**INTENDED USE**

The gram stain is used to stain microorganisms from cultures or specimens by the differential Gram method. It is a valuable aid to the microbiologist. It can provide preliminary information concerning the type of organisms present, the techniques that should be pursued to characterize them and the therapy to initiate while waiting for culture and sensitivity results.

**PRINCIPLE**

The mechanism of the Gram stain is not clearly understood. There is general agreement that a Gram positive organism retains the primary stain after decolorization because of a variety of cell wall factors including the permeability and the presence or absence of specific chemical compounds.

The test consists essentially of applying Gram crystal violet to a fixed smear from a culture or specimen; removing this stain after a suitable staining period and then applying Gram iodine as a mordant. The mordant is removed and Gram decolorizer is added to remove the primary stain where possible. Gram safranin is then added as a counter stain.

Organisms are judged to be Gram positive if they retain the primary stain after decolorization. Gram negative organisms are decolorized and appear pink to red because they take up the counter stain.

**MATERIALS AND EQUIPMENT:**

 **Reagents - purchased**

1. Methanol, absolute
2. Gram Crystal Violet Solution, Gram Primary Stain, approximately 0.4% crystal violet in an aqueous alcohol solution.
3. Gram Iodine Solution stabilized.
4. Gram Isopropyl Decolorizer Solution, Gram Stain Decolorizer, isopropyl alcohol and acetone, approximately three parts to one part, respectively.
5. Gram Safranin Solution, Gram Counter stain, approximately 0.25% safranin in 20% ethyl alcohol

 **Supplies**

1. Wax pencil or diamond-tipped glass marker
2. Frosted end glass slides
3. Sterile 0.85% saline or water
4. Sterile pipettes, wood applicator sticks, inoculating loops, or needles
5. Supplies for disposal of biological waste, including sharps
6. Sterile tubes with caps
7. Sterile scissors, scalpels, and forceps
8. Immersion oil
9. Microscope

**STORAGE OF REAGENTS**

Store gram stain reagents at 15-30ºC.

Some precipitation may occur in the crystal violet solution upon prolonged storage. If this appears to affect the quality of staining, with the dispensing closure open, briefly warm the bottle or quantities dispensed into the bottle in a 37⁰C water bath, then close and shake until the precipitate is dissolved or filter stain.

**PRECAUTIONS**

These reagents are harmful or fatal if swallowed and can cause eye irritation if contact is made. In event of eye

contact, flush eyes with an eye wash system or tap water for 15 min.

Gram decolorizer solution is flammable and its vapors may be harmful; use in a well-ventilated area away from

open flames.

**SPECIMEN**

1. Clinical specimens generally excluding throat swabs, nasal swabs, sputum from cystic fibrosis patients, fecal material, and prosthetic devices.

Direct smears are useful for wounds, eye lesions, sterile fluids, body tissues, and certain discharges.

1. Broth and blood cultures to determine growth, Gram reaction, or morphology of bacteria.
2. Colonies growing on solid media

**REJECTION CRITERIA**

1. Gram stains are of little value for direct smears of stool, throat, urine, and sputum from cystic fibrosis patients.

Gram stains are also not routinely performed on direct smears of blood due to low organism density. The exceptions are the detection of fulminant sepsis in suspected meningiococcemia or asplenic patients with suspected pneumococcemia.

1. Catheter tip specimens

**QUALITY CONTROL**

New lot numbers or new shipments of stain are checked for positive and negative results upon receipt before they are used for patient results.

**Daily**

1. Check appearance of reagents
2. If crystal violet has precipitate or crystal sediment, refilter before use.
3. Change working solutions regularly if not depleted with normal use.

**Note:** Stains can become contaminated. When contamination is suspected, use a new lot of stain.

1. **Non-Microbiology staff** (those who perform Gram stains infrequently) must run a positive and negative control slide daily or with each use.
2. **Gram stain and culture result comparison –** Compare final culture results with Gram stain reports to check for recovery of morphologies noted in the Gram stain but not recovered in the culture. Review both the smear and culture when organisms in 3 to 4+ quantities are recovered in culture but not observed on the gram stain. Organisms that fail to grow in culture but are seen on Gram stain are usually fastidious or anaerobic and require specific media and growth conditions.

**Note:** Discrepancies should be evaluated for adequacy of material on slide, for errors in smear evaluation or for indications for further culturing methods (e.g., anaerobic, fungal or AFB culture).

* Corrective Action - It may be necessary to prepare another slide.
* Errors in smear evaluation by technical personnel should be reported to the lead tech and reviewed by the tech who performed the Gram stain for competency and education.

**Weekly**

Known positive and negative controls are run weekly by Microbiology personnel.

**Corrective Action**

Restain slide or prepare a new slide when:

* Stained smear preparations show evidence of poor quality
* Stains are difficult to interpret
* Interpretations are inaccurate
* Slides are too thick or lack sufficient specimen

**Procedure for Making Control Slides**

1. Using a 24 hour culture of E. coli ATCC 25922, make a suspension in saline equal to a 2 McFarland Std.
2. Using a 24 hour culture of Staph epidermidis, make a suspension in saline equal to a 1 McFarland standard.
3. Add equal amounts of the E.coli and S. aureus to a tube
4. Lay out slides on the counter from a box of frosted slides
5. Add a drop of organism mixture to each slide. Allow to dry at R.T.
6. Label each slide as +/0. Heat fix the slides and return to the original box. Label the box with +/0

**Expected results**

Organisms used and expected results are:

* Staphylococcus epidermidis - Gram positive – deep violet to purple
* Escherichia coli – Gram negative – pink or red

If the control slide fails to give the appropriate reactions restain the slide and check slides stained with the control for appropriate reactions (restain if necessary).

**TEST PROCEDURE:**

**Slide Preparation**

1. Prepare gram stain smears in a BSL2 cabinet (biological safety hood).
2. Take a clean frosted-end glass microscope slide and place it on a flat surface. The location of the specimen inoculum may be marked by inscribing an area with a diamond-tipped glass marker or wax pencil.
3. Label the frosted end with the following information:
* Accession number
* Patient name (Last, first)
* Specimen type (abbreviation okay)
* Date

**Smear Preparation**

 For direct smears, prepare a monolayer of organisms sufficiently dense for easy visualization but sparse

 enough to reveal characteristic arrangements. In very thick smears, decolorization cannot be adequately evaluated

 or controlled; very thin smears are easily decolorized.

 **Note:** As a guideline, newspaper print should be visible through the smear

 **1. Preparation of smears from ESwab device**

1. Vortex or mix the BD ESwab tube containing the swab sample for 5 s to release the sample from the swab and evenly disperse and suspend the patient specimen in the Liquid Amies transport medium.
2. Unscrew the BD ESwab cap and using a sterile pipet, transfer 1 - 2 drops of Liquid Amies medium to the inscribed area on the glass slide.
3. Allow the specimen on the slide to air dry or place the slide on a 60° C slide warmer.
4. Smears may be fixed with heat or methanol. Methanol fixation may be preferable as it prevents lysis of red

 blood cells, avoids damage to all host cells and results in a cleaner background.

 **2. Body fluids, bronchoalveolar lavage fluid (BAL), and CSF** (specimens that are not grossly bloody or purulent)

1. Using a sterile pipette,place 4 drops or 200µl of sample into a cytospin specimen chamber. Refer to Cytofuge 2 Cytocentrifuge Procedure.

**Note:** Use of a cytospin slide centrifuge to concentrate body fluids increases the Gram stain sensitivity.

1. As an alternative for viscous, cloudy, or low volume specimens, 1 or 2 drops of the specimen may be placed on a slide with a sterile pipette.
2. **Urine Specimens -** One organism per oil immersion field is significant of infection (examine at least 20 fields) and is equal to >100,000 cfu/mL

a. Place 10µl of a well-mixed, uncentrifuged urine onto a glass slide marked with a wax pencil to indicate the

 location of the sample drop.

* One organism per oil immersion field is significant of infection (examine at least 20 fields) and is equal

 to >100,000 cfu/mL

1. Air dry without spreading (smear area should be less than the area of a dime)

 **4. Specimens received on swabs**

a. Request a separate swab for adequate smear preparation. Use Red top culturette with two swabs.

 b. Roll the swab gently across the slide to avoid destruction of cellular elements and disruption of cellular

 arrangements.

1. Alternatively, when only one swab is received, place the swab in a small amount of sterile saline (in a sterile tube) or broth, cap tube and vortex. The swab against the side of the tube, and use the swab to prepare smear. Use the remaining suspension to inoculate culture media.

 **5. Specimens not received on swabs; aspirates exudates, sputa, etc.**

a. Transfer specimen to the labeled slide.

* Select purulent or blood-tinged portions of pus or sputum with a sterile applicator stick, pipette or loop.
* Spread the sample over a large area of the slide to form a thin film.

b. For extremely thick or purulent specimens

* Dilute in a drop of saline on the slide for easier smear preparation

 **6. Dried material or very small amounts of clinical specimen**

 a. Emulsify specimen in 0.5 ml of sterile broth. Vortex if necessary.

 b. Use a sterile pipette to transfer 1 drop to a slide.

 c. Use the pipette tip to spread the drop into an even film.

 **7. Biopsy specimens and tissue sections**

 a. Touch preparation

* Place tissue in a sterile petri dish, and mince with sterile scissors or surgical scalpel.
* With sterile forceps to hold pieces, touch the sides of one or more of the minced fragments to a slide, grouping the touches together for easier examination. Discard fragment. (This fragment cannot be used for culture because the slide is not sterile)
1. **Thin-smear preparation**
* When a specimen is soft tissue or thick exudates, prepare a smear by taking a small portion of tissue, and place it on slide with a swab or sterile forceps.
* Place second slide over sample, and press slides together.
* Separate slide by sliding the two away from each other.

 **9. Ground specimen preparation**

a. Using a sterile pipette, place a drop of ground specimen on a labeled slide.

 b. Spread 1 drop to the size of a dime.

  **Note:** Homogenizing or grinding tissues before preparing Gram stain slide will often destroy characteristic

 cellular entities and bacterial arrangements.

 **10. Broth cultures**

 a. Use a sterile pipette (or a venting needle or safety needle and syringe for blood culture bottles) to transfer 1

 or 2 drops to a slide.

 b. Spread drop into an even film.

 **11. Colonies from solid media**

a. Place a small loopful of distilled water or sterile saline on a clean microscope slide. Distilled water may

 distort cellular morphology of fragile organisms.

 b. Touch a sterile inoculating loop to a colony, rub it into the droplet to obtain an even suspension of

 microorganisms and spread the suspension over the surface of the slide to obtain the correct density. The

 resulting smear should be slightly cloudy and homogenous.

**Smear fixation**

 **1.** Air dry slides in biosafety cabinet or covered on a slide warmer at 60⁰C until dry.

 **2.** **Heat fixation**

* Place on a 60⁰C slide warmer
* Allow slide to cool before staining

 **3**. **Methanol fixation** prevents the lysis of RBC’s, produces a cleaner background, and prevents washing

 off of liquid specimens.

1. Place a few drops of methanol on air-dried slide for 1 min, drain off remaining methanol without rinsing, and allow slide to air dry again.
2. Do not use heat before staining.

**Slide staining**

 1. Flood the fixed smears with Crystal Violet Stain and allow to stain for 30 s.

 2. Wash off excess crystal violet lightly with cold tap water

 3. Rinse off excess water with Iodine

 4. Flood with Iodine and allow to remain on the slide for 30 s.

 5. Remove iodine by gentle washing with tap water

 6. Flood off excess water with Decolorizer until solvent runs colorlessly from the slide. Adjust decolorization time to

 the thickness of the smear.

 7. Flood off excess decolorizer with Safranin or basic Fuchsin and allow to stain for 1 minute

 8. Wash off excess stain with cold tap water

 9. Blot off excess water with paper toweling or blotting paper or allow to air dry

 10. Examine under low power and oil immersion

 **NOTE:** Gram stain reagents containing alcohol or acetone can no longer be washed down the drain.

 a. Gram stain reagents on the slides are dumped into a bin beneath the staining rack.

 b. When finished the bin is drained into a labeled hazardous waste jug kept in the yellow Flammable Cabinet.

**Microscopic examination of direct smear**

 1. Evaluate smear under **low power (10X)**

 a. Observe for stain crystals

* If an excess of precipitated stain is observed, decolorize and restain slide.
* Alternatively, prepare another smear.
* If precipitate continues, use freshly filtered crystal violet or counter-stain in a clean container.

 b. Determine if the smear is properly decolorized.

* The background should be generally clear or gram negative (source dependent).
* WBC,s should appear completely gram negative.
* If the slide is overdecolorized, completely decolorize and restain the slide.

 c. Determine if the thickness of the smear is appropriate. For proper interpretation, areas must be no more than

one cell thick, with no overlapping cells.

Prepare new slide if unreadable.

 d. Smears from clinical specimens: Examine under low power for evidence of inflammation.

* Examine 10 fields for urine and 20-40 fields for other specimens
* Observe distribution of organisms and cells
* Look for areas of inflammation or purulence and areas of apparent contamination with squamous epithelial cells (SECs). If no purulence is seen, choose areas of apparent necrosis, inflammatory cell debris, and mucous.

 2. If cells are present, determine the average count of WBC’s and epithelial cells in 20-40 fields containing cells.

 3. Using the oil immersion lens (100X), examine 20-40 fields in a representative area with inflammation or purulence

 to observe cell morphology and Gram reaction.

1. If rare or no organisms ar seen from a normally sterile-site specimen, but the specimen appears purulent, or the specimen looks suspicious, perform a more extensive review of the slide..

 b. Should a slide need to be restained to repeat Gram stain or prepare a special stain to confirm findings

 suggested by a Gram stain, remove the immersion oil with xylene or a xylene substitute and decolorize

 smear. Then restain slide.

 4. Wipe microscope objective between smears, so there is no carry over of bacteria from one smear to another.

**Preservation of direct smear slides**

 Drain or gently blot excess oil from slide and save slides for further evaluation for a minimum of 1

 week to allow for confirmatory review

**Examination of broth and plate smears**

 1. Specify Gram stain morphology (e.g. gram-positive cocci in clusters).

 2. Specify probable genus or organism group based on Gram stain morphology, colonial morphology, and culture

 characteristics.

**Discarding stained smears**

* Handle as biological waste
* Treat as sharps. Discard in biohazard red sharps containers.

**INTERPRETATION**

**Low-power observations**

 1. WBC’s and RBC’s suggest infectious process.

 **Note:** Neutropenic patients may have few WBC’s, but they may hve necrotic debris and protein in the background.

 2. Squamous epithethlial cells, food debris, etc., suggest an improperly collected specimen.

 3. Certain microorganisms (parasitic forms, branching hyphae, etc.)

**Oil immersion observations**

 1. Observe microorganisms for characteristic morphology and presentation. See appendix for species consistent with

 each morphology.

 2. Gram Reaction

 a. Gram Positive: deep violet

 b. Gram negative: pink to red

 c. Gram variable: both gram-positive and gram-negative cells with the same morphology

 **Note:** This may result from a smear of uneven thickness, incomplete decolorization, overdecolorization,

 presence of older cells, damaged cell walls, or the gram-variable nature of the particular organism.

 d. Gram neutral: taking up neither crystal violet or safranin counterstain.

 **Note:** These cells appear colorless against a generally gram-negative background and may be intracellular.

 This reaction may be found when fungal elements or Mycobacterium are present.

 3. Staining, characteristics, predominant shapes of microorganisms, relative sizes, and characteristic arrangements.

**Indications of infection**

 1. The presence of microorganisms from abnormally sterile site is likely to indicate infection with an organism.

2. For unspun urine specimens, the presence of microorganisms is likely to indicate a bacterial count of >=105.

 3. The presence of large numbers of a single type of organism in a noninvasively collected specimen, especially

 associated with WBCs is likely to indicate infection.

**Rare microorganisms found on smears**

 Be wary of interpretations made from observing very few organisms (especially in the absence of inflammation or if the

 organisms are unevenly distributed), because collection tubes, slides, and media may harbor nonviable bacteria.

 *For critical specimens, where the results will define an infectious process (e.g., CSF smears), prepare a second smear*

 *to confirm rare findings of microorganisms.*

**REPORTING RESULTS**

 1. *Notify the caregiver, physician, or reffering laboratory of record of any clinically significant findings from a normally*

 *sterile site. Document notification, including date, time and individual notified.*

 2. Determine the number of cells and bacteria. Counts should be performed only in areas representative of

 inflammation or necrosis, if present.

 3. Computer reporting:

* No cells and no organisms seen: enter mnemonic **NCO** under GRAMRESULT analyte
* No organisms seen – **NOS**
* No cells seen – **NCS**
* For cells – Enter the appropriate count in each analyte by choosing from the Preferred List indicated by the down arrow in the Phrase Result entry bar.
* For organisms enter quantity 1+, 2+, 3+, 4+ and organism description – Choose from Preferred List located on

Phrase Result bar. Some of the most common entries are:

 **GPC –** gram positive cocci

 **GPR –** gram positive rods

 **GNR –** gram negative rods

 **GVR –** gram variable rods

 **GPCPR –** gram positive cocci in pairs

 **GPCPRCH –** gram positive cocci in pairs and chains

 **GPCCH –** gram positive cocci in chains

 **GPCCHCL –** gram positive cocci in chains and clusters

 **GPCCL –** gram positive cocci in clusters

 **GNDC –** gram negative diplococci

 **GNCB –** gram negative coccobacillus

1. Refer to appendix 1 and 2 for reporting of respiratory specimens and female genital specimens.

Reporting gram stain results- Examine 20-40 fields

|  |  |  |  |
| --- | --- | --- | --- |
| Enumeration of cells underlow-power objective (10X) | Description of the types of cells to report | Enumeration of bacteria under  Oil immersion objective |  Description of the morphology  of bacteria |
| Count each type of cell and report: No cells: None seen<10(few):<10/LPF10-25(moderate): 10- 25/LPF>25(many): >25/LPF | Epithelial cellsPMNsRBCsHost cellular material | Count bacteria and yeasts from  areas associated with cells  and report:1+ (rare or occ): <1/OIF2+ (few): 1-5/OIF3+ (moderate): 6-30/OIF4+ (heavy): >30/OIF | Gram positive Cocci in pairs and chains Cocci in clusters Large rods Small rods Branching rods Coryneform rods (diphtheriod-like) Gram negative Diplococci Rods Rods, filamentous, pleomorphic, or fusiform CoccobacillusGram variable Rods CoccobacillusBudding yeast cellsPseudohyphae  |

**LIMITATIONS**

* The sensitivity of the Gram stain is 105 cells/ml or 104/ml if the specimen has been prepared with the cytocentrifuge.
* Gram stain of cytocentrifuged BAL with one or more organisms per OIF correlates with active bacterial pneumonia.
* Use Gram stain results in conjunction with other clinical and laboratory findings. Use additional procedures (e.g., special stains, selective media, etc.) to confirm findings suggested by Gram-stained smears.
* Careful adherence to procedure and interpretive criteria is required for accurate results.
* Gram-stain positive, culture negative specimens may be the result of contamination of reagents and other supplies, presence of antimicrobial reagents, or failure of organisms to grow under usual culture conditions (medium, atmosphere, etc.)
* False Gram stain results may be related to inadequately collected specimens or delays in transit.
* The Gram stain provides preliminary identification information only and is not a substitute for culture studies on a specimen.

**REFERENCES**

BBL Differential Gram Stain Reagents and Kits Package Insert, Becton Dickenson Microbiology Systems, 250 Schilling Circle, Cockersville, MD 21030, 1992**.**

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**Appendix 1**

**REJECTION CRITERIA FOR SPUTUM AND ENDOTRACHEAL ASPIRATES FOR CULTURE**

1. **Rationale**

 Diagnostic studies to detect and identify the etiologic agent are insensitive. Poorly collected respiratory specimens are a wasteful use of laboratory resources and can lead to erroneous reporting and treatment of patients.

1. **Rejection Criteria**
2. Do not reject sputum and endotracheal aspirates for culture for Legionella and AFB, or specimens from cystic fibrosis patients.
3. Examine 20 to 40 fields from sputum smears under low power and endotracheal smears under both low power and oil immersion. Average the number of cells in representative fields that contain cells.

Reject the following for culture, as poorly collected or not consistent with a bacterial infectious process.

1. Sputum >=10 SECs/LPF(squamous epithelial cells)

**Exception:** Accept the specimen if the number of WBCs is 10 times the number of SECs and there is 3 to 4+ of a single morphotype of bacteria.

1. Tracheal aspirates from adults: >=10 SECs/LPF or no organisms seen.
2. Tracheal aspirates from pediatric patients: no organisms seen.
3. **Reporting**

When rejecting the specimen for culture select the appropriate rejection comment in order entry.

* 2 Sputum rejected due to oral contamination
* 2 Endotracheal aspirate rejected; smear is negative for bacteria.
1. **Follow-up**
2. Notify the caregiver that the specimen will not be cultured.
3. Charge for the smear but not for the culture.

**Note:** For **outpatient specimens** and those that are grossly purulent (thick yellowish-green), the culture should be inoculated immediately upon receipt without waiting for the results of the smear. Incubated plates set up on rejected specimens may be examined to validate the laboratory policy and staff competency. Representative plates should be placed with the saved plates in case there is a question or a need for results on an outpatient specimen.

**Appendix 2**

**REPORTING GRAM-STAINED VAGINAL SMEARS TO DIAGNOSE BACTERIAL VAGINOSIS AND VAGINITIS**

1. **Rationale**

Bacterial vaginosis (BV) is a clinical syndrome characterized by an abnormal vaginal discharge in women in childearing years accompanied by a rise in pH from 4.5 and a fishy smell, especially after addition of KOH to the discharge. The microbial flora of the vagina shifts from predominantly lactobacilli to a mixture of Gardnerella, Prevotella, Mobiluncus, and other anaerobes and Mycoplasma hominus. Clue cells can also be present.

 The Gram stain with scoring of relative amounts of microbial morphotypes is the definitive laboratory method for

 diagnosis. The relative numbers of lactobacilli (medium to large gram positive rods) compared to gram negative

 curved rods (Mobiluncus) and gram-variable coccobacilli (Gardnerella) aid in the diagnosis.

 The Gram stain can be useful in the diagnosis of candidiasis but vaginal culture for yeast is more sensitive than

 Gram stain. The Gram stain is less helpful in the diagnosis of purulent vaginitis, characterized by a yellowis-

 green, foul-smalling discharge containing >=30 WBCs/HPF. The most common etiologic agent of this infection is

 Trichomonas vaginalis, which cannot be seen on Gram stain.

1. **Specimen**

Collect vaginal sectrtions from posterior fornix using the ESwab Transport device

1. **Procedure**
2. Perform gram stain using basic fuchsin as a counterstain instead of safranin.
3. Examine for host cells and bacteria in the same manner as for routine gram stain.
4. *For women in childbearing years and post-menopausal women on estrogen replacement therapy*, score as indicated in the table below.

 Table 1 Standardized scoring method for evaluation of Gram stains for BV (modified from Nugent)

|  |  |
| --- | --- |
| Quantitation of bacterial morphotype | Points scored per morphotype |
| None | 1+ | 2+ | 3+ | 4+ |
| Medium to large gram-positive rods |  4 | 3 | 2 | 1 | 0 |
| Small gram-negative or gram-variable rods |  0 | 1 | 2 | 3 | 4 |
| Curved gram-negative or gram-variable rods |  0 | 1 | 1 | 2 | 2 |

1. Record the number in each row that corresponds to the quantitation visualized in the smear.
2. Add the numbers to arrive at total score.
3. **Interpretation**

Interpret total score as follows:

* Normal: 0 to 3
* Intermediate 4 to 6
* BV 7 to 10

 Cultures with 3 to 4+ lactobacilli should correlate with scores of 0 to 3. Cultures with 3 to 4+ Gardnerella should

 correlate with scores of 7 to 10.

1. **Reporting**

Follow routine Gram stain reporting method to enumerate and report the following.

* WBCs and RBCs
* Clue cells
* Yeasts
* Pathogenic morphotypes, such as intracellular gram-negative diplococcic consistant with Neisseria

Report one of the following based on the score in Table 1

 0 to 3 Bacterial flora consistent with normal vaginal flora. **Mnemonic: NVAG**

 4 to 6 Mixed bacterial flora consistent with transition from normal vaginal flora. **Mnemonic: MXVAG**

7 to 10 Bacterial flora consistent with bacterial vaginosis. **Mnemonic: BV**