

ABSTRACT

Advanced Analytical has developed a rapid, 30-minute test for accurately enumerating *Mycoplasma bovis* biomass and viable cells using the RBD 3000, a fully automated instrument specifically designed for counting microorganisms. Real-time enumeration of cell mass and viable cells in culture allows scientists to monitor growth and harvest cultures at optimal times for best product yield. Generally, mycoplasmas take days, and in some cases weeks, to culture on agar plates. Therefore, a rapid method of enumeration is significantly beneficial. *M. bovis* ATCC 25025 cultures were serially diluted in buffer to yield 2 to 5 log₁₀ cfu/mL, which demonstrates the enumeration range of *M. bovis* on the RBD 3000. Diluted samples were placed on the RBD 3000 for enumeration and within 30 minutes both the biomass and viable cell count results were obtained. The RBD 3000 viable *M. bovis* counts (n = 19) correlated well (R² = 0.975) with the plate count method. The RBD 3000 can be utilized for real-time monitoring of *Mycoplasma* spp., as well as a wide variety of microorganisms in the fermentation industry.

MATERIALS

The RBD 3000 is an instrument uniquely designed, developed and manufactured by Advanced Analytical Technologies, Inc. (Ames, IA), which employs the principles of flow cytometry to enumerate fluorescent-labeled microorganisms. The Nucleic Acid (NA) and Dead Cell Dyes were supplied by Molecular Probes (Eugene, OR). The *Mycoplasma bovis* used in the following experiment was obtained from the American Type Culture Collection and was designated as 25025 (Manassas, VA). The diluent used was 0.2 μm filtered 10 mM Sodium Phosphate Buffer (PB, pH 7.2 +/- 0.2). The media used were PPLO broth and agar containing *Mycoplasma* supplement (Becton, Dickinson and Company, Sparks, MD).

METHODS

A static *M. bovis* culture was serially diluted in PB to yield 2 to 5 log₁₀ cfu/mL. Duplicate samples for biomass and dead cell enumeration were dispensed in 3 mL volumes into 5 mL sample tubes. The samples were loaded into a sample tray and placed into the fully automated RBD 3000.

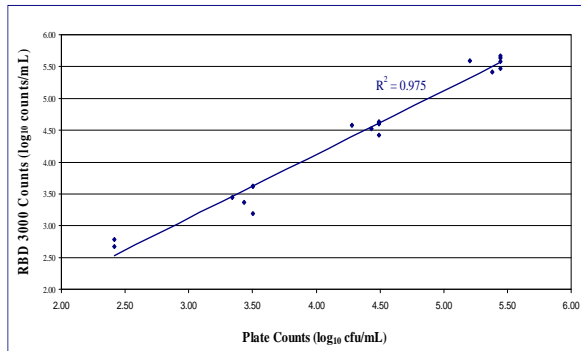
METHODS (continued)

The instrument automatically added the appropriate dyes (NA Dye or Dead Cell Dye) and enumerated the *M. bovis* in each sample. The NA Dye is a membrane permeable nucleic acid stain that provides information on the total number of cells (live and dead) in the analyzed sample. The Dead Cell Dye is a non-membrane permeable nucleic acid stain that provides information on the total number of dead cells only. The total number of viable *M. bovis* in each sample was determined by subtracting the Dead Cell Dye RBD 3000 count result from the corresponding NA Dye RBD 3000 count result, which can be calculated within the RBD 3000 software. Prior to RBD 3000 enumeration, each sample was spread-plated on PPLO agar containing *Mycoplasma* supplement for plate count comparison.

RESULTS

The RBD 3000 enumeration of viable *M. bovis* correlated well with the standard plate count results (R² = 0.975), where n = 19 (see Graph 1 and Table 1). Examples of RBD 3000 data output are shown in Figures 1A and 1B as Intensity Plots, where the fluorescence intensity is on the x-axis and scattered laser light is on the y-axis.

Graph 1: Correlation of log₁₀ RBD 3000 Counts vs. log₁₀ Plate Counts of *Mycoplasma bovis* in PB



RESULTS (continued)

Table 1: Viable log₁₀ RBD 3000 Count and log₁₀ Plate Count Results for *Mycoplasma bovis* in PB

Sample (approx. <i>M. bovis</i> level):	RBD 3000 Counts (log ₁₀ counts/mL):	Plate Counts (log ₁₀ cfu/mL):
<i>M. bovis</i> 10 ⁵	5.59	5.20
	5.42	5.38
	5.67	5.45
	5.64	5.45
	5.58	5.45
<i>M. bovis</i> 10 ⁴	5.47	5.45
	4.57	4.28
	4.53	4.43
	4.6	4.49
	4.64	4.49
<i>M. bovis</i> 10 ³	4.63	4.49
	4.43	4.49
	3.44	3.34
	3.37	3.43
	3.62	3.51
<i>M. bovis</i> 10 ²	3.62	3.51
	3.19	3.51
	2.67	2.41
	2.78	2.41

Figure 1A: *M. bovis* 10⁵ stained with NA Dye

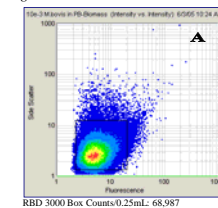
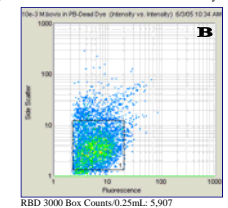


Figure 1B: *M. bovis* 10⁵ stained Dead Cell Dye



CONCLUSIONS

A rapid, 30 minute test has been developed using Advanced Analytical's RBD 3000 to accurately enumerate biomass, viable cells and/or non-viable *Mycoplasma bovis*.