Designation: F3504 – 21

Standard Practice for Quantifying Cell Proliferation in 3D Scaffolds by a Nondestructive Method[1]

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.*

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 File[4]

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**

[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.

[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.

start-up, by a manufacturer-qualified technician or by use of a calibrator plate from the manufacturer or other vendor, or both.

6.2 Black-walled, clear-bottomed, multi-well plates for fluorescence measurements (for example, 96-well plates) are recommended to reduce fluorescent signal crosstalk and background.

7. Reagents and Materials

7.1 Resazurin dye (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide). A common name for resazurin is Alamar Blue. The metabolized resazurin is called resorufin, which has a peak excitation wavelength of 579 nm and a peak emission wavelength of 584 nm.

7.2 Cells.

7.3 Scaffolds for 3D testing.

7.4 Cell culture plates for 2D testing.

8. Procedure

8.1 Resazurin is commercially available as a solution or as a powder and should be made into a stock solution using PBS (phosphate buffered saline) at 44 mM. If the powder is used, it is recommended that the dye solution be filtered through a 20-µm filter to sieve any undiluted particles, protected from light, and stored at –20 °C (for up to twelve months).

8.2 The resazurin working solution should be prepared by diluting the stock solution into the cell culture medium to obtain a 44-µM working solution (that is, a 1:1000 dilution). A 44-µM resazurin solution may be suitable for measurement of cells from low to high cell densities on a scaffold, such as between 1×10^3 cells/scaffold and 8×10^5 cells/scaffold, as described for a highly porous scaffold (**4**), without the need to dilute the reaction product due to high fluorescence readings outside of the linear range.

8.3 After plating the cells on the 3D scaffold, the assay cannot be performed until the constructs have been incubated long enough to allow the cells to adhere to the scaffolds. Cells generally take one to several hours to adhere to their substrate. The appropriate amount of adhesion time may be determined by preliminary experiments for each cell type and cell density range. Cells may have low activity after thawing and their activity may increase as they recover from freezing; hence, cells should be cultured for at least 24 h prior to seeding on scaffolds.

8.4 Before performing the resazurin assay, remove the old medium to eliminate any non-adherent living cells, rinse the scaffold twice with cell culture medium to eliminate any enzyme released by lysed cells, and move the scaffold into a new, empty well so that cells which came off the scaffold and cells adhered to the bottom of the well do not contribute to the resazurin metabolism.

8.5 Add an appropriate volume of the resazurin solution to entirely cover the scaffold.

8.6 Incubate in a humidified CO_2 incubator at 37 °C, at the desired oxygen conditions or any other specific culture condition, for an incubation time which is appropriate for the given cell density (often between 1 and 4 h). The appropriate incubation time and conditions should be determined by preliminary experiments for each cell type and cell density range.

8.7 The medium containing the metabolized resazurin (resorufin) should be collected by pipetting and 100 µL transferred into a 96-well plate for fluorescence intensity measurement with an excitation in the range of 530 nm to 570 nm and an emission range of 580 nm to 620 nm, with peak measurements at 570 nm for excitation and 584 nm for emission. The fixed reading volume (100 µL in a 96-well plate) enables a comparison between results from scaffolds of variable size if the total quantity of resazurin available for scaffolds has been the same.

8.8 Due to the variability of cells in culture, sufficient replicates to meet the statistical requirements of the experiment are recommended. The recommended minimum would be three biological replicates for each scaffold/condition and conducting the entire assay three times (three assay replicates). Replicates are necessary to assess experimental variations in cell viability, seeding, and other experimental factors.

8.9 To maintain the test sample in culture, the sample should be washed twice, or more if necessary (until the culture medium appears of its own color), with a sufficient volume of cell culture medium to cover the entire scaffold, to remove resazurin and resorufin. After washing, fresh culture medium should be added to cover the entire scaffold and the plate may be returned to the incubator for further culture or for recovering for at least 24 h before performing a new assay.

8.10 To create of a proliferation curve over time, repeat 8.5 – 8.9. An interval of time of at least 24 h should be considered between two resazurin assays on the same sample.

9. Hazards

9.1 The fluorescent light source on the microscope could cause eye damage.

9.2 Consult the resazurin Material Safety Data Sheet (MSDS) for safe use.

10. Sampling, Test Specimens, and Test Units

10.1 For each test sample, multiple aliquots of metabolized resazurin (resorufin) should be analyzed by the fluorescence intensity plate reader.

10.2 The units of analysis will be fluorescence intensity counts.

11. Controls and Calibration

11.1 Resazurin is reported to be generally nontoxic (**5**). However, a concentration of resazurin higher than 100 µM or an incubation time longer than 4 h may be toxic to cells or alter their natural metabolism. This kind of inhibition may be reversed by washing and addition of fresh medium.

11.1.1 Dye toxicity should be assessed with the cell of choice before use. A metabolic assay may be suitable for this where cells are incubated with a range of resaruzin concentration, incubated for appropriate time, and then assayed (**6**).

11.2 The working volumes need to be optimized for the scaffold of interest so that liquid will entirely cover the scaffold specimen. For example, filling each well of a 24-well plate filled with 1 mL of 44-μM resazurin in fresh medium should completely cover a 5-mm high scaffold. The appropriate volumes should be evaluated in preliminary testing.

11.3 The diffusion of the dye into the 3D scaffold interior may be hindered by the scaffold pore geometry or the presence of tissue generated by the cells, which may result in a low estimate of viable cell number. This may be a source of error for this assay.

11.3.1 The effect of the scaffold on diffusion of assay components can be tested. Known numbers of live cells can be seeded into scaffolds for short time periods, such as 1 h, where it can be assumed that cells have not proliferated. The resazurin assay can then be run and compared to controls where the same number of cells are seeded into empty wells with culture medium without scaffolds. If the same results are obtained for both treatments, then it may be concluded that the scaffold is not significantly affecting the results by impeding diffusion of assay components. If the results suggest that diffusion is affecting the results, then thinner scaffolds may reduce this effect.

11.3.2 If high cell and tissue density hinder diffusion in the constructs, assessing earlier time points when densities are lower may be helpful.

11.4 Cell viability may be affected by the nature of the scaffold in which the cells have been seeded. Different types of scaffolds may have different effects on cell viability attributes. This document assumes that the scaffold is nontoxic. To test for scaffold toxicity, please refer to ISO 10993.

11.5 Some cell types may not give a signal with the resazurin assay. The reason for this may be that the dehydrogenase or reductase enzymes are not accessible to the dye in some cell types or that the isoforms of dehydrogenase that are present in a particular cell type may not be able to catalyze the dye reaction. Preliminary testing with the cell of choice to ensure that a positive signal can be obtained is recommended.

11.6 When possible, it is recommended that automated pipets be used to conduct the assay, since manual pipetting is often a major source of uncertainty for biological assays (**6**). However, suitable results may be achieved with manual pipetting.

11.7 *Controls*—There are several controls that may be included in the assay to provide evidence that the assay has proceeded as expected and to help identify sources of error in the assay. A digital file[4] is available as an adjunct to this standard that provides a spreadsheet of synthetic data demonstrating a measurement that includes many of the suggested controls (see ADJF3504-EA). For the resazurin assay, there are two micro-well plates that should be considered for controls: the “cell” plate and the “plate reader” plate. The “cell” plate is a 24-well plate that contains the cells and scaffolds and will be placed in the cell culture incubator. The “plate reader” plate is a 96-well plate that contains the aliquots of metabolized resazurin from the “cell” plate and will be placed in the plate reader for the fluorescence measurements. Each control should have an appropriate number of replicates (three or more) as required by the intended use of the data. The user must decide which controls are appropriate for a given investigation.

11.7.1 Below are the controls that should be considered for the “cell” plate (the plate that will be placed in the cell culture incubator). These control wells should be treated exactly the same as the experimental wells, including medium changes and including incubation with resazurin solution with aliquots for plate reader measurements. With experience, users may find that some of the controls may only be required in preliminary testing and could be eliminated from routine runs if it is determined that they are not required for the purposes of the assay.

11.7.1.1 Wells with cell culture medium only. This control may determine if culture medium is providing an unwanted source of fluorescent signal in the assay. There may be dehydrogenase activity in the culture medium or dehydrogenase activity present in the culture medium may adsorb to the culture plates. If acidosis occurs, then low pH may cause a shift in the excitation/emission peaks of resorufin. There may be other mechanisms whereby the culture medium could provide a false signal in the assay.

11.7.1.2 Wells with scaffold only (no cells). This may be the most important control to run. This control may determine if the scaffold is providing an unwanted source of fluorescent signal in the assay. Dehydrogenase activity may be present in the culture medium or dehydrogenase that is present in the culture medium may adsorb to the scaffolds. There may be an interaction between the scaffold and the dye. The scaffold properties may change with incubation in medium, which may affect their ability to adsorb enzymes. These phenomena may provide an unwanted source of fluorescence signal in the assay. This control might also serve as the background signal for the assay, since the cells are the only major component that is lacking in comparison to the experimental wells. If this control were used as a background, then the signal in this well could be subtracted from the signal measured in the presence of cells on the scaffolds to obtain the signal induced by the cells.

11.7.1.3 For hydrogel scaffolds, it is important to consider that the liquid in the gel scaffold may dilute the resazurin solution and may change its concentration. Hydrogels may contain more than 90 % by volume liquid. This event can affect the comparison between results from scaffolds of variable size, even if the total quantity of resazurin available for scaffolds has been the same (see 8.7). If the criteria in 11.3.1 are met for different scaffold formulations and thicknesses, then direct comparison may be possible.

11.7.1.4 Wells with cells only (no scaffold). This control may serve many roles. A positive signal from this well establishes that the assay is working, that the cells being used in the test are viable, that they were able to adhere to the bottom of the culture well, and were able to metabolize the dye. When conducted in replicates, these wells may be used to assess well-to-well variability in the test results, which may be due to variability in cell aliquoting via pipet.

11.7.1.5 Wells with cells and scaffold. These are the test wells that are of primary interest in the assay.