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TECHNICAL ADVANCE

Droplet Digital PCR for Oncogenic *KMT2A* Fusion Detection

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Acute myeloid leukemia (AML) is an aggressive blood cancer diagnosed in approximately 120,000 individuals worldwide each year. During treatment for AML, detecting residual disease is essential for prognostication and treatment decision-making. Currently, methods for detecting residual AML are limited to identifying approximately 1:100 to 1:1000 leukemic cells (morphology and DNA sequencing) or are difficult to implement (flow cytometry). AML arising after chemotherapy or radiation exposure is termed therapy-related AML (t-AML) and is exceptionally aggressive and treatment resistant. t-AML is often driven by oncogenic fusions that result from prior treatments that introduce double-strand DNA breaks. The most common t-AML–associated translocations affect *KMT2A*. There are at least 80 known *KMT2A* fusion partners, but approximately 80% of fusions involve only five partners—*AF9*, *AF6*, *AF4*, *ELL*, and *ENL*. We present a novel droplet digital PCR assay targeting the most common *KMT2A*-rearrangements to enable detection of rare AML cells harboring these fusions. This assay was benchmarked in cell lines and patient samples harboring oncogenic *KMT2A* fusions and demonstrated a limit of detection of approximately 1:1,000,000 cells. Future application of this assay could improve disease detection and treatment decision-making for patients with t-AML with *KMT2A* fusions and premalignant oncogenic fusion detection in at-risk individuals after chemotherapy exposure. *(J Mol Diagn 2023, 25: 898–906; https://doi.org/10.1016/j.jmoldx.2023.09.006)*

Acute myeloid leukemia (AML) is an aggressive blood cancer driven by a diverse but finite set of oncogenic drivers.[1,2] Detecting persistent leukemic cells after treatment is essential for subsequent treatment decision-making and long-term prognostication. Currently, the methods for detecting measurable residual disease (MRD) after treatment for AML include bone marrow morphology, multiparameter flow cytometry, and DNA sequencing.[3] Morphologic assessment only detects leukemic cells at a 5% limit of detection. Multiparameter flow cytometry has a more sensitive limit of detection at 0.01% to 0.001% but is challenging to implement and interpret and is not standardized among laboratories. DNA sequencing approaches can identify leukemic cells by their somatic mutation profile but are expensive assays to implement and can be confounded by clonal hematopoiesis in nonleukemic blood cells.[4] For patients with AML with oncogenic fusions driving their disease, the fusion itself is a molecular marker than can be leveraged for sensitive MRD detection.

Already, oncogenic fusions are used for disease monitoring in hematologic malignant tumors, such as chronic myeloid leukemia (CML), which is driven by the *BCR-ABL1* fusion.[5] Quantitative real-time RT-PCR (RT-qPCR) sensitively identifies *BCR-ABL1* fusions by using primers that span the fusion.[6] Response to therapy is assessed by log-order decreases in transcript abundance measured by RT-qPCR with a 10^{-3} *BCR-ABL1* abundance classified as a major molecular response and $10^{-4.5}$ to 10^{-5} *BCR-ABL1* abundance marking a deep molecular response and the limit of detection for most available assays.[7] Because tyrosine kinase inhibitors have become more effective, individuals

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who clear their CML (molecular response $10^{-4.5}$ to 10^{-5}) can discontinue therapy, with approximately half of these individuals remaining disease free in the long term.[8,9] Although *BCR-ABL1* is almost universally associated with CML, there is no similar singular oncogenic fusion found in AML. The most common translocations associated with *de novo* AML, including *RUNX1-RUNX1T1*, *CBFB-MYH11,* and *PML-RARA*, can be detected with qPCR-based assays.[10,11] Droplet digital PCR (ddPCR) improves on qPCR by partitioning individual DNA molecules into microfluidic droplets, enabling absolute quantification of nucleic acids in a sample.[12] The improvements of ddPCR over qPCR are ease of assay implementation, improved lower limit of detection, high specificity, and absolute quantification (compared with relative quantification with a standard curve in qPCR). ddPCR has already demonstrated utility for detecting *BCR-ABL1* fusions associated with CML and *PML-RARA* fusions associated with acute promyelocytic leukemia.[13–15] However, these techniques are difficult to implement when the gene fusion involves many different partners and is not detectable with a single assay.

Therapy-related AML (t-AML) is a unique subpopulation of AML that arises after chemotherapy or radiation exposure usually used to treat an antecedent solid tumor or lymphoma. Prior work has demonstrated that cytotoxic therapy can select for preexisting premalignant hematopoietic stem and progenitor cells that lead to t-AML.[16,17] In other cases, the therapy itself creates the oncogenic initiating event. Topoisomerase II (TOP2) inhibitors are chemotherapeutics uniquely associated with oncogenic fusions that involve the *KMT2A* gene.[18] *KMT2A* fusions are potent drivers of leukemia. Mouse models show that introduction of these fusions into healthy bone marrow progenitor cells can drive an aggressive AML.[19] Moreover, in pediatric *de novo* leukemia, *KMT2A* fusions often arise without any cooperating mutations.[20] In patients with cancer who receive high doses of TOP2 inhibitor therapy, t-AML is a devastating complication that is difficult to treat and typically fatal. The ability to detect preleukemic *KMT2A* fusions during therapy or after treatment could identify patients at high risk of t-AML who may benefit from early intervention.

In contrast to most oncogenic fusions, there are at least 80 known *KMT2A* fusion partners. However, approximately 80% of *KMT2A* fusions involve only five partners—*AF9*, *AF6*, *AF4*, *ELL,* and *ENL*.[21] Given the varied fusion partners, it is difficult to detect these fusions by qPCR. This manuscript presents a novel ddPCR assay enabling the detection of the five most common *KMT2A* fusions, accounting for the vast majority of oncogenic *KMT2A* fusions found in patients with t-AML. The assay was benchmarked using cell lines and primary patient samples with *KMT2A* fusions. Together, this assay is an inexpensive, rapid, sensitive, and specific platform for *KMT2A* fusion detection that could improve MRD detection for patients with AML with *KMT2A* fusions and enable screening for patients at risk for developing t-AML after receiving TOP2 inhibitor therapy.

Materials and Methods

Cell Lines

Human cell lines known to harbor *KMT2A* fusions were used to design, benchmark, and validate the ddPCR assay. Cell lines used were THP-1 (*KMT2A-AF9*), MOLM-13 (*KMT2A-AF9*), MV4-11 (*KMT2A-AF4*), OCI-AML2 (*KMT2A-AF6*), and KOPN8 (*KMT2A-ENL*). A sample from a patient with t-AML harboring the *KMT2A-ELL* fusion was used to design and test the *KMT2A-ELL* reagents. Cell lines without *KMT2A* fusions were used as controls, including K562, HEL, Kasumi, Jurkat, and OCI-AML3. For each ddPCR experiment, cell lines harboring a *KMT2A* fusion and cell lines without a *KMT2A* fusion were used as controls. THP-1 and HEL cells were grown in RPMI 1640 medium (ATCC, Manassas, VA), 10% heat-inactivated fetal bovine serum (HI-FBS; Gibco, Billings, MT), and 1% penicillin-streptomycin (P/S; Gibco) with 0.05 mmol/L B-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) added to the THP-1 media. MOLM-13, Jurkat, and KOPN8 cells were grown in RPMI 1640 medium (Gibco), 10% HI-FBS, and 1% P/S. Kasumi cells were grown in RPMI 1640 medium (Gibco), 20% HI-FBS, and 1% P/S. The MV-4 to 11 cells were grown in IMDM (Gibco), 10% HI-FBS, and 1% P/S. OCI-AML2 and OCI-AML3 cells were grown in Mem Alpha (Gibco), 20% HI-FBS, and 1% P/S.

Patient Samples

Cryopreserved human samples from patients with t-AML were banked at Washington University in St. Louis, MO. All patients with t-AML provided written informed consent for tissue repository and genomic sequencing in accordance with protocol 201011766 approved by the Washington University in St. Louis Institutional Review Board. Deidentified control patient peripheral blood or bone marrow samples were obtained according to a protocol approved by the Washington University Human Studies Committee.

RNA Extraction and cDNA Synthesis

RNA was extracted from cell lines and patient samples using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Up to 1×10^6 cells were processed per experiment. RNA was extracted per manufacturer recommendations without modification. The RNeasy spin columns were eluted with 25 to 50 μL of RNase-free water. The RNA concentration was quantified using Qubit Fluorometric Quantification (Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized using SuperScript IV VILO (Thermo Fisher Scientific). Synthesized cDNA molecules were stored at −20 °C.

ddPCR Primer and Probe Design

Primer and Probe Design

The genomic locations for the translocation in each cell line harboring a *KMT2A* fusion were identified from the Cancer Dependency Map (*https://depmap.org/portal*, last accessed December 17, 2021) and used to design primer sequences to span the fusions (Figure 1A, Supplemental Figure S1, and Table 1). Primers and probes were designed using Primer3Plus (*https://www.primer3plus.com*).[22] Multiple exonic primers were designed for each translocation partner. Fusion-specific cDNA amplicons generated from cell lines harboring a *KMT2A* fusion were Sanger sequenced and mapped to the hg38 reference genome using BLAT[23] to verify the fidelity of the primer pairs (Supplemental Figure S1). PrimeTime fluorescent probes (Integrated DNA Technologies, Coralville, IA) were designed to anneal within the fusion-specific primers to add sensitivity and specificity to the assay (Figure 1A and Supplemental Figure S1 and Table 1). For each fusion, one fluorescein (FAM)−labeled probe was designed to anneal to *KMT2A* upstream of the fusion, and one hexachlorofluorescein (HEX) −labeled probe was designed to anneal to the fusion partner downstream of the fusion. A control primer-probe pair was designed to tag the wild-type *KMT2A* cDNA with FAM- and HEX-labeled probes annealing in *KMT2A* exons.

ddPCR Reaction Conditions

ddPCR experiments were conducted on the QX200 Droplet Digital PCR System (Bio-Rad Laboratories, Hercules, CA). For each ddPCR reaction, the input cDNA was diluted to ensure that <330 ng of cDNA was input per reaction. Each ddPCR reaction was composed of 10 μL of 2× ddPCR Supermix for Probes no dUTP, 1000 nmol/L fusion-specific primers, 250 nmol/L fusion-specific probes, cDNA (maximum, 330 ng), and RNase/DNase-free water to 20 μL total. Droplets were generated on the QX200 Droplet Generator (Bio-Rad) per manufacturer instructions. Droplet PCR amplification occurred using the following thermocycler conditions: 94 °C for 10 seconds, 40 cycles of 94 °C for 30 seconds, 60 °C for 1 minute, 98 °C for 10 minutes, and 4 °C hold. Amplification was followed by imaging on the QX200 Droplet Reader (Bio-Rad) and analyzed using the QuantaSoft Analysis Pro software package version 7 (Bio-Rad). Multiple negative (cell lines without known *KMT2A* fusions) and positive (cell lines with known *KMT2A* fusions) controls were used per experiment. For each sample analyzed for *KMT2A* fusions, a separate aliquot was analyzed using primers and probes that targeted the wild-type *KMT2A* locus spanning exon 7 to 9 to provide an estimate of wild-type transcript abundance (Table 1).

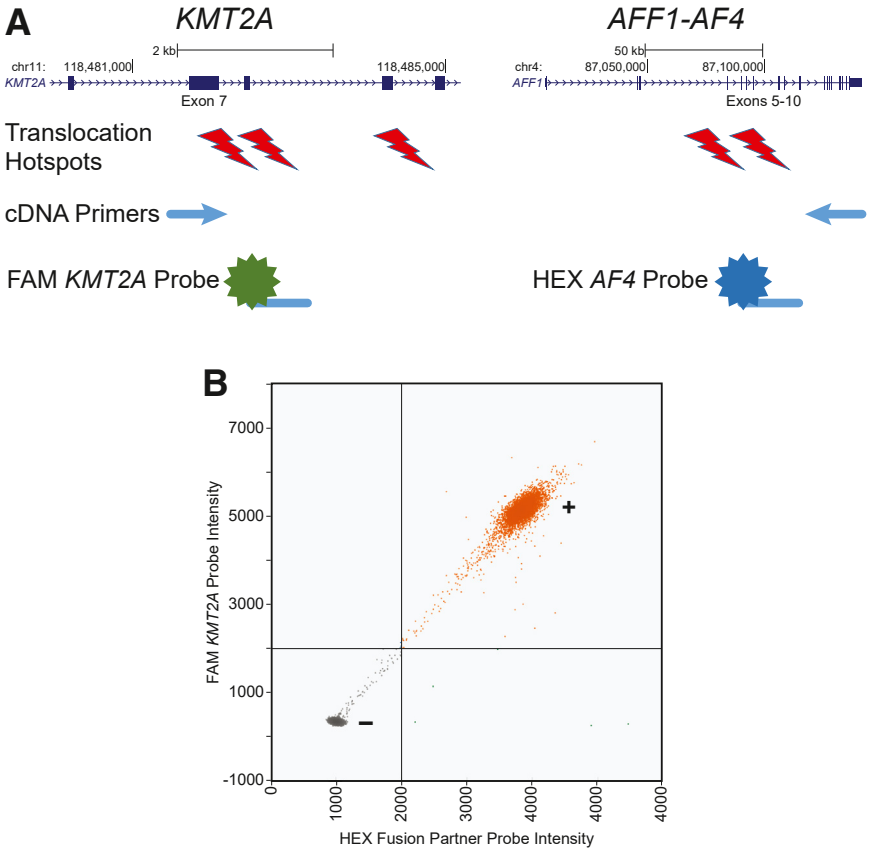

Figure 1 Design of droplet digital PCR (ddPCR) assay. **A:** Schematic of ddPCR assay. Primers spanned the fusion break points (*KMT2A-AF4* depicted). Nested fluorescently labeled probes recognized unique sequences in *KMT2A* (FAM) and the fusion partner *AFF1-AF4* (HEX). **B:** Example of ddPCR result depicting double-positive droplets containing cDNA with an oncogenic *KMT2A* fusion (+). Double-negative droplets contained wild-type *KMT2A* cDNA or no cDNA from *KMT2A* (−).

Table 1 Primer and Probe Sequences for *KMT2A* Fusion Detection

Primer-probe label	Nucleotide sequence and modifications
KMT2A_e7_fwd*	5′-CCACTCCAGCTTCCAGGAAG-3′
KMT2A_e9_fwd*	5′-CAGCACTCTCTCCAATGGCA-3′
KMT2A_e9_rev*	5′-TGCCATTGGAGAGAGTGCTG-3′
KMT2A_e11_rev*	5′-TTTGCAACGACGACAACACC-3′
AF4_AFF1_e5_rev*	5′-AACTTGGATGGCTCAGCTGT-3′
AF4_AFF1_e10_rev	5′-TTCTGACTCTGCACTGCTGG-3′
AF9_MLLT3_e6_rev*	5′-CTTGTTGCCTGGTCTGGGAT-3′
AF9_MLLT3_e8_rev	5′-TGCTTTCACTATCGCTGCCA-3′
AF6_AFDN_e2_rev*	5′-GTCGAAATTTCTCCGCGAGC-3′
AF6_AFDN_e2_rev2	5′-GGAGAGGACAGCATTCGCAT-3′
ENL_MLLT1_e2_rev	5′-CAGTCGTGAGTGAACCCCTC-3′
ENL_MLLT1_e4_rev	5′-GGTCGTAGGTGAAGCAGACC-3′
ENL_MLLT1_e7_rev*	5′-GTTCTGGGATGGCTCGAAGT-3′
ELL_ELL_e3_rev*	5′-GGCCGATGTTGGAGAGGTAG-3′
ELL_ELL_e4_rev	5′-CAGTCCAGGTGAACTTCCCC-3′
KMT2A_e7_FAMprobe	5′-/56-FAM/AGCAGGTCT/ZEN/CCCAGCCAGCA/3IABkFQ/-3′
AF4_AFF1_HEXprobe	5′-/5HEX/ACCCATTCA/ZEN/TGGCCGCCTCCTTTG/3IABkFQ/-3′
AF9_MLLT3_HEXprobe	5′-/5HEX/CCTGCCAGC/ZEN/TCCAGCTCCAG/3IABkFQ/-3′
AF6_AFDN_HEXprobe	5′-/5HEX/TCGGGTCTC/ZEN/TAGTACTGCCACCACTC/3IABkFQ/-3′
KMT2A_e9_HEXprobe	5′-/5HEX/ACCACCTCC/ZEN/GGTCAATAAGCAGGA/3IABkFQ/-3′
ENL_MLLT1_HEXprobe	5′-/5HEX/AGTCTGAGC/ZEN/TGGAGTCTGAG/3IABkFQ/-3′
KMT2A_e9_FAMprobe	5′-/56-FAM/CAGCAGATG/ZEN/GAGTCCACAGGATCAGAG/3IABkFQ/-3′
ELL_e3_HEXprobe	5′-/5HEX/AACGTCCGC/ZEN/GCCTCTGCG/3IABkFQ/-3′

All primers tested listed above.
*Primers moved forward for the droplet digital PCR assay.
fwd, forward; rev, reverse.

Pooled Assay Conditions

To enable simultaneous detection for all five *KMT2A* fusions targeted by the assay, a pooled ddPCR assay was designed and benchmarked. In a single reaction mixture, forward primers and probes for *KMT2A* exon 7 and *KMT2A* exon 9 were combined with reverse primers and probes for *AF9* exon 6, *AF4* exon 5, *AF6* exon 2, *ENL* exon 7, and *ELL* exon 3 (Table 2). The pooled primer-probe mixture was 10× concentrated, such that 2 μL of the pooled primer-probe mixture added to a 20-μL ddPCR reaction would yield a final concentration of 1000 nmol/L for each primer and 250 nmol/L for each probe. Droplet generation, thermocycler conditions, droplet imaging, and droplet analysis were performed as described above.

Dilution Series Experiments

Cell lines harboring *KMT2A* fusions were serially diluted into OCI-AML3 cells (*KMT2A* wild type). Cells were quantified by automated cell counter (Nexcelom Bioscience, Lawrence, MA). Beginning at a 50% mixture, 1,000,000 cells harboring a *KMT2A* fusion (eg, THP-1) were mixed with 1,000,000 OCI-AML3 cells. From this mixture, 200,000 cells were removed and added to 1,800,000 OCI-AML3 cells to create the 5% mixture. The serial dilution was repeated to 0.0005%, at which point it was estimated there would be <10 cells harboring a *KMT2A* fusion remaining in the mixture. RNA was extracted and converted into cDNA as described above. The cDNA was diluted to 40 ng/μL and stored at −20 °C. For the 50%, 5%, 0.5%, and 0.05% dilutions, 80 ng of cDNA was used in a single ddPCR reaction well for fusion detection. For the 0.005% dilution, 320 ng of cDNA was used per well for four ddPCR reaction wells (1280 ng total). For the 0.0005% dilution, 320 ng of cDNA was used per well for eight ddPCR

Table 2 Pooled Primers and Probes for 10× Concentrated Master Mix (Final Volume of 200 μL)

Reagent	Volume, μL
KMT2A_e7_fwd	20
KMT2A_e9_fwd	20
AF4_AFF1_e5_rev	20
AF9_MLLT3_e6_rev	20
AF6_AFDN_e2_rev	20
ENL_MLLT1_e7_rev	20
ELL_ELL_e3_rev	20
KMT2A_e7_FAMprobe	5
AF4_AFF1_HEXprobe	5
AF9_MLLT3_HEXprobe	5
AF6_AFDN_HEXprobe	5
ENL_MLLT1_HEXprobe	5
KMT2A_e9_FAMprobe	5
ELL_e3_HEXprobe	5
DNase-/RNase-free water	25

The stock primer and probes were 100 μmol/L. Each primer was 1000 nmol/L and each probe was 250 nmol/L in the final 20-μL ddPCR reaction.
fwd, forward; rev, reverse.

Table 3 sgRNA Molecules Designed to Introduce *KMT2A-AF9* Rearrangements Using CRISPR/Cas9

Label	sgRNA sequence
KMT2A_sgRNA	5′-UUAGAAAUGGAGGCUGGGCG-3′
AF9_sgRNA	5′-AUCUACUUUGCCUGCGCUAU-3′

sgRNA, single guide RNA.

reaction wells (2560 ng total). Increasing the amount of cDNA included in the reaction was necessary at the 0.005% and 0.0005% dilutions to ensure that enough cells were assayed to enable detection of the rare *KMT2A* fusions. These serial dilution experiments simulated detecting rare leukemia cells harboring *KMT2A* fusions in patients and established the limit of detection for the assay.

KMT2A Fusion Generation by Gene Editing

Prior work demonstrated that *KMT2A* fusions can be generated using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene editing.[24] This fusion break point occurs between exons 10 and 11 of *KMT2A*.[24] Guide RNA sequences targeting the intronic regions of *KMT2A* and *MLLT3/AF9* (Table 3) were synthesized (Synthego Corp., Redwood City, CA). Ribonucleoprotein complexes were formed by incubating guide RNAs (120 pmol) with Cas9 (Integrated DNA Technologies) for 20 minutes at room temperature.[25] Nucleofection was performed using the Neon Transfection System (Thermo Fisher Scientific). The ribonucleoprotein complex was combined with 250,000 cells in 10 μL of buffer R. Cells were electroporated using the settings 1700 V, 20 ms, and 1 pulse. After nucleofection, cells were cultured in appropriate media. From these cells, RNA was extracted using the above protocol and converted into cDNA. This cDNA was assayed using the *KMT2A* exon 9 and *AF9* primer-probe combination to detect oncogenic fusions.

Data Availability

No sequencing data were generated from this study. All reagents and protocols are listed in the *Materials and Methods*.

Results

Design of ddPCR Assay

The standard dual color ddPCR assay for mutation detection uses FAM- and HEX-labeled probes overlapping a region of interest that differ by a single nucleotide or small indel. Competitive annealing of the probes at the locus provides the specificity to distinguish between wild-type and mutated DNA molecules. This standard method for variant detection is not compatible with fusion detection. To enable low-frequency fusion detection, a novel cDNA-based dual color ddPCR assay was developed in which each fusion was identified by PCR primers spanning the fusion and nested fluorescently labeled probes flanking the fusion (Figure 1 and Table 1). In general, a FAM-labeled probe was designed to anneal within *KMT2A* upstream of the fusion and HEX-labeled probes were designed to anneal within the fusion partner downstream of the fusion.

ddPCR Benchmarking in Cell Lines

These primer-probe pairs were initially benchmarked in a dilution series experiment using bulk qPCR. Excellent performance was observed for both *KMT2A* FAM probe and fusion partner HEX probes across the panel (Supplemental Figure S2). For each qPCR experiment, a wild-type primer-probe pair was also incorporated into a separate reaction to estimate the abundance of the *KMT2A* wild-type transcript for comparison and ensure adequate sample preparation and loading. Once optimized, the primer-probe reagents were benchmarked on the ddPCR platform in a dilution series experiment (Supplemental Figure S3, Supplemental Table S1, and *Materials and Methods*). Cell lines harboring known *KMT2A* fusions were serially diluted into OCI-AML3 cells (*KMT2A* wild type) and analyzed using ddPCR. The appropriate cell type–specific *KMT2A* fusion was detected over 5 to 6 logs of dynamic range in this experiment. The fractional abundance of *KMT2A* fusion transcripts was determined by calculating the concentration of *KMT2A* fusion transcripts and dividing by the total number of *KMT2A* (fusion and wild-type) transcripts detected, and the expected *KMT2A* mutant cell line abundance was matched over the entire dilution series (Figure 2A). Primer-probe pairs designed to target a specific *KMT2A* fusion exhibited no off-target activity when assaying cell lines with different *KMT2A* fusions not targeted by the specific primer-pair pair (Supplemental Figure S4).

As proof of principle for *KMT2A* fusion detection in a setting that mimics patients receiving TOP2 inhibitor therapy, HEL cells with wild-type *KMT2A* were edited with CRISPR/Cas9 to introduce a *KMT2A* fusion. *KMT2A* fusions were detected 4 days after CRIPSR/Cas9 treatment and persisted at 14 and 21 days in culture. Interestingly, the fractional abundance of *KMT2A-AF9* fusion transcripts (relative to total *KMT2A* transcript abundance) remained stable for the duration of the experiment (Figure 2, B and C).

Development and Validation of a Pooled ddPCR Assay

Using a single primer-probe pair for tracking leukemic clones is useful when the oncogenic *KMT2A* fusion is known. However, in discovery settings when the KMTA2 fusion is unknown, for example, when screening for *KMT2A* fusions in patients receiving TOP2 inhibitors, simultaneous testing for all fusions is necessary. Because each reaction is limited by availability of patient sample RNA, a pooled primer-probe strategy was designed to

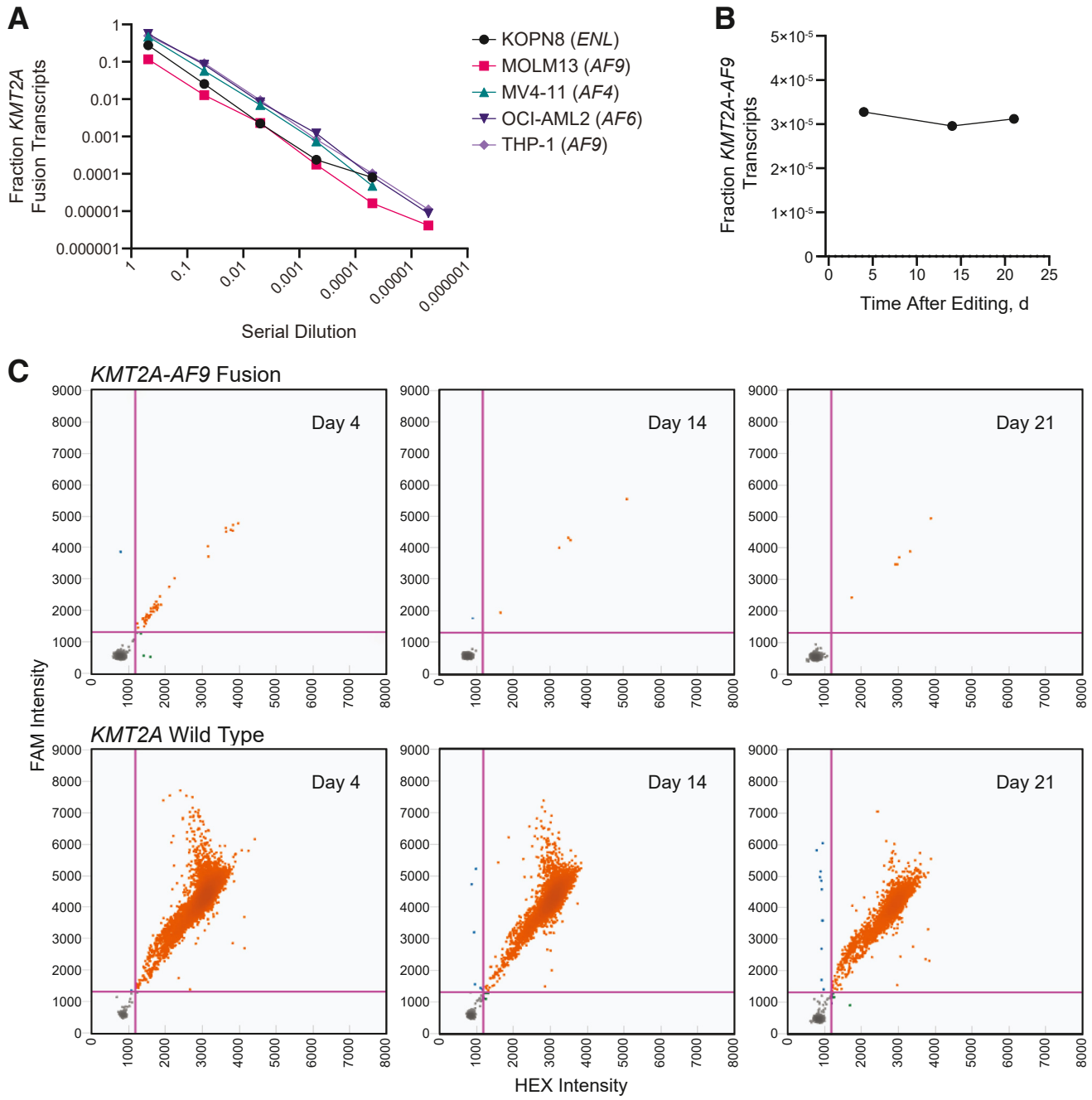

Figure 2 Droplet digital PCR (ddPCR) benchmarking in cell lines. **A:** Cell lines harboring *KMT2A* fusions were serially diluted over six orders of magnitude into OCI-AML3 cells (*KMT2A* wild type). Oncogenic *KMT2A* fusions were detected using primer-probe pairs targeting the cell type–specific *KMT2A* fusions. **B:** Quantification of *KMT2A-AF9* fusions in HEL cells edited with CRISPR/Cas9 using guides targeting the *KMT2A* and *AF9* loci, tracked over time and normalized to total (fusion and wild-type) *KMT2A* transcript abundance. **C:** ddPCR results for edited HEL cells. *KMT2A-AF9* fusion transcripts (**top panels**) and wild-type *KMT2A* transcripts (**bottom panels**) were detected at 4, 14, and 21 days. Horizontal and vertical lines demarcated the positive and negative cutoffs for fluorescent intensity, respectively.

enable discovery for the five most common *KMT2A* fusions in the same reaction (Table 2 and *Materials and Methods*). The pooled reagents detected *KMT2A* fusions in all cell lines known to harbor *KMT2A* fusions and found no evidence of *KMT2A* fusions in cell lines known not to harbor *KMT2A* fusions (Figure 3A and Supplemental Figure S5). The fraction of *KMT2A* transcripts originating from a *KMT2A* fusion was found to be 0.28 to 0.57 across the cell lines (Figure 3B).

The pooled reagents also detected *KMT2A* fusions in samples from patients with AML known to harbor *KMT2A* fusions (Supplemental Figure S6). The fractional abundance of *KMT2A* fusion transcripts was compared with orthogonal metrics of leukemia burden (Figure 3C and Supplemental

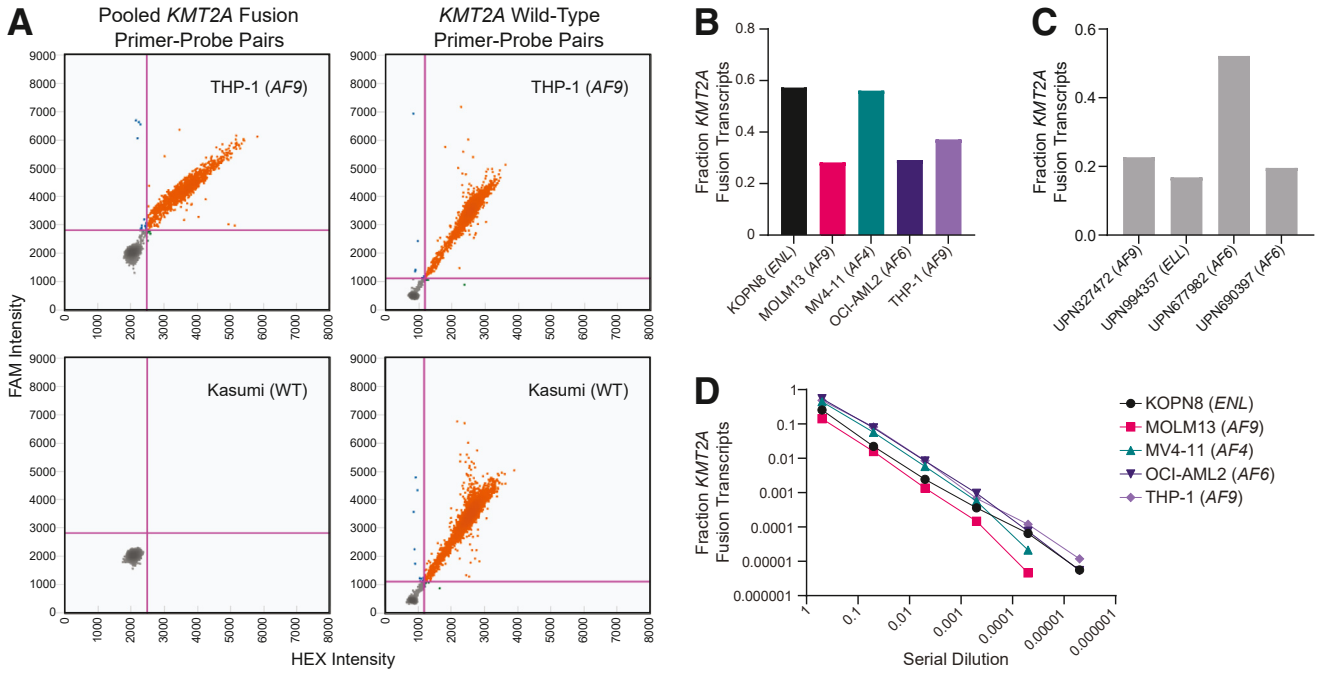

Figure 3 Development and validation of a pooled droplet digital PCR (ddPCR) assay. **A:** Example of ddPCR results using pooled primer-probe pairs targeting the five most common *KMT2A* fusions (**left panels**). *KMT2A* fusions were detected in THP-1 cells (*KMT2A-AF9*) but not detected in Kasumi cells [*KMT2A* wild-type (WT)]. *KMT2A* WT transcript abundance included for comparison (**right panels**). Horizontal and vertical lines demarcated the positive and negative cutoffs for fluorescent intensity, respectively. **B:** *KMT2A* fusion transcript abundance from multiple cell lines known to harbor *KMT2A* fusions normalized to the total *KMT2A* transcript abundance. **C:** *KMT2A* fusion transcript abundance from multiple patient samples known to harbor *KMT2A* fusions normalized to the total *KMT2A* transcript abundance. **D:** Dilution series experiment over six orders of magnitude using the pooled primer-probe pairs targeting the five most common *KMT2A* fusions in cell lines harboring *KMT2A* fusions.

Table S2). Four additional healthy control human samples were identified without any known *KMT2A* fusions, and the pooled primer-probe assay did not find any evidence of *KMT2A* fusions (Supplemental Figure S6).

Finally, a cell line dilution series experiment was conducted using the pooled primer-probe reagents. Cell lines known to harbor *KMT2A* fusions were serially diluted into OCI-AML3 cells (*KMT2A* wild type), and *KMT2A* fusions were detected using the same pooled primer-probe reagent for each cell line dilution experiment (Supplemental Figure S7 and Supplemental Table S3). The fraction abundance of *KMT2A* fusion transcripts matched the expected dilution across five to six orders of magnitude (Figure 3D).

Discussion

This article describes a novel method for *KMT2A* fusion detection by dual-color ddPCR. The assay was compared with prior techniques using bulk qPCR. Sensitive detection was found over several logs of dynamic range. The assay is specific, reliably excluding patient samples and cell lines that do not harbor *KMT2A* fusions. This assay improves on qPCR strategies because of its ease of use, accurate transcript quantification, ease of multiplex analysis, flexibility to modify or expand the target panel, and reproducibility. The assay does not require standard curves for calibration because the quantification is absolute. Prior efforts to develop a digital quantification for *KMT2A* fusions have been limited because of the promiscuous nature of *KMT2A* fusions with >80 known *KMT2A* fusion partners.[21] This assay targets the five most common *KMT2A* fusion partners that encompass approximately 80% of *KMT2A* rearranged AML cases. The limit of detection for this assay is variable based on the amount of input material, but from a limited number of dilution series experiments, a limit of detection of approximately 1:1,000,000 is estimated for this assay.

The primary limitations of this assay are the panel size and use of RNA as the biomarker. The assay targets a limited subset of 80 possible *KMT2A* fusions. However, the promiscuity of *KMT2A* to translocate with at least 80 known fusion partners has previously hindered the development of any sensitive RT-qPCR or ddPCR assays to detect *KMT2A* fusions. This assay can sensitively and specifically detect *KMT2A* fusions by ddPCR, even if limited to the most common fusions. Given the ease of assay development and validation, additional *KMT2A* fusions could be added to the assay with minimal cost and effort. The secondary limitation is use of RNA, which is less stable than DNA and requires fusion expression for detection. RNA is the necessary biomarker for this assay because of the heterogeneity of translocation break points within the *KMT2A* gene—occurring predominantly between exons 7 and 11.[26] A

similar DNA assay would require many more primer-probe pairs to cover the same set of translocation break points as this RNA-based assay. Other limitations of an RNA-based assay are the necessity of oncogene expression for detection and that transcript abundance does not necessarily correlate with leukemic burden. These limitations were mitigated by normalizing *KMT2A* fusion abundance to total (wild-type and fusion) *KMT2A* expression, which should have similar gene regulation. This normalization is supported by the cell line dilution experiments that used the fractional abundance of *KMT2A* fusion transcripts to total *KMT2A* transcripts to estimate the fraction of cells harboring *KMT2A* fusions, which matched abundance at each serial dilution. Alternatively, normalization of fusion transcript abundance to housekeeping genes is used in other ddPCR fusion assays.[15] Additionally, an RNA-based assay can underestimate disease burden if the primer-probe pairs do not perfectly capture the fusion transcript. This possibility was mitigated by covering the most common exons translocated in *KMT2A*; however, fusions harboring uncommon break points may require new primer-probe pairs to accurately detect their presence.

This platform is directly applicable for residual disease detection after treatment for AML harboring *KMT2A* fusions and for early *KMT2A* fusion detection in individuals at risk for developing *KMT2A* fusion-driven t-AML. For patients with AML with *KMT2A* fusion−driven disease, this assay could augment or replace standard methods for residual disease detection during treatment, after hematopoietic stem cell transplant or during long-term surveillance. This improvement would be similar to the marked improvements in CML treatment decision-making made possible by sensitive qPCR-based *BCR-ABL1* fusion detection. Future work will compare this assay directly with multiparameter flow cytometry and morphology for residual disease assessment during the treatment of AML. This diagnostic test could be incorporated long term into interventional clinical trials that use this biomarker for treatment decision-making. Additionally, this platform could identify premalignant *KMT2A* fusions in patients receiving TOP2 inhibitors and at risk for t-AML. Indeed, t-AML risk is highest for several cancers that affect children and adolescents, including Ewing sarcoma, Hodgkin lymphoma, and neuroblastoma.[18] It is terrible for these patients to be cured of their primary malignancy only to later develop t-AML. Although rare, t-AML is extremely difficult to treat in a population that has already received large lifetime doses of chemotherapy. Likewise, application of this assay would be essential for identifying at-risk individuals and, in the future, intervening before fulminant disease develops.

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Author Contributions

A.L.Y. conceptualized the study, designed the methodology, performed experiments, collected and analyzed data, and wrote the manuscript; H.C.D. performed experiments; and G.A.C. conceptualized the study, acquired funding, provided resources, supervised the study, and reviewed and edited the manuscript.

Disclosure Statement

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Supplemental Data

Supplemental material for this article can be found at *http://10.1016/j.jmoldx.2023.09.006*.

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