Sutter Health Sacramento Sierra Region
We Plus You

Origination:	*3/3/2014*
Effective:	*7/15/2019*
Final Approved:	*N/A*
Last Revised:	*6/10/2019*
Next Review:	*2 years after approval*
Owner:	*Lindsey Westerbeck: Dir, Lab*
Policy Area:	*Lab - Microbiology*
References:	
Applicability:	*Sac Sierra Region*

Performing a Gram Stain in the Hospital Laboratory, MI.ANA13.00-/-SS.xx

Purpose	This procedure describes how to stain and read a Gram stain smear.
Principle	The Gram stain is used to classify bacteria on the basis of their shapes, sizes, cellular morphologies and Gram reaction. It is an important test for the presumptive diagnosis of infectious agents and serves to assess the quality of clinical specimens. Bacteria will differentially retain crystal violet depending on their cell wall composition. Because of this property the Gram stain can be used to divide most bacterial species into two large groups: those that retain the basic dye, crystal violet (Gram positive) and those that allow the crystal violet dye to wash out easily with a decolorizer (Gram negative).
Policy	• STAT Gram stains are intended to provide a preliminary identification of organisms and evaluate specimen quality when appropriate. • STAT Gram stains will be performed on site and results reported within 1 hour of receipt. • STAT Gram stains on all sources must be ordered separately using a different accession number than the culture for the same specimen. • The accession number for the STAT Gram stain order only must be the one used for reporting. • Gram stain smears can be prepared by Lab Assistants, Senior Lab Assistants, Medical Laboratory Technicians (MLT) or Clinical Laboratory Scientists (CLS). • Interpretation of smears is done by CLS only. • STAT Gram stains that present as a critical result should be called to the physician or nurse in charge of the patient within 30 minutes of finalizing the report. • Following reporting of a STAT Gram stain, the smear and accompanying forms will be sent to Sutter Health Shared Lab (SHSL) Microbiology department. ◦ Gram stain results will be correlated with final culture results and will help guide work-up of cultures when appropriate by SHSL Microbiology.
Equipment	• Heat block (33-37°C or 55-60°C) or slide warmer (55-60°C) • Staining rack • Forceps • Drying rack • Bright field microscope with 10X and 100X Objectives

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<table>
<tr><td>Reagents</td><td>
<table>
<tr><th>Reagents</th><th>Storage & Stability</th></tr>
<tr><td>• Gram Crystal Violet Solution
• Gram Safranin Solution</td><td>• Store at Room Temperature (15-30˚C).
• Stable until manufacturer's expiration date.</td></tr>
<tr><td>• Grams Iodine (non-stabilized)</td><td>• Store at Room Temperature (15-30˚C).
• Protect from light, excessive heat, moisture, freezing, and exposure to air.
• Stable until manufacturer's expiration date.</td></tr>
<tr><td>• Acetone or Gram Decolorizer
• Methanol (optional fixative)</td><td>• Store at Room Temperature (15-30˚C).
• Excessive volumes of flammable fluids to be stored in flammable cabinet as per safety policy.</td></tr>
</table>
</td></tr>
<tr><td>Supplies</td><td>• Gram Stain QC slides (prepared manually or commercially)
• Paper towels
• Filter paper
• Immersion oil
• Slide holders</td></tr>
<tr><td>Specimen Requirements</td><td>• All clinical specimens are acceptable, except for stool, urine, throat, nasal and NP specimens.
• NOTE: Gram stains are of little value for direct smears of these specimen types. Requests will be honored, but it is recommended that the ordering provider be contacted about a more beneficial alternative test (i.e. culture).</td></tr>
<tr><td></td><td></td></tr>
<tr><td>Quality Control</td><td>• Gram stain QC will be performed with every patient slide, or with every batch of slides stained at one time.
• Gram stain QC will be performed on each new lot of stains prior to use.
• Evaluate the control slide for the presence of precipitate, contaminants in the stain (such as yeast or fungus), and for acceptable staining intensity.
• Acceptable QC Results: Gram positive cocci (purple) and gram negative rods (pink) should be observed.
<table>
<tr><th>If</th><th>Then</th></tr>
<tr><td>• Contaminants or excessive crystals/ precipitate present.
• Slide is grossly under-decolorized or over-</td><td>• Repeat procedure with new QC/patient slides or completely decolorizing original slides.</td></tr>
</table>
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<table>
<tr><td></td><td>
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<tr><td>decolorized.
• QC slide does not yield anticipated results (i.e. gram positive and/or gram negative organisms not seen).</td><td></td></tr>
<tr><td>Contaminants or crystals/ precipitate persist after initial repeat.</td><td>• Filter the crystal violet stain into a sterile container, using filter paper (grade 1, 12.5 cm diameter) and repeat procedure with new QC/ patient slides or decolorizing original slides.
◦ DO NOT centrifuge crystal violet.
<table>
<tr><td>If not resolved with</td><td>Then
(repeat using new QC/ patient slides or decolorizing original slides)</td></tr>
<tr><td>Filtering</td><td>Open new bottle/same lot of the crystal violet stain</td></tr>
<tr><td>New bottle/ same lot</td><td>Open new bottle/new lot of the crystal violet stain</td></tr>
</table>
• Document all corrective action taken on site-specific Out of Control report or Gram Stain log.</td></tr>
</table>
</td></tr>
<tr><td>Quality Control</td><td>
<table>
<tr><td>If</td><td>Then</td></tr>
<tr><td>Under-decolorized or over-decolorized slide or unacceptable QC results persist after initial repeat.</td><td>• Replace the entire set of reagent bottles and/or control slides.
• Repeat procedure with new QC/patient slides or completely decolorizing original slides.
• Document all corrective action taken on site-specific Out of Control report or Gram Stain log.</td></tr>
<tr><td>Unable to resolve poor smear quality and/or unacceptable QC.</td><td>• Do NOT report patient results.
• Notify the nursing unit/clinic of delay in testing.
• Send slide with accompanying Form A to SHSL Microbiology Lab on next courier run.
• Consult with vendor for troubleshooting and/or replacement reagents.
• Document all corrective action taken on site-specific Out of Control report or Gram Stain log.</td></tr>
<tr><td></td><td></td></tr>
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</td></tr>
<tr><td>Procedure: Slide</td><td>Follow the steps below to Gram stain a specimen smear.</td></tr>
</table>

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Staining

Step	Action
1	Prepare smear for staining. Refer to *Preparing Smears for Gram Stain in the Hospital Laboratory* procedure.
2	Fix the smear by one of the following methods:

Heat Fixation	Alcohol Fixation
• Place slide on 55-60°C heat block or slide warmer for 60 seconds ***or*** • Place slide on 33-37°C heat block for 2 minutes.	• Place a few drops of methanol on air-dried slide for 60 seconds. • Drain off remaining methanol without rinsing and allow to air dry. • **If alcohol fixation is used, do NOT also heat fix before staining**
Do NOT overheat, as it may cause gram positive bacteria to stain gram negative and distortion of cellular material.	Alcohol fixation is preferred, as it prevents lysis of RBCs, produces a cleaner background, and prevents washing off of liquid specimens.

3	On rack over sink, flood QC and patient slides with crystal violet and allow it to remain on the surface without drying for approximately 30 - 60 seconds.
4	Rinse slides gently with tap or DI water.
5	Flood the slides with Grams iodine and allow it to remain on the surface without drying for approximately 30 - 60 seconds.
6	Rinse slides gently with tap or DI water, shaking off all excess.
7	Decolorize **one slide at a time** by letting acetone or gram decolorizer flow over the smear while the slide is held at an angle for 1-5 seconds until the runoff is clear.
8	Rinse immediately with a gentle flow of tap or DI water. Repeat step 7 for each slide.
9	Flood slides with safranin and allow it to remain on the surface without drying for 30 - 60 seconds.
10	Rinse slides gently with tap or DI water.
11	Drain slides and place in an upright position on a paper towel to air dry.

Procedure: Reading a Gram Stain Smear

Follow the steps below to read a Gram stain smear.

Step	Action
1	Evaluate the general nature or quality of the smear under low power (10X). **Look for:** • Presence of stain crystals/precipitate • Proper decolorization

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<tr><td></td><td>

◦ The background should generally be clear or gram negative (pink).
◦ If WBCs are present, they should be completely gram negative.

• Appropriate thickness of smear

◦ For proper interpretation, there must be areas where cells are no more than one cell layer thick (no overlapping cells).

<table>
<tr><th>If smear</th><th>Then</th></tr>
<tr><td>Quality is acceptable.</td><td>• Circle "Y" at the "Smear/Stain Quality Acceptable:" prompt on Form A: Evaluating Stat Gram Stains for Consistency of Morphologic Observation.
• Proceed to Step 2.</td></tr>
<tr><td>Has stain crystals/ precipitate.</td><td>• Compare to QC slide to determine if debris is from specimen or stain.
• If on both QC and patient slides, follow troubleshooting steps in the Quality Control section.</td></tr>
<tr><td>Is under- or over-decolorized.</td><td>• Follow troubleshooting steps in the Quality Control section.</td></tr>
<tr><td>Is too thick.</td><td>• Prepare new smear and repeat staining.</td></tr>
</table>
</td></tr>
<tr><td>2</td><td>Under low power (10x), select areas containing inflammatory cells or purulence for examination. If no purulence is seen, then choose areas of apparent necrosis, inflammatory cell debris and mucus.
• If cells are present, quantify each type seen: WBCs, epithelial cells, RBCs etc.</td></tr>
</table>

Procedure: Reading a Gram Stain Smear

Step	Action
3	Apply drop of immersion oil to slide.
4	Examine 20 to 40 fields in areas selected in Step 2 using the 100X oil immersion objective. **Look for the presence of:** • Bacteria – if present, note: • Gram reactions • Morphologies (e.g. cocci, bacilli, etc.) • Yeast or fungal elements • Inflammatory cells (WBCs) • Other cells such as epithelial cells and RBCs. **If organisms or cells are present, quantify each type seen.**

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		NOTE: **DO NOT** attempt to identify or report a genus or species from a Gram stained smear.
	5	Record results on Form A: *Evaluating Stat Gram Stains for Consistency of Morphologic Observation*. • **The presence or absence of WBCs and organisms must always be recorded**. • Other cell types are recorded only if present. **Also include the following information on the form:** • Patient Name/MRN/Accession number • Source • HID • Tech Code/Date • Received Date/Time • Reported Date/Time • Called Date/Time (*if applicable*)
	6	Place the slide face down on paper towel to absorb excess oil.

Technical Notes

If	Then
Rare single gram positive cocci seen.	• Care must be taken not to confuse stain debris as gram positive cocci. • Scan an additional 20-40 fields looking for typical gram positive cocci clusters/chains **or** prepare a second slide to confirm.
Rare amount of organism is seen in critical specimen (*i.e. CSF, joint fluid).*	• Prepare a second slide to confirm. • It may be necessary to have a secondary review performed by another CLS to verify findings on critical specimens. • If QNS for second slide and: ◦ High confidence, report finding. ◦ Low confidence or uncertainty, consider reporting NOS, leave note on Form A for SHSL Microbiology for review.
Smear has very few organisms, or the organisms are unevenly distributed, especially in the absence of inflammation.	• Consider possible contaminants. (*collection tubes, slides, etc. may harbor nonviable bacteria*) • Prepare a second slide using other supplies if possible.
The patient is on antimicrobial therapy.	• Gram positive organisms may be

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	more susceptible to decolorization.

Interpreting Results

- Gram positive bacteria and yeast will stain purple.
- Gram negative bacteria will stain pink or red.
- WBCs will stain pink or red.
- Gram variable bacteria will show both gram positive & gram negative cells with the same morphology.
- Fungal elements may stain gram variable

If	Then
No WBCs are seen.	Record "No WBCs seen".
No organisms are seen.	Record "No organisms seen".

Interpreting Results

If	Then
Cells are seen • WBCs • Epithelial cells • RBCs • Other	Quantify and record by cell type as follows: Rare: <1 cells per LPF (10X) Few: 1-9 cells per LPF (10X) Moderate: 10-25 cells per LPF (10X) Many: >25 cells per LPF (10X) *Example:* • *Many WBCs* • *Rare Epithelial cells*
Organisms are seen	Quantify and record each organism type seen (i.e. gram negative rods, gram positive cocci, yeast, etc.) as follows: Rare: <1 organism per OIF Few: 1-5 organism(s) per OIF Moderate: 6-30 organisms per OIF Many: >30 organisms per OIF *Example:* • *Few gram positive cocci* • *Moderate gram negative rods*

Critical Limits

- Gram stains positive for bacteria or other organisms from normally sterile sources are considered critical results.
- See *Appendix A: Common Sterile and Non-Sterile Sources* for listing of sterile and non sterile sites.

Reporting Results

Follow the steps below to enter results Sunquest.

Step	Action

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	1	In Sunquest Gateway Microbiology Result Entry function, enter the patient accession number to be resulted.
	2	Using designated result keys < >, enter Gram stain results under the Direct Exam tab. Press <F8> to display the keyboard on screen. See *Appendix B: Microbiology Direct Exam Result Keyboards and Codes* for key listing. • **The presence or absence of WBCs and organisms must always be reported**. • *Other cell types are reported only if present*.

If	Then enter
No WBCs are seen	No WBCs seen <S>
No organisms are seen	No organisms seen <A>
Cells are seen • WBCs • Epithelial cells • RBCs • Other	• Quantity followed by cell description. • Repeat for each cell type seen. *Example:* • *Many WBCs* • *Few Epithelial cells*
Organisms are seen **and source is a sterile site**: • Blood • Body fluid (except urine) • Bones • CSF • Synovial fluid • Tissue/Valves • Surgical specimen • Vitreous Fluids See ***Appendix A: Common Sterile and Non-Sterile Sources*** for complete list.	• Quantity followed by **"P" code** organism description (flags as abnormal result). • Repeat for each type of organism seen. *Example: Few gram positive cocci seen in CSF, enter <6> <u>* • <u> (PGPC) Gram positive cocci (result flags as abnormal)

Reporting Results

Step	Action
2.	(*continued*)

If	Then
Organisms are seen **and source is a non-sterile site:** • Wound • Sputum • Eye • Ear • Urine See ***Appendix A: Common Sterile***	• Quantity followed by **"N" code** organism description (no result flag). • Repeat for each type of organism seen. *Example: Many gram positive cocci seen in sputum, enter <8> <U>*

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	and Non-Sterile Sources for complete list.	• <U> (NGPC) Gram positive cocci (no result flag)
3	Enter each observation on a separate line.	
4	Final the report by clicking the period key <.>.	
5	Enter critical call notification (*if applicable*) in Composed Text field prior to closing out of accession number and press Save.	
6	Verify the accuracy of result entry by using an inquiry function (IQ, GUI Laboratory Inquiry, or GUI Microbiology Inquiry) to review results. **Confirm by writing RVS and your initials on the result form.**	
7	Make a copy of the completed result form and retain copy in the designated binder.	
8	Send STAT Gram stain slide (in slide holder) and completed Form A to SHSL Microbiology on next routine courier run (also send specimen if available).	

Method Limitations	• False positive - Gram stain positive, culture negative specimens may be the result of: contaminated reagents and/or supplies, presence of antimicrobial agents or failure of organisms to grow under usual culture conditions (i.e. medium, atmosphere, etc.) • False negative - Gram stain negative, culture positive results may be the result of: improperly prepared slide(s) or small bacterial load in sample submitted.
Supporting Documents	• Form A: *Evaluating Stat Gram Stains for Consistency of Morphologic Observation* • Appendix A: *Common Sterile and Non-Sterile Sources* • Appendix B: *Microbiology Direct Exam Result Keyboards and Codes*
References	• Gram Stain Reagents Manufacturer's Insert, Becton, Dickinson and Co., 6/2008. • Gram Stain Kit/Reagents Manufacturer's Insert, Hardy Diagnostics, 1996-2013 At SMF Microbiology Laboratory: • *Bailey and Scott's Diagnostic Microbiology*, 11th ed, Barron, et al, 2002. • *Clinical Microbiology Procedures Handbook*, 3rd ed, Garcia, et al, 2010.

All revision dates: 6/10/2019, 3/12/2018, 3/3/2014

Attachments:

Appendix A: Common Sterile and Non-Sterile Sources

Appendix B: Microbiology Direct Exam Result Keyboards & Codes

Form A: Evaluating Stat Gram Stains for Consistency of Morphologic Observation

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Approval Signatures

Step Description	Approver	Date
Lab Medical Directors	Rowberry Ron: MD	pending
Lab Medical Directors	Marian Butcher: MD	6/19/2019
Lab Medical Directors	Jamie Cassity: MD	6/13/2019
Lab Medical Directors	Andrea Ong: MD	6/13/2019
Lab Medical Directors	Hannah Wong: MD	6/13/2019
Lab Medical Directors	Kristen Vandewalker: MD	6/12/2019
Lab Medical Directors	Mary Keohane: MD	6/12/2019
	Lindsey Westerbeck: Dir, Lab	6/12/2019

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