Plasmids & Transposable Elements

Lecture 3

BY

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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.

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- 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**.

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

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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

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- 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.

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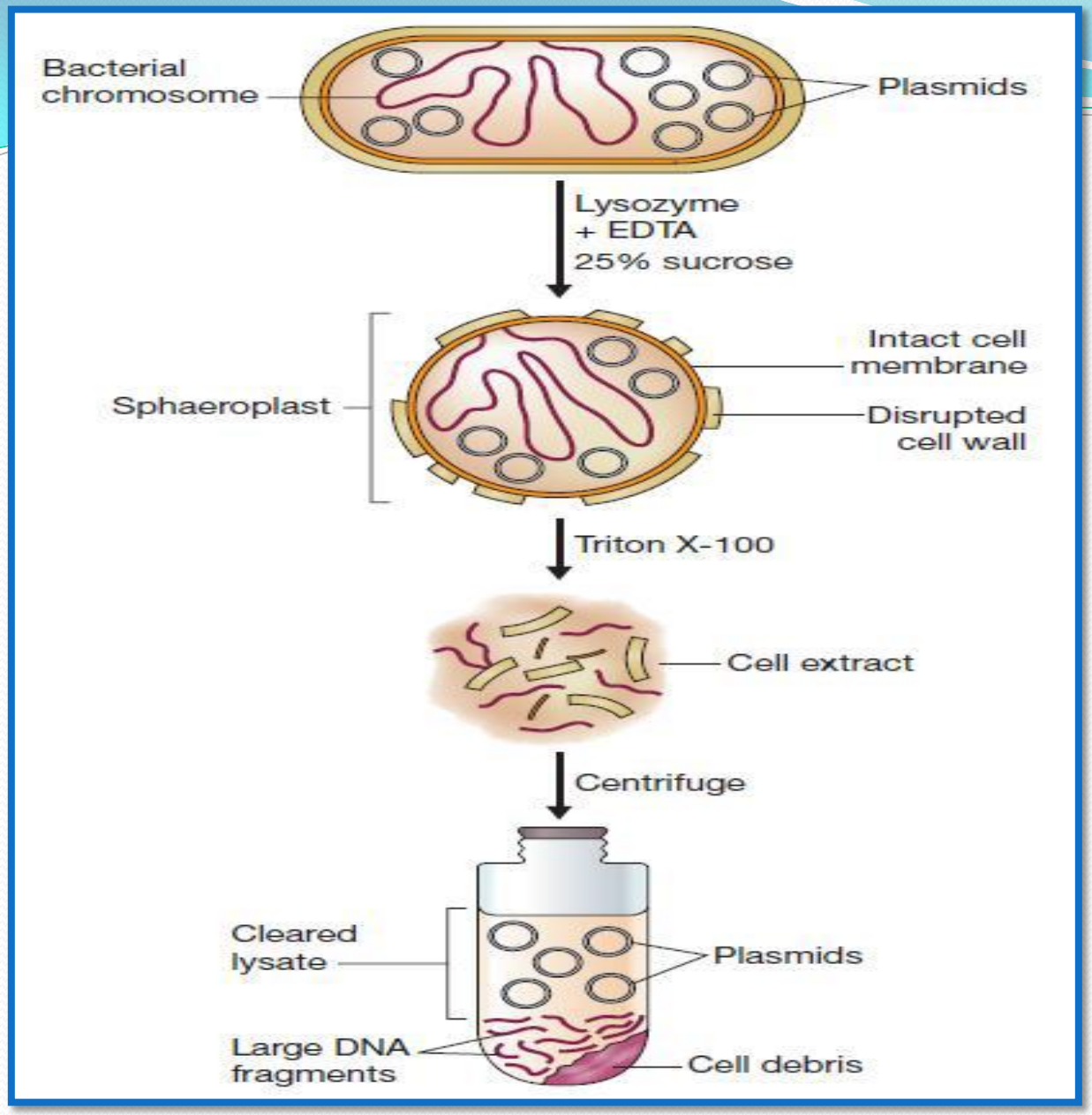

Size based separation

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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**.

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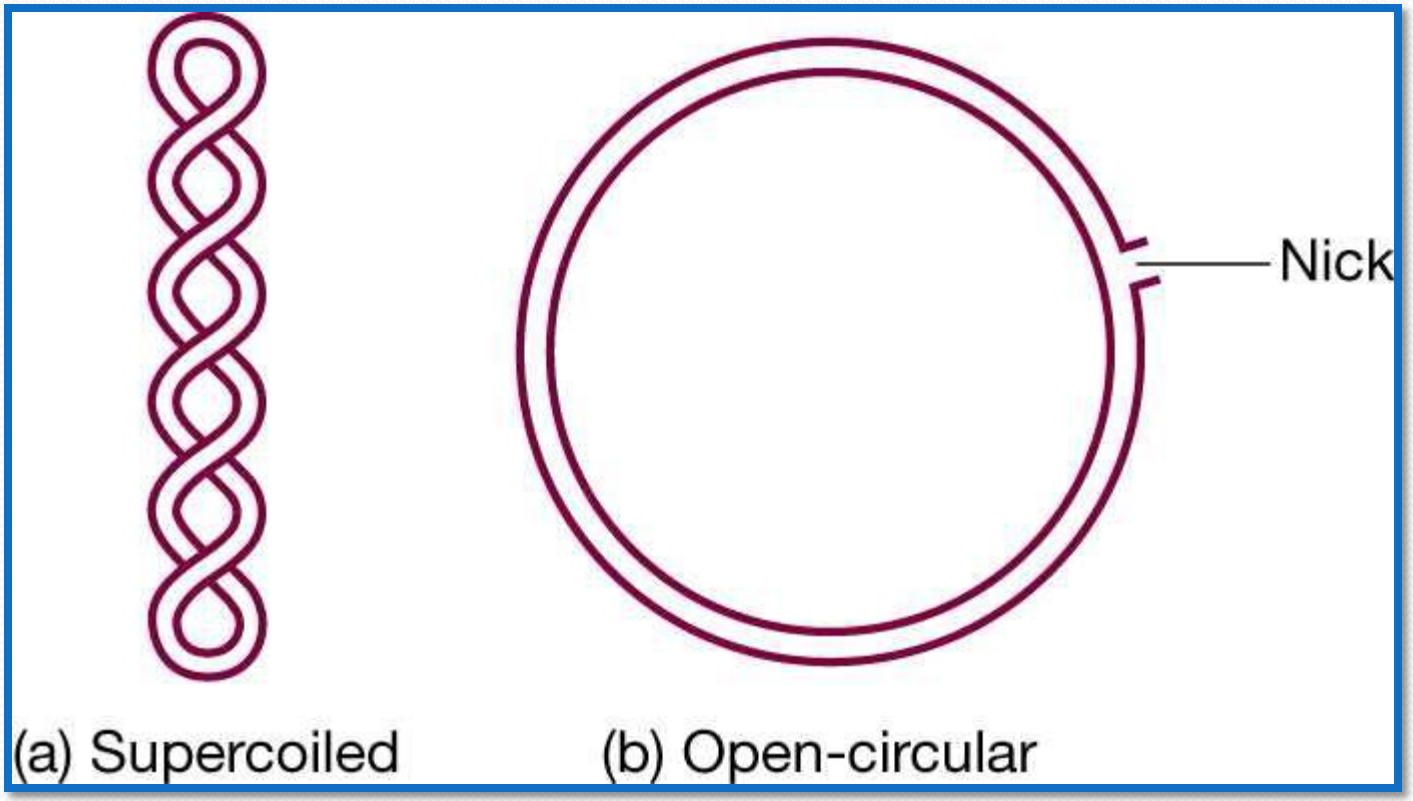
Conformations of DNA



(CCC) **(OC)**

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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 .

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- 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.

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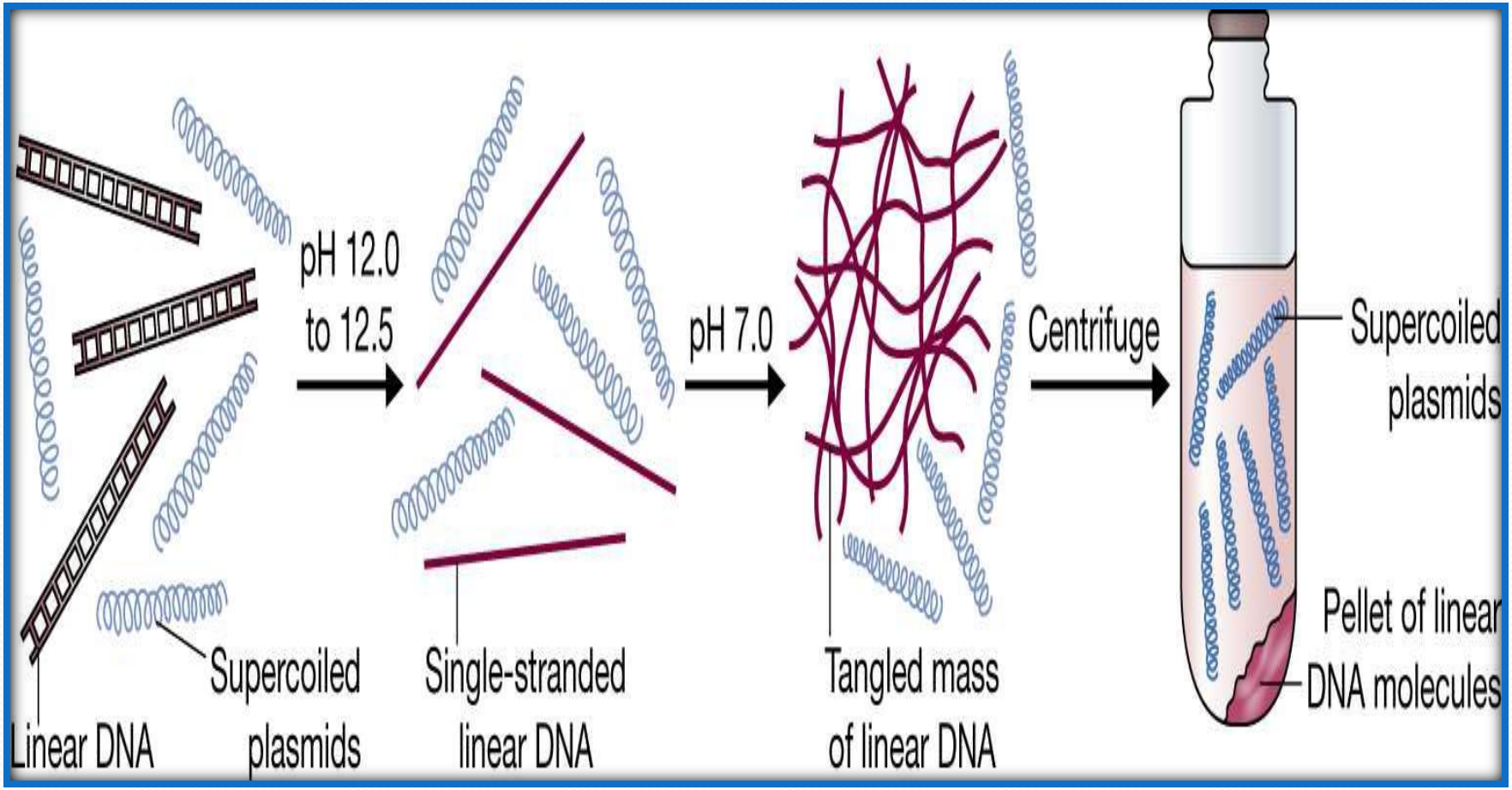

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Ethidium bromide-caesium chloride(EtBr-CsCl) density gradient centrifugation

- 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.

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- 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**).

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- 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.

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- 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 .

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- 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.

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- 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.

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- 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.

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- 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.

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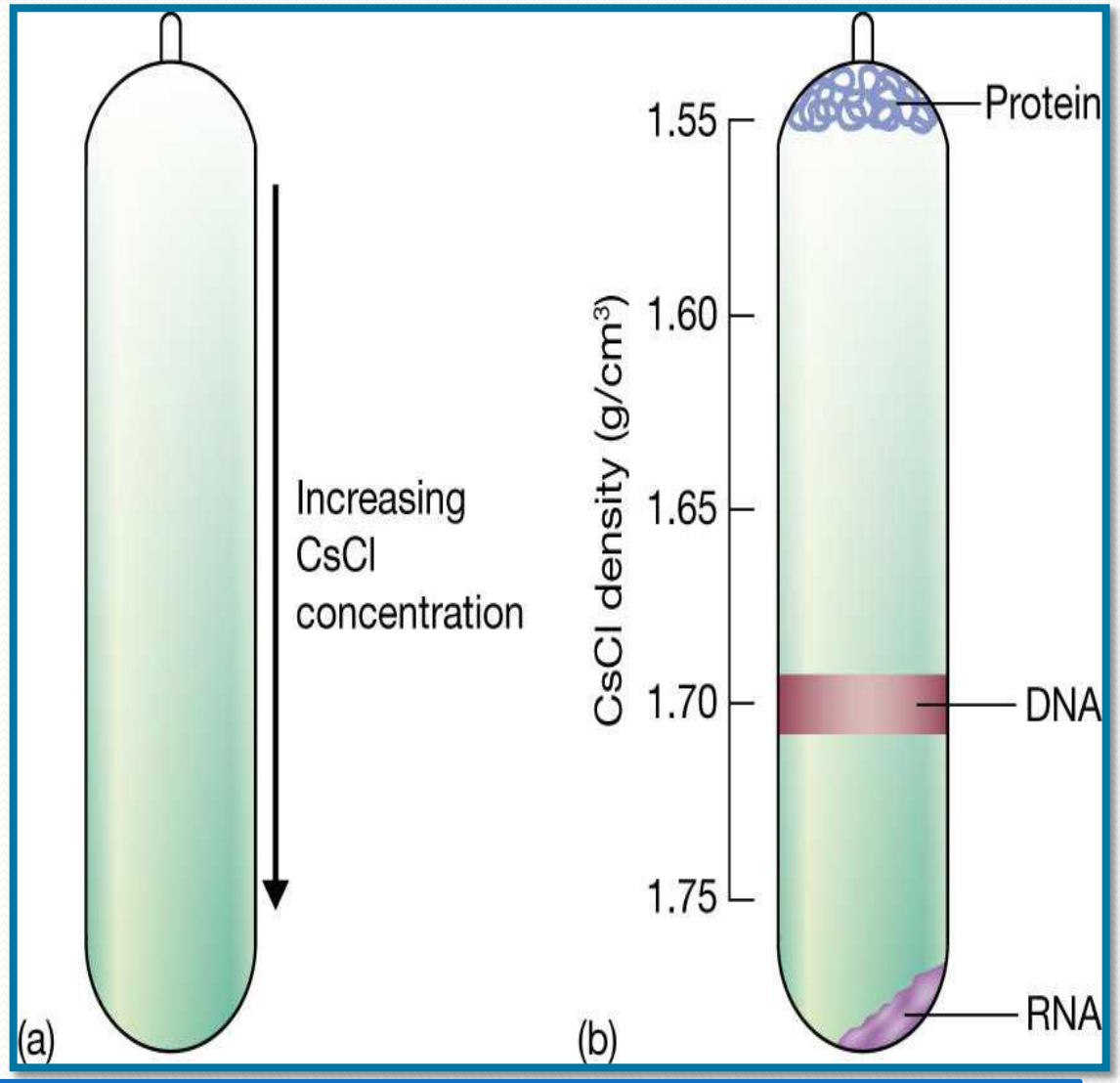
CsCl density gradient centrifugation



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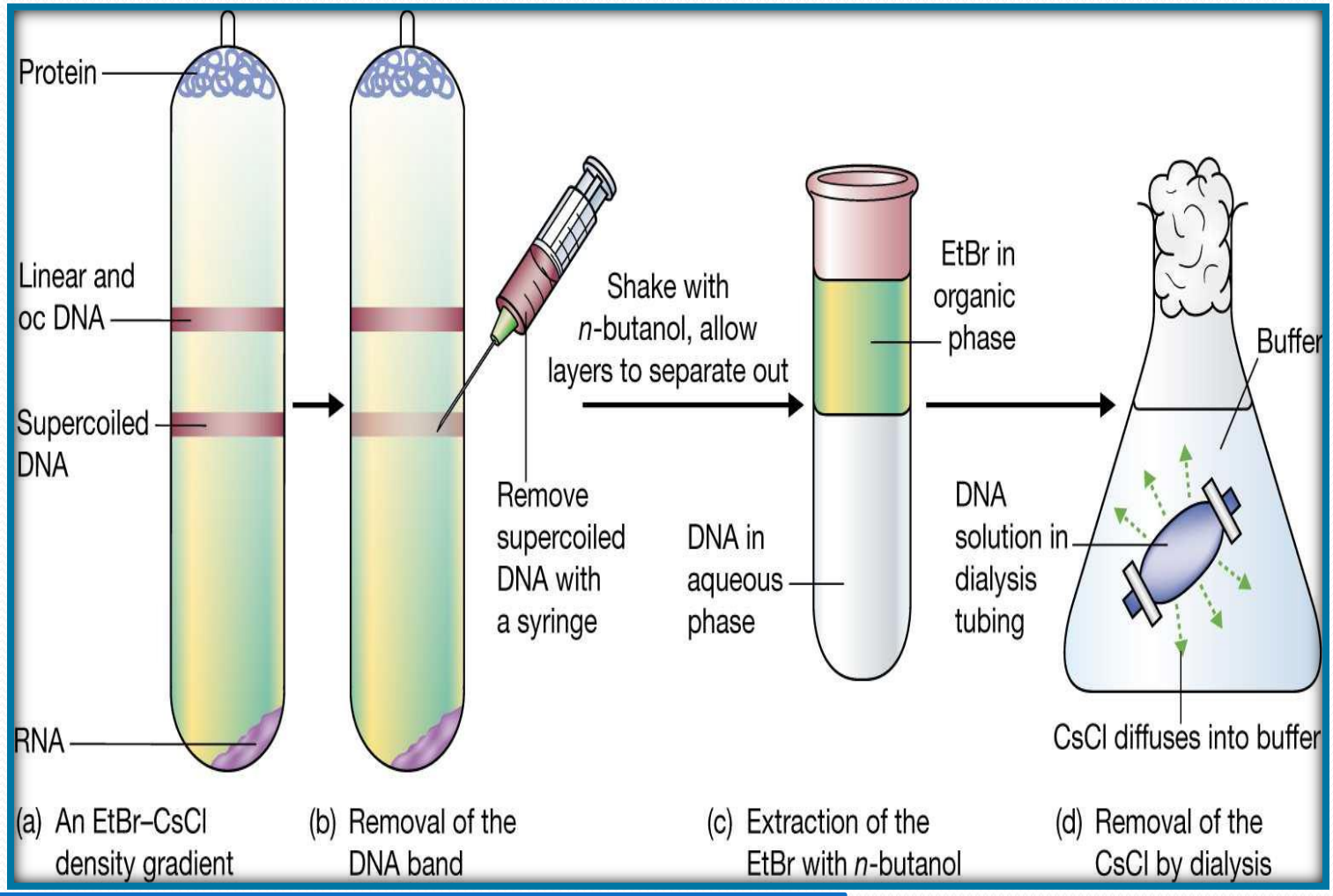
Purification of plasmid DNA with EtBr CsCl density gradient centrifugation



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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.**

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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**.

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

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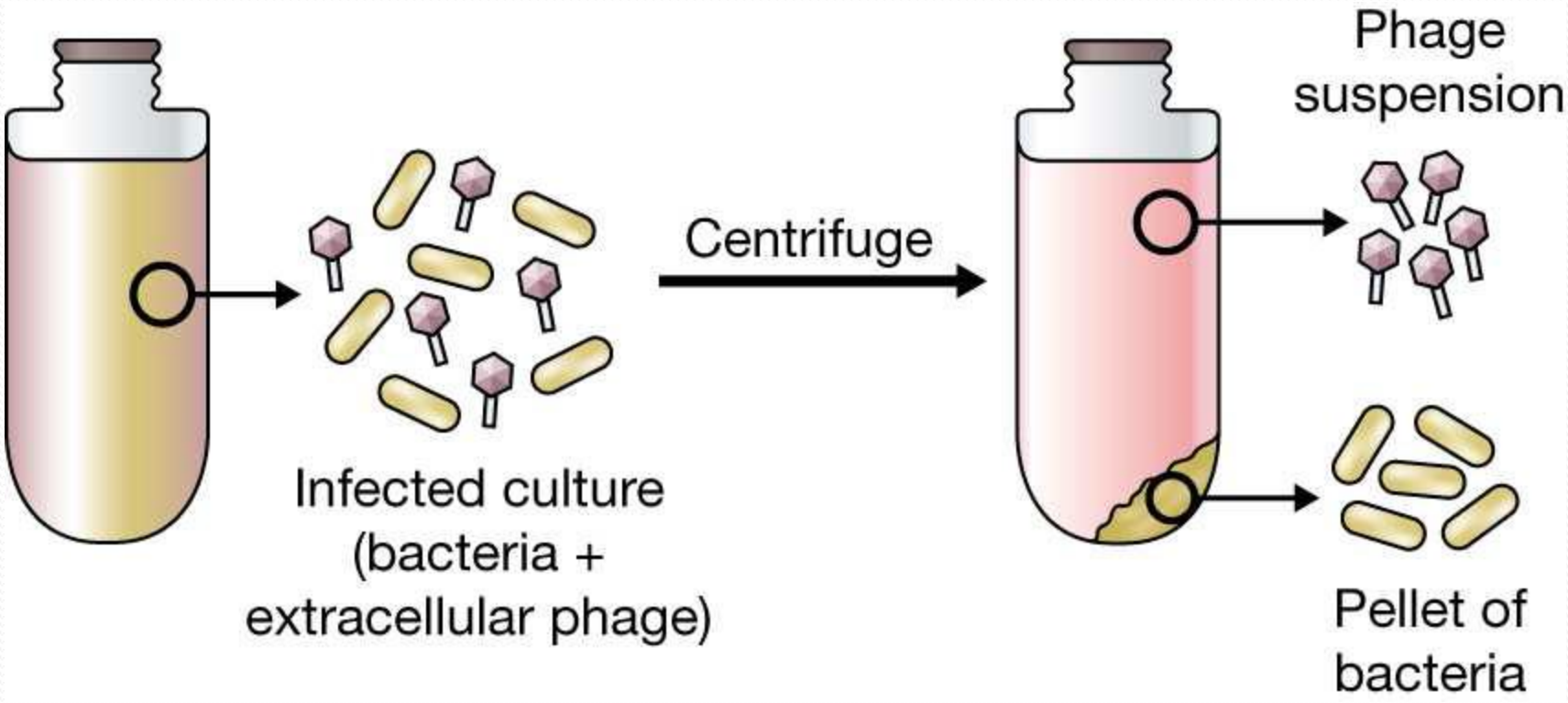

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- Exception:
- M13 is a virus that infects the bacterium Eschreichia coli bacteria .It is composed of a circular single-stranded DNA molecule .
- **M13 filamentous phage** have a single strand genome that exists temporarily inside infected E.coli cells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

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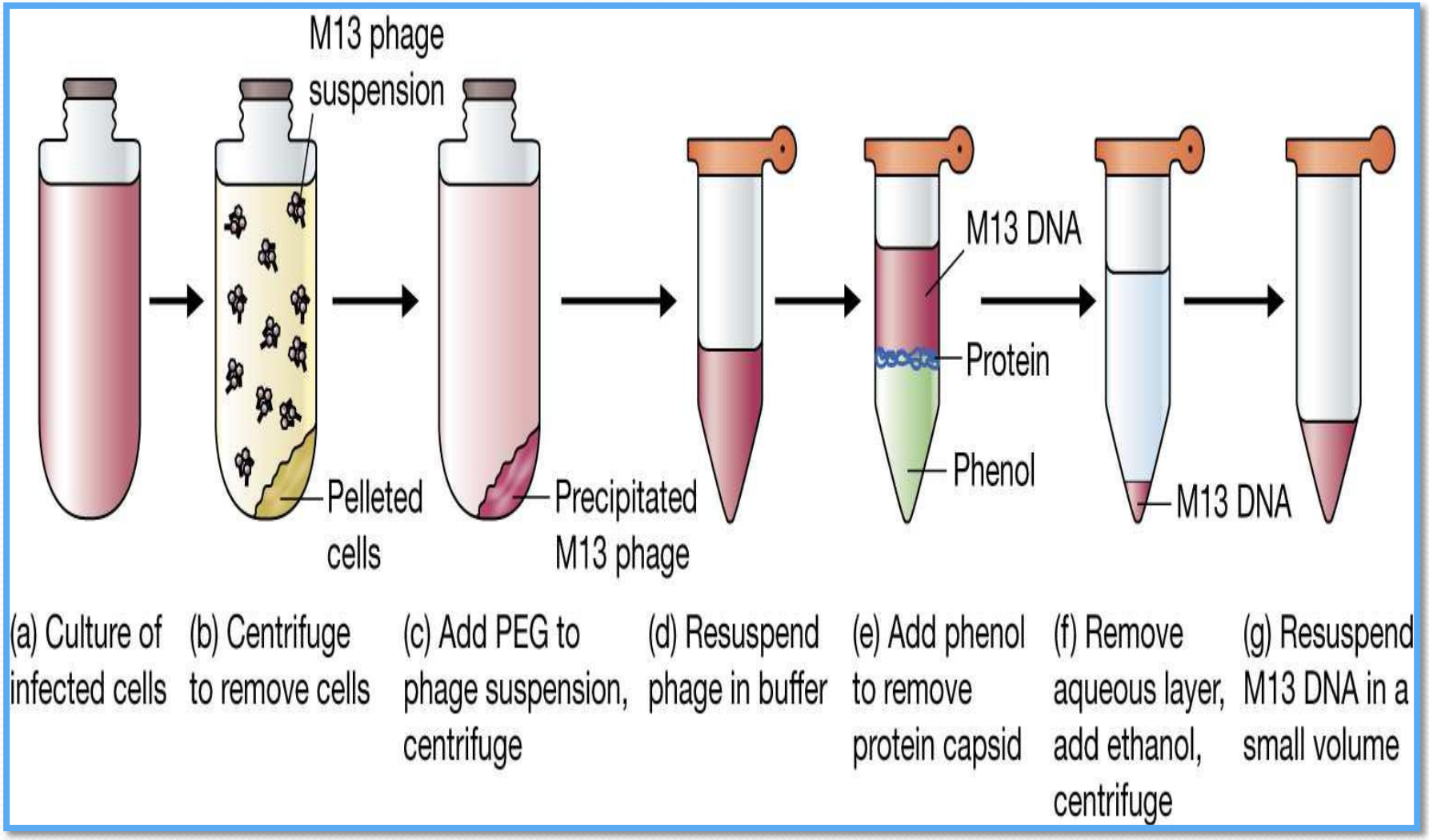
Preparation of ss-DNA from M13



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Good
Luck!