Validation study on fragment analysis of *FLT3*-ITD and sequencing of pGEM by capillary electrophoresis (Seq-studio)

Dissertation submitted in partial fulfillment for the degree of

Master of Science in Applied Microbiology

Submitted By

ANIMESH GOPE

1662005

KIIT School of Biotechnology, Campus- 11

KIIT University

Bhubaneswar, Odisha, India

Under the Supervision of

Dr. Sakthivel Murugan

MEDGENOME LABS LTD.

Narayana Health City, #258/A,

Bommasandra, Hosur Road,

Bangalore-560099, India

Tel: +91 (0)80 6715 4900

Organisation/company (General Manager letter head with logo)

CERTIFICATE

This is to certify the dissertation entitled " Validation study on fragment analysis of FLT-3 Internal Tandem Duplication and sequencing of pGEM by capillary electrophoresis (Seq-studio)" Submitted by Animesh Gope in partial fulfillment of the requirement for the degree of Master of Science in Applied Microbiology, KIIT School of Biotechnology, KIIT University, Bhubaneswar bearing Roll No.**1662005** &Registration No.1**6647451471** is a bonafide research work carried out by his/her under my guidance and supervision from 8th January 2018 to 17th May 2018.

Date:
Place: *Name and signature*

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CERTIFICATE

This is to certify that the dissertation entitled **"Validation study on fragment analysis of FLE-3 Internal Tandem Duplication and sequencing of pGEM by capillary electrophoresis(Seq-studio)"** submitted by **Animesh Gope** *Roll No.* **1662005** *Registration No.* **16647451471** to the KIIT School of Biotechnology, KIIT University, Bhubaneswar-751024, for the degree of Master of Science in Applied Microbiology is his original work, based on the results of the experiments and investigations carried out independently by his/her during the period from 8th January 2018 to 17th May 2018 of study under my guidance.

This is also to certify that the above said work has not previously submitted for the award of any degree, diploma, fellowship in any Indian or foreign University.

Date:
Place: *Supervisor name & signature*

DECLARATION

I hereby declare that the dissertation entitled **"Validation study on fragment analysis of FLE-3 Internal Tandem Duplication and sequencing of pGEM by capillary electrophoresis(Seq-studio) "** submitted by **Animesh Gope,** for the degree of Master of Science to KIIT University is a record of bonafide work carried by me under the supervision of, Mr. ***Ramesh Kumar Gnanavadivel Senior Research Associate,*** *Medgenome Labs Ltd, Bangalore, Karnataka, India*

Date: Animesh Gope
Place: *Name & Signature*

Abstract

FLT3 is a receptor tyrosine kinase that is expressed on early hematopoietic progenitor cells and plays an important role in stem cell survival and differentiation. *FLT3* mutations can be divided into 2 categories: (1) internal tandem duplications (*FLT3*/ITD mutations) in or near the juxtamembrane domain of the receptor and (2) point mutations resulting in single amino acid substitutions occurring within the activation loop of the tyrosine kinase domain (*FLT3*/TKD mutations).

Activating mutations in FMS-like tyrosine kinase 3 (*FLT3*), including internal tandem duplications (ITDs) and tyrosine kinase domain (TKD) mutations, are common in patients with acute myeloid leukemia (AML). Mutations in the FMS-like tyrosine kinase 3 (*FLT3*) gene represent one of the most frequently encountered, and clinically challenging, class of AML mutations. Patients with acute myeloid leukemia who harbor an FMS-like tyrosine kinase 3 (*FLT3*) mutation present several dilemmas for the clinician. The results of an *FLT3* mutation test, which can be influenced by several variables, need to be interpreted according to the clinical setting and there is a need for internationally standardized *FLT3* mutation assays.

Acute myeloid leukemia (AML) patients with mutated *FLT3* have a large disease burden at presentation and a dismal prognosis. First-generation and second-generation inhibitors are used to treat patients with *FLT3*-mutated AML in virtually all disease settings including induction, consolidation, maintenance, relapse, and after hematopoietic cell transplantation (HCT).
Detection of ITD and D835 FLT3 mutations is clinically important for several reasons. First, patients harboring these mutations generally have a worse prognosis and may benefit from aggressive up-front treatment interventions. Secondly, both ITD and D835 mutations may serve as markers for the detection of residual disease, which may become an important part of posttreatment disease monitoring. DNA template control pGEM 3Zf(+) results can help to determine whether failed reactions are caused by poor template quality or sequencing reaction failure. The results of the assay should always be interpreted in the context of clinical data and other tests performed for the patients.

Acknowledgements

I take this opportunity to express my profound gratitude and deep regards to my guide Dr. Sakthivel Murugan for his exemplary guidance, monitoring and constant encouragement throughout the course of this thesis. The blessing, help and guidance given by him time to time shall carry me a long way in the journey of life on which I am about to embark. I also take the opportunity to express a deep sense of gratitude to my manager Mr. Ramesh Kumar Gnanavadivel for his cordial support, valuable information and guidance, which helped me in completing this task through various stages.

I am obliged to lab members Mukesh Das and Somi Srivastava for their guidance and constant supervision as well as for providing necessary information regarding the project & also for their support in completing the project. I am grateful for their cooperation during the period of my thesis. Lastly, I thank almighty, my parents and my brother for their constant encouragement without which this thesis would not be possible.

Date:
Place: Bangalore

Abbreviations

DNA:Deoxy ribonucleic acid
RNA:Ribonucleic acid
FLT3:FMS-like Tyrosine
AML:Acute Myelogenous Leukemias
ALL:Acute Lymphoblastic Leukemias
ITD:Internal Tandem Duplications
JM-juxtamembrane
PCR:Polymerase Chain Reaction
TKD:Tyrosine kinase domain
ER:Endoplasmic reticulum
MEK:mitogen-activated protein kinase
ERK:extracellular-signal-regulated kinase
EBPalpha:estradiol-binding protein alpha
FL:*FLT3* ligand
RFS:relapse-free survival
OS:overall survival
CR:complete remission
HSCs:Hematopoietic stem cells
HPCs:Hematopoietic progenitor cells
MHPCs:Multipotent hematopoietic progenitor cell
FISH:Fluorescence ***in situ*** hybridization
APL:acute promyelocytic leukemia
PIA:Plasma inhibitory activity
VEGFR:vascular endothelial growth factor receptors
PDGFR:platelet-derived growth factor receptor
GST:Gastrointestinal Stromal Tumor
PNT:Pancreatic Neuroendocrine Tumor
SYK:Spleen Tyrosine kinase
FGF2:Fibroblast Growth Factor 2
HDACis:Histone Deacetylase Inhibitors
PFS: progression free survival
PR: partial response
QTcF: Fridericia-corrected QT interval
LTK: leukocyte tyrosine kinase
AE:Adverse event
ALK: naplastic Lymphoma Kinase
ALP: Alkaline Phosphatase
ALT :Alanine Aminotransferase
ANC: Absolute Neutrophil Count

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Introduction

The FMS-like tyrosine (*FLT3*) gene encodes a class III receptor tyrosine kinase, sharing structural and sequence homologies with family members, including c-kit, c-FMS, FLT1, and PDGF-βR. *FLT3* plays a key role in the control of hematopoiesis. High levels of WT *FLT3*-expression have been observed in various hematological malignancies including the majority of acute myelogenous leukemias (AMLs) and B-cell acute lymphoblastic leukemias (ALLs). In line with these findings, *FLT3* is clearly expressed in the majority of leukemia cell lines and, particularly, in monocytic cell lines. In addition to these frequent abnormalities, in 1996 it was discovered that in AMLs *FLT3* is frequently mutated; in fact, internal tandem duplications (ITDs) within the juxtamembrane domain of *FLT3* have been reported in about 25% of patients, making it one of the most single frequent mutations in adult AMLs.

Subsequently, it was shown that these *FLT3*-ITD mutations resulted in an uncontrolled receptor activation, characterized by ligand-independent receptor dimerization, constitutive *FLT3* signaling with consequent activation of STAT5 and of the RAS/MAPK and PI3 K pathways.

In 2001, another frequent class of *FLT3* mutations causing constitutive receptor activation occurred at the level of the activation loop of the second kinase domain; this group of mutations is represented by various abnormalities, such as substitutions, small deletions, or insertions mainly occurring at codons 835 and 836 and is detected in about 5–10% of AMLs.

Finally, more recently, a group of point mutations within the juxtamembrane domain of *FLT3* have been described in about 1% of AML patients; these mutations involve various amino acid residues, such as 579, 590, 591, and 594.

As it will be discussed in detail below, several studies have suggested that the presence of *FLT3*-ITD mutations in AMLs was associated with an increased risk of relapse and shorter overall survival. Furthermore, it was shown that low/absent expression of the normal *FLT3* allele was associated with a negative outcome.

The scope of this review is to briefly outline recent evidences indicating that *FLT3*-ITD mutations are leukemic driver mutations and, therefore, represent important therapeutic targets. In addition, several recent molecular studies have clearly identified subsets of AML patients, whose prognosis is considerably worsened by *FLT3*-ITD mutations and could considerably benefit from an efficacious *FLT3* pharmacological targeting.

Background and Context

Certain, but not all, chromosome translocations in childhood leukemia are known to be present at birth. This phenomenon of prenatal origin was initially presumed from twin studies where it was observed that mono-amniotic twins always harbored the same translocations. In addition, several studies have shown that specific mutations found at diagnosis in children with leukemia were present at birth, that is, "backtracked" to neonatal Guthrie Cards (blood spots used for newborn screens) (reviewed in. The *MLL* rearrangement in infant ALL "backtracks" in nearly all cases and *TEL-AML1* is found on 75% of Guthrie cards matched to leukemia cases with the translocation. The *E2A-PBX1* fusion generated by the t(1;19) translocation is a likely exception, with a postnatal origin, along with possibly others. These results collectively support a "two hit" model of leukemia, with one hit early in life or *in utero*, and another at a later date in temporal proximity to leukemia diagnosis.

The FLT3 gene is located on chromosome 13q12 and encodes a Type III membrane receptor kinase that regulates normal hematopoiesis. Mutations in FLT3 in AML occur in approximately 5-15% in children and 25-35% in adults, and account for the most common single gene defect in AML (reviewed in. Several studies have indicated that children and adults with AML and the FLT3 mutation have a very poor prognosis. FLT3 mutations have also been documented in adult and pediatric ALL. An initial report demonstrated a 14% frequency of FLT3 mutations among childhood ALL overall, with mutations concentrated among the cytogenetic subgroups high hyperdiploidy (> 50 chromosomes in diagnostic karyotype) and *MLL*-translocation; more recent studies have indicated a lower overall frequency in childhood ALL (in the 1-8% range) while consistently demonstrating a higher incidence among those with *MLL* rearrangement and high hyperdiploidy. The absolute incidence of FLT3 mutations in pediatric leukemia is of interest in part because of the existence of several promising *FLT3* inhibitors currently under development. such inhibitors are more effective in the presence of *FLT3* activation.

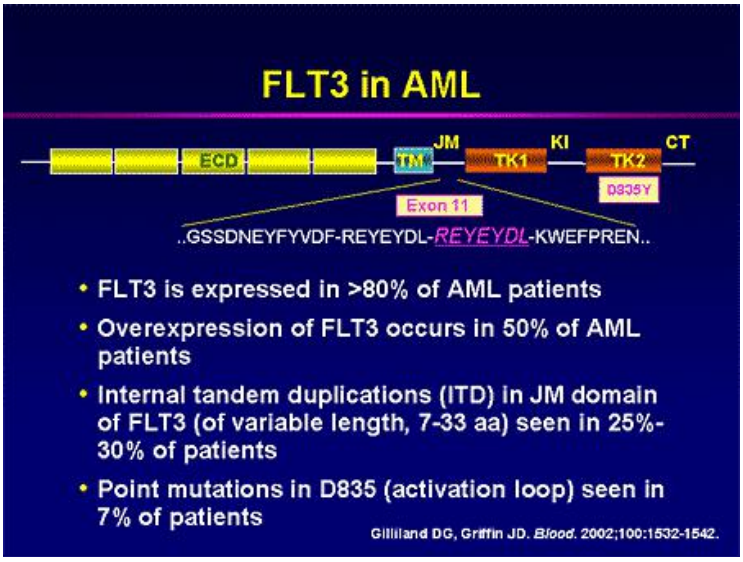
The first and best-studied FLT3 mutation is an internal tandem duplication (ITD) mutation. ITD mutations typically result from the duplication and tandem insertion of a portion of the juxtamembrane (JM) region (exons 11 to 12) of the FLT3 wild-type gene. The lengths of the duplicated segments have been reported to range in size from 6 to 180 bases and are always in frame. ITD mutations result in the constitutive autophosphorylation of the *FLT3* receptor and are thus gain-of-function mutations of the FLT3 proto-oncogene. FLT3 ITD mutations have been reported to occur in 20 to 30% of patients with AML and have been associated with an increased relapse risk, decreased disease-free survival, decreased event-free survival, and decreased overall survival. In a multivariate analysis of FLT3 ITD mutations, cytogenetic risk group, presentation white blood cell count, percentage BM blasts at diagnosis, age, gender, and FAB type in 854 AML patients, the presence of a FLT3 ITD mutation was the most significant factor adversely affecting relapse risk ($P < 0.0001$) and disease-free survival ($P < 0.0001$). FLT3 ITD mutations are amenable to polymerase chain reaction (PCR)-based molecular diagnostic DNA testing because they are limited to a small, predictable region of the FLT3 gene.

Recently, an additional type of FLT3 mutation has been described. These alterations are missense mutations that alter the wild-type aspartic acid residue at position 835 (D835) within the activation loop of the *FLT3* protein. Alteration of D835 also appears to result in constitutive activation of the *FLT3* receptor and portends a worse disease-free survival in at least some studies. D835 mutations have been reported to occur in ~ 7% of patients with AML, 3% of patients with myelodysplastic syndrome (MDS), and 3% of patients with acute

lymphocytic leukemia. D835 and ITD mutations appear to occur independently but not exclusively of one another and the presence of concurrent D835 and ITD mutations has been reported.The D835 wild-type gene sequence is located within an *Eco*RV restriction endonuclease cut site, a feature exploited by the reported assay.

Detection of ITD and D835 FLT3 mutations is clinically important for several reasons. First, patients harboring these mutations generally have a worse prognosis and may benefit from aggressive up-front treatment interventions. Secondly, both ITD and D835 mutations may serve as markers for the detection of residual disease, which may become an important part of posttreatment disease monitoring. Finally, investigators have recently developed specific tyrosine kinase inhibitors of the *FLT3* receptor for use as tumor-specific chemotherapeutic agents, analogous to the use of STI-571 [imatinib mesylate (Gleevac); Novartis, Basel, Switzerland] in the treatment of chronic myelogenous leukemia. Clinical trials are currently underway looking at the utility of *FLT3* inhibitors in the treatment of relapsed or refractory AML with FLT3 mutations. Clinical testing for FLT3 mutations in AML may thus become critical to the determination of appropriate therapeutic interventions in AML.

Here we describe a molecular diagnostic approach capable of detecting both ITD and D835 mutations of the FLT3 gene in a single multiplex PCR assay. We discuss the assay design strategy, assay validation, and our experience with the clinical application of the assay.



Tyrosine Kinase Receptor *FLT3*

FLT3 structure and function

FLT3, on chromosome 13q12, encodes a receptor tyrosine kinase (RTK) expressed on normal hematopoietic stem/progenitor cells. *FLT3* dimerizes and autophosphorylates upon binding of *FLT3* ligand (*FLT3*L), activating the intracellular tyrosine kinase domain (TKD), which causes phosphorylation of downstream molecules, thereby activating signaling cascades that promote transcription of genes regulating survival, proliferation and differentiation. FLT3 is silenced during hematopoietic differentiation.

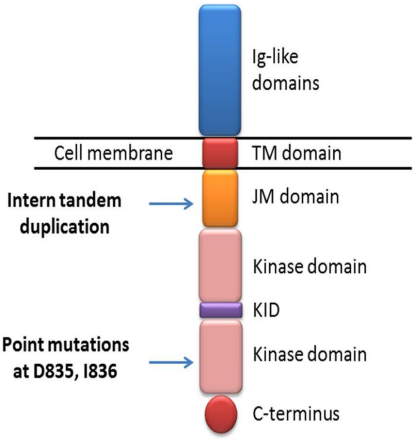


Fig:- Locatsion of Activating mutations of *FLT3.ITD* occurs in exons 14and 15 of the JM domain.

FLT3 mutations

FLT3 is expressed in AML cells of most patients and is mutated in AML cells of approximately 30%. Mutations include internal tandem duplicationa (ITD), present in AML cells of approximately 25% of patients, and point mutations in the tyrosine kinase domain (TKD), present in approximately 5%. Both ITD and TKD mutations are activating, causing ligand-independent, or constitutive, *FLT3* receptor signaling, and thereby promote cytokine-independent AML cell survival and proliferation.

In-frame internal tandem duplications within the FLT3 gene (*FLT3*-ITD) occur most commonly in exon 14, encoding the juxtamembrane (JM) domain. The JM domain inhibits activation of the receptor by steric hindrance, preventing the TKD from assuming an active conformation. Presence of an ITD causes loss of this inhibitory effect, resulting in activation of the TKD. ITDs are of variable size, ranging from 3 to 1,236 nucleotides; loss of *FLT3* inhibitory effect is independent of size of the duplication within the receptor. Additionally, *FLT3* signaling activated by ITDs is aberrant, notably activating signal transducer and activator of transcription (STAT) 5 and its downstream effectors, including Pim-1 kinase. Aberrant signaling occurs in association with partial retention of *FLT3*-ITD in the endoplasmic reticulum (ER), with trafficking of the receptor out of the ER-Golgi impaired by the presence of the duplicated domain .

Point mutations in the TKD are less common; they are present in AML cells of approximately 5% of patients. TKD point mutations cause amino acid substitutions producing changes in the activation loop that favor the active kinase conformation.

While both *FLT3*-ITD and *FLT3* TKD mutations result in constitutive activation of *FLT3* signaling, signaling pathways differ. *FLT3*-ITD activates *FLT3* signaling through STAT5, in addition to PI3 kinase (PI2K)/Akt and mitogen-activated protein kinase (MEK)/extracellular-signal-regulated kinase (ERK), while *FLT3* TKD mutations activate *FLT3* signaling through Akt and ERK, but not STAT5. Additionally *FLT3*-ITD suppresses CCAAT/estradiol-binding protein alpha (c/EBPalpha) .Transcription factors that promote myeloid differentiation, while *FLT3* TKD mutations do not.

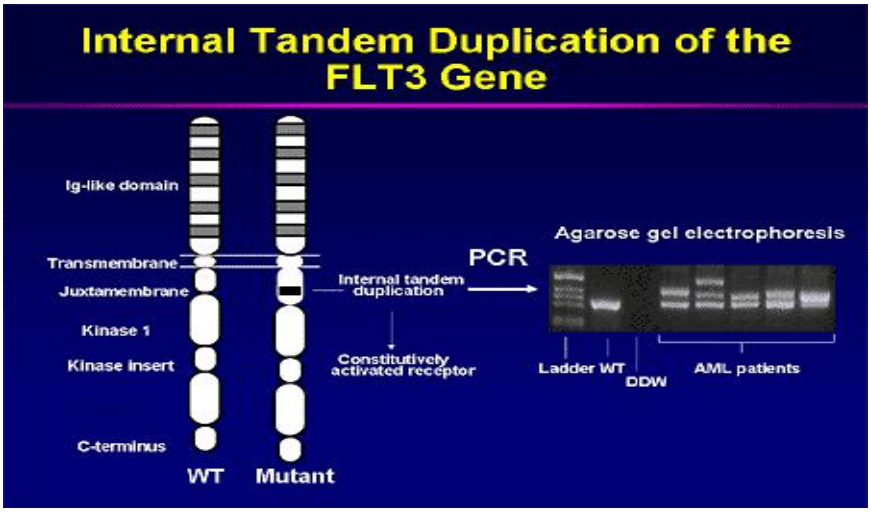


Fig:-Internal Tandem Duplication of FLT3 gene.

AML with *FLT3*-ITD usually presents with high blood blast counts and a normal karyotype, and has poor treatment outcomes, with initial treatment response, but high relapse rate and short relapse-free survival (RFS) and overall survival (OS). ITD locations and allelic ratios vary, as does size, as noted above; higher allelic ratios are associated with lower complete remission (CR) rate and shorter OS. *FLT3*-ITD is present in CD34+/CD38− AML stem cells, the cells that likely generate relapse. New structural cytogenetic abnormalities are frequently present at relapse of AML with *FLT3*-ITD, consistent with genomic instability. Genomic instability may result from increased DNA double-strand breaks associated with increased

reactive oxygen species generation and from error-prone DNA double-strand break repair. HSCT is the preferred treatment for *FLT3*-ITD AML patients in remission, but outcomes are inferior to those of patients without *FLT3*-ITD due to a high rate of early relapses, suggesting the potential utility of treatments targeting *FLT3* signaling after transplant.

In contrast to *FLT3*-ITD, *FLT3* TKD mutations are not associated with leukocytosis and only modestly negatively impact treatment outcomes. These clinical differences may be due to the difference in downstream signaling between *FLT3*-ITD and TKD mutations.

FLT3 and it's Ligand

The Fms-like tyrosine kinase 3 (*FLT3*) is a tyrosine kinase that is expressed in CD34+ hematopoietic stem/progenitor cells (HSCs/HPCs) and is important for both normal lymphoid and myeloid differentiation. Particularly, the *FLT3* is a type III receptor tyrosine kinase belonging to the same subfamily as c-kit, M-CSF, and PDGF receptors. This receptor was cloned in 1991 by two separate groups and it was reported its expression in undifferentiated stem/progenitor cells and its absent expression on the majority of differentiated hemopoietic cells. Human *FLT3* is a single peptide composed by 993 amino acids, with a molecular weight of about 155–160 KDa of the mature fully glycosylated protein.

The knockout of *FLT3* gene in mice elicited the generation of viable and fertile mice, exhibiting hematological abnormalities with reduced B-cell precursors in the bone marrow and with a defect in the generation of dendritic cells in lymphoid organs.

The ligand of *FLT3*, *FLT3* ligand (FL), is a homodimer existing both as a membrane-bound or a soluble protein. In vitro studies in human hematopoietic cells have shown that this cytokine acts as a multipotent growth factor allowing the expansion of multiple cell lineages. Mice lacking FL have a deficient hematopoiesis with a reduced cellularity at the level of hematolymphopoietic organs, a reduced number of bone marrow myeloid and lymphoid progenitors, a marked deficiency of natural killer cells in the spleen, and reduced numbers of both lymphoid-related and myeloid-related dendritic cells.

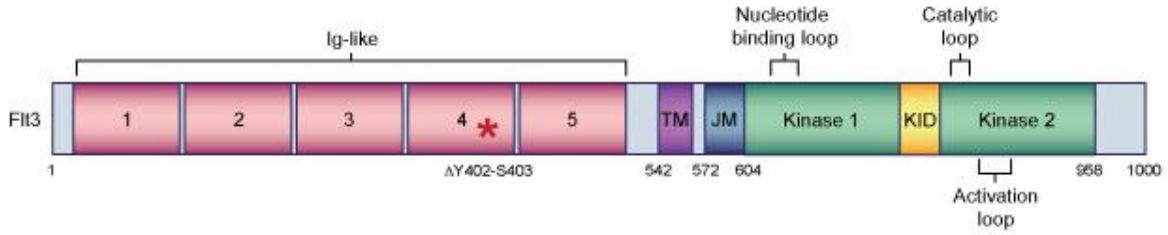


Figure 7. Domain structure of Flt3. The extracellular domain contains five Ig-like domains. Following the transmembrane domain (TM), the intracellular domain contains the juxtamembrane domain (JM) and the kinase domain, which is divided into two portions separated by the kinase insert domain (KID). The *warmflash* mutation results in deletion of Y402 and S403, located in the fourth Ig-like domain.

The gene knockout studies have failed to show the effect of *FLT3* loss on myelopoiesis. However, this lack of effect could be due to cytokine signaling redundancy. Some studies have shown a role for *FLT3* in the control of myelopoiesis. In fact, it was shown that a part of granulomonocyte progenitors possess surface *FLT3* receptors. This progenitor subpopulation was identified as early GM progenitors. A higher *FLT3* expression was clearly observed in early GM progenitors than in late GM progenitors. In line with this observation, it was found that early GM progenitors are decreased in FL knockout mice. In line with these findings, Gabbianelli and coworkers have previously reported that *FLT3* mRNA expression is very rapidly lost during erythroid differentiation, while it remained sustained during all the early stages of GM differentiation of purified human hematopoietic progenitor cells. Furthermore, FL greatly potentiates the proliferation and the expansion of monocytic progenitors/precursors purified from human bone marrow/peripheral blood.

The study of *FLT3* at the level of human hematopoietic stem cells and various progenitor subpopulations provided evidence that this receptor is expressed on repopulating HSCs, as well as in early lymphoid progenitors and in the common myeloid progenitor and in the granulocyte/macrophage progenitor. *FLT3* expression and signaling is required for the survival of these stem/progenitor cells. The studies carried out in mice have led to conclusions slightly different from those reached in human studies. In fact, through a lineage tracing mouse model, it was shown that hematopoietic stem cell origin and maintenance does not involve a *FLT3*+ stage, while differentiation of these cells into all hematopoietic lineages involves *FLT3*+, non-self-renewing, and multipotent hematopoietic progenitor cell.

Sanger Sequencing Methods

Sanger sequencing methods:-Sanger sequencing also knows as chain-termination sequencing or dideoxy sequencing has been the powerhouse of DNA sequencing since its invention in the 1970s.The process is based on the detection of labelled chain-terminating nucleotides that are incorporated by a DNA polymerase during replication of a template.

This method has been extensively used to advance the field of functional comparative genomics,evolutionary genetics and complex disease research.Notably the dideoxy method was employed in sequencing the first human genome in 2002.Because of its suitability for routine validation of cloning experiments and PCR fragments,Sanger sequencing remains a popular technique in many laboratories across the world.

Principle of Sanger dideoxy sequencing

The principles of DNA replication were used by Sanger et al. in the development of the process now known as Sanger dideoxy sequencing. This process takes advantage of the ability of DNA polymerase to incorporate 2′,3′-dideoxynucleotides—nucleotide base analogs that lack the 3′-hydroxyl group essential in phosphodiester bond formation. Sanger dideoxy sequencing requires a DNA template, a sequencing primer, DNA polymerase, deoxynucleotides (dNTPs), dideoxynucleotides (ddNTPs), and reaction buffer. Four separate reactions are set up, each containing radioactively labeled nucleotides and either ddA, ddC, ddG, or ddT. The annealing, labeling, and termination steps are performed on separate heat blocks. DNA synthesis is performed at 37°C, the temperature at which DNA polymerase has the optimal enzyme activity. DNA polymerase adds a deoxynucleotide or the corresponding 2′,3′-dideoxynucleotide at each step of chain extension. Whether a deoxynucleotide or a dideoxynucleotide is added depends on the relative concentration of both molecules. When a deoxynucleotide (A, C, G, or T) is added to the 3′ end, chain extension can continue. However, when a dideoxynucleotide (ddA, ddC, ddG, or ddT) is added to the 3′ end, chain extension terminates. Sanger dideoxy sequencing results in the formation of extension products of various lengths terminated with dideoxynucleotides at the 3′ end.

Components for Sanger sequencing
Sanger sequencing involves making many copies of a target DNA region. Its ingredients are similar to those needed for DNA replication in an organism, or for polymerase chain reaction (PCR), which copies DNA in vitro. They include:

1. A DNA polymerase enzyme
2. A primer, which is a short piece of single-stranded DNA that binds to the template DNA and acts as a "starter" for the polymerase
3. The four DNA nucleotides (dATP, dTTP, dCTP, dGTP)
4. The template DNA to be sequenced

Materials and Methods

Instrumentation
Instrumentation description and dyes Fragment analysis is run on the SeqStudio genetic analyzer 3730 instrument. This is an 4 capillary instrument with non proprietary software for DNA sequencing, STR (Short Tandem Repeat). The CEQ8800 has diode lasers that excite infrared dyes, and has four detection channels. For fragment analysis, alleles in each sample can be labeled with up to three separate dyes, the fourth dye is used for the size standard. WellRED dye labeled oligonucleotide can be purchased at ITS or Sigma. Three different WellRED dyes are available: D4-PA (650/670), D3-PA (685/706) and D2-PA (750/770). WellRED dyes have different signal strengths, therefore the signal strengths of amplification products will also depend on the dye employed for labeling. The Molar Extinction Coefficient of these dyes and subsequently their signal strengths are in order D4-PA>D3-PA>D2-PA. Therefore, when pooling reactions, roughly equal signal strengths are obtained by adding the following relative volume of PCR reaction products (assuming the same amplification efficiency for all the alleles)

All equipment should be properly maintained according to the manufacturer's instructions.
1.Cell Counter
2.DNA Isolation Automator
3.Spectrophotometer
4.Refrigerator capable of 2 °C to 8 °C storage
5.Freezer capable of -15 °C to -30 °C storage
6. Dead air box
7.Micro pipettes

Materials Required
1. DNA Size Standard Kit
2. Fragment Analysis Test Sample
3. DNA Separation Capillary Array
4. Samples Loading Solution
5. Molecular Biology Grade Nulease free water

6. Thermal cycler with heated lid
7. PCR enzyme and buffer
8.Hi-Di(Formamide)
9.Labeled primers
10. Thermal cycling plates and caps

Storage of all Fragment Analysis reagents (Size Standards, Test Sample, and Mobility Calibration Standard) must be in a -20°C non frost-free freezer.

DNA Extraction
Genomic DNA was extracted from whole blood specimens or tissue samples using a QIAsymphony instrument (Qiagen), according to the manufacturer's recommendations DNA from fresh-frozen and formalin-fixed, paraffin-embedded specimens was extracted using QIAamp DNeasy blood and tissue DNA extraction kits (Qiagen).

Qubit Reading of DNA
Extracted DNA from QIAsymphony is measured by Qubit to quantify that how much quantity of the DNA is present in test samples. DNA is also measured using a NanoDrop spectrophotometer (Thermo Scientific)for all samples;

Preparing of Test Sample
1. Prepare test samples enough for 8 wells by combining the following in an eppendorf tube; 200 µL of SLS,
3. after that taking the Qubit reading of the test sample.
4.Preparing the 9µL of Master mix and 1 µL Test sample. Mix the contents by pipetting up and down a few times.
5.Dilute a portion of the test DNA sample with low-TE buffer so that 1.0 ng of total DNA is in a final volume of 12.5 µL. Add 0.5ng-1ng of the diluted sample to the reaction mix.
6. Add 5ul of dilted sample with low-TE buffer to each of the 8 wells and load onto the instrument.

Preparation of the Fragments for Analysis
Prepare the PCR reactions in a thin wall microplate or tube. All reagents should be kept on ice while preparing the PCR reactions. Follow the recommendations of the enzyme manufacturer for the set up of the PCR reactions.

Final concentration of the labeled primers in the PCR reaction should be 2.5ul.

Thermal Cycling Program

Steps	Temperature	Time	Cycles
Initial Incubation	95˚C	11 min	1
Denature	94˚C	1min	28
Anneal	59˚C	1min	
Extend	72˚C	1min	
Final Extension	60˚C	60 min	1
Final Hold	4-25˚C	Hold	Hold

Fragment Analysis by SeqStudio

1. Prepare the samples for electrophoresis immediately before loading.
2. Dilute the STR PCR product to 1:5 with low-TE buffer
3. Calculate the volume of Hi-Di™ Formamide and size standard needed to prepare the samples:

Reagent	Volume per reaction
GeneScan™ 500 LIZ® Size Standard	3 µL
Hi-Di™ Formamide	7 µL
Diluted STR PCR product	1µL

OR

Reagent	Volume per reaction
GeneScan™ 600 LIZ® size Standard v2.0	5 µL
Hi-Di™ Formamide	5 µL
Diluted STR PCR product	1µL

4.Denature at 95C for 3 min and snap cool at 4C for 3 min. Keep for the run in instrument.

Assay Uses

***FLT3* Mutation Assays are useful for the study of**

1.Identifying *FLT3* mutations in patients with AML.

2.Discriminating between high and low risk patients. *FLT3* mutations portend a worse prognosis for patients with AML. Therefore patients testing positive for *FLT3* mutations may benefit from a more aggressive treatment regimen.

Specimen Requirements

This assay tests genomic DNA

1.5cc of peripheral blood, bone marrow biopsy, or bone marrow aspirate anti-coagulated with heparin or EDTA. Ship at ambient temperature;

2. Minimum 5mm cube of tissue shipped frozen; or at room temperature or on ice.

3. 2ug of genomic DNA;

4. Formalin-fixed paraffin embedded tissue or slides

Storage Conditions

PCR master mixes are sensitive to freeze/thaw cycles. Therefore, for any duration other than immediate use, our master mixes and assay kits should be stored at -65°C to -85°C

The reason for this is quite straightforward: Due to the high salt concentrations in our master mixes, the effective freezing and thawing temperature of the master mixes is approximately –10°C. The temperature in a standard laboratory –20°C freezer can easily reach –10°C or warmer during the day when these freezers are opened on a regular basis. At these temperatures, PCR master mixes may go through multiple freeze/thaw cycles, resulting in precipitation of the primers. Accordingly, to minimize the exposure of your master mixes to freeze/thaw cycles, IVS recommends that master mixes be stored at -65°C to -85°C.

Reagents Required But Not Included

PCR Amplification
1.Ampli*Taq* Gold DNA Polymerase or equivalent
2. Eagle*Taq* DNA Polymerase
3. Ampli*Taq* DNA Polymerase
4. EcoRV restriction endonuclease

ABI Fluorescence Detection
1.HI-DI Formamide with ROX size standards

Recommended Positive Controls

Master Mix	Target	Color	Control DNA
FLT3-ITD	*FLT3* Juxtamembrane Domain	Blue & Green	Valid Size Range IVS-0017 Clonal Control DNA
FLT3 D835	*FLT3* Kinase	Blue	Valid Size Range IVS-P001 Clonal Control DNA
Specimen Control Size Ladder	Multiple Genes	Blue	Valid Size Range IVS-0000 Polyclonal Control DNA

Fig:Recommended Positive Control

Reagent Preparation
1.All unknown samples should be tested using the template amplification control (Amplification Control or Specimen Control Size ladder) master mix. This is to ensure that no inhibitors of amplification are present, and there is DNA of sufficient quality and quantity to generate a valid result.
2.All samples should be tested in singles.
3.Positive, negative and no template controls should be tested for each of the master mixes.
4.Using gloved hands, remove the master mixes from the freezer. Allow the tubes to thaw; then gently vortex to mix.
5.In an containment hood or dead air box remove an appropriate aliquote to clean, sterile microcentrifuge tube.
6.Add the appropriate amount of Ampli*Taq* Gold or Eagle *Taq* polymerase (0.25l of Ampli*Taq* Gold or Eagle*Taq*)to each of the master mixes and gently mix by inverting several times or gently vortexing.
7.The master mixes are now ready for distribution to reaction tubes or plate, and addition of sample.

Sample Preparation
Using any method of DNA extraction, extract the genomic DNA from unknown samples. Resuspend DNA to final concentration of 100ug-400ug per ml in TE(10 mM Tris-HCl, 1mM EDTA, pH 8.0) or distilled water. This is a robust assay system. A wide range of DNA concentrations will generate a valid result. Therefore, quantifying and adjusting DNA concentrations is generally not necessary. Testing sample DNAs with the Amplification Control or Specimen Control Size Ladder master mix will ensure that DNA of sufficient quality and quantity was present to yield a valid result.

Amplification
1.Aliquot 45ug of the master mix/enzyme solutions into individual PCR wells or tubes.
2.Add 5ul of sample or control DNA to the individual tubes or wells containing the respective master mix reactions, listed for each master mix, below. Pipet up and down several times to mix.

***FLT3* ITD Master Mix**
1.Water
2.VS-0017 Clonal Control DNA
3.IVS-0000 Polyclonal Control DNA
4.Test Specimen DNA

***FLT3* D835 Master Mix**
1.Water
2.VS-0017 Clonal Control DNA I
3.VS-0000 Polyclonal Control DNA
Test Specimen DNA

Specimen Control Size Ladder
1.Water
2.VS-0000 Polyclonal Control DNA
3.Test Specimen DNA.

Detection
Not all detection formats are available for all assays

Available Template Amplification Controls
1.The Amplification Control master mix primers are labeled with a fluorescent dye (6-FAM). This label is detected as BLUE using the differential fluorescence software. The amplicons produced with this master mix are at 235 basepairs. The products of this master mix should be run separately.

2.The Specimen Control Size Ladder master mix primers are labeled with a fluorescent dye (6-FAM). This label is detected as BLUE using the differential fluorescence software. The amplicons produced with this master mix are at ~100, 200, 300, 400, and 600 basepairs. Please note that the ~100bp band is comprised of a 84bp and 96bp bands. Both of these bands co-migrate on a gel. The products of this master mix should be run separately.

Interpretation and Reporting

The size range for each of the master mixes has been determined testing positive control samples. For accurate and meaningful interpretation it is important to ignore peaks that occur outside of the proscribed/valid size range for each of the master mixes. Peaks that are outside of the range cannot be assumed to be valid.

Results can be reported as "Positive" or "Negative" for "Detection of mutations in the *FLT3* gene".

1. Samples that fail to amplify following repeat testing should be reported as "A result cannot be reported on this specimen because there was DNA of
insufficient quantity or quality for analysis".
2. It is acceptable to call a sample "Positive" when a product is generated in the valid size range yet the positive control for that master mix fails.
3. Samples that test negative should be repeated if the positive control reaction failed.
4. All assay controls must be examined prior to interpretation of sample results. If the controls do not yield the correct results, the assay is not valid and the samples should not be interpreted.

The following describes the analysis of each of the controls, and the decisions necessary based upon the results.

1.Negative Control

positive-If the negative control is Possible contamination of all PCR amplification reactions. Do not continue with the interpretation of results. Prepare fresh master mix and repeat amplification.

Negative- Continue with the analysis.

2.Positive Control If the positive control is

positive- Continue with the analysis

Negative- Repeat assay unless specimen tests positive

3.Specimen Control Size Ladder- If the amplification control is

positive- 100, 200, 300, 400, and 600 basepair products are seen. Because smaller PCR fragments are preferentially amplified, it is not unusual for the 600 basepair fragment to have a diminished signal or to be missing entirely. Continue with analysis.

Negative- Repeat assay unless specimen tests positive.

Sample Interpretation
Following the acceptance of the controls, the clinical samples are interpreted as follows

1. *FLT3* ITD Master Mix

Positive- Presence of product(s) larger than 335 basepairs are reported as: "Detection of internal tandem duplication mutation of the *FLT3* gene."

Negative- Presence of product(s) of approximately 331 basepairs are reported as: "No evidence of an internal tandem duplication mutation of the *FLT3* gene."

2.*FLT3* D835 Master Mix

Positive- Presence of product(s) of ~150 basepairs (undigested) and ~130 basepairs (EcoRV digested) are reported as: "Detection of an Aspartic Acid 835 (D835) mutation of the *FLT3* gene."

Negative- Presence of product(s) of ~150 basepairs (undigested) and ~81 basepairs (EcoRV digested) are reported as: "No evidence of an Aspartic Acid 835 (D835) mutation of the *FLT3* gene."

Study Objectives- Primary objectives of the study were to

1) establish agreement with respect to selection of *FLT3* mutant patients and the *FLT3* Mutation Assay by assessing the overall, positive, and negative percent agreement between the two assays and 2) to estimate midostaurin efficacy in the *FLT3* Mutation Assay positive population on both overall survival (OS).
2. Secondary objectives of the study were to 1) identify potential demographic and disease state covariates affecting the relationship between diagnostics and efficacy and 2) to present objective evidence that gDNA isolated from mononuclear cells (MNCs) isolated from either bone marrow (BM) or peripheral blood (PB) provide concordant results from both specimen types for the *FLT3* Mutation Assay through comparison of paired samples.
3. Other testing and analyses included the assessment of the presence or absence of *FLT3* mutation by next generation DNA sequencing using the high throughput DNA sequencing technology as an independent source of sequence information

3.Limitations Of Assay Procedure

1. Test only the indicated specimen types, as the *FLT3* Mutation Assay has been validated for use only with peripheral blood and bone marrow aspirate. Reliable results are dependent on appropriate storage and processing of the specimens; therefore, follow the procedures in this Package Insert.
2. *FLT3* Mutation Assay has been validated using only the included QIAamp DSP DNA Blood Mini Kit to extract genomic DNA
3.*FLT3* Mutation Assay will detect ITD mutations sized 3 bp to 323 bp; however, the assay is only validated to detect mutations sized 30 bp to 279 bp.
4.ITD insertions sized between 3 bp and 30 bp will be reported as ITD mutations.

5.ITD insertions sized between 279 bp and 323 bp will be reported as ITD mutations
6.ITD insertions sized greater than 323 bp, will not be reported as insertions

This assay may not detect *FLT3* mutations that present below the sensitivity level of the assay:

1. For ITD insertions sized 30 bp to 126 bp, inclusive, an allelic ratio of 0.08 will yield a positive *FLT3* Mutation Assay result.
2. For ITD insertions sized 129 bp to 279 bp, inclusive, an allelic ratio of 1 will yield a positive *FLT3* Mutation Assay result.
3. For TKD mutations that destroy the EcoRV site, an allelic ratio of 0.18 will yield a positive *FLT3* Mutation Assay result.
4. he results of the assay should always be interpreted in the context of clinical data and other tests performed for the patients.

5. Detection of a mutation is dependent on the number of mutant sequence copies present in the specimen and may be affected by specimen integrity, amount of DNA isolated, and the presence of interfering substances. PCR-based assays are subject to interference by degradation of DNA or to inhibition of PCR due to EDTA and other agents.
6. Use of this product must be limited to personnel trained in the techniques of PCR and the use of the *FLT3* Mutation Assay
8. *FLT3* Mutation Assay is a qualitative test. The test is not for quantitative measurements of ITD or TKD mutations
9. The allelic ratio of a specimen cannot be calculated, measured, or determined using this assay.

Non-Clinical Performance Evaluation

Evaluable Set- The accuracy of the *FLT3* Mutation Assay was determined by comparing the results of the *FLT3* Mutation Assay to a validated high throughput sequencing method using specimens from the clinical trial. The samples for the method comparison study were a subset of the *FLT3* bridging study samples which included all available and evaluable *FLT3* mutation positive samples and approximately 300 *FLT3* mutation negative samples. The negative sample subset was selected by a randomization algorithm with the proportion from each laboratory test site matching the proportion from that site in the overall study.

Analytical Sensitivity(Limit of Blank (LoB)- When samples containing wild type DNA only were tested in the *FLT3* Mutation Assay, the SR was 0.00 in the ITD assay and 0.00 to 0.01 in the TKD assay. This limit of blank is well below the clinical cutoff SR of 0.05.

Analytical Sensitivity- The assay was evaluated in two studies. The first study used contrived samples created by blending cell lines with leukocyte-depleted whole blood. Cell line samples were used to represent three ITD insert sizes: 30 bp insert, 126 bp insert, and a 279 bp insert .Additional cell lines containing either the D835 mutation or the I836 TKD mutation were also assessed. DNA was diluted to 5 ng/µL, 10 ng/µL, and 15 ng/µL and tested at two allelic ratios for each cell line. A second study with clinical specimens was conducted to confirm the LoD observations obtained with cell lines. Five clinical samples

were diluted with clinical negative samples in order to yield a targeted signal ratio (TSR) within the linear range of an appropriate cell line standard curve . Each specimen was diluted to 5 levels representing a low negative (LN), high negative (HN), near the cut-off (CO), a low positive (LP), and a medium positive (MP). These linear range samples were tested in the *FLT3* Mutation Assay and an average SR value was determined. Each clinical LoD sample dilution was tested 20 times for each dilution level over four nonconsecutive days (5 replicates per day) by one operator using one equipment set. The AR of each clinical LoD sample dilution was calculated using the AR estimated from the cell line standard curves. The ARs of the clinical LoD samples were estimated based on the study meeting the following acceptance criteria:

1.The SR and AR where *FLT3* mutations can be detected above the limit of blank (LoB) in ≥95% of replicates (Analytical LoD).
2.The AR near the clinical cut-off, a SR of 0.04 – 0.06 (Cut-off).
3. The AR and SR that is detected above the clinical cut-off in ≥95% of replicates (Above Cut-off).

Precision-

1.The precision of the *FLT3* Mutation Assay was determined by three operators independently testing 10 replicates each of ITD mutation samples with inserts ranging in size from 30 bp to 126 bp and TKD mutation samples. The 10 replicates were tested in batches of two 5 separate times.
2. For the ITD mutation samples, the SR %CV ranges for the 3 operators were 7.4% to 15.0%, 3.7% to 13.0%, and 4.2% to 8.8%.
3. For the TKD mutation samples, the SR %CV ranges for the 3 operators were 6.3% to 11.2%, 5.8% to 9.3%, and 5.5% to 8.3%.

Operator-to-Operator Reproducibility (clinical samples)

1. In a second study, precision was assessed using clinical DNA samples from 8 clinical samples with ITD lengths of 21 bp, 24 bp, 66 bp, 90 bp and 217 bp, TKD D835 substitution, TKD I836 deletion, and *FLT3* negative samples. DNA from *FLT3* negative clinical specimens was pooled and used to dilute the *FLT3* positive samples in order to achieve three target SR levels near the assay's clinical cut-off (i.e., high negative, low positive, and moderate positive).

2. The total %CV of all mutation types and levels are shown in table below and the %CV for all mutation types, except the long ITD insert (217 bp) sample, ranged from 4.2% to 16.1%. The sample with a 217 bp mutation %CV ranged from 26.9% to 27.2% (Table 11). The low positive (LP) dilution level %CV was 26.9% for 217 bp, therefore failing the study acceptance criteria of ≤25% CV for SR. Results show that acceptance criteria were met for both D835 and I836 TKD mutations and for ITD mutations up to 217 bp. Variation for the 217 bp ITD mutation exceeded 25%, thus indicating greater imprecision around the largest ITDs.

Clinical Performance Evaluation

Study Overview-

1.To support the safety and efficacy assessment of the *FLT3* Mutation Assay, clinical agreement was required to be demonstrated between samples with *FLT3* status determined from the A2301 Clinical Trial Assay (CTA) and the *FLT3* Mutation Assay in the intent-to-test population.
2. The *FLT3* Mutation Assay has been developed by *Invivo* scribe as a companion diagnostic to be used as an aid in the assessment of AML patients for whom midostaurin treatment is being considered. drug efficacy when stratified by the *FLT3* Mutation Assay was evaluated in this Bridging Study.

Conclusions

1.Overall these results support that the *FLT3* Mutation Assay identifies the same AML patient population as enrolled in the A2301 clinical trial with respect to *FLT3* ITD and TKD gene mutations.
2. The data from this study support the reasonable assurance of safety and effectiveness of the *FLT3* Mutation Assay when used in accordance with the indications for use.

Clinical dilemmas in the management of *FLT3*-mutated AML

Current *FLT3* testing landscape:
Historically, patients with AML were stratified into risk groups based on age, performance status, white blood cell count, and cytogenetics. Subsequently, gene mutations (e.g. NPM1, *FLT3*, TP53, and CEBPA) were recognized as important prognostic factors and thus included in testing recommendations in the United States and Europe. Until recently, *FLT3* testing was recommended as a prognostic marker only in patients with cytogenetically normal AML. However, new recommendations for *FLT3* testing in all patients with AML are a result of the approval of the first *FLT3*- targeted therapy, midostaurin, and the recognition that *FLT3* is a negative prognostic marker, regardless of cytogenetics. Importantly, results of *FLT3* testing should be made available within 48–72 h after the initial diagnosis of AML so that targeted therapy can be initiated in a timely manner.

testing rates (including *FLT3*) between academic centers and community referral sites, as suggested by the results of a single-institution retrospective chart review that analyzed molecular testing rates over time (2008–2012). Despite an increase in testing over time, testing rates were significantly higher at academic centers than at community sites (93% vs 41%; $p < .001$). Routine testing for *FLT3* in patients with cytogenetically normal AML had been recommended since at least 2010, which corresponds to the time at which molecular testing was routinely performed in 100% of patients at academic centers but not at community sites . This suggests that there is a lack of awareness or knowledge about the importance of molecular testing at community sites. More recently (2015), 294 members of professional societies in the United States and Europe were surveyed about their testing practices. Among responders, 51 and 46% indicated that they tested for *FLT3*-ITD in all patients and selected patients, respectively. This survey was intended to provide a baseline

for testing prior to the release of the diagnostic workup guidelines jointly issued by the College of American Pathologists and the American Society of Hematology in 2017. It would be expected that testing rates, particularly those for *FLT3*, will soon increase given that *FLT3*-targeted therapies are entering the market. One potential hurdle to widespread *FLT3* testing in the past was the lack of commercially available tests. It will be interesting to see whether testing rates at community sites will catch up to those at academic centers – especially now that commercially developed *FLT3* testing assays are routinely incorporated into clinical trials and are beginning to hit the market.

Methods for testing *FLT3*

PCR assays for *FLT3* mutations

Clinically validated *FLT3* mutation testing is performed with a PCR-based assay of genomic DNA isolated from a sample of the patient's leukemia cells. The most commonly used assay, which is available in commercial laboratories and in the certified laboratories of several tertiary care centers, uses genomic DNA prepared from whole blood or BM. The number of blasts within the sample tested is crucial because samples diluted with nonmalignant hematopoietic elements will lower the sensitivity of the assay. The most common assay method used is that reported by Murphy et al, which is actually a duplex assay testing for both types of *FLT3* mutations in the same tube: PCR primers (labeled with a fluorochrome) flanking the juxtamembrane coding sequence amplify *FLT3*/ITD mutations, whereas a second set of primers amplify the kinase domain sequence in the same reaction tube. The amplified fragments are separated using capillary gel electrophoresis and the fluorochrome signals are analyzed using a software program such as GeneScan. An EcoRV digestion of the PCR products will cleave the kinase domain fragment unless a TKD mutation at D835 (or I836) is present. It is important to note that neither PCR product is sequenced; the assay simply determines whether an *FLT3*/TKD mutation is present or, in the case of *FLT3*/ITD mutations, the length of the insertion. Because the prognostic effect of *FLT3*/TKD mutations remains unresolved, some laboratories only assay for *FLT3*/ITD mutations.

Limitations of the *FLT3*/ITD mutation assay

A major limitation of most PCR assays for *FLT3*/ITD mutations is their relative lack of sensitivity, at least compared with PCR assays for other AML-associated lesions such as AML-ETO. Gene fusions such as AML-ETO or BCR-ABL generate a unique sequence highly amenable to amplification over background. The sensitivity of these assays is simply a function of the amount of sample DNA and the number of PCR cycles. In the *FLT3*/ITD assay, however, increasing the number of cycles will not increase the sensitivity because the PCR primers used to amplify the mutant allele also amplify the wild-type allele. This would not be a problem if both mutant and wild-type alleles were amplified equally, but in this assay, the shorter wild-type allele has a competitive advantage. In a reaction using a typical patient specimen that is heterozygous for an *FLT3*/ITD mutation, the fragments generated in the PCR reaction consist of the 330-bp wild-type fragment and the longer mutant fragment, the length of which is increased over the wild-type length due to the inserted sequence. The increased length of the mutant sequence translates into a longer time required to complete a PCR cycle, which leads to the wild-type allele being amplified at a more rapid rate than the mutant allele. The result of this wild-type "PCR bias" is that even in a pure 1:1 mixture of mutant and wild-type templates, the mutant-to-wild-type

allelic ratio will be less than 1. The longer the insertion is, the greater the PCR bias. For example, an insertion of 120 bp will artificially lower the ratio to a greater degree than a 30-bp insertion. This bias can be minimized using fewer cycles of PCR, but this could affect sensitivity when there is a low burden of leukemia cells in the sample.

***FLT3*/ITD mutation length**

A few studies have examined the impact of the length of mutation on clinical outcome. The median length of a mutation in these studies ranged from 39 to 54 bp, and longer mutations are usually (but not always) found to be associated with reduced remission rate and/or worse overall survival. The structural consequence of a long duplication of a sequence beginning at or around the codon for arginine 595 is that the actual duplicated sequence starts within the kinase domain rather than within the juxtamembrane domain.

Allelic ratio

For *FLT3*/ITD mutations, the allelic ratio refers to the number of ITD-mutated alleles compared with the number of wild-type allele. This ratio is not only influenced by the amount of malignant versus nonmalignant cells in the sample tested, but also by the percentages of cells with mutated alleles. AML is a polyclonal disease at presentation and, whereas in most cases, the dominant cell in a diagnostic sample is heterozygous for the mutation, subpopulations within the sample can lack the mutation altogether or be biallelic (uniparental disomy). Still other cells can be hemizygous for the mutant allele either through outright loss of the chromosome 13 containing the wild-type allele (which is identifiable using conventional cytogenetic analysis) or through a smaller deletion of the wild-type (detectable using a single-nucleotide polymorphism array).

As *FLT3*/AML evolves from diagnosis to relapse, the mutant to wild-type allelic burden also typically evolves. Occasionally, the mutation is completely absent (or at least undetectable) at relapse. This typically is seen in cases in which the mutant allelic burden is low (eg, 5%-15%) at diagnosis. This phenomenon, coupled with the overall lack of sensitivity of the assay, is why *FLT3*/ITD mutations have been regarded as unsuitable for use as a marker of minimal residual disease. In most cases, however, the mutation originally detected at diagnosis is also present at relapse, and often at an even higher allelic ratio than at diagnosis. Several studies in which *FLT3*/ITD mutation testing was performed on banked specimens from clinical trials have concluded that higher mutant to wild-type allelic ratio is predictive of worse outcomes, with the worst scenario of all being loss of the wild-type allele altogether. The negative prognostic impact of a high mutant allelic burden hold true even in studies that have modified the assay to account for the PCR bias from mutation length. In the context of the polyclonal nature of AML at diagnosis.

Conclusions about *FLT3* mutation testing

To summarize, *FLT3*/ITD mutation testing should be performed in all AML patients at diagnosis for prognostic purposes and for guiding therapeutic decisions, but currently has little utility for minimal residual disease monitoring. It is relatively insensitive compared with other PCR assays and the results are dependent upon the blast percentage assayed and (depending on the number of PCR cycles used) on the length of the insertion. *FLT3*/ITD mutations are common in AML and, apart from APL, they usually have a pronounced negative impact on clinical outcomes at all stages of the disease. The clinician may be tempted to declare a patient intermediate risk when the mutant-to-wild-type allelic ratio is low (and when there is a coexisting NPM1 mutation), but such a classification should take PCR methodology into account. A safer default at this time, therefore, is still probably to classify all non-APL *FLT3*/ITD cases as generally poor risk and tailor therapy accordingly. The variations in results from different *FLT3*/ITD mutation testing methods argue for the development of an international standard to avoid some of the problems encountered with other oncogene testing (such as for BCR-ABL). This will be particularly important as targeted therapies against *FLT3* are investigated.

FLT3/ITD AML: how to treat?

A relapse waiting to happen

Newly diagnosed *FLT3*/ITD AML patients in the age range of 18 to 65 years receiving intensive cytarabine-based induction therapy generally achieve remission at or near the same rate as other AML patients. The exception to this rule comes from those patients presenting with a high allelic burden or who are hemizygous for the mutation: these patients are often refractory to induction.Regardless, a propensity to relapse—and relapse quickly—is the salient feature of *FLT3*/ITD AML patients in first remission. Their median time to relapse is 6 to 7 months compared with 9 to 11 months for patients with other AML subtypes. *FLT3*/ITD AML at relapse is an extremely dire situation. Although survival for AML patients in first relapse is generally poor, those with an *FLT3*/ITD mutation can be distinguished as having the shortest survival of any of them. This is presumably a reflection of the fact that the salvage rate for *FLT3*/ITD AML in first relapse is lower than in nonmutated cases (22% vs 64% in one study), and is probably also due to the generally aggressive, proliferative nature of the disease. In one trial of *FLT3*-mutated patients in first relapse treated with conventional salvage chemotherapy (high-dose cytarabine or mitoxantrone/etoposide/cytarabine), only 11% of patients with a first remission duration of 6 months or less achieved a second remission.

Role of allogeneic transplantation

These dire statistics starkly frame the dilemma facing a clinician deciding on a consolidation therapy for an *FLT3*/ITD AML patient in first remission: to transplant or not to transplant? For *FLT3*/ITD AML, the role of allogeneic transplantation as consolidation therapy has been a controversial issue. In most studies of AML patients, allogeneic transplantation confers a lower relapse risk compared with conventional chemotherapy.

Given that relapse risk is a central feature of *FLT3*/ITD AML, allogeneic transplantation would seem the logical consolidation choice if not for the fact that transplantation is typically associated with at least a 20% treatment-related mortality risk. Although there have as yet been no prospective studies to specifically address the role of allogeneic transplantation in *FLT3*/ITD AML, several retrospective analyses have been performed and a coherent picture is emerging.

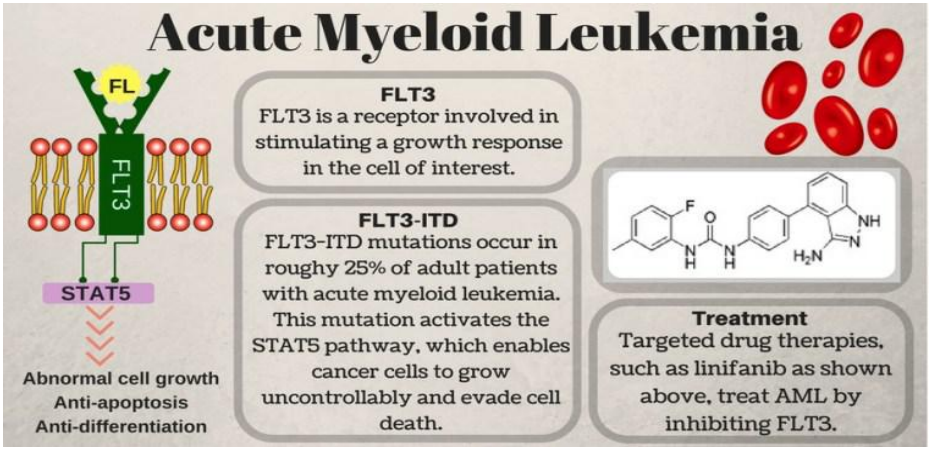


Fig:-Workflow of FLT3-ITD and Drug Therpy

patient in first remission should be offered an allogeneic transplantation as consolidation, assuming that a donor can be found and that the patient is an acceptable candidate for the procedure (in terms of age and comorbidities). Given the rapidity with which these patients relapse, it seems prudent to initiate HLA typing and a donor search immediately upon identifying an AML patient as having an *FLT3*/ITD mutation. At this time, there are no reliable data available regarding the relative benefits of myeloablative versus reduced-intensity conditioning for these patients.

Elderly patients

Although the maximum age for allogeneic transplantation continues to rise with the use of reduced-intensity conditioning, AML remains a disease most often encountered in the elderly. Therefore, there remains a significant fraction of *FLT3*/ITD AML patients ineligible for transplantation because of their age. Such patients are often unsuitable for intensive induction therapy and referral to a clinical trial, if possible, is recommended. *FLT3* inhibitors have been under active investigation for several years now, either administered as single-agent therapy or in combination with other agents. The hypomethylating agents (azacitidine and deoxy-5-azacitidine) have shown activity in newly diagnosed elderly AML patients. A recent trial in which azacitidine was combined with the tyrosine kinase inhibitor sorafenib (which has activity as a single agent in *FLT3*/ITD AML) showed promising results, and other trials of hypomethylating agents combined with *FLT3* inhibitors are under way.

Future Directions for *FLT3* Targeted Therapy

The major current questions in the field are which *FLT3* inhibitor(s) are most effective in different settings, and which combination regimens will enhance the efficacy of *FLT3* inhibitors.

The first-generation type I *FLT3* inhibitor midostaurin given to newly diagnosed AML patients 18 to 60 years old with *FLT3*-ITD or TKD mutations after induction and consolidation chemotherapy and as maintenance therapy showed significant efficacy in prolonging survival, compared to placebo. Therefore midostaurin will likely become the first *FLT3* inhibitor to be approved by the FDA, and treatment with midostaurin may therefore become the standard of care, in conjunction with chemotherapy, for newly diagnosed patients with AML with *FLT3* mutations. It is possible that midostaurin has particular efficacy in the newly diagnosed setting because of broad activity against AML with multiple leukemic clones and low *FLT3* mutation allelic burden. Nevertheless, it is also possible that a more potent and better tolerated inhibitor such as gilteritinib might be even more efficacious, and unfortunately answering this question would require another large randomized trial with long follow-up. It will be challenging to test new inhibitors against midotaurin with chemotherapy in the newly diagnosed setting. Additionally, HSCT

in first CR has become the standard or care, and it will be important to determine which *FLT3* inhibitors are well tolerated following HSCT and have efficacy in preventing relapse in that setting.

In contrast, the first-generation type I *FLT3* inhibitor lestaurtinib was ineffective, compared to placebo, in relapsed patients with *FLT3*-ITD or TKD mutations after reinduction chemotherapy. It is likely that more potent and specific *FLT3* inhibitors will be more efficacious following reinduction chemotherapy, given the common presence of a dominant clone with *FLT3* mutation at relapse. Diverse *FLT3* inhibitors will need to be tested against placebo, and then potentially against each other, following reinduction chemotherapy in the relapsed/refractory setting.

Numerous drug combinations with *FLT3* inhibitors are being explored, and will be essential for patients who are not candidates for chemotherapy or whose AML is refractory to chemotherapy. Combinations may also be effective post HSCT.

Summary of Clinical Experience

At the time of submission of this article, 147 clinical samples from 135 patients have been analyzed for *FLT3* mutations using the reported approach. The majority of the samples we have tested using this assay have been peripheral blood or bone marrow samples from patients with AML although we have also tested samples from patients with MDS, acute lymphocytic leukemia, myeloproliferative disorders, and biphenotypic leukemia. Many of these samples were from outside institutions and their diagnosis could not be confirmed. Of 110 patients reported to have AML, 22 (22 of 110, 20%) were positive for an ITD mutation. The ITD mutations identified in our clinical experience have ranged in size from 18 bp to

186 bp. Several of the mutant samples have been sequenced to confirm and characterize the ITD (data not shown). Three of the ITD-positive samples revealed the presence of two different ITD mutations.

The assay cannot distinguish whether these two mutations occurred in the same or different *FLT3* alleles. No *FLT3* ITD mutations were identified in the samples from patients with MDS (0 of 13) or other diagnoses (0 of 13).

Three of the 110 patients with AML tested positive for a D835 mutation (3 of 110, 2.7%). D835 mutant samples were subjected to sequence analysis to confirm and characterize the mutation. One of the samples positive for a D835 mutation also contained an ITD mutation. This assay does not distinguish whether the two

mutations are in the same or different *FLT3* alleles. No *FLT3* D835 mutations were identified in any of the samples from patients with MDS (0 of 13) or other diagnoses (0 of 12). Thus a total of 24 patients had identifiable *FLT3* mutations (21 ITD, 2 D835, 1 both ITD and D835), yielding an overall *FLT3* mutation rate of 24 of 110, 21.8%. No mutations have been identified in any cases with diagnoses other than AML.

Discussion

The reported *FLT3* molecular diagnostic assay provides a reliable and robust method to simultaneously detect the two different types of *FLT3* mutations currently known to be of importance in the natural history of AML. Experience reveals the assay to function reliably for both routine clinical testing and to be efficient enough to allow stat clinical testing. The reported assay has allowed us to achieve a 36-hour turnaround time from receipt of the clinical blood or bone marrow sample to written report of the diagnostic result. This format has facilitated rapid eligibility determination and enrollment into a *FLT3* inhibitor trial currently underway at our institution.

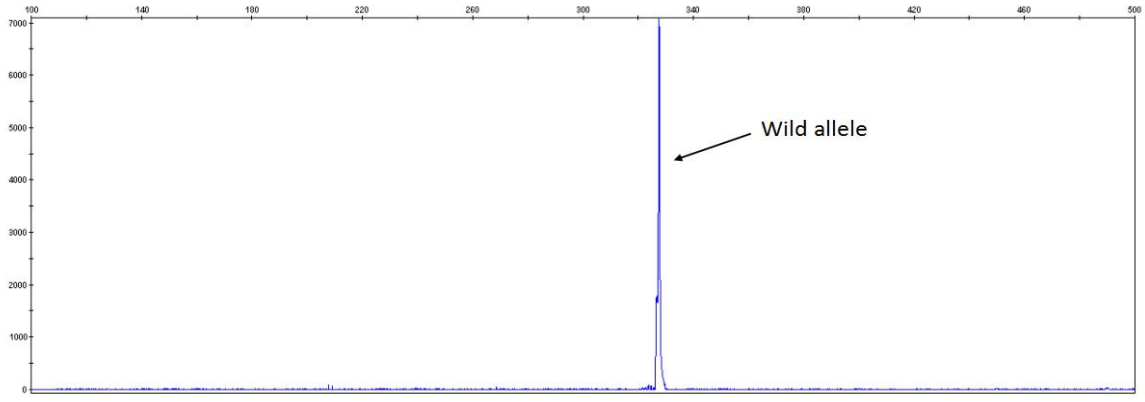


Fig:- FLT3 wild allele

Several features of this assay make it convenient for clinical diagnostic use. The multiplex PCR format of the assay provides for the simultaneous detection of both types of clinically relevant *FLT3* mutations. We chose to design the assay as a DNA-based PCR reaction rather than a RNA-based assay to avoid assay variability often associated with RNA

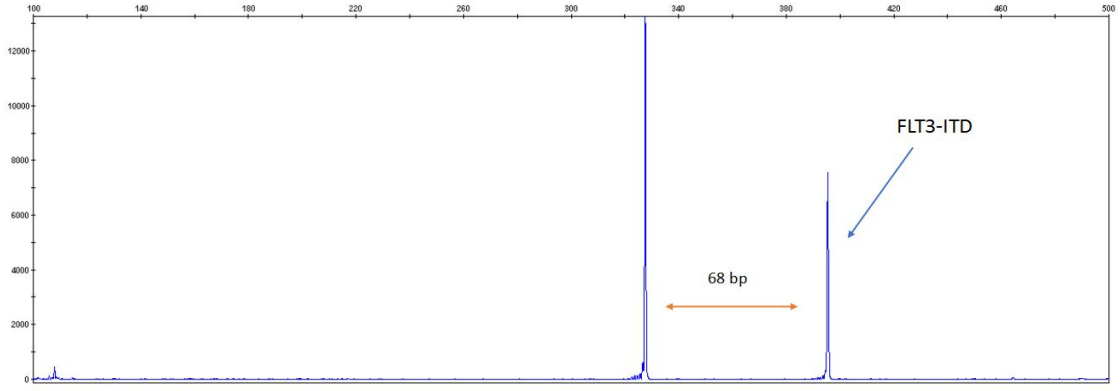


Fig:- FLT3-ITD mutant allele

Instability. The D835 portion of the assay is optimized for clinical use by the addition of an EcoRV cut site engineered into the D835 reverse

primer, providing an internal quality control measure of the success of the EcoRV restriction enzyme digestion. The addition of M13 sequence to both primers facilitates sequencing of D835-positive samples for mutation confirmation and characterization. Our clinical reports reflect the fact that any alteration of D835 or I836 yields a positive result in this assay. One insertion mutation has previously been reported that alters I836. Limited data suggest this mutation also results in constitutive activation of the *FLT3* receptor and may be of biological and clinical importance.

The ITD portion of this assay is clinically appealing because of the use of fluorescently labeled forward and reverse primers and the use of CE for separation of the PCR products. Double-labeling the PCR products decreases the likelihood of false-positives, increasing the specificity of the assay. Detection of PCR products by

CE allows for more accurate sizing of ITD mutations and the ability to resolve multiple ITD bands of similar size that cannot be resolved by PAGE. In addition, it appears that heteroduplex formation between wild-type and ITD PCR products can result in multiple bands on PAGE that are not independent ITD mutations. Because the CE format applied to this assay is denaturing, it detects only single-stranded PCR products, and thus does not suffer from this artifact. The significance of multiple ITD products is unclear at this time, however accumulation of this data may help establish the biological and clinical significance of this finding.

The assay we describe has been optimized for use as a qualitative diagnostic tool and is not optimal for quantitative assessment of minimum residual disease. Compared to assays that measure translocations (Bcr-Abl, PML-RARα), the *FLT3* assay has decreased sensitivity because of competition between wild-type and mutant *FLT3* alleles. Without the use of mutation (patient)-specific primers, an adequately high level of assay sensitivity is difficult to achieve in this testing situation. It is currently unclear what role *FLT3* mutation analysis will have as a tool for minimum residual disease detection in AML. Three groups have studied *FLT3* mutations in paired diagnostic and relapse samples. From these studies, it appears that a significant percentage of relapsed patients will have acquired an ITD

mutation that was not detectable at diagnosis, or will have lost an ITD mutation that was detected at diagnosis. Although the exact frequency at which *FLT3* mutation status is discrepant at diagnosis and relapse is still unclear, it is clear that molecular evolution may make minimum residual disease detection difficult in a subset of AML patients.

Although it has been reported that the ratio of ITD mutant peak height to the wild-type peak height provides additional prognostic information, we currently do not report this data. Clearly this ratio is highly dependent on the percentage of neoplastic cells in the population tested. In many clinical testing situations, this ratio may simply reflect the ratio of the normal to neoplastic cells in the mixture and therefore would have to be interpreted in that context. We do however report the absence or near absence of wild-type PCR products, indicating a *FLT3* loss of heterozygosity event. It has been reported that disease-free survival and overall survival are significantly inferior for patients with *FLT3* ITD mutations and loss of heterozygosity of the wild-type allele (*FLT3* −/−) compared to patients with *FLT3* wild-type (*FLT3* +/+) or *FLT3* ITD mutations without loss of heterozygosity (*FLT3* +/−).

Our experience has revealed a slightly higher rate of ITD mutations in our validation study set (38%) than expected (20 to 30%) that is also higher than that seen in our clinical experience with the assay (20%). This probably results from the use of banked

samples in the validation study that are likely to have a collection bias toward patients with high peripheral white blood cell counts. *FLT3* ITD mutations correlate with elevated white blood cell counts, likely explaining this result and highlighting the need to accumulate *FLT3*mutation data prospectively. The *FLT3* mutation rate in our clinical experience may be slightly lower than expected because of testing samples with blast counts below the limit of detection for this assay (10%).

The clinical identification of *FLT3* mutations in a prospective manner will yield important information about the incidence and natural history of *FLT3* mutations in AML. In addition, identification of *FLT3*mutations is likely to become important for optimization of patient care. Because *FLT3* ITD mutations portend a worse prognosis, it has been proposed that patients testing positive for a *FLT3* mutation may benefit from aggressive up-front treatment regimens such as an allogeneic bone marrow transplantation. On-going clinical trials will determine whether AML patients with *FLT3* mutations will also benefit from novel therapeutic strategies that target and inhibit *FLT3* tyrosine kinase activity.

Sequencing of pGEM

What is pGEM

1. pGEM is control DNA for S30 Extract system contains the Contains the β-galactosidase coding sequence downstream of E. coli wildtype lacZ promoter.
2. pGEM β-gal Use as positive control in the E. coli S30 Extract System for Circular DNA.
3. The wildtype LacZ promoter is not efficient for initiating transcription from a linear DNA template.

Introduction

The pGEM is Easy Vector Systems are convenient systems for the cloning of PCR products.The vectors are prepared by cutting Promega's pGEM 5Zf(+)(b) and pGEM Easy Vectors with EcoR V and adding a 3 terminal thymidine to both ends. These single 3-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases.

The high copy number pGEM Easy Vectors contain T7 and SP6 RNA Polymerase promoters flanking a multiple cloning site (MCS) within the a-peptide coding region of the enzyme 尾-galactosidase. Insertional inactivation of the a- peptide allows recombinant clones to be directly identified by color screening on indicator plates. The multiple cloning region of the two vectors includes restriction sites conveniently arranged for use with Promega's Erase-a-Base system for generating nested sets of deletions.

pGEM Easy Vector contain multiple restriction sites within the MCS. These restriction sites allow for the release of the insert by digestion with a single restriction enzyme. .The pGEM Easy Vector MCS is flanked by recognition sites for the restriction enzymes EcoR I, BstZ I and Not I, thus providing three single-enzyme digestions for release of the insert, while the pGEM Vector cloning site is flanked by recognition sites for the enzyme BstZ I. Alternatively, a double-digestion may be used to release the insert from either vector.

The pGEM Easy Vectors also contain the origin of replication of the filamentous phage f1 for the preparation of single-stranded DNA.

The pGEM vector is used as control DNA for cycle sequencing for sanger confirmation(Capillary Electrophoresis-SeqStudio).

Advantage of using pGEM

To simplify troubleshooting, Applied Biosystems recommends that you run controls with every run for multicapillary instruments or each set of runs on 310 instruments:

1.DNA template control pGEM 3Zf(+) Results can help you determine whether failed reactions are caused by poor template quality or sequencing reaction failure.
2.Sequencing standards Results can help you distinguish between chemistry problems and instrument problems.

Reagents used

1.Control DNA used as pGEM.
2.Big-dye
3.Primer-M13 *Taq*
4.Big-dye terminator sequencing buffer
5.Nuclease free water.

Capillary electrophoresis

Historically, DNA sequencing products were separated using polyacrylamide gels that were manually poured between two glass plates. Capillary electrophoresis using a denaturing flowable polymer has largely replaced the use of gel separation techniques due to significant gains in workflow, throughput, and ease of use. Fluorescently labeled DNA fragments are separated according to molecular weight. Because you do not need to pour gels with capillary electrophoresis, you can automate DNA sequence analysis more easily and process more samples at once.

Process overview
During capillary electrophoresis, the extension products of the cycle sequencing reaction enter the capillary as a result of electrokinetic injection. A high voltage charge applied to the buffered sequencing reaction forces the negatively charged fragments into the capillaries. The extension products are separated by size based on their total charge.

The electrophoretic mobility of the sample can be affected by the run conditions: the buffer type, concentration, and pH, the run temperature, the amount of voltage applied, and the type of polymer used.

Shortly before reaching the positive electrode, the fluorescently labeled DNA fragments, separated by size, move across the path of a laser beam. The laser beam causes the dyes attached to the fragments to fluoresce. The dye signals are separated by a diffraction system, and a CCD camera detects the fluorescence.

Because each dye emits light at a different wavelength when excited by the laser, all colors, and therefore loci, can be detected and distinguished in one capillary injection.

The fluorescence signal is converted into digital data, then the data is stored in a AB1 (.ab1) file format compatible with an analysis software application.

Cycle sequencing

Process overview

Like Sanger sequencing, fluorescence-based cycle sequencing requires a DNA template, a sequencing primer, a thermal stable DNA polymerase, deoxynucleoside triphosphates/deoxynucleotides (dNTPs), dideoxynucleoside triphosphates/dideoxynucleotides (ddNTPs), and buffer. But unlike Sanger's method, which uses radioactive material, cycle sequencing uses fluorescent dyes to label the extension products and the components are combined in a reaction that is subjected to cycles of annealing, extension, and denaturation in a thermal cycler. Thermal cycling the sequencing reactions creates and amplifies extension products that are terminated by one of the four dideoxynucleotides (Figure 3). The ratio of deoxynucleotides to dideoxynucleotides is optimized to produce a balanced population of long and short extension products.

Cycle sequencing kits

Applied Biosystems Cycle Sequencing Kits available for dye terminator chemistries are:

1.BigDye Terminator v1.1 and v3.1 Cycle Sequencing Kits
2.dGTP BigDye Terminator v1.0 and v3.0 Cycle Sequencing Kits
3.BigDye Direct Cycle Sequencing Kits.

Product description

1.The BigDye™ Terminator v3.1 Cycle Sequencing Kit provides pre-mixed reagents for Sanger sequencing reactions.
2.The kit includes BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer , which is specifically optimized for use with the BigDye™ Ready Reaction mixes.
3.The kit has been formulated to deliver robust performance across a wide variety of DNA sequences while maximizing readlengths. When used in combination with Minor Variant Finder Software, the kit can also be used to detect variants as low as 5% in a sample.

Run the sequencing reactions

1.Place the tubes or plate(s) in a thermal cycler and set the volume.

Parameter	Stage/step				
	Incubate	Cycling (25 cycles)			Hold
		Denature	Anneal	Extend	
Ramp rate	—	1°C/second.			
Temperature	96°C	96°C	50°C	60°C	4°C
Time (mm:ss)	01:00	00:10	00:05	04:00[1]	Until ready to purify

Perform cycle sequencing

1. Completely thaw the contents of the BigDye™ Terminator v3.1 Cycle Sequencing Kit and your primers and store on ice.
2. Vortex the tubes for 2 to 3 seconds, then centrifuge briefly (2 to 3 seconds) with a benchtop microcentrifuge to collect contents at the bottom of the tubes.
3. Label microcentrifuge tubes "forward" and "reverse" and add components as indicated
4.Seal the plate with MicroAmp™ Clear Adhesive Film.
5.Vortex the plate for 2 to 3 seconds, then centrifuge briefly in a swinging bucket centrifuge to collect contents to the bottom of the wells (5 to 10 seconds) at 1,000 x g.

Advantages-There are many advantages to performing cycle sequencing, including:

1. Protocols are robust, easy to perform, and effective for sequencing PCR products.
2. High temperatures reduce secondary structure, allowing for precise priming, template annealing, and thorough extension.
3. The same protocol can be used for double- and single-stranded DNA.
4.Difficult templates, such as bacterial artificial chromosomes (BACs), can be sequenced.

Dye terminator chemistry
With dye terminator chemistry, each of the four dideoxynucleotide terminators is tagged with a different fluorescent dye. One reaction is performed, containing the enzyme, nucleotides, and all dye-labeled dideoxynucleotides. The products from this reaction are injected into one capillary.

Dye primer chemistry
With dye primer chemistry, four separate tubes of sequencing primers are each tagged with a different fluorescent dye. Four separate reactions are performed, each containing the enzyme, nucleotides, a specific dye-labeled sequencing primer, and either A, C, G, or T dideoxynucleotides. The products from these four reactions are then combined and injected into one capillary.

Modified DNA polymerase
The cycle sequencing reaction is directed by highly modified, thermally stable DNA polymerases. These enzymes have been carefully selected to allow incorporation of dideoxynucleotides, to process through stretches of G-C-rich and other difficult sequences, and to produce uniform peak heights. The modified DNA polymerases are also formulated with a pyrophosphatase to prevent reversal of the polymerization reaction (pyrophosphorolysis).

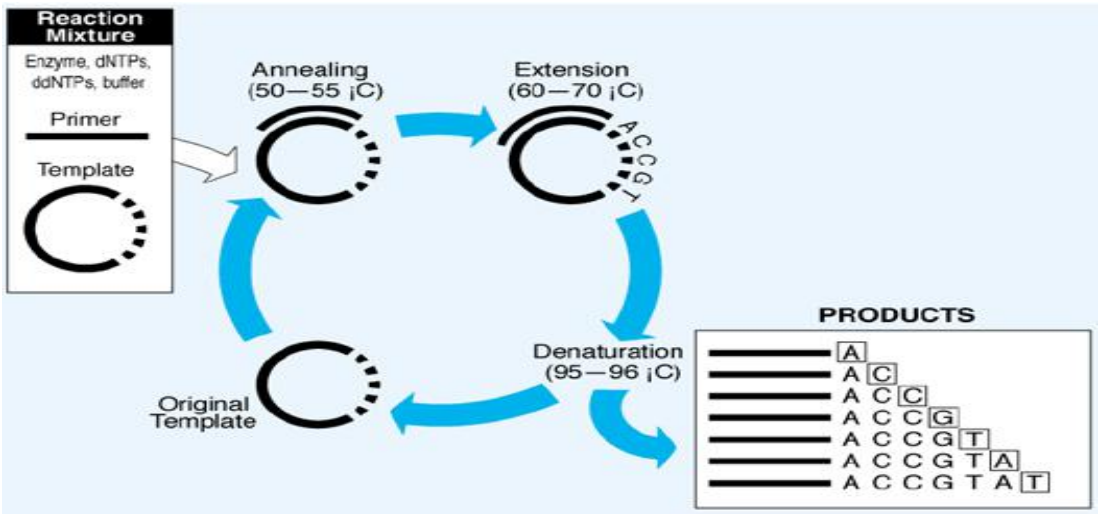


Figure 1: Cycle Sequencing

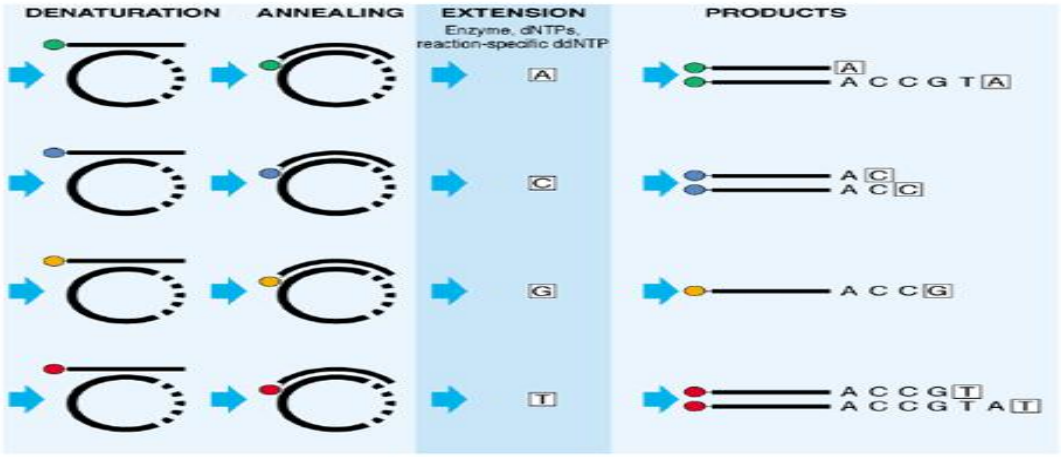


Figure 2: One Cycle of Dye Primer Cycle Sequencing

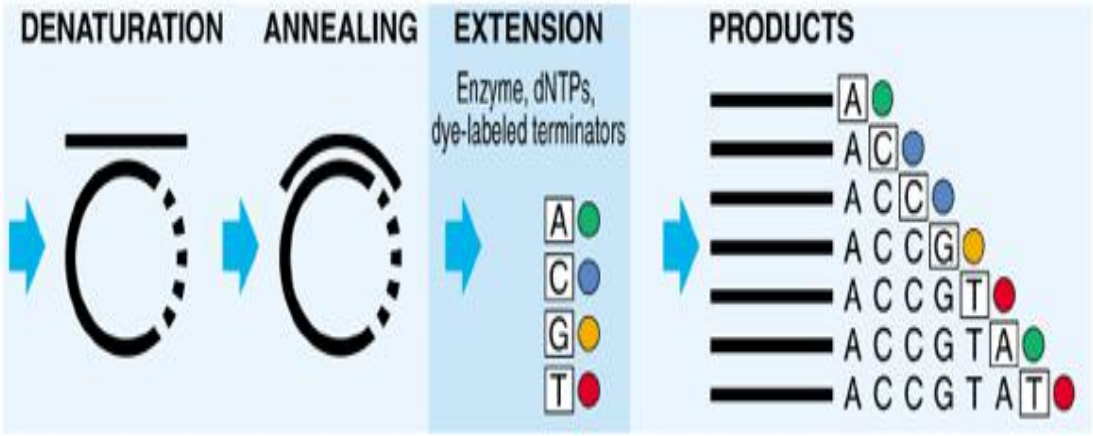


Figure 3: One Cycle of Dye Terminator Cycle Sequencing

Table:- Applied Biosystems genetic analyzers

Instrument	Number of capillaries	Capillary array length†‡ (cm)	Sample capacity 禮
3730xl DNA Analyzer	96	36, 50	96- and 384-well plates
3730 DNA Analyzer	48	36, 50	96- and 384-well plates
3500xL Genetic Analyzer	24	36, 50	96- and 384-well plates
3500 Genetic Analyzer	8	36, 50	96- and 384-well plates
3130xl Genetic Analyzer	16	36, 50, 80	96- and 384-well plates

Available instruments

Thermo Fisher Scientific offers the following automated Applied Biosystems genetic analyzers.

3730 DNA Analyzer

The 48-capillary 3730 DNA Analyzer is the Gold Standard in medium-to-high throughput genetic analysis. 3730 DNA Analyzer is used for DNA fragment analysis applications such as microsatellites, AFLP, SNP analysis, **mutation detection** and traditional DNA sequencing. Get the highest quality data at a low cost per sample. This is upgradeable to 96 capillaries**.**

Result

The chromatogram images below are from our sequencing standard controls (pGEM) that are run after every array change.

Sequence at the beginning of chromatogram:

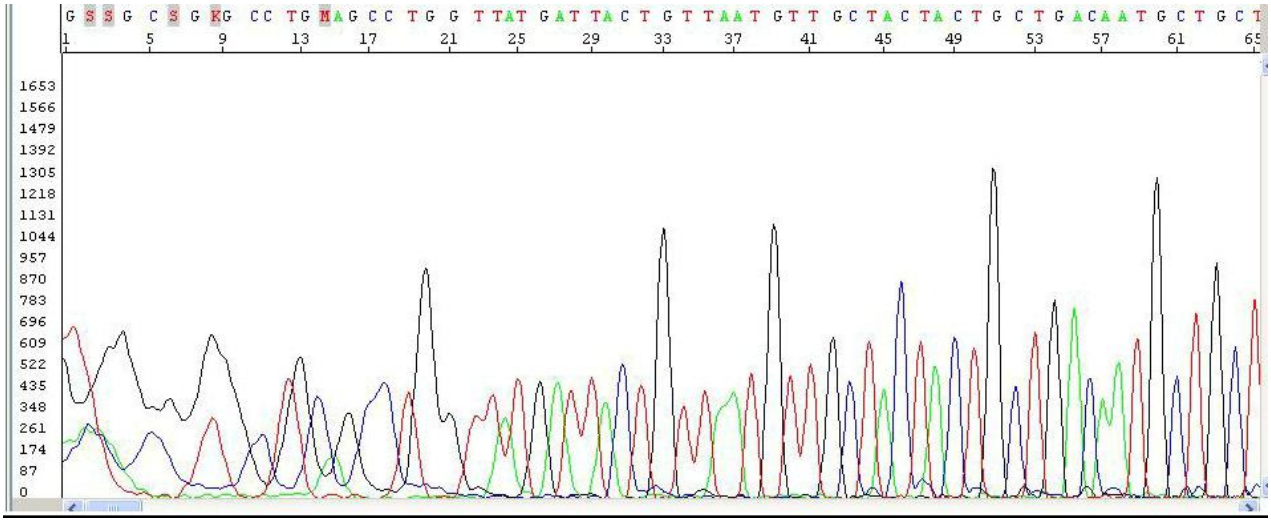
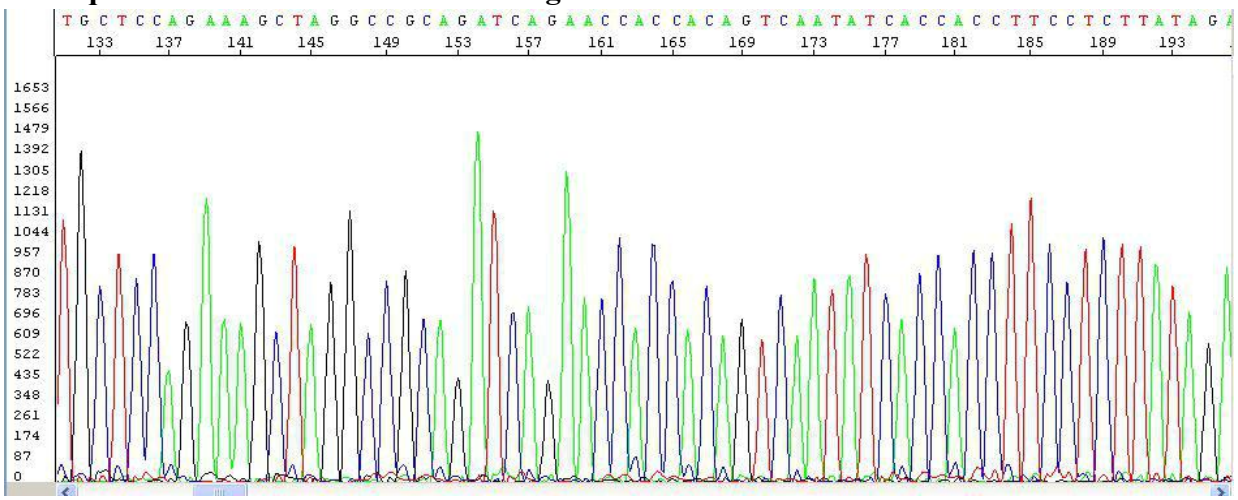


Fig:-Sanger Sequencing Data of the pGEM(Control DNA) by Capillary electrophoresis.

The first 25 bases or so give peaks that are round, crowded, and not resolved. Peak height is also smaller at the beginning (although sometimes very high due to primer dimers). Note that the 2 A's located at bp37 are not clearly resolved from each other. All of this is normal, but reinforces the suggestion of the BCL lab to place your primer at least 40-50 base pairs away from your sequence of interest. As you can see from the example above, even when using a high quality control plasmid, the early data is not very reliable and you will not be able to confidently read your sequence from base

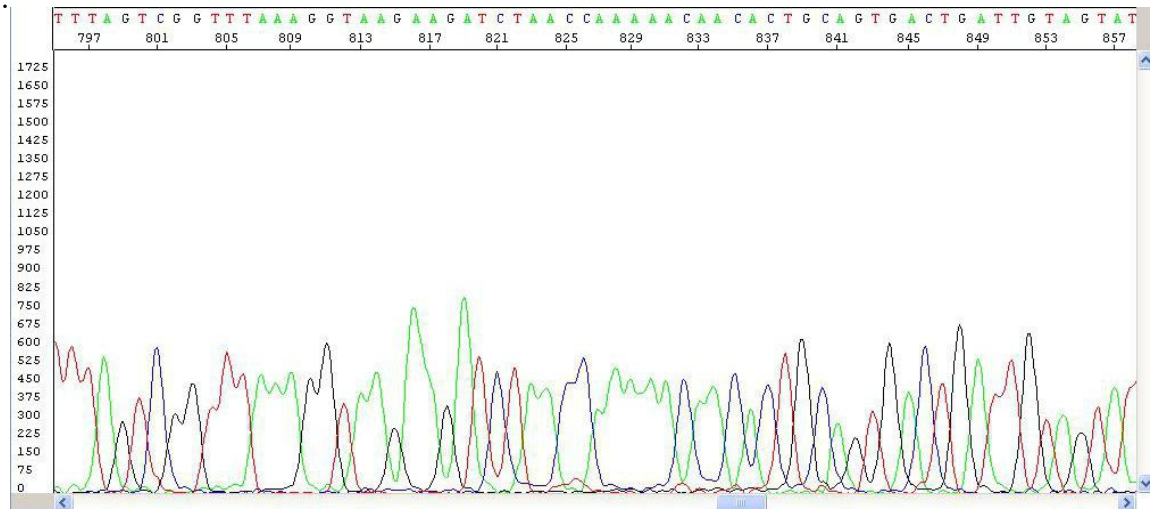
Sequence in the middle of chromatogram:



Peaks are sharp, well defined, with even spacing between them. Peak height is higher than the

earlier data with little or no background interference at the baselines. You will be able to confidently read this sequence manually with 100% accuracy.

Sequence towards the end of chromatogram:

1. 

This is where the sequence resolution begins to deteriorate. Peaks are broad and rounded in shape (they are also referred to as “rolling hills”), especially when there are multiples of the same nucleotide in a row. This region will still be called with 100% accuracy by the 3130 base-calling algorithm (KB base caller), although it may be difficult to manually interpret any heterozygote calls, especially in a sequence where high background is present.

Data analysis

Process overview: Data analysis software processes the raw data in the AB1 file using algorithms and applies the following analysis settings to the results:

1.Multicomponent analysis
2.Base calling
3.Mobility shift correction
4.Quality value determination (QV)

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