In Vitro Osteogenesis Assay Kit

Catalog No. ECM810

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Introduction

Bone matrix is a dense mixture comprised mainly of collagen fibers and calcium phosphate particles, with a population of living cells contained within. Despite its rigidity, bone continually undergoes a remodeling process that requires the coordinated activity of two types of cells, osteoclasts and osteoblasts [1]. Many diseases of bone including osteoporosis, a common phenomenon in postmenopausal women in which bone mass is greatly reduced, and osteogenesis imperfecta, also known as brittle-bone disease, are likely caused by the misregulation of osteoblasts and osteoclasts. Understanding the molecular mechanisms that underlie osteogenesis, the process by which new bone is formed, is thus of significant clinical importance [2].

Osteoclasts are related to macrophages, and erode the bone matrix by secreting acids and hydrolases to dissolve bone minerals and digest organic components. Osteoblasts arise from undifferentiated precursor cells, and deposit a mineralized matrix consisting of collagen, calcium, and phosphorous and other minerals, leading to the formation of new bone. Osteoblasts have also been shown to express factors which regulate the differentiation and function of osteoclasts [2]. The balance between the activity of osteoblasts and osteoclasts determines the mass and density of bone [1].

Studies of the mechanisms underlying osteogenesis have been greatly facilitated by the use of the undifferentiated preosteoblastic cell line MC3T3-E1, available from ATCC. This cell line was derived from mouse calvaria [3,4] and under defined culture conditions can be induced to undergo a developmental sequence leading to the formation of multilayered bone nodules [4,5]. This sequence is characterized by the replication of preosteoblasts followed by growth arrest and expression of mature osteoblastic characteristics such as matrix maturation and eventual formation of multilayered nodules with a mineralized extracellular matrix [5]. This cell line has become the standard *in vitro* model of osteogenesis and has found widespread use in studies examining many aspects and applications of osteogenesis, including transcriptional regulation [5], mineralization [3,6] and tissue engineering [7].

Millipore's *In Vitro* Osteogenesis Assay Kit provides the necessary reagents and protocols for efficient *in vitro* differentiation of MC3T3-E1 cells to mature osteoblasts, and for the end-point determination and quantification of this process.

Application

Millipore's *In Vitro* Osteogenesis Assay Kit contains all reagents necessary to efficiently differentiate MC3T3-E1cells to a mature osteoblastic lineage, as determined by staining for mineralization. In addition this kit provides all the necessary reagents and a protocol for quantifying osteogenesis using a standard plate reader. This product is useful for studying the effects of growth factors, drugs and toxic agents on bone formation and for screening candidate osteogenic pharmaceuticals. Also, studies examining the intracellular signaling pathways regulating osteoblast differentiation can be facilitated by use of this kit.

The kit includes the osteogenesis-inducing reagents: ascorbic acid 2-phosphate, β-glycerophosphate and melatonin, and Alizarin Red Solution, a staining solution used to detect the presence of calcium in bone. Additionally, we provide reagents and a protocol for performing quantitative analysis of Alizarin Red staining.

Using Millipore's *In Vitro* Osteogenesis Assay Kit, we routinely obtain >75% mature osteoblasts from a starting population of uninduced MC3T3-E1 cells obtained from ATCC. Efficiency of osteoblastic differentiation may vary, depending upon the quality of the cells used, and if variations to the protocol are introduced.

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Kit Components

1. Ascorbic Acid 2-Phosphate Solution (500X): Part No. 2004011
   One vial containing 500 µL of 100 mM ascorbic acid 2-phosphate in deionized water.
   Store at -20ºC.

2. Glycerol 2- Phosphate Solution (100X): Part No. 2004010
   Three vials containing 1 mL of 1 M glycerol 2-phosphate in deionized water.
   Store at -20ºC.

3. Melatonin Solution: Part No. 2004353
   One vial containing 6 µg of melatonin. Reconstitute before use with 500 µL distilled H2O for a final concentration of 50 µM. Store at -20ºC.

4. Alizarin Red S Stain Solution (1X): Part No. 2003999
   One bottle containing 50 mL Alizarin Red S Solution. Store at 2º-8ºC.

5. 10% Acetic Acid: Part No. 2004807
   One vial containing 20 mL of 10% Acetic Acid in deionized water.
   Store at 2º-8ºC.

6. 10% Ammonium Hydroxide: Part No. 2004809
   One vial containing 10 mL of 10% Ammonium Hydroxide in deionized water. Store at 2º-8ºC.

7. 10X ARS Dilution Buffer: Part No. 2004810
   One vial containing 5 mL of ARS (Alizarin Red Stain) Diluent. **Add 5mL of 80% Acetic Acid for a final volume of 10 mL before use.**
   Store at 2º-8ºC.

Materials Not Supplied

- MC3T3-E1 cells (available from ATCC; Catalog number CRL-2593)
- Cell culture medium appropriate for MC3T3-E1 cells (*e.g.* α-MEM with 10% Fetal Bovine Serum and Antibiotics)
- Cell culture plates
- Phosphate-Buffered Saline (1X PBS)
- Cell detachment solution (e.g. Trypsin/EDTA)
- Sterile pipettes and pipette tips
- Sterile cell culture hood
- CO2 incubator appropriate for subject cells
- Fixative for Alizarin Red Staining
  (e.g. 10% formaldehyde, or 4% paraformaldehyde, or 70% ethanol)
- Hemocytometer
- Microscope
- Plate reader capable of reading at $_{405}$ nm

Storage

Kit components require **two different storage temperatures.** Ascorbic Acid 2-Phosphate Solution, Glycerol 2-Phosphate Solution and Melatonin Solution should be stored at -20ºC. Alizarin Red Solution, 10% Acetic Acid, 10% Ammonium Hydroxide and ARS Dilution Buffer are stored at 2°- 8℃ up to expiration date.

Preparation of Reagents

Kit contains a sufficient amount of reagents to perform at least 24 assays using a 24-well plate format.

Osteogenesis Induction Media

Osteogenesis Induction Media #1 and #2 should be made fresh for each use or medium change. The recommended amount of medium for a 24-well plate is 1 mL/well.

Melatonin Solution
Make up a 50 µM solution of Melatonin by reconstituting supplied vial with 500 µL of distilled H_2O. Reconstituted Melatonin solution aliquots should be stored at -20ºC until needed.

Mix the following sterile ingredients to make 10 mL of medium. Scale up according to experimental design.

OSTEOGENESIS INDUCTION MEDIUM #1			
Component	**Stock Conc.**	**Amount**	**Final Conc.**
Cell culture medium (e.g. α-MEM containing fetal bovine serum and antibiotics)	100%	9.88 mL	99% (approx.)
Ascorbic Acid 2-Phosphate Solution	0.1 M	20 µL	0.2 mM
Glycerol 2-Phosphate Solution	1 M	100 µL	10 mM
OSTEOGENESIS INDUCTION MEDIUM #2			
Component	**Stock Conc.**	**Amount**	**Final Conc.**
Cell culture medium (e.g. α-MEM containing fetal bovine serum and antibiotics)	100%	9.87 mL	98% (approx.)
Ascorbic Acid 2-Phosphate Solution	0.1 M	20 µL	0.2 mM
Glycerol 2-Phosphate Solution	1 M	100 µL	10 mM
Melatonin Solution	50 µM	10 µL	50 nM

10X ARS Dilution Buffer: Part No. 2004810
One vial containing 5 mL of ARS (Alizarin Red Stain) Diluent is provided. **Add 5mL of 80% Acetic Acid for a final volume of 10 mL before use.**

Cell Culture

Perform the following steps in a sterile hood

1. Culture the cells in a T75 flask until they are 80-90% confluent. (MC3T3- E1 cells can typically be plated at 4 x 10^5 cells per T75 flask and passaged every third day.)

2. Aspirate the media.

3. Wash the flask twice with 5-10 mL of 1X PBS. Aspirate after each wash.

4. Apply cell detachment solution and incubate in a 37℃ incubator for 5-7 minutes.

5. Inspect the plate and ensure the complete detachment of cells by gently tapping side of the plate with the palm of hand.

6. Apply 10 mL of tissue culture medium containing fetal bovine serum.

7. Transfer the dissociated cell suspension into a 15 mL conical tube.

8. Centrifuge the tube at 300xg for 3-5 minutes to pellet the cells.

9. Aspirate and discard the supernatant.

10. Apply 2 mL of tissue culture medium to the tube and resuspend the cells thoroughly. **IMPORTANT: Do not Vortex the Cells.**

11. Count the number of cells using a hemocytometer.

12. Plate the cells at the desired cell density into appropriate flasks, plates or wells. A plating ratio exceeding 1:8 is not recommended. A typical plating density is 1 x 10^5 cells per well of 24-well plate.

Osteogenic Differentiation (for 24-well tissue culture plates)

1. Plate the cell suspension at desired density in 24-well culture dish with 1 mL volume of cell culture medium per well. A typical plating density is 1 x 10^5 cells per well of 24-well plate.

2. Incubate the cells at 37ºC in a 5% CO2 humidified incubator until cells are confluent, replacing cell culture media every 48-72 hrs as necessary.

3. When the cells are confluent, carefully aspirate the medium from each well and add 1 mL **Osteogenesis Induction Medium #1**. This medium change corresponds to Differentiation Day 0.

4. Replace medium with 1 mL fresh **Osteogenesis Induction Medium #1** every 2-3 days.

5. On Differentiation Day 6, replace medium with 1 mL fresh **Osteogenesis Induction Medium #2.**

6. Replace medium with 1 mL fresh **Osteogenesis Induction Medium #2** every 2-3 days.

7. After 12-20 Differentiation Days, cells can be fixed and stained with Alizarin Red Solution. **Note:** degree of mineralization will increase slightly with increased differentiation time.

Alizarin Red Staining Protocol

1. Carefully aspirate the medium from each well. Be careful to not aspirate the cells.

2. Wash cells 1X with 2 mL PBS or HBSS

3. Fix cells by covering with 10% formaldehyde and incubating at room temperature for 15 minutes.

4. Carefully remove the fixative and rinse cells three times (5-10 minutes each) with an excess of distilled water. Use care to wash gently as possible to avoid disturbing the monolayer.

5. Remove water and add 1 mL/well Alizarin Red Stain Solution.

6. Incubate at room temperature for at least 20 minutes.

7. Remove excess dye and wash four times with deionized H_2O. **Note**: It helps to wash with gentle rocking for 5 minutes with each wash.

8. Add 1 - 1.5 mL water to each well to prevent the cells from drying. The plate is now ready for visual inspection and/or image acquisition. Differentiated cells containing mineral deposits will be stained bright red by the Alizarin Red Solution.

Differentiation Results

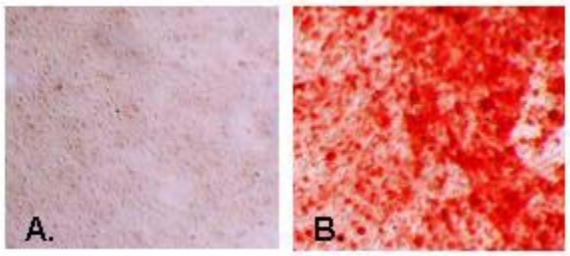


Figure 1. MC3T3-E1 after 15 days of osteogenic differentiation. Cells readily differentiate to a mature osteoblastic lineage as indicated by Alizarin Red staining (B). Alizarin Red staining was not observed in untreated MC3T3-E1 cells (A). The presence of Alizarin Red staining demonstrates mineral deposition throughout the culture.

Protocol for quantitative analysis of Alizarin Red Staining

For some applications, such as osteogenic compound screening, it may be useful to perform a quantitative analysis of Alizarin Red Staining [8]. This can be done by determining OD_{405} values of a set of known Alizarin Red concentrations and comparing these values to those obtained from unknown samples. This protocol is particularly versatile in that the dye can be extracted from the stained monolayer and quantified directly [8]. The sensitivity of the assay is improved by the extraction of the calcified mineral at low pH and, since the mineral is already stained in a quantitative manner, there is no requirement for an additional colorimetric quantification step [8].

1. Add 400 µL 10% acetic acid to each well of a 24-well plate and incubate for 30 minutes with shaking.

2. The monolayer will now be loosely attached. With the aid of a cell scraper, gently scrape the cells from the plate and transfer the cells and acetic acid to a 1.5 mL microcentrifuge tube.

3. Vortex vigorously for 30 seconds.

4. Heat to 85℃ for 10 minutes. **Note:** To avoid evaporation, microcentrifuge tube may be sealed with parafilm. Alternatively, sample may be overlaid with 200 µL mineral oil)

5. Transfer tube to ice for 5 minutes. Take care not to open the tube until fully cooled.

6. Centrifuge the slurry at 20,000xg for 15 minutes.

7. While centrifuging, make up Alizarin Red standards. Begin by diluting the 10X ARS Dilution buffer 1:10 in distilled H2O to obtain a 1X working ARS Dilution buffer. Next, dilute the 40 mM Alizarin Red solution 1:20 in 1X ARS dilution buffer (e.g. 50 µL Alizarin Red + 950 µL 1X ARS dilution buffer). This gives a 2 mM working stock. Standards can be constructed in a 'high range' or 'low range' set. Construct the 'high range' set by diluting the 2 mM working stock in 2-fold serial dilutions in 1.5-mL microcentrifuge tubes. To generate a 'low range' set, begin by first diluting the 2 mM working stock 1:66 (15 µL 2mM Alizarin Red solution + 985 µL 1X ARS dilution buffer) to achieve a 30 µM working stock. Construct the low range. set by further diluting this 30 µM working stock in 2-fold serial dilutions in 1.5-mL microcentrifuge tubes. The blank will consist of just the 1X ARS dilution buffer. Examples are shown below.

High Range

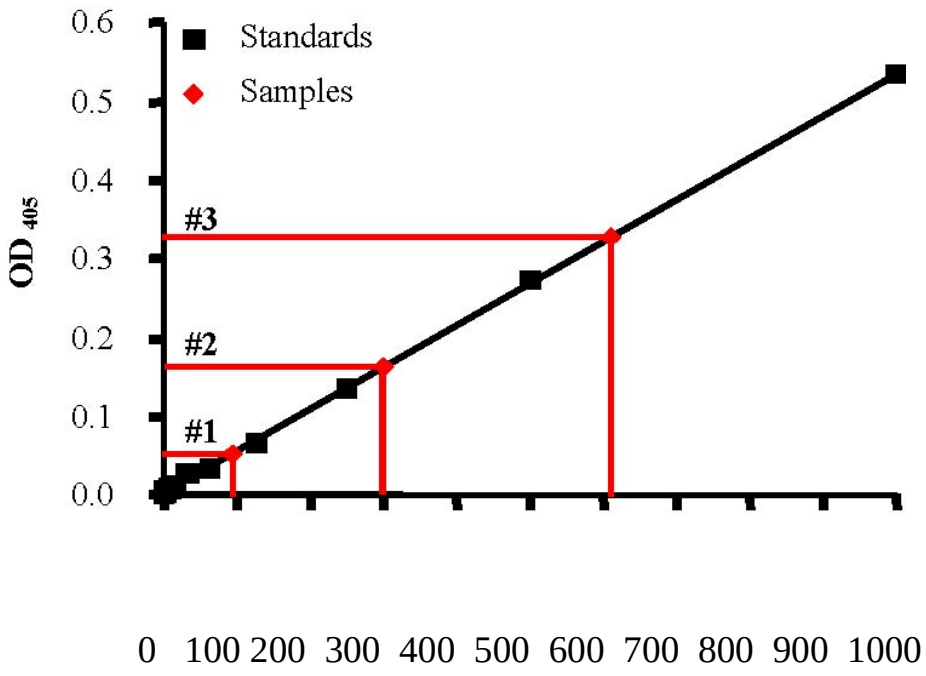
2mM	1mM	500µM	250µM	125µM	62.5µM	31.3µM	Blank

Low Range

30µM	15µM	7.5µM	3.75µM	1.88µM	0.94µM	0.47µM	Blank

8. When centrifugation is finished, remove 400 µL of the supernatant and transfer to a new 1.5 mL microcentrifuge tube.
9. Neutralize the pH with ~150 µL 10% Ammonium hydroxide. Take a small aliquot and test pH to ensure it falls within the range of 4.1 - 4.5.
10. Add 150 µL of the standard / sample to an opaque-walled, transparent bottom 96-well plate.
11. Read at OD_{405}.
12. Plot Alizarin Red concentration vs. OD_{405}.

An example of an Alizarin Red quantitation data set is shown in Figure 2 below.



Sample #	OD_{405}	Alizarin Red Conc. (µM)
1	0.053	93.85
2	0.162	298.57
3	0.328	610.33

Figure 2. Quantitation of three unknown samples using Alizarin Red standards.

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