Designation: ~~E2564 – 18~~ E2564 – 23

An American National Standard

Standard Practice for Enumeration of *Mycobacteria* in Metalworking Fluids by Direct Microscopic Counting (DMC) Method[1]

This standard is issued under the fixed designation E2564; 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~~ Scope*

1.1 This practice describes a direct microscopic counting method (DMC) for the enumeration of the acid-fast stained mycobacteria population in metalworking fluids. It can be used to detect levels of total mycobacteria population, including culturable as well as non-culturable (possibly dead or moribund) bacterial cells. This practice is recommended for all water-based metalworking fluids (Classification D2881).

1.2 *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, health, and environmental practices and determine the applicability of regulatory limitations prior to use.* For additional safety information, see *Laboratory Safety: Principle and Practices, 4th Edition.*[2]

1.3 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D2881 Classification for Metalworking Fluids and Related Materials

E2523 Terminology for Metalworking Fluids and Operations

3. Terminology

3.1 For definitions of terms used in this standard, refer to Terminology E2523.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *acid-fast bacteria, n*—a distinctive staining property of *Mycobacteria* due to their lipid-rich cell walls.

3.2.1.1 *Discussion*—

Once stained, mycobacteria resist decolorization when exposed to acidified organic solvents, and are therefore informally designated acid-fast.

3.2.2 *non-tuberculous Mycobacteria (NTM)*—environmental mycobacteria not associated with tuberculosis.

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[1] This practice is under the jurisdiction of ASTM Committee E34 on Occupational Health and Safety and is the direct responsibility of Subcommittee E34.50 on Health and Safety Standards for Metal Working Fluids.

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[2] Gilchrist, Mary J. R., “Biosafety Precautions for Airborne Pathogens,” in *Laboratory Safety: Principles and Practices*, ASM Press, 1995, pp. 67–76.

[3] 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.

***A Summary of Changes section appears at the end of this standard**

3.2.3 *microscopic factor (MF), n*—a calibrated conversion factor for calculating the mycobacterium count per mL sample.

3.2.3.1 *Discussion*—

The average number of mycobacterium cells per one microscopic field (or oil field, OIF) is multiplied by the MF to give the concentration of mycobacteria per mL of sample.

3.2.4 *oil immersion field (OIF), n*—the circular area of a microscopic field visible in the eyepiece of the microscope using oil immersion objective.

4. Summary of Practice

4.1 This practice describes a semi-quantitative test for enumerating acid-fast stained environmental mycobacteria (AFB) from metalworking fluids by direct microscopic counting (DMC) method.[4] It is used to determine total mycobacterium counts, including culturable and possibly dead or moribund cells in the sample. This practice cannot be used to determine the total viable mycobacterium population in the sample. A known sample volume (centrifuged or direct) is spread over a known area (1 cm^2 or similar) on a microscope slide (marked by frosted or painted circles). Following differential acid-fast staining,[5] the acid-fast cells are counted in several microscopic fields over the designated area. The calculation is based on using a calibrated microscope with a known microscopic factor (MF). The MF is determined by the microscopic area over which a known amount of sample was spread, the number of microscopic fields in the marked circle, and the volume of sample examined. The number of acid-fast stained mycobacterium cells per microscopic field multiplied by the MF gives the mycobacterium number per mL of sample.

5. Significance and Use

5.1 ~~During the past decade, it has become increasingly apparent that non-tuberculous mycobacteria are common members of the indigenous MWF bacterial population.~~ Measurement of mycobacterial cell count densities is an important step in establishing a possible relationship between mycobacteria and occupational health-related allergic responses, for example, hypersensitivity pneumonitis (HP) in persons exposed to aerosols of metalworking fluids. It is known that the viable mycobacteria count underestimates the total mycobacterial levels by not counting the non-culturable, possibly dead or moribund population that is potentially equally important in the investigation of occupational health-related problems. The direct microscopic counting method (DMC) described here gives a quantitative assessment of the total numbers of acid-fast bacilli. It involves using acid-fast staining to selectively identify mycobacteria from other bacteria, followed by enumeration or direct microscopic counting of a known volume over a known area. Although other microbes—particularly the Actinomycetes—also stain acid-fast, they are differentiated from the mycobacteria because of their morphology and size. Non-mycobacteria, acid-fast microbes are 50 to 100 times larger than mycobacteria. This practice provides quantitative information on the total (culturable and non-culturable viable, and non-viable) mycobacteria populations. The results are expressed quantitatively as mycobacteria per mL of metalworking fluid sample.

5.2 The DMC method using the acid-fast staining technique is a semi-quantitative method with a relatively fast turnaround time.

5.3 The DMC method can also be employed in field survey studies to characterize the changes in total mycobacteria densities of metalworking fluid systems over a long period of time.

5.4 The sensitivity detection limit of the DMC method depends on the MF and the sample volume (direct or centrifuged, etc.) examined.

6. Interferences

6.1 Some metalworking fluid formulations fail to completely dry or provide an uneven film on the microscope slide (for example, synthetic fluids and metalworking fluids with high trap tramp oil content and debris). For these samples, the results can be difficult to interpret, as heat fixing may not provide full adherence. These samples should be re-stained or a new slide may be prepared.

6.2 A negative acid-fast staining reaction does not necessarily indicate that a sample will be culturally negative for *Mycobacteria*, since the culture method has a lower detection limit (1 cell/mL) than the DMC method.

[4] *Standard Methods for the Examination of Dairy Products*, Chapter: 10: Direct Microscopic Methods for Bacteria or Somatic Cells, 16th ed., American Public Health Association, Inc., Washington, DC, 1978.

[5] Ebersole, L. L., "Acid-Fast Stain Procedures," pp. 3.5.1–3.5.11, in *Clinical Microbiology Procedures Handbook*, Vol 1, American Society for Microbiology, Washington, DC, 1994.

7. Apparatus

7.1 *Centrifuge,* ("microfuge"), 14 000 relative gravities.

7.2 *Centrifuge Tubes with Caps,* disposable, 1 mL to 2 mL capacity, such as Eppendorf Safe-Lock tube or any other suitable centrifuge tubes.

7.3 *Calibrated Variable Pipet,* with sterile tips: 5 µL, 10 µL, 1.0 mL, 5 mL.

7.4 *Microscope Slides,* with 100 mm^2 or similar areas, marked by frosted or painted circles and frosted labeling ends.

7.5 *Calibrated Stage Micrometer,* 0.01 mm or similar divisions.

7.6 *Compound Microscope,* with oil immersion lens.

7.7 *Microscope Eye Pieces,* 10× magnification, equipped with a net micrometer (10 by 10 mm) or similar.

7.8 *Slide Drying Apparatus,* (box) 50 to 60 °C, with level drying rack.

7.9 *Staining Hood.*

7.10 *Staining Rack and Running Water.*

7.11 *Hand Tally or Electrical Counter.*

7.12 *Kinyoun Acid-Fast Stain Kit,* (see 8.1).

7.13 *Analytical Balance.*

8. Reagents and Materials

8.1 *Staining Reagents for Acid-Fast Staining Procedure for Staining Mycobacteria by the Kinyoun (Cold) Acid-Fast Procedure:*

8.1.1 *TB Quick Stain Carbolfuchsin, Reagent A*—Basic fuchsin (alcoholic) 17.0 g, aqueous phenol 1000.0 mL.

8.1.2 *TB-Decolorizer*—Hydrochloric acid, 30.0 mL; denatured ethanol/methanol, 970 mL.

8.1.3 *TB Quick Stain Methylene Blue Reagent B*—Methylene Blue (alcoholic) 2.0 g, acid-alcohol 1000.0 mL; (acid-alcohol: 30 mL HCl 970 mL, 90 to 95 % ethanol) or Brilliant Green stain: Brilliant Green 2.0 g, sodium hydroxide 0.02 g, distilled water 1000 mL.

9. Hazards

9.1 The analyst must know and observe good laboratory practices and safety procedures required in the microbiology laboratory in preparing, using, and disposing of cultures, reagents, and materials.

10. Sampling, Test Specimens, and Test Units

10.1 Use sterile, screw-capped, plastic containers (100 to 200 mL) for microbiological sampling of metalworking fluids. The sample should be a random representative portion of 50 to 100 mL that is from the circulating tank, as opposed to a pooled spillover or stagnant hose contents. Refrigerate samples until analyzed. Maximum sample storage time is 24 h at refrigeration temperatures. Follow sample documentation procedure in accordance with good laboratory practices.