Application Note

Detection of Mycoplasma Using the Synergy™ 2 Multi-Mode Microplate Reader and the Lonza MycoAlert Assay

Important quality control for tissue cultures

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Mycoplasma contamination is a common problem with continuously cultured cell lines. Contamination, which may go undetected for long periods of time, can alter cellular proliferation, gene expression, and other cellular responses. Early detection of this insidious pest is an important part of good tissue culture quality control. Here we describe the detection of Mycoplasma using the Synergy™ 2 Multi-Mode Microplate Reader to run a MycoAlert® Mycoplasma detection kit from Lonza.

Mycoplasma are primitive prokaryotes, lacking a cell wall and consisting of only a plasma membrane, ribosomes, and a double stranded 580 kb genome. Due to their small size, mycoplasma can pass through 0.22 and 0.45 μm filters typically used to sterilize tissue culture reagents. Nor can they be observed using routine microscopy [1].

Mycoplasma are often slow-growing contaminants that typically exist in low numbers, but can cause experimental artifacts in cells. Mycoplasma contamination has been shown to alter cellular growth rates, affect amino acid and nucleic acid metabolism, affect cell antigenicity, and induce chromosomal and membrane aberrations [2, 3].

Detection of mycoplasma can be accomplished by either direct culture techniques of the organism or by indirect assay methods. Indirect methods, which include DNA staining, biochemical detection, nucleic acid hybridization, immunoassays, and polymerase chain reaction (PCR), have the advantage of detecting “noncultivatable” species.

The MycoAlert® assay is a biochemical test that screens for the presence of Mycoplasma specific enzymes that interact with reagents catalyzing the conversion of ADP to ATP. By measuring the ATP produced before and after the addition of a substrate, a ratio is obtained which is indicative of the presence or absence of mycoplasma. If mycoplasma enzymes are not present (mycoplasma negative), the second reading shows no increase over the first. However, if they are present their reaction with specific substrates leads to the generation of more ATP. ATP levels are monitored in samples using a luciferin/luciferase reaction that produces light (Equation 1).

$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow[Mg^{+2}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{LIGHT}$$

Eq. 1. The emitted light is linearly related to the amount of ATP present in the sample.

The Synergy™ 2 Multi-Mode Microplate Reader utilizes multiple sets of optics to provide optimal performance regardless of the detection technology. Absorbance measurements use a xenon-flash lamp with a monochromator for wavelength selection, allowing the selection of any wavelength for endpoint or kinetic measures from 200 nm to 999 nm. Fluorescence measurements are made using either a continuous tungsten-halogen lamp or a xenon-flash lamp with bandpass filters with or without dichroic mirrors for wavelength selection and PMT for detection. Fluorescence polarization is accomplished with the use of polarizing filters in conjunction with label specific dichroic mirrors for wavelength specificity. For time-resolved fluorescence measurements, the Synergy™ 2 integrates a high-energy xenon-flash-lamp with excitation and emission filters and PMT detector. Luminescence measurements are made using a liquid-filled optical fiber to capture light along with a low noise PMT.

Here we describe the detection of Mycoplasma controls using the Synergy™ 2 Multi-Mode Microplate Reader and a MycoAlert® Mycoplasma detection kit from Lonza.

Materials and Methods

MycoAlert® Mycoplasma Detection kit (cat no. LT07-318), MycoAlert® Assay control set (cat no. LT07-518), and MycoAlert® Assay buffer (cat no. LT27-218) were purchased from Lonza (Rockland, ME). Solid white flat-bottomed, non-treated microplates, catalogue number 3912, were purchased from Corning (Corning, NY).

The assay was performed according to the kit instructions. Lyophilized MycoAlert Reagent, Substrate, and Positive Control were reconstituted with the appropriate amount of MycoAlert Assay buffer. All reagents were allowed to equilibrate at room temperature for 15 minutes after reconstitution. A series of 1:2 dilutions of MycoAlert Control were made using the assay buffer as the diluent. Aliquots (100 µl) of each dilution were pipetted in replicates of 8 into wells of a microplate. To each well 100 µl of MycoAlert Reagent was pipetted. Using the delay feature of the Gen5™ Data Analysis Software, the plate was incubated in the dark for 5 minutes inside the Synergy™ 2 Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT). After incubation, the luminescence was measured using the Synergy™ 2 [Read A]. Once the initial luminescence measurement was completed, 100 µl of MycoAlert Substrate was added manually to all the wells. The plate was resubmitted to the reader and allowed to incubate inside the reader at room temperature for 10 minutes, after which the luminescence was read a second time using the Synergy™ 2 [Read B]. For both reads, the luminescence was measured from the top using a 1 second integration time and sensitivity setting of 200.

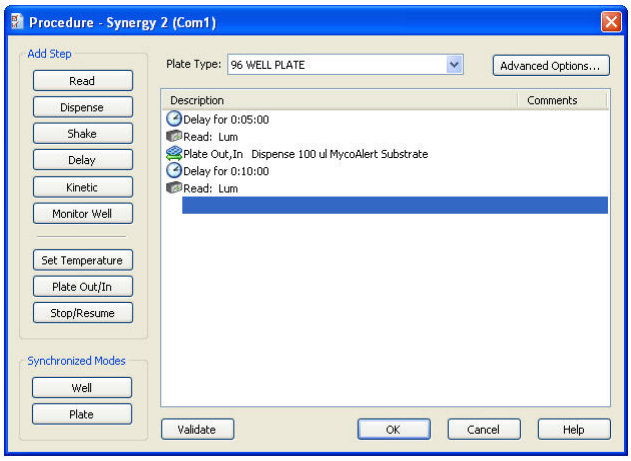

Figure 1. Synergy™ 2 Read Steps. Both incubations are controlled by Gen5™ Data Analysis Software and performed in the reader. The plate is ejected after the initial read such that the MycoAlert substrate can be added manually. Resubmission of the plate initiates the second incubation and read. The Gen5™ software then performs data analysis automatically.

Results

As demonstrated in Figure 2, there is a marked difference in the B/A ratio between the assay control samples and those of the buffer only samples. The average B/A ratio for 8 replicates of the negative control was determined to be 0.078, whereas the ratio for the positive control was 11.35. This represents a difference of greater than 145 fold.

When a series of dilutions of the positive control were assayed a linear relationship between the B/A ratio and control concentration is observed (Figure 3).

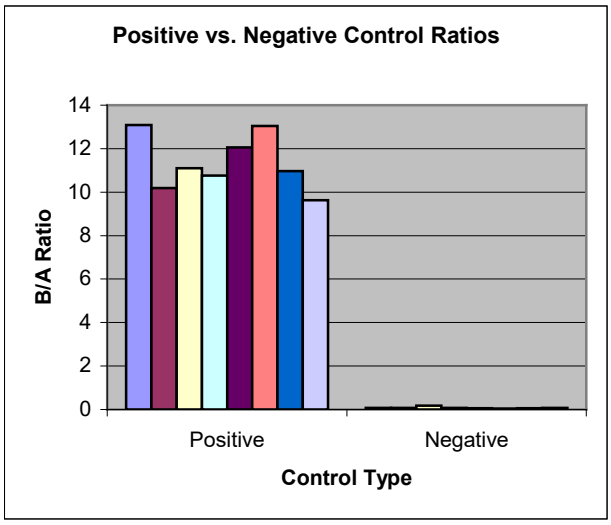

Figure 2. Comparison of Positive and Negative Control B/A Ratios. MycoAlert Assay Control samples and negative buffer samples were assayed according to the kit instructions and their B/A ratios compared.

The positive control can be serially diluted several times and still assays as a positive sample. When the linear regression of the data is interpolated, samples that have been diluted 1:10 still assay as positive samples.

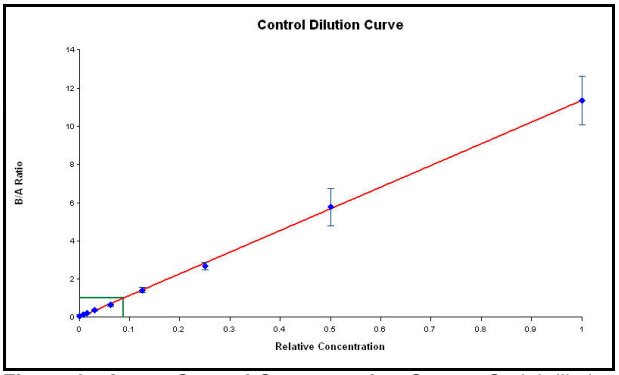

Figure 3. Assay Control Concentration Curve. Serial dilutions of the assay control were made using the assay buffer as the diluent. The dilutions were then assayed for mycoplasma. The green line represents the interpolation of the curve at the cut off. Sample having a ratio greater than 1.0 are considered positive.

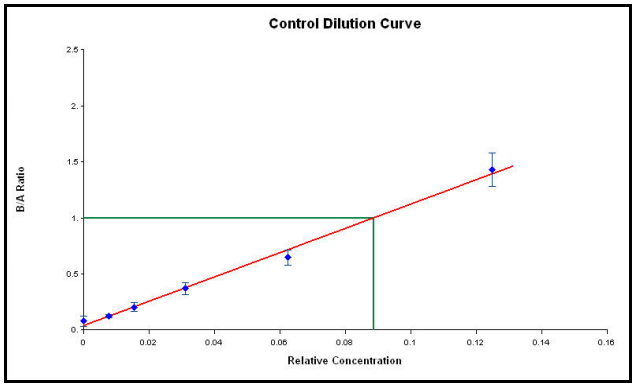

Figure 4. Low Concentrations of Assay Control. Serial dilutions of the assay control were made using the assay buffer as the diluent. The dilutions were then assayed for mycoplasma. The green line represents the interpolation of the curve at the cut off. Sample having a ratio greater than 1.0 are considered positive.

The Synergy™ 2 Multi-Mode Microplate Reader's luminescence sensitivity is demonstrated in Figure 4. When very low concentrations of the assay control are examined a linear relationship between the control concentration and the B/A ratio are still observed. Despite the sample being well below the assay cut off for being a positive sample a very linear relationship is observed. Because the assay control contains a solution of mycoplasma specific enzymes, rather than infective mycoplasma itself, ATP can be generated, but not at a rate sufficient to overcome the consumption of ATP by the assay over time.

Discussion

These data demonstrate that the Synergy™ 2 Multi-Mode Microplate Reader is capable of detecting mycoplasma using the MycoAlert® Assay kit. The reader can detect concentration differences of the positive control well below the assay cut off.

There are several things that the end-user can do to produce optimal results. The assay utilizes a very standard ATP assay in conjunction with mycoplasma specific substrates to detect true mycoplasma contamination. However, contamination of reagents with exogenous ATP can lead to falsely high readings. Skin surfaces are a big source of contaminating ATP. The use of latex gloves or the equivalent is suggested to reduce this source of contamination. The optimal temperature for the assay is at room temperature; refrigerated reagents should be equilibrated prior to use.

Because of the specificity of the assay, bacterial contamination may not be detected using this procedure. Typically a ratio of less than 1.0 indicates a negative test for mycoplasma. A ratio of less than 1.0 is produced by the ongoing consumption of ATP over the time course of the assay. Negative samples therefore would have less ATP and thus less light output with the second read of the assay. Viable bacteria, while able to produce more ATP, will not generate as much ATP as the presence of mycoplasma specific enzymes.

Reader sensitivity is an important issue with the MycoAlert® assay. The assay uses a ratio determination between two separate luminescent determinations. In samples that do not contain mycoplasma and therefore do not generate ATP the ratio would be expected to be 1.0 (i.e. no change). However ratios for negative samples are typically less than 1.0 as a result of consumption of ATP over the time course of the assay. Consistent ratios of 1.0 indicate that the PMT gain setting is not high enough or that the reader being used is not sensitive enough to detect these changes.

The ratiometric-dual reading nature of this assay serves as an effective internal control because each well is read twice and a ratio made reading from the same well. Well to well locational differences, as well as sample pipetting errors, are automatically corrected. For example, if two wells inadvertently received different amount of sample due to a pipetting error, the ratio determination would still be correct despite the raw data being significantly different due to the various fluid volumes. Because the ratio calculation is based on two discrete determinations on the same well, differences in signal as a result of sample sizes would be corrected. Provided that the same amount of reagent and substrate were added to all of the wells, one can still make comparisons based on the ratio determinations between wells.

BioTek's Gen5™ Data Analysis Software makes the reader control and data reduction for this assay very easy. Both incubations can be performed inside the reader, which allows for not only a stable temperature, but also a darkened environment. The dark environment allows for the white microplate to dark adapt, as these type of plates have a propensity to absorb energy and emit it as light once in the reading chamber. The software can control the plate carrier, allowing the manual addition of the MycoAlert assay substrate, as well as providing a visual message on the PC monitor to do so. The software also records both individual reads and performs the ratio and cut off calculation automatically.

References

[1] B.M. Martin (1994) Tissue Culture Techniques: An Introduction. Boston Birkhauser press.

[2] Miller et. al. (2003) Mycoplasma Infection Significantly Alters Microarray Gene Expression Profiles, Biotechniques 35(4):812-814.

[3] Razin et.al. (1998) Molecular Biology and Pathogenicity of Mycoplasmas. Microbiol and Molecular Biology Reviews, 1094-1156.

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