Designation: F2997 – 21

Standard Practice for Quantification of Calcium Deposits in Osteogenic Culture of Progenitor Cells Using Fluorescent Image Analysis[1]

This standard is issued under the fixed designation F2997; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This practice defines a method for the estimation of calcium content at multiple time points in living cell cultures that have been cultured under conditions known to promote mineralization. The practice involves applying a fluorescent calcium-chelating dye that binds to the calcium phosphate mineral crystals present in the live cultures followed by image analysis of fluorescence microscopy images of the stained cell cultures. Quantification of the positively stained areas provides a relative measure of the calcium content in the cell culture plate. A precise correlation between the image analysis parameters and calcium content is beyond the scope of this practice.

1.2 Calcium deposition in a secreted matrix is one of several features that characterize bone formation (*in vitro* and *in vivo*), and is therefore a parameter that may indicate bone formation and osteoblast function (that is, osteoblastic differentiation). Calcium deposition may, however, be unrelated to osteoblast differentiation status if extensive cell death occurs in the cell cultures or if high amounts of osteogenic medium components that lead to artifactual calcium-based precipitates are used. Distinguishing between calcium deposition associated with osteoblast-produced mineralized matrix and that from pathological or artifactual deposition requires additional structural and chemical characterization of the mineralized matrix and biological characterization of the cell that is beyond the scope of this practice.

1.3 The parameters obtained by image analysis are expressed in relative fluorescence units or area percentage (area%), for example, fraction of coverage of the area analyzed.

1.4 *Units*—The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.6 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

F2312 Terminology Relating to Tissue Engineered Medical Products

F3294 Guide for Performing Quantitative Fluorescence Intensity Measurements in Cell-based Assays with Wide-field Epifluorescence Microscopy

3. Terminology

3.1 Unless provided otherwise in 3.2, terminology shall be in conformance with Terminology F2312.

3.2 *Definitions:*

3.2.1 *calcium deposit, n*—a calcium phosphate-containing substance synthesized in cell cultures during mineralization or osteoblast differentiation assays that may be directly produced by osteoblasts or precipitated out of the solution without cell participation.

3.2.2 *mineralized matrix, n*—a calcium phosphate-containing substance produced by cells typically in the osteoblast, odontoblast, and calcifying chondrocyte lineages, which is composed of crystals of calcium phosphate and contains Type I collagen and other non-collagenous proteins.

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *osteoblast, n*—secretory mononuclear cell that will initiate the formation of a matrix containing characteristic proteins, such as collagen, and non-collageneous proteins such

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

as bone sialoprotein and osteocalcin, that will mineralize in the presence of a calcium and phosphate source.

4. Summary of Practice

4.1 This practice consists of *(1)* fluorescently staining the calcium deposits in a cell culture using the non-toxic calcium-chelating dye xylenol orange (XO), *(2)* collecting fluorescent microscopy images of the stained samples, *(3)* collecting images of intensity standards in bead form, and *(4)* conducting image analysis of thresholded images of the standards and the samples to determine area% and mean intensity of the stained areas.

4.2 The practice involves the testing and analysis of a fluorescent intensity standard in order to determine standardized image analysis settings for imaging of the calcified cell cultures. The use of a standard allows for the comparison between different samples or different time points. Methods for determining area% and mean intensity of the standard and the samples are described.

5. Significance and Use

5.1 *In-vitro* osteoblast differentiation assays are one approach to screen progenitor stem cells for their capability to become osteoblasts. The extent of calcium deposits or mineralized matrix that form *in vitro* may be an indicator of differentiation to a functional osteoblast; however, expression of osteogenic genes or proteins is another important measurement to use in conjunction with this assay to determine the presence of an osteoblast.

5.2 This practice provides a technique for staining, imaging, and quantifying the fluorescence intensity and area related to the mineralization in living cell cultures using the non-toxic calcium-chelating dye, XO. The positively stained area of mineralized deposits in cell cultures is an indirect measure of calcium content. It is important to measure the intensity to ensure that the images have not been underexposed or overexposed. Intensity and area do not correlate directly to calcium content.

5.3 XO enables the monitoring of calcium deposits repeatedly throughout the life of the culture without detriment to the culture. There is no interference on subsequent measurements of the mineralized area due to dye accumulation from repeated application (**1**).[3] Calcium deposits that have been previously stained may appear brighter, but this does not impact the area measurement. Calcein dyes may also be used for this purpose (**1**) but require a different procedure for analysis than XO (that is, concentration and filter sets) and are thus not included here. Alizarin Red and Von Kossa are not suitable for use with this procedure on living cultures since there is no documentation supporting their repeated use in living cultures without deleterious effects.

5.4 The practice may be applied to cultures of any cells capable of producing calcium deposits. It may also be used to document the absence of mineral in cultures where the goal is to avoid mineralization.

5.5 During osteoblast differentiation assays, osteogenic supplements are provided to induce or assist with the differentiation process. If osteogenic supplements are used in excess, a calcium deposit that is not osteoblast-mediated and is referred to as dystrophic, pathologic, or artifactual may occur in the cell cultures (**2**). For example, when higher concentrations of beta-glycerophosphate are used in the medium to function as a substrate for the enzyme alkaline phosphatase secreted by the cells, there is a marked increase in free phosphate, which then precipitates with Ca^{++} ions in the media to form calcium phosphate crystals independently of the differentiation status of the progenitor cell. Alkaline phosphatase production is associated with progenitor cell differentiation, and is frequently stimulated by dexamethasone addition to the medium, which enhances the formation of calcium deposits. These kinds of calcium/mineral deposits are thus considered dystrophic, pathologic, or artifactual because they were not initiated by a mature osteoblast. The measurement obtained by using this practice may thus result in a potentially false interpretation of the differentiation status of osteoprogenitor cells if used in isolation without gene or protein expression data (**3, 4**).

5.6 Due to the possibility of artifactual calcium deposits during mineralization assays (**2-4**), gene expression analysis or protein analysis techniques demonstrating the RNA message or the presence of osteocalcin and bone sialoprotein are recommended for use in conjunction with the calcium deposit quantification procedure described here in order to confirm the presence of mature osteoblasts that are in the process of secreting a mineralizing matrix.

5.7 The deposition of a mineralized substance in the culture dish does not confirm that the cells being cultured are capable of forming bone *in vivo*.

5.8 The pattern of mineralized matrix deposition in the culture dish will vary, depending on the number of times the cells have been passaged (that is, first passage primary cells versus cells that have been passaged several times, including cell lines). First passage primary cells typically form relatively large nodules of osteoprogenitor cells that differentiate and mineralize, while cells that have been passaged many times lead to the formation of diffuse, dispersed mineral throughout the culture dish. This practice is independent of the pattern of mineralization and can be used to analyze mineralized matrix in both primary cells and cell lines.

5.9 Since some cells proliferate slower than others and since some of the cell culture surfaces being tested may affect proliferation of the cells, the data can be normalized to total cell number. Since reduced proliferation typically reduces mineralization, normalization to cell number typically does not influence the outcomes. Total DNA content can be determined as an indirect measure of cell number. There are several commercially available kits for this purpose. Since DNA analysis is a destructive, toxic assay, additional cell cultures must be prepared if this assay is used.

[3] The boldface numbers in parentheses refer to the list of references at the end of this standard.

6. Interferences

6.1 XO does not photobleach during microscopy nor leach out of the stained mineral with time, and is stable for several months; thus, stained samples can be reanalyzed or analyzed at multiple time points without loss of identified areas due to previous dye application.

6.2 There is no interference on stained area measurements due to repeated application of XO.

6.3 The substrate on which the cells are grown can affect the quantitation if non-specific fluorescent dye absorption into the substrate occurs. Tissue culture plastic commonly used for culture of cells does not interfere with this practice, but calcium-containing substrates and scaffolds (such as calcium phosphate or calcium carbonate) will bind the calcium-chelating dye used to identify the cell-produced mineral and cause background fluorescence that will interfere with this practice (**5**). Background values from analysis of XO-stained substrates that have been exposed to osteogenic medium for the same length of time must be determined and subtracted from the obtained values. All substrates beyond tissue culture plastic should be tested for non-specific dye binding prior to initiating this practice.

6.4 This practice is designed for use with living cells. Dead cells may become calcified and take up the calcium-chelating dyes, leading to artifactual mineral deposition. Because the culture medium is changed immediately before imaging to avoid non-specific fluorescence from unbound dye, floating dead cells that may interfere are also removed; however, the user must confirm if the cell cultures are viable to avoid possible misinterpretation of the assay.

7. Apparatus

7.1 *Fluorescent Microscope and Digital Camera:*

7.1.1 A 10× objective is recommended with an additional 10× in the eyepiece that results in a total of 100× magnification.

7.1.2 Microscope filter sets specific for the dyes. XO has an excitation wavelength of 570 nm and emission wavelength of 610 nm and should be examined with a TRITC (tetramethyl rhodamine isothiocyanate) red filter. The filter set range shall be recorded and similar filters can be used as long as there is no bleed-through of another fluorophore in the culture, which can be detected by imaging control cultures without the XO.

7.1.3 Camera and image collection software specifications. Digital imaging system which can include either a greyscale monochrome camera or color camera. Images obtained with a monochrome camera will be of better quality. Minimum resolution of 1000 × 1000 pixels, a minimum of 12 bit. The camera and image collection software shall be capable of saving the image in a lossless file format (for example, tiff file).

7.1.4 Computer with image analysis software.

7.1.4.1 The image analysis can be conducted using a program such as the publicly available National Institutes of Health (NIH) program called ImageJ (https://imagej.net) to quantify the positively stained areas. ImageJ is image analysis software available through the NIH and does not require a license to use. It may be utilized on Linux, Mac OS X, and Windows. It is widely used and customizable for specific image analysis tasks. Many image file types are compatible with this software including: TIFF, GIF, JPEG, BMP, PGM, FITS, ASCII, and DICOM.

8. Reagents and Materials

8.1 Xylenol orange (XO) ($C_{31}H_{28}N_2O_{13}SNa_4$) (3,3'-Bis[N, N-bis(carboxymethyl)aminomethyl]-o-cresolsulfonephthalein tetrasodium salt) is a fluorochromatic calcium-chelating dye suitable for staining calcium deposits in cell cultures. This compound binds to calcium, allowing the calcium deposits to be visualized. This dye has proven to be reliable for assessing mineralization of osteoprogenitor cultures (**2**). At the concentrations used in this practice the dye is safe and non-toxic to cells and can be used without detriment to the cultures, enabling the analysis of multiple time points.

8.2 XO is commercially available as a powder and should be made into a stock solution using sterile distilled water at 20 mM and filtered through a 0.20 µm filter, protected from light, and stored at 4 °C for up to three months. It is important to use aseptic technique and sterile reagents since this is an assay on live cultures. The XO stock solution should be added directly into the cell culture medium at a concentration of 20 µM within the culture well for 12 h to 24 h before imaging. It is important to replace the medium with fresh medium that does not contain dye prior to imaging to limit background fluorescence. At this concentration the dye will effectively stain an area of mineralized matrix in a similar manner to von Kossa staining which is commonly used in osteogenic cultures (**2**). It is thus important to use XO at this concentration and not other untested dyes which may reduce or enlarge the mineralized areas from their actual size.

8.3 A fluorescent intensity standard in the form of a suspension of fluorescence microspheres is used to calibrate image intensity (InSpeck Red, excitation 580 nm, emission 605 nm, 2.5 µm diameter beads, ThermoFisher, Cat. # I7224). Beads can be purchased from many vendors. For consistency between groups, and between tests conducted on different days or in different labs, the same standards should be used. Each kit may include six separate suspensions of fluorescent microspheres with relative fluorescence intensities of 100 %, 30 %, 10 %, 3 %, 1 %, and 0.3 %. Beads of each one of the six types have the same diameter. Guidance from the manufacturer with respect to storage and shelf life should be followed.

8.4 *Cell Culture:*

8.4.1 Living cell cultures that have been exposed to mineralizing conditions, such as osteogenic supplements (**3-5**) to induce calcium deposits are needed for this practice.

8.4.2 Cell cultures that have *not* been exposed to mineralizing conditions, but cultured for the same time period, as well as tissue culture dishes containing medium only, should also be included as negative controls.

8.4.3 If biomaterial substrates are used, tissue culture wells containing the biomaterial substrates with osteogenic medium should also be prepared as controls and kept in the incubator for the same time as the samples with cells.