SGMC.M 1002 Gram Stain

Copy of version 1.0 (approved and current)

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Approval and Periodic Review Signatures

Type	Description	Date	Version	Performed By	Notes
Approval	Lab Director	3/3/2019	1.0	Nicolas Cacciabeve	
Approval	Micro Director approval	2/19/2019	1.0	Ronald Master	
Approval	QA approval	2/15/2019	1.0	Leslie Barrett	

Version History

Version	Status	Type	Date Added	Date Effective	Date Retired
1.0	Approved and Current	Initial version	2/15/2019	3/5/2019	Indefinite

Linked Documents

- AG.F24 Gram Stain QC Manual Method
- AG.F25 Gram Stain Quality Control for Wescor and Previ Stainers
- AG.F27 Microbiology Gram Stainer PM Chart

Technical SOP

Title	**Gram Stain**	
Prepared by	Ron Master	Date: 5/4/2010
Owner	Ron Master	Date: 5/4/2010

Laboratory Approval	**Local Effective Date:**	
Print Name and Title	**Signature**	**Date**
Refer to the electronic signature page for approval and approval dates.		

Review		
Print Name	**Signature**	**Date**

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1. TEST INFORMATION

Assay	Method/Instrument	Order Code
Gram Stain	Manual	GS

Synonyms/Abbreviations
Smear for bacteria, GS, Gram smear

Department
Microbiology

2. ANALYTICAL PRINCIPLE

The Gram Stain is used to classify bacteria on the basis of their forms, sizes, cellular morphologies, and Gram reaction. It is also a critical test for the rapid presumptive diagnosis of infectious organisms and serves to assess the quality of clinical specimens.

Bacteria vary in their ability to retain crystal violet after treatment with an organic solvent (either alcohol or acetone). Those organisms which retain crystal violet are considered gram positive; those which do not retain the crystal violet and are stained pink or red by the counter-stain are considered gram negative. The difference in the staining reaction can be directly related to the cell wall structure of gram negative and gram positive organisms, although the actual mechanism is still unclear.

3. SPECIMEN REQUIREMENTS

3.1 Patient Preparation

Component	Special Notations
Fasting/Special Diets	Not Applicable
Specimen Collection and/or Timing	Not Applicable
Special Collection Procedures	Refer to the online Laboratory Test Directory via hospital intranet for instructions on specimen collection and transport.
Other	Not Applicable

3.2 Specimen Type & Handling

Criteria	
Type -Preferred	1. Body Fluids and CSF: Specimens should be processed either by a cytocentrifuge (preferred) or regular centrifuge and sediment used to prepare a smear. Using slides with etched rings may help locate the inoculated area. Smear should be prepared in a BSC. 2. Urine Specimens: Place one drop of well-mixed, un-centrifuged specimen on a slide with a sterile Pasteur pipette (do not spread the drop). Allow the drop to dry. 3. Sputum Specimen – Always order a Gram stain with each sputum culture. Select the most purulent or most blood-tinged portion of the sample using sterile loops, rayon swabs, sticks or a pipette. **THE USE of COTTON TIPPED SWABS FOR SMEAR PREPARATION IS NOT OPTIMAL.** Smear should be prepared in a BSC. 4. Swab Specimens: • Note: If two swabs are submitted, use one to inoculate media and the other to prepare the smear.

Criteria	
	• If only one swab is received, inoculate the solid media first. (see section 8.1 step 2) • Smear should be prepared in a BSC.
Type-Preferred cont.	B. Specimens from secondary sources: 1. Liquid medium (e.g., thioglycolate broth) Prepare smear using sterile pipette as for urine. in 2. (above). Prepare smear in a BSC. 2. Cultures growing on solid media: Mix enough organisms from an isolated colony with a drop of saline on a slide to obtain a barely visible turbidity. 3. Blood cultures: Prepare smear using a venting needle (to avoid manipulating a needle and syringe) and transfer 1 to 2 drops to the slide. Smear should be prepared in a BSC.
-Other Acceptable	Tissues in transport media or in a small amount of sterile saline are acceptable.
Collection Container	Specimens may be collected using various devices such as sterile swabs, scalpels, syringes or Pasteur pipettes. Inform clinicians not to submit syringe with needle attached. If submitting syringe, the client must remove needle, expel air and recap syringe.
Volume - Optimum	2-3mL of fluid or 1-2 grams of tissue.
- Minimum	One properly prepared slide (air-dried and heat or methanol fixed) or any amount of material.
Transport Container & Temperature	• Air-dried smears should be submitted in a slide envelope, plastic or cardboard slide holder or culture swabs may be used. • Sterile screw cap cups or other sterile containers may be used for whole specimens.
Stability & Storage Requirements	• Air dried smears are stable for at least 10 days at room temperature. • Specimens on swabs in transport media are stable for 2 days at room temperature. • For Gram stain purposes, respiratory specimens are stable for up to 24 hours at room temperature and 48 hrs refrigerated. Stability for culture is less. • Body Fluids - Stable for 48 hrs at room temperature • Port-a-cult – Up to 72 hours room temperature
Timing Considerations	None

Criteria	
Unacceptable Specimens & Actions to Take	Reject: • Broken slides or smears too thick to read. • Smears fixed with Cytology fixative • Slides previously stained by cytology and cover slipped • Specimens in DNA probe transports or PVA transports • Dry swabs
Compromising Physical Characteristics	Not Applicable
Other Considerations	Not Applicable

NOTE: Labeling requirements for all reagents, calibrators and controls include: (1) Open date, (2) Substance name, (3) Lot number, (4) Date of preparation, (5) Expiration date, (6) Initials of tech, and (7) Any special storage instructions. Check all for visible signs of degradation.

4. REAGENTS

The package insert for a new lot of kits must be reviewed for any changes before the kit is used. A current Package Insert is included as a Related Document.

4.1 Reagent Summary

Manual Stain

Reagents / Kits	Supplier & Catalog Number	Quantity
Gram Stain Kit	BD 212524	4 X 250 mL
Or individual reagents:		
Gram Crystal Violet	BD 212525	4 X 250 mL
Gram Iodine	BD 212529	4 X 250 mL
Gram Decolorizer	BD 212527	4 X 250 mL
Gram Safranin	BD 212531	4 X 250 mL

Wescor Stainer

Reagents	Supplier & Catalog Number
Crystal Violet	Wescor SS-041C
Iodine	Wescor SS-041B
Decolorizer with Safranin	Wescor SS-041A
Reagent Grade Methanol	Wescor SS-MeOH

Previ Color Gram Stainer

Reagents	Supplier & Catalog Number
Crystal Violet – C	bioMerieux 29587
Iodine – B	bioMerieux 29586
Decolorizer with Safranin – A	bioMerieux 29582
Reagent Grade Methanol – E	Not specified

4.2 Reagent Preparation and Storage

4.2.1 Manual Stain

Reagent	Crystal Violet (1% aqueous solution) Store at room temperature
Container	Manufacturer's packaging
Storage	Store at room temperature.
Stability	Stable until expiration noted on bottle.
Preparation	None

Reagent	Gram's Iodine (Aqueous solution of iodine and potassium iodide)
Container	Manufacturer's packaging
Storage	Store at room temperature.
Stability	Working solution stable for 3 months from preparation.
Preparation	Working solution prepared by adding entire 2.5 mL ampule of Gram Iodine 100X to 250 mL bottle of Gram Diluent. Mix thoroughly. Write date prepared and your initials on the bottle. Caution: Iodine is corrosive. Avoid contact.

Reagent	Gram's Decolorizer (Mix of 50% acetone and 50% 0f 95% ethanol)
Container	Manufacturer's packaging
Storage	Store at room temperature. Store decolorizer in a flame proof cabinet away from sparks or high heat.
Stability	Stable until expiration noted on bottle.
Preparation	None

Reagent	Gram's Safranin (0.04% Safranin and water in reagent alcohol)
Container	Manufacturer's packaging
Storage	Store at room temperature.
Stability	Stable until expiration noted on bottle.
Preparation	None

4.2.2 Wescor Stainer

Reagent	**Crystal Violet**
Container	500 mL
Storage	Store at room temperature away from direct light.
Stability	Stable until expiration noted on bottle.
Preparation	Ready for use

Reagent	**Iodine**
Container	500 mL
Storage	Store at room temperature away from direct light.
Stability	Sensitive to light, heat, and oxygen. Stable until expiration noted on bottle.
Preparation	Ready for use.

Reagent	**Decolorizer with Safranin**
Container	500 mL
Storage	Store at room temperature away from direct light.
Stability	Photosensitive, may slowly form peroxidase. Stable until expiration noted on bottle.
Preparation	Ready for use

Reagent	**Reagent Grade Methanol**
Container	500 mL
Storage	Store at room temperature away from direct light. Store in a flame proof cabinet away from heat, flame, and sources of ignition.
Stability	Stable until expiration noted on bottle.
Preparation	Ready for use

4.2.3 Previ Color Gram Stainer

Reagent	**Crystal Violet**
Container	500 ml
Storage	Store at room temperature away from direct light.
Stability	Stable until expiration noted on bottle.
Preparation	Ready for use

Reagent	**Iodine**
Container	500 ml
Storage	Store at room temperature away from direct light.

Stability	Sensitive to light, heat, and oxygen. Stable until expiration noted on bottle.
Preparation	Ready for use.

Reagent	**Decolorizer with Safranin**
Container	500 ml
Storage	Store at room temperature away from direct light.
Stability	Photosensitive, may slowly form peroxidase. Stable until expiration noted on bottle.
Preparation	Ready for use

Reagent	**Reagent Grade Methanol**
Container	500 ml
Storage	Store at room temperature away from direct light. Store in a flame proof cabinet away from heat, flame, and sources of ignition.
Stability	Stable until expiration noted on bottle.
Preparation	Ready for use

5. CALIBRATORS/STANDARDS

5.1 Calibrators/Standards Used

Not applicable

6. QUALITY CONTROL

6.1 Controls Used

- Gram stain reagents should be tested using known gram-positive and gram-negative organisms.
    1. Gram Positive Control: *Staphylococcus aureus* ATCC25923
    2. Gram Negative Control: *Escherichia coli* ATCC25922

    Additional organisms such as a *Bacillus* species may be added in situations where problems with decolorization or interpretation are encountered.

    Use of a clinical specimen such as a sputum smear may also be considered.

6.2 Control Preparation and Storage

Refer to the control organism product insert sheet for preparation, storage and handling instructions. Make a lightly turbid suspension of both control organisms in 0.5 mL of saline. Pipette or apply several loops full of the suspension to the surface of a glass microscope slide and allow to air dry. Fixing with 95% methanol is preferred. Alternatively, heat fix (allow slides to cool before staining). Store smears in an appropriately labeled box at room temperature. Control slides are stable for 1 year from preparation.

Preparation	Prepare a suspension of mixed *Staphylococcus aureus*_ATCC 25923 and *Escherichia coli* ATCC 25922 in saline. Place a thin drop on the: surface of a slide and allow it to air dry. Fix the air-dried smear on the heat block no longer than 10 minutes and stain the smear in the same manner as the test slides. Examine microscopically.
Storage/Stability	Prepared slides can be made in advance. Store smears in an appropriately labeled box at room temperature. Control slides are stable for 1 year from preparation.

6.3 Frequency

- Quality control testing is performed before first patient use with each lot number and/or shipment of any reagent. Additionally, quality control is performed daily.
- Refer to specific test procedures or department QC plan for QC frequency requirements.

6.4 Tolerance Limits and Criteria for Acceptable QC

- Do not interpret test unless the controls yield expected results as below.

Control Organism	Expected Results
Staphylococcus species	Deep purple (gram positive) cocci must be observed.
Escherichia coli ATCC 25922	Pink (gram negative) rods must be observed.
Bacillus species	Purple (gram positive), may be stain irregularly (purple and pink)

- Corrective Action:
  - All steps taken in response to QC failures must be documented, including: a description of the QC failure, the root cause of the problem, actions taken to correct the problem, how patient samples were handled, and the date and initials of the person recording the information.

- Supervisors may override rejection of partial or complete runs based on findings in the corrective-action investigation and based on established override criteria that have been approved by the Medical Director. The rationale for the override must be thoroughly documented.
- See the SOP "QC Responsibilities" for more details.

6.5 Documentation

Refer to local policies and procedures for QC documentation and to Quest Diagnostics records management program for record retention requirements.

6.6 Quality Assurance Program

Refer to National and local policies and procedures for other quality assurance activities applicable to this procedure

7. EQUIPMENT and SUPPLIES

7.1 Assay Platform

Manual stain or an optional automated slide stainer may be used: See section 16, Related Documents.

7.2 Equipment

- Heat block or Slide warmer 45-62°C or Bacti Cinerator
- Drain rack
- Microscope
- Centrifuge
- Biosafety cabinet
- Wescor or Previ Color Gram stainer

7.2.1 Optional materials, depending on specimen source

- Sterile scalpel, forceps
- Cytocentrifuge
- Sterile tubes, screw cap
- Tissue grinder
- Vortex mixer
- Sterile saline, sterile broth

7.3 Supplies

- Glass slides, frosted ends desirable

- Slide labels
- Pencil
- Sterile Applicator Sticks or swabs.
- Pasteur pipettes, or wood applicator sticks (sterile)
- Sterile Disposable Inoculating Loops
- Absorbent paper
- Immersion oil
- Lens paper
- Lens cleaner

8. PROCEDURE

NOTE: For all procedures involving specimens, buttoned lab coats, gloves, and face protection are required minimum personal protective equipment. Report all accidents to your supervisor.

8.1	Specimen Prep and Manual Staining Procedure
1.	A smear for Gram stain may be prepared from clinical specimens, broth cultures, or colonies growing on solid media. Appropriate Personal Protective Equipment (PPE) must be utilized to prepare smear. A biological safety cabinet must be utilized to prepare all smears. Label slide using pencil with the accession number, patient last name, source, and date. Refer to section 3.2 for additional PPE and BSC requirements.
2.	**For clinical specimens on swabs**, roll the swab gently across a slide (cleaned with 85% alcohol) or use pre-cleaned slides to avoid destruction of cellular elements and disruption of bacterial arrangements, covering an area no more than the size of a quarter. *Note*: If only one swab is received, and a culture ordered as well, inoculate the solid media first, and then prepare the smear prior to placing the swab tip into liquid media. Alternately, vortex the swab in 0.5-1 ml of sterile saline and use the resulting suspension to inoculate media and prepare smear. Fix the slide with 95% methanol as this prevents the lysis of RBCs, avoids damage to the host cells, as well as bacteria and results in a clear background. Alternatively, heat fix at 45-62°C until dry for a maximum of 10 minutes and allow the smear to cool before staining. **For tissue specimens**, transfer sample to a sterile Petri dish lid and mince tissue with sterile scalpel, selecting purulent, necrotic, or bloody portions. Grind portions of the minced tissue and small quantity of culture broth or sterile saline with a sterile tissue grinder. Prepare a thin smear the size of a quarter using the tissue grindings. a. Air dry the smear b. Fix with 95% methanol for one minute, drain off remaining methanol without rinsing, and allow to air dry before staining. Alternatively, heat fix at 45-62°C until dry for a maximum of 10 minutes and allow the smear to cool before staining.

8.1	Specimen Prep and Manual Staining Procedure
3.	For culture growth: Use young, actively growing organisms (< 24 hours old, ideally grown on an enriched medium yield the most accurate results). Note: Never mix vigorously. Avoid creation of aerosols. For growth from blood culture bottles: Spread drop to an even thin film. (Refer to local blood culture procedure). Fix with 95% methanol. Alternatively, heat fix at 45-62°C until dry for a maximum of 10 minutes and allow the smear to cool before staining. Prepare smear in a BSC. For growth in other broth media: Prepare smear using sterile pipette as for urine in a BSC.
4.	Apply crystal violet to prepared slide. Let sit for 30-60 seconds, and then rinse slide gently with running tap water. ***Caution***: Excessive rinsing in this step could cause crystal violet to be washed from gram-positive cells.
5.	Apply iodine solution for 30 -60 seconds, and then rinse slide gently with running tap water.
6.	Decolorize with alcohol-acetone for up to 10-15 seconds or until run-off is clear while the slide is held at and angle. (Thickness or type of smear may have an impact on the decolorizer timing.) Rinse slide gently with running tap water.
7.	Counterstain slide with Safranin for 30-60 seconds. Rinse slide with a gentle flow of tap water and air dry or blot on absorbent paper.

8.2	Repeat Staining
1.	If after preliminary review, the smear does not appear adequately stained, re-staining may be performed.
2.	Gently blot off any oil.
3.	Flood smear with decolorizer
4.	Rinse gently with tap water.
5.	Repeat staining procedure steps 8.1.4 to 8.1.7 above

8.3	Automated Gram Stain Test Procedure - Wescor/Previ Stainer
1.	Air-dry smear.
2.	Heat fix on heat block/slide warmer no longer than 10 minutes.
3.	Check reagent level in each reagent bottle on the stainer to verify sufficient quantity for staining. Reagents must be placed front to back in the following order: a) Decolorizer with Safranin counterstain b) Iodine c) Crystal violet d) Deionized water e) Methanol
4.	Run a CLEAN cycle once per shift. Refer to section 8.4
5.	Load slides into carousel slots (Addendum 2) with smears facing clockwise. If loading fewer than 12 slides, balance the rotor by placing slides in opposing positions. Use blank slides as necessary to balance.

8.3	Automated Gram Stain Test Procedure - Wescor/Previ Stainer
6.	Replace carousel lid and place the carousel on the instrument hub. Close the lid. Caution: Do not lock carousel lid in place while carousel is sitting on the drive hub.
7.	Select decolorize cycle by pressing the keypad that corresponds with the desired cycle. Use setting 2 or 3 depending on smear thickness.
8.	Set alcohol fixation as desired. Press **PROG** to activate or deactivate the alcohol pre-wash for removal of fixation residue. Select **0** = OFF, **9** = ON.
9.	**For Previ stainer only**: Full Carousel is 12 slides. If less than 12 slides, enter the number of slides.
10.	Press **RUN** to begin cycle.
11.	An audible signal sounds at the end of the staining cycle. Remove the rotor and unload slides for reading.
12.	Examine microscopically under oil immersion.

8.4	Clean Cycle
1.	Remove all specimen slides before using the CLEAN or reprime cycles.
2.	At the end of each shift, or whenever the stainer will be idle for more than three hours, use the CLEAN cycle to maintain nozzle performance. See Addendum 3
3.	Place carousel in the stainer and close the lid.
4.	Press CLEAN
5.	Leave the stainer with "PRESS CLEAN TO REPRIME" on the display while it is idle.
6.	When you are ready to stain slides, make sure there is a carousel in the stainer and press CLEAN to reprime the stainer.
7.	After running the CLEAN cycle, spray nozzle faces with methanol.
8.	Use a nozzle brush to clean the nozzle orifices when patterns are less than optimal.

8.5	Monthly Maintenance for Reagent "B" Line (Iodine)
1.	Remove nozzle B press the TESTS button. Select Option 3 for B-LINE FLUSH.
2.	Place carousel in bowl and close lid.
3.	Load 500 ml of DI water in line B position and press the RUN button.
4.	Remove DI water bottle and load 200 ml of nozzle cleaning solution (SS029C or 29588). Press the RUN button and allow solution to sit for 1 hour up to 12 hours.
5.	Remove nozzle cleaning solution bottle and load 500 ml of DI water and press the RUN button.
6.	Remove DI water bottle and load at least 200 ml of Iodine reagent (B). Press the RUN button.
7.	Reinstall nozzle B and press RUN.
8.	Press CLEAN to reprime the reagents. Perform a Pattern and Volume test before running a new cycle.

8.6	Previ Color Gram Monthly Nozzle Cleaning
1.	Perform a CLEAN alcohol purge and remove the nozzle.

8.6	Previ Color Gram Monthly Nozzle Cleaning
2.	Disassemble the nozzle and clean the O-rings or discard if damaged.
3.	Place nozzle parts with 25 ml of nozzle cleaning solution in nozzle tube. Record nozzle location on the cap invert gently a few times.
4.	Place tubes in the appropriate position in the tube holder. Allow them to soak for at least 10 minutes or up to 12 hours.
5.	Slide the edge of a piece of paper through the swirl cone grooves and run the nozzle cleaning wire through the nozzle orifice to remove any debris.
6.	Thoroughly rinse nozzle parts in DI water and then with ethanol.
7.	Replace O-rings and apply silicone lubricant to nozzle threads. Reassemble and replace each nozzle in their original position.
8.	Reprime each reagent line and perform a Pattern and Volume test before running a new stain cycle.
9.	Perform QC after monthly maintenance.

8.7	Cytocentrifuge Operation
1.	The Cytocentrifuge function is used to sediment sample cells onto microscope slides in preparation for staining.
2.	Select the Cytopro® Cytocentrifuge Rotor (AC-060) with accompanying supplies. Using pre-cleaned slides, label two slides with the patient's name and accession number. Place each labeled slide into a slide bracket with the labeled side facing the rotor. The release levers need not be depressed to load slides
3.	Select a Cytopad® according to the sample: (Cytopads come in two absorption rates.) a) **Low viscosity or low cellularity samples**, such as cerebrospinal fluid: Use the slow (tan) pad b) **Viscous samples**, such as synovial fluid: Use the fast (white) pad.
4.	Using the chamber with selected Cytopad,® depress the release lever and insert a chamber assembly. Release the lever while gently pressing down on the top of the chamber frame to ensure the chamber is squarely seated.
5.	Add 4-5 drops of CSF or other non-viscous fluid to the chamber. Add 1-2 drops of viscous fluids such as Synovial Fluid. The total volume in chamber may not exceed 0.5 mL. The optimum cell recovery is obtained with sample volumes of 0.2 to 0.3 mL.
6.	Load the appropriate amount of sample and prewetting fluid, if needed. Gently apply a chamber cap to minimize contamination and accidents.
7.	Place the lid on the rotor by lifting the locking pin as you place the center pin into the rotor lid receptacle. Press down on the locking pin until it locks.
8.	Carefully transfer the rotor to the stainer, taking care not to bump or tilt the rotor while transferring. Gently lower the rotor into place on the drive hub. If necessary, rotate the rotor until it fits over the drive hub. Be sure the rotor is firmly seated on the hub.
9.	Close the instrument lid.
10.	Press the **CYTO/CENT** button and the pre-programmed settings for the Cytocentrifuge should display. (1000 RPM and 5 MIN)

8.7	Cytocentrifuge Operation
11.	Press **RUN**.
12.	After the tone signaling the end of the cycle, remove the rotor from the stainer and put it on a flat surface.
13.	Remove the rotor lid by pressing with one hand on the center of the rotor lid while lifting the locking pin with the other hand. Check the chambers for complete absorption of suspension fluid. If the fluid is not completely absorbed, rerun the sample.
14.	Completely depress the release lever and remove the chambers. Discard chambers in a biohazard container.
15.	Remove the slide and air dry until completely dry prior to staining. Stain as described in section 8.3.
16.	Check for any other pending tests on the sample. If not needed by another department, store the remainder of the sample.

9. CALCULATIONS

Not applicable

10. REPORTING RESULTS AND REPEAT CRITERIA

10.1 Interpretation of Data

Result	Interpretation
Purple or deep blue staining	Gram positive
Pink or light red staining	Gram negative
Both purple and pink staining	Considered gram variable when seen in smears of culture isolates. This is often seen with *Bacillus*, *Clostridium,* anaerobic cocci, *Moraxella and Gardnerella vaginalis*. It is suggested that fresh subcultures be performed and the stain repeated. A lead tech or supervisor must review the Gram stain before reporting as gram variable.

10.2 Clinical Specimens

Check that white cells, epithelial cells and proteinaceous material allow the crystal violet stain to wash out with decolorization. Smears that have been correctly prepared and stained should appear completely gram negative (pink). Smears which appear purplish-pink have not been adequately decolorized and should be restained. Thick areas of the smear may be stained purple, perhaps with precipitated crystal violet present. Do not read in these areas. After determining that the staining is adequate, proceed to look for bacteria. Do not mistake thin crystal violet precipitation needles

for gram-positive bacillus-shaped bacteria. Consider the shape and arrangement of organisms in assessing proper staining characteristics.

Examine the slide only in the central area where the specimen has been applied. Edges of slides may be contaminated with bacteria.

10.3 Smears of Isolates

Evaluate only in areas where the organisms are well dispersed as gram negative organisms may not adequately decolorize in thick areas. Avoid evaluating morphology in areas where the inoculating loop has scraped the smear as this can cause distortion of organisms such as *Neisseria gonorrhoeae*, making them appear rod-shaped or bizarre.

10.4 Units of Measure

Determine the average number of cells and bacteria in 20 to 40 fields of the smear. Skip fields where there are no cells or bacteria, and do not average these fields in the count if there are fields where cells and/or bacteria are present.
If cells and organisms are observed, use the following guidelines for quantitation and message codes to report

Examine smears prepared from clinical specimens under low power for evidence of inflammation. Enumerate host cells in a Gram smear using low-power objective (10x objective with 10x eyepiece) and report relative number accordingly. Use the following guidelines for quantitation and message codes to report:

Enumeration of White Blood Cells and Epithelial Cells		
Average Quantity Observed 10X LPF	**Report As**	**Sunquest Mnemonic**
<1 per lpf	Rare	RARE
1—9 per lpf	Few	FEW
10—24 per lpf	Moderate	MOD
>25 per lpf	Many	MANY

Count bacteria, yeast and/or hyphal elements under oil immersion (100x objective with 10x eyepiece) and report relative numbers from areas with cells. Use the following guidelines for quantitation:

Enumeration of Bacteria and Yeast	
Average Quantity Observed (100X)	**Report As Above**
<1 per oil immersion field	Rare
1 to 9 per oil immersion field	Few
10 – 24 per oil immersion field	Moderate
> 25 per oil immersion field	Many

10.5 Clinically Reportable Range (CRR)

Semi-quantitative test, reported with host cells and organisms. See section 10.4 and 10.7.

10.6 Review Patient Data

Review patient results for unusual patterns, trends or distributions in patient results, such as an unusually high percentage of abnormal results.

10.7 Repeat Criteria and Resulting

If the Gram stain is the initial positive blood culture for a patient:	Initially 2 technologists must read the Gram stain.* If the interpretation is not definitive, a lead technologist, supervisor, or video microscopy remote technologist must be used to determine the correct interpretation.
If the Gram stain is CSF, sterile body fluid or tissue and organisms or WBCs are present:	Initially 2 technologists must read the Gram stain.* If the interpretation is not definitive, a lead technologist, supervisor, or video microscopy remote technologist must be used to determine the correct interpretation.
If the Gram stain is for other specimen types:	If the interpretation is not definitive, a second technologist must read the Gram stain.* If interpretation is not definitive, a lead technologist, supervisor, or video microscopy remote technologist must be used to determine the correct interpretation.
* Document by adding second tech code in LIS.	

IF the body site is …	**THEN…**
Sites with Normal Flora (except Stool)	Report the presence and relative amount of epithelial cells, WBC's and any organism(s) seen. Report the presence of "Clue cells" in vaginal Gram stains. (See definition under Limitations of Method)
Tissues and Unspun Fluids	Report all organisms seen. Report the presence and relative amount of WBC's and any organism(s) seen.
Normally Sterile Fluids, BAL, Bronchial Brushings	Report presence and relative amount of epithelial cells, WBC's and any organism(s) seen.
Wounds and Abscesses (Non-Oral Cavity)	Report the presence and relative amount of epithelial cells, WBC's and any organism(s) seen.

IF the body site is …	THEN…
Male urethral (GC) smears	Report the presence of WBC's. Report presence or absence of gram negative diplococci and whether they are intra- or extra-cellular. A report of intracellular gram negative diplococci on a male patient is considered a diagnosis for gonorrhea.
Oral Cavity	Report the presence of WBC's. Report the relative amount of gram positive and gram negative organisms, also noting the presence of gram negative spirochetes (curved spirals) bacteria.
Sputum	**Evaluation of sputum specimens:** **If specimen is an induced sputum, perform Gram stain and culture.** **If specimen is not an induced sputum, perform the specimen quality examination as described below.** 1. Specimens are examined for quality or suitability for a lower respiratory culture by scanning the Gram stained smear microscopically under low power (10x objective). 2. Screen the specimen for squamous epithelial cells, using several fields to provide an overall average for the slide. Columnar epithelial cells must not be used for this determination as they are normally found in the lower respiratory tract. 3. Use the following criteria to assess sputum specimen quality: • If there are < 25 squamous epithelial cells per low power field, the specimen is considered acceptable for culture. Perform a complete smear evaluation according to step 4. • If ≥25 squamous epithelial cells per low power field (regardless of WBC or bacteria present in the smear), the specimen is unacceptable for culture. o Report Gram Stain as "SPUU" and do not enumerate cells or organisms. o Call the nursing unit and request they order an induced sputum for Gram stain and sputum culture. o Cancel the sputum culture in LIS. Use cancellation code "UNAC" (Invalid specimen) and include name of the person notified as free text.

IF the body site is …	THEN…
	4. Using 100x (oil immersion) examine for bacteria, yeast and/or hyphal elements. Focus your attention on areas where the WBCs are concentrated. Enumerate the microorganisms seen on the smear and report according to Section 10.4

11. EXPECTED VALUES

11.1 Reference Ranges

- Gram-negative organisms stain pink
- Gram-positive organisms stain deep violet
- The expected distribution of bacteria and cells is based on the body site and type of infection.

11.2 Critical Values

- The extremely serious nature of central nervous system infections demand that CSF specimens be treated as an emergency specimen and should be worked on right away. A Gram stain should always be performed and the smear result reported to the clinician immediately.
- Refer to the current Critical Values Procedure.

11.3 Standard Required Messages

None established

12. CLINICAL SIGNIFICANCE

Clinical significance varies with source. The Gram stain is a critical test for the rapid, presumptive diagnosis of infectious agents and also serves to assess the quality of clinical specimens.

13. PROCEDURE NOTES

13.1 FDA Status: Manual Method: LDT without message

FDA Status: Wescor/Previ Automated Stainer: Approved/Cleared

Validated Test Modifications: None

13.2 Possible Sources of Error

- Some specimens may yield negative results unless they are concentrated by centrifugation (e.g., Cytospin). Specimens of body fluids especially CSF require centrifugation.
- Thick specimens or specimens with a high mucous content are difficult to decolorize and may appear falsely gram positive.
- Use of glass slides that have not been pre-cleaned or degreased (Storing slides in a jar with 95% ethanol will ensure clean slides, drain excess alcohol before use).
- Do not over heat fix slide. Overheating leads to over-decolorization and will cause all cells to appear Gram-negative.
- Over-decolorization: Often caused by using a strong decolorizer or by leaving the decolorizer on the slide for too long.
- Prolonged washing between any of the steps can cause over-decolorization

13.3 Special Precautions

- Requests for Gram staining of material processed in Mycobacteriology must be denied as the NaOH causes lysis of gram negative organisms

14. LIMITATIONS OF METHOD

- Some organisms may give a Gram stain reaction which is opposite from what may be expected. In particular, some *Bacillus* and *Clostridium* species stain gram negative as the cultures age. Other species in these groups are frankly gram negative.
- Some organisms do not stain well with the Gram stain and can be overlooked. This is particularly true of some anaerobes and small gram negative rods (*Brucella, Campylobacter spp, Fusobacterium spp, Bacteroides spp, Bordetella spp, Franciscella spp, Haemophilus spp)* in addition to *Legionella spp*. Carefully observe the smear (particularly blood smears) so as not to overlook these organisms. Longer exposure to the counter stain can enhance faintly staining *Campylobacter* and *Fusobacterium spp*
- Staphylococci, streptococci and pneumococci in clinical preparations may decolorize and appear gram negative. Always ask the question "Is this organism reasonable from this source and is this morphology correct?"
- Some streptococci (*S. mutans*) may look like rods when stained from solid media. Gram stains from liquid media should show the more typical coccus morphology.
- Nutritionally-deficient streptococci and organisms recovered from patients receiving antibiotics may be pleomorphic.
- Clue cells are epithelial cells completely covered by tiny, gram variable bacilli and coccobacilli, suggestive of bacterial vaginosis.
- 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.).
- False Gram stain results may be related to inadequately collected specimens.

14.1 Analytical Measurement Range (AMR)

Not applicable

14.2 Precision

Not applicable

14.3 Interfering Substances

- Be wary of artifacts in Gram stains, particularly from clinical material. Precipitated crystal violet may appear as gram positive cocci, but close examination will show that these "cocci" vary in size and appear in "fused" groups. Smears from cultures of *Neisseria* may have areas where there appear to be gram negative rods. These areas are usually next to a "scrape" in the smear and probably represent proteinaceous material. If gram negative rods are present, they should be distributed throughout the smear.
- It has been noted that smears from eyes used for corneal transplants and from eye aspirates contain material which resemble gram positive coccobacilli. This fact should be kept in mind when evaluating Gram stains from eye specimens.

14.4 Clinical Sensitivity/Specificity/Predictive Values

Not applicable

15. SAFETY

Refer to your local and corporate safety manuals and Safety Data Sheet (SDS) for detailed information on safety practices and procedures and a complete description of hazards.

16. RELATED DOCUMENTS

- Laboratory Quality Control Program
- Laboratory Safety Manual
- Safety Data Sheets (SDS)
- *Gram Stain* (QDHOS701)
- Current Package Inserts for gram stain reagents
- Gram Stain Quality Control Manual Method (AG.F24)
- Gram Stain Quality Control for Wescor / Previ Stainers (AG.F25)
- Microbiology Gram Stainer PM Chart (AG.F27)

17. REFERENCES

1. Betty A. Forbes, Daniel F. Sahm, Alice S. Weissfeld. 2007. Bailey and Scott's Diagnostics Microbiology, 12th edition. Mosby, St. Louis, Mo.

2. McClelland, R. 2001. Gram's Stain: the Key to Microbiology. Med Lab Observer. April pp. 20- 31. Web site: http://www.microbiologyforum.org/PMFNews/PMFNews.12.02.0602.pdf
3. MSDS forms from the Becton Dickinson Web site.
4. Package insert for Gram Stain Kits and Reagents, BD Becton, Dickinson and Company, part 8820191JAA, 2008/06.
5. Miller, J. Michael. 1999. Specimen Management in Clinical Microbiology, Second edition, ASM press , Washington, DC
6. Isenberg, H.D. ed. 1992. Clinical Micro Procedures Handbook. American Society for Microbiology, Washington, DC.

18. REVISION HISTORY

Version	Date	Section	Reason	Reviser	Approval
			Supersedes SGAH.QDHOS701v1.5		

19. ADDENDA

Addendum	Title
1	Sunquest Codes
2	Loading Wescor or Previ Stainer

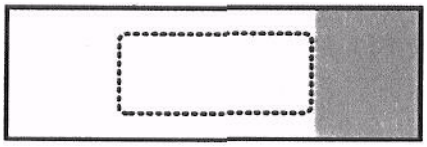
ADDENDUM 1

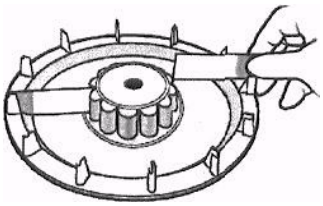
Message	Sunquest Mnemonic
* If no organisms are present, report "No organism seen"	NOS
WBC's	WBCS
Epithelial cells	EPIT
Gram negative bacilli	GNR
Gram negative coccobacilli	GNCB
Gram negative diplococci	GNDC
Gram positive cocci	GPC
Gram positive coccobacilli	GPCB
Gram positive cocci in chains	GPCC
Gram positive cocci in clusters	GPCL
Gram positive cocci in pairs	GPC PAR
Gram positive bacilli	GPR
Branching gram positive rods	BGPR
Gram variable rods	GVR
Yeast	YST

ADDENDUM 2

Loading the Wescor or Previ Stainer

LOAD CAROUSEL





Smears must face clockwise on **hub.**

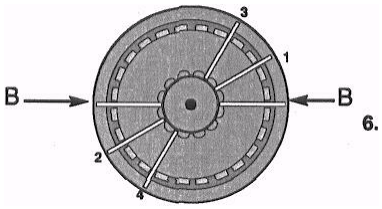
Press the center button to remove the carousel lid. Smears must face clockwise.
For best results, load slides with labels **away** from the **hub with specimen placement as shown.**

Full Carousel:	Place a slide in each slot.
7 to 12 Slides:	Load slides in balanced pairs (use a blank slide if necessary).
1 to 6 slides:	Load slides in sequence (in balanced pairs) into carousel slot 1 through 6.

Use blocking slides for uniform results: If there are empty slots in the carousel, place a blank slide in the first empty slot clockwise from a specimen slide, and another directly across the carousel. Leave blocking slides in the carousel unless running a full load.

SELECT NUMBER OF SLIDES

Enter the number of slides to be stained using the numbered keypads on the stainer's Iron! panel. Be sure slides are loaded properly. Stainer sprays 2, 4, 6, or 12 slides.



Blocking Slides (B)

Others shown are specimen slides.

LOAD INSTRUMENT
Replace and lock the carousel lid and place carousel onto drive hub. Close the lid.

BEGIN STAINING
Press RUN to start a staining cycle.