Droplet digital PCR for oncogenic KMT2A fusion detection

Andrew L Young  
*Washington University School of Medicine in St. Louis*  
Hannah C Davis  
*Washington University School of Medicine in St. Louis*  
Grant A Challen  
*Washington University School of Medicine in St. Louis*

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Acute myeloid leukemia (AML) is an aggressive blood cancer driven by a diverse but finite set of oncogenic drivers. 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. 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. 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. Quantitative real-time RT-PCR (RT-qPCR) sensitively identifies BCR-ABL1 fusions by using primers that span the fusion. 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. Because tyrosine kinase inhibitors have become more effective, individuals...
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.\textsuperscript{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 \textit{de novo} AML, including RUNX1-RUNXIT1, CBFB-MYH11, and PML-RARA, can be detected with qPCR-based assays.\textsuperscript{10,11} Droplet digital PCR (ddPCR) improves on qPCR by partitioning individual DNA molecules into microliter droplets, enabling absolute quantification of nucleic acids in a sample.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{19} Moreover, in pediatric \textit{de novo} leukemia, KMT2A fusions often arise without any cooperating mutations.\textsuperscript{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.\textsuperscript{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), 10\% 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 $1 \times 10^6$ 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 $-20^\circ C$. 

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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]. 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 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 (−).
were performed as described above. Droplet generation, ther-
concentrated, such that 2
concentrations targeted by the assay, a pooled ddPCR assay was
designing and benchmarking. In a single reaction mixture,
forward primers and probes for
exon 3 (Table 2). The pooled primer-probe mixture was
exon 6, m
L ddPCR reaction would
exon 7, and
ELL_e3_rev2
5’-GGAGAGGAGACCACTTGCAT-3’
5’-ACCACCTCC/ZEN/GGTCAATAAGCAGGA/3IABkFQ/-3
5’-CCTGCCAGC/ZEN/TCCAGCTCCAG/3IABkFQ/-3
5’-HEX/ACCCATTCA/ZEN/TGGCCGCCTTCTTTG/3IABkFQ/-3
AF4_AFF1_hexprobe
5’-/56-FAM/AGCAGGTCT/ZEN/CCCAGCCAGCA/3IABkFQ/-3’
Fusion Detection by DDPCR
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
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_e4_rev
20
ELL_MLLT1_e7_rev
20
KMT2A_e9_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 2  Pooled Primers and Probes for 10× Concentrated Master Mix (Final Volume of 200 µL)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume, µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>KMT2A_e7_fwd</td>
<td>20</td>
</tr>
<tr>
<td>KMT2A_e9_fwd</td>
<td>20</td>
</tr>
<tr>
<td>AF4_AFF1_e5_rev</td>
<td>20</td>
</tr>
<tr>
<td>AF9_MLLT3_e6_rev</td>
<td>20</td>
</tr>
<tr>
<td>AF6_AFDN_e2_rev</td>
<td>20</td>
</tr>
<tr>
<td>ENL_MLLT1_e7_rev</td>
<td>20</td>
</tr>
<tr>
<td>ELL_MLLT1_e7_rev</td>
<td>20</td>
</tr>
<tr>
<td>KMT2A_e9_FAMprobe</td>
<td>5</td>
</tr>
<tr>
<td>AF4_AFF1_HEXprobe</td>
<td>5</td>
</tr>
<tr>
<td>AF9_MLLT3_HEXprobe</td>
<td>5</td>
</tr>
<tr>
<td>AF6_AFDN_HEXprobe</td>
<td>5</td>
</tr>
<tr>
<td>ENL_MLLT1_HEXprobe</td>
<td>5</td>
</tr>
<tr>
<td>KMT2A_e9_FAMprobe</td>
<td>5</td>
</tr>
<tr>
<td>ELL_e3_HEXprobe</td>
<td>5</td>
</tr>
<tr>
<td>DNase-/RNase-free water</td>
<td>25</td>
</tr>
</tbody>
</table>

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

Pooled Assay Conditions
To enable simultaneous detection for all five KMT2A fu-
sions 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, ther-
mocycler 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 1 Primer and Probe Sequences for KMT2A Fusion Detection

<table>
<thead>
<tr>
<th>Primer-probe label</th>
<th>Nucleotide sequence and modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>KMT2A_e7_fwd*</td>
<td>5’-CCACTCCACCTCCAGAGGAAACG-3’</td>
</tr>
<tr>
<td>KMT2A_e9_fwd*</td>
<td>5’-CAGGACTCTCTCCACAGCAG-3’</td>
</tr>
<tr>
<td>KMT2A_e9_rev*</td>
<td>5’-TGCATGAGGAGAAGCTGCTG-3’</td>
</tr>
<tr>
<td>KMT2A_e11_rev*</td>
<td>5’-TTTGACAACGACGACAC-3’</td>
</tr>
<tr>
<td>AF4_AFF1_e5_rev*</td>
<td>5’-AAGGCTAGGGCGCTG-3’</td>
</tr>
<tr>
<td>AF4_AFF1_e10_rev</td>
<td>5’-TTTGGACCTCTGCACTGCTG-3’</td>
</tr>
<tr>
<td>AF9_MLLT3_e6_rev*</td>
<td>5’-CTTTGGCTGCTGCTGAGGAT-3’</td>
</tr>
<tr>
<td>AF9_MLLT3_e8_rev</td>
<td>5’-GGTCTGAGGATGAGCAGGAC-3’</td>
</tr>
<tr>
<td>AF6_AFDN_e2_rev*</td>
<td>5’-GTCGAATTCCCGCCAGGAC-3’</td>
</tr>
<tr>
<td>AF6_AFDN_e2_rev2</td>
<td>5’-GGAGAGGAGACCACTTGCAT-3’</td>
</tr>
<tr>
<td>ENL_MLLT1_e2_rev</td>
<td>5’-GAAGCTAGGGAGAAGCTGCTG-3’</td>
</tr>
<tr>
<td>ENL_MLLT1_e4_rev</td>
<td>5’-GTCGAAATTTCTCCCGCCAGGAC-3’</td>
</tr>
<tr>
<td>ENL_MLLT1_e7_rev*</td>
<td>5’-GTCGAAATTTCTCCCGCCAGGAC-3’</td>
</tr>
<tr>
<td>ELL_ELL_e3_rev*</td>
<td>5’-GCCGATGTTGGAGAGAGT-3’</td>
</tr>
<tr>
<td>ELL_ELL_e4_rev</td>
<td>5’-CAGGCTAGGGAACCTCCCC-3’</td>
</tr>
<tr>
<td>KMT2A_e7_FAMprobe</td>
<td>5’-56-FAM/ACCCAGTGCT/ZEN/CCAGCCAGCA/3IABkFQ/-3’</td>
</tr>
<tr>
<td>AF4_AFF1_HEXprobe</td>
<td>5’-5HEX/ACCAGTGCT/ZEN/CCAGCCAGCA/3IABkFQ/-3’</td>
</tr>
<tr>
<td>AF9_MLLT3_HEXprobe</td>
<td>5’-5HEX/CCTGCCACG/ZEN/TCCAGCTCCAG/3IABkFQ/-3’</td>
</tr>
<tr>
<td>AF6_AFDN_HEXprobe</td>
<td>5’-5HEX/TGGGCTCTC/ZN/TTACTGCTGACCCACCTC/3IABkFQ/-3’</td>
</tr>
<tr>
<td>KMT2A_e9_Hexprobe</td>
<td>5’-5HEX/CCACCTCC/ZN/GTCGAATAAGCAGGA/3IABkFQ/-3’</td>
</tr>
<tr>
<td>ENL_MLLT1_Hexprobe</td>
<td>5’-5HEX/AGTCTGGAC/ZN/TGCCAGCAGG/3IABkFQ/-3’</td>
</tr>
<tr>
<td>KMT2A_e9_FAMprobe</td>
<td>5’-56-FAM/CCAGCGATG/ZN/GAGTCCACGGAGGAT/3IABkFQ/-3’</td>
</tr>
<tr>
<td>ELL_e3_HEXprobe</td>
<td>5’-5HEX/AACGTCGGC/ZN/GTCCCTGCG/3IABkFQ/-3’</td>
</tr>
</tbody>
</table>

*Primers moved forward for the droplet digital PCR assay.
fwd, forward; rev, reverse.
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. This fusion break point occurs between exons 10 and 11 of KMT2A. 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. 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 CRISPR/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 KMT2A 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

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

<table>
<thead>
<tr>
<th>Label</th>
<th>sgRNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KMT2A_sgRNA</td>
<td>5′-UUAGAAAUUGGGCGCUUCA-3′</td>
</tr>
<tr>
<td>AF9_sgRNA</td>
<td>5′-AUCUACUUUGGUCCCUUU-3′</td>
</tr>
</tbody>
</table>

sgRNA, single guide RNA.
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 S6).

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.
KMT2A fusions in cell lines harboring transcript abundance. KMT2A transcript abundance. for fluorescent intensity, respectively. Horizontal and vertical lines demarcated the positive and negative cutoffs for fluorescent intensity, respectively. 

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 (WT)). KMT2A WT transcript abundance included for comparison (right panels). Horizontal and vertical lines demarcated the positive and negative cutoffs for fluorescent intensity, respectively. 

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. 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. 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. 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. 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.

References


