



Designation: F2944 – 12

Standard Test Method for Automated Colony Forming Unit (CFU) Assays—Image Acquisition and Analysis Method for Enumerating and Characterizing Cells and Colonies in Culture¹

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1. Scope

1.1 This test method, provided its limitations are understood, describes a procedure for quantitative measurement of the number and biological characteristics of colonies derived from a stem cell or progenitor population using image analysis.

1.2 This test method is applied in an *in vitro* laboratory setting.

1.3 This method utilizes: (a) standardized protocols for image capture of cells and colonies derived from *in vitro* processing of a defined population of starting cells in a defined field of view (FOV), and (b) standardized protocols for image processing and analysis.

1.4 The relevant FOV may be two-dimensional or three-dimensional, depending on the CFU assay system being interrogated.

1.5 The primary unit to be used in the outcome of analysis is the number of colonies present in the FOV. In addition, the characteristics and sub-classification of individual colonies and cells within the FOV may also be evaluated, based on extant morphological features, distributional properties, or properties elicited using secondary markers (for example, staining or labeling methods).

1.6 Imaging methods require that images of the relevant FOV be captured at sufficient resolution to enable detection and characterization of individual cells and over a FOV that is sufficient to detect, discriminate between, and characterize colonies as complete objects for assessment.

1.7 Image processing procedures applicable to two- and three-dimensional data sets are used to identify cells or colonies as discreet objects within the FOV. Imaging methods may be optimized for multiple cell types and cell features using analytical tools for segmentation and clustering to define

groups of cells related to each other by proximity or morphology in a manner that is indicative of a shared lineage relationship (that is, clonal expansion of a single founding stem cell or progenitor).

1.8 The characteristics of individual colony objects (cells per colony, cell density, cell size, cell distribution, cell heterogeneity, cell genotype or phenotype, and the pattern, distribution and intensity of expression of secondary markers) are informative of differences in underlying biological properties of the clonal progeny.

1.9 Under appropriately controlled experimental conditions, differences between colonies can be informative of the biological properties and underlying heterogeneity of colony founding cells (CFUs) within a starting population.

1.10 Cell and colony area/volume, number, and so forth may be expressed as a function of cell culture area (square millimetres), or initial cell suspension volume (millilitres).

1.11 Sequential imaging of the FOV using two or more optical methods may be valuable in accumulating quantitative information regarding individual cells or colony objects in the sample. In addition, repeated imaging of the same sample will be necessary in the setting of process tracking and validation. Therefore, this test method requires a means of reproducible identification of the location of cells and colonies (centroids) within the FOV area/volume using a defined coordinate system.

1.12 To achieve a sufficiently large field-of-view (FOV), images of sufficient resolution may be captured as multiple image fields/tiles at high magnification and then combined together to form a mosaic representing the entire cell culture area.

1.13 Cells and tissues commonly used in tissue engineering, regenerative medicine, and cellular therapy are routinely assayed and analyzed to define the number, prevalence, biological features, and biological potential of the original stem cell and progenitor population(s).

1.13.1 Common applicable cell types and cell sources include, but are not limited to: mammalian stem and progenitor cells; adult-derived cells (for example, blood, bone marrow,

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skin, fat, muscle, mucosa) cells, fetal-derived cells (for example, cord blood, placental/cord, amniotic fluid); embryonic stem cells (ESC) (that is, derived from inner cell mass of blastocysts); induced pluripotency cells (iPS) (for example, reprogrammed adult cells); culture expanded cells; and terminally differentiated cells of a specific type of tissue.

1.13.2 Common applicable examples of mature differentiated phenotypes which are relevant to detection of differentiation within and among clonal colonies include: hematopoietic phenotypes (erythrocytes, lymphocytes, neutrophils, eosinophiles, basophiles, monocytes, macrophages, and so forth), mesenchymal phenotypes (osteoblasts, chondrocytes, adipocytes, and so forth), and other tissues (hepatocytes, neurons, endothelial cells, keratinocyte, pancreatic islets, and so forth).

1.14 The number of stem cells and progenitor cells in various tissues can be assayed *in vitro* by liberating the cells from the tissues using methods that preserve the viability and biological potential of the underlying stem cell and/or progenitor population, and placing the tissue-derived cells in an *in vitro* environment that results in efficient activation and proliferation of stem and progenitor cells as clonal colonies. The true number of stem cells and progenitors (true colony forming units (tCFU)) can thereby be estimated on the basis of the number of colony-forming units observed (observed colony forming units (oCFU)) to have formed **(1-3)**² (Fig. A1.1). The prevalence of stem cells and/or progenitors can be estimated on the basis of the number of observed colony-forming units (oCFU) detected, divided by the number of total cells assayed.

1.15 The automated image acquisition and analysis approach (described herein) to cell and colony enumeration has been validated and found to provide superior accuracy and precision when compared to the current “gold standard” of manual observer defined visual cell and colony counting under a brightfield or fluorescent microscope with or without a hemocytometer **(4)**, reducing both intra- and inter-observer variation. Several groups have attempted to automate this and/or similar processes in the past **(5, 6)**. Recent reports further demonstrate the capability of extracting qualitative and quantitative data for colonies of various cell types at the cellular and even nuclear level **(4, 7)**.

1.16 Advances in software and hardware now broadly enable systematic automated analytical approaches. This evolving technology creates the need for general agreement on units of measurement, nomenclature, process definitions, and analytical interpretation as presented in this test method.

1.17 Standardized methods for automated CFU analysis open opportunities to enhance the value and utility of CFU assays in several scientific and commercial domains:

1.17.1 Standardized methods for automated CFU analysis open opportunities to advance the specificity of CFU analysis methods though optimization of generalizable protocols and

quantitative metrics for specific cell types and CFU assay systems which can be applied uniformly between disparate laboratories.

1.17.2 Standardized methods for automated CFU analysis open opportunities to reduce the cost of colony analysis in all aspects of biological sciences by increasing throughput and reducing work flow demands.

1.17.3 Standardized methods for automated CFU analysis open opportunities to improve the sensitivity and specificity of experimental systems seeking to detect the effects of *in vitro* conditions, biological stimuli, biomaterials and *in vitro* processing steps on the attachment, migration, proliferation, differentiation, and survival of stem cells and progenitors.

1.18 Limitations are described as follows:

1.18.1 *Colony Identification—Cell Source/Colony Type/Marker Variability*—Stem cells and progenitors from various tissue sources and in different *in vitro* environments will manifest different biological features. Therefore, the specific means to detect cells or nuclei and secondary markers utilized and the implementation of their respective staining protocols will differ depending on the CFU assay system, cell type(s) and markers being interrogated. Optimized protocols for image capture and image analysis to detect cells and colonies, to define colony objects and to characterize colony objects will vary depending on the cell source being utilized and CFU system being used. These protocols will require independent optimization, characterization and validation in each application. However, once defined, these can be generalized between labs and across clinical and research domains.

1.18.2 *Instrumentation Induced Variability in Image Capture*—Choice of image acquisition components described above may adversely affect segmentation of cells and subsequent colony identification if not properly addressed. For example, use of a mercury bulb rather than a fiber-optic fluorescent light source or the general misalignment of optics could produce uneven illumination or vignetting of tiles images comprising the primary large FOV image. This may be corrected by applying background subtraction routines to each tile in a large FOV image prior to tile stitching.

1.18.3 *CFU Assay System Associated Variation in Imaging Artifacts*—In addition to the presentation of colony objects with unique features that must be utilized to define colony identification, each image from each CFU system may present non-cell and non-colony artifacts (for example, cell debris, lint, glass aberrations, reflections, autofluorescence, and so forth) that may confound the detection of cells and colonies if not identified and managed.

1.18.4 *Image Capture Methods and Quality Control Variation*—Variation in image quality will significantly affect the precision and reproducibility of image analysis methods. Variation in focus, illumination, tile registration, exposure time, quenching, and emission spectral bleeding, are all important potential limitations or threats to image quality and reproducibility.

1.19 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

² The boldface numbers in parentheses refer to a list of references at the end of this standard.