency >2.5% of the total reads, which also had to be at least fivefold greater than the frequency of the fourth most detected clonotype. This threshold value was chosen based on a clinically significant clonal cell population of 5%, assuming that each clone usually presents one or two rearrangements. Sequences with one or two nucleotide differences were merged, retaining the one with the higher number of reads, which was done with the rate of error of the performed test taken into account. The sample was considered as oligoclonal if there were three or more clonotypes with frequency >2.5%. Otherwise, if none of the reads had a frequency >2.5%, or if there was not a sufficient gap in frequency from the polyclonal background, the sample was considered as polyclonal. It was necessary that a minimum of 10,000 reads be present to make a determination of clonality.

2.8. Statistical methods

For statistical analysis, CATmaker software (Center for Evidence-Based Medicine, Oxford University, http://www.cebm.net) was used to calculate ST, SP, positive predictive value (PPV), negative predicting value (NPV), positive likelihood ratio (LR+), negative likelihood ratio (LR-), and pre-test probability. In each of the performed analyses, the cutoff for significance was set at $P < 0.05$).

The correlation between the different tests was calculated using the Pearson correlation method and linear regression analysis, and the criteria for determining a significant correlation was a coefficient of determination ($R^2 > 0.50$) and a Pearson correlation with $P < 0.05$).

3. RESULTS

To assess the diagnostic accuracy of NGS-based methods for clonality evaluation in T-cell lymphoproliferative disorders, a total of 72 samples (cases) has been examined, of which all (72/72) had been analyzed using both standard PCR-based and NGS-based approaches, that is, BIOMED-2 multiplex assay and Invivoscribe LymphoTrack® Dx TRG MiSeq® assay, whereas 31/72 (43.1%) had been analyzed using another PCR-based approach, that is, IdentiClone® TCR Gamma Gene Rearrangement Assay 2.0 (Table 1).

In accordance with standardized criteria, the results obtained from the 72 cases using the BIOMED-2 master mixes were as follows: 35 were identified as mono/biclonal (35/72, 48.6%), 14 as oligoclonal (14/72, 19.5%), and 23 as polyclonal (23/72, 31,9%). Meanwhile, the results from the 31 cases using the IdentiClone® TCR Gamma Gene Rearrangement Assay 2.0 master mix were as follows: 15 were identified as mono/biclonal (15/31, 48.4%), 2 as oligoclonal (2/31, 6.4%), and 14 as polyclonal (14/31, 45.2%) (Figure 2).

Upstream, all MiSeq runs fulfilled these validity requirements: Q30 > 75% for 500 cycles, Q30 > 70% for 600 cycles, PF > 90%, cluster density > 600 K/cm^2, and the total number of reads per run > 10 million. The obtained runs had the following average values: Q30 = 97.4%, PF = 96.1%, cluster density = 742 K/cm2, and total reads = 14,496,020 (Figures 3-5).

In accordance with these criteria, the results obtained from the 72 examined cases using the *Invivoscribe LymphoTrack® Dx TRG MiSeq® assay* were as follows: 32 were identified as mono/biclonal (32/72, 44.4%), 13 as oligoclonal (13/72, 18.1%), and 27 as polyclonal (27/72, 37.5%) (Figure 6).

To determine the diagnostic accuracy of the molecular tests under investigation, the results from the NGS-based

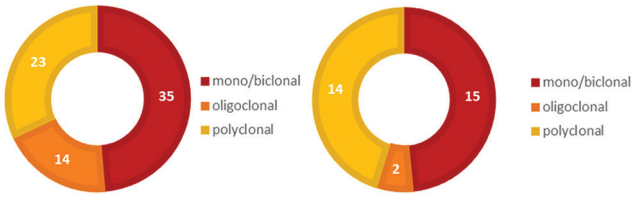

Figure 2. Clonality assessment results obtained through BIOMED-2 (left panel) and IdentiClone® (right panel) assays. All cases (72) were analyzed by BIOMED-2, and 31 were analyzed also with IdentiClone®.

Invivoscribe LymphoTrack® Dx TRG MiSeq® assay and from the PCR-based IdentiClone® TCR Gamma Gene Rearrangement Assay 2.0 were compared to the current gold standard PCR-based test BIOMED-2 multiplex assay, given that the ones identified as oligoclonal had been included in the polyclonal ones.

First, we compared the results from IdentiClone® TCR Gamma Gene Rearrangement Assay 2.0 to those from the

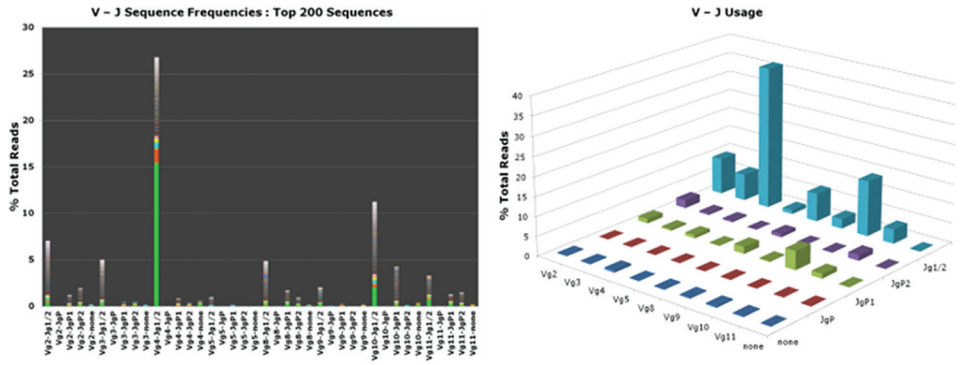

Figure 3. Example of monoclonal pattern of TRG clonality. The assessment was performed by NGS approach by utilizing LymphoTrack® MiSeq® software.

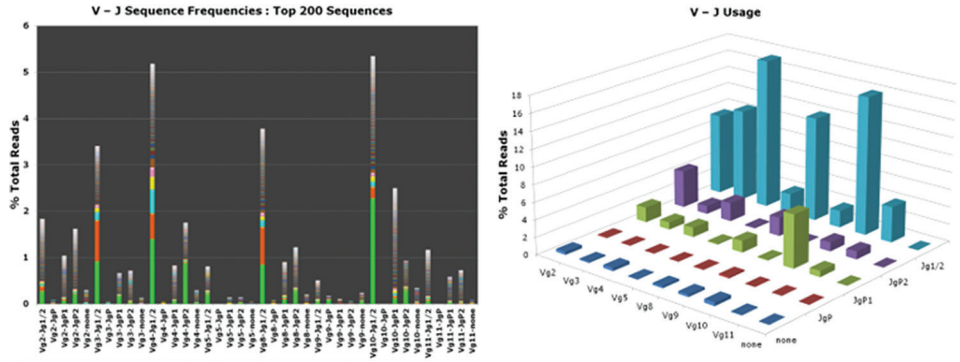

Figure 4. Example of oligoclonal pattern of TRG clonality. The assessment was performed by NGS approach by employing LymphoTrack® MiSeq® software.

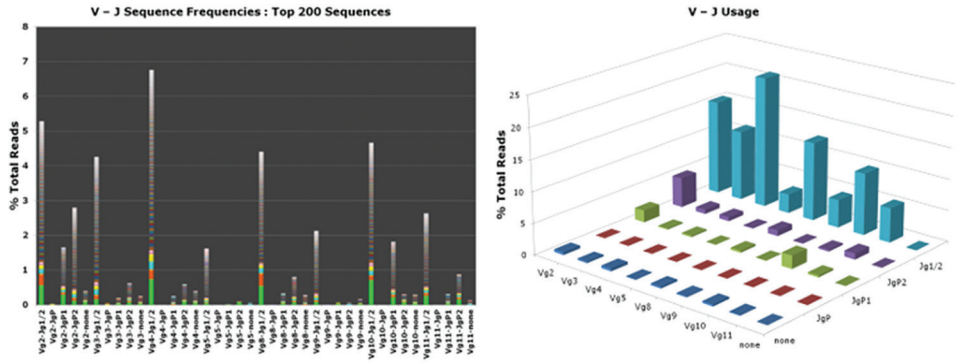

Figure 5. Example of polyclonal pattern of TRG clonality. The assessment was performed by next-generation sequencing approach using LymphoTrack® MiSeq® software.

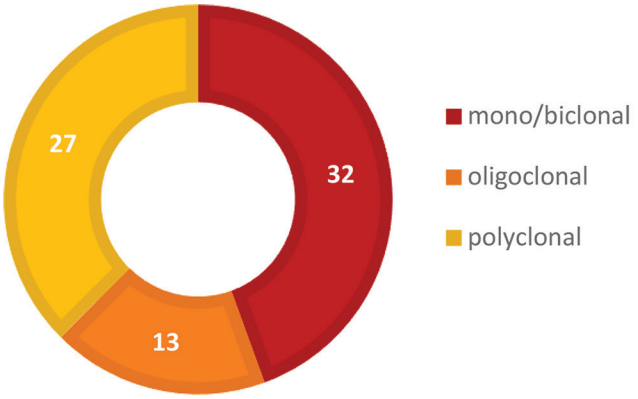

Figure 6. Clonality assessment results obtained through LymphoTrack® Dx TRG MiSeq® assay. Thirty-two cases were found to be mono/biclonal, 13 were oligoclonal, and 27 were polyclonal.

Table 2. Diagnostic accuracy of Invivoscribe LymphoTrack® Dx TRG MiSeq (Invivoscribe NGS) compared to BIOMED-2 and to reference diagnoses. The overall accuracy of NGS- versus PCR-based test was 90%. Clonal populations were detected in 82% of T-cell lymphomas

Invivoscribe NGS	BIOMED-2		Reference diagnoses	
	Mono/ biclonal	Oligo/ polyclonal	T-NHL	RLH
Mono/biclonal	30	2	40	2
Oligo/polyclonal	5	35	9	21

PCR: Polymerase chain reaction; NGS: Next-generation sequencing.

BIOMED-2 multiplex assay, to verify the reliability of the Invivoscribe chemistry (even though it has already been reviewed and validated by EuroClonality/BIOMED-2 group). Eleven out of 12 mono/biclonal samples and 15 out of 19 polyclonal samples yielded consistent results. One sample identified as mono/biclonal by the standard analysis was polyclonal according to the other PCR-based test, while four samples identified as polyclonal by the standard analysis was mono/biclonal according to the Invivoscribe one. Three out of five reference diagnoses of these discordant cases supported that the results obtained using the IdentiClone® TCR Gamma Gene Rearrangement Assay 2.0, since these three cases, identified as mono/biclonal, were actually diagnosed as T-NHL, whereas two out of five reference diagnoses of these discordant cases supported, the results obtained using the BIOMED-2 multiplex assay, since one of this cases identified as polyclonal and one identified as mono/biclonal, were indeed diagnosed as RLH and T-NHL, respectively.

At the statistical level, the general diagnostic accuracy of the PCR-based Invivoscribe test in comparison to its standard PCR-based counterpart was 92% ST, 79% SP, 73% PPV, and 94% NPV.

Once the substantial equivalence of the two chemistries (BIOMED2 vs. Invivoscribe) had been verified, we, thus, proceeded to compare the results from Invivoscribe LymphoTrack® Dx TRG MiSeq® assay to the BIOMED-2 multiplex assay ones. Results showed that 35 out of 37 polyclonal samples and 30 out of 35 mono/biclonal samples had a concordant result (Table 2), with an accuracy of 90.3%. Two samples identified as polyclonal by the standard analysis were mono/biclonal according to the NGS-based approach, while five samples identified as mono/biclonal by the standard analysis were identified to be polyclonal by the NGS-based approach. All but one reference diagnoses (made against the WHO Classification of Tumors of the Hematopoietic and Lymphoid tissues [44]) about these discordant cases supported the validity of the BIOMED-2 multiplex assay, since one case, identified as polyclonal, and five, identified as mono/biclonal, were indeed RLH and T-NHL, respectively, whereas one of the reference diagnosis supported the validity of the LymphoTrack® Dx TRG MiSeq® assay, since that case, identified as mono/biclonal, was actually diagnosed as having T-NHL.

At the statistical level, the general diagnostic accuracy of the NGS-based test in comparison to the standard PCR-based one was 86% ST, 95% SP, 94% PPV, and 88% NPV (Table 3).

Relatively homogeneous results were obtained from FFPE and fresh/frozen samples.

Specifically, a correspondence was observed in 89.8% of FFPE samples and a correspondence of 91.3% was found in fresh/frozen samples, with a ST of 86%, a SP of 95%, and with a remarkable PPV of 94% (within a pre-test probability of 49%) (Figure 7 and Table 3).

Furthermore, given that the focus of this study was to assess the diagnostic accuracy of the NGS-based test, the results obtained through LymphoTrack® Dx TRG MiSeq® assay were also compared to the reference diagnoses, finding out that 21 out of 23 samples diagnosed as RLH were identified as polyclonal, while 40 out of 49 samples diagnosed as T-NHL were identified as mono/biclonal (Table 2). The resulting comparison led to the following statistical values: 82% ST, 91% SP, 95% PPV, and 70% NPV (Table 3). Details of discordant cases are reported in Table 4.

4. DISCUSSION

At present, the assessment of TCR γ gene rearrangement is an indispensable approach for the diagnostic routine in a considerable percentage of doubtful cases in every hematopathology laboratory, not only to support the hypothesis of a determinate lymphoproliferative disorder but also for the establishment of an actual diagnosis in certain cases [2-4,6,15,44].

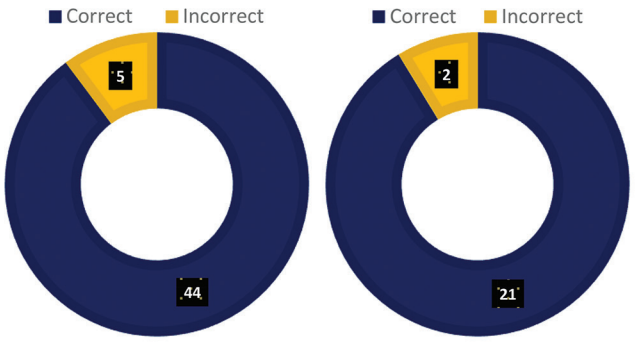

Figure 7. Diagnostic accuracy of LymphoTrack® Dx TRG MiSeq® compared to BIOMED-2 in formalin fixing and paraffin embedding (FFPE) samples (left panel) and in fresh/frozen sample (right panel). Altogether, there was a good agreement between the two methods, both in FFPE and fresh/frozen cases.

Table 3. Statistical values of LymphoTrack® Dx TRG MiSeq® (Invivoscribe NGS) compared to BIOMED-2 and to reference diagnoses by CATmaker software

Parameter	Value (%)	95% confidence intervals
Invivoscribe NGS versus BIOMED-2		
ST	86	74–97
SP	95	87–100
PTP	49	37–60
PPV	94	85–100
NPV	88	77–98
LR+	15.86	4.09–61.56
LR-	0.15	0.07–0.34
Invivoscribe NGS versus reference diagnoses		
ST	82	71–92
SP	91	80–100
PTP	68	57–79
PPV	95	89–100
NPV	70	54–86
LR+	9.39	2.48–35.53
LR-	0.20	0.11–0.37

ST: Sensitivity; SP: Specificity; PTP: Pre-Test Probability; PPV: Positive Predictive Value; NPV: Negative Predictive Value; LR+: Positive likelihood ratio; NGS: Next-generation sequencing; LR-: Negative likelihood ratio.

Starting from the 1980s, the gold standard method used for the molecular investigation of clonality was the Southern blot hybridization analysis (SBH) (Table 4). In the following years, the new techniques that gradually replaced the SBH as gold standard for clonality assessment have been the PCR-based approaches, that can be categorized as qualitative PCR, used for the clonality assessment, and quantitative PCR (like real-time PCR), useful for monitoring MRD and assessing therapeutic responses [23,24] (Table 4). These methods are based on the selective amplification of junctional regions of the rearranged TCR (or Ig) genes, using appropriate forward and reverse primers that usually target V and J regions, respectively, of those genes, to detect and characterize the over-representation of amplified V-J (or V-D-J in Ig) products, and also the clonality state of genetic rearrangements (it is therefore necessary to have a prior accurate knowledge of the targeted segments for the primer design). For this purpose, as explained, a subsequent analysis of the PCR products, such as Heteroduplex/CE and/or Sanger sequencing, is required for the determination of a neoplastic cell population (clonal pattern) or of a reactive lymphoid cell population (polyclonal pattern) [3,4,19-24]. Such approach is very reliable, specific, simple, fast, highly-sensitive, and feasible also with small FFPE archival biopsies (using also small amount of partially-degraded DNA), but the detectable genetic rearrangements are limited by the used primers which may not always be optimal and easily reproducible among different laboratories. In this perspective, in 2003, BIOMED-2 group defined standardized protocols and a set of optimized primers for a multiplex PCR clonality analysis that improved both efficiency and reproducibility of such approaches for most of B-cell and T-cell malignancies across worldwide laboratories, since standardization is crucial in routine diagnosis and these protocols and primers have been highly validated by many other research groups outside the EuroClonality consortium [12-19].

In recent years, starting from the beginning of this century, NGS technologies have found wide applications in diagnostic laboratories, and in the clonality assessment in

Table 4. Analysis of discordant cases

Case	Sample type	Pathological diagnosis	Clonality by BIOMED-2	Clonality by Invivoscribe NGS	Clonality by Invivoscribe PCR	DNA concentration (ng/mL)	Note
15	FFPE	T-NHL	Oligoclonal	Monoclonal	Clonal	361.4	NGS consistent with Sanger when the same primers were used
20	FFPE	T-NHL	Monoclonal	Polyclonal	Polyclonal	248.7	NGS consistent with Sanger when the same prmers were used
27	FFPE	T-NHL	Monoclonal	Polyclonal	Clonal	511.3	NGS apparently less sensitive
28	fresh/frozen	RLH	Polyclonal	Monoclonal	Polyclonal	280	NGS more sensitive?
58	FFPE	T-NHL	Monoclonal	Polyclonal	Not Done	62.6	NGS apparently less sensitive
63	fresh/frozen	T-NHL	Monoclonal	Polyclonal	Not Done	42.3	NGS apparently less sensitive
64	FFPE	T-NHL	Monoclonal	Polyclonal	Not Done	67.9	NGS apparently less sensitive

PCR: Polymerase chain reaction; NGS: Next-generation sequencing; FFPE: Formalin-fixed paraffin-embedded.

lymphoproliferative disorders (Table 5). These methods, as explained, are based on the preparation of a library which is then amplified by PCR using appropriate forward and reverse primers which usually target V and J regions of TCR (or Ig) genes, respectively. The amplified library is then sequenced and analyzed with bioinformatic approaches that return the proportion of the given sequences and assign them a rearrangement identity based on an alignment score [3,4,29-33]. These technologies involve the execution of complex procedures which can however be parallelized for the simultaneous analysis of several samples, obtaining thus high-throughput data.

Evidently different from other studies on NGS evaluation of TCR, the present study conducted the first formal phase 3 diagnostic accuracy study on this kind of assays. Specifically, we investigated the diagnostic accuracy of a commercially available NGS approach, Invivoscribe LymphoTrack® Dx TRG MiSeq® assay, for detection of TCR γ gene clonal rearrangements. Our criteria of interpretation were based on the results of the same cases, obtained through the gold standard PCR approach (and subsequent CE analysis), according to the highly-validated EuroClonality consortium guidelines. In compliance with STARD and QUADAS requirements for evidence-based medicine [45], all 72 studied cases were analyzed using both PCR and NGS approaches, the results of which were respectively blinded to the molecular pathologists performing the analyses. In addition, 31 out of 72 of the studied cases were analyzed using another PCR method on the basis of the same chemistry of the NGS-based one used, that is, IdentiClone® TCR Gamma Gene Rearrangement Assay 2.0, to exclude major differences in the pre-sequencing phases, consistently in accordance with STARD and QUADAS guidelines [45].

The results indicated that the test under investigation was highly reliable. In fact, our experience with Invivoscribe LymphoTrack® Dx TRG MiSeq® assay demonstrated an overall accuracy of 90.3% in the examined cases. Only seven cases yielded inconsistent results, as two samples identified as polyclonal by BIOMED-2 clonality assay were found to be mono/biclonal by NGS analysis whereas five samples identified as mono/biclonal by BIOMED-2 clonality assay were shown to be polyclonal by NGS. Six out of seven of the reference diagnoses confirmed the outcomes obtained through the PCR approach and this could be ascribed to different reasons: first of all, most of the inconsistent results were derived from FFPE tissues, implying that the extracted DNA was of lower quality due to the effect of formalin fixation on nucleic acids, with partial degradation that could lead to a lower confidence in the results [46,47]. Other reasons might include selection of primers and the operators' skills in performing the various stages of the library preparation and sequencing. Indeed, the recommended primers by BIOMED-2 protocols might have been more representative in these cases. Unfortunately, it was not possible to perform another NGS analysis on the cases in question due to the lack of genetic materials and it would have been useful in determining the mismatch reasons and the possible solutions (e.g., changing the used primers). Furthermore, in one case, reference diagnosis confirmed the result obtained through the NGS approach, but since this was a phase 3 diagnostic accuracy study, at statistical level, this would decrease the accuracy of the test under investigation, as it differed from the result obtained through the gold standard analysis.

Table 5. Comparison of the main different approaches for clonality detection. Both PCR and NGS methods showed clear superiority to Southern blotting, while NGS needs less DNA, as compared to PCR

Approach	Sensitivity	Specificity	Time	Material load*
Southern blotting	Low/medium	Very high	Days	10–20 µg
PCR	Very high	High	Hours	50–500 ng
NGS	Very high	High	Hours	10–20 ng

*Minimum load in standard protocols; for NGS, there was a significant correlation between input and sensitivity. PCR: Polymerase chain reaction; NGS: Next-generation sequencing.

Over the past few years, different prior studies have been evaluating the validity and reliability of NGS-based analyses compared to standard PCR/CE ones regarding clonality assessment of TRG gene rearrangements in hematological malignancies. However, to the best of our knowledge, this was the first study that was designed to completely fulfill the requirements of a phase 3 diagnostic accuracy study. Ho *et al*. [25] reported a correspondence in 75% (76/101) of samples analyzed by an Invivoscribe (EuroClonality-based) PCR assay and using the same Invivoscribe NGS assay, we yielded a lower ST (71%) but a better SP (100%). Sufficool *et al*. [26] reported an accuracy of 85% achieved by NGS assay in an assessment regarding mycosis fungoides (MF) detection in FFPE samples, but they compared it with histological analyses and not with the gold standard test. Moreover, the studied cases were less (n = 34) and all had already been diagnosed as MF, with no cases of reactive T proliferation included. Schumacher *et al.* [27] made a comparison between traditional PCR/CE analysis and an alternative NGS platform, namely, the Ion TorrentTM (IT) platform (Life Technologies, Carlsbad, CA, USA), about TRG assessment, and compared it also with the MiSeq® platform. They attained a remarkable 93% accuracy over 46 cases selected among archival samples that had already received a diagnosis. Kansal *et al.* [28] assessed the value of TRG clonality analysis by NGS, comparing the same commercial assay with a single-tube and two-tube PCR/CE methods. For this purpose they analyzed 41 samples with an impressive variety, providing also a thorough discrimination of the case studies based on

patients' pathological conditions, general characteristics, and previous clinical conditions, confirming the reliability and feasibility of NGS in clonality assessment. Nonetheless, we accomplished one of the highest accuracy rates relative to the case study size at present in this context, supporting the previous finding. Moreover, according to the study design, we analyzed unselected cases received for routine diagnostic.

Overall, although PCR methods are still the gold standard for clonality analysis, they have some disadvantages: the most relevant issues are the lack of objectiveness in the interpretation of some results, especially in samples that give rise to a non-Gaussian distribution (so the conclusions drawn from these are subject to the operator interpretation) and the impossibility of resolving a relevant peak relative to a sequence frequency, since a single peak can be the outcome of a single amplified sequence as well as the outcome of different same-sized amplified sequences. Moreover, this approach is not optimal with regard to the determination of the specific clonal V-J rearrangement, and that's fundamental to the monitoring of the disease progression and treatment response, especially in the detection of MRD, considering that the identification of the clone-specific DNA sequence can be used as patient-specific marker for the clonal tumoral cell population.

The introduction of NGS techniques, like Invivoscribe LymphoTrack® Dx TRG MiSeq® assay in the evaluation of antigen-receptor gene rearrangements, can definitively lead to useful advantages over the gold standard PCR-based ones, overcoming their main limitations, as aforementioned, starting from the possibility of distinguishing between the same-sized amplified sequences, given that sequencing separates them on the basis of their composition and not on their length alone and the possibility of better interpreting ambiguous results, such as non-uniform Gaussian distributions and better resolving a polyclonal background. Furthermore, this approach theoretically requires a small amount of DNA (recommended 10-20 ng vs. 100-500 ng). In this study, however, we used a broad range of DNA amounts (75 ng – 4.8 µg), mostly higher than that with the conventional method (median amount 208 ng vs. 100 ng). Intriguingly, three out of seven mismatches were recorded when the amount of DNA was within the lowest quartile (Table 4). This may indicate that, despite the feasibility with lower amounts of input DNA, to reach the highest performance, NGS may need higher amounts, if compared with standard methods. It is noteworthy that NGS has the ability to detect and quantify recurrent minor clones, from a small number of circulating tumor cells. As a consequence, NGS can improve the monitoring of the disease progression and treatment response, especially when it comes to MRD detection, the assessment of which could also be extended virtually to all patients [34-38]. Hence, these new techniques could lead to a better and more objective classification, stratification, and monitoring of lymphoid malignancies, which are essential features of routine diagnostic. Moreover, in recent years, a consortium has been formed to establish standard procedure for TCR and Ig NGS-based analyses, namely, the EuroClonality-NGS consortium (https://euroclonality.org/ngs/about/), again with the aim of optimizing and standardizing both the pre-analytical (sample preparation and study design) and post-analytical (bioinformatics procedures) stages, allowing for, across all international laboratories, an increased efficiency and reproducibility of this approach for the detection of lymphoproliferative malignancies. Actually, the multiplex protocol proposed by the EuroClonality-NGS has been validated by many recent studies concerning different aspects, from clonality assessment to MRD marker identification [39-41]. In addition, from the perspective of reducing as much as possible, the subjectivity of result reading, some of the most recent studies about clonality testing by NGS methods (and also precisely about the TRG rearrangement assessment) proposed new algorithms of result interpretation [42,43], and offered computational pipelines by which it is possible to call clonality in an automated fashion [42]. These procedures lead to an improved efficiency of these assays, with a significant increase in ST and a reduction in false-negative outcomes. Despite the different publications on clonality assessment by NGS analysis, no formal recommendation to use it whenever conventional methods fail was given. Our study somehow supports this idea and it is conceivable that, in future, this can be a reasonable option, also given that the NGS approach can potentially provide a better ST (anyway related to the reliability of the outcomes) in such analyses, thereby also giving the possibility to identify minor clones that could not be identified through conventional PCR/CE analyses.

However, despite the several advantages and the rigorous optimization and standardization of the past few years, the NGS-based methods still have some limitations. First of all, they are costly since NGS technologies require more expensive equipment and reagents, limiting their use, at present, to bigger and well-equipped laboratories that are capable of multiplexing. In fact, the cost-effectiveness of these techniques is also related to the amount of sample analyzed per run and the depth level of the analysis. Therefore, if an analysis involves only few samples without the necessity of the rearrangement identification, a PCR-based approach will still be more cost-effective. It should be noted, however, that NGS prices are continuously dropping and in bigger laboratories, where several samples are run routinely, NGS can be convenient.

Given previous findings, the myriad amount and complexity of data produced by NGS technologies pose a significant bioinformatic challenge, including high-level technical skills. Moreover, considering the multiple amplification steps and library preparation in NGS procedures, another problem is the risk of amplification bias, which leads to an uneven amplification of the analyzed sequences due to differences in nucleotide composition, primer binding efficiency, and template accessibility.

The present study had some limitations, such as case selection bias, since the selected samples were patients with suspected T-cell-related lymphoid disorders, and the failure to distinguish the various pathological cases. Unfortunately, the studied cases were enrolled over a period of 2 years on the basis of retrospective routine diagnostic analyses and were restricted by the statistical representation of lymphoproliferative disorders. Moreover, it is also important to keep it in mind that such analyses are subsequent steps of a previous cytohistological evaluation in the diagnosis of these disorders and are thus normally limited to suspicious situations.

5. CONCLUSIONS

Evaluation of the TCR γ gene rearrangements by Invivoscribe LymphoTrack® Dx TRG MiSeq® assay proved to be practically as effective as conventional and highly-validated PCR-based approaches, such as the BIOMED-2 one, providing significant advantages like increase in amplicon resolution and objectiveness regarding the interpretation of results. Given the efficiency and the depth level of these analyses, they have the potential to be used in molecular diagnostic in the near future and to contribute to the development of personalized medicine. Further, publications, starting from phase 4 diagnostic accuracy studies, will be able to regularly implement NGS-based clonality assessment in routine diagnostic.

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CONFLICT OF INTEREST

The funding agency had no role in the design of the study, the collection, analyses or interpretation of data, the writing of the manuscript, or the decision to publish the results. The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

Conceptualization: Pier Paolo Piccaluga
Data curation: Riccardo Donelli
Formal analysis: Riccardo Donelli, Anna Gazzola, Mohsen Navari, Pier Paolo Piccaluga
Investigation: Riccardo Donelli, Anna Gazzola, Pier Paolo Piccaluga
Methodology: Anna Gazzola, Claudia Mannu, Pier Paolo Piccaluga
Software: Maryam Etebari, Mohsen Navari
Validation: Anna Gazzola, Pier Paolo Piccaluga
Writing – original draft: Riccardo Donelli, Pier Paolo Piccaluga
Writing – review & editing: Mohsen Navari, Pier Paolo Piccaluga

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted on anonymized samples, according to the rules established by the ethics committees for clinical trials at the time of samples collection. In particular, we analyzed anonymous genetic sequences obtained for routine diagnostic purpose in a retrospective manner. Approval code of the ethics committee: 1/2011/U/Tess.

CONSENT FOR PUBLICATION

The study subjects gave consent to participate in this study.

AVAILABILITY OF DATA

Raw data cannot be provided due to privacy concerns.

SUPPLEMENTARY MATERIALS

Supplementary Tables 1-3 indicate STARD requirements, QUADAS 2 requirements, and REMARK requirements, respectively.

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