An AML targeted Duplex Sequencing assay can detect Measurable Residual Disease (MRD) at a sensitivity better than 0.01% Variant Allele Frequency

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Poster Number 122

Introduction

The majority of acute myeloid leukemia (AML) patients relapse after initially achieving remission following conventional therapies. Measurable residual disease (MRD) in AML has emerged as a strong prognostic factor important for managing therapy, which may include hematopoietic stem cell transplant, and for monitoring response and predicting clinical outcomes. Next-generation sequencing (NGS) assays have the potential for accurate detection of low frequency mutations in blood or bone marrow; however, PCR and sequencing errors limit accuracy at variant allele frequencies (VAF) below approximately 1%. Recent European LeukemiaNet (ELN) guidelines define AML NGS-MRD positivity as ≥ 0.1% VAF, but recent evidence suggests that identification of lower frequency variants may be informative for managing subsequent therapy. Thus, there is great need for NGS assays that incorporate error correction to achieve better variant detection sensitivity.

Duplex Sequencing (DS) is an error-corrected NGS (ecNGS) method that greatly reduces errors by comparing complementary DNA strands to each other to eliminate PCR and sequencing artifacts. Here, we report the analytical validation of the DuplexSeq™ AML MRD Assay, an updated 36-gene AML targeted assay informed by 2022 European LeukemiaNet (ELN) recommendations. Using this assay coupled with our updated DuplexSeq V2 Library Preparation Kit, we conducted Limit of Detection (LoD), Accuracy & Linearity, Intermediate Precision, and Limit of Blank (LoB) studies, testing samples consisting of contrived human genomic DNA carrying 25 targeted variants, plus DNA extracted from both healthy normal and AML-positive peripheral blood and bone marrow specimens.

With a demonstrated LoD below 0.01% VAF and an LoB of 0, this novel AML assay represents a highly sensitive and specific ecNGS test for detecting MRD in AML research. This Duplex Sequencing assay will be a valuable asset for investigators wishing to confidently detect ultra-rare mutations and advance understanding of disease progression.

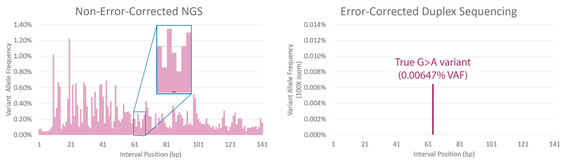
Sequencing Errors Obscure Truth

Next-Generation Sequencing (NGS)

Single Strand Error-Corrected NGS

Duplex Sequencing

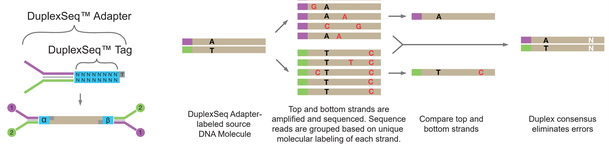
Duplex Sequencing reduces background noise, enabling detection of minority variants that other DNA sequencing methods miss.



Above is shown a portion of the *IDH1* gene sequenced by conventional, non-error-corrected sequencing (left) and with the DuplexSeq AML MRD Assay (right). Without error correction, every position across this interval appears mutated in approximately 0.043 - 1.2% of molecules sequenced. A very low-frequency G>A variant (Arg132Cys) shown in the darker shade is obscured by preponderance of sequencing and PCR errors (see inset on left). Duplex Sequencing eliminates the background noise, revealing the previously hidden true mutation (observed at 0.00647% VAF, right hand panel). Note the 100-fold change in y-axis from left to right; the true mutation is nearly invisible on the scale on the left.

Duplex Sequencing: How it Works

Duplex Sequencing eliminates errors by separately tagging, copying, and sequencing the forward and reverse DNA strands.



AML MRD Panel Content and Performance

Genes Targeted by the AML MRD Panel					
ASXL1	*CSF3R*	*GATA2*	*MPL*	*PTPN11*	*STAG2*
BCOR	*DDX41*	*IDH1*	*NPM1*	*RAD21*	*TET2*
BRAF	*DNMT3A*	*IDH2*	*NRAS*	*RUNX1*	*TP53*
CALR	*ETV6*	*JAK2*	*PHF6*	*SETBP1*	*U2AF1*
CBL	*EZH2*	*KIT*	*PPM1D*	*SF3B1*	*WT1*
CEBPA	*FLT3*	*KRAS*	*PTEN*	*SRSF2*	*ZRSR2*

Table 1: Genes targeted by the DuplexSeq AML MRD panel. These genes are recurrently mutated in adult AML, with 90-95% of adult patients predicted to have at least one mutation in at least one of these genes. This panel content (220 probe intervals, 80 kb) incorporates recent guidance from the research community and the European LeukemiaNet (ELN).

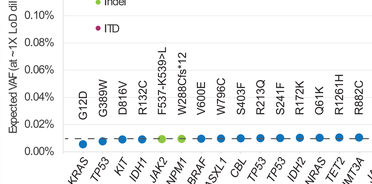


Figure 1: Performance of AML MRD targets from 2,000 ng samples. Each bar represents the mean duplex molecular depth (a measure of recovered source DNA molecules) of a target interval. The bars represent the average of four samples derived from four healthy normal female donors, with error bars representing standard deviation of the mean. Plum-colored bars indicate targets on X chromosome, and black bars indicate autosomal targets. Green and orange lines represent 80% and 20% (respectively) of the mean-of-mean duplex molecular depth from the entire panel. Across samples, mean duplex molecular depth is > 54,000x; a level sufficient to ensure > 98% of targets are covered at a duplex molecular depth >20,000x (blue line). This is achieved with 2x150 base paired-end sequencing on 1.25 billion clusters per sample.

Samples used in Analytical Validation Studies

Samples used in Analytical Validation studies					
Sample	Donor Age	Donor Sex	Specimen type	Genes mutated (expected)	Expected VAF range
AMLpos1[1*]	67	Female	BM	*FLT3* (ITD), *IDH2*, *RUNX1*, *SRSF2*	30.1 - 96.1%
AMLpos2[1**]	71	Male	PB	*FLT3* (SNV), *NPM1*, *SRSF2*, *TET2*	8.26 - 38.5%
AMLpos3[1]	61	Female	BM	*IDH2*, *KRAS*	3.30 - 36.6%
AMLpos4[1]	81	Female	BM	*CEBPA*, *EZH2*, *ZRSR2*	45.8 - 94.7%
AMLpos5[1]	62	Male	BM	*FLT3* (ITD)	≥1%***
AMLpos6[1]	62	Male	PB	*FLT3* (ITD)	≥1%***
AMLpos7[1]	76	Male	PB	*CBL*, *KRAS*, *NPM1*, *NRAS*	11.9 - 31.8%
AMLpos8[1]	69	Male	BM	*BCOR*, *DNMT3A*, *FLT3* (ITD), *KRAS*, *SF3B1*, *TET2*, *U2AF1*	3.62 - 53.7%
AMLpos9[1]	44	Female	PB	*BCOR*, *RUNX1*, *U2AF1*, *WT1*	20.2 - 66.3%
AMLpos10[1]	61	Male	PB	*KIT*	43.4%
Surrogate sample[1,2,3]	n/a	n/a	n/a	See Figure 2	0.00055 - 1.3%
negative control DNA[2]	18	Male	PB	see table caption	0%
healthy normal DNA (older cohort)[3,4]	63-75	Male & Female	PB & BM	none	0%
healthy normal DNA (younger cohort)[3,4]	21-36	Male & Female	PB & BM	none	0%

* DNA sample was diluted into healthy normal donor DNA to target VAFs just above and just below the assay LoD, based on *IDH2* expected VAF
** DNA sample was diluted into healthy normal donor DNA to target VAFs just above and just below the assay LoD, based on *NPM1* expected VAF
*** FLT3-ITD data from the orthogonal method were reviewed by a clinical laboratory director at FHCC and reported only as positive or negative, with a 1% VAF threshold
[1]Sample used in LoD study
[2]Sample used in Precision study
[3]Sample used in Accuracy & Linearity study
[4]Sample used in LoB study

Table 2: Description of samples used in Analytical Validation studies.
For AML-positive samples, expected VAFs were orthogonally determined using the Ion Torrent Oncomine Myeloid DNA Assay GX v2. Expected VAFs for variants present in the contrived/surrogate sample were orthogonally determined from vendor documentation, public databases, and published studies; see Figure 2 for more detail. Negative control DNA used throughout these studies was derived from an 18-year-old healthy normal donor; no AML-relevant variants are detected above assay LoD. Healthy normal samples used in the LoB study were also assessed by the Oncomine Myeloid DNA Assay GX v2; no variants at sites of interest were detected.
Some samples in these studies were sourced from the FHCC/UW Hematopoietic Disease Repository (protocols 1690 and 1713).

Figure 2: Variants present in surrogate sample contrived for use in Analytical Validation studies.
Well-characterized DNA samples harboring AML-relevant variants (Horizon Discovery Oncospan gDNA and Myeloid DNA Reference standard, cancer cell lines) were mixed into a background of negative control DNA from a young, healthy normal male donor. Mixing ratios were designed such that the majority of VAFs surrounded the expected assay LoD of 0.01% VAF at the "1X LoD dilution" level. After creating a stock of this sample at the "10X LoD dilution" level, it was further blended with healthy normal DNA such that it could be tested at a range of dilutions from 10X to 0.1X LoD, enabling interrogation of variants from 1.3% down to 0.00055% VAF. Grey dashed line: expected 0.01% VAF level.

The AML MRD Assay Limit of Detection is <0.01% VAF

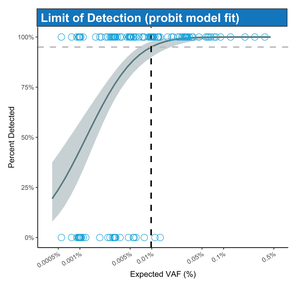


Figure 3. The DuplexSeq AML MRD assay exhibits a limit of detection of 0.0098% VAF.
A surrogate sample contrived to harbor known variants targeted by the AML assay was mixed with healthy normal control DNA at different ratios to achieve expected VAFs from 0.00055% to 0.37%. Each variant at each dilution level was independently evaluated 4 times (twice with each of 2 manufactured reagent lots) for a total of 500 total measurements. Application of a minimum Duplex Sequencing depth threshold of 20,000x reduced the total number of measurements to 480. From these data, a probit model (note fitted curve in teal) yielded a limit of detection (the VAF at which probability of detection is 95%), of 0.0098%. Shown here is the percent detection of each variant at a given VAF, using reagent lot 1 only. Black dashed vertical line: 0.0098% VAF level. Gray dashed horizontal line: 95% detection level. A 95% confidence interval around the fitted curve is shaded in gray.

Table 3. Limit of Detection estimates for the DuplexSeq AML MRD assay.
Limit of Detection was estimated separately using two different manufactured lots of DuplexSeq V2 Library Preparation Kit reagents. After application of a minimum Duplex Sequencing depth threshold of 20,000x, the LoD was estimated as 0.0098% and 0.0068% VAF for reagent lots 1 and 2 respectively. The higher LoD was conservatively chosen as the claimed assay limit of detection. When all targeted sites are assessed, the limit of detection estimates are 0.013% and 0.011% VAF for reagent lots 1 and 2 respectively. However, the vast majority of the panel achieved depths well above 20,000x duplex depth; the estimates from data with this threshold applied should be considered the better representation of overall panel performance.

Limit of Detection Estimation using probit model [log(expected VAF)]							
Data set	Reagent Lot	N sites	LoD estimate	95% CI around LoD		Intercept	Slope
all sites with duplex depth ≥ 20,000x	Lot 1	240	**0.0098%**	(0.0023%, 0.0412%)		5.30351	0.79107
	Lot 2	240	**0.0068%**	(0.0021%, 0.0215%)		6.56638	0.98595
all sites	Lot 1	250	**0.013%**	(0.0028%, 0.0569%)		4.90696	0.74493
	Lot 2	250	**0.011%**	(0.0028%, 0.0432%)		5.17486	0.78182

The AML MRD Assay is accurate and linear

Accuracy: percent agreement between methods					
Metric	N libraries	N sites*	N sites agree†	Estimate	95% CI**
OPA	37	1,962	1,934	98.60%	(97.8%, 99.3%)
PPA	16	97	96	99.00%	(97.0%, 101.0%)
NPA	37	1,865	1,838	98.60%	(97.7%, 99.4%)

†Expected call at each interrogated site is based on orthogonal method
* Data derived from all sites ≥20,000x duplex depth, all variants with expected VAF ≥ DuplexSeq AML MRD assay LoD
** CIs are based on cluster proportions across runs

Table 4: Accuracy of the DuplexSeq AML MRD assay vs an orthogonal method.
Across 16 sample libraries with expected variants present and 37 sample libraries with expected negative sites, there was very high percent agreement between the DuplexSeq AML MRD assay and the orthogonal Oncomine assay. The single DuplexSeq false negative was expected at very low frequency near LoD. All but 1 of the 27 DuplexSeq false positives were well below the orthogonal LoD and are very likely true positives that were not detected by the orthogonal method. One false positive (a T>TCTGC insertion in *NPM1*) was confidently called by the DuplexSeq assay; support for this call was also observed in the orthogonal .vcf file. The orthogonal call was filtered out of the report due to strand bias. No significant strand bias was observed in the DuplexSeq data at this locus.

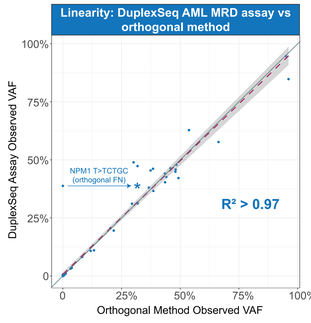


Figure 4: Linearity of the DuplexSeq AML MRD assay vs an orthogonal method.
Linearity of variant detection from the samples described above was assessed for the DuplexSeq AML MRD assay vs the orthogonal method. When all sites are considered, $R^2 = 0.952$ (N = 1,962). When the surfeit of true negatives are excluded, $R^2 = 0.943$ (N = 116).
*The expected VAF for the orthogonally-filtered NPM1 insertion (see above) can be estimated as 31.7% based on variant calls in the orthogonal .vcf file for this sample. When the expected VAF for this variant is set to 31.7%, **$R^2 > 0.97$**. FLT3-ITD variants were excluded from this analysis, as orthogonal VAF estimates are low confidence.

The AML MRD Assay is highly reproducible

DuplexSeq AML MRD assay overall reproducibility					
Metric	N libraries	N sites*	N agree†	Estimate	95% CI**
OPA	30	1,506	1,467	**97.40%**	(96.7%, 98.1%)
PPA	20	410	405	**98.80%**	(97.8%, 99.8%)
NPA	30	1,096	1,062	**96.90%**	(96.0%, 97.8%)

†Expected call at each interrogated site is based on majority call across replicate runs
* Data derived from all sites ≥20,000x duplex depth, all variants with
** CIs are based on cluster proportions across runs

Table 5: Reproducibility of the AML MRD Assay.
Intermediate precision was assessed across two library preparation operators, two manufactured lots of DuplexSeq V2 Library Preparation Kit reagents, and three independent runs of library preparation with start dates ≥21 days apart. The 3X LoD surrogate sample, 1X LoD surrogate sample, and negative control DNA were tested in each library preparation run. Repeatability was assessed with triplicate libraries from one library operator in one library preparation run; samples were tested in singlicate in all other runs. Reproducibility estimates were excellent, with all percent agreement metrics ≥96.9%.

FLT3, *NPM1* variants detected with 100% accuracy

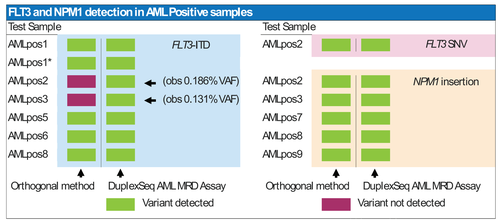
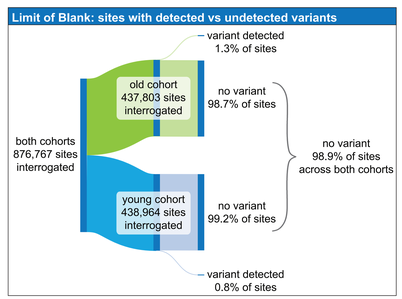


Figure 5: *FLT3* and *NPM1* variants are detected with 100% accuracy in AML-positive samples.
Within the ten AML-positive samples tested for the Accuracy & Linearity study shown to the left, 11 *FLT3* and *NPM1* variants are expected based on orthogonal assay testing. All of these variants are detected by the DuplexSeq AML MRD assay. In addition, two *FLT3*-ITDs are detected by the DuplexSeq assay with measured VAFs below the LoD of the orthogonal assay. These are likely true positive calls, but the orthogonal assay lacks the sensitivity to confidently detect variants at VAFs in this range.
*AML-positive sample 1 was tested in the undiluted state (top row) and diluted 1,767-fold into a background of negative control DNA (second row). The expected VAF of this variant in this diluted state is below the LoD of the orthogonal method, but this sample is considered positive for the purposes of this comparison.

The AML MRD Assay Limit of Blank is 0% VAF



Limit of Blank estimates		
age cohort	N sites	LoB estimate
all	876,767	**0.0000%**
older	437,803	**0.0000%**
younger	438,964	**0.0000%**

Table 6 (above): DuplexSeq AML MRD Assay Limit of Blank estimates.
When the older and younger cohorts are analyzed separately or combined, all Limit of Blank estimates are 0.0000% VAF (>98.7% of sites interrogated have no variant detected).

Figure 6: Sankey diagram of sites interrogated in Limit of Blank study.
Two sample donor cohorts of 10 individuals each (older donors vs younger donors) were tested in this study. Across the two cohorts, 867,767 sites achieved duplex depths above the 20,000x threshold. Roughly half these sites were in the older cohort samples and half in the younger cohort. In each cohort, and in the combined data, there was no variant detected in greater than 95% of sites, supporting a Limit of Blank estimate of 0.0000% VAF .

Study Design and Methods

Sequencing libraries were prepared from 2,000 ng DNA input using TwinStrand DuplexSeq V2 Library Preparation Kits. This kit comprises reagents enabling enzymatic fragmentation, end-repair/A-tailing, adapter ligation, library conditioning to remove damaged DNA molecules, PCR amplification, and target enrichment via hybrid capture. Libraries were sequenced on the Illumina® NovaSeq 6000 platform using S4 flow cells and reagents. Each library was allocated 1.25 billion sequencing clusters. Analysis of data gathered for the Limit of Detection study showed that the data best fit the probit regression model when a minimum duplex depth threshold of 20,000x was applied (assessed by Akaike's Information Criterion). This threshold was then applied to all libraries in these studies; 91% of the bases targeted by the AML MRD panel achieved depths above this threshold in ≥95% of these libraries.

Conclusions

- This updated AML MRD panel features refined and expanded content to drive AML MRD research.
- With a Limit of Detection below 0.01% VAF and a Limit of Blank of 0, this assay is a highly sensitive, accurate, and reproducible test enabling confident detection of rare clonal somatic AML-related mutations in your studies.
- As shown recently in a research study by Dillon LW and Higgins J, *et al.* (Haematologica. 2024 Feb 1;109(2):401-410), a TwinStrand AML panel outperformed Multiparametric Flow Cytometry in identifying adult AML relapse cases. The updated panel and updated DuplexSeq V2 Library Preparation Kit described here represent an analytically validated AML MRD assay suitable and available for similar studies.

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