EDUCATIONAL COMMENTARY – GRAM STAIN

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Learning Outcomes

Upon completion of this exercise, the participant will be able to:

- Explain the principle behind the Gram stain procedure.
- Describe common modifications to the Gram stain procedure.
- Discuss circumstances which may cause unexpected or erroneous Gram stain results.

Developed by Danish bacteriologist Christian Gram in 1884, the Gram stain is the most fundamental test used to differentiate bacteria. This simple, rapid stain separates most clinically important bacteria into 2 broad groups: gram-positive bacteria, which appear blue or purple, and gram-negative bacteria, which appear pink or red. The Gram stain is used both to characterize bacteria growing on culture media and to directly examine specimens submitted for culture. It is especially useful in helping to quickly confirm or refute a physician's presumptive diagnosis when infection is suspected, because culture results usually come too late to alter presumptive therapy. And, since white blood cells and epithelial cells also stain, the Gram stain provides a way to assess specimen quality and thus deduce whether culture results are likely to be useful.

Principle and Procedure

The differential staining seen with the Gram stain occurs because the two types of bacteria differ significantly in their cell envelope composition. The cell envelopes of both gram-positive and gram-negative bacteria contain a cell wall (also known as the murein layer) and a cytoplasmic membrane. In addition, the cell envelopes of gram-negative bacteria contain a periplasmic space (between the cell wall and cytoplasmic membrane) and an outer membrane.

It is the murein layer, or cell wall, that causes the differential staining seen with the Gram stain. The murein layer, which is composed of a peptidoglycan macromolecule, is much thicker in gram-positive bacteria than in gram-negative bacteria. Also, the cell walls of gram-positive bacteria contain teichoic acids and mycolic acids which fortify the murein layer. The thin cell walls of gram-negative bacteria, on the other hand, contain lipopolysaccharides which are extracted during decolorization.
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In the Gram stain procedure (see Table) 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 with acetone, absolute alcohol, or a mixture of the two. 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.

**Gram Stain Procedure**

1. Using either heat or methanol, fix the specimen onto the slide.

   **Heat fixation:** Gently pass the slide through a flame until all moisture is evaporated, or place on a slide warmer for at least 10 minutes.
   
   *Caution: Do not overheat. Overheating can cause over-decolorization.*

   **Methanol fixation:** Flood the slide with 70%-95% methanol for 1 minute. The methanol is then drained off, and the slides are air-dried.

2. Flood the slide with crystal violet. Allow to remain 30 seconds. Rinse with water.

   *Caution: Avoid excessive rinsing. This can cause over-decolorization because crystal violet is not bound to the cell until Gram’s iodine is added.*

3. Flood the slide with Gram’s iodine. Allow to remain 30-60 seconds, then rinse with water.

4. Decolorize the slide with acetone, alcohol, or a mixture of the two. Rinse with water as soon as the purple color disappears.

   *Caution: Avoid prolonged decolorization or excessive rinsing. These can wash the crystal violet-iodine complex from gram-positive cells.*

5. Flood the slide with safranin for 30-60 seconds. Rinse gently with water.

   *Caution: Do not leave the counterstain on longer than 1 minute. This can make gram-positive organisms appear gram-negative.*
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Modifications
Common modifications of the classic Gram stain procedure involve variations in fixation method, reagents, and timing. Fixation, which attaches the specimen to the slide before staining, can be done with heat or methanol. In heat fixation, the slide is gently warmed so that all moisture evaporates from the material. In methanol fixation, the slides are flooded with 70%-95% methanol for 1 minute. The methanol is then drained off, and the slides are air-dried. Although more labs use heat fixation, methanol fixation is superior for 5 reasons:

1. It preserves bacterial and human cell morphology.
2. It preserves red blood cells, which makes it especially useful with bloody specimens.
3. It provides greater control over the decolorization process, because organisms fixed with methanol are more resistant to decolorization.
4. It prevents liquid specimens from washing off the slide.
5. It leaves a clearer background.

Common variations in reagents involve the decolorizer and the counterstain. Slides can be decolorized with acetone, absolute alcohol, or a mixture of the two. Acetone works more rapidly, but the slide must be rinsed with water as soon as the purple color disappears to avoid over-decolorization. Slides are usually counterstained with safranin, but neutral red or dilute carbolfuchsin, which better stains anaerobes, are used sometimes. Some microbiologists also advocate adding a fast green and tartrazine step before the final counterstain with safranin to give a better contrast between gram-negative organisms and background material.

Finally, the duration of the steps in the Gram stain procedure may vary somewhat. Because anaerobes tend to stain weakly, they may stain better if the counterstain is left on for 1 minute instead of 30 seconds.

Precautions
Pitfalls in the Gram stain include both inherent limitations and technical errors. The Gram stain will not detect organisms which exist within host cells (e.g., Chlamydia spp), organisms with no cell wall (e.g., Mycoplasma spp and Ureaplasma spp), and organisms too small to be seen with light microscopy (e.g., spirochetes). Mycobacteria usually will not stain, and Legionella spp stain only when taken directly from culture. Gram-negative bacteria that stain poorly with safranin include Campylobacter spp, Legionella spp, Bacteroides spp, Fusobacterium spp, and Brucella spp.
Certain conditions are known to damage the cell wall, causing gram-positive bacteria to falsely appear
gram-negative or gram-variable. These include antibiotic treatment, cultures more than 48 hours old,
inflammatory responses in the host, and autolytic enzymes (e.g., *S. pneumoniae*). To minimize
ambiguous results, specimens should be collected before the patient begins antibiotic therapy. Also,
Gram stains should be performed on colonies taken from culture media that do not contain antibiotics,
preferably on colonies that are 18-24 hours old.

Finally, correct interpretation of Gram stains requires a theoretical background of bacteria and their
morphology, because improper technique or suboptimal reagents can cause unreliable results. Errors in
technique which can alter Gram stain results include the following:

- **Fixation with excessive heat** alters cell morphology and makes organisms more susceptible to
  over-decolorization.

- **Low concentrations of crystal violet** make gram-positive organisms more susceptible to over-
  decolorization.

- **Insufficient exposure to iodine** and **lack of available iodine** can prevent crystal violet from
  bonding firmly with the cell wall, thus making gram-positive organisms more susceptible to over-
  decolorization. To ensure reliable Gram stain results, only fresh iodine should be used.

- **Prolonged decolorization**, especially with acetone, can cause gram-positive bacteria to appear
  gram-negative. **Insufficient decolorization** can make gram-negative organisms falsely appear
  gram positive.

- **Insufficient counterstaining** can fail to stain gram-negative bacteria and background material,
  whereas **excessive counterstaining** will leach the crystal violet-iodine complex from gram-
  positive bacteria and stain them with safranin, thus making them falsely appear gram-negative.

- **Prolonged washing** between any of the steps can cause over-decolorization.
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Suggested Reading


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