Designation: F3088 – 14

Standard Test Method for Use of a Centrifugation Method to Quantify/Study Cell-Material Adhesive Interactions[1]

This standard is issued under the fixed designation F3088; 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 test method covers a centrifugation cell adhesion assay that can be used to detect changes in adhesive characteristics of cells with passage or treatments. This approach measures the force required to detach cells from a substrate. Adhesion, among many variables, may vary due to changes in the phenotype of the cells.

1.2 This test method does not cover methods to verify the uniformity of coating of surfaces, nor does it cover methods for characterizing surfaces.

1.3 The cells may include adult, progenitor or stem cells from any species. The types of cells may include chondrocytes, fibroblasts, osteoblast, islet cells, or other relevant adherent cell types.

1.4 This test method does not cover methods for isolating or harvesting of cells. This test method does not cover test methods to quantitate changes in gene expression, or changes in biomarker type or concentration, as identified by immunostaining. Nor does this test method cover quantitative image analysis techniques.

1.5 *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]

F2603 Guide for Interpreting Images of Polymeric Tissue Scaffolds
F2664 Guide for Assessing the Attachment of Cells to Biomaterial Surfaces by Physical Methods
F2739 Guide for Quantitating Cell Viability Within Biomaterial Scaffolds
F2944 Test Method for Automated Colony Forming Unit (CFU) Assays—Image Acquisition and Analysis Method for Enumerating and Characterizing Cells and Colonies in Culture

3. Summary of Test Method

3.1 *Centrifugation Cell Assay on Cell Populations*—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 (**1-3**).[3] The centrifugal force that the cells are subject to can be calculated according to the following formula assuming that the plates are 90° (normal) to the spindle of the centrifuge.

$$F_D = (p_{cell} - p_{medium})V_{cell}RCF \quad (1)$$

where:

F_D = detachment force applied per cell,
V_{cell} = cell volume,
p_{cell} = density of the cell,
p_{medium} = the density of the surrounding medium, and
RCF = Relative Centrifugal Forces = $r\varphi^2$, where r = centrifugation radius and φ = centrifugation speed.

See X1.6 for example of a calculation of "detachment force per cell."

3.1.1 Such tests are easy to conduct and the results represent a population average. The method can be performed in a moderately outfitted cell biology lab with an inverted microscope or a fluorescence microplate reader and a centrifuge with a microtiter plate holder. Three factors need to be considered when using this methodology: the potential influences of forces applied during the period of spin up time, the maximum spin speed, and the test duration. The method is only able to correlate with cell detachment with the maximum force applied after the centrifuge has reached its set spin speed. It should also be considered that the maximum force that can be applied is limited by the centrifuge, sample configuration (that is, configuration and type of multiwell plate) and time of centrifugation. In some instances, the cell adhesion strength will be

[1] This test method 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 May 1, 2014. Published June 2014. DOI: 10.1520/F3088-14.

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

greater than the applied forces. Therefore, for certain cell types that have attached for extended periods of time, the method may not be able to generate forces sufficient to detach these cells.

3.1.2 Cell attachment is a complex, time-dependent, process involving significant morphological and structural changes of the cell and deposition of a bed of extracellular matrix and is a function of cellular and substrate parameters. Cell adhesion to a surface depends on a range of biological factors that include culture history, species, the source and origin of the cells, embryological status of the cells (adult, progenitor, stem), histological types of cells (chondrocytes, fibroblasts, osteoblast, islet cells, etc.), purity, passage number, population doublings, and time after trypsinization. The adhesive strength also depends on the chemistry, surface chemistry/topography, and morphology of the underlying surface and adsorbed proteins or engineered ligands and, most importantly, time of adhesion.

4. Significance and Use

4.1 This test method describes a cell adhesion method that can be used to provide a detachment percent at a given RCF for cells that have adhered to a substrate, typically for a short time. The information generated by this test method can be used to obtain a semi-quantitative measurement of the adhesion of cells to either an uncoated or pre-coated substrate, when compared to a reference (adherent) cell type on the same substrate. As described in Reyes and Garcia (2003), it is recommended that the 50 % point be used for either ligand concentration or RCF for the most robust measurement of adhesion strength. The adhesion may vary due to changes in the phenotype of the cells or as a result of the specific properties of the surface. The substrate may include tissue culture-treated polystyrene, biomaterials or bioactive surfaces. If the substrate is a hydrogel, care must be taken to avoid cohesive failure in the hydrogel (that is, detached cells have pulled away fragments of gel). The coating may consist of (but is not limited to) the following: natural or synthetic biomaterials, hydrogels, components of extracellular matrix (ECM), ligands, adhesion or bioactive molecules, genes or gene products. Cell concentration is also critical, as use of too high a concentration of cells may result in cells detaching as a sheet, rather than as individual cells. This centrifugation approach, once validated, may be applicable for quality control (QC) and product development. However, until the method is correlated to other measures of cell attachment, the current method should be run in parallel with other known measures of cell adhesion.

4.2 This test method does not cover test methods to quantitate changes in gene expression, or changes in biomarkers, as identified by immunostaining. This test method additionally does not cover quantitative image analysis techniques. In some cases the change in adhesive properties may reflect on the degree of differentiation or de-differentiation of the cells. However, it is worth noting that adhesive interactions do not necessarily reflect the differentiation state of a particular cell type, although in many instances they do. (See X1.3 for application to the Adhesion of Chondrocytes.)

5. Interferences

NOTE 1—The following interferences may result in variable and inconsistent results from this method and care should be taken to minimize these conditions.

5.1 Cell concentration too high in multi-wells—If the cell concentration is too high, then the cells may detach as a sheet, instead of as individual cells.

5.2 Cells not evenly distributed in wells (that is, clumping).

5.3 Inconsistency in the fluid level within multi-wells—Wells must be completely filled with fluid (**4**).

5.4 Inconsistency in well coating and blocking protocols—Where possible, a negative substrate control (that is, non-tissue culture-treated polystyrene, or similar control which has been shown to limit cell adhesion) should be included.

5.5 Inconsistency in pipetting forces when washing wells.

5.6 Air bubbles in well prior to, or forming during, centrifugation. The air bubbles can result from improper pipetting of fluid within the wells, or an improper fluid level in the wells prior to sealing the top of the wells with an adhesive cover (that is, "reverse meniscus" not formed in well prior to sealing with acetate sealing tape).

5.7 Incomplete washing of wells to remove loosely adherent or dead cells.

5.8 Cross-over of fluorescent signal between adjacent wells in clear or translucent multi-well plates.

5.9 Centrifugation of multiple multi-well plates. A complex stress situation can result from spinning multiple multi-well plates (that is, stacking plates within a plate holder on the centrifuge), depending on the orientation of cells with respect to the centripetal force.

5.10 Photobleaching of fluorescence stain. Photobleaching may be reduced by limiting the exposure of the stained cells to room light or ultraviolet light.

5.11 Long cell adhesion times and variations in adhesive proteins—Use of long exposure times and/or specific ECM or protein may require adjustment of the protocol to optimize cell detachment conditions. Since detachment forces with this method are low, well spread, or strongly attached cells may not be detached by this method (**5**).

6. Apparatus

6.1 *Humidified CO_2 Incubator.*

6.2 *Rocker for Isolated Cells.*

6.3 *Hemocytometer.*

6.4 *Centrifuge.*

6.5 *Plate Adapters for Centrifuging Multi-well Plates.*

6.6 *Multi-well Pipetter.*

6.7 *Inverted Microscope* equipped with bright field/phase, as well as fluorescence optics.

6.8 *Microplate Reader.*

6.9 *Image Acquisition and Image AnalysisSsoftware* (that is, Zeiss AxioVision Software, Image J or equivalent).

7. Reagents and Materials

7.1 *Cell Type,* for adhesion assay, as well as control cell type (if applicable). A positive control cell type should consistently exhibit greater than 50 % adhesion on a given substrate, at a given RCF. Similarly, a negative control cell type should consistently exhibit less than 50 % adhesion on a given substrate at a given RCF.

7.2 *Proteins, Extracellular Matrix Ccomponents, Genes or Gene Products or Biomaterials* for pre-coating wells of multi-well plates.

7.3 *Medium* appropriate for either the cell type of interest or the control cell type, with or without the addition of antibiotic/antimycotic solution. Care should be taken to limit/avoid carryover of serum contents and adhesive proteins from media. Additionally, the trypsin protocol (trypsin concentration and trypsinization times) should be optimized.

7.4 *Trypan Blue* or equivalent (cell viability).

7.5 *Multi-well Plates* (96 well).

7.6 *Dulbecco's Phosphate Buffered Saline* (without calcium and magnesium), (D-PBS).

7.7 *Fluorescence Stain.*

7.8 *Acetate Sealing Tape,* or equivalent (to seal top of multi-well plate).

7.9 *Aluminum Foil* (to cover multi-well plate during incubation).

8. Hazards

8.1 Hoechst 33342 fluorescence stain (possible carcinogen).

8.2 Trypan blue dye (possible carcinogen).

9. Calibration and Standardization

9.1 *Calibration of Image System*—Any inverted microscope system equipped with appropriate image capture device and image analysis software may be used for the assay. For the purposes of illustration in this standard, we refer to a Zeiss AxioVert inverted microscope equipped with an Axio Cam digital camera for image capture and AxioVision software for data collection/analysis.

9.1.1 Using an inverted microscope, 5 × 5 digital tiled images (tiled overlap 10 %, total tiled image area = 3.177 mm^2) are captured for each of the three wells prior to centrifugation (pre-spin). Approximately 2400 to 3600 cells (800 to 1200 cells/well × 3 wells) are counted per sample.

10. Procedure

10.1 *Centrifugation Cell Adhesion Method*—A schematic of the method is shown in Fig. 1. The method was modified from the method of Reyes and Garcia (2003) (**2**).

10.1.1 Wells of a 96 multi-well plate are passively coated with protein or matrix protein. The coating concentration will depend on the ECM protein. The incubation time and incubation temperature (for example, room temperature versus 4°C versus 37°C) will vary with the substrate. In the example of fibronectin, the wells are passively coated for 30 min. to 1 h at room temperature.

10.1.2 *Optimization of Multi-Well Plate Coating*—The following procedure is suggested to obtain the optimal well-coating concentration and time of incubation for the coating. First, a series of test runs of the method should be performed where a broad concentration range (including a no-coating, zero concentration, control) of the desired coating substrate are coated on separate wells of the multi-well plate for various time periods at the appropriate temperature/relative humidity. Note that depending on the specific coating or intended outcome of the adhesion method, it may be appropriate to compare multi-well plates with and without "tissue culture treatment." A statistically significant number of replicates should be run to allow appropriate statistical analysis of the data. The data from the initial (broad concentration) test run will provide data to re-run the adhesion method using a more restrictive range for the coating concentration and for the time of incubation. Appropriate statistical analyses should be performed on the data. If an even more specific coating concentration is desired, then the procedure can then be repeated using data from the second set of test runs. If desired, incubation times for the coating may also be varied. The procedure shall be repeated for each new coating material to optimize the coating concentration and the time of incubation for the coating.

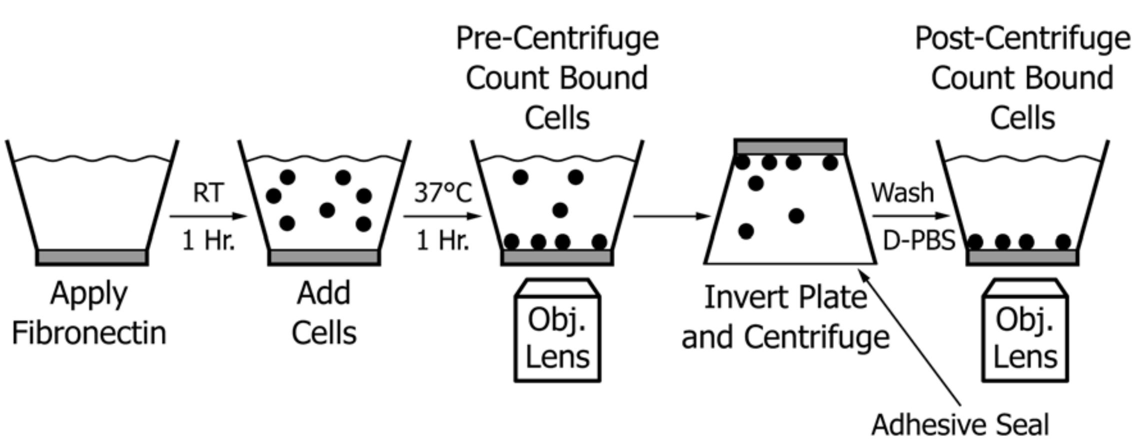

FIG. 1 Schematic of Centrifugation Cell Adhesion Assay