Designation: E 1532 – ~~93~~00

Standard Practice for ~~Indirect Detection~~ Detection of Mycoplasma Contamination of Cell Cultures by ~~DNA Binding with~~ Use of the Bisbenzamide DNA-Binding Fluorochrome ~~Stain~~[1]

This standard is issued under the fixed designation E 1532; 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 covers ~~procedures used for~~ the ~~detection~~ use of cell cultures and DNA-binding flurorochrome techniques to detect mycoplasma contamination ~~by DNA staining.~~ of cell cultures.

1.2 This practice does not cover axenic cultivation ~~methods for the detection of mycoplasma~~ or ~~other methods such as biochemical detection, hybridization methodology, histo-chemical stains, or immunochemical methods.~~

~~1.3 This practice does not cover methods for the~~ identification of ~~mycoplasma organisms.~~

~~1.4 The values stated in SI units are to be regarded as the standard. The values given in parentheses are for information only.~~

~~1.5~~ mycoplasmas.

1.3 *This standard does not purport to address all of the safety ~~problems~~, 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:*

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[1] This practice is under the jurisdiction of ASTM Committee E-48 on Biotechnology and is the direct responsibility of Subcommittee E48.02 on Characterization and Identification of Biological Systems.

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E 1531 Practice for ~~Direct~~ the Detection of Mycoplasma Contamination of Cell Cultures by ~~Broth Enrichment and Agar~~ Growth on Agarose Medium[2]

E 1533 Practice for Indirect Detection of Mycoplasma in Cell Culture by 4′-6-Diamindino-2-2 Phenylindole (DAPI) Staining[2]

E 1536 Practice for ~~Large Volume Testing~~ the Detection of ~~Serum for~~ Mycoplasma Contamination of Bovine Serum by the Large Volume Method[2]

3. Terminology

3.1 *Definitions:*

3.1.1 ~~*direct detection of mycoplasma*—detection of mycoplasma by~~axenic cultivation ~~i~~, *n*—cultu~~i~~ivation free from othe~~d~~r living organisms.

3.1.2 ~~*DNA fluorochrome stain*—staining~~direct mycoplasma detection, *n*—demonstration of ~~DNA specifically by the use of bisbenzamide fluorochrome stain[3] or other DNA fluorochromes of comparable quality and performance, such as DAPI (4′,6-diamidine-2-phenyl-indole-2HCl).~~ characteristic colonial growth on axenic agar medium.

3.1.3 ~~*indirect detection of mycoplasma*—detection of mycoplasma by DNA staining or any method other than cultivation.~~

~~3.1.4 *mycoplasma*—the smallest~~mycoplasma (Mollicute), *n*—smallest prokaryotes capable of ~~living freely, lacking a cell wall, having a circular double-stranded DNA relatively rich in adenine and thymine, and containing 16s and 23s ribosomal RNAs. They can be found as contaminants in cell cultures.~~ self replication.

4. Significance and Use

4.1 *Mycoplasma ~~c~~ hyorhinis*, cult~~amin~~var α strains **(1)**[3] do not grow on any of ~~cell cultures is a common problem that can affect the growth, metabolism, and function of cultured animal cells. The ability to detect~~ standard media used for mycoplasma

[2] *Annual Book of ASTM Standards*, Vol 11.05.

~~[3] Available from Calbiochem, P.O. Box 12087, San Diego, CA 92112-4180.~~

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

cultivation. These strains, which are found as contaminants in cell cultures provides an opportunity to ensure that cells cultures, are free of contamination, and to replace those that are not. For additional information, see Practices E 1531, E 1533, and E 1536. Strict adherence to well-established procedures is essential for reliable results. This practice was developed detected by Task Group E48.01.02 to assist in developing and maintaining an established regimen for mycoplasma detection by DNA binding with bisbenzamide fluorochrome stain **(1-6)**.[4] Cytoplasmic DNA staining is only an indication of mycoplasma contamination, as indirect methods.

4.2 A specialized medium has been described but it is not specific for mycoplasma DNA. Some hybridomas and EBV-transformed cell lines have presented false positive results.

4.2 This practice is intended for use yet in examining cultured mammalian cells for the presence of mycoplasma contamination. wide use **(2)**.

4.3 This practice is not intended for use should be used in the detection of mycoplasma contamination in serum, culture media, or systems other than cultures of mammalian cells.

4.4 All conjunction with Practice E 1531.

4.4 All cell cultures to be examined for mycoplasma should undergo a minimum of two passages in antibiotic-free tissue culture medium before testing.

5. Quality Control

5.1 Visually examine the bisbenzamide stain concentrate at the time of use for contamination. Sterilization by filtration may diminish the quality of fluorescence. Fresh stock should be prepared every two months.

5.2 Determine the pH of the mounting medium periodically to assure it remains at pH 5.5.

5.3 *Indicator Cells*:

5.3.1 Indicator cellsIndicator Cell Cultures

5.1 BHK-21, 3T6, and Vero are mycoplasma-free cells that are susceptible to infection by the common most widely used indicator cell culture contaminating mycoplasma species. They cultures. BHK 21 are used for positive and negative controls, maintained as well as test cultures.

5.3.2 Use continuous monolayer cultures, which are trypsinized to prepare, cell lines such suspensions as the African green monkey kidney needed **(4-6)**.

5.2 Fetal bovine is heat inactivated at 56°C for 30 minutes before it is used in cell line, Vero, American Type Culture Collection (ATCC CCL 81), 3T6 mouse fibroblast (ATCC CCL 96), or 3T3 mouse fibroblast (ATCC CCL 92) as indicator cells. These cells have culture medium.

5.3 Place previously sterilized 11 x 22-mm coverslips in a large cytoplasmic 10 x 35-mm plastic culture dishes.

5.4 Add 3.8 mL of cell suspension to nuclear area when attached.

5.3.3 Do not use cells each dish. The suspension should be dilute enough so that attach only loosely the resulting monolayer is subconfluent in 2 to 3 days. Growth medium is replaced and the coverslip cultures are ready for use.

5.5 Inoculate 0.1 mL of sample into each culture substratum dish.

5.6 For positive control, inoculate two dishes with *M. hyorhinis*, strain DBS 1050 (ATCC 29052). Additional control strains of *M. orale* or cells that produce extra nuclear fluorescence *M. pirum* are useful.

5.7 Two uninoculated dishes serve as indicators. negative controls.

6. Procedure Materials for Staining

6.1 *Preparation of Bisbenzamide Stain Concentrate*:

6.1.1 Add 5.0 mg bisbenzamide fluorochrome stainCarnoy's Fixative—Mix one part glacial acetic acid with three-parts methanol. This fixative may be made in advance and 10.0 mg thimersol to 100 mL Hanks' balanced salt solution without sodium bicarbonate or phenol red.

6.1.2 Mix thoroughly, using a magnetic stirrer for 45 min, stored at room temperature.

6.1.3 The stain is heat and light sensitive. Prepare and store the concentrate in an amber bottle wrapped in aluminum foil and store frozen at − 20°C or colder.

6.2 *Preparation of Mounting Medium McIlvane's Citrate-Phosphate Buffer, pH 5.5*:

6.2.1 *0.1 M Citric Acid Monohydrate (MW 210.14)*—Add 21 g to 1000 mL 0.1 of deionized water.

6.2.2 *0.2 M citric acid, 27.8 Dibasic Sodium Phosphate (MW 141.96)*—Add 34.08 g to 1200 mL of deionized water.

6.2.3 For working solution, combine 572 mL of 0.2 M dibasic sodium phosphate, and with 450 mL glycerol. Adjust the pH of the mixture to 5.5.

6.2.2 Store the medium 0.1 M citric acid.

6.2.4 Store buffer at 2 to 8°C.

6.3 *Preparation of Indicator Cell Cultures*:

6.3.1 Aseptically place a previously sterilized glass cover slip into each 60 × 15-mm culture dish or appropriately sized wells

6.3 Bisbenzamide (H-Stain) Stock Solution: dissolve 5.0 mg Hoechst 33258 (Calbiochem) in a multi-well plate. Prepare two 40 × 24-mm cover slips each for the negative and positive controls and four coverslips for each of the samples to be tested. Coverslips with a No. 1 thickness are recommended, and 22 × 22-mm coverslips can optionally be used.

6.3.2 Dispense 3 mL minimal essential medium (MEM) containing 10 % fetal bovine serum into each culture dish. Optionally, Dulbecco's modified MEM (DMEM) or Medium 199, both supplemented with fetal bovine serum, may be used. Make certain that each glass cover slip is submerged totally and not floating on top of the medium.

6.3.3 Prepare a single-cell suspension of indicator cells 100.0 ml deionized water. Portion in the above medium at a concentration of 1.0×10^5 cells/mL.

6.3.4 Inoculate 1 mL of the cell suspension into each culture dish or well.

6.3.5 Incubate the culture overnight in a 5 % CO_2-95 % air incubator at 35 to 37°C.

6.3.6 Microscopically examine the cultures to verify that the cells have attached to the glass cover slip. Number the culture dishes and the corresponding test samples and controls for identification. The culture medium may be replaced at this point, especially if cell death is exceptionally high.

6.3.7 Add 0.5 mL of sterile culture medium to each of two cultures as negative controls.

6.3.8 Add 0.5 mL of each test sample to each of two cultures. For suspension cells, the sample may be obtained directly from a frozen sample after thawing, or from a continuous culture. Approximately 3.0×10^6 cells should be used.

6.3.9 Add 0.5 mL of medium containing 100 cfu or higher *Mycoplasma hyorhinis* ATCC 29052 to each of two cultures, ml amounts and 0.5 mL *Mycoplasma arginini* 23838 to each of two cultures as positive controls.

6.3.10 Incubate all cultures in a CO_2 incubator for 3 to 6 days. store at –20°C.

6.4 *Preparation of StainingBisbenzamide (H-Stain) Working Solution*:

6.4.1 Add 1.0—Add 0.1 mL of the Bisbenzamide stock stain concentrate (see 6.1) solution to 100 mL Hanks' balanced salt solution without sodium bicarbonate of McIlvane's citrate-phosphate buffer. Protect from light and phenol red.

6.4.2 Prepare in an amber bottle wrapped with aluminum foil and mix thoroughly. Use within 24 h. use directly.

6.5 *Fixing the Cells*:

6.5.1 Remove the cultures from the incubator and remove the medium from each dish by aspiration after 3 to 7 days of incubation.

6.5.2 Do not allow the culture to dry between removal of the medium and addition of the fixative. The fixative should be added slowly at the periphery of the cover slip. Add 0.25Mountant—To 50 mL of a 1:3 mixture of freshly prepared glacial acetic acid:methanol to each culture for 5 min.

6.5.3 Aspirate or decant each culture and McIlvane's citrate-phosphate buffer, add 50.0 mL of the fixative for 10 min. Repeat this step one more time.

6.5.4 Aspirate or decant the fixative glycerol. Mix and allow the cultures to air dry.

6.6 *Staining of Cells*:

6.6.1 Add 5 mL of the staining solution (see 6.4) to each culture dish; cover and let stand at room temperature for 30 min.

6.6.2 Aspirate the stain and rinse each culture three times with distilled water.

6.6.3 After the third rinse, aspirate thoroughly and mount for observation.

6.7 *Mounting of Cover Slips*:

6.7.1 Assure that a labelled slide is ready for each specimen cover slip.

6.7.2 Place one drop of mounting medium (see 6.2) on a clean glass slide. Use a forceps to remove the glass cover slip containing the specimen from the culture dish, and place it cell side down on the mounting fluid.

6.7.3 To preserve the mounted slide, and facilitate the application of immersion oil for observation, seal the edges of the cover slip with lacquer or petroleum jelly.

6.8 *Observation and Recording of Results*:

6.8.1 Observe each specimen, including both the positive and negative controls, by fluorescence microscopy. Use 100× and then 500× magnification, or higher, if necessary. Examine using exciter filters emitting 400 to 450 mm from a mercury lamp (for example, Exciter B612/Barrier 50 (Zeiss[4])).

6.8.2 *Negative Controls*—Cell nuclei will appear as large oval fluorescing bodies.

6.8.3 *Positive Controls*—Cell nuclei will appear as large oval fluorescing bodies surrounded by small fluorescent particles demonstrating the DNA of the contaminating organisms.

6.8.4 Compare the test samples to the controls, and record the results as positive or negative for possible mycoplasma contamination.

6.9 *Frequency of Testing*:

6.9.1 Test all cell lines maintained dispense in the laboratory small dropper bottles. Store at least every three months.

6.9.2 Test all preserved cells at the time they are frozen.

6.9.3 Test cells after 4 2 to 6 weeks of cultivation in media containing a new lot of serum.

6.9.4 Test cells before the initiation of a new experiment.

6.10 *Complementary Testing*—Perform a direct test (see Practice E 1531) at the same time as the indirect test. 8°C.

7. Staining Procedure

7.1 Fix cultures by adding approximately 0.5 mL to 1 mL of Carnoy's fixative to the cell culture dishes containing medium.

7.2 Let stand two minutes then aspirate.

7.3 Reapply fixative, and let stand five minutes.

7.4 Aspirate and air-dry coverslips.

7.5 Apply sufficient volume of DNA-stain to immerse coverslip.

7.6 Let stand for ten minutes, then aspirate.

7.7 Wash with distilled water, then air dry.

7.8 Mount preparations by placing each coverslip (cell side up) onto a drop of mountant that has been placed on a microscope slide. Apply another drop of mountant to the surface of the specimen coverslip. Place a clean coverslip over the specimen coverslip so that is sandwiched between the top coverslip and the glass slide.

8. Examination

8.1 Observe with an oil-immersion 40X objective on Zeiss microscope equipped with a mercury vapor lamp, UG1 exciter filter, and no barrier filter or equivalent. Several suitable microscopes, which use halogen as well as mercury vapor, are available for fluorescent microscopy.

8.2 In the absence of mycoplasmas, the stained coverslips will show only the brightly fluorescent nuclei of the BHK 21 cells.

8.3 Infected coverslip cultures will display fluorescent mycoplasmas scattered over the cytoplasm of the indicator cells. The mycoplasmas are pleomorphic and will appear as single cells, clumps of cells, chains, and filaments.

9. Keywords

79.1 cell culture; DNA stain; indicator cells; mycoplasma

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