TECHNNOVIT® REAGENTS AND STAINS

Acquired from Kulzer Mitsui Chemicals Group, Technovit-Histology, Polymerization Systems for Histological Application. Also from Heraeus/Kulzer, Technovit 9100 Routine Staining, immune reactions, enzyme histochemistry, in-situ hybridization, 2004

HEMATOXYLIN-EOSIN

Staining process

1. Stain the sections in hematoxylin in accordance with Gill* (filter the dye solution)	15 min
2. Blue in tap water	10 min.
3. Rinse in distilled water	
4. Counterstain sections with Eosin	2–5 min.
5. Dehydrate through ethanol 96% and 100%	
6. Clarify with xylene and coverslip with a quick drying acrylic mounting media	

Result

Nucleus	blue
Basophilic cytoplasm	blue
Acidophilic cytoplasm	pink
Muscle tissue	pink
Connective tissue	pink

Solutions

Hematoxylin in accordance with Gill:	
Hematoxylin (C.I. 75290).	6 g
Sodium iodate	0.6 g
Aluminum sulfate	52.8 g
Distilled water.	690 ml
Ethylene glycol.	250 ml
Glacial acetic acid.	60 ml
Eosin:	
Eosin Y-(alcoholic) C.I. 45380	0.5 g
Ethanol 96 %	100 ml
Glacial acetic acid	2 drops

**After staining with hematoxylin (1) the plastic matrix can be decolorized with 0.5 ml of HCL (36%) in ethanol 70%; briefly submerse and then quickly process in tap water (2).*

TED PELLA, INC.
Microscopy Products for Science and Industry
P.O. Box 492477, Redding, CA 96049-2477, U.S.A
Telephone: 530-243-2200; 800-237-3526 (U.S.A. or Canada) • FAX: 530-243-3761
Email: sales@tedpella.com • Web Site: http://www.tedpella.com

PERIODIC ACID SCHIFF (PAS)

Staining process

1. 0.4% periodic acid	30 min, 56°C
2. Rinse in tap water	
3. Rinse in distilled water	3 times
4. Schiff's reagent	15 min
5. Rinse thoroughly in tap water	
6. Rinse in distilled water.	
7. Counterstain sections with hematoxylin in accordance with Gill*	10 min
8. Blue in tap water	10 min
9. Dehydrate, clarify with xylene and coverslip with a quick drying acrylic mounting media	

Note: To avoid a specific pink sheen, rinse with sulfite water instead of tap water (5), see also Feulgen.
**Gill's hematoxylin: see Hematoxylin-Eosin*

Result

Nucleus	blue
Glycogen	violet / red
Basement membranes	violet / red
Mucin	violet / red

Solutions

Schiff's reagent:	
Solution 1: Pararosaniline (C.I. 42500) 1 N hydrochloric acid	0.5 g 15 ml
Solution 2: Potassium metabisulphite ($K_2S_20_5$) Distilled water.	0.5 g 85 ml

Mix solution 2, solution 1. After 24 hours (in the dark) the light brown solution is decolorized with 200 mg of bone black (approx. 2 min.) and subsequently filtered.

Store the colorless reagent (leucofuchsin) in the refrigerator.

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FEULGEN

Staining process

1. Hydrolize in hydrochloric acid 5 N	20 minutes/RT
2. Rinse in distilled water	3 times
3. Schiff's reagent	15 minutes
4. Sodium hydrogen sulfite 0.5%	3 times, 2 minutes
5. Rinse thoroughly with tap water	
6. Dehydrate, clarify with xylene and coverslip with a quick drying acrylic mounting media	

Result

DNS	violet / red
Other tissue elements	colorless

Solutions

Schiff's reagent	
Hydrochloric acid 5 N	
Fill up with 42 ml of hydrochloric acid 36% up to 100 ml Natrium hydrogen sulfite	
NaHSO3	0.5 g
Distilled water	100 ml

GIEMSA

Staining Process

1. Stain sections in the Giemsa solution (20%) (Giemsa Merck: Dilute 1:5 with distilled water.)	1.5 hrs. RT
2. Briefly in acetic acid solution: (4 drops to 100 ml of distilled water)	2 seconds
3. Submerge in alcohol 96%	
4. Submerge in alcohol 96%	
5. Isopropanol	3 times, 2 min.
3 Clarify with xylene and cover in Malinol	

Results

Nucleus	violet
Cytoplasm	blue
Erythrocytes	pink

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PRUSSIAN BLUE REACTION IN ACCORDANCE WITH PERLS

Staining process

1. Potassium ferrocyanide First warm up the solution to 60°C and then filter filtrates	15 min
2. Rinse in distilled water	
3. Safranin O. 0.2%	2-5 min
4. Rinse in acetic acid 1%	
5. Dehydrate, clarify with xylene and coverslip with a quick drying acrylic mounting media	

Results

Nucleus	red
Hemosiderin	blue / green

Solutions

Potassium ferrocyanide solution:	
Potassium ferrocyanide	1 g
Distilled Water	50 ml
Hydrochloric acid 2 %	50 ml

Safranin-Solution:	
Safranin O. (C.I. 50240)	0.2 g
Acetic acid 1 %	100 ml

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PERIODIC ACID METHENAMINE SILVERC (PAMS) ACCORDING TO JONES

Note: It is recommended to stick on the plastic sections with Mayer's albumin.

Staining process

1. Periodic acid 1 %	30 min
2. Rinse in distilled water	3 times
3. Methenamine silver solution	60 min, 60 °C
4. Rinse in distilled water, microscopic test. Sections that have been too weakly stained again in 3	
5. If the sections refuse to dissolve despite pre-treatment, dry them on a hotplate at 60°C in accordance with Point 4.	
6. Gold chloride 0.2%	1-2 min
7. Rinse in distilled water	
8. Sodium thiosulfate 2%	5 min
6 Rinse in tap water	
7 If necessary, counterstain with HE or Safranine O	
8 Dehydrate, clarify with xylene and coverslip with a quick drying acrylic mounting media	

Result

Basement membranes	brown / black

Solutions

Methenamine silver stock solution:	
a) Hexamethylenetetramine 3 %	100 ml
b) Silver nitrate 5%	5 ml
a) and b) can be stored separately	
Methenamine silver stain solution:	
Stock solution	50 ml
Borax 5%	5 ml
Periodic acid 1% (Sigma No. P 7875)	
Gold chloride 0.2%	
Sodium thiosulphate solution 2% (Na2S2O3.5H20)	

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DETERMINING ENZYME ACTIVITY

Determination of the enzyme activity in tissues that are embedded in 2 hydroxyethyl methacrylate (GMA) - in particular Technovit® 7100

Freshly removed tissue is fixated in 4% neutral formaldehyde at 4°C for two hours (immersion). If perfusion fixations are made, very brief fixation times can be adhered to and the enzyme activity is better maintained.

Rinsing fluid

0.1 M cacodylate buffer pH 7.4; the material can possibly be left overnight at 4°C.

Dehydration

1. Alcohol 70 % Acetone 70 %, 30 min. at 4 °C
2. Alcohol 96 % acetone 96 %, 30 min. at 4 °C
3. Alcohol 100 % acetone 100 %, 30 min. at 4 °C

Pre-infiltration

Alcohol 100 % Technovit® 7100 1:1, 2 hrs. at 4 °C
or
Acetone 100 % Technovit® 7100 1:1, 2 hrs. at 4 °C

Infiltration

Technovit® 7100, 12 hrs. at 4°C

Polymerization

15 parts Technovit® 7100 (solution A)
1 part Technovit® 7100 hardener II, at 4°C

The tissue can be embedded in Histoforms S or Q, or in the Sorvall embedding system. Because polymerization starts at 4°C, it will occur slower than at room temperature. A polymerization time of 12 hours at 4°C must be adhered to ensure polymerization.

The 2-µ sections are also dried at room temperature with section floating on a drop of distilled water. Enzyme actions can be made without removing the plastic matrix.

Note: It is difficult to detect dehydrogenases.

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ALKALINE PHOSPHATASE IN ACCORDANCE WITH BURSTONE

Staining process

1. Incubate the plastic sections in the incubation medium. Note: In many cases a 2-hour incubation period is sufficient.	1-3 hrs
2. Rinse in distilled water	2 min
3. Counterstain the sections with nuclear fast red	5-10 min
4. Rinse in distilled water	
5. Air dry	
6. Cover in malinol	

Result

Nucleus	red
Enzyme activity area	blue

Note: In this reaction, the choice of medium used to cover the material is significant because crystals formation may occur in the reaction product.

Solutions

Buffer solution	
0.2 M tris-(hydroxymethyl)-aminomethane	2.4 g
Distilled water	100 ml
Set the pH value to 8.9 with diluted HCL and store the buffer at 4°C.	
Incubation medium	
Naphtol AS-MX phosphate, disodium salt (Sigma)	5 g
N,N dimethylformamide	0.25 ml
After dissolving, add:	
Distilled water	25 ml
Buffer solution (pH 8.9)	25 ml
$MgSO_4 \cdot 7H_2O$ 10 %	2 drops
Fast Blue BB (Sigma)	30 mg

Shake well and then filter before using.
Note: Always freshly prepare the incubation medium.

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ATP-ASE (WACHSTEIN AND MEISEL)

Staining process

1. Incubate the plastic sections in the incubation medium (filter before using) Note: In many cases a 2-hour incubation period is sufficient.	1-3 hrs, 37°C
2. Rinse in distilled water	2 min
3. Sodium sulfide solution	30 sec
4. Rinse in distilled water	
5. Counterstain the sections with nuclear fast red	5-10 min
6. Rinse in distilled water	
7. Air dry	
8. Coverslip with a quick drying acrylic mounting media or malinol	

Result

Nucleus	red
Enzyme activity area	brown

Solutions

1. Tris maleic acid buffer pH 7.2 solution A Maleic acid:	29g
Tris-(hydroxymethyl)-aminomethane	30.3g
Distilled water	500 ml
Add 2 g of activated carbon, shake for ten minutes and filter. Then add 40 ml of the stock solution A, 20 ml 1N NaOH, and fill with distilled water up to 100 ml (pH 7.2).	

2. Lead nitrate solution:	
Lead nitrate	2g
Distilled water	100 ml

3. Magnesium sulphate solution:	
$MgSO_4 \cdot 7H_2O$	1.2g
Distilled water.	100 ml

Incubation medium:	
Distilled water	22 ml
Disodium adenosine-5-triphosphate (Boehringer, Mannheim)	25 mg
Tris maleic acid buffer pH 7.2	20 ml
Magnesium sulfate solution	5 ml
Lead nitrate solution (add by drops, heat to 42°C and filter)	3 ml

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Sulfide Solution:	
Sodium sulfide	2g
Distilled water	100 ml
Adjust the pH value to 7.0-7.5 with 1 N of HCL (verify with pH paper).	

Technovit 9100 Routine Staining, immune reactions, enzyme histochemistry, in-situ hybridization

Heraeus/Kulzer, 2004

The following staining and detection reactions are only important examples of the processing hard-cut sections. They also apply to MMA thin sections.
Reagents, antibodies, probes, detection systems are variable.

Routine Staining

Counterstaining Sections for Immunohistochemistry and Enzyme Histochemistry	
Hematoxylin n. Mayer† flowing water in tap water transfer to distilled water.	30 sec / RT 10min / RT
Rinse nuclear fast red in distilled water.	10 min / RT
Methyl green (cleaned; see Romeis) Rinse with distilled water.	10-20 min / RT

HE staining	
Same as staining paraffin sections	

Giemsa Staining	
Deacrylate sections	
Giemsa sol. (Mix fresh!)	30-40 min./RT
Differentiate and dehydrate	
• Acetone / xylol (95:5)	
• Acetone / xylol (70:30)	
• Acetone / xylol (30:70)	
• Xylol	

† *Kiernan*

Masson Goldner Staining	
Deacrylate sections	
Haemalaun (Mayer†)	10 min / RT
Tap water	
Ponceau acid magenta azophloxin	45 min / RT
1% acetic acid	
Phosphomolybdic acid/ Orange G	7 min / RT
1% acetic acid	
Light green	40 min / RT
1% acetic acid	
Ascending alcohol series	
Xylol	
Coverslip with a quick drying acrylic mounting media	

Performing the immune reaction

Antibody Incubation	
Rinse the section with 0.01mol/l phosphate buffer, pH 7.4	
Primary antibody **or** diluted in DAKO-antibody diluent	16 h / 4 °C 30 - 45 min / RT
Rinse with buffer (see above)	
DAKO EnVision polyvalent antibody (goat-anti-mouse/goat-anti-rabbit) coupled to alkaline phosphatase	30 min / RT
Visualization	
Rinse with buffer	
Chromogenic substrate solution:	
Fast Red	15 - 20 min / RT
Counterstain with hematoxylin according to Mayer†	

† *Kiernan*

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Enzyme histochemical staining

With Acid and Alkaline Phosphatase	
Rinse sections with 0.1 mol/l Tris buffer, pH 9.4	10 min / RT
Incubate in substrate solution 0.1 mol/l Tris buffer pH 9.4 Fast Blue Naphthol-AS-BI-phosphate	2 hr / 37 °C
Rinse with distilled water	
Rinse in 0.1 mol/l acetate buffer, pH 5.6	10 min / RT
Incubate in substrate solution Hexonium-Pararosaniline solution Naphthol-AS-Bl-phospate	1 h / 37 °C
Rinse with distilled water	
Fix in 40% formalin	2 - 3 hr / RT
Rinse with tap water	
Counterstain with Methyl Green	
With Esterase Reaction using Naphthol-AS-D-chloracetate	
Rinse sections with 0.01 mol/l phospate buffer, pH 7.4	5 min / RT
Incubate in substrate solution 0.1 mol/l phosphate buffer, pH 6.5 Naphthol-AS-D-chloracetate Hexonium-Pararosaniline solution	1 hr / RT
Rinse with distilled water	
Counterstain with Hematoxylin according to Mayer	

† *Kiernan*

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REAGENTS

Buffers and Stock Solutions

SODIUM ACETATE STOCK SOLUTION – 2 mol/l.
74.13 g sodium acetate
5.5 ml glacial acetic acid
make up to 500 ml with distilled water.

SODIUM ACETATE BUFFER – 0.1 mol/l, pH 5.6
50 ml stock solution (see above)
950 ml distilled water adjust pH to 5.6 with either
sodium hydroxide (pH too low) or acetic acid (pH too high)

PHOSPHATE STOCK SOLUTION – 1 mol/l
112.5 g disodium hydrogen phosphate
30 g potassium dihydrogen phosphate
make up to 1 liter with distilled water.

PHOSPHATE BUFFER – 0.01 mol/l, pH 7.4
10 ml phosphate stock solution (see above)
980 ml distilled water adjust to pH 7.4 with o-phosphoric
acid or sodium hydroxide
make up to 1 liter with distilled water

0.04 mol/l PHOSPHATE BUFFERED 10% SUCROSE – pH 7.4
40 ml phosphate stock solution (see above)
100 g sucrose
1g sodium azide (e.g. 10 ml 10% NaN3-solution)
850 ml distilled water adjust pH to 7.4 (see above)
and make up to 1 liter with distilled water.

TRIS STOCK SOLUTION – 1 mol/l
121.4 g Tris(hydroxymethyl)aminomethane (Tris)
make up to 1 liter with distilled water.

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TRIS BUFFER – 0.1 mol/l , pH 9.4
100 ml Tris stock solution (see above)
850 ml distilled water adjust pH to 9.4 with hydrochloric acid and make up to 1 liter with distilled water.
Stock solutions are best stored in the dark in stoppered brown glass bottles to prevent microbial growth. Diluted buffers can be stored at 4 °C, stock solutions at room temperature.
Fixative Solutions.

BUFFERED FORMALIN SOLUTION (4%)
100 ml 37% formaldehyde (formalin)
4.5 g potassium dihydrogen phosphate
6.5 g disodium hydrogen phosphate
850 ml distilled water.
Adjust the pH to 7.0 with sodium hydroxide or o-phosphoric acid and make up to 1 liter with distilled water.

PARAFORMALDEHYDE STOCK SOLUTION – 8%
40 g paraformaldehyde
make up to 500 ml with distilled water

PARAFORMALDEHYDE SOLUTION – 1.4%
35 ml paraformaldehyde stock solution (see above)
65 ml distilled water
100 ml 0.04 mol/l phosphate buffered 10% sucrose, pH 7.4 (see above)
Reaction Mixtures

FAST RED SOLUTION
3 ml substrate solution
1 Fast Red tablet
120 µl Levamisole
Mix components in a 5 ml stoppered polystyrene or polyethylene test tube. The solution can be then used for approximately 60 min.

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ALKALINE PHOSPHATASE SUBSTRATE / REACTION MIXTURE
50 ml Tris buffer – 0.1 mol/l, pH 9.4
50 ml Fast Blue Solution
25 mg Naphthol-AS-BI-phosphate dissolved in 0.5 ml dimethyl sulphoxide (DMSO) / Triton X-100

ACID PHOSPHATASE SUBSTRATE / REACTION MIXTURE
50 ml acetate buffer – 0.1 mol/l, pH 5.6
500 µl Hexonium-Pararosaniline (250 µl Pararosaniline (C.I. 42500) in 2 mol/l hydrochloric acid + 250 µl 4% sodium nitrite in distilled water – Vortex and allow to react for 5 min before use)
25 mg Naphthol-AS-BI-phosphate in DMSO / Triton X-100 (see above)

NON-SPECIFIC ESTERASE SUBSTRATE / REACTION MIXTURE
50 ml phosphate buffer – 0.1 mol/l, pH 6.5
15 mg Naphthol AS-D-chloroacetate in DMSO / Triton X-100 (see above)
250 µl hexonium-pararosaniline (see above)

Staining Solutions
GIEMSA SOLUTION
3 ml Giemsa stock solution (Merck)
97 ml distilled water
1 –2 drops of glacial acetic acid.

LIGHT GREEN
1 g Light Green SF Yellowish
2 ml glacial acetic acid
Make up to 1000 ml with distilled water.

PHOSPHOMOLYBDIC ACID – ORANGE-G
30 g phosphomolybdic acid
20 g Orange-G
Make up to 500 ml with distilled water.
– add both solutions together
– filter

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PONCEAU-S – FUCHSIN – AZOPHLOXIN
100 ml Masson´s solution
20 ml Azophloxin solution
880 ml 0.2 % acetic acid
For Masson´s solution mix 1 part of Masson´s solution A with
2 parts of Masson´s solution B.
Masson´s Solution A: 1 g acid fuchsin
(fuchsin-S, acid magenta)
made up to 100 ml with distilled water
heat to boiling
add 1 ml glacial acetic acid
and filter.
Masson´s Solution B: 2g Xylidine Ponceau
(Ponceau 2R – C.I. 16150)
made up to 200 ml with distilled water
heat to boiling
add 2 ml glacial acetic acid
and filter.

AZOPHLOXIN SOLUTION
0.5 g azophloxin
made up to 100 ml with distilled water
and add 2 ml glacial acetic acid.

SOURCE DOCUMENTS:

Kulzer Mitsui Chemicals Group, *Technovit-Histology, Polymerization Systems for Histological Application*,
Kulzer GmbH, Division Technique Philipp-Reis-Straße 8/13, 61273 Wehrheim
Tel. +49 (0)6181.9689-2574 Fax +49 (0)6181.9689-3864
technik.wehrheim@kulzer-dental.com, www.technovit.de

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† "Histological & Histochemical Methods" Theory and Practice, 4th Edition, J.A Kiernan, page 150