Evaluation of Signature® NPM1 Mutations (RUO)* for the Rapid Multiplex Detection of NPM1 Exon12 Mutations

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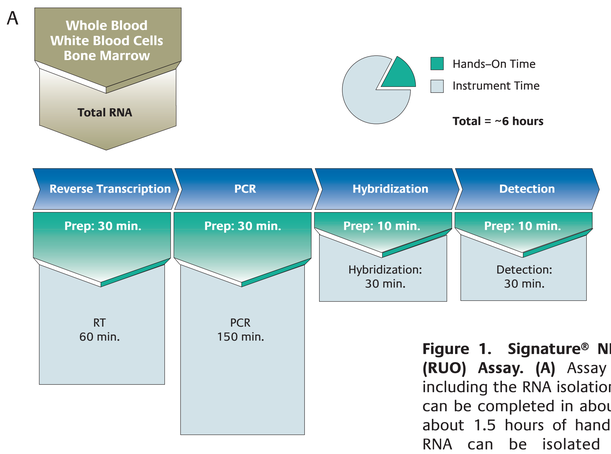
SUMMARY POINTS

- The Signature® NPM1 Mutations (RUO)* assay has a streamlined workflow allowing simultaneous detection of wild-type and mutants A, B, D and J NPM1 transcripts on a liquid bead array using total RNA specimens
- Analytical sensitivity reached 0.01% in this study by dilution of mutant-positive cell line RNA in HL-60 RNA or the equivalent of 1,000 to 2,000 copies of mutant transcripts*
- The assay is compatible with other laboratory-developed assays using Signature reagents for the simultaneous amplification and detection of NPM1 mutations and common AML-specific fusion transcripts
- Sensitivity and specificity were 100% in this study of 63 specimens with hematologic malignancies using capillary electrophoresis size fractionation as the reference method and sequencing to confirm positive specimens*.

INTRODUCTION

Nucleophosmin (NPM1) gene mutations represent the most common genetic alteration in adult acute myeloid leukemia (AML), accounting for about one third of all cases and 50-60% of AML with normal karyotype (AML NK). The vast majority of cases harbour type A (70-80%), B (~10%) or D (~5%) mutations in NPM1 exon 12. As AML patients carrying NPM1 mutations show distinctive biological, clinical and prognostic features, molecular screening and monitoring of NPM1 mutations may be beneficial to improved risk-stratified treatment approaches. However, because the heterozygous mutations result in 4 nucleotides repeats or insertions (Figure 1B) in a background of wild type sequences, sensitive and specific detection of NPM1 mutations by polymerase chain reaction (PCR) methods can be challenging.

Signature® NPM1 Mutations is a research-use-only kit for the rapid detection of common NPM1 mutations using total RNA purified from cultured cells, bone marrow or peripheral blood. The assay utilizes multiplex reverse transcription-PCR (RT-PCR) in combination with fluorescent bead-based detection to simultaneously identify transcripts for NPM1 mutations and wild type targets (Figure 1A). The objective of this study was to evaluate the performance of the Signature® NPM1 assay.



B

Wild type	TCTG . . . . GCAG	
Mut A	TCTGTCTGGCAG	80.3%
Mut B	TCTGCATGGCAG	9.2%
Mut D	TCTGCCTGGCAG	3.1%
Mut J (DD6)	TCTGTATGGCAG	~0.4%

Figure 1. Signature® NPM1 Mutations (RUO) Assay. (A) Assay workflow. Not including the RNA isolation step, the assay can be completed in about six hours with about 1.5 hours of hands-on time. Total RNA can be isolated with standard commercially available methods. The kit includes Negative and Positive Controls for NPM1 mutations. **(B)** NPM1 exon 12 transcript sequences detected by the Signature assay. Percent of mutations are from Thiede et al., Blood, 2006. Mutation J is also called DD6 in the literature.

MATERIALS AND METHODS

Peripheral blood or bone marrow from 63 patients with AML, ALL, CML, or MDS were collected as part of standard clinical care and following a protocol approved by the Johns Hopkins Medical Institutions IRB. Total RNA was isolated from peripheral blood or bone marrow using the QIAamp RNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. The Signature® NPM1 Mutations kit was evaluated using Signature® General Purpose Reagents following the NPM1 Package Insert. Briefly, total RNA (5µL) was reverse transcribed into cDNA and amplified by PCR using biotin-modified primers (45 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec). NPM1 wild type transcripts were co-detected in the same reaction to serve as endogenous internal controls. The PCR products were then sorted on the liquid bead array containing oligonucleotide probes specific for NPM1 and detected using the Luminex® 200™ system. The Mean Fluorescence Intensity (MFI) detected on at least 50 beads for each target was reported. For co-amplification and co-detection of NPM1 mutations and AML fusion transcripts, 1 µL of Signature® AML/ETO, inv(16)-ADE Primers (ASRs) and 5 µL of Signature® AML/ETO, inv(16)-ADE Probes (ASRs) were added to the NPM1 Amplification master mix and Hybridization mix, respectively.

RESULTS

Analytical Performance

A

	NPM1-WT	NPM1-Mut
NTC	53	58
HL-60 400ng	10126	206
Mut-A 20M	9258	10423
Mut-A 2M	9635	9669
Mut-A 200K	11776	7924
Mut-A 20K	10966	4592
Mut-A 2K	10611	1416

B

	NPM1-WT	NPM1-Mut
NTC	60	96
HL-60 1000ng	12905	126
Mut-A 100%	11912	11358
Mut-A 10%	11650	11134
Mut-A 1%	12699	8470
Mut-A 0.1%	13087	4427
Mut-A 0.01%	13531	1082

C

	NPM1-WT	NPM1-Mut
NTC	94	11
HL-60 1000ng	12427	126
Mut-A 1000ng	11058	10304
Mut-A 100ng	10592	10920
Mut-A 10ng	9025	10088
Mut-A 1ng	4756	8762
Mut-A 0.1ng	668	6288

D

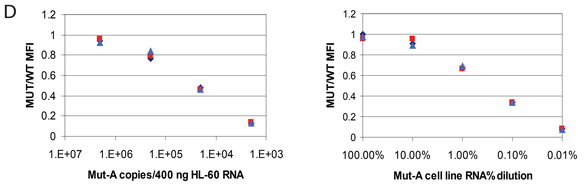


Figure 2. Analytical Performance.
(A) Sensitivity assessed by dilution of mutant A transcripts prepared by in vitro transcription (IVT) and diluted in 400 ng of HL-60 total RNA. The assay also detects mutations B, D and J but does not distinguish between them. A similar sensitivity (1-2,000 copies) was obtained with these IVT transcripts (data not shown). **(B)** Sensitivity assessed by dilution of total RNA purified from a cell line expressing mutation A in a background of HL-60 RNA and keeping the total amount of RNA constant (1,000 ng). **(C)** RNA input range assessed using total RNA purified from a cell line expressing mutation A. **(D)** Semi-quantitative analysis. Mutant A IVT transcripts (left) or mutant A cell line RNA (right) were diluted in a constant background of HL-60 total RNA and tested in triplicate. The resulting mutant to wild type NPM1 MFI ratios were plotted against the RNA inputs. Up to 4 Log decrease could be reproducibly measured with a linear response below ~10% dilution or ~0.5 millions copies of mutant A transcript.

A

	Signature	CE
HL60 400ng	Pos	Pos
NTC	Neg	Neg
Mut-A 100%	Pos	Pos
Mut-A 10%	Pos	Pos
Mut-A 5%	Pos	Pos
Mut-A 1%	Pos	Pos
Mut-A 0.1%	Pos	Neg
Mut-A 0.01%	Pos	Neg
Mut-A 20M	Pos	Pos
Mut-A 2M	Pos	Pos
Mut-A 200K	Pos	Pos
Mut-A 20K	Pos	Neg
Mut-A 2K	Pos	Neg

B

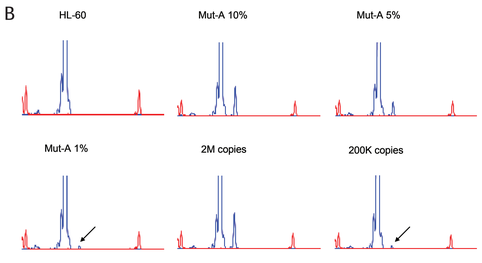


Figure 3. Comparison with size-based assay.
(A) Assay sensitivity was compared with a prototype capillary electrophoresis (CE) assay using total RNA purified from a cell line expressing mutant A or mutant A transcripts prepared by in vitro transcription (IVT) and diluted in a constant background of HL-60 total RNA (400 ng). The same RT products were used for both assays. For CE, the PCR consisted of 35 cycles followed by 10 min incubation at 72°C. PCR products were diluted 1:50 and analyzed on an ABI 3130x Genetic Analyzer. Similar sensitivity was obtained with mutant B and D transcripts (data not shown). **(B)** Representative examples of CE traces for the indicated samples. CE sensitivity reached at best 1% or about 200,000 copies of NPM1 mutant transcript.

Co-Detection of AML Fusion Transcripts

	Signature NPM1		Signature NPM1 + Signature AML/ETO, inv(16) ADE ASRs					
	NPM1-WT	NPM1-Mut	NPM1-WT	NPM1-Mut	AML1/ETO	inv16-A	inv16-D	inv16-E
NTC	100	53	68	85	43	81	57	49
HL60 400ng	11506	167	8117	94	10	70	44	45
NPM1 Mut-A 100%	10851	11636	7584	9512	52	68	25	41
NPM1 Mut-A 10%	11009	10481	8458	8734	52	69	64	129
NPM1 Mut-A 1%	11693	7149	8836	5660	61	90	46	91
NPM1 Mut-A 0.1%	11459	2444	8776	1734	96	80	54	57
AML1/ETO 100%	12466	201	3998	92	8446	68	64	40
AML1/ETO 10%	11679	180	5628	74	8349	94	61	66
AML1/ETO 1%	12487	134	7302	40	7917	57	70	65
AML1/ETO 0.1%	11927	154	7555	67	6763	73	44	96
CBFβ/MYH11-A 100%	11775	199	6441	77	41	6290	105	55
CBFβ/MYH11-A 10%	11306	125	8005	94	51	6233	59	68
CBFβ/MYH11-A 1%	11867	163	8306	90	55	5409	69	42
CBFβ/MYH11-A 0.1%	12031	168	8651	91	33	1517	61	52

Figure 4. Simultaneous amplification and detection of NPM1 mutations and AML-specific fusion transcripts.
Dilutions of total RNA purified from cell lines expressing either the NPM1 mutation A, AML1/ETO or CBFß/MYH11 transcripts and diluted in HL-60 total RNA were tested with the Signature® NPM1 Mutations (RUO) reagents alone or in combination with Signature® AML/ETO, inv(16)-ADE Primers (ASRs) and Probes (ASRs). NPM1 wild type transcripts were used as an internal endogenous control in both cases. No cross reactivity between the different targets was observed and sensitivity reached at least 0.1%.

Specimens

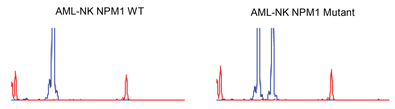
Specimens		Signature NPM1 RUO		
Classification	RNA (ng/uL)	# cases	Pos (Mutant)	Neg (WT)
AML normal karyotytpe	40-732	14	7	7
AML t(8;21) or inv(16)	92-250	6	0	6
Other AML	50-789	22	1	21
Non-AML	49-825	21	0	21
Total	40-825	63	8	55

Figure 5. Residual specimens used in this study.
Total RNA from 63 specimens was analyzed with the Signature NPM1 assay, with or without simultaneous detection of AML-specific fusion transcripts. There was no significant difference whether assays were combined or not (data not shown). The specimens consisted of 14 AML-NK, 4 AML t(8;21), 2 AML in(16), 22 other AML (20 cases with various chromosomal abnormalities other than t(8;21) or inv(16) and 2 acute bilineage leukemia cases), and 21 non AML (9 CML, 9 ALL and 3 MDS cases). Fifty percent (7/14) of AML NK and one AML with a del(7)(q22) were found positive for NPM1 mutations and were all confirmed by CE and sequencing analyses.

		NPM1-WT	NPM1-Mut	AML1/ETO	Inv16-A	Inv16-D	Inv16-E
NPM1 Positive Control		12567	11284	67	39	53	18
NPM1 Negative Control		10415	107	33	35	79	59
No Template Control		36	50	50	28	29	63
AML t(8;21)	462 ng input	3269	60	8210	14	98.5	58
AML t(8;21)	695 ng input	2959	82	8363	72	91.5	50
AML-NK	2000 ng input	9800	64	16	47.5	84.5	51
AML-NK	1500 ng input	12285.5	11416	34	33	9	46
AML-NK	1042 ng input	10683.5	10175	34	56.5	50	19
AML-NK	645 ng input	13094	11095	56.5	29	48	40.5
AML-NK	685 ng input	7962.5	33	45	81	87	68
AML del(7)	268 ng input	11856	11240	29	50	64.5	86
AML t(7;11)	2330 ng input	8367	69	36	89	67.5	61

Figure 6. Representative Signature results with clinical specimens.
Total RNA samples (5 µL) were tested for simultaneous detection of NPM1 mutations and AML-specific fusion transcripts. The cut-off for positivity was 400 MFI and was determined by a limit of detection analysis (data not shown).

A



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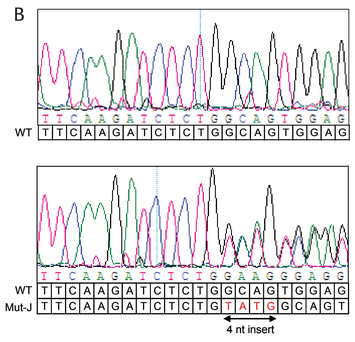

C

Signature NPM1	# cases	CE
Neg (WT)	55	100% (55 WT)
Pos (Mutant)	8	100% (8 mutants)

Signature NPM1	# cases	Sequencing
Neg (WT)	5	100% (5 WT)
Pos (Mutant)	8	100% (5A, 2J, 1D)

Figure 7. Capillary electrophoresis and sequencing analyses.
(A) Representative CE results for NPM1 wild type (left) and mutant positive (right) RNA specimens. The 63 total RNA samples were reverse transcribed, amplified and analyzed as described in Figure 3 legend. **(B)** Representative sequencing results for NPM1 wild type (top) and mutant J (bottom) RNA specimens. Thirteen (13) total RNA samples (5 NPM1 negative and 8 NPM1 positive) were reverse transcribed and amplified with a pair of NPM1-specific primers using standard RT-PCR conditions. Following treatment with exonuclease and alkaline phosphatase, PCR products were sequenced by the BigDye® terminator method. **(C)** Method agreement and data summary. There was 100% agreement between the Signature NPM1 assay and both the CE and sequencing methods. Among the 8 NPM1 positive specimens, 5 mutations A, 2 mutations J and 1 mutation D were identified.

CONCLUSION

The Signature® NPM1 Mutations (RUO)* assay is highly compatible with the molecular laboratory workflow. It presents several advantages such as rapid time to result, 96-well plate format, broad range of RNA input, inclusion of positive and negative controls, and single-well multiplexing compatibility with other Signature reagents for simultaneous detection of leukemia-specific fusion transcripts. Evaluation with 63 newly diagnosed leukemia specimens resulted in 100% sensitivity and specificity in this study. The assay analytical sensitivity (0.01%) and dynamic range (4 Logs) warrant further evaluation to determine its potential clinical utility for monitoring residual disease during treatment of AML patients.

*For Research Use Only. Not for use in diagnostic procedures. The performance characteristics of this product have not been established.