CHAPTER 3

METHODOLOGY

This study was divided into three parts: Part I, the proliferation assay; Part II, the dentino-/osteogenic capacity; and Part III, a quantitative RT-PCR to compared the mineralization markers of 3Mix-treated cells to untreated cells.

Dental pulp and apical papilla cells were isolated from non-infected third molars. For experimental group in all parts, DPCs/APCs were treated with 0.39 µg/mL for seven days. After the treatment period, complete regular media was used to culture both type of cells in PartI. These cells were further investigated for their proliferation compared to control groups using a colorimetric quantification of 3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyl tetrazolium bromide (MTT) at days 1, 3, 5 and 7.

Both control groups and experimental groups of DPCs and APCs in PartII were cultured in dentino-/osteogenic differentiating media for 7, 14 and 21 days and further analyzed for their mineralization potential using spectrophotometrically quantitative Alizarin-red S de-staining method. Additionally, both type of cells were observed for their cell morphology using Hematoxylin and Eosin disclosed.

Eventually, the mRNA expression of mineralization markers of 3Mix-treated DPCs/APCs compared to untreated DPCs/APCs using quantitative RT-PCR (Roche Lightcycler® 480II, Basel, Switzerland). The mineralization genes which expected to study were BSP, ALP, DMP-1**.** The flow chart to explain the steps for conducting the experiment is shown in Figure 16.

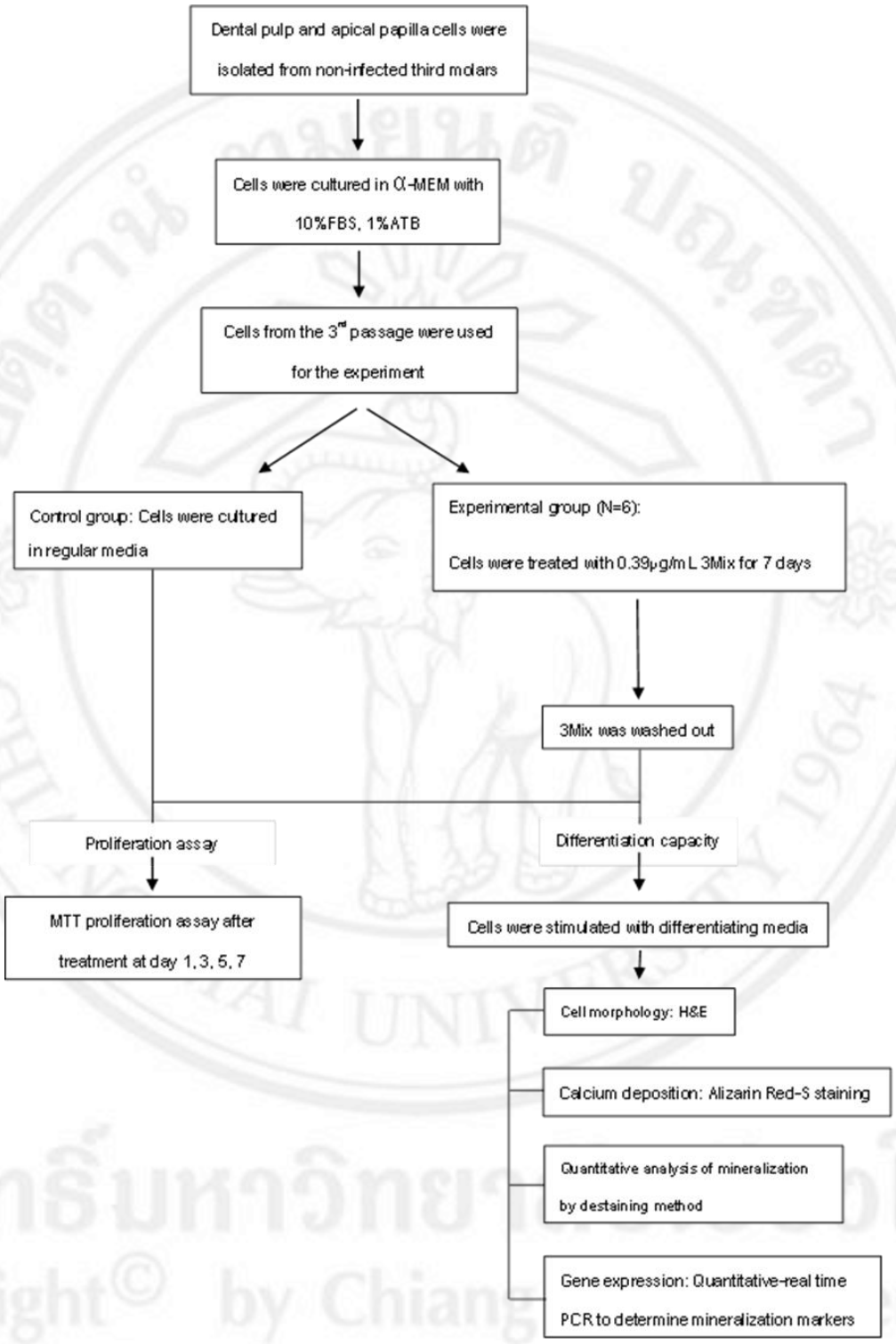

Figure 16: *Flow chart showing the steps for cell culture, group categorization, proliferation assay and differentiation capacity.*

1. Patient Recruitment

This study was approved by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand. After verbal and written informed consent, non-pathologic impacted third molars with/without immature roots from healthy patients (aged 18-25 years) were collected.

Inclusions criteria

- Third molars without diseases from healthy patients aged 18-25 years

Exclusions criteria

- Volunteers who had severe underlying diseases e.g. AIDS, Cancer.
- Volunteers who were under or over the selected age range.
- Teeth with pulpal or periapical disease (irreversible pulpitis, pulp necrosis with/without periapical lesion)

2. Tissue harvesting

The teeth were rinsed using sterile normal saline solution and stored on ice in separate containers with serum-free media. To obtain pulp tissue in mature teeth, teeth were soaked in 5.25% Sodium hypochlorite except for 3 mm from the root apex. The teeth were longitudinally sectioned into buccal and lingual parts (Figure 17), then pulp tissue was removed using a sterile endodontic spoon and immediately transferred into serum-free Alpha Minimum Essential Medium (α-MEM) (Sigma-Aldrich, St Louis, MO).

Figure 17: *Pulp tissue harvesting. The tooth was longitudinally sectioned into buccal and lingual parts.*

For immature teeth, the entire tooth surface was thoroughly cleaned and irrigated with sterile phosphate-buffered saline solution (PBS) (Sigma-Aldrich). The root apical papilla was gently separated from the root surface by tweezers (Figure 18), copiously irrigated with sterile normal saline and then transferred into serum-free α-MEM transport medium (Sigma-Aldrich). After that, the teeth were soaked in 5.25% sodium hypochlorite except for 3mm from apical foramina and used the same protocol for harvesting pulp tissue in mature teeth. Eventually, all samples were at once transferred to the laboratory for further cell processing.

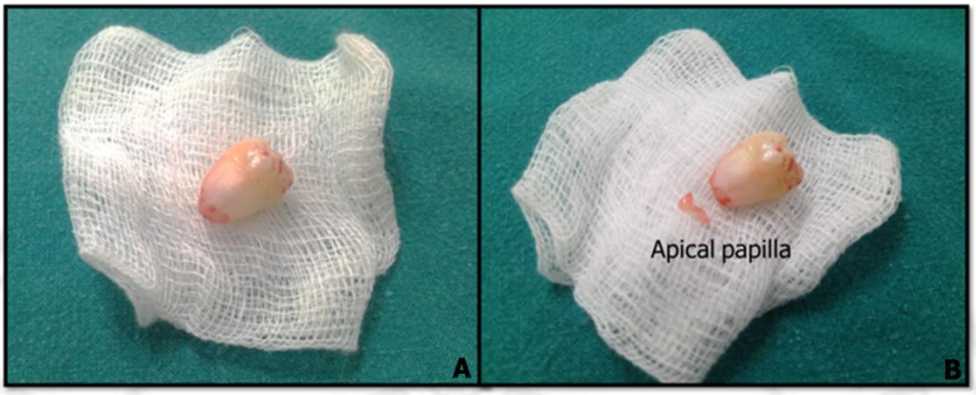

Figure 18: Apical papilla cell harvesting, the root apical papilla was gently separated from the root surface by tweezers.

3. Culture of primary human dental pulp cells and apical papilla cells

Pulp tissue and apical papilla were minced and digested separately in 3.00 mg/mL collagenase type I (Gibco/Invitrogen; Gaithersburg, MD) and 4.00 mg/mL dispase (Sigma-Aldrich) for 45 minutes at 37 °C. After that, cells were centrifuged at 1500 rpm at 25 °C for five minutes (Figure 19). The extracted cells were cultured in complete α-MEM (Sigma-Aldrich) containing 10% fetal bovine serum (Gibco/Invitrogen), 1% penicillin-streptomycin (Sigma-Aldrich) (100.00 U/mL Penicillin, 100.00 µg/ml Streptomycin) and 100 µmol/L L-ascorbic (Sigma-Aldrich) in a humidified atmosphere of 5% CO_2 and 95% air. Cells from the 3rd Passage were used in the experiment.

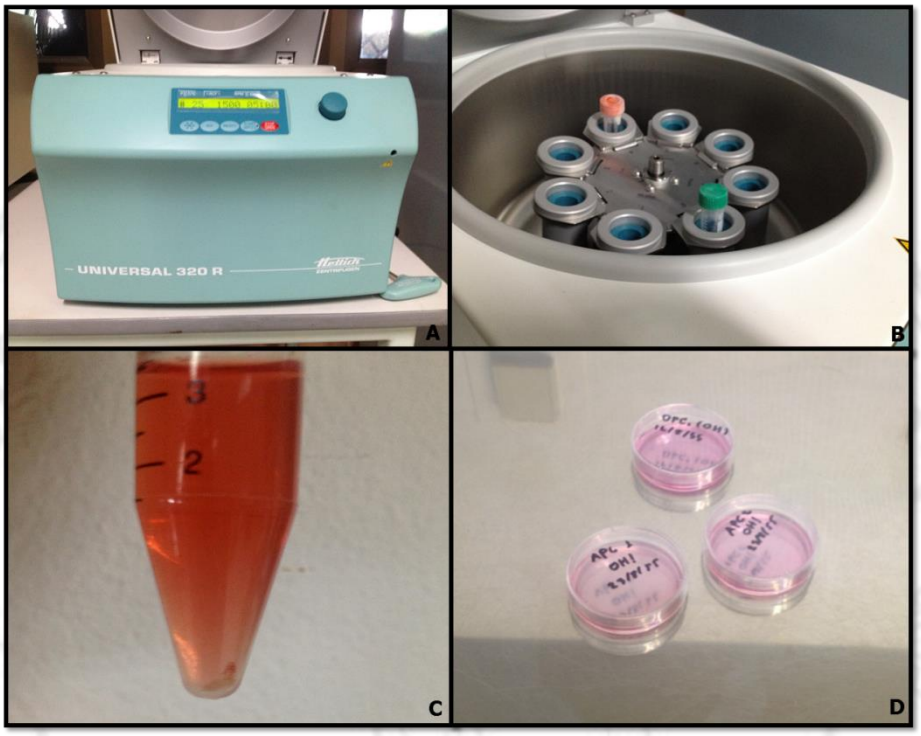

Figure 19: *Cell extraction using enzymatic technique. Both types of cells were centrifuged at 1500 rpm at 25 ˚C for five minutes (A, B). The expecting cells were precipitated at the buttom of volumetric tube (C). The extracted cells were cultured in complete α-MEM until use (D).*

Both types of cells were monitored using an inverted-light microscope and photographs were taken by a DP-12 Olympus camera. The culture medium was regularly replaced every three days. The sub-cultured process at a 1:3 ratio was achieved when both types of cells reached their confluence. DPCs and APCs from the third passage were used for this experiment.

4. Three-mix preparation and treatments

Preparation of antibiotics stock solution

To prepare a 150 µg/ml stock solution, ciprofloxacin 250mg/tablet (Khandelwal, Mumbai, India), metronidazole 200 mg/tablet (Piramel Healthcare, Gujarat, India) and minocycline 50 mg/capsule (Qualimed, Samut Prakarn, Thailand) were weighed and dissolved separately in de-ionized distilled water (DI)(Figure 20, 21), then sterilized by passing through 2 µm filtering papers (Whatman, Maidstone, Kent, England) and 0.2 µm microfilters (Corning, Oneonta, NY). Then a known concentration of 3Mix solution was prepared by mixing each drug at equal volume (Figure 22). All antibiotics stock solution was prepared at room temperature, and refrigerated at -20.00 ◦C until used.

Table 1. *The proportion of antibiotics and DI water volume used for preparing a stock solution of single antibiotics at the concentration of 150.00 µg/mL*

Antibiotic	DI water volume (mL)
ciprofloxacin 250mg/tablet	1666.67
metronidazole 200 mg/tablet	1333.33
minocycline 50 mg/capsule	333.33

Figure 20: *Preparation of 3Mix powder. Ground and crushed each antibiotic to a fine powder in a separated mortar.*

Figure 21: *Preparation of 3Mix in water. Each antibiotic powder was dissolved in measured DI water and unified using magnetic stirrer in a separated beaker.*

Figure 22: *Preparation of 3Mix stock solution. The dissolved single antibiotic was sterilized by passing through 2 µm filtering papers (A, B) and 0.2 µm microfilters (C). Then a known concentration of 3Mix solution was prepared by mixing each drug at equal volume to a final concentration of 150.00 µg/mL 3Mix stock solution (D).*

4.1 Preparation of Three-mix for the experiment

The stock solutions of 3Mix was freshly diluted to 0.39 µg/mL using complete culture media on the day of the experiment

5. The three-mix treatment for DPCs and APCs in experimental groups

Generally, a clinical protocol recommended for revascularization technique includes placement of intra-canal medication for some period. Therefore, to mimic the situation in patients, DPCs/APCs were treated either with 3Mix or without 3Mix for

seven days before further investigation. The cells were divided into two groups as follows:

1) Control group: DPCs/APCs were cultured in regular complete media for seven days
2) Experimental group: DPCs/APCs were cultured in complete media containing 0.39 µg/ml of 3Mix for seven days.

In all groups, after seven days, culture media were replaced with regular α-MEM. Then samples of 30,000 cells were plated into 6-well plates and samples of 5,000 cells into 24-well plates for further investigation.

6. MTT cell proliferation assay

3Mix-treated and untreated DPCs/APCs, which were plated and cultured in regular α-MEM, were used to determine their proliferative capacity. Briefly, DPCs and APCs were separately seeded into 24-well plates at 5,000 cells/well in regular complete media (n=6, triplicates). Cell proliferation was measured at the specific time intervals of 1, 3, 5 and 7 days using colorimetric quantification of 3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyl tetrazolium bromide (MTT)(Sigma-Aldrich). A freshly mixed MTT solution (5mg/mL MTT in phosphate-buffered saline solution) was added to growing cells at 80 µL/well, incubated at 37 °C and 5%CO_2 for three hours. After that, the medium was removed, each well was flushed with sterile PBS and 800µL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was added into each well.

One hundred microliters of the mixed solution was transferred into a 96-well plate, and absorbance at 550 nm was measured using a spectrophotometer (Sunrise; Tecan, Mannerdorf, Switzerland) (Figure 23).

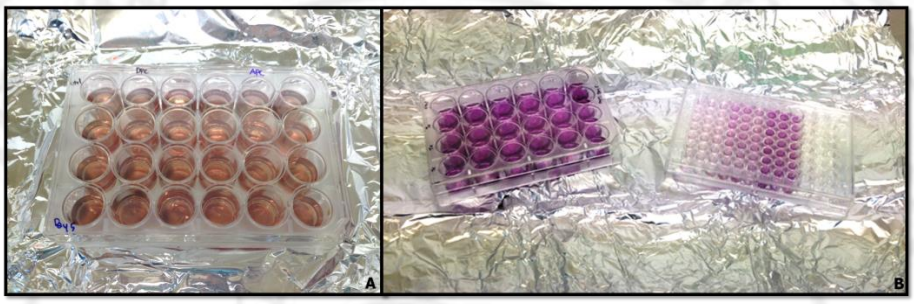

Figure 23: *MTT cell proliferation assay. A freshly mixed MTT solution was added to growing cells (A). The mixed solution of MTT and DMSO was transferred into a 96-well plate (B), and absorbance at 550 nm was measured using a spectrophotometer.*

7. Dentinogenic/Osteogenic differentiation capacity

3Mix-treated DPCs/APCs and control groups (without 3Mix treatment) were seeded at a density of 5,000 cells/well on 24-well plates. After 70% confluence in culture, the culture medium was replaced by differentiation medium containing complete α-MEM, 50 µg/mL ascorbic acid (Sigma-Aldrich), 10nmol/mL dexamethasone (Sigma-Aldrich) and 10 mmol/mL ß-glycerophosphate (Sigma-Aldrich). Then cells were cultivated for 7, 14 and 21 days in a humidified atmosphere of 5%CO_2 and 95% air with medium changed regularly every three days. The cells were monitored every day under an inverted-light microscope (DP12; OLYMPUS, Melville, NY).

7.1 Alizarin red-S staining and quantification assay

Alizarin red-S staining was used to detect calcium deposits. 3Mix-treated cells and control groups in dentinogenic differentiation medium were evaluated at 7, 14, and 21 days. All specimens were fixed with 4% paraformaldehyde in phosphate-buffered saline solution (4% PFA in PBS). Then, they were rinsed with 1 mL PBS/well and 0.5 mL of the alizarin red-S solution at pH 4.2 (Sigma-Aldrich) was added into each well. The specimens were then incubated at room temperature for five minutes with gentle rocking (Figure 24). Then the excess dye was thoroughly washed from the wells with deionized water. The specimens were visualized under an inverted-light microscope (OLYMPUS).

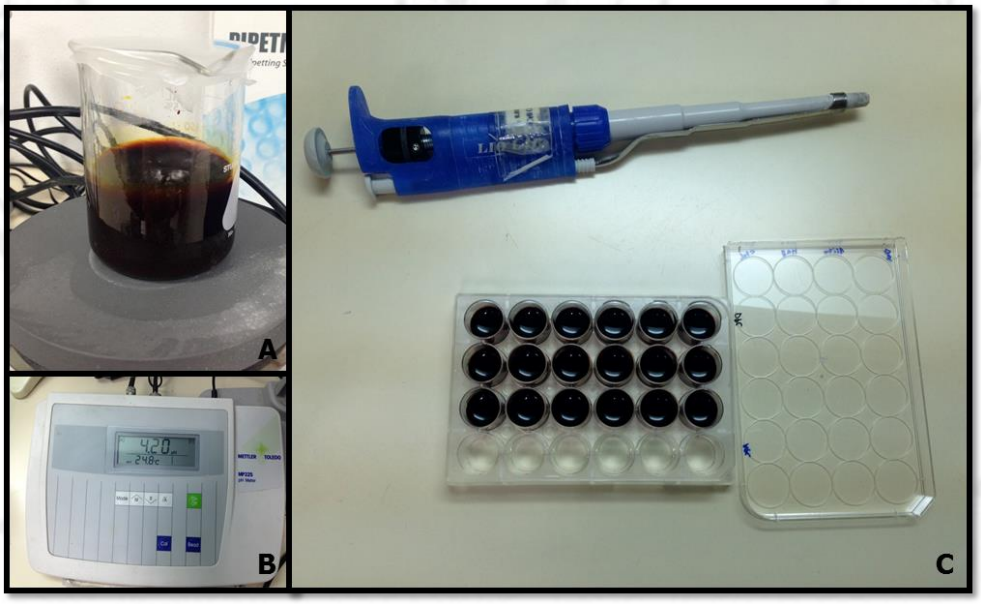

Figure 24: *The alizarin red-S staining method for mineralization assay. The alizarin red-S solution was prepared at pH 4.2 (A,B). The specimens were rinsed with that solution for 5 minutes (C) with gentle rocking.*

After all of the samples were air-dried at room temperature for one week, the amount of calcium deposit was quantitatively analyzed by de-staining with 10% cetylpyridinium chloride monohydrate (Sigma-Aldrich) in 10 mM sodium phosphate at room temperature for 20 minutes. One hundred microliters of de-stained solution were transferred into 96-well plates. The absorbance was measured spectrophotometrically at 550 nm (Figure 25).

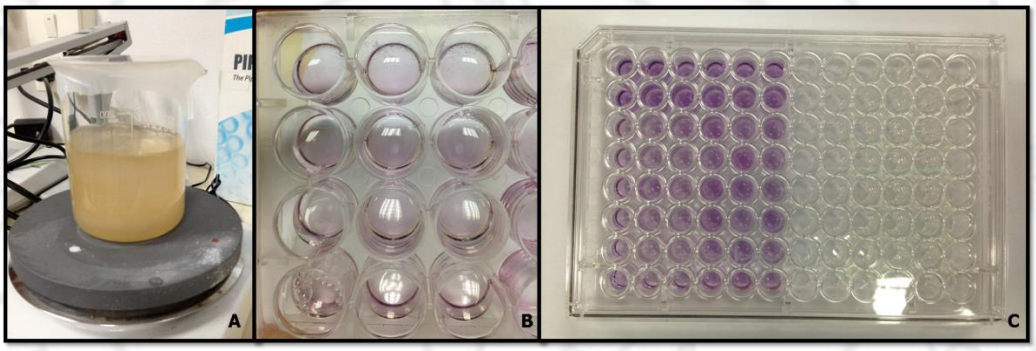

Figure 25: *Alizarin red-S de-staining and quantification assay. The amount of calcium deposit was quantitatively analyzed by de-staining with 10% cetylpyridinium chloride monohydrate in 10 mM sodium phosphate (A, B). One hundred microliters of de-stained solution were transferred into 96-well plates (C). The absorbance was measured spectrophotometrically at 550 nm.*

8. Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction (Real time RT-PCR)

3Mix-treated cells and those in the control group, all of which were cultured in dentinogenic differentiation medium at 30,000 cells/well were evaluated at 7, 14, and 21 days. Total RNA was collected using TRIZOL Reagent (Invitrogen, Burlington,

Canada) by adding 1 ml of reagent to all samples directly. Then the cell lysate was passed several times through a pipette and vortexed thoroughly. Samples were kept at -20 ◦C until used.

The mRNA extraction and first-strand cDNA synthesis were performed using an Illustra RNAspin Mini RNA isolation Kit (GE Healthcare, Buckinghamshire, UK). Then real time RT-PCR was carried out according to the manufacturer's instructions using human specific primers sets: Dentin matrix protein-1 (DMP-1) (sense: 5'-CCCTTGGAGAGCAGTGAGTC-3', antisense: 3'-CTCCTTTTCCTGTGCTCCTG-5'), Bone sialoprotein (BSP) (sense: 5'- AAAGTGAGAACGGGGAACCT-3', antisense: 3'-GATGCAAAGCCAGAATGGAT-5'), Alkaline phosphatase (ALP) (sense: 5'-CCACGTCTTCACATTTGGTG-3', antisense: 3'-AGACTGCGCrGGGTAGTTGT-5'). Glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) (sense: 5'-CAAGGCTGAGAACGGGSSGC-3', antisense: 3'-AGGGGGCAGAGATGATGACC-5') was used as an internal control. Quantitatively, SsofastTM Evagreen® Supermix (Bio-rad, Applied Biosystem, Berkeley, CA) was used together with the following thermal cycling conditions; 55 cycles at 95◦C for two minutes, 95 ◦C for 15 seconds, 60◦C for one minute, and 72◦C for 25 seconds. The relative expressions of DMP-1, BSP and ALP were calculated using the formula 2^[-(delta)(delta)Ct], and statistically analyzed.

Table 2. *Summary of the human specific primer sets used for the experiment.*

Gene name	Primer sequence (5'-3')
DMP-1	CCCTTGGAGAGCAGTGAGTC CTCCTTTTCCTGTGCTCCTG
BSP	AAAGTGAGAACGGGGAACCT GATGCAAAGCCAGAATGGAT
ALP	CCACGTCTTCACATTTGGTG AGACTGCGCrGGGTAGTTGT
GAPDH	CAAGGCTGAGAACGGGSSGC AGGGGGCAGAGATGATGACC

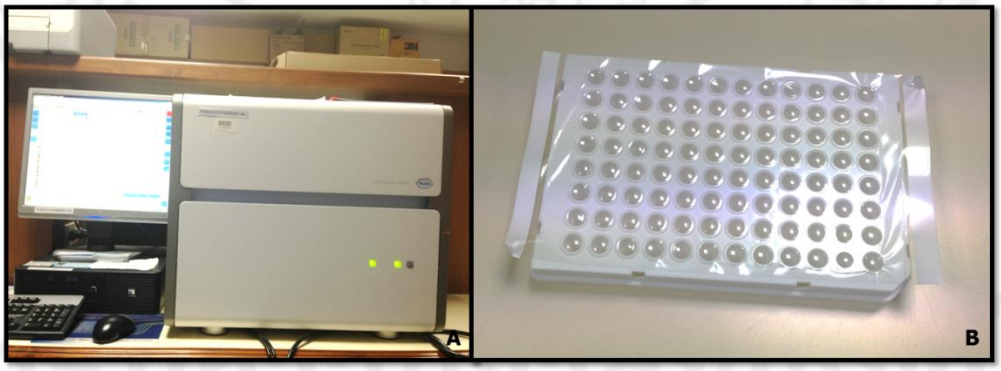

Figure 26: *Real time RT-PCR. The mRNA extraction and first-strand cDNA synthesis were performed using an Illustra RNAspin Mini RNA isolation Kit (GE Healthcare, Buckinghamshire, UK). Then real time RT-PCR was carried out (A, B) according to the manufacturer's instructions (Roche Lightcycler® 480II, Basel, Switzerland).*