Fluorescence microscopy for disease diagnosis and environmental monitoring

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and environmental monitoring**

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Foreword

Fluorescence microscopy has, for some time now, enhanced the microscopic diagnosis and monitoring of both communicable and noncommunicable diseases. It has entered many other fields also, especially those in the health sciences, including food safety and research. The latest fluorescent microscopes are much less expensive and more easily transported than the older versions, making the technology affordable now for small laboratory and public health settings in developing countries.

This comprehensive publication provides laboratory specialists with all the up-to-date information necessary to purchase and use a fluorescence microscope, including suppliers to approach. It addresses, in detail, technical issues such as the equipment itself and the reagents used, principles of immuno-fluorescence microscopy and last, but certainly not least, the use of such microscopy in clinical laboratory diagnosis.

Fluorescence microscopy for diagnosis and environmental monitoring confirms fluorescence microscopy as a rapid and cost-effective diagnostic tool for countries with limited resources, and as an invaluable technique that has been tested and validated by experts around the world and, above all, by laboratory practitioners in the field. I strongly recommend its use among central public health laboratories in developing countries.

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Preface

In countries with limited resources simple, rapid and cost-effective diagnostic techniques are a key element for improving medical and public health laboratory services at district and peripheral levels. In these laboratories, bright-field microscopy still remains the primary diagnostic technique. Rarely has it been displaced by other, more modern techniques because these are usually more complex and require a continuous line of logistical support. However, there are certain technologies that do fulfil the technical and logistical requirements of these laboratories and should enjoy a routine role in improving diagnostic efficiency. One of these is fluorescence microscopy.

Fluorescence microscopy has proved to be a useful and cost-effective procedure for surveillance of disease outbreaks and for diagnosis of a wide range of both communicable and noncommunicable diseases. Fluorescence microscopy is almost as simple and rapid to do as bright-field microscopy, and often it is also more specific. However, fluorescence microscopy requires additional investment in equipment. In the past, the high cost of fluorescence microscopes has prevented the wider application of this method. More recently, less expensive fluorescence microscopes have been developed, and accessories now are available that convert an existing bright-field microscope into a fluorescence microscope. This development places fluorescence microscopy in a favourable position as a method that can be used by smaller laboratories with limited resources to enhance their effectiveness at affordable cost.

The World Health Organization's Regional Office for the Eastern Mediterranean is convinced that laboratories should take more advantage of fluorescence microscopy. This manual provides basic information on fluorescence microscopy and an overview of many simple and cost-effective applications for diagnosing diseases and monitoring environmental contamination by laboratories, even at district or peripheral levels.

Abbreviations

Ab   antibody

AFB   acid-fast bacilli

Ag   antigen

BAM   bright-field acid-fast microscopy

BSA   bovine serum albumin

CBS   carbonate-buffered saline

CFU   colony forming unit

DANS   1-dimethylaminonaphthalene-5-sulfonic acid

DAPI   4,6-diamidino-2-phenylindol hydrochloride

DFA   direct fluorescent antibody

DNA   deoxyribonucleic acid

EMEM   Eagle's minimum essential medium

FA   fluorescent antibody

FAM   fluorescence acid-fast microscopy

FDA   fluorescein diacetate

FITC   fluorescein isothiocyanate

FM   fluorescence microscopy

IF   immunofluorescence

IFA   indirect fluorescence antibody

im.   intramuscular

iv.   intravenous

L   litre

M   molar

mL   millilitre

NA   numerical aperture

NAC   N-acetylcysteine

nm   nanometre

NSS   non-specific staining

PBS   phosphate-buffered saline

RB 200   rhodamine B 200

RNA   ribonucleic acid

sc.   subcutaneous

TRITC   tetramethylrhodamine isothiocyanate

URI   upper respiratory tract infections

UV   ultraviolet

Glossary

Affinity: tendency of certain substances or their compounds to unite with other substances and form new compounds.

Antigen: a substance, usually a protein or carbohydrate, capable of stimulating an immune response in an animal.

Antibody: any of an animal's immunoglobulins that are produced in response to an antigen and are able to counteract the affects of the stimulating antigen by neutralizing toxins, agglutinating cells or precipitating soluble antigen; i.e., an animal's body defence mechanism.

Autoantibody: an antibody directed against a patient's own body tissue.

Autofluorescence: synonymous with self, natural or primary fluorescence. The appearance of fluorescence in tissues or materials that have not been treated with labelled conjugates.

Fluorochrome: a chemical compound that emits fluorescent light after exposure to shorter wavelength (higher energy) light (usually ultraviolet, purple, blue or green).

Heterologous: derived from a different substance, i.e. antigen or antibody.

Homologous: reaction of an antibody or antigen with the corresponding specific antigen or antibody.

Monoclonal antibody: antibody derived from a single cell clone.

Monovalent antigen or antibody: antigen or antibody having only one site at which it can become attached to an antibody or antigen, respectively.

Nonspecific staining (NSS): a result of nonimmunological staining by fluorochrome

1. Introduction

Fluorescence is light produced by a substance when it is stimulated by another light. It is "cold" light because it does not come from a hot source such as the filament of an incandescent light bulb. Fluorescence is a short-term event; the fluorescent substance produces light only while it is being stimulated.

Fluorescence microscopy is a way to discover facts about specimens that often are not visible to bright-field microscopy. In bright-field microscopy, specimens are illuminated from the outside, from below or above. In bright-field microscopy dark objects are seen against a light background. In fluorescence microscopy, specimens are self-illuminated by internal light, so fluorescence shows bright objects in vivid colour against a dark background. Bright objects against dark backgrounds are more easily seen. Since the light comes from within the specimen, internal structures and certain substances can be seen and located. These characteristics make fluorescence microscopy a very sensitive and specific tool.

Fluorescence microscopy is used in medicine, environmental studies, food sanitation, biological research, education and industry. One of the main applications is for rapid medical diagnosis. Fluorescent staining is commonly used to improve tuberculosis diagnosis efficiency as well as for malaria diagnosis, for early detection of bacteria in blood cultures, and to detect and identify nucleic acids by colour. Fluorescent antibodies provide a wide variety of immunologically specific, rapid diagnostic tests for infectious diseases. In certain cases, detecting and identifying disease-causing organisms is only possible by fluorescence microscopy because some organisms cannot be cultured easily or the organisms in a specimen are dead.

Air and water samples are easily tested for microbial contamination using fluorescence microscopy. Organisms trapped from these specimens on the surface

of membrane filters can be quickly detected and identified in place, without need for culture. Environment monitoring by this method is easy and very efficient.

Fluorescence microscopy is efficient for direct counting of microorganisms in food and dairy products. It is used to test both raw and pasteurized milk and is about 100 times more sensitive than standard culture methods. Fluorescence detection of microorganisms on membrane filters is also used to control the quality of wine and potable water.

The advantages of fluorescence microscopy are due to its sensitivity, specificity, adaptability and easy use. Reliability is high because it is simple and can be well controlled.

This makes fluorescence microscopy a very useful tool for teaching biology. The brilliant colours of fluorescence seen in illuminated specimens are very memorable for students.

The equipment needed for fluorescence microscopy ranges from very complex microscopes to relatively simple devices, most bright-field microscopes can be easily converted into fluorescence microscopes.

Using fluorescence microscopy can reduce laboratory testing costs. Fluorochrome dyes are effective at very dilute concentrations, 1:10 000 or greater. Thus, the cost per test is low. When detecting bacteria in blood cultures, fluorescence microscopy can replace expensive and slow subculturing, saving both time and expensive culture media. It considerably reduces time per test, further reducing costs. For example, examining a tuberculosis sputum slide by the standard Ziehl–Neelsen stain requires 15–20 minutes. Using fluorescent acid-fast stain, the time for investigation can be reduced to 2–3 minutes. In many other ways, specific and sensitive fluorescence microscopy can replace certain conventional bacteriological culture uses, reducing costs. Laboratories can easily improve their services by using fluorescence microscopy, which saves time in laboratory testing to detect pathogenic microorganisms that other methods may miss.

1.1 Principle of fluorescence

Light is electromagnetic energy that moves through space as massless particles called photons. Light may also be thought of as moving in waves with high

and low points. The distance between a point on one wave, for example the peak, and the same peak on the next wave is the called the wavelength. The energy is inversely proportional to the wavelength—the shorter the wavelength, the more energy. Wavelengths of the visible light spectrum range from about 400 nm to 750 nm. Different light wavelengths of visible light are seen by humans as different colours. The wavelengths of some basic colours are: violet: 420 nm; blue: 480 nm; green: 520 nm; yellow: 590 nm; orange: 630 nm; and red: 660 nm. Longer wavelengths of light above 750 nm are invisible light called infrared Shorter wavelengths of light below 400 nm are a higher energy invisible light called ultraviolet. Near ultraviolet means wavelengths just shorter than 400 nm, and far ultraviolet means even shorter wavelengths, about 350 nm and less. Infrared light has relatively low energy and low penetrating power, while the shorter-wavelength ultraviolet light has greater energy and penetrating power. The energy spectrum continues, and radiation with wavelengths shorter than ultraviolet light, such as gamma rays and X-rays, have still more energy and deeper penetrating power.

When light radiation of high enough energy strikes a substance that can fluoresce, the substance absorbs that energy and converts a small part of it into vibrational energy (i.e. heat). The energy that is not absorbed by the substance is emitted again as light (Figure 1.1). The emitted light is called fluorescent light. The wavelength of the emitted light is always longer than the wavelength of the generating light. This phenomenon is called Stokes shift. Fluorescence is short duration, and fluorescent substances emit light without noticeable delay and only while being stimulated.

1.2 Primary and secondary fluorescence

A fluorescent substance can be excited by invisible ultraviolet light, and it will emit longer-wavelength visible light, for example, violet, blue, green or red light. However, many fluorescent substances are also excited by visible violet, blue or green light. In fact, visible light is more often used to stimulate fluorescence than invisible ultraviolet light. The commonly used dye fluorescein is a good example. Exciting this fluorescent dye with blue light yields yellow-green fluorescence light.

Substances that can be activated to fluoresce are called fluorochromes. Fluorochromes may be naturally present in biological materials or may be artificially
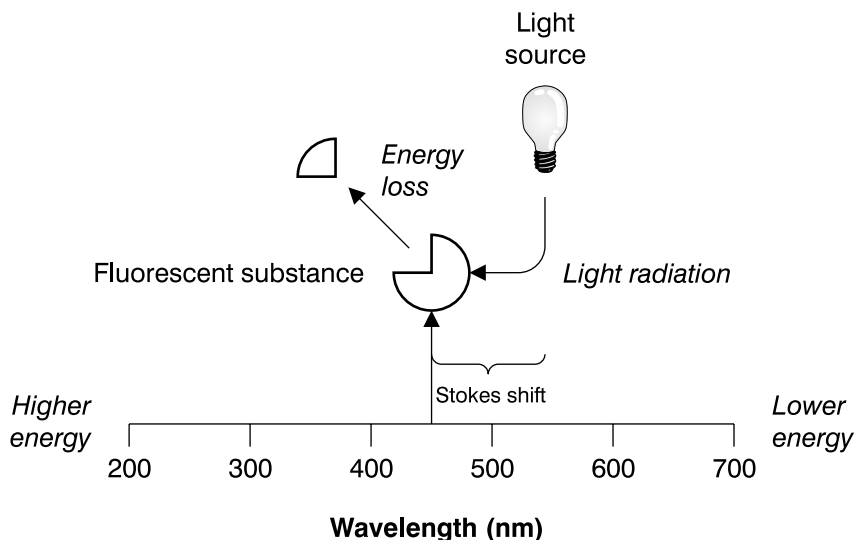
Figure 1.1 **Scheme showing the origin of fluorescence radiation**

introduced into these materials. Fluorescence emission from untreated materials is known as primary, natural, self- or autofluorescence. When fluorescent substances are artificially introduced into a specimen, the emitted light is called secondary fluorescence. Fluorochromes should, when possible, be specific for those structures or molecules that are of special diagnostic interest.

1.3 Fluorescence in microscopy specimens

Most biomedical specimens (cells, tissues and microorganisms) do not have useful primary fluorescence. Thus, to observe fluorescence in most biological specimens by microscopy, they first must be stained with a fluorochrome. The ultimate goal of staining specimens with fluorochromes is to get specific fluorescence that makes certain areas of a specimen clearly visible.

Structures in some plant tissues show primary fluorescence in different colours making these structures stand out in vivid detail. However, at times natural primary fluorescence (autofluorescence) can interfere with clinical specimen or animal

tissue section examination. Protein in tissue sections or specimen slide smears often fluoresces a dull blue. This autofluorescence can be overcome with a fluorescent counterstain. On the other hand, autofluorescence can be helpful for locating the field of view, especially in negative slides. Thus, autofluorescence is a mixed blessing.

2. Applications of fluorescence microscopy

A fluorescence microscope delivers the exciting light to the specimen, separates the emitted fluorescence light from the exciting light and provides a high contrast image. It permits users to easily see very small cells and their contents in microscopic specimens with outstanding detail. Fluorescence microscopy often reveals features in a specimen that cannot be seen by standard bright-light microscopy, especially internal structures. As a result of the exceptionally low fluorochrome concentrations needed for secondary fluorescence (1:10 000 or higher), fluorochromes are safely used to examine living tissue and microorganisms.

Fluorescence microscopy applications are outlined in Table 2.1

Fluorescence microscopy offers a number of advantages, especially for medical diagnostic tests. These are as follows.

- *Easy*. Preparing and staining fluorescence test specimens is as easy as, or easier than, conventional slide staining. Examining fluorescent specimens is easy because the contrast is excellent.

- *Sensitive*. Due to the relatively bright fluorescent image against a dark background, small objects are easy to see.

- *Specific*. By using modern fluorochromes and interference light filters, high specificity for details is possible.

- *Rapid*. Specimens can be examined directly on a slide, often without any prior purification or concentration.

- *Reliable*. Good reliability and accuracy of microscopic analysis are due to the high sensitivity and specificity of fluorescence microscopy techniques. Risks

of wrong observations or differences in test interpretation are reduced, even when done by less qualified technicians.

- *Universal*. Fluorescence microscopy can be applied to a wide range of biomedical disciplines such as bacteriology, mycology, virology, parasitology, serology, immunology, autoimmunology, cytology, cell biology and histochemistry.

- *Adaptable*. Fluorescence microscopy methods can replace and improve many conventional bright-field microscopy tests. Most compound microscopes can be used for fluorescence microscopy, provided the instruments are equipped with a few fluorescence accessories.

Table 2.1 **Fluorescence microscopy applications**

Infectious disease diagnosis	*Industrial process control*
Agent detection and identification	Monitoring of clean rooms
Antibody titration	Detection of fluid contamination
Toxin detection	Sampling of food and beverages
Autoimmune disease diagnosis	*Disease diagnosis*
Antibody detection and identification	Etiological agent detection and identification
Antibody titration	Antibody titration
Environment monitoring	*Environment microorganism monitoring*
Microbial aerosol monitoring	Air testing (aerosols)
Water analysis	Water analysis (bacteriological safety)
Limnology	Sewage treatment control
Emergency support	*Biological attack defence*
Casualty diagnosis	Rapid diagnosis of casualties
Microorganism aerosol detection and	Detection of biological aerosols
identification	Sampling of suspicious munitions
Biological research	*Industrial process control*
Location and identification of cells	Monitoring of clean rooms (electronics)
Identification of tissues	Detection of fluid contamination
Detection of nucleic acids	(electronics, pharmaceuticals)
	Detection of product contamination
	(food, beverages)

2.1 Medical disease diagnosis and monitoring

The most common use of fluorescence microscopy is for medical diagnosis. Rapid, simplified infectious disease diagnosis is possible with fluorescence microscopy. Often, it is the most effective, or the only, way to make diagnosis. Microorganisms such as bacteria, viruses, fungi and parasites can be detected and generally or specifically identified. This approach provides very rapid and sensitive diagnosis by using fluorochromes that are specific for an infecting agent of disease. Examples are fluorescent antibody (serologically specific staining) and the fluorescent acid-fast stain. Fluorescent antibody tests are available for a wide variety of infectious disease agents. Fluorescent acid-fast stains are very useful for diagnosing important diseases such as leprosy and tuberculosis.

It is not always necessary to have particulate antigens such as bacteria or protozoa to study disease. Soluble toxins and viruses can also be demonstrated in tissues or specimens with fluorescent antibody. For example, the technique has been used to show staphylococcal enterotoxin in an animal's body after ingestion and locate its action in the brain and other organs where it causes toxic symptoms. Similar findings are possible with other soluble antigens.

Another important medical use of fluorescence microscopy is to detect, identify and titrate host antibodies to infective agents. This is done by the indirect fluorescent antibody (immunofluorescence) test. Certain autoantibodies or anti-tissue antibodies cause diseases in which the body's immune system attacks the body itself. These autoimmune diseases can be detected and identified by indirect fluorescent antibody test. Distinctive patterns of staining are seen in the tissues involved or in other cells that react with specific antibodies in a patient's blood that are characteristic of a disease.

2.2 Monitoring of the environment

Public health laboratories routinely monitor microorganisms in the environment. Water, air and soil are tested. Samples are normally examined by bacterial culture, but they can also be investigated by fluorescence microscopy. Often, more microorganisms are detected in a sample by fluorescence microscopy than by culture. This is because some organisms in a sample may be dead or damaged or

are difficult to culture. Fluorescence microscopy used for this purpose is economical because fluorochrome dyes are inexpensive and analysis time is short. Expensive culture media and bacteriological equipment are not needed to do the tests. Perhaps the most significant advantage of fluorescence microscopy for sample analysis is a fast result. Instead of days to obtain a result, as with culture, fluorescence microscopy provides results within hours or minutes. Fluorescence microscopy is used to detect microorganisms in potable water, fresh water and seawater. It is the preferred analysis method to detect *Cryptosporidium* protozoa in finished potable water.

In most water analysis applications the sample is drawn through a membrane filter that has sub-micron-sized pores. The microorganisms are trapped on the surface of the filter where they may be grown in place for colony counts or directly observed by microscopy, especially epi-illumination fluorescence microscopy (see Section 4.2).

Air samples are also collected and tested by fluorescence microscopy. Air is passed through a filter, and subsequently the filter is examined for microorganisms by epi-illumination fluorescence microscopy. This can be done directly or by first collecting the air sample in a liquid using a device called a liquid impinger. Following collection, the sample in the liquid may be filtered, and the microorganisms trapped on the filter surface are observed by epi-illumination fluorescence microscopy. Air sampling with fluorescence microscopy is used in industrial settings as well in the open air to detect microbial aerosols. Other liquids are examined by fluorescence microscopy on filters as well, including parenteral fluids and dilute disinfectants.

2.3 Food sanitation and safety

The method primarily used by the food and beverage industries to detect microbial contamination is direct counting by epi-illumination fluorescence microscopy on solid foods and liquid foods. It is particularly useful for dairy products, but other liquids such as wine are analysed for progression of the process using fluorescence microscopy. It has been used to test both raw and pasteurized milk.

Microbial contamination of animal carcasses during meat processing can be monitored quickly and easily by fluorescence microscopy. For example, pathogens

such as *Escherichia coli* O157:H7 and *Listeria monocytogenes* can be detected. A standardized method, the direct epifluorescent filter technique (DEFT), uses either fluorescent antibody or the fluorochrome acridine orange. The DEFT method is used to detect the presence of microorganisms on food processing surfaces and also to assess the effectiveness of cleaning methods for food processing equipment and areas. Thus, epi-illumination fluorescence microscopy is a valuable tool in the food industry that efficiently helps keep food products safe.

2.4 Biological research

Fluorescence microscopy in biological research reveals the exact location, development and functions of tissues and cells. Fluorochromes and immunologically specific fluorescent antibodies can be used as markers. Certain fluorochromes also distinctively stain subcellular structures of an organism, for example cell wall structure, composition and nucleic acids, depending on various factors in the specimen such as chemicals, viscosity and pH.

Use of fluorescence microscopy can provide basic biological data for locating DNA and RNA in tissues, cells and organisms. The most common fluorescent probe used is acridine orange. Another method, called fluorescence *in situ* hybridization (FISH), visually demonstrates DNA and RNA sequences, providing further insight into basic biology. Acridine orange is a vital stain. When used at very high dilutions, it does not kill organisms, and thus it allows the study of microorganisms in their living state with their functions intact. For example, studies have been made of motile paramecia and other protozoa. The organelles on motile protozoa can be easily seen and differentiated by various fluorescent colours. Fluorescent markers are also used to study redox reactions in respiring bacteria. Viable cells are examined for their ability to differentiate. Leukocytes reveal their phagocytic and microbiocidal activities when examined by fluorescence microscopy.

Fluorescence microscopy permits quantitative measurement of various substances in cells and tissues. This includes toxins and viruses that can be specifically visually located in the parts of the tissues or cells that they affect. For example, viruses infecting cells may be located in the nucleus or various parts of the cytoplasm. Different viruses inhabit different compartments of cells, and this

may be important when developing therapy for infections or for diagnostic tools. In cancer studies, fluorescence microscopy has been used to demonstrate the development of polyps and cancers of the colon. In basic biology of the environment, fluorescence microscopy has been used to demonstrate viable, but non-culturable, salmonella in soil. It also has been used to demonstrate the interaction between microorganisms and the roots of plants.

3. Equipment for fluorescence microscopy

3.1 Basic fluorescence microscope parts

The equipment needed for fluorescence microscopy comprises:

- compound microscope (basic platform and lenses)
- high-power illuminator (excitation light)
- exciter light filter (selects light colour)
- dichroic mirror (reflects or passes certain light colours—epifluorescence only)
- barrier filter (blocks low wavelength light).

A *compound microscope* is the platform that brings together all the other parts and provides magnification and resolution of the specimen image.

A *light source* supplies bright light of a suitable wavelength (colour) to stimulate fluorescence.

An *excitation* (*primary*) *filter* transmits only the light wavelength (colour) needed to excite fluorescence of the fluorochrome used. It blocks other wavelengths from the light source that are not needed.

A *dichroic mirror* further reflects the exciting light colour, but it passes the higher wavelength fluorescence light.

A *barrier* (*secondary*) *filter* blocks any exciting light that may escape the other filters, and it also passes the higher wavelength fluorescence light.

Figure 3.1 illustrates the schema described above.

Excitation   Fluorescent   Barrier
filter   substance   filter

Fluorescence light

Excitation light

Observer

White light (many colours)
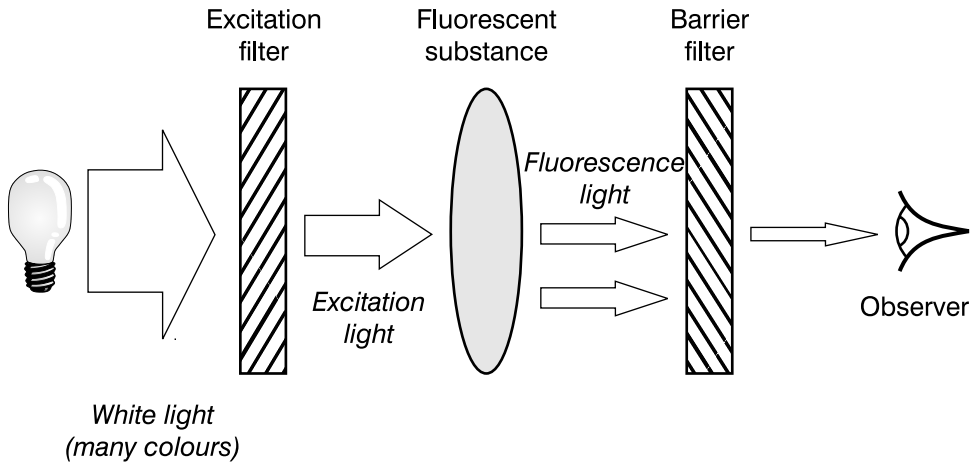
Figure 3.1 **Schematic diagram of a fluorescence microscopy system**

3.2 Compound light microscopes for fluorescence microscopy

A standard compound microscope fitted with an appropriate excitation light source and suitable light filters can be used for fluorescence microscopy. There are two types of fluorescence microscope:

- transmitted light (excitation illumination through the specimen, usually from below)

- epi-illumination (incident light shining on the surface of the specimen).

Transmitted-light fluorescence microscopy usually needs a dark-field condenser. This type of condenser provides a dark background against which fluorescence is easily seen. Dark-field condensers concentrate the exciting light on the specimen. However, since the light passes through the slide and the specimen, it can be compromised by factors such as slide thickness, slide material and the opaqueness of the specimen itself. Since all specimens must be transparent for examination by transmitted light fluorescence microscopy, it makes examination of specimens collected on filter surfaces difficult. Thus, air and liquid specimens collected by filtration are best examined by epi-illumination fluorescence.

It is important to understand that a primary concern in fluorescence microscopy is to maximize the fluorescence image. The light energy needed to excite fluorescence is far greater than the fluorescence light produced, so all primary factors must be optimized. These primary factors are:

- wavelength of the exciting light

- numerical aperture (NA) of the objective lens

- total magnification.

In order to see the relatively weak fluorescence light, it is necessary to suppress unwanted wavelengths of the exciting light. This requires an efficient barrier filter to allow the fluorescence light to pass to the observer while blocking out the excitation light. Various types of satisfactory light filter are available (see section 3.4).

The NA of the objective lens is very important. The primary function of the objective lens is to gather light; and the larger the NA, the more light that is gathered. In epi-illumination fluorescence microscopy, the objective lens also acts as the condenser lens. Because the objective in epi-illumination first concentrates the excitation light and then gathers fluorescence light, the fluorescence image intensity varies directly with the fourth power of the numerical aperture. This is a significant increase. Light intensity decreases with the square of the magnification. The formula is as follows:

$$I = \frac{NA^4}{M^2}$$

where *I* is the light intensity, *NA* is the numerical aperture and *M* is the total magnification.

Therefore examining specimens at medium magnifications, ×400 to ×600, provides the best combination of object size and bright fluorescence. Because of the basic nature of fluorescence, it is possible to examine fluorescent specimens better at lower magnifications than by bright-field microscopy.

A fluorescence image appears much brighter with an objective lens of a certain magnification and NA than it does with the same magnification lens with a smaller NA. For example, most ×40 objective lenses are 0.65 NA, but some manufacturers

make a ×40 objective lens (oil immersion) with 1.0 NA or even 1.3 NA. These objectives yield much brighter fluorescence images than the dry objective[1]. Furthermore, since fluorescence images are brighter at lower magnification using a ×6 or ×8 ocular lens with a given power objective lens will produce a brighter image than if using the more common ×10 or ×12.5 oculars.

Some medium-power objectives (×40 to ×63) are designed with large NAs— 0.95 to 1.4. The best ones are used for very critical fluorescence microscopy, and these are fluorite or apochromat lenses. These types of objective also give superior colour correction and image clarity. However, these objectives are expensive.

An important technical point is that the NA of the condenser lens should be as close as possible to the NA of the objective lens. Therefore, dark-field condensers must be changed to provide suitable NAs when using different magnification objective lenses in transmitted light fluorescence microscopy.

Most disadvantages of transmitted-light fluorescence microscopy are overcome by epi-illumination fluorescence microscopy, a simpler technology that does not need a dark-field condenser. In epi-illumination fluorescence microscopy the objective lens itself acts as the condenser, so the NA of the "condenser" exactly matches any objective lens being used. This improves efficiency, promotes simplicity and is more economical because a separate condenser lens is not needed. Also, because epi-illumination deals with the surface of the specimen, it is intrinsically more efficient and provides improved fluorescence. The optical arrangement of transmitted and epi-illumination fluorescence microscopy are compared in Figures 3.2a and 3.2b.

Various types of objective lenses are satisfactory for fluorescence microscopy including achromat, plan-achromat, fluorite, and apochromat objective lenses. Modifications of these objectives are also satisfactory for fluorescence microscopy, but these types of objectives are expensive and only used in special research applications. For most purposes, standard achromat objectives are satisfactory and are the ones most commonly used. Plan-achromat lenses are used when a flat field of view is needed, and these lenses are common today.

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[1] Dry objectives cannot have NA greater than 1.0, the refractive index of air.

Observer

Ocular lens

Fluorescence light

Barrier filter

Objective lens

Fluorescent
specimen
on slide

Microscope stage

Dark-field
condenser

Excitation filter

Excitation light

Light source
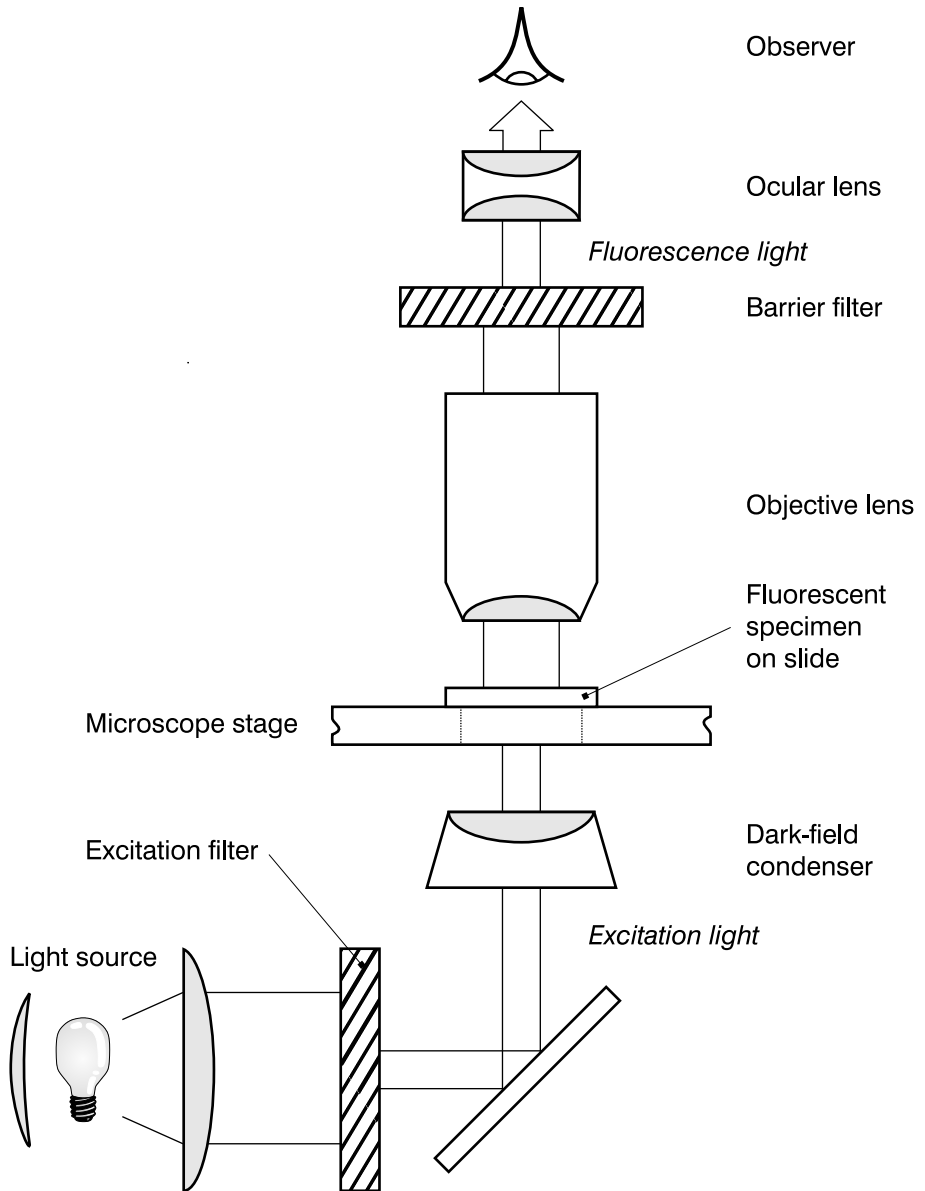
Figure 3.2a **Optical arrangement of transmitted fluorescence microscopy**

Observer

Fluorescence light

Ocular lens

Barrier filter

Excitation light

Light source

Dichroic beam splitter

Excitation filter

Objective lens (also condenser lens)

Fluorescent specimen on slide

Microscope stage
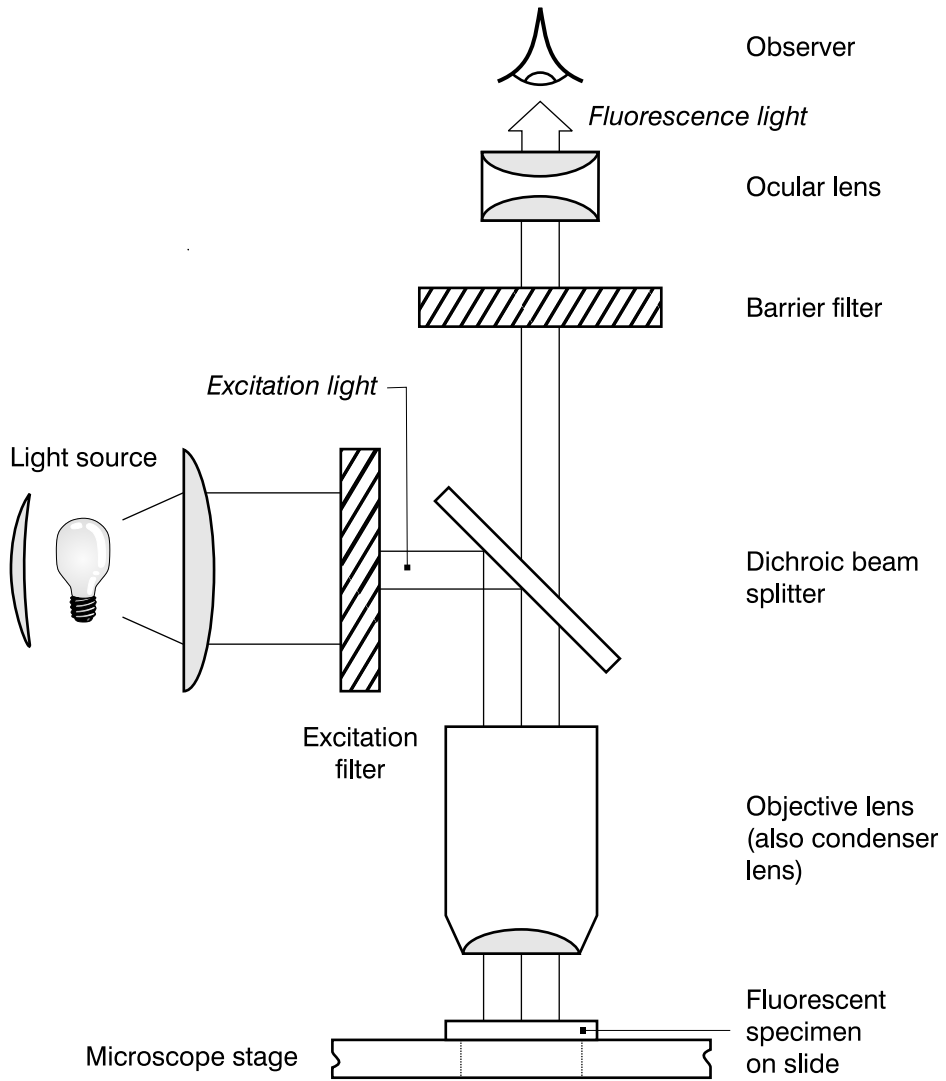
Figure 3.2b **Optical arrangement of epi-illumination fluorescence microscopy**

Fluorite and apochromat objectives offer several advantages, large NA and complete spectrum colour correction. Larger NA in an objective lens increases light gathering power and thus improves the fluorescence image, especially in low fluorescence specimens. Complete spectrum colour correction is important in certain photomicrography applications. These advantages of fluorite and apochromat objectives come at considerably higher cost than achromats, and fluorescence microscopists must balance the need for advantages with this increased cost.

Objective lenses with high NA are usually immersion objectives. These objectives require a liquid between the lens and the slide to avoid the change in refraction of light passing through a different medium (air). Most immersion objectives are designed for use with oil. The oil should be synthetic non-fluorescent oil with a refractive index of 1.515, which is the refractive index of the glass in the objectives. While most high NA objectives are oil immersion, objective lenses are available that are corrected for water immersion or glycerol immersion. These objectives are useful for examining wet mounts and living organisms. They are also good for fieldwork because these lenses are easily cleaned without using solvents.

Certain objective lenses may fluoresce, thus degrading the fluorescent image from the specimen. Achromat objectives rarely fluoresce, but some older apochromats do. The fluorescence in older apochromat objectives is caused by ultraviolet light stimulation. For epi-illumination fluorescence microscopes using quartz–halogen lamps this is not a practical issue; only mercury or xenon excitation light sources emit ultraviolet light.

Choice of ocular lenses (eyepieces) is also important for fluorescence microscopy. The most common types are the standard Huygens and wide-field eyepieces. Compensating oculars are needed when using apochromat objectives. Lower power oculars with ×5 to ×8 magnification are desirable for fluorescence microscopy. Microscope manufacturers offer immersion objective lenses with magnifications of ×40 to ×60, and these have NAs ranging from 0.95 to 1.4. These lenses are excellent for fluorescence microscopy when used with ×10 oculars but even better with lower power oculars. Unfortunately these objective lenses tend to be expensive. A relatively inexpensive alternative combination is to use the commonly available ×100 (oil) objective, NA 1.25–1.3, with a low power ocular, for

example ×5 or ×6. This combination yields very satisfactory bright fluorescent images at minimal cost.

Compound microscopes used for fluorescence microscopy are usually standard upright models. However, fluorescence microscopy also can be done with inverted microscopes such as those used for examining tissue cultures in flasks or dishes. Since the bottom of the container may be thick, inverted microscopes often have long working distance objectives to focus through the container to the cells.

There may be a brighter image with a monocular microscope than with a binocular microscope because there are fewer lens and prism surfaces in the light path to cause light loss. However, the slight loss of light intensity is insignificant when using mercury or xenon light sources, and choice may be dictated by the greater viewing comfort of a binocular microscope. When using a monocular microscope, the unused eye can be covered to provide viewing comfort. For fieldwork, a monocular microscope is less bulky and lighter, so it is more easily transported.

3.3 Light sources for fluorescence microscopy

Three types of excitation light source are commonly used for fluorescence microscopy. These light sources are quartz–halogen, mercury vapour and xenon. Each provides light across the visible spectrum, and the mercury and xenon lamps give near-ultraviolet light as well (Figure 3.3). Quartz–halogen lamps give little or no ultraviolet light. Therefore, they cannot be used for fluorochromes that need ultraviolet excitation. However, they are safer to use than mercury or xenon light sources, as there is no radiation hazard.

Blue and blue–violet light excite fluorescence in the most commonly used fluorochromes for medical and environmental studies including fluorescein isothiocyanate (FITC), auramine O and acridine orange. All three types of lamp yield enough blue or blue–violet light for satisfactory epi-illumination fluorescence microscopy with these fluorochromes. Mercury and xenon lamps provide more intense light output than quartz–halogen lamps, and these provide the strong green light needed to excite fluorescence in rhodamine-type fluorochromes. Quartz–halogen lamps are not satisfactory for use with rhodamine-type fluorochromes. Recently, laser diodes have been successfully used as light sources for small,
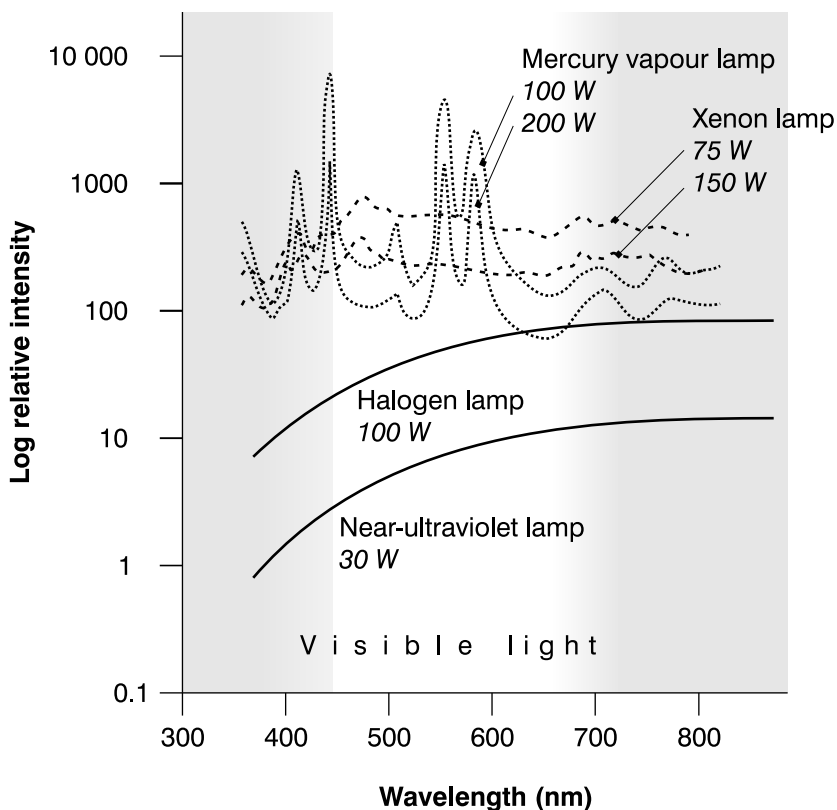
Figure 3.3 **Comparison of light spectral output from different types of light source**

portable fluorescence microscopes [source: private communication, D. Jones, University of Marburg].

When installing any light bulb, it is important to avoid touching the glass envelope with the fingers. This leaves an oily residue that becomes permanent when the lamp is turned on, and it reduces the light output. Only handle a lamp with holders that are sometimes provided or using a clean cloth. When removing or changing a lamp, wait until it is completely cooled. Mercury and xenon vapour lamps have high internal pressure and become extremely hot when on. Handling the lamps when cold will avoid burns and minimize the possibility of a lamp bulb exploding.

Arc lamps, such as mercury and xenon vapour lamps, must be set up to fill the objective field with excitation light. Therefore the lamp image and its mirror image are arranged side by side in the field. Manufacturers provide adjustment mechanisms to move the lamp and reflecting mirror to do this. Follow the lamp manufacturer's instructions carefully.

Caution:

• Never operate the lamp without its housing in place.

• Never look directly at the lamp or its image when it is on.

• Never touch the lamp when it is on or hot.

When the lamp is first turned on, give it a few minutes to warm up and get to full intensity. Light output from arc lamps and some halogen lamps is controlled with a field diaphragm or by putting neutral density filters in the light path. A rheostat on the lamp housing can control the intensity of some quartz–halogen lamps. When this type of lamp is first turned on, leave it at the low position for several minutes before increasing the intensity. This will lengthen bulb life.

3.4 Light filters for fluorescence microscopy

Light filters are essential in fluorescence microscopy for selecting appropriate colour wavelengths to stimulate fluorescence and to block unwanted excitation light from the observer's eye. Filters provide wavelength colour selection. They can be changed for different microscopy applications. Various types of light filters are used. In some situations, a certain type of filter is required while in others, choices may be made to gain good results balanced with economy. Light filters fall into several types; short band pass, long band pass, wide band pass, narrow band pass and dichroic. These are compared in Table 3.1.

A short band pass filter passes light up to a selected wavelength. It does not allow light of higher wavelengths to pass through the filter. In practice, these filters usually pass ultraviolet, purple, blue and green light. A long band pass filter only passes light above the selected wavelength; thus these tend to pass green, yellow, orange and red light. Wide band pass and narrow band pass filters are self-explanatory: many light wavelengths are passed through the wideband pass filter

Table 3.1 **Light filters and their uses in fluorescence microscopy**

Filter type	Use	Filter method
Short band pass	Excitation	Colour absorption or interference
Long band pass	Barrier (blocking)	Colour absorption or interference
Wide band pass	Excitation	Colour absorption or interference
Narrow band pass	Excitation	Barrier (blocking), interference
Dichroic	Beam-splitting mirror	Colour beam-splitting (epi-illumination)

and few through the narrow band pass. Interference filters and narrow band pass filters are used in epi-illumination fluorescence microscopy, especially for the excitation light.

A dichroic beam-splitting mirror is essential for epi-illumination fluorescence microscopy. This device reflects short wavelength light and passes long wavelength light. Typically, the dichroic mirrors used are at least 90% efficient for reflectance and transmission. For example, if a fluorochrome absorbs low wavelength blue–violet light and emits fluorescence in the green–yellow range, the blue–violet dichroic mirror light reflects, and the green–yellow fluorescence light passes to the eye of the microscopist. This relationship is shown in Figure 3.4.

Two kinds of filter are used for fluorescence microscopy, colour absorption and interference. Colour absorption filters were mainly used for transmission fluorescence microscopy, but interference filters have replaced these for epi-illumination fluorescence microscopy (Table 3.2). An exception is the barrier filter. For this, some manufacturers still use colour absorption long band pass barrier filters for their high light transmittance and lower cost.

Colour absorption filters are usually made of glass, but gelatin filters are also used. A wide range of colours is available. With glass filters, the amount of absorption depends on the thickness of the glass. This is not possible with gelatin filters as they are all the same thickness. However, gelatin filters are inexpensive and easily cut to shape or size. For special needs, they can serve as temporary supplementary filters.
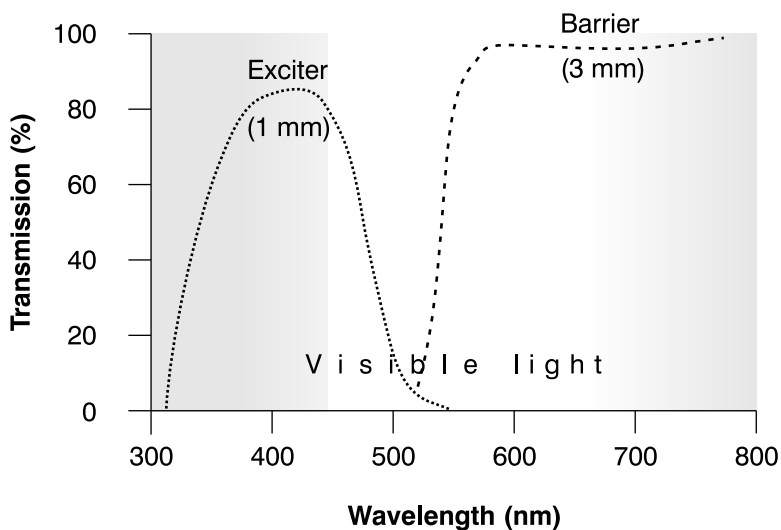
Figure 3.4 **Principle of complementary filters**

Table 3.2 **Filter types for fluorescence microscopy**

Excitation system	Application	Filter type
Epi-illumination	Exciter	Interference
	Barrier	Interference (uncommon) Coloured glass Gelatin (uncommon)
Transmitted light	Exciter	Coloured glass Interference
	Barrier	Coloured glass Gelatin Interference (uncommon)

4. Practical fluorescence microscopy

4.1 Bright-field versus fluorescence microscopy

The basic difference between bright-field and fluorescence microscopy lies in the way microscopic objects are viewed. With bright-field microscopy, dark objects are viewed against a light background. In dark-field or fluorescence microscopy, bright objects are viewed against a dark background. The human eye sees bright objects against dark backgrounds more easily.

As a result of improved object viewing, a small object, such as a bacterial cell, can be seen at lower magnification with fluorescence microscopy than by bright-field microscopy. This means that lower power objectives and ocular lenses can be used that provide larger fields of view. For example, a ×20 objective will show approximately four times the field area as that shown using a ×40 objective. Thus, a technician can examine more area of a slide specimen in a shorter time. This is important when examining specimens like sputum slides for acid-fast bacilli or blood smears for malaria parasites. Fluorescence microscopy increases diagnostic efficiency. This means that efficient examination of slide specimens such as sputum for acid-fast bacilli can be done much faster by fluorescence microscopy than by bright-field microscopy.

4.2 Epi-illumination fluorescence microscopy

Epi-illumination fluorescence microscopy allows lower output excitation light sources such as quartz–halogen lamps to be used. These are inexpensive compared with arc-type lamps. The efficiency of the epi-illumination fluorescence system is due in part to the fact that the exciting light stimulates fluorescence on the surface of

the specimen. Moreover, since the excitation light passes through the same objective lens to the specimen and the fluorescent light passes back through the objective, the objective lens serves as its own condenser. This amplifies the fluorescent light by several orders of magnitude. This means a perfect match of NA and elimination of a separate condenser lens.

4.3 Basic equipment and supplies for epi-illumination fluorescence microscopy

Equipment

- Fluorescence microscope, epi-illumination (FITC filter sets for FITC conjugates, acridine orange, and auramine O and/or TRITC filter sets for rhodamine-type conjugates), halogen or mercury vapour light source

- Centrifuge, general laboratory type (optional depending on test)

- Refrigerator

- Freezer, –20 °C

- Ventilated safety cabinet (for highly pathogenic agent work)

- pH meter, thin combination electrode

- Supplies and reagents

- Applicator sticks

- Basic laboratory tools (bacteriological loops, forceps, diamond scriber, wax glass marker, etc.)

- Absorbent paper (bibulous paper book or filter paper)

- Bunsen burner or alcohol lamp

- Cotton gauze squares or a roll of gauze

- Cover glasses, No. 1 or No. 1.5, 22 mm square or 22 × 50 mm

- Ethanol, 95%

- Humid chamber (Petri dish with wet filter paper in the base and applicator sticks to support slides)

- Immersion oil (non-fluorescent, synthetic) refraction index 1.515

- Microscope slides (plain or pre-etched with two or three circles), 25 × 75 mm

- Buffered glycerol mounting medium, pH 8.5–9.0

- Nail polish (cover glass sealant)

- Phosphate buffered saline, pH 7.4 ± 0.1

- Stain rack

Comments

Microscope slides

Standard slides can be used with epi-illumination fluorescence microscopes, but some manufacturers offer special slides for fluorescence microscopy. These slides have various sizes of etched or plastic masked circles on them. The multi-well slides expedite the performance and decrease the cost of some routine illumination fluorescence testing. They retain the stain and limit the amount needed to specific spots of the specimens on the slides.

Cover glasses

The microscopist should try to have as homogeneous an optical system as possible in order to maximize image quality. The thickness of cover glasses used for fluorescence microscopy is critical. Commercial cover glasses are rather uniform in refractive index but vary widely in thickness. No. 1 or No. 1.5 cover glasses are preferred. Thicker cover glasses sometimes may cause a hazy, bleached image appearance.

Immersion oil

For fluorescence microscopy, non-fluorescent immersion oil, such as Cargille type A low-fluorescence non-drying immersion oil for microscopy, must be used. It has a refractive index of 1.515 at 23 °C. Various manufacturers produce similar synthetic oils that do not dry on the objective lens.

Specimen-mounting medium

The refractive index of the cover glass–mounting medium should closely match that of the cover glass. If the refractive index of the mounting medium differs considerably from that of the cover glass, it may have an adverse affect on image quality. Mounting media should be adjusted to the pH suitable for optimal fluorescence of the fluorochrome used. For FITC it should not be lower than pH 7.2 because FITC fluorescence decreases rapidly below this pH. Oxidation and absorption of CO_2 occur in stored mounting medium and thus decrease the pH of the glycerol in the mounting fluid. Therefore, pH of the mounting fluid should be at least pH 8.0, preferably between pH 8.5 and 9.0. The pH should be checked at least once a month, preferably weekly.

Buffered glycerol (9 parts of glycerol to 1 part [v/v] of 0.5 M carbonate buffer [pH 9.0]) is commonly used as a cover glass mounting medium. The edges of cover glasses may be sealed with nail polish, and some preparations sealed this way may be preserved at 4 °C for weeks or months. However, this varies with the specimen and stain used. As results are not always consistent after storage, slides should be examined as soon as possible.

4.4 Set-up and adjusting an epi-illumination fluorescence microscope

Setting up most epi-illumination fluorescence microscopes is not complicated. Each manufacturer provides clear directions, and these should be followed carefully.

The basic steps to operate an epi-illumination fluorescence microscope and examine specimens are as follows.

1. Select a microscopy viewing area with subdued light, not completely dark. This allows the technician's eyes to adjust to low light levels.

2. Turn on the excitation light source. Allow it to warm up for several minutes.

3. Swing the desired magnification objective into place directly above the centre of the microscope stage. (Sometimes it is convenient to start with a lower-power objective to more easily find the field and then to switch objectives for maximum magnification desired.)

4. Place a slide with a specimen in the centre of the stage. Secure it with the mechanical stage clip on the mechanical stage arm.

5. Lower the objective to the slide while observing its approach from the side. Avoid the objective touching the slide. The proper distance from the slide for each objective—working distance—will be learned from experience. (Some microscopes have a mechanical stop that can be set near the proper focal plane. The same thickness slide must be used each time.)

6. Increase the brightness of the excitation light to a practical level. (This can be done by opening the diaphragm, changing neutral density filters, or turning the rheostat knob, if available.)

7. Carefully focus up or down to bring the fluorescing specimen into focus. It is best to use the fine focus knob for this.

Note: sometimes it is hard to find the plane of the specimen when using fluorescence microscopy, especially with specimens that have few organisms or are negative. In these cases, it may be helpful to first focus on the specimen by bright-field using the substage lamp. Then, switch to fluorescence.

4.5 Using an epi-illumination fluorescence microscope

Fluorescence light brightness is low compared with light used in bright-field microscopy. Although little light falls on the eye of the observer, the system can be adjusted so that this light is easy to see. This is because fluorescence light is seen against a contrasting dark background. In many specimens, only a small part of the total field actually emits fluorescence light, and the brightness of this fluorescence light decreases or fades over time. To efficiently use available light in fluorescence microscopy, the following principles should be followed.

• Choose a solid place for the microscope that is free from vibration and shocks in a dust-free room that can be darkened. In most cases use epi-illumination (incident light) for fluorescence microscopy rather than transmitted light.

• Either transmitted light or epi-illumination light can be used to do fluorescence microscopy. In most cases, epi-fluorescence is the more effective and easier method. Transmitted-light fluorescence microscopy, usually with dark-field

light excitation, offers few advantages. Epi-illumination also is better for doing photomicrography.

- For specimens of average fluorescence brightness, epi-fluorescence methods allow exposure times of only 10–20 seconds (high-speed colour film) instead of several minutes as with fluorescence microscopes using transmitted light.

- Align the light source, microscope optics and specimen exactly. Each manufacturer's instructions should be followed carefully.

- Use objective lenses with as large NA as possible. (Fluorescence intensity increases exponentially with numerical increase in NA.)

- Use low magnification eyepieces (oculars), ×6 or ×8 instead of ×10 or ×12.5. (Fluorescence light intensity decreases exponentially with increase in total magnification.)

- Position the fluorescence microscope away from windows. (Fluorescence microscopy done in a subdued light area allows the user's eye to adapt to the low light level. Avoid complete darkness.)

- Use a microscope with a short, simple light path. (Light losses occur at all free surfaces of lenses, prisms and mirrors along the light path.)

- Make records after any changes to the apparatus, such as changing the light bulb.

- Do not put frosted ground glass in the excitation light path. (Ground glass severely reduces light levels.)

4.6 Inexpensive transmitted-light fluorescence microscopy

Originally, fluorescence microscopy was done by light transmitted through a microscope dark-field condenser, the slide and the specimen. The more efficient and simpler equipment used to do epi-illumination fluorescence microscopy later replaced this system. Thus, transmitted-light fluorescence microscopy gradually fell out of favour and is seldom used today.

However, there is an exception, due to the observations of Fumihiko Kawamoto [Kawamoto F, 1991]. He showed that transmitted-light fluorescence microscopy

was possible using an interference filter for the excitation filter, the standard bright-field condenser, and the light from a 50-watt or greater halogen lamp. This excited good fluorescence in acridine orange–stained malaria parasites in thin blood smears.

The advantages of this approach are that the need for a dark-field condenser is eliminated, and conversion of standard microscopes to transmitted light fluorescence microscopes is easy and inexpensive. Various sources of excitation light can be used. Part of the reason this system works well is due to fluorescence efficiency of acridine orange stain.

The excitation filter used is an interference filter passing blue or blue–violet light. The interference filter can be placed on an external halogen lamp, and the blue light projected off the microscope mirror. Some condensers have a filter holder where the interference filter can be placed. It also can be attached to the underside of the condenser by means as simple as tape.

A yellow colour filter of glass or gelatin is used as a barrier filter, and this is placed anywhere in the microscope light path above the objective, for example, in the microscope body or in the oculars. This system has been used successfully in several African countries. The system is shown in Figure 4.1.
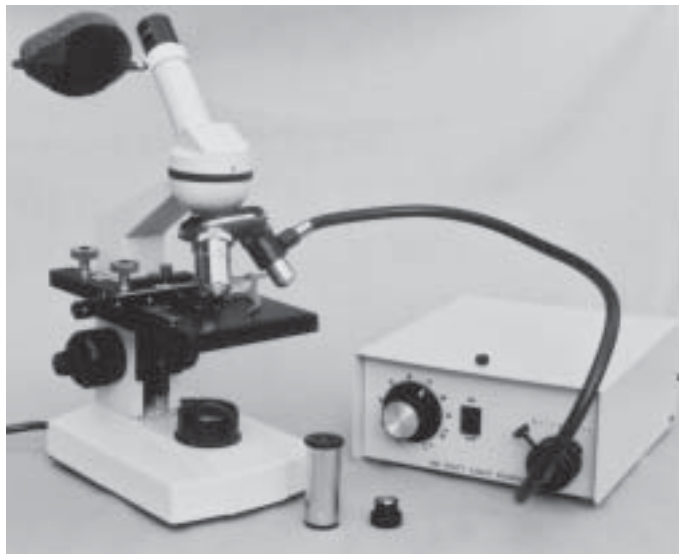
Figure 4.1. **Inexpensive transmitted-light fluorescence microscope**

4.7 Concentrating specimens for fluorescence microscopy

Many specimens for analysis may contain only a few microorganisms. Concentrating microorganisms into a smaller specimen volume or removing them from the specimen for direct examination increases their sensitivity to detection and identification. This can be done several ways:

- centrifugation

- membrane-filter concentration

- immunomagnetic particle separation.

4.7.1 Centrifugation

The most commonly used concentration method is centrifugation. Most laboratories have centrifuges in order to concentrate particles suspended in liquids, for example, cellular elements in urine, bacteria in cerebrospinal fluid, or helminth ova in faecal specimens. The principle of centrifugation is simple. A specimen is centrifuged at high speed so that centrifugal force sends particles of higher specific gravity than the suspending liquid to the bottom of the container, usually a specially designed test tube. A centrifuge's sedimentation capability is measured as "relative centrifugal force". This is expressed as multiples of gravitational (*g*) force—the acceleration due to the earth's gravity at sea level, about 9.8 m/s^2. Factors involved in sedimentation efficiency are speed of rotation, radius of rotation and time. For sedimenting small particles like bacteria or to clarify fluorescence antibody conjugates, use a centrifuge capable of at least 10 000*g*. The time of centrifugation needed varies by specimen. Determine this by experiment. In some instances, as when sedimenting *Mycobacterium tuberculosis* bacteria in sputum, the bacteria may be the same specific gravity as, or even less than the suspending fluid. In these instances, the bacteria will not sediment.

4.7.2 Membrane-filter concentration

Membrane-filter concentration is an excellent way to concentrate microorganisms for detection by epi-illumination fluorescence microscopy. Membrane filters have pore sizes usually ranging from 0.45 μm to 0.20 μm. Use polycarbonate filters

because they have a smooth surface that makes cells lying on it easy to see. A polycarbonate filter is a physically strong material that resists many solvents, so it is adaptable to various handling and staining methods. Polycarbonate filters and various designs of holding devices are available from a number of manufacturers (see Annex 12.5).

Materials

- Membrane filter, 0.45 µm or 0.20 µm pore size (polycarbonate, black preferred for low background fluorescence).

- Filter holder (many designs and sizes are available).

- Vacuum source (a small vacuum pump or a large suction syringe are commonly used).

- Sample (liquid or gaseous).

- Diluent and washing liquid (commonly filtered water).

- Epi-illumination fluorescence microscope.

- Fluorochrome dye or fluorescence antibody reagent.

- Buffer solution.

- Cover glass (optional depending on specimens or methods used).

- Mounting medium (for cover glass).

- Immersion oil (low fluorescence, non-drying).

Method

1. Set up the filter apparatus with the membrane filter in place. Usually, the holder is attached to the neck opening of a side-arm vacuum flask.

2. Add the sample (if the sample is small, first dilute it in a larger volume of filtered water to aid efficient distribution of microorganisms in the filtration process).

3. Draw the diluted sample through the filter via vacuum.

4. Wash the filter and filter holder with filtered, deionized H_2O or an appropriate filtered buffer.

5.   Draw the wash liquid through the filter.

6.   Remove the filter with trapped particles on its surface from the holder.

7.   Place it in a convenient container such as a Petri dish.

8.   Stain the filter with a fluorochrome or fluorescent antibody conjugate, and then rinse it.

9.   Mount the filter on a slide with a drop of low fluorescence immersion oil.

10. Examine the filter with an epi-illumination fluorescence microscope.

4.7.3 Immunomagnetic particle absorption

In the immunomagnetic separation method, magnetic particles or beads are coated with specific antibodies. The sensitized particles are mixed with a liquid suspension of the antigen sought, for example, cholera bacteria in a diluted stool specimen or CD-4 cells in blood. The antigen attaches to the antibody-coated magnetic particles (beads), and then a magnetic device is used to extract the particles from the suspension. This method is quite sensitive. It is capable of efficiently collecting organisms when only a few are present in a large specimen.

The magnetic particles (beads) are coated with a material that adsorbs antibody, for example, agarose, latex, polyacrylamide or polystyrene. Experimentally determine the type to be used with each antigen–antibody system. After reaction, remove the magnetic particles carrying the target antigen from the liquid suspension using a magnetic collecting device. These range from automatic or semi-automatic commercially available instruments to a simple magnetic wand swirled in the suspension.

Detect and identify the antigen on the magnetic beads by one or another of various means. These can be culturing (if bacteria), serological tests including fluorescence antibody, and fluorescence microscopy using acridine orange. Methods for concentrating antigens vary with manufacturers of the magnetic beads. Refer to each manufacturer's instructions for specific test methods. Magnetic beads are available commercially (see Annex 12.5).

5. Fluorochromes

5.1 Properties of fluorochromes

A fluorochrome is a chemical dye that fluoresces (emits light) when it is stimulated (excited) by certain wavelengths (colours) of light. There are many hundreds of fluorochromes, and new ones are being synthesized to meet new requirements. Some are general fluorescent stains, staining a wide variety of tissues and substances. Others specifically stain certain cells, tissues or structures based on factors such as specimen pH, viscosity or the presence of certain chemicals. Others differentiate between living and dead cells. Some are specific for white blood cells, mitochondria or chromosomes. Certain fluorochromes can be attached (conjugated) to antibodies to form immunologically specific stains. This combination is called a fluorescent antibody, and the technique is known as immunofluorescence. This stain is particularly useful in medical laboratory diagnosis.

For the most part, fluorochromes are used in basic biological and medical research and applied work. The most common application is for medical diagnosis, detecting and identifying disease-causing microorganisms and antibodies in immunological diseases. These are also used in environmental studies to detect and specifically identify microorganisms in water and air samples.

Some commonly used fluorochromes are listed in Table 5.1. This table includes peak absorption and emission wavelengths (colours), as well as fluorochrome applications. While each fluorochrome has at least one absorption peak, some may be stimulated to yield practical fluorescence by several peak wavelengths of excitation light. An example is Evans blue, which is used as a red fluorescing counterstain in fluorescent antibody tests using green fluorescing fluorescein

isothiocyanate (FITC). Although Evans blue is maximally excited by green light, it also fluoresces red when stimulated by blue light, the best colour light to excite FITC. Thus, when using combinations of fluorochromes, actual experimentation may be needed to find the best light wavelengths to use for the excitation light.

Other fluorochromes commonly used are acridine orange and auramine O. Acridine orange has many uses, and some of these are described in detail below. Auramine O is used mainly to detect acid-fast bacilli in sputum specimens. Acridine orange also can be used to do this, or it can be used as a contrasting counterstain for auramine O acid-fast stains as described below. Fluorescent acid-fast stains are diagnostically very efficient.

Table 5.1 **Commonly used fluorochromes, their characteristics and applications**

Fluorochrome	Absorption peaks (nm)	Emission peaks (nm)	Staining applications
Acridine orange	460, 500	525, 650	DNA, RNA, cells, general bacteria, acid-fast bacteria, fungi
Auramine O	460	550	Acid-fast bacteria
Berberine sulfate	430	550	General bacteria
Calcophor white	365 (UV)	435	Fungi in tissue
Coriphosphine O	460	575	Leukocytes, bacteria, mucus
Dimethylaminonaphthalene-5-sulfonic acid (DANS)	340 (UV)	525	Antigen–antibody reactions
Evans blue	550	610	Fluorescent counterstain (red)
Fluorescein isothiocyanate (FITC)	490	525	Antigen–antibody reactions
Lissamine-rhodamine B (RB 200)	570	590	Antigen–antibody (RB-200) reactions, fluorescent counterstain (red)
Texas Red	595	615	Antigen–antibody reactions
Tetramethylrhodamine isothiocyanate (TRITC)	555	620	Antigen–antibody reactions

5.2 Filter sets for commonly used fluorochromes

The most commonly used fluorochromes are FITC, acridine orange, and auramine O. Less frequently used fluorochromes are RB 200, TRITC, and Calcofluor white. In order to use these fluorochromes for fluorescence microscopy, companies offer a variety of filter combinations. Basically, these excite the dyes with ultraviolet, violet, blue–violet, blue or green light. More expensive fluorescence microscopes, mainly used for research, offer a wide range of filter sets, while the smaller, more economical microscopes usually have only a few choices. However, for routine epi-illumination fluorescence microscopy, these filter sets are quite adequate.

On the less expensive microscopes the filter sets are usually semi-permanently or permanently installed. In more sophisticated microscopes, most use filter-cube systems. These are plastic holders in the shape of a cube that contain the exciter and barrier filters along with a dichroic mirror. Often, the filter cubes are mounted on a slide that carries more than one filter cube. This slide in the light path makes changing filter cubes for different applications very easy. The system is mainly designed for research applications, and it is seldom needed for routine diagnostic work.

While the exciter filter and dichroic mirror used in epi-illumination fluorescence microscopy must be the interference type, the barrier filter can be either an interference filter or a colour filter, glass or gelatin. The main advantage of the interference type barrier filter is a sharper wavelength cut-off, while the advantage of the colour filters is lower cost. From a practical point of view for the barrier filter there is little technical difference, and cost may be the deciding factor.

FITC, auramine O, and acridine orange all can be used with the same filter set. Excitation is with blue–violet or blue light, and some companies use a broader band exciter filter that includes ultraviolet. Wavelengths in the near ultraviolet and up to about 460 nm will stimulate blue autofluorescence in tissues. Using an exciter filter passing wavelengths from 450 nm to 490 nm generally reduces or eliminates autofluorescence. In the final analysis, for routine medical diagnosis, a filter set from any manufacturer indicated for the fluorochrome being used can be applied with confidence.

6. Use of fluorochromes

Fluorochromes can be used alone or in combination with other fluorochromes to create multiple contrasting fluorescent colours in a specimen to clearly differentiate parts. Fluorochromes used singly include acridine orange and fluorescent antibody, but these can also be used in combination with other fluorochromes. Acridine orange can give different colours in a specimen depending on its content. For example, when acridine orange is used to stain a cerebrospinal fluid specimen from a patient suffering from meningitis, the bacteria stain red in contrast to a green or yellow background. When used to stain protozoa, the nuclei are often green while the cytoplasm is red. Combinations of fluorochromes are used when a counterstain is needed to produce a contrasting colour background. One version of the fluorescent acid-fast stain uses auramine O as the primary stain and acridine orange as the counterstain. This results in yellow fluorescing acid-fast bacilli against an orange fluorescing background of sputum debris. The fluorescing background is especially useful when dealing with negative specimens. It makes the specimen field easy to find.

In some research applications, two or three fluorescent antibody preparations made with different fluorochromes are used to stain various structures in different colours of fluorescence. This technique vividly shows the relationships between specific parts of a cell or tissue specimen. This technique is beyond the scope of this manual.

Green fluorescent antibody stains are often used with contrasting red or orange counterstains. The fluorochromes Evans blue and Congo red have been successfully applied. Also, lissamine-rhodamine-conjugated bovine serum albumin makes a good counterstain when mixed with an FITC conjugate. A particularly useful counterstain is Eriochrome black. It does not cover the specimen fluorescence, and

it suppresses non-specific staining. Since there are a wide range of fluorochromes that produce different colours, many combinations can be tried to gain the desired result.

Other fluorochromes stain specific structures in tissue and so are useful to locate positions of known structural elements in a specimen. This makes alignment or orientation easier. The most successful of these are:

• propidium iodide (5 [3-(diethylmethylammonio)propyl]-3, 8-diamino-6-phenylphenanthridinium diiodide)[1]

• brilliant cresyl blue

• DAPI (4, 6-diamidino-2-phenylindole hydrochloride).

Viewing nuclei in tissue specimens can be done using propidium iodide or DAPI[2].

Ethidium bromide is also an efficient fluorochrome for visualizing nuclei. However it has been abandoned because of its carcinogenic effect.

6.1 Acridine orange—diagnostic uses

Acridine orange is often used for fluorescent staining of nucleic acid–containing cells including nucleated blood cells, bacteria and protozoa. The following sections describe applications of acridine orange for a variety of diagnostic investigations.

_____

[1] Propidium iodide stock solution: 2 mg/mL in 0.15M saline.
Keep the stock solution (2 mg/mL) of propidium iodide refrigerated after dissolving it in saline, 0.15 M.
Add the embedding medium to the stock solution to give a concentration of 1 μg/mL.

[2] DAPI stock solution: 25 mg/mL in H_2O (distilled).
Dilute 3 μL of the stock solution in 1 mL phosphate buffered saline.
Add a drop of the diluted solution directly to the conjugate on the preparation 5 minutes before the end of the last incubation.

6.1.1 RNA/DNA identification

Purpose

Staining of RNA and DNA differentially in tissue and cells.

Materials and reagents

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Acetic acid 1%.

Ether–ethanol.

Ethanol series (80%, 70% and 50% to water).

Acridine orange, 1:10 000 (w/v).
Note: for preparation, see Annex 13.

Phosphate buffer, pH 6.0, 0.15 M.

Distilled water.

$CaCl_2$ 0.1M.

Method

1. Fix the specimen slide in ether–ethanol, 3 minutes.

2. Hydrate the specimen: 80%, 70%, 50% ethanol and distilled water, 1 minute each step.

3. Rinse in 1.0% acetic acid, 10 seconds.

4. Rinse in distilled water, two changes, 30 seconds each.

5. Stain with 0.01% (1:10 000) acridine orange, 3 minutes.

6. Rinse in phosphate buffer, pH 6.0, 30 seconds.

7. Differentiate in 0.1M $CaCl_2$, 2 minutes (the time may have to be increased in some systems for good differentiation).

8. Rinse in phosphate buffer, pH 6.0, two changes.

9. Mount in phosphate buffer, pH 6.0, under a No. 1 coverslip.

10. Examine the slide using a fluorescence microscope with FITC filters.

Results

Reading: Green or yellow–green = DNA

Red or orange = RNA.

References

Bertalanffy L, Bertalanffy FD, 1960; Bertalanffy FD, Nagy KP, 1962.

6.1.2 Blood smears for the diagnosis of bacterial septicaemia

Purpose

Detection of difficult-to-stain microorganisms in blood smears (such as *Borrelia*).

Approach

Fluorescence microscopy detects spirochetes in blood by use of contrasting colour fluorescence at low pH.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Acridine orange stain, acetate buffer, pH 3.5, 0.15M.
Note: for preparation, see Annex 13.

Methanol, absolute.

Formaldehyde.

Method

1. Prepare peripheral blood smears.

2. Fix the smears with:
    a. Formaldehyde vapour, 20 minutes, or
    b. Methanol, 2–5 minutes.

3. Stain the smears with acridine orange stain, 2–5 minutes.

4. Wash with tap water.

5. Air-dry the smears.

6. Examine smears with a fluorescence microscope (FITC filters) at about ×600 magnification. Confirm spirochete morphology at ×1000.

Results

Borrelia spirochetes are found in thick parts of the smears. They appear bright orange against a green or black background.

Note: acridine orange to detect spirochetes in peripheral blood smears is simple and more sensitive than Wright–Giemsa stain.

Reference

Sciotto CG et al., 1983.

6.1.3 Cerebrospinal fluid specimens for the diagnosis of bacterial meningitis

Purpose

Detection of bacteria in cerebrospinal fluid specimens.

Approach

Acridine orange at low pH is used to stain cerebrospinal fluid and other normally sterile clinical specimens.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Acridine orange stain, acetate buffer pH 3.5, 0.1 M.

Note: for preparation, see Annex 13.

Methanol, absolute.

Method

1. Make a slide smear of the cerebrospinal fluid specimen.

2. Air-dry the smear.

3. Fix the smear in methanol, 1–2 minutes.

4. Flood the fixed smear with acridine orange stain, 2 minutes.

5. Wash the smear with tap water.

6. Air-dry the smear.

7. Examine the stained smear with an epi-illumination fluorescence microscope (FITC filters).

8. Survey the smear at ×100 to ×400, no more than 5 minutes per slide.

9. Confirm suspect positive bacteria at about ×500 to ×800 with an oil immersion objective.

10. Confirm positives, when indicated, with Gram stain so that the Gram reaction of the bacteria is known.

Results

Bacteria stain brilliant orange against a black, light green, or yellow background. Many bacteria can be identified this way.

Note: the acridine orange stain is more sensitive than Gram stain on cerebrospinal fluid specimens. It is effective when the bacteria count in cerebrospinal fluid is 10^4/mL, 10 times less than with Gram stain. Since acridine orange fluorescence microscopy can be done at lower magnifications, specimens can be adequately examined in no more than 5 minutes.

References

Kleiman MB et al., 1984; Lauer BA et al., 1981.

6.1.4 Urine for the diagnosis of bacteriuria

Purpose

Fluorescence microscopy for the efficient diagnosis of bacteriuria.

Approach

Urine is alkalinized and stained with acridine orange in wet mounts followed by examination for red fluorescing bacteria with a fluorescence microscope.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Thoma bacterial counting chamber.

Borate buffer, pH 9.8.

Acridine orange aqueous solution.
Note: for preparation, see Annex 13.

Method

1. Measure 0.8 mL of borate buffer solution into a small test tube.

2. Add 0.6 mL of urine and gently mix.

3. Add 25 µL of acridine orange solution.

4. Incubate the tube at 40 °C for 5 minutes (water bath preferred).

5. Remove an aliquot from the tube, and place it in a Thoma bacterial counting chamber.

6. Examine the chamber with an epi-illumination fluorescence microscope, using TRITC filters at about ×500 magnification. (An immersion objective and water, glycerol or oil may be used.)

Results

The bacteria are marked as dots with bright fluorescence.

References

Manson R et al., 1985; Scholefield J et al., 1985.

6.1.5 Buffy coat for diagnosis of bacterial septicaemia

Purpose

Rapid diagnosis of neonatal bacteraemia.

Approach

Buffy coat leukocyte samples stained with acridine orange are examined by fluorescence microscopy to detect bacteria.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Haematocrit capillary tube, 75 mm, heparinized.

Haematocrit centrifuge.

Blood collection supplies.

Acridine orange in acetate buffer, pH 4.0, 1:10 000 (w/v).
Note: for preparation, see Annex 13.

Method

1. Collect blood by venepuncture.

2. Fill the capillary tube.

3. Centrifuge the capillary blood specimen in a haematocrit centrifuge at 10 500 rpm (= 7500g) for 2 minutes.

4. Score and break the capillary tube at the buffy coat–red cell interface.

5. Make a smear of the buffy coat on a slide.

6. Heat fix the slide smear.

7. Flood the slide with acridine orange for 2 minutes at room temperature.

8. Rinse the slide with tap water (dechlorinated water is preferable).

9. Air dry the slide.

10. Examine the slide with an epi-illumination fluorescence microscope, FITC filters, ×500 to ×1000 magnification.

Results

Bacterial cells are bright red–orange, inside and outside white cells. Leukocytes are yellow–green. Bacterial morphology is distinct.

Note: this method is superior to Gram stain and takes less time to prepare the smears and observe them.

References

Kleiman MB et al., 1984a; Kleiman MB et al., 1984b.

6.1.6 Malaria diagnosis from blood smears

Purpose

Rapidly, efficient and inexpensive diagnosis of malaria.

Approach

Acridine orange staining is used to detect and identify malaria parasites in blood smears.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Capillary blood collection supplies.

Phosphate buffered saline, pH 7.2–7.4

Acridine orange in phosphate buffered saline, pH 7.2–7.4 (acridine orange concentrations from 20 µg/mL to 100 µg/mL can be used).

Methanol, absolute.

Sodium heparin (optional).

EDTA (optional).

H_2O, distilled or deionized (chlorine-free).

Method

1. Collect blood from a finger stick, and prepare a thin blood smear.

2. Air-dry the blood smear.

3. Fix the smear in methanol.

4. Air-dry the fixed smear.

5. Stain the fixed smear with acridine orange solution[2].

6. Examine the stained smear using an epi-illumination fluorescence microscope with FITC filters.

Results

Parasite cytoplasmic RNA fluoresces red, while nuclear DNA fluoresces green, all against a dark background. Thus, the *Plasmodium* trophozoites appear as red signet rings with green stones.

Acridine orange staining for malaria diagnosis is significantly more sensitive than Giemsa (or Wright–Giemsa) staining. The greater sensitivity of is particularly evident when parasitaemias are below 5000 per μL. However, it is less specific, and sometimes Giemsa staining is needed to precisely identify the species of malaria parasite found. Acridine orange staining is very inexpensive, costing about €0.05 per test (Figure 6.1).

Notes

1. *Concentrations of acridine orange higher than 100 μg/mL are not advised because parasite nuclei may fluoresce red instead of green. A stock solution of 0.5% acridine orange can be used to prepare the use dilutions. Solutions can be protected from bacterial growth by adding a few drops of sodium azide solution.*

2. *Three ways to stain blood smears with acridine orange:*
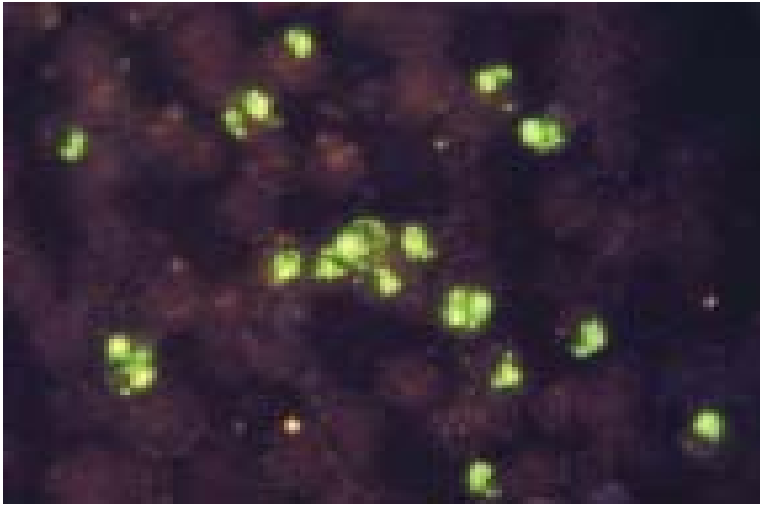
Figure 6.1 **Fluorescent antibody stain of *P. falciparum* in a blood smear**

a) Direct smear staining
1. *Cover the smear with acridine orange solution, 30–60 seconds.*
2. *Rinse the smear with H_2O.*
3. *Place a coverslip on the wet smear.*

b) Coverslip staining
1. *Pipette the acridine orange solution onto a coverslip laid over two applicator sticks.*
2. *Invert the slide with the fixed blood smear, and carefully place it on the coverslip.*
3. *Invert the slide with the attached coverslip and acridine orange (the acridine orange solution acts as a mounting medium).*

c) Dip staining
1. *Dip the slid smear in the acridine orange solution, 1 second.*
2. *Mount a coverslip on the stained smear (the acridine orange solution acts as a mounting medium).*
3. *Acridine orange-stained slides can be washed with methanol and restained with Giemsa or Wright–Giemsa stain.*

References

Gay F et al., 1996; Kawamoto F, 1991; Lowe BS et al., 1996.

6.1.7 Screening for trichomoniasis

Purpose

Convenient and sensitive screening test for *Trichomonas vaginalis* infection.

Approach

Acridine orange is used to stain slide smears of fresh vaginal specimens and those mailed to the laboratory.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Acridine orange, 0.1% aqueous.
Note: this stock solution is made monthly and stored in a brown bottle in a refrigerator.

Phosphate buffer, 0.067 M, pH 6.0.

$CaCl_2$, 1.1% aqueous.

Ethanol, 50%.

Saline, 0.85%.

Spray-Cyte.
Note: Spray-Cyte™, Clay-Adams, New Jersey.

Cotton-tipped swab.

Method

1. Obtain a vaginal specimen with a cotton-tipped swab.

2. Place the swab in a test tube with 1 mL saline, and twirl it in the saline.

3. Press the swab onto a microscope slide.

4. Allow the smear to dry until tacky.

5. Spray the smear with Spray-Cyte (this fixed smear may be mailed to the lab).

6. Dip the slide in 50% ethanol 5 times.

7. Air-dry the slide.

8. Stain the slide smear with acridine orange, 4 minutes.

    Note: excessively heavy smears need extra soaking in saline for several minutes. This allows the nuclei of the protozoa to be more easily seen.

9. Dip the slide in phosphate buffer, 1 minute.

10. Dip the slide in $CaCl_2$ solution, 40 seconds.

11. Dip the slide in phosphate buffer.

12. Mount a coverglass on the stained slide in saline.

13. Examine the slide with a fluorescence microscope, FITC filters.

Results

Trichomonas cells appear as orange balls with a yellow–green banana-shaped nucleus.

Note: the method is superior to wet mounts examined immediately by phase-contrast, even when slides spent several days in the mail.

Reference

Hipp SS et al., 1979.

6.1.8 Early detection of microorganisms in blood cultures

Purpose

Early and economical detection of positive blood cultures.

Approach

Acridine orange fluorescence microscopy is used to examine samples from blood culture bottles.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Broth blood culture incubated 6, 24 and 48 hours.

Acridine orange stain, pH 4.0.

Syringe, and needle (2–3 mL) or Pasteur pipette (sterile and clean).

Bacteriological loop.

Methanol, absolute.

Method

1. Incubate the broth blood culture at 35–37 °C.

2. Mix the blood culture before sampling it at 6, 24 and 48 hours.

3. Remove a small sample of the culture with a sterile syringe and needle or Pasteur pipette.

4. Place a drop of the sample on a slide, and make a thin smear (a fluorescence slide with masked wells may be used).

5. Air-dry the smear for 30 minutes.

6. Fix the smear in absolute methanol, 2 minutes.

7. Air-dry the fixed smear.

8. Cover the smear with acridine orange, 1 minute.

9. Wash the slide in water.

10. Air-dry the slide.

11. Examine the slide by epi-fluorescence with FITC filters at ×400–×600. Confirm suspect positives at ×1000.

Results

Orange organisms are seen against a green or dull yellow background. The colour of the background varies with the type of broth used. White cells stain green, and red cells do not stain.

Note: many investigators find the acridine orange method to examine blood cultures much more efficient than Gram stain or subculturing. The acridine orange method is recommended to replace subculturing to detect positive blood cultures. Using acridine orange staining to check blood cultures is very economical.

References

Burdash NM et al., 1983; Hunter JS, 1983; Larson AM, 1994; Mirrett S, 1982; Tierney BM, 1983.

6.2 Fluorescent acid-fast staining for diagnosing acid-fast bacterial infections

Acid-fast staining and microscopic examination of sputum smears and other specimens are usually the first steps in diagnosis of tuberculosis because it is a simple, effective and rapid diagnostic method. Fluorescent acid-fast microscopy can be done at relatively low magnifications giving large fields of view for fast, efficient slide scanning. Thus, as compared with fuchsin-based acid-fast stains, detecting tuberculosis bacilli in sputum of active tuberculosis cases with fluorescence microscopy is about 10 times faster, and it is more sensitive, especially where few tuberculosis bacilli are in the specimen, as in tuberculosis/AIDS cases. Fluorescent acid-fast stains use the fluorochromes rhodamine or acridine orange.

Tuberculosis bacilli can infect bone. The lesion is softened bone and dead tissue. In order to do diagnostic tests, the bone is exposed by surgery, and scrapings of the lesion are spread thinly on a glass slide. These may show acid-fast bacilli.

Tuberculosis bacilli also cause meningitis. Sometimes acid-fast bacilli are found in the cerebrospinal fluid, but they are few and difficult to detect. This is usually done by culture. Tuberculosis bacilli can be concentrated from cerebrospinal fluid by filtration through a polycarbonate filter. If stained on the filter by fluorescent acid-fast stain, this may yield an early diagnosis of tuberculosis meningitis (see the filtration method below).

Skin lesions caused by mycobacteria are usually due to the following: *Mycobacterium leprae*, *M. ulcerans* and *M. marinum*. Leprosy is caused by *M. leprae*. In the lepromatous form of the disease, the organisms are found in the

skin and sometimes other parts of the body as well. Microscopy is the only way to detect these bacilli, since they cannot be grown in culture.

In some tropical areas, disfiguring Buruli ulcers are caused by *M. ulcerans*. These begin as small swellings under the skin that enlarge until the overlying skin dies, and then spreading, open ulcers form. Strongly staining acid-fast bacilli are found in the subdermal tissue under the edge of the ulcer. Do not take specimens for detecting these acid-fast bacilli from the centre of the lesion, as acid-fast bacilli are not found there.

M. marinum can infect punctured or scraped skin that is exposed to the organism, usually in open water or water in fish tanks. Acid-fast bacilli are found in exudates from these ulcers, but the ulcers frequently heal with no complications.

6.2.1 Safety

Technician safety is a major concern in tuberculosis laboratories because tuberculosis is very infectious. Aerosol droplets containing acid-fast bacilli that are breathed into the lungs cause tuberculosis infection. Aerosols are created by coughing, sneezing and talking, or by certain laboratory operations like stirring or mixing liquids, breaking bubbles, or vibrating laboratory tools while working with liquids. Centrifuging specimens in open containers releases aerosols. Since liquids and moist materials spatter when heated in a flame, inoculating needles and loops must be properly handled (see below). Be very careful when working with tuberculosis specimens.

General rules to prevent laboratory infection

- Avoid making aerosols.

- Control aerosols that are made.

- Provide airflow in the work area to carry aerosols away from technicians

- Exhaust laboratory air away from other people.

- Wear protective clothing.

- Use respiratory protective masks.

- Use appropriate disinfectants and sterilizing equipment.

- Do not keep, eat or drink food or beverages in the laboratory.

- Do not smoke in the laboratory.

- Wash hands after doing bench work and before leaving the laboratory.

- Use a ventilated safety cabinet, if available, or an enclosed ventilated safety box.

    To avoid making aerosols in the laboratory:

- do not make or break bubbles in liquid specimens or cultures

- do not vibrate or wave about inoculating equipment (i.e. loops and needles)

- centrifuge tuberculosis specimens only in unbreakable plastic, tightly sealed, screw-cap centrifuge tubes (a 30–50 mL size tube is convenient for collection and processing of sputum specimens)

- clean loops and needles in a flask containing 70%–95% alcohol in sand before flame sterilizing (see Figure 6.2). Wire loops used to make sputum smears may be reused after dipping them in 70%–95% ethanol or methanol in sand and then holding them in a flame until they glow. Dipping the wire loop in ethanol dehydrates the specimen so that it does not spatter in the flame. The sand helps remove large particles. The flame should be colourless or blue, because an orange or red flame is usually not hot enough to sterilize the loop or needle.

Figure 6.2 **Sterilizing a bacteriological loop safely**

Commercial ventilated safety cabinets are very efficient, but expensive. They should be used whenever available. Alternatively, a simple boxed-in area on a tabletop with a front slot or armholes for working inside it can be used. It should have an exhaust fan and vent in the back to draw air from the box to the outdoors. This arrangement can give some protection to the laboratory technician.

In district hospital laboratories, optimum safety equipment may not be available, but specimens must be processed. Breezes can supply air currents needed to carry aerosols away from the immediate work area. An isolated one-room building with free airflow through openings on all sides is good for doing tuberculosis work. Technicians should work facing and close to a large open window on the downwind side so that a good airflow comes from behind the technician and exits immediately outside, away from others. Fans also can direct airflow. Cloth or paper strips hung in the openings will show the airflow direction.

Wear effective protective clothing when preparing slide smears. Protective clothing means lab coats or gowns, rubber or plastic gloves, bacteriological filter masks (not ineffective cloth or gauze masks) and shoes used only in hazardous laboratory areas.

Decontaminate all equipment exposed to possible contamination by infectious materials and aerosols by disinfecting, autoclaving, boiling for 10–15 minutes or burning before being discarded or processed for reuse. Some cleaning and disinfecting solutions contain phenolic compounds. These work well for killing tubercle bacilli if intended for that purpose (Table 6.1). Be sure the manufacturer tested the disinfectant on tuberculosis bacteria.

Sputum specimens that are not to be used for culture can be made much more safe for processing by first treating them with hypochlorite solution. This treatment both kills the acid-fast bacilli in the specimen and liquefies the sputum for easier processing. Sodium hypochlorite solution (NaOCl) is usually used. It helps when concentrating the specimen and greatly reduces laboratory infection hazards. Common household bleach is suitable, 5% to 6% sodium hypochlorite.

The sodium hypochlorite treatment technique is as follows.

1. Mix equal volumes of sputum and sodium hypochlorite solution in a screw-cap centrifuge tube (the sodium hypochlorite solution may be added to the collection container and used to help remove the sputum).

Table 6.1 **Disinfectants for *Mycobacterium tuberculosis***

Disinfectant	Concentration	Time	Remarks
Effective			
Phenol derivatives (pH 2–5)	0.4%–5% (aqueous)		skin disinfection
Ethanol	70%–80%	30 sec	skin disinfection
Isopropanol	60%–95%	30 sec	skin disinfection
4-dichlorobenzyl-alcohol	0.2%	30 sec	skin disinfection
Hypochlorite[3, 4] (free chlorine)	500–1000 ppm	20 min	corrosive
Formaldehyde[1, 3] (pH 4–9)	1%–8%		carcinogenic
Glutaraldehyde[1, 2] (pH 4–9)	2%–3.2%		tissue disinfection
Hydrogen peroxide	3%		wound disinfection
Acetic acid	2.5%	90 min	instruments, benches
Slightly effective			
Iodine			skin disinfection
Iodophor			skin disinfection
Not effective			
Alkali			
Methanol			
Quaternary ammonium compounds			

2.   Tighten the cap, and shake the tube, mixing the sputum and hypochlorite solution.

3.   Let the mixture stand at least 10 minutes, but not more than 30 minutes.

4.   Add water to near the top of the tube, and balance the tubes for centrifugation.

5.   Centrifuge at about 2000*g* for 15 minutes.

6.   Pour off the supernatant liquid into a proper disposal container and keep the sediment.

7.   Mix the sediment in several drops of water.

8.   Make the slide smear from this suspension.

Note: much of the solid organic debris in the specimen is broken down by the sodium hypochlorite. This material and the crystals that form as the slide smear dries wash off during staining, but the acid-fast bacilli stay on the heat-fixed slide smear.

6.2.2 Specimen collection and handling

Collecting a specimen and getting it to the laboratory in proper condition is as important as testing it in the laboratory. A poor quality specimen may yield bad or misleading results. Handle specimens correctly.

Use clean containers, preferably glass or plastic. Dirt, wax, or oils may look like acid-fast artefacts on the smear or may react with non-acid-fast bacteria and cause them to look acid-fast, so be sure that specimen containers are completely cleaned of these contaminants.

Specimen containers must be:

•   sturdy and leakproof

•   clean

•   have an identification label on the container, not on the lid.

Identifying information is:

•   patient name or number

•   date

•   patient location, i.e. address or hospital.

The sputum collection container should have an opening about 5 cm or more across, with at least 30 mL capacity. Ideally, this container is the one that also is used for processing the specimen, such as a large screw-cap plastic centrifuge tube. Process sputum specimens on the day collected.

Work with tuberculosis patients in the open air or in a well ventilated area. Medical personnel should wear bacteriological safety masks and rubber or plastic gloves, if available, when working with patients and collecting specimens.

Get a sputum specimen early in the day, before the patient has eaten. To collect sputum, instruct the patient as follows (Figure 6.3).

1.   Rinse your mouth with water before giving the specimen.

2.   Cover your mouth and nose with a tissue or handkerchief and cough up the specimen from deep in your chest. [The specimen must be sputum from the lungs and not saliva or mucus from the mouth or nasal area. If the patient does

Figure 6.3 **Collecting a sputum specimen**

not cough spontaneously, have the patient take several deep breaths and then hold his/her breath. Repeating this several times should induce coughing.]

3. Hold the specimen container to your lower lip and gently release the sputum from your mouth into the container. Don't spill any sputum.

4. Put the cap on the container.

Sputum may be thick and mucoid or fluid with fine chunks of dead tissue from a lung lesion. The colour may be a dull white or a dull light green. Bloody specimens are red or brown. Thin, clear saliva from the mouth or nasal area is not acceptable and is not used for laboratory examination.

Laryngeal swabs are usually not desirable for tuberculosis diagnosis, but swabbing may be the only method to get specimens from small children and very ill patients. Use a separate swab for each side of the throat. Hold the tongue down with a tongue depressor and swab the throat area behind the tongue as far down as can be reached easily. Make slide smears with the swabs.

Lepromatous leprosy is confirmed by the "scraped incision" method. Pinch the skin toward the incision site from either side to prevent bleeding or oozing. While pinch pressure is maintained, a small incision is made with a sterile scalpel, 4–5 mm long and 2–3 mm deep. Wipe away any blood or plasma, and scrape the sides of the incision with the edge of the blade held at a right angle to the incision. Spread the tissue specimen on a glass slide. Dress the incision with a small adhesive bandage.

6.2.3 Slide preparation

Glass microscope slides for acid-fast staining of sputum specimens should be new and clean. Used slides may be contaminated with material from previous smears that could cause a false positive. Write the patient's name or identification number on one end of the slide. Some slides have a frosted end so that a pencil can be used. For plain slides, scratch on identification with a diamond scriber or tungsten carbide-tipped stylus. Spread the specimen material over approximately 2×1 cm area with a 3 mm wire loop, applicator stick, swab or small pipette. Use applicator sticks, swabs and pipettes for only one specimen and then discard them.

Smears for acid-fast microscopy are best made from sediment of specimens concentrated by centrifugation or filtration through a polycarbonate membrane filter. When a centrifuge or filter is not available, make a direct smear from the specimen. Chunks of tissue, pus or caseous material (fine, pale-white, moist, granular material) are the most likely places where tuberculosis bacilli can be found. Seek these in the sputum specimen for preparing the slide smear. To make the caseous material easier to see, pour the sputum gently into a small dark-coloured dish or a clear Petri dish placed on a dark background.

A direct or concentrated sputum smear should appear cloudy before staining. The smear is too thick if you cannot read print in a newspaper through the smear when it is held 5–10 cm from the print. Smears that are too thick often wash off during staining, or the acid-fast bacilli may be hidden by sputum debris. Figure 6.4 is a schematic for preparing a sputum slide.
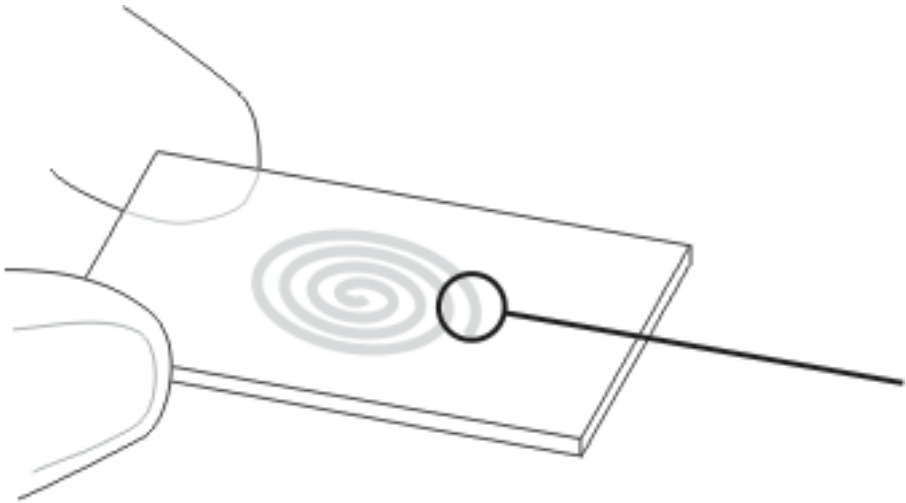
Figure 6.4 **Preparing a slide smear of sputum**

Note: acid-fast bacilli lose the acid-fast property when exposed to ultraviolet light, direct sunlight, overheating during flame fixing, or autoclaving. Avoid these problems.

After the specimen is spread on the glass microscope slide, dry the smear without heat and then heat-fix it. Use an electric slide warmer at 65–75 °C for at least 2 hours, or quickly heat it over a flame as for other bacteriological slide smears. Use a colourless or blue flame (produced by an alcohol or a gas burner) with the smear side up. Immediately after the slide is passed over the flame, it should be hot enough to cause slight pain when touched to the back of the hand. If there is no pain, the slide is too cold; if there is much pain, the slide is too hot. Technicians learn to judge how long to hold the slide over the flame to heat-fix the smear.

Warning. Heat-fixing does not always kill the acid-fast bacilli. Any of the smear rubbed from the slide may be a potential source of infection.

6.2.4 Membrane-filter concentration of tuberculosis bacilli from sputum for staining

Purpose

Increasing the sensitivity of detecting acid-fast bacilli in sputum specimens.

Approach

A sputum specimen for tuberculosis diagnosis is filtered through a polycarbonate membrane filter; a slide smear prepared from the filter surface is stained with fluorescent acid-fast stain and examined by epi-fluorescence microscopy.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Polycarbonate membrane filter (PMF), 1.0 and 0.45 µm pore size, 25 mm diameter.

Filter apparatus.

Vacuum source.

Centrifuge tube, plastic, 50 mL, screw-cap.

Sodium hypochlorite (NaOCl) 5%–6%, (liquid laundry bleach).

Ethanol, 96% (filtered through a PMF, 0.45 µm pore size).

H_2O, deionized (filtered through a PMF, 0.45 µm pore size).

Agar adhesive (0.1 g agar boiled in 100 mL filtered deionized H_2O).

Glass microscope slide.

Auramine-phenol acid-fast stain.

Acridine orange counterstain (0.01 g acridine orange, 0.01 g Na_2HPO_4, 100 mL distilled H_2O).

Acid–alcohol (0.5 mL HCl in 100 mL of 70% ethanol).

Method

1. Place 2 mL of sputum specimen in the 50 mL plastic centrifuge tube.

2. Add NaOCl to the sputum specimen in the centrifuge tube in the following amounts:

    4 mL: clear to light-coloured and liquid or moderately mucoid sputum; total 6 mL

    8 mL: dark or very mucoid sputum specimens; total 10 mL.

    Note: for highly mucoid sputum specimens, add an equal volume of NaOCl to the sputum specimen, and thoroughly mix them. Then, transfer 4 mL of this mixture to the centrifuge tube. Add 6 mL of NaOCl.

3. Mix the sputum specimen and NaOCl solution by capping the centrifuge tube tightly and inverting it up and down until the mixture is complete, about 1 minute maximum.

4. Filter the mixture through the PMF, 1.0 µm pore size, on the filter holder using vacuum.

5. Wash the filter with 1 mL of ethanol followed by 1 mL H_2O.

6. Place 2 drops of hot agar adhesive in the middle of a microscope slide.

7. Remove the filter from the holder, and place it with the top (sediment side) down on the agar adhesive. Be sure it lies flat on the slide.

8. Stain the filter (in place on the slide) with the auramine O acid-fast stain, 15 minutes.

9. Remove the filter from the slide, carefully peeling it off.

10. Rinse the slide with deionized H_2O.

11. Flood the slide smear with acid–alcohol, 2 minutes.

12. Rinse the slide with deionized H_2O.

13. Drain it.

14. Flood the slide smear with acridine orange counterstain, 2 minutes.

15. Rinse the slide in tap water.
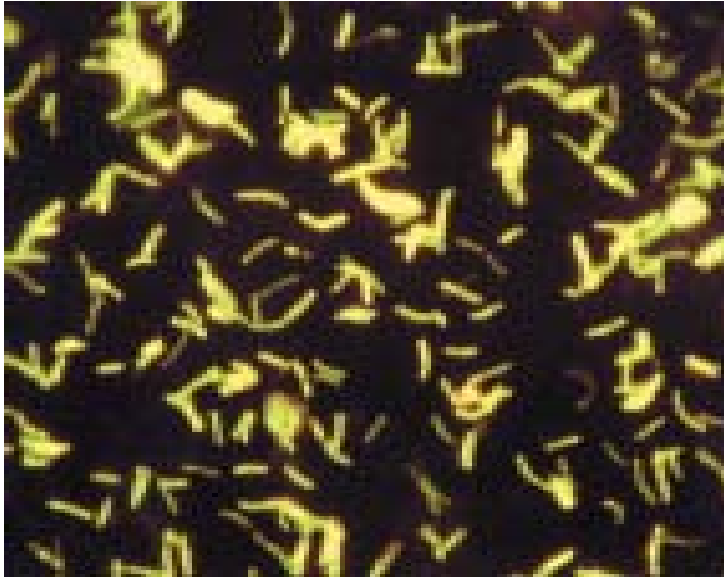
Figure 6.5 **Tuberculosis bacilli concentrated on a polycarbonate filter stained with auramine O acid fast stain and counterstained with acridine orange, magnification ×600**

16. Air-dry the smear.

17. Examine the slide smear with an epi-illumination microscope, FITC filters.

Results

Positive: yellow fluorescing acid-fast bacilli are seen against an orange fluorescing background.

Figure 6.5 shows tuberculosis bacilli concentrated on a polycarbonate filter stained with auramine O acid fast stain and counterstained with acridine orange, magnification ×600.

Note: this method traps most of the acid-fast bacilli in a sputum specimen on the surface of the filter. It is about as sensitive as culture, but much faster. It can be used as a substitute for culture to diagnose tuberculosis. It is significantly more sensitive for detecting acid-fast bacilli than are sputum specimens centrifuged or directly applied to slides.

References

Smithwick RW, David HL, 1971; Smithwick RW, Stratigos CB, 1981.

6.2.5 Blair fluorescent acid fast stain

Purpose

Rapid, efficient detection of acid-fast bacilli in sputum smears.

Approach

Fluorescence acid-fast stain is used to brightly stain acid-fast bacilli against a dark or black background of sputum debris.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Auramine O–phenol stain.

Acid–alcohol.

Potassium permanganate, 0.5%.

The preparation of the reagents for the Blair method is described in Annex 13.

Method

1. Prepare a sputum smear on a glass slide and fix by heating.

2. Flood the slide smear with auramine O–phenol, 15 minutes.

3. Rinse with distilled or deionized water (other clean water may be used as long as it does not contain chlorine).

4. Flood the slide smear with acid–alcohol, 2 minutes.

5. Rinse the slide smear, and drain it.

6. Flood the slide smear with potassium permanganate solution, 2 minutes.

7. Rinse, drain, and air-dry the slide smear (do *not* heat the smear).

8. Examine the stained smear with an epi-illumination fluorescence microscope, FITC filter system.
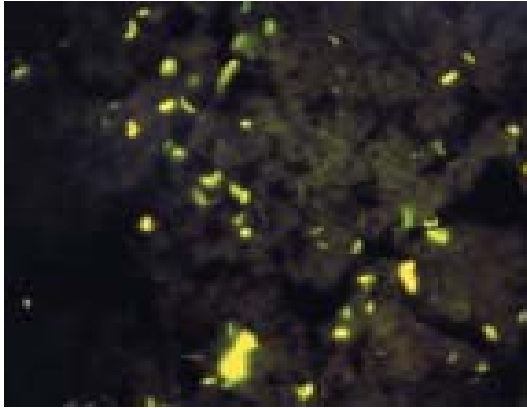
Figure 6.6 **Tuberculosis bacilli concentrated by centrifugation, stained with auramine O fluorescence stain, and then treated with potassium permanganate, magnification ×200**

Results

Yellow fluorescing bacilli are seen against a dark or black background.

Figure 6.6 shows tuberculosis bacilli concentrated by centrifugation, stained with auramine O fluorescence stain, and then treated with potassium permanganate, magnification ×200.

Note: a number of authors have shown that fluorescent auramine O acid-fast stain is superior to fuchsin–phenol bright-field stain.

Reference

Blair EB et al., 1969.

6.2.6 Smithwick fluorescent acid fast stain

Purpose

Detection of acid-fast bacilli in sputum smears with a colour contrast background.

Approach

Sputum smears are stained with auramine O to make acid-fast bacilli fluoresce yellow, and they are counterstained with acridine orange at alkaline pH to stain the background debris a contrasting orange.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Auramine O-phenol stain.

Acid–alcohol.

Acridine orange counterstain.

The preparation of the reagents for the Smithwick method is described in Annex 13.

Method

1. Prepare and heat-fix a sputum smear on a glass slide.

2. Flood the smear with auramine O–phenol stain, 15 minutes.

3. Rinse the smear with clean water (chlorine-free) and drain it.

4. Flood the smear with acid–alcohol, 2 minutes.

5. Rinse the slide with clean water and drain it.

6. Flood the smear with acridine orange counterstain, 2 minutes.

7. Rinse, drain and air-dry the slide (do *not* heat).

8. Examine the smear with a fluorescence microscope, FITC filter system.

9. Scanning may be done at ×200–×250 with confirmation of suspect acid-fast bacilli at ×400–×500.

Results

Yellow fluorescing acid-fast bacilli are seen against a background of red fluorescing sputum debris.
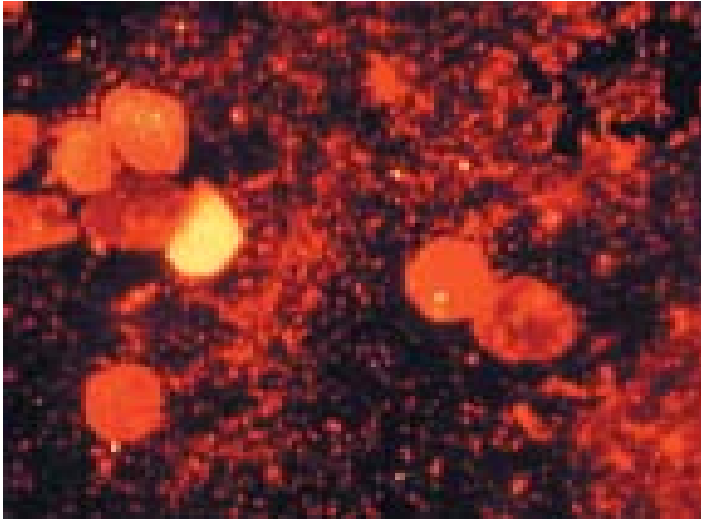
Figure 6.7 **Tuberculosis bacilli in sputum stained Smithwick auramine O acid-fast stain and counterstained with acridine orange, magnification ×200**

Figure 6.7 shows tuberculosis bacilli in sputum stained using the Smithwick method.

Note: the acridine orange fluorescent counterstain permits easy location of the microscopic plane of the specimen. This is particularly helpful with negative sputum smears.

Reference

Smithwick RW, David HL, 1971.

6.2.7 Phenolic acridine orange acid-fast stain

Purpose

Detection of acid-fast bacilli with efficient fluorescent acid-fast stain in sputum and other specimens with a contrasting fluorescence colour background.

Approach

Fluorescent acid-fast staining of sputum smears is done using only two reagents instead of three for simplicity and acid-fast bacilli detection efficiency.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Acridine orange–phenol stain.

Acid–alcohol–methylene blue.

The preparation of the reagents for this method is described in Annex 13.

Method

1. Prepare a slide smear of a sputum specimen and air-dry it.

2. Heat-fix the smear.

3. Cover the smear with the acridine orange stain, 15 minutes.

4. Rinse the smear with deionized water, and drain it.

5. Cover the stained smear with the destain solution, 2 minutes.

6. Rinse the smear with deionized water, drain it, and allow it to air-dry.

7. Observe the smear with an epi-illumination fluorescence microscope, scanning at ×200.

8. Confirm bacterial morphology at ×400–×600, no immersion oil.

Results

Red to orange stained acid-fast bacilli are seen against a pale yellow general background that contrasts with the red acid-fast bacilli. Figure 6.8 shows tuberculosis bacilli in direct sputum smear stained with acid-fast acridine orange, magnification ×200.

Note: reported results show this stain to be superior to auramine O acid-fast staining.

Reference

Smithwick RW, et al. 1995.
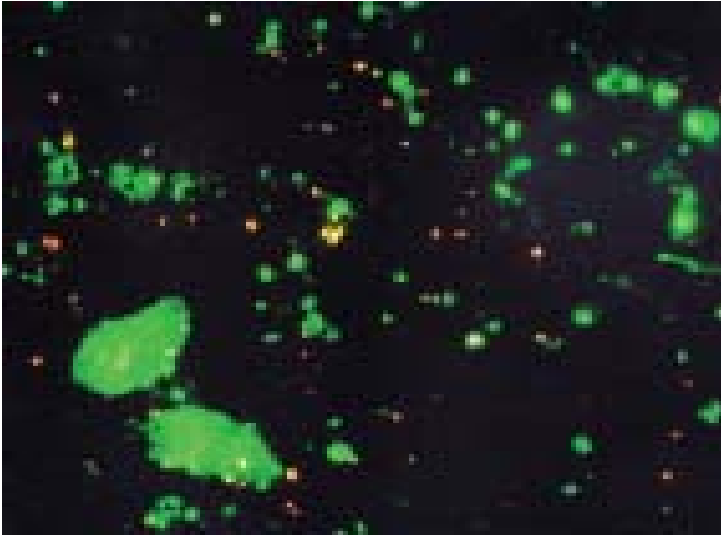
Figure 6.8 **Tuberculosis bacilli in direct sputum smear stained with acid-fast acridine orange, magnification ×200**

6.2.8 Fluorescent acid-fast staining notes

Certain items need particular attention in fluorescent acid-fast staining.

- Stain smears on staining racks, not in staining jars or dishes. Avoid transferring acid-fast bacilli from one smear to another. A few acid-fast bacilli transferred from one acid-fast positive smear to a negative one could cause the specimen to be incorrectly reported as positive, and the patient might suffer improper treatment.

- Use water to rinse away solutions before the next solution is applied. After each rinse, tilt the slide slightly to drain off excess rinse water that would dilute the next solution.

- Do not blot acid-fast stained smears (acid-fast bacilli may be transferred from one smear to another).

- Store staining solutions in dark bottles or closed cabinets to avoid exposure to light.

- Discard solutions contaminated with bacterial or fungal growth. Clean the staining and stock bottles with acid–alcohol. Sterilize them before reuse.

- Remove precipitates or other solid material from fresh staining solutions by filtering them through glass wool or filter paper.

- Store stained slides only for a short time. Fluorochrome-stained smears will not retain their fluorescence in long-term storage. For short-term storage, place slides in the refrigerator.

- Use control smears of mycobacterial cultures for stain controls and training. A known positive sputum smear or a smear from a culture should be stained as a control each day. Controls assure the technician that the staining solutions and microscope are working properly.

- Avoid heating auramine O. The fluorochrome is inactivated by temperatures above 40 °C and should not be heated to prepare staining solutions or when staining slides. In tropical areas, it should be protected from heat in storage.

- Interference with staining or fluorescence reduction may be due to:
    – smears that are too thick
    – using absorbent paper during staining
    – high chlorine content in rinse water
    – excess exposure of stained smear to potassium permanganate
    – exposing fluorescent stains to solutions of heavy metal ions.

- Examine fluorochrome-stained smears as soon as possible. They usually lose their fluorescence with time. Refrigeration will slow this process, but these smears should be observed within 24 hours of staining. Do not stain smears unless they can be examined soon after staining.

- A fluorescent acid-fast–stained smear can be restained with a fuchsin–phenol staining method to confirm observations. However, a fuchsin–phenol–stained smear cannot be restained for fluorescence microscopy.

7. Principles of immunofluorescence microscopy

The terms *immunofluorescence* and *fluorescent antibody* describe the combination of the high sensitivity of fluorescence microscopy with the strict specificity associated with immunological methods. It is not only important that an antigen–antibody complex be seen by immunofluorescence microscopy but also where it is seen. Often the shape of fluorescence is important. In the fluorescent antibody technique, antigens are reacted with immunoglobulins that are labelled (conjugated) with chemically stable fluorochromes. Fluorescent antibody conjugates are used both for fluorescence microscopy and flow cytometry.

Immunofluorescence microscopy offers a number of advantages over other ways of detecting and identifying antigens and antibodies, as well as advantages over diagnostic culture methods. These are as follows.

- *Rapid results*. Results are often obtained within one hour.

- *Pure antigen not needed*. Specific antigens of organisms in mixtures of bacteria and antigens in tissues can be detected and identified.

- *Wide application*. Specimens unsatisfactory for analysis by other methods often can be used for fluorescent antibody tests. Dead or difficult-to-grow microorganisms in specimens can be detected and identified.

- *High sensitivity*. A few pathogens among many contaminating organisms can be detected and identified. Theoretically, one specific cell in a specimen can be found and identified.

- *Intracellular organisms are detectable*. Organisms protected from detection by other methods because they are intracellular can be found by fluorescent antibody.

- *Soluble antigen detection*. Fluorescent antibody is suitable to locate and identify soluble antigens, native antigens, and autoantibodies.

- *Simplicity*. Fluorescent antibody techniques are simple because they need only a few steps.

- *Low cost*. The cost per test is low once the basic equipment is procured because only small amounts of reagents are needed.

  The disadvantages of the fluorescent antibody method are:

- initial cost of fluorescence microscopy equipment

- getting or training technical operators.

7.1 Procedures for fluorescent antibody staining

There are two basic methods of immunofluorescence microscopy, direct fluorescent antibody (DFA) and indirect fluorescent antibody (IFA).

DFA is usually used to detect and identify antigens; for example, infectious agents. IFA can detect and identify either antigens or antibodies. The difference lies in which is the unknown factor.

DFA is the simplest and most commonly used method. Antigen preparations such as fixed cells, bacteria, viruses, fungi, or histological sections on slides are treated with antigen-specific fluorochrome-labelled antibody (the conjugate). Then the slide is washed with buffered saline. Ideally, when the reaction is specific, only those parts of the preparation that contain the antigen of interest and immunologically bind the fluorescent antibody conjugate are visible with a fluorescence microscope (Figure 7.1).

IFA is done in several steps. First the target antigen is fixed to a slide. Then, a primary unlabelled antibody, specific for the antigen, is reacted with the antigen on the slide. The third part is a secondary antibody specific for the species of globulin used for the primary antibody. This antiglobulin antibody is labelled with the fluorochrome. For example, if the specific antibody was made in a rabbit, then an anti-rabbit serum conjugate is reacted with the antigen-antibody complex fixed on the slide. IFA is also called a "layering" or "sandwich" method. This "layering" often produces a brighter image than a direct fluorescent antibody test because
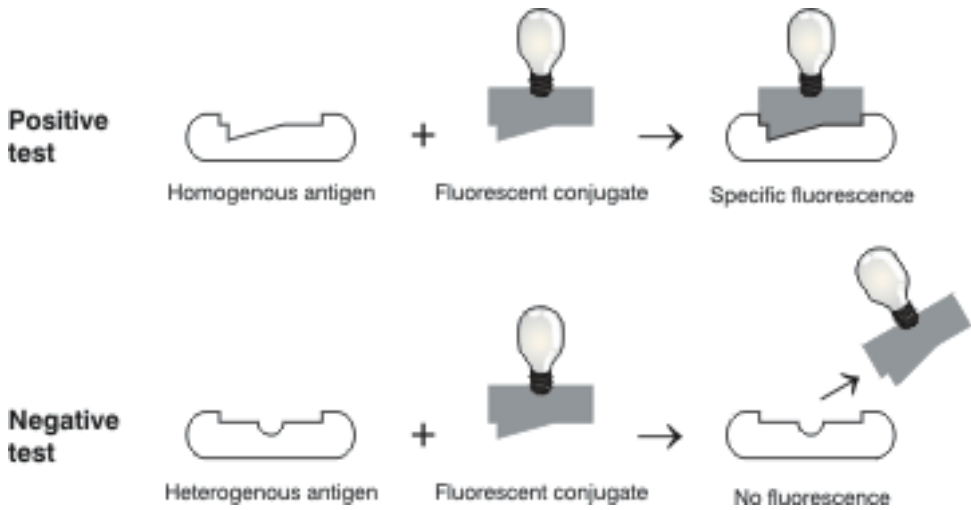
Figure 7.1 **Principle of the direct fluorescent antibody method (DFA)**

more fluorochrome molecules are present. Thus, the method is more sensitive than DFA. Some investigators estimate it to be 10 times more sensitive. However, IFA needs more controls than DFA because it is more subject to non-specific reactions (Figure 7.2).

The main advantage of the IFA technique is that only one fluorescent conjugate (secondary antibody) is needed to test for many different antigens. It is only necessary to use a primary (non-labelled) antibody for each new antigen that is prepared in the same animal species for which the secondary labelled antibody is specific.

IFA also is used to demonstrate antibodies in blood serum, other body fluids and tissue. To do this, a known antigen is fixed to a slide, and then it is reacted with the unknown antibody. This can be done to detect and titre infectious disease antibodies as well as antibodies to autoimmune diseases. IFA is most commonly used to help diagnose autoimmune disease. In this use, patterns of antibody immunofluorescence are shown in various types of tissue used as known antigen substrates fixed on slides.

STEP 1

STEP 2

Positive test
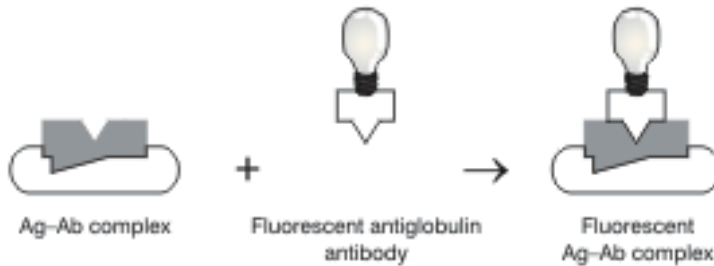
Negative test
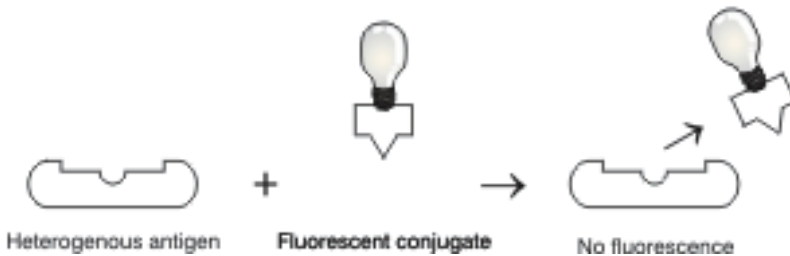
Figure 7.2 **Principle of the indirect fluorescent antibody method (IFA)**

The layering method can detect cell-bound antibody. Cells containing antibody can be fixed to keep the antibody from leaching during the experiment. After adding the antigen, the cells are incubated with fluorochrome-labelled antibody raised against the antigen. Cells that carry a specific antigen can be identified under the fluorescence microscope. This method is shown in Figure 7.3.

Complement fluorescent antibody staining is a modification of IFA. This also allows identification of either antigen or antibody. It is a complement fixation test with fluorescence as the end point. In this method, the fluorescent antiglobulin conjugate, instead of being directed to the antibody, is directed specifically to anti–

guinea pig complement in the reaction mixture. It is a two-step method. In the first step, the antigen, antibody and complement are reacted together. After incubation and subsequent washing, the fluorescent-labelled anti–guinea pig globulin is reacted with the complex formed from the three parts, and if it is positive, the result is observed with a fluorescence microscope as fluorescence. Depending on the
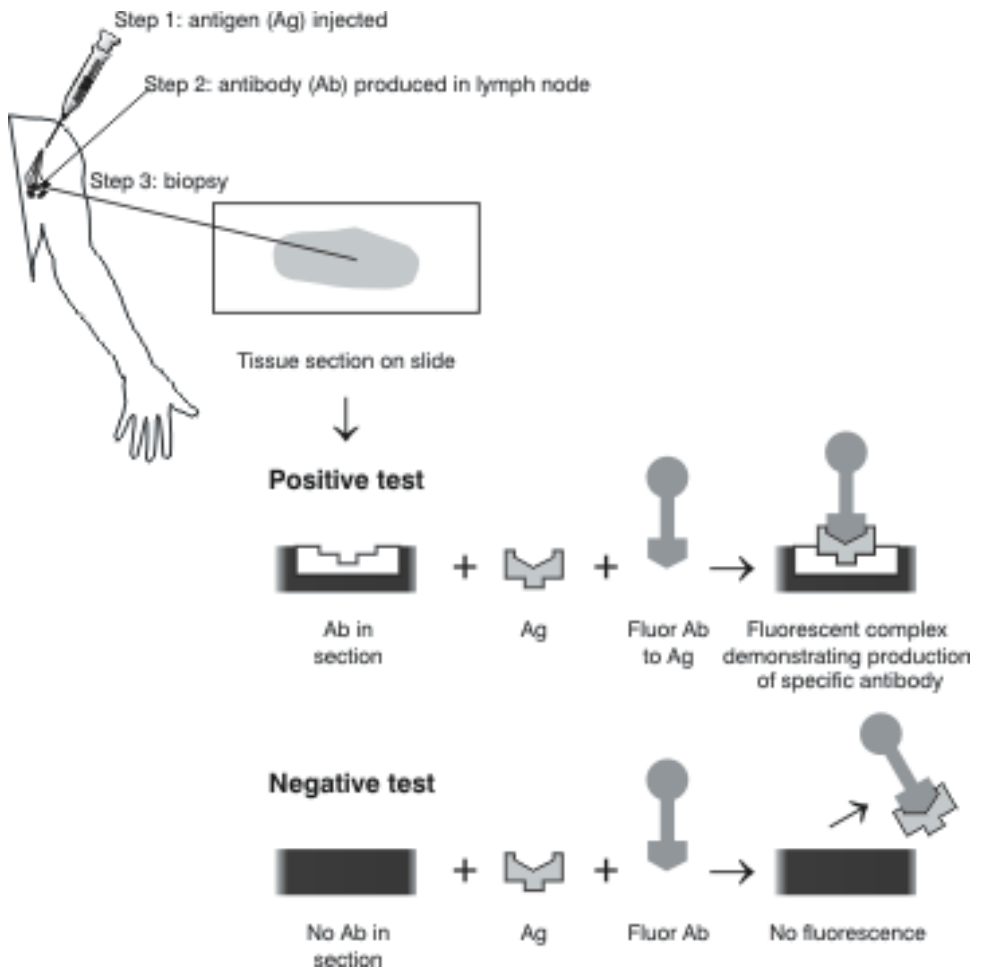
Figure 7.3 **Principle of the layering fluorescence method to detect cell bound antibody**
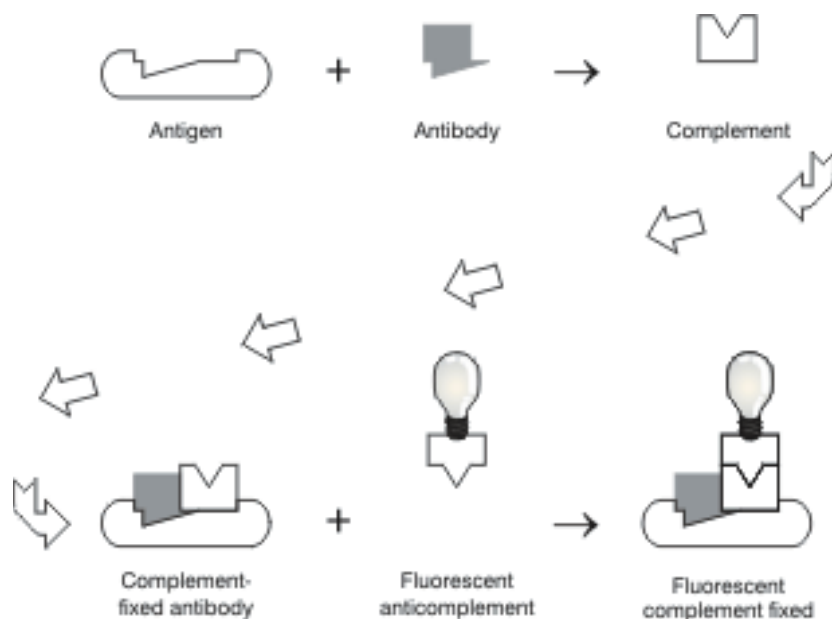
Figure 7.4 **Principle of the fluorescent complement fixation test**

known component of the reaction mixture, either antigen or antibody is specifically identified. The procedure is shown in Figure 7.4.

Fluorescent antibody preparations should be examined immediately, especially when photographic or fluorimetric recordings are to be made. However, preparations can be stored overnight in a refrigerator, but they must be warmed up for about 10 minutes to room temperature before use. The controls, provided with code numbers, should be examined first. If differences are observed, then clarification is needed before the remaining test preparations are examined. When doing tissue studies for autoimmune disease, comparison with a phase-contrast or interference-contrast image is useful to exactly locate fluorescence on the tissue specimen.

7.2 Fluorochromes for fluorescent antibody conjugates

There are many fluorochromes that yield fluorescent colours across the entire visible spectrum. However, only a few of these are practical to make immunologically specific fluorescent antibody conjugates. The most common fluorochromes used for fluorescent antibody are:

- fluorescein isothiocyanate (FITC)

- lissamine rhodamine B (RB 200)

- tetramethylrhodamine isothiocyanate (TRITC)

- Texas Red

- 1-dimethylaminonaphthalene-5-sulfonic acid (DANS).

Other fluorochromes give less efficient fluorescence than those above or result in unstable conjugates. The most commonly used fluorochrome is FITC. It fluoresces a bright yellowish green. This is easily seen under a fluorescence microscope.

The fluorochromes RB 200, TRITC and Texas Red are used to make fluorescent antibody conjugates that fluoresce orange or red. These are used in protein-tracing studies of tissues to define different areas and structures and to differentiate these from other parts fluorescing green with FITC conjugates.

DANS is rarely used today because FITC is more efficient and can be stimulated to produce fluorescence by blue–violet light. DANS fluoresces yellow when excited by UV light.

The excitation and emission curves of FITC, TRITC and RB 200 are shown in Figure 7.5.

7.3 Counterstains and counterstaining

Fluorescent counterstains for FITC conjugates reduce the effects of autofluorescence or non-specific staining and can increase contrast. Counterstaining is done with contrasting colour fluorochromes, usually red or orange. There are various ways to increase contrast and reduce autofluorescence or non-specific staining of a sample. Some are listed below.

- Treating with Eriochrome black (CI 14645) used as a 1:30 or 1:60 w/v solution in distilled water for immunofluorescence of lymphocytes. One drop of this solution is added to the preparation after the last wash and, after 10 seconds, washed off with phosphate buffered saline.

- Counterstaining with rhodamine-labelled serum albumin mixed with the FITC conjugate.
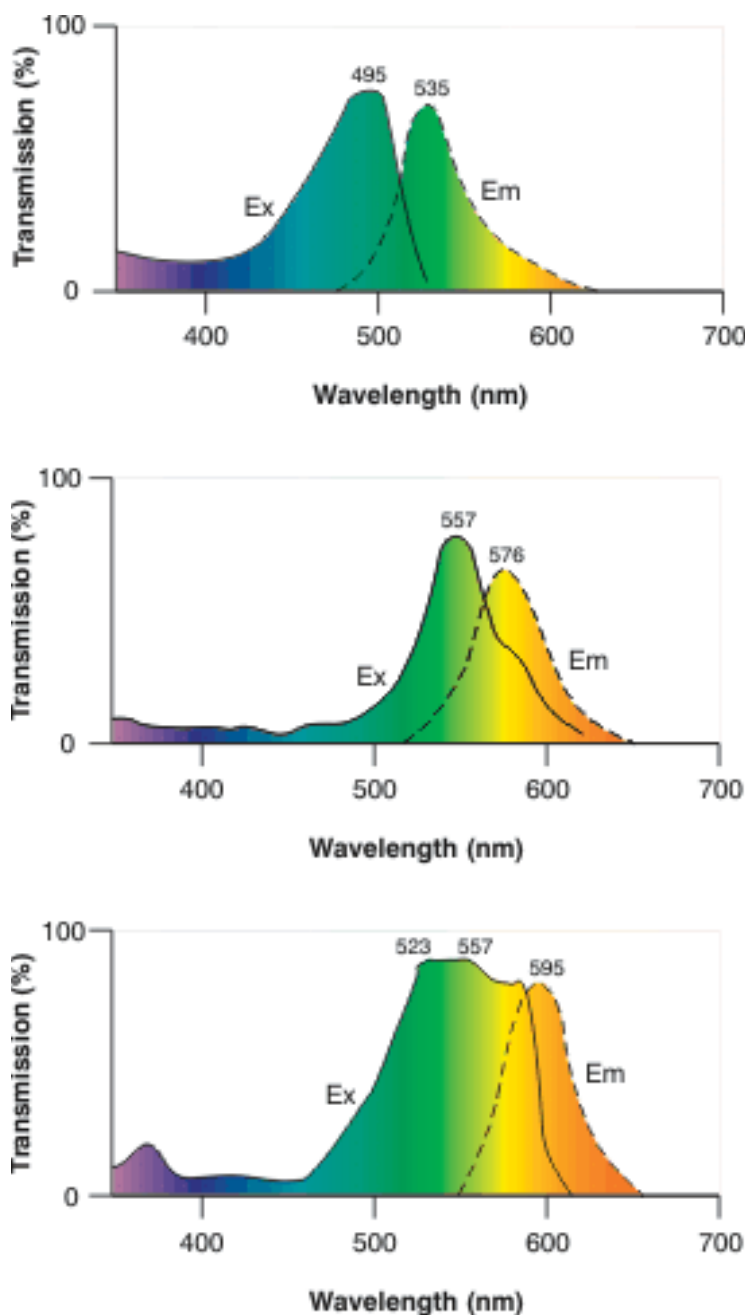
Figure 7.5 **Excitation and emission spectra of FITC (top), TRITC (middle) and RB 200 (bottom)**

- Treating the preparation with treated with a 0.01% to 0.1% w/v solution of Evans blue for 1–5 minutes after incubation, followed by washing with phosphate buffered saline.

- Adding 0.02% w/v brilliant cresyl blue (CI 51010) or eosin (CI 45400) to the last wash.

The procedures for counterstaining are described in Section 9.8.

7.4 Controls of undesirable fluorescence and non-specific reactions

False negative as well as false positive observations may be made during fluorescence microscopy. Such observations may result from inappropriate antigen substrates or antibodies, the methods used, the microscope or even the investigator. False negative observations can only be recognized if pure antigen and antibody, as well as standard or reference substances and defined antigen substrates, are available for controls. False positive observations can be caused for reasons that are not always identified. Usually, a positive result is characterized by structure-specific fluorescence of typical colour, with faint controls. In certain cases clear observation can be difficult or impossible because of unwanted or background fluorescence.

Unwanted fluorescence can be caused by:

- autofluorescence

- staining by free dye in the labelled serum

- nonspecific staining of eosinophils, basophils or other structures by acidic or degraded proteins, especially those with high F/P ratios, and for conjugates that are relatively insoluble in aqueous solutions

- nonspecific reactions with cross-reacting or polyvalent antibodies (i.e. undesired specific staining)

- binding of immunoglobulins to physiological receptors, for example, Fc receptors.

Controls of specificity should be carried out in defined test systems. Testing of suitability using special test procedures gives the best protection against false positives. The following controls for specificity are recommended.

- Staining should occur only in preparations that contain antigen. The staining should affect only the antigen.

- No staining should occur with fluorochrome-labelled non-immune serum.

- On preincubation with non-labelled immune serum prior to incubation with labelled immune serum there should be a reduction in fluorescence intensity whereas this should not occur after preincubation with non-immune serum.

- If the labelled serum is absorbed using the homologous antigen, staining should be inhibited, but not when a heterologous antigen is used.

- Fc-free antibody cleavage products should be used, for example, Fab or F(ab')2, to exclude binding to Fc receptors.

  Simple controls based on histochemical criteria are summarized below.

7.4.1 Controls during direct immunofluorescence (example: kidney biopsy)

- Haematoxylin and eosin (HE) or other staining procedure for correct orientation and identification of tissue elements.

- "Blank" sections to establish levels of autofluorescence: incubation with phosphate buffered saline alone.

- "Negative" control: a preparation that does not contain the sought-after antigens, e.g. immunoglobulins.

- "Positive" control: a preparation that contains the antigens.

- Incubation with a conjugate directed against other proteins, e.g. antihuman albumin, which point, for example, to the presence of an exudate.

7.4.2 Controls during indirect immunofluorescence for the detection of auto-antibodies

- Haematoxylin and eosin (HE) staining should be done for correct orientation and identification of tissue elements.

- "Blank" sections should be included to establish levels of autofluorescence-incubation with phosphate buffered saline alone.

- Conjugate controls (incubation with conjugate alone) should be included to establish the levels of non-specific staining especially with high fluorochrome content.

- Normal serum or "negative" controls: incubation should be included in the presence of normal serum at low dilutions, in order to establish non-specific binding by proteins that do not have a specific antibody function. (Some are able to bind antigen at high concentrations and cannot be removed during washing: this may occur with a high antibody content for the conjugate.)

- "Positive" controls: incubation should be done with a reference or standard serum that must be positive at a predetermined titre.

7.4.3 Controls for detecting complement-binding antibodies

- Haematoxylin and eosin (HE) or other stain for correct orientation and identification of tissue elements.

- Incubation with test serum, inactivated complement and conjugate (labelled anti-complement).

- Incubation with normal serum, complement and conjugate.

- Incubation with test serum and conjugate.

- Incubation with complement and conjugate.

- Incubation with conjugate alone.

Additional controls must be carried out, or the conjugate changed, if no clarification is achieved using the above controls. The number of controls for specificity depend on the particular investigation. One must keep in mind, however, that the specificity and sensitivity of immunofluorescence cannot be better than that of the antisera and conjugates used.

7.5 Control of pH

A very important factor when using fluorescent antibody with FITC conjugates is the pH of the system and reagents, especially the pH of the cover glass mounting medium. At pH 7.0, fluorescence of FITC diminishes fast, and becomes very dim below pH 7.0. Today, most fluorescent antibody testing done with FITC conjugates

is at a pH between 8.5 and 9.0, achieved with carbonate buffers, especially in the buffered glycerol mounting medium. The optimum is pH 8.6. A pH above 9.0 may interfere with immunological reaction and detailed morphology of some tissue sections. At a pH range of 8.5–9.0, fluorescence fading is also reduced. It is recommended to check the pH of the mounting medium at least monthly, preferably at weekly intervals. Oxidation and CO_2 absorption reduce the pH of glycerol. Since the pH of the buffers used in fluorescent antibody is critical, a pH meter is essential equipment for immunofluorescence microscopy.

7.6 Preventing fluorescence fading (photobleaching)

When a fluorochrome is excited by light to fluoresce, the fluorescence is bright at first and then begins to fade with continued excitation. This is because a molecule of fluorochrome can emit only a certain number of photons during its photochemical lifetime. Fluorescence fading is caused by the interaction of light and oxygen on the stained specimen. Oxygen bleaches the fluorochrome faster or more slowly, depending on the light intensity. Low light level excitation does not prevent fading; it simply reduces the rate of fading.

Fluorescence of different fluorochromes fades at different rates and for some, changes colour. FITC conjugates fade to half their intensity in about 30 seconds at pH 7.2. For RB 200 conjugates this happens in about two minutes. Also, RB 200 conjugates change colour from red–orange to yellow. This yellow fluorescence tends to last.

Fading is not very important during direct observation of fluorescent specimens for disease diagnosis because fluorescence brightness usually lasts long enough to allow adequate observation. It is possible to shift to a nearby microscope field on the slide that will have bright fluorescence. However, it does become a problem during photomicrography. Protection from fading (slowing the process) can be done three ways:

• reduce exposure time to the excitation light

• reduce intensity (brightness) of the excitation light

• deoxygenate the environment of the fluorochrome-stained specimen.

Fluorescence fading occurs two ways: short-term fading during observation or photomicrography and long-term fading over time during storage of fluorochrome-

stained slides. When preparing for photomicrography, the specimen should be exposed to excitation light as little as possible, just to get orientation on the specimen and do focusing. Then the excitation light is removed. A light stop in the light path of mercury or xenon lamps is convenient. Halogen lamps can simply be turned off. Full exposure of the stained specimen to the excitation light should await actual photography.

Photomicrographs are usually taken using fast colour film to reduce exposure time, but there is some loss of sharpness with faster films due to increased grain. Films of ASA 200 and above are often used. Epi-illumination reduces the time needed for exposure, as does using a high numerical aperture objective with a relatively low power ocular, ×5 to ×8. With anti-fading agents, photomicrography can be done with longer exposures using slower fine-grain films to give sharper images. Digital cameras can be used for photomicrographs. Advantageously, when using digital imaging, development of colour films is no more required.

Anti-fading agents are added to cover glass mounting media to reduce the rate of fading. These make good fluorescence last 10 to 20 times longer than mounting media without them. Unfortunately, some of these chemicals tend to quench the initial fluorescence in a specimen, making initial fluorescence less bright. However, other chemicals actually increase the initial fluorescence. Practical anti-fading chemicals are:

- *p*-phenylenediamine (PPD)

- *n*-propyl-gallate (NPG; trade name is Mowiol)

- 1,4-diazabicyclo(2.2.2)-octane (DABCO)

- sodium dithionite (sodium hydrosulfite) (SDT).

The preferred mounting medium to dissolve these chemicals is 90% glycerol, buffered at pH 8.5–9.0. The buffer may be either carbonate or TRIS-based. However, glycerol mounting medium buffered at pH 7.4–7.6 is also satisfactory for fluorochromes that are less sensitive to pH than FITC, such as rhodamine dyes. The pH 8.5–9.0 mounting media used with some of the anti-fading agents tend to give brighter background fluorescence.

Each anti-fading chemical has favourable and unfavourable characteristics. Characteristics are listed in Table 7.1. It is up to individual investigators to choose the one that suits their needs best.

Table 7.1 **Characteristics of anti-fading reagents**

Chemical	Fading reduction	Initial fluorescence	Storage conditions needed	Toxic
PPD	Very good	Reduced	−20 °C, dark	Yes
NPG (Mowiol)	Good	Reduced	Room temperature, light OK (precipitates form in two weeks)	No
DABCO	Good	Increased	Room temperature, light OK	No
SDT	Very good	Increased	Room temperature, light OK	No

Some of these chemicals are difficult to dissolve in glycerol. PPD and NPG are dissolved in 90% glycerol with constant stirring in about 2 hours. Alternatively, NPG can be tumbled overnight in 90% glycerol and the buffer added the next day. DABCO requires gentle heat at 37 °C to dissolve it in glycerol.

For fluorescein and acridine orange, treatment with sodium dithionite (SDT) increases initial fluorescence for about 5 minutes before it begins to fade slowly. SDT is an effective anti-fading agent, but it has not received much attention.

PPD is a very good fluorescence fading retardant, but it reduces initial fluorescence. DABCO is a less effective retardant but increases initial fluorescence. Mixing in the mounting medium with both reagents together increases high initial fluorescence and slows fading. Amounts of the anti-fading chemicals can be varied over a range. Recommended amounts to use are as follows.

Chemical	Mounting medium (g/L)
PPD	1
NPG	20
DABCO	6
SDT	3.5

Preparation of mounting media containing anti-fading agents is as follows.

PPD in buffered glycerol

1. Dissolve 100 mg of PPD in 10 mL of buffered saline, phosphate buffered saline or carbonate buffered saline.

2. Add this solution to 90 mL of glycerol dropwise while stirring. Avoid exposure of PPD solution to light.

3. Store suitable sized aliquots of stock PPD solution in the freezer at –20 °C.

NPG in buffered glycerol

1. Mix 5 g of NPG in 90 mL of glycerol.

2. Stir this mixture on a magnetic stirrer for about 2 hours to dissolve the NPG (alternatively, shake it in a stoppered test tube overnight).

3. Add 10 mL of buffered saline (phosphate buffered saline or carbonate buffered saline) and mix well.

DABCO in buffered glycerol

1. Dissolve 2.5 g of DABCO in 90 mL of glycerol in a 37 °C water bath.

2. Add 10 mL of buffered saline (phosphate buffered saline or carbonate buffered saline) and mix well.

SDT in phosphate buffer

1. Dissolve 3.5 g of SDT in 100 mL of phosphate buffer, pH 5.5.

2. Add the buffered SDT to glycerol at a 1:9 ratio.

3. Alternatively, dissolve SDT in carbonate buffered glycerol, 3.5 grams per litre.

Notes

1. *For FITC, adjust the solutions to pH 8.6 with 0.5 M bicarbonate buffer, pH 9.0, and/or NaOH, 1 M.*

2. *Protect these solutions from light. Keep them in clear glass bottles wrapped in aluminium foil. Do not use brown bottles, as this may obscure precipitates that might form.*

7.7 Fixatives and fixation methods

An essential step in preparing organisms and specimens for examination by fluorescent antibody is fixation. This process immobilizes soluble antigens in place

and makes specimens adhere to slides during the staining process. Antigens are fixed by various chemicals or simple heat for certain bacteria and fungi specimens. Four variable factors are involved in fixation, namely:

- chemical used

- concentration of chemical

- temperature

- time.

For each antigen fluorescent antibody system the best fixation method must be found experimentally. Successful methods are indicated as guidelines for choice of fixation method (Table 7.2).

- *Chemical used.* Methanol is a powerful fixative for many antigens. Ethanol is also recommended, as is acetone. Commonly, 95% ethanol for 30 minutes or acetone for 10 minutes, each at room temperature, is used. Formalin, alone or in combination with pH-neutralizing chemicals, also is a commonly used fixative.

Table 7.2 **Fixation methods for various antigens in specimens**

Fixative and/or method	Antigen
Acetone	Bacteria, fungi, viruses
Carbon tetrachloride	Viruses
Ethanol, absolute (0–2 °C)	Tissue antigens
Ethanol, 95%	Bacteria, fungi, viruses
Ethanol, 70%	Bacteria
Ethanol–ether	Immunoglobulin
Ethanol–glacial acetic acid	Viruses
Formalin, 10%	Viruses
Formalin, neutral (1%, 4% or 10%)	Tissue sections, rickettsiae, viruses
Formol–saline, 10%	Tissue sections
Heat, flame	Bacteria, fungi
Heat, slide heater	Bacteria, fungi
Methanol, absolute	Fungi
Osmic acid	Viruses

- *Concentration*. Concentration of chemical is an important variable in some cases. Ethanol is often used at 95%, but 70% is thought to do the most fixation. Absolute ethanol at 100% is less active. Acetone and methanol usually are used at 100%. Formalin, alone or with various other chemicals to neutralize or assist action, is used at 1%, 4%, or 10% concentrations, depending on the test system.

- *Temperature*. In general, the lower the temperature of the fixative, the less fixation happens. Fixation with solvents is mostly done at room temperature, but temperatures such as 37 °C, room, –20 °C, and even –76 °C have been suggested. Delicate labile antigens are better preserved at lower temperatures, but fixation takes longer. For most bacterial and fungal culture preparations, simple heat-fixation is adequate. A slide smear of a specimen or culture is gently heated in a flame until it is almost too hot to touch. This also can be done on a slide heater at a lower, controlled temperature for a longer period.

- *Time*. Various experts recommend several seconds to a few hours for fixation of various antigens. If the fixation period is very short, only adhesion to the slide and dehydration is achieved.

Some fixatives commonly used in histology, histochemistry and electron microscopy are not suitable for fluorescent antibody studies. These include Carnoy fluid, Bouin fluid, Zenker fluid and gluteraldehyde. They are unsuitable because they cause non-specific fluorescence, suppress fluorescence or disrupt antigen–antibody reactions.

7.8 Direct fluorescent antibody tests for infectious disease diagnosis

7.8.1 Detection of pathogenic enteric bacteria in faecal suspensions (example: pathogenic *Escherichia coli*)

Purpose

Detection and identification of bacteria in a contaminated specimen.

Approach

Direct fluorescent antibody is done on faecal specimens for specific bacteria.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Reagents

Phosphate buffered saline (phosphate buffered saline), pH 7.4–7.5.

Saline, 0.5%.

Mounting medium, pH 8.5–9.0 (carbonate buffered).

Anti–*E. coli* fluorescent antibody conjugate (rabbit serum) (conjugate specific for type sought, for example O157:H7).

Normal rabbit globulin conjugate (negative control).

Faecal specimen swab samples or rectal swab samples.

Method

1. Add 0.5 mL saline to test tubes.

2. Place the faecal swab in the saline. Agitate the swabs and squeeze liquid from the swab on the side of the tube.

3. Etch two circles about 10–12 mm diameter slide using a diamond scriber, or use commercial slides with masked circles.

4. Prepare duplicate smears on the slide using a loop to spread the suspension. Air-dry and flame-fix the smears.

5. Place a drop of anti–*E. coli* conjugate in one circle and a drop of the normal globulin conjugate in the other circle.

6. Use an applicator stick to gently spread the drop over each smear. (Take care to avoid mixing the drops.)

7. Stain the slide for 15 minutes in a moist chamber.

8. Shake off excess stain, and rinse the slides in phosphate buffered saline for 10 minutes.

9. Drain the slide and blot it with bibulous paper.
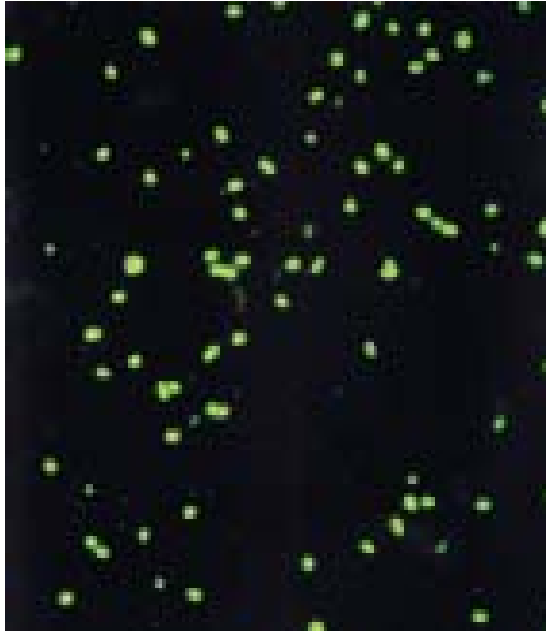
Figure 7.6 **Fluorescent antibody stain of *E. coli* in a faecal suspension**

10. Mount cover glasses on the stained smears with mounting fluid, pH 8.5–9.0.

11. Examine the slide under the microscope with a ×40 objective and with a ×100 (oil) objective for confirmation (Figure 7.6).

7.8.2 Diagnosis of diphtheria from throat swabs (*Corynebacterium diphtheriae*)

Purpose

Rapid, specific diagnosis of diphtheria.

Approach

Fluorescent antibody is used to identify bacteria following enrichment culture of specimens.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Reagents

Anti–*C. diphtheriae* FITC conjugates.

Normal rabbit globulin FITC conjugate.

Heart infusion broth + 0.5% glucose, 1 mL (slants of *C. diphtheriae* selective agar may also be used, as may Loeffler agar slants).

Method

1. Place the throat sample swab into the heart infusion broth (alternatively, place it on a Loeffler agar slant).

2. Incubate the swab in broth for four hours at 37 °C (incubate slants for 18–24 hours at 35–37 °C).

3. Remove the swab from the broth, and express the liquid on the side of the tube (discard the swab safely).

4. Centrifuge the broth culture to pack the cells. Discard the supernatant liquid.

5. Resuspend the sediment in phosphate buffered saline, a few drops only.

6. Prepare duplicate smears in 10–12 mm circles scored on the slide. Gently heat-fix the smears in a flame.

7. Add one drop of anti–*C. diphtheriae* conjugate to one circle.

8. Add one drop of normal globulin conjugate to the other circle.

9. Use an applicator stick held at an angle resting on one edge of the slide to spread each drop. Do not touch the surface of the smear. Do not allow the drops to mix.

10. Wash the slide in phosphate buffered saline, 10 minutes. Change the phosphate buffered saline once (a coplin jar is useful).

11. Blot the slide with bibulous paper.

12. Examine the slide with the fluorescence microscope. Compare results in both circles.

7.8.3 Diagnosis of whooping cough (*Bordetella pertussis* or *B. parapertussis*)

Purpose

Detection and identification of *Bordetella pertussis* and *B. parapertussis* from respiratory specimens.

Approach

Direct fluorescent antibody is done on nasopharyngeal swab specimens following a brief culture pre-incubation.

Materials

Basic equipment for fluorescence microscopy.

Basic fluorescence microscopy.

Conjugated anti–B. *pertussis*.

Conjugated anti–B. *parapertussis*.

Conjugated normal chicken globulin.

Reagents

Phosphate buffered saline, pH 7.4.

Cover glass mounting medium, pH 8.5–9.0.

Casamino acids, 1%.

Ethanol, 95%.

Collection supplies.

Cotton or Dacron swab.

Specimen preparation

1. Take a nasopharyngeal swab specimen.

2. Place the swab in 0.3 mL of Casamino acid solution.

3. Incubate this culture at 37 °C for one hour, preferably in a water bath.

4. Spread the incubated specimen on three slides a, b and c.

5. Allow the slides to air-dry.

6. Fix the slides in 95% ethanol for 1 minute, or *gently* heat-fix the slide in a flame.

Fluorescent antibody test method

1. Add a drop of conjugate to each slide:

    a. anti–*B. pertussis*

    b. anti–*B. parapertussis*

    c. normal chicken globulin.

2. Spread the conjugates across the smear with a clean applicator stick rested on one edge. Do not touch the smear.

3. Incubate the slides at room temperature for 30 minutes in a moisture chamber.

4. Wash the conjugates from the slides with phosphate buffered saline.

5. Place the slides in a staining jar of phosphate buffered saline. Agitate gently.

6. Change the phosphate buffered saline rinse bath twice.

7. Remove the slides from the phosphate buffered saline and gently blot them dry with bibulous paper. Allow them to completely air-dry.

8. Place a drop of mounting medium, pH 8.5–9.0, on each slide.

9. Place a cover glass on each drop. Avoid trapping air bubbles.

10. Examine each smear with a fluorescence microscope.

11. Magnification of ×500 to ×600 (oil immersion) is used, but it may help to confirm suspected positives at ×1000 (oil).

Reference

Halperin SA et al., 1989.

7.8.4 Detection of virus antigens in circulating leukocytes (encephalitis virus)

Purpose

Diagnosis of virus infections in which the virus antigens can be found in white blood cells of various types.

Approach

Leukocytes are separated and concentrated from a blood specimen, and direct fluorescent antibody is used to detect encephalitis virus antigen in the leukocytes.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Syringe and needle, 5 mL.

Test tube, 16 × 125 mm, screw cap (alternatively, 17 × 100 mm stoppered tube).

Water bath, 37 °C.

Wintrobe pipettes with rubber bulb.

Membrane filter apparatus.

Membrane filters, 47 mm diameter, 0.45 µm pore size.

Reagents

Sodium heparin solution, 100 units per 1.0 mL.

Bovine fibrinogen solution, 6%.

Saline solution, 0.15M.

Acetone.

Fluorescent antibody conjugates.

Anti–Japanese B encephalitis virus (rabbit origin).

Normal rabbit gamma globulin.

Antiserum (blocking control): anti–Japanese B serum.

Counterstain: Evans blue.

Specimen processing

1. Draw 5 mL of blood.

2. Expel the blood gently into a 16 × 125 mm screw cap test tube (optional 17 × 100 mm plastic tube with a stopper) containing 500 units (0.5 mL) of sodium heparin solution (1000 IU/mL). Cap the tube and mix it thoroughly but *gently*.

3. Add an equal volume (5.5 mL) of 6% bovine fibrinogen solution to the heparinized blood.

4. Mix the tube thoroughly but *gently*.

5. Incubate the mixture at 37 °C for approximately 15–30 minutes (water bath).
    Note: the time depends on the rate of erythrocyte settling. The supernatant fluid is carefully removed when the erythrocytes have settled to about half the vertical distance of the mixture in the vial.

6. Transfer the *cloudy* supernatant fluid to another screw-cap test tube.

7. Centrifuge the *tightly capped* tube at 800*g* for 5 minutes.

8. Decant and discard the *clear* supernatant fluid.

9. Resuspend (*gently*) the sediment in 10 mL of membrane-filtered 0.15 M NaCl solution.

10. Centrifuge again as in step 7 above.

11. Decant 9.5 mL of the *clear* supernatant fluid.

12. Resuspend the sediment in the remaining 0.5 mL of fluid.

13. Prepare slide smears from this suspension. Air-dry the smears.

14. Fix the smears in cold acetone, 1–4 °C for 30 minutes. (An ice bath may be used to cool the acetone.)

15. Air-dry the smears. (Slides should be stained and examined as soon as possible. Fixed slides may be stored in the refrigerator if necessary, but for no longer than one week.)

Conjugate treatment

1. Treat both anti–Japanese B and normal conjugates in an identical manner.

2. Thaw frozen conjugate. If it is cloudy, check its pH, being sure the pH is above pH 7.2. If necessary, adjust the pH using 0.1 N NaOH.

3. Weigh out 100 mg of human placenta tissue powder for each 1 mL of conjugate and mix the tissue powder with an excess of phosphate buffered saline in a centrifuge tube.

4. Centrifuge the mixture at the highest possible speed.

5. Decant and discard the supernatant phosphate buffered saline.

6. Add the conjugate to the tissue powder. Mix thoroughly.

7. Incubate the powder–conjugate mixture at room temperature, preferably around 25 °C, for 30 minutes.

8. Resuspend the powder at least every 5 minutes.

9. Centrifuge the tube at the highest possible speed.

10. Decant the conjugate, and filter it with a membrane, pore size 0.45 μm.

11. Measure the volume of the conjugate, and add 1 part of RB 200–bovine serum albumin counterstain per 10 parts of conjugate. The conjugate is now ready for use. If it is sterile, it may be kept at 4 °C for up to two weeks without significant loss of potency. Discard a prepared conjugate after two weeks.

Fluorescent antibody staining procedure

1. Fluorescent antibody stain three smears:

   a. anti–Japanese B encephalitis virus conjugate

   b. negative conjugate control

   c. blocking control.

2. Stain the air-dried, fixed smears in a moisture chamber, 35–37 °C for 30 minutes.

3. Rinse the stained slides for 2–3 minutes in each of three changes of carbonate–bicarbonate buffer, pH 9.0.

4. Dip the slides quickly in and out of a filtered distilled water bath, and mount cover glasses on the slides using carbonate–bicarbonate buffered glycerol.

Specimen slide examination

1. Examine the slides using the fluorescence microscope: ×10 ocular, ×50 (oil) objective, alternatively a ×5 or ×6 ocular with the ×100 (oil) objective.

2. Compare the amount, degree, and morphology of yellow-green fluorescence in leukocytes in the two smears.

3. Record all results.

4. Examine coded slides, including the controls, under the epi-fluorescence microscope, and record the results as indicated in 1, 2, and 3 above.

Fluorescent antibody controls

*   *Negative conjugate control*. Use normal rabbit globulin conjugate to stain leukocyte smears in the same manner as staining using the anti–Japanese B conjugate. The normal conjugate is prepared from normal rabbit serum in precisely the same manner as the anti–Japanese B conjugate. Before use, it should be tissue powder–absorbed and mixed with counterstain as with the anti–Japanese B conjugate. Do not use other normal conjugates for this control.

    Expected result: little or no green fluorescence in leukocytes.

*   *Blocking control*. Before fluorescent antibody staining by the standard procedure for staining a fixed leukocyte smear with the anti–Japanese B conjugate, a 1:2 dilution of a specific antiserum is reacted with the smear for 30 minutes, and then washed off in several changes of phosphate buffered saline. Then fluorescent antibody stain the smear.

    Expected result: reduction or elimination of positive green specific staining.

*   *Positive control*. Positive leukocyte smears should be used. One such smear should be incorporated in each slide series if possible. The staining by the normal rabbit conjugate is negative or minimal. However, remember that leukocytes present some special non-specific fluorescence problems. These are partially overcome by the method of conjugate preparation, placenta tissue

powder absorption and counterstaining. However, careful interpretation and controls are still required.

7.8.5 Diagnosis of leptospirosis in blood, urine, or cultures (*Leptospira* spp.)

Purpose

Detection and identification of *Leptospira* spp. in clinical specimens.

Approach

Direct fluorescent antibody is used to detect the organisms in urine, other body fluids and cultures.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Reagents

Anti–*Leptospira* spp.

Normal rabbit globulin.

Support reagents.

Phosphate buffered saline, pH 7.4.

Mounting medium, pH 8.5–9.0.

Stewart medium base.

Leptospira enrichment medium.

Ethanol, absolute.

Controls

Positive control: *Leptospira* culture or commercial *Leptospira* serotypes.

Non-specific staining: normal rabbit globulin conjugate on specimen.

Specimen preparation

A.   *Specimens to examine*

   1.   Urine (*after* the first week of illness).

   2.   Cerebrospinal fluid (first week to 10 days of illness).

   3.   Blood (first week to 10 days of illness).

   4.   Culture in Stewart medium.

B.   *Procedure*

   1.   Smear the specimen on a slide.

   2.   Allow the smear to air-dry.

   3.   Fix the smear in absolute ethanol, 10 minutes.

C.   *Specimen processing*

   1.   Blood.

      a.   collect 5 mL of blood into 0.5 mL 1% sodium oxalate or 1.0 mL of 1% sodium heparin

      b.   centrifuge at 500*g* for 15 minutes

      c.   transfer the supernatant to another tube

      d.   centrifuge this tube at 1500*g* for 30 minutes

      e.   discard the supernatant

      f.   prepare a smear from the sediment

      g.   fix the air-dried smear 10 minutes in absolute ethanol.

   2.   Cerebrospinal fluid, urine or broth culture.

      a.   centrifuge the specimen at 1500*g* for 15 minutes

      b.   prepare a slide smear from the sediment

      c.   fix the air-dried slide smear in absolute ethanol for 10 minutes.

Fluorescent antibody test method

1.   React the slide specimen with the fluorescent antibody conjugates (anti–

Leptospira spp. and normal rabbit globulin) in a moist chamber for 2 hours at 37 °C.

2. Wash the slide in phosphate buffered saline, pH 7.4, for 30 minutes. Do two changes of phosphate buffered saline in this period.

3. Drain the slide.

4. Mount a cover glass on the slide with a drop of mounting medium, pH 8.5–9.0.

5. Examine the slide with the fluorescence microscope (FITC filters) at ×600 magnification.

6. Check suspect positives for morphology at ×1000 magnification.

Reference

White FH, Ristic M, 1959.

7.8.6 Virus detection in mucoid respiratory specimens (respiratory syncytial virus)

Purpose

Improved detection of respiratory syncial virus (RSV) in clinical respiratory specimens.

Approach

Indirect fluorescent antibody to detect and identify viruses in cells from nasopharyngeal secretions processed with dithiothreitol followed by centrifugation through Percoll to separate cells from mucus.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Automatic pipette, 5 mL tip, bore widened for mucus suction.

Centrifuge tube, 15 mL.

Pasteur pipette and rubber bulb.

Reagents

Fluorescent antibody reagents.

Anti-RSV (bovine origin).

Anti-bovine conjugate.

Phosphate buffered saline, pH 7.4.

Phosphate buffered saline, pH 7.4 + dithiothreitol (10 mM).

Percoll, 45%.

Percoll, 20%.

Acetone, cold.

Sodium azide, 5%.

Specimen preparation and processing

1. Collect respiratory specimens (nasopharyngeal secretion, sputum, bronchoalveolar lavage).

2. Dilute the specimen 1:2 to 1:5 in phosphate buffered saline–dithiothreitol. (Higher dilutions are used for thicker mucus specimens.)

3. Aspirate the specimen and phosphate buffered saline–dithiothreitol several times in and out of a 5 mL pipette to mix it well.

4. Transfer the diluted specimen (2–10 mL) to a centrifuge tube.

5. Mix the diluted specimen with half the volume of 45% Percoll (final concentration of Percoll: 15%).

6. Place a 1 mL Percoll (20%) cushion under the specimen in the tube *slowly* with a Pasteur pipette (through the specimen).

7. Centrifuge the tube at 700*g* for 5 minutes.

8. Remove the supernatant fluid by aspiration.

9. Resuspend the cell pellet in the bottom of the centrifuge tube in 0.1–1.0 mL of phosphate buffered saline.

10. Pipette drops of the cell suspension on to a glass slide.

11. Air-dry the slide.

12. Fix the cells on the slide in cold acetone, 5 minutes.

Fluorescent antibody staining (indirect)

1. React the cells with bovine anti-RSV antiserum, 10 minutes.

2. Wash the anti-serum from the slide with several changes of phosphate buffered saline.

3. React the cells with anti-bovine conjugate.

4. Wash the slide several times in phosphate buffered saline.

5. Mount a cover glass on the cell spots with mounting medium.

6. Observe the slide with a fluorescent microscope at about ×400 (dry).

Note: the method works well with various respiratory tract specimens containing mucus. Fluorescent antibody examination of the specimens is more sensitive than enzyme immunoassay and is much simpler. It is applicable to various viruses simply by changing the antibody used. Direct fluorescent antibody is also possible with specimens prepared in this manner.

References

McDonald JC, Quennec P, 1993; Unnonen P, Julkunen I, 1987.

7.8.7 Detection of protozoa in sputum specimens (*Pneumocystis carinii*)

Purpose

Detection and identification of *Pneumocystis carinii* in immunocompromised patients with pneumonia.

Approach

Sputum or bronchoalveolar lavage fluids are examined by direct fluorescent antibody using monoclonal antibody conjugate.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Coated slide with wells, 10–14 mm diameter circles.

Nebulizer.

Vortexer (mixer).

Water bath, 37 °C.

Reagents

Dithiothreitol, 0.0065 mol/L.

Monoclonal fluorescent antibody conjugate, anti–*Pneumocystis carinii*.

Saline, 3%.

H_2O, distilled or deionized.

Evans blue counterstain.

Method

1. Select a sputum specimen (not saliva; see below).

2. Concentrate the sputum by mucolysis with dithiothreitol and centrifugation.

3. Overlay 2 mL of sputum specimen with 2 mL of dithiothreitol in a 15 mL conical screw cap centrifuge tube (plastic).

4. Vortex the specimen.

5. Incubate the vortexed specimen at 37 °C, 4 minutes, in a water bath with agitation.

6. Repeat steps 4 and 5 several times until the specimen is partially, but not completely, liquefied.

7. Prepare slide smears with 50 µL of concentrated sputum specimen.

8. Air-dry the slides.

9. Fix the smear slides in acetone, 10 minutes.

10. Stain the slides with anti–*P. carinii* conjugate, 30 minutes at 37 °C in a humid chamber. (Evans blue counterstain may be mixed with the conjugate.)

11. Drain the slides, and rinse in deionized or distilled water.

12. Mount a coverglass on each slide with buffered mounting fluid. (A fluorescence anti-quencher may be included in the mounting fluid.)

13. Examine the slide with an epi-fluorescence microscope, FITC filters. Use about ×250 magnification.

14. Confirm suspect positives at ×500.

Sputum collection

It is important to get sputum from deep in the lungs, not saliva from the mouth. For this reason, sputum collection should always be directly observed by a nurse, medical attendant or laboratory technician in order to assure a quality specimen. It is important to avoid getting extraneous material from the oral cavity in the specimen. For this reason, patent preparation should be instituted before collecting sputum by any method, as follows.

The patient should:

• eat no solid food for eight hours before providing sputum

• just before collection, brush the teeth, gingival areas, tongue and inside of the mouth (buccal surfaces) with water or saline

• rinse the mouth thoroughly with water.

• gargle at least twice, expelling the water each time.

This procedure removes general debris, bacteria clumps and loose epithelial cells. Thus, these cannot obscure a clear microscopic view of the test field on the slide prepared from the sputum.

Three methods may be used to obtain sputum for fluorescence microscopy testing. These are:

Method A: WHO method

Method B: nebulized 3% saline, inhaled

Method C: bronchoalveolar lavage.

Method A is the simplest way to collect a sputum specimen, and it may be satisfactory for use where equipment for the other methods is not readily available.

1. Collect sputum specimens early in the morning.

2. Give the patient a clean (not necessarily sterile), dry, wide-necked, leakproof container.

3. Use reusable jars or stiff paper boxes made in the laboratory for on-the-spot collection.

4. Ask the patient to take a deep breath and then cough deeply, spitting what he or she brings up into the container.

   Note: liquid frothy saliva and secretions from the nose and pharynx are not suitable for examination. Ask the patient to produce another specimen.

5. Check that a sufficient amount of sputum has been produced.

6. Screw on the top and label the bottle with the name and number of the patient.

7. Examine the sputum with the naked eye and then by microscopy.

   The sputum of a person suffering from a bacterial infection usually contains:

- thick mucus with air bubbles

- threads of fibrin

- patches of pus

- occasional brownish streaks of blood.

   After visual inspection, report the appearance of the sputum as:

- purulent: greenish, containing pus

- mucopurulent: greenish, containing both pus and mucus

- mucoid: containing mostly mucus

- mucosalivary: containing mucus with a small amount of saliva

- reddish from blood present.

Method B is induction of sputum by inhaling a nebulized spray (fine mist) of 3% saline. Commercial nebulizers deliver 3–7 mL of saline solution per minute. Between 5 and 10 minutes of inhalation is needed to induce sputum production of about 2–5 mL. The sputum is collected in a sterile container, a wide-mouth screw cap jar or cup.

Method C is bronchoalveolar lavage. This is an effective but more complex procedure. Only an experienced physician in an institution properly equipped for this procedure should do it. A small-diameter fibre optic bronchoscope is needed to do the procedure following examination of the tracheobronchial tree by X-ray. Between 15 mL and 70 mL of saline is instilled to collect the specimen. Chest X-ray and arterial blood gas measurements should be made to evaluate the patient periodically after the procedure.

Results

Cysts and trophozoites appear apple green, singly and in clusters.

References

Gill VJ et al., 1987; Kovacs JA et al., 1988; Ng VL et al., 1990 (IFA); Ng VL et al., 1990 (DFA).

7.8.8 Detection of protozoa in faecal specimens (*Cryptosporidium parvum* / *Giardia lamblia*)

Purpose

Detection of *Cryptosporidium* or *Giardia lamblia* infection in immuno-compromised patients with diarrhoea.

Approach

Faecal specimens are examined by fluorescent antibody after washing and centrifuging them.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Centrifuge, 500g capability.

Centrifuge tube, screwcap.

Slide adhesive, glycerol–agar
Note: preparation and use of slide adhesive

Adhesive formula
Refined agar, 0.1 g
Glycerol, 5 mL
H_2O, distilled, 100 mL

Boil this mixture. Dispense it in small amounts in screw-cap tubes. Autoclave the tubes. Store the tightly sealed tubes at room temperature.

Adhesive use
1.Place one drop of slide adhesive on a corner of the slide.
2.Place a square of filter paper over the drop.
3.Smear the adhesive over the slide with the filter paper square.
4.Air-dry the slide at room temperature (about 30 minutes) or for 5 minutes at 37 °C.

Slide, Teflon-masked with three 7 mm circles or a standard slide with diamond etched circles.

Pasteur pipette (optional).

Filter paper, square, 25 × 25 mm, Whatman 40.

Reagents

Formalin, 10%.

Potassium hydroxide, 10%.

Cryptosporidium–Giardia direct fluorescent antibody detection kit *or Cryptosporidium* monoclonal fluorescent antibody conjugate/*Giardia* monoclonal fluorescent antibody conjugate.

Eriochrome black.

Methanol, absolute.

Cryptosporidium positive control antigen.

Giardia positive control antigen.

Controls

Negative control (formalized normal faecal suspension).

Method

1. Collect the faecal specimen in 10% formalin (mix it well to suspend the faeces. Treat the faecal suspension with 10% formalin, 30 minutes minimum; it may contain HIV.).

2. Allow the faeces to settle by gravity.

3. Pour off the supernatant fluid, or use a Pasteur pipette to decant it.

4. Wash the sediment with 10% formalin.

5. Centrifuge the suspension at 500*g* for 10 minutes.

6. Decant the supernatant fluid.

7. Place one drop of sediment (10 µL) in a circle on a slide treated with glycerol–agar slide adhesive.

8. Spread the drop thinly in the circle, and allow it to air-dry.

9. Heat-fix the slide for 10 minutes on a slide warmer (very gentle flame fixation may be used).

10. Fix the slide in absolute methanol, 5 minutes, room temperature.

11. Stain the slide with the direct fluorescent antibody reagent, 20 minutes, room temperature.

12. Wash the slide with phosphate buffered saline, pH 7.4.

13. Mount a cover glass on the slide with buffered glycerol, pH 8.5.

14. Examine each well on the slide by epi-illumination fluorescence microscopy within 1 hour at ×100.

15. Confirm cysts or oocysts at ×250 or ×400.

Results

Cysts and oocysts fluoresce apple green. Sensitivity and specificity for both *Cryptosporidium* and *Giardia* should be 100%. *Giardia* cysts are oval, approximately 11 by 14 µm, and *Cryptosporidium* oocysts are round, about 4–6 µm in diameter.

Safety precautions

Faecal specimens from these patients may contain HIV. They should be handled with caution, and rubber gloves should be worn. Specimens should be suspended in 10% formalin for at least 30 minutes before testing.

Avoid skin contact with formalin. If contact occurs, wash the area thoroughly with water.

References

Garcia LS et al., 1987; Garcia LS et al., 1992.

7.8.9 Quantitating protozoa in faecal specimens (*Cryptosporidium parvum/Giardia lamblia*)

Purpose

Estimation of the parasite load in immunocompromised patients with protozoan diarrhoea.

Approach

A standard amount of faecal material is partially purified, and the protozoan cells in a measured volume are counted.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Centrifuge, 50 mL tube capacity, 1500*g*.

Centrifuge tube, screwcap, 50 mL.

Vortexer (optional).

Cheesecloth.

Funnel.

Pipette, 20 μL.

Reagents

Methanol, absolute.

H_2O, distilled.

Formalin, 10%.

Glycerol–agar slide adhesive (see Section 7.8.8).

Method

1. Take a 2 g faecal sample.

2. Suspend this in 10 mL of 10% formalin. Allow the mixture to fix for at least 30 minutes.

3. Filter the suspension through cheesecloth, 1 layer.

4. Centrifuge the filtered suspension at 1500*g*, 10 minutes.

5. Resuspend the pellet in 4 mL of distilled water.

6. Vortex or mix this by hand, thoroughly.

7. Pipette 20 µL of this suspension on to a treated slide.

8. Spread this as a faecal smear.

9. Fix the smear in absolute methanol, 5 minutes at room temperature.

10. Stain the smear using a direct fluorescent antibody test kit for *Cryptosporidium* and/or *Giardia*.

11. Search the entire smear by epi-illumination fluorescence microscopy at ×100 magnification.

12. Count *Cryptosporidium* oocysts and *Giardia* cysts.

13. Confirm suspect organisms found at ×250 or ×400.

14. Multiply the oocysts and cysts found on the entire smear by 100.

    Note: if the total oocysts or cysts counted exceeds 40 000, dilute the faecal suspension 10 or 100 times, depending on the count, and repeat steps 7–13. Multiply the actual count by the dilution factor

Results

The quantitative fluorescent antibody method was superior to both sucrose gradient

flotation and zinc sulfate flotation for detecting *Giardia* cysts. For detecting *Cryptosporidium* oocysts, the quantitative fluorescent antibody method is much faster and at least 100 times more sensitive than the haemocytometer method.

Note: the quantitative fluorescent antibody method provides a suitable and efficient way to quantitate Giardia *and* Cryptosporidium *infections in AIDS patients. Faecal specimens from immunocompromised patients may contain HIV. Wear rubber gloves when handling specimens and related materials.*

Reference

Xiao L, Herd RP, 1993.

7.8.10 Special pathogens

Today, preparedness against intentionally used infective agents must be part of the responsibility of public health systems. Medical and public health laboratories are among the first responders to a misuse of highly contagious, pathogenic microorganisms. While having the potential of being misused, these pathogens are also well known in natural situations (Table 7.3). It is important to detect early and identify rapidly these microorganisms to know whether the situation where they are found is normal or intentionally caused. Specific, early diagnosis of a small cluster of cases, or only a single case in an unnatural location, might be critical for choice and timely institution of effective epidemic disease control measures and initiation of protective measures

Other microorganisms also may be potentially misused, but most experts agree that these are the ones of primary concern. Salmonellosis is not usually considered a "special pathogen disease", but it is included in the list because it was actually used in recent times [Torok TJ et al., 1997].

Precautions against intentionally used microorganisms include rapid diagnosis of victims and detecting the microorganisms in the environment. Fluorescence microscopy with acridine orange and fluorescent antibody can play an important role and add important information. Fluorescence microscopy and fluorescent antibody can be used in various areas of protection. Fluorescence microscopy is a simple and inexpensive way to test the environment for aerosols, water pollution or surface contamination. In some situations, the technique is not as definitive for

precise microbial identification as a newer technology such as polymerase chain reaction (PCR) or DNA sequencing, but it is much simpler and faster than these methods and can quickly yield very useful preliminary information.

Table 7.4 outlines fluorescence microscopy diagnostic approaches for these microorganisms. Differences in test methods relate to the specimen in question. Some diseases take different forms depending on the route of infection; for example, cutaneous and inhalation anthrax. The form of the disease can bear heavily on the severity of the disease and outcome.

Anthrax bacilli can be detected easily with acridine orange, but some other *Bacillus* spp. resemble *Bacillus anthracis*. Also, members of the genus *Bacillus* share many antigens, so it is difficult to make a specific fluorescent antibody conjugate for *B. anthracis*. Monoclonal antibodies improve the examination but still may not be entirely specific. Nevertheless, a patient with inhalation or gastrointestinal anthrax is likely to have bacilli in the blood or spinal fluid, so finding large bacilli circulating in clinical specimens that normally are sterile yields a strong presumptive diagnosis.

The situation is different with plague, salmonellosis and tularaemia. Highly specific and sensitive fluorescent antibody conjugates are available commercially that can rapidly detect and identify these bacteria. In most instances, fluorescent antibody provides rapid, specific diagnosis of these infections. Furthermore, fluorescent antibody detects and specifically identifies these bacteria in environmental samples.

Table 7.3 **Diseases of potential primary concern for intentional misuse**

Disease	Agent type	Used previously
Anthrax	Bacteria	×
Botulism	Bacterial toxin	
Haemorrhagic fever	Virus	
Plague	Bacteria	×
Salmonellosis	Bacteria	×
Smallpox	Virus	×
Tularaemia	Bacteria	

Table 7.4 Fluorescence microscopy for rapid diagnosis of intentionally used infective agents

Disease (etiological agent)	Test specimen (disease form)	Fluorescence method	Technical approach	Test specificity	Test time
Anthrax (*Bacillus anthracis*)	Blood (intestinal, pulmonary)	AO[1]	Direct exam	Non-specific, definitive form	1 hour
	Cerebrospinal fluid (pulmonary)	AO	Direct exam	Non-specific, definitive form	1 hour
	Faeces (intestinal)	AO	Direct exam	Non-specific, presumptive form	1 hour
	Haemorrhagic fluid	FA[2]	Direct exam	Cross-reactions, definitive form	1 hour
	Nasal swab	FA, AO	Direct exam	Cross-reactions, presumptive form	1 hour
	Vesicular exudate (cutaneous)	FA, AO	Direct exam	Cross-reactions, definitive form	1 hour
	Agar culture from specimens	FA	Microcolony[4]	Cross-reactions, definitive form	12–18 hours
	Serum	IFA[3]	Antibody titre[5]	Cross-reactions	2 hours
Botulism (*Clostridium botulinum*)	Faeces	N/A	N/A	N/A	N/A
	Gastric contents	N/A	N/A	N/A	N/A
	Serum	N/A	N/A	N/A	N/A
Plague (*Yersinia pestis*)	Blood	FA	Direct exam	Specific	1 hour
	Bubo aspirate	FA	Direct exam	Specific	1 hour
	Sputum (pulmonary)	FA	Direct exam	Specific	1 hour
	Agar culture from specimens	FA	Microcolony	Specific	12–18 hours
	Serum	IFA	Antibody titre	Specific?	2 hours
Smallpox (variola virus)	Lesion scrapings	FA	Direct exam	False positives, other pox viruses	1 hour
	Vesicular fluid	FA	Direct exam	False positives, other pox viruses	1 hour
	Serum	IFA	Antibody titre	Cross-reactions, other pox viruses	2 hours
Tularaemia (*Francisella tularensis*)	Sputum (pulmonary)	FA	Direct exam	Specific	1 hour
	Ulcer exudate	FA	Direct exam	Specific	1 hour
	Agar culture from specimens	FA	Microcolony	Specific	12–18 hours
Viral haemorrhagic fevers (Marburg, Ebola, Lassa, Jenin)	Slide impressions (liver, spleen, kidney)	FA	Direct exam	Specific	1 hour
	Tissue culture	FA	Tissue culture	Specific	1–7 days
	Serum	IFA	Antibody titre	Specific	2–14 days
Salmonellosis[6] (*Salmonella spp.*)	Blood broth culture	FA	Selective culture	Specific	12–18 hours
	Faeces broth culture	FA	Selective culture	Specific	12–18 hours
	Urine broth culture	FA	Selective culture	Specific	12–18 hours
	Agar culture from specimens	FA	Microcolony	Specific	12–18 hours

1. Acridine orange fluorochrome dye 2. Fluorescent antibody test for antigen (Ag), direct or indirect 3. Indirect fluorescent antibody test for antibody (Ab) 4. Slide impression of early bacterial colony from agar culture plate 5. Antibody titration by indirect fluorescent antibody test. N/A = not applicable .

Viral diseases like smallpox and haemorrhagic fevers pose different diagnostic problems, but fluorescence microscopy is helpful for rapid diagnosis and, in some cases, it is quite specific. Among the poxviruses, there are serological cross-reactions. Thus, when poxvirus infection is suspected, fluorescent antibody tests must be interpreted carefully in combination with other diagnostic criteria.

If viral haemorrhagic fever is suspected in a patient, either direct and indirect fluorescent antibody can provide a specific diagnosis. Direct fluorescent antibody is done on tissue impressions from organs collected at autopsy to detect and identify viral antigens. Direct fluorescent antibody also detects and identifies viral antigens in tissue cultures. This is done on spot slides made from tissue culture cells.

Indirect fluorescent antibody (IFA) is used to titre antibodies to viral haemorrhagic fevers, and this is a way to rapidly diagnose patients. In some diseases, IFA detects antibodies earlier than other serological tests, and therefore it has been the preferred serological method. IFA is done on spot slides of known virus infected tissue cultures. Antibodies become detectable in Lassa fever within a few days after onset and with Marburg and Ebola viruses within 10 days. For other viral infections it can be used later when antibody titres appear.

Monoclonal and polyclonal antibodies are commercially available for *B. anthracis*, *F. tularensis*, *Salmonella* spp., and *Y. pestis*. Fluorescent antibody conjugates can be made with these, or they can be used in indirect fluorescent antibody tests. Commercial antisera are also available for vaccinia virus, and this may be useful in diagnosing smallpox, since poxvirus antisera cross-react because of close relationships within this group. Antisera to viral haemorrhagic fevers are not commercially available, but they have been prepared and used at infectious disease research institutes such as the Centers for Disease Control and Prevention and USAMRIID in the USA, as well as the Centre for Applied Microbiology and Research in England.

DANGER! Acute phase blood and sera from haemorrhagic fever patients contain highly infectious virus. These specimens must be processed only in laboratories specially equipped for maximum biological containment.

Safety must be the paramount concern when doing any laboratory work with these viruses. Any specimens suspected to contain haemorrhagic fever viruses must be considered highly infectious and dangerous. Specimens and tissue cultures

from these diseases must be handled only in maximum biocontainment facilities, Biosafety Level 4 laboratories.

Laboratories without these biosafety facilities and biological containment equipment such as closed negative pressure safety cabinets should not attempt fluorescent antibody diagnostic tests for these viruses. Acetone fixation of spot slides made from tissue cultures does not completely inactivate these viruses. Therefore, such slides must be considered infectious and hazardous. Viruses in tissue cultures can be biologically inactivated by gamma irradiation—the preferred method. However, this method is not generally available. Another successful inactivation method is treatment with β-propiolactone (Van der Groen and Elliot, 1982). Once spot slides from tissue cultures of these viruses are properly and completely inactivated, fluorescent antibody can be used to detect and identify virus antigen using completely inactivated virus material in normal laboratories. For more detailed information about how to handle viral haemorrhagic fever specimens, refer to Murray PM et al (2003), chapter 90: filoviruses and arenaviruses.

7.9 Indirect immunofluorescence tests (IFA)

7.9.1 Detection of humoral antibodies

7.9.1.1 Introduction and general method
Antibodies are immunoglobulins that can be classified in various ways based on:

- pathogenicity

- organ, tissue and cell specificity

- disease specificity

- cell components that are likely to be target antigens

- clinical significance.

The diagnostic value of humoral antibodies as indicators has been confirmed for various communicable and noncommunicable diseases. Antibodies are of particular interest to clinicians for the diagnosis of an infection by a microorganism even when the infectious agent is no longer detectable. In individual cases there is

little evidence of the function of a detected antibody, i.e. whether it is pathogenic, protective or of no significance to the patient. In such cases a clinical conclusion can be made only by an analysis of all retrievable details, including the age of the patient and the clinical, biochemical, morphological and immunological findings. The following aspects should be assessed when detecting antibodies:

- type of antibody (precise differentiation of various types, immunoglobulin class, possibly antibody subclass)

- antibody titre

- reactions with autogenous material or that already deposited in vivo

- time course of detectablility of the antibody—constantly or transiently detected, possible dependence on the course of the disease or on therapy

- complement binding

- simultaneous occurrence of other antibodies, possibly in a stable combination.

A typical method for detection of antibodies against an antigen of an infectious microorganism is the fluorescent *Treponema pallidum* antibody absorption test (FTA-ABS). The procedure is described in the following section.

7.9.1.2 Fluorescent treponemal antibody absorption (FTA-ABS test)

Purpose

Specific detection and identification antibody to *Treponema pallidum* to confirm a diagnosis of syphilis.

Approach

Indirect fluorescent antibody is used to detect specific anti–*T. pallidum* antibody in human serum that has been absorbed to remove cross-reacting non-specific antibodies (Figure 7.7).

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

56 °C water bath.

Reagents

FTA supplies.

FTA antigen (standardized killed suspension of *T. pallidum*, Nichols strain).

Reactive serum.

Sorbent antigen (extract of nonpathogenic Reiter treponeme).

Tween 80, 2% (2 mL Tween 80 in 98 mL phosphate buffered saline, pH 7.4).

FITC-conjugated anti-human globulin (rabbit origin).

Rhodamine-labelled anti–*T. pallidum* antibody.

Phosphate buffered saline, pH 7.4.

Cover glass mounting medium, pH 8.5–9.0.

Test specimen preparation

1. Scribe two circles, 10 mm diameter, on each clean microscope slide. Commercial pre-etched circle slides also may be used.

2. Smear one loopful (2 mm loop) of FTA antigen in each circle.

3. Allow the slide to dry completely for 15 minutes or more at room temperature.

4. Fix the slide smears in acetone, 10 minutes.

Note: slides may be made in advance, fixed in acetone, and stored frozen at –20 °C. Two slides are required as controls for each test run (group of test slides). One slide is required for each patient serum.

Serum preparation

1. Heat the patient and control sera at 56 °C for 30 minutes on the testing day. Previously heated sera should be heated at 56 °C for 10 minutes only.

Controls

4+ control serum diluted 1:5 in phosphate buffered saline, pH 7.4.

Figure 7.7 **Fluorescent antibody fluorescence of *Treponema pallidum* (Nichols strain) with adsorbed antibodies**

1+ control serum diluted and previously tested to give this reaction.

Non-specific serum control.

Sorbent control serum diluted 1:5 in phosphate buffered saline, pH 7.4.

Sorbent control serum diluted 1:5 in sorbent.

Non-specific antigen controls.

Antigen smear reacted with phosphate buffered saline, pH 7.4.

Antigen smear reacted with sorbent.

Method

1. Use freshly prepared slides or fixed slides from the freezer. The latter must be thawed and completely dry.

2. Prepare 1:5 dilutions of the test and 4+ control sera in sorbent. Dilute the 1+ control serum appropriately.

3. Cover each antigen smear in a circle with the appropriate serum dilution.

4. Incubate the slides in a moist chamber at 35–37 °C for 30 minutes.

5. Rinse the slides in running phosphate buffered saline for 5 seconds, and then soak them in phosphate buffered saline for 5 minutes. Agitate them occasionally in the phosphate buffered saline.

6. Change the slides to a new bath of phosphate buffered saline, and agitate them for 5 minutes.

7. Rinse the slides with H_2O (distilled) for 5 seconds, and blot them dry with bibulous paper.

8. Dilute the anti-human globulin FITC conjugate to its working dilution in 2% buffered Tween 80.

9. Cover each circled smear with conjugate.

10. Repeat steps 4, 5, 6 and 7.

11. Mount coverglasses on the smears with mounting fluid.

12. Examine the slides with the fluorescence microscope as soon as possible, certainly within 4 hours.

13. Record the degree of fluorescence using the 1+ control slide as a reference.

14. Report as follows:

2+ to 4+	Reactive
1+	Reactive (?)
Less than 1+	Borderline
Negative	Non-reactive

15. Retest any serum giving 1+ or less fluorescence.

References

Larsen SA, Hunter EF, Kraus SJ, 1990; Larsen SA, Steiner BM, 1995.

7.9.2 Autoantibodies and autoimmune disease

Autoantibodies are indicators of an autoimmune reaction. They may also be the cause of an autoimmune disease. Autoantibodies, for example against mitochondria in primary biliary cirrhosis, may be present before a diagnosis can be made by other means. Therefore, the presence of autoantibodies should also be considered when investigating the preclinical stage of a disease in a patient. During the early stage of a disease a test may be negative because the antibodies are not circulating in a patient's plasma but are stored in the primed organ.

Problems associated with the diagnostic value of autoantibodies include the following:

- different classifications published for similar or identical disease scenarios

- insufficient standardization, which especially concerns "new" methods, such as the immunoblotting procedure

- difficulties in nomenclature, for example, for ribosomes and mitochondria that also contain DNA

- difference in the affinity and specificity of the antibodies.

- method dependent factors for example with immunofluorescence using an antigen substrate (species, sex, tissue treatment, etc.).

7.9.2.1 Basic test procedures and materials preparation for autoimmune and autoantibody testing

In the indirect fluorescent antibody (IFA) test, serum of a patient with suspected disease is incubated with tissue that contains a diagnostic marker antigen. Antibodies present in the patient's serum bind to corresponding antigenic structures on the tissue substrate. The reacted antibodies are detected with a fluorochrome-labelled anti-human globulin, normally IgG, although for certain antibodies (e.g. anti–connective tissue antibodies) an anti-human IgA conjugate must also be used. The reaction is easily visible under the fluorescence microscope. The following chapter describes examples of indirect immunofluorescence microscopy used for detecting circulating autoantibodies that can be found in human serum of patients with certain diseases.

Basic materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Fluorescence microscope.

Cryotome.

Dissection kit.

Glue for cryotome sections.

Tweezers.

Cotton.

Stain rack.

Vortex mixer.

Rotating shaker.

Balance.

Absorbent paper.

Tray, washing.

Beaker, 100 mL.

Moist chamber.

Tube rack.

Tubes for dilution of serum specimens.

Graduated cylinders (5 mL, 100 mL, 1000 mL).

Micropipettes (5–50 µL, 50–200 µL, 200–1000 µL).

Waste container.

Microscope slides.

Reagents

Ethanol, 90%.

Ether.

Distilled water.

Physiological saline.

Evans blue solution.

Control sera.

Glycerol mounting medium, pH 7.6.

Method

1. Place the slides with the tissue sections or cultured cell substrates in a moist chamber.

2. Place patient serum on the substrate.

3. Incubate the slides for 30 minutes at room temperature.

4. Remove the serum and rinse the slides quickly with a gentle stream of phosphate buffered saline.

5. Wash three times, each time with fresh phosphate buffered saline for 10 minutes in a coplin jar.

6. Add one drop of anti-human IgG (or IgA) fluorescent conjugate.

7. Wash the slides three times, each time with fresh phosphate buffered saline, 10 minutes each wash.

8. Add Evans blue counterstain (5–6 drops of Evans blue in 150 mL of phosphate buffered saline).

9. Blot the slide with absorbent paper.

10. Put glycerol mounting medium on each slide and cover it with a cover glass.

11. Examine the slide under the fluorescence microscope.

12. Examine the controls first to be sure that the method and the antibodies work properly. Use a low magnification first to see that all parts of the tissue specimen are present on the slide.

Controls

Positive control sera (e.g. 4+ and 1+ positive sera from an experimentally determined dilution).

Negative control (normal serum to detect non-specific serum reactions).

"Conjugate" control (incubation only with the conjugate to detect an unwanted fluorescence of the conjugate).

"Buffer" control (incubation only with phosphate buffered saline, pH 7.4, to detect other non-specific fluorescence).

Semi-quantitative determination of autoantibodies

1. Dilute the patient's serum with phosphate buffered saline from 1:20 to 1:2560, or more if necessary.

2. Incubate each serum dilution with tissue substrate as described above.

Preparation of organ tissue substrates: tissue substrate

   Method

   1. Anaesthetize a male Wistar rat, 150 g (starved for 18 hours) with ether and decapitate the animal to bleed it.

   2. Remove the organs of interest (e.g. kidneys, liver and stomach). Clean the stomach of its contents.

   3. Wash the organs several times with physiological saline.

   4. Cut the liver into small cubes about 2 mm on a side.

   5. Cut the stomach into longitudinal strips.

   6. Cut the kidneys longitudinally. Be sure that you have both the cortex and medulla.

   7. Roll each piece of kidney with a piece of liver in a strip of stomach.

   8. Add a sufficient amount of embedding medium* to connect the organ slices to prepare an air-tight, densely packed composite (triple substrate) that can be easily cut by the cryotome.

      * Optimum controlled temperature compound.

9. Wrap each triple substrate in aluminium foil.

10. For storage, freeze the tissue immediately in liquid nitrogen; or use isopentane cooled by a dry ice/acetone bath.

11. Store the triple substrate at –70 °C or in liquid nitrogen.

Cutting triple organ sections for test substrates

1. Degrease and wash slides with 90% ethanol.

2. Degrease the cryotome knife and blade.

3. Adjust the cryotome temperature to about –20 °C.

4. Fix the tissue preparation with glue on the object carrier of the cry-microtome.

5. Adjust the cutter of the cryotome to get a transverse section of 4–5 mm thickness.

6. Collect the sections carefully from the knife blade, and place them on a microscope slide.

7. Dry the slides in the air, use them immediately for immunofluorescence microscopic examination, or wrap them in aluminium foil and store them frozen at –70 °C for up to one month in an air-tight container with desiccant.

Preparation of organ tissue substrates: granulocyte substrate

Materials

Pasteur pipettes (very narrow tip) (if necessary, pull the tip fresh through a flame thereby softening the glass to make the hole of the tip smaller).

Rubber pipette bulb.

Syringe, 10 mL.

Flask, Erlenmeyer, 25–50 mL.

Glass beads, 10.

Test tubes, polystyrene, 10 mL.

Pipette, 5 mL.

Safety pipette.

Centrifuge.

Slides.

Refrigerator.

Freezer, –20 °C.

Reagents

Dextran–metrizoate solution (for preparation see Annex 13).

Phosphate buffered saline, pH 7.4 (for preparation see Annex 13).

Phosphate buffered saline–albumin, 1% (for preparation see annex 13).

Ethanol, absolute.

Phosphate buffered saline–glycerol mounting medium (for preparation see Annex 13).

Anti-fading agent (*p*-phenylenediamine, 1 mg/mL, or DABCO, 25 mg/mL).

Heparin.

Formalin, 10%.

Method

1. Collect 10 mL of blood from a healthy donor.

2. Place the blood specimen in a small flask with 10 glass beads, and shake it well (no anticoagulant is used).

3. Put the defibrinated blood in a 10 mL polystyrene test tube.

4. Add 250 IU of heparin.

5. Distribute 5 mL of dextran–metrizoate solution into each of 5 polystyrene test tubes, and add 2 mL of defibrinated blood to each test tube.

6. Mix each tube, and allow them to stand at room temperature, 45 minutes.

7. Pipette the cloudy plasma containing the leukocytes from each test tube into another 10 mL polystyrene test tube (avoid taking up red cells).

8. Centrifuge the test tube at about 200*g*, 10 minutes.

9. Decant the clear plasma, and resuspend the leukocyte pellet in 10 mL phosphate buffered saline-albumin.

10. Repeat steps 8 and 9.

11. Decant the supernatant fluid until only about 150–200 μL remains above the pellet.

12. Resuspend the leukocytes in the remaining fluid.

13. Place droplets of the suspension on a clean slide, each droplet about 1 mm diameter. (Use a Pasteur pipette with a fine tip.)

14. Make smears from the droplets within 10 seconds.

15. Air-dry the slides.

16. Fix a slide in absolute ethanol at 4 °C, 5 minutes.

17. Fix a slide in 10% formalin, 5 minutes.

7.9.2.2 Anti-nuclear antibodies on human larynx epithelioma cells (HEp-2 cells)

Disease relationship

Anti-nuclear antibodies are a heterogenous group of antibodies against different components of cell nuclei. They are detectable in serum of healthy people at low concentrations, and in patients with autoimmune in higher concentrations.; in particular they occur in patients suffering from systemic lupus erythematosus, rheumatoid arthritis, Sjögren syndrome, scleroderma, dermatopolymyositis, Raynaud syndrome, and autoimmune hepatitis type 1. The titre of ANAs and the distinctive fluorescent pattern are to some extent related to the disease.

The most common method today to detect ANAs is indirect immunofluorescence microscopy on HEp-2 cells. However it can also be done on rat triple organ substrate, and this was the routine procedure in the past.

Control sera for ANA

ANA negative.

ANA positive homogenous.

ANA positive speckled or granular.

ANA positive nucleolar.

ANA positive centromere (kinetochore).

Method

1. Use the general immunofluorescent staining method on slides with HEp-2 cells as an antigenic substrate.

Fluorescence patterns

Different fluorescence patterns can be seen (Figures 7.8 and 7.9). A nucleolar and/or speckled (granular) pattern is predominantly found in patients with scleroderma and Sjögren syndrome A homogenous pattern is predominantly observed in patients with active lupus erythematosus. The centromere (kinetochore) pattern is predominantly seen in patients with Raynaud syndrome and primary biliary cirrhosis.

7.9.2.3 Anti-DNA antibodies using **Crithidia luciliae** *as antigenic substrate*

Disease relationship

The anti-DNA antibodies are detected by indirect immunofluorescence microscopy on *Crithidia luciliae* as antigenic substrates. *Crithidia luciliae* is a trypanosome haemoflagellate that contains DNA in a large mitochondrion, the kinetoplast (Figure 7.10). Anti-DNA antibodies are predominantly found in systemic lupus erythematosus.

Control sera for DNA-antibodies

DNA-negative.

DNA-positive.
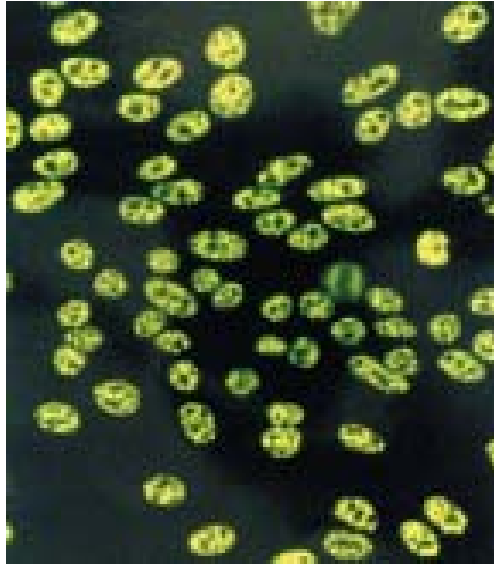
Figure 7.8 **Speckled fluorescence pattern of HEp-2 cells**
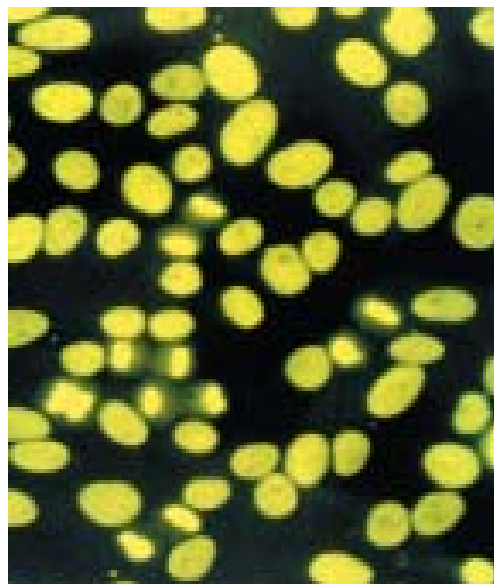
Figure 7.9 **Homogenous fluorescence pattern of HEp-2 cells**

Figure 7.10 **Kinetoplasts of *Crithidia luciliae* stained with fluorescent anti-DNA antibodies**

Method

1. Apply the general method using slides of *Crithidia luciliae* as an antigenic substrate.

Fluorescence pattern

The typical pattern is a homogenous staining of the kinetoplast.

7.9.2.4 Anti-mitochondrial antibodies (AMA)

Disease relationship

At least 10 classes of AMA have been identified, and they are classified from 1 to 10. Of these antibodies type 2 (AMA-2) is clinically important. A high AMA-2 titre is indicative for primary biliary cirrhosis and non-suppurative destructive cholangitis, a precursor stage of primary biliary cirrhosis.

Control sera for AMA

AMA negative.

AMA-2 positive.

AER/LKM-1 positive.

Method

1.   Apply the general method using slides with prepared triple substrate.

Fluorescence pattern

Typical patterns of AMA-2:

*   strong cytoplasmic staining of renal distal tubules (+++).

*   granular cytoplasmic staining of hepatocytes (++).

*   weak staining of the first and second part of renal proximal tubules (++).

*   weak staining of renal glomerula and the third portion of proximal tubules (+).

*   brilliant staining of parietal cells of the stomach (+++). The main cells of the stomach are weakly stained (+ to ++).

7.9.2.5 Anti-endoplasmatic reticulum antibodies (AER) and liver/kidney microsome antibodies (LKM-1)

Disease relationship

AER-or LKM-1 antibodies react with membranes of the rough and smooth endoplasmic reticulum. Strong reactions occur on liver and kidney cells (Figure 7.11). The predominant antigen is cytochrome P 450. More than six different antibodies have been described. At present only type 1 of these antibodies is diagnostically important. AER or LKM-1 are strongly indicative of type 2 autoimmune hepatitis (see also ANA for autoimmune hepatitis).

Control sera for AER (LKM-1)

AER/LKM-1 negative.
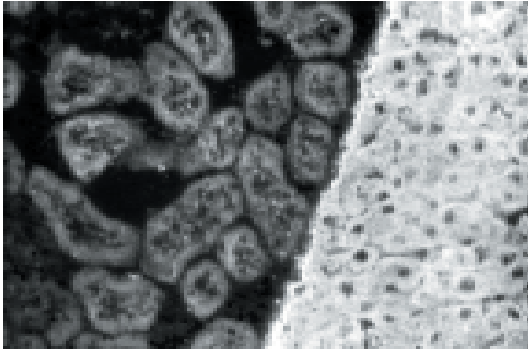
Figure 7.11 **Anti-microsomal antibody (LKM) immunofluorescence in rat kidney (left; weak staining) and liver (right; strong staining)**

AER/LKM-1 positive.

AMA-2 positive.

Method

1.   Use the general method as described using slides with triple substrate.

Fluorescence pattern

   Typical patterns:

•   homogenous brilliant cytoplasmic staining of hepatocytes (+++).

•   weaker homogenous cytoplasmic staining of the third portion of proximal tubules of the kidney (++).

7.9.2.6 Anti-smooth-muscle antibodies (ASMA)

Disease relationship

   ASMA reacts with proteins of the cytoskeleton (Figures 7.12 and 7.13). More than 200 antibodies are known, but only high-titre antibodies reacting with actin are used for the differential diagnosis of autoimmune hepatitis type 1. High titres of SMA-T and SMA-G are characteristic of autoimmune hepatitis type 1 (very often in combination with ANA).

Control sera for ASMA

ASMA negative.

ASMA positive.

Fluorescence patterns

Three different patterns can be distinguished on kidney sections:

- SMA-V: fluorescence of vessel walls only.

- SMA-G: fluorescence of vessel walls and renal glomeruli.
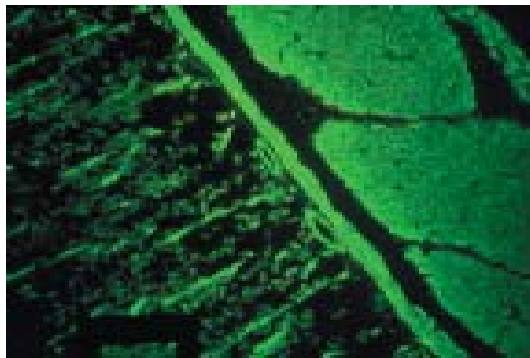
Figure 7.12 **ASMA (actin) type immunofluorescence on stomach (left) and liver (right) tissue. Magnification ×250**
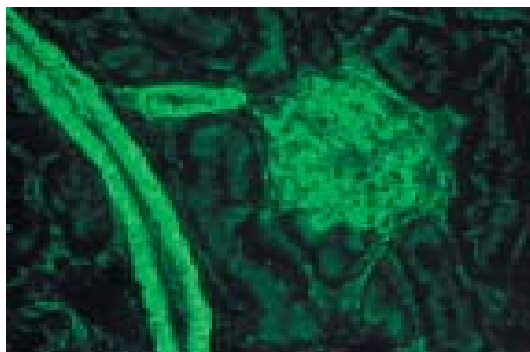
Figure 7.13 **ASMA (actin) type immunofluorescence on kidney artery, glomerulus and tubuli. Magnification ×500**

- SM-T: fluorescence of vessel walls, renal glomeruli and the brush border and peritubular fibrils.

7.9.2.7 Anti-neutrophil cytoplasmatic antibodies (ANCA)

Disease relationship

ANCA binds to cytoplasmic constituents of human polynuclear leukocytes (neutrophils, basophils and eosinophils) and monocular blood cells except lymphocytes (Figures 7.14 and 7.15). Two fluorescence patterns are related to disease:

- c-ANCA (cytoplasmic or classical ANCA) for Wegener disease.

- p-ANCA (perinuclear ANCA) for renal vasculitis.

All other fluorescence patterns are summarized as atypical (a-ANCA).

The main antigens for c-ANCA are proteinase 3, and for p-ANCA myeloperoxidase of neutrophils.

Control sera for ANCA

ANCA negative.

c-ANCA positive.

p-ANCA positive.

a-ANCA positive.

Method

1. Do the general method using slides of granulocytes fixed with ethanol as the substrate. Repeat the general method with slides fixed in 10% formaldehyde to compare the fluorescence patterns.

Fluorescence patterns

At least three patterns are seen:

- c-ANCA: fine granular diffuse cytoplasmatic staining very often brighter centrally.
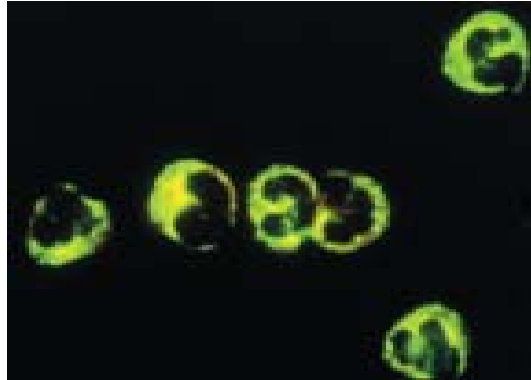
Figure 7.14 **c-ANCA fluorescence pattern of human neutrophils**

Figure 7.15 **p-ANCA fluorescence pattern of human neutrophils**

- p-ANCA: perinuclear accentuated cytoplasmatic staining without staining of the nucleus and the cell periphery.

- a-ANCA: atypical fluorescence: all other fluorescence patterns.

Note: nuclear patterns may also be seen.

7.9.2.8 Anti-endomysium (connective tissue type 3) antibodies (ACTA-3)

Disease relationship

Anti-endomysium (connective tissue type 3) antibodies are directed against tissue transglutaminase. The antibodies may be IgA and IgG. The antibodies, particularly IgA, are found in coeliac disease (gluten intolerance). The disease occurs in an acute form (particularly in children) and in a silent form (mainly in adults). The antibodies were also named anti-reticulin antibodies in the early literature. The antibodies are also used to monitor therapy.

Control sera for ACTA-3

IgG ACTA-3 negative.

IgA ACTA-3 negative.

IgG ACTA-3 positive.

IgA ACTA-3 positive.

Method

1.   Apply the general IFA method using slides of monkey oesophagus (lower part); alternatively, slides with rat triple substrate are also suitable.

Note: it is important to investigate the tissue under the microscope after exposure both to anti-human IgA and IgG conjugates.

Fluorescence patterns

Typical patterns:

•   Monkey oesophagus at ×250 magnification: "honeycomb" pattern (endomysial pattern) of the muscularis mucosae (Figure 7.16).

•   Rat triple substrate: at ×1000 magnification: "honeycomb" pattern on the stomach muscularis:
    –   fluorescence of all vessel endothelia of liver, kidney and stomach
    –   fluorescence of the Bowman capsule of the kidney
    –   fluorescence of the perivascular and renal peritubular tissue.
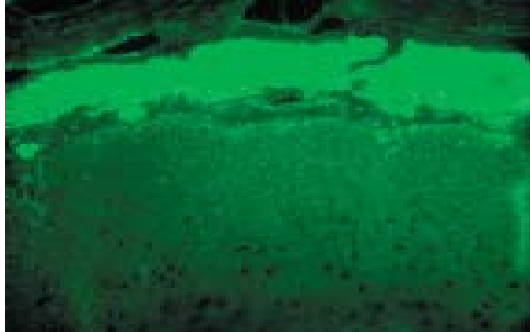
Figure 7.16 **ACTA fluorescence pattern on monkey oesophagus, magnification ×250**

8. Fluorescence microscopy for environmental monitoring

8.1 Examination for microorganisms in the environment

Microorganisms in the environment are traditionally counted by culturing. Two methods commonly used are counting colonies on agar plates and the statistical most probable number (MPN) method, done by dilutions of sample in culture broth. These methods have several limitations:

- many microorganisms in the environment are stressed and are viable but not culturable

- for certain microorganisms no suitable culture medium is available.

An alternative to culture methods for detecting microorganisms in the environment is fluorescence microscopy. It does not suffer from the above limitations.

Epi-fluorescence microscopy is an excellent way to detect and identify contaminating particles, including bacteria and mould spores, in the environment. It is simple, rapid and inexpensive. It is direct, giving a result of total organisms. Depending on the fluorochrome or fluorescent antibody conjugate used, fluorescence microscopy can be used to do general detection as well as very specific identification of organisms collected.

A major advantage of using fluorescence microscopy to detect microorganisms in the environment is that results are quickly obtained. Culture methods need 24–48 hours to yield results. Fluorescence microscopy provides more accurate microbial counts in less than two hours; in some tests only 30 minutes.

No standard widely accepted methods exist for doing these tests. Approaches vary depending on the microorganism, where it is located, and the fluorochrome used. Experimentation is essential in order to choose the right stain and specimen processing methods to get useful results. Assistance with developing protocols can be found in the references provided.

Primarily, epi-fluorescence microscopy is used to detect microorganisms such as bacteria and fungal spores in water, soil or air samples. In water samples, epi-fluorescence microscopy also detects and identifies protozoan parasites. Furthermore, fluorescence microscopy can detect inanimate particles such as various chemical dusts.

Three factors are involved in direct counting of microorganisms in environment samples:

- particle-free reagents

- method for cell concentration

- epi-illumination fluorescence microscope.

In short, the method calls for collecting a sample, stabilizing the microorganism population, presenting the particles on a flat surface, staining the microorganisms and examining that surface for trapped fluorescing particles by epi-fluorescence microscopy. The surface used is a membrane filter, usually made of polycarbonate. Earlier, cellulose acetate filters were used but were not satisfactory because many microorganisms entered the filter and could not be seen. Membrane filters made of polycarbonate are preferred by most investigators because they have a very smooth surface, are strong and resist many chemicals. This allows a range of fixation, staining and processing methods. Also, they come in black or white for different applications. Black filters are preferred for epi-fluorescence microscopy because they have low or no background fluorescence.

A newer type filter coming into use is the Anopore (0.2 μm pores), an inorganic filter made of aluminium oxide. It has a very flat surface and a high flow rate. It does not fluoresce. This filter is reported to yield 21% to 33% higher cell counts than polycarbonate filters, and a lower vacuum can be used [Jones, 1989].

Particle-free reagents are unique to this kind of testing. It is not enough to have sterile reagents in which all microorganisms are dead because fluorescence microscopy methods detect both living and dead microorganisms. Also, non-microbial particles and other debris in a sample can be stained. Thus, particle-free stains, buffers and wash water are needed. This can be done easily by passing liquid reagents and stains through membrane filters, pore size 0.2–0.22 µm. Filtered reagents should be stored in sterile screwcap bottles that have been rinsed with sterile, filtered water. Store reagents at 4 °C.

Another important factor is concentration of microbial particles in a sample. This is measured by membrane filtration. By passing the sample through a membrane filter, particles are trapped on the filter's surface. A few microorganisms in large sample volumes can be found in this way.

To examine water, or other liquids, the sample is filtered through a membrane held in a holder. A vacuum draws the liquid through the membrane filter. Following filtration of the sample, the filter and its holder are usually rinsed with sterile filtered water or buffer, and this also is drawn through the filter. The filter may be stained in place on the holder, or it may be removed for staining with fluorochrome. Sometimes the test sample is stained with fluorochrome before filtration.

Air and other gases also can be sampled by a direct sampling method, much the same as liquids, by drawing the sample through the filter in a holder. The filter then is removed from its holder. Particles trapped on the surface of the filter are stained with fluorochrome and examined by epi-illumination fluorescence microscopy.

In another air sampling method, particles in the air sample are first captured in a liquid. Usually, a device called a liquid impinger is used. For certain applications, this is the preferred method because other tests on contents collected in the liquid sampling medium may be needed. After the sampling is completed, the liquid containing the trapped particles is drawn through a membrane filter and treated in the same way as a water sample for epi-illumination fluorescence microscopy.

Soil or sediment samples are dispersed in a suspending liquid by a variety of mechanical methods. The large particles are allowed to settle. Then, the supernatant fluid is decanted, stabilized with formalin or other fixative, and then filtered, stained and examined much as in a water test.

Fluorescent stains used in environment monitoring fall into three categories:

- general fluorochromes

- immunologically specific stains

- physiological fluorochrome stains.

General fluorochromes act on structural parts of microorganisms. Most of these stain nucleic acids. Many are reported in the literature, but by far the most commonly used general fluorochrome is acridine orange. Methods using acridine orange are in both *Standard methods for the examination of water and wastewater* [Clesceri et al., 1989] and *Standard methods for the examination of dairy products* [Wehr and Frank, 2004]. An appealing feature of acridine orange is that it is excited by essentially the same wavelengths of light as FITC and auramine O. It can be used with the same light filter sets and an inexpensive halogen-type lamp. Most of the other general fluorochromes need UV excitation. Other features are that acridine orange is inexpensive and a very efficient fluorochrome.

Acridine orange fluoresces green when reacted with double-stranded DNA, and reddish orange when reacted with RNA and single-stranded DNA. Variations in the staining pH or destaining procedures may also result in the green or red staining of the microorganisms regardless whether they are live or dead. Therefore, under strictly controlled conditions of examination acridine orange provides potentially useful differentiation, although it is generally recommended in environment studies only for total counts of microorganisms.

In recent years, 4,6-diamidino-2-phenylindole (DAPI) is increasingly used to stain microorganisms in environmental samples. DAPI is also a DNA stain. When bound to DNA, it fluoresces blue or bluish white when excited by near-UV light at 365 nm. It fluoresces yellow when unbound or bound to non-DNA materials. Thus, a UV excitation light source is needed to use DAPI, and a halogen lamp such as can be used with acridine orange will not do. Both acridine orange and DAPI staining allow differentiation of bacteria on the basis of shape and size. This is important when analysing a sample for microorganisms because other things in the sample may fluoresce as well.

Immunologically specific stains are fluorescent antibodies that allow the investigator to determine the identity of a microorganism. Fluorescent antibody

testing in environmental studies is used to detect specific pathogens. The ones most commonly studied include *Salmonella* spp., *E. coli* O157:H7, *Legionella* spp., giardia and cryptosporidium. These bacteria and protozoa are found in water and air samples from various environmental niches.

Physiological fluorochrome stains are fluorochromes that stain microorganisms differentially depending on whether they are alive or dead. The most common are:

- propidium iodide

- fluorescein diacetate

- 5-cyano-2,3-ditolyl chloride (CTC).

Propidium iodide does not enter living cells that have intact membranes. Therefore, fluorescence of a living microorganism treated with propidium iodide is not seen with a microscope. A general fluorochrome is used as a counterstain to prove the presence of living cells.

Fluorescein diacetate enters the cell, where it is split by enzymes, and a fluorescent compound, free fluorescein, is formed. However, certain bacteria cannot bring the dye into their cells, so this chemical is not suitable for differentiating living from dead for many microorganisms.

5-cyano-2,3-ditolyl chloride (CTC) is colourless in its oxidized form. When CTC enters a living microorganism, the compound is reduced and converted to CTC-formazan, which is deposited inside the cell wall as a fluorescent red inclusion.

Many issues are involved in fluorescent microscopy analysis of environmental samples, especially for water and soil. All of these affect counts of microorganisms in environmental samples. These factors must be carefully controlled if results are to be meaningful and realistic conclusions are to be made. The major factors in epi-fluorescence environmental testing are:

- fluorochrome choice
  - acridine orange
  - DAPI
  - propidium iodide
  - fluorescein diacetate
- sample type

- air
- soil
- sediment (different marine and freshwater locations)
- water (fresh, marine, brackish, sewage)

- preservation of specimens
  - formalin
  - gluteraldehyde

- dispersion of microorganisms from specimen
  - blending
  - stomaching
  - surfactants
  - ultrasound

- filter type
  - polycarbonate
  - aluminium oxide (Anopore)

- counting methods
  - magnification
  - fields
  - grids
  - chambers

- concentration of stain
  - sample dependant

- time of staining
  - acridine orange (shorter)
  - DAPI (longer).

Other factors may play a role, but these are the major ones. All these factors must be carefully controlled, recorded, and reported to ensure that results are meaningful and useful (see Kepner and Pratt, 1994; McFeters et al. 1995).

8.2 Testing water for microorganisms

8.2.1 Collecting environmental water samples for testing

Most of the principles of water collection for bacteriological testing also apply to water for examination by epi-fluorescence microscopy. However, there are a few significant differences. Since culture is usually not involved, protection against the toxic effects of chlorine or heavy metals is usually not a major concern. Thus, dechlorinating or chelating agents are usually not needed. If special situations dictate otherwise, consult Section 9060 of *Standard methods for the examination of water and wastewater*.

Sampling methods

Use a non-reactive glass or plastic bottle. It may be a screwcap bottle or stoppered with a rubber or Neoprene stopper. If closed with a stopper, the stopper and top of the container should be covered with a hood of paper secured with string. For general microorganism testing, the container must be particle-free. Clean and rinse the container thoroughly. The final rinse should be with distilled or deionized water that has been filtered through a membrane filter of 0.2–0.22 µm pore size. After the final rinse it may be sterilized or simply protected from outside contamination. If the test is for specific microorganisms, as for fluorescent antibody testing, a final rinse of the sample container with filtered water may not be needed.

Use a sampling container large enough to allow air space on top of the sample for mixing (at least 2.5 cm). Keep the sampling container covered until actual use. Then take off the cap and its hood (if used) together. Fill the container without rinsing. Immediately replace the cap or stopper. Secure the cover, if used, over the cap or stopper with string.

Sample amount

The cleaner the water sample is expected to be, as from clear streams or lakes, the larger the sample that must be collected and tested, typically 100 mL to 200 mL. If sewage is to be tested, the amount needed to test is usually much smaller, 1 to 2 mL. However, a reasonably sized sample still should be collected, 25 to 50 mL, to be representative. The actual amount to be tested will be decided in the laboratory.

Sometimes water that appears to be clear contains algae that clog the test filter. In this case the laboratory must test smaller samples, but this decision is the laboratory's, not the sampler's. A more than adequate sample must always be taken.

Sample location

Take samples that are truly representative of the water to be tested. Do not take water samples close to shore or from the bottom (unless studying sediments). Avoid sampling water near drainage or sewage outfalls. (However, sewage samples can be collected at sewage outfalls.) If sampling source water for potable water systems, take the sample at the depth of the inlet pipe. Sometimes, when sampling at depth, it is helpful to attach a weight to the bottle to lower it by a string into the water. Attach a second string to the stopper to pull it out at the desired depth. Remember that pressure increases with depth, and if too deep, this can make the stopper hard to pull out. If sampling much below 10 metres, be sure that the stopper is seated lightly in the mouth of the sample container so that it can be pulled out with the string.

Sampling method

When sampling water from a river, stream or lake, hold the container near its base, remove the hood and container top, and plunge the sample container, opening downward, below the water surface. Then turn the container upward in a direction so that the opening faces the current. If sampling in a lake, move the container forward to create an artificial current. If in a boat, take the sample on the upstream side.

When sampling sewage or water suspected to contain pathogenic microorganisms, for example tropical streams that may be contaminated with leptospira from animal urine, wear protective clothing. Avoid direct contact with the sewage or potentially contaminated water by wearing rubber or plastic gloves.

Sample identification

Label each sample immediately after collection with the date, location, depth and other data that properly and completely describe and identify the sample. A pencil is usually best for fieldwork.

Transport of samples

Take the sample to the testing place as soon as possible, preferably within four hours. If possible, refrigerate the water sample. If the protocol calls for chemical preservation of the sample, for example with formalin, add the proper amount immediately after collection. This stabilizes the microorganism population in tests for total microorganism counts. Water samples properly fixed in this way will retain numbers of microorganisms within statistical limits for about three weeks when stored at 4 °C.

Special sampling methods

When testing for pathogenic microorganisms that are rare in water samples, but still medically significant, very large volumes of water must be collected. This is the case when testing water sources for potable water distribution in cities. The organisms usually being sought in these cases are cryptosporidium and giardia because these are resistant to standard water purification methods. In this case, the US Environmental Protection Agency protocol calls for collecting a 10 litre water sample in a plastic carboy [http://www.epa.gov/waterscience/methods/1623.pdf]. Few guidelines are provided for collecting 10 litres of water in one large container. In these instances the collector must improvise, but the principles above should be followed as much as possible. The laboratory procedures that are done on samples of this type are quite specific, and basic collection methods are adequate. There does not need to be great attention to removal of particles or sterilization. A good cleaning and rinse with distilled water should be adequate for these large containers. However, these samples must be delivered to the laboratory without delay.

8.2.2 Detecting and identifying *Legionella* spp. in water

Purpose

Specific detection and identification of bacteria in aquatic environments.

Approach

Microorganisms in water are trapped on a membrane filter. They are washed off the filter, a sample of the wash suspension is fixed on a slide, and the slide is examined by epi-fluorescence microscopy (Figure 8.1).
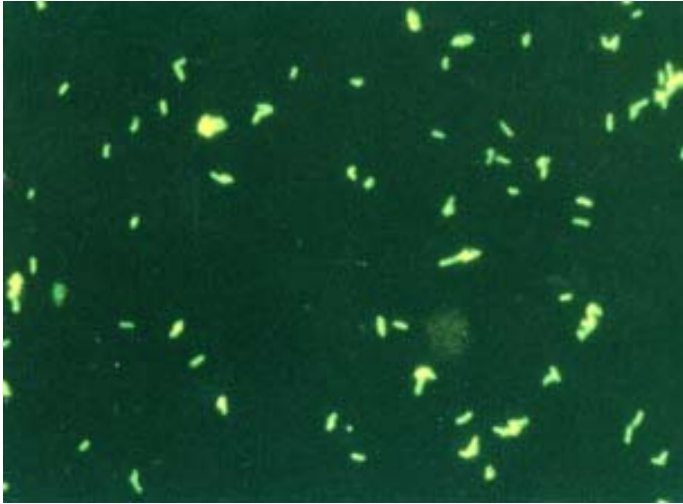
Figure 8.1 **Legionella pneumophila in water, magnification ×800**

Materials

Basic fluorescence microscopy equipment.

Basic fluorescence microscopy supplies.

Vortex mixer.

Membrane filter, 0.45 µm pore size (polycarbonate, polysulfone, polyvinylidene difluoride).
Note: any smooth-surfaced filter is satisfactory.

Filter holding apparatus.

Vacuum source (electrical pump, hand pump).

Sample collection containers (sterile, particle-free).

Test tubes, screwcap, 13×100 mm.

Microscope slides (glass slides with 6–8 wells are convenient).

Pipettes, 20 µL.

Bunsen burner.

Fluorescent antibody conjugates (*Legionella* polyclonal, *Legionella* various species–specific).

Incubator.

Humidity chamber (Petri dish with moist filter paper in the bottom and applicator stick slide supports).

Reagents

H_2O, distilled or deionized.

Formalin.

Phosphate buffered saline, pH 7.4–7.6.

Method

1. Collect the water sample in a clean, sterile container previously rinsed with filtered (0.2 µm pore size) distilled water.

2. Set up the filter apparatus with a smooth surface membrane filter in place.

3. Filter the sample by applying vacuum. Sample size depends on the expected level of contamination, 10 mL of sewage or 100 mL of ocean or freshwater. This volume may be adjusted based on experimental experience.

4. Place the filter in a tube of distilled water, 2 mL.

5. Vortex the tube for 30 seconds to dislodge the cells from the filter surface.

6. Remove the filter from the tube.

7. Add formalin to the suspension in the tube, final concentration 2% formalin.

8. Place 20 µL of the suspension in a small spot, 5–8 mm diameter, on a slide (a commercial multi-well slide is convenient to use).

9. Air-dry and heat-fix the slide.

10. Place 20 µL of anti-*Legionella* conjugate on the fixed spot.

11. Incubate the slide for 30 minutes, 37 °C, in a humidity chamber.

12. Rinse the slide with phosphate buffered saline, followed by a final rinse in distilled water.

13. Air-dry the slide.

14. Examine the slide and count fluorescing cells with an epi-illumination fluorescence microscope.

Other bacteria such as *Salmonella* spp., *E. coli* O111:H7, etc., may be tested for in the same manner by simply changing the specific fluorescent antibody conjugate.

References

Palmer CJ et al., 1993; Palmer CJ et al., 1995.

8.2.3 Fluorescent antibody test to detect enteric bacteria in environmental water

Purpose

Rapid and specific detection of selected enteric bacteria (for example, *Salmonella* spp.) in water from environmental sources.

Approach

Water samples are filtered through polycarbonate filters; a defined area on the filter is stained by indirect fluorescent antibody using a specific antibody; and the filter is examined by epi-fluorescence microscopy.

Materials

Basic fluorescence microscopy equipment.

Basic fluorescence microscopy supplies.

Membrane filtration system (filter holder, vacuum container, vacuum source).

Refrigerator.

Humidity chamber

Incubator.

Water sample collection supplies (screwcap container, sterile).

Membrane filter, polycarbonate, 0.2 µm pore size, black.

Reagents

Distilled water.

Phosphate buffered saline, pH 7.4.

Immersion oil, non-fluorescent.

Antiserum, polyclonal absorbed or monoclonal (for example, anti-*Salmonella*).

Anti-species globulin, FITC conjugate (example: goat anti-rabbit globulin if the anti-*Salmonella* serum is made in rabbits).

Method

Fluorescent antibody test

1. Collect the water sample (sterile screw-cap containers are satisfactory for fluorescent antibody testing).

2. Filter a measured volume of the water sample through a polycarbonate filter, 0.2–0.22 µm pore size.

    Note: choose the appropriate sample size depending on the expected sample contamination, i.e. small sample for sewage, large sample up to 100 mL for expected clean water.

3. Mark an 8 mm diameter circle on the membrane filter.

4. Spot a 20 µL drop of anti-*Salmonella* serum on this circle.

5. Incubate the filter in a humid chamber, 30 minutes, 37 °C.

6. Rinse the filter in excess phosphate buffered saline, about 30 mL.

7. Place a 20 µL drop of anti-species fluorescent antibody conjugate on the spot on the filter where the antiserum was placed.

8. Incubate the filter in a humidity chamber, 30 minutes, 37 °C.

9. Rinse the filter in phosphate buffered saline.

10. Place a drop of non-fluorescent immersion oil on a slide.

11. Place the filter, top face up with the trapped particles, on this immersion oil drop.

12. Place a drop of non-fluorescent immersion oil on the filter

13. Observe the spot on the filter with an epi-illumination fluorescence microscope, ×1000 magnification.

Acridine orange modification for total bacteria count

1. The water sample must be collected in a container that was rinsed with filtered distilled water, filter, 0.2–0.22 µm pore size.

2. Use a buffered acridine orange solution, about pH 7.5.

3. Filter the sample.

4. Overlay the filter in the filter holder with 2 mL of acridine orange solution, 1 minute.

5. Draw the stain through the filter with vacuum.

6. Rinse the sample with filtered distilled water and draw this through the filter.

7. Observe the filter by epi-fluorescence microscopy.

The sensitivity threshold of the fluorescent antibody-filtration method to detect salmonella in water samples is less than 104 cells per mL, 20 counted fields from a 1 mL sample.

Reference

Desmontes C et al., 1990.

8.2.4 Direct epi-fluorescent total count of bacteria in water

Purpose

Rapidly detection and counting of bacteria in natural and treated water.

Approach

Water samples, fresh or fixed, are filtered through a polycarbonate filter, captured residue stained with acridine orange, and the filter is examined by epi-fluorescence microscopy.

Materials

Basic fluorescence microscopy equipment.

Basic fluorescence microscopy supplies.

Counting graticule, calibrated (in ocular).

Stage micrometer.

Vortex mixer (or blender).

Polycarbonate filters, 25 mm diameter, 0.2–0.22 µm pore size, black.

Vacuum source (electrical or hand pump).

Sample collection containers (sterile, particle-free).

Microscope slides

Syringe, disposable, 3–5 mL

Syringe filters, 0.2–0.22 µm pore size

Test tubes, 13×125 mm, screw-capped

Reagents

H_2O, distilled or deionized, filtered through a filter, 0.2–0.22 µm pore size

Phosphate buffer, pH 7.2.

Gluteraldehyde, 5% w/v (optional).

Acridine orange, 0.1% in phosphate buffer.

Method

1. Collect the water sample as discussed in Section 8.2.1 above. Use a clean, sterile container.

2. Optional. Fix the sample by adding 1.0 mL of 5% gluteraldehyde to 9 mL of water sample. (Fixed samples can be stored up to three weeks at 4 °C.)

3. Set up the filter apparatus with a polycarbonate filter in place. (Pre-clean the filter apparatus with filtered water.)

4. Disperse the sample with a vortex mixer or blender to get an even distribution of microorganisms.

5. Optional. Make serial 10-fold dilutions of the sample in phosphate buffer if high counts are expected.

6. Put 1 mL of the water sample or dilution on the filter in the filter holder.

7. Add 1 mL of fluorochrome and wait for 2 minutes.

Alternative to steps 6 and 7: Add the fluorochrome to the sample, and allow these to react for several minutes. Then place this mixture on the filter.

8. Add about 3 mL of phosphate buffer to the filter.

9. Apply a vacuum to filter the stained sample (maximum vacuum should be 47.4 kPa or 36 cm Hg).

10. Turn off the vacuum after the sample has passed through.

11. Remove the filter with filter forceps and air-dry it for several minutes.

12. Cut the filter in half and save one half for reference.

13. Place one-half of the dried filter, face up, on a drop of immersion oil on a slide.

14. Add one drop of immersion oil to the top of the filter half.

15. Cover the filter gently with a cover glass.

16. Examine a minimum of 10 random fields by epi-fluorescence with the ×100 (oil) objective in order to assure an even distribution of microorganisms.

17. Count the cells in 20 squares using the calibrated counting graticule. (A count of 10–50 cells per field is preferred. If higher than 50 per field, select an appropriate 10-fold dilution and repeat the above staining and filtering process.)

18. Calculate an average number of cells per filter. (Total cells per mL = average cells per square × squares per filter × dilution factor ÷ sample volume in mL.)

Notes

1. *All diluents, stains, and buffers coming in contact with the water sample must be filtered through filters, 0.2–0.22 µm-pore size.*

2. *All sample containers, dilution tubes and the filter apparatus must be pre-rinsed with distilled water that has been filtered through a filter, 0.2–0.22 μm pore size.*

3. *This approach to total microbial testing can be done with any filterable liquid or washings made from equipment or small components.*

4. *An inorganic filter, the Anopore, is reported to give a flatter view field, faster filtration and less stain retention than the polycarbonate filters [Jones, 1989; Williamson, 1989].*

5. *Calibrate the counting graticule using the stage micrometer.*

6. *Phosphate buffer:*

    KH_2PO_4   *13.6 g*

    H_2O, distilled   *1000 mL*

    Adjust this to pH 7.2 if necessary.

    Filter this through a filter, 0.2–0.22 μm pore size.

References

Clesceri LS et al., 1998; Francisco DE et al., 1973; Hobbie JE et al., 1977; Jones SE et al., 1989; Williamson FA et al., 1989.

8.2.5 Detecting bacteria and viruses in natural water samples by DNA-specific fluorochrome staining

Purpose

DNA-specific counting of bacteria and/or virus particles in natural raw waters.

Approach

Seawater (or other raw water) is passed through a polycarbonate filter (various pore sizes), the filter stained with DAPI, and the sample examined with an epifluorescence microscope.

Materials

Basic fluorescence microscopy equipment (mercury vapour light source) (microscope light filter system: excitation: 350–410 nm; dichroic mirror: 455 nm; barrier: 470 nm).

Basic fluorescence microscopy supplies.

Formalin, 1% v/v (pre-filtered through filter, 0.2–0.22 µm pore size).

Water sample collection supplies (see Section 8.2.1).

Vacuum filtration system (filter holder and controllable pressure vacuum pump)

Polycarbonate filters, 0.2 µm (bacteria) or 0.015 µm (viruses) pore size, black (Nuclepore), 24–25 mm diameter.

Cellulose filter, 0.45 µm pore size, 25 mm diameter.

DAPI 10 µg/mL, aqueous (pre-filtered through a 0.2 µm pore size filter).

H_2O, distilled or deionized (filtered through a 0.2 µm pore size filter).

Immersion oil (non-fluorescent).

Microscope slides.

Water sample (seawater or other natural water).

Method

1. Collect the water sample.

2. Stabilize the water sample with an equal volume of 1% formalin. Keep it at 4 °C until testing is done.

3. Set up the vacuum filtration system with the polycarbonate filter on the filter platform. (Place a cellulose filter under the polycarbonate filter as a support and damp backing filter.)

4. Add the water sample to the filter surface (2 mL if testing for virus, 10–100 mL if testing for bacteria).

5. Apply vacuum to the filter (1.33 kPa = 10 mm Hg for the 0.015 µm filter, higher for the 0.2 µm filter). (It will take 1–1.5 hours to pass a 2 mL sample completely through the 0.015 µm filter.)

6. Add 0.5 mL of DAPI solution to the filter, and apply vacuum to filter it. (When using the 0.2 µm pore size filter, allow the DAPI solution to rest on it before applying vacuum.)

7. Add 2 mL of pre-filtered distilled/deionized water to the filter, and apply vacuum to draw this through the filter.

8. Remove the polycarbonate filter from the filter holder and place it on a drop of non-fluorescent immersion oil on a microscope slide.

9. Add a drop of oil to the filter and place a cover glass on it, pressing down gently to flatten the filter.

10. Examine the polycarbonate filter surface with an epi-illumination fluorescence microscope. (Use the excitation light source and filters recommended.)

Note: during the filtration, staining and washing process, cover the filter apparatus with aluminium foil to exclude light.

Results

Particles or specks seen that fluoresce blue–white or slightly greenish are interpreted as being biological, viruses (specks) or bacteria (small dots). The 0.015 µm filter captures ultramicrobacteria as well as viruses. These ultramicrobacteria may pass through the 0.2 µm pore size filter, but the virus specks on the 0.015 µm pore size filter are 3 to 20 times the number of bacteria. This method provides a relatively rapid and simple way to assess virus load in natural waters.

References

Hara S, et al., 1991; Hobbie JE, et al., 1977; Pyle BH et al., 1995.

8.3 Testing air for microorganisms

Purpose

Detection and identification of microorganisms in air samples, especially bacteria and mould spores.

Approach

Air is drawn through a polycarbonate filter by means of a vacuum, and the filter surface is stained and examined by epi-fluorescence microscopy.

Materials

Polypropylene filter, black, 25, 37 or 47 mm diameter, 0.2–0.45 µm pore sizes.

Filter holder, aerosol; 25, 37 or 47 mm diameter.

Flow-limiting orifice (used for measured volume sampling).

Vacuum hose.

Vacuum pump, electrical, vacuum 85.3 kPa = 64 cm Hg.

Aerosol adapter (filter holder to hose).

Acridine orange, pH 4 in acetate buffer.

Epi-fluorescence microscope.

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Forceps, blunt smooth tip.

Microscope slide.

Immersion oil, non-fluorescent.

Method (general)

1. Assemble the filter into the filter holder. (Various types of holder may be used.)

2. Place the flow-limiting orifice at some location in the vacuum line before the vacuum pump. (Some orifices screw into the aerosol filter holder, or they can be placed in the vacuum hose.)

3. Connect the vacuum hose to the filter holder and vacuum pump.

4. Turn on the vacuum pump.

5. Collect the air sample for the desired time period. (This time will be longer for relatively clean environments and shorter for more contaminated environments—see Table 8.1).

6. Open the filter holder, and remove the filter with filter forceps, grasping it carefully by the edge.

7. Place a drop of non-fluorescent immersion oil in the centre of a microscope slide.

8. Place the filter on the oil. (A 25 mm filter will fit on the slide, but 37 mm and 47 mm filters must be cut in half.)

9. Stain the filter with acridine orange.

10. Examine the stained filter under an epi-fluorescence microscope.

11. Count the bacterial cells and fungus spores.

References

Clesceri LS et al., 1998; Francisco DE et al., 1973; Hobbie JE et al., 1977; Jones SE et al., 1989; Williamson FA et al., 1989.

Table 8.1 **Suggested sampling times of air in representative areas**

Sampling area (air)	Sample size	Air flow (L/minute)*	Time (minutes)
Country	140 L	10	14
City	30 L	2	15
Dusty	14 L	2	7
Clean room	280 L	10	28

These are general estimates. Investigators must base actual sampling times of air flow on preliminary experiments done in the place being sampled.
*Flow-limiting orifices are available in 2, 3, 10, and 14 L per minute flow rates.

9. Preparation and fluorochrome labelling of antibodies

Antiserum quality plays a major role in the success of immunofluorescence investigations. Adequate quantities of sufficiently pure antisera may be available commercially, but there may still be the need to prepare special antisera.

Several basic principles are involved in production of specific polyclonal antisera for use in fluorescent antibody conjugates or for indirect fluorescent antibody tests. The purer the antigen used for animal inoculation, the more specific the antiserum is likely to be. Longer immunization series will usually yield higher titre antisera. However, this high titre will be at the expense of a broader reactivity, i.e. loss of specificity. This may be due to the reaction of the animal to minor impurities in the antigen, or it may be due in part to an endogenous response by the animal. This reduced specificity is not necessarily a problem for most fluorescent antibody systems, and higher titre is usually more important when producing satisfactory fluorescent antibody conjugates.

Various animals have been used successfully for antibody production, including mammals and birds. The preferred mammal for antibody production is the rabbit because of its good immune response and convenient size and handling. For larger batches of antiserum, especially anti-globulin antisera, the goat is very satisfactory. It is a distinct advantage to have a large batch of a single antiserum of known quality and characteristics to compare in experiments done over an extended time.

Sometimes it is useful to have polyvalent conjugates, for example to detect a group of similar organisms such as *Salmonella* spp. or to trace various globulins in tissue. In this instance, it is not practical to prepare all antibodies needed by injecting them into a single animal. It is unlikely that the proportions of the various antibodies in the antiserum produced would be satisfactory. It is best to make the component

Table 9.1 **Fluorescent antibody conjugate preparation scheme**

Step	Method
1. Prepare pure antigen:	a. Microbial culture b. Soluble antigen
2. Produce antiserum:	a. Suitable animal
3. Test antiserum for specificity and titre:	a. Serologic tests
4. Fractionate antiserum:	a. Ammonium sulfate b. Methanol c. DEAE cellulose d. Sodium sulfate
5. Analyse immunoglobulin fraction protein:	a. Biuret test
6. Conjugate immunoglobulin fraction with dye:	a. FITC (dialysis) b. FITC (dry dye addition) c. RB 200–albumin
7. Separate conjugate and unreacted dye:	a. Gel filtration (Sephadex) b. Dialysis
8. Other conjugate purification (optional):	a. Tissue powder absorption b. Chromatography (DEAE cellulose)

9. Characterize conjugate:	a. Titration
	b. Dye–protein ratio
	c. Non-specific staining
	  (Background staining)
	  (Serologic cross-reactions)

10. Apply conjugate to test antigen (with or without counterstain)	

parts separately in different animals and pool them in appropriate proportions based on titrations of each antiserum to gain the desired titre of each in the polyvalent fluorescent antibody conjugate.

Monoclonal antibodies are particularly attractive for many fluorescent antibody studies. They offer a very high degree of specificity and can be used both for direct fluorescent antibody and indirect fluorescent antibody tests. The procedures for preparing monoclonal antibodies are too complex to be described in detail in this manual. However, a great range of monoclonal antibodies is commercially available, and many of these sources are listed in the Annexes. By using these for indirect fluorescent antibody tests with high titre anti-globulin conjugate, monoclonal antibodies can be extended to many fluorescent antibody tests.

Animal and human serum may contain antibodies in sufficiently high titre after an infection to be used in fluorescent antibody tests in order to detect infecting organisms. However, such serum must be very carefully characterized before such use, since it is also likely to contain antibodies to other antigens as well.

The general steps for preparing fluorescent conjugated antibodies are outlined in Table 9.1.

9.1 Representative methods for antiserum preparation

9.1.1 Intense intravenous injection series

Purpose

Antiserum against *Neisseria meningitidis* serogroups for use in agglutination identification and fluorescent antibody tests.

Approach

Antiserum is produced against *Neisseria meningitidis* in rabbits by an intense series of intravenous inoculations interspersed by short rest periods. The same procedure is suitable for producing antisera against other bacteria.

Materials

N. meningitidis groups A, B, C, D, W-135.

Mueller–Hinton agar.

Mueller–Hinton broth.

Trypticase soy broth.

Balanced salt solution (Hanks without glucose and indicators).

Photometer.

Rabbits, 2 to 2.5 kg.

Inoculation equipment.

Equipment and supplies for culture incubation at 30 °C in 10% CO_2.

Blood collection and serum processing equipment and supplies.

Antigen preparation

1. Inoculate several Mueller–Hinton agar plates with thawed frozen stock culture and incubate them overnight at 30 °C (10% CO_2).

2. Harvest cells with either Trypticase soy broth or Mueller–Hinton broth (5 mL per plate) and check them for purity by Gram stain.

3. Seed several flasks (500 mL) containing 150 mL Mueller–Hinton broth with 1–5 mL of cell suspensions, and incubate them on a rotary shaker for 4–5 hours at 30 °C.

   Note: serogroup D is slow growing and usually requires a larger inoculum.

4. Remove the cells by centrifugation (10 minutes at 3000*g*), decant supernatant, and resuspend the cells aseptically to an optical density of 1.0 (520 nm) in modified Hanks balanced salt solution (no indicator or sugar).

5. Place the cell suspensions into sterile vaccine bottles and freeze immediately at –50 °C or below.

Note: the distribution of cells in the vaccine bottles is designed for thawing only one bottle each day for immunization.

Antiserum production

The rabbits are injected intravenously as follows:

Day	Procedure
1–5	0.5 mL cell suspension each day
6–8	Rest
9–13	1.0 mL cell suspension each day
14–18	Rest
19–20–21	Bleed by cardiac puncture

Rabbits are taken off food 1 day before bleeding. The blood is allowed to clot in glass test tubes at room temperature for 1 hour, the clot ringed with an applicator stick, and the tube placed in the refrigerator overnight. The tubes are then centrifuged, and the serum is removed with a pipette. Serum is stored at –20 °C.

Serum test methods

The titration method is slide agglutination. Each rabbit antiserum is checked for specificity against prototype organisms of all other types. All type specific sera are then pooled, and the type specificity is rechecked. The antisera are tested for the appropriate working dilution that gives 4+ agglutination and shows specificity.

Note: 4+ is the strongest agglutination of the bacteria that can be achieved.

Reference

Vedros NA, 1980.

9.1.2 Multiple injections of purified antigen by different inoculation routes

Purpose

Highly specific and high titre antiserum against *Vibrio cholerae* for coagglutination reagents, fluorescent antibody conjugates, and other serological tests.

Approach

Antiserum is raised against *Vibrio cholerae* using a purified antigen and applying different routes of injection. The same procedure is suitable for producing antiserum against other antigens.

Materials

V. cholerae El Tor Inaba, non-haemolytic, strain 929 (source: Institut Pasteur, Unité du Choléra et des Vibrions).

Note: other strains may be suitable, but they must be tested to assure this.

Heart infusion agar.

Rabbits, 2 kg or larger.

Inoculation equipment.

Serum separation and processing equipment.

Antigen preparation

Purified antigen from *V. cholerae* Ch 1+2 fraction is prepared as follows. Use the recommended strain of *V. cholerae* for this antiserum production. This is strain 9292, isolated at Dakar in 1971. It is *V. cholerae* El Tor Inaba, non-haemolytic.

Note: the antigen is in the first and second fraction collected on a molecular separative gel of the bacterial lysate.

1. Grow the bacteria on heart infusion agar, pH 8.2, 18 hours at 37 °C in Roux bottles. Alternatively, Petri dishes may be used.

2. Harvest the bacterial cells from the agar surface with distilled water, and concentrate them by centrifugation. Bring this suspension to a concentration of 8×10^8 cells per mL. (See the McFarland turbidity standards, Section 5.2 [McFarland J, 1907].)

3. Lyse the cells by ultrasound at 500 kHz for 4 hours, followed by centrifugation at 3000g for 20 minutes.

4. Save the supernate (antigen) and adjust the nitrogen content to 8 g/L.

5. Separate the antigenic fractions from the whole lysate using Sephadex G200 (fine). Use a column 25 mm in diameter and 450 mm long.

6. Fill the column with 205 mL of G200 (40–120 mesh) gel.

7. Elute the column with phosphate buffer, 0.15 M, pH 7.2.

8. Collect the eluate in 3 mL samples at 20 °C. It is convenient to use a fraction collector.

9. Measure the protein in the samples at 280 nm. Fractions (samples) numbers 20 to 29 should contain the antigen.

Note: two other methods of cell lysis may be used.

a. *Freeze the cell suspension at –70 °C, then warm to 37 °C. This is done for six cycles. Centrifuge the mixture and save the supernatant liquid (antigen).*

b. *Suspend the bacteria in salt–glucose solution at a concentration of 8×10^8 cells per mL. Warm the suspension at 37 °C for 4 hours. The pH falls to 5.8. Centrifuge the suspension, and save the supernate (antigen).*

Antiserum raising

1. Animal selection: use rabbits weighing at least 2 kg.

2. Immunization schedule

Five routes of immunization are used. Injections are given at five-day intervals. The schedule is as follows:

Day	Route	Amount	Notes
1	Intradermal	2 mL	20 shots, 0.1 mL each*
6	Intramuscular	2 mL	
11	Subcutaneous	2 mL	
16	Intraperitoneal	2 mL	
21	Intravenous	2 mL	
36	Bleed for serum		

* Mix the antigen with Freund adjuvant, 20%. Intradermal injections are given along the back of the rabbit. Shaving the rabbit's back makes it more convenient.

Antiserum collection

1. Bleed the immunized rabbit by cardiac puncture.

2. Express the blood from the syringe into glass test tubes.

3. Allow the blood to clot 1 hour at room temperature. Ring the clot from the sides of the tube with an applicator stick or glass rod.

4. Store the clotted blood at 4 °C overnight to allow the clot to retract.

5. Decant the serum from the blood clot carefully.

6. Centrifuge the serum to remove any remaining blood cells.

7. Store the serum at –20 °C.

Reference

Dodin A, Wiart J, 1975.

9.1.3 Long intravenous injection series of nonviable antigens

Purpose

Legionella antiserum for use in fluorescent antibody conjugates.

Approach

The inoculation schedule begins with an intramuscular injection of bacteria in complete Freund adjuvant followed by a second intramuscular injection in Freund incomplete adjuvant. Then, a long series of injections is given intravenously until test bleedings show that the antibody titre plateaus. At this point, the serum is harvested.

Materials

Rabbits, 2 kg minimum size.

Legionella bacteria (killed) in saline suspension, 4×10^9 cells per dose.

Freund complete adjuvant (Difco).

Freund incomplete adjuvant (Difco).

Inoculation equipment (syringes, needles, etc.).

Antibody titration systems (agglutination, indirect fluorescent antibody).

Immunization schedule

Day	Action
1	Intramuscular dose in complete Freund adjuvant
31	Intramuscular dose in incomplete Freund adjuvant
38	Intravenous dose
42	Intravenous dose
46	Intravenous dose
50	Test bleed and titre serum. Give intravenous dose
54	Test bleed and titre serum. Give intravenous dose
58 etc.	Intravenous doses given every 4 days until the serum agglutination titre plateaus
	Bleed from the heart 4–7 days after the last dose.

Reference

McKinney RM, Cherry WB, 1985.

9.1.4 Single intravenous antigen injection in roosters

Purpose

High-titre specific antiserum for pathogenic organisms produced in roosters.

Approach

Live pathogenic bacteria are injected intravenously into roosters to produce specific antibodies in high titre in a short time. These can be used for direct fluorescent antibody conjugates and indirect fluorescent antibody tests. The following procedure describes the production of antibodies against *Francisella tularensis*. The same procedure can also be used for the production of other antigens (e.g. for *Pseudomonas* spp.).

Materials

Roosters, 4–5 kg, at least 22 weeks old.

Francisella tularensis culture.

Glucose cysteine blood agar (GCBA) or other suitable culture medium.

Method

1. Antigen preparation

    a. Bacteria are grown on appropriate agar media (*F. tularensis* grown on GCBA) for 24 hours at 37 °C.

    b. Cells are harvested into saline and adjusted to the chosen concentration (5×10^7 viable cells per mL for *F. tularensis*).

2. Inoculation schedule

    Day 1: 0.1 mL of viable *F. tularensis* cell suspension is inoculated intravenously into a rooster using a wing vein.

    Day 9: Exsanguinate the rooster.

Serum titration

Use slide agglutination tests for titration. If cross-reaction occurs, it can be eliminated by heterologous adsorption.

Notes

1. *The White Leghorn rooster seems best suited to antibody production. Hens should be avoided because their serum may be opalescent, making some procedures difficult.*

2. *If live, highly pathogenic organisms are used for the inoculum, the procedures must be done in safety containment facilities, and the birds must be quarantined in safety containment facilities. Disposal of birds should be by incineration.*

3. *A high-titre specific antibody response can be produced in chickens with a single antigenic injection. Titres can be increased by further antigen injections but at the expense of loss of specificity. Thus, an excellent and specific antibody can be raised in roosters with a single antigenic injection. The procedure can be applied to many antigens, including* Yersinia pestis, Pseudomonas spp., Venezuelan equine encephalomyelitis virus, Rift Valley fever virus, Staphylococcus *enterotoxin and the serum globulins of most species.*

References

Abramoff P, Wolfe HR, 1959; Wolfe HR et al., 1957; Yager RH et al., 1961.

9.1.5 Short series of intravenous injections using non-viable whole bacteria

Purpose

Salmonella "O" antiserum for agglutination and fluorescent antibody conjugates.

Approach

Salmonella strains with appropriate "O" antigens are selected. These are heat-treated to destroy "H" antigens, and killed salmonella cells are inoculated intravenously into rabbits. The procedure is also suitable for producing other bacterial antisera.

Materials

Salmonella culture (appropriate "O" antigen).

MacConkey agar plate.

Nutrient agar slant.

Saline, 0.15 M.

McFarland No. 7 turbidity standard.

Merthiolate, 1% stock solution.

Rabbits, 2 kg or larger.

Inoculation equipment.

Serum collection and processing equipment.

Procedure

A. *"O" antigen preparation*

1. Streak the *Salmonella* strain out on a MacConkey agar plate and incubate it overnight.

2. Transfer an isolated smooth colony on to a nutrient agar slant. Incubate for 18–20 hours at 37 °C.

3. Wash the growth off the slant in 0.85% saline.

4. Dilute the suspension so that there are approximately 2×10^9 bacteria per millilitre (McFarland No. 7).

5. Boil the suspension for 2½ hours. Cool it and preserve it with 1% final merthiolate concentration.

6. Store the preserved antigen in the refrigerator at 4 °C.

B. *Inoculation schedule*

1. Use rabbits, minimum 2 kg.

2. Rabbits are given 4 intravenous injections of the suspension at 4-day intervals. The amounts administered are: 0.5 mL, 1 mL, 2 mL, and 3 mL. (It may be necessary to add one last injection of 3 mL of the antigen suspension in order to obtain a sufficiently high titre of antiserum.)

3. Bleed the rabbits 6–8 days after the last injection. The titre by tube agglutination test should be 1:1600 to 1:6400.

C. *Serum collection and processing*

1. Bleed the rabbits by cardiac puncture.

2. Collect the blood in sterile glass tubes (potato tubes).

3. After clotting occurs, about 1 hour, ring and loosen the clot from the walls of the tubes with an applicator stick or a glass rod.

4. Allow the tubes to stand overnight at 4 °C (in the refrigerator).

5. Pour the serum off and centrifuge it until it is clear of blood cells.

6. Preserve the serum with merthiolate (thimerosal), final concentration 0.01% (0.1 mL of 1% stock solution of merthiolate in phosphate buffered saline for each 10 mL of serum).

Reference

LeMinor L, Rohde R, 1978.

9.1.6 Antibody preparation against immunoglobulins in goats

Purpose

Preparation of a large batch of antiglobulin antiserum for use in direct fluorescent antibody tests.

Approach

A goat is given multiple site intramuscular injections of purified IgG and then bled for anti-IgG antibody.

Materials

Goat (medium-sized adult, not old).

IgG immunoglobulin (prepared by DEAE cellulose fractionation).

Phosphate buffered saline, pH 7.4.

Blood collection supplies (20–50 mL syringes, 18–20 gauge needles, large centrifuge tubes or bottles, 70% methanol, etc.).

Centrifuge.

Refrigerator.

Freund complete adjuvant.

Inoculation supplies (5–10 mL syringes, 20–21 ga needle, 70% ethanol, etc.).

Method

1. Take a pre-immunization blood sample, 100–200 mL.

2. Allow the blood to clot. Ring it from the tube sides with an applicator stick or glass rod, and place it in the refrigerator overnight to retract the clot.

3. Decant the serum.

4. Clarify the serum of any blood cells by centrifugation.

5. Save this pre-immunization serum for control studies.

6. Immunize the goat.

    a. Inject the goat with IgG (or other purified globulin) at four intramuscular sites; total dose: 0.5 mg IgG per kg of body weight.

    b. Emulsify serum globulin at 0.5% solution in phosphate buffered saline in an equal volume of Freund complete adjuvant;

7. Repeat the same dose at 6 weeks.

8. Bleed the goat 3 weeks after the second dose, from the jugular vein, about 400–500 mL.

9. Allow the blood to clot, as in step 2 above, decant the serum, and clarify it by centrifugation, as in steps 3 and 4 above.

10. Give a booster immunization 6–12 weeks after the second dose.

11. Bleed the goat out 3 weeks later. This should yield more than 1 L of anti-IgG antiserum.

References

Nairn RC, 1968; Proom H, 1943.

9.1.7 Antibody preparation against immunoglobulins in rabbits

Purpose

Production of high-titre rabbit anti-human globulin antisera.

Approach

Antibodies are produced in rabbits by subcutaneous injection of human immunoglobulins.

Materials

Pure IgG.

Complete and incomplete Freund adjuvant.

Sephadex G25.

Phosphate buffered saline, pH 7.4.

Beakers, mixer, crushed ice.

5 mL syringes.

Rabbits, 2 to 2.5 kg.

10% w/v potassium aluminium sulfate (alum) solution.

1 N sodium bicarbonate solution.

Method for anti-IgG antibodies

1. Disperse 2 mg IgG and 50 mg Sephadex G 25 in 2 mL phosphate buffered saline.

2. Add 2 mL of Freund complete adjuvant drop by drop, keeping the mixture on ice and stirred well. If no suitable mixer/stirrer is available, the suspension can be transferred to and fro between two 5 mL syringes connected by tubing, until a stiff emulsion is obtained.

3. Place a drop of the emulsion on the surface of lukewarm water (body temperature). The drop should not disperse or run.

4. Inoculate each rabbit with approximately 0.2 mL of the suspension subcutaneously between the toes, with 2 mL subcutaneously under the shaved skin of the back, and the remainder given intramuscularly into the upper thigh.

5. Repeat the procedure after 10 days using half the volume.

Method for anti–L chain antibodies

To produce antibodies against L chains, the antigen is given intravenously after 20 days rest.

1. Dissolve 10 mg IgG in 2.5 mL phosphate buffered saline, mix with 15 mL 1 N $NaHCO_3$ solution and add 2.5 mL of a 10% w/v solution of potassium aluminium sulfate (alum) dropwise within 10 minutes.

2. Allow the suspension to stand overnight at 4 °C in a refrigerator.

3. After centrifugation for 30 minutes at 15 000*g*, resuspend the precipitate in 4 mL phosphate buffered saline.

4. Inject the suspension intravenously into a rabbit in the following quantities twice weekly over a three-week period:

    Week 1:   0.05 mL and 0.10 mL

    Week 2:   0.10 mL and 0.20 mL

    Week 3:   0.20 mL and 0.40 mL

5. Take the first blood samples, and test it by Ouchterlony diffusion 1–2 weeks after the last injection.

6. Store the serum at –20 °C.

Note: antibodies produced during the early phase of an immune response (the primary response) usually have a higher specificity but lower avidity than those produced during the later phases (the secondary response), when this relationship is reversed. Furthermore, it is often more effective to allow a longer period to elapse (five–six weeks) after the first injection before giving the second injection.

Reference

Storch WB, 2000.

9.2 Purification of gamma globulins

9.2.1 Ammonium sulfate precipitation

Purpose

Conservation of expensive dye and assuring efficient conjugation of antibodies in sera to be used for immunofluorescence.

Approach

Ammonium sulfate salt solution salts out the globulin, which precipitates.

Materials

Magnetic stirrer and stirring magnet.

Beaker, 500 mL.

Burette.

Refrigerator.

Centrifuge.

Reagents

Saturated $(NH_4)_2SO_4$ (at 4 °C):

> $(NH_4)_2SO_4$, 750 g (approximately)

> H_2O, distilled to 1000 mL

>   Dissolve the ammonium sulfate at room temperature. Cool and store it in the refrigerator at 0–4 °C.

>   Combine equal volumes of the supernatant from the saturated solution and distilled water. Store this at 0–4 °C.

NaCl solution, 0.15 M.

Phosphate buffered saline, pH 7.4.

Nessler reagent (see Annex 14).

Merthiolate stock solution, 1% in phosphate buffered saline.

Method

1. Dilute the antiserum with an equal volume of 0.15 M NaCl solution in a beaker.

2. Chill the solution to 0–4 °C.

3. Place it on a mechanical stirrer.

4. Add, dropwise while stirring, cold saturated $(NH_4)_2SO_4$ equal to the volume of the serum–saline solution.

5. Stir this solution for 30 minutes.

6. Centrifuge for 15–30 minutes at 1000–1500g (15 cm radius), 4 °C.

7. Discard the clear supernatant liquid.

8. Wash the precipitate with the half-saturated $(NH_4)_2SO_4$ solution.

9. Centrifuge as above.

10. Discard the clear supernatant liquid.

11. Dissolve the precipitate in one-third of the original serum volume using cold 0.15 M NaCl.

12. Dialyse the solution against frequent changes of phosphate buffered saline, pH 7.4, at 4°C, until the dialysate is free of ammonium ions, 3–7 days. Test the dialysate following 12 hours of dialysis. Use Nessler reagent to detect ammonium ions in the dialysate (see Annex 14).

13. Recover the globulin solution from the dialysis sac, and freeze it or preserve. It with merthiolate, 1:10 000 final concentration, stored at 4 °C until ready for conjugation.

Reference

Coons AH, 1957.

9.2.2 DEAE–Sephadex fractionation

Materials

DEAE–Sephadex A-50, medium grade.

NaCl, 2 M

Phosphate buffer, 0.02M, pH 7.6.

Solution 1: $NaH_2PO_4 \cdot H_2O$, 2.76 g.

        H_2O, distilled, to 1000 mL.

Solution 2: Na_2HPO_4, 2.84 g.

        H_2O, distilled, to 1000 mL.

Add solution 1 to solution 2 in a ratio to yield pH 7.6 in a beaker under pH meter control while stirring. (The ratio will be approximately 1:9.)

Method

1. Prepare and pack the chromatography column in accordance with directions of the manufacturer.

2. Equilibrate the column using phosphate buffer (four column volumes) at 4 °C.

3. Place the serum on the column.

4. Elute the gamma globulin using phosphate buffer (check fractions or samples for protein using a spectrophotometer at 280 nm or biuret tests.)

5. Combine the protein-containing fractions (gamma globulin).

6. Concentrate the gamma globulin using PVP to the desired concentration, if necessary.

7. Elute the remaining proteins from the column using 2 M NaCl solution. Discard these proteins or save them, if required for other work.

References

Dedmon RE et al., 1965; Levy HB, Sober HA, 1960.

9.3 Globulin concentration with polyethylene glycol

Purpose

Concentration of dilute globulin solutions before conjugation with fluorochrome.

Approach

Several methods can be used. These are:

- dialysis against water-soluble polymers such as polyethylene glycol (Carbowax series)

- lyophilization

- pressure-filtration or negative-pressure dialysis using semi-permeable membranes

- adsorption on to protein A-Sepharose, followed by elution with 0.58% w/v acetic acid in 0.15 M NaCl and dialysis against phosphate buffered saline.

Dialysis against polyethylene glycol is inexpensive, simple to do, and generally applicable. The method is described below.

Materials

Polyethylene glycol (Carbowax 2000) 20%–25% w/v in phosphate buffered saline.

Dialysis equipment (tank or graduated cylinder, twirling apparatus).

Dialysis tubing and string.

Refrigerator.

Globulin solution.

Method

1. Soak the dialysis tubing in phosphate buffered saline.

2. Secure one end of the tubing by tying knots in it. (Use two knots in succession on the tubing for security.)

3. Fill the dialysis tubing with the globulin solution to be concentrated and secure the end with a string knot (do this over a beaker in case something goes wrong).

4. Place the tubing directly into the bath of previously prepared polymer solution (at least 10 volumes). The time required for dialysis depends on the quantities involved and the degree of concentration desired. It can be anywhere from a few hours to several days.

Reference

Lipp W, 1961.

9.4 Conjugation of immunoglobulin with fluorochromes

9.4.1 Conjugation of globulin with fluorescein isothiocyanate (FITC)

9.4.1.1 Direct reaction method

Purpose

Preparation of FITC-antibody conjugate for use in fluorescent antibody tests.

Approach

Dry crystalline FITC is added slowly to the globulin solution while stirring in the cold.

Materials

Gamma globulin solution.

Fluorescein isothiocyanate (FITC), isomer 1, 9 % or greater.

Phosphate buffered saline, pH 7.4.

Carbonate buffer, pH 8.5 (see Section 12, Annex 13).

NaCl, 0.15 M.

Analytical balance.

Magnetic stirrer and stirring magnet.

Refrigerator, 4 °C.

Beakers, various sizes.

Dialysis tubing and string.

Chromatography column.

Sephadex G-25.

Method

1. Find the concentration of protein in the sample in terms of mg/mL.

2. Calculate the total protein content of the sample.

3. Find the final total volume of the conjugation reaction mixture at the protein concentration desired. (Generally this is 10 mg/mL, 20 mg/mL, or 25 mg/mL.)

   Note: at 10 mg/mL protein concentration, the reaction mixture volume will be 1/10 of the total protein in the original sample.
   Example: sample is 15 mL at 17 mg/mL, protein = 255 mg total protein
     Total protein   255 mg
     Volume at 10 mg/mL   25.5 mL
     Volume at 20 mg/mL   12.3 mL.

4. Set up calculations for the conjugation mixture as follows:

   Total volume at _____mg/mL   = _____mL   A

   Sample volume   = _____mL   B

   (10% of total volume)   = _____mL   C

   0.15M NaCl (to total volume)   = _____mL   D

   Note: carbonate–bicarbonate buffer A = B + C + D

5. Prepare the reaction mixture in accordance with the calculations in 4 above.

6. Add FITC in the dye/protein ratio desired (1:20, 1:40, 1:80 are common ratios.). Add dye slowly.

Ratio	Dye	Protein
1:20 =	0.05 mg per	1.0 mg
1:40 =	0.025 mg per	1.0 mg
1:80 =	0.0125 mg per	1.0 mg

7. Store the complete reaction mixture for the desired time period at 0–4 °C in the refrigerator.

Protein concentration	Dye/protein ratio	Reaction time
10 mg/mL	1:20	14–18 hours
20 mg/mL	1:40–1:80	4–8 hours
25 mg/mL	1:40–1:80	4–8 hours

8. Pass the conjugate through a Sephadex G-25 gel column. Collect the first coloured band. This is the conjugate.

    Alternatively, remove conjugation mixture from the stirrer, place it in a dialysis tube, and dialyse it against frequent changes of phosphate buffered saline (0.15 M NaCl, pH 7.4) for 4–7 days (until there is no more fluorescein in the dialysate following 12 hours of dialysis). Use a UV light such as a Wood lamp to check for fluorescence in the dialysate.

9.4.1.2 Dialysis method

Purpose

Gentle, simple conjugation of FITC to gamma globulin.

Approach

Gamma globulin solution is dialysed against an alkaline solution of FITC at 4 °C.

Materials

Gamma globulin solution in saline (0.15 M NaCl).

Fluorescein isothiocyanate (FITC) (isomer 1, 90%, is preferred).

Carbonate–bicarbonate buffer (0.025 M), pH 8.5 or pH 9.0 (see Section 12, Annex 13).

Dialysis tubing, approximately 19 mm diameter.

Graduated cylinder, 50 or 100 mL.

Magnetic stirrer with magnet stirring bar.

Chromatography column.

Sephadex G-25.

Phosphate buffered saline, pH 7.4.

Refrigerator.

Freezer, –20 °C.

Method

1. Adjust the globulin solution to 1% (10 mg/mL) with the carbonate–bicarbonate buffer. Measure the final volume.

2. Prepare a solution of FITC, 0.1 mg/mL, in carbonate–bicarbonate buffer, 10 times the volume measured in step 1 above.

3. Place the globulin solution in a dialysis bag.

4. Hang the dialysis bag in a graduated cylinder filled with the FITC solution.

5. Stir the solution in the graduated cylinder around the dialysis bag with a magnetic mixer.

6. Continue the reaction for 24 hrs at 4 °C.

7. Remove the conjugated globulin from the dialysis bag and pass it through the Sephadex G-25 column equilibrated with phosphate buffered saline. The fast moving coloured fraction is the conjugate. Dialysis of the conjugate against many changes of phosphate buffered saline may also be used to remove the unreacted FITC instead of gel filtration if desired. There will be little free unreacted FITC in the conjugate.

8. Adjust the conjugate to the desired concentration with phosphate buffered saline, usually 1:2 of the original volume passed through the Sephadex column. Filter the conjugate through a membrane filter (0.45 µm pore size) and divide it into convenient aliquots in vials or test tubes.

9. Store the aliquots of conjugate at –20 °C.

Note: this conjugation method is gentle on the antibody, and it leaves little unreacted FITC in the conjugate. Dialysis or gel filtration of the conjugate to remove unreacted dye may not be needed. The drawback to this method is that it uses more FITC dye than the direct reaction conjugation method.

Reference

Clark HF, Shepard CC, 1963.

9.4.1.3 Dimethyl sulfoxide (DMSO) method

Purpose

Preparation of a green fluorescent antibody conjugate with FITC.

Approach

FITC is dissolved in dimethyl sulfoxide solution and added slowly to purified IgG.

Materials

Serum globulin fraction, purified IgG (see Section 9.2.1 or 9.2.2).

Fluorescein isothiocyanate (FITC) , isomer 1 (crystalline, minimum 90%).

Dimethyl sulfoxide (DMSO).

Carbonate buffer, pH 9.3.

Phosphate buffer, 0.01 M, pH 8.0.

Dialysis tubing and string.

Magnetic stirrer and stirring magnet.

Refrigerator.

Beakers, various sizes.

Dropping pipette (Pasteur).

Chromatography column.

Biogel P-6 (BioRad laboratories).

Method

1. Dialyse the purified serum IgG fraction against carbonate buffer, pH 9.3, overnight in the refrigerator.

2. Adjust the concentration of the IgG fraction to about 6 mg/mL in 10 mL total volume.

3. Stir this mixture slowly in a small beaker on a magnetic stirrer, room temperature.

4. Dissolve FITC in DMSO at a ratio of 1:100 (3 mg FITC in 3 mL DMSO).

5. Add the FITC/DMSO solution to the IgG solution dropwise, while stirring. Allow the reaction to take place for 2 hours at room temperature.

6. Pass the mixture through a column of Biogel P-6 and collect the first coloured band. This is the conjugate.

Reference

Goding JW, 1976.

9.4.2 Conjugation of globulin or bovine serum albumin with lissamine rhodamine B (RB 200)

Purpose

Red fluorescent stain for fluorescent antibody tests and counterstain for FITC conjugates.

Approach

Serum proteins, including whole serum, globulin fractions and bovine serum albumin are conjugated with RB 200 to yield red fluorescing specific-antibody conjugates or bovine serum albumin that can be mixed with green fluorescing FITC conjugates to act as a contrasting colour counterstain.

Materials

Dry box.

Fume hood.

Ice bath.

Dessicator jar.

Refrigerator.

Magnetic stirrer and stirring magnet.

pH meter.

Mortar and pestle.

Erlenmeyer flask, 125 mL.

RB 200 (lissamine rhodamine B) 1.0 g.

$PC1_5$ (dry), 2.0 g.
CAUTION. This chemical is corrosive and toxic. It is first dried in a dessicator jar over P_2O_5. When handling and weighing $PC1_5$, use a dry box. If this is not available, try to work on a low humidity day.

Acetone (dry), 10.0 mL.
Note: Add several grams of anhydrous $CaCl_2$ to 50 mL of acetone and let the mixture stand several hours with occasional shaking.

Carbonate–bicarbonate buffer, 0.5 M, pH 9.0.

Phosphate buffered saline, pH 7.4.

Phenol saline (1.5M NaCl, 0.5% phenol).

Serum protein solution in phenol saline.
Note: let these solutions equilibrate at 4 °C for five days before conjugation.

Whole serum. Mix equal parts of serum and NaCl, 0.15 M, containing 1% phenol.

Serum globulins. Reconstitute fractionated precipitates from antisera in phenol saline at pH 8.0.

Bovine serum albumin. Dissolve bovine serum albumin in 0.15 M NaCl containing 0.5% phenol at pH 8.0 to a bovine serum albumin concentration of 15 mg/mL.

Sephadex G-25 (coarse bead).

Method

1. Grind the RB 200 and $PC1_5$ together in a mortar for about 5 minutes. Do this step in a fume hood because of corrosive fumes.

2. Scrape as much of the dye powder as possible into a flask. Using a portion of the dry acetone, wash the rest of the dye powder from the mortar into the flask.

Cap the flask loosely, and mix the solution occasionally for 10 minutes.

3. Filter the solution through filter paper.

4. Mix 1 volume of carbonate–bicarbonate buffer with each 2 volumes of the serum protein–phenol saline solution (or serum globulin or bovine serum albumin). Cool this mixture in an ice bath.

5. Stir at 3–5 °C, and slowly add the acetone solution of RB 200. (Add this over about 15 minutes.) Be sure that the mixture remains alkaline during the procedure. Keep constant pH control using a pH meter.

    a. Whole serum: 0.1 mL RB 200 solution per 2 mL of serum–phenol saline solution

    b. Globulins or bovine serum albumin: 0.1 mL RB 200 solution per 60 mg total protein.

        Note: when conjugating a solution of fractionated serum globulins, prepare the reaction mixture by adding one-third of the original serum volume of carbonate–bicarbonate buffer. Adjust the mixture to 10 mg of protein per mL. Add the RB 200 solution at the rate of 0.1 mL per 60 mg total protein.

6. Continue stirring in the cold on a magnetic stirrer 14–18 hours.

7. Dialyse against phosphate buffered saline, pH 7.4, for 24 hours with several changes of dialysate.

8. Pass the conjugate through a Sephadex G-25 (coarse bead) column using phosphate buffered saline, pH 7.4, as an eluant.

9. Store small aliquots frozen for use.

Use

1. Mix the RB 200–bovine serum albumin conjugate with fluorescein isothiocyanate conjugates as a counterstain in an appropriate ratio, usually between 1:20 and 1:30.

2. Mix serum or globulin conjugates containing RB 200-bovine serum albumin with the antigen or specimen in question.

3. Proceed as in the fluorescent antibody test.

References

Chadwick CS et al., 1958; Smith CW et al., 1959.

9.4.3 Conjugation of globulins with tetramethylrhodamine isothiocyanate (TRITC)

Purpose

Preparation of red fluorescing antibody conjugates.

Approach

Globulin fractions of antisera are conjugated with TRITC in an alkaline solution using DMSO as a solvent.

Materials

Refrigerator.

Magnetic stirrer and stirring magnet.

Beakers.

Dialysis equipment.

Carbonate buffer, pH 9.3.

TRITC, crystalline.

IgG serum fraction.

Dimethyl sulfoxide (DMSO).

Gel filtration column.

Biogel P-6 (or Sephadex G25).

Method

1. Dilute the IgG fraction in carbonate buffer to approximately 6 mg/mL in a total volume of 10 mL.

2. Dialyse the IgG fraction against carbonate buffer overnight at room temperature while stirring the dialysate bath using a magnetic stirrer.

3. Dissolve 3 mg TRITC in 3 mL DMSO (1 mg per mL).

4. Add the TRITC solution to the IgG *dropwise* with continuous stirring.

5. Allow the solution to stand at room temperature *in the dark*.

6. Purify the solution by gel chromatography over Biogel P-6.

References

Bergqvist NR, Nilsson P, 1974; Goding JW, 1976.

9.5 Removal of unreacted dye from fluorescent antibody conjugates

9.5.1 Gel filtration

Free dye is separated from labelled conjugate by chromatography using cross-linked dextran or polyacrylamide gels (Sephadex or Biogel). After being soaked in aqueous solutions, these function as molecular sieves. Small molecules, such as free FITC, penetrate the pores and are slowed as they pass down the column, whereas larger molecules, such as protein-bound FITC, are not slowed and pass through the column quickly. Sephadex is usually used, but Biogel polyacrylamide gel series should be used for separations when it is important to avoid carbohydrate contamination or protein binding problems.

Apparatus

Chromatography column, with or without a cooling water jacket.

Commercial columns are available, but one can be made using a simple glass or plastic tube with a sintered glass platform and a stopper in the bottom end. Alternatively, 0.5–1 cm glass wool held in place by glass beads at the bottom of the cylinder is a sufficient platform for the gel. A 50 or 100 mL plastic syringe barrel can be used for a column. A drain in the bottom of the cylinder allows liquid to be drawn off. Flow is controlled with a stopcock or an adjustable clamp on a section of rubber hose attached to the column drain (Figure 9.1).
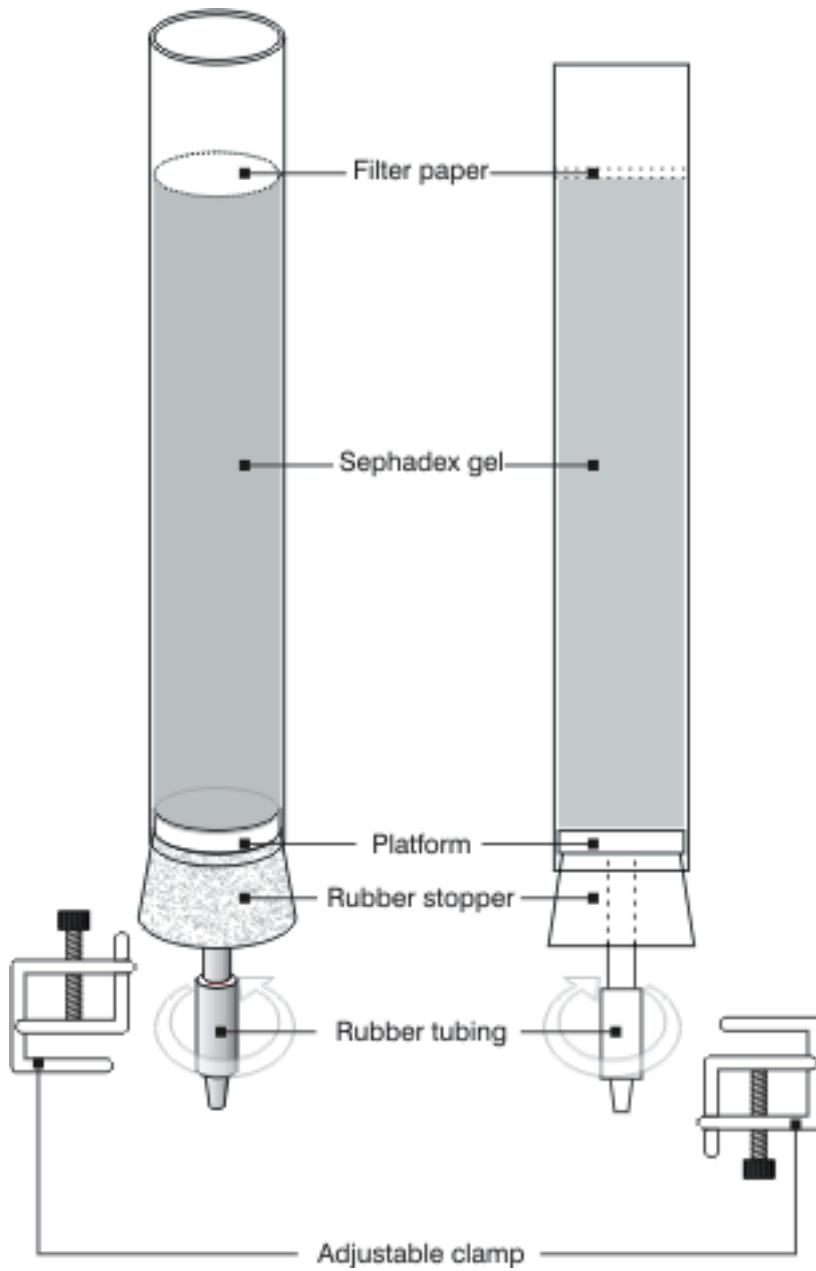
Figure 9.1 **Column for gel chromatography**

Materials and reagents

Phosphate buffered saline, pH 7.2–7.4.

Sephadex G-25 or G-50 medium grade.

Conjugate, freshly prepared.

Gel preparation

Prepare the column 1–2 days before use. Depending on the column used (length: diameter = 10:1 to 30:1), 6–10 g of Sephadex will be needed to purify 10 mL of conjugate. Disperse the dry powder in the phosphate buffered saline by stirring it in sufficient phosphate buffered saline in a beaker so that, after settling, the supernatant liquid volume is approximately five times that of the gel layer. After soaking for about 5 hours, remove small particles by washing with phosphate buffered saline and decanting a number of times. Degassing is rarely needed, but when required, do it using a water vacuum pump.

Column preparation

Mount the chromatography column vertically, wash it with phosphate buffered saline, and leave phosphate buffered saline in one-third of the column volume. Suspend the swollen Sephadex in an equal volume of buffer, and, in one movement, pour it into the column. Open the column drain only after the gel has started to settle by gravity. When the gel has completely settled, cover the top of the gel bed with either a circle of filter paper or a nylon net applicator disk.

Filtration

1. Drain off most of the phosphate buffered saline above the top of the gel column.

2. Carefully allow the remainder of phosphate buffered saline to run exactly to the gel surface.

3. Lay the conjugate, carefully dropwise, onto the top of the gel.

4. Allow it to penetrate into the gel by gravity. It may be necessary to open the drain slightly.
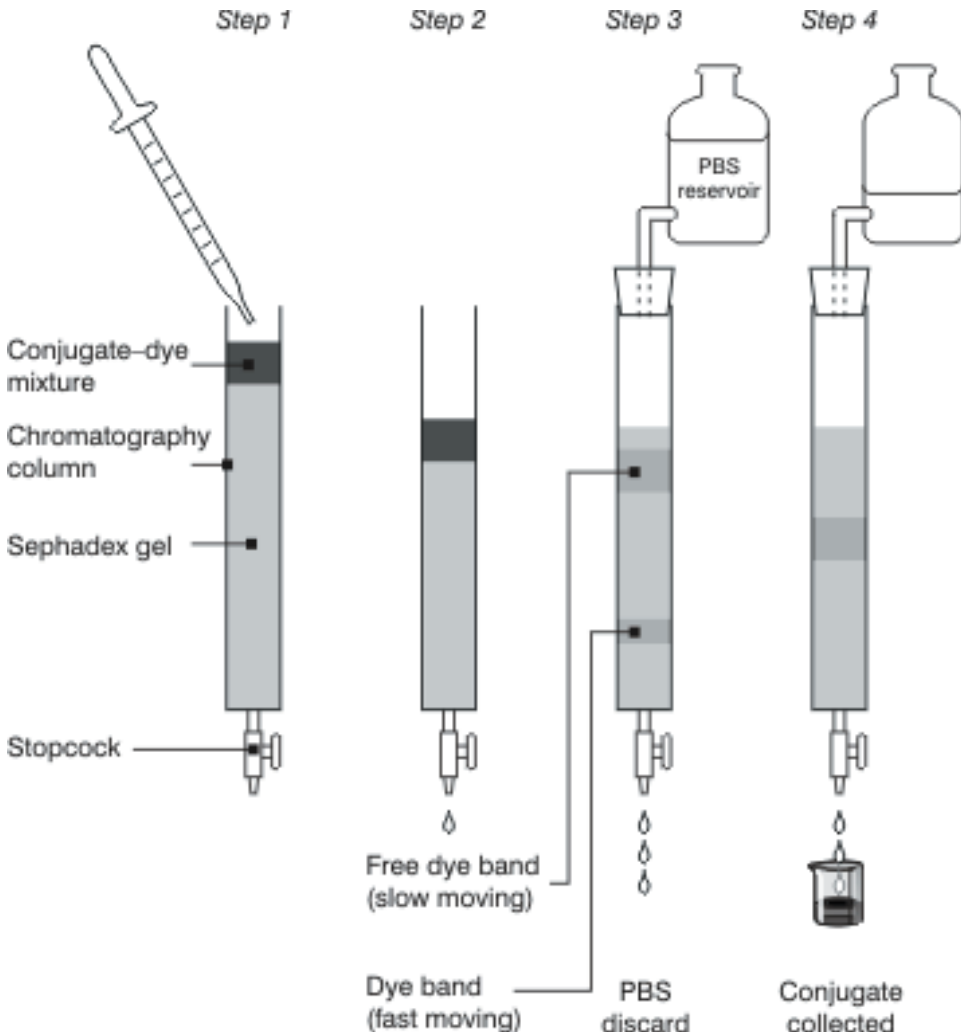
Figure 9.2 **Labelled conjugate passing through a Sephadex column**

5.   Add more phosphate buffered saline to the top of the gel column and connect a buffer reservoir (optional) to the top of the column so phosphate buffered saline can flow in from the top while the column is drained. (Alternatively, add phosphate buffered saline to the space on top of the column by hand as the level goes down.)

6. Drain the column slowly, carefully controlling the flow with the drain stopcock so that phosphate buffered saline flows in from the reservoir. Separation starts immediately. Free dye remains at the top of the column, while the conjugate band moves rapidly down the column.

7. Collect the conjugate in a small beaker. (It comes through with a high degree of purity. A fraction collector is not needed, since the coloured conjugate band is easily seen.) The process is shown in Figure 9.2.

8. If necessary, the conjugate can be concentrated.

Note: the column can be reused if the top layer of gel containing free dye is removed and/or the remaining dye is eluted with phosphate buffered saline. When the Sephadex column is not in use, add 0.01% w/v sodium azide to the phosphate buffered saline in it as a preservative.

9.5.2 Dialysis

Purpose

Dialysis of a newly prepared conjugate to remove unreacted fluorochrome.

Approach

The newly prepared conjugate is dialysed against multiple changes of phosphate buffered saline.

Materials

Dialysis tank (large beaker).

Stirring device (magnetic or mechanical).

Refrigerator.

Phosphate buffered saline, pH 7.2–7.4.

Dialysis tubing.

Conjugate.

Pipette, 5–10 mL.

Method

1. Fill the dialysis tank with phosphate buffered saline. Place it on a stirring device (magnetic bar stirrer preferred). Cool these in the refrigerator.

2. Soak a length of dialysis tubing in phosphate buffered saline for several hours. (The tubing should be about 20% longer than needed to hold the volume of conjugate.)

3. Tie two knots in succession in one end of the wet dialysis tubing.

4. Pipette the newly prepared fluorescent antibody conjugate into the dialysis tubing.

   Note: to prevent conjugate loss in case of filling problems, do the pipetting into the dialysis tubing over an empty beaker.

5. Tie two knots in succession in the top end of the dialysis tubing.

   Note: leave empty space between the conjugate and the knots, as the liquid volume in the dialysis tubing will increase during dialysis.

6. Dialyse the conjugate in the dialysis tubing in the phosphate buffered saline bath while stirring the bath in the refrigerator. (The dialysis bag can be secured with a string to a bar across the top of the bath to prevent it being drawn into the turning magnet.)

7. Replace the phosphate buffered saline in the bath two or three times daily for the first few days of dialysing, and thereafter daily.

8. Test the dialysate in the bath with an ultraviolet light source in a dark room to observe fluorescence. The dialysis is finished when fluorescence can no longer be seen in the dialysate after 2–3 hours of dialysis.

9. Remove the dialysed conjugate from the dialysis bag by cutting the bottom of the bag with scissors over an empty beaker.

10. Centrifuge the conjugate to remove any trace of precipitate.

11. Preserve the conjugate with merthiolate, 1:10 000 final concentration.

12. Store the conjugate at 0–4 °C.

9.6 Preventing non-specific staining

In fluorescent antibody preparations, any fluorescence that is not specific to the antigen under study is called non-specific staining. Non-specific staining is most often seen in fluorescent antibody studies of tissue sections, tissue cultures, and certain clinical specimens containing much cellular and mucoid material like purulent cerebrospinal fluid, sputum or nasopharyngeal secretions. Sometimes non-specific staining is strong enough to obscure weak specific fluorescent antibody fluorescence. The main sources of non-specific fluorescence are:

- natural fluorescence in the specimen (for example, protein in tissue sections)

- unreacted free fluorochrome dye in the conjugate

- non-antibody conjugated serum proteins in the conjugate

- heterologous (other) conjugated antibodies in the conjugate

- leukocyte (granulocyte and histiocyte) primary fluorescence.

The causes of non-specific staining in fluorescent antibody preparations are complex (see Nairn RC, ed., 1976). Some leukocytes, particularly granulocytes and histiocytes, exhibit natural fluorescence. At times, this makes it difficult to see fluorescent antibody stained antigens of infecting agents that may be in these cells.

Various methods of treatment of specimens and conjugates are used to reduce or even eliminate non-specific staining. In most cases, non-specific staining can be controlled sufficiently to allow satisfactory examination of specimens and clear showing of specific fluorescent antibody fluorescence. The methods commonly used to eliminate or control non-specific fluorescent antibody staining are:

- removing unreacted dye from the conjugate

- using purified, specific antibody to make the conjugate

- absorbing conjugates or antisera with heterologous antigen

- pre-treating specimens with chemicals

- pre-treating specimens with enzymes

- absorbing conjugates with tissue powder

- counterstaining specimens.

Protein fluorescence. When excited by UV light or violet light at about 460 nm, protein in tissues fluoresces blue. This is usually a low-level fluorescence, but it may obscure weak FITC fluorescence of specific antigen in a specimen. It can be avoided by using excitation light above 460 nm with interference filters. Since the maximum absorbance of the most important fluorochromes, FITC, acridine orange, and auramine O is in the 480–495 nm range, this problem is easily overcome. However, blue background non-specific staining also can be helpful. In some situations, this primary blue fluorescence of tissues can help locate the fields of view in some specimens.

Unreacted dye in the conjugate. Early in the use of fluorescent antibody, non-specific staining by unreacted free fluorochrome in the conjugate was a major problem. Dialysis after conjugation did not remove all the unreacted dye. With the use of gel filtration with Sephadex or Biogel to purify the conjugates of unreacted dye, this problem is solved.

Non-antibody conjugated serum proteins. Antibody should be as pure and specific as possible to make conjugates for use in fluorescent antibody tests. The sensitivity of fluorescent antibody is very high, so small impurities in the conjugate can cause non-specific staining. Using gamma globulin fractions to make conjugates instead of whole serum is helpful, and it makes more economic use of expensive fluorochromes.

Heterologous conjugated antibodies. Antibodies to antigens other than the one of interest may be in the gamma globulin fractions of polyclonal antisera. Also, organisms closely related to the one of concern may show cross-reactions. Absorption of the conjugate with heterologous antigens may remove non-specific staining and leave the specific stain reactions in place. For example, cross-reactions between *E. coli* and *Shigella* may be removed from an anti-*Shigella* conjugate by suspending about 0.1 mL of packed and washed *E. coli* cells in an anti-*Shigella* fluorescent antibody conjugate, incubating for a period, and removing the cells by centrifugation. If the organisms are antigenically similar, this may reduce both non-specific and specific staining, but sufficient specific fluorescent antibody staining should remain to give satisfactory fluorescent antibody tests. Monoclonal antibodies are very pure and specific and can be used in fluorescent antibody systems. They

can be used to make conjugates for direct fluorescent antibody or for the specific antibody when doing indirect fluorescent antibody tests.

Chemical pre-treatment. Treating specimens with certain chemicals prior to staining with fluorescent antibody conjugates often reduces non-specific staining. Direct examination of fluorescent antibody stained respiratory specimens is often hampered by non-specific fluorescence. The mucolytic agents dithiothreitol and N-acetylcysteine are used for chemical pre-treatment, as well as dilute formalin (see Sections 9.6.1. and 9.6.2).

Enzyme pre-treatment. Pre-treatment of specimens like tissue sections or tissue cultures with certain enzymes can reduce or eliminate non-specific staining. The enzyme most commonly used is trypsin, but protease, pronase, pepsin and papain also have been used. Papain met with limited success and is seldom used.

Additionally, enzymes are instrumental in unmasking antigens of microorganisms in formalin-fixed tissues or tissue cultures, especially formalin-fixed tissues preserved in paraffin blocks. Enzymes can even enhance fluorescent antibody reactions in sections from these specimens. This approach has been successful for a variety of antigens, such as viruses, fungi, and protozoan parasites. While mild enzyme treatment does reduce or remove non-specific staining from tissue sections and other specimens, its more important role is to unmask antigens of infectious agents in formalin-fixed paraffin-embedded tissues and formalin-fixed tissue cultures. For details of enzyme treatment, see Section 9.1.

9.6.1 N-acetylcysteine pretreatment of respiratory specimens

Purpose

Reduction of non-specific staining by mucus and clumped respiratory cells to detect virus infection.

Materials

Sputum.

Viruses used (others may be also successful):

respiratory syncytial virus

influenza A and B viruses

parainfluenza viruses

adenovirus.

Reagents

N-acetylcysteine solution (NAC):

N-acetylcysteine, 0.5g

Sodium citrate, 1.5g

H_2O, distilled, 100 mL.

Saline, 0.15 M, sterile:

NaCl   , 0.88g

H_2O,   100 mL.

Eagle minimal essential medium (EMEM).

Method

1. Collect a sputum specimen or nasopharyngeal washings.

2. Dilute the specimen 1:5 in saline solution. Mix as well as possible.

3. Mix the diluted specimen with an equal volume of NAC.

4. Agitate this mixture for 20 seconds, preferably on a mechanical mixer.

5. Incubate the mixture at room temperature for 20 minutes.

6. Mix the suspension again.

7. Centrifuge the mixture at about 150g for 5 to 10 minutes.

8. Remove and discard the supernatant liquid.

9. Suspend the pellet in about 0.2 mL of EMEM. (If this is not available, use saline.)

10. Prepare a slide smear from the pellet suspension and air-dry it.

11. Fix the slide smear in acetone, room temperature, 20 minutes.

12. Stain the fixed smear by direct or indirect fluorescent antibody for the virus.

13. Examine the stained smear by epi-fluorescence microscopy.

Result

Separated cells should be seen, and background fluorescence should be minimal.

9.6.2 Sodium nitrite treatment of tissue cultures

Purpose

Reduction of non-specific staining in virus-infected tissue cultures.

Materials

Specimen: tissue culture (for example: HeLa cells).

Reagents

Sodium nitrite solution, 1%

Sodium nitrite, 1 g

Acetic acid, glacial, 2 mL

H_2O, distilled, 98 mL.

Fluorescent antibody conjugate (anti-virus).

Phosphate buffered saline, pH 7.4.

Method

1. React the acetone-fixed virus–tissue culture with the virus-specific fluorescent antibody conjugate.

2. Wash the tissue culture with phosphate buffered saline, and drain most of the phosphate buffered saline. Do not allow the tissue culture to dry.

3. Flood the tissue culture with sodium nitrite solution, and allow it to react for 30 seconds to one minute. (Longer reaction times make little or no difference.)

4. Wash the tissue culture with phosphate buffered saline.

5. Examine the tissue culture with an epi-illumination fluorescence microscope.

9.6.3 Absorption of fluorescent antibody conjugates with tissue powder

Purpose

Reduction of non-specific staining factors in fluorescent antibody conjugates.

Approach

Tissue powder (or tissue culture cells) is mixed with a fluorescent antibody conjugate, and the mixture is separated by centrifugation.

Materials

Acetone dried tissue powder (tissue appropriate to the specimens to be examined—liver, brain, muscle, etc.) See Section 12, Annex13 for preparation of tissue powder. Alternatively, use commercial tissue powder.

Centrifuge tube (plastic preferred).

Centrifuge (10 000g capability preferred).

Refrigerator.

Balance, 10 mg sensitivity.

Applicator stick.

Syringe and long needle (or Pasteur pipette with rubber bulb).

Fluorescent antibody conjugate.

Phosphate buffered saline, pH 7.4 ± 0.1.

Method

1. Weigh out 100 mg tissue powder for each mL of conjugate to be absorbed (treated).

2. Place the tissue powder in a centrifuge tube.

3. Add phosphate buffered saline to the tissue powder, about the same volume or a little more than that of the conjugate to be sorbed.

4. Mix the phosphate buffered saline and tissue powder with an applicator stick.

5. Centrifuge the mixture at 10 000*g* for 15 minutes.

6. Decant all the supernatant liquid.

7. Add the conjugate to the wet tissue powder and mix it with an applicator stick.

8. Put the mixture in the refrigerator for 1 hour. Stir the mixture every 15 minutes.

9. Centrifuge the mixture at 10 000*g* for 15 minutes.

10. Remove the absorbed conjugate from the packed tissue powder carefully. *Do not disturb the sediment* (use a syringe and needle or Pasteur pipette).

11. Store the absorbed conjugate in the freezer at –20 °C.

Notes:

1. *If sorbing with tissue culture cells, use 1 mL of centrifuged, packed cells per each mL of conjugate.*

2. *It is not necessary to wet the tissue culture cells with phosphate buffered saline. More than one sorption may be necessary with some conjugates.*

References

Coons AH, Kaplan MH, 1950; Nairn RC, 1969.

9.7 Counterstain

One of the ways to overcome non-specific staining is by counterstaining the background material in a specimen. Counterstains not only cover up non-specific staining in the specimen's background, but they provide contrasting fluorescent colour that may reveal useful specimen morphology. For fluorescent antibody stains using FITC (yellow–green fluorescence), the counterstain usually has a contrasting red or orange fluorescence. However, sometimes it is brownish or even dark grey, depending on the counterstain and the pH of the reaction system. The most commonly used counterstains are the following:

- Evans blue

- Eriochrome black

- RB 200–conjugated bovine serum albumin.

Fluorescent counterstains increase contrast and reduce effects of autofluorescence or non-specific staining. Counterstaining is done with contrasting colour fluorochromes, usually red or orange. There are various ways to increase contrast and reduce autofluorescence or non-specific staining of a sample, for example:

- treating the preparation with a 0.01% to 0.1% w/v solution of Evans blue for 1 to 5 minutes after incubation, followed by washing with phosphate buffered saline

- treating the preparation with eriochrome black (CI 14645) used as a 1:30 or 1:60 w/v solution in distilled water for immunofluorescence of lymphocytes. Add one drop of this solution to the preparation after the last wash procedure and, after 10 seconds, wash it off with phosphate buffered saline

- RB 200–bovine serum albumin mixed with the FITC conjugate in a ratio of 1:10 to 1:20.

9.7.1 Evans blue

Evans blue is a popular counterstain for reducing non-specific staining because it is simple to use, effective in many situations and inexpensive. It is used two ways: as a regular counterstain added to the specimen after fluorescent antibody staining, or be mixed with the fluorescent antibody conjugate. This is usually only done with antispecies conjugates in indirect fluorescent antibody tests.

Concentrations of Evans blue used range from 0.001% to 2%. The dye can be dissolved in distilled water or phosphate buffered saline. Evans blue is prepared as a 0.5%–1% stock solution, and a use dilution made from this. The stock solution can be stored at 4 °C for up to six months, or indefinitely at –20 °C. It is convenient to make and store aliquots of 1–5 mL of the stock solution for daily dilution for use. The staining time used by various investigators ranges from 30 seconds to 20 minutes, and this is done at 37 °C or room temperature. With such a wide range of conditions reported in the literature, it is obvious that different test systems need

different concentrations and titres. Each investigator should experiment with Evans blue counterstain to find the most suitable conditions for the system under study.

Evans blue in conjugate

Materials

Anti-virus antiserum (bovine origin).

Anti-bovine conjugate with 0.005% Evans blue.

Virus-containing specimen (smear, tissue culture, tissue).

Phosphate buffered saline.

Method

1. Make a slide smear with the specimen, and fix it.

2. Cover the smear with antiserum and incubate it.

3. Wash the smear with phosphate buffered saline.

4. React the smear with antispecies conjugate containing Evans blue, 30 minutes at 37 °C.

5. Wash the stained smear in multiple changes of phosphate buffered saline and finally, water.

6. Mount a cover glass on the smear.

7. Examine it with an epi-illumination fluorescence microscope.

Evans blue as a counterstain

Materials

Specimen containing antigen.

Fluorescent antibody conjugate.

Evans blue (0.1% in phosphate buffered saline).

Phosphate buffered saline.

Buffered glycerol mounting medium.

Method

1. Fix the specimen on a slide.

2. Stain the specimen on the slide with fluorescent antibody conjugate.

3. Wash the slide in several changes of phosphate buffered saline.

4. Cover the slide specimen with Evans blue counterstain, 5 minutes.

5. Wash the slide specimen in several changes of phosphate buffered saline.

6. Mount a cover glass on the specimen.

7. Examine the specimen with an epi-illumination fluorescence microscope.

9.7.2 Eriochrome black

Eriochrome black is an excellent counterstain to reduce or eliminate non-specific staining in fluorescent antibody-stained tissue sections or other specimens. It appears to work in two ways. It stains the background pink, red, reddish brown, or light brown depending on the tissue and system involved. It also appears to quench the background non-specific staining by some mechanism that is not understood. At the same time, Eriochrome black has no effect on specific fluorescent antibody–FITC staining, unlike other counterstains such as Evans blue and RB 200–bovine serum albumin, which do exert some masking or suppressing effect on weak specific fluorescent antibody staining.

Some of the Eriochrome black counterstaining conditions reported by various investigators are in the following table:

Antigen	Specimen	Dilution	Time	Temperature
Bacteria	Tissue culture	3% in H_2O	20 minutes	Room
Virus	Tissue section	3% in H_2O	20 minutes	Room
	Clinical specimen	3% in H_2O	20 minutes	Room
Protozoa	Blood	1% in PBS	5 minutes	Room
Bacteria	Slide smear	2% in H_2O	3 minutes	Room
	Tissue section	2% in H_2O	to 1 minute	Room
Bacteria	Tissue section in H_2O	0.3%–0.7% (not critical)	10 seconds	Room
Blood vessel collagen	Tissue section	0.3% in PBS	5 minutes	Room
Fungi	Tissue section	0.4% in H_2O	3 minutes	Room

Eriochrome black can be used before or after staining tissue or specimens with specific fluorescent antibody-FITC conjugates. It is not mixed with the fluorescent antibody conjugate. Eriochrome black can be used in a wide range of concentrations from 0.3% to 3%, dissolved in either distilled water or phosphate buffered saline.

The staining reaction is stopped by washing the Eriochrome black-stained material with excess phosphate buffered saline. Cover glasses are mounted in buffered glycerine, pH 7.4–9.0, depending on the system. Each investigator must test this.

9.7.3 RB 200–bovine serum albumin

RB 200–bovine serum albumin is a conjugate of bovine serum albumin and the fluorochrome Lissamine-Rhodamine RB 200. Use it mixed with the specific fluorescent antibody conjugate at a 1:20 or 1:30 ratio. When the specimen is stained with the conjugate, the target antigen and its background are stained at the same time. (See Section 9.4.2 for conjugation of RB 200 with bovine serum albumin.)

9.7.4 Brilliant cresyl blue counterstain

Brilliant cresyl blue has been used successfully as a fluorescent counterstain for fluorescent antibody studies of cell populations. Potworowski and Nairn (1967) used it in an indirect fluorescent antibody (sandwich) technique to differentiate thymus cells in an aqueous suspension. Brilliant cresyl blue is used as a 0.02% (1:5000) solution in phosphate buffered saline as the final wash in the staining procedure. Cells not stained by the FITC conjugate are stained to give a reddish fluorescence. This helps assure that all cells in a preparation can be seen.

9.8 Immunohistochemical staining of pre-stained tissue sections

Sometimes it is important to do retrospective studies to find the true etiology of a disease. It is a routine procedure to collect tissue samples from biopsies and autopsies for histopathology study. These show tissue and cellular changes, but they

seldom specifically identify etiological agents. Fluorescent antibody can do this. In certain circumstances, fluorescent antibody can stain conventionally treated and stained histological slide sections. Enzymatic digestion of tissue sections allows this to be done.

Slides of tissue sections stained by the following methods can be used:

• haematoxylin and eosin

• Giemsa

• Brown and Brenn.

Tissues stained with most other histological stains are not satisfactory for immunofluorescence microscopy following the initial staining.

Method

1. Heat the slide *gently* over a Bunsen burner to soften the mounting medium.

2. Remove the cover glass carefully with a toothpick.

3. Dissolve and remove the mounting medium with xylene. Two changes and gentle agitation may be needed.

4. Rehydrate the slide through an ethanol series; 3 minutes each in 95%, 90%, 85%, 80%, 70% ethanol, and water.

5. Treat the slide with acid alcohol (97 mL of ethanol 95%, 3 mL of concentrated HCl), 30 seconds.

6. Place the slide in phosphate buffered saline, two changes, 30 minutes each.

7. Digest with trypsin 1%, and proceed with fluorescent antibody staining.

Note: this methood assumes that the tissue section is mounted on the slide with an effective adhesive.

Reference

Gordon MA, Lapa EW, 1984.

9.9 Unmasking antigens in formaldehyde-fixed paraffin-embedded tissues

Early in fluorescent antibody studies, antigens of microorganisms were localized in tissues. However, most antigens are difficult to stain by fluorescent antibody in conventional formalin-fixed tissues. The antigenicity of many antigens fixed by formalin is altered to the point that fluorescent antibody tests cannot detect them, while others yield very weak specific fluorescence. Formalin fixation affects antigenic determinants by changing amino groups and protein cross-linking. Thus, receptors for fluorescent antibodies are altered. This is the reason for difficulties in fluorescent antibody staining of formalin-fixed tissues.

Investigators used fresh or frozen cryostat-cut tissue sections in order to detect microbial antigens in these tissues. However, there are disadvantages to using fresh or frozen tissue sections. Some of these are:

- morphological features may be lost

- cell types may be obscured

- distribution of intracellular antigens may be unclear

- definition of inclusions, intracellular and intranuclear, cannot be made

- affected tissue areas in small specimens may be missed

- infectious hazards to laboratory workers may come from unfixed specimens

- shipping tissues frozen or on ice is difficult in many places

- storage problems limit retrospective studies

- special frozen section cutting equipment and skills are needed.

Enzymatic digestion of formalin-fixed paraffin-embedded tissues "unmasks" antigens of microorganisms in the tissues and makes them reactive in fluorescent antibody tests. The enzymes commonly used are:

- trypsin

- protease (Pronase, Nagarase)

- pepsin.

Note: enzymes are heat labile. Stock solutions should be stored frozen at –20 °C; use solutions at 4 °C.

Various investigators used protease to unmask fluorescence. Pronase, a protease obtained from *Streptomyces griseus*, enhances fluorescence. Pronase is a non-specific protease. For that reason, some investigators preferred the gentler trypsin (Huang, 1976). Protease "Type VII: Bacterial" is no longer available, but new replacement proteases exist. One is Sigma "Type XXVII" Nagarase, consistent with material offered before 1975. Material offered after 1975 is consistent with Sigma "Type XXIV: Bacterial". Investigators wishing to replicate results from the literature before and after 1975 should use these new proteases.

Gentle digestion with trypsin is by far the most commonly used method today. Working solutions vary from 0.0625% to 1.0%, most being from 0.1% to 0.25%. Thus, the concentration is not very critical, except for deep fungal infections where 1% trypsin digestion is used. However, trypsin alone is not best for all antigens. Sometimes a combination of enzymes is used, such as trypsin and pepsin.

Formaldehyde treatment followed by paraffin embedding preserves antigens very well. Using enzyme digestion of formalin-fixed paraffin-embedded blocks of tissues, many antigens were easily detectable by fluorescent antibody after 10–20 years of storage. Indeed, Korean haemorrhagic fever virus was detected after 31 years of such storage.

Conditions of enzymatic digestion of formalin-fixed tissue sections vary considerably. Basic formaldehyde fixation of tissues and some successful methods are outlined below.

9.9.1 Basic formadehyde fixation and processing of tissues

Purpose

Preparation of fixed specimens in paraffin for fluorescence microscopy.

Approach

Small tissue blocks or biopsy specimens are fixed in buffered formalin, dehydrated, cleared, infused with melted paraffin and sectioned on a microtome.

Materials

Microtome.

Oven.

Refrigerator or cold plate.

Flotation water bath, 44 °C (or up to 56 °C).

Oven, 60 °C.

Laboratory tools: scalpel, forceps, scissors, etc.

Formalin, 10% (4% formaldehyde) in phosphate buffered saline, pH 7.2–7.4.

Phosphate buffered saline, pH 7.2–7.4.

Ethanol, absolute (100%).

Ethanol series , 95%, 80%, 70%, 50%.

Chloroform.
Note: xylene may be substituted for chloroform.

Xylene.

Liquid paraffin, 60 °C.

Tissue specimen (caution: this may contain infectious agents).

Method

1. Take an appropriate tissue specimen for the antigen or disease to be studied.

2. Place a small block of it in buffered 10% formalin. Blocks of $2 \times 4 \times 4$ mm are suggested.

3. Allow the formalin to act on the tissue for at least 2 days.
    Note: various authors recommend 3 to 10 days for tissues containing rabies virus, 3 days for adenovirus and 2 days for *Chlamydia psittaci*–infected tissue.

4. Dehydrate the formalin-fixed tissue specimen through a graded ethanol series and clearing agents:

    70% ethanol, 1 hour

    80% ethanol, 1 hour

> 95% ethanol, 2 changes, 1 hour each
>
> absolute ethanol, 2 changes, 1 hour each
>
> absolute ethanol + chloroform, equal parts, 1 hour
>
> chloroform, 2 changes, 1 hour each.

5. Embed the processed tissue in paraffin at 60 °C.

6. Harden the paraffin block in the refrigerator or on a cold plate.

7. Cut sections of the paraffin-embedded tissue on a microtome. Depending on the antigen under study, these can be 2–9 μm thick.

8. Float a tissue section onto a slide from a tissue flotation water bath at 44–56 °C.

9. Dry the section on the slide at 60 °C for 20 minutes to several hours.

10. Deparaffinize the sections in two changes of xylene, 3 minutes each.

11. Rehydrate the sections by passing them through two changes each of absolute ethanol, 95%, 80% and 50% ethanol and water.

12. Place the slide section in phosphate buffered saline.

13. Proceed with enzyme digestion and fluorescent antibody staining.

Note: laboratories may wish to vary this general method based on their experience.

9.9.2 Trypsin (0.25%) digestion of formaldehyde treated tissue sections

Purpose

Enzyme digestion of formaldehyde treated tissue sections to unmask antigenic sites for fluorescent antibody tests.

Approach

Trypsin, 0.25%, is used to unmask a variety of virus antigens in formalin-fixed paraffin-embedded tissue sections for detection by indirect fluorescent antibody.

Materials

Incubator, 37 °C

Tissue section flotation water bath, about 56 °C

Tissue sections, deparaffinized and rehydrated.

Antisera to viruses, inactivated at 56 °C, 30 minutes.

Anti-species globulin conjugate.

Trypsin solution (store at 4 °C or frozen).

Trypsin (1:250) 0.25% solution in water.

Note : The ratio 1:250 indicates that 1 part of trypsin digests 250 parts of casein under the conditions of the USP test.

Ethanol series 95%, 80%, 70%, 50%.

Chloroform.

$CaCl_2$, 0.02 g.

Phosphate buffered saline, pH 7.6–7.7.

Method

1. Coat slides with a suitable adhesive.

2. Float tissue sections (2–3 µm thick) on a 55–57 °C water bath, and pick them up on the slides.

3. Dry the tissue section slides at 37 °C for 24 hours.

4. Remove the paraffin with several changes of xylene.

5. Rehydrate the sections in an ethanol series from 95% down to water.

6. Wash the slides in phosphate buffered saline and distilled water, 5 minutes each.

7. Digest the tissue on the slide with 0.25% trypsin solution, 37 °C, 3 hours (a Petri dish or coplin jar may be used).

8. Rinse the slides in phosphate buffered saline, 15 minutes.

9. Stain the slides by direct or indirect fluorescent antibody.

10. Examine the slides for specific virus fluorescence with an epi-illumination fluorescence microscope.

Notes

1. *Fixation in 10% formalin for about 7 days is best. Fixation of the tissue for a year in formalin makes the viral antigens more difficult to detect.*

2. *Viral antigens in tissues fixed for the shorter period above and then embedded in paraffin retain their ability to be unmasked by trypsin digestion for many years.*

3. *This approach has been used for many viruses. Details vary for time of fixation, type of virus and length of storage. Each investigator must work out the details for the system under study.*

4. *Formalin fixation kills many viruses, but for the most dangerous viruses (for example, Lassa, Ebola, Marburg, Crimean–Congo, Rift Valley fever, and Hanta), investigators must make sure of complete inactivation by using gamma radiation of tissue sections or spot smears on slides. ß-propiolactone treatment may also be used.*

References

Hondo R et al., 1982; Kurata T et al., 1983; Swoveland PT, Johnson KP, 1979.

9.9.3 Trypsin (1%) digestion of formaldehyde treated tissue sections

Approach

Fungi causing deep mycoses are detected in formalin-fixed tissues after 1.0% trypsin digestion.

Materials

Tissue sections containing fungus cells.

Tissue section flotation water bath, about 56 °C.

Trypsin (1:250) 1.0% solution in water (see Section 12, Annex 13)

Screwcap freezer vials, 10 mL.

Freezer, –20 °C.

Centrifuge, refrigerated; 3500g capability.

Epi-illumination fluorescence microscope.

Ethanol series, 95%, 80%, 70%, 50%

Phosphate buffered saline, pH 7,6 - 7,7 (see Section 12, Annex 13)

Method

1. Float a paraffin section on to a slide treated with Elmer's "Glue-All" glue.

2. Deparaffinize tissue sections on slides, and rehydrate them through an ethanol series to water.

3. Dip the slide into phosphate buffered saline, and then soak it in two changes of phosphate buffered saline, 30 minutes each.

4. Immerse the slide in 1% trypsin solution, 30 minutes, in a 37 °C water bath.

5. Drain the slide, and gently heat-fix it in a flame or on a slide warmer to stop the trypsin digestion.

6. Stain the slide with an FITC conjugate (counterstains such as Eriochrome black may be used to quench non-specific staining).

7. Mount a cover glass on the slide in buffered glycerol, pH 9.0.

8. Examine the slide with an epi-illumination fluorescence microscope.

Reference

Gordon MA, Lapa EW, 1984.

9.9.4 Pepsin and trypsin digestion of formaldehyde treated tissue sections

Approach

Rabies virus antigen in brain tissue is unmasked by pepsin and trypsin digestion.

Materials

Tissue section, formaldehyde-fixed and paraffin-embedded (fixation with 10% neutral formalin for 10 days).

Xylene.

Ethanol.

Phosphate buffered saline (Ca^{2+} free, Mg^{2+} free), 0.1 M, pH 7.6.

Trypsin 0.25% and 0.1% $CaCl_2$ in distilled water.

Pepsin 0.4% in distilled water.

Anti-rabies fluorescent antibody conjugate.

Method

1. Mount the tissue section on a slide. (Float it on from a flotation water bath at about 56 °C. An adhesive substance may be used to stick the tissue section to the slide.)

   Note: Histostix coating is used as an adhesive on the slide, and the slide is dried at 60 °C, 24 hours. Elmer's Glue-All also may be used as an adhesive if not too thin or too thick.

2. Dry the slide for 24 hours at 60 °C.

3. Deparaffinize the section with xylene.

4. Rehydrate the section in a graded ethanol series.

5. Rinse the section in phosphate buffered saline, 5–10 minutes.

6. Digest the section in pepsin solution, 0.4%, 1 hour, room temperature.

7. Digest the section in trypsin solution, 0.25%, 90 minutes, room temperature.

8. Wash the digested section in phosphate buffered saline, 30 minutes.

9. Stain the section with anti-rabies conjugate, wash it in phosphate buffered saline and mount a cover glass on it with buffered glycerine, pH 8.5–9.0.

   Note: onjugate is used at 4× the normal staining titre to get 4+ fluorescence.

10. Observe the section with an epi-illumination fluorescence microscope.

Notes

1. *This test method is* not *recommended to replace the standard direct fluorescent antibody test done on fresh tissues for rabies diagnosis. However, it may be useful in retrospective studies of formalin-fixed paraffin-embedded tissues, or it may help make diagnoses when there is no fresh brain specimen to test. Sometimes shipment of brain tissue in ice is not possible. Formalin fixation can be used in these situations.*

2. *This approach may be useful in studies of other viral diseases like encephalitis when single enzymatic digestion is not successful.*

References

Reid FL et al., 1983; Brozman M, 1978; Enestrom S et al., 1980.

9.9.5 Protease type XXVII digestion of formaldehyde fixed tissue sections

Approach

Formalin-fixed paraffin-embedded tissue sections are pretreated with protease in order to reduce non-specific staining and enhance specific fluorescent antibody fluorescence.

Materials

Tissue with target antigen in formalin-fixed paraffin-embedded liver biopsy specimens. (Antigens were hepatitis B surface antigen [HBsAg] and hepatitis B core antigen [HBcAg].)

Xylene.

Ethanol.

Phosphate buffered saline, pH 7.4.

Nagarase (Sigma).

Protease type XXVII (Sigma), 0.1%, pH 7.4 in distilled water.

Tissue flotation water bath, 56 °C.

Fluorescent antibody reagents specific for HBsAg and HBcAg.

Fluorescent antibody supplies and equipment.

Method

1. Cut tissue into 4 µm sections, and float the sections on to slides. Use slide adhesive to hold them on the slides during the digestion process.
    Note: Histostix or Elmer's Glue-All may be used. See Section 9.11.

2. Dry the sections on the slides at 60 °C, 1 hour.

3. Remove the paraffin with several changes of xylene.

4. Rehydrate the sections through an ethanol series to water.

5. Incubate the slides in the protease solution, 37 °C for 1 hour.

6. Wash the slides in phosphate buffered saline, several changes.

7. Drain the slides, but do not allow them to completely dry.

8. Stain the slides with specific fluorescent antibody by standard methods. Use either direct or indirect fluorescent antibody test methods.

9. Observe the slides with epi-illumination fluorescence microscope.

References

Huang S, 1975; Huang S et al., 1976; Radaszkiewicz T et al., 1979.

9.10 Storage of reagents, conjugates, antisera, and blood serum specimens

Immunological reagents must neither change their properties nor cause cleavage of antibodies or antigens. Use a particular method for storage, and keep the conditions constant as much as possible. Long-term storage of all serological reagents should be at –20 °C or lower. Repeated freezing and thawing results in a reduction of antibody activity. To avoid this, appropriate small quantities (aliquots) of conjugates, positive controls and negative controls should be kept frozen. The amount in each vial should be chosen to be consistent with normal use.

Antibody conjugates. A conjugate, such as FITC–anti-human IgG, should be dispensed in volumes of 100 mL in Eppendorff tubes and frozen. Immediately before use, a tube is taken from the deep freeze, thawed and diluted to the working concentration, e.g. 1:40, with phosphate buffered saline. Four mL of conjugate are sufficient for 40 tissue sections.

Positive controls. Serum containing antimitochondrial antibodies (titre = 1:800) is diluted 1:10 with phosphate buffered saline, and then frozen in 0.05 mL aliquots. Before use, 0.95 mL phosphate buffered saline is added to result in an initial dilution of 1:200. Serial dilutions of 1:200, 1:400, 1:800 and 1:1600 are made and used as positive controls.

Negative controls. A negative control such as normal serum is kept frozen, without dilution, in 0.05 mL aliquots. The control must be diluted 1:20 by addition of 0.95 mL phosphate buffered saline.

Patient serum. This specimen should not contain products of haemolysis. The samples are frozen in volumes between 0.5 and 10 mL. Each tube or container should be marked with an ID number and entered in a sample register.

Centrifugation may be necessary after thawing to separate impurities or precipitated protein.

9.11 Sticking tissue sections to slides

Sometimes it is difficult to make tissue sections adhere to slides during histological processing, especially during enzyme digestion of formalin-fixed sections. A number of adhesives have been recommended to solve this problem. These are:

* collodion in ether

* Histostix

* Neoprene in toluene

* gelatin-coated slides

* Elmer's Glue-All (casein glue)

* LePage Bondfast (resin glue).

Adhering tissue sections to slides that are going through lengthy digestion and staining procedures is a problem. When the tissue section slips off the slide, the procedure usually must start again, beginning with cutting new tissue sections. Sometimes this is critical because only very small tissue specimens are available. Because of this, many investigators put forward a variety of ways to stick paraffin-embedded tissue sections to slides.

Few comparisons have been made among these methods. Gordon and Lapa (1983) report that Histostix performed as well as did their Elmer's Glue-All method. However, Reid et al. (1983) reported difficulties with the Elmer's Glue-All method. In their hands, too little glue on the slide was ineffective, while too much glue gave excessive autofluorescence. Phenol–gelatin solution, another method, sometimes would not hold the tissues on the slides. They indicated routine success with Histostix. The methods using the flammable solvents ether and toluene present laboratory hazards that might better be avoided. If others can confirm it, the simple LePage Bondfast method seems satisfactory for most tissues, as the glue is simply applied with no special preparation and is non-fluorescent.

Since there is little agreement as to which method is best, investigators wishing to do this work must experiment and choose the method that is most suitable in their hands.

Collodion treatment

Materials

Collodion, 0.83 mL.

Ether, 10 mL.

Method

1. Place the tissue section on the slide.

2. Cover the section with collodion solution.

CAUTION: this solution is highly flammable, and the fumes are potentially explosive. Avoid open flames and electrical sparks. Store ether in a well stoppered container only in an explosion-proof refrigerator.

Histostix treatment

Material

Histostix (Accurate Chemical and Scientific Corp., Westbury, New York).

Method

1. Coat the slide with Histostix according to the manufacturer's directions.

2. Mount the tissue section on the slide from a tissue flotation water bath.

3. Dry the mounted tissue section at 60 °C, 24 hours.

Neoprene in toluene treatment

Materials

Neoprene (polychloroprene), 0.1 g.

Toluene, 100 mL.

Method

1. Coat the slide with neoprene–toluene by dipping it in the solution.

2. Dry the coated slide for 30 minutes at room temperature.

3. Place the tissue sections in a flotation water bath, 55–57 °C.

4. Float the tissue section on to the slide.

5. Dry the tissue section on the slide at 37 °C, 24 hours or more.

CAUTION. Toluene is flammable. Avoid flames.

Gelatin coating

Materials

Gelatin (laboratory grade), 5 g.

H_2O, distilled, hot, 800 mL.

Chromic potassium sulfate, 0.5 g.

Method

1. Dissolve the gelatin in hot distilled water.

2. Add the chromic potassium sulfate.

3. Filter the solution through filter paper.

4. Dip slides in the solution several times.

5. Drain the slides, and dry them at room temperature in a vertical position.

6. Mount the tissue sections on the gelatin-coated slides from a flotation water bath.

7. Incubate mounted tissue sections at 56 °C, overnight.

Elmer's Glue-All coating

Materials

Elmer's Glue-All (Borden, Inc.), 4 drops.

H_2O, deionized, sterile, 2 mL.

Method

1. Add the Elmer's Glue-All to the water.

2. Mix the solution thoroughly.

3. Coat a slide lightly. Use a brush or finger.

4. Float a paraffin-embedded tissue section onto the slide.

5. Dry the section on the slide at 37 °C, overnight.

LePage Bondfast glue coating

Material

LePage Bondfast resin glue

Method

1. Preclean the slide to remove any oil residue.

2. Smear the glue on the face of the slide thinly. Use a water-wetted finger.

3. Immediately, float the paraffin-embedded tissue section onto the slide from a 55–57 °C water bath.

4. Drain excess water from the tissue section on the slide.

5. Dry the tissue section slide at 60 °C, 1 hour.

10. Characterization of antisera and conjugates

Using conjugates having the same specificity and similar properties is essential for reproducible and comparable results. Appropriate reference antigens and antisera must be available in order to make standardization possible. Also, there must be a consensus on the methods of analysis.

False negative and false positive results occur because the antigen substrates, antibodies, methods and microscope may not be suitable or the investigator may not have sufficient experience. False negative results can be recognized only when known pure antigen and antibody standards or references are available for testing. False positive results may occur for reasons that are not clear. A true positive result with tissues or microorganisms is recognizable by structure-specific fluorescence of the correct colour, with faint or non-fluorescent negative controls. For example, the morphology or anatomy of microorganisms or tissues should be recognizable in a colour that is typical for the fluorochrome being used. In some cases, a conclusion as to whether the result is positive or negative may be difficult or impossible to reach because of unwanted or background fluorescence.

10.1 Methods for characterizing conjugates

10.1.1 Assessment of free dye in the conjugate

Purpose

Detection of unreacted dye after purification of a conjugate (see Section 9.5.1) that could cause non-specific staining.

Approach

Thin-layer chromatography of the conjugate is done in miniature on a microscope slide.

Materials

Conjugate.

Sephadex G-25 or G-50.

Phosphate buffered saline.

Microscope slide.

Petri dishes (2).

Filter paper strips, 25 mm $\times$ 75 mm (2).

Micropipette.

Method

1. Suspend Sephadex G-25 or G-50 in phosphate buffered saline and allow it to sediment for about 15 minutes.

2. Remove the supernatant liquid.

3. Spread a drop of the concentrated Sephadex suspension as a thin layer on a microscope slide.

4. Place the slide on the edges of two Petri dishes, one beside and above the other, making a slope of 10° to 20°.

5. Use strips of filter paper to connect the layer of gel on the slide with the two Petri dishes.

6. Fill the higher of the two dishes with phosphate buffered saline.

7. Wait 3 minutes.

8. Apply a few microlitres of the conjugate to the gel layer, approximately 1 cm from its upper end.

9. Observe the result after about 20 minutes. The labelled antibody will have moved down the slide a few centimetres, but any free dye will stay at the

application point. If free dye remains, it must be removed by gel filtration. (Usually the conjugate has already been passed through a Sephadex gel column, so a free dye result is unlikely.)

10.1.2 Assessment of the fluorescein/protein ratio of FITC conjugates

Measurement of the molar concentration of protein-bound FITC is difficult because the absorption maxima of the dye change when FITC is coupled to protein. Differences in dye quality cause additional problems. This explains why different procedures have been used for determining the fluorescein/protein ratio.

Measuring protein concentration

Approach

Use pure bovine serum albumin or a standard protein solution as a reference to determine the protein concentration (mg/mL). The choice of method may be influenced by the concentration of the protein, the presence of interfering substances and the absorption method. The absorption maxima used for the test should not interfere with, or be interfered by, the fluorochrome. The following wavelengths are suitable for quantitative work:

Biuret   540–560 nm

Lowry–Folin   500 nm.

Measuring protein-bound FITC

Approach

FITC concentration is measured by spectrophotometry using fluorescein diacetate. This compound is used as the standard because FITC is unstable in solution.

Materials

Spectrophotometer.

Water bath.

500 mL volumetric flask.

Polyethylene bottles, 10–25 mL.

Method

   Preparing FDA standard solution (100 mg/L)

   1.   Add 3 g of NaOH to 40 mL 95% ethanol under reflux while stirring in a boiling water bath.

   2.   Decant the solution from excess NaOH that does not dissolve.

   3.   Add 50 mg FDA to 25 mL of the hot alcoholic NaOH solution.

   4.   Heat the alcoholic NaOH stock solution (25 mL) and the FDA (50 mg) in a water bath until all the FDA has dissolved, then cool the solution to room temperature.

   5.   Transfer the FDA solution to a 500 mL volumetric flask, after first washing the flask with distilled water.

   6.   Make the solution up to 500 mL with distilled water.

This stock solution contains 10 mg FDA/100 mL, i.e.100 µg/mL. It can be stored in the refrigerator (cool and dark) for up to two years in small polyethylene bottles of suitable size to prepare dilutions for use.

Preparing an FDA standard curve

   1.   Dilute the FDA stock solution with NaOH, 0.1 N, to give final FDA concentrations in the range 1–4 µg/mL (for example, diluted 1:100 = 1 µg/mL; 1:50 = 2 µg/mL; 1:25 = 4 µg/mL).

   2.   Measure the extinction at 490 nm for each standard dilution, using 0.1 N NaOH in the reference cuvette (10 mm glass).

   3.   Convert the FDA concentrations to FITC concentrations by multiplying by 1.07, as a correction factor, for the lower extinction coefficient of FITC.

   4.   Plot the values to prepare a calibration curve (Figure 10.1).
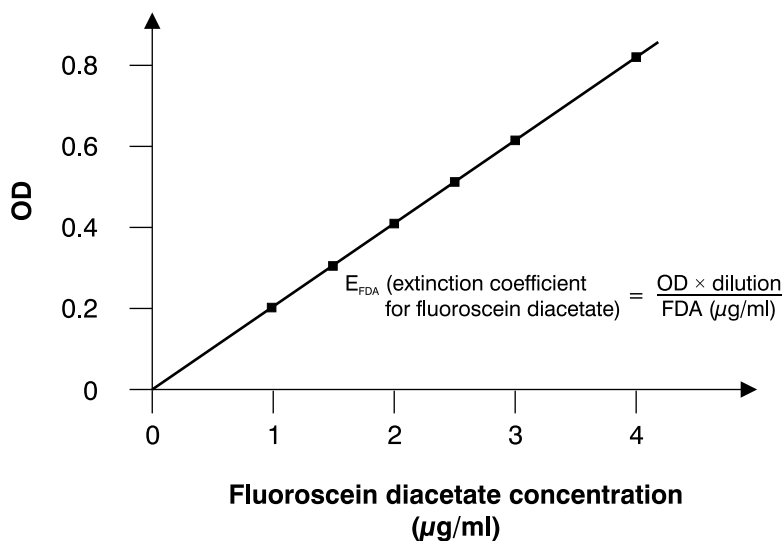
Figure 10.1 **Change of optical density (OD) of a fluorescein diacetate (FDA) standard at different concentrations**

Determination of the amount of FITC in the conjugate

Only FITC concentrations between 1 mg/mL and 6 mg/mL can be measured accurately. Therefore, protein solutions must be diluted. For example, with a protein content of 30 µg/mL (3% w/v) and based on a mean value for the fluorescein/protein ratio, the expected value for the dye content would be $5 \times 10^3 \times 30$ µg/mL = 150 mg/mL. A dilution of 1:50 with 0.1 N NaOH is needed. Adjust the reaction to pH 13, and measure the extinction at 490 nm.

Find the concentration of protein-bound FITC in mg/mL by multiplying the measured FITC concentration by the serum dilution factor. If no dilution was made, the value should be multiplied by a correction factor of 1.07.

Calculation of the fluorescein/protein ratio

1. Express protein concentrations in mg/mL.

2. Find the FITC amount using the optical extinction at 490 nm in 0.1 N NaOH with reference to a pure FDA standard.

3. Calculate the fluorescein/protein ratio and express the result as milligrams of FITC per milligram of protein.

For conversion into the molar ratio, use a factor of 0.411. The factor is calculated from the molecular weight of FITC (389) and IgG (160 000).

Note: commercial conjugates may contain unlabelled proteins for stabilization and reduction of non-specific staining. Therefore it is necessary to identify the type and amount of the added protein first to find the correct fluorescein/ protein ratio.

References

Bradley, 1976; Lowry et al., 1951; Weichselbaum, 1946.

10.1.3 Assessment of the the molar fluorescein/protein ratio of TRITC conjugates

The correct wavelength must be chosen for quantitative determinations, since there is no stable reference material available to substitute for TRITC. Amorphous TRITC has two absorption maxima, at 515 nm and 550 nm, whereas crystalline TRITC has only one maximum (555 nm).

Use the following expression for calculating the molar fluorescein/protein based on a molecular weight of 160 000. Ratio of IgG conjugates to crystalline TRITC:

Molar fluorescein/protein = 6.6 × OD 555 nm protein concentration (mg/mL)

The factor 6.6 is the ratio of the extinction coefficient of 1 M TRITC (0.041) and the factor for converting IgG concentration in mg/mL to molarity (0.00625). A correction factor *a* must be applied to find the protein concentration.

IgG concentration (mg/mL) = $(OD_{280\,nm} - [a \times OD\,\lambda max])/1.4$

The correction factor *a* for TRITC = 0.56.

10.1.4 Determination of staining titres of conjugates for direct fluorescent antibody tests

Purpose

Finding the specific fluorescent antibody optimum conjugate staining titre with homologous antigen.

Approach

A two-fold serial dilution is made of the conjugate, and these dilutions are reacted with the specific antigen and a negative (heterologous) antigen control. Brightness of specific staining is assessed for each dilution.

Materials

Basic equipment for fluorescence microscopy.

Basic supplies for fluorescence microscopy.

Conjugate, specific.

Antigen, homologous to the conjugate, fixed appropriately on slides (specific staining control).

Note: this may be bacterial smears, tissue culture spot slides, or infected tissue.

Antigen, heterologous to the conjugate (non-specific staining control).

Phosphate buffered saline.

Test tubes.

Serological pipettes, 1 and 5 mL.

Fixation method (heat or chemical appropriate for the antigen).

Method (example: bacteria)

1. Prepare slide smears of 18–24 hour cultures of bacteria.
    Note: suspensions of bacteria in distilled water make good substrates.

2. Heat-fix the smears.

3. Make serial two-fold dilutions of the conjugate in phosphate buffered saline.
    Note: the maximum dilution tested is learned by experience with the antigen–antibody system used.

4. React smears of the bacteria with conjugate dilutions according to standard fluorescent antibody test methods.

5. Examine the smears by epi-illumination fluorescence microscopy, and grade the fluorescence intensity as 0, 1+, 2+, 3+ (bright) or 4+ (brilliant).

Note: it is preferable that one person code the test smears and another person do the slide fluorescence assessment without knowing the code (blind reading).

Results

The staining titre is the highest two-fold dilution of conjugate that yields 3+ specific fluorescence. The control, heterologous bacteria or other antigen, should not stain at all, or only slightly at low dilutions (more concentrated).

Use dilution

This is usually one two-fold dilution below (more concentrated) than the titre dilution. (For example, if the highest dilution of conjugate that gives 3+ fluorescence is 1:16, then the use dilution is 1:8.)

11. History of fluorescence microscopy

The history of fluorescence microscopy started in 1904 when Köhler examined biological materials under a microscope using ultraviolet light. In 1938, Haitinger summarized the actual knowledge about the application of fluorochromes in microscopy. The birthday of immunofluorescence microscopy (fluorescent antibody) was in 1941, when Coons, Creech and Jones conjugated the fluorescent compound ß-anthryl isocyanate to pneumococcal antiserum. This fluorescent antiserum considerably improved detection of pneumococcal antigen in tissue. The original labelling of antibody with fluorescein isocyanate was quite complex, and this limited its use to more sophisticated laboratories. In 1958, Riggs et al. reported a more stable fluorochrome, fluorescein isothiocyanate (FITC). This was more easily conjugated to antibody and made fluorescent antibody methods available for general use.

All this original work was done by transmitted light fluorescence microscopy. Brumberg was the first to describe the epi-illumination microscopy technique in 1959. This was used and preferred in the USSR and eastern Europe for several years before it was appreciated in the West, when Leitz and Zeiss were the first to produce epi-illumination fluorescence microscopes. In 1967, Ploem reported a vertical illuminator and a dichroic mirror system that permitted passage of selected-wavelength light through a dichroic mirror and reflected other wavelengths. In this technique the low-wavelength excitation beam is reflected downward and focused on the specimen through the objective lens, and the higher-wavelength emitted light is transmitted back through the objective to the eye through the dichroic mirror.

With the epi-illumination system two microscopic techniques could be combined; for example, to examine living cells stained with fluorochromes or

fluorescent antibodies by both fluorescence and phase contrast microscopy simultaneously. Furthermore, epi-fluorescence microscopy was more efficient and simpler to use.

The introduction of halogen lamps for fluorescence microscopy made it possible to provide more economical light sources for epi-fluorescence microscopes. This meant that fluorescence microscopy could be used under more difficult working conditions and in smaller laboratories for a wide range of rapid diagnostic tests.

In 1967, Tomlinson reported a filter and lamp system to view fluorescent antibody preparations with a 100 watt quartz–iodine lamp. In 1968, Johnson and Dollhopf expanded on Tomlinson's work. Interestingly, the filter combination they used gave satisfactory bright orange fluorescence from rhodamine conjugates. Others reported a lack of fluorescence in rhodamine fluorochromes with iodine–quartz illumination when using interference filters. This orange fluorescence was achieved by transmitted light fluorescence microscopy using colour glass and gelatin filters. Perhaps their success lay in the filter systems used.

Nairn summarized the development of fluorescence microscopy in the fourth edition of his publication *Fluorescent protein tracing*. Since then, development of more sophisticated techniques, such as laser fluorescence microscopy and confocal fluorescence microscopy, shows that fluorescence microscopy technology has not reached its end.

References

Brumberg EM, 1959; Coons AH et al., 1941; Haitinger M, 1959; Johnson GD, Dollhopf FL, 1968; Köhler A, 1904; Nairn RC, 1975; Ploem JS, 1967; Riggs JL et al., 1958; Tomlinson AH, 1967; Wijnaendts van Resandt RW et al., 1985.

12. Annexes

SUPPLIERS
12.1 Suppliers of fluorescence microscopes

Helmut Hund GmbH (formerly Will Wetzlar GmbH)
Wilhelm-Will-Strasse 7
D-35580 Wetzlar
Germany
Tel.:   +49 644 120 040
Fax: +49 644 120 0444
www.hund.de

Leica Microsystems AG
Ernst-Leitz-Strasse 17–37
D-35578 Wetzlar
Germany
Tel.:   +49 6441 29 0
Fax: +49 6441 29 2590
www.leica-microsystems.com

Lomo America, Inc.
15 East Palatine Road
Unit 104
Prospect Heights IL 60070
USA
Tel.:   +1 847 215 8800
Fax: +1 847 215 9073
www.lomoplc.com

Note: Russian microscope made in St Petersburg.

LW Scientific, Inc.
4727-G North Royal Atlanta Drive
Tucker GA 30084
USA
Tel.:   +1 770 270 1394
Fax: +1 770 270 2389
www.lwscientific.com

Nikon Corporation
9–16 Ohi 3-chome
Shinagawa-ku
Tokyo 140-8505
Japan
Tel.:   +81 3 3773 8121
www.nikon.com

Olympus Corporation
Shinjuku Monolith
3–1 Nishi-Shinjuku 2-chome
Shinjuku-ku
Tokyo 163-0914
Japan
Tel.:   +81 3 3340 2111
www.olympus-global.com/en/global/index.html

Portable Medical Laboratories, Inc.
PO Box 667
Solana Beach CA 92075-0667
USA
Tel.:   +1 858 755 7385
Fax: +1 858 259 6022
www.portable-medical-lab.com

Seiler Instrument & Manufacturing Company,
Inc.
Microscope Division
170 East Kirkham Avenue
St Louis MO 63119
USA
Tel.:   +1 314 968 2282
Fax: +1 314 968 2637
www.seilerinst.com

Thomas Scientific
99 High Hill Road
PO Box 99
Swedesboro NJ 08085-0099
USA
Tel.:   +1 856 467 2000
Fax: +1 856 467 3087
www.thomassci.com

United Products & Instruments, Inc.
182 Ridge Road
Suite E
Dayton NJ 08810
USA
Tel.:   +1 732 274 1155
Fax: +1 732 274 1151
www.unicoupi.com

Wang BioMedical Europe
Bowlespark 30–30a
6701 DS Wageningen
Netherlands

Tel.:   +31 317 416 025
Fax: +31 317 427 278
www.wangbiomed.com

Western Scientific Co., Inc.
4104 24th Street #183
San Francisco CA 94114
USA
Tel.:   +1 415 826 5732
Fax: +1 415 826 5738
www.wescomicroscopes.com

Carl Zeiss, Inc.
Microscopy & Imaging Systems
1 Zeiss Drive
Thornwood NY 10594-1939
USA
Tel.:   +1 914 747 1800
Fax: +1 914 681 7446
www.zeiss.com/micro

Carl Zeiss Mikroskopie
Königsallee 9–21
D-37081 Göttingen
Germany
Tel.:   +49 551 50 60 660
Fax: +49 551 50 60 480
www.zeiss.de/micro

Zirconium, Inc.
1744 rue William bureau 108
Montreal
Quebec H3J 1R4
Canada
Tel.:   +1 514 334 0322
Fax: +1 514 334 1455
www.zirkonium.ca

12.2 Suppliers of fluorescence conversion kits for microscopes

Portable Medical Laboratories, Inc.   Tel.:   +1 858 755 7385
PO Box 667   Fax: +1 858 259 6022
Solana Beach CA 92075-0667
USA

12.3 Suppliers of fluorescent antibody conjugates, kits and antisera

ABI   Advanced Biotechnologies, Inc.
        Rivers Park 2
        9108 Guilford Road
        Columbia MD 21046-2701
        USA
Tel.:   +1 410 792 9779
Fax: +1 301 497 9773
        www.abionline.com

ACC   Accurate Chemical Scientific Co.
        300 Shames Drive
        Westbury NY 11590
        USA
Tel.:   +1 516 333 2221
Fax: +1 516 997 4948
        www.accuratechemical.com

ACE   Access Biomedical
        6362 Ferris Square
        San Diego CA 92121
        USA
Tel.:   +1 858 452 7700
Fax: +1 858 452 7701
        www.accessbiomed.com

ADI   Advanced Immunochemical, Inc.
        105 Claremont Avenue
        Long Beach CA 90803
        USA
Tel.:   +1 562 434 4676
Fax: +1 562 494 3776
        www.advimmuno.com

AMQ   American Qualex Antibodies, Inc.
        920A Calle Negocio
        San Clemente CA 92673
        USA
Tel.:   +1 949 492 8298
Fax: +1 949 492 6290
        www.americanqualex.com

AMR   American Research Products, Inc.
        489 Common Street
        Belmont MA 02478
        USA
Tel.:   +1 617 489 1120
Fax: +1 617 489 5120
        www.arp1.com

ANT   Antibodies, Inc.
        PO Box 1560
        Davis CA 95617-1560
        USA
Tel.:   +1 530 758 4400
Fax: +1 530 758 6307
        www.antibodiesinc.com

ARG   Argene SA
        Parc Technologique Delte Sud
        09120 Varilhes
        France
Tel.:   +33 56 16 96 100
Fax: +33 56 16 96 101
        www.argene.com

BCM   Biochemed Corp.
        9552 Telhan Drive
        Huntington Beach CA 92646
        USA
Tel.:   +1 714 963 4117
Fax: +1 714 964 8881

BET   Bethyl Laboratories, Inc.
        PO Box 850
        Montgomery TX 77356
        USA
Tel.:   +1 936 597 6111
Fax: +1 936 597 6105
        www.bethyl.com

BGN   Biogenesis Ltd.
       Technology Road
       Poole BH17 7DA
       Dorset
       United Kingdom
Tel.:   +44 1202 660 006
Fax: +44 1202 660 020
       www.biogenesis.co.uk/homejcc.stm

BID   Biodesign International
       60 Industrial Park Road
       Saco ME 04072
       USA
Tel.:   +1 207 283 6500
Fax: +1 207 283 4800
       www.biodesign.com

BMX   bioMérieux SA
       F-69280 Marcy l'Etoile
       Charbonnières-les-Bains
       France
Tel.:   +33 4 78 87 20 00
Fax: +33 4 78 87 20 90
       www.biomerieux.com

BND   The Binding Site, Inc.
       5889 Oberlin Drive
       Suite 101
       San Diego CA 92121
Tel.:   +1 858 453 9177
Fax: +1 858 453 9189

       The Binding Site Ltd.
       PO Box 11712
       Birmingham B14 4ZB
       United Kingdom
Tel.:   +44 121 436 1000
Fax: +44 121 430 7061
       www.bindingsite.co.uk

BSR   Biosource International
       542 Flynn Road
       Camarillo CA 93012
       USA
Tel.:   +1 805 987 0086
Fax: +1 805 987 3385
       www.biosource.com

CHM   Chemicon International, Inc.
       28835 Single Oak Drive
       Temecula CA 92590
       USA
Tel.:   +1 909 676 8080
Fax: +1 909 676 9209
       www.chemicon.com

CHR   Chromaprobe, Inc.
       Dorsett–Fee Fee Center
       378 Fee Fee Road
       Maryland Heights MO 63043
       USA
Tel.:   +1 314 738 0001
Fax: +1 314 738 0001
       www.chromaprobe.com

CLA   Cellabs Pty. Ltd.
       PO Box 421
       Brookvale NSW 2100
       Australia
Tel.:   +612 9905 0133
Fax: +612 9905 6426
       www.cellabs.com.au

CMB   Cambio Ltd.
       Irwin Centre
       Scotland Road
       Dry Drayton
       Cambridge CB3 8AR
       United Kingdom
Tel.:   +44 1954 210 200
Fax: +44 1954 210 300
       www.cambio.co.uk

       Corgenix United Kingdom Ltd.
       International Division
       75 Broadway
       Peterborough PE1 1SY
       Cambridgeshire
       United Kingdom
Tel.:   +44 1733 296 800
Fax: +44 1733 296 809
       www.corgenix.co.uk

COR   Cortex Biochem
       1933 Davis Street
       Suite 321
       San Leandro CA 94577
       USA
Tel.:   +1 510 568 2228
Fax: +1 510 568 2467
       www.cortex-biochem.com

DAK   DakoCytomation A/S
       Produktionsvej 42
       DK-2600 Glostrup
       Denmark
Tel.:   +1 45 44 85 95 00
Fax: +1 45 44 85 95 95

       DakoCytomation, Inc.
       6392 Via Real
       Carpinteria CA 93013
       USA
Tel.:   +1 805 566 6655
Fax: +1 805 566 6688
       www.dakocytomation.com

DIF   Becton Dickinson Diagnostic Systems
       7 Loveton Circle
       Sparks MD 21152
       USA
Tel.:   +1 410 316 4000
Fax: +1 410 527 0244
       www.bd.com, www.difco.com

DNS   Denka Seiken Company, Ltd.
       3–4–2 Nihonbashi
       Kayaba-cho
       Chuo-ku
       Tokyo 103-0025
       Japan
Tel.:   +81 3 3669 9421
Fax: +81 3 3669 99390
       www.denka-seiken.co.jp/english/en-
index.htm

DSL   Diagnostic Systems Laboratories
       445 Medical Center Boulevard
       Webster TX 77598-4217
       USA

Tel.:   +1 281 332 9678
Fax: +1 281 338 1895
       www.dslabs.com

EYL   E-Y Laboratories
       107–127 North Amphlett Boulevard
       San Mateo CA 94401
       USA
Tel.:   +1 650 342 3296
Fax: +1 650 342 2648
       www.eylabs.com

FII   Fitzgerald Industries International, Inc.
       34 Junction Square Drive
       Concord MA 01742-3049
       USA
Tel.:   +1 978 371 6446
Fax: +1 978 371 2266
       www.fitzgerald-fii.com

MRL   Focus Technologies, Inc.
       Diagnostics Client Services
       10703 Progress Way
       Cypress CA 90630-4717
       USA
Tel.:   +1 714 220 1900
Fax: +1 714 220 1820
       www.MRLinfo.com,
       www.focusanswers.com

GBO   Genbio
       15222-A Avenue of Science
       San Diego CA 92128
       USA
Tel.:   +1 858 592 9300
Fax: +1 858 592 9400
       www.genbio.com

HAS   Harlan Sera-Lab Ltd.
       Dodgeford Lane
       Belton
       Loughborough LE12 9TE
       Leicestershire
       United Kingdom
Tel.:   +44 1530 222 123
Fax: +44 1530 224 970
       www.harlanseralab.co.uk

HDS   HD Supplies
44 Rabans Close
Rabans Lane Industrial Estate
Aylesbury HP19 3RS
Buckinghamshire
United Kingdom
Tel.:   +44 1296 431 920
Fax: +44 1296 392 121

HEM   Hemagen Diagnostics, Inc.
34–40 Bear Hill Road
Waltham MA 02451
USA
Tel.:   +1 781 890 3766
Fax: +1 781 890 3748
www.hemagen.com

HYT   Hytest Ltd.
Pharmacity
Fifth floor
Itainen Pitkakatu 4C
20520 Turku
Finland
Tel.:   +358 2 512 0900
Fax: +358 2 512 0909
www.hytest.fi

IMA   Immunsystem AB
Dag Hammarskjölds väg 26
S-751 83 Uppsala
Sweden
Tel.:   +46 18 53 89 09
Fax: +46 18 53 89 97
www.immunsystem.se

IPP   Institut Pasteur
Aventis Pasteur
2 Avenue Pont Pasteur
F-69367 Lyon CEDEX 07
France
Tel.:   +33 43 73 70 100
Fax: +33 43 73 77 737
www.aventispasteur.com

IMN   Immunodiagnostics, Inc.
21 F Olympia Avenue
Woburn MA 01801
USA

Tel.:   +1 781 938 6300
Fax: +1 781 938 7300
www.immunodx.com

KIR   Kirkegaard & Perry Laboratories, Inc.
2 Cessna Court
Gaithersburg MD 20879
USA
Tel.:   +1 301 948 7755
Fax: +1 301 948 0169
www.kpl.com

KMI   KMI Diagnostics, Inc.
8201 Central Avenue NE
Suite P
Minneapolis MN 55432
USA
Tel.:   +1 763 780 2955
Fax: +1 763 780 2988
www.kmidiagnostics.com

LAM   Lampire Biological Laboratories
PO Box 279
Pipersville PA 18947
USA
Tel.:   +1 215 795 2838
Fax: +1 215 795 0237
www.lampire.com

LEL   Lee Laboratories
1475 Athens Highway SW
Grayson GA 30017
USA
Tel.:   +1 770 972 4450
Fax: +1 770 979 9570
www.leelabs.com

MBS   Maine Biotechnology Services
1037R Forest Avenue
Portland ME 04103
USA
Tel.:   +1 207 797 5454
Fax: +1 207 797 5595
www.mainebiotechnology.com

MCA Medica, Inc.
     336 Encinitas Boulevard
     Suite 200
     Encinitas CA 92024
     USA
Tel.: +1 760 634 5440
Fax: +1 760 634 5442
     www.medica-dx.com

MEN Meridian Diagnostics, Inc.
     3471 River Hills Drive
     Cincinnati OH 45244
     USA
Tel.: +1 513 271 3700
Fax: +1 513 272 5432
     www.meridianbioscience.com

MIB Microbix Biosystems
     341 Bering Avenue
     Toronto
     Ontario M8Z 3A8
     Canada
Tel.: +1 416 234 1624
Fax: +1 416 234 1626
     www.microbix.com

NOR Nordic Immunological Laboratories BV
     Langestraat 55–61
     5308 SC Tilburg/PO Box 22
     5000 AA Tilburg
     Netherlands
Tel.: +31 13 535 10 85
Fax: + 31 13 536 45 89
     www.nordiclabs.nl

NOV Novocastra Laboratories Ltd.
     Balliol Business Park West
     Benton Lane
     Newcastle upon Tyne NE12 8EW
     United Kingdom
Tel.: +44 191 215 0567
Fax: +44 191 215 1152
     www.novocastra.co.uk

OEM O.E.M. Concepts, Inc.
     1000 Industrial Way North
     Unit C
     Toms River NJ 08755
     USA

Tel.: +1 732 341 3570
Fax: +1 732 286 3173
     www.oemconcepts.com

OBT Oxford Biotechnology Ltd.
     Biotech House
     Bankside
     Kidlington
     Oxford OX5 1JE
     United Kingdom
Tel.: +44 1865 852 740
Fax: +44 1865 852 741
     www.immunologicalsdirect.com

PAE Paesel + Lorei GmbH
     Moselstrasse 2b
     D-63452 Hanau
     Germany
Tel.: +49 61 81 18 70 0
Fax: + 49 61 81 18 70 70
     www.paesel-lorei.de

PBP PanBio Pty. Ltd.
     116 Lutwyche Road
     Windsor
     Brisbane
     Queensland 4030
     Australia
Tel.: +61 7 3357 1177
Fax: +61 7 3357 1222
     www.panbio.com.au

RES Research Diagnostics, Inc.
     Pleasant Hill Road
     Flanders NJ 07836
     USA
Tel.: +1 973 584 7093
Fax: +1 973 584 0210
     www.researchd.com

SAB Sanbio BV/Monosan
     Postbus 540
     5400 AM Uden
     Netherlands
Tel.: +31 413 25 11 15
Fax: +31 413 26 66 05
     www.sanbio.nl

Sanquin Blood Supply Foundation
PO Box 9190
Diagnostic Services
1006 AD Amsterdam
Netherlands
Tel.:   +31 20 512 3333
Fax: +31 20 512 3332
  www.sanquin.nl/sanquin-eng/
  sqn_home_eng.nsf

SCI   Scimedx Corporation
400 Ford Road
Denville NJ 07834
USA
Tel.:   +1 973 625 8822
Fax: +1 973 625 8796
  www.scimedx.com

SER   Serotec Ltd.
22 Bankside
Station Approach
Kidlington
Oxford OX5 1JE
United Kingdom
Tel.:   +44 1865 852 700
Fax: +44 1865 373 899
  www.serotec.co.uk

SIG   Sigma Chemical Co.
PO Box 14508
St Louis MO 63178
USA
Tel.:   +1 314 771 5765
Fax: +1 314 791 5757
  www.sigma-aldrich.com

SOU   Southern Biotechnology Associates
PO Box 26211
Birmingham AL 35226
USA
Tel.:   +1 205 945 1774
Fax: +1 205 945 8768
  www.southernbiotech.com

SPR   Spring Valley Laboratories, Inc.,
PO Box 242
Woodbine MD 21797
USA

Tel.:   +1 410 795 2222
Fax: +1 410 795 2242
  www.svlab.com

TMO   Thermo Electron (Shandon Products)
171 Industry Drive
Pittsburgh PA 15275
USA
Tel.:   +1 412 788 1133
Fax: +1 412 788 1138
  www.thermo.com

TRN   Trinity Biotech, Inc.
PO Box 1059
Jamestown NY 14702-1059
USA
Tel.:   +1 716 483 3851
Fax: +1 716 488 1990

Trinity Biotech plc
IDA Business Park
Bray
County Wicklow
Ireland
Tel.:   +353 1 276 9800
Fax: +353 1 276 9888
  www.trinitybiotech.com

UNL   Catholic University of Louvain
Experimental Immunology Unit
UCL 30–56
Clos Chapelle-aux-Champs 30
1200 Bruxelles
Belgium
Tel.:   +32 2 764 34 30
Fax: +32 2 764 39 46
  rch.adre.ucl.ac.be/browse/list_alpha/
  IMEX/en

VIC   Vircell SL
Plaza Dominguez Ortiz 1
Poligono Industrial Dos De Octubre
18320 Santa Fe
Granada
Spain
Tel.:   +34 958 44 12 64
Fax: +34 958 51 07 12
  www.vircell.com

VIO   Virogen
       200 Dexter Avenue
       Watertown MA 02472
       USA
Tel.: +1 617 926 9167
Fax: +1 617 926 9157
       www.virogen.com

VIR   Virostat
       PO Box 8522
       Portland ME 04104
       USA
Tel.: +1 207 856 6620
Fax: +1 207 856 6864
       www.virostat-inc.com

VMR   VMRD, Inc.
       PO Box 502
       Pullman WA 99163
       USA
Tel.: +1 509 334 5815
Fax: +1 509 332 5356
       www.vmrd.com

WAM   Wampole Laboratories
       2 Research Way
       Princeton NJ 08540
       USA

Tel.: +1 609 627 8000
Fax: +1 609 627 8013
       www.wampolelabs.com

WAS   Washington Research Foundation
       2815 Eastlake Avenue East
       Suite 300
       Seattle WA 98102
       USA
Tel.: +1 206 336 5600
Fax: +1 206 336 5615
       www.wrfseattle.org

WTR   Waterborne
       6047 Hurst Street
       New Orleans LA 70118-6129
       USA
Tel.: +1 504 895 3338
Fax: +1 504 895 3338
       www.waterborneinc.com

ZYM   Zymed Laboratories
       561 Eccles Avenue
       South San Francisco CA 94080
       USA
Tel.: +1 650 871 4494
Fax: +1 650 871 4499
       www.zymed.com

12.4 Suppliers of chemicals and fluorochromes

Gallard-Schlesinger Industries, Inc.
245 Newtown Road
Suite 305
Plainview NY 11803
USA
Tel.:   +1 516 683 6900
Fax: +1 516 683 6990
www.gallard-schlesinger.com

EMD Chemicals, Inc.
480 South Democrat Road
Gibbstown NY 08027
USA
Tel.:   +1 856 423 6300
Fax: +1 856 423 4389
www.emdchemicals.com

Fisher Scientific Co.
4500 Turnberry Drive
Hanover Park IL 60103
USA
Tel.:   +1 630 259 1200
Fax: +1 630 259 4444
www.fishersci.com

Merck KGaA
Frankfurter Strasse 250
D-64293 Darmstadt
Germany
Tel.:   +49 6151 72-0
Fax: +49 6151 72 2000
www.merck.de

9F. Hoffmann-La Roche Ltd.
Diagnostics Division
Grenzacherstrasse 124
CH-4070 Basel
Switzerland
Tel. +41 61 688 1111
Fax: +41 61 6 91 9391
www.roche-diagostics.com

Sigma-Aldrich Co.
PO Box 14508
St Louis MO 63178-9916
USA
Tel.:   +1 314 771 5750
Fax: +1 314 771 5757
www.sigmaaldrich.com

VWR International
1310 Goshen Parkway
West Chester PA 19380
USA

Tel.:   +1 610 431 1700
Fax: +1 610 436 1762
www.vwrsp.com

VWR International AG
Rüchligstrasse 20
Postfach 964
CH-8953 Dietikon
Switzerland
Tel. +41 1 745 1111
Fax: +41 1 745 1100
www.vwr.com

ZLB-Behring GmbH
Emil-von-Behring-Strasse 76
D-35401 Marburg
Germany
Tel.:   +49 6421 3912
Fax: +49 6421 39 4825
www.aventisbioservices.com,
www.zlbbehring.com

12.5 Suppliers of fluorescence microscopy supplies

BioRad Laboratories, Inc.
2000 Alfred Nobel Drive
Hercules CA 94547
USA
Tel.:   +1 541 741 1000
Fax: +1 541 741 5800
www.biorad.com

Fisher Scientific International, Inc.
Export Division
50 Fadem Road
Springfield NJ 07081-3193. USA
Tel.:   +1 973 467 6511
Fax: +1 973 376 1546
www.fishersci.com

Pharmacia Diagnostics AB
SE-751 37 Uppsala
Sweden

Tel.:   +46 18 16 30 00
Fax: +46 18 14 03 58
www.diagnostics.com

Thomas Scientific
PO Box 99
Swedesboro NJ 08085
USA
Tel.:   +1 856 467 2000
Fax: +1 856 467 3087
International sales fax: +1 856 467 0512
www.thomassci.com

VWR International
1310 Goshen Parkway
West Chester PA 19380
USA
Tel.:   +1 610 431 1700
Fax: +1 610 436 1762
VWR International BVBA/SPRL

Haasrode Researchpark Zone 3
Geldenaaksebaan 464
B-3001 Leuven
Belgium
Tel.:   +32 16 385 011
Fax: +32 16 385 385
www.vwr.com

Pall Life Sciences
600 South Wagner Road
Ann Arbor MI 48103-9019
USA
Tel.:   +1 734 665 0651
Fax: +1 734 913 6114
www.pall.com

Membrane filters and filter apparatus
Millipore Corporation
80 Ashby Road
Bedford MA 01730
USA
Tel.:   +1 781 533 6000
Fax: +1 781 533 3110
www.millipore.com

Whatman, Inc.
200 Wells Avenue
Newton MA 02459-3304
USA
Tel.:   +1 781 753 6590
Fax: +1 781 753 6555
www.whatman.com

12.6 Suppliers of auto-immune disease test reagents

Anti-nuclear antibody FITC conjugate kits

MCA   Medica, Inc.
       336 Encinitas Boulevard
       Suite 200
       Encinitas CA 92024
       USA
Tel.:   +1 760 634 5440
Fax: +1 760 634 5442
       www.medica-dx.com

SCI   Scimedx Corp.
       400 Ford Road
       Denville NJ 07834
       USA
Tel.:   +1 973 625 8822
Fax: +1 973 625 8796
       www.scimedx.com

Anti-DNA antibody FITC conjugate kits

ACC   Accurate Chemical & Scientific Co.
       300 Shames Drive
       Westbury NY 11590
       USA

Tel.:   +1 516 333 2221
Fax: +1 516 997 4948
       www.accuratechemical.com

ANT   Antibodies, Inc.
       PO Box 1560
       Davis CA 95617-1560
       USA
Tel.:   +1 530 758 4400
Fax: +1 530 758 6307
       www.antibodiesinc.com

BND   The Binding Site Ltd.
       PO Box 11712
       Birmingham B14 4ZB
       UK
Tel.:   +44 121 436 1000
Fax: +44 121 430 7061
       www.bindingsite.co.uk

HEM   Hemagen Diagnostics, Inc.
       9033 Red Branch Road
       Columbia MD 21045
       USA
Tel.:   +1 443 367 5500
Fax: +1 410 997 7812
       www.hemagen.com

CONJUGATES, ANTIBODIES AND TEST KITS
12.7 Monoclonal fluorescent antibody conjugates to detect infectious antigens

Etiological agent	*Disease*	*Conjugate source*
Adenovirus	URI	ACC, ACE, ARG, BGN, COR, HAS, VIR
Bluetongue virus	Bluetongue, cattle	VMR
Candida albicans	Candidiasis	COR
Canine distemper	Distemper	VMR
Chlamydia spp.	Trachoma, STD	ARG, PAE
C. pneumoniae	Pneumonia	WAS
C. trachomatis	Trachoma	ACC, COR, VIR
Clostridium spp.	Septicaemia, food-poisoning	VMR
Cryptosporidium spp.	Diarrhoea	BGN, HOV, VMR, WTR
Cytomegalovirus	CNS abnormalities	ACC, BGN, CHM, COR, DAK, HAS, SHA, VIR
Encephalitozoan spp.	Diarrhoea	WTR
Enterovirus VP 1	Aseptic meningitis, febrile illness	NOV
Epstein–Barr virus	Infectious mono-nucleosis, tumorigenic	ACC, VIR
Escherichia coli O157	Haemorrhagic colitis	ACC, COR, VIR
Feline leukaemia virus	Viral leukaemia	VIR
Hepatitis A virus	Hepatitis	CHR, VIR
Hepatitis B virus	Hepatitis	CHR, VIR
Hepatitis C virus	Hepatitis	VIO, VIR
Herpes simplex virus	Herpes	ACC, ARG, BGN, COR, HAS, VIR
Human immunodeficiency virus	HIV/AIDS	AMR, BID, BGN, CHR, COR, IMN
Influenza virus	Influenza	ACC, ARG, BGN, COR, FII, HAS, NOV, VIR
Legionella pneumophila	Pneumonia	AMR, BGN, PAE
Measles virus	Measles	ACC, BGN, COR, VIR
Mumps virus	Mumps	ACC, BGN, COR, VIR
Mycoplasma pneumoniae	Pneumonia	ACC, BGN, COR, VIR
Neisseria gonorrhoeae	Gonorrhoea	ACC, COR, VIR
Parainfluenza virus	Influenza	ACC, ARG, BGN, COR, HAS, VIR
Pneumocystis carinii	Pneumonia	ACC, COR, VIR
Respiratory syncytial virus	Bronchiolitis, pneumonia	ACC, ARG, BGN, CAL, COR, FII, HAS, NOV, SAB, VIR
Rubella virus	German measles	CHM

Rubeola virus	Measles	ACC, COR, VIR
Salmonella spp.	Diarrhoea	ACC, BGN, COR, VIR
Simian immunodeficiency virus	AIDS-like disease	AMR
Streptococcus Gr. A	Pharyngitis, endocarditis, arthritis, glomerulonephritis	ACC, COR, VIR
Toxoplasma gondii	Lymphadenopathy, chorioretinitis, CNS disease	ACC, BGN, COR, VIR
Trichomonas vaginalis	Vaginitis, STD	ACC, BDP, COR, VIR
Trypanosoma brucei	Sleeping sickness	ACC, CED
Varicella/zoster virus	Chickenpox, shingles	BGN, NOV
Yersinia enterocolitica	Diarrhoea	AMR, DAE

12.8 Polyclonal fluorescent antibody conjugates to detect infectious antigens

Etiological agent	*Disease*	*Conjugate source*
Adenovirus	URI	ACC, BGN, CHM, COR, VIR
Aspergillus	Aspergillosis	ACC, BGN, VIR
Bordetella pertussis	Whooping cough	DIF
B. parapertussis	Whooping cough	DIF
Borrelia burgdorferi	Lyme disease	ACC, CHM, COR, FII, KIB, VIR, VMR
Bovine adenovirus	URI	VMR
Bovine herpes virus	Herpes	VMR
Bovine infectious rhinotracheitis virus	Rhinotracheitis	BGN
Bovine parainfluenza	Influenza	VMR
Bovine parvovirus	Exanthema	VMR
Bovine reovirus	Calf diarrhoea	VMR
Bovine respiratory syncytial virus	Pneumonia	VMR
Bovine viral diarrhea	Diarrhoea	BGN, VMR
Campylobacter spp.	Diarrhoea	ACC, BGN, KIR, VIR
Candida albicans	Candidiasis	ACC, BGN, COR, VIR
Canine corona virus	Gastroenteritis	VMR
Canine distemper	Distemper	VMR
Canine herpes	Herpes	VMR
Chlamydia trachomatis	Trachoma, VD	ACC, BGN, CHM, CHR, COR, VIR
Clostridium chauvoei	Herbivore enterotoxaemia	VMR

C. novyi	Gas gangrene	VMR
C. septicum	Neutropenic enterocolitis	VMR
C. sordelli	Food poisoning	VMR
Corynebacterium diphtheriae	Diphtheria	ACC, BGN, DIF, VIR
Cryptosporidium parvum	Diarrhoea	CLA
Cytomegalovirus	Neonatal CNS disease	ACC, BGN, COR, VIR
Dirofilaria immitis	Canine heartworm	ACC, VIR
Encephalitozoan spp.	Diarrhoea	WTR
Epstein–Barr virus	Infectious mononucleosis	BGN
Equine rhinopneumonitis	URI	VMR
Escherichia coli	Urinary tract infection	ACC, BGN, COR, VIR
E. coli O157	Diarrhoea	BGN, KIR, FII
Feline herpes	Herpes	VMR
Feline infectious peritonitis	Peritonitis	VMR
Feline leukaemia virus	Leukaemia-like infection	ACC, COR, VMR, VIR
Giardia lamblia	Diarrhoea	CLA, VIR
Helicobacter pylori	Stomach ulcer	ACC, KIR, VIR
Hepatitis B virus	Hepatitis	ACC, BGN, CEN, CHM, COR, VIR
Hepatitis C virus	Hepatitis	ACC, BGN, VIR
Hepatitis delta virus	Hepatitis	ARG, BGN
Herpes simplex virus	Herpes	ACC, BGN, CHM, COR, DAK, SHA, VIR
Human immunodeficiency virus	HIV/AIDS	AMR, ACC, BGN, COR, IMN, VIR
Influenza virus	Influenza	ACC, BGN, CHM, COR, FII, VIR
Klebsiella spp.	Pneumonia, sepsis	OXB
Legionella longbeachae	Legionnaire disease	COR
L. micdadei	Legionnaire disease	COR
L. pneumophila	Legionnaire disease	ACC, COR, VIR
Leishmania spp.	Leishmaniasis	CLA
Leptospira spp.	Leptospirosis	ACC, BGN, COR, VIR
Listeria monocytogenes	Listeriosis	ACC, BGN, DIF, KIR, VIR
Mycobacterium tuberculosis	Tuberculosis	ACC, VIR
Mycoplasma pneumonia	Pneumonia	ACC, BGN, COR, VIR
Neisseria gonorrhoeae	Gonorrhoea	ACC, BGN, DIF, VIR
N. meningitidis	Meningitis	ACC, BGN, CIR, VIR
Neospora caninum	Helminth infection	VMR
Parainfluenza virus	Influenza	ACC, BGN, VIR, COR, CHM
Pneumocystis carinii	Pneumonia	CLA
Porcine adenovirus	URI	VMR

Porcine parvovirus	Exanthema	VMR
Rabies virus	Rabies	VMR, IPP, CHM
Renibacterium salmoninarum	Salmon infection	KIR
Respiratory syncytial virus	URI	ACC, BGN, CHM, COR, VIR
Rotavirus	Diarrhoea	ACC, BGN, CHM, COR, VIR
Rubella virus	German measles	ACC, BGN, CHM, COR, VIR
Salmonella spp.	Salmonellosis	ACC, BGN, CHM, COR, VIR
Shigella spp.	Shigellosis	ACC, BGN, COR, DIF, VIR
Staphylococcus aureus	Septicaemia, various infections	OXB
Streptococcus Gr. A	Strep throat, arthritis, rheumatic fever	ACC, BGN, CHM, COR, VIR
Streptococcus pneumoniae	Pneumonia, meningitis	VIR
Toxoplasma gondii	Toxoplasmosis	ACC, BGN, VIR
Treponema pallidum	Syphilis	ACC, COR, VIR
Transmissible gastroenteritis virus	Gastroenteritis	VMR
Vaccinia virus	Cowpox	ACC, BGN, COR, VIR
Varicella/Zoster virus	Chickenpox	BGN
Vibrio cholerae	Cholera	ACC, BGN, VIR

12.9 Monoclonal and polyclonal antibodies for indirect fluorescent antibody tests

Etiological agent	*Disease*	*Source*
Adenovirus	URI	MBS
Bacillus anthracis	Anthrax	AMQ, FII, OBT, OEM, OXB
Bordetella pertussis	Whooping cough	ABG, BGN, CMB, OXB
Borrelia burgdorferi	Lyme disease	MBS
Bunya virus	Haemorrhagic fever	CHM
Burkholderia pseudomallei	Melioidosis	ADI, AMQ, HYT
Campylobacter jejuni	Diarrhoea	ACC, BGN, BID, MBS, OXB
Candida albicans	Thrush, throat ulcers	MBS, OBT, OXB
Canine distemper virus	Distemper	OBT, OXB
Chlamydia spp.	STD, blindness	CHM, MBS, OXB
Clostridium botulinum	Botulism	ADI, AMQ, BGN, HYT, OEM
C. difficile	Diarrhoea	ICN, MBS, OBT, OXB, TEC
Corynebacterium diphtheriae	Diphtheria	ACC, ANQ, ARG, MBS
Coxsackievirus	Myocarditis	BGN, CHM
Cryptococcus neoformans	Meningitis	LAB

Cryptosporidium parvum	Diarrhoea	OBT, OXB
Cytomegalovirus	CNS abnormalities	CHM, MBS, OXB
Dengue virus	Dengue	CHM, FII, MIB, OBT, OXB
Eastern equine encephalitis (EEE)	Encephalitis	CHM
Entamoeba histolytica	Dysentery	AMR, BGN, QED
Epstein–Barr virus	Mononucleosis	CHM, FII
Escherichia coli O157	Gastroenteritis	MBS
Feline immunodeficiency virus	AIDS-like	OXB
Francisella tularensis	Tularaemia	BGN, CMB, HYT, OEM
Feline leukaemia virus	Leukaemia-like infection	MBS, OBT, OXB
Giardia lamblia	Diarrhoea	ACC, BGN, MBS, OBT, OXB, VIR
Hantavirus	Haemorrhagic encephalopathy	MBS
Helicobacter pylori	Stomach ulcer	ACC, BGN, CNX
Hepatitis virus: A, B, C, delta	Hepatitis	CHM, OBT
Herpes simplex virus	Herpes	CHM, MBS, NOV
Human immunodeficiency virus	HIV/AIDS	MBS
Influenza virus	Influenza	ACE, ARG, BGN, CHM, COR, FII, MBS, NOV OEM, PAE
Japanese B encephalitis virus	Encephalitis	MIB
Leishmania spp.	Leishmaniasis	ACC, BGN, CED
Legionella pneumophila	Pneumonia	MBS, NOV, OBT
Leptospira spp.	Weil disease, jaundice	WHO-4
Listeria spp.	Listeriosis	MBS
Mycobacterium tuberculosis	Tuberculosis	MBS, NOV
Neisseria gonorrhoeae	Gonorrhoea	MBS, OBT
Neisseria meningitidis	Meningitis	ACC, MBS, OBT
Papilloma virus	Warts, neoplasia	ACE, BGN, CHM, OBT
Plasmodium falciparum	Malaria	BGN, COR
Pneumocystis carinii	Pneumonia	MBS, NOV
Poliovirus	Polio	NOV
Prion protein	BSE, scrapie	CHM, MBS, OBT
Pseudomonas aeruginosa	Ear infections, meningitis	BGN
P. mallei (*Burkholderia mallei*)	Glanders	ADI, AMQ, HYT, OEM
Rabies virus	Rabies	ACC, ACE, AMR, OBT
Respiratory syncytial virus	CNS disease	MBS

Rotavirus	Gastroenteritis	ACE, AMQ, CHM, MBS, OBT
Rubella virus	German measles	MBS
Salmonella spp.	Diarrhoea	MBS, OBT
Salmonella paratyphi A	Enteric fever	CAP, ICN
S. typhi	Typhoid fever	BGN, MBS, OBT, STA
Shigella spp.	Dysentery	CHM, MBS
Staphylococcal enterotoxin	Food poisoning	ACC, ARG, MBS, VIR
Streptococcus Grs. A, B	Strep throat, sepsis	CHM
Streptococcus pneumoniae	Pneumonia, meningitis	CHM, MBS
Toxoplasma gondii	Lymphadenopathy	OBT, MBS
Treponema pallidum	Syphilis	ACE, BGN, MBS, OBT, OEM
Trichomonas vaginalis	STD	MBS, OBT
Vaccinia virus	Cowpox	MBS
Venezuelan equine encephalitis (VEE)	Encephalitis	CHM
Verotoxins	Diarrhoea	OBT
Vibrio cholerae	Cholera	ACC, AMQ, BGN
Western equine encephalitis (WEE)	Encephalitis	CHM
Yellow fever virus	Yellow fever	BGN, CHM, OBT
Yersinia pestis	Plague	ACC, AMQ, AMR, BGN, CMB, HYT, COR, MBS, OBT, OEM, RES, VIR

12.10 Direct fluorescent antibody test kits for detecting infectious antigens

Etiological agent	*Disease*	*Kit source*
Adenovirus	URI	ACC, ARG, CHM, DAK
Borrelia burgdorferi	Lyme disease	WAM
Chlamydia pneumoniae	Pneumonia	ACC, DAK
C. trachomatis	Trachoma (VD)	ACC, AMR, DAK, TRN
Coxsackievirus	Neonatal sepsis, myocarditis, haemorrhagic conjunctivitis	CHM
Cryptosporidium parvum	Diarrhoea	MEN, WTR
Cytomegalovirus	CNS abnormalities	ARG, TRN, CHM
Echovirus	Neonatal sepsis	CHM
Ehrlichia spp.	Ehrlichiosis	PBP
Enterovirus	Diarrhoea	CHM
Giardia lamblia	Diarrhoea	MEN, WTR
Herpes simplex virus	Herpes	ABI, ACC, CHM, DAK, TRN
Influenza virus	Influenza	ACC, CHM, DAK
Legionella spp.	Pneumonia	AMR, SCI, TRN

Parainfluenza virus	Influenza	ACC, CHM, DAK
Plasmodium falciparum	Malaria	IPP
Pneumocystis carinii	Pneumonia	AMR, CHM, MEN, VIC
Pneumonia screening	Pneumonia	VIC, CHM
Rabies virus	Rabies	CHM
Respiratory syncytial virus	Pneumonia	ACC, CHM, DAK, TRN
Rickettsia spp.	Rickettsioses	IND
Toxoplasma gondii	Toxoplasmosis	IPP
Trichomonas vaginalis	Trichomoniasis	CHM
URI screen	URI	IPP
Viral respiratory	Respiratory disease	TRN

12.11 Indirect fluorescent antibody test kits for infectious disease antibodies

Antibody	*Disease*	*Source code*
Anti-adenovirus	Respiratory disease	VIC
Anti-arbovirus screen	Encephalitis	MRL, VIC
Anti–*Bartonella henselae*	Cat scratch fever	AMR, MRL
Anti–*Borrelia burgdorferi*	Lyme disease	AMR, KMI, MRL, WAM
Anti–*Brucella canis*	Brucellosis	AMR
Anti-*Brucella* spp.	Brucellosis	BMX
Anti-*Candida*	Candidiasis	KMI
Anti–*Chlamydia pneumoniae*	Pneumonia	AMR, MRL, VIC
Anti–*C. psittaci*	Ornithosis	AMR
Anti–*C. trachomatis*	Trachoma, VD	DIF, HEM
Anti–*Coxiella burnetii*	Q fever	AMR, MRL, VIC
Anti-cytomegalovirus	CNS disease, birth defects	HEM, KMI, WAM
Anti–dengue virus	Dengue	AMR, PBP
Anti–Epstein–Barr virus	Infectious mononucleosis	AMR, HEM, GUL, KMI, MRL, VIC
Anti–*Erlichia* spp.	Erlichiosis	MRL
Anti-hantavirus	Haemorrhagic nephropathy	MBS
Anti–herpes simplex virus	Herpes	GBO, HEM, KMI
Anti-HIV	AIDS	KMI
Anti–influenza virus	Influenza	TRN, VIC
Anti–*Legionella pneumophila*	Pneumonia	MRL, SCI, VIC, WAM
Anti–*Leishmania donovani*	Visceral leishmaniasis	VIC
Anti–*Leptospira* spp.	Leptospirosis	AMR

Anti–measles virus	Measles	HEM, KMI
Anti–mumps virus	Mumps	HEM, KMI
Anti–*Mycoplasma pneumoniae*	Pneumonia	AMR, VIC, WAM
Anti–parainfluenza virus	Influenza	TRN, VIC
Anti–*Plasmodium falciparum*	Malaria	BMX
Anti–respiratory synctial virus	Bronchiolitis, pneumonia	HEM, KMI, TRN, VIC
Anti–*Rickettsia conorii*	Boutonneuse fever	AMR, BMX, MRL, VIC
Anti–*R. prowazekii*	Louse-borne typhus	AMR, IND, MRL, PBP
Anti–*R. rickettsii*	Rocky Mountain spotted fever	AMR, IND, MRL, PBP
Anti–*R. tsutsugamushi*	Scrub typhus	AMR, IND, PBP
Anti–*R. typhi*	Murine typhus	MRL, PBP, IND
Anti–rubella virus	German measles	HEM
Anti–rubeola virus	Measles	GUL
Anti–*Toxoplasma gondii*	Toxoplasmosis	BMX, GUL, GBO, HEM, IPP, WAM
Anti–*Treponema pallidum*	Syphilis	MX, DIF, HEM, KMI, SCI, WAM
Anti–varicella/zoster virus	Chickenpox, shingles	GUL, KMI, WAM
Anti–West Nile virus	Encephalitis	PBP
Anti–yellow fever virus	Yellow fever	PBP

12.12 Anti-species globulin conjugates for indirect fluorescent antibody tests

Anti–chicken gamma globulin (gg)	ACC, AMQ, BET, HAS, KIR, SIG, SOU
Anti–bovine gg	ACC, AMQ, BET, COR, EYL, KIR, SOU
Anti–goat gg	ACC, BET, COR, EYL, NOR, SIG
Anti–guinea pig gg	ACC, BET, COR, IMC, NOR, SIG, ZYM
Anti–horse gg	ACC, AMQ, BET, COR, ICN, IMC, KIR
Anti–human gg	ACC, AMQ, BET, ICN, IMA, NOR, SIG, UNL, VEC, ZYM
Anti–mouse gg	ACC, BSR, EVL, HAS, SIG, SOU, UNL, ZYM
Anti–rabbit gg	ACC, AMQ, BSR, HAS, NOR, SIG, SPR, UNL, ZYM
Anti–rat gg	ACC, BSR, COR, HAS, NOR, SIG, UNL

12.13 Preparation of saline solutions, buffers, fluorochrome stains, miscellaneous reagents and tests

Contents

Preparation procedures

A. General purpose saline solutions

1. Saline, 0.15 M

2. Phosphate buffered saline (PBS), pH 7.1–7.2

3. Phosphate buffered saline, pH 7.4

3.a PBS–albumin, 1%

3.b PBS–glycerol mounting medium

4. Carbonate buffered saline (CBS), pH 8.6–9.0

B. Buffers, general and special purpose

1. Carbonate–bicarbonate buffer, 0.5 M, pH 8.5 and pH 9.0

2. Carbonate–bicarbonate buffer, 0.025 M, pH 9.0

3. Phosphate buffer, 0.2 M, pH 7.6

C. Fluorochrome stains

1. Acridine orange	*Application*
a. Acridine orange (acetate, 0.15 M); pH 3.5	Body fluids, blood culture, cerebrospinal fluid
b. Acridine orange (acetate, 0.1 M); pH 3.5	Blood smear, bacteria
c. Acridine orange (acetate, 0.2 M); pH 4.0	Blood culture, cerebrospinal fluid, bacteria
d. Acridine orange (phosphate, 0.15 M); pH 6.0	RNA/DNA differentiation
e. Acridine orange (PBS); pH 7.2–7.4	Malaria parasites in blood smear

f.   Acridine orange (tris-HCl); pH 7.4   Malaria parasites in
                                                 blood smear

g.   Acridine orange (potassium   Water (bacteria)
     dihydrogen phosphate) pH 8.3

h.   Acridine orange (borate); pH 9.8   Urine (bacteria)

i.   Acridine orange 0.5% (aqueous)   Malaria (stock solution)

j.   Acridine orange 0.1% (aqueous)   Vaginal swab (*Trichomonas
                                                 vaginalis*)

2. Fluorescent acid-fast stains

a.   Blair fluorescent acid-fast stain

b.   Smithwick fluorescent acid-fast stain

c.   Phenolic acridine orange acid-fast stain

D. Miscellaneous reagents

1.   Ammonium sulfate globulin precipitation solution

2.   Calcium chloride (0.1 M) differentiation solution

3.   Indirect fluorescent antibody test substrate *Crithidia luciliae*

4.   Merthiolate (thimerosal) preservative, pH 7.4

5.   Merthiolate (thimerosal) preservative, borated

6.   Acetone dried tissue powder

E. Reagents and tests

1.   Biuret reagent and test for protein

2.   Ammonium ion test for dialysate from globulin precipitation

Preparation procedures

A. General purpose saline solutions

1. Saline, 0.15

Purpose

Diluting reagents, dialysing and specimen preparation.

Parts

NaCl 8.77 g.

H_2O, distilled, 1000 mL.

Preparation

1.   Dissolve the NaCl in the distilled water.

2.   Filter solution through a 0.2 or 0.22 µm pore size filter.

2. Phosphate buffered saline, pH 7.1–7.2

Purpose

General purpose diluent for antisera, conjugates and reagents.

Parts

Na_2HPO_4, anhydrous, 1.07 g.

$NaH_2PO_4 \cdot 2H_2O$, 0.39 g.

NaCl, 8.50 g.

H_2O, distilled, to 1000 mL.

Preparation

1.   Dissolve the salts in distilled water and make it up to 1000 mL.

2.   Make minor adjustments in pH with 1 M NaOH.

3.   Filter solution through a 0.2 or 0.22 µm pore size filter.

Note: merthiolate (thimerosal) may be added at a 1:10 000 concentration to suppress microbial contamination.

3. Phosphate buffered saline, pH 7.4 (PBS)

Purpose

Preparing and distilling reagents, dialysing and specimen preparation.

Parts

Stock solution (0.1 M)

$NaH_2PO_4·H_2O$, 2.07 g.

Na_2HPO_4, anhydrous, 12.07 g.

NaCl, 80 g.

KCl, 2 g.

H_2O, distilled or deionized, to 1000 mL.

Use solution (0.01 M)

1.   Dilute the stock solution 1:10 with distilled water.

Note: thimerosal (merthiolate) may be added at a ratio of 1:10 000 or 1:5000 to suppress microbial contamination.

3.a PBS–albumin, 1%

Parts

Stock solution PBS, pH 7.4, 10 mL.

Human serum albumin, 1 g.

H_2O, distilled, to 100 mL.

Preparation

1.   Dissolve human serum albumin in PBS solution at room temperature and add distilled water up to 100 mL.

3.b PBS–glycerol mounting medium

Parts

Stock solution PBS, pH 7.4, 3.4 mL.

Glycerol, 6 mL.

Sodium azide, 1 g.

H_2O, distilled, 31.6 mL.

Preparation

1. Dilute stock solution PBS in distilled water; mix with potassium-free glycerol. Add sodium azide as preservative.

4. Carbonate buffered saline, pH 8.6–9.0 (CBS)

Preparation

By dry weight component

pH	Na_2CO_3	$NaHCO_3$	*NaCl*
8.6	0.45 g	8.05 g	8.77 g
8.8	0.83 g	7.74 g	8.77 g
9.0	1.38 g	7.31 g	8.77 g

All solutions are brought to 1000 mL with distilled H_2O by mixing stock solutions.

Stock solution 1

Na_2CO_3, 0.6 g.

NaCl, 8.8 g.

H_2O, distilled, to 1000 mL.

Stock solution 2

$NaHCO_3$, 8.4 g.

NaCl, 8.8 g.

H_2O, distilled, to 1000 mL.

Filter solutions through a 0.2 or 0.22 µm pore size filter.

pH	*Stock solution 1*	*Stock solution 2*

8.6	42.1 mL	957.9 mL
8.8	78.2 mL	921.8 mL
9.0	130 mL	870 mL

Minor adjustments in the ratios may be required for exact pH.

B. Buffers, general and special purpose

1. Carbonate–bicarbonate buffer 0.5 M; pH 8.5 and pH 9.0

Purpose

Preparation of FITC–immunoglobulin conjugates.

Parts

Solution 1

NaHCO$_3$, 42g/L (0.5 M).

Solution 2

Na$_2$CO$_3$, 3 g/L (0.5 M).

pH	Solution 1	Solution 2
8.5	980 mL	20 mL
9.0	925 mL	74 mL

Preparation

1. Dissolve the chemicals in the distilled water.

2. Keep in a tightly stoppered bottle.

3. Make small batches fresh for use rather than large batches.

Note: keep the stock solutions tightly closed in a bottle to prevent absorption of CO$_2$ from the air.

2. Carbonate–bicarbonate buffer 0.025 M, pH 8.5 and pH 9.0

Purpose

Conjugation of serum proteins by the dialysis method.

Parts

Solution 1

    Na_2CO_3, anhydrous, 2.65 g.

    H_2O, distilled, to 1000 mL.

Solution 2

    $NaHCO_3$, 2.10 g.

    H_2O, distilled, to 1000 mL.

Preparation

Mix to yield the desired pH:

	Solution 1	Solution 2
pH 8.5	1 part	49 parts
pH 9.0	1 part	12.5 parts

Add Solution 2 to Solution 1 while stirring under the electrode of a pH meter.

3. Phosphate, 0.2 M, pH 7.6

Purpose

1. Gammaglobulin extraction from serum with DEAE Sephadex.

2. Trypsin digestion of tissue sections.

Parts

Solution 1

    $NaH_2PO_4 \cdot H_2O$, 2.76 g.

    H_2O, distilled, to 1000 mL.

Solution 2

Na$_2$HPO$_4$, anhydrous, 2.84 g.

H$_2$O, distilled, to 1000 mL.

Filter solutions through a 0.2 or 0.22 µm pore size filter.

Preparation

Add Solution 1 to Solution 2 while stirring under the electrode of a pH meter to yield pH 7.6. The ratio will be about 1 to 9.

C. Fluorochrome stains

1. Acridine orange
1.a Acridine orange in acetate buffer 0.15 M, pH 3.5

Purpose

Detection of bacteria in body fluids and blood cultures.

Parts

Acetate buffer pH 3.5.

Sodium acetate (C$_2$H$_3$O$_2$Na·3H$_2$O), 20.4 g.

Acetic acid, glacial, 9.0 mL.

H$_2$O, distilled, 1000 mL.

HCl, 1 M (adjust the solution to pH 3.5.).

Acridine orange stain.

Acetate buffer, pH 3.5, 190 mL.

Acridine orange, 20 mg.

Preparation

1. Add the acridine orange powder to the buffer.

2. Stir to dissolve the dye.

3. Filter solution through a 0.2 or 0.22 µm pore size filter.

4. Store the stain in a brown bottle at room temperature.

1.b Acridine orange in acetate buffer 0.1 M, pH 3.5

Purpose

Detection of bacteria in blood smears.

Parts

Buffer

 Sodium acetate ($C_2H_3O_2Na\cdot3H_2O$), 3.6 g.

 H_2O, distilled, 100 mL.

 HCl, 1 M, 90 mL.

Stain

 Acetate buffer, 190 mL.

 Acridine orange, 20 mg.

Preparation

1. Dissolve the sodium acetate on distilled water.

2. Add the HCl slowly while stirring.

3. Adjust the pH to 3.5, if necessary, with 0.1 M HCl.

4. Add the acridine orange powder to the buffer.

5. Stir to dissolve the dye in the buffer.

6. Store the stain in a brown bottle at room temperature.

(Final concentration of acridine orange is about 100 mg/L.)

1.c Acridine orange in acetate buffer, 0.2 M, pH 4.0

Purpose

Detection of bacteria in blood cultures and cerebrospinal fluid.

Parts

Buffer

Sodium acetate ($C_2H_3O_2Na·3H_2O$), 27.2 g.

Acetic acid, glacial, 12.0 mL.

H_2O, distilled, to 1000 mL.

Stock solution

Acridine orange, 1 g.

H_2O, distilled, 1000 mL.

Store at 4 °C (stable for two years).

Use stain solution

Stock solution, 0.5 mL.

Acetate buffer, 5.0 mL.

Preparation

1. Dissolve the sodium acetate in the distilled water.

2. Add the glacial acetic acid slowly while stirring.

3. Dissolve the acridine orange in distilled water while stirring.

4. Add the acridine orange stock solution to the acetate buffer (1:100).

1.d Acridine orange in phosphate buffer 0.15 M, pH 6.0

Purpose

Differentiation of DNA and RNA in cells and microorganisms.

Parts

Stock buffer 1

NaH_2PO_4, anhydrous, 8 g.

H_2O, distilled, q.s. to 1000 mL.

Stock buffer 2

Na_2HPO_4, anhydrous, 21.3 g.

H_2O, distilled, q.s. to 1000 mL.

Buffer solution for use

Stock buffer 1, 87.8 mL.

Stock buffer 2, 12.2 mL.

Preparation

1. Mix the parts.

2. Slight adjustments to this ratio may be necessary to get pH 6.0.

3. Test the pH with a pH meter.

Stock acridine orange solution

Acridine orange, 0.1 g.

H_2O, distilled, 1000 mL.

Acridine orange stain for use

Buffer solution, pH 6.0, 9 mL.

Stock acridine orange solution, 1 mL.

Preparation

1. Prepare stain fresh for use.

2. Store stock acridine orange solution in the dark at 4 °C.

1.e Acridine orange in PBS, pH 7.2–7.4

Purpose

Detection of malaria parasites in blood smears.

Parts

Acridine orange   0.1 g

PBS, pH 7.2–7.4   1000 mL

Preparation

1. Dissolve the acridine orange in the PBS.

2. Mix thoroughly.

3. Store in a brown bottle.

1.f Acridine orange in tris-HCl buffer, 0.05 M, pH 7.4

Purpose

Detection of malaria parasites in blood smears.

Parts

Tris-HCl, 6.61 g.

Tris base, 0.97 g.

H_2O, distilled, to 1000 mL.

Acridine orange, 10–50 mg per 1000 mL.

Preparation

1. Dissolve the salts in the distilled water.

2. Check the pH with a pH meter.

3. Dissolve the acridine orange in the buffer.

1.g Acridine orange buffer in KH_2PO_4 buffer, pH 7.2

Purpose

Differential staining of bacteria in natural water samples.

Parts

KH_2PO_4, 13.6 g.

H_2O, distilled, to1000 mL.

Preparation

1. Dissolve the KH_2PO_4 in the distilled water.

2. Adjust the pH to 7.2, if necessary.

3. Filter this buffer solution through a 0.2 or 0.22 µm pore size filter.

4. Store the filtered buffer in a sterile container that has been rinsed with filtered water or buffer.

Acridine orange stain

   KH_2PO_4 buffer solution, 100 mL.

   Acridine orange, 0.1 g.

Preparation

1. Mix the acridine orange in the buffer.

2. Store the stain in a particle free bottle, in the dark.

1.h Acridine orange in borate buffer, pH 9.8

Purpose

Differential staining of bacteria in urine specimens.

Parts

Buffer solution

   $Na_2B_4O_7$ (borax), 7.6 g.

H_2O, distilled, 1000 mL.

EDTA, 4 g.

Triton X100, 0.1 mL.

Formaldehyde, 37%–39%, 77 mL.

NaOH, 1 M   to pH 9.8.

Stain

H_2O, distilled, 100 mL.

Acridine orange, 0.5 g.

H_2O, distilled, 100 mL.

Preparation

1. Prepare the 0.2 M borax solution.

2. Add the other ingredients except the sodium hydroxide.

3. While stirring under the electrode of a pH meter, adjust the solution to pH 9.8 with sodium hydroxide, 1 M.

4. Dissolve the acridine orange in distilled water.

5. Filter both solutions through 0.45 mm pore size membrane filters.

6. Store the acridine orange solution in an amber bottle at room temperature.

1.i Acridine orange, aqueous 0.5%

Purpose

Detection of malaria parasites in blood smears.

Parts

Acridine orange, 500 mg.

H_2O, distilled, 100 mL.

1.j Acridine orange, aqueous 0.1%

Purpose

Detection of *Trichomonas vaginalis* in vaginal swab specimens.

Parts

Acridine orange, 100 mg.

H_2O, distilled, 100 mL.

2. Fluorescent acid-fast stains

2.a Blair fluorescent acid-fast stain

Purpose

Rapid, efficient detection of acid-fast bacilli in sputum smears.

Reagents

Auramine O–phenol

  Auramine O, 0.1 g.

  Ethanol, 95% (alternative: absolute methanol), 10 mL.

  Phenol crystals (colourless), 3 g.

  H_2O, distilled, 50 mL.

Preparation

1. Dissolve the auramine O in the ethanol.

2. Dissolve the phenol crystals in the distilled water.

3. Mix the two solutions.

Acid–alcohol

  HCl, concentrated, 0.5 mL.

  Ethanol, 70% (alternative: methanol), 100 mL.

1. Add the HCl to the ethanol.

Potassium permanganate (KMnO$_4$) background quencher

KMnO$_4$, 0.5 g.

H$_2$O, distilled, 100 mL.

1. Dissolve the potassium permanganate in the distilled water.

Alternative counterstains for auramine O acid-fast stain

Evans blue counterstain

Evans blue, 0.1 g.

H$_2$O, distilled, 100 mL.

Stain washed smear for 1 to 2 minutes.

Background fluoresces reddish.

Ink, blue counterstain

Ink, blue (Pelikan 4001), 100 mL.

H$_2$O, distilled, 900 mL.

Stain washed smear for 1 minute.

Background fluoresces reddish.

Methylene blue counterstain

Methylene blue, 0.3 g.

H$_2$O, distilled, 100 mL.

Stain washed smear for 1 to 2 minutes.

Background fluoresces greenish.

Thiazine red counterstain

Thiazine red, 0.1%, pH 7.0, 850 mL.

Magnesium chloride, 2%, 100 mL.

Aqueous phenol (90%), 50 mL.

Stain washed smear for 1 to 2 minutes.

Background fluoresces reddish.

2.b Smithwick fluorescent acid-fast stain

Purpose

Detection of acid-fast bacilli in sputum smears with a colour contrast background.

Reagents

Auramine O–phenol fluorescent stain

    Auramine O, 0.1 g.

    Ethanol, 95% (or absolute methanol), 10 mL.

    Phenol crystals (colourless), 3 g.

    H_2O, distilled, 87 mL

Preparation

1. Dissolve the auramine O in ethanol.

2. Dissolve the phenol crystals in the water.

3. Mix the two solutions.

Acid alcohol

    HCl, concentrated   0.5 mL

    Ethanol, 70% (or absolute methanol)   100 mL

1. Add the hydrochloric acid to the ethanol.

Acridine orange counterstain

    Na_2HPO_4, anhydrous, 0.01 g.

    H_2O, distilled, 100 mL.

    Acridine orange, 0.01 g.

1. Dissolve the disodium hydrogen phosphate in the water.

2. Dissolve the acridine orange in this solution.

3. Store this counterstain in an amber bottle at room temperature.

2.c Phenolic acridine orange acid-fast stain

Purpose

Efficient fluorescent acid-fast stain for detection of acid-fast bacilli in sputum and other specimens with a contrasting fluorescence colour background.

Reagents

Acridine orange reagent

Phenol crystals (colourless), 5 g.

H_2O (distilled or deionized), 50 mL.

Glycerol, 25 mL.

Ethanol, 95%, 25 mL.

Acridine orange, 0.1 g.

Preparation

1. Mix the first four ingredients, then add the acridine orange.

2. Allow the solution to rest overnight, and stir it again.

3. Store the stain in a brown bottle at room temperature.

Acid–alcohol destain solution

    Ethanol, 95%, 74 mL

    H_2O, deionized, 26 mL

    HCl, concentrated, 0.5 mL

    Methylene blue, 0.2 g

1. Mix the ingredients in order.

2. Store the destain solution in a brown bottle at room temperature.

D. Miscellaneous reagents

1. Ammonium sulfate globulin precipitation solution

Purpose

Precipitation of serum globulins.

Stock solution of saturated ammonium sulfate

$(NH_4)_2SO_4$, anhydrous, 75.4 g.

H_2O, distilled, 100 mL.

Working solutions of ammonium sulfate

Saturated Stock parts	H_2O parts (distilled)	Use dilution % $(NH_4)_2SO_4$	Final % in serum
10	0	100	50
9	1	90	45
8	2	80	40
7	3	70	35
6	4	60	30

2. Calcium chloride (0.1 M) differentiation reagent

Purpose

Enhanced differentiation of DNA and RNA in tissue.

Parts

$CaCl_2$, 1.47 g

H_2O, distilled, 100 mL

3. Indirect fluorescent antibody test substrate, Crithidia luciliae

Purpose

Detection of anti-DNA antibodies (See Section 7.9.2.3).

Crithidia luciliae preparations are available from the American Tissue Type Culture Collection and from commercial sources. The microorganisms must be stored at –70 °C. They are grown at 20–25 °C.

Preparation of Crithidia luciliae *for testing*

1. Wash *Crithidia luciliae* suspension three times with PBS at pH 7.4.

2. Centrifuge the suspension at 3000*g*.

3. Resuspend in distilled water to give a concentration of 20 million organisms/ mL.

4. Place 10 L of the suspension on a slide.

5. Dry in ventilated air.

6. Fix with 96% ethanol and incubate with patient's serum to be tested.

4. Merthiolate (thimerosal) preservative, pH 7.4

Purpose

Suppression of microbial growth in conjugates, serum, and reagents.

Stock solution, 1%

Thimerosal, 1.0 g.

PBS, pH 7.4, q.s. to 100 mL.

Store in a dark bottle at 0–4 °C.

Use (final concentration 1:10 000)

a. Serum, conjugates, etc.

0.01 mL of stock solution per 1.0 mL of sample.

b. PBS

0.1 g thimerosal powder per 1000 mL of PBS.

5. Merthiolate (thimerosal) preservative, borated

Purpose

Preservation of serum, conjugates, and buffered saline.

Stock solution, 1%

$Na_2B_4O_7$ (borax), 1.4 g.

Thimerosal, 1.0 g.

H_2O, distilled, to 100 mL.

Use

Final concentration 1:10 000 thimerosal.

Serum, conjugate, etc., 0.01 mL per 1 mL of sample.

6. Acetone dried tissue powder

Purpose

Tissue powders are prepared for sorption of conjugate to remove non-specific staining.

Reagents

Normal tissue (brain, liver, etc.), about 100–500 g.

Phosphate buffered saline, pH 7.4 at 4 °C.

Pre-cooled acetone, 4 °C.

Pre-cooled distilled water, 4 °C.

Preparation

1. Weigh the tissue and record the weight.

2. Add a volume of pre-cooled distilled water equal to the weight of the tissue.

3. Homogenize the tissue in a Waring (or other) blender. Turn the blender on and off in short intervals during the grinding to avoid heating.

4. Pour the homogenate into a large beaker (about 5 times the volume of the homogenate). Place the beaker on a magnetic stirrer.

5. Add four volumes of pre-cooled acetone and continue the mixing for 5 minutes.

6. Centrifuge the mixture at 500g for 10 minutes in a refrigerated centrifuge, 4 °C. Use large (150–250 mL) centrifuge bottles.

7. Decant the supernatant and add cold PBS, pH 7.4, while stirring the sediment well.

8. Centrifuge at 500g for 10 minutes as above. Decant the supernatant.

9. Repeat steps 7 and 8 until the supernatant is clear.

10. Resuspend the sediment in a minimal amount of cold PBS.

11. Strain the suspension through fine cheesecloth into a large beaker on a magnetic stirrer.

12. Rinse the bottles with cold PBS and pour the rinsing through the cheesecloth.
    (Note: it may be necessary to rub the material through the cheesecloth with the aid of a stirring rod.)

13. Add four volumes of precooled acetone to the strained material. (Volumes are based on the original volume of tissue homogenate.)

14. Centrifuge the suspension as before and decant the supernatant.

15. Resuspend the sediment in pre-cooled acetone.

16. Centrifuge the suspension again.

17. Repeat steps 13 and 14 until the supernatant is clear.

18. Resuspend the sediment in a small amount of acetone, and filter it through a Buckner funnel with suction using Whatman #52 filter paper.

19. Wash the material on the filter paper with several volumes of cold acetone. Stir the material during this phase, being careful not to tear the filter paper. When dry, the tissue powder should have a powdery feeling when rubbed between the thumb and forefinger.

20. Transfer the powder to Petri dishes and place it in the 37 °C incubator overnight.

21. Transfer the tissue powder to a dark brown bottle and store in a refrigerator.

Note: tissue powder preparations of whole organs are used to reduce non-specific staining. It is impractical, however, to prepare powders of tissue culture material in an effort to reduce non-specific staining of tissue culture infected material. Wet packed normal tissue culture cells may be used. In this case, there may be some dilution of the conjugate.

Use

1. Measure an aliquot of conjugate into a plastic centrifuge tube.

2. Add 100 mg of tissue powder per 1 mL of conjugate and mix.

3. Agitate the mixture for 1 hour at room temperature.

4. Centrifuge the mixture, 30 000g at 0–15 °C for 30 minutes.

5. Decant the supernatant conjugate and measure it into another plastic centrifuge tube.

6. Repeat steps 2, 3 and 4.

7. Filter the conjugate, if desired (0.45 mm pore size membrane filter).

8. Place the conjugate in an appropriate storage container. Add thimerosal (1:10 000 final concentration) for preservation.

9. Store the conjugate in small aliquots in a freezer (–20 °C) or in a refrigerator (0–4 °C).

Reference

Coons AH, 1958.

E. Reagents and tests

1. Biuret reagent and test for protein

Purpose

Biuret test for amount of serum protein.

Materials

$CuSO_4 \cdot 5H_2O$, 1.5 g.

$NaKC_4H_4O_6 \cdot H_2O$ (sodium potassium tartarate), 6.0 g.

KI, 1.0 g.

NaOH (10% solution, carbonate-free), 300 mL.

H_2O, distilled, q.s. to 1000 mL.

Method

1. Dissolve the copper sulfate and the sodium potassium tartarate in a 1L volumetric flask, in about 500 mL of distilled water.

2. Dissolve the potassium iodide.

3. Add, while swirling, 300 mL of 10% NaOH.

4. Bring the solution to 1000 mL with distilled water.

5. Mix thoroughly, and store the reagent solution in the dark in a plastic bottle (not glass). Discard the solution if a black or red precipitate forms.

Protein determination

Method

1. Prepare a 1:10 dilution (0.2 mL sample + 1.8 mL of 0.15 M NaCl solution) or a 1:20 dilution (0.1 mL + 1.9 mL) of the sample.

2. Prepare a blank using 2.0 mL of 0.15 M NaCl.

3. Add 8 mL biuret reagent to the samples and the blank.

4. Let stand for 30 minutes.

5. Read % transmittance of the samples in relation to the blank at 540 nm on a spectrophotometer.

6. Determine sample protein concentrations from the standard protein curve.

Preparation of protein calibration curve

1. Use a protein standard in the range of 6 to 10 g per 100 mL.

2. Prepare dilutions in the range of 80, 60, 40, 20 and 10 mg/mL.

3. Use these dilutions to develop a curve.

4. Plot a curve of % transmittance against protein concentrations. Use semi-log graph paper.

5. Prepare a new curve for each new batch of biuret reagent.

Reagents

0.15 M NaCl

    NaCl, 8.77 g.

    H_2O, distilled, q.s. to 1000 mL.

Biuret reagent

    $CuSO_4 \cdot 5H_2O$, 1.5 g.

    $NaKC_4H_4O_6 \cdot 4H_2O$ (sodium potassium tartrate), 6.0 g.

    KI, 1.0 g.

    NaOH (10% solution, carbonate-free), 300 mL.

    H_2O, distilled, q.s to 1000 mL.

References

Gornall AG et al., 1949; Dedmon RE et al., 1965.

2. Ammonium ion test for dialysate from globulin precipitation

Purpose

Testing of dialysate from dialysis of ammonium sulfate–precipitated globulin solutions for freedom from ammonium ions.

Reagents

Ammonium sulfate, 1:50 000 (positive control).

Weigh 1 g of $(NH_4)_2SO_4$ into a volumetric flask, and q.s. to 1000 mL with PBS (yields 1:1000 stock solution)

1 mL 1:1000 stock + 49 mL PBS = 1:50 000

1 mL 1:1000 stock + 24 mL PBS = 1:25 000

1 mL 1:1000 stock + 9 mL PBS = 1:10 000

Phosphate buffered saline (PBS), pH 7.4

KOH, 20%.

Nessler reagent.

HgI_2 45.5 g.

KI, 34.9 g.

KOH, 10 M 200 mL (112 g q.s. to 200 mL).

H_2O, distilled, q.s. to 1000 mL.

Preparation

1. Dissolve the salts in about 500 mL of distilled water.

2. Add the KOH solution, and dilute to 1000 mL.

3. Decant the clear supernatant liquid.

4. Store it in a plastic bottle or in a paraffin lined glass bottle.

Method

Set up three test tubes containing the solutions as shown in the following chart:

		Controls	
Item	*Mixture*	*Positive*	*Negative*
Dialysate	15.0 mL		
1:50 000 $(NH_4)_2SO_4$*		15.0 mL	
PBS, pH 7.4			15.0 mL
Nessler reagent	2.0 mL	2.0 mL	2.0 mL
20% KOH	2.5 mL	2.5 mL	2.5 mL
	Mix	Mix	Mix

A yellow-brown colour equals a positive test. Compare the test mixture to the controls.

*Positive controls at other concentrations may be used if desired.

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13.1 Fluorochrome dye fluorescence microscopy applications

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