IFU

Instructions for Use

HARDY DIAGNOSTICS
A Culture of Service™

TRICHROME STAIN KIT AND REAGENTS

Cat. no. VXF019	Trichrome Starter Kit	8 reagents; 10 Coplin jars/kit
Cat. no. VXR010	Trichrome Starter Kit Refill	8 reagents/kit
Cat. no. T016	Trichrome Stain	One 16oz. bottle/package
Cat. no. MT016	Trichrome Blue Stain, Modified	One 16oz. bottle/package
Cat. no. VXM016	Schaudinn's Fixative, Modified	One 16oz. bottle/package
Cat. no. VXM032	Schaudinn's Fixative, Modified	One 32oz. bottle/package
Cat. no. VXI016	Iodine Alcohol	One 16oz. bottle/package
Cat. no. VXI128	Iodine Alcohol	One gallon/box
Cat. no. VXB032	Ethanol 70%	One 32oz. bottle/package
Cat. no. 3616A128	Ethanol 70%	One gallon/box
Cat. no. VXC032	Ethanol 95%	One 32oz. bottle/package
Cat. no. 3719A128	Ethanol 95%	One gallon/box
Cat. no. 3720A32	Acid Alcohol 90%	One 32oz. bottle/package
Cat. no. VXZ016	Carbol Xylene	One 16oz. bottle/package
Cat. no. 134B16	Xylene	One 16oz. bottle/package
Cat. no. 134B128	Xylene	One gallon/box

INTENDED USE

Hardy Diagnostics Trichrome Stain products are used in the differentiation and staining of intestinal parasites. These products may also be used as a counterstain in histological sections when performing the Gomori procedure.

SUMMARY

Masson, in 1929, introduced the first trichrome stain. It provided a fast yet easy means to stain tissue samples, as compared to the procedures in use at the time. A single solution trichrome stain was developed in 1949 by Gomori. It was stated to be a counterstain for cytological smears and hematoxylin stained tissue sections. In 1951, Wheatley stained *E. histolytica* with chromotrope 2R, and thus developed the Wheatley methodology for fecal and other intestinal tract specimens. This procedure was a modification of traditional fixation and dehydration steps combined with a trichrome stain, and resulted in a staining method that stains flagellates and amebae easily and quickly. The Trichrome Stain Kit is recommended for use according to the Gomori procedure for tissue samples and the Wheatley procedure for parasites.[1-8] Hardy Diagnostics Modified Trichrome Blue Stain is recommended for the detection of the microsporidia from fresh or preserved stool specimens.

In the Gomori procedure, paraffin imbedded specimens are sectioned, deparaffinized, and dehydrated with distilled water. Specimens are then stained with hematoxylin and counterstained with the Trichrome Stain, and mounted, after which they may be stored indefinitely.[5,6]

The Wheatley procedure requires fresh or fixed fecal smears. The slide is passed through several alcohol solutions, and stained to provide a permanent slide for the observation and identification of parasites. Once mounted, these slides may be stored indefinitely.[3,5-8]

The Modified Trichrome Blue Stain is used in the Ryan modified trichrome stain procedure, which is designed specifically to detect microsporidia. As penetration of the microsporidial spore is difficult, the dye content and the staining time are increased in the Modified Trichrome Blue Stain protocol.[3,5-8]

REAGENT FORMULA

	CAS No.	PRECAUTIONS
Trichrome Stain		May be harmful if ingested.
Chromotrope 2R	4197-07-3	
Light Green SF	5141-20-8	
Fast Green FCF	2353-45-9	
Phosphotungstic Acid	12501-23-4	
Acetic Acid - Glacial	64-19-7	
Deionized Water		

	CAS No.	PRECAUTIONS
Trichrome Blue Stain, Modified		May be harmful if ingested.
Chromotrope 2R	4197-07-3	
Aniline Blue	66687-07-8	
Phosphotungstic Acid	12501-23-4	
Acetic Acid - Glacial	64-19-7	
Deionized Water		

	CAS No.	PRECAUTIONS
Schaudinn's Solution		**HIGHLY TOXIC!**
Mercuric Chloride 7	487-94-7	Corrosive liquid. Contains mercury.

		Wash thoroughly after handling. May be fatal if ingested!
Isopropyl Alcohol	67-63-0	
Deionized Water		

	CAS No.	PRECAUTIONS
Iodine Alcohol		**FLAMMABLE!** Warning: Avoid sources of heat, open flames, or sparks. Cannot be made non-poisonous.
Alcohol Diluent, Ethanol	67-14-5	
Iodine Concentrate		
Iodine Crystals	7553-56-2	
Ethanol	67-14-5	

	CAS No.	PRECAUTIONS
Ethanol Solutions		**Flammable Liquids**
Ethanol, 70%	67-14-5	
Ethanol, 95%	67-14-5	

	CAS No.	PRECAUTIONS
Acid Alcohol Solution		**Flammable Liquids**
Ethanol, 90%	67-14-5	
Acetic Acid	64-19-7	

	CAS No.	PRECAUTIONS
Carbol Xylene Solution		**Flammable Liquids and Poisonous** Avoid open flames, sparks.
Phenol	108-95-2	
Xylene	1330-20-7	

	CAS No.	PRECAUTIONS
Xylene Solution		**Poisonous, Caustic, and Flammable**
Xylene	1330-20-7	

	CAS No.	PRECAUTIONS
PVA Fixative		
Polyvinyl Alcohol		
Mercuric Chloride, Sat. Sol.		
Ethanol, Absolute		
Glycerol		
Glacial Acetic Acid		

STORAGE AND SHELF LIFE

Storage: Upon receipt, store at 15-30ºC. away from direct light. Product should not be used if there are any signs of deterioration, discoloration, contamination, or if the expiration date has passed.

The expiration dating 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 quality control incubation times.

Refer to the document "Storage" for more information.

PRECAUTIONS

This product may contain components of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not guarantee the absence of transmissible pathogenic agents. Therefore, it is recommended that these products be treated as potentially infectious, and handle observing the usual universal blood 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.

Warning: Solutions contained within this kit are hazardous in nature. Wear appropriate safety apparel when working with the solutions contained within this kit.

LIMITATIONS

If a fixed specimen is used, slides must be completely dry before staining. Air dry only - do not heat, as heating of the slide may cause the organisms to distort. An exception to this is Schaudinn's fixed slides. These slides should not be allowed to dry before staining.

After immersing the slide in fixative and/or Schaudinn's Solution, the slide is immersed in alcohol-iodine to remove the mercuric chloride within these solutions. If dark crystalline artifacts occur on the slide, the alcohol-iodine solution has become saturated. Change this solution often; it should be a strong tea color.

Unsatisfactory staining of the nucleus and/or cytoplasm may be due to incomplete fixation of the smear, when improper emulsification of the specimen in the fixative occurs.

To ensure proper staining characteristics of paraffin smears or sections, proper technique in hydration and dehydration must be carried out.

PROCEDURE

Specimen Collection:
This product is used in conjunction with other biochemical tests to identify cultures of isolated organism. Consult listed

references for information on specimen collection.[3-8]

Reagent Preparation when using the Trichrome Stain Kit:
All reagents in the Trichrome Stain Kit are provided ready to use, except as noted below.

1. Schaudinn's Solution: Add 1/10 volume of acetic acid to 9/10 volume Schaudinn's Solution (a 1:10 dilution) on the day of use. Stable for one day.

2. Iodine Alcohol: Mix iodine concentrate (small bottle) with the diluent (large bottle) prior to use. Stable for approximately one month.

Smear Preparation for the Gomori Procedure:
Smears may be fixed, the preparation spread in Alcohol (Alcohol-Ether), or embed tissue blocks in paraffin and section at 3-5mm.

Smear Preparation for the Wheatley Staining Procedure:
Using fresh fecal material or a fixed specimen only, make a smear on a clean glass slide. A drop of saline may be used to emulsify the specimen if necessary. If fresh specimen is to be used, while the smear is still wet immerse in a Coplin jar containing Schaudinn's Solution for one hour. The fixing time may be shortened to 5 minutes if the Schaudinn's Solution is heated to 50 degrees C. However, heating the fixative is rarely used in the routine clinical laboratory. If a fixed specimen is to be stained, allow the smear to air dry overnight and stain the following day. Or, the slides can be dried for approximately 30 to 60 minutes in the incubator (do not use a heating block). Proceed to the staining procedure.

Smear Preparation for the Modified Trichrome Staining Procedure:
The specimen can be fresh stool or stool that has been previously preserved as well as from body fluid or urine. Using a 10um aliquot of concentrated preserved liquid stool, prepare the smear by spreading the material over an area 45 by 25mm. Allow the smear to air dry. Place the smear in absolute methanol for 5 or 10 minutes. Allow the smear to air dry. It is highly recommended that the smear be prepared from concentration sediment to enhance the sensitivity of the stained smear (500xG for 10 minutes).

Detailed Procedure for Using Mercury-Based Fixatives[3,5-8]

Note: In all staining procedures for fecal and gastrointestinal tract specimens, the term "Xylene" will be used in the generic sense. Xylene substitutes are recommended for the safety of all personnel performing these procedures.

1. Prepare slide for staining.

2. Remove the slide from Schaudinn's Fixative (30 minutes fixation time), and place it in 70% ethanol for 5 minutes.

3. Place the slide in 70% ethanol plus iodine for 1 minute for fresh specimens or 5 to 10 minutes for PVA air-dried smears.

4. Place the slide in 70% ethanol for 5 minutes.*

5. Place the slide in a second container of 70% ethanol for 3 minutes.*

6. Place the slide in trichrome stain for 10 minutes (staining times may vary on the thickness of the fecal material and/or the number of slides stained at one time).

7. Place the slide in 90% ethanol plus acetic acid for 1 to 3 seconds. Immediately drain the rack, and proceed to the next step. Do not allow slides to remain in this solution.

8. Dip the slide several times in 100% ethanol. Use this step as a rinse.

9. Place the slide in two changes of 100% ethanol for 3 minutes each.*

10. Place the slide in xylene for 5 to 10 minutes.*

11. Place the slide in a second container of xylene for 5 to 10 minutes.*

12. Mount the coverslip (No. 1 thickness), using mounting medium (e.g., CytoSeal™, Cat. no. 83114).

Note: An alternative method that does not require the use of mounting medium as follows:

a. Remove the slide form the last xylene container, place it on a paper towel (flat position), and allow it to air dry. Remember that some of the xylene substitutes may take a bit longer to dry.

b. Approximately 5 to 10 minutes before examining the slide, add a drop of immersion oil to the dry fecal film. Allow the oil to absorb into the film for a minimum of 10 to 15 minutes. If the smear appears to be refractile upon microscopic examination, add more oil to the film.

c. Once you are ready to examine the slide, place a No.1 (22x22mm) coverslip onto the oiled smear, add another drop of immersion oil to the top of the coverslip, and examine with the oil immersion lens (100X objective).

d. Ensure that the coverslip is added to the slide; the dry fecal material on the slide often becomes very brittle after dehydration. Without the addition of the protective coverslip, the surface of the oil immersion lens may become scratched.

13. Allow the smear to dry overnight or for 1 hour at 37ºC.

14. Examine the smear microscopically with the 100X objective. Examine at least 200 or 300 oil immersion fields before reporting a negative result.

* Slides may be held up for 24 hours in these solutions without harming the quality of the smear for stainability of organisms.

Detailed Procedure for Using Non-Mercury-Based Fixatives (Iodine-Alcohol Step and Alcohol Rinse Not Required)[5]

1. Prepare a slide for staining as described above.

2. Place the slide in 70% ethanol for 5 minutes.*

3. Place the slide in trichrome stain for 10 minutes. Some people prefer to place the dry smear directly into the stain and eliminate step 2.

4. Place the slide in 90% ethanol plus acetic acid for 1 to 3 seconds. Immediately drain the rack and proceed to the next step. Do not allow the slides to remain in the solution.

5. Dip the slide several times in 100% ethanol. Use this step as a rinse.

6. Place the slide in two changes of 100% ethanol for 3 minutes each.*

7. Place the slide in xylene for 5 to 10 minutes.*

8. Place the slide in a second container of xylene for 5 to 10 minutes.*

9. Mount with a coverslip (No. 1 thickness), using mounting medium (e.g., CytoSeal™, Cat. no. 83114). An alternative method to using mounting medium is given above.

10. Allow the smear to dry overnight or for 1 hour at 37ºC.

11. Examine the smear microscopically with the 100X objective. Examine at least 200 to 300 oil immersion fields before reporting a negative result.

* Slides may be held up for 24 hours in these solutions without harming the quality of the smear for stainability of

organisms.

Detailed Procedure for Using a Modified Trichrome Stain Method Using Non-Mercury-Based Fixatives

(Iodine-Alcohol Step and Alcohol Rinse Not Required)[3,5-8]

1. Prepare a slide for staining as described above. Place the slide in absolute methanol for 5 or 10 minutes.

2. Allow the smear to air dry.

3. Place in the modified trichrome stain for 90 minutes.

4. Rinse in acid-alcohol for no more than 10 seconds.

5. Dip slides several times in 95% alcohol. Use this step as a rinse (no more than 10 seconds).

6. Place slides in 95% alcohol for 5 minutes. Rinse, reimmerse slides in fresh 95% alcohol for an additional 5 minutes.

7. Place in 100% alcohol for 10 minutes.

8. Place in xylene substitute for 10 minutes.

10. Mount with a coverslip (No. 1 thickness), using mounting medium (e.g., CytoSeal™, Cat. no. 83114).

11. Examine smears with the 100X objective (oil immersion) and read at least 100 fields; the examination time will probably be at least 10 minutes per slide.

INTERPRETATION OF RESULTS

Fecal Smears with Trichrome:
Artifacts and background material will stain green. Yeast, vegetable fibers, etc., also stain green. Red blood cells and bacteria usually stain red. Chromatin material and karyosomes of the nuclei will stain red to purple, as will helminth larvae and eggs. Cysts and organisms stain blue-green with a purple tinge. It is important to remember that there is a great deal of color variation in both the background and actual parasites; this is normal (Cat. no. SL1010).

Fecal Smears with Modified Trichrome Blue Stain:
The microsporidial spore wall should stain pink to red in color, with the interior of the spore being approximately 1-2um in size and clear or perhaps showing a horizontal or diagonal stripe representing the polar tube. The background will appear blue in color. Other bacteria, some yeast cells, and some debris will stain pink to red; the shapes and sizes of the components are useful in differentiating the spores from other structures. The results from this staining procedure should be reported only if the positive control smears (Cat. no. 369) are acceptable.

Tissue Smears or Sections:
Muscle and cytoplasm stains red. Connective tissue stains green. Nuclei of the cells stains blue.

LIMITATIONS

The permanent stained smear is not recommended for staining helminth eggs or larvae; they are often too dark (excess stain retention) or distorted. However, occasionally they are recognized and identified. The wet smear preparation from the concentrate is the recommended approach for identification of helminth eggs and larvae.

The smear should be examined with the oil immersion lens (100X) for the identification of protozoa, human cells, Charcot-Leyden crystals, yeast cells, and artifact material. These cells and other structures are normally quantitated from the examination of the permanent stained smear, not the wet-smear preparations (direct wet smear or concentration wet smear) (rare, few, moderate, many, packed). With the exception of *Blastocystis hominis*, intestinal protozoa are not quantitated on the report.

This high-magnification (oil immersion, total magnification, 1000X) examination is recommended for protozoa, particularly for confirming species identification.

With low magnification (10X objective), one might see eggs or larvae; however, this is not recommended for the permanent stained smear as a routine approach.

In addition to helminth eggs and larvae, *I. belli* oocysts are best seen in wet preparations (concentration wet smears prepared from formalin-preserved, not PVA-preserved, material).

Cryptosporidium spp. oocysts will generally not be recognized on a trichrome stained smear (modified acid-fast stains or the immunoassay reagent kits are recommended).[3,5-8]

All solutions need to be changed periodically, to prevent carry-over and/or watering down of the solutions. Carry-over of the solutions may cause lack of contrast and/or cloudiness on the slide.

If the Trichrome Stain weakens, it may be strengthened by exposing it to air overnight, or by replenishing with fresh stain.

When using the Modified Trichrome Blue Stain, data has indicated that centrifugation at 500xG for 10 minutes dramatically increases the number of microsporidial spores available for staining from concentrate sediment.[3,5-8]

MATERIALS REQUIRED BUT NOT PROVIDED

Standard microbiological supplies and equipment such as slides microscopes, cover slips, Coplin jars*, Mayer's Albumin and Mounting Fluid, etc., are not provided.

* Ten plastic Coplin jars are included with Cat. no. VXF019.

USER QUALITY CONTROL

It is recommended that each new lot or shipment of reagent be tested with known positive and negative controls and retested each month of use thereafter.[4] Use of prepared smears of known positive specimens allows the effectiveness of the stains and the staining techniques to be monitored. Inconsistencies in the control slides indicate that the technique is incorrect or that a defect in the stains is present.

PHYSICAL APPEARANCE

- Trichrome Stain should appear dark blue/green in color.
- Modified Trichrome Blue Stain should be opaque and appear dark purple in color.
- Ethanol, Acid Alcohol, Xylene Solution, and Schaudinn's Solution all should appear colorless in color.
- Iodine Concentrate should appear dark amber to brown in color (diluted iodine should appear like a strong tea color).
- PVA Fixative should appear clear and colorless.
- Carbol Xylene Solution should appear clear to light amber in color.

REFERENCES

1. Anderson, N.L., et al. *Cumitech 3B; Quality Systems in the Clinical Microbiology Laboratory,* Coordinating ed., A.S. Weissfeld. American Society for Microbiology, Washington, D.C.

2. Balows, A., et al. 1991. *Manual of Clinical Microbiology*, 5th ed. American Society for Microbiology, Washington, D.C.

3. Clinical and Laboratory Standards Institute. 2005. *Procedures for the Recovery and Identification of Parasites from the Intestinal Tract.* Approved Guideline, M28-2A. Clinical and Laboratory Standards Institute, Wayne, PA.

4. Forbes, B.A., et al. 2007. *Bailey and Scott's Diagnostic Microbiology*, 12th ed. C.V. Mosby Company, St. Louis, MO.

5. Garcia, L.S. (Coordinating Editor). 2003. *Cumitech 30A; Selection and Use of Laboratory Procedures for Diagnosis of Parasitic Infections of the Gastrointestinal Tract.* ASM Press, Washington, D.C.

6. Garcia, L.S. 2007. *Diagnostic Medical Parasitology*, 5th ed. ASM Press, Washington, D.C.

7. Garcia, L.S. 2009. *Practical Guide to Diagnostic Parasitology*, 2nd ed. ASM Press, Washington, D.C.

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

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

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