# Gram Staining of Bacterial Cells

## 1. INTRODUCTION

1.1. A gram stain is performed to distinguish between gram-positive (+) and gram-negative (–) bacteria. Both types of bacteria are stained with crystal violet. The addition of iodine forms a crystal violet-iodine complex within the cell. When a solution of decolorizer is added, the thick cell wall of the gram-positive (+) bacteria is dehydrated by the alcohol. This makes the cell wall less porous and allows the bacterial cell to retain the crystal violet-iodine complex and block out the safranin counter stain. Gram-negative (–) bacteria have an extra lipid-rich outer layer and thin cell wall, which increases in porosity when the decolorizer is added. This facilitates the removal of the crystal violet-complex and allows the safranin counter stain to permeate the cell.

## 2. EQUIPMENT AND SUPPLIES

2.1. Sterile dropper

2.2. Sterile glass slides

2.3. Sterile inoculation loop (wire or plastic)

2.4. Bunsen burner

2.5. Metal tongs

2.6. Striker

2.7. Metal tray with rack

2.8. Timer

2.9. Absorbent paper/paper towels

2.10. Kimwipes

2.11. Compound microscope

2.12. Permanent black marker

2.13. Labels

## 3. REAGENTS AND STANDARDS

3.1. Deionized (DI) water

3.2. Gram stain kit – includes Crystal violet stain, Gram iodine solution, Decolorizer solution and Safranin Stain

3.3. *Escherichia coli* (ATCC 11775) and *Enterococcus faecalis* (ATCC 100411) control cultures

3.4. Immersion oil


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## 4. SLIDE PREPARATION

4.1. Place a small drop of sterile DI water onto a labeled, clean slide.

4.2. Using a sterile inoculating loop add a small amount of bacterial growth to the drop and mix. Spread the smear evenly on the slide.

4.3. Air dry the smear. Using the tongs, heat fix by quickly passing the slide several times through the flame of the Bunsen burner. Allow the slide to cool slightly between passes to prevent burning the bacteria.

4.4. For QC, prepare separate smears of gram-positive *E. faecalis* and gram-negative *E. coli*.

## 5. GRAM STAINING

5.1. Place metal tray with rack over a sink or waste container.

5.2. Place prepared slides on rack in metal tray.

5.3. Flood the slides with Gram crystal violet stain and let stand for one minute.

5.4. Rinse the slides in a gentle stream of DI water.

5.5. Flood with Gram Lugol's iodine solution and let it stand for one minute.

5.6. Gently rinse the slides with DI water.

5.7. Gently rinse the slides with concentrated decolorizer solution for about 10 seconds. Some gram stain kits use a diluted decolorizer and will need a longer rinse time (20-30 seconds). Do not over-decolorize.

5.8. Gently rinse the slides with DI water.

5.9. Flood the slides with the safranin counterstain and let stand for 30 seconds.

5.10. Gently rinse the slides with DI water.

5.11. Place the slides on absorbent paper/paper towels. Gently blot dry with Kimwipes.

## 6. INTERPRETATION

6.1. Examine the slides under an oil immersion lens. Gram-positive bacteria retain the crystal violet–iodine stain and have a blue/purple color. Gram-negative bacteria retain the safranin stain and are pink in color.

6.2. Use the control cultures for comparison. *E. coli* are gram-negative pink rods. *E. faecalis* are gram-positive purple cocci. If controls do not produce proper reactions, restart the procedure with new slides.

## 7. REFERENCES

7.1. Standard Methods 9221B-2006

7.2. Protocol Gram stain kit instructions



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## APPENDIX OF CHANGES

| | |
|---|---|
| 07/16/2008 | Changed SOP format; added introduction section; added references |
| 01/27/2015 | Formatted for 508 Compliance |
| 05/21/2015 | Added equipment and supplies to the list; added a sentence saying to use tongs while heat fixing the slides; added the use of the metal tray in section 5. |
| 12/30/2016 | Removed “Blank” from Section 2.13.; included labels in Section 4.1.; sink/waste container has been added to Section 5. |
| 10/01/2018 | Corrected grammatical errors. |
| 08/22/2019 | Updated ATCC number of *E. faecalis;* added caution not to over decolorize slides in 5.7; updated safranin staining time to 30 seconds in 5.9. |
| 01/02/2020 | Corrected SM 9221B version. |


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