DEVELOPMENT OF A PROTOCOL TO MEASURE GENE EXPRESSION IN THE MOUSE TIBIA

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PROJECT INFORMATION

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ABSTRACT

DEVELOPMENT OF A PROTOCOL TO MEASURE GENE EXPRESSION

IN THE MOUSE TIBIA

Daniel Hoover

Numerous molecular factors active in bone tissue direct fracture repair and remodeling which can be altered by disease conditions such as Peripheral Arterial Disease (PAD) and Osteoporosis. Methods of molecular biology are commonly applied to investigate the expression and role of these molecular factors. This project presents a robust three-step protocol for examining gene expression in the mouse tibia. The protocol begins with isolating RNA from a flash frozen tibia sample. The isolated RNA is reverse transcribed into cDNA. Finally, PCR is performed to indentify expressed genes. Establishing this protocol will allow further research into the mechanisms of bone remodeling and repair. For example, this approach can be used to examine the relationship between osteocyte apoptosis and angiogenesis in bone tissue, with applications to ischemic conditions and osteoporosis.

Keywords: osteocyte, angiogenesis, microcirculation, osteoporosis, fracture, molecular,

gene expression, remodeling, electrophoresis, PCR, reverse transcription, isolation, RNA

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“It is my deepest belief that only by giving our lives do we find life.”

-Cesar Estrada Chavez

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I Introduction

Project Goal

The purpose of this project was to develop a method for studying gene expression in mouse tibiae. Specifically, a protocol for analyzing gene expression of the mouse tibia was needed to pioneer a new area of research into the molecular factors governing osteocyte behavior under disease conditions such as PAD or Osteoporosis. This protocol was built on a collection of established methods and required a quarter of experimentation designed to adjust the existing protocols to overcome specific difficulties associated with bone tissue. A reliable and accurate method of studying gene expression in the mouse tibia was created to ensure that molecular biology continues to complement ongoing research into angiogenesis and bone microcirculation. Bone-vasculature interactions are many and manifold, but the immediate areas of application of this project are to the pathologies of Peripheral Arterial Disease (PAD) and Osteoporosis.

Background: Osteoporosis and PAD

Osteoporosis

Overview of Osteoporosis

The World Health Organization quantifies the impact of a disease by the number of disability-adjusted life years (DALYs) it costs the population. In American and Europe, fractures resulting from osteoporosis create about 2.8 million DALYs each year [6]. Half of postmenopausal women are predicted to have an osteoporosis related fracture and older men are also at risk. The fracture itself has less life impact than the pain, loss of

independence, impaired ambulation, depression, and nursing home admission that often follow [7]. Biologically, osteoporosis has several characteristics. First, there is an overall decrease in bone density resulting in weaker bone. Second, osteocytes suffer a loss of viability. Osteocyte function is critical for bone health and their deterioration has a serious impact on bone remodeling and bone composition regulation [1]. Third, in cancellous bone, osteoporosis results in fewer, narrower trabeculae that are no longer interconnected in the strong lattice framework of healthy bone [2].

Normal Bone Structure

Two types of bone tissue are present in the human body: cortical or compact bone, and cancellous or trabecular bone. The basic structural unit of compact bone is the osteon, consisting of concentric ring-like layers of matrix, termed lamellae, surrounding a central canal (see Figure 1 below) [1]. Collagen fibers interweave through compact bone tissue and vasculature clusters run through canals in the matrix, supplying oxygen and nutrients. Osteocytes inhabiting tiny lacunae sense strain conditions on bone and regulate remodeling activities. Trabecular bone is less dense than compact bone and features spindly trabeculae, thin spines, and plates of bone lattice that develop along principal-stress directions. Within the trabecular framework are cavities containing bone marrow. Trabecular bone is optimized for strength with a minimum weight of bone. It is found at the ends of long bones and in the middle of many other bone structures always enclosed by compact bone [1].

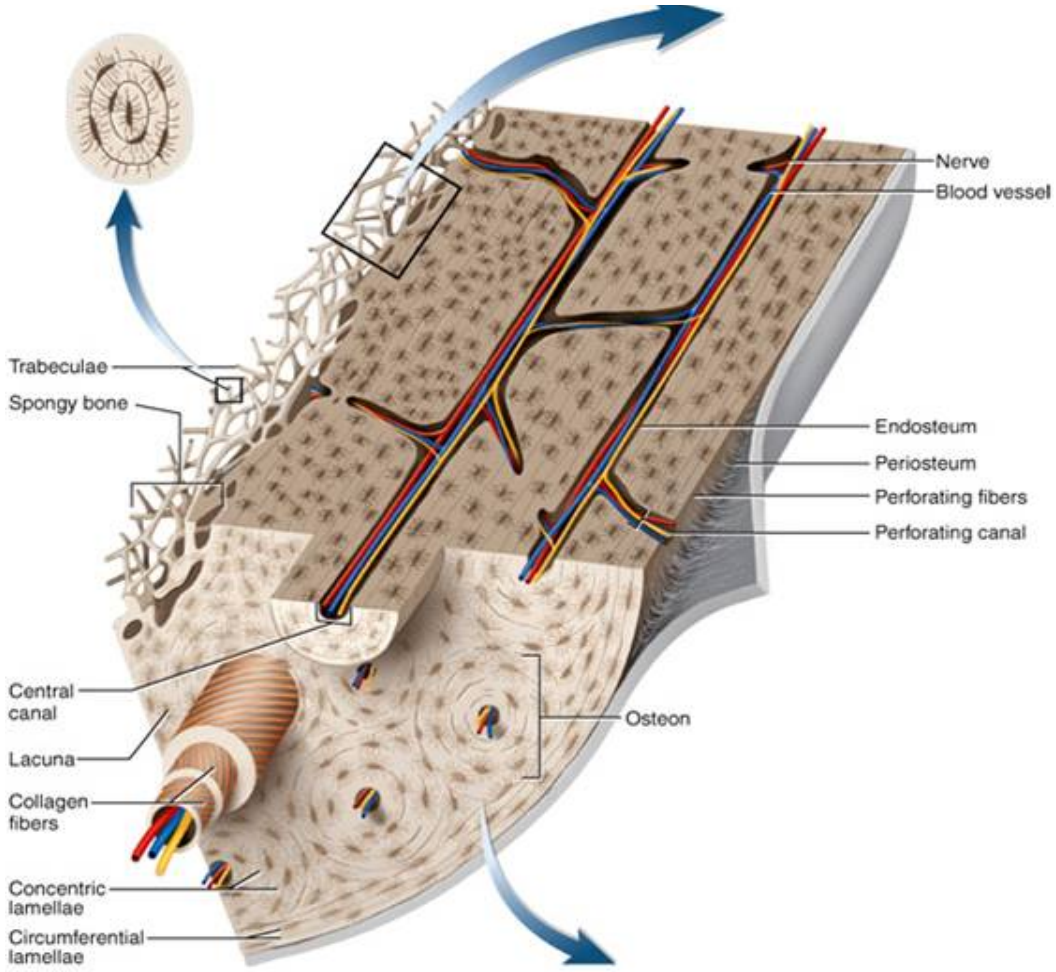

Figure 1 - Histology of Osseous Tissue [1].

Normal Bone Remodeling

Bone remodeling is both a normal response to changing stress conditions and a standard repair mechanism to compensate for daily wear. Remodeling is initiated by osteocytes responding to their environment [2]. Osteocytes perceive stresses and strains that correspond to microcracks in the bone, and release signaling factors that trigger remodeling. In damaged or growing regions, bone endothelium recruits osteoclasts using the RANKL/OPG system which will be discussed in more detail later [2, 3]. Bone remodeling is performed by the Basic Multicellular Unit (BMU) which is made up of

osteoclasts, osteoblasts, and osteocytes [3]. Osteoclasts function to resorb old or damaged bone (see Figure 2). They produce VEGF for the early stages of remodeling, which acts in a variety of capacities to encourage remodeling [3]. Osteoblasts deposit new bone (see Figure 2). Remodeling occurs in cycles beginning with resorption by the osteoclasts and ending with osteoblast deposition of fresh bone. In normal cancellous bone, remodeling takes place on the surface of the trabeculae with an average cycle duration of 200 days (see Figures 5 and 6 below). Resorption occurs relatively quickly, in 30-40 days, while bone formation occurs over a period of 150 days [3]. In cortical bone, osteoclasts travel in resorbing "cutting cones" and are directly followed by osteoblasts that refill the resorption cavity with new bone as shown in Figures 3 and 4 below. The remodeling cycle in cortical bone is shorter than the remodeling cycle in trabecular bone; in cortical bone, it has a median length of 120 days [3, 4].

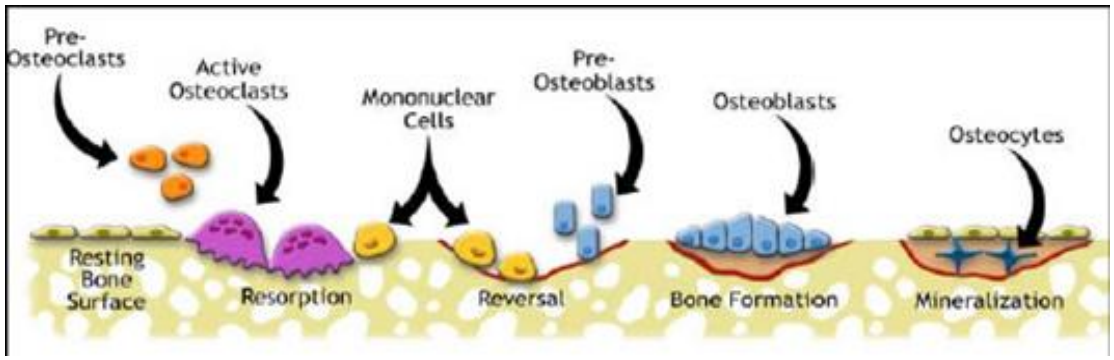

Figure 2 - Cell Types and Their Roles in Bone Remodeling [5].

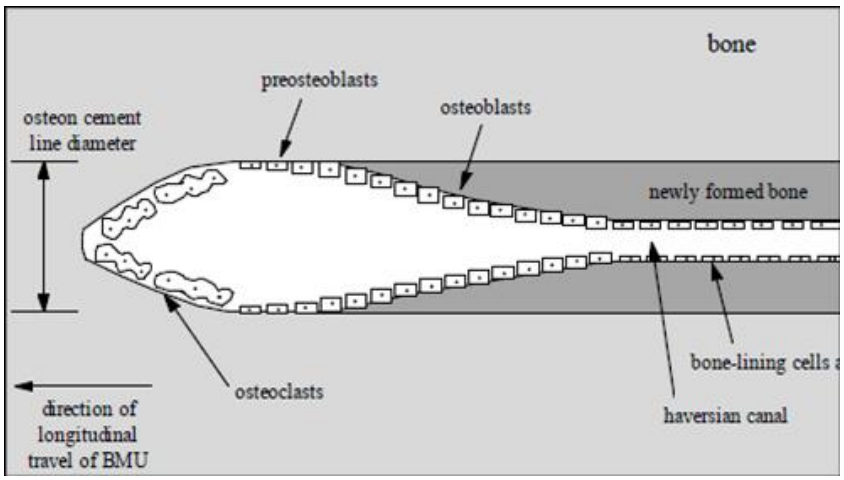

Figure 3 – Diagram of Remodeling in Cortical Bone [5].

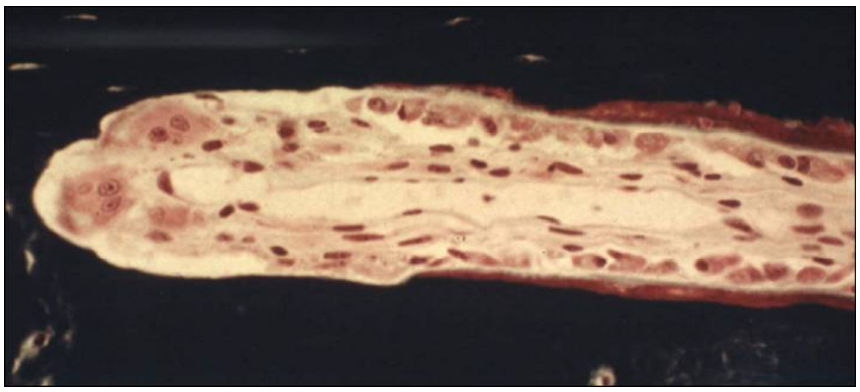

Figure 4 – Photomicrograph of Remodeling in Cortical Bone [5].

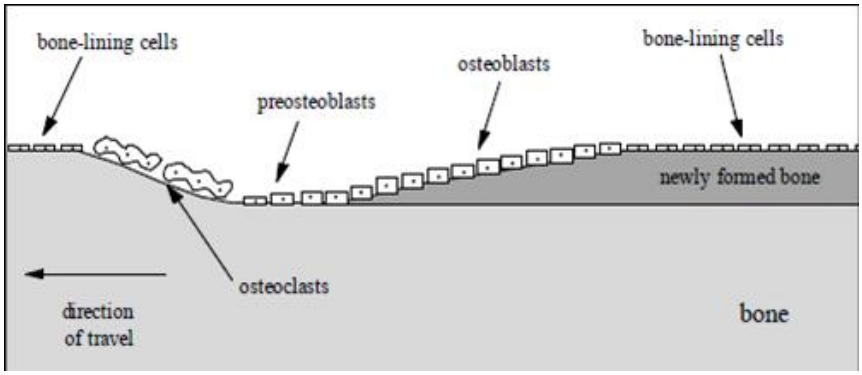

Figure 5 – Diagram of Remodeling in Trabecular Bone [5].

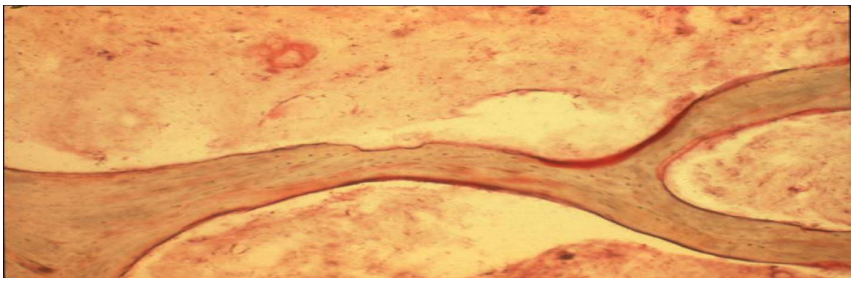

Figure 6 – Photomicrograph of Remodeling in Trabecular Bone [5].

Osteoporotic Bone Remodeling and Treatments

Bone remodeling continues under osteoporotic conditions, but it contributes to the diseased state rather than restoring bone structure. Osteoblast function is impaired, lagging far behind counterpart osteoclast activity. In normal bone remodeling, the remodeling cycle ends with a complete refilling of the resorption lacuna; in osteoporotic bone, the osteoblasts are unable to refill the entire cavity. Bone density is lost with each cycle of remodeling [3]. There are several treatments currently available for osteoporosis that target various biochemical pathways in bone. Treatment with Bisphosphonates targets the inhibition of osteoclast activity. Preventing osteoclast resorption effects a healthy increase in bone density. Unfortunately, complex instructions for administering this treatment result in inconsistent adherence to long term therapy [8]. In addition, there is concern that bisphosphonates over-inhibit bone remodeling, impairing fracture repair and allowing bone microcracks to accumulate [9]. Treatment with Strontium Ranelate increases bone formation and decreases bone resorption [10]. Its mechanism of action is not well understood and may have unforeseen drawbacks as is the case with other modern treatment strategies. There are no treatments for osteoporosis that focus on the impaired vascular system even though the vasculature heavily influences bone remodeling [2].

Peripheral Arterial Disease (PAD)

Peripheral arterial disease (PAD) is a serious chronic condition of the vasculature. Blood vessels are occluded by atherosclerotic plaques, impairing blood circulation. The disease's effects are most noticeable in the extremities; in the early stages, PAD causes claudication: intermittent pain during ambulation. In the late stages, PAD progresses to ischemic ulceration and gangrene ultimately resulting in amputation in 30 percent of cases [11]. Plaques are formed over time by the accumulation of ox-LDL, oxidized low density lipoprotein, in the subendothelial space [2]. The body responds with acute dilation of collateral vessels to redirect some of the blocked flow. When PAD persists, the chronic response of the vasculature is to remodel itself to provide less collateral resistance. The end result however is an exhaustion of the vasculature's ability to compensate and a severe reduction in the body's vasodilation and vessel repair capacity [2].

Link Between PAD and Osteoporosis

The conception of a link between these two pathologies is fascinating and compelling for several reasons. The diseases share common risk factors such as older age, physical inactivity, smoking, and diabetes mellitus [12]. The intermittent claudication of PAD restricts physical activity and results in less mechanical stimulation of the bone and less impetus for remodeling [2]. PAD often occurs concurrent with low bone mineral density (BMD). Decreases in bone formation and overall bone mass have been previously linked to deficiencies in the vasculature network and PAD-affected vasculature is deficient in many ways [19].

The potential for PAD and osteoporosis interaction exists because they share the same biological pathways and similar marker molecules [12]. In order for bone regeneration to take place, angiogenesis must occur to some degree. PAD affected vasculature is impaired in its ability to undergo both angiogenesis and arteriogenesis. Thus, PAD's adverse affects on bone remodeling may contribute to osteoporosis. There may exist a specific connection centering on the relationship between osteoprotegerin (OPG), RANK, and RANKL [2]. These three molecules work together to regulate remodeling. The excess osteoclast activity of osteoporosis stimulates OPG production to suppress that osteoclast activity. The increase in OPG also disrupts the RANK / RANKL interaction that ordinarily stimulates angiogenesis, leading to a potential lack of the angiogenesis that ordinarily should accompany bone remodeling [2]. Finally, both disease states are vitamin D deficient and vitamin D therapy has been very successful in patients with PAD and osteoporosis [2, 12]. The reason for these successes could be the general physical inactivity of patients suffering from each disease. Physical inactivity is common associated with a lack of vitamin D absorption from the sun. In conclusion, although there is more correlation than causal evidence at present, this hypothesized relationship between PAD and osteoporosis is well worth exploring.

Background: Genes of interest:

For this study, six osteocyte genes of interest were selected to design primers (see Table 1 below). These are expressed in bone tissue at varying times during the progression of PAD, the disease of osteoporosis, and fracture healing. Studying the

expression of these and similar factors under different experimental conditions will advance the current knowledge of molecular mechanisms in bone.

Table 1 - Signal factor molecules related to Osteoporosis, PAD, and fracture healing.

Signal Factor / Molecule	Functions
Cytokines (TNF-α) IL-1, IL-6, TNF-α	Cytokines contribute to osteoclast activation and bone resorption [2]. TNF-α stimulates OPG production to suppress osteoclast activity [2]. They are expressed at high levels during days 1 through 3 of bone remodeling. Cytokines increase extracellular matrix synthesis and attracts other inflammatory cells. They promote angiogenesis as well as bone resorption [15].
TGF-β (TGFβ-1) Transforming Growth Factor Beta Superfamily	The factors of the TGF-β superfamily are key regulators of bone development and repair [14]. They target mesenchymal stem cells and osteoprogenitors and incite cell proliferation of bone forming cells. TGF-β increases the density of the extracellular matrix (ECM) and is released throughout fracture healing process [15].
BMPs (BMP2) Bone Morphogenetic Proteins	BMPs promote differentiation of mesenchymal cells into chondrocytes and osteoblasts, and osteoprogenitors into osteoblasts [15]. In addition, they may stimulate VEGF synthesis [15]. Their expression during fracture healing takes many patterns.
VEGFs (VEGFa) Vascular Endothelial Growth Factors	The degree of collateral arteriogenesis depends on the extent of VEGF and VEGF-A gene expression [2]. For angiogenesis, VEGF incites proliferation and differentiation of endothelial cells (ECs). The higher the concentration of VEGF, the more EC proliferation and differentiation. VEGF also stimulates EC proliferation during endochondral formation in the case of fracture [15]. It can increase the rate of fracture repair when administered exogenously [20].
Angiopoietins (Ang1 & Ang2)	The angiopoietins affect the development of larger vessels and structures such as collateral branches [15]. The angiopoietin pathway co-regulates angiogenesis with VEGF.

Project Plan

As alluded to previously, the behavior of a tissue can be characterized to some extent by studying the gene expression of its cells. In this case, the protocol was created to analyze those signaling factors and molecules active in bone tissue by studying the gene expression of the bone cells. These bone cells contain genetic code in the form of DNA that is capable of encoding the factors of interest upon activation. The actual expression of a gene depends on the input the cell is receiving at that point in time. If the cell is triggered to produce a given factor by external stimuli, then it will transcribe messenger RNA from the DNA that codes that factor. The messenger RNA will be translated by ribosomes that assemble the protein or enzyme of interest. The point at which molecular biologists often intervene is after the DNA has transcribed mRNA. The protocol presented here begins with the isolation of mRNA from the bone cells.

To obtain gene expression data from the mRNA, three steps were performed post-isolation. First, the fragile mRNA was reverse transcribed into double stranded dsRNA or cDNA. cDNA is less vulnerable to destruction by environmental RNases and can also be used as starting material for PCR, Polymerase Chain Reaction. Second, a PCR reaction was run on the newly created cDNA. PCR functions to amplify a low concentration of cDNA fragments into quantities large enough to visualize [21]. Primers bind specific genetic material from the assortment of cDNA fragments. For this study, primers were selected to bind cDNAs corresponding to the genes of interest in Table 1. These cDNAs were amplified during PCR and then visualized by electrophoresis and imaging under UV light. PCR amplicons were identified by base pair length, corresponding to distance

traveled during electrophoresis. If an amplicon was present for a given gene, then that gene was being expressed, encoding mRNA to produce a certain protein or enzyme by translation.

RNA is difficult to extract from highly mineralized bone matrix but special reagents such as QIAzol make extraction possible. The contingency plan, if the Qiagen reagents failed, was to use a Trizol two step method of RNA isolation.

In conclusion, the project plan was to develop a protocol for analyzing the gene expression in mouse tibia; its essential components were RNA isolation, the reverse transcription of cDNA, primer design and PCR, and gel electrophoresis. This protocol will augment research into osteocyte activity in Osteoporosis and PAD.

II Methods

Methods Overview

Each tibiae was surgically extracted from the mouse and frozen for storage to prevent degradation. RNA isolations were performed on the frozen samples. RNA quality was tested via nanodrop spectrophotometry. If the sample contained quality RNA, it was reverse transcribed to make cDNA. Finally, cDNA was used as the starting material for PCR and selected primers were added. The PCR products were visualized on an electrophoresis tray under UV light.

Methods

The tibia sample was taken from a mouse specimen during surgery. IACUC guidelines were strictly observed and the animal remained anesthetized. After exposing the tibia, bone scissors were used to extract the tibia sample. The dissected sample was frozen to inactivate RNases and preserve the tissue. See Table 2 for an overview of this procedure.

Table 2 - Tibia Extraction Overview

Starting Material:	Mouse specimen
Ending Product:	Frozen 50 mg mouse tibia sample
Reagents:	-
Equipment:	Bone scissors, test tube

For the RNA isolation, first all benchtops were wiped down with RNase Zap wipes to eliminate RNases. Liquid nitrogen was used to flash freeze the sample and cool the Biopulverizer. Tibia samples were pulverized in the Biopulverizer and placed in a

centrifuge tube containing Qiazol solution. The sample was disrupted and homogenized under the fume hood using the Tissue Tearor. In the fume hood, chloroform was added to the lysate, the tube was vortexed thoroughly, and the solution was incubated, centrifuged, and separated. A series of washes and centrifugations purified the RNA in a Qiagen spin column. The RNA was eluted with the addition of RNase-free water and an absorbance reading was taken using the nanodrop spectrophotometer to assay RNA concentration. See Table 3 for an overview of this procedure.

Table 3 - RNA Isolation Overview

Starting Material:	Frozen 50 mg mouse tibia sample
Ending Product:	Purified RNA
Reagents:	RNeasy Mini Tissue Kit (Qiagen #74104), QIAzol (Qiagen #79306), liquid nitrogen, β-mercaptoethanol
Equipment:	BioSpec Biopulverizer, BioSpec Tissue Tearor, forceps, heating block, centrifuge, liquid nitrogen container, ice bucket, RNase Zap wipes, micropipettes, Nanodrop spectrophotometer

To begin the process of reverse transcription, all solutions were thawed, vortexed, and centrifuged. RNA samples and oligo(dT) primers were added and the reaction was incubated in the thermocycler. The remaining reagents were added. The reaction was completed in the thermocycler and the product cDNA was stored at -20°C for future PCR. See Table 4 for an overview of this procedure.

Table 4 - Reverse Transcription Overview

Starting Material:	Purified Quality RNA
Ending Product:	cDNA
Reagents:	AffinityScript Multiple Temperature cDNA Synthesis RT Kit (Agilent #200436)
Equipment:	Ice bucket, RNase Zap wipes, micropipettes

Following cDNA synthesis, PCR was performed using primers designed for genes of interest. A master mix was prepared containing all the components necessary for PCR except template cDNA and primers: 10x PCR Buffer, dNTP mix, HotStarTaq DNA Polymerase, and RNase-free water. Template cDNA was added to the mix, which was then divided among PCR tubes; the primers were added in forward and reverse pairs. PCR was performed in the thermocycler, following a cycling protocol similar to the one in Table 5, but adjusted to be primer specific. After the PCR reaction completed, the products were analyzed by electrophoresis. See Table 6 for an overview of the entire PCR procedure.

Table 5 - Thermocycler Protocol Design

Initial Activation Step:		15 min	95°C	HotStarTaq DNA Polymerase requires this heating to activate.
3-step cycle	Denaturation:	0.5-1 min	94°C	
	Annealing:	0.5-1 min	50-68°C	Aim 5°C below T_m of primers.
	Extension:	1 min	72°C	
	# of Cycles:	35		
Final Extension:		10 min	72°C	

Table 6 - PCR Overview

Starting Material:	cDNA
Ending Product:	Primer-selected amplified cDNA
Reagents:	HotStarTaq DNA Polymerase Kit (Qiagen #203203), dNTPs, forward and reverse PCR primers (IDT)
Equipment:	BioRad Gradient Thermocycler C1000, PCR tubes, ice bucket, micropipettes

The gel electrophoresis visualized the amplified cDNA products of PCR. An agarose gel was prepared with ethidium bromide. Loading dye was mixed with each PCR product and with the standard ladder. The samples were loaded into the gel matrix. After electrophoresis, the finished gel matrix was imaged under UV light. By comparing the fluorescent bands present in the PCR product lanes to the standard ladder markings, the primer-selected genes (if they were present) were identified. See Table 7 for an overview of this procedure.

Table 7 - Gel Electrophoresis Overview

Starting Material:	Primer-selected amplified cDNA
Ending Product:	Visualized and identified cDNA: gene expression data
Reagents:	Loading dye, standard ladder, agarose, ethidium bromide, TAE buffer
Equipment:	Electrophoresis: Electrophoresis tray and accessories, electrophoresis power supply, microwave, digital balance, spatula, Erlenmeyer flask Imaging: UV imager, Canon G10 camera, laptop with camera software

III Results

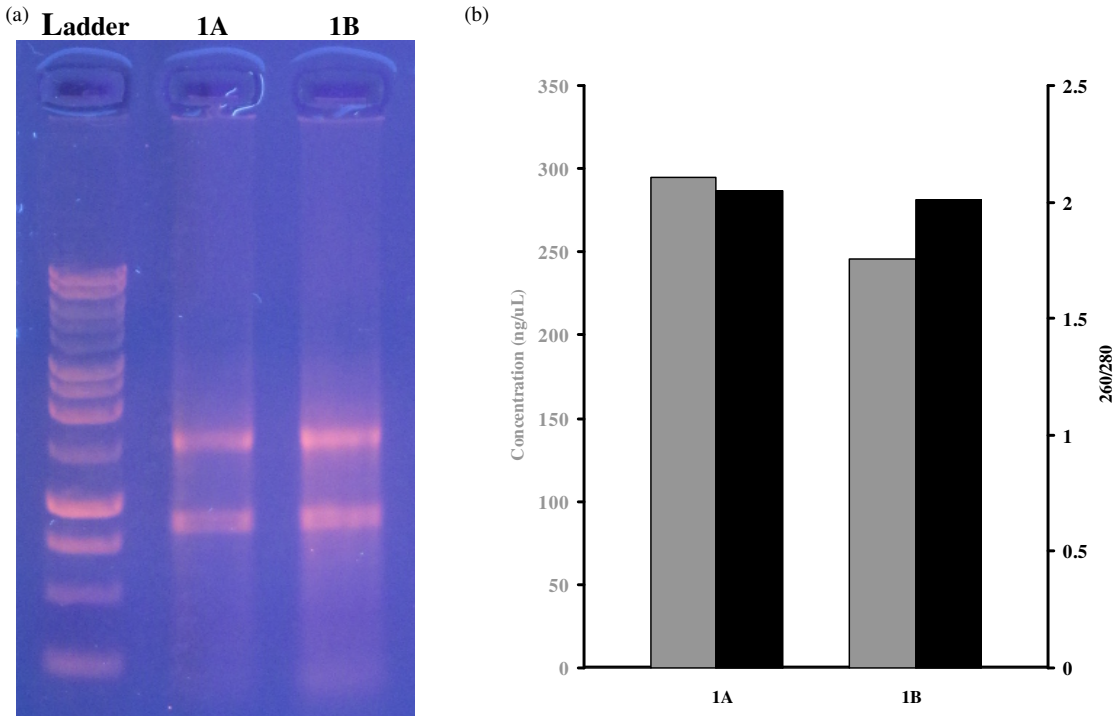

Figure 7 - (a) Quality RNA imaged via gel electrophoresis. Left: standard ladder. Middle: sample 1A. Right: sample 1B. (b) Column graph of RNA concentration (ng/μL) and RNA purity (A260/280).

Figure 7a is an image of two quality RNA samples obtained using the tibia isolation protocol. Both samples displayed the crisp 18S and 28S bands indicative of a quality RNA isolate. In theory, the 28S band should be approximately twice as bright as the 18S band. For both samples, the 28S band is brighter than the 18S band as expected, but without using an alternate technique, the brightness could not be measured quantitatively. Figure 7b is a graph of each samples' nucleic acid concentration and 260/280 ratio as measured with the nanodrop spectrophotometer. The concentration was high enough to use the RNA in a reverse transcription reaction. In quality RNA, the 260/280 ratio should be approximately 2.0; the sample ratios presented here are excellent.

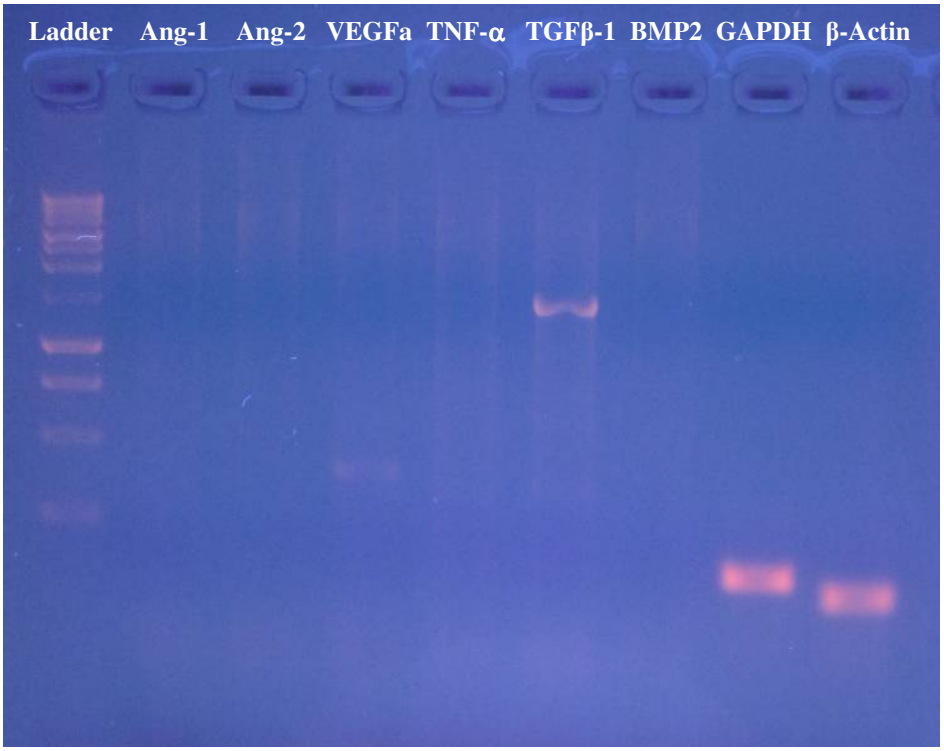

Figure 8 - PCR amplicons using cDNA reverse transcribed from RNA isolate 1B.

Table 8 - Primers

Gene	5' F Primer 3'	5' R Primer 3'	Tm
Ang-1	CCAGAGAATGCCACTCACAATC	CAACCACCACAATCACCAGAAT	62.0°C
Ang-2	CGGAAACTGACTGATGTGGAAG	ATTCCAGAGATGTGGGCATTTT	61.9°C
VEGFa	AAGCCTGACATGAAGGAAGAGG	CACCGATCTGGGAGAGAGAGAT	62.0°C
TNF-α	ACATTGACAGGTCCAGCAAGAA	AAGCACACAGAAAAGCTGCAAG	62.0°C
TGFβ-1	AAGTCAGAGACGTGGGGACTTC	CTTCTCTGTGGAGCTGAAGCAA	62.0°C
BMP2	TGCCCCCTAGTGCTTCTTAGAC	CTTCATGTGCTGGAGTTGAACC	62.0°C
GAPDH	TATGTCGTGGAGTCTACTGGTGTC	AGTTGTCATATTTCTCGTGGTTCAC	56.0°C
β-Actin	CTGACAGACTACCTCATGAAGATCC	GTCTAGAGCAACATAGCACAGCTTC	58.0°C

Figure 8 above displays the successful results of a PCR performed on a sample of cDNA. The cDNA sample was prepared from "DH Tibia RNA 1B" shown in figure 7. The PCR was run using a temperature gradient 56-58°C. TGFβ-1 primers created a strong amplicon in lane 6 around 1400 base pairs, matching the expected product length of 1437 base pairs. GAPDH primers created a very strong amplicon in lane 8 corresponding to their expected product length. β-Actin primers created a very strong amplicon in lane 9 corresponding to their expected product length. Primer details are specified in table 10.

IV Discussion

Success of Methodology

The three stages of RNA isolation, reverse transcription, and PCR were completed successfully. For the RNA isolation, homogenizing the tissue and extracting RNA was the most difficult step. The Qiagen RNeasy mini kit protocol was not adapted for bone tissue and produced a low yield of RNA, but homogenizing the tibia in Qiazol reagent and separating with chloroform solved this problem. This approach yielded a high concentration of RNA and avoided degradation. Reverse transcription followed easily. PCR proved difficult at first, but optimizing annealing temperature and using previously validated primers eventually resulted in success. Future experiments now have a robust protocol for investigating gene expression in bone tissue.

Potential Improvements

The protocol has room for improvement, however. First, the PCR method employed only yields qualitative gene expression data. In order to assay the extent of mRNA transcription, quantitative real time PCR must be performed. Transitioning the existing protocol to real time PCR will not be difficult; the microcirculation lab already uses real time PCR for other molecular biology applications. A second need for improvement is to handpick osteocyte gene targets of interest and optimize these primers. The primers used in this proof-of-concept study were chosen broadly to look for amplification of any kind. Future work will require primers specific to certain osteocyte

gene targets of interest. The proper annealing temperatures for these primers will need to be established through primer optimization.

Future Research

Future research will focus on the connections between osteocyte gene expression, angiogenesis, bone remodeling, and osteoporosis. Osteocyte apoptosis is known to stimulate angiogenesis, presumably by releasing molecular signaling factors. The microcirculation lab plans to examine the angiogenic factors produced by osteocyte apoptosis in bone stimulated by axial loading. This is an appropriate follow on to current work studying the effects of mechanical stimulus induced osteocyte apoptosis on vascular and mechanical properties of bone tissue. A second potential application for the microcirculation lab is studying osteocyte gene expression in a mouse model that simulates osteoporosis, for instance an aged mouse with impaired remodeling. This will yield more information regarding the progression of osteoporosis and the molecular reasons for the impaired remodeling observed in cortical and trabecular bone.

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VI Appendix A: Protocols

Bone RNA Isolation Qiagen Mini Lipid Tissue Kit

Date _____ **Bone RNA Isolation Qiagen Mini Lipid Tissue Kit** Initials_____

Description:
Isolate high quality total RNA from mouse tibia

Materials:
Qiazol, 70% EtOH, chloroform, sterile pipet tips, gloves, Biopulverizer, Tissue Tearor, Qiagen MinElute Cleanup kit, forceps, liquid nitrogen, eye protection

Reagents required per sample:
____1. RPE buffer w/ 100% EtOH added
____2. 70% EtOH
____3. Qiazol
____4. Chloroform
____5. Liquid N_2

Preparations Protocol:
____1. Make sure RLT is dissolved (can be kept at room temp)
____2. Add 4 volumes of Ethanol (100%) to RPE Buffer

Procedure Protocol:
____1. RNase Zap counters, pipettes, instruments
____2. Cool centrifuge to 4°C in preparation for step 11
____3. Retrieve sample from -80°C and transport in liquid N_2 (get enough for biopulverizer too)
____4. Prepare 2mL round-bottom microcentrifuge tube with ~900 µL Qiazol
____5. Super-cool biopulverizer and pulverize sample
____6. Collect sample into Qiazol solution
____7. Homogenize thoroughly with Tissue Tearor (in fume hood)
____8. Place on benchtop at RT for 5 min
____9. Add 200 µL Chloroform (in fume hood) and vortex for 15s
___10. Place on benchtop at RT for 2-3 min
___11. Centrifuge at 12000xg for 15 min at 4°C
___12. Heat centrifuge back to RT
___13. Transfer the upper aqueous layer (roughly 600 µL) into a new 1.5 mL tube, add 1 volume 70% EtOH, and vortex thoroughly
___14. Transfer ~600 µL of sample to an RNeasy Mini spin column and centrifuge at ≥8000xg for 15s
___15. Repeat step 14 using the rest of the sample
___16. Add 700 µL RW1 Buffer to the spin column and centrifuge at ≥8000xg for 15s (short)
___17. Add 500 µL RPE Buffer to the spin column and centrifuge at ≥8000xg for 15s (short)
___18. Add 500 µL RPE Buffer to the spin column and centrifuge at ≥8000xg for 2 min
___19. Place the RNeasy spin column in a new 2 ml collection tube and spin at full speed for 1 min.
___20. Place the RNeasy spin column in a 1.5 mL supplied collection tube, add 50 µL RNase-free water directly onto the column membrane, and centrifuge at ≥8000xg for 1 min
___21. Repeat step 20, reusing the collection from the previous step for higher concentration
___22. Nanodrop RNA to determine RNA concentration and (opt) assay quality by gel electrophoresis
___23. Store RNA isolate at -80°C

Iso Date	Sample ID	Ex Date	Nucleic cid conc.	A260	A280	260/280	260/230
			ng/µL				
			ng/µL				
			ng/µL				
			ng/µL				
			ng/µL				
			ng/µL				

Reverse Transcription Agilent AffinityScript Kit

Date _____ **Reverse Transcription Agilent AffinityScript Kit** Initials_____

Description:
Synthesize cDNA from RNA

Materials:
sterile pipet tips, gloves, microcentrifuge tubes, ice bucket, ice, RNase Zap wipes, Agilent AffinityScript Multiple Temperature cDNA Synthesis Kit

Reagents required per sample
____1. RT Kit Reagents, ice, RNA sample

Preparations Protocol:
____1. Calculate the amount of RNA sample to use for RT from its Nanodrop-measured concentration
____2. Amount of sample cannot be greater than 15.7µL

Procedure Protocol:
____1. Thaw kit reagents on ice
____2. Vortex briefly and spin down each reagent in a microcentrifuge
____3. Add 1ng-5µg of total RNA sample.
____4. Add RNase-free water to total volume of 15.7µL
____5. Add 1.0 µL of oligo(dT) primer OR 3.0 µL of random primers
____6. Incubate rxn at 65°C for 5 mins
____7. Cool rxn at room temp for ~10 mins
____8. Add 2.0 µL of 10x AffinityScript RT Buffer.
____9. Add 0.8 µL of dNTP mix
___10. Add 0.5 µL of RNase Blocker
___11. Add 1.0 µL of AffinityScript multi-temp RT
___12. (If using random primers) incubate rxn at 25°C for 10 mins. Otherwise, go to step 13
___13. Mix gently and incubate rxn at 42-55°C for 60 mins
___14. Terminate the rxn at 70 °C for 15 mins.
___15. Store cDNA at -20 °C or on ice if proceeding directly to PCR

Sample type

_____

Date	RNA Sample ID	RNA concentration	Sample Vol. Added to rxn	RNA Amt. (must be ≤ 5 µg)	Primer type	Rxn Temp

Gel Electrophoresis and Spectrophotometry

Date_____ **Gel Electrophoresis and Spectrophotometry** Initials _____

Gel Electrophoresis of RNA samples Materials:

____1. Agarose (0.5g)
____2. TAE (50 mL)
____3. 2 µL Ethidium Bromide
____4. Electrophoresis station
____5. Flask
____6. Graduated cylinder

Protocol

____1. Measure out 50 mL TAE
- Actual:_____

____2. Weigh 0.5g Agarose on parafilm paper
- Actual:_____

____3. Add Agarose to TAE and boil in microwave
- Stop and swirl

____4. Add 2 µL of EtBr to TAE agarose mix
- Let cool for a while (until it can be touched)

____5. Tape of the edges of the tray
____6. Pour TAE EtBr mix into tray
- Wait for it to polymerize

____7. Remove tape
____8. On a sheet of parafilm, mix sample and loading dye
- 2-5 µL sample + 2 µL loading dye

____9. Pipet samples into gel wells
____10. Use TAE to fill machine, cover gel
____11. Place in electrophoresis tray and run at 125 V for at least 30 minutes

Nucleic Acid Spectrophotometry Materials:

____1. RNase free water
- not DEPC (RNase free) make working solution

____2. Kim wipes
____3. P10

Protocol

____1. Open Nanodrop 2000 software
- Select nucleic acid
- Select "ok" when prompted
- Select RNA from type pull down on right side
- Label sample ID (name and date)
- Load 1 µL of RNase free water
  - Select blank in upper left
- Use kim wipe to clean upper and lower pedestal
- Apply sample and select measure
- Repeat (blank optional between samples)

Nano drop software
- Blank (F3)
- Measure (F1)
- 260/280 should be around 2

Gel Imaging
- Leave in tray to make sure its ready
- Open remote capture software
- Save file in "gel image" folder on desktop

NanoDrop results

Sample	Nucleic acid Conc	A260	A280	260/280	260/230

Notes:

Design and Order Primers

Date _____ **Design and Order Primers** Initials_____

Description:
Design and Order Primers

Design Primers Protocol:
____1. Open internet browser window
____2. Navigate to http://www.ncbi.nlm.nih.gov/genbank/
____3. Search "Entrez" for [gene name] and click "Go"
____4. Click "Nucleotide"
____5. Narrow results by filtering by "mus musculus" in the side pane or add "mus musculus" to the search box
____6. Select gene from search results and click its title
____7. Click "Fasta" at the top of the page and copy the gene sequence to the clipboard
____8. Open a new tab/window and navigate to Primer3 at http://frodo.wi.mit.edu/
____9. Paste source sequence. Verify that "Pick Left Primer" and "Pick Right Primer" are checked
____10. Specify values approximately as in table below depending on the desired PCR conditions

Product Size Ranges:	700-2000		
General Primer Picking Conditions:			
	Min	Opt	Max
Primer Size	20	22	25
Primer Tm	58	62	65
Primer GC%	35	50	64

___11. Click "Pick Primers"
___12. Choose one of the primers under "ADDITIONAL OLIGOS" that meets the appropriate conditions. Left and Right primers should have similar Tm's and the product size should be in the target range.
___13. Leaving that window open, return to the NCBI window and click "Run Blast" under "Analyze this sequence"
___14. Paste in primer sequences one at a time for Left and Right primers in the "Enter accession number(s)…" dialog box
___15. Ensure that the database selected is "Nucleotide collection nr/nt." Click the "BLAST" button
___16. Screen search results for any genes with low E values that are not variants of the intended gene product. (Low E value signifies high specificity.) If genes other than the intended product may be produced, choose a different primer. Otherwise, proceed to order primers.

Order Primers Protocol:
____1. Navigate to http://www.idtdna.com/site
____2. "Products → DNA/RNA Synthesis → Custom DNA Oligos
____3. Under "Ordering" choose multiple entry and enter the "Number of Items." (One and left and one Right Primer would be 2.) Click "Go"
____4. Change Scale (Primer concentration) to "25 nmolar DNA oligo"
____5. Leave Change Purification as default value
____6. Change Normalization to "LabReady"
____7. Enter gene name in "Sequence Name" field
____8. Enter Forward/Reverse and Tm in "Notes" field
____9. Enter Primer sequence copied from the Primer3 window into the 5'-3' field
___10. Add to Cart and check out on credit card
___11. Save receipts for reimbursement

VII Appendix B: Experimental Setup

RNA Isolation Equipment

Figure 9 - BioSpec Pulverizer (left) and Tissue Tearor (right).

Figure 10 - Nanodrop spectrophotometer for assaying RNA concentration and purity.

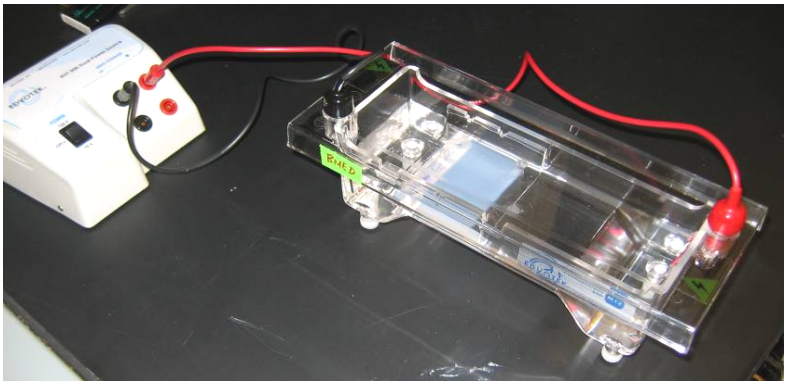

Figure 11 - Gel electrophoresis apparatus.

RT and PCR Equipment

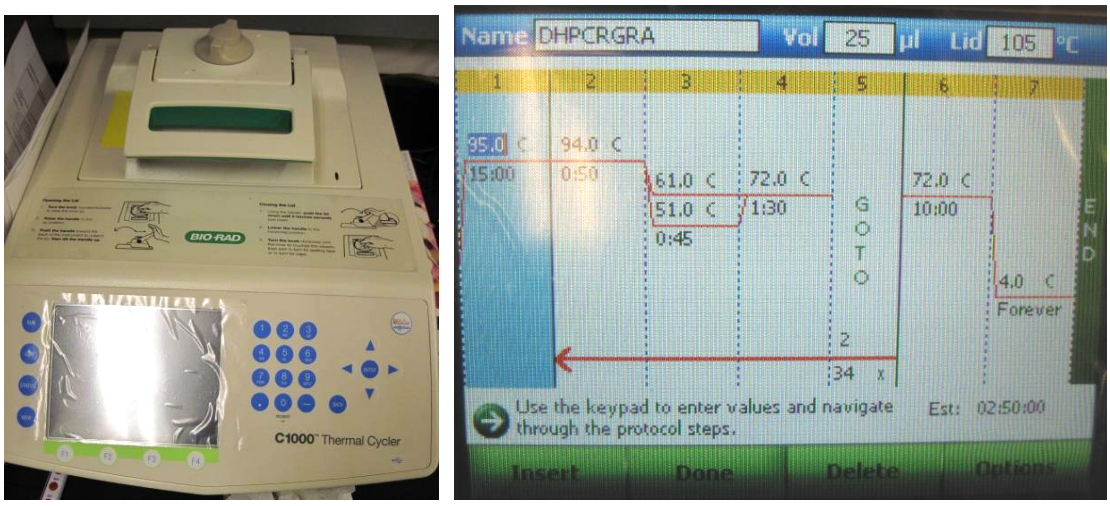

Figure 12 - BioRad Gradient Thermocycler C1000 (left) and display (right).

Comprehensive Reagents/Equipment List

Table 9 - Reagents/Equipment List

Reagents	Category	Source	Cost
Agarose	Gel	Fisher	-
dNTPs	PCR	Qiagen	-
ethidium bromide	Gel	Fisher	-
forward and reverse PCR primers	PCR	IDT	$138.40
liquid nitrogen	Isolation	Cal Poly	-
Loading dye	Gel	Fisher	-
Oligo-dT Primers	RT	Qiagen	-
HotStarTaq DNA Polymerase Kit #203203	PCR	Qiagen	-
AffinityScript Multiple Temperature cDNA Synthesis Kit, (50) #200436	RT	Agilent	$259.00
RNeasy Mini Tissue Kit (10) #74104	Isolation	Qiagen	-
QIAzol 79306	Isolation	Qiagen	-
RNase free water	All	Fisher	-
RNase inhibitor	RT	Qiagen	-
standard ladder	Gel	Fisher	-
TAE buffer	Gel	Fisher	-
Equipment			
BioRad Gradient Thermocycler C1000	PCR		-
BioSpec Biopulverizer	Isolation		-
BioSpec Tissue Tearor	Isolation		-
bone scissors	Extraction		-
Canon G10 camera	Gel		-
centrifuge	Several		-
digital balance	Gel		-
electrophoresis power supply	Gel		-
electrophoresis tray and accessories	Gel		-
Erlenmeyer flask	Gel		-
forceps	Isolation		-
heating block	Several		-
ice bucket	All		-
laptop	Spec, Gel		-
liquid nitrogen container	Isolation		-
micropipettes	All		-
microwave	Gel		-
Nanodrop 2000 software	Spec		-
Nanodrop spectrophotometer	Spec		-
PCR tubes	PCR		-
RNase Zap wipes	Iso, RT	Applied Biosystems	-
spatula	Gel		-
test tubes	All		-