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Standard Guide for Quantifying Cell Viability and Related Attributes within Biomaterial Scaffolds[1]

This standard is issued under the fixed designation F2739; 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 guide is a resource of cell viability test methods that can be used to assess the number and distribution of viable and non-viable cells within porous and non-porous, hard or soft biomaterial scaffolds, such as those used in tissue-engineered medical products (TEMPs).

1.2 In addition to providing a compendium of available techniques, this guide describes materials-specific interactions with the cell assays that can interfere with accurate cell viability analysis, and includes guidance on how to avoid or account for, or both, scaffold material/cell viability assay interactions.

1.3 These methods can be used for 3-D scaffolds containing cells that have been cultured *in vitro* or for scaffold/cell constructs that are retrieved after implantation in living organisms.

1.4 This guide does not propose acceptance criteria based on the application of cell viability test methods.

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.6 *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.*

1.7 *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]

F748 Practice for Selecting Generic Biological Test Methods for Materials and Devices

F2149 Test Method for Automated Analyses of Cells—the Electrical Sensing Zone Method of Enumerating and Sizing Single Cell Suspensions

F2315 Guide for Immobilization or Encapsulation of Living Cells or Tissue in Alginate Gels

F2998 Guide for Using Fluorescence Microscopy to Quantify the Spread Area of Fixed Cells

2.2 *ICH Document:*[3]

ICH Q2(R1) Validation of Analytical Procedures: Text and Methodology

2.3 *FDA Document:*[4]

U.S. Food and Drug Administration (FDA) Center for Biologics Evaluation and Research (CBER), 2015 Analytical Procedures and Methods Validation for Drugs and Biologics—Guidance for Industry

3. Terminology

3.1 *Definitions:*

3.1.1 *non-viable cell, n*—a cell not meeting one or more of the criteria for a viable cell.

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

3.1.2.1 *Discussion*—The use of the term viable herein only applies at the instant at which the measurement is conducted and is not meant to indicate anything about the future state of the cell.

[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.

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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 International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), ICH Secretariat, 9, chemin des Mines, P.O. Box 195, 1211 Geneva 20, Switzerland, http://www.ich.org.

[4] Available from U.S. Food and Drug Administration (FDA), 10903 New Hampshire Ave., Silver Spring, MD 20993, http://www.fda.gov.

4. Summary of Guide

4.1 It is the intent of this guide to provide a compendium of the commonly used methods for quantifying the number and distribution of viable and non-viable cells within, or on, a biomaterial scaffold, because cell viability is an important parameter of tissue-engineered products used to regenerate or repair lost or diseased tissue. The methods can be applied to cells residing within an intact 3-D scaffold or matrix (that is, non-destructive methods) or to cells that have been removed from the scaffold or matrix (that is, destructive methods). It should be noted that not all cells require a scaffold, and some cell types, such as hematopoietic cells, cannot be cultured or grown on an adherent surface.

4.2 Most of the methods originate from analysis of cell number on 2-D surfaces, but have been adapted for the analysis of cells within 3-D constructs that are typically used in regenerative medicine approaches. The mechanisms and the sensitivity of the assays are discussed. The limitations of the assays due to using standard curves generated from cells on 2-D surfaces are described in this document. In addition, the ways in which the biomaterial scaffold itself can affect the viability assays are described.

4.3 This guide describes test methods which, when used together, may enable accurate measure of the number and distribution of viable and non-viable cells. Different viability assays have different measurands, which means that the results from different assays may not correlate with one another. For instance, cell membrane integrity tests and cell metabolic tests measure fundamentally different cell properties. Although both tests are related to cell viability, they may not correlate with one another.

5. Significance and Use

5.1 The number and distribution of viable and non-viable cells within, or on the surface of, a biomaterial scaffold is one of several important characteristics that may determine *in vivo* product performance of cell/biomaterial constructs (see 5.7); therefore, there is a need for standardized test methods to quantify cell viability.

5.2 There are a variety of static and dynamic methods to seed cells on scaffolds, each with different cell seeding efficiencies. In general, static methods such as direct pipetting of cells onto scaffold surfaces have been shown to have lower cell seeding efficiencies than dynamic methods that push cells into the scaffold interior. Dynamic methods include: injection of cells into the scaffold, cell seeding on biomaterials contained in spinner flasks or perfusion chambers, or seeding that is enhanced by the application of centrifugal forces. The methods described in this guide can assist in establishing cell seeding efficiencies as a function of seeding method and for standardizing viable cell numbers within a given methodology.

5.3 As described in Guide F2315, thick scaffolds or scaffolds highly loaded with cells lead to diffusion limitations during culture or implantation that can result in cell death in the center of the construct, leaving only an outer rim of viable cells. Spatial variations of viable cells such as this may be quantified using the tests within this guide. The effectiveness of the culturing method or bioreactor conditions on the viability of the cells throughout the scaffold can also be evaluated with the methods described in this guide.

5.4 These test methods can be used to quantify cells on non-porous or within porous hard or soft 3-D synthetic or natural-based biomaterials, such as ceramics, polymers, hydrogels, and decellularized extracellular matrices. The test methods also apply to cells seeded on porous coatings.

5.5 Test methods described in this guide may also be used to distinguish between proliferating and non-proliferating viable cells. Proliferating cells proceed through the DNA synthesis (S) phase and the mitosis (M) phase to produce two daughter cells. Non-proliferating viable cells are in some phase of the cell cycle, but are not necessarily proceeding through the cell cycle culminating in proliferation.

5.6 Viable cells may be under stress or undergoing apoptosis. Assays for evaluating cell stress or apoptosis are not addressed in this guide.

5.7 While cell viability is an important characteristic of a TEMP, the biological performance of a TEMP is dependent on additional parameters. Additional tests to evaluate and confirm the cell identity, protein expression, genetic profile, lineage progression, extent of differentiation, activation status, and morphology are recommended.

5.8 The main focus of this document is not scaffold toxicity or the toxicity of the scaffold raw materials. This document is meant to address the situation where a scaffold that is thought to be cytocompatible is cultured with cells and the user desires to assess the viability of cells within the construct. Prior to conducting the tests described herein, the raw materials used to make the scaffold should be assessed as described in Practice F748. This testing may include assessment of the release of toxic leachables from the raw materials.

5.9 Methods that remove the cells from a 3-D scaffold may reduce the cell number and viability due to the manipulation required.

5.10 Some scaffold constructs may prevent reliable measurements of cell viability within the scaffolds using the methods described herein. Scaffolds may limit diffusion of assay components into and out of the scaffolds. This is especially problematic for methods that require dyes to penetrate into the scaffold, that require detergents or other cell-lysing agents to diffuse into the construct, that require lysed-cell components to diffuse out of the constructs, or that require assay reactants to diffuse into or out of the scaffold. Diffusion in scaffolds and assay results may also be affected by dense cell populations in scaffolds, the generation of tissue-like structures by the cells within the scaffold, and the presence of cell-generated extracellular matrix (ECM) in the scaffold. The formation of tight junctions between cells and cell-ECM interactions may also limit diffusion, especially in the case of hard tissues such as bone.

5.11 Assay results may be affected by interactions between assay components and the scaffold. Assay components may adsorb to the surface of the scaffold which would affect their participation in the assay and the resulting assay signal.

Biochemical interactions between the scaffold and assay components may cause activation or inhibition of the assay chemistries.

5.12 Different cell viability tests may measure different things and may not agree with one another. A large variety of cell viability assays have been developed to measure different aspects of the cell death process. Some of the common measurements include penetration of dyes into the cell, cell metabolic activity, cellular ATP, and leakage of intracellular components out of the cell. Each of these phenomena are related to the state of cell viability in different ways, and may represent different attributes of the cell death process. The mechanism of cell death will also affect the results for these different types of viability measurements. Necrosis, oxygen depravation, starvation, chemical toxicity, apoptosis, anoikis, and mechanical damage represent some of the causes of cell death. Each of these mechanisms may have different effects on the different aspects of cell death that are measured by cell viability assays.

6. Selection of Test Methods

6.1 Table 1 is a compendium of methods that can be used to quantify cell viability on surfaces or in biomaterial scaffolds. Importantly, a combination of the methods listed in Table 1 is required to determine viable and non-viable (or live and dead) cells quantitatively, and additional tests must be completed to quantify the subset of proliferating viable cells within the total number of viable cells. Proliferating cells are viable, but viable cells are not necessarily proliferating. Non-viable cells can be identified, even if they are not intact structurally or metabolically, by intact nuclei, DNA stains, or dye entry into the cell through a disrupted cytoplasmic membrane.

6.2 Development of assays to assess cell viability in scaffolds should consider ICH Q2(R1), which describes assay characteristics of accuracy, precision, specificity, detection limit, quantification limit, linearity, and range. Another useful resource is the FDA Guidance for Industry on Analytical Procedures and Methods Validation for Drugs and Biologics. These documents are applicable to cell-scaffold constructs intended for clinical use where cell viability quantification is used as part of the process to establish identity, strength, quality, purity, and potency.

6.3 The total number of cells, both alive and dead, within a 3-D construct may be determined by DNA analysis (7.2) after the cells are removed destructively (lysis) from the biomaterial scaffold and solubilized (with detergents or sonication, for example). It may not be possible to completely recover all cell material that is located deep within scaffold pores due to diffusion limitations.

6.4 Counting cells harvested (by trypsinization or passaging, for example) from scaffolds may not be reliable if the scaffold specimens are small (from 96-well or 48-well plates, for example). The dilutions with cell harvesting medium or buffers may yield cell concentrations that are too low to be effectively counted (by hemocytometer, for example).

6.5 If cells in a suspension are to be counted, electrical sensing zone test method (Test Method F2149) or flow cytometry may be useful.

6.6 To determine the quantity of live cells only, the use of a fluorescent or colorimetric metabolic indicator that fluoresces or changes color in response to cell metabolic activity may be used (7.2). Metabolic assays are available in both destructive

TABLE 1 Methods for Quantifying Cell Viability

	Destructive (Requires cell removal from scaffold or matrix)	Non-destructive (Cells remain in scaffold or matrix during test)
I. Total Cell Number		
DNA assay	X	
Crystal violet	X	
II. Live Cell Number		
Metabolic assays	X	X
Tetrazolium salt uptake: MTT, MTS, WST, XTT	X	
Alamar Blue (resorufin)		X
Neutral Red	X	
Glucose Consumption	X	X
Cell proliferation (DNA synthesis)		
[3H] Thymidine or BrDu (Bromodoeoxyuridine) labeling	X	
Dye exclusion assays		
Trypan blue, erythrosin, and nigrosin	X	
III. Live/Dead Ratios		
Live/Dead assays using dual fluorescent stains for plasma membrane integrity	X	
Non-fluorescent dye exclusion assays	X	
IV. Imaging—density, morphology and spatial distributions of cells		
Histological sectioning	X	
Confocal microscopy	X	X
Scanning electron microscopy	X	

and non-destructive forms. The MTT (3-[4,5-dimethylthiazol-2]-2,5-diphenyltetrazolium bromide) or MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assays (7.2.1) are destructive, commonly used methods that can be read with a spectrophotometer. The Alamar Blue assay (resorufin) (7.2.2) is a non-destructive method that requires a fluorimeter. Cell metabolism in a 2-D environment may differ from than in a 3-D environment, even when the same cell numbers are the same. Accordingly, results for 3-D cell numbers can be erroneous when growth curves of cells cultured in 2-D are used for calibration (**1**).[5] It is important to note that metabolic assays are direct measures of intracellular enzyme activity produced by cells. Although the level of enzyme activity may be directly proportional to the number of viable cells, it is possible that specific culture conditions may affect the production and activity of the enzyme being assayed or that the scaffold may interfere with the measurement (matrix effects). In this situation, the metabolic measurement may not be directly proportional to cell number.

6.7 The quantity of live cells within the total cell population may be determined by a proliferation or metabolic assay (7.3). It may be helpful to verify quantitative results with an imaging technique (7.4) in order to provide visual evidence of live or dead cells. Visual evidence assures that the quantitative measurements can be trusted and did not arise due to experimental artifacts (such as the scaffold reacting with assay reagents and causing a false positive reading). Imaging also provides information on the spatial distribution of live cells within a construct.

6.8 Non-destructive methods to determine cell viability of an entire cell population within a scaffold or bioreactor are included in this guide and are useful for conducting kinetic studies of cell number and distribution over time within the same sample.

6.9 Since scaffolds can interfere with cell viability assays, a blank scaffold, without cells, should always be included as a control. Assay components may bind or adsorb to the scaffold, which can affect results. The scaffold may activate assay components, causing a false positive reading. If the assay is affected by the presence of the scaffold, then either the interference should be subtracted or an alternative assay should be selected. Notes on known interferences are included in each of the assay descriptions below.

6.10 Cell density could impact accuracy of quantification. Cells grown at low density are generally harder to wash off than cells grown to confluency, where a whole sheet of cells may be rather easy to displace. Many scaffolds are seeded at as high a cell density as possible. High densities may also affect dye binding. Also, cell density generally impacts the "health" of the whole culture, since cell-to-cell interactions are important effectors of cell state.

6.11 In many instances, a mixed population of cells may be present. Metabolic assays will not accurately quantify mixed cultures of cells because some cells are more metabolically active than others. There is a similar problem with dyes: nuclear sizes may not be identical (though they may be similar). Cell cytoplasm volumes may be very different, as could be the number of cellular processes. In a mixed population of cells, some cells may be proliferating rapidly, whereas others might be post-mitotic.

6.12 Some scaffolds will be translucent, others opaque. Some may be rigid, others very fragile. For more fragile scaffolds, cells may fall off during handling, so it would be preferable to use a method that minimizes handling. Scaffolds break down over time. Edges of scaffolds might be softer than internal portions. Scaffolds may not have uniform thickness or density, which may affect statistical sampling.

6.13 It is important that any test of cell viability clearly defines how cell viability is measured. There are many tests for cell viability, and confusion often arises when the methods used to assess cell viability are not clearly defined.

7. Specific Test Methods for Determining Cell Viability

7.1 *Dye Exclusion Technique to Distinguish Live from Dead:*

7.1.1 One of the simplest methods to approximate cell viability is the dye exclusion technique. This approach is based on the assumption that viable cells must have an intact membrane, which is required for life-associated cellular processes such as the conversion of food sources into energy, growth, and reproduction. This method utilizes an indicator dye to demonstrate cell membrane damage. Cells which absorb the dye become stained and are considered non-viable. Dyes such as trypan blue, erythrosin, and nigrosin are used commonly, with trypan blue being the most common in preliminary cell isolation procedures. Cells must be removed from the scaffold, mixed with the dye, and then counted manually with a hematocytometer. Cells must be analyzed shortly after the addition of 0.4 % trypan blue, since trypan blue is cytotoxic. There are large standard deviations with increasing cell densities; therefore, samples should be diluted to the densities recommended in the hematocytometer instructions.

7.2 *Determination of Total Cell Number:*

7.2.1 *DNA Assay*—DNA analysis is a commonly used method for determining cell number because the amount of DNA per cell is relatively constant. There are several commercially available kits for assessing DNA content. It is important to fully extract the cells from the scaffold prior to analysis, using for example, a solution of 0.125 mg/mL papain and 10 mmol⁄L L-cysteine dihydrochloride in phosphate-buffered ethylenediaminetetraacetic acid (EDTA) in a 60 °C water bath for 10 h (**2**). The process lyses the cells to yield soluble DNA for detection by the assay and the papain and EDTA inactivate nucleases to prevent DNA degradation. A freeze/thaw cycle (-20 °C for at least 1 h) can rupture cell membranes to improve DNA recovery. For natural ECM-based biomaterial scaffolds or constructs with tissue-like content having high cell densities, enzymatic treatment for protein digestion, using, for example, proteinase K digestion for 2 h to 6 h at 60 °C, can be used to

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