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Research Article

Qualitative and Quantitative Detection of Runt-Related Transcription Factor 1 and Eight-Twenty-One Oncoprotein Gene Fusion Products using Thermal Fingerprint and Cycle Threshold Value by Employing a DNA Intercalating Dye and Fluorescent PCR for Detecting Acute Myeloid Leukaemia (AML)

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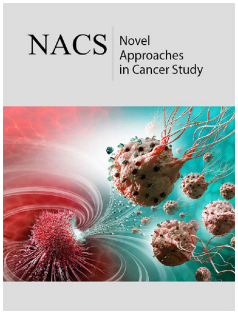

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Abstract

An intercalating dye-based qualitative and quantitative real time PCR assay was developed to detect and estimate the AML1/ETO fusion transcript in patients suffering from Acute myeloid leukaemia. Quantitation standard was prepared by molecular cloning of the fusion transcript in a multicopy cloning vector followed by its propagation in Escherichia coli. Nucleotide sequencing confirmed the AML1/ETO fusion point within the cloned insert and further, its serial dilution resulted in a predicted increase in cycle threshold value which was used to generate a standard curve. Secondary calibration of the standard with an CE-IVD approved, quantified control DNA allowed quantitative detection of AML1/ETO and ABL copy numbers of an AML1/ETO testing panel of samples and the percentage of the AML1/ETO was successfully ascertained. Qualitative detection data of the fusion transcripts from the panel indicated 100% concordance of data when compared with a published protocol that used hydrolysis probes (TaqMan) for detecting the AML1/ETO fusion transcript percentage in clinical samples. The study indicated the potential of using Evagreen as an intercalating dye for qualitative and quantitative detection of AML1/ETO and ABL transcripts for estimating Minimal Residual Disease (MRD) in AML patients.

Keywords: Evagreen; AML1/ETO; RT-PCR; Quantitation; Cloning

Abbreviations: E-RT-PCR: Evagreen Real Time PCR; CT: Cycle Threshold

Introduction

In hematopoietic and lymphoid tumours, chromosomal translocation events are a common phenomenon [1] Such genome rearrangements provide specific advantages in the growth of cells and also generate congenial cellular environments for birth of subsequent mutations in stem or progenitor cells that may finally lead to the development of malignant tumours. In chromosomal translocation, the genetic shuffling alters the original location of the proto-oncogenes that is one of the primary reasons for onset of an oncogenic event. Specifically, this has been found to occur in two defined ways [2&3]. The first and most important one is the formation of novel fusion proteins that have oncogenic potential. One of the best example of this category is the well-known translocation event between the chromosome 9 and 22 [t (9;22)], also known as the Philadelphia (Ph) chromosome that is seen in Chronic Myeloid Leukaemia (CML), which is born due to translocation of the proto-oncogene ABL located at chromosome 9 to the BCR gene present on chromosome 22. The resultant new (onco) protein

is associated with tumorigenesis of CML and acute lymphoblastic leukaemia (ALL) [4].

The other way is when protooncogenes are positioned close to new cis-regulatory elements. Juxtaposition of the c-MYC in Burkitt lymphoma disease, caused as a result of a t (8;14) translocation, onto the Immunoglobulin Heavy chain (IGH). Regulatory elements are a classic example of this type [5]. Such an event of chromosomal re shuffling that balance regions on two different chromosomes such as the one expressed as t (8;21) (q22; q22), occurs in the human population due to fusion of the AML1 (CBFa) gene present on human chromosome 21 and the ETO (MTG8, CDR8) gene residing on chromosome number 8 [6]. The chimeric fusion gene thus formed gets transcribed from the derivative chromosome number 8 [7&8]. This is an outcome of events occurring at chromosomal breakpoint cluster resident within intron 5 of the AML1 gene and that within exon 2 of the ETO gene that orchestrates together to give birth to an in-frame fusion transcript [9&10]. However, it may be mentioned that additional out-of-frame splicing variants too are reported for AML disease in isolated cases [11&12].

The event of t (8;21) translocation has an incidence rate of around 7-12% of all AML cases but the observation is primarily in AML-M2 [13-15] as per the French American-British (FAB) classification of AML [16]. AML patients harbouring a 't (8;21)' have a favourable prognosis and the patient acquires complete remission (CR) in almost 98% of the cases. Further, the overall survival rate at 5 years for all AML patients is around 69% while it is 83% at 3 years in cases of children [13]. Studies suggest that although the AML1/ETO gene fusion is related to a favourable prognosis, qualitative RT-PCR does indicate the presence of the AML1/ETO fusion transcript even when remission is in place following chemotherapy and autologous or allogenic transplantation of the bone marrow [17-19]. Converse to this, there exist reports also where remission is perfectly correlated with absence of the fusion transcript as is evident from a negative RT-PCR report in CR patients [20-22]. One such study reported good prognosis with absence of the fusion transcript in AML patients [23]. Another report provided encouraging results that supported quantitative PCR assay for AMl1/ETO using competitive RT-PCR technique for monitoring minimal residual disease (MRD) [24-26]. Another study described a statistically valid correlation between the quantity of the AML1/ETO transcript and remission as well as enhanced risk of relapse in AML patients [25].

However, competitive RT PCR is a labour-intensive technique that requires significant time to complete and has limited dynamic range for quantification. These properties diminish its candidature as a potential technique for routine diagnosis to monitor AML1/ETO fusion transcript levels. This problem has been effectively solved by the introduction of the reverse transcription real time PCR or the RQ-RT-PCR. This method is fast, reliable, accurate and has excellent reproducibility. These features make this a technique of choice for AML studies related to MRD. In fact, comparative study between competitive RT PCR and RQ-RT-PCR has shown a high degree of correlation [27]. The specialty of RT PCR is its unique ability to monitor the progress of a reaction as it happens. This is achieved by specific types of chemistries that are key to the PCR reaction occurring inside the machine. These chemistries primarily comprise of either DNA binding intercalating dyes such as SYBR Green 1 or hydrolysis & hybridization probes including molecular beacons, peptide nucleic acid light-up, sunrise and scorpion probes [28-31]. Dual labelled TaqMan probes are one of the most popular tools for sensitive detection of targets in a real time PCR. Studies have shown parallel detection of different fusion genes numbering as many as 45 and associated with leukaemia that can be detected using these dual-colour fluorescence probes on a real-time PCR platform [32].

However, some of the major disadvantages of TaqMan probe based real time PCR tests are the high cost of synthesis and the need for a unique probe for each specific target. On the other hand, intercalating dyes such as SYBR Green 1 operate by binding with the minor groove of the double stranded DNA thereby emitting fluorescence which is 1000-fold higher than that during unbound conditions [33]. With exponential synthesis of double stranded DNA during PCR, proportionate quantities of the dye molecules get intercalated thereby resulting in increasing quantum of fluorescence. One of the advantages of the intercalating dye chemistry is the cost benefit and its ease of use. It does not require single or dual labelled probes and can be adopted at ease with ordinary desalted oligonucleotide primers if the intercalating dye is available. However, specificity remains a concern when intercalating dyes are used since it can bind to any amplified product, specific or otherwise, that are generated during the process of PCR. In this study we demonstrate the development of an EvaGreen dye based real time PCR assay for the detection and absolute as well as relative quantification [34] of AML1/ETO fusion product using ABL as the control gene, by analyzing cycle threshold (Ct) value and the thermal melt curve that is generated at the end of the PCR protocol. Our study indicates that adequate optimization can make DNA intercalating dye such as Evagreen as useful as dual labelled probes for supporting qualitative and quantitative RT-PCR for monitoring MRD in AML patients.

Material and Methods

Patients

Peripheral Blood (PB) samples of 15 patients with suspected de novo AML and stored at the sample depository section of SN GeneLab (a molecular diagnostic laboratory located at Surat, India) were used in this study.

Extraction of total RNA from peripheral blood (PB)

Mononuclear cells were isolated from PB samples and used to extract total RNA employing a spin column-based human blood RNA extraction kit (Wobble Base Bioresearch, India). RNA was eluted in 50µL of the elution buffer and 7µL was used for generating cDNA.

cDNA synthesis and PCR

cDNA was synthesized from extracted human peripheral blood RNA using Prime Script 1st strand cDNA Synthesis Kit (Takara

Bio, Japan) according to manufacturer's instructions and 7µL of synthesized cDNA solution was used for subsequent PCR using gene specific primers (GSPs).

Designing the GSPs

GSPs specific for the AML1 gene portion spanned from position 634 to 654 of the AML1 gene (Sequence ID: NM_001754.5). The sequence of this primer was identical to the top strand of the reference gene sequence. For the ETO gene, the primer sequence corresponded to position 404 to 424 of the ETO gene with GenBank accession number NM_175635.3. The nucleotide sequence of this primer was identical to the bottom strand of the reference sequence.

Control gene

In order to compensate for variations of RNA integrity and trace variables in the reverse transcription step, the AML1/ETO fusion transcript was normalized to a control gene. For this, the human ABL gene was chosen after referring to the reports of the 'Europe Against Cancer (EAC) Program' [35].

Evagreen-RT-PCR (E-RT-PCR) and dissociation curve analysis

For amplification of the AML1/ETO fusion transcript gene segment by E-RT-PCR, the reaction condition comprised of 2µL of synthesized cDNA, 1µL of each forward (AML1) and reverse (ETO) primers (7.5pMoles each), 12.5µL of 2X Evagreen Master Mix (Wobble Base Bioresearch, India) topped up with sterile double distilled water to a final reaction volume of 25µL. The thermal cycling conditions were as follows: 940C-5 mins hold followed by 35 cycles, each comprising 940 °C -45 seconds, 67 °C - 30 seconds and 720 °C -30 seconds. This was followed by the melt curve analysis [36-38]. For amplification of the control gene (ABL) all parameters were identical as mentioned above except that the primer concentrations were 10 pMoles each and the annealing temperature was 570 °C.

Gold standard

For the gold standard assay, the method, as described by Fujimaki et al. [38], for qualitative detection of AML1/ETO fusion transcript was followed. Briefly, cDNA synthesized from patient RNA was used as a template and both AML1-ETO fusion transcript and the ABL gene segments were amplified from the same cDNA. For real time PCR, universal primers and (hydrolysis) probes for AML1-ETO with ABL as the control gene were used as described by the authors.

Real Time PCR was performed in 25µL reaction volume which comprised of Premix Ex Taq Master Mix (Takara, Japan), 300nM of each primer, 200nM of ABL or 100nM of AML1-ETO probe (Applied Biosystems, Weiterstadt, Germany), and 1µl of cDNA. Rhodamine derivative ROX was used as a passive reference. The cycling conditions were 2 minutes at 50 °C (UNG treatment), 30 seconds at 95 °C (UNG inactivation & activation of Taq Polymerase) followed by 40 cycles, each comprising of 4 seconds at 95 °C (denaturation) and 32 seconds at 60 °C (annealing and extension). All experiments were performed on a Rotorgene Q PCR machine (Qiagen, Germany).

Molecular cloning of gene target

GSPs were used to amplify the AML1/ETO fusion gene transcript using cDNA as a template. The amplicon generated was directly ligated to a commercial T-tailed vector (pGEM T Easy (pGEM®-T Easy Vector Systems; Promega Corporation, USA) and the ligated mixture used to transform a chemically competent JM109 Escherichia coli (E.coli) cells. Transformed cells were selected using S-Gal-appended media containing ferric ammonium citrate [36]. Transformed cells harbouring the cloned DNA fragment was confirmed by colony PCR, followed by DNA sequencing of the insert. For preparing standards, pure plasmids were extracted using a silica spin column-based plasmid DNA extraction kit (Wobble Base Bioresearch, India). A similar verified recombinant clone for ABL (cloned in pGEM T Easy vector) was already available in the laboratory and was used as the control target.

Secondary calibration and generating quantitation controls

Secondary calibration of the recombinant plasmid DNAs, namely AML1/ETO and ABL were done using the ABL and RUNX1-RUNX1T1 Fusion Gene Standards available with ipsogen RUNX1-RUNX& ABL 1T1 Kit (Cat. No. / ID: 675013), Qiagen, Germany. Briefly, the recombinant plasmid DNA (AML1/ETO) were linearized using Not1 and purified using a silica spin column-based DNA purification kit (Wobble Base Bioresearch, India). The linearized DNA was estimated by recording optical density at 260nm using a spectrophotometer (Labman Scientific Instruments, India). The purified linear plasmids were then run against the ipsogen standards and calibrated followed by serial dilutions to generate 105, 104, 103, 102 and 101 copies/µL for the AML1/ETO and ABL plasmid DNAs, respectively, for use as quantitation standard in E-RT-PCR.

Determining the thermal DNA fingerprint of the AML1/ETO and ABL gene fragments

The thermal DNA fingerprint of the target amplicons for AML1/ETO fusion gene and the ABL gene was determined by dissociation curve analysis on a Rotor-Gene real time PCR platform (Qiagen, Germany). For this, the target was amplified using GSPs and subjected to melt curve analysis using the default parameters available in the Rotor-Gene Q software. Melt peaks were detected and their temperature signature identified from the X axis of the graph and recorded.

Optimization of E-RT-PCR

The E-RT-PCR was optimized by titrating the primer and MgCl2 concentrations such that a No Template Control (NTC) consistently generated nil Ct value when run across the full number of thermal cycles. The analytical sensitivity was determined by serially diluting purified AML1/ETO target fragments with wild type human genome and estimating the lowest dilution that could be detected by the protocol. For clinical samples from the test panel, positive results were reported as a ratio between copies of t (8;21) transcript and the normal control gene (ABL) detected.

Result

Molecular cloning of the AML1/ETO fusion product

The GSP pair amplified a 395 bp amplicon (Figure 1A) when cDNA, synthesized from blood RNA of a patient positive for AML1/ETO fusion, was used as a template. Identical amplicon was generated when the same GSP was used for amplification using the AML1/ETO recombinant plasmid DNA as template (Figure 1B).

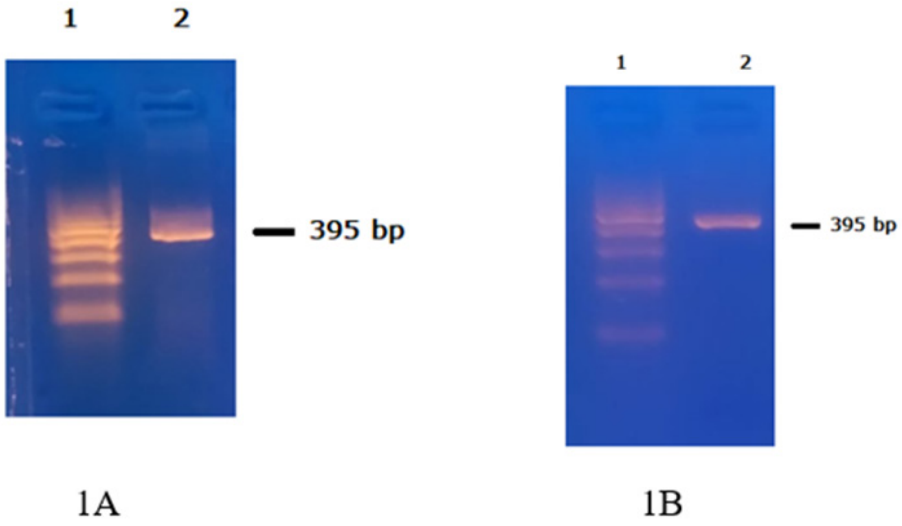


Figure 1:

A. AML1/ETO fusion transcript amplicon (395 bp) using cDNA from AML1/ETO positive RNA as template.

B. PCR amplicon generated using a positive recombining clone for AML1/ETO as template. This amplicon was used for confirmation of identity by nucleotide sequencing.

Fluorescent DNA sequencing of the AML1/ETO cloned insert

Fluorescent DNA sequencing of the AML1/ETO cloned insert indicated the fusion point of the AML1 and the ETO genes with a distinct breakpoint in the middle of the cloned DNA fragment (Figure 2). A Basic local alignment search tool [37] analysis indicated successful alignment of the sequence with the nucleotide sequence of AML1/ETO fusion protein mRNA submitted to GenBank by another author and with accession number OK063977.1 (Figure 3).

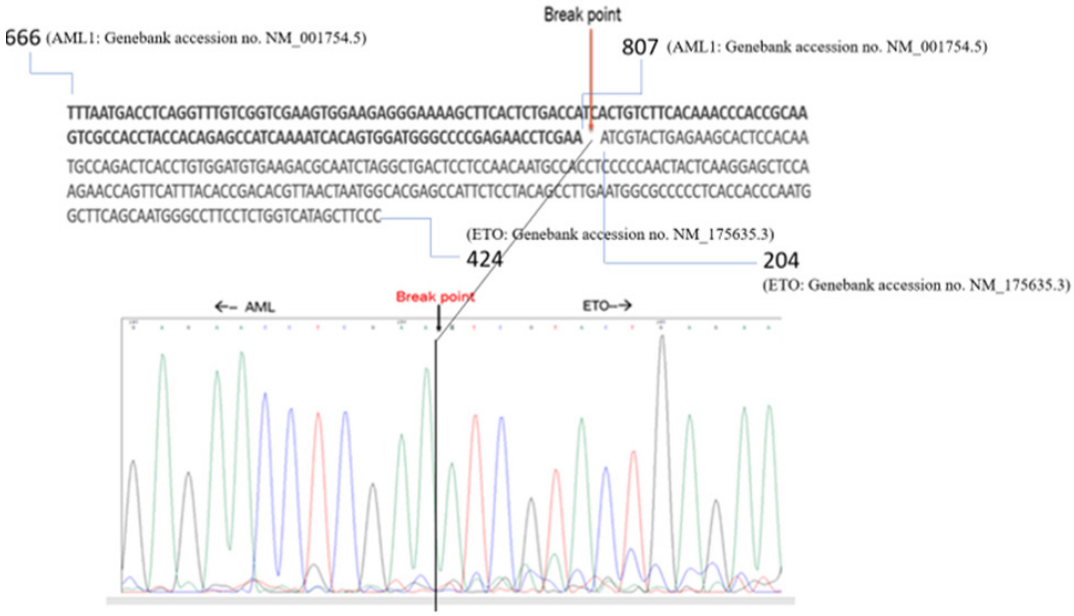


Figure 2: Nucleotide sequencing of the AML1/ETO fusion transcript insert cloned in a multicopy E. coli cloning vector. The fusion point between the AML1 and the ETO gene is indicated. The nucleotide sequence section shows the position of the start and end bases of AML1 and the ETO gene portion in the corresponding GenBank accession number.

Homo sapiens AML1-ETO fusion protein mRNA, partial cds

Sequence ID: OK063977.1 Length: 276 Number of Matches: 1

Range 1: 1 to 276 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
503 bits(272)	9e-140	275/276(99%)	1/276(0%)	Plus/Plus

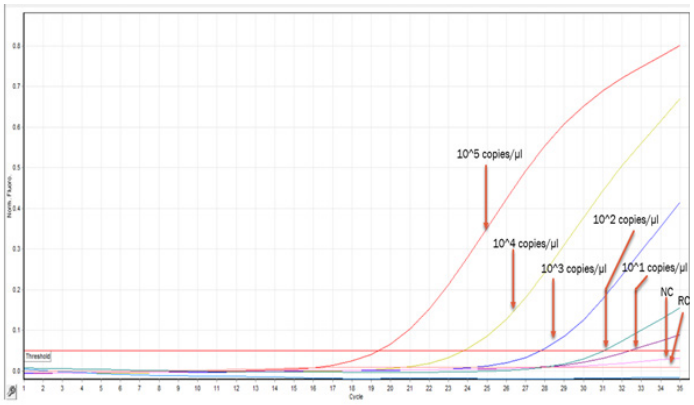
```
Query  41   AAAGCTTCACTCTGACCATCACTGTCTTCACAAACCCACCGCAAGTCGCCACCTACCACA  100
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    AAAGCTTCACTCTGACCATCACTGTCTTCACAAACCCACCGCAAGTCGCCACCTACCACA  60

Query  101  GAGCCATCAAAATCACAGTGGATGGGCCCCGAGAACCTCGAAATCGTACTGAGAAGCACT  160
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GAGCCATCAAAATCACAGTGGATGGGCCCCGAGAACCTCGAAATCGTACTGAGAAGCACT  120

Query  161  CCACAATGCCAGACTCACCTGTGGATGTGAAGACGCAATCTAGGCTGACTCCTCCAACAA  220
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CCACAATGCCAGACTCACCTGTGGATGTGAAGACGCAATCTAGGCTGACTCCTCCAACAA  180

Query  221  TGCCACCTCCCCCAACTACTCAAGGAGCTCCAAGAACCAGTTCATTTACACCGAC-ACGT  279
            |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  181  TGCCACCTCCCCCAACTACTCAAGGAGCTCCAAGAACCAGTTCATTTACACCGACAACGT  240

Query  280  TAACTAATGGCACGAGCCATTCTCCTACAGCCTTGA  315
            ||||||||||||||||||||||||||||||||||||
Sbjct  241  TAACTAATGGCACGAGCCATTCTCCTACAGCCTTGA  276
```

Figure 3: Nucleotides sequence alignment between the AML1/ETO fusion transcripts amplicon and a matching sequence available in NCBI GenBank (Accession no. OK063977.1) indicating specificity of the cloned AML1/ETO insert used as control in this study.

Generating quantitation standards

Secondary calibrated and serially diluted linearized plasmid DNAs of AML1/ETO & ABL indicated a dynamic range of detection ranging from 105 copies to 101 copies of target/µL (Figure 4A). Corresponding melt curves for AML1/ETO control indicated a thermal fingerprint of 83.5-84.50C (Figure 4B). The shifting of Ct value from low to high corresponded with comparable reduction in peak height of the dissociation curve (Figure 4A & 4B). Similar data was observed for the control ABL gene except that the signature thermal melt point ranged between 82.5-83.50C (Figure 5A & 5B).

(A)



(B)

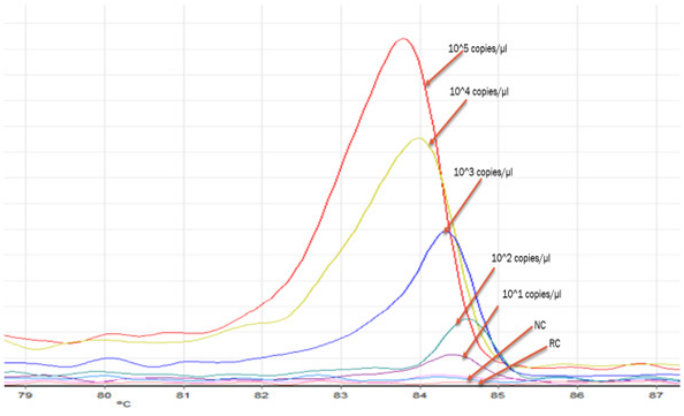


Figure 4:

A. E-RT-PCR data of serially diluted AML1/ETO fusion transcript target showing gradual shifting of the Ct value to the right with increasing value. The negative control (NC) and the reagent control (RC) graphs are below the threshold and hence generated nil Ct value.

B. Reducing height of the dissociation curve peak corresponding to each dilution run shown in figure 4A. The Specific thermal melt signature was seen in all dilutions. Please see the result section for details.

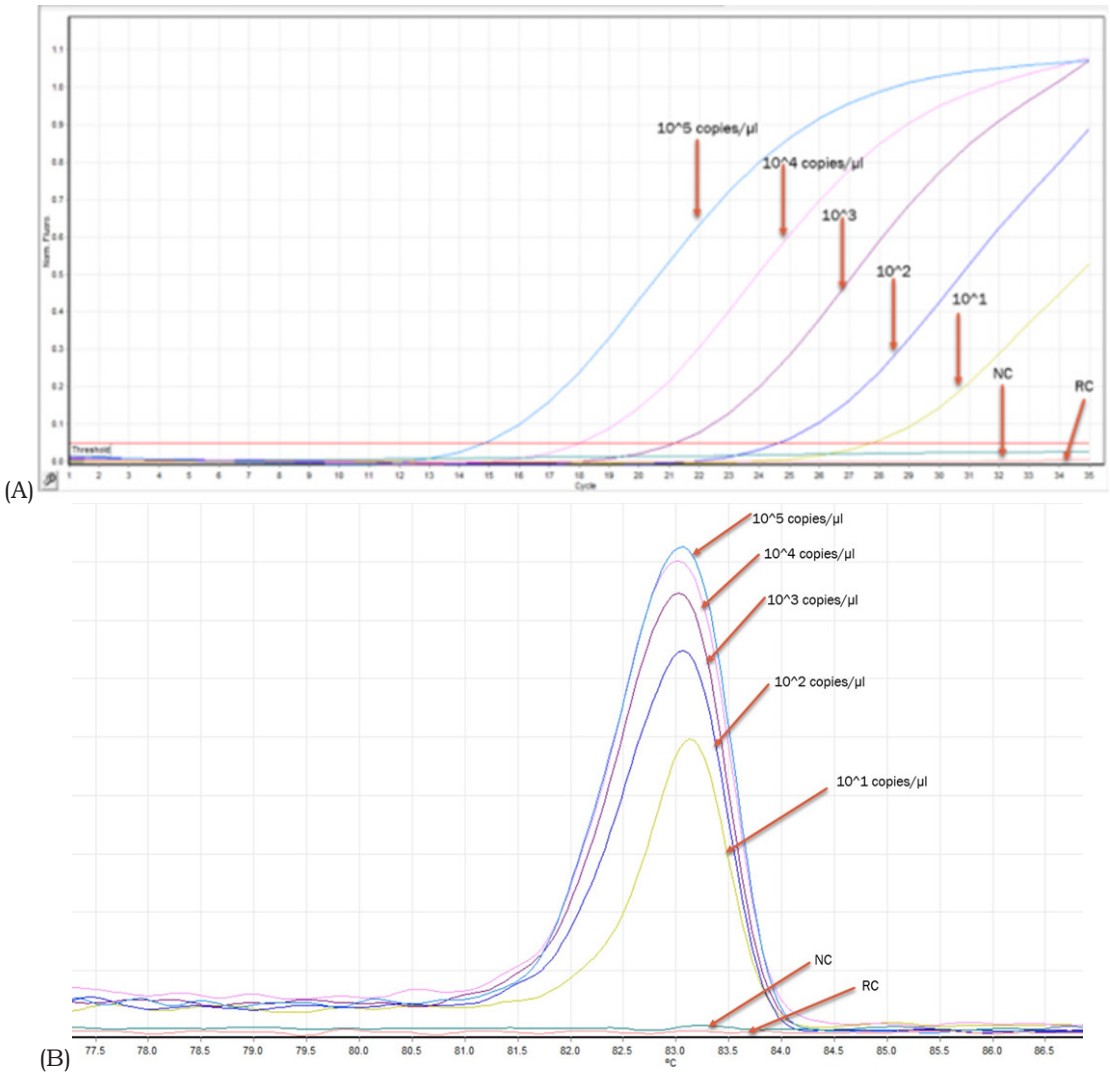


Figure 5:

A. E-RT-PCR data of serially diluted ABL transcript target showing gradual shifting of the Ct value to the right with increasing value. The negative control (NC) and the reagent control (RC) graphs are below the threshold and hence generated nil Ct value.

B. Reducing height of the dissociation curve peak corresponding to each dilution run shown in figure 4A. The thermal melt signature of each dilution peak is detected. Please see the result section for details.

Resource population used in this study

The resource population used in this study and data generated from them by RT-PCR are summarized in Table 1. The data generated from E-RT-PCR was analyzed by two different methods. First, the qualitative data generated was compared with a Taqman probe-based test following a published protocol [38] and the results were compared for concordance. Second, after secondary calibration of the AML1/ETO and ABL controls (this study) with ipsogen-quantified controls, the AML1/ETO and ABL transcript ratio was estimated for all the positive samples available in the test panel.

Table 1: Real time PCR data generated from a panel of 15 clinical samples included in this study.

Serial No.	Sample ID	Gold standard (Qualitative; Fujimaki et al. [38])	E-RT-PCR quantitative* results				TOTAL COPY NO.	% AML1/ETO detected
			AML1/ETO		ABL			
			CT	COPY NO.	CT	COPY NO.		
1	AE1	AML1/ETO fusion Positive	13.1	3000263	18.7	1572297	4572560	65.61
2	AE2	AML1/ETO fusion Positive	15.8	627715	18.6	1672401	2300116	27.29
3	AE5	AML1/ETO fusion Positive	19.6	100484	24.5	43541	144025	69.77
4	AE10	AML1/ETO fusion Positive	22.1	24266	21.4	287005	311271	7.8
5	AE8	AML1/ETO fusion Positive	32.6	860	18.3	1940110	1940970	0.04

6	AE12	AML1/ETO fusion Positive	30	1479	15.6	10652254	10653733	0.01
7	AE9	AML1/ETO fusion Positive	24.4	45908	12.8	59893149	59939057	0.08
8	AE11	AML1/ETO fusion Positive	21.3	304500	14.6	1245614	1550114	19.64
9	AE4	AML1/ETO fusion Positive	18.2	210888	20	73170	284058	74.24
10	AE14	AML1/ETO fusion Negative	0	0	15.1	1398325	1398325	0
11	AE15	AML1/ETO fusion Negative	0	0	12.3	8002620	8002620	0
12	AE6	AML1/ETO fusion Negative	0	0	19.2	85000	85000	0
13	AE7	AML1/ETO fusion Negative	0	0	25.1	40053	40053	0
14	AE13	AML1/ETO fusion Negative	0	0	15.6	10652254	10652254	0
15	AE3	AML1/ETO fusion Negative	0	0	12.8	59893149	59893149	0

Analytical sensitivity

The analytical sensitivity of the E-RT-PCR developed in this study was detection of 1 target in the background of 100,000 wild genome copies.

Discussion

From the clinical point of view, AML disease with t (8;21) translocation appears to be prevalent in early adulthood and is associated with a superior prognosis compared to most other categories of AMLs. An enhanced marrow granulopoiesis coupled with inhibition of erythropoiesis is a consistent pathological signature of this disease [39]. Overall survival rate (OS) associated with AML has been demonstrated to improve when dynamic monitoring of minimal residual disease is followed along with corresponding optimized treatment [40]. Failure of treatment resulting due to relapse of the disease in AML continues to remain a serious challenge to the onco-clinicians [41]. This indicates the need for a sensitive method of monitoring MRD in AML for proper treatment and prevention of relapse cases.

As of now, the primary techniques for determining MRD in AML include Multiparameter flow Cytometry (MFC) and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) [42]. It has been shown that detection of high MRD level on a MFC platform broadly correlated with an inferior prognosis [43] but this technology is not fully standardized yet, because of constraints associated with low sensitivity and antigenic changes following treatment [44]. Compared to this, RT-qPCR has been demonstrated to be superior with enhanced sensitivity for adequate monitoring of MRD in AML patients which can also be used to predict the risk of relapse [45&46]. Two methods of real time PCR analysis are well known in the domain of a gene expression study. These are the SYBR Green (intercalating dye) and the TaqMan (hydrolysis probes) chemistries. The TaqMan method is expensive due to the high cost of synthesis of the dual labelled probes and that of the commercial PCR master mixes that are most often required for TaqMan probe-based PCR. Further, the TaqMan probe-based PCR assays are less adaptable to new assays since every target requires a sequence-specific TaqMan probe to be synthesized. However, one of the significant advantages of TaqMan probe-based PCR is its high specificity that results in near-nil false positive and negative calls.

On the other hand, intercalating dye-based PCR is known to have comparable and similar precision as considered to TaqMan probe-based PCR [30] but its specificity remains a concern because the intercalating dye binds to any double stranded DNA generated during the course of amplification in contrast to TaqMan probes that are designed only to measure sequence-specific amplification. In this study we demonstrated that carefully optimized intercalating dye based real time PCR tests for detecting and estimating AML1/ETO fusion transcripts can be incorporated in routine diagnostics. It can add convenience and economy to the test and generate comparable data as compared to hydrolysis and similar probe-based assays. The GSPs successfully and repeatedly amplified the desired fusion region from cDNA synthesized from RNA which were obtained from AML1/ETO-positive patients. Following molecular cloning of this fragment in a multi copy E. coli vector and subsequent propagation in a laboratory strain of E. coli (JM109), the sequence from the rescued insert from the recombinant clone matched its pre-cloning counterpart (Figure 2). Further, an online alignment analysis revealed a perfect match between the nucleotide sequence obtained from the cloned insert with another which was submitted to GenBank by another author (Figure 3). Analysis of the electropherogram showed the exact point of fusion of AML1 and the ETO gene. This was independently confirmed through sequence-alignment analysis of the left (AML1) and the right (ABL) part of the fusion gene sequence with AML1 and ETO gene sequences available at the GenBank.

Fujimaki et al. [38] reported an efficient methodology for qualitative detection of AML1/ETO fusion transcript using TaqMan chemistry. This protocol can be used for estimating MRD in AML1/ETO positive patients. This protocol has been followed by other authors also working in this domain [47]. In this study we used this TaqMan probe-based protocol as the gold standard for qualitative detection of AML1/ETO fusion transcript and determination of MRD. Out of the 15 samples tested in this study, there was cent per cent concordance of data generated between the [38] qualitative detection protocol and that used in this study for E-RT-PCR for the detection of AML1/ETO fusion transcripts (Table 1). In a study by [48], it was observed that there was no trend where higher rates of relapse of AML patients were associated with higher levels of AML1/ETO fusion transcripts. However, with reducing levels of AML1/ETO fusion transcripts in patients, there was a reducing probability of relapse. Importantly, once complete remission was achieved, an enhanced AML1/ETO at any given point of time was associated with enhanced probability of relapse. The study effectively demonstrated that both qualitative as well as quantitative estimation of AML1/

ETO fusion transcripts have high prognostic value in monitoring of MRD and that a negative or continued low level of AML1/ETO expression was related to increased disease-free survival.

Table1 summarizes the comparative data on qualitative detection of AML1/ETO fusion transcript generated from E-RT-PCR (this study) and a Taqman protocol-based qualitative detection of AML1/ETO fusion transcript in a panel of 15 clinical samples. There was 100% concordance of data between the two sets of measurements. The cloned AML1/ETO and ABL gene segments in this study were secondary calibrated with a CE and IVD approved control DNA (Ipsogen, Qiagen, Germany) and used to generate quantitative data on AML1/ETO fusion transcript in samples investigated in this study (Table 1). Serial dilution of the controls (developed in this study) indicated gradual shifting of Ct to a higher value (corresponding to reducing concentration of analyte). This is reflected in the corresponding reduction of peak height of the dissociation curves associated with each dilution for both AML1/ETO and ABL standards, respectively (Figure 4B & 5B). While the melt peak height did not correlate with concentration of the analyte in a linear fashion, the Ct values maintained a linear relation with concentration of the analyte (control DNA) over a wide range (Figure 4A & 5A).

Intercalating dye-based real time PCR has been shown to face challenges in addressing adequate target specificity during amplification [49]. This is primarily because the detector dye can generate signal from any double stranded DNA without sequence-specific discrimination as opposed to hydrolysis probe-based assays where signal is generated after discrete sequence-based discrimination. However, the feature of dissociation curve analysis in intercalating dye-based real time PCR offers the opportunity to generate unique 'thermal' DNA fingerprint for each specific target amplicon that can be used to assess the specificity of the amplicon generated and detected by the intercalating dye. In this study this thermal melt point temperature of the specific amplicon was found to be 82-83 °C for ABL and 83.5 °C - 84.5 °C for AML1/ETO fusion transcript, respectively. This thermal signature allowed confirmation of the specificity of the PCR product generated and detected in a run. The detection of 1479 copies of AML1/ETO fusion transcript against the background of 10652254 copies of ABL transcript in sample no. AE12 is encouraging. This corresponded to an AML1/ETO % of 0.01 which can be considered sensitive as compared to traditional methods of detection such as FISH [50].

Appropriate optimization of the E-RT-PCR indicated no detectable fluorescence when a negative control or a no template control was used (Figure 4 & 5). This indicated that no detectable double stranded DNA products were formed in the E-RT-PCR when a specific target was absent in the reaction tube. It is important to note that while the level of fluorescence against a given quantum of analyte vary when a hydrolysis probe, such as a TaqMan probe, is used compared to that of an intercalating dye, such as Evagreen or SYBR Green, a secondary calibration can bring both the sensitivity quotients under the measurement of a single scale. In such a case, both the detection chemistries can generate comparable data provided the slope, r2 value and efficiency of the reaction remains within acceptable limits [51].

In conclusion, this study demonstrated adaptability of Evagreen as an intercalating dye in real time PCR for qualitative detection of AML1/ETO fusion transcript against the background of ABL transcripts as the control. The assay has the potential to be used in routine diagnostics and can offer economical and quality MRD solutions for patients suffering from AML disease.

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