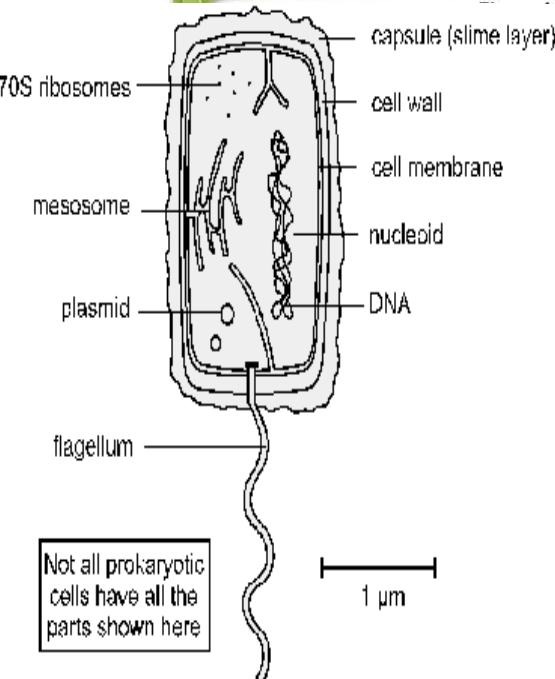
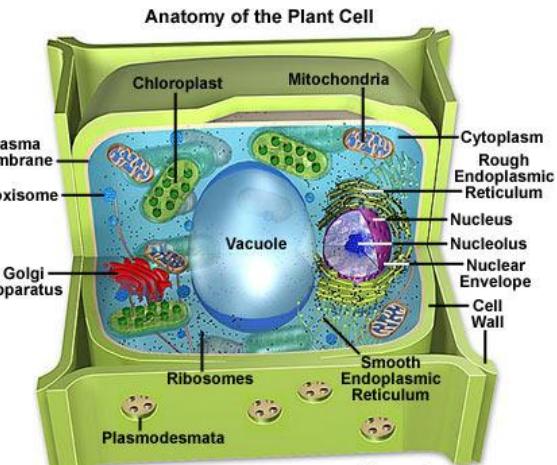
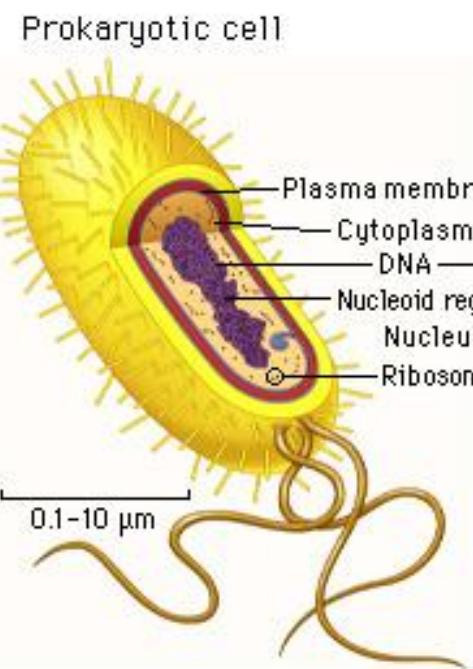
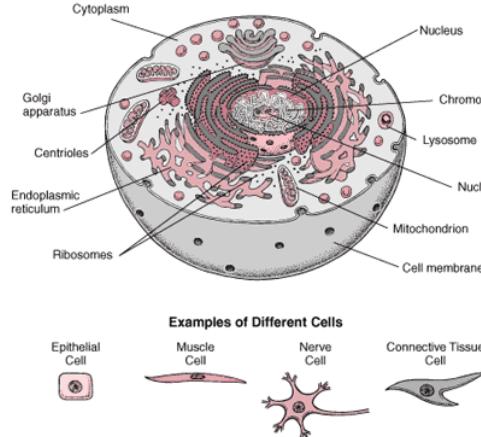

ISOLASI DNA & RNA

Oleh :
Dra. Fatchiyah, M.Kes. Ph.D

Sumber DNA atau RNA



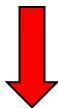
-
- Melisikan DNA dari(nukleus)
 - Penggerusan
nukleus
 - Memisahkan DNA
dari komponen-
komponen sel yang
lain
 - Mortar dan pestle
atau lainnya
 - Bufer ekstraksi
SDS, CTAB
 - Sentrifugasi
10.000 rpm lebih

Extraction/Precipitation Method

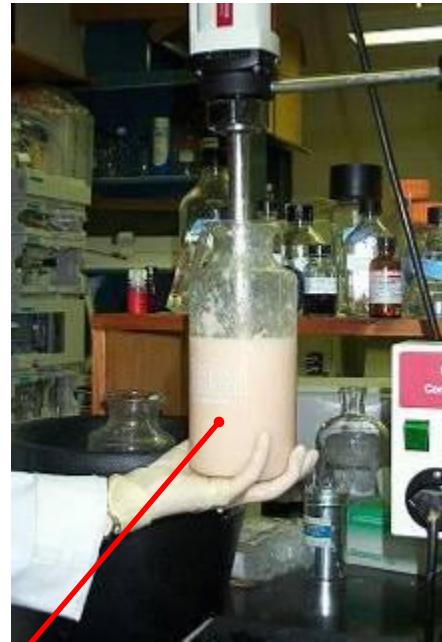
Step 1: Disruption of cell walls by grinding



Grind sample into a fine powder to shear cell walls and membranes

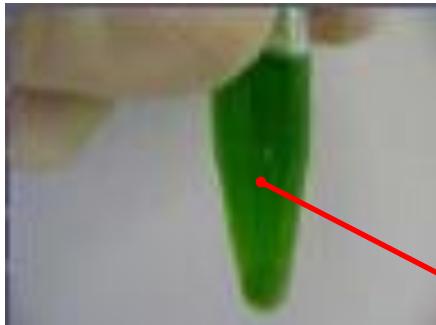


Step 1+2: mechanical disruption and homogenization in extraction buffer



A homogenizer allows cells to be mechanically disrupted within the extraction buffer

Step 2: Lysis of cells in extraction buffer



Mix thoroughly with extraction buffer to dissolve cell membranes and inhibit nuclease activity

Crude lysate

-
- Digunakan fenol (pendenaturasi protein)
 - Fenol + kloroform (1:1) = deproteinasi lebih efektif jika digunakan 2 macam pelarut organik. Kloroform juga menghilangkan lipid dan pada tahap akhir ekstraksi, membantu menghilangkan sisa fenol.
 - Isoamil alkohol membantu pemisahan fase, menurunkan jumlah material kontaminan yang terikut pada fase cair dan interfase serta membantu menurunkan busa.

-
- Garam (NH_4^+ , Na^+ , NaOAc , NaCl , NHOAc)
 - Dan ethanol 100% atau isopropanol
 - ethanol sebanyak 2-2,5 x volume
 - isopropanol 0.6-07 X volume

untuk Isopropanol sering digunakan untuk presipitasi pertama, tetapi tidak untuk tahap akhir karena cenderung mengendapkan garam dibanding ethanol.

Garam yang biasa ditambahkan dalam presipitasi alkohol

Secara umum $\text{Na}^+ = 3 \text{ M}$; $\text{NH}_4^+ = 7.5 \text{ M}$

Jenis garam	Larutan stok	Konsentrasi akhir
Na asetat	2.5 M (pH 5,2-5,5)	0.25 M - 0.3 M
NaCl	5,0 M	0,1 M (optimal < 0,15 M)
Amonium asetat*	4,0 M	1,3 M

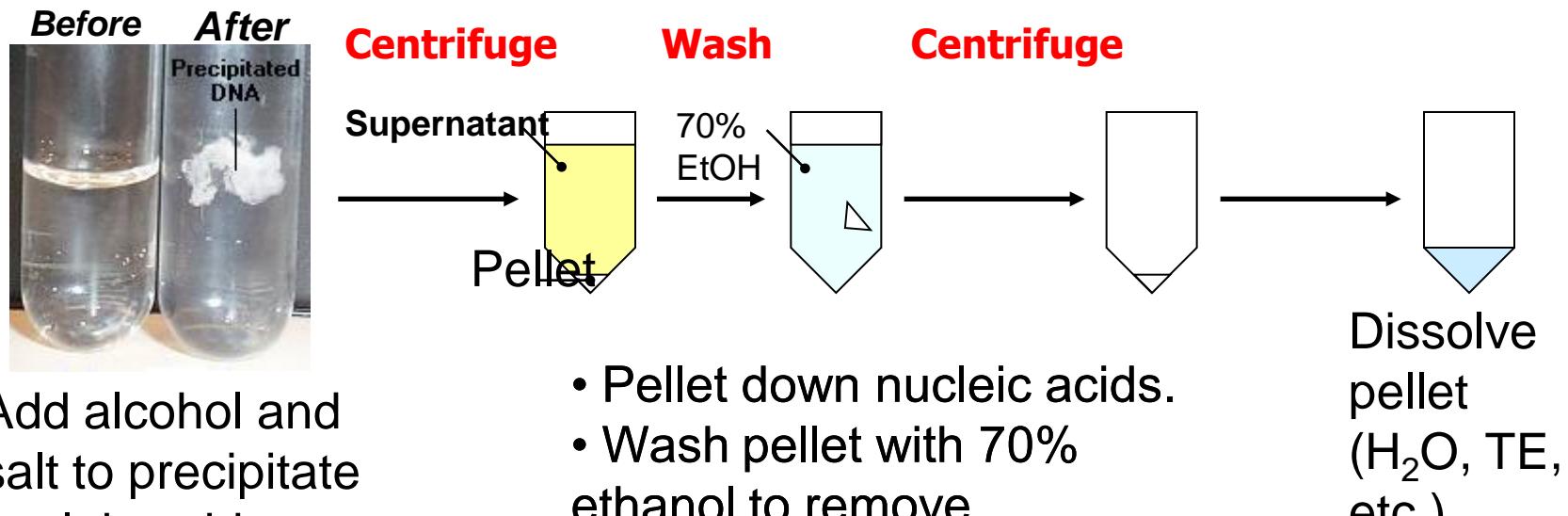
Komponen	Sifat	Pengaruh
pH		Penghambat enzim degradative
Tris	bufer	Menjaga pH
Ethylene diamine tetraacetate (EDTA)		Mengkelat kation (Mg), menghambat enzim yg tgt logam (nuklease, DNase)
Na/K	garam	Menstabilkan inti
Proteinase K	enzim	Mendigest protein
Sodium dodecyl sulfat (SDS)	Detergent anionik	Merusak membran sel, mendenaturasi protein, menghambat aktivitas nuklease

Komponen	Sifat	Pengaruh
Cetyltrimetilammonium bromide (CTAB)	Detergen kationik	Merusak membran sel, mendenaturasi protein, membentuk kompleks dengan DNA, menghambat aktivitas nuklease
Chloroform-isoamyl alkohol (CIA)		Ekstraksi protein
Isopropanol)		Presipitasi kompleks CTAB-DNA
Hidroksikuinolin	Agen pereduksi	Menghambat proses oksidasi, melindungi DNA dari quinone, disulfit, peroxidase, polyphenoloksidase, mencegah pencoklatan oleh polifenolik
Glukosa, cystein		
β -merkaptoethanol		
Glutation, $\text{Na}_2\text{S}_2\text{O}_5$		
Dithiothreitol		
Asam askorbat		

Komponen	Sifat	Pengaruh
Polyvinilpirolidon (PVP)	Absorben polyfenol	Mengurangi efek polifenol, quinon, tanin
Bovin serum albumin (BSA)		BSA = denaturasi enzim degradatif
Dietylpyrocarbonate	Poliamin	Melindungi dari RNase
Bentonite		
Spermine		
Spermidin		
Dietylditiocarbamic acid (DIECA)	Inhibitor fenoloksidase	Mengkelat Cu ²⁺
Cyanide		Melindungi dari enzim2 oksidase logam berat
NH		Melindungi dari H ⁺

Extraction/Precipitation Method

Step 4: Nucleic Acid Precipitation

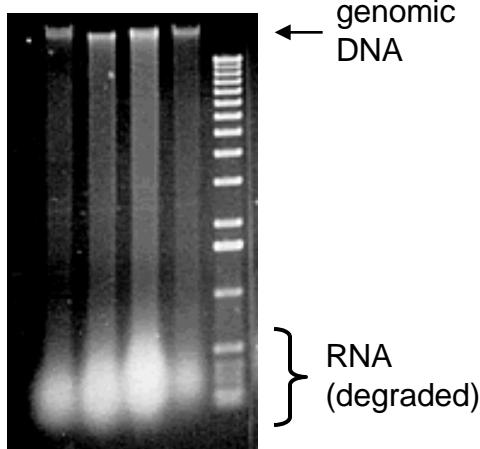


Add alcohol and salt to precipitate nucleic acids from the aqueous fraction

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

-
- DNA yang diperoleh selanjutkan dinilai kemurniannya dan dikuantifikasi menggunakan spektrofotometer-UV.
 - Kemurnian DNA dinilai dari perbandingan absorbansi pada λ 260 dan 280 nm.
 - Konsentrasi
$$[\text{DNA}] = \text{A}_{260} \times \text{faktor pengenceran} \times 50\mu\text{g/ml}$$
 - Kemurnian
$$\text{A}_{260}/\text{A}_{280}$$
 antara 1.8 – 2.0
$$\text{A}_{230}/\text{A}_{260}$$
 sekitar 0.5 (only from plant)

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.

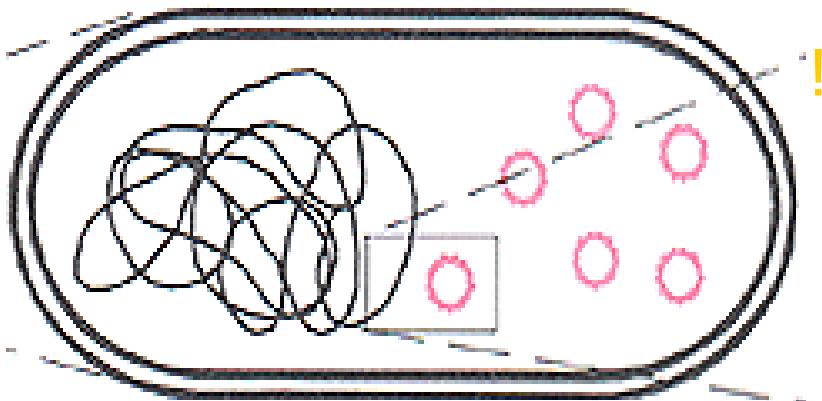
Plasmid Isolation

- First, separate cells from growth medium
- Lyse host cells to release the plasmid DNA but not the genomic DNA
 - usually done in alkaline solution and SDS
- Neutralize with acid to allow genomic DNA to renature and aggregate
 - Large DNA and proteins bound to SDS will ppt

Isolating Recombinant Plasmids

- Cells are resuspended in buffer (P1) containing Tris, EDTA, and RNase.
- EDTA stabilizes the cell membrane by binding divalent cations (Mg^{++} and Ca^{++}).
- RNase destroys the cell's RNA.

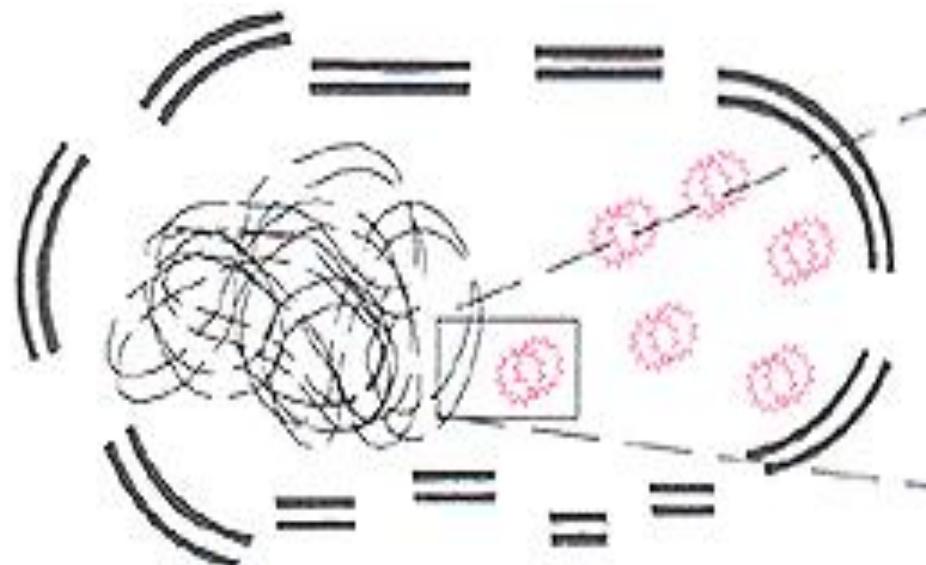
E. coli CELL



Source: DNA Science: The First Course in Recombinant DNA Technology

Isolating Recombinant Plasmids

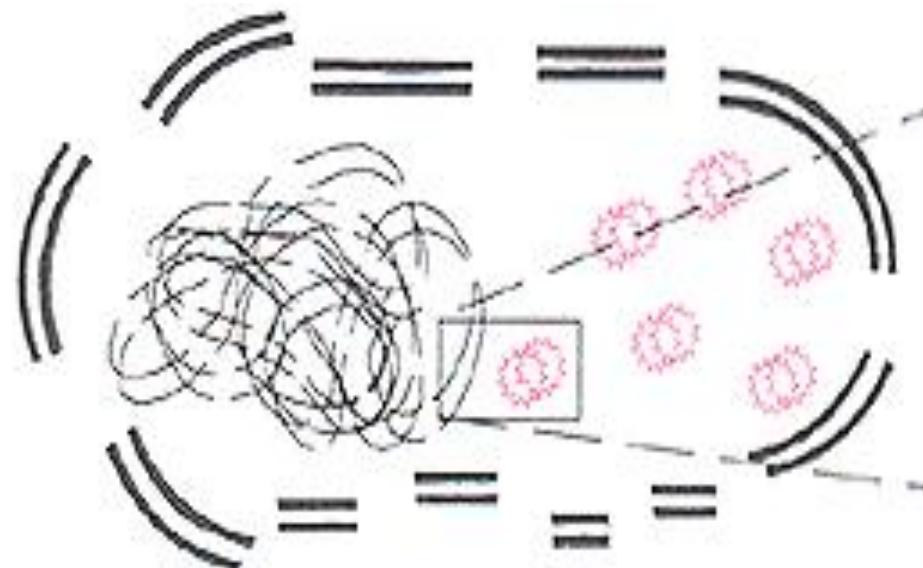
- The resuspended cells are then treated with a SDS and sodium hydroxide (P2).
- SDS dissolves the phospholipid and protein components of the cell membrane.
- The cell membrane lyses, releasing the cell contents.



Source: DNA Science: The First Course in Recombinant DNA Technology

Isolating Recombinant Plasmids

- Sodium hydroxide denatures both plasmid and chromosomal DNA into single strands.
- Chromosomal DNA separates completely into individual strands.
- The single-stranded plasmid loops remain linked together like interlocked rings.



Source: DNA Science: The First Course in Recombinant DNA Technology

Quantifying Plasmid DNA

Quantify DNA using UV absorbance

- DNA UV absorbance peaks at 260 nm
- protein UV absorbance peaks at 230 nm (peptide bond) and 280 nm (aromatic amino acids)
- The ratio of the absorbance at 260 nm/280 nm is a measure of the purity of a DNA sample; it should be between 1.65 and 1.85.

Isolasi RNA

- Prosedur isolasi RNA **HARUS** dilakukan dalam kondisi **RNase-free**.
- Sampel yang akan diisolasi RNanya harus bebas dari kontaminasi dengan *ribonucleases* (RNase).
- Peralatan yang dipergunakan harus terlebih dahulu diautoklaf atau ditreatment (disemprot ethanol 70%) untuk mencegah kontaminasi RNase.
- Selama melakukan isolasi RNA, peneliti harus menggunakan sarung tangan (*gloves*) baru untuk melindungi pengguna dan melindungi RNA hasil isolasi dari *nucleases* yang terdapat pada kulit.

**DEPC: diethyl pyrocarbonate → RNase-Free
untuk semua larutan & peralatan yg akan
digunakan**

Getting Prepared: Creating a Nuclease-Free Environment

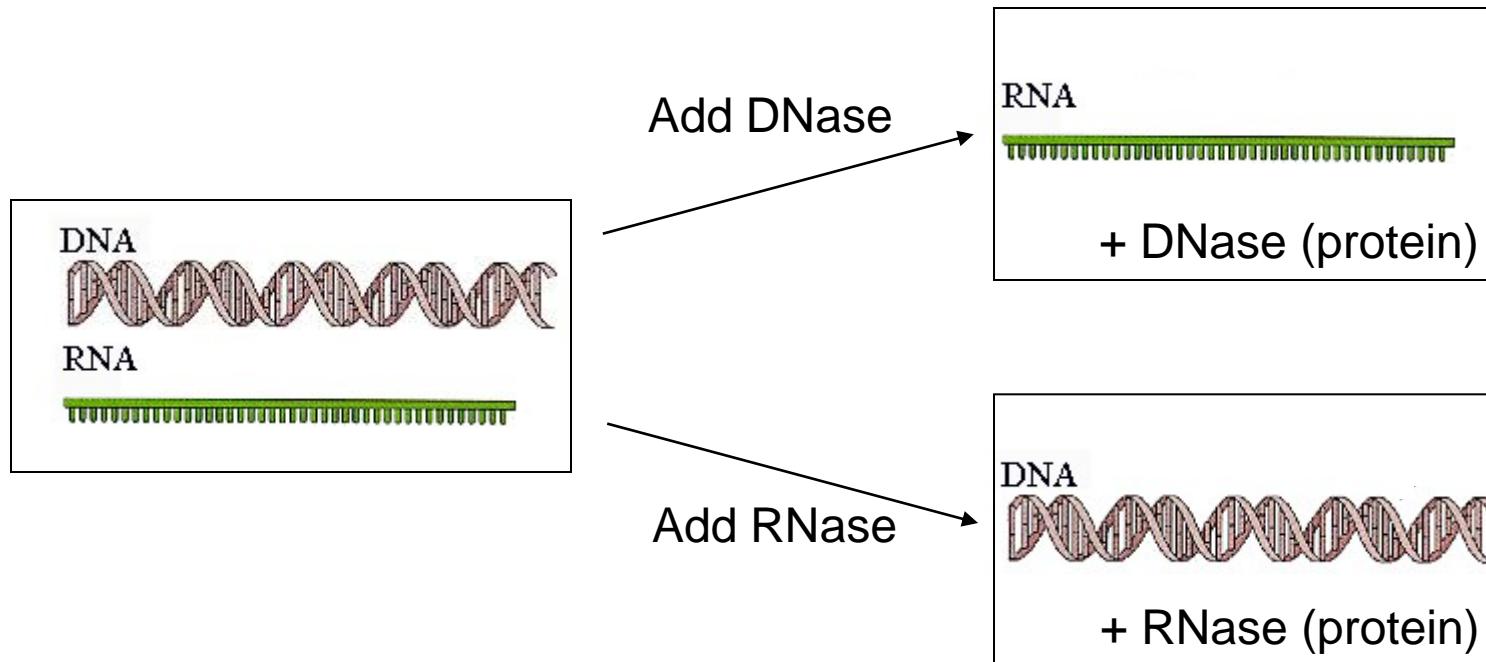
Living organisms produce several enzymes designed to degrade DNA and RNA molecules. There are several things you can do to minimize the risk of exposing your samples to external DNases and RNases.

- 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. You should be doing this anyway for safety reasons, but skin cells also produce RNase7, a potent RNA-degrading enzyme.

- Bake glass, metal, or ceramic equipment at high temp.

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).

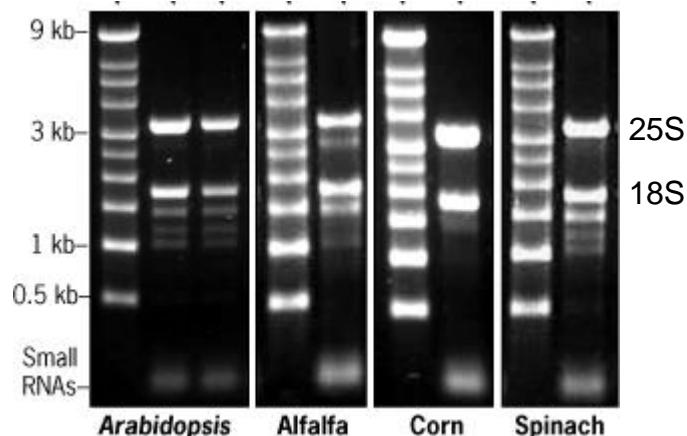
Solution for RNA isolation

- RNA diisolasi dengan cara menghomogenasi jaringan pada buffer ekstraksi yang mengandung *Guanidinium Thiocyanate* (GTC) untuk melisiskan sel dan menonaktifkan RNase endogenous.
- *Lithium Chloride* (LiCl) yang ditambahkan pada homogenasi yang kedua berfungsi sebagai *selective precipitation* pada *isopycnic centrifugation* yang menggunakan *Caesium Trifluoroacetate* (CsTFA).
- *Isopycnic centrifugation* adalah suatu mekanisme pemisahan molekul berdasarkan perbedaan Berat Jenis (BJ) antara DNA (1.5 – 1.7 g/ml) dengan RNA (1.7 – 2 g/ml).
- Sesudah sentrifugasi, RNA berada pada pellet, protein berada di atas supernatan, DNA terlarut dalam supernatan. Pellet RNA selanjutnya dicuci untuk menghilangkan kontaminan protein dan DNA.

Kuantifikasi dan Kemurnian RNA

- RNA yang diperoleh selanjutkan dinilai kemurniannya dan dikuantifikasi menggunakan spektrofotometer-UV. Kemurnian RNA dinilai dari perbandingan absorbansi pada λ 260 dan 280 nm. Sampel RNA murni mempunyai rasio A260/A280 sebesar 2 ± 0.05 .
- Kuantifikasi RNA dilakukan berdasarkan absorbansi pada λ 260 nm.
- Konsentrasi RNA pada absorbansi dengan nilai 1 adalah 40 $\mu\text{g}/\text{ml}$.
- Konsentrasi RNA pada sampel dapat dihitung menggunakan rumus berikut.
- **[RNA] = A260 x D x 40 $\mu\text{g}/\text{ml}$**
- **D = faktor pengenceran**

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

Electrophoresis for nucleic acid

- Agarose Gel Electrophoresis
- Purification for Specific Fragment of DNA
 - DNA Electro-elution
 - Electrophoresis onto DEAE-cellulose membranes
- Polyacrylamide Gels
- Pulse-field Gel Electrophoresis (PFGE)

Agarose Gel Electrophoresis

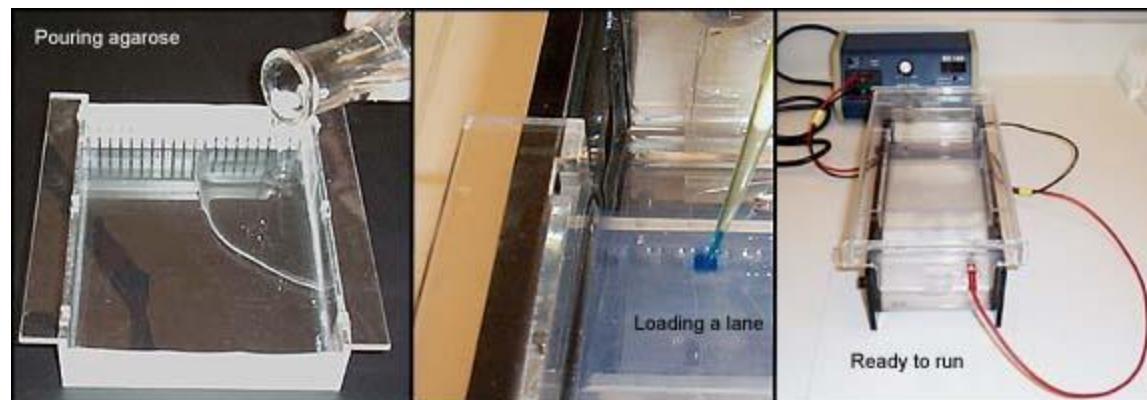
- ❑ Standard method used to separate, identify, and purify DNA fragments is electrophoresis through agarose gel.
- ❑ The technique is simple, rapid to perform, and capable of resolving mixtures of DNA fragment that cannot be separated adequately by other sizing procedures, such as density gradient centrifugation.

Preparing and Running Standard Agarose DNA Gels

- **The equipment and supplies necessary for conducting agarose gel electrophoresis are relatively simple and include:**
- **An electrophoresis chamber and power supply**
- **Gel casting trays**, which are available in a variety of sizes and composed of UV-transparent plastic. The open ends of the trays are closed with tape while the gel is being cast, then removed prior to electrophoresis.
- **Sample combs**, around which molten agarose is poured to form sample wells in the gel.
- **Electrophoresis buffer**, usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).
- **Loading buffer**, which contains something dense (e.g. glycerol) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring of how far the electrophoresis has proceeded.

More preparation ...

- Ethidium bromide, a fluorescent dye used for staining nucleic acids. *NOTE: Ethidium bromide is a known mutagen and should be handled as a hazardous chemical - wear gloves while handling.*
- Transilluminator (an ultraviolet lightbox), which is used to visualize ethidium bromide-stained DNA in gels. *NOTE: always wear protective eyewear when observing DNA on a transilluminator to prevent damage to the eyes from UV light.*



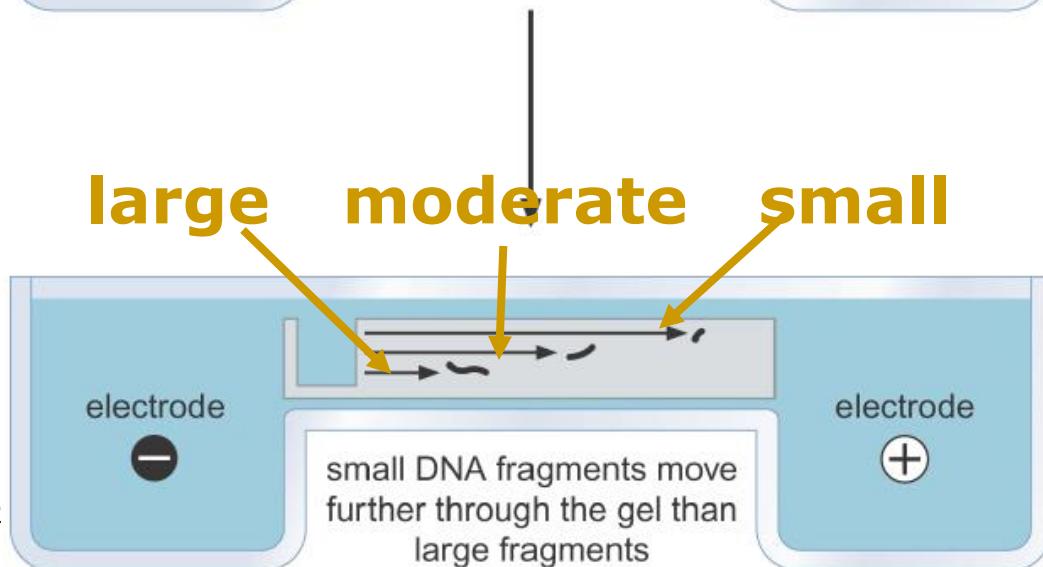
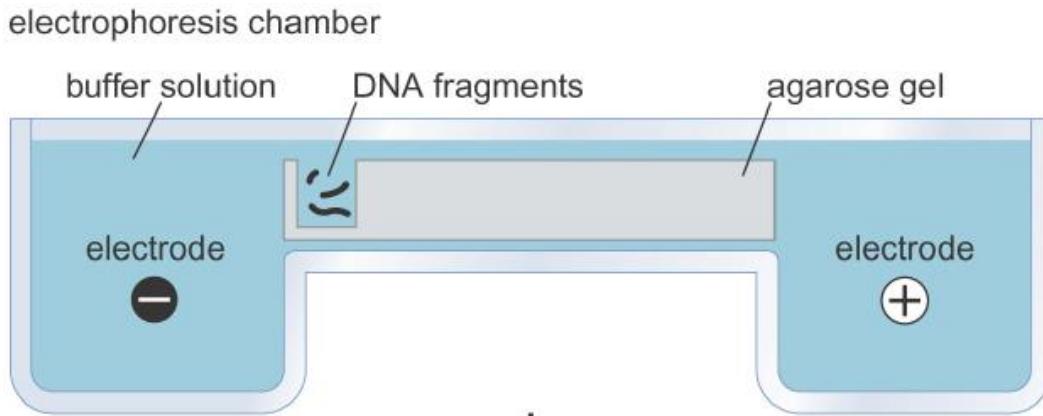
Electrophoresis

- DNA and RNA molecules are negatively charged, thus move in the gel matrix toward the positive pole (+)
- Linear DNA molecules are separated according to size
- The mobility of circular DNA molecules is affected by their topological structures. The mobility of the same molecular weight DNA molecule with different shapes is:
supercoiled > linear > nicked or relaxed

Migration of DNA Fragments in Agarose

- ❑ **Fragments of linear DNA** migrate through agarose gels with a mobility that is inversely proportional to **the log₁₀ of their molecular weight**. In other words, if you plot the distance from the well that DNA fragments have migrated against the log₁₀ of either their molecular weights or number of base pairs, a roughly straight line will appear.
- ❑ **Circular forms of DNA** migrate in agarose distinctly differently from linear DNAs of the same mass. **Typically, uncut plasmids will appear to migrate more rapidly than the same plasmid when linearized**. Additionally, most preparations of uncut plasmid contain at least two topologically-different forms of DNA, corresponding to supercoiled forms and nicked circles.

DNA Migration

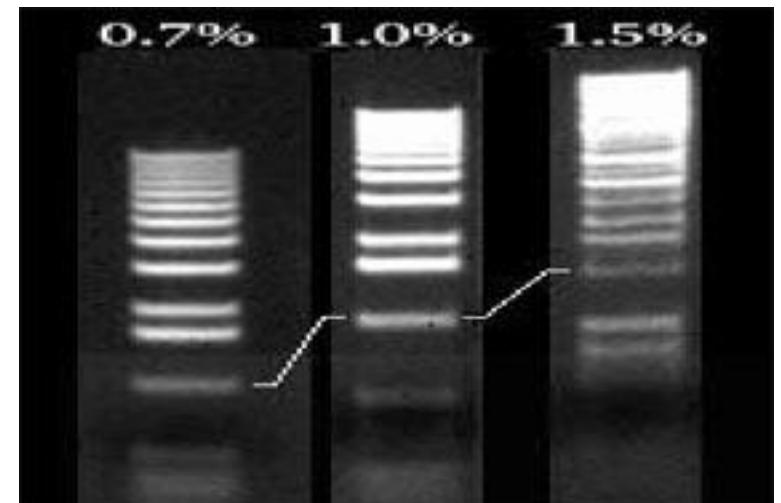


Picture of DNA separation by gel electrophoresis

Factors of DNA Migration

- Several additional factors have important effects on the mobility of DNA fragments in agarose gels, and can be used to your advantage in optimizing separation of DNA fragments.
Chief among these factors are:
 - Agarose Concentration
 - Voltage
 - Electrophoresis buffer
 - Effects of Ethidium Bromide

Fig. Agarose Concentration



Purification for Specific Fragment of DNA

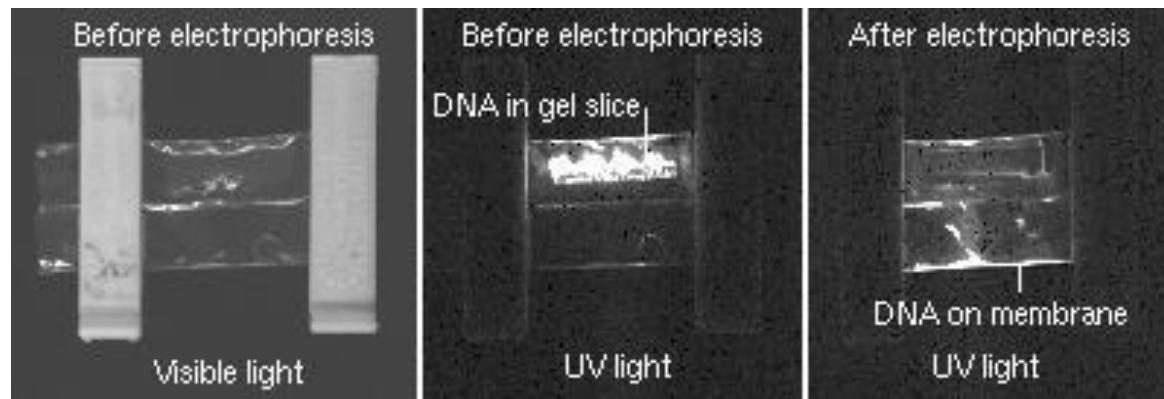
- In addition to its importance as an analytical tool, gel electrophoresis is widely used for isolating and then purifying specific fragments of DNA, usually in preparation for subcloning
- Several techniques can be used to purify DNA from agarose gels, and choosing between them is, to some extent, a matter of personal preference. They all start out by excising the desired "band" from an ethidium-stained gel viewed with a UV transilluminator. Because UV light can fragment DNA, it is best to work expeditiously and keep exposure time to a minimum.
- Cut out the desired piece of agarose using a razor blade or scalpel blade, and try to get as little extra agarose as possible. The block of agarose containing DNA is then subjected to any of the following.

DNA Electroelution

- The block of agarose is placed in a piece of dialysis tubing with a small amount of fresh electrophoresis buffer, the ends sealed with clamps, and the bag placed into an electrophoresis chamber. Application of current will cause the DNA to migrate out of the agarose, but it will be trapped within the bag.
- Progress can be monitored using a transilluminator, as shown below. When the DNA is out of the agarose, the flow of current is reversed for a few seconds to knock the DNA off of the side of the tubing.
- The buffer containing the DNA is then collected and the DNA precipitated with ethanol.

DNA Electroelution . .

Electroelution is more time consuming than some of the other techniques, but works well and is probably the best technique for recovery of large (> 5 kb) fragments of DNA.



TERIMAKASIH