A Real-Time Quantitative PCR Assay for Quantification of c-Myc DNA in Patients who Suffers from Leukemia

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Abstract: The MYC cancer gene contains instructions for the production of the c-Myc protein. The c-Myc protein is known as a transcription factor or a regulator of other genes. It is a protein that binds DNA at specific sites and instructs genes whether or not they should be transcribed into messages for cells to make additional or other new proteins. Quantitative real-time PCR (qRT-PCR) addresses the evident requirement for quantitative data analysis in molecular medicine, biotechnology, microbiology, archaeometry and diagnostics and has become the method of choice for the quantification of cDNA and nDNA. Therefore, we used Polymerase chain reaction (PCR)-based assays can target either DNA (the genome) or cDNA, namely used for research both DNA. We optimized a method for monitoring quantitative real-time PCR (qRT-PCR) of c-Myc cancer gene in patients with leukemia. We describe qRT-PCR a series of protocols that illustrate the essential technical steps required to generate quantitative data that are reliable and reproducible. In addition, our aim is to also quantify extracted DNA and determine its purity and the validation of extracted DNA from patients with leukemia including active Myc gene family. We also believe these protocols will be accessible to the researchers to provide them reliable data in this protocol. These analytical methods are essential for accurate gene quantification. With reference to, advantages of qRT-PCR are a large dynamic range of quantification, no requirement for post-PCR sample handling and the need for very small amounts of starting material. The specificity, reproducibility and detection limit of the assay was examined. The assay was used to monitor c-myc DNA levels in patients with leukemia.

Keywords: qRT-PCR, cDNA, leukemia, c-myc gene.

INTRODUCTION

The c-Myc gene is located on human chromosome 8q24, consisting of three exons. Its transcription may be initiated at one of three promoters. Translation at the AUG start site in the second exon produces a major 439 amino acid, 64 kDa c-Myc protein. The c-Myc gene is mapped to this region of chromosome, and the gene family play an important role in the regulation of cellular proliferation and differentiation. Overexpression of the c-Myc oncoprotein is observed in a large number of hematopoietic malignancies have revealed a potent role for c-Myc in the generation of leukemias and lymphomas. However, the reason for high c-Myc protein levels in most cases is unknown. The fundamental importance of nucleic acid amplification methods, pharmacogenomics and molecular diagnostics [1] continues to direct efforts aimed at improving current methodologies as well as the development of novel technologies. Quantitative real-time PCR has become one of the most popular tools in molecular biology research. It allows precise quantitative and qualitative detection of nucleic acids in this study. To this end, quantitative real-time PCR is used for many different purposes, particularly for quantifying nucleic acids in this study. The major advantages of quantitative real-time PCR compared with other methods for the quantification of nucleic acids are the extremely wide dynamic range and the significantly higher reliability of the results compared with conventional PCR. We have studied quantitative real-time polymerase chain reaction assay based on SYBR Green methodology to quantify MYC gene of cDNA and nDNA levels in homogeneous total solutions prepared from tumor samples. This recently developed method of nucleic acid quantification in homogeneous solution has the potential to become a reference in terms of performance, accuracy, sensitivity, wide dynamic range, and high throughput capacity and eliminates the need for tedious post-PCR processing. Above all, this method is suited to the development of new target gene assays with a high level of inter-laboratory standardization and yields statistical confidence values [2]. We used this technique to measure and determined whether MYC gene overexpression correlated with DNA overrepresentation at the MYC locus, as determined previously using quantitative real-time PCR method [3]. The assay relies on measuring the increase in fluorescent signal, which is proportional to the amount of DNA produced during each PCR cycle. In the RT-qPCR applications, it is vital that study the detection and quantification of amplification products in real time. Although it has become the method of choice for the quantification of cDNA [4, 5], there are several concerns that have a direct impact on the reliability of the assay. Principally,

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these are that results depend on template quantity, quality and optimization of the assay. The reverse transcription reaction is not standardized, hence can be very variable [6, 7], and data analysis is highly subjective. In the case of carriying out inappropriately, it may lead confusions of the results obtained from the assay [8]. Consequently, it is essential to minimize variability and maximize reproducibility by quality assessing every component of the RT-qPCR assay and adhering to common guidelines for data analysis. Individual reactions are characterized by the PCR cycle at which fluorescence first rises above a defined or threshold background fluorescence, a parameter known as the threshold cycle (Ct) or crossing point (Cp). The more target there is in the starting material, the lower the Ct value. This correlation between fluorescence and amount of amplified product permits accurate quantification of target molecules over a wide dynamic range, while retaining the sensitivity and specificity of conventional end-point PCR assays. Furthermore, many of the applications of real-time qPCR include measuring mRNA expression levels, DNA copy number, transgene copy number and expression analysis, allelic discrimination, and measuring viral titers [9]. As it is understood, applications of RT-qPCR are numerous. They include DNA copy number measurements in genomic [9, 10, 11], transgene copy number [2], and allelic discrimination assays [12, 13], and confirmation of microarray data [14, 15]. Some of the most recent applications, which demonstrate the sensitivity of this technology when applied to expression analysis of limited samples, include expression analysis of specific splice variants of genes [16].

DNA Copy Number Measurements in Leukemia

DNA copy number measurements are important in determining the extent of genomic imbalance that underlies most malignancies. There are numerous techniques available for measuring DNA copy number in tumors; each method has specific advantages and disadvantages. Chromosomal CGH can detect imbalances across the entire genome, but at relatively low resolution. Fluorescent in situ hybridization (FISH) can provide copy number measurements in a cell-specific manner, but it is difficult to perform in high throughput and is difficult to count approximately 25 or more DNA copies. PCR has been used for determining allelic imbalance (or loss of heterozygosity) using polymorphic simple-sequence repeats; however if it is performed as an end-point PCR assay, quantitative conclusions can be misleading. RT-qPCR has been used in several studies [3, 9, 17, 18], in which allelic imbalance is determined. Quantifying the DNA copy number of specific genes or markers at many different loci enumerated regions of chromosomal imbalance. With reference to, advantages of RT-qPCR are a large dynamic range of quantification, no requirement for post-PCR sample handling and the need for very small amounts of starting material. The specificity, reproducibility and detection limit of the assay was examined. The assay was used to monitor c-myc DNA levels in patients with leukemia.

MATERIAL AND METHODS

Cases

Blood samples of patients with leukemia were derived from the archives of the Department of Hematology in Faculty of Medicine at the University of Selcuk, Turkey. Namely, specimens of 50 blood of patients with leukemia were used in this study. Age range was 6-24 years, 50% of patients were male and female. Control samples were obtained from natural or healthy blood samples of the same cases.

Molecular Materials

We have described a RT-qPCR reference assay, which we have named c-Myc primer pairs. It identifies inhibitors of the reverse transcription or PCR steps by recording the Cts characteristic of a defined number of copies of a sense-strand amplicon: an artificial amplicon (c-Myc) is amplified using two primers (c-Myc F) and (c-Myc R).

In this study, PCR primers for the c-Myc gene target were as follows:

c-Myc forward primer: 5'-TCAAGAGGTGCCAC GTCTCC-3' and c-Myc reverse primer: 5'-TCTTGGCA GCAGGATAGTCCTT-3'.

In this study, cDNA were used for SYBR green quantitative PCR (Quantifast SYBR green PCR kit; QIAGEN, Switzerland) and c-Myc primer pairs, for control λDNA (bacteriophage-ROCHE) to form standard curve. We used Eppendorf Mastercycler Realplex 2S equipment for Quantification of being isolated DNA (By Biorobot EZ1, QIAGEN) to determination of Myc gene activation in the individuals with leukemia patient. The cDNA was then diluted to 100 µl with water and stored at -20°C.

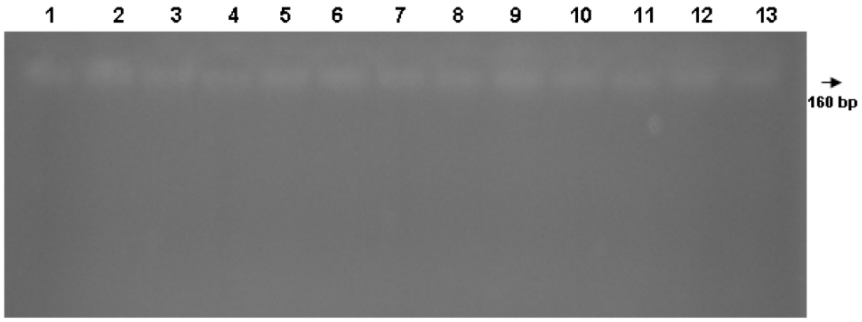

Figure 1: Genomic DNA was isolaled from blood samples of patients with leukemia respectively, Lane 1, 2, 3-13 with Bio Robot EZ1. DNA samples submitted to electrophoresis in 1% agarose gel.

DNA Quality

DNA quality and quantity encompasses both its purity (absence of protein and RNA contamination, absence of inhibitors) and its integrity. Traditionally DNA quality has been determined by analysis of the A260/A280 ratio and/or analysis of the cDNA bands on agarose gels. In other words, five microliter of each DNA was analyzed on a 1% agarose gel (TAE buffer), including a molecular weight marker or 1000 bp (Figure **1**) and then stained with ethidium bromide (0.5 µg/ml) for 30 min and then agarose gel washed in double-distilled and UV-irradiated H_2O. Analysis of DNA fragmentation was performed by ethidium-bromide stained agarose gel electrophoresis. The ethidium bromide luminescence from the CCD camera is integrated for 1-2 s into the computer memory directly from the gel on the UV Transilluminator using Gel Doc. 1000 system (Bio Rad). One of the most common methods for nucleic acid detection is the measurement of solution absorbance at 260 nm (A260) due to the fact that nucleic acids have an absorption maximum at this UV wavelength. Although a relatively simple and time-honored method, A260 suffers from low sensitivity and interference from nucleotides and single-stranded nucleic acids. Furthermore, compounds commonly used in the preparation of nucleic acids absorb at 260 nm leading to abnormally high quantitation levels. However, these interference and preparation compounds also absorb at 280 nm leading to the calculation of DNA purity by performing ratio absorbance measurements at A260/A280.

Polymerase Chain Reaction and Reverse Transcription

The RT-qPCR assay can be performed either as a one-tube single RT and PCR enzyme method or a separate RT and PCR enzyme technique using one or a two tubes. we have included protocols using oligo-dT and gene-specific primers in the RT section.

Quantitative Real-Time PCR (RT-qPCR)

Following the PCR, a melting curve analysis was performed to test the specificity of the amplification. The PCR reaction is performed by temperature cycling. Each cycle starts with an incubation at high temperature 95°C to separate the strands, the

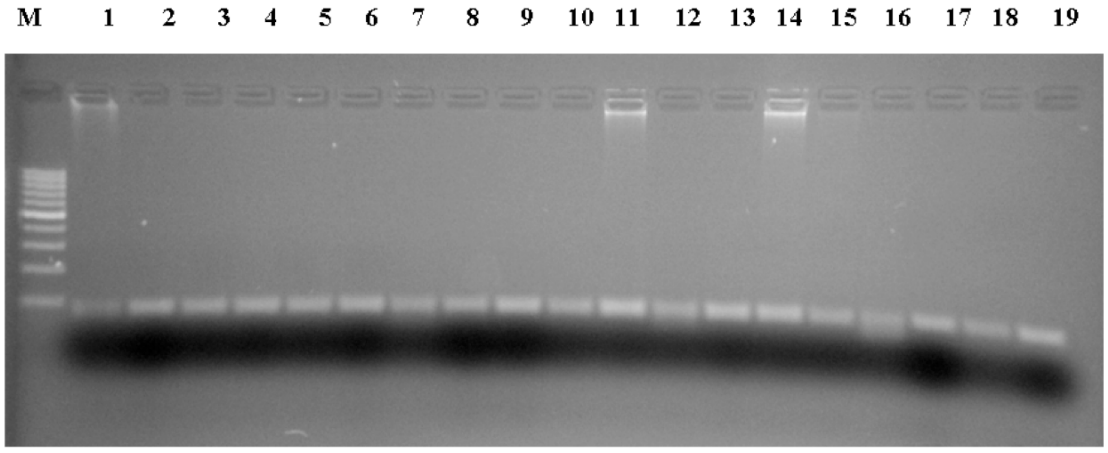

Figure 2: Representative results obtained using primer pairs c-myc gene (see materials and methods). The 282 bp c-Myc variant was detected in patients with leukemia.

Table 1: Run in RT-qPCR İnstrument Using the Following Three-Step Protocol

1 cycle	Activation	95 °C for 5 min
35 cycle	Denaturation	95 °C for 10 s
	Annealing+extension (Fast PCR)	60 °C for 20 s
1 cycle	Melting Curve	Between 60 °C and 95 °C

temperature is then lowered to allow the primers to anneal to the template, and then set at 60°C, at which temperature the primers are extended by dNTP incorporation, the product is formed. The generation of the product is determined by measuring the SYBR Green I fluorescence signal (Table **1** and Figures **3**, **4**, **5**). The increase in SYBR Green I fluorescence is directly proportional to the amount of double-stranded

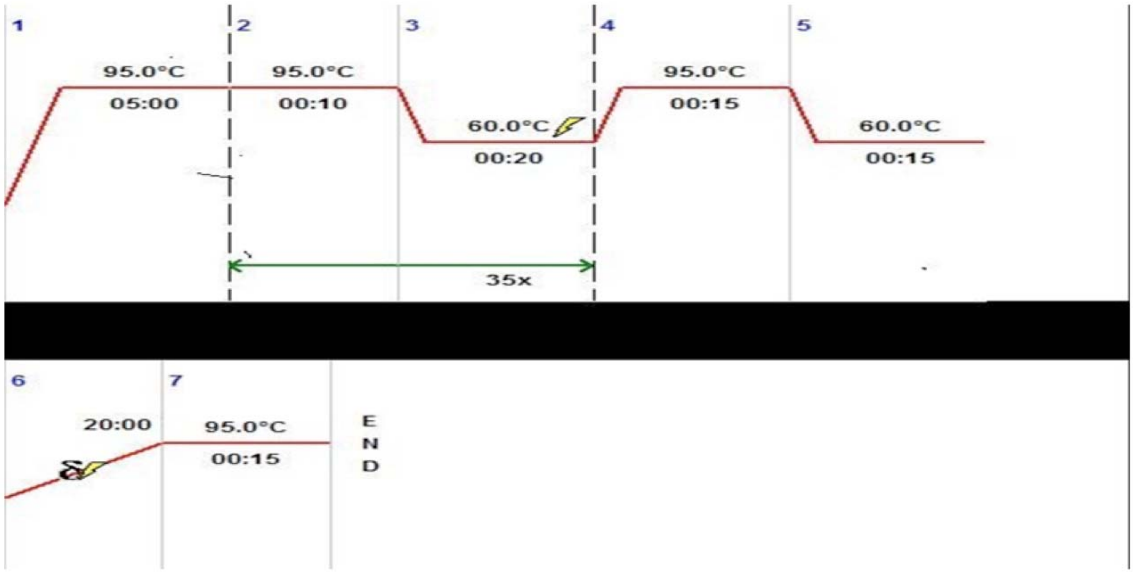

Figure 3: Optimization of qPCR reaction (from Table **1**).

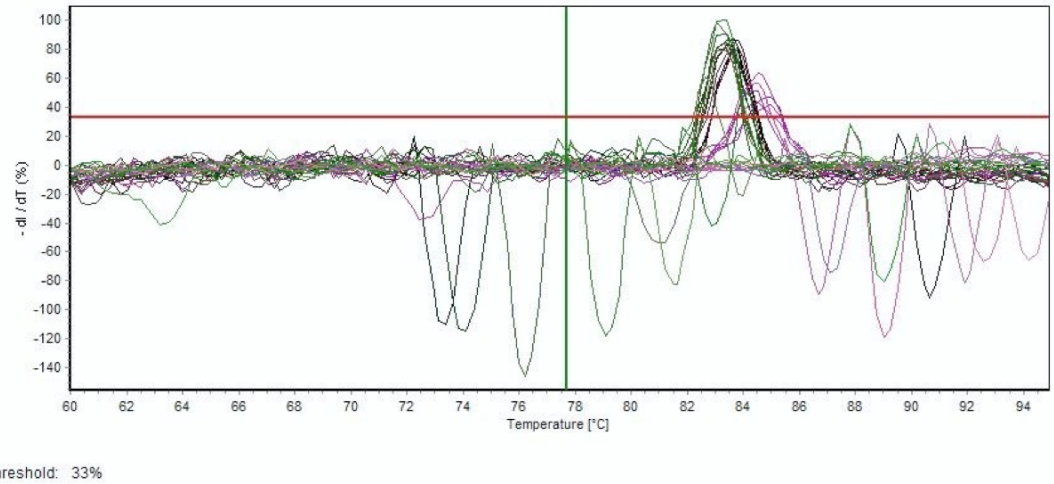

Figure 4: Melting Curve: Revealing the specific product at specific temperature called Tm. (from the figure Tm of the target is approximately 84.5 °C). SYBR Green can detect any double stranded DNA including primer dimers, contaminating DNA, and PCR product from misannealed primer. From the melting curve figure it is understood that the desired amplicon was detected. As it is seen from the curve there are 2 shoulders appeared. The first and the sharpest one belongs to λDNA which is amplified together with c-Myc in the same reaction with different primers and the second one belongs to c-Myc cDNA. We can conclude that there is no contamination and primer-dimer artifacts in this reaction.

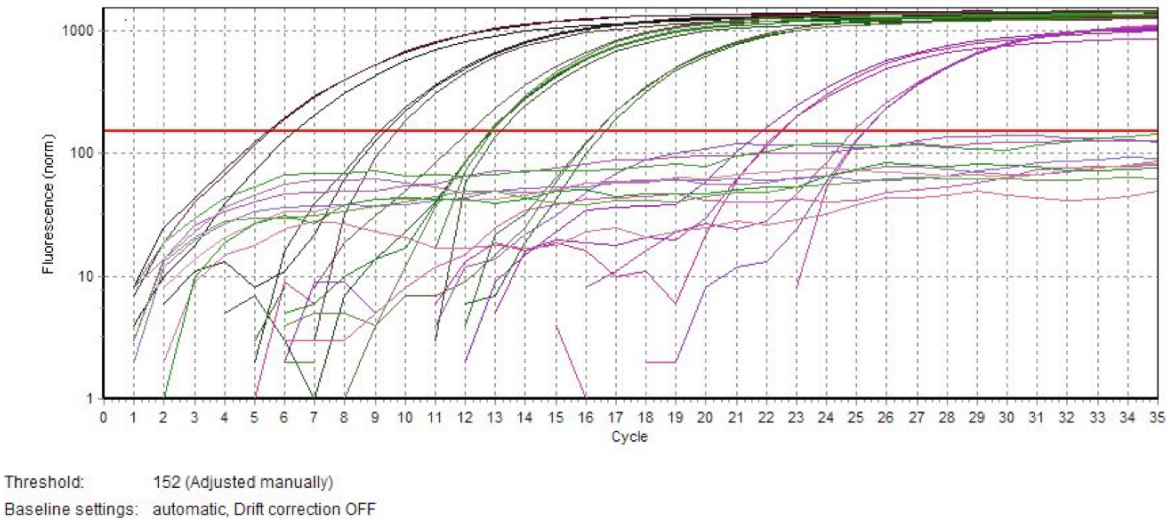

Figure 5: Real-time PCR amplification curves. A threshold level is set sufficiently above background and the number of cycles required to reach threshold, Ct, are registered (Quantification).

product formed. After the PCR has been completed, a melting curve analysis can be performed to prove that only the desired PCR product has been amplified. This is done by increasing the temperature gradually to 95°C, which is the temperature when the double stranded DNA separates, making the dye come off and fluorescence drops rapidly. The PCR product can also be run on a 1.5% agarose/0.5XTBE gel containing ethidium bromide to confirm that the product is of expected size. SYBR Green I assay is a fairly inexpensive to run, which is an advantage when require to test the expression of a number of genes. PCR reactions were performed in 20 µl mixtures, containing 2 µl cDNA (diluted 1:4) and 18 µl FastStart SYBR Green I PCR Mix. For the detection of c-Myc gene, Real-Time qPCR was performed in 10 µl reaction mixture in each well using the QuantiFast SYBR Green PCR Kit. Furthermore, 10 µl of each dilution was used as template DNA in Lambda assay. Both assays were run together at different wells in the same reaction.

Statistical Analysis

We performed statistical analysis using SPSS (13 version) statistical analyses software. In this study, the differences were statistically not significant ($p>0.05$). Our results, although statistically not significant, indicate, consistently with literature, that the positive

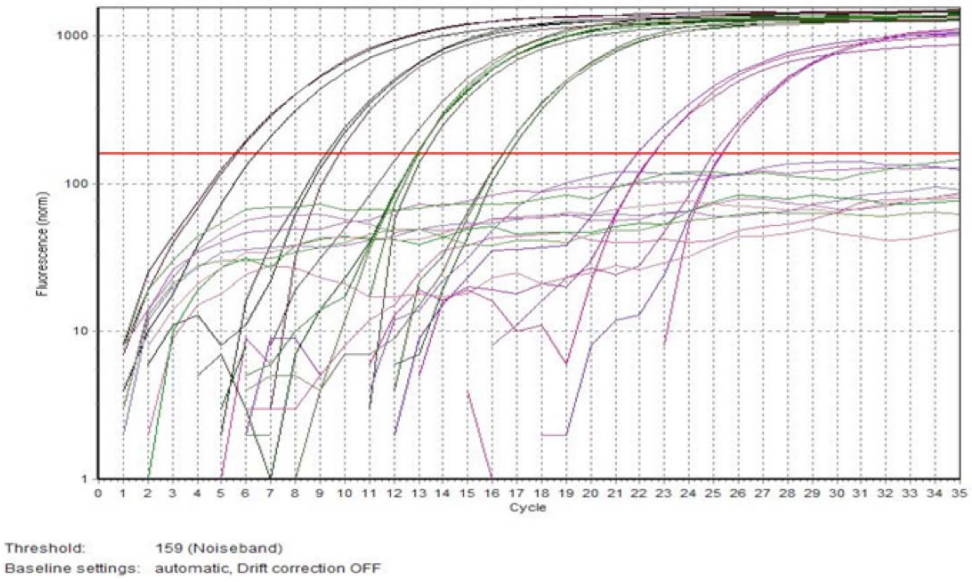

Figure 6: Positive/Negative assaays: Showing that there is no contaminant in the environment because negative controls are not amplified.

and/or negative variant genotypes are less frequent in leukemia patients (Figure **6**). Positive/Negative assay reveals that negatives are controls do not contain any target DNA (c-myc DNA) and positives contain target DNA. It can be concluded that there occured any contaminants.

RESULTS AND DISCUSSION

The study was reported to confirm developed a fast and reliable genomic DNA extraction protocol and RT-qPCR optimization for quantity and quality identification of isolated DNA from blood samples (Figures **5** and **6**). PCR differs significantly from simply looking for a band on a gel (Figures **1** and **2**). The threshold is calculated as a function of the amount of background fluorescence and is plotted at a point in which the signal generated from a sample is significantly greater than background fluorescence. Therefore, the fractional number of PCR cycles required to generate enough fluorescent signal to reach this threshold is defined as the cycle threshold, or Ct. The standard curve is constructed from a measure of Ct (y-axis) against log template quantity (x-axis); the Ct of unknown samples can then be compared to this curve to determine the amount of starting template. On the instrument software, use the plate set up facility and define the appropriate wells as ‘‘standards’’ and specify the concentration in those wells. The standard curve is then constructed automatically. This defines the working dynamic range for the assay. One measure of assay efficiency is made by comparison of the relative Ct values for subsequent dilutions of sample. The efficiency of the reaction can be calculated by the equation: E ¼ 10 (-1/slope). The efficiency of the PCR should as close to 100% as possible, corresponding to a doubling of the target amplicon at each cycle. Using this measure an assay of 100% efficiency will result in a standard curve with a gradient of -3.323 (also see http://www.gene-quantification.de/efficiency.html). An optimized assay will result in a standard curve with a slope between -3.2 and -3.5. Reproducibility of the replicate reactions also reflects assay stability, with R2 values of 0.98 or above being indicative of a stable and reliable assay. These Ct values are directly proportionate to the amount of starting template and are the basis for DNA copy number measurements (Figures **4** and **5**). The amplicon needs to be designed either within an exon or including a portion of the intron for it to be specific to genomic DNA. Both relative and standard-curve quantitation methods can be used. The standard used for generating the standard curve can be as that of RT-qPCR assays, but the choice of endogenous control genes can be different for the relative quantitation method. The DNA purity is the primary concern for template preparation of both fixed archival and fresh samples [19]. Ideally, each DNA sample should be tested with a standard curve to determine if there are any PCR inhibitors present, which may prevent accurate quantitation. Quantitative analyses with precisions of less than 15% within a dynamic range of more than six orders of magnitude make this technique a valuable tool in nearly all investigations in which the amounts or concentrations of known nucleic acid target sequences in biological samples have to be determined. This real-time PCR method for quantitation of gene expression is also labor intensive, requiring pipetting of numerous reagents in minute quantities. The isolation of RNA and first strand cDNA synthesis are time consuming, difficult to perform for multiple samples and poorly suited to automation. The role of real-time PCR in the detection and quantitation of disease-specific genes is expanding, and it has been suggested that this technology may revolutionize the monitoring of minimal residual disease in hematological malignancies [20, 21]. SYBR Green is the most frequently used dsDNA-specific dye in real-time PCR today. In contrast to ethidium bromide, intercalation of cyanine dyes is negligible under the assay conditions of real-time PCR experiments. Quantitative real time PCR (qRT-PCR) of c-Myc cancer gene in patients with leukemia [22], public the paper” c-Myc is an important direct target of Notch1 in T cell acute lymphoblastic leukemial/ lymphoma.” QPCR was performed using the SYBR green system to tested c-Myc gene. Instead, SYBR Green largely binds sequence independently to the minor groove of dsDNA. The binding affinity is more than 100 times higher than that of ethidium bromide. The fluorescence of the bound dye is more than 1000-fold higher than that of the free dye and, therefore, is well suited for monitoring the product accumulation during PCR [23, 14]. Nucleic acids were extracted from blood samples using a Qiagen RNA/DNA purification kit (Qiagen, Hilden Germany). The MYC copy number and level of expression using TaqMan 5' nuclease fluorigenic real-time quantitative PCR assay were measured as previously described [23, 24]. Ct values (defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe crosses a fixed threshold) were determined. Calibration curves plotting Ct against reference DNA quantity or cDNA quantity were generated and the gene copy number or cDNA level for the samples were determined by extrapolation. Assuming a certain amplification efficiency, which

Table 2: Positive and Negative Analysis

Position	Name	Result
A1	lambda dna_1E7/1	Positive
A2	lambda dna_1E7/2	Positive
A3	lambda dna_1E7/3	Positive
A7	Easyone	Positive
A8	Easyone	Positive
A9	Easyone	Positive
B1	lambda dna_1E6/1	Positive
B2	lambda dna_1E6/2	Positive
B3	lambda dna_1E6/3	Positive
B7	Easyone2	Positive
B8	Easyone2	Positive
B9	Easyone2	Positive
C1	lambda dna_100000/1	Positive
C2	lambda dna_100000/2	Positive
C3	lambda dna_100000/3	Positive
D1	lambda dna_10000/1	Positive
D2	lambda dna_10000/2	Positive
D3	lambda dna_10000/3	Positive
E1	unknown	Positive
E2	unknown	Positive
E3	unknown	Positive
E7	NTC	Negative
F1	NTC	Negative
F2	NTC	Negative
F3	NTC	Negative

Threshold detection parameters:
Threshold 159 (Noiseband).
Baseline Settings automatic.
Drift Correction OFF.

typically is close to a doubling of the number of molecules per amplification cycle, it is possible to calculate the number of DNA molecules of the amplified sequence that were initially present in the sample. However, for genes that are not leukemia-specific, the accurate quantitation of gene expression may be hampered by the presence of contaminating normal cells. A polymerase chain reaction (PCR) assay using a set of specific primers and SYBR Green was developed to quantify the amount of DNA in the patients with leukemia. In this study, we determined as Ct value approximately 21.81 and the gene copy number 293 bp (the least amount was 30.1 copies from standart curve at well B7) value for DNA or cDNA quantity of the samples isolated from patients with leukemia (Table **2**, Figures **7** and **8**). Furthermore, we used RT-qPCR amplification curves for c-Myc DNA with $4x10^6$ of control DNA. We found our method more sensitive, reproducible and accurate than other similar recently described assays and comparable to the more expensive probe-based assays. The use of real-time qPCR for molecular diagnostics is attractive because it is objective, rapid, versatile, and cost-effective and can be performed on small tissue samples. The amplification plots reveal the huge differences in Ct, slope and plateau obtained using identical templates. Each amplification plot represents a different combination of primer concentration. The horizontal red

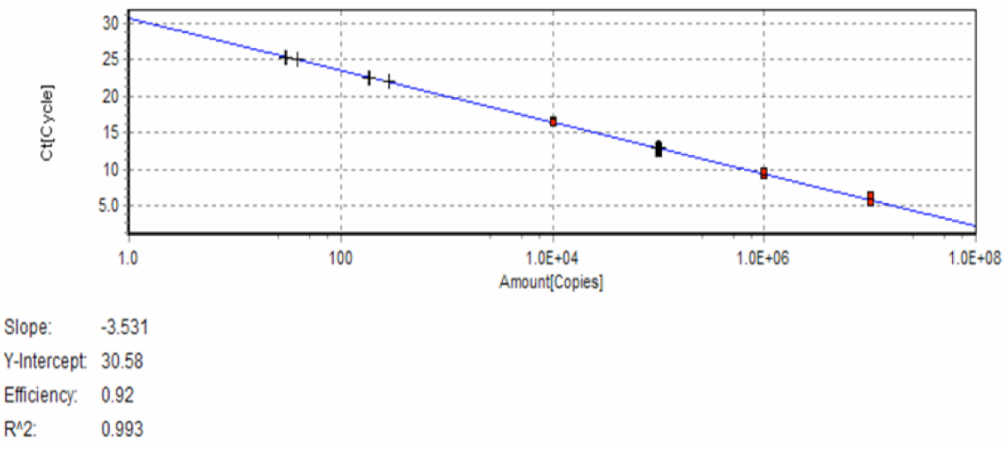

Figure 7: The standart curve produces linear relationship between Ct values and amount of total cDNA and also allows to determine the concentrations of unknowns based on Ct values.

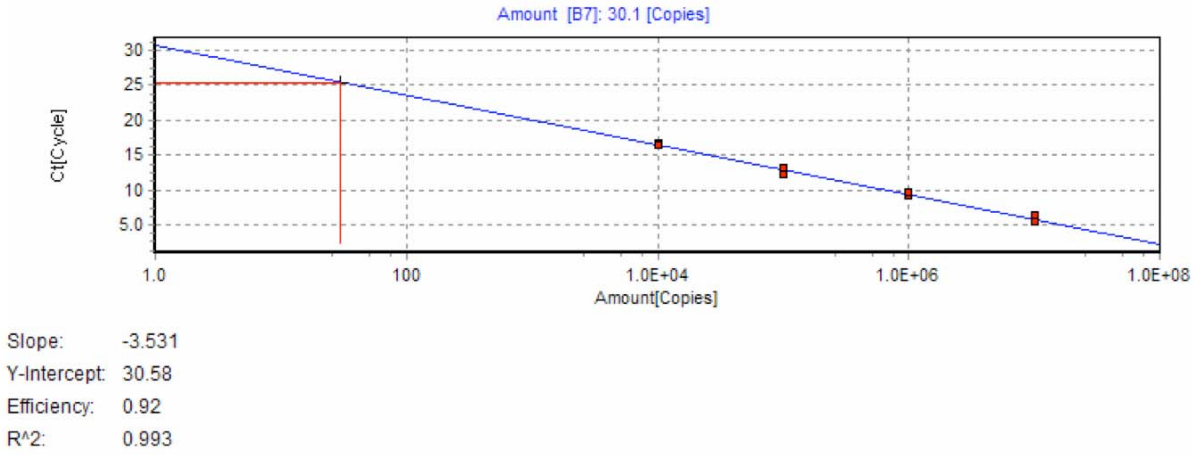

Figure 8: Imaging of the least amount was 30.1 copies from standart curve at well B7 value for DNA or cDNA quantity of the samples isolated from patients with leukemia.

line represents the threshold, which was set automatically by the instrument. The real-time quantitative PCR assay seemed to be highly sensitive and specific which might be used to rapidly detect the quantity of activated DNA be of myc gene in various patients with leukemia and to early diagnose patients with cancer.

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