An Optimized NGS Workflow for Detection of FLT3 Internal Tandem Duplication (ITD) in AML Samples

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Introduction

FLT3 ITD, a prognostic marker and drug target, is present in ~19% of AML patients. FLT3 ITD is a tandem repeat of the entire or partial FLT3 exon 13 to exon 15 region ranging in size from 3bp to above 300bp, inserted into the FLT3 juxtamembrane domain or nearby tyrosine kinase domain. Recent publications showed FLT3 ITD can be detected at single nucleotide resolution by NGS panels. However, detection of larger FLT3 ITD and accurate reporting of ITD frequency remain challenging for NGS methods. False negative ITD results or inaccurate mutant/wild type allele ratio measurements could negatively alter treatment decisions for AML patients.

The Illumina TruSight Myeloid panel (TMP) is a broadly used off-the-shelf NGS assay that covers 54 genes involved in myeloid malignancies. Although the TMP assay can detect SBS and small indel, medium to large size FLT3 ITD cannot be detected by the assay. Here we present an enhanced workflow to overcome these challenges.

In addition, low-level mutations were detected by the TMP assay and further confirmed with an independent assay. A long deletion in the exon 9 of CALR was also successfully detected by the custom pipeline. CEBPA drop-off was covered by a custom designed Nextera based PCR amplicon sequencing assay.

Materials and Methods

Dynamic range, accuracy, and inter-run reproducibility were validated for the detection of SBS, indel, and FLT3 ITD using reference standards and DNA extracted from cell lines and EDTA blood and bone marrow aspirate samples from AML patients. NGS libraries were prepared using 50 ng extracted genomic DNA following Illumina's standard TMP protocol. 275bp X2 sequencing instead of the standard 150bp X2 was run on MiSeq flowcell v3. Data analysis was performed using a MolecularMD custom pipeline for SBS and small indel detection as well as for the analysis of FLT3 ITD. FLT3 ITD data analysis was conducted using four methods including three off-the-shelf programs, Pindel, ITDseek and ITDetector, as well as Cigar, the MolecularMD developed ITD algorithm.

PCR products targeting FLT3 ITD region generated from the original extracted DNA were analyzed by the Caliper assay and a reference capillary electrophoresis (CE) method for ITD size and frequency confirmation. The ArcherDX Core AML panel was also used to detect the FLT3 ITD. Other genes of interest were analyzed on the MiSeq using a custom Nextera XT assay.

Results

Limit of Detection and Inter-run Reproducibility for FLT3 ITD

FLT3 ITDs at 126 bp and 21 bp in PL-21 and MOLM-13 cell lines, respectively, were detected by TMP assay using the three algorithms (ITDseek, Pindel, and Cigar). In contrast to ITDseek which only reports ITD sequence without frequency information, Pindel and Cigar report both ITD sequence and frequency. Compared to Pindel, ITD frequency reported by the custom algorithm Cigar is closer to that determined by fragment analysis using the Caliper assay (Table 1).

Table 1. TMP Performance for FLT3 ITD Detection in Cell Line DNA

Sample	TMP (ITDseek, Pindel, Cigar)			Mutation Frequency (%)		
	ITD Position	ITD Sequence	ITD Size	TMP		Caliper
				Pindel	Cigar	
PL-21	Insertion of GATTTGA between the duplication of 13:28,608,204-28,608,322	ACATTCCATTCTTACCAAACTCTAAATTTTCTCTTGGAAACTC CCATTTGAGATCATATTCATATTCTCTGAAATCAACGTAGAAG TACTCATTATCTGAGGAGCCGGTCACCTGTACCGATTTGA	126 bp	10.38	18.34	22.63
MOLM-13	Chr13: 28608261	ATTCATATTCTCTGAAATCAA	21 bp	62.57	59.40	67.44

PL-21 DNA was serially diluted into NA12878 DNA at 1:1 ratio to prepare five dilution mixtures (mix-1 to mix-5). FLT3 ITD with mutation frequencies from ~5% to ~10% were expected in mix-1 and <1% to ~5% in further dilutions. The TMP analysis was repeated three times using aliquots of the 5 mixtures. The FLT3 ITD mutation was detected in all 5 mixtures from all 3 runs. The changes in mutation frequencies from mix-1 to mix-5 also correlated with the dilution factor. The limit of detection of the 126 bp ITD is ~0.5%. FLT3 ITD mutation frequencies from all mixtures were confirmed by the Caliper assay which was in closest agreement with the Cigar analysis. All 3 runs detected the same ITD location with the same sequence (Table 2).

Table 2. Dynamic Range of FLT3 ITD Detection and Assay Reproducibility

Sample	Mutation Frequency (%)								
	TMP								Caliper
	Pindel				Cigar				
	Run-A	Run-B	Run-C	Mean ± SD	Run-A	Run-B	Run-C	Mean ± SD	
PL-21	10.38	NT	NT		18.34	NT	NT		22.63
mix-1	5.58	4.29	5.42	5.10 ± 0.70	9.21	6.62	8.7	8.18 ± 1.37	8.48
mix-2	2.78	2.42	3.53	2.91 ± 0.57	4.18	3.75	5.4	4.44 ± 0.86	5.47
mix-3	1.12	1.45	1.99	1.52 ± 0.44	2.00	2.28	3.2	2.49 ± 0.63	3.32
mix-4	0.74	0.47	0.88	0.70 ± 0.21	1.11	0.75	1.4	1.09 ± 0.33	1.64
mix-5	0.33	0.36	0.35	0.35 ± 0.02	0.53	0.61	0.6	0.58 ± 0.04	0.55
NT, Not Tested.									

Assay Accuracy

DNA from a set of 16 AML blood samples was analyzed using the TMP assay, as well as the Caliper and ArcherDX Core AML assays for detection of FLT3 ITD mutation. A 54 bp FLT3 ITD mutation was detected in sample AML-S2 by the TMP assay. Fragment analysis of FLT3 ITD using the Caliper assay and ArcherDX Core AML panel also detected the same ITD in AML-S2 (Table 3). The ITD frequency was 43.40% measured by the Caliper assay. The ArcherDX Core AML panel reported the ITD at 52 bp length with a small difference in ITD sequence from that reported by TMP. No FLT3 ITD was detected in the remaining 15 samples using the ArcherDX and Caliper assays. The two independent analyses of FLT3 ITD by Caliper and Archer assays demonstrate that there was no false negative detection of FLT3 ITD by TMP in this sample set.

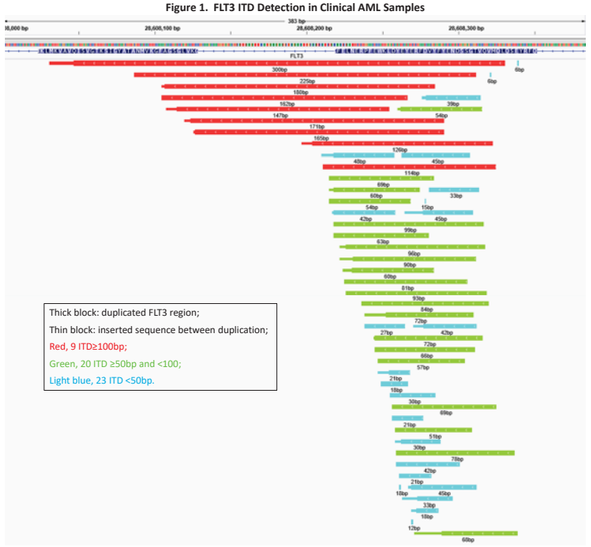
Table 3. FLT3 ITD Detection in AML Samples (pilot study)

Sample	Assay	ITD Region	ITD Sequence	ITD Size	Mutation Frequency (%)	
AML-S2	TMP	chr13: 28,608,215-28,608,268	TTACCAAACTCTAAATTTTCTCTTGGAAACT CCCATTTGAGATCATATTCATAT	54 bp	33.26 (Pindel)	38.60 (Cigar)
	Caliper	NR	NA	55 bp	43.40	
	Archer	chr13: 28,608,219-28,608,270	CAAACTCTAAATTTTCTCTTGGAAACTCCCA TTTGAGATCATATTCATATTC	52bp	NR	
NA, Not Applicable; NR, Not Reported.						

FLT3 ITD Detection in Clinical AML Samples and Cell Lines

52 unique FLT3 ITDs ranging in size from 6bp to 300bp were detected in 79 clinical AML samples (a total of 230 clinical AML samples were tested) and 10 cell line samples using our enhanced TMP workflow.

Figure 1. FLT3 ITD Detection in Clinical AML Samples



Algorithm Comparison for FLT3 ITD Analysis

Four different algorithms were used to call FLT3 ITDs in 58 samples that were sequenced using MolecularMD's enhanced workflow for the TMP assay. The MolecularMD algorithm, Cigar, detected 100% (76/76) of the ITDs. The ITDs ranged from small 6bp to large 300bp in size. Cigar identified potential ITDs by searching for and parsing the soft clip regions in alignment which are significantly different from control samples. In comparison, the publicly available methods such as Pindel, ITDseek, and ITDetector detected 53-82% of the FLT3 ITDs in this sample set (Table 4).

Table 4. Methods Comparison to Call FLT3 ITD

Methods	# of ITD called	# of ITD not called	Notes
Cigar	76	0	
Pindel	62	14	
ITDseek	50	26	Most called at the 5' end of ITD
ITDetector	40	36	5 large size ITDs are weak calls with incorrect ITD length, and 29 ITDs are not called due to alignment as insertion. ITDetector only call those aligned as soft clip.

Long FLT3 ITD Detection

A 165 bp large ITD (Figure 2) was successfully detected with an accurate mutation frequency call by our enhanced analysis. More interestingly, a 300bp FLT3 ITD (Figure 3) is captured by a novel amplicon 155bp in size from tile 1 and tile 3 probes. This amplicon is generated by the extension of tile 1 upstream probe and ligation of the extended DNA strand to the downstream probe of tile 3, which is a different case compared to a 165bp long ITD that only occurs with the tile 2 probes.

Figure 2. A 165bp FLT3 ITD Captured by Tile 2 Amplicon

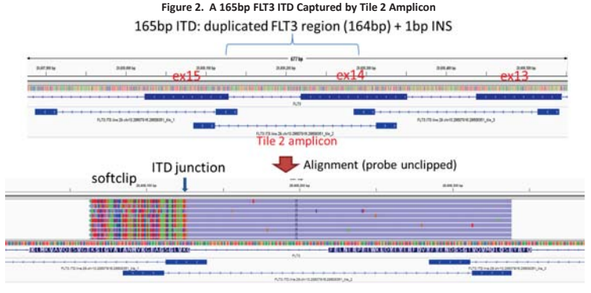
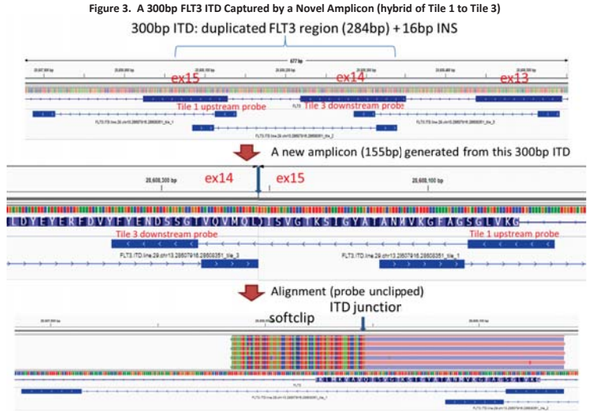


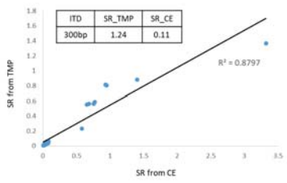
Figure 3. A 300bp FLT3 ITD Captured by a Novel Amplicon (hybrid of Tile 1 to Tile 3)



Correlation of the FLT3 ITD Signal Ratio (SR) between TMP Assay and CE Method

32 samples with known SR values detected by a CE method were compared with that of the TMP assay. Except for the sample with 300bp FLT3 ITD, there was a good correlation between CE and TMP assays (R^2 = 0.88) for the other 31 samples. In general, ITD SR measured by the TMP NGS assay is lower than the reference CE result because the larger amplicon size caused by the inclusion of the duplicated region in the PCR amplicon results in lower PCR efficiency compared to wild type amplicon. One exception in this study was the 300bp ITD where the SR measured by TMP assay was close to the true result, although the CE assay significantly underestimates the SR because of the large amplicon size.

Figure 4. SR Correlation between TMP and CE Assays



Samples with Multiple ITDs

Multiple ITDs in single sample were detected by MolecularMD's enhanced TMP assay. The SR ratio was concordant with the reference CE result.

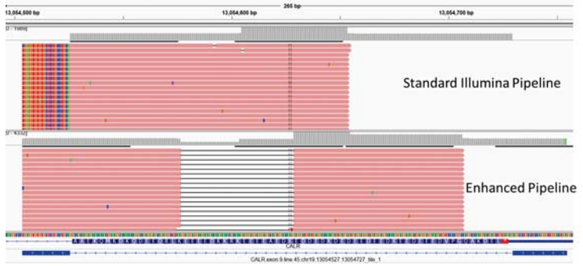
Table 5. Samples with Multiple ITDs

No. of ITD per sample	Sample	ITD size (bp)	Frequency	SR	Total SR
2	S1	6	1.0%	0.0101	0.3633
		15	26.1%	0.3532	
	S2	18	5.74%	0.0609	0.0957
		33	3.36%	0.0348	
	S3	30	12.5%	0.1429	0.231
		42	8.1%	0.0881	
	S4	45	1.1%	0.0111	0.2899
		96	21.8%	0.2788	
	S5	60	25.5%	0.3423	0.4061
		69	6.0%	0.0638	
	S6	72	1.01%	0.0102	0.0115
		84	0.13%	0.0013	
	S7	72	1.7%	0.0173	0.1774
		171	13.8%	0.1601	
3	S8	6	1.50%	0.0152	0.5633
		27	13.74%	0.1593	
		39	27.57%	0.3806	
	S9	21	0.24%	0.0024	0.0066
		42	0.22%	0.0022	
		90	0.20%	0.0020	
5	S10	42	0.97%	0.0098	0.2303
		57	0.73%	0.0074	
		68	0.91%	0.0092	
		78	5.15%	0.0543	
		114	13.01%	0.1496	

CALR 52bp Deletion Detection

CALR is known to have long deletions in exon9. Our data demonstrated that long deletions were missed using the standard TMP assay due to incorrect alignment. To overcome this issue, we directly aligned the fastq files from 151bp paired sequencing run with BWA-MEM. The untrimmed probe region at 5' end enables BWA-MEM to correctly align the long deletion and the variants can be further called by VarScan and Pindel (Figure 5).

Figure 5. CALR 52bp Deletion Detected by the Enhanced TMP Assay



Low-Level Mutations, Drop-off and Uncovered Regions

Low-level mutations, i.e. NRAS G12D at 1.7%, NRAS G13D at 2%, and IDH2 R140Q at 1.4%, were detected by TMP panel from AML samples and further confirmed with an independent Nextera XT assay, which could sequence the custom designed PCR target regions. Drop off regions, such as the GC enriched region within CEBPA, were also recovered by the custom Nextera XT assay (Table 6).

Table 6. Low-Level Mutations, Drop-off and Uncovered Regions Detection

Gene	CDS	Amino Acid	Mutation Frequency (%)	
			TMP	Nextera XT
DNMT3A	c.1786C>T	p.R596W	8.88	9.34
IDH2	c.419G>A	p.R140Q	1.43	2.14
NRAS	c.35G>A	p.G12D	1.68	2.66
NRAS	c.38G>A	p.G13D	2.02	3.26
CEBPA	whole exon		partial drop-off	covered

Conclusions

- MolecularMD's enhanced TMP workflow allows for accurate detection and reporting of FLT3 ITD ranging in size from 6bp to above 300bp.
- Low ITD frequency at <0.5% can be reproducibly detected by the enhanced TMP assay.
- Cigar, the MolecularMD developed ITD algorithm, is more accurate and reliable than most off-the-shelf programs for FLT3 ITD detection.
- The enhanced TMP workflow was fully validated for dynamic range, accuracy, and inter-run reproducibility.
- The calculated SR from the enhanced TMP assay has good concordance with the SR determined by a reference CE method.
- Low-level hotspot mutations (<2%) can be accurately detected by the enhanced TMP assay and confirmed using an independent custom designed Nextera XT assay.
- Drop-off and uncovered regions could be recovered by the custom designed Nextera XT assay.
- Short and long indel can be detected using the custom pipeline in the enhanced TMP assay.

References

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