Improving NGS Library Coverage Uniformity Using Single Molecule Droplet PCR for the Molecular Assessment of Acute Myeloid Leukemia

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Abstract

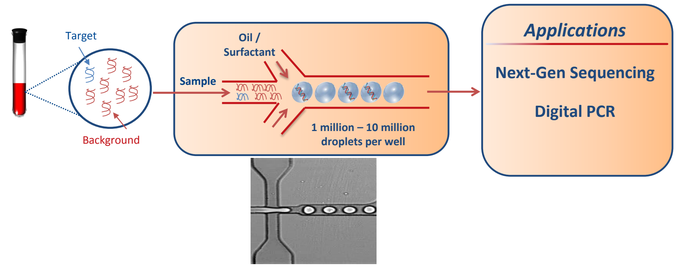
INTRODUCTION: Library preparation methods are the backbone in any NGS assay. Specifically with targeted gene panels, the process of generating a library relies on multiplex primer-based enrichment chemistries to generated hundreds of discreet amplicons simultaneously. Currently, a handful of commercially available library preparation kits provide mutational analysis of acute myeloid leukemia (AML)-specific molecular abnormalities. While targeted panels library preparation paired with next generation sequencing (NGS) technologies have emerged as a powerful duo in the clinical setting, these methods have their limitations. Not surprisingly, it is difficult to optimize or "fine tune" the amplification efficiency for individual amplicons in multiplex PCR assays. Single molecule droplet PCR is a robust methodology for generating NGS libraries with superior amplicon coverage uniformity.

MATERIALS AND METHODS: 51 bone marrow samples previously characterized by a NGS methods from patients with acute myeloid leukemia/myelodysplastic syndromes were compared in a double blind fashion to the results obtained using an AML gene panel with 548 amplicons covering clinical relevant regions in 49 genes using single molecule droplet PCR (ThunderBolts™ Myeloid Panel, RainDance™ Technologies. Billerica, MA). Sequencing was performed using a MiSeq® bench top sequencer (Illumina® Inc. San Diego, CA), and bioinformatic analysis was perform using NextGENe® 2nd generation sequence analysis software (SoftGenetics®. State College, PA)

RESULTS: The panel successfully called all single nucleotide variants (SNV) and small insertions/deletions (indels) in all 51 samples. *FLT3*-internal tandem duplications were not initially detected with NextGENe software and an indel detection algorithm is needed for their successful detection. Both mean and median amplicon coverage (read depth) of *CEBPA* gene was greater than 2,500X. Dilution of AML patient DNA showed this panel could detected an allelic frequency down to 5% with a high level of assay reproducibility.

CONCLUSION: Applying a single molecule droplet methodology for library preparation enrichment allowed for the detection of relevant somatic mutations using a simplified workflow with a 7-day turn-around time, requires only 75 ng of patient DNA, while providing superior coverage uniformity, especially in difficult to amplify GC-rich regions such as the *CEBPA* gene.

Droplet Technology

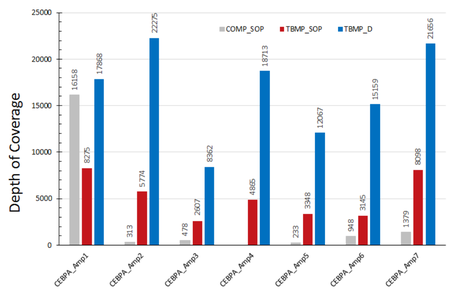


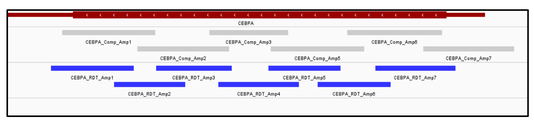
Precise 5 picoliter partitions. Controlled reactions.
Single target quantitation.

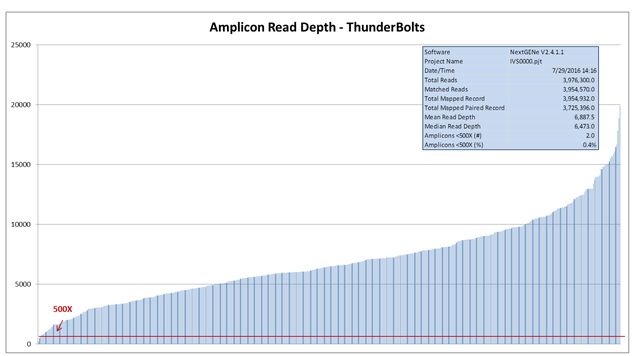
Myeloid NGS Panel Content

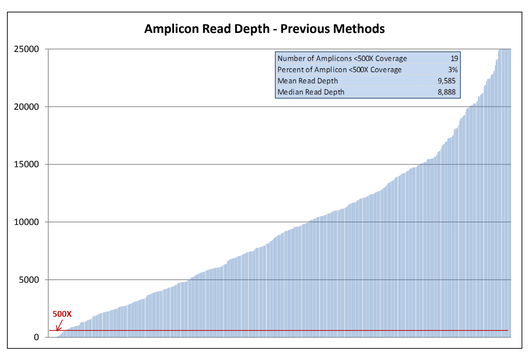
Gene	Exon(s) Covered	Gene	Exon(s) Covered	Gene	Exon(s) Covered	Gene	Exon(s) Covered
ASXL1	1-3, 5-12	*GATA1*	2	*MEK1*	2 and 3	*SF3B1*	13-16
BCOR	2-15	*GATA2*	2-6	*MPL*	10	*SMC1A*	1-25
BCORL1	2-12	*GNAS*	8 and 9	*MYD88*	3-5	*SMC3*	10, 13, 19, 23, 25, 28
BRAF	11-15	*HRAS*	1-4	*NOTCH1*	26-28, 34	*SRSF2*	1
CALR	9	*IDH1*	4	*NPM1*	11	*STAG2*	3-35
CBL	8 and 9	*IDH2*	4	*NRAS*	2-4	*TET2*	3-11
CBLB	9-10	*JAK1*	10 and 14	*PHF6*	2-10	*TP53*	2-11
CEBPA	All	*JAK2*	12 and 14	*PML*	3	*U2AF1*	2 and 6
CSF3R	14-17	*JAK3*	13	*PTEN*	5 and 7	*WT1*	7 and 9
DNMT3A	2-19	*KDM6A*	1-29	*PTPN11*	3 and 13	*ZRSR2*	2-11
ETV6	2-8	*KIT*	8-11, 13, 17	*RAD21*	2-14		
EZH2	2-20	*KMT2A*	1, 3, 5, 6, 7, 8, 27	*RUNX1*	2-9		
FLT3	14, 15, 20	*KRAS*	2-4	*SETBP1*	4		

CEBPA Depth of Coverage









Results

Validation Summary

Gene	Metric	Value	Total Samples	Diagnostic Sensitivity	Diagnostic Specifictiy	Diagnostic Accuracy
FLT3-ITD	TP	1	57	100%	100%	100%
	TN	56				
	FP	0				
	FN	0				
FLT3 -D835	TP	3	57	100%	100%	100%
	TN	54				
	FP	0				
	FN	0				
IDH1	TP	5	57	100%	100%	100%
	TN	52				
	FP	0				
	FN	0				
IDH2	TP	8	57	100%	100%	100%
	TN	49				
	FP	0				
	FN	0				
JAK2	TP	1	57	100%	100%	100%
	TN	56				
	FP	0				
	FN	0				
CALR	TP	1	57	100%	100%	100%
	TN	56				
	FP	0				
	FN	0				
NPM1	TP	3	57	100%	100%	100%
	TN	54				
	FP	0				
	FN	0				
CEBPA	TP	1	57	100%	100%	100%
	TN	56				
	FP	0				
	FN	0				

Conclusions

- The ThunderBolts™ Myeloid Panel was able to detect relevant somatic mutations using a simplified workflow
- This NGS panel demonstrates significant advantages over previous panel used in our laboratory
- The current version of the panel has superior coverage for the *GC*-rich *CEBPA gene*

Acknowledgements

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