NGS better discriminates true MRD positivity for the risk stratification of childhood ALL treated on MRD-based protocol

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SUPPLEMENTARY METHODS

NGS Sequencing

The total DNA input in the first PCR NGS library preparation step was normalized to the equivalent of approximately 150,000 nucleated cells based on the DNA concentration measured with Qubit dsDNA HS assay (ThermoFisher Scientific, Waltham, MA, USA), in order to reach MRD sensitivity of 10^{-5}. For samples with very low DNA concentration, the maximal input volume was capped at 15 µl. Two sets of indices were combined in order to barcode up to 384 samples per one sequencing run. The majority of final libraries were sequenced in two independent runs on the NovaSeq 6000 instrument using the 2x250 bp SP kit, the remaining libraries were sequenced on the MiSeq instrument using the 2x250 v2 kit (all Illumina, San Diego, CA, USA).

MRD markers' specificity analysis

To confirm the specificity of each IG/TR MRD marker, all reads with a matching nucleotide sequence were extracted from all the libraries prepared from the corresponding IG/TR locus and the counts were assigned to the corresponding sample. Each IG/TR MRD marker was then classified based on the matching read counts and the indices used for NGS library barcoding to control for possible primer contamination issues or errors during demultiplexing of the sequencing data.[1] IG/TR markers with no matching sequence identified in other NGS libraries were classified as specific, those with a matching sequence identified in an NGS library sharing one of the indices were classified as specific with demultiplexing/contamination errors arising from pooling a large number of NGS libraries in a single run. Nonspecific background was then assessed similarly to the EuroMRD qPCR interpretation guidelines[2] and IG/TR MRD markers with a matching read count in an unrelated NGS library within one log difference to the patient's library were classified

as nonspecific. Because the true MRD positivity of these markers could not be assessed in patient samples, they were considered negative based on the same rationale that is used in the interpretation of qPCR results with nonspecific background amplification according to the EuroMRD criteria. Representative MRD markers for the classification categories are shown in **Supplementary Figure 1**.

Data visualization

Processing of the data was performed in R (R Core Team, R: A language and environment for statistical computing. R Foundation for Statistical Computing, https://www.R-project.org/.) and the plots were generated using ggplot2[3], ggalluvial (Jason Cory Brunson and Quentin D. Read, ggalluvial: Alluvial Plots in "ggplot2". R package version 0.12.3. http://corybrunson.github.io/ggalluvial/) and ggupubr (Alboukadel Kassambara, ggpubr: "ggplot2" Based Publication Ready Plots. R package version 0.4.0. https://CRAN.R-project.org/package=ggpubr) packages.

Risk stratification

Only the MRD level at day 33 and no other criterion was used for the assumed risk stratification of the patients for the purpose of this study with a threshold of 10^{-3} for assignment to the high risk (HR) group, any positivity below 10^{-3} for the medium risk (MR) group and negativity for the standard risk (SR) group.

RESULTS

IG/TR MRD marker specificity

Since with NGS the complete junctional nucleotide sequence can be evaluated directly, including its germline V(D)J origin and the resulting segmentation, many false-positive results arising in qPCR systems can be avoided. Still, the low complexity of the junctional sequence and the short N regions of some IG/TR MRD markers can hamper the distinction between the malignant clonotype and IG/TR rearrangements of nonmalignant lymphocytes that randomly arise. Here we show that this occurs most frequently with MRD markers from the IGK, TRD a TRG gene loci

(**Supplementary Figure 2A**). We have compared the complexity of the junction between the nonspecific markers and those that were safely classified as specific for the genetic loci with the highest number of nonspecific markers (IGK-VJ-Kde: 11 vs 25; TRD: 15 vs 30 and TRG: 22 vs 20) and found a significant difference between the N region length in these groups (**Supplementary Figure 2B**). Only two out of these 54 markers were in the quantitative range by qPCR and 20 were classified as PnQ by qPCR with a median NGS MRD value of 2.9E-5. Nonspecific background amplification within one Ct of the patient's sample or even with a lower Ct was detected by qPCR for 16 of these 54 nonspecific markers, leading to their negativity by qPCR according to the EuroMRD guidelines.

Even though high MRD positivity can still be safely detected by these nonspecific markers, they should be avoided during the selection process for both NGS and qPCR MRD analysis. Large IG/TR NGS datasets from BM samples – as the one arising from our retrospective study – can be used to predict the specificity of individual IG/TR MRD markers detected at diagnosis and prevent selecting an MRD marker with low QR and sensitivity of the qPCR assay.

Re-screening

One patient was assigned to the SR group by NGS, having only one TRG MRD marker that was highly positive by qPCR and not detected in the NGS data. Upon re-screening the diagnostic sample of this patient, we identified a major clonal TRG rearrangement, that was also detected by NGS at day 33 and that was not discovered with the classical Sanger sequencing at diagnosis. This patient would have been stratified in the HR group based on this TRG MRD marker identified by NGS-based IG/TR MRD marker screening. This illustrates the importance of using NGS for both diagnostic screening and MRD evaluation and the limitation of this study combining both methods. We then re-screened the diagnostic samples of seven patients, for whom we were not able to identify an IG/TR MRD marker from the Sanger sequencing data and optimize a qPCR assay, mainly due to mixed sequences coming from a biallelic or biclonal rearrangement of the same locus. With NGS, we were able to successfully identify at least two independent markers for six out of these seven patients. This is in line with the previously published data and supports the importance of using NGS for reliable IG/TR MRD marker screening at diagnosis.[4]

REFERENCES

1. Bartram J, Mountjoy E, Brooks T, et al. Accurate Sample Assignment in a Multiplexed , Ultrasensitive , High-Throughput Sequencing Assay for Minimal Residual Disease. *J. Mol. Diagnostics*. 2016;18(4):494–506.

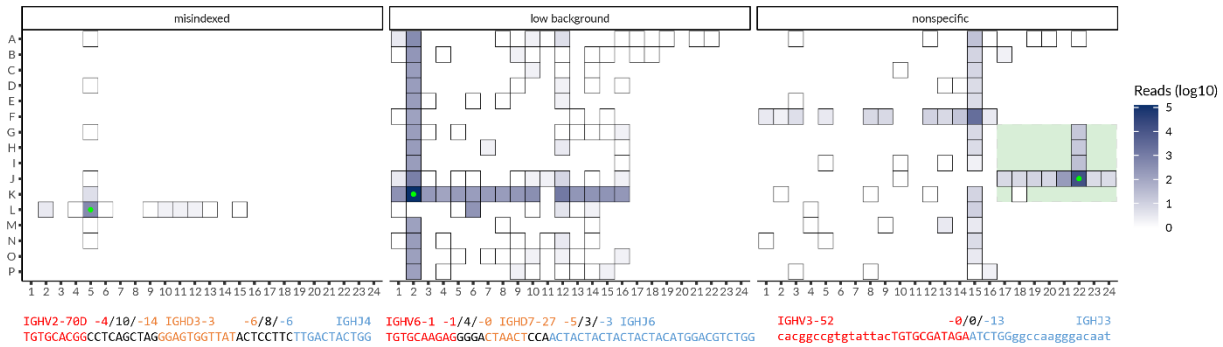
2. van der Velden VHJ, Cazzaniga G, Schrauder A, et al. Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. *Leukemia*. 2007;21(4):604–611.

3. Wickham H. ggplot2: Elegant Graphics for Data Analysis. New York: Springer-Verlag New York; 2016.

4. Brüggemann M, Kotrová M, Knecht H, 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(9):2241–2253.

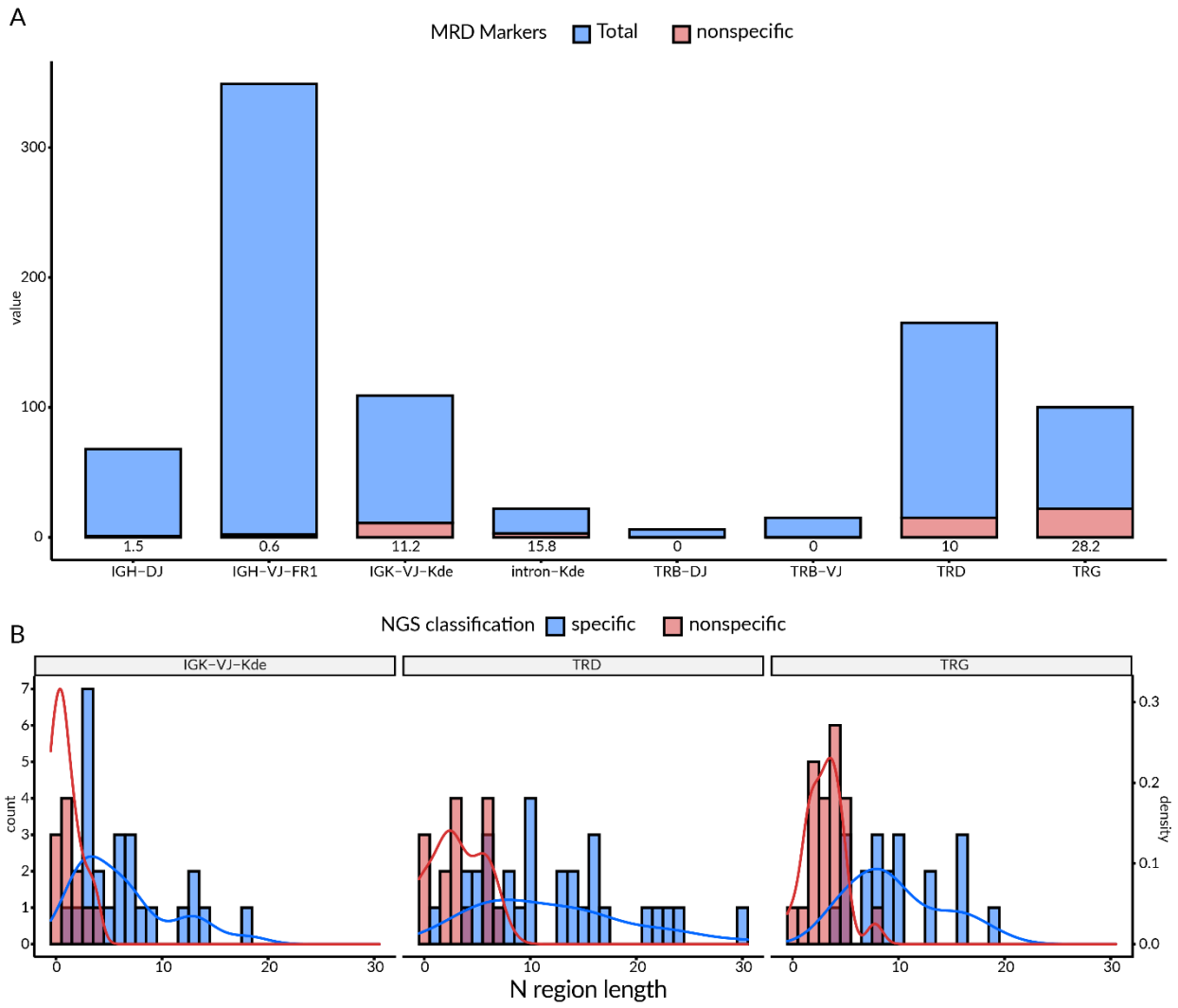
SUPPLEMENTARY FIGURES

Supplementary Figure 1.



Supplementary Figure 1. NGS-based MRD marker specificity analysis of representative markers for each category. Number of reads with an identical sequence to the V(D)J junction of the selected IG/TR MRD marker, that were identified across all IGH-VJ NGS libraries is shown as a heatmap on a logarithmic scale. The *x* and *y* axes correspond to the forward (A-P) and reverse (1-24) barcodes used for sequencing library preparation. Each MRD marker and its segmentation is shown below and the junction nucleotide sequence that was traced across these libraries is capitalized. The correct patient-specific sample for each marker is indicated by a green dot. An identical sequence to the traced junction of MRD markers classified as *misindexed* or having *low background* was observed in unrelated samples at very low levels, due to the technical limitations of multiplexing large number of NGS libraries in a single run and barcode leakage. Whereas the *nonspecific* marker shown here can be traced at high levels (within 1 log) in unrelated samples from 2 independent NovaSeq experiments (all libraries prepared together with the patient-specific library are highlighted in green).

Supplementary Figure 2.



Supplementary Figure 2. Results of the NGS MRD marker specificity analysis. (A) Percentage of MRD markers classified as nonspecific from each IG/TR rearrangement locus is shown below each bar and is represented in red. **(B)** Comparison of the N region length between MRD markers classified as nonspecific (red) and MRD markers found uniquely in the patient's sample or at a low level in samples sharing one of the barcodes and classified as specific (blue) is shown as a histogram with the count of the MRD markers shown by bars plotted on the left-axis and relative density curves plotted on the right-axis.