Countable Labs.

Highly multiplexed detection and quantification of 18 biologically relevant *KMT2A* rearrangements using single-molecule counting.

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Introduction

Acute myeloid leukemia (AML) with *KMT2A* rearrangements (*KMT2A*-r) is an aggressive leukemia subtype characterized by poor prognosis, chemotherapy resistance, and high relapse rates. Although more than 90 *KMT2A* fusion partners have been identified, the majority of cases involve a relatively small number of recurrent fusion partners. The recent development and approval of menin inhibitors have further stimulated research interest in *KMT2A*-r AML, highlighting the need for sensitive and quantitative molecular tools to investigate fusion transcript expression, treatment response, and disease progression.

Digital PCR (dPCR) and next-generation sequencing (NGS) are widely used for sensitive detection and molecular characterization of *KMT2A*-r. However, conventional dPCR is limited by its multiplexing capacity, while NGS typically requires more complex workflows, longer turnaround times, and higher costs. These limitations present challenges for research studies involving multiple fusion transcripts and large sample sets. Therefore, a rapid, highly multiplexed, sensitive, and quantitative approach capable of simultaneously detecting the most prevalent *KMT2A* fusion transcripts is needed to facilitate *KMT2A*-r AML research and the evaluation of emerging targeted therapies.

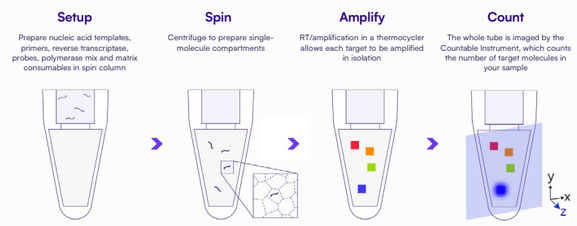


Figure 1. Single-molecule quantification of RNA using Countable PCR. Schematic diagram of Countable PCR workflow. In Countable PCR, target nucleic acid molecules are partitioned into ~30 million compartments within an optically clear matrix. Subsequent RT, PCR amplification, and counting all occur within a single tube. Unlike conventional dPCR, the Countable system provides true single-molecule counting across a wide dynamic range, enabling high multiplex detection of target sequences.

Materials and methods

Two highly multiplexed panels were developed using the Countable PCR platform to enable simultaneous detection and quantification of *KMT2A*-r transcripts. Each panel targets three major *KMT2A*-r partners and their prevalent breakpoint exons, collectively covering six fusion partners and 18 biologically relevant *KMT2A*-r. Each fusion transcript was assigned a distinct optical signature, enabling specific identification and quantitative discrimination of individual rearrangement within a multiplexed reaction, without dependency of exact breakpoints.

Assay performance was evaluated under standardized one-step reverse transcription Countable PCR conditions using synthetic DNA (gBlocks), in vitro-transcribed (IVT) RNA, and total RNA extracted from cultured cell lines carrying *KMT2A* fusion using the QIAGEN RNeasy Plus Mini Kit. Analytical performance was assessed using these reference materials, and the feasibility of detecting *KMT2A* rearrangements in patient-derived specimens was further investigated using 12 well-characterized clinical research samples.

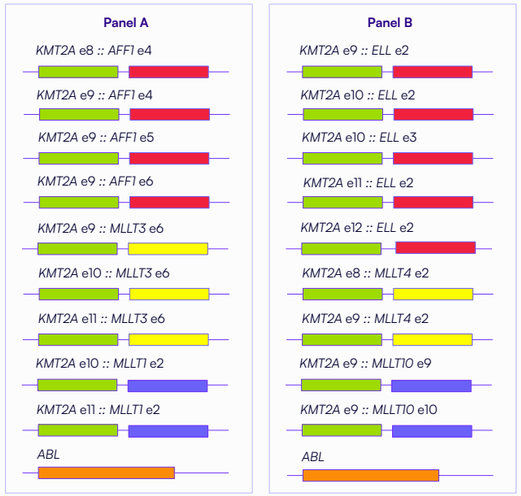


Figure 2. Optical signature of each of the 18 fusion transcripts and *ABL1* control transcript in the *KMT2A*-r Countable PCR panel. Each *KMT2A* fusion partner is identified by a unique optical signature, enabling simultaneous detection and quantification of three fusion partners per panel, with *ABL1* serving as an internal reference.

Results

Analytical specificity. Synthetic reference materials were used to evaluate intra-panel and inter-panel cross-reactivity. Each assay specifically detected its corresponding *KMT2A* fusion transcript, with no meaningful cross-reactivity against other *KMT2A* fusion targets within or across the two panels.

Table 1a. Fusion counts detected for synthetic templates in Panel A.

		Assays in Panel A			
Synthetic Template Name		*AFF1*	*MLLT3*	*MLLT1*	*ABL*
Imaging control developed for Panel A	*KMT2A::AFF1* fusion	76,342	0	0	0
	KMT2A::MLLT3 fusion	0	65,190	0	0
	KMT2A::MLLT1 fusion	0	0	33,953	0
	ABL1	0	0	0	23,026
Imaging control developed for Panel B	*KMT2A::MLLT4* fusion	1	0	0	0
	KMT2A::ELL fusion	0	0	0	0
	KMT2A::MLLT10 fusion	0	0	0	0
	ABL1	0	0	0	90,174

Table 1b. Fusion counts detected for synthetic templates in Panel B.

		Assays in Panel B			
Synthetic Template Name		*MLLT4*	*ELL*	*MLLT10*	*ABL1*
Imaging control developed for Panel B	*KMT2A::MLLT4* fusion	43,936	0	0	0
	KMT2A::ELL fusion	0	108,881	0	0
	KMT2A::MLLT10 fusion	1	1	38,397	0
	ABL1	0	0	0	99,665
Imaging control developed for Panel A	*KMT2A::AFF1* fusion	1	0	0	0
	KMT2A::MLLT3 fusion	0	0	0	0
	KMT2A::MLLT1 fusion	0	0	0	0
	ABL1	0	0	0	23,711

Analytical sensitivity & linearity.

Figure 3. Analytical linearity and sensitivity of the *KMT2A* rearrangement detection panels. Both Panel A (left) and Panel B (right) demonstrated linearity across serial dilutions of *KMT2A* fusion reference materials, with R^2 values of 0.9986 and 0.995, respectively. Linearity was evaluated using cell-extracted RNA from *KMT2A* fusion carrying cell (Panel A) or in vitro-transcribed (IVT) fusion RNA (Panel B) spiked into an HL60 RNA background. Based on the limit of detection study, *KMT2A* fusion transcripts were detected at levels as low as 0.005% relative to *ABL1*, demonstrating the high analytical sensitivity and broad dynamic range of both panels.

Analysis with clinical research specimens.

Table 2. Evaluation of *KMT2A* rearrangement detection in clinical research specimens. The two-panel assay was evaluated using 12 well-characterized clinical research specimens, categorized by prior qPCR results as high-burden or low-burden samples. With adequate input, all three high-burden samples and five low-burden samples showed fusion partner results concordant with qPCR. Samples with low fusion transcript abundance or insufficient *ABL1* reference counts highlighted the importance of adequate sample input for reliable quantification. No meaningful counts were detected in the no-template control (NTC).

Sample	Type	Expected fusion	Detected	Fusion counts	*ABL1* counts	% ratio	Call
CL-001	High-burden	*KMT2A::MLLT3*	*MLLT3*	5,969	24,050	24.82%	Concordant
CL-002	High-burden	*KMT2A::MLLT1*	*MLLT1*	39,588	37,068	106.80%	Concordant
CL-004	High-burden	*KMT2A::MLLT4*	*MLLT4*	783	2,511	31.18%	Concordant
CL-008	Low-burden	*KMT2A::MLLT3*	*MLLT3*	338,203	835,245	40.49%	Concordant
CL-009	Low-burden	*KMT2A::ELL*	*ELL*	26,815	92,083	29.12%	Concordant
CL-010	Low-burden	*KMT2A::MLLT10*	*MLLT10*	929	48,975	1.897%	Concordant, low input
CL-006	Low-burden	*KMT2A::AFF1*	*AFF1*	36	23,383	0.154%	Concordant, low input
CL-005	Low-burden	*KMT2A::ELL*	*ELL*	25	15,151	0.165%	Concordant, low input
CL-012	Low-burden	*KMT2A::MLLT3*	—	2	7,861	—	Low input
CL-003	Low-burden	*KMT2A::MLLT10*	—	0	23,430	—	Low input
CL-011	Low-burden	*KMT2A::MLLT10*	—	0	12,052	—	Low input
CL-007	Low-burden	*KMT2A::AF1*	—	1	112,367	—	NA *(not in panel)*
NTC	—	—	—	0	≤ 2	—	Clean

Conclusion

Two highly multiplexed Countable PCR panels were developed to detect and quantify 18 biologically relevant *KMT2A* rearrangements involving six major fusion partners. Both panels demonstrated high analytical specificity, sensitivity down to 0.005% relative to *ABL1*, and high linearity ($R^2 \geq 0.994$). Evaluation of 12 clinical research specimens demonstrated concordant fusion detection in samples with adequate input, supporting the feasibility of this approach for quantitative *KMT2A* fusion profiling and future AML research applications.

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We thank Dr. Dongbin Xu from HematoLogics, Inc. for providing clinical research specimens for this study.

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