

GRAM STAIN SET

Intended Use

To differentiate bacterial species into two groups based on the characteristics of their cell walls. Provide insight into the morphology and arrangement of microorganisms.

Principle and Results

The procedure outlined is based on the Hucker modification of the original Gram staining method. Both Gram-positive and Gram-negative organisms are initially subjected to staining with crystal violet. The subsequent addition of iodine results in the formation of a crystal violet-iodine complex within the cell wall. The application of a decolorizer extracts lipids, which increases the permeability of the cell wall and leads to the loss of the dye complex in the thin peptidoglycan layer characteristic of Gram-negative organisms. Conversely, Gram-positive organisms undergo dehydration, which effectively traps the crystal violet-iodine complex within the cell. Ultimately, safranin is employed as a counter stain, imparting a pink or red coloration to the Gram-negative organisms.

Specimen Collection

Liquid Media: Add a drop of suspension onto a clean glass slide using a sterile transfer pipette. Allow air drying before fixing.

Solid Media: Place a drop of saline on a clean glass slide. Using a sterile loop, pick up a small colony and transfer to the saline drop. Spread the suspension onto the slide and allow air drying before fixing. Heat-fix by placing on a ~42°C heat block for at least 5 minutes. Or methanol fix by submerging slide(s) into methanol for a minimum of 30 seconds and allowing it to dry before staining.

Precautions

Gram Stain Set is for *In Vitro* diagnostics use and should be used by properly trained individuals. Precautions should be taken against the dangers of microbiological hazards by properly sterilizing and using aseptic techniques. Refer to SDS for complete warnings, precautions, hazard and precautionary statements, and disposal information.

Autostainer Configuration

This stain set may be adapted for use on most open platform autostainers. A minimum of four baths is required to perform this procedure. Iodine can decrease in intensity when left in an open well. Crystal violet can precipitate when left in an open well. Replenish reagents each 8-hour shift, at a minimum.

Quality Control

Ethos Biosciences tests each lot using appropriate microorganisms following CLSI document M22-A3 Quality Assurance of Commercially Prepared Microbiological Culture Media. It is recommended that end-users perform their own quality control suggested by their facility. Organisms used in testing: *Staphylococcus aureus* ATCC 25923 for Gram-positive and *Escherichia coli* ATCC 25922 for Gram-negative.

Interpretation of Test

| | |
|---------------|------------------|
| Gram-positive | Dark blue/purple |
| Gram-negative | Red/pink |

| PROCEDURE | | | | | | |
|-----------|--------|----------------|------|------|-----|--|
| # | ACTION | SOLUTION | HEAT | TIME | | DETAILS |
| | | | | MIN | SEC | |
| 1 | FLOOD | CRYSTAL VIOLET | — | 1 | — | FLOOD OR SUBMERGE SLIDE |
| 2 | RINSE | RUNNING WATER | — | — | — | RINSE UNTIL WATER RUNS CLEAR |
| 3 | FLOOD | IODINE* | — | 1 | — | FLOOD OR SUBMERGE SLIDE |
| 4 | RINSE | RUNNING WATER | — | — | — | RINSE UNTIL WATER RUNS CLEAR |
| 5 | RINSE | DECOLORIZER | — | — | — | RINSE UNTIL SLIDE RUNS CLEAR. ADJUST TIMING BASED ON ACETONE CONCENTRATION |
| 6 | RINSE | RUNNING WATER | — | — | — | |
| 7 | FLOOD | SAFRANIN | — | — | 30 | FLOOD OR SUBMERGE SLIDE |
| 8 | DRY | | — | — | — | ALLOW SLIDE TO DRY BEFORE USING IMMERSION OIL |

*For traditional iodine, before use mix the iodine concentrate with the diluent.

Limitations

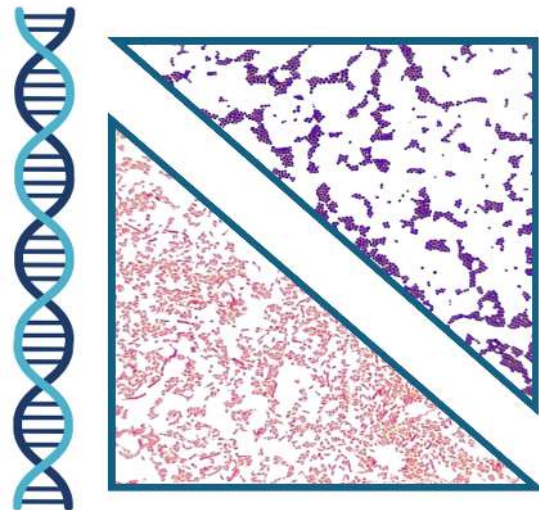
1. Antibiotic treatment of the patient, cultures more than 48 hours old, and autolytic enzymes can damage the cell wall causing gram-positive organisms to appear gram-negative or gram-variable.
2. Fixation with excess heat can alter cell morphology, cause over-decolorization, and can lead to cells sloughing off the slide during rinsing.
3. Insufficient exposure to iodine or lack of available fresh iodine can prevent crystal violet from binding in the Gram-positive organisms. Keep iodine bottles closed when not in use.
4. Excessive counterstaining can cause leaching of crystal violet – iodine complex from gram-positive organism causing false gram-negative appearance.
5. Over rinsing with water can cause over-decolorization of gram-positive organisms.
6. Adjusting stain times might be needed depending on specimen type, Use facility guidelines or recommendations of lab director/manager, when necessary.

Pro-Tip

1. Decolorization time depends on acetone concentration. 50/50 decolorizer will decolorize slower than a 75% decolorizer. It is best to pick a slower decolorizer for inexperienced technicians.
2. Thick specimens, such as sputum or joint fluid, will hold onto crystal violet more. Spread specimens as thin as possible. Smears should not be so thick that newsprints are not visible through the slide.
3. Temperature can affect decolorization. Using warm water while rinsing can decrease decolorization times.
4. Use of cytospin for smear preparation can improve sensitivity of Gram stains; however, it can lead towards overly thick smears.
5. Inappropriate use of eSwabs can contribute to discrepant Gram stain results.

References

1. Manual of Microbiological Methods, p. 15-18. 1957. Mc Graw-Hill.
2. Mangels, J.I., M.E. Cox and L.H. Lindley. 1984. Methanol fixation. An alternative to heat-fixation of smear. *Diag. Microbiol. Infect. Dis.* ; 2:129-137
3. Isenberg, H.D. *Clinical Microbiology Procedures Handbook*, Vol. I, II & III. American Society for Microbiology, Washington, D.C. Bartholomew, J.W. and Mittwer, T., 1952. *The Gram Stain*. Department of Bacteriology, University of Southern California. Vol 16. 1 – 2



PRODUCT INFORMATION

| CAT. # | DESCRIPTION |
|---------|----------------------------------|
| 6060-02 | Gram Stain Set 2oz |
| 6250 | Gram Stain Set 8oz, Traditional |
| 6400 | Gram Stain Set 8oz, Stabilized |
| 6500 | Gram Stain Set 16oz, Traditional |
| 6600 | Gram Stain Set 16oz, Stabilized |

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