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OsteoImage™ Mineralization Assay

Instructions for Use

Receiving Instructions

Protect from light, store kit at 4°C.

Safety Statements

THESE PRODUCTS ARE FOR RESEARCH USE ONLY. Not approved for human or veterinary use, for application to humans or animals, or for use *in vitro* diagnostic or clinical procedures.

Assay Components

Assay contains sufficient materials for assessment of five 96 well plates (500 tests) and can be scaled to other multi-well plate formats. Assay contents include:

1. 500 µl OsteoImage™ Staining Reagent
2. 50 ml Staining Reagent Dilution Buffer
3. 50 ml OsteoImage™ Wash Buffer (10x)

Introduction

Osteoblast differentiation is marked by the formation of mineralized nodules composed of inorganic hydroxyapatite (HA) ($Ca_{10}(PO_4)_6(OH)_2$) and organic components including type I collagen. *In vitro* mineralization by cultured osteoblasts is typically assessed using histochemical methods including von Kossa or Alizarin Red. Neither method is HA specific, as the von Kossa stain reacts with the anionic portion of phosphates, carbonates, and other salts[1], while Alizarin Red reacts with the calcium portion to form a chelate[2]. In addition to not being HA specific, the von Kossa stain requires multiple steps and is non-quantitative. Although Alizarin Red can quantitate calcium deposits, tedious extraction steps are required.

The OsteoImage™ Assay is based on the specific binding of the fluorescent OsteoImage™ Staining Reagent to the hydroxyapatite portion of the bone-like nodules deposited by cells. *In vitro* mineralization can be rapidly assessed qualitatively by fluorescent microscopy and quantitatively using a plate reader.

The OsteoImage™ Assay can quantitate *in vitro* mineralization by osteogenic stem cells, primary osteoblasts, and osteoblast-like cell lines (Figure 1). The assay is sufficiently sensitive to detect the time-dependent increases of mineralization in differentiating osteoblast cultures (Figure 2).

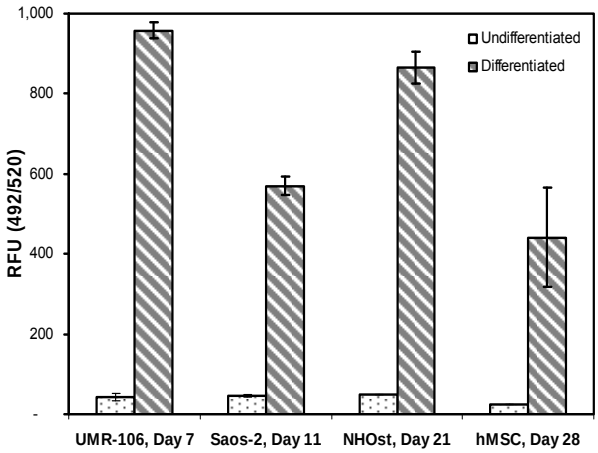

Figure 1. Osteoblast-like cell lines (UMR-106, Saos-2), primary Norman Human Osteoblasts, and Mesenchymal Stem Cells cultured for various lengths of time with differentiation factors or without (undifferentiated control cells). Mineralization evaluated by staining cells using the OsteoImage™ Assay. 96 well plates read on a plate reader using 492nm excitation and 520nm emission wavelengths.

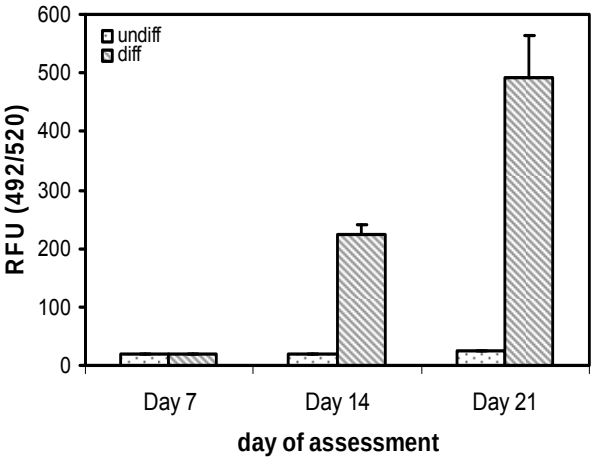

Figure 2. Normal Human Osteoblasts were seeded at 3,200 cells/well in a 96-well plate. Cells were cultured as undifferentiated control cells or with differentiation factors. Mineralization was quantitated on a plate reader after staining with the OsteoImage™ Assay on days 7, 14, and 21.

Protocol Details

- The OsteoImage™ Assay requires a fluorescence microscope or plate reader capable of excitation/emission at approximately 492nm and 520nm, respectively.
- Black-walled plates are recommended for assays using OsteoImage™; however, clear plates can be used.
- OsteoImage™ performs equally well on cells fixed with either cross-linking or denaturing fixatives (e.g. formaldehyde or alcohols).

Cell Culture Notes

- Different osteoblast cell types require varying methods and lengths of time for differentiation and for mineralization.
- If using a related Lonza cell product, refer to the Instructions for Use specific for that cell type for culture suggestions.

Preparation of reagents:

1. Calculate the total volume of OsteoImage™ Wash Buffer required for 5 wash steps (see Table 1 for suggested volumes per well). Dilute the 10x stock Wash Buffer 1:10 in deionized water at the final volume calculated (alternatively, the entire stock can be diluted for a 500 ml total volume and stored at room temperature or 4°C for future use).
2. Calculate the total volume of OsteoImage™ Staining Reagent needed based on number of wells and well size (see Table 1). Dilute Staining Reagent 1:100 in Staining Reagent Dilution Buffer to the final calculated volume. ***Mix well and keep protected from light***

Protocol for Staining & Assay:

1. When cells are ready to be evaluated for mineralization, remove culture plate from incubator and allow cooling to room temperature.
2. Remove media and wash once with PBS.
3. Fix cells using appropriate fixative method (e.g. Incubation with ethanol for 20 minutes).
4. After fixation, rinse 1-2 times with diluted (1X) Wash Buffer.
5. Add appropriate amount (See Table 1) of diluted Staining Reagent to each well.
6. Incubate at room temperature, protected from the light, for 30 minutes.
7. After incubation step, remove the Staining Reagent from wells and discard. Wash 3 times with the appropriate volume (see Table 1) of diluted Wash Buffer, leaving wash buffer in the wells for ~5 minutes per wash.
8. After final wash, add Wash Buffer to each well for microscope viewing or plate reader analysis according to volumes in Table 1.
9. View under appropriate excitation and emission settings on fluorescence microscope (e.g. Fluoroscein filter set).
10. If performing quantitative assay using fluorescent plate reader, choose appropriate excitation/emission wavelengths (492/520) and plate layout before reading plate.

Table 1

Wells/ Plate	Volume of Diluted Staining Reagent/well	Volume of Diluted Wash Buffer/well
6-well	1.0ml	2.0ml
12-well	0.75ml	1.5ml
24-well	0.5ml	1.0ml
48-well	0.2ml	0.4ml
96-well	0.1ml	0.2ml

Expected result:

Samples should exhibit green fluorescent staining proportional to the amount of mineralization present in the culture.

References

1. Bonewald, L.F. *et al.* Von Kossa staining alone is not sufficient to confirm that mineralization *in vitro* represents bone formation. *Calcif. Tissue Int.* 72, 537-547 (2003).
2. Wang, Y.H. *et al.* Examination of mineralized nodule formation in living osteoblastic cultures using fluorescent dyes. *Biotechnol. Prog.* 22, 1697-1701 (2006).

Ordering Information

PA-1503	OsteoImage™ Mineralization Assay	500 tests

Related Products

PT-2501	hMSC, Human Mesenchymal Stem Cells, cryopreserved	≥750,000 cells
CC-2538	NHOst, Normal Human Osteoblasts, cryopreserved	≥500,000 cells