

DEPMEDS LABORATORY PROCEDURES
DEPARTMENT OF CLINICAL SUPPORT SERVICES
U.S. ARMY MEDICAL DEPARTMENT CENTER AND SCHOOL
FORT SAM HOUSTON, TEXAS 78234-6137

MCCS-HCM

STANDING OPERATING PROCEDURE

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GRAM STAIN

1. INTRODUCTION:

The Gram stain allows bacteria to be seen when utilizing bright-field microscopy. Unstained bacteria appear colorless and can not be differentiated. Stained bacteria are distinguished based on morphological and staining differences such as Gram stain reaction, cell shape, and cell arrangements. Staining also provides a presumptive identification of bacteria, aiding in the selection of antimicrobial therapy and culture media.

2. PRINCIPLE:

- a. The Gram reaction distinguishes between two major subgroups of bacteria in which the cell walls have a different structure and chemical composition. On microscopic examination, not only are the form, size, and other structural details made visible, but the microorganisms present can be grouped into gram-positive and gram-negative types by their reactions. This is an important diagnostic tool in subsequent identification procedures.
- b. At physiological pH, the bacterial protoplasm is basophilic; that is, it stains readily only with basic fuchsin, safranin, and methylene blue. The staining reaction may be considered as a chemical reaction between the negatively charged proteins and the positively charged basic dye; that is, a simple neutralization of the positive and negative charges.
- c. Iodine is used as a mordant in the Gram Stain, and the crystal violet-iodine complex is formed. The characteristic of a good mordant is its affinity for both the cellular material and the dye, binding the two together.
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- e. The cell wall of a gram-positive bacterium forms a complex with the crystal violet and Gram's iodine reagents, thus creating a barrier which prevents the leaching of the crystal violet-iodine complex. Therefore, this crystal violet-iodine complex remains in gram-positive bacteria when the decolorizer is applied.
- f. Gram-negative bacteria, presumably because of a higher lipid content of their cell wall, lose the crystal violet primary stain when treated with the decolorizer. These bacteria will retain the counter stain, safranin to their cell walls, appearing red or pink on microscopic exam.

3. REAGENTS AND MATERIALS:

- a. Clean glass microscope slides.
- b. Crystal Violet
- c. Gram's iodine solution
- d. Acetone-alcohol decolorizer
- e. Safranin O
- f. Methanol -- can be used alternatively as slide fixative.
- g. Reagents and stains may be stored at room temperature.
- h. The DEPMEDS equipment list includes a commercial Gram stain reagent kit. However, if the Gram stain reagents must be prepared from in-house chemicals, prepare them as follows:

(1) CRYSTAL VIOLET REAGENT:

Crystal Violet	5.0 g
Ethyl Alcohol, 95%	50.0 mL
Ammonium Oxalate	2.0 g
Distilled Water	200.0 mL

Dissolve the crystal violet in the ethyl alcohol; dissolve the ammonium oxalate in the water. After dissolving, mix the two solutions.

(2) IODINE REAGENT:

Iodine	1.0 g
Potassium Iodide	2.0 g

Distilled Water 300.0 mL

Dissolve the iodine and potassium iodide in a small amount of water (5-10 mL), then q.s. to 300 mL.

(3) DECOLORIZER:

Acetone 300.0 mL
Ethyl Alcohol, 95% 700.0 mL

NOTE: The decolorization action increases with increasing concentrations of acetone.

(4) SAFRANIN O REAGENT:

Safranin O 0.5 g
Distilled Water 100.0 mL

Dissolve the safranin O in a small amount of distilled water. Then, q.s. to 100 mL with distilled water. Store all reagents at room temperature in brown bottles to protect from exposure to light.

NOTE: Since decolorizer is used more rapidly than are the other Gram stain reagents, additional acetone and ethyl alcohol are provided in the supply list to allow for its preparation.

4. SPECIMEN:

- a. Preliminary STAT Gram stain reports on CSF, pleural, pericardial, peritoneal, amniotic and synovial fluid, as well as all surgical specimens, will be provided by the DEPMEDS lab. All Gram stains not identified as "STAT" will be done routinely in order of receipt.
- b. Any specimen where organisms encountered on review of a Wright's stained smear will also have a Gram stain performed.
- c. All STAT Gram stain requests on other specimens must be approved by the NCOIC or the Laboratory OIC.
- d. Specimens collected on swabs should be transported immediately to the laboratory. Do not allow the specimens to dry out.

5. PROCEDURE:

- a. Prepare the smear.

- (1) Use an inoculating loop, sterile pipet, or needle and syringe to place a drop of a liquid specimen or broth on the center of a labeled slide. Roll swabs across the slide's surface.
 - (2) Prepare smears from colonies growing on agar media by placing a small drop of saline or distilled water onto the center of a labeled slide. Touch the top center of a colony with a sterile inoculating needle and transfer a small amount of the bacterial colony to the drop of saline. Use the needle to mix the bacteria with the saline. Spread the mixture over an area approximately the size of a dime.
 - (3) Allow smears to air dry.
- b. Fix the smear.
- (1) Methanol fixation (recommended method).

NOTE: This method is preferred for smears from patient specimens, especially wounds, sputa, and buffy coats.

- (a) Flood or immerse the entire slide with methanol (methyl alcohol) and allow it to stand for one minute.
 - (b) Pour off excess methanol and allow smear to air dry.
- (2) Heat fixation (alternate method). Briefly pass the slide through a burner flame 2 or 3 times. Use care to prevent overheating. DO NOT BOIL! Keep the smear side of the slide up when passing it through the flame.
- c. Cover the fixed smear with the crystal violet allowing the stain to remain for one minute.
- d. Decant crystal violet and rinse slide with gently flowing tap water until clear.
- e. Flood the slide with iodine solution for one minute; rinse with gently flowing tap water.
- f. While holding the slide in a tilted angle (about 45° angle), apply a few drops of decolorizer to the upper end of the slide, and allow the decolorizer solution to flow-over the smear for 2 to 5 seconds.
- g. Stop the decolorization after 2-5 seconds with a gentle flow of water. Do not apply decolorizer until the color stops running off because that will over-decolorize the bacteria.

- h. Apply the safranin O counterstain for 30 to 60 seconds and remove the excess with a gentle flow of tap water.
 - i. Drain slide and carefully blot dry with bibulous paper. (paper towel is acceptable, DO NOT wipe.
 - j. Examine the smear microscopically using the oil immersion objective (100x).
6. RESULTS: (See DEPMEDS Microbiology Procedures Manual and/or ANNEX A of this SOP for further identification criteria.)
- a. Gram-positive bacteria will stain blue to purple.
 - b. Gram-negative bacteria will stain pink to red.

NOTE: Structural detail (form, size, etc) are also made visible. Report cell shape (coccus, bacillus, etc.), arrangement (clusters, pairs, chains, etc.), and any additional details that appears significant (e.g. presence of spores or relative size).

- c. Positive control -- from stock cultures of S. aureus, Gram Positive(purple) cocci in clusters
 - d. Negative control -- from stock cultures of E. coli, Gram Negative (red rods bacilli), e.g.,E. coli.
7. QUALITY CONTROL:
- a. TRAINING AND PROFICIENCY TESTING: All DEPMEDS laboratory personnel will be trained to perform this task. Proficiency will be monitored when reviewing results.
 - b. A control slide (commercially prepared or in-house) should be performed each day a Gram stain is done.
 - c. COMMERCIAL CONTROL SLIDES:
 - (1) A Gram stain control slide is a specially designed microscope slide intended to be part of a quality control program to monitor stains and staining techniques. Each slide contains one block of heat-fixed gram-positive control (Staphylococcus aureus), and one block of heat-fixed gram-negative control (Escherichia coli).

NOTE: For best results, repeat the methanol-fixation of the control blocks before staining.

- (2) Store the Gram stained slides at room temperature (20-30°C). Do not freeze or expose to excess heat. Use until expiration date indicated on the label. Stain a control slide once a day when staining slides prepared from clinical material.
 - (3) Stain exactly as you do a patient slide. See procedure above.
- d. **IN-HOUSE PREPARED CONTROL SLIDES:** Quality control slides may be prepared by adding one tiny drop/smear area of a 24-hour broth culture of a Gram-positive organism (Staphylococcus aureus or epidermidis and a tiny drop of a 24-hour culture of a Gram-negative organism e.g. Escherichia coli) to a glass slide and allowing the broth to dry onto the surface of the slide. Methanol fix each slide and store in a slide box until ready for QC testing. A slide containing separate drops of gram-positive organism and gram-negative organism should be stained each day to test the quality of Gram stain reagents. Alternatively, a mixture containing both gram-negative and gram-positive organisms can be used in preparing the control slides.
 - e. **CORRECTIVE ACTION:** If the control slide is improperly stained, repeat with a new control slide and a slide prepared from clinical material. Because most problems are due to personal techniques, adjust staining time and technique. However, if proper results are not obtained after those adjustments, consider a possible problem with the reagents. Notify supervisor if unable to obtain correct results.
8. **REPORTING:**
- a. Record results on Gram Stain log sheet and annotate results on the patients request slip.
 - b. Pull the middle copy of the requisition and retain in the Microbiology section of the laboratory.
 - c. The original and back copies will be forwarded to the specimen processing point in the laboratory for verification and dissemination to the proper requesting service.
 - d. The original Gram stained slide will be retained in the Microbiology section for future reference and/or review.

9. SAFETY:

Preparation of smears directly from patient specimens should be handled in biosafety cabinet to prevent exposure to airborne pathogens. If hood is unavailable, utilize a mask and face-shield. Preparation of smears from materials other than the original patient sample may be made outside of a biosafety cabinet. All staining may be performed outside of a biosafety cabinet. Follow universal precautions when handling potentially dangerous materials.

10. PROCEDURAL NOTES:

- a. Great care must be taken to examine the Gram stained smear as some organisms are few in number.
- b. Relate as closely as possible what you see on the slide as pertains to numbers of cells and types, relative numbers of organisms, and their morphology.
- c. If unsure of slide interpretation, secure the help of a supervisor.

11. REFERENCES:

- a. Bailey, W.R. and Scott, E.G., Diagnostic Microbiology. 7th ed., St. Louis: C.V. Mosby Co., 1986.
- b. Henry, J.B., Clinical Diagnosis and Management by Laboratory Methods. 18th ed., Philadelphia: W. B. Saunders, 1991.