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A Histological Procedure for Precise Determinates With Tuberculate Ureide and Enzyme Transformations of *Canavalia Ensiformis* (L) DC.

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Abstract

A procedure for the rapid processing and staining of plant material is presented. Sharply defined histology of plant material in excellent paraffin sections is now possible in a relatively short time.

Introduction

The foundations of modern microscopy were laid by Botanists. Most of the work in microscopy prior to 1849, done by plant scientists and the "father" of histochemistry, Raspail[1], concentrated on botanical work during the 1830's. In our modern day of genetic engineering with recombinant DNA and hybridomas, plant microtechnique is once again important since there are homologous histochemical determinates that are common to microbe, plant, and animal cellular organelles[2]. Examples of these are the purine conversions for adenine- uric acid-allantoin- ureidoglycolates. These are not only of particular interest in animal and human uremic disorders but are governing components for symbiotic nitrogen fixation and xylem transport of ureides within the unique legume, Jackbean, *Canavalia ensiformis* (L) DC. Mature Jackbean meal contains about 0.14% urease and is the principle source of the pure crystalline enzyme utilized extensively for industrial biologics and pharmaceuticals. Cotyledon content of about 4% Concanavalin A is the sole source of this important phyto-hemagglutinin lectin essential for biomedical diagnostics[3].

The difficulties in processing botanical material and the time involved, plus the relatively poor microscopic preparations which result are probably a large factor in the relative dearth of microscopic material supporting research. This paper describes a method which features overnight processing and excellent thin sections, all of which can be accomplished within a 24 hour period.

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Material and Methods

The material in this study consisted of root nodules of the Jackbean, *Canavalia ensiformis* (L) DC. Planted seeds were inoculated with *Rhizobium leguminosarum* and grown in pots containing 5 kilograms of fertile sandy soil. Selected plants were harvested at 72 days (anthesis) and at 149 days (full seed set). The root nodules were fixed in Perfix (Fisher Scientific, Pittsburgh, PA), the commercial fixative solution of isopropyl alcohol, TCA, and paraformaldehyde. Nodules larger than 4.0 mm in greatest diameter were bisected for better penetration of the processing solutions and the nodules placed in a plastic cassette. The cassettes were then placed on the Tissue-Tek V.I.P.3000 (Vacuum Infiltration Processor, Ames Division of Miles Laboratories, Inc.) and processed overnight on the routine schedule for surgical material. At the end of the processing schedule, the nodules were embedded in Ameraffin (Stephens Scientific Division of Cornwell Corporation. Distributed by American Scientific Products, McGaw Park, IL) and the blocks rapidly chilled.

The blocks were then sectioned using an American Optical Model 840 microtome (Reichert-Jung, Buffalo, NY) using Accu-Edge blades (Ames Division, Miles Laboratories, Inc.). For the purpose of this study, sections were cut at 5 micra and 4 adjacent sections were mounted on 4 glass slides.

All specimens were stained with a polychrome stain modified from Johansen[4] as a routine overview stain. Selected specimens were stained with a modified Giemsa stain, others with the toluidine blue stain of Vassar and Culling[5], and others with a Sudan Black B stain for lipofuscin.

Modified Polychrome Stain

Solutions

2.0% aqueous Safranin O
0.5% aqueous crystal violet
1.0% fast green FCF in 95% alcohol
Orange G-acridine orange solution

To prepare, add orange G to clove oil to saturation. When clove oil is saturated with a small amount of orange G on the bottom, add acridine orange again to saturation.

Staining Procedure

1. Decerate and hydrate to distilled water.
2. Place in 2% aqueous Safranin 0 which has been preheated in a 56-58° C. water bath. Leave solution containing slides in the water bath.
3. Rinse slide quickly in tap water. From this point on, slides should be stained one at a time. The other slides may remain in the Safranin while one slide is being completed.
4. Immerse slide in crystal violet 10 seconds.
5. Rinse well in tap water until water runs clear.
6. Dip slide several times in 50% acetone.
7. Rinse slide well in 90% acetone.
8. Immerse slide in fast green solution for 5 seconds.
9. Rinse slide quickly through three changes of 100% alcohol.
10. Place slide on staining rack and flood section with the orange G-acridine orange mixture in clove oil for 10 seconds.
11. Drain stain from slide into waste container and rinse with clove oil (use dropper) into waste container. Place slide on rack and flood section with fresh clove oil for 3 minutes.
12. Drain oil from slide into waste container, and using a dropper rinse slide with a 1:1 mixture of clove oil and xylene.
13. Rinse slide with xylene from a dropper.
14. Take slide through several changes of xylene.
15. Mount with a synthetic resin.

Results

This procedure provided sharply defined histology with marked contrasts in nuclear staining of bacteroid engorged (nitrogenase active) and noninfected cortex cells. Vivid contrasts resulted for predominately pleomorphic X, Y, and T forms at anthesis and of the swollen rod, nonpleomorphic morphology with sparse cell vacuolation at seed maturity. Specific cytochemical differentiation resulted in brilliant red-violet metachromasia of ureide (Purine) moieties with the Giemsa stains. These were conspicuous within the interstitials and peripheral parenchyma at anthesis and concentrated within the mesothelial sclerenchyma at maturity (Figure 1). These precise determinates directly correlated the microscopy with the cytochemistry of specific cytosol enzyme activity levels governing the ureidoglycolate transformations.

Discussion

The excellent quality of the slides produced for this study are, in the opinion of the authors, a result of the use of the Tissue-Tek Vacuum Infiltration Processor (Ames Division of Miles Laboratories, Inc.). This machine features alternating pressure and vacuum during the processing cycle which results in a far superior exchange of fluids in the dehydration, clearing, and infiltration of the material to be sectioned. As stated previously in the Methods section, the routine surgical processing schedule was used with these particular specimens. It is felt that with a V.I.P. dedicated solely to botanical material, a schedule could be formulated which would consistently produce superior results. Cotyledons, meristems, and materials other than root nodules have also been processed in this manner with the same excellent results.

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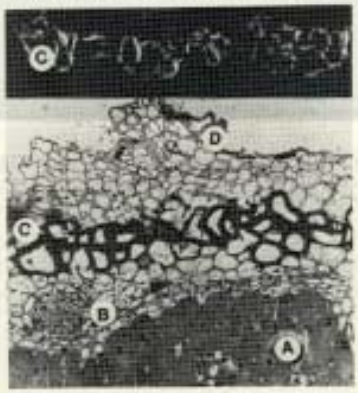

Figure 1: Representative median transverse nodule section of mature *Cannabis sativiformis*.

Lower: (A) Bacteroidal engorged cells, elongated with enlarged nuclei and sparse interstitials. (B) Prominent well defined vasculars within the peripheral parenchymatous cortex. (C) Entodermic stained mesothelial sclerenchyma circumferential to the vasculars. (D) Protruberant epidermal lenticels. Upper: (C) the iridescent moieties of anisotrophic ureides within the mesothelial sclerenchyma visualized with cross-polarized, dark field microscopy with a Leitz fluorite 54/0.95 objective and UV 366nm illumination.

Biological Stain Commission Meets In Rochester, New York June 11-12, 1987

Editors Note

Following are highlights of various aspects of the Biological Stain Commission's annual meeting.

Scientific Session, 8:00-11:00 AM

Dr. Richard Horobin, Reader, Department of Anatomy and Cell Biology, University of Sheffield, United Kingdom, gave a presentation titled "Structure-Staining Relationships". Dr. Jules Elias, Professor, Department of Pathology, State University of New York at Stony Brook, gave a talk titled "The Need for Quality Control of Immunostaing Reagents".

General Business Meeting, 11:00-12:00 Noon

The annual general meeting of the Biological Stain Commission was called to order by Frederick Kasten, President. In attendance were the following Trustees and Members: F. Kasten, A. LaVelle, J. Longley, L. Luna, E. McDowell, R. Mowry, S. Nettleton, E. Schenk, H. Schenider, T. Bonfiglio, C. Churukian, R. Dapson, J. Elias, F. Green, H. Hoch, A. Mallick, A. Villanueva and C. Willis.

E. A. Schenk, Secretary, presented a report on the activities of the Assay Laboratory for 1986. A total of 126 dye samples were received for certification; 115 of these were found to be acceptable, 11 were rejected because of low dye content, poor staining or solubility problems. Ninety-eight dye samples were received for "recheck".

Vendors also purchased 58,365 labels in 1986. The total weight of dyes certified in 1986 was 10,033 pounds. The big 8, namely Wright stain, eosin Y, hematoxylin, safranin, basic fuchsin, malachite green, crystal violet and methylene blue, accounted for approximately 75% of the total poundage. Eighteen different companies submitted dyes for certification and/or purchased labels.

The Secretary also reported that the Commission currently has 66 members, 10 are Trustees, and 14 scientific societies are represented.

E. Stotz prepared the Treasurer's Report which was given by E. Schenk. The financial picture of the Commission continued to be favorable for the year 1986.

James B. Longley, Editor of STAIN TECHNOLOGY reported that the journal is doing well despite a persistent yearly decrease in subscriptions and in the number of manuscripts received for publication.

Frederick Kasten, reporting for the Publications Committee, advised that the new revision of BIOLOGICAL STAIN has been scheduled for 1988 and that good progress is being made with the updating of STAINING PROCEDURES, which will have multiple new co-authors and topics and is scheduled for 1989.

President's Forum, 2:00-5:00 PM

F. Kasten indicated that the role of representatives is to establish a two-way communication between the Commission and the Society; to bring to the Commission problems and needs expressed by Society members and to bring to them a sense of the Commission's objectives, accomplishments, current activities and future plans.

C. Churukian gave an update on the ever increasing use of microwave ovens in staining.

D. Files discussed the topic of the use of *dyes used as therapeutic agents*. While there is an obvious need for high purity, the Commission has not been involved in testing materials used for this purpose.

J. Bertsch encouraged the certification of *Acridine orange*.

G. Nettleton has developed an HPLC assay method, but staining protocols are not yet in hand.

J. Bertsch also mentioned that there has recently been an increased demand for *Azure C*. No one could shed any light on what this is being used for.

E. Schenk presented a problem encountered with a sample of *Azure B* received for certification. This sample was found to have a dye content of 83% and poor solubility, which led to rejection.

F. Kasten reviewed data from *dye solubility studies* which he has carried out in his laboratory. The solubility of over 100 dyes in water and 95% alcohol has been evaluated.

D. Dapson presented a detailed report on the solubility of a *specially prepared oil red O*.

R. Horobin presented information on the newly formed *EUROPEAN COMMITTEE FOR CLINICAL LABORATORIES STANDARDS (ECCLS)*.

A. Mallik passed out a list of 24 dyes used in molecular biology and pointed out that only a few of these were currently certified.

Board of Trustees Meeting

June 12, 1987
9:00-12:00 Noon

Items of Interest

The Trustees recommended that the charge for labels be increased from 45¢ to 55¢ as of January 1988. G. Stephen Nettleton has been appointed Editor in Chief of STAIN TECHNOLOGY as of January 1988. James B. Longley, who has held that position for many years, will stay on as Associate Editor.

The following were elected to membership in the Commission: Dr. Richard Horobin, Department of Anatomy and Cell Biology, University of Sheffield, United Kingdom; Dr. James N. Turner, New York State Department of Health, Albany, NY; Dr. Terrence M. Hammill, Biology Department, SUNY, Oswego, NY. Dr. Floyd Green was appointed Consultant to the Trustees.

The following statement was drafted for dissemination to dye users:

> "Purchasers of dyes and stains need to be warned not to mistake such terms as 'Certistain' or the equivalent in catalogs and advertisements, for certification of dyes by the Biological Stain Commission. All dyes certified by the Commission have a label bearing a certification number, affixed to each individual bottle sold."

The annual meeting in 1988 will be held in Washington, D.C. Lee G. Luna will be the host and organizer.

Biological Stain Commission Board of Trustees
Front row (L-R): Dr. Eric A. Schenk, Dr. G. Stephen Nettleton, Dr. Frederick H. Kasten, Dr. Elmer H. Stotz. Back row (L-R): Mr. Lee G. Luna, Dr. Robert W. Mowry, Dr. Henry Schneider, Dr. James B. Longley, Dr. Arthur LaVelle, Dr. Elizabeth M. McDowell.

Microwave Trouble Shooting

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Editors Note: The author is now employed by Reichert-Jung, Inc., P.O. Box 123, Buffalo, New York 14240.

The following are some of the problems which could arise from staining using a microwave oven. These problem and solution situations are the result of many trial and error efforts and will hopefully benefit those who are new to the microwave staining concept.

Problem

Published staining times do not seem to work for your particular microwave unit. Either the solutions become too hot and boil out of the coplin jar or do not get hot enough.

Solution

Commercial microwave ovens vary in their power supply. Units may vary from 400 watts to above 1000 watts. The temperature of the solution exposed in a microwave is dependent upon three variables: volume of liquid exposed, time of exposure and wattage of the microwave unit. Maintaining the volume as a constant, the other two can be varied. The higher the wattage the less time necessary for incubation.

Problem
The Grocott's methenamine silver has too much background staining with the silver. Reticulin fibers are also demonstrated but, they should not be.

Solution
One potential answer here is that there could be insufficient chromic acid oxidation. Either the time of exposure was insufficient for complete oxidation or the solution is old and needs to be changed. After several exposures in the microwave, the solution will change from an orange color to a dark brown. When this occurs, the chromic acid is no longer effective as an oxidizer.

Problem
Extensive silver precipitation from the GMS on the slide and tissue. A mirror effect has been produced.

Solution
Depending upon the extent of silver precipitation, this could be reduced by extended time in gold chloride. A mirror effect is most likely caused by leaving the slides in the hot silver solution too long. The only prevention for this is to remove the slides from the solution sooner.

Problem
Frozen sections float off slides after chromic acid oxidation.

Solution
Several possibilities exist to this problem. The concentration of the chromic acid could be too strong. The concentration could be reduced to 2.5% and still be effective. Gelatin, or albuminized slides can be used for frozen sections and help prevent floating.

Problem
After removal from the microwave oven there is uneven silver deposition on the tissue sections. The sections at the top of the slide are darker than those on the lower area.

Solution
An unusual phenomenon which occurs in a coplin jar exposed in the microwave, is that the solution gets hotter at the top of the coplin jar than at the bottom. There can be as much as a 5° *to* 8° difference which can account for greater silver deposition at the higher temperature. This can be alleviated by agitating the solution after removal from the microwave and thus equilabrating the temperature. This will cause more even staining.

Problem
The control sections do not demonstrate the organisms after incubation in the GMS solution or the organisms stain very lightly.

Solution
Exposure to the silver solution is insufficient. After microscopically examining the control section, it can be immediately placed back into the microwave and exposed for an additional 10 seconds. Another alternative is to allow the sections several additional seconds standing time.

Problem
Argyrophil granules are not demonstrated in the control sections when the Grimelius is performed.

Solution
Several possibilities exist with this problem. The most likely answer is that the reducer is not sufficiently hot enough to induce silver reduction. If reduction does not occur with the initial attempt, it may be necessary to incubate slides in the hot silver solution a second time followed by another exposure to the reducing solution. This should produce adequate staining.
Experience has shown that if the reducing solution is sufficiently heated argyrophil granules should be demonstrated. A temperature above 70° should induce proper reduction.

Problem
Legionella and spirochetes are not demonstrated on control sections after the Dieterle reaction is performed.

Solution
The Dieterle reaction is argyrophilic in nature as is the Grimelius. After incubation in the silver solution, it is necessary to expose the slides to a hot reducing solution. Since the reducing solution contains pyridine, it is advisable to heat the reducing solution in a zip-lock baggie in the microwave oven. This will contain any spillage which might occur if the solution is heated too long. The reducing solution should be exposed long enough to raise the temperature of the solution to about 70° to 80° C. This should be sufficient to reduce the silver at the sites of the organisms.

Problem
Copper staining is not intense enough after a microwave exposure and a few minutes standing time

Solution
Allow sections to remain in the hot incubating solution for 5 to 10 minutes and microscopically examine the control slide for quality. Slides may also be reexposed in the microwave for an additional 5 to 10 seconds and allowed to have a few additional minutes standing time.

Problem
Incubation of Schiff's reagent causes the top of the solution to become pink. Is the solution reusable?

Solution
Solutions exposed in the microwave often become hotter at the top than they do at the bottom. When this happens

to the Schiff's reagent, it can become hot enough at the top to become pink. Once the heat is more evenly distributed throughout the solution, the color will be absorbed and the staining reaction will be more even. The solution can be reused by placing it in the refrigerator. It will then resume its original amber or colorless appearance.

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A Method for Sectioning Ticks

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The JOURNAL OF HISTOTECHNOLOGY (Vol. 1, No. 1, pp. 25-26, 1977) contained an excellent method by Trombetta and Forbes[1] for processing sclerotized insects. The information contained herein is to report on my experience with the use of this method for processing and sectioning ticks. The technique proved equally good for both Argasidae (soft bodied ticks) and Ixodidae (hard bodied ticks).

Periodically there is a need to process ticks which accompany tissue specimens being diagnosed for a tick bite associated disease; i.e., Rocky Mountain Spotted Fever. The species of tick would normally determine how the specimen is to be processed and sectioned. The male of both species has a distinctive cuticular area, whereas the female has a narrower cuticle plate. Because of this sclerotized characteristic, the microtomist should use exceptional care in sectioning: Adequate water soaking followed by proper chilling of the faced paraffin block, is one of the essential requirements for good microtomy.

Prior to the introduction of the method by Trombetta and Forbes, I processed soft ticks in a conventional manner with the use of ethyl alcohol, xylene and embedding in paraplast wax. A longer infiltration period of up to 4 hours in vacuum was often necessary. There were times when the processing fluids hardened the exterior of the tick bodies. However, satisfactory sections could be obtained after long periods of soaking the specimen surface with warm water or weak ammonia-water solution and use of a well sharpened microtome knife.

For hard bodied ticks, the glycerol-formalin solution was often used in a 50-50 ratio for softening the cuticular area. Specimens were left in this solution from 24-72 hours, then placed in 10% buffered formalin and processed. Another means of softening cuticular areas is by exposure to a depilatory solution (i.e., Nair or Neet) for several hours or overnight. In this case thorough washing is then necessary before processing. Although we were able to obtain quality cut sections with this method, the staining results were often rather poor. This, I believe, was due to the action of the depilatory on tissue constituents.

The N-butyl alcohol-celloidin paraplast plus (BCPP) double infiltration method of Trombetta and Forbes is valuable in Processing **both** species of ticks, since the method avoids contact with ethyl alcohol and xylene which tend to harden these tissues.

Celloidin has long been used for delicate and hard tissues and is particularly useful in double embedding. I used nitrocellulose rather than celloidin in this modification, since the nitrocellulose solutions are of low viscosity and penetrate quicker. For infiltration I used Paraplast instead of Paraplast Plus as recommended in the article by Trombetta and Forbes.

Since this procedure is lengthy, it is sometimes necessary to alter the time to suit your needs. At some processing stages, the specimens can be left overnight and even over the weekend in the celloidin-methyl benzoate (CMB) solutions. When specimens are placed in CMB, they may float but will gradually sink to the bottom of the container.

The largest tick processed in our laboratory measured 1.5 cm in length by .75 cm in width. This large specimen processed exceptionally well but required additional infiltration in paraffin with the use of vacuum. (The original technique suggests that openings be made in the cuticle of the tick to allow penetration of fluids.) The exterior cuticle and limbs of the tick are the most difficult to demonstrate since they are brittle and difficult to section, but excellent results can be obtained by either the original Trombetta and Forbes method or by incorporating the above suggested modifications.

The routine H&E stain employing Mayer's hematoxylin and phloxine-eosin differentiates the interior organs well.

The use of special stains makes particular features of the tick section more discernible. For example, the Russell

modification of the Movat Pentachrome clearly defines the spirocler as intense emerald green. The columnar cells of the intestine stain blue, muscle bright red and other areas of the cuticle and limbs are seen as transparent-like golden-brown.

Reference

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Trypsin Digestion of Retinas of Laboratory Rats

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Fixation

10% buffered formalin or other desired fixatives.

Microtomy

See step two of method.

Solutions

Tris Buffer—0.15M (pH 7.8)

Tris buffer (Sigma 7-9, M.W. 121-136).. 18.17 gm
Distilled water ..... 1000.0 ml
Hydrochloric acid (concentrated to adjust pH)

Trypsin Digestion Solution
Make Fresh—Use Immediately

Trypsin ..... 3.0 gm or (1.5 gm) or (.75 gm)
Tris buffer ..... 100.0 ml or (50 ml) or (25 ml)
Merthiolate* (preservative) ..... 1 pinch
*Thimerosal may be used as preservative if Merthiolate is not available.

Method

1. Fix eyes in fixative of choice for at least 12 to 14 hours or longer to achieve proper fixation. Longer fixation in most fixatives will not harm the eye or affect the staining results.
2. Rinse the eyes in water for a few minutes.
3. Cut the eye into 2 pieces while holding with forceps and save posterior (optic nerve end) portion of the eye for digestion.
4. Gently remove the retina from the eye (slight scooping motion) with a small spatula.
5. Remove retina and place into a vial of trypsin digestion solution with an eye dropper.
6. Place vial into a shaker water bath at 37° C for 1 to 2 hours, checking often. Regular water bath will work, just shake vial periodically. Change solution when it becomes cloudy and repeat the change if necessary.
7. Remove digested retina from the vial with an eye dropper and rinse in water to remove excess trypsin solution.
8. Remove retina from water and place onto a clean microscopic slide with an eye dropper. Manipulate the retina with a teasing needle to remove any wrinkles which may have formed.
9. Air dry slides or dry in a medium heat dryer for 10 to 20 minutes.
10. Stain slides with Periodic Acid Schiff's/Hematoxylin method below.

Periodic Acid Schiff's/Hematoxylin Staining Method

1. Periodic acid 0.5% for 5 minutes.
2. Rinse in water.
3. Stain in Schiff's reagent for 15 minutes.
4. Rinse in water for 10 minutes.
5. Counterstain in hematoxylin for 5 minutes.
6. Glacial acetic acid (1%) for 2 or 3 dips.
7. Blue in tap water for 5 minutes.
8. Dehydrate through absolute alcohol, clear in xylene and mount coverslip with resinous media.

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Acknowledgments

Kathy Jones, HT (ASCP) for her initial training of this technique. Special thanks to Dr. David Fairchild, DVM, Diplomate, for his help and support of this project.

Helpful Hint For Frozen Sections

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I would like to share my technique for saving time while preparing a frozen section. Keep a bottle of Optimum Cutting Temperature (OCT) in the refrigerator at 5° C and when a frozen section is required, the OCT is already cool and doesn't take as long to freeze. This technique has proven very beneficial for frozen sections in our laboratory.

HISTOTECHNOLOGY
QUALITY CONTROL TRADE-OFF

"EVERY TIME YOU ATTEMPT TO SOLVE A PROBLEM IN HISTOPATHOLOGICAL TECHNIQUE, YOU CREATE A POTENTIAL NEW PROBLEM."

LEE G. LUNA

A Modified Gram Positive and Gram Negative Procedure

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Following is a modified method for demonstrating gram-positive and gram-negative bacteria. The method has been used successfully in our laboratory. The pathologists find it as useful as the Brown-Brenn or the MacCallum-Goodpasture methods. It is being presented here, in the hope others will find it beneficial.

Fixation
10% buffered neutral formalin

Technique
Cut paraffin sections at 4-6 microns

Solutions
Gram (Bacto) crystal violet solution*
Gram (Bacto) Safranin solution*

*These solutions can be obtained from Difco, P.O. Box 1058, Detroit, MI 48432

Gram's Iodine Solution

Iodine	1.0 gm
Potassium iodide	2.0 gm
Distilled water	300.0 ml

Alcoholic-Acetone Solution

95% ethyl alcohol	70.0 ml
Acetone	30.0 ml

Staining Procedure

1. Deparaffinize and hydrate slides to distilled water.
2. Stain slides in the crystal violet solution (filtered) for 2 minutes.
3. Drain slides but do not wash.
4. Apply Gram's iodine solution for 1 minute.
5. Rinse slides in distilled water.
6. Differentiate slides in alcohol-acetone solution until blue color no longer streams from the section (approximately 10-20 seconds).
7. Rinse slides in distilled water.
8. Stain slides with Gram's Safranin for 4-8 seconds. Do not overstain.
9. Wash slides in distilled water.
10. Dehydrate slides quickly in 95% alcohol, absolute alcohol and clear in xylene, two changes each.
11. Mount coverslip with resinous mounting media.

Results

Gram positive bacteria	-purple
Gram negative bacteria	-reddish pink
Background	-light pink

To receive your own copy of HISTO-LOGIC, or to have someone added to the mailing list, submit home address to: **Ames**, Division of Miles Laboratories, Inc., P.O. Box 70, Elkhart, Indiana 46515. Printed in U.S.A.

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