# Protocol list

# Chapter 2
# Microbiological techniques for molecular biology: bacteria and phages

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## 1 Introduction: techniques for handling microbes

### 1.1 Basic microbiological techniques

Recombinant DNA technology depends on the manipulation of particular strains of bacteria and bacteriophages (phages). Expertise in isolating, checking, growing, and analysing these strains is crucial for the success of genetic engineering. Many experiments are now permitted under conditions of 'good microbiological technique' and anyone embarking on research in this area should check exactly what is meant by this phrase. Essentially, all of the basic techniques depend on the culturing of a particular microbe in the absence of other organisms (sterile or aseptic technique), the isolation of a genetically pure culture or clone derived from a single cell (single-colony or single-plaque isolation) and the characterization of the known genetic features of the strain. For safety, experiments should be carried out in such a way that recombinant microbes do not escape into the environment or infect the experimenter or others. The basis of sterile technique is the efficient sterilization of all equipment and growth media followed by the protection of the media from contamination during manipulations. If contamination of a culture is suspected then colony morphology, smell of the culture or staining of the cells followed by microscopic examination can be useful. Growth of the strains in liquid or solid media allows larger numbers of cells to be cultured and it is important to understand the various phases of the growth curve, as different types of cells are required for different experiments (1). Plating techniques such as dilution and streak plates can give rise to single colonies or plaques derived from individual cell or phage particles. Selective techniques are often used to isolate particular strains or to detect rare events, for example in transformation (see Chapter 8), and such techniques are very sensitive to low levels of contamination by unrelated strains which happen

to have the same characteristics as the desired strain: this may be resistance to antibiotics or nutritional independence.

## 1.2 Basic techniques of microbial genetics

Genetic purity of strains, plasmids or bacteriophages is critical and it should be clearly understood that no strains can ever be completely stable (2). Growth can lead to rearrangements of DNA, mutation of essential genes or infection by foreign DNA. Strains should therefore be carefully checked to make sure that they are of the correct genotype before starting crucial experiments. Chemicals and enzymes are not self-replicating and they are therefore usually purchased from suppliers. In contrast, strains of bacteria and phages are frequently subcultured many times and passed from one research group to another. Quality control and frequent checking is necessary or significant alterations in the genome of the organism may occur. It is particularly important to make a careful note of any deliberate changes or improvements in particular strains. This means recording the pedigree of the strains and using a unique numbering system, and detailing the full genotype. The dangers here are that essential gene mutations such as *recA* (deficient for the major recombination system) may have reverted to wild type and transposons may have caused rearrangements. An empirical approach can be adopted such that if the technique works the strain must be correct, but this can delay the problem until a later stage. Unless you are absolutely sure of the origin of the strain it is much safer to obtain the correct culture from a reputable supplier. When the correct strain has been successfully characterized it is essential that it is preserved efficiently. Any growth can lead to mutation and any desiccation or contamination can lead to death of the culture. The basis of preservation is to stop growth by lyophilization or freezing in the presence of cryoprotectants such as glycerol. Duplicate cultures should be kept in different locations to avoid dangers of apparatus failure.

## 1.3 Safety in the molecular biology laboratory

Although *Escherichia coli* K12 is not considered to be pathogenic, there are other strains of *E. coli* which are dangerous and can cause septicaemia or kidney infections. The development of disease symptoms depends on the type and number of organisms which gain access to the body, as well as on the efficiency of the immune system of the laboratory worker. Most healthy individuals can cope with small numbers of microbes but depression of the immune system by illness or infection or the use of immunosuppressive drugs can markedly increase the risks of a serious infection. It is safer to assume that all microbes are potential pathogens and to treat them with respect. The basic safety precautions are shown below and should be followed along with local and national regulations (3, 4).

(1) Laboratory overalls, which should ideally be side-or back-fastening, must be worn.

(2) Avoid all hand-to-mouth operations, such as licking pencils or labels.

(3) Make a habit of washing hands before leaving the laboratory.

(4) Before starting work, protect all cuts with adequate waterproof dressings.

(5) Cultures spilt on the bench, floor, apparatus or on yourself or others, should be treated with disinfectant. Material used to wipe up should be discarded for incineration or sterilization.

(6) Do not eat, drink, smoke or apply cosmetics in the laboratory.

(7) All contaminated apparatus should be sterilized before washing or disposal.

(8) All contaminated glassware such as pipettes and tubes should be discarded into disinfectant prior to sterilization and washing.

(9) All contaminated disposable plasticware should be discarded into strong autoclavable bags for sterilization or incineration.

(10) All apparatus for autoclaving or incineration should be carried in leak-proof containers.

(11) All apparatus used for the culture of microbes should be clearly labelled before inoculation. Apparatus to be left in communal areas should show your name, the organism and the date.

(12) All contaminated syringe needles should be discarded into special 'sharps' containers for special sterilization and disposal.

(13) Every effort should be made to avoid the production of aerosols. These are produced, for example, by blenders, centrifugation, ultrasonication and movement of liquids against surfaces. If the microbes present a hazard then the equipment used should be placed in a suitable safety cabinet.

(14) Records should be kept of the storage and transport of all microbes.

A more detailed treatment of this subject will be found in reference works by Collins (5, 6). In the UK, the regulations for the Control of Substances Hazardous to Health (7) clearly define microorganisms as a health hazard and state that an assessment should be made of the risks created by microbiological work and of the steps that need to be taken to ensure safe practice. Essentially, exposure to microbes by inhalation, ingestion, absorption through the skin or mucous membranes or contact with the skin must be either prevented or controlled. Thus, safety should be treated seriously and precautions taken to limit the access of microbes to the environment, laboratory workers, and the public. Safety is not the blind following of regulations but an awareness of the hazards and the methods which can be used to minimize them.

This chapter is intended to provide a basic introduction to the techniques involved in the handling of *E. coli* and its phages λ and M13. It does not include techniques in microbial genetics such as mutant induction, gene mapping and replica plating, which will be found in a variety of other manuals (8, 9).

## 1.4 Sterilization and disinfection

It is important to distinguish between these two processes. The aim of steriliza-

tion is to eliminate all microbes from laboratory equipment or materials, whereas disinfection aims to eliminate organisms which may cause infection. Space does not permit a theoretical treatment of this subject (5) but the important practical principles will be given.

Sterilization can be achieved by heat, chemicals, radiation, or by filtration. Nichrome loops are sterilized by flaming in a Bunsen burner, and disposable plastics can be sterilized by incineration. Glassware can be sterilized either by autoclaving or by dry heat. Autoclaving which uses wet heat is much more efficient than dry heat as hydrated microbes are killed more easily. Autoclaves vary from domestic pressure cookers to large, industrial-size motorclaves. It is very important that the operating instructions are followed and in particular that the autoclave is not overloaded, as the central region may not reach the necessary temperature. It is important to remove all of the air from the autoclave because the presence of air will depress the final temperature reached. Autoclave tape which changes colour after the correct time and temperature is a useful check. Loosen the caps of all bottles and do not autoclave completely-sealed bags in small autoclaves. Always make sure that there is sufficient water in pressure cookers and check that the correct procedure for autoclaving and recovery of materials is followed. Autoclavable plastic tubes such as pipette tips and microfuge tubes should be wrapped in autoclavable nylon bags. Dry-heat sterilization is normally used for flasks and glass pipettes, which should be left on a 6–12 h cycle at 160 °C. Sterilization with chemicals and radiation are impractical in the average laboratory. Sterile plasticware is normally produced by $\gamma$ irradiation.

Disinfection procedures will vary from laboratory to laboratory: 2% (v/v) Hycolin (Adams Healthcare) is a general-purpose disinfectant that turns from green to blue when no longer effective; an alternative is Virkon (Philip Harris). Any contaminated glassware or unwanted cultures should be immersed in disinfectant before autoclaving and washing. Contaminated disposable plasticware should be placed in autoclavable bags or bowls, which must be leak-proof. This material should be disposed of by incineration. Detailed procedures are the responsibility of the local safety officer.

## 1.5 Basic principles of aseptic technique

There are two basic principles of aseptic technique: protection of yourself and others, and protection of cultures and apparatus from contamination by unwanted microbes. In normal laboratory areas, microbes are everywhere—in the air, in dust, on your fingers. It is very difficult to produce an environment that is completely free from microbes, and special equipment such as laminar-flow cabinets and sterile areas is required. In a clean laboratory, with reasonable precautions, it is not necessary to use inoculating cabinets for the preparation of media and the manipulation of cultures. However, if you have trouble with contamination or your cultures are particularly slow-growing then a cabinet can be useful. Safety cabinets should not be needed for experiments classified as needing only 'good microbiological technique'. Experiments at higher levels of

containment need different precautions in different countries (3, 4) and you should consult your local safety officer for guidance.

Unless laboratory air has been efficiently filtered, it will contain many suspended bacterial cells and spores, fungal spores, and, in some laboratories, air-stable phage particles. This population of particles is added to by air movements which resuspend dust particles from bench surfaces. These airborne particles will settle on to any exposed surface and this is a major source of contamination, therefore anything which is to be kept sterile must be exposed to the air for a minimum period of time. Dust and aerosol particles tend to settle rather than drift sideways unless there is a draught of air. Consequently, containers and Petri dishes should not be left open, surface upwards, although tubes opened at an angle are less at risk. All apparatus which cannot be flamed in a Bunsen burner immediately prior to use should be left in the wrappers or containers in which they have been sterilized until needed. No sterile equipment should be allowed to come into contact with non-sterile surfaces. Plugs and caps from sterile tubes and bottles should not be placed on the bench, although they can be placed on a tile swabbed with a disinfectant.

The commonest source of contamination in the laboratory is the access of non-sterile air to the apparatus. This is increased by draughts and general movement of air and it follows that every effort should be made to work in still-air conditions. Windows and doors should be closed and all rapid movements in the laboratory should be eliminated. It is obvious that the laboratory should be free from dust which could be resuspended by air movement but it should be remembered that cleaning techniques such as sweeping and dusting can be a serious source of aerial contamination. The major advantage of inoculating cabinets is that they give protection from these air movements and allow a small volume of air to be sterilized by UV radiation or by a 70% (v/v) alcohol spray. The principles of spraying the air in a cabinet or over a bench is that it settles dust particles on to the bench, from where they can be removed with a paper towel. Fungal spores are adapted to aerial transmission and particular care should be taken when handling fungi or disposing of apparatus contaminated by fungi. Fungal spores released into the environment can take up to 7 h to settle and therefore can be a source of contamination for a considerable period.

Skin, hair, breath, and clothing are all sources of microbes and it is particularly important that you do not touch sterile surfaces such as the tips of pipettes and the inside of containers. Do not bend over your equipment such that skin scales or dust from your hair might fall into your cultures. Problems have arisen from contamination with yeasts traced to home baking of bread. Where strict asepsis is required (as in operating theatres) sterile caps, gloves and gowns should be worn.

## 2 Culturing of *Escherichia coli*

A culture of an *E. coli* strain will normally be received as a broth culture, on a Petri dish or as a freeze-dried culture from a supplier. The first step is to make a

careful record of the strain number and genotype of the strain. From this you will be able to identify an appropriate medium on which it will grow well and any additions such as antibiotics which are necessary to ensure the stability and maintenance of plasmids. Prepare some well-dried agar plates, as described in *Protocol 1*. It is necessary to dry the plates because *E. coli* is motile and will swim across the plate in the thin film of water. In addition, contaminants will also spread more easily across the plate and the desired single colonies will not be isolated. Cooling the agar at 50 °C reduces the condensation of water on the inside of the lids. Drying the plates overnight at 37 °C has the advantage that contaminated plates can be detected and discarded, but care should be taken to avoid microcolonies on the plate. This is particularly critical with spread plates where the contaminant colonies will be spread over the plate. To avoid this problem it is better to dry the plates in an oven.

## Protocol 1

## Preparation of agar plates

### Equipment and reagents

- Agar medium
- Steamer or microwave oven
- Water bath at 50 °C
- Petri dishes

### Method

1. Select the ingredients for the required agar medium.
2. Loosen the top of the agar bottle and melt the agar in a steamer or in a microwave oven. Do not use metal caps in a microwave oven.
3. Swirl the liquid gently to check that the agar is fully melted. Take care that superheated agar does not boil over.
4. Allow the agar to cool for about 10 min at room temperature and then place in a water bath at 50 °C for at least 20 min.
5. Place the concentrated nutrient medium in the same water bath to equilibrate to the same temperature.
6. Flame the tops of the glass bottles containing agar and concentrated nutrients and pour the nutrient liquid into the bottle containing the agar. Screw the cap tight and shake to ensure complete mixing.
7. Return the bottle to the water bath and allow time for any air bubbles to disappear.
8. Arrange the sterile Petri dishes on a level surface and label the base of each plate to indicate the medium prepared.
9. Remove the bottle from the water bath and wipe the outside carefully with a paper towel (water baths can be contaminated).
10. Flame the neck of the bottle and pour the required amount of medium into the plate. This will vary between 10 ml for a thin plate for short-term bacterial culture to 40 ml for phage culture where large plaques are required.

**Protocol 1** continued

**11** Allow the plates to set. Dry the surface of the plates by overnight incubation at 37 °C (check for contamination the following morning) or by opening the plates and placing them, medium surface down, in an oven at 45–55 °C. The lids should also face downwards separately from the base of the plate. Leave plates at 45–55 °C for 15 min.

**12** Most nutrient plates can be stored for at least a few weeks at 4 °C following wrapping in parafilm or sealing in a plastic bag.

*Escherichia coli* can be cultured on slopes, on plates, in broth, or in stab cultures. The first step is to isolate single colonies usually by a streak plate method (see *Protocol 2*). A single colony is used to produce a series of identical broth cultures and a check is made of the phenotype of the strain. The culture can then be used for experimental purposes. As soon as it is determined that the culture is correct, every effort should be made to preserve it. This can be done on plates, in broths, or in stab cultures where protection from contamination and desiccation is particularly important (Section 4). Freezing is necessary for long-term storage.

## 2.1 Single-colony isolation

The principle of this technique is to streak a suspension of bacteria until single cells are separated on the plate. Each individual cell will then grow in isolation to produce a clone of identical cells known as a colony. The vast majority of these cells will be genetically identical, although mutation can occur during the growth of even a single colony to give low levels of mutant cells. This technique assumes that there are no clumps of cells in the culture; however, it is not unknown for contaminant organisms to stick to bacterial cells via an extracellular polysaccharide. Any clumps will be visible upon examination of a culture under the microscope (see Section 3.8). If they are present, every effort should be made to disrupt them by suspension in PBS followed by agitation or gentle ultrasonication treatment. Repeated single-colony isolations should result in a pure culture.

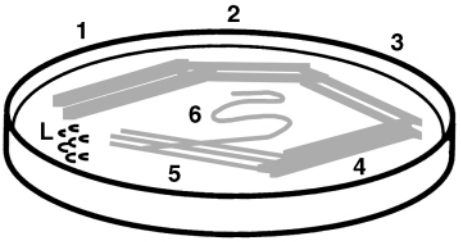


**Figure 1** Procedure for the production of single colonies by the streak plate technique, as described in *Protocol 2*.

## Protocol 2

## Streak plate method for single-colony isolation (refer to *Figure 1)*

### Equipment and reagents

- Nichrome loop
- Mixer
- *Either* PBS (8.0 g NaCl, 0.34 g $KH_2PO_4$, 1.21 g $K_2HPO_4$ per 1 l). pH should be 7.3; sterilize by autoclaving
- *or* Ringer's solution

### Method

**1** Flame a Nichrome loop which is about 3 mm across and has a stem of about 6 cm. Allow the loop to cool, or cool by immersion in a sterile area of agar.

**2** Flame the neck of an overnight broth culture and remove a loopful of cells. Alternatively, make a suspension of cells directly from an agar plate in PBS or dilute Ringer's solution. Vortex and remove a loopful of cell suspension.

**3** Streak the cells at one side of a well-dried agar plate at position 1 as shown in *Figure 1*. Streak several times close together.

**4** Flame the loop and cool carefully at one side of the plate (position L).

**5** Streak again at position 2 on *Figure 1*.

**6** Flame the loop and cool as before. Repeat steps 4 and 5 as indicated at positions 3, 4, 5 and 6 on *Figure 1*.

**7** Incubate the plate at 37 °C with the agar facing downwards to minimize contamination and to reduce the chance of droplets of condensation falling on the agar surface.

The plates are then examined for colony morphology and the presence of possible contaminants. If all of the colonies are of a uniform size and appearance it can be assumed that you have a pure culture. Subculture of almost any colony should give the required strain and the plate is worth keeping as a future source of a purified culture. When plasmid-containing strains are plated on a medium supplemented with an antibiotic it is often observed that colonies of a variety of sizes are obtained. It is not always immediately obvious which is the correct strain and it may be necessary to subculture a representative range of colonies on to fresh medium for further checking. A careful note should be made of the characteristics of the correct strain for future reference. Re-streak the correct colony to give a plate containing a uniform colony size and use this as a source of purified culture.

In a number of experiments, such as transformation and the preparation of plasmid or cosmid genomic libraries, it is necessary to have as many single colonies on the same plate as possible. This involves a dilution series and spread plates:

## Protocol 3

## Dilution series and the spread-plate method for single-colony isolation

### Equipment and reagents

- PBS or Ringer's solution (see *Protocol 2*)
- LB agar plates (10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl, 15 g bacto-agar per 1 l). Check the pH and adjust to 7.0–7.2 with NaOH; sterilize by autoclaving at 121 °C, 103.5 kPa (15 lb/in$^2$), for 20 min
- Glass spreader and ethanol

### Method

1. Prepare a series of six tubes containing 9 ml of PBS or dilute Ringer's solution. Label the tubes –1, –2, –3, –4, –5, and –6. Prepare three LB agar plates labelled –4, –5, and –6.
2. Take 1 ml of the test culture (assumed to be about $10^8$ cells/ml) and add to tube –1. This is a $10^{-1}$ dilution. Vortex the tube to mix the cells.
3. With a fresh pipette or disposable tip, take 1 ml from the –1 tube and add to the –2 tube. This is a $10^{-2}$ dilution.
4. Repeat this operation from –2 to –3 and so on down the series until tube –6 is reached.
5. Take 100 μl of the –6 dilution and add to an LB agar plate.
6. Dip a glass spreader into ethanol in a glass Petri dish and pass rapidly through a Bunsen burner to burn off the alcohol.
7. Cool the spreader on the agar surface and spread the suspension evenly over the plate. Repeat for tubes –5 and –4.
8. Incubate the plate overnight at 37 °C. There should be about 1000, 100, and 10 colonies on the –4, –5, and –6 plates respectively. Plate lower dilutions for more dilute suspensions of cells.
9. A variation of this method, which is more economical on plates, is to add drops of 20 μl on marked places on the same plate without spreading. This is the Miles–Misra technique. The plate should be incubated at 25 °C for 2 days to give smaller colonies, which are easier to count and to subculture.

## 2.2 Small-scale broth culture

Suspend an appropriate single colony in 0.5 ml of PBS or dilute Ringer's solution (see *Protocol 2*) and vortex the suspension. Use this to inoculate a series of three or four tubes containing 5 ml of a suitable liquid nutrient medium. Incubate the tubes at 37°C overnight and then store the tubes at 4 °C until required. If higher cell densities are required the tubes can be shaken at 250 r.p.m. during over-

night growth. These cultures can be used over a period of weeks to provide a source of purified cultures. Alternatively, before each experiment take a single colony from the original plate and use it to inoculate a single 5 ml broth culture, shake overnight and then use to seed a larger-scale culture.

## 2.3 Large-scale broth culture

These cultures are prepared when more cells are required for plasmid extraction or as a source of competent cells for transformation. The scale of the culture depends on the number of cells required. A typical yield would be $5 \times 10^8$ cells/ml so that a 20 ml culture would give a total of $10^{10}$ cells (dry weight 4 mg) whereas a 500 ml culture would produce $2.5 \times 10^{11}$ cells (dry weight 100 mg). A 3–5 ml culture is suitable for a mini-preparation of plasmid DNA whereas a 500 ml culture is required for a large-scale plasmid preparation (see Chapter 3, Section 2.3).

## Protocol 4

## Large-scale broth culture of *E. coli*

### Equipment and reagents

- Sterile media
- Spectrophotometer and cuvettes

### Method

1. Prepare a 5 ml broth culture from a single colony as described above (Section 2.2) and use this as a seed culture for the large-scale culture.
2. Prepare conical flasks containing the appropriate volume of sterile medium in flasks roughly 10 times the volume of the medium (i.e. 25 ml in a 250-ml flask or 200 ml in a 2-l flask). This ratio has been shown to give maximum aeration so that oxygen is not the growth-limiting factor. However, it is possible to obtain good yields of cells using 500 ml of broth in a 2-l conical flask.
3. Dilute the seed culture 1 in 20 into the large-scale culture and shake the flask overnight at 37 °C on a rotary shaker at 250–300 r.p.m.
4. Harvest the cells by centrifugation at 2000–4000 **g**, depending on the tightness of the pellet required and the characteristics of the strain.
5. Resuspend the pellet of cells in a suitable buffer—this will vary according to the purpose of the experiment.
6. Recentrifuge and resuspend the pellet for further use.
7. To follow a growth curve (see Section 2.3.1), wait until the culture is just visibly turbid (usually about 2–3 h) and remove a small sample (1–2 ml depending on culture size).
8. Assay OD at 550 nm in disposable cuvettes using the uninoculated growth medium as a blank.
9. Continue sampling approximately every 30 min until the desired OD is reached.

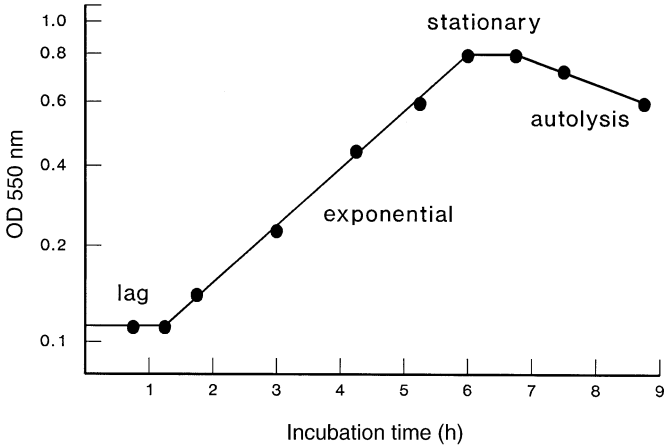


**Figure 2** Typical growth curve of *Escherichia coli* in a shake flask at 37°C. Samples are taken about every 30 min and the OD at 550 nm measured. The length of the lag, stationary and autolysis phases will vary.

## 2.3.1 The bacterial growth curve

A knowledge of bacterial growth kinetics is essential for a number of techniques in recombinant DNA technology (1). *Figure 2* shows a typical growth curve for *E. coli*, the basic features of which are a lag phase of about 1.5 h followed by a period of exponential growth, a deceleration phase, a stationary phase and finally a decline or autolytic phase. When an overnight culture is diluted 1 in 20 into fresh medium, there is typically a lag phase when no growth can be detected. This can vary from 1.5 h to 3 h, depending on the strain, its growth rate, and the number of cells inoculated. The easiest way to monitor growth is by use of spectrophotometry. Essentially, this is a measure of the turbidity (OD) of the

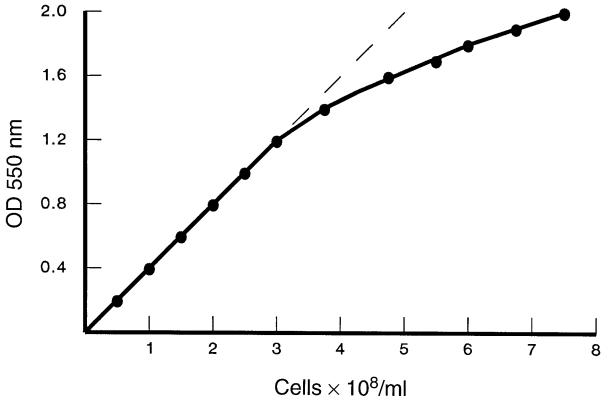


**Figure 3** Typical standard curve for the correlation of cell numbers and OD. Cell numbers can be estimated by the dilution and spread-plate techniques described in *Protocol 3*. The standard curve will vary with strain, cell size and spectrophotometer used. It is useful to obtain dry weight estimates for a similar range of samples.

culture estimated on instruments which record absorbance. When a beam of light is passed through a suspension of bacterial cells, light is scattered so less light passes through the suspension in comparison with a control sample. The intensity of the light decreases exponentially as the bacterial concentration increases linearly over a limited range. Ideally, this should be checked for a particular strain with a known spectrophotometer. In a complete analysis there should also be a check of OD against cell numbers or dry weight of cells (*Figure 3*). In practice, it is normally sufficient to note values of OD for particular strains which give good competent cells or adequate yields of cells for plasmid extraction or growth of phage λ. Until you are used to a particular strain it is recommended that you follow OD at intervals and plot this on semi-log paper. This will show the lag and exponential period of growth and it will allow you to predict when the cells will be ready for harvesting.

# 3 Characterization of bacterial strains

## 3.1 Genotypes and strain nomenclature

The first step in characterizing a strain is to check its phenotype and, indirectly, its genotype. Some expertise is required in understanding symbols, which in general are based on a system proposed by Demerec *et al.* (10). A summary of the major points of this proposal follows (see also ref. 11).

(1) Each locus of a wild-type strain is designated by a three-letter, lower-case italicized symbol (e.g. *arg* is the gene determining and regulating arginine biosynthesis).

(2) Different loci, any one of which may mutate to produce the same gross phenotypic change, are distinguished from each other by adding an italicized capital letter immediately following the three-letter lower-case symbol (e.g. *argA*, *argB*).

(3) A mutation site should be designated by placing a serial isolation number after the locus symbol. If it is not known in which of several loci governing related functions the mutation has occurred, the capital letter is replaced by a hyphen (e.g. *argA1*, *argB2*, *arg-3*).

(4) Plasmids should be designated by symbols which are clearly distinguishable from symbols used for genetic loci.

(5) Mutant loci and mutational sites on plasmids should be designated by symbols of the same type as those used for loci on the chromosome.

(6) Phenotypic traits should be described in words, or by the use of abbreviations which are defined the first time they appear in a given paper. The abbreviations should be clearly distinguished from the genotype symbols (e.g. the $Arg^-$ phenotype is an arginine requirement, associated with the *argA* locus).

(7) Strains should be designated by simple serial numbers. To avoid duplications, different laboratories should use different letter prefixes. Strain designations should not be italicized (e.g. HB101).

(8) When a strain is first mentioned in a publication its genotype should be described and relevant phenotypic information should be given. The genotype includes a list of all mutant loci and/or mutant sites, a list of plasmids, and information concerning the state and location of any episomes (plasmids or prophage).

A careful record should be kept of strain designations, the origin of the strains and details of the genotype. It is not enough simply to label the strain with the plasmid which it contains as you will need to grow the strain under conditions that allow good growth without the loss of the plasmid. Maintenance of the plasmid often depends on a selective technique; for example, by incorporating an antibiotic such as ampicillin in the medium (see Section 3.3). In some cases, a wild-type gene on the plasmid will complement a deletion or mutant gene on the chromosome. In these cases, growth on a medium deficient in the growth factor will be necessary to ensure survival of the plasmid (Section 3.2). Similarly, the knowledge that the host strain possesses a deletion of the lactose operon may be essential in analysing plasmids which carry the normal β-galactosidase gene. These various points are illustrated by considering the genotype and phenotype of *E. coli* strain HB101.

$F^-$ Lacks the sex factor F.

*hsdS20* A mutant of type ($r_B^- m_B^-$) in the site recognition gene for the strain B restriction endonuclease system. This makes the strain deficient for both restriction and modification of the DNA.

*recA13* Deficient for major recombination protein. The strain is UV sensitive and lacks the major *E. coli* recombination system. This reduces the chance of rearrangements and transfer of recombinant DNA.

*ara-14* Unable to utilize arabinose as the sole source of carbon and energy.

*proA2* Requirement for proline in the medium.

*lacY1* Mutation in the permease for the uptake of lactose.

*galK2* Unable to utilize galactose.

*rpsL20* Resistance to streptomycin ($Sm^r$) owing to a mutation in the ribosome.

*xyl-5* Unable to utilize xylose.

*mtl-1* Unable to utilize mannitol.

*supE44* Carries a suppressor for the amber chain-terminating triplet.

$(\lambda)^-$ Non-lysogenic for bacteriophage λ. Does not carry the λ prophage.

This genotype tells us that the strain carries no plasmids or phage λ and so can be used for plasmid transformation or assay of phage. It can be transformed efficiently by plasmid DNA and then used for the production of large-scale preparations of plasmid DNA. It can grow on glucose but is unable to grow on a range of sugars as sole sources of carbon and energy. A chemically defined medium would need to have proline added, but this would normally be supplied by peptone in complex media. Although this strain is $Lac^-$ it does carry a wild-type

gene for β-galactosidase (*lacZ*$^+$) so this strain is unsuitable for use as a host for plasmids which depend on lactose fermentation to give a blue coloration with X-gal (Section 3.5). The *supE* locus means that it will suppress amber mutations, for example in phage λ, thus allowing it to produce plaques.

Space does not permit a complete glossary of a wide range of symbols and the reader is referred to *Appendix 1* and to the *Molecular Biology Labfax* (11). The latter contains genotypes of all relevant strains as well as a list of gene symbols.

## 3.2 Characterization of nutritional mutants

It is normally only essential to check for genetic characters which you intend to use in a particular experiment. Thus if absence of β-galactosidase activity is essential then you should check that the strain is Lac$^-$. Similarly if you needed to select for the Pro$^+$ phenotype then you would need to check that the strain is proline requiring. A method illustrating the general points is given in *Protocol 5*. However, if you suspect that your strain has mutated, reverted or is a contaminant, it may by necessary to do a more thorough check of other markers using the same general approach.

## Protocol 5

## Analysis of nutritional mutants

### Reagents

- PBS or Ringer's solution (see *Protocol 2)*

### Method

**1** Prepare a stock solution of a chemically defined minimal agar medium lacking a carbon and energy supply or growth factors.

**2** Examine the genotype of the strain(s) for analysis and identify the growth factors to be analysed. The system will be illustrated for HB101 and the *pro*, *lac*, and *mtl* markers only.

**3** Label four tubes (20 ml capacity) with the letters A, B, C, and D and make additions as indicated:

| | A | B | C | D |
|---|---|---|---|---|
| proline | + | + | + | – |
| glucose | + | – | – | + |
| lactose | – | + | – | – |
| mannitol | – | – | + | – |

Use a 1% (w/v) proline stock solution and dilute 1 in 100; use 20% (w/v) sugar stocks diluted to 0.1% final volume.

**4** Add the contents of each tube to 20 ml agar medium and pour four plates (see *Protocol 1*).

**Protocol 5** continued

5 Suspend single colonies of HB101 and a wild-type control in 0.5 ml PBS or dilute Ringer's solution and vortex.

6 Streak a loopful of HB101 and the wild-type control on to all four media at previously marked positions.

7 Incubate for 16–24 h and record growth. Always compare the relative growth of the mutant strain on the media with the growth of the wild-type strain.

8 Analysis of mutants defective in the utilization of different nitrogen, sulphur or phosphorus sources requires a minimal medium lacking these supplements.

## 3.3 Characterization of antibiotic resistance

The resistance of bacterial strains to antibiotics is a very useful selective technique. Genes controlling this resistance can be carried on the bacterial chromosome or on a plasmid and the latter are very useful in ensuring that a particular plasmid is present (see Chapter 8, Section 3.1). However, it should be remembered that plating large numbers of sensitive cells on media containing an antibiotic can select rare spontaneously-resistant mutants due to chromosomal mutation rather than to the receipt of a resistant plasmid. Similarly, contaminants may also be naturally resistant to the same antibiotic and even a low level of contamination will be readily revealed on the selective plates. Because antibiotics are often thermolabile they cannot be sterilized by autoclaving, therefore solutions should be membrane filtered. Most antibiotics are supplied sterile and it is possible to make up solutions using aseptic technique in sterile distilled water. The actual concentration of antibiotic required depends on the conditions used. In general, higher concentrations are required for high density of cells on agar plates while low concentrations are needed for low densities of cells growing in liquid medium. Detailed techniques for checking antibiotic resistance are shown in *Protocol 6* and *Appendix 2* lists some of the points to note about particular antibiotics.

## Protocol 6

## Analysis of antibiotic resistance

### Reagents

- Stock solutions of antibiotics (see *Appendix 2*)
- LB agar plates (see *Protocol 3*)
- PBS or Ringer's solution (see *Protocol 2*)

### Method

1 Make up stock solutions of antibiotics as described in *Appendix 2*, using aseptic technique and sterile distilled water. Filter through a sterile membrane, pore size 0.45 μm, if desired.

**Protocol 6** continued

2 Distribute in aliquots of 200 μl into labelled 1.5-ml microfuge tubes and store at −20 °C until required.

3 Make up a suitable nutrient medium such as LB agar and cool to 50 °C. Pour and dry a few plates (see *Protocol 1*) without the antibiotic, to act as controls.

4 Add the antibiotic to the remainder of the nutrient medium to give the desired concentration and mix well.

5 Pour and dry the antibiotic plates. Most antibiotic plates will keep for at least 2–3 weeks.

6 Mark each set of agar plates with a suitable grid and label with the strains to be tested. It is best to include control strains which are sensitive to the antibiotic to test the efficiency of the antibiotic activity.

7 Suspend a single colony of a strain to be tested in 200 μl of PBS or dilute Ringer's. Vortex to suspend the cells.

8 Flame and cool a Nichrome loop about 3–4 mm diameter and remove a loopful of cell suspension.

9 Streak this suspension over a distance of 1–2 cm on each antibiotic plate. Repeat on the medium without antibiotic.

10 Repeat steps 7–9 with each strain to be tested, including the control strain.

11 Incubate at 37 °C for 16–36 h depending on the strain and the antibiotic. Compare the growth of the strains on the antibiotic and control media. Note that if antibiotic activity is low or absent (due possibly to inactivation because the agar was not cooled sufficiently in step 3) then the control strain will grow on both media.

12 For large-scale checking of transformants or other recombinants, it is more convenient to pick off individual colonies with sterile toothpicks and to streak plates with these directly.

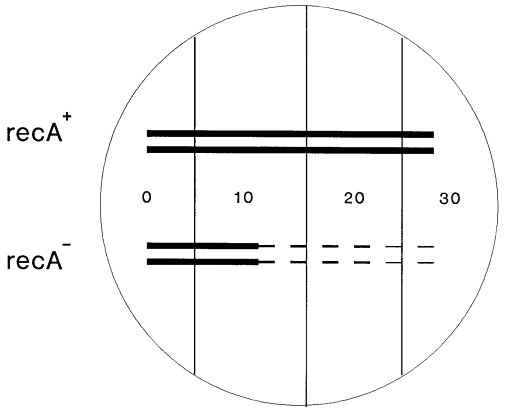


**Figure 4** Procedure for characterizing *recA* strains. The marked areas show the expected growth after irradiation and incubation.

## 3.4 Characterization of *recA* and UV-sensitive mutants

Recombination-deficient mutants, such as *recA*, are used to minimize recombination between cloned genes and homologous regions on the chromosome. In addition, the use of *recBC* mutants can minimize the loss of repetitive DNA in gene libraries. It is difficult to check for recombination deficiency, but luckily there is an associated phenotype of UV-sensitivity which is easy to analyse. As with any genetic character, it is important to have both *recA*$^-$ and *recA*$^+$ strains so that a comparison can be made. The technique is as given in *Protocol 7*.

## Protocol 7

## Checking strains for the Rec$^-$ phenotype (see *Figure 4*)

### Equipment and reagents

- LB agar plates (see *Protocol 3*)
- Bactericidal UV lamp (254 nm)

### Method

1. Prepare overnight broth cultures from single colonies of Rec$^+$ and Rec$^-$ cultures.
2. Using a flamed Nichrome loop, streak each strain across a well-dried LB plate such that the streak is about 6 cm. Allow the liquid to dry.
3. Mark the base of the agar plate so that each streak is divided into four segments labelled, left to right, 0, 10, 20 and 30 s.
4. Cover the plate with a piece of card such that only the 30 s segment is uncovered. Remove the lid of the agar plate and expose to a bactericidal UV lamp (254 nm) at a height of about 30 cm for 10 s. **Caution: wear UV safety goggles and gloves during these operations.**
5. Move the card across so that the 20 s segment is exposed for 10 s.
6. Move the card across so that the 10 s segment is exposed for 10 s.
7. Switch off the lamp, wrap the plate rapidly in foil to avoid light-induced repair processes and incubate at 37 °C overnight.
8. The segments will have received 0, 10, 20 or 30 s exposure to UV and there should be a clear distinction between the two phenotypes.

## 3.5 Characterization of the utilization of lactose: X-gal

There are several ways of detecting the utilization of sugars such as lactose. One method makes use of a chemically defined medium in which lactose is the sole source of carbon and energy. It is used to replace glucose and, in this medium, only Lac$^+$ strains will be able to grow. Alternatively, a nutrient medium with lactose can be used which contains the indicator dyes eosin and methylene blue. When lactose is fermented, acid is produced and the indicators change colour: Lac$^+$ colonies with β-galactosidase activity give dark purple colonies with a green

fluorescent sheen whereas Lac$^-$ colonies are pink. A more expensive way of analysing for this characteristic is to use the substituted β-galactoside sugar X-gal. Media are normally prepared containing this compound, together with IPTG which fully induces the *lacZ* gene but which is not a substrate for the enzyme. Details are given in Chapter 8, Section 3.2.

## 3.6 Detection of lysogeny

Bacteria carrying the prophage of temperate phages such as λ and P2 have been exploited in a variety of ways. The λ lysogens have been used to produce packaging extracts for isolation of genomic DNA libraries and P2 lysogens are important in the Spi$^+$ selective system for some types of recombinant λ phages (Chapter 8, Section 3.4). When it is necessary to check for lysogeny it is essential to have a strain which is sensitive to the phage. Thus, for a (P2)$^+$ lysogen it is necessary to have a second strain which is sensitive to phage P2 as well as, ideally, a sample of phage P2 itself. The simplest check is to make a single streak of each strain, such as *E. coli* Q359 (P2)$^+$ and Q358 (P2)$^-$, on an LB agar plate. The liquid is allowed to dry and a small volume of a P2 stock is spotted on the streak. Q359 is immune to the phage due the presence of a repressor, while Q358 is lysed. If you do not have any P2 phage then prepare pour-plates of each strain and spot overnight broth cultures of Q358 and Q359 on to each lawn. Q359 produces low numbers of free phages, owing to spontaneous lysis, and these appear as a thin halo of lysis round the spot of Q359 on the lawn of Q358 (*Figure 5*). Similar techniques will also work for λ phage and lysogens.

## 3.7 Screening for plasmids

It is essential to have a good accurate map of the plasmid with details of its genotype. The most popular plasmid vectors are described in Chapter 9, and detailed compilations have been published (11). The easiest characters to detect are antibiotic resistance. For example, the pUC and pGEM series of vectors are resistant to ampicillin, so a simple check can therefore be made by streaking on

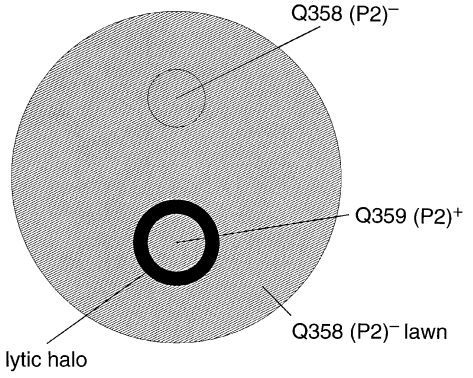


**Figure 5** Detection of lysogeny by spot testing of strains on a bacterial lawn.

media containing ampicillin. It is advisable to include the selective antibiotic, whether this is ampicillin or some other compound, in all media used for the growth and maintenance of plasmid-containing bacteria because the *par* locus, which controls regular partition, has been deleted in most plasmids. Cells lacking plasmids can therefore be generated when cells divide but these will not survive if the selective agent is present in the medium. Further preliminary screening depends on other markers carried by the plasmid, and final checking will depend on extraction, restriction and electrophoresis of the plasmid DNA.

## 3.8 Microscopic examination of cultures

A visual check of a culture may suggest contamination, or it may be that a rare transformant simply does not look right. Rather than extracting and analysing plasmid DNA it may be worth having a look at the organism to check that it is actually Gram-negative and rod-shaped. It is even quicker to do a simple smear followed by a methylene blue stain as this will quickly eliminate budding yeasts and cocci. *Protocol 8* describes the basic methods.

## Protocol 8

## Techniques for the microscopic examination of strains

### Equipment and reagents

- Loeffler's methylene blue, crystal violet, Lugol's iodine, ethanol and dilute fuchsin
- Microscope with oil immersion lens, plus microscope slides

### Method

1. Clean a microscope slide by immersing it in alcohol and flaming off in a Bunsen burner.
2. Either place a loopful of a cell suspension onto the slide or suspend cells from an agar plate in a small drop of water on the slide.
3. Spread the suspension thinly and evenly over the slide. This is best done with the edge of a second microscope slide. The smear should be only just visible and the aim is to produce a monolayer of cells.
4. Leave the slide to air-dry or hold high over a Bunsen burner to dry the smear slowly.
5. Fix the cells by passing the dried slide slowly three times through the Bunsen flame. The bacteria will adhere to the slide.
6. Place the smear on a staining rack (two glass tubes held together by short pieces of rubber tubing). Flood the slide with methylene blue and leave for 5 min.
7. Wash the slide under the tap and blot dry with blotting or filter paper.
8. Add a small drop of immersion oil and view down the microscope using the oil immersion lens. Look for areas where the cells are well spread out.

**Protocol 8** continued

**9** Alternatively, use the Gram stain:

(a) Stain in crystal violet for 1 min, then wash well under running water and blot dry.

(b) Wash with Lugol's iodine and then stain for 1 min with fresh Lugol's iodine.

(c) Wash with tap water and blot dry. Decolourize with ethanol until no more dye is washed out.

(d) Wash with tap water and counterstain with dilute fuschin for 30 s.

(e) Wash and blot dry. Gram-negative cells will appear red whereas Gram-positive cells will be dark purple. It is best to have examples of both types when you are practising the technique.

## 4 Preservation of stock cultures

A culture which has been checked and used successfully is a valuable laboratory asset and should be preserved as efficiently as possible. It is sensible to preserve the culture by a number of methods as you will require the strain for short-term and long-term use. The broth and plate cultures already described can be used for several weeks or even months, but there are problems inherent in using cultures stored at 4°C. Cultures may become contaminated or lose viability; they can also mutate or be infected by phages or other plasmids. Some microbiologists believe that the only stable strain is a dead strain. This is true, but we can get close to complete lack of growth and slow chemical change in frozen cultures. Alternatively, freeze-dried cultures are convenient to keep and are not susceptible to damage following electrical breakdown. There are several good reviews of preservation methods (12, 13), therefore detailed techniques on freeze-drying will not be given here.

### 4.1 Preservation of short-term cultures

Broth cultures of *E. coli* will retain viability for several weeks if stored in sealed containers at 4°C. This can be recommended for cultures which lack plasmids and show little variation, such as *E. coli* HB101. The use of broth cultures older than a few days for strains carrying unstable plasmids is not recommended as the antibiotics used to select the plasmid may have been inactivated and growth of cells without the plasmid may have occurred. It is much safer to use an agar plate with single colonies which have previously been checked. The major problems with agar plates are desiccation and contamination. These problems can be reduced by sealing the edge of the plates with parafilm or adhesive tape. Plates like this can be used successfully for 2–3 months, but for particularly critical experiments you should check your strains again by re-streaking on a selective or diagnostic medium.

## 4.2 Stab cultures

A very simple and effective method for keeping bacterial strains is to stab actively-growing cells into a nutrient medium containing 0.6% (w/v) agar. A conveniently-sized tube is about 4 × 1 cm with a water-tight cap. Wrap the tubes separately from their caps and autoclave for 15 min at 121 °C (check that the caps are autoclavable). Fill the tubes two-thirds full with sterile nutrient agar at 50°C and cap the tubes lightly. When set, incubate the tubes at 45 °C for 1–2 h to remove moisture. Label the tubes and inoculate with a loopful of an overnight broth culture of a checked strain. Stab the loop straight down the middle of the agar. Incubate the tube overnight at 37 °C with the cap loose and check for contamination. This can be seen as disc- or lens-shaped colonies suspended in the agar. Discard contaminated stab cultures. Check that the culture has grown in the stab region, then tighten the cap, and seal with candle wax or melted paraffin wax if a more effective air-tight seal is required. Store the tubes mounted in polystyrene sheets in cupboards or drawers away from direct sunlight. Viable cultures have been isolated from such tubes after periods of up to 10 years.

## 4.3 Preservation of cultures with glycerol or DMSO

Cultures can be preserved very effectively if frozen in the presence of a cryoprotectant which reduces damage from ice crystals (13). Temperatures above −20 °C are not very effective because of the formation of eutectic mixtures exposing cells to high salt concentrations. The simplest way to preserve a culture is to add 15% (v/v) glycerol to the culture and then to store it at −20 °C or −80 °C in a freezer or at −196 °C in liquid nitrogen. In practice, the survival of cells also depends on the freezing rate and efforts should be made to control this at the optimum for the strains used. *Protocol 9* shows a method for preserving broth cultures with glycerol. This method can be extended to nutrient plates containing 15% (v/v) glycerol overlaid with a nitrocellulose or nylon filter containing bacterial colonies. The whole system is wrapped in a nylon bag and stored in a −80°C freezer. This is particularly useful for gene libraries in bacterial cells, as a template or a dense plate of colonies can be stored conveniently. Another useful approach is to coat glass beads with the glycerol mix. On partial thawing, each glass bead is a convenient inoculum to grow up fresh cultures. DMSO can also be used as a cryoprotectant at 5–7% (v/v).

## Protocol 9

## Preservation of cultures by freezing with glycerol

### Reagents

- LB broth (10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl per 1 l). Check the pH and adjust to 7.0–7.2 with NaOH; sterilize by autoclaving at 121°C, 103.5 kPa (15 lb/in$^2$), for 20 min
- Glycerol, 60% (v/v) solution
- Glass beads, 2 mm diameter

**Protocol 9** continued

### Method

1. Grow a 5 ml overnight broth culture of the strain to be preserved.
2. Autoclave a 60% (v/v) solution of glycerol and add 1.6 ml to the overnight broth culture. Mix well.
3. (a) Dispense 500 μl into a number of sterile, screw-top, freezer-proof vials and place a series of vials at −20 °C. Freeze the rest of vials in an ethanol/dry-ice mix and place the vials in a −80 °C freezer or into liquid nitrogen.
   (b) Alternatively, wash and sterilize aliquots of 20–30 glass beads (2 mm diameter). Add the glycerol/broth mix and aspirate to remove air bubbles and to make sure that the beads are well wetted. Decant the excess liquid, dispense and store as before.
4. Recovery of cultures is by partial thawing and removal of a few ice crystals or a glass bead into fresh LB broth suitably supplemented. Freezing and thawing of the stocks will reduce viability and it is therefore best to store replicate cultures that are left untouched.

## 4.4 Freeze-dried cultures

This is a relatively specialized technique that requires some expertise. It will not be dealt with here because the technique varies for different types of apparatus and there are specialized manuals available (12). It a technique worth considering if strains are not to be used for a number of years or if they need to be sent to other laboratories. Some commercial suppliers of bacterial strains will carry out custom freeze-drying on a batch basis.

# 5 Culturing of bacteriophages λ and M13

## 5.1 Theoretical background

Phages are viruses and this means that they are dependent on the host for their synthetic activities (1). This is at the root of many techniques for handling phages. Bacterial cells have to be actively growing if phage replication is to occur so the basic processes affecting growth described in Section 2 also apply here. A major difference between phages and their host is that phages do not have a cell wall and are therefore more susceptible to ionic strength. Osmotic shock will simply burst the phage head and release the phage chromosome with an instant drop in viability. Phages are also sensitive to detergents and other chemical contaminants on glassware.

It should be remembered that λ is a temperate phage and can exist in two cycles: the lytic cycle which involves infection, replication and lysis with the release of progeny phage and the lysogenic cycle in which the phage chromosome recombines with the host chromosome to become a prophage. A strain

carrying a temperate phage is said to be lysogenic and is given the symbol $(\lambda)^+$ whereas a non-lysogenic strain is given the symbol $(\lambda)^-$. In the prophage state there is a complex system of regulation which results in the inactivation of most genes on the phage chromosome except for the repressor gene itself (14).

The λ vectors are very different from the original wild-type phage. The region controlling the regulation system is dispensable and is not required for plaque formation, so in many vectors it has either been deleted or is replaced by recombinant DNA during the production of gene libraries (see Chapter 9, Section 4). The choice of hosts for particular strains of λ is important, as was mentioned in Section 3.6.

Bacteriophage M13 is a virulent, male-specific filamentous phage which contains a circular single-stranded DNA molecule. The attraction of the phage is that it produces single-stranded DNA which is useful as a template for DNA sequencing reactions. Consequently, the replication control region of M13 has been incorporated into hybrid vectors known as phagemids (see Chapter 9, Section 3.3). One unusual feature of M13 is that the host does not lyse to release phage. Instead, the phage particles are released through the wall without killing the host. Plaques are still produced because there is inhibition of the growth of the host, resulting in a reduction in the number of bacteria in the area of the plaque.

## 5.2 Factors affecting the growth and survival of phage λ

In a suitable suspending medium (SM, see *Protocol 10*) λ is very stable and will survive for months at 4°C assuming that there is no contamination. The phage needs $Mg^{2+}$ and a low level of gelatin for stability. In general, $MgSO_4$ or $MgCl_2$ must be present in all growth media used for λ or greatly reduced yields will be obtained. The growth of λ involves three main techniques:

(1) The assay of a phage stock to produce separate plaques

(2) Growth in liquid culture to produce high titre stocks

(3) The induction of lysogens to produce a lytic cycle

There are some variations on this, for example in plating at high density which can be used to produce phage that can be screened for specific sequences with gene probes or monoclonal antibodies.

The main factor underlying a number of these growth techniques is the ratio of phage to bacteria. This is known as the multiplicity of infection ($m$):

$$m = \frac{\text{number of phage particles}}{\text{number of bacteria}}$$

A value of $m$ of 0.01 means that there will be $10^8$ phage for every $10^{10}$ bacteria. Very high values of $m$ ($> 1000$) result in lysis externally, which means that the cells are killed without the release of progeny phage. There is a further complication with λ, and that is that phage can infect and lysogenize rather than

lyse. Lysogenic bacteria are immune to further infection because of the presence of a repressor. Consequently, these bacteria will continue to grow even in the presence of free phage. The aim of experiments to produce phage stocks is to maximize the lytic cycle by infecting actively-growing cells with an *m*-value of 1 or less. Cells growing slowly are more likely to be lysogenized and active growth is therefore essential. Some λ phage vectors lack an active repressor gene and produce clear plaques, consequently, lysogeny is not a major problem with these.

In assaying λ phage to produce single plaques, it is essential to have a very low level of *m*, with about 100 phage to about $10^7$ bacteria. In this situation each phage particle will infect a single bacterium and act as a centre of infection. After several rounds of replication and spread of the released phage particles a circle of lysed bacteria appears on the plate as a plaque. The size of this plaque is determined by the rate of phage replication and release, and the period for which the host is growing. As soon as the bacterium stops growing, the size of the plaque is fixed. This means that plaques can often be seen after about 6 h of incubation and well before full overnight growth. The size of the plaque can be controlled by manipulating the density of bacterial cells and the thickness of the nutrient agar plate. Thin plates are used for small plaques during screening with DNA probes and thick plates should be used during subculture for phage stock production.

Another factor which affects phage infection and growth is adsorption of the phage to the cell wall. The tail fibres of λ phage bind to the *lamB* receptor (a maltose-binding protein). This is induced by maltose but repressed by glucose and therefore for maximum adsorption 0.2% (w/v) maltose should be included in the medium. $Mg^{2+}$ has already been mentioned as being required for the integrity of the phage, but it is also needed for adsorption. Both maltose and $Mg^{2+}$ should therefore be present when assaying phages by the plaque method (see Section 5.3). However, when preparing phage stocks by the broth method (see Section 5.5), reduction in titre can occur by adsorption of the phage on to the receptors in the wall debris formed by lysis of the cells. This effect can be minimized by omitting maltose from the medium. Sufficient receptors are produced for numerous rounds of the lytic cycle without overproducing receptors, which would reduce the final titre. $CaCl_2$ at 5 mM can also be added as this has been observed to increase yields (15).

## 5.3 General techniques for the assay of phages by the plaque method

The basic technique for the assay of phage is applicable to a variety of phages. If the initial titre is unknown then a dilution series down to about $10^{-10}$ is prepared as described in *Protocol 3*. This will cover most of the phage titres encountered. A bacterial lawn is prepared by pouring a cell suspension in 0.6% (w/v) agar over a dried nutrient agar plate. The phage dilutions are then spotted on the surface of the plate in the same way as for the Miles–Misra technique described in *Protocol 3*,

step 9. This gives an approximate titre which is used to make a full assay by suspending a known phage concentration with the bacterial suspension in the overlay. When the titre is known in advance a limited range of dilutions can be prepared. The details of these techniques are given in *Protocol 10*.

# Protocol 10

## Assay of phages by the plaque method

### Reagents

- SM (λ storage and dilution medium: 5.8 g NaCl, 2 g $MgSO_4.7H_2O$, 50 ml 1 M Tris-HCl, pH 7.5, 5 ml 2% [w/v] gelatin solution, water to make 1 l). Sterilize by autoclaving
- DYT agar plates (16 g bacto-tryptone, 10 g bacto-yeast extract, 5 g NaCl, 15 g bacto-agar per 1 l). Check the pH and adjust to 7.0–7.2 with NaOH; sterilize by autoclaving
- LTS top agar (same recipe as DYT except with 6 g agar/l)

### Method

1. Add 1 ml of SM to a series of sterile plastic vials. Label the tubes –2, –4, –6, –8 and –10.
2. Add 10 μl of phage stock to tube –2 and mix well. This dilution is $10^{-2}$. Repeat this operation to produce the dilution series.
3. Prepare a thick agar plate of a rich medium such as DYT on a level area of bench and dry the plate well. Warm the plate by incubating at 37 °C.
4. Prepare 3 ml of LTS top agar, melt, and maintain at 50 °C.
5. Add 200 μl of an overnight broth culture[a] of a suitable host strain to the molten agar at 50 °C.
6. Mix the bacteria and the agar. Wipe the outside of the tube and pour rapidly over the DYT plate (again on a even surface). Rock and swirl the plate to spread the agar evenly over the surface (this must be done immediately).
7. Allow the agar to set, and dry the plate for about 15 min at 45 °C.
8. At marked places, spot 20 μl of the six dilutions and the neat suspension on the overlay plate. Allow the liquid to dry before inverting the plate. Incubate at 37 °C overnight.
9. Count the plaques where there are 5–20 per drop. The titre of the phage/ml is 50 times the count multiplied by the dilution factor.
10. For a more accurate assay, or when a complete plate at the same dilution is required:
    (a) Prepare as many nutrient plates as necessary. If large plaques are required for assay or production of stocks use thick plates (40 ml) while for screening use thin plates (20 ml).
    (b) Calculate the required volume of diluted phage for between 30 and 300 phage particles (or 1000+ for screening) and add this to 3 ml of molten agar medium at 50°C as described in step 4.

**Protocol 10** continued

(c) Add 200 μl of indicator bacteria and mix bacteria, phage and agar.

(d) Pour quickly over the plate as before, allow to set and incubate at 37°C overnight. Count the plaques as before.

[a] Bacterial cultures for λ should be grown on a medium containing 0.2% (w/v) maltose (see *Appendix 2*) to induce receptors. The LTS agar should also contain 0.2% maltose. The broth culture can be stored at 4°C for up to 1 month. For M13, male bacterial strains (Hfr or $F^+$) must be used; better results will be obtained if the lawns are prepared from actively-growing cultures.

## 5.4 Purification of phage by single-plaque isolation

All phage stocks should be purified periodically by single-plaque isolation and checked for appropriate genotypes or DNA restriction patterns. This is equivalent to single-colony isolation for bacteria (see Section 2.1). The same technique is used to purify a phage containing cloned DNA. Where the purity of a stock is critical it is best to choose well-separated plaques on fresh plates, as phages may diffuse sideways in the agar. A second purification step will avoid these problems. Stab an appropriate plaque, prepared by the technique in *Protocol 10*, with the tip of a sterile Pasteur pipette and rock from side to side so that a plug of agar containing the plaque is removed in the pipette tip. With a rubber teat or pipette dispenser, blow the plug into 1 ml of SM (*Protocol 10*) in a sterile plastic tube. Leave for 1 h so that the phage diffuses into the SM. An average plaque will contain about $10^6$ phages. Plate a suitable dilution of the phage suspension to obtain single plaques and then prepare a fresh phage stock by one of the methods detailed below.

## 5.5 Preparation of small-scale phage stocks

The plate method depends on producing almost confluent lysis on a plaque assay plate. It has the advantage that it is reliable and predictable but the disadvantage that agar has to be removed from the preparation. Agar contains inhibitors which can interfere with restriction, ligation, and other enzymes involved in DNA manipulations. If this is found to be a major problem it is better to replace the agar with agarose.

# Protocol 11

## The plate method for preparing a λ phage stock

### Reagents

- SM (see *Protocol 10*)
- Chloroform

### Method

**1** Prepare a series of up to five agar plates displaying almost confluent lysis by the method described in *Protocol 10*, step 10. The number of phage particles required will vary depending on the size of the plaques but normally will be between 5000

**Protocol 11** continued

and 100 000. Plaques should just be visible in isolated areas. Plates with complete lysis are less reliable as lysis without phage replication may have occurred.

**2** If mutant plaques are likely to be a problem (e.g. clear plaque mutants in a wild-type stock) then use the minimum number of phages required for almost confluent lysis and process the plates at either 6 h or about 15 h.

**3** Add 5 ml of SM to each plate and scrape the 0.6% agar off the plates into a sterile 250-ml conical flask with a sterile glass spreader.

**4** Add 0.5 ml of chloroform, vortex the flask for 1 min and leave to stand for 1 h so that the phage can diffuse from the agar.

**5** Decant the mixture into a centrifuge tube (resistant to chloroform) and centrifuge at 5000 ***g*** for 10 min to bring down bacterial debris and agar fragments.

**6** Remove the supernatant carefully and filter to sterilize or add to a sterile plastic tube over a few drops of chloroform.

**7** Assay the stock by one of the methods described in *Protocol 10*.

Notes

(a) The method can be modified by using agarose instead of agar or by allowing the phage to diffuse into the SM by gentle shaking of the intact plate for 3 h. Both of these methods will reduce the contamination of the stock by inhibitors.

(b) The method is useful when rapid lysis mutants or clear plaque mutants are a potential problem as diffusion is restricted by the agar and mutants will not overgrow the required phage, as can happen in broth culture methods.

The broth method is cleaner because it is only necessary to remove bacterial debris, but it is less predictable because it is essential to have the correct value for $m$ to give maximum lysis (see Section 5.2). If a stock culture is required quickly then you will need to set up a series of cultures with different ratios of phage and bacteria.

## Protocol 12

## The broth method for preparing λ phage stocks

### Reagents

- LB broth (see *Protocol 9*). For this procedure add $CaCl_2$ to a final concentration of 5 mM from a filter-sterilized stock solution immediately before use
- Chloroform

### Method

**1** This method can be used on any scale from 5 ml to 500 ml cultures. For λ use LB broth containing 5 mM $CaCl_2$ but no $MgCl_2$/$MgSO_4$ or maltose. The method can also work with more complex media.

**Protocol 12** continued

2 Grow an overnight culture of a suitable host and add 100 μl to 5 ml in a 20 ml tube, or 10 ml to 500 ml in a 2-l conical flask.

3 Incubate at 37 °C at 250 r.p.m. until the OD at 550 nm is about 0.2. This should be approximately $10^8$ exponentially growing cells/ml. Incubation should take about 2–3 h.

4 Add phage to give an *m* of 0.01. For the 5 ml culture this means adding the phage from two or three plaques (removed as agar plugs; see Section 5.4), or for the 500 ml culture the phage from one or two plates showing almost confluent lysis (prepared by the method in *Protocol 11*). The latter can be done simply by scraping the agar overlay into the flask. Alternatively, prepare a phage stock from the plates and add the agar-free stock directly. It is important to remove all traces of chloroform by aeration before adding.

5 Continue shaking until the culture lyses, which will normally take between 2 and 4 h. This can be detected by a rapid drop in OD accompanied by the production of 'ropes' of lysed bacteria. If this does not occur after 5 h, either continue incubation overnight or add chloroform (200 μl per 5 ml culture or 20 ml per 500 ml culture). Continue shaking until lysis occurs.

6 The production of efficient lysis is essential for a high phage yield. The presence of clearing and 'rope' formation is a reliable indication of a high titre stock. If this does not occur it is better to repeat the procedure until good lysis is detected.

7 If not already added, add chloroform to lysed cultures as in step 5 above. Shake for 10 min and then harvest the cultures into centrifuge tubes which will resist chloroform. Centrifuge for 15 min at 11 000 ***g*** to remove cell debris and agar.

8 Remove the supernatant and store with a few drops of chloroform at 4 °C.

## 5.6 Preparation of large-scale phage stocks

As described in *Protocol 12*, the broth method for preparation of small-scale phage stocks can be scaled up to 500 ml cultures in 2-l flasks.

## 5.7 Purification of λ phage particles

For many purposes, phage preparations need to be free from bacterial nucleic acids and other contaminants such as bacterial carbohydrates or agar. Bacterial DNA can be incorporated into gene libraries and other contaminants can interfere with ligase or restriction enzyme activities. Intact phage particles are resistant to nucleases, so a treatment with RNase and DNase can remove these contaminants. Phage particles can be purified by differential centrifugation but damage to the particles occurs. PEG has been used effectively to precipitate the phage particles making centrifugation easier. The most efficient way of purifying the phage particles is on a CsCl gradient, as this separates the particles from carbohydrates. A method for purifying λ phage is given in *Protocol 13*. Extraction of DNA from these particles is straightforward (see Chapter 3, Section 2.2).

# Protocol 13

## Purification of phage λ

### Reagents

- DNase I (stock solution of 1 mg/ml prepared in 20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, stored at −80 °C)
- RNase I (stock solution of 10 mg/ml prepared in 10 mM Tris-HCl, pH 7.5, 15 mM NaCl)
- PEG 6000
- SM (see Protocol 10)
- Chloroform

### Method

**1** Prepare a high-titre stock (at least $10^{10}$/ml) by the method described in *Protocol 12*. Measure the volume and add 1 μg/ml of both DNase I and RNase I to remove bacterial nucleic acids. Incubate at room temperature for 30 min.

**2** Add 40 g solid NaCl per litre and dissolve by gentle agitation.

**3** Add 140 g PEG 6000 per litre. Add this slowly with constant gentle mixing on a magnetic stirrer at room temperature.

**4** Leave at 4 °C overnight to precipitate the phage.

**5** Swirl the flask gently to resuspend any sediment.

**6** Centrifuge at 11 000 ***g*** for 10 min to collect the precipitated phage.

**7** Decant the supernatant and discard. Invert the centrifuge tube and drain well. Wipe the inside of the tube with a paper tissue.

**8** Resuspend the pellet in 16 ml of SM (per litre of original stock). Add an equal volume of chloroform and stir on a magnetic shaker at 37 °C for 1–2 h until the pellet is fully resuspended. Care should be taken at this stage as losses can occur.

**9** Centrifuge at 2000 ***g*** to separate the phases and carefully remove the upper aqueous layer containing the phage, which can be stored as a stock.

**10** Losses can occur at a number of stages and it is useful to do a Miles–Misra assay (see *Protocol 3*, step 9) for phage at various steps such as original stock, the supernatant after PEG precipitation and the final stock. Supernatants should be retained until the results are known so that further recovery can be made and phage stocks pooled.

**11** Add 0.75 g CsCl per ml of stock and dissolve by inversion. This should give a density of 1.5 g/ml. Centrifuge at 100 000 ***g*** for 24 h and phage should produce a bluish band half-way down the tube. Remove the band with a 21-gauge syringe needle.

**12** Dialyse against 2 l of SM overnight and store at 4 °C with a few drops of chloroform.

## 5.8 Induction of λ lysogens

Traditionally, lysogenic cultures were induced by UV irradiation. Damage to the DNA occurred followed by derepression of the *recA* gene product which then destroyed the λ *c*I repressor. The use of UV is not recommended as it can induce mutations in phage particles, and most lysogens are now induced by a temperature-sensitive system. With many λ strains the repressor protein is thermolabile at 45 °C and thus lysogenic cultures can be induced to undergo the lytic cycle by a brief treatment at this temperature. The $S^+$ gene product is required for natural lysis and some phage strains have amber mutations or chain-terminating triplets in this gene. If the host strain has the equivalent suppressor then natural lysis will occur, otherwise it has to be induced with chloroform.

## Protocol 14

## Induction of λ lysogens

### Equipment and reagents

- Shaking water baths at 37°C and 45°C
- LB agar plates (see *Protocol 3*)
- LB broth (see *Protocol 9*)
- SM (see *Protocol 10*)
- Chloroform

### Method

**1** Prepare duplicate streak plates of the lysogenic culture on LB plates and incubate at 30 °C and 42 °C. No growth should occur at 42 °C owing to induction of the lytic cycle.

**2** Use two shaking water baths at 37 °C and 45 °C, respectively. Grow an overnight 20 ml LB broth culture at 37 °C and inoculate into 400 ml of LB in a 2-l flask. Shake at 250 r.p.m. at 37 °C until the OD at 550 nm reaches 0.6 (about $3 \times 10^8$ cells per ml).

**3** Rapidly increase the temperature in the broth culture to 45 °C. This is best done in a large volume of water at 70 °C. Monitor the temperature, and when this reaches 45 °C, transfer the flask to the water bath at 45 °C. Incubate with vigorous shaking for 15 min.

**4** Transfer the flask to the 37 °C water bath and continue shaking.

**5** Monitor for lysis (if suppressor mutations are present), as in *Protocol 12*, step 5. Samples can be withdrawn and tested for lysis with a small volume (0.05 volume) of chloroform. Vigorous shaking should be followed by lysis seen as translucence or rope-like debris.

**6** Strains which do not suppress amber mutations will not lyse spontaneously. After about 2 h at 37 °C, collect the cells by centrifugation at 3000 ***g*** and suspend in 10 ml of SM. Add 500 μl of chloroform and vortex vigorously.

**7** Stand the suspension for up to 1 h at room temperature.

**8** Centrifuge to remove cell debris and treat as for standard λ phage stock, as described in *Protocols 12* and *13*.

## 5.9 Techniques involving phage M13

Many of the techniques which apply to phage λ also apply to M13. However, a few points should be noted. The bacterial host must be a male strain containing the F factor as the phage infects via the sex pilus (an appendage coded by the sex factor). Plaques can be produced either by transforming competent *E. coli* cells with the double-stranded replicative form of the DNA (Chapter 8, Sections 2.1 and 2.2) or by infecting cells with intact phage particles containing a single-stranded circular molecule. Since phage particles do not lyse the host, it is possible to prepare stocks of M13 simply by inoculating a broth culture of an $F^+$ strain with phage particles and growing to stationary phase. The bacteria are removed by centrifugation at 10 000 ***g*** for 15 min. The supernatant contains free phage particles released through the wall of the host. The phage can be purified by a PEG precipitation method similar to *Protocol 13*, as described in Volume II, Chapter 6.

## 5.10 Methods for preserving phage stocks

The simplest method for keeping a phage stock is to seal a Petri dish containing separate plaques of a purified phage type. Parafilm or tape is used to seal the edges of the plate, which is kept at 4 °C. Viable phage can be isolated from single plaques for several months, but this method is not recommended if there is a variety of different phage types in the same area, since cross-diffusion may occur. Once a high-titre stock has been prepared in SM this can be kept over a small volume of chloroform at 4 °C for many months. The chloroform may evaporate with time and should be replenished or the stock may become contaminated with fungi or bacteria. Stocks can also be preserved by the addition of 7% (v/v) DMSO. Add DMSO and mix gently, then freeze rapidly in liquid nitrogen or a dry-ice–alcohol mix and store at −70 °C. Scrape the surface with a sterile Nichrome loop and spot on to a overlay lawn of a sensitive host to test for viability.

# 6 Troubleshooting

## 6.1 General principles

Techniques are always easy when you know how or when you can watch an expert using the method. However, it is not uncommon to find that the technique is simply not working. The speed of solving these technical problems makes a big impact on the time available for research and rate of progress. One basic principle is to include controls whenever possible, since these can show clearly what has gone wrong. Solving more difficult problems is very much like detective work—following up clues, eliminating possible suspects and testing theories. It makes sense to ask others who have managed to get the technique to work and to show them your plates or photographs of puzzling results. In microbiology, you should be fully aware of where microorganisms can grow: they can grow wherever conditions are not inhibitory. Algae and bacteria have been found growing in distilled water, and phosphate buffer with a low glucose concentration is

an ideal growth medium. Solutions which were sterile last week may have been opened and left on the bench, and be heavily contaminated. If they are, you can be sure that the contaminant produces a nuclease which will destroy your precious DNA.

## 6.2 Contamination

### 6.2.1 Contaminated cultures

The source of contaminated cultures may be the air, the apparatus or the growth medium. The culture may have been contaminated when it was received. A visual inspection of a streak plate should show the most obvious contaminants and these can also be checked by their phenotypes and microscopic properties. More subtle changes are difficult to check and the failure of a technique to work may be the only clue.

If you are not sure which colony on a plate is the correct strain you may have to prepare a range of cultures and carry out some simple tests to see which are, for example, sensitive to λ phage or which can conjugate with a known Hfr strain. Genetic variation is the most difficult to detect and may not become apparent for some time. Chromosomal rearrangements such as transposition may only become apparent on genetic mapping or on analysis of antibiotic resistance. Gene mutation or reversion will only be detected if you check for these particular genetic markers. If all else fails and the technique is still not working, ask someone else for an alternative culture or order a substitute from a commercial supplier.

### 6.2.2 Contaminated media, solutions and equipment

Your strains may be correct but you may be contaminating them every time you attempt to perform an experiment. The inclusion of uninoculated controls should indicate the source of the problem. Media with disc-shaped colonies probably means that one of the media components is contaminated. If you suspect that solutions and buffers are contaminated then spread them on nutrient media and look for colonies after incubation. Wherever possible, solutions should be sterilized, stored at 4°C and opened using sterile technique. If a solution is used frequently it is much better to sterilize it in suitable aliquots and discard material once it has been opened a few times. Contaminated equipment implies that your quality control on sterilization or storage is inadequate. Autoclave tape may indicate that your use of the autoclave is at fault or it could be that the environment is contaminating the equipment after sterilization. Glass pipettes and other glassware generally can be flamed before use, but this is not possible for plasticware previously sterilized by gamma radiation. If a particular piece of apparatus, such as a centrifuge tube, is suspected then add some sterile nutrient medium, incubate and look for turbidity.

## 6.3 Poor growth of bacteria or phage

Techniques often quote a time-course for a particular experiment; if your cultures are growing much slower than this then you may suspect that there are

factors limiting growth. The first component to suspect is that the growth medium is inadequate. A check of the genotype of the bacterial strain should reveal if there are growth factor requirements which may be absent from the medium; for example, peptone may be deficient in arginine and/or lyrine, and vitamins may be absent from yeast extract. Alternatively, the concentration of an antibiotic may be too high and inhibition may have occurred. Components of the medium can be altered and the growth rate of the strain checked again. A low level of contamination can also affect growth, particularly if the contaminant is a streptomycete that produces an antibiotic which inhibits the strain under study. Another factor which affects growth is the aeration of the culture as large volumes of media with inadequate shaking can rapidly become oxygen-limited. Ideally, a flask of 250 ml capacity should have no more than 25 ml of medium to avoid oxygen-limitation. In practice, the earlier phases of growth are less likely to be oxygen-limited and larger volumes can be used without affecting doubling times. A common source of problems is inhibition by chemicals contaminating the glassware. Glassware is often used many times and may have been washed in a dish-washer that has left residues of salts and detergents. These can rapidly destroy phage λ and, consequently, plastic tubes are best for the handling of phages. You are strongly advised to wash your own glassware and to make sure that rinse cycles are sufficient to remove these chemical contaminants. Poor growth of phages can be due to a variety of factors such as resistance of the host to phage infection and absence of cofactors essential for adsorption and infection. Sudden lysis of cultures may be due to the presence of air-stable phages in the environment. A rich medium with a good carbon source will obviously give better bacterial growth than a chemically defined medium where the organism must synthesize almost everything for itself. Good bacterial growth will normally give good phage growth but note should be taken of the optimum conditions for handling λ, as discussed in Section 5.

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