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Noninvasive personalized fusion measurable residual disease detection in acute myeloid leukemia using digital PCR

Running Title. Improving Fusion AML MRD Detection

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Acute myeloid leukemia (AML) is an aggressive hematologic malignancy that requires accurate assessment of disease burden to guide effective care[1]. Detection of leukemia cells below the limit of detection (LOD) of conventional morphology is termed measurable residual disease (MRD) detection and is predominantly assessed using multi-parameter flow cytometry (MFC) or quantitative PCR (qPCR)[2]. MFC detects residual leukemia cells by identifying a leukemia-associated immunophenotype or a "different-from-normal" immunophenotype, which enables a 0.01% LOD[2]. Despite this low reported LOD, 10-20% of patients who are MRD negative by MFC relapse within a few months of testing[3,4]. Sequencing-based MRD approaches show promise for tracking leukemia-associated single nucleotide variants and small insertions/deletions but are often limited by cost and the persistence of pre-leukemic clonal hematopoiesis mutations which complicate interpretation[5]. Quantitative PCR (qPCR) has emerged as an effective MRD technology for detecting a subset of leukemia-associated genomic alterations such as *NPM1* insertions and recurrent gene fusions such as *RUNX-RUNX1T1*[6,7]. Unfortunately, these assays are only available for the handful of common genomic alterations driving AML and there are hundreds of less common AML gene fusions that do not have a molecular MRD assay[8].

To address this diagnostic gap, we developed a platform for personalized gene fusion MRD detection using digital PCR (dPCR) and have previously shown the analytical validity of the assay[9]. Here, we utilized patient-specific fusion assays for serial MRD monitoring in peripheral blood (PB) or bone marrow (BM) samples from patients undergoing treatment for AML. We focused specifically on *KMT2A* fusion-driven leukemia given the significant heterogeneity in fusion partners that precludes widespread qPCR-based molecular monitoring and the pressing need to develop clinical assays to assess the efficacy of novel therapies target *KMT2A* fusion-driven leukemia[10,11]. However, our approach is broadly applicable to any fusion-driven leukemia. We demonstrate the feasibility and clinical performance of non-invasive dPCR-based MRD detection.

Patients with gene fusion-driven leukemia were identified and provided written informed consent under protocols 201011766 and 202509075 approved by the Washington University Institutional Review Board. This study was observational and performed in parallel to standard of care treatment; no results were returned to patients or providers. From clinical whole genome sequencing (WGS) obtained at diagnosis[12], the genomic coordinates of the translocation breakpoints were utilized to design personalized dPCR assays and validated in high disease burden specimens (e.g. diagnosis PB or BM) as described previously (**Figure 1A**)[9]. For subsequent samples obtained after initial diagnosis (during or after treatment), whole PB or BM specimens underwent RNA extraction, RNA quantification, cDNA conversion and dPCR preparation as described previously (**Figure 1B**)[9]. Assays were run on the Bio-Rad (Hercules, California, USA) QX200 platform using 20-100ng of cDNA for diagnostic specimens and up to 1200ng for MRD testing specimens, split over multiple wells. Each 20µL dPCR reaction contained dPCR Supermix for Probes (Bio-Rad #1863023), 1000nM primers, 250nM probes, and cDNA as described above. Partitions were generated on the Bio-Rad QX200 Droplet Generator and analyzed on the Bio-Rad QX200 Droplet Reader. Disease burden was reported as relative transcript abundance—the concentration of fusion transcripts divided by all transcripts (fusion and wild-type) for the 5-prime partner gene as described previously[9].

A total of 20 patients were included (age range, 19–77 years; median, 49). The disease phenotypes included 16 *de novo* AML, 2 therapy-related AML (t-AML), 1 acute leukemia, and 1 acute lymphoblastic leukemia (ALL; **Table 1**, **Supplementary Table 1**). Seven patients experienced disease recurrence as determined by standard-of-care (SOC) assessment and had specimens banked prior to relapse available for dPCR-based MRD testing. The *KMT2A*-

rearrangements encompassed a diverse set of fusion partners including *AF9*, *AF4*, *AF6*, *MLLT11*, *ELL*, *AF10* and *SEPTIN6* (**Table 1, Supplementary Table 1**).

In total, 26 bone marrow (BM) and 107 peripheral blood (PB) samples were banked (median 5 samples per patient; range 1–21). At diagnosis, personalized fusion-specific dPCR assays detected high fusion transcript abundance in all patients. Interestingly, for UPN510086 the diagnostic sequencing identified a *KMT2A::EP300* fusion that was initially undetectable by dPCR; but a reciprocal *EP300::KMT2A* also identified by WGS was highly expressed in the leukemia cells, detectable by dPCR and suitable for MRD monitoring (**Supplementary Figure 1A**). This highlights the utility of WGS to define multiple fusion breakpoints to inform personalized MRD assay development, even for non-canonical fusions.

Among 10 patients with serial sampling during initial therapy, seven experienced treatment failure. For those seven patients, patient-specific fusion transcripts were detected prior to relapse as assessed by SOC testing or clinical progression, with a median lead time of 92 days and range of 36–145 days (**Figure 2A-D, Supplementary Figure 1B-D**).

Several representative cases highlight the utility of serial blood-based MRD monitoring for the early detection of disease recurrence. In UPN530934, dPCR was positive in PB 44 days before a bone marrow biopsy (BMBx) with positive MFC MRD testing (**Figure 2A**). In UPN435200, dPCR was positive in PB 106 days before a FISH positive (0.5%) BMBx and, notably, MFC MRD testing had been negative two weeks before that BMBx (**Figure 2B**). UPN922368 was positive at end of induction by dPCR when SOC testing was negative and 107 days before the patient was FISH positive during consolidation (but MFC MRD negative) and 168 days before recurrence was confirmed by multiple modalities, prompting a change in therapy (**Figure 2C**). For UPN614333 disease recurrence was detected during long-term surveillance in the PB 92 days before the patient clinically relapsed (**Figure 2D**). Additional patients demonstrated detectable disease at end of induction or during consolidation, which preceded treatment failure detection by SOC modalities or overt relapse (**Supplementary Figures 1B-D**). Conversely, for patients who cleared their fusions and remained negative by dPCR no disease recurrence was observed (**Figures 2E, 2F; Supplementary Figure 1E**). One patient relapsed (UPN159941), but only had the diagnosis and relapse specimens available, which confirmed high disease burden at both timepoints (**Supplementary Figure 1F**). For patients with MFC MRD testing and dPCR performed on the same BM specimen (n=15), five specimens were negative by both modalities, six specimens were positive by both modalities, and four specimens were positive by dPCR and negative by MFC MRD (**Supplementary Figure 1G**).

In several long-term survivors who were years out from their initial diagnosis, a personalized fusion MRD assay detected their disease at diagnosis and remained negative in available specimens from extended surveillance (**Supplementary Figure 2**), similarly to what has been previously reported in a cohort of long remission patients[13].

Collectively, these findings demonstrate that personalized serial PB monitoring by dPCR can detect persistent or rising patient-specific fusion transcripts months before disease is detect by SOC testing or overt clinical relapse (**Figure 2G**). For UPN614333 molecular recurrence was detected in the PB more than two years after diagnosis and more than a year after their last BM biopsy, which highlights the value of non-invasive long-term surveillance when routine BM-based testing is no longer performed.

This approach is feasible because personalized dPCR assays are straightforward to design and deploy, while offering extremely high sensitivity even in PB samples. When PB and BM samples were obtained at similar times, fusion transcript abundance was highly concordant between the sample types (**Figure 2H**). By enabling a low LOD of 1:100,000 with high specificity, the negative results may provide greater reassurance compared to SOC MRD testing which are associated with a 10-20% false negative rate[3,4].

These findings highlight the utility of frequent longitudinal molecular MRD testing to accurately track the residual disease *trajectory* in patients at risk of relapse. Detecting treatment failure early, when the disease burden is low, enables an escalation in therapy before patients clinically decompensate. Once patients decompensate, an escalation in therapy is less effective and often not tolerated. In contrast, current MRD strategies rely on increasingly sensitive testing at predefined treatment timepoints (e.g., pre-transplant) to predict outcomes, yet they often fail to detect recurrence before it becomes clinically overt. While we initially focused on *KMT2A*-rearranged leukemia due to its molecular heterogeneity and emerging targeted therapies, this approach is broadly adaptable to any fusion-driven leukemia. Other prior studies have focused on technical optimization of these types of personalized assays[9,14,15]. We have extended these prior works by showing the utility of frequent, serial, blood-based monitoring to augment or replace BM-based MFC MRD testing in fusion-driven leukemia. We demonstrate that personalized dPCR-based fusion MRD detection accurately quantifies disease burden, can identify residual disease missed by MFC and enables non-invasive serial blood-based MRD monitoring. Long-term this approach could improve treatment decision-making and lead to better outcomes for patients with fusion-driven leukemia.

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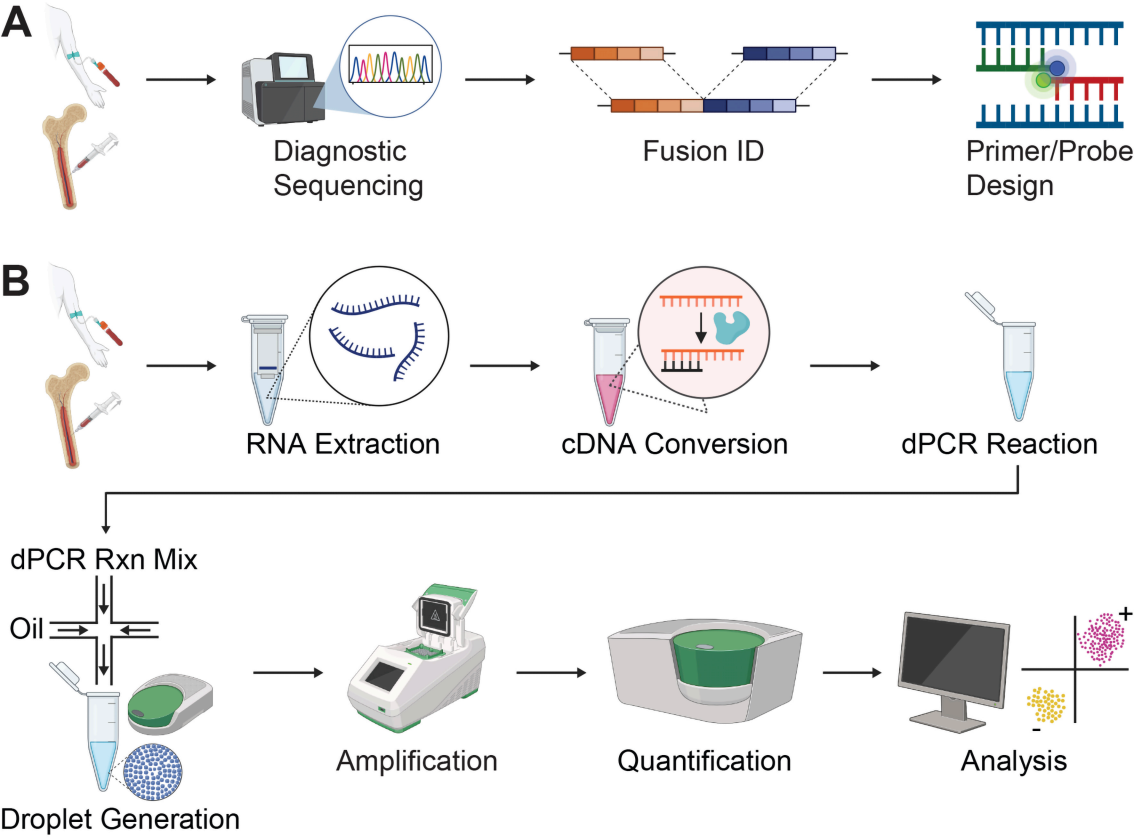
Table 1. Study Population Characteristics.

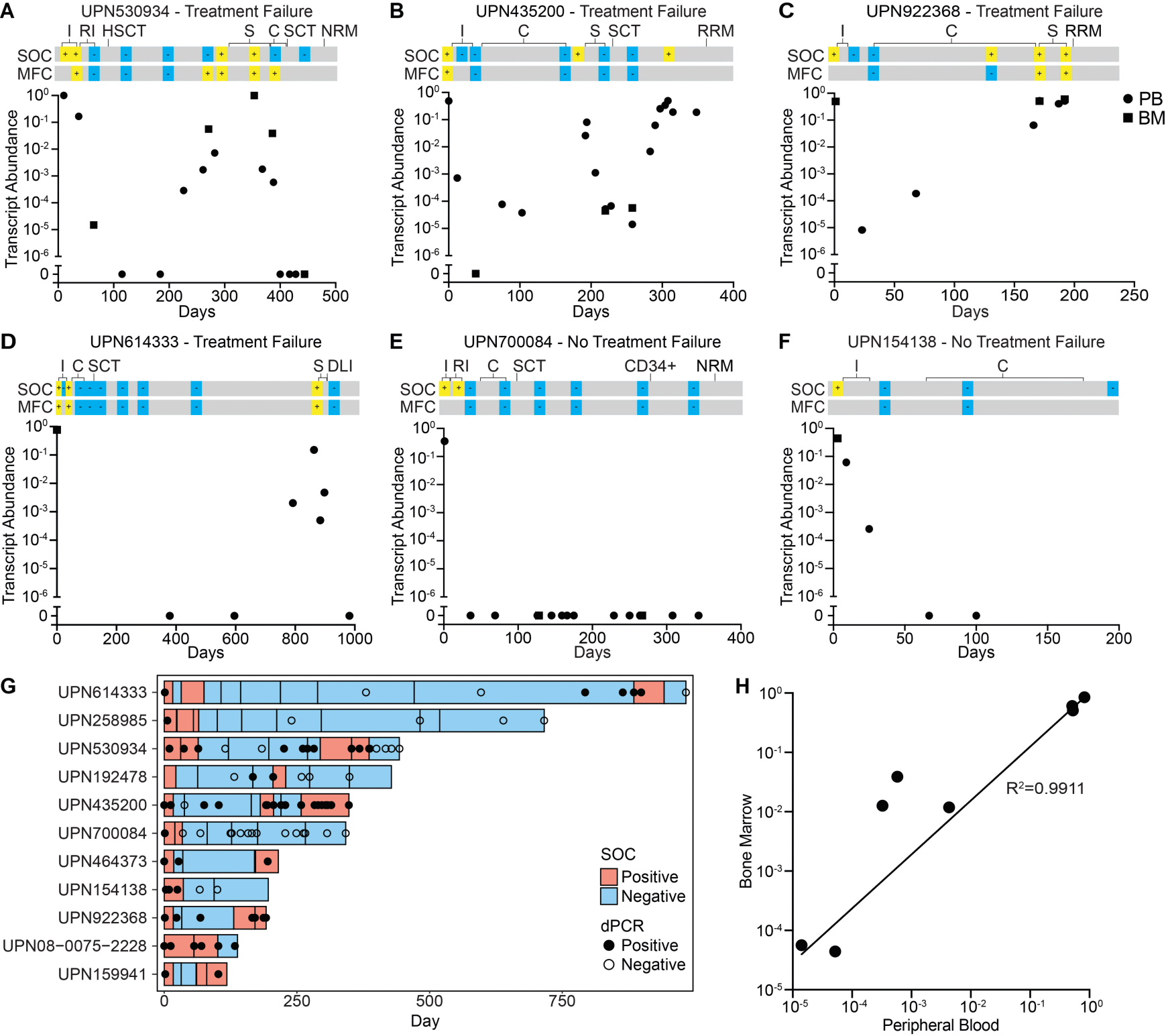
Characteristic	Value	
Participants	20	
Median Samples per Participant (Range)	5 (1-21)	
BM Samples	26	
PB Samples	107	
Median Age	43 years	
Male (%)	5 (25%)	
Female (%)	15 (75%)	
Relapsed/Treatment Failure	8	
Genomic Alteration	Fusion	Count
t(4;11)(q21.3;q23.3)	*KMT2A*::*AF4*	1
t(6;11)(q27;q23.3)	*KMT2A*::*AF6*	2
t(9;11)(p21.3;q23.3)	*KMT2A*::*AF9*	10
t(10;11)(p12.31;q23.3)	*KMT2A*::*AF10*	3
t(11;19)(q23.3;p13.11)	*KMT2A*::*ELL*	1
t(X;11)(q24;q23.3)	*KMT2A*:*SEPTIN6*	1
t(1;11)(q21.3;q23.3)	*KMT2A*::*MLLT11*	1
t(11;22)(q23.3;q13.2)	*EP300*::*KMT2A*	1

Figure Legends

Figure 1. Workflow for patient-specific fusion dPCR assay development and MRD monitoring in AML. (A) Schematic of assay design. At diagnosis, clinical sequencing of peripheral blood or bone marrow identified patient-specific gene fusions. The fusion breakpoint coordinates were utilized to design custom primers and probes targeting the patient-specific gene fusion. **(B)** dPCR workflow for MRD monitoring. Peripheral blood or bone marrow samples were collected longitudinally. RNA was extracted and reverse transcribed into cDNA. Fusion-specific primers and probes were combined with cDNA and dPCR master mix, followed by droplet generation to partition the reagents into thousands of nanoliter droplets. PCR amplification was performed and droplets were analyzed to distinguish positive from negative droplets.

Figure 2. Longitudinal dPCR-based fusion transcript quantification in AML patients. (A–F) Serial monitoring of fusion transcript abundance over time by dPCR. Each point represents a single peripheral blood (circle) or bone marrow (square) sample. The treatment course description includes induction (I), re-induction (RI), consolidation (C), salvage (S), hematopoietic stem cell transplantation (SCT) and/or donor lymphocyte infusion (DLI). Relapse related mortality (RRM) and non-relapse related mortality (NRM) were also listed. Standard of care (SOC) testing (all modalities and MFC MRD alone) results were indicated as positive (yellow) or negative (blue). **(G)** Swimmer plot of dPCR and clinical testing results for 11 patients with serial testing available early during their treatment course. Horizontal bars represented disease status based on clinical testing in red (positive) or blue (negative). Symbols mark dPCR testing result with solid circles (positive) and open circles (negative). **(H)** Comparison of patient-specific fusion transcript abundance in paired bone marrow and peripheral blood by dPCR.

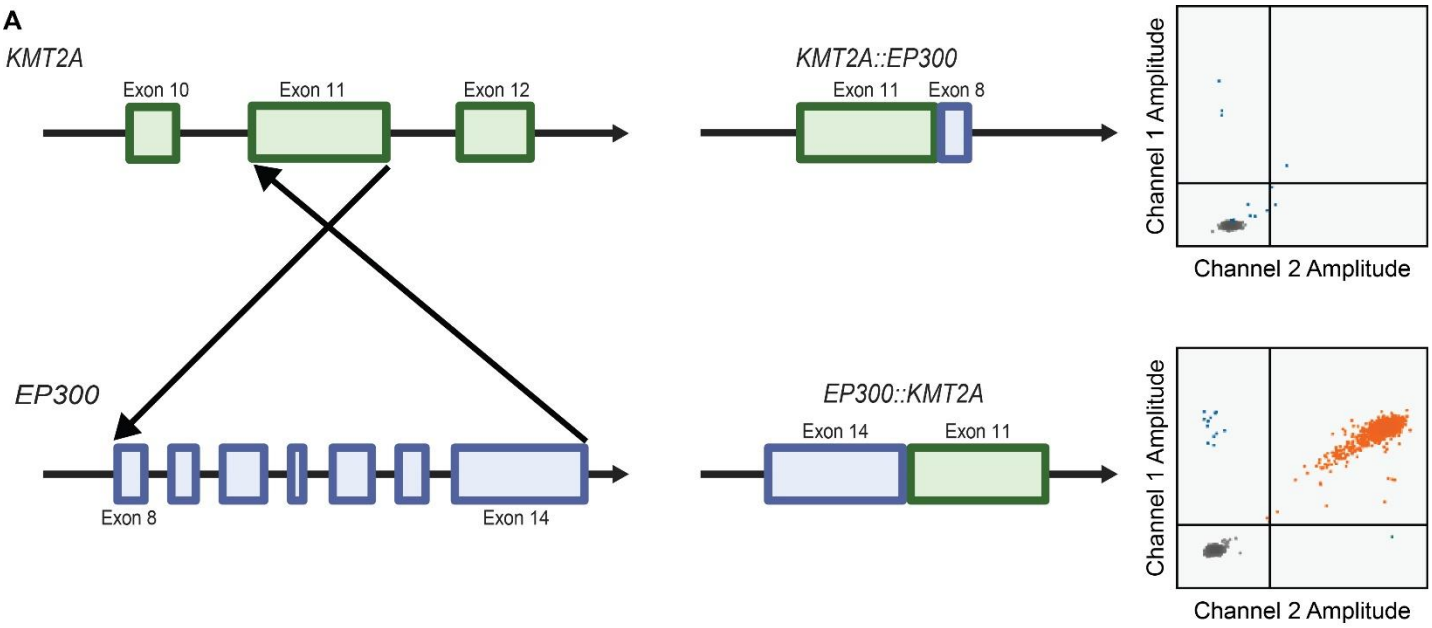

A
Diagnostic Sequencing
Fusion ID
Primer/Probe Design
B
RNA Extraction
cDNA Conversion
dPCR Reaction
dPCR Rxn Mix
Oil
Droplet Generation
Amplification
Quantification
Analysis
+
-

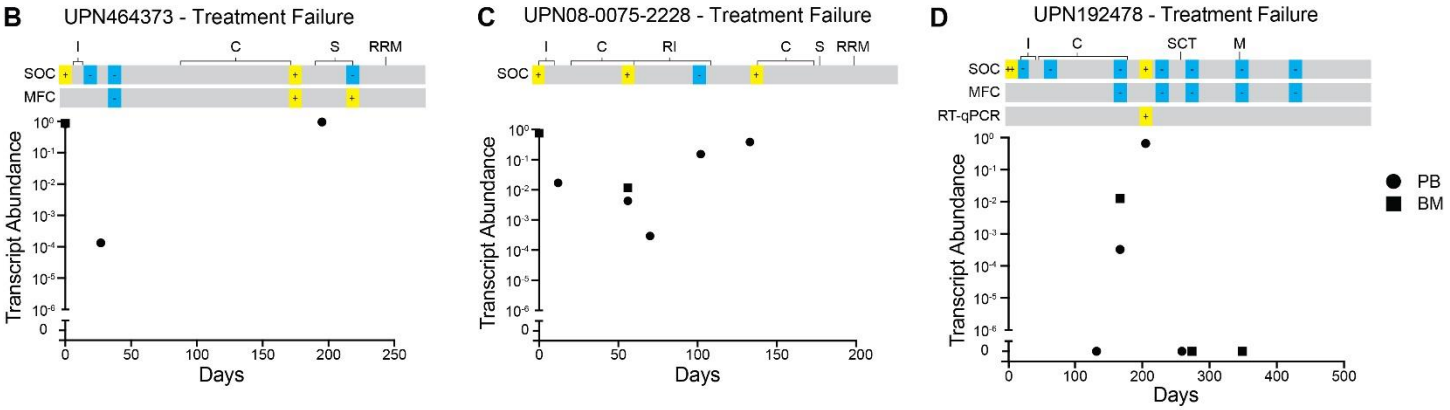

A
UPN530934 - Treatment Failure
I RI HSCT S C SCT NRM
SOC
MFC
Transcript Abundance
Days
B
UPN435200 - Treatment Failure
I C S SCT RRM
C
UPN922368 - Treatment Failure
I C S RRM
PB
BM
D
UPN614333 - Treatment Failure
I C SCT S DLI
E
UPN700084 - No Treatment Failure
I RI C SCT CD34+ NRM
F
UPN154138 - No Treatment Failure
I C
G
UPN614333
UPN258985
UPN530934
UPN192478
UPN435200
UPN700084
UPN464373
UPN154138
UPN922368
UPN08−0075−2228
UPN159941
Day
SOC
Positive
Negative
dPCR
Positive
Negative
H
Bone Marrow
Peripheral Blood
R²=0.9911

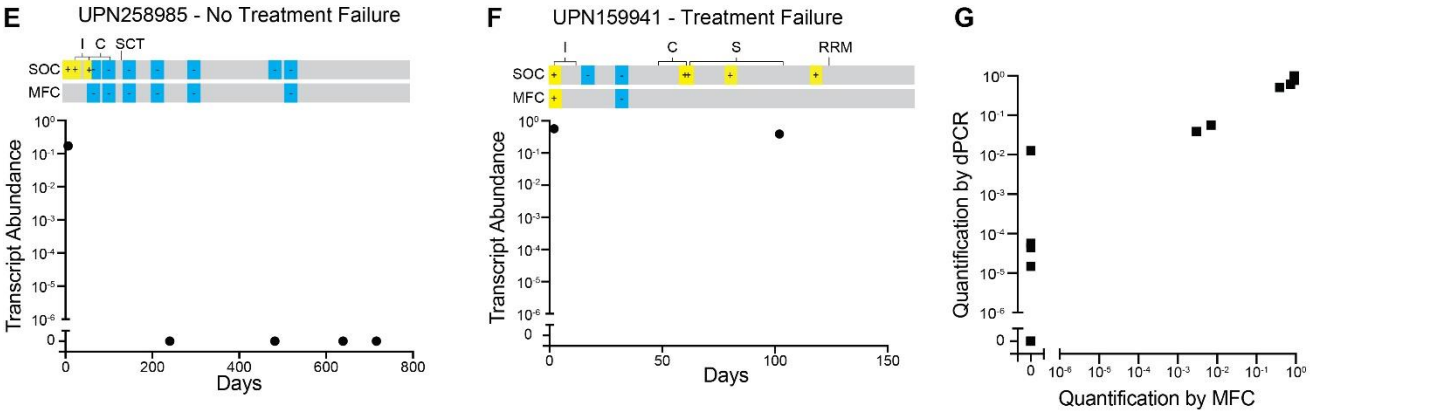
Supplementary Table 1: Unique Patient Number (UPN), disease type and exon-level fusion breakpoint information.

UPN	Translocation	Fusion	5' Partner	3' Partner	Diagnosis
UPN08-0075-2228	t(4;11)(q21.3;q23.3)	*KMT2A::AF4*	*KMT2A* exon 7	*AF4* exon 6	ALL
UPN440617	t(6;11)(q27;q23.3)	*KMT2A::AF6*	*KMT2A* exon 7	*AF6* exon 2	AML
UPN530934	t(6;11)(q27;q23.3)	*KMT2A::AF6*	*KMT2A* exon 35	*AF6* exon 2	acute leukemia
UPN154138	t(9;11)(p21.3;q23.3)	*KMT2A::AF9*	*KMT2A* exon 9	*AF9* exon 6	AML
UPN192478	t(9;11)(p21.3;q23.3)	*KMT2A::AF9*	*KMT2A* exon 9	*AF9* exon 6	AML
UPN258985	t(9;11)(p21.3;q23.3)	*KMT2A::AF9*	*KMT2A* exon 9	*AF9* exon 6	AML
UPN435200	t(9;11)(p21.3;q23.3)	*KMT2A::AF9*	*KMT2A* exon 7	*AF9* exon 6	AML
UPN763371	t(9;11)(p21.3;q23.3)	*KMT2A::AF9*	*KMT2A* exon 9	*AF9* exon 6	T-AML
UPN902615	t(9;11)(p21.3;q23.3)	*KMT2A::AF9*	*KMT2A* exon 7	*AF9* exon 6	AML
UPN918315	t(9;11)(p21.3;q23.3)	*KMT2A::AF9*	*KMT2A* exon 7	*AF9* exon 6	AML
UPN922368	t(9;11)(p21.3;q23.3)	*KMT2A::AF9*	*KMT2A* exon 7	*AF9* exon 6	AML
UPN614333	t(9;11)(p21.3;q23.3)	*KMT2A::AF9*	*KMT2A* exon 9	*AF9* exon 6	AML
UPN924089	t(9;11)(p21.3;q23.3)	*KMT2A::AF9*	*KMT2A* exon 7	*AF9* exon 6	AML
UPN159941	t(10;11)(p12.31;q23.3)	*KMT2A::AF10*	*KMT2A* exon 7	*AF10* exon 5	AML
UPN464373	t(10;11)(p12.31;q23.3)	*KMT2A::AF10*	*KMT2A* exon 5	*AF10* exon 14	AML
UPN700084	t(10;11)(p12.31;q23.3)	*KMT2A::AF10*	*KMT2A* exon 7	*AF10* exon 9	AML
UPN242129	t(11;19)(q23.3;p13.11)	*KMT2A::ELL*	*KMT2A* exon 9	*ELL* exon 6	AML
UPN228699	t(X;11)(q24;q23.3)	*KMT2A:SEPTIN6*	*KMT2A* exon 9	*SEPTIN* exon 6	AML
UPN585442	t(1;11)(q21.3;q23.3)	*KMT2A::MLLT11*	*KMT2A* exon 9	*MLLT11* exon 2	AML
UPN510086	t(11;22)(q23;q13)	*EP300::KMT2A*	*EP300* exon 14	*KMT2A* exon 11	T-AML

Supplementary Figure 1. Detection and longitudinal monitoring of canonical and non-canonical *KMT2A* fusion transcripts by dPCR. (A) Non-canonical *EP300::KMT2A* rearrangement identified in UPN510086 with dPCR showing low *KMT2A::EP300* and high *EP300::KMT2A* transcript abundance. **(B–F)** Longitudinal dPCR-based quantification of fusion transcript abundance during therapy in peripheral blood (circles) and bone marrow (squares) samples. Treatment course and disease status was included with induction (I), re-induction (RI), consolidation (C), salvage (S), HSCT, maintenance (M), and relapse-related mortality (RRM). Yellow (+) and blue (-) boxes indicated MRD positivity and negativity by SOC testing or MFC MRD testing. **(G)** Disease burden quantification of bone marrow specimens that were assessed by MFC and dPCR at the same timepoint.







Supplementary Figure 2. dPCR fusion transcript quantification for patients several years out from initial therapy and in long-term remission. dPCR-based quantification of fusion transcript abundance at diagnosis and during remission after completing therapy. Transcript abundance is shown on a log10 scale (y-axis) over time in days (x-axis). Circles represent peripheral blood (PB) samples and squares represent bone marrow (BM) samples. These patients do not overlap with patients presented in Figure 2 or Supplementary Figure 1.