Development of a Synthetic RNA Control Panel for Monitoring Detection of 'Type A' NPM1 Mutant Transcripts

Matthew Held, Michael Escott, Emma Farrell, Joan Gordon, Loren Krott, Dan Magoon, Abbey Olsen, Bretna Parker, Arie Rietdyk, John Ross, and Tania Spenlinhauer

Maine Molecular Quality Controls Inc., Saco, Maine, USA

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Introduction

Nucleophosmin-1 (NPM1) is a versatile chaperone protein with chief roles in nucleocytoplasmic protein shuttling, ribosome biogenesis, and cell survival[1]. Tetranucleotide insertions in *NPM1*'s endmost exon 12 occur in one-third of adult acute myeloid leukemia cases[1]. The 'Type A' *NPM1* (*NPM1mutA*) insertion is a TCTG tandem duplication (c.860_863dupTCTG) and represents approximately 80% of all *NPM1* mutations[1,2]. The result is a frameshift leading to aberrant accumulation of NPM1 in the cytoplasm and instability of tumor-suppressors p53 and ARF[3].

Notably, NPM1 mutant leukemias are recognized as a distinct class by the World Health Organization, representing nearly 30% of adult AML[2]. Recent studies suggest NPM1 mutations occur typically as secondary events following initial oncogenic driver mutations in other frequently mutated oncogenes such as DNMT3A, IDH1, TET2, and NRAS[3]. Other co-occurring mutations with NPM1 have been observed in myeloid lineage critical genes including PTPN11, FLT3, and GATA2[3]. Previous survival studies indicated a more favorable prognosis for NPM1-mutant AML, however it is now becoming evident that this depends on the specific genetic alterations concomitant with those in NPM1[3,4].

Diagnostic assays for *NPM1mutA* quantitation are valuable tools for defining treatment responses in patients. To monitor performance of NPM1 assays, a novel synthetic control panel was developed comprising a set of relevant concentrations of wild-type *ABL1* and *NPM1* transcripts mixed with varying levels of *NPM1mutA* within a stabilizing matrix formulation.

Methods

Partial sequences of ABL1, NPM1 and NPM1mutA genes were synthesized, ligated into engineered vectors, and transformed to generate stable frozen clones. Bi-directional Sanger sequencing confirmed correct sequences for all clones used in this study. *In vitro* transcripts (IVT) were generated, quantified by UV-spectrophotometry and combined with MMQCI's proprietary stabilizing formulation to create a control panel consisting of 5 levels of NPM1mutA to ABL1; i.e. 0%, 0.1%, 5%, and 20% NPM1mutA. Xpert NPM1 Control Panel C194 has been assigned the % ratios of NPM1 Type A mutant RNA transcript to ABL1 RNA transcript listed in Table 1.

For reproducibility studies, three lots of the NPM1 control panel were tested across 5 manufactured lots of Xpert® NPM1 Mutation Assay cartridges (Cepheid) with 4 different operators. A total of 150 cartridges were tested on the GeneXpert Dx System (ver. 900-0400 Rev B), with 30 cartridges tested per NPM1mutA level. Linear regression analysis demonstrated R^2 values >0.98 for each Xpert cartridge lot, and >0.97 across all 5 cartridge lots. Combining data from all 3 NPM1 control panel lots showed high accuracy and precision at all positive *NPM1mutA* levels: 20% level, 95% CI [18.22, 21.11]; 5% level, 95% CI [4.69, 5.72]; 1% level, 95% CI [0.89, 1.08]; 0.1% level, 95% CI [0.113, 0.139].

For repeatability studies, the 1% and 5% NPM1mutA levels were tested on one lot of Xpert NPM1 Mutation Assay cartridges (12 cartridges each) on the same day using the same operator. Descriptive statistics were generated using the Analysis ToolPak module in Microsoft Excel (ver. 2208).

References

[1] Grisendi S, Mecucci C, Falini B, Pandolfi PP. Nucleophosmin and cancer. *Nat Rev Cancer*. 2006, 6(7):493-505.

[2] Falini B, Brunetti L, Sportoletti P, Martelli MP. NPM1-mutated acute myeloid leukemia: from bench to bedside. *Blood*. 2020; 136(15):1707-1721.

[3] Panuzzo C, Signorino, E, Calabrese C, Ali MS, Petiti J, Bracco E, Cilloni D. Landscape of Tumor Suppressor Mutations in Acute Myeloid Leukemia. *J Clin Med*. 2020, 16;9(3):802

[4] Heath EM, Chan SM, Minden MD, Murphy T, Shlush LI, Schimmer AD. Biological and clinical consequences of NPM1 mutations in AML. *Leukemia*. 2017, 31(4):798-807.

Results

Figure 1. Overview of the NPM1 Type A Mutant Control Panel development. (A) Domain map illustrations of wildtype NPM1 (*upper panel*) and Type A mutant NPM1 (*lower panel*) with location of the core, acidic, basic and aromatic domains, and the 4-bp TCTG duplication (red font in mRNA sequence) resulting in frameshift and de novo peptide sequence (blue amino acids in protein sequence). Bold tryptophan (W) amino acids in wildtype protein sequence are critical for nucleolar localization and lost upon the Type A insertion. NES, nuclear export signal; NLS, nuclear localization signal; NOLS, nucleolar localization signal. **(B)** Development included synthesis of wildtype and Type A mutant NPM1 sequences, cloning and purification of NPM1 plasmids, generation of *in vitro* transcripts (IVTs), formulation testing and scale-up validation.

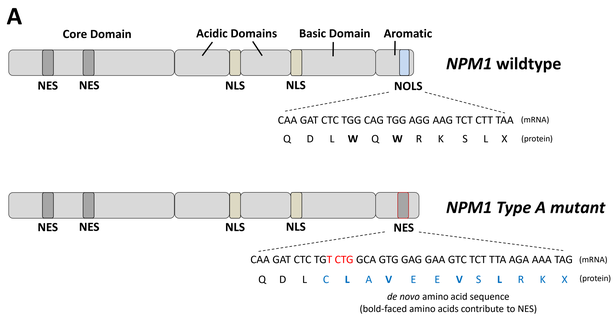
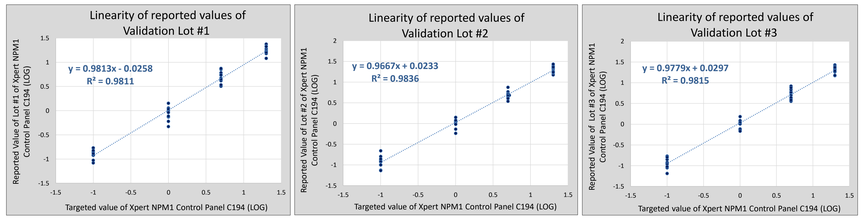


Figure 2. Reproducibility studies demonstrate high run-to-run precision of all NPM1mutA levels. Three manufactured lots of the NPM1 Control Panel were tested across 5 lots of Xpert NPM1 Mutation Assay cartridges (Cepheid). **(A)** Linear regression of Validation Lot #1, #2 and #3, showing slope values between 0.9 and 1.1 and Pearson correlation coefficient, R^2 >0.98. **(B)** Average cycle threshold (Ct) values for each level of NPM1mutA transcript to ABL1 transcript, with provided 95% confidence interval (CI) values showing less than a single cycle range for all control levels. **(C)** Average delta Ct values, and **(D)** average % NPM1mutA to ABL1 transcript ratios, with 95% CI ranges.

A



B

0% Level	AVG Ct	95% CI, Ct	
ABL	14.26	13.95	14.58
NPM1mutA	n/a	n/a	n/a
0.1% Level	**AVG Ct**	**95% CI, Ct**	
ABL	13.90	13.62	14.24
NPM1mutA	24.76	24.40	25.12
1% Level	**AVG Ct**	**95% CI, Ct**	
ABL	14.08	13.81	14.34
NPM1mutA	21.78	21.47	22.10
5% Level	**AVG Ct**	**95% CI, Ct**	
ABL	13.92	13.62	14.23
NPM1mutA	19.06	18.68	19.45
20% Level	**AVG Ct**	**95% CI, Ct**	
ABL	13.79	13.51	14.01
NPM1mutA	16.87	16.54	17.20

C

Level	AVG ΔCt	95% CI, ΔCt	
0.1%	-10.83	-10.95	-10.70
1%	-7.70	-7.82	-7.59
5%	-5.14	-5.28	-5.00
20%	-3.09	-3.20	-2.98

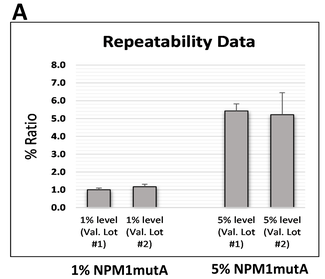
D

Level	AVG % Ratio	95% CI, % Ratio	
0.1%	0.126	0.113	0.139
1%	0.98	0.880	1.080
5%	5.21	4.689	5.720
20%	19.66	18.215	21.108

Results

Figure 3. Intra-run repeatability studies of the NPM1 Control Panel. Two scaled-up manufactured NPM1 Control Panel lots were tested on a single NPM1 Mutation Assay cartridge lot (Cepheid) at the 1% and 5% NPM1mutA control levels. **(A)** Lots #1 and #2 each demonstrated high accuracy of the targeted percentage. **(B)** Combined repeatability data from Lots #1 and #2 demonstrate narrow 95% CI ranges of ABL and NPM1mutA Ct values, as well as delta Ct, and % NPM1mutA:ABL ratios.

A



B

1% NPM1mutA control

	ABL Ct	NPM1mutA Ct	Delta Ct	% ratio
Avg [95% CI]	**13.2** [12.6, 13.8]	**21.0** [20.3, 21.7]	**-7.8** [-7.9, -7.7]	**1.087** [0.995, 1.179]
StDev	0.97	1.04	0.2	0.14
%CV	7.37	4.94	2.48	13.34

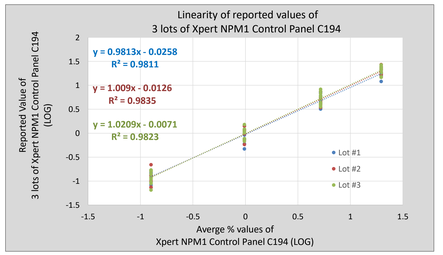
5% NPM1mutA control

	ABL Ct	NPM1mutA Ct	Delta Ct	% ratio
Avg [95% CI]	**13.3** [12.6, 13.7]	**18.6** [17.9, 19.2]	**-5.3** [-5.5, -5.2]	**5.50** [4.76, 5.88]
StDev	0.88	1.03	0.3	0.81
%CV	6.60	5.53	5.23	14.77

Table 1. Xpert NPM1 Control Panel C194 Average % Ratio. The average % ratios were determined by testing 3 lots of Xpert NPM1 Control Panel C194 across 5 unique Xpert NPM1 Mutation Assay cartridge lots on the Cepheid GeneXpert System.

Xpert NPM1 Control Panel C194 Component	Average % Values
Xpert NPM1 MUT A 0%	Negative (Sufficient ABL transcript)
Xpert NPM1 MUT A 0.1%	0.126%
Xpert NPM1 MUT A 1%	0.98%
Xpert NPM1 MUT A 5%	5.21%
Xpert NPM1 MUT A 20%	19.66%

Figure 4. Reported % ratios for three lots of Xpert NPM1 Control Panel C194 tested on one lot of Xpert NPM1 Mutation Assay cartridges on the GeneXpert System compared to the average % ratio values, demonstrating linearity across all levels of all three lots. Ten replicates of each level were tested and 0.1%, 1%, 5% and 20% levels were plotted.



Conclusions

- The synthetic NPM1 Control Panel demonstrated high accuracy, precision and linearity with slope values between 0.9 and 1.1 and Pearson correlation coefficient, R^2 >0.98 when tested across 5 Xpert NPM1 Mutation Assay cartridge lots.
- Stability data for Xpert NPM1 Control Panel along with historical data of similar products containing MMQCI's proprietary matrix formulation supports stability for 1 year when stored at -20°C. Studies to determine stability at -20°C for 2+ years are ongoing.
- Reported % values for Xpert NPM1 Control Panel C194 may vary among laboratories, test systems and reagent lots, however the use of the synthetic external control panel enables a laboratory to establish acceptable % ranges and confirm linearity across all testing levels.

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