Countable Labs.

Development of a multiplexed *NPM1* panel by single-molecule RNA counting for differential detection of Type A, B, and D mutations for AML MRD research applications.

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Introduction

Acute myeloid leukemia (AML) harboring *NPM1* mutations represents approximately 30% of adult AML cases. Type A, B, and D insertion mutations account for ~90% of all *NPM1* mutations and are absent in healthy hematopoietic cells, making them ideal minimum residual disease (MRD) targets. Persistent or increasing MRD following induction therapy or transplantation is associated with relapse and inferior outcomes. Tracking the patient's index mutation type is essential to distinguish true clonal relapse from secondary or donor-derived *NPM1* events, underscoring the research need for mutation type-specific rather than pan-*NPM1* detection.

Current approaches (allele-specific qPCR, RT-qPCR, and fragment analysis) offer high sensitivity and rapid turnaround but require separate reactions per mutation type and rely on calibration standards. NGS enables simultaneous multi-variant detection but is limited by higher cost, turnaround, and greater bioinformatics complexity, restricting its utility for serial monitoring.

Here, we developed a multiplex *NPM1* mutation panel on the Countable PCR platform, a single-molecule nucleic acid counting platform, supporting its potential for sensitive, quantitative longitudinal research assessment of AML disease burden.

Materials and methods

Single-molecule quantification of nucleic acid using Countable PCR.

- The Countable PCR platform generates ~30 million compartments, ensuring single-molecule occupation and a dynamic range up to 1 million RNA molecules. This enables high sample input, such as for MRD research applications.
- The Countable *NPM1* RNA assay utilizes a 1-step RT workflow, integrating the RT and PCR reactions into a single reaction tube. This approach minimizes hands-on time and reduces the risk of sample contamination by simplifying the workflow.
- In this study, we used this 1-step RT workflow with Countable PCR to detect three major mut*NPM1* insertion mutations (Type A, Type B, and Type D) alongside an *ABL1* control.
- Here we show that this 4-color, 4-plex assay demonstrates sensitive detection and precise quantification of mut*NPM1* isoforms across a wide dynamic range.

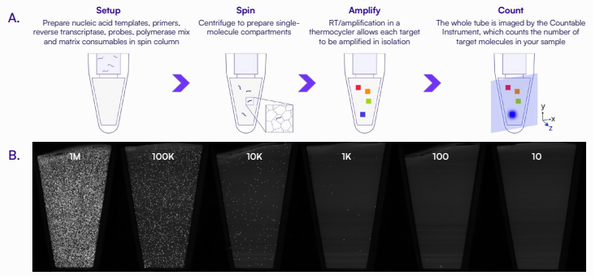


Figure 1. (A) Schematic diagram of Countable PCR workflow. In Countable PCR, target nucleic acid molecules are partitioned into ~30 million compartments within an optically clear matrix. Subsequent RT, PCR amplification, and counting all occur within a single tube. Unlike dPCR, the Countable system provides true single-molecule counting across a wide dynamic range, enabling high multiplex detection of target sequences. (B) Cross-sections of a Countable PCR reaction containing HL60 RNA at various target concentrations across the platform's dynamic range.

Results

Countable *NPM1* RNA assay demonstrates good specificity for each mutant isoform in a single reaction.

Table 1. Specificity of each mut*NPM1* target in the Countable 4-plex assay.

WT HL60 RNA spiked with:	Type A		Type B		Type D	
	counts/50µL	%*ABL1*	counts/50µL	%*ABL1*	counts/50µL	%*ABL1*
OCI-AML3 RNA (Type A)	775,602	88.98%	2	0.0002%	36	0.0042%
Type B IVT RNA	0	0.0000%	479,432	61.91%	33	0.0043%
Type D IVT RNA	83	0.0102%	26	0.0032%	497,820	61.39%

RNA extracted from mut*NPM1*-negative HL60 cell line material were added to a Countable PCR reaction, spiked with either RNA from OCI-AML3 (Type A positive), Type B *in vitro* transcribed (IVT) RNA, or Type D IVT RNA. Unlike drop off PCR assays, this panel can distinguish between each isoform with good specificity.

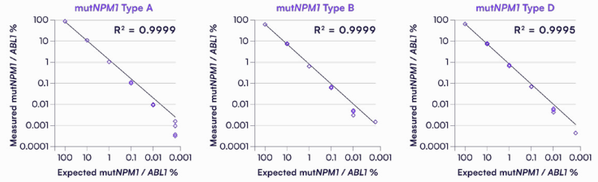
Countable *NPM1* RNA assay has high analytical sensitivity across all three mut*NPM1* isoforms.

- Using the 24 replicates of HL60-only reactions, a limit of blank (LOB) and limit of detection (LOD) were calculated for each *NPM1* mutant isoform.
- LOB is calculated as average blank + 1.645*stdev (blank).
- LOD was calculated as LOB + 1.645*stdev (low input counts).

Table 2. Analytical sensitivity of each Type A, Type B, and Type D assays in the Countable *NPM1* RNA panel. Counts from mutant isoform targets remained low despite high WT HL60 input.

		LOB (n-24)	LOD	*ABL1* input (from LOB)
Type A	Counts/50µL	1	8	806,550
	%*ABL*	0.0001%	0.0010%	–
Type B	Counts/50µL	4	13	806,550
	%*ABL*	0.0005%	0.0013%	–
Type D	Counts/50µL	0	3	806,550
	%*ABL*	0.0000%	0.0004%	–

Countable PCR achieves linear quantification and high precision across all three mut*NPM1* targets.



Dilution	Type A counts/50µL	%CV (n=4)
1	775,602	0.46%
2	84,176	2.81%
3	9,246	5.13%
4	855	8.70%
5	81	7.06%
6	7	64.94%
7	0	N/A

Dilution	Type B counts/50µL	%CV (n=4)
1	479,432	2.46%
2	57,545	4.59%
3	4,971	3.40%
4	483	6.74%
5	32	22.53%
6	5	121.85%
7	1	N/A

Dilution	Type D counts/50µL	%CV (n=4)
1	497,820	3.57%
2	55,429	2.36%
3	5,624	5.56%
4	481	6.04%
5	39	19.42%
6	2	86.60%
7	0	N/A

Figure 2. Linearity of precision of *NPM1* Type A, Type B, and Type D assays across a 6 log dynamic range. Each concentration point was calculated as the average of four technical replicates.

- Linearity was evaluated by spiking mut*NPM1* Type A (OCI-AML3-derived RNA) and Type B and D (IVT RNA) into mut*NPM1*-negative HL60 RNA with an input near 1 million *ABL1* counts per 50 µL.
- The Countable *NPM1* RNA assay demonstrates good precision at mut*NPM1* input counts above 100 with <10%CV.
- Each mut*NPM1* target demonstrates good linearity (r^2>0.999) across a six-log dynamic range.

Countable *NPM1* RNA panel detects mutations across blood and bone marrow specimen types.

- To demonstrate compatibility with clinical research sample types, *NPM1*-positive blood and bone marrow samples were processed on the Countable *NPM1* RNA panel.
- Results from the Countable *NPM1* RNA panel were concordant with mutation status confirmed by sequencing. Notably, the panel resolved two distinct mut*NPM1* Type A insertion variants within the same sample type.

Table 3. Tabulated results from 10 clinical research samples run with the Countable *NPM1* RNA panel. Results from Countable PCR were concordant with results reported from the clinic. Furthermore, the Countable *NPM1* RNA panel was able to detect two different types of mut*NPM1* Type A insertion mutations.

Sample	Origin	Expected mutation	Input per reaction (ng)	mut*NPM1* detected	*ABL1* counts per 50µL	mut*NPM1* counts per 50µL	%Ratio mut*NPM1*/*ABL1*
1	Bone marrow	Type A, 860_863dup	31.6	Type A	15,519	83,549	538.4%
2	Blood	Type A, 860_863dup	29	Type A	17,262	181,483	1,051.3%
3	Bone marrow	Type A, 860_863dup	48.9	Type A	27,805	319,148	1,147.8%
4	Blood	Type A, 863_864insTCAG	34.9	Type A	10,377	63,977	616.5%
5	Bone marrow	Type A, 860_863dup	44	Type A	16,816	249,520	1,483.8%
6	Blood	Negative	34.4	Not detected	3,483	Not detected	N/A
7	Blood	Negative	30	Not detected	11,031	Not detected	N/A
8	Blood	Negative	48	Not detected	9,156	Not detected	N/A
9	Blood	Negative	35	Not detected	17,893	Not detected	N/A
10	Blood	Negative	33	Not detected	13,398	Not detected	N/A

Conclusion

- We developed a four-plex assay simultaneously targeting mut*NPM1* Type A, Type B, Type D, as well as an *ABL1* wild-type control, enabling direct variant discrimination in a single reaction.
- Single-molecule RNA counting enabled sensitive detection of rare mutant *NPM1* Type A, Type B, and Type D transcripts, achieving an LOD ≤ 0.002% mut*NPM1* target/*ABL1*.
- Linear quantification is demonstrated up through 100% mut*NPM1*/*ABL1* across all three mutant subtypes (r^2 > 0.999 in all targets), with high measurement precision when measuring above 100 mut*NPM1* counts per 50 µL reaction (<10%CV)
- The Countable *NPM1* RNA panel demonstrates compatibility with both bone marrow and blood sample types, supporting its potential as a streamlined tool for *NPM1* mutation AML monitoring research.

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Clinical research specimens were kindly provided by Dr. Pamela Ward At the University of Southern California Keck School of Medicine

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Learn more about how Countable PCR simplifies quantitative AML MRD monitoring research.

Visit countablelabs.com/resources/npm1-multiplex-panel-aml-mrd-mutation-detection