



## GRAM STAIN REAGENTS

- For in vitro use only -

Catalogue No. SG51-55

Our Gram-Stain Reagents are intended to be used as a differential stain for the microscopic examination of bacterial cultures and laboratory specimens.

Gram staining is the single most useful test in the microbiology laboratory given its simplicity and ability to differentiate bacteria into two main groups: gram-positive organisms and gram negative organisms. Hans Christian Joachim Gram first devised the original procedure in the late 19<sup>th</sup> century, and although modifications have since been made, the basic principles and results remain the same. Our formulation is often referred to as the Hucker Modification.

The staining spectrum includes almost all bacteria, many fungi, and parasites such as *Trichomonas*, *Strongyloides*, and protozoan cysts. The notable exceptions include intracellular pathogens such as *Chlamydia* and *Rickettsia*, and those organisms lacking a true cell wall such as *Mycobacterium*, *Mycoplasma*, and *Ureaplasma*.

The differential properties of the staining process are attributed to the differences in composition between gram-positive and gram-negative cell walls. Gram-positive cell walls contain a thick layer of peptidoglycan, numerous teichoic acid cross-linkages and low lipid content. These structural elements make the cell less permeable to organic solvents making it resistant to the decolorizing process. Conversely, the cell walls of gram-negative organisms show a higher lipid content and an increased permeability to decolorizer, and thus lose the crystal violet dye.

The staining procedure occurs in four parts: The first step is the addition of the primary stain, crystal violet, this initial step stains the contents of the slide purple. After the rinse, Gram's iodine is added to chemically bond the alkaline dye to the bacterial cell wall, if possible. The third step is the decolorizing step where the slide is exposed to an alcohol wash to take off any unbound crystal violet.

The last step is the application of a counterstain. The most common counterstain is safranin, which colors decolorized cells pink. An alternate counterstain is basic fuchsin, which gives the decolorized cells more of a bright pink or fuchsia coloration. The basic fuchsin counterstain works particularly well for anaerobic bacteria, but poorly for *Legionella* and *Bordetella* species.

### Formulation per Litre

#### SG51 Gram Crystal Violet

Crystal Violet.....	20.0 g
Ammonium Oxalate .....	8.0 g
Methanol .....	200.0 mL

#### SG52 Gram Iodine

Iodine Crystals.....	3.33 g
Potassium Iodide.....	6.67 g

#### SG53 Gram Decolorizer

Ethanol.....	500.0 mL
Acetone .....	500.0 mL

#### SG54 Gram Safranin

Safranin O.....	0.25 g
Ethanol.....	10.0 mL

#### SG55 Gram Basic Fuchsin

Basic Fuchsin.....	0.7 g
Phenol .....	3.5 mL
Ethanol.....	14.0 mL

## Recommended Procedure

1. Fix the smear to the slide by flooding the slide with 95% methanol for 1-2 minutes or alternatively, the specimen can be heat fixed by passing the slide several times over a flame until the slide is too hot to touch comfortably. Do not overheat the smear and ensure the slide is cool before staining.
2. Flood the fixed smear with Gram Crystal Violet and let it stand for approximately 45 seconds. Rinse the slide using cold tap water.
3. Flood the slide with Gram Iodine and let it stand for approximately 45 seconds. Rinse the slide using cold tap water.
4. Briefly flood the slide with Gram Decolorizer until no more excess dye runs off the smear (approximately 10 seconds). Immediately rinse with cold tap water.
5. Flood slide with Gram Safranin and let it stand for approximately 30 seconds. Rinse the slide thoroughly with cold tap water.
6. Remove excess moisture using blotting paper or paper towels or alternatively, allow the slide sufficient time to air dry.
7. Examine the smear microscopically under an oil immersion lens.

## Interpretation of Results

In a properly stained smear using safranin as a counterstain, gram-positive cells appear blue to purple, and gram-negative cells appear pink to red. If basic fuchsin is used instead of safranin, gram-positive cells appear bright purple to purplish-black, while gram-negative cells appear bright pink to fuchsia.

When examining the prepared slide using oil immersion, the slide is evaluated for the presence of bacterial cells, as well as their gram-reactions (gram-positive or gram-negative), their shape (i.e. cocci or rods), and cellular arrangements (i.e. clusters, chains, pairs). This information provides a preliminary diagnosis of the infectious agent.

If the smear is from a patient specimen, examine the stained smear for the presence of host cells such as inflammatory and epithelial cells, which may provide information on the state of infection, and quality of the specimen. In general, host cells stain gram-negative and appear pink. Other infectious agents such as yeasts and molds may also be present on the slide and should be documented.

- *Overheating slides during heat fixation can distort the appearance of the organisms*
- *The procedure given is based on an ideal thin smear of cells. Staining and decolorization times may vary depending on the sample and its thickness*
- *The decolorization step is the most important step in the gram-staining process. Overdecolorization results in a abundance of bacteria that appear gram negative, while underdecolorization results in too many bacteria that appear to be gram-positive*
- *Only fresh cultures and specimens should be gram-stained since cell wall integrity of older cells may give improper gram-staining characteristics. Gram-positive organisms that have lost cell wall integrity because of old age or antibiotic treatment may appear pink*
- *Spirochetes will not appear due to their insufficient dimensions and the limitations of light microscopy*
- *Proper smear preparation is key to obtaining good gram staining results. Avoid excessive material or thick smears which may interfere with the passage of light and lead to distortion of images*

## Quality Control

Internal quality control of the Gram stains and reagents must be performed regularly on known reference organisms to ensure the performance of the stains.

## Storage and Shelf life

Our Gram Stain Reagents should be stored at room temperature and protected from light. Under these conditions it has a shelf life of 52 weeks from the date of manufacture, with the exception of Gram Iodine, which has a shelf life of 26 weeks. The solutions are working solutions and do not require dilution prior to use.

## References

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4. Heineman HS, Chawla JK, Lofton WM. Misinformation from sputum cultures without microscopic examination. J Clin Micro 1977; 6:518-27.
5. Murray PR, Washington II, JA. Microscopic and bacteriologic analysis of expectorated sputum. Mayo Clinic Proc 1975; 50:339-44.
6. Mangels JI, Cox ME, Lindley LH. Methanol fixation. An alternative to heat-fixation of smear. Diag Micro Infect Dis 1984; 2:129-37.
7. Forbes BA, Sahm DF, Weissfeld AS. Bailey and Scott's diagnostic microbiology. 10th ed. St. Louis: Mosby, 1998.
8. Murray PR, Baron E, Pfaller M, Tenover F, Tenover FC, Eds. Manual of clinical microbiology. 7th ed. Washington: ASM, 1999.

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