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Molecular MRD Assessment in Acute Myeloid Leukemias

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Abstract

Detection of measurable residual disease (MRD) is of significant value in the management of acute myeloid leukemia (AML) patients. Along with multicolor flowcytometry (MFC), molecular techniques form an integral tool in AML MRD detection. Multiple studies have reiterated the role of molecular MRD evaluation in AML at defined timepoints during the course of therapy, helping in risk stratification, prediction of relapse, and as guide for pre-emptive therapy. The latest World Health Organization (WHO) classification (WHO-HEME5) has refined the classification of AML bringing forth newer entities defined by molecular abnormalities, especially fusions. AML is a clonally heterogeneous disease characterized by a spectrum of multiple molecular abnormalities including gene mutations and fusions. Accordingly, the molecular methods employed are also diverse and need robust technical standardization in clinical laboratories. Real-time quantitative polymerase chain reaction (PCR), digital PCR, and next-generation sequencing (NGS) are the major molecular platforms for AML MRD. The European LeukemiaNet (ELN) MRD Working Party consensus document recently updated in 2021 for the first time has reflected on the technical recommendations for NGS MRD in AML and stressed the value of an integrated approach. It is, therefore, desirable for physicians, scientists, and pathologists alike to thoroughly understand these molecular methods for appropriate utilization and interpretation. In this article, we discuss the various facets of molecular methods for MRD detection in AML including technical requirements, advantages, drawbacks, and applications.

Keywords
- molecular MRD
- error corrected next-generation sequencing
- measurable residual disease

Introduction

Acute myeloid leukemia (AML) constitutes approximately 1% of all cancer cases as per the Surveillance, Epidemiology, and End Results Program (SEER) Program in the United States and considered a disease with dismal prognosis. It is one of the most common leukemias in adults and not a very uncommon leukemia in children. Over the last two decades, our understanding of disease biology and pathogenic mechanisms has improved and has resulted in newer targeted therapies in AML. Detection of the residual disease burden beyond conventional morphological techniques known as measurable residual disease (MRD) is a standard of care in leukemia patients and plays a crucial role in disease prognostication, predicting and monitoring of response. MRD positivity during complete morphological remission is known to be associated with increased relapse rates and poor survival outcomes in AML.[1,2] In addition, MRD status is also explored for treatment decisions.

The European LeukemiaNet (ELN) MRD Working Party recommendations are widely followed for AML MRD, which were last updated in 2021.[3] The two major modalities for MRD detection in AML are multiparametric flow cytometry (MFC)

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and molecular MRD testing. Each of these two techniques has its merits and demerits. MFC is widely available and can be applied in almost all cases of AML. Assessment of CD34+/CD38-leukemic stem cell burden in combination with aberrant markers by MFC has also been used for MRD detection. However, the detection of true minimal residual disease against a background of regenerative hematopoiesis and chemotherapy effects (such as immunophenotypic shifts), along with the technical and analytical expertise required, together pose practical challenge for AML MRD detection by MFC. This is further complicated by the fact that unlike in precursor B lineage acute lymphoblastic leukemia, the frequencies of leukemia-associated immunophenotypes in AML do not always permit a clear detection of the leukemic clone.

AML is a heterogeneous disease marked by the presence of numerous clones and subclones of leukemic cells harboring multiple molecular changes that undergo fluctuations during the disease course and therapy. Tracking of the molecular aberrations detected at diagnostic timepoint forms the basis of molecular MRD testing. The list of newer AML entities defined by molecular methods continues to expand, as evident in the latest World Health Organization (WHO) classification of hematolymphoid neoplasms (WHO-HEME5).[4] The implementation of molecular techniques for MRD monitoring is therefore need of the hour for hematopathology laboratories. We review the molecular methods for AML MRD considering the recent ELN AML MRD recommendations[3] and also discuss their clinical utility in this paper.

Overview of the Molecular Aberrations and Methods of Detection

Molecular studies performed at diagnostic timepoint help in the identification of molecular aberrations in most AML patients, each of which can be potentially tracked for MRD using molecular techniques. The molecular aberrations broadly include

a) Chimeric gene fusions as a result of chromosomal translocations, for example, *RUNX1::RUNX1T1*, *CBFB::MYH11*, *PML::RARA*
b) Insertions and deletions (Indels), for example, nucleophosmin 1 (*NPM1*), *FLT3*-internal tandem duplication (ITD)
c) Single nucleotide variants (SNVs), for example, *TP53* R273H, *KRAS* G12D
d) Increased gene expression levels, for example, *WT1*, *ERG*, *BAALC*, *PRAME*, and *MN1*.[5–11]

Given the genomic heterogeneity of AML, a variety of molecular techniques are employed for monitoring MRD. These range from real-time quantitative polymerase chain reaction (RT-qPCR) and digital (dPCR) to next-generation sequencing (NGS)-based approaches. The latter include conventional "bulk" NGS (for single genes as well as multigene panels) and error corrected NGS. Other methods that have been reported in literature but not widely used for the purpose of MRD detection include chimerism studies post-transplant[12] and immunoglobulin heavy chain and T cell receptor gene rearrangements.[13] Exploratory methods for AML MRD include sequencing of cell free DNA, single cell genomics, and metabolomic profiling.[14,15] ►**Fig. 1** provides an overview of the common molecular aberrations and the molecular methods used to detect AML MRD.

Needless to say, technical standardization and stringent validation in the laboratory are utmost challenging and irreplaceably required for implementation and reporting in a clinical setting.

Technical Considerations: Preanalytical

Sample Considerations

The recommended sample for molecular MRD in AML as per ELN 2021 recommendations is either bone marrow (BM) or peripheral blood (PB). The volume of draw for BM aspirate should be up to 5 mL, while a volume of more than or equal to 10 mL can be required when using PB.[3] In addition, the white cell count and the assay to be performed also need to be considered while determining the required volume of sample drawn. First pull of marrow aspirate should be preferred to avoid hemodilution.

Although ELN recommends both ethylenediaminetetraacetic acid and heparin as the anticoagulants for sample collection, heparin is better avoided given its inhibitory effect on PCR reactions. Cell isolation techniques to reduce hemodilution by granulocytes can be used (e.g., Ficoll separation), provided that the method followed in laboratory is consistent, as leukemic cell percentage can be altered based on it.

Peripheral Blood versus Bone Marrow

The use of BM yields up to one log higher sensitivity compared to PB.[16] Multiple studies have also shown concordance between the two sample types. Recently, Skou et al reviewed literature concerning the utility of PB versus BM for molecular AML MRD and suggested that PB may be sufficient for predicting impending AML relapses.[17] The ELN 2021 MRD document, however, currently recommends the use of both samples (PB and BM) at diagnostic timepoint (as comparators) if using log reduction for MRD calculation. PB sample is preferred at post-induction (PI) timepoint in *NPM1* mutated and core binding factor (CBF) AMLs, while BM sample is recommended for post-consolidation (PC) timepoint. Subsequent follow-up can either be done on PB or BM.[3]

Other Considerations

Once the sample is received by the laboratory and sample requirements are met, it is essential to confirm timepoint of testing, the diagnostic molecular results, and the current disease status on morphology. These factors can influence the choice of assay to be performed and the interpretation.

Technical Considerations: Analytical

Single Biomarker Methods

RT-qPCR and digital PCR are the two major techniques recommended for molecular MRD assessment for the

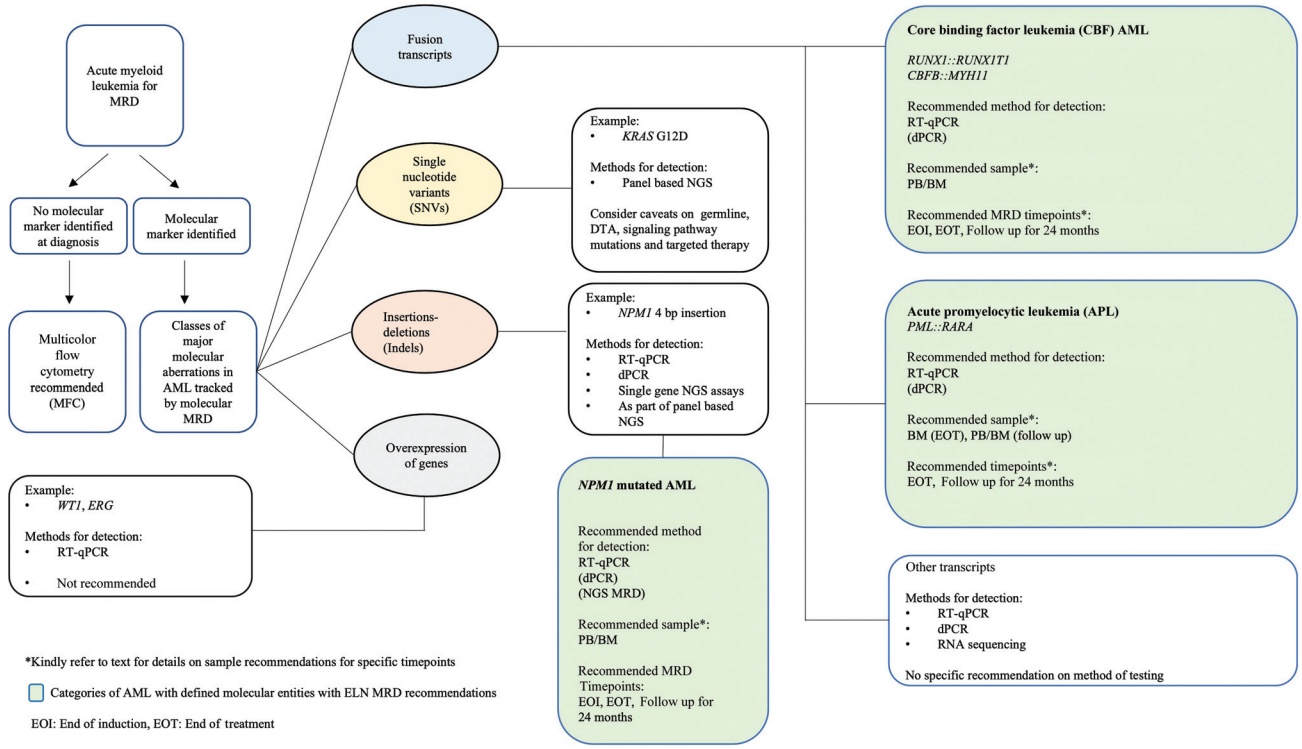

Fig. 1 Overview of molecular aberrations in acute myeloid leukemia (AML) tracked by molecular methods and their major methods of detection along with recommended methods. BM, bone marrow; dPCR, digital PCR; MRD, measurable residual disease; NGS, next-generation sequencing; PB, peripheral blood; PC, post-consolidation; RT-qPCR, real-time quantitative polymerase chain reaction.

detection of CBF-AML, acute promyelocytic leukemia (APL), and *NPM1* mut AML.[3] For patients with no molecular abnormality identified, the recommended method remains MFC.[3]

RT-qPCR: General Considerations

RT-qPCR has been the most widely adopted method for molecular MRD assessment and involves quantitative detection using fluorescent probes designed specifically to target the regions of interest. The molecular abnormalities detected by RT-qPCR broadly include (a) fusion transcripts, (b) single gene variants and indels, and (c) genes with altered expression levels in AML.

RNA is usually the starting material for detecting fusions and differential expression of genes, while both RNA and DNA have been employed for the detection of gene mutations using RT-qPCR, for example, *NPM1*, *FLT3*-ITD, *IDH1*, *IDH2,* and *DNMT3A*.[18–24]

The technical requirements established by Europe Against Cancer (EAC) in 2003 remain the resort for the implementation of molecular MRD in AML by RT-qPCR.[3,25,26] Along with the region of interest, a house keeping gene (e.g., *ABL1*) is also amplified (minimum 10,000 copies) and detected using specifically designed primers and probes. The obtained copy number of target gene/ fusion transcript is then normalized against that of a housekeeping gene or the wild-type counterpart of the respective mutated gene (e.g., *NPM1*).

A positive RT-qPCR result is defined as amplification with cycle threshold (Ct) values below 40 in at least two of the three (triplicates) results of the sample tested, when the value of cycling threshold is 0.1.[3,25,26] Assay sensitivity needs to be determined especially while reporting a negative result. The sensitivity achieved by RT-qPCR ranges between 10^{-4} and 10^{-5}. ►**Table 1** highlights the major studies where RT-qPCR has been used for the detection of molecular MRD in AML in major molecularly defined entities.

RT-qPCR for Uncommon Fusion Transcripts

While the RT-qPCR for CBF AMLs and APL is performed using absolute quantification using the respective standards, the reporting of uncommon fusion transcripts (e.g., *KMT2A::MLLT3, NUP98::NSD1*) may require the use of relative quantification method. As these transcripts are rare, it is not practically feasible for clinical laboratories to procure and sustain standards for absolute quantitation of all of them. Relative quantification involves comparison of baseline and follow-up samples calculating delta delta Ct (ΔΔ Ct).[25]

RT-qPCR for Common Gene Mutations

Mutations in *NPM1* gene constitute over 30 to 40% of all AMLs and are the "driver mutations." Being stable over course of disease, *NPM1* mutations form a suitable target for MRD detection. There are more than 50 types of *NPM1* mutations reported, depending on which 4 bp insertion is present in the exon 11 (NM_002520) of *NPM1* gene. The type A *NPM1* mutation (TCTG) is the most common (~75–80%).[41,42] Interpretation of RT-qPCR requires prior knowledge of mutation type, as type-specific probes would be required. Failure to use specific type of primer-probes can lead to a false negative MRD result. Currently, commercial plasmid standards are available only for the three most common subtypes (A, B, and

Table 1 Major studies that utilized RT-qPCR for AML MRD detection in CBF-AML, APL, and NPM1 mutated AMLs

Marker	Study	Interpretation of results	Sensitivity	Highlights
Core binding factor leukemia	Viehmann S 2003[27]	10 copies reliably detected	10^{-5}	- 92 follow-up samples from 15 pediatric AML cases studied - 2–4 log decrease at start of consolidation in most patients - Serial monitoring technique suggested for prediction of relapse
	Perea G 2006[28]	Ct <40 for RUNX1::RUNX1T1 Ct <39 for CBFB::MYH11	10^{-4} to 10^{-5}	- Higher CIR for cases with >10 copies at end of treatment
	Corbacioglu A 2010[29]	–	10^{-3} to 10^{-4}	- PC MRD in CBFB::MYH11 AML identifies patients with high relapse risk - Showed that PB can be used for monitoring in patients with negative MRD after consolidation and early follow-up
	Yin JA et al 2012[30]	As per EAC criteria	10^{-5}	- PI BM MRD >3 log, BM copies >500 and PB >1000 informative for RUNX1::RUNX1T1 - PC BM MRD ≥4 log reduction associated with lower CIR, and presence of >500 RUNX1::RUNX1T1 copies predicted relapse - Presence of >10 copies of CBFB::MYH11 in PB associated with higher CIR
	Zhu HH 2013[31]	As per QinY-ZLiJLZhuHH et al	Not mentioned	- Positive MRD after 2nd consolidation discriminated patients with high relapse risk
	Jourdan E 2013[32]	As per EAC criteria	Not mentioned	- PC MRD (after 2nd consolidation) could best discriminate patients with high relapse risk
	Wang Y 2014[33]	As per EAC criteria	Not mentioned	- Post-HSCT monitoring of RUNX1::RUNX1T1 at 1, 2, 3 months
	Willekens et al 2016[34]	0.001%	10^{-5}	- 94 paired PB and BM samples prospectively - Two years follow-up - Serial PB MRD of *RUNX1::RUNX1T1* monitoring predicted hematological relapse
Acute promyelocytic leukemia	Grimwade D 2009[35]	As per EAC criteria	10^{-4}	- 406 patients monitored prospectively - Sequential MRD post-consolidation predicted relapse and helps guide pre-emptive therapy
NPM1	Gorello P 2006[36]	–	10^{-4} to 10^{-5}	- *NPM1* types A and B mutations studied - *NPM1* shown as stable MRD marker
	Chou WC 2007[21]	–	10^{-5}	- DNA based study - Ten years follow-up of 194 samples from 38 AML patients - Patients with at least 2 log reduction PC showed better OS and PFS
	Papadaki 2009[37]	–	10^{-5}	- RNA based - 51 patients with Type A mutation studied - Both PB and BM studied - 9.5% relapsed cases showed loss of *NPM1* mutation

(*Continued*)

Table 1 (*Continued*)

Marker	Study	Interpretation of results	Sensitivity	Highlights
	Schnittger S 2009[38]	–	10^{-5}	- Paired diagnostic/relapse samples of 84 patients studied - 17 types of *NPM1* mutations studied - A threshold of 0.1% found relevant for EFS at four timepoints - No impact on relapse risk or OS
	Krönke J 2011[39]	–	10^{-5} to 10^{-6}	- RNA-based, six types of *NPM1* detected - 245 young adult patients - Cutoff of 200 copies/10^4 ABL copies predicted relapse
	Shayegi 2013[40]	–	10^{-5}	- *NPM1* types A, B, and D included - LNA clamping strategy used - MRD detectable above 1% at CR1 significantly correlated with worse DFS and OS
	Ivey A 2016[19]	As per EAC criteria	10^{-4}	- 2,569 samples from 346 patients. - MRD positivity associated with inferior 3-year survival and higher relapse rates

Abbreviations: BM, bone marrow; CBF-AML, core binding factor-acute myeloid leukemia; DFS, disease-free survival; EAC, Europe Against Cancer; HSCT, hematopoietic stem cell transplant; MRD, measurable residual disease; NPM1, nucleophosmin 1; OS, overall survival; PB, peripheral blood; PC, post-consolidation; PFS, progression-free survival; PI, post-induction; RT-qPCR, real-time quantitative polymerase chain reaction.

D) of *NPM1* mutated AML.[43] Also, the possible cross reactivity of probes between wild-type and mutated *NPM1* alleles is also an important consideration while using RT-qPCR for *NPM1* MRD.[41,42] Similarly, RT-qPCR studied for *FLT3*-ITD MRD[44] also carries drawbacks especially ascribed to the variable length of ITD in every case.

Although fragment length analysis (GeneScan) is conventionally used for the detection of *NPM1* mutation and *FLT3*-ITD at diagnostic timepoint, its utility in MRD detection is limited. In context of *FLT3*-ITD, however, fragment length analysis does offer an advantage of detecting multiple mutant peaks and calculation of allelic ratio (calculated as Area Under Curve of mutant peaks divided by that of wild-type peak).

Limitations of RT-qPCR

Although RT-qPCR is considered "gold standard" for molecular MRD and is highly sensitive, cost-effective, and widely available, there are limitations to the method as well. Drawbacks of RT-qPCR include limited applicability, requirement of high-quality validated standards, prior knowledge of the molecular abnormality, need for specific primer-probes, separate assays for every molecular abnormality, and practical difficulty for application in tracking insertions deletions (indels). Moreover, application of RT-qPCR can be extended to only around half of all AML patients, limiting its utility in substantial proportion of cases.[45]

Digital PCR

Digital PCR forms a lucrative alternative to RT-qPCR with potentially higher sensitivity and specificity. PCR is conducted in numerous sub partitions in the form of solid chambers known as chips or water-in-oil droplets (digital droplet PCR). Absolute quantitation of target gene copy number is obtained by this technique. Unlike RT-qPCR, the need for standards is bypassed. However, the requirement to set up individual assays for different molecular targets still remains. Guidelines for performing quantitative digital PCR have been published in 2013 and updated in 2020 by the Minimum Information for Publication of Quantitative Digital PCR Experiments (dMIQE) group.[46,47] The role of dPCR based AML MRD has been studied at different timepoints for gene mutations, for example, *NPM1*, *DNMT3A*, *IDH1*, *IDH2* mutations as also for transcripts like *PML::RARA*.[19,23,48–54] Though the appropriate threshold remains to be established in larger studies, provisionally, a variant allele fraction (VAF) of more than or equal to 0.2% is defined as dPCR positivity for genomic DNA. There is no suggested optimal threshold positivity using cDNA currently. Drawbacks of dPCR include subsampling errors, partitioning errors, and the need to develop individual assays for each genetic alteration.[3,43]

Limit of Detection

The limit of detection (LOD) is the measure of lowest concentration of the analyte that can be reliably measured by an assay, with a specific degree of confidence. A LOD of 10^{-3} or lower is recommended for molecular MRD assessment.[3] It is required that LOD be established for every marker targeted by the MRD assay, both RT-qPCR and NGS. Along with this, limit of blank (LOB) also needs to be determined using healthy/ negative controls.[3] As numerous factors can affect the calculation of LOD of PCR assays, it is recommended to follow the EAC guidelines for establishment of the same.

Next-Generation Sequencing

High-throughput sequencing or NGS makes the detection of multiple mutations (panel based) possible for multiple

samples in a single assay, unlike the PCR-based methods. This is especially relevant for AML, a clonally heterogeneous disease showing dynamic fluctuations in a patient over the course of therapy. Apart from panel-based sequencing, NGS assays have also been developed for single genes, for example, *NPM1* and *FLT3*-ITD. For the first time, ELN MRD document in 2021 has reflected upon technical requirements for NGS MRD in AML.[3]

Panel-Based NGS (DNA)

Targeted NGS performed at the diagnostic timepoint can detect somatic mutations (SNVs, indels) in nearly all patients

Table 2 Summary of major panel-based NGS-based molecular MRD studies in AML

Study	Method	Interpretation of results	Highlights
Klco et al 2015[55]	- Targeted amplicon-based sequencing - Ion Torrent platform	- VAF of 2.5% and 1% studied as cut offs	- Day 30 remission FFPE BM biopsy samples of 25 patients. - Increased relapse risk and reduced OS in MRD positive cases
Getta et al 2017[56]	- Amplicon capture-based NGS - 28 gene panel	- VAF $<$5% was defined as allele clearance	- Pre-HSCT MRD studied in 47 patients - Presence of MRD shown to be associated with post-transplant relapse and survival - Compared with MFC MRD
Jongen-Lavrencic 2018[57]	- Targeted NGS - 54 genes	- Maximum sensitivity of 0.02% achieved	- 482 patients - PI MRD positivity associated with increased rates of relapse, RFS and OS - DTA mutations excluded - Compared with MFC MRD
Kim et al 2018[58]	- Targeted NGS - 84 gene panel - Mean on target coverage 1725.6x	- Minimum VAF of 0.02% called	- 104 patients - Post-HSCT day 21 VAF of $\geq$ 0.2% observed to be associated with increased relapse and worse 3 years OS
Morita et al 2018[59]	- Haloplex HS molecular barcode sequencing - 32 genes	- Mutation clearance defined as per VAF at CR (2.5%, 1%, undetectable)	- 131 patients - Cases with VAF less than 1% and undetectable mutations at PI had better EFS, OS, and lower CIR at median follow-up of 35.2 months - CHIP mutations found noninformative
Thol et al 2018[16]	- Targeted UMI based error corrected sequencing - 24 genes	- Sensitivity of $<$0.02%	- 96 cases in CR pre-transplant - MRD positivity shown to be associated with higher CIR and lower PFS at median follow-up of 6.2 years - Comparable results in PB and BM
Press et al 2019[60]	- Ampliseq panel of 42 gene - PGM sequencer platform - Mean coverage 1900x	- Lower limit of detection (LLOD) of 0.24%	- 42 cases pre-HSCT ($\leq$27 days) - MRD positivity shown to independently predict relapse and associated with significantly shortened PFS
Hourigan et al 2019[61]	- Ultra-deep, error-corrected sequencing - 13 gene panel	- 0.001% VAF	- 190 cases - RIC conditioning associated with increased relapse rates decreased OS and compared to MAC in pre-transplant NGS MRD positive cases
Balagopal et al 2019[62]	- Hybrid capture based error corrected targeted NGS incorporating UMIs - 22 genes targeted	- VAF of 0.1% for SNVs and 0.001% for indels	- Retrospective analysis of 30 relapsed patients post-HSCT using pre recurrence samples - NGS MRD effective for

(*Continued*)

Table 2 (*Continued*)

Study	Method	Interpretation of results	Highlights
			surveillance post-HSCT to identify relapse - NGS more useful than STR PCR and MFC - Suggest that CHIP mutations can be used post-transplant
Onecha et al 2019[63]	- 32 genes - $\geq$ 1000000 read depth - Ion Torrent platform	- 10 – 4 (SNVs) 10 – 5 (indels)	- Samples from 63 patients studied at PI and PC timepoints - CHIP mutations excluded - Sensitivity compared to dPCR
Patkar et al 2021[64]	- 34 gene panel - smMIPS based - *FLT3*-ITD studied using gene specific NGS assays - Median coverage 14,728x	- 0.05% for SNVs and 0.03% for *FLT3*-ITD 0.002%	- 201 patients - Presence of positive NGS-MRD predictive of inferior OS and RFS for PI and PC timepoints - Median follow-up of 42.3 months - Compared with MFC MRD

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; CHIP, clonal hematopoiesis of indeterminate potential; EFS, event free survival; HSCT, hematopoietic stem cell transplant; MFC, multicolor flowcytometry; MRD, measurable residual disease; NGS, next-generation sequencing; NPM1, nucleophosmin 1; OS, overall survival; PB, peripheral blood; PC, post-consolidation; PFS, progression-free survival; PGM, personal genome machine; PI, post-induction; RFS, relapse free survival; RIC, reduced intensity conditioning; RT-qPCR, real-time quantitative polymerase chain reaction; smMIPS, single molecular inversion probe system; SNV, single nucleotide variants; UMI, unique molecular identifier; VAF, variant allele fraction.

of AML[43] and hence can be potentially applied for MRD detection in most patients like MFC-based MRD. ►**Table 2** summarizes major studies utilizing panel-based NGS for AML MRD detection.

Conventional NGS versus Error Corrected NGS

MRD detection being a rare event analysis, distinguishing a true call from false call, is of utmost significance. Conventional NGS technology is prone to errors (0.1–1%), owing either to the sequencing technique itself or can be PCR generated.[65] Hence, application of error correction methods is important to ensure appropriate results while using NGS for AML MRD. Error correction methods are broadly physical (involving changes in library preparation and processing steps) or computational.[65–67] ►**Fig. 2** illustrates error correction in brief (2A) and summarizes the major error correction methods (2B).

Library Preparation

Library preparation involves sample processing steps to make the sample ready for sequencing, and broadly includes fragmentation, end repair, A-tailing, and adaptor ligation. Modifications in library preparation are required for MRD detection in order to achieve higher sensitivity and correct variant calls. Incorporation of unique molecular identifiers (UMI) is the most widely used technique that can be performed using commercial or laboratory developed methods (►**Fig. 2B**). Broadly, the methods for UMI incorporation are either single molecular inversion probe system (smMIPS) based or PCR based. Further, the UMIs can be incorporated on

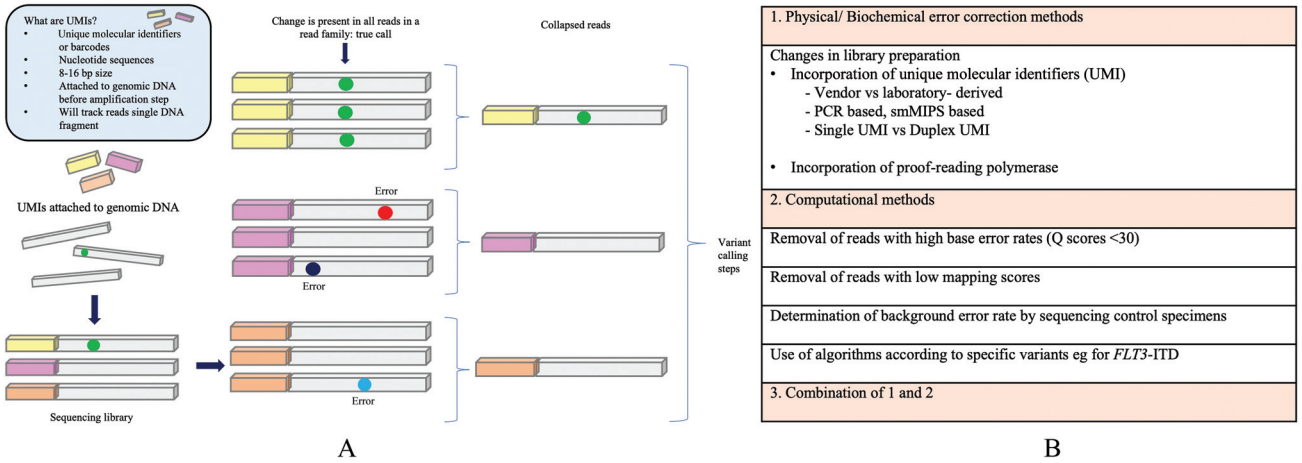

Fig. 2 Illustration of error correction in next-generation sequencing (**A**) and overview of error correction techniques applied for measurable residual disease detection in NGS (**B**).[65–67]PCR, polymerase chain reaction; smMIPS, single molecular inversion probe system.

single strand or both strands (duplex), with duplex method providing higher error correction.[65,66] We have demonstrated the utility of error-corrected NGS using in house laboratory developed 34- gene panel using smMIPS and single gene assays for *NPM1* and *FLT3*-ITD.[64]

LOD for NGS Assays

Results of NGS are expressed in terms of VAF, defined by proportion of reads containing the mutant allele out of the total reads obtained for that particular locus. The LOD for conventional NGS methods applied at diagnostic timepoints ranges from 2 to 5%, which lowers down to the desirable 0.1 to 0.5% when error correction is applied.[66]

Sequencing Depth

Achievement of high sensitivity in MRD requires a higher sequencing depth (>20,000x) while using non-error corrected sequencing.[3,66] A read depth high enough to discriminate true call from background noise should be targeted.[3] Deeper approach is usually possible using a limited set of genes and tracking mutations detected earlier in the patient. Use of smaller panels with deeper sequencing leads to compromise on "breadth" leading to chance of missing mutations: (a) present at diagnostic timepoint but not targeted in the MRD panel and (b) newer mutations that could potentially lead to relapse.

Markers for Panel-Based NGS-MRD

Potentially every gene mutation detected at diagnostic timepoint can be targeted for MRD detection by targeted NGS. A panel of 23 commonly mutated genes in AML has been suggested by ELN.[3] Four important considerations while choosing markers for analysis of NGS MRD are:

(i) DTA mutations (*DNMT3A*, *TET2*, *ASXL1*) associated with clonal hematopoiesis of indeterminate potential (CHIP): The mutations associated with CHIP should not be considered for MRD analysis as they are known to persist post-remission and also may not be part of leukemic clone.[57,64,68] If DTA mutations are the only detected mutations at the diagnostic time point, MFC based MRD or PCR-based MRD should be applied.[3]

(ii) Germline mutations: Mutations in genes known to be associated with germline predisposition to myeloid malignancies example, *CEBPA*, *DDX41*, are detected at near heterozygous VAF (~50%) at baseline and persist at further timepoints. These variants are noninformative and should not be used as MRD markers.[3] A potential exception to this situation could be post allogenic hematopoietic stem cell transplant (allo-HCT)[65]

(iii) Signaling pathway genes: Mutations in the signaling pathway, for example, *FLT3*-ITD, *FLT3*-TKD, *KIT*, *KRAS*, *NRAS,* are likely secondary events in leukemogenesis and are mostly subclonal. When detected at MRD timepoint, they represent true MRD positivity; however, their absence in isolation should not be taken as evidence of MRD negativity.[3]

(iv) Targeted therapy: When performing MRD in a patient who has received targeted therapy, for example FLT3 inhibitors or IDH1/2 inhibitors, a marker apart from that targeted should also be included in the MRD analysis.[3]

Single Gene NGS: *NPM1* and *FLT3*-ITD

The limitations of RT-qPCR (as discussed in previous sections) are addressed to a large extent with the use of NGS for *NPM1* MRD detection as patient-specific primers are not required.[41,42,69] The *FLT3*-ITD mutations in AML involve insertions in the exon 14 (NM_004119) of variable length affecting the juxta membrane domain of the *FLT3* gene. With the widespread use of targeted therapy in the treatment of *FLT3*-ITD AML, monitoring forms an essential part of patient management. Sensitive PCR-based assays would require patient specific *FLT3*-ITD primers limiting its practical utility. Additionally, the variable length of ITDs also limits the use of conventional NGS algorithms.[70] Specific NGS assays for monitoring of *FLT3*-ITD targeting the exon 14 have been designed and implemented.[64,70–72] As emphasized in a previous section, ELN recommends integration of an additional marker in MRD analysis while interpretating a negative *FLT3*-ITD MRD, owing to the unstable and possible subclonal nature of the *FLT3*-ITD mutations.[73,74]

Defining MRD Positivity by NGS

Presence of a mutation at VAF more than or equal to 0.1% provisionally defines NGS-MRD positivity. As a VAF lower than 0.1% might also be possibly associated with adverse outcome, it is recommended to be reported as MRD-LL (low level).[3]

Informatics Considerations

Bioinformatics involves computational methods to process sequencing raw data and derive results for analysis. Calculation of error rate, reducing false positive calls, and calculation of sensitivity are crucial elements in bioinformatics in context of AML MRD. Derivation of background error rate involves calculation of the largest VAF of nucleotides that flank the target (excluding primer sequence). Sensitivity for a sample is calculated by dividing the mean background error by number of read families (or reads) expressed as percentage.[3]

There are currently no uniform recommendations on the use of specific bioinformatics pipeline, and this area still requires harmonized efforts for standardization. It is important to note that tools for detection of indels are required to be different from the regular alignment tools for SNVs.

ELN recommends that for a sample to be evaluable when error-corrected sequencing approach is used, at least 10,000 read families and more than 10 mutant reads should be present. At least three reads should be present in each read family. For a sample to be evaluable for non-error-corrected sequencing approaches, the total reads and mutant reads recommended are more than or equal to 60,000 and more than 60, respectively. When background error correction is applied, MRD positivity is defined by a VAF greater than the sum of mean background error and 3x standard deviation of

the background error. There can be other methods to define MRD positivity.[3]

Targeted RNA Sequencing

As previously discussed, RT-qPCR forms the gold standard for molecular MRD detection of common fusion transcripts, while the follow-up of uncommon fusions relies on relative quantification. Targeted RNA sequencing using UMI correction forms a promising alternative for molecular MRD of fusion transcripts possible in a single assay for multiple fusion transcripts.[75] Dillon et al demonstrated a sensitivity of 1 in 100,000 for detection of molecular MRD in AML using UMI-based multiplexed RNA sequencing assay.[76] The utility of RNA sequencing has also been explored by Kim et al in cases of CBF-AML, where they demonstrated that the reduction in disease burden was comparable between RNA sequencing and RT-qPCR.[77] However, unlike DNA-based NGS MRD, the literature on RNA sequencing based MRD for follow-up of fusion transcripts is still sparse and requires further exploration.

Limitations of NGS

While high sensitivity, throughput, and wider applicability give NGS an edge over other techniques, lack of standardization across laboratories and establishment of clinically relevant cutoffs are some issues which need addressal. Other factors influencing interpretation of NGS MRD include possibility of finding certain gene mutations in healthy individuals, and the fact that some genetic abnormalities can persist at low levels while patient is clinically stable can pose interpretation problems while reporting.[45,78] Consequently, NGS as a standalone technique is not currently recommended by ELN.[3]

Clinical Practice Considerations

Definitions of Response

The recommended definitions of response and relapse based on MRD are summarized in ►**Table 3**.[3]

Timepoints for Testing MRD

Specific timepoints for MRD testing have been recommended by ELN for established AML entities including *NPM1* mutated AML, CBF AML, and APL.[3] Disease burden in PB at diagnosis can be used as a baseline comparator if blast percentage is more than or equal to 20%. If log reduction is being used as a method to calculate MRD response, both PB and BM samples are required to be processed at baseline. PB MRD testing after two cycles of induction is the recommended first follow-up timepoint for *NPM1* and CBF-AML, BM MRD testing at the end of treatment or PC as second timepoint, followed by a total of 2 years' follow-up (every 3 months if using BM or 4–6 weekly if using PB).

For APL, the PC timepoint directly forms the MRD timepoint. Following this, no further follow-up is recommended for low-risk APL if PC MRD is negative. A further follow-up of 2 years is recommended for high-risk APL similar to *NPM1* mutated and CBF AML.

Clinical Impact

Numerous studies have highlighted the value of MRD detection in AML in the prognostication of patients using intensive as well as less toxic chemotherapeutic regimes and at various treatment timepoints. MRD positivity has been shown to be associated with higher relapse rates and inferior survival outcomes.[79]

Table 3 Definitions of response based on AML MRD (ELN 2021 recommendations)[3]

Response category	Response criteria
CR with negative MRD (CR MRD-)	- Complete morphologic remission (CR) and - MRD negativity by all MRD technologies used a. BM MFC-MRD negative (if MFC-MRD was used). b. MRD negative by RT-qPCR in BM (or in PB after cycle 2 for NPM1 and CBF-MRD) (if qPCR-MRD used). c. MRD negative by NGS in BM (if NGS-MRD used).
CR with positive MRD (CR MRD+)	1. Morphological CR and 2. MRD positivity in PB and/or BM either by MFC or NGS-MRD or qPCR-MRD positive.
CR with molecular MRD detection at low level (CR-MRD-LL)	- Morphologic CR and - Presence of PB and/or BM MRD detectable by molecular methods at low level (qPCR for NPM1 $<2\%$ or NGS-MRD $<0.1\%$, but above the LOD of assay).
MRD relapse	1. Conversion of a negative MRD result to positive by any MRD method or 2. increase in MRD copy numbers ≥ 1 $\log_{10}$ between any 2 positive samples in the cases with CR-MRD-LL monitored by qPCR. 3. Above results should be repeated on a second sample and confirmed, BM preferably.

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; LOD, limit of detection; MFC, multicolor flowcytometry; MRD, measurable residual disease; NGS, next-generation sequencing; NPM1, nucleophosmin 1; PB, peripheral blood; PC, post-consolidation; RT-qPCR, real-time quantitative polymerase chain reaction.

A meta-analysis of 81 studies on AML MRD reported by Short et al emphasizes the significance of MRD as a prognostic and surrogate marker for 5-year disease-free survival and overall survival in AML.[80] Important literature concerning therapeutic implications of AML MRD has been recently reviewed by Aitken et al.[81]

The MRD status also guides in recategorization of a patient at PI timepoint, for example, a PI MRD positivity in a favorable risk patient would newly categorize the patient into intermediate risk, especially seen in *NPM1* mutated AML.[79]

It is crucial to note that relapse can occur in cases who are MRD negative. Conversely, a consistent low level MRD positivity can be associated with a stable clinical course, of note in *NPM1* and *RUNX1::RUNX1T1*.[79] The implication of MRD-LL, which would be technically reported as MRD negative, is currently unclear in view of insufficient clinical evidence.[3,79] Importantly, the literature pertaining to NGS MRD is widely diverse in context of technical aspects like methods of error correction, DNA inputs, genes included in panel and cutoffs used. Taking all these factors into account, the broad directions concerning clinical intervention in the form of preemptive therapy based on molecular MRD are difficult to be generalized and would require individualized decisions.

Future Directions

In addition to larger studies utilizing the discussed molecular methods for MRD detection, newer methods are also being widely studied and could find a place in clinical practice. The role of circulating cell free DNA by targeted NGS using PB samples has been explored by Short et al[82] and others.[83,84] Techniques like whole exome and whole genome sequencing can detect new alterations implicated in MRD negative relapse; however, lower coverage obtained in them currently hampers the implementation for deeper sequencing. More recently, single-cell mutational profiling has also found its way to the armamentarium of AML MRD techniques,[15] thus giving a glimpse of rapid upgrades in near future in this field.

Conflict of Interest
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