Duplex Sequencing™ for Minimal Residual Disease (MRD) Detection in Acute Myeloid Leukemia

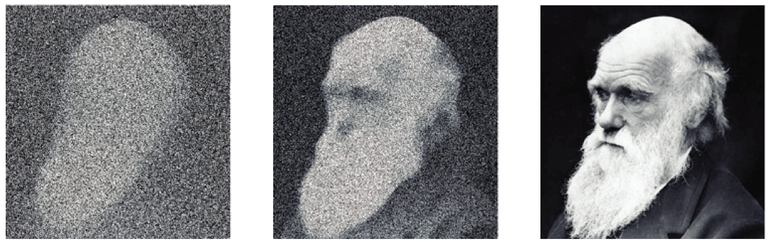
Jake Higgins PhD[1] – Lindsey N. Williams PhD[1] – Christopher S. Hourigan MD, PhD[2] – Jerald P. Radich MD[3,4] – Jesse J. Salk MD, PhD[1,3]

TwinStrand Biosciences, Seattle, WA[1] – NHLBI[2], National Institutes of Health, Bethesda, MD, 20892 – University of Washington, Department of Medicine, Division of Medical Oncology University of Washington, Seattle, WA 98195 – Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109[4]

DNA Sequencing of Ultra-Rare Mutations

The presence of minimal residual disease (MRD) after therapy in acute myeloid leukemia (AML) is the strongest predictor of relapse. Emerging molecular approaches for MRD detection use the persistence of somatically acquired mutations in leukemic cells as a marker of residual disease. PCR-based methods are very sensitive, but must be customized for each patient. Next generation sequencing (NGS) can interrogate many genes simultaneously, but technical noise obscures mutations below approximately 1% frequency. Unique molecular identifiers (UMI) in single-strand consensus sequencing (SSCS) can decrease background, but will not allow detection of ultra-rare mutations. Duplex Sequencing uses information from both strands of DNA molecules to achieve a very high degree of error correction, which reduces the miscall rate to below one-in-ten-million. Here we present a broadly applicable Duplex Sequencing assay for AML MRD quantification. We demonstrate detection of AML mutations at levels from 1/100 (1%) to below 1/100,000 (0.001%) with high accuracy and reproducibility.

Duplex Sequencing Eliminates Technical Background

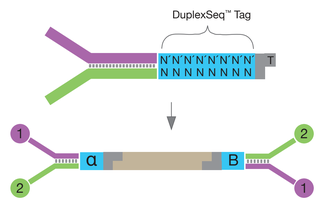


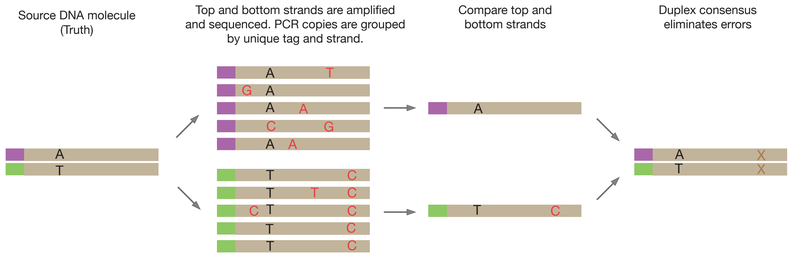
Next-Generation Sequencing (NGS) | Single Strand Error-Corrected NGS | Duplex Sequencing

TwinStrand Duplex Sequencing™ Technology

A DuplexSeq™ Adapter has:

1. Identical (or relatable) degenerate tags in each strand.
2. An asymmetry allowing independent strand identification.



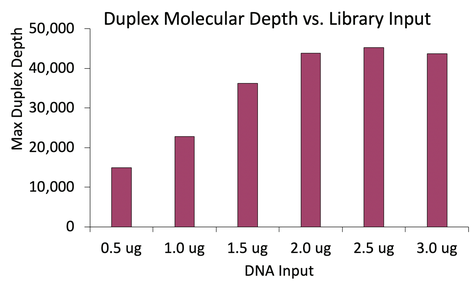


Genes in AML Capture Panel

ASXL1	FAM5C (BRINP3)	IDH2	NPM1	RAD21	TET2
CBL	FLT3	KIT	NRAS	RUNX1	TP53
CEBPA	GATA2	KRAS	PHF6	SMC1A	U2AF1
DNMT3A	HNRNPK	MLL (KMT2A)	PTEN	SMC3	WT1
EZH2	IDH1	MYH11-CBFB	PTPN11	STAG2	

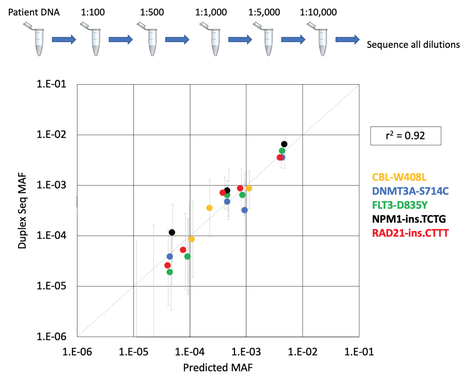
151 exons or hotspot codons in 29 genes recurrently mutated in AML are targeted with hybrid capture probes. If expected mutations are known, custom panels or sub-panels can be used for more efficient sequencing. More than 93% of AML patients carry a mutation in at least 1 panel gene.

Duplex Depth = True Molecular Depth



Duplex Sequencing libraries were prepared using genomic DNA inputs of 0.5 ug to 3.0 ug. The Duplex depth per library represents the number of original DNA molecules with sequences recovered from both strands. Duplex depths of more than 45,000x can be achieved in a single reaction. The limit of detection (LOD) in a DS assay is inversely proportional to the total Duplex depth of a sample at the locus of interest: the greater the depth, the greater the sensitivity.

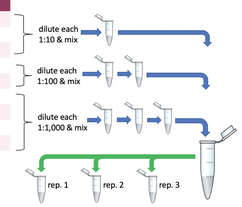
DS Accurately Detects Mutations Below 1/50,000 in a Patient-Based MRD Model



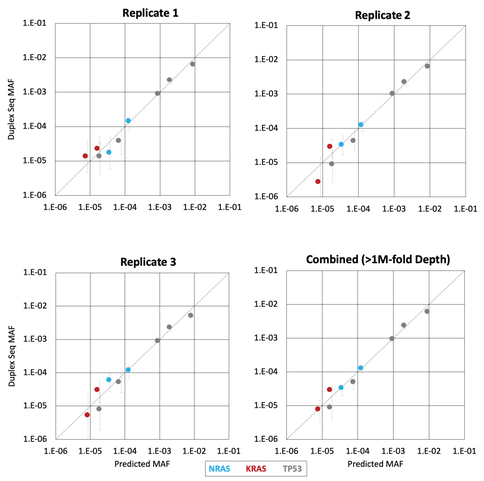
DNA was obtained from an AML patient with 5 clonal mutations known at the time of diagnosis. This was diluted 1:100, 1:500, 1:1,000, 1:5,000 and 1:10,000-fold into normal peripheral blood DNA to model a mixture of low-frequency mutations in MRD. DS was performed on all dilutions using a targeted hybrid capture panel to a maximum Duplex depth of 52,169x. MRD was accurately detected at all dilution factors. Only the 2 lowest frequency individual mutations below the LOD at this sequencing depth were not detected. Error bars show Wilson 95% binomial confidence intervals.

DS Accurately and Reproducibly Detects Mutations from 1/100 to <1/100,000 in a Cell Line MRD Model

Cell Line	Gene	Mutation	Dilution	Predicted MAF
K-562	TP53	Q136fs	1:100	$1x10^{-2}$
BT-549	TP53	R249S	1:500	$2x10^{-3}$
CA46	TP53	R248Q	1:1,000	$1x10^{-3}$
SW1271	NRAS	Q61R	1:5,000	$2x10^{-4}$
LS1034	TP53	G245S	1:10,000	$1x10^{-4}$
OCI-AML3	NRAS	Q61L	1:25,000	$4x10^{-5}$
SK-BR-3	TP53	R175H	1:50,000	$2x10^{-5}$
LS180	KRAS	G12D (het.)	1:50,000	$1x10^{-5}$
SW620	KRAS	G12V	1:100,000	$1x10^{-5}$

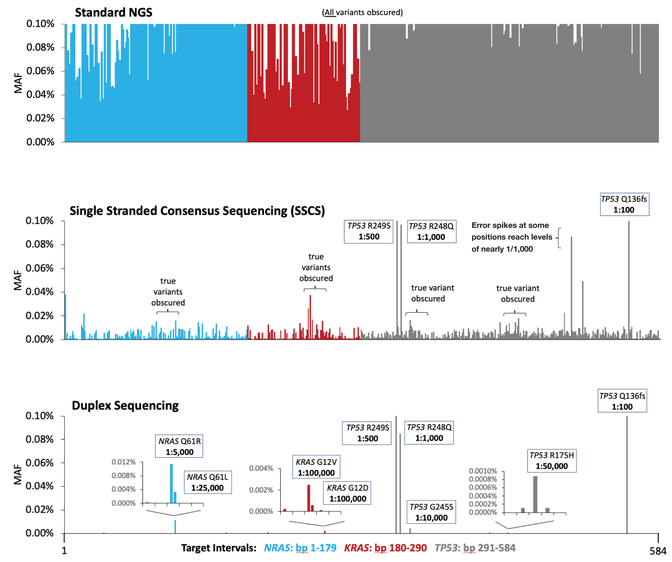


DNA from 9 cell lines with unique mutations in AML genes was serially diluted into normal peripherlal blood DNA from a healthy 18 year old at ratios from 1:100-1:100,000 in a single mix. The DNA mixture was prepped as DS libraries and sequenced in 3 independent runs. Supplemental sequencing brought the total to over 1 million Duplex molecular depth. All mutations were detected in all runs. The 3 replicate runs had r^2 values of 0.98, 0.96 and 0.95, with maximum Duplex depths of 257,058, 404,837 and 300,570, respectively. Error bars represent Wilson 95% binomial confidence intervals. Predicted MAF values were adjusted for known copy number alterations in cell lines.



	Overall background frequency	Minimum background frequency	Maximum background frequency	Note
Raw	$1.82x10^{-3}$	$1.73x10^{-4}$	$2.72x10^{-2}$	All base positions had background
SSCS	$4.50x10^{-5}$	$1.20x10^{-6}$	$8.66x10^{-4}$	All base positions had background
Duplex Sequencing	$5.84x10^{-7}$	0	$1.11x10^{-5}$	411/577 non-spike-in positions had 0 variant counts. Highest "background" mutation causes missense at NRAS Gly48, a residue mutated in colorectal cancer, and therefore may represent a minor functional clone.

Duplex Sequencing Reveals Mutations Below 1/100,000



The cell line mixture data were analyzed with standard NGS software, by SSCS or by Duplex Sequencing. The x-axis shows base positions in the 584 bp custom capture panel. The y-axis displays mutant allele frequency (MAF) at each position (axis truncated at 0.1%).

All positions had background errors with standard NGS, with many positions >0.1%. Background was reduced with SSCS, but all positions had background errors and true mutations <0.1% were obscured. DS revealed spiked-in mutations <0.001% (1/100,000) and every expected mutation was detected. The majority of DS positions had 0 background counts, even at extreme sequencing depths.

Conclusions

- Duplex Sequencing (DS) combines extreme sensitivity with the ability to flexibly capture and sequence any number of target genes.
- DS reveals low-frequency mutations that are obscured by errors in both standard NGS and other error-correction techniques.
- DS can confidently detect mutations at levels below 1/100,000.
- Duplex depths of >45,000x can be generated in a single library.
- Our standard DS AML gene panel is generalizable to >93% of patients.
- Duplex Sequencing is uniquely well suited to translational and clinical AML MRD detection, and validation against clinical outcomes with hundreds of patient samples among four clinical trials sample sets is ongoing.

Contact: https://twinstrandbio.com/contact/

PUBLICATIONS

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