# Chapter 3

# Purification of DNA from Living Cells

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3.1 Preparation of total cell DNA
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3.3 Preparation of bacteriophage DNA

The genetic engineer will, at different times, need to prepare at least three distinct kinds of DNA. First, **total cell DNA** will often be required as a source of material from which to obtain genes to be cloned. Total cell DNA may be DNA from a culture of bacteria, from a plant, from animal cells, or from any other type of organism that is being studied. It consists of the **genomic DNA** of the organism along with any additional DNA molecules, such as plasmids, that are present.

The second type of DNA that will be required is pure plasmid DNA. Preparation of plasmid DNA from a culture of bacteria follows the same basic steps as purification of total cell DNA, with the crucial difference that at some stage the plasmid DNA must be separated from the main bulk of chromosomal DNA also present in the cell.

Finally, phage DNA will be needed if a phage cloning vector is to be used. Phage DNA is generally prepared from bacteriophage particles rather than from infected cells, so there is no problem with contaminating bacterial DNA. However, special techniques are needed to remove the phage capsid. An exception is the double-stranded replicative form of M13, which is prepared from *E. coli* cells in the same way as a bacterial plasmid.

## 3.1 Preparation of total cell DNA

The fundamentals of DNA preparation are most easily understood by first considering the simplest type of DNA purification procedure, that where the entire DNA complement

*Gene Cloning and DNA Analysis: An Introduction*. 6th edition. By T.A. Brown. Published 2010 by Blackwell Publishing.

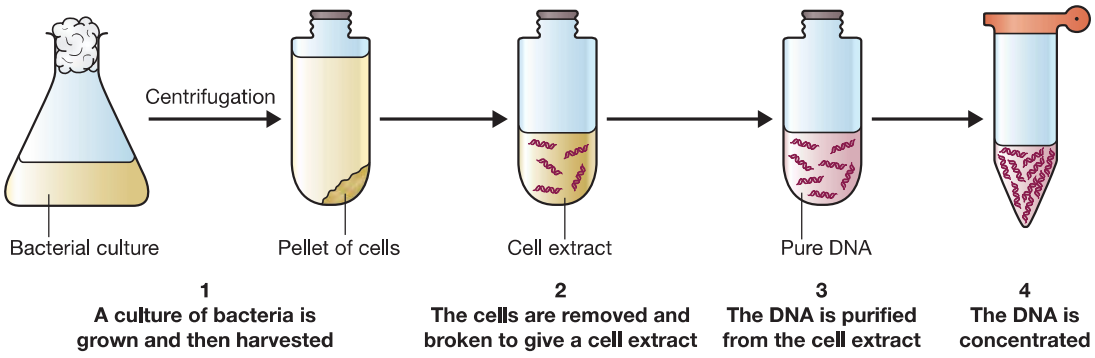



**Figure 3.1**

The basic steps in preparation of total cell DNA from a culture of bacteria.

of a bacterial cell is required. The modifications needed for plasmid and phage DNA preparation can then be described later.

The procedure for total DNA preparation from a culture of bacterial cells can be divided into four stages (Figure 3.1):

1 A culture of bacteria is grown and then **harvested**.
2 The cells are broken open to release their contents.
3 This **cell extract** is treated to remove all components except the DNA.
4 The resulting DNA solution is concentrated.

## 3.1.1 Growing and harvesting a bacterial culture

Most bacteria can be grown without too much difficulty in a liquid medium (**broth culture**). The culture medium must provide a balanced mixture of the essential nutrients at concentrations that will allow the bacteria to grow and divide efficiently. Two typical growth media are detailed in Table 3.1.

M9 is an example of a **defined medium** in which all the components are known. This medium contains a mixture of inorganic nutrients to provide essential elements such as

**Table 3.1**

The composition of two typical media for the growth of bacterial cultures.

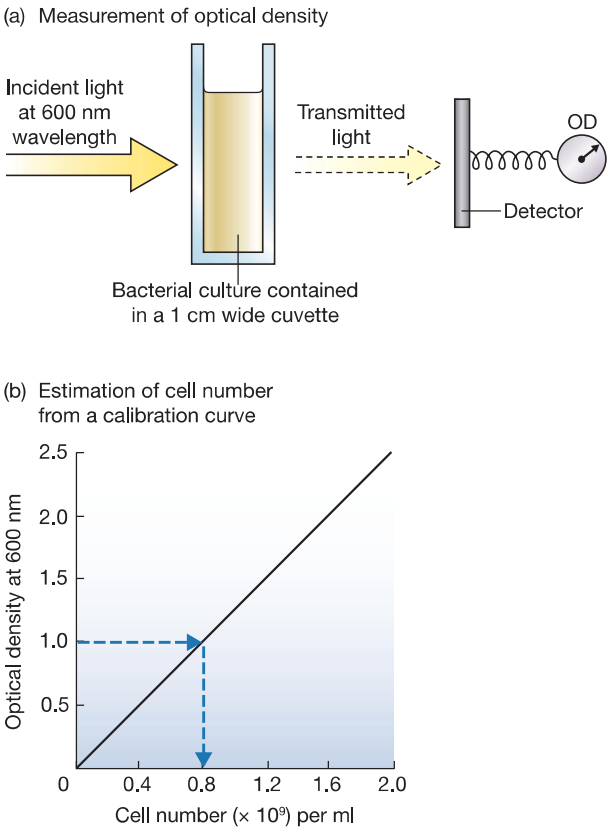
| MEDIUM | COMPONENT | g/l OF MEDIUM |
|---|---|---|
| M9 medium | $Na_2HPO_4$ | 6.0 |
| | $KH_2PO_4$ | 3.0 |
| | NaCl | 0.5 |
| | $NH_4Cl$ | 1.0 |
| | $MgSO_4$ | 0.5 |
| | Glucose | 2.0 |
| | $CaCl_2$ | 0.015 |
| LB (Luria-Bertani medium) | Tryptone | 10.0 |
| | Yeast extract | 5.0 |
| | NaCl | 10.0 |

nitrogen, magnesium, and calcium, as well as glucose to supply carbon and energy. In practice, additional growth factors such as trace elements and vitamins must be added to M9 before it will support bacterial growth. Precisely which supplements are needed depends on the species concerned.

The second medium described in Table 3.1 is rather different. Luria-Bertani (LB) is a complex or **undefined medium**, meaning that the precise identity and quantity of its components are not known. This is because two of the ingredients, tryptone and yeast extract, are complicated mixtures of unknown chemical compounds. Tryptone in fact supplies amino acids and small peptides, while yeast extract (a dried preparation of partially digested yeast cells) provides the nitrogen requirements, along with sugars and inorganic and organic nutrients. Complex media such as LB need no further supplementation and support the growth of a wide range of bacterial species.

Defined media must be used when the bacterial culture has to be grown under precisely controlled conditions. However, this is not necessary when the culture is being grown simply as a source of DNA, and under these circumstances a complex medium is appropriate. In LB medium at 37°C, aerated by shaking at 150–250 rpm on a rotary platform, *E. coli* cells divide once every 20 min or so until the culture reaches a maximum density of about $2–3 \times 10^9$ cells/ml. The growth of the culture can be monitored by reading the optical density (OD) at 600 nm (Figure 3.2), at which wavelength 1 OD unit corresponds to about $0.8 \times 10^9$ cells/ml.

In order to prepare a cell extract, the bacteria must be obtained in as small a volume as possible. Harvesting is therefore performed by spinning the culture in a centrifuge (Figure 3.3). Fairly low centrifugation speeds will pellet the bacteria at the bottom of the centrifuge tube, allowing the culture medium to be poured off. Bacteria from a




## Figure 3.2

Estimation of bacterial cell number by measurement of optical density (OD). (a) A sample of the culture is placed in a glass cuvette and light with a wavelength of 600 nm shone through. The amount of light that passes through the culture is measured and the OD (also called the absorbance) calculated as:

$$1 \text{ OD unit} = \log_{10} \frac{\text{intensity of transmitted light}}{\text{intensity of incident light}}$$

The operation is performed with a spectrophotometer. (b) The cell number corresponding to the OD reading is calculated from a calibration curve. This curve is plotted from the OD values of a series of cultures of known cell density. For *E. coli*, 1 OD unit = $0.8 \times 10^9$ cells/ml.

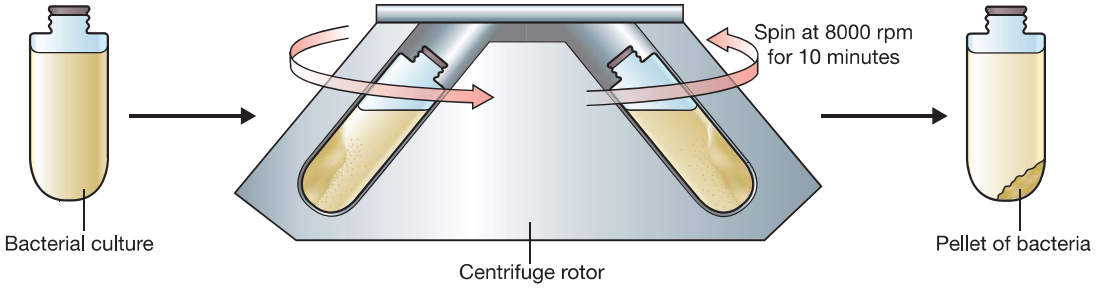



**Figure 3.3**
Harvesting bacteria by centrifugation.

1000 ml culture at maximum cell density can then be resuspended into a volume of 10 ml or less.

### 3.1.2 Preparation of a cell extract

The bacterial cell is enclosed in a cytoplasmic membrane and surrounded by a rigid cell wall. With some species, including *E. coli*, the cell wall may itself be enveloped by a second, outer membrane. All of these barriers have to be disrupted to release the cell components.

Techniques for breaking open bacterial cells can be divided into physical methods, in which the cells are disrupted by mechanical forces, and chemical methods, where cell lysis is brought about by exposure to chemical agents that affect the integrity of the cell barriers. Chemical methods are most commonly used with bacterial cells when the object is DNA preparation.

Chemical lysis generally involves one agent attacking the cell wall and another disrupting the cell membrane (Figure 3.4a). The chemicals that are used depend on the species of bacterium involved, but with *E. coli* and related organisms, weakening of the cell wall is usually brought about by **lysozyme**, ethylenediamine tetraacetate (EDTA), or a combination of both. Lysozyme is an enzyme that is present in egg white and in secretions such as tears and saliva, and which digests the polymeric compounds that give the cell wall its rigidity. EDTA removes magnesium ions that are essential for preserving the overall structure of the cell envelope, and also inhibits cellular enzymes that could degrade DNA. Under some conditions, weakening the cell wall with lysozyme or

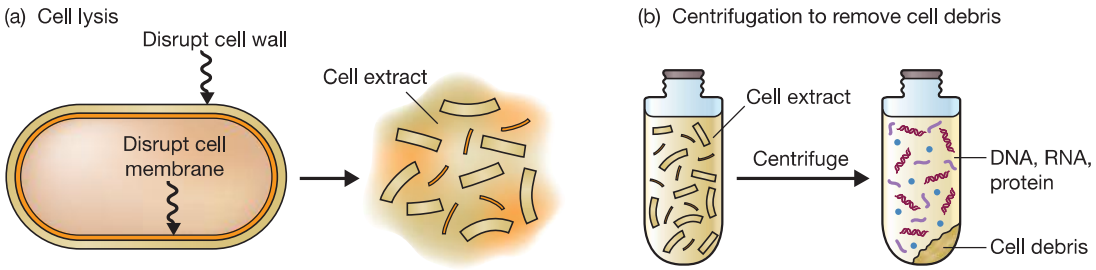



**Figure 3.4**
Preparation of a cell extract. (a) Cell lysis. (b) Centrifugation of the cell extract to remove insoluble debris.

EDTA is sufficient to cause bacterial cells to burst, but usually a detergent such as sodium dodecyl sulphate (SDS) is also added. Detergents aid the process of lysis by removing lipid molecules and thereby cause disruption of the cell membranes.

Having lysed the cells, the final step in preparation of a cell extract is removal of insoluble cell debris. Components such as partially digested cell wall fractions can be pelleted by centrifugation (Figure 3.4b), leaving the cell extract as a reasonably clear supernatant.

## 3.1.3 Purification of DNA from a cell extract

In addition to DNA, a bacterial cell extract contains significant quantities of protein and RNA. A variety of methods can be used to purify the DNA from this mixture. One approach is to treat the mixture with reagents which degrade the contaminants, leaving a pure solution of DNA (Figure 3.5a). Other methods use **ion-exchange chromatography** to separate the mixture into its various components, so the DNA is removed from the proteins and RNA in the extract (Figure 3.5b).

### *Removing contaminants by organic extraction and enzyme digestion*

The standard way to deproteinize a cell extract is to add phenol or a 1 : 1 mixture of phenol and chloroform. These organic solvents precipitate proteins but leave the nucleic acids (DNA and RNA) in aqueous solution. The result is that if the cell extract is mixed gently with the solvent, and the layers then separated by centrifugation, precipitated protein molecules are left as a white coagulated mass at the interface between the aqueous and organic layers (Figure 3.6). The aqueous solution of nucleic acids can then be removed with a pipette.

With some cell extracts the protein content is so great that a single phenol extraction is not sufficient to completely purify the nucleic acids. This problem could be solved by carrying out several phenol extractions one after the other, but this is undesirable as each mixing and centrifugation step results in a certain amount of breakage of the DNA molecules. The answer is to treat the cell extract with a **protease** such as pronase or

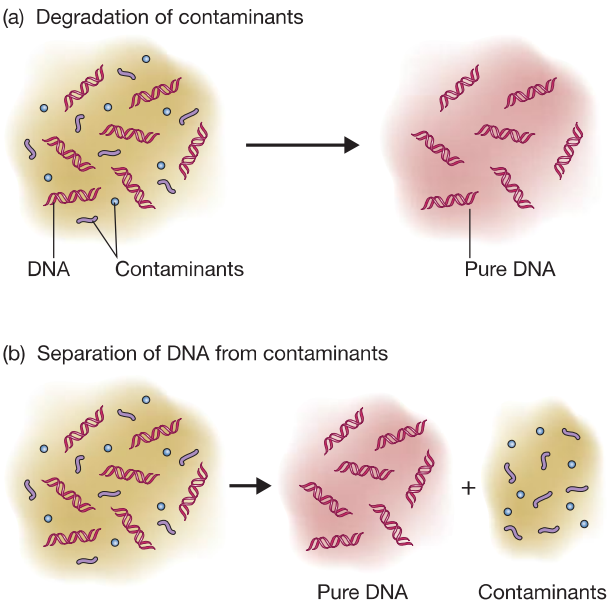



**Figure 3.5**
Two approaches to DNA purification. (a) Treating the mixture with reagents which degrade the contaminants, leaving a pure solution of DNA. (b) Separating the mixture into different fractions, one of which is pure DNA.

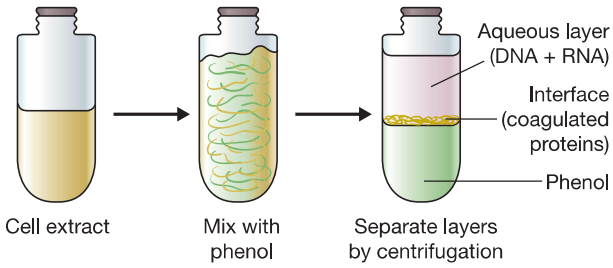



**Figure 3.6**
Removal of protein contaminants by phenol extraction.

proteinase K before phenol extraction. These enzymes break polypeptides down into smaller units, which are more easily removed by phenol.

Some RNA molecules, especially **messenger RNA (mRNA)**, are removed by phenol treatment, but most remain with the DNA in the aqueous layer. The only effective way to remove the RNA is with the enzyme **ribonuclease**, which rapidly degrades these molecules into ribonucleotide subunits.

*Using ion-exchange chromatography to purify DNA from a cell extract*

Biochemists have devised various methods for using differences in electrical charge to separate mixtures of chemicals into their individual components. One of these methods is ion-exchange chromatography, which separates molecules according to how tightly they bind to electrically charged particles present in a chromatographic matrix or **resin**. DNA and RNA are both negatively charged, as are some proteins, and so bind to a positively charged resin. The electrical attachment is disrupted by salt (Figure 3.7a), removal of the more tightly bound molecules requiring higher concentrations of salt. By gradually increasing the salt concentration, different types of molecule can be detached from the resin one after another.

The simplest way to carry out ion-exchange chromatography is to place the resin in a glass or plastic column and then add the cell extract to the top (Figure 3.7b). The extract passes through the column, and because this extract contains very little salt all the negatively charged molecules bind to the resin and are retained in the column. If a salt solution of gradually increasing concentration is now passed through the column, the different types of molecule will **elute** (i.e., become unbound) in the sequence protein, RNA, and finally DNA. However, such careful separation is usually not needed so just two salt solutions are used, one whose concentration is sufficient to elute the protein and RNA, leaving just the DNA bound, followed by a second of a higher concentration which elutes the DNA, now free from protein and RNA contaminants.

## 3.1.4 Concentration of DNA samples

Organic extraction often results in a very thick solution of DNA that does not need to be concentrated any further. Other purification methods give more dilute solutions and it is therefore important to consider methods for increasing the DNA concentration.

The most frequently used method of concentration is **ethanol precipitation**. In the presence of salt (strictly speaking, monovalent cations such as sodium ions ($Na^+$)), and at a temperature of −20°C or less, absolute ethanol efficiently precipitates polymeric nucleic acids. With a thick solution of DNA the ethanol can be layered on top of the sample, causing molecules to precipitate at the interface. A spectacular trick is to push a glass rod through the ethanol into the DNA solution. When the rod is removed,

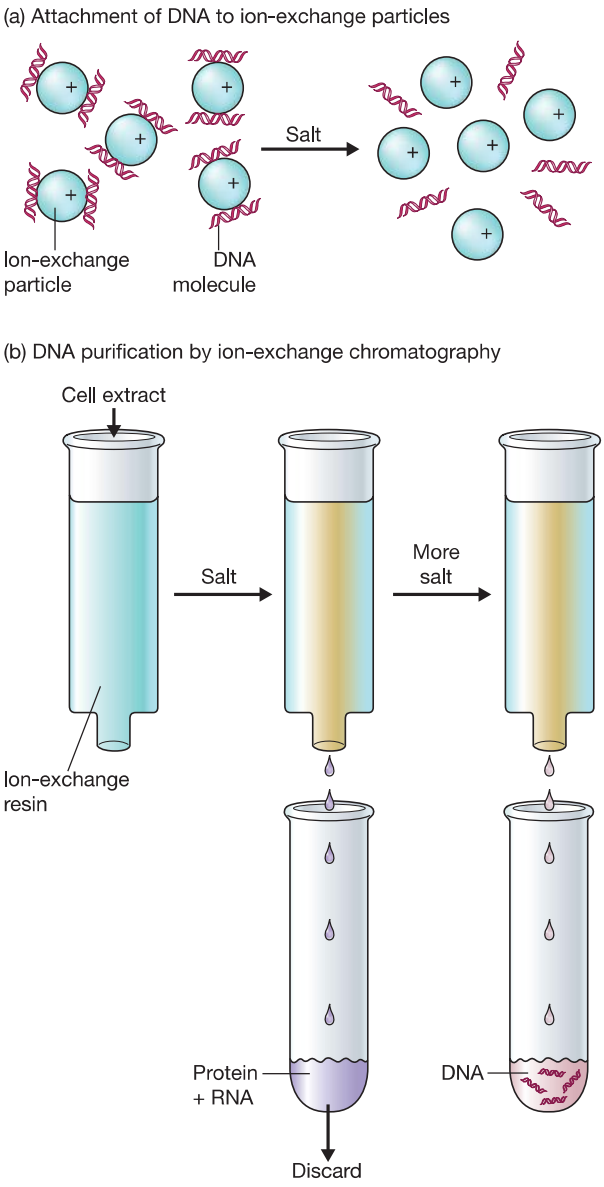



**Figure 3.7**

DNA purification by ion-exchange chromatography. (a) Attachment of DNA to ion-exchange particles. (b) DNA is purified by column chromatography. The solutions passing through the column can be collected by gravity flow or by the **spin column** method, in which the column is placed in a low-speed centrifuge.

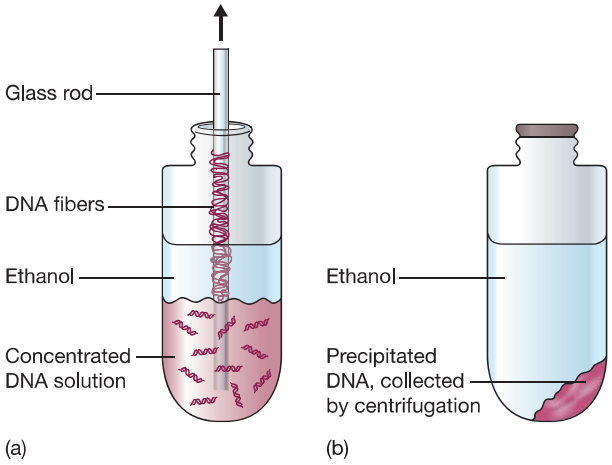
DNA molecules adhere and can be pulled out of the solution in the form of a long fiber (Figure 3.8a). Alternatively, if ethanol is mixed with a dilute DNA solution, the precipitate can be collected by centrifugation (Figure 3.8b), and then redissolved in an appropriate volume of water. Ethanol precipitation has the added advantage of leaving short-chain and monomeric nucleic acid components in solution. Ribonucleotides produced by ribonuclease treatment are therefore lost at this stage.

### 3.1.5 Measurement of DNA concentration

It is crucial to know exactly how much DNA is present in a solution when carrying out a gene cloning experiment. Fortunately DNA concentrations can be accurately measured by **ultraviolet (UV) absorbance spectrophotometry**. The amount of UV radiation absorbed by a solution of DNA is directly proportional to the amount of DNA in the

**Figure 3.8**

Collecting DNA by ethanol precipitation. (a) Absolute ethanol is layered on top of a concentrated solution of DNA. Fibers of DNA can be withdrawn with a glass rod. (b) For less concentrated solutions ethanol is added (at a ratio of 2.5 volumes of absolute ethanol to 1 volume of DNA solution) and precipitated DNA collected by centrifugation.




sample. Usually absorbance is measured at 260 nm, at which wavelength an absorbance ($A_{260}$) of 1.0 corresponds to 50 mg of double-stranded DNA per ml. Measurements of as little as 1 µl of a DNA solution can be carried out in spectrophotometers designed especially for this purpose.

Ultraviolet absorbance can also be used to check the purity of a DNA preparation. With a pure sample of DNA, the ratio of the absorbances at 260 and 280 nm ($A_{260}/A_{280}$) is 1.8. Ratios of less than 1.8 indicate that the preparation is contaminated, either with protein or with phenol.

### 3.1.6 Other methods for the preparation of total cell DNA

Bacteria are not the only organisms from which DNA may be required. Total cell DNA from, for example, plants or animals will be needed if the aim of the genetic engineering project is to clone genes from these organisms. Although the basic steps in DNA purification are the same whatever the organism, some modifications may have to be introduced to take account of the special features of the cells being used.

Obviously growth of cells in liquid medium is appropriate only for bacteria, other microorganisms, and plant and animal cell cultures. The major modifications, however, are likely to be needed at the cell breakage stage. The chemicals used for disrupting bacterial cells do not usually work with other organisms: lysozyme, for example, has no effect on plant cells. Specific degradative enzymes are available for most cell wall types, but often physical techniques, such as grinding frozen material with a mortar and pestle, are more efficient. On the other hand, most animal cells have no cell wall at all, and can be lysed simply by treating with detergent.

Another important consideration is the biochemical content of the cells from which DNA is being extracted. With most bacteria the main biochemicals present in a cell extract are protein, DNA and RNA, so phenol extraction and/or protease treatment, followed by removal of RNA with ribonuclease, leaves a pure DNA sample. These treatments may not, however, be sufficient to give pure DNA if the cells also contain significant quantities of other biochemicals. Plant tissues are particularly difficult in this respect as they often contain large amounts of carbohydrates that are not removed by phenol extraction. Instead a different approach must be used. One method makes use of a detergent called cetyltrimethylammonium bromide (CTAB), which forms an insoluble complex with nucleic acids. When CTAB is added to a plant cell extract the

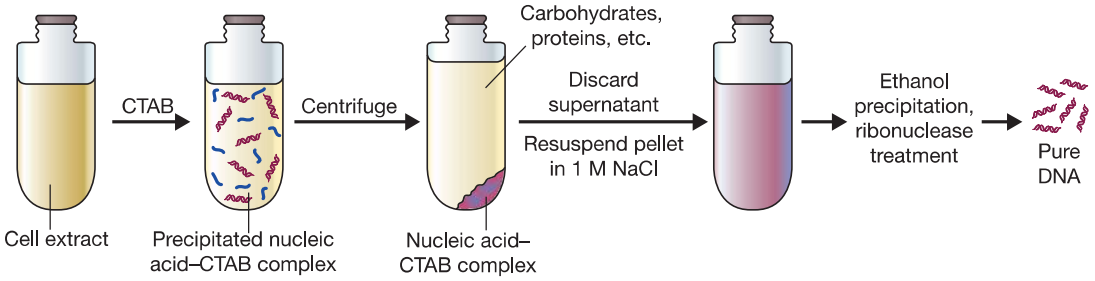



**Figure 3.9**

The CTAB method for purification of plant DNA.

nucleic acid–CTAB complex precipitates, leaving carbohydrate, protein, and other contaminants in the supernatant (Figure 3.9). The precipitate is then collected by centrifugation and resuspended in 1 M sodium chloride, which causes the complex to break down. The nucleic acids can now be concentrated by ethanol precipitation and the RNA removed by ribonuclease treatment.

The need to adapt organic extraction methods to take account of the biochemical contents of different types of starting material has stimulated the search for DNA purification methods that can be used with any species. This is one of the reasons why ion-exchange chromatography has become so popular. A similar method involves a compound called guanidinium thiocyanate, which has two properties that make it useful for DNA purification. First, it denatures and dissolves all biochemicals other than nucleic acids and can therefore be used to release DNA from virtually any type of cell or tissue. Second, in the presence of guanidinium thiocyanate, DNA binds tightly to silica particles (Figure 3.10a). This provides an easy way of recovering the DNA from the denatured mix of biochemicals. One possibility is to add the silica directly to the cell extract but, as with the ion-exchange methods, it is more convenient to use a chromatography column. The silica is placed in the column and the cell extract added (Figure 3.10b). DNA binds to the silica and is retained in the column, whereas the denatured biochemicals pass straight through. After washing away the last contaminants with guanidinium thiocyanate solution, the DNA is recovered by adding water, which destabilizes the interactions between the DNA molecules and the silica.

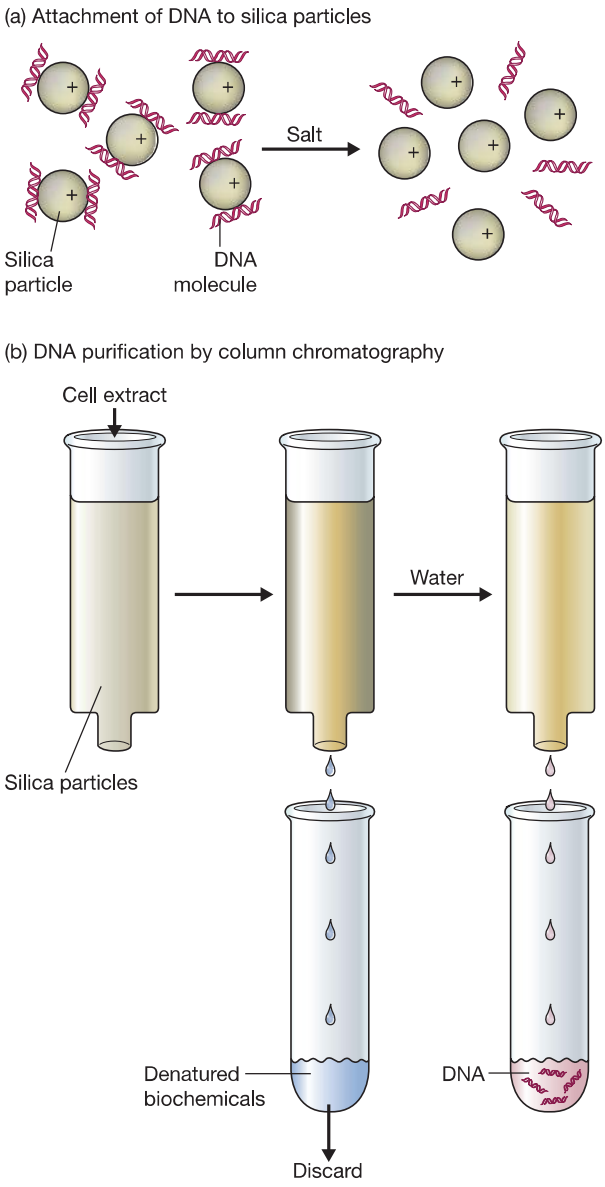
## 3.2 Preparation of plasmid DNA

Purification of plasmids from a culture of bacteria involves the same general strategy as preparation of total cell DNA. A culture of cells, containing plasmids, is grown in liquid medium, harvested, and a cell extract prepared. The protein and RNA are removed, and the DNA probably concentrated by ethanol precipitation. However, there is an important distinction between plasmid purification and preparation of total cell DNA. In a plasmid preparation it is always necessary to separate the plasmid DNA from the large amount of bacterial chromosomal DNA that is also present in the cells.

Separating the two types of DNA can be very difficult, but is nonetheless essential if the plasmids are to be used as cloning vectors. The presence of the smallest amount of contaminating bacterial DNA in a gene cloning experiment can easily lead to undesirable results. Fortunately several methods are available for removal of bacterial DNA during

**Figure 3.10**

DNA purification by the guanidinium thiocyanate and silica method. (a) In the presence of guanidinium thiocyanate, DNA binds to silica particles. (b) DNA is purified by column chromatography.




plasmid purification, and the use of these methods, individually or in combination, can result in isolation of very pure plasmid DNA.

The methods are based on the several physical differences between plasmid DNA and bacterial DNA, the most obvious of which is size. The largest plasmids are only 8% of the size of the *E. coli* chromosome, and most are much smaller than this. Techniques that can separate small DNA molecules from large ones should therefore effectively purify plasmid DNA.

In addition to size, plasmids and bacterial DNA differ in **conformation**. When applied to a polymer such as DNA, the term conformation refers to the overall spatial configuration of the molecule, with the two simplest conformations being linear and circular. Plasmids and the bacterial chromosome are circular, but during preparation of the cell extract the chromosome is always broken to give linear fragments. A method for separating circular from linear molecules will therefore result in pure plasmids.

### 3.2.1 Separation on the basis of size

The usual stage at which size fractionation is performed is during preparation of the cell extract. If the cells are lysed under very carefully controlled conditions, only a minimal amount of chromosomal DNA breakage occurs. The resulting DNA fragments are still very large—much larger than the plasmids—and can be removed with the cell debris by centrifugation. This process is aided by the fact that the bacterial chromosome is physically attached to the cell envelope, so fragments of the chromosome sediment with the cell debris if these attachments are not broken.

Cell disruption must therefore be carried out very gently to prevent wholesale breakage of the bacterial DNA. For *E. coli* and related species, controlled lysis is performed as shown in Figure 3.11. Treatment with EDTA and lysozyme is carried out in the presence of sucrose, which prevents the cells from bursting immediately. Instead, **sphaeroplasts** are formed, cells with partially degraded cell walls that retain an intact cytoplasmic membrane. Cell lysis is now induced by adding a non-ionic detergent such as Triton X-100 (ionic detergents, such as SDS, cause chromosomal breakage). This method causes very little breakage of the bacterial DNA, so centrifugation leaves a **cleared lysate**, consisting almost entirely of plasmid DNA.

A cleared lysate will, however, invariably retain some chromosomal DNA. Furthermore, if the plasmids themselves are large molecules, they may also sediment with the

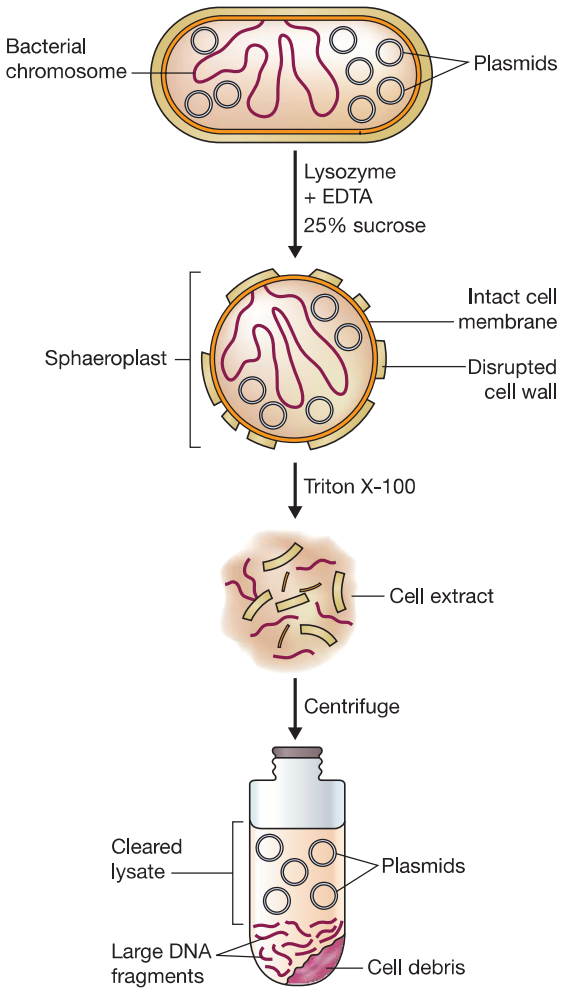
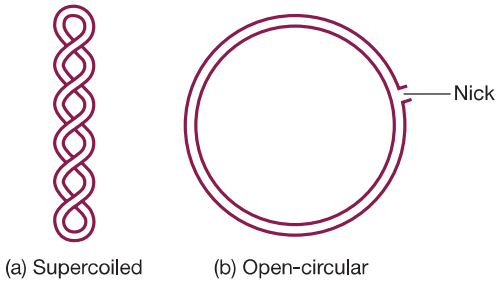



**Figure 3.11**
Preparation of a cleared lysate.

**Figure 3.12**
Two conformations of circular double-stranded DNA: (a) supercoiled—both strands are intact; (b) open-circular—one or both strands are nicked.




cell debris. Size fractionation is therefore rarely sufficient on its own, and we must consider alternative ways of removing the bacterial DNA contaminants.

## 3.2.2 Separation on the basis of conformation

Before considering the ways in which conformational differences between plasmids and bacterial DNA can be used to separate the two types of DNA, we must look more closely at the overall structure of plasmid DNA. It is not strictly correct to say that plasmids have a circular conformation, because double-stranded DNA circles can take up one of two quite distinct configurations. Most plasmids exist in the cell as **supercoiled** molecules (Figure 3.12a). Supercoiling occurs because the double helix of the plasmid DNA is partially unwound during the plasmid replication process by enzymes called topoisomerases (p. 69). The supercoiled conformation can be maintained only if both polynucleotide strands are intact, hence the more technical name of **covalently closed-circular (ccc) DNA**. If one of the polynucleotide strands is broken the double helix reverts to its normal **relaxed** state, and the plasmid takes on the alternative conformation, called **open-circular (oc)** (Figure 3.12b).

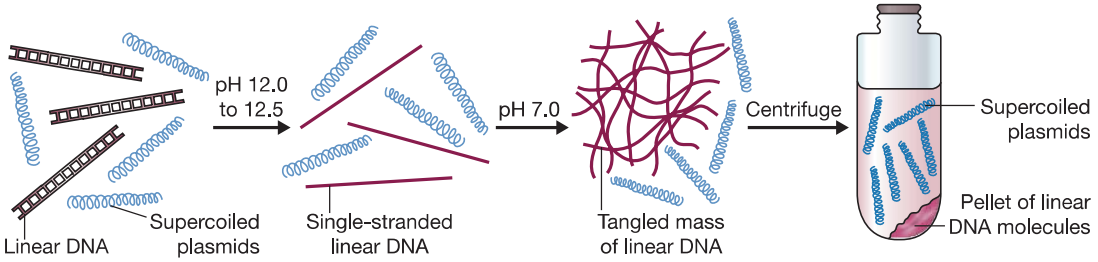
Supercoiling is important in plasmid preparation because supercoiled molecules can be fairly easily separated from non-supercoiled DNA. Two different methods are commonly used. Both can purify plasmid DNA from crude cell extracts, although in practice best results are obtained if a cleared lysate is first prepared.

### *Alkaline denaturation*

The basis of this technique is that there is a narrow pH range at which non-supercoiled DNA is denatured, whereas supercoiled plasmids are not. If sodium hydroxide is added to a cell extract or cleared lysate, so that the pH is adjusted to 12.0–12.5, then the hydrogen bonding in non-supercoiled DNA molecules is broken, causing the double helix to unwind and the two polynucleotide chains to separate (Figure 3.13). If acid is now added, these denatured bacterial DNA strands reaggregate into a tangled mass. The insoluble network can be pelleted by centrifugation, leaving plasmid DNA in the supernatant. An additional advantage of this procedure is that, under some circumstances (specifically cell lysis by SDS and neutralization with sodium acetate), most of the protein and RNA also becomes insoluble and can be removed by the centrifugation step. Further purification by organic extraction or column chromatography may therefore not be needed if the alkaline denaturation method is used.

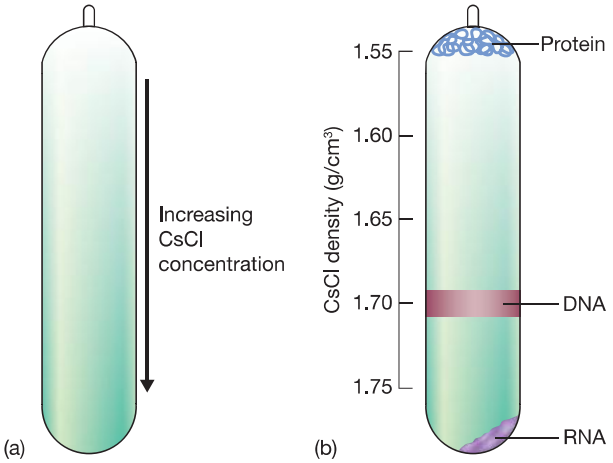
### *Ethidium bromide–caesium chloride density gradient centrifugation*

This is a specialized version of the more general technique of equilibrium or **density gradient centrifugation**. A density gradient is produced by centrifuging a solution of




## Figure 3.13

Plasmid purification by the alkaline denaturation method.




## Figure 3.14

Caesium chloride density gradient centrifugation. (a) A CsCl density gradient produced by high speed centrifugation. (b) Separation of protein, DNA, and RNA in a density gradient.

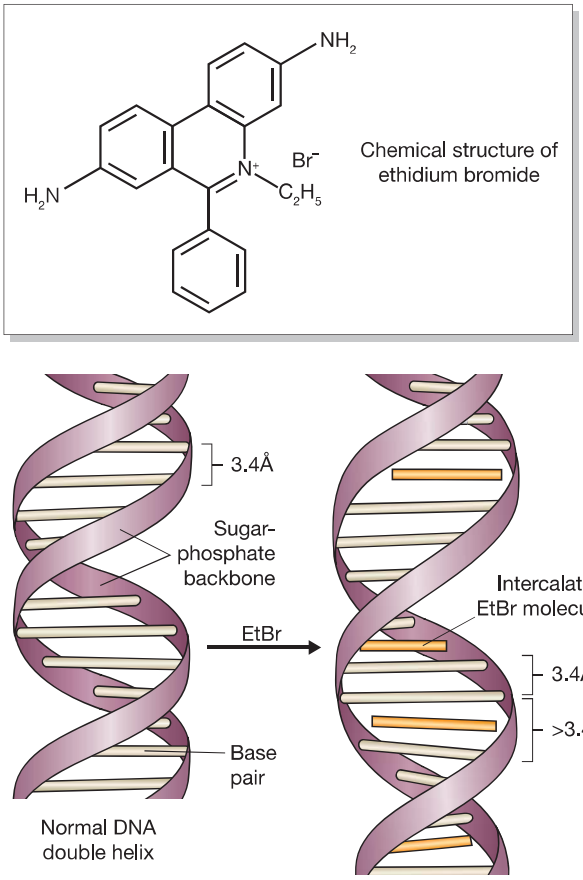
caesium chloride (CsCl) at a very high speed (Figure 3.14a). Macromolecules present in the CsCl solution when it is centrifuged form bands at distinct points in the gradient (Figure 3.14b). Exactly where a particular molecule bands depends on its **buoyant density**: DNA has a buoyant density of about 1.70 g/cm$^3$, and therefore migrates to the point in the gradient where the CsCl density is also 1.70 g/cm$^3$. In contrast, protein molecules have much lower buoyant densities, and so float at the top of the tube, whereas RNA forms a pellet at the bottom (Figure 3.14b). Density gradient centrifugation can therefore separate DNA, RNA, and protein and is an alternative to organic extraction or column chromatography for DNA purification.

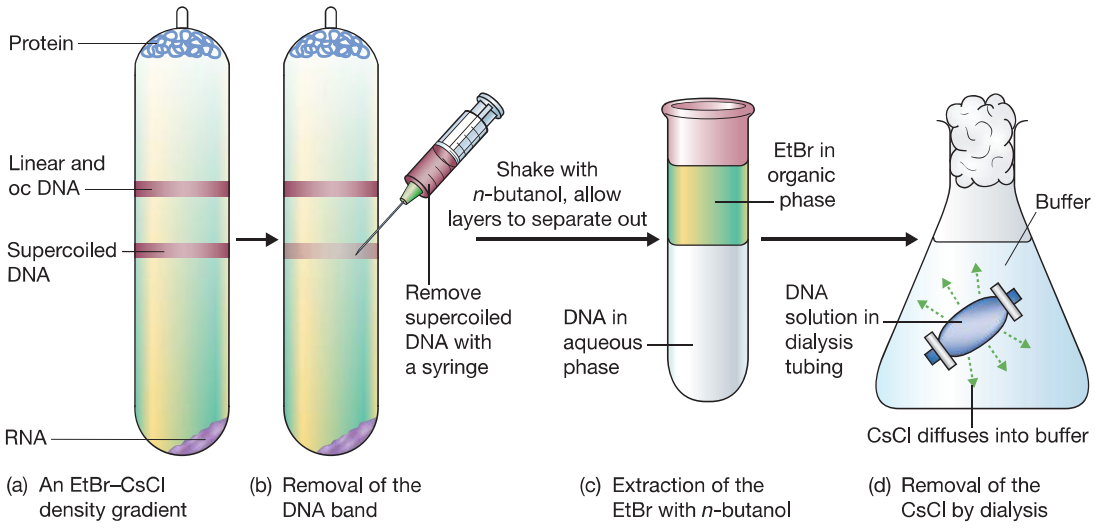
More importantly, density gradient centrifugation in the presence of **ethidium bromide (EtBr)** can be used to separate supercoiled DNA from non-supercoiled molecules. Ethidium bromide binds to DNA molecules by intercalating between adjacent base pairs, causing partial unwinding of the double helix (Figure 3.15). This unwinding results in a decrease in the buoyant density, by as much as 0.125 g/cm$^3$ for linear DNA. However, supercoiled DNA, with no free ends, has very little freedom to unwind, and can only bind a limited amount of EtBr. The decrease in buoyant density of a supercoiled molecule is therefore much less, only about 0.085 g/cm$^3$. As a consequence, supercoiled molecules form a band in an EtBr–CsCl gradient at a different position to linear and open-circular DNA (Figure 3.16a).

Ethidium bromide–caesium chloride density gradient centrifugation is a very efficient method for obtaining pure plasmid DNA. When a cleared lysate is subjected to this procedure, plasmids band at a distinct point, separated from the linear bacterial DNA,

## Figure 3.15

Partial unwinding of the DNA double helix by EtBr intercalation between adjacent base pairs. The normal DNA molecule shown on the left is partially unwound by taking up four EtBr molecules, resulting in the "stretched" structure on the right.




## Figure 3.16

Purification of plasmid DNA by EtBr–CsCl density gradient centrifugation.

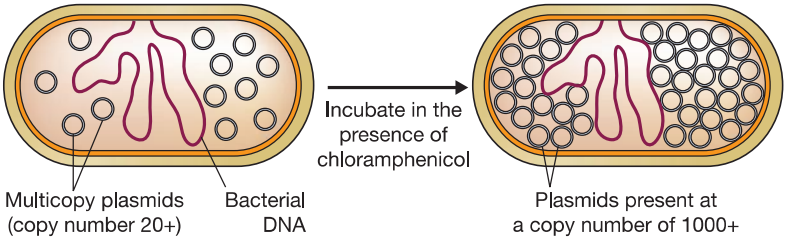



**Figure 3.17**
Plasmid amplification.

with the protein floating on the top of the gradient and RNA pelleted at the bottom. The position of the DNA bands can be seen by shining ultraviolet radiation on the tube, which causes the bound EtBr to fluoresce. The pure plasmid DNA is removed by puncturing the side of the tube and withdrawing a sample with a syringe (Figure 3.16b). The EtBr bound to the plasmid DNA is extracted with *n*-butanol (Figure 3.16c) and the CsCl removed by dialysis (Figure 3.16d). The resulting plasmid preparation is virtually 100% pure and ready for use as a cloning vector.

### 3.2.3 Plasmid amplification

Preparation of plasmid DNA can be hindered by the fact that plasmids make up only a small proportion of the total DNA in the bacterial cell. The yield of DNA from a bacterial culture may therefore be disappointingly low. **Plasmid amplification** offers a means of increasing this yield.

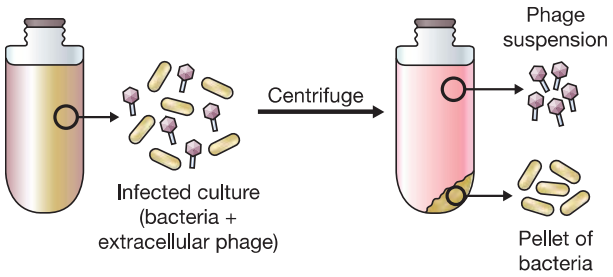
The aim of amplification is to increase the copy number of a plasmid. Some **multicopy plasmids** (those with copy numbers of 20 or more) have the useful property of being able to replicate in the absence of protein synthesis. This contrasts with the main bacterial chromosome, which cannot replicate under these conditions. This property can be utilized during the growth of a bacterial culture for plasmid DNA purification. After a satisfactory cell density has been reached, an inhibitor of protein synthesis (e.g., chloramphenicol) is added, and the culture incubated for a further 12 hours. During this time the plasmid molecules continue to replicate, even though chromosome replication and cell division are blocked (Figure 3.17). The result is that plasmid copy numbers of several thousand may be attained. Amplification is therefore a very efficient way of increasing the yield of multicopy plasmids.

## 3.3 Preparation of bacteriophage DNA

The key difference between phage DNA purification and the preparation of either total cell DNA or plasmid DNA is that for phages the starting material is not normally a cell extract. This is because bacteriophage particles can be obtained in large numbers from the extracellular medium of an infected bacterial culture. When such a culture is centrifuged, the bacteria are pelleted, leaving the phage particles in suspension (Figure 3.18). The phage particles are then collected from the suspension and their DNA extracted by a single deproteinization step to remove the phage capsid.

This overall process is more straightforward than the procedure used to prepare total cell or plasmid DNA. Nevertheless, successful purification of significant quantities of

**Figure 3.18**
Preparation of a phage suspension from an infected culture of bacteria.




phage DNA is subject to several pitfalls. The main difficulty, especially with λ, is growing an infected culture in such a way that the extracellular phage titer (the number of phage particles per ml of culture) is sufficiently high. In practical terms, the maximum titer that can reasonably be expected for λ is $10^{10}$ per ml; yet $10^{10}$ λ particles will yield only 500 ng of DNA. Large culture volumes, in the range of 500–1000 ml, are therefore needed if substantial quantities of λ DNA are to be obtained.

### 3.3.1 Growth of cultures to obtain a high λ titer

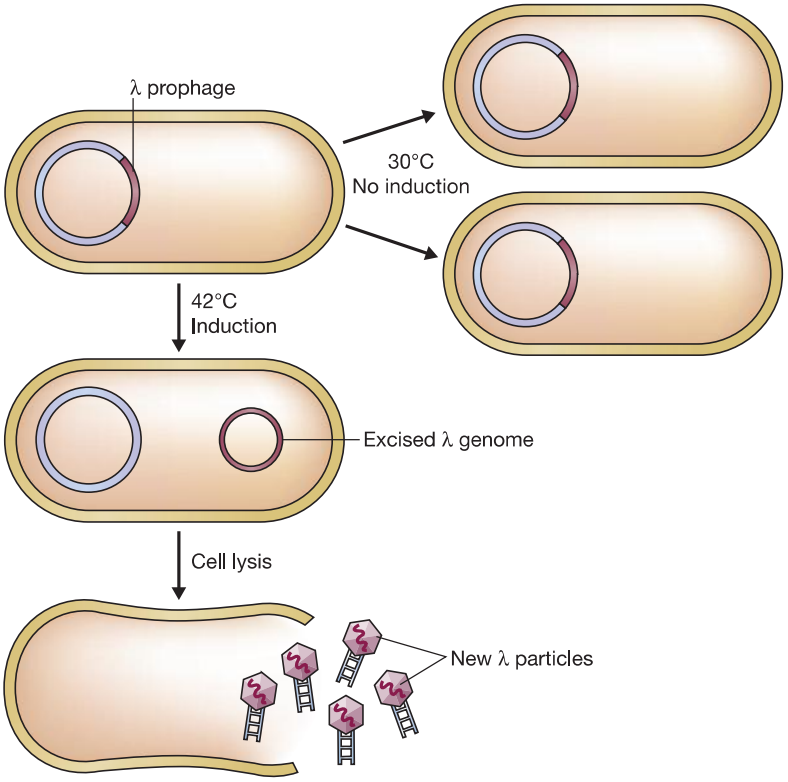
Growing a large volume culture is no problem (bacterial cultures of 100 liters and over are common in biotechnology), but obtaining the maximum phage titer requires a certain amount of skill. The naturally occurring λ phage is lysogenic (p. 19), and an infected culture consists mainly of cells carrying the prophage integrated into the bacterial DNA (see Figure 2.7). The extracellular λ titer is extremely low under these circumstances.

To get a high yield of extracellular λ, the culture must be **induced**, so that all the bacteria enter the lytic phase of the infection cycle, resulting in cell death and release of λ particles into the medium. Induction is normally very difficult to control, but most laboratory strains of λ carry a **temperature-sensitive (ts) mutation** in the *c*I gene. This is one of the genes that are responsible for maintaining the phage in the integrated state. If inactivated by a mutation, the *c*I gene no longer functions correctly and the switch to lysis occurs. In the *cIts* mutation, the *c*I gene is functional at 30°C, at which temperature normal lysogeny can occur. But at 42°C, the *cIts* gene product does not work properly, and lysogeny cannot be maintained. A culture of *E. coli* infected with a λ phages carrying the *cIts* mutation can therefore be induced to produce extracellular phages by transferring from 30°C to 42°C (Figure 3.19).

### 3.3.2 Preparation of non-lysogenic λ phages

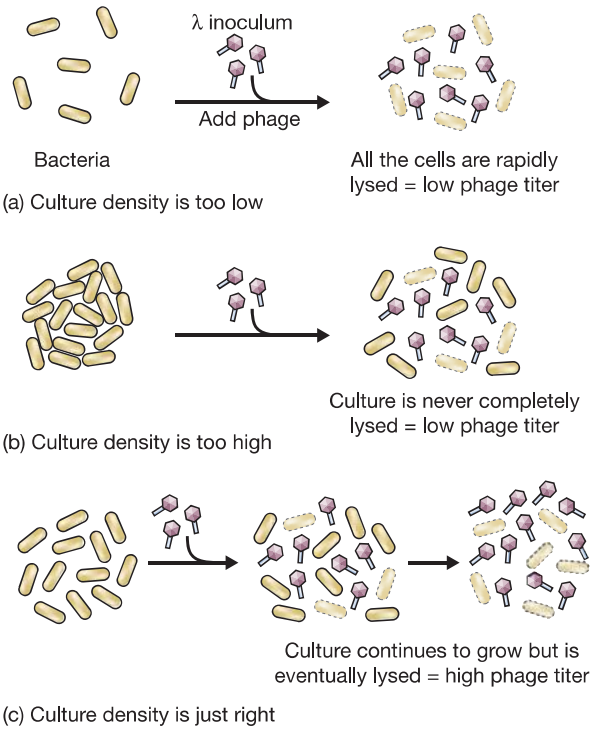
Although most λ strains are lysogenic, many cloning vectors derived from λ are modified, by deletions of the *c*I and other genes, so that lysogeny never occurs. These phages cannot integrate into the bacterial genome and can infect cells only by a lytic cycle (p. 18).

With these phages the key to obtaining a high titer lies in the way in which the culture is grown, in particular the stage at which the cells are infected by adding phage particles. If phages are added before the cells are dividing at their maximal rate, then all the cells are lysed very quickly, resulting in a low titer (Figure 3.20a). On the other hand, if the cell density is too high when the phages are added, then the culture will never be completely lysed, and again the phage titer will be low (Figure 3.20b). The ideal situation is when the age of the culture, and the size of the phage inoculum, are balanced such that the culture continues to grow, but eventually all the cells are infected




## Figure 3.19

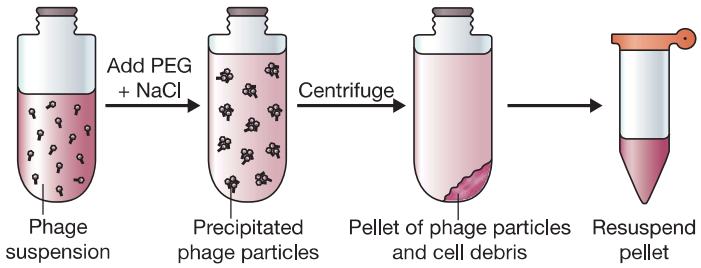
Induction of a λ *cIts* lysogen by transferring from 30°C to 42°C.




## Figure 3.20

Achieving the right balance between culture age and inoculum size when preparing a sample of a non-lysogenic phage.

**Figure 3.21**
Collection of phage particles by polyethylene glycol (PEG) precipitation.




and lysed (Figure 3.20c). As can be imagined, skill and experience are needed to judge the matter to perfection.

### 3.3.3 Collection of phages from an infected culture

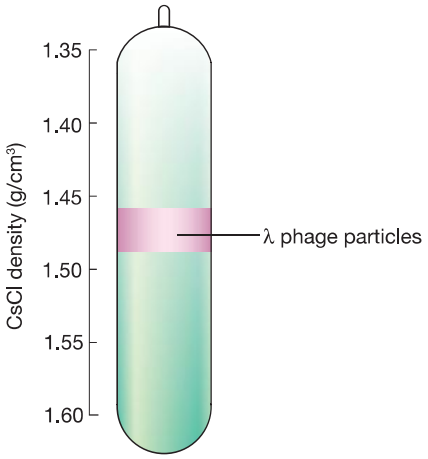
The remains of lysed bacterial cells, along with any intact cells that are inadvertently left over, can be removed from an infected culture by centrifugation, leaving the phage particles in suspension (see Figure 3.18). The problem now is to reduce the size of the suspension to 5 ml or less, a manageable size for DNA extraction.

Phage particles are so small that they are pelleted only by very high speed centrifugation. Collection of phages is therefore usually achieved by precipitation with **polyethylene glycol (PEG)**. This is a long-chain polymeric compound which, in the presence of salt, absorbs water, thereby causing macromolecular assemblies such as phage particles to precipitate. The precipitate can then be collected by centrifugation, and redissolved in a suitably small volume (Figure 3.21).

### 3.3.4 Purification of DNA from λ phage particles

Deproteinization of the redissolved PEG precipitate is sometimes sufficient to extract pure phage DNA, but usually λ phages are subjected to an intermediate purification step. This is necessary because the PEG precipitate also contains a certain amount of bacterial debris, possibly including unwanted cellular DNA. These contaminants can be separated from the λ particles by CsCl density gradient centrifugation. The λ particles band in a CsCl gradient at 1.45–1.50 g/cm$^3$ (Figure 3.22), and can be withdrawn from

**Figure 3.22**
Purification of λ phage particles by CsCl density gradient centrifugation.



the gradient as described previously for DNA bands (see Figure 3.16). Removal of CsCl by dialysis leaves a pure phage preparation from which the DNA can be extracted by either phenol or protease treatment to digest the phage protein coat.

### 3.3.5 Purification of M13 DNA causes few problems

Most of the differences between the M13 and λ infection cycles are to the advantage of the molecular biologist wishing to prepare M13 DNA. First, the double-stranded replicative form of M13 (p. 23), which behaves like a high copy number plasmid, is very easily purified by the standard procedures for plasmid preparation. A cell extract is prepared from cells infected with M13, and the replicative form separated from bacterial DNA by, for example, EtBr–CsCl density gradient centrifugation.

However, the single-stranded form of the M13 genome, contained in the extracellular phage particles, is frequently required. In this respect the big advantage compared with λ is that high titers of M13 are very easy to obtain. As infected cells continually secrete M13 particles into the medium (see Figure 2.8), with lysis never occurring, a high M13 titer is achieved simply by growing the infected culture to a high cell density. In fact, titers of $10^{12}$ per ml and above are quite easy to obtain without any special tricks being used. Such high titers mean that significant amounts of single-stranded M13 DNA can be prepared from cultures of small volume—5 ml or less. Furthermore, as the infected cells are not lysed, there is no problem with cell debris contaminating the phage suspension. Consequently the CsCl density gradient centrifugation step, needed for λ phage preparation, is rarely required with M13.

In summary, single-stranded M13 DNA preparation involves growth of a small volume of infected culture, centrifugation to pellet the bacteria, precipitation of the phage particles with PEG, phenol extraction to remove the phage protein coats, and ethanol precipitation to concentrate the resulting DNA (Figure 3.23).

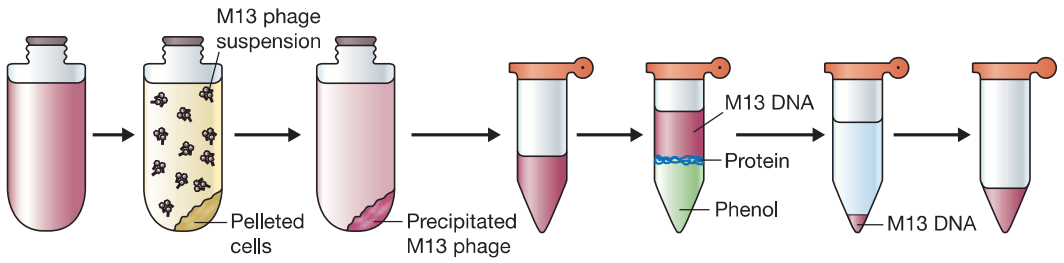



**Figure 3.23**

Preparation of single-stranded M13 DNA from an infected culture of bacteria.

# Further reading

FURTHER READING

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