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Supplemental Materials

for

Development of Gamified, Interactive, Low-Cost, Flexible Virtual Microbiology Labs That Promote Higher-Order Thinking during Pandemic Instruction

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BIOL2516K Virtual Lab

Gram Stain Technique

Read The Objectives & Introduction

Read the Protocol Steps

Watch Technique Videos

Perform the Technique

Gram Stain Technique – Objectives

Learning Objectives

1. Prepare and evaluate a Gram stain with 1000X magnified samples using a compound microscope photomicrographs.
2. Explain how the Gram stain reagents affect Gram positive and Gram negative cell walls at each step.
3. Predict the color of a Gram positive and Gram negative cell after each step of the staining process.

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Gram Stain Technique– Introduction

- Differential staining methods like the Gram stain divide bacteria into two groups based on their cell wall characteristics, and also aid in the identification of cell morphology, arrangement and size.
- It differentiates bacteria as Gram-positive or Gram-negative according to their type of cell wall.
  - Gram-positive bacteria: cell wall is a thick peptidoglycan layer and stain purple
  - Gram-negative bacteria: cell wall is a thick outer membrane plus a thin peptidoglycan layer and stain red/pink

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Gram Stain Technique – Introduction

- There are four basic steps to the Gram stain (after preparing and heat fixing a smear):

1. Primary stain: crystal violet (purple)
2. Mordant: Gram's iodine (yellow-brown)
3. Decolorizer: 85% ethanol (colorless)
4. Counterstain: safranin (reddish pink)

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Gram Stain Technique – Introduction

- The primary stain (crystal violet) is applied. This is followed by a brief rinse with water to remove excess stain.
  - All cells are stained purple at this time.

Gram Positive Cells

Gram Negative Cells

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Gram Stain Technique – Introduction

- The mordant (Gram's iodine) is applied, fixing the remaining primary stain in all cells. Iodine complexes with crystal violet to precipitate inside cells. This is followed by a brief rinse with water to remove excess iodine.
  - All cells are still stained purple at this time.

Gram Positive Cells

Gram Negative Cells

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Gram Stain Technique – Introduction

- The decolorizer (95% ethanol) is applied very briefly, dissolving lipids in the outer membrane and allowing the stain to rinse free. This is followed by a brief rinse with water to remove excess alcohol.
  - Gram positive cell walls are unaffected by brief decolorization and the cells remain purple.
  - Gram negative cells walls are dissolve by brief decolorization and the cells appear colorless.

Gram Positive Cells

Gram Negative Cells

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Gram Stain Technique – Introduction

- The counterstain (safranin) is applied, filling any cells that had been previously fully decolorized.
  - Gram positive cells are already filled with the crystal violet- iodine complex and the cells remain purple.
  - Gram negative cells are washed free of the crystal violet-iodine complex during decolorization, so the cells take up the safranin and appear reddish pink.

Gram Positive Cells

Gram Negative Cells

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Gram Stain Technique – Introduction

- The steps of the Gram stain must be performed in the correct order.
- Understanding how the different types of cell walls are affected by the reagents at each step can help you predict how cells will appear if the steps are done incorrectly.

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Gram Stain Technique – Introduction

- If the iodine is added first and crystal violet second, the crystal violet – iodine dye complex will not form and the stain will not be locked into the Gram-positive cells. Gram positive cells will not retain the purple stain when decolorization is performed. When the safranin is added, any colorless cells will be stained red/pink.
  - Gram positive cells will stain pink and be a false result.
  - Gram negative cells will stain pink as usual.

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Gram Stain Technique – Introduction

- If decolorizer is left on Gram positives too long, it will begin to degrade the cells walls and lead to Gram positive loss of due crystal violet – iodine complex from within the cells. When the safranin is added, any colorless cells will be stained red/pink.
  - Gram positive cells will stain pink and be a false result.
  - Gram negative cells will stain pink as usual.

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Gram Stain Technique – Introduction

- If decolorizer is not left on Gram negatives long enough, their cell walls will not be dissolved and the crystal violet – iodine complex will not be washed from within the cells. When the safranin is added, there is no room for the reddish/pink stain to enter the cells
  - Gram positive cells will stain purple as usual.
  - Gram negative cells will stain purple and be a false result.

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Gram Stain Technique – Introduction

- If cultures are older than 24 hrs, cell walls will begin to degrade. Degraded cells walls cannot fully retain the crystal violet –iodine dye complex and lead to partial or full decolorization of Gram positive cells. When the safranin is added, any colorless cells will be stained red/pink.
  - Gram positive cells will stain pink and be a false result.
  - Gram negative cells will stain pink as usual.

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Gram Stain Protocol

Gram Stain Technique - Protocol

Gram stain of a fixed smear:

1. Obtain a properly prepared and heat-fixed bacterial smear.
2. Place your slide smear (prepared above) on the rack above the drainage tub.
3. Cover smear with crystal violet for 1 minute. (Primary stain)
4. Rinse off the stain with distilled water from a wash bottle.

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Gram Stain Technique - Protocol

Gram stain of a fixed smear (cont.):

5. Cover smear with crystal violet for 1 minute. (Primary stain)
6. Rinse off the stain with distilled water from a wash bottle.
7. Cover smear with Gram's iodine for 1 minute. (Mordant)
8. Rinse off the iodine with distilled water from a wash bottle.

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Gram Stain Technique - Protocol

Gram stain of a fixed smear (cont.):

9. Cover smear with 95% ethanol for 10 – 20 seconds. Often, the slide is held at an angle and the alcohol is allowed to run down the length of the slide. (Decolorizer)
10. Rinse off the alcohol with distilled water from a wash bottle.
11. Cover smear with safranin for 1- 2 minutes. (Counterstain)
12. Rinse off the stain with distilled water from a wash bottle.

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Gram Stain Technique - Protocol

Gram stain of a fixed smear (cont.):

13. Blot dry with bibulous paper.
14. Observe under 1000X microscope and record your observations for morphology and cellular arrangement and stain color.
15. Evaluate the accuracy of the staining technique by comparing your results to the known results of bacterial species.

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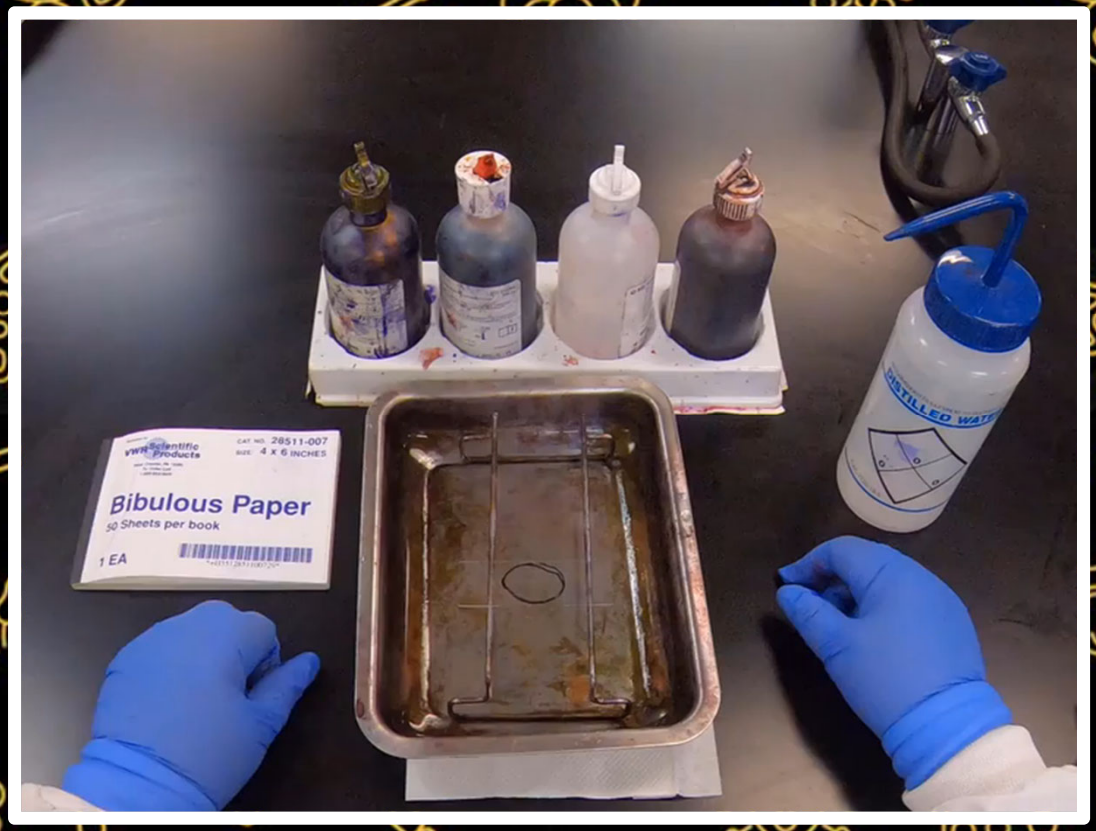
Watch Video – Gram Stain

Gram Stain Technique – Video



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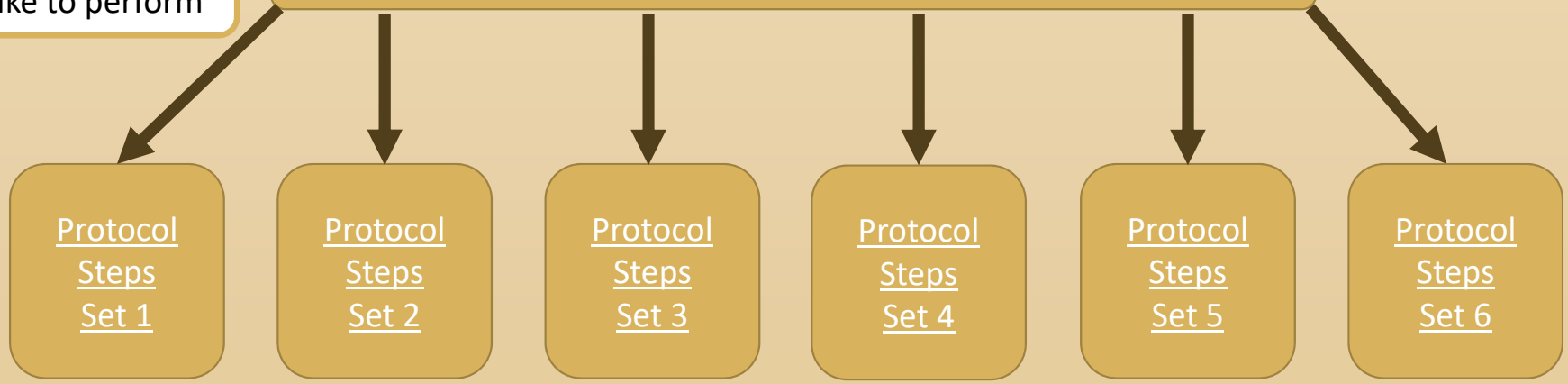
Review Gram Stain Protocol

Perform Gram Stain

Gram Stain Technique – Perform

Click on the path you would like to perform

1. Obtain a properly prepared and heat-fixed bacterial smear.



Review Gram Stain Protocol

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Gram Stain Technique: Protocol Steps Set 1

2. Place your slide smear (prepared above) on the rack above the drainage tub.
3. Cover smear with crystal violet for 1 minute, then rinse off the stain with distilled water from a wash bottle.
4. Cover smear with Gram's iodine for 1 minute, then rinse off the iodine with distilled water from a wash bottle.
5. Cover smear with 95% ethanol for 10 – 20 seconds then, rinse off the alcohol with distilled water from a wash bottle.
6. Cover smear with safranin for 1- 2 minutes then, rinse off the stain with distilled water from a wash bottle.
7. Blot dry with bibulous paper and observe under 1000X microscope.
8. Record your observations.

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Perform This Set of Protocols

Gram Stain Technique: Protocol Steps Set 2

2. Place your slide smear (prepared above) on the rack above the drainage tub.
3. Cover smear with crystal violet for 1 minute, then rinse off the stain with distilled water from a wash bottle.
4. Cover smear with Gram's iodine for 1 minute, then rinse off the iodine with distilled water from a wash bottle.
5. Cover smear with 95% ethanol for 1 minute then, rinse off the alcohol with distilled water from a wash bottle.
6. Cover smear with safranin for 1- 2 minutes then, rinse off the stain with distilled water from a wash bottle.
7. Blot dry with bibulous paper and observe under 1000X microscope.
8. Record your observations.

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Perform This Set of Protocols

Gram Stain Technique: Protocol Steps Set 3

2. Place your slide smear (prepared above) on the rack above the drainage tub.
3. Cover smear with crystal violet for 1 minute, then rinse off the stain with distilled water from a wash bottle.
4. Cover smear with Gram's iodine for 1 minute, then rinse off the iodine with distilled water from a wash bottle.
5. Cover smear with safranin for 1- 2 minutes then, rinse off the stain with distilled water from a wash bottle.
6. Blot dry with bibulous paper and observe under 1000X microscope.
7. Record your observations.

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Perform This Set of Protocols

Gram Stain Technique: Protocol Steps Set 4

2. Place your slide smear (prepared above) on the rack above the drainage tub.
3. Cover smear with crystal violet for 1 minute, then rinse off the stain with distilled water from a wash bottle.
4. Cover smear with 95% ethanol for 10 – 20 seconds then, rinse off the alcohol with distilled water from a wash bottle.
5. Cover smear with safranin for 1- 2 minutes then, rinse off the stain with distilled water from a wash bottle.
6. Blot dry with bibulous paper and observe under 1000X microscope.
7. Record your observations.

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Perform This Set of Protocols

Gram Stain Technique: Protocol Steps Set 5

2. Place your slide smear (prepared above) on the rack above the drainage tub.
3. Cover smear with safranin for 1 minute, then rinse off the stain with distilled water from a wash bottle.
4. Cover smear with Gram's iodine for 1 minute, then rinse off the iodine with distilled water from a wash bottle.
5. Cover smear with 95% ethanol for 10 – 20 seconds then, rinse off the alcohol with distilled water from a wash bottle.
6. Cover smear with crystal violet for 1- 2 minutes then, rinse off the stain with distilled water from a wash bottle.
7. Blot dry with bibulous paper and observe under 1000X microscope.
8. Record your observations.

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Perform This Set of Protocols

Gram Stain Technique: Protocol Steps Set 6

2. Place your slide smear (prepared above) on the rack above the drainage tub.
3. Cover smear with Gram's iodine for 1 minute, then rinse off the stain with distilled water from a wash bottle.
4. Cover smear with crystal violet for 1 minute, then rinse off the iodine with distilled water from a wash bottle.
5. Cover smear with 95% ethanol for 10 – 20 seconds then, rinse off the alcohol with distilled water from a wash bottle.
6. Cover smear with safranin for 1- 2 minutes then, rinse off the stain with distilled water from a wash bottle.
7. Blot dry with bibulous paper and observe under 1000X microscope.
8. Record your observations.

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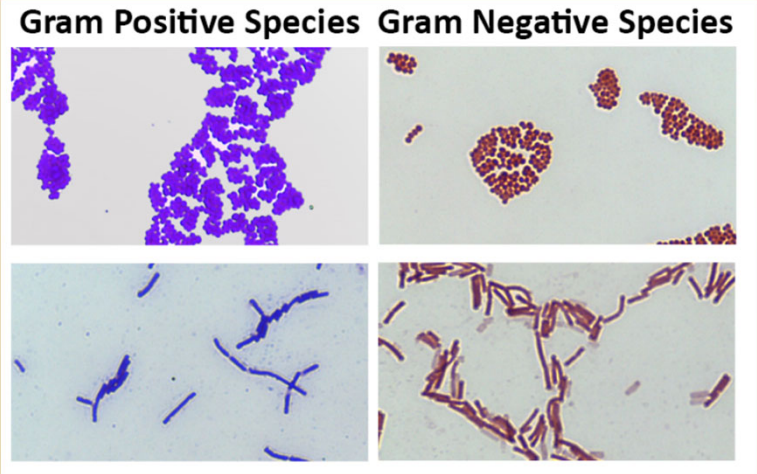
Perform This Set of Protocols

Gram Stain Technique: Protocol Steps Set 1

- You followed the **CORRECT** protocol!
- You can see the expected colors for each species!



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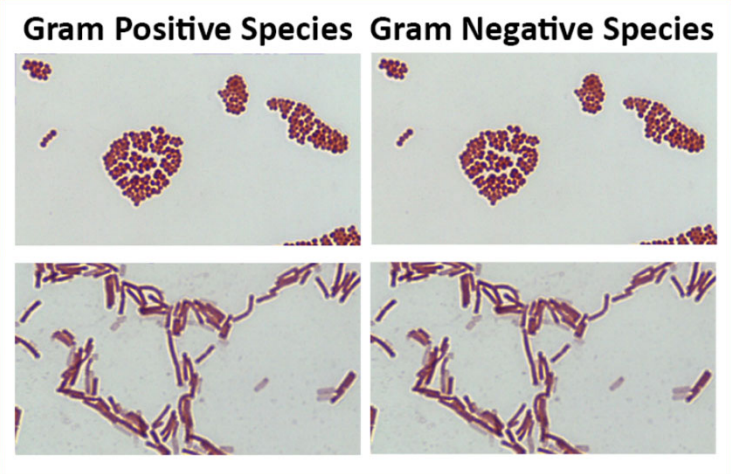
Gram Stain Technique: Protocol Steps Set 2

- You followed an **INCORRECT** protocol!
- You left the 95% ethanol on too long and all cells were completely decolorized!



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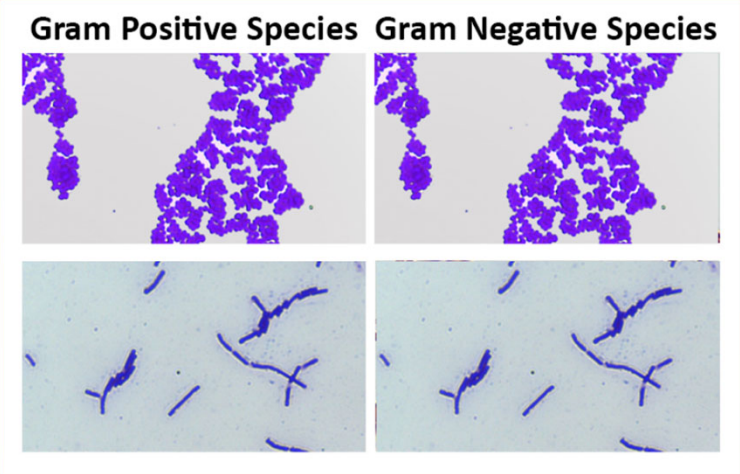
Gram Stain Technique: Protocol Steps Set 3

- You followed an **INCORRECT** protocol!
- You skipped the 95% ethanol step and no cells were decolorized!



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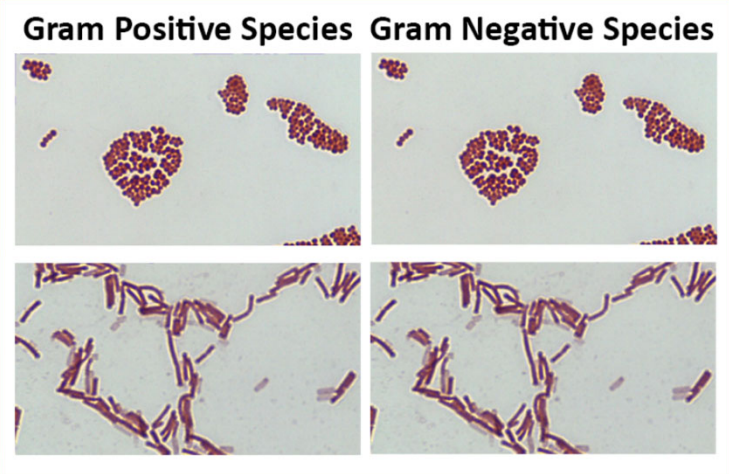
Gram Stain Technique: Protocol Steps Set 4

- You followed an **INCORRECT** protocol!
- You skipped the iodine step; the primary stain was not fixed inside Gram + cells!



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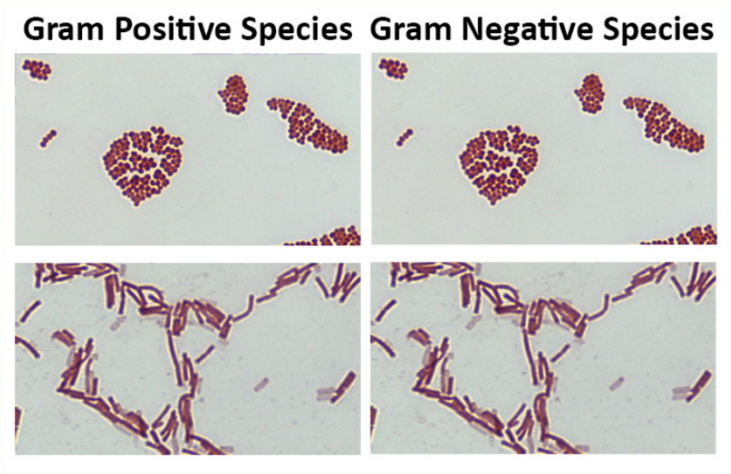
Gram Stain Technique: Protocol Steps Set 5

- You followed an **INCORRECT** protocol!
- You used counterstain first instead of primary stain!



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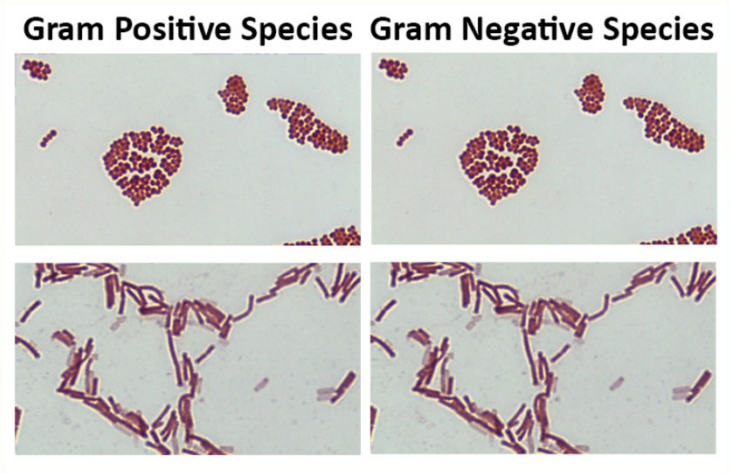
Gram Stain Technique: Protocol Steps Set 6

- You followed an **INCORRECT** protocol!
- You used mordant first instead of primary stain!



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Appendix 2: Virtual Lab Design Process and Construction

The virtual labs were created using Microsoft PowerPoint (presentations extension) as the delivery platform. Utilization of this program facilitated a more equitable distribution since most students have access to PowerPoint and because this program works on a variety of devices. Each virtual lab presents content and virtual lab experience in which the student can decide how he or she interacts throughout the lab. Each lab consists of common sections such as an introduction and a set of objectives, a protocol, a video demonstrating the technique, and an opportunity to perform the technique. Each slide has click buttons that have embedded navigation links that allow for returning to the previous slide, advancing to the next slide, or visiting different sections of the lab (Figure 1). For example, a student in the "Perform the Technique" section could choose to return to the "Protocol" section before performing the technique. When performing the technique, the student must choose from different protocol sets with each set containing slightly different steps or, in some cases, omitted steps. After making a choice, the student sees the results of that choice. The student is able to return and perform each protocol set, experiencing each outcome as dictated by that set of steps. In addition, the student could choose to return to any section of the lab. Including inoculations, performing the correct protocol, performing incorrect options, and setting up the PowerPoint module, each virtual lab required approximately 6-8 hours to develop, excluding film editing time.

The "Introduction and Objectives" were presented over several slides highlighting how the technique works and the significance of the technique. Understanding the relevance of the technique and the importance of performing it correctly were of paramount importance in this part of the lab and appropriate discussion was provided. Pictures and cartoons were added as appropriate to emphasize concepts.

The students were given a thorough set of "Protocol" steps in the subsequent slides following the introduction and objectives. The protocol steps provided details and each step was thoroughly explained.

The technique videos were completed in the campus microbiology lab. The videos carefully followed the protocol given to students and were captured using a GoPro as the instructor was performing the technique. The videos were edited and audio narration was added using Camtasia software(https://www.techsmith.com/video-editor.html) and compressed using Handbrake (https://handbrake.fr/) to reduce download sizes. To avoid necessitating students to stream the completed PowerPoint modules, the files were saved in presentation format (.PPSX) so that downloads could be played without further internet connection.

In the "Perform the Technique" portion of the lab, different sets of protocols were constructed and presented in steps, similar to the manner in which the protocol was provided. One of the protocol sets included the correct steps, in the correct order. The other protocol sets included common mistakes students make in performing the technique. In preparation for this part, images were taken of the correct and incorrect results. After making a choice, a link in the perform technique button will advance to a slide that advises if the correct choice was made and displays a picture of the result.

Appendix 3: Expectations of Students and Sample Questions

Students were advised to download the virtual lab and use the lab in presentation view. The students were instructed to migrate through all sections and to analyze the results from each protocol set option. Determining the mistake (if applicable) and how this mistake affected the result were assessed in a post-lab online quizzes and lab practicals. A few examples of post-lab and lab practical questions are included:

Class Discussion:

1. Explain the significance the decolorization step in the gram stain process. Describe the appearance of gram-negative and gram-positive bacteria if this step is omitted.
2. Discuss the importance of using fresh (under 24 hr old) cultures when performing the Gram stain technique. Predict the appearance and interpretation of performing the procedure correctly on >24 hr old Gram positive bacilli as well as >24 hr old cultures of Gram negative cocci, noting whether these would be false (inaccurate) or true (accurate) interpretations.

Case Scenarios:

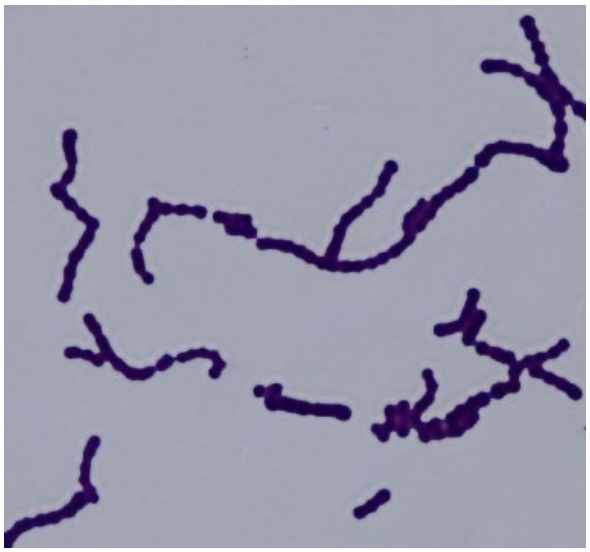
1. Jamal was asked to perform a Gram stain on a mixed culture from a 18-24 hr old broth. He made a smear from the culture, air dried and heat fixed it, then performed the Gram stain exactly as his lab manual outlined, paying close attention to the timing of his decolorization step and the following rinse to maintain accuracy. (In order: crystal violet for 1 min, water wash, Grams iodine for 1 min, water wash, 95% ethanol for 30 seconds, water wash, safranin for 2 min, water wash, blot & air dry.) When observing the stain under 1000x with immersion oil, Jamal noted the presence of a thick mass of cells with few observable cells visible: Gram positive bacilli along with some Gram positive staphylococci. His instructor had noted the culture mixture as a combination of *Escherichia coli* and *Staphylococcus epidermidis*. Jamal reported his results to his lab partner, who indicated that he should try the stain again, starting with a new smear preparation. Were his results accurate? Should he do as his partner suggested? Why or why not?
2. Amanda was performing a gram stain on *Staphylococcus aureus* bacteria. Unfortunately, she was distracted by one of her peers in lab. She knows that she performed the crystal violet step, but cannot remember if she performed the mordant step. She proceeded with the decolorization and counterstain steps with the hopes that she had successfully completed both steps 1 and 2. What should she observe if she had correctly performed all the steps of the gram stain? What will she observe if she did not add the mordant after the crystal violet step?

Multiple Choice:

1. Which of the following is the most time sensitive step in the Gram stain process?
    a. Mordant
    b. Counterstain
    c. Decolorization
    d. Primary stain
2. When performing the gram stain, if the student has swapped the Mordant step with the primary stain step on a Gram positive culture (<24 hr old), but has done all of the other steps correctly, predict the observation of the Gram reaction that the student should expect.
    a. Cells would appear pink/red
    b. Cells will still appear purple
    c. Cells will have no visible color at all
    d. Some cells will appear pink/red and some will appear purple

Virtual Lab Practical:

1. Observe the specimen on the micrograph in front of you. Identify A) the cell morphology, B) the cell arrangement, and C) the Gram reaction (write out the word positive or negative) of this organism. Use the appropriate scientific terms!



2. A. If you were performing a Gram stain on a Gram-positive organism and mixed up steps 2 and 3 (did step 1, then step 3, then step 2, and then step 4), what color would the cells be when viewing the slide using the microscope?

   B. If you were performing a Gram stain on a Gram-negative organism and mixed up steps 2 and 3 (did step 1, then step 3, then step 2, and then step 4), what color would the cells be when viewing the slide using the microscope?

   C. If you were performing a Gram stain on a Gram-negative organism and forgot to do step 3 (did step 1, then step 2, and then step 4), what color would the cells be when viewing the slide using the microscope?