

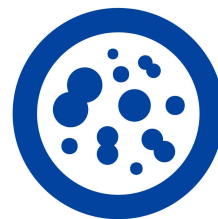
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Version 2

Bacterial Staining and Microscopy V.2

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Carlos Goller¹

¹Biotechnology Teaching Program (BIT), North Carolina State University

BIT-Protocols

Tech. support phone: +91 95134-135 email: ccgoller@ncsu.edu



Carlos Carlos Goller

Biotechnology Teaching Program (BIT), North Carolina State U...

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We use this protocol and it's working

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Abstract

Overview and Goals

Now that you have streaked your unknown bacteria for isolation, you will take steps to identify certain properties of the organisms via a variation of the gram stain. Broadly, bacteria can be categorized into gram-positive and gram-negative groups based on the presence or absence of an outer cell membrane and a thick peptidoglycan layer (cell wall).

Learning Objectives

After completing this lab, you will gain the following lab skills:

- Lab safety and proper personal protective equipment (PPE)
- Use of gram staining/ gram staining alternative to identify the gram status of a bacterial isolate
- Use of a fluorescent microscope for the detection of red/green fluorescence

Before starting

Review the complete protocol before beginning. Several of the steps in this procedure are time and/or temperature-sensitive, so you must know what to expect and how to manage your time. Work as a team! There are also several types of tubes with specific names that need to be used for specific protocol steps. They are shown in the illustration below to help you keep track of these materials.

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Guidelines

Clean-Up Instructions

- All liquids containing bacteria must be poured into a beaker for bleach decontamination (can be found on the blue bench paper by this sink labeled "liquid").
- All bacterial growth plates can be thrown away in the biohazard trash
- Return all reusable materials to our storage drawers.
- Wipe down all surfaces with Conflit and then ethanol.
- Remove PPE.
- Wash hands.

Materials

Each group will need:

- Slides and cover slips
- Bacterial culture/plate for isolates
- BUG agar plate with bacteria (incubated overnight at 33°C from your TSA plates)
- **Invitrogen LIVE BacLight kit**
- **MycoLight[®] rapid fluorescence bacterial gram stain kit**
- Micropipette tips for p200, p20, and p10
- Wipes
- Tip disposal container

Safety warnings

! Ask your instructor for instructions on how to dispose of the unused dye solutions.

Before start

Review the complete protocol before beginning. Several of the steps in this procedure are time and/or temperature-sensitive, so you must know what to expect and how to manage your time. Work as a team! There are also several types of tubes with specific names that need to be used for specific protocol steps. They are shown in the illustration below to help you keep track of these materials.



Protocol 1: Invitrogen BacLight Kit

53m

- 1 Take bacteria from a plate using a sterile swab. 1m
- 2 Combine equal volumes of Component A and Component B in a microfuge tube and mix thoroughly. 2m
- 3 Add  3 μL of the dye mixture per mL of bacterial suspension. 3m
- 4 Mix thoroughly and incubate at room temperature in the dark for 15 minutes.
 00:15:00 in the dark at room temperature
  15m
- 5 Trap  5 μL of each stained bacterial suspension between a slide and an 18 mm square coverslip. 2m
- 6 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. 15m
 

	A	B	C
	-	SYTO 9	Hexidium iodide
	Excitation/Emission	480/500 nm	518/600 nm
	Standard filter set	GFP	RFP
	Storage conditions	-20°C, protect from light	-20°C, protect from light

Table 1. Live gram-negative organisms should fluoresce green and gram-positive bacteria should fluoresce red. Source: Invitrogen LIVE BacLight kit.

- 7 **Cleanup Instructions.** All liquids containing bacteria must be poured into a beaker for bleach decontamination (can be found on the blue bench paper by this sink labeled "liquid"). 5m

- 7.1 All bacterial growth plates can be thrown away in the biohazard trash 1m



7.2 Return all reusable materials to our storage drawers.

1m

7.3 Wipe down all surfaces with Conflkt and then ethanol.

1m

7.4 Remove PPE.

1m

7.5 Wash hands.

1m

8 Save images and analyze results.

5m

Note

Critical Thinking Questions: Microscopy Protocol 1

1. What advantages and disadvantages can you think of when using fluorescent dyes instead of crystal violet (the traditional gram stain dye)?
2. Why do you think it is important that SYTO9 and Hexidium Iodide not have significantly overlapping emission spectra?
3. An iodine and then an alcohol wash are the final steps of a gram stain. Based on your understanding of lipid membranes and cell walls, what do you think the purpose of these reagents is? (Hint: iodine creates a high molecular weight complex with crystal violet)
4. Why might it be important to use a gram stain or acceptable alternative to identify the gram status of a bacterial isolate in a medical setting?

Protocol 2: MycoLight Kit

1h

9

5m



Safety information

Review the protocol. Please review the complete protocol before beginning. Several of the steps in this procedure are time—and/or temperature-sensitive, so it's important that you know what to expect and how to manage your time.

We will thaw all the kit components at room temperature before use and spin down briefly. We have created single-use aliquots to avoid freeze-thaw cycles and stored them at -20°C after preparation. We prepared the MycoLight™ Red stock solution previously by adding 100 µL of ddH₂O into one vial of MycoLight™ Red (Component B) and mixing well.

Instructors have also prepared the MycoLight™ dye working solution by mixing equal volume of MycoLight™ Green (Component A) and MycoLight™ Red stock solution in a tube.

- 9.1 Grow isolates overnight by picking a colony from an agar plate and inoculating  2.5 mL of Tryptic Soy Broth (TSB). Grow at  30 °C for 16-18 hours. Dilute in fresh medium and grow for 2-3 hours. The instructors will perform this step.   
- 9.2 We will prepare bacteria samples with concentrations around 10⁷ cells/ml. Grow bacteria into late log phase in an appropriate medium. **Note:** According to the manufacturer, for *E. coli* culture, OD600 = 1.0 equals 8 × 10⁸ cells/ml. The instructors will help with this step.  
- 9.3 Remove medium by centrifugation at  10.000 x g, 00:10:00 and re-suspend the pellet in ddH₂O, adjust bacteria concentration to ~ 10⁷ cells/ml. 10m
- 10 Add  2 µL MycoLight™ dye working solution to  100 µL of the bacterial suspension. 2m
- 11 Mix well and incubate in the dark for  00:15:00 at room temperature. You can store the solution in a 1.5 ml tube in your drawer. 15m
- 12 Remove the working solution by centrifugation at  10000 x g, 00:10:00 10m
- 13 Resuspend the bacteria pellet in  100 µL volume of ddH₂O. 1m

- 14 Add  10 μL of stained cells to a clean slide and cover with a coverslip. Provide the rest of the stained cells to your instructor for imaging. **Table 2.** MycoLight Bacterial Staining

2m



	A	B	C
		MycoLight Green	MycoLight Red
Excitation/Emission		488/530 nm	650/669 nm
Standard filter set		FITC	Cy5
Storage conditions		-20°C, protect from light	-20°C, protect from light
Staining		Gram-negative. Example: Escherichia coli	Gram positive. Example: Bacillus subtilis cells

Table 2. MycoLight Bacterial Staining

- 15 Monitor the fluorescence of bacteria with a fluorescent microscope or the Agilent LionHeart.

15m



Note

Critical Thinking Questions: Microscopy Protocol 2

1. What do your results suggest about your isolate?
2. What shape does your organism have?
3. Do your results support the previous staining procedure we used? Why or why not?