



*Handbook*  
*on*  
*Isolation*  
*Characterization*  
*and*  
*Cryopreservation*  
*of*  
*Leishmania*

Edited by

David Evans



UNDP/World Bank/WHO  
Special Programme for Research and Training in Tropical Diseases

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**Handbook on Isolation, Characterization and  
Cryopreservation of *Leishmania***

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## Foreword

For several years, the Scientific Working Group on Leishmaniasis of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) has been actively promoting the establishment of laboratories in the endemic countries where identification and cryopreservation of *Leishmania* may be accomplished. This is essential primarily because the epidemiology of leishmaniasis is diverse and specific control tools are required for different forms of the disease.

Several laboratories have played an essential role in characterizing the *Leishmania* isolated from different parts of the world and providing training, reference strains and identification services. In particular, the WHO Reference Centres in London, (The London School of Hygiene and Tropical Medicine); in Jerusalem, (The Kuvim Centre for the Study of Infectious and Tropical Diseases, Hadassah Medical School); and in Montpellier (Laboratoire d'Ecologie médicale et Pathologie Parasitaire, Faculté de Médecine) have been pioneers in this work. As a result, some laboratories around the world have successfully established these techniques and are identifying the isolates locally in communication with the Centres. New regional and national laboratories have also been established which are providing identification services.

The present manual, intended for beginners, is another attempt to promote identification, preservation and exchange of parasites amongst scientists with the view to establishing a standardized methodology so that results from different laboratories become comparable. It is hoped that all laboratories involved will establish ties with one another and with the International Reference Laboratories to keep abreast with new developments and for exchange of reagents and information. A list of Reference Laboratories is shown on page 33.

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# Contents

	Page
1. INTRODUCTION _____	1
2. ISOLATION OF <i>LEISHMANIA</i> _____	1
2.1 Basic Requirements _____	2
2.2 Human Cutaneous Leishmaniasis _____	2
2.3 Human Visceral Leishmaniasis _____	6
2.4 Isolation from Mammals Other than Man _____	7
2.5 Isolation from Wild Vectors _____	9
2.6 <i>In vitro</i> Isolation _____	9
3. LABORATORY MAINTENANCE OF ORGANISMS _____	10
3.1 Cultivation <i>in vitro</i> _____	10
4. PREPARATION OF SAMPLES FOR ELECTROPHORESIS _____	15
4.1 Harvesting Promastigote Cultures _____	15
4.2 Extraction of Soluble Enzymes _____	15
4.3 Beading in Liquid Nitrogen _____	17
5. PRINCIPLES OF ENZYME ELECTROPHORESIS AND STAINING _____	17
5.1 Enzyme Electrophoresis _____	17
5.2 Enzyme Staining _____	18
6. CELLULOSE ACETATE ELECTROPHORESIS _____	20
6.1 List of Specialised Equipment _____	20
6.2 Summary of Procedure _____	20
6.3 Detailed Preparations _____	21
6.4 Sample Application _____	24
6.5 Electrophoresis _____	25
6.6 Staining _____	25
6.7 Paper Underlay Development _____	26
6.8 Recording Results _____	27
Appendix A. Solutions Culture Media Etc. _____	28
Appendix B. Reference Centres _____	33
Appendix C. Reference Strains _____	34
Appendix D. Requirements for Cellulose Acetate Electrophoresis _____	35
Appendix E. Cellulose Acetate Electrophoresis Buffer Solutions _____	37
Appendix F. Preparation of Agar for Developers _____	41
Appendix G. Artefacts and Control _____	42
Appendix H. Transport and Import of Cultures _____	44
Appendix I. Labelling of <i>Leishmania</i> Isolates _____	45



## 1. INTRODUCTION

The global importance of human leishmanial infection has been realised mainly in recent years. It has become increasingly apparent that accurate identification of the causative organisms is of fundamental importance to our understanding of this group of diseases and that it is no longer sufficient to identify leishmanial organisms using criteria such as the clinical picture or the geographical area where the infection was acquired.

This manual which has been written primarily for workers new to the field of leishmaniasis, represents a practical guide to the isolation, cultivation, cryopreservation, transportation, and isoenzyme identification of leishmanial organisms. Isoenzymic methods of identification have been chosen in preference to newer methods (e.g. those involving the use of DNA probes or monoclonal antibodies) because they are relatively simple to use, reproducible, well established and are the standards against which the newer methods are assessed.

## 2. ISOLATION OF *LEISHMANIA*

Initial isolation is the process whereby leishmanial organisms are removed from their host and transferred either to an experimental animal (*in vivo* isolation) or into culture (*in vitro* isolation) so that multiplication can occur. It is the most important step in the whole chain of procedures described in this manual because, at present, isoenzyme identification methods require relatively large numbers of organisms. The choice of isolation method will depend on the immediate circumstances, and to some extent on the technical capability and experience of the staff.

*In vitro* isolation offers certain advantages over the *in vivo* methods:

- a) Cultures become positive more rapidly, often in as short a time as 5-7 days, in contrast to weeks or months for a lesion to appear on an animal.
- b) The materials are less expensive.
- c) The cultured organisms can be cryopreserved, thus reducing the time and personnel required for maintenance.

Disadvantages of *in vitro* isolation are:

- a) Some leishmanias are extremely difficult to culture.
- b) Sterile conditions may be difficult to attain in the field.

Injection of isolates into the foot pad of susceptible hamsters or mice and removal of samples for inoculation into cultures 7-10 days later, combines the advantages of *in vivo* and *in vitro* methods. Nevertheless, adequate conditions

for *in vitro* isolation can be achieved with organisation and a little ingenuity. One can work with the flame of a camping gas cooker or even of a cigarette lighter. Body surfaces, both human and animal, from which organisms are to be isolated, should be thoroughly cleansed with alcohol or other antiseptics, and sterile instruments must be used.

Under adverse conditions, use both methods of isolation, and if you have to choose a single method for field use, choose the *in vivo* one. Its main advantage is that strict aseptic technique is not required; its main disadvantage is that not all species of *Leishmania* infect any one kind of laboratory animal.

## 2.1 Basic Requirements

### 2.1.1 Equipment and materials

The following list includes much of what is required for isolation by any method, (refer to the particular method for additional equipment and materials).

- gas or spirit burner
- bottles (10-20 ml) of sterile saline (0.9% NaCl) or proline balanced salts solution (*see Appendix A*).
- tubes of culture media
- sterile Pasteur pipettes
- sterile tissue grinders (optional)
- cottonwool, alcohol, tube rack, marking pens, syringes
- needles, punches, scissors, forceps, microscope slides

For field use, each instrument is separately wrapped, either in paper or aluminium foil before sterilization by autoclaving or dry heat; alternatively heat-seal into autoclavable plastic bags before autoclaving.

### 2.1.2 Records and record-keeping

A record book is essential. Be prepared to establish a simple but reliable coding system for the primary isolations. It is most convenient to give each attempted isolation a code number. Against this number, record all other pertinent data, such as date, host, signs of infection, location of lesions, geographic source of specimen, etc. Ensure that this code is subsequently always marked on all containers with the isolate, and is recorded against all experimental animals. If isolation is eventually successful, a full code, which can include your own code, should be assigned to the isolate according to international recommendations (*see Appendix I*).

## 2.2 Human Cutaneous Leishmaniasis

Lesions are usually either ulcerative (open) or nodular (closed); use the same approach for isolating leishmanias for either case. Take tissue samples from those areas of the lesion most likely to contain leishmanial amastigotes. Generally the reddened, swollen edge of a cutaneous lesion is the best site.

Several methods are useful for taking samples:

- a) Biopsy of the lesion edge. This normally requires a local anaesthetic and is a standard dermatological technique. The sample is excised with a small scalpel or removed with a dermatological punch.
- b) Methods not normally requiring local anaesthesia include:
  - i) slit-skin preparation.
  - ii) dental broach biopsy.
  - iii) aspiration of the lesion edge.

### 2.2.1 Sampling Methods

The larger the piece of tissue taken as a sample, the greater is the chance of growing leishmanial organisms, so if possible, use methods 2.2.2.1. or 2.2.2.2. below for maximum amounts of tissue.

Since cutaneous lesions are often secondarily infected with microorganisms other than *Leishmania*, a thorough initial cleansing of the lesion is most essential. With lesions that have heavy secondary infections, it is useful to administer topical or systemic antibiotics for several days before attempting to isolate *Leishmania*.

During the attempt:

- a) Avoid the open area of an ulcerated lesion; take the tissue sample from the swollen edge.
- b) Remember that the amastigotes occur mainly in the shallow layers of the dermis.
- c) Remove crusts from the lesion and eliminate any pus.
- d) Thoroughly clean the edge of the lesion and surrounding skin with sterile gauze or cottonwool soaked in 70% ethanol or isopropanol ('rubbing alcohol'). The alcohol must not be allowed to enter the open part of the lesion; allow to dry before proceeding further.
- e) Swab secondarily infected open lesions with 6% (20 volume) hydrogen peroxide.

Avoid using substances like mercurochrome, which may kill the leishmanias. If iodine is used for disinfecting, remove every trace by cleaning with alcohol-soaked swabs before the biopsy is made.

With the following methods it should be possible to use the samples taken from the lesion for the preparation of smears as well as for animal and/or culture inoculation.

2.2.2.1 *Skin biopsy*: excise a 2-4 mm wedge of tissue from the edge of the lesion.

2.2.2.2 *Slit-skin technique*:

- a) Pinch firmly, between thumb and forefinger, the skin over the area to be sampled.
- b) Make a shallow slit about 1mm deep in the pinched skin, using a narrow pointed scalpel. Pinch the skin tightly to avoid bleeding.
- c) With the sharp edge of the scalpel blade, scrape the cut edges of the the slit from below upwards.
- d) Release the pressure on the sides of the slit and immediately apply a sterile dressing to the cut surface. Hold this tightly over the wound for 1-2 minutes, by which time any bleeding should have ceased.
- e) Use the material that collects on the blade for inoculations and stained smears. (see sections 2.2.4 and 2.2.3)

2.2.2.3 *Dental Broach Biopsy*

- a) Select a sterile, fine gauge dental broach, as is used for removing the nerve from the root canal of a tooth.
- b) Starting from the healthy skin side of the lesion, work the pointed end of the broach into the edge of the lesion.
- c) When all the barbs have entered the skin, press the thumb on to the skin where the broach was inserted.
- d) Remove the broach with a quick, sharp pull.
- e) Inoculate culture tubes by agitating the broach in the liquid portion of the culture medium; the adhering material is thus transferred to the medium.
- f) Preferably with another sterile broach, and if possible using the same hole, take another sample; if necessary use the original broach.
- g) Twist the broach 2 or 3 times, withdraw it, and again inoculate culture medium. Repeat this process twice; use material collected on the final insertion to prepare a smear (2.2.3.2).

2.2.2.4 *Aspiration of the lesion edge*

- a) Approach the lesion from the side of the healthy skin.

- b) Inject 0.1-0.2 ml of sterile saline or PBSS (*Appendix A*) into the edge of the lesion from a 2ml syringe (a 1 ml 'tuberculin' syringe is not suitable as you cannot obtain sufficient suction) fitted with a short needle; 0.5 in/20 gauge is ideal.
- c) Rotate the needle 2 or 3 times whilst it is in the skin; this cuts small pieces of tissue from the edge of the needle wound.
- d) Aspirate as much liquid as possible. Use the aspirated liquid to inoculate cultures and animals, and to prepare smears (2.2.3.3). Do not inoculate cultures with aspirates that contain more than a trace of blood because they rarely grow; instead make another aspiration.

### 2.2.3 Stained smears for microscopy

#### 2.2.3.1 Biopsy samples:

- a) Grasp, with a pair of forceps, a small piece of tissue that is not required for isolation purposes.
- b) Dry the freshly cut surface of the specimen by blotting on a sheet of filter paper.
- c) Press the dried surface of the specimen firmly on to the surface of a clean, dry microscope slide. Make 2 or 3 such impression smears (touch preparations), each one on a separate slide. Label, then dry, fix and stain as below (2.2.3.4).

#### 2.2.3.2 Dental broach samples. (These are often hardly visible to the naked eye, but successful smears can be made.)

- a) Place a drop of saline or PBSS, about the size of a pinhead, on a slide. Rub the barbed part of the broach backwards and forwards in the drop, keeping the smear to a small area.
- b) Allow to dry, fix with methanol and stain as below (2.2.3.4.).

#### 2.2.3.3 Aspirated material: Allow a drop of the aspirate to dry on a microscope slide. Fix and stain as below (2.2.3.4.).

#### 2.2.3.4 Giemsa staining

- a) Allow the smears to dry; **Do not heat or blot dry.**
- b) Fix in methanol 95% for 30 seconds. Stain for 20 min. with Giemsa (diluted 1 in 10 with 0.02M sodium phosphate buffer, pH 6.8-7.2)
- c) Rinse in tap water. Allow to dry. Examine without a coverslip under the oil immersion objective (x100) of a microscope.

#### 2.2.4 *In vitro* cultivation

- a) Use aseptic precautions throughout.
- b) Introduce the tissue sample into the culture medium. If biphasic medium is used, inoculate into the liquid portion.
- c) With a large piece of tissue, press it against the inside of the culture vessel with a large sterile needle or similar implement. Alternatively, grind the tissue in a small, sterile tissue grinder, and inoculate the resulting liquid.

#### 2.3 Human Visceral Leishmaniasis

Isolations are usually made from either bone marrow or splenic aspirates; occasionally venous blood is used. Samples of bone marrow and spleen must only be taken by medically qualified, experienced personnel. Make stained smears from the samples (2.2.3.4.) and inoculate culture media (2.3.2.) and animals.

##### 2.3.1 *Sampling methods*

###### 2.3.1.1 *Bone marrow*

- a) Puncture the sternum or iliac crest and aspirate marrow, using a syringe containing an anticoagulant such as heparin or EDTA. Marrow clots extremely rapidly in the absence of an anticoagulant.
- b) Use a drop or two of marrow to prepare smears; prepare and stain these as you would a thin blood film.
- c) Use the remainder of the marrow for culture. Contrary to sampling from cutaneous lesions, DO NOT inoculate culture tubes with large volumes of marrow. Certainly the volume must not be in excess of the amount of liquid overlay in the culture medium; bone marrow contains substances inhibitory to the growth of promastigotes. Only 2, or at most 3, drops from a syringe or Pasteur pipette should be inoculated into each culture tube. Inoculate several culture tubes.
- d) Alternatively, centrifuge the marrow for 10 min at 1500-2000 g to sediment the cellular components. Discard the supernatant and resuspend the pellet in a buffered salts solution such as PBSS (*Appendix A*). Centrifuge again and resuspend the pellet in 1 ml or less of PBSS. Use this to inoculate cultures and/or animals and make smears.

###### 2.3.1.2 *Splenic aspirates*

The aspirates are treated in a similar way to marrow samples (2.3.1.1.). Again, DO NOT inoculate culture tubes with large amounts of material containing a high proportion of non-cellular blood components.

### 2.3.1.3 Blood culture

Occasionally successful isolations have been made from the blood of patients with visceral leishmaniasis. Mostly, these isolations have been from patients in the endemic kala-azar region of Eastern India and in Kenya.

- a) Take 10 ml of venous blood into a sterile syringe containing anticoagulant.
- b) Centrifuge the blood at 1500-2000 g for 10 min. to sediment the cells. Carefully pipette off the buffy layer which collects above the red cells. Use this to inoculate cultures and/or animals, and prepare stained smears.

### 2.3.2 In vitro culture

The organisms from patients with visceral leishmaniasis can be very difficult to cultivate. Try whenever possible to inoculate experimental animals as well. When attempting cultivation, use a blood agar-based medium -- preferably NNN, otherwise USAMRU or modified Tobie's medium (*Appendix A*). Sometimes, even when the initial isolation is successful, the organisms may die when subcultured. This seems especially common when the initial isolation has been into a rich medium such as USAMRU or modified Tobie's. Often this can be overcome if subcultures are made into less nutritionally rich media such as NNN, or one of the semi-solid media such as 'sloppy Evans' or semi-solid Locke-blood agar (*Appendix A*).

## 2.4 Isolation from Mammals Other than Man

The equipment and materials required are basically the same as for sampling from man (2.1). Use, according to your needs, the techniques already described for staining smears (2.2.3.) and isolating the organisms (2.2.4; 2.3.2).

### 2.4.1 Domestic dog

#### 2.4.1.1 Lymph node puncture

The popliteal lymph nodes are almost invariably enlarged and parasitized in canine visceral leishmaniasis; these are the glands most commonly sampled for diagnostic purposes. Occasionally the prescapular lymph node is punctured. The procedure for this is as set forth below except the dog is maintained seated.

- a) Muzzle the dog with a non-elastic, 50 mm wide strip of cloth, e.g. a linen belt, bandage etc. Wrap this around the muzzle and tie firmly under the jaw. Tie for a second time up and behind the ears.
- b) Lay the dog on its side; an assistant must hold its head firmly.
- c) Cut and shave the hair from the skin above the node. Swab the exposed skin with a series of alcohol-soaked cottonwool pads, until a pad remains clean.

- d) Infiltrate the skin around the lymph node with 3–4 ml of 1.0% lignocaine hydrochloride (local anaesthetic).
- e) Palpate the lymph node and immobilise it between the thumb and forefinger.
- f) Puncture the node using a 50mm, 19 gauge needle attached to a syringe containing 1 ml saline with antibiotics (*Appendix A*).
- g) Pull back the plunger of the syringe and, without leaving the node, draw the needle back. Then push it in another direction so as to explore all parts of the node. Withdraw the syringe and needle.
- h) Use the contents of the syringe for culturing, inoculating animals and preparing stained smears.

#### 2.4.1.2 Skin biopsy

Ulcerated lesions are treated as described for man (2.2.4). Snip biopsies are made after careful shaving and disinfection. Treat them as described below (2.4.2.1).

#### 2.4.2 Wild rodents

Wild rodents can have leishmanias in either damaged or normal-looking skin; there may or may not be visceral involvement.

- a) Examine the skin carefully as skin lesions are often not obvious.
- b) The skin is usually heavily contaminated with fungi and bacteria. If the animal is dead, scrub the skin with soap and water; rinse thoroughly under running water before shaving the skin and swabbing with alcohol. With live animals (usually anaesthetised), thoroughly cleanse both lesions and apparently normal skin with alcohol, preferably on several occasions over 2 to 3 days before the biopsy is to be performed.
- c) For ulcerated lesions, take a biopsy from the dry skin with a punch or scalpel; use scissors and forceps for ear lesions and apparently normal skin.
- d) Transfer the tissue to a Petri dish containing sterile saline or PBSS (*Appendix A*), with a high concentration of penicillin (100 000 IU/ml).
- e) Dissect off any remaining fur with sterile instruments.
- f) Wash the remaining tissue by passage through several changes of saline or PBSS containing penicillin (100,000 IU/ml).
- g) Cut the tissue into very thin slices or else grind it in a tissue grinder.

h) Inoculate culture media and animals, and prepare stained smears.

## 2.5 Isolation from Wild Vectors

The method depends on whether or not the insects can be transported alive back to a laboratory. The direct inoculation of sandfly guts into culture medium has a high probability of causing fungal contamination of any resulting culture, and the process is particularly risky in the field.

Again the equipment and materials required are similar to those recommended for isolating from man (2.1), with the addition of instruments suitable for the dissection of sandflies. The staining and isolation techniques have already been described (2.2.4; 2.3.2).

### 2.5.1 Laboratory isolation

- a) Rinse the sandflies thoroughly in sterile saline or PBSS (*Appendix A*).
- b) Place the fly into a drop of sterile saline on a sterile microscope slide, dissect out the gut and examine under a microscope.
- c) When a gut is found which contains promastigotes, either, i) express the contents into the PBSS used for dissection, then inject directly into culture medium, or, ii) drop the entire gut into the medium.

A safer method is to inoculate an experimental animal such as a hamster (2.6) with the gut contents of the infected sandfly and reisolate the *Leishmania* from the hamster.

### 2.5.2 Field isolation

If flies have to be dissected in the field, *in vitro* isolation is usually extremely difficult because of problems with bacterial and fungal contamination of cultures inoculated under adverse conditions. Successful isolations into culture can be made, but require a great deal of expertise. *In vivo* isolations are likely to be far more successful under field conditions. Inoculate any positive guts directly into snouts, feet and peritoneal cavities of hamsters. Proceed then as in 2.6.

## 2.6 *In vivo* Isolation

This involves the inoculation of material containing leishmanial organisms into susceptible laboratory animals, the most suitable animal being the golden hamster. Other animals, such as various strains of in-bred (particularly BALB/c) and out-bred mice are used on occasions, but none of these are as uniformly susceptible to different species of infection as the hamster.

The hamster is inoculated either intradermally in the nose and dorsal surfaces of the rear feet for dermatrophic leishmanias, or intraperitoneally for visceral

organisms. *Leishmania* are then isolated into culture from the hamster in one of the following ways:

a) Wait until a lesion becomes apparent (cutaneous leishmaniasis) at the site of inoculation, then culture from it (2.2.).

b) Note exactly where the hamster was inoculated; wait 7 to 14 days, then kill the animal. Excise the tissue into which the inoculation was made. Culture from the tissue in the usual way. The method has the distinct advantage that the long wait is avoided for a lesion to become apparent; this may be as long as 1 year with some strains of *Leishmania braziliensis*.

c) Kill the animal, and make stained smears from liver and spleen. If positive, remove portions of positive tissue aseptically and inoculate culture medium.

### 3. LABORATORY MAINTENANCE OF ORGANISMS

#### 3.1 Cultivation *in vitro*

Apart from being an efficient method for the parasitological diagnosis of leishmaniasis, *in vitro* cultivation is an essential step in most procedures for distinguishing between the various leishmanias. The most difficult part is usually the initial establishment of the organism in culture when the amastigotes have to transform into promastigotes which then begin multiplication.

During the initial period the correct choice of culture medium is especially important. Unfortunately it is difficult to predict which *Leishmania* will grow easily in any one type of culture medium. The recipes for media that have been found to be widely applicable are given in *Appendix A*. Probably no two experienced workers will make the same choice of medium, as most individuals have their own favourites. If in doubt, choose one or more of the blood-agar based media in *Appendix A*; these are widely used for Old and New World leishmanias.

##### 3.1.1 Inoculation of culture medium

a) Label culture tubes with the code given when the organism was first isolated (2.1.2). This is vitally important at this point; if left until later, confusion will occur.

b) Using strict aseptic precautions, inoculate into the tubes of medium the material suspected of containing leishmanias, collected and prepared as described in Section 2. Introduce the inoculum into the liquid portion of a biphasic medium, Not into the blood agar. If sufficient material is available, inoculate as many tubes as possible, as this increases the chances of obtaining growth. Try several different culture media. Do not inoculate material into large volumes of

culture medium. With biphasic media restrict the volume of the liquid phase to 0.5 ml or less. When using liquid media, use 1 ml or less.

### 3.1.2 Temperature of incubation

After inoculation of culture tubes they should be transferred to a constant temperature incubator as soon as possible. Remember that more *Leishmania* cultures are killed by heat than by cold. Routinely maintain cultures at 22°C as this temperature is ideal for most leishmanias; consider 25°C as the maximum temperature. In hot climates where cooled incubators are not available, an air-conditioned room should provide a suitable ambient temperature. If, however, the air-conditioning is turned off at night, the temperature may increase to an unacceptably high level. A good place during such periods is the warmest part of a refrigerator; for example the box often found at the bottom of a domestic refrigerator, or the racks on the inside of the door. Otherwise, use any other suitably cool place.

### 3.1.3 Routine examination

Using aseptic precautions, examine the cultures regularly, preferably every 2 or 3 days, but never less than once a week. Frequent inspection during the early stages is important, especially where there is a risk of contamination; fungi and bacteria discovered at an early stage are much easier to control. If contamination is observed, subinoculate immediately into fresh culture medium containing higher concentrations of different antibacterial or antifungal agents. Alternatively inoculate a susceptible laboratory animal (2.6) with the contaminated culture, with the hope that only the leishmanial organisms will grow.

a) Remove a small drop of medium with a sterile Pasteur pipette or similar instrument and place on a microscope slide under a coverslip.

b) Examine under a microscope. Low power objectives x10 or x20 with x10 eyepieces are quite adequate. If possible use phase contrast or dark field illumination, otherwise use standard illumination with the condenser in a low position and the iris diaphragm nearly closed.

c) As soon as motile promastigotes are seen, transfer 2 or 3 drops into fresh medium. When the organisms increase in number, add small volumes (approx. 0.5 ml) of fresh medium to the culture tubes.

d) When the organisms are growing well in subculture, reserve at least one tube for cryopreservation (3.1.5).

e) Primary cultures which remain negative after 2 to 3 weeks incubation should be blind-passaged into fresh medium or into a selection of different media. Incubate these together with the primary culture for at least 2 more weeks; discard them if still negative after this time.

### 3.1.4 International coding for isolates

Once the organisms have been established *in vivo* they must be assigned an International Code; details are given in *Appendix I*.

### 3.1.5 Cryopreservation

It is important to preserve the organisms as soon as conveniently possible after initial isolation. The frozen sample is known as a stabilate. Cryopreserved leishmanias can be stored in low temperature mechanical freezers (-70°C), in solid carbon dioxide containers (-76°C) or in liquid nitrogen containers (-196°C). Each method has drawbacks; mechanical deep-freezers break down or else the electricity supply is not reliable; liquid nitrogen and carbon dioxide storage both rely on regular supplies being easily available.

Take great care to avoid cryogenic burns and accidents from exploding containers when freezing, storing or thawing samples. Always wear protective gloves, together with a total face mask or safety spectacles. Handle frozen tubes and ampoules with blunt-ended forceps.

#### 3.1.5.1 Record keeping

This is vitally important, and a cryopreservation record book or chart **MUST** be kept. It must contain details of the samples stored, their location in the cryobank, and a brief history of each stabilate with its International Code number. All tubes, ampoules, and capillaries in the bank must be clearly labelled; any material that has lost its label must be discarded.

Keep a careful record of what is removed from the cryobank; **Do not** allow general access. Give one person the overall responsibility for all issues and receipts from the bank, together with the concomitant record-keeping. This person together with one or possibly two assistants, should be the only people with direct access to the bank; all must know the method of record-keeping.

#### 3.1.5.2 Freezing

This is a simple process with the leishmanias and does not require sophisticated apparatus. Freezing is carried out slowly in the presence of a cryoprotectant.

a) Using aseptic technique, transfer a measured volume of culture into a sterile glass tube or other container kept on ice. Actively dividing promastigotes in mid-logarithmic growth phase seem to survive freezing and thawing better than non-dividing cells. Ideally, one would freeze cultures with a minimum promastigote concentration of 1 million per ml. Stabilates with lower concentrations take longer to re-establish in culture.

b) Add sterile cryoprotectant to give the required final concentration - glycerol (sterilised by autoclaving) to give a concentration of 7.5% to 10% in the

final volume, or filter sterilised dimethyl sulphoxide (DMSO) to a final concentration of 5.0%-7.5%. Mix thoroughly.

c) Transfer the cryoprotected samples to the sterile containers in which they are to be frozen. These may be 2 ml (38.0 x 12.5 mm) plastic freezing tubes with air-tight screw caps (eg. NUNC cryotubes), hard glass, heat-sealed ampoules, or capillaries made of glass or plastic. Do not overfill -- no more than two-thirds full. Label the containers.

d) Seal the containers. Ensure that those made of glass are correctly heat-sealed; failure to do so can result in violent explosions when the containers are thawed after storage in liquid nitrogen. It is safer to store glass containers in the vapour phase above the surface of liquid nitrogen, but special liquid nitrogen refrigerators are required for this. Slowly freeze the containers with the stabilates at about 1°C per minute. This can be done in several ways:

i) Place the containers in an insulated vessel; for example, a glass or metal tube surrounded by a jacket made of expanded polystyrene, or similar insulating material, about 3 cm thick. Place in a -70°C freezer overnight.

ii) Cool the samples to 4°C and keep them at this temperature for a minimum of 1 hour; they can be left overnight if necessary. Transfer to a -20°C freezer and leave for 24 hours, then remove to a -70°C freezer for at least 24 hours. They can be permanently stored at this temperature, or else transferred into liquid nitrogen (-196°C) or solid carbon dioxide (-76°C).

iii) The containers can be placed in a special vessel which fits into the mouth of a liquid nitrogen Dewar flask, where slow freezing takes place in liquid nitrogen vapour over a period of 24 hours.

iv) Programmable freezing units may be used if available. In this case, the rate of freezing is 1°C/min from 25°C to 2°C, then 5°C/min from 2°C to -18°C; 10°C/min from -18°C to -70°C and below.

v) Quickly transfer the containers with their stabilates to their final storage place, ensuring they do not become warm during the transfer.

Before discarding the cultures from which the stabilates were made, thaw the contents of one of each isolate frozen (3.1.6). Culture from it to check the viability of the stabilate. If no growth is obtained, make a fresh stabilate from the original culture.

### 3.1.6 Thawing stabilates

a) Thaw the stabilates rapidly by plunging the sealed container into warm water at about 25°C.

b) Transfer the thawed organisms aseptically into fresh culture medium, preferably of the same kind in which they were growing before freezing.

c) Inspect a sample of the thawed material under the microscope; motility of the organisms is a good indicator of viability. However, even if the promastigotes are immobile, they may nevertheless recover and grow in culture.

d) Check culture daily, and if no viable parasites appear after 72h, subculture again to eliminate possible residual DMSO.

### 3.1.7 Prolonged maintenance *in vitro*

Avoid prolonged maintenance by serial subculture *in vitro* as far as possible. There are several reasons for this: (i) increased risk of contaminating one culture with another; (ii) the risk of a mutant population spreading rapidly through the culture; (iii) loss of infectivity to laboratory animals; (iv) increasing the amount of labour involved in maintaining a large collection of cultures.

If you have neither the facilities to cryopreserve your stocks nor to maintain them *in vivo*, the following suggestions may be helpful:

a) Choose a medium in which the organisms will survive for as long as possible without subculture. A semi-solid medium (*Appendix A*) is probably best for this purpose; otherwise use a biphasic medium. Avoid liquid media.

b) Be especially careful over labelling of cultures and in keeping records.

c) When subculturing, or processing in any way, work on only one stock at a time; keep this away from all others. Mistakes such as picking up the wrong container can easily happen, and have happened, even in the best organised laboratories.

d) Keep the cultures cool (10°-15°C); the organisms will grow more slowly.

e) Donate any valuable cultures to one of the WHO Collaborating Centres for Leishmaniasis (*see Appendix B*) and ask them to preserve it for you. They do not charge for this service and will not use the material experimentally or pass it on to a third party without your permission.

### 3.1.8 Mass cultivation

Many of the methods in use for the biochemical identification of leishmanias require large numbers of promastigotes for their application (eg. isoenzymes and most DNA methods);  $10^{10}$  organisms are required in some cases. To produce such numbers the scale of *in vitro* cultivation has to be increased, and may involve the use of different culture media from those used in the isolation procedures. Liquid culture media are usually preferred to the more cumbersome biphasic media. Whatever the medium used, the same guidelines apply.

Build up the total volume of culture gradually; do not inoculate large volumes of medium with small volumes of inoculum. Subculture into successively larger culture vessels containing increasing volumes of medium; use almost the entire

contents as an inoculum for larger vessels. Use several vessels rather than one very large one. Thus, if the content of one is lost through contamination or such mishap, not all of your material is lost; four culture vessels each containing 50 ml is much safer than one with 200 ml.

When sufficient numbers of organisms have been obtained, proceed to harvest them and extract the water-soluble enzymes, as described in Section 4.

#### 4. PREPARATION OF SAMPLES FOR ELECTROPHORESIS

##### 4.1 Harvesting Promastigote Cultures

After mass cultivation as described in Section 3, promastigotes are usually harvested in late logarithmic growth phase, and almost invariably by centrifugation. Collecting and cleaning promastigotes presents few difficulties if they have been grown in a liquid medium. If grown on a biphasic medium, pieces of agar often become detached and contaminate the liquid phase.

Remember you are still handling live human pathogens, so take all the necessary safety precautions until the organisms are dead.

a) For promastigotes grown in a purely liquid culture medium, centrifuge at 2000 g for 20 min at 4°C in suitable capped centrifuge tubes.

b) For those grown on biphasic media, pour off the overlay containing the organisms; rinse the agar with PBSS (*Appendix A*). Filter the combined solutions through fine gauze to remove any small pieces of agar. Centrifuge as in (a) above. For isoenzyme electrophoresis, a small amount of red cell contamination is not important.

c) Discard the supernatant, and resuspend the pelleted organisms in about 5 ml of PBSS. Combine in one 50 ml conical centrifuge tube (or a 15 ml tube for small volume cultures) and centrifuge as before.

d) Resuspend the resulting pellet in 0.5-1.0 ml of PBSS. Transfer to a 2 ml plastic centrifuge tube and centrifuge again.

e) Carefully remove all the supernatant fluid using a finely drawn-out Pasteur pipette.

f) Proceed immediately to extract the water-soluble enzymes from the organisms as below.

##### 4.2 Extraction of Soluble Enzymes

Currently two methods are used to extract the water-soluble proteins, including enzymes, from leishmanias; in each case the organisms are lysed. Great care must be taken to carry out all parts of the process in the cold so as to avoid heat denaturation of the enzymes.

#### 4.2.1 Freeze-thaw lysis in water

##### 4.2.1.1 Stabilizer solution

a) Prepare in advance the following ingredients for the stabilizer solution. Weigh into 2 separate containers:

- Reagent A. 0.0308 g dithiothreitol (DTT)
- Reagent B. 0.0262 g  $\epsilon$ -aminocaproic acid (ACA)

These can be stored indefinitely in a refrigerator, and it is convenient to prepare a number of containers with the reagents for future use.

b) Prepare a 200 mM solution of EDTA by dissolving 7.445 g of disodium EDTA dihydrate in 50 ml of distilled water. Adjust the pH of the solution to pH 7.0 with 1.0 M NaOH. Check that the solution is clear, then make up the volume to 100 ml. Dispense into small bottles, each containing about 5 ml. Store indefinitely at  $-20^{\circ}\text{C}$ .

c) Add 1.0 ml of the EDTA solution to Reagent A, shaking to dissolve. Add this solution to Reagent B to give 200 mM concentration of each ingredient. This stock stabilizer solution can be kept at  $4^{\circ}\text{C}$  for one week.

##### 4.2.1.1 Lysis

a) Dilute the 200 mM stock stabilizer solution with distilled water to 2 mM (e.g. 0.1 ml to 10 ml) just before use. Discard any 2 mM stabilizer solution not used.

b) Add a volume of the 2 mM stabilizer solution approximately equal, as judged by eye, to that of the pelleted organisms in the microcentrifuge tube (4.1.(e)). Mix.

c) Freeze and thaw the mixture 3 times in liquid nitrogen. Alternatively, leave the original mixture with the stabilizer in a  $-20^{\circ}\text{C}$  freezer overnight; thaw the next day and then freeze and thaw twice in liquid nitrogen.

d) Centrifuge the lysate at approx. 30,000 g for 30 min at  $4^{\circ}\text{C}$  in a refrigerated centrifuge.

#### 4.2.2 Detergent lysis

The following method may be used as an alternative to freeze-thaw lysis in water.

To one volume of packed centrifuged organisms, add an equal volume of a 0.05% w/v solution of Triton X-100. Mix well, then carry out the beading procedures as in 4.3. below.

### 4.3 Beading in Liquid Nitrogen

- a) With caution, pour liquid nitrogen into a 100 ml plastic beaker to about 1.0 cm from the top.
- b) Fit two interlocking pieces of thin card into the beaker so that it is divided into four sections.
- c) Using a cardboard collar, suspend the beaker over more liquid nitrogen in a suitable container, such as a larger beaker or a small liquid nitrogen Dewar flask.
- d) Place LABELLED screw capped glass or plastic tubes into the lower container to cool. Labelling must be permanent and waterproof. It is essential that the caps of the tubes have a small hole to allow the excess nitrogen to escape.
- e) Using a micropipette (20 $\mu$ l) withdraw portions either of the supernatant from 4.2.1.1 (take care not to disturb the pellet), or of the whole lysate from 4.2.2. Holding the micropipette above the small beaker of liquid nitrogen, allow 15 $\mu$ l drops to fall into each section in sequence. As each drop enters the liquid a solid bead forms. The separate sections prevent the beads freezing to each other as they form. However, once fully formed the beads will remain separate; thus, the formation of more beads can continue in sequence in each section of the divided beaker.
- f) When all the sample is beaded, pour off most of the liquid nitrogen from the beaker, remove the card divider and transfer the beads with plastic forceps one by one to the pre-cooled labelled tubes. Cap the tubes and transfer quickly to a suitable liquid nitrogen bank.
- e) Record all relevant details in the record book, e.g. date, strain code, donor, storage position in the bank.

The beads can be kept indefinitely until you are ready to carry out electrophoresis as described in the remaining sections of the manual.

## 5. PRINCIPLES OF ENZYME ELECTROPHORESIS AND STAINING

### 5.1 Enzyme Electrophoresis

The precise techniques are described in detail later. Briefly, soluble enzymes are extracted from the organisms; a small amount of the extract is then placed in an inert supporting substance, the matrix, containing a buffer at a fixed pH. The matrix described in this manual is absorbent cellulose acetate, but it could equally well be starch, acrylamide or agarose. The pH of the buffer in the matrix is chosen so that usually the isoenzymes are negatively charged.

A direct current is passed through the matrix so that one end is positive and the other negative. The current is carried by the ions in the buffer. The anode,

or positive end of the matrix, will attract those isoenzymes that are negatively charged. At the opposite end, the negatively charged cathode will attract the positively charged isoenzymes.

The isoenzymes will migrate through the matrix according to the overall, or net, charge on each. Although the variants of one enzyme can all be negative at a certain pH, the net charges can be quantitatively different. Thus, during the same period of time, the one with the greatest negative charge will travel the furthest towards the anode, while the least negatively charged isoenzyme will travel the shortest distance.

## 5.2 Enzyme Staining

The simple water extract used contains many enzymes together with numerous other proteins. When electrophoresis is completed, most proteins will have moved in the matrix towards the anode. If stained at this stage with a general protein stain, very many bands will be seen. If extracts from 2 different organisms were run next to each other on the same matrix and the proteins stained, the staining patterns would be so complicated that the recognition of homologous proteins in each sample would be extremely difficult. However, the high substrate and co-factor specificity of enzymes has allowed developers to be devised that will stain only the enzymes required. Hence, the electrophoretic mobility of one particular enzyme can be compared between several organisms. In this way, precise comparisons can be made between organisms. The stained matrix with its collection of stained isoenzyme bands, is known as a zymogram.

The enzyme itself is not stained, but the products of its highly specific activity are. The method is extremely sensitive because one enzyme molecule may process many thousands of substrate molecules.

Normally the activity of only one enzyme is stained on each zymogram; hence, the normal practice is to run as many extracts as possible (e.g. 8-10) on each matrix. One or more extracts from reference organisms, in which the enzyme banding patterns are well documented, are included to aid the interpretation of results.

### 5.2.1 Dehydrogenase reactions

The majority of enzymes used for characterization purposes are stained by methods incorporating a dehydrogenase reaction. In many reactions the enzyme substrate is oxidised by removal of hydrogen or its equivalent. The coenzyme, nicotinamide adenine dinucleotide (NAD), or the closely related nicotinamide dinucleotide phosphate (NADP) must be present. Most dehydrogenase reactions of this type are highly specific involving just one of these coenzymes. During the reaction when the enzyme removes the hydrogen from the substrate, the NAD or NADP is correspondingly reduced by accepting the hydrogen; respectively, NADH or NADPH is formed.

For example, malate dehydrogenase (MDH) (E.C.1.1.1.37) catalyses the reaction:



Many such dehydrogenase activities are reversible, although some reactions proceed much faster in one direction than the other. Consequently, there are two different types of stain depending on which direction is most practical for revealing the activity.

#### 5.2.1.1 Tetrazolium staining for dehydrogenases

Tetrazolium salts are used when the specific enzyme reaction favours the formation of NADH or NADPH. The most common of these salts used is methylthiazole tetrazolium (MTT) which is yellow in solution. It is reduced by NADH or NADPH in the presence of an intermediary electron carrier, phenazine methosulphate (PMS), to an insoluble purple precipitate called formazan. In this manner the site of enzymic activity on a zymogram is clearly shown as a purple-staining area.

#### 5.2.1.2 Fluorescent staining for dehydrogenases

This method is used when the enzyme reaction favours the production of NAD<sup>+</sup> or NADP<sup>+</sup>. It is based on the fact that NADH or NADPH fluoresces under UV light whereas the oxidised forms, NAD<sup>+</sup> and NADP<sup>+</sup>, do not. Hence, when NAD<sup>+</sup> or NADP<sup>+</sup> is produced, the activity at an isoenzyme position is seen as a dark band of quenched fluorescence against a bright fluorescent background.

#### 5.2.1.3 Dehydrogenases as linking enzymes

Some enzymes can be detected by using a dehydrogenase as a linking enzyme, the dehydrogenase reacting with one of the products resulting from the other enzyme's activity. For example glucose phosphate isomerase (GPI) is detected with the tetrazolium method by incorporating the enzyme glucose-6-phosphate dehydrogenase (G6PD), with NADP<sup>+</sup> into the reaction mixture. Aspartate aminotransferase (ASAT) is similarly detected by including MDH and NADH in the reagent mixture.

Sometimes more than one linking enzymes has to be added in order to achieve a detectable reaction. For example, in the stain for mannose phosphate isomerase (MPI), both GPI and G6PD are required to achieve the coloured formazan reaction.

#### 5.2.2 Stains for hydrolases

Hydrolases catalyse the hydrolysis of a wide variety of compounds. They are classified according to their substrates into esterases, glycosidases, peptidases etc. For most of these enzymes, specially devised synthetic substrates are used which on hydrolysis yield either a fluorescent or a dye-reactive product.

#### 5.2.2.1 Esterases and glycosidases

These enzymes are demonstrated using the appropriate ester and glycoside derivatives of 4-methylumbelliferone. The substrates are non-fluorescent, but fluorescent 4-methylumbelliferone is released by enzymic hydrolysis. Under UV light, isoenzyme bands are easily seen as fluorescent bands on a dark background.

Another type of stain uses  $\alpha$ - and  $\beta$ -naphthyl ester or glycoside. Hydrolysis by the enzyme releases  $\alpha$ - and  $\beta$ -naphthol which react with diazo salts to give red, blue or mauve dyes.

#### 5.2.2.2 Peptidases

Many peptidases have overlapping substrate specificities in that several enzymes will react with the same peptide substrate. Thus, complex multiple-banded patterns are produced which are difficult to interpret.

### 6. CELLULOSE ACETATE ELECTROPHORESIS

Electrophoresis on cellulose acetate offers the prime advantage of speed. The equipment is readily available commercially, and the apparatus is designed for ease of use. The resolution of some of the enzymes commonly used for the identification of *Leishmania* is less good on cellulose acetate than on other matrices such as starch, but in general the system is perfectly satisfactory.

The following is a step by step account of the use of the Hellena 'Zip Zone' cellulose acetate electrophoresis apparatus.

#### 6.1 List of Specialised Equipment

See *Appendix D* for details.

- a) Electrophoresis chambers
- b) Sample applicator
- c) Alignment base
- d) Sample well plate
- e) Soaking and staining set
- f) Cellulose acetate plates
- g) Paper wicks

#### 6.2 Summary of the Procedure

##### 6.2.1 Day 1

Prepare the running and staining buffers for the enzymes to be examined. Do not add the sucrose to the running buffers.

### 6.2.2 Day 2

- a) If agar underlays are required, melt the agar and store in a water bath at 60°C.
- b) Add sucrose to the running buffers.
- c) Place the appropriate buffers in the electrophoresis chambers and soaking tanks.
- d) Pour water or ice into the central compartments of the electrophoresis chambers. Place the wicks in position.
- e) Label the cellulose acetate (CA) plates and lower into the soaking chambers.
- f) Prepare the developers. Place the agar underlays in the dark, and put the other developers on ice.
- g) Check that the power supply unit(s) are ready and in position.
- h) Arrange the samples in the listed order.
- i) Apply the samples to the presoaked CA plates.
- j) Place the loaded plates, cellulose acetate side downwards, on the wicks in the electrophoresis chambers. Weigh the plates down with microscope slides.
- k) Check that the samples are at the cathodic (-) side of the chamber.
- l) Switch on the current and set the voltage. Note time and current.
- m) Switch off at required time. Remove plates and develop.
- n) Incubate and start timing for fluorescent developers. Monitor development.
- o) At optimal development, photograph or preserve CA plates as records.
- p) Clean the equipment. Tidy up.

### 6.3 Detailed Preparations

#### 6.3.1 Day 1

Prepare the buffers according to which enzymes are to be examined (see *Appendix E*). Do not add the sucrose. Store the buffers at 4°C.

### 6.3.2 Day 2

#### 6.3.2.1 Preliminary preparation

- a) Because of the short running times (20–40 minutes), it is essential that the appropriate enzyme developer is prepared before starting the electrophoresis. Wearing rubber gloves, cut filter papers to the correct size for applying certain fluorescent stains.
- b) If a photograph is required, check (i) the camera contains film, (ii) the correct lens filter (if appropriate) is in position, and (iii) the correct lighting system is available and working.
- c) Prepare a wetting agent for the applicator wash plate; add 5 drops of sodium dodecyl sulphate solution (0.1g/100 ml) to 100 ml distilled water.
- d) Melt the appropriate amount of 1.2% agar (*see Appendix F*). Check that it is free of lumps.
- e) Maintain the agar at 60°C in a water bath until required.

#### 6.3.2.2 Preparation of buffers

- a) Thaw frozen buffers and substrates.
- b) The less stable ingredients (*see Appendix E*) eg NADH, must be weighed and dissolved.
- c) Pipette solutions of linking enzymes and reagents. **Do not pipette by mouth.** If using automatic pipettes, always use a new tip for each solution to avoid cross-contamination. With other types of pipettes, always use a clean one for each solution.
- d) Keep the reagents off your skin; some such as MTT and PMS are potential carcinogens.
- e) Prepare the developer (*Appendix E*) Note whether the one you require is applied as an agar underlay (MTT developers and other colour reactions), or as a paper overlay (UV fluorescent methods).

#### 6.3.2.3 Agar developer underlays

- a) Label the outside corner of a clear plastic dish (a square Petri dish approx 100 mm<sup>2</sup> is ideal) with the initials of the enzyme to be examined. Lay the dish on a flat surface.
- b) Put the correct amounts of reagents, *except* the agar, into a bottle or small plastic beaker. If it is a tetrazolium reaction, do not add the MTT and PMS until just before adding the agar.

- c) When you are ready to pour the underlay, check again that all the ingredients have been added.
- d) Add to the reagent mixture an equal volume of molten 1.2% agar at 60°C; for a 100 mm<sup>2</sup> Petri dish 10 ml of reagent and 10 ml of agar are required. Mix and pour quickly into the labelled dish. Make sure no air bubbles are trapped and that the bottom of the dish is completely covered with the agar mixture.
- e) Leave the developer to set. Cover with an opaque box lid or something similar to exclude light.
- f) After 5-10 minutes, when the agar has set, cover with a Petri dish lid. Keep in the dark, or in a black bag; avoid tilting the dish. Store until required.

#### 6.3.2.4 Tank preparation

- a) Add sucrose to the tank buffers to a concentration of at least 10%. The total amount of buffer required is approximately 500ml (100 ml for the electrophoresis chamber and 400 ml for the soaking tank).
- b) Unless specified otherwise, soak the plates in the same buffer.
- c) Label the electrophoresis chamber and soaking tank with the enzyme initials.
- d) Place 50 ml buffer into each outer electrode compartment of the electrophoresis chamber, and fill the soaking tank with the remainder.
- e) Place 30 ml of distilled water into each of the two centre compartments of the chamber. This helps prevent the CA plate drying out during electrophoresis. Alternatively, crushed ice can be used for especially heat-labile enzymes.
- f) Wet the two paper wicks with buffer by submerging them in the electrode compartments. Place them lengthways in position along each outer divider; they should dip into the buffer on one side and overlap by about 5 mm on the other side. The wicks must not touch the water in the centre of the chamber.
- g) Position the power unit(s) within reach of the tank leads.

#### 6.3.2.5 Preparation of the CA plates

- a) Each CA plate consists of a cellulose acetate matrix deposited on a stiff plastic sheet. The matrix side on which the samples are applied has a white, dull appearance while the reverse side is the shiny plastic backing. The plastic provides a useful surface for marking the plate.
- b) To avoid damaging or contaminating the matrix, handle the CA plates only by their edges.

- c) Place the CA plate with the matrix side (dull white surface) face down onto a clean sheet of paper. Using a fine tipped permanent waterproof marker pen, label the plastic back of the plates from right to left as described below. Keep the labelling small and close to the edge of the plate. Make sure the marker used does not (a) wash off (b) fluoresce under UV light. The numbers at the bottom indicate the positions of the first and last of the eight samples. When the samples are applied on the matrix side, they will read correctly from left to right. The bar across the top of the numbers is a reminder of the cathode (-) edge of the plate. Other information should include the enzyme, date type of buffer, pH, and a recording code.
- d) Using fine forceps, lower the labelled CA plate slowly and carefully into the slots on each side of the soaking tank.

If you have to use your fingers remember to hold the plate only by its edges. If the plate is dropped quickly into the tank air bubbles will be trapped on the surface of the matrix and the plate rendered useless. If a slotted tank is unavailable, use a rectangular glass or plastic tank of suitable capacity; four plates can be soaked in such a container by leaning two plates back-to-back at opposite sides.

#### 6.4 Sample Application

- a) Decide on the order in which you wish to run your samples, and note this in your record book.
- b) The frozen samples in bead form are transferred with plastic forceps from their storage bottles in liquid nitrogen to the wells of a microtitre plate or something similar, labelled 1 to 8. Ensure that the correct sample is placed in each well.
- c) Allow the beads to thaw in their wells and then pipette approximately 10  $\mu$ l of each sample into the corresponding wells of the cold, eight well sample plate. Cover the plate to avoid evaporation of the samples, and keep on ice until the loading procedure begins.
- d) Prepare a second well plate for washing purposes. Fill each well with the wetting agent. This is for rinsing the applicator to ensure efficient picking up of the sample.
- e) Clean the applicator by lowering it onto a flat piece of filter paper, repeating this process until the wet imprints of the applicator teeth look uniform.
- f) With fine forceps remove the CA plate from the soaking buffer, and place it, label side downwards, on a paper towel. Lightly blot the matrix side with 2 layers of Whatman No.1 filter paper or on one Helena blotting pad.
- g) Quickly transfer the blotted plate to the base aligning plate with the matrix side uppermost. Align the edge of the CA plate marked 1 to 8 on the back

with the line on the applicator plate marked 'cathode application'. Note that for enzymes such as SOD which have some cathodic-moving bands the alignment is somewhat different and the edge of the CA plate is aligned against the line marked 'centre alignment'.

- h) Lower the clean applicator 2 to 3 times into the sample wells, pause and remove. Slot it carefully into position on the aligning plate. Press down the applicator on to the CA plate and then release it. The samples have now been loaded on to the CA plate.
- i) Quickly remove the CA plate and place it, shiny side uppermost, across the two wicks in the electrophoresis chamber. Make sure that both side markers 1 and 8 are nearest the cathode (-) end of the tank.
- j) If only one CA plate is being run, place it in the centre of the chamber. If 2 plates are run, leave a gap of about 0.5 cm between them, and equal gaps on either side.
- k) Place 2 microscope slides on to each side of the CA plate to ensure good contact between the plate and the wicks.

## 6.5 Electrophoresis

- a) Fit the lid onto the tank. Connect the leads to the power supply unit. Set the voltage according to the enzyme schedule (*see Appendix E*) and switch on. Make a note of the time.
- b) Check the ammeter on the power supply unit. If it reads zero, no current is passing through the plate. Check the connections and ensure the lid is correctly positioned on the chamber.
- c) A reading of more than 12 mA per plate will cause overheating. Aim for 3-10 mA per plate, as a slight increase in current will occur as electrophoresis takes place. If 12 mA per plate is exceeded then reduce the voltage and run for a longer time (e.g. if 300v/20 min is recommended, change to 200v/30 min).
- d) Get the developers ready (6.3.2.2).
- e) At the end of the run, switch off the current, disconnect the power leads and remove the CA plate.

## 6.6 Staining

### 6.6.1 Agar underlay development

- a) Remove prepared agar underlay from the dark and place it on the bench.
- b) Lightly blot the matrix side of the CA plate with filter paper.

- c) With the labelled side of the CA plate uppermost and tilted at an angle of about 45 degrees, apply one of the shorter edges close to the edge of the developer agar. Holding the CA plate with forceps, or with fingers by the edges, lower the CA plate on to the surface of the agar carefully so as to avoid trapping air.
- d) Replace the cover on the developer plate and incubate in the dark at 37°C. View periodically until band development is clear.
- e) Avoid tilting the developing plate, as any movement of the CA plate on the surface of the agar is liable to produce artifactual double-banding.

#### 6.7 Paper Underlay Development

With certain developers (eg. for esterases) the enzyme reaction, when viewed under UV light, is seen as fluorescent bands against a dark background. With others (eg. ALAT and ASAT), the opposite is seen, dark bands against a fluorescent background.

- a) The developer will have been prepared and stored on ice (*see Appendix E*). Five minutes before the end of the electrophoretic run, pipette 3 ml onto a glass plate.
- b) Lower a piece of Whatman No.3 filter paper on to the pool of developer and allow it to soak up the buffer; wear rubber gloves when cutting and handling this paper.
- c) Rock the glass plate backwards and forwards to spread the developer evenly over the paper. Tip off the excess developer. Do not allow the paper to dry.
- d) Blot the edges of the CA plate very gently with filter paper.
- e) Hold the CA plate with the shiny side uppermost, and lower the matrix side gently on the filter paper on the glass plate. Do this carefully as described in 6.5(c) so as to exclude air bubbles.
- f) Place a piece of dry Whatman No 1 filter paper (cut to approx 9 x 12 cm) over the plate. Remove the excess developer by running the edge of a short ruler firmly across the filter paper and plate. Do this several times in different directions across the paper.
- g) Replace the top filter paper with a similar one moistened with distilled water. Place a second glass plate (or piece of aluminum sheet) on top and clamp everything together with a strong spring clip such as a 'Bulldog' clip.
- h) Incubate the plates either at room temperature or at 37°C, depending on the anticipated rate of reaction. The development of ALAT and ASAT is usually rapid, e.g. 4-10 minutes at room temperature. With these enzymes check, without removing the glass plate, under UV illumination for quenched (ie.

dark) fluorescent bands on the developer paper side. As soon as dark bands are faintly visible the reaction must be stopped quickly, as the bands will show up strongly on the CA plate.

- i) Unclip the plates. Carefully remove the CA plate from the two filter papers. Dry the plate with the dull side uppermost, on a paper towel in an oven at 60°C -- this takes about 10 min. The bands will not be clearly visible until the plate is completely dry. Avoid excessive heating as this can destroy the banding patterns.
- j) Using the same procedure, other enzymes, such as esterases, show up as brilliantly fluorescent bands against a non-fluorescent background. The incubation time for these enzymes is, however, considerably longer than for ALAT and ASAT. At least 30 min incubation at 37°C is required, and the development should be monitored periodically.

## 6.8 Recording Results

### 6.8.1 Photography

- a) Photographs of visible coloured bands are best taken by transmitted light, using a camera with a close-up lens. If using roll film, carefully record the sequence of photographs to avoid confusion after the film is developed. A Polaroid camera is especially useful as it provides an instant print.
- b) Photograph the bands immediately after optimal development. If one or two strong bands develop quickly, photograph the plate, then reincubate until the weaker bands show and rephotograph.
- c) The photography is best done in a darkened room with the plates illuminated from below -- a light box or X-ray film illuminator is an ideal light source. The procedure is somewhat different when UV light is used; illuminate the plate from above with a UV light source with a wavelength in the range 340-370 nm. Note that most photographic films will require the use of a yellow filter when photographing plates illuminated with UV light.

### 6.8.2 Preserving the CA plate

The CA plate, already labelled, makes a useful record and saves the expense of a photograph.

- a) To preserve the MTT-stained (tetrazolium) plates, remove them from the agar underlays. Fix in 5% acetic acid for 5-10 min. Rinse in distilled water; blot and dry at room temperature. The bands will appear much fainter on the dried plates, but can be restored to their former intensity by lowering them gently into a beaker of distilled water.
- b) Fluorescent plates cannot be preserved satisfactorily, so in the absence of a photographic record, place the dried plates under UV light and outline the bands with a ball-point pen.

## Appendix A

## SOLUTIONS CULTURE MEDIA ETC.

## Proline balanced salts solution (PBSS)

(A useful general-purpose salts solution and liquid phase for biphasic culture media for *Leishmania*.)

KCl .....	0.4 g
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O.....	0.06 g
KH <sub>2</sub> PO <sub>4</sub> .....	0.06 g
CaCl <sub>2</sub> ·2H <sub>2</sub> O .....	0.185 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O .....	0.1 g
MgCl <sub>2</sub> ·6H <sub>2</sub> O .....	0.1 g
NaCl .....	8.0 g
L-Proline .....	1.0 g
* Phenol red .....	0.001 g
Distilled water .....	1000 ml

Dissolve the ingredients, one at a time, in approx. 750 ml of distilled water. Adjust the pH to 7.2 with solid Tris (Tris(hydroxymethyl)aminomethane), make up the volume to 1000 ml, dispense into convenient screw-cap containers and autoclave at 121°C for 15 min. Store preferably at 4°C, but will withstand several months at room temperature.

## Biphasic Blood Agar-Based Media

## NNN Medium

## Preparation:

Solid phase: the agar is made by heating the following ingredients together in a flask:

Agar (plain, non-nutrient) .....	1.4 g
NaCl .....	0.6 g
Distilled Water .....	90 ml

*Method:* Heat the contents of the flask until the agar melts, keep the contents well mixed otherwise the agar will burn on the bottom of the flask. Transfer the appropriate amount of molten agar directly into the culture vessels. Sterilize the agar by autoclaving the culture tubes at 121°C for 15 min. Allow the agar to cool to about 50°C and add aseptically collected, defibrinated rabbit blood to a final concentration of approximately 15%. Mix the blood agar by rolling the

\* May be omitted

tubes in an upright position between the hands. Place the tubes in a sloped position until the agar has set, then stand them upright and transfer to a refrigerator or into iced water.

*Liquid phase:* This consists of the water that condenses at the bottom of the slopes, no additional liquid phase is added. The rapid cooling of freshly made slopes by transfer to a refrigerator or iced water greatly increases the amount of water of condensation that will accumulate.

**USMARU Medium ('Difco' Blood agar medium)**

A very much richer medium than 3N, which is especially useful in the isolation for the nutritionally more fastidious organisms such as those of the *L. braziliensis* group.

*Solid phase:*

'Bacto' Blood Agar Base (Difco) .....	4 g
Distilled Water .....	100 ml

*Method:* as for 3N medium

*Liquid phase:* As for 3N medium, or if additional liquid is required a few drops of sterile distilled water may be added.

**Evans Modified Tobie's Medium**

A rich biphasic medium which has been used successfully for the isolation of a great variety of leishmanias from both Old and New World sources.

*Solid phase:*

Beef extract (Oxoid Lab-Lemco L29) .....	0.3 g
Bacteriological peptone (Oxoid L37) .....	0.5 g
NaCl .....	0.8 g
Agar (Oxoid purified) .....	2.0 g
Distilled water .....	100 ml

*Method.* Mix and Heat the ingredients in a flask as for 3N medium. Transfer the molten agar into culture tubes and autoclave at 121°C for 15 min. Cool the sterilized agar to about 55°C, then add defibrinated horse blood (inactivated by heating at 56°C for 30 min) to give a final concentration of approximately 15%. Mix and slope as for 3N medium.

*Liquid phase:* A liquid phase other than the simple water of condensation is used, and this is proline-containing balanced salts solution (PBSS), [see above].

Add 0.2 - 0.3 ml of the liquid phase to the agar slope immediately before inoculation.

*Note on the use of blood other than rabbit blood in biphasic medium:* Quite often rabbit blood is not easily available for inclusion in biphasic media such as 3N or USAMRU. In such cases mammalian blood other than rabbit may be used. Sheep, horse, and human blood have all been used, but it is worth experimenting with whatever bloods are easily available. With bloods other than rabbit use them either defibrinated or with an anticoagulant, but always heat-inactivate them (56°C:30 min) and increase the concentration of agar-agar in the medium to 2%.

#### *Sterility checking of blood agar*

Incubate freshly made blood agar medium at 37°C for 24 hours and examine the surface of the blood agar for signs of bacterial growth. Discard immediately any medium showing signs of bacteria.

#### *Storage*

Store at 4°C, and if a separate liquid phase is to be added, do not add this until the medium is to be used. These media are best used within one week of making up. Discard after 3 weeks at 4°C.

#### **Schneider's *Drosophila* Medium**

This is a commercially available, liquid, insect tissue culture medium which, when supplemented with 10, 20 or even 30% foetal calf serum, has been widely used for the isolation and bulk cultivation of *Leishmania* spp. It is a very expensive medium and somewhat variable in performance.

• Schneider's <i>Drosophila</i> medium (revised)	
with L-glutamine (Gibco) .....	100 ml
Heat-inactivated (56°C for 30 min.)	
foetal calf serum .....	10, 20 or 30 ml.

#### **Media for maintenance and bulk cultivation**

Liquid media are more convenient for large volume cultures than are the biphasic ones.

#### **MEM:FCS:EBLB Medium**

A nutritionally rich liquid medium suitable for the growth (but not the isolation) of almost any *Leishmania*.

MEM medium with Earle's salts (modified, autoclavable)	
obtained from Gibco .....	100 ml
Sodium bicarbonate solution (7.5% w/v).....	3 ml
Evans Blood Lysate Broth (EBLB) .....	5 ml
Heat-inactivated Foetal Calf Serum .....	10 ml

**Preparation of EBLB****Ingredients:**

Tryptose (Oxoid L47) .....	1.5 g
Casein Hydrolysate (Oxoid L41) .....	1.0 g
Liver Digest (Oxoid L27) .....	1.0 g
L-Proline .....	0.15 g
KH <sub>2</sub> PO <sub>4</sub> .....	0.68 g
NaOH .....	0.17 g
Distilled Water .....	100 ml

Final pH 7.3 - 7.4 (adjust if necessary with either 1M HCl or 1M NaOH).

Dissolve the solid ingredients in the distilled water and sterilize by autoclaving at 121°C for 15 min. After cooling, add 15 ml of an aseptically prepared blood lysate (human, equine, ovine, caprine or rabbit bloods all seem to be equally good). Prepare the lysate from aseptically collected whole blood taken either into an anticoagulant or else defibrinated. Sediment the blood cells by centrifugation at approximately 3000 g for 10 min and remove the liquid portion (serum or plasma) and wash the packed blood cells twice by resuspension in an equal volume of either sterile isotonic saline or proline balanced salts solution (*see above*) and recentrifugation (3000 g/10 min). Lyse the washed blood cells by adding an equal volume (i.e. equal to the volume of packed cells) of sterile distilled water. Mix the water and blood cells thoroughly and use the mixture as the blood lysate to complete the EBLB medium. The medium will be very cloudy at this point due to the cellular debris added in the blood lysate, so clarify it by aseptic centrifugation at 15 000 g for at least 30 min. Decant off the clear supernatant taking care not to disturb the pellet. Bottle and store the supernatant (EBLB) at 4°C.

**Semi-solid media**

These are extremely valuable as transport media and for reviving ailing cultures.

**'Sloppy Evans'**

Proline Balanced Salts Solution (PBSS) [see above] .....	80 ml
Bacteriological Peptone (Oxoid) .....	0.1 g
Beef Extract (Oxoid, Lab-Lemco) .....	0.03 g
Washed packed horse blood cells <sup>1</sup> .....	10 ml
Heat-inactivated foetal calf serum .....	10 ml
Agar (plain non-nutrient) .....	0.3 g

<sup>1</sup>Harvest blood cells from aseptically collected defibrinated horse blood by centrifugation (3000 g:10 min), decant off the supernatant and wash the packed cells by resuspension in at least an equal volume of PBSS. Recentrifuge (3000g:10 min) and then repeat the washing process two more times. Note: the packed cell:FCS mixture can be replaced by 20 ml of defibrinated rabbit blood.

*Method:* Mix the ingredients together (omit the packed cells and FCS) in a flask or screw-capped bottle. Sterilize by autoclaving (121°C:15 min), cool to about 50°C and add the packed cells and FCS; mix well and dispense whilst still molten into suitable sterile culture tubes.

***Semi-solid Locke-blood agar medium***

*Locke's solution*

NaCl .....	9.2 g
CaCl <sub>2</sub> .....	0.24 g
NaHCO <sub>3</sub> .....	0.15 g
KCl .....	0.42 g
D-Glucose .....	1.0 g
Distilled water.....	1000 ml

*Blood agar base:*

Agar (plain non-nutrient) .....	2.5 g
Bacteriological peptone .....	1.0 g
NaCl .....	0.5 g
Distilled water .....	100 ml

*Method:* mix 7 parts of Locke's solution with 1 part of molten blood agar base, adjust the pH to 7.4 and autoclave to sterilize. Allow to cool to about 50°C and add defibrinated rabbit blood to a concentration of approximately 10%. Mix thoroughly, then dispense into sterile culture vessels.

## Appendix B

### REFERENCE CENTRES

#### Reference strains

The strains listed in APPENDIX C, together with details of their origins and life histories are available free of charge from:

Department of Medical Parasitology  
London School of Hygiene and Tropical Medicine  
Keppel Street  
London WC1E 7HT  
UK

#### Identification services

Centres offering a *Leishmania* identification service are:

Department of Medical Parasitology  
London School of Hygiene and Tropical Medicine  
Keppel Street, London WC1E 7HT, UK

Laboratoire d'Ecologie Médicale et de Pathologie Parasitaire  
Faculté de Médecine, rue Auguste Broussonet, 3400 Montpellier,  
France

The Gamaleya Institute of Epidemiology and Microbiology  
18, Gamaleya str., 123098 Moscow, USSR

Walter Reed Army Institute of Research  
Washington D.C., USA

Fundação Oswaldo Cruz  
Caixa Postal 926, 21040 Rio de Janeiro, RJ, Brazil

Alexander Von Humboldt Institute  
Universidad Peruana Cayetano Heredia  
Lima 100, Peru

Centro Internacional de Investigaciones Medicas  
CIDEIM, Tulane University Colciencias  
Apartado aereo 5390, Cali, Colombia

## Appendix C

## REFERENCE STRAINS

When attempting to identify leishmanial organisms using isoenzyme methods described in this manual, it is essential that the unknown organisms are compared with appropriate reference strains with known isoenzyme profiles. Such strains are readily available from the WHO International *Leishmania* Reference Centre, at the London School of Hygiene and Tropical Medicine (see Appendix B).

At least one of the following strains recommended by WHO should be used in addition to any stocks of your own:

*L.(L.) donovani* MHOM/IN/80/DD8  
*L.(L.) infantum* MHOM/TN/80/IPT1  
*L.(L.) chagasi* MHOM/BR/74/PP75  
*L.(L.) major* MHOM/SU/73/5ASKH  
*L.(L.) tropica* MHOM/SU/74/K27  
*L.(L.) aethiopica* MHOM/ET/72/L100  
*L.(L.) mexicana* MHOM/BZ/82/BEL21  
*L.(L.) amazonensis* MHOM/BR/73/M2269  
*L.(V.) braziliensis* MHOM/BR/84/LTB300  
*L.(V.) guyanensis* MHOM/BR/75/M4147  
*L.(V.) panamensis* MHOM/PA/71/LS94

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**Appendix D**
**REQUIREMENTS FOR CELLULOSE ACETATE ELECTROPHORESIS****1. Specialist Equipment**

Suggested supplier: Helena Laboratories, 1530 Lindburg Drive, P.O. Box 752, Texas 77704-0752. USA. Telex No. TWX 910- 8845171

Alternative suitable equipment may be available from other manufacturers.

	<u>Helena Catalogue No.</u>
1.1 'Zip Zone' electrophoresis chambers (preferably 3 or 4)	1283
1.2 Super CPK application kit (includes 8-sample applicator, alignment base, 2x sample well plates, wash solution)	4029
1.3 Pack of 2 Super CPK sample well plates (extra to above kit)	4095
1.4 Spare tips for applicator	4039
1.5 Soaking and staining kit (1000)	5122
1.6 Cellulose acetate plates. Titan III 76 x 94 mm	3024
1.7 Paper wicks (500/box)	5081

**2. Miscellaneous Equipment**

- 2.1 Whatman No. 1 chromatography paper, or similar, for blotting plates.
- 2.2 Whatman No. 3 MM chromatography paper for development of certain enzymes.
- 2.3 Small oven (50-60°C) or hair dryer, for drying fluorescent plates.
- 2.4 Square Petri dishes (100 x 100 x 8 mm. Sterilin or similar) for agar development.
- 2.5 Twelve glass or rigid plastic (eg. Perspex) plates approx 120 x 100 mm and 12 large stationary clips (Bulldog clips or similar) for fluorescent development.

- 2.6 Viewing box system of white transmitted light and overhead UV light for looking at completed zymograms.
- 2.7 A camera is useful for recording results, but is **not** essential as the CA plates themselves can be kept as permanent records in plastic envelopes.
- 2.8 The remaining equipment required is similar to that used for general laboratory work, eg. centrifuge, laboratory glass or plastic ware, pipettes (automatic if possible), liquid nitrogen storage system, incubator etc.

## Appendix E

## CELLULOSE ACETATE ELECTROPHORESIS BUFFER SOLUTIONS

## 1. Tank Buffers

1.1 *Tris barbital/sodium barbital pH 9.0 buffers.*

Tris-barbital buffer pH 9.0 is obtained in preweighed solid form in plastic sachets (Helena Laboratories, Beaumont, Texas, USA). The contents of one sachet dissolved in 500 ml of distilled or deionised water gives a stock solution of ionic strength  $I = 0.1$  from which tank buffers 1, 2 and 3 are prepared by dilution. When the tank buffers are being prepared, add any extra ingredients such as magnesium acetate from stock 1.0 M solutions. Remember to add sucrose to a final concentration of 10% (w/v) to ALL electrode buffer solutions **immediately** before use in the electrophoresis chambers.

- BUFFER 1. Tris barbital/sodium barbital pH 9.0,  $I = 0.075$ , 1mM magnesium acetate.
- BUFFER 2. Tris barbital/sodium barbital pH 9.0,  $I = 0.02$ , 1mM magnesium acetate.
- BUFFER 3. Tris barbital/sodium barbital pH 9.0,  $I = 0.02$ .
- BUFFER 4. 0.1M Tris/0.0057M citric acid/0.002M  $\text{Na}_2\text{EDTA}$ / 0.002M Mg acetate pH 8.6.
- BUFFER 5. 0.2M  $\text{KH}_2\text{PO}_4$  adjusted to pH 7.0 with KOH then diluted 3:37 with distilled water.
- BUFFER 6. 0.008M  $\text{NaH}_2\text{PO}_4$ /0.192M  $\text{Na}_2\text{HPO}_4$  pH 8.0 diluted 3:37 with distilled water.
- BUFFER 7. 0.1M Tris/0.1M maleic acid/0.01M  $\text{Na}_2\text{EDTA}$ /0.01M Mg acetate adjusted to pH 7.4 with NaOH then diluted 1:7 with distilled water.
- BUFFER 8. 0.1M Tris/0.0176M maleic acid/0.01M  $\text{Na}_2\text{EDTA}$ / 0.01M Mg acetate pH 7.4, diluted 1:4 with distilled water.
- BUFFER 9. 0.25 Tris/0.05M  $\text{Na}_2\text{EDTA}$ /0.055M boric acid/ 0.0075M Mg acetate pH 9.0, diluted 1:2 with distilled water.

Storage of buffers. Tank buffers without sucrose can usually be stored for up to one week at +4°C. Both may be stored for long periods frozen.

## Staining conditions for isoenzymes

Enzyme	Buffer (see p.40)	H <sub>2</sub> O	Addn. ions etc.	Co- enzyme	Linking enzyme	Substrates	Visual- ization method
ALAT	B 3ml +20% sucrose			NADH 1.5mg	LDH 25 units	2-oxoglutarate 5 mg; L-alanine 60 mg	Filter- paper overlay UV
ASAT	B 3ml + 20% sucrose			NADH 1.5mg	MDH 25 units	2-oxoglutarate 5 mg; L-aspartic acid 12 mg	Filter- paper overlay UV
ES	B 3ml + 20% sucrose					4-methylumbelli- feryl butyrate 0.5mg in 1ml acetone	Filter- paper overlay UV
GPI	A 6.5 ml	0.5ml	MgCl <sub>2</sub> 1M, 0.2ml	(10mg/ml) NADP 0.5ml	G6PD 5 units	D-fructose-6 phosphate (10mg/ml) 0.75ml	*MTT 1ml **PMS 0.5ml ***Agar 10ml
MDH	A 3.5ml	3.2ml		(10mg/ml) NAD 0.5ml		L-malic acid 1M neutralised with NaOH 0.6 ml	*MT 1ml **PMS 0.5ml ***Agar 10ml
MPI	D 6.5ml		MgCl <sub>2</sub> 1M, 0.1ml	(10mg/ml) NADP 0.2ml	GPI 15 units G6PD 5 units	Mannose-5- phosphate (Na <sub>2</sub> ) 2.5mg	*MTT 1ml ***PMS 0.5ml ***Agar 10ml
NH	C 6.5 ml	2.0ml			Xanthine oxidase 0.13unit	Inosine 10mg	*MTT 1ml **PMS 0.5ml ***Agar 10ml
PGM	A 6.5ml	0.5ml	MgCl <sub>2</sub> 1M, 0.2ml	(10mg/ml) NADP 0.2ml	G6PD 5 units	Glucose-1- phosphate containing 1% glucose 1,6,bi- phosphate, 1ml	*MTT 1ml **PMS 0.5ml ***Agar 10ml
6PGD	D 6.5ml	1.0ml	MgCl <sub>2</sub> 1M, 0.25ml	(10mg/ml) NADP 0.25ml		6-phospho- gluconate (10mg/ml),0.5ml	*MTT 1ml ***PMS 0.5ml ***Agar 10ml
PEP D	D 6.5	3.5	MnCl <sub>2</sub>		Peroxid- ase 0.5mg L-amino acid ox- idase 1mg	L-leucyl-L- proline 10mg	3-amino- 9-ethyl carbazole 12 mg in 0.75ml ethanol Agar 10ml (UV)

/continued

Staining Conditions for Isoenzymes (continued)

Enzyme	Buffer	H <sub>2</sub> O	Addn. ions etc.	Co-enzyme	Linking enzyme	Substrates	Visual-ization method
PK	D 6.5ml	1.5ml	MgCl <sub>2</sub> 1M, 0.3ml; KCl 1M, 1.5ml; Fruct-1, 6 diphos. 30mg	ADP 20mg NADH 25mg	LDH 25 unit	Phosphoenol pyruvate (K+ salt) 6mg	***Agar 10ml (UV)
SOD	E 9.4ml + 0.1ml 200mM EDTA neutralized					Riboflavin 0.25mg	<sup>+</sup> NBT (5mg/ml) 0.5ml (special incubation procedure****)

\* MTT = methylthiazole tetrazolium, 5mg/ml  
 \*\* PMS = phenazine methosulphate, 2mg/ml  
 \*\*\* Agar 1.2%  
 \*\*\*\*Incubate at 37° in the dark for 1 to 4 min, then expose to a uniform light source.  
 Light bands appear against a blue background.  
<sup>+</sup>NBT = nitroblue tetrazolium

**2. Developer (staining buffers)**

BUFFER A. 0.3M Tris/HCl pH 8.0

BUFFER B. 0.019 M Na<sub>2</sub>HPO<sub>4</sub>/0.081M Na<sub>2</sub>HPO<sub>4</sub> pH 7.4

BUFFER C. 0.3M Tris/HCl pH 7.0

BUFFER D. 0.3M Tris/HCl pH 7.4

BUFFER E. 0.004M NaH<sub>2</sub>PO<sub>4</sub>/0.096M Na<sub>2</sub>HPO<sub>4</sub> pH 8.0.

**Running conditions for cellulose acetate electrophoresis**

<i>Enzyme</i>	<i>Tank Buffer</i>	<i>Voltage (V/cm)</i>	<i>Running time (mins)</i>
ALAT	3	36	30
ASAT	3	36	30
ES	3	36	30
GPI	4	28	30
MDH	1	21	40
MPI	5	36	25
NH	6	36	30
PGM	7	36	35
6PGD	8	36	25
PEP D	3	36	20
PK	7	28	40
SOD	9	36	30

*Notes:*

1. Incubate the cellulose acetate plates with developer at 37°C (except ALAT and ASAT where they should be left at room temperature as they develop extremely rapidly. Minimise exposure of MTT plates to light to prevent darkening of the background. Note the special incubation procedure for SOD.
2. Protect MTT and PMS solution from light.
3. It is essential that the linking enzymes LDH and MDH are in 50% glycerol and NOT in (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solution, otherwise artefactual bands of glutamic dehydrogenase may appear.

## Appendix F

### PREPARATION OF AGAR FOR DEVELOPERS

To make up 100 ml of agar:

1. Weigh out 1.2 g of plain high purity agar such as Oxoid L28 or one of similar quality.
2. Add the agar slowly to 100 ml of distilled or de-ionised water, stirring continuously.
3. Continue to stir whilst heating the suspension of agar in water to near boiling point. A hot plate with a magnetic stirrer is ideal for this. Do not allow to boil.
4. When the agar has completely dissolved, allow to cool to about 70°C and then dispense into 20 ml quantities in screw- capped glass bottles.
5. If the agar is required for use that day transfer the bottles to a water bath at about 60°C in order to keep the agar molten. If the agar is to be stored, sterilise by heating to 121°C in a pressure cooker (15lb/sq in) for 15 min, first making sure that the cap of the bottle is loose. Once out of the pressure cooker, tighten the lid of the bottle. Store at room temperature.
6. Melt solidified agar solutions before preparing the developer solutions. This may be done, after loosening the screw cap, either by heating in a boiling water bath (slow - can take an hour or more), or heating to 121°C for 10 min in a pressure cooker, or in a microwave oven provided the bottles are not more than half full and do not have metal caps.

## Appendix G

### ARTEFACTS AND CONTROLS

When staining for a particular enzyme after electrophoresis, control plates are run periodically to check whether or not any of the bands observed on the zymogram could be artefacts.

Control plates are prepared, loaded and run under the same conditions alongside the normal plate for the enzyme concerned. When the control plate is developed certain reagents are omitted from the reaction mixture. For enzymes such as the dehydrogenases, eg. MDH, ME, 6PGD etc which require a comparatively simple developer, one control plate is sufficient: the reagent omitted is the principal substrate, eg. malate for MDH and ME. Enzymes such as ALAT or MPI which require complex developers with linking enzymes require several control plates. A substrate is omitted in one plate, and a linking enzyme in another; if several linking enzymes are used, the substrate and each enzyme must be left out of the developer on separate control plates.

Obviously it is uneconomical on samples, time and reagents to include control plates with each electrophoretic run. In reality one runs a series of such controls when introducing a new enzyme and then periodically as a check for contaminants in reagents and buffers.

#### Artefacts that may be encountered on zymograms

##### *Causes of artefactual bands*

Artefactual bands usually belong to a second enzyme and appear for a variety of reasons. Those encountered so far in this particular field are summarized below.

##### *1. Potential substrates present as contaminants in gel buffers*

An example of this is found if matrix buffers containing citric acid are used when screening for enzymes requiring NADP in the developer (eg. dehydrogenases such as ME, G6PD etc.). Artefactual bands of isocitrate dehydrogenase present in the sample stain due to traces of isocitric acid in the citric acid which react with NADP in the developer.

##### *2. Potential substrates in suspending media*

2.1 Linking enzymes are commonly supplied in three different forms: lyophilised, in 50% glycerol solution or suspended in strong ammonium sulphate solution. Artefactual bands of endogenous glutamate dehydrogenase (GDH) are observed in the development of ALAT and ASAT when the respective linking

enzymes LDH and MDH are used in ammonium sulphate suspension rather than in 50% glycerol solution. The ALAT and ASAT developers contain two of the ingredients for GDH development and the addition of the ammonium salt completes the reaction mixture.

2.2 The use of ethanol to help dissolve MTT for NADP linked dehydrogenase reactions can produce artefactual bands due to endogenous alcohol dehydrogenase (NADP). MTT is now dissolved, albeit slowly, in water only.

### 3. *Interference by endogenous enzymes*

Endogenous enzymes can sometimes replace linking enzymes omitted from control plates, giving unexpected positive results. For example the control for ASAT prepared by the omission of the linking enzyme MDH from the developer gives a result similar to that from the normal developer. This is due to the presence of endogenous MDH streaking across the plate during electrophoresis. A similar problem is encountered when staining for aconitase activity, where controls omitting the linking enzyme, isocitrate dehydrogenase, stain as normal due to the presence of endogenous enzyme.

### 4. *Developers staining for more than one enzyme*

These reactions are of two different types.

4.1 The first is due to certain reagents in the developer other than the principal substrate which will react with a second enzyme. An example of this is the tetrazolium developer for pyruvate kinase (PK) which also stains adenylate kinase (AK). Omission of the principal substrate for PK reveals the bands due to AK. Another reaction sometimes seen with a tetrazolium stain if the background darkens, is that of distinct colourless bands due probably to superoxide dismutase (SOD).

4.2 The second type of reaction is one where the principal substrate reacts with more than one enzyme. For example, with the ALAT developer additional bands appear due to the enzyme ASAT, since the broad specificity of ASAT allows the enzyme to utilize alanine as well as aspartate. ALAT, however, is highly specific and does not react with the ASAT developer. On comparison of ALAT and ASAT plates run under the same conditions, the ASAT bands on the ALAT plate can easily be recognised and discounted. A further, more extreme, example of overlapping substrate specificity is shown by the peptidases. Very few peptidases show narrow specificity, although proline peptidase (peptidase D) is an exception, which is the reason for its usefulness in characterization. The majority of peptidases have broad substrate specificities where, for example, a dipeptide such as leucyl-alanine can act as substrate for 6 different peptidases, producing a zymogram with extremely complex banding patterns which defy interpretation. The usual controls cannot unravel this type of reaction and until more specific substrates are discovered for the individual peptidases involved, these enzymes are best disregarded.

## Appendix H

### TRANSPORT AND IMPORT OF CULTURES

It is especially important to adhere closely to national regulations on the movement of pathogenic organisms, both within and between countries. As these regulations vary considerably from country to country, it is pointless trying to summarise them. However, note the following points:

1. If cultures are being imported from one country into another, Check if a special import permit is required for cultures of *Leishmania*.
2. Some countries, eg. the United Kingdom, are very strict on the importation of any animal blood and blood products. Check what is in the culture medium in which the organisms will be sent when applying for permits.
3. Wherever possible, send cultures in plastic tubes. Avoid thin-walled glass culture tubes if possible; if only these are available, **Do not** use cottonwool bungs.
4. Some countries e.g. USA lay down strict packaging instructions for the transportation of pathogens, follow these precisely. In the absence of specific instructions, wrap the tubes in 2 sealed plastic bags. Pack in a metal or strong cardboard or wooden box: include absorbent material in the packing, in case a tube leaks.
5. Many different culture media have been used for the transportation of *Leishmania*, but probably the most reliable are the ones based on semi-solid blood agar such as 'Sloppy Evans' (*Appendix A*). Cultures in such medium have survived delays of 6 weeks or more in delivery.
6. Deep-frozen stabulates of *Leishmania* may also be transported. When the journey time is in excess of 24 hours there is, however, a considerable risk of the stabulates thawing. If the material is accompanied, then it is feasible to carry it in a small Dewar flask containing liquid nitrogen or solid carbon dioxide. If travelling by air, there are special IATA regulations relating to the packing of the containers; consult an airline office! Stabulates in solid carbon dioxide are not in such danger from spillage of the coolant as those in liquid nitrogen; they also do not necessarily require a Dewar flask. Insulated containers made from materials such as expanded polystyrene may be used to hold solid carbon dioxide, and these are quite adequate for short journeys. If the contents of the container can be immersed in liquid nitrogen beforehand and placed in a second insulated container for transportation, the life of the solid carbon dioxide is considerably extended. If you are at all in doubt as to how long the coolant will last, try it out first.

Transporting frozen material is always more troublesome than transporting organisms in culture. Moreover, material carried by hand is the most likely to arrive alive.

Appendix I

LABELLING OF *LEISHMANIA* ISOLATES

In accordance with the recommendations of the UNDP/World Bank/WHO Workshop on the Biochemical Characterization of *Leishmania*, Washington, DC, 1980, the code for the description of isolates should consist of 4 elements, separated by oblique strokes:

(i) The type of host animal or vector from which the strain was isolated. A four letter code should be used: first indicating the class to which the animal or vector belongs (M for Mammalia and I for Insecta) followed by 3 letters indicating the generic name of the host or OOO if the host has not yet been identified. Tables of these code letters are given in *The Leishmaniases: Technical Report Series 701* published by WHO, Geneva 1984.

(ii) The country where isolation was made. The country of isolation is indicated by a 2 letter code - a table of codes is on p.128 of Technical Report Series 701.

(iii) The year of isolation is indicated by the last 2 digits or 00 if unknown.

(iv) The original laboratory code given to the isolate.

Thus an isolate made from man in France in 1979 and coded CERV-1 would have the following code:

MHOM/FR/79/CERV-1

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