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LIVE BacLight™ Bacterial Gram Stain Kit (L-7005)

Quick Facts

Storage upon receipt:

- Components A and B: –20°C, protect from light
- Component C: room temperature

Note:

Do not use Component C as an immersion oil.

Introduction

The LIVE *Bac*Light™ Bacterial Gram Stain Kit provides a novel one-step fluorescence assays for the determination of gram sign in *living* bacteria. This kit allows researchers to easily classify bacteria as gram-negative or gram-positive in minutes without the use of fixatives. The gram stain is one of the most important and widely used differential stains for the taxonomic classification of bacteria in both clinical and research settings. The original method involves several steps — heat fixation of the bacteria, a two-step staining protocol, alcohol extraction and counterstaining. Over the years, several improved gram-staining techniques have been developed, but most involve cell fixation or cell-membrane permeabilization steps that kill the bacteria being tested. In addition, fixation steps can be a significant drawback if the bacteria are to be characterized further. The one-step LIVE *Bac*Light Bacterial Gram Stain Kit overcomes several of the problems inherent in these labor-intensive, fixation-dependent procedures.

The LIVE *Bac*Light Bacterial Gram Stain Kit utilizes mixtures of our green fluorescent SYTO® 9 and red fluorescent hexidium iodide nucleic acid stains.[1] These stains differ both in their spectral characteristics and in their ability to label live gram-negative and gram-positive bacteria. The SYTO 9 stain labels both live gram-negative and gram-positive bacteria, whereas hexidium iodide preferentially labels live gram-positive bacteria. In gram-positive bacteria that are exposed to both dyes, the hexidium iodide stain effectively displaces the SYTO 9 stain. The excitation/emission maxima for these dyes are about 480 nm/500 nm for SYTO 9 stain and 480 nm/625 nm for hexidium iodide. Thus, when a mixed population of live gram-negative and gram-positive bacteria is stained with this mixture of dyes, the gram-negative bacteria fluoresce green and the gram-positive bacteria fluoresce red. Any dead bacteria present may stain variably.

Although the LIVE *Bac*Light Bacterial Gram Stain Kit has only been tested on a limited number of bacterial types (see *Note*), it has yielded consistent results over that range of organisms. We must point out, however, that the bacteria tested were from logarithmically growing laboratory cultures; other conditions have not been tested.

Materials

Kit Contents

- **Component A**, 300 µL of 3.34 mM SYTO 9 in anhydrous DMSO
- **Component B**, 300 µL of 4.67 mM hexidium iodide in anhydrous DMSO
- **Component C**, 10 mL of *Bac*Light mounting oil, for bacteria immobilized on membranes. Refractive index at 25°C is 1.517 ± 0.003. DO NOT USE FOR IMMERSION OIL.

Number of Tests Possible

At the recommended reagent dilutions and volumes, the kit con tains sufficient material to perform ~1000 individual tests in 96-well assay microplates, many more tests by fluorescence microscopy or ~200 tests by flow cytometry.

Storage and Handling

DMSO stock solutions should be stored frozen at -20°C and protected from light. Allow reagents to warm to room temperature and centrifuge briefly before opening the vials. Before refreezing, seal all vials tightly. The *Bac*Light mounting oil may be stored at room temperature. When stored properly, these stock solutions are stable for at least one year.

Caution: No data are available addressing the mutagenicity or toxicity of these reagents. Because SYTO 9 and hexidium iodide stains bind to nucleic acids, they should be treated as potential mutagens and used with appropriate care. The DMSO stock solutions should be handled with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues. We strongly recommend using double gloves when handling the DMSO stock solutions. As with all nucleic acid stains, solutions containing these reagents should be poured through activated charcoal before disposal. The charcoal must be incinerated to destroy the dyes.

Fluorescence Microscopy Protocols

General Considerations

The following protocols are provided as examples to guide researchers in the development of their own bacterial staining procedures. Researchers at Molecular Probes have used these procedures and found them to be simple and reliable for discriminating several representative species of gram-negative and gram-positive eubacteria.

In a patented procedure described by Sizemore and colleagues,[2,3] a fluorescent derivative of wheat germ agglutinin (WGA) is used for discriminating gram-positive from gram-negative bacteria. With appropriate licensing, Sizemore's staining technique may be combined with Component B of this kit as a confirmatory test for gram sign. The stains provided in the LIVE *Bac*Light Bacterial Gram Stain Kit are chemically compatible with many of

Molecular Probes' fluorophore-conjugated WGA derivatives. Our ViaGram™ Red+ Bacterial Gram Stain and Viability Kit (V-7023) combines a bacterial viability test and WGA-based gram-sign determination in a simple, three-color assay.

Care must be taken to remove traces of growth medium before staining bacteria with the SYTO 9 and hexidium iodide reagents. Nucleic acids and other media components can bind these reagents in unpredictable ways, resulting in unacceptable variations in staining. A single wash step is usually sufficient to remove significant traces of interfering media components from the bacterial suspension. Phosphate wash buffers are not recommended as they appear to decrease the staining efficiency.

Selection of Optical Filters

The fluorescence from both live gram-negative and live gram-positive bacteria may be viewed simultaneously with any standard fluorescein longpass optical filter set. Alternatively, the live gram-negative (green fluorescent) and live gram-positive (red fluorescent) cells may be viewed separately with fluorescein and Texas Red® bandpass optical filter sets. A summary of the fluorescence microscope filter sets recommended for use with the LIVE *Bac*Light Bacterial Gram Stain Kit is shown in Table 1.

Culture Conditions and Preparation of Bacterial Suspensions

1.1 Grow a culture of bacteria to late log phase (usually 10^8–10^9 bacteria/mL) in an appropriate nutrient medium.

1.2 Add 50 µL of the bacterial culture to 1 mL of filter-sterilized water in a microcentrifuge tube.

1.3 Concentrate by centrifugation for 5 minutes in a microcentrifuge at 10,000 × g.

1.4 Remove the supernatant and resuspend the pellet in 1 mL of filter-sterilized water. Note that the water used should be filtered through a 0.2 µm pore–size filter to sterilize and to remove particulate matter that might interfere with the assay.

Optimization of Bacterial Staining in Suspension

The LIVE *Bac*Light Bacterial Gram Stain assay may be optimized for a particular fluorescence microscope by testing several different proportions of Component A to Component B in the specific application. The fluorescence of bacteria labeled with the green-fluorescent nucleic acid stain and those labeled with the red-fluorescent nucleic acid stain may undergo different rates of photobleaching, depending on the intensity of the excitation illumination, which varies considerably between fluorescence microscopes. Note that compensation for photobleaching is not as important when using a fluorometer or fluorescence microplate reader because the excitation illumination is much less intense. We suggest beginning with a 1:1 mixture of Component A and Component B as described below, observing the results and subsequently adjusting the mixture for optimum performance. A 1:1 component mixture results in the same final concentration of dyes. When used at the recommended dilutions, the reagent mixes will contribute 0.3% DMSO to the staining solution. Higher DMSO concentrations may adversely affect staining.

2.1 Combine equal volumes of Component A and Component B in a microfuge tube and mix thoroughly.

2.2 Add 3 µL of the dye mixture per mL of bacterial suspension.

2.3 Mix thoroughly and incubate at room temperature in the dark for 15 minutes.

2.4 Trap 5 µL of each stained bacterial suspension between a slide and an 18 mm square coverslip.

2.5 Observe in a fluorescence microscope equipped with any of the filter sets listed in Table 1. Live gram-negative organisms should fluoresce green and gram-positive bacteria should fluoresce red (see *Note*).

2.6 To optimize the staining pattern, repeat steps 2.1 through 2.5 with different mixtures of Component A and Component B. The volume of the dye mixture added to the cell suspension in step 2.2 may also be varied; however, adding more than 3 µL of DMSO (from the dye mixture) per mL of bacterial suspension may adversely affect staining.

Staining Bacteria Immobilized on Glass Surfaces

3.1 Prepare 1 mL of a 0.1 mg/mL solution of >100,000 MW poly-L-lysine in water that has been filtered through a 0.2 µm pore–size filter to sterilize and to remove particulate matter.

Table 1. *Characteristics of common filters suitable for use with the LIVE BacLight Bacterial Gram Stain Kit.*

Omega filters *	Chroma filters *	Notes
XF25, XF26, XF115	11001, 41012, 71010	Longpass and dual emission filters useful for simultaneous viewing of SYTO 9 and hexidium iodide stains
XF22, XF23	31001, 41001	Bandpass filters for viewing SYTO 9 alone
XF32, XF43 XF102, XF108	31002, 31004 41002, 41004	Bandpass filters for viewing hexidium iodide alone
***** Catalog numbers for recommended bandpass filter sets for fluorescence microscopy. Omega® filters are supplied by Omega Optical Inc. (www.omegafilters.com). Chroma filters are supplied by Chroma Technology Corp. (www.chroma.com).		

3.2 Add 20–30 µL of poly-L-lysine solution to the center of a clean glass microscope slide and spread to cover a circle about 1 cm in diameter.

3.3 Incubate at room temperature for 10 minutes.

3.4 Rinse the slide with filter-sterilized water.

3.5 Combine Components A and B in optimal proportions (as determined from optimization procedures in *Optimization of Bacterial Staining in Suspension*) in a microfuge tube and add 3 µL of this solution per mL of bacterial suspension.

3.6 Apply 5 µL of stained bacteria to the poly-L-lysine–coated area of the slide.

3.7 Place an 18 mm square coverslip over the suspension and seal with melted paraffin or other sealant.

3.8 Incubate the preparation for 5–10 minutes at room temperature in the dark to allow bacteria to adhere to the slide.

3.9 Observe the sample in a fluorescence microscope that is equipped with any of the filter sets described in Table 1.

Staining Bacteria Immobilized on Membranes

Bacteria may be stained either before or after filtration, although staining prior to filtration generally produces superior results. Filters with low dye binding and superior flatness should be used. Blackened polycarbonate filter membranes with a 13 mm–diameter and 0.2 µm pores are typically used in conjunction with drain disc-support membranes, which are placed beneath the filters to promote uniform distribution of bacteria on the filter surface.

4.1 Prepare and stain bacteria as in *Culture Conditions and Preparation of Bacterial Suspensions* and *Optimization of Bacterial Staining in Suspension*.

4.2 For vacuum filtration, filter the bacteria onto a 13 mm–diameter membrane under low vacuum using a stainless steel vacuum filtration apparatus. For pressure filtration, filter the bacteria using a 13 mm–diameter filter membrane secured in a stainless steel Swinney filter holder that is attached to a syringe apparatus.

4.3 Place 4 µL of sterile water on a glass microscope slide.

4.4 Remove the filter and drain disc together and place both, bacteria side up, on top of the water droplet.

4.5 Add 6–10 µL of *Bac*Light mounting oil to the top of the filter.

4.6 Place an oversized coverslip (22 mm square) on top of the mounting oil and apply gentle pressure to spread the fluid over the filter. Do not spread the mounting oil past the edge of the filter.

4.7 Seal the coverslip with melted paraffin or other suitable sealant.

4.8 Observe the sample in a fluorescence microscope equipped with any of the filter sets listed in Table 1.

Note

The LIVE *Bac*Light Bacterial Gram Stain Kit has been tested on the gram-negative bacterial species *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella oranienburg* and *Shigella sonnei* and the gram-positive species *Bacillus cereus, Micrococcus luteus, Staphylococcus aureus* and *Streptococcus pyogenes.* Tests were performed on logarithmically growing cultures of these organisms.

Fluorescence Spectroscopy

Overview

The relative proportions of Components A and B required for fluorescence spectroscopy of bacterial cells will typically be significantly lower than those recommended for fluorescence microscopy. Consequently, when bacteria are stained according to the following protocol, determination of their gram sign by fluorescence microscopy will be less conclusive than if the bacteria were stained according to the fluorescence microscopy protocol (see *Fluorescence Microscopy Protocols*). Because bacteria may vary in size and nucleic acid content, it is important to note that the cell densities (bacteria/mL) of different species of bacteria are typically not equal in suspensions that exhibit equal fluorescence intensities. Instead, the ratio of green to red fluorescence emission is proportional to the *relative amount of fluorescence* of the two bacterial suspensions. Such assays can be used to measure shifts in populations of gram-negative and gram-positive organisms, but the absolute numbers of bacteria in the suspension must be adjusted to balance their relative contributions to the total fluorescence of the mixed population. The following protocols are provided as examples for potential applications of the LIVE *Bac*Light Bacterial Gram Stain Kit. They may require modification for use with different organisms.

Culture Conditions and Preparation of Bacterial Suspensions

5.1 Grow 30 mL cultures of *Escherichia coli* (gram-negative) and *Staphylococcus aureus* (gram-positive) to late log phase in nutrient broth (e.g., DIFCO catalog number 0003-01-6).

5.2 Concentrate 25 mL of each bacterial culture (separately) by centrifugation at 10,000 × g for 10–15 minutes.

5.3 Remove the supernatants and resuspend the pellets from each bacterial culture in 2 mL of filter-sterilized water (filtered through a 0.2 µm pore–size filter to remove particulate matter).

5.4 Dilute each concentrated bacterial suspension to a final volume of 20 mL with filter-sterilized water.

5.5 Determine the optical density at 670 nm (OD_{670}) of a 3 mL aliquot of each bacterial suspensions in an absorption cuvette (1 cm pathlength).

5.6 Adjust the *E. coli* suspension to 5×10^7 bacteria/mL (~0.015 OD_{670}) and the *S. aureus* suspension to 2.5×10^6 bacteria/mL (~0.037 OD_{670}). *S. aureus* suspensions typically should be 20-fold less concentrated than *E. coli* for this fluorometric test.

Staining Bacterial Suspensions

6.1 Mix five different proportions of *E. coli* and *S. aureus* (prepared in step 5.6) in acrylic, glass or quartz fluorescence cuvettes (1 cm pathlength) (Table 2). The total volume of each of the five samples will be 3 mL.

6.2 Prepare 1.67 mM SYTO 9 dye, 0.17 mM hexidium iodide working solution. Mix 25 µL of Component A, 1.8 µL of Component B and 23 µL DMSO.

6.3 Add 9 µL of 1.67 mM SYTO 9, 0.17 mM hexidium iodide solution prepared in step 6.2 to each of the five samples (5 samples × 9 µL = 45 µL total) and mix thoroughly by pipetting up and down several times.

6.4 Incubate at room temperature in the dark for 15 minutes.

Fluorescence Spectroscopy and Data Analysis

7.1 Measure the fluorescence emission spectrum (excitation 470 nm, emission 480–700 nm) of each cell suspension (F_{cell}) in a fluorescence spectrophotometer (Figure 1a).

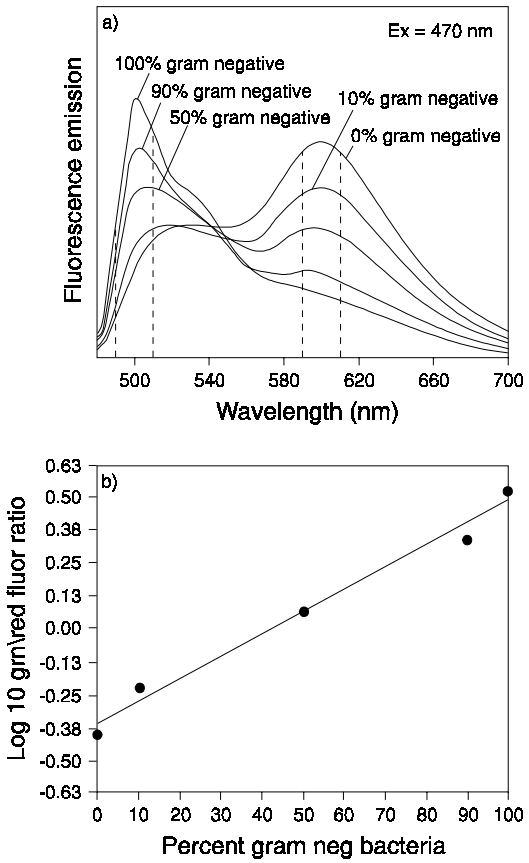

Figure 1. *Analysis of the proportion of E. coli in suspension by fluorescence spectroscopy. a) Emission spectra of suspensions of various proportions of E. coli and S. aureus suspensions were obtained from samples prepared and stained as outlined in section 5.2–5.3. Integrated fluorescence emission intensities (see Figure 1b) were determined from the spectral regions indicated by dashed vertical lines. b) Integrated intensities of the green (490–510 nm) and red (590–610 nm) emission were acquired and the green/red fluorescence ratios ($Ratio_{G/R}$) were calculated for each proportion of E. coli suspension according to the equation in 7.2. The line is a least-squares fit of the relationship between % gram-negative bacteria (E. coli, x) and log_{10} $Ratio_{G/R}$ (y).*

Table 2. *Volumes of E. coli and S. aureus bacterial suspensions to mix to achieve various proportions of the suspensions.*

Ratio of *E. coli* : *S. aureus* Bacterial Suspensions	mL *E. coli* Cell Suspension	mL *S. aureus* Cell Suspension
0 : 100	0	3.0
10 : 90	0.3	2.7
50 : 50	1.5	1.5
90 : 10	2.7	0.3
100 : 0	3.0	0

7.2 Calculate the ratio of the integrated intensity of the portion of each spectrum between 490–510 nm (em1; green) to that between 590–610 (em2; red) for each bacterial suspension.

$$\text{Ratio}_{G/R} = \frac{F_{cell,em1}}{F_{cell,em2}}$$

7.3 Plot the log_{10} of the ratio of integrated green fluorescence to integrated red fluorescence ($Ratio_{G/R}$) versus percentage of *E. coli* suspension (Figure 1b).

Fluorescence Microplate Readers

Overview

Conditions required for measurement of fluorescence in microplate readers are very similar to those required for fluorescence spectroscopy of bacterial cell suspensions. As in fluorescence spectroscopy experimental protocols, reagent concentrations are typically lower than those recommended for fluorescence microscopy, and the ratio of green to red fluorescence emission is proportional to the *relative amount of fluorescence* of the two bacterial populations even though the absolute cell densities may be very different.

Culture Conditions and Preparation of Bacterial Suspensions

8.1 Grow 30 mL cultures of *E. coli* and *S. aureus* to log phase in nutrient broth (e.g., DIFCO catalog # 0003-01-6).

8.2 Concentrate 25 mL of each bacterial culture (separately) by centrifugation at 10,000 × g for 10–15 minutes.

8.3 Remove the supernatants and resuspend the pellets from each bacterial culture in 2 mL of filter-sterilized water (filtered through a 0.2 µm pore–size filter to remove particulate matter).

8.4 Dilute each concentrated bacterial suspension to a final volume of 20 mL with filter-sterilized water.

8.5 Determine the optical density at 670 nm (OD_{670}) of a 3 mL aliquot of each bacterial suspension in an absorption cuvette (1 cm pathlength).

8.6 Adjust the *E. coli* suspension to 1×10^8 bacteria/mL (~0.030 OD_{670}) and the *S. aureus* suspension to 5×10^6 bacteria/mL (~0.074 OD_{670}). *S. aureus* suspensions typically should be 20-fold less concentrated than *E. coli* in these experiments.

8.7 Mix five different proportions of *E. coli* and *S. aureus* (Table 3) in 16 × 125 mm borosilicate glass culture tubes. The total volume of each of the five samples will be 2 mL.

Staining Bacterial Suspensions

9.1 Prepare a 2X stain solution by adding 40 µL of 1.67 mM SYTO 9 dye, 0.17 mM hexidium iodide (working solution prepared in step 6.2) to 6.6 mL of filter-sterilized water in a 16 × 125 mm borosilicate glass culture tube and mix well.

9.2 Pipet 100 µL of the bacterial cell suspension into each test well of a 96-well flat-bottom microplate. The outside wells (rows A, G and H and columns 1 and 12) are usually kept empty to avoid spurious readings (Table 4).

9.3 Using a new tip for each row, pipet 100 µL of the 2X staining solution (from step 9.1) to each appropriate well.

9.4 Incubate with continuous mixing on a microplate shaker at room temperature in the dark for 15 minutes.

Table 3. *Volumes of E. coli and S. aureus bacterial suspensions to mix to achieve various proportions of the suspensions.*

Ratio of *E. coli* : *S. aureus* Bacterial Suspensions	mL *E. coli* Cell Suspension	mL *S. aureus* Cell Suspension
0 : 100	0	2.0
10 : 90	0.2	1.8
50 : 50	1.0	1.0
90 : 10	1.8	0.2
100 : 0	2.0	0

Table 4. *Suggested microplate configuration.*

Row	Columns	Contents
row B	columns 2–11	0% *E. coli* suspension
row C	columns 2–11	10% *E. coli* suspension
row D	columns 2–11	50% *E. coli* suspension
row E	columns 2–11	90% *E. coli* suspension
row F	columns 2–11	100% *E. coli* suspension

Fluorescence Measurements and Data Analysis

10.1 With the excitation wavelength centered at about 485 nm, measure the fluorescence intensity at a wavelength centered at about 530 nm (emission 1; green) for each well of the entire plate.

10.2 With the excitation wavelength still centered at about 485 nm, measure the fluorescence intensity at a wavelength centered about 620 nm (emission 2; red) for each well of the entire plate.

10.3 Analyze the data by dividing the fluorescence intensity of the stained bacterial suspensions (F_{cell}) at emission 1 by the fluorescence intensity at emission 2.

$$\text{Ratio}_{G/R} = \frac{F_{cell,em1}}{F_{cell,em2}}$$

10.4 Plot the $\text{Ratio}_{G/R}$ versus the percentage of live cells in the *E. coli* suspension (Figure 2).

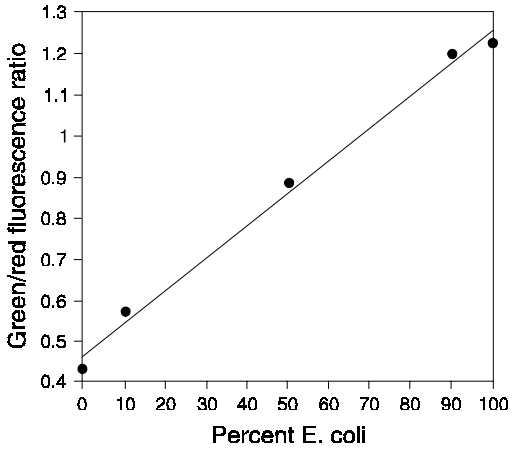

Figure 2*. Analysis of the proportion of E. coli in suspension determined using a fluorescence microplate reader. Samples of E. coli were prepared and stained as outlined in section 6.2–6.3. The integrated intensities of the green (530 ± 12.5 nm) and red (620 ± 20 nm) emission of suspensions excited at 485 ± 10 nm were acquired and the green/red fluorescence ratios ($Ratio_{G/R}$) were calculated for each proportion of E. coli suspension according to the equation in 9.2. The line is a least-squares fit of the relationship between % gram-negative bacteria (x) and $Ratio_{G/R}$ (y).*

References

Appl Environ Microbiol 56, 2245 (1990)

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
L-7005	LIVE *Bac*Light™ Bacterial Gram Stain Kit *for microscopy and quantitative assays* *1000 assays* .	1 kit
V-7023	ViaGram™ Red+ Bacterial Gram Stain and Viability Kit *200 assays* .	1 kit

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