
INTENDED USE

Sigma Diagnostics Trichrome Stains (Masson) are intended for use in the study of connective tissue, muscle and collagen fibers.

**BACKGROUND AND
PRINCIPLE OF TEST**

"Trichrome" stains are used primarily for distinguishing collagen from muscle tissue.¹ In general, they consist of nuclear, collagenous and cytoplasmic dyes in mordants such as phosphotungstic or phosphomolybdic acid. Historically, the first trichrome system was attributed to Mallory.^{2,3} Further modifications were introduced by Masson and Gomori.^{3,4} The procedure described here is based on the work of Masson as modified by Lillie⁵ using aniline blue as a collagen stain instead of a green dye. Tissue sections are treated with Bouin's solution to intensify the final coloration. Nuclei are stained with Weigert's iron hematoxylin, and cytoplasm¹ and muscle are then stained with Beibrich scarlet-acid fuchsin. After treatment with phosphotungstic and phosphomolybdic acid, collagen is demonstrated by staining with aniline blue. Rinsing in acetic acid after staining renders the shades of color more delicate and transparent.⁴ Included is a trichrome stain technique for rapid staining in microwave ovens.⁶⁻⁹

REAGENTS

BIEBRICH SCARLET-ACID FUCHSIN SOLUTION, Catalog No. HT15-1
Biebrich scarlet, 0.9%, acid fuchsin 0.1%, in acetic acid, 1.0%.

PHOSPHOTUNGSTIC ACID SOLUTION, Catalog No. HT15-2
Phosphotungstic acid, 10%.

PHOSPHOMOLYBDIC ACID SOLUTION, Catalog No. HT15-3
Phosphomolybdic Acid, 10%

ANILINE BLUE SOLUTION, Catalog no. HT15-4
Aniline blue, 2.4% and acetic acid, 2%.

PRECAUTIONS:

Trichrome Stain reagents are for "In Vitro Diagnostic Use". Normal precautions exercised in handling laboratory reagents should be followed. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Take off immediately all contaminated clothing. Wear suitable protective clothing, gloves and eye/face protection. Dispose of waste observing all local, state and federal laws.

CAUTION: Avoid contact and inhalation of Biebrich Scarlet-Acid Fuchsin. Phosphotungstic Acid and Phosphomolybdic Acid are **CORROSIVE**. Causes burns.

Aniline Blue Solution is an **IRRITANT**. Irritating to eyes, respiratory system and skin.

Refer to Material Safety Data Sheets for any updated risk, hazard or safety information.

PREPARATION:

Prepare **WORKING PHOSPHOTUNGSTIC/PHOSPHOMOLYBDIC ACID SOLUTION** by mixing 1 volume of Phosphotungstic Acid Solution, Catalog No. HT15-2, and 1 volume Phosphomolybdic Acid Solution, Catalog No. HT15-3, with 2 volumes of deionized water. Discard after one use.

STORAGE AND STABILITY:

Store reagents at room temperature (18–26°C). Reagent labels bear expiration dates. Formation of a precipitate in Phosphomolybdic Acid Solution, Catalog No. HT15-3, does not affect performance.

SPECIMEN COLLECTION

It is recommended that specimen collection be carried out in accordance with NCCLS document M29-A. No known test method can offer complete assurance that human blood samples will not transmit infection. Therefore, all blood derivatives should be considered potentially infectious.

Any well fixed paraffin section cut at 5–6 microns may be used. Incorporate appropriate control slides.

PROCEDURE

MATERIALS PROVIDED:

Trichrome Stains (Masson), Catalog No. HT15

REAGENTS/EQUIPMENT REQUIRED BUT NOT PROVIDED:

Bouin's Solution, Catalog No. HT10-1

Weigert's Iron Hematoxylin Set, Catalog No. HT10-79. Prepare working solution according to label instructions

Acetic Acid, 1% Prepare by diluting 1 part Acetic Acid, Catalog No. 545-7, with 9 parts water

Microscope

Microscope slides, coverslips and staining dishes

FOR MICROWAVE PROCEDURE ONLY:

Hematoxylin Solution, Gill No. 3, Catalog No. GHS-3

Scott's Tap Water Substitute Concentrate, Catalog No. S 5134. Prepare working solution according to label instructions

Coplin Jar (plastic) with vented lids, Catalog No. C 8338

ACCUMATE™ H2100 Microwave Oven, Catalog Nos. A 9084 (110 v) or A 9209 (220 v)

STANDARD PROCEDURE:

1. Deparaffinize slides to deionized water.
2. Mordant in preheated Bouin's Solution, Catalog No. HT10-1, at 56°C for **15 minutes** or at **room temperature overnight**.
3. Cool slides in tap water (**18–26°C**) contained in a Coplin jar.
4. Wash in running tap water to remove yellow color from sections.
5. Stain in Working Weigert's Iron Hematoxylin Solution for **5 minutes**.
6. Wash in running tap water for **5 minutes**.
7. Rinse in deionized water.
8. Stain in Biebrich Scarlet-Acid Fuchsin, Catalog No. HT15-1, for **5 minutes**.
9. Rinse in deionized water.
10. Place slides in Working Phosphotungstic/Phosphomolybdic Acid Solution for **5 minutes**.
11. Place slides in Aniline Blue Solution, Catalog No. HT15-4, for **5 minutes**.
12. Place slides in Acetic Acid, 1%, for **2 minutes**. Discard solution.
13. Rinse slides, dehydrate through alcohol, clear in xylene and mount.

ACCUMATE™ H2100 MICROWAVE PROCEDURE:

1. Deparaffinize slides and hydrate to deionized water.
2. Place slides in **40 mL** of Bouin's Solution contained in a plastic Coplin jar. Loosely cover Coplin jars with lid before placing in microwave oven or use Coplin jars with holes drilled into the lids.
3. Microwave on **600 watts** for **25 seconds**. Gently mix solution with beral pipet or applicator stick, incubate slides in heated Bouin's Solution for **5 minutes** in a fume hood or well ventilated area.
4. Rinse slides in running tap water until yellow color disappears.
5. Place slides in **40 mL** Hematoxylin Solution, Gill No. 3, contained in plastic Coplin jar.
6. Microwave on **800 watts** for **5 seconds**.
7. Rinse well in running tap water for **30 seconds** to **1 minute**.
8. Blue in Working Scott's Tap Water Substitute at **room temperature**.
9. Rinse well in running tap water.
10. Place slides in **40 mL** Biebrich Scarlet-Acid Fuchsin Solution contained in plastic Coplin jar.
11. Microwave on **600 watts** for **20 seconds**. Mix gently with beral pipet or applicator stick. Let incubate for **2 minutes**.
12. Rinse quickly in several changes of deionized water.
13. Place slides in **40 mL** Phosphotungstic-Phosphomolybdic Acid Solution contained in plastic Coplin jar.
14. Microwave on **600 watts** for **20 seconds**. Immediately remove slides and rinse in several changes of deionized water.
15. Place slides in **40 mL** Aniline Blue Solution contained in a plastic Coplin jar.
16. Microwave on **600 watts** for **15 seconds**. Mix gently with a beral pipet or applicator stick. Let incubate for **1 minute**.
17. Rinse well in deionized water.
18. Place slides in 1% Acetic Acid for **30 seconds** to **1 minute** at **room temperature**.
19. Rinse slides, dehydrate through alcohol, clear and coverslip.

MASSON TRICHROME STAINING

(Michelle's protocol, 9/01)

REAGENTS NEEDED:

Sigma Accustain Trichrome Stain Kit (Catalog #HT15) contains:

Biebrich Scarlet-Acid Fuchsin Solution (# HT15-1, 0.9% biebrich scarlet, 0.1% acid fuchsin, 1% acetic acid),

Phosphotungstic Acid Solution (#HT15-2, 10% phosphotungstic acid),

Phosphomolybdic Acid Solution (#HT15-3, 10% phosphomolybdic acid), and

Aniline Blue Solution (#HT15-4, 2.4% aniline blue, 2% acetic acid)

Bouin's Solution (Sigma Catalog #HT10132-1L or HT101128-4L)

Weigert's Iron Hematoxylin Set (Sigma catalog #HT10-79)

1. Deparaffinize slides and rehydrate sections:

3 x 3'	Xylene (<i>blot excess xylene before going into ethanol</i>)
3 x 3'	100% ethanol
1 x 3'	95% ethanol
1 x 3'	80% ethanol
1 x 5'	deionized H ₂ O

2. Mordant in Bouin's Solution at room temperature overnight in a hood. Be careful, Bouin's solution is hazardous and the picric acid, when in less than 10% water, is very explosive. Used Bouin's solution should be placed in an appropriate waste container.

** Bouin's Solution intensifies the final coloration of the tissue.

3. Wash slides in running tap water to remove yellow color from sections. Rinse briefly in distilled water.

4. Stain in Working Weigert's Iron Hematoxylin Solution for 5 minutes. Make Hematoxylin Solution fresh by adding equal volumes of Solution A (1% Hematoxylin in 95% EtOH) and Solution B (1.2% Ferric Chloride and 1% Acetic Acid in distilled water). The working solution is good for approximately 10 days.

** Hematoxylin stains nuclei blue-black.

5. Wash in running tap water for 5 minutes. Rinse in deionized water.

6. Stain in Biebrich Scarlet-Acid Fuchsin for 5 minutes.

Decreased red staining usually indicates that the staining solution has aged or been overused and should be discarded.

** Beibrich scarlet-acid fuchsin stains cytoplasm and muscle red.

7. Rinse in deionized/distilled water.

8. Place the slides in Phosphomolybdic/Phosphotungstic Acid Solution for 5-10 minutes. Freshly prepare Working Phosphotungstic/Phosphomolybdic Acid Solution by mixing 1 volume of Phosphotungstic Acid Solution and 1 volume of Phosphomolybdic Acid Solution with 2 volumes of distilled water. Discard after one use. Formation of a precipitate in Phosphomolybdic Acid Solution does not affect performance.

** This allows for uptake of the aniline blue stain.

9. Stain sections in Aniline Blue Solution for 5 minutes.

**Aniline blue stains collagen blue.

10. Rinse slides briefly in distilled water.

11. Place slides in 1% acetic acid solution for 3-5 minutes. Discard this solution.

** Rinsing in acetic acid after staining renders the shades of color more delicate and transparent.

** If blue staining of connective tissue appears faded, the section has probably been overdifferentiated in the acetic acid solution.

12. Dehydrate to xylene.

2 x 3'

95% ethanol

2 x 3'

100% ethanol (*blot excess ethanol before going into xylene*)

3 x 5'

Xylene

13. Leave slides in xylene overnight to get good clearing of the ethanol.

14. Coverslip slides using Permount or Polymount (xylene based).

•Place a drop of Permount on the slide using the glass rod, taking care to leave no bubbles.

(don't stir the Permount with the rod too much and make sure that xylenes still cover the slide)

•Angle the coverslip and let fall gently onto the slide. Allow the Permount to spread beneath the coverslip, covering all the tissue.

•Dry overnight in the hood or at 37°C.

Representative Images:

