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| Gram Stain |
| **Purpose** | This procedure provides instruction for staining and reading Gram Stains. |
| **Principal and Clinical Significance** | The Gram stain is used to classify bacteria on the basis of their size, shape, arrangements, and Gram reactions. It is also used for the rapid presumptive diagnosis of infectious agents and serves to assess the quality of the specimen. The Gram stain can be used to divide bacteria into two groups: those that take up the basic dye crystal violet (gram positive) and those that allow the crystal violet to wash out easily with a decolorizer such as acetone (gram negative). The difference in composition between Gram-positive and Gram-negative cell walls and cell wall permeability is involved in the differential retention of crystal violet iodine complex. |
| **Policy Statements** | This procedure applies to Microbiologists who perform culture set-up and plate reading. |
| **Test Code** | GRAM |
| **Materials** |  |  |  |  |
|  | **Reagents** | **Supplies** | **Equipment** |
|  | BBL™* Gram Crystal Violet (primary stain) Cat. No. 212525
* Gram Iodine (mordant) Cat. No. 212542
* Gram decolorizer (decolorizer)

Cat. No. 212527* Gram Safranin (counterstain) Cat. No. 212531
 | * Glass slide
* Frosted glass slide
* 0.85% sterile saline
* Sterile disposable pipette
 | * Centrifuge
* Cytospin centrifuge
* Incinerator
* Inoculating loop
* Microscope
* Slide warmer
* Vortex mixer
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| **Specimen** | 1. Acceptable specimens: Most specimens submitted for culture
2. Smears may also be prepared from broth cultures or colonies growing on solid media.
3. Specimen quality can be assessed on any specimen that may become contaminated during collection by normal flora from adjacent sites. The presence of WBCs suggests an inflammatory process while many squamous epithelial cells suggests contamination.
* See Interpretation section for assessment of Sputum gram stains.
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| **Special Safety Precautions** | Microbiologists are subject to occupational risks associated with specimen handling. Refer to the safety policies located in the safety section of the *Microbiology Procedure Manual***.**1. [*Biohazard Containment*](file:///G%3A%5CLab%20Procedures%5CMicrobiology%5C1NEW%20Micro%20Procedure%20Manual.%20%28same%20as%20in%20Starnet%29%5CMCVI%203%20Safety%5CMCVI%203.1%20Biohazard%20Containment.docx)
2. [*Biohazardous Spills*](file:///G%3A%5CLab%20Procedures%5CMicrobiology%5C1NEW%20Micro%20Procedure%20Manual.%20%28same%20as%20in%20Starnet%29%5CMCVI%203%20Safety%5CMCVI%203.4%20Biohazardous%20Spills.docx)
3. [*Safety in the Microbiology Laboratory*](file:///G%3A%5CLab%20Procedures%5CMicrobiology%5C1NEW%20Micro%20Procedure%20Manual.%20%28same%20as%20in%20Starnet%29%5CMCVI%203%20Safety%5CMCVI%203.2%20Safety%20in%20the%20Microbiology%20Lab.docx)
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| **Storage** | 1. Gram Crystal Violet / Gram Safranin / Gram Iodine
2. Store reagents at room temperature 15-30ºC until expiration date.
3. The expiration date is for unopened bottles.
4. Do not open until ready to use.
5. Decolorizer
6. Store at room temperature 15-30ºC.
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| **Quality Control** | 1. Run controls weekly. Record on daily QC form.
2. If performed infrequently, perform positive and negative control with each specimen tested.
3. Positive control: *Staph aureus* ATCC 25923
4. Negative control: *E.coli* ATCC 25922
5. Perform QC with each new lot or shipment before put into service. Record results in QC manual.
6. If there is a QC failure, document observation, notify technical specialist and call BBL technical service at 1-800-638-8663.
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| **Procedure** | 1. **Smear preparation:** Label the slide with an aliquot (foot) label or use pencil if not available to label with accession # and date, source.
2. Specimens on swabs
3. Emulsify swab in 1.0 ml of SLNE by vortexing well. Squeeze the swab against the side of the tube to express remaining fluid and then discard.
4. First inoculate all culture media and then transfer 1-2 drops of the suspension onto a slide.
5. If for Gram Stain only, roll the swab gently across the slide to avoid destruction of cells and bacterial arrangements.
6. Specimens not on swabs: aspirates, exudates
7. Vortex specimen if appropriate.
8. Select purulent or blood-tinged portions by using a loop or pipette. For extremely thick specimens, dilute with 1-2 drops of sterile saline and vortex.
9. Spread specimen on slide to form a thin film.
10. Body fluids: centrifuge or use a Cytocentrifuge
11. Centrifuge specimen, remove supernatant leaving 0.5 ml.
12. Vortex sediment to resuspend.
13. Transfer a small drop with a sterile disposable pipette to glass slide.
14. Do not spread drop.
15. Urine
16. Do not centrifuge. Mix specimen well.
17. Using a sterile disposable pipette, transfer one drop to a frosted glass slide.
18. Do not spread the drop.
19. Colonies from solid media
20. Place a drop of sterile saline or water on a slide.
21. Transfer a small portion of the colony from an 18-24 hr culture with a loop or needle.
22. Gently mix to emulsify.
23. Broth cultures (THIO)
24. Using a sterile disposable pipette or loop, transfer one drop from the cloudy portion of the tube onto a frosted glass slide.
25. Do not spread the drop.
26. **Staining procedure**

**Note Hazardous waste:** Discard all stain reagents down the sink into a hazardous waste collection container (**HWC**). Refer to the procedure [MCVI 3.3 Hazardous Waste Management](file:///G%3A%5CLab%20Procedures%5CMicrobiology%5C1NEW%20Micro%20Procedure%20Manual.%20%28same%20as%20in%20Starnet%29%5CMCVI%203%20Safety%5CMCVI%203.3%20Hazardous%20Waste%20Management.docx) in the Microbiology Laboratory for additional information.1. Heat fix smear by holding slide against the front of the incinerator for 5-10 seconds or place on a 60ºC slide warmer. To avoid distortions, do not overheat.
2. Flood slide with crystal violet for 30-60 seconds.
3. Rinse slide briefly with tap water from squirt bottle. Caution: *Excessive rinsing in this step will cause the crystal violet to be washed from the gram-positive cells.*
4. Rinse excess crystal violet with iodine solution and then flood with fresh iodine solution. Allow to stand for 30-60 seconds.
5. Rinse with tap water from squirt bottle.
6. Decolorize with acetone-alcohol by letting the reagent run over the smear while the slide is at an angle. Run the decolorizer down the front of slide until the runoff is clear.
7. Remove excess decolorizer with tap water from squirt bottle.
8. Flood the slide with safranin for 30-60 seconds.
9. Rinse with tap water from squirt bottle.
10. Stand slide to air-dry, gently blot the slide dry with paper towels or use a slide drier.
11. **Examine the slide microscopically:**
12. Evaluate the general nature of the smear under low power (100X) before applying immersion oil.
13. Observe for stain crystals. If an excess of precipitated stain is observed, decolorize and restain the slide.
14. Determine if stain has been properly decolorized. Background should be generally clear or gram negative. If WBCs are present, they should appear gram-negative. If the slide has been over decolorized, completely decolorize and restain slide.
15. Determine if the thickness is appropriate. If it is too thick, organisms may be missed. The smear should not be more than one cell thick.
16. Evening and night staff will document on the Gram Stain Review sheet if sample and stain quality is acceptable by indicating Pass or Fail.
17. Examine several fields (20 – 40) under low power (100x) to determine the average count of white cells and epithelial cells. Quantitate each type of cells using table:

|  |  |
| --- | --- |
|  Grade  | White cells and epithelial cells per low power field |
| 1+ :  | Less than 1 |
| 2+ :  | 1-9  |
| 3+ :  | 10-25  |
| 4+ :  | Greater than >25  |

1. **Sputum**: add code **SALV** if 3+ or greater epithelial cells are observed. Refer to section **Sputum Examination** for further instructions.
2. Use area associated with cells to count bacteria and yeast.
3. Examine several fields (20 – 40) under oil immersion (1000x) for bacteria and yeast. Quantitate using table:

|  |  |
| --- | --- |
|  Grade  | Bacteria and yeast per high power field with oil |
| 1+ :  | Less than 1 |
| 2+ :  | 1-5 |
| 3+ :  | 6-30 |
| 4+ :  | Greater than >30 |

1. Blot excess oil from slide. Hold slide for one week.
2. If a Gram stain QA failure should occur, review slide and culture.
3. **St Paul Gram Stains:**
4. After resulting CSF or Body Fluid Gram stains, send slide to Minneapolis with stat courier for stat review by additional tech. Refer to procedure [MCVI 2.2 St Paul Microbiology Specimen Processing.](file:///G%3A%5CLab%20Procedures%5CMicrobiology%5C1NEW%20Micro%20Procedure%20Manual.%20%28same%20as%20in%20Starnet%29%5CMCVI%202%20Specimen%20Management%5CMCVI%202.2%20St%20Paul%20Microbiology%20Specimen%20Processing.docx)
5. All St Paul gram stains need to be reviewed. When CSF or Body Fluid slides are received from St Paul, it must be reviewed on a **stat** basis. Evening and nights, document review on Gram Stain Review sheet on clipboard by scope. Leave slide in slide holder for day shift.
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| **Interpretation** | 1. **Gram-positive** reaction: Purple-black cells.
2. **Gram-negative** reaction: Pink to red cells.
3. Observe for shape and arrangement of bacteria, i.e., bacilli, cocci, in pairs, clusters, tetrads, chains, etc.
4. Gram-variable reaction: both gram positive and gram negative with same morphology. May be due to:
5. Incomplete de-colorization
6. Over-decolorization
7. Gram variable nature of the particular organism
8. Damaged or older cells.
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| **Critical Values** | 1. **Positive** **BC** (Blood Cultures) are reported immediately by phone to the Physician or nurse, excluding those with pending BioFire FilmArray BCID results.
2. **Positive** **BF** (Body Fluid Culture) **gram stains that show organisms** are reported immediately by phone to the Physician or nurse.
3. **Positive CSF** grams stains are reported immediately by phone to the Physician or nurse.
4. Document the person phoned -- first name and first initial of last name as well as credentials**.**
5. Report the presence or absence of **both** bacteria and WBCS
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| **Infection Prevention Notification** | **Call Infection Prevention at 651-220-5555 or the On Call Pager at 952-260-9021 with gram stain results that appear to be gram negative cocci/gram-negative diplococci.** |
| **Sputum Examination** | 1. **Sputum**: Examine the slide under low power (10X objective) to access specimen quality.
* Examine a minimum of 20-40 fields concentrating on areas with WBCs.
* An acceptable specimen will have less than 10 squamous epithelial cells (SECs) per low power field.
* In general, the ratio must be ≥ 2:1, WBCs to SECs or mucus threads present with <10 cells (WBCs or SECs). The number of WBCs may not be relevant if the patient is neutropenic
1. If ≥10 SECs/low-power field are observed, report results and add the Sunquest code **SALV:** SPECIMEN MICROSCOPICALLY RESEMBLES SALIVA; suggest repeat specimen.
2. DO NOTreject sputum specimens on CF patients. Rejection on the basis of specimen quality is inappropriate because the organisms associated with CF disease and their presence in culture are significant regardless of the Gram stain findings.
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| **Limitations** | 1. The age of a culture affects its degree of Gram positivity. Young actively growing cultures retain the crystal-violet-iodine complex more avidly than older cultures.
2. Overheating the slide may cause gram-positive bacteria to stain gram negative.
3. Antimicrobial agents may make gram positive organisms more susceptible to de-colorization.
4. The gram stain should be used only to provide taxonomy information. It should not be used as a substitute for culture.
5. Gram-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.
6. Gram stain is not useful for throat or feces except to screen for the presence of WBC and overgrowth of yeast.
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| **Method Performance Specifications** | **Reagent Preparation**1. Decolorizer
2. Add 500 ml 95% alcohol to 500 ml of acetone.
3. Mix thoroughly.
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| **Result Reporting** | 1. Record Gram stain results in Sunquest MRE *Direct Exam* tab in Observations or Workups by using customized keyboards or by entering a code in the result box.
2. Report bacteria and WBC results quantitatively, i.e., 1+, 2+, 3+ or 4+.

 Example: Observations: 1. 4+ GRAM NEGATIVE RODS key A 2. 3+ GRAM POSITIVE COCCI key S 3. 3+ GRAM NEGATIVE COCCI key D 4. 2+ GRAM POSITIVE RODS key F 5. 2+ WBC’s key W 6. 1+ EPITHELIAL CELLS key E FINAL 070903 key /1. Use the following quantitation method for reporting **bacterial** and **yeast:**

 1+ : Less than one organism per oil immersion field 2+ : 1-5 organisms per oil immersion field 3+ : 6-30 organisms per oil immersion field 4+ : Greater than >30 organisms per oil immersion field1. Use the following quantitation method for reporting **cells**:

 1+ : Less than one WBC/epithelial cells per low power field 2+ : 1-9 WBC/epithelial cells per low power field 3+ : 10-25 WBC/epithelial cells per low power field 4+ : Greater than >25 WBC/epithelial cells per low power field1. If no organisms are seen, report as “No organisms seen”, Sunquest code **NOS,** key (`).
2. If no WBC are seen, report as “No WBC’s seen”, Sunquest coded **NWBC,** (comma key).
3. Enter a Gram stain culture workup result under the appropriate workup using the Code:  **GMS**.

 Workups: Wkup # 1 Workup Components Med : SB SC: SB MAC  Desc: GRAY-SM GMS: GMNR ID: GNR1. If a Gram stain QA failure (when gram stain results do not correlate to culture results) should occur, review the slide and the culture. Hold culture plates an additional day if necessary. Correct the Gram stain results if a there is discrepancy between what is observed and what is reported.
2. If a culture requires a correction, the code **CORR** (corrected report) must be reported on an observation line in the Direct Exam or Culture Entry tab. Refer to policy [MCVI 5.1 LABELING ERRORS/SPECIMEN MIX-UP](file:///G%3A%5CLab%20Procedures%5CMicrobiology%5C1NEW%20Micro%20Procedure%20Manual.%20%28same%20as%20in%20Starnet%29%5CMCVI%205%20Computer%5CMCVI%205.1%20Labeling%20Errors-Specimen%20Mix-up.docx) for Sunquest report entry information located in the Microbiology Computer Manual and/or Microbiology Manual.
3. Review **Culture Summary** for accuracy before filing report.
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| **References** | 1. BD Gram Stain Kits and Reagents, [Product Insert] September 2017, Becton, Dickinson and Company, Sparks, MD., 21152, USA.
2. Leber, A. L. Clinical Microbiology Procedures Handbook. 4th Edition (3.2.1), 2016 ASM Press Washington, DC
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| **Training Plan/ Competency Assessment** | **Training Plan** | **Initial Competency Assessment** |
| 1. Employee must read the procedure.
2. Employee will observe trainer performing the procedure.
3. Employee will demonstrate the ability to perform procedure, record results and document corrective action after instruction by the trainer.
 | 1. Direct observation.
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| **Historical Record** |  |  |  |  |
|  | **Version** | **Written/Revised by:** | **Effective Date:** | **Summary of Revisions** |
| 1.0 | Pat Ackerman | 1973 | Initial Version |
| 1.1 | Pat Ackerman | 05/1975 |  |
| 1.2 | Pat Ackerman | 02/06/1992 |  |
|  | 1.3 | Pat Ackerman | 09/06/2003 |  |  |  |
| 1.4 | Pat Ackerman | 01/12/2008 | Updated Sunquest 6.2 reporting information. Revised CORR statement. Update procedure Specimen Processing #1, Specimens on swabs. Added hazardous waste information to staining procedure. Added Hyper-link to Labeling policy and Haz waste procedure. |
| 1.5 | Becky Carlson | 07/19/2012 | Revised Iodine reagent preparation 🡪 stabilized Iodine. |
|  | 1.6 | Becky Carlson | 04/15/2014 | Added Stool PMNS procedure. |
|  | 1.7 | Tina Gronquist | 06/20/2014 | Updated into online format. |
|  | 1.8 | Becky Carlson | 08/14/2014 | Added WBC quantitation clarification |
|  | 1.9 | Becky Carlson | 1/23/2015 | Added Zn PVA Stool preservative for PMNS |
|  | 2 | Becky Carlson | 4/18/2015 | Re-numbered from MC 701 for CMS load. Added Critical Value and Critical test reporting. |
|  | 3 | Susan DeMeyere | 8/7/2018 | Add perform QC with each patient if performed infrequently. Remove PMNS testing. Move slide interpretation to procedure section.  |
|  | 4 | Susan DeMeyere | 6/14/2019 | Added instructions for St Paul gram stain review. |
|  | 5 | Susan DeMeyere | 2/4/2020 | Added instructions for evaluation of sample preparation and stain quality.  |
|  | 6 | Susan Demeyere | 9/8/2020 | Added enumerate cells on low power, adjust numbers for grading. |
|  | 7 | Susan DeMeyere | 9/15/2023 | Removed calling negative CSF Gram stains. |