



Standard Guide for Quantitating Cell Viability Within Biomaterial Scaffolds¹

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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, and/or account for, 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 and health practices and determine the applicability of regulatory limitations prior to use.*

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

3. Terminology

3.1 *Definitions:*

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

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

4. Summary of Guide

4.1 It is the intent of this guide to provide a compendium of the commonly used methods for quantitating the number and distribution of viable and non-viable cells within, or on, a biomaterial scaffold, because cell viability is important parameter of tissue engineering 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).

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 combinations of test methods which, when used together, will ensure the most accurate measure of the number and distribution of viable and non-viable cells.

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 quantitate cell viability.

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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.

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 on to 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 number 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 quantitated 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 quantitate cells on hard or soft 3-D biomaterials, such as ceramics and polymer gels. 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 dependant 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 Fundamental biocompatibility testing of the scaffold material itself as described in Practice F748 should be completed prior to using the biomaterial with cells.

6. Selection of Test Methods

6.1 Table 1 is a compendium of methods that can be used to quantitate 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 quantitate 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 or dye entry into the cell through a disrupted cytoplasmic membrane.

6.2 The total number of cells, both alive and dead, within a 3-D construct is typically determined by DNA analysis (7.2) after the cells are removed destructively from the biomaterial scaffold and solubilized. Since many cell types adhere very well to a scaffold, significant cell lysis can occur during cell removal and simple manual counting of intact stained or unstained cells (7.1) is not very reliable.

TABLE 1 Methods for Quantitating 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		X
Neutral Red	X	
Glucose Consumption	X	X
Cell proliferation (DNA synthesis)		
[3H] Thymidine or BrDu labeling	X	
Dye exclusion assays		
Trypan blue, erythrosin, and nigrosin	X	
III. Live/Dead Ratios		
Live/Dead assays using dual fluorescent stains	X	
for plasma membrane integrity		
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	

6.3 If cells in a suspension are to be counted, Test Method **F2149** may be useful.

6.4 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 chemical reduction of growth media resulting from cell metabolic activity may be used (7.2). Metabolic assays are available in both destructive and non-destructive forms. The MTT or MTS assay (7.2.1) is a destructive, commonly used method that can be read with a spectrophotometer. The Alamar Blue assay (7.2.2) is a non-destructive method that requires a fluorimeter. Cell metabolism in a 2-D environment, compared with a 3-D environment, is significantly different, even with the same cell numbers. Accordingly, results for 3-D cell numbers can be erroneous when growth curves of cells cultured in 2-D are used for calibration (1).³ It is important to note that metabolic assays are direct measures of intracellular enzyme activity produced by cells. Although the level of enzyme activity is typically linearly proportional to the number of viable cells, it is possible that specific culture conditions can affect the production and activity of the enzyme being assayed or that in certain circumstances, substrate may not be limiting. In this situation, the metabolic assay would not necessarily be linearly proportional to cell number. Despite these limitations, these methods are still commonly used.

6.5 The quantity of live cells within the total cell population may be determined by a proliferation or metabolic assay (7.3). However, this will not provide information on the distribution of live cells within a construct; hence, an imaging technique (7.4) to visualize the morphology and spatial distribution of dye-labeled live and dead cells is typically utilized.

6.6 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.

6.7 The scaffolding material may interfere with any of the following assays and must be included within the assay, typically as a control, to determine whether it has an effect. If the assay is affected by the presence of the scaffold, then either the interference should be subtracted out or an alternative assay should be selected. Notes on known interferences are included in each of the assay descriptions below.

6.8 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 of our products may be seeded at as high a density as possible. High densities may also affect dye binding.

6.9 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 are probably

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.

6.10 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.

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 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 for a few minutes, and then counted manually with a hemacytometer. Cells must be analyzed shortly (3 to 5 min) 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 hemacytometer instructions.

7.2 Determination of Total Cell Number:

7.2.1 **DNA Assay**—DNA analysis is a commonly used method for determining total cell number, including both viable and non-viable cells. There are several commercially available kits for assessing DNA content, all of which can be used. 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 EDTA in a 60°C water bath for 10 hours to extract cells from a polymer matrix (2). If the cell fluorescence will be measured, using a proteinase K digestion step ablates any residual endogenous fluorescence of the cells (3).

7.2.2 **Crystal Violet Staining**—Another cell stain used for determining total cell number is crystal violet which binds to the DNA of viable and non-viable cells. Cells must be removed from the biomaterial scaffold prior to analysis. Cells are washed in phosphate buffered solution (PBS), stained with 0.1 to 0.2 % crystal violet in methanol for 15 min at 37°C, and then washed extensively prior to analysis. Absorbance is measured at a wavelength of 590 nm using an ELISA plate reader.

7.3 Proliferation or Metabolic Assays for Quantitating Live Cell Number:

7.3.1 **MTT, MTS, XTT or WST Tetrazolium Salt Assays**—Metabolic activity of cells is commonly monitored colorimetrically by assaying the cell-based alteration of tetrazolium salts such as MTT (3-[4,5-dimethylthiazol-2]-2,5-diphenyltetrazolium bromide). The cells take up the crystals and convert the tetrazolium salt to formazan crystals by the

³ The boldface numbers in parentheses refer to the list of references at the end of this standard.

succinate-tetrazolium reductase system in the mitochondria. The amount of formazan dye formed correlates directly to the number of metabolically active cells in the culture. A standard ELISA plate reader is used to read the results. The seeded scaffolds should be rinsed in either a serum-free medium or PBS to remove unattached cells before beginning the assay. Since there can be a chemical interaction of the biomaterial scaffold with the assay components or an absorbance from the scaffold itself, an unseeded scaffold must be used as a control. Standard curves are established from known cell numbers. A large, but not excessive variability between absorbance values of similar samples can be expected due to natural variability in cell output. The dissolution of the formazan crystals can be slow and difficult in the original MTT assay, and requires alcohol, which may damage the scaffold and cells; therefore, an aqueous MTS assay that does not require dissolving the formazan crystals is now available. These kits contain a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)] and an electron coupling reagent (phenazine ethosulfate; PES). The XTT kit, which uses XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) instead of MTT, is also easier to run than the MTT because it produces a soluble dye. A non-destructive version of a tetrazolium salt-based proliferation assay is the WST. The WST method utilizes the tetrazolium salt WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium). The redox indicator resazurin can also be used for non-destructive monitoring of proliferation.

7.3.2 ATP Content—ATP content and oxygen uptake have been shown to be highly predictive of pancreatic islet performance, for example, and it is possible to assess high energy phosphoryl metabolism, particularly of ATP and creatine phosphate in living tissue, using non-destructive P-31 NMR or, to obtain a more detailed picture of overall metabolic dynamics, stable isotope labeling with O-18 and mass spectrometry.

7.3.3 Alamar Blue Assay—The Alamar Blue assay uses a non-cytotoxic oxidation-reduction metabolic indicator that fluoresces and changes color in response to chemical reduction of growth media resulting from cell growth. Since the assay is not cytotoxic it is non-destructive and can be used repeatedly to assess cell viability and cell proliferation. It is applicable to non-proliferating cells as well. An advantage of this method is that cells do not have to be removed from the scaffold prior to analysis. Standard curves are established from known cell numbers exposed to the reagent (per unit time). A fluorimeter or a standard spectrophotometer is used to read the results.

7.3.4 Neutral Red Assay—The neutral red assay is based on the rapid uptake of the dye into the lysosomes of viable cells. After cells are incubated with the dye, the cells are lysed and the dye uptake is determined spectrophotometrically. When significant changes in cell viability occur, irreversible changes in the lysosome and dye uptake mechanism occur. The dye uptake is compared to that of a control sample and the % viability with respect to control cells is reported. This assay is used routinely in high-throughput screening for cytotoxic

compounds. This dye becomes toxic with light, so the reagents must be handled with safety precautions.

7.3.5 ³H-Thymidine or BrdU Labeling—Incorporation of tritiated thymidine into cellular DNA during the synthesis phase of the cell cycle is a widely used and sensitive protocol to monitor rates of DNA synthesis and hence cell proliferation. Cells are seeded and incubated with tritiated thymidine. During each cell division, the nucleotide becomes incorporated into the newly synthesized chromosomes. The more cell divisions (or the higher the proliferation rate), the more radioactivity is incorporated into cell DNA. After incubation, the cells are harvested, the cells are disrupted, and DNA is released. The cell fragments and intact DNA are collected on a filter. The higher the proliferation rate of the cells during incubation, the more cells are harvested along with their radioactive DNA. The filter membrane is dried and the amount of radioactivity (which corresponds to the number of cell divisions during incubation) is counted in a scintillation counter. Note that there is evidence demonstrating the ability of this radiochemical to induce cell cycle arrest and apoptosis, leading to erroneous results if the rate of proliferation is being determined (4). This assay is best when used as a terminal or endpoint assay. Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) is an analogue of thymidine often used in place of ³H-Thymidine because it is also incorporated into the newly synthesized DNA of replicating cells. BrdU is commonly used in the detection of proliferating cells in living tissues. Antibodies specific for BrdU can then be used to detect the incorporated chemical by flow cytometry, immunocytochemistry, or ELISA-type assays. Binding of the antibody is achieved by denaturation of the DNA, usually by exposing the cells to acid or heat. Caution should be used as this step may damage the biomaterial scaffold.

7.3.6 Glucose Consumption—The consumption of glucose by cultured cells is another measure of cell growth and metabolism. Glucose levels are measured before and after incubation with a given cell type and number to establish standard curves. Commercial spectrophotometric assays are available to quantify glucose depletion. The method of analysis must be selected so as to avoid spectral interference from the phenol red and calf sera present in cell culture media.

7.3.7 Enzymatic Cleavage of Reporter Substrates—There are several colorimetric, fluorescent, and luminescent assays for viability that take advantage of the ability of enzymes in culture supernatant to cleave reporter substrates. Enzymes such as glucose 6-phosphate dehydrogenase (G6PD) and lactate dehydrogenase (LDH) are released by cells with damaged membranes, and are thus an indication of cell death. These tests have the advantage that they can be performed on aliquots of existing cultures.

7.3.8 Concerns About Proliferation Assays Used for Determining Viability Within Three-Dimensional Constructs:

7.3.8.1 Variability in metabolic output for cells seeded on thick (more than 1 mm) 3-D scaffolds, and even on sheets of cells, has been observed due to variability of nutrient access into the inner portions of the scaffold/cell layer (1). Therefore, despite increasing DNA content, metabolic activity does not increase as well in parallel. There is currently no way to account for this variability, so calibration curves from 2-D

constructs must be used. Hollow fiber culture systems may offer a means of preparing 3-D control scaffolds.

7.3.8.2 Scaffold thickness may affect the diffusion of the assay substances into the scaffold and hence the cellular processing of the assay components. For replicate samples it may not be possible to have every scaffold sample the exact same size or dimensions, so some measure of scaffold volume or weight must be taken into consideration.

7.3.8.3 Polylactic acid is a common resorbable material commonly used in scaffolds for TEMPs. As these scaffolds degrade they release the metabolic product lactic acid. The release of such chemicals or others might impact assays sensitive to changes in pH or dependent on metabolic activity.

7.3.8.4 The dye reduction assays depend primarily on mitochondrial metabolism. This may not be useful for tissues that generally function anaerobically.

7.4 *Imaging or Visual Methods to Determine Cell Viability:*

7.4.1 *General*—To determine spatial distributions of the cells within the biomaterial scaffold it is necessary to visualize the cells using a microscope, such as a traditional light microscope, confocal microscope, or electron microscope.

7.4.2 *Histological Sectioning*—Traditional histological sectioning and staining followed by counting of cell numbers provides a measure of cell seeding uniformity.

7.4.3 *Fluorescent Markers for Live and Dead Cells*—Live/Dead assays are most commonly used to determine the ratio of live to dead cells. Other chemistries have been developed for this use, but commonly calcein AM is taken up by live cells and is seen as a green fluorescence, while an ethidium homodimer is taken up by dead cells with damaged membranes and is seen as a red product. There are assays available for both *in vitro* and *in vivo* use. Sections can be made, particularly through soft biomaterials, enabling quantitation by a plate reader on the thin slabs of approximately 1 mm. Cells may also be stained with a variety of commercially available fluorescent markers for proliferating viable or non-viable cells and then counted and classified using flow cytometry. Cells may be genetically manipulated to express fluorescence, in which case the net fluorescence could be quantitated through the use of a 2-D culture and a dose range of cells.

7.4.4 *Confocal Microscopy*—After labeling the live and dead cells with fluorescent markers, confocal microscopy can be used to distinguish distribution of live and dead cells within the construct. Since confocal microscopes typically only penetrate into a construct a few hundred microns at most, multiple sections are necessary to quantitate the proportion of viable cells and their distribution throughout the construct. Two photon confocal microscopy (or imaging) may not be practical for routine analysis. To address the issue of specimen thickness, which limits the applicability of confocal microscopy to some extent, multi-photon imaging is a possibility, albeit one that requires fairly expensive instrumentation. Depending on thickness of the sections, non-confocal microscopes can also be used to quantitate numbers of viable cells and to provide information on the 3-D cell population within a construct.

7.4.5 *Magnetic Resonance Imaging*—For very thick scaffolds (mm to cm), high field MRI might be possible, using

either iron oxide or gadolinium tags. This approach would have the advantage that it is designed for 3D analysis, but is severely limited by resolution and contrast.

7.4.6 *Scanning Electron Microscopy (SEM)*—The extent and uniformity of the cell seeding or cell ingrowth within a biomaterial can be analyzed by SEM or an environmental-SEM (E-SEM). The cells must be fixed using traditional tissue fixation and dehydration methods for electron microscopy (5). Less preparation is required for E-SEM (for example, cacodylate-buffered 2.5 % glutaraldehyde (v/v) for 1 h prior imaging (6)). With a Cryo-SEM no sample fixation is needed. Samples are freeze dried and transferred directly to the chamber for analysis. Typically a sample through the middle of the 3-D construct is analyzed, as well as the outer surface.

7.4.7 *Some Concerns About Visual Observation of Cells Within Scaffolds:*

7.4.7.1 Uniform staining cannot be assumed and must be confirmed through visual observation.

7.4.7.2 If used with a scaffold, the scaffold may absorb the dye or have a background autofluorescence.

7.4.7.3 Quantifying cells within a scaffold or even on thick sections of a scaffold is difficult because of the shallow depth of field of imaging devices and microscopes. Scaffolds do not always lie flat. Some cells will be in focus and others out of the focal plane. Confocal analysis partially gets around this problem, but there may be counting errors. The most accurate method would be a 3D reconstruction of serial sections. Some software packages can perform this, though they can be expensive and slow.

7.5 *Image Analysis:*

7.5.1 *General*—In addition to using image analysis software to quantitate the amount of fluorescently labeled cells, there are more complex analyses that can be completed, such as determining cell density, cell spreading, and spatial uniformity of live and dead cells.

7.5.2 *Cell Density*—The cells must be stained prior to analysis. Typically a stain for cell nuclei, such as 4',6-diamidino-2-phenylindole (DAPI), is utilized so that the location of cells can be identified. Multiple color images at successive depths along the cross-sections of the constructs are taken (7). However, not all scaffolds are easy to section. Some may be brittle. If cells are mostly growing on or outside of scaffold, then quantification may be trickier than if they are evenly distributed on the surface. Cell packing could affect accuracy of counts. If cells clump or form aggregates upon, or within the scaffold, cell number estimates might be inaccurate. This could be an issue for islets or neurospheres, for example, where one group of cells could contain thousands of cells tightly packed together.

7.5.3 *Cell Spreading*—The cells must be stained prior to analysis (for example, Texas Red-C2-Maleimide to stain entire cell interiors and 4',6-diamidino-2-phenylindole (DAPI) as a nuclear counter stain (8, 9)). Cell density and cell spreading can be quantified within a given area using measures of object area, roundness, aspect ratio, and perimeter (9). Although these stains work well with cells on 2-D surfaces, stain retention in a 3-D scaffold can significantly increase background levels and

reduce cell edge contrast. Extensive washing may be required to remove the stain from the non-cellular regions of a 3-D scaffold.

7.5.4 Spatial Uniformity Determinations—In addition to determining cell density, a statistical measure of spatial uni-

formity of the cells within a particular scaffold can be calculated (10). The method involves calculating the coefficient of variation determined from statistical analysis of cell location (geometrical parameters) determined by an image analysis program.

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