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| **Alcian blue pH 2.5 for Mucin** |

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| Sample type | Animal |
| Application | Mucin-acidic mucosubstances |
| Specimen(s) | Mucin-acidic mucosubstances. Routine paraffin processed, formalin or "prefer" fixed tissue sections. |
| Submitted by | [Rick Giberson](mailto:rick_giberson@tedpella.com), [Ted Pella, Inc.](http://www.tedpella.com) and Bruce Caukins, formerly with Pathology Sciences Medical Group, Chico, CA |
| Instrument used | BioWave™, Ted Pella, Inc. Microwave system with PELCO ColdSpot®, vacuum chamber, and variable wattage. |
| Sample size | 3-5 µm sections on slides |
| Sample container | Microwave-safe plastic coplin jars (Thermo-Shandon#1001361) or other microwave safe staining containers should be used. Place sample container on PELCO ColdSpot®, and place temperature probe in sample container and set TR. |
| Contrl | Small intestine, appendix, or colon, fixed like specimen and cut at 4 microns. Run a control slide with each stain batch. If negative, repeat procedure (check expiration dates of reagents). |

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| **Step** | **Reagent** | **Power** | **Time** | **TR** |
| 1 | 50 ml Coplin Jar, xylene Note #2 | 165 W | 4 min |  |
| 2 | 50 ml Coplin Jar, 95-100% ETOH Note #2 | 165 W | 1 min |  |
| 3 | Wash in tap water to clear | Bench step |  |  |
| 4 | 50 ml Coplin Jar, 3% Acetic Acid | 165 W | 30 sec | 45°C |
| 5 | 50 ml Coplin Jar, Alcian Blue solution | 165 W | 90 sec | 45°C |
| 6 | Wash in DI water, 3 changes |  | Bench step |  |
| 7 | 50 ml Coplin Jar, Nuclear Fast Red or Brazilliant, Anatech | 165 W | 90 sec | 45°C |
| 8 | Wash in DI water, 3 changes |  | Bench step |  |
| 9 | Dehydrate through ETOH's, clear and mount |  | Bench step |  |

##### Detailed reagent prep information

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| 3% acetic acid solution | * 3 ml of glacial acetic acid * 97 ml dd-H2O |
| Alcian Blue, pH 2.5 | Solution may be filtered back and reused, stable for about 2 months.   * 1 g of Alcian Blue 8GX * 97 ml deionized water * 3 ml glacial acetic acid * Mix, filter. Check pH and adjust to 2.5 * Add a few crystals of thymol as a preservative. |
| Nuclear fast red | See Iron Stain (it is soluble in about 0.25% in water) |

##### Comments

Acidic mucosubstances are stained blue by the Alcian blue technique. This procedure is used in this laboratory to aid in the diagnosis of Barret's esophagus to distinguish glandular epithelium with intestinal metaplasia. Microwaves cause most reactions and staining to proceed more quickly.

This procedure can easily be combined with the PAS reaction to differentiate between neutral mucosubstances and acid mucosubstances.

##### Results

Exclusively acid mucosubstances - blue

Nuclei - pale red

##### References

Carson, F.L., Histotechnology: A Self-Instructional Text, ASCP Press, 1990, pg 130-131

Sheehan and Hrapchak, Theory and Practice of Histotechnology, 2nd Edition, 1980

Mosby Preece, A Manual for Histologic Technicians, 3rd Edition, 1972, Little, Brown & Co.

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| **Grocott's methenamine silver nitrate - fungus and pneumocystis** |

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| --- | --- |
| Sample type | Animal |
| Application | Organisms |
| Specimen(s) | Fungi and pneumocystis carinae. Routine paraffin, formalin or "prefer" fixed tissue sections or air-dried smears. |
| Submitted by | [Rick Giberson](mailto:rick_giberson@tedpella.com), [Ted Pella, Inc.](http://www.tedpella.com) and Bruce Caukins, formerly with Pathology Sciences Medical Group, Chico, CA |
| Instrument used | BioWave™, Ted Pella, Inc. Microwave system with PELCO ColdSpot®, vacuum chamber, and variable wattage. |
| Sample size | 3-6 µm sections on slides |
| Sample container | Microwave-safe plastic coplin jars (Thermo-Shandon#1001361) or other microwave safe staining containers should be used. Place sample container on PELCO ColdSpot®, and place temperature probe in sample container and set TR. |
| Control | Fungus or Pneumocystis carinae known positive tissue, formalin-fixed and cut at 4 microns. Run a control slide with each stain batch. If negative, repeat procedure (check expiration dates of reagents). |

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| **Step** | **Reagent** | **Power** | **Time** | **TR** |
| 1 | 50 ml Coplin Jar, xylene | 165 W | 4 min |  |
| 2 | 50 ml Coplin Jar, 95-100% ETOH | 165 W | 1 min |  |
| 3 | Wash in DI water to clear |  | Bench step |  |
| 4 | 50 ml Coplin Jar, 5% Chromic Acid | 502 W | 6 min | 60°C [1] |
| 5 | Wash in DI water |  | Bench step |  |
| 6 | 50 ml Coplin Jar, Sodium bisulfite sol. |  | Bench step, 1 min |  |
| 7 | Rinse in tap water, then 3-4 changes in DI Water |  | Bench step |  |
| 8 | 50 ml Coplin Jar, Methenamine silver | 315 W | 4 min 30 sec | 60°C [2] |
| 9 | Rinse in tap water, then 3-4 changes in DI water |  | Bench step |  |
| 10 | 50 ml Coplin Jar, 1% gold chloride | 165 W | 40 sec | 40°C [3] |
| 11 | Wash in DI water |  | Bench step |  |
| 12 | 50 ml Coplin Jar, 2% sodium thiosulfate | 165 W |  | 40°C [3] |
| 13 | Wash in DI water |  |  |  |
| 14 | 50 ml Coplin Jar, Light green solution | 165 W |  | 40°C [3] |
| 15 | Dehydrate through ETOH's, clear and mount |  |  |  |

[1] Temperature should reach 60°C in approximately 3 minutes. Change wattage, if required, to smooth heating curve.

[2] Temperature should reach 60°C in the first 2 minutes of the time period. Adjust wattage to smooth heating curve. Also, repeat step for 2 minutes or less to improve silver development. Keep the TR at 60°C.

[3] Temperature does not need to reach 40°C during the time period.

##### Detailed reagent prep information

|  |  |
| --- | --- |
| 5% chromic acid | * 3 g chromium trioxide * 100 ml dd-H2O |
| 1% sodium metabisulfite | In distilled water |
| Stock methanamine silver | * 100 ml 3% methenamine * 5 ml 5% silver nitrate   Keep refrigerated at 4°C |
| Working methanamine silver | * 25 ml stock methenamine silver * 25 ml 1% borax (sodium borate) |
| 1% gold chloride stock | Use same bottle as for Fontana |
| Working 0.1% gold chloride | * 5 ml stock * 45 ml DI Water |
| 2% sodium thiosulfate | In distilled water |
| Light green stain | American MasterTech Inc. Light Green Solution CAT#STLGCPT |

##### Comments

*For principle:*

For demonstration of fungi and pneumocystis carinae. Chromic acid oxidizes polysaccharide such as glycogen, mucin, and fungal cell walls to aldehyde groups. The polysaccharide/aldehyde oxidation products selectively reduce the alkaline silver solution to black metallic silver deposits, rendering them visible. Gold chloride tones the tissue, eliminating yellow tones. Sodium thiosulfite fixes the silver reaction in the tissue by stopping all previous reactions and removing unreduced silver nitrate. Light green acts as a counterstain. Microwaves cause most reactions and staining to proceed more quickly.

*Hazards*

Chromium trioxide and silver nitrate dry powders are strong oxidizers: use safety glasses, gloves, and lab coat when preparing solutions. Chromium trioxide is a potential carcinogen. **HOT CHROMIC ACID CREATES VERY CAUSTIC FUMES. COVER CONTAINER WITH PARAFILM. REMOVE FROM OVEN TO FUME HOOD TO REMOVE SLIDES TO RINSE WATER.**

*For staining*

* 5% Chromic acid should be clear, bright orange. It is exhausted (reduced) when the color starts to turn dark brown, usually after 2-3 uses.
* 0.1% Gold chloride: usually good for several uses. Replace when it turns cloudy, dirty, or precipitates (may be filtered).
* Light green solution: replace when 3 minutes is insufficient staining time.
* Silver nitrate, chromic acid, and gold chloride are to be disposed of as hazardous waste in the marked, separate containers in the hazardous waste corner of the lab. **DO NOT FLUSH DOWN DRAIN OR POUR IN FLAMMABLE ORGANICS DRUM.**
* Other reagents should be poured up fresh weekly.
* Working solutions are kept in 50 or 75 ml Coplin jars in the stain cupboard unless refrigerated.

##### Results

Fungi and Pneumocystis - dark brown to black

Mucin - taupe to dark grey

Background - light green

##### References

Sheehan and Hrapchak, Theory and Practice of Histotechnology, 2nd Edition, 1980

Preece, A Manual for Histologic Technicians, 3rd Edition, 1972, Little, Brown & Co. page 335, with modifications

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| **Iron-prussian blue stain for ferric iron** |

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| --- | --- |
| Sample type | Animal |
| Application | Organisms |
| Specimen(s) | Bone marrow, spleen and liver. Routine paraffin, formalin or "prefer" fixed tissue sections on air-dried smears. |
| Submitted by | [Rick Giberson](mailto:rick_giberson@tedpella.com), [Ted Pella, Inc.](http://www.tedpella.com) and Bruce Caukins, formerly with Pathology Sciences Medical Group, Chico, CA |
| Instrument used | BioWave™, Ted Pella, Inc. Microwave system with PELCO ColdSpot®, vacuum chamber, and variable wattage. |
| Sample size | 4 µm sections on slides |
| Sample container | Microwave-safe plastic coplin jars (Thermo-Shandon#1001361) or other microwave safe staining containers should be used. Place sample container on PELCO ColdSpot®, and place temperature probe in sample container and set TR. |
| Control | Known Fe positive tissue fixed like specimen and cut at 4 microns. Excessive amounts of iron are not desirable because the reaction product is slightly soluble and may contaminate the incubating solution, giving a background stain in all sections. Coplin jars inadequately cleaned after iron hematoxylin may also contaminate the staining solution. l; Run a control slide with each stain batch. If negative, repeat procedure (check expiration dates of reagents). |

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| **Step** | **Reagent** | **Power** | **Time** | **TR** |
| 1 | 50 ml Coplin Jar, xylene | 165 W | 4 min |  |
| 2 | 50 ml Coplin Jar, 95-100% ETOH | 165 W | 1 min |  |
| 3 | Wash in tap water to clear |  | Bench step |  |
| 4 | Wash in DI water, 3 changes |  | Bench step |  |
| 5 | 50 ml Coplin Jar, Ferrocyanide/HCL | 315 W | 2 min | 45°C |
| 6 | Wash in DI water, 3 changes |  | Bench step |  |
| 7 | 50 ml Coplin Jar, Nuclear Fast Red | 315 W | 90 sec | 45°C |
| 8 | Wash in DI water, 3 changes |  | Bench step |  |
| 9 | Dehydrate through ETOH's, clear and mount |  | Bench step |  |

##### Notes

* + Place temperature probe in ColdSpot® port when temperature restriction (TR) is "none" and set TR to >50°C.
  + Steps #5 and #7 do not require reaching 45°C
  + Use nonmetallic forceps and chemically-cleaned glassware

##### Detailed reagent prep information

|  |  |
| --- | --- |
| 2% potassium ferrocyanide | * + 10 g potassium ferrocyanide   + 500 ml deionized water |
| 2% hydrochloric acid | * + 10 ml hydrochloric acid   + 490 ml deionized water |
| Working potassium ferrocyanide/HCL solution | Mix just before use, equal volumes of:   * + 2% potassium ferrocyanide   + 2% hydrochloric acid   Note: ALWAYS ADD ACID TO THE FERROCYANIDE - reduces chance of acid splashes. |
| Nuclear-fast Red (Kernechtrot) solution | Brazilliant from Anatech, Inc. |

##### Comments

*For principle*

Ferric iron is normally found in small amounts in the bone marrow, spleen, and liver. Abnormally large deposits may be seen in hemochromatosis and hemosiderosis. This method detects the ferric iron in loosely bound protein complexes (as in hemosiderin). Iron that is strongly bound, as in hemoglobin, will not react. In the Prussian blue reaction, sections are treated with an acidic solution of potassium ferrocyanide and any ferric iron present reacts to form an insoluble bright blue pigment called Prussian blue. Microwaves cause most reactions and staining to proceed more quickly.

*For staining*

* + Some iron may be dissolved by some of the acidic fixatives and decalcification solutions.
  + It is important to use iron-free reagents, especially iron-free hydrochloric acid.
  + If excess nuclear-fast red counterstain is not adequately removed with running water before beginning dehydration, cloudy slides will result. Run back to water if necessary.
  + Poisonous HCN gas is released only with long-term storage of the potassium ferrocyanide/HCL mixture. Add 5 ml of sodium carbonate to approx. 50 ml of the staining mixture. The color should turn yellow; carbon dioxide bubbles may form. The pH is about 7.0 and may be flushed down drain with tap water.

##### Results

Hemosiderin (iron) - blue   
Nuclei and hemofuschin - red   
Background - pink

##### References

Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology, 3rd Edition, Lee Luna (editor), 1968

Carson,F.L., Histotechnology: A Self-Instructional Text, ASCP Press, 1990

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| **PAS-periodic acid-Schiff reaction** |

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| --- | --- |
| Sample type | Animal |
| Application | Organisms |
| Specimen(s) | Routine paraffin, formalin or "prefer" fixed tissue sections. |
| Submitted by | [Rick Giberson](mailto:rick_giberson@tedpella.com), [Ted Pella, Inc.](http://www.tedpella.com) and Bruce Caukins, formerly with Pathology Sciences Medical Group, Chico, CA |
| Instrument used | BioWave™, Ted Pella, Inc. Microwave system with PELCO ColdSpot®, vacuum chamber, and variable wattage. |
| Sample size | 3-5 µm sections on slides. |
| Sample container | Microwave-safe plastic coplin jars (Thermo-Shandon#1001361) or other microwave safe staining containers should be used. Place sample container on PELCO ColdSpot®, and place temperature probe in sample container and set TR. |
| Control | Appendix or colon mucosa fixed like specimen and cut at 3-5 microns. Run a control slide with each stain batch. If negative, repeat procedure (check expiration dates of reagents). |

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| --- | --- | --- | --- | --- |
| **Step** | **Reagent** | **Power** | **Time** | **TR** |
| 1 | 50 ml Coplin Jar, xylene | 165 W | 4 min |  |
| 2 | 50 ml Coplin Jar, 95-100% ETOH | 165 W | 1 min |  |
| 3 | Wash in DI water to clear |  | Bench step |  |
| 4 | 50 ml Coplin Jar, 0.5% Periodic Acid | 315 W | 2 min 30 sec | 60°C |
| 5 | Wash in DI water, 3 changes |  | Bench step |  |
| 6 | 50 ml Coplin Jar, Schiff Reagent [1] | 165 W | 2 min | 45°C |
| 7 | 50 ml Coplin Jar, Warm DI Water | 165 W | 4 min | 45°C |
| 8 | Wash in DI water |  | Bench step |  |
| 9 | 50 ml Coplin Jar, Gill#1 Hematoxylin | 165 W | 40 sec | 45°C |
| 10 | Wash in DI water |  | Bench step |  |
| 11 | Blue in Scott's water |  | Bench step, 1 min |  |
| 12 | Wash in DI water |  | Bench step |  |
| 13 | Dehydrate through ETOH's, clear and mount |  | Bench step |  |

[1] Use fresh reagent - Destain with 0.55% Potassium Metabisulfite if needed after step #6 (bench step) (see reference)

##### Detailed reagent prep information

|  |  |
| --- | --- |
| Gill #1 Hematoxylin | IMEB Cat# GL- |
| 0.5% periodic acid | * + 2.5 g periodic acid   + 500 ml deionized water |
| 2% potassium metabisulfite | * + 2.75 g potassium metabisulfite   + 500 ml deionized water |
| Schiff reagent | Richard Allen #88017 |

##### Comments

*For principle*

The reaction is based on oxidation of certain tissue elements to aldehydes by periodic acid. The most common reactive group is the 1,2 glycol group, but other groups are also selectively oxidized by periodate. Schiff reagent is prepared by treating parasaniline with sulfurous acid. Reduction causes the loss of the quinoid structure, and a colorless compound, referred to as leucofuschin, is formed. Following the Schiff reaction, washing in running water causes the loss of the bound sulfurous acid group attached at the central carbon atom, the restoration of the quinoid structure in the dye bound by the aldehyde, and the visualisation of the typical Schiff color. Metabisulfite rinses are used to remove excess Schiff reagent and prevent false colorization of the tissue elements due to oxidation of any adsorbed reagent. Microwaves cause most reactions and staining to proceed more quickly.

*Procedure notes*

* + Reid and Culling state that, contrary to the generally held assumption, Schiff staining after periodate oxidation does not necessarily mean that carbohydrate residues are absent. They conclude that the intensity of staining in the routine PAS reaction is due to a combination of the following factors:
    - The number of available 1:2 glycol groups
    - The reactivity of Schiff reagent with the reaction product
    - The structure of the polymer oxidized
    - The exact procedural reaction conditions
  + Sulfite rinses are considered essential to remove any uncombined leucofuchsin following exposure to the Schiff reagent. Highly chlorinated water is capable of oxidation, and if the sections are transferred directly to tap water, any loosely adsorbed Schiff reagent may be reoxidized to basic fushsin, which may then nonspecifically stain the section.
  + Washing in tap water is very important after the sulfite rinses, for color development.
  + Longer staining times may be necessary for thinner sections such as kidney biopsies or bone marrows.
  + Prolonging the staining time in the Schiff reagent can compensate for a weaker solution; however, it should be discarded when a pink color develops.
  + If the Schiff reagent is weak, reactivity can be restored by adding 1 ml of 12.5% sodium metabisulfite to the 50 ml Coplin jar of working Schiff reagent.
  + Test for Schiff reagent quality: place 10 ml of 37-40% formaldehyde in a beaker and add a few drops of Schiff reagent. If the solution turns rapidly reddish purple or magenta, it is good. If the reaction is delayed and the color is a deep blue-purple, the solution is breaking down and should be discarded.

##### Results

Glycogen, neutral mucosubstances, certain epithelial sulfomucin and sialmucins, colloid material of the thyroid and pars intermedia of the pituitary, basement membranes, and fungal walls - magenta or rose to red

Nuclei - blue

##### References

Carson, F.L., Histotechnology: A Self-Instructional Text, ASCP Press, 1990, pg 119

Sheehan and Hrapchak, Theory and Practice of Histotechnology, 2nd Edition, 1980.

H.R.A Cabral, I.T.C Novak, Z.M. Rabino and G.B. Robert, *A Simple Method to Restore Exhausted Schiff Reagent*, The Journal of Histotechnology 20:1(79-80) (1997)

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| **Masson's trichome stain for collagen and smooth muscle** |

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| --- | --- |
| Sample type | Animal |
| Application | Organisms |
| Specimen(s) | Collagen and smooth muscle. Routine paraffin, Bouin's processed, formalin or "prefer" fixed tissue sections. |
| Submitted by | [Rick Giberson](mailto:rick_giberson@tedpella.com), [Ted Pella, Inc.](http://www.tedpella.com) and Bruce Caukins, formerly with Pathology Sciences Medical Group, Chico, CA |
| Instrument used | BioWave™, Ted Pella, Inc. Microwave system with PELCO ColdSpot®, vacuum chamber, and variable wattage. |
| Sample size | 4-5 µm sections on slides. |
| Sample container | Microwave-safe plastic coplin jars (Thermo-Shandon#1001361) or other microwave safe staining containers should be used. Place sample container on PELCO ColdSpot®, and place temperature probe in sample container and set TR. |
| Control | Small intestine, uterus, appendix, or fallopian tube, fixed like specimen and cut at 4 microns. Run a control slide with each stain batch. If negative, repeat procedure (check expiration dates of reagents). |

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| **Step** | **Reagent** | **Power** | **Time** | **TR** |
| 1 | 50 ml Coplin Jar, xylene | 165 W | 4 min |  |
| 2 | 50 ml Coplin Jar, 95-100% ETOH | 165 W | 1 min |  |
| 3 | Wash in DI water to clear |  | Bench step |  |
| 4 | 50 ml Coplin Jar, 0.5% Bouin's Solution | 315 W | 6 min | 60°C |
| 5 | Wash in DI water to yellow |  | Bench step |  |
| 6 | 50 ml Coplin Jar, Gill #2 Hematoxylin | 315 W |  | 40°C |
| 7 | Wash in DI water to blue |  | 1 min 20 sec |  |
| 8 | 50 ml Coplin Jar, Biebrich Scarlet-Acid Fuchsin | 315 W | 40 sec | 40°C |
| 9 | Wash in DI water, 3 changes |  | Bench step |  |
| 10 | 50 ml Coplin Jar, Phosphotungstic - Phosphomolybdic Solution | 315 W | 1 min | 40°C |
| 11 | 50 ml Coplin Jar, Analine Blue Solution | 165 W | 40 sec | 40°C |
| 12 | Wash in DI water, 3 changes |  | Bench step |  |
| 13 | 50 ml Coplin Jar, 1% Acetic Acid Solution |  | Bench step, 30 sec |  |
| 14 | Dehydrate through ETOH's, clear and mount |  | Bench step |  |

##### Detailed reagent prep information

|  |  |
| --- | --- |
| Bouin's Solution | IMEB Cat#S129 |
| Gill #2 Hematoxylin | IMEB Cat# GL-232 |
| Biebrich scarlet-acid fuchsin, working solution | * + 45 ml of 1% aqueous biebrich scarlet   + 5 ml of 1% aqueous acid fuchsin   + 0.5 ml glacial acetic acid |
| Phosphomolybdic-phosphotungstic acid solution | * + 5 g phosphomolybdic acid   + 5 g phosphotungstic acid   + 200 ml deionized water |
| Aniline blue solution | * + 2.5 g aniline blue   + 2 ml acetic acid   + 100 ml deionized water |
| 1% acetic Acid Solution | * + 1 ml glacial acetic acid   + 99 ml dd-H2O |

##### Comments

*For principle*

To differentiate between collagen and smooth muscle in tumors, and to identify increases in collagenous tissue in diseases such as cirrhosis of the liver. Trichrome is so named because three dyes are used. Sections are first stained with acid dye such as Biebrich's scarlet; all acidophilic elements, such as cytoplasm, muscle, and collagen, will bind the acid dyes. The sections are then treated with phosphotungstic/phosphomolybdic acid. Because cytoplasm is much less permeable than collagen, phosphotungstic/phosphomolybdic acids cause the Biebrich scarlet to diffuse out of the collagen but not out of the cytoplasm. Phosphotungstic/phosphomolybdic acids have numerous acidic groups that most likely act as a link between the decolorized collagen and aniline blue, the collagen dye. Probably the pH of the phosphotungstic/phosphomolybdic acid solution also increases selective collagen staining and aids in the diffusion or removal of Biebrich scarlet. Microwaves cause most reactions and staining to proceed more quickly.

*For staining*

* + Use the longer staining times for thinner (3 micron) sections.
  + Decreased red staining usually indicates that the staining solution has aged or been overused and should be discarded.
  + If blue staining of the collagen appears faded, the section has probably been overdifferentiated in the acetic acid solution, or the phosphotungstic/phosphomolybdic acid solution should be changed.
  + Pathologically-altered collagen, such as that seen in burns, may lose its affinity for aniline blue and bind the acid dye instead.

##### Results

Nuclei - blue-black

Collagen - blue

Cytoplasm, keratin, muscle fibers, intercellar fibers - red

##### References

Carson, F.L., Histotechnology: A Self-Instructional Text, ASCP Press, 1990, pg 119

Sheehan and Hrapchak, Theory and Practice of Histotechnology, 2nd Edition