Programma di Laboratorio

1. 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.

2. 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 from mammalian cells
3. PCR to detect presence of Alu repeat
4. 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:
Plasmid prep for DNA cloning, PCR, restriction, sequencing, fingerprinting, library construction etc.;

The type of DNA:
genomic vs plasmid (size)

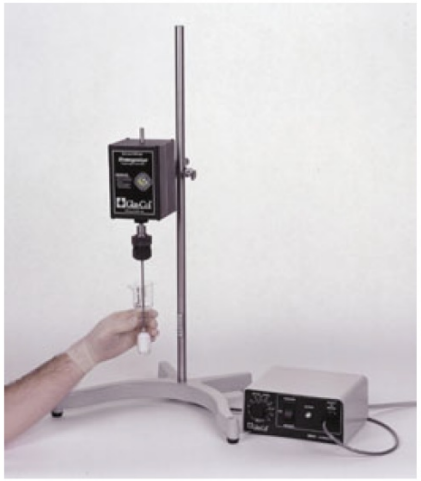
Why to prepare DNA

- 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 (human, mouse, etc..)

Resistant structure
Homogenization (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), high salt concentration
Yeast/fungi - zymolase

b) Cell membrane rupture
-Detergents: SDS, sarcosine, triton X-100, CTAB Proteinases; Proteinase K, Pronase E
-Chelators → EDTA (binds bivaltent ions)
- 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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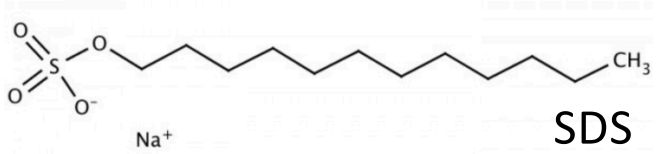
GENOMIC DNA Preparation from Cells
The organic method

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



SDS

•Lyse cells in buffer with SDS/PK /(DTT) (500 ul)

- SDS = detergent (Sodium dodecyl sulfate, 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

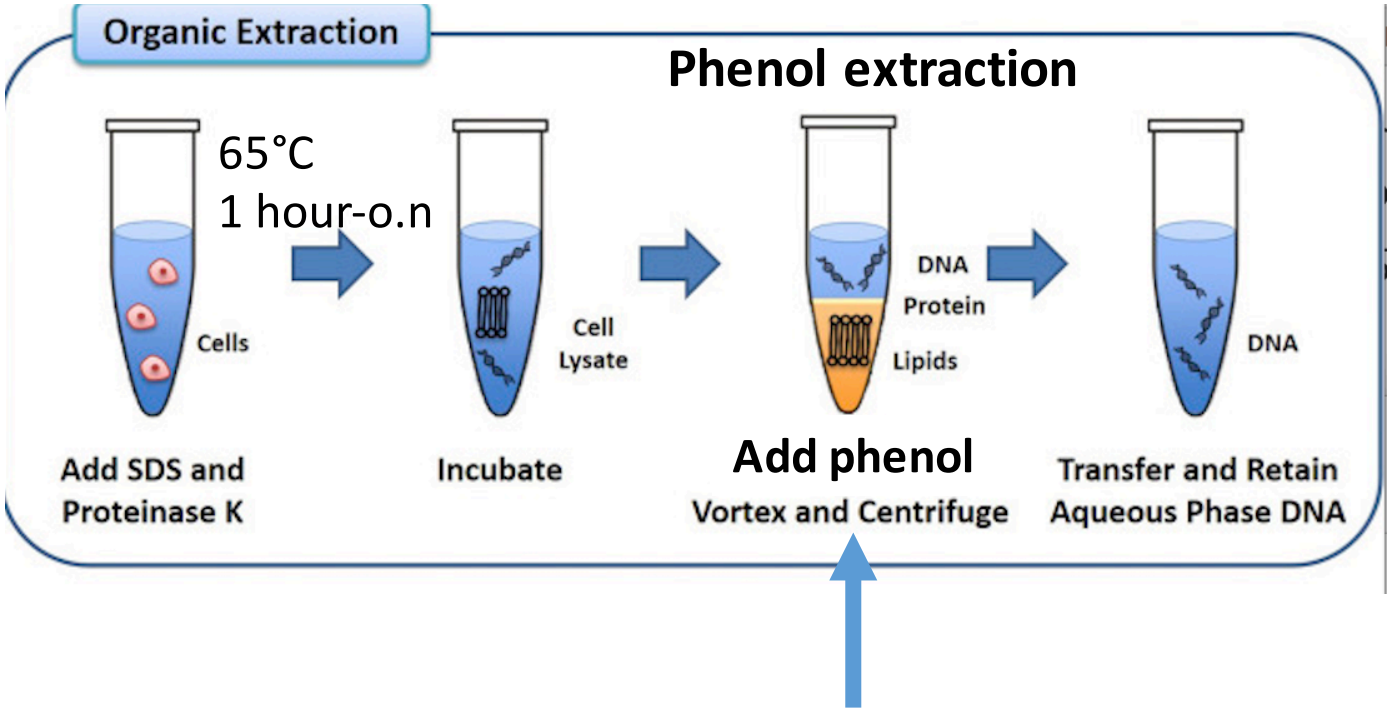
•Add equal volume of phenol (500 ul)

- 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

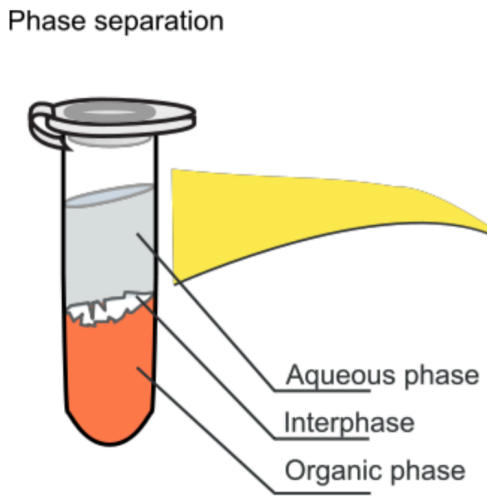
SDS: Sodium dodecyl sulfate

DNA Preparation from Cells – The organic method

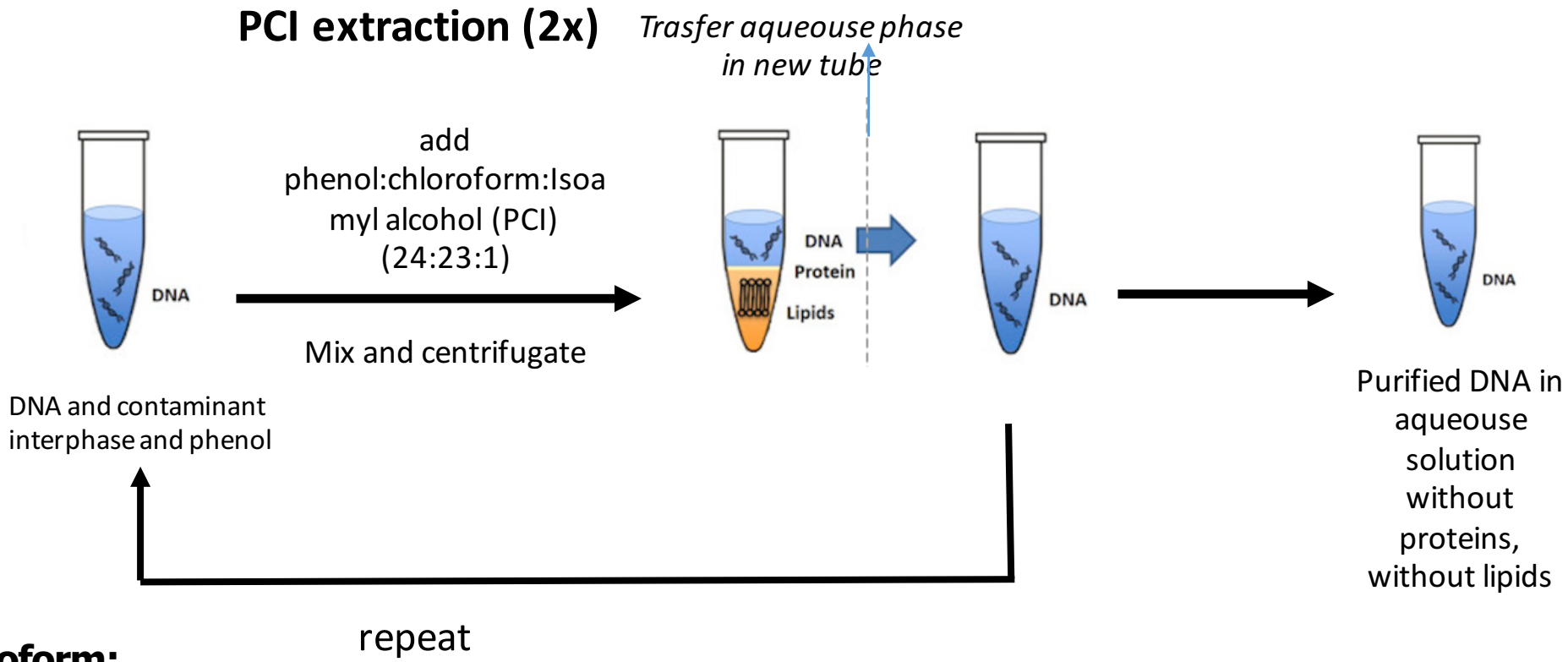


Aqueous phase contains DNA (+RNA) and rests of phenol and rests from interphase

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



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:

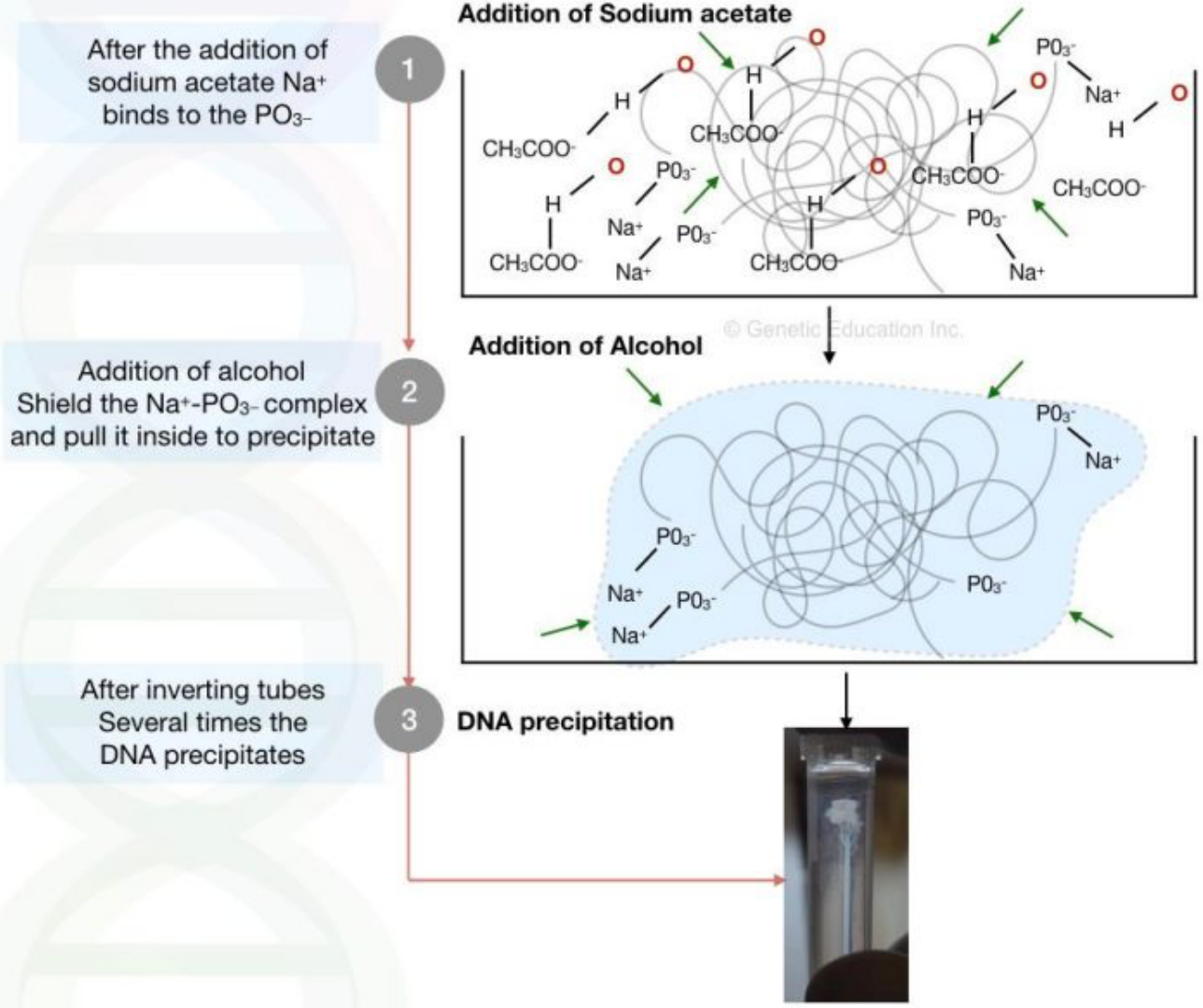
500 ul DNA solution
+ 55,5 ul 3M Na-Acetate
(final conc. 0,3M)

+ 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: H_2O in 70% Ethanol solves salt (NaCl or Na-Acetate) that can co-precipitate

DNA storage

Buffer

• DNA, is typically 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 DNA 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, PCI 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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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

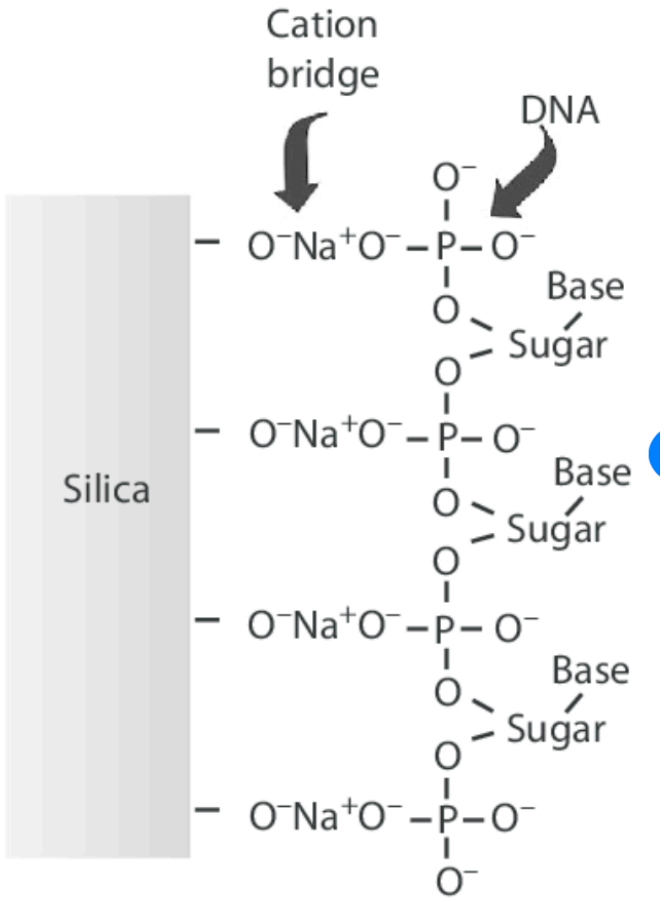
- Silica

Advantages

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

Disadvantage

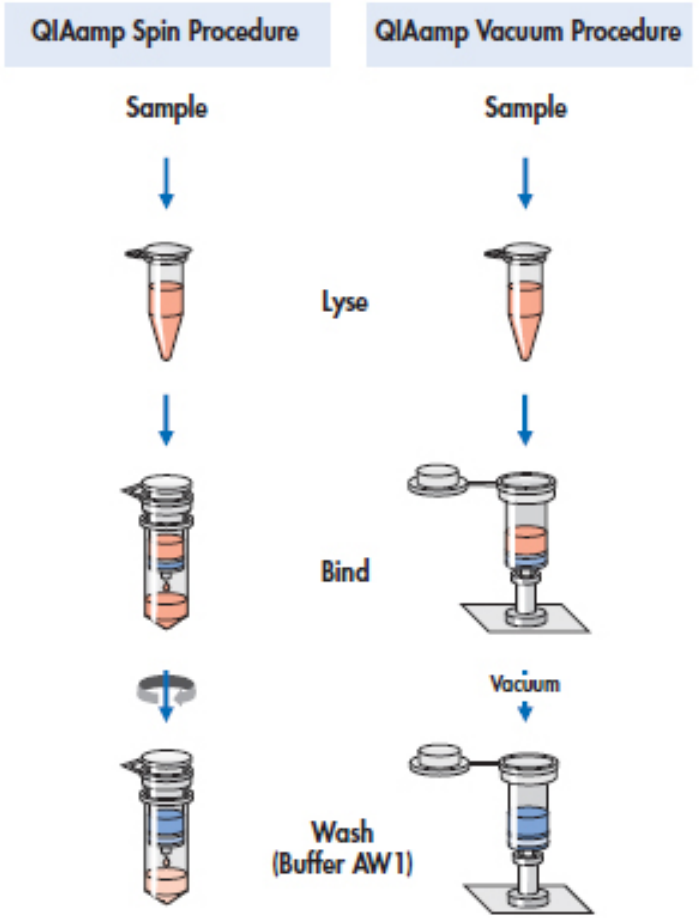
- DNA fragmentation
- Low yield



Binding at high salt concentrations
Elution at low salt concentations

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:

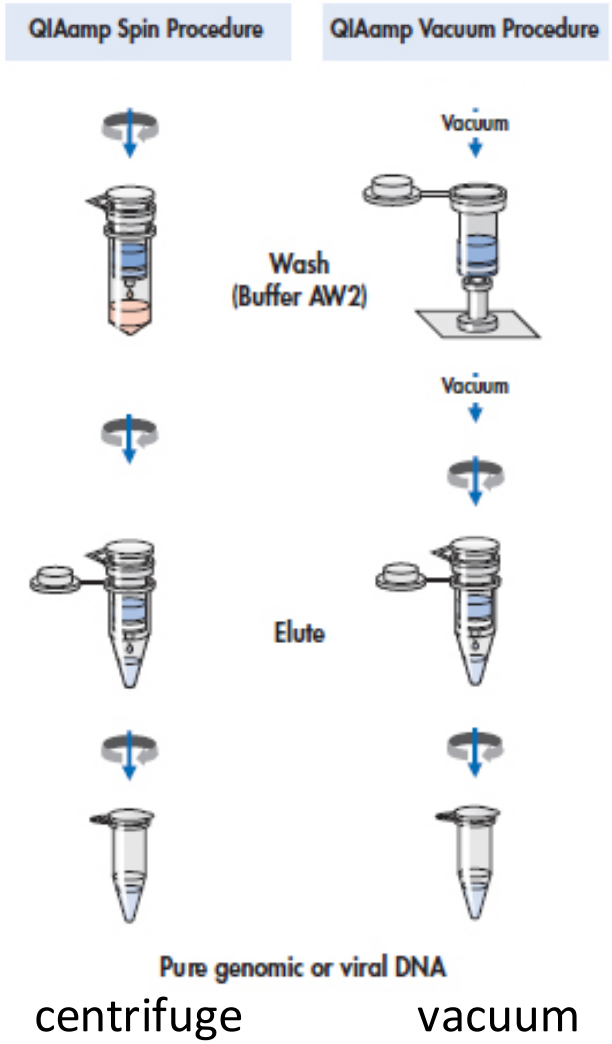
Cell lysis in buffer containing high concentration of SDS and high concentration of EDTA (binds bivalent ions such as Mg2+)

Passage on an insoluble resin (silica resins) that specifically binds DNA in the presence of salts and pH<7

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?)

DNA preparation using silica columns



centrifuge vacuum

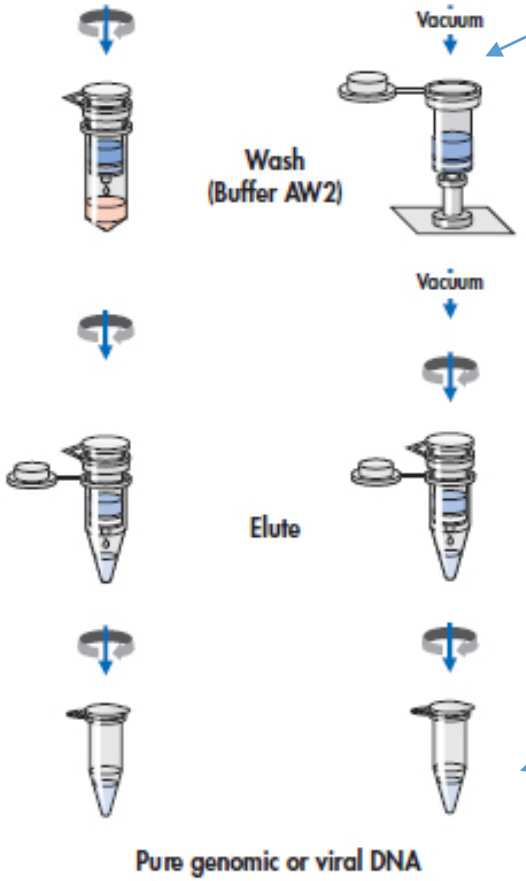
More Resin washing with buffer that allow separation from contaminants (proteins, etc…)

Elution of DNA from the resin with water or solutions with a low concentration of salts that facilitate its detachment; pH >7

DNA preparation

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

RNA contamination in DNA preparations



Option 1: -Addition of RNase to DNA bound to column for 10min; wash column extesively. Proceed with elution

Option 2: -Addition of RNase to DNA preparation. follwoed by PCI extraction or silica column purification

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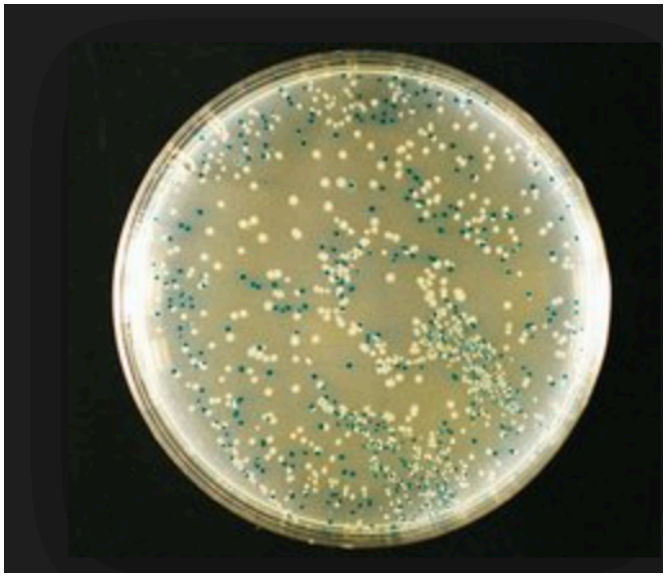
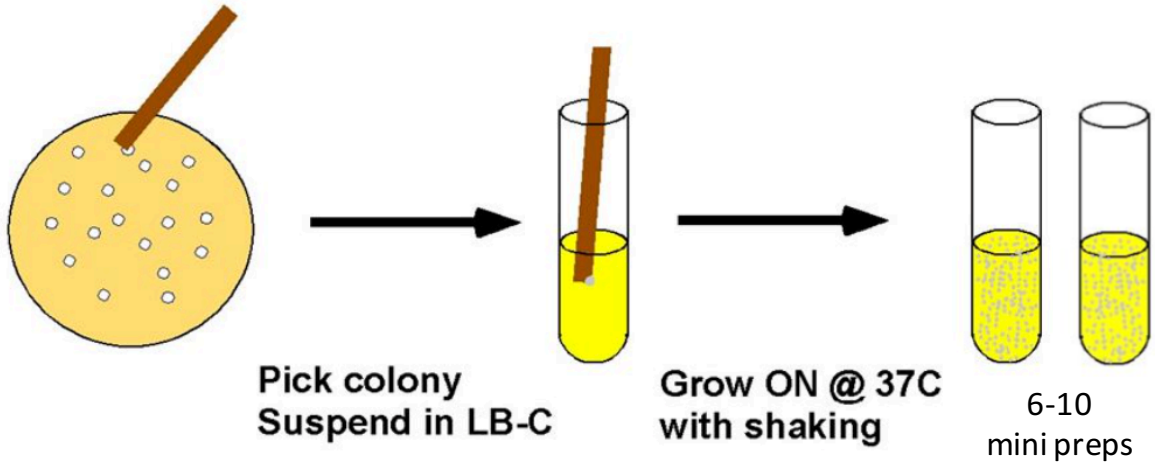
DNA PREPARATION AND CONTROL DIGEST



In general: pick 6-10 white colonies with sterile pipette tip

Preparation. Grow the bacteria

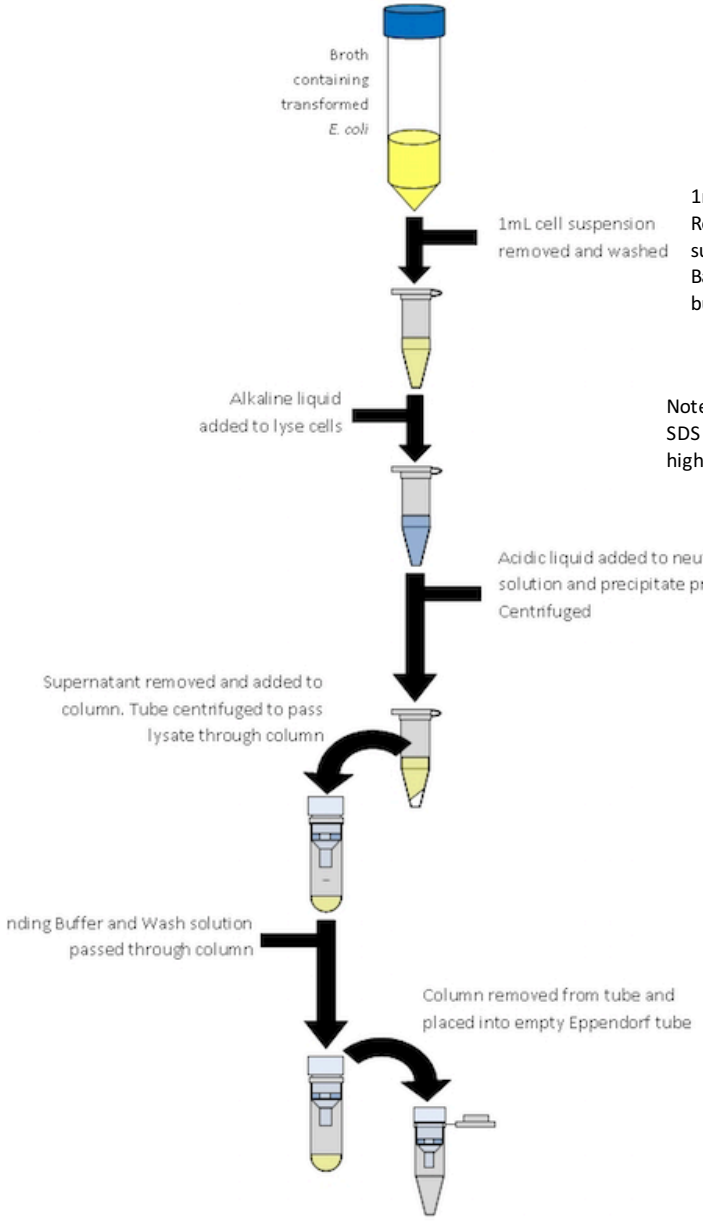
Grow an overnight (ON) culture of the desired bacteria in 2-5 ml of LB medium containing the appropriate antibiotic for plasmid selection. Incubate the cultures at 37°C with vigorous shaking.



Next day: harvest bacteria by centrifugation and prepare plasmid DNA

2. DNA PREPARATION AND CONTROL DIGEST

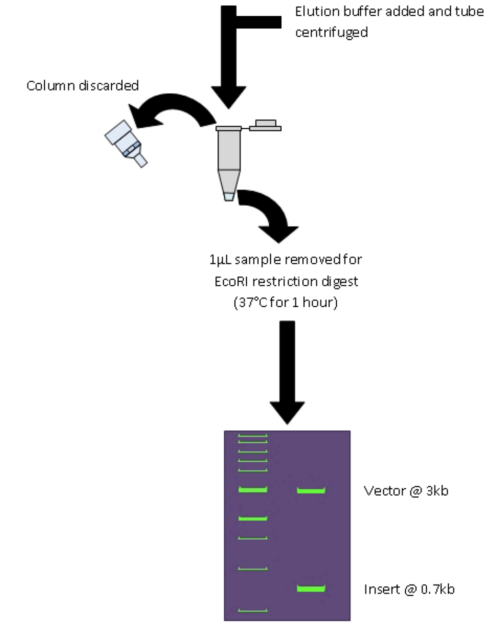
Alkaline lysis with columns



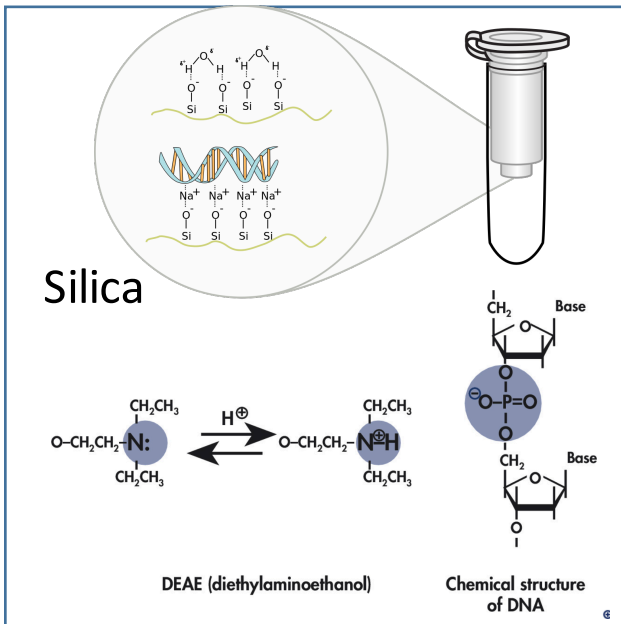
1ml of overnight culture Removed, spinned and supernatant removed. Bacteria pellet resuspendet in buffer that does not kill cells

Note: Alkaline liquid: mix of NaOH and SDS if DNA is too long in solution with high pH: Hydolysis → destroyed

The lysate is neutralized by the addition of acidic potassium acetate; The high salt concentration causes Potassium 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 **Centrifugation an high speed** (ca. 13.000 rpm); cell debries and genomic DNA precipitate; small DNA molecules (plasmid remain in supernatant)



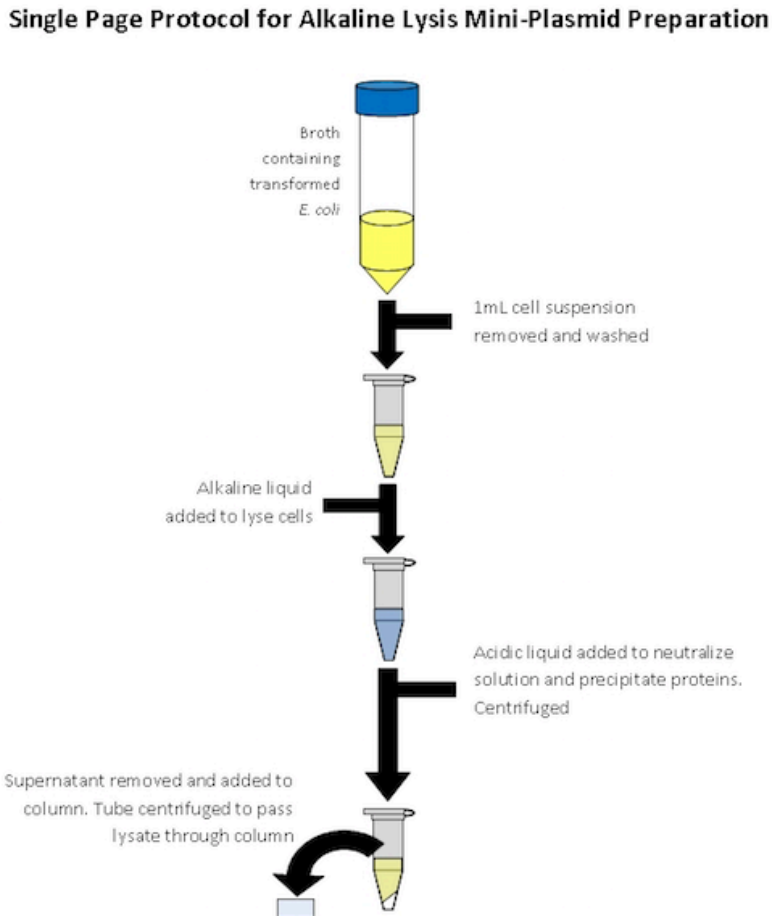
The use of columns Results in very pure plasmid DNA. "**sequence grade**"



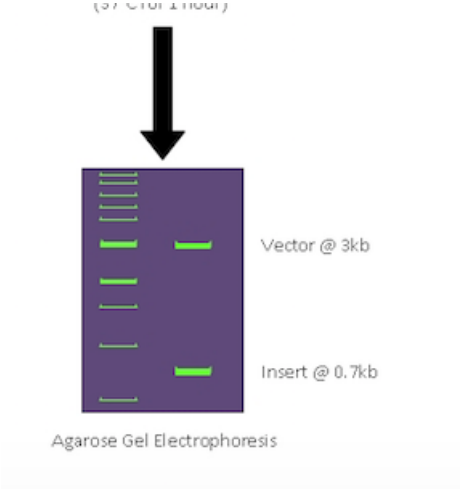
Resin in column Is positivley charged: Binds negative charge of plasmid DNA backbone

2. DNA PREPARATION AND CONTROL DIGEST

Alternative method without columns



- put supernatant in new tube
- add salt (final 0,5M NaCl)
- add Isopropanol
- put at -20C for 1 hour
- centrifuge
- plasmid DNA will preciptate



Plasimid is not very clean; sufficent for digestion with restriction enzyemes; not usable for DNA sequencing
"**not sequencing grade**"

→ **Much cheaper; you can test many colonies for correctness of plasmid**
→ **Takes some more time**

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:

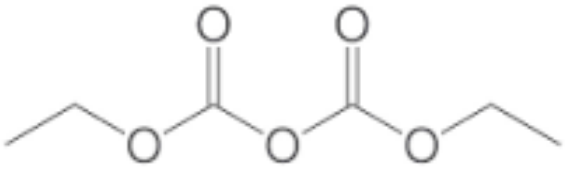
RNA is a molecule that is easily degraded by ribonucleases (RNases), enzymes that can highly abundant – also in the laboratory environment.

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**



Diethyl pyrocarbonate DEPC