Gram Stain Procedure

Principle:

The Gram stain is used to classify bacteria based on their forms, sizes, cellular morphologies, and Gram reaction. Bacterial cell wall composition is the key to the gram reaction, organisms are classified as Gram positive or Gram negative based on the cell wall color when gram stained.

The cell walls of Gram negative organisms are thin and high in lipid content. The cell walls of Gram positive organisms are thick and have a low lipid content, in addition they contain teichoic acids and mycolic acids which fortify the murein layer of the cell wall.

In the Gram stain procedure the slide is flooded with crystal violet (the primary stain), followed by Gram’s iodine (the mordant), which chemically bonds the alkaline crystal violet to the cell wall. The slide is then decolorized most commonly with a mixture of acetone and absolute alcohol. The decolorizer substantially damages the thin cell walls of gram-negative bacteria and allows the crystal violet-iodine complex to wash out, whereas the thicker cell walls of gram-positive bacteria are more resistant to damage and therefore retain the stain complex. In the final step, the slide is flooded with safranin (the counterstain), which stains the decolorized gram-negative bacteria pink or red.

In the clinical setting, the gram stain is useful for presumptive diagnosis of infections based on gram stain reaction and morphology as well as assessing the quality of specimen collected.

Specimens:

1. Clinical specimens
   a. Urine
   b. CSF
   c. Sterile Body Fluids
   d. Aspirates
   e. Exudates
   f. Tissue
   g. Sputums, BALS, etc.
   h. Swabs from infected areas
   i. Stools less than 24 hrs old – for WBC’s only

2. Broth cultures, or colonies growing on solid media. Young cultures (<24 hr) from non inhibitory media and fresh clinical specimens yield the best results. For certain morphological considerations, broth culture smears are required.

Reagents, Supplies and Equipment:

1. Reagents:
   a. Crystal Violet Solution
      (Caution! Combustible May irritate eyes, skin and resp. tract)
   b. Gram Iodine Solution
      (Danger! Poison May irritate skin, eyes, and resp. tract. Avoid breathing.)
   c. Gram Decolorizer, ethyl alcohol 95% and acetone in equal parts
      (Warning! Flammable. May irritate skin, eyes, and resp. tract)
d. Gram Safranin Solution
   (Caution! Combustible. May irritate skin, eyes, and resp. tract. Avoid breathing.)

2. Supplies:
   a. Frosted precleaned microscopic slides
   b. Paper towels
   c. Sterile 0.85 % Saline
   d. Microbiological loops, sterile wood applicator sticks
   e. Immersion Oil
   f. Biohazard bins

3. Equipment:
   a. Cytospin centrifuge
   b. Incinerators
   c. Slide warmer
   d. Tissue grinders
   e. Sterile scalpels
   f. Forceps
   g. Staining rack
   h. Vortex mixer
   i. Microscopes

Storage:

1. Store all reagents at 15 -30 °C.
2. Protect the Gram’s iodine reagent from the light when not in use.

Quality Control:

1. Background material in clinical specimens should stain pink to red.
2. New lot or weekly QC:
   a. Prepare a smear of known QC organisms, fix and stain.
      i. Escherichia coli 25922 – stains red/pink, gram negative
      ii. Staphylococcus aureus 25923 – stains purple, gram positive

Procedure:

1. Slide preparation: Properly label the frosted end of the slide with patient or specimen information. (Pencil or patient label)
   a. Swabs –
      i. Roll swab gently across the slide to avoid destruction of cellular elements and disruption of bacterial arrangements.
      ii. If only one swab received, roll swab on sterile slide for gram stain, inoculate plates, and place swab in broth last.
   b. Aspirates, exudates, sputum, stool, etc.
      i. If specimen received in syringe, transfer contents to sterile vial. Vortex specimen if necessary.
      ii. A representative area of the specimen (purulent or blood-tinged portions) should be selected using a sterile swab.
iii. Roll the swab gently across the slide to avoid destruction of cellular elements and disruption of bacterial arrangements.

iv. Extremely thick specimens may be diluted in drop of saline.

c. **CSF or sterile body fluids:**
   
i. All clear body fluids including CSF greater than 1 ml should be centrifuge at 2,500 rpm for 10 minutes to concentrate any organisms present.
   
   - Cloudy specimens may not be centrifuged.
   
   - Use a sterile pipette to transfer 1 drop of sediment to a slide. Do not spread out.

   - Alternatively, a cystospin prep can be prepared. The specimen is concentrated into a small area on a slide.

   ii. If less than 1 ml is received the specimen can be inoculated directly to the slide.

   - Use a sterile pipette to transfer 1 drop to a slide. Do not spread out.

d. **Tissue specimens:**
   
i. Prepare a “touch prep” to preserve characteristic cellular elements and bacterial arrangements.

   - Mince tissue with sterile scissors or scalpel.

   - Use sterile forceps to hold pieces of tissue, touch the sides of one or more of the minced fragments to a sterile glass slide.

e. **Urine specimens:**
   
i. Not routinely done

   ii. Mix or vortex specimen

   iii. Use a sterile Pasteur pipette to transfer 1 drop to a slide. Do not spread out.

   iv. Allow drop to dry.

f. **Stool Specimens:**
   
i. For WBC’s only

   ii. A representative area of the specimen (purulent or blood-tinged portions) should be selected using a sterile swab.

   iii. Roll the swab gently across the slide to avoid destruction of cellular elements and disruption of bacterial arrangements

g. **Broth cultures:**
   
i. Use a sterile pipette to transfer 1 to 2 drops to a slide

   ii. See blood culture procedure for preparation of smears from positive blood cultures.

   iii. Allow drop to dry.

h. **Positive blood cultures:**
   
i. Direct gram stains are not performed on blood specimens.

   ii. Gram stains are performed on flagged positive blood cultures (broth specimens).

   iii. Wipe septum of bottle with sterile alcohol pad.

   iv. Using a subculturing unit or safety needle unit, insert the needle through the center of the disinfected septum (this will vent the blood culture bottle).

   v. Gently agitate vented bottle(s) to evenly disperse contents.

   vi. If using a subculturing unit, remove the plastic sheath to expose the blunt-ended subculture needle.

   vii. Tip the bottle(s) and place 1 drop on the slide(s) or draw up with the syringe/safety needle unit and place 1 drop on the slide.
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viii. Use sterile stick to spread the blood over the slide to make a thin smear.
ix. Place gram stain slide(s) on slide warmer to dry.

i. **Colonies on solid media:**
   i. Place a drop on sterile saline to a slide
   ii. Transfer a small portion of the colony to the slide using a sterile applicator stick, wire needle or loop.
   iii. Gently mix to emulsify.
   iv. Spread out to make a thin smear

2. Smear Fixation:
   a. Specimen gram stain
      i. Allow slide(s) to air dry.
      ii. Methanol fixation: Place slides in absolute methanol for 1 – 2 minutes.
         • Methanol fixation prevents lysis of RBC’S and results in a cleaner background.
   b. Broth culture including positive blood cultures or colonies growing on solid media
      i. Heat fixation: Place on a slide warmer for 1 minute.
         • Caution: Do not overheat. Overheating can cause over-decolorization.

3. Staining:
   a. Once the slide has been methanol or heat fixed, place the slide on a slide holder rack at the sink.
   b. Avoid applying stains, water or decolorized directly to the specimen area, allow reagents to flow to the specimen area.
   c. Flood the slide with Crystal Violet solution for 30 to 60 seconds
   d. Rinse the slide gently with running tap water. Avoid excessive rinsing as the crystal violet can be removed from the Gram positive cell walls.
   e. Flood the slide with Gram’s Iodine solution for 30 to 60 seconds.
   f. Rinse the slide gently with running tap water.
   g. Pick up the slide
   h. Decolorize the slide by letting decolorizer flow over smear while slide is held at angle. Usually 1 -2 quick squirts, until no more color is being washed from the smear.
   i. Quickly, rinse off any remaining decolorizer with running tap water.
      i. Avoid excessive rinsing as this can wash the crystal violet-iodine complex from the gram positive cell walls.
   j. Flood the slide with Gram’s Safranin for 30 to 60 seconds.
      i. Extend staining time to 2 minutes for poor staining organisms or substitute carbolfuschin for the safranin.
   k. Rinse the slide gently with running tap water.
   l. Drain slide, gently blot to dry with a paper towel.
   m. Carefully wipe excess stain form the back of the slide.
   n. Let the slide dry completely before adding immersion oil and examining microscopically.
4. Interpretation:
   a. Examine smear under low power (10x) for overview of smear and evidence of inflammation.
      i. Background material, RBC’s and WBC’s in clinical specimens should stain pink to red.
      ii. Scan the slide to observe distribution of organisms and cells.
      iii. Identify a good part of slide to evaluate bacteria and cell morphology (background material is pink to red).
      iv. Avoid areas with stain precipitate or cells that still appear blue-purple.
      v. Sputum samples: Evaluate sputum for acceptance of the sample
         - Scan ten representative fields under low power (10x) for areas in which PMN’s are clustered.
         - Enumerate the average number of squamous epithelial cells per low power field
         - Reject specimens with >25 squamous epithelial cells per low power field
         - Notify caregiver to recollect specimen
   vi. Cells are quantitated on low power. Scan the slide for squamous epithelial cells, RBC’s, WBC’s and other cell types.
      - Examine several fields before deciding upon the average quantity of each cell. Note the presence of bronchial epithelial cells in respiratory specimens.
      - You may need to switch to high power (oil immersion) to differentiate cells.
      - RBC’s may be difficult to see as they appear washed out (ghost cells)
      - Always note the presence or absence of WBC’s in the final report
         a. No WBC’s seen
   vii. Use the following key for quantitation of cells on low power field (10x)
      - Rare = <1-2 cells/10x field
      - Few = 1-10 cells/10x field
      - Moderate = 11-25 cells/10x field
      - Many = >25 cells/10x field
   b. Examine smear on oil immersion (100x) objective for bacteria
      i. Background material, RBC’s and WBC’s in clinical specimens should stain pink to red.
      ii. Scan the slide to observe distribution of organisms and cells.
      iii. Concentrate on areas with WBC’s when looking for organisms.
      iv. Identify a good part of slide to evaluate bacteria and cell morphology (background material is pink to red) and PMN’s are clustered if present.
      v. Avoid areas with stain precipitate or cells that still appear blue-purple.
      vi. Bacteria and other organisms such as yeast are quantitated on oil immersion (100x).
      vii. Bacteria are classified based on gram reaction, morphology (coccus, rod/bacillus, coccobacillus, fusiform) and arrangements.
         - Gram reaction
            a. Gram positive – blue/purple color
            b. Gram negative – pink/red
            c. Gram variable - staining irregularly or inconsistently by Gram's stain, cells may appear both blue purple and pink
• **Gram morphology**
  
  a. Bacilli or rod (abbreviated R) = rod shaped  
  
  b. Cocci (abbreviated C) = spherical shaped  
  
  c. Lancet shape = elongated spherical shape  
  
  d. Coccobacilli (abbreviated CB) = short rods almost spherical in shape  
  
  e. Diplococci (abbreviate DC) = in the clinical lab refers to an gram negative organism that appears cocci in shape usually fat with a slight indent (dip) into the cocci shape, almost coffee bean in shape, the organism are usually found in pairs  
  
  f. Fusiform = bacilli with tapered, pointed ends  
  
  g. Pleomorphic = varies in size and shape with pure culture  
  
• **Arrangements**  
  
  a. Gram Positive cocci –  
  
  i. Single = appear singly  
  
  ii. Pairs = paired spherical bacteria  
  
  iii. Chains = spherical bacterial in a line  
  
  iv. Clusters = grape like in arrangement  
  
  v. Tetrads = groups of four
b. Gram positive bacilli/rods –
   i. Large
   ii. Pallisading = aligned side by side (Chinese letters pattern)
   iii. Other arrangements such as chains in rods are not clinically useful and therefore are not usually noted in the report.
   iv. The presence of spore is usually noted in the report.

   c. Gram negative bacilli/rods
   i. Arrangements in gram negative bacilli/rods are not clinically useful and therefore are not usually noted in report.

   viii. Examine several fields before deciding upon the average quantity of each organism.

   ix. Use the following key for quantitation of bacteria/other organisms on oil immersion (100x)
       • Rare = 1 or 2 elements in some but not all fields
       • Few = 1 – 10 elements in every oil immersion field
       • Moderate = 11 – 25 elements per oil immersion field
       • Many = greater than 25 elements per field

5. Specimen Reporting:
   a. Amount of WBC’s or No WBC’s seen in the specimen
   b. Amount of other cells present in the specimen
   c. Amount of bacteria and other organisms (elements) in the specimen
      i. Gram reaction, morphology, and arrangement if applicable
   d. Do not quantitate cells or organisms in flagged positive blood culture specimen, only list the gram reaction, morphology and arrangement of the organisms seen.
   e. Sample report:
      i. Many WBC’s seen
      ii. Many Gram positive cocci in chains (GPC in chains)
   f. Acceptable abbreviations:
      i. GPC = gram positive cocci
      ii. GPCB = gram positive coccobacilli
      iii. GPR = gram positive rod
      iv. GNC = gram negative cocci
      v. GNCB = gram negative coccobacilli
      vi. GNR = gram negative rod
      vii. GNDC = gram negative diplococcic
6. Procedure Notes:
   a. Excessively thick smears will be difficult to decolorize appropriately, and portions of the specimen may "wash off".
   b. Gram positive organisms that are old or that have been treated with antibiotics may appear gram negative.
   c. Irregularly shaped Gram positive areas usually represent precipitated stain and not gram positive cocci.

7. References:
   d. Remel, Lenexa, KS, Gram stain – Crystal violet IFU, TI No. 40052, Revised 10/17/99

8. Gram Stain Key Quick Reference:
   a. Use the following key for quantitation of cells on low power field (10x)
      • Rare = <1-2 cells/10x field
      • Few = 1-10 cells/10x field
      • Moderate = 11-25 cells/10x field
      • Many = >25 cells/10x field
   b. Use the following key for quantitation of bacteria/other organisms on oil immersion (100x)
      • Rare = 1 or 2 elements in some but not all fields
      • Few = 1 – 10 elements in every oil immersion field
      • Moderate = 11 – 25 elements per oil immersion field
      • Many = greater than 25 elements per field
   c. Do not quantitate cells or organisms in flagged positive blood culture specimens, only list the gram reaction, morphology and arrangement of the organisms seen.