Biotechnology Science for the New Millennium by Ellyn Daugherty

Characterizing Bacteria Using Gram Staining

(Lab 4L)

(Cat. # BE-202)

Developed in partnership with

Characterizing Bacteria Using Gram Staining (Lab 4L)

Teacher's Guide

The following laboratory activity is adapted from "Laboratory 4L: Characterizing *E. coli* Using a Light Microscope and Gram Staining" from *Biotechnology: Laboratory Manual* by Ellyn Daugherty. For more information about the program, please visit www.emcp.com/biotechnology. This kit is produced under license from Paradigm Publishing, Inc., a division of New Mountain Learning.

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About Ellyn Daugherty: Ellyn Daugherty is a veteran biotechnology educator and recipient of the Biotechnology Institute's National Biotechnology Teacher-Leader Award. She is the founder of the San Mateo Biotechnology Career Pathway (SMBCP). Started in 1993, SMBCP has instructed more than 10,000 high school and adult students. Annually, 30-40 SMBCP students complete internships with mentors at local biotechnology facilities.

About G-Biosciences: In addition to the Biotechnology by Ellyn Daugherty laboratory kit line and recognizing the significance and challenges of life sciences education, G-Biosciences has initiated the BioScience Excellence™ program. The program features hands-on teaching kits based on inquiry and curiosity that explore the fundamentals of life sciences and relate the techniques to the real world around us. The BioScience Excellence™ teaching tools will capture the imagination of young minds and deepen their understanding of various principles and techniques in biotechnology and improve their understanding of various social and ethical issues.

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Upon receipt, store the materials as directed in the package literature.

MATERIALS INCLUDED

This kit has enough materials and reagents for 8 lab groups ((8 student pairs or 4 groups of 32 students).

- 1 vial *Kocuria rhizophilia* Agar Stab
- 1 vial *Bacillus subtilis* Agar Stab
- 1 vial *E. coli* K-12 Agar Stab
- 3 Inoculating loops, sterile, plastic
- 3 vials sterile LB Broth
- 3 Transfer pipettes (large)
- 8 vials of Gram Crystal Violet (stain), 2 ml
- 8 vials of Gram Iodine (mordant), 2 ml
- 8 vials of Gram Stain Decolorizer (alcohol), 2 ml
- 8 vials of Gram Safranin (stain), 2 m
- 24 Glass Microscope Slides
- 32 Transfer pipettes (small)

ADDITIONAL EQUIPMENT & MATERIALS REQUIRED

- Shaking incubator, 37°C or equivalent
- Bunsen Burner and flint igniter
- Compound microscopes
- Paper towel
- Permanent lab markers
- Beakers (filled with tap water)
- 10% bleach solution (disinfectant), freshly prepared and disinfectant towels

OPTIONAL MATERIALS:

- 100X objectives and immersion oil

SPECIAL HANDLING INSTRUCTIONS

- Store all agar stabs at 4°C until ready to prepare overnight cultures. All other components can be stored at room temperature until ready to use.
- Briefly centrifuge all small vials before opening to prevent waste of reagents.
- Dispose of all plastic vessels that have contained bacteria cultures by soaking them in 10% bleach for 30 minutes, then placing them in a biohazard bags to be autoclaved and disposed, or treated by another appropriate procedure.
- The following items need to be used with caution and bottles must be kept away from open flame.

Part #	Name	Hazard
G051	Gram Crystal Violet	Flammable
G081	Gram Stain: Decolorizer Solution	Flammable

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GENERAL SAFETY PRECAUTIONS

- The reagents and components supplied in the *Biotechnology by Ellyn Daugherty*™ kits are considered non-toxic and are safe to handle (unless otherwise noted), however good laboratory procedures should be used at all times. This includes wearing lab coats, gloves* and safety goggles mean measuring and using chemicals.
- Rubber or latex gloves should not be used when using an open flame such as a Bunsen burner.
- The de-colorizer in this kit contains alcohol (EtOH), which is flammable and should not be used by hot plates or open flames.
- When using a Bunsen burner, safety goggles must be worn, and hair longer than chin length must be tied back. Anything flammable should be at least 1 foot away from the Bunsen burner. Sometimes it is hard to see the Bunsen burner flame; adjust the overhead light so the Bunsen burner flame is visible. Make sure the gas is shut off after using the Bunsen burner.
- The teacher should 1) be familiar with safety practices and regulations in his/her school (district and state) and 2) know what needs to be treated as hazardous waste and how to properly dispose of non-hazardous chemicals or biological material.
- Students should know where all emergency equipment (safety shower, eyewash station, fire extinguisher, fire blanket, first aid kit etc.) is located and be versed in general lab safety.
- Remind students to read all instructions including Safety Data Sheets (SDSs) before starting the lab activities. A link for SDSs for chemicals in this kit is posted at www.gbiosciences.com
- At the end of the lab, all laboratory bench tops should be wiped down with a 10% bleach solution or disinfectant to ensure cleanliness. Remind students to wash their hands thoroughly with soap and water before leaving the laboratory. All contaminated culture vessels, pipet tips, and inoculating loops must disinfected in bleach and autoclaved in a biohazard bag or equivalent procedure. All tubes and broth cultures containing bacteria samples are considered biohazards. To prevent ecological damage and a public health threat, all plates, tubes, and bottles containing these substances are to be disposed of only in red biohazard bags. All items that have been in contact with bacteria must be autoclaved before disposal. Make sure that all students wash their hands thoroughly with soap and water before and after working with any bacteria.

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TEACHER'S PRE EXPERIMENT SET UP

- Briefly centrifuge all small vials before opening to prevent waste of reagents.
- Preheat a shaking incubator to 37°C prior to starting the preparation of the bacterial broth cultures.
- Wipe down the lab tabletop with disinfectant towels before starting broth cultures.
- Place all bacteria-contaminated pipets and tips into a 10% bleach solution. Allow contaminated items to soak for 30 minutes before disposing.

1. In the presence of the class, label the LB Broth vials with the bacteria to be cultured. Using a sterile micropipette, transfer 0.8ml LB broth from one of the 2ml LB Broth vial to the bacterial agar stab and incubate at 37°C for 30 minutes.
2. Vigorously shake or vortex for 1-2 minutes, then transfer, with a sterile micropipette, 0.6ml LB broth from the agar stab to the original labelled LB Broth vial.
3. Cap the vial and swirl the vial to distribute the bacteria throughout the broth. Place the vial in a 37°C shaking incubator or water bath for 4-6 hours or leave at room temperature overnight, swirling it to mix several times through the day.
4. Repeat, the steps above to inoculate and incubate the other vials of LB broth with each of the other bacteria samples. Label each vial.
5. Give each group the 1-2 clean glass beakers, a lab marker, paper towels and distribute other reagents prior to starting the Gram staining.
6. Briefly centrifuge all small tubes and vials containing the stains/reagents before opening to prevent waste of reagents. Distribute the following to each group:
    - 1 vial of Gram Crystal Violet (stain), 2 ml
    - 1 vial of Gram Iodine (mordant), 2 ml
    - 1 vial of Gram Stain Decolorizer (alcohol), 2 ml
    - 1 vial of Gram Safranin (stain), 2 ml
    - 3 Glass Microscope Slides
    - 4 Small Transfer pipettes (1 each for the 2 stains, mordant, and de-colorizer)
7. At a disinfected table top, place each of the labeled 3 bacterial cultures on paper towel matting. Place the cultures about 2 feet apart to avoid confusion. Place a large transfer pipettes on the paper towel next to a culture for students to use take a sample for their slide preparation.
8. Review all the steps in the Procedure with students pointing out the safety hazards (fixing the bacteria can be a burn hazard) and the de-colorizer is flammable.
9. Remind students that starting with very clean slides is very important since dust will look like bacteria on a slide. Remind students not to let their slides sit in the flame and get too hot otherwise their bacteria will melt. The

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slides are just passed through the flame. Students must decolorize until no crystal violet stain is running off the slide. Otherwise everything will look purple.

10. Good microscopy is very important in studying the slides. Make sure that the tiny flecks of bacteria are centered and in sharp focus on 100X before going to 400X, and center and focused on 400X before adding the immersion oil and going to 1000X. Students often use too much light when using a microscope. Decreasing the diaphragm will increase the contrast. Have them start near the lowest light setting.

11. Place all bacteria-contaminated pipets and tips into a 10% bleach solution. Allow contaminated items to soak for 30 minutes before disposing. Autoclave bacteria cultures to sterilize or fill a culture with an equal volume of 10% bleach and leave overnight before discarding.

TIME REQUIRED

- 30 minutes, the day before Gram staining, to start the overnight cultures
- 30 minutes for distribution of materials and pre-lab discussion
- 1 hour to prepare Gram-stained slides
- 1 hour for microscopy to observe and analyze slides/samples

NEXT GENERATION SCIENCE STANDARDS ADDRESSED

- HS-LS1: From Molecules to Organisms: Structures and Processes
- LS1.A: Structure and Function

For more information about Next Generation Science Standards, visit: http://www.nextgenscience.org/

EXPECTED RESULTS

E. coli cells are Gram$^-$ bacillus bacteria and should appear as tiny individual reddish-pink rods at 400X magnification or higher.

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OBJECTIVES

After Gram staining, what is the difference in appearance between Gram– and Gram+ cells?

What is the shape and Gram-staining group of *E. coli* cells?

BACKGROUND

When bacteria cells are collected or cultured, how does a researcher have confidence that a sample contains a particular bacterium of interest?

Scientists use a variety of methods to study bacteria characteristics and collect data used in bacteria identification. These methods include microscopy, contrast staining, and nutritional, reproductive, and genetic studies. Once their traits are characterized, it is easier to recognize and identify a species of bacteria.

Light microscopy is used to study cell structure (morphology), cell shape, and growth pattern (whether bacteria grow as individual cells, or pairs, or in chains or groups). There are three common shapes of bacteria: coccus (spherical), bacillus (rod-shaped), and spirillum (spiral). Using a compound, light microscope, these shapes are easily seen on high power (a total magnification of 400X or greater) and may be used in bacteria identification to put a sample into one of the three shape groups.

Staining bacteria samples further separates bacteria into different groups. Selective staining (picking certain cell stains to use) increases the contrast of structures in microscopic samples. One type of selective staining, called Gram staining, is used to group bacteria into two groups based on the characteristics of their cell wall.

The Gram staining method was first described in 1844 by the Danish bacteriologist Hans Christian Gram, after whom the test was named. The Gram staining test for bacteria is one of the most important tests in microbiology and is often one of the first tests performed in the identification of bacteria.

Gram staining separates the three shape groups into two color groups: purple or red coccus, bacillus, or spirillum. Gram-stained samples can be separated into one of these six groups.

The purple or red coloration is determined by the bacterium's cell wall type. Bacteria that have a thick cell wall of peptidoglycan with a relatively low lipid content retain a purple stain, called crystal violet, and turn dark purple in color. These are called $Gram^{+}$ bacteria because they take up and retain the crystal violet stain.

Bacteria that have a cell wall with a relatively high lipid content (and lower peptidoglycan content) lose the purple color during de-colorization and must be stained with a contrasting red counterstain, called safranin. These bacterial cells appear reddish-pink and are called $Gram^{-}$ bacteria because they have not retained the purple color of the crystal violet stain.

When properly Gram-stained, *Kocuria rhizophilia* appears as purple spheres ($Gram^{+}$ cocci) and *Bacillus subtilis* appears as light red rods ($Gram^{-}$ bacilli) at 400-1000X magnification. These two bacteria will be used as positive controls for comparison when Gram-staining a sample of *E. coli*.

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MATERIALS FOR EACH GROUP

Supply each group with the following components. The bacterial cultures are shared by the whole class and should be kept on a communal table.

- 1-2 clean glass beakers, 250 ml, ½ full of tap water
- 1 vial of Gram Crystal Violet (stain), 2 ml
- 1 vial of Gram Iodine (mordant), 2 ml
- 1 vial of Gram Stain Decolorizer (alcohol), 2 ml
- 1 vial of Gram Safranin (stain), 2 ml
- 3 Glass Microscope Slides
- 4 Small Transfer pipettes (1 each for the 2 stains, mordant, and de-colorizer)

NOTE: Have students label the transfer pipettes with appropriate solution name to prevent cross contamination of reagents.

ADDITIONAL MATERIALS FOR EACH GROUP

The following standard lab equipment should be available for each group.

- Permanent lab marker
- Deionized water
- Paper towels
- Bunsen burner
- Microscope, compound, with 100X objective lens if available
- 10% bleach solution (disinfectant), freshly prepared and disinfectant towels

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PROCEDURE

1. On the left side of the slide, with a permanent marker, label three slides with K, B, or E (to represent the 3 bacteria being tested) plus your group ID.

2. Swirl each bacteria culture before taking a sample to suspend the bacteria.

3. Using the labeled, large transfer pipet, place a small drop of the *Kocuria rhizophilia,* overnight culture on the appropriately labeled slide. Use the side of the transfer pipet to spread the sample over the entire slide to form a thin film of bacteria.

4. Repeat step 3 with each bacteria sample until all three slides have a thin film of the appropriate bacteria.

5. Allow the film on each slide to air dry completely.

6. To "fix" the *Kocuria rhizophilia* bacteria to its slide, hold the slide specimen side up, by its edge and quickly pass the slide across a Bunsen burner flame 10-20 times. Make sure the slide does not stop in the flame or it will be overheated. Repeat this fixing step with the other 2 slides.
   CAUTION: *Ensure that the students are carefully supervised during this stage. Or fix the bacteria to the slides for the students to minimize the dangers of a naked flame.*

7. Place the 3 slides, specimen side up, on a large piece of paper towel. Make sure you can still see each label. If not, re-label the slides. Be ready to fill and refill a beaker with clean tap water for use in step 8.

8. One at a time, cover the entire area of bacteria on each slide with Gram Crystal Violet (about 500µl) and leave at room temperature for 1 minute. After, 1 minute, rinse the crystal violet off each slide by gently dunking the slide up and down for 5 seconds in a beaker of clean tap water. The specimen should appear blue-violet when observed with the naked eye.

9. Place the 3 slides, specimen side up, back on a large piece of paper towel. One at a time, cover the entire area of bacteria on each slide with the Gram Iodine and leave at room temperature for 1 minute. Rinse each slide for 5 seconds by gently dunking the slide up and down for 5 seconds in a beaker of clean deionized water. then immediately proceed to the next step. At this point the specimen should still be blue-violet.

10. Holding one of the slides over an empty beaker, add the Gram Stain: Decolorizer Solution drop-wise until the blue-violet color is no longer visualized on the sample. Repeat with each sample slide.

11. Rinse each slide by gently dunking the slide up and down for 5 seconds in a beaker of clean tap water.

12. Place the 3 slides, specimen side up, back on a large piece of paper towel. Cover the bacteria one each slide with Gram Safranin and leave at room temperature for 1 minute. After, 1 minute, rinse the safranin off each slide by gently dunking the slide up and down for 5 seconds in a beaker of clean tap water.

13. Allow the slides to air dry (best if left overnight) before viewing under a compound light microscope. 400x magnification is adequate for viewing bacteria on the slide, however an oil immersion objective (100x) may be

used for high magnification and better resolution. A drop of oil can be placed directly on the slide. Observe the center of the slide where the bacteria has been treated with the Gram stains.

14. What color does the *Kocuria rhizophilia* bacteria appear under microscope? It is Gram positive bacteria and should appear purple.

15. What color does the *Bacilius subtilis* bacteria appear under microscope? It is Gram negative bacteria and should appear reddish-pink.

16. What color does *E. coli K-12* bacteria appear under microscope? Is it Gram positive or negative? Are *E. coli K-12* bacteria cocci, bacilli, or spirilla?

DATA ANALYSIS AND CONCLUSION:

Do the cells in the *E. coli K-12* bacteria culture appear to be Gram+ or Gram– bacilli? Give evidence for your answer. Describe the issues that make it difficult to distinguish tiny bacteria cells through microscopy and Gram staining.

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