Supplementary Material

Bone Marrow Mesenchymal Stem Cells Promote Repairing the Bruised Tissue via Regulating mR NA Expression of Molecular Biomarkers and the Apoptotic Rate

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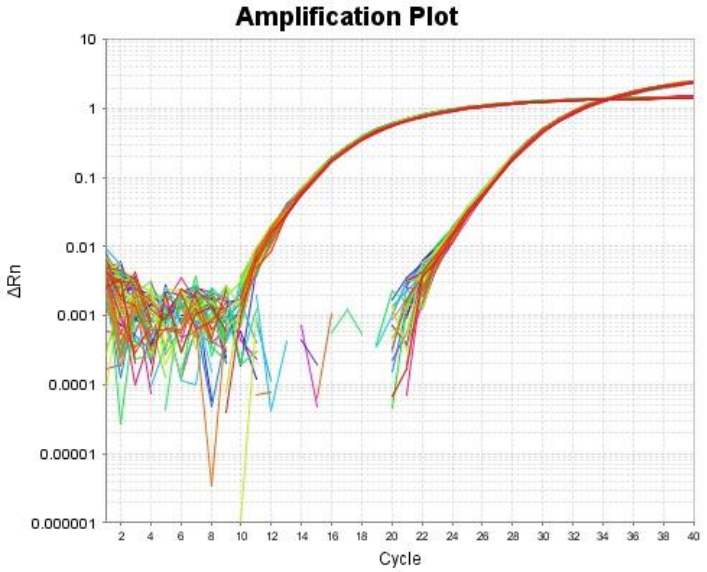
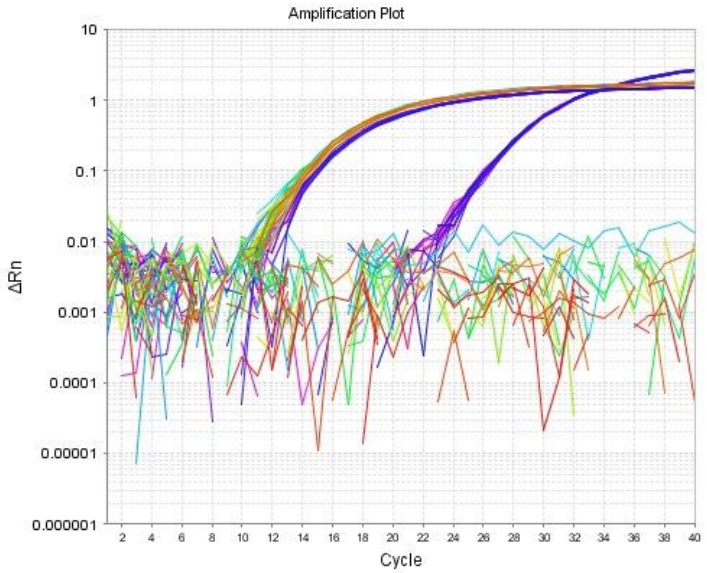
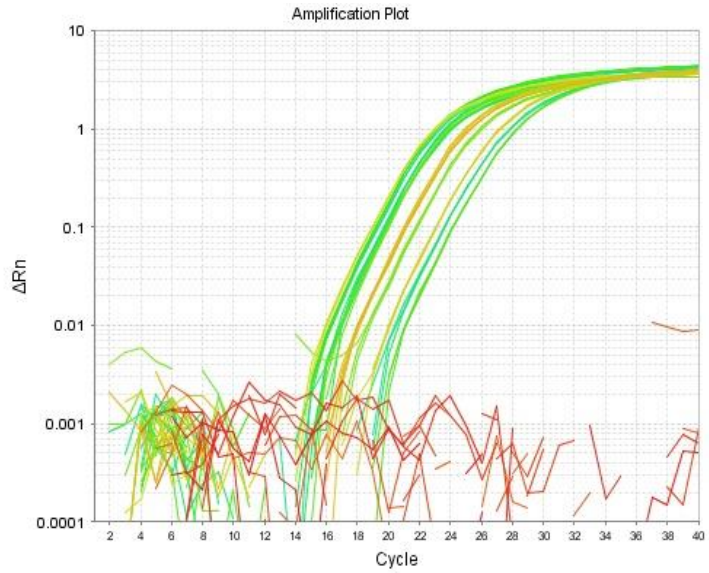


Amplification blot curves for quantitative real time PCR of HSP-90 gene vs β-actin as house keeping gene for model Bruise.



Amplification blot curves for quantitative real time PCR of MMPs gene vs β-actin as house keeping gene for model Bruise.



Amplification blot curves for quantitative real time PCR of miRNA gene vs 6UB as house keeping gene for model Bruise.

Apo-BrdU *In Situ* DNA Fragmentation Assay Kit

(Catalog #K401-60; Store Components Separately-see below)

I. INTRODUCTION:

Internucleosomal DNA fragmentation is a hallmark of apoptosis in mammalian cells. BioVision's **Apo-BrdU *In Situ* DNA Fragmentation Assay Kit** provides complete components including positive and negative control cells for conveniently detecting DNA fragmentation by fluorescence microscopy or flow cytometry. The kit utilizes Br-dUTP (bromolated deoxyuridine triphosphate nucleotides) which is more readily incorporated into DNA strand breaks than other larger ligands (e.g., fluorescein, biotin or digoxigenin). The greater incorporation gives rise to brighter signal when the Br-dUTP sites are identified by a fluorescein labeled anti-BrdU monoclonal antibody.

II. KIT CONTENTS:

Component	Cap Color	Volume	Store Temp.	Part Number
Positive Control Cells	brown	5 ml	-20˚C	K401-60-1
Negative Control Cells	natural	5 ml	-20˚C	K401-60-2
Wash Buffer	blue	120 ml	+4˚C	K401-60-3
Reaction Buffer	green	0.6 ml	+4˚C	K401-60-4
TdT Enzymes	yellow	45 µl	-20˚C	K401-60-5
Br-dUTP	violet	0.48 ml	-20˚C	K401-60-6
Rinse Buffer	red	120 ml	+4˚C	K401-60-7
Anti-BrdU-FITC Antibody	orange	0.3 ml	+4˚C	K401-60-8
PI/RNase Staining Buffer	amber bottle	30 ml	+4˚C	K401-60-9

III. APOBRDU ASSAY PROTOCOL FOR CULTURED CELLS:

A. Cell Fixation

1. Induce apoptosis in cells by desired method. Concurrently incubate a control culture without induction.
2. Pellet 1-5 x 10^6 cells and resuspend in 0.5 ml of PBS.
3. Fix the cells by adding 5 ml of 1% (w/v) paraformaldehyde in PBS and place on ice for 15 minutes.
4. Centrifuge the cells for 5 min at 300 x g and discard the supernatant.
5. Wash cells in 5 ml of PBS and pellet the cells by centrifugation. Repeat one time the wash and centrifugation step.
6. Resuspend the cells in 0.5 ml of PBS.
7. Add the cells to 5 ml of ice-cold 70% (v/v) ethanol. Let cells stand for a minimum of 30 min (or overnight if you prefer) on ice or in the freezer.
8. Store the cells in 70% (v/v) ethanol at –20˚C until use. Cells can be stored at –20˚C for several days before use.

B. Detection by Flow Cytometry and Fluorescence Microscopy:

The procedures can be used for both control cells and your testing cells.

1. Resuspend the fixed cells by swirling the vials. Remove 1 ml aliquots of the cell suspension (~1 x 10^6 cells per ml) and place in 12 x 75 mm tubes. Centrifuge (300 x g) for 5 min and carefully remove the ethanol by aspiration.
2. Resuspend the cells with 1 ml of **Wash Buffer** (blue cap). Centrifuge as before and remove supernatant carefully by aspiration.
3. Repeat one time the washing step (step 2).
4. Resuspend in 50 µl of the **DNA Labeling Solution** prepared as below:

DNA Labeling Solution	1 assay	10 assays
TdT Reaction Buffer (green cap)	10 µl	100 µl
TdT Enzyme (yellow cap)	0.75 µl	7.5 µl
Br-dUTP (violet cap)	8 µl	80 µl
ddH_20	32.25 µl	322.5 µl
Total Volume	51 µl	510 µl

5. Incubate the cells in the **DNA Labeling Solution** for 60 min at 37˚C. Shake cells every 15 min to resuspend.
6. Add 1 ml of **Rinse Buffer** (red cap) to each tube and centrifuge for 5 min. Remove supernatant by aspiration.
7. Repeat one time the rinsing step (step 6).

8. Resuspend cells in 0.1 ml of the **Antibody Solution** prepared as below:

Antibody Solution	1 assay	10 assays
Anti-BrdU-FITC Antibody (orange cap)	5 µl	50 µl
Rinse Buffer (red cap)	95 µl	950 µl

9. Incubate the cells with the **Antibody Solution** in the dark for 30 min at room temperature.
10. Add 0.5 ml of **Propidium Iodide/RNase A Solution** (amber bottle).
11. Incubate the cells in the dark for 30 min at room temperature.
12. Analyze the cells by fluorescence microscopy using FITC and rhodamine filters (apoptotic cells show green staining over an orange-red PI counter-staining) or flow cytometry (Ex/Em = 488/520 nm for FITC, and 488/623 nm for PI). Cells should be analyzed within 3 hours of staining.

IV. APO-BRDU ASSAY PROTOCOL FOR TISSUE SECTIONS:

A. Tissue Section Preparations:

The protocol describes the preparation of formalin-fixed, paraffin-embedded tissue section mounted on glass slides. For information on fixing and embedding techniques, see Ben-Sasson *et al*., (Methods Cell. Biol. 46:29-39, 1995). Most steps are performed in Coplin Jars.

Note: If using fresh-frozen tissue sections, proceed directly to step 7.

1. Remove paraffin by immersing slides in a Coplin jar containing fresh xylene. Incubate at room temperature for 5 minutes.

2. Repeat in a second Coplin jar containing fresh xylene.

3. Immerse the slides in a Coplin Jar containing 100% ethanol and incubate at room temperature for 5 min.

4. Rehydrate the slides by sequential 3-min, room temperature incubations in Coplin jars containing:

- 100% ethanol
- 95% ethanol
- 85% ethanol
- 70% ethanol
- 50% ethanol

5. Immerse the slides in a Coplin jar containing 0.85% NaCl and incubate at room temperature for 5 min.

6. Immerse the slides in a Coplin jar containing PBS and incubate at room temperature for 5 minutes.

7. Fix the slides by immersing them in a Coplin jar containing fresh 4% formaldehyde/PBS, and incubate at room temperature for 15 min.

8. Wash the slides by immersing them in a Coplin jar containing PBS, and incubate at room temp. for 5 min.

9. Transfer to another Coplin jar containing PBS, and incubate at room temperature for 5 min.

10. Allow the liquid to drain thoroughly and place slides on a flat surface.

11. Prepare 20 µg/ml of Proteinase K Solution (combine 2 µl of 10 mg/ml Protease K and 998 µl of 100 mM Tris-HCl, pH 8.0, 50 mM EDTA) and cover each section with 100 µl of it. Incubate at room temperature for 5 min.

12. Immerse the slides in Coplin jar containing PBS, and incubate at room temperature for 5 min.

13. Transfer the slides to a Coplin jar containing 4% formaldehyde/PBS and incubate at room temperature for 5 minutes.

14. Wash the slides by immersion in Coplin jar containing PBS, and incubate at room temperature for 5 min.

B. Detection by Fluorescence Microscopy:

1. Remove slides from PBS and tap gently to remove excess liquid. Cover the cells in 100 µl of Wash buffer (blue cap).
2. Use forceps, gently place a piece of plastic coverslip on top of the cells to evenly spread the liquid, incubate for 5 min. Remove plastic coverslip and gently tap the slides to remove excess liquid.
3. Repeat step 2. Carefully blot dry around the edges with tissue paper.
4. Gently place 50 µl of the DNA Labeling Solution (prepared as in Section IIIB, Step 4) on the cells.
5. Use forceps, gently place a piece of plastic coverslip on top of the cells to evenly spread the liquid.
6. Place the slides in a dark, humidified 37˚C incubator for 60 minutes.

   Note: Ensure high humidity by placing wet paper towels in the bottom of the dry incubator.

7. Using forceps, remove the plastic coverslips. Rinse the slides to a fresh Coplin jar filled with PBS for 5 min.
8. Repeat step 7. Carefully blot dry around the edges with tissue paper.
9. Place 100 µl of the Antibody Solution (Prepared as in Section IIIB, step 8).
10. Use forceps, gently place a piece of plastic coverslip on top of the cells to evenly spread the liquid.
11. Incubate the cells with the antibody solution in a humidified incubator for 30 min at room temperature.
12. Carefully remove the solution from slides. Add 100 µl of Propidium Iodide/Rnase A solution (amber bottle).
13. Use forceps, gently place a piece of plastic coverslip on top of the cells to evenly spread the liquid.
14. Incubate the slides in the dark in a humidified incubator for 30 min at room temperature.
15. Wash the cells by transferring the slides to a fresh Coplin jar filled with ddH_2O and incubate at room temperature for 5 min.
16. Repeat Step 15.
17. [Optional] Add a drop of anti-Fade solution and cover the treated portion of the slide with a glass coverslip.
18. [Optional] Seal the edges of the coverslip with rubber cement or clear nail polish.
19. View slides as soon as possible using FITC and rhodamine filters. Apoptotic cells will exhibit strong nuclear green fluorescence. All cells should be stained with PI and exhibit strong red counter staining.

RELATED PRODUCTS:

Apoptosis Detection Kits & Reagents
- Annexin V Kits & Bulk Reagents
- Caspase Assay Kits & Reagents
- Mitochondrial Apoptosis Kits & Reagents
- Nuclear Apoptosis Kits & Reagents
- Apoptosis Inducers and Set
- Apoptosis siRNA Vectors

Cell Fractionation System
- Mitochondria/Cytosol Fractionation Kit
- Nuclear/Cytosol Fractionation Kit
- Membrane Protein Extraction Kit
- Cytosol/Particulate Rapid Separation Kit
- Mammalian Cell Extraction Kit
- FractionPREP Fractionation System

Cell Proliferation & Senescence
- Quick Cell Proliferation Assay Kit
- Senescence Detection Kit
- High Throughput Apoptosis/Cell Viability Assay Kits
- LDH-Cytotoxicity Assay Kit
- Bioluminescence Cytotoxicity Assay Kit
- Live/Dead Cell Staining Kit

Cell Damage & Repair
- HDAC/HAT Fluorometric & Colorimetric Assays & Drug Discovery Kits
- DNA Damage Quantification Kit
- Glutathione & Nitric Oxide Fluorometric & Colorimetric Assay Kits

Signal Transduction
- cAMP & cGMP Assay Kits
- Akt & JNK Activity Assay Kits
- Beta-Secretase Activity Assay Kit

Adipocyte & Lipid Transfer
- Recombinant Adiponectin, Survivin, & Leptin
- CETP & PLTP Activity Assay & Drug Discovery Kits
- Cholesterol, HDL, LDL, Triglyceride Quantification Kits

Molecular Biology & Reporter Assays
- siRNA Vectors
- Cloning Insert Quick Screening Kit
- Mitochondrial & Genomic DNA Isolation Kits
- 5 Minutes DNA Ligation Kit
- 20 Minutes Gel Staining/Destaining Kit
- Beta-Galactosidase & Luciferase Assay Kits

Growth Factors and Cytokines

Monoclonal and Polyclonal Antibodies

RNA extraction:

RNA was extracted utilizing nucleic acid extraction kit (NucleoSpin®) purchased from Macherey- Nagel GmbH & Co. KG- Germany (REF 740955.50) as following: (The steps summarized in table 1)

Cells lysis:

350 µl of buffer RA1 and 3.5 µl of β-mercaptoethanol added to cell pellet and vortex vigorously.

Lysate filtration:

To reduce the viscosity and clear the lysate filtration was done using NucleoSpin®Filter (violet ring) placed in collection tube (2ml) and centrifuged for 1 min at 11,000 rpm.

Adjustment of RNA binding conditions:

The NucleoSpin®Filter discarded and 350 µl of 70% ethanol added to lysate and mixed by pipetting up and down (5 times).

RNA binding:

NucleoSpin® RNA Column (light blue ring) placed in a collection tube then the lysate loaded to the column and centrifuged for 30 s at 11,000 rpm.

Washing and drying the silica membrane:

600 µl of buffer RA3 added to NucleoSpin® RNA Column and centrifuged at 11,000 rpm for 30 s. the flow- through discarded then the column placed back into the collection tube.

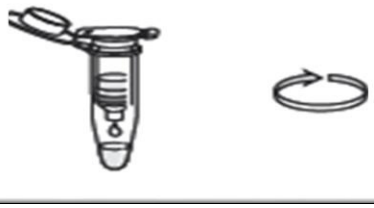
A second wash was done by adding 250 µl of buffer RA3 to NucleoSpin® RNA Column and centrifuged at 11,000 rpm for 2 min to dry the membrane completely.

RNA elution:

NucleoSpin® RNA Column placed into a nuclease free collection tube (1.5 ml, supplied with kit) then RNA eluted in 60 µl DNase –free H2O, and centrifuged at 11,000 rpm for 1 min.

The purity (*A*260/*A*280 ratio) and the concentration of RNA were obtained using spectrophotometry (dual wave length Beckman, Spectrophotometer, USA). The extracted and purified DNA samples were stored at –80 °C for further use.

Table 1: steps of RNA extraction

Cells lysis:		350µl RA1 3.5µl β-mercaptoethanol Mix
Lysate filtration:		11,000 rpm 1 min
Adjustment of RNA binding conditions:	350 µl 70% ethanol Mix	
RNA binding:		load sample 11,000 rpm 30 s
Desalting the silica membrane:		350 µl MDB 11,000 rpm 1 min
Washing and drying the silica membrane:		1st wash 600 µl RA3 11,000 rpm 30 s 2nd wash 250 µl RA3 11,000 rpm 2min
RNA elution:		60 µl DNase free H2O 11,000 rpm 1 min

Quantitative real time PCR of all studied genes:

The kit was provided by Vivantis, ViPrimePLUS One Step Taq RT-qPCR Green Master Mix I with ROX (SYBR Green Dye) (cat no #QLMM14-R).

- **Description:**

- The master mix is prepared in 2X concentrated solution and contains unique thermostable M-MuLV (Moloney Murine Leukemia Virus) reverse transcriptase enzyme, Taq DNA Polymerases, SYBR Green dye, ROX dye as well as MgCl2 and buffer components at optimal concentrations.
- The M-MULV enzyme has an optimal operating temperature and a higher affinity for primer template duplexes which allows very rapid processing during RT step.
- SYBR Green dye emits fluorescence when bound to double-stranded DNA. Detection of PCR product is monitored by the increase in fluorescence.

- **Kit components and master mix:**

Kit includes 3 x 0.6ml aliquots of master mix.

Table (?): ViPrime PLUS One Step Taq RT-qPCR Green Master Mix I with ROX:

Component	**Reaction(1X)**
Taq One Step RT-qPCR Green Master Mix I	10µl
Primers	1µl
Template	5µl
Nuclease free water	4µl
Final Volume	20µl

Procedure:

Primers sequences:

Gene symbol	Forward	Reverse	Gene bank
HSP-90	TGTTGGGACCAGCAACTCAA	TTTGAGGCTCAGTGGTAGCC	NM_021576.2
MMP-9	GGCAGCTTCAACAACCATCA	GGATGGACTAGATCGGAGCC	XM_032900290.1
β-actin	TGACAGGATGCAGAAGGAGA	TAGAGCCACCAATCCACACA	NM_031144.3
MiRNA 21	TAGCTTATCAGACTGATGTTGA	GAATCGAGCACCAGTTACGC	
6UB	AACGCTTCACGATTTGCGT	CTCGCTTCGGCAGCACA	XR_003710259.1

The prepared reaction mix samples were applied in real time PCR (Step One Applied Biosystem, Foster city, USA) (figure????????). ViPrime PLUS One Step Taq RT-qPCR Green Master Mix I with ROX Kit was compatible with three-step cycling (table ?????)

Table (???): The Thermal profile cycling of RT-qPCR:

Step	**Cycle**	**Temp.**	**Time**
Reverse Transcription	1	55°C	10 mins
Enzyme activation	1	95°C	8 mins
Denaturation	40	95°C	10 secs
Annealing and extension		60°C	60 secs

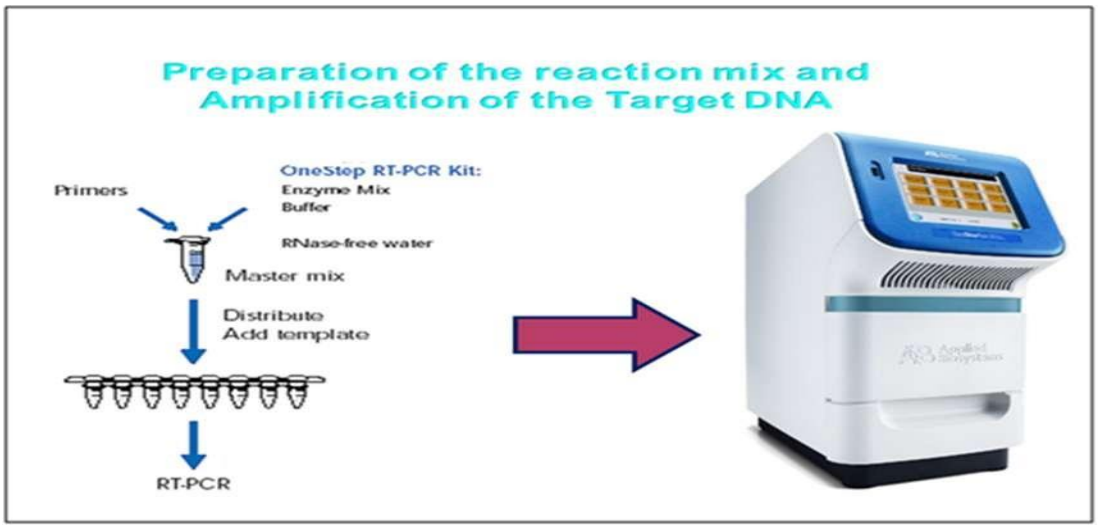


Figure (?): Preparation of master mix and amplification of the target DNA

Calculation of Relative Quantification (RQ) (relative expression):

After the RT-PCR run the data were expressed in Cycle threshold (Ct). The PCR data sheet includes Ct values of assessed gene (HSP-90, MMP-9 and the house keeping gene (β-actin), miRNA21 and 6UB)

In order to measure the gene expression of certain gene, a control sample should be used. The RQ of each target gene is quantified according to the calculation of delta-delta Ct (ΔΔCt). We calculated the RQ of each gene by taking $2^{-\Delta\Delta Ct}$ as following:

ΔΔCt = [(Ct target, Sample)-(Ct ref, Sample)]-[(Ct target, Control)-(Ct ref, Control)] Where:

Ct target, Control = Ct value of gene of interest in control DNA.

Ct ref, Control = Ct value of reference gene in control DNA.

Ct target, Sample = Ct value of gene of interest in tested sample.

Ct ref, Sample = Ct value of reference gene in tested sample.