Programma di Laboratorio

1. AMPLIFICAZIONE PCR DEL GENE G6PD CON LE SUE VARIANTI ALLELICHE: Un locus che ospita varianti alleliche del gene G6PD sarà amplificato mediante PCR. I prodotti di PCR saranno purificati dopo elettroforesi su gel e sottoposti a digestione utilizzando enzimi di restrizione. Il DNA digerito verrà separato mediante elettroforesi su gel.

2. AMPLIFICAZIONE DELLE VARIANTE DI RIPETIZIONE ALU ATTRAVERSO PCR: Le ripetizioni del numero di Alu su un locus del cromosoma 16 sarà determinata mediante PCR specifica. L'elettroforesi su gel di agarosio verrà utilizzata per monitorare le differenze nel numero di ripetizioni di Alu.

3. ANALISI DEI DATI E DISCUSSIONE: L'analisi Chi-square sarà utilizzata per confrontare le frequenze del genotipo di Alu all'interno del gruppo di studenti con quelle previste dall'equazione di Hardy-Weinberg. Le frequenze genotipiche della popolazione di classe possono anche essere confrontate con le frequenze genotipiche di un'altra popolazione in il database

1. Plasmid Mini preparation: postive andnegative control for G6PD variants (silica resina)
2. Preparation of genomic DNA
3. PCR to detect presence of Alu repeat
4. Purification of PCR product (silica resina)
5. Restriction digest of PCR fragment to detect G6PD variant
6. Agarose gel electrophoresis

Isolation of genomic DNA

The isolation method of choice is dependent upon:

The source of the DNA:
cells, tissue, bacteria, virus etc.;

The final application:
Plasmide prep for DNA cloning, PCR, restriction, sequencing, fingerprinting, library construction etc.;

The type of DNA:
genomic vs plasmid (size)

Why to prepare DNA

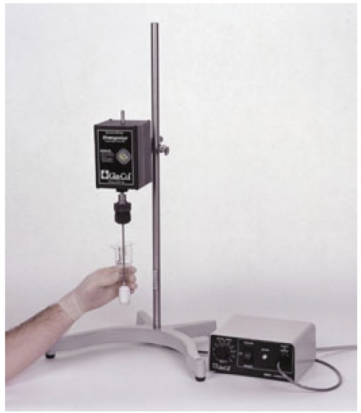
- Many applications require purified DNA.
- Purity and amount of DNA required (and process used) depends on intended application.
- Example applications:

➢ Tissue typing for organ transplant
➢ Detection of pathogens
➢ Human identity testing
➢ Genetic research
➢ Genotyping of mice
➢ Detection of mutations in cancer cells
➢ Measurement of modifications of DNA (DNA methylation)
➢ Cloning of “interesting” DNA elements (promoter, enhancer….)

DNA isolation

Tissue

Homogenise, chemically or mechanically
a) Ultrasould vibration (Vibrazioni ultrasoniche)
b) Homogenization (omogeneizzatore)
c) Freeze/thaw (Congelamento/scongelamento)



OMOGENIZZATORE

SONICATORE

Single cell suspension (bacteria, cells in cell culture or blood)

a) Cell wall rupture
Bacteria (Gram-) enzymatic (lysozyme) or SDS, NaOH (pH)
Yeast/fungi - zymolase

b) Cell membrane rupture
-Detergents: SDS, sarcosine, triton X-100, CTAB Proteinases; Proteinase K, Pronase E
-Chelators - EDTA
- Guanidine thiocyanate/chloride, urea

General steps in DNA Isolation

1. Genomic DNA

- SDS/Proteinase K (Organic method)
- Silica columns
- Alkaline method
- Automated method

2. Plasmid DNA

- Alkaline/SDS method
- Silica column method

3. Bacteriophage DNA

- PEG/Salt precipitation method

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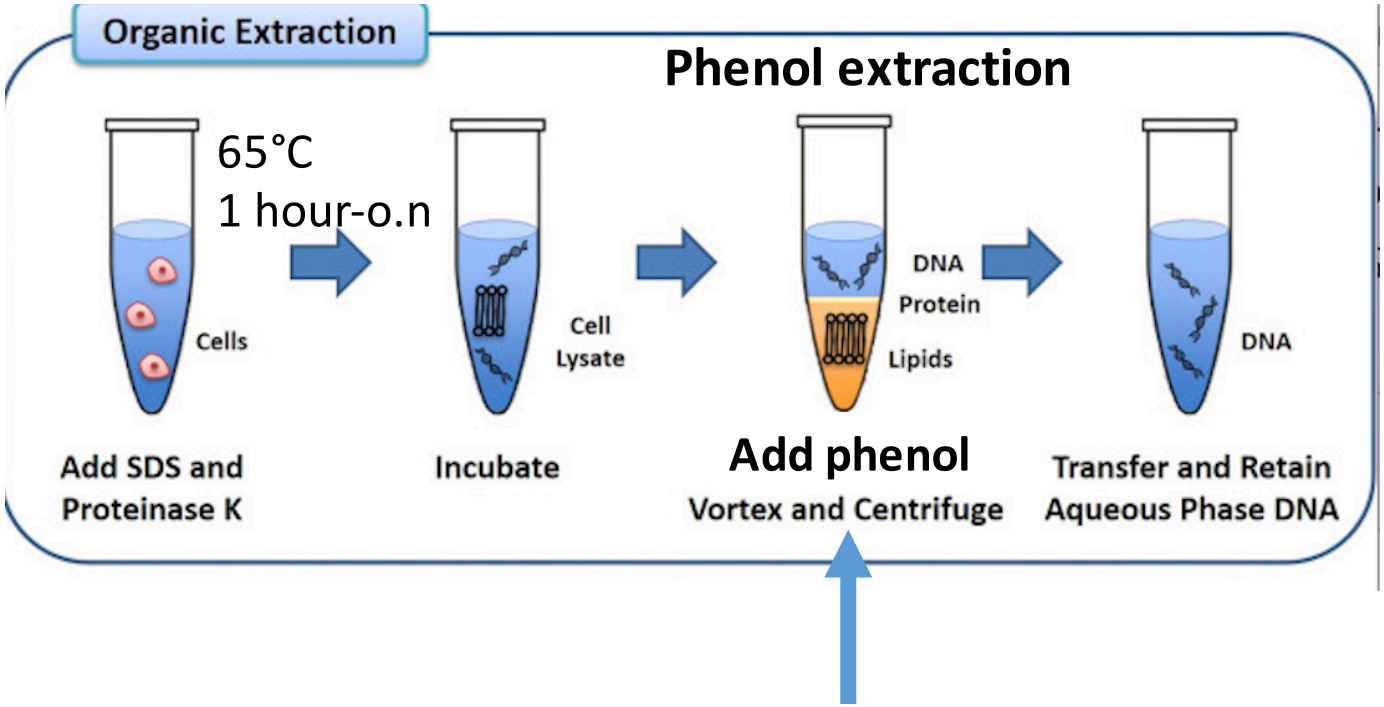
DNA Preparation from Cells – The organic method

- Open cells, digest proteins, extraction,
- Concentration of DNA - takes advantage of highly negative charge on nucleic acids

- **Lyse cells in buffer with SDS/PK /(DTT) (500 ul)**
  - SDS = detergent (solubilizes cell membrane)
  - PK = proteinase K (degrades proteins
  - DTT = reducing agent - breaks disulfide bonds in folded proteins
  - 65°C, agitation, 1 hour - over-night (depending on amount of DNA)
  - Why at 65°C?PK remains active but DNases are denatured and most RNA is degraded
- **Add equal volume of phenol (500 ul)**
  - Protein fragments and lipids attracted to hydrophobic phenol
  - Nucleic acids attracted to water
  - → separates aqueous phase (DNA, RNA) from organic phase (lipids, proteins)
- **Proceed with PCI extraction and DNA precipitation**

SDS: Sodium dodecyl sulfate

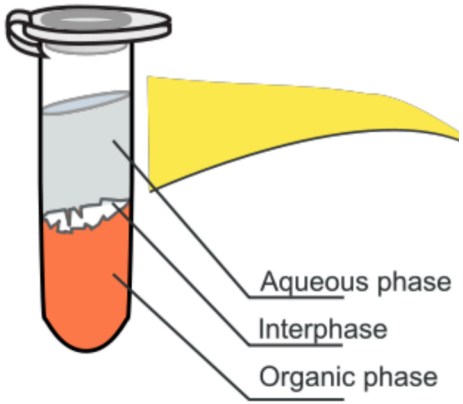
DNA Preparation from Cells – The organic method



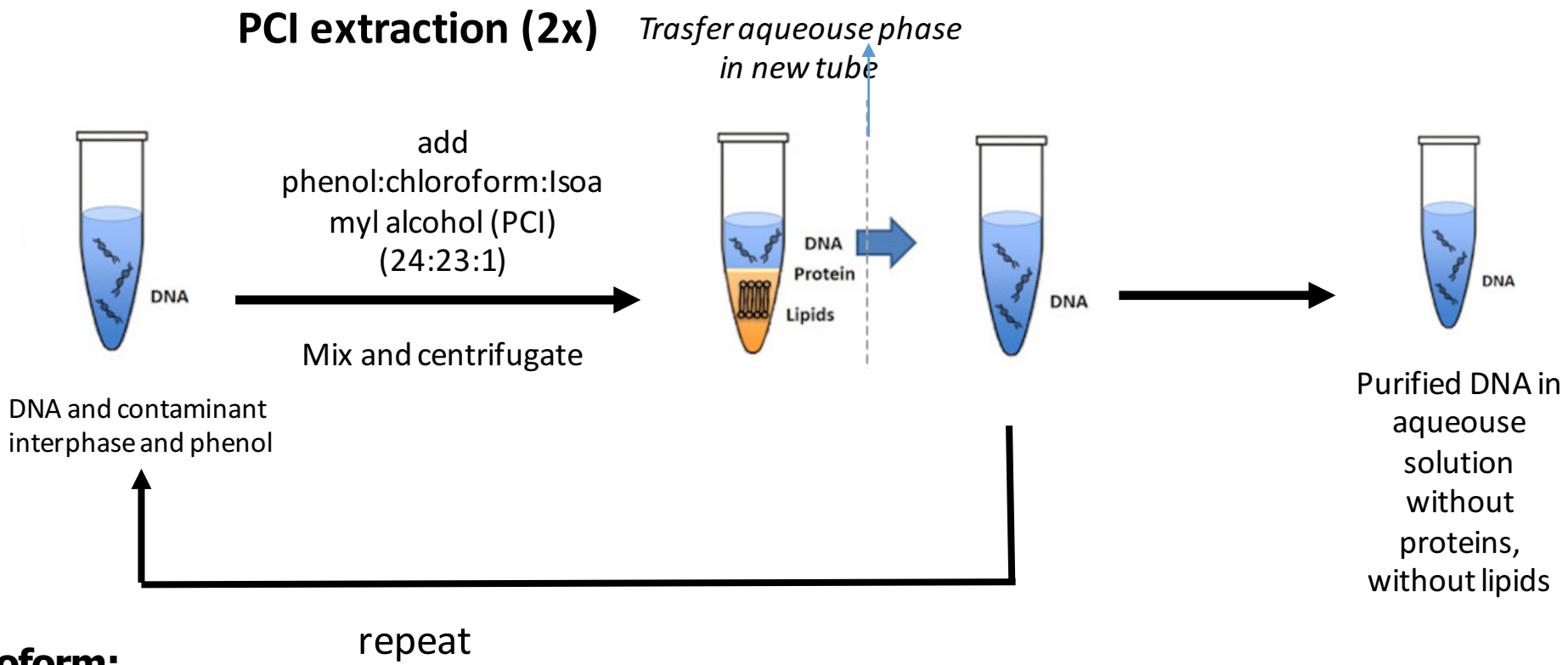
Aqueous phase contains DNA (+RNA) and rests of phenol and rests from interphase

Phenol:
is a strong denaturant of the protein; Denatured proteins, with hydrophobic groups exposed, become soluble in the phenolic phase or precipitate at the phenol-water interphase. It is a solvent of lipids and RNA molecules containing long sections of poles (A).
PHENOL IS HIGHLY TOXIC – WORK IN FUME HOOD

Phase separation



DNA Preparation from Cells – The organic method



Cloroform:

- completes protein denaturation
- removes lipids
- its high density it facilitates the separation of the aqueous phase (containing the deproteinized DNA) from the organic one (phenolic) stabilizing the interface between the two phases.

NOTE: DNA at relative low concentration, still some organic solvents can be presnet (PCI)

Isoamyl alcohol:

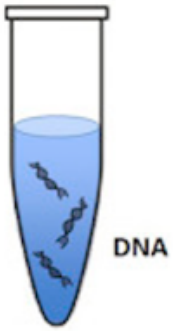
Reduces the foam that forms during the course extraction.

PCI IS HIGHLY TOXIC – WORK IN FUME HOOD

DNA Precipitation

1. Precipitation

DNA is rendered insolable → precipitates and can be isolated → Precipitate is dissolved in storage buffer

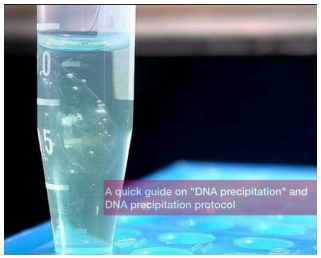


Set set up of DNA precipitation

1. Na-Acetate: 0,3M final conc
+ 1x volume Isopropanol
2. Na-Acetate: 0,3M final conc
+ 3x volume Ethanol
3. NaCl: 0,5M final conc
+1x volume Isopropanol
4. Nacl: 0,5M final conc
+3x volume Ethanol

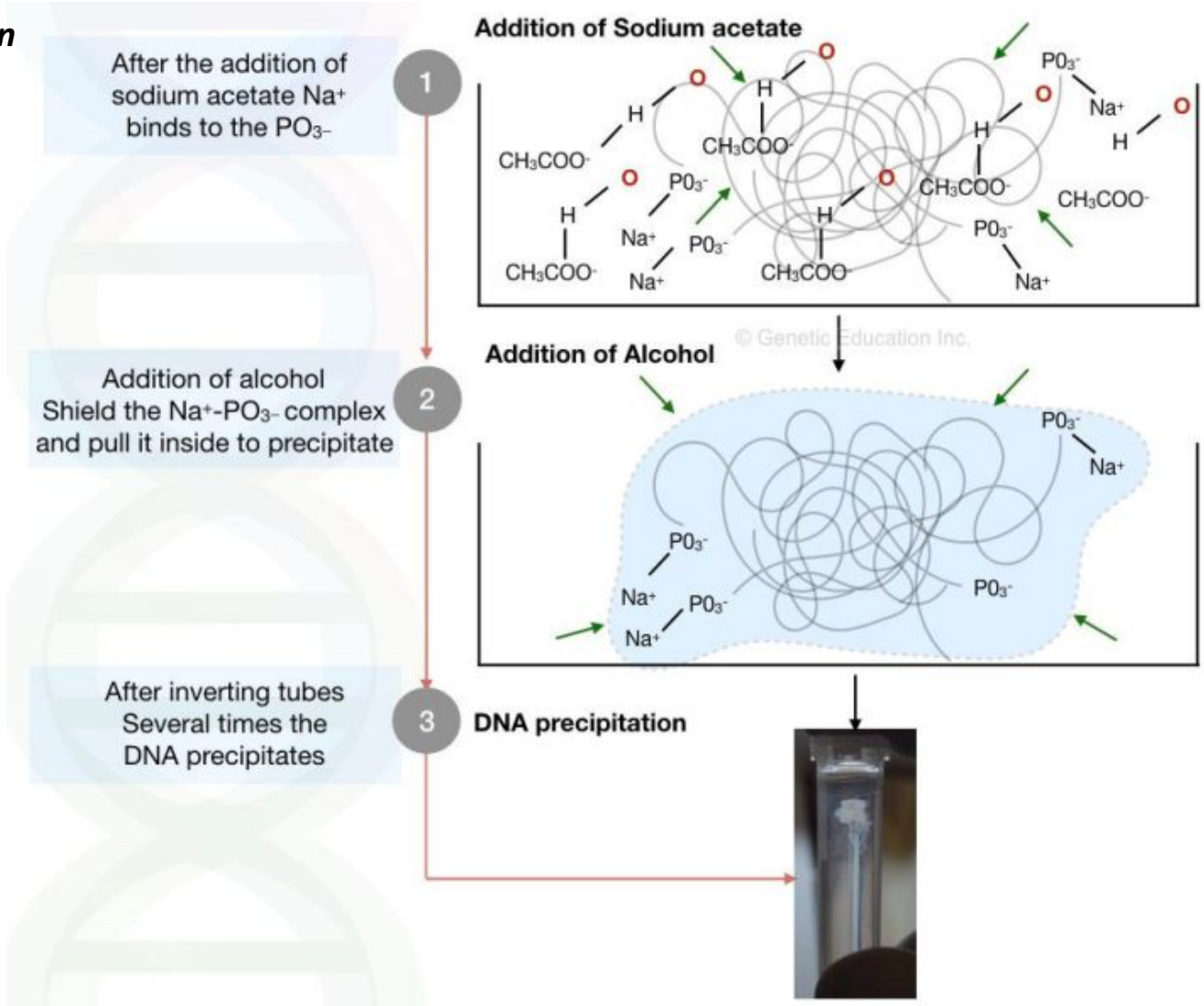
Practical example:

500 ul DNA solution
+ 55,5 ul 3M Na-Acetate
(final conc. 0,3M)
+ 555,5 ul Isopropanol
-20°C for 1 hour (DNA precipitates)
Centrifuge 13.000rpm 30 minutes at 4°C



centrifugation

pellet



DNA Precipitation

2. Washing of with 70% Ethanol

- Remove liquid from tube
- Carefully add 70% Ethanol
- Pellet does not dissolve
- Centrifuge for 10 seconds to force pellet to bottom ot tube
- Remove supernatant
- Let pellet air dry at room temperature for 5-10 minutes
- Add storage buffer

Why: 70% Ethanol disolves salt (NaCl or Na-Acetate) that can co-precipitate

DNA storage

Buffer

• DNA, RNA and oligonucleotide are storage in 1xTE solution (1 mM EDTA, Tris-HCl, pH 7.2): pH should always be <7,5 (risk of alkaline hydrolysis)

Temperature:

•Everyday use: +4°C (generic samples)
•Storage for long time: -20°C or -80°C; (long term storage, valuable samples)
•A precipitate in 70% ethanol, DNA/RNA can be stored at +4°C almost indefinitely, without loosing integity

Note: Phenol and DNA precipitation can be used for other purposes:

- ***Preparation of DNA from cells and tissues***

- ***To remove proteins from DNA***

  For example restriction digest plasmid:

  - **digest with EcoRI**
  - **phenol extraction**
  - **DNA precipitation**
  - **70% EtOH wash**
  - **dissolve DNA in new buffer**
  - **digest with BamHI**

- ***To change buffer of DNA solution***

  - **You need DNA in another buffer with different salt concentration**
  - **DNA precipitation**
  - **70% EtOH wash**
  - **dissolve DNA in new buffer**

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   - Automated method

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3. **Bacteriophage DNA**

   - PEG/Salt precipitation method

DNA preparation using silica columns

New 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

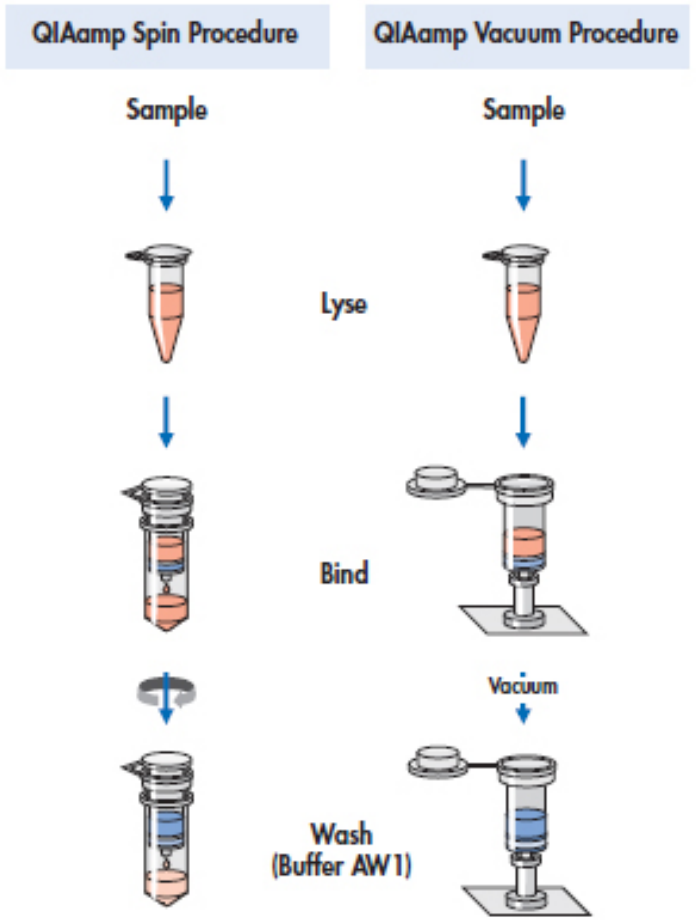
- Speed and convenience
- No organic solvents
- Amenable to automation/miniaturization

Disadvantage

- DNA fragmentation
- Low yield

For example: https://www.qiagen.com/it/resources/resourcedetail?id=62a200d6-faf4-469b-b50f-2b59cf738962&lang=en

DNA preparation using silica columns



centrifuge vacuum

Main steps in genomic DNA preparation:

Cell lysis in buffer containing high concentration of SDS and high concentration of EDTA (binds bivalent ions such as Mg2+)

Passage on an insoluble resin (silica resins) that specifically binds DNA in the presence of salts

Resin washing with buffers that allow separation from contaminants (proteins, etc…)

Note: same principal like in mini-prep for plasmids, however genomic DNA is eliminated in mini prep… (how?)

General steps in DNA Isolation

1. Genomic DNA

- **SDS/Proteinase K (Organic method)**
- **Silica columns**
- **Alkaline method**
- **Automated method**

2. Plasmid DNA

- **Alkaline/SDS method**
- **Silica column method**

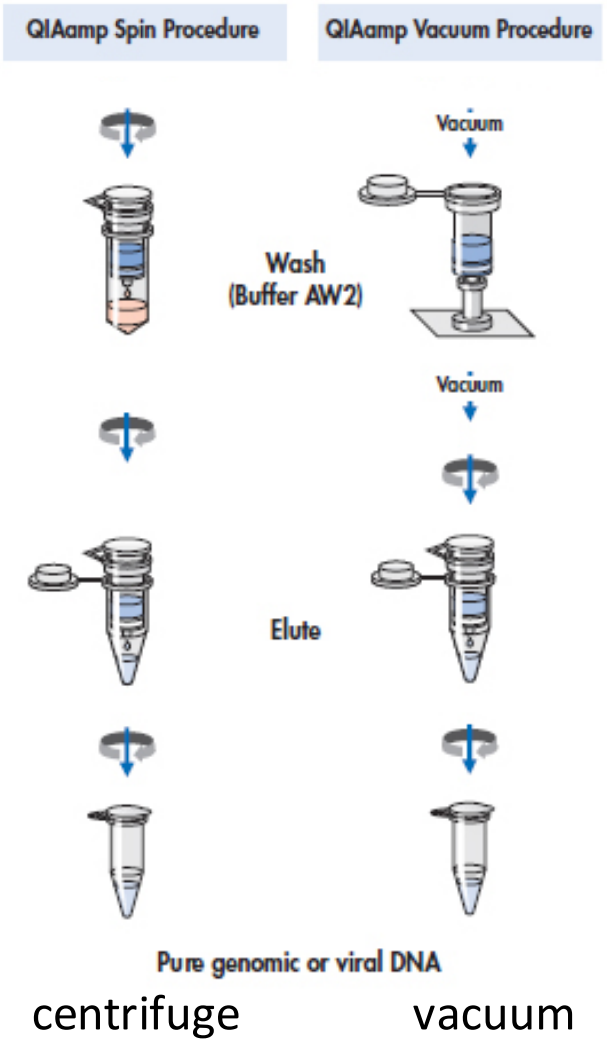
Check lecture on plasmid prep - cloning

3. Bacteriophage DNA

- **PEG/Salt precipitation method**

Not relevant for us

DNA preparation using silica columns



centrifuge vacuum

More Resin washing with buffer that allow separation from contaminants (proteins, etc…)

Elution of DNA from the resin with water or solutions with a low concentration of salts that facilitate its detachment

DNA preparation

Alternative resin: anion exchange resins with DEAE groups (diethylaminoaethyl) to bind DNA (eluizione con sali e precipitazione).

RNA contamination in DNA preparations

- Addition of Rnase to DNA preparation to remove RNAses – purify DNA after RNAase treatmetn with column

Isolation of RNA

Total RNA contains:

- Messenger RNA (mRNA): 1-5%
  Serves as a template for protein synthesis

- Ribosomal RNA (rRNA): >80%
  Structural component of ribosomes

- Transfer RNA (tRNA): 10-15%
  Translates mRNA information into the appropriate amino acid

- other small RNAs: miRNAs, siRNAs, snoRNAs, snRNAs, etc…

RNA is unstable:

RNA is a molecule that is easily degraded by ribonucleases (RNases), enzymes that can highly abundant – also in the laboratory environment.

RNases do not require enzymatic cofactor, resitant to high temperatures, abundantly present

ALWAYS WHEN WORKING WITH RNA:
….to minimize their action and reduce risk of contamination with RNases:

- Be careful not to introduce exogenous RNases (wear gloves).
- Use only solutions and materials that are sterile or treated with DEPC (diethylpyrocarbonate; TOXIC!!)) and autoclaved– binds covalently to histidine, lysine, cystein and tyrosin - protein inactivation
- Store the RNA samples on ice during their handling.
- Use dedicated materials (test tubes, tips, filters, etc.) and solutions for RNA prep
- Clean working surface

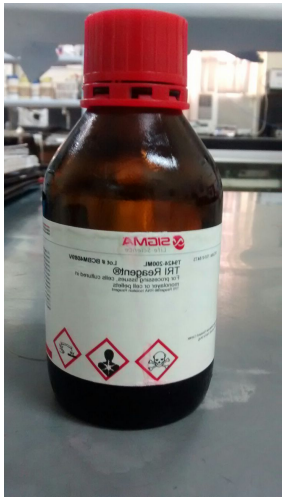
RNase Inhibitors:
- **add proteins that act RNAse inhbitors to reactions involving RNA**
- **Treat solutions when possible with DEPC+autoclave**

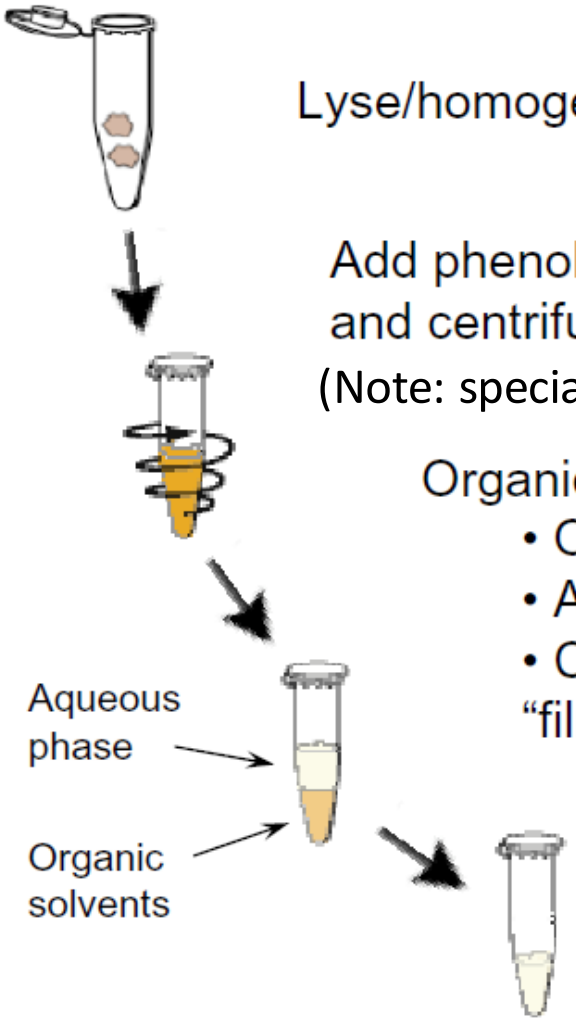
RNA purification techniques

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

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

Organic extraction of total RNA





Lyse/homogenize cells

Add phenol:chloroform:isoamyl alcohol to lysed sample, and centrifuge

(Note: specialized reagetns exist and are communy used: Trizol – Trireagent)

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

Remove RNA solution to a clean tube; repeat PCI extraction precipitate RNA and wash with ethanol, then resuspend RNA in water

Conditions for precipitations: the same like for genomic DNA

Note: NO Proteinase K digest; chromatin precipitates at interphase

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 DNA

DNase treatment – Phenol extraction – precipitation
DNase treatment – purification via resin

RNA purification techniques

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  - Affinity purification

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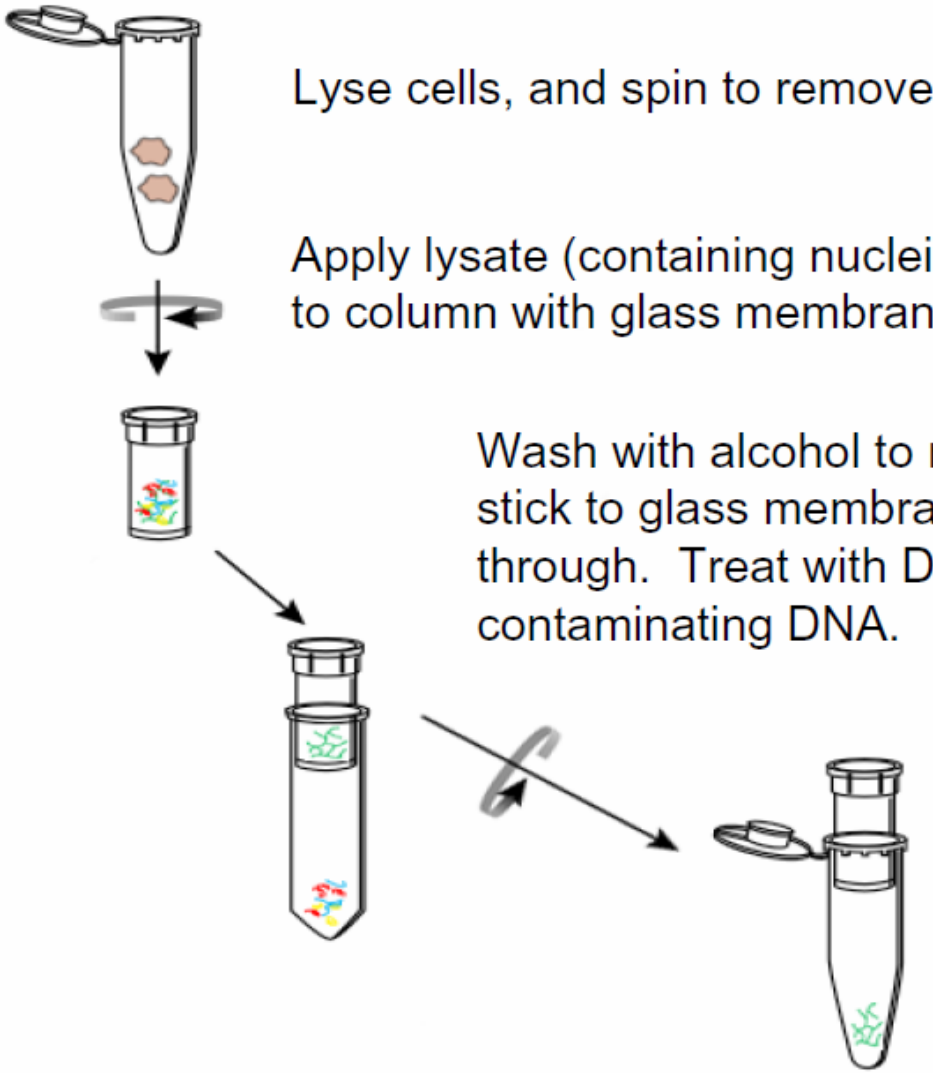
Affinity purification of total RNA



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

Affinity purification of total RNA

Advantages

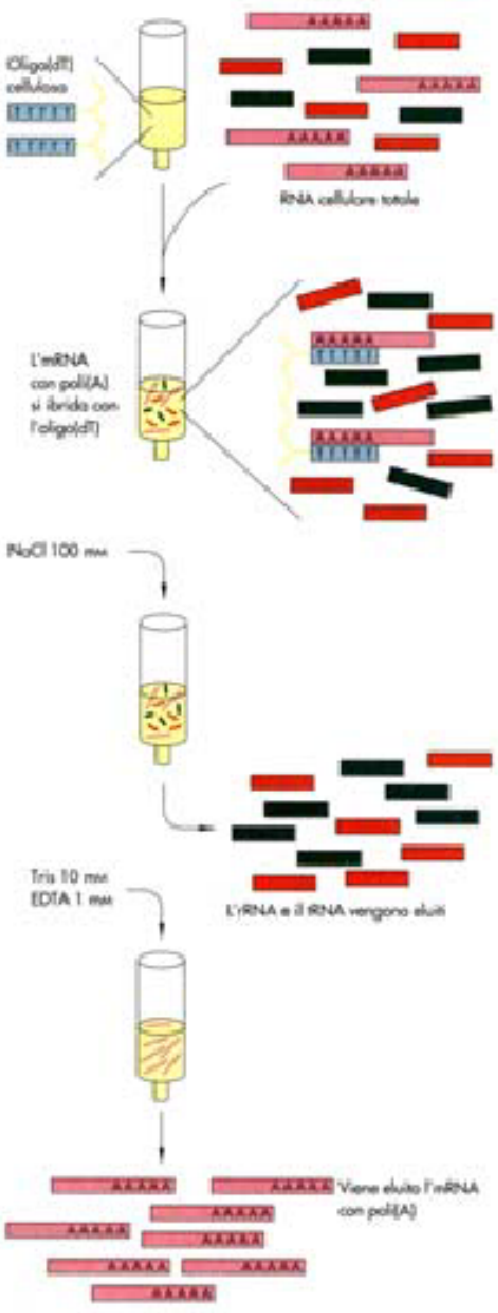
- Eliminates need for organic solvents
- Compatible with a variety of sample types (tissue, tissue culture cells, white blood cells, plant cells, bacteria, yeast, etc.)
- DNase treatment eliminates contaminating genomic DNA
- Excellent RNA purity and integrity

RNA purification techniques

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

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

mRNA purification



Solo gli mRNA possiedono "code" di poli(A) lunghe 30-150 residui. Le sequenze di oligo(dT) sono immobilizzate su supporti solidi, in genere di cellulosa.

L'RNA totale viene denaturato e quindi applicato alla colonna in una soluzione salina concentrata (NaCl 500 mM).

Si effettuano molti lavaggi con una soluzione salina meno concentrata (NaCl 100 mM), per rendere più selettivo il legame tra RNA e l'oligo(dT).

Aggiungendo una soluzione di TE (Tris/EDTA) si recupera l'RNA messaggero legato alla colonna.

Eventualmente si ripete il ciclo applicazione-lavaggi-eluizione.

La frazione poli(A)+ RNA contiene sempre una minima parte di RNA non poliadenilato.

Total RNA preparation is passed through a column consisting of a coated polymer with oligo (dT).

Only polyadenylated mRNA will hybridize with oligo (dT), while other species will be eliminated by washing with low concentration pads saline.

The final eluate will be made up of mixture of all mRNA species present in the cell at the time extraction.

Storage of RNA

Buffer

- 1xTE solution (1 mM EDTA, Tris-HCl, pH 6.5): pH should always be <7 (high risk of alkaline hydrolysis)

– no DEPC treatment possible (DEPC destroys Tris)

- RNase free water (DEPC treated); check pH of water: <7.0

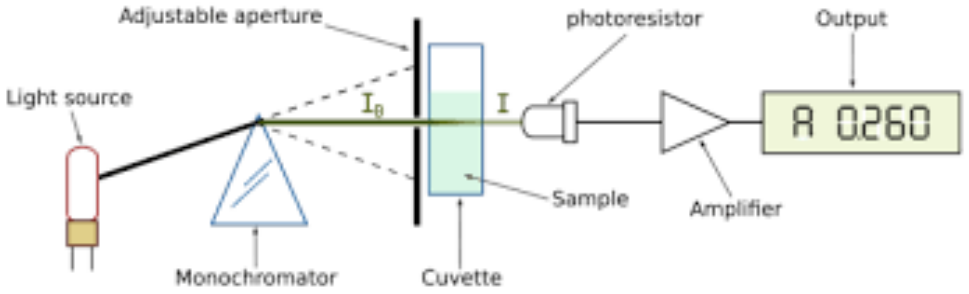
Temperature:

- Always -80°C when aqueous solution
- As precipitate in 70% ethanol, DNA/RNA can be stored at -20°C almost indefinitely, without loosing integity

Quantification of DNA and RNA concentration and purity

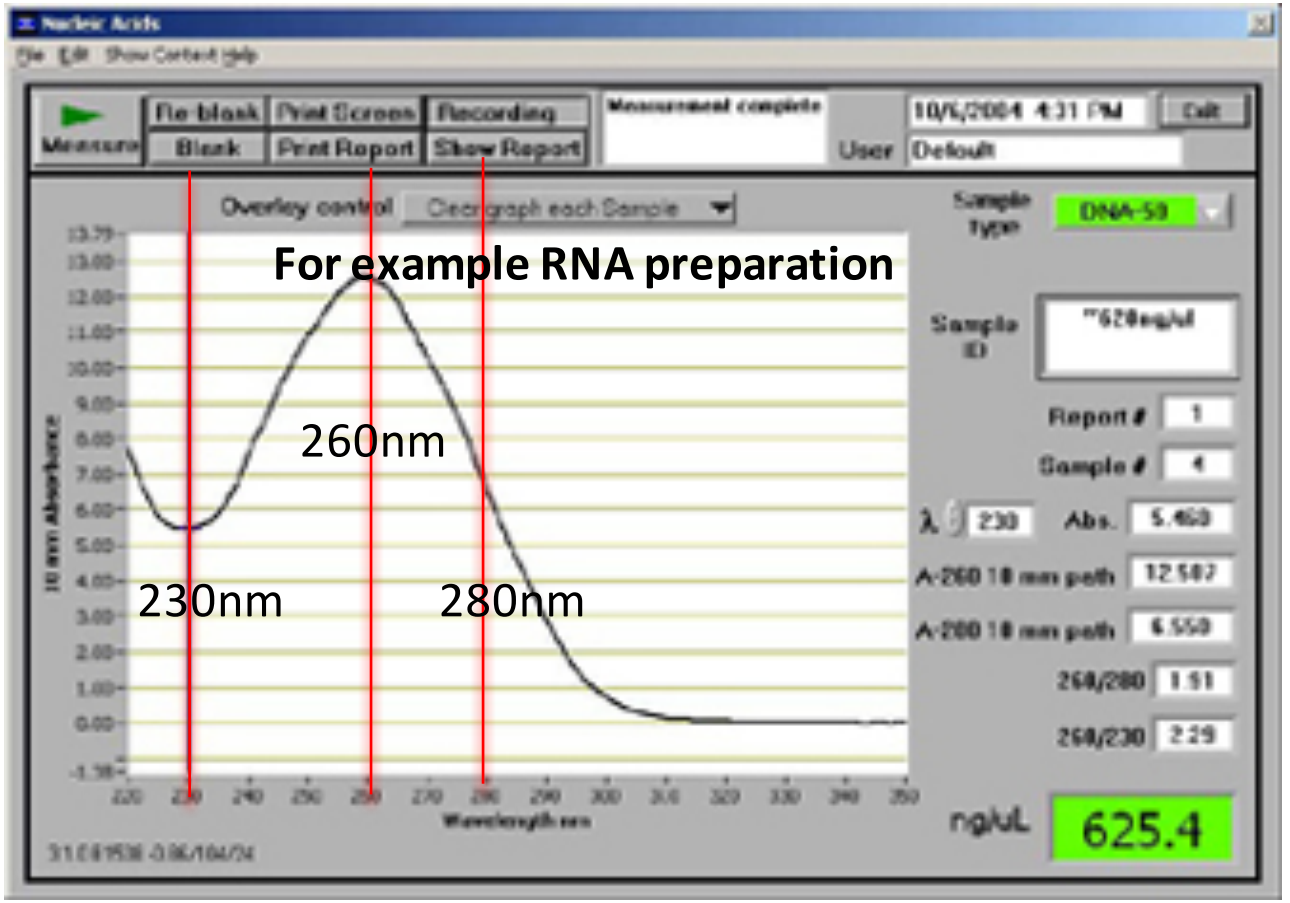
It operates on Beer' s law:

When monochromatic light (light of a specific wavelength) passes through a solution there is usually a quantitative relationship (Beer's law) between the solute concentration and the intensity of the transmitted light, that is, the more concentrated the specimen is, the less light is transmitted through it.



Measurement with a SPECTROPHOTOMETER

→ **Measure absorbance of DNA/RNApreparation = concentration**



Maximum absorbance:

RNA, DNA: 260 nm

Protein: 280 nm

Solvents/carbohydrates: 230 nm

Quantification of DNA and RNA concentration and purity

- **Concentration**

Parameters: Dillution and Absorbance (260 nm)

- DNA (mg/mL)= A_{260} x dillution x50

Example: take 5 ul from preparation and dillute in 500 ul = Dillution: 1:100;

Absorbance at 260 nm measured: 0,234;

concentration = 1,170mg/mL

- **RNA (mg/mL)= A_{260} x dillution x40**

Example: take 5 ul from preparation and dillute in 500 ul = Dillution: 1:100;

Absorbance at 260 nm measured: 0,344;

concentration = 1,376mg/mL

Note: Slide corrected!

Quantification of DNA and RNA concentration and purity

- **Purity** (contamination with proteins/carbohydrates/solvent)

- **ratio A_{260}/A_{280}**
- **ratio A_{260}/A_{230}**

Nucleic acids absorb at A=260nm

Proteins absorb at A=280 nm

Ratio A260/A280 = quantification of protein contamination

For DNA preparations: 1.6-1.8

For RNA preparations: 1.8-2.0,

Il ration is above value: **contamination with proteins**

Ratio A260/A230 = quantification of contamination with carbohydrates and phenol (solvents)

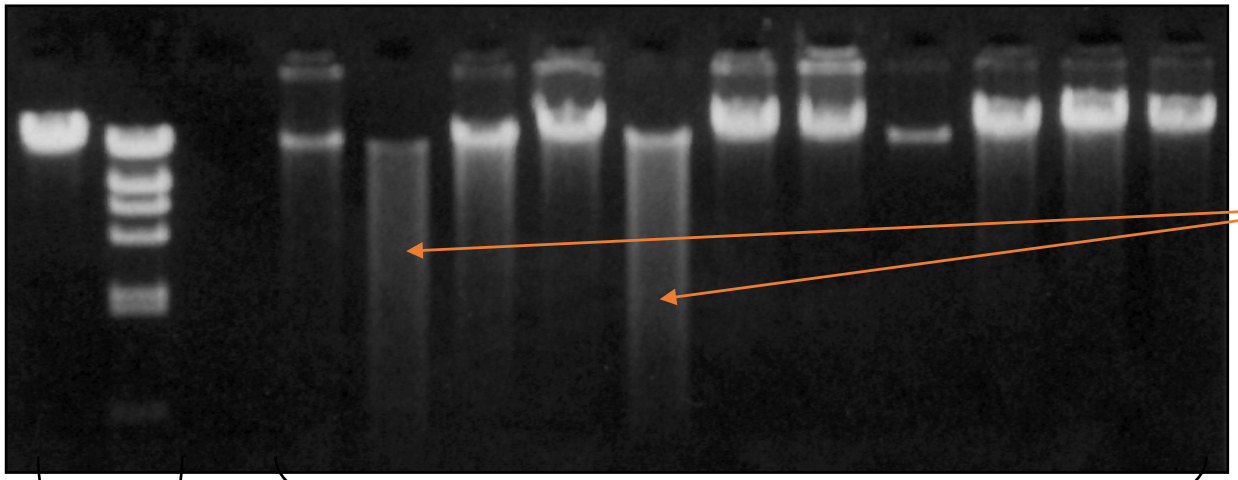
For RNA and DNA: ratio must be ca. 2.2

ratio < 2,2: contamination with solvent/carbohydrate

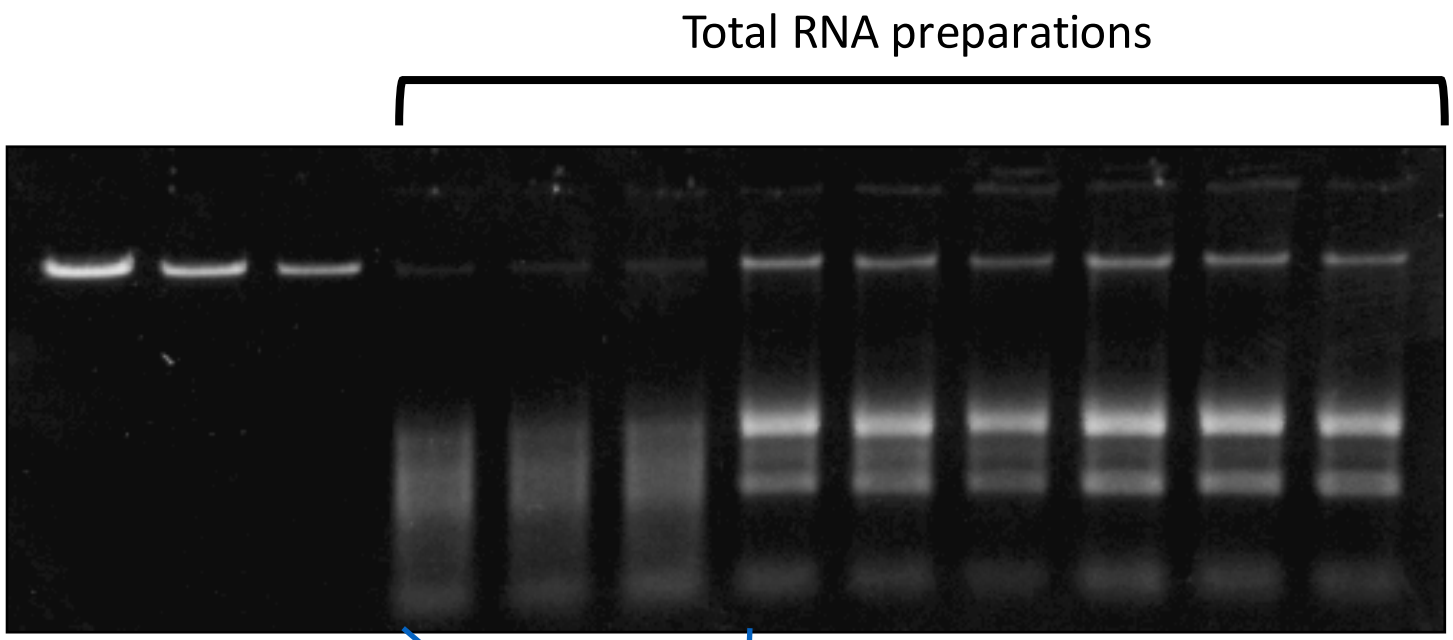
Determination of DNA integrity

Easiest method: Gel electrophoresis

Gel di agarosio, le bande di DNA sono state messe in evidenza introducendo nel gel etidio bromuro una sostanza che si inserisce tra le basi del DNA diventando fuorescente.

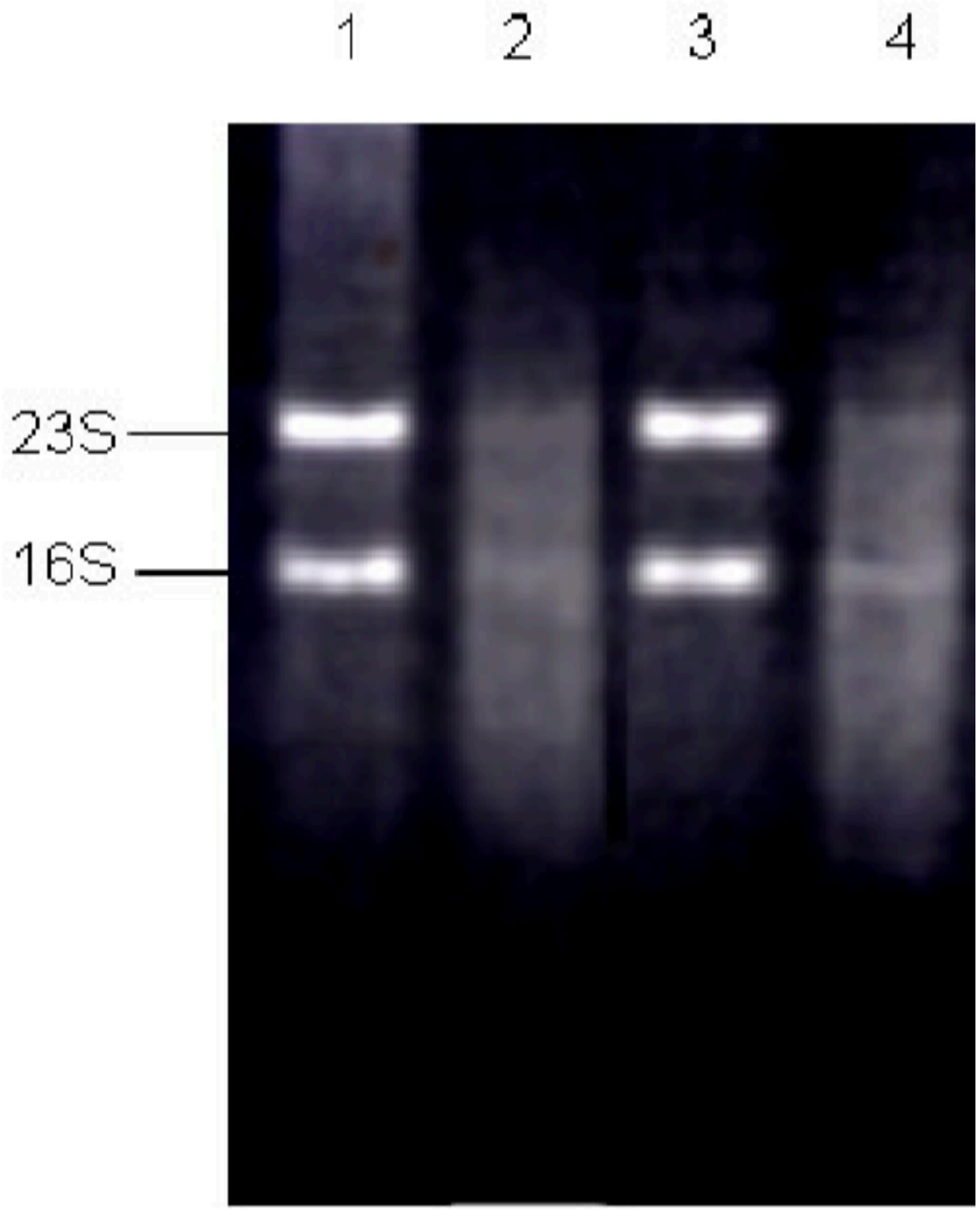


Determination of RNA integrity



Attenzione: Se si è purificato mRNA, esso appare come una scia

Determination of RNA integrity – poly A RNA (mRNA)



polyA RNA (mRNAs)

Lane 1+2 Patient 1 blood cells used to prepare RNA

Lane 1: total RNA

Lane 2: poly A RNA fraction prepared from total RNA

→ smear! (scia)– **normal pattern for poly A** RNA (no degradation!!)

→ **Lane 3+4 Patient 1 blood cells used to prepare RNA**

Lane 3: total RNA

Lane 4: poly A RNA fraction prepared from total RNA

→ smear! (scia)– **normal pattern for poly A** RNA (no degradation!!)