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| Gram Stain |
| **Purpose** | 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 read Gram stains. |
| **Test Code** | GRAM  |
|  | **Reagents** | **Supplies** | **Equipment** |
| **Materials** | 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
* Store at 15-30ºC
 | * 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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| **Sample** | 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 Collection and Transport: Refer to [*Microbiology Specimen Collection and Handling Manual.*](http://www.childrensmn.org/Manuals/Lab/Chapters.asp?account=MicroBioViral)*.*
4. 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/virologists are subject to occupational risks associated with specimen handling. Refer to the safety policies**:**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. [Safety in the Microbiology/Virology 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)
* [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)
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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: Staph aureus ATCC 25923
4. Negative: 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 supervisor 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 acc# 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 in a hazardous waste collection container (**HWC**). Refer to the procedure MCVI 3.3 Hazardous Waste Management in the Microbiology and Virology 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. Decant in HWC.
3. Rinse slide briefly with tap water. 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. Decant in HWC.
5. Rinse with tap water.
6. Holding the slide over the HWC, 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, down the back of the slide and then down the front once again until the runoff is clear.
7. Remove excess decolorizer with tap water.
8. Flood the slide with safranin for 30-60 seconds. Decant in HWC.
9. Rinse with tap water.
10. Stand slide to air-dry, gently blot the slide dry with paper towels or use a slide drier.
11. Evaluate the general nature of the smear under low power, before applying immersion oil.
12. Observe for stain crystals. If an excess of precipitated stain is observed, decolorize and restain the slide.
13. Determine is 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.
14. 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.
15. Examine several fields (20 – 40) under oil immersion (1000x) to observe cell morphology and Gram reaction.
16. Examine several fields (20 – 40) under oil immersion (1000x) for white cells, epithelial cells, bacteria, and yeast.
17. Quantitate bacteria, yeast and cells.
18. Blot excess oil from slide. Hold slide for one week.
19. If a Gram stain QA failure should occur, review slide and culture.
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| **Interpretation/ Results** | 1. Gram-positive reaction: Purple-black cells.
2. Gram-negative reaction: Pink to red cells.
3. Gram-variable reaction: both gram positive and gram negative with same morphology. May be due to:
4. Incomplete decolorization
5. Overdecolorization
6. Gram variable nature of the particular organims
7. Damaged or older cells.
8. Observe for shape and arrangement of bacteria, i.e., bacilli, cocci, in pairs, clusters, tetrads, chains, etc.
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| **Critical Values** | 1. All **positive** **BC** (Blood Cultures) are reported immediately by phone to the Physician or nurse.
2. All **BF** (Body Fluid Culture) **gram stains that show organisms** are reported immediately by phone to the Physician or nurse.
3. **ALL CSF grams must be phoned to a care provider at the level of RN or greater.**
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 651-629-4444 with gram stain results that appear to be gram negative cocci/gram-negative diplococci.** |
| **Sputum Contaminated with Saliva** | 1. Sputum gram stain interpretation: Stain smear and examine the slide under low power (10X objective) to access specimen quality.
2. Examine a minimum of 10 fields concentrating on areas with WBCs.
3. An acceptable specimen will yield 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
4. If unacceptable, report results and add the Sunquest code **SALV:** SPECIMEN MICROSCOPICALLY RESEMBLES SALIVA; suggest repeat specimen.
5. 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 decolorization.
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.
* Reagent Storage
1. Gram Crystal Violet / Gram Safranin / Gram Iodine
2. Store reagents at room temperature 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.
7. 1 year expiration
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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+.

 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 cell morphotypes:

 1+ : Less than one organism/WBC per oil immersion field 2+ : 1-5 organisms/WBC 3+ : 5-10 organisms/WBC 4+ : Greater than 10 organisms/WBC1. 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 MW 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.
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. *Bailey and Scott’s “Diagnostic Microbiology*”, Baron, E. J.; Forbes, B.A.; Sahm, D.R.; and Weissfield,

 A.S.; C. V. Mosby, Inc. twelfth edition, 2007,.1. BD Gram Stain Kits and Reagents, BBL circular 88-20191, Revised May. 2017, Becton, Dickinson

 and Company, Sparks, MD., 21152, USA .3. Leber, A. L. Clinical Microbiology Procedures Handbook. 4th Edition, 2016 ASM Press Washington, DC  |
| **Appendices** | WORKLABEL MEDIA-FORM DEFINITION BATTERY: GRAM SPEC MEDIA 0 SM |
| **Training Plan/ Competency Assessment** | **Training Plan** |  **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.
 | Direct observation1. Day Micro staff: Complete Gram stain proficiency surveys 3 times per year.
2. Evening and Night shift staff: Complete Gram stain proficiency surveys 3 times per year.
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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.  |