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Bacterial Staining and Microscopy V.3

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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 Conflikt and then ethanol.
- Remove PPE.
- Wash hands.

Materials

Each group will need:

- Slides and cover slips
- Sterile PBS
- 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 disposeof 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 (1 or 2 colonies) from a plate using a sterile swab or loop. 1m

2 Mix bacteria into 1 mL PBS (bacterial suspension).

3 Combine equal volumes of Component A and Component B in a microfuge tube and mix thoroughly. You were given 5 µL of each component. 2m

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

5 Mix thoroughly and incubate at room temperature in the dark for 15 minutes. 15m
00:15:00 in the dark at room temperature

6 Trap 20 µL of each stained bacterial suspension between a slide and a 24×50 mm rectangular coverslip. 2m

7 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.

8 **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 5m

"liquid").

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

8.2 Return all reusable materials to our storage drawers. 1m

8.3 Wipe down all surfaces with Conflikt and then ethanol. 1m

8.4 Remove PPE. 1m

8.5 Wash hands. 1m

9 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

10 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.

10.1 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.

10.2 We will prepare bacteria samples with concentrations around 10^7 cells/ml. Grow bacteria into late log phase in an appropriate medium. **Note:** According to the manufacturer, for *Escherichia coli* culture, OD600 = 1.0 equals 8 × 10^8 cells/ml. The instructors will help with this step.

10.3 Remove medium by centrifugation at 10.000 x g, 00:10:00 and re-suspend the pellet in ddH2O, adjust bacteria concentration to ~ 10^7 cells/ml. 10m

11 Add 2 µL MycoLight™ dye working solution to 100 µL of the bacterial suspension. 2m

12 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

13 Remove the working solution by centrifugation at 10000 x g, 00:10:00 10m

14 Resuspend the bacteria pellet in 100 µL volume of ddH2O. 1m

15 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

16 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?