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***Department of Laboratory***

***Policy / Procedure***

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| **Title: MIC 1100.02—GRAM STAIN PROCEDURE/POLICY** | | **Original Date:**  01/14/2006 | **Page:** | |
| **Section: Microbiology/Immunology** | | **Reviewed Date:**  01/12/2019 | **Revised Date:**  2/7/2006, 06/28/2016, 01/12/2017 | |
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| **Keywords: Gram stain,** | |  | | |
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**PURPOSE:**

**The Gram stain is used to classify bacteria based on their cell wall structure and allows observation of their size and cellular morphology. It can be used to assess the quality of the clinical specimen and a critical test for rapid and presumptive diagnosis of infective agents directly from specimens.**

**Gram-positive species have a thick peptidoglycan layer and large amount of teichoic acids which make them unaffected by alcohol decolorization and retain their initial deep violet stain if not affected by age, antimicrobial agents, or other factors.**

**Gram-negative bacteria have a single peptidoglycan layer attached to an asymmetric lipopolysaccharide-phospholipid bilayer outer membrane interspersed with proteins. This outer layer is damaged by the alcohol-based decolorizer, allowing the crystal violet to leak out and be replaced by a counterstain, usually pink in color.**

**POLICY:**

Gram stains are incorporated into such procedures as: RESP (lower respiratory cultures—sputums), WD2 (wound culture with gram stain), SURG (surgical culture which includes a wound and gram stain along with an anaerobic culture), TIS (tissue cultures), BF (body fluids—cytospin Gram stain), CSF (spinal fluid—also cytospin GS), and can be ordered separately as “GS” in Meditech.

**Policy on STAT Gram stains:**

**Specimens for which STAT Gram stains are necessary or optimum:** Gram stains are performed “STAT” for: positive blood cultures, blood culture bottles inoculated with other fluids (such as peritoneal, abdominal, or pleural fluid), wounds that are suspected to be gas gangrene, necrotizing fasciitis, or systemic toxemia, CSF gram stains, any body fluid direct Gram stains, and any that are requested “STAT” from surgery or elsewhere.

Gram stains on sputum cultures should be done on the shift in which they are received so as to detect acceptability for culture or a single organism.

Gram stains, in addition to being ordered with primary culture, are often done from colonies growing on culture media or in nutrient broth.

**(SPECIAL OR NURSING) CONSIDERATIONS: If a Gram stain is requested as “STAT”, please call the Micro. Dept. to be sure it is done in a reasonable time.**

**EQUIPMENT:** If using large volumes of stain, please decant reagents into smaller bottles for daily use and replacing the smaller bottles monthly for the crystal violet and the counterstain to avoid precipitation of stain on slides.

**Methanol (absolute) for fixation or heat**

**Crystal Violet Gram’s Iodine**

**Decolorizer containing acetone-alcohol Safranin**

**Precleaned glass slides—alcohol wipes Wax pencil**

**SPECIMEN:** Clinical specimens, generally **exclude** throat swabs, sputum from cystic fibrosis patients, fecal material, and prosthetic devices. Direct smears are particularly useful for wounds, eye lesions, sterile fluids, abscesses, and body tissues.

**QUALITY CONTROL**: Prepare a faintly turbid broth culture of*Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923). Divide slide in half. Make slides for Quality Control by using 2 drops per slide, about the size of a dime. Fix in methanol and store at room temperature. Run QC weekly and with each new lot of reagent. For lab staff that perform Gram staining infrequently, if may be useful to stain a control slide with each patient.

**PROCEDURE: Preanalytical**

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| **Step 1** | Take 2 clean slides and wipe with alcohol pads. Allow to dry completely. Label slides with patient information: Name, Date, M#(Micro #), source. |
| **Step 2** | **For direct smears**, prepare a monolayer of organisms sufficiently dense for easy visualization but sparse enough to reveal characteristic arrangements. As a guideline, newspaper print should be visible through the smear.  NOTE: When working with the same pipette or swab, always **prepare the Gram stain before sequentially inoculating culture plates,** so that the smear is representative of the original cellular and microbial composition of the clinical specimen. |
| **Step 3** | **If there are two swabs sent**, one swab may be used for the smear. Roll the swab gently across the slide to avoid destruction of cellular elements and disruption of bacterial arrangements. |
| **Step 4** | **If only a single swab is received**, either “TNP” (test not performed) the Gram stain or place the swab in a small amount of sterile saline or culture broth, cap the tube and vortex. Squeeze the swab against the side of the tube, and use the swab to prepare the smear. Use the remaining suspension to inoculate the culture media. |
| **Step 4** | **Aspirates, exudates, sputa, etc.,** select purulent or blood-tinged portions of pus or sputum with a sterile swab, pipette, or loop. Spread the sample over a large area of the slide to form a film. |
| **Step 5** | **For extremely thick or purulent specimens**   1. Dilute in a drop of sterile saline for easier smear preparation. 2. Alternately, place specimen on one slide, cover it with a second slide, press slides together, and pull them apart.   Remove excess specimen from sides of slides with a disinfectant-soaked  paper towel. |
| **Step 6** | **Tissue and biopsy specimens**   1. **Touch preparation**, place tissue in sterile petri dish and mince with scapel.   Using sterile forceps to hold pieces, touch the sides of one or more of the  minced fragments to a sterile glass slide, grouping the touches for easier  examination.   1. **Thin-smear preparation**, take a piece of soft tissue or thick exudatewith a swab or sterile forceps. Place a second slide over the sample, and press slides together. Slide the two slides apart. 2. **Ground specimen preparation**, If there is no other option, use a ground specimen**,** spreading 1 drop to the size of a dime. |
| **Step 7** | **Dried material of very small amounts of clinical specimen**   1. Emulsify specimen in 0.5 mL of sterile THIO broth. Vortex if necessary. 2. Use a sterile pipet to transfer 1 drop to a slide. 3. Use the pipette tip to spread the drop into an even film. |
| **Step 8** | **E-swab Gram stains**   1. Label 2 slides with Patient information: Name, Date, Micro #, and source 2. Place slides on flat surface inside BSC. 3. Vortex or mix the BD ESwab tube containing swab sample for 5 seconds. 4. Unscrew the BD ESwab cap and using a sterile pipet, transfer 1-2 drops of Liquid Amies medium to the slide. Spread specimen with end of pipet to form an even, thin film. |

**PROCEDURE: Analytical**

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| **Step 1** | All slides should be allowed to air dry in a biosafety cabinet prior to staining. |
| **Step 2** | Smears may be fixed with heat or methanol.  **Heat fixation** may be performed by holding the air-dried slide in front of the micro-incinerator for 5-10 seconds or placing on slide dryer for 30 seconds. Allow slide to cool before staining.  **Methanol fixation** is preferable as it prevents lysis of red blood cells, avoids damage to all the host cells and results in a cleaner background. If using methanol, place a few drops on air-dried slide for 1 minute, drain off remaining methanol without rinsing and allow slides to air dry again. Do not heat before staining. |
| **Step 3** | Do not apply stains, decolorizer or water directly to the specimen area. Apply drops near the frosted end of the slide, allowing reagent to flow over the remaining surface. |
| **Step 4** | Flood the fixed smear with crystal violet solution. Allow the stain to remain for 30 seconds. |
| **Step 5** | Decant crystal violet and gently rinse slide under running tap water. Avoid excessive rinsing that could cause crystal violet from being washed from Gram-positive cells. |
| **Step 6** | Rinse off the excess water with Gram’s iodine solution, and then flood the slide with fresh iodine solution. Allow iodine to remain for 30 seconds. |
| **Step 7** | Rinse off iodine gently with tap water. |
| **Step 8** | Decolorize with acetone-alcohol solution by letting the reagent flow over the smear while the slide is held at an angle. Stop when the runoff becomes clear. Adjust decolorization to thickness of the slide. |
| **Step 9** | Remove excess decolorizer with gentle flow of water. |
| **Step 10** | Flood the slide with safranin counterstain and allow to remain for at least 30 seconds. |
| **Step 11** | Remove excess counterstain with a gentle flow of tap water. |
| **Step 12** | Drain slide, and air dry in an upright position. Slides may be gently blotted with filter paper to remove excess water, but care should be taken to avoid wiping the stained material from the slide. |

**PROCEDURE: Post Analytical**

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| **Step 1** | **Evaluate the general nature of the direct smear microscopically under the low power**.   1. Observe for stain crystals. If an excess of precipitated stain is observed, decolorize and re-stain slide. Or, prepare another Gram –stained smear. 2. If precipitate continues, use a freshly filtered crystal violet or safranin in a clean container. |
| **Step 2** | **Determine if the slide has been properly decolorized.**   1. Depending on the source, the background should be generally clear or Gram negative. 2. If WBC’s are present, they should appear completely Gram negative. 3. If slide is over-decolorized, completely decolorize and re-stain slide or make a new Gram-stained smear. |
| **Step 3** | **Determine if thickness is appropriate.** For proper interpretation, areas must be no more than one cell thick, with no overlapping of cells. Prepare a new slide if unreadable. |
| **Step4** | **For smears of clinical specimens, examine several fields (10-20 for urine, 20-40 for other specimens) under low power for inflammation.**   1. Observe distribution of organisms and cells. 2. Determine areas representative of inflammation or purulence and areas of apparent contamination with squamous epithelial cells. If no purulence is found, choose areas of apparent necrosis, inflammatory cell debris, and mucus. 3. Skip fields where there are no cells or bacteria and do not average these fields in the counts **if** there are fields where cells and/or bacteria are present. |
| **Step 5** | **If cells are present**, determine the average count of WBCs and epithelial cells in 20-40 representative fields that contain cells. |
| **Step 6** | **In an area of purulence or inflammation,** switch to the 100x oil lens and examine 20-40 fields to observe cell morphology and Gram reaction. |
| **Step 7** | **Grade the number of organisms.** If rare or no organisms are seen in a normally sterile site specimen, but the specimen appears purulent, it may be necessary to view the entire slide.Critical specimens should have the entire slide read, as well, to establish a negative result.  **Ignore one or two microorganisms on the entire slide, unless the results can be reproduced on a second smear and only then if it is from an invasively collected specimen.** |
| **Step 8** | Enumerate **cells** on **Low Power Field** and **bacteria and yeast on Oil Immersion Objective:**  **Cells Bacteria & Yeast**   * **Rare: <1**/**LPF** **Rare: <1/OIF** * **Few:** **1-9/LPF**  **Few: 1-5/OIF** * **Moderate:** **10-25/LPF** **Moderate: 6-30/OIF** * **Many: >25/LPF Many: >30/OIF** |
| **Step 9** | Drain or blot oil from slide and keep at least 1 week to allow confirmatory review. |
| **Step 10** | After interpretation and resulting, please leave the result “Preliminary” and leave the slide for review by Microbiology staff. |

**INTERPRETATION/RESULTS: See Procedure: MIC 1102.00FC and 1103.00FC for Gram stain interpretation and resulting.**

**QUALITY ASSURANCE:** Gram stains performed on off-shifts by non-microbiology personnel, will be released in the computer as “PV”—Preliminary Verified results. They will be reread and confirmed by Microbiology staff in the morning, marked as “reviewed by:” and left as “PV” until culture is finalized at which time it will be “FV” final verified along with the culture. This is the only way that it shows as we are reading the culture. Any correction will be noted in the computer and called to the attention of the physician or nursing unit, as well as the technologist who sent the original result.

**METHOD LIMITATIONS:** Bacterial culture results should be correlated to the Gram smear results from the original clinical specimen. Organisms that fail to grow in culture or are seen on the Gram stain may represent fastidious bacterial species (including anaerobes) that require specific media and growth conditions to be viable.

**PROCEDURE NOTES:**

**DOCUMENTATION: See Interpretation of Gram Stained Smears, MIC 1104.01.**

**REFERENCE:**

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