Designation: F1027 – 86 (Reapproved 2012)

Standard Practice for Assessment of Tissue and Cell Compatibility of Orofacial Prosthetic Materials and Devices[1]

This standard is issued under the fixed designation F1027; 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 practice describes a procedure to assess the cytotoxic potential of materials for use in the construction of medical materials and devices using human excised donor (HED) tissues and their derived primary cells taken from the orofacial region.

1.2 This practice may be used either directly to evaluate materials or as a reference against which other cytotoxicity methods may be compared.

1.3 This practice is one of a series of reference methods for assessment of cytotoxic potential, employing different techniques.

1.4 Assessment of cytotoxicity is one of several procedures employed in determining the biological response to a material, as recommended in Practice F748.

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

1.6 *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]

D883 Terminology Relating to Plastics

F604 Specification for Silicone Elastomers Used in Medical Applications (Withdrawn 2001)[3]

F703 Specification for Implantable Breast Prostheses

F748 Practice for Selecting Generic Biological Test Methods for Materials and Devices

F813 Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices

3. Terminology

3.1 Nomenclature relating to the physical, mechanical, and chemical characteristics of plastics shall be in accordance with Terminology D883.

3.2 The nomenclature and glossary of terms related to tissue culturing shall conform to that of the Tissue Culture Association (**1**).[4]

3.3 For other definitions used in this practice, see Annex A1.

4. Summary of Practice

4.1 Primary human orofacial tissue or cells and established human cell lines are cultured in Medium A3 or any medium supporting primary cell growth with homologous processed human serum or serum components in cell culture flasks or appropriate containers. The following series of cultures is set up:

4.1.1 Test material placed in contact with the cell layer.

NOTE 1—One or more replicates of 4.1.1 may be necessary.

4.1.2 Primary control wherein no material contacts the cell layer.

4.1.3 Positive control wherein the cell layer is contacted by a material eliciting a known cytotoxic response, such as a toxic chemical published in the Toxic Substances List **(2).**

4.1.4 Negative control wherein the cell layer is contacted by polystyrene used in tissue culture labware.

4.2 The test culture shall be observed daily for growth and signs of toxicity. The test shall be terminated upon the attainment of confluency.

NOTE 2—For an established cell line cultured with Holmes alpha growth factor (AGF), confluency is usually achieved in slightly more than 5 days.

NOTE 3—For first passage cells from human excised donor (HED)

[1] This practice is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devicesand is the direct responsibility of Subcommittee F04.16 on Biocompatibility Test Methods.

Current edition approved Oct. 1, 2012. Published October 2012. Originally approved in 1986. Last previous edition approved in 2007 as F1027 – 86 (2007). DOI: 10.1520/F1027-86R12.

[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 last approved version of this historical standard is referenced on www.astm.org.

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

Copyright © ASTM International, 100 Barr Harbor Drive, PO Box C700, West Conshohocken, PA 19428-2959. United States

cultures, confluency is usually achieved between 10 to 20 days with an upper limit of 30 days.

5. Significance and Use

5.1 This practice is useful for assessing the cytotoxic potential both when evaluating new materials or formulations for possible use in medical applications, and as part of a quality control program for established medical devices.

5.2 This practice is used for assessing the cytotoxic potential of materials intended for the fabrication of inserts or implants in the orofacial region.

5.3 This practice is restricted to normal non-transformed, human orofacial tissues using cells cultured in human serum factors and does not depend upon cells and serum from non-human sources.

5.4 This practice incorporates procedures to monitor the quality of ingredient materials and the uniformity of the production process for formulating stock compositions.

5.5 This practice may be useful to determine the effects of age and radiation, and the state of carcinogenicity on the sensitivity of HED tissues to materials and devices used for orofacial prostheses.

6. Apparatus

6.1 *Incubator,* capable of maintaining a temperature of 37 ± 1°C and an atmosphere of 95 % air and 5 % CO_2 with at least 90 % relative humidity.

6.2 *Plastic and Glassware,* that is specified by chemical type and is traceable to its source of supply by catalog number or trade designation of the manufacturer or vendor.

6.3 *Laminar Flow Cabinet,* that meets the Class 100 clean room requirements of the U. S. Federal Standard 209B or the National Standard Foundation Standard NSF 49.

6.4 *Fluid Filters,* capable of removing 95 % of particles 0.22 µm or larger.

6.5 *Water Purification System,* with filtration capability for organic contaminants, capable of producing water with resistivity of 18 MΩ-cm or greater.

6.6 *Inverted Stage Microscope,* with phase contrast optics.

6.7 *Bright Field Microscope,* or a photomicroscope with magnification to 200×.

7. Reagents

7.1 *Medium A3*—Chemically defined medium A3 described by Holmes **(3).**

NOTE 4—Other chemically defined media shall be acceptable provided the test human cell adapts within 1 to 3 days to a steady growth rate from low cell density for a period of 7 to 30 days.

7.2 *Trypsin 0.25 % Solution,* stored in lyophilized form at 3 to 5°C. A solution may be prepared as needed and used at 37°C.

7.3 *Insulin,* 6.6 U/100 mL, used as supplement for primary cell and cell line cultures.

7.4 *Miscellaneous Fixatives,* dehydrating solutions, stains, and so forth, for making permanent record microscopic slides.

8. Human Serum

8.1 The human serum shall be processed in accordance with the method described in Annex A2.

NOTE 5—The dialysis treatment serves to remove suspect toxicants, ingested medication, unneeded adventitiae, and unidentified growth inhibitants with exclusion up to a molecular weight of 3500 Daltons.

9. Cell Growth Factors

9.1 *Alpha Growth Factor (AGF)*—AGF, separated from the dialyzed human serum as described in Annex A3, shall be used as needed to enhance cell growth.

NOTE 6—Initially designated alpha-1-protein **(4),** AGF can be used in place of whole serum to maintain the reference established cell line (ECL) cultures for the 7 to 30 day test period when added to a chemically defined medium (See 7.1).

10. Reference ECL Cells

10.1 Human non-transformed established cell line (ECL) cell obtainable from a repository source, such as the American Type Culture Association (ATCC), shall be used as a reference to monitor the procedural details for uniformity of the testing system and for an indication of quality and reliability of culture medium, human serum preparation, and quality of selected growth factors.

NOTE 7—For interlaboratory comparison of these procedural details, the clinically accessible gingival orofacial tissue cell, as well as the mucosal (nasal, maxillo, and so forth), shall be selected and appropriately designated.

11. (HED) Cells

11.1 Human tissues of the orofacial region, obtained from patient donors, shall be cultured as explants until sufficient cell density is attained for succeeding passages into a valid primary cell line.

12. Preparation of Specimens

12.1 Aseptic techniques shall be used throughout the procedure.

12.2 Warm all solutions and materials to a temperature of 37 ± 2°C before placing in contact with cells.

12.3 *Specimens:*

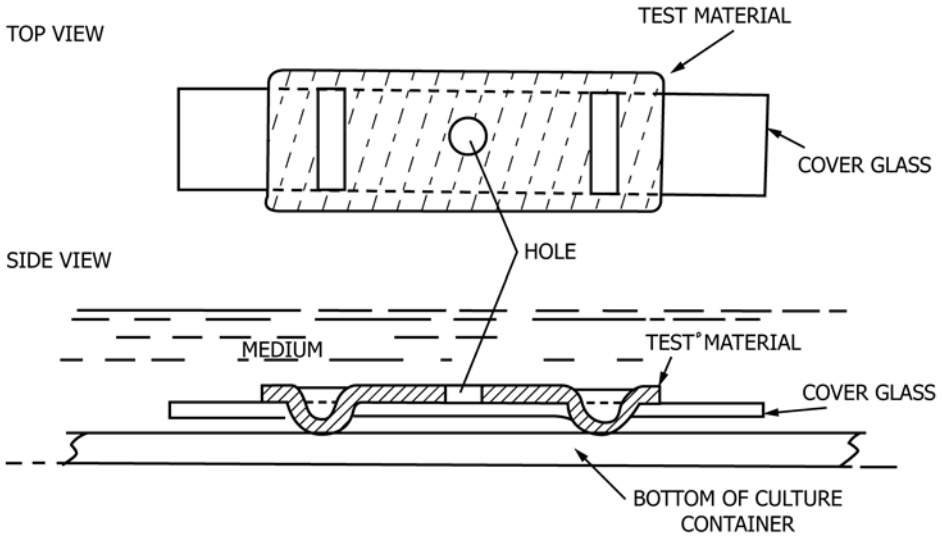
12.3.1 Test materials shall range from 0.1 to 1 mm in thickness, cut into square or triangular geometries, 10 to 15 mm on a side.

12.3.2 Test specimens shall be sterilized by the method used in the preparation of the finished device.

12.3.3 The test arrangement shall provide total immersion and immobilization of specimens (see Fig. 1). A pair of slots is cut in the specimen and a suitably cleaned cover glass (9 by 50 mm, No. 1) is threaded through the slots. One or more round holes (3 mm in diameter) are pre-cut in the center of the specimen. This provides an area of high leaching concentration as well as a focus for a photomicrographic record.

NOTE 8—If contamination of the assembled test material-microslip is suspected, it may be autoclaved before insertion into a sterile culture flask.

12.4 Conventional practices of maintaining contaminant-free working conditions shall be applied in handling tissues,



NOTE 1—Test specimens less than 1 mm in thickness tend to float. The figure depicts one means of maintaining submerged contact between specimens and cell cultures.

NOTE 2—Dimensions and configuration of the hole, serving for initial cell seeding, may be optionally modified and appropriately specified.

FIG. 1 Arrangement for Submersing and Immobilizing Specimens

cells, and glassware with well-established techniques as already prescribed in numerous texts and handbooks. In this connection, see the work by Paul (**5**) as one of several texts dealing with prevention of aerial and fluid contamination in cell culture practices.

13. Preparation of (ECL) Cultures

13.1 The reference cell line (see 10.1) shall be routinely maintained as stock cultures, either in completely chemically defined A3 medium or in Annex A3 medium containing the alpha growth factor (AGF), or in A3 medium supplemented with 10 % processed human serum.

13.2 Medium changes are made every 48 to 72 h or on a triweekly schedule, such as Mondays, Wednesdays, and Fridays. Cultures are checked microscopically at the time and observed for any morphological changes or contamination, delayed or incidental.

13.3 Cell stock cultures of an established cell line are maintained not only as a source of cells for the biocompatibility assessment but also as a means to ascertain and verify the quality and titer of the production lots of processed human serum and preparation lots of separated AGF.

14. Preparation and Maintenance of Primary Human Cells

14.1 Place the human excised donor tissue (explant) aseptically onto a sterile petri dish holding gauze covered with A3 medium containing 10 % processed human serum.

14.2 Dissect explant immediately into small pieces, cut into 1 to 2 mm thin slices approximately 2 to 4 mm^2 in cross-sectional area.

14.3 Incubate at 37 ± 1°C in a 5 % CO_2 and 95 % air atmosphere in quadruplicate series of culturing containers until a monolayer is formed and confirmed microscopically.

14.4 Trypsinize with fresh, ready prepared 0.25 % trypsin solution.

NOTE 9—Other methods of enzyme treatment may be utilized provided the outcome of the assay has been substantially equivalent.

14.5 Place the trypsinized cells in sterile culture flasks to prepare a stock of first passage cells for the biocompatibility test (see Section 17).

14.6 Check the first passage cultured cells for native contamination by virus, bacteria, and pleuropneumonia-like organisms (PPLO). Discard if present and identified and replace with new donor explants.

NOTE 10—Such microorganisms are often entrapped in oral mucosal tissues as contaminants, which could compromise the validity of the test result by imposing foreign, nonspecific cytotoxicity in the procedure in Section 17.

14.7 Harvest the cultured propagated cells for use in the amounts needed in 17.3. Store any unused portion in glycerol or dimethyl sulphoxide (DMSO) at −70°C for new sets of tests, using the procedure described in Chapter XIX of Ref. (**5**).

15. Ascertaining Minimum Effective Titer of Growth Factor

15.1 The selected reference human ECL cell shall be adapted to grow in the Holmes A3 medium or in a culturing medium of equivalent effectiveness using:

15.1.1 Initial low density cell level of 10^3 to 10^4 cells/mL,

15.1.2 One percent processed (Annex A2) human serum,

15.1.3 A series of alpha growth factor comprising 0, 0.1, 1.0, and 10 µg (dry basis) per millilitre of media.

15.1.4 Grown to confluent monolayer with a schedule of three fresh media maintenance replenishments per week as indicated in 13.2.

NOTE 11—It is essential to recognize the various phases of cell growth, which includes adaptation (I), usually with decreasing cell population for

one or more days, followed by log growth (II), usually referred to in population doubling time, leading to monolayer confluency (III), and ultimately to a decline (IV) in cell population by reason of senescence or toxicity. In this connection, appropriate, periodic cell counts to confluency (IV) can be applied as described by Lontz (**6**).

15.2 Following the attained confluency in the above AGF 0 to 10 µg/mL range, the minimal supplementation by AGF is noted for use in the ensuing procedure for biocompatibility of prosthetic material samples (Section 17).

16. Reference Control and Materials

16.1 The *negative* control shall be a material that consistently does not inhibit cell growth as observed visibly or by an appropriate increase in cell count during growth to confluency. The following material may be used:

16.1.1 USP Negative Control Plastic Reference Standard (**7**).

TABLE 1 Test Cell Type, Medium, and Serum Options

Cell Type	Medium	Human Serum (*P*) Maximum Level(*), %
(*A*) Human Orofacial (primary)	A3 with 6.60 Medium	10
(*B*) Established Cell Line[A]	A3	(Replaced by 0.1 to 10 % AGF)
(*C*) Established Cell Line	A3	10

[A] The combinations (*B*) and (*C*) of this table are used to ensure cell viability and procedural consistency.

16.1.2 Fluorocarbon film or sheeting.

NOTE 12—Satisfactory sheetings are Teflon FEP fluorocarbon, 10 mil (0.010 in.) in thickness and a copolymer of tetrafluorethylene and hexafluoropropylene of commercial prominence; This grade of fluorocarbon polymer is uniquely useful because of (*a*) exceptional chemical inertness, (*b*) high specific gravity, higher than most of the nutrient media, and (*c*) exceptional clarity for viewing cell structure.

16.1.3 Polystyrene culturing flask used in the test procedure.

16.2 The *positive* control shall be a material as required in other cell culture test methods, such as Practice F813, section 8.2.2, or specially compounded at a level of 1 %, mixed in RTV grade of commercial silicone with known toxic agent, such as listed in Registry of Toxic Substance (**2**).

NOTE 13—Although phenol is a common reference toxicant (Practice F813, Section 8.7.1), its aqueous solubility and hence leachability is too rapid. For suitable, less soluble alternatives, use any of its aryl substituted chloro or nitro derivatives listed in the Registry.

16.3 Prosthetic material used in the device shall be in the chemically converted form, appropriately polymerized or otherwise consolidated to the final fabricated stage of the prosthetic device.

16.4 All prosthetic test materials, including the positive and negative controls, shall be in the substantially similar or equivalent form of a thin film or sheeting in the range of 0.1 to 1.0 mm thickness, within a range of surface area of 2 to 4 cm^2 with disclosure of each of the test material specimens in terms of:

16.4.1 Shape description, that is, square, rectangle, and so forth.

16.4.2 Density or specific gravity.

16.4.3 Thickness (mm).

16.4.4 Weight (mg).

16.4.5 Surface area (m^2).

16.4.6 Volume (mL).

16.4.7 Surface/volume ratio.

16.5 In case of low-density test material specimens that tend to float on the culture medium and away from the developing, culturing monolayer, submerge the test specimen with an appropriate weighting, such as depicted in Fig. 1.

17. Assessment of Biocompatibility of Prosthetic Material

17.1 Conduct concurrently the biocompatibility assessment of the prosthetic material samples by using the three combinations (a, b, and c) of cell type, medium and serum options shown in Table 1.

17.2 Conduct the biocompatibility assessment of the prosthetic material sample in accordance with the format shown in Table 2.

17.3 Suspend the stock human primary cells, (see 14.7), dislodged either by scraping or by trypsin treatment, into fresh A3, or equivalent medium, with optimal additives of serum, insulin, and growth factor described in 17.1 and Table 1.

17.4 Place an aliquot portion adjusted to a level of 10^3 to 10^4 cells/mL, determined by counting using a hemocytometer, to each of the quadruplicate series of Table 2, with the appropriate A3 medium or its equivalent.

17.5 Replenish the culture medium at a maintenance feeding schedule of three times weekly or every 48 h for at least 7 days.

17.6 Examine microscopically at each maintenance feeding and compare the cell growth with regard to evidence of cell growth viability and, where indicated, cell abnormality compared to the control series (*A*) of Table 2.

NOTE 14—These abnormalities shall include visual evidence of (*a*) malformation or degeneration in structure, (*b*) cell lysis, or (*c*) reduced cell population or actual cell count.

TABLE 2 Biocompatibility Format[A] for Primary Human Cells (for 12.4.7) for 7 or 30 Days (optional)[B]

Test Material in Culturing Series	Number of Culture Flasks	
	Morphological Assessment	Optional[C] Biochemical Assessment
(*A*) None—Procedural Control[D]	4	4
(*B*) Prosthetic Material Test Specimen	4	4
(*C*) Positive Control (cytotoxic reference)	4	4
(*D*) Negative Control (nontoxic reference)	4	4

[A] The cell stocks in this test matrix shall be drawn from a pool of stock cultures maintained in the same A3 or equivalent medium as supplemented and selected for this format.

[B] The optional 30-day test period, or longer, shall be acceptable with cells or from media modifications where diverse human cell growth adaptation may require extended periods.

[C] For use as collateral, adjunctive assessment for cell viability or cell transformations, or both.

[D] When using sterile polystyrene plastic containers of established non-cytoxicity, series (*A*) may be used as the negative control in lieu of series (*D*).

17.7 Prepare photomicrographs at 100 to 200 magnification of each Table 2 series at culture days of 1, 3, 5, and 7 days for qualitative rating of the cells, applying an appropriate system rating of toxicity.

NOTE 15—The appropriate system of rating shall be any of the following conventional, precedental methods of grading or scoring described by Johnson and Northrop (**8**) and Horres et al (**9**). Both references apply a grade or rating of 0 for nontoxicity to 1 + to 4 + for degree of cell degeneration or inhibiton.

17.8 Where required for continued monolayer confluency beyond the 7-day maintenance period, continue the maintenance of the culture series with cytotoxic grading and microphotographs at approximately weekly intervals to 30 days or until terminated due to total of severe (more than 75 %) cell demise.

17.9 At the end of the 7-day or extended 30-day test period, the cells grown on the coverslip depicted in Fig. 1 shall be, according to the conventional matter, fixed with formalin, dehydrated, and stained with hematoxylin and eosin.

17.10 An optional, corollary biochemical and biophysical assessment, as indicated in Table 2, shall be applied for evidence of cell transformations during the course of the 7 to 30 days maintenance, and as a check on potential or possible cytogenetic changes of stock primary and stock reference cells.

NOTE 16—Such biochemical assessments shall include key enzyme activities, for instance, lactose dehydrogenase and chromosomal aberrations such as described by Lontz et al (**10**).

17.11 The cultured cells for optional biochemical assessment and or cell count shall be rinsed at least twice with 4 mL of fresh A3 medium, or its selected equivalent, containing no additives.

17.12 The rinsed cells, on the completion or termination of a test phase, shall be collected from the culture flask in 1 mL of A3 medium, or its selected equivalent, for the selected biochemical assessment with the further option of freezing for storage at −70°F until used for the biochemical assessment or cell counts, or both.

18. Validity and Standardization

18.1 The rating of biocompatibility in accordance with 17.7 shall be deemed valid only when the morphological responses are evident with the negative and positive controls.

18.2 Wherever cell counting is applied during the 7-day or the extended 30-day culturing periods, conventional statistical assessment shall be by appropriate P^* (null hypothesis) values for paired comparisons with nontoxic and toxic reference controls.

18.3 Cytotoxicity shall be based on any one of the following criteria:

18.3.1 Visible cell lysis or cell demise, or both, in the vicinity of the test material.

18.3.2 Marked reduction in cell count by direct counting or equivalent means, such as DNA analysis.

18.3.3 Marked changes in enzyme levels when compared to controls, as determined by analyses of selected target enzymes.

18.4 Nontoxicity shall be so stated based on non-evident toxic assessments in 17.6 or 17.7, or both.

19. Report

19.1 Report the following information:

19.1.1 Test cells utilized.

19.1.1.1 Designate the established cell line utilized (Table 1 (*a*) and (*b*)).

19.1.1.2 Designate the human cell utilized; anatomical classification, viz, facial skin, lip, gingiva, palate, nasal pharyngeal, and so forth.

19.1.1.3 Prior history from primary culturing, media adaptation, passages, and so forth.

19.1.1.4 Results in accordance with Table 2.

19.1.2 Media.

19.1.3 Growth Factor(s), that is AGF or substitutes, or both.

19.1.3.1 General preparation method, whether human plasma or from serum, and the concentration or assay employed.

19.1.3.2 Commercially available source of media, cell growth factors, and so forth, with designated concentration used in test.

19.1.4 Prosthetic device test sample.

19.1.4.1 General chemical designation of principal polymer or elastomer component according to nomenclature established by the American Chemical Society Chemical Abstracts.

19.1.4.2 Commercial designation of principal component, if provided as article of commerce, by classification, type or grade, with lot or batch identity.

19.1.4.3 Molecular characterization of principal component, in terms of molecular weight, infrared (IR) group frequency spectra, and differential thermal analyses (DTA) or differential scanning calorimetry (DSC).

19.1.4.4 Component additives, such as catalyst, stabilizers, fillers, and pigments or colorants with conventional chemical designation.

19.1.5 General method of processing or conversion of ingredient materials into fabricated prosthetic device.

19.1.6 Principal end-use tensile properties and tear resistance on: (*a*) fabricated unexposed test specimen, and (*b*) test specimens appropriately exposed to selected metabolite replicating reagents, namely, at least (*1*) glyceride selected from an edible vegetable oil, and (*2*) lactic acid.

19.1.7 Test specimen quantification and mensuration.

19.1.7.1 Density of specimen (g/cm^3 at stated temperature);

19.1.7.2 Weight, g;

19.1.7.3 Thickness (cm), width (cm), length (cm);

19.1.7.4 Volume (cm^3) from 19.1.7.3;

19.1.7.5 Volume (cm^3) of test medium per maintenance;

19.1.7.6 Total volumes (cm^3) of 19.1.7.5 to rated cytotoxicity;

19.1.7.7 Calculated ratios (quotients) for:

(1) Surface area/sample weight from 19.1.7.3/19.1.7.2;

(2) Sample weight/volume of test medium from 19.1.7.2/19.1.7.6.

19.1.7.8 Provide summarizing statement with a graded rating, as per 17.6, from 0 for nontoxicity to + 4 for extreme toxicity;

19.1.7.9 Permanent mounted slide stained and containing the test material, or photographs of the slide mentioned above and any other photographs taken during the test duration which may serve as a permanent record of the results;

19.1.7.10 All quantitative data derived from cell counts, DNA analyses, enzyme analyses, and so forth.

ANNEXES

(Mandatory Information)

A1. STANDARD DEFINITIONS OF TERMS RELATING TO TISSUE CULTURE TESTING FOR CYTOTOXICITY

A1.1 *Introduction*—The following is a list of specific terms taken with modifications from Appendix 4 of Ref. **(11)**, based on recommendations of the Committee on Nomenclature of the Tissue Culture Association, **(1),** and supplemented by definitions taken from the text of the *Medical Dictionary* **(12).**

A1.2 *List of Terms*:

cell—a general non-specific term describing a small microscopic mass protoplasm bounded externally by a semipermeable membrane; should be used with descriptive and specific modifier as listed sequentially herein.

cell alteration—a change in the character of a cell line (seq.) usually associated with the emergence of an established cell line with alteration of morphology, contact inhibition, karyotype, viral susceptibility, and ability to grow in suspension.

cell culture—the growing of cells *in vitro* in which the cells are no longer organized into tissues.

cell line—a "cell line" arises from a primary culture at the time of the first subculture and implies that cultures from it consist of numerous lineages of the cells with further characterizations so as to form and chromosome constitutions.

cell senescence—a marked change in any significant growth and/or morphology indicative of a pronounced lack of cell vitality.

cell strain—a type of cell derived from either a primary culture or from a cell line (*vide supra*) having specific characteristics, notably biochemical or biophysical markers, or both, that persist during subsequent culturing. In describing a cell strain, its specific features should be defined, as for instance, a cell strain with a certain marker chromosome, or a cell strain which has acquired resistance to a certain virus, or a cell strain having a specific antigen, and so on.

chemically-defined medium (media)—a medium (media) composed entirely of known chemical components in which cells may be cultured without any undefined additives.

cytotoxicity—an effect or range of effects, microscopic and/or visual, linked to the presence of test materials. These range from impairment in growth to complete cell lysis or dissolution when test cultures are compared to the reference blank.

cytotoxic effect—an effect on cell culture that may include decreased plating efficiency, cell lysis, inhibition of macromolecular synthesis and cell growth and detachment of cells from the substrate.

cytotoxic substance—any substance that inhibits or prevents the function of cells, or causes destruction of cells or both.

dialysis—the process of separating crystalloids and colloids in solution by the difference in their rates of diffusion through a semipermeable membrane.

established cell line—a cell line having and demonstrating the potential to be subcultured indefinitely *in vitro.*

explant—an excised fragment of a tissue or organ used to initiate a culture.

granulation—the visual microscopic formation of minute discrete masses within a cell.

growth—an increase in the mass of living substance and/or the number of cells.

lysis—the breaking up or disintegration of cells.

medium renewal or maintenance—all or part of the used medium is removed and replaced with fresh medium.

medium—a mixture of substances which are essential, beneficial, or harmless to cells in culture.

mitotic figure—microscopic appearance of the achromatic spindle with chromosomes attached; it is an indication of cell division.

monolayer—a single layer of cells growing on a surface.

passage—this term is synonymous with subculture, and can denote the passage of cells from one culture vessel to another.

PPLO—initials standing for pleuropneumonia-like organisms, which are a group of filterable microorganisms, belonging to the genus *Mycoplasma.* This term may be used loosely to include *L*-forms of a variety of bacterial species.

primary culture—a culture started from cells, tissues, or organs taken directly from an organism; a primary culture may be regarded as such until it is subcultured for the first time. It then becomes a cell line.

rounding—the auto-conversion of a cell culture from a monolayer into loosely attached or floating spherically shaped cells.

serum—the fluid portion of the blood obtained after dialysis and coagulation.

subculture—the transfer of cells from one culture vessel to another.

tissue culture—the maintenance of tissue fragments *in vitro*; culture conditions may or may not be designed to preserve primordal tissue morphology.

A2. PREPARATION OF HUMAN SERUM FOR USE IN CULTURE MEDIUM FOR THE SEPARATION AND PREPARATION OF CELL GROWTH FACTOR

A2.1 The best serum is obtained from freshly clotted human blood containing no additives. However, this type of serum is difficult to obtain and satisfactory results may be obtained from plasma furnished by a Blood Bank, even though this plasma contain the anticoagulant acid citrate dextrose (ACD).

A2.2 Whole blood shall be utilized no later than 48 h after its expired shelf life. The plasma is obtained by allowing whole blood to settle at 2 to 4°C (35 to 39°F) in the original blood pack for at least 48 h.

A2.3 The plasma is decanted and centrifuged at 17 210 g (Sorval RC-2) for 20 min at 20°C (68°F) to remove any residual blood cells.

A2.4 The plasma is dialyzed against a balanced salt solution consisting of 84 g NaCl, 4.8 g KCl, 2.4 g $MgSO_4 \cdot 7H_2O$, 1.68 g $CaCl_2 \cdot 2H_2O$, 1.37 g $Na_2HPO_4 \cdot 7H_2O$, and 0.72 g KH_2PO_4 in 12 L of deionized or distilled water in the cold. The dialysis is carried out in Spectrapor membrane tubing of 3500 molecular weight exclusion. The total dialysis time is 48 h. The balanced salt solution is changed after the first 24 h period.

A2.5 At the termination of the dialysis, the plasma is clotted within the tubing as the result of removing anticoagulant and the introduction of calcium into A2.4. The resulting serum is squeezed from the tubing and stored cold (2 to 4°C) for either direct use in culture or fractionation on a glass bead column.

NOTE A2.1—Other human plasma or serum-derived cell growth factors shall be appropriate for use as a substitute, particularly as available commercially with appropriate specifications and analysis. As an example, alternate growth factors derived from blood components, such as the heat-stable human platelet-derived growth factor **(13),** may be considered.

A3. PRODUCTION OF ALPHA GROWTH FACTOR (AGF)

A3.1 *Preparation of the Glass Microbead:*

A3.1.1 Place the dry beads in a 2-L glass beaker to a volume of 500 mL.

A3.1.2 Add 50 g of Haemosol enzyme active (EA) grade followed by 1 L of hot (65 to 70°C) distilled or deionized water while stirring for 1 min.

A3.1.3 Allow the slurry to stand at room temperature overnight or approximately 16 h.

A3.1.4 Remove the water the next day by decantation or suction from the setted microbeads.

A3.1.5 Wash the microbeads four times with 1 L of distilled or deionized water to remove the surfactant Haemosol.

A3.1.6 Add 250 mL of concentrated hydrochloric acid and adjust the total volume (including the microbeads) to 1 L with added distilled or deionized water.

A3.1.7 Stir the resulting suspension of microbeads for 2 min and let stand overnight, appropriately covered to contain the acid (HCl) vapor.

A3.1.8 Remove the acid liquid with at least 5 rinses of 1 L of distilled or deionized water.

NOTE A3.1—The washed microbeads are stored at this stage for ensuing or future use for an extended period of time.

A3.2 *Preparation of Microbead Column:*

A3.2.1 Glass Column Description (See Fig. A3.1):

A3.2.1.1 *Length*—40 cm.

A3.2.1.2 *Diameter*—5 cm.

A3.2.1.3 *Effluent Drain.*

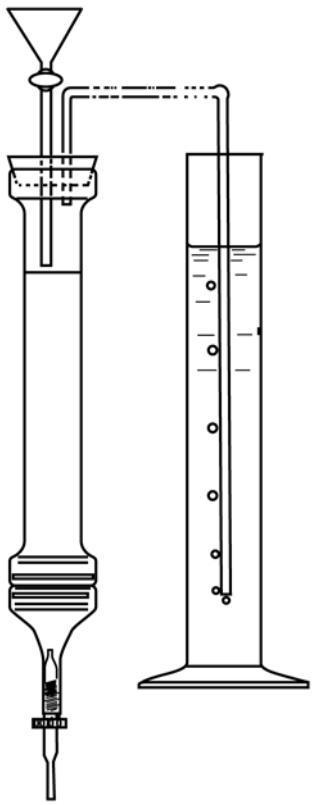


FIG. A3.1 Schematic Diagram for Chromatographic Separation of Holmes Alpha-1-Protein Growth Factor (AGF) from Dialyzed Human Serum

A3.2.1.4 *Pressure Head.*

A3.2.2 Wash the column with 50 mL Haemosol and rinse with 500 mL of distilled or deionized water, with the effluent flow at 2 to 5 mL/minute.

A3.2.3 Prior to packing into the column, wash the prepared, stored microbeads with 2 L of 0.6 *M* sodium bicarbonate ($NaHCO_3$) solution.

NOTE A3.2—This pretreatment neutralizes the chance acidity in the acid-washed stock of microbeads, thus preventing formation of entrapped bubbles in the column.

A3.2.4 Pour the microbead slurry conditioned with 0.6 *M* sodium bicarbonate ($NaHCO_3$) into the column to the depth of 25 cm.

A3.2.5 Place a 5 cm disk of high grade chromatography paper (Whatman 3 mm thickness, basic weight 185 g/m^2), on top of the microbead as settled.

NOTE A3.3—The paper must be floated onto the column so that no air bubbles are trapped between it and the microbead layer.

A3.2.6 Drain off the superfluent above the settled microbeads bicarbonate solution to the boundary of the filter paper–microbead interface, shutting off the flow by the bottom Teflon adaptor.

A3.2.7 Place a preconditioning solution of 200 mL of 0.6 *M* sodium bicarbonate ($NaHCO_3$), pH 8.0, on top of the column and allow it to run through by gravity at the rate of 30 to 40 drops/min.

NOTE A3.4—Make sure that the column is never allowed to dry. To assist the flow rate, pressure the column using compressed air with no more than 18 in. of water head (Fig. A3.1, C).

A3.2.8 When the superfluent bicarbonate layer reaches the paper–head interface, stop the flow by closing off the Teflon adaptor drain; the column is now ready for fractionation of the dialyzed human serum (Annex A2).

A3.3 *Preparation of Solutions of Column Elution*—Prepare the series of stock solutions listed in Table A3.1 and Table A3.2, stored in stoppered bottles and useable for a maximum of 12 weeks.

A3.4 *Column Separation Procedure:*

A3.4.1 Prepare beforehand the series listed in Table A3.3 of progressive additions added to the microbead column.

A3.4.2 Add gently 20 mL of dialyzed human serum (A2.4) in two 10-mL pipette portions following the preliminary column conditioning with 0.8 *M* sodium bicarbonate (A3.2.3).

A3.4.3 Once the 20 mL of serum has settled in the column, add 200 of 0.6 *M* sodium bicarbonate ($NaHCO_3$), pH 8.0, solution with an initial 10-mL pipette portion directed to the internal wall of the column to rinse off the absorbed serum.

A3.4.4 Once the initial 10 mL of bicarbonate has settled down into the column, the remaining 190 mL is carefully poured into the column, making sure not to disturb the lay of the filter paper and the underlying microbeads.

A3.4.5 Adjust the flow rate to 30 to 40 drops per minute by means of the Teflon stopcock in a series of 10 to 12 mL test tube fraction collection system.

NOTE A3.5—Any conventional type fraction collector with a variable wavelength detector to locate peak collection for the alpha growth factor at 280 mm may be used.

A3.4.6 With the depletion of the sodium bicarbonate elution, proceed next with the 100 mL of $NaHCO_3$-Na_2CO_3 solution with continued fraction collection and absorbance monitoring at 280 mm.

A3.4.7 Immediately following the depletion of the $NaHCO_3$-Na_2CO_3 solution, proceed next with 150 mL distilled water elution.

A3.4.8 Immediately following the depletion of the distilled water elution, proceed next with the 200 mL $KHCO_3$-K_2CO_3 elution.

A3.4.9 Immediately following the depletion of the $KHCO_3$-K_2CO_3 elution, proceed next with the last, distilled water elution.

NOTE A3.6—It is necessary to elute a total of 670 mL from the column in order to ensure that all fractions of the active growth factor have been eluted in the $KHCO_3$-K_2CO_3 buffer elutant.

A3.5 *Identification and Collection of AGF Fractions*:

A3.5.1 Using the 280 mm detection of the AGF fraction from A3.4.8 and following the potassium ion (pH 9.6) buffer, collect and consolidate the 280 nm absorptive fractions, usually in the range of 6 to 10 test tubes.

A3.5.2 Place the consolidated fractions in a dialysis membrane (Spectra/Por 2. Membrane, 12 000 to 14 000 MWCO).

A3.5.3 Suspend the dialysis membrane contents in 8 to 10 L of distilled or deionized water at 4°C with constant stirring.

TABLE A3.1 Stock Solutions for Elution Buffers

Solution	Stock Quantity	pH
0.6 *M* $NaHCO_3$	100.0 g/2 L	8.0
0.2 *M* Na_2CO_3	42.4 g/2 L	Unadjusted
0.4 K_2CO_3	130.6 g/2 L	Unadjusted
1.2 $KHCO_3$	340.2 g/2 L	Unadjusted
0.2 *M* Na_2CO_3	42.4 g/2 L	Unadjusted
0.4 K_2CO_3	130.6 g/2 L	Unadjusted
1.2 $KHCO_3$	340.2 g/2 L	Unadjusted

TABLE A3.2 Elution Buffer Preparation

Solution	Volume	Final pH	Volume per/rim
0.6 *M* $NaHCO_3$	200 mL	...	...
0.2 *M* Na_2CO_3	390 mL	9.5 ± 0.02	100 mL
0.4 *M* K_2CO_3	429 mL	...	...
1.2 *M* $KHCO_3$	572 mL	9.6 ± 0.1	200 mL

TABLE A3.3 Additions to Column

Solution	Procedure Section	Volume
Human Serum	A3.4.2	20 mL
$NaHCO_3$	A3.4.3	200 mL
$NaHCO_3$ – Na_2CO_3	A3.4.3	100 mL
Distilled Water	A3.4.7	150 mL
$KHCO_3$ – K_2CO_3	A3.4.8	200 mL
Distilled Water	A3.4.9	250 mL

A3.5.4 After a minimum of 6 h, change the water and continue dialysis for 16 h.

A3.5.5 Sterilize the dialyzed AGF factor by filtering through a 0.2 mm filter.

A3.5.6 Store the AGF factor in a sterile container.

APPENDIX

(Nonmandatory Information)

X1. RATIONALE FOR ASSESSMENT OF CELL GROWTH COMPATIBILITY OF PROSTHETIC MATERIALS AND DEVICES

X1.1 This practice utilizes normal, non-transformed human excised donor (HED) tissue cells in a homologous human cell – human serum culturing system as a means for assessing biocompatibility of prosthetic materials directly relevant to specific orofacial reconstructive prosthetic devices. This HED tissue cell culturing system, with alternatives and options on the use of separated human serum growth factors and corollary use of established cell lines, takes cognizance of the extensive variability of human source tissues and human serum and its separated components.

X1.2 This practice utilizes human serum processed to remove native and adventitious molecular entities by specified dialysis and further separations of the processed serum by buffered ionic elutions through glass microbead chromatography for specified cell growth factors. Thus, the human serum processing and separation provide a ready means for monitoring, with an established cell line for quality and uniformity of the serum preparations, serving as a reference standard for the ensuing cultures with specific HED orofacial tissue cell culturing for biocompatibility. The corollary use of established cell line reference, with defined growth factors, provides a means of comparison of relative sensitivity of the HED tissue cell culture test system.

X1.3 Accordingly, this practice using appropriately processed homologous human cell-human serum provides significant relevance on which to assess the biocompatibility and safety of the prosthetic material for the specific orofacial device.

X1.4 *Precedental Use of Adult Human Cell Culture Methods*—The procedure relates to the precedental usage disclosed in the published literature, listed in Refs. (**14**) thru (**15**) using adult human tissues, and heeds the suggestion of employing actual cell types at the anatomical sites at which the specific biomaterial is to be used (**16**). The published literature provides numerous studies of *in-vitro* cell culture methods and procedures of normal, non-transformed human cells, exclusive of cells derived from neoplastic tumor or cancer cells, derived from various anatomical regions in a variety of culture system media supplemented with heterologous (bovine) and homologous (human) serum derivations, as well as culturing in chemically defined media with specific cell growth factors. This method provides for the procedural details that are in consonance with this precedental trend toward assessing biocompatibility in relation to anatomical site prosthetic application.

X1.5 Table X1.1 provides a summary of the continued development and modifications of cell culture methods, some salient features of which include: the employment of adult cells that are attributed with more clinical cogency than embryo cells; the involvement of not just the stabilized confluent stage of the test, but rather at the cell division stage with low cell density that must be taken into account as being more sensitive

TABLE X1.1 Development and Modifications of Human Cell Culture Methods

Designation Authorship	Species Source	Cell Type	Prime Nutrient	Serum Type	Cell Growth Test Stage	Test Days	Toxicity Criteria	Clinical Implication
A. Heterologous Cell-Serum Culturing Methods								
ASTM F813 – 83 (Autian) (1968)	Mouse	Connective tissue	MEM	Bovine	Confluency	1	Morphologic inhibition, lysis, etc.	General safety screening
Wilsnack (1976)	Human fetal	Lung	MEM	Bovine	Confluency	1	Morphologic inhibition, lysis, etc.	General safety screening
		Foreskin	MEM	Bovine	Confluency			
Hensten-Pettersen (1977)	Human adult	Gingival cell lines	MEM	Bovine	Proliferation	3	Morphologic, inhibition, 51-Cr rel.	Dental resins safety to human tissues
VA (Lontz, et al) (1977)	Human adult	Orofacial	MEM	Bovine	Proliferation	30	Morphologic, inhibition, lysis, etc.	Maxillofacial and oral (at site safety)
B. Homologous Human Cell-Human Serum Culturing Methods								
Davison, et al (1980)	Human newborn	Endothelial, foreskin	MEM	Human	Proliferation	10	Morphologic, growth rate, patterns,	(Potential)
Davison, et al (1983)	Human adult	Endothelial, (melo-plasty, mammo-plasty)	MEM	Human + (growth factors)	Proliferation	16	Morphologic, growth rate, age variable.	(Proposed assay)
VA (Lontz, et al (1978)	Human adult	Orofacial	MEM and A-3 med.	Human + (growth factors)	Proliferation	30	Morphologic, inhibition, lysis, etc.	Maxillofacial and oral (at site safety)

than that at matured confluency; and the extension of the short-term, one-day test exposure to long-term involvement of adaptation to media, log growth, and possible senescence or toxicity, or both, **(17)** and **(18).**

REFERENCES

(1) Federeoff, S., *Experimental Cell Research*, Tissue Culture Association, 46, 1967, p. 642.

(2) *Registry of Toxic Substances*, National Institute for Occupational Safety and Health, Vol II, 1977.

(3) Holmes, R., *Journal of Biophysical and Biochemical Cytology,* Vol. 6, 1959, pp. 535–536.

(4) Holmes, R., *Journal of Cell Biology*, Vol 32, No. 2, 1967, pp. 297 –308.

(5) Paul, John, *Cell and Tissue Culture*, 5th Ed., Chapter IX, Churchill Livingstone, New York, 1975.

(6) Lontz, J. F., et al, *Determining Cell Dynamic Constants for Assessing and Comparing Efficacy of Nutrient Media Modification,* Abstract, 35th Annual Meeting, Tissue Culture Association, Texas, 1984.

(7) *USP Negative Control Plastic Reference Standard*, U.S. Pharmacopeia, Vol XXIII, Mack Publishing, Easton, PA, 1995, p. 1652–1653. Use latest supplement to ensure current cumulative revisions are used.

(8) Johnson and Northrop, *Tissue-Culture Biocompatibility* Testing Program, pp. 25–34.

(9) Horres, et al, "Evaluation of Materials Used for the Fabricating of Medical Devices: Cytotoxicity Testing," *Cell Culture Test Methods, ASTM STP 810,* ASTM, 1983, pp. 35–50.

(10) Lontz, et al, "Use of Human Excised Donor Tissue Culture Systems to Evaluate Safety of Polydimethyl-Siloxane for Orofacial Prosthesis," *Biomaterials,* John Wiley and Sons, London, 1980, pp. 805–810.

(11) Paul, John, *Cell and Tissue Culture*, 5th Ed., Churchill Livingstone, New York, 1975, Appendix 4, pp. 466–470.

(12) *Medical Dictionary,* Stedman, Williams, and Wilkens Co., Baltimore, 1961.

(13) Nardone, R. M., "Toxicity Testing in Vitro," *Growth, Nutrition and Metabolism of Cells in Culture,* Academic Press, 1977, Chapter 11, pp. 471–495.

(14) Wilsnack, R. E., Meyer, F. S., and Smith, J. G., "Human Cell Culture Toxicity Testing of Medical Devices and Correlation to Animal Tests," *Biomaterials, Medical Devices, and Artificial Organs,* Vol. 1, 1973, pp. 543–562.

(15) Antoniades, H. N., "Platelet-Derived Growth Factor and Malignant Transformation," *Biochemical Pharmacology,* Vol. 33, No. 18, 1984, pp. 2823–2828.

(16) Oser, Z., Abodeely, R. A., and McGunnigle, R. G., "Evaluation of Elastomers for Biomedical Applications Utilizing *in vivo* and *in vitro* Model Systems—State of the Art and Future Trends," *Intern. J. Polymeric Mater.,* Gordon Breach Science Publishers, Ltd. 1977, Vol. 5, pp. 177–187.

(17) Lontz, J. F., Nadijcka, M. D., Gugel, E. A., Mohamed, N., and Hanson, V. A.," Determining Cell Dynamic Constants for Assessing Efficacy of Nutrient Media Modifications," Abstract No. 169, *In Vitro,* Vol. 20, No. 3, Part II, March 1984, pp. 409–415. Based on long term, 11 days culturing.

(18) Trewyn, R. W., and Gatz, H. B., "Altered Growth Properties of Normal Human Cells Induced by Phorbol 12, 13 Didecanoate," *In Vitro,* Vol. 20, No. 5, May 1984, pp. 409–415. Depicts long term cell growth curver up to 8 days from low cell count to confluency.

(19) Wilsnack, R. E., "Quantitative Cell Culture Biocompatibility Testing of Medical Devices and Correlation to Animal Tests," *Biomaterials, Medical Devices, and Artificial Organs,* Vol. 4, 1976, pp. 235–261, quoted from the Abstract, "Positivity (toxicity) in animal tests (USP XIX up to 7 days) did not correlate quantitatively to cytotoxic titers in cell culture (1 day). Discrepancies between cell culture tests and animal tests, specifically rubber compounds, were attributable, in some instances, to differentials in elution efficiency between saline, cottonseed, and complete MEM culture medium."

(20) Rheinwald, J. G., and Green, H., "Serial Cultivation of Strains of Human Epidermal Keratinocytes: The Formation of Keratinizing Colonies," *Cell*, Vol. 6, 1975, pp. 331–344.

(21) Hensten-Pettersen, Arne, and Helgelan, Kristen, "Evaluation of Biologic Effects of Dental Materials Using Four Different Cell Culture Techniques" *Scand. J. Dent. Res.,* 1977: 85, pp. 291–296, quoted from the Abstract, "It is concluded that the effects obtained by the (four) cell culture techniques did not mimic the reactions obtained when the (dental) materials are tested under conditions which reflect their clinical use."

(22) Lontz, J. F., and Schweiger, J. W., "Maxillofacial Restorative Biomaterials and Techniques," *Bull. Prosth. Res.,* Fall 1977, pp. 183–184.

(23) Lechner, J. F., Shankar-Narayyan, K., Ohnuki, T., Babcock, M. S., Jones, L. W., and Kaighn, M. E., "Replicative Epithelial Cell Cultures from Normal Human Prostate Gland," *Journal National Cancer Institute,* Vol. 60, 1978, pp. 797–802.

(24) Autrup, H., Barrett, L. A., Jackson, F. E., Jesudason, M. L., Stoner, G., Phelps, P., Trump, B. F., and Harris, C. C. "Explant Culture of Human Colon," *Gastroenterology,* Vol. 74, 1978, pp. 1248–1257.

(25) Trump, B. F., and Harris, C. C., "Human Tissues in Biomedical Research," *Human Pathology,* Vol. 10, No. 3, 1979, pp. 245–248.

(26) Davison, P. M., Bensch, K., and Karasek, M. A., "Isolation and Growth of Endothelial Cells from Microvessels of the Newborn Human Foreskin in Cell Culture," *J. Invest. Dermat.,* 75, 1980, pp. 316–321.

(27) Lontz, J. F., and Schweiger, J. W., "Maxillofacial Restorative Biomaterials and Techniques," *Bull. Prosth. Res.,* Fall 1978, pp. 230–40; also Spring 1981, pp. 165–71.

(28) Davison, P. M., Beensch, K., and Karasek, M. A., "Isolation and Long-term Serial Cultivation of Endothelial Cells from the Microvessels of the Adult Human Dermis," *In Vitro,* Vol. 19, 1983, pp. 937–435.

(29) Spiegel, J., Carey, T. E., Shimura, S., and Krause, C. J., "In Vitro Sensitivity and Resistance of Human Squamous Carcinoma Cells to cis-Platinum and Methotrexate," *Otolaryngology—Head and Neck Surgery,* Vol. 92, No. 5, October 1984, pp. 524–531. Depicts long term cell growth up to 12 days from moderate cell count to confluency and, in toxic modifications, to severe toxic loss of cell count.

(30) Merchant, D. J., Kahn, R. H., and Murphy, W. H., *Handbook of Cell and Organ Culture,* 2nd Ed., Burgess Publishing Co., Minneapolis, MN, 1964.

(31) *Manual of Cell, Tissue and Organ Culture Procedures*, Tissue Culture Association, Vol 7 and antecedents, ISSN 0271–8057, see Procedure No. 27072.

(32) The American Type Culture Collection, ATCC, *Catalog of Strains II,* 12031 Parklawn Drive, Rockville, MD, 10852.

ASTM International takes no position respecting the validity of any patent rights asserted in connection with any item mentioned in this standard. Users of this standard are expressly advised that determination of the validity of any such patent rights, and the risk of infringement of such rights, are entirely their own responsibility.

This standard is subject to revision at any time by the responsible technical committee and must be reviewed every five years and if not revised, either reapproved or withdrawn. Your comments are invited either for revision of this standard or for additional standards and should be addressed to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee, which you may attend. If you feel that your comments have not received a fair hearing you should make your views known to the ASTM Committee on Standards, at the address shown below.

This standard is copyrighted by ASTM International, 100 Barr Harbor Drive, PO Box C700, West Conshohocken, PA 19428-2959, United States. Individual reprints (single or multiple copies) of this standard may be obtained by contacting ASTM at the above address or at 610-832-9585 (phone), 610-832-9555 (fax), or service@astm.org (e-mail); or through the ASTM website (www.astm.org). Permission rights to photocopy the standard may also be secured from the Copyright Clearance Center, 222 Rosewood Drive, Danvers, MA 01923, Tel: (978) 646-2600; http://www.copyright.com/