User Manual

Osteogenesis Quantitation Kit

Catalogue Number ECM815

Pack Size 1 KIT

Store at 2-8 °C

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures. Not for Human or Animal Consumption

Introduction

Osteogenesis is the process of laying down new bone material by osteoblasts.[1]

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. Many diseases of bone including osteoporosis, a common phenomenon in post-menopausal women in which bone mass is greatly reduced, and osteogenesis imperfecta, also known as brittle-bone disease, are likely caused by the mis-regulation of osteogenesis. Understanding the molecular mechanisms that underlie osteogenesis is thus of significant clinical importance.[2]

Studies of osteogenesis have been facilitated by the development of in vitro models of this process. Examples of in vitro models used to study osteogenesis include the preosteoblastic MC3T3-E1 cell line,[3] the osteoblastic UMR 106-01 BSP cell line,[4] stromal stem cells from dental pulp,[5] mesenchymal stem cells,[6] and embryonic stem cells.[7]

In studies using each of these cell types, osteogenesis has been determined by staining with Alizarin Red Solution, which can be used to visually detect the presence of mineralization in bone tissue. In addition to non-quantitative visual detection, it has been demonstrated that, using appropriate reagents and protocol, osteogenesis can be quantified by extraction of the stain and subsequent measurement of Alizarin Red uptake.[4, 8]

Application

Our Osteogenesis Quantitation Kit provides all the necessary reagents and a protocol to enable quantification of 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 Alizarin Red Solution, a staining solution which can be used to visually detect the presence of mineralization in bone tissue. Also included are reagents for extraction of Alizarin Red from stained cells, and a protocol for performing quantitative analysis of the extracted Alizarin Red. Enough reagents are provided to perform 48 quantitation assays on 24-well plates.

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Storage and Handling

All components should be stored at 2-8 °C expiration date printed on label

Preparation of reagents

10X ARS Dilution Buffer: Cat. No. 2004810

One vial containing 5 mL of ARS (Alizarin Red Stain) Diluent is provided. Add 5 mL of 80% Acetic Acid for a final volume of 10 mL before use.

Materials Required

- Phosphate-Buffered Saline (1X PBS)
- Fixative for Alizarin Red Staining (for example 10% formaldehyde, or 4% paraformaldehyde, or 70% ethanol)
- Hemocytometer
- Microscope
- Plate reader capable of reading at 405 nm
- 80% Acetic acid

Kit Components

- Alizarin Red S Stain Solution (1X): Cat. No. 2003999. One bottle containing 50 mL Alizarin Red S Solution. Store at 2-8 °C.
- 10% Acetic Acid: Cat. No. 2004807. One bottle containing 20 mL of 10% Acetic Acid in deionized water. Store at 2-8 °C.
- 10% Ammonium Hydroxide: Cat. No. 2004809.One bottle containing 10 mL of 10% Ammonium Hydroxide in deionized water. Store at 2-8 °C.
- 10X ARS Dilution Buffer: Cat. No. 2004810. One vial containing 5 mL of ARS (Alizarin Red Stain) Diluent. **Add 5 mL of 80% Acetic Acid for a final volume of 10 mL before use**. Store at 2-8 °C.

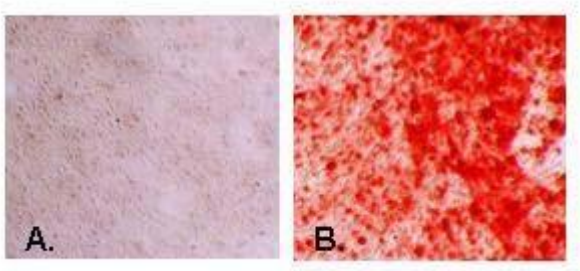
Protocols

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.

Sample Staining Results



Alizarin Red staining of MC3T3-E1 cells after 15 days of osteogenic differentiation. Using our Osteogenesis Quantitation Kit (Cat No. ECM815), differentiation of MC3T3-E1 cells to a mature osteoblastic lineage is readily detectable. Alizarin Red staining was not observed in undifferentiated MC3T3-E1 cells (A), whereas in MC3T3-E1 cells differentiated using our In vitro Osteogenesis Kit (Cat No. ECM810), the presence of Alizarin Red staining demonstrates mineral deposition throughout the culture

Protocol for Quantitative Analysis of Alizarin Red Staining

Quantitative analysis of Alizarin Red Staining can be performed 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. 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.

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 °C 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 20,000 x g for 15 minutes.
7. While centrifuging, make up Alizarin Red standards. Standards can be constructed in a 'high range' or 'low range' set.
8. **High Range:** Begin by diluting the 10X ARS Dilution buffer 1:10 in distilled H_2O to obtain a 1X working ARS Dilution buffer.
9. Next, dilute the 40 mM Alizarin Red solution 1:20 in 1X ARS dilution buffer (for example 50 µL Alizarin Red + 950 µL 1X ARS dilution buffer). This gives a 2 mM working stock.
10. Construct the 'high range' set by diluting the 2 mM working stock in 2-fold serial dilutions in 1.5 mL microcentrifuge tubes.
11. **Low Range:** To generate a 'low range' set, begin by first diluting the 2 mM working stock, 1:66 (15 µL 2 mM Alizarin Red solution + 985 µL 1X ARS dilution buffer) to achieve a 30 µM working stock.
12. 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 shown below.

High Range

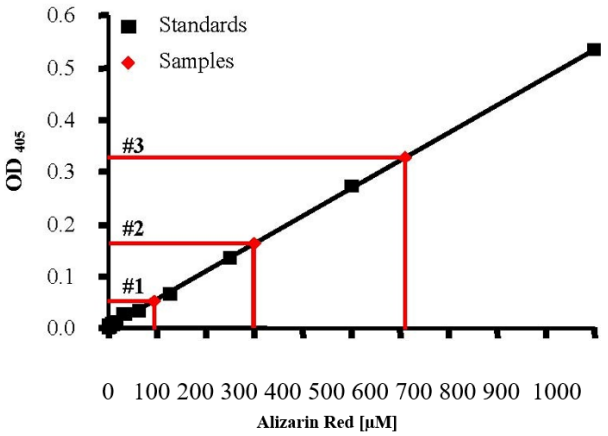
2mM	1mM	500µM	250µM	125µM	62.5µM	31.3µM	Blank

Low Range

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

13. When centrifugation is finished, remove 400 µL of the supernatant and transfer to a new 1.5 mL microcentrifuge tube.
14. 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.
15. Add 150 µL of the standard/sample to an opaque-walled, transparent bottom 96-well plate.
16. Read at OD_{405}.
17. Plot Alizarin Red concentration vs. OD_{405}.

An example of an Alizarin Red quantitation data set is shown in the figure below.



Quantitation of three unknown samples using Alizarin Red standards

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

References

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