



Designation: **F2944 – 12 F2944 – 20**

## Standard Test Method Practice for Automated Colony Forming Unit (CFU) Assays—Image Acquisition and Analysis Method for Enumerating and Characterizing Cells and Colonies in Culture<sup>1</sup>

This standard is issued under the fixed designation F2944; 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 ( $\epsilon$ ) indicates an editorial change since the last revision or reapproval.

### 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 discrete 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.

<sup>1</sup> This test method practice 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 March 1, 2012; April 1, 2020. Published April 2012; June 2020. Originally approved in 2012. Last previous edition approved in 2012 as F2944-12. DOI: 10.1520/F2944-12; 10.1520/F2944-20.

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, 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, eosinophils, basophils, 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)**<sup>2</sup> (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 through 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 tile 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.

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

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.

1.20 *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. Terminology

### 2.1 Definitions:

2.1.1 *cell number, n*—number of cells counted within a culture area based upon a ubiquitous, separable cell marker (that is, nuclear stain).

2.1.2 *colony, n*—a cluster 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).

2.1.3 *colony area, n*—sum of all pixels within a given colony multiplied by the pixel resolution (square millimetres).

2.1.4 *colony aspect ratio, n*—ratio of colony major and minor axes (1 = perfect circle).

2.1.5 *colony centroid, n*—central pixel determined using all x- and y-coordinates of pixels within given colony (may also be calculated using center of best-fit ellipse or box).

2.1.6 *colony forming efficiency (CFE), n*—the probability of converting a tCFU to an oCFU, where a probability of 1.0 represents 100% conversion. Therefore the relationship between tCFU to an oCFU can be defined by the relationship:  $tCFU \times CFE = oCFU$ .

2.1.7 *colony major axis, n*—longest dimension of the best-fit box (or ellipse) around a given colony (millimetres).

2.1.8 *colony minor axis, n*—shortest dimension of the best-fit box (or ellipse) around a given colony (millimetres).

2.1.9 *colony or colony forming unit (CFU), n*—a single cell, which when placed into *in vitro* culture will survive and proliferate to create progeny which become manifest as a colony of lineage-related cells derived from the founding CFU.

2.1.10 *effective proliferation rate (EPR), n*—the proliferation rate that would be necessary to produce the number of cells found in a given colony during the time in culture ( $EPR = \log_2(\text{cell number})/\text{time in days}$ ).

2.1.11 *observed CFU (oCFU), n*—the number of cells in a given sample that form a colony of interest under the conditions used.

2.1.12 *prevalence, n*—number of colonies per cell plated (often expressed in colonies per million cells).

2.1.13 *proliferation rate, n*—the current incidence of mitosis within a population of cells over a defined period of time. *Note*—The proliferation rate may change over time.

2.1.14 *secondary marker, n*—any marker in addition to the nuclear marker or cell localization marker that provides information related to the genotype, phenotype, biological activity, biochemical features or lineage history of a colony or cell.

2.1.15 *true CFU (tCFU), n*—the number of cells in a given sample that are capable of forming a colony of interest under some optimal condition.

## 3. Significance and Use

3.1 *The Manual Observer-Dependent Assay*—The manual quantification of cell and CFU cultures based on observer-dependent criteria or judgment is an extremely tedious and time-consuming task and is significantly impacted by user bias. In order to maintain consistency in data acquisition, pharmacological and drug discovery and development studies utilizing cell- and colony-based assays often require that a single observer count cells and colonies in hundreds, and potentially thousands of cultures. Due to observer fatigue, both accuracy and reproducibility of quantification suffer severely (5). When multiple observers are employed, observer fatigue is reduced, but the accuracy and reproducibility of cell and colony enumeration is still significantly compromised due to observer bias and significant intra- and inter-observer variability (4, 8). Use of quantitative automated image analysis provides data for both the number of colonies as well as the number of cells in each colony. These data can also be used to calculate mean cells per colony. Traditional methods for quantification of colonies by hand counting coupled with an assay for cell number (for example, DNA or mitochondrial) remains a viable method that can be used to calculate the mean number of cells per colony. These traditional methods have the advantage that they are currently less labor intensive and less technically demanding (9, 10). However, the traditional assays do not, provide colony level information (for example, variation and skew), nor do they provide a means for excluding cells that are not part of a colony from the calculation of mean colony size. As a result, the measurement of the mean number of cells per colony that is obtained from these alternative methods may differ when substantial numbers of cells in a sample are not associated with colony formation. By employing state-of-the-art image acquisition, processing and analysis hardware and software, an accurate, precise, robust and automated analysis system is realized.

3.1.1 *Areas of Application*—Cell and colony enumeration (CFU assay) is becoming particularly important in the manufacture, quality assurance/control (QA/QC), and development of product safety and potency release criteria for cell-based regenerative medicine and cellular therapy. In 2006, the Food and Drug Administration (FDA) released a guidance document foreshadowing the importance of the colony assay in establishing an audit trail for the aforementioned steps of product development in this industry (11). Corroborating this document, the FDA released an additional guidance document in 2009 proposing the potential significance of the colony assay with regard to functional potency of placental and umbilical cord blood-derived stem cells in regenerative medicine and therapeutic applications (12). Since cell source validation and QA/QC comprise approximately 50 % of the manufacturing cost of cellular therapies (13), developing a precise, robust, and cost-effective means for enumerating cells and colonies is vital to sustainability and growth in this industry. The broad areas of use for automated analysis of colony forming unit assays include:

3.1.1.1 Characterization of a cell source by correlating biological potential and functional potency with CFU formation.

3.1.1.2 Characterization of the effect of processing steps or biological or physical manipulation (for example, stimuli) on cells or colony formation.

3.1.1.3 Cell and colony characterization using specific fluorescent and non-fluorescent (differentiation) markers.

3.1.1.4 Extrapolation of the biological potency (for example, differentiation, proliferative, and so forth) of a larger sample from application of colony forming assay to sub-samples.

3.1.1.5 Provision of criteria for sub-colony selection of preferred colonies (specific tissue type, proliferation rate, and so forth) for use and/or further expansion.

3.2 *The Technology (image acquisition, processing, and analysis)*—Current standards utilize user input for defining the presence and location of colonies based on visualization of an entire culture surface at low magnification through the eyepieces of a microscope. In this case, the sample may be viewed in transmission light mode (unstained or with a histochemical marker) or fluorescently with a dye or antibody. For this test method, the colony count is the only measurable output parameter. Utilizing a microscope-based imaging system to stitch together high resolution image tiles into a single mosaic image of the entire culture surface and subsequently “clustering” segmented cells using image processing algorithms to delineate colonies, provides a fully automated, accurate, and precise method for characterizing the biological potential and functional potency of the cultured cells. Furthermore, extracted parameters in addition to colony number provide means of further characterization and sub-classification of colony level statistics. These parameters include, but are not limited to, cell/nuclear count, cell/nuclear density, colony morphology (shape and size parameters), secondary marker coverage, effective proliferation rates, and so forth (Fig. A1.2). In addition to Human Connective Tissue Progenitors (CTPs), this test method and technology has been implemented in the cell and colony identification and characterization of several cell and tissue types including: Cartilage Progenitor Cells (Fig. X1.1); Umbilical Cord Blood Hematopoietic Stem Cells (Fig. X1.2); Adipose-derived Stem Cells (Fig. X1.3); and Human Epidermal (Fig. X1.4) and Dermal (Fig. X1.5) Stem Cells.

ASTM F2944-20

3.3 *Benefits of Automated Analysis of CFU Assays*—Automated analysis is expected to provide more rapid, reproducible, and precise results in comparison to the manual enumeration of cells and colonies utilizing a microscope, hemocytometer, and so forth. In addition to being highly time and labor intensive and subjective, manual enumeration has been shown to have a significant degree of intra- and inter-observer variability, with coefficients of variation (CV) ranging from 8.1 to 40.0 % and 22.7 to 80 %, respectively. Standard CVs for cell viability assessment and progenitor (colony) type enumeration have been shown to range from 19.4 to 42.9 % and 46.6 to 100 %, respectively (4, 8, 14). In contrast, studies focusing on bacteria, bone marrow-derived stem cells and osteogenic progenitor cells have collectively concluded that automated enumeration provides significantly greater accuracy, precision, and/or speed for counting and sizing cells and colonies, relative to conventional manual methodologies (4-6). Automated methods for enumerating cells and colonies are less biased, less time consuming, less laborious, and provide greater qualitative and quantitative data for intrinsic characteristics of cell and colony type and morphology.

3.4 *Selection of Cell Culture Surface Area and Optimal Cell Seeding Density*—When performing a CFU assay, optimizing the cell culture surface area and cell seeding density is critical to developing methods for generating reliable and reproducible colony- and cell-level data. If seeding density is too low, then the frequency of observed colonies is decreased. This can result in a sampling size that is inadequate to characterize the population of CFUs in the sample. If seeding density is too high, the colonies that are formed may be too closely spaced. Overlapping colony footprints compromise colony counting and characterization. Because the intrinsic range of CFU prevalence in a given cell source may vary widely, in many cases, a trial and error approach to optimizing cell seeding density (or range of densities) that are needed for a given cell source will be necessary. It is important to note that the more heterogeneous the cell source (for example, bone marrow), the more colonies that are needed to accurately represent the stem and progenitor cell constituents. Further, the cell type, effective proliferation rate (EPR) and specific cell culture conditions (for example, media, serum, factors, oxygen tension, and so forth) can impact colony formation. For example, the automated CFU Assay depicted in Fig. A1.2 employs a six-day culture period, two media changes, 20 % oxygen tension, alpha-MEM media (with 25 % fetal bovine serum, ascorbate, dexamethasone and streptomycin), an optimized cell seeding density of 250 000 nucleated cells per cm<sup>2</sup> (250 000 cell per 1 mL of cell culture medium) and a cell culture surface area of 22 by 22 mm (dual-chamber Lab-Tek culture slides) (15, 16).

#### 4. Interferences

4.1 *Nuclear Aggregation*—Depending upon the specific implementation of morphologically-based filters utilized to separate cells stained with a particular nuclear marker (that is, watershed), there is a possibility of over- or under-estimation of cell number that could potentially affect cell clustering for colony identification.

4.2 *Localization of Secondary Markers*—While various processing filters may be utilized to separate distinct, regular shaped objects such as nuclei, cellular bodies visualized via cytoplasmic markers are generally amorphous in size and shape posing problems for accurate separation of cells in contact. Thus, while the staining area of these markers may be determined for a particular colony or culture surface area, the amount of staining cannot always be determined for a specific cell.

4.3 *Cellular and Non-cellular Debris*—In general debris (non-cellular or apoptotic), may be segmented using morphological-based criteria (that is, area, length, aspect ratio, and so forth) or intensity (that is, lack of gradient, gray-level intensity, and so forth) and subtracted from the large FOV image of the cell culture surface prior to nuclear clustering. Debris that is consistent with the size and shape of nuclei may not be easily removed unless visible in another portion of the fluorescence spectrum where neither the nuclear marker nor secondary markers are present.

4.4 *Colony Forming Efficiency*—While a frequent use for the standard method proposed is to determine the true number of CFUs in a given sample or cell source, it must be recognized that the number of true CFUs (tCFU) will only be the same as the observed number of CFUs (oCFU) if all potential CFUs form colonies under conditions of the assay. This is only true if the efficiency of converting a tCFU to an oCFU is 1.0 or 100 %. Therefore, the relationship between a tCFU to an oCFU is dependent upon the colony forming efficiency (CFE) of the assay conditions used and can be represented by the relationship:  $tCFU \times CFE = oCFU$ . In any given cell source, variables that influence CFE can be systematically explored by culturing identical samples under varying conditions. Using this experimental design, since tCFU in the starting samples is constant; changes in oCFU are the result of changes in CFE.

#### 5. Apparatus

5.1 *Imaging Requirements*—In the setting of image capture using the strategy described in 1.12, the components of instrumentation necessary for generating large FOV images may include a microscope stand with 5 to 40× magnification objective lenses, a fluorescence filter turret with basic filter cube sets, a fluorescence energy source (mercury, xenon, and so forth), an x-, y-, z- motorized (linearly-encoded) stage, a charge-coupled device (CCD) camera, and imaging software to acquire, process, and save images acquired by the CCD camera. Each of these components is available from multiple commercial vendors.

5.2 *Computational Requirements*—Image processing steps (that is, clustering) require access to the entire, contiguous pixel dataset stored in each high resolution, large FOV image, short-term random-access memory (RAM) (>2 gigabytes (GB)) and long-term memory storage (>200 GB) is needed. This requirement will vary depending on the area or volume of the FOV required and on the resolution (pixel or voxel size) required for the individual application.

<https://standards.iteh.ai/catalog/standards/sist/30a95a80-e652-4c23-b81f-124ace9881d4/astm-f2944-20>

#### 6. Hazards

6.1 *Warning (Electrical)*—High voltages are present inside this instrument. Instrument shall be sited on a firm, dry work space and be properly grounded.

6.2 *Warning (Biological)*—Institutional-, state-, and Occupational, Safety and Health Administration (OSHA)-approved safety action plans shall be followed.

#### 7. Procedure

7.1 Tile images are acquired by raster scanning across a cell culture surface using a motorized microscope fitted with multiple fluorescence filters and light source, x-, y-, z- motorized scanning stage, high magnification objective lenses (may range from 5 to 40×), and a CCD camera (for example, monochrome, quantum efficiency of 55 % @ 500 nm, 7 by 7 μm pixel size, and 1600 by 1200 resolution):

7.1.1 For cell cultures forming three-dimensional colonies (that is, cells in matrix such as methyl cellulose), z-axis image slices (predefined range where the number of slices correlates with slice thickness and the depth of the colony) are also acquired at each tile's x,y position:

7.1.2 Scanning shall be performed for at least one nuclear marker and repeated for other secondary markers. Image resolution should be high enough (0.7 to 2.8 μm) to provide at least 40 pixels for each nucleus.

7.2 To clean and prepare the image tiles for stitching and subsequent analysis, uneven illumination and vignetting in each scanned tile image shall be background corrected (a single black/blank tile image is smoothed and divide into every image tile) and flattened (removal of any uneven illumination from light source).

7.3 Background corrected and flattened tile images are stitched together into a single, large field-of-view (FOV) image.

7.4 Individual cell nuclei are segmented using a predefined global threshold, local neighborhood examination of pixel(s) intensity, or spectral filters.

7.5 Objects that do not fall within the predefined area range for the nuclei morphology (for example, size, shape, and so forth for a given cell type – human nuclei =  $18.7 \mu\text{m}^2$ ) of a given cell type shall be rejected and digitally removed from the large FOV image.

7.6 Objects that are not consistent with the general elliptical shape of a nucleus (that is, aspect ratio of  $>3.0$ ) shall be rejected and digitally removed from the large FOV image.

7.7 Objects that do not exhibit a radial pixel intensity gradient that increases toward their respective centroids shall be rejected and digitally removed from the large FOV image (removal of object with uniform pixel intensity, for example, lint or apoptotic debris).

7.8 The remaining objects, cell nuclei, shall be clustered/grouped to define larger scale colony objects using a predefined criteria. (For example, at least eight cells whose nuclei are separated by less than or equal to  $5 \mu\text{m}$ .)

7.9 Colony outlines (border or perimeter of the object) and masks (entire “footprint” of an object, for example, area within the object outline) are generated.

7.10 Colony masks are applied/multiplied to secondary marker images.

7.11 Global thresholds shall be used to define the percentage coverage (that is, overlap) of these markers for each cell and/or colony based on the amount of overlap/correlation between the colony and secondary marker masks.

7.12 The resulting qualitative and quantitative parameters are output for subsequent organization and analysis using graphing and statistical software.

7.12.1 The qualitative and quantitative parameters include, but are not limited to:

7.12.1.1 Colony number;

7.12.1.2 Prevalence;

7.12.1.3 Number of cells for a given colony;

7.12.1.4 Total cell number (within and not within a colony);

7.12.1.5 Effective proliferation rate;

7.12.1.6 Cell density per colony;

7.12.1.7 Percent expression of a given differentiation marker per colony;

7.12.1.8 Number of cells positive/negative for a given differentiation marker (for example, per colony or per area of culture surface).

## 8. Precision and Bias

8.1 The precision and bias using this test method are expected to vary depending on the quality of the images used for analysis. The quality of the images will be dependent upon the quantum efficiency and resolution of the camera used, numerical aperture of the objectives used, and the emission and excitation profiles of the filters used. Precision and bias will also be dependent upon the cell type being analyzed (for example, cell morphology, growth pattern) and the sensitivity and specificity of the biological markers which are utilized to identify the cells and cellular features.

8.2 Improved precision and reduced bias have been empirically demonstrated in the case of colony and cell identification and classification in human bone marrow-derived connective tissue progenitors (CTPs). The automated method for colony identification agreed with multiple skilled observers on individual colonies over 85 % of the time. Moreover, concordance in colony and cell identification between individual skilled observers and the automated system was 2.7 times greater than concordance between the skilled observers. Colony numbers obtained using this automated method demonstrated a correlation coefficient of 0.99 with data from skilled observers.

8.3 Precision and reproducibility between image acquisition systems has been characterized in the analysis of human bone marrow-derived CTP colony formation. Multiple large field-of-view (FOV) motorized microscopes were used to assess intra- and inter-hardware precision and reproducibility. A detailed description of the experimental approach and results will be appended here upon availability.

## 9. Keywords

9.1 automated cell and colony enumeration; automated colony forming unit assays; biological potential; cells; cell-based assays; cell source validation; cell therapies; CFU; colonies; colony area; colony cell density; colony morphology; colony size; counting; differentiation markers; drug developments; drug discoveries; FDA; image acquisition; image analysis; image processing; pharmaceuticals; progenitor cells; proliferation rates; quality control; quality assurance; QA/QC; stem cells

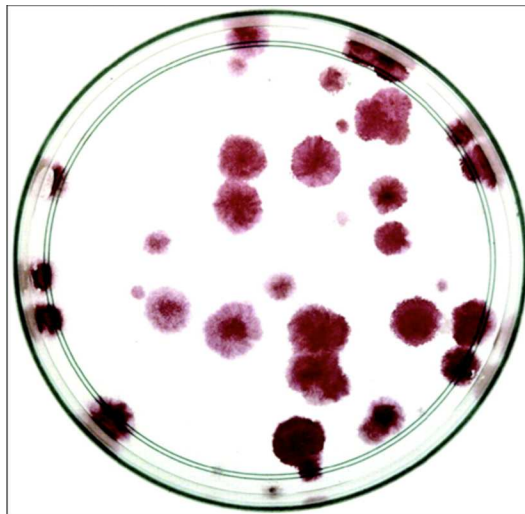
**A1. SAMPLE IMAGE ANALYSIS PROCEDURE**

A1.1 See [Fig. A1.1](#) and [Fig. A1.2](#).

iTeh Standards  
(<https://standards.iteh.ai>)  
Document Preview

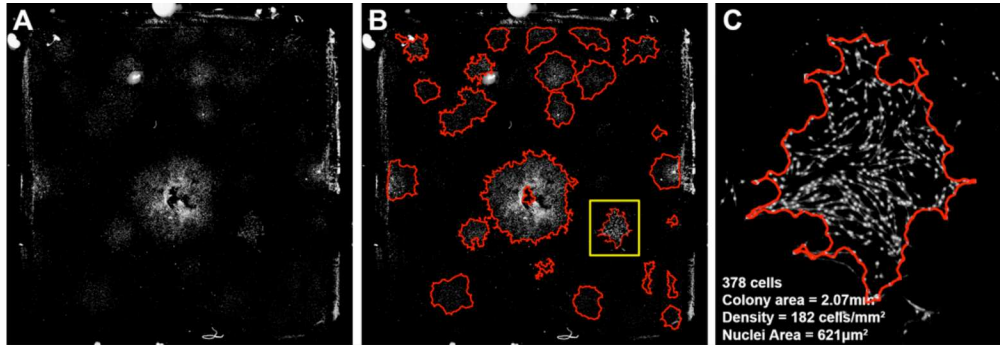
[ASTM F2944-20](#)

<https://standards.iteh.ai/catalog/standards/sist/30a95a80-e652-4c23-b81f-124ace9881d4/astm-f2944-20>



NOTE 1—Colonies are displayed on tissue culture plastic and are manually enumerated by hand counting by multiple observers.

**FIG. A1.1 Traditional CFU Assay for Manual Analysis by Multiple Observers**



NOTE 1—(A) Large FOV images (480 image tiles stitched together) of Connective Tissue Progenitors (CTPs) seeded on 22 mm by 22 mm chamber slides and stained with 4',6-diamidino-2-phenylindole (DAPI). The images shown here have been background corrected (flattened illumination for each tile) and processed for the removal of artifacts. (B) Images processed for colony segmentation (red outline) using various automated algorithms. (C) Magnified colony (delineated with yellow box in B indicating various quantitative parameters that may be extracted.

FIG. A1.2 Automated CFU Analysis of Primary Adult Stem Cell from Bone Marrow

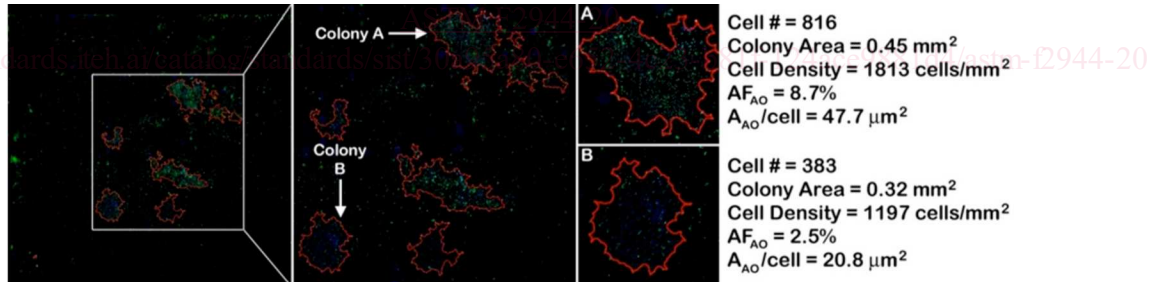
APPENDIX

(Nonmandatory Information)

X1. ILLUSTRATIVE EXAMPLES OF QUANTITATIVE IMAGE ANALYSIS APPLIED TO THE CHARACTERIZATION OF VARIOUS CELL POPULATIONS

(<https://standards.iteh.ai>)  
Document Preview

X1.1 See Figs. X1.1-X1.5.





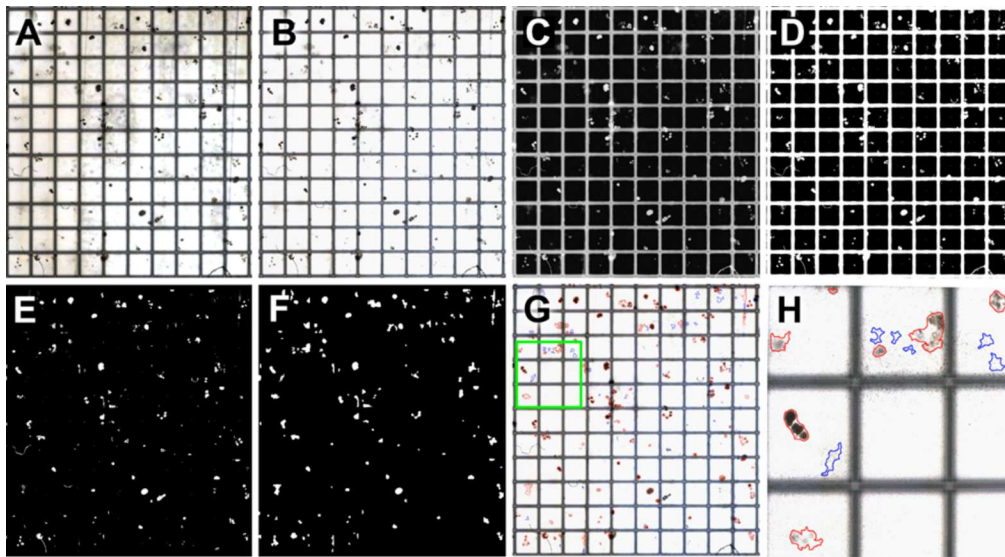
- NOTE 1—Analysis of Chondrogenesis in 2D Colony Assay. ~~Cartilage-derived~~Cartilage-derived CTPs were cultured on 2 cm by 2 cm Lab-tek glass slides under chondrogenic conditions for six days. At day six, cells were stained in situ with acridine orange (AO) and counterstained with DAPI. Large FOV imaging and quantitative image analysis software was used to identify, count, and analyze colonies. Outcome parameters include (but are not limited to) cells per colony ( $N_C$ ), colony area (A), cell density (D) (cells per area), area fraction of AO staining ( $AF_{AO}$ ). As we have found in each tissue we have assessed, colonies varied significantly with respect to each of these ~~parameters~~parameters. For example,  $AF_{AO}$  ranged from 5 % to over 80 %.

**iTeh Standards**  
**(<https://standards.itih.ai>)**  
**Document Preview**

[ASTM F2944-20](#)

<https://standards.itih.ai/catalog/standards/sist/30a95a80-e652-4c23-b81f-124ace9881d4/astm-f2944-20>

FIG. X1.1 Automated CFU Analysis of Cartilage-derived Cartilage-Derived Connective Tissue Progenitors



iTeh Standards  
(<https://standards.iteh.ai>)  
Document Preview

[ASTM F2944-20](https://standards.iteh.ai/catalog/standards/sist/30a95a80-e652-4c23-b81f-124ace9881d4/astm-f2944-20)

<https://standards.iteh.ai/catalog/standards/sist/30a95a80-e652-4c23-b81f-124ace9881d4/astm-f2944-20>