Development of an External Control Panel to Confirm Linear Dynamic Range of Xpert NPM1 Mutation Assay

Emma Farrell, Joan Gordon, Olivia Hamilton, Mathew Held, Loren Krott, Dan Magoon, Isreal Moreno, Abbey Reilly, and Tania Spenlinhauer

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

Introduction

Nucleophosmin-1 (NPM1) is a multifunctional protein with roles in nucleocytoplasmic shuttling, ribosome biogenesis, and cell cycle progression[1]. Approximately 30% of patients with acute myeloid leukemia have mutations in exon 12 of NPM1. Tetranucleotide insertions disrupt the NPM1 nucleolar localization signal, resulting in aberrant NPM1 cytoplasmic accumulation[2].

The World Health Organization recognizes NPM1 mutant leukemias as a distinct class, therefore quantifying NPM1 mutations is a valuable diagnostic tool for defining treatment responses in patients. Type A (TCTG) insertion accounts for 80% of NPM1 mutations while Type B (CATG) and Type D (CCTG) are less common[3]. Such assays measuring NPM1 mutations in peripheral blood by RT-PCR requires verification of the entire dynamic range in order to validate and monitor an assays performance.

An external control panel consisting of these three NPM1 variants, as well as a linearity panel representing levels across the entire dynamic range of Xpert NPM1 Mutation assay is being developed to monitor assay performance and confirm linearity across the reportable range.

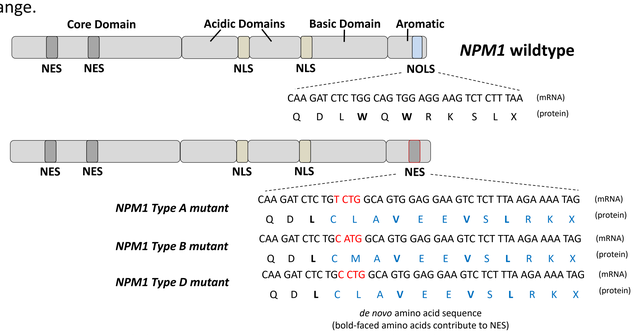


Figure 1. Overview of the NPM1 Control and Linearity Panel development. Domain map illustrations of wildtype NPM1 and NPM1 mutations with location of the core, acidic, basic and aromatic domains, and the 4-bp duplication (red font in mRNA sequence) resulting in frameshift and de novo peptide sequence (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, B, or D insertions. NES, nuclear export signal; NLS, nuclear localization signal; NOLS, nucleolar localization signal.

Methods

Partial gene sequences of ABL1, NPM1wt, NPM1mutA, NPM1mutB and NPM1mutD were synthesized, ligated into engineered vectors, and transformed to generate stable frozen clones. All sequences were confirmed via bi-directional Sanger sequencing. *In vitro* transcripts were generated, quantified by UV-spectrophotometry and combined with proprietary stabilizing matrix to create a panel of NPM1mutA to ABL1 levels, as well as NPM1mutB to ABL1 and NPM1mutD to ABL1 levels. NPM1wt transcript was included at the same concentration as ABL1 to better mimic patient samples. The panel has been designed to report 0%, 0.1%, 1%, 5%, 20%, 100%, and 450% NPM1mutA and includes 1% NPM1mutB, 1% NPM1mutD. Three lots of each NPM1 level were manufactured and tested across multiple lots of Cepheid Xpert® NPM1 Mutation Assay to confirm linearity and reproducibility.

For reproducibility studies, three lots of the NPM1 control and linearity panels were tested across two lots of Xpert NPM1 Mutation Assay cartridges with five different operators. A total of 118 cartridges were tested on the GeneXpert Dx System (ver. 900-0400 Rev B).

For repeatability studies, the 1% levels of NPM1mutA, NPM1mutB, and NPM1mutD and 5% NPM1mutA level were tested on one lot of Xpert NPM1 Mutation Assay cartridges (12 cartridges each) on the same day by 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.

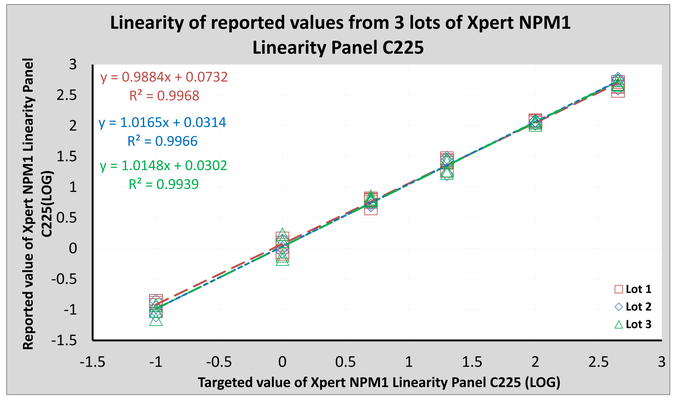
[2] Panuzzo C, Signorino, E, Calabrese C, Ali MS, Petiti J, Bracco E, Cilloni D. Landscape of Tumor Suppressor Mutations in Acute Myeloid Leukemia. *J Clin Med*. 2020, 16;9(3):802.

[3] Bacher U, Porret N, Joncourt R, Sanz J, Aliu N, Wiedemann G, Jeker B, Banz Y, Pabst T. Pitfalls in the Molecular Follow Up of NPM1 Mutant Acute Myeloid Leukemia. *Haematologica*. 2018, 103(10):e486-e488.

[4] Grubbs F. Procedures for detecting outlying observations in samples. *Technometrics* 11, 1–21 (1969).

Reproducibility

A



B

MutA Level	Target	AVG Ct	95% CI, Ct	
			Lower	Upper
0%	ABL	12.4	12.0	12.9
	NPM1	n/a	n/a	n/a
0.1%*	ABL	12.6	12.2	13.0
	NPM1mutA	23.5	23.1	24.0
1%	ABL	12.5	12.2	12.8
	NPM1mutA	20.2	19.9	20.6
5%	ABL	12.4	12.1	12.7
	NPM1mutA	17.6	17.2	18.0
20%	ABL	12.4	12.1	12.8
	NPM1mutA	15.5	15.1	15.9
100%*	ABL	12.4	11.9	12.9
	NPM1mutA	13.2	12.8	13.7
450%	ABL	12.6	12.1	13.1
	NPM1mutA	11.3	10.7	11.9

C

Level	AVG % Ratio	95% CI, % Ratio	
		Lower	Upper
0.1%*	0.112	0.098	0.126
1%	1.098	0.928	1.268
5%	5.877	5.472	6.282
20%	24.552	21.762	27.342
100%*	115.608	111.837	119.379
450%	495.323	456.658	533.987

Figure 2. Reproducibility studies demonstrate high run-to-run precision of all NPM1mutA levels. Three manufactured lots of the NPM1 Linearity Panel were tested across two lots of Xpert NPM1 Mutation Assay cartridges (Cepheid). **(A)** Linear regression of Validation Lots #1, #2 and #3, showing slope values between 0.9 and 1.1 and Pearson correlation coefficient, R^2 >0.99. **(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 levels, except the 450% level. **(C)** Average % NPM1mutA to ABL1 transcript ratios, with 95% CI ranges. * 0.1% and 100% Levels had one outlier removed based on Grubb's outlier test[4].

NPM1mutA Repeatability

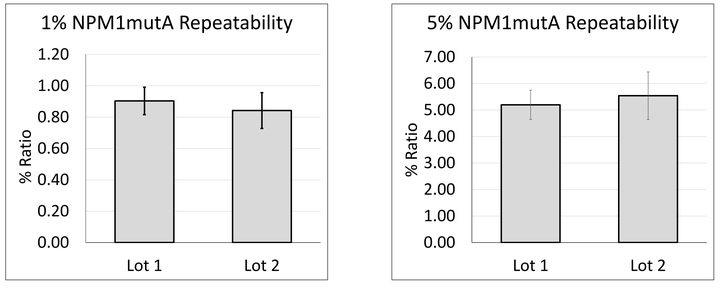


Figure 3. Intra-run repeatability studies of the NPM1 Linearity Panel. Two lots of 1% NPM1mutA and 5% NPM1mutA were tested on a single Xpert NPM1 Mutation Assay cartridge lot (Cepheid).

Acknowledgements: We would like to thank Cepheid (Sunnyvale, CA) for providing reagents for this study.

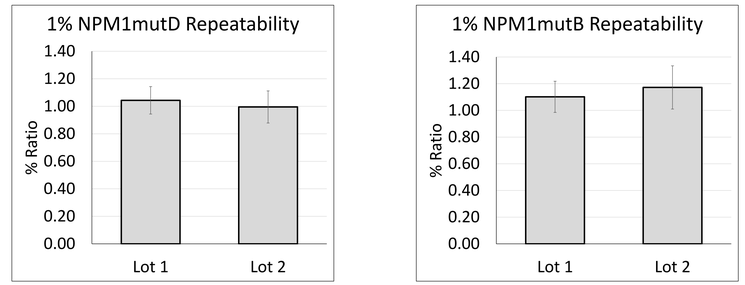
NPM1mutB and NPM1mutD Reproducibility and Repeatability

Table 1. Reproducibility studies demonstrate high run-to-run precision of NPM1mutB and NPM1mutD. Three manufactured lots of the NPM1 Control Panel were tested across two lots of Xpert NPM1 Mutation Assay cartridges (Cepheid).

	Target	AVG Ct	95% CI, Ct	
1% NPM1mutB	ABL	12.3	11.9	12.7
	NPM1mutB	19.7	19.3	20.1
1% NPM1mutD	ABL	12.3	11.9	12.6
	NPM1mutA	19.7	19.3	20.0

Level	AVG % Ratio	95% CI, % Ratio	
1% NPM1MutB	1.273	1.112	1.435
1% NPM1MutD	1.293	1.141	1.446

Figure 4. Intra-run repeatability studies of the NPM1 Control Panel. Two lots of 1% NPM1mutB and 1% NPM1mutD were tested on a single Xpert NPM1 Mutation Assay cartridge lot (Cepheid).



Summary

Reproducibility: Linear regression analysis of NPM1mutA levels demonstrated R^2 values >0.99 for each NPM1 lot. Combining data from all 3 NPM1 manufactured lots showed high accuracy and precision of each level tested.

Repeatability: Repeatability studies demonstrated each of the NPM1 levels tested performed with highly repeatable results of which the average %CV of the Ct's was below 20%.

Xpert NPM1 MUT A Component	Average % Values
Xpert NPM1 MUT A 0.1%	0.112
Xpert NPM1 MUT A 1%	1.098
Xpert NPM1 MUT A 5%	5.877
Xpert NPM1 MUT A 20%	24.552
Xpert NPM1 MUT A 100%	115.608
Xpert NPM1 MUT A 450%	495.323

Xpert NPM1 MUT B or D	Average % Values
Xpert NPM1 MUT B 1%	1.273
Xpert NPM1 MUT D 1%	1.293

Table 2. Xpert NPM1 Panels Average % Ratio. The average % ratios were determined by testing 3 lots of Xpert NPM1 Control and Linearity Panels across two unique Xpert NPM1 Mutation Assay cartridge lots on the Cepheid GeneXpert System.

Conclusions

- The synthetic Xpert NPM1 Control and Linearity Panels demonstrated high accuracy, precision and linearity with slope values between 0.9 and 1.1 and Pearson correlation coefficient, R^2 >0.99 when tested across two Xpert NPM1 Mutation Assay cartridge lots. Further testing of these 3 manufactured lots on at least 3 additional Xpert NPM1 Mutation Assay cartridges (Cepheid) is desired for full validation of these panels.
- Stability data for Xpert NPM1 Control Panel along with historical data of similar products containing MMQCI's proprietary matrix formulation supports stability for 2 years when stored at -20°C.
- Reported % values for Xpert NPM1 Controls 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.