G-Biosciences ♦ 1-800-628-7730 ♦ 1-314-991-6034 ♦ technical@GBiosciences.com

A Geno Technology, Inc. (USA) brand name

Bacterial Gram Staining

Teacher's Guidebook

(Cat. # BE-202)



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MATERIALS INCLUDED WITH THE KIT

This kit has enough materials and reagents for 24 students (six groups of four students).

- 1 vial K.R. Specimen Agar Stab
- 1 vial E.C. K-12 Agar Stab
- 2 vials LB Broth
- 1 bottle Gram Crystal Violet
- 1 bottle Gram Iodine
- 1 bottle Gram Stain: Decolorizer Solution
- 1 bottle Gram Safranin
- 48 Glass Slides
- 4 Transfer pipettes (Large)

SPECIAL HANDLING INSTRUCTIONS

- Store K.R. Specimen and E.C. K-12 Agar Stabs at 4°C.
- All other reagents can be stored at room temperature.
- Briefly centrifuge all small vials before opening to prevent waste of reagents.

The majority of reagents and components supplied in the *BioScience Excellence*™ kits are non toxic and are safe to handle, however good laboratory procedures should be used at all times. This includes wearing lab coats, gloves and safety goggles.

For further details on reagents please review the Material Safety Data Sheets (MSDS).

The following items need to be used with particular caution.

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

ADDITIONAL EQUIPMENT REQUIRED

- Shaking Incubator
- Bunsen Burner
- Microscope

TIME REQUIRED

- 1-2 hours

OBJECTIVES

- Perform bacterial Gram staining.
- Visualize bacteria under microscope.
- Differentiate Gram-positive and Gram-negative bacteria.

BACKGROUND

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.

The primary stain used in this kit for the Gram staining is crystal violet; however methylene blue is an adequate substitute. The microorganisms (bacteria) that retain the primary crystal violet stain appear as a purple brown color under the microscope. The stained bacteria are referred to as **Gram-positive**. The microorganisms that do not retain the primary stain and are a red color are **Gram-negative**.

The basic principle of Gram staining is the properties of certain bacteria cell walls to retain the crystal violet dye. The cell walls for Gram-positive microorganisms have a higher peptidoglycan and lower lipid content than Gram-negative bacteria.

The mechanics of the Gram staining method is that the bacteria cell walls retain the crystal violet and subsequently added iodine, which complexes with the crystal violet, preventing the easy removal of the dyes. This step is known as the "*fixing the dye*" step. During the subsequent addition of a decolorizer, a mixture of acetone and ethanol solvents, Gram-positive cell walls dehydrate, closing the pores in the cell wall, resulting in the retention of the crystal violet: iodine complexes. In contrast, the decolorizer dissolves the higher lipid content of Gram-negative bacteria and the primary stain is able to leach into the solvent, essentially washing away the dye, leaving the Gram-negative bacteria unstained.

The length of the decolorization stage is critical as prolonged decolorizing will remove the primary stain from the Gram-positive cells and this will lead to false negatives during characterization of the microorganisms.

Finally, in order to visualize the unstained Gram-negative bacteria, a counter stain is added. This kit uses safranin, a basic stain that stains bacteria red. Some bacteria stain weakly with Safranin and the alternative counter stain Fuchsin is used.

TEACHER'S PRE EXPERIMENT SET UP

Prepare Bacterial Stocks

1. The day before experimentation, label the LB Broth vials with either K-12 or K.R. Specimen.

2. Using aseptic techniques, transfer 0.8ml LB broth to the bacterial agar stab and incubate at 37°C for 30 minutes.

3. Vigorously shake or vortex for 1-2 minutes and transfer 0.25ml LB broth from the agarose stab to the labeled LB Broth vials.

4. Incubate at 37°C overnight in a shaking incubator.

MATERIALS FOR EACH GROUP

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

- K.R. Specimen, overnight culture (shared with class)
- E.C. K-12 Specimen, overnight culture (shared with class)
- Gram Crystal Violet with large transfer pipette (shared with class)
- Gram Iodine with large transfer pipette (shared with class)
- Gram Stain: Decolorizer Solution with large transfer pipette (shared with class)
- Gram Safranin with large transfer pipette (shared with class)
- 8 Glass Slides (2 for each student)

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

PROCEDURE



Wear gloves throughout the staining procedure.

1. Label the two slides with K.R. Specimen or E.C. K-12 Specimen and your name for identification.

2. Place a small drop of K.R. Specimen on the labeled glass slide and a small drop of E.C. K-12 Specimen on the second labeled glass slide.

3. Spread the sample over a large surface with a pipette tip to form a thin film.

4. Allow the suspension to completely air dry.

5. To fix the bacteria to the 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 is not overheated each time.

   ***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.*

6. Cover the entire area of bacteria with Gram Crystal Violet and leave at room temperature for 1 minute. Rinse the slide for 5 seconds under slow running water using a wash bottle.

   The specimen should appear blue-violet when observed with the naked eye.

7. Cover the bacteria with the Gram Iodine and leave at room temperature for 1 minute. Rinse the slide for 5 seconds under slow running water then ***immediately*** proceed to the next step.

   At this point the specimen should still be blue-violet.

8. Add the Gram Stain: Decolorizer Solution drop-wise until the blue-violet color is no longer visualized on the sample.

9. Rinse the slide for 5 seconds under slow running water using a wash bottle.

10. Cover the bacteria with the Gram Safranin and leave at room temperature for 1 minute. Rinse the slide for 5 seconds under slow running water to remove any excess dye.

11. Blot the slide gently with absorbent paper or allow it to air dry before viewing under a bright field microscope. 600 x magnification is adequate for viewing bacteria on the slide. Observe the center of the slide where bacteria has been treated with all Gram Stains. An oil immersion objective may be used for high magnification. A drop of oil can be placed directly on the slide.

RESULTS, ANALYSIS & ASSESSMENT

1. What color does the K.R. Specimen appear under microscope? Is it Gram positive or negative?

K.R. Specimen *(Kocuria rhizophilia) appears blue-violet color.*

It is Gram-positive.

2. What color does E.C. K-12 Specimen appear under microscope? Is it Gram positive or negative?

E.C. K-12 Specimen *(Escherichia coli K-12) appears pink color.*

It is Gram-negative.

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The length of the decolorization stage is critical as prolonged decolorizing will remove the primary stain from the Gram-positive cells and this will lead to false negatives during characterization of the microorganisms.

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- Gram Safranin with large transfer pipette (shared with class)
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2. Place a small drop of K.R. Specimen on the labeled glass slide and a small drop of E.C. K-12 Specimen on the second labeled glass slide.

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4. Allow the suspension to completely air dry.

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   The specimen should appear blue-violet when observed with the naked eye.

7. Cover the bacteria with the Gram Iodine and leave at room temperature for 1 minute. Rinse the slide for 5 seconds under slow running water then ***immediately*** proceed to the next step.

   At this point the specimen should still be blue-violet.

8. Add the Gram Stain: Decolorizer Solution drop-wise until the blue-violet color is no longer visualized on the sample.

9. Rinse the slide for 5 seconds under slow running water using a wash bottle.

10. Cover the bacteria with the Gram Safranin and leave at room temperature for 1 minute. Rinse the slide for 5 seconds under slow running water to remove any excess dye.

11. Blot the slide gently with absorbent paper or allow it to air dry before viewing under a bright field microscope. 600 x magnification is adequate for viewing bacteria on the slide. Observe the center of the slide where bacteria has been treated with all Gram Stains. An oil immersion objective may be used for high magnification. A drop of oil can be placed directly on the slide.

RESULTS, ANALYSIS & ASSESSMENT

1. What color does the K.R. Specimen appear under microscope? Is it Gram positive or negative?

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2. What color does E.C. K-12 Specimen appear under microscope? Is it Gram positive or negative?

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