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Development of a Standardized, DNA-Based Next-Generation Sequencing Assay for Assessment of Measurable Residual Disease (MRD) in Acute Myeloid Leukemia (AML)

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Background

- *NPM1* mutations (*NPM1*m) are a well-characterized biomarker for measurable residual disease (MRD) detection in acute myeloid leukemia (AML).
- Our recent meta-analysis across multiple trials[1] suggest the level of MRD based on detection of *NPM1m* transcripts by reverse transcriptase-mediated quantitative polymerase chain reaction (RT-qPCR) in patients that achieve complete response (CR) after 2 cycles of intensive induction chemotherapy confers prognostic value.
- While *NPM1*m MRD detection based on RT-qPCR is considered the gold standard because of its high sensitivity, DNA-based next-generation sequencing (NGS) may complement RT-qPCR for assessment of residual disease, providing certain advantages.
- NGS allows for detection of all *NPM1*m variant alleles in a single assay (more closely approximating the variant allelic fraction [VAF] from leukemic cells vs quantitation of *NPM1*m transcripts). Further, longer stability of DNA during transit alleviates logistic challenges related to sample processing in different labs and transport worldwide to a central testing site in clinical trials.

Purpose

- To develop a standardized and highly sensitive DNA-based NGS assay for the detection of MRD in *NPM1*m AML and to assess its performance against an established RT-qPCR method.

Methods

- Assay performance was assessed by serially diluting *NPM1*m synthetic DNA or *NPM1*m genomic DNA (gDNA) extracted from OCI/AML3 (*NPM1*m) cells in a background of wildtype bone marrow (BM) gDNA (range: 0.008%–0.0005% VAF) using a sample input of 400,000 gene copies.
- DNA sequencing analysis based on the Inivata MRD platform (median read depth: 5.5×10^6) was performed on the Illumina NextSeq 550 instrument (Illumina, Inc.) using a proprietary bioinformatic pipeline.
- A set of AML samples consisting of peripheral blood (PB; N=31) and BM (N=55) from patients enrolled in the UK National Cancer Research Institute AML Study Group AML-19 trial (ISRCTN 2014-002195-90) were tested for comparison of MRD by NGS with an orthogonal *NPM1* RT-qPCR method.[2]
- To assess the level of agreement between both platforms, we used Pearson correlation statistics including 95% confidence intervals (CIs). Assay performance was evaluated by determining the area under the receiver operating characteristic curve (ROC_{AUC}).
- NGS results are expressed as percent variant allele frequency (%VAF) and RT-qPCR results as *NPM1*m/10^4 *ABL1* transcripts (normalized copy number [NCN], as reported by investigators).

Summary of Test Models Used to Assess Feasibility of *NPM1m* NGS MRD Assay

Model	Mutant DNA	Mutation targeted by PCR
1	HCC1395	Ins_chr8_12322022_GGGCTAC
2	NPM1_gBlock1	NPM1: c.860_863dup (type A)
3	OCI-AML3	NPM1: c.860_863dup (type A)

Table 1. A total of 3 test models were developed to establish the workflow and understand the assay performance characteristics. HCC1395 gDNA is derived from a breast carcinoma cell line. *NPM1*_gBlock1 is a synthetic DNA template covering approximately 500 base pairs of the human *NPM1* gene with a type A *NPM1*m. OCI-AML3 gDNA is derived from a cell line isolated from a patient with AML containing a type A *NPM1*m.

Input Study Demonstrates 100% Sensitivity Across a Range of 200,000–600,000 Total Genome Copies at 0.002% VAF (20 ppm)

(A)

Dilution	VAF (%)	VAF (ppm)	Mutant Molecules per 50,000 Diploid Genomes
d8	0.008	80	4
d9	0.004	40	2
d10	0.002	20	1
d11	0.001	10	0.5
d12	0.0005	5	0.25
d0	0	0	0

(B)

Dilution	VAF (ppm)	Sensitivity (%)			
		40,000 Copies	200,000 Copies	400,000 Copies	600,000 Copies
d8	80	80	100	100	100
d9	40	95	100	100	100
d10	20	0	100	100	100
d11	10	0	95	100	95
d12	5	0	95	30	70
d0	0	0	0	0	0

Table 2. An input study was conducted in order to evaluate the sensitivity of the assay when using a range of total genomic input copies at different dilutions of target DNA. **(A)** Summary of DNA serial dilutions used to generate contrived MRD samples with varying percentages of VAF. The conversion of theoretical VAFs to ppm is shown as well as the equivalent theoretical number of mutant copies per 50,000 diploid genomes. **(B)** Summary of results observed in the feasibility study. The table shows the assay sensitivity results (% sensitivity) at different total DNA inputs using model 1 (Table 1) diluted in a background of human gDNA, a commercially available DNA prepared from the blood of normal male donors (Promega). The experimental sensitivity of the assay was determined to be 100% in the range of 200,000–600,000 input copies per sample. The performance of the assay at LoD_{95} of 20 ppm, using an input of 400,000 copies, was robust (100% sensitivity) with a margin of ± 50% change in DNA input. Copies indicate DNA input per sample. Sensitivity was assessed by bootstrapping (n=20); calling was performed on groups of 4 randomly selected barcodes from the same dilution.

d=dilution; LoD_{95}=limit of detection (concentration detected in at least 95% of replicates); ppm=parts per million.

Feasibility Study Demonstrates LoD_{95} ≤0.002% VAF (20 ppm)

Dilution	VAF (ppm)	Sensitivity (%)					
		Model 1 HCC1295		Model 2 NPM1_gBlock		Model 3 OCI-AML3	
		Promega	BM	Promega	BM	Promega	BM
d8	80	100	100	100	100	100	100
d9	40	100	100	100	100	100	100
d10	20	100	100	100	100	100	100
d11	10	100	75	85	94	55	85
d12	5	45	85	35	78	0	0
d0	0	0	0	0	0	0	0

Table 3. A feasibility study was performed to assess the sensitivity of the assay in 3 different models using contrived samples. Each model utilized target DNA serially diluted into normal background DNA as described in Table 2. Sensitivity was assessed by bootstrapping (n=20); calling was performed on groups of 4 randomly selected barcodes from the same dilution. Two MRD replicates per dilution point were used to test the LoD of the assay using a fixed input of 400,000 copies per sample.

Pre-validation LoD Study Indicates LoD_{95} <0.002% VAF (20 ppm)

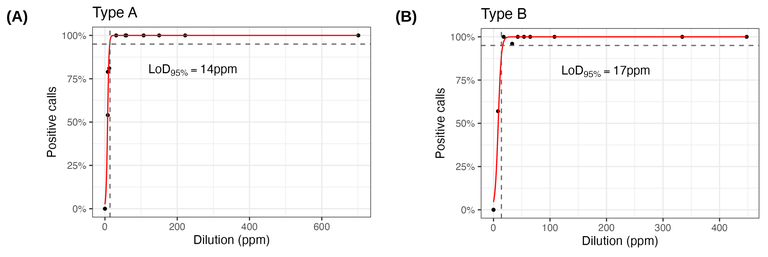

Figure 1. Pre-validation LoD study demonstrating the sensitivity of the *NPM1*m NGS MRD assay. **(A)** *NPM1*m type A or **(B)** type B mutation-positive DNA isolated from AML patient BM aspirates were spiked into mutation-negative buffy coat gDNA at different target %VAFs, ranging from 0.06% (600 ppm)–0.0004% (4 ppm). Each sample was tested at 400,000 total genome input copies.

*NPM1*m MRD Detection by NGS Shows Strong Correlation to RT-qPCR

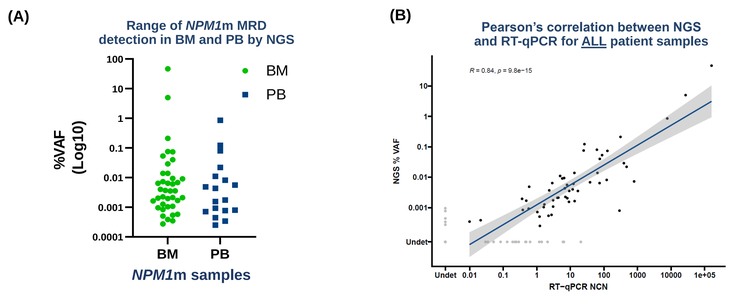

Figure 2. (A) In the AML-19 study patient set, the level of MRD detected by NGS varied between 0.00025%–46% VAF (median: 0.0042%) and by RT-qPCR between 0.01–1.65×10^5 NCN (median: 8.03), **(B)** Pearson correlation showing a strong linear relationship between NGS and RT-qPCR when considering all (ie, BM and PB) *NPM1*m-positive samples (R=0.84, p=$9.8e^{-15}$).

NCN=normalized copy number; Undet=undetected.

Table 4. Contingency table comparing ALL NGS vs RT-qPCR *NPM1*m allele calls

	NGS		
	Detectable (N=57)	Undetectable (N=29)	Overall (N=86)
RT-qPCR			
Detectable	52 (91.2%)	18 (62.1%)	70 (81.4%)
Undetectable	5 (8.8%)	11 (37.9%)	16 (18.6%)

Table 5. *NPM1*m variants detected by NGS vs RT-qPCR

	NGS Variant Calls				
	Type A (N=41)	Type B (N=3)	Type D (N=7)	Other (N=1)	Overall (N=52)
RT-qPCR					
Type A	41 (100%)	0 (0%)	0 (0%)	1 (100%)	42 (80.8%)
Type B	0 (0%)	3 (100%)	0 (0%)	0 (0%)	3 (5.8%)
Type D	0 (0%)	0 (0%)	7 (100%)	0 (0%)	7 (13.5%)
Other	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Table 4. *NPM1*m was detectable in 52 of 70 (74.3%) samples by both platforms. In 18 of 70 (25.7%) samples, *NPM1*m was not detected by NGS but detected by RT-qPCR, with NCN between 0.03 and 20.5 (median: 0.37), confirming the expected higher sensitivity of RT-qPCR given the measurement of RNA transcripts as opposed to mutant DNA alleles by NGS. Conversely, *NPM1*m was detected in 5 of 16 samples by NGS but not detected by RT-qPCR (VAFs were below technical LoD_{95} in all cases). **Table 5.** NGS assay demonstrates 98% agreement on variant allele type compared to RT-qPCR. Of 52 samples, 41 were called type A, 3 of 3 type B, and 7 of 7 type D *NPM1*m variants in both assays. Data are in n (%).

Receiver Operating Characteristic (ROC) Analysis Shows Optimal NGS Threshold of 0.003% VAF When Applying a Cutoff of >10 NCN by RT-qPCR

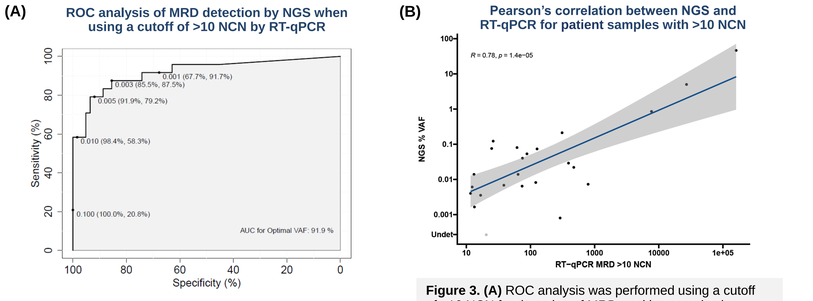

Figure 3. (A) ROC analysis was performed using a cutoff of >10 NCN for detection of MRD-positive samples by RT-qPCR, demonstrating good concordance (AUC=91.9%) and identifying an optimal NGS cutoff of 0.003% VAF (specificity= 85.5%, sensitivity=87.5%). **(B)** The NGS assay could detect MRD positivity in almost all samples with an NCN >10 copies (23 of 24 samples, 95.8%) with a statistically significant Pearson correlation (R=0.78, p=$1.4e^{-05}$).

Table 6. Contingency table comparing NGS vs RT-qPCR *NPM1*m allele calls using NGS cut-off of (0.003% VAF)

	NGS 0.003% VAF		
	Positive (N=30)	Negative (N=56)	Overall (N=86)
RT-qPCR >10 NCN			
Positive	21 (70.0%)	3 (5.4%)	24 (27.9%)
Negative	9 (30.0%)	53 (94.6%)	62 (72.1%)

Table 6. Contingency table using the optimal NGS cutoff (0.003% VAF) determined by the ROC analysis. When applying this cutoff, NGS MRD was called positive in 21 of 24 (87.5%) and negative in 53 of 62 (85.5%) samples.

*NPM1*m AML Patients With MRD-Negative CR in BM Have Better EFS/OS Than Patients With MRD-Positive CR

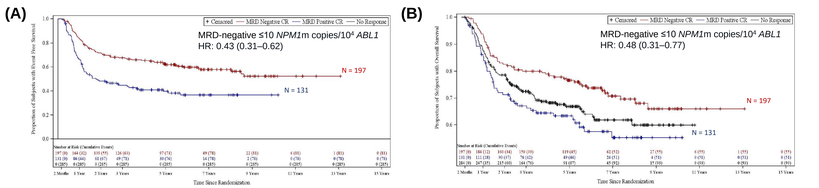

Figure 4. Data were extrapolated from a retrospective analysis of 3 cooperative group-studies confirming a survival advantage for *NPM1*m patients achieving MRD-negative CR in BM after intensive induction.[1] Kaplan-Meier estimates of **(A)** event-free survival (EFS) and **(B)** overall survival (OS) for patients who achieved or remained in morphologic CR and underwent molecular MRD assessment by RT-qPCR within 42 days of the start of chemotherapy cycle 2. Patients are classified as MRD positive if 10 *NPM1*m copies per 10^4 ABL1 (ie, >10 NCN) were measured.

No response=No CR (CR with incomplete count recovery, CR with partial hematologic recovery, partial response, morphologic leukemia-free state, other).

Conclusions

- Our newly developed *NPM1*m NGS-MRD assay demonstrates high sensitivity and specificity with a technical LoD_{95} of at least 0.002% VAF (ie, 1 *NPM1*m allele in 50,000 wildtype copies or 2.0×10^{-5}). This is consistent with the 2021 European LeukemiaNet[3] recommendation that MRD assessment using molecular techniques achieve LoD ≤10^{-3}.
- *NPM1*m MRD measured by NGS showed a high degree of correlation with an orthogonal RT-qPCR MRD assay, measuring *NPM1*m transcripts. The assay can robustly and reliably identify MRD-positive samples above a RT-qPCR threshold of 10 NCN (*NPM1*m/10^4 *ABL1* transcripts).
- While an *NPM1*m MRD threshold with definitive prognostic value after 2 cycles of intensive chemotherapy has yet to be defined, a sensitivity analysis demonstrated that a threshold of 10 NCN (*NPM1*m/10^4 *ABL1* transcripts) in BM by RT-qPCR can provide valuable prognostic information for patients with *NPM1*m AML **(Abstract 2799)**.[1]
- These data suggest *NPM1*m MRD assessment by NGS may complement RT-qPCR with high degree of sensitivity and specificity in order to qualify MRD-negative CR as a surrogate endpoint reasonably likely to predict clinical benefit.

References

1. Döhner K, et al. Presented at: ASH Annual Meeting; December 10-13, 2022; New Orleans, LA. Abstract 2799.
2. Ivey A, et al. *N Engl J Med*. 2016;374(5):422-433.
3. Heuser M, et al. *Blood*. 2021;138(26):2753-2767.

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