



Designation: F3504 – 21

Standard Practice for Quantifying Cell Proliferation in 3D Scaffolds by a Nondestructive Method¹

This standard is issued under the fixed designation F3504; 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 describes how to conduct a nondestructive proliferation test for mammalian cells based on metabolic activity that can be used to assess the number of viable cells within three-dimensional (3D) scaffolds for regenerative medicine and in tissue-engineered medical products (TEMPs).

1.2 This practice provides a detailed explanation of the resazurin cell metabolic activity method in terms of reagent concentrations, incubation times, cell culture media composition, calibration curve, controls, assay linearity, and limitations of the assay.

1.3 This practice describes factors that can interfere with accurate cell proliferation assessment.

1.4 Since the assay has washing steps, it is limited to assessing cells that are immobilized, such as by adhesion to a culture dish, adhesion to a scaffold, or encapsulation in a hydrogel.

1.5 The assay is limited to cell types that can metabolize resazurin to provide a signal in the assay.

1.6 This document does not propose acceptance criteria for a cell-based product based on the application of a cell proliferation test method.

1.7 *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.*

1.8 *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.*

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. Referenced Documents

2.1 ASTM Standards:²

F2312 Terminology Relating to Tissue Engineered Medical Products

F2664 Guide for Assessing the Attachment of Cells to Biomaterial Surfaces by Physical Methods

F2739 Guide for Quantifying Cell Viability and Related Attributes within Biomaterial Scaffolds

F3163 Guide for Classification of Cellular and/or Tissue-Based Products (CTPs) for Skin Wounds

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

2.2 ISO Standard:³

ISO 10993 Biological Evaluation of Medical Devices

2.3 ASTM Adjuncts:

Digital Spreadsheet File⁴

3. Terminology

3.1 Definitions:

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

3.1.2 *non-viable cell, n*—a cell not meeting one or more of the criteria for viability given in 3.1.6. **F2739**

3.1.3 *proliferation competent cell, n*—cell capable of replication. **F3163**

3.1.4 *senescence, n*—in vertebrate cell cultures, the property attributable to finite cell cultures, namely, their inability to grow beyond a finite number of population doublings. Neither invertebrate nor plant cell cultures exhibit this property. This term is synonymous with *in vitro* senescence. **F2664**

3.1.5 *stem cell, n*—progenitor cell capable of self-replication, proliferation, and differentiation to produce cells that take on more specialized functions. **F2312**

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.

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

⁴ Available from ASTM International Headquarters. Order Adjunct No. **ADJF3504-EA**.

3.1.6 *viable cell, n*—a cell capable of metabolic activity that is structurally intact with a functioning cell membrane. **F2739**

3.2 Definitions of Terms Specific to This Standard:

3.2.1 *cell differentiation, n*—developmental process of multi-cellular organisms, through which a cell becomes specialized in order to perform a specific function for further building different tissues and organs. (For example, bone marrow stromal cells may differentiate into chondrocytes and/or osteoblasts.)

4. Summary of Practice

4.1 This practice consists of: (1) incubating cells cultured on three-dimensional scaffolds with a solution of a metabolic dye, resazurin, which emits fluorescence after being metabolized by living cells; (2) collecting the metabolized/reduced dye solution in a multi-well plate (for example, a 96-well plate); (3) measuring the fluorescence intensity using a multi-plate reader; and (4) conducting fluorescence intensity analysis to determine cell proliferation and viability.

4.2 The resazurin molecule can penetrate the cells by passing through the cell membrane into the cytoplasm where it is reduced by cytosolic, microsomal, and mitochondrial dehydrogenase or reductase enzymes, producing the highly fluorescent resorufin. Resorufin diffuses out of cells and back into the culture medium, which is collected and measured. Resazurin reduction is not carried out by a single enzyme, as incubation with different subcellular fractions (cytosolic, microsomal, and mitochondrial) leads to the formation of resorufin (1).⁵ There are multiple isoforms of dehydrogenase and reductase. Dehydrogenase and reductase activity vary among different cell lines, which may influence the assay conditions (2). Enzymes that may participate in resazurin reduction include alcohol and aldehyde oxidoreductases, nicotinamide adenine dinucleotide (NADH) dehydrogenase, NAD(P)H:quinone oxidoreductase, flavin reductase, and cytochromes (3).

5. Significance and Use

5.1 *In-vitro* cell proliferation assays are used to screen the capability of cells to proliferate and self-renew within scaffolds for regenerative medicine and tissue-engineering applications. The cell proliferation *in vitro*, in conjunction with other characteristics of the cells such as gene expression, can be used to determine if the cells have maintained their properties.

5.2 Cell proliferation may be an important parameter to test as a quality attribute of a cell-scaffold construct. This test helps to assess cell colonization within a scaffold.

5.3 This method provides a technique for vital assessment and quantification of the fluorescence intensity related to dye metabolism by living and proliferating cells. This method assumes that viable cells will have an active metabolism, which is required to support life-associated cellular processes such as the conversion of nutrient sources into energy and proliferation. There may be cells that are not actively proliferating, yet are still viable within the construct. The

methods described within this practice enable nondestructive testing for monitoring the cell proliferation kinetics throughout the culture period by repeated analysis at multiple time points on the same test sample with minimal toxicity. This standard practice is written only for resazurin dye, a non-cytotoxic reagent that should not affect cell viability and proliferation at low concentration. This is a distinct advantage over many other reagents used to measure cell number, such as measurements of the intracellular components (such as DNA, protease, or ATP) which require cell lysis and can therefore only be used for endpoint analysis.

5.4 Resazurin, which has low fluorescence, may be metabolized by cells into resorufin, which is highly fluorescent. An increase in fluorescence caused by the conversion to resorufin may correlate with increased dehydrogenase activity, which may correlate with an increase in cell number and therefore proliferation. Plotting the signals measured at multiple time points enables the generation of proliferation curves. It is important to note that metabolic assays are intended to be measurements of intracellular dehydrogenase or reductase enzyme activity produced by cells. The level of enzyme activity may be directly proportional to the number of viable cells within a range of cell number per volume (or per scaffold) identified by a calibration curve. This is because cell metabolism rate may decrease without a loss in cell viability when cells have reached confluency or when they are differentiating. Some cells may be quiescent but still viable. Furthermore, certain cell types have different metabolic activity. In these situations, the relationship between cell metabolism and cell number may not be linear and other assays may be considered.

5.5 The method may be applied to planar 2D cell cultures and 3D scaffold cell cultures. This assay is intended for 96, 48, and 24-well plates but could work for other size plates. Size and thickness of cell scaffold construct where the test can be applicable should be tested with control experiments. In Reference (4), a 5 mm thick scaffold in a 24-well plate was used.

5.6 The method may also be used to document the absence of cell proliferation in cultures.

NOTE 1—The absence or suppression of proliferation under the tested conditions may be a result of lack of reagent/nutrient diffusion through the scaffold. If so, the same result may not be observed if diffusion is improved by, for example, changing from a 96-well plate to other cell culture formats.

5.7 The dye is not cell type specific; hence, cell identification cannot be based on this method.

5.8 The assay as described herein is not designed to assess cell distribution in scaffolds. It is possible that this could be achieved by sectioning the scaffolds prior to staining and analysis.

6. Apparatus

6.1 Fluorescence reader (for example, microplate fluorescence reader, etc.) allowing excitation in the range of 500 nm to 600 nm and emission in the range of 550 nm to 700 nm. The plate reader should be qualified and calibrated. This is typically done by automated self-checks that occur during plate reader

⁵ The boldface numbers in parentheses refer to a list of references at the end of this standard.