Designation: E 1533 – 00

Standard Practice for Indirect Detection of Mycoplasma in Cell Culture by 4′-6-Diamidino-2-2 Phenylindole (DAPI) Staining[1]

This standard is issued under the fixed designation E 1533; 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 of mycoplasma contamination by indirect DNA staining.

1.2 This practice does not cover direct methods for the detection of mycoplasma or other indirect methods such as enzymatical detection or DNA probes.

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 *This standard does not purport to address all of the safety problems, 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 Detection of Mycoplasma Contamination of Cell Cultures by Growth on Agrose Medium[2]

E 1532 Practice for Detection of Mycoplasma Contamination of Cell Cultures by Use of the Bisbenzamide DNA-Binding Flurorochrome[2]

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

3. Terminology

3.1 *Definitions:*

3.1.1 *DAPI staining*—staining of DNA in particular by using DAPI fluorochrome stain.

3.1.2 *direct detection of mycoplasma*—detection of mycoplasma by cultivation in culture media.

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 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.

4. Significance and Use

4.1 Mycoplasma contamination of cell cultures is a common problem that can affect the growth, metabolism, and function of cultured animal cells. The ability to detect mycoplasma in cell cultures provides an opportunity to ensure that cells are free of contamination, and to replace those that are not. For additional information, see Practices E 1531, E 1532, and E 1536. Strict adherence to established, well-tested procedures is necessary. This practice was developed by Task Group E48.01.02 to assist in developing and maintaining an established regimen for mycoplasma detection by indirect 4′-6-Diamidino-2-Phenylindole (DAPI) fluorochrome staining.

4.2 This practice is intended for use in examining cultured animal cells for the presence of mycoplasma contamination.

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

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 DAPI stain concentrate routinely for contamination. Fresh stock should be prepared periodically.

5.2 *Indicator cells*:

5.2.1 Indicator cells support the growth of mycoplasma species and provide positive and negative controls.

5.2.2 Use continuous cell lines such as the African green monkey kidney cell line, Vero, American Type Culture Collection (ATCC CCL81) as indicator cells as described in this practice; 3T6 mouse fibroblast (ATCC CCL 96) may also be used.

5.2.3 Do not use transformed cells as indicators since they produce large amounts of extra nuclear fluorescence.

6. Procedure

6.1 *Preparation of DAPI Stain Concentrate*:

6.1.1 Add 1.0 mg DAPI stain to 100 mL sterilized distilled water and mix thoroughly at room temperature.

6.1.2 The stain is heat and light sensitive. Prepare the concentrate in a bottle wrapped completely in aluminum foil,

[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.

and store at 2 to 8°C.

6.2 *Preparation of Indicator Cell Cultures*:

6.2.1 Sterilize Leighton tubes containing a glass cover slip of 32 × 4 mm.

6.2.2 By trypsinization, prepare a cell culture containing 10^5 cells/mL.

6.2.3 Dispense 2 mL of recently (same day) prepared cell culture in each Leighton tube, taking care that the glass cover slip is submerged totally in the medium. Number the tubes.

6.2.4 *Inoculation*:

6.2.4.1 Inoculate 0.2 mL of previously frozen cell sample, thawed immediately prior to use, into each of two tubes containing indicator cells. Register the corresponding number of tube and sample.

6.2.4.2 Inoculate 0.2 mL of a broth culture of *M. hyorhinis* into each of two tubes for positive controls. Register the tube numbers.

6.2.4.3 Inoculate 0.2 mL of sterile culture medium to each of two tubes containing indicator cells only. Register the tube numbers. These tubes are used as negative controls.

6.2.5 Incubate the cultures at 37°C for three days in a 5 % Co_2 + 95 % air incubator.

6.3 *Preparation of Staining Solution*:

6.3.1 Add 1.0 mL of the stock stain concentrate (see 6.1) to 100 mL sterilized distilled water and mix thoroughly at room temperature.

6.3.2 Keep the solution away from light until the moment of using.

6.4 *Fixing the Cells*:

6.4.1 Take the cultures from the incubator and remove the medium from each Leighton tube.

6.4.2 Wash twice with 2 mL phosphate buffered saline (PBS) pH 7.2.

6.4.3 Fix 1 min with 2 mL absolute alcohol.

6.4.4 Wash twice with 2 mL PBS pH 7.2.

6.5 *Staining of Cells*:

6.5.1 Add 2 mL DAPI staining solution to each tube.

6.5.2 Incubate for 30 min at 37°C.

6.5.3 Wash twice with 2 mL PBS pH 7.2.

6.6 *Mounting of Cover Slips*:

6.6.1 Remove the cover slip from the Leighton tube and place it on a glass slide. The monolayer must be directed to the glass slide. Do not allow the culture to dry.

6.6.2 Label each slide to identify the specimen being tested.

6.7 *Observation and Recording of Results*:

6.7.1 Observe each specimen, including both the positive and negative controls, by fluorescence microscopy at 250× magnification.

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

6.7.3 *Positive Controls*—Cell nuclei will appear as large oval fluorescing bodies, and, in addition, numerous small fluorescent particles are observed elsewhere on the monolayer. This extra nuclear fluorescence is due to the DNA of the contaminating organism.

6.7.4 Compare the test samples to the controls, and record the results as positive or negative for possible mycoplasma contamination. Positive results must be confirmed by cultivation or another appropriate test method.

6.8 *Frequency of Testing*:

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

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

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

6.8.4 Test cells before the initiation of a new experiment.

6.9 *Complementary Testing*—The DNA staining as an indirect test for infections with mycoplasma offers only an indication concerning the presence of mycoplasma in the cell culture. A confirmation of the results is absolutely necessary, and one cannot forget that some cases of negative results in the DNA-staining and positive in the agar cultivation.[3, 4, 5, 6]

7. Keywords

7.1 DAPI staining; indicator cells; mycoplasma

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[3] Russel, W. C., Newman, C., and Williamson, D. H., "A Simple Cytochemical Technique for Demonstration of DNA in Cells Infected with Mycoplasmas and Viruses," *Nature*, Vol 253, 1975, pp. 461–462.

[4] Bonissol, C., Gilbert, M., and Ivanova, L. M., "Valeur de la technique de coloration d'ADN pour le depistage de contamination des cultures cellulaires par les mycoplasmas," *Annuals Microbiology*, Vol 129B, 1978, pp. 245–265.

[5] Polak-Vogelgang, A. A., Brugman, J., and Reijgers, R., "Comparison of Two Methods for Detection of Mollicutes (Mycoplasmatales and Acholeplasmatales) in Cell Cultures in The Netherlands," *Antonie van Leeuwenhoek*, Vol 53, 1987, pp. 107–118.

[6] McGarrity, G. J., Steiner, T., and Vanaman, V., "Detection of Mycoplasmal Infection of Cell Cultures by DNA Fluorochrome Staining," *Methods in Mycoplasmology*, Vol. II, Academic Press, 1983, pp. 183–190.