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Original Article

Optimisation of polymerase chain reaction conditions for detection of mineralization markers in isolated odontoblasts from human teeth

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Abstract The present study aimed to determine the best polymerase chain reaction (PCR) conditions for amplification of odontoblast markers; alkaline phosphatase (*ALP*), dentin matrix protein 1 (*DMP1*), dentin sialophosphoprotein (*DSPP*) and osteopontin (*OPN*). Informed consent was obtained from the individuals prior to tooth extraction. RNA was extracted from odontoblasts obtained from extracted teeth using innuPREP RNA Mini kit (Analytik Jena, Germany). Five selected target factors in enhancing PCR: primer concentration, extension time, number of cycles, annealing time, and annealing temperature were manipulated to yield the correct size of amplicons. One step reverse transcriptase PCR reactions were performed using MyTaq One-Step RT-PCR kit (Bioline, USA) with a C1000 Thermal Cycler (Bio-Rad, USA) in a 25 μL reaction, keeping the amount of 2 ng/μL RNA, 0.25 μL reverse transcriptase, 0.5 μL RiboSafe Rnase inhibitor and 1X MyTaq One-Step Mix, constant. The optimal conditions were determined to be 400nM of primers for *DMP1* and *DSPP*, 200 nM for *ALP* and *OPN*; 30 seconds of extension time and 35 PCR cycles for all genes; 10 seconds of annealing time for *ALP*, *DMP1* and *DSPP*, 7 seconds for *OPN*. The annealing temperature were 56.4°C for *ALP*, 58.6°C for *DMP1*, 52.7°C for *DSPP*, and 56.3°C for *OPN*, respectively. The optimized PCR protocols produced the correct size of odontoblast markers.

Keywords: odontoblast marker, optimisation, reverse transcriptase polymerase chain reaction.

Introduction

Polymerase chain reaction (PCR) is a powerful, relatively easy and inexpensive technique, of which a DNA segment can be multiplied over a million-fold with only a small amount of starting DNA. Because of this great sensitivity, PCR has been used in a wide range of applications. Although PCR has been the tool of choice for amplification of genetic sequences, developing a PCR protocol is not a straightforward procedure because a successful PCR protocol requires optimisation. However, an established PCR protocol that had been optimised and successful for the amplification of a certain DNA segment might not work with another different region of DNA. Poor amplification could be a result of one of these causes: the annealing temperature, annealing time, PCR cycle, extension time and primer concentration (Ausubel *et al.,* 1991). The optimal conditions for all PCR parameters which resulted with the best yield are usually determined by trial and error. An optimised PCR protocol would improve both product yield and reproducibility between reactions, besides reducing amplification of non-specific products (Møller, 2000). PCR product from an optimised PCR protocol would produce a bright single band with minimal background when electrophoresed on an agarose gel.

Cell surface marker is an antigenic determinant molecule found on the surface of cells that often function to characterize cell types (Cartwright, 2007). These markers allow cells to be defined based on what molecules are present on their surface. In vertebrates, odontoblast is a type of cell originating from neural crest that is part of the outer surface of the dental pulp. It

functions in dentinogenesis, which is the formation of dentin. There are several cell surface markers expressed by odontoblasts, namely *DSPP* (dentin sialophosphoprotein), *DMP1* (dentin matrix protein1), *ALP* (alkaline phosphatase), and *OPN* (osteopontin). The expression of these markers on odontoblast enables researchers to identify odontoblasts from other cells. Therefore, the present study aimed to optimise the PCR protocol for amplification of *DSPP*, *DMP1*, *ALP* and *OPN* using RNA extracted from odontoblasts obtained from human dental pulps.

Materials and methods

Collection of teeth

A total of 30 human teeth were obtained from healthy individuals who came for teeth extraction at the Dental Clinics, School of Dental Sciences, Universiti Sains Malaysia, Kelantan, Malaysia. The present study was approved by the Research and Ethics Committee, Universiti Sains Malaysia (USM/JEPeM/15050161), and informed consent was obtained from the donors prior to teeth collection.

Isolation of odontoblast cells

Odontoblast cells were obtained from the extracted human teeth. All teeth used in the experiments were extracted during normal treatment of patients. The procedures employed were modified from Tjäderhane *et al.* (1998). Initially, the attached periodontal ligament of the teeth was removed. Then, the teeth were cut using hard tissue cutter, crown vertically and the root horizontally. Dental pulp tissue inside the teeth was then taken out with extreme care and discarded. Following that, the teeth were soaked in TRIzol® reagent (Ambion, Life Technologies, USA) for 10 minutes. Odontoblast cells lining the pulp wall was then scratched into TRIzol® reagent with needle. The TRIzol® reagent containing odontoblast cells was then collected into a microtube, and subjected to RNA extraction.

RNA extraction

RNA extraction was done using innuPREP RNA Mini Kit (Analytikjena, Germany) according to manufacturer's protocols. The concentration of the extracted RNA was then quantified using BioPhotometer Plus (Eppendorf, Germany).

Optimisation of PCR protocol

One step reverse transcriptase PCR was performed using MyTaq™ One-Step RT-PCR kit (Bioline, USA) with a C1000 Thermal Cycler (Bio-Rad, USA) in a 25 μL reaction, with the amount of 2 ng/μL RNA, 0.25 μL reverse transcriptase, 0.5 μL RiboSafe RNase inhibitor and 1x MyTaq One-Step mix, was kept constant. The sequence of the primers with their references and respective product sizes are presented in Table 1. The PCR protocol generally consists of 35 cycles of three different steps: denaturation at 95°C for 10 seconds, annealing at various temperature for different time (in seconds), and extension at 72°C for 30 seconds. Different PCR conditions for each gene were optimized according to five selected target factors: primer concentration, extension time, number of cycles, annealing time, and annealing temperature. The concentration of primers tried was between 200 to 400 nM. On the other hand, the annealing temperature was tested between 50 - 60°C, while the annealing time was tested between 7 to 10 seconds.

Gel electrophoresis of amplicons

A total of 2 µl of the PCR products were mixed with 2 µl of loading buffer (10 X BlueJuice™ Gel Loading Buffer (Thermo Fisher Scientific, USA), and the amplicons were electrophoresed on different concentrations of agarose gel in LB buffer at 70V (Power Pac HC, Bio-Rad, USA) for 60 minutes. Agarose gel with concentration of 0.8% was used to electrophorese *OPN*, while a concentration of 1.2% was used for *DSPP*. Amplicons of *ALP* and *DMP1* were electrophoresed on 1.5% agarose gel. All gels were visualised and photographed under UV light using digital image analyser (Gel Doc XR System, Bio-Rad, USA). Appropriate product size of specific genes was indicated by the production of discrete single band on the respective gel.

Results

The present results have shown that the optimal concentration of primer for *DMP1* and *DSPP* was 400 nM and 200 nM for *ALP* and *OPN*. The extension time of 30 seconds was found to be ideal for all genes, while the total number of PCR cycles was 35. The optimum annealing temperature yielding the correct PCR products were 56.4°C for *ALP*, 58.6°C for *DMP1*, 52.7°C for *DSPP*, and 56.3°C for *OPN*. The ideal annealing time was 10 seconds for *ALP*, *DMP1* and *DSPP*, and 7 seconds for *OPN*. The PCR protocol for amplification of each odontoblast marker is presented in Table 2, while the summary of optimised PCR conditions is tabulated in Table 3.

The amplified PCR products for *ALP* and *DMP1* are shown in Fig. 1 which were best visualised on 1.5 % agarose gel. The amplicon of *DSPP* is shown in Fig. 2. Amplicon from the optimised PCR protocol of *DSPP* gave clear band when electrophoresed on 1.2% agarose gel. Fig. 3 shows the results from optimisation of the annealing temperature for OPN. Based on the bands obtained on the 0.8% agarose gel, the most ideal annealing temperature was chosen to be 56.3°C.

Table 1 Sequence of primers for the different odontoblast markers and their respective product sizes

Gene	Primer Sequences	bp	Annealing Temperature (°C)	References
Dentin Sialophosphoprotein (*DSPP*)	F: 5'-TGTCGCTGTTGTCCAAGAAG-3' R: 5'-ATTCTTTGGCTGCCATTGTC–3'	496	F: 55.3 R: 53.9	Kitagawa *et al*. (2007)
Dentin Matrix Protein 1 (*DMP1*)	F: 5'-CAGGAGCACAGGAAAAGGAG-3 R: 5'-CTGGTGGTATCTTGGGCACT-3	213	F: 55.6 R: 56.9	Kim *et al*. (2010)
Alkaline Phosphatase (*ALP*)	F: 5'-CCACGTCTTCACATTTGGTG-3' R: 5'-AGACTGCGCCTGGTAGTTGT-3'	196	F: 53.4 R: 58.2	Chadipiralla *et al*. (2010)
Osteopontin (*OPN*)	F: 5'-CATCTCAGAAGCAGAATCTCCTA-3' R: 5'-GGAAAGTTCCTGACTATCAATCA-3'	659	F: 56.0 R: 54.2	Khodavirdi *et al*. (2006)

Table 2 PCR protocol for amplification of the different odontoblast markers

		PCR conditions			
		DSPP	*DMP1*	*ALP*	*OPN*
Reverse transcription		45°C 20 mins	45°C 20 mins	45°C 20 mins	45°C 20 mins
Polymerase activation		95°C 20 mins	95°C 20 mins	95°C 20 mins	95°C 20 mins
Cycles	Denaturation	95°C 10 secs	95°C 10 secs	95°C 10 secs	95°C 10 secs
	Annealing	52.7°C 10 secs	58.6°C 10 secs	56.4°C 10 secs	56.3°C 7 secs
	Extension	72°C 10 secs	72°C 10 secs	72°C 10 secs	72°C 10 secs

Table 3 Summary of optimised PCR conditions for the amplification of different odontoblast markers

Variables	Gene			
	DSPP	***DMP1***	***ALP***	***OPN***
Annealing temperature (°C)	52.7	58.6	56.4	56.3
Primer concentration (nM)	400	400	200	200
Annealing time (seconds)	10	10	10	7
Extension time (seconds)	30	30	30	30
Number of PCR cycles	35	35	35	35

Table 4 RT-PCR conditions as recommended for MyTaq One-Step RT-PCR kit (Bioline, USA)

Steps	Number of cycles	Temperature (°C)	Time
Reverse transcription	1	45	20 minutes
Polymerase activation	1	95	1 minutes
Denaturation	40	95	10 seconds
Annealing		60	10 seconds
Extension		72	30 seconds

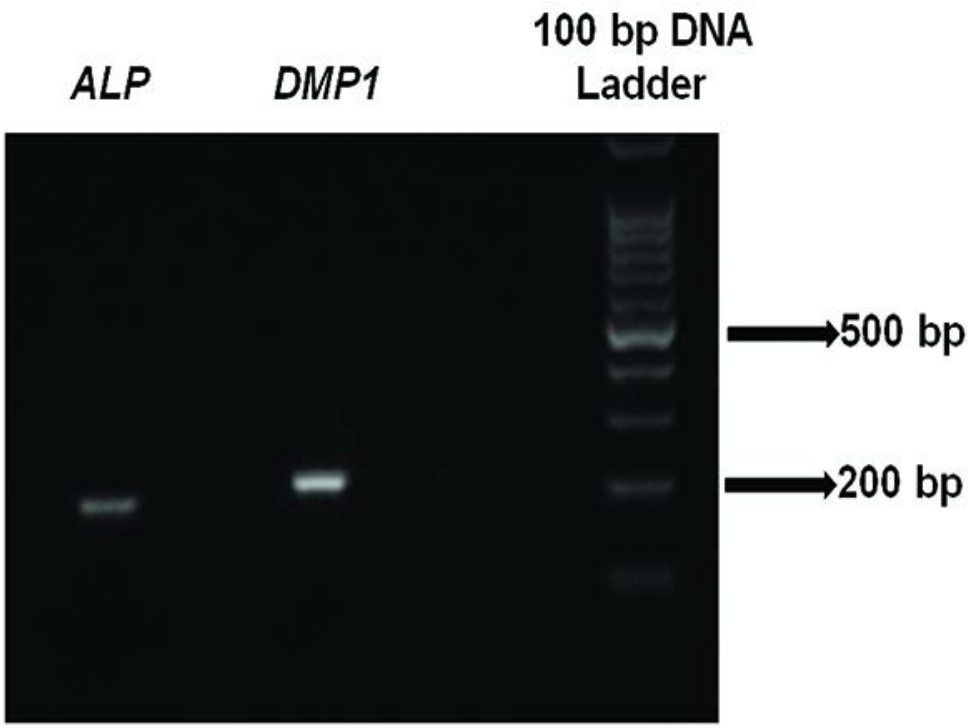

Fig. 1 Gel electrophoresis on 1.5% agarose at 70V for 60 minutes showing PCR products of ALP (196 bp) and DMP1 genes (213 bp), respectively.

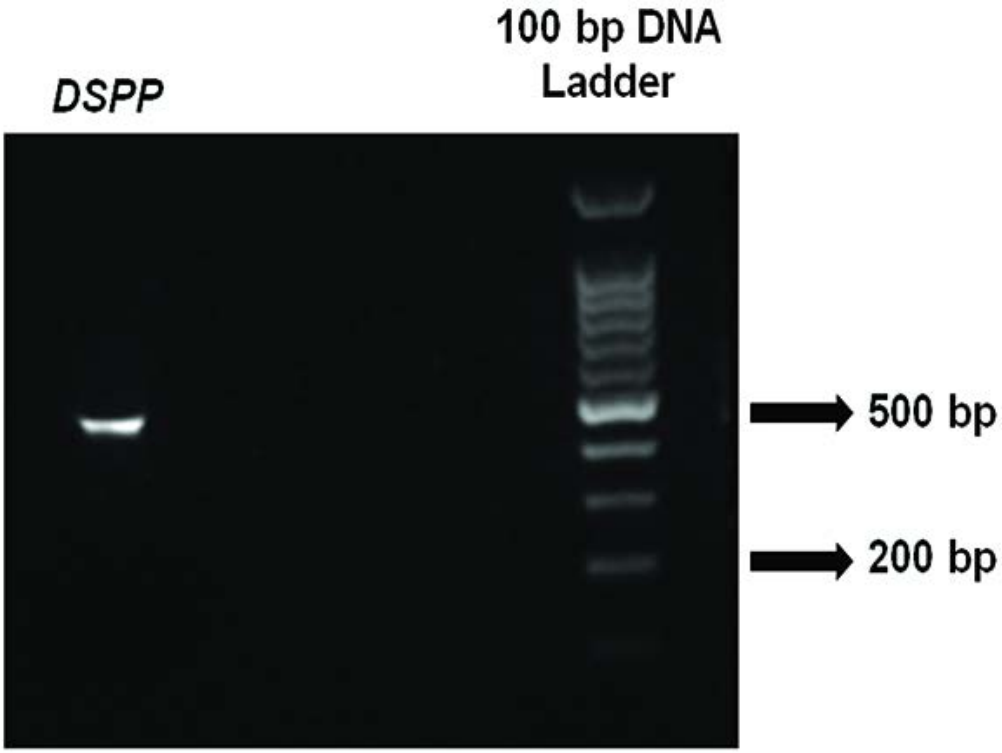

Fig. 2 Gel electrophoresis on 1.2% agarose at 70V for 60 minutes showing PCR product of DSPP gene (496 bp).

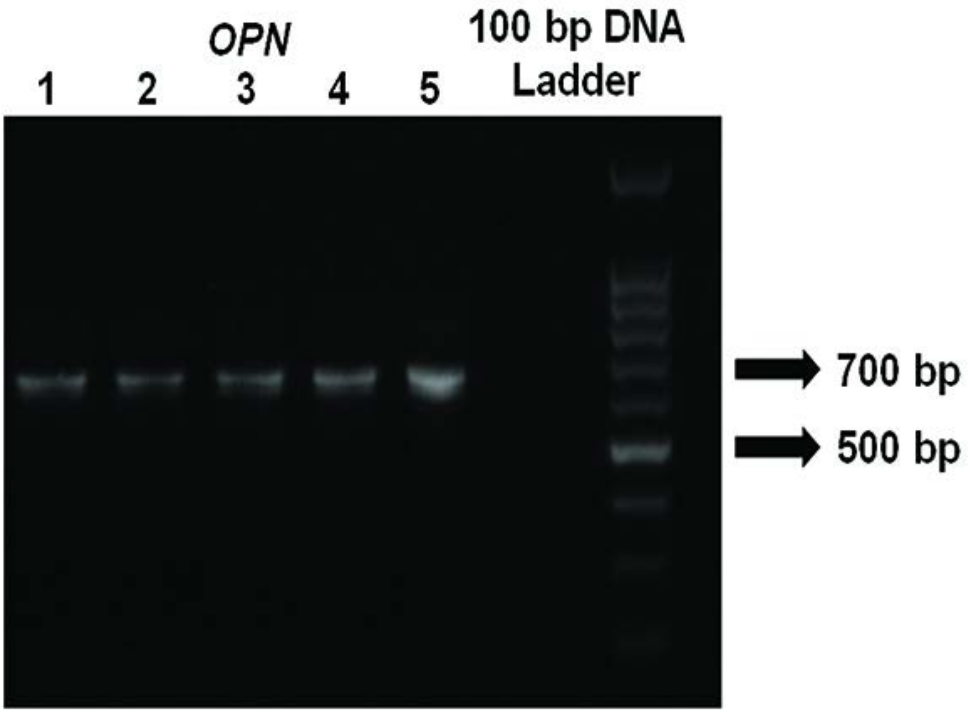

Fig. 3 Gel electrophoresis on 0.8% agarose at 70V for 60 minutes showing PCR products of *OPN* gene (659 bp). Lanes 1, 2, 3, 4 and 5 are five distinct amplicons from temperature 56.3°C, 58.3°C, 60.3°C, 62.3°C and 64.3°C. Temperature used in Lane 1 was chosen as the optimised annealing temperature for PCR protocol for amplification of *OPN* gene.

Discussion

During mature teeth development, a highly-specialized cell called odontoblast, lining the pulp cavity of a tooth, is responsible for synthesis and secretion of its collagenous and non-collagenous extracellular matrix (ECM) components (Goldberg and Smith, 2004). Odontoblasts are originally differentiated from mesenchymal papilla cells during human craniofacial development by migration of neural crest cells (Arana-Chavez and Massa, 2004). Among those, dentin sialoprotein and dentin phosphoprotein are dentin specific and others like alkaline phosphatase, osteopontin and dentin matrix protein 1 are commonly expressed by differentiated odontoblasts (Lee *et al.,* 2012).

Dentin sialophosphoprotein

DSPP is a secreted precursor protein that is rapidly cleaved post-translationally into two dentin products: dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) (Ritchie *et al.,* 1994; MacDougall *et al.,* 1997). DSP and DPP are polyionic macromolecules that contain large amounts of carbohydrate and sialic acid and relatively high level of aspartic acid and phosphoserine. This feature proposed an important role of dentin ECM in dentin mineralization and mineral nucleation (George *et al.,* 1996). Several experimental data have demonstrated high expression of DSPP in odontoblasts, transiently in preameloblasts, and low level expression in bone (D'Souza *et al.,* 1997; Baba *et al.,* 2004; Qin *et al.*, 2002). Altogether, these data suggest the key role of DSPP in tooth formation and mineralization.

Dentin matrix protein 1

DMP1 is an acidic phosphoprotein that regulates many important cellular processes including differentiation and ECM production which is normally expressed by both osteoblasts and odontoblasts (George *et al.,* 1993). This non-collagenous protein is highly composed of aspartic acid, glutamic acid, and serine residues (George *et al.,* 1994; MacDougall *et al*., 1998). DMP1 constitutes a crucial component of bone and dentin matrix and therefore believed to play an important role in the initiation of mineralization through the regulation of mineral crystallinity as well as its morphology (Linde and Goldberg, 1993). Moreover, early expression DMP1 gene can be observed during the commitment of neural crest-derived cells into odontoblasts (Narayanan *et al.,* 2006).

Osteopontin

OPN is an acidic, rich phosphorylated glycoprotein, and highly expressed by cells residing in bone, teeth, and its expression is often present in several pathologic calcification sites of soft tissues (Rathinam *et al.,* 2015). In bone, OPN is a major component found in cell-matrix interfacial structures called cement lines and lamina limitans (McKee and Nanci, 1996). In normal bone remodelling, OPN is responsible for the regulation of bone cell adhesion, migration, osteoclast function, cell survival and deposition of bone matrix (Sodek *et al.,* 2000). OPN also induces pulp mineralization in the early stages of matrix formation and helps the initiation of reparative dentinogenic process. The function of OPN is also implicated in diverse biological and molecular events taking place during the differentiation of their progenitor cells into new odontoblast-like cells (Kuratate *et al.,* 2008).

Alkaline phosphatase

ALP is an endogenous enzyme present in many organs, particularly detectable in high quantities in liver, bile duct, kidney, bone, intestinal mucosa (Rosalki and Mcintyre, 1999). ALP activity is most often involved in the beginning of matrix mineralization and has been proposed as an early marker in the cascade of osteo/odontoblast differentiation (Min *et al.,* 2010). Pang *et al*. (2006) reported that the activity of ALP in odontoblast-like cells is higher than undifferentiated cells. Therefore, ALP is believed to stimulate the formation of mineralized nodule and calcium deposition in most cells differentiated into odontoblasts, as it is considered as an early marker of odontoblastic differentiation (Pang *et al.,* 2006).

Optimisation of PCR protocol for odontoblast markers

Reverse transcriptase PCR (RT-PCR) optimisation strategy has several steps that can be classified into pre-PCR and PCR, and each of the steps is equally important to obtain optimal amplification.

Pre-PCR steps in improving the quality of RNA sample

Quality, purity and integrity of the starting RNA play an important role in determining the validity and accuracy of gene expression analysis by RT-PCR (Fleige *et al.,* 2006). Low quality RNA may likely to compromise the results of downstream applications which are often labour intensive, time consuming and expensive (Raeymaekers, 1993; Imbeaud *et al.,* 2005). Thus, great care should be taken during sampling, extraction, and dilution of RNA. In the present study, quantity and quality check of RNA samples were performed prior to optimisation of PCR protocol using biophotometer and gel electrophoresis (result not shown).

PCR steps in improving the quality of RNA sample

There are numerous variables in PCR optimisation. In the present study, five selected target factors that are most crucial were chosen to ensure successful PCR amplification: annealing temperature, primer concentration, annealing time, extension time, and number of cycles. Each factor is independent to each other. The adjustment of each factor was made by taking the manufacturer's suggested RT-PCR condition for MyTaq One-Step RT-PCR kit as baseline protocol for the present study. The manufacturer's suggested RT-PCR condition for MyTaq One-Step RT-PCR kit is shown in Table 4.

Annealing temperature

Annealing temperature (Tm) is one of the most important parameters that need adjustment in the PCR reaction. In the present study, the annealing temperature was optimised by using the primers' manufacturer given temperature (Table 1) as a reference point. Tm is affected by the individual PCR buffer components and even the primer and template concentrations. Tm provided by the primers' manufacturer would give a first approximation of the optimum annealing temperature for a given set of reaction conditions. In the present study, several PCR reactions were run at a range of temperature in gradient of 10°C above and below the given Tm. The resulted PCR products were analysed using agarose gel electrophoresis. In the present study, temperature gradient steps were also performed to optimise the annealing temperature. The optimal Tm selected was the one that resulted in the specific product without any non-specific amplification.

Primer concentration

Primer concentration should be enough to allow sufficient sensitivity, specificity and fidelity within the reaction. Increased concentration of primer would increase the risk of primer-dimer formation. Primer concentrations between 200nM and 600nM are recommended for successful PCR amplification. In the present study, 400nM was selected as the starting concentration. It is recommended that the primer concentration of the gene with lower gene expression level is increased so that similar PCR band is observed on the gel, and a comparison of different PCR products is possible (Persson *et al.,* 2005). From the present results (not shown), it was found that the PCR products for *ALP* and *OPN* showed multiple bands on agarose gel, and hence, the concentration of primer for both genes were reduced to 200nM.

Annealing time

The annealing times were also changed accordingly in the PCR cycles to optimise the PCR condition for the odontoblast markers. Annealing times greater than 10 seconds would produce smeared PCR amplified products (Mamedov *et al.,* 2008). Hence, annealing time was tested at 10 seconds for all genes of interest in the present study. Prolonged annealing improves yield and the annealing time must be sufficiently long to form the ternary complexes at the correct template site; however, excessive annealing time might

create the opportunity for ternary complexes to form at incorrect binding sites leading to non-specific band (Subramanian *et al.,* 2008) Thus, PCR protocols must adopt shortened annealing times to optimize the efficiency of annealing. In the present study, the annealing time for *OPN* was reduced to 7 seconds due to the presence on non-specific band at 10 seconds.

Extension time

In the present study, the PCR protocols were run for 30 seconds of extension time following the manufacturer's protocol. The activity of *Taq* DNA polymerase is approximately 150 nucleotides per second. Extension time is increased considering the reaction conditions, template quality, and inhibitors. When larger DNA fragments are amplified, the extending time is also increased, as shorter extension time would not be sufficient for a complete replication of the target (Higuchi, 1989). However, it is important to prevent excessively long extension times, as they will increase the likelihood of creating artefacts associated with the intrinsic exonuclease activity of *Taw* DNA polymerase (Longley *et al.,* 1990; Bell and DeMarini, 1991).

PCR cycles

In the present study, the numbers of PCR cycles were optimised to obtain the best PCR product. The number of PCR cycles depends on the amount of template DNA and the expected yield of the PCR product. For less than 10 copies of template DNA, 40 cycles should be performed. For higher quantity of template DNA, 25 - 35 cycles of PCR are usually sufficient. Using too few PCR cycles could lead to insufficient amplification (Debnath *et al.,* 2010). In the present study, the use of 35 PCR cycles in the PCR protocols for all the genes of interest was found to be efficient.

Conclusion

Generally, the most favourable concentrations for all PCR components and the most optimal thermal cycling parameters are achieved after experimenting rigorously. This is necessary not only to improve product yield, but also to enhance the reproducibility of the reactions and to minimise non-specific product amplification. In the present study, the PCR protocols have been optimised for the odontoblast markers, *DSPP, DMP1, ALP* and *OPN* which yielded the correct size of PCR products.

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