Gram Stain Determination

Introduction

Bacteria can be classified according to the results of a procedure known as a Gram stain. Gram staining is often the first step in identifying an unknown bacterial species. The Gram staining technique requires relatively few reagents, is well defined, and can be successfully practiced with a few simple steps.

Concepts

- Microbiology
- Gram stain
- Gram (+) vs. Gram (–) microorganisms

Background

Gram staining is one of the most valuable tools for identifying bacteria. Gram staining is often used to separate many species into two groups based on the composition of their cell wall. *Gram-positive* bacteria have simple walls which contain a relatively large amount of peptidoglycan. Peptidoglycan is a complex which consists of polysaccharides and peptides which cross-link and form the shape of the bacteria. The walls of *gram-negative* bacteria have thin layer of peptidoglycan surrounded by a membrane bilayer which contains phospholipids and lipopolysaccharides, carbohydrates bonded to lipids. Upon gram stain treatment, gram-positive bacteria will appear purple and gram-negative bacterium will appear a pink/magenta color.

In order to obtain accurate results when gram staining bacterial cultures it is extremely important to practice *aseptic technique*. When working with microorganisms in the laboratory, it is important isolate the particular organism of interest from other microorganisms to obtain a *pure culture*. Aseptic technique is the procedure designed to keep unwanted microorganisms from contaminating sterile materials or pure cultures of microorganisms. To accurately practice aseptic technique all instruments must be sterilized either in an autoclave or, if an autoclave is unavailable, flame sterilization method may be used.

Materials

Crystal violet solution, 5–10 mL
Disinfectant such as Lysol® or a 10% bleach solution
Ethyl alcohol, 95%, 5 mL
Gram iodine, 5–10 mL
Gram safranin, 5–10 mL
Immersion oil, low viscosity
Unknown microorganisms, 3
Water, distilled or deionized
Beaker, 250-mL
Beral pipet, thin-stem
Bunsen burner
Compound microscope with oil immersion lens
Kimwipe®
Marker
Microscope
Slides, 3

Safety Precautions

Gram stain materials will stain skin and other materials. Care should be taken when sterilizing the inoculating loop and when flame-fixing slides. Wear chemical splash goggles and chemical-resistant gloves. Wash hands thoroughly with soap and water before leaving the laboratory. Follow all laboratory safety guidelines. Please review current Material Safety Data Sheets for additional safety, handling and disposal information.

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Procedure

Part A. Transferring the Microorganism to the Slide

1. Thoroughly disinfect the lab table by applying a disinfectant such as Lysol and allow it to air dry.
2. Obtain a clean dry slide, inoculating loop and a culture tube containing an unknown microorganism. If necessary, clean the microscope slide by wiping with a Kimwipe saturated with 95% ethyl alcohol.
3. Using a marker, label the slide with the appropriate unknown sample number.
4. Light the Bunsen burner and adjust to obtain a blue flame.
5. Loosen the culture tube cap but do not remove it.
6. Pass the inoculating loop through the flame causing the loop to become red hot (see Figure 1). *Note:* The tip of the loop is red hot when sterilized. Do not wave the loop back and forth or blow on it to cool it more quickly. Such action will increase the likelihood of airborne microorganisms adhering to the loop and contaminating the specimen.
7. Remove the tube cap and hold it between your little finger and ring finger of the hand holding the loop (see Figure 2).
8. Briefly flame the lip of the tube rotating the tube to ensure no microorganisms remain on the lip (see Figure 3).
9. Dip the inoculating loop into the culture. *Note:* If this tip is placed in the culture media while it is still hot it may kill the microorganisms. If a sizzling sound is heard when the loop enters the media it is too hot, repeat steps 6–8.
10. Briefly reflame the culture tube lip again, rotating the tube; recap and set aside.
11. Transfer the bacteria from the loop to the slide by rubbing the loop in a circle about the size of a dime in the center of the microscope slide.
12. Reflame the loop and repeat steps 5–11.
13. Allow the smear to evaporate. *Note:* Do not heat until the slide appears dry.
14. Once dry, briefly pass the prepared slide over the flame to kill the bacteria and fix the bacteria to the slide. The slide will "cloud" then dry as the bacteria become fixed to the glass of the slide.
15. Repeat steps 1–14 using the remaining two unknown samples.

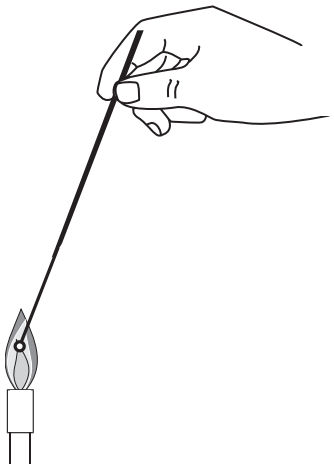

Figure 1. Flaming an inoculating loop.

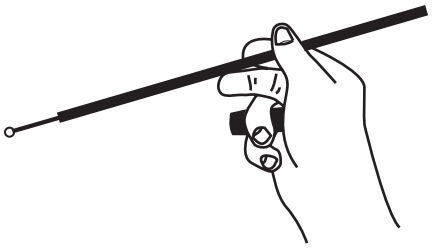

Figure 2.

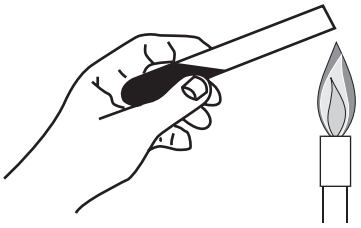

Figure 3. Flaming a test tube.

Part B. Gram Stain Unknown Prepared Slides

16. Apply Gram crystal violet stain from an eye-dropper or pipet onto and slightly beyond the smear area on the slide. Allow to sit for *one minute*.
17. Tilt the slide on an angle and gently rinse the slide with DI water from a dropper bottle by dropping water above the smear and allowing it to rinse the stain off the smear. Rinse until the solution dripping from the slide is clear.
18. Repeat steps 16–17 using the Gram iodine (stain serves as a mordant).
19. Decolorize microbial smears with 95% ethyl alcohol by applying drop-wise for approximately 30 seconds or to a tipped slide until no more color runs off. Decolorization time is determined primarily by the thickness of the smear. It is very important not to over-decolorize. Rinse with water.
20. Apply Gram safranin counter stain and let stand 30–45 seconds. Safranin serves to stain bacterium which are gram-negative that do not hold crystal violet after step 20. Rinse briefly with water as above. Allow the slide to air dry and examine under the oil-immersion lens of a compound microscope.

21. Thoroughly disinfect the lab table as directed in step 1.

Disposal

Please consult your current *Flinn Scientific Catalog/Reference Manual* for general guidelines and specific procedures governing the disposal of laboratory wastes. Gram stain solutions may be disposed of down the drain with an excess of water according to Flinn Suggested Disposal Method #26b. Iodine solution may be disposed of according to Flinn Suggested Disposal Method #12a.

Connecting to the National Standards

This laboratory activity relates to the following National Science Education Standards (1996):

Unifying Concepts and Processes: Grades K–12
Evidence, models, and explanation
Form and function
Content Standards: Grades 5–8
Content Standard C: Life Science, structure and function in living systems
Content Standards: Grades 9–12
Content Standard C: Life Science, the cell

Tips

- The instructor may wish to grow additional cultures of each microorganism before students begin activity to speed up the process so each student does not need to obtain the specimen from the same culture tube.
- Broth cultures are easier for beginners to work with. Overloading and gouging are common with solid cultures.
- Many students may find it helpful to see the instructor demonstrate this activity to reinforce proper aseptic technique before beginning.
- This activity is written with the three microorganisms being unknown so students will not be able to simply find what the answer without doing the lab. The names of the microorganisms may be given if desired. Suggested microorganisms include: *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*.

References

Campbell, N.A. *Biology;* Benjamin Cummings: San Francisco, CA; 2002; 6th Edition, p 528–529.

McHale, B. *Learning About Microbes A Laboratory Manual;* Portland, ME; 1998; p 5.

Materials for *Gram Stain Determination* are available from Flinn Scientific, Inc.

Catalog No.	Description
LM1000	Bacillus cereus, bacterial culture
LM1003	Bacillus subtilis, bacterial culture
LM1006	Escherichia coli, bacterial culture
N0064	Prepared Culture Media–Nutrient Agar

Consult your *Flinn Scientific Catalog/Reference Manual* for current prices.