Next-Generation Sequencing–Based Clonality Assessment of Ig Gene Rearrangements

Michiel van den Brand, Jos Rijntjes, Markus Möbs, Julia Steinhilber, Michèle y van Der Klift, Kim C Heezen, Leonie I Kroeze, Tomas Reigl, Jakub Porc, Nikos Darzentas, et al.

▶ **To cite this version:**

Michiel van den Brand, Jos Rijntjes, Markus Möbs, Julia Steinhilber, Michèle y van Der Klift, et al.. Next-Generation Sequencing–Based Clonality Assessment of Ig Gene Rearrangements. Journal of Molecular Diagnostics, 2021, 23 (9), pp.1105 - 1115. 10.1016/j.jmoldx.2021.06.005 . hal-03354017

HAL Id: hal-03354017
https://hal.sorbonne-universite.fr/hal-03354017v1

Submitted on 24 Sep 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

The Journal of Molecular Diagnostics, Vol. 23, No. 9, September 2021

the Journal of Molecular Diagnostics

jmdjournal.org

See related Guest Editorial on page 1043

Next-Generation Sequencing—Based Clonality Assessment of Ig Gene Rearrangements

A Multicenter Validation Study by EuroClonality-NGS

Michiel van den Brand,* Jos Rijntjes,* Markus Möbs,† Julia Steinhilber,‡ Michèle Y. van der Klift,§ Kim C. Heezen,§ Leonie I. Kroeze,* Tomas Reigl,¶ Jakub Porc,¶ Nikos Darzentas,¶‖ Jeroen A.C.W. Luijks,* Blanca Scheijen,* Frédéric Davi,** Hesham ElDaly,††‡‡ Hongxiang Liu,§§ Ioannis Anagnostopoulos,† Michael Hummel,† Falko Fend,‡ Anton W. Langerak,§ and Patricia J.T.A. Groenen* on behalf of the EuroClonality-NGS Working Group

From the Department of Pathology, Radboud University Medical Center, Nijmegen, the Netherlands; the Institute of Pathology,† Charité-Universitätsmedizin, Berlin, Germany; the Institute of Pathology and Neuropathology,‡ University Hospital Tübingen, Tübingen, Germany; the Laboratory Medical Immunology,§ Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, the Netherlands; the Molecular Medicine Program,¶ Central European Institute of Technology, Brno, Czech Republic; the Department of Hematology,‖ University Hospital Schleswig-Holstein, Kiel, Germany; the Hematology Department,** Hospital Pitié-Salpêtrière and Sorbonne University, Paris, France; the Histopathology Department,†† Coventry University Hospitals National Health Service Trust, Coventry, United Kingdom; the Clinical Pathology Department,‡‡ Cairo University, Cairo, Egypt; and the Haematopathology and Oncology Diagnostics Service,§§ Addenbrooke's Hospital, Cambridge University Hospitals National Health Service Foundation Trust, Cambridge, United Kingdom*

Accepted for publication June 1, 2021.

Address correspondence to Michiel van den Brand, M.D., Ph.D., Department of Pathology, Radboud University Medical Center, Geert Grooteplein Zuid 10, 6525 GA, Nijmegen, the Netherlands. E-mail: michiel.vandenbrand@radboudumc.nl.

Ig gene (IG) clonality analysis has an important role in the distinction of benign and malignant B-cell lymphoid proliferations and is mostly performed with the conventional EuroClonality/BIOMED-2 multiplex PCR protocol and GeneScan fragment size analysis. Recently, the EuroClonality-NGS Working Group developed a method for next-generation sequencing (NGS)—based IG clonality analysis. Herein, we report the results of an international multicenter biological validation of this novel method compared with the gold standard EuroClonality/BIOMED-2 protocol, based on 209 specimens of reactive and neoplastic lymphoproliferations. NGS-based IG clonality analysis showed a high interlaboratory concordance (99%) and high concordance with conventional clonality analysis (98%) for the molecular conclusion. Detailed analysis of the individual IG heavy chain and kappa light chain targets showed that NGS-based clonality analysis was more often able to detect a clonal rearrangement or yield an interpretable result. NGS-based and conventional clonality analysis detected a clone in 96% and 95% of B-cell neoplasms, respectively, and all but one of the reactive cases were scored polyclonal. We conclude that NGS-based IG clonality analysis performs comparable to conventional clonality analysis. We provide critical parameters for interpretation and discuss a first step toward a quantitative scoring approach for NGS clonality results. Considering the advantages of NGS-based clonality analysis, including its high sensitivity and possibilities for accurate clonal comparison, this supports implementation in diagnostic practice. *(J Mol Diagn 2021, 23: 1105—1115; https://doi.org/10.1016/j.jmoldx.2021.06.005)*

Supported by the Dutch Health Insurers' Innovation Fund project number 17-179; and the revenues of the previously obtained patent (PCT/NL2003/000690), which is collectively owned by the EuroClonality/BIOMED-2 Consortium and licensed to Invivoscribe.

Disclosures: The EuroClonality-NGS Working Group is part of the EuroClonality Consortium, which is an independent scientific consortium that aims at innovation, standardization, and education in the field of diagnostic clonality analysis. The revenues of the previously obtained patent (PCT/NL2003/000690), which is collectively owned by the EuroClonality/BIOMED-2 Consortium and licensed to Invivoscribe, are exclusively used for EuroClonality Consortium activities, such as for covering costs of the consortium meetings, collective experiments, the External Quality Assessment schemes, and the EuroClonality Educational Workshops.

Copyright © 2021 Association for Molecular Pathology and American Society for Investigative Pathology. Published by Elsevier Inc.
This is an open access article under the CC BY license (*http://creativecommons.org/licenses/by/4.0*).
https://doi.org/10.1016/j.jmoldx.2021.06.005

Ig gene (IG) clonality analysis is an important technique in the diagnosis of lymphoid diseases to aid in the distinction between benign and malignant B-cell lymphoid proliferations. The current gold standard technique for IG clonality analysis is the standardized and validated EuroClonality/BIOMED-2 multiplex PCR protocol, followed by GeneScan fragment length analysis.[1–3] The availability of next-generation sequencing (NGS) techniques opens up new possibilities for clonality analysis, allowing detection of small clones and accurate clonal comparison. Also, the large amount of data that is generated allows a mathematical/statistical approach to interpretation of the results of clonality analysis.

Recently, the EuroClonality-NGS Working Group reported on a technically feasible method for NGS-based IG gene analysis of the IG heavy chain (IGH) and kappa light chain (IGK) genes.[4–6] Herein, we report the results of an international multicenter biological validation of this NGS IG clonality technique based on 209 specimens of reactive and neoplastic lymphoproliferations using the EuroClonality/BIOMED-2 protocol as benchmark technology.

The B-cell neoplasms that were included in the study were mostly of germinal center or post-germinal center type and reflect the types of lymphoma that are most often tested for clonality in diagnostic practice, such as follicular lymphoma (FL) and marginal zone B-cell lymphoma. In addition, this provides insight in test performance of somatically hypermutated lymphoma subtypes, also including diffuse large B-cell lymphoma (DLBCL). For comparison, a small number of unmutated cases of chronic lymphocytic leukemia/small lymphocytic lymphoma was included. In addition, a large number of reactive lesions, both tonsils and reactive lymph nodes, was included to determine if small clones are detected in reactive lymphoproliferations. This is particularly important because of the high analytical sensitivity of NGS-based IG clonality testing by which clonal rearrangements for IGHV-IGHD-IGHJ, IGHD-IGHJ, and IGKV-IGKJ could be traced back at 2.5% dilutions,[4] but which may also cause a false-positive (over)interpretation.

Materials and Methods

Sample Collection and Pathology Review

DNA samples ($n = 209$) were collected from the archives of the Departments of Pathology at the University Hospital Tübingen (Tübingen, Germany), Charité-Universitätsmedizin Berlin (Berlin, Germany), Cambridge University Hospital (Cambridge, UK), Erasmus MC University Medical Center (Rotterdam, the Netherlands), and Radboud University Medical Center (Nijmegen, the Netherlands) and from the Hematology Department at the Pitié-Salpêtrière and Sorbonne University Hospital (Paris, France) in accordance with the Declaration of Helsinki. DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue ($n = 150$), frozen tissue ($n = 41$), or peripheral blood ($n = 18$) samples.

BIOMED-2 multiplex PCRs and GeneScan analysis were performed according to standard procedures[1] in the participating centers that provided the samples. All conclusions per target, final molecular interpretation, GeneScan result files, and DNA samples were subsequently sent to the coordinating laboratory.

For all cases with tissue available, central pathology review was performed by four hematopathologists (M.B., I.A., H.E., and F.F.) during a joint session at a multiheaded microscope. Cases were diagnosed as B-cell neoplasia ($n = 124$), reactive lymphoproliferation ($n = 82$), or inconclusive between neoplasia and a reactive condition ($n = 3$), as based on the available material. B-cell neoplasia samples were classified according to the 2017 revised fourth edition of the World Health Organization classification.[7] Supplemental Table S1 presents a more detailed specification of the included diagnoses.

Study Design of Clonality Detection by Next-Generation Sequencing

PCR library preparation and sequencing were performed as described previously.[4] Sequencing data were analyzed and visualized with the ARResT/Interrogate platform.[8]

From each sample, DNA was sent to two of the four participating laboratories that locally performed both the wet laboratory procedures and data interpretation. For each sample analyzed, an overall conclusion and a conclusion per target (IGHV-IGHJ, IGHD-IGHJ, IGKV-IGKJ, IGKV-KDE/intronRSS-KDE) was scored by the participating laboratory and subsequently submitted to the coordinating laboratory. The concordance between the laboratories (interlaboratory concordance) was scored for the overall interpretation and the conclusion per target. In case of a complete failure of the analysis, the analysis of the entire sample was repeated. In case of failure of a single target, this was scored as discordant due to failure of the target in a single laboratory.

Data Interpretation and Analysis

Data interpretation was performed at multiple levels. First, NGS results were compared between different laboratories. Second, results from the NGS-based clonality analysis were compared with the results from conventional EuroClonality/BIOMED-2 GeneScan clonality analysis. Third, the NGS-based conclusion was compared with the panel diagnosis.

Technical scoring of the NGS-based IG clonality analysis was done per target. Like in the conventional analysis, no quantitative cutoff was used, but the data were interpreted according to the EuroClonality uniform scoring system for the technical description of the targets (described in the EuroClonality/BIOMED-2 guidelines[3]). The categories for the technical description per target were as follows: clonal

Table 1 IG Clonality Analysis Concordance Assessment

Interlaboratory concordance	Value, *N* (%)
Interlaboratory concordance for NGS-based analysis, overall molecular conclusion	
Concordant	207 (99)
Discordant	2 (1)
Interlaboratory concordance for NGS-based analysis, per target conclusion	
IGHV-IGHJ	
Concordant	193 (92)
Discordant	16 (8)
*Different sequencing result**	*11*
Failure to sequence target in one laboratory	*5*
IGHD-IGHJ	
Concordant	181 (87)
Discordant	28 (13)
*Different sequencing result**	*12*
Failure to sequence target in one laboratory	*15*
Different clonotypes, low template amount	*1*
IGKV-IGKJ	
Concordant	201 (96)
Discordant	8 (4)
*Different sequencing result**	*8*
Failure to sequence target in one laboratory	*0*
IGKV-KDE/intronRSS-KDE	
Concordant	196 (94)
Discordant	13 (6)
*Different sequencing result**	*8*
Failure to sequence target in one laboratory	*5*
Concordance for NGS-based vs GeneScan analysis, overall molecular conclusion	
Concordant	205 (98.1)
Discordant	3 (1.4)
Not evaluable	1 (0.5)
Concordance for NGS-based vs GeneScan analysis, per target conclusion	
IGHV-IGHJ	
Concordant	173 (83)
Discordant	34 (16)
Clonal result in NGS, not in GS	*28*
Clonal result in GS, not in NGS	*6*
Not evaluable	2 (1)
IGHD-IGHJ	
Concordant	170 (81)
Discordant	23 (11)
Clonal result in NGS, not in GS	*9*
Clonal result in GS, not in NGS	*6*
Interpretable result in NGS, not in GS	*8*
Not evaluable	16 (8)
IGKV-IGKJ	
Concordant	186 (89)
Discordant	22 (11)
Clonal result in NGS, not in GS	*19*
Clonal result in GS, not in NGS	*3*
Not evaluable	1 (0.5)
IGKV-KDE/intronRSS-KDE	
Concordant	190 (91)
Discordant	17 (8)
Clonal result in NGS, not in GS	*7*
Clonal result in GS, not in NGS	*0*
Interpretable result in NGS, not in GS	*10*
Not evaluable	2 (1)

(*table continues*)

The interlaboratory concordance was in line with the interlaborabory concordance evaluated by GeneScanning, as reported in the general testing phase, being >80% for IGHD-IGHJ and approximately 90% for IGK rearrangements.[1]

*Clonal versus non-clonal.

IG, Ig gene; IGH, IG heavy chain gene, IGK, IG kappa light chain gene, NGS, next-generation sequencing.

(optional with more detailed descriptions: weak clonal rearrangement or presence of background), polyclonal (optional: irregular polyclonal), no specific products, or multiple products. Especially for the category multiple products, a duplicate measurement, coming from the other laboratory, was essential to see whether the same dominant clonotypes were identified between the two laboratories. In case of a discordant NGS-based result, a third NGS-based analysis was performed, and the majority result was used for comparison with GeneScan results. If the three NGS-based analyses resulted in three different results, the target was excluded from NGS-based versus GeneScan comparison.

The final molecular conclusion was based on the integration of the technical evaluations of the different targets, which was done according to the EuroClonality/BIOMED-2 guidelines,[3] resulting in five main categories: clonal, multiple clones/oligoclonal, polyclonal, no specific product, or pseudoclonal. More detailed molecular interpretations were provided as well.[3] For the overall analysis of concordance, interpretations of particular molecular conclusions were grouped as follows: clonal, clonal with (some) polyclonal background, clonal (bi-allelic), or clonal (biclonal) were all categorized as clonal; polyclonal, polyclonal irregular, multiple products, or oligoclonal were categorized as not clonal; very small clones (ie, <5%) with a high polyclonal background or polyclonal profiles with a clone of unknown significance were categorized as polyclonal.

Results

High Interlaboratory Concordance for NGS-Based IG Clonality Analysis

For NGS-based IG clonality testing, a panel of primer sets targeting the IG heavy [framework (FR) 3] and light chain (κ) genes (ie, IGHV-IGHJ FR3, IGHD-IGHJ, IGKV-IGKJ, IGKV-KDE/intronRSS-KDE) was applied. The median number of reads per case was 44,832 for IGHV-IGHJ, 17,663 for IGHD-IGHJ, 66,606 for IGKV-IGKJ, and 9869 for IGKV-KDE/intronRSS-KDE. The overall molecular

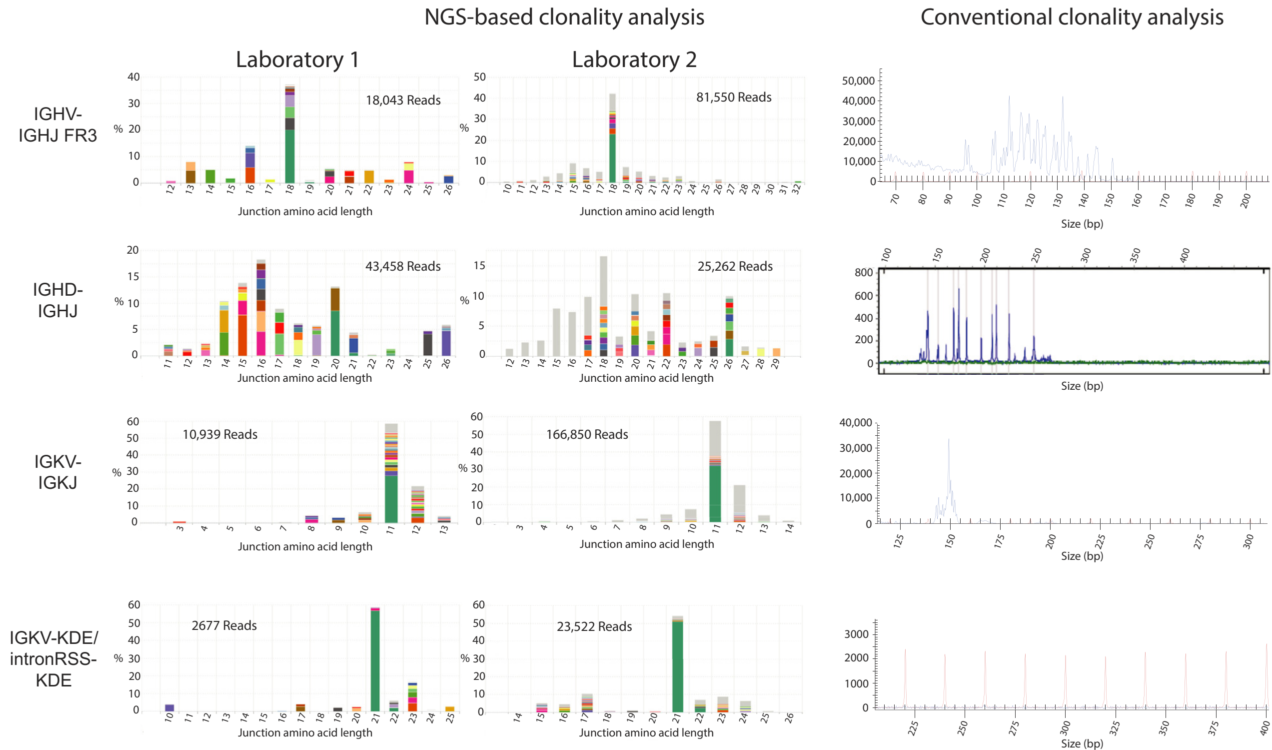

Figure 1 Next-generation sequencing (NGS)—based Ig gene (IG) clonality result in a follicular lymphoma (case EC-175). In this follicular lymphoma, conventional IG clonality analysis with GeneScan gave a polyclonal result for IGHV-IGHJ framework (FR) 3, IGHD-IGHJ, and IGKV-IGKJ, and no (clonal) result for IGKV-KDE/intronRSS-KDE. NGS-based IG clonality analysis gave a clonal result for IGHV-IGHJ FR3, IGKV-IGKJ, and IGKV-KDE/intronRSS-KDE (green bars representing dominant clonotypes). For conventional clonality analysis, IGHD-IGHJ was performed in the coordinating center, whereas the three other PCRs were performed at the center that submitted the case, explaining the different *y*-axis intensities, which are due to different fragment size analyzers. For NGS-based IG clonality analysis, the percentage of reads attributable to a specific clonotype is shown on the *y* axis. The 50 most abundant clonotypes are shown in different colors, and less frequent clonotypes in the background are shown in gray. The *x* axis shows the amino acid length of the junction. For conventional (GeneScan) analysis, the *y* axis shows the signal intensity and the *x* axis shows the fragment size in base pairs.

conclusion of NGS-based IG clonality analysis was concordant between the laboratories in 207 of 209 cases (99% interlaboratory concordance) (Table 1). In two cases, the two laboratories reached a discordant overall molecular conclusion. One case (EC-105) concerned a grade 3B FL with transformation to DLBCL in a lymph node, in which clonal IGKV-IGKJ and IGKV-KDE/intronRSS-KDE products were detected in one of the two laboratories, whereas the other laboratory detected a polyclonal result (Supplemental Figure S1A). Results for IGHV-IGHJ and IGHD-IGHJ were polyclonal in both laboratories. Subsequent analysis of the IGK targets by a third laboratory also gave a polyclonal result, resulting in a final polyclonal score. The other case (EC-207) concerned a peripheral blood analysis of a patient with Sjögren syndrome in whom a clonal product for IGHD-IGHJ was detected in only one laboratory. All other targets gave a polyclonal result in both laboratories. Repeated analysis of IGHD-IGHJ gave a polyclonal result (Supplemental Figure S1B), again resulting in a final score of polyclonality.

When comparing the NGS-based clonality results per target, a high level of concordance (87% to 96%) was shown (Table 1). Discordances were observed in 8% of the target comparisons, which were solved by repetitive testing. This discordance rate per target is in line with the laboratory discordances reported in the general testing of the BIOMED-2 primers by GeneScanning.[1] Because of the complementarity of the different targets and the concordance of the other targets, the discordance of a target did not affect the overall conclusion.

Although carryover of clonal sequences from sample to sample is a potential risk, signs of slight carryover were only occasionally noticed in a small subset of PCRs, and this never influenced the final scoring of the targets.

High Concordance in Overall Molecular Conclusion between Conventional (GeneScan-Based) and NGS-Based IG Clonality Analysis

Results from conventional GeneScan clonality analysis were retrieved from the initial diagnostic workup or, if unavailable, performed specifically for this study. Comparison of the overall molecular conclusion was possible for all but one case (EC-204) in which insufficient DNA was left to perform GeneScan analysis, leaving 208 cases for further analysis. The comparison was restricted to analysis of those targets that were shared between GeneScan and NGS-based clonality (ie, IGHV-IGHJ framework 3; IGHD-IGHJ;

Table 2 Complementarity of IG Targets for Clonality in B-Cell Lymphomas (Number of Targets with Clonal Result)

NGS-based IG clonality analysis

Variable		IGH						IGK									
		VH-JH FR3		DH-JH		VH-JH FR3 + DH-JH		VK-JK		VK-KDE/ intron-RSS-KDE (KDE)		VK-JK + KDE		IGH + IGK (FR3, DH-JH, VK-JK, KDE)		IGH DH-JH + KDE	
	n	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
All B-cell lymphomas	120	79	66	52	43	93	78	96	80	75	63	110	92	115	96	96	80
FL	53	29	55	15	28	35	66	40	75	37	70	48	91	50	94	43	81
DLBCL	34	22	65	19	56	28	82	29	85	15	44	30	88	32	94	24	71
CLL/SLL	13	13	100	9	69	13	100	12	92	7	54	13	100	13	100	11	85
MZL	12	10	83	5	42	12	100	11	92	10	83	12	100	12	100	11	92

Conventional (GeneScan-based) IG clonality analysis

Variable		IGH						IGK									
		VH-JH FR3		DH-JH		VH-JH FR3 + DH-JH		VK-JK		VK-KDE/ intron-RSS-KDE (KDE)		VK-JK + KDE		IGH + IGK		IGH DH-JH + KDE	
	n	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
All B-cell lymphomas	120	55	46	44	37	77	64	83	69	70	58	106	88	114	95	93	78
FL	53	20	38	12	23	28	53	35	66	35	66	46	87	50	94	42	79
DLBCL	34	12	35	18	53	23	68	24	71	13	38	27	79	31	91	24	71
CLL/SLL	13	11	85	7	54	12	92	12	92	8	62	13	100	13	100	11	85
MZL	12	7	58	4	33	9	75	8	67	9	75	12	100	12	100	10	83

CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; FR, framework; IG, Ig gene; IGH, IG heavy chain gene; IGK, IG kappa light chain gene; MZL, marginal zone B-cell lymphoma; NGS, next-generation sequencing; SLL, small lymphocytic leukemia.

IGKV-IGKJ; and IGKV-KDE/intronRSS-KDE). GeneScan and NGS-based IG clonality analysis resulted in the same overall molecular conclusion in 205 of 208 cases (98%) (Table 1). The three discordant cases were all lymphomas (two FLs and one DLBCL). In one case (FL; EC-171) (Supplemental Table S2), a clonal result was obtained with GeneScan for IGHD-IGHJ only, but not with NGS. In the other two cases [FL (EC-175); and DLBCL (EC-198)], clonal IGHV-IGHJ and IGK results were obtained with NGS-based analysis, whereas GeneScan could not detect clonal rearrangements (Figure 1).

In addition, the results from GeneScan and NGS-based IG clonality assessment were also evaluated per individual target. This showed concordance in 83% to 91% of comparisons (Table 1). Discordant results were most often (*n* = 81 comparisons) due to a detectable clonal product or an interpretable result with NGS-based analysis, whereas GeneScan analysis failed to detect a clonal rearrangement or was interpreted as no specific product. In only 15 comparisons, a clonal rearrangement could be detected with GeneScan but not with NGS-based analysis. Altogether, the detection rate of clonal rearrangements per target was higher in NGS-based IG clonality analysis compared with GeneScan. This was observed for the IGHV-IGHJ, IGHD-IGHJ, IGKV-IGKJ, and IGKV-KDE/intronRSS-KDE targets in FL and DLBCL, and in chronic lymphocytic leukemia/small lymphocytic leukemia and marginal zone B-cell lymphoma in particular for the IGH targets (Table 2).

NGS-Based IG Clonality Analysis Is Highly Concordant with the Histologic Diagnosis

The result of NGS-based IG clonality analysis was evaluated in the context of the histologic diagnosis for 204 cases, based on the concept that lymphomas in principle are clonal diseases. In five cases, evaluation was not possible due to an inconclusive pathology diagnosis (*n* = 3; EC-208, EC-220, and EC-224) or a failure of the NGS clonality analysis (*n* = 2; EC-036 and EC-037). Overall, in 197 of 204 cases (97%), the NGS-based clonality result was in line with the histologic diagnosis (ie, clonality detected in lymphomas, and polyclonality detected in reactive cases). In only seven cases (3%), a discordance was observed. In all but one case, this was due to polyclonal NGS results in B-cell neoplasia cases, comprising FL (*n* = 3; EC-084, EC-105, and EC-

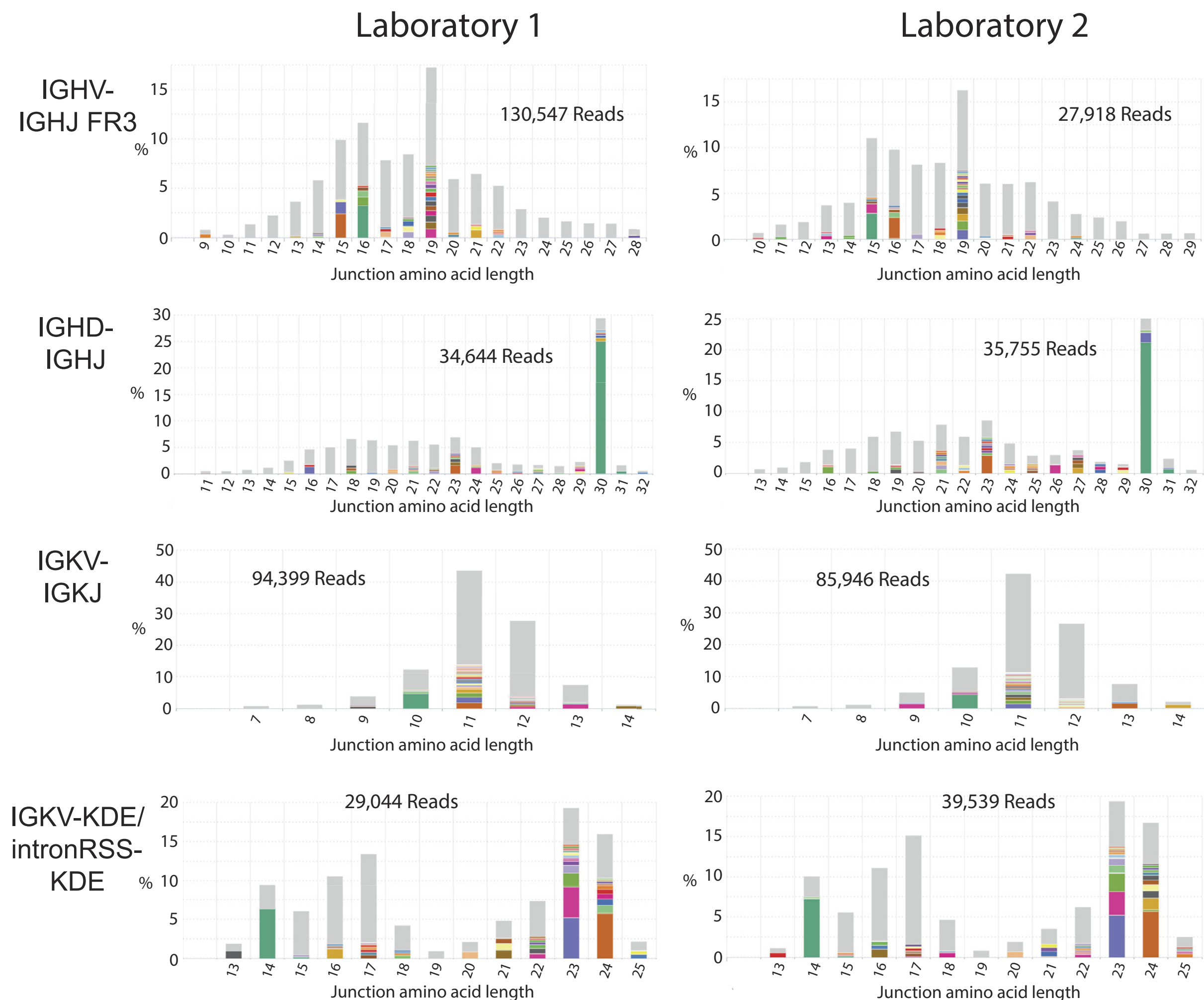

Figure 2 Reactive lymphoproliferation with a clonal result (case EC-226). In this reactive lymphoproliferation that could not be further specified, next-generation sequencing—based Ig gene (IG) clonality analysis detected a clone in a polyclonal background in duplicate for IGHV-IGHJ framework (FR) 3, IGHD-IGHJ, IGKV-IGKJ, and IGKV-KDE/intronRSS-KDE (green bars representing dominant clonotypes). With conventional GeneScan IG clonality analysis, only in the IGHV-IGHJ FR3 target, a minor clone was detected (data not shown).

171), DLBCL ($n = 2$; PT13 and PT14), and monoclonal B-cell lymphocytosis ($n = 1$; EC-212) (Supplemental Table S2). In four of these six cases of B-cell neoplasia that were polyclonal with the NGS clonality analysis, a (weak) clonal rearrangement could be detected with conventional clonality analysis for IGHV-IGHJ FR1 and/or FR2 (which are not included in this NGS-based assay). These four cases had good DNA quality, showing amplification of the 300- and/or 400-bp band of the control PCR. Thus, for 3.3% of the total number of B-cell lymphomas in this cohort, consisting of mainly germinal center and post-germinal center lymphomas derived from FFPE tissue, including IGHV-IGHJ FR1 and FR2, would have additional value. Another case (EC-226) with a discordant result was a reactive lymphoproliferation in which all NGS-PCR targets were suggestive of a clone in a reactive background, probably reflecting the higher sensitivity of NGS-based clonality analysis (Figure 2). Minor clones were observed in seven other reactive cases and added as a comment, but in these cases the final molecular conclusion was not clonal.

When focusing on B-cell lymphomas only, NGS-based clonality analysis was able to detect a clonal B-cell proliferation in 96% of the cases versus 95% by conventional IG clonality analysis (Table 2).

Frequency of Most Abundant Clonotypes in Reactive Lymphoproliferations

Clonality results should always be interpreted in the context of the clinical presentation and histopathology; hence, it is impossible to provide a fixed cutoff for clonality. Nevertheless, one aspect of NGS-based clonality is that it does allow a more quantitative evaluation of the different clonotypes present. The background level of dominant clonotypes was therefore evaluated in reactive lymphoproliferations by

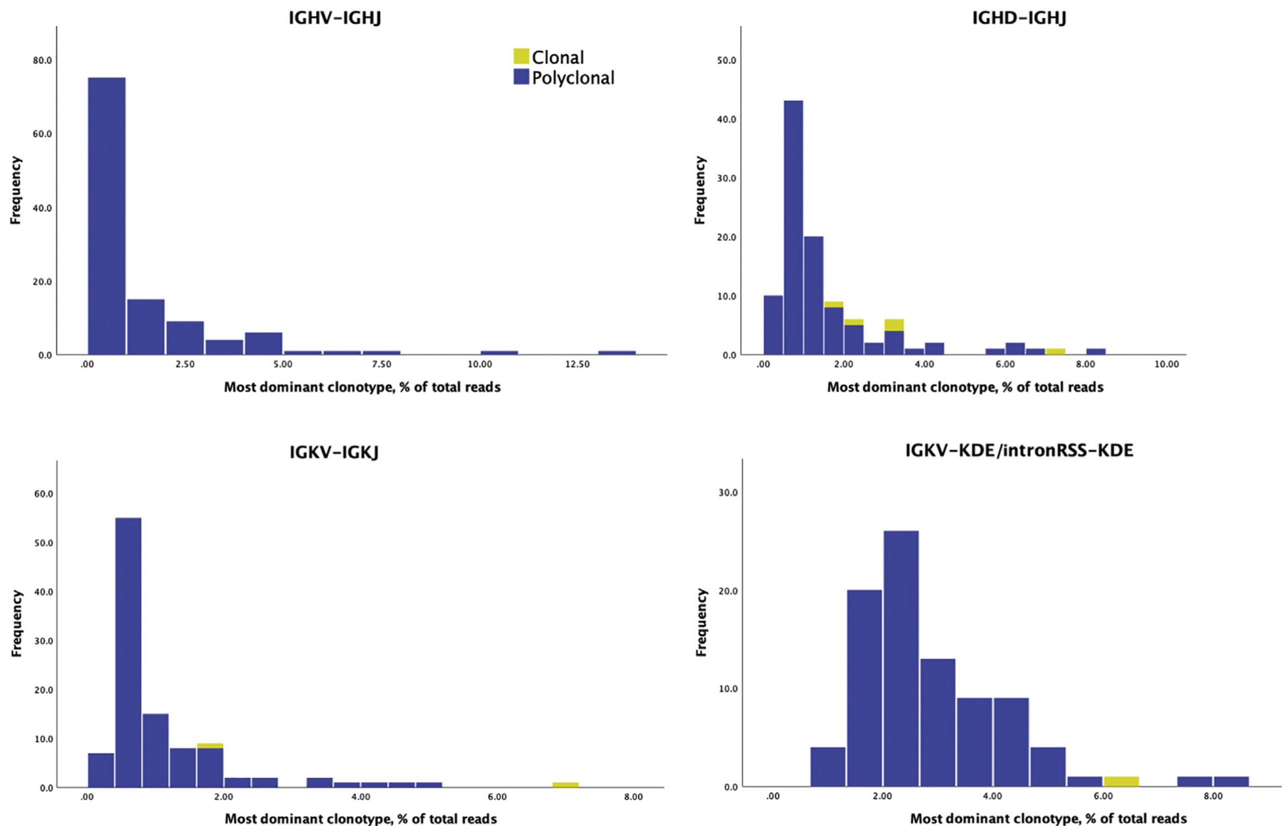

Figure 3 Distribution of the most abundant clonotypes in next-generation sequencing—based clonality analysis of reactive lymphoproliferations. These figures show the distribution of the percentage in which the most abundant clone was detected per sample for the different Ig gene (IG) targets in samples with a panel diagnosis of a reactive lymphoproliferation. Only samples with a DNA quality of ≥300 bp and at least 1000 reads were included. In all targets, the large majority of samples had a most dominant clonotype consisting of <5% of the reads, but all targets also showed some outliers. However, in only a small part of these outliers, the molecular conclusion was finally scored as clonal, indicating that not only the percentage of the dominant clonotype, but also the composition of the background is important for scoring of clonality.

assessing the distribution of the most dominant clonotype per target in reactive lymph node and tonsil samples. Only samples with a DNA quality of ≥300 bp (as assessed by a size ladder PCR, according to the BIOMED-2 assay[1]) were included in this analysis, and at least 1000 reads per target were required for a target to be evaluable. Data on clonotype distribution show that in most reactive samples the most abundant clonotype showed a frequency of <5% (Figure 3). For all targets, some outliers were observed, but the results of most of these targets were scored as polyclonal. This underlines the idea that not only the percentage of the most dominant clonotype, but also recognition of the overall pattern, is important for interpretation (an example for case EC-053 is shown in Supplemental Figure S2). For IGKV-KDE/intronRSS-KDE targets, more dominant clonotypes, with a frequency of >5%, were observed than for the other targets, which can be explained by the limited variation in intronRSS-KDE rearrangements. This implies that dominant intronRSS-KDE products in NGS-based clonality data should be interpreted with caution, especially when other targets do not show clonality.

Toward a Quantitative Approach for Scoring of NGS-Based Clonality Results

A quantitative measure of a clonal result could help in the interpretation of NGS-based clonality data. In an attempt to find relevant indicators of clonality, the percentage of the most dominant clonotype over the background was correlated, using pattern scores of the results per target (clonal versus non-clonal) as benchmark. For an individual target to be included in this analysis, at least 1000 reads were required. Ratios of the most dominant clonotype divided by different measures of the background were calculated by dividing the read percentage of the most dominant clonotype (number 1 clonotype) by either the read percentage of clonotypes 2 to 8 or by an average of these background clonotypes. With these ratios, receiver-operating characteristic curves were calculated, and the area under the curve was determined (Supplemental Figure S3). This allowed identification of the measure with the highest discriminatory value between a clonal result and a non-clonal result (ie, either polyclonal or

Table 3 Critical Parameters for Interpretation of NGS-Amplicon—Based Clonality Analysis of Ig Genes

Critical parameters for interpretation	Technical aspects	More detailed considerations
Input of DNA per multiplex PCR	40, 20, or 10 ng of input DNA for each multiplex PCR is highly recommended.[4]	• The essential input was determined by evaluation of the diversity rearrangement pattern in a polyclonal sample. Note with 5 ng input of DNA, the pattern starts to change to a less diverse pattern; 2.5-ng input results in pseudoclonality. • An input of 5, 2.5, and even 1.25 ng per multiplex PCR is sufficient to detect clonal rearrangement in clonal B-cell lymphoma samples (containing 80% and 10% of tumor cells).
Estimated tumor load by histology or flow cytometry data	Clonal rearrangements can be detected at a dilution of 10%, 5%, and 2.5% in polyclonal background[4] (tested with 40 ng of DNA input).	• Note the detection will be less sensitive when the input of DNA/multiplex PCR is <40 ng.
Amount of DNA	Limited template DNA may be due to: • only few B cells present • and/or failure to amplify a prominent neoplasia (due to somatic hypermutation) with resulting amplification of the background • and/or low amount of input DNA	• Because of the limited diversity in the template DNA, the analysis could provide a sufficient number of reads but with a clonal/multiple clones-like pattern. • Correlation with the input DNA, tumor cell percentage, and results from other targets will allow recognition of this situation. • A duplicate analysis will help to distinguish pseudoclonality from true clonality.
Number of reads/target	Reads >1000: can be interpreted Reads <500: cannot to be interpreted Reads 500—1000: interpreted with caution, dependent on the PCR targets; the PCR targets IGHD-IGHJ and IGKV-KDE/intronRSS-KDE may lead to a limited number of reads because of the ordered process of Ig heavy and light chain rearrangements[12—14]	• Note: cutoffs cannot be generalized as they depend on many conditions, including the sequencing platform used and the number of samples per chip. • For this study, we have used up to 24 samples per Ion Torrent 318 chip. • Because unique molecular identifiers are not included in this technique, it is unknown how many DNA molecules in the original sample form the basis of the result. • Samples with <500 reads may result in patterns with limited diversity, which could be scored as clonal or multiple clones but which should not be interpreted as such because of the low number of reads.
Duplicate assessment	A duplicate analysis is helpful in case of ambiguous results. On the basis of the results in this study, we recommend doing a duplicate analysis at least in the following situations: • A clonal result in only a single target. • A result of multiple products in one of the PCR targets that may affect the conclusion of the case.	Duplicate analyses can be performed standard or only in specific situations.

(*table continues*)

Table 3 (*continued*)

Critical parameters for interpretation	Technical aspects	More detailed considerations
Clinical, immunologic, and histopathologic context	NGS-based IG clonality analysis should be interpreted in the context of the clinical presentation and histopathology	• Small clones can be detected, also in reactive conditions. • A small clone could be relevant in a case with an expected low tumor cell percentage. • In a sample with a high number of cells suspicious for lymphoma, detection of a small clone with NGS-based clonality should not necessarily lead to a diagnosis of lymphoma.

IG, Ig gene; IGH, IG heavy chain gene; IGK, IG kappa light chain gene;NGS, next-generation sequencing.

multiple products). High area under the curve values could be achieved for all four targets (0.996 for IGHV-IGHJ; 0.998 for IGHD-IGHJ; 0.997 for IGKV-IGKJ; and 0.978 for IGKV-KDE/intronRSS-KDE) (Supplemental Table S3). For each target, one of the ratios with the highest area under the curve was used to determine cutoff ratios to differentiate between a clonal and a non-clonal result. Altogether, this resulted in sensitivities ranging from 94% to 97% and specificities ranging from 95% to 99% (Supplemental Table S4).[9]

Discussion

This biological validation study of NGS-based IG clonality testing shows that NGS-based IG clonality and conventional EuroClonality/BIOMED-2 IG clonality analysis employing GeneScan provide highly similar results; the two techniques arrived at the same overall conclusion in 98% of the analyses. In addition, NGS-based IG clonality showed a high interlaboratory concordance of 99% for the overall conclusion of the analysis. These results were obtained despite the enrichment of the samples for FFPE material and (post-) germinal center B-cell lymphomas. Our multicenter validation study thus shows that NGS-based IG clonality can reliably be applied as an alternative for conventional GeneScan clonality analysis in molecular lymphoma diagnostics. Of note, plasma cell neoplasms were not included in this study. Also, the NGS assay, as used in this study, does not utilize IGHV FR1/FR2 forward primer sets analogous to those in BIOMED-2/GeneScan analysis, and may yield polyclonal results in a small number of cases that would have been clonal by the latter assay (if the DNA quality of these cases allows amplification of the larger amplicons). The three samples with a discordant result between NGS-based clonality analysis and GeneScan clonality analysis were histologically all lymphomas. In one of these, GeneScan but not NGS-based analysis was able to detect a clonal B-cell proliferation. In the other two, NGS-based analysis but not GeneScan demonstrated the clonal B-cell population. These discordances are probably a result of the different primer design for NGS- and GeneScan-based clonality analysis; a new set of primers was designed for NGS-based clonality detection to generate smaller amplicons, which makes the technique more suitable for analysis of FFPE tissue. Indeed, although the overall results were highly similar, when comparing the results from GeneScan- and NGS-based clonality assessment for the different targets (ie, IGHV-IGHJ, IGHD-IGHJ, IGKV-IGKJ, and IGKV-KDE/intronRSS-KDE), NGS-based clonality was more often interpretable and was more often able to demonstrate a clonal product in B-cell neoplasia (Table 2). In addition to the smaller amplicon size that facilitates the detection of rearrangements in suboptimal DNAs from FFPE tissues, primer design of the NGS-based assay was performed on the basis of different parameters than for the first BIOMED-2 primers. The new set of primers was designed to be gene specific,[5] whereas the BIOMED-2 primers were designed as gene family—specific primers.[1] Clearly, this new primer design with more optimal primer binding sites for NGS-based assays resulted in a higher detection rate of the different targets in both pre-germinal and post-germinal center B-cell lymphomas. When comparing our current NGS-based approach with the results obtained in the original BIOMED-2 GeneScan clonality analysis studies, the BIOMED-2 assay shows a higher detection rate of clonality in B-cell lymphoma (99% versus 96%). One could argue that in the BIOMED-2 study,[10] fresh-frozen material was used, whereas in the current study, most DNA was derived from FFPE material with suboptimal DNA integrity. Nevertheless, in the B-cell neoplasms in which NGS clonality could not detect a clonal B-cell proliferation ($n = 6$), the DNA was derived from fresh-frozen tissue/peripheral blood ($n = 4$) or from FFPE material with sufficient DNA quality ($n = 2$) (Supplemental Table S2). Moreover, comparison of the detection rate of a clonal result in lymphoma showed a better performance using DNA derived from FFPE material than from fresh-frozen material (98% versus 89%) (Supplemental Table S5). A more likely explanation therefore seems to be that the BIOMED-2 study included

multiple IGHV-IGHJ targets (ie, FR1, FR2, and FR3), whereas only one (IGH FR3) was analyzed in this study. Indeed, additional analyses of IGH FR1 and FR2 PCRs gave a clonal result in four of the six cases of B-cell neoplasia in which analysis with only IGH FR3 could not detect a clonal rearrangement (Supplemental Table S2). In addition, the BIOMED-2 study contained a larger number of lymphomas with lower levels of somatic hypermutation (ie, chronic lymphocytic leukemia and mantle cell lymphoma).

Analysis of reactive lymphoproliferations showed that the most dominant clonotype in that setting is often present in <5% of the reads, although it can be present in as much as 14% of reads. Nevertheless, most of the targets with a high percentage of dominant clonotypes were still scored as polyclonal in the light of the entire clonotype pattern, thus stressing the importance of evaluation of the pattern and not only the percentage of the most dominant clonotype. In most B-cell lymphomas, the most dominant clonotype was represented by a much higher percentage of the reads, albeit that this is of course primarily dependent on the tumor cell percentage. In 81 of 82 reactive cases, the NGS-based clonality assay was helpful in confirming the polyclonal character of the reactive lesions. In 1 of the 82 reactive cases, the final molecular result was reproducibly scored as clonal using the NGS-based clonality assay based on dominant clonotypes in all four different gene targets. Detection of small clones in reactive lesions has been described previously[11] and should result in a more detailed pathologic review that might explain the presence of a small B-cell clone.

As the NGS-based approach for clonality analysis allows quantitative assessment of the results, a first effort was made to find cutoff values to distinguish between a clonal and a polyclonal result. Indeed, using the percentages of the different clonotypes, the score of a particular target (clonal versus nonclonal) could be predicted with high specificity and sensitivity. Although this is only a first and preliminary attempt to quantitatively assess clonality results, the results suggest that quantitative cutoff points could theoretically be useful to help interpret NGS-based clonality analysis. It has to be stressed that a quantitative analysis by strict cutoff values can never replace a careful assessment of the clonality analysis. This is because the DNA input, the DNA quality of the sample, and the PCR target are important parameters, as summarized in Table 3, which influence the number of reads and the clonotype pattern so that these do not necessarily reflect the actual distribution of the different clonotypes in the tissue. Also, there are minor differences in efficiency of the different primer sets. In combination with the knowledge that Ig V, D, and J genes are utilized with unequal frequencies,[15–18] an entirely equal, unbiased, detection of V, D, and J genes is not possible. Moreover, histomorphology, the suspected tumor cell percentage, and immunologic aspects[11] should also be taken into consideration on evaluation of clonality results, as summarized in Table 3. Implementation of NGS-based clonality analysis in the diagnostic laboratory has multiple potential advantages. First of all, the technique is highly sensitive, allowing the detection of small clones in a polyclonal background. The limit of detection via NGS-based clonality of 2.5% is not possible with the conventional assays because the clonal products will disappear in the polyclonal background. Second, the NGS-based clonality assay has been designed especially for FFPE specimens with suboptimal DNA quality in contrast to the conventional assays (with size windows between 100 and 420 nucleotides for detection of clonal rearrangements), via which potential clonal rearrangements in suboptimal DNAs will not be detected. Third, a more accurate comparison of clonal B-cell populations is possible because the exact sequences can be compared rather than comparing peak sizes in GeneScan analysis. This is extremely valuable in determining clonal relatedness in patients with multiple lymphomas. Fourth, some targets are more easily scored with the NGS-based approach. Especially IGK can be difficult to score with GeneScan analysis because of the narrow gaussian curve. With the NGS-based approach, each clonotype is visualized individually, allowing easier interpretation. The large amount of data that is generated with NGS-based clonality analysis opens up new possibilities. It carries the potential for a quantitative approach to the scoring of clonality analysis rather than scoring by pattern recognition only. Also, new applications become possible on a larger scale (eg, assessment of the Ig repertoire).

The introduction of NGS-based clonality testing needs an adapted workflow in a laboratory. Currently, in a diagnostic (pathology) laboratory, many tests are NGS based; therefore, switching to NGS for clonality assessment can simplify the workflow from many different technologies to a few NGS-based workflows. Also, maintaining different technologies (NGS, GeneScan, and/or heteroduplex analysis) in a laboratory requires validation of the workflows and the different assays under the accreditation standards.

The workflow for NGS-based clonality testing will probably affect the turnaround time, although this is highly dependent on the local situation. In general, it can be expected that the workflow for the conventional method of clonality analysis involving the EuroClonality/BIOMED-2 clonality assay with GeneScan has a turnaround time of 1 to 1.5 days, excluding analysis time, whereas NGS-based clonality has an estimated turnaround time of 2.5 days for the wet laboratory part and sequencing time, followed by the bioinformatics analysis.

Depending on the local situation, the clonality sequencing samples (with amplicons of around 200 bp) might be combined with other sequencing samples with similarly sized amplicons, which will save sequencing costs and reduce turnaround time. This will of course need local testing and validation. The sequencing costs will be highly dependent on the instrument platform, the number of samples, and the maximum acceptable turnaround time.

In summary, in this study, we have investigated the clonality profiles in 124 World Health Organization–defined mature B-cell neoplasms, most of which concerned FFPE tissue. We showed that the NGS-based IG clonality assay, based on the combination of IGHV-IGHD-IGHJ FR3, IGKV-IGKJ, and the

unmutated targets IGHD-IGHJ and IGKV-KDE/intronRSS-KDE, performs extremely well in mature B-cell neoplasms using good, but also suboptimal, quality DNA. Even though more objective parameters for interpretation could be formulated, it remains important to evaluate clonality testing in the context of clinical, histologic, and immunophenotypic information.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2021.06.005*.

References

1. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, Delabesse E, Davi F, Schuuring E, Garcia-Sanz R, van Krieken JH, Droese J, Gonzalez D, Bastard C, White HE, Spaargaren M, Gonzalez M, Parreira A, Smith JL, Morgan GJ, Kneba M, Macintyre EA: Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003, 17:2257−2317
2. van Krieken JH, Langerak AW, Macintyre EA, Kneba M, Hodges E, Sanz RG, Morgan GJ, Parreira A, Molina TJ, Cabecadas J, Gaulard P, Jasani B, Garcia JF, Ott M, Hannsmann ML, Berger F, Hummel M, Davi F, Bruggemann M, Lavender FL, Schuuring E, Evans PA, White H, Salles G, Groenen PJ, Gameiro P, Pott C, Dongen JJ: Improved reliability of lymphoma diagnostics via PCR-based clonality testing: report of the BIOMED-2 Concerted Action BHM4-CT98-3936. Leukemia 2007, 21:201−206
3. Langerak AW, Groenen PJ, Bruggemann M, Beldjord K, Bellan C, Bonello L, Boone E, Carter GI, Catherwood M, Davi F, Delfau-Larue MH, Diss T, Evans PA, Gameiro P, Garcia Sanz R, Gonzalez D, Grand D, Hakansson A, Hummel M, Liu H, Lombardia L, Macintyre EA, Milner BJ, Montes-Moreno S, Schuuring E, Spaargaren M, Hodges E, van Dongen JJ: EuroClonality/BIOMED-2 guidelines for interpretation and reporting of Ig/TCR clonality testing in suspected lymphoproliferations. Leukemia 2012, 26:2159−2171
4. Scheijen B, Meijers RWJ, Rijntjes J, van der Klift MY, Mobs M, Steinhilber J, Reigl T, van den Brand M, Kotrova M, Ritter JM, Catherwood MA, Stamatopoulos K, Bruggemann M, Davi F, Darzentas N, Pott C, Fend F, Hummel M, Langerak AW, Groenen P, EuroClonality-NGS Working Group: Next-generation sequencing of immunoglobulin gene rearrangements for clonality assessment: a technical feasibility study by EuroClonality-NGS. Leukemia 2019, 33:2227−2240
5. Bruggemann M, Kotrova M, Knecht H, Bartram J, Boudjogrha M, Bystry V, et al: Standardized next-generation sequencing of immunoglobulin and T-cell receptor gene recombinations for MRD marker identification in acute lymphoblastic leukaemia; a EuroClonality-NGS validation study. Leukemia 2019, 33:2241−2253
6. Knecht H, Reigl T, Kotrova M, Appelt F, Stewart P, Bystry V, Krejci A, Grioni A, Pal K, Stranska K, Plevova K, Rijntjes J, Songia S, Svaton M, Fronkova E, Bartram J, Scheijen B, Herrmann D, Garcia-Sanz R, Hancock J, Moppett J, van Dongen JJM, Cazzaniga G, Davi F, Groenen P, Hummel M, Macintyre EA, Stamatopoulos K, Trka J, Langerak AW, Gonzalez D, Pott C, Bruggemann M, Darzentas N, EuroClonality-NGS Working Group: Quality control and quantification in IG/TR next-generation sequencing marker identification: protocols and bioinformatic functionalities by EuroClonality-NGS. Leukemia 2019, 33:2254−2265
7. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri S, Stein H, Thiele J: WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Rev ed 4. Lyon, France, International Agency for Research on Cancer, 2017
8. Bystry V, Reigl T, Krejci A, Demko M, Hanakova B, Grioni A, Knecht H, Schlitt M, Dreger P, Sellner L, Herrmann D, Pingeon M, Boudjoghra M, Rijntjes J, Pott C, Langerak AW, Groenen P, Davi F, Bruggemann M, Darzentas N, EuroClonality NGS: ARResT/-Interrogate: an interactive immunoprofiler for IG/TR NGS data. Bioinformatics 2017, 33:435−437
9. Youden WJ: Index for rating diagnostic tests. Cancer 1950, 3: 32−35
10. Evans PA, Pott C, Groenen PJ, Salles G, Davi F, Berger F, Garcia JF, van Krieken JH, Pals S, Kluin P, Schuuring E, Spaargaren M, Boone E, Gonzalez D, Martinez B, Villuendas R, Gameiro P, Diss TC, Mills K, Morgan GJ, Carter GI, Milner BJ, Pearson D, Hummel M, Jung W, Ott M, Canioni D, Beldjord K, Bastard C, Delfau-Larue MH, van Dongen JJ, Molina TJ, Cabecadas J: Significantly improved PCR-based clonality testing in B-cell malignancies by use of multiple immunoglobulin gene targets: report of the BIOMED-2 Concerted Action BHM4-CT98-3936. Leukemia 2007, 21:207−214
11. Langerak AW, Molina TJ, Lavender FL, Pearson D, Flohr T, Sambade C, Schuuring E, Al Saati T, van Dongen JJ, van Krieken JH: Polymerase chain reaction-based clonality testing in tissue samples with reactive lymphoproliferations: usefulness and pitfalls: a report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2007, 21:222−229
12. Maki K, Nagata K, Kitamura F, Takemori T, Karasuyama H: Immunoglobulin beta signaling regulates locus accessibility for ordered immunoglobulin gene rearrangements. J Exp Med 2000, 191: 1333−1340
13. Korsmeyer SJ, Hieter PA, Ravetch JV, Poplack DG, Waldmann TA, Leder P: Developmental hierarchy of immunoglobulin gene rearrangements in human leukemic pre-B-cells. Proc Natl Acad Sci U S A 1981, 78:7096−7100
14. Langerak AW, Nadel B, De Torbal A, Wolvers-Tettero IL, van Gastel-Mol EJ, Verhaaf B, Jager U, van Dongen JJ: Unraveling the consecutive recombination events in the human IGK locus. J Immunol 2004, 173:3878−3888
15. Pascual V, Wilson P, Liu YJ, Banchereau J, Capra JD: Biased VH4 gene segment repertoire in the human tonsil. Chem Immunol 1997, 67: 45−57
16. Brezinschek HP, Foster SJ, Brezinschek RI, Dorner T, Domiati-Saad R, Lipsky PE: Analysis of the human VH gene repertoire: differential effects of selection and somatic hypermutation on human peripheral CD5(+)/IgM+ and CD5(-)/IgM+ B cells. J Clin Invest 1997, 99:2488−2501
17. de Wildt RM, Hoet RM, van Venrooij WJ, Tomlinson IM, Winter G: Analysis of heavy and light chain pairings indicates that receptor editing shapes the human antibody repertoire. J Mol Biol 1999, 285: 895−901
18. Arnaout R, Lee W, Cahill P, Honan T, Sparrow T, Weiand M, Nusbaum C, Rajewsky K, Koralov SB: High-resolution description of antibody heavy-chain repertoires in humans. PLoS One 2011, 6: e22365