Designation: E 1532 – 00

Standard Practice for Detection of Mycoplasma Contamination of Cell Cultures by Use of the Bisbenzamide DNA-Binding Fluorochrome[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 the use of cell cultures and DNA-binding flurorochrome techniques to detect mycoplasma contamination of cell cultures.

1.2 This practice does not cover axenic cultivation or identification of mycoplasmas.

1.3 *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:*

E 1531 Practice for the Detection of Mycoplasma Contamination of Cell Cultures by 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 the Detection of Mycoplasma Contamination of Bovine Serum by the Large Volume Method[2]

3. Terminology

3.1 *Definitions:*

3.1.1 *axenic cultivation*, *n*—cultivation free from other living organisms.

3.1.2 *direct mycoplasma detection*, *n*—demonstration of characteristic colonial growth on axenic agar medium.

3.1.3 *mycoplasma (Mollicute)*, *n*—smallest prokaryotes capable of self replication.

4. Significance and Use

4.1 *Mycoplasma hyorhinis*, cultivar α strains **(1)**[3] do not grow on any of the standard media used for mycoplasma cultivation. These strains, which are found as contaminants in cell cultures, are detected by indirect methods.

4.2 A specialized medium has been described but it is not yet in wide use **(2)**.

4.3 This practice should be used in 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. Indicator Cell Cultures

5.1 BHK-21, 3T6, and Vero are the most widely used indicator cell cultures. BHK 21 are maintained as monolayer cultures, which are trypsinized to prepare, cell suspensions as needed **(4-6)**.

5.2 Fetal bovine is heat inactivated at 56°C for 30 minutes before it is used in cell culture medium.

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

5.4 Add 3.8 mL of cell suspension to each dish. The suspension should be dilute enough so that 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 dish.

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

5.7 Two uninoculated dishes serve as negative controls.

6. Materials for Staining

6.1 *Carnoy's Fixative*—Mix one part glacial acetic acid with three-parts methanol. This fixative may be made in advance and stored at room temperature.

6.2 *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 of deionized water.

6.2.2 *0.2 M 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 with 450 mL of 0.1 M citric acid.

6.2.4 Store buffer at 2 to 8°C.

6.3 Bisbenzamide (H-Stain) Stock Solution: dissolve 5.0 mg Hoechst 33258 (Calbiochem) in 100.0 ml deionized water. Portion in 0.5 ml amounts and store at –20°C.

6.4 *Bisbenzamide (H-Stain) Working Solution*—Add 0.1 mL of Bisbenzamide stock solution to 100 mL of McIlvane's

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

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

citrate-phosphate buffer. Protect from light and use directly.

6.5 *Mountant*—To 50 mL McIlvane's citrate-phosphate buffer, add 50.0 mL glycerol. Mix and dispense in small dropper bottles. Store at 2 to 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

9.1 cell culture; DNA stain; indicator cells; mycoplasma

REFERENCES

(**1**) Del Giudice, R. A., Gardella, R. S., and Hopps, H. E., "Cultivation of formerly noncultivable strains of *Mycoplasma hyorhinis*," *Curr. Microbiol.*, Vol 4, 1980, pp. 75–80.

(**2**) Del Giudice, R. A., "M-CMRL a new axenic medium to replace indicator cell cultures for the isolation of all strains of *M. hyorhinis*," *In Vitro Cell. Dev. Biol.-Animal*, Vol 34, 1998, pp. 88–89.

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(**5**) Del Giudice, R. A., Hopps, H. E., "Microbiological methods and fluorescent microscopy for the direct demonstration of mycoplasma infection of cell cultures," In: McGarrity, G. J., Murphy, D. G., Nichols, W. W., ed., Mycoplasma infection of cell cultures, New York: Plenum Press, 1978:57–69.

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