Culture-Based Microbial Sterility Testing Using Water-Glo™ Reagents and GloMax® Instruments

Promega Corporation

Kit :
Water-Glo™ Microbial Water Testing Kit 96-Well Batch (Reagent Only; Cat.# AM1003)
Includes:

- Water-Glo™ Substrate, 1 vial
- Water-Glo™ Buffer, 1 × 60ml
- Water-Glo™ Lysis Reagent, 1 × 65ml
- Water-Glo™ ATP Standard, 1 × 2ml
- Luminometer Injector Tubing Cleaning Kit (Cat.# AM1110). Includes:
  - Injector Cleaning Solution, 70% Ethanol, 100ml (Cat.# AM1091)
  - Injector Cleaning Solution, ATP-Free Water, 100ml (Cat.# AM1101)

Recommended Culture Media:

- Letheen Broth
- Fluid Thioglycollate Medium
- Muller Hinton II (Cation-Adjusted)
- TAT (Tryptone-Azolectin-Tween®) Broth, also known as Casein-Peptone Lecithin Polysorbate Broth

Storage of Reagents:
All components can be stored at room temperature (20–25°C) prior to initial use. The reconstituted Water-Glo™ Detection Reagent can be stored at 4°C for 6 months or at room temperature (23°C) for 2 weeks without significant loss of performance. All other components can be stored at room temperature after initial use.

Materials Required:

- Water-Glo™ Microbial Water Testing Kit 96-Well Batch (Reagent Only; Cat.# AM1003)
- 96-Well Filter Plate (e.g., 96-Well Filter Plate, 2ml, 0.2µm, wwPTFE, Cat.# 8782; AcroPrep™ Advance 2ml 96 Filter Plate, PALL Cat.# 8784)
- Solid Flat Bottom 96-Well White Luminometer Plate (e.g., Corning Cat.# 3912)
- Robotic Reservoir (Cat.# AM1300)
- Vacuum Manifold for Water-Glo™ 96 (Cat.# AM5017)
- GloMax® Navigator (Cat.# GM2010) or GloMax® Explorer (Cat.# GM3500; with injector Cat.# GM3030) or GloMax® Discover (Cat.# GM3000; with injectors Cat.# GM3030)

Introduction

Monitoring of microbial testing for sterile and non-sterile products, as well as sterility testing, requires microbial culturing with or without filtration. Traditional compendial microbial methods are based on culturing techniques which are labor-intensive and time-consuming. Such tests require several days of incubation for the microbial contamination to be detected. Therefore, management is seldom able to take proactive corrective action. In addition, culturing conditions may impact sensitivity, accuracy and reproducibility. To overcome these challenges, culture-independent, rapid microbiological methods are often considered. One of the methods relies on detection of ATP, the cellular energy currency. Cellular ATP levels can be effectively correlated to viable biomass or metabolic activity.

Standard methods USP<1223>, EP<5.6.12> and PDF TR33 lay the framework for the use of rapid microbiological methods. The assay can be performed in two ways: 1) for samples that are aqueous and can be filtered through a 0.45µm filter, a filtration-based approach in conjunction with a short culturing step is implemented and, 2) for samples that cannot be filtered, only a culturing step is implemented. The system uses a 96-well format assay allowing high-throughput analysis. GloMax® Navigator, Explorer and Discover Instruments have two injectors, one injector can be used for dispensing Water-Glo™ Lysis Reagent and the other for dispensing Water-Glo™ ATP Detection Reagent.

This application note describes an overview of different workflows for rapid microbial analysis. Validation of the method to identify the most suitable workflow for culturing, filtering or no filtering, is dependent on the sample type and must be performed prior to implementation.

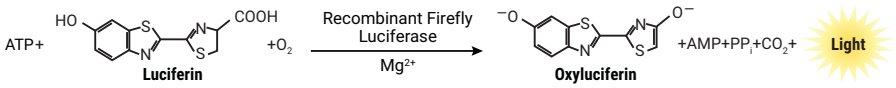
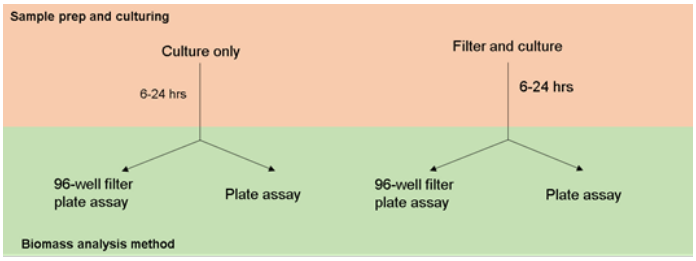


Figure 1. Schematic diagram of Water-Glo™ technology.

Growth medium is a factor that could contribute to background luminescence and affect the luciferase reaction in terms of both signal level and signal stability. We have found that MH II Broth (cation-adjusted Mueller Hinton Broth; Becton, Dickinson and Company Cat.# 297963) supports growth of most common aerobic and facultative anaerobic bacteria, and is selected for use in food testing and antimicrobial susceptibility testing by the Food and Drug Administration and the National Committee for Clinical Laboratory Standards (NCCLS).

MH II Broth has low luminescence background and good batch-to-batch reproducibility. For test methods where cultured sample is directly used for biomass analysis and not filtered, it may be useful to use MH II Broth with 0.5% polysorbate 80 and 0.1% Lecithin to neutralize preservatives in food, cosmetics, pharmaceuticals and consumer products and allow microbial growth.

Protocol



Sample Preparation and Culturing

1. Samples that cannot be filtered should be cultured in recommended culture media. The time of culturing (6–24 hours) should be optimized during the method validation process.
   a. Take 0.01g (or 0.01ml), 0.1g (or 0.1ml) and 1g (or 1ml) in 20ml of a recommended culture media. For solids, use weight in grams. For liquids, use volume in ml. Test multiple replicates (n>3) of each dilution.
   b. Mix the sample and incubate at 37°C for 6–24 hours.
2. For large volume liquid samples that can be filtered, the sample can be concentrated on a filter and then cultured. Use a 0.2µm or 0.45µm filter.
   a. Filter 100ml of liquid sample through a 47mm filter using a filter funnel adaptor and manifold.
   b. Aseptically take the filter membrane using forceps and place it in a tube or a plate containing 10–20ml of culture media.
   c. Incubate the tube or plate at 37°C for 6–24 hours.

Luminometer Instrument Check

1. Prepare the Water-Glo™ Detection Reagent by adding the Water-Glo™ Buffer to the lyophilized Water-Glo™ Substrate.
2. Prior to use, clean the two injectors of the GloMax® Instrument by first flushing five times with ATP-Free Water, then flushing five times with 70% Ethanol, and then an additonal five times with ATP-Free Water.
3. Prime one injector twice with 1ml of Water-Glo™ Detection Reagent.
4. Prime the injector twice with 1ml of Water-Glo™ Lysis Reagent.
   Note: If the 96-well filter plate format is used, priming with Lysis Reagent is not needed as the filter plate method extracts the cell lysates from biomass captured in the filter membrane.
5. Take a white 96-well luminometer plate, add 100µl of Lysis Reagent in four wells and 100µl of ATP Standard in another four wells.
6. Place the white 96-well plate containing the Lysis Reagent (negative control) and ATP Standard (positive control) into the GloMax® Instrument. Start the Water-Glo™ 96 protocol (Kinetics mode; inject 100µl; Speed 200µl/min; Wait: 2 sec; Luminescence: 2 sec integration time; Reading: 1; Interval: 2 sec).
7. Record the RLU from the Lysis Reagent and ATP Standard. This information can be used to analyze reagent performance, reagent contamination and reagent activity.

Biomass Analysis

96-Well Filter Plate Assay

For samples that can be filtered, using a filtration step can reduce the culturing time to arrive at an actionable outcome.

1. Assemble the filtration setup following the manufacturer's instructions for using the Vacuum Manifold. Place a 96-Well Filter Plate on a waste reservoir.
2. Filter up to 2ml of the culture media containing the grown bacteria through the 96-Well Filter Plate. If the culture media is optically turbid, reduce the volume of sample to be filtered.
3. In addition, filter 2ml of the culture media only (no bacteria) through the 96-Well Filter Plate. This is the media-only control (negative control). Record the wells used for the media-only control.
4. Apply vacuum until all the liquid is passed through the filter. The biomass in the water sample is collected on the filter surface while the media is collected in the waste reservoir and can be discarded.
5. Optional: This step is to reduce background luminescence from the culture media if needed. Place the 96-Well Filter Plate on the waste reservoir again. Assemble the plates in the Vacuum Manifold following the manufacturer's instructions. Add 2ml of ATP-Free Water to the wells to wash off any culture media from the filter. Apply vacuum until all the water is passed through the filter. Discard the waste reservoir.

6. Place the 96-Well Filter Plate on a clean, white 96-well plate and the spacer block. Assemble the Vacuum Manifold following the manufacturer's instructions. Add 100µl of Water-Glo™ Lysis Reagent to each well of the 96-Well Filter Plate. Incubate for 1 minute.
7. Apply vacuum until all the liquid is passed through the filter and collected in the white 96-well plate.
8. Clean the GloMax® Instrument injector: Flush the injector five times with ATP-Free Water, then five times with 70% Ethanol, then five more times ATP-Free Water and a final five times with air.

   Note: To prevent biofilm buildup in the injector which may result in high background, we recommend occasional cleaning with acid and base solutions. See Section 4.D of the *Water-Glo™ System Technical Manual* #TM547 for detailed instructions.
9. Place the white 96-well plate containing the lysate into the GloMax® Instrument. Start the Water-Glo™ 96 protocol.
10. Export the data from the GloMax® Instrument to the desktop computer for analysis of microbial contamination. To calculate ATP concentration, copy exported data into the Water-Glo™ ATP Calculator Tool, available at: **www.promega.com/resources/tools/water-glo-atp-calculator-tool**

Plate Assay Without Filtering

1. Pipette 50µl of culture medium with samples cultured at 37°C for 6–24 hours in wells of a white 96-well plate.
2. Pipette 50µl of culture media only in at least four wells. Record the wells used. This is for the media-only control (negative control).
3. Add 50µl of Lysis Reagent to each well. Incubate for 1 minute.
4. Clean the GloMax® Instrument injector: Flush the injector five times with ATP-Free Water and then five times with air. **Note:** To prevent biofilm buildup in the injector which may result in high background, we recommend occasional cleaning with acid and base solutions.
5. Place the white 96-well plate containing the sample into the GloMax® Instrument. Start the Water-Glo™ 96 protocol.
6. Export the data from the GloMax® Instrument to the desktop computer for analysis of microbial contamination.
7. To calculate ATP concentration, copy exported data into the Water-Glo™ ATP Calculator Tool, available at: **www.promega.com/resources/tools/water-glo-atp-calculator-tool**

Analysis

From the Excel® data report, use the following scheme as general guidelines to determine possible contamination. We recommend establishing your own criteria prior to implementation.

No Contamination	<1.5 times RLU of media-only control
Caution	1.5–2 times RLU of media-only control
Contamination	>2 times RLU of media-only control

Protocol for Cleaning the Injector

Biofilm buildup in the GloMax® Instrument injector can result in high background signal (>1,000 RLU for Water-Glo™ Lysis Reagent only). Biofilm buildup can be minimized by cleaning the tubings before and after use. After use, remember to pass air through the tube to remove any residual liquid. This section describes a cleaning cycle with acid and base solutions to remove and prevent biofilm buildup.

1. Optional: Flush the injector five times with 1M HCl, then five times with ATP-Free Water.
2. Flush the injector five times with 1M NaOH.
3. Flush the injector five times with ATP-Free Water.
4. Confirm cleaning: Add Lysis Reagent into at least 5 wells of a clean white 96-well plate. Place the plate into the GloMax® Instrument and run the Water-Glo™ 96 Protocol. An average signal under 1,000RLU suggests the cleaning removed the biofilm. If the average signal is over 1,000RLU, repeat the cleaning cycle including the optional 1M HCl wash (Step 1), or replace the injector tubing (See Section 9 of the *Water-Glo™ System Technical Manual* #TM547 for replacement tubing ordering information) or clean the injector tubing with 10% bleach solution.

   Note: For instructions on how to replace the injector tubing, please refer to the appropriate GloMax® Instrument operating manual. The most current version is available at **www.promega.com/protocols**

   Instructional videos for replacing the injector tubing on the GloMax® Navigator, Discover or Explorer Microplate Readers are also available at **www.promega.com/resources/multimedia**

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