

NATIONAL STANDARD METHOD

STAINING PROCEDURES

BSOP TP 39

Issued by Standards Unit, Evaluations and Standards Laboratory
Centre for Infections

Association of Medical Microbiologists
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STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 1 of 29

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Suggested citation for this document:

Health Protection Agency (2007). *Staining procedures*. National Standard Method Issue 1. http://www.hpa-standardmethods.org.uk/pdf_sops.asp.

STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 2 of 29

Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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INDEX

| | |
|---|----|
| STATUS OF NATIONAL STANDARD METHODS | 2 |
| INDEX | 3 |
| AMENDMENT PROCEDURE | 4 |
| SCOPE OF DOCUMENT | 5 |
| INTRODUCTION | 5 |
| TECHNICAL INFORMATION | 5 |
| 1 ACRIDINE ORANGE STAIN | 6 |
| 2 AURAMINE-PHENOL STAIN-1 | 7 |
| 3 AURAMINE-PHENOL STAIN-2 | 9 |
| 4 CALCOFLUOR STAIN | 10 |
| 5 FIELD'S STAIN..... | 11 |
| 6 GIEMSA'S STAIN (FOR <i>DIENTAMOEBIA FRAGILIS</i> , <i>BLASTOCYSTIS</i>)..... | 13 |
| 7 GIEMSA'S STAIN (FOR <i>PLASMODIUM</i> SPECIES) | 14 |
| 8 GRAM'S STAIN | 16 |
| 9 SANDIFORD'S MODIFICATION OF GRAM'S STAIN | 17 |
| 10 LUGOL'S IODINE..... | 18 |
| 11 MODIFIED TRICHROME STAIN..... | 19 |
| 12 MODIFIED COLD ZIEHL-NEELSEN'S STAIN | 20 |
| 13 NIGROSIN (INDIA INK) PREPARATION | 21 |
| 14 RAPID FIELD'S STAIN | 22 |
| 15 SPORE STAINS | 23 |
| 16 VINCENT'S STAIN | 24 |
| 17 ZIEHL-NEELSEN'S STAIN | 25 |
| ACKNOWLEDGEMENTS AND CONTACTS..... | 27 |
| REFERENCES | 28 |

STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 3 of 29

Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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AMENDMENT PROCEDURE

| | |
|--------------------------------------|----------------------------|
| Controlled document reference | BSOP TP 39 |
| Controlled document title | Staining procedures |

Each National Standard Method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from standards@hpa.org.uk.

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.

| Amendment Number/ Date | Issue no. Discarded | Insert Issue no. | Page | Section(s) involved | Amendment |
|---------------------------|------------------------|------------------------|------|---------------------|-----------|
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STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 4 of 29

Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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STAINING PROCEDURES

SCOPE OF DOCUMENT

This test procedure contains the methods for the staining procedures commonly used in Clinical Microbiology Laboratories for the identification of pathogens.

INTRODUCTION

Quality Assurance

Many of the stains that are described in this National Standard Method (NSM) are commercially available. Users should ensure that commercially prepared stains have been subject to stringent quality control. When using commercial stains it is important for quality control purposes to keep records of the batch numbers of the stains and the dates when they were used.

Stains prepared or diluted in house should be controlled to ensure that there is no contamination by environmental organisms.

Positive and negative control slides should be used every time the staining procedure is performed except for Gram staining where positive controls may be enough unless a new batch of stain is made. If the control slides do not prove satisfactory, the staining procedure is not accepted. Positive and negative slides may be prepared using known or reference strains.

TECHNICAL INFORMATION

Duration of each step may vary depending on the concentration and formulation of staining solutions and other reagents. Follow manufacturer's instructions when possible.

STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 5 of 29

Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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1 ACRIDINE ORANGE STAIN

(for *Trichomonas vaginalis*)

1.1 INTRODUCTION

This technique may be used for the demonstration of *Trichomonas vaginalis* in vaginal smears.

1.2 SAFETY CONSIDERATIONS

Follow local COSHH and risk assessments when performing all staining procedures.

1.3 METHOD²

- Prepare a smear and air dry (slides should be processed within 24 h)
- Stain the slides with acridine orange solution for 5 - 10 seconds
- Wash off the stain, and decolourise the smear with alcoholic saline for 5 - 10 seconds
- Rinse the smear with physiological saline and place the slide in a drying rack
- Add a drop of saline or distilled water to the smear and cover with a cover glass
- Examine the smear by fluorescence microscopy with BG 12 excitor filter and No. 44 and No. 53 barrier filters

1.4 INTERPRETATION

Trichomonas vaginalis is usually pear shaped with average dimensions of approximately 10 x 7 μm^3 .

Positive: Trophozoites of *Trichomonas vaginalis* stain brick red with green banana-shaped or rounder nucleus.

Negative: Yeasts stain red with a bright green nucleus.

Epithelial cells fluoresce light green with a bright green nucleus and the nuclei of leucocytes fluoresce bright green.

1.5 QUALITY CONTROL

Positive: *Trichomonas vaginalis*.

Negative: A proven negative vaginal smear may be used as the negative control.

1.6 TECHNICAL INFORMATION⁴

Acridine orange staining has been shown to be more sensitive than wet-mount examination when detecting *Trichomonas vaginalis*⁵.

STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 6 of 29

Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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2 AURAMINE-PHENOL STAIN-1

(for acid fast bacilli)

2.1 INTRODUCTION

This staining technique is used to demonstrate the presence of acid fast bacilli (*Mycobacterium* species). These organisms have waxy envelopes that make them difficult to stain and decolourise. A fluorescent stain is used in this method and the method is more sensitive than Ziehl-Neelsen's method.

2.2 SAFETY CONSIDERATIONS

All suspected *Mycobacterium* species must be processed in a Class 1 exhaust protective cabinet in a Containment Level 3 room.

Follow local COSHH and risk assessments when performing all staining procedures.

Disposable gloves must be worn.

Diamond markers are not recommended; frosted slides marked with a pencil are recommended.

Smear material should be fixed by placing the slides on an electric hotplate prior to staining (65°C to 75°C). This procedure should be performed in a Class 1 exhaust protective cabinet until the smeared material is dried and fixed to the slide. They should then be placed in a rack or suitable holder.

Note: Heat fixing does not kill *Mycobacterium* species and slides should be handled with care.

See BSOP 40 - Investigation of specimens for *Mycobacterium* species.

2.3 METHOD⁶

- Prepare a smear and heat to fix
- Flood the slide with Auramine-phenol (1:10 v/v) and leave for 10 min
- Gently rinse with tap water (ensure water is free from acid alcohol fast bacteria or use filtered water)
- Decolourise with 1% acid alcohol for 3 - 5 min
- Gently rinse with tap water as above
- Repeat acid alcohol step until no further stain seeps from the film
- Counterstain with 0.1% potassium permanganate or thiazine red for 15 sec (this ensures a dark background for the fluorescing AAFB which are easier to see). KMNO₄ stains all epithelial cells etc making it more difficult to see AAFB
- Gently rinse with tap water as above and air dry. Do not blot dry
- Examine slides using ultra violet epi-fluorescence microscopy at 25 x or 40 x magnification (the use of a 40 x magnification non-cover-glass (NCG) objective lens will avoid the need to apply a cover glass)

NOTE: Follow manufacturer's procedure if commercial kits are used.

STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 7 of 29

Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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2.4 INTERPRETATION

Positive: Acid fast bacilli vary from 0.5 to 10 µm in length⁶ and stain bright yellow-green against a dark background.

Negative: No fluorescence observed.

2.5 QUALITY CONTROL

Positive: *Mycobacterium* species.

Negative: A proven negative smear may be used as the negative control.

2.6 TECHNICAL INFORMATION

It is important to ensure that the rinsing water and the water that is used to make up the stain is not contaminated with environmental acid alcohol fast bacteria. This is frequently found in tap water and with the use of rubber tubing. Filtered water should be used if necessary.

STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 8 of 29

Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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3 AURAMINE-PHENOL STAIN-2 (for *Cryptosporidium* species)

3.1 INTRODUCTION

This fluorescent staining technique is used for the demonstration of oocysts of *Cryptosporidium* species in faeces.

3.2 SAFETY CONSIDERATIONS

Follow local COSHH and risk assessments when performing all staining procedures.

3.3 METHOD⁷

- Prepare a smear and air dry (smears should be medium to thick)
- Fix in methanol for 3 min
- Flood the slide with Auramine-phenol* solution and leave for 10 min
- Rinse with tap water
- Decolourise the slide with 3% acid methanol and leave for 5 min
- Rinse with tap water
- Counterstain the slide with 0.1% potassium permanganate and leave for 30 sec
- Rinse with tap water, drain and air dry. Do not blot because some blotting materials may fluoresce
- Examine with x 20 objective and a x 10 eyepiece lens and an incident-light fluorescence microscope. The filters should be blue or FITC with excitation (690 nm) and emission (510 nm) wavelengths. A minimum of 50 fields should be examined

*Auramine 0.3 g, phenol 3.0 g, distilled/deionised water 97 mL. Dissolve the phenol in water with gentle heat. Add the auramine gradually and shake vigorously until dissolved. Filter and store in a dark stoppered bottle⁸.

3.4 INTERPRETATION

Positive: *Cryptosporidium* oocysts (4 to 6 µm diameter) are ring or doughnut-shaped and fluoresce greeny-yellow (depending on the filter wavelengths) against a dark background. Putative oocysts may be measured by increasing the bright field light intensity and measuring the oocysts with a calibrated eye-piece graticule.

Negative: No fluorescence observed.

3.5 QUALITY CONTROL

Positive: *Cryptosporidium* species. Positive control material can be obtained from the *Cryptosporidium* Reference Unit.

Negative: A proven negative smear may be used as the negative control.

3.6 TECHNICAL INFORMATION

Confirmation of staining results should be made by staining a new smear using modified Ziehl-Neelsen's stain.

STAINING PROCEDURES

4 CALCOFLUOR STAIN

(for microsporidia)

4.1 INTRODUCTION

Calcofluor stain binds to the chitin in the endospore layer of the spore wall of microsporidia and fluoresces a brilliant blue-white. This staining technique is used for the demonstration of microsporidia in faeces.

4.2 SAFETY CONSIDERATIONS

Follow local COSHH and risk assessments when performing all staining procedures.

4.3 METHOD⁹

- Prepare a very thin smear and air dry
- Fix the smear in methanol for 5 min
- Stain the smear with 1 to 2 drops of Calcofluor solution (0.5% w/v) and leave for 2 - 3 min
- Rinse under slow running water
- Counterstain with Evans blue solution (0.1%) for 1 min
- Rinse under slow running water
- Air dry
- Add 1 or 2 drops of mounting fluid to the slide and mount with a coverslip
- Examine microscopically under a fluorescence (395 - 415 nm) microscope

4.4 INTERPRETATION

Positive: Spores of microsporidia are typically ovoid or piriform and fluoresce brilliant blue-white. Dimension of spores vary by species and range from 1 to 20 μm ¹⁰.

Negative: No fluorescence observed.

4.5 QUALITY CONTROL

Positive: Microsporidia species

Negative: A proven negative smear may be used as the negative control.

4.6 TECHNICAL INFORMATION

Fungal spores may contain chitin, and some experience is required to differentiate spores of microsporidia from those of fungi.

STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 10 of 29

Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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5 FIELD'S STAIN

(for *Plasmodium* species)

5.1 INTRODUCTION

This technique is used for the demonstration of *Plasmodium* species in thick and thin blood films¹¹.

5.2 SAFETY CONSIDERATIONS

Follow local COSHH and risk assessments when performing all staining procedures.

5.3 METHOD¹²

5.3.1 RAPID FIELD'S STAINING FOR THIN FILMS

- Prepare a thin film
- Air dry the film
- Fix in methanol for 1 min
- Cover the slide with 1 mL of diluted Field's stain B (1 in 4 in buffered water pH 7.2)
- Immediately add an equal volume of Field's stain A and mix
- Leave to stain for 1 min
- Rinse the slide with clean water and drain dry

5.3.2 FIELD'S STAINING FOR THICK FILMS

Caution: Thick blood films are not fixed and the stains do not kill the parasites, viruses or other pathogens which may be present in the blood.

- Hold the slide with the dried thick film facing downwards
- Dip the slide in the Field's stain A for 3 sec
- Drain the excess stain by touching a corner of the side against the side of the container
- Wash gently for about 3 sec in clean water and gently agitate
- Drain off the excess water
- Dip the slide in Field's stain B for 3 sec
- Drain off the excess stain
- Wash gently in clean water
- Wipe the back of slide clean and place it upright in a draining rack for the film to air dry

Note: If after staining, the whole film appears yellow-brown (a sign that too much blood has been used), too blue or too pink, do not attempt to examine it. Re-stain it by dipping the slide in the Field's stain A for 1 second, followed by a gentle wash in clean water, dip in Field's stain B for 1 second and finally wash gently in clean water.

STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 11 of 29
Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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5.4 INTERPRETATION

5.4.1 FIELDS' STAINING FOR THIN FILMS

Positive:

| | |
|--------------------------------|-------------|
| Chromatin of parasite | Dark red |
| Cytoplasm of parasite | Blue |
| Schüffner's dots/James's dots | Red |
| Maurer's dots (clefts) | Red-mauve |
| Malaria pigment in white cells | Brown-black |

Negative:

| | |
|--------------------------------|-------------------------|
| Red cells | Grey to pale mauve-pink |
| Reticulocytes | Grey-blue |
| Nuclei of neutrophils | Dark purple |
| Cytoplasm of mononuclear cells | Blue-grey |
| Granules of eosinophils | Red |

5.4.2 FIELDS' STAINING FOR THICK FILMS

Positive:

| | |
|-----------------------|------------|
| Chromatin of parasite | Dark red |
| Cytoplasm of parasite | Blue-mauve |
| Schüffner's dots | Pale red |

Negative:

| | |
|---|------------------------------|
| <i>Plasmodium vivax</i> and <i>Plasmodium ovale</i> parasites | |
| Malaria pigment | Yellow brown or yellow black |
| Nuclei of small lymphocytes | Dark purple |
| Nuclei of neutrophils | Dark purple |
| Granules of eosinophils | Red |
| Cytoplasm of mononuclear cells | Blue-grey |
| Reticulum of reticulocytes | Blue-grey |

5.5 QUALITY CONTROL

Positive: *Plasmodium* species.

Negative: A proven negative smear may be used as the negative control.

5.6 TECHNICAL INFORMATION

This is a useful method for rapid presumptive species identification of malaria parasites. This method shows adequate staining of all stages including stippling. However staining with Giemsa is always the method of choice for definitive species differentiation.

With thick preparations, the end of the smear closest to the edge of the slide that was draining should be looked at. The edges of the film will also be better than the centre where the film may be too thick or cracked.

STAINING PROCEDURES

6 GIEMSA'S STAIN (FOR *DIENTAMOEBEA FRAGILIS*, *BLASTOCYSTIS*)

6.1 INTRODUCTION

Giemsa's stain is used to demonstrate the presence of *Dientamoeba fragilis* and *Blastocystis hominis* in faeces and *Pneumocystis jirovecii* in broncho-alveolar lavage.

6.2 SAFETY CONSIDERATIONS

Follow local COSHH and risk assessments when performing all staining procedures.

6.3 METHOD

- Prepare a 1 in 10 dilution of Giemsa's stain in buffered water. This should be freshly prepared
- Prepare a faecal smear and allow to air dry
- Fix in methanol for 60 sec
- Tip off the methanol
- Flood the slide with diluted Giemsa's stain and leave for 20 - 25 min
- Run tap water on to the slide to float off the stain and to prevent precipitation on the smear
- Allow to air dry

6.4 INTERPRETATION

Positive: Parasite nuclei and chromatin stain red

Negative: Leucocyte nuclei stain purple, cytoplasm stains bluish-grey, bacteria and yeasts stain dark-blue.

Note: Giemsa's stain does not stain the cyst walls of *Pneumocystis* but does allow trophic forms to be seen.

6.5 QUALITY CONTROL

Positive: *Dientamoeba fragilis* and *Blastocystis hominis*.

Negative: A proven negative smear may be used as the negative control.

6.6 TECHNICAL INFORMATION

It is not possible to see the typical fragmented nuclei when using this method as the nuclear contents often coalesce¹³.

Staining for *Pneumocystis jirovecii* is more commonly done by specific immunofluorescence antibody methods or by silver staining. Alternative diagnostic methods such as PCR are used increasingly.

STAINING PROCEDURES

7 GIEMSA'S STAIN (FOR *PLASMODIUM* SPECIES)

7.1 INTRODUCTION¹¹

Giemsa's stain is used to demonstrate the presence of *Plasmodium* species in thick and thin blood films. A thick film is about 30 times more sensitive than a thin film, detecting about 20 parasites per μL . Thick films are therefore the most suitable method for the rapid detection of the parasite. A thin film is required to confirm the *Plasmodium* species if this is not clear from the thick film. Thin films are also of value in assessing whether a patient with falciparum malaria is responding to treatment in areas where drug resistance is suspected.

7.2 SAFETY CONSIDERATIONS

Follow local COSHH and risk assessments when performing all staining procedures.

7.3 METHOD^{11,14}

Thin films of blood or marrow

- Fix in methanol for 5 minutes
- Place in a Coplin jar containing Giemsa's stain diluted 1:10 for 20 minutes
- Rinse in distilled water
- Drain and dry at room temperature
- Mount in DPX or leave unmounted and use oil immersion

Thick films for malaria parasites

- Air dry the slides
- Place in a Coplin jar containing Giemsa's stain diluted 1:50 at pH7.2 for one hour
- Wash with distilled water (flushing the stain from the slides and the staining container is necessary to avoid the films being covered with a fine deposit of stain)
- Differentiate in 1:1,500 acetic acid (control by viewing at intervals under a microscope. Sections should have an overall pink colour, with the nuclei blue and eosinophil granules red)
- Rapidly rinse in distilled water
- Air dry

7.4 INTERPRETATION

Positive:

| | | |
|------------------------|-----|-----------|
| Chromatin of parasite | | Dark red |
| Cytoplasm of parasite | | Blue |
| Schüffner's dots | Red | |
| Maurer's dots (clefts) | | Red-mauve |

Negative:

| | | |
|--------------------------------|--|--------------------|
| Red cells | | Grey to pale mauve |
| Reticulocytes | | Grey blue |
| Nuclei of neutrophils | | Dark purple |
| Granules of neutrophils | | Mauve purple |
| Granules of eosinophils | | Red |
| Cytoplasm of mononuclear cells | | Blue-grey |

STAINING PROCEDURES

7.5 QUALITY CONTROL

Positive: *Plasmodium* species.

Negative: A proven negative smear may be used as the negative control.

7.6 TECHNICAL INFORMATION

Rapid diagnostic tests (RTDs) are available as alternatives for microscopy. These tests detect three main groups of antigens including Histidine-rich protein 2 (HRP2) specific to *P. falciparum*, plasmodium lactate dehydrogenase (pLDH), and Aldolase. These products are available in the forms of plastic cassettes, cards, dipsticks, and hybrid cassette-dipsticks. Factors such as parasite prevalence, availability of skilled personnel and resources, the capacity for maintaining quality assurance of microscopy and RDT, and the need for quantitative assessment of parasite density need to be considered when selecting microscopy or an RTD as an identification method¹⁵.

STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 15 of 29
Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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8 GRAM'S STAIN

8.1 INTRODUCTION

Organisms are classified according to their Gram-staining reaction. Gram-positive bacteria have thicker and denser peptidoglycan layers in their cell walls. Iodine penetrates the cell wall in these bacteria and alters the blue dye to inhibit its diffusion through the cell wall during decolourisation. Gram-positive bacteria must have an intact cell wall to produce a positive reaction. Gram-negative cells which do not retain the methyl/crystal violet are stained by a counterstain¹⁶. Neutral red, safranin or carbol fuchsin may be used as the counterstain¹⁶.

8.2 SAFETY CONSIDERATIONS

Follow local COSHH and risk assessments when performing all staining procedures.

8.3 GRAM'S METHOD FOR EXAMINATION OF SMEARS^{16,17}

- Prepare a smear and heat gently to fix
- Flood the slide with 0.5% methyl/crystal violet and leave for 30 sec
- Tilt the slide, pour on sufficient (1%) Lugol's iodine to wash away the stain, cover with fresh iodine and allow to act for 30 sec
- Tilt the slide and wash off the iodine with 95 - 100% ethanol or acetone until colour ceases to run out of the smear
- Rinse with water
- Pour on 0.1% counterstain (neutral red, safranin or carbol fuchsin) and leave to act for about 2 min
- Wash with water and blot dry

8.4 INTERPRETATION

Positive: Gram-positive organisms stain deep blue/purple.

Negative: Gram-negative organisms stain pink/red.

8.5 QUALITY CONTROL ORGANISMS

A culture containing Gram-positive and Gram-negative organisms may be used for quality control.

8.6 TECHNICAL INFORMATION

Some Gram-positive bacteria regularly appear Gram-negative, in whole or in part eg rapidly growing *Streptococcus* species, involution forms of *Streptococcus pneumoniae* and some strains of *Bacillus* species.

Some gracile Gram-negative bacteria such as *Haemophilus* species might easily be missed if stained by the Gram method (see Sandiford's modification).

When clinical material is strongly suspected to contain bacteria but none are visible by Gram's stain, use of alternative counter stains (such as Sandiford's or Giemsa's), negative stains such as India ink, or wet preparations may be useful.

STAINING PROCEDURES

9 SANDIFORD'S MODIFICATION OF GRAM'S STAIN

9.1 INTRODUCTION

Sandiford's modification of Gram's staining technique was originally used for demonstrating the presence of Gram-negative diplococci intracellularly¹⁸. The counterstain also enhances the appearance of Gram-negative and Gram-variable organisms.

9.2 SAFETY CONSIDERATIONS

Follow local COSHH and risk assessments when performing all staining procedures.

9.3 METHOD¹⁹

- Spread a loop of clinical specimen thinly on a degreased slide. Air dry.
- Stain with crystal violet stain for 2 min
- Rinse in tap water.
- Counter stain with Iodine solution for 2 min
- Rinse in tap water
- Blot dry
- Differentiate in acetone-alcohol for 10 - 15 sec
- Wash in running tap water
- Blot dry
- Counter stain with Sandiford's malachite green solution (mixture of pyronin Y and malachite green) and leave for 3 min
- Flood the slide with water (do not wash)
- Air dry

9.4 INTERPRETATION

Positive: Gram-positive organisms stain deep blue/purple.

Negative: Gram-negative or Gram-variable organisms stain pink against a blue green background.

9.5 QUALITY CONTROL ORGANISMS

A culture containing Gram-positive and Gram-negative organisms may be used for quality control.

9.6 TECHNICAL INFORMATION

N/A

STAINING PROCEDURES

10 LUGOL'S IODINE

(for parasites)

10.1 INTRODUCTION

1% Lugol's iodine, when diluted, is used to stain ova and protozoan cysts in wet mounts. This method enhances their internal structures.

10.2 SAFETY CONSIDERATIONS

Follow local COSHH and risk assessments when performing all staining procedures.

10.3 METHOD²⁰

- Mix a small amount of faeces with 1 drop of 0.85% NaCl on the left hand side of the slide and one drop of the iodine reagent on the right hand side of the slide
- Cover with coverslip and examine

10.4 INTERPRETATION

Positive: Protozoan nuclei take up the iodine and stain pale brown while cytoplasm remains colourless.

Negative: N/A

10.5 QUALITY CONTROL

Positive: A proven positive smear may be used as the positive control.

Negative: A proven negative smear may be used as the negative control.

10.6 TECHNICAL INFORMATION

Some workers prefer to make saline and iodine mounts on separate slides. There is less chance of getting fluids on the microscope stage if separate slides are used.

The microscope light should be reduced for low power observations since most organisms will be overlooked by bright light. Illumination should be regulated so that some of the cellular elements in the faeces show refraction. Most protozoan cysts will refract light under these conditions.

For this method to work effectively the 1% Lugol's iodine solution should be a fresh preparation (10 - 14 days)

STAINING PROCEDURES

11 MODIFIED TRICHROME STAIN

(for microsporidia)

11.1 INTRODUCTION

This technique is used for the demonstration of microsporidia in faeces.

11.2 SAFETY CONSIDERATIONS

Follow local COSHH and risk assessments when performing all staining procedures.

11.3 METHOD²¹

- Prepare a very thin smear from a suspension of unconcentrated liquid stool in 10% formalin (1:3 ratio) and air dry
- Fix the smear in methanol for 5 min
- Flood the slide with Chromotrope-based stain* and leave for 90 min
- Rinse under a running tap for 1 min to remove excess stain
- Rinse in acid alcohol (0.45% glacial acetic acid in ethyl alcohol) for 10 sec
- Rinse briefly in 95% alcohol
- Place the slide in 95% alcohol for 5 min
- Place the slide in 100% alcohol for 10 min
- Place the slide in Hemo-De (a xylene substitute) for 10 min
- Air dry and examine using a high power objective

*Chromotrope 2R 6 g, fast green 0.15 g, phosphotungstic acid 0.7 g, , leave in 3 mL of glacial acetic acid for 30 min, then mix with 100 mL of distilled water.

11.4 INTERPRETATION

Positive: Spores of species of microsporidia that infect mammals including humans tend to be small, ranging in size from 1.0 to 3.0 µm X 1.5 to 4.0 µm²². They are ovoid and refractile. The spore walls stain bright pink-red. Occasionally the spores stain with a red “belt” across the centre of the spore.

Negative: No spore material observed.

11.5 QUALITY CONTROL

Positive: Microsporidia species.

Negative: A proven negative smear may be used as the negative control.

11.6 TECHNICAL INFORMATION

Screening of 100 oil immersion fields with average reading time of 10 min per slide is recommended for establishing diagnosis. Screening fewer fields might result in false negative results for patients who excrete small numbers of spores²¹.

STAINING PROCEDURES

12 MODIFIED COLD ZIEHL-NEELSEN'S STAIN

(for *Cryptosporidium* species)

12.1 INTRODUCTION

This technique is used for the demonstration of oocysts of *Cryptosporidium* species in faeces. Alternatively, the modified auramine-phenol stain may be used (refer section 3).

12.2 SAFETY CONSIDERATIONS

Follow local COSHH and risk assessments when performing all staining procedures.

12.3 METHOD²³

- Prepare a medium to thick smear and air dry
- Fix in methanol for 3 min and air dry
- Flood the slide with modified Kinyoun's acid fast stain (3% carbol fuchsin) and leave for approximately 15 min
- Rinse with tap water
- Flood the slide with 1% acid methanol to decolourise and leave for 15 to 20 seconds
- Rinse with tap water
- Counterstain with 0.4% malachite green or alternative and leave for 30 seconds
- Rinse with tap water and air dry
- Examine using x 40 or x 50 objective and x 10 eyepiece lenses. Morphology may be examined more closely with a high power objective

12.4 INTERPRETATION

Positive: *Cryptosporidium* species are 4 to 6 µm and spherical. Oocyst staining is variable, and some oocysts may appear unstained. Internal structures may take up the stain to varying degrees. Sometimes the crescent shape of the sporozoites may be seen under high power magnification. Isospora species stain red, measure 32 x 16 µm and are elongated oval bodies tapered at both ends, containing a granular zygote or two sporoblasts. *Cyclospora* species oocysts stain pinkish red, are spherical 8 to 10 µm and contain a central morula. Staining is variable and some oocysts may appear unstained. The oocysts seen in faeces are usually unsporulated. Yeasts, other biota and faecal debris may also take up the stain.

Negative: Parasite not detected.

12.5 QUALITY CONTROL

Positive: Species of *Cryptosporidium*. Positive control material can be obtained from the *Cryptosporidium* Reference Unit.

Negative: A proven negative smear may be used as the negative control.

12.6 TECHNICAL INFORMATION

Care should be taken because spores and artifacts may stain with Ziehl-Neelsen's stain and appear as positive to untrained eyes.

STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 20 of 29
Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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13 NIGROSIN (INDIA INK) PREPARATION

13.1 INTRODUCTION

Nigrosin staining is a negative staining technique because the background is stained whereas the organisms remain unstained. Capsules displace the dye and appear as halos surrounding the organism¹⁶. This technique is particularly recommended for the demonstration of the capsule of *Cryptococcus neoformans* and it can also be used to demonstrate the presence of bacterial and yeast capsules.

13.2 SAFETY CONSIDERATIONS

Follow local COSHH and risk assessments when performing all staining procedures.

13.3 METHOD¹⁶

- Place a drop of India ink on to a clean slide
- Add 1 drop of specimen or liquid culture or rub a speck of material on the slide surface just beside the ink before mixing it into the ink
- Cover with a cover slip, press it down through a sheet of blotting paper so that the film becomes very thin and pale in colour, and examine

13.4 INTERPRETATION

Positive: Organisms possessing a capsule appear highly refractile, surrounded by a clear zone against a dark background.

Negative: No clear zone around the organism is observed.

13.5 QUALITY CONTROL

Positive: *Cryptococcus neoformans*, or other capsulate organisms.

Negative: A proven negative smear may be used as the negative control.

13.6 TECHNICAL INFORMATION

The correct concentration of India ink is critical for showing the capsular zone.

STAINING PROCEDURES

14 RAPID FIELD'S STAIN

(for *Dientamoeba fragilis*, *Blastocystis hominis* and *Pneumocystis jirovecii*)

14.1 INTRODUCTION

This is a staining technique to demonstrate the presence of *Dientamoeba fragilis* and *Blastocystis hominis* in faeces, and *Pneumocystis jirovecii* in bronchoalveolar lavage.

14.2 SAFETY CONSIDERATIONS

Follow local COSHH and risk assessments when performing all staining procedures.

14.3 METHOD

- Prepare a smear and allow to air dry
- Fix in methanol for 60 sec
- Flood the slide with Field's stain B (diluted 1 in 4 with buffered water pH 6.8)
- Immediately add an equal volume of Field's stain A (undiluted), mix and leave for 60 sec
- Rinse with tap water, drain and air dry

14.4 INTERPRETATION

Positive: Parasite nuclei and chromatin structures stain red. Cyst walls of *P. jirovecii* will not be stained but trophic forms will.

Negative: Bacteria and yeasts stain dark-blue. Leukocyte nuclei stain purple and leukocyte cytoplasm stains bluish-grey.

14.5 QUALITY CONTROL

Positive: *Dientamoeba fragilis*, *Blastocystis hominis* and *Pneumocystis jirovecii*.

Negative: A proven negative smear may be used as the negative control.

14.6 TECHNICAL INFORMATION

It is not possible to see the typical fragmented nuclei when using this method as the nuclear contents often coalesce¹³.

Staining for *Pneumocystis jirovecii* is more commonly done by specific immunofluorescence antibody methods or by silver staining. Alternative diagnostic methods such as PCR are used increasingly.

STAINING PROCEDURES

15 SPORE STAINS

15.1 INTRODUCTION

The following methods may be used for the demonstration of spores in Gram-positive bacilli.

15.2 SAFETY CONSIDERATIONS

Follow local COSHH and risk assessments when performing all staining procedures.

15.3 METHODS

15.3.1 SCHAEFFER AND FULTON'S METHOD (MODIFIED BY ASHBY)²⁴

- Prepare a smear and heat gently to fix
- Place the slide over a beaker of boiling water, resting it across the rim with the bacterial smear uppermost
- When large droplets of water appear on the underside of the slide, flood it with the malachite green solution and leave it to act for 1 min while the water is still boiling
- Wash with cold water
- Treat with safranin or basic fuchsin for 30 sec
- Wash and dry

15.3.2 WIRTZ-CONKLIN'S METHOD²⁵

- Flood the slide with malachite green solution
- Steam for 3 - 6 min
- Rinse under tap water
- Counterstain with safranin for 30 sec
- Wash and dry

15.4 INTERPRETATION

Positive: Bacterial spores stain green.

Negative: Bacterial cells stain red.

15.5 QUALITY CONTROL

Positive: *Bacillus* species

Negative: Non-spore producing organisms eg *E.coli*

15.6 TECHNICAL INFORMATION

N/A

STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 23 of 29
Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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16 VINCENT'S STAIN

(for oral bacteria)

16.1 INTRODUCTION

This technique is used to stain *Borrelia vincentii* (a spirochaete causing Vincent's angina) from oral and throat swabs. Presence of large numbers of *Borrelia vincentii* in conjunction with barred fusiform bacilli and Gram-negative rods together with polymorphonuclear leucocytes indicates infection.

16.2 SAFETY CONSIDERATIONS

Follow local COSHH and risk assessments when performing all staining procedures

16.3 METHOD

Procedure for Vincent's stain is similar to that of Gram stain except that the counterstain (1% carbol fuschin) is applied for 30 sec.

16.4 INTERPRETATION

Positive: *Borrelia vincentii* appear as pale pink staining spirals together with pink cigar shaped fusiforms.

Note: Presence of both organisms is needed for establishing the diagnosis of Vincent's disease.

Negative: N/A

16.5 QUALITY CONTROL ORGANISMS

Borrelia vincentii are large spirochaetes which vary between 10 - 30 µm in length²⁶.

Positive: *Borrelia vincentii*.

Negative: A proven negative smear may be used as the negative control.

16.6 TECHNICAL INFORMATION

Correct concentration of the stain is critical in producing accurate results.

STAINING PROCEDURES

17 ZIEHL-NEELEN'S STAIN

(for acid fast bacilli)

17.1 INTRODUCTION

This staining technique is used to demonstrate the presence of acid and alcohol fast bacilli (AAFB) which have waxy envelopes that make them difficult to stain and decolourise. Auramine-phenol staining is more sensitive than Ziehl-Neelsen's and is thus more suitable for assessment of smears from clinical specimens. Ziehl-Neelsen's staining provides morphological details and is more useful for confirming the presence of AAFB in positive cultures.

17.2 SAFETY CONSIDERATIONS

All suspected *Mycobacterium* species must be processed in a Class 1 exhaust protective cabinet in a Containment Level 3 room.

Follow local COSHH and risk assessments when performing all staining procedures.

Disposable gloves must be worn.

Diamond markers are not recommended and frosted slides marked with a pencil are recommended.

Smear material should be fixed by placing the slides on an electric hotplate prior to staining (65°C to 75°C). This procedure should be performed in a Class 1 exhaust protective cabinet until the smeared material is dried and fixed to the slide. They should then be placed in a rack or suitable holder.

Note: Heat fixing does not kill *Mycobacterium* species and slides should be handled with care.

See BSOP 40 - Investigation of specimens for *Mycobacterium* species.

17.3 METHOD⁶

- Flood the slide with strong carbol fuchsin
- Heat gently, and once slide is just "steaming" leave for 3 - 5 min
- Rinse well with water
- Decolourise for 2 - 3 min with a (3% v/v) acid-alcohol solution, rinse with water, then replace with fresh acid-alcohol for 3 - 4 min until the slide remains a faint pink colour
- Rinse well with water
- Counter stain with (1% w/v) methylene blue or malachite green for 30 sec
- Rinse with water and allow to dry
- Apply immersion oil and read with a transmitted light microscope

NOTE: Follow manufacturer's procedure if commercial kits are used.

17.4 INTERPRETATION

Positive: Acid fast bacilli vary from 0.5 to 10 µm in length⁶ and stain red. Some may appear beaded.

STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 25 of 29
Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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Negative: All other organisms and background material stain green if malachite green counterstain is used or blue if methylene blue counterstain is used.

17.5 QUALITY CONTROL

Positive: *Mycobacterium* species.

Negative: A proven negative smear may be used as the negative control.

17.6 TECHNICAL INFORMATION²⁷

Ziehl-Neelsen's staining is less sensitive than Auramine-phenol staining. This method provides morphological details and is more useful for confirming the presence of AAFB in positive cultures, but should not be used to "confirm" results from clinical specimens which are positive by Auramine-phenol.

STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 26 of 29
Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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ACKNOWLEDGEMENTS AND CONTACTS

This National Standard Method has been developed, reviewed and revised by the Standard Methods Working Group for Bacteriology (http://www.hpa-standardmethods.org.uk/wg_bacteriology.asp). The contributions of many individuals in clinical bacteriology laboratories and specialist organisations who have provided information and comment during the development of this document, and final editing by the Medical Editor are acknowledged.

The National Standard Methods are issued by Standards Unit, Evaluations and Standards Laboratory, Centre for Infections, Health Protection Agency London.

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STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 27 of 29
Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 28 of 29
Reference no: BSOP TP 39i1

This SOP should be used in conjunction with the series of other SOPs from the Health Protection Agency

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STAINING PROCEDURES

Issue no: 1 Issue date 14.03.07 Issued by Standards Unit, Evaluations and Standards Laboratory Page 29 of 29
Reference no: BSOP TP 39i1

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