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HARDY DIAGNOSTICS
A Culture of Service™

Instructions for Use

GRAM STAIN KITS AND REAGENTS

Cat. no. C008A	Advanced Crystal Violet™	8oz bottle
Cat. no. C128A	Advanced Crystal Violet™	One gallon
Cat. no. I008	Iodine, Stabilized Gram's	8oz bottle
Cat. no. I128	Iodine, Stabilized Gram's	One gallon
Cat. no. I008N	Iodine, Non-stabilized Gram's	8oz bottle
Cat. no. D008	Decolorizer, Intermediate (50% Acetone)	8oz bottle
Cat. no. D128	Decolorizer, Intermediate (50% Acetone)	One gallon
Cat. no. D128S	Decolorizer, Slow (25% Acetone)	One gallon
Cat. no. D008F	Decolorizer, Fast (75% Acetone)	8oz bottle
Cat. no. D128F	Decolorizer, Fast (75% Acetone)	One gallon
Cat. no. S008	Safranin	8oz bottle
Cat. no. S016	Safranin	16oz bottle
Cat. no. S128	Safranin	One gallon
Cat. no. S008A	Advanced Counterstain™	8oz bottle
Cat. no. S128A	Advanced Counterstain™	One gallon
Cat. no. GK400A	Gram Stain Kit Advanced™, (with Advanced Crystal Violet™ and Advanced Counterstain™)	4 x 8oz bottles
Cat. no. BF008	Basic Fuchsin	8oz bottle

INTENDED USE

Hardy Diagnostics Gram Stain Kits and Reagents are used to stain microorganisms from cultures or specimens by the differential Gram method.

SUMMARY AND PRINCIPLES

In 1883, Karl Friedlander investigated differential staining of bacterial cells in tissue. The following year, Hans Christian Gram, while working with Friedlander at his lab in Berlin, published a detailed account of a new, innovative staining procedure. Various modifications of the Gram's original procedure have been proposed over the years. Nearly all clinically important bacteria can be detected using this method, the only exceptions being those organisms that exist almost exclusively within host cells (e.g. chlamydia), those that lack a cell wall (e.g. mycoplasma and ureaplasma), and those of insufficient dimensions to be resolved by light microscopy (e.g. spirochetes).

Crystal Violet is taken up equally well by both gram-positive and negative bacterial cell walls. A crystal violet-iodine complex is formed within the peptidoglycan layer of the cell wall of each type of bacteria upon addition of iodine. When a decolorizer is applied, lipids are extracted from the cell walls of gram-negative bacteria. Lipid extraction causes an increase in cell wall permeability and results in the loss of the dye complex in the thin peptidoglycan layer. The effect of decolorizer on gram-positive bacteria is dehydration. This decreases the cell wall permeability and increases the retention of the crystal violet-iodine complex in the very thick peptidoglycan layer of the Gram positive bacteria. The result is gram-positive bacteria appearing violet due to retention of the crystal violet-iodine complex, and gram-negative bacteria appearing pink red due to the staining from the Safranin counterstain.

Both Hardy Diagnostics Stabilized and Non-stabilized Gram's Iodine are premixed and ready to use. Stabilized Iodine is thicker and requires a longer decolorization step or a "faster" decolorizer (see below). Stabilized Iodine is less sensitive to light and has a longer shelf life. Non-stabilized Iodine has a shorter shelf life, is thinner and decolorizes more quickly than Stabilized Iodine.

Hardy Diagnostics recommends using Intermediate Decolorizer (Cat. no. D008, 50% Acetone) or Fast Decolorizer (Cat. no. D008F, 75% Acetone) with Stabilized Iodine. Our Gram Stain Kit Advanced™ with Stabilized Iodine (Cat. no. GK400A) includes Fast Decolorizer.

Hardy Diagnostics Gram Stain Kit Advanced™ (Cat. no. GK400A) is comprised of Advanced Crystal Violet™, Stabilized Iodine, Fast Decolorizer and Advanced Counterstain™. Advanced Crystal Violet™ has been shown to provide superior, consistent and brighter staining of gram-positive organisms, especially those which decolorize easily. Advanced Counterstain™, a stronger counterstain than Safranin, will stain the gram-negative organisms a very deep red. Contrast between gram-positive organisms and gram-negative organisms in a mixed field is greatly enhanced. The advanced system helps to guard against accidental over-decolorization, thus reducing the possibility of mistaking a gram-positive organism for a gram-negative one.

STORAGE AND SHELF LIFE

Storage: Upon receipt store products at 15-30ºC. away from direct light. Products should not be used if there are any signs of deterioration (contamination, discloration) or if the expiration date has passed. Products are light and temperature sensitive; protect from light, excessive heat, moisture, freezing, and exposure to air.

The expiration date on the product label applies to the product in its intact packaging when stored as directed. The product may be used and tested up to the expiration date on the product label and incubated for the recommended incubation times as stated below.

Refer to the document "Storage" for more information.

PRECAUTIONS

Do not ingest, inhale, or allow to come into contact with skin.

This product is for *in vitro* diagnostic use only. It is to be used only by adequately trained and qualified laboratory personnel. Observe approved biohazard precautions and aseptic techniques. All laboratory specimens should be considered infectious and handled according to "standard precautions." Refer to the document "Guidelines for Isolation Precautions" from the Centers for Disease Control and Prevention.

For additional information regarding specific precautions for the prevention of the transmission of all infectious agents from laboratory instruments and materials, and for recommendations for the management of exposure to infectious disease, refer to CLSI document M-29: *Protection of Laboratory Workers from Occupationally Acquired Infections: Approved Guideline.*

Sterilize all biohazard waste before disposal.

Refer to the document "Precautions When Using Media" for more information.

Some components of the Gram Stain(s) are poisonous and may be harmful or fatal if swallowed. Avoid contact with eyes, skin, or clothing. Avoid breathing the fumes or vapors. Use only in an adequately ventilated room. Wear protective gloves and wash thoroughly after use.

Acetone and alcohol are flammable; therefore, keep away from heat, flames or sparks.

FIRST AID: In case of eye or skin contact, immediately flush thoroughly with plenty of water for at least 15 minutes. If swallowed, call a physician, hospital or poison control center immediately. Induce vomiting if swallowed. Never give anything by mouth to an unconscious person.

PROCEDURE

Specimen Preparation:

1. Apply the test specimen to a clean glass slide (Cat. no. PF72P) in a manner that will yield a thin, uniform smear. Emulsify colonies from an 18-24 hour culture in sterile saline (Cat. no. R45) if necessary to obtain the proper density.

2. Fix smear to slide using one of the following fixation technique:

> Methanol fix slide by flooding with absolute methanol (95%) for 1-2 minutes, then tilt the slide to drain off methanol and allow to air dry.
> **Note:** For proper fixation, absolute methanol should be stored in brown screw-capped bottles and the working supply should be replenished every two weeks.
>
> **Note: Do not use heat fixation.** Methanol fixation is superior to heat fixation for five reasons:[5,9]
> 1. It preserves bacterial and human cell morphology.
> 2. It preserves red and white 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 over-decolorization.
> 4. It prevents specimens from washing off the slide. Cells will adhere to the slide much better.
> 5. It leaves a clearer background with much less debris.

Staining Procedure:

1. Cover slide with Crystal Violet Reagent for one minute.

2. Rinse slide with deionized or tap water.

3. Cover slide with Iodine Reagent for one minute.

4. Gently rinse the slide with deionized or tap water and allow to drain.

5. Tilt the slide and flood with a few drops of Decolorizer until no violet color runs off. This will usually take 10 seconds or less depending on the thickness of the specimen. Do not over decolorize.

6. Rinse slide gently with deionized or tap water.

7. Cover slide with Safranin, Advanced Counterstain™, or Basic Fuchsin counterstain for one minute.

8. Rinse slide gently with deionized or tap water. The rinse water in this step should be slightly pink. Do not wash excessively.

9. Allow slide to drain and air dry, or gently dry with lintless bibulous paper (Cat. no. 28511007) or paper towel.

10. Examine slide under lens (1,000X magnification) using immersion oil (Cat. no. Z95).

Note: Use caution so that slides are not over-decolorized with the acetone-alcohol, causing gram-positive bacteria to appear gram-negative.

INTERPRETATION OF RESULTS

Bacteria appearing dark blue to violet in color after staining are considered gram-positive. Bacteria appearing pink to red in color are gram-negative.

LIMITATIONS

The Gram Stain provides preliminary identification information only and is not a substitute for cultural studies of the specimen.

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 of the patient, 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.

Precipitated crystal violet can occasionally appear as coccoid shapes or fungal elements, as well as other artifacts or background material, which may interfere with interpretation.

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, makes organisms more susceptible to over-decolorization, and can lead to the cells sloughing off the slide during the rinse steps. Always use methanol fixation.[5,9]

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

- **Insufficient exposure to iodine** and **lack of available fresh 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.

MATERIALS REQUIRED BUT NOT PROVIDED

Standard microbiology supplies and equipment such as microscope slides (Cat. no. PF72P), methanol (Cat. no. VMT032), control slides (Cat. no. Z302), bacteriological loops, swabs, blotting paper (Cat. no. 28511007), microscopes, oil immersion lens, immersion oil (Cat. no. Z95), etc., are not provided.

QUALITY CONTROL

Hardy Diagnostics tests each lot of commercially manufactured media using appropriate quality control microorganisms and quality specifications as outlined on the Certificate of Analysis (CofA) and the CLSI document M22-A3 *Quality Assurance for Commercially Prepared Microbiological Culture Media*. The following microorganisms are routinely used for testing at Hardy Diagnostics:

Test Organisms	Reaction
Staphylococcus aureus ATCC® 25923	Gram-positive cocci, violet
Escherichia coli ATCC® 25922	Gram-negative rods, pink to red

USER QUALITY CONTROL

End users of commercially prepared culture media should perform QC testing in accordance with applicable government regulatory agencies, and in compliance with accreditation requirements. Hardy Diagnostics recommends end users check for signs of contamination and deterioration and, if dictated by laboratory quality control procedures or regulation, perform quality control testing to demonstrate growth or a positive reaction and to demonstrate inhibition or a negative reaction, if applicable. Hardy Diagnostics quality control testing is documented on the certificate of analysis (CofA) available from Hardy Diagnostics Certificate of Analysis website. Also refer to the document "Finished Product Quality Control Procedures," and the CLSI document M22-A3 *Quality Assurance for Commercially Prepared Microbiological Culture Media* for more information on the appropriate QC procedures. See the references below.

PHYSICAL APPEARANCE

- Advanced Crystal Violet™ should appear dark violet.
- Gram's Iodine should appear dark amber to brown in color.
- Decolorizer should appear colorless in appearance.
- Safranin, Advanced Counterstain™, and Basic Fuchsin should appear red.

REFERENCES

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5. Mangels, J.I., M.E. Cox and L.H. Lindley. 1984. Methanol fixation. An alternative to heat-fixation of smear. *Diag. Microbiol. Infect. Dis.* ; 2:129-137.

6. Isenberg, H.D. *Clinical Microbiology Procedures Handbook,* Vol. I, II & III. American Society for Microbiology, Washington, D.C.

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8. Centers for Medicare and Medicaid, *Appendix C, Survey Procedures and Interpretive Guidelines for Laboratories and Laboratory Services*. Subpart K - Quality System for Non-Waived Testing. 493;1200-1265. www.cms.hhs.gov/clia.

9. Minnerath et al. 2009. Comparison of Heat Versus Methanol Fixation for Gram Staining Bacteria. *Bioscene: Journal of College Biology Teaching;* 35:36-41.

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IFU-10450[E]

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The Hardy Diagnostics manufacturing facility and quality management system is certified to ISO 13485.

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