A Novel NGS Assay to Detect Any *KMT2A* Fusion Transcript at Low Levels

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Abstract

Introduction: Measurable residual disease (MRD) detection is associated with relapse. Leukemias with *KMT2A* rearrangement (*KMT2A*r) have an adverse prognosis with more than 80 possible fusion partner genes (FPG) identified. With the advent of effective targeted therapies for *KMT2A*r leukemia such as menin inhibitors, there is an unmet need for sensitive assays to detect these various fusions, not only to guide and monitor therapy, but also to predict and intercept relapse. Multicolor flow cytometry (MFC) is the only readily available test for MRD in *KMT2A*r acute myeloid leukemia (AML), not requiring prior knowledge of exact fusions and breakpoints for design of patient-specific assays. Standard next-generation sequencing (NGS) methods cannot accurately detect genetic alterations at low frequencies because of inherent technical errors and the need for high sequencing depth. Blocker Displacement Amplification (BDA) technology enables selective detection of such rare alterations with NGS (Wu LR, Nat Biomed Eng 2017). Here, we demonstrate the effective detection of KMT2Ar and its various FPG with a novel NGS assay leveraging BDA.

Methods: The NuProbe RNA fusion proprietary technology uses BDA (FusionBDA) where primers flank any known exon junction of cDNA corresponding to transcripts of the gene of interest, while non-extensible oligonucleotides (blockers) span the wildtype (WT) exon junction, therefore suppressing amplification of WT while differentially amplifying fusion junctions. As a result, final libraries are enriched with reads mapping to fusion genes. This allows de novo calls and minimizes read requirements. We designed a custom panel targeting 35 exons of *KMT2A* (forward and reverse) and developed a pipeline using 3 previously established fusion detection methods (Arriba, STAR-Fusion, FusionCatcher). We validated this assay in a *KMT2A*r cell line. Limit-of-detection (LoD) was assessed by blending RS4;11 (leukemia with *KMT2A::AFF1*) with HT-1197 cells (bladder cancer, no *KMT2A*r), to attain contrived samples with 0.001% to 100% tumor fractions (400ng input). Limit-of-input (LoI) was assessed using cDNA with 1- 400ng RNA from 100% and 0.1% tumor fraction samples. In a blinded analysis, we tested 50 bone marrow samples from 35 patients with *KMT2A*r AML, chosen deliberately with various 11q23 translocations including rare ones.

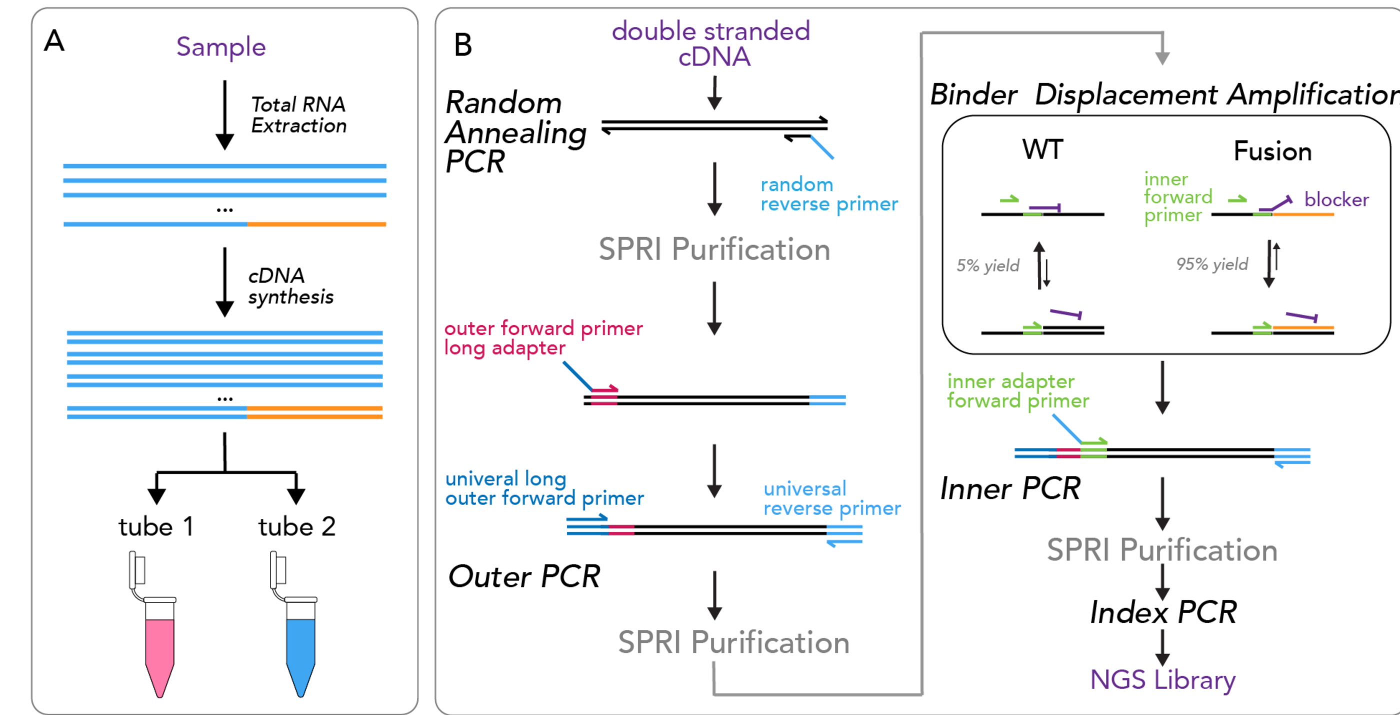
Results: Following LoD experiment, *KMT2A*r was detected accurately down to 0.005% tumor fraction. Subsequently, LoI assay revealed the ability to detect *KMT2A*r in 100% VAF tumor fraction samples using as low as 1ng of input RNA and using 400ng in 1% VAF samples. Among samples with morphologic evidence of leukemia, FusionBDA detected various *KMT2A*r in 44/45 samples (97.8%) except 1 sample with a t(11;19)(q23;p13.1). This sample had a suboptimal amount and quality of cDNA loaded. We evaluated concordance of predicted FPG based on bands by cytogenetics (CG) and the published literature on KMT2A recombinome. We found that FusionBDA identified the same predicted FPG (same band) in 41/45 samples (91%). In samples with various t(11;19) translocations, different FPG were identified all located on 11p13 within a few bands from ones by CG, likely representing the accurate FPG given the low resolution of CG and the associated error in determining arm band accurately. Finally, we explored the potential of this assay for MRD detection by examining 5 samples at morphologic remission following treatment. All samples where fluorescence in situ hybridization (FISH) detected a KMT2A fusion had FusionBDA+ (31/31 samples including 1 in morphologic remission). 1 sample with no detectable KMT2A fusion by FISH and MRDneg by MFC, had detectable fusion by FusionBDA, indicating possibly an improved sensitivity. 3 samples with MRD+ by MFC also had detectable KMT2Ar by FusionBDA (lowest MRD+ level by flow was 0.5%). 1 sample was MRD+ by MFC and had a detectable KMT2Ar by FusionBDA in 2/3 callers (detectable by FusionCatcher and StarFusion not Arriba), therefore not meeting our stringent criteria though likely concordant with MRD by MFC. Further analysis in the MRD setting is planned.

Conclusion: FusionBDA is a novel, sensitive, and specific assay for KMT2Ar acute leukemia. This assay is able to agnostically detect KMT2Ar with various fusions at a predicted sensitivity of at least 0.005%, therefore allowing both MRD detection and target identification.

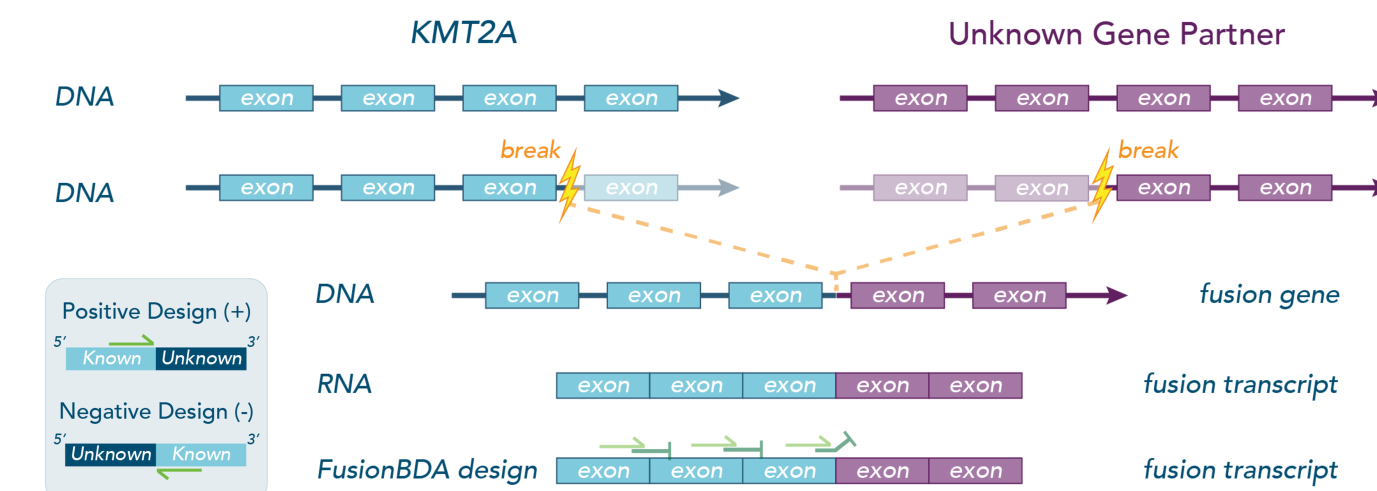
Introduction

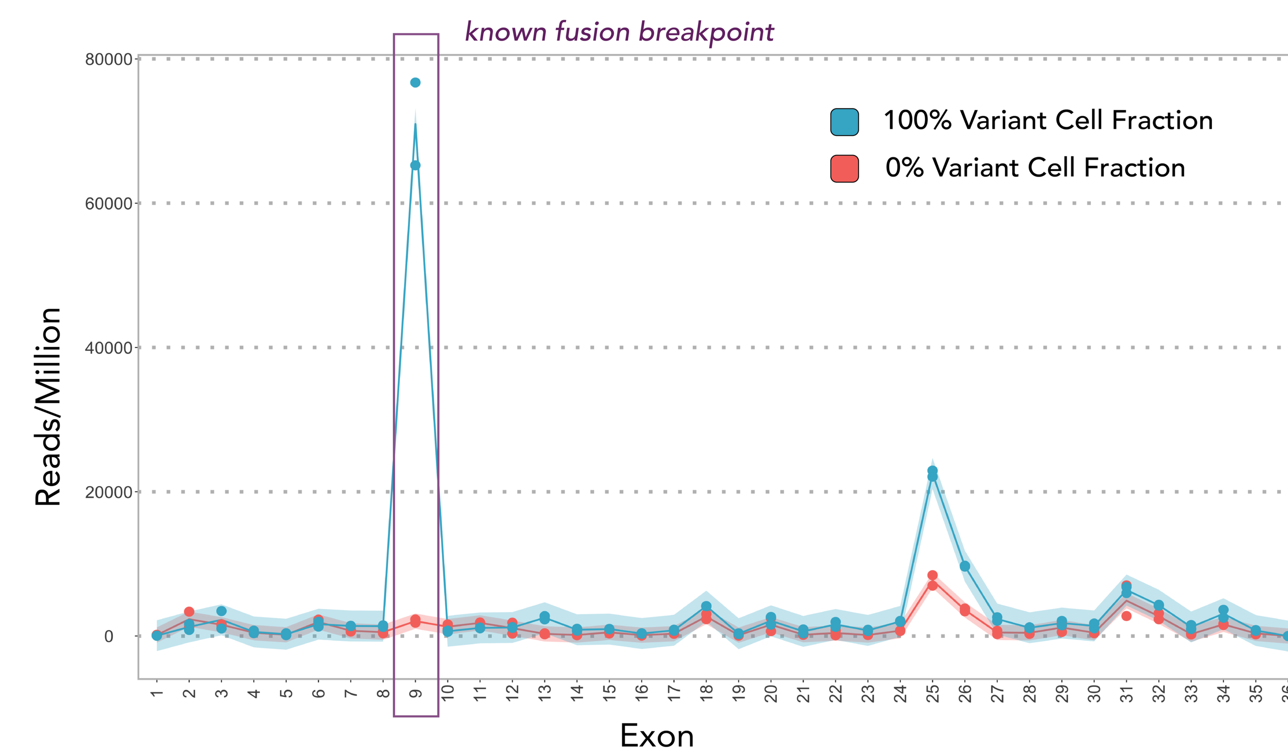
- Leukemias with *KMT2A* rearrangement (*KMT2Ar*) have an adverse prognosis with >80 fusion partner genes (FPG) identified.
- There is an unmet need for sensitive assays to guide, and monitor therapy and to predict and intercept relapse in this subset.
- We demonstrate effective detection of *KMT2Ar* with a novel NGS assay leveraging Blocker Displacement Amplification (BDA) technology (Wu LR, Nat Biomed Eng 2017) for detection of genomic alterations at low frequencies while minimizing NGS errors.
- This assay does not require prior knowledge of exact fusions and breakpoints.

Methods



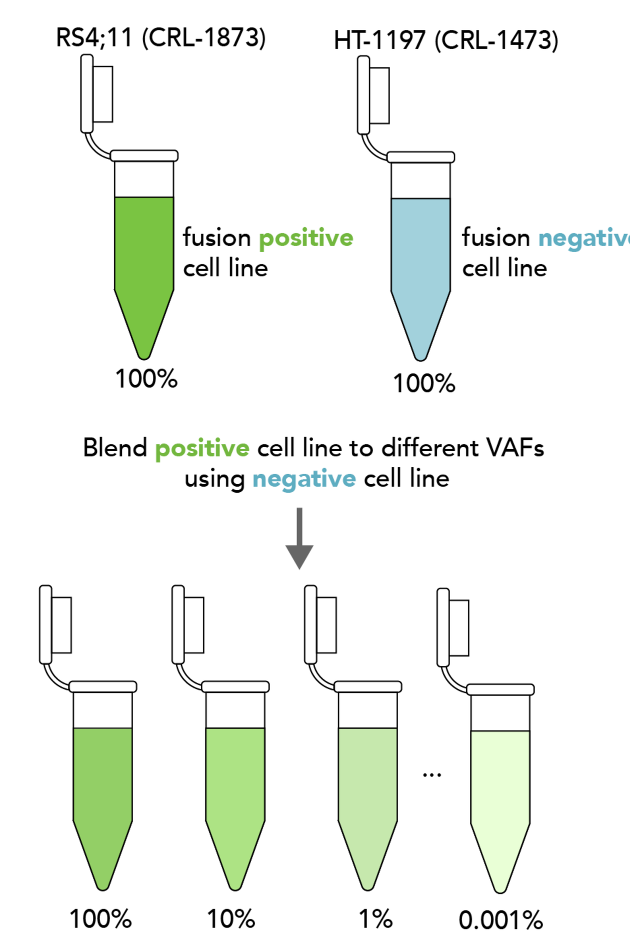
Workflow. A. cDNA is split between two tubes, Tube 1 for detecting fusions on the positive strand (known exon on 5'), and Tube 2 for the negative strand (known exon on 3'). B. Blocker (purple) is designed for WT exon junctions. In fusion genes, the forward primer preferentially displaces the blocker leading to differential amplification. We designed a custom panel targeting 35 exons of *KMT2A* (forward and reverse) and developed a pipeline using 3 previously established fusion detection methods (Arriba, STAR-Fusion, FusionCatcher).



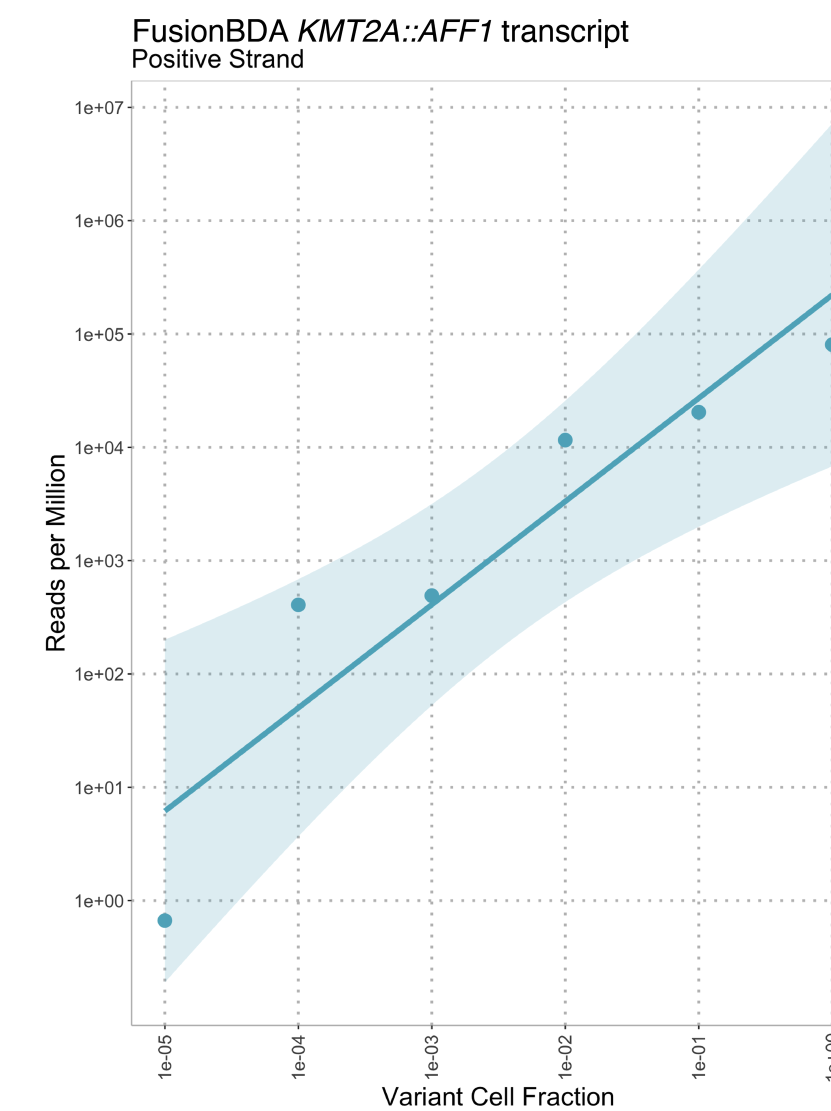


Design. A. Forward primers are designed to flank all known exon junctions of the gene of interest with primers for the positive (5'-3') and negative(3'-5') strands. Non-extensible oligonucleotides (blockers) span the known exon junctions, suppressing amplification of the wildtype gene. B. FusionBDA differential amplification of KMT2A exon 9 in RS4;11 cell line (*KMT2A::AFF1* fusion) based on correct start reads (20nt region 5' of exon junction)

Results



	No BDA Positive	No BDA Negative	Final (+ or -)	BDA Positive	BDA Negative	Final (+ or -)	Total variant cells in pipetting
100%	4/4	4/4	4/4	4/4	4/4	4/4	40000
50%	2/2	2/2	2/2	2/2	2/2	2/2	20000
10%	2/2	2/2	2/2	2/2	2/2	2/2	4000
1%	4/4	1/4	4/4	4/4	4/4	4/4	400
0.5%	0/2	1/2	1/2	2/2	2/2	2/2	200
0.1%	0/4	1/4	1/4	2/4	2/4	4/4	40
0.05%	1/2	0/2	1/2	1/2	1/2	2/2	20
0.01%	1/2	0/2	1/2	1/2	2/2	2/2	4
0.005%	0/2	0/2	0/2	1/2	2/2	2/2	2
0.001%	0/2	0/2	0/2	0/2	0/2	0/2	0.4



Limit of Detection: Two cell lines (HT1197 a bladder cancer cell line used as a negative control, and RS4;11, a leukemia cell line with *KMT2A::AFF1* as a positive control) were blended together in varying quantities to produce VAFs down to 0.001%. After blending, RNA was extracted and cDNA was synthesized with 400ng RNA. Library preparation was performed on all blended VAFs using FusionBDA with 3M reads allocated. The *KMT2A* translocation was detected in samples down to 0.005% VAF.

Predicted Class	True Class: Positive	True Class: Negative
Positive	TP = 24	FP = 0
Negative	FN = 2	TN = 4/4

11q23 translocation by CG	N	Predicted FPG	Identified FPG	Identified translocation	Identified/Tested
t(9;11)(p21.3;q23.3)	21	*MLLT3*	*MLLT 3*	t(9;11)(p21.3;q23.3)	21/21 (100%)
t(11;19)(q23;p13.1)	5	*ELL*	*ELL*	t(11;19)(q23;p13.1)	3/5 (60%)
			MLLT1	t(11;19)(q23;p13.3)	1/5 (20%)
			Not detected	No translocation	1/5 (20%)
t(11;19)(q23;p13.3)	2	*MLLT1*	*ELL*	t(11;19)(q23;p13.1)	2/2 (100%)
t(11;19)	3	*ELL* *MLLT1*	*ELL*	t(11;19)(q23;p13.1)	2/3 (67%)
			MLLT1	t(11;19)(q23;p13.3)	1/3 (33%)
t(6;11)(q27;q23)	8	*AF6*	*AF6*	t(6;11)(q27;q23)	8/8 (100%)
t(11;15)(q23;q15)	3	*KNL1*	*KNL1*	t(11;15)(q23;q15)	3/3 (100%)
t(11;17)(q23;q12)	1	*ACACA* *MLLT6*	*ACACA*	t(11;17)(q23;q12)	1/1 (100%)
t(11;17)(q23;q25)	1	*SEPT9*	*SEPT9*	t(11;17)(q23;q25)	1/1 (100%)
t(11;22)(q23;q11)	1	*SEPT5*	*SEPT5*	t(11;22)(q23;q11)	1/1 (100%)

CG, cytogenetics. N, number of samples tested from different patients. FPG, fusion partner gene. Predicted fusion partner based on published literature for the corresponding translocation.

Detection of *KMT2A fusion transcripts* in patient samples using FusionBDA: In a blinded analysis, we tested 50 bone marrow samples from 35 patients with *KMT2Ar* AML, chosen deliberately with various 11q23 translocations including rare ones.

MRD Detection:

- Examined 5 samples at morphologic remission following treatment. FusionBDA was + in all samples where FISH detected a *KMT2A* fusion.
- FusionBDA was + in 3 samples with MRD+ by multicolor flow cytometry (MFC). One sample with no detectable *KMT2A* fusion by FISH and MRD- by MFC, had detectable fusion by FusionBDA, indicating possibly an improved sensitivity.

Conclusion

- FusionBDA is a novel, sensitive, and specific assay for *KMT2Ar* acute leukemia.
- This assay can agnostically detect *KMT2Ar* with various fusions at a predicted sensitivity of at least 0.005%, therefore allowing both MRD detection and target identification.

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