Primer Design Using Polymerase Chain Reaction for SNPs Analysis in *SLC22A1* rs622342 Encoding OCT1 as Metformin Main Transporter

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Abstract: Organic Cation Transporter 1 (OCT1) is the primary transporter of metformin mainly located in hepatocytes, which plays an important role in metformin action to inhibit gluconeogenesis so as to reduce blood glucose. Genetic polymorphism of *SLC22A1* that encodes OCT1, one of which is rs622342, has been widely reported and proven to decrease the antidiabetic effect of metformin. This study aimed to design primers and to obtain an optimum condition for polymerase chain reaction (PCR) process that can detect the genetic polymorphism of *SLC22A1* rs622342. Primers were computationally designed in primer 3 webpage and analyzed with Primer BLAST and Oligo Analyzer. Optimization of PCR condition was conducted for temperatures of denaturation, annealing, and elongation as well as for the number of cycles in PCR process. Sensitivity test was performed on PCR condition using a variety of volumes and DNA template qualities undergoing multiple freeze-thaw cycles. The obtained pair, forward primer (5'- CAG AGA GAA TCA GTG AGC TGT G-3') and reverse primer (5'- CCC AGG CTG GTC TTT TTA AG-3'), was proven to be capable of amplifying DNA sequence containing SNPs in rs622342 at 96°C denaturation, 60°C annealing, and 72°C elongation temperatures with a 30-cycle iteration. Such PCR condition could amplify DNA with 0.2 µL of template volume and 7 freeze-thaw cycles. Therefore, in addition to the selected primer pairs and PCR condition to analyze SNPs in rs622342, this study also recommends that the volume of DNA template having undergone multiple freeze-thawing be increased if the amplicon PCR products are unqualified.

Keywords: Metformin, PCR, Primer design, OCT1, rs622342.

INTRODUCTION

Metformin lowers plasma glucose level by mainly inhibiting the gluconeogenesis process in hepatocytes. It is transported to penetrate hepatocyte membrane by Organic Cation Transporter1 (OCT1) [1]. This transporter is encoded by *SLC22A1* gene on chromosome 6q25.3 with a span of approximately 37kb [2]. A study of 122 patients with Type-2 DM in South India who administered metformin monotherapy found differences in response after 12 weeks of therapy [3]. The presence of Single Nucleotide Polymorphism (SNP) in rs622342 at intron 9 of *SLC22A1* gene was found to be the causative factor. Patients with either homozygous or heterozygous mutant allele showed a deteriorating response to metformin compared to those with wild-type allele who had 5-6 times higher antiglycemic response [3]. Another study of 102 Caucasian patients in the Netherlands found 0.53% reduction in the average HbA1C level of patients with homozygous wild-type allele (AA), 0.32% in those with heterozygous wild-type allele (AC), and only 0.02% decrease in patients having homozygous mutant allele (CC) [4]. In addition, Becker *et al*. (2009) were the first to report the MAF value of rs622342 SNP in Dutch population reaching 0.37 and 0.216 in Xhosa population in South Africa that increased to 0.23 in 2014 [5]. A high frequency of genetic variations has made several pharmacogenetics studies of metformin therapy in different races involve such polymorphism using PCR with various techniques for genotype analysis.

Polymerase Chain Reaction (PCR) refers to an enzymatic technique in molecular biology that has been extensively used for DNA amplification due to its quick, inexpensive, simple process [6, 7]. This technique sensitive because it only requires a few DNAs to amplify but generates plenty of products to analyze using a simple method in the laboratory [7]. To amplify DNA sequence, PCR technique requires several components, including nucleotide, DNA polymerase, DNA template, and primer. Primer is a short single-stranded DNA fragment, which is specifically complementary to the DNA sequence targeted for amplification. Nucleotide consists of bases Adenine (A), Thymine (T), Cytosine (C), and Guanine (G), which function as a building block for PCR process to create new DNA strands [6, 7]. DNA polymerase is an enzyme that will arrange nucleotides according to the targeted sequence of DNA template to form new strands as the product of PCR [6]. As a short single-stranded DNA

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with a sequence complementary to DNA template and required as a marker of polymerase enzyme to commence PCR process, a primer must be able to bind specifically to the target sequence [8].

In the meantime, sophisticated technology of whole-genome scans, DNA sequencing, and DNA methylation studies requires a certain volume (in micrograms) of high-quality DNA [9]. Additionally, the freeze-thaw process is inevitable in genomic studies. Therefore, specific primer design, optimum PCR condition using the minimum volume, as well as quality of DNA template having undergone multiple freeze-thaw cycles become crucial to DNA sequence amplification for analysis of polymorphism that does not result in false-results. This study aimed to obtain primers and optimum PCR condition for the amplification of DNA sequence containing rs622342 in *SLC22A1* gene encoding OCT1 with a variety of volumes and qualities of DNA template experiencing multiple freeze-thaw cycles.

MATERIALS AND METHODS

The present research designed primer pairs and optimum PCR condition to amplify DNA sequence that holds SNPs of rs622342for OCT1.The study was conducted in the laboratory of biochemistry of Universitas Islam Indonesia.

Subjects

The research subjects were type-2 diabetes mellitus patients aged 36-60 years old who used metformin 500 mg twice daily. The Ethics Committee of the Faculty of Medicine, University of Gadjah Mada approved this study through a letter numbered KE/FK/648/EC, and all procedures were conducted according to the Declaration of Helsinki. All subjects have given their written informed consent.

DNA Extraction

DNA was isolated from patients' buffy coat samples using Geneaid® Genomic DNA Reagent Mini Kit with the silica gel method. The extraction procedure consisted of sample preparation, cell lysis, DNA binding, washing, and elucidation.

Primer Design and Analysis

Primers were designed using the Primer3 website (http://bioinfo.ut.ee/primer3-0.4.0/) by inputting the target DNA sequence sourced from NCBI website (http://www.ncbi.nlm.nih.gov/snp) on 1 January 2016. Primer pairs were selected if they could specifically bind to the target sequence. Specificity was obtained from the analysis using Primer BLAST in NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Computational analysis of the physicochemical properties, including the number of bases, GC content, melting temperature (Tm), Tm hairpin, self-dimer, and hetero-dimer of the selected primer pairs, was performed in Oligo Analyzer website (http://sg.idtdna.com/calc/analyzer).

Optimization of PCR Condition

A mixture was prepared as much as 25μL containing Promega® PCR Master Mix, DNA, forward and reverse primers each at 10μM concentration, and nuclease-free water. Optimization was undertaken for denaturation temperature, annealing temperature, elongation temperature, and number of iterative amplification cycles.

Sensitivity Test of PCR Condition towards DNA Template Volume

As much as 25μL mixture of Promega® PCR Master Mix, 10μM of each forward and reverse primers, nuclease-free water, and DNA template at 0.1μL-2μL of volume was used. Amplification was performed under the obtained optimum condition.

Sensitivity Test of PCR Condition towards DNA Template Quality

A total of 25μL PCR mix containing Promega® PCR Master Mix, forward and reverse primers each for 10μM, nuclease-free water, and DNA template having undergone 7 freeze-thaw cycles was prepared. Under the achieved optimal condition, amplification was conducted.

Analysis

The amplification products were analyzed using electrophoresis with 2% agarose and 1x TBE buffer for 120 minutes at 80 Volt. The results were then visualized with UV light at 254nm wavelength.

RESULTS AND DISCUSSION

Both specific primer design and identification of optimum PCR condition with varied volumes and DNA template qualities are necessary to ensure that the DNA sequences produced remain specific in efficient volumes of high-quality DNA undergoing repeated freeze-thaw processes. Not only to expand the

benefits, information of DNA-template minimum volumes is also requisite to assure that the analysis process evades false-findings. In addition, the maximum number of freeze-thaw cycles that still provide high-quality amplicon products can guarantee the quality of DNA template after iterative freeze-thaw cycles.

Primer Design and Analysis

Sequence of DNA template, which encodes OCT1, with 285bp of length containing rs622342 from NCBI website (http://www.ncbi.nlm.nih.gov/snp) was used to design specific primers in Primer3 website (http://bioinfo.ut.ee/primer3-0.4.0/). The most specific primer pairs were resulted from the analysis in NCBI Primer BLAST website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) as shown in Figure **1**. The sequence of forward primer was 5'- CAG AGA GAA TCA GTG AGC TGT G-3' and 5'- CCC AGG CTG GTC TTT TTA AG-3' for reverse primer, resulting in 216bp-long amplified product whose binding is illustrated in Figure **2**. The position of SNP in rs622342 was at 125th base of the first C bases.

Table **1** shows the results of computational analysis for the physicochemical properties of the selected primer pairs from Oligo Analyzer website (http://sg.idtdna.com/calc/analyzer). The number of bases of recommended primer is usually around 15-35bp [10, 11]. A long primer has better specificity but less efficiency because there are plenty of nucleotides in a long primer that can increase the melting temperature (Tm) but reduce the primer stability [12]. Tm refers to the temperature in which half of the primer strand is flanked by the target sequence [12]. It is affected by the nucleotide base of the primer, and it will determine the annealing process. A difference in Tm inside a primer pair will affect primer specificity and efficiency. At a lower annealing temperature, primer with higher Tm will misprime, leading to decreased specificity, while at a higher temperature, primer with low Tm will not be flanked optimally or even not being flanked at all by the template [11]. To balance primer specificity and efficiency, it is therefore recommended to employ primers with Tm > 54°C and 40-60% GC content or at least higher than the GC content of DNA template [12, 13]. High value of GC content will make the template difficult to amplify [14]. A temperature

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CAGAGAGAATCAGTGAGCTGTG	22	58.49	50.00	5.00	1.00
Reverse primer	CCCAGGCTGGTCTTTTTAAG	20	56.31	50.00	7.00	3.00

Products on target templates

>NC_000006.12 Homo sapiens chromosome 6, GRCh38.p2 Primary Assembly
product length = 216
Features associated with this product:
solute carrier family 22 member 1 isoform X1

solute carrier family 22 member 1 isoform X2

Forward primer	1	CAGAGAGAATCAGTGAGCTGTG	22
Template	160151710	.....	160151731
Reverse primer	1	CCCAGGCTGGTCTTTTTAAG	20
Template	160151925	.....	160151906

Figure 1: Results from Primer BLAST.

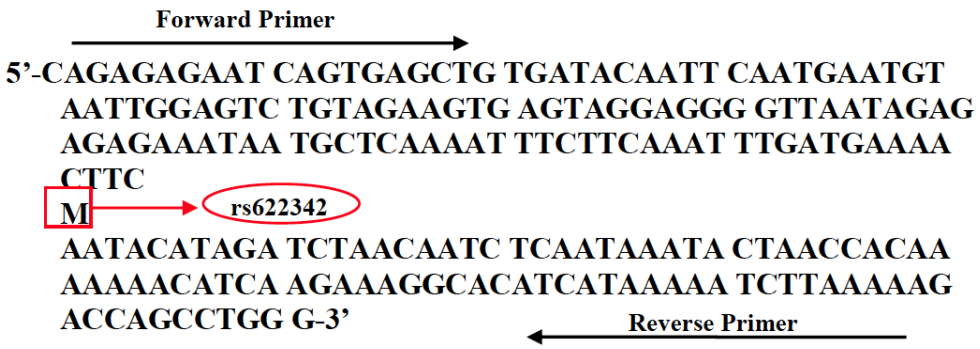

Figure 2: Illustration of primer pair binding.

Table 1: Physicochemical Properties of Primer Pair

Parameter	Forward Primer	Reverse Primer
Sequence	CAG AGA GAA TCA GTG AGC TGT G	CCC AGG CTG GTC TTT TTA AG
Number of bases	22bp	20bp
GC content	50%	50%
Tm	55°C	53.6°C
Tm Hairpin	37.4°C	33.3°C
Self-Dimer	ΔG max : -36.48 kcal/mole ΔG1 : -6.34 kcal/mole (match 4bp)	ΔG max : -39.32 kcal/mole ΔG1 : -6.62 kcal/mole (match 4bp)
Hetero-Dimer	ΔG max : -39.32 kcal/mole ΔG1 : -4.74 kcal/mole (match 3bp)	

shown by Tm hairpin should not be used in PCR process to avoid the formation of hairpin as a secondary structure that can eliminate primer from PCR process as it cannot be flanked by the template and elongated. Other secondary structures, self-dimer and hetero-dimer, have a change in Gibbs free energy (ΔG), indicating spontaneity and stability of complement form. More negative ΔG means more spontaneous and stable complement of the established secondary structure [11]. The value of ΔG max shows that the change in Gibbs free energy of primer sequence is perfectly complementary [15]. The recommended primer to be used had at least -9 kcal/mole of ΔG in both self-dimer and hetero-dimer [12]. The value of ΔG1 indicates the highest probability of self-dimer and heterodimer formation because it has the most spontaneous and stable reaction of formation. Meanwhile, the far more negative value of ΔGmax shows that the reaction of primer complement and template occurs much more spontaneously and stably. Therefore, the obtained primer pairs could acceptably be used in PCR process to amplify the target sequence containing rs622342.

Optimum PCR Condition for rs622342 Analysis

Optimum PCR condition will result in desired amplification products. To amplify DNA sequence in PCR process, several components should be mixed, such as dNTPs, TaqDNA polymerase, $MgCl_2$, DNA template sequence, forward and reverse primers, as well as buffer. This study used Promega® 2XPCR Master Mix containing TaqDNA polymerase, dNTPsand MgCl2. Table **2** describes the composition of PCR mix used for this present experiment.

Figure **3** shows the electrophoresis results of amplification products from various PCR conditions using the composition in Table **2**. The variety includes denaturation, annealing, and elongation temperatures, as well as number of PCR cycles. Optimum PCR condition will result in one specific electrophoresis band that meets the desired target. In Figure **3**, such result is shown by the single band in Lane 7 sized 216bp with its PCR condition described in Table **3**. The optimum annealing temperature reached 60°C. Extremely low annealing temperature will lead to unspecific products [16] as is shown in Lane 1-6 where <60°C annealing temperature produced more than one electrophoresis bands. In contrast, too high annealing temperature will reduce amplification products [16] as indicated by Lane 8-12 that the resulted electrophoresis bands become thinner along with increased annealing temperature. A clear electrophoresis band is obtained from 30 cycles of iteration.

Table 2: Composition of Mixed Materials for Amplification Using PCR

Material	Quantity (µL)
Promega® 2X PCR Master Mix	12.5
10µM Forward Primer	1
10µM Reverse Primer	1
DNA template	2
Nuclease-free water	8.5
Total	25

Sensitivity of PCR Condition

The sensitivity test result of optimum PCR condition towards DNA template volume in PCR mix is shown in Figure **4**. A small volume of DNA template, only 0.1 µL, was well amplified using the obtained optimum condition. This indicates that the obtained optimum PCR condition could be sensitively used to amplify DNA template sequence at up to 0.1 µL of volume.

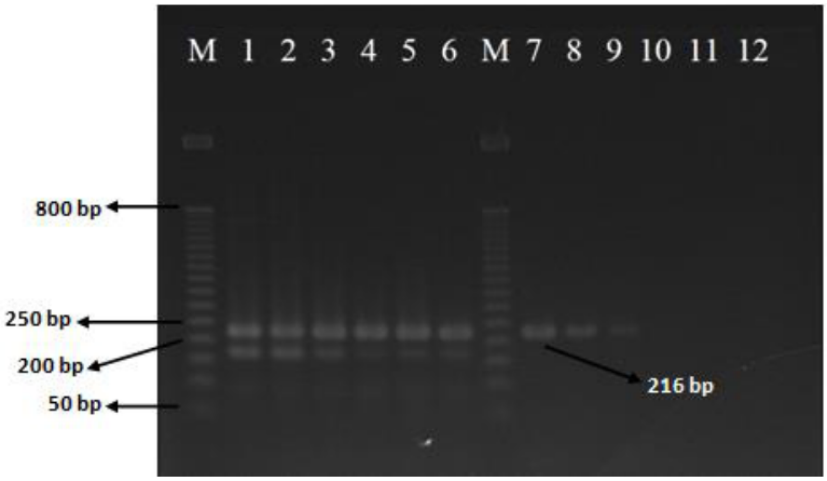

Figure 3: Result of PCR Condition Optimization.

Note: M = 50bp Ladder; 1-6 = PCR products at 94°C pre-denaturation (2 min), 94°C denaturation (40 sec), 48°C; 49.8°C; 52.9°C; 54.8°C; 58°C; 59.7°C consecutive annealing , 72°C elongation (1 min) and 72°C post-elongation (3 min) with 35 cycles of denaturation-elongation; 7-12 = PCR products at 96°C pre-denaturation (3 min), 96°C denaturation (1 min),60°C; 61.2°C; 63.2°C; 64.6°C; 66.6°C; 68°C consecutive annealing, 72°C elongation (30 sec) and 72°C post-elongation (1min) with 30 cycles of denaturation-elongation.

Table 3: Optimum PCR Condition

Stage	Temperature (°C)	Time (seconds)	Number of Cycles
Pre-denaturation	96.0	180.0	1
Denaturation	96.0	60.0	30
Annealing	60.0	40.0	30
Elongation	72.0	30.0	30
Post-elongation	72.0	60.0	1
Hold	4.0	∞	1

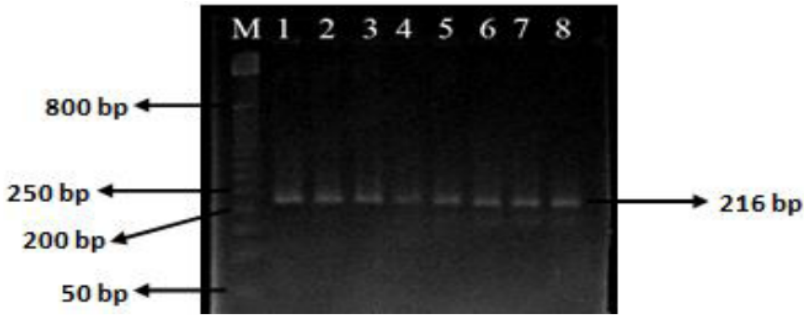

Figure 4: Results of PCR Condition Sensitivity Test towards DNA Template Volume.

Note:M = 50bp Ladder; 1-8 = PCR products with differences in DNA template volumes (1 = 2µL; 2 = 1.5µL; 3 = 1µL; 4 = 0.5µL; 5 = 0.4µL; 6 = 0.3µL; 7 = 0.2µL; 8 = 0.1µL).

Not only towards DNA template volume, sensitivity test for the obtained optimum PCR condition was also conducted towards the quality of DNA template undergoing repeated freeze-thaw cycles. A previous study suggested that iterative freeze-thaw process can degrade DNA structures [17, 18]. Figure **5** shows that 2µL DNA template having undergone 7 cycles of freeze-thaw process could be well amplified, resulting in a specific product that met the target using optimum PCR condition. Different from the PCR process using 2µL DNA template, the sensitivity test for 0.1µL of DNA template volume showed unstable process in repeated freeze-thaw cycles indicated by the produced bands in Lane 10 and Lane 13 with 3 and 5 respective freeze-

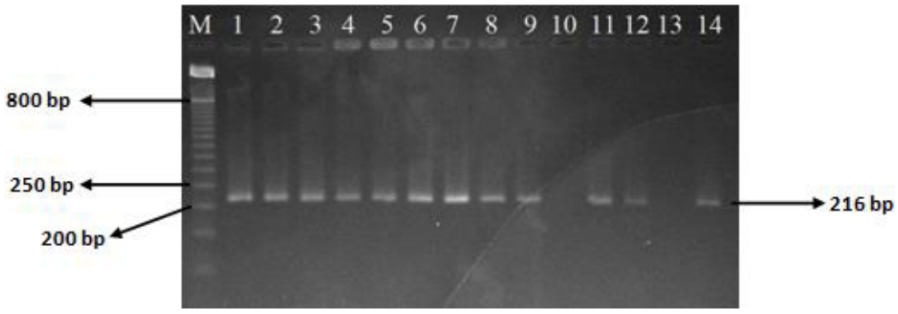

Figure 5: Electrophoresis Result of PCR Condition Sensitivity Test towards DNA Template Quality at 2μL and 0.1 μL of Volumes.

Note: M = 50bp Ladder; 1-7 = template amplification products at 2μL with 1-7 cycles; 8-14 = template amplification products at 0.1μL with 1-7 cycles.

thaw cycles. Therefore, amplification in optimum condition using 0.1μL DNA template volume could only yield expected products in maximum 2 freeze-thaw cycles. Figure **6** shows amplification results using 0.2μL DNA template in the same condition. Stable and specific products desired were yielded up to 7 cycles of freeze-thaw process. DNA template undergoing more than 2 freeze-thaw cycles required a higher volume, reaching 0.2μL, while the sample with less than 2 cycles of freeze-thaw could still be amplified at 0.1μL of volume. This is because freeze-thawing can damage DNA in terms of both DNA from the cell nucleus and mitochondrial DNA [19]; as a result, higher volume of DNA template is needed in higher frequency of freeze-thawing.

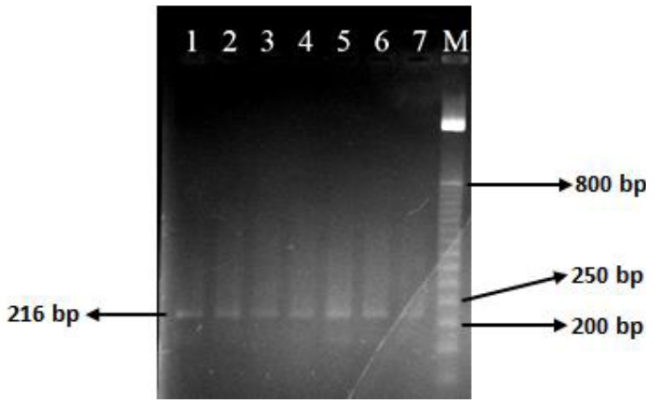

Figure 6: Electrophoresis Results of PCR Condition Sensitivity Test towards DNA Template Quality at 0.2 μL of Volume.

Note: M = 50bp Ladder; 1-7 = template amplification products at 0.2μL with 1-7 cycles.

CONCLUSION

The pair of forward primer (5'- CAG AGA GAA TCA GTG AGC TGT G-3') and reverse primer (5'- CCC AGG CTG GTC TTT TTA AG-3') was able to amplify specifically the DNA sequence containing SNPs in rs622342 using the PCR process at 96°C denaturation temperature, 60°C annealing temperature, and 72°C elongation temperature with 30 cycles of iteration. The obtained PCR condition could be used to amplify DNA template at up to 0.1μL of lowest volume and 2 freeze-thaw cycles, while 0.2μL of volume could amplify DNA template with 7 freeze-thaw cycles. Therefore, in addition to the selected primer pairs and PCR condition to analyze SNPs in rs622342 encoding OCT1, this study also recommends that the volume of DNA template having undergone multiple freeze-thawing be increased if the amplicon PCR products are unqualified.

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REFERENCES

[1] Viollet B, Guigas B, Sanz Garcia N, *et al*. Cellular and molecular mechanisms of metformin: an overview. Clin Sci Lond Engl 1979 2012; 122: 253-270. https://doi.org/10.1042/CS20110386

[2] National Center for Biotechnology Information. *SLC22A1* solute carrier family 22 (organic cation transporter), member 1 [Homo sapiens (human)] - Gene - NCBI. *SLC22A1*http://www.ncbi.nlm.nih.gov/gene/6580 (2015, accessed 27 October 2015).

[3] Umamaheswaran G, Praveen RG, Damodaran SE, *et al*. Influence of *SLC22A1* rs622342 genetic polymorphism on metformin response in South Indian type 2 diabetes mellitus patients. Clin Exp Med 2014; 15: 511-7. https://doi.org/10.1007/s10238-014-0322-5

[4] Becker ML, Visser LE, van Schaik RHN, *et al*. Genetic variation in the organic cation transporter 1 is associated with metformin response in patients with diabetes mellitus. Pharmacogenomics J 2009; 9: 242-247. https://doi.org/10.1038/tpj.2009.15

[5] Jacobs C, Pearce B, Du Plessis M, *et al*. Genetic polymorphisms and haplotypes of the organic cation transporter 1 gene (*SLC22A1*) in the Xhosa population of South Africa. Genet Mol Biol 2014; 37: 350-359. https://doi.org/10.1590/S1415-47572014005000002

[6] Joshi M, Deshpande JD. Polymerase chain reaction: methods, principles and application. Int J Biomed Res; 2. Epub ahead of print 11 October 2011. https://doi.org/10.7439/ijbr.v2i1.83

[7] Garibyan L, Avashia N. Research Techniques Made Simple: Polymerase Chain Reaction (PCR). J Invest Dermatol 2013; 133: e6.

[8] Borah P. Primer designing for PCR. Sci Vis 2011; 11: 134-136.

[9] Gail MH, Sheehy T, Cosentino M, *et al*. Maximizing DNA Yield for Epidemiologic Studies: No More Buffy Coats? Am J Epidemiol 2013; 178: 1170-1176. https://doi.org/10.1093/aje/kwt079

[10] Jones N. PCR. In: Theophilus BM, Rapley R (eds) PCR Mutation Detection Protocols. Humana Press, pp. 37-46.

[11] Álvarez-Fernández R. Explanatory Chapter: PCR Primer Design. In: Methods in Enzymology. Elsevier, pp. 1-21.

[12] Rychlik W. Selection of Primers for Polymerase Chain Reaction. In: White B (ed) Methods in Molecular Biology. Humana Press, pp. 31-40.

[13] Dieffenbach CW, Lowe TM, Dveksler GS. General concepts for PCR primer design. Genome Res 1993; 3: S30-S37.

[14] Benita Y, Oosting RS, Lok MC, *et al*. Regionalized GC content of template DNA as a predictor of PCR success. Nucleic Acids Res 2003; 31: e99.

[15] Integrated DNA Technologies. Homo-Dimer Analysis. Oligo Analyzer https://sg.idtdna.com/calc/analyzer (2016, accessed 25 June 2016).

[16] Rychlik W, Spencer WJ, Rhoads RE. Optimization of the annealing temperature for DNA amplification *in vitro*. Nucleic Acids Res 1990; 18: 6409-6412. https://doi.org/10.1093/nar/18.21.6409

[17] Davis DL, O'Brien EP, Bentzley CM. Analysis of the degradation of oligonucleotide strands during the freezing/thawing processes using MALDI-MS. Anal Chem 2000; 72: 5092-5096. https://doi.org/10.1021/ac000225s

[18] Shao W, Khin S, Kopp WC. Characterization of effect of repeated freeze and thaw cycles on stability of genomic DNA using pulsed field gel electrophoresis. Biopreservation Biobanking 2012; 10: 4-11. https://doi.org/10.1089/bio.2011.0016

[19] Amor H, Zeyad A, Alkhaled Y, *et al*. Relationship between nuclear DNA fragmentation, mitochondrial DNA damage and standard sperm parameters in spermatozoa of fertile and sub-fertile men before and after freeze-thawing procedure. *Andrologia*. Epub ahead of print 12 March 2018. https://doi.org/10.1111/and.12998

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