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Document Title: III. GRAM STAINED SMEARS

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CHANGE CONTROL FORM

Document Title: III. GRAM STAINED SMEARS

Document Number: SH.CP.MC.BAC.0003.000 Version: from 5 To 6

Submitted By: Debra Jesien Date: 10/24/2012

1. Check one:

New procedure ☐	New process ☐	New form ☐	New flow chart ☐
Revised procedure X	Revised process ☐	Revised form ☐	Revised flow chart ☐
New job aid ☐	New labels ☐		
Revised job aid☐	Revised label ☐		

Brief description of change:

a) Gram stains are routinely performed on Sinus specimens. (see I.A for complete list)
b) Gram stains are performed by request only on: Bartholin cyst and Semen. (see I.B. for complete list)
c) Gram stains are not performed on Shunts or Implements such as screws, electrodes, mesh, metal plates, etc.... TNP as "inappropriate specimen for test requested". (see I. C. for complete list)
d) For Sterile fluids submitted in BactAlert bottles, when additional sample has been submitted in a separate container a GS is ordered in the LIS along with the FBTL. The slide will be prepared, read and reported per Sterile fluid protocol.
e) A protocol for performing gram stain on Culture positive platelets has been included.
f) Slides for gram stain of Joint fluid, Pleural fluid and viscous or bloody fluids will be prepared as follows:
   - Place one drop of the specimen in the center of a clean glass microscope slide.
   - Gently spread the specimen on the slide, distributing it evenly.
   - For extremely thick specimens it may be necessary to make a second slide diluted with 1 drop of sterile saline.
   - Air dry.
f) Gram stain procedure was updated to include Gram's decolorizer.

1. S. O. P. validation needed? (Circle one) NO YES If yes, attach validation sheet

2. Process validation needed? (Circle one) NO YES If yes, attach process validation documentation

3. Associated procedure and other documents (list those that need to be written or revised): _____

4. Table of Content (TOC) update needed? (Circle one) NO YES If yes, attach Updated TOC

Author: Paula Migneault Date: _____
Revised by: Debra Jolen Date: 10-24-12
Approved by: Dwight J. Hardy, PhD Date: 10/30/2012

GRAM STAINED SMEARS

Principle:
The gram stain is used to classify bacteria based on color, size, form and cellular morphology. Bacteria stain either gram positive or gram negative based on differences in cell wall composition. Gram positive bacteria retain crystal violet and appear purple due to a thick peptidoglycan layer that resists decolorization by alcohol. Gram negative bacteria are damaged by the alcohol decolorizer so that crystal violet-iodine complexes leak out and are replaced by safranin counterstain, giving them a red appearance.

I. Occasion for gram-stained smears

A. Gram stains are routinely performed on the following specimen types:

1) Sterile body fluids
2) Tissues and surgical specimens
3) Sputum, BAL, Bronchial washes, Bronchial brushes, Tracheal aspirates, Transtracheal Aspirates etc...
4) **Sinus**
5) Abscesses from any internal organ (brain, liver, lung, etc...)
6) Breast abscesses
7) Any quantity of pus
8) Superficial wounds and drainages
9) Cul-de-sac if fluid is received

B. Gram stains are performed by request on the following specimens types:

1) Urine
2) Urethra
3) **Bartholin cyst**
4) **Semen**

C. Gram stains are not performed on the following specimen types: (TNP as "inappropriate specimen for test requested".)

1) Stool, rectal abscesses, rectal or anal swabs
2) Oral, mouth or throat specimens
3) Nasal and nasopharyngeal swabs
4) Skin and scalp
5) Sterility cultures such as: Donor cornea, bone bank, water, media, etc...
6) Catheter **or Shunt** tips
7) **Implements such as screws, electrodes, mesh, metal plates, etc....**

D. Routine gram stains are **not** performed on Vaginal or cervical swabs (unless requesting Nugent score). TNP using the following cancellation comment:

"Gram stain not performed due to poor correlation with culture."
(choice |XBCX on cancellation keypad)

E. Sterile fluids submitted in BactAlert bottles

1) **When additional sample has been submitted in a separate container a GS is ordered in the LIS along with the FBTL**

F. Culture positive platelets

1) **GS is ordered in LIS along with FBTL**
2) **From GS key pad - choose "?fluid in bottles |>SORG "**

GRAM STAINED SMEARS

3) **Report descriptively**
(Example: gram positive cocci in clusters with quantity. Do not add resembles Staphylococci
4) **From FBTL keypad - select appropriate plate media**

II. Slide preparation

Note: Slides prepared from specimens with AFB precaution must be air dried in the Biosafety cabinet and exposed to UV light for a full 10 minutes before being removed from the cabinet. Slides are then heat fixed at 85°C for 15 minutes prior to staining.

A. CSF, Urine and non-viscous fluids:
1) Place one drop of the fluid in the center of a clean glass microscope slide.
2) Air dry.

B. **Abscesses, Pus, Joint fluid, Pleural fluid and viscous or bloody fluids:**
1) Place one drop of the specimen in the center of a clean glass microscope slide.
2) Gently spread the specimen on the slide, distributing it evenly.
3) For extremely thick specimens it may be necessary to make a second slide diluted with 1 drop of sterile saline.
4) Air dry.

C. Respiratory specimens
1) Using a sterile swab select most purulent portion of material.
2) Gently roll swab across a clean glass microscope slide, distributing it evenly.
3) Air dry. Heat fix slide at 85°C before staining.

D. Specimens received on swabs
1) Gently roll swab across a clean glass microscope slide.
2) Air dry.

E. Tissue samples
1) Grind specimen in sterile saline according to specimen processing guidelines (refer to Set-up Procedure Manual)
2) Place one drop of the ground specimen in the center of a clean glass microscope slide.
3) Gently spread the specimen on the slide, distributing it evenly.
4) For extremely thick specimens it may be necessary to make a second slide diluted with 1 drop of sterile saline.
5) Air dry.

III. Procedure for performing a Gram stain[1]

A. Heat-fix the inoculated slide.
B. Flood slide with Crystal Violet (10 - 30 seconds).
C. Rinse with tap water, shaking off excess water.
D. Flood slide with Gram's Iodine (20 - 60 seconds).
E. Rinse with tap water, shaking off excess water.
F. Holding the slide at an angle, carefully flood with **Gram's decolorizer, and rinse immediately with tap water**. Thick slides may require prolonged decolorization.
G. Shake off excess water.
H. Flood slide with Safranin (30 seconds).
I. Rinse with tap water, shaking off excess water.
J. Air dry or gently blot dry the slide before examining microscopically.

GRAM STAINED SMEARS

IV. Distribution

A. Day shift

The technologist at each station is ultimately responsible for reading smears that belong to his/her station. However, Monday – Friday is usually a person assigned to read all Gram-stained smears. **When there is no assigned reader or the workload is heavy, scheduled technologists from all areas should assist with the reading of smears.**

B. Other shifts

When personnel qualified to read Gram stains are present, requested, "sterile" specimen smears, and sputum and/or tracheal aspirate smears must be read as soon as possible; other slides as time permits.

V. Review of slides

A. Smears from the following specimens need not be routinely reviewed by a second reader:

- Sputum, **Tracheal aspirates, Bronchial Washings, ETT and Deep Tracheal Lavage**
- Urine
- Fluids/tissues/pus/exudates with ≤10 PMNs/LPF and no organisms seen (NOS)
- Exudates other than syringes or volumes of pus and abscesses from internal organs

B. Slides from the following specimen types should always be independently (unbiased) reviewed by a second person, if possible, before being reported. This person may be a senior technologist, a supervisor, or another qualified staff member.

- Smears from fluids/tissues with >10 PMNs/LPF
- Cytospin preparations
- Brain abscesses, abscesses from internal organs, syringes or volumes of pus
- Urogential smears
- "positive" gram stains which require a call to a healthcare provider

C. Any gram stained smear with a questionable interpretation or an unusual organism must be reviewed. Record coordinates on those smears with very few organisms present. These findings should be reviewed by a Tech V, supervisor, or director.

D. The reader and the reviewer should initial the entry on the worksheet.

E. DO NOT CHANGE ANY RESULTS that have been reported unless you have discussed the matter with a supervisor.

Note: These guidelines apply to all shifts. If there is no other member on your shift who can act as a checker (e.g. midnight to 8 am shift), report and leave slides and corresponding worksheet entries of specimens included in the above categories out for review by the dayshift ASAP the following morning. For Gram stains requiring review the next morning:

- Enter results
- Skip a line
- Enter "Results to be considered preliminary until review in A.M."
- Give "Preliminary" status.
- Save.
- View your report for accuracy.

VI. Examination of Gram-stained smear

A. Gross examination

Note where the stained material is on the slide and whether or not the smear is too thick or improperly decolorized.

B. Scan using 10x objective (low power).

1. Gain general impression of smear and look for best areas (where inoculum is adequate).

2. Look for cells using low power (10x objective)

a. **Epithelial Cells:**
Report the presence (quantitate) or absence of squamous epithelial cells only on Respiratory specimens. (See specific source-driven guidelines IV.) Do not mention epithelial cells on smears other than respiratory specimens.

Quantitate (#) squamous epithelial cells as follows:

≤10/low power field
>10/low power field

b. **PMN's:**
Report presence (quantity) or absence of PMN's from all specimen types, including bone, bone marrow, and lymph node specimens.

Estimate PMNs/low power field (lpf).
If only PMN cell fragments are seen (PMNs lacking a cell membrane) on smears, report:

"(#) cell fragments"

c. **Nucleated white cells other than PMNs (NPMNs)**:
Reported as "nucleated cells, not PMNs". These are reported on Sterile Fluid specimens only.

C. **Quantitation (#) of PMNs, and other cells:**
use low power (10x objective) to quantitate, but confirm cell morphology using 40x or 100x

1. Expectorated sputum and tracheal aspirates with >10 epithelial cells/low power field as follows:

<25/low power field.....(**not** read for bacteria)
>25/low power field.....(read for bacteria)
too numerous to count (TNTC)/low power field.....(read for bacteria)

2. For all other sources:

0/low power field
<1/low power field
1-10/low power field
10-25/low power field
>25/low power field

D. Look for presence of hyphae

Because of their large size, uneven distribution, relatively small numbers present, **and characteristic uneven or negative staining**, hyphae can easily be overlooked or missed on high power. However, low power scanning can sometimes spot these elements. This is especially true of respiratory specimens, including lung tissue and sinuses.

E. Be alert to problems with smear/stain preparation

a. Underdecolorization – cells (esp. PMNs) with gram-positive nuclei is a clue

b. Overdecolorization – suspect especially when entire smear is gram-negative

c. Abnormal color of stain or gram-variable organisms of same morphology

d. Contamination – a possibility, especially if grossly uneven distribution of organisms

F. Quantitation of bacteria (using 100x oil objective):

1. Under oil immersion, examine carefully for various morphologies and types of bacteria and yeast. Report relative numbers of gram-negative bacilli, gram-negative diplococci, gram-positive cocci, gram-positive bacilli, and yeast.

many = several per oil immersion field
moderate = one per field
few = one per several/many fields
very few = one or two/slide

Note: For sterile specimen types when only a few organisms are seen on the entire slide, document organism coordinates in LIS as a hidden comment.

2. **For all specimen types (including respiratory and non-sterile exudates) examine for, and report, all morphotypes seen.**

Example:

(#) PMNs
(amount) gram-negative bacilli
(amount) gram-positive cocci in pairs and chains
(amount) gram-positive bacilli
(amount) yeast

G. Cytospin preparation

1. Prepare on "**sterile**" fluid or "**sterile"** abscess only if:

   - ≥10 PMNs/low power field, but no organisms seen on direct smear.....**(add cytospin - test code: GSCY)**
   - ?whether or not organisms are seen on direct smear .....**(add cytospin – test code: GSCY)**
   - when requested on a sterile fluid by physician.....**(add cytospin – test code: GSCY)**

2. Prepare routinely on all:

   - CAPD fluid, Peritoneal Dialysate fluid (PD), Ascites fluid
   - CSF obtained from shunt
   - BALs and Protected Bronchial Brush specimens

   Note: For grossly bloody specimens, dilute by placing 1 drop of specimen into a sterile tube containing upto 1 ml of sterile saline. Use the diluted suspension to perform the cytospin.

3. Cytospin Procedure Report

   a. If no organisms are seen on routine Gram stain, but organisms are seen on cytospin, report: **(No need to report cell picture)**

      (few, moderate or many) (organism description)

   b. If no organisms are seen on routine Gram stain and cytospin preparation, report: **(No need to report cell picture)**

      No organisms seen

   c. If cytospin prep is done to confirm positive findings of routine Gram stain, report the results for both smears.

   d. Report results of a cytospin performed on a CAPD fluid, Peritoneal Dialysate fluid (PD), Ascites fluid or CSF shunt as follows:

      (few, moderate or many) PMNs
      (report quantity of NPMNs if present. Do not report absence of NPMNs)
      (few, moderate or many) (organism description)

VII. Smears of respiratory specimens

A. Sputa and tracheal aspirates

1. If ≤10 epithelial cells/lpf **the specimen is acceptable for culture**. Record and report:

   c. ≤10 epithelial cells/low power field

   d. # PMNs/low power field

   e. Amount and description of each type of organism.

   Examples:

   ≤10 epithelial cells/low power field
   >25 PMNs/low power field
   gram-negative bacilli
   many
   gram-positive cocci in pairs
   few

   ≤10 epithelial cells/low power field
   1-10 PMN's/low power field
   gram-positive cocci in chains
   few

   Retrieve sputum and from Biosafety cabinet. Label BAP/CHOC/MAC with patient labels and place at Set-up bench to be plated.

2. If >10 epithelial cells/low power field **the specimen in unacceptable for culture.** Read for PMN's and select appropriate choice:

   ≤25 PMNs/low power field ...not read for bacteria*

   >25 PMNs/low power field (but not TNTC) ...read for and report bacteria*

   TNTC PMNs/low power field ...read for and report bacteria*

   *The following comment will be automatically added to the report when the appropriate choice is selected from the keypad:

   " The presence of large numbers of squamous epithelial cells indicates that the specimen is contaminated with oropharyngeal microorganisms. Specimen not cultured for organisms colonizing the upper respiratory tract airways or mouth."

   The specimen will be rejected for routine bacterial culture.
   Cancel the AER test in the LIS. BCYE will still be set up to culture for *Legionella*.

Note: The patient location must be notified that the specimen is not being cultured. Inpatient reports must be printed to the floor. Outpatient reports may be faxed to the location. If a fax number is unavailable, the report must be phoned.

B. Exceptions to IV.A.2 include the following:

1. **Cystic Fibrosis (CF) sputum or deep throat specimens**:
   - Quantitate epithelial cells as in III.B.b and PMNs as in III.B.e.
   - Specimens from CF patients with <10 EPIs are always read for bacteria.
   - Specimens from CF patients with >10 EPIs and/or <10 PMNs ... report cell picture with "Not examined for bacteria".
   - **These are never rejected for culture.**

2. If **Nocardia** or aerobic **Actinomycetes** is requested prepare two smears (1 Gram stain, 1 modified Kinyoun stain). Perform Gram stain, read cell picture and enumerate all bacteria. Examine the entire smear on high power. If characteristic organisms are observed (filamentous, branching, beaded staining gram-positive bacilli), report:

   (#) cell picture
   branching gram-positive bacilli
   (quantity)

   A "MODK" test will reflex when the branching gram-positive bacilli is reported in the LIS.

   Give slide for modified Kinyoun stain to Mycology/Mycobacteriology personnel ASAP. If appropriate staff is not available, leave slide clearly marked for modified Kinyoun in the fungal stain box. Leave a note for the Mycology technologist.

3. Smear preparations that are too thick to be read ... repeat smear.

C. **Bronchial washes, Unprotected Bronchial Brushes, Deep Tracheal Lavages and Transtracheal aspirates, Endotracheal aspirates:**

1. Always read for cells and bacteria, report cells and bacteria regardless of epithelial count.
2. Quantitate epithelial cells as in III.B.b and PMNs as in III.B.e.
3. **These are never rejected for culture.**

D. **BALs and Protected Bronchial Brush specimens:**

1. Prepare cytospin smear.
2. Quantitate epithelial cells as in III.B.b.
3. Report relative amounts (few, moderate, many) of PMNs and relative amounts and types of bacteria seen.
4. Report the percent of PMNs observed to contain intracellular organisms.
5. **These are never rejected for culture.**

VIII. **GC smears (GC specified)**

A. Report the cell picture. (PMNs, cell fragments)

B. Examine for and report presence (few, moderate or many) or absence of gram-negative diplococci, intracellular or extracellular. The presence of a cell membrane is necessary for the use of the word "intracellular". If PMN cell fragments are seen, but no intact PMNs, report: "(#) cell fragments.

C. Note: Gram stains of cervical/vaginal swabs are not routinely read for gram-negative diplococci in our lab. If a request is made, prepare a smear but do not read. Cancel as: "Gram stain not performed due to poor correlation with culture" (XBCX). The smear may be examined for the presence of intracellular gram-negative diplococci if further discussion with the physician indicates it is appropriate to do so. **For requests made to gram stain vaginal swabs for the diagnosis of bacterial vaginitis, refer to VII.**

D. Examples of reports:

>25 PMNs/low power field
intracellular gram-negative diplococci
few

many cell fragments
extracellular gram-negative diplococci
many

0 PMNs/low power field
no intracellular or extracellular gram-negative diplococci seen

O PMNs/low power field
Scant inoculum on slide. Specimen is not adequate to determine presence of
Intracellular gram negative diplococci.

IX. Urine smears[2]

A. Report cell picture, i.e., PMNs/low power field

B. Report organisms

1. ">100,000 (organisms)/ml" if ≥1 per oil immersion field."
2. "<100,000 (organisms)/ml" if <1 per oil immersion field."
3. "No organisms seen"

X. Interpretation of Vaginal Smears Using Nugent Criteria[3]

A.. Slides should be delivered to the Mycology Laboratory (or placed in the fungal smear box).

B. Heat fix and stain as usual.

C. Examine for presence (quant.) or absence of:

PMNs/low power field
gram-positive bacilli consistent with lactobacilli/oil immersion field
gram-negative to gram-variable bacilli consistent with *Gardnerella*/oil immersion field
gram-variable curved bacilli consistent with *Mobiluncus*/oil immersion field
Clue cells/high dry field
Yeast/oil immersion field

D. Interpretation of the Gram stain

Large gram-positive bacilli (large with flat ends) are the lactobacilli morphotype. The *Lactobacillus* and *Gardnerella* morphotypes often decolorize and appear gram-negative. This should not be confused with predominantly gram-negative flora. Enteric gram-negative bacilli rarely predominate in the vagina. Clue cells are squamous epithelial cells that have a dense coating of adherent bacteria. The epithelial cells are irregular, almost polygonal, in shape and are about 100 x 50 µM in size. The dense adherent bacteria almost entirely obscure any cellular detail. In normal epithelial cells, the ellipsoidal cell nucleus (10 x 5 µM) is commonly visible.

E. Quantitation

Each morphotype and clue cell is quantitated from 0 to 4+ with regard to the number of morphotypes per oil immersion field, except clue cells which should be counted on high dry and PMNs per low power field:

0 = none seen
1+ = less than one per field
2+ = 1 – 4 per field
3+ = 5 – 30 per field
4+ = ≥30 per field

F. Calculating the Nugent score

Score	*Lactobacillus*	*Gardnerella/Bacteroides*	*Mobiluncus*
0	4+	0	0
1	3+	1+	1+ or 2+
2	2+	2+	3 or 4+
3	1+	3+	
4	0	4+	

Example:

	Score
<1 PMN/low power field	
1+ gram-positive bacilli consistent with Lactobacilli	3
4+ gram-negative bacilli consistent with *Gardnerella*	4
2+ gram-variable bacilli consistent with *Mobiluncus*	1
0 yeast	

Nugent Score = 8

The scoring criteria sums the weighted quantitation of the 3 morphotypes to yield a score of 0 to 10. The criterion for bacterial vaginosis is a score of ≥7, a score of 4-6 is considered intermediate, and a score of ≤3 is considered normal.

XI. Entering Gram Stain reports into LIS

A. Access the "Gram Stain Worklist" in the LIS.
Gram stain reports for all specimens should be entered using this list. In addition, this list should be checked at the beginning, end, and throughout each shift. All specimens on the list must be accounted for.

B. When entering gram stain results for sterile fluids, obtain the following information from the Gram Stain Worksheet and enter into the LIS:

- Enter volume of fluid received
- If specimen was bloody, indicate that in the LIS
- If specimen was spun, indicate that in the LIS
- If a BacTAlert bottle or Isolator was inoculated, indicate the volume of fluid placed into the bottle/tube in the LIS.

C. When entering gram stain results for tissue samples remember to enter the grind charge, as appropriate.

D. When entering gram stain results for smears that have been reviewed by a second reader indicate the checkers initials in the LIS.

E. When entering gram stain results which were called to a patient location document in the LIS the following:

- The first and last name of the person taking the report
- That the report was read back and verified

References:

[1]Forbes, Betty A., D. F. Sahm, A.S.Weissfeld. 1998. Diagnostic Microbiology, 10th ed. St. Louis, Missouri.

[2]Cumitech 2A, Laboratory Diagnosis of Urinary Tract Infections; American Society for Microbiology; March 1987.

[3]Nugent R P, et. al. Reliability of Diagnosing Bacterial Vaginosis is Improved by a Standardized Method of Gram Stain Interpretation. J Clin Micro. 29:297-301, 1991.

Gram Stain Review

The following smears should be independently (unbiased) reviewed by a second person, if possible, before being reported. This person may be a senior technologist, a supervisor, or another qualified staff member.

- **Smears from fluids/tissues with >10 PMNs/LPF or any organism**
- **"positive" gram stains which require a call to a health care provider**
- **Cytospin preparations**
- **Brain abscesses, abscesses from other organs, syringes or volumes of pus**
- **Urogenital smear**

- **Any gram stained smear with a questionable interpretation or an unusual organism (branching gram positive bacilli, fungal elements, ?Cryptococcus, etc...) should be reviewed by a Tech V (or specialist) supervisor or director.**

Smears from the following specimens need not be routinely reviewed by a second reader:

Sputum, Tracheal Aspirates, Bronch Wash, ETT, Deep Trach Lavage
Urine
Fluids/tissues with ≤10 PMNs/LPF and no organisms seen (NOS)
Non-sterile Exudates

RESPIRATORY GRAM STAINS

Sputums and Tracheal aspirates- rejected specimens require a phone call to outside locations. For inpatients, send a report to the unit.

<=10 Epi and any PMN count	Read for bacteria	Cultured
>10 Epi and <25 PMN keypad choice }>E<P	Not read for bacteria	Rejected
>10 Epi and >25 PMN keypad choice }>E>P	Read for bacteria	Rejected
>10 Epi and TNTC PMN keypad choice }E>>P	Read for bacteria	Rejected

Cystic Fibrosis specimens (always cultured)

<=10 Epi and any PMN count	Read for bacteria
>10 Epi and any PMN count	Not read for bacteria (BG1)

BAL, BW, BB, Deep Trach Lavage, ETT and Transtracheal aspirate (always cultured)

Read and record Epi and PMN counts.
Always read for bacteria

Debra Jesien 10/24/12
Debra Jesien Date

Dwight J. Hardy, PhD 10/31/2012
Dwight J. Hardy, PhD, Director Effective Date