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Accurate and Sensitive Analysis of Minimal Residual Disease in Acute Myeloid Leukemia Using Deep Sequencing of Single Nucleotide Variations

Erik Delsing Malmberg,* Anna Rehammar,† Mariana B. Pereira,† Jonas Abrahamsson,‡ Tore Samuelsson,§ Sara Ståhlman,¶ Julia Asp,* Anne Tierens,‖ Lars Palmqvist,*¶ Erik Kristiansson,† and Linda Fogelstrand*¶

From the Departments of Clinical Chemistry and Transfusion Medicine and Medical Biochemistry and Cell Biology,§ Institute of Biomedicine, and the Department of Pediatrics,‡ Institute of Clinical Sciences, Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden; the Department of Mathematical Sciences,† Chalmers University of Technology and University of Gothenburg, Gothenburg, Sweden; the Department of Clinical Chemistry,¶ Sahlgrenska University Hospital, Gothenburg, Sweden; and the Laboratory Medicine Program,‖ University of Toronto and University Health Network, Toronto, Ontario Canada*

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Address correspondence to Linda Fogelstrand, M.D., Ph.D., Department of Clinical Chemistry, Sahlgrenska University Hospital, Bruna Straket 16, SE-413 45 Gothenburg, Sweden. E-mail: Linda.Fogelstrand@clinchem.gu.se.

Minimal residual disease (MRD) in acute myeloid leukemia (AML) is of major prognostic importance. The genetic landscape of AML is characterized by numerous somatic mutations, which constitute potential MRD markers. Leukemia-specific mutations can be identified with exome sequencing at diagnosis and assessed during follow-up at low frequencies by using targeted deep sequencing. Our aim was to further validate this patient-tailored assay for substitution mutations. By applying a statistical model, which corrects for position-specific errors, a limit of detection for single nucleotide variations of variant allele frequency (VAF) of 0.02% was achieved. The assay was linear in MRD range (0.03% to 1%) with good precision [CV, 4.1% (2.2% to 5.7%) at VAF 1% and 13.3% (8.8% to 19.4%) at VAF 0.1%], and low relative bias [7.9% (2.5% to 15.3%) at VAF 1%]. When applied to six childhood AML cases and compared with multiparameter flow cytometry for MRD analysis, deep sequencing showed concordance and superior sensitivity. Further high concordance was found with expression of fusion transcripts *RUNX1-RUNX1T1* and *KMT2A-MLLT10*. The deep sequencing assay also detected mutations in blood when VAF in bone marrow exceeded 0.1% ($n = 19$). In conclusion, deep sequencing enables reliable detection of low levels of residual leukemic cells. Introduction of this method in patient care will allow for highly sensitive MRD surveillance in virtually every patient with AML. *(J Mol Diagn 2019, 21: 149—162; https://doi.org/10.1016/j.jmoldx.2018.08.004)*

Acute myeloid leukemia (AML) is the most common form of acute leukemia. One of the most important risk factors for relapse in both adults and children with AML is persistence of measurable leukemic cells, or minimal residual disease (MRD), after induction therapy. The role of MRD analysis in trials and clinical decision making is therefore increasing. With the use of multiparameter flow cytometry (MFC),

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assessment of MRD is feasible in 85% to 90% of patients.[1–3] However, usually only a subpopulation of the leukemic cells carries the leukemia-associated immunophenotype (LAIP) used to identify MRD with MFC (MFC-MRD analysis) and antigen expression can change during treatment, resulting in immunophenotypic shifts in the cells. Therefore, the sensitivity and negative predictive value of MFC for relapse prediction is low; 20% to 40% of patients that are MFC-MRD negative after end of induction therapy eventually relapse.[3–6] In addition, the clinical use of MFC for MRD analysis is hampered by the requirement of experienced operators, a large array of antibodies, and difficulties in standardization.[7] An alternative approach for identification of leukemic cells is to take advantage of their genetic aberrations. This enables not only assessment of response to treatment but also monitoring after completed therapy. In cases in which a leukemia-specific recurrent fusion gene or mutation (such as in *NPM1*) is present, quantitative RT-PCR (RT-qPCR) can be used for MRD monitoring with higher sensitivity than MRD analysis using MFC.[8–10] However, a considerable number of patients lack fusion genes or recurrent mutations that can be monitored with RT-qPCR.[1] Therefore, to increase the applicability and efficacy of MRD analysis for treatment stratification and monitoring, and hence the outcome for AML patients, other methods are warranted.

Because of the genetically heterogeneous nature of AML, nonrecurrent mutations are numerous, and among these, single nucleotide variations (SNVs) are the most common.[11] With the advent of cost-effective and reliable benchtop sequencers, the use of next-generation sequencing (NGS) in the clinical setting has become feasible. With this technique available, also nonrecurrent mutations constitute possible targets for leukemia surveillance. With the use of amplification of clonal Ig/TCR gene segments deep sequencing for MRD analysis has been described for acute and chronic lymphocytic leukemia.[12–14] In AML, mutations have recently been shown as valid markers for disease burden after induction and consolidation,[15–18] and deep sequencing for quantification of mutations has been reported for *NPM1, RUNX1*, *DNMT3A*, and *IDH1/2*, with varying applicability and sensitivity.[19–23] Leukemia-specific mutations also in nonrecurrently mutated genes can be identified with exome sequencing at diagnosis and assessed during follow-up at low frequencies with the use of patient-tailored deep sequencing.[23]

In the recently published consensus document on minimal/measurable residual disease from the European LeukemiaNet, assays for molecular MRD should be able to detect leukemic cells to a level of 0.1%.[24] With NGS-based approaches for assessment of leukemia burden, this level has so far been difficult to reach, especially for SNVs, the markers of interest in AML cases without recurrent genetic aberrations. This is mostly because of sequencing errors, which have a higher impact on SNVs than on oligonucleotides as measured in Ig/TCR rearrangements. The predominant source of sequencing errors of the Illumina MiSeq platform, although less prevalent than in most alternative platforms, is substitution type miscalls.[25] Sequencing errors are derived from multiple sources, including difficulties in signal separation due to fluorophore emission spectral overlap and sequence-specific errors (SSEs), depending on the nucleotide context.[26] In this study, we developed and implemented a statistical model based on reference samples to correct for position-specific errors, thus adjusting relative abundance of SNVs for both SSEs and other forms of sequencing errors. With this approach, it could be shown that deep sequencing of leukemia-specific SNVs has high sensitivity, high accuracy, good precision, and good concordance with MFC-MRD analysis. Introduction of this method paves the way for sensitive MRD analysis in all patients with AML.

Materials and Methods

Deep Sequencing: Library Preparation, Sequencing, and Data Processing

For deep sequencing, the library preparation and sequencing were performed as previously described.[23] Unless otherwise specified, the target regions were amplified from 100 ng of DNA by using the Illumina Truseq-library preparation system, and PhiX bacteriophage genome (Illumina, San Diego, CA) was added to the libraries to increase diversity (mean, 22% aligned) (Table 1). Sequencing was performed on the MiSeq platform (Illumina) with the use of paired-end reads (2 × 150 bp). The output files were remultiplexed to allow zero mismatches by applying the configureBclToFastq.pl script,–mismatches 0 (Illumina CASAVA software version 1.8.2; Illumina, Hayward, CA). Paired-end reads were merged with PEAR version 0.9.6 (*https://sco.h-its.org/exelixis/web/software/pear*) by using default parameters.[27] Quality filtering of the merged reads was performed by using the FASTX-Toolkit (Hannon Lab; URL *http://hannonlab.cshl.edu/fastx_toolkit*, last accessed January 2, 2017), removing merged reads in which <85% of the bases had a quality score >30. The merged reads that passed quality filtering were then aligned to the correct region (build hg19, University of California, Santa Cruz) with the Burrows-Wheeler Aligner in single end mode (commands *aln* followed by *samse*) with default parameters.[28] Only merged reads with a perfect match of at least 10 bp in each direction from the position of the leukemia-specific mutation were considered in the analysis. The resulting variant allele frequency (VAF) for each sequenced sample was calculated as the number of reads with the variant allele divided by the total number of analyzed reads. Further data processing for the estimation of the underlying VAF in the patient sample is described in the paragraph below. The median coverage for sequencing of patient mutations after processing was 945,542 reads/targeted mutation (range, 418,548 to 2,244,255 reads/targeted

Table 1 List of Genes and Gene-Specific Primers Used for Targeted Deep Sequencing of DNA Reference Standards and Patient Samples

Gene	Position and alteration	Mutation type	Patient mutations	TRUQ7 reference DNA	Forward primer	Reverse primer
ALK	NM_004304.3:c.3522C>A:p.Phe1174Leu	Substitution		X	5′-CTCGGAGGAAGG-ACTTGAGG-3′	5′-AGATTTGCCCAGAC-TCAGCT-3′
BRAF	NM_004333.4:c.1799T>G:p.Val600Gly	Substitution		X	5′-GCCTCAATTCTT-ACCATCCACA-3′	5′-TCATAATGCTTGCTC-TGATAGGA-3′
BRAF	NM_004333.4:c.1799T>A:p.Val600Glu	Substitution		X	5′-GCCTCAATTCTT-ACCATCCACA-3′	5′-TCATAATGCTTGCTC-TGATAGGA-3′
CCDC115	NM_032357.2:c.375G>A:p.Trp125Xaa	Substitution	X		5′-CCAAACAACACG-GTCCACTC-3′	5′-CAGCCTCCTCCAAC-CTTTCT-3′
CCNA1	NM_001111045:c.583G>T:p.Glu195Xaa	Substitution	X		5′-GGGTTAGTGGAT-TGAACAAATGT-3′	5′-AGAAGGTGGGAAAA-CTTACTTCA-3′
CLCC1	NM_001048210.1:c.527A>T:p.Asp176Val	Substitution	X		5′-GGAAACTTAATG-ACGTTGCCCT-3′	5′-GAAACCAGGTGCCT-TGGATG-3′
CPA3	NM_001870:exon2:c.144+1G>C	Substitution	X		5′-CCACAAAGGGAGA-AGGTGTT-3′	5′-CCCATGGGCATTGA-CAGTAA-3′
EGFR	NM_005228.3:c.2235_2249delGGAATTAAGAGAAGC:p.Glu746_Ala750del	Deletion		X	5′-CTGGATCCCAGAA-GGTGAGA-3′	5′-CAGCTGCCAGACAT-GAGAAA-3′
EGFR	NM_005228.3:c.2573T>G:p.Leu858Arg	Substitution		X	5′-GGGCATGAACTAC-TTGGAGG-3′	5′-TGTCAGGAAAATGC-TGGCTG-3′
EGFR	NM_005228.3:c.2582T>A:p.Leu861Gln	Substitution		X	5′-GGGCATGAACTAC-TTGGAGG-3′	5′-TGTCAGGAAAATGC-TGGCTG-3′
FER1L5	NM_001113382:c.880G>A:p.Asp294Asn	Substitution	X		5′-CCTGTGACCTCCC-CATTACC-3′	5′-GGACTACCGCTGAC-TTGAAG-3′
GNA11	NM_002067.2:c.626A>T:p.Gln209Leu	Substitution		X	5′-CGTCCTGGGATTG-CAGATTG-3′	5′-GGCGACGAGAAACA-TGATGG-3′
IKZF2	NM_016260.2:c.848A>T:p.Lys283Met	Substitution	X		5′-ACCTTGCAAAGAAA-CAAAAGCA-3′	5′-GCCTTTTGAGAGAC-CTGCTG-3′
KCNK9	NM_016601.2:c.847C>T:p.Arg283Trp	Substitution	X		5′-CTTGGCGCTGAAGG-AGTTC-3′	5′-TGAACAGTGAGGAT-GAGCGG-3′
KRAS	NM_004985.3:c.35G>C:p.Gly12Ala	Substitution		X	5′-TGTATCAAAGAATG-GTCCTGCAC-3′	5′-GGCCTGCTGAAAAT-GACTGA-3′
KRAS	NM_004985.3:c.34G>T:p.Gly12Cys	Substitution		X	5′-TGTATCAAAGAATG-GTCCTGCAC-3′	5′-GGCCTGCTGAAAAT-GACTGA-3′
LDLRAD1	NM_001276392.1:c.20A>T:p.Glu7Val	Substitution	X		5′-TGTTCATGTCTTCG-GTTTCCTG-3′	5′-CTGCTTGTCCCTCC-TCCAG-3′
LRCH1	NM_001164213.1:c.1899G>A:p.Ala633Ala	Substitution	X		5′-CCCACGGTCGGTTG-CAAG-3′	5′-TAAACCAACAACAG-TGCCCC-3′
LRP1B	NM_018557.2:c.2961C>T:p.Cys987Cys	Substitution	X		5′-CTTGGCTCTGCAAG-ATTCCC-3′	5′-TGAGCCACTAACCC-AATTCG-3′
NHLH1	NM_005598.3:c.160G>A:p.Gly54Ser	Substitution	X		5′-GTCGGGCTTCAGTG-ACTGTG-3′	5′-GTGCGGTACTTGGC-TGTG-3′
NKX2-3	NM_145285.2:c.174A>G:p.Gly58Gly	Substitution	X		5′-AGCACCACTTCCAC-TCTGC-3′	5′-CTGCGGCTAGTGAG-TTCAAA-3′
NPM1	NM_002520:exon11:c.859_860insTCTG:p.Leu287fs	Insertion	X		5′-TGTCTATGAAGTGT-TGTGGTTCC-3′	5′-GCATTATAAAAAGG-ACAGCCAGA-3′
RIMS3	NM_014747.2:c.301C>T:p.Arg101Cys	Substitution	X		5′-CTGTTGCTGTTGG-TGCTCC-3′	5′-CACCAAGAAGCTGC-GCAG-3′
TECTA	NM_005422.2:c.3782C>T:p.Ser1261Leu	Substitution	X		5′-TGTCCATCACAGT-CCCTCG-3′	5′-CACAGGACGGACAG-CGGT-3′
TGM7	NM_052955.2:c.439+2T>C	Substitution	X		5′-ACACGCTACTACA-TTGCATAGC-3′	5′-GATCTCTCAGGGCC-AAGGTC-3′
ZBTB33	NM_006777.3:c.1556delAGA	Deletion	X		5′-TGCAAAAGGTCAT-ATGTCTGTCT-3′	5′-ACACTGATACCTTC-GCTCCC-3′

NM accession numbers can be retrieved from GenBank (*https://www.ncbi.nlm.nih.gov/genbank*).

mutation). The threshold for MRD positivity with the use of deep sequencing was set to 0.02% based on the determined limit of detection (LOD), described in detail in *Results*.

Estimation of VAFs: Model Formulation and Parameter Estimation

For each mutation a position-specific error corrected VAF (VAF^{EC}) was calculated as the difference in VAF between a patient/normal sample and a reference sample, with the calculation based on a model that included different sources of variation. Specifically, after the data processing steps described in the section above, the counts of the variant allele y_m and the total number of allele counts N were recorded for each sample. Assuming that DNA fragments are picked at random and independently for sequencing and that N is fixed, the variant allele counts follow a binomial distribution. Because N is large (typically between 10^5 and 10^6), the binomial distribution was approximated with a normal distribution, that is,

$$y_M \sim \text{norm}[Np_M, Np_M(1-p_M)],$$

where p_M denotes the probability of observing a variant allele. The probability p_M depends both on the underlying VAF in the patient sample, denoted f, and the probability of observing the variant allele because of errors in the sequencing process. To estimate the error probability, a reference sample based on DNA from a healthy individual was sequenced, using the exact same protocol as for the patient sample and analyzed at the same genomic position. The estimator $\widehat{f}$ for the underlying VAF in the patient sample was defined as

$$\widehat{f} = \widehat{p}_M - \widehat{p}_M^{ref} = \frac{y_M}{N} - \frac{y_M^{ref}}{N^{ref}}.$$

The estimate of f is referred to as the VAF^{EC}. The variance of $\widehat{f}$ was divided into two components. The first variance component, denoted σ_1^2, was the sample specific variability that follows from the random sequencing of DNA fragments and was calculated by

$$\sigma_1^2 = \frac{p_M(1-p_M)}{N} + \frac{p_M^{ref}\left(1-p_M^{ref}\right)}{N^{ref}}.$$

The second variance component, denoted σ_2^2, was modeled as an additive component and corresponded to the between-sample variability caused by factors such as discrepancies in sample handling and quality, preparation and error rates between sequencing runs.

Thus, the distribution of $\widehat{f}$ was modeled as

$$\widehat{f} \sim \text{norm}\left(f, \sigma_1^2 + \sigma_2^2\right)$$

where f and σ_1^2 are sample specific, while σ_2^2 is assumed to be common for samples with the same underlying VAF f and run under the same conditions. The parameter σ_2^2 was estimated based on a set of samples using numerical maximum likelihood. Descriptions of data used for estimations of variances are given in *Statistical Analysis*.

Experimental Setup

To construct a validation data set for establishment of linearity, precision, and accuracy the Horizon HDx Tru-Q7 (1.3% Tier) and Tru-Q0 (100% wild-type) reference standard DNA were used (Horizon, Waterbeach, UK). For linearity tests, the reference standard Tru-Q7 DNA was diluted in series with Tru-Q0 DNA at 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128, in three independent experiments (Table 1). Estimation of precision and accuracy were performed through repeated measurements of genetic variants in the HDx Tru-Q7 (undiluted and for precision also diluted 1:10). To determine whether the amount of input DNA in the PCR reaction affects the result, three SNVs (*EGFR* c.2573T>G, *EGFR* c.2582T>A, and *ALK* c.3522C>A) present at approximately VAF 1% in the DNA reference standard Tru-Q7, but not in Tru-Q0 (both Horizon), were analyzed with 100 and 500 ng of DNA input in the PCR reaction at 1% (Tru-Q7), 0.1% (Tru-Q7 diluted 1:10 with Tru-Q0), and 0% (Tru-Q0) VAF levels. To estimate the LOD, 15 DNA samples extracted from blood from healthy individuals (normal samples) were each analyzed for 1 of the 15 different leukemia-specific SNVs used for MRD detection (Table 1). Mutations in positions in which reference and normal samples consistently showed a signal that exceeded a VAF of 0.05% were excluded from the analysis (two mutations in total in the study). For specificity testing, three leukemia-specific SNVs (*CPA3* c.144+1G>C, *CCDC115* c.375G>A, and *LDLRAD1* c.20A>T), present in one of the included patients, were analyzed in two follow-up samples per case in five other cases. For analysis of carryover, DNA extracted from a diagnostic bone marrow (BM) sample (AML with *NPM1* mutation, type A insertion at VAF of approximately 50%) and *NPM1* wild-type samples were analyzed in the same pool. To estimate the degree of carryover from the diagnostic sample to the normal samples, either two unique 8-bp indexes (four samples) or one unique index and one shared with the diagnostic sample (four samples), were used for the normal samples.

Patient Samples

Diagnostic and follow-up samples from six children (three male, three female; median age, 6 years; range, 1 to 17 years) diagnosed with AML at the Department of Clinical Chemistry, Sahlgrenska University Hospital, during 2014 to 2016 were analyzed. For diagnostic samples, sorting of leukemic cells and lymphocytes with fluorescence-activated cell sorting, exome sequencing, and identification of variants suitable for MRD analysis were performed as previously described.[23] Briefly, identification of leukemia-specific variants was performed with Mutect[29]

Table 2 Antibody Panel used for Flow Cytometric MRD Analyses

Tube	Horizon V450	Horizon V500	FITC	PE	PerCP-Cy5.5	PC7	APC	APC-H7
1	HLA-DR BD (Franklin Lakes, NJ), Clone L243	CD45 BD, Clone HI30	CD56 BD, Clone NCAM16.2	CD13 BD, Clone L138	CD34 BD, Clone 8G12	CD117 Beckman Coulter (Fullerton, CA), Clone 104D2D1	CD33 BD, Clone P67.6	CD11b BD, Clone ICRF44
2	HLA-DR BD, Clone L243	CD45 BD, Clone HI30	CD36 BD, Clone CLB-IVC7	CD64 BD, Clone 10.1	CD34 BD, Clone 8G12	CD117 Beckman Coulter, Clone 104D2D1	CD33 BD, Clone P67.6	CD14 BD, Clone MφP9
3	HLA-DR BD, Clone L243	CD45 BD, Clone HI30	CD15 BD, Clone MMA	NG2 Beckman Coulter, Clone 7.1	CD34 BD, Clone 8G12	CD117 Beckman Coulter, Clone 104D2D1	CD2 BD, Clone S5.2	CD19 BD, Clone SJ25C1
4	HLA-DR BD, Clone L243	CD45 BD, Clone HI30	CD7 Dako (Carpinteria, CA), Clone DK24	CD96 eBioscience (San Diego, CA), Clone NK92.39	CD34 BD,Clone 8G12	CD117 Beckman Coulter, Clone 104D2D1	CD123 Miltenyi Biotec (Auburn, CA), Clone AC145	CD38 BD, Clone HB7
5	HLA-DR BD, Clone L243	CD45 BD, Clone HI30	CD99 Bio-Rad (Hercules, CA), Clone DN16	CD11a BioLegend (San Diego, CA), Clone TS2/4	CD34 BD, Clone 8G12	CD117 Beckman Coulter, Clone 104D2D1	CD133 Miltenyi Biotec, Clone AC133	CD4 BD, Clone SK3

APC, allophycocyanin; APC-H7, allophycocyanin-cyanine; BD, Becton Dickinson; Cy, cyanine; FITC, fluorescein isothiocyanate; HLA-DR, human leukocyte antigen- antigen D related; PC7, phycoerythrin cyanin 7; PE, phycoerythrin; PerCP, peridinin chlorophyll protein complex.

for SNVs and with Strelka[30] and VarScan2[31] for short insertions/deletions. To filter out subclonal mutations, which were not considered suitable as MRD markers, a 95% CI was applied around the allele frequency of each mutation, and all variants were removed with a CI <0.5.[23] The treatment protocol used was NOPHO-DBH AML 2012 from the Nordic Society of Paediatric Haematology and Oncology, EUdract number 2012-002934-35. It consists of two intensive induction courses, followed by risk-adapted consolidation with three courses of conventional chemotherapy for standard risk patients and allogeneic stem cell transplantation for high-risk patients. The study was performed according to the Declaration of Helsinki and approved by the Regional Ethical Review Board in Gothenburg. Informed consent was obtained from guardians and when age appropriate from the patients.

MFC

Assessment of MRD with MFC was performed as part of clinical routine according to MFC guidelines in the NOPHO-DBH AML-2012 study. Briefly, MFC analysis was performed on the first portion (1 to 2 mL) of the BM aspirate to minimize blood dilution, using bulk lysis, staining with an eight-color panel (Table 2) and standardized settings according to Euroflow.[32] Cells were analyzed with a FACSCantoII flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and data processing performed with FACSDiva version 8.0.1 (Becton Dickinson). The LOD for MFC was run-specific, but in line with NOPHO-DBH AML-2012 guidelines for treatment stratification, MRD positivity in the comparisons was defined as ≥0.1% cells with LAIP, corresponding to at least 50 cells with LAIP in 50,000 viable cells.

RT-PCR

Assessment of MRD with RT-qPCR for *RUNX1-RUNX1T1* and *KMT2A-MLLT10* was performed on BM aspirate samples as part of the clinical routine with RNA isolation and cDNA synthesis as previously described.[33] For *RUNX1-RUNX1T1*, the Rotorgene platform was used with primers and probes as described in guidelines from the Europe Against Cancer program with *ABL1* as reference gene.[34,35] For *KMT2A-MLLT10*, the KAPA SYBR FAST system was used, as previously described with slight modifications; exon 7 forward primer 5′-GCCTGAATCCAAACAGGCCAC-3′ and exon 10 reverse primer 5′-TCTTCCAAGCGCTTCAAT-3′ with *GUSB* as reference gene.[36] The limit of quantification, used as threshold for MRD positivity, was determined based on the reference gene copy number for each run and defined as 10 (lowest point of the calibration curve for fusion gene)/reference gene copy number. For each follow-up sample, the MRD value was defined as fusion transcript/reference gene at the sampled time point divided with fusion transcript/reference gene at diagnosis.

Statistical Analysis

Statistical analyses were performed by using R version 3.1 (R Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria; *http://www.r-project.org*, last accessed January 2, 2017). Graphs were constructed with GraphPad Prism software version 7.0 (GraphPad Software, La Jolla, CA). The LOD was determined as the absolute value of the mean VAF^{EC} + 3 SDs in samples without mutations according to the International Union of Pure and Applied Chemistry guidelines.[37] To calculate the SD, the two

variance components in the model needed to be estimated. The second variance component σ_2^2 was estimated under the assumption of $f = 0$. An overall estimate of σ_1^2 was then calculated, based on a fixed sequencing depth and taking the mean over the resulting 15 sample-specific variance components. Together, this gives a LOD for a fixed sequencing depth. The probability of observing a VAF^{EC} above the LOD, at an underlying VAF in the patient of $f = 0.05\%$, was calculated by using the normal distribution, with p_M^{ref} set to the median over the 15 reference samples, p_M set to $f + p_M^{ref}$ and σ_2^2 set to the value estimated above. For estimation of precision, the CV was calculated from replicated measurements done on samples with the same underlying VAF f and having the same leukemia-specific mutation. To calculate the variance component σ_2^2, f was estimated to the mean VAF^{EC} for the replicates. Because the sequencing depth, and thus the variance component σ_1^2, varies between samples, a joint σ_1^2 was then estimated by taking an average

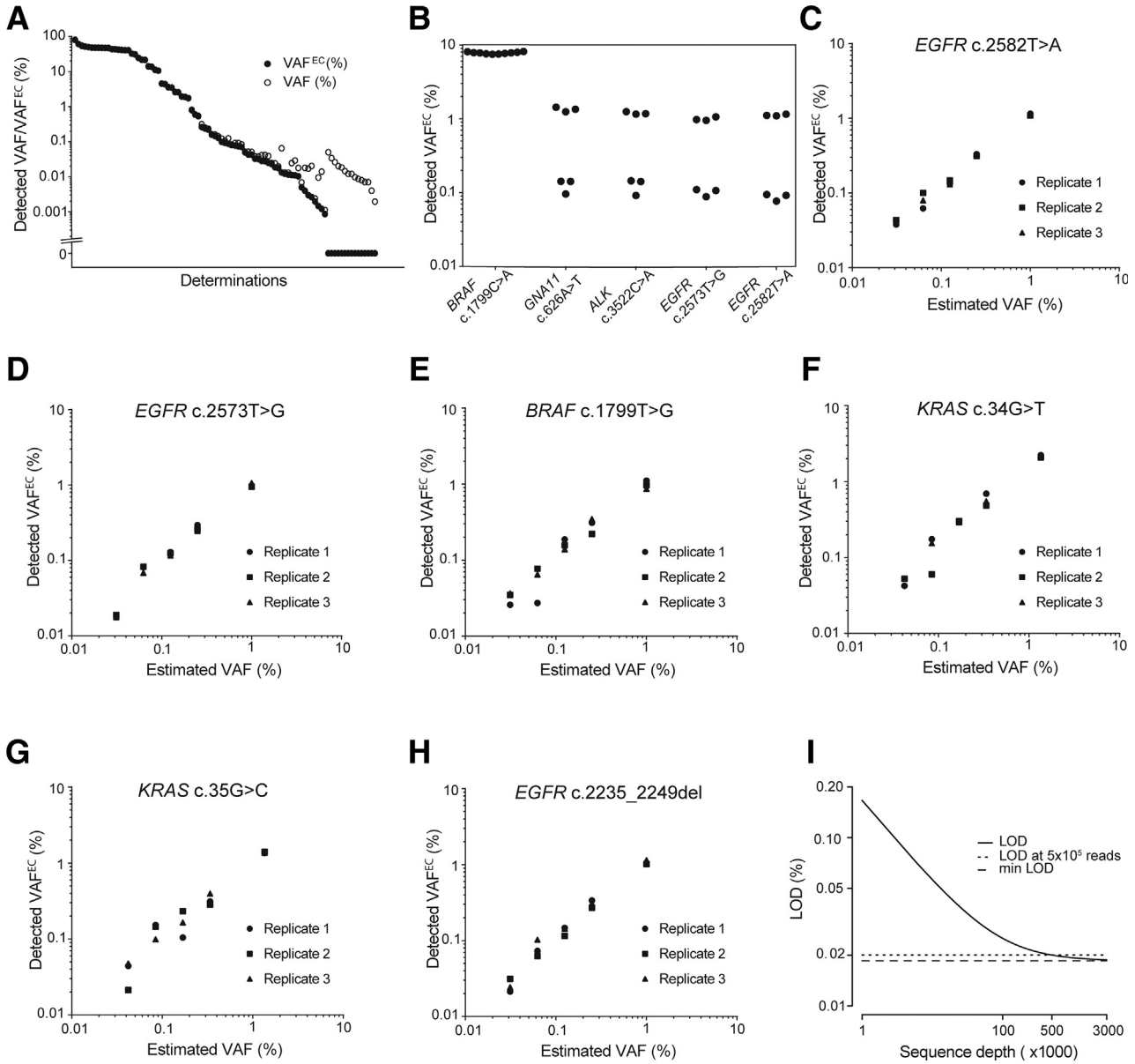


Figure 1 Determination of mutation variant allele frequency (VAF) was refined to a sequencing error-corrected VAF (VAF^{EC}) with a resulting limit of detection (LOD) of VAF^{EC} 0.02%. **A:** VAF and corresponding VAF^{EC} of mutations in bone marrow (BM) samples. Results from 91 determinations are shown in order of decreasing VAF^{EC}. **B:** Repeated measurements of variants at known frequencies are shown (8%, 1.3%, 1.0%, 0.13%, and 0.1%). Variants $\leq$1.3% are displayed as VAF^{EC} and the 8% variant is displayed as VAF. (**C**–**H**) Linearity of the method was established by using serial dilution of the DNA reference standard Tru-Q7 at 1:1, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128, followed by deep sequencing of single nucleotide variations (SNVs) T→A, T→G, A→C, C→A, C→G and a 15-bp deletion with only values above the determined LOD shown. **I:** The LOD as a function of sequencing depth is shown, whereby the **dotted line** represents the determined LOD at a sequencing depth of 5.0×10^5 and the **dashed line** represents the lowest LOD achievable (ie, when σ_1^2 was set to 0).

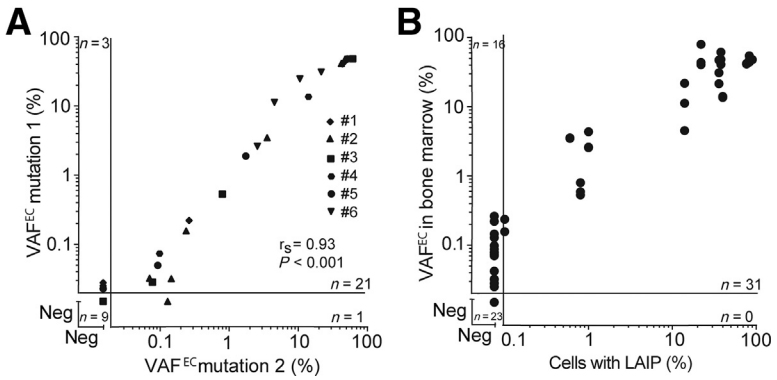


Figure 2 High correlation between mutation error-corrected variant allele frequency (VAF^{EC}) levels measured in the same samples. **A** and **B:** Relationship between VAF^{EC} levels of mutations measured within the same patient and time point (**A**) and between VAF^{EC} levels and cells with leukemia-associated immunophenotype (LAIP; **B**). Lines denote the limit of detection at 0.02% for deep sequencing and 0.1% for multiparameter flow cytometry. neg, negative (ie, below the limit of detection).

over the replicates. Analyses of correlations were performed with Spearman's rank correlation coefficient and estimated with partial correlation adjusted for patient effects. Values below LOD were set to zero. For the estimation of correlation between VAF^{EC} for different mutations, samples taken at time of diagnosis were excluded, and two mutations were randomly selected per patient. For the estimation of correlation between VAF^{EC} in BM and blood, one mutation was randomly selected per patient. The Cohen's κ coefficient was used to measure the agreement between MRD status with deep sequencing and MFC (samples taken at time of diagnosis excluded), and the McNemar's test was used to assess discrepancies between the two methods. For comparison between paired samples analyzed with different amount of DNA input, CI was calculated under model assumptions, assuming variance homogeneity and with second variance component estimated as described for CV calculations. P values < 0.05 were considered statistically significant.

Results

Effects of Sequence-Specific Error Correction in Samples with Low Mutation Burden

In NGS, many sequencing errors are SSEs and depend on the genomic context.[38,39] To improve the determination of the true VAF for each position of interest, the result was corrected for the background signal in the specific position. The background signal was determined by the VAF of the position of interest in a reference sample from a healthy individual. The resulting position-specific error corrected value was denoted VAF^{EC}. To investigate the effect of this error correction, the total variability in the assay was estimated by calculating the sample SD with and without error correction for 15 normal samples analyzed for 15 different mutations. The value was lowered from 0.0123% to 0.0060% by using VAF^{EC}, hence showing a 51% reduction in between-sample noise level. VAF and VAF^{EC} were then determined in patient samples with different mutation loads (Figure 1A). This comparison showed that applying the position-specific error correction was especially important at lower VAF levels, whereby the relative differences between corrected and uncorrected VAFs were large (Figure 1A).

Precision, Accuracy, and Linearity at MRD Levels

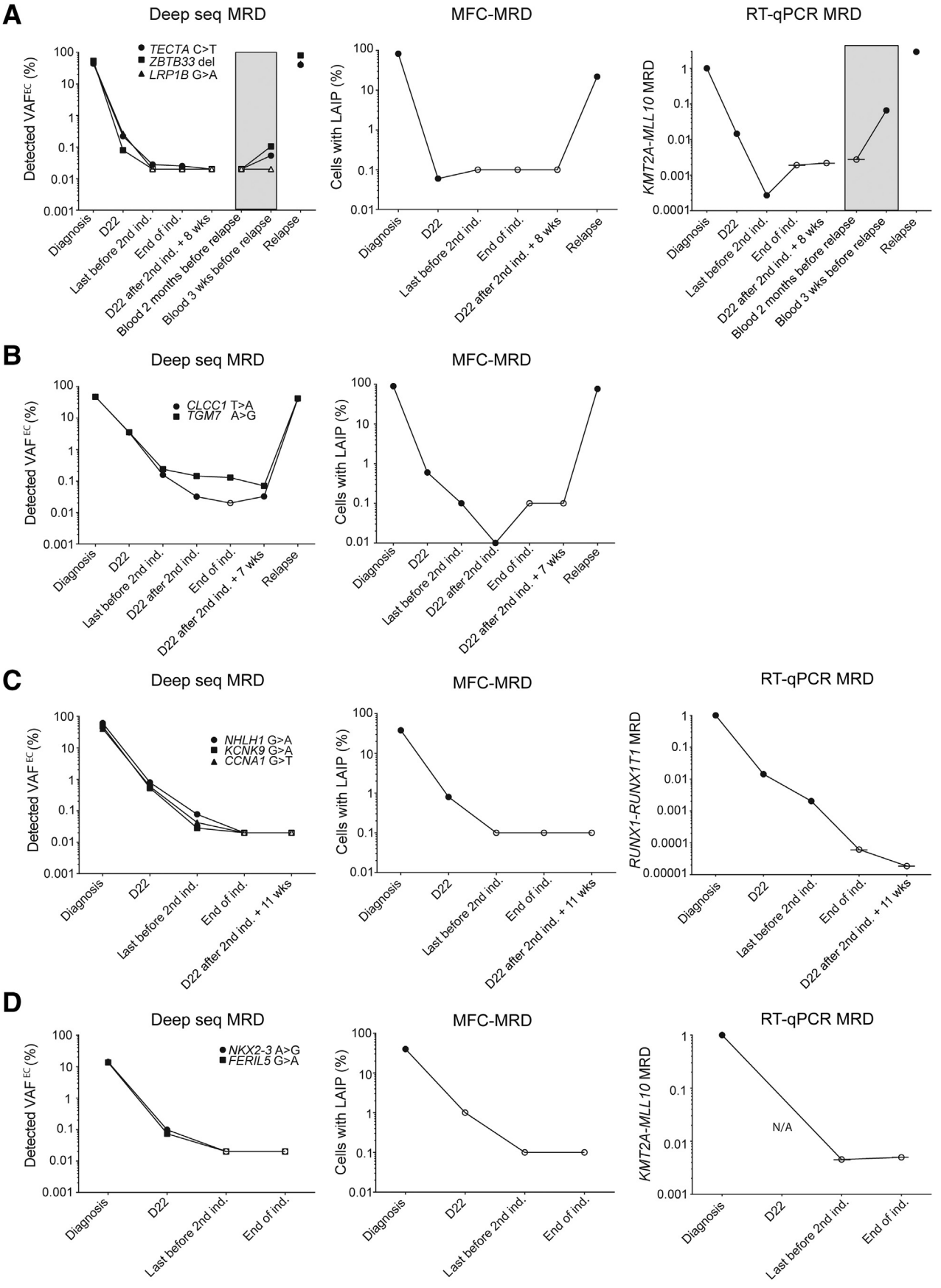
Because MRD refers to measurable levels of leukemic cells in ranges below the detection of microscopy differential count, precision, accuracy, and linearity were determined at low mutation levels. For precision and accuracy, a reference standard DNA that contained specific mutations at known allele frequencies was used. Measurements were performed in triplicate for four SNVs at VAF at approximately 1% (1.0/1.3) and 0.1% (0.10/0.13) with a resulting median CV of 4.1% (range, 2.2% to 5.7%) for SNVs at VAF 1% and 13.3% (range, 8.8% to 19.4%) at VAF 0.1%. The median relative bias for SNVs at VAF 1% was 7.9% (range, 2.5% to 15.3%). Further, one SNV present at 8% VAF was examined in 10 samples with resulting CV of 2.8% and median relative bias of 2.4% (range, 0.5% to 6.5%) (Figure 1B). To investigate if the amount of input DNA in the PCR reaction would affect the results, 100 ng (as for all other experiments) and 500 ng of reference standard DNA were analyzed for three SNVs each at expected 0.1% and 1% VAF levels; *EGFR* c.2573T>G, *EGFR* c.2582T>A, and *ALK* c.3522C>A. For 500 ng of DNA input, the median relative bias for SNVs at VAF 1% was 14.5% (range, 11.0% to 17.3%). No statistically significant difference was found

Table 3 Comparison of Deep Sequencing and Multiparameter Flow Cytometry MRD Analyses

Analysis	MFC MRD^+, *n*	MFC MRD^-, *n*
Deep sequencing MRD^+		
Samples	12	9
Determinations	31	16
Deep sequencing MRD^-		
Samples	0	6
Determinations	0	23

Sample MRD^+ defined as at least one detectable mutation of two to three determinations per sample.

MFC, multiparameter flow cytometry; MRD, minimal residual disease.


A
Deep seq MRD
MFC-MRD
RT-qPCR MRD
Detected VAF EC (%)
TECTA C>T
ZBTB33 del
LRP1B G>A
Cells with LAIP (%)
KMT2A-MLL10 MRD
Diagnosis
D22
Last before 2nd ind.
End of ind.
D22 after 2nd ind. + 8 wks
Blood 2 months before relapse
Blood 3 wks before relapse
Relapse
B
Deep seq MRD
MFC-MRD
CLCC1 T>A
TGM7 A>G
D22 after 2nd ind.
D22 after 2nd ind. + 7 wks
C
Deep seq MRD
MFC-MRD
RT-qPCR MRD
NHLH1 G>A
KCNK9 G>A
CCNA1 G>T
RUNX1-RUNX1T1 MRD
D22 after 2nd ind. + 11 wks
D
Deep seq MRD
MFC-MRD
RT-qPCR MRD
NKX2-3 A>G
FERIL5 G>A
N/A

between results obtained with 500 ng and 100 ng (mean relative difference, 6.8%; 95% CI, −4.0% to 17.6%). To estimate the linearity at MRD levels, the reference standard DNA was used to make dilutions to VAF of 0.008% to 1% for five SNVs and one 15-bp deletion. The dilutions confirmed linearity down to levels of approximately VAF^{EC} of 0.03% (Figure 1, C–H), in line with what has been previously described for insertion in *NPM1*.[23]

Low LOD and High Specificity Using Post-Sequencing Data Processing

To estimate the LOD of deep sequencing of SNVs, a statistical model was developed that took both the within- and between-sample variability into account. The LOD was then estimated from 15 normal samples, each sequenced for a different mutation. Because a higher number of sequenced DNA fragments reduce the within-sample variability, the resulting LOD decreased with increased sequencing depth (Figure 1I). At a sequencing depth of 5.0×10^5 the LOD was estimated to VAF^{EC} of 0.02%, corresponding to one mutated cell in 2500. Having higher sequencing depths than 5.0×10^5 only lowered the LOD marginally, down to a minimum value of 0.0185%. At a sequencing depth of 5×10^5, the probability of obtaining a result above the LOD in a patient sample with VAF of 0.05% was >99.9%. In other words, if a sample contained >0.1% leukemic cells with a heterozygous mutation, there was a >99.9% probability that it would be detected by the method. In addition, when increasing the DNA input to 500 ng, negative samples were scored negative; reference-standard DNA with expected VAF of 0% for the SNVs *EGFR* c.2573T>G, *EGFR* c.2582T>A, and *ALK* c.3522C>A showed VAF^{EC} well below the LOD of 0.02% for all three SNVs. To determine the specificity obtained when using LOD of 0.02%, 10 follow-up samples from five childhood AML cases (two for each case) were analyzed for the presence of three leukemia-specific SNVs (with different nucleotide substitutions) identified at diagnosis in a sixth case ($n = 30$). In 29 of 30 samples the VAF^{EC}s were below the LOD of 0.02%. In one sample, one of the mutations displayed a VAF^{EC} of 0.055%, thus falsely determined positive (for 1 of 3 mutations in the sample). These results corresponded to a specificity of 97% when the LOD of the assay was set at VAF^{EC} of 0.02%.

Dual Unique Indexing Reduces Carryover from Samples with High Mutation Burden

In NGS, incorrect assignment of reads multiplexed in the same sequencing reaction will result in carryover between samples. To test if this could be avoided by using a stricter index design, DNA from a patient with AML with a 4-bp insertion in the *NPM1* gene (VAF^{EC} of 47%) was sequenced in multiplex with normal samples that did not contain the *NPM1* mutation. The normal samples were barcoded with either one unique index combined with one index shared with the diagnostic AML sample or with two unique indexes. The insertion type mutation was chosen to avoid influence from sequencing errors, because insertion-type errors were infrequent on the MiSeq platform. The normal samples with dual unique indexes displayed a 10-fold decrease in carryover from the diagnostic sample (median VAF^{EC}, 0.0015%; range, 0.0009% to 0.0029%), compared with the samples that shared one index with the diagnostic sample (median VAF^{EC}, 0.014%; range, 0.008% to 0.026%). From the LOD defined above, at least one of the normal samples with one shared index would have been scored positive because of carryover between samples. Thus, to reach the high sensitivity of the method described here, dual unique indexing was needed.

Deep Sequencing MRD Analysis Is Concordant with MFC but Has a Higher Sensitivity

To test the deep sequencing method for monitoring of MRD during treatment, 34 BM samples were analyzed from six children with AML. In diagnostic samples, leukemia-specific somatic mutations were identified through exome sequencing of sorted leukemic cells and lymphocytes. For each patient two to three mutations, determined suitable for MRD analysis with the previously described filtering system, were chosen for analysis with deep sequencing.[23] The VAF^{EC} of different mutations in each sample was highly correlated (correlation coefficient, 0.93; $P < 0.001$) (Figure 2A). Twenty-three samples were analyzed for three leukemia-specific mutations of which 19 samples had concordant MRD assignments for all mutations. Another 11 samples were analyzed for two leukemia-specific mutations of which 10 samples had concordant MRD assignments for both mutations. BM samples from the same time points were analyzed with MFC as part of clinical routine for 27 of the 34 samples. When the deep sequencing result for each analyzed mutation was compared with MFC, 54 of 70 determinations were concordant and 16 were MRD^+ with deep sequencing only (Figure 2B and Table 3). Samples were then dichotomized into MRD^+ and MRD^- results with MRD^+ sample defined as at least one detectable mutation with deep sequencing. Sample dichotomization showed concordant results in 18 of 27 samples (Cohen's κ coefficient, 0.3; $P = 0.02$, diagnostic samples not included in the

Figure 3 Good concordance between deep sequencing (Deep seq) and other methods for minimal residual disease (MRD) detection. **A–D:** Four patients were monitored for MRD during treatment by using Deep seq and flow cytometry of which three were also surveilled by using quantitative RT-PCR (RT-qPCR), values shaded in gray constitute blood samples. Filled shapes denote quantifiable levels, and open shapes denote measurements below the limit of detection (LOD) for Deep seq and multiparameter flow cytometry (MFC) and below limit of quantification (LOQ) for RT-qPCR. The LOD and LOQ for MFC and RT-qPCR are run specific. D22, day 22 after start of induction course; ind., induction; LAIP, leukemia-associated immunophenotype.

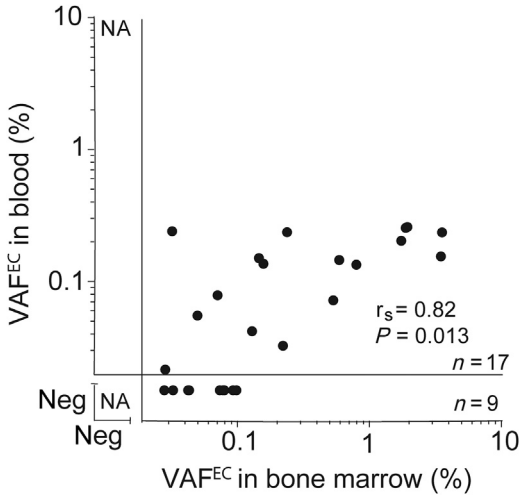


Figure 4 Mutations >0.1% error-corrected variant allele frequency (VAF^{EC}) in bone marrow (BM) were also detected in peripheral blood. VAF^{EC} levels for mutations positive in BM (VAF^{EC} < 5%) are plotted against the corresponding VAF^{EC} levels in blood. Lines denote the limit of detection (LOD) at 0.02% for deep sequencing and 0.1% for multiparameter flow cytometry (MFC). NA, not applicable; neg, negative (ie, below the limit of detection).

analysis) (Table 3). Nine samples were MRD^+ with deep sequencing but MRD^- with MFC, corresponding to a significant systematic discrepancy between the two methods ($P = 0.008$, McNemar's-test) and implicating a higher sensitivity of deep sequencing. Notably, no samples/mutations showed MRD^- with deep sequencing and MRD^+ with MFC. In Figure 3, the kinetics of leukemia in four patients is shown. The two patients with MRD^+ samples with deep sequencing and MRD^- by flow cytometry after end of induction both relapsed (Figure 3, A and B). Two patients who were MRD^- at end of induction with deep sequencing and MFC remained in first remission after completion of therapy (Figure 3, C and D). Two additional patients were investigated, of which one underwent stem cell transplantation in first remission because of high-risk classification (*FLT3*-ITD without *NPM1* mutation) (Supplemental Figure S1A). For this patient, no data from MFC-MRD analysis (no LAIP distinguishable from normal regenerating BM at diagnosis) or RT-qPCR were available for comparison. One patient with secondary AML received other therapy but showed comparable kinetics with deep sequencing and MFC (Supplemental Figure S1B). In summary, deep sequencing was concordant with MFC but had a higher sensitivity.

Deep Sequencing MRD Analysis Is Concordant with RT-qPCR but Has a Higher Precision

For three of the AML cases, RT-qPCR of fusion transcripts *RUNX1-RUNX1T1* or *KMT2A-MLLT10* was also performed. By comparing results from deep sequencing with results from RT-qPCR, 13 of 14 samples showed concordant MRD assignments (eight MRD^+ with both methods, five MRD^- with both methods). One sample was MRD^+ (VAF^{EC}, 0.025%) with the use of deep sequencing and MRD^- with the use of RT-qPCR for *KMT2A-MLLT10* (limit of quantification, 1.9×10^{-3}). The limit of quantification was higher in the BM samples analyzed with the *KMT2A-MLLT10* assay (median of diagnostic level, 2×10^{-3}; range of diagnostic level, 1×10^{-3} to 5×10^{-3}) than with the *RUNX1-RUNX1T1* assay (range of diagnostic level,1.9×10^{-5}; range of diagnostic level, 8×10^{-6} to 6.1×10^{-5}; $P = 0.006$). The precision for RT-qPCR was determined from 40 runs of two positive controls (at levels of *RUNX1-RUNX1T1/ABL1* at 2.1% and 21.2%), resulting in CV of 33% and 25%, respectively. Thus, deep sequencing was concordant with RT-qPCR but had a higher precision.

Deep Sequencing Allows Detection of MRD in Peripheral Blood

To explore the applicability of the method for MRD detection in peripheral blood, blood samples were analyzed from the same time points as for 12 BM samples that were MRD^+ during treatment with deep sequencing (26 mutations in 12 samples; VAF^{EC}, 0.028% to 3.6%). The results from the blood analysis correlated with those of BM (correlation coefficient, 0.82; $P = 0.013$), and 17 of 26 mutations were MRD^+ also in blood (Figure 4). In fact, all mutations with $VAF^{EC} > 0.1\%$ in BM were MRD^+ in blood, and at least one mutation was detected in 10 of the 12 investigated blood samples. Further, for seven blood samples, results from RT-qPCR of *RUNX1-RUNX1T1* and *KMT2A-MLLT10* were also available (three MRD^+ and four MRD^-). Deep sequencing showed concordant MRD assignments in six of these samples (three MRD^+ and three MRD^-), and one sample was MRD^+ with deep sequencing but MRD^- with RT-qPCR of *KMT2A-MLLT10*. Thus, deep sequencing has potential for clinical applicability in blood and in BM.

Discussion

In this study, patient-tailored MRD analysis was validated and optimized by using individualized targeted deep sequencing of leukemia-specific mutations. With this method, determination of VAF^{EC} could quantify SNVs at low VAFs in a linear manner with high accuracy and good precision. Concordance was found between results obtained with deep sequencing and MRD analyses with MFC and RT-qPCR, but deep sequencing allowed for a MRD detection with higher sensitivity than MFC-MRD analysis. Strikingly, all samples determined to be MRD^+ with MFC-MRD analysis could also be verified with deep sequencing. In addition, the precision of deep sequencing at MRD levels superseded that of RT-qPCR. This shows promise for

accurate and sensitive molecular MRD analysis with deep sequencing in virtually all patients with AML.

The obtained LOD of 0.02% with deep sequencing with position-specific error correction enabled more sensitive detection of MRD than MFC in AML. It also fulfills the newly published European LeukemiaNet consensus requirement of detection of down to 0.1% leukemic cells.[24] The LOD of a deep sequencing assay depends on several parameters, including the genetic context of the target mutation (causing SSE), general sequencing errors, sequencing depth, index assignment, and amount of sample. Several benchtop sequencing platforms are available, such as Illumina MiSeq, Ion Torrent Proton, and PGM. They all have fast run times, but they differ in error rates and possibilities for paired-end sequencing.[25,38] The LOD reported here is lower than expected from sequencing on the platform used (Illumina MiSeq), as a result of several factors. The paired-end sequencing enables sequencing of each read twice, allowing for post-sequencing correction of substitution errors. Because the error frequency increases toward the end of the reads, the amplicons were constructed with the mutation site centered.[26] This strategy also maximized the read overlap between the paired-end reads, allowing for merging into a single consensus sequence. The software PEAR, which is used for merging of the paired-end reads, has previously been shown to reduce substitution error rates by 18% to 97%.[26] The distribution of sequencing errors from the MiSeq platform is biased toward characteristic base-calling errors and SSEs.[26,39] Several groups have reported that sequencing errors are more frequent after GC-rich sequences in MiSeq data, particularly GGC motifs,[38,39] implying that errors are not completely random. The issue of general errors and SSEs was addressed by using a reference sample for each investigated position to calculate the VAF^{EC}. This is as opposed to making precedent general assumptions about error distribution, such as equal error frequencies among the different nucleotides. The relative impact of this correction was highest in the samples with low VAF and had only minor effect in samples with higher VAF.

Being able to process multiple samples in the same run, that is, multiplexing, is an advantage of NGS-based techniques over MFC or RT-qPCR. Although sample multiplexing enhances the possibilities of simultaneous processing, it also increases the risk of mis-assignment that results in carryover. First, there is a potential risk of index contamination at the manufacturer if the primer purification is done sequentially, for example, using high performance liquid chromatography. Polyacrylamide gel electrophoresis purification of the primers was therefore used. Second, sequencing errors in indexes can lead to mis-assignments of reads. Preventive measures have been taken by the designer in the construction of index-sequences to avoid false assignment due to sequencing errors. However, the MiSeq demultiplexing step by default allows for one mismatch in the index, which slightly increases the risk of mis-assignment in the case of sequencing errors.[40] The demultiplexing step was therefore modified to allow zero mismatches despite a minor loss of sequencing data. Application of dual unique indexing instead of single unique indexing resulted in a 10-fold decrease in carryover. Bartram et al[14] similarly showed a fourfold reduction in mis-assignment by using dual unique indexing. By using dual unique indexing, the identity of the sample origin is independently determined twice and is additionally a measure to minimize the risk of sample bleeding through mis-assignment. Finally, most mis-assignments derive from either overlapping clusters or from clusters originating from two different templates, because of limitations in signal separation of the sequencing platform.[40,41] To increase template diversity, the libraries were spiked with PhiX bacteriophage genome, and to increase cluster separation samples were sequenced at a relatively low cluster density.[40] The estimated specificity by using the deep sequencing approach for SNVs was 97%. An alternative way of increasing the specificity of a deep sequencing assay is to tag the template DNA molecules with unique molecular identifiers before PCR amplification. The use of unique molecular identifiers shows promise for error removal in deep sequencing data, because reads that contain PCR or sequencing errors could be identified.[42,43] However, it requires two consecutive PCR reactions, and uniform tagging of all DNA molecules in the sample and is associated with a risk that the tags themselves interfere with the PCR reaction or are afflicted by PCR errors.[44]

Because 90% to 96% of mutations with VAF >40% at diagnosis are present at relapse, mutations with high allelic frequency at diagnosis should be selected for MRD follow-up to maximize the sensitivity for predicting relapse.[17,45] Patient-tailored deep sequencing was therefore applied to leukemia-specific mutations present in all leukemic cells and not in subclones.[23] Moreover, because mutation loss at relapse has been described (eg, for *FLT3*-ITD, *KRAS*, *NRAS*), the analysis should preferably include more than one mutation, as in this study.[46] In the sample series from children with AML, patients that were MRD^{+} for at least one mutation at the end of induction therapy later relapsed with all tested mutations remaining. In adult AML, mutation clearance <2.5% has been shown to be a more precise way of determining remission than using BM morphology.[15] Recently, clearance of mutations <0.5% and 0.01% (using deep sequencing and droplet digital PCR, respectively) was shown to be associated with decreased risk of relapse.[17,18] Morita et al[47] reported significantly better 2-year overall survival for AML patients with mutation clearance VAF <1% as well as complete mutation clearance in complete remission, but not if the VAF of residual mutations was <2.5%. The combined use of MFC-MRD and mutation clearance in complete remission was shown by Jongen-Lavrencic et al[48] to confer additive prognostic value for relapse rate and overall survival compared with either method alone. Hirch et al[16] confirmed the prognostic value of mutation clearance only when at least two early

mutations of the leukemic clone were cleared to <0.4% of leukemic cells (level corresponding to their LOD of VAF of 0.2%). This illustrates one of the obstacles in using mutation clearance in adult AML, namely the persistence of preleukemic mutations after end of treatment, frequently found in the genes *ASXL1*, *DNMT3A*, *U2AF1*, *TET2,* and *SRSF2*. Thus, such preleukemic mutations should probably be avoided as targets for MRD analysis with patient-tailored deep sequencing, as applied in the recent studies by Jongen-Lavrencic et al[48] and Morita et al.[47] This remains to be shown, because the impact of such mutations might depend on age[49] and also on monitoring purposes. Another potential obstacle for mutation monitoring of AML is the different patterns of clonal evolution at relapse, whereby one such pattern is the acquirement of additional mutations at relapse.[50] The targeted nature of the assay described herein does not allow for detection of such mutations.

Costs and turnaround times for the assay have been described previously.[23] Specifically, the process to identify MRD suitable mutations takes approximately 2 working weeks, including fluorescence-activated cell sorting (1 day), exome sequencing (3 days), bioinformatic analysis (2 days), and design and validation of PCR primers (3 days). For each MRD analysis, the library preparation takes approximately 2 days, and deep sequencing, including bioinformatic analysis, 2 days. Taken together, the time and resources required are comparable with that used for TCR/Ig PCR for MRD assessment in acute lymphoblastic leukemia and probably less if mutation identification is performed with a targeted panel. This is likely appropriate for adults, rather than exome sequencing that most probably is needed for children.

Conclusions

The deep sequencing analysis of leukemia-specific mutations described herein can quantify substitution type mutations at low VAFs in a linear manner with high accuracy and good precision. It enables MRD detection at lower levels than MFC, and its sensitivity in blood implies that it can be of value for monitoring after the end of treatment, which currently is only available for patients with recurrent gene fusions or mutation in *NPM1*. Introduction of this method in clinical care thus paves the way for MRD surveillance in virtually every patient with AML.

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E.D.M. planned the study, performed experiments, analyzed data, and wrote the manuscript; A.R. and M.B.P. developed the statistical model, performed bioinformatic analyses, and edited the manuscript; J.Ab. provided patient samples and edited the manuscript; T.S. performed bioinformatic analyses and edited the manuscript; S.S. performed experiments and edited the manuscript; J.As. and L.P. provided routine analyses and edited the manuscript; A.T. designed the multiparameter flow cytometry−MRD analysis, analyzed data, and edited the manuscript; E.K. planned parts of the study, supervised the development of the statistical model, and edited the manuscript; L.F. planned the study, analyzed data, and wrote the manuscript.

Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.jmoldx.2018.08.004*.

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