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Fragment analysis

Research Protocol Guide: FLT3-ITD mutation detection using capillary electrophoresis

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Introduction

Acute myeloid leukemia (AML) accounts for approximately one-third of all adult leukemias[1] with a poor 5 -year survival rate. FLT3 (FMS-related tyrosine kinase-3, CD135), a receptor protein tyrosine kinase, mutations are present in ~30% of newly diagnosed patients. These mutations include ITD (internal tandem duplication) and TDK (tyrosine kinase domain) mutations. FLT3 receptor has an extracellular ligand-binding domain, five immunoglobulin-like domains, and a transmembrane domain. The intracellular portion of the receptor has a juxta membrane (JM), two intracellular kinase subdomains (TKD) and a C-terminal portion (Fig 1).

Under normal physiological conditions FLT3 exists as a monomer. Upon binding to the FLT3 ligand it forms a dimer and becomes activated. This results in a signaling cascade leading to cell survival, differentiation, proliferation.[2,3] Both ITD and TDK mutations result in dimerization and constitutive activation of FLT3 and the subsequent activation of downstream signaling like STAT5.[6]

The ITD mutation within the receptor's autoinhibitory JM is the most common and is associated with poor prognosis. ITD length of mutations is variable ranging from 3 to more than 400 bp; they involve an in-frame duplication of a fragment of the JM in a head-to-tail orientation. Insertions can occur in different regions of the JM as well.

Given the significant role of FLT3 mutations in AML, targeting FLT3 has emerged as a promising therapeutic strategy. Various FLT3 inhibitors have been developed and are being evaluated in clinical trials to specifically target and inhibit the aberrant FLT3 signaling in AML. With analytical sensitivity down to as little as a single nucleotide and relative quantification, fragment analysis by CE is an excellent tool for the study of ITD mutations.

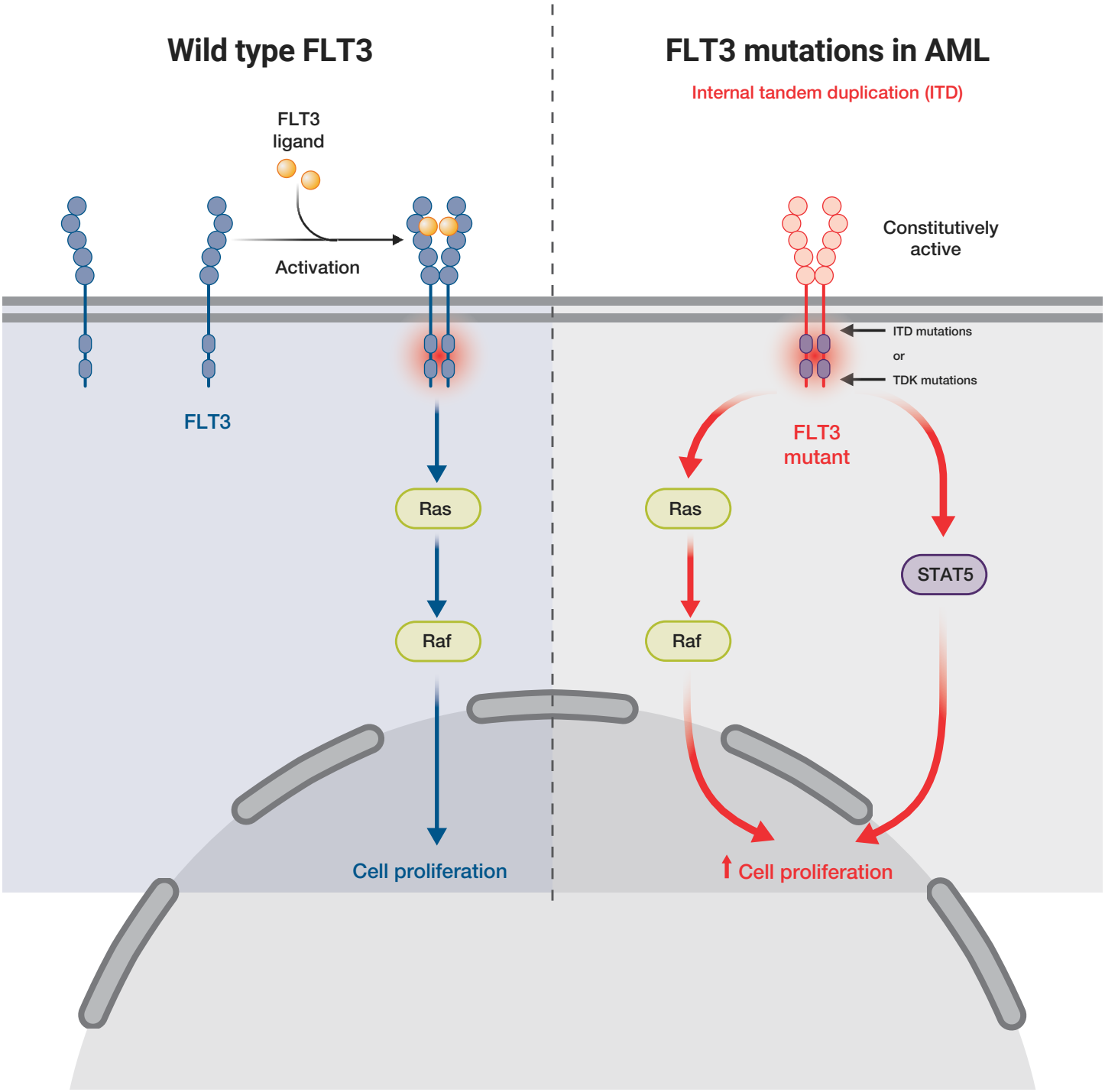

Figure 1. Wild type FLT3 is a monomer which becomes a dimer after binding to FLT3 ligand leading to activation of signaling pathways. Mutated versions of FLT3 (ITD and TKD) form dimers without ligand binding. They are constitutively activated leading to the activation of downstream signaling pathways. This leads to STAT5 activation which facilitates leukemia progression.

FLT3-ITD detection and characterization using fragment analysis

Internal tandem duplication mutations (ITD) are the most common FLT3 mutations in patients with AML. These occur in the juxtamembrane (JM) region within exons 14 to 15 of the FLT3 wild-type gene. The lengths of the duplicated sequences vary but are always in-frame and at least 3 bp with most between 6 and 180 bp. Since FLT3-ITD mutations are important to understanding disease progression, analytically sensitive and accurate research tools are imperative. In this study we amplified samples using the standard delta-PCR method and compared it to a tandem duplication (TD)-PCR method. In this protocol comparison we demonstrate that the TD-PCR shows greatly improved analytical sensitivity and specificity[5], to a limit of detection of only a few molecules of an ITD. In this comparison, all the amplified products (fragments) are fluorescently labeled and detected by capillary electrophoresis (CE).

Delta-PCR uses 3 primers for detection, 1 forward and two reverse[4] (figure 2a). Reverse primers are labeled with different fluorescent dyes (6-FAM and Hex). The two reverse primers generate two amplicons with a size difference of 19 bp (delta 19 bp), where in the shorter PCR product serves as a confirmation probe for PCR analytical sensitivity. With delta-PCR two peaks are detected for wild type and two more peaks, larger than wild-type amplicons are detected for FLT3-ITD positive samples. The difference between the WT and ITD peaks is variable per sample as the insertion length is variable (figure 2b).

TD-PCR, used for the detection of FLT3-ITD, is a variation of inverse PCR. Because of the arrangement of tandem duplication, PCR primers facing away from each other can be used for detection of ITD. As shown in figure 2c, the reverse primer is upstream of the forward primer, therefore, both primers only face each other in a tandem duplicated sequence, allowing for amplification to occur across the insertion junction. Because ITDs vary in length a second “internal” reverse primer is used as a probe to support PCR analytical sensitivity. Both reverse primers are labeled with different fluorescent probes (6-FAM and HEX, here) to aid in detection. With the TD-PCR approach no template specific amplification of wild-type allele should occur. Like delta-PCR a pair of amplicons with a delta of 19 bp is considered as positive for ITD mutation (figure 2d).

The standard delta-PCR method utilizes a competitive amplification method which amplifies both the wild-type allele and the mutant allele, resulting in competition for amplification and reduced mutant signal. In contrast the TD-PCR is a singleplex method amplifying only the mutant allele which results in higher sensitivity (figure 6).

In this protocol guide, we compare delta-PCR and TD-PCR amplification with CE detection. We were able to detect ITDs in <6 hours with:

- **Analytical sensitivity as low as 4 copies**
- **Information about ITD size**
- **Simple data analysis and scalable workflow**

Research assay design for the detection of FLT3-ITD mutations

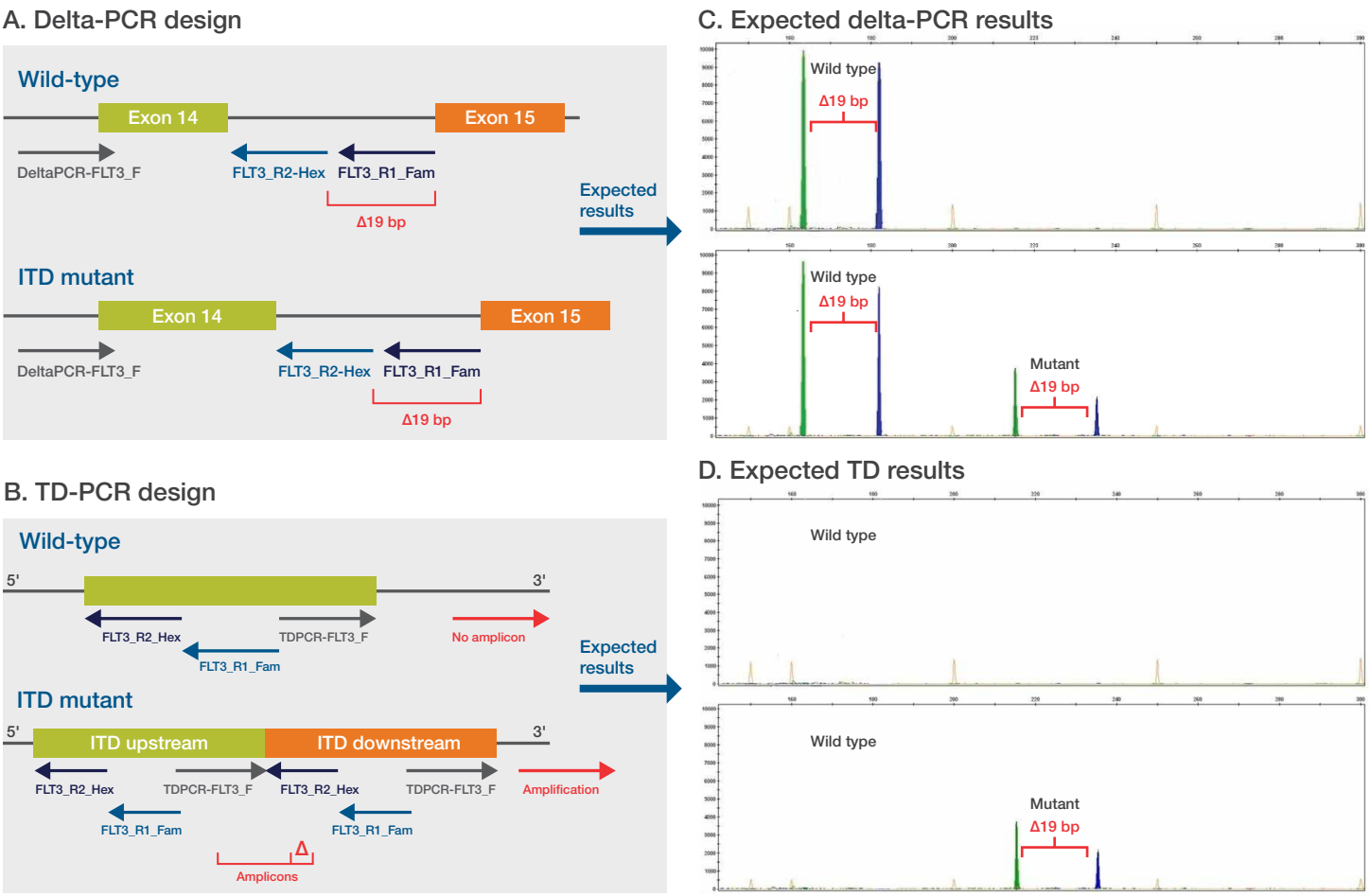

Figure 2. Detection of FLT3-ITD and theoretical results.

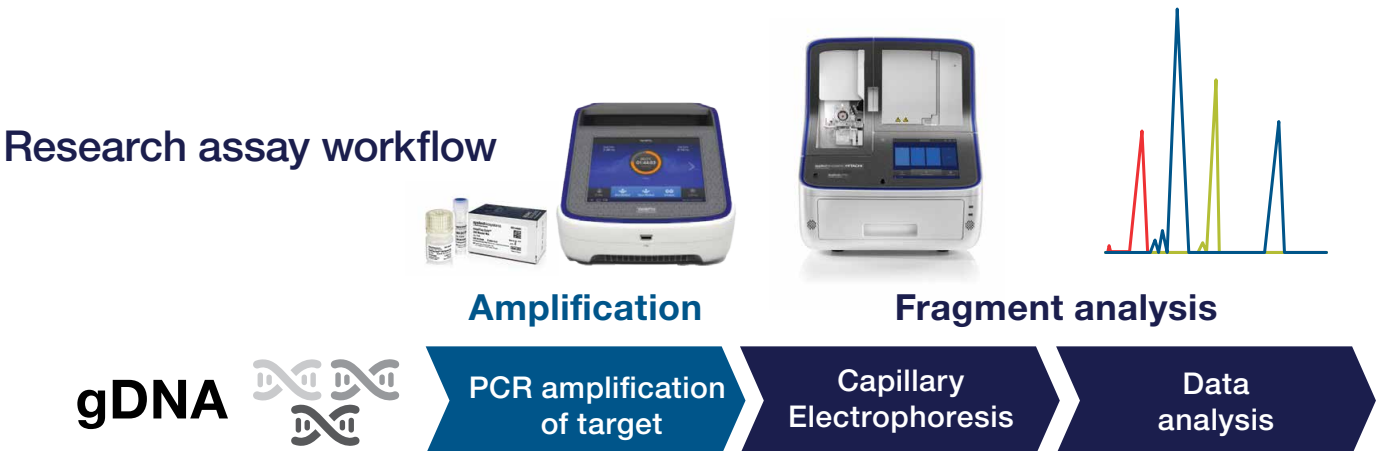

Figure 3. FLT3 fragment analysis workflow.

Protocol

Important: This protocol is very sensitive; therefore, utmost care must be taken to prepare stock solutions and set up amplification reactions in an amplicon-free environment.

Materials needed

Equipment

Product	Supplier	Cat. No.
Applied Biosystems™ VeritiPro™ Thermal Cycler, 96-well	Thermo Fisher Scientific	A48141
Applied Biosystems™ SeqStudio™ 24 Flex Genetic Analyzer	Thermo Fisher Scientific	A53630
Applied Biosystems™ SeqStudio™ Flex Genetic Analyzer 24-Capillary Array	Thermo Fisher Scientific	A49107
Thermo Scientific™ Basic Vortex Mixers	Thermo Fisher Scientific	88882011
Single-channel and multichannel micropipettes of various sizes capable of pipetting volumes from 1.00 µL to 1000.0 µL	Major Lab Supplier (MLS)	Any
Cold block or ice	MLS	Any
Microcentrifuge and mini centrifuge	MLS	Any
Plate Centrifuge	MLS	Any

Reagents, kits, and consumables

Product	Supplier	Cat. No.
AmpliTaq Gold™ 360 Master Mix	Thermo Fisher Scientific	4398881
Invitrogen™ Nuclease-Free Water (not DEPC-Treated)	Thermo Fisher Scientific	AM9932
Applied Biosystems™ BigDye™ Direct Cycle Sequencing Kit	Thermo Fisher Scientific	4458689
Applied Biosystems™ BigDye XTerminator™ Purification Kit	Thermo Fisher Scientific	4376487
Control DNA (from CEPH Individual 1347-02)	Thermo Fisher Scientific	403062
Applied Biosystems™ POP-7™ Polymer, for 3500/SeqStudio™ Flex	Thermo Fisher Scientific	4393708
Applied Biosystems™ MicroAmp™ Optical 96-Well Reaction Plate	Thermo Fisher Scientific	N8010560
Applied Biosystems™ Anode Buffer Container (ABC), for 3500/SeqStudio™ Flex	Thermo Fisher Scientific	4393927
Applied Biosystems™ Cathode Buffer Container (CBC), for 3500/SeqStudio™ Flex	Thermo Fisher Scientific	4408256
Applied Biosystems™ Hi-Di™ Formamide	Thermo Fisher Scientific	4311320
Applied Biosystems™GeneScan™ 500 ROX™ dye Size Standard	Thermo Fisher Scientific	4310361
Applied Biosystems™ DS-30 Matrix Standard Kit (Dye Set D)	Thermo Fisher Scientific	4345827
Applied Biosystems™ MicroAmp™ Clear Adhesive Film	Thermo Fisher Scientific	4306311
50 µL PCR tubes, clean	MLS	Any
Sterilized aerosol barrier (filter) pipette tips	MLS	Any
Myeloid DNA Reference Standard	Horizon Discovery	HD829

Note: May use above listed products or relevant substitute.

1. PCR amplification of targets

In our experiments we used purchased control gDNA samples. For a positive control Myeloid Reference Standard HD829 was obtained from Horizon Discovery. HD829 gDNA is isolated from cell lines which contain 22 variants across 19 genes relevant to myeloid cancer including a 300 bp ITD. This mutation has an expected allelic frequency of 5%. For a wild-type control gDNA from a cell line (CEPH 1347-02) was used. For biological samples we recommend purifying the DNA with a genomic DNA (gDNA) purification kit (such as Invitrogen™ PureLink™ Genomic DNA Mini Kit Applied Biosystems™ MagMAX™ DNA Multi-Sample Ultra 2.0 Kit). Purified gDNA should be quantified using an established quantification method such as Qubit or NanoDrop. Adjust concentration to 0.25 to 25 ng/µL. Use serial dilutions to determine the optimal input for detection. Postive and negative controls are recommended for all assays.

1.1. Prepare PCR Assays

1.1.1. Make two PCR primer working solutions for each type of PCR by adding 0.8 µL of each reverse primer and 1.6 µL of forward primers from the paired primer pair to 196.8 µL of DNA suspension buffer (this provides sufficient primer mix for about 66 samples, If you are running more samples 3 µL of primer mixture is needed per sample).

1.1.2. Create labels for assay tubes

1.1.3. Vortex and centrifuge primer 2x before combining

1.1.4. Mix primers accordingly

Assay name	Primer Name	Sequence
FLT3-DeltaPCR	DeltaPCR-FLT3_F	gcaatttaggtatgaaagccagc
	FLT3_R1_Fam	caatggaaaagaaatgctgcag
	FLT3_R2_Hex	cagaaacatttggcacattcca
FLT3-TD_PCR	TDPCR-FLT3_F	aatgcacgtactcaccatttgtc
	FLT3_R1_Fam	caatggaaaagaaatgctgcag
	FLT3_R2_Hex	cagaaacatttggcacattcca

1.1.5. Prepare PCR amplification reaction master mix. Adjust quantities based on number of samples.

Reagent	1x (10ul)	10X
dH2O	1 µL	10 µL
PCR Primer Assay (0.8 µm ea)	3 µL	30 µL
AmpliTaq Gold 360 Master mix	5 µL	50 µL
Total	9 µL	90 µL

1.1.6. Set up PCR plate as follows with Myeloid DNA Reference Standard samples which contain a 300 bp ITD.

1.1.6.1 Prepare samples for a 1 µL sample volume. Triplicates and 3 dilutions are recommended for each sample. In our example we started 25 ng/µL DNA. 1 µL of 25 ng/µL DNA was added to 9 µL dH2O and mixed to create the 2.5 ng sample. The 2.5 ng sample was diluted 1:10 create the 0.25 ng sample.

1.1.7 Set up PCR plate as follows add 9 µL of indicated master mix and 1 µL of the indicated gDNA sample to each well. For the no template control (NTC) at 1 µL dH2O instead of sample. Prepare an NTC control for each primer set used as indicated. Plate example is for our positive and negative controls run in study.

	1	2	3	4
	Delta-PCR	**TD-PCR**		
A	HD829-25 ng	HD829-25 ng		
B	HD829-2.5 ng	HD829-2.5 ng		
C	HD829-0.25 ng	HD829-0.25 ng		
D	CEPH-25 ng	CEPH-25 ng		
E	CEPH-2.5 ng	CEPH-2.5 ng		
F	CEPH-0.25 ng	CEPH-0.25 ng		
G				
H	NTC	NTC		

1.2. Run PCR on VeritiPro Thermal Cycler with the following cycle conditions:

PCR Cycle Conditions

Cycle	Temp	Time
1x	95	10-min
40x	95	30-s
	57	30-s
	72	60-s
1x	72	7-min
1x	4	hold

2. Perform capillary electrophoresis

2.1 Spectral calibration for the SeqStudio Flex

DS-30 Matrix Standard (re#4345827, Lot#, exp:01250854)	
DS-30 dye	6 µL
Hi:Di	294 µL

2.1.1 Aliquot 10 µL, into 24 wells of column 1-3.

2.1.2 Heat to 95°C for 5 mins, cool to 4 for 2 mins using the thermocycler

2.1.3 Load the plate onto the SeqStudioFlex

2.1.4 Set Maintenance → calibrate select spectral → calibration tab → chemistry standard → matrix std → Dye set DS-30- → start run.

2.1.5 At the end of the run accept the results after visual inspection of pass fail.

2.2 Prepare samples for capillary electrophoresis

Note: Prepare the samples for electrophoresis immediately before loading.

2.2.1 Prepare the mix of Hi-Di™ Formamide and ROX500 Size Standard for the required number of reactions plus 3 additional reaction for overage.

2.2.1.1 Per reaction prepare: ROX 500 0.5 µl + Hi Di™ Formamide 8.5 µL for a total volume of 9 µL per rxn. For 20 samples, mix 10 µL ROX500 with 170 µL Hi-Di (adjust as needed based on your sample number).

2.2.1.2 Thoroughly mix the components by vortexing 3–5 seconds, then centrifuge 3–5 seconds before use.

2.2.2 Prepare the fragment analysis reactions. To a MicroAmp™ Optical 96-Well Reaction Plate, add the following components.

Component	Volume per reaction
HiDi™ and ROX500	9 µL
PCR product	1 µL
Total Volume	10 µL

2.2.2.1 Seal the reaction plate with MicroAmp™ Clear Adhesive Film.

2.2.2.2 Thoroughly mix the components by vortexing 3–5 seconds, then centrifuge 10–20 seconds before use.

2.2.2.3 Denature the DNA fragments:

2.2.2.4 Incubate the mixture at 95°C for 3 mins.

2.2.2.5 Incubate the mixture at 4°C, or on ice, for 2 mins

2.2.2.6 Centrifuge the plate for 10–20 sec to ensure that all sample mixtures are at the bottom of the wells.

2.2.2.7 Remove the MicroAmp™ Clear Adhesive Film, then seal the plate with a septa

2.2.2.8 Assemble the plate with the retainer and base, then load on the instrument.

2.2.2.9 Run reactions on the Applied Biosystems genetic analyzer.

2.2.2.10 Use Dye Set DS-30, 50cm array, Pop7, Fragment analysis Run module

Analysis of results using GeneMapper Software

Results for Detection of ITD mutation for FLT3 gene using delta-PCR assay

Expected results with Wildtype and Mutants

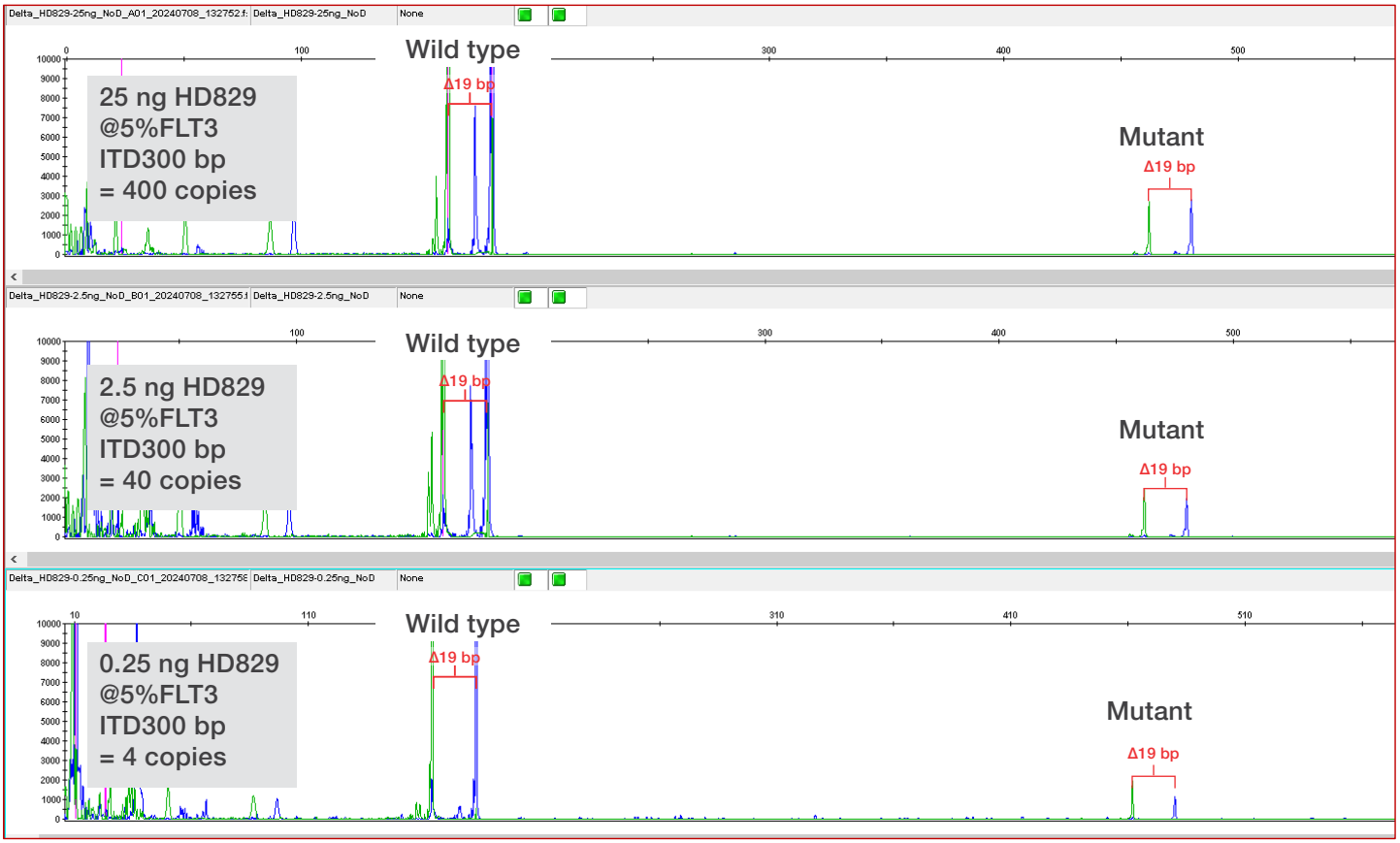

Figure 4. Detection of FLT3-ITD mutations using delta-PCR assay. Mutants can be detected down to 4 copies.

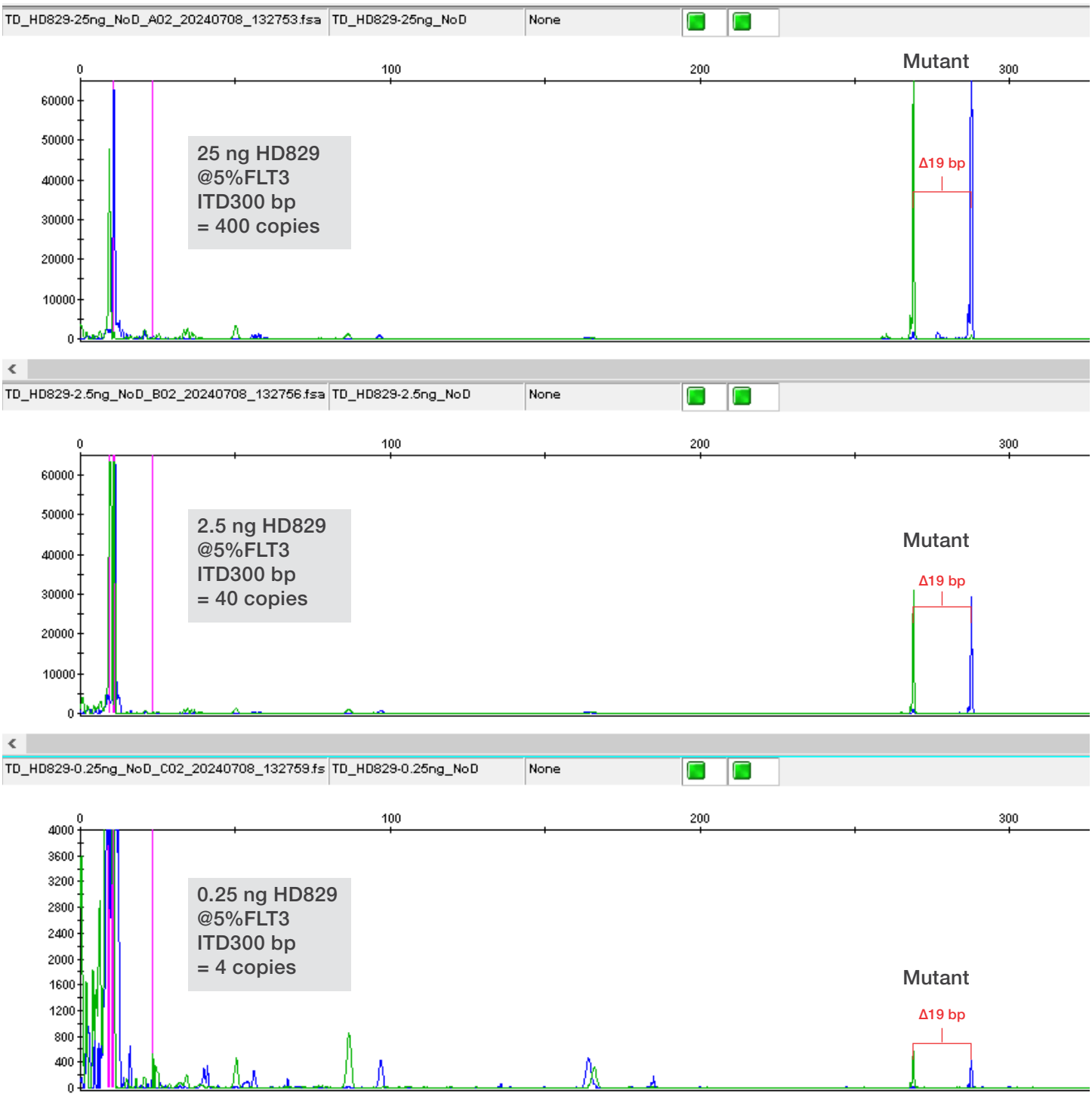

Figure 5. Detection of FLT3-ITD mutations using TD-PCR assay. Mutants can be detected down to 4 copies

Expected results with Wildtype and Mutants (cont.)

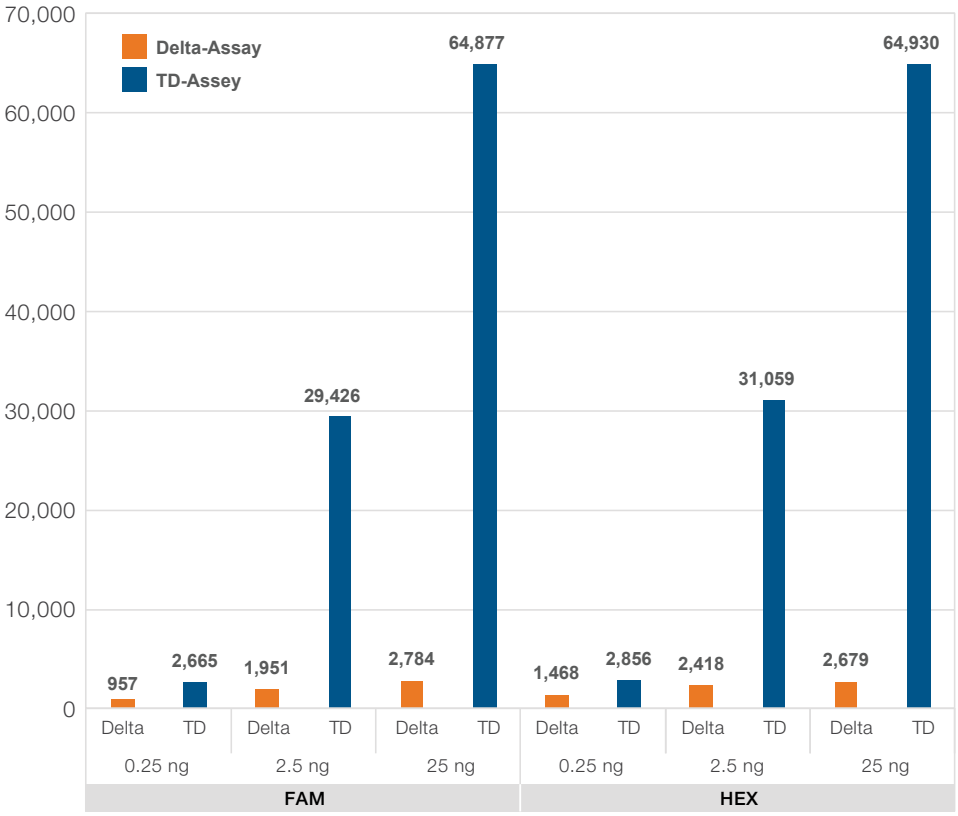

Fig. 6 TD-PCR assay is more analytically sensitive than delta-PCR assay for the detection of FLT3 ITD mutation detection.

Conclusion

Research tools for the rapid and analytically sensitive detection of FLT3-ITD mutations is critical to evaluating potential AML treatments under development. With the ability to distinguish very small differences in nucleotide lengths, fragment analysis by CE is an excellent tool for the study of FLT3-ITD. We compared two methods to amplify the FLT3-ITD prior to fragment analysis, TD-PCR and Delta PCR.

- **Both methods detected as little as 4 copies of the mutant allele.**
- **TD-PCR seems to be more analytically sensitive generating a stronger signal than delta-PCR.**
- **This protocol guide offers a fast, analytically sensitive and simple protocol for the detection of FLT3-ITD.**

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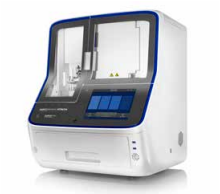
Choose the genetic analyzer that is right for you

	SeqStudio Genetic Analyzer **Easy-to-use, flexible system** 	SeqStudio Flex Series genetic analyzers **Easy-to-use, flexible, connected system** 	3730*x*/ DNA Analyzer **Maximum throughput, scalability, and flexibility** UPGRADED 
Number of capillaries	4	8 or 24	48 or 96
Capillary array length (cm)	28	36, 50	36, 50
Sample capacity	1 plate; 96-well plate; 8-strip tube (compatible)	4 plates; 96- or 384-well plates; 8-strip tube (compatible)	16 plates; 96- or 384-well plates
Continual plate loading	No	Yes	Yes
Sample reprioritization	No	Yes	No
Polymer type	POP-1, integrated into click-in cartridge	POP-6, POP-7, and POP-4	POP-6, POP-7, and conformational analysis polymer
Radio-frequency ID	Yes	Yes	No
Configuration	Integrated computer with touchscreen; optional desktop	Integrated computer with touchscreen; optional desktop	External desktop required
Remote monitoring and data sharing	Yes	Yes	No
Integrated remote troubleshooting tools	No	Yes	No
Connectivity	USB, Ethernet ports, and Wi-Fi dongle	USB, Ethernet ports, and Wi-Fi dongle	Ethernet port
Connectivity with Thermo Fisher™ Connect Platform	Yes	Yes	Yes

References:

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6. 6. Liu J, Gu J. (2024) Importance of PTM of FLT3 in acute myeloid leukemia. Acta Biochim Biophys Sin (Shanghai). 56(8):1199. doi:10.3724/abbs.2024112 56(8):1199. doi:10.3724/abbs.2024112

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