Designation: F2149 – 16

Standard Test Method for Automated Analyses of Cells—the Electrical Sensing Zone Method of Enumerating and Sizing Single Cell Suspensions[1]

This standard is issued under the fixed designation F2149; 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, provided the limitations are understood, covers a procedure for both the enumeration and measurement of size distribution of most all cell types. The instrumentation allows for user-selectable cell size settings and is applicable to a wide range of cell types. The method works best for spherical cells, and may be less accurate if cells are not spherical, such as for discoid cells or budding yeast. The method is appropriate for suspension as well as adherent cell cultures (**1**).[2] Results may be reported as number of cells per milliliter or total number of cells per volume of cell suspension analyzed. Size distribution may be expressed in cell diameter or volume.

1.2 Cells commonly used in tissue-engineered medical products (**2**) are analyzed routinely. Examples are chondrocytes (**3**), fibroblasts (**4**), and keratinocytes (**5**). Szabo et al. used the method for both pancreatic islet number and volume measurements (**6**). In addition, instrumentation using the electrical sensing zone technology was used for both count and size distribution analyses of porcine hepatocytes placed into hollow fiber cartridge extracorporeal liver assist systems. In this study (**7**), and others (**6, 8**), the automated electrical sensing zone method was validated for precision when compared to the conventional visual cell counting under a microscope using a hemocytometer. Currently, it is not possible to validate cell counting devices for accuracy, since there not a way to produce a reference sample that has a known number of cells. The electrical sensing zone method shall be validated each time it is implemented in a new laboratory, it is used on a new cell type, or the cell counting procedure is modified.

1.3 Electrical sensing zone instrumentation (commonly referred to as a Coulter counter) is manufactured by a variety of companies and is based upon electrical impedance. This test method, for cell counting and sizing, is based on the detection and measurement of changes in electrical resistance produced by a cell, suspended in a conductive liquid, traversing through a small aperture (see Fig. 1(**9**)). When cells are suspended in a conductive liquid, phosphate-buffered saline for instance, they function as discrete insulators. When the cell suspension is drawn through a small cylindrical aperture, the passage of each cell changes the impedance of the electrical path between two submerged electrodes located on each side of the aperture. An electrical pulse, suitable for both counting and sizing, results from the passage of each cell through the aperture. The path through the aperture, in which the cell is detected, is known as the "electronic sensing zone." This test method permits the selective counting of cells within narrow size distribution ranges by electronic selection of the generated pulses. While the number of pulses indicates cell count, the amplitude of the electrical pulse produced depends on the cell's volume. The baseline resistance between the electrodes is due to the resistance of the conductive liquid within the boundaries of the aperture. The presence of cells within the "electronic sensing zone" raises the resistance of the conductive pathway that depends on the volume of the cell. Analyses of the behavior of cells within the aperture demonstrates that the height of the pulse produced by the cell is the parameter that most nearly shows proportionality to the cell volume.

1.4 Limitations are discussed as follows:

1.4.1 *Coincidence*—Occasionally, more than a single cell transverses the aperture simultaneously. Only a single larger pulse, as opposed to two individual pulses, is generated. The result is a lower cell count and higher cell volume measurement. The frequency of coincidence is a statistically predictable function of cell concentration that is corrected by the instrument. This is called coincidence correction (**8**). This phenomenon may be reduced by using lower cell concentrations.

1.4.2 *Viability*—Electrical sensing zone cell counting enumerates both viable and nonviable cells and cannot determine percent viable cells. A separate test, such as Trypan blue, is required to determine percent viable cells.

1.4.3 *Cell Diameter*—This is a function of the size range capability of the aperture size selected. Measurements may be made in the cell diameter range of 0.6 µm to 1200 µm. Setting the counting size range on the instrument can affect the test

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

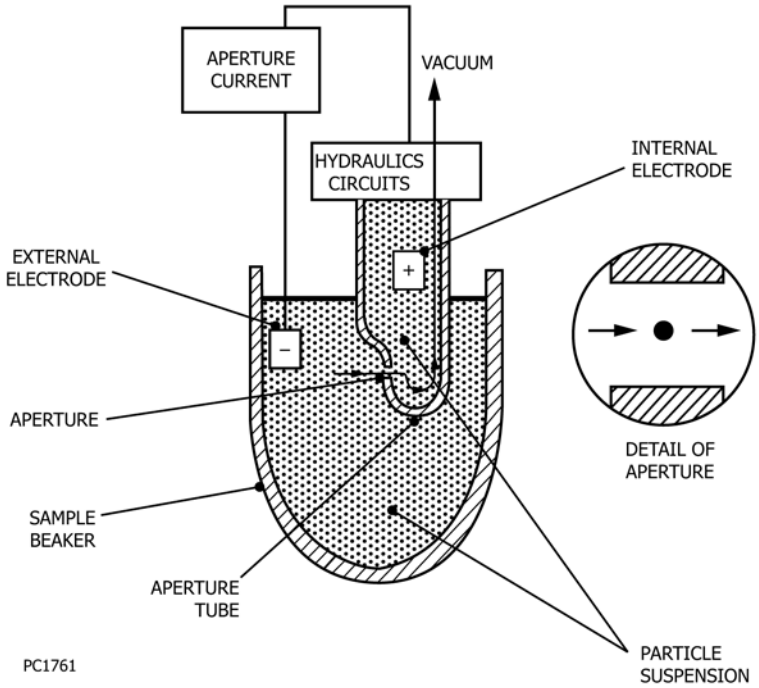

FIG. 1 Cell, Suspended in a Conductive Fluid, Traversing Through a Small Aperture

results, especially if the cell size has a large distribution, and should be carefully controlled to help achieve repeatability.

1.4.4 *Size Range of the Aperture*—The size range for a single aperture is proportional to its diameter. The response has been found to depend linearly on diameter over a range from 2 % to 80 % of the diameter. However, the aperture tube may become prone to blockage at levels greater than 60 % of diameter. Therefore, the practical operating range of the aperture is considered to be 2 % to 60 % of the diameter.

1.4.5 *Humidity*—10 % to 85 %.

1.4.6 *Temperature*—10 °C to 35 °C.

1.4.7 *Electrolyte Solution*—The diluent for cell suspension shall provide conductivity and have minimal effect on cell size. The electrolyte of choice is commonly phosphate-buffered saline.

2. Terminology

2.1 *Definitions:*

2.1.1 *channelyzer, n*—a pulse height analyzer; places voltage pulses into appropriate size bins for the size distribution data.

2.1.2 *coincidence, n*—more than one cell transversing the aperture at the same time.

2.1.3 *corrected count, n*—the cell count corrected for coincidence.

2.1.4 *electrolyte, n*—diluent, offering slight conductivity, in which cells are suspended.

2.1.5 *femtoliter, n*—a cubic micrometer; a measurement of cell volume.

2.1.6 *raw count, n*—the enumeration of the cell population not corrected for coincidence.

2.1.7 *ruggedness, n*—the degree of reproducibility of the same sample under a variety of normal conditions; for example, different operators.

2.1.8 *size thresholds, n*—the instrument's lower and upper size settings for the particular cell population; adjustable "size gate." Cells or fragments outside the size settings are excluded from the analyses.

3. Significance and Use

3.1 The electrical sensing zone method for cell counting is used in tissue culture, government research, and hospital, biomedical, and pharmaceutical laboratories for counting and sizing cells. The method may be applicable to a wide range of cells sizes and cell types, with appropriate validation (**10**).

3.2 The electrical sensing zone methodology was introduced in the mid-1950s (**9**). Since this time, there have been substantial improvements which have enhanced the operator's ease of use. Among these are the elimination of the mercury manometer, reduced size, greater automation, and availability of comprehensive statistical computer programs.

3.3 This instrumentation offers a rapid result as contrasted to the manual counting of cells using the hemocytometer standard counting chamber. The counting chamber is known to have an error of 10 to 30 %, as well as being time-consuming (**11**). In addition, when counting and sizing porcine hepatocytes, Stegemann et al concluded that the automated, electrical sensing zone method provided greater accuracy,

precision, and speed, for both counts and size, compared to the conventional microscopic or the cell mass-based method (7).

4. Interferences

4.1 *Debris and Cellular Fragments*—When these are in the cellular size ranges, they will be analyzed. Correct cell size threshold settings help to correct inclusion of debris or fragments in the analyses. A count of culture medium without cells can be used to assess background counts. For adherent cells, a sample of the medium from a culture may be counted (before trypsinization).

4.2 *Cellular Aggregation*—Aggregates will be enumerated as a single cell. They will be sized larger than their individual cell components. Commercially available enzyme solutions aid in the preparation of single cell suspensions. Efforts to disaggregate cells may affect cell viability. Cell sample preparations may exhibit settle, resulting in decreased counts. Gently mixing the cells often and immediately before counting will produce more repeatable results.

4.3 *Adherence*—Cells may adhere to the sides of the sample vessel, thus affecting count accuracy.

4.4 *Line Noise*—Instruments shall be located on a bench away from flickering lights and other equipment such as centrifuges.

4.5 *Bubbles*—May cause a false increase in cell counts.

4.6 *Current*—Excessive current across the aperture may damage cellular membranes. The current can be adjusted on the instrument.

5. Hazards

5.1 *Warning (Chemical)*—Do not use nonaqueous electrolyte solutions (that in which the cells are suspended). Care shall be taken when mixing some electrolyte solutions. Violent reactions may occur. Azide shall not be used in acid solutions. Flammable electrolyte solutions and organic solvents, as noted above, shall not be used.

5.2 *Warning (Electrical)*—High voltages are present inside the instrument. Instrument shall be sited on a firm, dry work bench and shall be grounded correctly.

5.3 *Warning (Biological)*—Institutional, state, and OSHA approved safety action plans shall be followed.

6. Procedures

6.1 The details in this procedure should be used as guidelines since modifications may be required for the cell type, instrument, or procedure being employed. Commercial instrumentation among manufacturers may vary as to the instrument's recommended cell size range and aperture diameters available. Certain manufacturers provide instrumentation in which both instrument function control and data analyses are computer controlled. Some models provide cell count only, while others also provide cell size distribution.

6.2 Calibrate the instrument following the instrument manufacturer's procedure. Instrument calibration should be performed monthly or after the unit has been serviced. For all cell types, a commercial calibrator of known volume should be used. Light microscopy can be used to verify reference particle size or cell size.

6.3 Dilute the cell suspension in the electrolyte, typically phosphate-buffered saline. Gently mix the sample. The mixing method should be carefully considered and controlled since variability in this step may increase variability in the test result. The count should be performed as soon as possible after mixing as cell settling may occur (even within a few seconds depending on cell type). It may be necessary to assess the effect of time from mixing to counting on the test result to establish the appropriate time frame that gives a consistent result.

6.4 Set the size range on the instrument for the particular cell population being analyzed. Choose the correct aperture size. The manufacturer's operator manual will contain appropriate protocols.

6.5 Select the volume of the suspension to be analyzed. Generally, this may be 0.5 mL. Place the cell sample onto the sample stand.

6.6 Press Start. To determine aperture blockage, one can monitor the analyses time for the volume of cells to be analyzed. This may be 13 s for 500 µL; however, most instrumentation contains an aperture viewing screen on which blockages may be seen. In addition, most instrumentation provides an automated aperture unblock function.

6.7 Record the results of the analysis. Account for both the dilution factor and sample volume to determine the concentration of the original cell sample. For instance, 10 000 cells in 500 µL would be 20 000 cells/mL. For a 1:500 dilution, the original sample concentration is 1×10^7 cells/mL. Most instrumentation now automatically provides this calculation.

6.8 To ensure result quality, commercial controls in a variety of concentrations should be run periodically. It is up to each laboratory to determine control protocols. Regulatory agencies may require certain laboratories to analyze controls on a more frequent basis.

6.9 When implementing the electrical sensing zone counting method for a new cell type, it is important test how variability in the measurement process affects the test result (commonly referred to as ruggedness testing). Variability in test parameters, such as cell concentration, the volume of the cell suspension being counted, pipetting technique, cell washing steps, the counting buffer used, cell mixing procedure, time between mixing and counting, and methods for disaggregating the cells, may affect the test result. An understanding of how changes to the measurement process influence the test result allows users to design process controls and test specifications to ensure that test results are reliable.

7. Precision and Bias

7.1 The precision of replicate measurements of the same sample expressed as a coefficient of variation (CV) has been observed as 3 % for porcine hepatocytes (7), 3.3 % for pancreatic islets (6) and 0.24 % to 0.72 % for red blood cells (8). An instrument's manual may list precision of representative cell types. A statement of bias is not available since there is not

an accepted procedure for preparing a cell suspension of a known cell count. The electrical sensing zone method of cell counting is the reference method for the enumeration of both red blood cells (RBCs) and white blood cells (WBCs) as set forth by the International Council for Standardization in Hematology (**12**).

8. Keywords

8.1 automated cell counting; electrical sensing zone; size distribution

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