Designation: F2664 – 19ε1

Standard Guide for Assessing the Attachment of Cells to Biomaterial Surfaces by Physical Methods[1]

This standard is issued under the fixed designation F2664; 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.

ε[1] NOTE—Editorial corrections were made throughout in November 2019.

1. Scope

1.1 This guide describes protocols that can be used to measure the strength of the adhesive bond that develops between a cell and a surface as well as the force required to detach cells that have adhered to a substrate. Controlling the interactions of mammalian cells with surfaces is fundamental to the development of safe and effective medical products. This guide does not cover methods for characterizing surfaces. The information generated by these methods can be used to obtain quantitative measures of the susceptibility of surfaces to cell attachment as well as measures of the adhesion of cells to a surface. This guide also highlights the importance of cell culture history and influences of cell type.

1.2 *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.*

1.3 *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]

D4410 Terminology for Fluvial Sediment

F22 Test Method for Hydrophobic Surface Films by the Water-Break Test

F2312 Terminology Relating to Tissue Engineered Medical Products

F2603 Guide for Interpreting Images of Polymeric Tissue Scaffolds

2.2 *ISO Standards:*[3]

ISO 4287 Geometrical Product Specifications (GPS)—Surface Texture: Profile Method—Terms, Definitions and Surface Texture Parameters

ISO 13565-1 Geometrical Product Specifications (GPS)—Surface Texture: Profile Method; Surfaces Having Stratified Functional Properties—Part 1: Filtering and General Measurement Conditions

3. Terminology

3.1 *Definitions:*

3.1.1 *biocompatibility, n*—material may be considered biocompatible if the materials perform with an appropriate host response in a specific application. **F2312**

3.1.2 *biomarker, n*—biochemical feature or facet that can be used to measure the progress of disease or the effects of treatment.

3.1.3 *biomaterial, n*—any substance (other than a drug), synthetic or natural, that can be used as a system or part of a system that treats, augments, or replaces any tissue, organ, or function of the body. **F2312**

3.1.4 *detachment, n*—process whereby an adhered cell or group of cells is actively detached from a surface.

3.1.5 *hydrophilic, adj*—having a strong affinity for water, wettable. **F22**

3.1.6 *implant, n—in medicine*, object, structure, or device intended to reside within the body for diagnostic, prosthetic, or other therapeutic purposes.

3.1.7 *laminar flow, n*—well-ordered, patterned flow of fluid layers assumed to slide over one another. See Ref (**1**).[4]

[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 Oct. 1, 2019. Published November 2019. Originally approved in 2007. Last previous edition approved in 2011 as F2664 – 11. DOI: 10.1520/F2664-19E01.

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

3.1.8 *lay, n*—direction of the predominant surface pattern. **ISO 13565-1**

3.1.9 *passage, n*—transfer or transplantation of cells, with or without dilution, from one culture vessel to another. It is understood that any time cells are transferred from one vessel to another, a certain portion of the cells may be lost and, therefore, dilution of cells, whether deliberate or not, may occur. This term is synonymous with the term *subculture*. See Ref (**2**).

3.1.10 *passage number, n*—number of times the cells in the culture have been subcultured or passaged. In descriptions of this process, the ratio or dilution of the cells should be stated so that the relative cultural age can be ascertained. See Ref (**2**).

3.1.11 *Reynolds number, n*—dimensionless number expressing the ratio of inertia forces to viscous forces in a moving fluid. The number is given by VLr/m where V, is the fluid's velocity, L is a characteristic length or distance such as pipe diameter, r is the fluid's mass density, and m is the fluid's dynamic viscosity. **D4410**

3.1.12 *scaffold, n*—support, delivery vehicle, or matrix for facilitating the migration, binding, or transport of cells or bioactive molecules used to replace, repair, or regenerate tissues. **F2312**

3.1.13 *surface profile, n*—surface profile formed by the intersection of a real surface by a specified plane. It is customary to select a plane that lies perpendicular to the direction of lay unless otherwise indicated. **ISO 13565-1 and ISO 4287**

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *adhesion*—physiochemical state by which a cell is coupled to a non-cell surface by interfacial forces, which may consist of covalent or ionic forces.

3.2.2 *senescence, n—in vertebrate cell cultures*, 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*. See Ref (**2**).

3.2.3 *shear stress, n*—components of stress that act parallel to the plane of the surface. See Ref (**3**).

3.2.4 *tack, n*—ability of an adhesive to form a bond to a surface after brief contact under light pressure.

4. Significance and Use

4.1 Cell attachment or, lack of it, to biomaterials is a critical factor affecting the performance of a device or implant. Cell attachment is a complicated, time-dependent, process involving significant morphological changes of the cell and deposition of a bed of extracellular matrix. Details of the adhesive bond that is formed have been reviewed by, for example, Pierres et al (2002) (**4**), Lukas and Dvorak (2004) (**5**), and Garcia and Gallant (2003) (**6**). The strength of this coupling can be determined either by monitoring the force of attachment between a cell and a substrate over time or by measuring the force required to detach the cell once it has adhered.

4.2 Cell adhesion to a surface depends on a range of biological and physical factors that include the culture history, the age of the cell, the cell type, and both the chemistry and morphology of the underlying surface and time. These elements need to be considered in developing a test protocol.

4.3 Devising robust methods for measuring the propensity of cells to attach to different substrates is further complicated since either cell *adhesion* or *detachment* can be assessed. These processes are not always similar or complementary.

4.4 Most studies of cell attachment focus on obtaining some measure of the time-dependent force required to detach, or de-adhere, cells that have already adhered to a surface (James et al, 2005) (**7**). More recently investigators have begun to measure the adhesive forces that develop between cells and the underlying surface during attachment (Lukas and Dvorak, 2004) (**5**). From a practical point of view, it is much easier to measure the force required to detach or de-adhere cells from a surface than to measure those that develop during attachment. However, in both cases, the experimental data should be interpreted with a degree of caution that depends on the intended use of the measurements. The methods of measuring cell adhesion described herein are measures of the force required to detach an adherent cell.

4.5 The purpose of this guide is to provide an overview of current generic test methods and identify the key factors that influence the assessment of cell adhesion and detachment. It is anticipated that this guide will form the basis for producing a series of standards that will describe these test methods in more detail.

5. Cell Attachment Assays

5.1 Table 1 provides examples of common cell adhesion assays, including a brief description of the forces applied. These assays are discussed in more detail in Section 6.

5.2 Cell attachment assays can be performed using single cells or a population of cells. Single cell techniques can provide quantitative measures of the adhesive force that

TABLE 1 Assays for Measuring Cell Detachment from Surfaces

Cell Requirements	Assay	Assay Description	Section
Single Cell	Micromanipulation	Measurement of the Force developed during attachment via an AFM	6.1.1-6.1.2
Single Cell	Micromanipulation	Forces applied via a micropipette, microprobe or AFM	6.1.3
Cell Population	Gravity	Detect the number of cells that remain attached after turning the culture vessel upside down	6.2.1
	Wash	Wash off adhered cells	6.2.2
	Centrifugation	Detachment of cells using centrifugal force	6.2.3
	Hydrodynamic Flow	Detachment of cells using shear forces generated by laminar flow over cells	6.2.4

develops between a cell and a substrate with time or that required to detach an adhered cell from a substrate. Individual ligand-surface interactions can be measured directly using, for example, a cell mounted on an atomic force microscope (AFM) tip. Single cell measurements do have their disadvantages. Variations in adhesive strength are not averaged out over a population and sophisticated equipment, such as an AFM, is required.

5.3 Cell population based assays average out variations in cell-to-substrate adhesiveness compared with measurements performed on a single cell. This variation arises both because of variations in biomaterial surface properties, and variations in cell phenotype used as the probe (Appendix X1 and Appendix X2). Cell population techniques provide a usable measure of the biomaterial's adhesiveness for a given batch of cells and test conditions. Cell population techniques are attractive in that they provide robust measurements based on a large number of cells, which is an important consideration given the inherent variance of biological systems. Measurements that are based on large numbers of cells reduce the influences of local variations in surface chemistry and texture and in the adhesiveness of the cells themselves.

6. Measurement of Cell Detachment

NOTE 1—In principle, the strength of the adhesive bond that develops between the cell and underlying substrate will increase with time, although in practice this will depend on the cell-surface interactions. These measurements can be performed on either populations of cells or single cells. It should also be noted that it is not possible to conduct a series of measurements over time on the same cell, as these tests are destructive. Each test described below carries its own unique sources of statistical error. Users should familiarize themselves with the appropriate assay system and should consult with appropriate statistical staff to determine the necessary statistical parameters to ensure statistical significance. These parameters may include, but are not limited to: sample size, power of study, number of image fields counted (for microscope-based assays), number of cell lots tested, variability between users, what is the most appropriate statistical analysis (that is, analysis of variance, Tukeys test, *t*-test, etc.) and determination of a standard curve for analysis of detached cells.

6.1 *Micromanipulation:*

6.1.1 *Micromanipulation Methods (Single Cells)*—Single cells can be used to measure the force required to uncouple cells from the underlying substrate (measure of detachment), as a result of a time-dependent adhesion. Such measurements are made using micromanipulation or micropipettes. Cells can be seeded onto a small block of material mounted on an AFM tip, attached to a coated AFM tip or to the tip directly. The cell-coated tip can then be used to measure the tack force that develops over time.

6.1.2 There are some practical issues that need to be addressed when using this direct approach to force measurement:

6.1.2.1 Care should be taken to ensure that the measurements relate to a single cell and not to contributions from a number of cells. This is a particular issue when a block of material is mounted onto the tip.

6.1.2.2 Care should be taken to ensure that the measurement relates to the detachment force and is not a measure of cell membrane strength. If the cell adheres very tightly to both the culture substrate and the block of test material, then the cell may be torn apart during the measurement. In this case, the mechanical properties of the cell body would be measured instead of the cell adhesion force to the test material. This can be checked by examining the cell in the microscope after the adhesion measurement to make sure the cell looks healthy and is intact.

6.1.2.3 These measurements need to be made using a wet cell AFM. Problems have been reported with protein adsorption on the cantilever having an adverse effect on its reflectivity.

6.1.3 Micropipettes, microprobes, and AFMs have been used to measure the force required to suck or pull single cells away from the substrate to which they are attached (for example, Shao et al, 2004) (**8**). All these methods provide quantifiable sensitive and real-time direct measures of the force required to detach the cell that is typically less than 10 mN (for example, Lee et al, 2004) (**9**). Control over the magnitude of the force and the rate at which it is applied can be used to explore the process of cell detachment in detail. Practical issues that need to be considered when using these methods include:

6.1.3.1 Specialized equipment, which must be calibrated to ensure that data are repeatable and reproducible, is required for such sensitive measurements.

6.1.3.2 Consideration should be given as to the direction of the applied force (tensile, shear, or some combination of the two) and the magnitude of the applied stress. Larger area pipette tips will subject the cell to a lower stress than the tip of an AFM for a given applied force.

6.1.3.3 The period of time between exposing the cells to a surface and that at which measurements are made.

6.2 *Cell Detachment Measurements on Cell Populations:*

6.2.1 *Gravity*—Gravity can be used to differentiate between cells that are attached to a substrate and those that have not by turning the cell culture vessel upside down. Prior to using this approach, the user should consider the buoyancy of the cells with respect to medium to ensure that it is negative. Consideration should be given to the test duration to improve the consistency of repeat measurements.

6.2.2 *Wash Assays*—A simple, convenient, widely used assay that readily provides qualitative information on adhesion of cells to a substrate is to wash off non-adherent cells using culture medium. This approach may take many forms from mild shaking of the culture vessel to sluicing of the culture well. Clearly the simplicity, speed and low cost of these approaches are attractive, although lack of control of the applied force in terms of both its magnitude and the nature of the applied stress limits the sensitivity of the measurement, and hence reproducibility. For this reason comparisons between successive tests are subject to large unquantifiable uncertainties. Checks should also be made to ensure that the adherent surface is not removed or damaged during the assay.

6.2.2.1 This assay can be used to monitor cell attachment to a surface under different culture conditions, used as a measure of the biocompatibility or as a route to gauging how well cells are attached to a substrate. This approach is also a destructive method, so measurements should only be made using samples that have not been previously tested. This protocol will remove

any contributions from residual extra-cellular matrix of fragments of cell membrane that may impact on the adhesiveness of the surface.

6.2.3 *Centrifugation*—A conventional centrifuge can be used to apply a normal or shear force to cells depending on the orientation of the cells with respect to the centrifugal force (for example, Heneweer et al, 2005) (**10**). The force that the cells are subject to can be calculated according to the following formula:

$$F = VdR\omega^2 \quad (1)$$

where:

F = centrifugal force,
V = cell volume,
d = difference in density between a cell and the surrounding medium,
R = centrifugation radius, and
ω = centrifugation speed.

6.2.3.1 Such tests are easy to conduct, do not require specialized equipment or training and the results represent a population average. Factors that need to be considered when using this methodology include the test duration and the potential influence of forces applied during the period of spin up. The assay only correlates cell detachment with the maximum force applied after the centrifuge has reached its set spin speed.

6.2.4 *Hydrodynamic Flow Assays*—The basis of hydrodynamic test methods is to apply a known force to a population of cells by means of controlled movement of fluid. The assays rely on forces generated by fluid flow over adhered cells. There are several subtypes of hydrodynamic flow assays: *(1)* parallel plate flow chambers, *(2)* spinning disk chambers, and *(3)* radial flow chambers. The geometry of the flow cell and mode of operation influence both the magnitude of the applied force and its complexity, as discussed below.

6.2.4.1 The stresses that the cells are subjected to are complex and difficult to quantify. Typically cells will be subjected to a combination of shear stress and hydrodynamic drag leading to the development of torque. The geometry of the cell (that is, the amount of spreading and the presence of focal adhesions) will cause the actual stress that the cell experiences to be different from the calculated wall stress and therefore must be considered during any quantitative analysis.

6.2.4.2 *Parallel and Convergent Plate Flow Chambers*—Fig. 1 consists of parallel plates that are a known distance apart. Flow of fluid through the chamber is laminar (Reynolds number is less than 2300). In this configuration the cells are subjected to a wall shear stress, τ_w, that is, the shear stress at the wall-fluid interface according to the following equation:

$$\tau_w = \frac{6\mu Q}{wh^2} \quad (2)$$

where:

Q = flow rate of the fluid,
w = width (channel dimension),
h = height (channel dimension), and
μ = fluid viscosity.

(1) This function applies to Newtonian fluids, of which water is an example and assumes no influences from edge effects. Care should be taken to ensure that these requirements are met for particular test geometries and culture media.

(2) The key element of this approach is to ensure that the fluid flow over the cells is laminar. The wall shear stress applied to the cells can be constant or variable, depending on the design of the flow cell. A controlled static shear stress gradient can easily be generated by converging either one or both pairs of parallel sides of the flow cell.

(3) The wall shear stress at a given point along the length of the cell is given by Eq 2.

6.2.4.3 *Spinning Disc*—The spinning disk arrangement shown in Fig. 2 can be used to subject the cells to a centripetal force and complex flow field that equates to a wall shear stress, the magnitude of which increases with increasing distance away from the pole according to:

$$\tau_w = 0.8r\sqrt{\rho\mu\omega^2} \quad (3)$$

where:

ω = rotational speed,
ρ = density of the culture medium,
r = radial position, and
μ = fluid viscosity.

6.2.4.4 *Radial Flow Cell*—The wall stress in the radial flow cell shown in Fig. 3 is given by:

$$\tau_w = \frac{3\mu Q}{\pi rh^2} \quad (4)$$

where:

Q = flow rate,
r = radial position,
h = gap between the plates, and
μ = viscosity of the fluid.

(1) This function is the same as that for the parallel plate cell shown in Eq 2. The highest wall shear stress in this configuration will be in the vicinity of the entrance port. A consequence of this will be that cells detached by the highest wall shear stress may influence detachment of cells in the lower wall stress zone. Unlike the parallel plate laminar flow

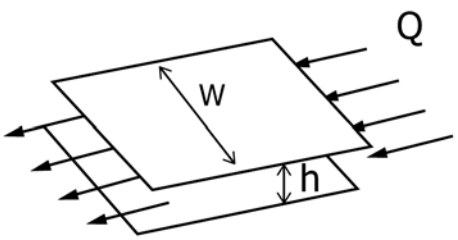

FIG. 1 A Simple Parallel-Sided Flow Cell Can Be Used to Apply a Known Shear Stress to a Bed of Adhered Cells

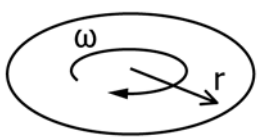

FIG. 2 A Schematic Representation of a Spinning Disk