

Targeted Next-Generation Sequencing for Detecting *MLL* Gene Fusions in Leukemia

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Abstract

Mixed lineage leukemia (*MLL*) gene rearrangements characterize approximately 70% of infant and 10% of adult and therapy-related leukemia. Conventional clinical diagnostics, including cytogenetics and fluorescence *in situ* hybridization (FISH) fail to detect *MLL* translocation partner genes (TPG) in many patients. Long-distance inverse (LDI)-PCR, the "gold standard" technique that is used to characterize *MLL* breakpoints, is laborious and requires a large input of genomic DNA (gDNA). To overcome the limitations of current techniques, a targeted next-generation sequencing (NGS) approach that requires low RNA input was tested. Anchored multiplex PCR-based enrichment (AMP-E) was used to rapidly identify a broad range of *MLL* fusions in patient specimens. Libraries generated using Archer FusionPlex Heme and Myeloid panels were sequenced using the Illumina platform. Diagnostic specimens ($n = 39$) from pediatric leukemia patients were tested with AMP-E and validated by LDI-PCR. In concordance with

LDI-PCR, the AMP-E method successfully identified TPGs without prior knowledge. AMP-E identified 10 different *MLL* fusions in the 39 samples. Only two specimens were discordant; AMP-E successfully identified a *MLL-MLLT1* fusion where LDI-PCR had failed to determine the breakpoint, whereas a *MLL-MLLT3* fusion was not detected by AMP-E due to low expression of the fusion transcript. Sensitivity assays demonstrated that AMP-E can detect *MLL-AFF1* in MV4-11 cell dilutions of 10^{-7} and transcripts down to 0.005 copies/ng.

Implications: This study demonstrates a NGS methodology with improved sensitivity compared with current diagnostic methods for *MLL*-rearranged leukemia. Furthermore, this assay rapidly and reliably identifies *MLL* partner genes and patient-specific fusion sequences that could be used for monitoring minimal residual disease. *Mol Cancer Res*; 16(2); 279–85. ©2017 AACR.

Introduction

Chromosomal translocations involving the human mixed lineage leukemia (*MLL*) gene [recently renamed *Lysine Methyltransferase 2A* (*KMT2A*)] result in oncogenic fusion genes. Expressions of chimeric transcripts from these fused genes are considered to be clinically important drivers of acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) (1, 2). The incidence of *MLL*-rearranged leukemia varies with age, is more common in children (3–5), and this subtype accounts for approximately 70% of infant

acute leukemia cases (6). More than 130 different translocation partner genes (TPG) of *MLL* have now been identified, and approximately 94 fusions have been characterized, of which *AF4* (*AFF1*), *AF6* (*MLLT4*), *AF9* (*MLLT3*), *AF10* (*MLLT10*), *ENL* (*MLLT1*), and *ELL* are the most frequent partners (4, 7, 8). Importantly, there is evidence that the particular fusion and TPG have prognostic importance, with fusions other than t(9;11) and t(11;19) being associated with poorer outcomes in childhood AML with t(10;11) and t(6;11) having particularly high relapse rates (9).

The diagnostic workup to detect *MLL* rearrangements typically involves cytogenetics, including FISH and RT-PCR. However, these methods have intrinsic limitations. Conventional cytogenetics can provide information on structural and numerical abnormalities but often cannot detect cryptic rearrangements (3). Although FISH with *MLL* break apart probes can usually detect the presence of an *MLL* fusion, FISH is unable to identify the partner gene in about one third of patients (10), and RT-PCR is routinely used to detect only the most frequent *MLL* fusions (such as *MLL-AF4*). Untargeted next-generation sequencing (NGS) methods, such as whole genome and transcriptome sequencing, have the potential to identify multiple gene fusions without prior knowledge of fusion events, but they are expensive for routine use. Long-distance inverse-polymerase chain reaction (LDI-PCR) can identify the genomic breakpoints in *MLL* fusion genes and has been used to characterize the precise chromosomal rearrangements in individual *MLL*-rearranged patients. Although LDI-PCR has successfully identified gene rearrangements that are not detected by conventional methods, such as FISH, this method

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also has intrinsic limitations that reduce the applicability of this method. It requires a relatively large input of patient genomic DNA (~1 µg) with blast cell percentages of at least 20% to 30% to achieve sufficient sensitivity, and the workflow is relatively laborious and time consuming (4, 11). There is a currently unmet clinical need to improve both the accuracy and speed with which *MLL* fusions can be detected in a routine diagnostic workup.

Recently, a new NGS-based assay has been introduced, which claims to be able to rapidly identify gene fusions in clinical specimens that may be unsuitable for analysis by other methods without the prohibitive costs associated with untargeted NGS approaches (12). This assay, commercialized by ArcherDx as the FusionPlex assay (12), uses anchored multiplex PCR (AMP-E) to rapidly enrich a defined set of fusion transcripts that is subsequently subjected to sequencing. Therefore, TPGs and transcriptional fusion points can be determined without requiring prior knowledge of the TPGs.

Here, using the Archer Heme and Myeloid FusionPlex kits, which target panels of fusion genes that have been previously associated with hematologic malignancies, including *MLL*, we describe the application of the Archer FusionPlex assay to a diverse cohort of childhood leukemia patient samples with known *MLL* rearrangements to evaluate the sensitivity, specificity, and potential clinical utility of this assay.

Materials and Methods

Study design and sample selection

We obtained a cohort of 39 diagnostic samples from patients known to have *MLL* rearrangements from the Queensland Children's Tumour Bank and the Sydney Children's Tumour Bank Network. Ethics approval for the collection and use of these samples was granted by each bank's Institutional Review Board, and informed consent was obtained in accordance with the Declaration of Helsinki. All samples were known to bear a disrupted *MLL* gene based on conventional cytogenetic analysis with FISH. DNA from all samples had also been subjected to LDI-PCR for characterization of the *MLL* TPG. One High BCR-ABL p210 control from Ipsogen BCR-ABL Mbc Control Kit (Qiagen) and two patient samples known to express a *BCR-ABL1* fusion were used as positive controls as the Heme/Myeloid Kits also detect this fusion event. Four cytogenetically normal, *MLL* wild-type ALL remission samples and one normal human blood leukocyte total RNA (BioChain) sample were used as negative controls in each panel. In addition, four leukemia cell lines were used as controls for fusion transcript detection and assay sensitivity tests. The leukemia cell lines used were Kasumi-1, derived from a childhood AML wild type for *MLL* but harboring a *RUNX1-RUNX1T1* fusion (13), MV4-11, derived from a childhood myelomonocytic leukemia with a *MLL-AFF1* fusion (14), ML-2, derived from an adult AML expressing *MLL-MLLT4* (15) and THP-1, derived from a childhood AML expressing *MLL-MLLT3* (16). Cell lines were sourced, validated, and cultured as described previously (17).

FISH

FISH analyses were performed by an accredited diagnostic pathology laboratory according to routine diagnostic procedures.

LDI-PCR

Long-distance inverse PCR was performed as described previously (11, 18).

AMP-E

RNA was extracted from patient samples using the Qiagen RNeasy Kit as per the manufacturer's instructions. Samples with a total of 100 ng RNA and RNA integrity number ≥ 4.1 were used as input for AMP-E library preparation. Briefly, after the first- and second-strand complementary DNA (cDNA) synthesis, a PreSeq RNA quality control assay was performed to ensure an effective library preparation. Any samples with a Cq value more than 28.5 were excluded due to insufficient library quality. Double-stranded cDNA was then cleaned with Agencourt AMPure XP beads (Beckman Coulter) and subjected to end repair, adenylation, and ligation with a universal Archer molecular barcode adapter that allowed amplification of gene specific primers (GSP) in one direction only. Postligation, the libraries were cleaned up and subjected to two rounds of nested PCR for target enrichment. These nested primers each had the 5' end tagged with a common sequencing adapter, which, in combination with the first half-functional universal adapter, amplifies target amplicons for multiplexing. The amplicons were clonally amplified and then sequenced. Libraries were quantified using quantitative PCR (Kapa Biosystems), normalized, and processed respectively for sequencing on a MiSeq or NextSeq, (Illumina) according to the manufacturers' instructions (12). Twelve samples were sequenced on MiSeq (Illumina) to the minimum depth suggested by the manufacturer (1.5 million reads/sample). For the remaining samples, NextSeq, (Illumina) was used and a depth of 3 million reads/sample was obtained. Sequencing was performed by the Institute for Molecular Bioscience Sequencing Facility (The University of Queensland, Queensland, Australia).

Sanger sequencing

PCR was used to amplify a 245-bp amplicon using cDNA to identify the transcriptional fusion breakpoint in patient 17. The amplicons were then cleaned up and incorporated with fluorescently labeled nucleotides using BigDye terminator (Thermo Fisher Scientific). The reactions were then sequenced using the capillary electrophoresis sequencer ABI Prism 3130xl Genetic Analyzer (Applied Biosystems).

Generation of a standard curve of *MLL-MLLT3* expression

To measure the expression level of the *MLL-MLLT3* fusion transcript in patient 17, we established a standard curve for the transcript using cDNA from patient 32, as LDI-PCR detected the same breakpoint in these 2 patients. Briefly, the standard curve was generated by 9 reactions using absolute quantifications. The quantity of cDNA input for the 9 reactions was serially reduced by 2-fold from 25 ng. This strategy resulted in the lowest cDNA input for the standard curve to be 0.098 ng. For patient 17, a total of 1.65 ng of cDNA was used as templates to amplify the fusion transcript alongside the 9 standard curve reactions. The qPCR was performed using the probe and primers specific to *MLL-MLLT3* fusion transcript on the ViiA7 thermocycler (Applied Biosystems) with default cycling conditions for TaqMan assay.

Determination of cell-based sensitivity of the Archer platform

MV4-11 cells (*MLL-AFF1*) were serially diluted 1 in 10 with Kasumi-1 cells (*RUNX1-RUNX1T1*) to achieve cell ratios from 10^{-2} to 10^{-7} . RNA was extracted, as described above, from each of the mixed cell populations. A total of 100 ng of each RNA sample was then subjected to AMP-E amplification.

Absolute quantification of fusion transcript

RNA (1 µg), extracted from each of the mixed MV4-11/Kasumi-1 cell populations at different ratios (10^{-2} to 10^{-7}) and from patient 17 was reverse transcribed in a 20 µL reaction using SensiFast cDNA Synthesis Kit (BioLine). The same amount of RNA, extracted from pure Kasumi-1 cells (*RUNX1-RUNX1T1*, *MLL-wt*) and THP-1 cells (*MLL-MLLT3*), was also transcribed to serve as negative controls. A total of 5 µL of the cDNA was used as templates in a 25 µL ddPCR reaction, with 1× droplet PCR Supermix (Bio-Rad), 0.25 µmol/L of a FAM-labeled fusion-specific probe and 0.5 µmol/L of a fusion-specific primer pair. A total of 20 µL of the 25 µL reaction was used to generate droplets using QX200 Droplet Generator (Bio-Rad). The droplet PCR was then performed using the Bio-Rad thermocycler with an initial enzyme activation step at 95°C for 10 minutes, followed by 40 cycles of denaturing at 94°C for 30 seconds and annealing/extension at 61°C for 1 minute. The cycling was finished with an enzyme deactivation step at 98°C for 10 minutes, and positive fluorescence droplets of each reaction were then read using QX200 Droplet Reader (Bio-Rad) under absolute quantification settings. The analysis was performed using QuantaSoft software (Bio-Rad). The fluorescent levels detected in the Kasumi-1 and THP-1 samples were used as a threshold to exclude false-positive droplets. Fluorescent signals above the threshold were considered positive. The assay was done in duplicates for each sample. The concentration of fusion transcript in a given sample was calculated as the mean of the duplicate assays.

Data processing and statistical analysis

Raw sequencing reads were analyzed using the Archer Analysis 4.1 software with default parameters. Briefly, this software aligns reads to a reference genome, identifies regions that map to noncontiguous regions of the genome, and applies filters to exclude probable false-positive events and annotate previously characterized fusion events. Some events were also investigated by manual inspection of the aligned reads.

The binary diagnostic test was used to determine sensitivity and specificity of each method (19), and both Fisher and Barnard exact tests were used to calculate the *P* value, describing the ability of each method to identify known fusion events and discriminate between fusion-positive and fusion-negative samples (20, 21).

Results

Characterization of the study population

For evaluation of the AMP-E method, 39 patients with childhood leukemia and a known *MLL* rearrangement were selected from two pediatric tumor banks. Patient characteristics are summarized in Table 1. This study was limited to childhood leukemia with the median age of the cohort being 5.3 years (range, 0.3–16). The proportion of male and female patients was approximately equal (59% male, 41% female). A total of 67% of patients had ALL, while 33% were diagnosed with either primary or secondary AML. Blast cell percentages in either peripheral blood or bone marrow ranged from 8%–98% with a mean of 84%.

Fusion transcript identification by AMP-E

AMP-E identified *MLL* fusion transcripts in 38 of 39 samples in our study. The analysis software reported the transcript-level breakpoints and translocation partner genes in each case.

Table 1. Study population characteristics

Characteristics	Group
Number of samples	39
FISH data available	39
LDI-PCR data available	38
Age (mean ± SD, years)	5.2 ± 5.8
Sex	
Male (n, %)	23 (59%)
Female (n, %)	16 (41%)
Diagnosis	
ALL	26 (67%)
AML	13 (33%)
Secondary AML	2 (5.1%)
Blast % (mean ± SD)	85% ± 20%

AMP-E identified 10 different *MLL* fusions, including *MLL-AFF1* (n = 14), *-MLLT3* (10), *-MLLT1* (3), *-MLLT10* (3), *-ELL* (3), *-MLLT4* (1), *-EPS15* (1), *-DCP1A* (1), *-AFF3* (1), and *-TNRC18* (1) (Fig. 1). It also revealed that 47% of these patients expressed multiple transcripts of a *MLL* fusion gene (Table 2).

Comparison of performance of LDI-PCR and AMP-E

Comparison of the AMP-E with LDI-PCR results showed a high concordance between the methods (Table 2). AMP-E was able to correctly identify the TPG and breakpoints in all samples except one (patient 17). LDI-PCR correctly identified the TPG and breakpoints in all samples except one (patient 21; Supplementary Table S1).

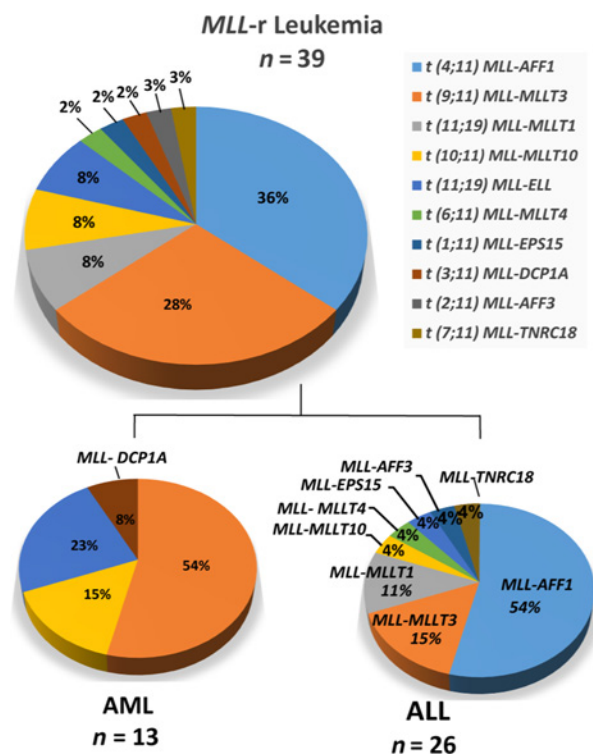


Figure 1.

Spectrum of *MLL* fusions in the entire sample cohort and by leukemia type. The pediatric patient cohort was grouped into ALL and AML. Gene names and percentages are written in black.

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Table 2. Comparison of *MLL* fusions detected by LDI-PCR and AMP-E

<i>MLL</i> fusion	Samples positively detected by	Samples positively detected by	Samples with multiple fusion transcripts detected by	Number of samples tested
	LDI-PCR	AMP-E	AMP-E	
<i>MLL-AFF1</i>	14	14	8	14
<i>MLL-MLL3</i>	11	10	3	11
<i>MLL-MLL1</i>	2	3	2	3
<i>MLL-MLL10</i>	3	3	1	3
<i>MLL-MLL4</i>	1	1	0	1
<i>MLL-EPS15</i>	1	1	0	1
<i>MLL-AFF3</i>	1	1	1	1
<i>MLL-TNRC18</i>	1	1	0	1
<i>MLL-ELL</i>	3	3	3	3
<i>MLL - DCP1A</i>	1	1	0	1

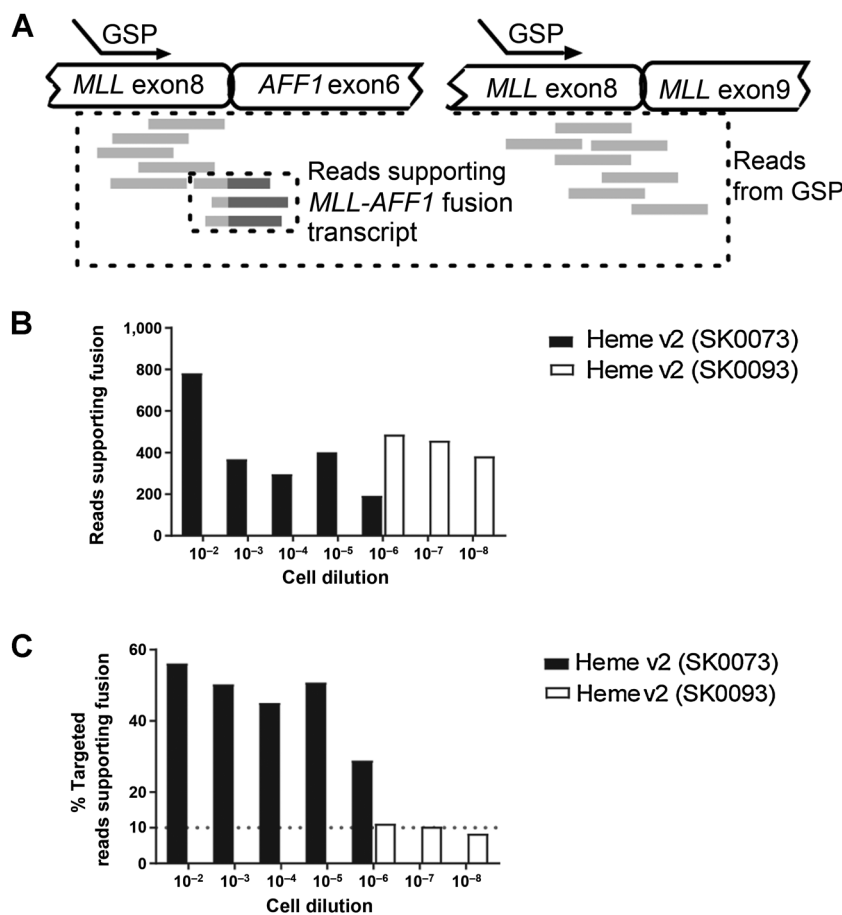
Verification of a discordant *MLL-MLL3* fusion event

A *MLL-MLL3* fusion event was identified in patient 17 at the DNA level (with LDI-PCR) but not at the RNA level (using AMP-E). This prompted us to determine whether these discordant observations were due to a failure of AMP-E to detect the fusion transcript or due to a very low level of fusion expression. To test the first possibility, we examined whether there were mutations that interfered with GSP binding during library preparation. We characterized the expected fusion transcript with Sanger sequencing. Primers and probes were designed on the basis of the fusion transcripts expressed in patient 32, as both samples had

a *MLL-MLL3* fusion involving intron 11 and intron 5 by LDI-PCR and patient 32 was correctly identified by the Archer FusionPlex Heme panel, which revealed a *MLL-MLL3* fusion transcript involving exon 10 and exon 6. Sanger sequencing did not reveal any mutations at the GSP-binding sites in patient 17 (data not shown), suggesting that a fusion transcript, if present, could be successfully amplified. We established a standard curve for the fusion transcript identified in the patient 32 using qPCR. Given that patients 17 and 32 shared the same fusion breakpoint and TPGs, we used this standard curve to quantify the expression levels of the fusion transcript in the patient 17. Our results demonstrated that patient 17 did express the same *MLL* fusion transcript, albeit at an extremely low level; the transcript was only detected after 33 cycles of PCR amplification (Supplementary Fig. S1). This result showed that the expression of the fusion transcript was even lower than the levels in the 9th standard curve dilution, where 0.09765625 ng of cDNA was used as templates. The amount of patient 17's cDNA (1.65 ng) was in between the cDNA quantity of the 4th (3.125 ng) and the 5th reaction (1.5625 ng) of the standard curve. Therefore, our results demonstrated that the expression level of the *MLL-MLL3* fusion transcript in patient 17 was more than 32 times (2^{9-5+1}) lower than the levels in patient 32.

Cellular detection limit of AMP-E

To determine the sensitivity of the AMP-E method for detecting *MLL* fusion transcripts in a heterogeneous mixture of cells, we

**Figure 2.**

Cell-based limit of detection of AMP-E. The cell-based limit of detection of AMP-E has been determined in two batches using the previous Archer Heme v2 Kit (reference number SK0073) for the cell dilutions ranging from 10^{-2} to 10^{-6} , and the current Archer Heme v2 Kit (reference number SK0093) for the cell dilutions of 10^{-6} to 10^{-8} . **A**, Shows a schematic of the reads that are used to determine the existence of a fusion transcript; light gray bars represent all reads are generated from the GSP corresponding to *MLL* exon 8; some of these reads align to the wild-type transcript, while others align to a *MLL/AFF1* fusion transcript; the light gray/dark gray bars represent the subset of these reads (targeted reads supporting fusion) that support the *MLL/AFF1* fusion transcript because they cross the breakpoint. **B**, Demonstrates the number of reads supporting the *MLL/AFF1* fusion transcript present in MV4-11 cells at different cell dilutions. **C**, Shows the percentage of reads supporting *MLL/AFF1* fusion transcript (from all reads generated by the *MLL* exon 8 GSP) present in MV4-11 cells at different cell dilutions. Dashed line, threshold of calling fusions (10%).

performed serial dilutions of MV4-11 (*MLL-AFF1*) cells in Kasumi-1 (*RUNX1-RUNX1T1*) cells for a ratio ranging from 10^{-2} to 10^{-7} . RNA extracted from each dilution point was subjected to AMP-E using the Heme panel. Results showed AMP-E successfully detected the presence of both the *RUNX1-RUNX1T1* fusion and the *MLL-AFF1* (*MLL* exon8 - *AFF1* exon6) fusion transcript at every dilution tested, suggesting that AMP-E can detect *MLL-AFF1* at an effective blast cell percentage of 0.0001% (10^{-7}). Figure 2A–C shows the percentage of reads that are targeted to this specific genomic region (*MLL1* exon 8) that support the fusion. At all dilution levels, the fusion event is supported by 60%–80% of the reads; the remaining reads map to events that are consistent with transcriptional readthrough into a downstream gene or events do not meet the criteria for a true fusion transcript, both of which are identified by the Archer software as false-positive events. Given the strong evidence for the known *MLL-AFF1* event in the cell dilution up to 10^{-7} , we concluded that the sensitivity of AMP-E is at 10^{-7} based on fusion transcript expression levels in an exemplar *MLL*-rearranged cell line.

Transcript copies detection limit of AMP-E

We concluded that the Archer AMP-E platform can reliably detect one MV4-11 leukemic cell in 10^7 Kasumi-1 cells, achieving the cellular sensitivity of detection of 10^{-7} . However, it is possible that such a high sensitivity was, in part, attributed to an inherently high transcriptional level of the *MLL-AFF1* fusion in MV4-11 cells. To interrogate the transcriptional limit of the Archer platform in fusion detection, we quantified the absolute number of copies of the *MLL-AFF1* (MV4-11) fusion transcript, using ddPCR, in 6 cell dilution samples, ranging from 10^{-2} to 10^{-7} . These were the same dilutions used to test the AMP-E cellular sensitivity. The ddPCR results demonstrated that the copies of the *MLL-AFF1* (MV4-11) fusion decreased 10 times with a 10 times dilution in MV4-11 cellularity with $R^2 = 0.9998$, confirming that our cell dilutions were accurate and our assay amplifying the *MLL-AFF1* (MV4-11) fusion transcript was specific (Fig. 3). The number of copies of the fusion transcript at a dilution level of 10^{-7} , the limit of the AMP-E cellular sensitivity, was determined to be 0.005 copies/ng of total RNA (Fig. 3). These data illustrate that AMP-E is able to detect one copy of fusion transcript in as little as 20 ng of total RNA. Considering quantification using ddPCR is not dependent on the amplification efficiency of the assay per se, this detection limit, in theory, can be applied to any fusion. On the basis of these results, the number of copies of the fusion transcript in patient 17, for which AMP-E did not detect an *MLL* fusion transcript, is likely to be lower than 0.005 copies/ng. To confirm this, we quantified the copies of the fusion transcript in patient 17 and found that, indeed, the expression of the fusion transcript in the patient 17 sample was 0.0001 copies/ng, 50 times lower than the AMP-E detection limit (Fig. 3).

Discussion

We sought to determine whether a targeted NGS approach for detecting fusion transcripts could improve the diagnostic identification of *MLL* rearrangements in leukemia. Specifically, we aimed to determine whether the AMP-E method could: (i) identify the *MLL* translocation partner gene in patients where routine FISH failed to do so; and (ii) sensitively and specifically identify *MLL* rearrangements in all patients, including those with a low blast cell percentage. We found that both LDI-PCR

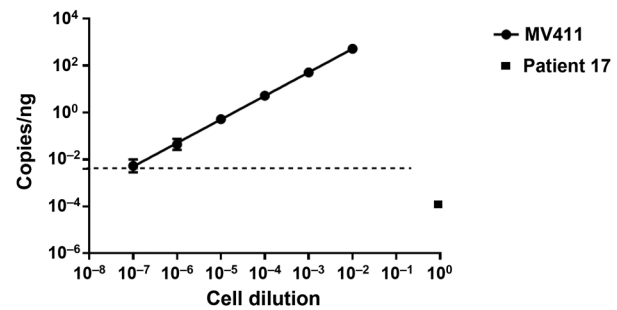


Figure 3.

Transcriptional limit of Archer in fusion detection. Absolute quantification of the MV4-11 fusion transcript (●) in different cellularity samples (10^{-2} to 10^{-7}) was determined by ddPCR. The cellularity limit of AMP-E in fusion detection is indicated by the dashed line, equivalent to 0.005 copies/ng of total RNA. The copies of the patient 17 fusion transcript in 1 ng of the patient's total RNA, extracted from the bone marrow sample with 90% blasts, was 0.0001, shown as a square (■). Data are presented as mean and SD.

and AMP-E were able to identify the translocation partner gene involved in each *MLL* fusion event in 97% of our samples, whereas routine FISH was able to do so in only 77%. AMP-E identified both the *MLL* fusion event and the TPG using the standard workflow with minimal sequencing. Furthermore, by comparing the transcript breakpoint of the *MLL* fusion events identified by AMP-E with the genomic breakpoints identified by LDI-PCR, we were able to confirm that the fusion transcripts are consistent with translocation events at the genomic level. Current diagnosis of *MLL* fusions often involves multiple cytogenetic techniques (e.g., karyotyping, FISH) and standard RT-PCR assays. However, routine detection by RT-PCR analysis is only available for the most recurrent *MLL* fusion transcript of an *MLL* rearrangement with a known fusion partner gene. (22). When prior knowledge of the fusion partner gene is lacking, conventional techniques need to couple with LDI-PCR to identify the partner genes (11). AMP-E is able to simultaneously investigate hundreds of potential gene fusions (including *MLL*) and relevant point mutations that are commonly known to be associated with hematologic malignancies. Therefore, AMP-E has the potential to replace the need for multiple and laborious RT-PCR assays and FISH, by detecting the spectrum of potential *MLL* fusion partners as well as other molecular events in AML and ALL samples in a single NGS assay. Given the prognostic impact of *MLL* rearrangements in acute leukemia can vary according to the TPG (4, 8, 9, 23), sensitive and specific detection of *MLL* fusion transcripts by AMP-E may improve risk stratification for therapy.

Interestingly, in 47% of our patient samples AMP-E identified multiple, patient-specific fusion isoforms transcribed from each *MLL* fusion gene. Although we have yet to fully characterize these unexpected findings and determine the functional significance of these additional isoforms, biochemical experiments show that even differences as small as nine nucleotides can affect domain stability and binding affinity in proteins translated from both wild-type and rearranged *MLL* fusion transcripts (24). The functional role and prognostic impact of multiple patient-specific fusion isoforms in *MLL*-rearranged leukemia is not known. However, these transcript isoforms may potentially be useful for minimal residual disease (MRD) tracking in individual patients.

Any additional fusions detected by AMP-E could also potentially be evaluated for prospective MRD detection.

AMP-E successfully identified known *MLL* fusion events in 38 of 39 patients and did not call *MLL* fusion events in the controls, 12 wild-type *MLL* leukemia patients, and one normal leukocyte sample, resulting in a sensitivity of 97.4% and a specificity of 100% (Table 2). We investigated the one case where AMP-E failed to detect the *MLL* fusion (patient 17) to determine the cause. Because we had already identified the genomic breakpoint and TPG in this patient using LDI-PCR, and we identified a very similar event in another patient (patient 32), we postulated that this fusion event was detectable by AMP-E. To investigate the cause of the failed detection, we determined that no mutations were present in the patient transcript at the FusionPlex gene-specific priming site so that if fusion transcripts were present, they could be successfully amplified. By quantifying the level of fusion transcript in this patient, we found that it was present at an extremely low level, suggesting that this fusion event was missed because the expression level was below the detection limit of the AMP-E assay requiring us to establish that limit. By using ddPCR, we determined the AMP-E detection limit, 0.005 copies/ng, and that the expression of the fusion transcript was 0.0001 copies/ng, 50 times lower than that limit. As the expression of this fusion transcript is so low, it is arguable whether it has clinical significance and whether the failure to detect it is clinically relevant.

The NGS libraries are generated in less than 9 hours and are compatible with low input amounts of nucleic acids (~20–200 ng). One advantage of the FusionPlex assay is that, unlike LDI-PCR, the Heme/Myeloid Kit AMP-E primers are not restricted to the *MLL* "breakpoint cluster region" (BCR) that encompasses exons 8 to 14 of the *MLL* gene (18). The BCR defines the site where the majority of, although not all, *MLL* fusion breakpoints have been observed allowing this assay to capture a broader range of *MLL* fusion events compared with other methods that are currently in use. A limitation of AMP-E, however, is that this method is unable to detect reciprocal fusion transcripts, known to be present in 9 of our samples. The Heme and Myeloid panel GSPs are designed to target the 5' ends of *MLL* exons 2 and 3 and the 3' ends of *MLL* exons 4 to 35, rendering this method blind to complex rearrangements. However, Archer does support the design of custom primers making the capture of these events possible if required. *MLL*-partial tandem duplication (PTD), where internal rearrangement of the *MLL* gene within the breakpoint cluster region occurs, often involves multiple exons duplication of the gene. Such rearrangement challenges the NGS techniques that are based on small sequencing fragments. Therefore, AMP-E is not able to robustly call *MLL*-PTD, although *MLL*-PTD-like events, where duplication of a single exon within the breakpoint cluster region could be identified. A capture-based RNA sequencing approach has been previously used to

successfully detect *MLL* fusion genes in a cohort of 6 samples hinting at the promise of NGS platforms for fusion detection (25). However, the methodology described by Sevov and colleagues lacks the sensitivity and breadth of the FusionPlex assay, demonstrated in our larger cohort of 39 patients.

In conclusion, the Archer FusionPlex AMP-E NGS assay is a relatively simple and efficient technique for the detection of *MLL* gene rearrangements in ALL and AML. This technique has the potential to significantly reduce the time required to detect *MLL* gene rearrangements and characterize the TPG without compromising specificity. AMP-E also provides information on the presence of any additional fusion events involving other genes included in the FusionPlex panel. Furthermore, AMP-E can identify patient-specific fusion transcript variants expressed within a sample, providing markers that could potentially be used in RQ-PCR or ddPCR assays for MRD monitoring and disease prognostication. An easy workflow, fast turnaround time and the ability to accurately characterize a broad range of *MLL* gene rearrangements in patient samples could make AMP-E an attractive alternative to current methods used.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Afrin, C.R.C. Zhang, C. Meyer, R. Marschalek, J.L. Fink, A.S. Moore

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