Designation: F 2149 – 01

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 F 2149; 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, hence, this test method is not restricted to specific cell types. The method is appropriate for suspension as well as adherent cell cultures **(1)**.[2] This is a quantitative laboratory method not intended for on-line or field use. Results may be reported as number of cells per millilitre or total number of cells per volume of cell suspension analyzed. Both count and size distribution may be expressed in cell micron diameter or volume, femtolitres.

1.2 Cells commonly used in tissue-engineered medical products **(2)** routinely are analyzed. 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 clearly validated for superior accuracy and precision when compared to the conventional manual method, visual cell counting under a microscope using a hemocytometer. This validation has been demonstrated over a wide variety of cell types. In addition, the automated procedure is rapid, rugged, and cost effective; it also minimizes operator-to-operator variability inherent in manual techniques.

1.3 This instrumentation is manufactured by a variety of companies; however, the principle used in all is 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 very 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 minimized, thus ensuring greater result accuracy, by using relatively low cell concentrations, around the 5 % level.

1.4.2 *Viability*—Automated cell counting enumerates both viable and nonviable cells. It does not measure percent cell viability. To measure the percent cell viability, either a vital dye or nonvital dye, such as trypan blue, procedure must be performed.

1.4.3 *Size Variation of the Cell Sample*—Up to 30 to 1 by cell diameter in microns; 27 000 to 1 by cell volume. This is simply a function of the size range capability of the particular aperture size selected. Using this technology, measurements may be made in the range of about 0.6 to 1200 µm. The lower size limit is restricted only by thermal and electronic noise.

1.4.4 *Size Range of the Aperture*—The size range for a

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

single aperture is proportional to its diameter, *D*. The response has been found to depend linearly on *D* over a range from 0.02 *D* to 0.80 *D*; however, the aperture tube may become prone to blockage at levels greater than 0.60 *D*. The practical operating range, therefore, 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 to 35°C.

1.4.7 *Electrolyte Solution*—The diluent for cell suspension must provide conductivity and have no effect on cell size. The electrolyte of choice is most often physiologic 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 *femtolitre*, *n*—a cubic micron; a measurement of cell volume.

2.1.6 *micron (μ)*, *n*—0.001 mm, also known as a micrometre; measurement of cell diameter.

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

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

2.1.9 *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 This assay is used in university tissue culture laboratories, government research, and hospital, biomedical, and pharmaceutical laboratories to automate cell counting and sizing. This instrumentation provides very rapid, accurate, and precise results for any tissue culture facility. In addition, as noted, since the cell sizes to be analyzed by the instrument are set by the user, the analyses may be done on virtually any species of cells and cell type; it is not restricted to human cells or blood cells.

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 standard counting chamber, hemocytometer. The counting chamber is known to have an error of 10 to 30 %, as well as being very time consuming **(10)**. In addition, when counting and sizing porcine hepatocytes, Stegemann et al concluded that the automated, electrical sensing zone method provided significantly 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.

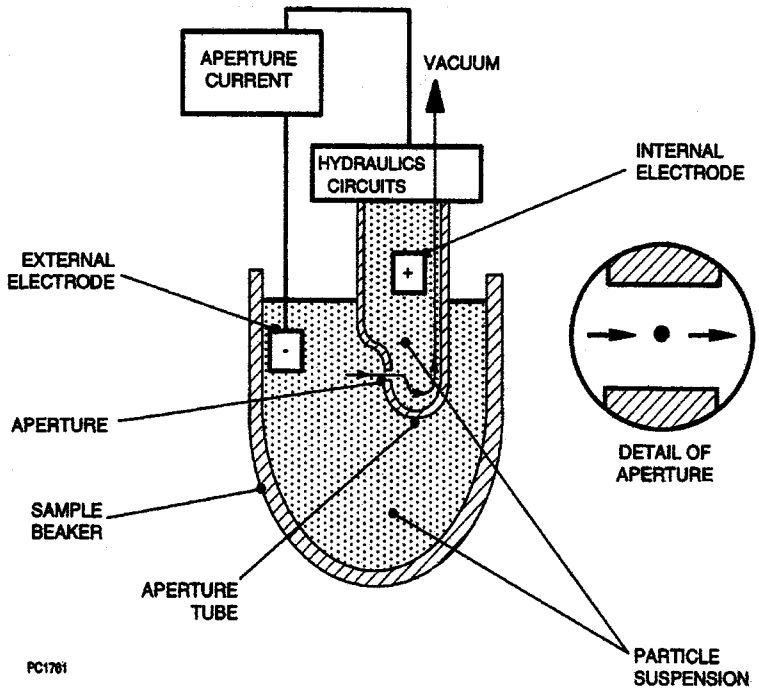

FIG. 1 Cell, Suspended in a Conductive Fluid, Traversing Through a Small Aperture

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.

4.3 *Settling*—Cell sample preparations may exhibit settling resulting in decreased counts. Inverting the sample container several times will produce more accurate and precise results.

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

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

4.6 Excessive current across the aperture may damage cellular membranes. The optimum current is set by 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, must 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 must be grounded correctly.

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

6. Procedures

6.1 The procedure for this test method is similar regardless of the brand of commercial instrumentation used. Commercial instrumentation among manufacturers may vary as to the instrument's size range, number of aperture diameters available, and data acquisition capabilities using various printers or computer data acquisition. Certain manufacturers provide instrumentation in which both instrument function control and data analyses are computer controlled. In addition, some models will provide cell counts only; however, other models contain a channelyzer enabling the instrument to determine cell size distribution in addition to count results.

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 MCV should be used.

6.3 Dilute the cell suspension in the electrolyte, typically physiologic phosphate-buffered saline. Gently mix the sample by inverting it seven or eight times. Perform the analyses within 20 min after sample preparation.

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 should 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. Typically, for 500 µL, this would be 13 s; however, most instrumentation contains an aperture viewing screen on which any blockages may easily be seen. In addition, most contemporary instrumentation now provide an automatic 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 provide 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.

7. Precision and Bias

7.1 Reproducibility on replicate samples of porcine hepatocytes is ±5 % (average ±3 %) **(7)**. Lehmann reported a CV of 3.0 % on pancreatic islets **(6)**. Evaluation of this methodology, using red blood cells, by the International Committee for Standardization in Hematology resulted in a CV in the range of 0.24 to 0.72 % **(8)**. The instrument operator's manual, from certain manufacturers, lists precision on representative cell types. Instructions for instrument installation qualification, operational qualification, and performance qualification are available. A statement of bias is not available; however, this technology 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 in 1988 **(11)**.

8. Keywords

8.1 automated cell counting; electrical sensing zone; size distribution

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