MB 210 : MODULE 2 - B

- **Extraction of Nucleic Acids**
  (Genomic DNA, mRNA and Plasmid DNA)

Isolation of Nucleic Acids

Goals:
- removal of proteins
- DNA vs RNA
- isolate specific type of nucleic acid

Types of Methods:
- differential solubility
- 'adsorption' methods
- density gradient centrifugation

Types :
- genomic (chromosomal)
- organellar (satellite)
- plasmid (extra-chromosomal)
- phage/viral (ds or ss)
- complementary (mRNA)

Extraction of total genomic DNA

- Plant, animal or bacteria cells, tissues (spleen, liver), whole organisms.
- Pretreatment depends on the nature of cell wall present.
- No pretreatment for mammalian cells & *E. coli*
  - simple nuclei lysis.
- Gram-positive bacteria cells have thick cell wall need pretreatment - digestion of cell wall with lysozymes.
- Genomic DNA purification kit are available to isolate genomic DNA

Nucleic Acid Extraction Requirements

Disruption of cell wall and membranes to liberate cellular components.

Inactivation of DNA- and RNA-degrading enzymes (DNases, RNases).

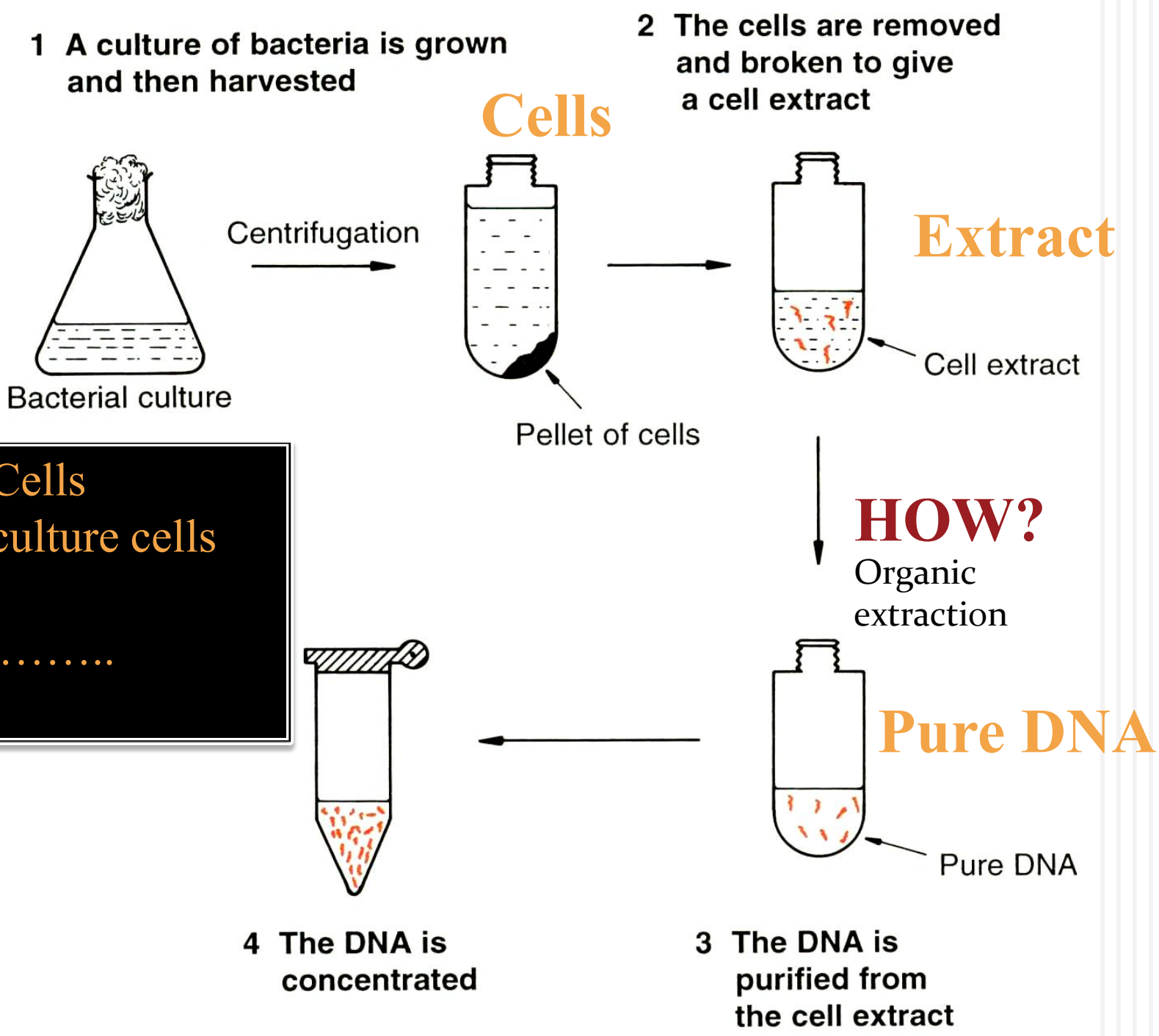
Separation of nucleic acids from other cellular components.

- Extraction/Precipitation method
- Adsorption Chromatography method

Getting Prepared: Creating a Nuclease-Free Environment

There are several things you can do to minimize the risk of exposing your samples to external DNases and RNases.

- **Autoclave solutions.** This is usually sufficient for getting rid of DNases, and most RNases as well.
- Treat solutions with 0.1% **DEPC**. DEPC inactivates nucleases by covalently modifying the His residues in proteins. Generally considered unnecessary for DNA extraction. Not compatible with solutions containing Tris or HEPES.
- Have a dedicated set of pipettors or use aerosol barrier tips.
- **Wear gloves (especially for RNA)**. You should be doing this anyway for safety reasons, but skin cells also produce RNases, a potent RNA-degrading enzyme.
- **Bake** glass, metal, or ceramic equipment at high temp.



Bacterial Cells
Or tissue culture cells
Or blood
Or flies…….

High MW Genomic DNA Isolation

Typical Procedure

1 Harvest cells

2 Cell Lysis

- 0.5% SDS + proteinase K (55° several hours)

3 Phenol Extraction

- gentle rocking several hours

4 Ethanol Precipitation

5 RNAse followed by proteinase K

6 Repeat Phenol Extraction and EtOH ppt

Detail of step 3
Phenol Extraction

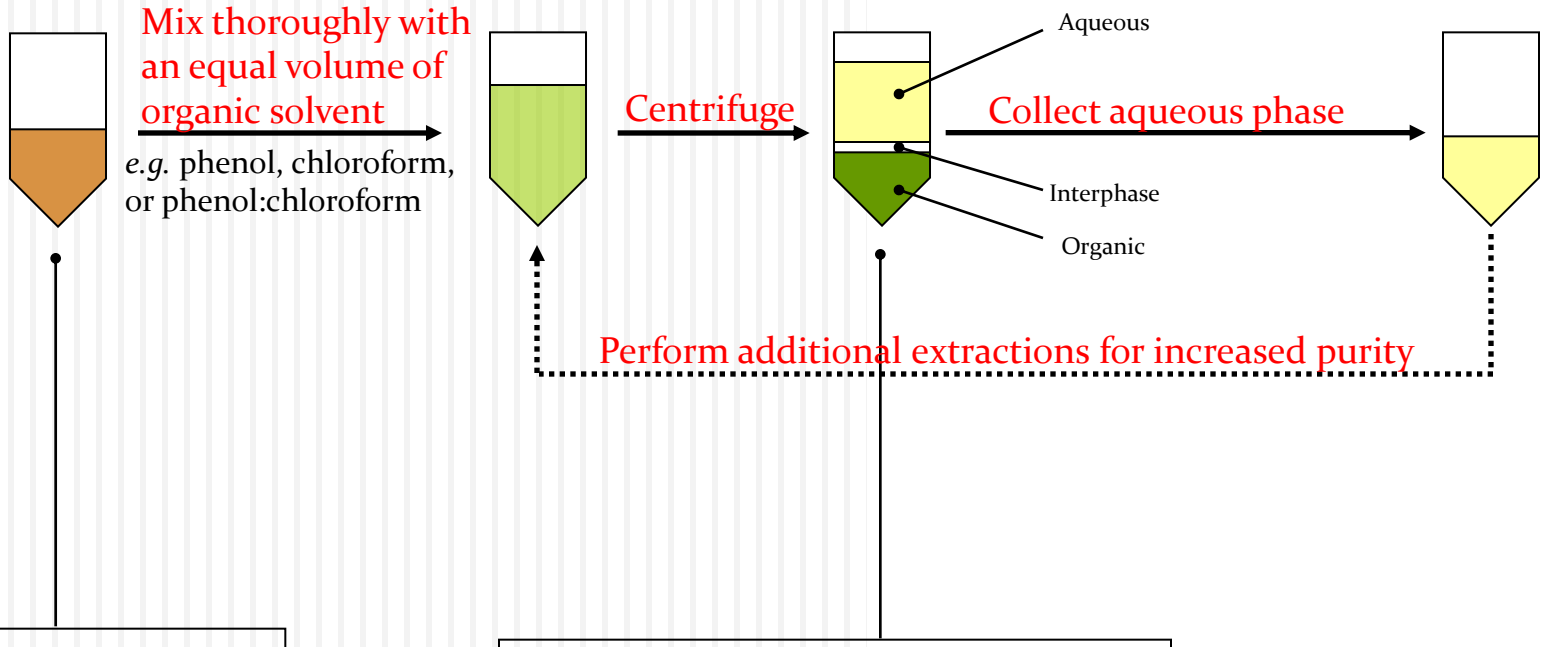
- mix sample with equal volume of sat. phenol soln
- retain aqueous phase
- optional chloroform/isoamyl alcohol extraction(s)

aqueous phase (nucleic acids)

(proteins)

Extraction/Precipitation Method

Step 3: Organic extraction



Crude lysate containing nucleic acids and other cell constituents

The **aqueous phase** contains water-soluble molecules, including nucleic acids. Proteins and lipids become trapped in the **organic phase**, and are thus separated away. Insoluble debris become trapped in the **interphase** between the two layers

High MW Genomic DNA Isolation

Typical Procedure

1 Harvest cells

2 Cell Lysis

- 0.5% SDS + proteinase K (55° several hours)

3 Phenol Extraction

- gentle rocking several hours

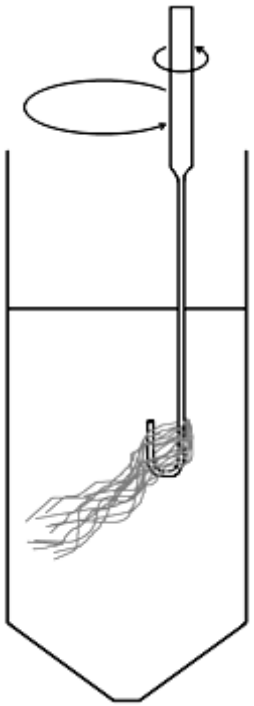
4 Ethanol Precipitation

5 RNAse followed by proteinase K

6 Repeat Phenol Extraction and EtOH ppt

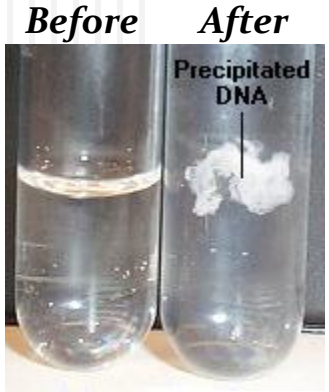
Detail of step 4 EtOH Precipitation

- 2-2.5 volumes EtOH, -20°
- high salt, pH 5-5.5
- centrifuge or 'spool' out

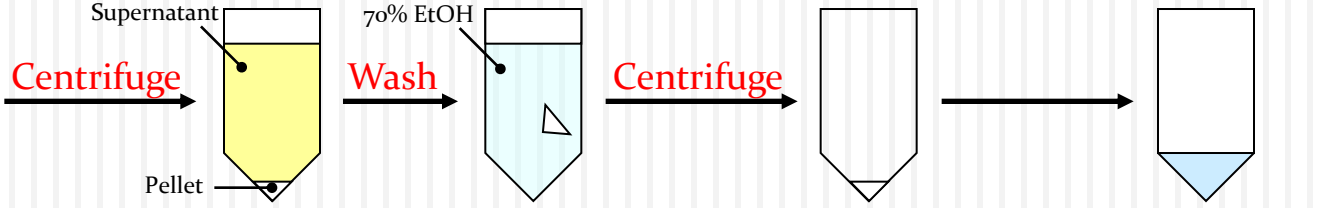


Extraction/Precipitation Method

Step 4: Nucleic Acid Precipitation

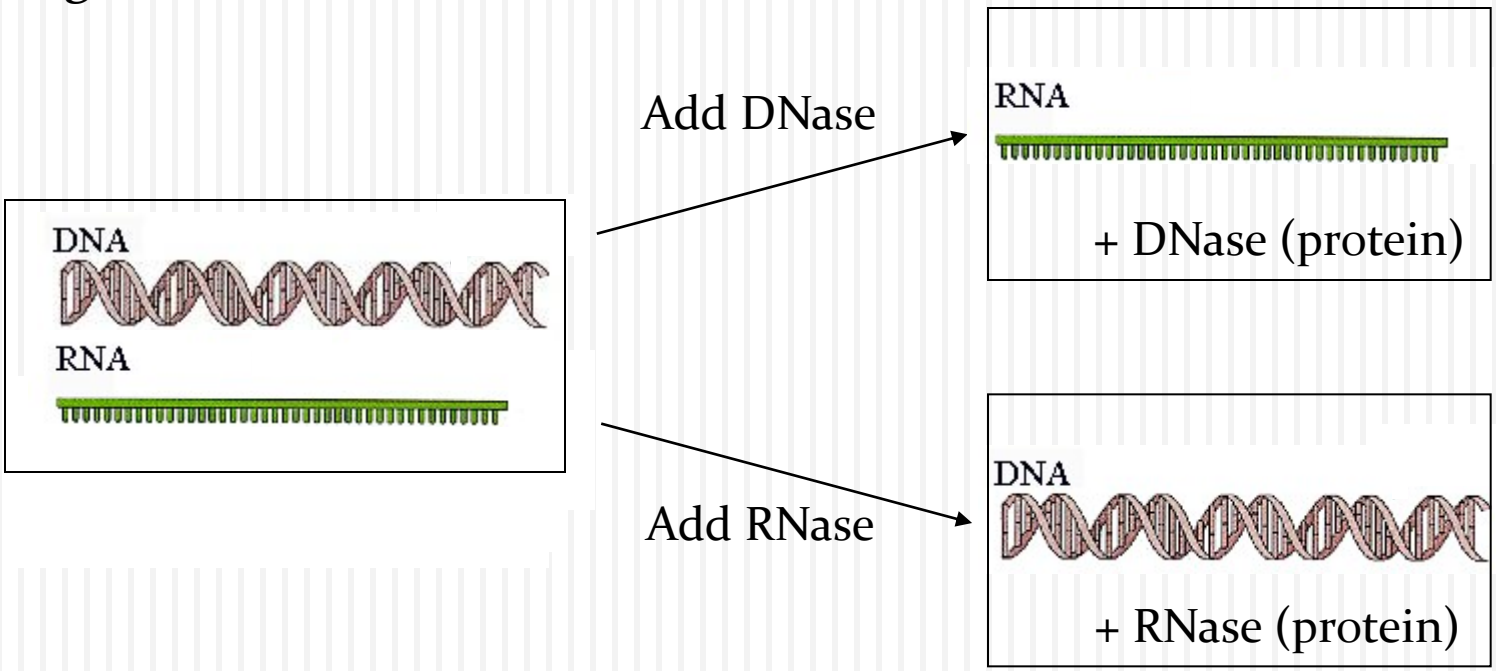


Add alcohol and salt to precipitate nucleic acids from the aqueous fraction



- Pellet down nucleic acids.
- Wash pellet with 70% ethanol to remove residual salts and other contaminants.
- Discard ethanol and allow pellet to dry.

Detail of step 5
Using Nucleases to Remove Unwanted DNA or RNA

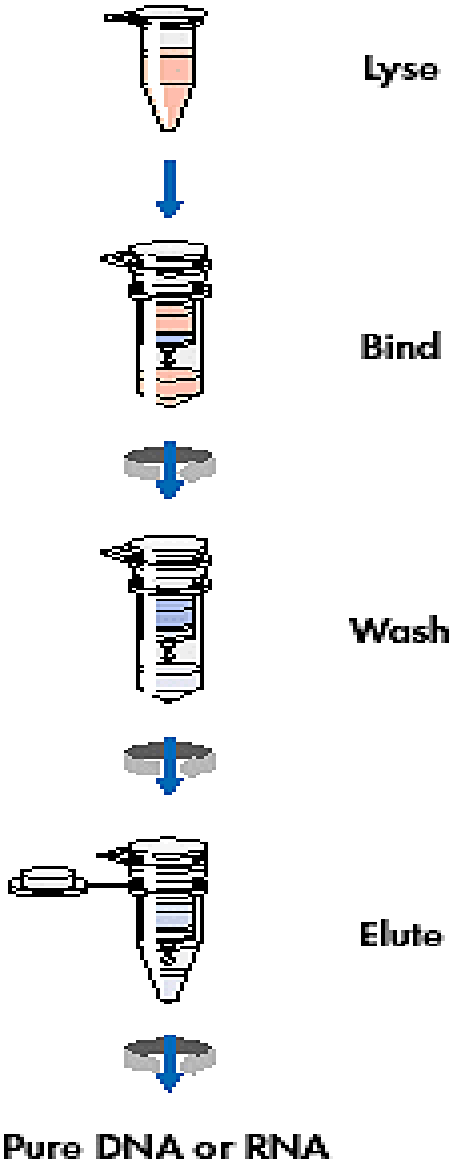


Depending on when nuclease treatment is performed, it may be necessary to repeat purification steps for protein removal (e.g. phenol/chloroform extraction).

Preparing genomic DNA from whole organisms (eg. Drosophila) / tissues

1. Homogenization: whole organisms / tissue - snap frozen in liquid Nitrogen using a mortar and pestle.
2. Cell and Nuclei Lysis: resuspend homogenized material in nuclei lysis **Buffer** (Tris HCL, EDTA, 1% SDS).
3. Digestion with Proteinase K: to degrade proteinaceous components (100 mg/ml for 1-2h @ 37oC with occassional swirling).
4. Phenol-Chloroform extraction: to remove degraded proteins (forms a while interphase between top aqueous layer (contain DNA) and the bottom organic layer (phenol/chloroform).
5. Precipitate the DNA: from the aqueous phase by adding half vol 7.5M NH4OAc and equal vol of isopropanol; mix gently, leave o/n @ -20^{o}C
6. Pellet DNA: by centrifuging and rinse DNA pellet in 70% EtOH and air dry briefly
7. Resuspend genomic DNA in TE buffer.
8. RNase A treatment: @ 100mg/ml; 37^{o}C, 30 min
9. Repeat phenol-chloroform extraction to remove residue RNase and NaOAc/EtOH ppt.
10. Store DNA in Tris-EDTA buffer (pH 8.0), at 4^{o}C (short term) or 20^{o}C (long term)

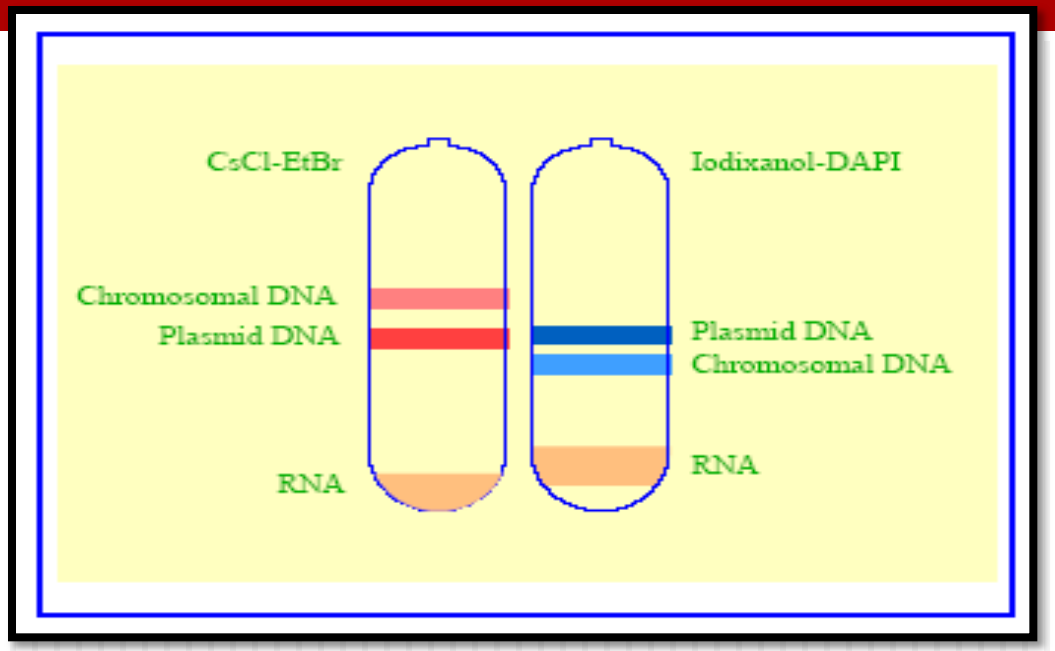
Preparing genomic DNA from cells or tissues using commercially available kits (eg. Qiagen / Promega)



1. Harvest cells / tissues (homogenize)
2. Add Nuclei Lysis Solution and pipette to lyse the cells - no visible cell clumps remain
3. Treat nuclear lysate with RNase Solution, mix the sample by inverting the tube 2.5 times (15 min at 37°C)
4. Add Protein Precipitation Solution and vortex vigorously at high speed for 20 sec.
6. Centrifuge to pellet the precipitated protein (white pellet)
7. Carefully separate the supernatant containing the DNA
8. Isopropanol precipitation of DNA can be replaced by placing the supernatant onto a spin column, DNA captured on the positively charged silica column, washed and eluted in TE buffer/ H_2O.

Work with Plasmid DNAs

Isolation and Purification

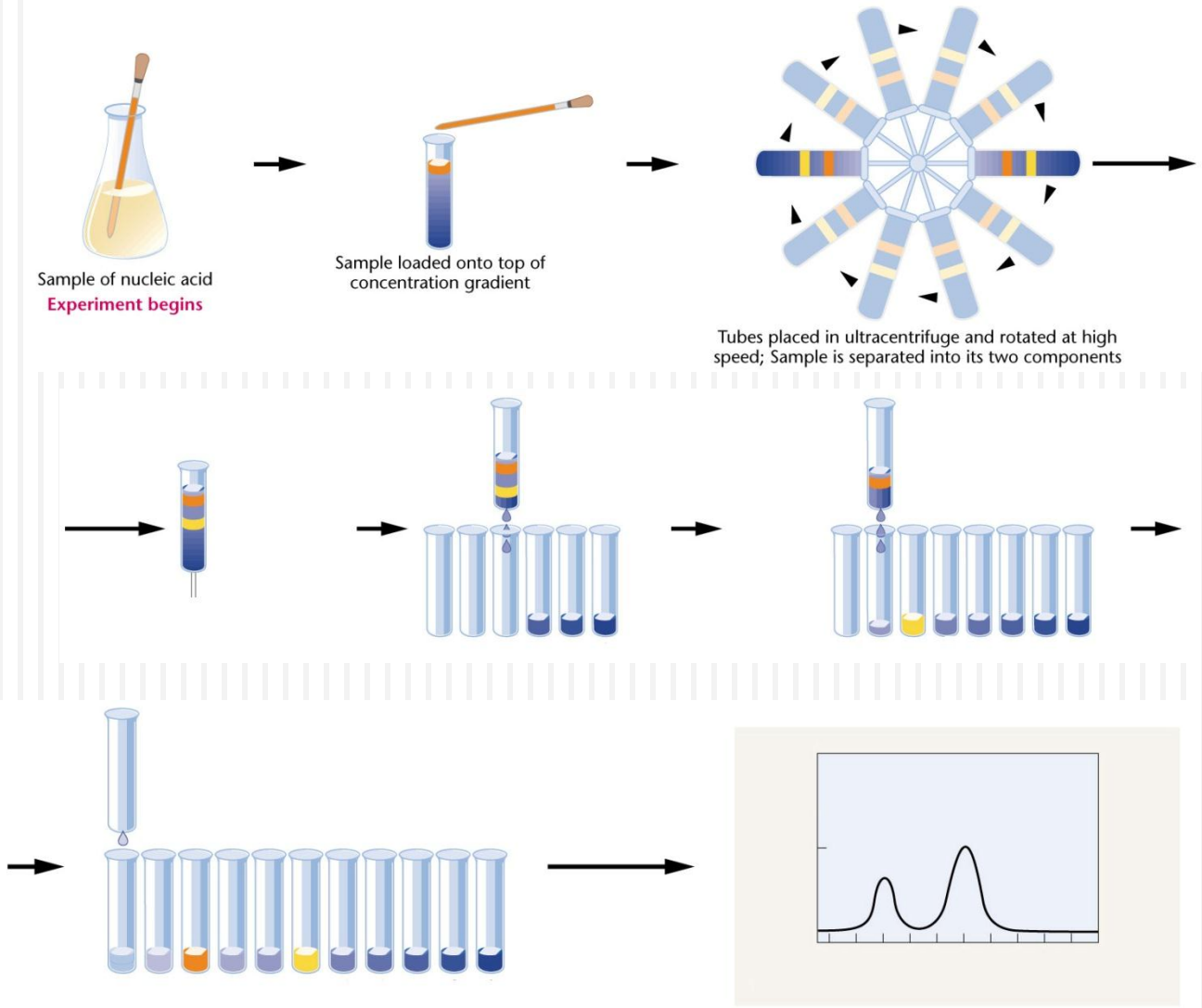


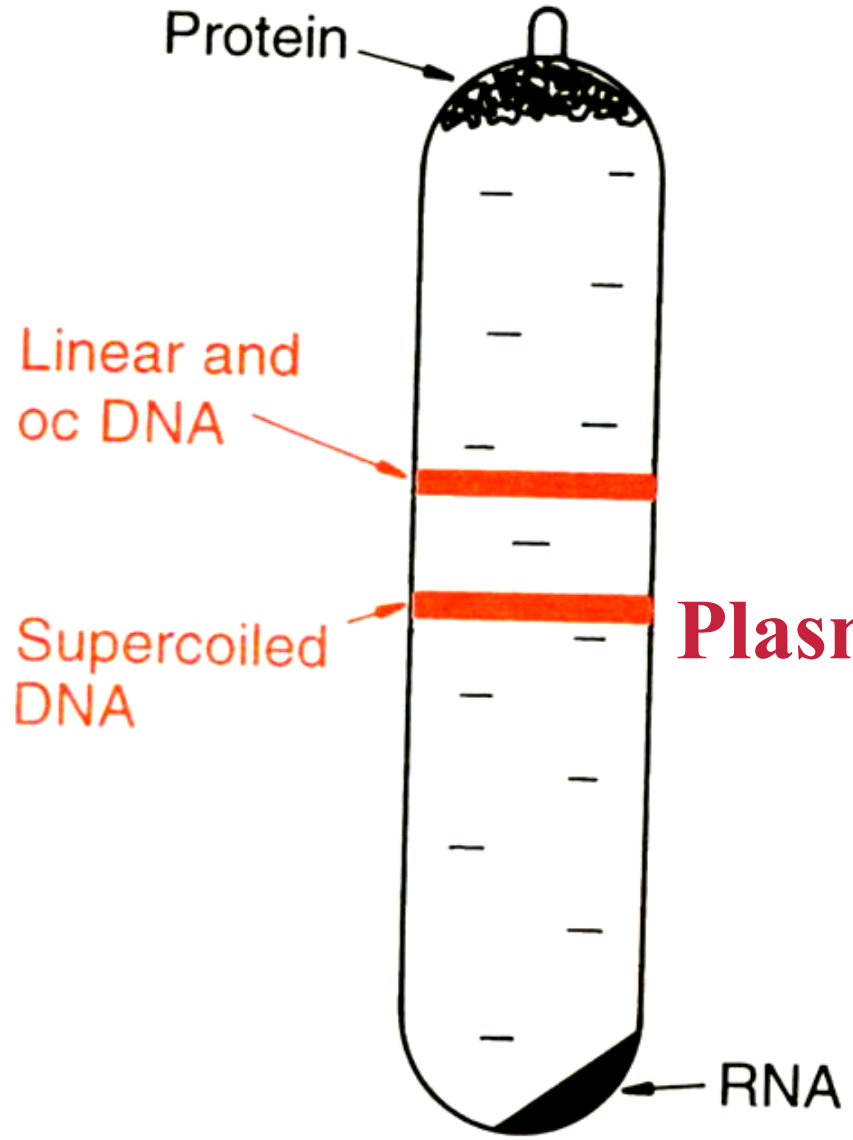
After 10 hrs centrifugation at 100,000 rpm (450,000 x*g*), two distinct bands, corresponding to **linear nuclear DNA** above and **circular mitochondrial DNA** below, are visible under ultraviolet light.

Banding of plasmids and chromosomal DNAs in CsCl-EtBr and in iodixanol-DAPI gradients.

CsCl Gradient centrifugation or CsCl dye-bouyant density method

Separation of Nucleic Acids by CeCl Gradient Centrifugation

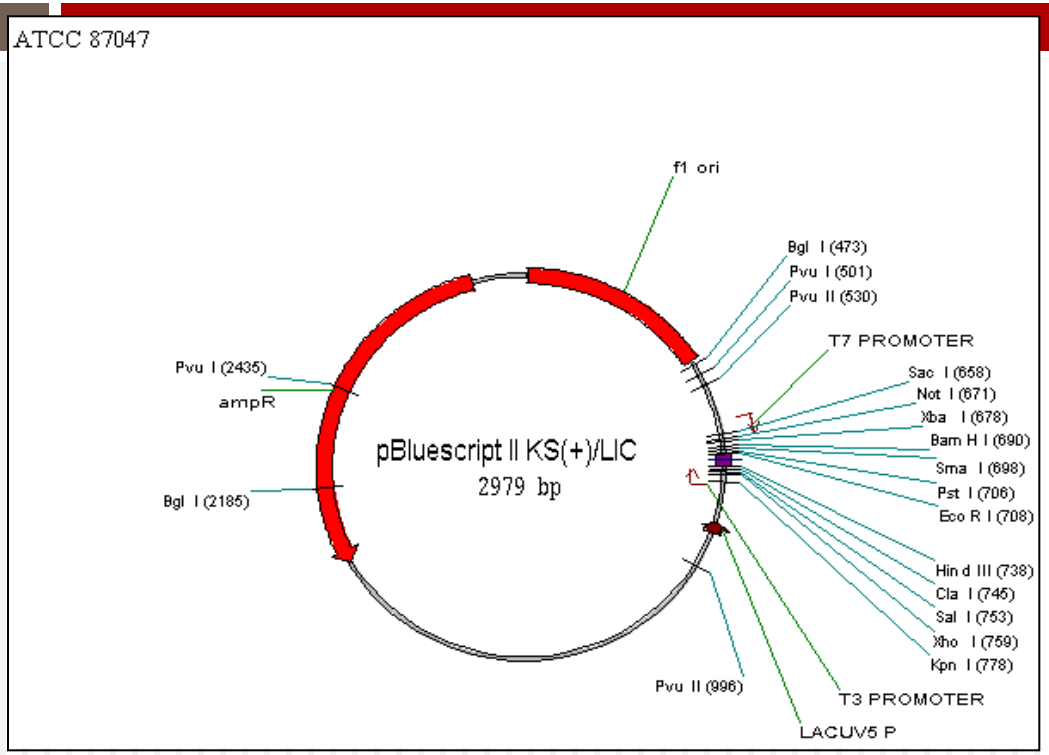


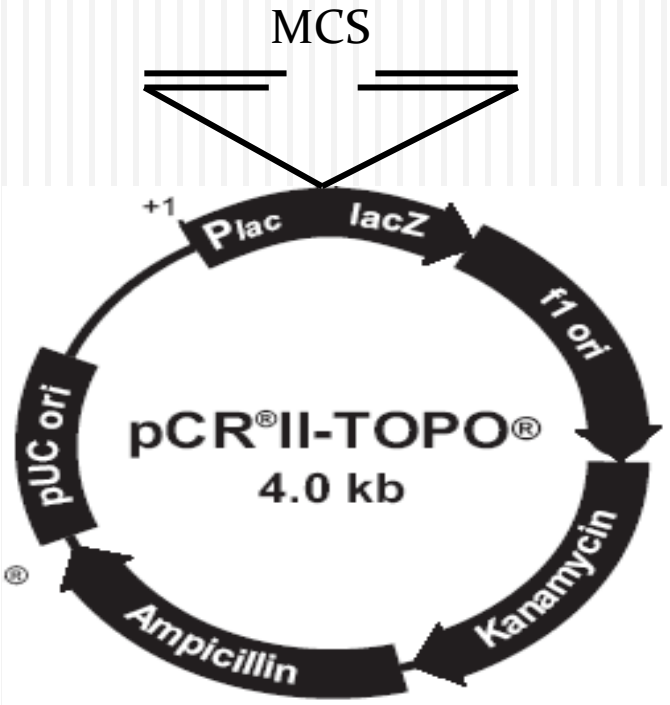


(a) An EtBr-CsCl density gradient

Plasmid DNA

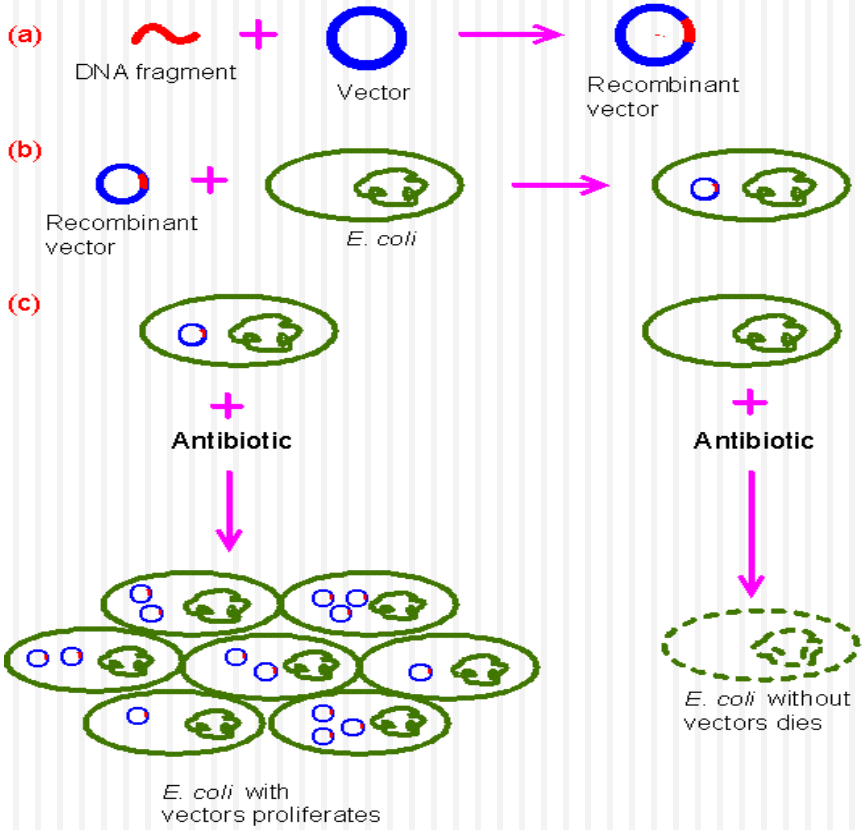
Plasmid Vectors





Application of Plasmid Vectors

In Molecular Cloning



(d) Isolation of recombinant DNA clones

How it works?

(a) Initially, the gene to be replicated is inserted in a plasmid or vector.

(b) The plasmids are next inserted into bacteria by a process called *transformation*.

(c) Bacteria are then grown on specific antibiotic(s).

(d) As a result, only the bacteria with antibiotic resistance can survive and will be replicated.

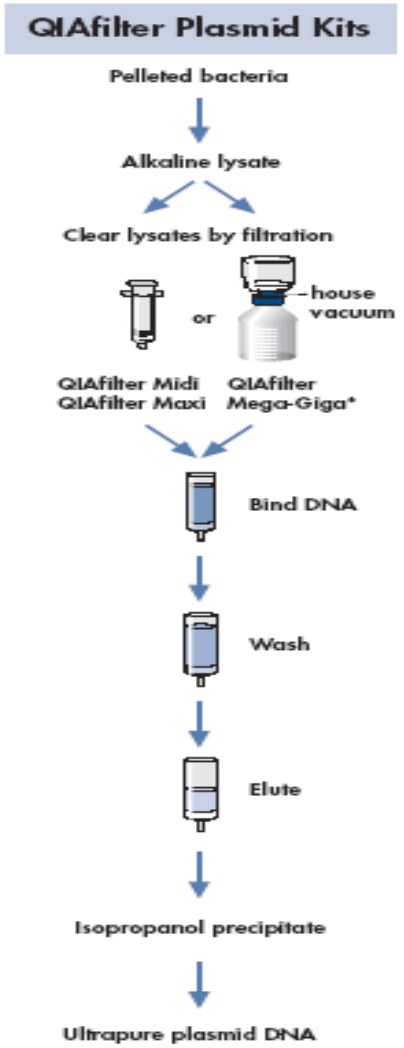
Plasmid DNA Isolation continued

Tranditional Ways

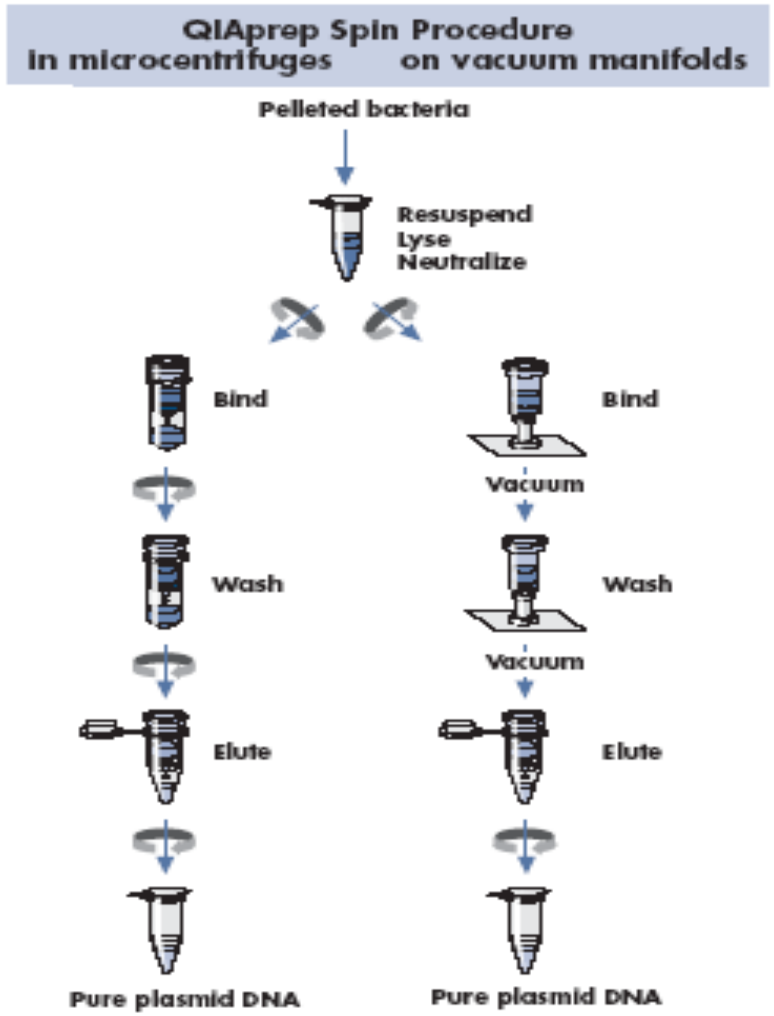
(d) collecting plasmid DNA by centrifugation (after ethanol precipitation or through filters - positively charged silicon beads),

(e) check plasmid DNA yield and quality (using spectrophotometer and gel electrophoresis).

Midi Prep



Mini Prep

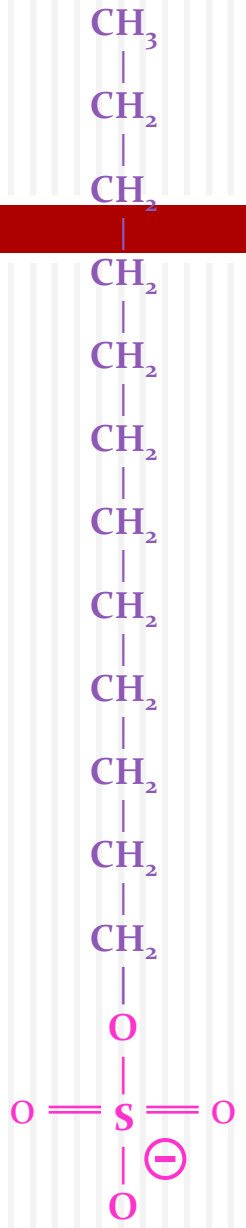


spectrophotometer and gel electrophoresis

Isolation of plasmid DNA

- About 10 years ago, this method was modified so that very pure plasmid DNA can be purified using a column specially packed with glass fiber.

- The plasmid DNA purified through those columns can be used directly for PCR, cloning, sequencing, *in vitro* transcription, synthesize of labelled hybridization probes, microinjection, electroporation and transfection, etc.



Sodium dodecyl sulfate (SDS)

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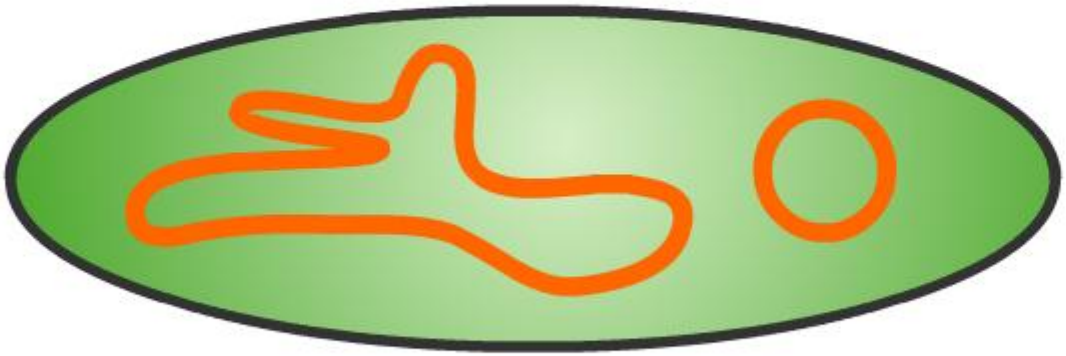
- SDS is a strong ionic detergent with a **negative charge.**
- At high pH, SDS can open the bacterial cell wall, denature chromosomal DNA and proteins, and release plasmid DNA into the supernatant.



Click on animation to start playing

Absorption of UV light by different molecules

Molecules	Absorption
DNA	260 nm
RNA or single-stranded oligo	260 nm
Organic compounds: phenolate ion and thiocyanates.	230 nm
Protein containing aromatic amino acids such as tyrosine, tryptophan and phenylalanine	280 nm
Non-specific absorption produced by light scattering	320 nm

How pure is your sample?

The A_{260}/A_{280} ratio is ~1.8 for dsDNA, and ~2.0 for ssRNA. Ratios lower than 1.7 usually indicate significant protein contamination.

The A_{260}/A_{230} ratio of DNA and RNA should be roughly equal to its A_{260}/A_{280} ratio (and therefore ≥ 1.8). Lower ratios may indicate contamination by organic compounds (e.g. phenol, alcohol, or carbohydrates).

Turbidity can lead to erroneous readings due to light interference. Nucleic acids do not absorb light at the 320 nm wavelength. Thus, one can correct for the effects of turbidity by subtracting the A_{320} from readings at A_{230}, A_{260} and A_{280}.

DNA Quantification

- The absorption spectra of DNA and RNA are maximal at 260 nm.
- It is often a good practice to measure the absorption of nuclei acid solution at multiple wavelengths.
- Most spectrophotometers used in research laboratories at the HKUST are equipped to measure absorbance at various wavelengths simultaneously.

DNA Electrophoresis

The process using electro-field to separate macromolecules in a gel matrix is called electrophoresis.

DNA, RNA and proteins carry negative charges, and migrate into gel matrix under electro-fields.

The rate of migration for small linear fragments is directly proportional to the voltage applied at low voltages.

At low voltage, the migration rate of small linear DNA fragments is a function of their length.

At higher voltages, larger fragments (over 20kb) migrate at continually increasing yet different rates.

Large linear fragments migrate at a certain fixed rate regardless of length.

In all cases, molecular weight markers are very useful to monitor the DNA migration during electrophoresis.

Conformations of Plasmid DNAs

Plasmid DNA may appear in the following five conformations:

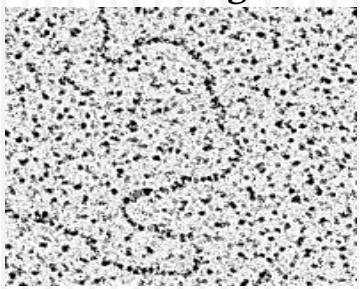
Super Coiled

1) "Supercoiled" (or "Covalently Closed-Circular") DNA is fully intact with both strands uncut.

2) "Relaxed Circular" DNA is fully intact, but "relaxed" (supercoils removed).

SC

Relaxed region



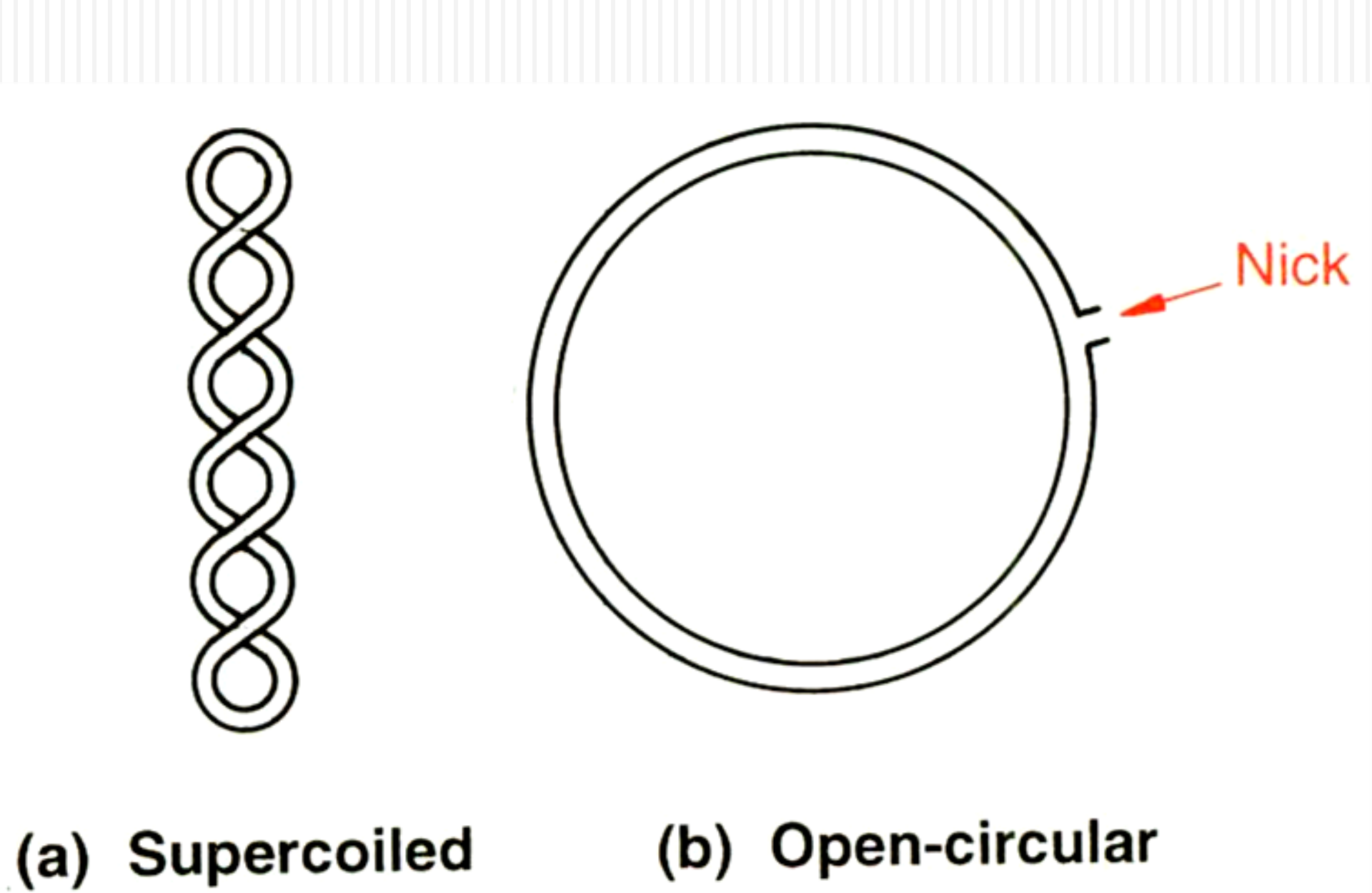
3) "Supercoiled Denatured" DNA. small quantities occur following excessive alkaline lysis; both strands are uncut but are not correctly paired, resulting in a compacted plasmid form.

4) "Nicked Open-Circular" DNA has one strand cut.

Nicked DNAs

5) "Linearized" DNA has both strands cut at only one site.

Linear DNA


Nick
(a) Supercoiled
(b) Open-circular

Conformation of Plasmid DNAs

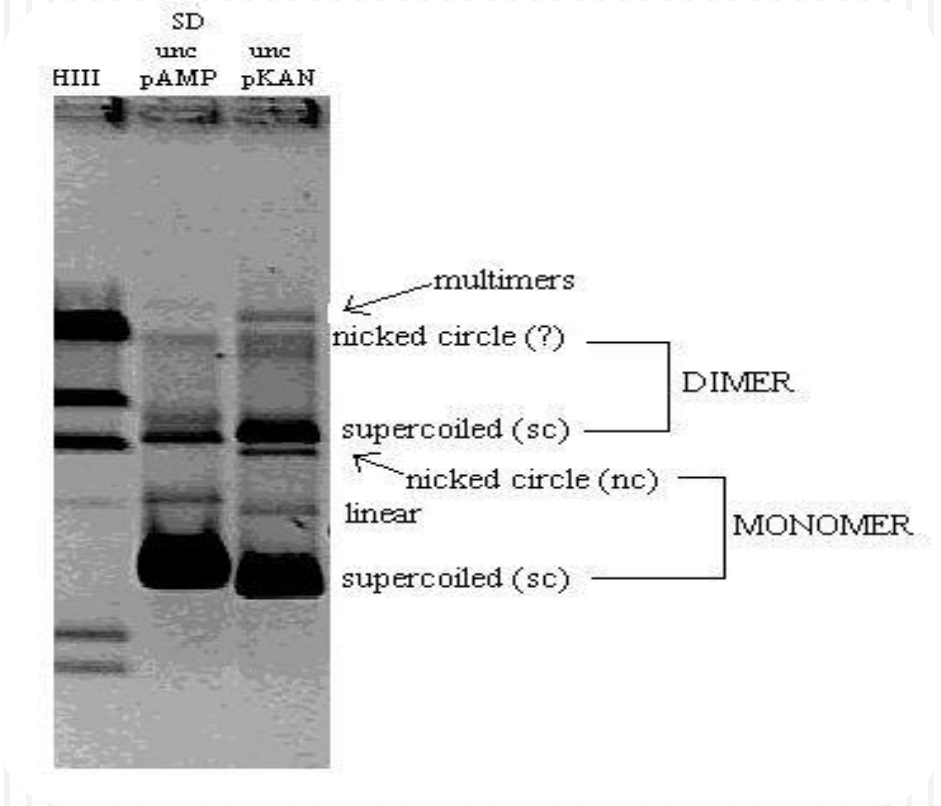
Nicked Open Circular (slowest)

Linear

Relaxed Circular

Supercoiled Denatured

Supercoiled (fastest)



DNA Quantification

- A common standard for determining the purity of isolated nuclei acids is the ratio of absorbance at 260 nm to the absorbance at 280 nm. (A_{260}:A_{280})
- A *pure* DNA solution has a A_{260}:A_{280} of around 1.8.
  A *pure* RNA solution has a A_{260}:A_{280} of around 2.0.
- By measuring the amount of DNA in a solution, we can determine how much of the isolated DNA to use for the restriction digestion analysis.

Procedure for determining DNA concentration

1. Dilute the plasmid DNA isolated with Miniprep DNA Kit in water for 100 fold. To do this, add 99 µl of autoclaved water into a centrifuge tube and add 1 µl of the plasmid DNA to the tube. Mix the sample by pipetting several times.

2. Add 100 µl of water into a cuvette and use it as a blank.

3. Measure the absorbance of the sample at 260 and 280 nm, respectively.

Procedure for determining DNA concentration (2)

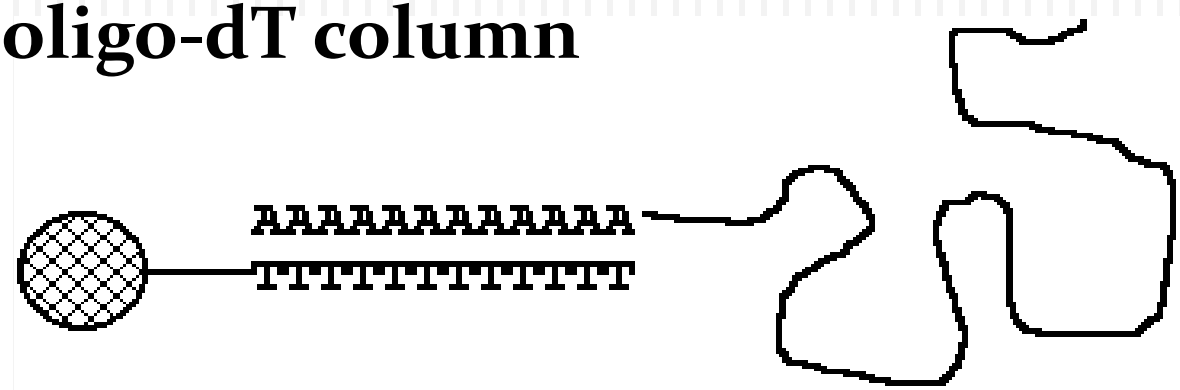
4. Calculate the concentration of plasmid DNA using the following formula:

DNA concentration = OD_{260} x Dilution Factor (100) x 50 µg/ml

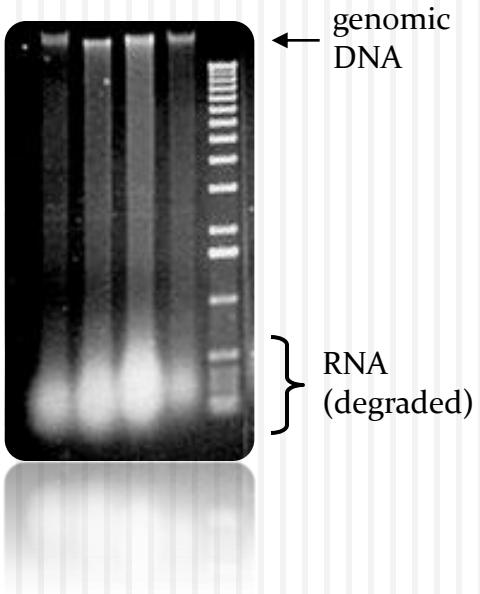
- Most spectrophotometers allow multiple sample reading against one reference blank.

Isolation of RNA
Special Considerations

- RNAse inhibitors!
- extraction in guanidine salts
- phenol extractions at pH 5-6
  - (pH 8 for DNA)
- treatment with RNase-free DNase
- selective precipitation of high MW forms (rRNA, mRNA) with LiCl
- oligo-dT column

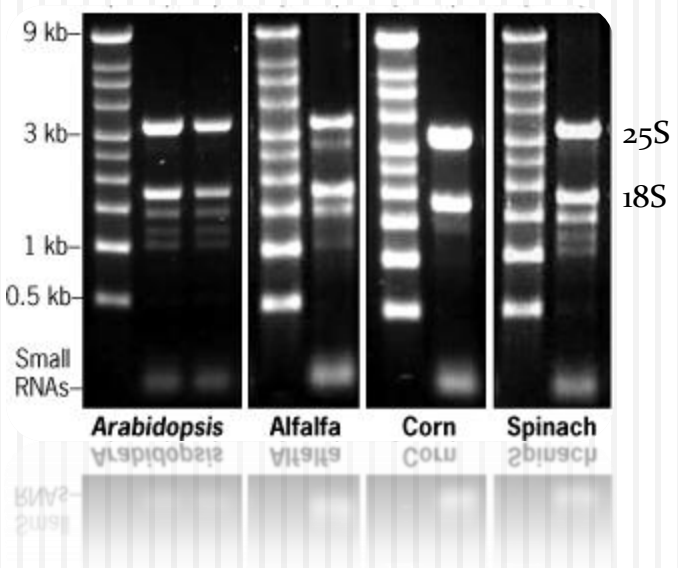


Checking for Degradation: DNA



Running your sample through an agarose gel is a common method for examining the extent of DNA degradation. Good quality DNA should migrate as a high molecular weight band, with little or no evidence of smearing.

Checking for Degradation: RNA



Ribosomal RNA (rRNA) makes up more than 80% of total RNA samples. Total RNA preps should display two prominent bands after gel electrophoresis. These correspond to the 25S and 18S rRNAs, which are 3.4 kb and 1.9 kb in Arabidopsis (respectively).

Good quality RNA will have:
No evidence of smearing
25S/18S ratio between 1.8 - 2.3

DNA PURIFICATION & QUANTITATION

DNA Purification Requirements

- Many applications require purified DNA.
- Purity and amount of DNA required (and process used) depends on intended application.
- Example applications:
  - Tissue typing for organ transplant
  - Detection of pathogens
  - Human identity testing
  - Genetic research

DNA Purification Challenges

1. Separating DNA from other cellular components such as proteins, lipids, RNA, etc.
2. Avoiding fragmentation of the long DNA molecules by mechanical shearing or the action of endogenous nucleases.

   Effectively inactivating endogenous nucleases (DNase enzymes) and preventing them from digesting the genomic DNA is a key early step in the purification process. DNases can usually be inactivated by use of heat or chelating agents.

Quality is Important

- Best yields are obtained from fresh or frozen materials.
- Blood/Tissues must be processed correctly to minimize destruction of DNA by endogenous nucleases.
- DNA yield will be reduced if endogenous nucleases are active.
- Prompt freezing, immediate processing or treatment with chelating agents (such as EDTA) minimizes nuclease effects.

Nucleic Acid Purification

There are many DNA purification methods. All must:

1. Effectively disrupt cells or tissues
   (usually using detergent)
2. Denature proteins and nucleoprotein complexes
   (a protease/denaturant)
3. Inactivate endogenous nucleases
   (chelating agents)
4. Purify nucleic acid target away from other nucleic acids and protein
   (could involve RNases, proteases, selective matrix and alcohol precipitations)

Disruption of Cells/Tissues

Most purification methods disrupt cells using lysis buffer containing:

- **Detergent** to disrupt the lipid bilayer of the cell membrane
- **Denaturants** to release chromosomal DNA and denature proteins

Additional enzymes are required for lysis of some cell types:

- Gram-positive bacteria require lysozyme to disrupt the bacterial cell wall.
- Yeasts require addition of lyticase to disrupt the cell wall.
- Plant cells may require cellulase pre-treatment.

Disruption of Cells: Membrane Disruption

- Detergents are used to disrupt the lipid:lipid and lipid:protein interactions in the cell membrane, causing solubilization of the membrane.
- Ionic detergents (such as sodium dodecyl sulfate; SDS) also denature proteins by binding to charged residues, leading to local changes in conformation.

Protein Denaturation

Denaturation = Modification of conformation to unfold protein, disrupting secondary structure but not breaking the peptide bonds between amino acid residues.

Denaturation results in:

- Decreased protein solubility
- Loss of biological activity
- Improved digestion by proteases
- Release of chromosomal DNA from nucleoprotein complexes ("unwinding" of DNA and release from associated histones)

Protein Denaturing Agents

- Ionic detergents, such as SDS, disrupt hydrophobic interactions and hydrogen bonds.
- Chaotropic agents such as urea and guanidine disrupt hydrogen bonds.
- Reducing agents break disulfide bonds.
- Salts associate with charged groups and at low or moderate concentrations increase protein solubility.
- Heat disrupts hydrogen bonds and nonpolar interactions.

- Some DNA purification methods incorporate proteases such as proteinase K to digest proteins.

Inactivation of Nucleases

- Chelating agents, such as EDTA, sequester Mg^{2+} required for nuclease activity.
- Proteinase K digests and destroys all proteins, including nucleases.

- Some commercial purification systems provide a single solution for cell lysis, protein digestion/denaturation and nuclease inactivation.

Removal of RNA

- Some procedures incorporate RNase digestion during cell lysate preparation.
- In other procedures, RNase digestion is incorporated during wash steps.

Separation of DNA from Crude Lysate

DNA must be separated from proteins and cellular debris.

Separation Methods

- Organic extraction
- Salting out
- Selective DNA binding to a solid support

Separation by Organic Extraction

- DNA is polar and therefore insoluble in organic solvents.
- Traditionally, phenol:chloroform is used to extract DNA.
- When phenol is mixed with the cell lysate, two phases form. DNA partitions to the (upper) aqueous phase, denatured proteins partition to the (lower) organic phase.

- DNA is a polar molecule because of the negatively charged phosphate backbone.
- This polarity makes it more soluble in the polar aqueous phase.

More about how phenol extraction works at: **bitesizebio.com/2008/02/12/**

Separation by Salting Out

- Salts associate with charged groups.
- At high salt concentration, proteins are dehydrated, lose solubility and precipitate.
  Usually sodium chloride, potassium acetate or ammonium acetate are used.
- Precipitated proteins are removed by centrifugation.
- DNA remains in the supernatant.

DNA Lab
Part 2

Ethanol Precipitation of DNA

- Methods using organic extraction or salting-out techniques result in an aqueous solution containing DNA.
- The DNA is precipitated out of this solution using salt and isopropanol or ethanol.
- Salt neutralizes the charges on the phosphate groups in the DNA backbone.
- The alcohol (having a lower dielectric constant than water) allows the sodium ions from the salt to interact with the negatively charged phosphate groups closely enough to neutralize them and let the DNA fall out of solution.

Separation by Binding to a Solid Support

Most modern DNA purification methods are based on purification of DNA from crude cell lysates by selective binding to a support material.

Support Materials

- Silica
- Anion-exchange resin

Advantages

- Speed and convenience
- No organic solvents
- Amenable to automation/miniaturization

DNA purification column containing a silica membrane

Silica

- DNA binds selectively to silica in the presence of high concentrations of chaotropic salts (e.g., guanidinium HCl).
- Protein does not bind under these conditions.
- Silica membranes or columns are washed with an alcohol-based solution to remove the salts.
- DNA is eluted from the membrane with a low-ionic-strength solution, such as a low-salt buffer or water.

Advantages

- Fast purification
- Amenable to automation
- No centrifugation required (can use vacuum)
- No organic solvents or precipitation steps

Magnetic Separation: Silica or Charge-Based

Several commercial systems are based on capture of DNA from solution using magnetic particles.

Magnetic Particle Types

- Silica-based (bind/release DNA depending on salt concentration)
- Charge-based (particle charge changes based on pH of solution, binding/releasing negatively charged DNA).

Advantages

- Fast
- No organic extraction or precipitations
- Amenable to automation

Anion-Exchange Columns

- Based on interaction between negatively charged phosphates in DNA and positively charged particles.
- DNA binds under low-salt conditions.
- Protein and RNA are washed away using higher salt buffers.
- DNA is eluted with high salt (neutralizes negative charge on DNA).
- Eluted DNA is recovered by ethanol precipitation.

Advantages

- No organic solvents
- Fast, but more hands-on than silica (requires ethanol)

Summary

DNA purification methods all do the following:

- Disrupt cells and denature/digest of proteins
- Separate DNA from proteins, RNA and other cellular components
- Prepare a purified DNA solution

Older methods relied on laborious organic extraction and precipitation procedures.

Newer methods are faster, using selective binding of DNA to silica or magnetic beads, and are amenable to automation and miniaturization.