



Designation: F3106 – 22

Standard Guide for *in vitro* Osteoblast Differentiation Assays¹

This standard is issued under the fixed designation F3106; 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 document provides guidance on how to conduct *in vitro* osteoblast differentiation assays with progenitor stem cells including mesenchymal stromal cells.

1.2 This document describes the roles of various osteogenic supplements that are added to the cell culture medium of an osteoblast differentiation assay to encourage and support the differentiation of progenitor cells into matrix-producing osteoblasts.

1.3 This document provides recommendations for the concentrations of osteogenic supplements that may prevent the precipitation of artifactual mineral deposits that are not directly produced by osteoblasts, nor correlated with osteoblastic gene expression of the cells.

1.4 *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.*

1.5 *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](#)

[F2944 Practice for Automated Colony Forming Unit \(CFU\) Assays—Image Acquisition and Analysis Method for Enumerating and Characterizing Cells and Colonies in Culture](#)

¹ This guide 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.

Current edition approved April 1, 2022. Published April 2022. Originally approved in 2014. Last previous edition approved in 2014 as F3106 – 14. DOI: 10.1520/F3106-22.

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.

[F2997 Practice for Quantification of Calcium Deposits in Osteogenic Culture of Progenitor Cells Using Fluorescent Image Analysis](#)

2.2 ISO Standards:³

[ISO 21709 Biotechnology—Biobanking—Process and Quality Requirements for Establishment, Maintenance and Characterization of Mammalian Cell Lines](#)

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 deposits, 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 collagen Type I and other non-collagenous proteins.

3.2.3 *osteoblasts, n*—secretory mononuclear cells that will initiate the formation of a matrix containing characteristic proteins, such as collagen, and non-collagenous proteins such as bone sialoprotein and osteocalcin, that will mineralize in the presence of a calcium and phosphate source.

4. Significance and Use

4.1 This guidance document describes the components and conditions used for *in vitro* osteoblast differentiation assays that can be used to screen for the osteogenic capability of progenitor stem cells from various human or animal sources, including mixed tissue-derived connective tissue progenitor populations, or cell populations that may be selectively isolated or manipulated through culture expansion, processing, transfection, or genetic modification.

4.2 The osteoblast differentiation assay may be referred to as an osteogenesis assay or a mineralization assay.

³ Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, <http://www.ansi.org>.

4.3 It is important to carefully select the components and conditions used for *in vitro* osteoblast differentiation assays since high amounts of osteogenic medium components can lead to dystrophic, pathologic, or artifactual calcium-based precipitates that do not indicate differentiation of the cells in culture to functional osteoblasts (1).⁴ For example, when high 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 (2, 3).

4.4 Alkaline phosphatase production is an early event associated with osteoblast differentiation, but it can also be stimulated in other cell types by the addition of the osteogenic supplement dexamethasone to the medium. Alkaline phosphatase enhances the formation of calcified deposits prior to their natural occurrence in bone that typically coincides with bone sialoprotein and osteocalcin expression by mineralized matrix-producing osteoblasts. These kinds of calcified/mineral deposits are thus considered dystrophic, pathologic, or artifactual because they were not initiated by a mature osteoblast. A calcium measurement, such as that described in Practice F2997 for the Quantification of Calcium Deposits in Osteogenic Culture of Progenitor Cells Using Fluorescent Image Analysis, 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.

4.5 In addition to screening for multipotentiality of undifferentiated stem cells, osteoblast differentiation assays are useful for assessing the osteoinductivity of cell culture substrates or biomaterial scaffolds or drugs or biomolecules, such as cytokines or growth factors.

4.6 *In vitro* osteoblast differentiation assays are not predictive of *in vivo* bone formation, but are useful for comparison purposes to standardize performance between different types, sources or passages of progenitor cells, biomaterials, or types and concentrations of biomolecules.

5. An Overview of the *in vitro* Osteoblast Differentiation Assay Procedure

5.1 Briefly, progenitor stem cells are seeded in monolayer on treated tissue culture plastic cell culture dishes or plates, or on biomaterial substrates, and allowed to proliferate in proliferation medium until they reach confluency with bi- or tri-weekly medium changes. After confluency is reached, cell culture medium is then changed from proliferation medium to differentiation medium that contains supplements to promote the osteogenic differentiation of the progenitor cells and the formation of a mineralized matrix. The cells are cultured for up to 28 days in total with medium changes bi-weekly or every other day. Cells will undergo apoptosis during *in vitro* mineralization (4).

5.2 To assess the extent of differentiation of the progenitor cells after the osteoblast differentiation assay, the calcium deposits can be quantified using the nondestructive fluorescent image analysis as described in Practice F2997 or terminated and measured directly by conducting a total calcium content analysis using one of the many commercial colorimetric kits available for this purpose. Calcium deposition alone should never be used as a measurement of osteogenic potential.

5.3 Quantifying the expression of osteogenic genes or proteins is another important measurement to use in conjunction with measurement of calcified deposits to confirm the presence of osteoblasts. Both gene and protein measurements should be performed at multiple time points, as many peak and then later decrease.

6. *In vitro* Osteoblast Differentiation Assay Components

6.1 Cell Sources:

6.1.1 Mixed tissue-derived connective tissue progenitors from various tissues (for example, from marrow, bone, fat, synovium, periosteum, cartilage, muscle, vascular and perivascular cells, and cord blood).

6.1.2 Progenitor populations that have been selectively isolated or manipulated through culture expansion, processing, transfection, or genetic modification.

6.1.3 Osteoprogenitors obtained from the pool of cells that grow out of bone chips during explant culture or from collagenase digestions of bone.

6.1.4 Embryonic stem cells, induced pluripotent stem cells or their progeny.

6.1.5 It should be noted that optimal conditions for osteogenesis vary between species, with human cells typically more resistant to differentiation, while rodent are more reproducible. Outcomes also differ between human and rodent cells, with rodent cells more likely to form discrete calcified nodules particularly when harvested from neonatal tissues by enzymatic digestion.

6.2 Media for Osteoblast Differentiation Assays:

6.2.1 *Proliferation Phase Cell Culture Medium*—Dulbecco's Modified Eagle Medium (DMEM), including low glucose DMEM, or DMEM F-12 supplemented with 10 % Fetal Bovine Serum (FBS) is commonly used for the proliferation or expansion phase of the osteoblast differentiation assay. Some cells may have been propagated or expanded in alpha-MEM and should therefore remain in that medium for the proliferation phase, even although it contains ascorbate which can promote osteoblast differentiation.

6.2.2 *Differentiation Phase Cell Culture Medium*—For osteogenic differentiation cultures, α -MEM is commonly used during the differentiation phase, although, as mentioned, it can be used for proliferation as well. Low-glucose DMEM with additional osteogenic supplements has also been used for human mesenchymal stem cell osteogenic differentiation.

6.3 Fetal Bovine Serum (FBS):

6.3.1 Fetal bovine serum (FBS), previously called fetal calf serum (FCS), is typically used at 10 % of the medium volume during osteoblast differentiation assays in both the proliferation medium and the differentiation medium.

⁴ The boldface numbers in parentheses refer to the list of references at the end of this standard.