GENETICS AND MOLECULAR BIOLOGY FOR ENVIRONMENTAL ANALYSIS

MOLECULAR ECOLOGY
LESSON 3: NUCLEIC ACID ISOLATION

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NUCLEIC ACID EXTRACTION METHODS

- **Purpose:**

- To release nucleic acid from the cell for use in other procedures
- Must be free from contamination with protein, carbohydrate, lipids or other nucleic acids.
- Used pure nucleic acids for testing.

- Routinely isolated from human, fungal, bacterial and viral sources.
- Pretreat to make nucleated cells available,
  - whole blood
  - Tissue samples
  - Microorganisms
- Need sufficient sample for adequate yield.

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.
3.
4. 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.

There are many DNA purification methods. All must:

Effectively disrupt cells or tissues

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

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.

DNA ISOLATION

DNA must be separated from proteins and cellular debris.

Separation Methods

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

ORGANIC SOLUTIONS

- 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.

ORGANIC SOLUTIONS

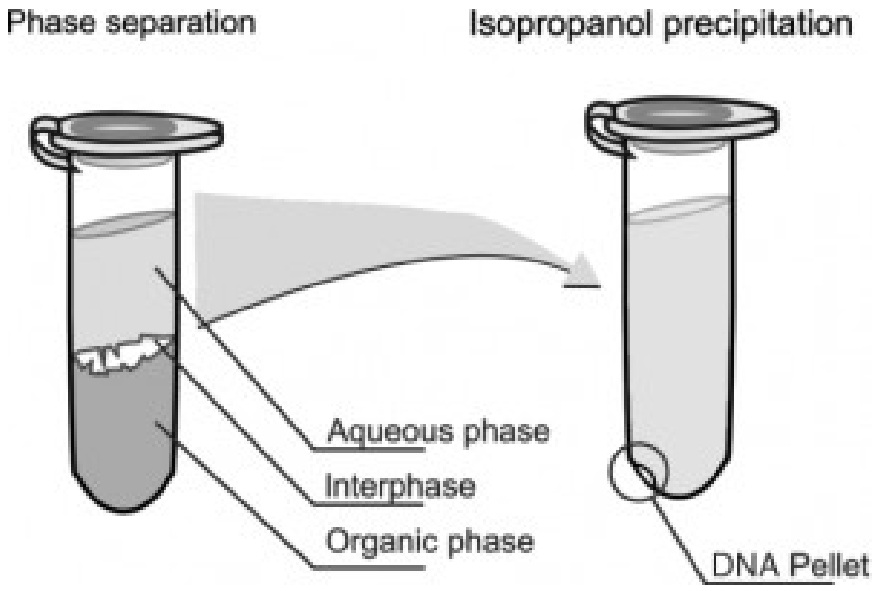
1:1 **phenol** : **chloroform**

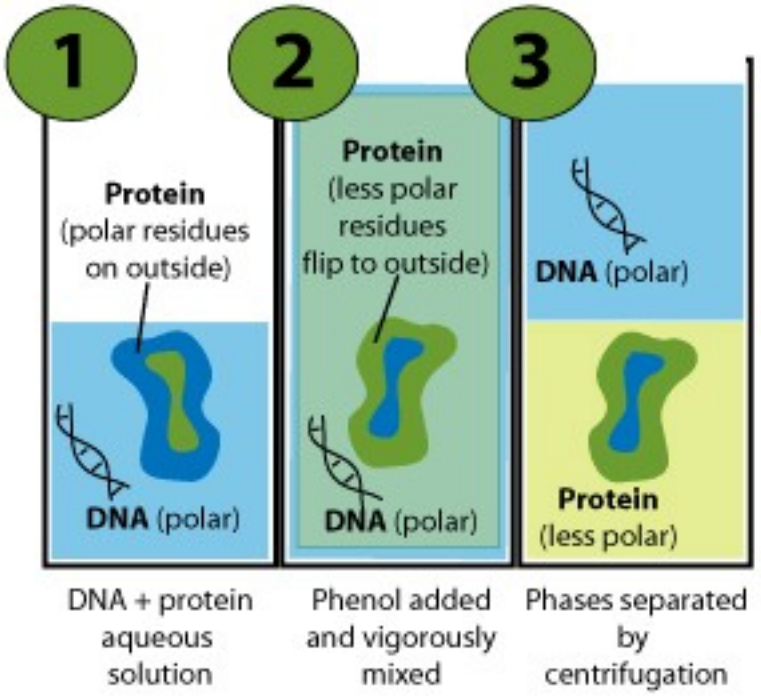
or

25:24:1 **phenol** : **chloroform** : **isoamyl alcohol**

- **Phenol**: denatures proteins, precipitates form at interface between aqueous and organic layer
- **Chloroform**: increases density of organic layer
- **Isoamyl alcohol**: prevents foaming

Genomic DNA is isolated as pieces up to 1 Mbp!


Phase separation
Isopropanol precipitation
Aqueous phase
Interphase
Organic phase
DNA Pellet


1
2
3
Protein
(polar residues
on outside)
DNA (polar)
Protein
(less polar
residues
flip to outside)
DNA (polar)
DNA (polar)
Protein
(less polar)
DNA + protein
aqueous
solution
Phenol added
and vigorously
mixed
Phases separated
by
centrifugation

DNA PRECIPITATION

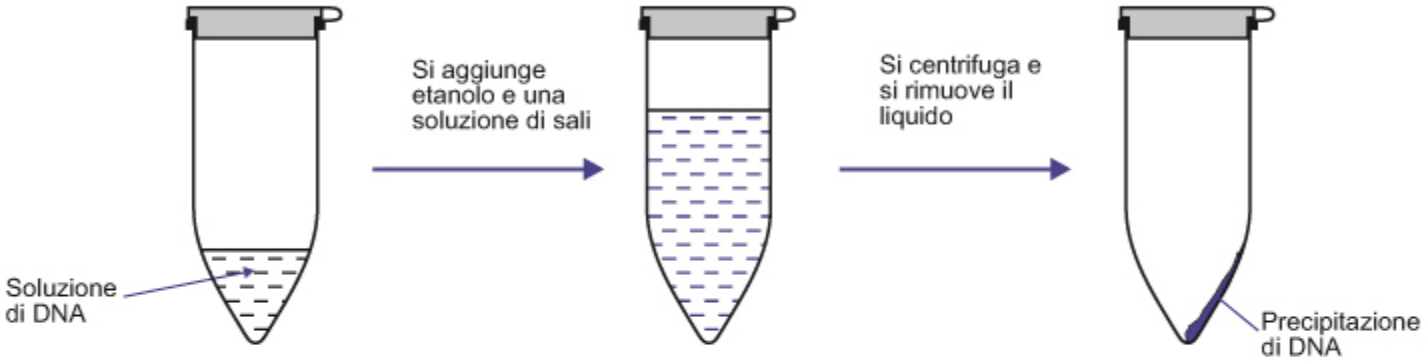
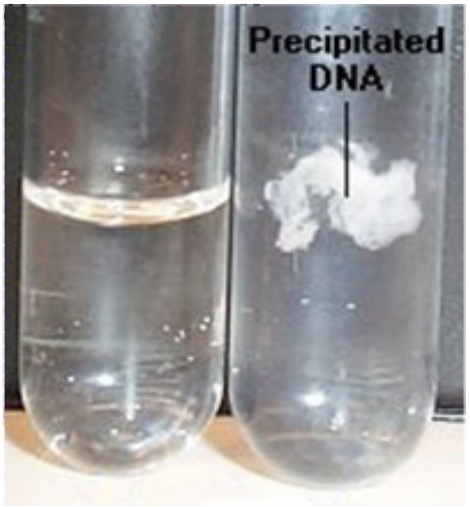
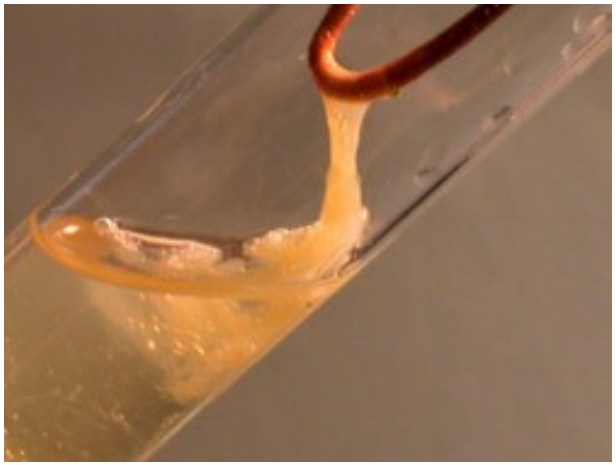


Figura 2.4 Precipitazione in etanolo. Le provette sono centrifugate in un rotore ad angolo fisso, in modo tale che il precipitato sia su un lato della parte inferiore della provetta.

INORGANIC ISOLATION METHODS

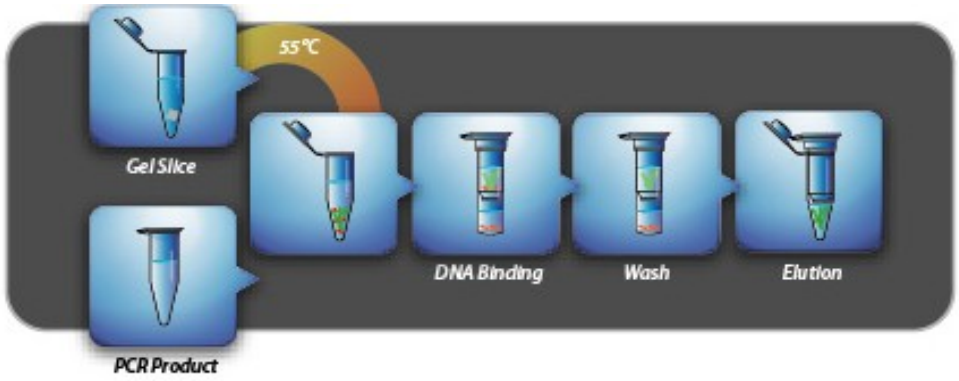
- Also called “salting out”.
- Uses low pH and high salt condition to selectively precipitate proteins.
- DNA is left in solution.
- Precipitate out DNA with isoproproanol.
- I know this is not scientific but the precipitated out DNA is usually referred to as





BINDING TO A SUPPORT MATERIAL

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



Support Materials

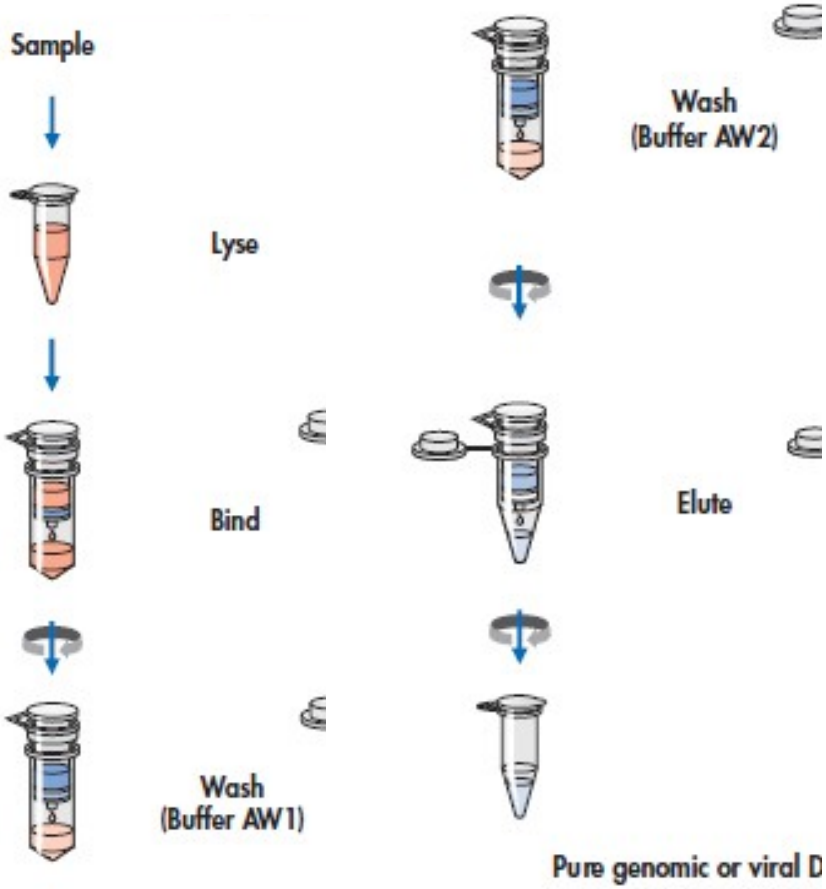
- Silica
- Anion-exchange resi

Advantages

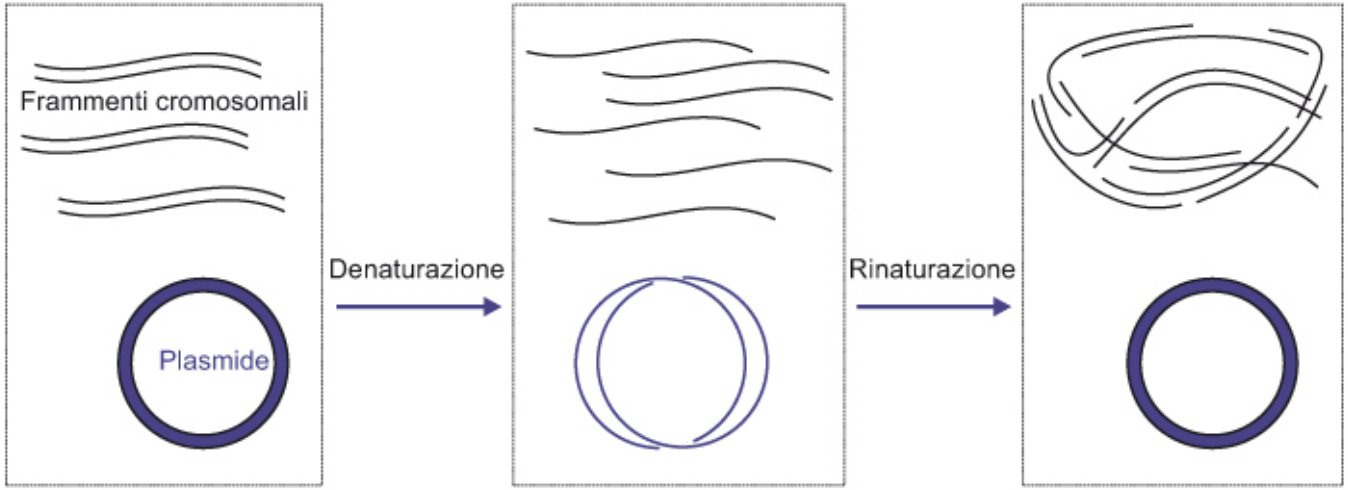
- Speed and convenience
- No organic solvents
- Amenable to automation/miniaturization

Disadvantage

KIT


Sample
Lyse
Bind
Wash
(Buffer AW1)
Wash
(Buffer AW2)
Elute
Pure genomic or viral D

PLASMID DNA PREP

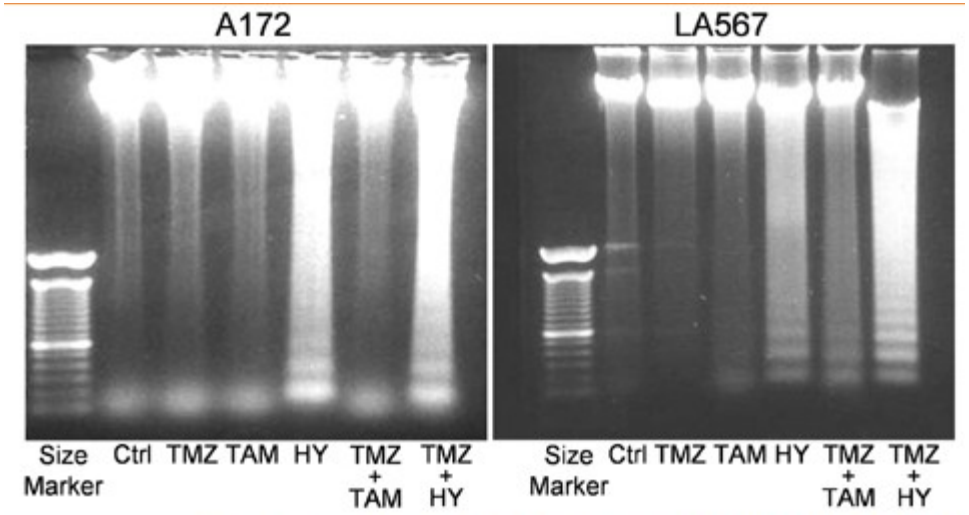


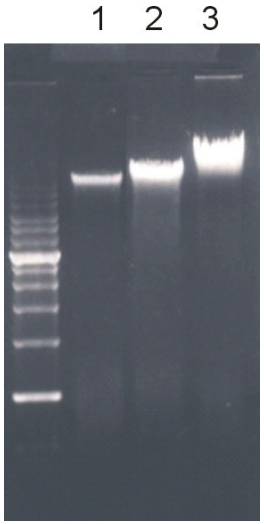
DNA cromosomale sotto forma di frammenti lineari; plasmide sotto forma di DNA circolare covalentemente chiuso

I filamenti di DNA del plasmide denaturato rimangono concatenati; i frammenti di DNA cromosomale lineari si dissociano

I filamenti concatenati del plasmide rinaturano; i frammenti cromosomali formano aggregati

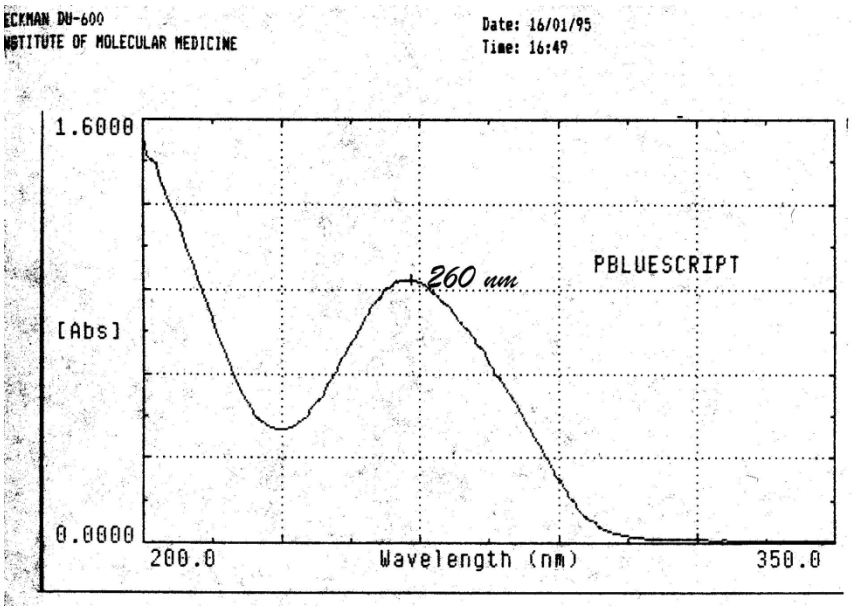
GENOMIC DNA ANALYSIS





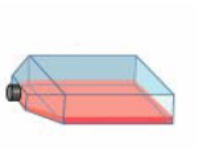
CONCENTRATION MEASUREMENT

- Photometric measurement of DNA concentration
- UV 260 nm
- Conc=50ug/ulxOD$_{260}$

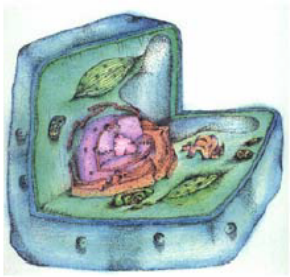


RNA ISOLATION

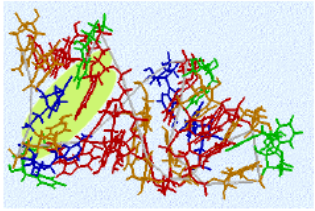
Tissue



Cells



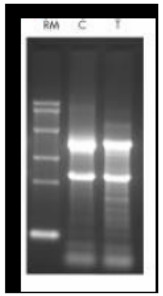
Break open the cell



RNA Purification

Purity

Quality Parameters



Integrity

WHAT RNA IS NEEDED FOR?

- *Messenger RNA synthesis is a dynamic expression of the genome of an organism. As such, mRNA is central to information flow within a cell.*

- • **Size** – examine differential splicing
- • **Sequence** – predict protein product
- • **Abundance** – measure expression levels
- • **Dynamics of expression** – temporal, developmental, tissue specificity

RNA ISOLATION

- **Total RNA from biological samples**
  - – Organic extraction
  - – Affinity purification

- **• mRNA from total RNA**
  - – Oligo(dT) resins

- **• mRNA from biological samples**
  - – Oligo(dT) resins

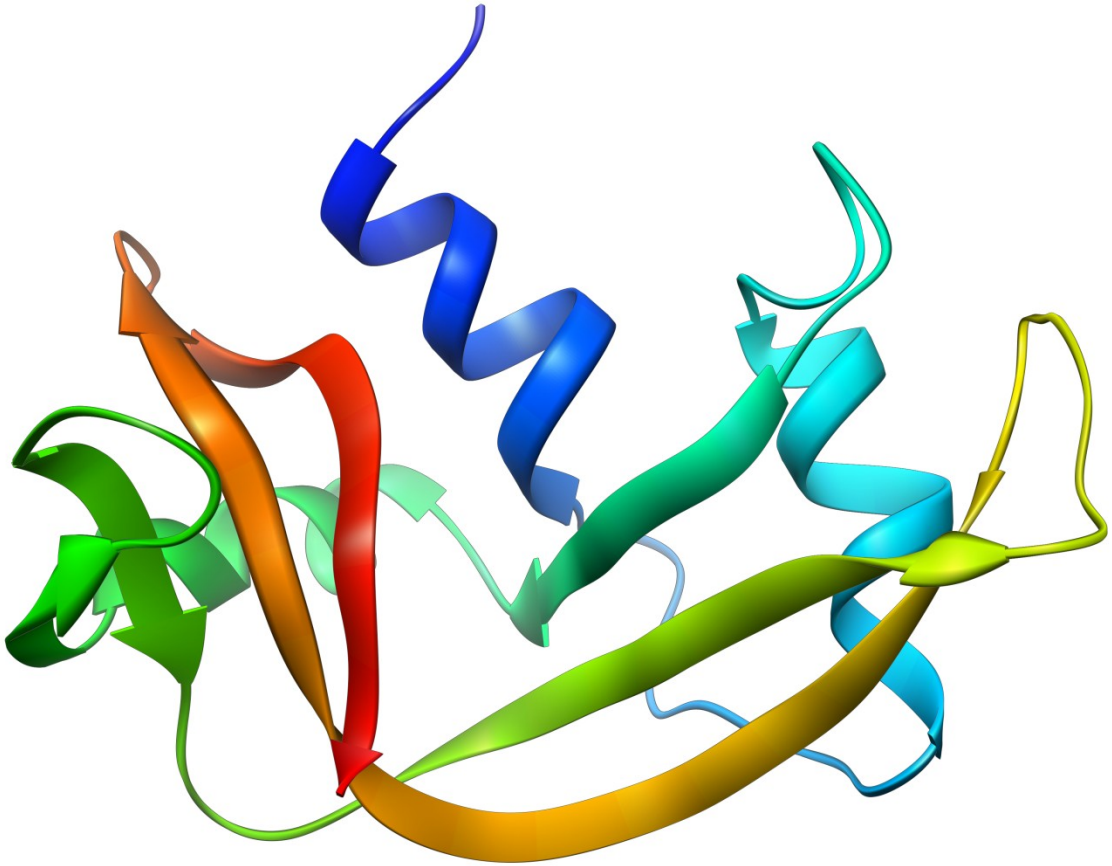
TOTAL RNA PURIFICATION

- **Goal: Isolate RNA from cellular components**
  - – Cells or tissue must be rapidly and efficiently disrupted
  - – Inactivate RNases
  - – Denature nucleic acid-protein complexes
  - – RNA selectively partitioned from DNA and protein

- **• Isolation from different tissues/sources raises different issues**

THE BAD GUY: RNASE

- RIBONUCLEASES



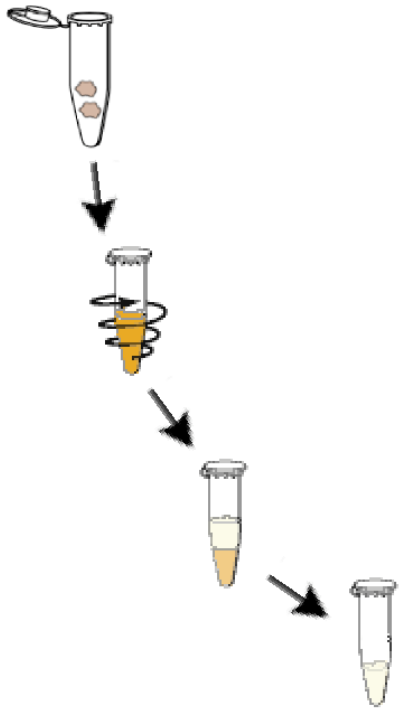
PROTECTING AGAINS RNASES

Wear gloves at all times!

- • Use RNase-free tubes and pipet tips
- • Use dedicated, RNase-free, chemicals
- • Pre-treat materials with extended heat (180°C for several hours), wash with DEPC-treated water, NaOH
- or H_2O_2
- • Supplement reactions with RNase inhibitors
- • Include a chaotropic agent (guanidine) in the procedure
- – Chaotropic agents such as guanidine inactivate and precipitate RNases and other proteins

ORGANIC EXTRACTION OF TOTAL RNA

1. Lyse/homogenize cells
2. Add phenol:chloroform:isoamyl alcohol to lysed sample, and centrifuge
3. Organic phase separates from aqueous phase
   - Organic solvents on bottom
   - Aqueous phase on top (contains total RNA)
   - Cellular debris and genomic DNA appears as a “film” of debris at the interface of the two solutions
4. Remove RNA solution to a clean tube; precipitate RNA and wash with ethanol, then resuspend RNA in water

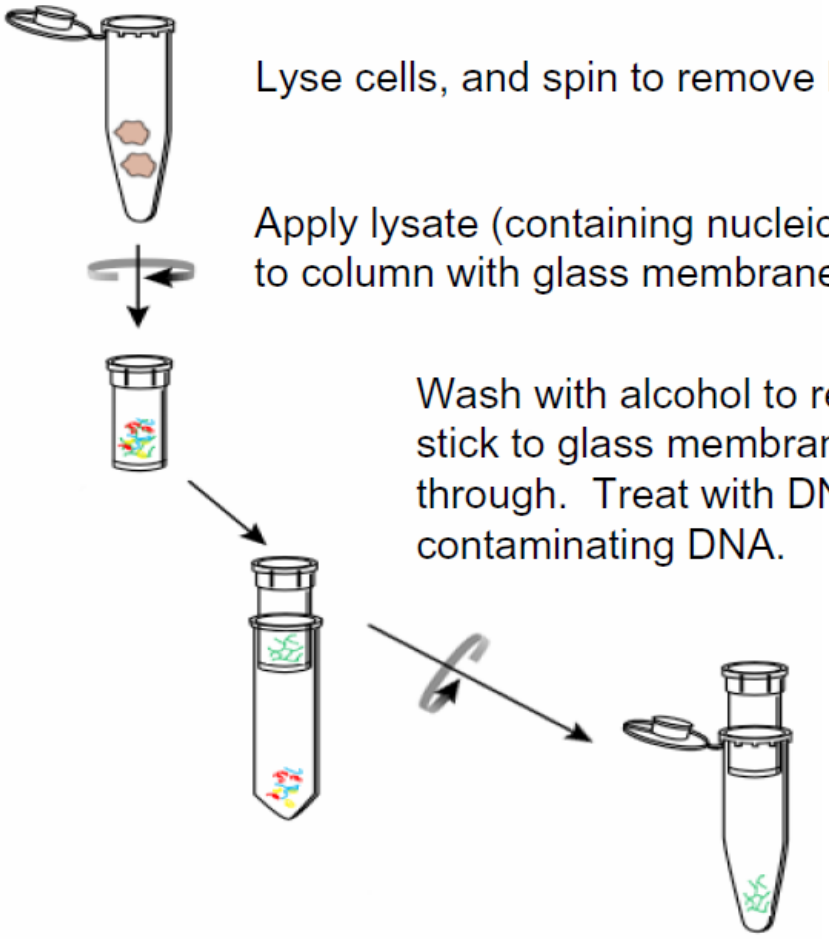


ORGANIC EXTRACTION OF TOTAL RNA

- **Advantages**
  - Versatile - compatible with a variety of sample types
  - Scalable - can process small and large samples
  - Established and proven technology
  - Inexpensive

- **Disadvantages**
  - Organic solvents
  - Not high-throughput
  - RNA may contain contaminating genomic

AFFINITY PURIFICATION OF TOTAL RNA



Lyse cells, and spin to remove large particulates/cell debris

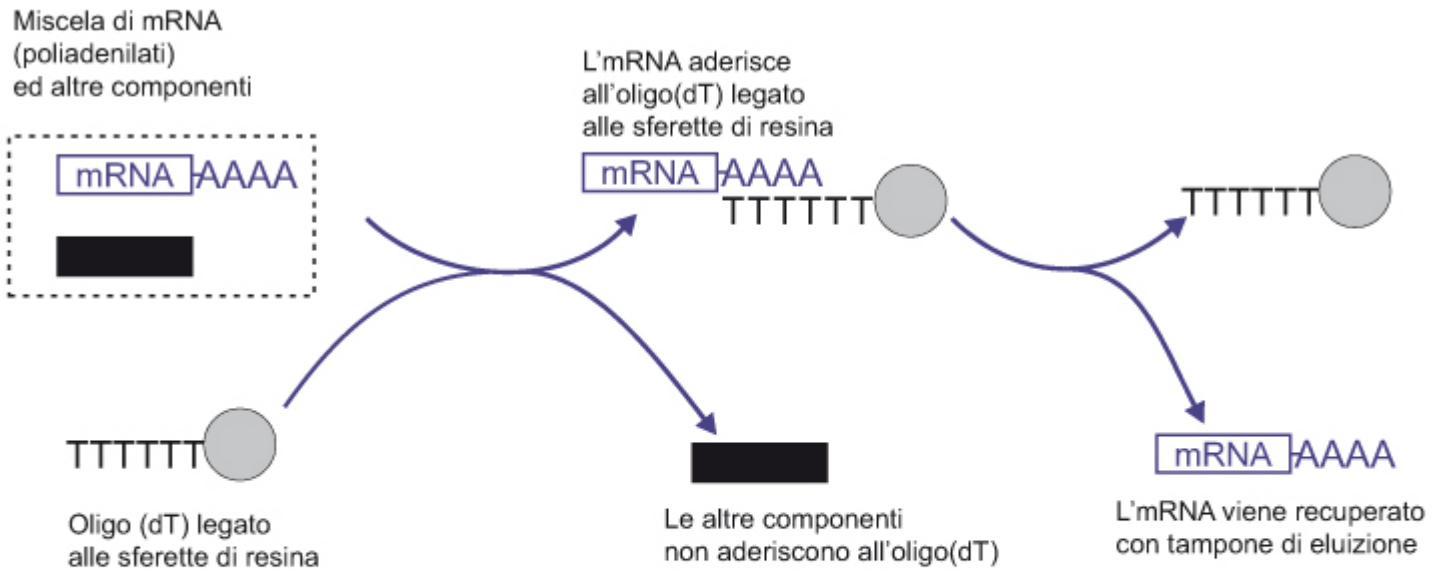
Apply lysate (containing nucleic acids and cellular contaminants) to column with glass membrane

Wash with alcohol to remove contaminants; nucleic acids stick to glass membrane while contaminants wash through. Treat with DNase enzyme to remove contaminating DNA.

Apply water to the column; purified RNA washes off the glass and is collected

ISOLATION OF POLYA (MESSENGER) RNA

- Only 2.5-5%
- mRNA molecules have a tail of A's at the 3' end (polyA tail)
- Oligo(dT) probes can be used to purify mRNA from other RNAs
- mRNA can be eluted from oligo(dT) matrix using water or low-salt buffer



RNA ANALYSIS

- Photometric measurement of RNA concentration
- UV 260 nm
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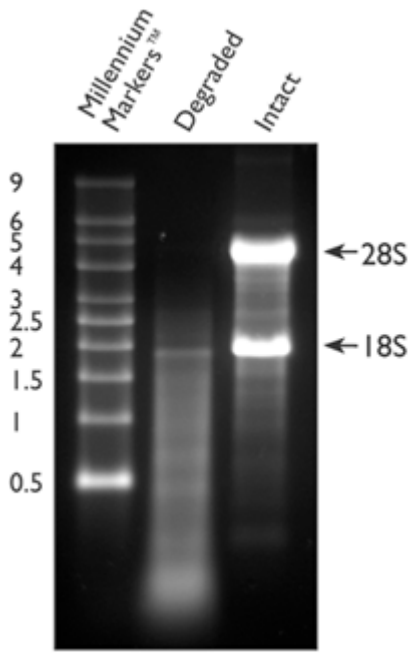

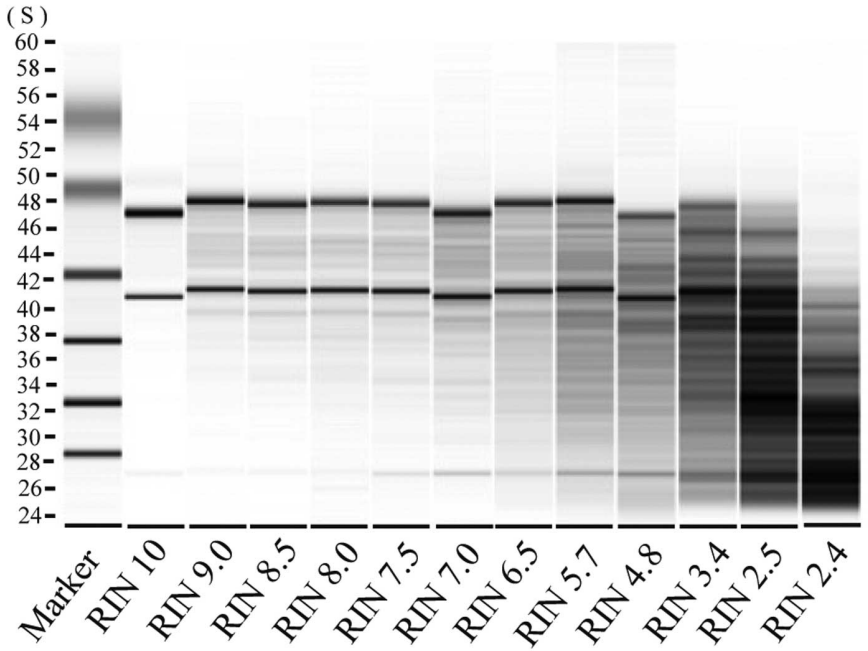

Electrophoresis

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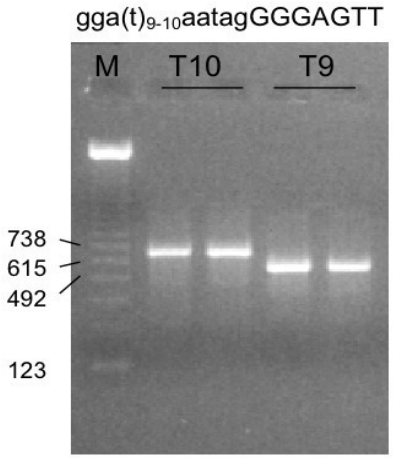
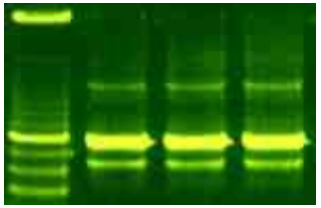
Staining with Ethidium Bromide and Sybr Green





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Spectrophotometry

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Determining Concentration

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  -

Determining Purity

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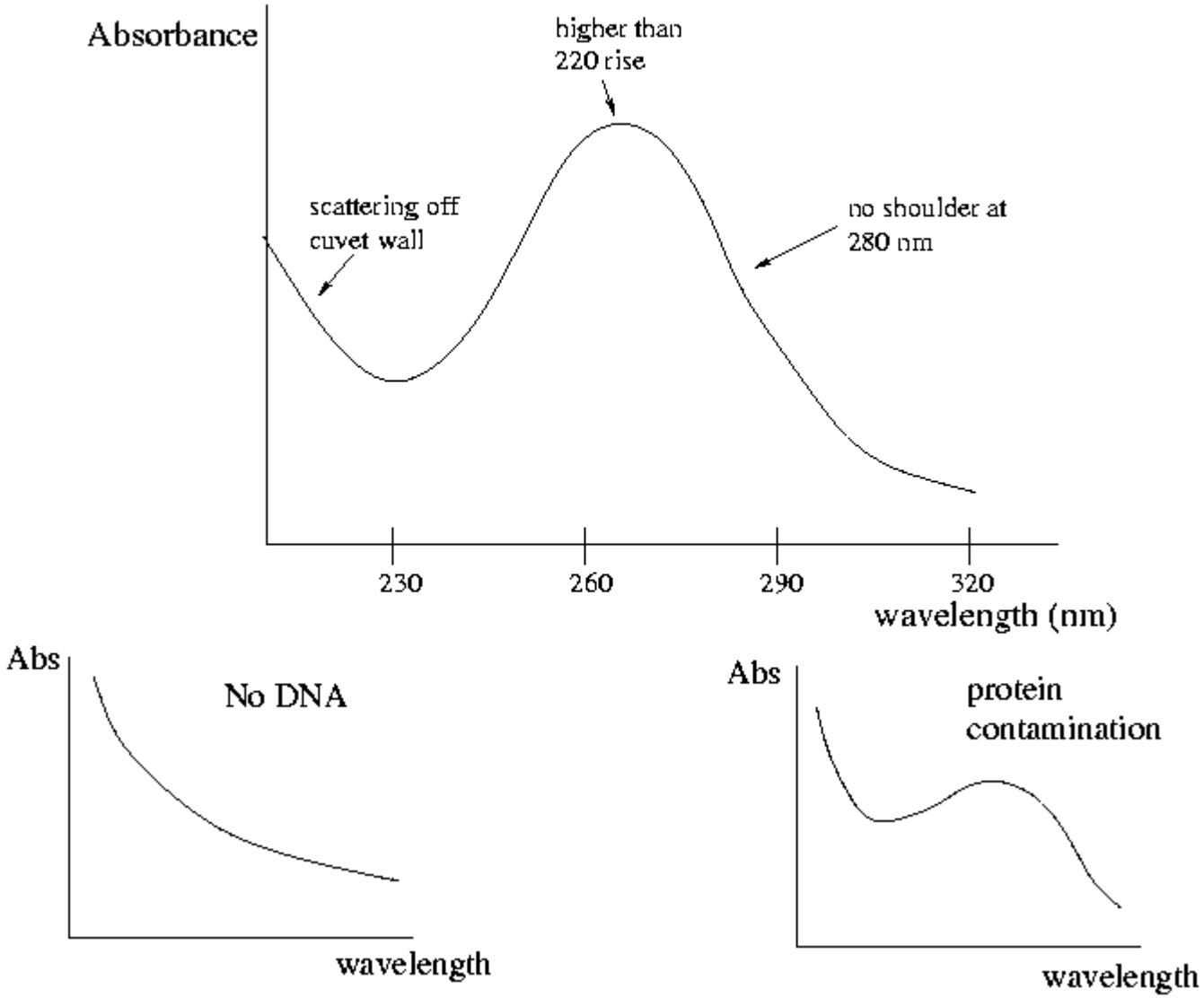
-
-

Absorbance of a pure DNA sample



Fluorometry

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Fluorometry

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Popular instruments

BIOANALYSER