Identification of Bacteria Using the Gram Staining Technique

Department of Microbiology

Faculty of Medicine

UWUSL

Standard Operating Procedure (SOP)
Identification of Bacteria Using the Gram Staining Technique

Title:
Identification of Bacteria Using the Gram Staining Technique

Issued By:
Faculty of Medicine, Uva Wellassa University of Sri Lanka

(1) Purpose
To establish a standardized procedure for performing Gram staining in microbiology practical sessions to differentiate bacteria into Gram-positive and Gram-negative groups based on cell wall characteristics.

(2) Scope
This SOP applies to all medical students, academic staff, and laboratory personnel involved in microbiology practical sessions, including:

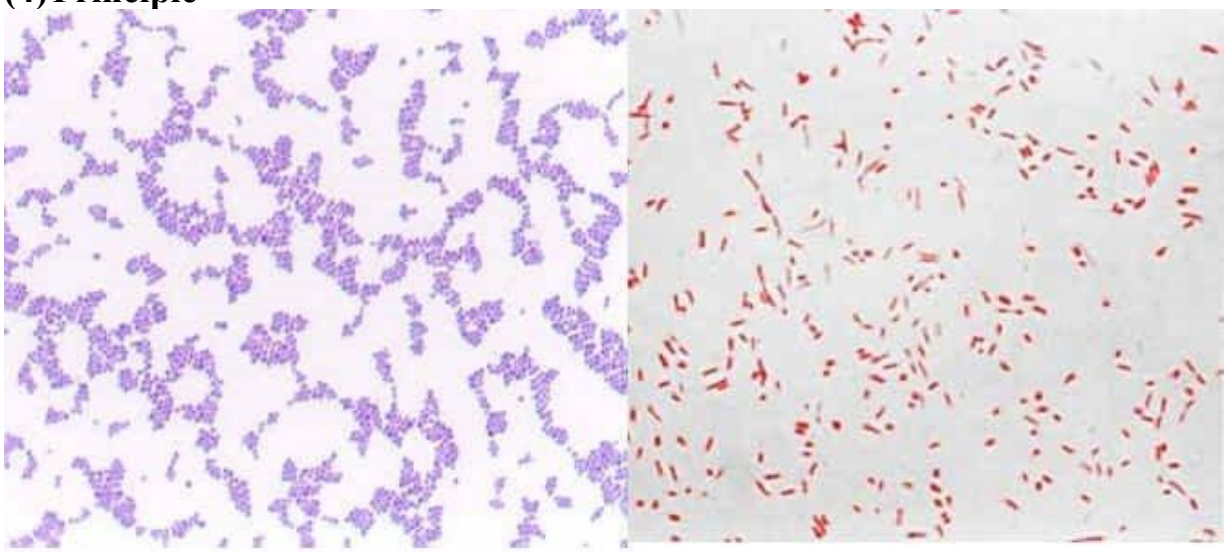
- Preparation of bacterial smears
- Performing Gram staining
- Microscopic identification of bacteria

(3) Responsibilities
3.1 Students
- Follow correct staining procedures
- Handle reagents and equipment safely
- Accurately observe and record findings

3.2 Demonstrators / Lecturers
- Supervise staining procedure
- Ensure correct technique is followed
- Assist in interpretation of results

3.3 Laboratory Staff
- Provide reagents and materials
- Ensure proper functioning of equipment
- Maintain laboratory safety standards

(4) Principle



Gram +ve Bacteria Gram -ve Bacteria

Gram staining is a differential staining technique where bacteria are stained with crystal violet and iodine, then decolorized and counterstained.

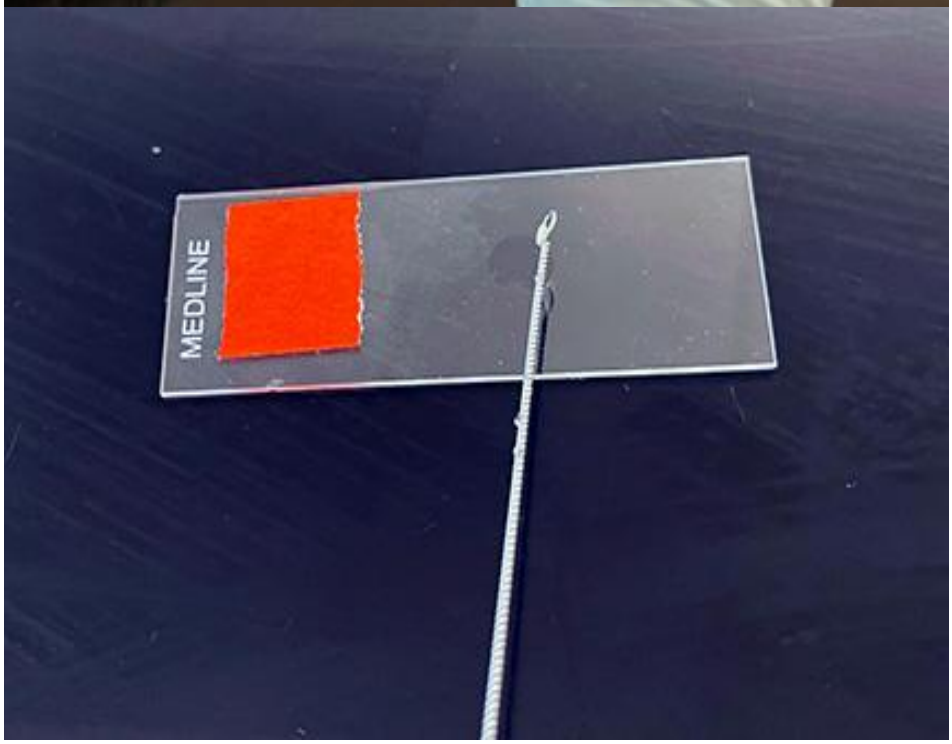
- Gram-positive bacteria retain the crystal violet–iodine complex and appear purple.
- Gram-negative bacteria lose the complex during decolorization and take up the counterstain, appearing pink.

(5) Equipment and Materials

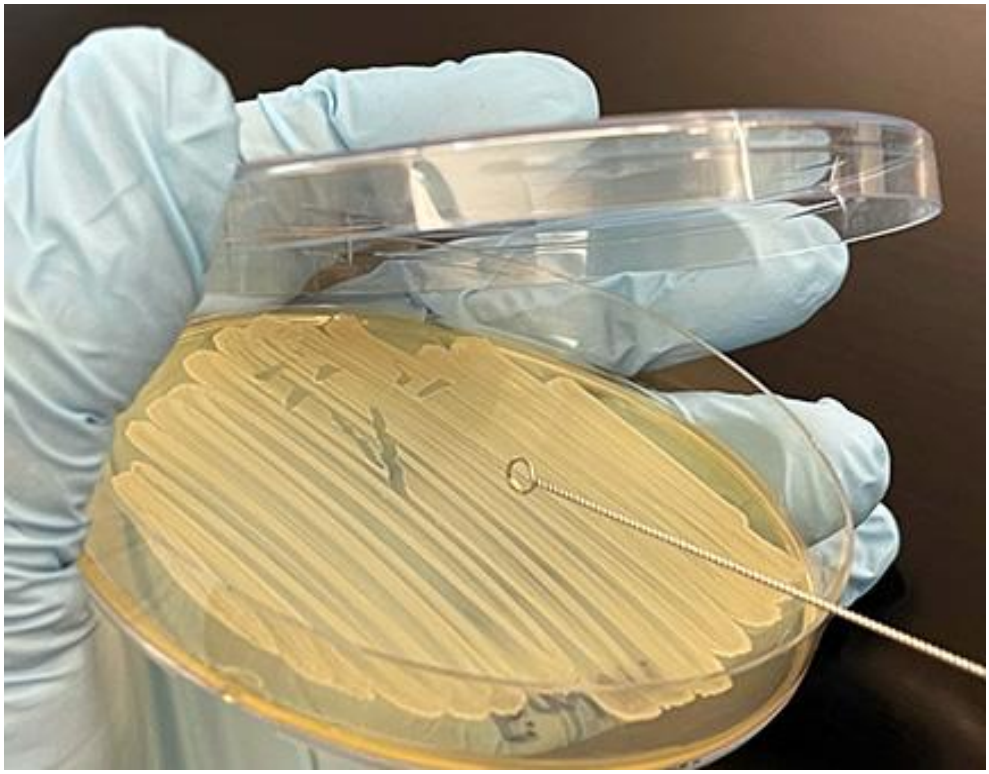
- Sterile inoculating loop
- Crystal violet stain
- Gram's iodine
- Iodine Acetone or Acetone alchohol (decolorizer)
- Dilute Carbol Fuchsin (counterstain)
- Distilled water
- Bunsen burner
- Glass slides and cover slips
- Compound microscope

(6) Procedure

Step 1: Smear Preparation & Heat Fixing
Label one end of a clean, dry glass slide using a grease pencil to ensure proper identification and prevents loss of labeling during staining. Place a small drop of distilled water on a clean slide.



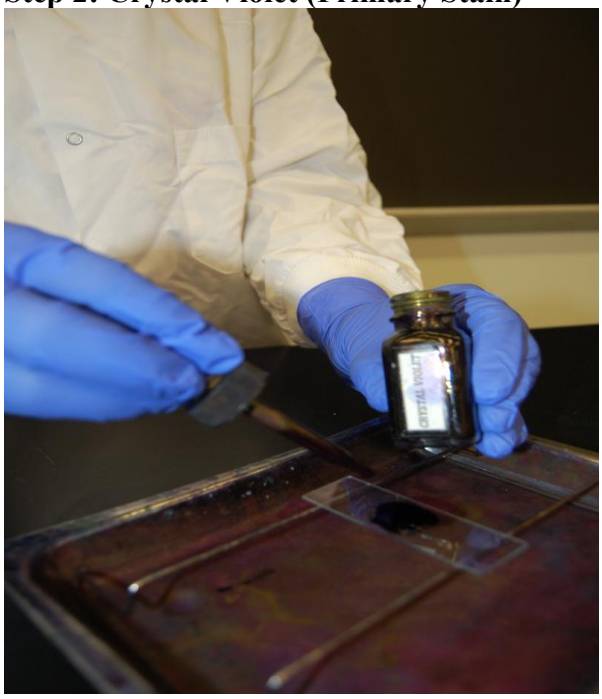
Using a sterile loop, transfer a small amount of bacterial culture, and mix to form a thin smear then allow it to air dry completely.

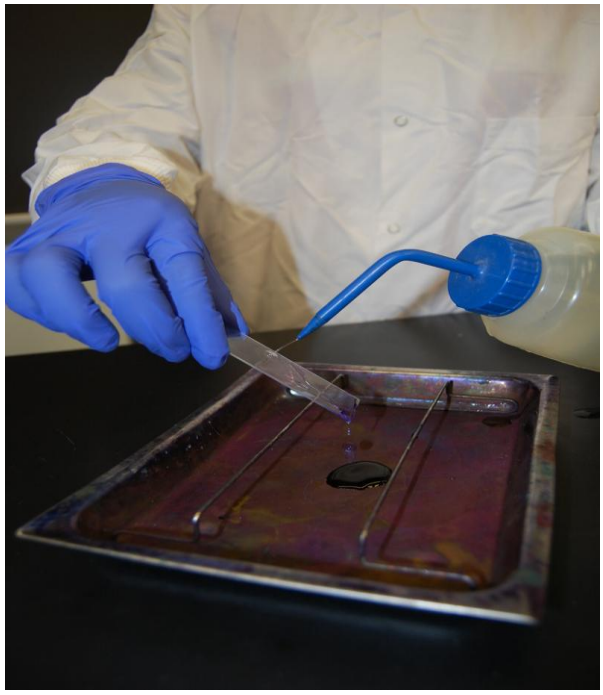


Pass the slide briefly through a flame 2–3 times to heat-fix.

Explanation: Heat fixing kills bacteria, adheres them to the slide, and preserves cell morphology for staining.

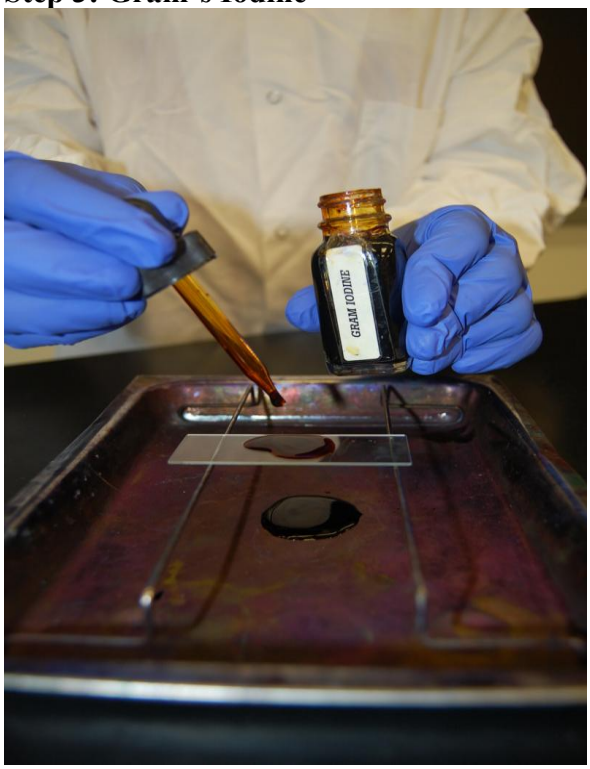
Step 2: Crystal Violet (Primary Stain)





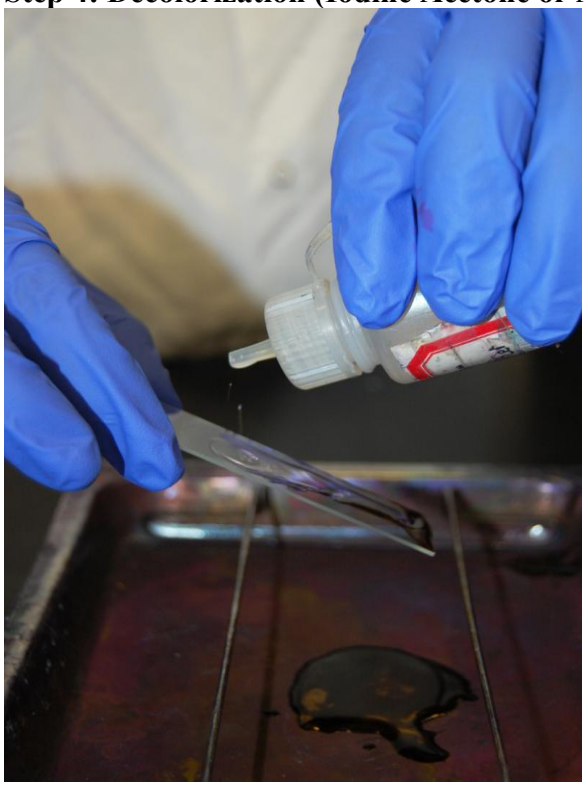
Flood the smear with crystal violet for 30 seconds, then rinse gently with water.
Explanation: Crystal violet, a basic dye, penetrates the bacterial cell wall and cytoplasm, staining all bacteria purple.

Step 3: Gram's Iodine



Flood the smear with Gram's iodine for 1 minute, then rinse with water.
Explanation: Iodine forms a crystal violet–iodine complex, making the dye less soluble and more firmly retained in Gram-positive bacteria.

Step 4: Decolorization (Iodine Acetone or Acetone Alcohol)



Apply decolorizer dropwise for 1-5 seconds (until the runoff becomes clear), then immediately rinse with water.

Explanation: Acetone-based agents act almost instantly compared to ethyl alcohol. They dissolve the outer membrane of Gram-negative bacteria to remove the dye complex.

Step 5: Dilute Carbol Fuchsin (Counterstain)

Flood the smear with Dilute Carbol Fuchsin for 30 seconds to 1 minute, then rinse and air dry.
Explanation: In Sri Lanka, Dilute Carbol Fuchsin is the preferred counterstain because it provides a much more intense contrast than Safranin, making it easier to identify Gram-negative organisms.

Step 6: Microscopic Observation

1. Place the stained slide on the microscope stage.
2. Focus under the low power (10x) to locate the smear.
3. Switch to high power (40x) and observe.
4. Add a drop of immersion oil and observe under oil immersion (100x)
5. Observe and record:
    - Shape (cocci, bacilli)
    - Arrangement (chains, clusters)
    - Gram reaction (purple/pink)

(7) Results Interpretation

Gram-positive bacteria → Purple
Gram-negative bacteria → Pink

(8) Precautions

- Use thin smear
- Do not over-decolorize (Acetone acts very rapidly)
- Heat-fix gently (avoid overheating)
- Use clean slides and fresh reagents
- Follow aseptic technique
- Handle flame carefully

(9) Post Practical Procedure

- Dispose of slides safely
- Clean microscope stage
- Turn off light source
- Clean work area

(10) Records and Documentation

- Practical number and title
- Date of experiment
- Observations (shape, arrangement, Gram reaction)
- Student name and signature
- Demonstrator verification

Reference for images:
BioLibreTexts. Gram Staining Procedure. Microbiology Labs II. Available at: https://bio.libretexts.org/Learning_Objects/Laboratory_Experiments/Microbiology_Labs/Microbiology_Labs_II/06%3A_Gram_Stain_and_Capsule_Stain/6.02%3A_Gram_Staining_Procedure (Accessed April 2026).

Prepared by:

Dr. Lakmal Hewage
Acting Head of the Department of Microbiology
Uva Wellassa University of Sri Lanka

Dr. Mahen Kothalawala
Consultant Clinical Microbiologist
National Hospital of Sri Lanka (NHSL)

Snr. Prof. Muditha Vidanapathirana
Dean
Faculty of Medicine
Uva Wellassa University of Sri Lanka

Dr. Nadeesha Senevirathne
Temporary Demonstrator
Department of Microbiology

Date of Implementation:
2026.05.06