

GRAM STAIN

Preanalytical Considerations

I. PRINCIPLE

The Gram stain is used to classify bacteria on the basis of their forms, sizes, cellular morphologies, and Gram reactions; in a clinical microbiology laboratory, it is additionally a critical test for the rapid presumptive diagnosis of infectious agents and serves to assess the quality of clinical specimens. The test was originally developed by Christian Gram in 1884, but was modified by Hucker in 1921. The modified procedure provided greater reagent stability and better differentiation of organisms. Other modifications have been specifically developed for staining anaerobes and for weakly staining gram-negative bacilli (*Legionella* spp., *Campylobacter* spp., *Bacteroides* spp., *Fusobacterium* spp., *Brucella* spp.) by using a carbol-fuchsin or basic fuchsin counterstain. Interpretation of Gram-stained smears involves consideration of staining characteristics and cell size, shape, and arrangement. These characteristics may be influenced by a number of variables, including culture age, media, incubation atmosphere, staining methods, and the presence of inhibitory substances. Similar considerations apply to the interpretation of smears from clinical specimens, and additional factors include different host cell types and possible phagocytosis.

Gram stain permits the separation of all bacteria into two large groups, those which retain the primary dye (gram-positive) and those that take the color of the counterstain (gram-negative). The primary dye is crystal violet and the secondary dye is usually either safranin O or basic fuchsin. Some of the more common formulations include: saturated crystal violet (approximately 1%), Hucker's crystal violet, and 2% alcoholic crystal violet.

II. SPECIMENS

Smears for Gram stain may be prepared from clinical specimens, broth cultures, or colonies growing on solid media. Young cultures (<24 h) from noninhibitory media and fresh clinical specimens yield the most accurate results, and for some morphological parameters, broth culture smears are recommended.

III. MATERIALS

A. Reagents: Reagents may be purchased commercially or prepared in the laboratory. The various Gram stain modifications and used are seen in Table 1.

- a. Hucker's modification
 - i. Crystal violet
 - ii. Gram's iodine: *Caution: Iodine is corrosive. Avoid inhalation, ingestion, or skin contact.*
 - iii. Decolorizers
 1. Slowest: ethanol, 95%

2. Intermediate: acetone-alcohol; mixture of ethanol, 95% (100 ml) and acetone (reagent grade, 100 ml) Combine in a brown-glass bottle, label with 1-year expiration date, and store at room temp.
3. Fastest: acetone (reagent grade) *Caution: Ethanol and acetone are flammable.*

B. Supplies

- a. Glass slides (25 by 75 mm), frosted ends desirable
- b. 0.85% NaCl, sterile
- c. Pasteur pipettes and wood applicator sticks, sterile
- d. Microbiological loops, inoculating needles
- e. Supplies for disposal of biological waste, including “sharps”
- f. Microincinerator or Bunsen burner
- g. Immersion oil

C. Equipment: Optional materials, depending on specimen source of laboratory protocol

- a. Electric slide warmer, 60°C
- b. Centrifuge
- c. Cytospin centrifuge
- d. Vortex mixer
- e. Sterile tubes, screw cap
- f. Sterile scissors, scalpels, forceps
- g. Tissue grinder
- h. Methanol, absolute: store methanol in brown screw-cap bottles. A working supply may be stored in plastic containers if replenished every 2 weeks.
- i.

Analytical Considerations

IV. QUALITY CONTROL

A. Check appearance of reagents daily

- a. If crystal violet has precipitate or crystal sediment, refilter before use even when purchased commercially. **NOTE:** Some stains, especially basic fuchsin and safranin, can become contaminated. When suspected, either culture or start with fresh material in a clean bottle.
- b. Evaporation may alter reagent effectiveness; working solutions should be changed regularly if not depleted with normal use.

B. Daily and when a new lot is used, prepare a smear of *Escherichia coli* (ATCC 25922) and *Staphylococcus epidermidis* (ATCC 12228) or *Staphylococcus aureus* (ATCC 25923). Fix and stain as described. Expected results

- a. Gram-negative bacilli, pink
- b. Gram-positive cocci, deep violet **NOTE:** An alternative QC source is material scraped from between teeth with a wooden applicator stick; both gram-positive and -negative organisms will be present.

C. The following are some common causes of poor Gram stain results

- a. Use of glass slides that have not been precleaned or degreased **NOTE:** Storing slides in a jar with 95% ethanol will ensure clean slides. Drain excess alcohol or flame slide before use.

- b. Smear preparations that are too thick
- c. Overheating of smears when heat fixation is used
- d. Excessive rinsing during the staining procedure

D. To ensure accuracy of interpretation, set up a system for reviewing Gram stain reports.

- a. Daily review of selected Gram stains by supervisory personnel may help determine competency/retraining needs and will help in correlating relevant clinical information.
- b. Compare final culture results with Gram stain reports. NOTE: Not all organisms seen on a smear can be cultured.
- c. A set of reference slides should be available for training and comparison.

V. PROCEDURE

A. Smear preparation: Proper smear preparation should produce a monolayer of organisms sufficiently dense for easy visualization but thin enough to reveal characteristic morphological characteristics. Use clean, new glass slides. NOTE: When using the same pipette or swab, always inoculate culture media first before preparing the smear.

- a. Clinical specimens: **NOTE:** Wear latex gloves and other protection required for universal precautions when handling clinical specimens. Observe other BSL 2 recommendations.
 - i. Specimens received on swabs (A separate swab should preferably be submitted).
 - 1. Roll the swab gently across the slide to avoid destruction of cellular elements and disruption of bacterial arrangements.
 - 2. Alternatively, when only one swab is received, place the swab in a small amount of saline and vortex. Squeeze the swab against the side of the tube, and use the swab to prepare a smear. Use the remaining suspension to inoculate culture media.
 - ii. Specimens not received on swabs: aspirates, exudates, sputa, stools
 - 1. If the specimen is received in a syringe, first transfer entire amount to a sterile tube; vortex specimen if appropriate. **NOTE:** Do not accept syringes with attached needles. Establish this policy and educate all medical personnel regarding the need to remove all needles before transport; observe universal precautions.
 - 2. Select purulent or blood-tinged portions by using a sterile applicator stick, pipette, or wire loop. Very thick or purulent specimens may be diluted in a drop of saline on the slide for easier smear preparation.
 - 3. Spread the sample over a large area of the slide to form a thin film.

- iii. CSF and other body fluids requiring centrifugation: Some laboratories may choose to use a Cytospin slide centrifuge to concentrate body fluids for smear preparation. This method has been recommended to increase Gram stain sensitivity and to decrease centrifugation and examination time for more rapid results. **NOTE:** Using slides with etched rings helps to locate the inoculated area.
 - 1. After centrifugation, use a sterile pipette to remove supernatant to a sterile tube, leaving approximately 0.5 ml as sediment.
 - 2. Vortex or forcefully aspirate the sediment in and out of a sterile Pasteur pipette several times.
 - 3. Use the Pasteur pipette to transfer a small drop of the sediment to a clean slide.
 - 4. Do not spread the drop out; allow it to air dry.
- iv. Urine specimen
 - 1. Do not centrifuge; mix specimen well.
 - 2. Use a sterile Pasteur pipette to transfer 1 drop to a slide; do not spread the drop.
 - 3. Allow the drop to dry
- v. Dried material or very small amounts of clinical specimens
 - 1. Emulsify specimen in 0.5 ml of sterile saline; vortex if necessary.
 - 2. Use a sterile Pasteur pipette to transfer 1 drop to a slide
 - 3. Use the pipette tip to spread the drop into an even thin film
- vi. Biopsies and tissue sections: A “touch prep” and/or ground specimen preparation
 - 1. Mince with sterile scissors or scalpel
 - 2. Prepare a “touch prep” - use sterile forceps to hold pieces, and touch the sides of one or more of the minced fragments to a sterile glass slide, grouping the touches together for easier examination.
 - 3. For homogenized ground specimens, place 1 drop onto the slide and spread the material within a 1 inch circle.
- b. Broth cultures: One smear per slide is recommended to avoid wash-off from one area to another during staining.
 - i. Use a sterile Pasteur pipette (or a venting needle for containers with septa, such as blood culture bottles, to avoid manipulating a needle and syringe) to transfer 1 or 2 drops to the slide
 - ii. Spread drop into an even thin film
- c. Colonies from solid media

- i. Place a drop of sterile saline or water on the slide
- ii. Transfer a small portion of colony with a sterile applicator stick, wire needle, or loop.
- iii. Gently mix to emulsify. **NOTE:** Never mix too vigorously; avoid creation of aerosols.

B. Smear fixation: Smears may be fixed with heat or methanol

- a. Heat
 - i. Air dry smears on a flat surface, or place them on a 60°C electric slide warmer until dry.
 - ii. If smears are air dried, pass them two or three times through a flame, or hold the slide against the front of a microincinerator for 5 to 10 s. To avoid distortions, do not overheat.
 - iii. Allow slide to cool before staining.
- b. Methanol fixation prevents the lysis of RBCs, avoids damage to all host cells, and results in a cleaner background. It is strongly recommended for all clinical material, especially urines, and it also prevents wash-off of urine specimens.
 - i. Air dry the smear on a flat surface.
 - ii. Place a few drops of methanol on a slide for 1 min, drain off remaining methanol without rinsing, and allow slide to air dry.
 - iii. Do not use heat before staining.

C. Staining procedures

- a. Hucker's modification
 - i. Flood the fixed smear with the crystal violet solution; allow the stain to remain for 30 s.
 - ii. Decant crystal violet, and rinse slide gently with running water. *Caution: Excessive rinsing in this step could cause crystal violet to be washed from gram-positive cells. The flow of water may be applied to the underside of the angled slide to ensure a gentle flow across the smeared side.*
 - iii. Rinse off excess water with iodine solution, and then flood the slide with fresh iodine solution. Allow the iodine to remain for 3- s.
 - iv. Rinse gently with flowing tap water.
 - v. Decolorize by letting the reagent flow over the smear while the slide is held at an angle. Stop when the runoff becomes clear. Adjust decolorization time to thickness of the smear and type of decolorizer used.
 - vi. Remove excess decolorizer with gentle flow of tap water. **NOTE:** A properly decolorized smear appears with an almost olive-green hue and without observable evidence of crystal/gentian violet.

- vii. Flood the slide with safranin and allow counterstain to remain for 30 s.
 - viii. Remove excess counterstain with a gentle flow of tap water.
 - ix. Drain slide, and air dry in an upright position. Clean off the bottom of the slide by wiping the alcohol or acetone on a tissue or paper towel.
 - x. Examine the smear microscopically.
- b. Basic/carbol-fuchsin counterstain (see Table 1 for timing). Basic/carbol-fuchsin is recommended for detecting faintly staining gram-negative organisms.
 - c. Kopeloff's modification is recommended for better visualization and differentiation of anaerobes, which may easily overdecolorize and stain faintly with Hucker's modification (see Table 1).

Postanalytical Considerations

VI. REPORTING RESULTS

A. Results and interpretation

- a. Evaluate the general nature of the smear under low-power magnification (10X).
 - i. Determine if smear has been properly decolorized. Depending on the source of the specimen, the background should be generally clear or gram negative. If WBCs are present, they should appear completely gram negative. Do not mistake thin crystal/gentian violet precipitate needles for gram-positive bacillus-shaped bacteria.
 - ii. Determine if thickness of smear is appropriate. For proper interpretation, areas must be no more than one cell thick with no overlapping of cells.
- b. Examine smears prepared from clinical specimens under low power for evidence of inflammation. If appropriate for culture source, note the following:
 - i. Relative amounts of PMNs, mononuclear cells, and RBCs
 - ii. Relative amounts of squamous epithelial cells, bacteria consistent with normal microbiota, which may indicate an improperly collected specimen
 - iii. Location and arrangements of microorganisms
- c. Examine several areas of the smear under oil immersion for presence of microorganisms.
 - i. If no microorganisms are seen in a smear of a clinical specimen, report "No microorganisms seen."
 - ii. If microorganisms are seen, report relative numbers and describe morphology.
- d. Observe predominant shapes of microorganisms

- i. Overall shape: coccus, coccoid, coccobacillary, bacilli, filament, yeastlike
- ii. Appearance of ends; rounded, tapered, flattened, clubbed (swollen), concave, swelling of sides can suggest the presence of spores but can also be caused by vacuoles, marked pleomorphism, or irregular staining.
- iii. Appearance of sides: parallel, ovoid (bulging), concave, irregular
- iv. Nature of axis: straight, curved, spiral
- v. Pleomorphism (variation in shape): the descriptive term “diphtheroid” or “coryneform” is used to describe gram-positive bacilli that are pleomorphic, club shaped, or irregularly staining or that have palisading and/or angular arrangements (V and L shapes).
- vi. Branching or cellular extensions

B. Recording observations: Each laboratory must standardize specific reporting policies. Clinically significant findings should be called to the attention of the attending physician.

- a. Smears of clinical specimens: for urine cultures, examine 20 or more fields. Report as positive if an average of one or more organisms are seen per oil immersion field. This correlates with a colony count of $\approx 10^5$ CFU/ml.
 - i. Record relative amounts of observed cells and microorganisms. Commonly used quantitation systems include the following.
 - 1. Numerical
 - a. 1+ (< 1 per oil immersion field, 100x)
 - b. 2+ (1 per oil immersion field)
 - c. 3+ (2 to 10 per oil immersion field)
 - d. 4+ (predominant or >10 per oil immersion field)
 - 2. Descriptive
 - a. Rare (<1 per oil immersion field)
 - b. Few (1 to 5 per oil immersion field)
 - c. Moderate (5 to 10 per oil immersion field)
 - d. Many (>10 per oil immersion field)
 - ii. Record the morphology of observed bacteria

C. Gram stain review

- a. After interpretation of smears, hold slides long enough to allow a confirmatory review, especially if culture or other lab test results are inconsistent.
 - i. Drain or gently blot excess oil
 - ii. For slide libraries and teaching collections that will be stored for longer periods, immersion oil can be removed with xylene solution and the slides can be coverslipped using Permount to prevent fading.

- iii. When discarding stained smears, handle as biological waste and use biohazard containers as required by local/state regulations. Slides may puncture biohazard bags and should be treated as “sharps.” Cardboard boxes or other protective containers may be used.
- b. If a repeat Gram stain or a special stain to confirm findings suggested by a Gram smear interpretation is required when extra unstained smears are not available, a Gram-stained smear may be destained.
 - i. Remove immersion oil on the slide with xylene solution.
 - ii. Flood slide with acetone-alcohol until smear appears colorless.
 - iii. Restain.

VII. LIMITATIONS OF THE PROCEDURE

- A. Use results of Gram stains in conjunction with other clinical and laboratory findings. Use additional procedures (e.g., special stains, direct antigen tests, inclusion of selective media, etc.) to confirm findings suggested by Gram-stained smears.
- B. Careful adherence to procedure and interpretative criteria is required for accurate results. Accuracy is highly dependent on the training and skill of microscopists.
- C. Additional staining procedures are recommended for purulent clinical specimens in which no organisms are observed by the Gram stain method.
- D. Gram stain-positive, culture-negative specimens may be the result of contamination of reagents and other supplies, presence of antimicrobial agents, or failure of organisms to grow under usual culture conditions (media, atmosphere, etc.)
- E. False Gram stain results may be related to inadequately collected specimens.

SUPPLEMENTAL READING

Gram, C. 1884. *Fortschr. Med.* 2:185.

Isenberg, H.D. (ed). 1992. *Clinical Microbiology Procedures Handbook*. American Society for Microbiology, Washington, D.C.

Isenberg, H.D. (ed). 1995. *Essential Procedures for clinical Microbiology*. American Society for Microbiology, Washington, D.C.

APPENDIX

Gram stain reagents available from Medical Chemical Corporation are as follows:

| REAGENT | CATALOG NUMBER | SIZE AND CATALOG NUMBER |
|--|----------------|---|
| Acetone alcohol, 25% v/v | 304A | 304A 8 oz (decolorizer) 304A 1 gal (decolorizer) |
| Acetone alcohol, 50% v/v | 305A | |
| Crystal violet, 2% alcoholic | 321, 583A | |
| Crystal violet, Hucker's | 514A | |
| Crystal violet, saturated (approximately 1%) | 512A | 512A 8 oz 512A 1 gal |
| Gram's iodine | 322, 604A | 604A 8 oz 604A 1 gal |
| Gram's iodine, stabilized Has a longer shelf life, but is more difficult to use. | 605A | 605A 8 oz 605A 1 gal |
| Reagent alcohol | 323, 374A | |
| Safranin O, 0.4% w/v | 7965A | 7965A 8 oz 7965A 1 gal |
| Safranin O, 1% w/v | 324, 7972A | |
| Basic Fuchsin, 0.13% w/v | 435A | For better anaerobe staining substitute basic fuchsin for safranin. |
| Gram's stain kit, 2 oz Contains the following: Crystal violet, 2% alcoholic Gram's iodine Reagent alcohol decolorizer Safranin O counterstain, 1% w/v | 320 | |
| Gram's stain kit, 8 oz Contains the following: Crystal violet, saturated Gram's iodine Acetone-alcohol decolorizer Safranin O counterstain, 0.4% w/v | 607K | |