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Short communication

FRET primers for quantitative real-time PCR: Optimization for genomic and cDNA templates

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The chemistry of FRET (fluorescence resonance energy transfer) primers real-time thermal cycling technique depends on internally labeled primers with FRET dyes. Fluorescent dyes are linked internally close to the 3` end of both primers. FRET primers technique was tested and optimized on PCR products as an amplification template. It showed high efficiency, correlation coefficient and wide dynamic range in short amplification time. FRET primers technique was not tested on real sample materials such as genomic and cDNA. The main objective of this study was to test and optimize FRET primers on genomic and cDNA as an amplification templates to be used in research and diagnosis. Two steps amplification protocol (denaturation step and annealing-elongation step) showed higher efficiency, correlation coefficient and wider dynamic range compared to three steps protocol in case of genomic and cDNA templates. Moreover, shorter amplification time is needed for two steps protocol that makes FRET primers suitable and alternative choice for research and diagnostic purposes.

Keywords : Real-time PCR, FRET Primers, labeled primers, quantitative real-time PCR, FAM, Texas Red

Quantitative real-time PCR is one of the most potent techniques to detect and quantify as low as 1-5 copies of target nucleic acid. Although non specific real-time PCR chemistry is cheap and easy to design and optimize, it lacks specificity due to non specific complexation between fluorescent dye and double stranded nucleic acid species (Higuchi et al., 1993; Wittwer et al., 1997). SYBR Green I (Wittwer et al., 1997; Morrison et al., 1998), BOXTO (Ahmad, 2007) and BOXTO-MEE and BOXTO-BETIBO (Ahmad and Ghasemi, 2007a) and other fluorescent dyes have been excessively studied as a reporter dyes for nonspecific real-time PCR. Moreover, several additives, enhancers and buffers have been used to increase dynamic range and signal to noise ratio and to enhance sensitivity and specificity (Sarkar et al., 1990; Varadaraj and Skinner, 1994; Ahmad and Ghasemi, 2007b). This chemistry still nonspecific and showed poor sensitivity and efficiency when short templates (40-45 bp) are amplified because dye/base pair ratio (dbpr) which is important to achieve significant increase in fluorescence signal is low (Zipper et al., 2004; Karlsson et al., 2003; Holland et al., 1991). Specific real-time PCR techniques (FRET Primers (Ahmad and Ghasemi, 2007c), TaqMan Probes (Livak et al., 1995; Tyagi and Kramer, 1996), Molecular Beacons (Caplin et al., 1999), Hybridization Probes (Kutyavin et

al., 2000), TaqMan MGB Probes (Irina et al., 2002a), MGB Eclipse Probes (Irina et al., 2002b; Whitcombe et al., 1999), Scorpions Primers (Irina et al., 2002c) and LUX Primers (Fuad et al., 2001)) showed high specificity, sensitivity and wide dynamic range for nucleic acid detection and quantification, in spite of being expensive and difficult to design and optimize. These chemistries cannot be used in case of short template (40-45 bp) due to the presence of labeled probes that make PCR product long.

FRET primers real-time PCR technique (Figure 1) has been shown to be the fastest and the most sensitive and specific technique for nucleic acid detection and quantification. FRET primers technique based on labeling both forward and reverse primers with fluorescent dyes. Fluorescent dyes must be in resonance to behave as FRET dyes (fluorescence resonance energy transfer). Moreover, primers should be designed to maintain 5 to 7 nucleotides distance between dyes (optimal is 6). FRET dyes are immobilized at a specific position close to the 3`-end of primers in order to keep dyes at specific proximity to achieve highest orbital overlapping for optimal energy transfer and strongest signal acquisition (Ahmad and Ghasemi, 2007c). FRET primers technique was partially optimized for PCR product as a template and was not tested on real samples such as genomic and cDNA (Ahmad and Ghasemi, 2007c). Usually, low efficiency and high cycle threshold (Ct) values are obtained when cDNA (gene expression quantification) and genomic DNA (detecting and quantifying pathogenic genes) are used as templates, and in some cases PCR additives or nested PCR are used. Therefore; the present study is the first study to test FRET primers technique on real samples (genomic and cDNA) and to optimize it by combining the annealing and elongation PCR steps in order to increase the sensitivity and efficiency of PCR and reduce run time because annealing step is the most critical PCR step.

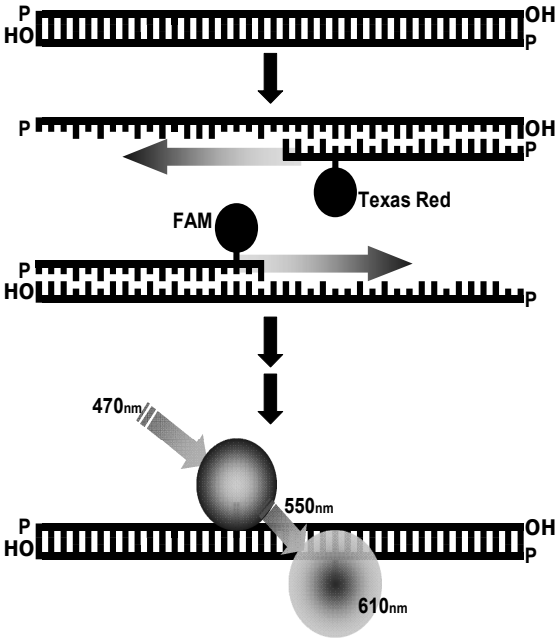

Figure. 1. Schematic diagram for the FRET primer real-time PCR.

Materials and methods

Labeled Primers.

The web interfaces, Primer3Plus (http://primer3plus.com) (Untergasser et al., 2007) was used to design primers for human β-actin and GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase). Group of primers were analyzed and tested for the presence of nonspecific base pairing and secondary structures using oligoanalyzer (https://eu.idtdna.com/calc/analyzer) and netprimer (http://www.premierbiosoft.com/netprimer/index.html) to select best primers. Chosen primers must contain thymine (dT) close to the 3`, because the only nitrogenous base where fluorescent dyes can be internally linked is thymine (Table 1). Labeled primers were ordered from MWG-Biotech (Ebersberg, Germany) and used according to manufacturer description without further purification.

Table 1. Labeled Primers.

Gene	Forward Primers (5`→3`)	Reverse Primers (5`→3`)
β-Actin	CCAGTCCTCTCCCAAG**T*****CC	ATGCTATCACCTCCCC**T***GTG
GAPDH	AGGTCATCCATGACAACTTTGG**T***ATC	GGACTGTGGTCATGAGTCCT**T***CC

T* is Fluorescein labeled for forward primers and Texas Red labeled for reverse primers.
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

Real-Time PCR Conditions

PCR materials (PCR buffer, $MgCl_2$, dNTPs, Taq polymerase) were purchased from Sigma-Aldrich (Steinheim, Germany). Human universal reference total cDNA was purchased from BD Biosciences Clontech (California, USA) and human genomic DNA was purchased from Promega (Stockholm, Sweden). RotorGene3000 (Corbett Research, Sydney, Australia) iCycler (BIO-RAD, California, USA) and LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) were used for real-time thermal cycling.

1- Three steps amplification: denaturation at 95°C (touchdown to 85°C after 10cycles) for 0 sec, annealing at 58°C for 7 sec and elongation at 72°C for 2 sec.

2- Two steps amplification: denaturation at 95°C (touchdown to 85°C after 10cycles) for 0 sec and annealing-elongation step at 64°C for 9 sec.

Signal was acquired at the end of the elongation (in case of 3 steps) or annealing-elongation (in case of 2 steps) step by exciting reaction mixture with FAM (6-Carboxyfluorescein) excitation channel (490nm filter) and signal emitted from reporter dye was acquired by Texas Red detection channel (610nm filter). Real-time PCR master mixture contained 1x PCR buffer, 3 mM $MgCl_2$, 200 mM dNTPs, 0.15 mM labeled primers and 0.03U/µl Taq polymerase as final concentrations, 1nM fluorescein was included when iCycler (BIO-RAD) was used.

Results and discussion

Two versus three steps real-time PCR

Labeled primers for β-actin gene were tested by two and three steps real time PCR protocols using PCR product and human cDNA as templates and RotorGene3000 as a real-time thermal cycler. Table 2 shows that PCR products can be used as a template with two and three steps protocols without significant effects and changes on efficiency and Cq (cycle of quantification) values. In case of using cDNA as an amplification template, two PCR protocol showed higher efficiency and lower Cq value than that generated by three steps protocol. Working with cDNA needs more optimization steps and mostly PCR additives and enhancers are used to improve results (Fuad et al., 2001; Musso et al., 2006; Piero et al., 1998). It is clearly shown in this study that using FRET primers needs less efforts in optimizing reaction conditions because very short product is generated. The efficiency and Cq values were improved by combining annealing and elongation steps in FRET primers technique, because longer time for annealing is required when cDNA is used as a template, at the same time very short time is required to elongate 20 nucleotides. In addition to that the PCR product is very short; therefore, the product will be less stable at 72°C which will affect the acquired signal. Therefore; combining annealing and elongation steps enable faster cycling, because all PCR machines take long ramping time to move from temperature to another (Table 2).

Table 2. Three compared to two steps real-time PCR protocols using β-actin primers and templates as PCR product and human cDNA

	Three Steps Real-Time PCR		Two Steps Real-Time PCR	
	PCR Product Template	cDNA Template	PCR Product Template	cDNA Template
Efficiency (%)	97	89	98	97
Cycle Threshold	15	22	14	20

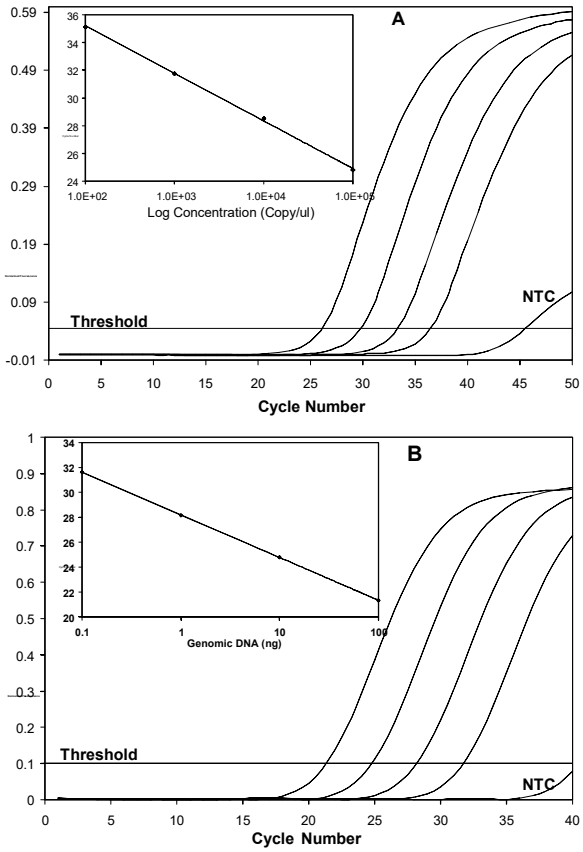

Figure. 2. (A) Quantification of 10 fold serial dilutions (10^5 to 10^2 copy/µl) of human cDNA as template with two steps real-time PCR using LightCycler. (Insert) Standard curve (efficiency = 96.6%, correlation coefficient = 0.999). (B) Quantification of 10 fold serial dilutions (100 to 0.1 ng/µl) of human genomic DNA as template with two steps real-time PCR using RotorGene3000. (Insert) Standard curve (efficiency = 98%, correlation coefficient = 0.999).

Table 3. PCR efficiency, correlation coefficient and cycling time for GAPDH FPRT primers on different platforms

Platform	Efficiency (%)	Correlation Coefficient	Time (min/40 Cycles)
RotorGene 3000	98.0	0.999	30
LightCycler	96.6	0.999	13
iCycler	95.0	0.999	37

Quantification of cDNA by FRET Primers

Two steps real-time thermal cycling was used to test GAPDH labeled primers with human cDNA as a template using LightCycler (10^5 to 10^2 copy/µl cDNA), iCycler (2.5×10^5 to 2.5×10^2 copy/µl cDNA) and RotorGene3000 (2×10^5 to 2×10^2 copy/µl cDNA). Table 3 shows that two steps protocol is the fastest real-time thermal cycling which is suitable for gene expression quantitation with high efficiency and correlation coefficient. Figure 2 (A) shows linear data analysis generated by LightCycler for real-time thermal cycling, using 10 fold serial dilutions (10^5 to 10^2 copy/µl) of human cDNA as a template. Standard curve (Figure 2 (A) insert) was generated from linear data analysis at a specific threshold of quantification, shows that the new conditions can be used to quantify high and low level expressed genes with high efficiency (96.6%) and correlation coefficient (0.999) within 13 for 40 cycles.

Genomic DNA as a Template

β-actin labeled primers were tested with 10 fold serial dilutions of human genomic DNA (100 to 0.1 ng/ µl) as a template by applying two steps protocol using RotorGene3000 for real-time thermal cycling. Figure 2 (B) shows linear data analysis and standard curve that is generated at specific threshold of quantification. The new two steps thermal cycling protocol can be used with human genomic DNA as template to detect and quantify specific target genes with high efficiency (98%) and correlation coefficient (0.999) within 30 min for 40 cycles.

Conclusion

FRET primers real-time PCR technique can be successfully used to quantify genomic and cDNA as a real sample materials. High efficiency, correlation coefficient and wide dynamic range were achieved in a very short thermal cycling time varied from 13 to 37 min depending on the thermal cycling device. Moreover, two steps real-time thermal cycling protocol is more efficient and faster than 3 steps protocol with FRET primers.

Acknowledgment

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