Plasmids & Transposable Elements

Lecture 2

BY

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DNA Used in Cloning Experiments and its Purification

DNA Used in Cloning Experiments

- Total Cell DNA
- Plasmid DNA
- Bacteriophage DNA

Isolation and Purification of DNA

- DNA is the basic constituent of any living being, and some viruses too. The isolation and purification of DNA is a significant step in any molecular biological technique.

Total Cell DNA

- 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

Preparation of Total Cell DNA

The procedure can be divided into the following 4 steps :

- **Step 1: Growing and harvesting a bacterial culture**
- **Step 2: Cell wall rupture and preparation of cell extract**
- **Step 3: Purification of DNA from the cell extract**
- **Step 4: Concentration of DNA solution**

1.Bacterial culture and growth

- Most bacteria can be grown without much difficulty in a **liquid medium** otherwise known as **broth culture**.
- The culture medium must provide a balanced mixtures of essential nutrients at concentrations that will allow the bacteria to grow and divide effectively.

a.Defined media

- **Defined media** must be used when the bacterial culture has to be grown under precisely controlled conditions.
- In this case we know the exact concentration of all the chemicals used in the media preparation.

- This media contains mixtures of **inorganic nutrients** to provide essential elements like **N**, **Mg**, **Ca** as well as **glucose** (source of carbon and energy).
- Additional growth factors like ***trace elements*** and ***vitamin*** may be added to provide additional nutrients to the bacteria depending upon the bacterial species.
- **M9** is an example of a known defined medium

Undefined Media

- In this case the precised identity and the quantity of its components are not known.
- Ingredients like **tryptone** and **yeast extract** are used which are complicated mixture of unknown chemical compounds.
- It is known as **tryptone** contains small peptides and amino acids like whereas **yeast extract** supplement provides N_2 and glucose requirement to the bacteria.
- This complex media needs no further supplementation and support growth of wide range of bacterial species .
- **LB (Luria-Bertain)** is a complex undefined medium

- In order to prepare a cell extract, the bacteria must be obtained in as small a volume as possible “**minipreps**” .
- Harvesting is therefore performed by spinning the culture in a centrifuge.
- Low centrifugation speeds will pellet the bacteria at the bottom of the centrifuge tube, allowing the culture medium to be poured off.

Preparation of cell extract

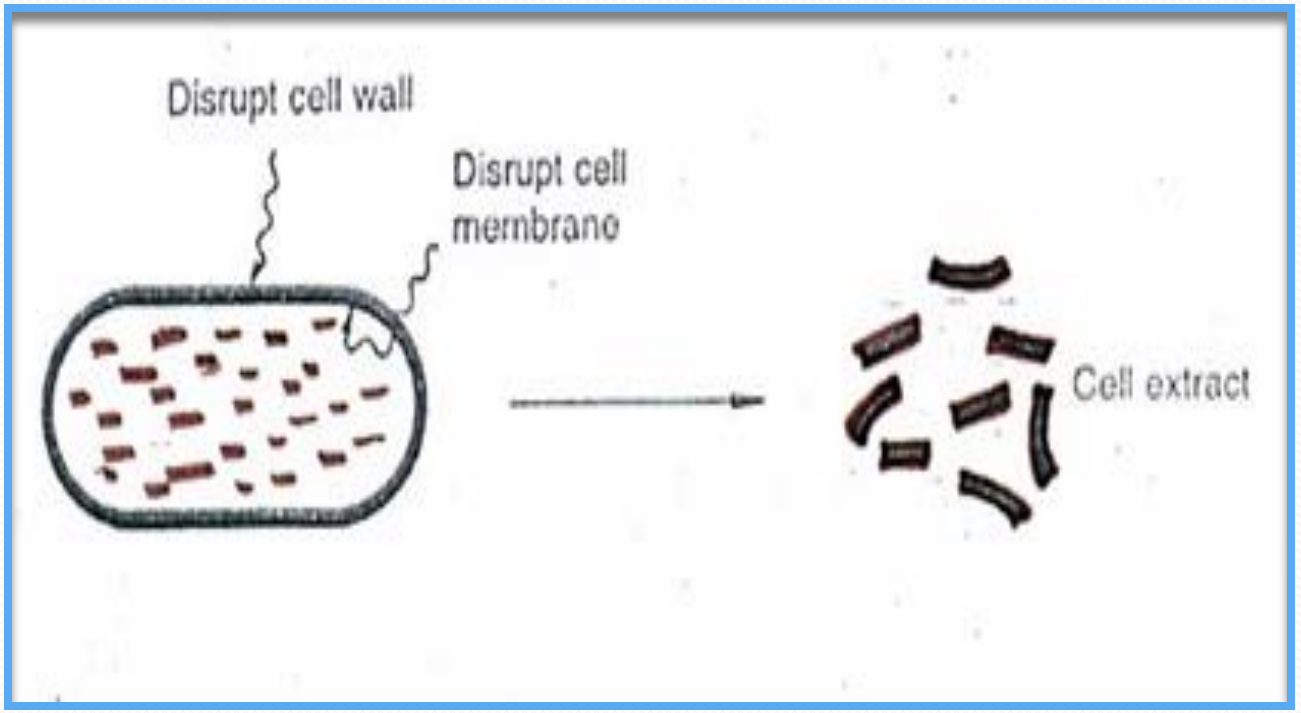
- The bacterial cell is enclosed in a cytoplasmic membrane and surrounded by rigid cell wall.
- In some species like *E. coli* , the cell wall itself may be engulfed by the second outer membrane.
- All these barriers have to be disrupted to release the cell components.

- **Physical method :** Use of beads and other physical methods to rupture the cell wall by mechanical forces

- **Chemical method**

  - Chemical methods are most commonly used with bacterial cells during DNA preparation.

  - Cell lysis by chemical method is brought about by one chemical that attacks the cell wall while the other disrupts the cell membrane .

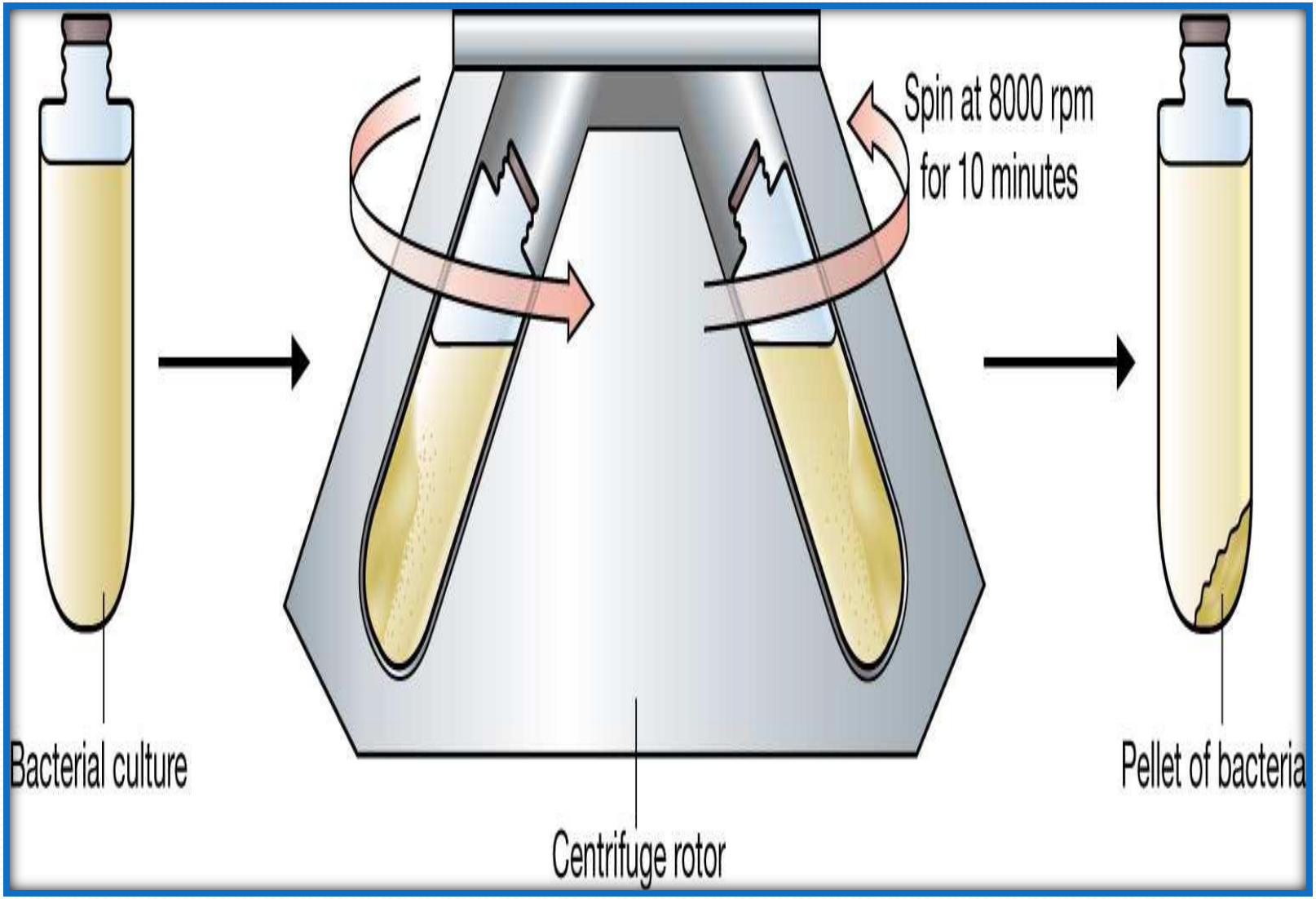

Disrupt cell wall
Disrupt cell membrano
Cell extract

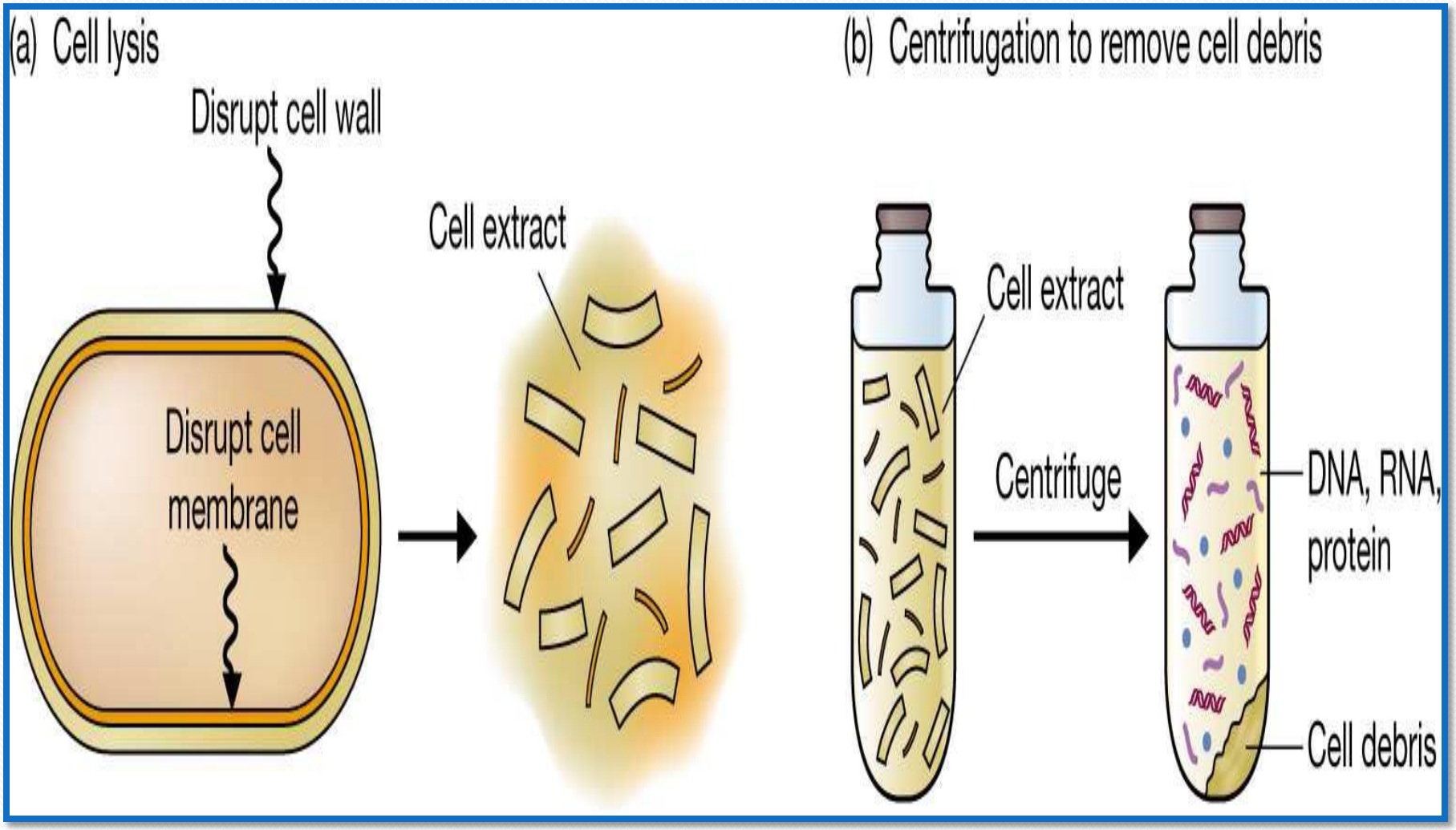
- Cell wall weakening is brought about by **lysozyme, ethylenediamine tetraacetate (EDTA)** or a combination of both.
- **Lysozyme**
- Lysozyme is present in secretion by saliva, tears egg-white .
- Lysozyme digests the polymeric compounds that give the cell wall its rigidity.

- **EDTA (Ethylene Diamine Tetra-acetic Acid),** it removes the Mg^{2+} ions that are essential for preserving the overall structure of the cell also inhibits cellular enzymes that could degrade DNA.
- **SDS (Sodium Dodecyl Sulphate).**

  Sometimes a detergent such as **sodium dodecyl sulphate (SDS)** is added along with the chemicals, because detergents aid the process of lysis by removing lipid molecules and thereby cause disruption of the cell membranes.

- Generally mixture of **EDTA** and **lysozyme** is used.
- The insoluble cell debris can be pelleted down by **centrifugation** of this mixture at a speed of **8000 rpm** for **10 mins**.
- It causes the precipitation of cell forming and the pellets in the test-tube leaving the **cell extract** as a reasonably **clear supernatant(lysate)** .
- Finally the cell extracts are extracted from cell debris.

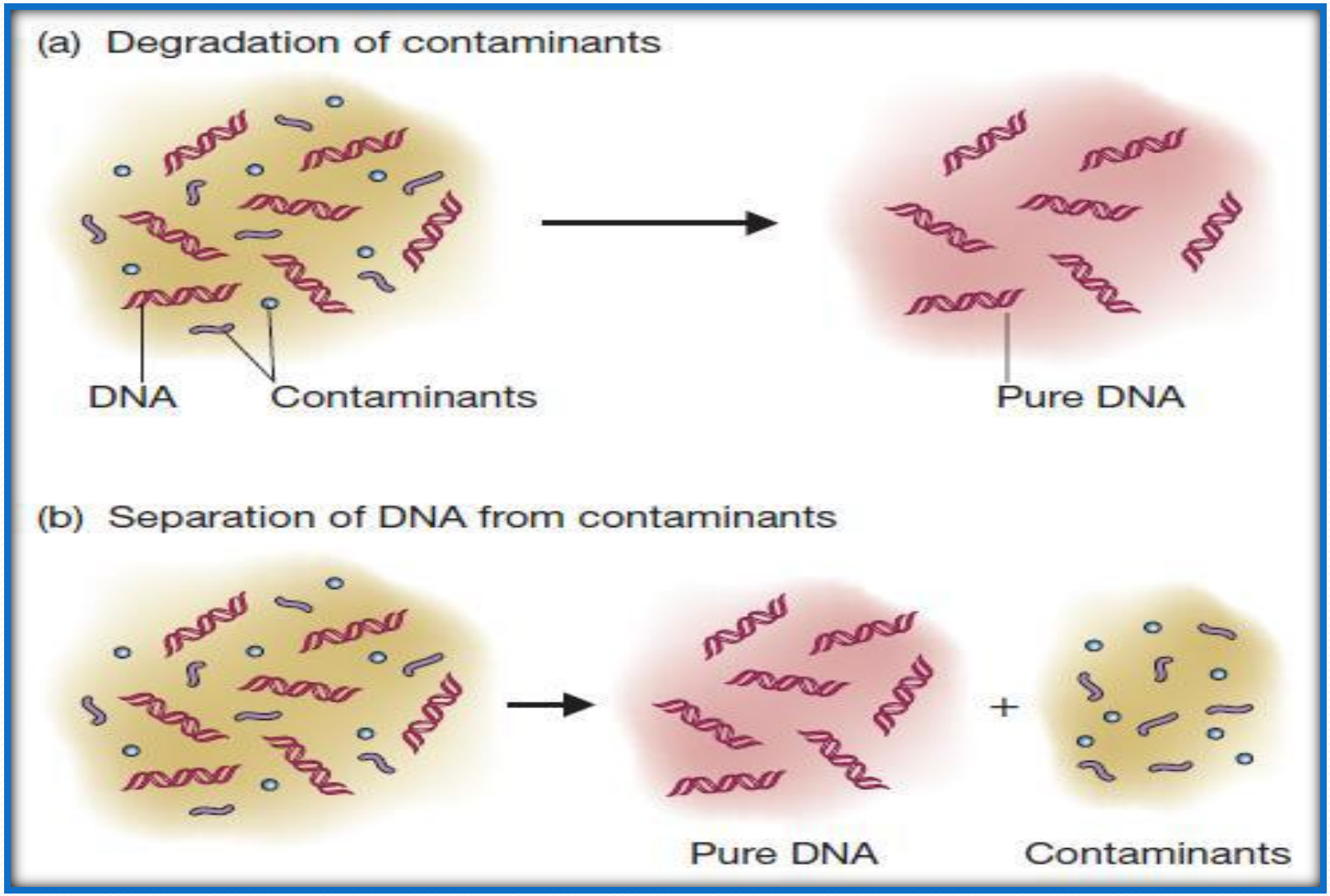

Spin at 8000 rpm
for 10 minutes
Bacterial culture
Centrifuge rotor
Pellet of bacteria


(a) Cell lysis
Disrupt cell wall
Disrupt cell membrane
Cell extract
(b) Centrifugation to remove cell debris
Cell extract
Centrifuge
DNA, RNA, protein
Cell debris

Purification of DNA from cell extract

- In addition to **DNA** , a bacterial cell extract contains significant quantities of **protein** and **RNA** .
- For obtaining pure DNA, bacterial cell extract is purified from significant quantities of Protein and RNA.
- DNA associated proteins, as well as other cellular proteins, may be degraded with the addition of a **protease**.
- Cell extract is treated with **protease** such as **pronase** or **proteinase K** before extraction .
- These enzymes will break polypeptides into smaller units thus making phenol easier to remove them.

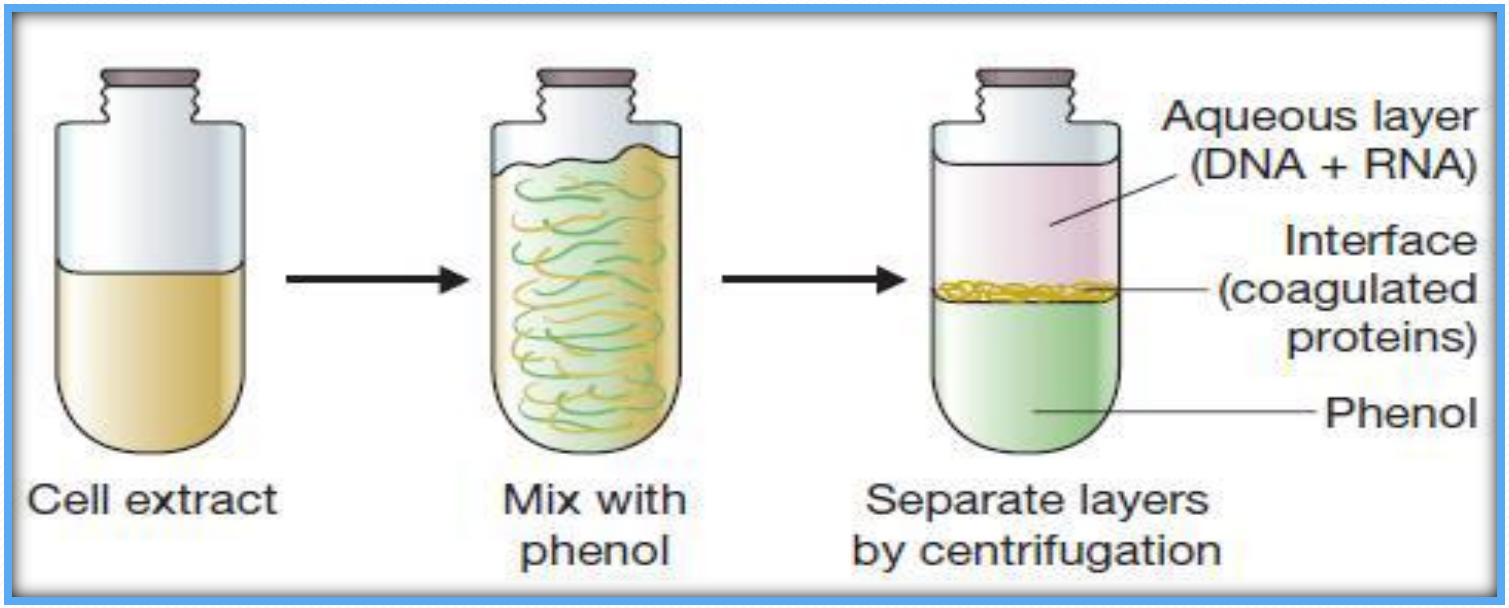
Two approaches to DNA purification:



- **Precipitation** of the protein is aided by the addition of a **salt** such as **ammonium** or **sodium acetate**.
- The standard way to de-proteinize a cell is vortexes the cell extract with **phenol** or a 1:1 mixture of **phenol and chloroform.**
- The **organic solvents** precipitate proteins but leave the nucleic acids (DNA and RNA) in an aqueous solution.

The cell extract is mixed gently with the solvent , and the layers then separated by centrifugation.

The result is that the precipitated proteins left as **white coagulated mass** will remain at the interface between the **aqueous** and **organic layers** and can be drawn off carefully .

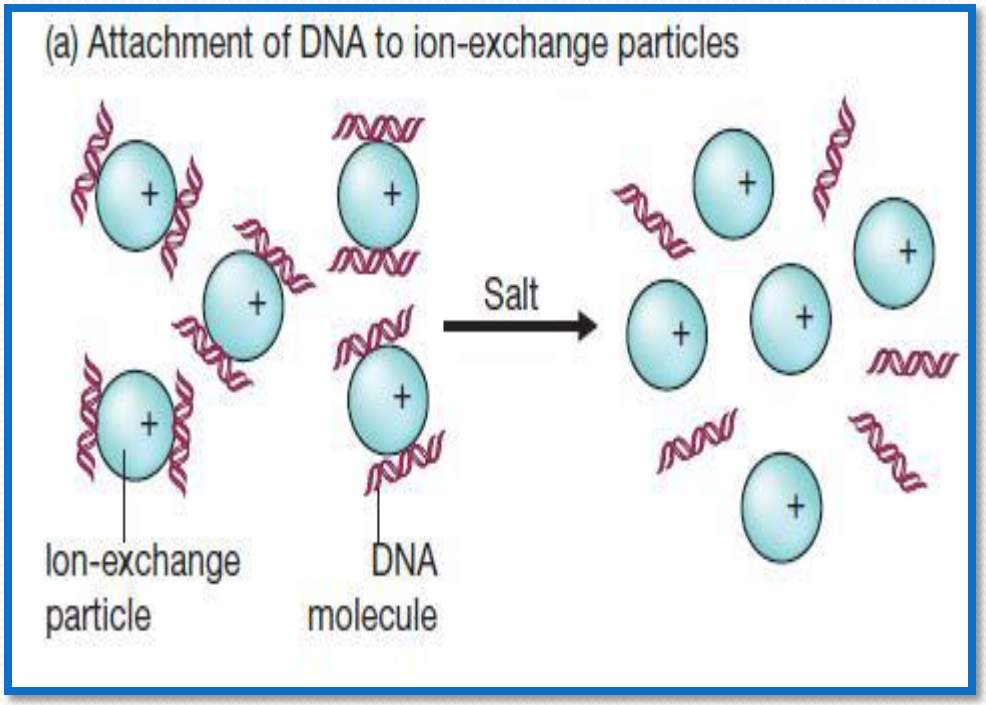


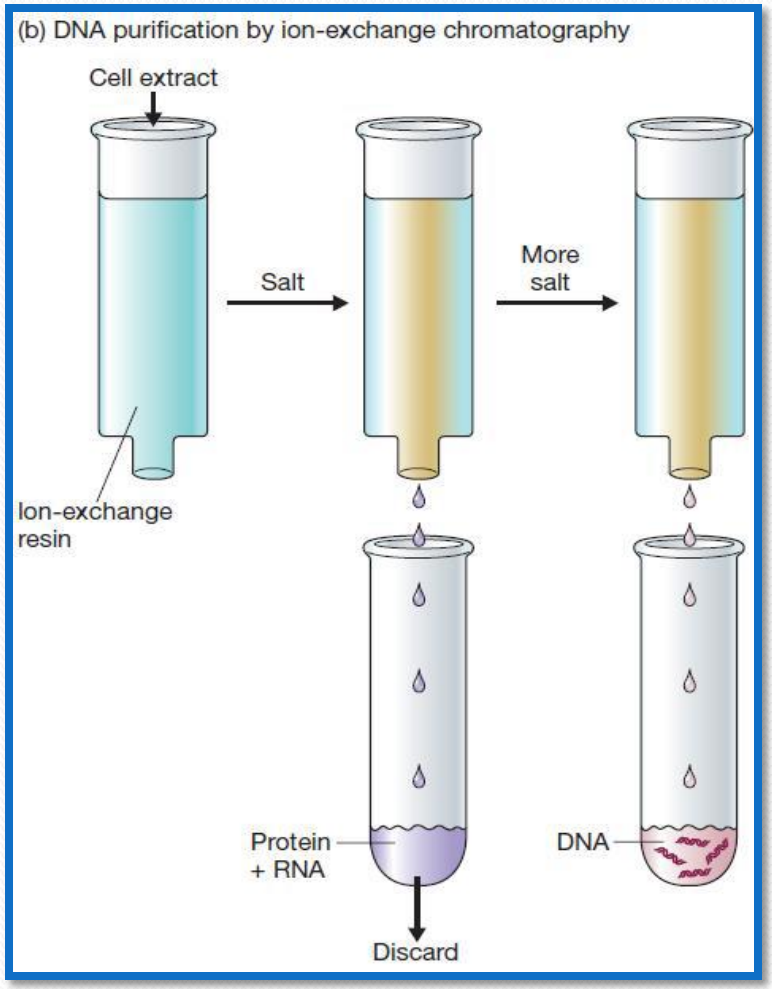
- The aqueous solution of nucleic acids can then be removed with a white pipette.
- The only effective way to get rid of RNA is the use of **Ribonuclease enzyme .**

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

- Ion-exchange chromatography 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 and so bind to a positively charged resin.

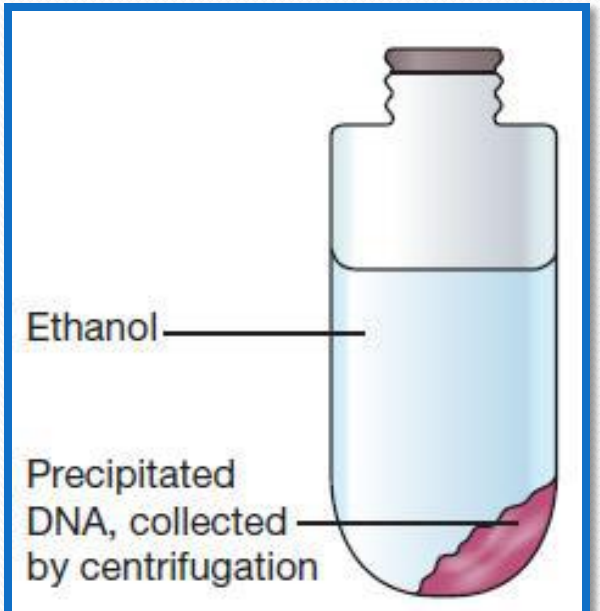
- The electrical attachment is disrupted by salt, removal of the more tightly bound molecules requiring higher concentrations of salt.





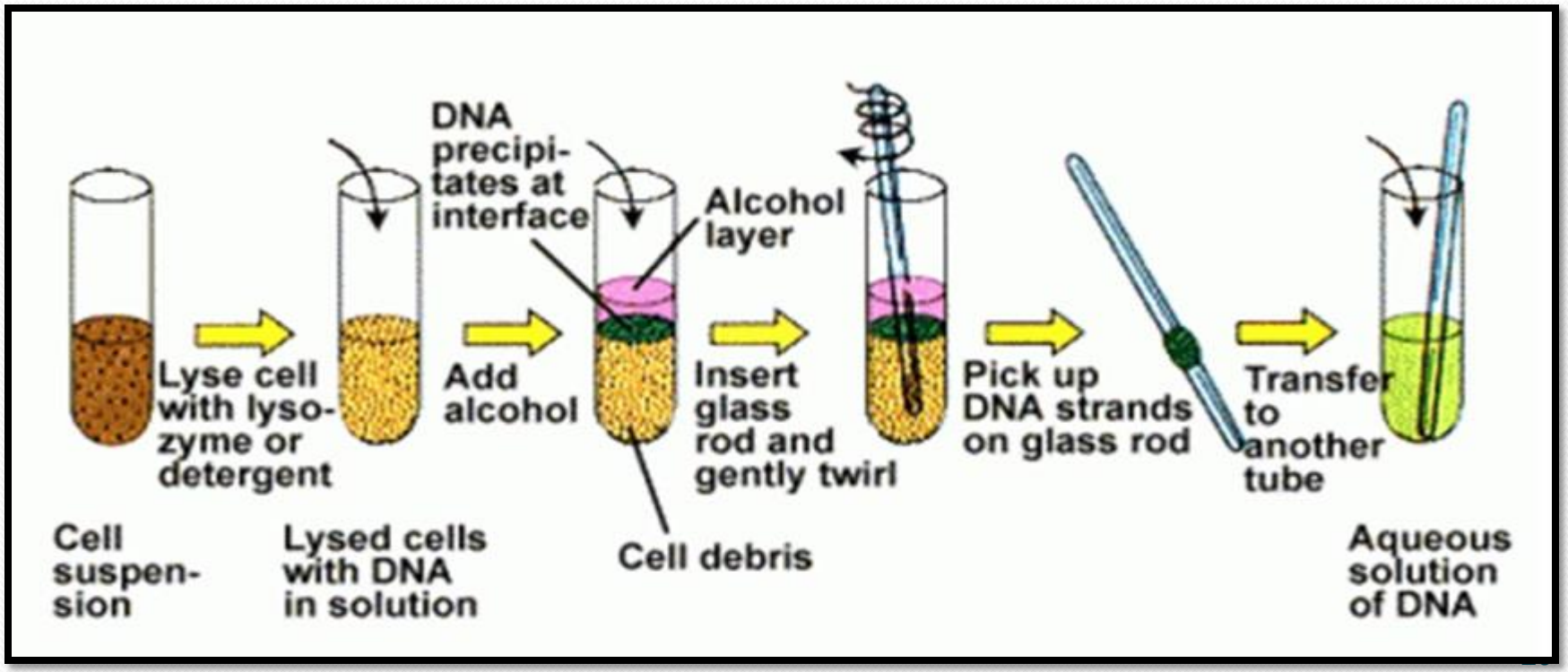
Concentration of DNA samples

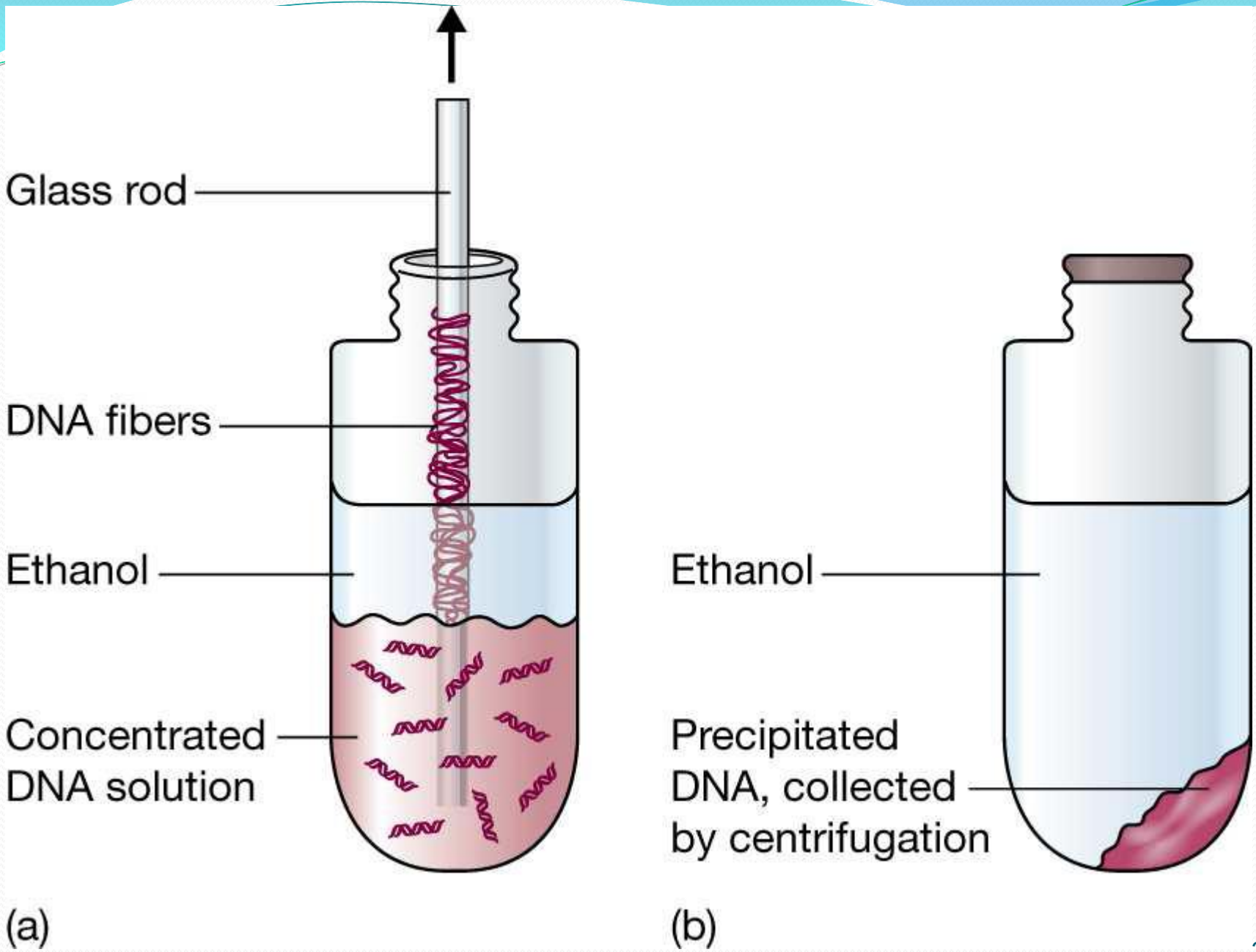
- The most frequently used method of concentration is **ethanol precipitation.**
- In the presence of salt (Na+) and a temperature of -20°c or less absolute ethanol efficiently precipitate polymeric nucleic acids.



- With a **thick solution** of DNA, the ethanol can be layered on the 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, DNA molecules will adhere and be pulled out of the solution in the form of long fiber.

-If ethanol is mixed with a **dilute solution**, the precipitate can be collected by centrifugation , and then redissolved in an appropriate volume of water.




Glass rod
DNA fibers
Ethanol
Concentrated
DNA solution
(a)
Ethanol
Precipitated
DNA, collected
by centrifugation
(b)

Measurement of DNA concentration

- DNA **concentrations** can be accurately measured by **ultraviolet** (UV) **absorbance spectrophotometry**.
- The amount of ultraviolet radiation absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample.
- Absorbance is measured usually at 260nm, at which wavelength an absorbance (A_{260}) of **1.0** corresponds to **50µg** of double stranded DNA /ml.

purity of a DNA preparation

- Ultraviolet absorbance can also be used to **check the purity of a DNA** preparation.
- With a pure sample of DNA indicates the ratio of absorbances at **260** and **280 nm** is **1.8** i.e. , **A_{260}/A_{280}** is **1.8** , for a **pure sample of DNA**.
- Ratio less than 1.8 indicate that the preparation is contaminated, either with protein or with phenol.

DNA preparation from animal and plant cells

- Preparation of DNA from plant and animal cells is different from bacterial cell.
- Bacterial cell wall degradating enzyme lysozyme has no effect on plant cell wall.
- whereas most animal cells have no cell wall at all, and can be lysed simply by treating with detergent.
- Plant tissues consist of large amount of carbohydrates which are not removed by phenol extraction.

- In this case a **detergent** called **cetyl trimethyl ammonium bromide (CTAB)** is used which forms an insoluble complex with nucleic acids .

- When CTAB is added to a plant cell extract the nucleic acid-CTAB complex precipitates, leaving carbohydrate, protein and other contaminants in the supernatant.

- The precipitate is then collected by **centrifugation** and **resuspended** in 1M Nacl, which causes the complex to breakdown
- the **RNA** removed by **ribonuclease** treatment.


Good
Luck!

Plasmids & Transposable Elements

Lecture 3

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Plasmid DNA preparation

- In order to use a vector for cloning, sequencing, etc., it is necessary to isolate the vector in a **highly purified form**.
- This is an important technique, and is routinely done by most labs.
- There are several ways to purify plasmids.
- Is same as total cell DNA preparation but importantly distinct in one aspect that in plasmid DNA 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.

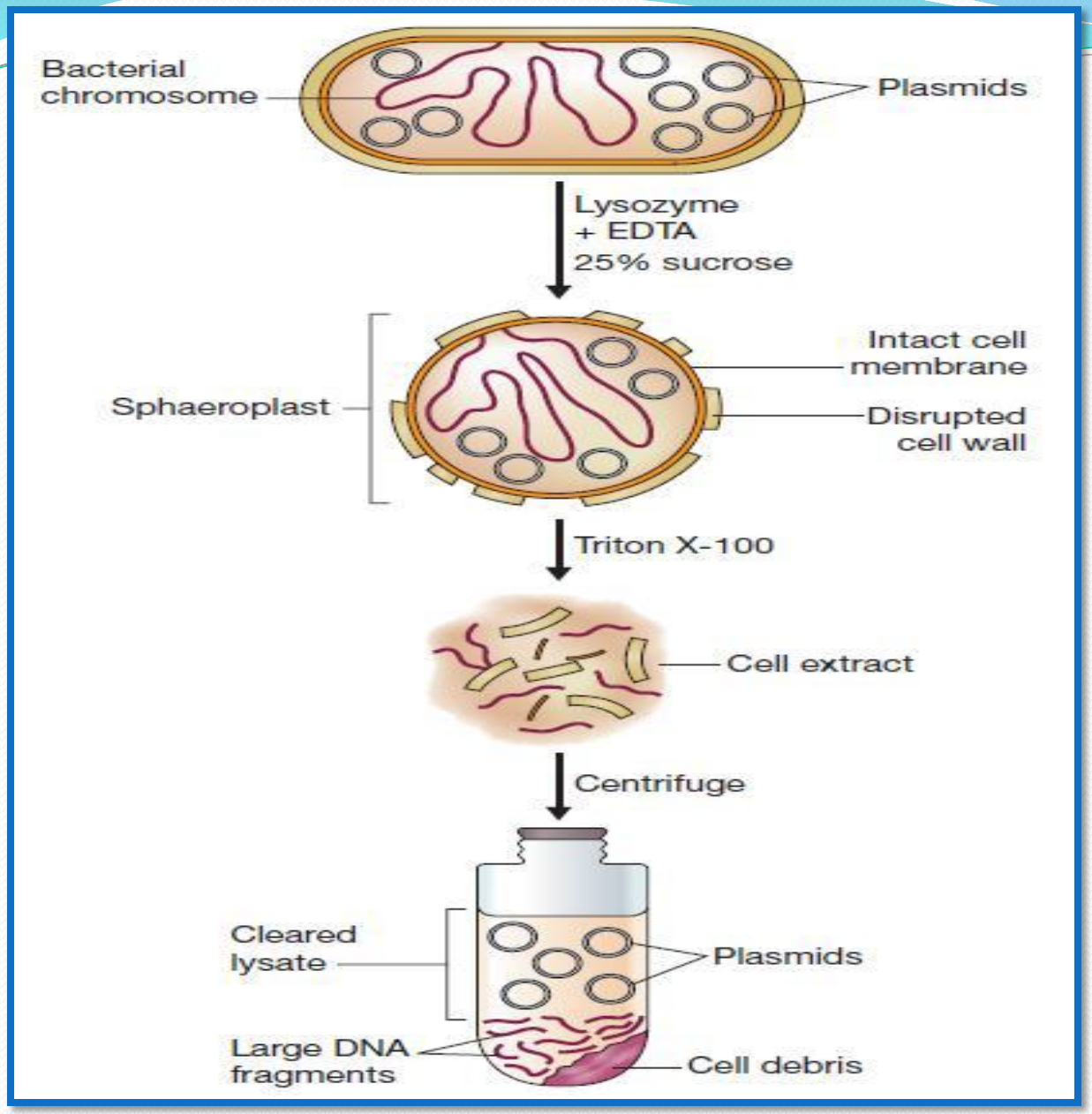
- In addition to **size**, plasmids and bacterial DNA differ in **conformation**.
- Plasmids and the bacterial chromosome are **circular**, but during preparation of the cell extract the **chromosome** is always **broken , so the double helix reverts to its normal relaxed state (**oc isoform) to give **linear fragments** .
- A method for separating **circular** (ccc pDNA) from **linear molecules** will therefore result in **pure plasmids**.

- To obtain a homogeneous plasmid DNA preparation, different pDNA purification strategies aim at capturing ccc pDNA and eliminating the oc isoform.

Size based separation

- Bacterial cell disruption is carried out very gently to prevent wholesale breakage.
- Treatment with **EDTA** and **lysozyme** is carried out in the presence of **sucrose**, which prevents the cell from bursting.
- Sphaeroplasts (partially wall cells) are formed that retain an intact cytoplasmic membrane

- Cell lysis is induced by adding a **non-ionic detergent Triton X-100** which causes minimal breakage of the bacterial DNA , therefore centrifugation will leave a cleared lysate, consisting almost entirely of plasmid DNA.
- A clear lysate will however, invariably retain some chromosomal DNA .
- **Size fractionation** does not sufficiently help to remove contaminants, and therefore alternative ways for it must be considered.

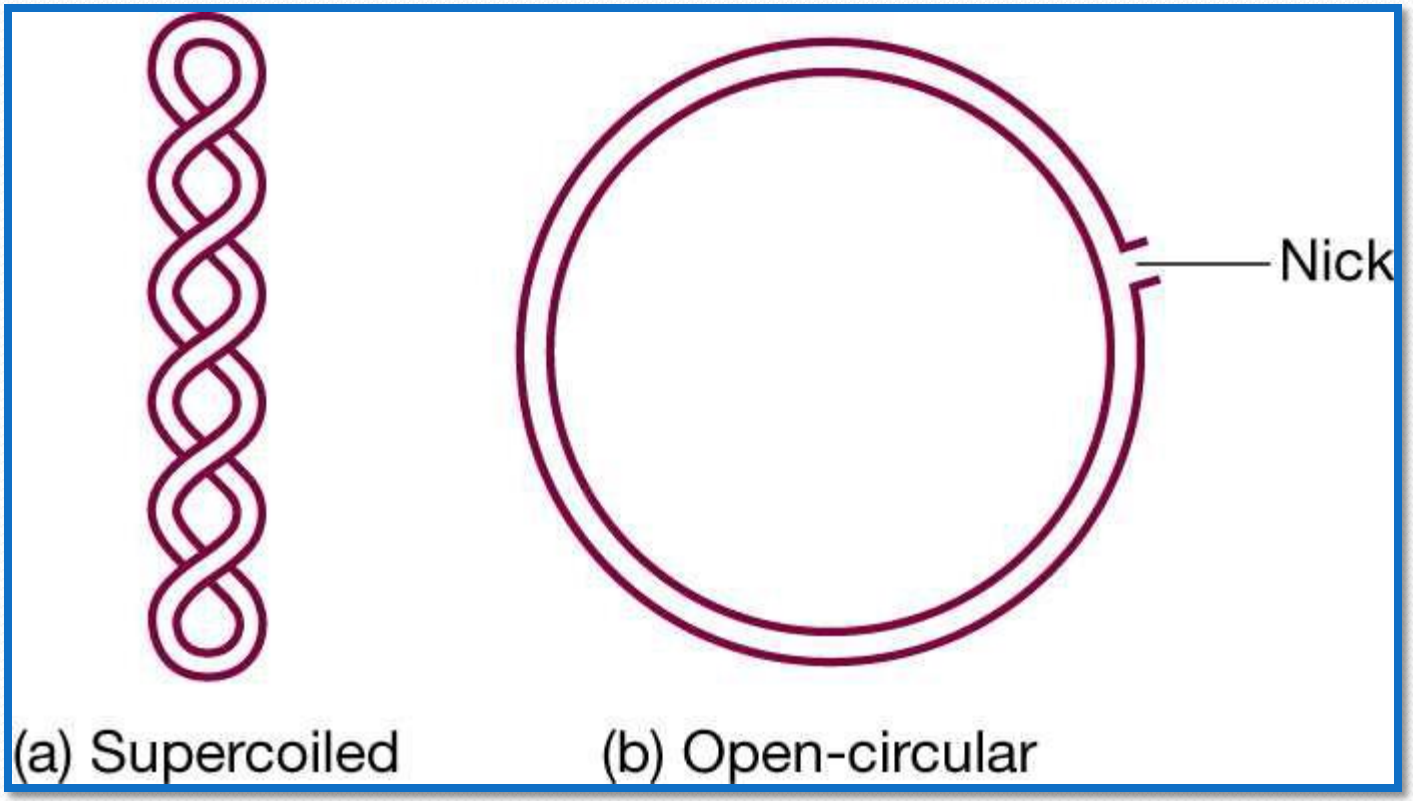


Size based separation

Conformation based separation

- Most plasmids exist in the cell as supercoiled molecules called covalent closed circle (ccc).
- Supercoiled molecules can be easily separated from non supercoiled DNA .
- Two different types of conformation based separation are **alkaline denaturation** and **EtBr-CsCl density gradient centrifugation**.

Conformations of DNA



(CCC)

(OC)

Alkaline denaturation

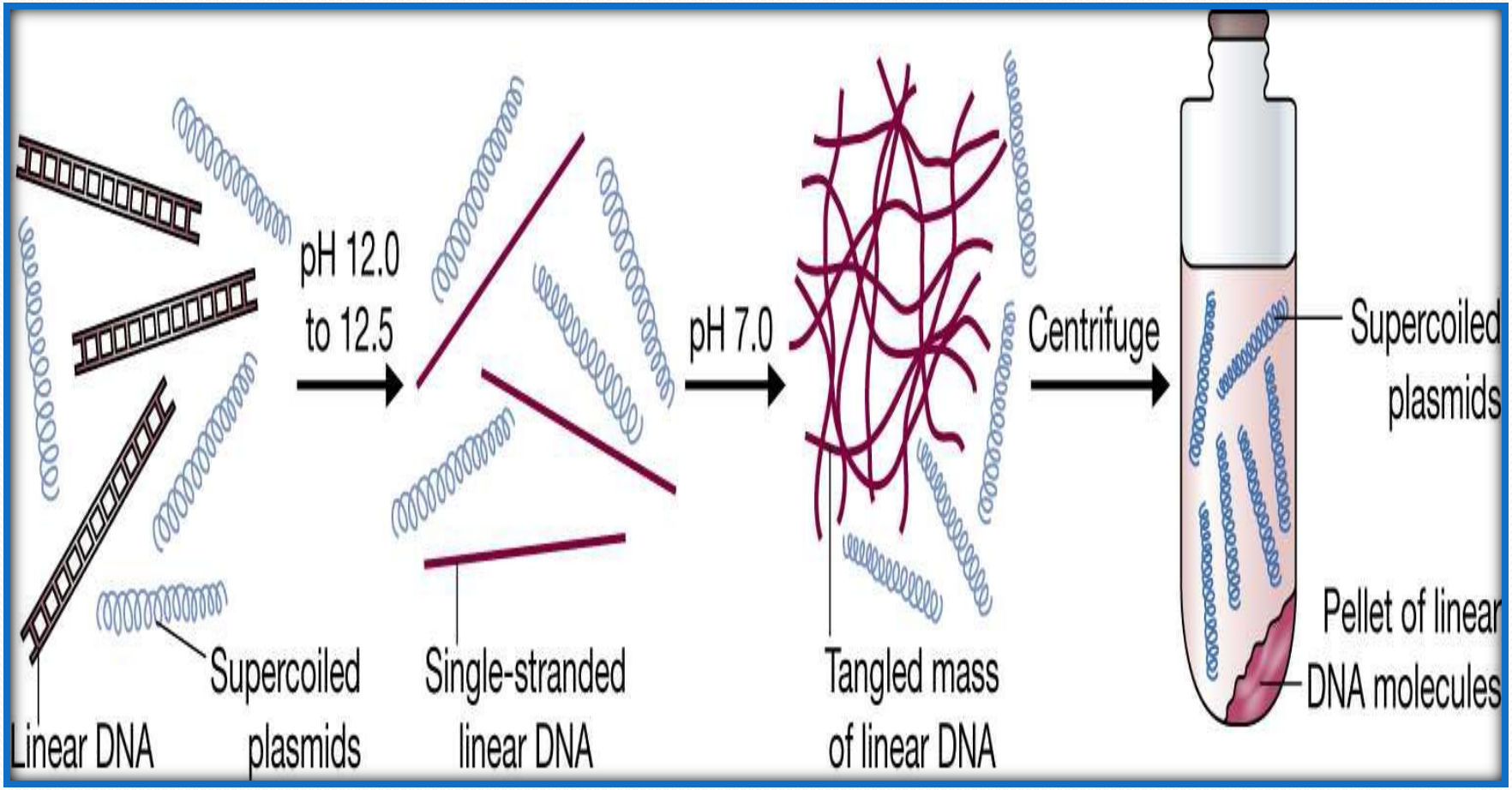
- Non-supercoiled DNA is denatured at a narrow pH range.
- If pH of a cell extract or cleared lysate is increased (12.0-12.5) by addition of NaOH , the cells are brought to a high pH to not only lyse the cells, but also to denature the DNA .Thus , the hydrogen bonding in non supercoiled DNA molecules is broken , causing the unwinding of double helix and finally separation of two polypeptide chains .

- The DNA solution is then neutralized .

- Since plasmid DNA is circular and supercoiled, when the pH is brought back down to neutral, the plasmid DNA snaps back to being double-stranded.

- By contrast, genomic DNA is so large that it is broken into linear pieces . The linear DNA denatures in alkali and forms precipitates when the pH is lowered (denatured DNA strands will re-aggregate into a tangle mass by the addition of acid .

- With the help of centrifugation, the insoluble network can be pelleted, leaving pure plasmid DNA in the supernatant.
- Under some circumstances (cell lysis by SDS and neutralization with sodium acetate) , most of the proteins and RNA also becomes insoluble and can be removed by centrifugation.


pH 12.0
to 12.5
pH 7.0
Centrifuge
Supercoiled
plasmids
Pellet of linear
DNA molecules
Linear DNA
Supercoiled
plasmids
Single-stranded
linear DNA
Tangled mass
of linear DNA

Ethidium bromide-caesium chloride(EtBr-CsCl) density gradient centrifugation

- Under high centrifugal force (very high speed) , a solution of **cesium chloride (CsCl)** molecules will dissociate, and the heavy **Cs^+ atoms** will be forced towards the outer end of the tube, thus forming a shallow density gradient.

- Macromolecules present in the **CsCl solution** when it is **centrifuged** will migrate to the point where they have the same density (the **isopycnic point**) and form **bands** at **distinct points** in the gradient.
- The gradient is sufficient to separate **types of DNA** with slight differences in **density** due to differing **(G+C) content**, or **physical** form (e.g., **linear** versus **circular molecules**).

- Density gradient centrifugation in the presence of **ethidium bromide (EtBr)** can be used to separate supercoiled DNA from non-supercoiled molecules .
- EtBr binds to DNA molecules by intercalating between adjacent base pairs, causing partial unwinding of the double helix.

- This unwinding results in a decrease in the buoyant density , by as much as 0.125 g/cm3 for linear DNA .
- 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 molecules is therefore much less , only about 0.085 g/cm3 . As a consequence , supercoiled molecules form a band in an EtBr-CsCl gradient at a different position to linear and open-circular DNA .

- Density gradient centrifugation can separate DNA, RNA and protein and is an alternative to phenol extraction and ribonuclease treatment for DNA purification.
- EtBr-Cscl 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
- pDNA has a buoyant density of about 1.7 g/cm3 , and therefore migrates to the point in the gradient where the CsCl density ia also 1.7 g/cm3 .

separated from the linear bacterial DNA,

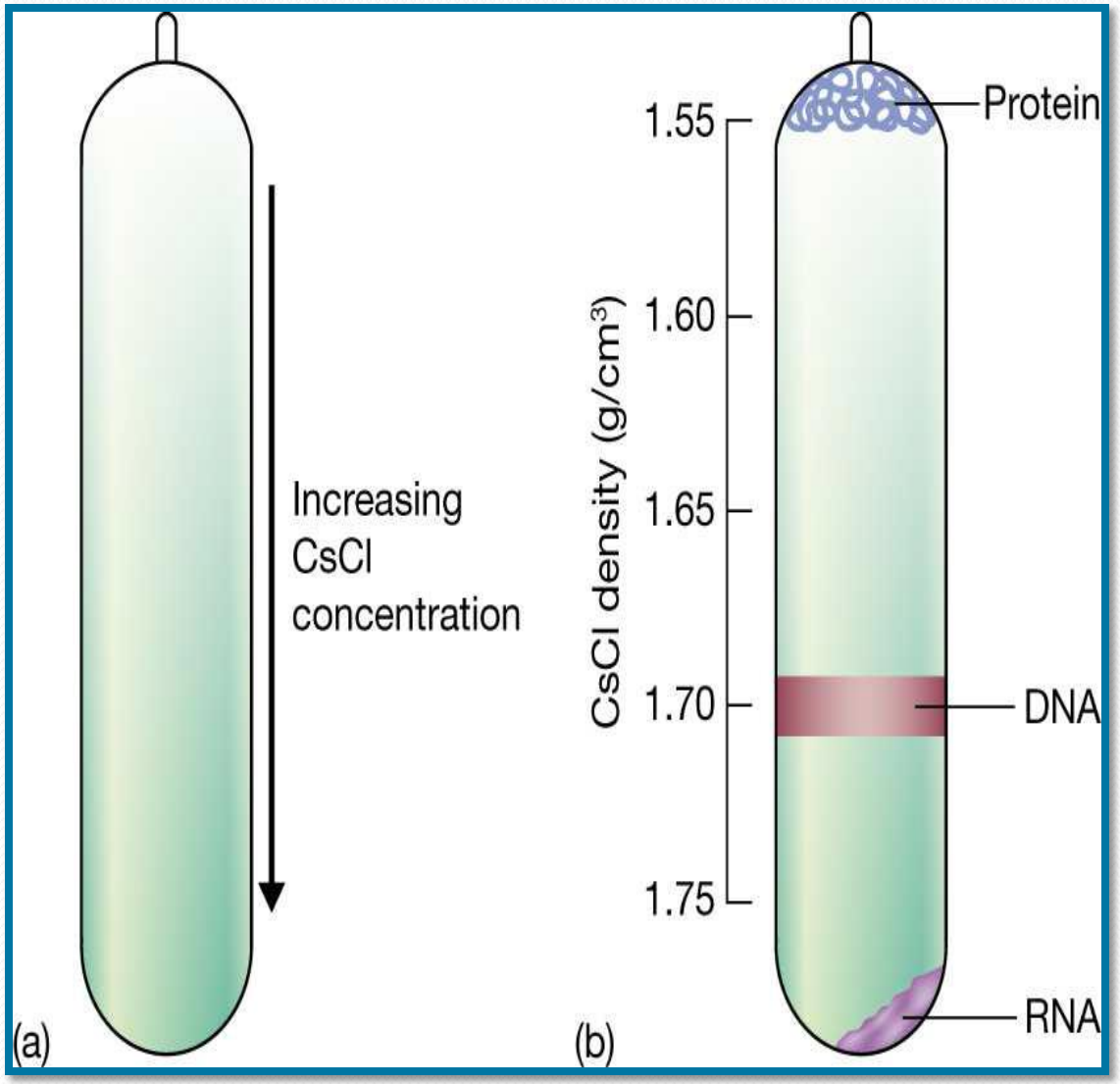
with the protein floating at the top of the gradient

and RNA pelleted at the bottom.

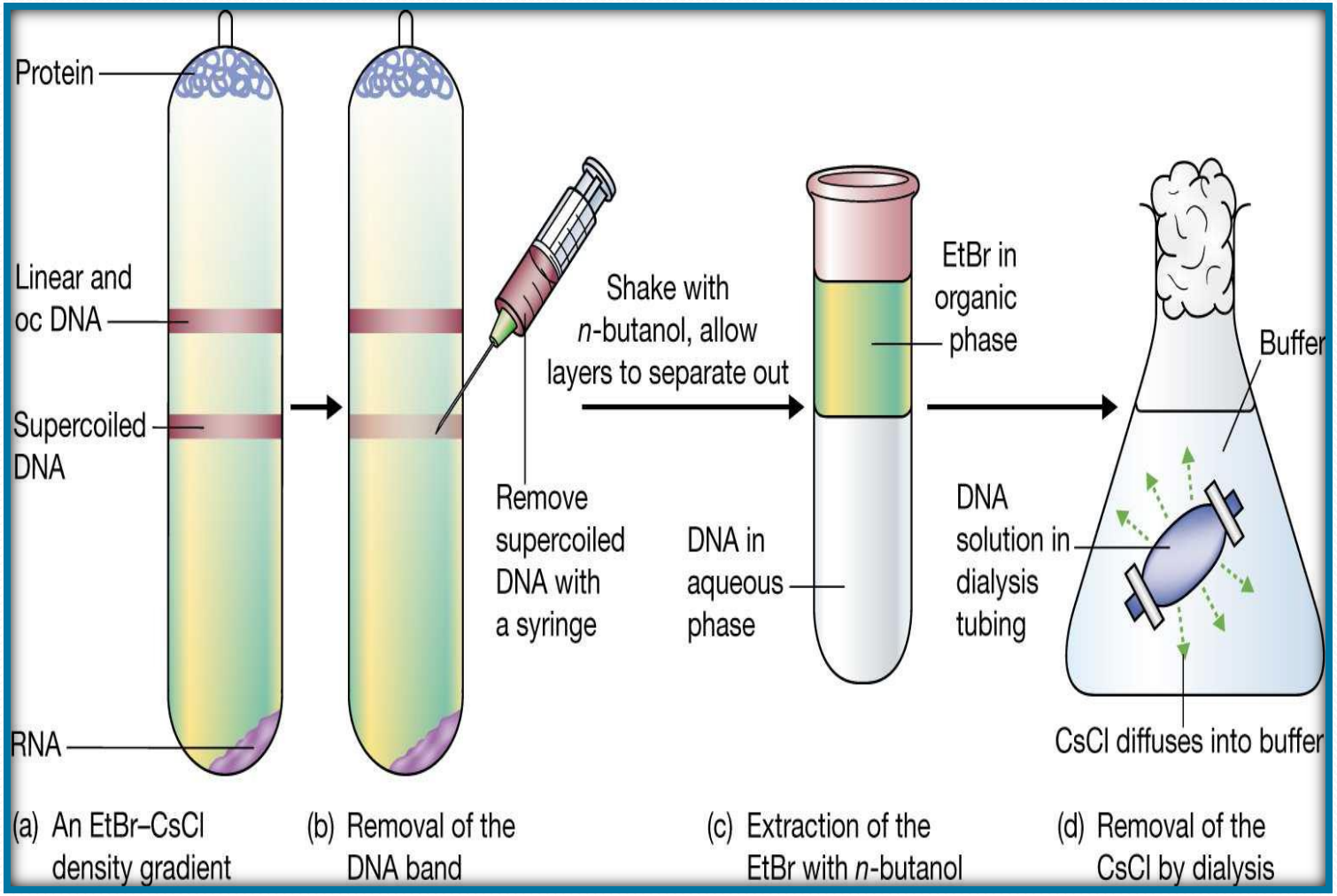
- The position of the **DNA bands** can be seen by shining ultraviolet radiation on the tube, which causes the bound EtBr to fluoresce.
- The pure plasmid DNA is removed by puncturing the side of the tube and withdrawing a sample with a syringe.

- The **EtBr bound** to the **plasmid DNA** is extracted with **n-butanol** .
- the **CsCl** removed by **dialysis** .
- The resulting plasmid preparation is **pure** and can be used in cloning.

CsCl density gradient centrifugation



Purification of plasmid DNA with EtBr CsCl density gradient centrifugation



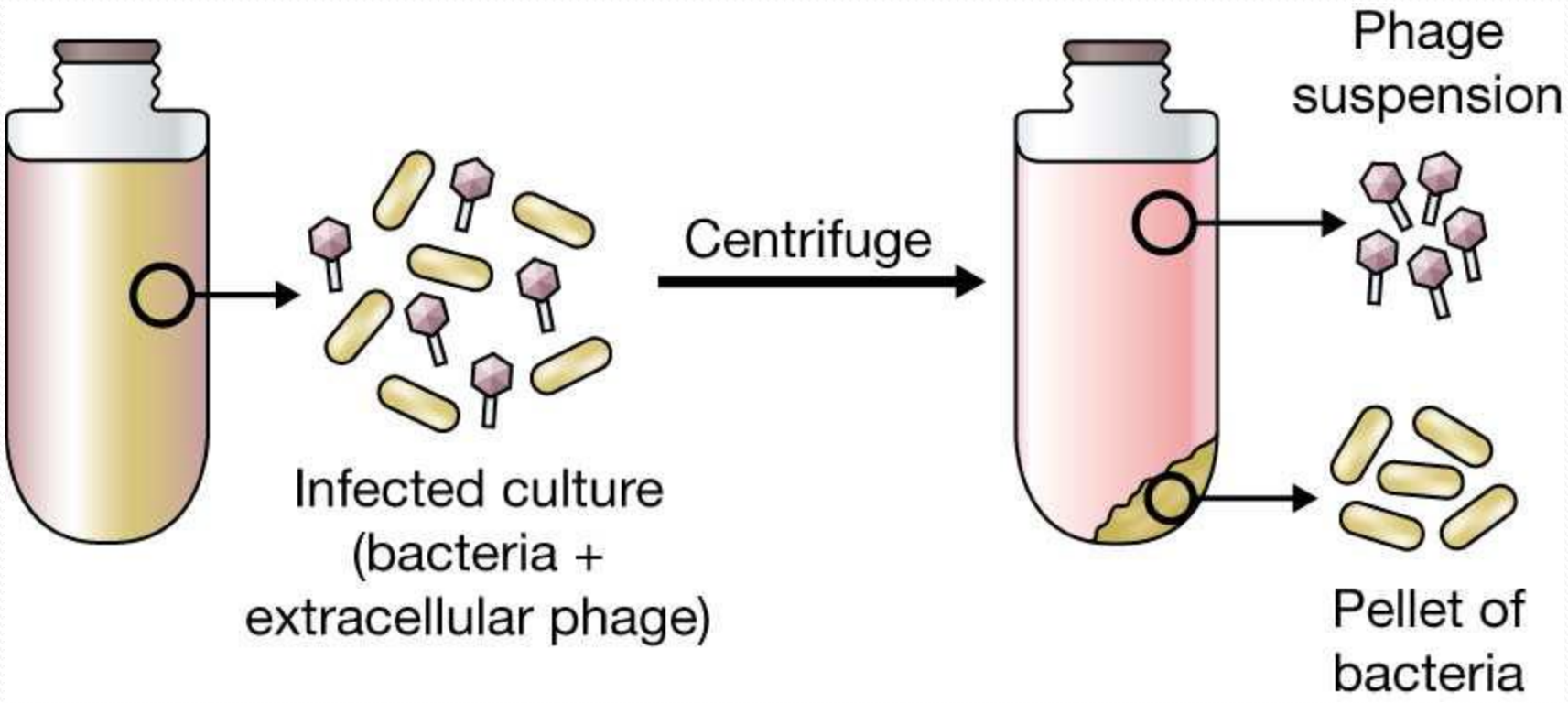
Bacteriophage DNA preparation

- Bacteriophages are viruses that specifically infect bacteria.
- Phages are very simple in structure, consisting merely of a DNA (or RNA) molecule carrying a number of genes, including several for replication of the phage, surrounded by a protective coat or **capsid made up of protein molecules.**

In bacteriophage DNA preparation

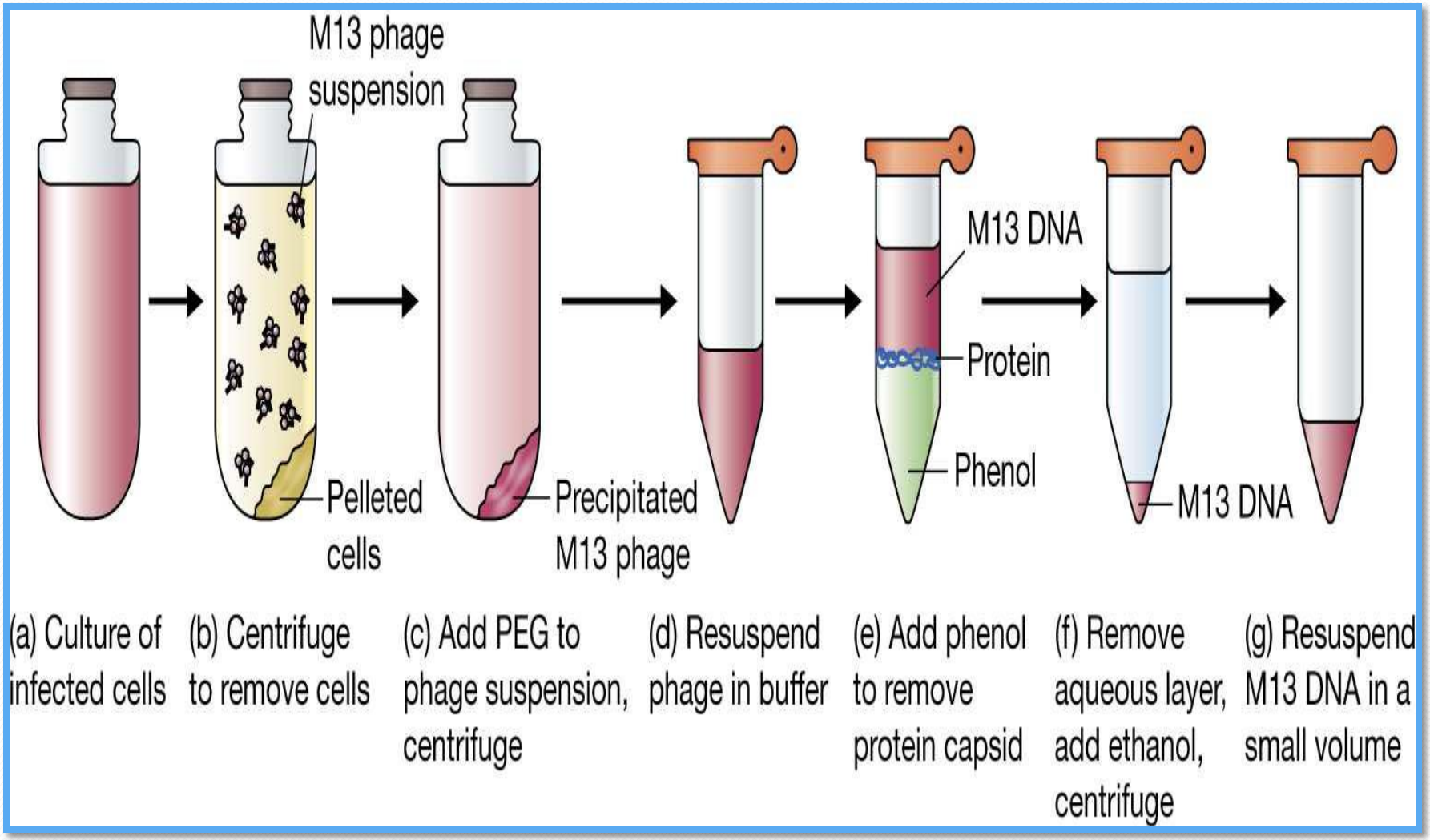
- a cell extract is not the starting material, because bacteriophage particles can be obtained in large numbers from the **extracellular medium** of an **infected bacterial culture**.
- When such a culture is centrifuged, the bacteria are pelleted , leaving the **phage** particles in **suspension**.

- The phage particles are then collected from the suspension and their DNA extracted by a single deproteinization step to remove the phage capsid.


Phage suspension
Centrifuge
Infected culture (bacteria + extracellular phage)
Pellet of bacteria

- Exception:
- M13 is a virus that infects the bacterium Eschreichia coli bacteria .It is composed of a circular single-stranded DNA molecule .
- **M13 filamentous phage** have a single strand genome that exists temporarily inside infected E.coli cells as a double strand plasmid.
- *E. coli* infected with M13 grow more slowly

Preparation of ss-DNA from M13




Good
Luck!