Development of New Generation Fluorescent In Situ Hybridization Probes by PCR

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ABSTRACT

Leukemia is the sixth most common cause of cancer deaths worldwide. There are several types of leukemia, such as CML (Chronic Myeloid Leukemia), AML (Acute Myeloid Leukemia) and MDS (Myelodysplastic Syndromes). Fluorescent In Situ Hybridization (FISH) is the most common technique used in leukemia diagnosis. CML patients usually carry a translocation that results in fusion of ABL in 9q34 and BCR in 22q11. In AML, there occurs a fusion of PML in 15q24 and RARA in 17q21. Lastly, MDS patients carry an EGR1 deletion in 5q. In this study, we aim to produce FISH probes with PCR method to detect common translocations and deletions in CML, AML and MDS. For CML, primer sets were designed and synthesized in order to produce 10 amplicons of 1 kb size in BCR gene and 10 amplicons of 1 kb size in ABL gene. Similarly, primer sets were designed and synthesized to produce 10 amplicons of 1 kb size in PML and RARA genes for AML and in EGR1 gene for MDS. PCR amplifications with these primers were optimized in terms of PCR conditions such as annealing temperature and a total of 50 amplicons were produced by PCR in high amounts and purified. In order to label PCR products, florescent dUTP molecules with two different chemistries were used in PCR reactions. Different concentrations of florescent dUTP and different Taq polymerases were tested for labelling, however, a very low yield of florescent dUTP incorporation into PCR products was obtained. Therefore, labelling studies with alternative approaches such as chemical or enzymatic labeling are still ongoing to produce PCR FISH probes with post PCR florescent labeling.

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INTRODUCTION

FISH (fluorescent in situ hybridization) is a technique based on the principle of observing chromosomal or gene regions of interest using fluorescent probes (Wolf et al., 2007, Lambros et al., 2007). Due to this feature, it is frequently used commercially in the determination of many diseases.

Chromosomal abnormalities occur frequently in leukemia types (Mitelman et al., 2007, Rowley 2008). First detected one, also known as Philadelphia chromosome, was seen in leukemic cells of Chronic Myeloid Leukemia (CML) patients (Nowell and Hungerford 2004). Later studies revealed that t(9;22)(q34;q11) translocation combines the upstream portion of the BCR and the downstream portion of the ABL, as a result of translocation between chromosomes 9 and 22 (Rowley 1973, Klein et al., 1982, Bartram et al., 1983, Goffen et al., 1984). The breakpoints frequently observed in CML patients are regions called major breakpoint cluster regions (Groffen et al., 1984).

The presence of the fusion gene between the PML gene (# 15q24.1) and the RARA gene (# 17q21.2) through t(15;17) translocation is the hallmark of Acute Promyelocytic Leukemia (APL), also known as Acute Myeloid Leukemia (AML) type M3 (Pandolfi et al., 1992). Myelodysplastic Syndromes (MDS), which is another type of leukemia, partial or complete deletion of chromosome 5 is present in 10-15% of patients with MDS and is the most documented cytogenetic abnormality (Bernasconi et al., 2007).

In this study, FISH probes specific to chromosomal abnormalities in these three leukemia types were amplified by PCR and fluorescent labeling was investigated.

In silico methods

Using SnapGene software, primer designs specific to all specific gene regions (ABL, BCR, PML, RARA and EGR1) were made manually considering major breakpoint regions, and 10 primer pairs for each gene region were obtained. Cross dimer, hairpin and self dimer structures were examined by checking the primer pairs using BeaconDesigner software. Since the FISH probe to be produced within the scope of this study will be commercially available and due to the know-how policy of the company, primer pairs and those that may be related were not given.

Primer Production and PCR Protocol

The designed primer pairs (50 pairs of primers in total) were synthesized by Dr. Oligo device in SENTEBIOLAB company. Primer pairs produced at a concentration of 50 mmol by OPC purification technique. After the primers were produced, optimum annealing temperatures were determined using the gradient PCR method. The reaction mixture and conditions were set in accordance with the manufacturer's recommendations, using Thermo Taq Polymerase (ThermoFisher, EP0402) in the PCR step.

Table 1. Reaction Mixture

10X Taq Buffer	5 µL	2 µL
dNTP Mix (10mM each)	1 µL	0,4 µL
Forward Primer (10 mM)	1 µL	0,4 µL
Reverse Primer (10 mM)	1 µL	0,4 µL
25 mM MgCl2	4 µL	1,6 µL
Template DNA	1 µL	0,4 µL
Taq DNA polymerase	1,25 U= 0,25 µL	0,1 µL
Nuclease free water	36,75	14,7 µL
Total volume	50 µL	20 µL

Table 2. Reaction Conditions

Initial Denaturation	95°C	1 min	
Denaturation	95°C	30 sec	35 cycle
Annealing	Tm (Primer)	30 sec	
Extension	72°C	1 min	
Final Extension	72°C	5 min	

PCR with Florescent Labeled dUTP

After optimizing the PCR conditions for all sets (ABL, BCR, PML, RARA and EGR1), fluorescent labeling studies of amplicons were started. For this purpose, fluorescent labeled dUTPs were used during PCR. PCR was performed using 2 mM dNTP (Hybrigen) and 6-FAM-11-dUTP (Metkinen Chemistry) at different ratios. A different Taq polymerase (NEB M0273) was also tested at this stage. Reaction conditions were carried out as recommended by the manufacturer.

RESULTS

The optimum working conditions of the primers were studied using gradient PCR and optimum annealing temperatures were determined. PCR results were visualized and confirmed using agarose gel electrophoresis (Figures 1, 2, 3, 4 and 5).

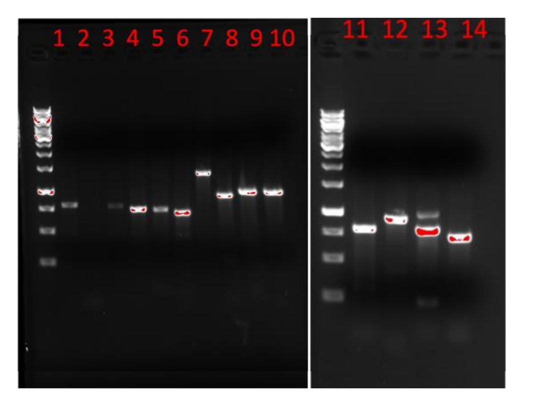

Figure 1. PCR results of primers designed for the ABL gene. Obtained amplicons will be used as ABL probes. From left to right; 1kb marker; ABL primer sets; 1) 1st set primer Tm 54°C,2) 2nd set primer Tm 56°C, 3) 3rd set primer Tm 60°C, 4) 4th set primer Tm 56°C, 5) 5th set primer Tm 52°C, 6) 6th set primer Tm 52°C, 7) 7th set primer Tm 52°C, 8) 8th set primer Tm 56°C , 9) 9th set primer Tm 54°C,10) 10th set primer Tm 58°C,11) 1st set primer Tm 52°C, 12) 2nd set primer Tm 60°C, 13) 3rd set primer Tm 60°C,14) 6th set primer Tm 52°C.

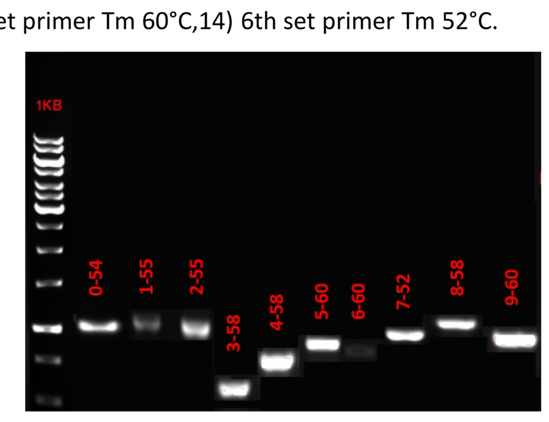

Figure 2. PCR results of primers designed for the BCR gene. Numbers indicate primer set number and tested annealing temperature, respectively.

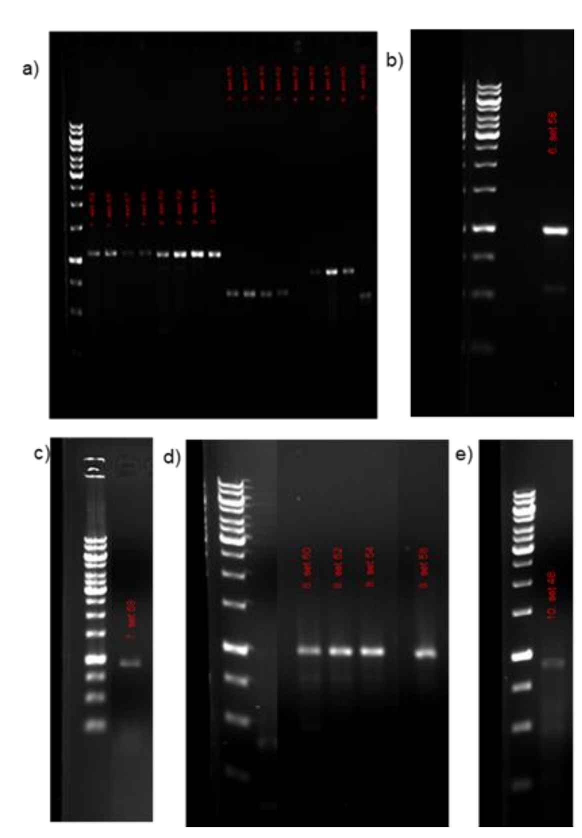

Figure 3. Gradient PCR results of primers (a-e) designed for the RARA gene. Numbers indicate primer set number and tested annealing temperature, respectively

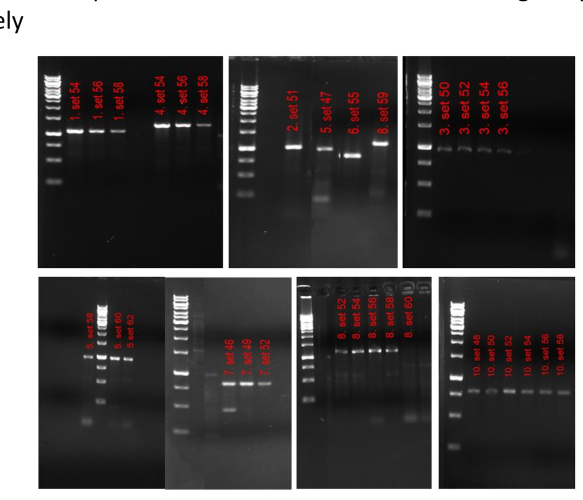

Figure 4. Gradient PCR results of primers (a-g) designed for the PML gene. Numbers indicate primer set number and tested annealing temperature, respectively.

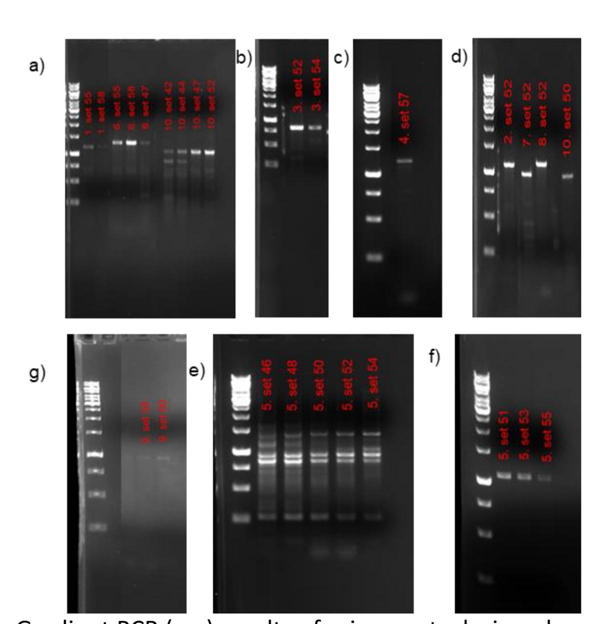

Figure 5. Gradient PCR (a-g) results of primer sets designed specifically for the EGR1 deletion region (del5q). Numbers indicate primer set number and tested annealing temperature, respectively.

RESULTS

Next, PCR method was performed using florescently labeled FAM-dUTP in order to label PCR amplicons. PCR reactions were set by using two different Taq polymerases and FAM-dUTP/dTTP at a ratio of 1:3. PCR amplicons were imaged using agarose gel electrophoresis. Two filters were used to visualise ethidium bromide and florescent signals of amplicons. Results were given in Figures 6 and 7. It was found that there was a very low level of florescent dUTP incorporation into PCR amplicons during PCR under our experimental conditions.

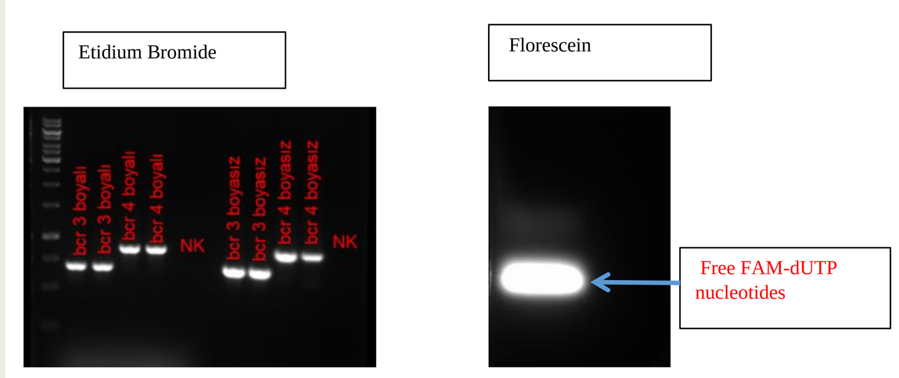

Figure 6. First trial with florescent labeled dUTP (1:3 ratio, ThermoFisher Taq Polymerase), ethidium bromide and fluorescence images.

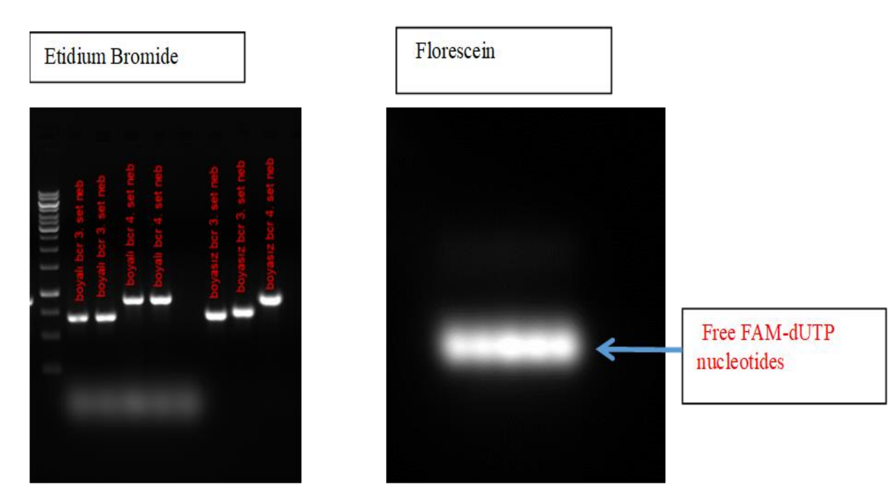

Figure 7. Second trial, ethidium bromide and fluorescence images using florescent labeled dUTP and another Taq polymerase (NEB Standard Taq Polymerase).

CONCLUSIONS

Designed primer pairs for the target regions yielded the desired amplicon sizes. However, it was observed that FAM-dUTP could not be integrated into the PCR products in the next step. The use of different ratios of FAM-dUTP/dTTP and different Taq polymerases were tested, but no results were obtained. Studies on Post-PCR labeling, which is considered as the next method, are still ongoing.

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