RACE AGAINST THE CLOCK: VALIDATING A RAPID NGS MYELOID PANEL

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INTRODUCTION

- Acute leukemia (AL) requires rapid (3–5 days) multi-gene results for accurate subtyping.
- Traditional workflows rely on multiple send-outs and overlapping assays, extending turnaround and delaying genomically guided treatment decisions.
- The Hematological Expedited Sequencing (HEXS) streamlines testing into a single next-generation sequencing (NGS) panel covering 58 key genes relevant to myeloid neoplasms.
- This study validated HEXS on both the MiSeq and MiSeq i100 Plus (i100) platforms to deliver comprehensive, rapid genomic results for hematologic malignancies.

METHODS

- HEXS is based on the Pillar oncoReveal® Myeloid Panel (Figure 1).
- Data were analyzed using Pillar's PiVAT bioinformatics pipeline (v.2024.2.3) for variant calling.

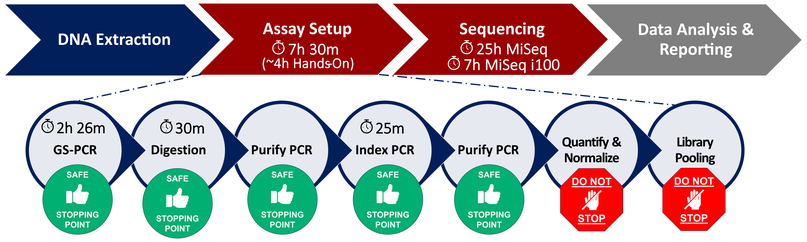

Figure 1: HEXS assay workflow.

HEXS Gene Coverage									
ABL1	*BRAF*	***CEBPA***	***ETV6***	*HRAS*	***KDM6A***	*NPM1*	*PTEN*	*SMC1A*	***TP53***
ANKRD26	*CALR*	*CSF3R*	***EZH2***	*IDH1*	*KIT*	*NRAS*	*PTPN11*	*SMC3*	*U2AF1*
ASXL1	*CBL*	*CUX1*	*FLT3*	*IDH2*	*KMT2A*	*PDGFRA*	***RAD21***	*SRSF2*	*WT1*
ATRX	*CBLB*	*DDX41*	*GATA1*	***IKZF1***	*KRAS*	***PHF6***	***RUNX1***	*STAG1*	***ZRSR2***
BCOR	*CBLC*	***DNMT3A***	***GATA2***	*JAK2*	*MPL*	***PIGA***	*SETBP1*	***STAG2***	
BCORL1	***CDKN2A***	*ETNK1*	*GNAS*	*JAK3*	*NF1*	*PPM1D*	*SF3B1*	***TET2***	

Table 1: 58 genes covered by HEXS. Bold: Full coding DNA sequence; Red: ELN and NCCN required testing at diagnosis; Blue: ELN recommended for testing at diagnosis.

- Performance was established using 61 positive and 18 negative samples sourced from residual bone marrow, peripheral blood and bone core specimens previously tested on NGS and/or capillary electrophoresis methods, and covered SNVs, indels, and *FLT3*-ITDs.
- One comprehensive control, which acts as both a positive and negative control, was included in every run.
- Precision, limit of input (LOI), and limit of detection (LOD), were established by up to 10 replicates using 4 clinical samples and 1 reference standard (Horizon HD829).
- Receiver operating characteristic (ROC) analysis was performed to optimize sequencing depth and establish cutoffs for accurate variant detection.
- Performance equivalency testing was completed using the i100.

RESULTS

1. Accuracy

- Good correlation between MiSeq and i100 VAF% and Read Depth, confirming comparable variant detection and sequencing performance.

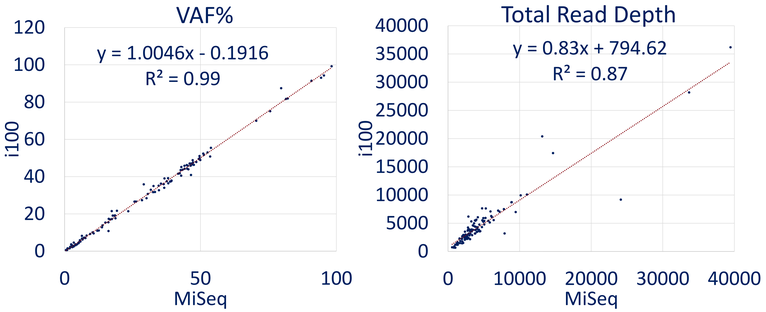

Figure 2: Linear regression for VAF% and read depth for SNVs, indels, and *FLT3*-ITDs between MiSeq and i100.

2. Sensitivity and Specificity

- HEXS demonstrates strong concordance across variant types, with few false positives.

Variant Type	Sensitivity (PPA) (True Positive/Expected)		Specificity (PPV) (True Positive/True Positive + False Positive)	
	MiSeq	i100	MiSeq	i100
SNVs	99.5% (195 / 196)	100% (203 / 203)	96.3% (206 / 214)	99.5% (210 / 211)
Indels <10bp / "Delins"	100% (64 / 64)	100% (65 / 65)	98.5% (66 / 67)	97.0% (65 / 67)
Indels ≥10bp	94.1% (16 / 17)	100% (22 / 22)	87.5% (21 / 24)	87.5% (21 / 24)
FLT3-ITDs	100% (17 / 17)	100% (21 / 21)	100% (22 / 22)	100% (24 / 24)

Table 2: Sensitivity and Specificity across sequencing platforms with data shown after discrepancy resolution.

3. Precision

- Reproducible qualitative variant detection across VAFs; high %CVs due to expected variability near LOD.

Instrument	Precision Type	Total Replicates	% Concordant (VAF% Range)	Overall %CV Range (All Samples and Variant Types)
MiSeq	Intra-Assay	36	100% (2.3 - 49%)	0.4–23.7%
	Inter-Assay	36	100% (1.1-49%)	0.5–49.2%
i100	Intra-Assay	36	100% (1.0-47.8%)	0.7–25.3%
	Inter-Assay	36	100% (0.9-47.8%)	0.7–44.6%

Table 3: HEXS intra- and inter- assay reproducibility data for SNVs, indels, and *FLT3*-ITDs.

4. ROC Analysis and Limit of Detection

- ROC analysis identified 500x as the optimal read-depth cutoff, while LOD studies confirmed reliable detection at ~1–2 % VAF for SNVs/Indels and 0.3 % for *FLT3*-ITDs.
- False positivity limited to variants with low-VAF calls or homopolymer regions (Figure 3).

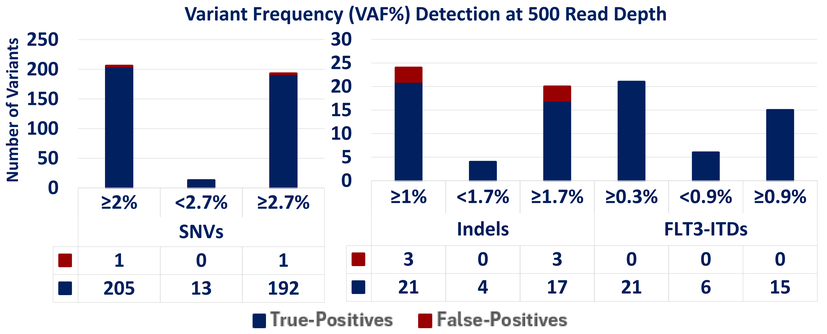

Figure 3: HEXS performance at 500x read depth across VAF bins. True-positive (blue) and false-positive (red) counts are shown for SNVs, Indels, and *FLT3*-ITDs.

LIMITATIONS

- Variants in homopolymer or low-complexity regions with <500× coverage or VAFs <4 % (SNVs) or <3 % (indels) increase false positivity.
- DNA input of 20–60 ng is optimal for reliable performance. 10 ng can be used, but should be reported with caution, due to increased false positivity at lower inputs.
- Detection is limited by vendor-defined filters in regions of low complexity. SNVs, Indels <10bp, and indels ("delins") ≥10bp are filtered at 2%. All other indels are filtered at 1%.
- *FLT3*-ITDs ≥21 bp are detected down to 0.3 % VAF, while ITDs >84 bp fall outside the validated detection range.

CONCLUSIONS

- Overall results were concordant and reproducible, confirming manufacturer cutoffs of 2%, 1% and 0.3% for SNVs, indels and *FLT3*-ITDs, respectively.
- HEXS provides a streamlined, and clinically actionable workflow for rapid genomic characterization of hematologic malignancies.
- HEXS performed on the i100 delivers equivalent or better analytical performance than the MiSeq and reduces sequencing time by 17 hours.
- HEXS enables 3–5-day myeloid NGS reporting and supports rapid clinical decision-making.