Skin specimens

Small pieces of skin are examined for onchocerciasis (river blindness), a filarial infection of human beings in Africa, Central and South America, and parts of the eastern Mediterranean region. The worms live in nodules in the subcutaneous tissues. Corneoscleral punches are most commonly used to take bloodless skin snips.

Materials and reagents

- Coverslips
- Gauze pads
- Microscope slides, or microtitration plates
- Needle, 22 gauge
- Scalpel, razor blade, or 2 mm punch
- Ethanol, 95%
- Saline solution, isotonic (reagent no. 24) or distilled water.

Collection of specimens

Patients with nodules

Look for nodules:

- on the chest (over the ribs),
- on the hips,
- on the legs (calves),
- on the back (shoulder-blades).

The nodules are round and hard, 1–5 cm in diameter; when pushed with the fingertips they slide about under the skin. Take the specimen from the skin in the centre of the nodule.

Patients without nodules

Take the skin specimen from:

- the top of the buttocks (the upper outer part where intramuscular injections are given),
- the calf (upper outer part),
- the back (centre of shoulder-blade).

It is recommended that 6 specimens (2 from buttocks, 2 from calves, 2 from shoulder-blades) be examined.

1. If a corneoscleral punch is not available, use a sterile, disposable scalp knife, and needles or punches. If disposable equipment is not available, the apparatus must be sterilized before use on each patient. This can be done by placing the scalpel, razor blade, punch or needle in a little alcohol and setting fire to it.
2. Disinfect the skin area with a gauze pad dipped in alcohol.

1 The punch is supplied by: Karl Storz, Tuttingen, Germany.
3. Push the point of the needle 2–3 mm into the skin and lift up.

4. Place the cutting edge of the scalpel or razor blade on the stretched skin above the point of the needle. Cut with a quick stroke the piece of skin pulled up by the point of the needle, as close to the needle as possible. The specimen should be about 2–3 mm wide. It should remain attached to the tip of the needle.

The specimen should not be blood-stained. The biopsy must be bloodless to avoid possible contamination with blood parasites.

**Examination of specimens**

1. Put a drop of distilled water or saline solution on a microscope slide.
2. Put the small piece of skin in the drop and place a coverslip over it. Do not press on the skin or coverslip. If there is not enough saline to cover the skin, add some at the edge of the coverslip, so that it will run under.
3. Let the mount stand for 30 minutes, then examine under the microscope with the ×10 objective. If microfilariae are present, they can usually be seen wriggling around in the saline. If they are not present, skin snips should be allowed to stand in saline solution for 4 hours at room temperature, then re-examined.

Quantitative examination can be achieved as follows:

1. Weigh a skin specimen with a balance (1–10 mg).
2. Transfer the specimen into one hole of a microtitration plate.
3. Add 0.1 ml of saline solution.
4. Cover the plate to avoid evaporation of water and leave to incubate at ambient temperature for 24 hours.
5. Count the number of microfilariae in the saline by microscopy using the ×10 objective, and express the results per wet weight of the specimen.

Collagenase digestion of the skin over more than 24 hours may increase the sensitivity of examination in specimens from patients with low parasite loads. The digestion can also be performed on ethanol-fixed material stored at ambient temperature.
Section 2

Identification of parasite species
Intestinal parasites

*Helminths*

There are three groups of medically important helminths—nematodes (roundworms), cestodes (tapeworms), and trematodes (flukes). The usual diagnostic stages are eggs and larvae. Less frequently, adult worms like *Ascaris* and *Enterostrongylus* may be seen and segments or proglottids are used for diagnosing certain of the tapeworms. However, for the majority of the worm infections, eggs are used for identification.

**Key to identification of eggs**

The characteristics used to identify species of eggs are as follows:

1. *Size.* The length and width are measured and are generally within a specific range.
2. *Shape.* Each species has its own particular shape.
3. *Stage of development when passed.* In some species, the eggs consist of a single cell, in some, there may be several cells; and some species are usually embryonated (i.e., they contain a larva) when passed in the faeces. Occasionally, if the stool specimens are several hours or 1–2 days old, eggs may develop to more advanced stages. *Ascaris* eggs usually have only 1 cell when passed in the faeces; however, the single cell may divide and, in old specimens, eggs with 2 or 4 cells may be seen. Hookworm eggs in specimens that are several hours old may contain 16, 32, or more cells. In 12–24 hours, the egg may be embryonated and later still the larvae may hatch. Therefore, when observing the stage of development of helminth eggs, be sure that the stool specimen is freshly passed. If it is several hours or a day old, expect to see changes in the stage of development of some species. Ideally only fresh samples should be accepted for diagnosis.
4. *Thickness of the egg shell.* Some species, like *Ascaris*, have thick egg shells; others, like hookworm, have thin shells.
5. *Colour.* Some eggs are colourless (e.g., hookworm, *Enterostrongylus*), others are yellow or brown (*Ascaris*, *Trichuris*).
6. Presence of characteristics like opercula (lids), spines, plugs, hooklets, or mammillated outer coats.

If an egg, or an object that looks like an egg, is found, these features should be carefully observed in order to make a specific identification. Occasionally, atypical or distorted eggs will be seen. In such cases, it will be necessary to look for more typical forms in order to make a reliable diagnosis. Remember that more than one species of helminth may be present in an individual patient.

The following key (Fig. 3) and the diagrams of helminth eggs shown in Fig. 4, will be helpful in identifying species.

---

**REMEMBER**

Look at the size, shape, stage of development, thickness of the egg shell, and colour, and for the presence of special structures like opercula, spines, plugs, hooklets, and bumpy outer coat to identify species of eggs.
Fig. 3 Key to the identification of helminth eggs

Eggs

| Not encapsulated Plug at each end? | Encapsulated (30-50 µm x 27-50 µm) Diphyllobothrium latum (posterior end broad and round (25-35 µm x 11-22 µm)) | Length of egg < 35 µm (25-35 µm x 8-12 µm) Clonorchis sinensis (posterior end broad and round) with spine (28-30 µm x 15-17 µm) Ovophorus heterophyes (small knob at posterior end) with operculum maximal length of egg? Length of egg < 35 µm No shoulderering Metagonimus yokogawai (28-30 µm x 15-20 µm) Ovoid, gold yellow thick shell, not embryonated (58-70 µm x 40-51 µm) Diphyllobothrium latum (posterior end broad and round) golden brown, not embryonated thick shell, ovoid, with flattened operculum? Yes Paragonimus westermani (68-118 µm x 40-51 µm) Echinostoma sp. (83-120 µm x 56-90 µm) No Length of egg > 120 µm ovoid, thin shell, yellowish brown Clearly protruding plugs (49-65 µm x 20-30 µm) Lemon shaped, no clearly protruding plugs (36-45 µm x 21 µm) Lemon shaped, but slightly narrowing in the middle (36-45 µm x 20-30 µm) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Length of egg > 110 µm Spine terminal without spine, thin or thick shell? | Rounded anterior end, usually in urine or bladder biopsy (112-170 µm x 40-70 µm) Schistosoma haematobium (seem in faeces) Only in faeces |
| Length of egg < 100 µm Lateral minute spine (often invisible), in faeces (68-100 µm x 45-80 µm) Schistosoma japonicum OR Schistosoma mekongi (seem in faeces) |
| Prominent lateral spine, transparent shell (114-180 µm x 45-73 µm) Schistosoma mansoni |
| Shell thick smooth, egg symmetrical or asymmetrical? Egg dark brown (38-64 µm x 22-30 µm) Dicrocoelium lanceatum (contains miracidium) Egg colourless, shell consists of 4 layers (50-60 µm x 20-32 µm) Enterobius vermicularis (contains larva) Symmetrical Egg round to nearly round, shell with two membranes, with or without embryophore? With embryophore, pale yellow to brown, (diam.: 44-77 µm) Taenia solium OR Taenia saginata (distinguished by examination of proglottids) Without embryophore, with or without polar filaments? With polar filaments (40-60 µm x 30-50 µm) Hymenolepis nana Without polar filaments (70-86 µm x 60-80 µm) Hymenolepis diminuta |
Fig. 4. Identification of intestinal parasites—helminths

RELATIVE SIZES OF HELMINTH EGGS *

* Schistosoma mekongi and Schistosoma intercalatum have been omitted.
Worm larvae

In fresh stool specimens, the larvae seen are usually rhabditiform (= first stage) larvae from *Strongyloides stercoralis*. However, if the stool has been passed for more than 12 hours, the larvae may hatch into filariform larvae (infective stage); these must be differentiated from hookworm larvae, which may hatch in stool within 12–24 hours. The appearance of filariform larvae of *Strongyloides stercoralis* may indicate a systemic hyperinfection.

The characteristics used to separate the species are shown in Fig. 5 and in Table 4.

In iodine preparations, the genital primordium will be more visible. Iodine will kill the larvae and you will be able to see the features better. You will need to use high-power, dry magnification to see these structures.

- If you see a larva with a short mouth opening and a prominent (clearly visible) genital primordium, it is *Strongyloides*.
- If you see a larva with a long mouth opening and do not see a genital primordium, it is *hookworm*.

**Fig. 5. Helminth larvae**

<table>
<thead>
<tr>
<th>Hookworm</th>
<th>Strongyloides</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Filariform larvae</strong></td>
<td><strong>Filariform larvae</strong></td>
</tr>
<tr>
<td>Size 500 x 14–20 μm</td>
<td>Size 500 x 14–20 μm</td>
</tr>
<tr>
<td>Sheathed</td>
<td>Unsheathed</td>
</tr>
<tr>
<td>Tail tapered</td>
<td>Tail forked or blunt</td>
</tr>
<tr>
<td>Oesophagus one-third of body length with no swelling</td>
<td>Oesophagus half of body length with no swelling</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Rhabditiform larvae</strong></th>
<th><strong>Rhabditiform larvae</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Size 100–150 x 15–17 μm</td>
<td>Size 200–300 x 15–18 μm</td>
</tr>
<tr>
<td>Buccal cavity long (15 μm)</td>
<td>Buccal cavity short (4 μm)</td>
</tr>
<tr>
<td>Oesophagus one-third of body length with two swellings</td>
<td>Oesophagus one-third of body length with two swellings</td>
</tr>
<tr>
<td>Genital primordium small (7 μm)</td>
<td>Genital primordium large (22 μm)</td>
</tr>
<tr>
<td>Anal pore 80 μm from posterior end</td>
<td>Anal pore 50 μm from posterior end</td>
</tr>
</tbody>
</table>
Protozoa

Intestinal protozoa include amoebae and flagellates. Two diagnostic stages are recognized: the vegetative or trophozoite stage and the dormant cyst stage. Both stages may be passed in the faeces. Trophozoites are usually found in diarrhoeal or loose stools; cysts are usually found in formed stools. However, both stages may be present in the same stool specimen.

Trophozoites and cysts can be seen in saline mounts of fresh faeces. On occasions, species identification may require stained preparations. The types of preparation usually used for detection and identification of protozoa and the characteristics that can be seen in each type of mount are shown in Table 5.

Amoebic trophozoites

Saline wet mount

Motile amoebic trophozoites may be seen in saline mounts of freshly passed faeces. These will be slightly greenish and refractile (shiny). With high-power, dry

Table 5. Characteristics of protozoa seen in different types of preparation

<table>
<thead>
<tr>
<th></th>
<th>Temporary stains</th>
<th>Permanet stain (Trichrome)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>BMB*</td>
</tr>
<tr>
<td><strong>Amoebae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophozoites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inclusions</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nucleus</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Cysts</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclei</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Chromatoid bodies</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycogen</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Flagellates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trophozoites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Shape</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nucleus</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Cysts</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shape</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nuclei</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fibrils</td>
<td>+ f</td>
<td>+</td>
</tr>
</tbody>
</table>

* BMB stain is used only to stain living amoebic trophozoites.
* Iodine is used to stain cysts, although flagellate trophozoites may also stain.
* Permanent staining techniques are not routinely used in diagnostic laboratories, but are reserved for special circumstances as detailed in the text (e.g., diagnosis of cryptosporidiosis).
* Chromatoid bodies in amoebic cysts are better seen in saline mounts than in iodine mounts. They are best seen in permanent stained smears.
* Glycogen is dissolved during the staining process, and in permanently stained smears only a clear space (vacuole) will be seen.
* Fibrils (filaments) can sometimes be seen in saline mounts.
magnification you can see the type of motility and inclusions like red blood cells and ingested yeasts. You will not be able to see the nucleus. (Macrophages may also contain red blood cells, and may move.)

If a trophozoite moves quickly in one direction and forms pseudopods rapidly, it may be *Entamoeba histolytica*. Other species of amoebae do not usually move like this. If the trophozoite moves as described and if red blood cells are present in the cytoplasm, it can be assumed that it is *E. histolytica*. On occasions it may be necessary to use BMB to stain the nucleus for confirmation.

```
Saline mount:
DEFINITE DIRECTIONAL MOTILITY
+ INGESTED RED BLOOD CELLS
= E. histolytica
```

**BMB wet mount**

If you suspect the presence of amoebic trophozoites, examine a BMB mount (see Table 5). The trophozoites may curl up and no longer be motile but the nucleus and inclusions will stain dark blue, while the cytoplasm will stain light blue. Look for peripheral nuclear chromatin granules (granules in the membrane around the nucleus); if present, it is an *Entamoeba* species. If there is no peripheral chromatin, it is not *Entamoeba*. When peripheral nuclear chromatin is seen, you must identify the species. Use the “Key for the identification of amoebic trophozoites in stained smears” (Fig. 6).

*E. histolytica* trophozoites are about 12–40 μm long. The nucleus will appear as a dark blue circle with a dark dot (the karyosome) towards the centre. If the peripheral nuclear chromatin granules are fine and even and there is a small central karyosome, it is probably *E. histolytica*, but you must look at several organisms to be sure. If red blood cells are seen in the cytoplasm, this will help confirm the diagnosis. The blood cells will be dark blue in BMB stained mounts.

*Entamoeba coli* is about the same size as *E. histolytica* and also has a nucleus with peripheral chromatin. It has rough, coarse cytoplasm, often containing bacteria or moulds. The peripheral nuclear chromatin is irregular and the karyosome is not in the centre.

**Trichrome stained smears**

In stained smears, trophozoites may be round, elongated, or irregular in shape. Amoebic trophozoites usually stain green or blue-green with purple or red nuclei. The nuclei of *Entamoeba* species have peripheral chromatin granules (as described for BMB mounts) which may appear as a circle, or as a beaded circle. The karyosome may be seen as a red or purple dot within the nucleus. In *E. histolytica*, the karyosome is usually in the centre of the nucleus, but may occasionally be eccentric and you must look at several organisms to decide whether the species is *E. histolytica* or *E. coli*.

In addition to amoebic trophozoites, other cells resembling trophozoites may be seen. These must be differentiated from the amoebae. Compare the objects you see in the stained smears with the figures in the key and on p. 79, "Problems of
identification”, to decide whether they are amoebic trophozoites, or tissue cells, or some other structure.

Trophozoites may be identified as *E. histolytica* if you see:

- peripheral nuclear chromatin
  
  *and*
  
  - red blood cells in cytoplasm

  **OR**

  - uniform peripheral nuclear chromatin
    
    *and*
    
    - small central karyosome in nucleus
      
      *and*
      
      - finely granular, smooth cytoplasm
        
        *and*
        
        - length of trophozoite = 2–6 × red blood cell diameter

Amoebic cysts

Accurate measurement of cysts is essential for correct identification.

**Saline wet mount**

When examining with the ×40 objective, focus up and down and look for shiny round objects with a diameter roughly equal to 1–3 red blood cells. Look also for chromatoid bodies (rod-shaped structures), which are best seen with the high-power objective. They are more distinct in the saline mounts than in the iodine mounts. These bodies are characteristic in appearance, and occur in *E. histolytica* and *E. coli* cysts. In *E. histolytica*, the rod-shaped bodies have blunt rounded ends, in contrast to the pointed ends of the chromatoid bodies in *E. coli*. These structures are seen less frequently in cysts of *E. coli* than in those of *E. histolytica*.

Nuclei are not easily visible in saline, but are well seen in iodine mounts. The appearance of the nucleus is important in differentiating species of amoebae. Therefore, if cysts (or things that look like cysts) are seen in the saline mount, examine the iodine mount.

Measure any cysts found.

**Iodine wet mount**

Focus on the mount with the ×40 objective and search for cysts. When you find cysts, or objects that look like cysts, switch to the high-power dry objective to see the details necessary to identify the species. Measure the cysts.

Use the "Key to identification of amoebic and flagellate cysts" in iodine mounts and stained smears (Fig. 7) and the following description to identify the species of cysts found.

*E. histolytica* cysts measure 10–15 μm in diameter. Mature *E. histolytica* cysts have four nuclei that have uniform peripheral nuclear chromatin and a central karyosome. Usually the nuclei are not in the same plane, so it is necessary to focus up and down to see all four. Carefully move the objective up until the cyst is just out of focus.
Fig. 6. Key for the identification of amoebic trophozoites in stained smears

Trophozoite with or without peripheral nuclear chromatin

- with peripheral nuclear chromatin
  - coarse granulated cytoplasm, bacteria & yeast, no red blood cells
  - finely granulated cytoplasm, red blood cells absent or present, no bacteria

- without peripheral nuclear chromatin
  - one nucleus in all trophozoites
    - nucleus with peripheral granules
    - nucleus without peripheral granules, large endosome

- two nuclei in more than 50% of trophozoites
  - large chromatin mass in clear space

Entamoeba coli
  size >15 μm
  - small endosome, peripheral granules in nucleus regularly arranged

- size 8 -15 μm
- size 15 - 60 μm

Dientamoeba fragilis
  size 5 - 15 μm
  - large irregular endosome

Iodamoeba bütschlii

Endolimax nana

Entamoeba hartmanni

Entamoeba histolytica

Focus carefully and continue to move the objective slowly downward to see all levels of the cyst. Count the nuclei as they become visible.

Sometimes immature E. histolytica cysts are seen. These may have 1, 2, or 4 nuclei. If only one nucleus is present, it is usually quite large. Often cysts with one nucleus have large glycogen masses and several small chromatoid masses. (The chromatoid masses have not yet formed into the typical rod-shaped bodies.) Measure the cyst.

E. coli cysts are usually larger than those of E. histolytica (15–30 μm). E. coli cysts have eight nuclei that have irregular peripheral chromatin and karyosomes that are not central. They are usually on different planes, so you must focus up and down carefully to see and count them.
Fig. 7. Key to identification of amoebic and flagellate cysts

- **Cyst**
  - With filaments inside flagellate cyst
    - Pear-shaped, 1 nucleus, size 4 - 7 μm
    - Lemon-shaped, 1 nucleus, size 5 - 9 μm
    - Oval cyst, thick wall
    - Retortamonas intestinalis
    - Chilomastix
  - Without filaments, amoebic cyst
    - Peripheral nuclear chromatin absent
      - 4 nuclei, large chromatin masses
      - Large eccentric nucleus, iodine-staining vacuole
      - Endolimax nana
      - Iodamoeba bütschlii
    - Peripheral nuclear chromatin present
      - 1, 2 or 4 nuclei, glycogen mass
      - 2 or 4 nuclei, glycogen mass
      - Entamoeba hartmanni
      - Entamoeba coli
  - Enteromonas hominis
  - Giardia
Immature *E. coli* cysts may be seen and should not be confused with *E. histolytica*. The most commonly seen immature form is a cyst with two nuclei (*E. coli* cysts with one nucleus are rarely seen), a large glycogen mass and several small chromatoid masses. As with *E. histolytica*, the masses have not yet formed into typical chromatoid bodies. Accurate measurement will help distinguish cysts of *E. coli* from those of *E. histolytica*.

Cysts from *E. hartmanni* are similar to those from *E. histolytica*. However, they are smaller (7–9 μm).

In iodine preparations, cysts of *Iodamoeba bütschlii* show a compact glycogen body, and this differentiates them from *E. histolytica*. Cysts of *I. bütschlii* have no chromatoid bodies and contain a single nucleus.

If at first you see immature cysts, look for mature forms. Mature cysts can usually be found and the species identified. If you see cysts with five or more nuclei, they are *E. coli*.

In recent years, several cases of primary amoebic meningoencephalitis caused by free-living amoebae, *Acanthamoeba* spp and *Naegleria* spp, have been reported throughout the world. Most infections have been traced to polluted water. Laboratory diagnosis is made by microscopical examination of the purulent cerebrospinal fluid containing polymorphonuclear cells but no bacteria. The amoebae can be seen in the Giemsa stained smear.

*Blastocystis hominis*, a protozoan that is also found in stools, can be distinguished from amoebic cysts because the centre stains green (or sometimes clear) with refractile granules around the edge.

Flagellates

**Saline wet mount for detection and identification of trophozoites**

Flagellate trophozoites (Fig. 8) are best identified by the way they move in saline mounts. The flagella will usually not be seen and nuclei will not be visible.

- *Giardia intestinalis* trophozoites (pathogenic) move like a “falling leaf”; they flip back and forth.
- *Chilomastix mesnili* trophozoites (nonpathogenic) rotate as they move.
- *Trichomonas hominis* trophozoites (rarely pathogenic) have a jerky movement.

**NOTE**

BMB does not stain flagellate trophozoites, so a BMB mount is not of value in their diagnosis.

**Iodine wet mount for detection and identification of cysts**

Iodine solutions are used primarily to stain cysts and make it possible to see the structure of the nuclei. Use the “Key to identification of amoebic and flagellate cysts” (Fig. 7) for identification.
Fig. 8. Key to identification of flagellate trophozoites in stained smears

- Trophozoite with 2 or more flagellae
  - 2 flagellae, 1 nucleus, size 4 - 9 μm
  - 4 flagellae, size 4 - 8 μm, 1 nucleus
  - 5 flagellae, 1 nucleus, undulated membrane (rarely visible), size 8 - 20 μm
  - 5 flagellae, 1 nucleus, undulated membrane (rarely visible), size 8 - 20 μm
  - Pearson-shaped trophozoite, 4 pairs of flagellae, 2 nuclei, parabasal bodies, size 10 - 20 μm

- Retortamonas intestinalis
- Enteromonas hominis
- Chilomastix mesnilli
- Trichomonas hominis
- Giardia lamblia

Note: *Trichomonas hominis* does not have a cyst stage.

*Giardia intestinalis* trophozoites and cysts in stained smears

*Giardia* organisms are characteristic and usually match the illustrations in manuals and text books. They are not hard to identify in stained smears.

The other species of intestinal flagellates are rarely pathogenic.

*Balantidium coli*

This is the only ciliate parasite of human beings and infections are not common. It has not been discussed elsewhere in this manual because human cases are rare. (It is primarily a parasite of swine and monkeys.) However, it may occasionally be seen in human stool specimens, so a brief description is included here.

*Balantidium coli* has a trophozoite and a cyst stage. The trophozoites are large (50-200 μm long by 40-70 μm wide) and very active, and can easily be seen in saline mounts using the low-power objective (see p. 78). They are covered with short hairs (cilia) that beat rapidly and cause the organisms to move quickly. They die very quickly outside the body, so stool specimens must be examined within 1 hour after passage.

The cysts are 45-75 μm in diameter. Neither the trophozoites nor the cysts stain
well with iodine or permanent stains. The best technique, therefore, is the saline wet mount.

Isospora belli and Cryptosporidium spp

*Isospora belli* and *Cryptosporidium* are intestinal coccidian parasites. Human infection with *Isospora belli* is uncommon, rarely serious and often asymptomatic. Infection with *Cryptosporidium* is a significant cause of diarrhoea in children below the age of 5 years and is particularly severe in immunocompromised patients.

**Laboratory diagnosis**

Oocysts of *Isospora belli* can be detected in direct faecal smears. If required the oocysts can be concentrated by the formalin–ether technique (see Section 1, pp. 16–17).

Mix a small amount of faeces in saline at the end of a slide with a small amount of iodine solution at the other end. Oocysts from *Isospora belli* are oval (about 32 × 16 μm) and contain a central individual mass of protoplasm (see diagrams below).

NOTE
Prepare iodine solution by mixing 10 ml of Lugol’s iodine (reagent no. 16) with 10 ml of 25% v/v acetic acid. This solution gives good staining of nuclei.

*Cryptosporidium* oocysts are examined in stained faecal smears (see Section 1, pp. 17–18).

**Toxoplasma gondii**

*Toxoplasma gondii* is an animal parasite that causes toxoplasmosis. Human toxoplasmosis is often asymptomatic. It can cause fever, a rash, enlargement of lymph glands, and lymphocytosis. The most serious form of human infection is congenital toxoplasmosis, which often causes severe cerebral damage in the fetus. Infection occurring in early pregnancy may result in abortion, while infection in late pregnancy may cause symptoms of infection in the infant 2–3 months after birth. Clinical manifestations of *Toxoplasma* infection often occur in patients with acquired immunodeficiency syndrome (AIDS).
Laboratory diagnosis of toxoplasmosis is by:

1. Serological tests, which include the Sabin–Feldman dye test, the indirect fluorescent antibody test (IFAT), the indirect haemagglutination test (IHA), a complement-fixation test (CFT), and more recently the enzyme-linked immunosorbent assay (ELISA), which is claimed to be more specific. Further discussion is outside the scope of this manual.

2. Microscopical investigation is occasionally useful for diagnosis of an acute infection, using Giemsa or Field’s stained preparations of lymph node aspirates, bone marrow aspirates, cerebrospinal fluid, peritoneal, or pleural fluids.

The parasites are crescent-shaped and small (3 × 7 μm). One end is rounded, the other end is pointed. The cytoplasm stains blue, the dark red-stained nucleus is at the rounded end. Occasionally the organisms may appear round and resemble *Leishmania* amastigotes in tissue sections.

**Problems of identification**

Many things in stool specimens look like parasites but are not.

Epithelial cells and macrophages can be confused with amoebic trophozoites, especially macrophages that show slight amoeboid movement and may contain red blood cells. The nuclei, which can be seen in BM stained mounts, appear much larger than nuclei of amoebae and usually contain several granules or particles of chromatin (see below).

![Epithelial cells and Macrophage](image)

Pus cells can be confused with amoebic cysts. The nuclei appear as 3 or 4 rings and usually stain heavily. The cytoplasm is ragged and the cell membrane is often not seen. Amoebic cysts have a distinct cell wall.

Hairs and fibres may be confused with larvae. However, hairs and fibres do not have the same internal structure as larvae.

Plant cells (e.g., moulds or yeasts) can be confused with cysts or eggs. Plant cells usually have a thick wall; cysts have a thin wall. Yeasts and moulds are usually smaller than amoebic cysts and do not have nuclei such as are seen in amoebic cysts.

**NOTE**

*E. histolytica* trophozoites and cysts are often not typical, and several organisms should be examined to be certain of the species.

**IDENTIFICATION MAY NEED CAREFUL STUDY OF SEVERAL CYSTS OR TROPHozoITES. MEASUREMENT IS ESSENTIAL**
Blood parasites

Malaria

Identification of malaria parasites in thin blood films

In examining thin blood films for malaria, you must look at the infected red blood cells and the parasites inside the cells.

Infected red cells

- Look at the size of the infected red cells.
- Is Schüffner’s stippling present or not present?

<table>
<thead>
<tr>
<th>Infected red cells</th>
<th>→ Suggests P. vivax or P. ovale.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enlarged:</td>
<td>Look at parasites.</td>
</tr>
<tr>
<td>Schüffner’s stippling present¹</td>
<td></td>
</tr>
<tr>
<td>Normal:</td>
<td>P. malariae or P. falciparum</td>
</tr>
<tr>
<td>Schüffner’s stippling not present</td>
<td></td>
</tr>
<tr>
<td>(infected cells may be smaller than normal in P. malariae infections)</td>
<td></td>
</tr>
</tbody>
</table>

Parasites

Rings of the four main species may look alike. If you see rings, look for older stages. In patients with P. falciparum only rings are usually seen; older stages are present only in severe infections.

NOTE
If Plasmodium falciparum is seen, the percentage parasitaemia (number of infected red cells per hundred) must be reported.

See Tables 6 and 7, and Fig. 9, 10, 11, and 12, for the diagnostic features to look for in thin and thick blood films.²

Identification of malaria parasites in thick blood films

In stained thick blood films, the red blood cells are lysed, so diagnosis is based on the appearance of the parasite. In thick films, organisms tend to be more compact and denser than in thin films.

¹ In poorly stained slides, Schüffner’s dots may not be visible, so it is essential that correct staining methods are used. Also Schüffner’s dots may not be present in the early rings of P. vivax or P. ovale.
² Table 7 and Fig. 9–13 are taken from Bench aids for the diagnosis of malaria, Plates No. 1–8, Geneva, World Health Organization, 1968.
## Table 6. Morphological features of malaria parasites in thin blood films

<table>
<thead>
<tr>
<th>Stage</th>
<th><em>P. vivax</em></th>
<th><em>P. ovale</em></th>
<th><em>P. malariae</em></th>
<th><em>P. falciparum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected red cell</td>
<td>Enlarged; Schüffner’s dots present</td>
<td>Enlarged; may be oval with filmbriae; Schüffner’s dots present</td>
<td>Size normal or smaller than normal</td>
<td>Size normal; Maurer’s clefts may be seen</td>
</tr>
<tr>
<td>Ring stage (early trophozoite)</td>
<td>Quite large; one or two chromatin dots; may be two rings per rbc</td>
<td>Compact; two rings per rbc; rare</td>
<td>Compact; two rings per rbc; rare</td>
<td>Small and delicate; often two chromatin dots; often two or more rings per rbc; accolé forms common</td>
</tr>
<tr>
<td>Late trophozoite</td>
<td>Large; amoeboid; pigment seen as fine rods</td>
<td>Small; not amoeboid; pigment coarse</td>
<td>Small; compact; often band-shaped; pigment coarse</td>
<td>Moderate size; usually compact; pigment granular</td>
</tr>
<tr>
<td>Mature schizonte</td>
<td>Large; merozoites large (12-24 in number); coalescent pigment</td>
<td>Smaller than <em>P. vivax</em>; 6-12 merozoites; pigment darker than in <em>P. vivax</em></td>
<td>Small but merozoites (6-12) large; “daisy head” appearance characteristic; pigment coarse</td>
<td>Rare in peripheral blood; merozoites (8-26) small; single pigment mass</td>
</tr>
<tr>
<td>Gametocytes</td>
<td>Spherical; compact; single nucleus; pigment diffuse and coarse</td>
<td>Similar to, but smaller than, <em>P. vivax</em></td>
<td>Resemble <em>P. vivax</em> but smaller, less numerous and Schüffner’s dots absent</td>
<td>Crescent shaped; single nucleus</td>
</tr>
</tbody>
</table>

## Methods of counting malaria parasites in thick blood films

### Parasites per microlitre

The following is a practical method of adequate accuracy. It is based on the number of parasites per µl of blood in a thick film, these being counted in relation to a predetermined number of leukocytes. An average of 8000 leukocytes per µl is taken as the standard. Despite inaccuracies due to variations in the number of leukocytes between individuals in normal health, and greater variations in ill health, this standard allows for reasonable comparisons. Before counting begins, the equivalent of 0.25 µl of blood (about 100 fields, using a x7 ocular and a x100 oil-immersion objective) should be examined in the thick film to determine the parasite species and the stages that are present. When this has been done, the following counting method should be employed for positive blood films.

1. Two tally counters are required to count parasites and leukocytes separately.
2. (a) If, after 200 leukocytes have been counted, 10 or more parasites have been identified, record the results in the record form, showing the number of parasites per 200 leukocytes.
   (b) If, after 200 leukocytes have been counted, 9 or fewer parasites have been counted, continue counting until 500 leukocytes have been counted and record the number of parasites per 500 leukocytes.
3. In each case, the parasite count in relation to the leukocyte count can be converted to parasites per µl by the simple mathematical formula:

   \[
   \frac{\text{No. of parasites} \times 8000}{\text{No. of leukocytes}} = \text{No. of parasites per µl}
   \]

This means that if 200 leukocytes are counted, the number of parasites counted is multiplied by 40, and if 500 leukocytes are counted the number of parasites is multiplied by 16.

(continued on p. 88)
## Table 7. Species identification of malaria parasites in Giemsa-stained thick blood films

<table>
<thead>
<tr>
<th>Species</th>
<th>Stage of parasite in peripheral blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trophozoite</td>
</tr>
<tr>
<td><em>Plasmodium falciparum</em></td>
<td>Young, growing trophozoites and/or immature gametocytes usually seen.</td>
</tr>
<tr>
<td><em>P. vivax</em></td>
<td>All stages seen, Schüffner’s stippling in growing stages, host red cells, especially at budding.</td>
</tr>
<tr>
<td><em>P. ovale</em></td>
<td>All stages seen, prominent Schüffner’s stippling in growing stages, host red cells, especially at budding.</td>
</tr>
<tr>
<td><em>P. malariae</em></td>
<td>All stages seen.</td>
</tr>
</tbody>
</table>

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Fig. 9. *Plasmodium falciparum*
Fig. 10. *Plasmodium vivax*
Fig. 11. *Plasmodium malariae*
Fig. 12. *Plasmodium ovale*
Fig. 13. Appearance of cellular elements in Giemsa-stained thin and thick blood films: effect of pH on Giemsa staining of malaria parasites

Thin Film LEUKOCYTES Thick Film
N = Neutrophil, E = Eosinophil, M = Monocyte, L = Lymphocyte, P = Platelets

Thin Film ERYTHROCYTES Thick Film
NC = Normocyte, MC = Microcyte, PM = Polychromatic macrocyte, PC = Poikilocyte, PB = Punctate basophilia, CR = Cabot’s ring, HJ = Howell-Jolly bodies, RC = Reticular ‘clouds’ and chromatoid bodies in severe anaemia

<table>
<thead>
<tr>
<th>pH</th>
<th>6.4</th>
<th>6.8</th>
<th>7.2</th>
<th>7.6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

MALARIA STAINING AND pH
4. It is normal practice to count all the species present and to include both sexual and asexual parasites together. Occasionally, a separate count is made of the gametocytes of *Plasmodium falciparum*, but when this is done, they should still be included in the general parasite count. It is rarely possible to separate the gametocytes of *P. vivax* or *P. malariae* from the asexual parasites with sufficient accuracy to justify a gametocyte count.

**The plus system**

A simpler method of enumerating parasites in thick blood films is to use the plus system. This indicates the relative parasite count and entails using a code of 1–4 pluses, as follows:

- ++ = 1–10 parasites per 100 thick-film fields
- +++ = 11–100 parasites per 100 thick-film fields
- ++++ = 1–10 parasites per thick-film field
- ++++ = more than 10 parasites per thick-film field

This system should be used only when it is not possible to undertake the more acceptable parasite count per μl of blood.

**Things that may be confused with malaria parasites**

In blood films, the following things may look like malaria parasites (see also Fig. 13):

- platelets adhering to red blood cells in thin films,
- clumps of platelets,
- fragments of white cells in thick films,
- precipitated stain on red blood cells,
- debris from the patient’s skin, dust, bacteria, yeasts, spores, and other organisms that get on the blood film while it is drying,
- algae and other organisms from contaminated staining solutions.

All of these things may look like malaria parasites in stained blood films and can be mistakenly identified as malaria. Usually, however, they do not have all three components of a malaria organism: blue staining cytoplasm, red or purple staining chromatin, and brown or black pigment. Except for rings (which lack pigment), you should see all three characteristics before you identify any structure as a parasite. If you are uncertain whether a particular object or structure is a malaria parasite, look for more typical organisms. If you do not find things that are clearly malaria parasites, report the film as “No malaria parasites found,” preferably after making, staining, and examining another film. Diagrams of some of the things that can be confused with malaria parasites are included in Fig. 13.

You should keep the blood films protected while they are drying to prevent dust and organisms, such as spores or bacteria, from contaminating the film. Also, keep the staining solutions (stock stain and buffered water) stoppered or closed so that dust and organisms in the air do not get into them, and then on to the film when you stain it.

**Trypanosoma**

Trypanosomes may be distorted in thick films. If organisms cannot be recognized in thick films, look for them in the thicker areas of the thin film. They are between the red blood cells. Look at the length, the shape, and the size of the kinetoplast of the parasites.
Remember

1. In Africa, the two *Trypanosoma* subspecies that infect man are identical. You cannot determine the subspecies from the appearance in stained films.
2. In South and Central America, *T. cruzi* and *T. rangeli* must be distinguished from each other.
3. *T. rangeli* is longer than *T. cruzi*.
4. *T. cruzi* has a very large, prominent kinetoplast and is often seen in C, U, or S shapes in stained films.

**Microfilariae**

The following characteristics are used for the identification of microfilariae (see Fig. 14):

- presence or absence of a sheath,
- presence or absence of nuclei in the tip of the tail,
- innerbody—can or cannot be demonstrated,
- size of the microfilaria.

Sometimes other characteristics have to be used to identify species. Unfortunately, not all the diagnostic features can be seen in Giemsa stained preparations, so occasionally special stains like Delafield's haematoxylin must be used to demonstrate them.

**Remember**

1. Use a ×10 objective to locate microfilariae.
2. Search the blood film systematically.
3. Use high-power, dry (×40) or oil-immersion objectives to examine microfilariae for specific identification.

A. *Wuchereria bancrofti* (Asia, Africa, Central and South America, West Indies)

Sheath may or may not stain with Giemsa; does stain with haematoxylin stains. Discrete nuclei. Empty space between the nuclei and the body wall. No nuclei in tip of tail. Innerbody rarely visible in Giemsa. Does not stain with haematoxylin. Cephalic space as long as it is broad. Tip of tail may be bent back underneath the body. Found in blood.

B. *Brugia malayi* (South-East Asia, India)

Kinked microfilaria. Sheath stains deep pink with Giemsa stain. Does stain with haematoxylin stains.
Fig. 14. Microfilariae found in human beings

- **With a sheath**
  - Cephalic space
  - Anal pore
  - R1 cell
  - R2 cell
  - Excretory pore
  - Excretory cell
  - Nerve ring

- **Without a sheath**

**In blood**
- With sheath
  - Nuclei extending to tip of tail
  - Tail swollen with 2 distinct nuclei, cephalic space twice as long as broad

- Without sheath
  - Nuclei not extending to tip of tail, cephalic space as long as broad
  - Tail uniform, cephalic space as long as broad

**In skin**
- With sheath
  - Nuclei extending to tip of tail, tail blunt
  - Small, thin filaria

- Without sheath
  - Nuclei not extending to tip of tail, small, thin filaria, hooked tail
  - Thick filaria

**Species**
- *Brugia malayi*
- *Loa loa*
- *Wuchereria bancrofti*
- *Mansonella perstans*
- *Mansonella ozzardi*
- *Mansonella streptocerca*
- *Onchocerca volvulus*
Nuclei crowded and fill the whole body. Empty space between nuclei and body wall.
Cephalic space twice as long as it is broad.
Innerbody may or may not stain; when it does, it is prominent. Found in blood.

C. *Loa loa* (only in Africa)
Kinked and sheathed microfilaria.
Sheath does not stain with Giemsa stain; does stain with haematoxylin stains.
Nuclei crowded extending to tip of tail; tip of tail tapers. Cephalic space as long as it is broad.
Innerbody does not usually stain. Found in blood.

D. *Mansonella perstans* (Africa and South America)
Small, thin microfilaria.
Does not have a sheath.
Nuclei extend to end of tail; last nucleus bigger; tip of tail is blunt.
Nuclei stain deeply and "run together". Found in blood.

E. *Mansonella ozzardi* (Central and South America)
Small thin microfilaria.
Does not have a sheath.
Nuclei do not extend to end of tail; tip of tail tapers.
Stains very lightly; tip of tail difficult to see. Found in blood and skin.

F. *Mansonella streptocerca* (West and Central Africa)
Small, thin, microfilaria.
Does not have a sheath.
Nuclei extend to end of tail.
Tail is hooked; its tip is rounded or forked. Found only in skin.

G. *Onchocerca volvulus*
Thick microfilaria. Does not have a sheath.
Head often spatulate.
Nuclei do not extend to tip of tail.
Found only in skin.
Bibliography


¹ Available on request from Division of Control of Tropical Diseases, World Health Organization, 1211 Geneva 27, Switzerland.
² Available on request from Diarrhoeal Diseases Control, World Health Organization, 1211 Geneva 27, Switzerland.
ANNEX 1

Equipment and materials for diagnostic parasitology in health centres and district hospital laboratories

**Equipment**

Microscopes with adequate illumination, preferably built-in light sources. Microscopes with adjustable mirrors will be needed if the light source is separate, e.g., sunlight or lamp. Microscopes should have an adjustable iris diaphragm and substage condenser; ×10 oculars, and ×10, ×40, and oil-immersion objectives.

Centrifuge—either table model or floor model—with head and cups to hold 15-ml centrifuge tubes. Sealed buckets are preferred.

Microhaematocrit centrifuge.

Refrigerator, 4–5 °C.

**Materials**

Adhesive tape, transparent, 2 cm wide, for anal swabs
Applicator sticks, wooden
Block, wooden, with grooves to hold slides
Bottles, 1000 ml
Bottles, small, 25 ml, 30 ml, 50 ml, and 100 ml, with rubber stoppers or dropper-top and screw-cap
Bottles, dispensing or plastic “squeeze”, 100 ml, 250 ml, and 500 ml
Bottles, glass, with glass stoppers, 250 ml, 1000 ml
Centrifuge tubes, conical, graduated, 15 ml
Cotton swabs
Cellophane, wettable, 40–50 μm thick, 25 × 30–35 mm strips
Coverslips, 20–22 mm square
Cylinders, graduated, 10 ml, 25 ml, 50 ml, 100 ml, and 1000 ml
Dishes for staining
Dropping bottles for saline, iodine, buffered methylene blue, and methanol (for blood staining).
Filter, brass wire, 40 mesh (425 μm) 7.5 cm diameter
Flasks, 100 ml, 250 ml, 500 ml, 1000 ml, and 5000 ml
Flask, conical, for urine collection
Forceps
Funnel
Gauze pads, sterile, for cleaning fingers
Glass rod
Hot plate (metal plate with spirit lamp beneath)
Immersion oil, low viscosity
Labelling pens or markers
Labels
Lancets, disposable, individual
Membrane filter, 12 μm or 15 μm and filter holder
Microscope slides—25 × 75 mm, 50 × 75 mm (optional)
Needle for splenic aspiration
Needle, 25 gauge, 0.5 × 16 mm, for subcutaneous injection
Needle for venepuncture
Petroleum jelly
Pipettes, capillary, about 14 cm long with rubber bulbs (for concentration procedure and general use)
Pipettes, serological, 1 ml, 5 ml, 10 ml capacity with rubber bulbs
Pipettes, Pasteur, with rubber bulbs
Pipettes, Sahli
Rack to hold centrifuge tubes
Rack for staining, glass rods or small jars
Rack, wooden, for drying blood smears
Reagent tubes
Register or record forms
Scalpel or razor blades
Screen, stainless steel, nylon or plastic, 60–105 mesh
Stoppers, rubber, to fit 15-ml centrifuge tubes (usually size 0 or size 1)
Syringe, plastic, 5 or 10 ml
Template, stainless steel, plastic or cardboard, size 20–50 mm
Test tubes, small, 100 × 13 mm, with cotton plugs or screw-caps
Test tubes, large, 150 × 13 mm, for boiling
Timer (clock)
Toilet paper, facial tissue or lens paper
Tongue depressor (or plastic spoons)
Towel, cotton, lint-free
Towels, paper or sponges
 Tubes, capillary
 Vials, 20 ml, with tight-fitting screw-caps

Chemicals and solutions for the preparation of reagents (preparation is described in Annex 2)

Disodium hydrogen phosphate (Na₂HPO₄) (for buffered water)
Distilled water
Dyes (stain powder):
- Azur 1—for Field's stain A
- Chromotrope 2R—for trichrome stain
- Eosin—for Field's stain B
- Light Green SF—for trichrome stain
- Fast Green FCF—for trichrome stain
- Malachite green
- Methylene blue

Ethanol (ethyl alcohol), 70%, 95%, 100% (absolute)
Ether, anaesthesia or technical grade, or ethyl acetate
Formalin (formaldehyde)
Glacial acetic acid
Glycerol
Hydrochloric acid, concentrated (HCl)
Iodine crystals (I₂)
Isopropanol (isopropyl alcohol)
Mercuric chloride crystals (HgCl₂)
Methanol (methyl alcohol)
Phenol crystals (carbolic acid)
Phosphotungstic acid crystals [H₃[PO₄(W₁₂O₃₆)], 5H₂O]
Polyvinyl alcohol (PVA)
Potassium iodide crystals (KI)
Potassium dihydrogen phosphate (KH₂PO₄) (for buffered water)
Sodium acetate powder (CH₃COONa) or sodium acetate crystals
(CH₃COONa . 3H₂O)
Sodium chloride (NaCl)
Sodium citrate crystals (C₆H₇O₇Na₃ . 2H₂O)

Stains:
- Buffered methylene blue
- Field’s stain A
- Field’s stain B
- Giemsa, stock
- Glycerol–malachite green; glycerol–methylene blue
- Delafield’s haematoxylin, stock
- Safranin solution
- Trichrome, stock

Xylene

If Delafield’s haematoxylin is prepared in the laboratory the following will be needed:
- aluminium ammonium sulfate to prepare a saturated aluminium ammonium sulfate solution
- haematoxylin powder or crystals.
ANNEX 2
Reagents and solutions and their preparation

**Acetic acid alcohol destain solution (No. 1)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol, 95%</td>
<td>600 ml</td>
</tr>
<tr>
<td>Glacial acetic acid (CH₃COOH)</td>
<td>4 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>350 ml</td>
</tr>
</tbody>
</table>

Pour the 95% ethanol into a 1000-ml graduated cylinder. Add the distilled water making a total quantity of 950 ml. Pour into a 1000 ml bottle. Add the glacial acetic acid and mix.

Label the bottle: ACID ALCOHOL DESTAIN and write the date. Store on a shelf or in a cabinet. This solution will remain good for a year or more.

**Warning:** Glacial acetic acid is highly corrosive.

**Buffered methylene blue solution (No. 2)**

**Solution A (acetic acid solution)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glacial acetic acid (CH₃COOH)</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>98.8 ml</td>
</tr>
</tbody>
</table>

Measure the 98.8 ml of distilled water into a clean flask or bottle, add the 1.2 ml of glacial acetic acid and mix well. Store in a clean bottle.

**Warning:** Glacial acetic acid is highly corrosive.

**Solution B (sodium acetate solution)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium acetate (CH₃COONa)</td>
<td>1.6 g</td>
</tr>
<tr>
<td>(If you are using crystalline sodium acetate (CH₃COONa.3H₂O)</td>
<td>2.6 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Measure the 100 ml of distilled water into a small flask. Weigh out 1.6 g of sodium acetate (or 2.6 g of crystalline sodium acetate) and dissolve in water; mix thoroughly. Store in a clean bottle.

**Working solution (for stain)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A (acetic acid solution)</td>
<td>46.3 ml</td>
</tr>
<tr>
<td>Solution B (sodium acetate solution)</td>
<td>3.7 ml</td>
</tr>
<tr>
<td>Methylene blue dye</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

Pour the 50 ml of distilled water into a flask or beaker of about 150 or 200 ml capacity. Add solutions A and B and mix well. Add about 0.5 g of methylene blue dye and shake or stir until the dye has dissolved. If all the dye does not dissolve in about 15 minutes, the solution should be filtered. Pour or filter into a clean stoppered bottle and label: BUFFERED METHYLENE BLUE SOLUTION. Write
the date on the label. This solution will keep indefinitely. If stain particles settle in
the bottom of the bottle, the solution should be filtered. Pour a small amount
(20 ml) into a dispensing or dropping-bottle for ready use. The dropping-bottle
should have a pipette with a rubber bulb.

Note: Acetate buffers with a pH of 3.6 are most satisfactory for this stain.

**Buffer solutions for malaria staining (No. 3)**

A phosphate buffer solution, balanced to pH 7.2, is essential for Giemsa staining of
malaria parasites.

**Preparation of a solution for daily use**

1. Dissolve 1.0 g of anhydrous disodium hydrogen phosphate (Na₂HPO₄) and
   0.7 g of potassium dihydrogen phosphate (KH₂PO₄) in 1 litre of distilled or
deionized water. Filtered rainwater or even tap-water may be used if no other is
available.
2. Check the pH with a pH meter or a colour-based indicator, such as the Lovibond
   comparator.
3. If the pH is below 7.2, add small quantities of a 2% Na₂HPO₄ solution; if it is
   above 7.2, add small quantities of a 2% solution of KH₂PO₄.
4. When balanced to pH 7.2, store in a tightly stoppered bottle, preferably of dark
   glass, in a cool place away from direct sunlight.

This solution will remain good for some weeks, but needs to be regularly checked
to ensure that growths or moulds do not become established. This may be done by
shaking the solution; if cloudy, discard.

**Preparation of a concentrated stock solution (useful for field trips or dispatch to distant stations)**

1. Dissolve 3.0 g of anhydrous Na₂HPO₄ and 2.1 g of KH₂PO₄ in 25 ml of
distilled or deionized water.
2. Adjust the pH to 7.2 in the way described in point 3 above.
3. Store in a dark bottle away from direct sunlight; this will remain good for several
weeks.
4. To make up a working solution, dilute 1 ml of the concentrate with 20 ml of
distilled or deionized water.

**Preparation of preweighed packs**

The two phosphate salts can be preweighed and placed together in a clearly
labelled, tightly stoppered tube, bottle, or well sealed plastic bag, and stored in a
screw-capped jar. To make the solution, add the contents of the packet to 1 litre of
water, and adjust the pH to 7.2.

**Carbol–fuchsin solution (No. 4)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic fuchsin</td>
<td>10 g</td>
</tr>
<tr>
<td>Ethanol, absolute, technical grade</td>
<td>100 ml</td>
</tr>
<tr>
<td>Phenol (carbol)</td>
<td>50 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 litre</td>
</tr>
</tbody>
</table>
Preparation

1. Weigh the basic fuchsin powder and transfer it into a 1.5-litre bottle.
2. Add 100 ml of absolute ethanol and dissolve the dye completely.
3. Weigh the phenol in a beaker and dissolve in a small volume of distilled water.
4. Add the aqueous phenol solution to the dye solution and mix well.
5. Add the rest of the water, mix well, and label the bottle. The dye solution will be stable indefinitely.

Note: Ethanol is flammable. Phenol is toxic and corrosive.

Carbol–xylene solution (No. 5)

Note: This should be prepared at an intermediate level laboratory because of the dangerous reagent involved. Loosen the top of a jar of phenol¹ (carbolic acid) crystals and put the jar in a water bath. Heat the water to liquefy the crystals. DO NOT HEAT PHENOL CRYSTALS DIRECTLY OVER A FLAME. ALWAYS PUT THE JAR INTO A WATER BATH.

<table>
<thead>
<tr>
<th>Liquid phenol</th>
<th>200 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene</td>
<td>600 ml</td>
</tr>
</tbody>
</table>

Measure the liquid phenol and pour into a 1000-ml glass-stoppered bottle. Add the xylene. Use an unopened bottle of xylene, if possible. Mix by shaking the bottle.

Label the bottle: CARBOL–XYLENE and write the date. Store in a cabinet or on a shelf away from direct light. The solution is good for a year or more, but the bottle must be kept tightly closed. Adhesive tape can be wrapped around the stopper to keep moisture out. If moisture gets into the solution, it will be unsatisfactory.

Warning: Phenol is highly corrosive and poisonous.

Delafield's haematoxylin stain (No. 6)

<table>
<thead>
<tr>
<th>Haematoxylin crystals</th>
<th>1 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol, absolute</td>
<td>10 ml</td>
</tr>
<tr>
<td>Saturated solution of aluminium ammonium sulfate</td>
<td>100 ml</td>
</tr>
<tr>
<td>(NH₄)₂Al(SO₄)₃ in distilled water</td>
<td>100 ml</td>
</tr>
<tr>
<td>Glycerol (C₃H₆(OH)₂)</td>
<td>25 ml</td>
</tr>
<tr>
<td>Methanol, absolute</td>
<td>25 ml</td>
</tr>
</tbody>
</table>

Dissolve the haematoxylin crystals in the absolute ethanol. Add a few drops at a time to the saturated aluminium ammonium sulfate solution. Leave this solution unstoppered in direct sunlight or in a 37 °C incubator for 3–4 months to oxidize the haematoxylin to haematein.²

Stopper and label the bottle: DELAFIELD'S HAEMATOXYLIN and write the date. When reopened, filter and add the glycerol and methyl alcohol, and the stain is then ready for use. Keep stoppered to prevent evaporation. The stain will remain good for at least 18 months.

¹ Liquid phenol available from commercial sources must not be used. It contains water which makes it unsatisfactory for staining.
² This is not the same as the blood product haematin.
**Ethanol, 70% (No. 7)**

Ethanol, 95% ............................................. 726 ml  
Distilled water ........................................... 274 ml  

Pour the ethanol into a 1000-ml graduated cylinder. Add the distilled water making a total amount of 1000 ml. Pour into a 1000-ml glass-stoppered bottle.

Label the bottle: 70% ETHANOL and write the date. This solution will remain good indefinitely but keep the bottle tightly closed. Store on a shelf or in a cabinet.

**Warning:** Ethanol is inflammable.

**Ether–alcohol fixative (No. 8)**

Ether ....................................................... 20 ml  
Ethanol, 95% ............................................. 20 ml  

Add the ether to the alcohol in a graduated cylinder and mix. Pour into a jar with a screw-cap lid or a lid with a ground-glass rim.

**Warning:** Ether is highly inflammable and potentially explosive.

**Field’s stain (No. 9)**

**Field’s stain A**

*Preparation from prepared powders:*

Field’s stain A powder ................................ 5.9 g  
Hot distilled water ..................................... 600 ml  

Mix until dissolved. Filter when cool.

*Preparation from original stains and chemicals:*

Methylene blue (medicinal) ................................ 1.6 g  
Azur I ..................................................... 1.0 g  
Disodium hydrogen phosphate \( \text{Na}_2\text{HPO}_4 \) anhydrous .................. 10.0 g  
Potassium dihydrogen phosphate \( \text{KH}_2\text{PO}_4 \) .................. 12.5 g  
Distilled water .......................................... 1000 ml  

Dissolve the two phosphates in the water. Pour about half of the phosphate solution into a 1-litre bottle containing a few glass beads. Add the stain powders and mix well. Add the remainder of the phosphate solution. Mix well and filter.

**Field’s stain B**

*Preparation from prepared powders:*

Field’s stain B powder ................................ 4.8 g  
Hot distilled water ..................................... 600 ml  

Mix until dissolved. Filter when cool.
**Preparation from original stain and chemicals:**

- Eosin (yellow, water-soluble) ........................................... 2.0 g
- Disodium hydrogen phosphate \((\text{Na}_2\text{HPO}_4)\), anhydrous ........... 10.0 g
- Potassium dihydrogen phosphate \((\text{KH}_2\text{PO}_4)\) ............... 12.5 g
- Distilled water ............................................................... 1000 ml

Dissolve the two phosphates in the water. Pour into a 1-litre bottle. Add the eosin. Mix until dissolved. Filter.

**Formalin solutions (No. 10)**

**10% Formalin solution for preserving faeces**

- Formalin (neutral formaldehyde, at least 37%) .......................... 100 ml
- Distilled water ............................................................. 900 ml

Measure the aqueous formalin solution into a graduated measuring cylinder and pour it into a 1000-ml bottle with a glass stopper or screw-cap. Add the distilled water to the bottle and mix.

Label the bottle: 10% FORMALIN and write the date. Store on a shelf or in a cabinet. The solution will remain good for two years or more.

**2% Formalin (concentration method for microfilariae)**

- Formalin (neutral formaldehyde, at least 37%) .......................... 20 ml
- Distilled water ............................................................. 980 ml

Measure the aqueous formalin solution into a graduated measuring cylinder and pour it into a 1000-ml bottle with a glass stopper or screw-cap. Add the distilled water to the bottle and mix.

Label the bottle: 2% FORMALIN and write the date. Store on a shelf or in a cabinet. The solution will remain good for two years or more.

**Warning:** Formalin is corrosive and poisonous.

**Giemsa stain (stock solution) (No. 11)**

Giemsa is the standard stain for reliability in the routine staining of blood films for malaria diagnosis. However, the quality of this stain, in ready-made solution or powder form, varies according to its source of supply and it is advisable to obtain it from a reputable manufacturer. Even so, you will need to establish stain quality by testing each batch after it has been made up and prior to routine staining of large numbers of blood films.

- Giemsa powder ........................................................... 3.8 g
- Methanol ................................................................. 250 ml
- Glycerol ................................................................. 250 ml

A dark bottle is preferred, but if one is not available, use a chemically clean and dry, clear, hard glass or polyethylene bottle of suitable size. You will also need about 50 solid glass beads of about 5 mm in diameter.
1. Put the glass beads in the bottle; pour in the measured amount of methanol and add the stain powder.
2. Tightly stopper the bottle. Allow the stain powder to sink slowly through the methanol until it settles to the bottom. Shake the bottle in a circular motion for 2–3 minutes.
3. Add the measured amount of glycerol and repeat the shaking process. Continue to shake for 2–3 minutes at half-hourly intervals, for at least six times.
4. Leave the bottle for 2–3 days, shaking it 3–4 times each day until the stain is thoroughly mixed. Keep some of this stock solution in a small bottle for routine use to avoid contamination of the stock solution.

Each newly prepared batch of stain should be properly labelled, including date of preparation, and should be tested for optimum stain dilution and staining time. Always keep the bottle tightly stoppered, in a cool place, away from direct sunlight. Clear glass stock bottles can be covered with a thick dark paper jacket to keep out the light.

**Glycerol–malachite green solution or glycerol–methylene blue solution (No. 12)**

Glycerol .................................................. 100 ml
3% aqueous malachite green, or 3% aqueous methylene blue .... 1 ml
Distilled water ........................................... 100 ml

Grind some malachite green or methylene blue powder with a pestle in a clean, dry mortar. Weigh out 3 g of the powder, pour it into a bottle and add distilled water to give 100 ml. Seal and label the bottle: 3% AQUEOUS MALACHITE GREEN or 3% AQUEOUS METHYLENE BLUE. Store in a cabinet away from light.

To prepare the solution: pour 1 ml of the 3% aqueous solution into a 250-ml bottle. Add 100 ml of glycerol and 100 ml of distilled water and seal the bottle; mix thoroughly before use.

**Hydrochloric acid–ethanol solution (No. 13)**

Hydrochloric acid (concentrated) .................................. 1 ml
Ethanol, 95% .................................................. 100 ml

Put 100 ml of 95% ethanol into a clean 250-ml bottle with glass stopper. Add 1 ml of concentrated hydrochloric acid and mix.

**Warning:** Hydrochloric acid is highly corrosive. Ethanol is flammable.

**Hydrochloric acid–methanol solution (No. 14)**

Hydrochloric acid (concentrated) .................................. 3 ml
Methanol, absolute ........................................... 100 ml

Measure 100 ml of absolute methanol and pour into a clean 250-ml bottle with glass stopper. Add 3 ml of concentrated hydrochloric acid and mix.

**Warning:** Hydrochloric acid is highly corrosive. Methanol is flammable.
Hydrochloric acid–water destain (No. 15)

Hydrochloric acid (HCl), concentrated ................. 0.5 ml
Distilled water ............................................. 100 ml

Measure the distilled water and pour into a 1000-ml glass-stoppered bottle. Add the hydrochloric acid. Mix thoroughly.

Label the bottle: 0.5% HCl and write the date.

Warning: Hydrochloric acid is highly corrosive.

Iodine–alcohol solution (No. 16)

Ethanol, 70% ................................................. 40 ml
Iodine crystals .............................................. a few

Pour the 70% alcohol into a small flask. Using two applicator sticks held together, pick up some iodine crystals and add to the alcohol. Shake or stir the mixture and add more iodine if necessary until the solution has the colour of strong tea. The colour is important.

The purpose of iodine–alcohol is to remove mercuric chloride left by the fixative and it must be the correct strength to do this. If the iodine–alcohol is not strong enough, it will not remove the mercuric chloride residue on the preparation, which will interfere with examination. If the iodine–alcohol is too strong, the iodine will penetrate the protozoa and prevent staining by the trichrome solution.

Label the bottle: IODINE–ALCOHOL SOLUTION and write the date. Iodine–alcohol solutions must be prepared fresh every three weeks.

Lugol’s iodine (stock 5% solution) (No. 17)

Iodine ......................................................... 5 g
Potassium iodide (KI) ........................................ 10 g
Distilled water .............................................. up to 100 ml

Weigh the iodine in a porcelain dish or a watch glass. Grind the dry iodine and potassium iodide in a mortar. Add water, a few millilitres at a time, and grind thoroughly after each addition until the iodine and iodide dissolve. Put the solution into an amber glass bottle with the remainder of the distilled water.

Alternatively: Dissolve the potassium iodide in about 30 ml of the water. Add the iodine and mix until dissolved. Add a further 70 ml of water and mix well. Store in a brown bottle.

Lugol’s iodine (1% solution for wet mounts) (No. 18)

Lugol’s iodine stock solution is too strong for wet mounts of stool. It will cause the faecal material to clump and organisms may get trapped and not be seen. Therefore, the stock Lugol’s iodine solution should be diluted.

Lugol’s iodine (stock, 5% solution) (No. 17) ................. 5 ml
Saline solution, isotonic (No. 24) .......................... 20 ml
Measure the isotonic saline into a dispensing or dropping bottle. Add the 5% Lugol's iodine stock solution. Mix thoroughly. This will give a 1% iodine solution which will satisfactorily stain cysts.

Label the bottle: LUGOL'S IODINE 1% and write the date. The 1% solution must be prepared fresh every 14 days.

**Methylene blue–phosphate solution (No. 19)**

Methylene blue powder: \( \text{1 g} \)  
Disodium hydrogen phosphate (\( \text{Na}_2\text{HPO}_4 \)): \( \text{3 g} \)  
Potassium dihydrogen phosphate (\( \text{KH}_2\text{PO}_4 \)): \( \text{1 g} \)  
Distilled water: \( \text{300 ml} \)

Weigh out the methylene blue powder and put in a clean dry mortar. Add the disodium hydrogen phosphate and potassium dihydrogen phosphate. With a pestle, grind the dye and phosphate powders together and mix thoroughly. Weigh 1 g portions of the mixture and put in small well-stoppered vials.

Label the vials: METHYLENE BLUE-PHOSPHATE and write the date. The dry mixture will keep for a long time, if the vials are kept tightly closed. Put a piece of adhesive tape around the stopper to seal the vial and keep out moisture.

**To prepare the solution**

Put 1 g of the mixture in a 500-ml flask. Add the distilled water and shake the flask or stir to dissolve the dye mixture. Filter through filter paper into a 500-ml clean, dry, glass-stoppered bottle.

Label the bottle: METHYLENE BLUE-PHOSPHATE SOLUTION and write the date. Store in a cabinet away from the light. The solution will remain good for two years or more.

**Methylene blue–saline (No. 20)**

Methylene blue: \( \text{0.1 g} \)  
Isotonic saline: \( \text{100 ml} \)

Weigh the methylene blue and transfer it to a clean bottle. Add the saline and mix until the dye crystals are completely dissolved.

For use: Filter a small amount of the stain solution into a dropper bottle.

**Potassium iodide solution, 10% (No. 21)**

Potassium iodide (KI): \( \text{100 g} \)  
Distilled water: \( \text{1000 ml} \)

Weigh out the potassium iodide. Measure the distilled water into a clean glass-stoppered bottle. Dissolve the potassium iodide in the water.

---

1 Medicinal methylene blue powder is preferred, but any good quality methylene blue can be used.
Label the bottle: 10% POTASSIUM IODIDE and write the date. (Put a piece of paper or string in the neck of the bottle to prevent the stopper sticking.) Store in a cabinet or on a shelf out of the light. On standing, the solution may become slightly yellow, but this does not interfere with its use.

**PVA-fixative preparation (No. 22)**

**Note:** This should be prepared at an intermediate level laboratory, because of the dangerous reagents involved.

**Modified Schaudinn’s fixative**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercuric chloride crystals (HgCl₂)</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Ethanol, 95%</td>
<td>31.0 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

Dissolve the mercuric chloride in the ethanol in a stoppered flask (50 or 125 ml) by swirling at intervals. Add the acetic acid, stopper, and mix by swirling.

**Warning:** Mercuric chloride is highly poisonous. Glacial acetic acid is highly corrosive.

**PVA mixture**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Polyvinyl alcohol (PVA) powder (low viscosity)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>62.5 ml</td>
</tr>
</tbody>
</table>

In a small beaker, add the glycerol to the PVA powder and mix thoroughly with a glass rod until all particles appear coated with the glycerol. Scrape the mixture into a 125-ml flask. Add the distilled water, stopper, and leave at room temperature for 3 hours or overnight. Swirl mixture occasionally to mix.

PVA powder and PVA-fixative solutions are available from several commercial sources. There are many grades of PVA powder on the market, but the grades with high hydrolysis and low or medium viscosity are most satisfactory for preparing PVA-fixative for protozoa.

**PVA-fixative working solution**

1. Heat a water-bath (or large beaker of water) to 70–75 °C. Adjust the heat to maintain this temperature range.
2. Place the loosely stoppered flask containing the PVA mixture in the bath for about 10 minutes, swirling frequently.
3. When the PVA powder appears to be mostly dissolved, pour in the modified Schaudinn's fixative solution, restopper and swirl to mix.
4. Continue to swirl mixture in the bath for 2–3 minutes to dissolve the remainder of the PVA, to allow bubbles to escape, and to clear the solution.
5. Remove the flask from the water-bath and let cool. Store the PVA-fixative in a screw-cap or glass-stoppered bottle. Label: PVA-FIXATIVE and write the date. Fixative will keep for 6–12 months.

For mixing, a high-speed agitator that will produce a whirlpool in the fixative solution is highly desirable.
**Safranin solution (No. 23)**

Stock solution

Safranin 0 ......................................................... 2.5 g  
Ethanol, 95% ..................................................... 100 ml  

Weigh the dye powder and dissolve in 100 ml of ethanol. Store in a labelled bottle.

Working solution

Stock solution ...................................................... 10 ml  
Distilled water .................................................. 90 ml  

**Saline solution, isotonic (No. 24)**

Sodium chloride (NaCl) ........................................... 8.5 g  
Distilled water ................................................... 1000 ml  

Weigh out the sodium chloride. Measure the distilled water into a clean, glass-stoppered bottle. Dissolve the sodium chloride in the water and mix thoroughly. Put a piece of string or a narrow strip of paper between the glass stopper and the neck of the bottle, to keep the stopper from sticking.

Label the bottle: ISOTONIC SALINE and write the date. Store on a shelf or in a cabinet. Pour some saline into a dispensing or dropping-bottle for daily use. Write the date on the label. The dispensing bottle should have a pipette with a rubber bulb.

**Schaudinn's fixative (No. 25)**

*Note:* This should be prepared at an intermediate level laboratory, because of the dangerous reagents involved.

Stock solution

Mercuric chloride, saturated, aqueous (HgCl) ................................. 600 ml  
Ethanol, 95% ..................................................... 300 ml  

Measure out the saturated aqueous mercuric chloride and pour into a 1-litre glass-stoppered bottle. Add the 95% ethanol and mix by shaking the bottle.

Label the bottle: SCHAUDINN'S FIXATIVE–STOCK and write the date. The stock solution will remain good indefinitely (a year or more).

Working solution for staining

Schaudinn's fixative stock solution ........................................... 100 ml  
Glacial acetic acid (CH₃COOH) ......................................... 5 ml  

1 Add about 80 g of mercuric chloride crystals to 1000 ml of distilled water and heat to dissolve the crystals. Allow to cool. Some crystals should form in the bottom of the flask if the solution is saturated.
Measure the Schaudinn's fixative stock solution and pour into a 250-ml glass-stoppered bottle. Add the glacial acetic acid and mix by shaking the bottle.

Label the bottle: SCHAUDINN'S FIXATIVE WITH ACETIC ACID, and write the date. This solution will remain good for 2–3 months.

**Warning:** Mercuric chloride is highly poisonous. Glacial acetic acid is highly corrosive. Ethyl alcohol is inflammable.

**Sodium citrate solution, 2% (No. 26)**

Sodium citrate crystals \((C_6H_7O_6Na_3 \cdot 2H_2O)\) ........................................ 20 g
Distilled water .................................................. 1000 ml

Measure the distilled water and pour into a clean bottle (glass-stoppered or screw-cap). Weigh out the sodium citrate crystals and add to the water. Stir until dissolved.

Label the bottle: SODIUM CITRATE SOLUTION, 2% and write the date. Store on a shelf or in a cabinet. The solution will remain good for one year or more.

**Trichrome stain solution (No. 27)**

Chromotrope 2R .................................................... 6.0 g
Light green SF ...................................................... 1.5 g
Fast green FCF ...................................................... 1.5 g
Phosphotungstic acid crystals \((H_2[PO_4(W_2O_5)] \cdot 5H_2O)\) ...................... 7 g
Glacial acetic acid \((CH_3COOH)\) .................................. 10 ml
Distilled water .................................................. 1000 ml

Weigh each dye powder separately. Put the dyes into a 1-litre flask.

Weigh out the phosphotungstic acid crystals and add to the flask with the dyes. Measure the glacial acetic acid and pour into the flask. Swirl the flask so that the acetic acid wets the dyes. Let stand for 30 minutes. Add the distilled water and mix. Pour the stain into a 1-litre, clean, glass-stoppered bottle.

Label the bottle: TRICHROME STAIN and write the date. Store on a shelf or in a cabinet away from the light. Good trichrome stain is a deep purple-black colour. The stain will remain good for a year or more.

**Warning:** Glacial acetic acid is highly corrosive.
ANNEX 3
Preparation of culture media

*Schneider's enriched medium for the in vitro culture of Leishmania*

**Materials**

- Schneider's *Drosophila* medium\(^1\) \(80\) ml
- Fetal calf serum\(^1\) \(20\) ml
- Antibiotic-antimycotic solution\(^1\) \(1.2\) ml

The antibiotic-antimycotic solution contains penicillin, streptomycin, and amphotericin.

**Preparation**

1. Inactivate the fetal calf serum for 30 minutes at 56 °C and allow to cool.
2. Mix 20 ml of inactivated fetal calf serum with 80 ml of Schneider's medium.
3. Add 1.2 ml of the antibiotic-antimycotic solution, mix, aseptically dispense the medium in 3-ml amounts into sterile 16 × 100 mm tubes, and stopper.
4. Label and freeze at \(-20\) °C. The medium can be kept for up to 1 year at \(-20\) °C. It can be kept for up to 6 weeks at \(4-6\) °C.

**Note:** The medium should be prepared in aseptic working conditions.

**Use**

1. Warm 2 tubes of medium to room temperature.
2. Inoculate each tube with 0.1 ml of the specimen.
3. Incubate the cultures at 24 °C (±2 °C) in the dark for up to 14 days. Room temperature is usually suitable.
4. Examine daily for promastigotes. Transfer a drop of the culture to a slide for examination using a wire loop. Cover with a coverslip and look for motile, flagellated promastigotes.

**Note:** Negative cultures must be subcultured after 4 days into fresh medium and examined daily for a further 10 days.

*Novy Nicolle–McNeal (NNN) culture medium for the in vitro culture of Leishmania*

**Materials**

- Difco blood agar base\(^2\) \(8\) g
- Distilled water \(200\) ml
- Defibrinated rabbit blood \(0.6\) ml in each 5 ml of medium

---

1 All the ingredients are obtainable from the Institut Pasteur, 3 boulevard Raymond Poincaré, BP 3, F-92430 Marnes-la-Coquette, France.
2 Difco Blood Agar Base is obtainable from Difco Laboratories, PO Box 10587, Detroit, Michigan 48233, USA.
Preparation of defibrinated rabbit blood

Collect 20 ml of rabbit blood into a sterile flask containing about 100 glass beads of 4 mm diameter. Defibrinate the blood by rotating the flask for 5 minutes. Add 200 IU of penicillin, 200 mg of gentamicin, and 2 mg of streptomycin per ml of defibrinated blood.

Preparation

1. Pour 200 ml of water into a flask, add the agar to the water, mix, and warm the flask in boiling water until the agar is completely dissolved.
2. Dispense the medium in 5 ml amounts into screw-cap bottles (20 ml capacity). Sterilize by autoclaving (with caps loosened) at 121 °C for 15 minutes and allow the agar to cool to 45–50 °C.
3. Add 0.6 ml of sterile defibrinated rabbit blood to each bottle and mix gently. Allow the medium to solidify with the bottles in a sloped position.
4. Leave the bottles in an upright position at room temperature for 24 hours to allow fluid of condensation to form. The bottles should be stored at 4–6 °C until required.

Note: The medium should be prepared in aseptic working conditions.

Use

1. Inoculate about 0.1 ml of specimen aseptically into the fluid of condensation of each of 2 bottles at room temperature.
2. Incubate the cultures at 24 °C (± 2 °C) in the dark.
3. Examine every 4 days. Transfer a drop of the culture using a sterile wire loop to a slide for examination for promastigotes.

Note: Negative cultures must be subcultured after 8 days into fresh medium and examined every 4 days for a further 20 days.
ANNEX 4

Cleaning and storage of microscope slides

Cleaning

The availability of clean, good quality glass slides for the preparation of blood specimens for microscopic examination needs to be emphasized. All slides must be scrupulously clean and free from grease or moisture. This will prevent most of the artefacts which confuse malaria diagnosis and will avoid the detachment and washing away of thick blood films during the staining process. Reject defective materials such as:

- slides with an iridescent bloom or frosted appearance;
- imperfectly cleaned slides, whether new or old;
- old slides with surface scratches or notched edges.

New slides

It is prudent to clean all new slides (including commercially pre-cleaned slides) by soaking in water with a reliable detergent¹ and then placing them in running tap-water, or several changes of clean water, for some hours. Each slide should be wiped dry and polished with a dry, clean, lint-free cloth. Always handle the cleaned slides by the edges to avoid finger marks.

Used slides

Used slides must be soaked for at least 60 minutes in hypochlorite solution before washing. They should be washed in hot soapy water and both sides scrubbed with a brush. Wash only a few at a time to avoid scratching or chipping. The slides should then be cleaned one by one with gauze or cotton wool. Then the slides should be transferred to a fresh solution of detergent and later to running water, or several changes of clean water, before drying with a clean cotton cloth. Slightly scratched slides that are considered unsuitable for blood films may still be usefully passed on to the entomology section for routine laboratory use.

Storage of slides

In the humid tropics, glass slides should not be kept in the ambient climate for more than a few weeks. Otherwise they will adhere to each other because of entrapped moisture and there will be a loss of transparency due to “frosting”. After cleaning, slides are best stored in a dry place or a warm air cabinet.

It is recommended that cleaned slides be stored in packages of 10, which have been wrapped in thin paper and secured with adhesive tape or rubber bands, so that they are ready for use. Packages of slides can be put in the original cardboard boxes or other suitable boxes for mailing or transportation, but should be protected with corrugated cardboard, expanded polystyrene, or cotton wool.

¹ The use of acid-bichromate as a cleaning solution is not recommended.
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