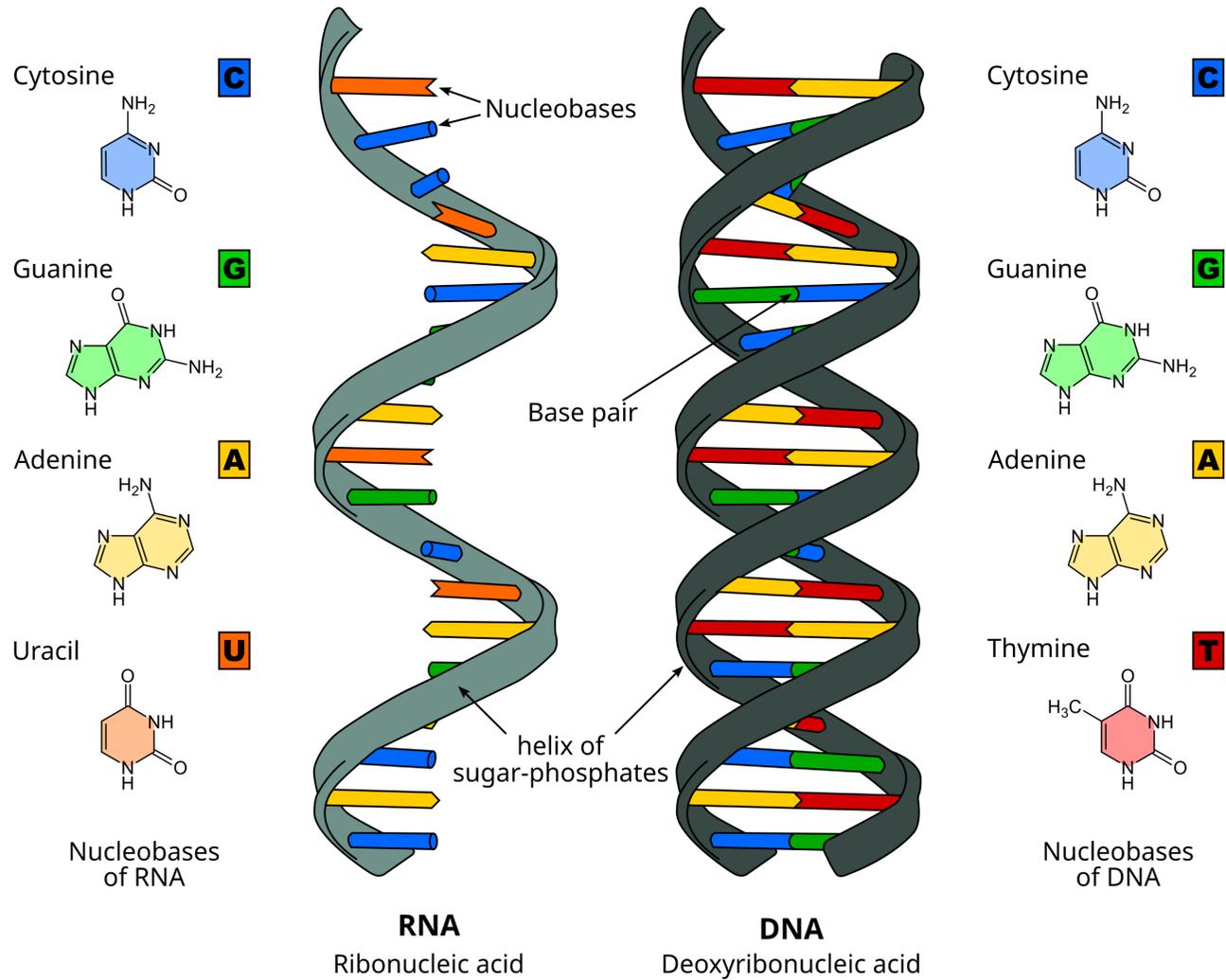
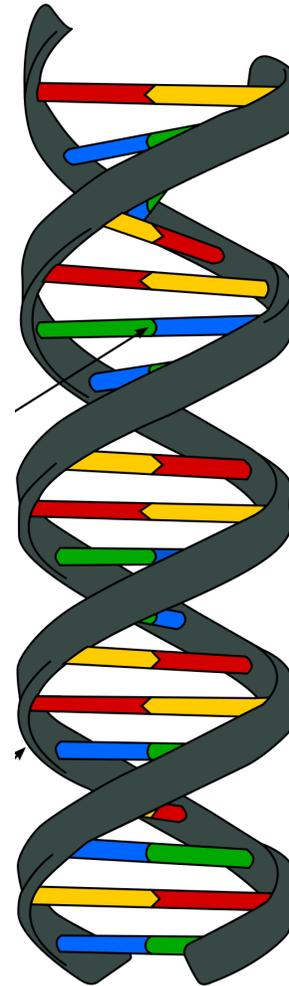


TOPIC 1: DNA and RNA Preparation



TOPIC 1: DNA Preparation

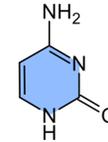


DNA

deoxyribonucleic acid

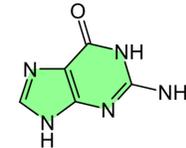
Cytosine

C



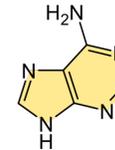
Guanine

G



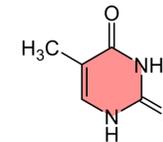
Adenine

A



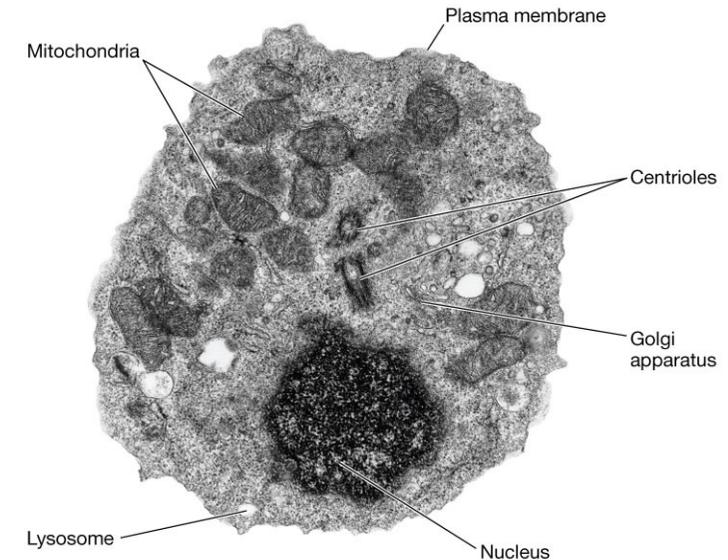
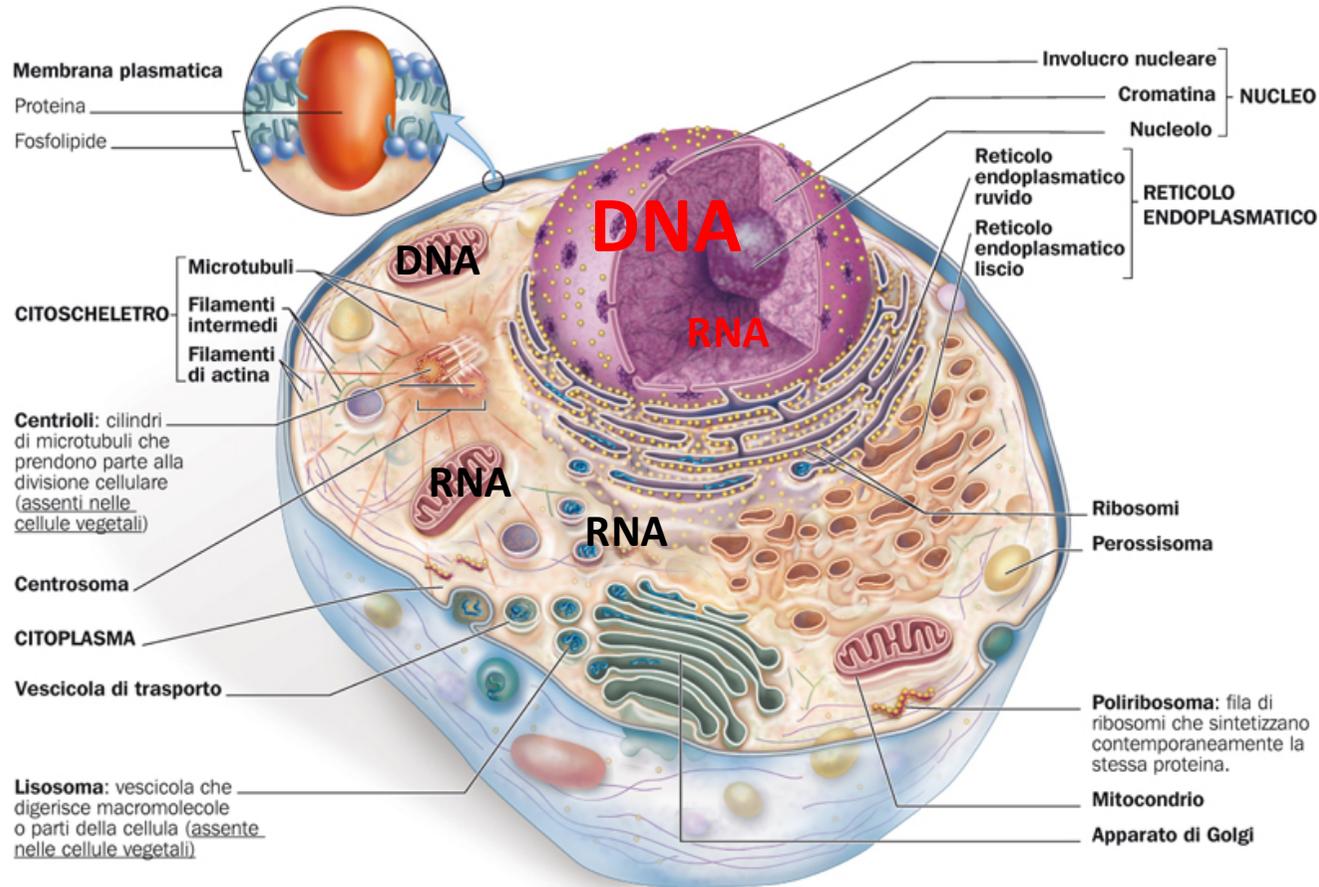
Thymine

T



Nucleobases
of DNA

LA CELLULA EUCARIOTE



10-100µm

**Genoma umano:
 3,289,000,000 nucleotidi**

- Dimensioni: circa dieci volte più grandi delle cellule procariotiche (10-100 µm)
- La **membrana plasmatica** racchiude il materiale cellulare, lo separa dall'ambiente e regola il passaggio di sostanze cellula/esterno
- **Compartimentazione interna:** all'interno della membrana si trova il **citoplasma**, l'insieme del contenuto cellulare, comprendente il **citosol** (soluzione acquosa di piccole e grandi molecole) ed una serie di **organuli**, compartimenti funzionalmente specializzati delimitati da membrana o comunque strutturalmente separati (Apparato di Goghi; Mitocondrio; Reticolo endoplasmatico)

EUCARIOTI

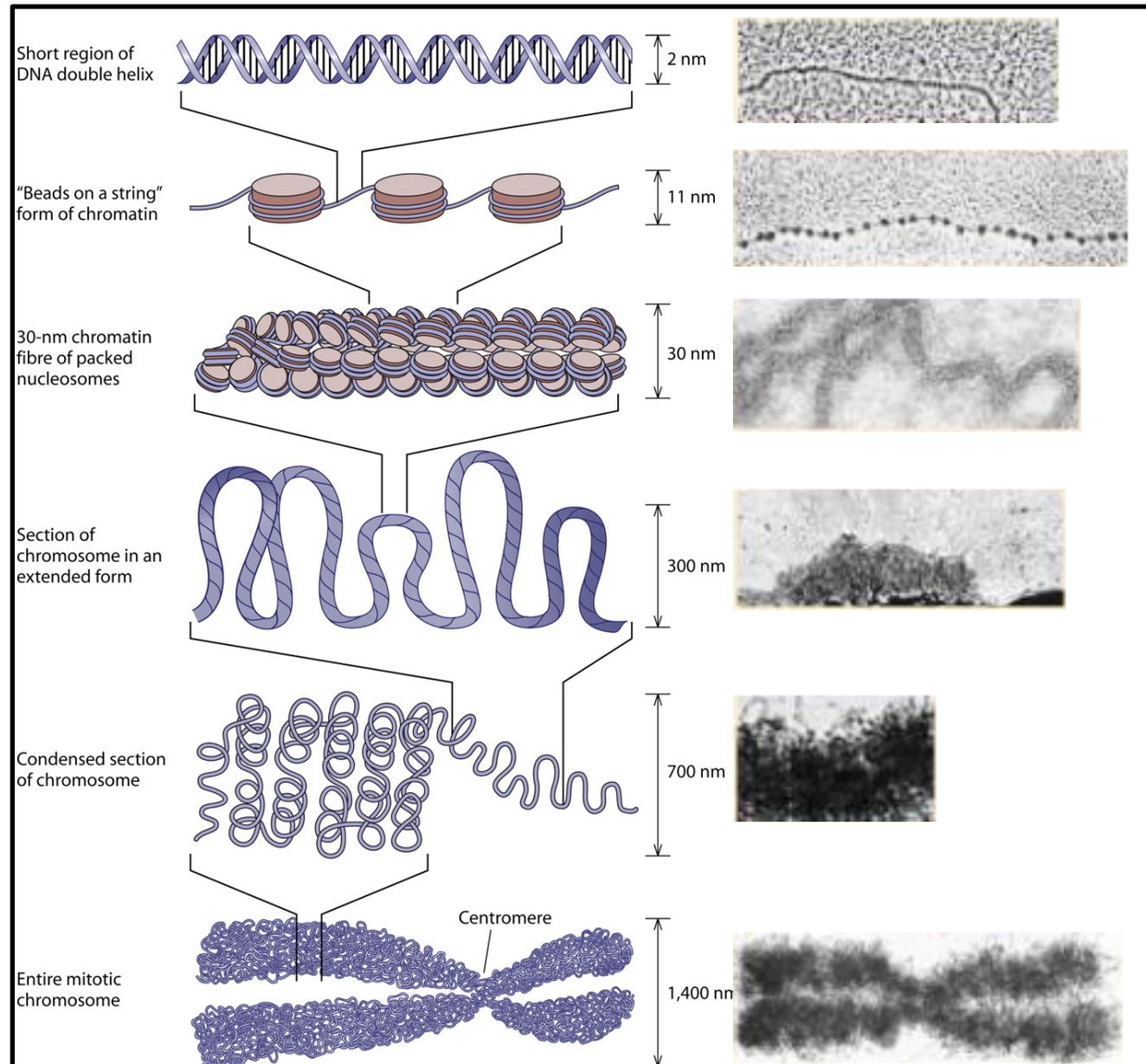
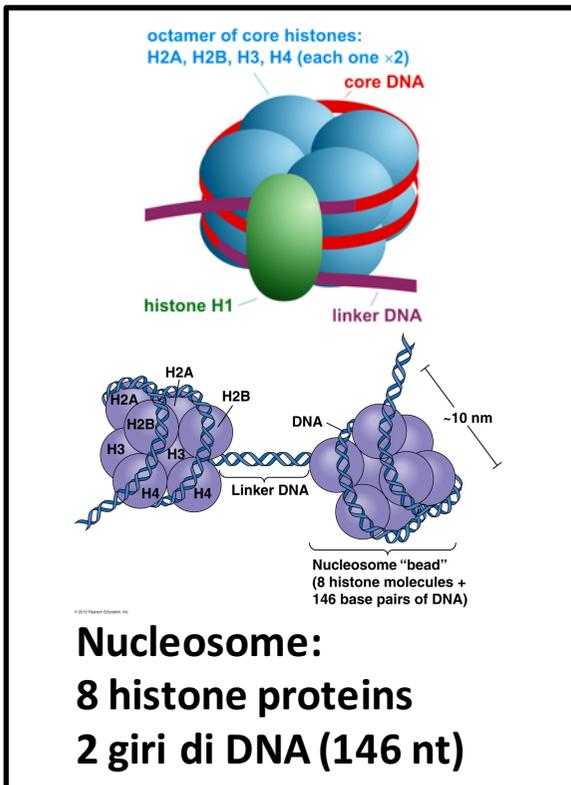
1. DNA GENOMICA + PROTEINE ASSOCIATE = Cromatina

La **cromatina** è la forma in cui gli acidi nucleici si trovano nella cellula.

Funzione:

- impacchettamento del DNA
- rafforzare il DNA per permettere la mitosi
- prevenire danni al DNA
- controllare la replicazione del DNA e l'espressione (attività) del gene

Interazione DNA - proteine: forte

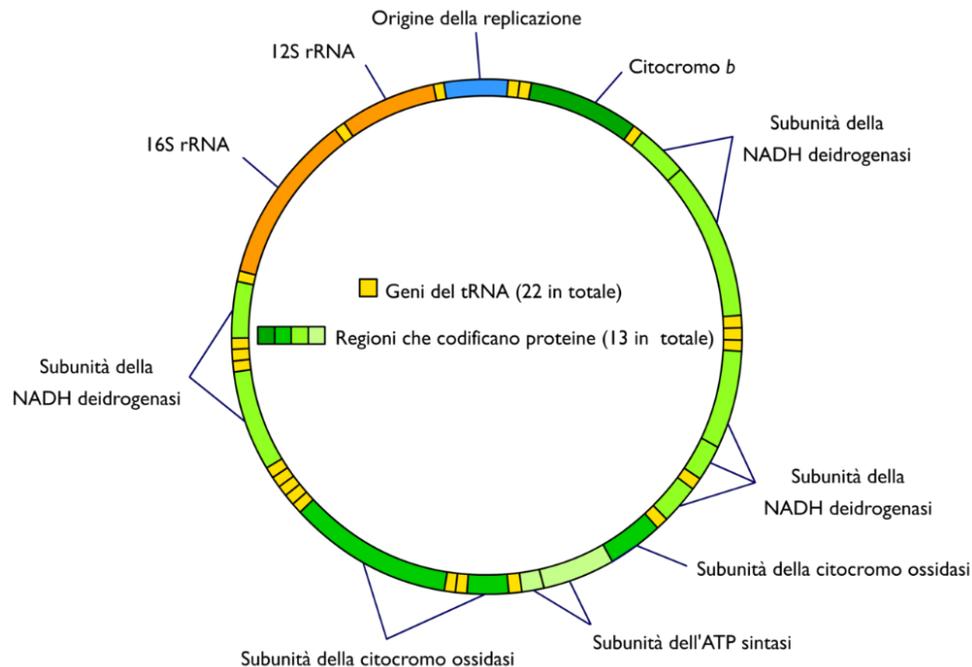


EUCARIOTI

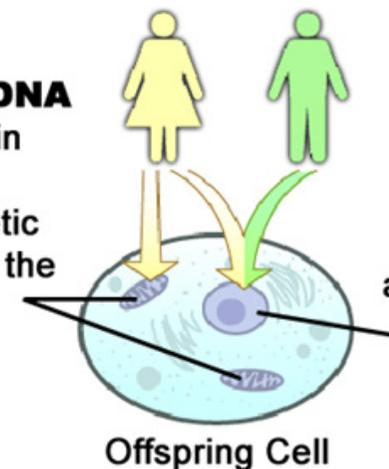
2. DNA MITOCONDRIALE

DNA mitocondriale dell'uomo:

- 1000 – 2000 mitocondri per cell
- 16569 paia di basi e 37 geni (codificano per 13 polipeptidi sintetizzati dal ribosoma mitocondriale)
- 22 tRNA e 2 rRNA), coinvolti nella produzione di proteine necessarie alla respirazione cellulare.
- La presenza di mitocondri dà un vantaggio energetico alle cellule eucariote da 3 a 4 ordini di grandezza in più

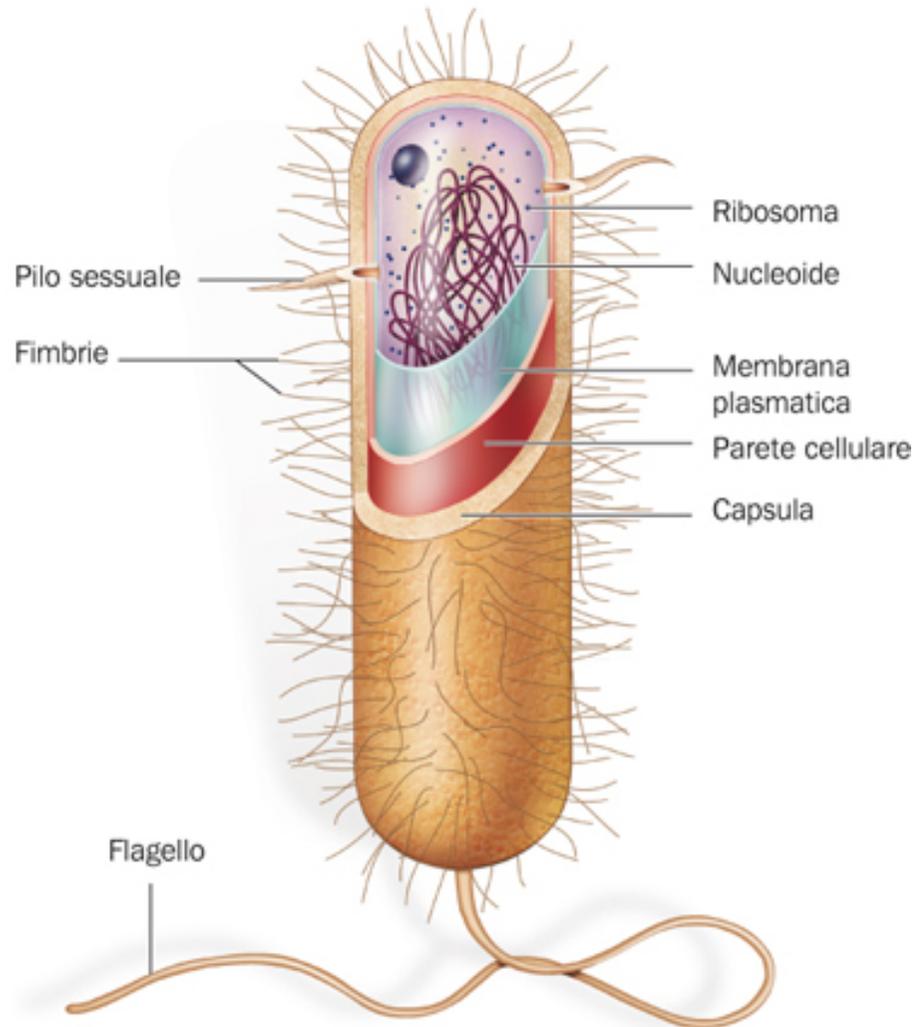


Mitochondrial DNA (mtDNA) is found in cell mitochondria and contains genetic material only from the **mother**.



Nuclear DNA (nuDNA) is found in the cell nucleus and contains genetic material from **both parents**.

PROCARIOTI



Le cellule procariotiche (da *pro*, prima e *karyon*, nucleo) sono **prive di un nucleo** racchiuso da una membrana.

Gli organismi unicellulari costituiti da cellule procariotiche, i **procarioti**, sono classificati in due domini:

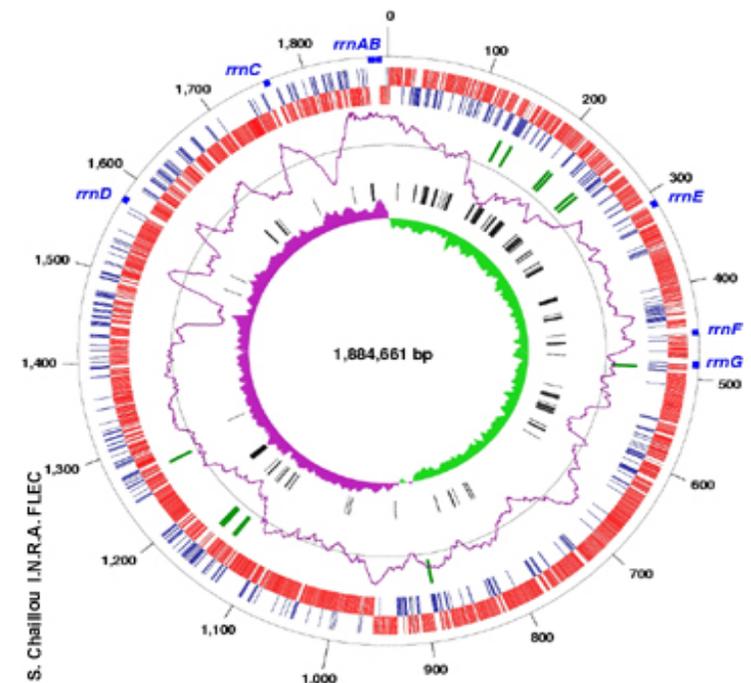
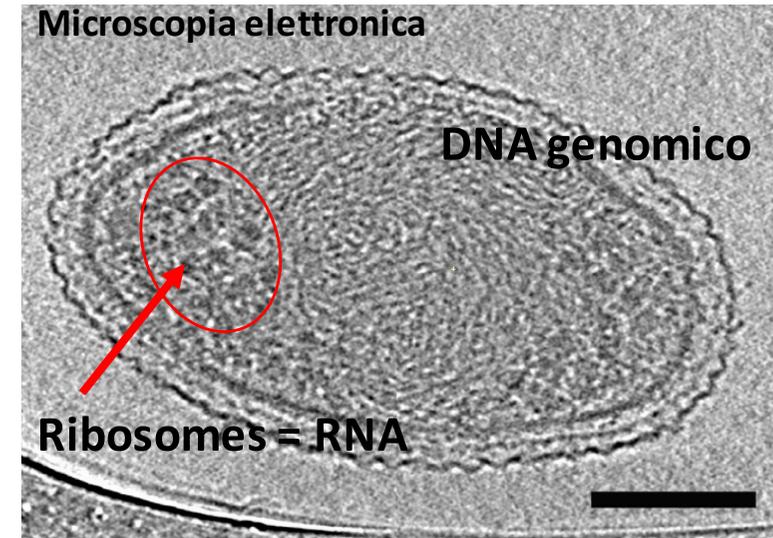
- ***Archaea* (archei);**
- ***Bacteria* (batteri).**

PROCARIOTI

1. DNA GENOMICO PROCARIOTI

Il materiale genetico, il DNA, e' organizzato in un **singolo cromosoma circolare** localizzato nell'area nucleare o **nucleoide**.

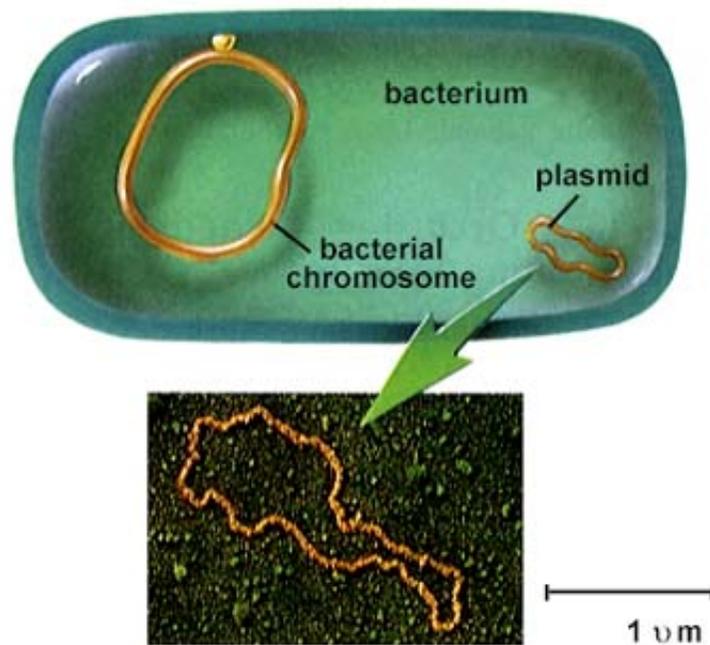
organism	genome size (base pairs)	protein coding genes	number of chromosomes
model organisms			
model bacteria <i>E. coli</i>	4.6 Mbp	4,300	1
budding yeast <i>S. cerevisiae</i>	12 Mbp	6,600	16
fission yeast <i>S. pombe</i>	13 Mbp	4,800	3
amoeba <i>D. discoideum</i>	34 Mbp	13,000	6
nematode <i>C. elegans</i>	100 Mbp	20,000	12 (2n)
fruit fly <i>D. melanogaster</i>	140 Mbp	14,000	8 (2n)
model plant <i>A. thaliana</i>	140 Mbp	27,000	10 (2n)
moss <i>P. patens</i>	510 Mbp	28,000	27
mouse <i>M. musculus</i>	2.8 Gbp	20,000	40 (2n)
human <i>H. sapiens</i>	3.2 Gbp	21,000	46 (2n)
viruses			
hepatitis D virus (smallest known animal RNA virus)	1.7 Kb	1	ssRNA
HIV-1	9.7 kbp	9	2 ssRNA (2n)
influenza A	14 kbp	11	8 ssRNA
bacteriophage λ	49 kbp	66	1 dsDNA
<i>Pandoravirus salinus</i> (largest known viral genome)	2.8 Mbp	2500	1 dsDNA
organelles			
mitochondria - <i>H. sapiens</i>	16.8 kbp	13 (+22 tRNA +2 rRNA)	1
mitochondria - <i>S. cerevisiae</i>	86 kbp	8	1
chloroplast - <i>A. thaliana</i>	150 kbp	100	1
bacteria			
<i>C. ruddii</i> (smallest genome of an endosymbiont bacteria)	160 kbp	182	1
<i>M. genitalium</i> (smallest genome of a free living bacteria)	580 kbp	470	1
<i>H. pylori</i>	1.7 Mbp	1,600	1
Cyanobacteria <i>S. elongatus</i>	2.7 Mbp	3,000	1
methicillin-resistant <i>S. aureus</i> (MRSA)	2.9 Mbp	2,700	1
<i>B. subtilis</i>	4.3 Mbp	4,100	1
<i>S. cellulosum</i> (largest known bacterial genome)	13 Mbp	9,400	1
archaea			
<i>Nanoarchaeum equitans</i> (smallest parasitic archaeal genome)	490 kbp	550	1
<i>Thermoplasma acidophilum</i> (flourishes in pH<1)	1.6 Mbp	1,500	1
<i>Methanocaldococcus (Methanococcus) jannaschii</i> (from ocean bottom hydrothermal vents; pressure >200 atm)	1.7 Mbp	1,700	1
<i>Pyrococcus furiosus</i> (optimal temp 100°C)	1.9 Mbp	2,000	1
eukaryotes - multicellular			
pufferfish <i>Fugu rubripes</i> (smallest known vertebrate genome)	400 Mbp	19,000	22
poplar <i>P. trichocarpa</i> (first tree genome sequenced)	500 Mbp	46,000	19
corn <i>Z. mays</i>	2.3 Gbp	33,000	20 (2n)
dog <i>C. familiaris</i>	2.4 Gbp	19,000	40
chimpanzee <i>P. troglodytes</i>	3.3 Gbp	19,000	48 (2n)
wheat <i>T. aestivum</i> (hexaploid)	16.8 Gbp	95,000	42 (2n=6x)
marbled lungfish <i>P. aethiopicus</i> (largest known animal genome)	130 Gbp	unknown	34 (2n)
herb plant <i>Paris japonica</i> (largest known genome)	150 Gbp	unknown	40 (2n)



PROCARIOTI

2. DNA EXTRACROMOSOMICO = PLASMIDI NAURALI

In aggiunta al DNA principale i batteri possono contenere piccole molecole di DNA circolare, dette **plasmidi**, che codificano per enzimi catabolici, per la resistenza ad antibiotici o legati a meccanismi per lo scambio di materiale genetico tra organismi. Replicazione autonomo, numero di copie è controllato



Class	Organism	Plasmid (Genbank #)	Size (kb)
Beta Proteobacteria	<i>Burkholderia cenocepacia</i> HI2424	1 (CP000461)	164.857
	<i>Burkholderia cenocepacia</i> J2315	pBCJ2315 (AM747723)	92.661
	<i>Burkholderia vietnamiensis</i> G4	pBVIE01 (CP000617)	397.868
	<i>Burkholderia vietnamiensis</i> G4	pBVIE05 (CP000621)	88.096
Gamma Proteobacteria	<i>Escherichia coli</i> O157:H7 EDL933	pO157 (AF074613)	92.077
	<i>Escherichia coli</i> DU1040	NR1 (DQ364638)	94.289
	<i>Escherichia coli</i> EH41	pO113 (AY258503)	165.548
	<i>Escherichia coli</i>	pMAR7 (DQ388534)	101.558
	<i>Escherichia coli</i>	pC15-1a (AY458016)	92.353
	<i>Escherichia coli</i> K12	Clodf13 (X04466)	9.957
	<i>Salmonella enterica</i>	pAM04528 (FJ621587)	158.213
	<i>Salmonella choleraesuis</i>	pMAK1 (AB366440)	208.409
	<i>Salmonella typhi</i>	pHCM1 (AL513383)	218.160
	<i>Salmonella typhimurium</i>	R64 (AP005147)	120.826
	<i>Salmonella dublin</i>	pOU1115 (DQ115388)	74.589
	<i>Pseudomonas aeruginosa</i>	pMATVIM7 (AM778842)	24.179
	<i>Pseudomonas aeruginosa</i>	RMS149 (AJ877225)	57.121
	<i>Pseudomonas aeruginosa</i>	pBS228 (AM261760)	89.147
Epsilon Proteobacteria	<i>Klebsiella pneumoniae</i>	pCTXM360 (EU938349)	68.018
	<i>Klebsiella pneumoniae</i>	pKPN3 (CP000648)	175.879
	<i>Helicobacter pylori</i> P29	pHE15 (AF469113)	18.291
	<i>Helicobacter pylori</i>	pAL202 (AY584531)	12.120
	<i>Helicobacter pylori</i>	pHP489 (AF027303)	1.222
	<i>Helicobacter pylori</i>	pHP51 (AY267368)	3.955
	<i>Helicobacter pylori</i>	pHP666 (DQ198799)	8.108
	<i>Helicobacter pylori</i>	pHP69 (DQ915941)	9.153
	<i>Helicobacter pylori</i>	pHE14 (AF469112)	10.970
	<i>Helicobacter pylori</i> HPMS	pHPM8 (AF275307)	7.817
Actinobacteria	<i>Streptomyces coelicolor</i> A3(2)	SCP1 (AL589148)	356.023
Bacilli	<i>Bacillus cereus</i> E33L	pE33L466 (CP000040)	466.370
	<i>Bacillus anthracis</i>	pX01 (CP001216)	181.773
	<i>Bacillus subtilis</i>	p1414 (AF091592)	7.949
	<i>Bacillus thuringiensis</i>	pBMB67 (DQ363750)	67.159
Deinococci	<i>Deinococcus radiodurans</i> R1	MP1 (AE001826)	177.466
	<i>Deinococcus radiodurans</i> R1	CP1 (AE001827)	45.704
	<i>Thermus thermophilus</i>	pTF62 (DQ058601)	10.402

I plasmidi hanno origine di replicazione e possono essere presenti in molte copie in un batterio.

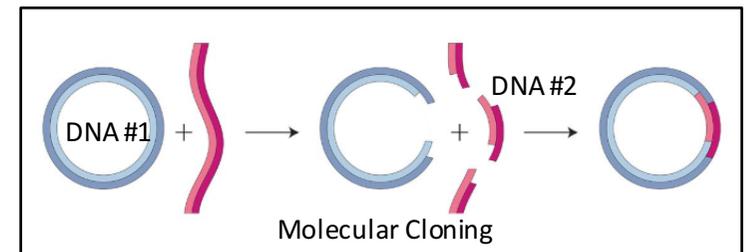
Plasmidi *modificati* servono in laboratorio come veicoli, chiamati anche **vettori, per amplificare le molecole di DNA (in batteri) per scopi di ricerca e biotecnologici = DNA ricombinante**

DNA RICOMBINANTE

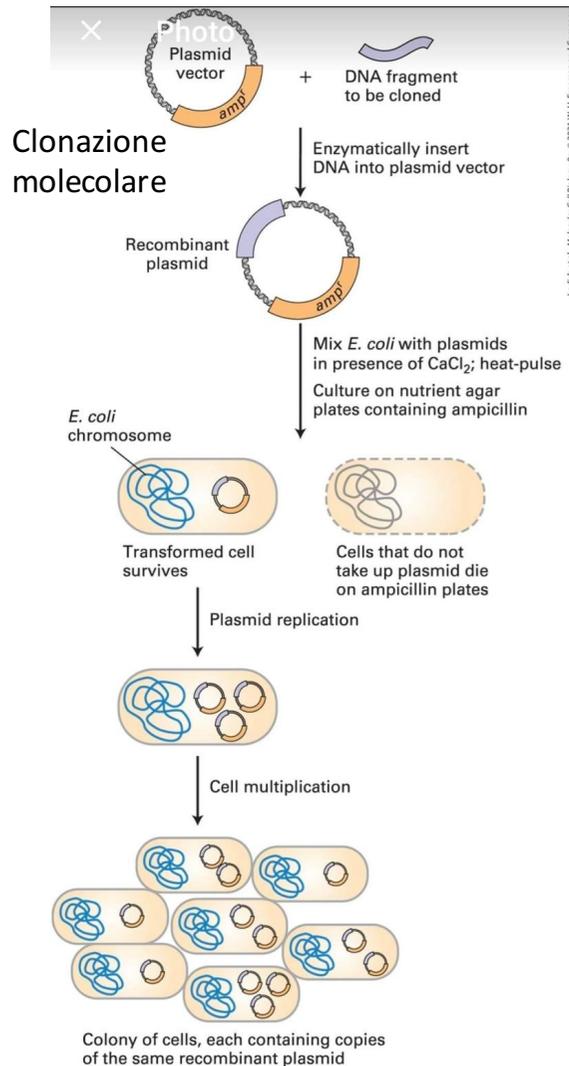
DNA ricombinante si riferisce a molecole di DNA create artificialmente combinando materiale genetico proveniente da fonti diverse. Questo processo viene solitamente effettuato in laboratorio tramite tecniche come la clonazione molecolare.

Un tecnica che permette di

- **ottenere brevi segmenti di DNA clonati in plasmidi, amplificali in batteri e di studiarne la sequenza nucleotidica**
- **di introdurre mutazioni nel DNA e di studiarne gli effetti in cellule**
- **sintesi artificiale di nuovi geni**
- **studiare la funzione di una proteina/gene**
- **di trasferire segmenti di DNA nel genoma di altre cellule**
- **creazione di vettori per la produzione di proteine ricombianti (ricerca, medicina, biotecnologie)**



Plasmids a vehicles to stabilize and amplify a DNA sequence of interest in bacteria (plasmid + additional DNA sequene = recombiant DNA)



Use plasmids to clone a DNA sequence of interest

Transfer recombinant DNA (plasmid) into bacteria (*E. coli*)

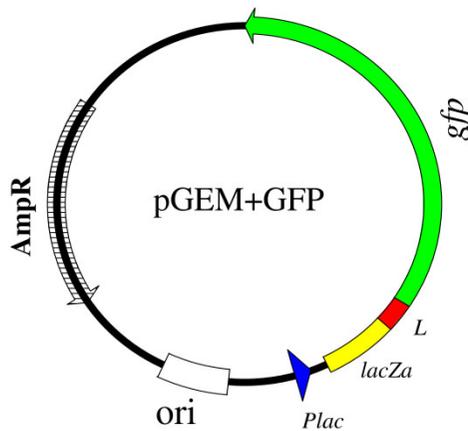
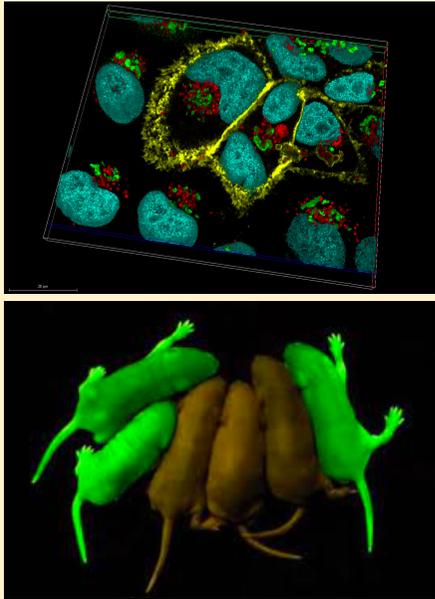
Amplify the recombinant DNA in bacteria using the bacteria's high fidelity DNA polymerase

Prepare plasmid DNA and use plasmid DNA for further purposes (research or biotec...)

A General Strategy to study or use recombinant DNA

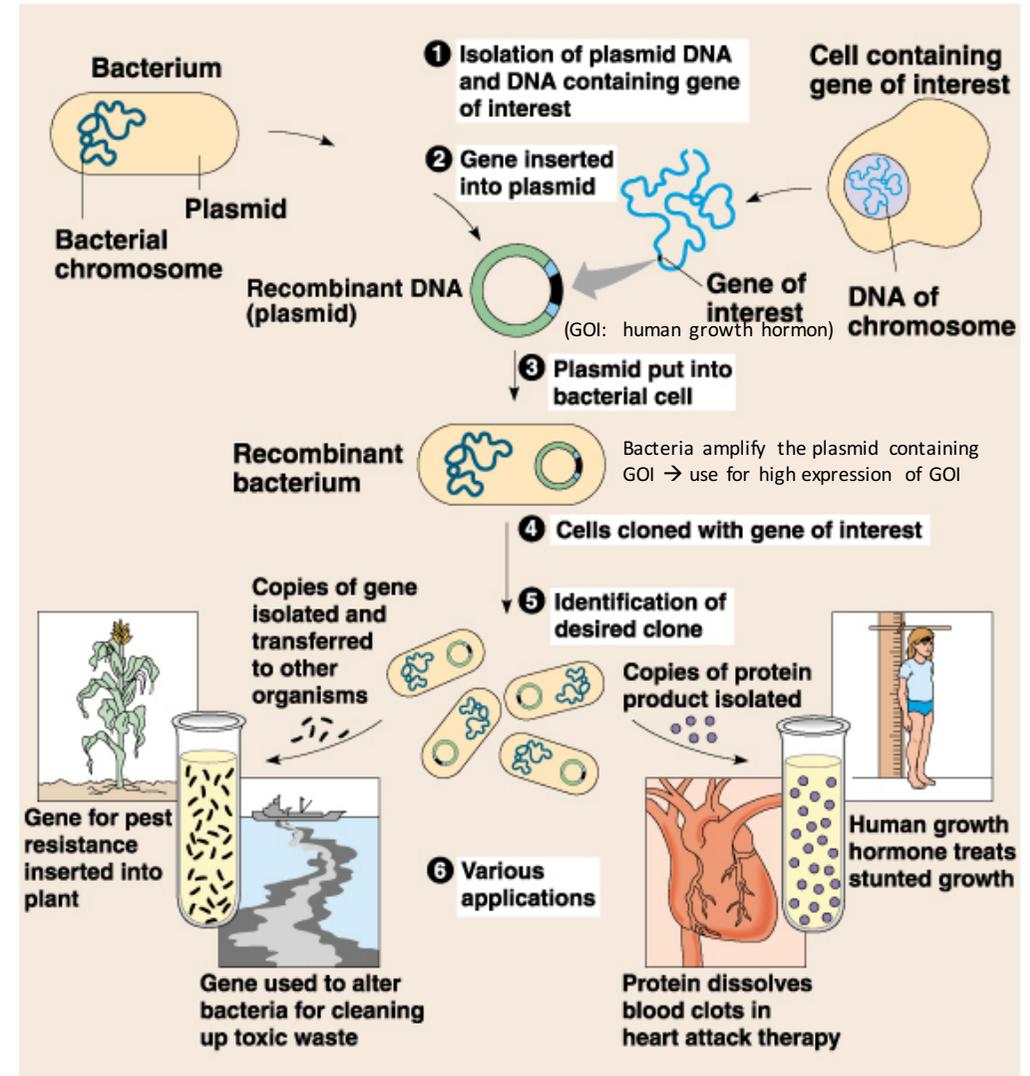
Plasmid encoding GFP inserted into genomic DNA

Introduce into a model system and investigate the function of a gene in development and disease (example vector encoding GFP inserted into cells (top) or mice (transgenic, bottom))



Gfp: green fluorescent protein

Use plasmids to produce proteins of interest (for example a growth hormone)



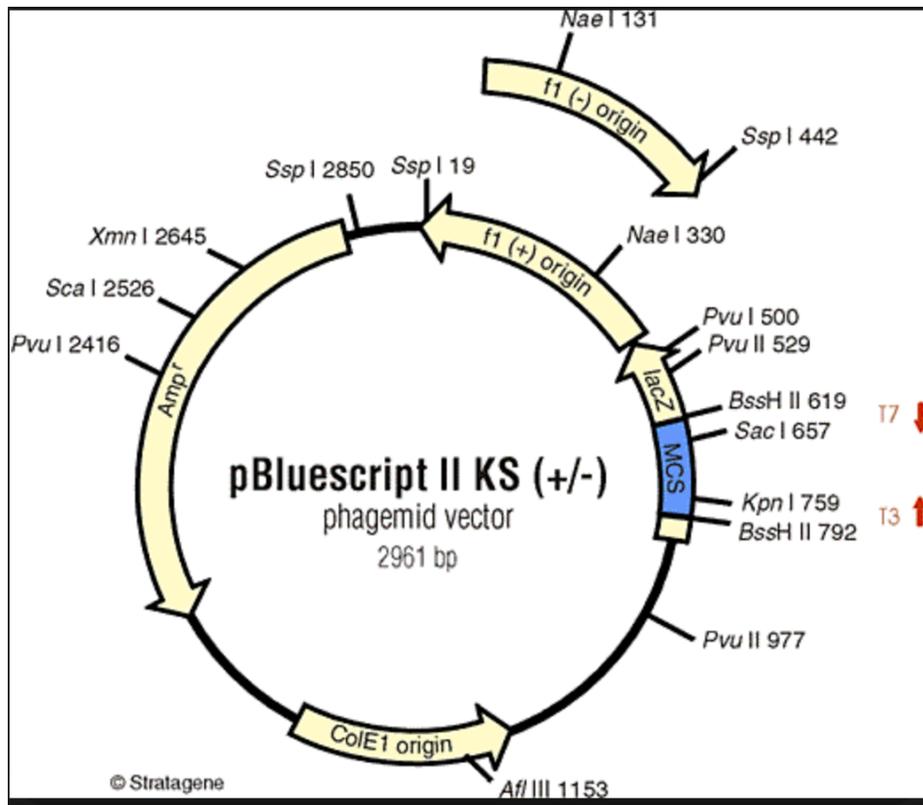
Preparation of nucleic acids

1. Plasmid DNA
2. Genomic DNA from cells (mammals)
3. RNA preparation (mammals)

Preparation of nucleic acids

1. **Plasmid DNA**
2. Genomic DNA from cells (mammals)
3. RNA preparation (mammals)

Plasmids engineered for the use in the laboratory

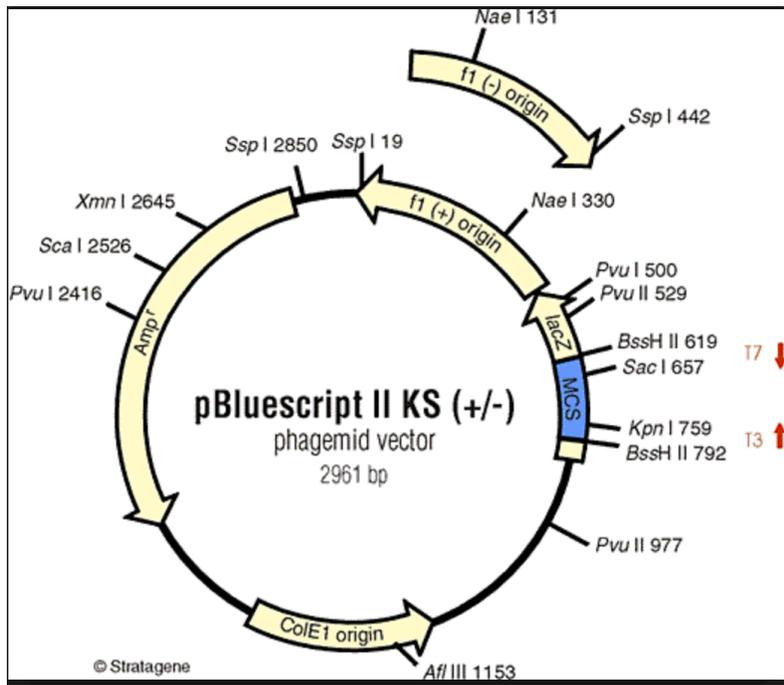


VECTOR MAP
PLASMID MAP

Laboratory plasmids are «recombinant DNA» by themselves (segments are derived from different sources: bacteria, phages...):

- **ColE1 origin:** obtained from a natural plasmid
- **Resistance to ampicilin:** obtained from natural plasmid
- **f1-/+ origin:** obtained from f1 phage (allows single stranded DNA production and packaging)
- **LacZ:** beta galactosidase (from bacteria encoded also by mammals, yeast, and funghi)
- **MCS:** multiple cloning site, synthetic DNA enriched with sites for cleavage by endonucleases (to insert genes of interest)
- Diversi siti di restrizione

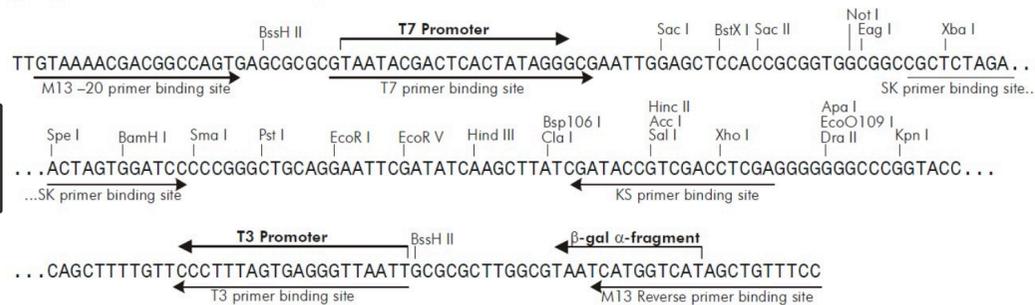
Plasmids engineered for the use in the laboratory



What makes a good plasmid for cloning (generating recombinant DNA)??

- **oriC**, an origin of replication. Gotta start making new plasmid somewhere.
- a **selectable marker**: This is usually an antibiotic resistance of some sort, to give the bacteria with plasmids a selective advantage in specific media. Example: pBluescript: Ampicilin
- a **multiple cloning site (MCS) inside a scorable marker**. The MCS allows us to cut the plasmid, insert new DNA, and re-ligate; the scorable marker allows us to see if the plasmid does indeed have an insert, because the insert will disrupt expression of the marker. This is seen in the use of the lac-Z-alpha fragment in blue/white screening.
- and it should be **small**, with a high **copy number**.

pBluescript II KS (+/-) Multiple Cloning Site Region (sequence shown 598–826)



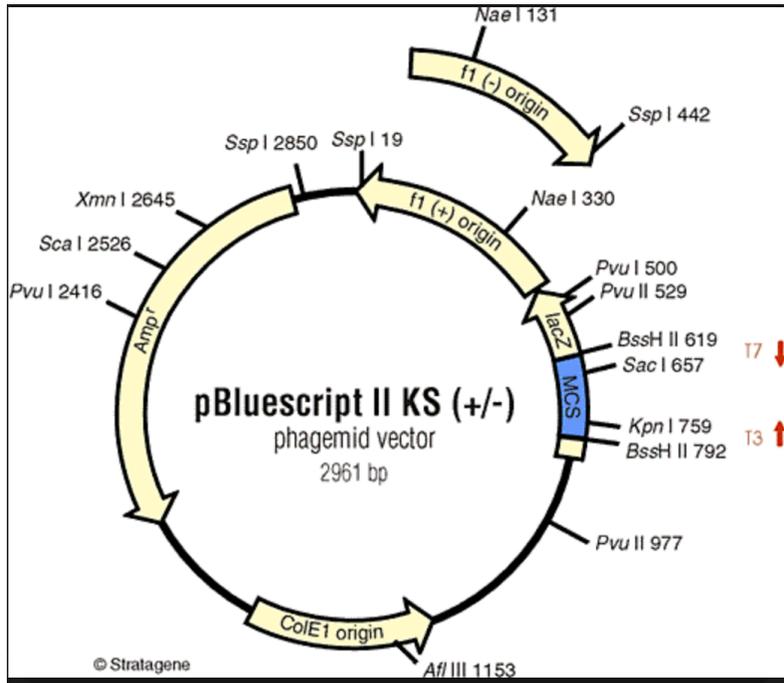
Inserts of interest can be cloned onto the MCS of the plasmid

HOW ARE PLASMIDS TRANSFERRED INTO BACTERIA IN THE LAB?

HOW ARE PLASMIDS AMPLIFIED IN BACTERIA?

HOW ARE PLASMIDS PURIFIED FROM BACTERIA?

1. PLASMID PREPARATION

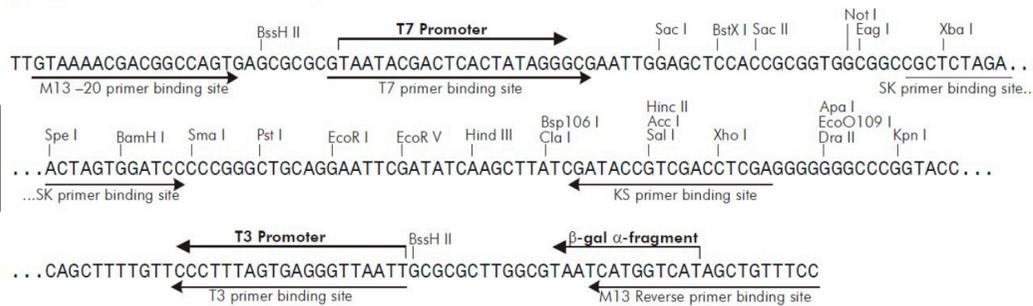


BACTERIA ARE USED AS TOOLS TO AMPLIFY PLASMIDS THAT CONTAIN A CLONED SEQUENCE OF INTEREST:

Q1: HOW ARE PLASMIDS TRANSFERRED INTO BACTERIA IN THE LAB?

Q2: HOW ARE PLASMIDS AMPLIFIED IN BACTERIA?

pBluescript II KS (+/-) Multiple Cloning Site Region (sequence shown 598-826)



Q3: HOW ARE PLASMIDS PLASMIDS PURIFIED FROM BACTERIA?

(COURSE)

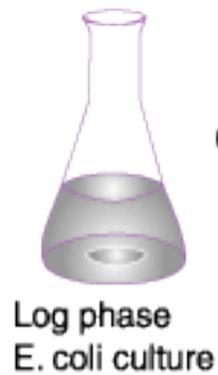
TRANSFORMATION: TRANSFER OF PLASMID INTO BACTERIA - METHOD 1:

Trasformazione batterica

Treatment with CaCl_2 (cloruro di calcio) and cold environment makes bacteria competent for DNA uptake

= **CHEMOCOMPETENT BACTERIA - metodo del CaCl_2**

(Can be stored at -80°C for years (normally >100 aliquots are prepared))



Centrifuge

Resuspend bacterial
pellet in CaCl_2 solution
50mM

- Cell surface of bacteria has negative charge
- CaCl_2 is bivalent (Ca^{2+}) and bridges between cell surface and negative charge of DNA
- Repulsive charges neutralized – DNA stabilized around bacteria
- Production of membrane proteins that bind DNA



Chill on ice

Aliquot competent cells



Store at -80°C

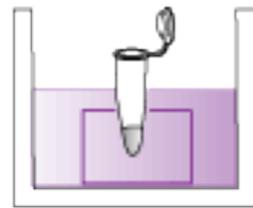
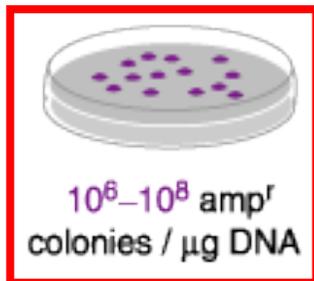
Preparation of
**CHEMO -
COMPETENT
BACTERIA**



amp^r plasmid DNA

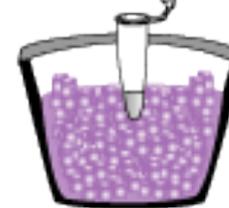
Add recovery
medium (SOC)
and plate on
LB+Amp

37°C



42°C H_2O bath

Heat
shock



Chill on ice

TRANSFORMATION

Competent bacteria are put on ice until bacteria are thawed; add plasmid/vector; induce heat shock (42°C) to **destabilize membrane** DNA can enter the bacteria; add liquid media to allow bacteria to recover; plate on media plate containing ampicillin (37°C)

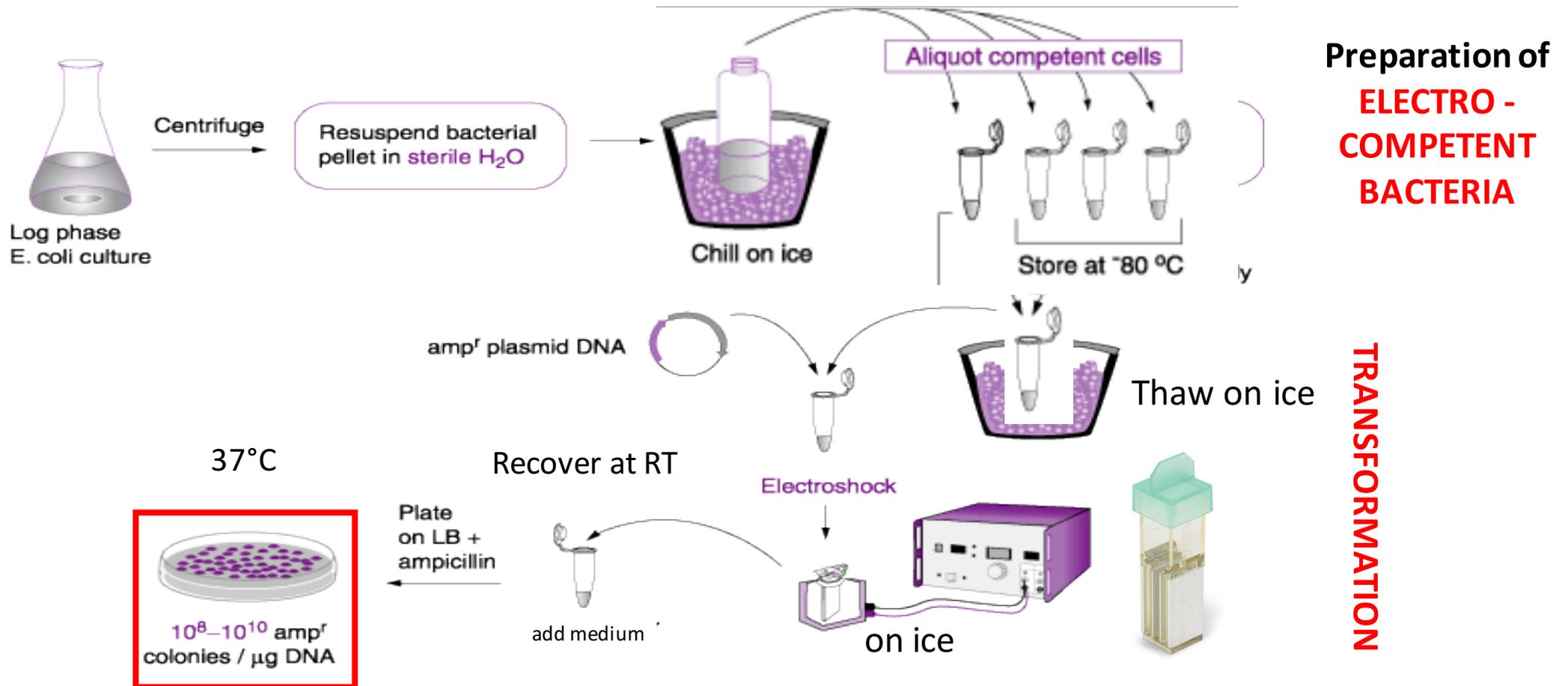
<https://www.youtube.com/watch?v=lr2s8zpVKtg>

TRANSFORMATION: TRANSFER OF PLASMID INTO BACTERIA - METHOD 2:

H₂O and cold environment makes membrane permeable without killing the cells

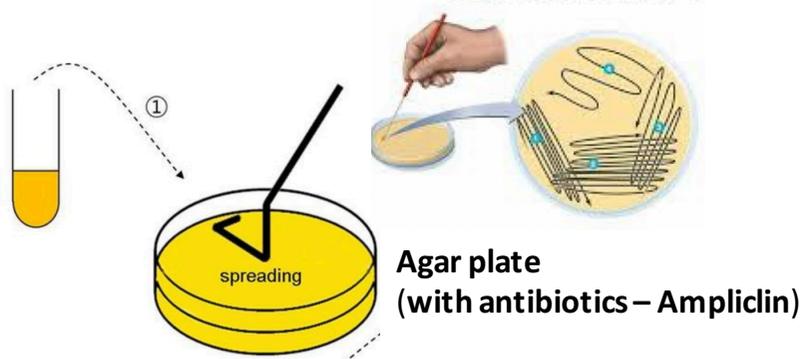
= **ELECTROCOMPETENT BACTERIA**

(Can be stored at -80°C for years (normally >100 aliquots are prepared))



Competent bacteria are put on ice until bacteria are thawed; add plasmid; induce electroshock; electric field destabilizes membrane DNA can enter the bacteria; add liquid media to allow bacteria to recover; plate immediately on media plate containing ampicillin

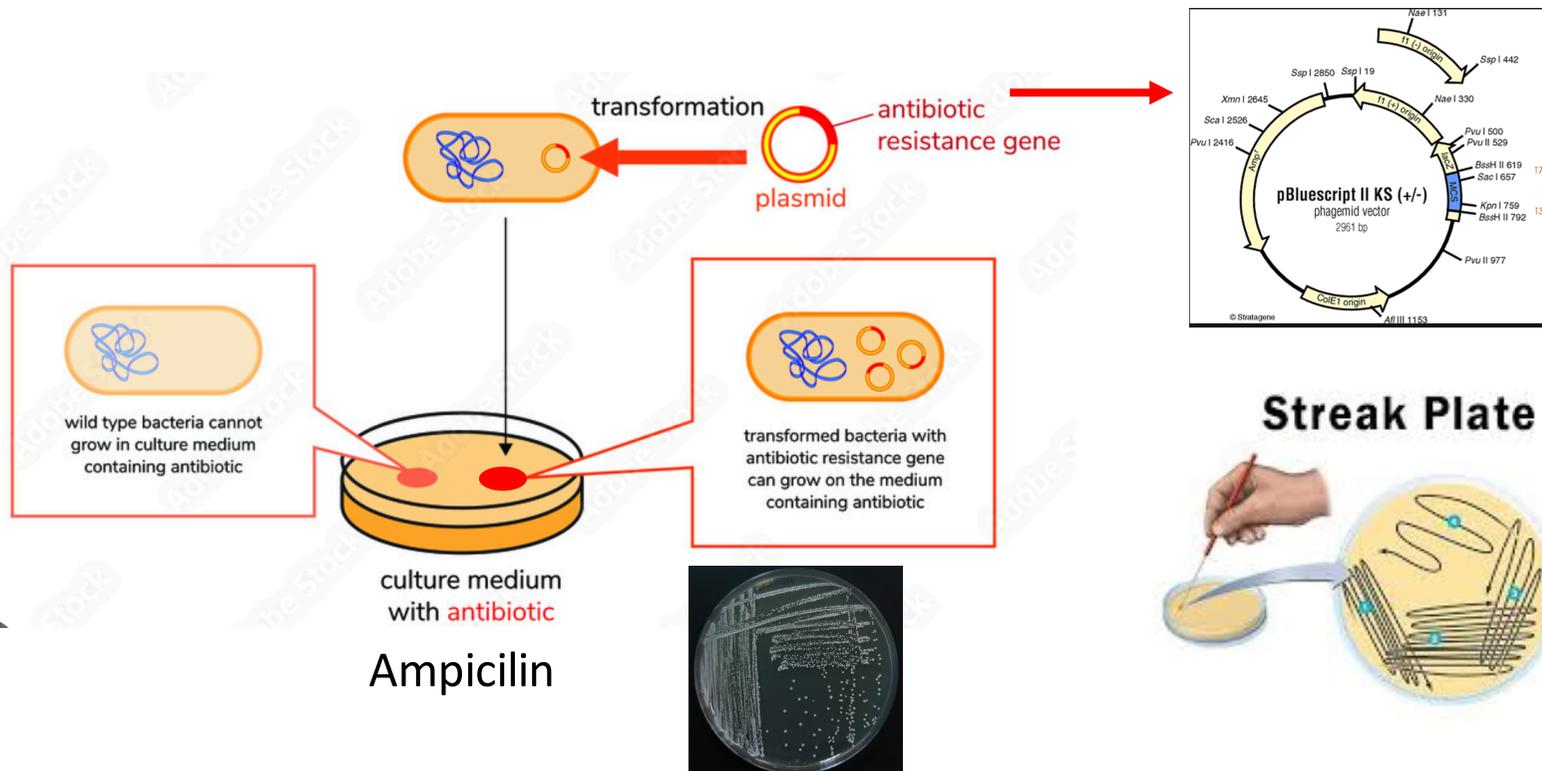
PLATING OF TRANSFORMED BACTERIA AND SELECTION



37°C
overnight

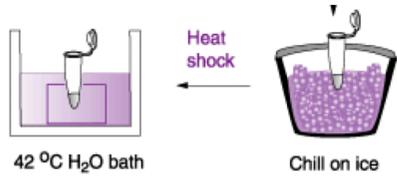


Bacteria after transformation
(mix of transformed and non-transformed bacteria)



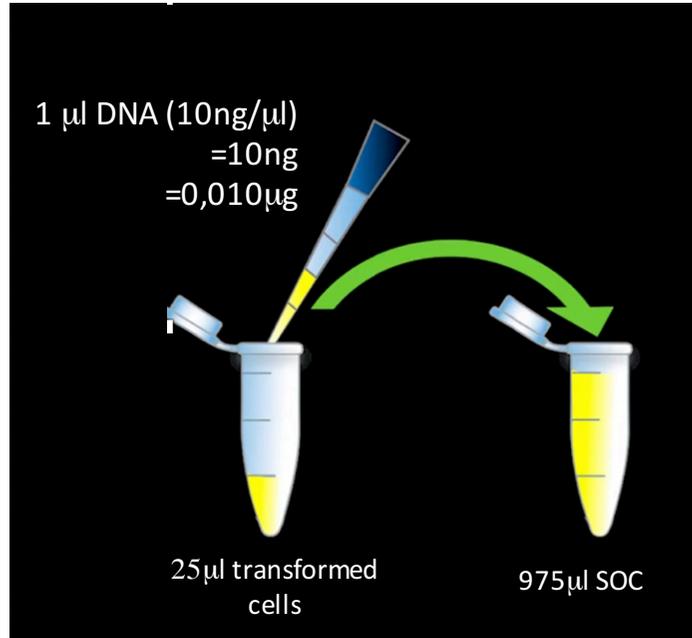
CALCULATION OF TRANSFORMATION EFFICIENCY

Transformation Efficiency (TE) is: **TE = Colonies (CFU)/ μg** of transformed DNA

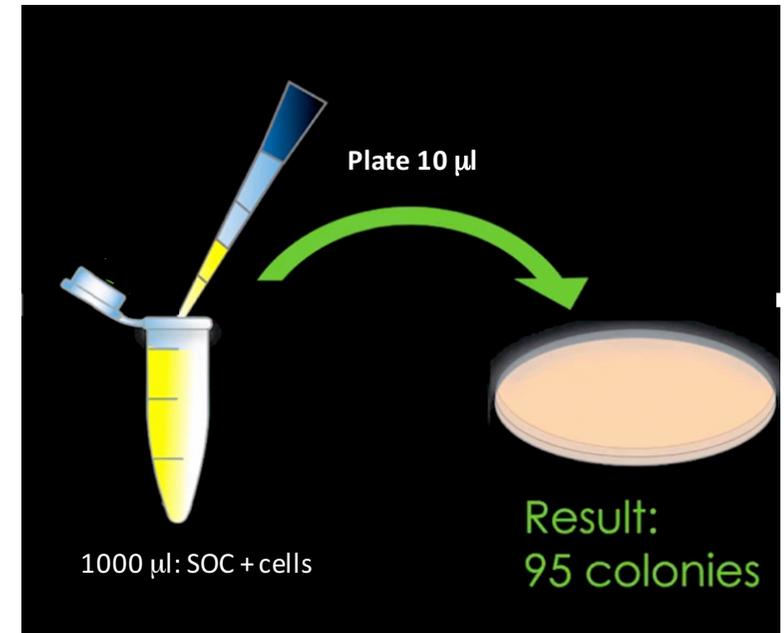


STEP 0

Transformation of chemocompetent bacteria with 1 μl DNA with concentration of (10ng/ μl)



STEP 1



STEP 2

Determine the transformation efficiency using the following formula:

$$\frac{\text{Number of transformants}}{\mu\text{g of DNA}} \times \frac{\text{final vol at recovery (mL)}}{\text{vol plated (mL)}} = \text{Number of transformants per } \mu\text{g}$$

$$\frac{95 \text{ colonies}}{0,01 \mu\text{g}} \times \frac{1000 \mu\text{l}}{10 \mu\text{l}} = 950000 \text{ CFU}/\mu\text{g} \quad (9,5 \times 10^5 \text{ CFU}/\mu\text{g})$$

CFU: colony forming units

CALCULATION OF TRANSFORMATION EFFICIENCY

Transformation Efficiency (TE) is: $TE = \text{Colonies}/\mu\text{g}$

95 colonies

Determine the transformation efficiency using the following formula:

$$\frac{\text{Number of transformants}}{\mu\text{g of DNA}} \times \frac{\text{final vol at recovery (mL)}}{\text{vol plated (mL)}} = \text{Number of transformants per } \mu\text{g}$$

$$\frac{95 \text{ colonies}}{0,01\mu\text{g}} \times \frac{1000\mu\text{l}}{10\mu\text{l}} = 950000 \text{ CFU}/\mu\text{g} \quad (9,5 \times 10^5 \text{ CFU}/\mu\text{g})$$

1 colonia

Determine the transformation efficiency using the following formula:

$$\frac{\text{Number of transformants}}{\mu\text{g of DNA}} \times \frac{\text{final vol at recovery (mL)}}{\text{vol plated (mL)}} = \text{Number of transformants per } \mu\text{g}$$

$$\frac{1 \text{ colonia}}{0,01\mu\text{g}} \times \frac{1000\mu\text{l}}{10\mu\text{l}} = 10000 \text{ CFU}/\mu\text{g} \quad (1 \times 10^4 \text{ CFU}/\mu\text{g})$$

950 colonies

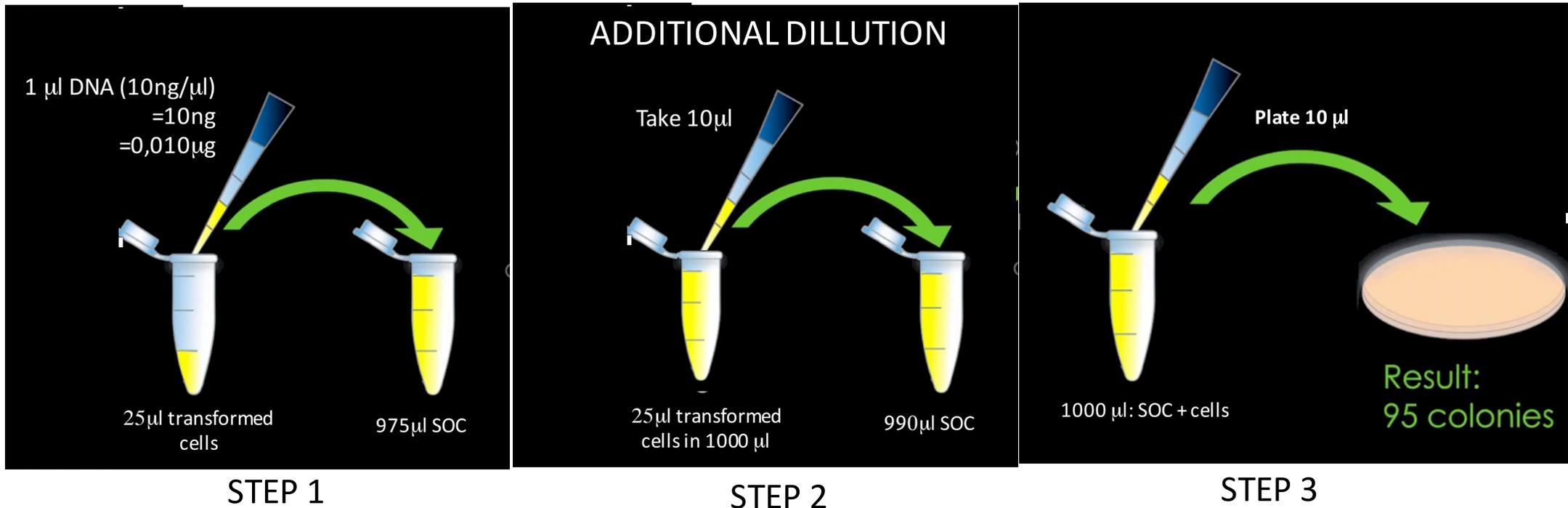
Determine the transformation efficiency using the following formula:

$$\frac{\text{Number of transformants}}{\mu\text{g of DNA}} \times \frac{\text{final vol at recovery (mL)}}{\text{vol plated (mL)}} = \text{Number of transformants per } \mu\text{g}$$

$$\frac{950 \text{ colonies}}{0,01\mu\text{g}} \times \frac{1000\mu\text{l}}{10\mu\text{l}} = 95000000 \text{ CFU}/\mu\text{g} \quad (9,5 \times 10^6 \text{ CFU}/\mu\text{g})$$

CALCULATION OF TRANSFORMATION EFFICIENCY

Transformation Efficiency (TE) is: $TE = \text{Colonies}/\mu\text{g transformed DNA}$



Determine the transformation efficiency using the following formula:

$$\frac{\text{Number of transformants}}{\mu\text{g of DNA}} \times \frac{\text{final vol at recovery (mL)}}{\text{vol plated (mL)}} = \text{Number of transformants per } \mu\text{g}$$

$$\frac{95 \text{ colonies}}{0,01\mu\text{g}} \times \frac{1000\mu\text{l}}{10\mu\text{l}} \times \frac{1000\mu\text{l}}{10\mu\text{l}} = 95000000 \text{ CFU}/\mu\text{g} \quad (9,5 \times 10^7 \text{ CFU}/\mu\text{g})$$

Commercial electrocompetent competent bacteria: ca 1×10^{10} cfu/ μg
 Commercial chemocompetent bacteria: ca 1×10^8 cfu/ μg

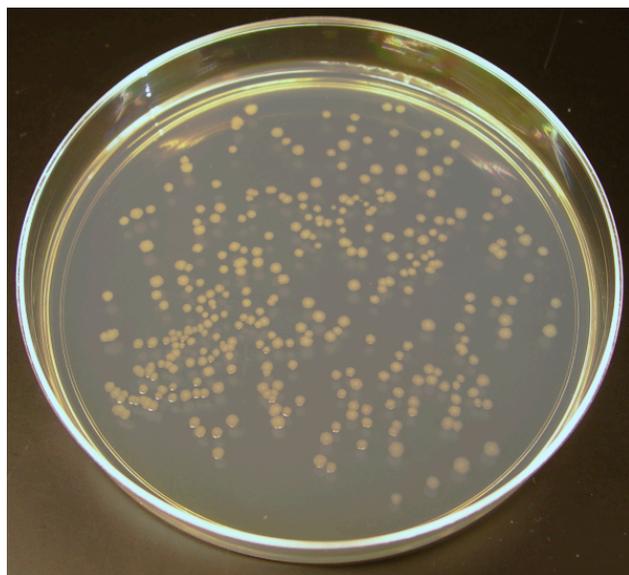
AMPLIFICATION OF PLASMID HOSTING BACTERIA

= proliferation from a single bacteria forms a colony = **clonal population of cells** (genetically identical)

Plasmid is maintained by the application of antibiotics to the agar

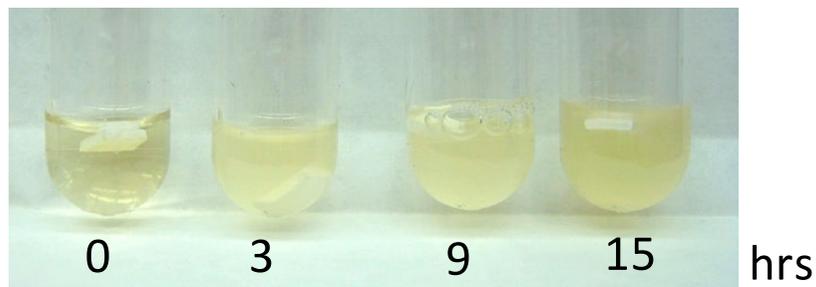
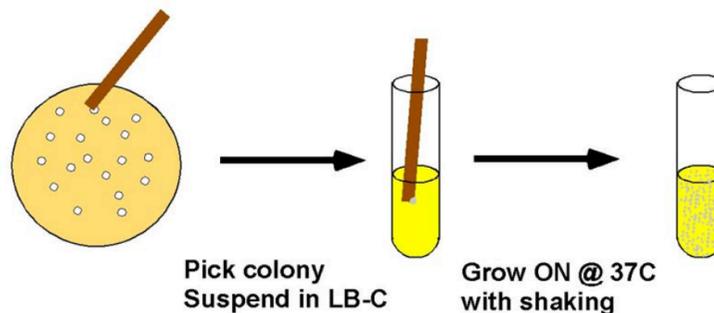
oriC recruits replication machinery. Laboratory plasmids carry modified origins of replication to obtain high copy numbers (ca. 500 copies/cell)

(the antibiotics resistance gene in the plasmid ensures maintenance of plasmid in cells)



Preparation. Grow the bacteria

Grow an overnight (ON) culture of the desired bacteria in 2-5 ml of LB medium containing the appropriate antibiotic for plasmid selection. Incubate the cultures at 37°C with vigorous shaking.



In general:
Pick **single** white colony with sterile pipette tip



Shaker Incubator

AMPLIFICATION OF PLASMID HOSTING BACTERIA

Next day:

1. after overnight culture, harvest bacteria by centrifugation – PELLET OF BACTERIA

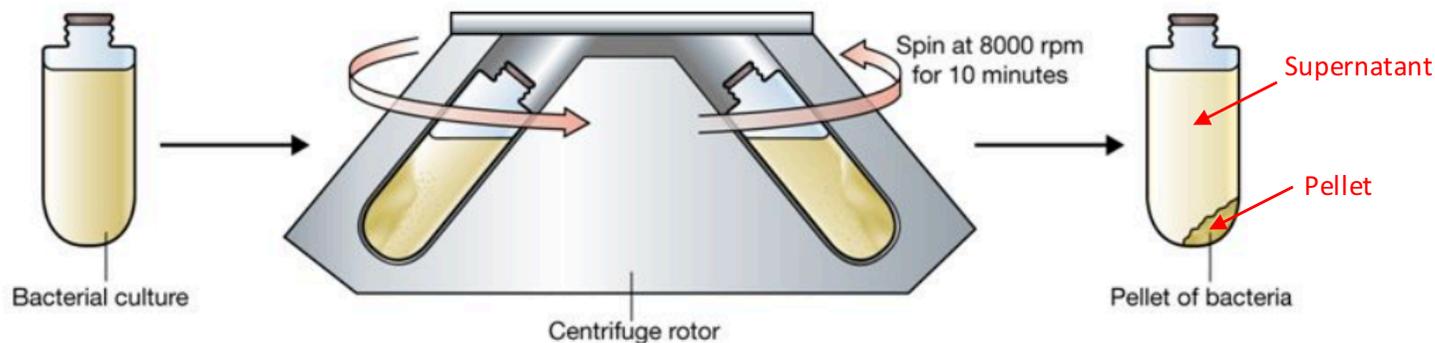
Diverse volumes possible:

2 ml bacterial culture (mini prep)

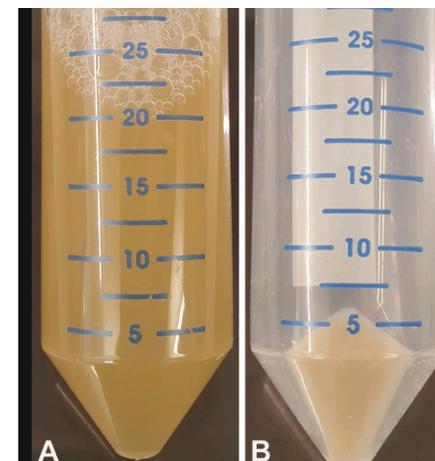
20-50 ml bacterial culture (midi prep)

100 -200 ml bacterial culture (maxi prep)

...choose appropriate centrifuge



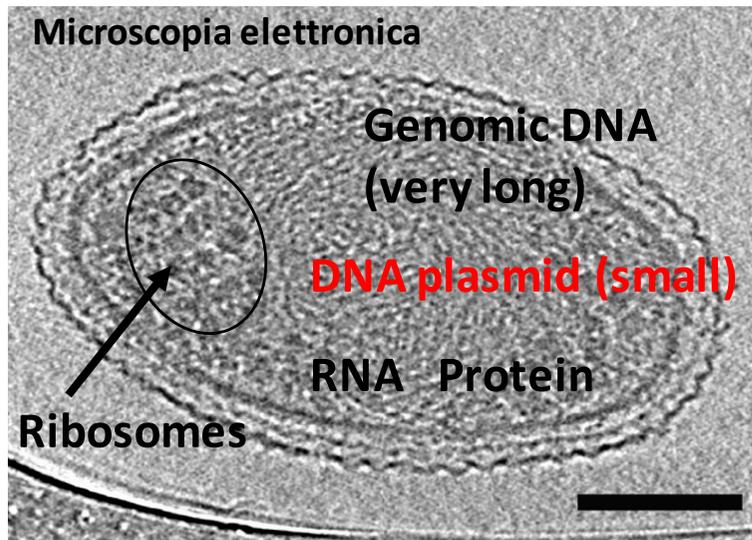
2. Discard **supernatant** (medium) and use **pellet** of bacteria for plasmid preparation



Pellet of bacteria containing plasmid DNA

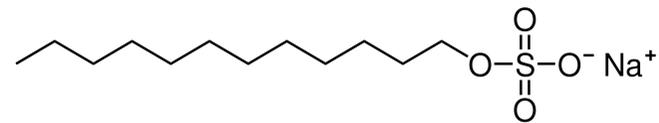
PURIFICATION OF PLASMID – ALKALINE LYSIS

Alkaline lysis – most common method



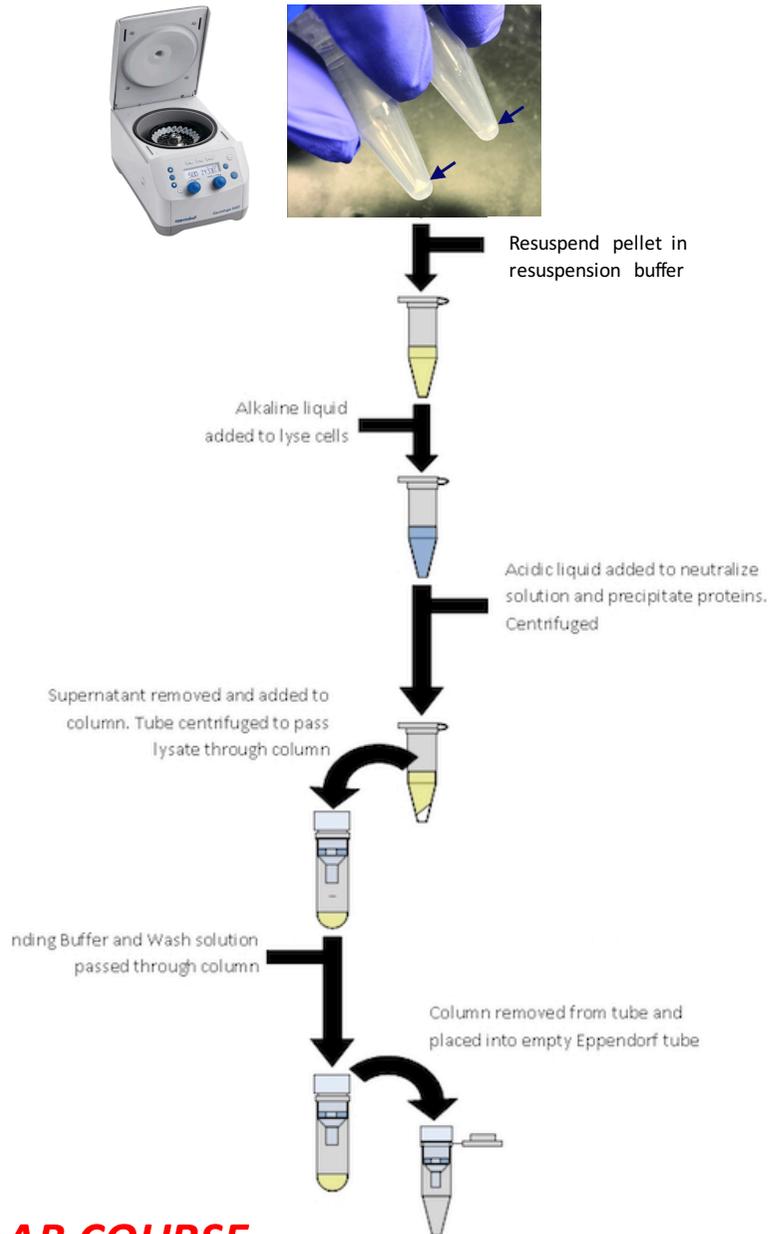
- Bacteria are lysed in basic solution (idrossido di Sodio; NaOH, pH 12-13) and SDS
- Chromosomal DNA: precipitate
- Proteins: precipitate
- RNA: hydrolyzed (low pH)
- **Plasmid DNA: remains stable (for reasonable time)**
- Isolation of plasmid DNA from slurry of lysed cells
 - Precipitation
 - Silica column

Sodium Dodecylsulfate (SDS; dodecilsolfato di sodio) is used in laboratory as a component of buffer for cell lysis, cell lysis during DNA and protein extraction. SDS is an anionic detergent applied to protein samples to, disrupt protein complexes to linearize proteins and to impart a negative charge to linearized proteins, solubilizes membranes.



PURIFICATION OF PLASMID DNA WITH RESIN (COLUMN)

Alkaline lysis with columns



«Mini» prep: 2ml of overnight culture
Removed, spinned and supernatant removed.

1. Bacteria pellet resuspended in buffer that does not kill cells (**resuspension buffer**)

2. Add **lysis buffer (contains NaOH, SDS)**
→ **LYSIS OF BACTERIA** (integrity of all cell structures destroyed)
Note: Alkaline liquid: mix of NaOH and SDS if plasmid DNA is too long in solution with high pH: Hydrolysis

3. The lysate is neutralized by the addition of **acidic potassium acetate/acetato di potassio (neutralization buffer)**; The high salt concentration causes Sodium dodecyl sulfate to precipitate, and the denatured proteins, chromosomal DNA, and cellular debris become trapped in salt-detergent complexes. Plasmid DNA, being smaller and covalently closed, renatures correctly and remains in solution in the supernatant

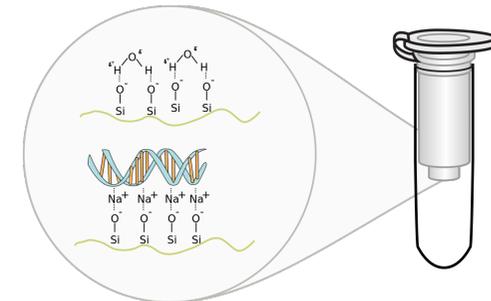
3. **Centrifugation at high speed** (ca. 13.000 rpm); cell debris and genomic DNA pellet; small DNA molecules (plasmid remain in supernatant)

4. **Apply supernatant to resin that traps plasmid DNA (Silica, DEAE)**
At high salt conditions at low pH (6,5), phosphosugar backbone binds silica resin

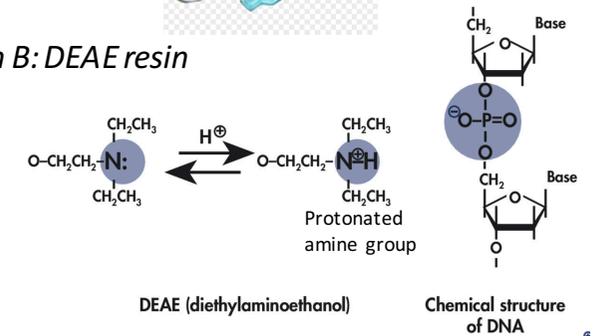
5. **Wash contaminants from column (washin buffer)**

Option A: Silica resin

irregular tridimensional framework of alternating silicon and oxygen atoms (SiO_2) with nanometer-scale voids and pores

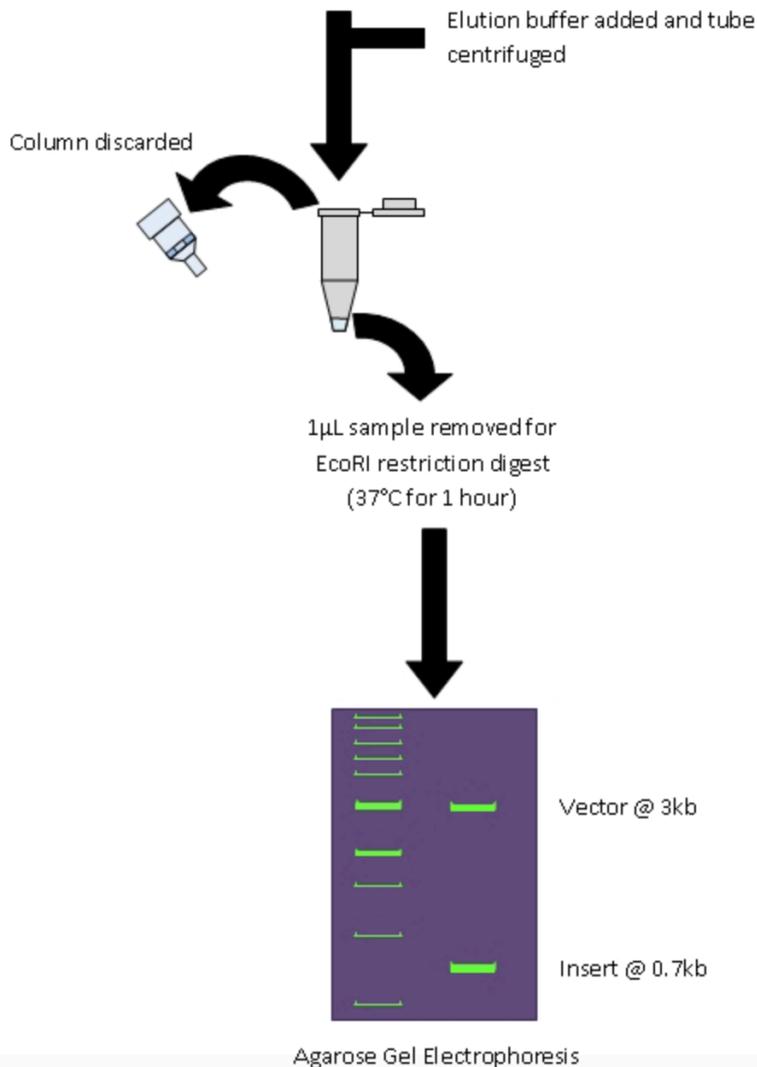


Option B: DEAE resin



Resin in column is positively charged:
Binds negative charge of plasmid DNA backbone

PURIFICATION OF PLASMID AND CONTROL DIGEST (COLUMN)



6. Elution of DNA at low salt conditions and at increased pH (7,0) – competes interaction resin – plasmid DNA

The use of columns results in very pure plasmid DNA also frequently called: “sequence grade plasmid DNA”

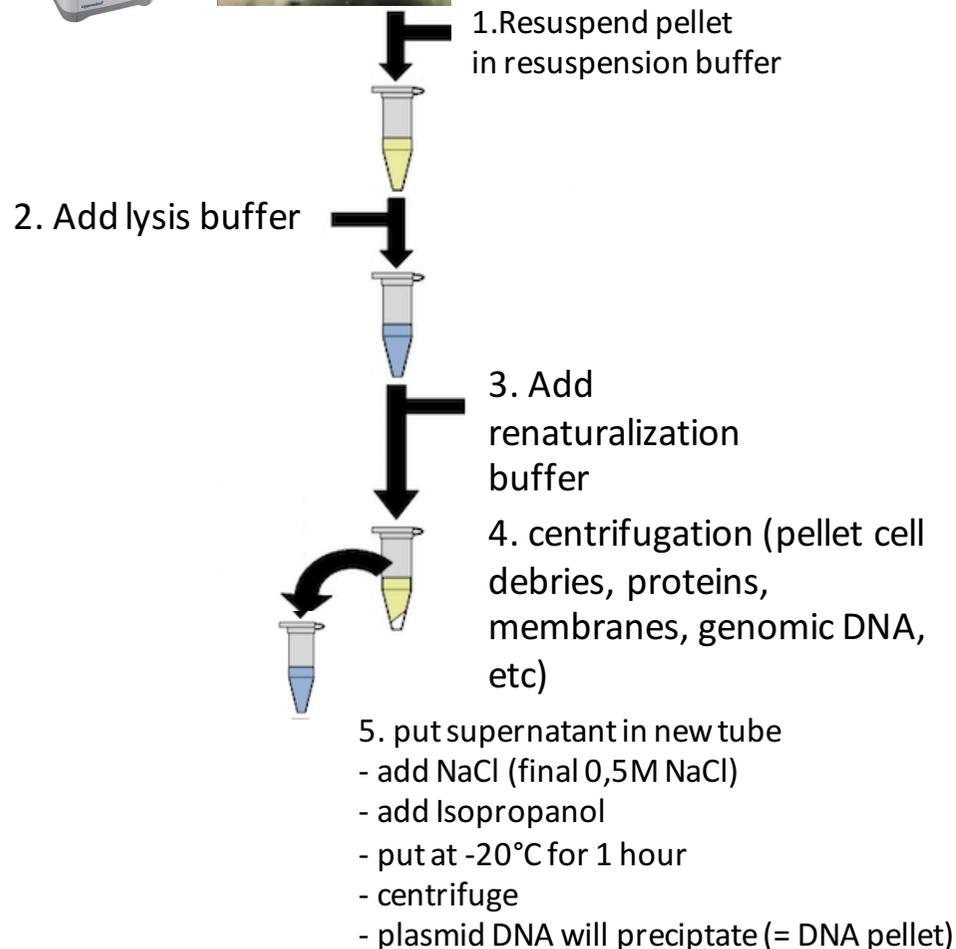
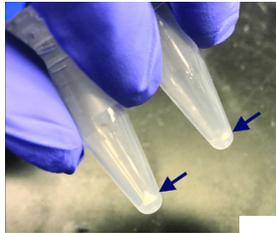
DNA is pure enough for all downstream applications that involve enzymes

https://www.youtube.com/watch?app=desktop&v=We9_UI8XQ6E

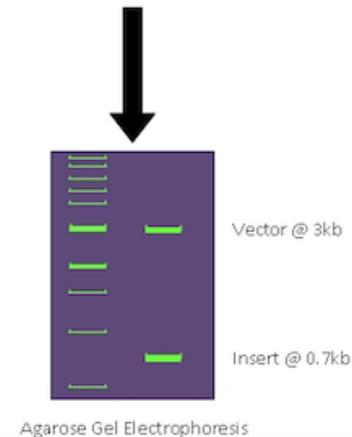
Alternative plasmid preparation without columns (via precipitation)

Alternative method without columns

Single Page Protocol for Alkaline Lysis Mini-Plasmid Preparation



- plasmid DNA will precipitate (= DNA pellet)
- Remove supernatant
- Wash pellet with 70% EtOH
- Remove EtOH (70%), let air-dry
- Resuspend in H₂O/buffer



Plasmid is not very clean; sufficient for digestion with restriction enzymes; not usable for DNA sequencing
“not sequencing grade”

- **Much cheaper; you can test many colonies for correctness of plasmid (after «building» recombinant DNA)**
- **Fast**
- **Low purity**
- **Note useful for downstream applications with delicate enzymes (not sequencing grade)**

Preparation of nucleic acids

1. Plasmid DNA
- 2. Genomic DNA from cells (mammals)**
3. RNA preparation (mammals)

2. PREPARATION OF GENOMIC DNA (mammals)

The isolation method of choice is dependent upon:

The source of the DNA:

cells, tissue, bacteria, virus etc.;

The final application:

PCR, restriction, sequencing, binding studies, library construction etc.;

The type of DNA:

genomic vs plasmid (size)

Why to prepare genomic 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
 - Preparation of DNA from genetically modified organisms
 - Preparation of DNA from patient (diseased/normal tissue) to study mutations
 - Detection of pathogens
 - Human identity testing (paternity)
 - Genetic research (mutations of inherited disease)
 - Material for the amplification of region of research interest (by PCR)

Sources for the isolation of Genomic DNA

Processing of biological material prior to DNA purification

Solid tissue (i.e. mouse organ)

Homogenise, chemically or mechanically

- a) Ultrasound vibration (Vibrazioni ultrasoniche)
 - b) Homogenization (Omogeneizzatore)
 - c) Freeze/thaw (Congelamento/Scongelamento)
- (note difference: Alkaline lysis for bacterial plasmids)

} Lysate



OMOGENIZZATORE



SONICATORE

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

a) Cell wall rupture

Bacteria (Gram-) – lysozyme or SDS, NaOH (pH alto)
Yeast/fungi - zymolase

} Lysate

b) Cell membrane rupture

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

} Lysate

General steps in DNA Isolation from lysates

1. Genomic DNA from cell lysates

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

2. Plasmid DNA

- Alkaline lysis method with or without silica columns

3. Bacteriophage DNA

- PEG/Salt precipitation method

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

DNA Preparation from cell lysates

“The organic method”

Step 1: DNA preparation: Open cells, digest proteins, extraction of genomic DNA

Step 2: Precipitation of DNA → concentrate DNA

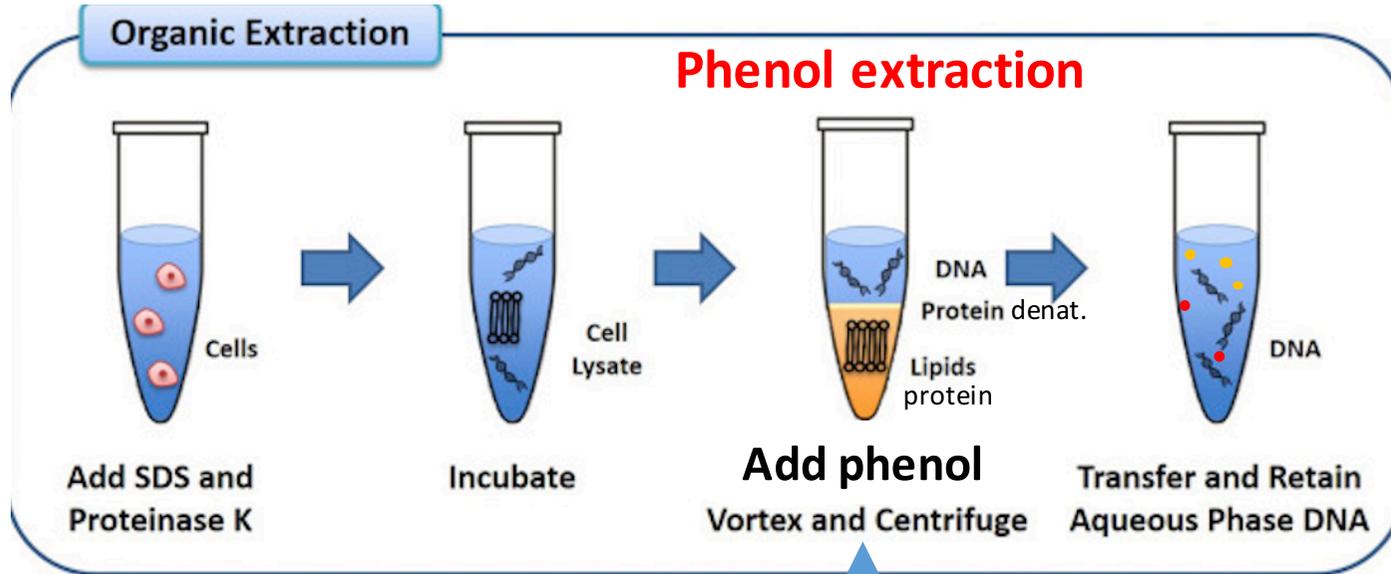
- **Lyse cells (or tissues) in buffer with SDS/PK /(DTT) (laboratory typically: 500 ul)**
 - SDS (sodium dodecyl sulfate) = detergent (solubilizes cell membrane, proteins)
 - PK = proteinase K (proteinasi K) proteins)
 - DTT = reducing agent - breaks disulfide bonds in folded proteins
 - 65°C, agitation, 1 hour - over-night (depending on amount of DNA) to degrade proteins
 - At 65°C over-night most RNA is degraded (alternatively add RNase to lysis buffer and incubate for 1 hour before adding PK))
- **Separation of hydrophobic and hydorphilic components: add equal volume of phenol (500 ul); mix**
 - Protein fragments and lipids attracted to hydrophobic phase (phenol)
 - Nucleic acids attracted to water
 - → separates aqueous phase (DNA, RNA) from organic phase (lipids, proteins)

SDS: Sodium dodecyl sulfate

DNA Preparation from cell lysates

“The organic method”

Step 1: DNA preparation: Open cells, digest proteins, extraction of genomic DNA



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

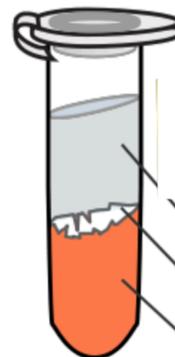


Phenol:

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. Lipids from membrane dissolve in phenol.

PHENOL IS HIGHLY TOXIC – WORK IN FUME HOOD WITH PROTECTION GLASSES

Phase separation



Aqueous phase	Fase acquosa (RNA, DNA)
Interphase	Interfaccia (Proteine denaturate)
Organic phase	Fase fenolica (Proteine solubile, lipidi)

DNA Preparation from cell lysates

“The organic method”

Step 1: DNA preparation: Open cells, digest proteins, extraction of genomic DNA



DNA and
Residual contaminants:
proteins, lipids,
phenol from aqueous phase

•Elimination of rests of proteins, lipids, phenol from aqueous phase:

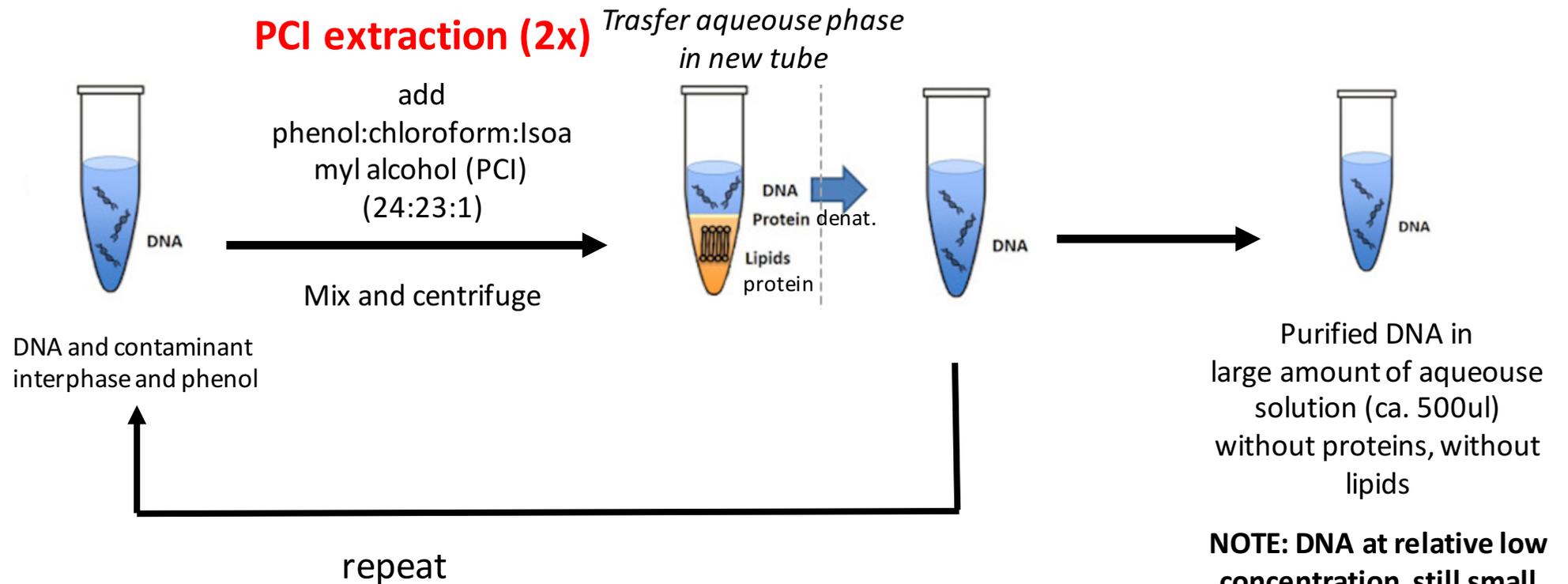
add equal volume (500 ul) of PCI; mix

→ PCI: mix of Phenole, Chloroform, Isoamylacohol

DNA Preparation from cell lysates

“The organic method”

Step 1: DNA preparation: Open cells, digest proteins, extraction of genomic DNA



Chloroform:

- 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.

Isoamyl alcohol:

- Reduces the foam that forms during the course extraction.

PHENOL, CHLOROFORM ARE HIGHLY TOXIC – WORK IN FUME HOOD WITH PROTECTION GLASSES

DNA Preparation from cell lysates

“The organic method”

Step 1: DNA preparation: Open cells, digest proteins, extraction of genomic DNA,
Step 2: Precipitation of DNA → to concentrate DNA

- Creating a condition that renders DNA insoluble (precipitation) !!!!
- Generate a DNA pellet in tube by centrifugation
- Wash pellet to remove contaminants (salts and contaminants from extraction)
- Dissolve in aqueous buffer

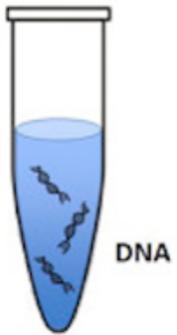
DNA Precipitation

Step 1: DNA preparation: Open cells, digest proteins, extraction of genomic DNA,

Step 2: Precipitation of DNA → to concentrate DNA

1. Precipitation:

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



3 options to set set up of DNA precipitation using high salt conditions in alcohol

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 exmple:

500 ul DNA solution

1. Add + 55,5 ul 3M Na-Acetate

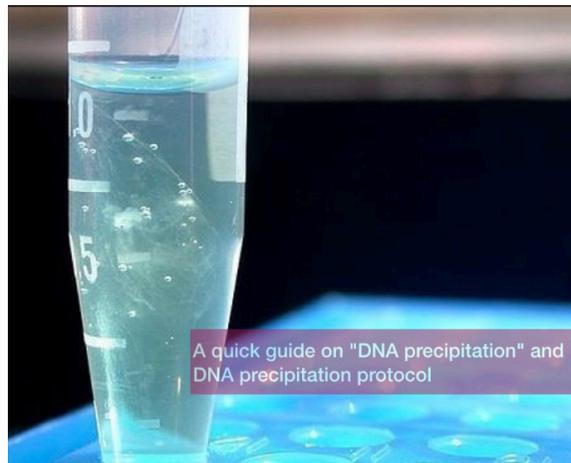
(final conc. 0,3M; vol. totale 555,5ul)

2. Add + 555.5 ul Isopropanol

3. Place in freezer at -20°C for 1 hour (DNA precipitates)

4. Centrifuge 13.000rpm 30 minutes at 4°C

+ Salt
+ Isopropanol or EtOH
→ DNA precipitate forms



DNA-precipitate in suspension
(cloudy appearance)

centrifugation
at min. 8000g at 4°C



**DNA
pellet**

DNA pellet:

- DNA
- Residual contaminants (salt, eventually traces of PCI)

NaCl: cloruro di sodio
NaAc: acetato di sodio

DNA Precipitation

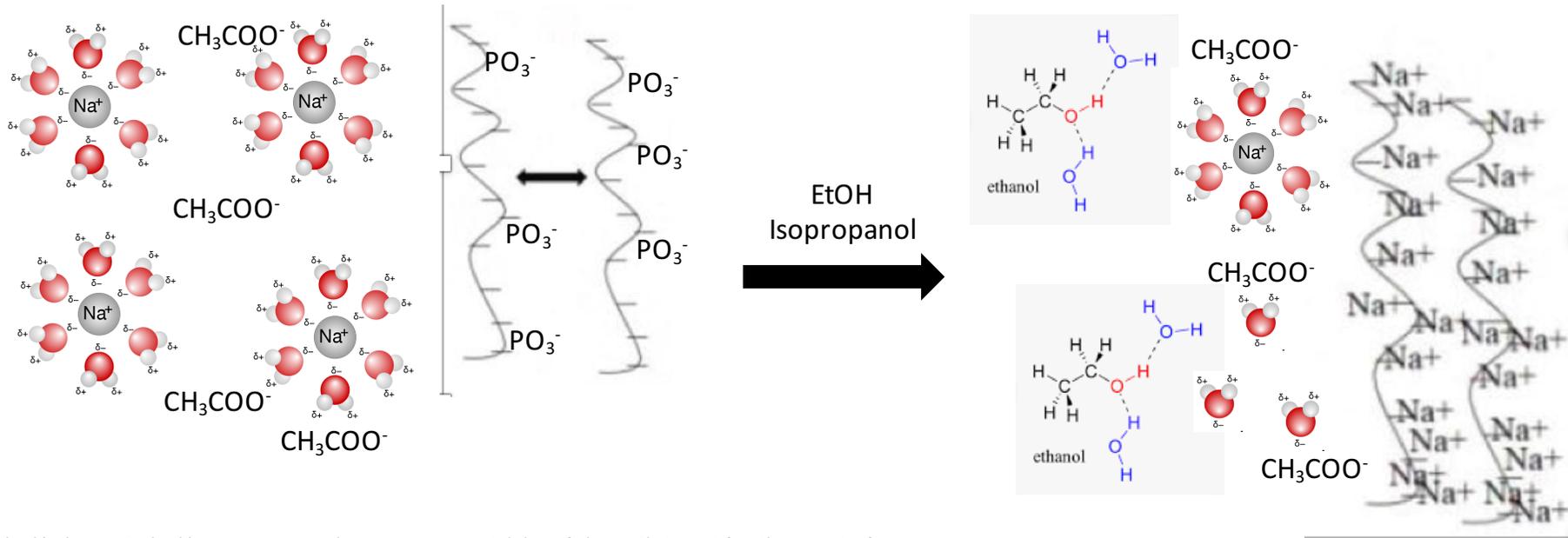
Step 1: DNA preparation: Open cells, digest proteins, extraction of genomic DNA,

Step 2: Precipitation of DNA → to concentrate DNA

The role of salt in the protocol is to neutralize the charges on the sugar-phosphate backbone. This renders DNA/RNA insoluble and leads to precipitation

<https://www.youtube.com/watch?v=MFvdrMgCnFA>

1. Addition of sodium acetate or NaCl: In aqueous solution, ionic bonds of salt breaks up into Na^+ and $[\text{CH}_3\text{COO}]^-$ or Cl^- .
2. H_2O has a high dielectric constant, and shields Na^+ ions. Na^+ is not captured by PO_3^- in the DNA backbone. DNA stays in solution PO_3^-
3. Ethanol/Isopropanol are polar, small sized alcohols that are soluble in water. They have a much lower dielectric constant. Mixing of water with EtOH or Isopropanol reduces the dielectric constant in the environment.
4. Reduced dielectric constant enables Na^+ to interact with the PO_3^- . This shields its charge and makes the nucleic acid less hydrophilic, thus causing it to drop out of the solution



Costante dielettrica – dielectric constant

Coulomb's law, or Coulomb's inverse-square law, is an experimental law of physics that quantifies the amount of force between two stationary, electrically charged particles. The electric force between charged bodies at rest is conventionally called electrostatic force or Coulomb force.

DNA Precipitation

Step 1: DNA preparation: Open cells, digest proteins, extraction of genomic DNA,

Step 2: Precipitation of DNA → to concentrate DNA

1. Precipitation:

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

+ Salt
+ Isopropanol
or EtOH
→ DNA
precipitate
forms



DNA-precipitate in suspension
(cloudy appearance)

centrifugation
at min. 8000g at 4°C



**DNA
pellet**

DNA pellet:

- DNA
- Residual
contaminants
(salt, eventually
traces of PCI)

DNA Precipitation

Step 1: DNA preparation: Open cells, digest proteins, extraction of genomic DNA,

Step 2: Precipitation of DNA → to concentrate DNA

2. Washing of with 70% Ethanol

- Remove supernatant (mix of salt, buffer and alcohol) from top of pellet
- Carefully add 70% Ethanol (in H₂O) on top of pellet
(now residual salt from precipitation will be dissolved in H₂O)
- Pellet does not dissolve; remains visible
- Centrifuge for 10 seconds to force pellet to bottom of tube
- Remove carefully supernatant
- Let pellet air dry at room temperature for 5-10 minutes to allow the evaporation of EtOH
- Dissolve pellet in storage buffer (TE buffer = 10mM TrisHCl, pH 7,2; 1mM EDTA)

Why: 70% Ethanol dissolves salt (NaCl or Na-Acetate) that can co-precipitate during centrifugation.

Contaminating salt in the DNA preparation can have negative effect on enzymatic reactions using the prepared DNA!!!!

PRECIPITATION METHOD IS APPLICABLE TO ALL TYPES OF NUCLEIC ACIDS (RNA, genomic DNA, plasmid DNA)

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 (otherwise 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 can be stored at +4°C almost indefinitely, without losing DNA/RNA integrity



Note: Organic method is not limited to de-novo DNA preparation; also for DNA purification purposes after enzymatic reactions

- *To remove proteins from DNA*

- You have cut DNA with the restriction enzyme EcoRI
- Now you want to purify DNA
- Make Phenol, PCI extraction (to remove restriction enzyme, stays in hydrophobic phase)
- DNA precipitation (Buffer component remains in aqueous supernatant)
- 70% EtOH wash
- dissolve DNA in storage buffer

- *To change buffer of DNA solution*

You have used DNA for an enzymatic reaction using a defined buffer; for another enzymatic reaction you need a different buffer

Purify DNA:

- Phenol, PCI extraction (to remove enzyme; stays in hydrophobic phase)
- DNA precipitation (buffer component remains in aqueous supernatant)
- 70% EtOH wash
- dissolve DNA in new buffer
- Start second enzymatic reaction

General steps in DNA Isolation

1. Genomic DNA

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

2. Plasmid DNA

- Alkaline/SDS method
- Silica column method

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 (see earlier) by selective binding to a support material.

Support Materials

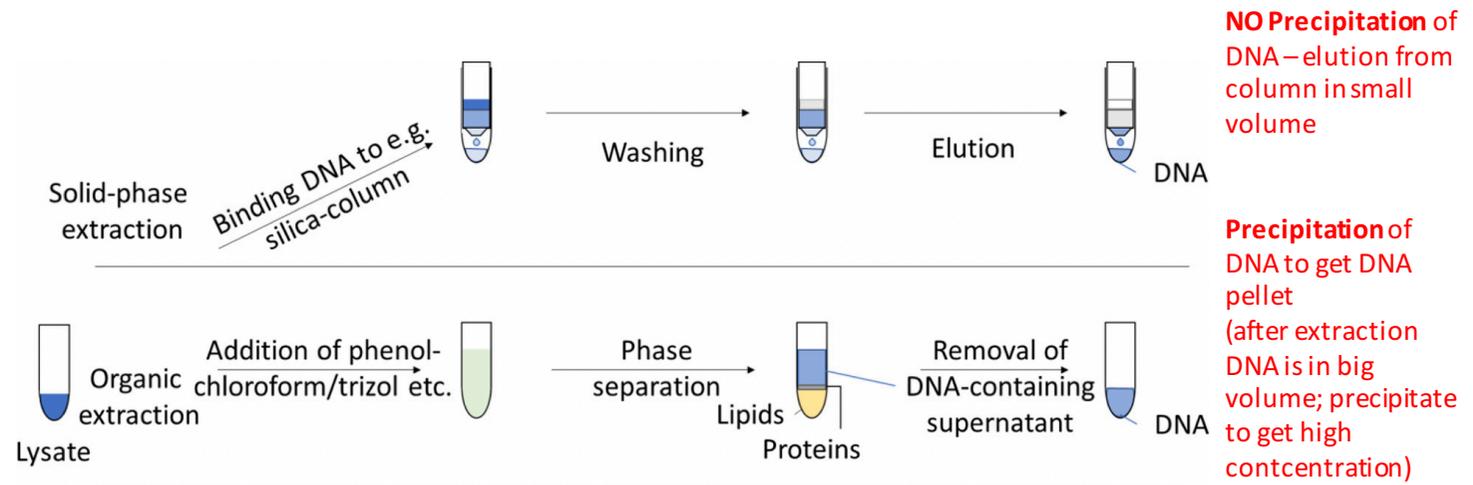
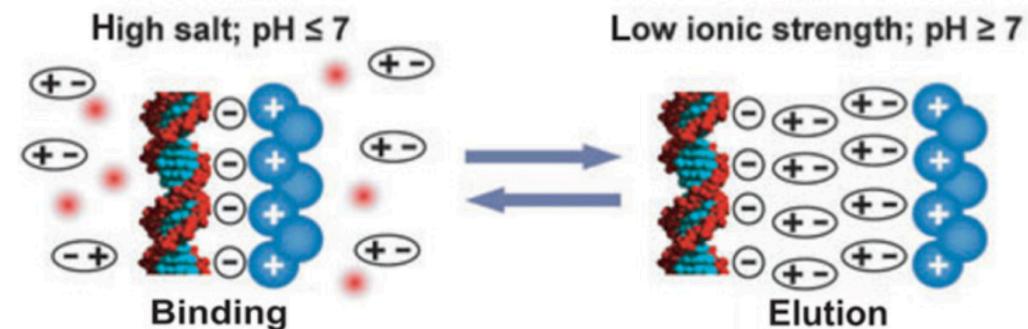
- Silica
- Anion-exchange resin

Advantages

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

Disadvantage

- DNA fragmentation
- Low yield



Xenotransplantation, Volume: 28, Issue: 1, First published: 15 September 2020, DOI: (10.1111/xen.12643)

DNA preparation using silica columns

QIAamp Spin Procedure

Sample



Lyse



Bind



centrifuge

QIAamp Vacuum Procedure

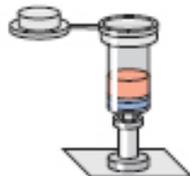
Sample



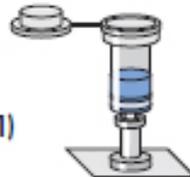
Lyse



Bind



Vacuum



Wash
(Buffer AW1)

vacuum

Main steps in genomic DNA preparation:

1. Cell lysis in buffer containing high concentration of SDS and high concentration of EDTA (binds bivalent ions such as Mg^{2+})

2. Passage on an insoluble resin (silica resins) that specifically binds DNA in the presence of salts (see column concept in mini prep)

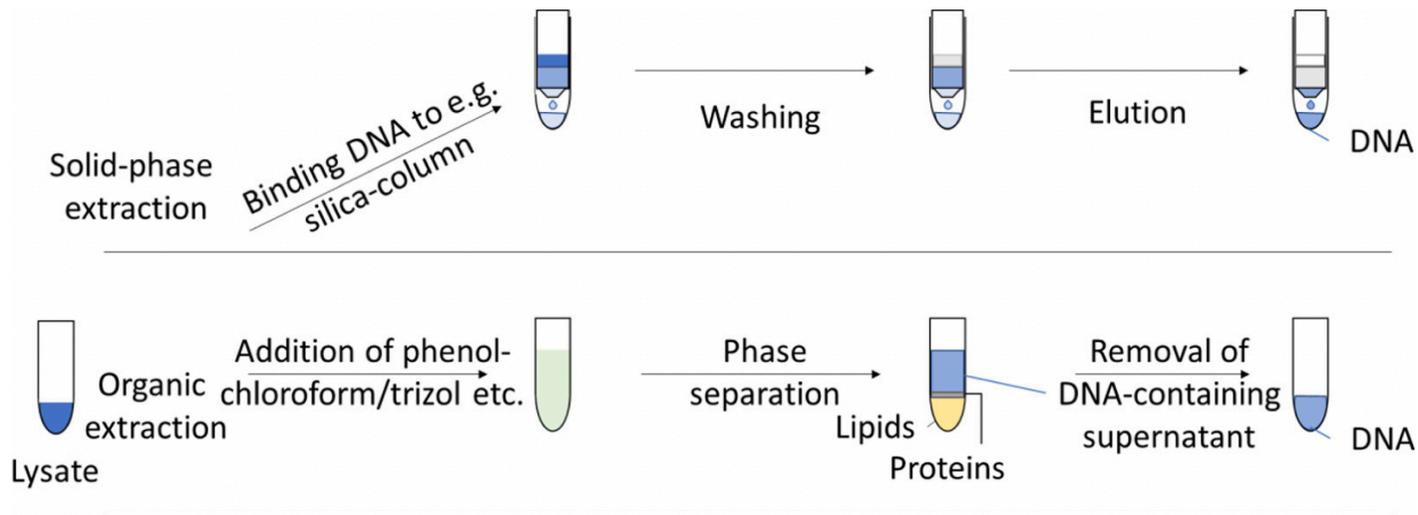
3. Wash resin using specialized washing buffers (pH) to remove contaminants (proteins, etc...)

4. Elute bound DNA with elution buffer (that also serves as storage buffer)

Typically, all components come in a kit

Note: same principal like in mini-prep for plasmids, however genomic DNA is eliminated in mini prep... (why: in mini prep alkaline lysis causes the precipitation of genomic DNA, but not plasmid DNA)

Comparison column method and organic method



NO Precipitation of DNA – elution from column in small volume
High purity → sequencing grade
Cost intensive

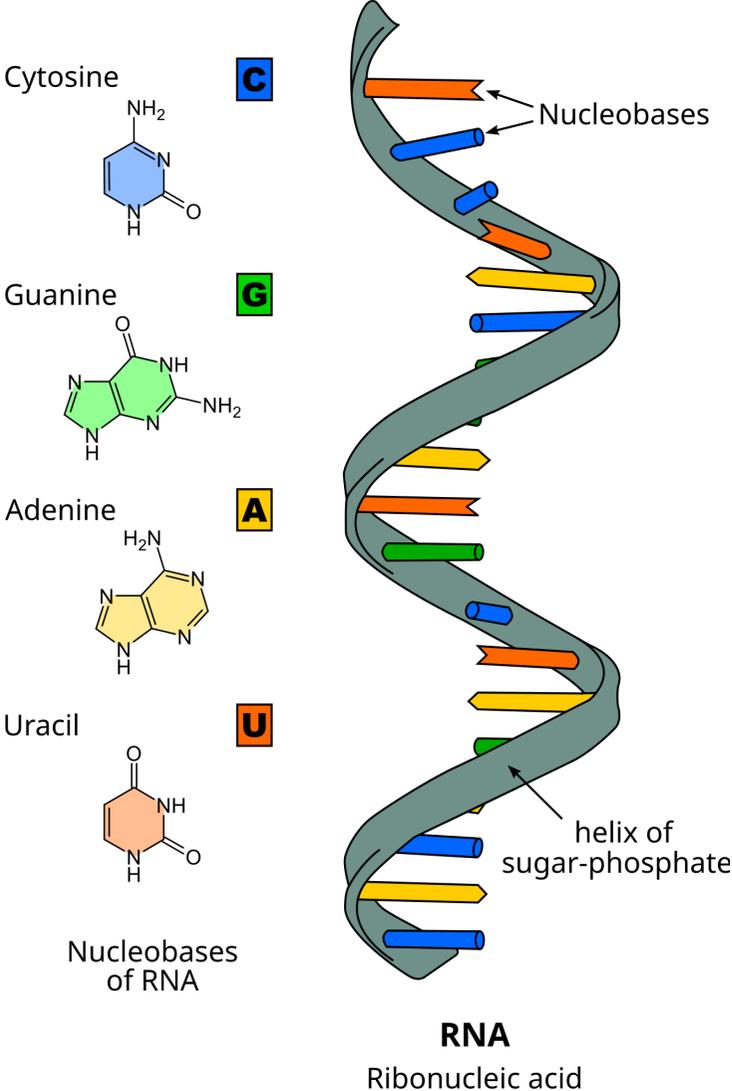
Precipitation of DNA to get DNA pellet
(after extraction DNA is in big volume; precipitate to get high concentration)

Low cost
Risk of contamination with organic substances
Not suitable for NGS

Preparation of nucleic acids

1. Plasmid DNA
2. Genomic DNA from cells (mammals)
- 3. RNA preparation (mammals)**

TOPIC 1: DNA and RNA Preparation



3. Isolation of RNA

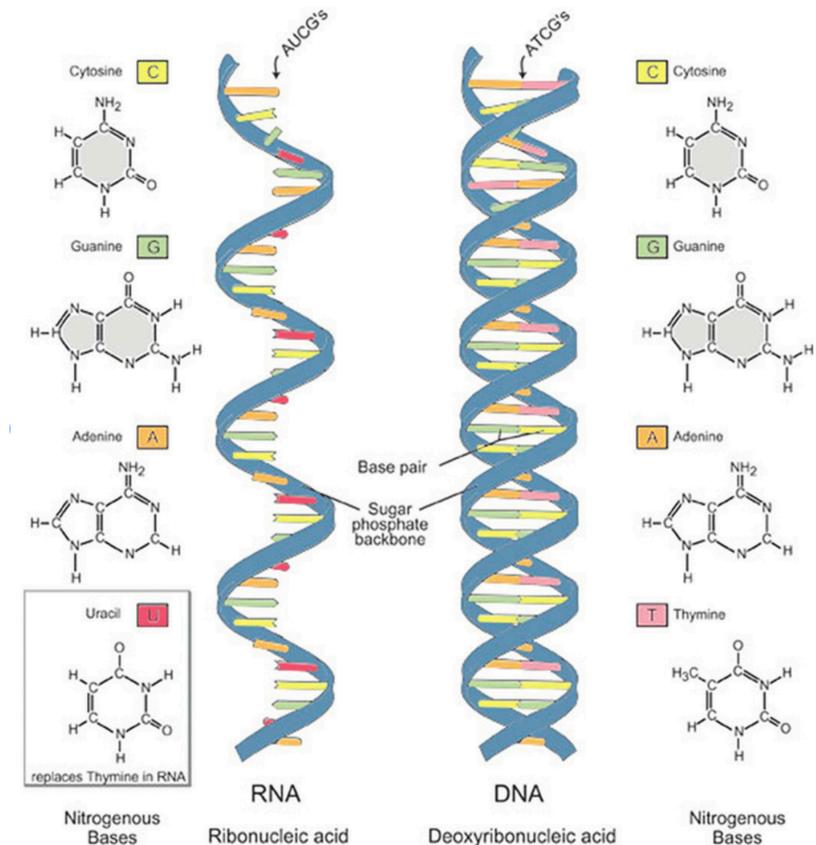
Total RNA contains:

- 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: 10-30 pg per cell (vertebrate)

DNA: 6 pg per cell (vertebrate)

Human body: ca. 37.2 trillion cells



RNA is unstable:

RNA is a molecule that is easily degraded by high pH or ribonucleases (RNases). RNases are highly abundant in the environment – also in the laboratory!

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

ALWAYS WHEN WORKING WITH RNA:

.... reduce risk of contamination with RNases:

- Be careful not to introduce exogenous RNases (wear gloves, work on cleaned surface or fume hood).
- Use only solutions and materials that are sterile or treated with DEPC (diethylpyrocarbonate; TOXIC!!!) and autoclaved– binds covalently to histidine, lysine, cysteine and tyrosine - protein inactivation. Alternatively, RNase free solutions can be bought from lab-reagent suppliers.
- Store the RNA samples on ice during their handling.
- When not used, store RNA preparations at -80°C or as precipitate (4°C or -20°C)
- Use dedicated materials (test tubes, tips, filters, etc.) and solutions for RNA prep
- Clean working surface

RNase Inhibitors:

- **add proteins that act as RNase inhibitors to reactions involving RNA**
- **Treat solutions when possible with DEPC+autoclave**

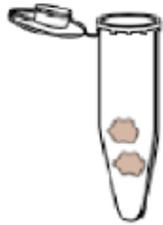
RNA purification techniques

- **Total RNA from biological samples**
 - Organic extraction
 - Affinity purification
- **mRNA from total RNA**
 - Oligo(dT) resins

Organic extraction of total RNA



Organic reagent for RNA preparation



Option 1: Collect cells as pellet (for example Hela cells cultivated in plates)

- Apply specialized, commercial available reagents for RNA preparation: Trizol – Trireagent) = solution of guanidinium thiocyanate-phenol-chloroform
- Reagent lyses cells (if tissue: homogenize in Trizol/Trireagent)



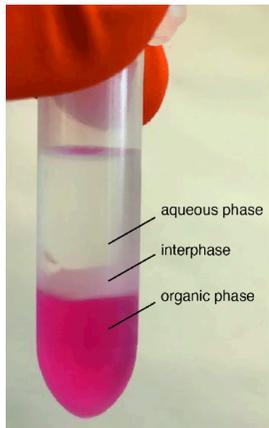
Option 2: Collect tissue pieces (for example mouse liver)

- Apply reagent for RNA preparation: Trizol – Trireagent) and mechanically dissociate tissue
- Reagent lyses cells
- Add chloroform - mix



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 (interfaccia)



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

} Same procedure like for genomic DNA

Note: NO Proteinase K digest; chromatin precipitates at interphase

Guanidinium thiocyanate ideal to lyse cells and virus particles in RNA and DNA extractions, where its function, in addition to its lysing action, is to prevent activity of RNase enzymes and DNase enzymes by denaturing them. These enzymes would otherwise damage the extract.

MOVIE

<https://www.youtube.com/watch?v=RmPsLoIPRwc>

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

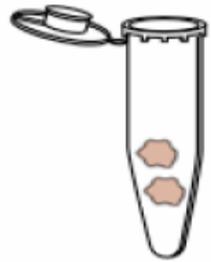
DNase treatment – Phenol extraction – precipitation

DNase treatment – purification via resin

RNA purification techniques

- **Total RNA from biological samples**
 - Organic extraction
 - **Affinity purification**
- **mRNA from total RNA**
 - Oligo(dT) resins

RNA purification techniques



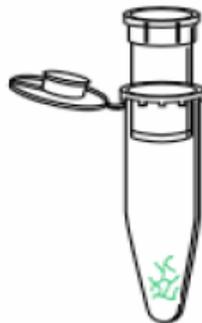
Lyse cells, and spin to remove large particulates/cell debris



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



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



Apply water to the column; purified RNA washes off the glass and is collected (alternatively, as specialized elution buffer can be used)

RNA can be stored at -80°C (or alternatively precipitated and stored in 70% EtOH at 4°C or -20°C)

Typically, all components come in a kit

Affinity purification of total RNA

Advantages

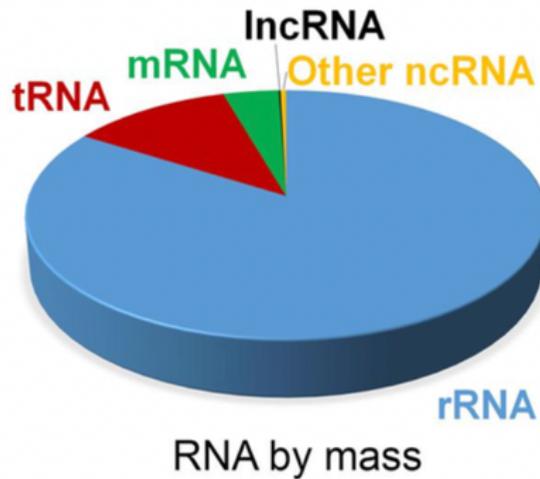
- Eliminates need for organic solvents
- Compatible with a variety of sample types (tissue, tissue culture cells, white blood cells, plant cells, bacteria, yeast, etc.)
- DNase treatment eliminates contaminating genomic DNA
- Excellent RNA purity and integrity

RNA purification techniques

- **Total RNA from biological samples**
 - Organic extraction
 - Affinity purification
- **mRNA from total RNA**
 - Oligo(dT) resins

RNA purification techniques

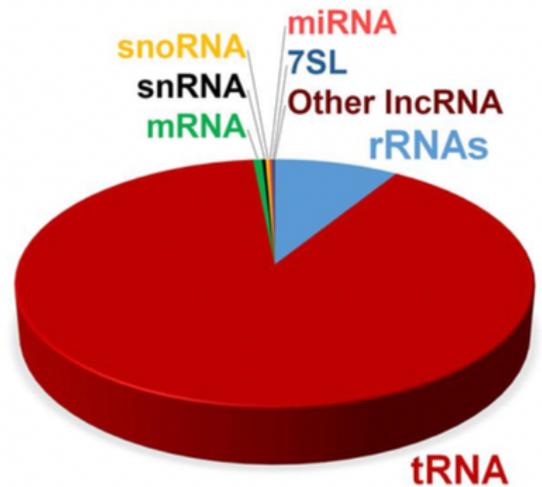
A



...how to purify an RNA species from total RNA

Type	Percent of total RNA by mass	Molecules per cell	Average size (kb)	Total weight picograms/cell	Notes	Reference
rRNAs	80 to 90	3–10 × 10 ⁶ (ribosomes)	6.9	10 to 30		Blobel and Potter (1967), Wolf and Schlessinger (1977), Duncan and Hershey (1983)
tRNA	10 to 15	3–10 × 10 ⁷	<0.1	1.5 to 5	About 10 tRNA molecules /ribosome	Waldron and Lacroute (1975)
mRNA	3 to 7	3–10 × 10 ⁵	1.7	0.25 to 0.9		Hastie and Bishop (1976), Carter et al. (2005)
hnRNA (pre-mRNA)	0.06 to 0.2	1–10 × 10 ³	10*	0.004 to 0.03	Estimated at 2–4% of mRNA by weight	Mortazavi et al. (2008), Menet et al. (2012)
Circular RNA	0.002 to 0.03	3–20 × 10 ³	~0.5	0.0007 to 0.005	Estimated at 0.1–0.2% of mRNA**	Salzman et al. (2012), Guo et al. (2014)
snRNA	0.02 to 0.3	1–5 × 10 ⁵	0.1–0.2	0.008 to 0.04		Kiss and Filipowicz (1992), Castle et al. (2010)
snoRNA	0.04 to 0.2	2–3 × 10 ⁵	0.2	0.02 to 0.03		Kiss and Filipowicz (1992), Cooper (2000), Castle et al. (2010)
miRNA	0.003 to 0.02	1–3 × 10 ⁵	0.02	0.001 to 0.003	About 10 ⁵ molecules per 10 pg total RNA	Bissels et al. (2009)
7SL	0.01 to 0.2	3–20 × 10 ⁴	0.3	0.005 to 0.03	About 1–2 SRP molecules/100 ribosomes	Raue et al. (2007), Castle et al. (2010)
Xist	0.0003 to 0.02	0.1–2 × 10 ³	2.8	0.0001 to 0.003		Buzin et al. (1994), Castle et al. (2010)
Other IncRNA	0.03 to 0.2	3–50 × 10 ³	1	0.002 to 0.03	Estimated at 1–4% of mRNA by weight	Mortazavi et al. (2008), Ramsköld et al. (2009), Menet et al. (2012)

B



*The size for the average unspliced pre-mRNA is 17 kb; however, most pre-mRNAs are partially spliced at any given time, and the average size of hnRNA is estimated at 10 kb (Salditt-Georgieff et al., 1976).

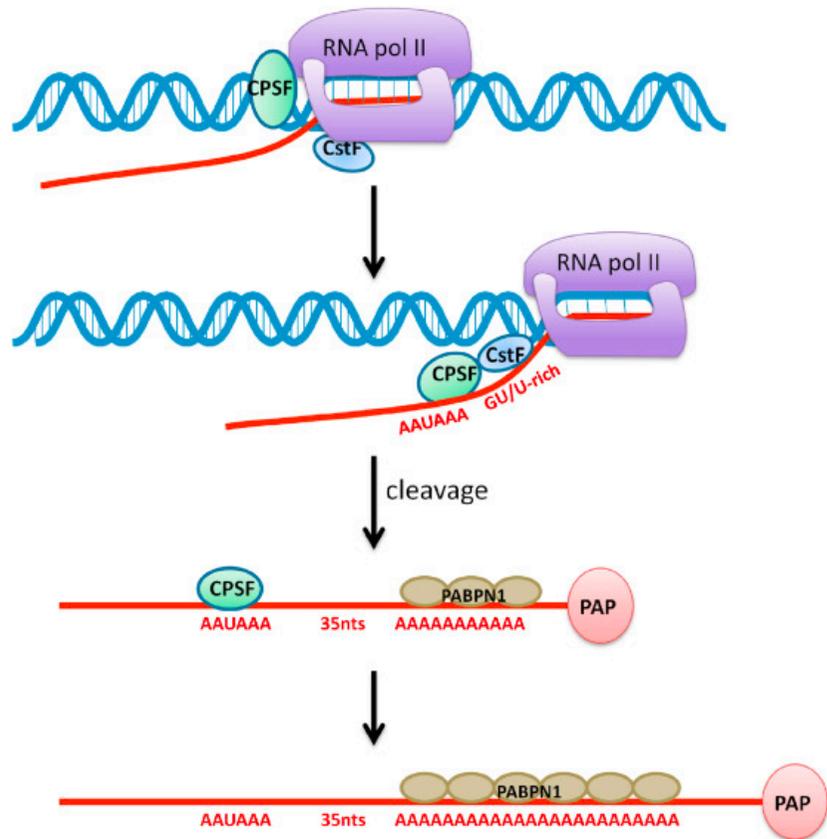
**Based on the finding that 1–2% of all mRNA species generate circular RNA, which is present at 10% of the level of the parental mRNA.

RNA purification techniques

Selective preparation of polyadenylated transcripts

Transcripts produced by RNA Pol II are polyadenylated

- mRNA (coding RNAs)
- lncRNA (long non-coding RNAs)



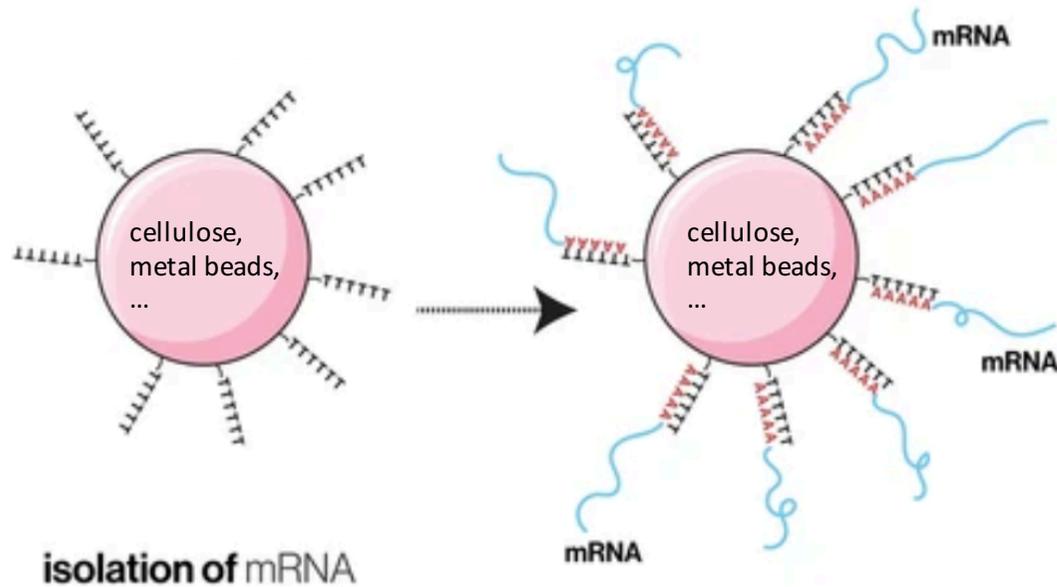
First, the cleavage and polyadenylation specificity factor (CPSF) and cleavage stimulation factor (CstF) protein complexes begin to bind at carboxy-terminal domain (CTD) of RNA polymerase II (RNAP II). When RNAP II progresses through the **polyadenylation signal (PAS)** and CstF is transferred to the new mRNA precursor, CPSF will bind to the PAS, and CstF will bind downstream of PAS. Meanwhile, CFIm complex binds to 20–30 nt upstream of PAS, and Pcf11 (a subunit of CFIm) binds to the CTD of RNAP II. Then **CPSF and CstF initiate cleavage** approximately 35 nt after the PAS. Immediately the **polyadenylation polymerase (PAP)** begins to synthesize poly(A) tails. Almost at the same time, the polyadenylation binding protein in the nucleus (PABPN1) binds to the newly-formed poly(A) sequence. CPSF begins to dissociate, while PAP continues to catalyze polyadenylation and synthesize poly(A) tails. PABPN1 will be a molecular scale defining when polyadenylation stops. Then PAP begins to dissociate, and PABPN1 continues to maintain the binding state.

RNA purification techniques

Selective preparation of polyadenylated transcripts

Transcripts produced by RNA Pol II are polyadenylated

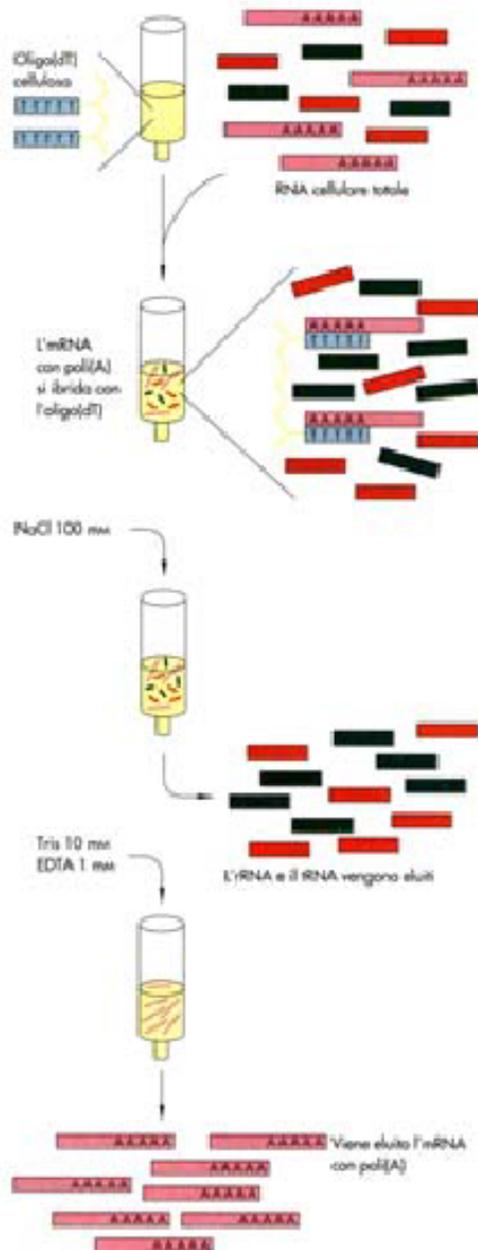
- mRNA (coding RNAs)
- lncRNA (long non-coding RNAs)



Allow to pair polyA RNA to oligo dT coupled beads

Obtain beads with paired RNA (mRNA, lncRNA)

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-eluzione.

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.

CRITICAL PARAMETERS OF DNA AND RNA PREPARATIONS

- 1. Quantity (Concentration)**
- 2. Purity**
- 3. Integrity**

CRITICAL PARAMETERS OF DNA AND RNA PREPARATIONS

1. Quantity (Concentration)

2. Purity

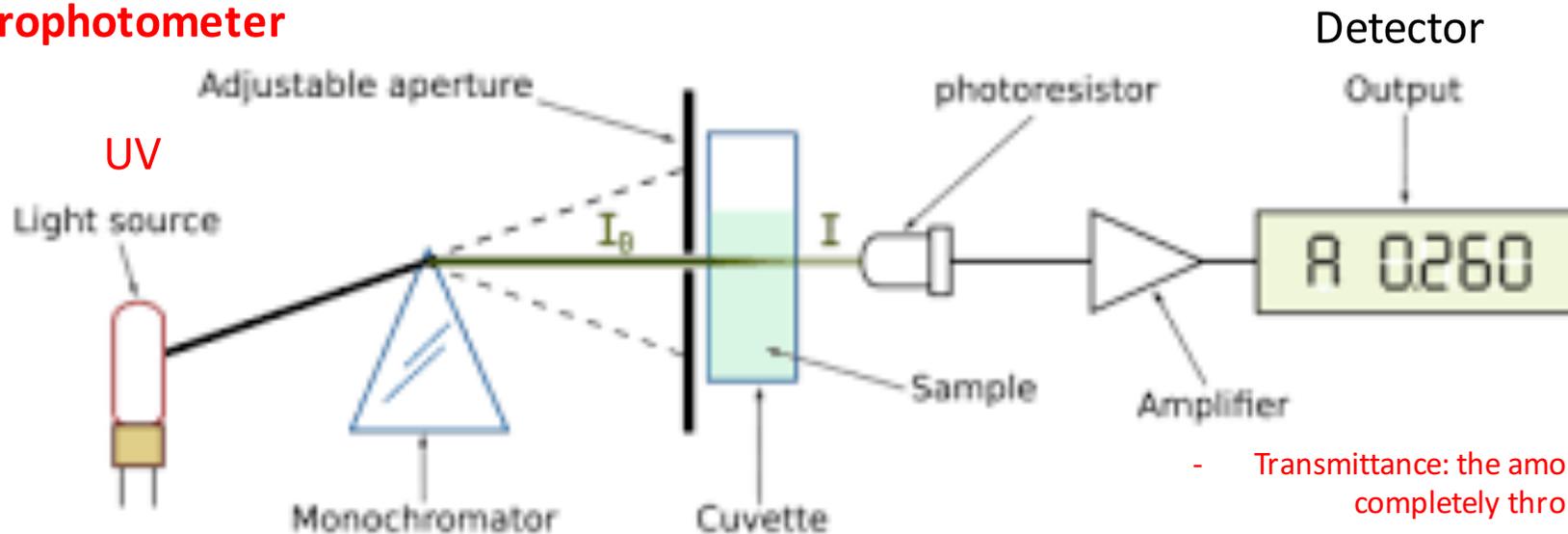
3. Integrity

1. Quantification of DNA and RNA concentration

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.

Spectrophotometer



- Transmittance: the amount of light that passes completely through the sample

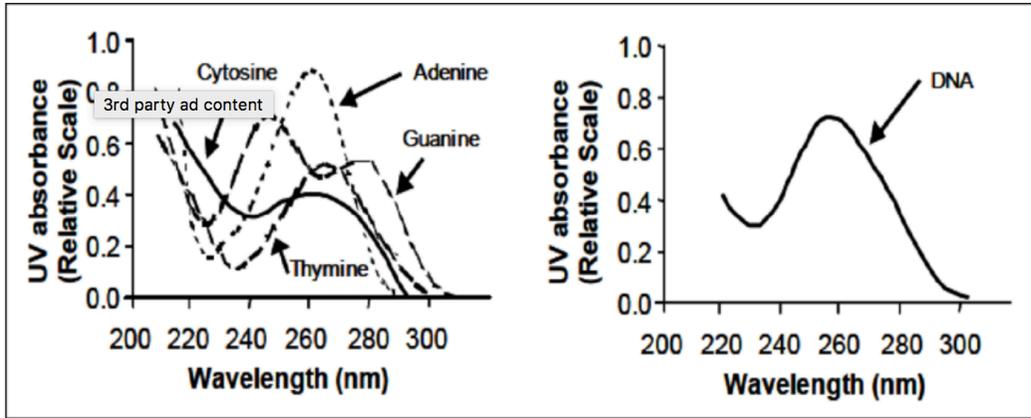
Absorbance: measurement of light that is absorbed by the sample

Monochromator:
Selects precise wavelength

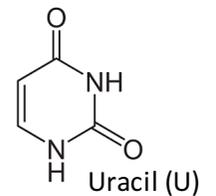
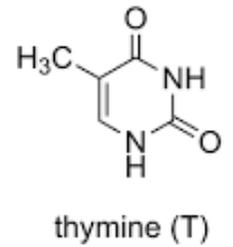
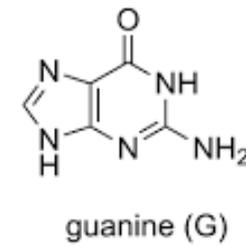
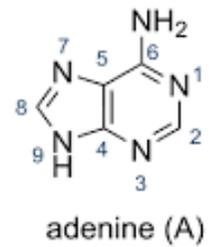
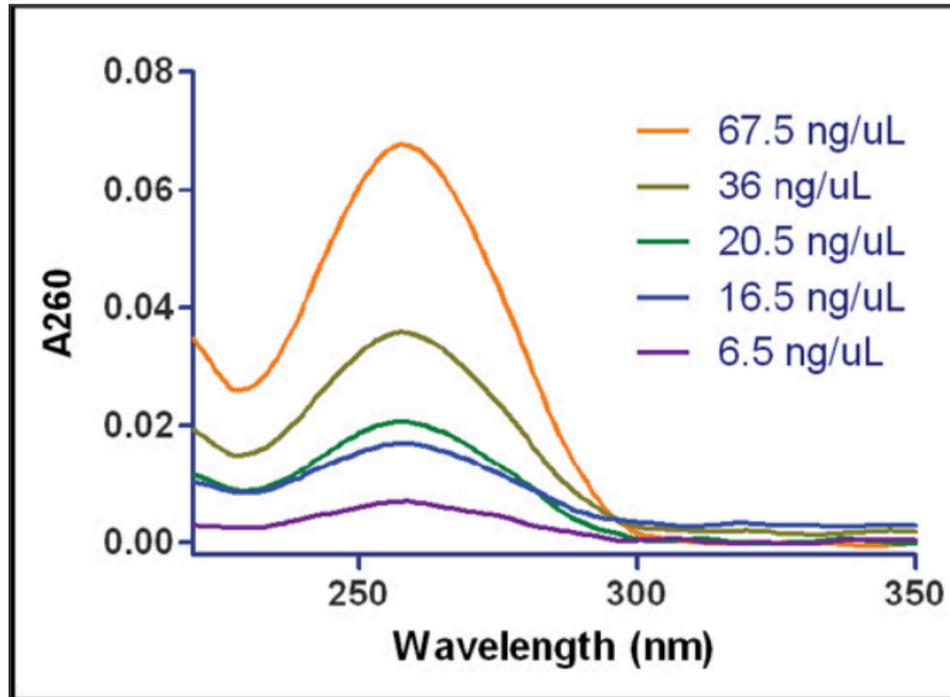
Optical density (OD) = Log (Intensity of incident light / Intensity of Transmitted light)

OD depends on type of molecule, wavelength and concentration

Concentration measurements using Spectrophotometers



5: UV absorbance of Nucleotides (left) and Nucleic Acid (right) at pH7

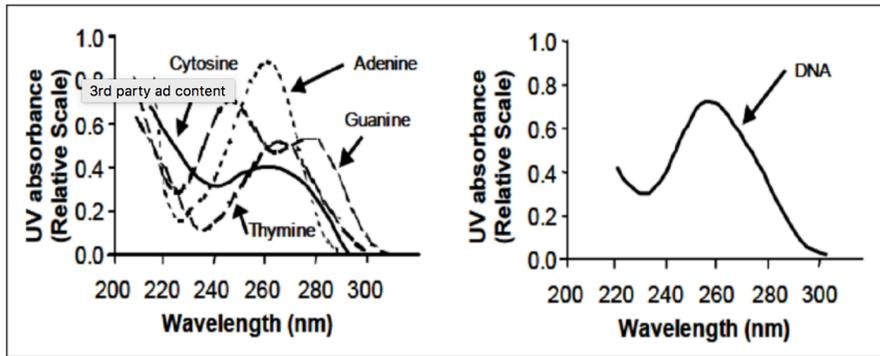


Heterocyclic rings of nucleic acids in DNA show maximum absorbtion at 260 nm

Note RNA has a higher absorbion 260 ratio **due presence of uracil compared to thymine in DNA**

Low Absorbtion value: low nucleic concentration
High Absorbtion value: high nucleic concentration

Concentration measurements using Spectrophotometers



5: UV absorbance of Nucleotides (left) and Nucleic Acid (right) at pH7

Heterocyclic rings of nucleic acid show maximum absorbtion at 260 nm

- Spectrophotometer measures absorbance (A): measurement of light that is absorbed by the sample
- Molecules have specific extinction coefficients (ϵ). For nucleic acid concentration measurements, ϵ , at 260 nm wavelength is used.
- Beer's Law converts absorbance (A) to concentration and path length (l)

$$A = \epsilon \times c \times l \quad \longrightarrow \quad c = A \left(\frac{1}{\epsilon \times l} \right)$$

(ϵ) dsDNA: $0.020 (\mu\text{g/ml})^{-1} \text{cm}^{-1}$

→ at $50 \mu\text{g/ml}$ absorbtion (260nm) measured: $A=1$

$$c = 1 \left(\frac{1}{0,02 * 1 \text{ cm}} \right) = 50 \mu\text{g/ml}$$

(ϵ) ssDNA: $0.027 (\mu\text{g/ml})^{-1} \text{cm}^{-1}$,

→ at $33 \mu\text{g/ml}$ absorbtion (260nm) measured: $A=1$

$$c = 1 \left(\frac{1}{0,027 * 1 \text{ cm}} \right) = 33 \mu\text{g/ml}$$

(ϵ) RNA: $0.025 (\mu\text{g/ml})^{-1} \text{cm}^{-1}$

→ at $40 \mu\text{g/ml}$ absorbtion (260nm) measured: $A=1$

$$c = 1 \left(\frac{1}{0,025 * 1 \text{ cm}} \right) = 40 \mu\text{g/ml}$$

Concentration measurements using Spectrophotometers

(ϵ) dsDNA: $0.020 (\mu\text{g/ml})^{-1} \text{ cm}^{-1}$

→ at $50\mu\text{g/ml}$ absorbtion (260nm) measured: $A=1$

$$c = 1 \left(\frac{1}{0,02 * 1 \text{ cm}} \right) = 50\mu\text{g/ml}$$

(ϵ) ssDNA: $0.027 (\mu\text{g/ml})^{-1} \text{ cm}^{-1}$,

→ at $33 \mu\text{g/ml}$ absorbtion (260nm) measured: $A=1$

$$c = 1 \left(\frac{1}{0,027 * 1 \text{ cm}} \right) = 33\mu\text{g/ml}$$

(ϵ) RNA: $0.025 (\mu\text{g/ml})^{-1} \text{ cm}^{-1}$

→ at $40\mu\text{g/ml}$ absorbtion (260nm) measured: $A=1$

$$c = 1 \left(\frac{1}{0,025 * 1 \text{ cm}} \right) = 40\mu\text{g/ml}$$

Practical aspects from the laboratory

1. Nucleic samples are diluted before measuring the concentration (typically 1:100 or 1:50)
2. Pathlength (l) in photometer is typically 1cm

(ϵ) dsDNA: $0.020 (\mu\text{g/ml})^{-1} \text{ cm}^{-1}$

→ at $50\mu\text{g/ml}$ absorbtion (260nm) measured: $A=1$

$$c (\mu\text{g/ml}) = A \times 50 \times \text{dilution factor}$$

(ϵ) ssDNA: $0.027 (\mu\text{g/ml})^{-1} \text{ cm}^{-1}$,

→ at $33 \mu\text{g/ml}$ absorbtion (260nm) measured: $A=1$

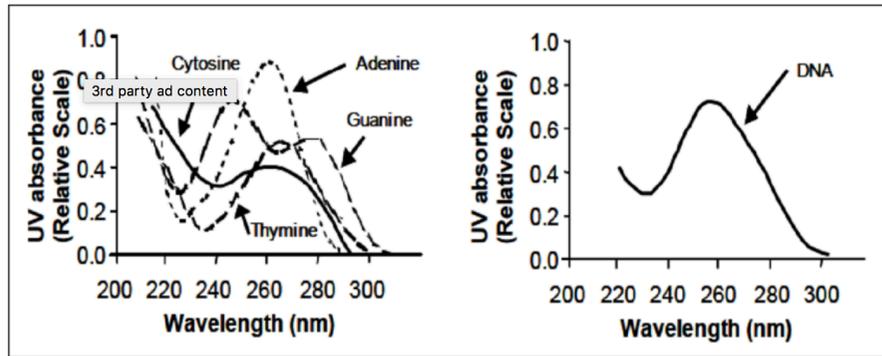
$$c (\mu\text{g/ml}) = A \times 33 \times \text{dilution factor}$$

(ϵ) RNA: $0.025 (\mu\text{g/ml})^{-1} \text{ cm}^{-1}$

→ at $40\mu\text{g/ml}$ absorbtion (260nm) measured: $A=1$

$$c (\mu\text{g/ml}) = A \times 40 \times \text{dilution factor}$$

Concentration measurements using Spectrophotometers



5: UV absorbance of Nucleotides (left) and Nucleic Acid (right) at pH7



Heterocyclic rings of nucleic acid show maximum absorbtion at 260 nm

Typical situation in the laboratory: Plasmid Mini prep done

Now determine the concentration of DNA in the prep:

Note: concentration depends on copy number, number of bacteria, efficacy of preparation

Take 3 ul of mini prep (total volume 30 μ l) and dillute **100 fold** (3 μ l mini prep + 297 μ l water)

Transfere dilluted DNA prep into cuvette

Measure OD at 260nm = "A260"

RESULT A260: A = 0,200

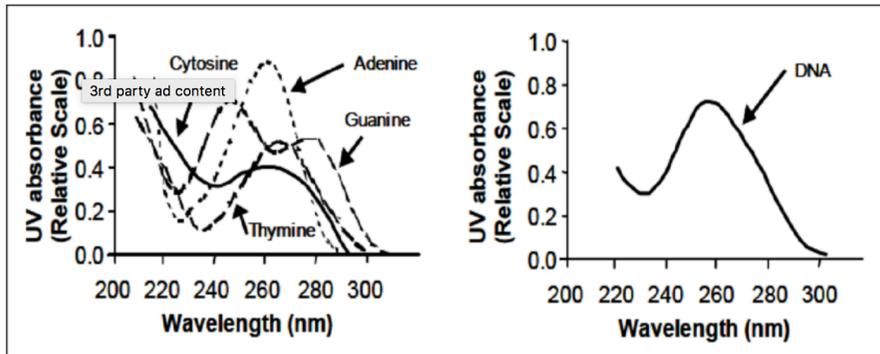
$$c \text{ (}\mu\text{g/ml)} = A \times 50 \times \text{dilution factor}$$

$$1000 \text{ (}\mu\text{g/ml)} = 0,200 \times 50 \times 100$$

DNA concentration is: 1000 (μ g/ml) = 1 μ g/ μ l

Total amount of DNA (30 μ l) in mini prep: 30 μ g

Concentration measurements using Spectrophotometers



5: UV absorbance of Nucleotides (left) and Nucleic Acid (right) at pH7



Heterocyclic rings of nucleic acid show maximum absorbtion at 260 nm

Typical situation in the laboratory: RNA prep done; now determine the concentration of RNA

Take 3 ul of RNA prep (total volume 60 μ l) and dillute **100 fold** (3 μ l RNA prep + 297 μ l water)

Transfere dilluted DNA prep into cuvette

Measure OD at 260nm = "A260"

Absorbtion at 260 nm: A = 0,200

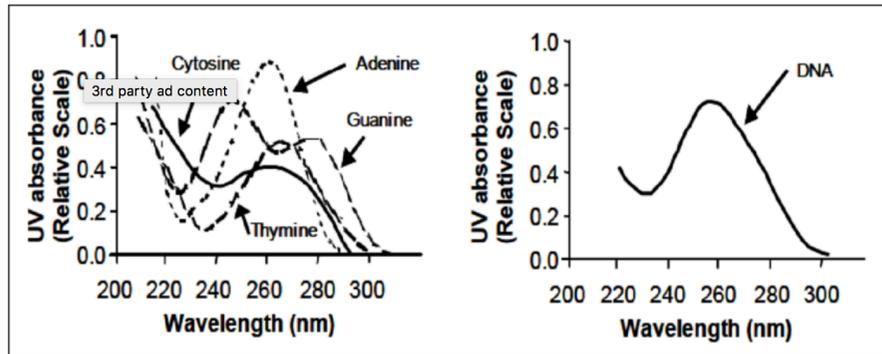
$$c (\mu\text{g/ml}) = A \times 40 \times \text{dilution factor}$$

$$800 (\mu\text{g/ml}) = 0,200 \times 40 \times 100$$

DNA concentration is: 800 (μ g/ml) = 0,8 μ g/ μ l

Total amount of RNA : 48 μ g

Concentration measurements using Spectrophotometers



5: UV absorbance of Nucleotides (left) and Nucleic Acid (right) at pH7



Heterocyclic rings of nucleic acid show maximum absorbance at 260 nm

Typical situation in the laboratory: determination of the concentration of primers (ssDNA)

20 μ l of primers in buffer, concentration not known.

Dilute 1:100 and transfer diluted DNA prep into cuvette

Measure OD at 260nm = "A260"

Absorbance at 260 nm: A = 0,300

$$c (\mu\text{g/ml}) = A \times 33 \times \text{dilution factor}$$

$$990 (\mu\text{g/ml}) = 0,300 \times 33 \times 100$$

DNA concentration is: 990 ($\mu\text{g/ml}$) = 0,99 $\mu\text{g}/\mu\text{l}$

Total amount of DNA : 19,8 μg

CRITICAL PARAMETERS OF DNA AND RNA PREPARATIONS

1. Quantity (Concentration)

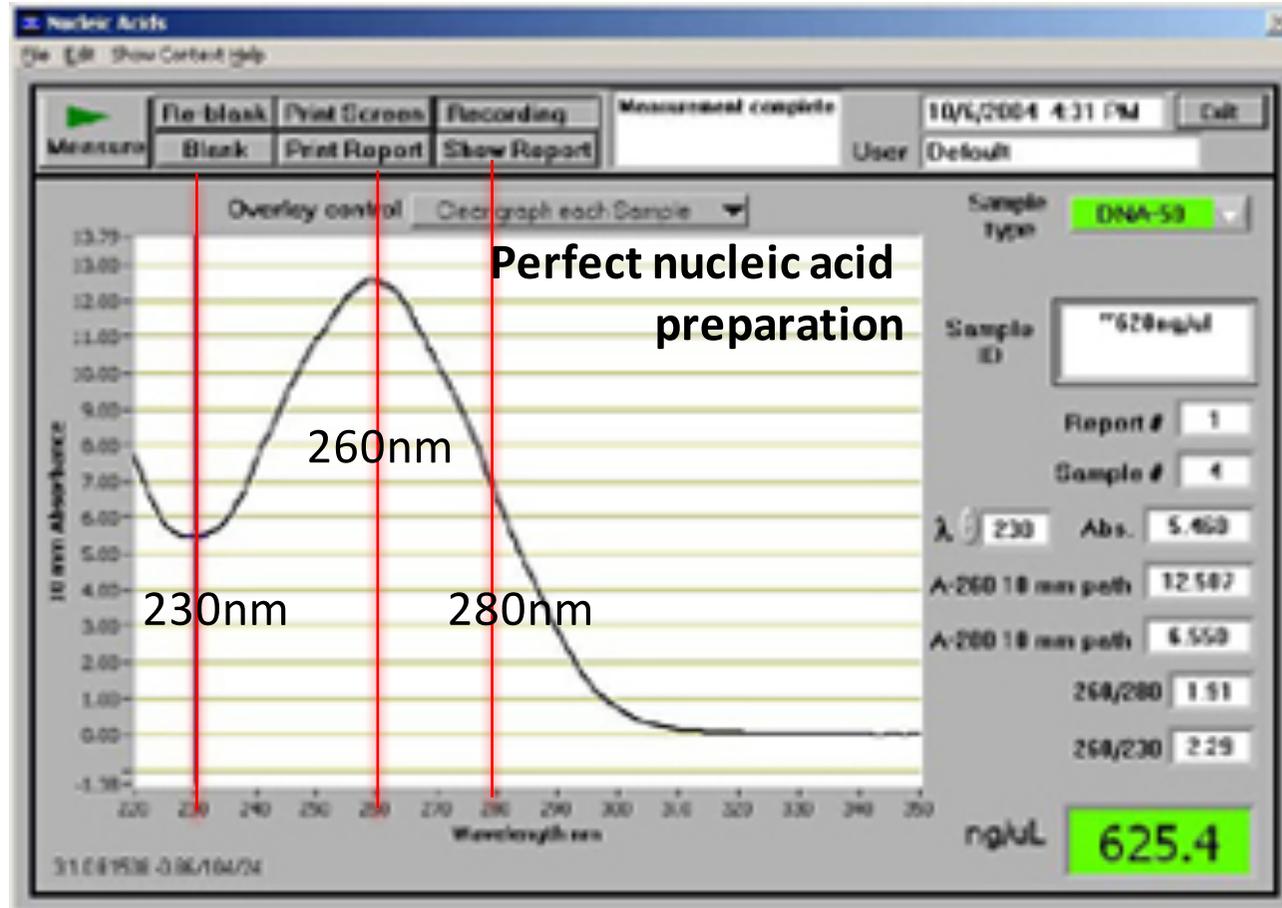
2. Purity

3. Integrity

2. Determination of DNA and RNA purity

Measuring absorbance (A) using a SPECTROPHOTOMETER at different wave lengths

→ Measure absorbance of DNA/RNA preparation = concentration



Maximum absorbance:

Molecule of interest:

RNA, DNA: max A at 260 nm

Contaminants:

1. Protein: A280nm

2. Organic compounds

/salt/carbohydrates: A230 nm



Determination of DNA and RNA purity

Measuring absorbance (A) using a SPECTROPHOTOMETER at different wave lengths

→ Measure absorbance of DNA/RNA preparation = concentration

Maximum absorbance:

Molecule of interest:

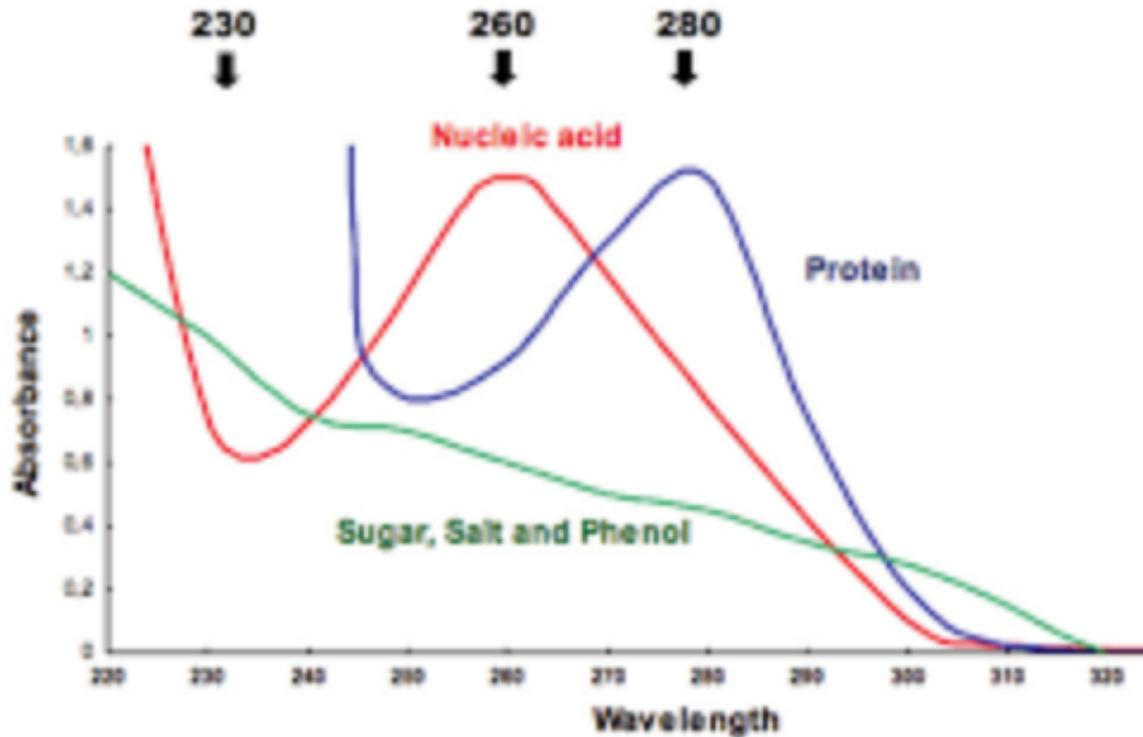
RNA, DNA: 260 nm

Contaminants (inprecise purification)

1. Protein: A280 nm

2. Organic compounds

/salt/carbohydrates: A230 nm

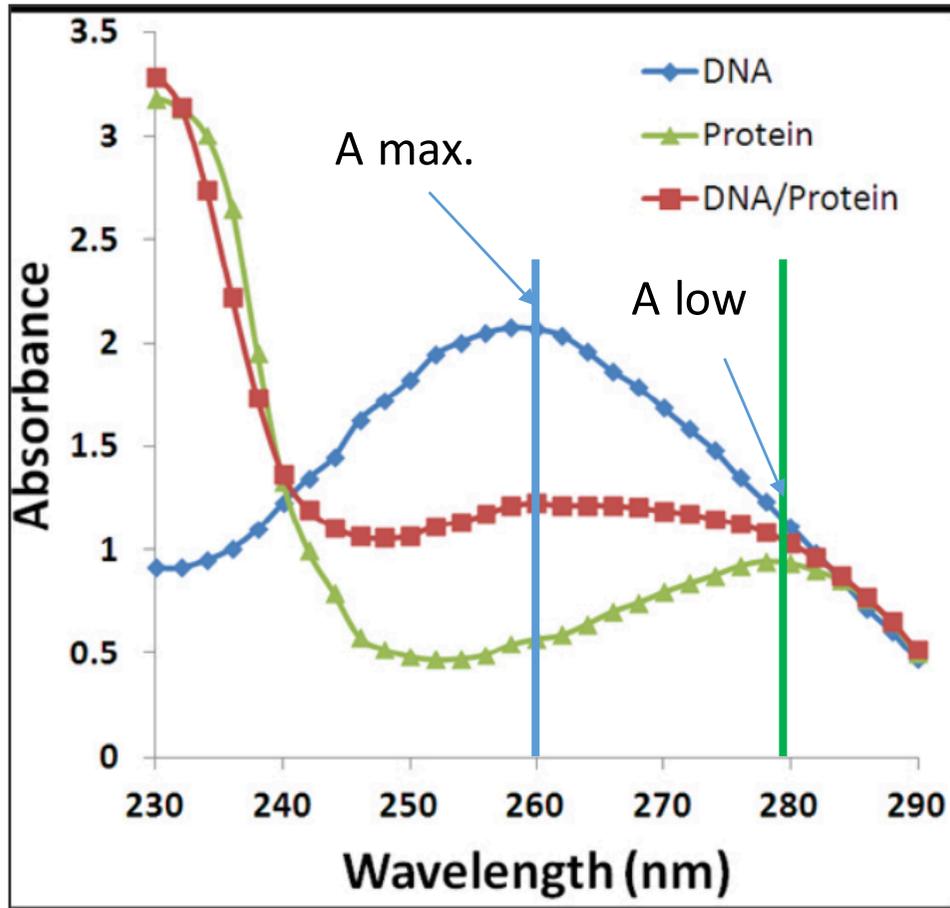


Determination of DNA and RNA purity

Measuring absorbance (A) using a SPECTROPHOTOMETER at different wave lengths

→ Measure absorbance of DNA/RNA preparation at 230nm, 260nm, 280nm

PROTEIN CONTAMINATION in nucleic acid preparation



Molecule of interest: RNA, DNA: 260 nm
Contaminants (imprecise purification)

Protein: 280 nm

Use ratio A_{260}/A_{280} as indicator for protein contamination

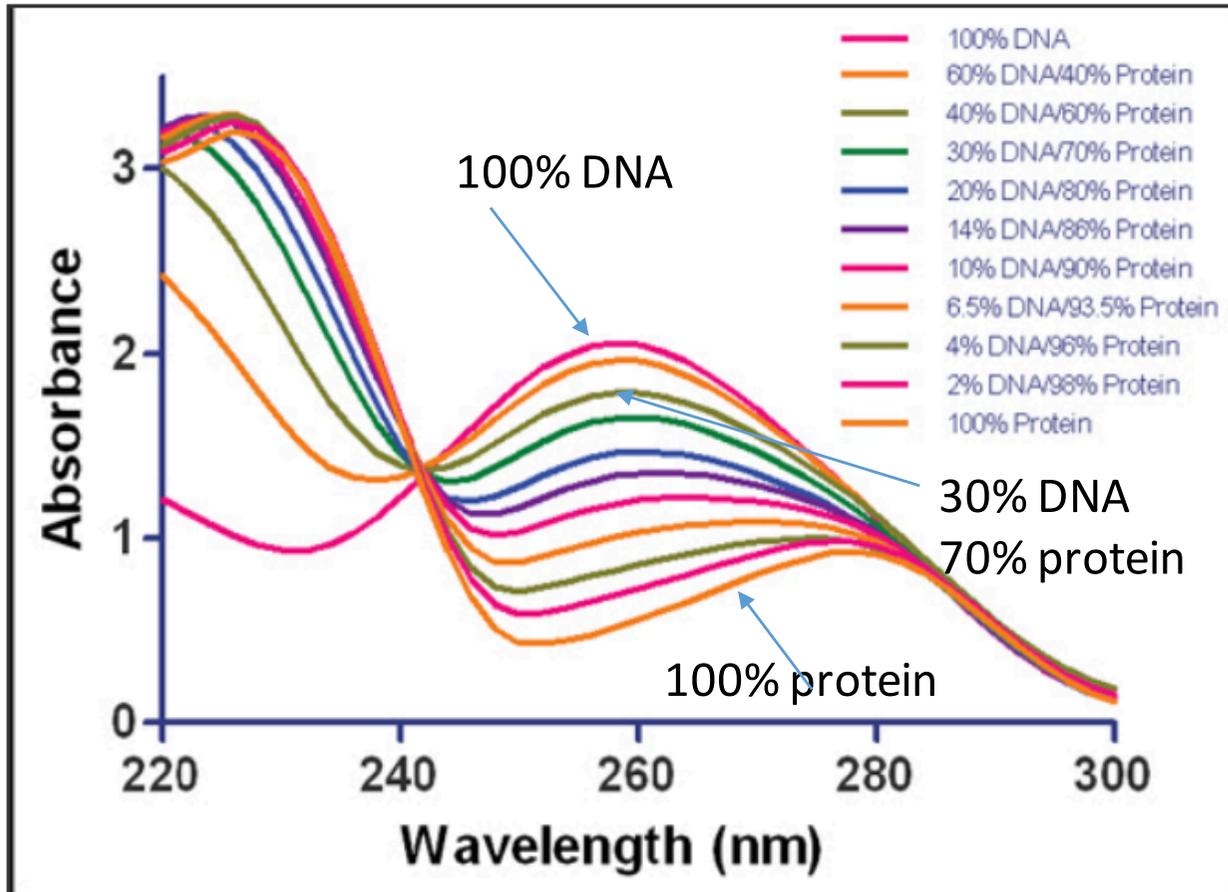


Determination of DNA and RNA purity

Measuring absorbance (A) using a SPECTROPHOTOMETER at different wave lengths

→ Measure absorbance of DNA/RNA preparation at 230nm, 260nm, 280nm

PROTEIN CONTAMINATION in nucleic acid preparation



Maximum absorbance:

Molecule of interest: RNA, DNA: 260 nm

Contamination with protein: absorption relevant at 280 nm



Calculate: ratio A_{260nm}/A_{280nm}

Ratio A_{260nm}/A_{280nm} high: low/no protein contamination

Ratio A_{260nm}/A_{280nm} low: protein contamination

Determination of DNA and RNA purity

Measuring absorbance (A) using a SPECTROPHOTOMETER at different wave lengths

→ Measure absorbance of DNA/RNA preparation = concentration

Maximum absorbance:

Molecule of interest:

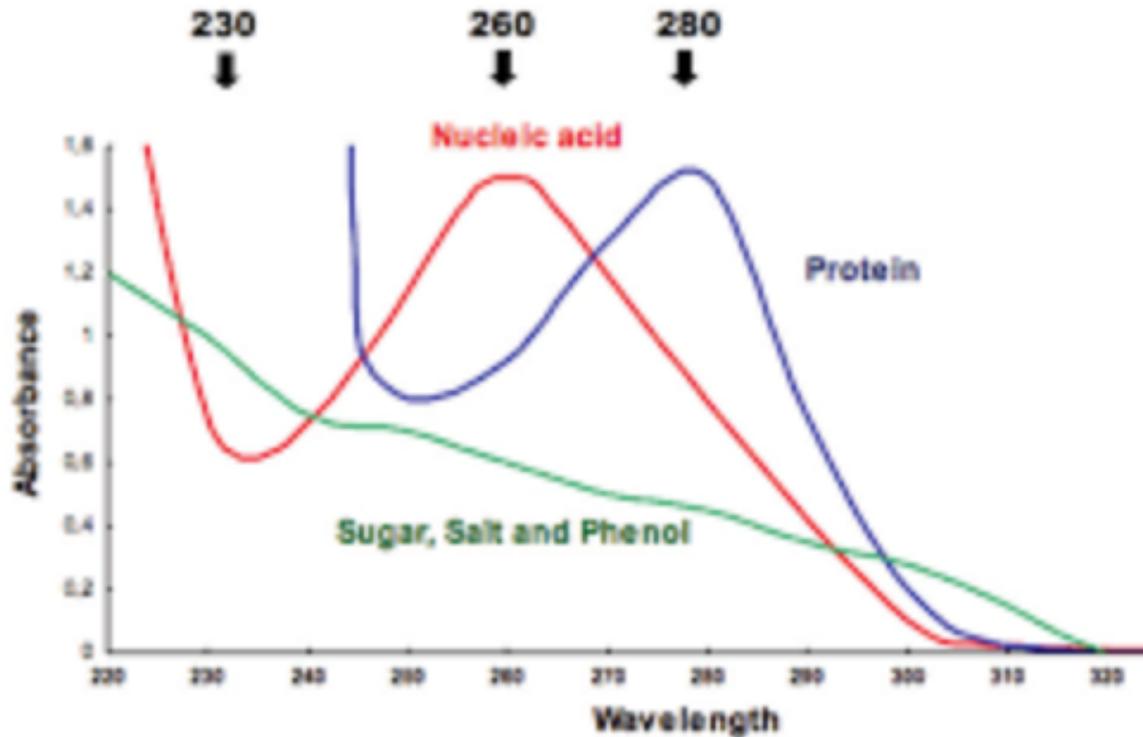
RNA, DNA: 260 nm

Contaminants (inprecise purification)

1. Protein: A280 nm

2. Organic compounds

/salt/carbohydrates: A230 nm

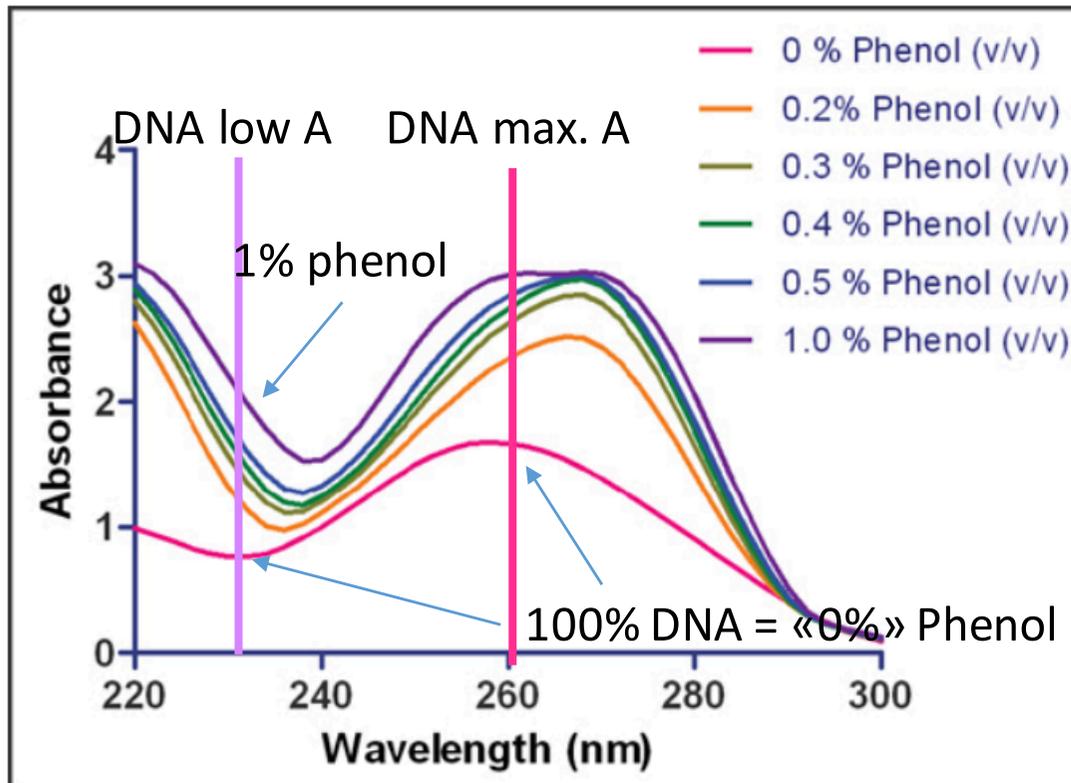


Determination of DNA and RNA purity

Measuring absorbance (A) using a SPECTROPHOTOMETER at different wave lengths

→ Measure absorbance of DNA/RNA preparation at 230nm, 260nm, 280nm

Organic compounds /salt/carbohydrates contamination in nucleic acid preparation



Maximum absorbance:

Molecule of interest: RNA, DNA: 260 nm

Contamination with organic compounds /salt/carbohydrates: absorption relevant at A230 nm

Calculate: ratio $A_{260\text{nm}}/A_{230\text{nm}}$

Ratio $A_{260\text{nm}}/A_{230\text{nm}}$ high: low/no contamination

Ratio $A_{260\text{nm}}/A_{230\text{nm}}$ low: contamination

3. Determination of DNA and RNA purity

- ratio $A_{260}/A_{280} \rightarrow$ PROTEIN CONTAMINATION
- ratio $A_{260}/A_{230} \rightarrow$ ORGANIC COMPOUND, SALT, CARBOHYDRATE CONTAMINATION

Ratio A_{260}/A_{280} = quantification of protein contamination

For DNA preparations: **ideal A_{260}/A_{280} ratio: 1.6-1.8**

Example DNA: $A_{260}=0.234$; $A_{280}=0,137$: ratio =1,7 \rightarrow OK

Example DNA: $A_{260}=0.198$; $A_{280}=0,142$: ratio =1,4 \rightarrow NO

For RNA preparations: **ideal A_{260}/A_{280} ratio: 1.8-2.0**

If ratio is out of range : **contamination with proteins**

Example RNA: $A_{260}=0.234$; $A_{280}=0,119$: ratio =1,96 \rightarrow OK

Example RNA: $A_{260}=0.201$; $A_{280}=0,119$: ratio =1,69 \rightarrow NO

Ratio A_{260}/A_{230} = quantification of contamination with carbohydrates and phenol (solvents)

For RNA and DNA: ratio must be ca. 2.0 - 2.2

if ratio is too low (high absorption at 230nm) contamination with solvent/carbohydrate

Example: $A_{260}=0.234$; $A_{230}=0,111$: ratio =2,1 \rightarrow OK

Example: $A_{260}=0.234$; $A_{230}=0,200$: ratio =1,17 \rightarrow NO

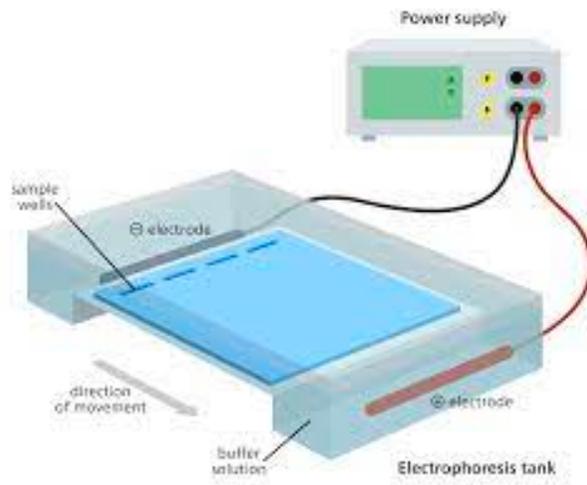
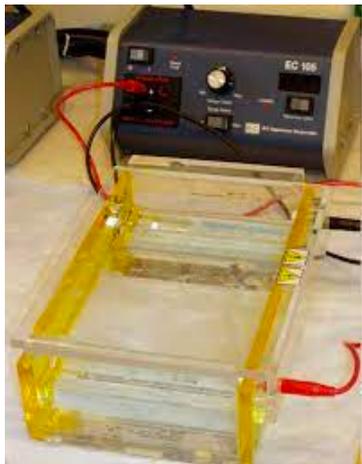
CRITICAL PARAMETERS OF DNA AND RNA PREPARATIONS

- 1. Quantity (Concentration)**
- 2. Purity**
- 3. Integrity**

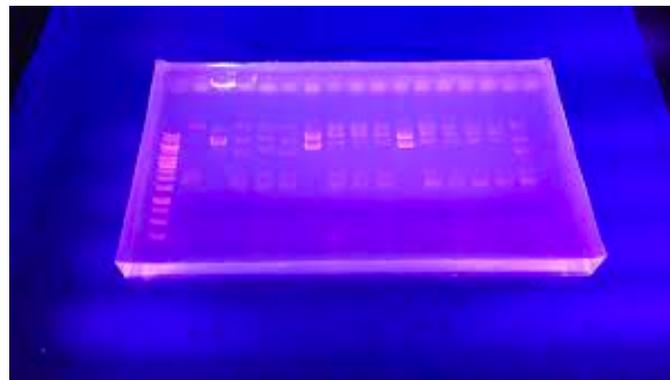
3. DNA / RNA Integrity

Easiest method: Gel electrophoresis

Agarose gel: Agarose is a heteropolysaccharide, generally extracted from certain red seaweed. It is a linear polymer made up of the repeating unit of agarobiose, which is a disaccharide made up of D-galactose and 3,6-anhydro-L-galactopyranose. Agarose is one of the two principal components of agar, and is purified from agar by removing agar's other component, agarpectin. Agarose powder is mixed with buffer, boiled and poured into a tray, inserting a «comb» creates slots (pozzetti) for loading DNA/RNA samples. DNA/RNA stains are added to the gel or directly into the RNA/DNA sample



- Stain directly in DNA/RNA sample
- Stain in agarose gel, running through the gel, nucleic acids are saturated with the dye present in gel



Exposure of gel to UV light:

DNA stain absorbs UV light and emits light in the visible nm range; take a picture

(Ethidium Bromide: classic DNA stain, however carcinogen; substituted by safer dyes)

Gel preparation

<https://www.youtube.com/watch?v=wXiiTW3pfIM>

Gel run

https://youtu.be/U2-5ukpKg_Q

Movie: agarose gel electrophoresis

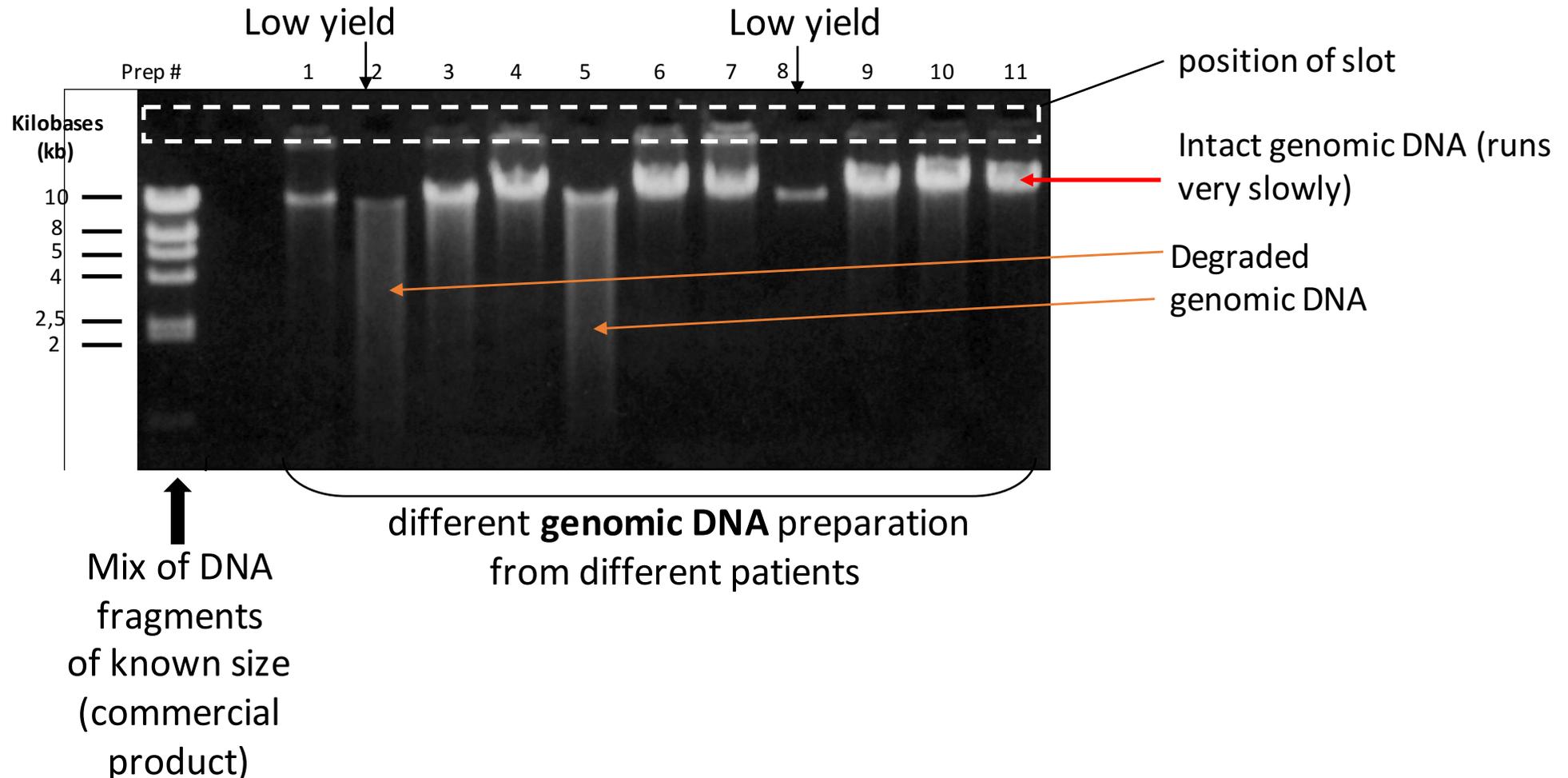
<https://www.youtube.com/watch?v=vq759wKCCUQ>

Why are there so many bubbles? H_2 and O_2 . Once the electric current is applied, the anode and cathode are involved in redox reactions that remove electrons from water molecules in the running buffer, resulting in gas formation.

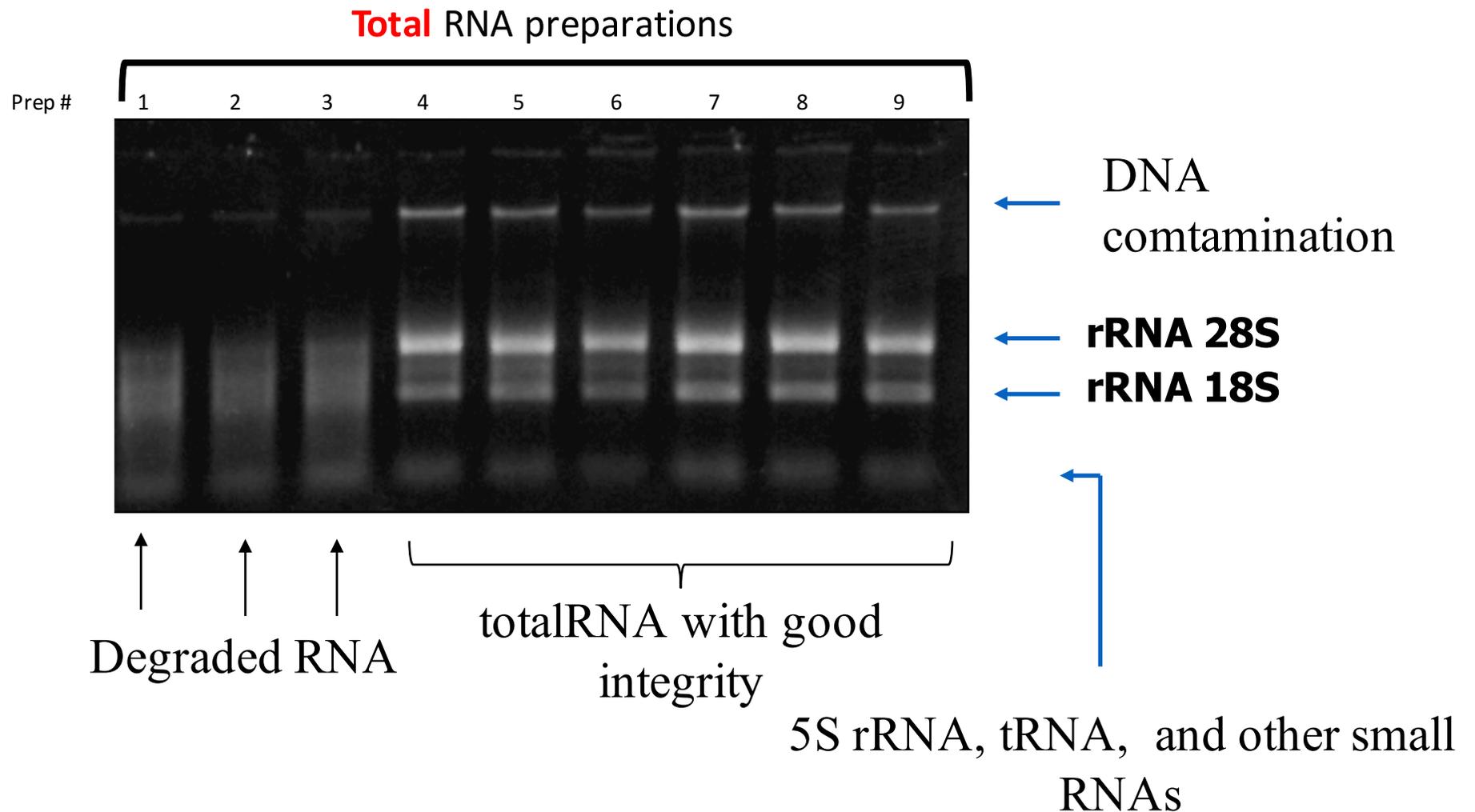
3. DNA Integrity

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 fluorescente.



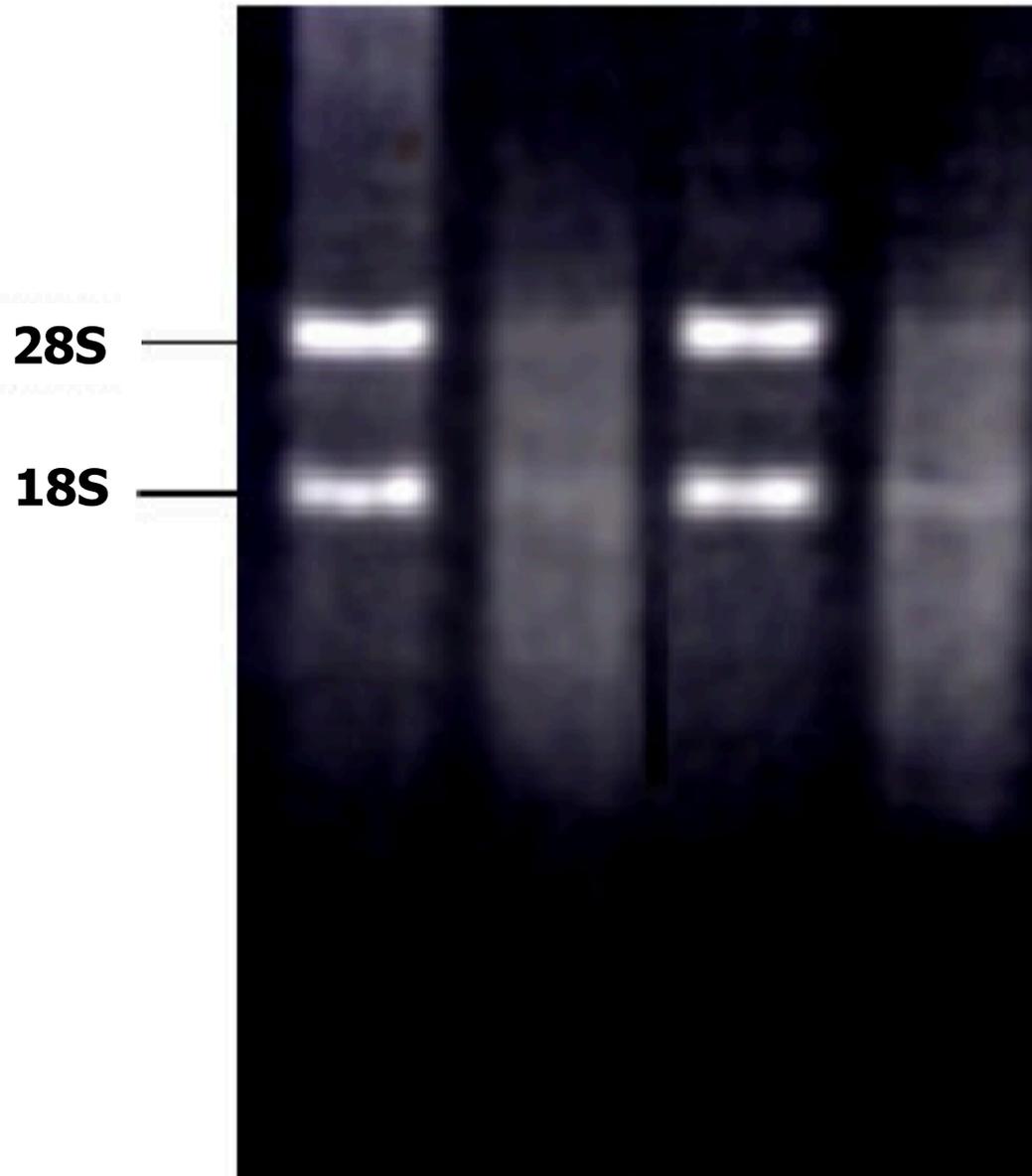
Determination of RNA integrity



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

Determination of RNA integrity – poly A RNA (mRNA)

1 2 3 4



polyA RNA (mRNAs)

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

Lane 1: total RNA (5 μ g)

Lane 2: poly A RNA fraction prepared from total RNA (5 μ g)

→ 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!!)