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ARTICLE

Reproducibility of low-level residual myeloma immunoglobulin detection using ultra-deep sequencing

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Multiple myeloma, a mature B-cell neoplasm, is the second most common hematologic malignancy. Despite advancements in treatment, the disease remains incurable, with more than 100,000 annual deaths worldwide. As recommended by the International Myeloma Working Group, measurable residual disease (MRD) should be addressed at a 10^{-5} sensitivity level or beyond for practical purposes. Next-generation sequencing (NGS) has provided new opportunities with deep sequencing of clonal rearrangements of the immunoglobulin heavy chain (IGH) locus in B-cell malignancies. Although the ability to resolve one cancerous cell in a million other B cells is becoming attractive as a prognostic indicator in sustained patients who are MRD-negative, reaching consistent sensitivity levels is challenging because of sample stochasticity and the substantial amount of deoxyribonucleic acid (DNA) required for library preparation. Thus, in the presented study, we implemented ultra-deep sequencing of rearranged IGH to investigate the reproducibility and consistency aimed at the 10^{-5} sensitivity level. In this controlled setup, our data provided stable MRD detection of 1.2 clonal cells per 100,000 analyzed cells and longitudinal reproducibility. We also demonstrated a low false-negative rate using 4–5 replicates and 700–800 ng DNA per sequencing replicate. In conclusion, adding an internal control to the replicates enabled clonal cell normalization for MRD evaluation as a stable reference. These findings may guide MRD-level reporting and comparisons between laboratories. © 2023 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/)

HIGHLIGHTS

- Detection of low-level myeloma residual disease using deep sequencing of clonal immunoglobulin rearrangements is reproducible in a controlled setup using the LymphoTrack assay.
- Residual myeloma cells can consistently be quantified by employing an internal deoxyribonucleic acid control.
- The nontechnical replicate failure rate of low-level residual disease detection by sequencing is a stochastic process.

Multiple myeloma (MM) is a mature B-cell neoplasm in which activated clonal plasma cells proliferate and secrete immunoglobulins, accumulating primarily in the bone marrow. MM is the second most common hematologic malignancy, accounting for more than 100,000 annual deaths worldwide. Despite new effective treatments, the disease remains incurable. The typical course of MM is periods of active disease with the need for treatment followed by periods of remissions of variable durations [1,2].

The International Myeloma Working Group (IMWG) has established measurable residual disease (MRD) negativity as a criterion to assess disease response in MM [3]. MRD negativity, and particularly sustained negativity, has been proved to predict prolonged progression-free survival and overall survival in newly diagnosed patients with MM [3–9].

The MRD can be assessed by different methodologies, including flow cytometry, allele-specific oligonucleotide (ASO) quantitative polymerase chain reaction (qPCR), or next-generation sequencing (NGS) of immunoglobulin variable, diversity, and joining (VDJ) sequences. More recent advancements have enabled highly sensitive and specific quantification of MRD through deep sequencing of clonal immunoglobulin heavy chain (IGH) or light chain (IGL) rearrangements, thereby attaining significant clinical potential, and several method comparisons have been performed or reviewed [3,10,11].

MRD evaluation by NGS still necessitates the use of PCR primer sequences, such as those developed by EuroClonality/BIOMED-2 group with subsequent deep sequencing, or NGS-based assays from service providers, such as US Food and Drug Administration-approved clonoSEQ (Adaptive Biotechnologies) or the commercially

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available LymphoTrack assay (Invivoscribe) [12−17]. Collectively, experimental design and data analyses lack some standardization, despite previous thorough work and ongoing efforts (more information is given in [13,16,18−22]).

Sensitive detection of MRD has shown prolonged progression-free survival and overall survival in patients who were MRD-negative [8]. Therefore, reaching as low as 10^{-6} has become attractive for monitoring MRD, and even sensitivity levels down to 10^{-7} have been published [23]. Although reaching a sensitivity of 10^{-6} is attractive from a clinical perspective, achieving this may be challenging, as discussed previously [24]. Thus, in this article, we evaluated the technical aspects of the LymphoTrack assay, focusing on the 10^{-5} sensitivity level recommended by IMWG [3], we assessed the number of reads and replicates and the amount of deoxyribonucleic acid (DNA) required for reliable MRD detection. No clear guidelines currently exist on how to report or calculate MRD. In this study, we implemented an internal DNA control to evaluate the reproducibility and consistency of MRD clonotyping.

METHODS

Samples and DNA Preparation

Eight diagnostic samples from patients diagnosed with MM at Odense University Hospital, Denmark, were included to demonstrate reproducible sensitivity levels down to approximately one clonal cell among 100,000 analyzed cells. CD138-positive cells were isolated from bone marrow samples of each patient using CD138 microbeads (StraightFrom Whole Blood CD138 microbeads, Miltenyi Biotec) with an AutoMACS (Miltenyi Biotec) and placed in lysis buffer (MagNA Pure LC mRNA isolation kit I, lysis buffer, Roche Diagnostics GmbH). DNA was extracted using the MagNA LC DNA isolation kit (Roche) on a MagNA Pure LC robot (Roche). DNA from the diagnostic samples (Supplementary Table E1) was diluted into a background of leukocyte DNA consisting of a pool of DNA from 13 control donors. Fresh DNA preparations were made for each LymphoTrack sequencing run and replicate series. DNA from control donors was extracted directly from 20 mL of blood according to the manufacturer's protocol (QIAamp DNA Blood Midi/Maxi kit). Donor DNA was precipitated by adding sodium acetate to reach 0.3 M final concentration in a 2.5 vol/vol 96% ethanol ratio. The DNA was then centrifuged at 16,100*g* for 20 min at 4°C and washed two times by centrifuging DNA for 10 min at 16,100*g* at 4°C in 77% ethanol. The DNA was dissolved in 10 μL H_2O. Concentrations were determined using Qubit 2.0 Fluorometer (Invitrogen, ThermoFisher Scientific) with the Qubit dsDNA HS assay kit for patients and the Qubit dsDNA BR assay kit for the donors.

Library Preparation and NGS

NGS was performed as described in the LymphoTrack Dx IGH (FR1/FR2/FR3) assay S5/PGM user guide (Invivoscribe, San Diego, CA, USA, 280388, Rev. D, Dec. 2018). In brief, mastermix from the LymphoTrack Dx IGH FR1 assay S5/PGM and EagleTaq DNA polymerase (Roche) were used. After 29 amplification cycles, the PCR products were cleaned using AMPure XP beads (Beckman Coulter), eluted in tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetic acid buffer (Promega), and quantified with the 2100 Agilent Bioanalyzer using the DNA 1000 kit (Agilent Technologies). The libraries were prepared in tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetic acid buffer (100 pM). The pooled libraries were diluted in H_2O (Sigma) to a final concentration of 33 pM, loaded on the Ion 530 chip using the Ion Chef system, and sequenced on an Ion Gene Studio S5 Prime sequencer (Ion Torrent; ThermoFisher Scientific). The clonotypes were determined using the LymphoTrack software (version 2.4.6, Invivoscribe) for each of the diagnostic samples.

For the MRD analysis, a control sample (*LymphoTrack B cell Low Positive Control*, Invivoscribe) corresponding to a clonal control DNA diluted into a polyclonal background DNA from tonsils to a final concentration of 10^{-4} was added on the PCR plate together with the internal B-cell DNA control. This was done to determine the MRD sensitivity level (Invivoscribe, 280473, Rev. B, Jan. 2020). Fresh dilutions from the concentrated patient samples were prepared from stock for most of the experiments, aiming at 10^{-4} and 10^{-5} dilutions.

Based on the results retrieved using the LymphoTrack MRD software (version 1.2.0, Invivoscribe), MRD was defined as the estimated cell equivalent (ECE) calculated as the ratio of clonal reads (R_{clone}) to the internal control reads ($R_{control}$) from approximately 100 cells, normalized to represent equivalents per 100,000 cells (Equation 1).

$$\mathrm{ECE_{norm}} = \frac{\text{cells}}{10^5} \times \frac{R_{\text{clone}}}{R_{\text{control}} \times 10^{-2}} \qquad \text{(Eq. 1)}$$

In total, 173 samples were sequenced in this study: 8 diagnostic samples, 13 control donor samples, 1 pooled control sample, 139 MRD replicates, and 12 samples for longitudinal assessment. The lack of pronounced monoclonality for donors was confirmed by sequencing, with a mean of 0.48% for the most abundant immunoglobulin sequence (Supplementary Table E1).

Statistical Analyses

Statistical analyses were performed in Mathematica (version 11.3, Wolfram Research). Calculations regarding detection confidence were based on Poisson distribution $\lambda^k e^{-\lambda} k!^{-1}$, where detecting a true-positive rearrangement is expressed as a probability (Equation 2).

$$p(k) = \left(1 - \frac{{\lambda_{\mathrm{NGS}}}^k e^{-\lambda_{\mathrm{NGS}}}}{k!}\right)\left(1 - \frac{{\lambda_{\mathrm{DNA}}}^k e^{-\lambda_{\mathrm{DNA}}}}{k!}\right) \qquad \text{(Eq 2)}$$

Because positivity is defined as all observations above 0 ($k = 0$) with sequence coverage aimed at exceeding the sensitivity level by an order of magnitude (λ_{NGS}>>sensitivity), the calculations are simplified to $p = 1 - e^{-\lambda_{\mathrm{DNA}}}$, where λ_{DNA} defines the amount of input DNA relative to the intended sensitivity level. Conversely, the risk of a false negative is $e^{-\lambda_{\mathrm{DNA}}}$. As elaborated previously [24], the $\mathrm{PCR_{Eff}}$ ratio is based on the PCR amplification efficiency (E) of the clonal sequence versus the polyclonal background rearrangements. In situations where the risk of slightly skewed PCR cycle (c) amplification exists, this may be incorporated into the theoretical probability of detecting the clone (p, Equation 3).

$$\mathrm{PCR_{Eff}} = \frac{(E_{\text{clonal}} + 1)^c}{(E_{\text{background}} + 1)^c} \rightarrow p = 1 - e^{-\mathrm{PCR_{Eff}}\,\lambda_{\mathrm{DNA}}} \qquad \text{(Eq 3)}$$

Finally, our study implemented multiple replicates (n), affecting the overall detection confidence to the power of replicates (Equation 4).

$$p_n = 1 - \left(e^{-\mathrm{PCR}_{\mathrm{Eff}}\ \lambda_{\mathrm{DNA}}}\right)^n \qquad \text{(Eq 4)}$$

RESULTS

The presented methodology reflected our aim of performing a technical assessment of assay reproducibility at fixed sensitivity levels. Specifically, the results empirically extend our previous theoretical framework [24], which describes a probabilistic confidence model for detecting clonal rearrangements.

All eight patients with MM harbored a high-frequency clone ranging from 24.71%−83.17% (read length 265−298 bp, Supplementary Table E1) detected with the LymphoTrack Dx IGH FR1 assay, whereas the highest background of the 13 donor samples ranged from 0.09%−2.11% (116−295 bp). The samples used to assess 10^{-5} sensitivity were prepared by diluting DNA from each patient in a pool of DNA from donors. Disregarding the very low relative fraction of DNA derived from spike-in and patient monoclonal cells, the estimated polyclonal B cells from donors were concordant with the expected number of B cells present in the blood. These constituted approximately 5.6% of the leukocytes (quartiles [Q1; Q2; Q3] = [3.5; 5.6; 6.9], Figure 1A, Supplementary Table E2).

Sequencing Metrics

Collectively, the median number of sequenced reads per sample was 1.6 million ([Q1; Q2; Q3] = [1,196,433; 1,618,568; 1,954,284]) using the Torrent 530 chip (enabling 20 million/chip). The percentage of usable reads analyzed with the LymphoTrack software was 60% ([Q1; Q3] = [51%; 65%]). As expected, the diagnostic samples (DNA from CD138+ plasma cells) had a significantly increased median number of usable reads compared with control donor DNA samples from total blood leukocytes (61% vs. 28%, 716,588 usable reads vs. 364,559, Figure 1B and Supplementary Table E3). Furthermore, the low-positive control had a higher percentage of usable reads than the in-house-prepared patient samples, including all 10^{-4} and 10^{-5} samples (68% vs. 60%, Supplementary Tables E4 and E3, respectively). Among the 139 samples for the MRD reproducibility assessment, none of the sequencing replicates failed in the 700−800 ng subsets, whereas seven 1,000−2,000 ng replicates failed to reach 100,000 reads (Supplementary Table E5). Because of the obtained usable sequencing depth in the MRD samples (770,031; 991,250; 1,234,920), generally severalfold higher than required for

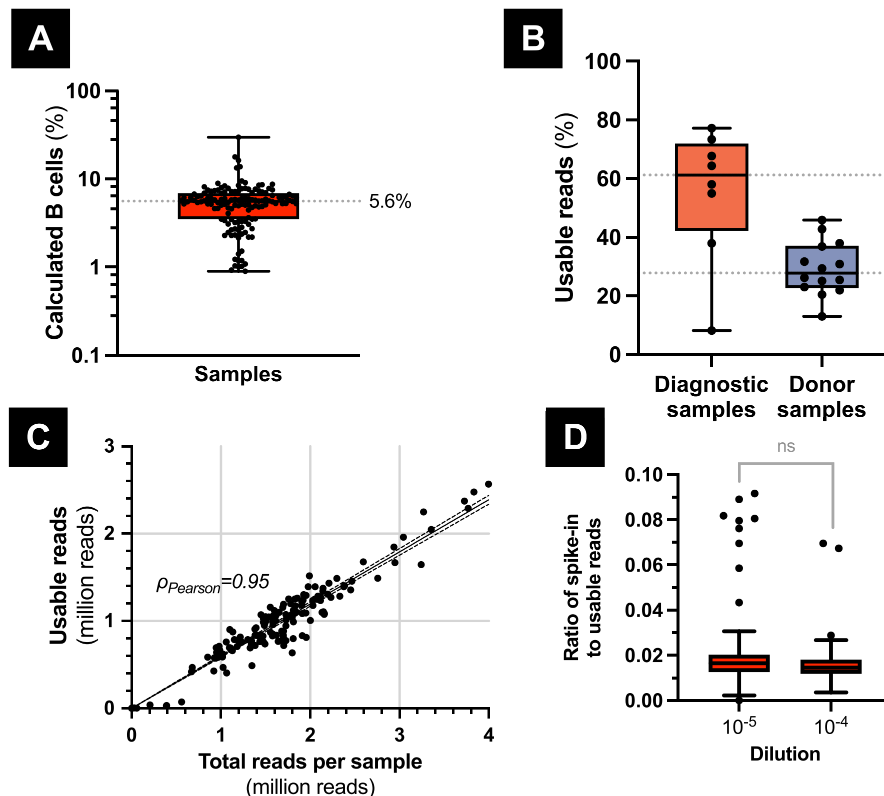

Figure 1 **Sample quality assessment.** The estimated percentage of polyclonal B cells in the samples was 5.6%, ranging from 0.9%−29.8% (**A**). A higher fraction of usable reads (61.2% vs. 27.8%) was evident for the diagnostic multiple myeloma samples compared with control donor samples (**B**). The total and usable number of reads obtained varied per sequenced sample with a high consistency between the two parameters (**C**, $\rho = 0.95$, $p < 10^{-4}$, $R^2 = 0.90$, 95% confidence interval band shown). Also relevant for consistent and quantitative evaluation of cellular equivalents, no significant difference in the ratio of spike-in and usable reads was found between 10^{-5} and 10^{-4} dilution subsets (**D**, $p = 0.094$).

the 10^{-5} sensitivity level, the risk of low coverage being a limiting factor was considered negligible. Although a different number of total reads were sequenced for 700, 800, 1,000, and 2,000 ng (1.08, 0.84, 0.73, and 2.25 million reads, respectively), a direct correlation between the total and usable reads was found, regardless of input (Figure 1C, Pearson $\rho = 0.95$, t-statistics $p < 0.0001$, $R^2 = 0.90$). Also, no significant difference in the relative ratio of spike-in control reads to the total number of usable reads was found (Figure 1D, Mann-Whitney U test: $p = 0.094$).

Reproducibility

Longitudinal consistency was assessed from 12 successive samplings of a low-positive control (175 days, Figure 2A and Supplementary Table E4), based on 1.4 million usable reads per sample [1.3; 1.6]. The calculated equivalent was 2.6 clonal cells (1.8; 3.8) diluted in polyclonal tonsillar DNA of approximately 16,297 polyclonal B cells (15,394; 19,310), roughly corresponding to 1−2 clonal cells per 10,000 cells (median of 1.3, Figure 2B). Overall, the findings reflected a longitudinal consistency to the actual dilution of 10^{-4}.

In the relative comparison of the 10^{-5} levels with the dilutions of 10^{-4} (62 vs. 32 sequencing runs for 700−800 ng, Supplementary Table E5), an eightfold difference was found between the cell equivalents (Figure 2C, 9.7 [6; 14.2] vs. 1.2 [0.; 3.6], Mann-Whitney U test: $p < 0.0001$, Supplementary Table E5). None of the ECEs at 10^{-5} or 10^{-4} dilutions differed significantly from the theoretical 1 per 100,000 cells or 1 per 10,000 cells (one-sample Wilcoxon signed rank test, $p = 0.07$ and $p = 0.37$). The empirical observations were thus in reasonable agreement with the theoretical reference.

Failure Rates of Replicates Versus Increased DNA Amounts

We then assessed whether replicates hold an empirical advantage to employing increasing amounts of DNA. From this assessment, pooled analysis of the 700 ng assays at a 10^{-5} sensitivity level, 38 samples in total (replicates of 4−5; Supplementary Table E5)

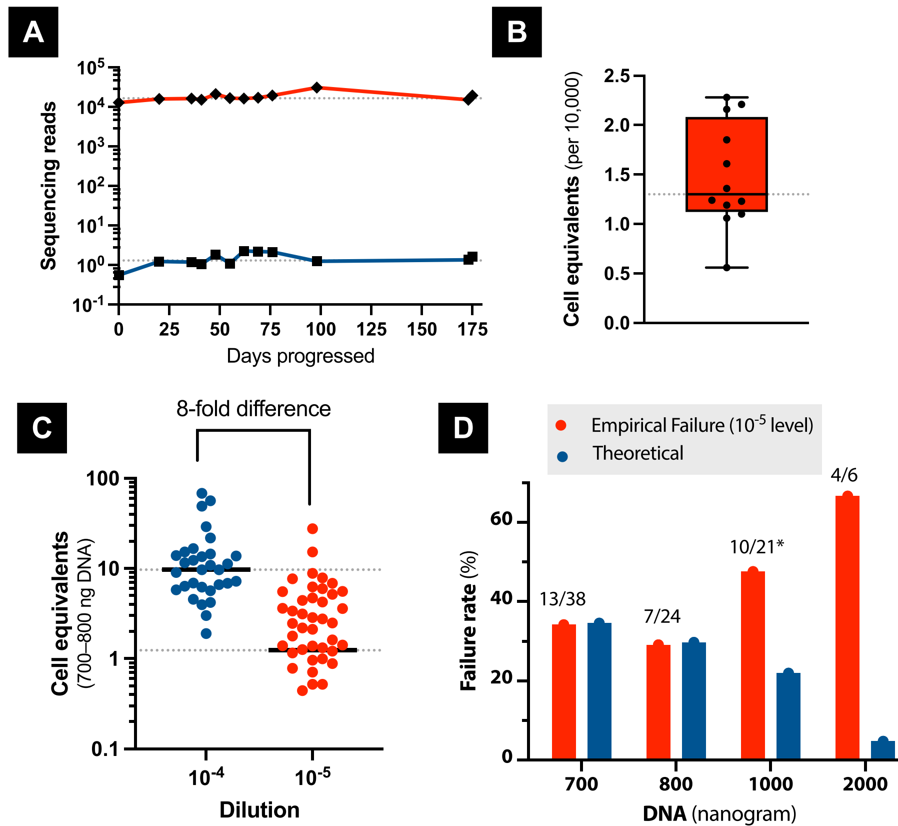

Figure 2 **Reproducibility and consistency of low-level clonotyping.** Assessment of longitudinal reproducibility (red: total reads; blue: clonal B cells per 10,000) demonstrated reasonable consistency between 12 successive samplings (**A**, 175 elapsed days) with a calculated median cell equivalent of 1.3 cells per 10,000 (**B**, range 0.6−2.3). An overall eightfold difference (9.7 vs. 1.2) between 10^{-4} and 10^{-5} was observed in contrast to the theoretical one order of magnitude difference (**C**). Estimated cell equivalents did not significantly differ from the theoretical 1 and 10 per 100,000 cells. The empirical detection failure rates, i.e., false negatives, were in agreement with the theoretical ($e^{-\lambda_{DNA}}$) for the 700−800 nanogram assays (**D**) but not for 1,000−2,000 ng. *Six samples of the 1,000 ng subset did not reach 100,000 usable reads and were excluded because of technical bias. Without this exclusion, detection failure was 15/27 (56%).

showed a median normalized cell equivalent (Equation 1) of 1.2 (0; 5.2) per 100,000 cells with a detection failure rate of 13/38 (34%, Figure 2D, Supplementary Table E5). One sample was marked by 0 clonal reads in all replicates (#136), which may suggest technical bias. The exclusion of this sample led to a median cell equivalent of 1.7 (0.4; 5.4) (detection failure rate of 8/33, 24%). Detection failure was defined as null observations, except for one sequencing run with a low number of reads (Supplementary Table E5). Similarly, the median cell equivalent of the 800 ng assays (in four replicates each) was calculated to be 1.1 (0; 2.2) at a failure rate of 7/24 (29%). No statistical difference was found between these calculated cell equivalences for 700 versus 800 ng (Mann-Whitney U test: $p > 0.05$, $10^{-5}/10^{-4}$ level). The empirically determined sample failure rates were in agreement with the theoretical risk of obtaining false negatives at 35%−38% and 30%−34%, respectively, based on 6.6 pg of DNA per cell and employing 100%−90% relative PCR amplification efficiency range (Equation 3). Only a single sample at 700−800 ng input faulted at the 10^{-4} level, also failing to reach 10^{-5} in all five replicates, thus corresponding to an empirical failure rate of 3% (0% without #136) versus the theoretical 0% (Equation 4).

In contrast, the higher input of 1 and 2 μg DNA, provided in triplicates and duplicates, resulted in a detrimental empirical failure rate of 19/32 (59%) with a median of 0 cell equivalents at the 10^{-5} level (16/29, 55%, excluding #136). Collectively, this revealed significantly altered performance between the low and high input assays (χ^2 test: $p = 0.01$, Fisher exact test: $p = 0.01$), favoring replicates of 700 −800 ng derived from approximately 100,000 cells.

DISCUSSION

Reaching MRD sensitivity levels consistently below 10^{-5} is challenging because the assays are affected by stochasticity and require a substantial amount of high-quality DNA. To push low-level residual myeloma immunoglobulin detection by deep sequencing into clinical practice, it is imperative to demonstrate and characterize assay reproducibility. Hence, we addressed the 10^{-5} sensitivity range for assessing clonotype MRD quantification and replicability using rearranged IGH locus. Because this assessment required a substantial amount of DNA, varying between patient samples, the study layout in terms of the number of replicates and input was influenced by the sample availability.

The recent study by Medina et al. [10] thoroughly demonstrated clonotype concordance between the LymphoTrack assay, using the MiSeq platform, and Sanger sequencing together with cross-center reproducibility. Here, we added to the series of recent studies, such as the others showing a close correlation between flow cytometry and NGS assays [25,26], by demonstrating the replicability of low-level MRD detection using an equivalent sample cohort size (139 MRD replicates). Our results showed a high reproducibility when using 700 800 ng DNA per sequencing replicate. Moreover, the implemented internal control, corresponding to 100 cells added per sample for longitudinal quantification of low-positive control, proved stable over time. A dedicated spike-in control enables quantitative and comprehensible MRD estimation, such as the 1.2/100,000 cells reported here.

A common limitation of the MRD assays pertains to the number of cells or the amount of DNA used as input. Theoretically reasoned from the Poisson distribution, as Rustad et al. [27] previously discussed, to reach a given sensitivity level with 95% probability, the total number of cells to be analyzed must be at least three times the intended sensitivity [24]. Thus, theoretically, 300,000 cells corresponding to approximately 2,000 ng DNA are required to achieve a sensitivity of 10^{-5}. Our results here show that it is advantageous to direct the required amount of DNA into replicates. Although data may indicate that a high DNA load inhibits the PCR reaction or cause problems in downstream library preparation, the most obvious advantage of employing replicates is the direct inverse correlation between the effective failure rate and the number of replicates. Importantly, we show that it is possible to estimate the failure rate, at least under optimized conditions.

Although a high PCR amplification efficiency is assumed, it is advisable to consider the theoretical risk of a suboptimal PCR amplification rate. Even a minor difference of a 5%−10% decrease in the relative PCR efficiency of the clonotype to that of the polyclonal background may lead to a marked reduction in the effective assay sensitivities. This skewness is augmented exponentially according to the number of PCR rounds, where only a slight decrease in efficiency, e.g., from 1−0.95, causes the sensitivity to drop by 50% after 27 rounds (Equation 3). Consequently, the theoretical estimation of the confidence may not reflect the clinical applicability when biased amplification of the polyclonal background to that of the MRD clone occurs. Based on our consistent results, we conclude that low PCR efficiency has not been a pronounced issue.

Studies by Yao et al. [18,19], addressing 10^{-5} sensitivity with triplicates of 1,000 ng, demonstrated clonal cells in most replicates. Our setup indicates that implementing a higher number of replicates with less DNA could be more robust. However, reaching one million reads per replicate, using DNA from approximately 100,000 cells, our calculations based on the Poisson distribution show a theoretical detection probability for the 10^{-5} sensitivity of 65%−70% per replicate. In addition, a low overall false-negative rate of 1% or less was demonstrated for each sample's replicates combined (4−5 replicates). Thus, the theoretical forecast was in close agreement with the results. In our study, failure denotes the risk of not establishing the desired sensitivity as a stochastic event and does not encompass operator bias. It may therefore be necessary to address this issue for a more robust MRD setup for clinical implementation.

Although the empirical results put forth here are in concordance with the theoretical confidence, the proposed model provides the opportunity to directly extend the Poisson distribution to include a lower-level confidence threshold, such as suboptimal relative PCR efficiency, low coverage, or input DNA. We confirm that mathematical modeling is possible, to a certain extent, and may be implemented in the study design to forecast the assay sensitivity and empirical failure rate. Notwithstanding, the validation of a comprehensive statistical model of the effective sensitivity levels would require several factors to be assessed empirically, such as the aforementioned relative PCR amplification efficiency, operator bias, DNA loss from assay carryover, and setting a threshold of the minimum number of clonal reads acquired.

Currently, the MiSeq platform (Illumina) provides a maximum of 25 million paired-end sequenced reads per chip, as used by Yao et al. [18,19], whereas Ion 530 chip used here potentially allows for 20 million reads per sample variation and not all reads will be used for the analyses. We assume that unusable reads may arise from truncated fragments, primer-dimers, or from amplification and sequencing of nonspecific high-affinity targets, which may vary according to the

concentration of immunoglobulin sequences. As such, a significant difference in the number of usable reads was observed between diagnostic samples containing a high proportion of B cells because of the malignancy and control donor blood samples, in which the proportion of B cells and thus of rearranged IGH VDJ represents a smaller fraction in blood leukocytes. Likewise, it may be extrapolated that fewer B cells, and thus immunoglobulin sequences, will be present and sequenced in samples from patients with B-cell depletion because of treatment or B-cell malignancy-associated humoral immunodeficiency. The low-positive control demonstrated the highest number of usable reads compared with the in-house prepared patient samples, reflecting its commercial-grade application as a 10^{-4} sensitivity control sample. This control was based on tonsillar DNA derived primarily from B cells [28,29].

The use of read frequency for monitoring follow-up samples over time requires analysis of the same number of cells to attain compatible sensitivities, which may not always be possible in a clinical situation. This issue stresses the importance of including an internal control to attenuate the variability arising from a low-total cell input and to normalize the clonal cells to a stable reference. To monitor MRD and aid the comparison of reported MRD levels between laboratories, we thus propose the use of internal control, when using LymphoTrack or similar assays to normalize the number of clonal cells. Also, the transparency of MRD calculations, the amount of DNA analyzed, and the number of reads obtained per replicate are important.

Although assessing assay robustness is critical, our data, in objective terms, demonstrated reproducibility under controlled conditions using the LymphoTrack assay. Thus, further studies focusing on more patient-oriented investigations should be performed to reflect the clinical potential.

Conflict of Interest Disclosure

The authors do not have any conflicts of interest to declare in relation to this work.

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Ethical Considerations

The project was approved by The Regional Committee on Health Research Ethics in Southern Denmark (S-20160069), and data were handled in accordance with requirements by the Danish Data Protection. We obtained a dispensation from written consent by the Regional ethical committee, as the material was collected as excess material from the diagnostic routine. Control donors provided informed consent.

Author Contributions

Contribution: OC, CN, and NA designed the study. OC and SD performed the experiments. OC, MH, and CN performed data analyses and drafted the manuscript. NA, SD, and TK discussed the results and commented on the manuscript. NA and CN obtained all funding for the project. All authors contributed to the final version and approved the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.exphem.2023.01.002.

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