Designation: E 1531 – ~~93~~00

Standard Practice for ~~Direct Detection~~ Detection of Mycoplasma Contamination of Cell Cultures by ~~Broth Enrichment and Agar~~ Growth on Agarose Medium[1]

This standard is issued under the fixed designation E 1531; 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 the procedures used for detection of mycoplasma contamination by direct microbiological culture.

1.2 This practice does not cover indirect methods for detection of mycoplasma such as DNA staining, biochemical detection, or genetic probes.

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

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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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1.4 This practice will not detect cultivar α strains **(1)**[2] of *Mycoplasma hyorhinis*.

1.5 This practice is not intended for use in detection of mycoplasma contamination in sera, culture media, vaccines, or other systems.

1.6 *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:*

E 1532 Practice for ~~Indirect~~ Detection of Mycoplasma Contamination of Cell Cultures by the Use of the Bisbenzamide DNA Binding ~~with Bisbenzamide~~ Fluorochrome ~~Stain~~[24]

E 1533 Practice for Indirect Detection of Mycoplasma in Cell Culture by 4′-6-Diamidino-2-2 Phenylindole (DAPI) Staining[3]

E 1536 Practice for ~~Large Volume Testing~~ Detection of ~~Serum for~~ Mycoplasma Contamination of Bovine Serum by the Large Volume Method[3]

3. Terminology

3.1 *Definitions:*

3.1.1 *direct mycoplasma detection, n*—demonstration of ~~mycoplasma—detection of mycoplasma by cultivation in culture media.~~ characteristic colonial growth on axenic agar medium.

3.1.2 *indirect detection of mycoplasma, n*— detection of mycoplasma by DNA staining or any method other than cultivation.

3.1.3 *mycoplasma (Mollicute), n*—~~the~~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 contamination~~

4.1 The demonstration of ~~cell cultures~~ characteristic colonial growth on axenic solid medium is a ~~common problem that can~~

~~*Annual Book*~~

[2] The boldface numbers in parenthesis refer to the list of ~~ASTM Standards, Vol 11.05.~~ references at the end of this standard.

affect the growth, metabolism, sensitive and function of cultured animal cells **(Refs 1-3)**.[3] The ability specific method to detect mycoplasma infection of cell cultures provides an opportunity to ensure that cells are free of contamination. Strict adherence to well established procedures and it is necessary the standard detection method **(4, 5) (2). This practice was developed by Task Group E48.01.02**

4.2 When mycoplasmas contaminate cell cultures they usually grow to develop high titer (10^8 colony forming units/mL) and maintain an established regimen for when inoculated onto agar medium they produce abundant and easily detectable growth **(3)**.

4.3 *M. hyorhinis* cultivar α strains do not grow on conventional mycoplasma detection by direct microbiological media **(1)** but require an indicator cell culture according system to well documented sources.

4.2 This practice is intended for use in examining cultured cells for the detect their presence of mycoplasma contamination. The methodology (see Practice E 1532). Alternatively, a specialized axenic medium is intended suitable for use in the detection direct isolation of the presence of mycoplasma in cultivar α from infected cell cultures.

4.3 This practice is not intended for use in detection of cultures **(4)**.

4.4 Immunofluorescent procedures are used to identify mycoplasma contamination in serum, culture media, or other culture systems. isolates **(5)**.

5. DM-1 Solid Medium Preparation

5.1 Dissolve CMRL-1066 powder (CMRL-1066 powder Formula No. 78–5156EF[4], packaged for 10L), in 5000 mL of distilled water. This is one-half the volume of water specified on the package. Add 47.6 g HEPES[5], and 9.35 g NaCl.

5.2 Adjust the pH to 7.3 and filter sterilize (450 nm). Store this 2X CMRL in the refrigerator in 500 mL amounts.

5.3 Dissolve 10.0 g of Myosate[6] and 12 g of agarose[7] in 400 mL of distilled water. Autoclave at 121°C for 15 minutes. Cool the autoclaved solution to about 50°C, and combine with 500 mL of 2X CMRL and 100 mL of sterile horse serum[8] (both ingredients also warmed to 50°C).

5.4 Aseptically dispense medium in 5 mL amounts in petri dishes and allow to solidify. Store at 4°C and prevent drying. The DM-1 plates have a shelf-life of eight weeks.

6. Quality Control

5.1 Test the growth promoting properties of the mycoplasma broth

6.1 It is important that solid medium using *Mycoplasma pneumoniae* American Type Culture Collection, (ATCC) No. 15531, *Mycoplasma arginini* ATCC No. 23838, and *Mycoplasma orale* ATCC No. 23714. Prepare a 100 mL logarithmic phase broth culture of each organism and freeze 1.5 mL aliquots of each stock at −70°C or lower. For used for isolation have high plating efficiency; therefore, quality control tests, thaw one vial should include titration of each of three frozen stock strains and perform serial ten-fold dilutions (through at least nine dilutions) for each organism in a) standard culture mycoplasma suspensions onto the solid medium formulation, and b) to determine the new formulation highest dilution to produce colonies.

6.2 Test mycoplasma strains should not be adapted to artificial media. Instead, cell culture grown mycoplasma strains should be used. The broth formulations Test strains should contain appropriate growth promoting components (glucose and arginine) and a phenol red pH indicator (see formula below). Adjust the medium for include: *M. pneumoniae hyorhinis*, BTS-7; to pH 7.8 and the media for *M. orale*, CH 19299; or*M. pirum*, 70–159; *M. arginini*, G230; to pH 7.0. Incubate the two dilution series for *M. fermentans*, PG-18; Mycoplasma infected BHK-21 cell cultures are stored at –70°C.

6.3 Prepare test batches of DM-1 medium with each organism. After 14 days, record the last tube or vial in each series showing a color change (color changing units/mL or CCU/mL). Compare color-changing units/mL between the standard broth and new formulation. Avoid using a new formulation that shows a decline lot of more than one ten-fold dilution from the control broth.

5.2 Test the agarose, myosate, horse serum for mycoplasma contamination using the large volume broth culture procedure (see Practice E 1536; Barile and Kern **(2)**.

6. Procedure

6.1 *Preparation of Culture Media* :

6.1.1 *Stock Solution*:

6.1.1.1 Add dextrose (50 g), L-arginine HCl (10 g), thymic DNA (0.02 g), choline chloride (0.922 g), i-inositol (0.110 g), niacinamide (0.024 g), D-calcium pantothenate (0.024 g), pyridoxal HCl (0.020 g), folic acid (0.013 g), riboflavin (0.10 g), cyanocobalamin (0.003 g), D-biotin (0.002 g), and thiamine HCl (0.010 g) to 900 mL distilled water.

6.1.1.2 Mix at 37°C until dissolved and bring final volume to 1000 mL.

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

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

[4] Available from Calbiochem, P.O. Box 12087, San Diego, CA 92112-4180. Life Technologies, Gaithersburg, MD.

[5] Available from Research Organics, Cleveland, OH.

[6] Available from BBL Microbiology Systems, Cockeysville, MD.

[7] Available from SeaPlaque agarose, FMC Bioproducts, Rockland, MA.

[8] Available from Whittaker Bioproducts, Walkersville, MD.

~~6.1.1.3 Sterilize the solution by filtration using a 0.22 µm filter.~~

~~6.1.2 *Preparation of Broth Medium* :~~

~~6.1.2.1 Add 14.7 g mycoplasma broth base (BBL 11458) and 0.2 g phenol red to 600 mL distilled water and heat to dissolve.~~

~~6.1.2.2 Sterilize the solution by autoclaving for 15 min at 121°C and allow to cool to room temperature.~~

~~6.1.2.3 Aseptically add 200 mL horse~~ serum, ~~100 mL fresh yeast extract and 100 mL thawed stock solution (6.1.1) and mix thoroughly.~~

~~6.1.2.4 Dispense 10 mL aliquots~~ or CMRL-1066.

6.4 Inoculate ten-fold dilutions of the ~~broth medium into sterile test tubes and cap. Store broth tubes at 2–8°C for no longer than four weeks.~~

~~6.1.3 *Preparation*~~ *battery* ~~*of Agar Medium* :~~

~~6.1.3.1 Add 15.0 g mycoplasma agar base (BBL 11458) to 600 mL distilled water. Add 8.0 g Noble agar (Difco), Ionagar No. 2 (Oxoid), or agarose. Dissolve by bringing solution to a boil.~~

~~6.1.3.2 Sterilize the solution by autoclaving for 15 min at 121°C.~~

~~6.1.3.3 Place the~~ quality control strains with known titers onto medium ~~into a water bath at 50°C. Place 200 mL horse serum, 100 mL yeast extract,~~ plates and ~~100 mL stock solution (6.1.1) in a water bath at 37°C. Allow the solutions~~ compare to ~~equilibrate at the respective temperatures.~~

~~6.1.3.4 Aseptically add the horse serum, yeast extract, and stock solution to the medium and mix well.~~

~~6.1.3.5 Dispense the~~ DM-1 prepared ~~medium in 10 mL portions into 60 mm petri dishes, or in 15 mL portions into 90 mm petri dishes and allow to solidify. Store the petri dishes at 2–8°C for no longer than four weeks.~~

~~6.2 *Preparation*~~ *with previously tested components.*

6.5 A new lot of ~~Cells from Test Sample:~~

~~6.2.1 Culture cells without any antibiotics for two subcultures before testing.~~

~~6.2.2 If bacteria contamination~~ material is ~~a problem when subcultures are made in antibiotic-free culture media, discard the cells and examine culture media, laboratory techniques, or other factors for the origin of the contamination.~~

~~6.2.3 Select a culture for testing that~~ acceptable if there is ~~near confluency and has not had~~ no more than a ~~fluid change within three days of testing.~~

~~6.3 *Inoculation of Test Sample* :~~

~~6.3.1 For monolayer cultures, remove and discard all but 3 to 5 mL of the culture medium from a T25 flask or equivalent. Scrape a portion of the cell monolayer into the remaining culture medium using a sterile pipet.~~

~~6.3.2 For suspension cultures, take the test sample directly from a heavily concentrated culture (at least 10^5 cells/mL) that has not received a fresh medium supplement or renewal within the last three days.~~

~~6.3.3 Inoculate 1.0 mL of the test cell culture suspension into two tubes each of two mycoplasma broth cultures and 0.1 mL of the test suspension onto each of two mycoplasma agar culture plates.~~

~~6.3.4 Incubate one broth culture aerobically and one in an atmosphere of 5 % CO_2 and 95 % nitrogen at 37°C and observe daily for development of turbidity or shift in pH, or both.~~

~~6.3.5 Incubate one agar plate aerobically and one in an atmosphere of 5 % CO_2 and 95 % nitrogen at 37°C in a humidified atmosphere atmosphere of 5 % CO_2-95 % nitrogen.~~

~~6.3.6 After 5–7 days of incubation, and again after 10 to 14 days of incubation, remove a 0.1 mL sample from the broth culture and inoculate a new agar plate. Incubate these plates anaerobically at 37°C.~~

~~6.3.7 Microscopically examine the agar plates weekly for at least three weeks for mycoplasma colony formation and growth before scoring them as negative. Observe the plates at 40, 100, and 300 magnification using an inverted microscope. Mycoplasma colonies can be positively identified directly by subculturing or indirectly by staining.~~

~~6.3.7.1 Subculture a small section (1 cm^2) of the suspicious area of the agar culture into a new broth culture and observe for turbidity and pH changes.~~

~~6.3.7.2 Use the Dienes stain (Hayflick, 1973) to stain colonies. True mycoplasma colonies will absorb the stain.~~

~~6.3.8 In view of the~~ ten-fold difference between number of ~~factors which can cause a shift in the pH of the broth, any pH changes should only be considered a" presumptive" positive until verified by colony formation in agar.~~

~~6.4 *Indications and Frequency of Testing*:~~

~~6.4.1 Test all cell lines maintained in the laboratory every three months.~~

~~6.4.2 Test all preserved cells at the time they are frozen.~~

~~6.4.3 Test cells after 4–6 weeks of cultivation in media containing a new lot of serum.~~

6.4.4 ~~Compare~~ colonies on test ~~results to the positive~~ and ~~negative controls.~~

~~6.5 *Complementary tests*—An indirect test for non-cultivable mycoplasmas using bisbenzamide fluorochrome stain[4] or another DNA fluorochrome of comparable quality and performance (see Practice E 1532) should be performed along with the direct test.~~ control media plates.

7. Mycoplasma Isolation

7.1 Samples include monolayer, suspension or frozen cell cultures that have been grown in antibiotic free medium. Monolayer cultures should be scraped with a pipette to provide a cell suspension.

7.2 Inoculate one DM-1 medium plate with 0.1 mL of the cell culture sample, and incubate anaerobically at 36°C.[6]
7.3 Microscopically examine the plate at 5 and 14 days. Most isolates develop colonies in 5 days.

8. Keywords

78.1 cell culture; cultivation; mycoplasma

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