

## FIXATIVES, REAGENTS & STAINS

APACOR

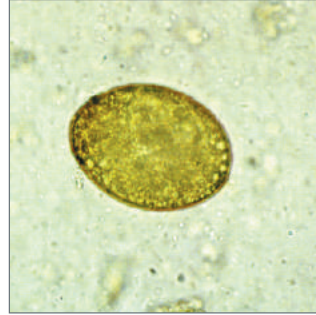
Parasitology  
FIXATIVES, REAGENTS & STAINS



Hymenolepis nana



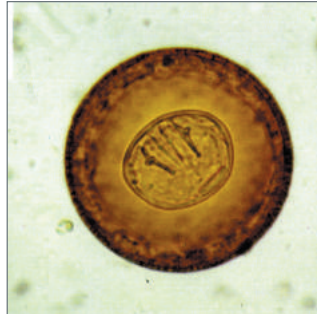
Paragonimus species



Diphyllobothrium latum



Clonorchis



Hymenolepis diminuta



Trichuris trichiura



Taenia species



Hookworm



Fasciola species

## Fixatives

### 10% Formalin in Buffered Saline

This preserves the morphology of cysts and ova. Trophozoites do not preserve well in Formalin-Saline and parasite morphology is not maintained adequately for a permanently stained faecal smear. Can be used in the Parasep® (Ridley Allen) method. Method - It is recommended that 1 part of stool be mixed with 3 parts of Formalin/Formalin-Saline preservative for the storage of bulk specimens.

### 10% Formalin Fixative in Water Part No. 1460

This preservative is a good overall fixative and will fix both ova and cysts although it only preserves the internal morphology of the cysts for up to 6 months, after which the cytoplasm of the organism becomes granular with poor nuclear definition.

Trophozoites do not preserve well in formalin and parasite morphology is not maintained adequately for a permanently stained faecal smear. Preferred to 10% Formalin in Saline when to be used in Parasep® (Ridley Allen concentration method).

### Sodium Acetate Acetic Acid Formalin (SAF) Part No. 1461

Fresh stools may be preserved using SAF especially when a delay may occur between the passage of the faeces and its delivery for subsequent laboratory examination.

SAF fixed material is suitable for direct examination, concentration (Formalin/Ethyl Acetate) and permanent staining.

Method - A "pea sized" sample (0.5g) of faeces (include external and internal portions) or 1ml of loose stool should be added to about 5ml of SAF fixative. Mix thoroughly and if necessary break up solid material to ensure it is well dispersed in SAF.

### Preparation of SAF preserved smears for permanent staining

1. Wash specimen at least twice and preferably three times in Saline to remove all traces of SAF. Centrifuge after each wash at 1000g for at least 1 minute. (10 minutes for Cryptosporidia and Isospora).
2. Shake the Mayer's Glycerin/Albumin to ensure the albumin and glycerin are well mixed. Place a small drop on the centre of a prelabelled slide.

3. Transfer a drop of the washed sediment onto the Mayer's Glycerin/Albumin and mix the two together well with an applicator stick.
4. Using the applicator stick, spread the mixture over the slide so there are alternating thick and thin bands. The thick areas are made with a rolling motion of the wrist. Holding the applicator stick in the middle, closer to the slide, may provide more control when making the smear. If the smear looks too thick or is improperly made, the specimen can easily be redistributed on the slide.
5. Allow the slides to dry thoroughly before staining. The smear should not appear shiny or have a wet appearance, otherwise it may flake off during the staining procedure.
6. Proceed to Staining Method.

### Preparation of smears for staining from SAF preserved stool specimens.

The Formalin/Ethyl Acetate concentration method for ova and cysts should be employed e.g. using Parasep®.

1. Each stool specimen should be treated on an individual basis depending on its composition. Although straining is appropriate for most specimens, mucous strands containing Cryptosporidia, other coccidia, or Giardia lamblia may become trapped in the gauze. Therefore, mucoid specimens and strands of mucous are processed without staining and the mucous itself should be used to make the smear. In these cases, the specimen is still washed in Saline to remove the SAF. Mucoid specimens are also processed without straining, and the mucous itself is applied as the smear.
2. Centrifugation for 10 minutes when processing the stool specimen increases the likelihood of detecting organisms such as Cryptosporidia and Isospora particularly in fatty stool specimens. Smears are made from the washed sediment, not the concentrate, since the organisms are often caught in the ethyl acetate plug.
3. When using SAF as a preservative, it is essential to wash the specimen two or three times to remove the SAF otherwise the specimen may flake off the slide. Removal of the excess debris provides a cleaner specimen.
4. Preparing slides for haematoxylin staining requires some practice before ideal slides are made. Albumin is used as a protein fixative to ensure the specimen adheres to the slide. When a slide is properly made, thin and thick bands are formed. The end product should be a smear thin enough to read a newspaper through. The thick areas aid in the detection of the trophozoites and cysts as well as ova such as unfertilised Ascaris and Hymenolepis nana which do not always concentrate well.

*With acknowledgements to J. Williams, LSH & TM & A. Moody, LHTD.*

## Fixatives

### Schaudinn's Fixative

Add mercuric chloride to the water and place in a water bath, preferably in an extraction hood, until it is dissolved. Allow to cool and form crystals.

\* **Health hazard - POISON**, harmful by ingestion and skin contact. Danger of cumulative effects. Irritating to eyes and skin.

#### Stock solution

Saturated mercuric chloride	600ml
95% ethyl alcohol	300ml

**Method** - Add 5ml glacial acetic acid to 95ml of the stock solution. Prepare faecal smears without allowing the smears to dry and place them immediately in Schaudinn's fixative for 1 hour. The reagents used in the preparation of this fixative are hazardous and should be handled with care.

The working reagents should be prepared fresh for use. This fixative is particularly good for making permanent stained faecal smears of protozoan trophozoites

### Bayers Solution

This following technique is useful for the preservation of cyst morphology.

**Method** - Dilute stock solution 1 in 10 with distilled water before use. Mix 1 part of faeces with 1 part of Bayer's solution.

### Merthiolate-Iodine Formalin (MIF) Part No. 1465

#### For use

Stock solution	4.7ml
Lugol's iodine (see below)	0.3ml

**Method** - Add approximately 1 gram of faeces to the 4.7ml of MIF/0.3ml iodine solution and emulsify well. Ova, cysts and larvae can be preserved in MIF for several months.

Formol- Ethyl Acetate concentration methods can be performed on samples preserved by any of the above.

### Polyvinyl Alcohol (PVA)

**Method** - To use, emulsify 1 part of faeces in 3 parts of PVA solution.

This method will preserve ova, larvae and trophozoites well, but cysts may show some distortion. However some ova and cysts do not concentrate well when preserved in PVA. Faecal smears made from the faeces/PVA mixture and allowed to dry can be used for the permanent staining of trophozoites. Before staining, the slides must be placed in 70% ethyl alcohol containing 5 – 10 drops of Lugol's Iodine (see below) to remove the mercuric chloride.

## Reagents

### Mayer's Glycerin/Albumin

Mayer's Glycerin/Albumin is used when preparing slides for staining. The Albumin helps to ensure that the specimen adheres to the slide and the Glycerin retains sufficient moisture to prevent distortion or disruption of organisms on drying.

#### Method

1. Shake the Mayer's Glycerin/Albumin to ensure it is well mixed. Place a drop in the centre of the slide. Add a drop of washed sediment to the Mayer's Glycerin/Albumin and mix well, using an applicator stick.
2. Using the same applicator stick spread the mixture on the slide ensuring that there are alternative thick and thin bands of mixture.
3. Allow the slides to dry well at room temperature before staining.

### Physiological Saline

Buffered saline solution.

### Triton X Solution Part No. 1472

Used to emulsify parasites in faeces, for use in standard Parasep® protocols.

### Ethyl Acetate Part No. 1473

A solvent that removes fat from faeces, for use in standard Parasep® protocols.

### Acetone

Solvent.

### Neutral Red

Aqueous solution and dye, can be used in the Gram's technique.

### DPX Mounting Media

### Immersion Oil

For use in immersion microscopy.

## Stains

### Eosin/Saline

This stain is useful for the detection of motile trophozoites of *Entamoeba* species.

**Method** - Emulsify faeces directly in a warm 37°C solution of Eosin in Saline. The amoebae are easily seen unstained against a pink background. The coarse, granular endoplasm can be differentiated from the clear, colourless ectoplasm.

### Acridine Orange (Acetate Buffered)

The addition of Acridine Orange to a faecal concentrate highlights the chromidial bars of *Entamoeba coli*, *Entamoeba histolytica/dispar* and *Entamoeba hartmanni*, which fluoresce bright green.

#### Working solution

Stock Acridine Orange	1ml
Glacial acetic acid	0.5ml
Buffered water pH 6.8	8.5ml

**Method** - Add an equal volume of stain to the concentrate. Examine the deposit under UV light after 30 minutes.

### Auramine Phenol (Lempert)

#### Method

1. Make faecal smears as for ZN and fix in methanol.
2. Stain with Auramine-Phenol (Lempert) for 10 - 15min.
3. Rinse thoroughly in tap water.
4. Decolourise in acid alcohol (as for ZN).
5. Rinse thoroughly in tap water.
6. Counterstain with 0.1% potassium permanganate for 30 sec.
7. Rinse thoroughly in tap water, allow to air dry. Do not blot dry, many brands of blotting paper will fluoresce!

#### Results

Oocysts appear as bright yellow discs against a dark background.

*Condensed from ACP broadsheet 128, June 1991, 'Laboratory methods for diagnosing Cryptosporidiosis.'*

### Field's Stain - Solution A and Solution B Part No. 1482/83

**N.B.** Both solutions are ready for use and should not be diluted.

This technique is a rapid Field's Stain method, which enables rapid staining of fixed thin films of various clinical samples. This particular method is very useful for staining films of unformed faeces, faecal exudates, duodenal aspirates etc.

#### Method

1. Make a thin film of faeces/exudate and allow to dry.
2. Fix in methanol for 1 min.

3. Flood slide with 1ml of Field's Stain B (Code: 1483).
4. Immediately add an equal volume of Field's Stain A (Code: 1482) mix well on slide and allow to stain for 1 min.
5. Rinse well in tap water and drain dry.
6. Examine the film using the oil immersion objective and Immersion Oil.

#### Results

Parasite nuclei and structures containing

Chromatin -	Red
Cytoplasm -	Bluish-grey
Leucocyte nuclei -	Purple
Yeasts and bacteria -	Dark blue

*Reference: Moody A.H. and Fleck S.L. (1985) Versatile Field's Stain. J.Clin. Pathol. 38(7), 842-3.*

### Giemsa Stain Rapid Part No. 1484

Giemsa Stain can also be used to stain films of unformed faeces, faecal exudate, duodenal aspirates etc.

#### Method

1. Make a thin film of faeces/exudate. Allow to dry.
2. Fix in methanol for 1 min.
3. Tip off the methanol and flood the slide with Giemsa Stain (Code: 1484) diluted 1:10 with buffered distilled water pH 7.2. The diluted stain must be freshly prepared each time.
4. Stain for 20-25 min.
5. Run tap water on to the slide to float off the stain and prevent deposition of precipitate on to the film. Allow to drain dry.
6. Examine the film using the oil immersion objective and Immersion Oil.

#### Results

Parasite nuclei and structures containing

Chromatin -	Red
Cytoplasm -	Bluish-grey
Leucocyte nuclei -	Purple
Yeasts and bacteria -	Dark blue

### Lugol's Iodine - Aqueous Part No. 1486

Temporary Stain for Protozoa.

#### Method

1. Make wet preparations following concentration by the formol-ether method.
2. Add an equal volume of Lugol's Iodine to 25% glacial acetic acid.
3. Place a drop of the wet preparation on a slide and add a drop of the Iodine/acetic acid mixture prepared in (2) above.
4. Cover slip and examine.

#### Result

Iodine Stains:

Glycogen -	Brown
Nuclear Chromatin of Amoebic Cysts -	Brown/Black.



# Stains

## Iron Haematoxylin Solution A and Solution B Part No. 1487/1488

### Preparation of Working Iron Haematoxylin Solution - Notes

#### Method

1. Mix equal volumes of the two solutions and filter.
2. Allow to stand at least two hours (preferably overnight) before use in order that the chemical reaction is complete. Staining is optimum 3-5 days after preparation.

If the Iron Haematoxylin Solution is used immediately after preparation the parasites may be stained an intense blue with little nuclear detail differentiation. When the Iron Haematoxylin Solution is mature (usually 3-5 days after preparation) the background should stain grey with the protozoa light blue, and the nuclei blue-black. The background staining of the slide depends on the composition of the specimen.

The stain is normally useable for a week. Shelf life can be extended by storage in a stoppered bottle in the dark after each use.

The Picric Acid removes more stain from non-parasitic matter than parasitic matter, and more from the cytoplasm of protozoa than the nucleus. Some cells retain Picric Acid. Complete removal of the picric acid will result in an understained slide. Smears such as these containing Picric Acid will fade with time.

If a slide appears cloudy, then dehydration has been inadequate. Agitation in the final alcohols can improve the clarity of the smear. Thick areas may contain more water.

Never examine microscopic slides when the DPX is still wet, since the resolution is affected. Drying the smears at 37°C is acceptable but care must be taken that air bubbles do not form during drying.

### Iron Haematoxylin Staining Procedure

#### Method

1. Prepare smear using Mayers Albumin.
2. Treat with 95% ethanol or IMS for at least 10 minutes to coagulate albumin, fixing smear to slide. Glycerin is removed at this point and care should be taken to ensure slides do not dry out from this stage on.
3. Slides should be brought to water stepwise through 70% and 25% alcohols. The slides should be treated for at least 10 minutes at each step. Running tap water is best for removal of all residual alcohol. An additional 2 minutes in alkalised water (by addition of a drop of ammonium hydroxide) is recommended.
4. Stain slides for 10 minutes in Iron Haematoxylin Solution (Codes 1487, 1488) - see notes on preparation.
5. Wash for 30 seconds in distilled or de-ionised water.
6. Treat for 10 minutes using 50% saturated Picric Acid which preferentially destains background.

7. Wash slides in running tap water for at least 10 minutes to remove Picric Acid, stopping destaining.
8. Transfer to alkalised tap water for 2 minutes.
9. Treat for 10 minutes in alkalised 70% alcohol (+ 2 drops ammonium hydroxide).
10. Bring to 100% alcohol stepwise through 95% and absolute alcohol (twice) leaving slides in each alcohol for at least 10 minutes.
11. Clear in Xylene for at least 10 minutes, twice.
12. Mount in DPX or any similar neutral mounting medium.
13. Examine using Immersion Oil under oil immersion lens.

## Trichrome for Microsporidia Part No. 1489

#### Method

1. Make smears from unconcentrated stool specimens in 10% Formalin (1:3 ratio). NOTE: ensure that the smears are extremely thin.
2. Fix in methanol for 5 mins.
3. Stain in modified Trichrome stain (Code: 1489) for 90mins.
4. Rinse in acid alcohol for 10 secs.
5. Rinse briefly in 95% alcohol.
6. Place in 95% alcohol for 5 mins.
7. Place in 100% alcohol for 10 mins.
8. Clear in Xylene for 10 mins.
9. Examine under X 100 oil immersion objective, using Immersion Oil.

#### Interpretation

Microsporidial spores are ovoid and refractile and the spore wall stains bright pinkish-red. Occasionally the cellular content of some spores does not stain and appears transparent, others show a pinkish-red stained belt girding the spores either diagonally or equatorially. The spores are approximately 1.5 by 0.9µ. The background debris and bacteria are counterstained faint green.

#### References

Weber R et al (1992) Improved light-microscopical detection of Microsporidia spores in stool and duodenal aspirates. *New Eng. J. Med.* 326(3): 161-166.

With acknowledgements to J. Williams, LSH & TM & A. Moody, LHTD GRAM STAIN PROTOCOL

## Stains

### Trichrome for Protozoa Part No. 1490

May be used to stain fresh faeces, prefixed faeces (only certain fixatives) or cultured organisms. The method varies slightly depending on the sample preparation used.

#### Method

1. Make thin smears of fresh stool or cultured amoebae on a clean microscope slide and immediately fix in Schaudinn's Solution\* - do not allow slide to dry. Leave for at least 30mins, preferably overnight. \*Schaudinn's Fixative contains Mercuric Chloride which is very poisonous via mouth and skin contact.
2. Transfer to 70% ethanol for 5 mins (omit this step if using PVA fixed material).
3. Transfer to alcoholic-iodine for 2 mins.
4. Transfer to 70% ethanol for 5 mins.
5. Transfer to 70% ethanol for 2-5 mins.
6. Transfer to Trichrome stain (Code: 1490) for 10 mins.
7. Differentiate in 0.5% acetic acid/alcohol for 12 secs.
8. Transfer to 100% ethanol for 1 sec only.
9. Transfer to 100% ethanol for 2-5 mins.
10. Transfer to 100% ethanol for 2.5 mins.
11. Transfer to xylene for 2-5 mins.
12. Transfer to xylene for 2 mins.
13. Mount with DPX or similar neutral mountant.

NB: Do not allow the slide to dry at any time during processing.

*With acknowledgments to J. Williams, LSH & TM & A. Moody, LHTD.*

### Carbol Fuchsin for Modified Z/N Stain (Cold Kinyoun)

#### Suggested Method

1. Flood the fixed smears with Carbol Fuchsin (Kinyoun) and stain for 2 minutes without heating.
2. Wash with tap water and decolourise with acid-alcohol until the dye no longer runs off the slide.
3. Wash with tap water and counterstain for 10-30 seconds with Methylene Blue or Malachite Green.
4. Wash, blot dry and examine.

Acid fast organisms stain red, the background and other organisms stain blue.

### Malachite Green Part No. 1501

Malachite Green is a green counterstain used to differentiate bacteria.

### Methylene Blue

Methylene Blue is a blue counterstain used to differentiate bacteria.

### Modified Z/N Stain Pack (Cold Kinyoun) Part No. 1503

#### Suggested Method

1. Flood the heat fixed smears with Carbol Fuchsin (ZN) and steam gently for 5 minutes. Add more stain if necessary to prevent drying.
2. Wash with water and decolourise with acid-alcohol until the dye no longer runs off the slide.
3. Wash with water and counterstain for 10-30 seconds with Methylene Blue or Malachite Green.
4. Wash, blot dry and examine.

Acid fast organisms stain red, the background and other organisms stain blue

# General Microbiology

## Stain Reagents

### Gram Stain Protocol

Gram's Iodine	Crystal Violet
Safranin O (Gram)	Neutral Red
Carbol Fuchsin (Gram)	Gram's Decolouriser

#### Introduction

Gram's Stain distinguishes between the two major classes of bacteria due to the differences in cell wall structure; Gram-positive bacteria, remain coloured after the staining procedure, and gram-negative bacteria, which do not retain dye.

In the staining technique, cells on a microscope slide are heat-fixed and stained with a basic dye, Crystal Violet, which stains all bacterial cells blue. Iodine solution is then added that allows the iodine to enter the cells and form a water-insoluble complex with the Crystal Violet dye. The preparation is then treated with a decolourise solvent, in which the Iodine-Crystal Violet complex is soluble.

Following solvent treatment, only gram-positive cells remain stained, possibly because of their thick cell wall, which is not permeable to solvent. After the staining procedure, cells are treated with a counterstain which may be Safranin O (Gram), Carbol Fuchsin (Gram) or Neutral Red. Counterstained gram-negative cells appear red, and gram-positive cells remain blue.

Although the cell walls of gram-negative and gram-positive bacteria are similar in chemical composition, the cell wall of gram-negative bacteria is a thin layer sandwiched between an outer lipid-containing cell envelope and the inner cell membrane, whereas the gram-positive cell wall is much thicker, lacks the cell envelope, and contains additional substances, such as teichoic acids, polymers composed of glycerol or ribitol.

The difference in reactivity between gram-positive and gram-negative bacteria is linked with differences in physiological properties of the two groups. Gram-positive bacteria are generally more sensitive to growth inhibition by dyes, halogens, many antibiotics, and to attack by phagocytosis and are more resistant to digestion by the enzymes pepsin and trypsin and enzymes in animal sera.

#### Suggested Method

1. Heat fix sample
2. Flood heat fixed sample with Crystal Violet solution for 1 minute
3. Rinse with tap water
4. Stain with Gram's Iodine for 1 minute
5. Rinse with tap water

6. Decolourise with Gram's Decolouriser (typically 3-5 seconds)
7. Wash with tap water
8. Counterstain with one of the following:
9. Safranin O (Gram) for 30 seconds **or**
10. Neutral Red for 1 minute **or**
11. Carbol Fuchsin (Gram) for 1 minute
12. Flush with tap water until no further stain can be flushed out
13. Air dry and examine

#### Examination

The Gram's staining procedure will stain all Gram +ve cells violet. The Neutral Red counterstain will stain all cells, but will be masked by the violet colour of the Gram's stain. Hence all Gram -ve cells will appear red stained.

### Gram's Stain Pack

#### Contents

- A Crystal Violet
  - B Gram's Iodine Diluent
  - C Gram's Iodine Concentrate
  - D Gram's Decolouriser
  - E Counterstain: choice of (Safranin O (Gram), Neutral Red, or Carbol Fuchsin (Gram)) \*
- \* Please specify on order which counterstain required.

#### Note

The Gram's Iodine Concentrate (C) should be added to the Diluent (B) and mixed well before use. All solutions are now ready to use in dropper bottles.

#### Suggested Method

1. Flood the heat fixed smears with Crystal Violet (A) and allow to stain for up to 1 minute.
2. Wash with tap water
3. Stain with Gram's Iodine (B+C) for 1 minute.
4. Wash with tap water
5. Decolourise (D) until no further stain runs from the slide.
6. Wash thoroughly with tap water
7. Counterstain (E) with Safranin O (Gram), Neutral Red, or Carbol Fuchsin (Gram) for up to 1 minute.
8. Wash, air dry and examine.

#### Expected result

Gram positive organisms - purple  
Gram negative organisms - red

### Lugol's Iodine Part No. 1486

The addition of iodine to a stool concentrate highlights the internal inclusions of cysts; e.g. the nuclei and glycogen mass, thus aiding their identification.

For example, the addition of iodine enhances refraction of the nuclei of *Endolimax nana*, stains the peripheral chromatin of the nuclei of *Entamoeba* species and demonstrates the well-defined glycogen mass which is a feature of pre-cysts or immature cysts of *E. coli* and cysts of *Iodamoeba butschlii*.

Iodine does not stain the body of *Entamoeba* species.

# General Microbiology Stain Reagents

## Crystal Violet

Primary gram stain Crystal Violet Solution is a violet stain used to differentiate bacteria.

## Safranin O (Gram)

Safranin O (Gram) is a red stain used to differentiate bacteria, used in the Gram's technique.

## Carbol Fuchsin (Gram) Part No. 1495

Used in the Gram's technique

## Lugol's Iodine Part No. 1486

Used in the Gram's technique

## Gram's Decolouriser

Used in the Gram's technique

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