Performance Characteristics of an NPM1 p.W288fs RT-qPCR Assay for MRD Detection in AML.

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Introduction

Approximately 35% of all acute myeloid leukemia cases harbor pathogenic frameshift variants (p.W288fs) within the terminal exon of the nucleophosmin 1 (NPM1) gene. At multiple milestones, NPM1 measurable residual disease (MRD) positivity is associated with increased risk of relapse and shorter overall survival. NPM1 MRD monitoring via sensitive RT-qPCR provides helpful information to guide therapeutic decisions. Here we present our laboratory's validation experience and performance characteristics of a cost effective, reverse transcription quantitative PCR (RT-qPCR) assay. This method is designed to support clinical diagnostic laboratories and hematology services managing AML patients.

Methods

RNAs extracted from blood and bone marrow were analyzed using the NPM1 One-Step MRD assay (EntroGen) on the ABI 7500 fast (Applied Biosystems) qPCR platform. The three most common NPM1 variants (types A, B, and D) are quantitatively detected. The quantitative reporting units are normalized percentage of variant NPM1 transcripts relative to total NPM1 transcripts (%mutNPM1/totNPM1) calculated according to the delta-delta Ct method relative to a standardized single-point calibrator. Analytical performance was evaluated by determining the limit of blank (LOB), lower limit of detection (LLOD), and limits of quantitation (LLOQ, ULOQ). The 95% LLOD was determined via serial dilution of patient samples ranging from 10^{1} to 10^{-6}. A quantitative cutoff defined at LOB was applied to the study cohort to evaluate accuracy, sensitivity, and specificity.

The validation dataset is comprised of 23 unique true negative samples, 31 unique true positive samples (replicate measurements and dilution series not counted), and plasmid calibrators prepared at three levels (Anchor Molecular). True positive patients were previously identified to harbor NPM1 p.W288fs (CCDS4376.1) variants located in exon 11 by concurrent molecular testing. In total, including replicate measures and controls, RT-qPCR data was collected for 200 reactions over 10 independent, 96-well plates on an ABI 7500 Real Time qPCR instrument.

Mutant NPM1 Type A, Type B, and Type D are amplified via three separate reaction mixes: RxnA, RxnB, RxnD respectively. Each reaction mix contains multiplexed primers to amplify mutant NPM1 (FAM) and total NPM1 (VIC) transcripts (wild-type plus mutant). PCR amplification efficiency was assessed for all amplicons using plasmid calibrators (Anchor Molecular) and for mutant NPM1 in patient samples. Amplification efficiency ranged from 92% -110% and was considered acceptable.

The following NPM1 variants were assessed in this validation:
Type A p.W288Cfs*12 (chr5-170837548--TCTG)
Type B p.W288Cfs*12 (chr5-170837548--CATG)
Type D p.W288Cfs*12 (chr5-170837548--CCTG)
non-ABD p.W288Cfs*12 (chr5-170837548--CTTG)
non-ABD p.W288Vfs*12 (chr5-170837545--TGTA)
non-ABD p.W288Cfs*12 (chr5-170837548--CAGA)
non-ABD p.W288Cfs*12 (chr5-170837548--TAAG)
non-ABD p.W288Cfs*12 (chr5-170837548--TCGA)

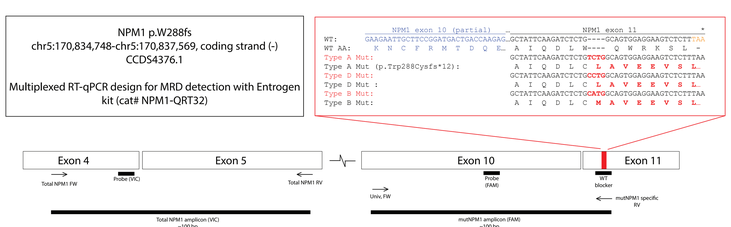

Figure 1. Assay design.

Performance characteristics

		Type A			Type B			Type D		
	Overall reaction counts	No amplification	Not tested	Positive	No amplification	Not tested	Positive	No amplification	Not tested	Positive
NTC	9	8	1	0	5	4	0	5	4	0
Negative control RNA	9	8	1	0	5	4	0	5	4	0
True negative RNA	29	28	1	0	27	2	0	27	2	0

Figure 2. The limit of blank defines the highest signal expected when no analyte is present, and therefore distinguishes true analytical signal from background noise. The LOB was assessed for each reaction mix (RxnA, RxnB, RxnD) independently in 29 true negative samples derived from normal blood. Amplification of mutant NPM1 was not observed in any true negative reactions. The LOB for this assay is undetectable mutant NPM1 transcripts with a FAM Ct <40 cycles threshold applied. Samples with a FAM Ct value greater than 40 are considered below the background of this assay.

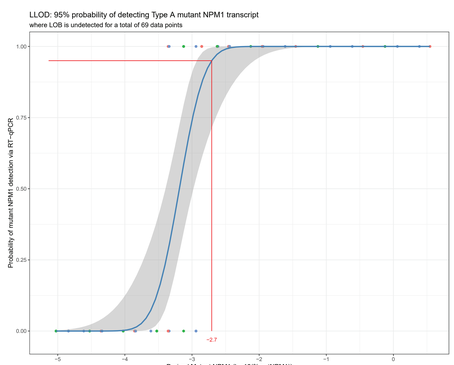

Figure 3. The lower limit of detection (LLOD) is the lowest concentration of analyte that can be detected with acceptable repeatability. Herein, the lower limit of detection (LLOD) was established at 95% statistical probability of detection via a binomial regression fitted to NPM1 type A mutant dilution data. Serial dilutions were prepared from 3 independent patient samples determined to be true positive NPM1 mutant via NGS analyses. Low level dilutions were measured in triplicate for a total of 69 data points. Samples were coded "positive" if mutant NPM1 (FAM) was measured above the LOB (<40Ct) for Rxn A. Binomial regression was fit to log10 transformed data and the 95% probability of detecting Type A mutant NPM1 transcript above the LOB was derived from the fitted curve (95% confidence interval shaded in gray). The 95% LLOD for NPM1 Type A corresponds to -2.71 (log10) equating to 0.002% mutant NPM1 transcripts (ie 10^-2.71).

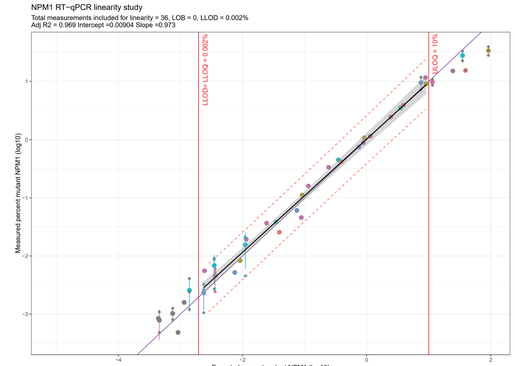

Figure 4. Linearity is the assays ability to provide results that are directly proportional to the concentration of the measurand in the test sample. The linear range is bounded by the upper and low limits of quantitation (ULOQ, LLOQ). Serial dilutions were prepared from 5 independent patient samples determined to harbor NPM1 mutant transcripts (3 Type A pts, 1 each Type B and Type D patients) via NGS analyses. Across all dilution levels, the 95% confidence interval (gray shading) for the fitted linear regression (black line) overlaps the expected line for a perfect correlation (purple line denoting where x=y) with a slope near 1 and adjusted R2 of 0.969. Indicating these data accurately model the expected quantitative value and are sufficiently linear over the diluted range. Thus, for this assay the linear range is 0.002% - 10% mutant NPM1 transcripts, where LLOQ= LLOD = 0.002% and ULOQ = 10% mutant NPM1 transcripts.

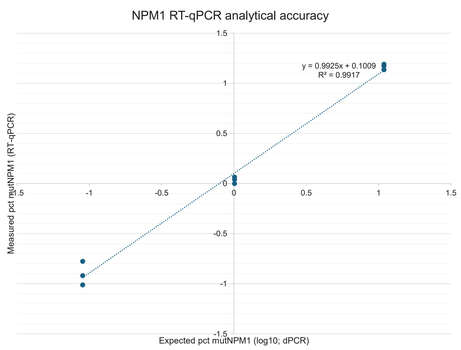

Figure 5. Analytical accuracy is validated by comparing measured results to a definitive or reference method, or an established comparative method. Contrived plasmid calibrators (purchased from Anchor Molecular as a custom product) were prepared at 10%, 1%, and 0.1% mutant NPM1 molecules relative to "total" NPM1 molecules. Absolute quantity was established via digital PCR measurement of the plasmid calibrators provided by the manufacturer with a certificate of analysis. Calibrators were measured by this RT-qPCR protocol in triplicate.

Analytical precision was validated by repeat measurement of samples at varying concentrations or activities within-run and between-runs (n=42 replicate measures). Precision was relatively consistent across the reportable range of this assay, averaging at 46.6% (range:0.06%-116%) coefficient of variation.

		Measured percent mutant NPM1 by RT-qPCR					
	Expected %mutNPM1	Replicate 1	Replicate 2	Replicate 3	Average measured value	Standard deviation	%CV
0.1% calibrator	**0.1 +/- 0.02**	0.10	0.12	0.17	0.13	0.04	27.8
1% calibrator	**1.0 +/- 0.2**	1.0	1.2	1.1	1.1	0.1	7.5
10% calibrator	**10 +/- 2**	13.7	14.8	15.5	14.7	0.9	6.3

NPM1 type	Specimen Type	Rxn A FAM Ct	Rxn B FAM Ct	Rxn D FAM Ct	Total NPM1 Ct (VIC)	QC Check	Percent mutant NPM1	Interpretation
Type A	Blood	23.173	36.4181	27.3564	20.654	QC_pass	30.8	above ULOQ
Type B	Bone Marrow Aspirate	30.3887	22.0378	24.4749	19.9614	QC_pass	33.8	above ULOQ
Type D	Bone Marrow Aspirate	25.2171	26.3756	24.829	21.1617	QC_pass	19.9	above ULOQ
Type D	Bone Marrow Aspirate			24.7752	20.4997	QC_pass	15.4	above ULOQ
Type A	Bone Marrow Aspirate	25.5607	37.659	30.4106	21.5899	QC_pass	8.6	8.57
Type D	Blood	24.8279	26.1178	24.1454	20.2927	QC_pass	20.9	above ULOQ
Type A	Bone Marrow Aspirate	22.5863	33.529	26.8206	19.6831	QC_pass	18.0	above ULOQ
Type A	Bone Marrow Aspirate	30.7419	Undetermined	37.9603	21.1913	QC_pass	0.2	0.18
Type A	Bone Marrow Aspirate	28.5253	Undetermined	33.5956	21.1741	QC_pass	0.8	0.82
Type B	Blood	36.2014	24.0406	27.4124	19.7176	QC_pass	9.5	9.51
Type A	Bone Marrow Aspirate	35.1174			21.4158	QC_pass	0.0	0.01
Type A	Bone Marrow Aspirate	30.483			20.5179	QC_pass	0.2	0.19
Type A	Bone Marrow Aspirate	34.9278			21.9088	QC_pass	0.0	0.02
Type A	Blood	25.1227			21.3925	QC_pass	14.6	above ULOQ
Type A	Bone Marrow Aspirate	23.7363	36.6189	31.9902	20.9061	QC_pass	33.0	above ULOQ
Type B	Bone Marrow Aspirate	34.5852	27.4177	33.5028	22.1115	QC_pass	5.1	5.09
Type A	Bone Marrow Aspirate	25.948	38.2397	34.4004	21.6283	QC_pass	11.8	above ULOQ
Type B	Bone Marrow Aspirate	Undetermined	33.3972	Undetermined	22.0833	QC_pass	0.1	0.08
Type D	Bone Marrow Aspirate	29.7148	33.0973	33.0379	23.0864	QC_pass	0.2	0.24
Type A	Bone Marrow Aspirate	20.3918	35.5133	29.6023	17.0756	QC_pass	23.6	above ULOQ
Type B	Bone Marrow Aspirate	32.8602	24.551	29.9252	20.5673	QC_pass	12.7	above ULOQ

Figure 6. Representative quantitative results in patient samples.

NPM1 RT-qPCR	"Truth" samples	Positive	Negative
	Positive	21	0
	Negative	0	21

Sensitivity & PPA	PPV	Specificity	Accuracy
100%	100%	100%	100%

Figure 7. Sample-level sensitivity refers to the ability of the assay to correctly identify true positive results. Specificity refers to the ability of the assay to correctly identify true negative. Accuracy is the ratio of correct predictions over the total number of predictions made. After applying quality control measures, 42 unique samples (21 true negative samples, 12 Type A, 5 Type B, 4 Type D) were submitted to the sample level performance metric (Sensitivity, Specificity, Accuracy) calculations.

Results

The assay demonstrated a limit of blank (LOB) of undetectable amplification earlier than 40 PCR cycles in all true negative samples. The lower limit of detection (LLOD) was 0.002%, and the limits of quantitation ranged from 0.002% (LLOQ) to 10% (ULOQ). Linearity was observed across the dilution range from the LLOQ to ULOQ (R^2 = 0.97). Precision studies showed relatively consistent between run reproducibility across the linear range, with an average of 46.6% (range:0.06%-116%). Using a qualitative cutoff corresponding to the LOB, the assay achieved 100% accuracy, 100% sensitivity and 100% specificity, with overall concordance of 100 % compared to reference methods.

Conclusion

The NPM1 RT-qPCR assay demonstrated robust analytical performance, including high sensitivity, reproducibility, and accuracy across clinically relevant mutation types. The assay reliably detects measurable residual disease at levels consistent with a 5-log reduction, supporting its use in longitudinal AML monitoring. Its cost effective and rapid workflow makes it suitable for implementation in clinical diagnostic laboratories.