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Next-Generation Sequencing of *NPM1* for Minimal Residual Disease Monitoring in Leukemia Patients

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Introduction

The nucleophosmin (*NPM1*) gene is an important marker for acute myeloid leukemia (AML) stratification. *NPM1* is one of the most commonly mutated genes in AML, with mutations seen in roughly 35% of patients at diagnosis, and in approximately 60% of adult cytogenetically normal AML patients. Importantly, *NPM1* mutations generally confer a slightly more favorable outcome in AML patients and can mitigate poor prognoses when they occur concurrently with activating mutations in *FLT3*. As a commonly mutated gene with prognostic value in AML, *NPM1* is an appropriate biomarker for minimal residual disease (MRD) monitoring. MRD detection has proven to be valuable in the clinical management of patients with AML. *NPM1* is of particular importance, as it was recently shown that the presence of *NPM1* mutants after chemotherapy were associated with a greater risk of relapse. Thus, the development of a sensitive and reliable assay to detect *NPM1* mutations at low frequencies represents a significant advancement in guiding treatment for AML patients.

Materials

LOD & linearity:

- A panel of samples was created consisting of cell line DNA (IVS-0046) containing a known *NPM1* 4 bp insertion mutation (COSM17559) diluted into DNA from a cell line known to be negative for *NPM1* mutations (IVS-0061). The panel contains samples at dilutions that range from: $1.0x10^{-5}$ to $5.0x10^{-1}$.

Sensitivity & specificity:

- Using the same panel as above, we were able to calculate the sensitivity and specificity of this *NPM1* MRD assay at various input mutation frequencies, including at and above the assay's LOD claim of $5.0x10^{-5}$.

Detection of mutations in clinical samples:

- We tested 5 clinical samples for *NPM1* mutations and assessed the assay's concordance with a CAP/CLIA validated capillary electrophoresis *NPM1* assay.
- Since no clinical samples were available with *NPM1* mutations at MRD levels, we diluted two positive clinical samples as much as 1 in 1,000 to test detection of mutations closer to the assay's LOD.

Methods

NPM1 target:

- This next-generation sequencing (NGS) MRD assay targets exon 12 of the *NPM1* gene using a single, optimized PCR amplification that was developed to overcome inherent challenges caused by repetitive sequence across this locus (**Figure 1**).

DNA input and sequencing depth (Figure 1):

- To optimize the assay to sensitively detect mutations at a LOD of $5.0x10^{-5}$, we utilize:
  - An input of 700 ng of high quality DNA (> 100,000 cell equivalents)
  - Sequencing depth > 240,000x (this allows us to test 24 samples per run)

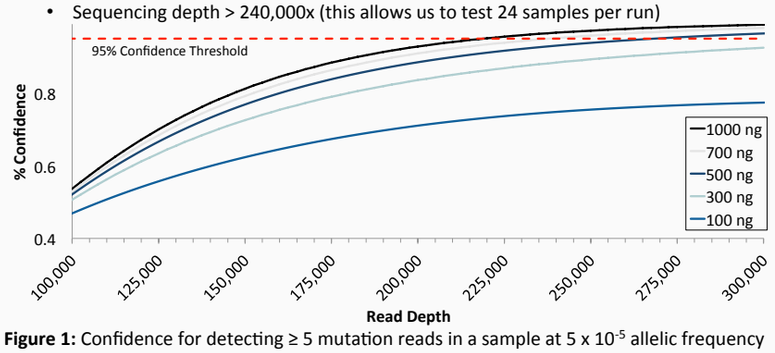

Figure 1: Confidence for detecting ≥ 5 mutation reads in a sample at 5×10^{-5} allelic frequency
Data for this figure was calculated using a statistical model that does not incorporate any PCR bias. An input of 700 ng of DNA would require a ≥ 240,000x for confident detection of a mutation at 5×10^{-5}. Decreased input DNA amount would require additional read depth to maintain a 95% confidence for detecting a mutation at this frequency.

Data analysis:

- All generated data was analyzed by the proprietary *NPM1* MRD Data Analysis Tool software developed by Invivoscribe.

Results: Limit of Detection and Linearity

To establish the limit of detection (LOD) and linearity of *NPM1* variant detection, we used a panel of samples consisting of a cell line containing a known *NPM1* 4 bp insertion mutation (IVS-0046; COSM17559) diluted into a background cell line containing wild-type *NPM1* exon 12 (IVS-0061). We tested our ability to detect mutations at a LOD as low as $1x10^{-5}$ and used a range of dilutions up to 5×10^{-1} to test for linearity in the mutation detection (**Figure 2**).

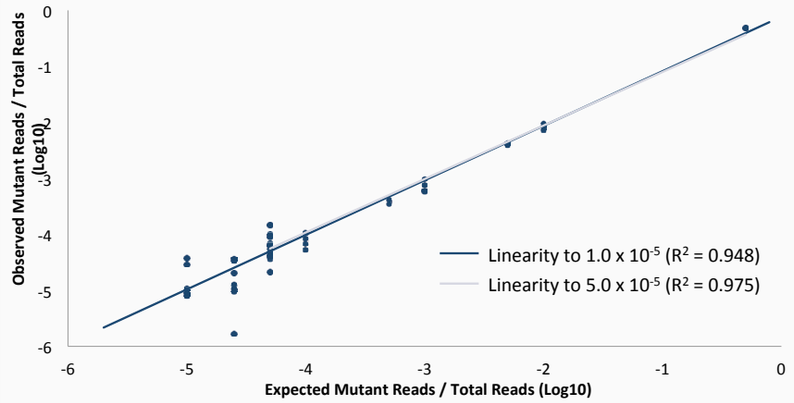

Figure 2: Linearity of *NPM1* MRD assay
This figure examines linearity for the detection of the COSM17559 4 bp insertion mutation using dilutions of a positive control (IVS-0046).

Table 1: Results of the *NPM1* MRD assay at decreasing input mutation frequencies

Expected Mutation Frequency	Number of Tests (N)	% of Tests Positive† for the Expected Mutation	Mean Observed Mutation Frequency
5.0×10^{-1}	3	100%	4.9×10^{-1}
1.0×10^{-2}	3	100%	8.4×10^{-3}
5.0×10^{-3}	2	100%	4.1×10^{-3}
1.0×10^{-3}	4	100%	7.3×10^{-4}
5.0×10^{-4}	2	100%	3.9×10^{-4}
1.0×10^{-4}	4	100%	7.8×10^{-5}
5.0×10^{-5}	20	100%	7.2×10^{-5}
2.5×10^{-5}	7	71.4%	1.8×10^{-5}
1.0×10^{-5}	6	83.3%	1.8×10^{-5}
0.0	11	0%	0.0

† A positive call requires the detection of a minimum of 5 NGS reads containing a mutation.

Limit of Detection:
The *NPM1* 4 bp insertion mutation was detected at every dilution level, down to $1x10^{-5}$. However, the lowest dilution level where 100% of the tests were positive for the expected mutation (100% sensitivity).

- We established an optimal final LOD of 5×10^{-5} for this *NPM1* MRD assay

Linearity:
The data suggests strong linearity for the detection of mutations by the *NPM1* MRD assay.

- The data has an R^2 value of 0.948.
- If we only include data down to the LOD of 5×10^{-5}, the R^2 increases to 0.975, suggesting slightly superior linearity in the assay's detection range.

Precision and Reproducibility:
The assay shows acceptable precision and reproducibility both at and above the assay's 5×10^{-5} LOD (data not shown).

Results: Sensitivity and Specificity

To determine the sensitivity and specificity at different input mutation frequencies, we used the data that was generated for LOD and linearity. We determine that the assay has 100% sensitivity and 100% specificity for mutations at or above a LOD of 5×10^{-5} (**Table 2** and **Figure 3**).

Expected Mutation Frequency	Number of Tests (N)	True Positives	False Positives	True Negatives	False Negatives
5.0×10^{-1}	3	3	0	0	N/A
1.0×10^{-2}	3	3	0	0	N/A
5.0×10^{-3}	2	2	0	0	N/A
1.0×10^{-3}	4	4	0	0	N/A
5.0×10^{-4}	2	2	0	0	N/A
1.0×10^{-4}	4	4	0	0	N/A
5.0×10^{-5}	20	20	0	0	N/A
2.5×10^{-5}	7	5	0	2	N/A
1.0×10^{-5}	6	5	0	1	N/A
0.0	11	N/A	0	N/A	11

		Expected Mutations: Positive	Expected Mutations: Negative	
Observed Mutations	Positive	38	0	PPV = 100%
	Negative	0	11	NPV = 100%
		Sensitivity = 100%	Specificity = 100%	

Figure 3: Sensitivity and Specificity Table
This figure shows the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for this study's mutation detection at or above the LOD of 5×10^{-5}.

At the established LOD of 5×10^{-5}, this assay has 100% sensitivity and specificity for the detection of a 4 bp insertion mutation in *NPM1*. At lower detection limits, our sensitivity and NPV drop, but specificity and PPV remain at 100% due to the lack of false positives in this study.

Results: Concordance with Clinical Samples

We tested 5 clinical samples from AML patients. Of these, 2 had previously been found to be positive for an *NPM1* mutation using a CAP/CLIA-capillary electrophoresis (CE) method.

- The *NPM1* MRD assays results match the expected results from the CE assay.
- We diluted the 2 positive samples and again confirmed the mutation at lower frequencies.

Table 3: Results from clinical samples tested using the *NPM1* MRD assay

Sample	Dilution	CE Assay	Observed Mutation	Observed Frequency
AML-04	None	Positive	4 bp insertion (TGTA)	4.7×10^{-1}
	1 in 10			3.5×10^{-2}
	1 in 100			3.2×10^{-3}
	1 in 1,000			2.7×10^{-4}
AML-12	None	Positive	4 bp insertion (TGCA)	4.6×10^{-1}
	1 in 10			5.2×10^{-2}
	1 in 100			5.5×10^{-3}
AML-03	None	Not Detected	Not Detected	0.0
AML-57	None	Not Detected	Not Detected	0.0
AML-58	None	Not Detected	Not Detected	0.0

In addition, we also tested DNA from 4 normal controls and found no false positive mutations.

Conclusions

The ***NPM1* MRD NGS assay** is a highly specific test that can detect *NPM1* mutations with a sensitivity at least two orders of magnitude greater than current commercially available assays.

- Strong linearity and 100% sensitivity and specificity at and above a LOD of 5×10^{-5}.
- Assay is concordant with CAP/CLIA-CE assay for clinical samples with no false positives in normal controls.

This assay provides a reliable tool to assess MRD in AML patients.