



Designation: F2997 – 21

Standard Practice for Quantification of Calcium Deposits in Osteogenic Culture of Progenitor Cells Using Fluorescent Image Analysis¹

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-collagenous proteins such

¹ This practice is under the jurisdiction of ASTM Committee **F04** on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee **F04.43** on Cells and Tissue Engineered Constructs for TEMPs.

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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.