Estrazione Acidi Nucleici

Scelta del protocollo di estrazione dipende da:

- *Tipo di tessuto*
- *Resa del metodo*
- *Purezza e grandezza degli acidi nucleici isolati*
- *Costo*
- *Possibilità di automazione*

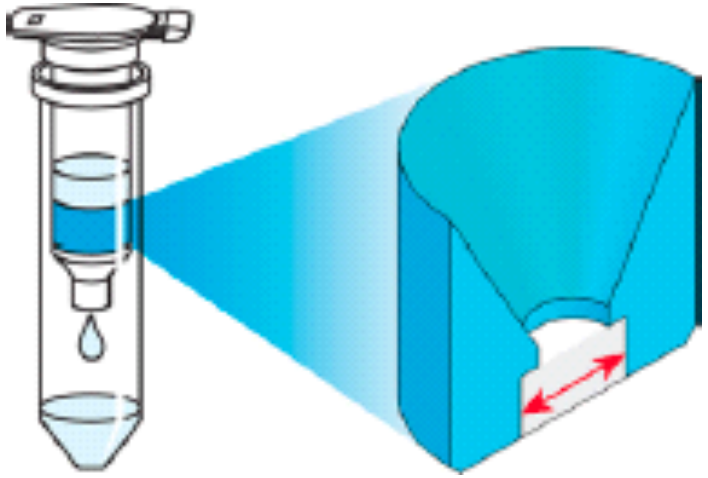
Procedimento generale: Fase liquida

- *Lisi cellule: digestione membrane cellulari e nucleari e proteine in generale (Proteasi K)*
- *Separazione degli acidi nucleici dalle proteine (estrazione con fenolo:cloroformio): fase acquosa/ interfase/fase fenolo:cloroformio*
- *Precipitazione DNA/RNA (alcoli)*
- *Quantificazione spettrofotometrica e misura della purezza (A260 nm e A280 nm)*

Fase solida

Estrazione con kit che impiegano colonnine con filtri specifici per il legame degli acidi nucleici:

- *Digestione con proteinasi*
- *Lavaggi per eliminazione proteine*
- *Eluizione DNA*



DNA extraction from fresh tissues

§-*Tissue homogenization (or cells washing)*

- Tissue homogenization or pellet cells and wash it with PBS.

§-*Proteinase K digestion*

-Put in 10X volume Proteinase K solution with SDS and enzyme.

Proteinase K solution: Proteinase K final 1 mg/ml + 10 mM Tris-HCl pH 7.4 + 10 mM EDTA + 150 mM NaCl + 0.4 % SDS (add just before use)

-O.N. incubation at 37-45^{0}C with stirring (minimum 4 hrs).

§-*Phenol/Chloroform extraction*

-Extraction with phenol-Tris (pH 8)/chloroform (50%/50%) 1:1 in volume.

§-*DNA precipitation*

-Precipitate aqueous phase w/ 1 volume of isopropanol. Wash w/ 70% ethanol.

§-*DNA pellet solubilization*

-Recover pellet in H_2O or TE buffer (better) and store at -20˚ C.

DNA extraction from blood

§- *Red cells lysis (blood in citrate, heparin or EDTA)*

-Add 45 ml lysis buffer to 5 ml blood, mix by inversion and leave at RT for 5'. Centrifuge X 1000 g at 4^0C for 6'.

Red cells lysis buffer: 0.32 M saccarose + 10 mM tris-HCl pH 7.5 + 5 mM $MgCl_2$ + 1% triton X 100.

-Wash the pellet with PBS and centrifuge as before.

§- *Proteinase K digestion*

-Recover pellet in 600 µl of buffer A and add SDS final 0.5% and proteinase K 1 mg/ml. Incubation at 37^0C O.N. (at least 4 hrs).

Buffer A: 10 mM Tris-HCl pH 8 + 400 mM NaCl + 2 mM EDTA.

§- *Protein salting-out*

-Add 220 µl of sature solution of NaCl, vigorous stirring for 15" and centrifuge 15' X 14000 rpm at 4° C.

§- *DNA precipitation*

-Recover supernatant and precipitate with 1 volume isopropanol at rt with immediate precipitation. Centrifuge and wash pellet with 1 ml of 70% ethanol.

§- *DNA pellet solubilization*

-DNA is solubilized in 500 µl of TE or H_2O.

DNA extraction from blood- silica use

1. 200 µL whole blood mixed with 100 µL lysis buffer[a] **or** 25 µL mononuclear cells were added to 100 µL binding solution[b]. Mix and incubate for 3 min at room temperature. Centrifuge for 15 s at 65× *g* and discard supernatant.
2. Add 100 µL lysis buffer and mix. Centrifuge for 15 s at 65× *g* and discard supernatant. Repeat once.
3. Add 100 µL washing solution[c] and mix. Centrifuge for 15 s at 65× *g* and discard supernatant. Repeat once.
4. Add 100 µL absolute ethanol and mix. Centrifuge for 15 s at 65× *g*. Discard supernatant and vacuum-dry the pellet.
5. Add 50 µL elution buffer[d], resuspend the pellet, and incubate for 3 min at 65°C. Centrifuge for 1 min at 65× *g*, and transfer supernatant in a clean tube.

[a]3 M guanidine thiocyanate, 20 mM EDTA, 10 mM Tris-HCl, pH 6.8, 40 mg/mL Triton® X-100, 10 mg/mL DTT
[b]40 mg/mL silica (Sigma) directly suspended in the lysis buffer
[c]25% absolute ethanol, 25% isopropanol, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0
[d]10 mM Tris-HCl, pH 8.0, 1 mM EDTA
All solutions were prepared in double-distilled water and sterilized by filtration and remain stable for more than six months.

RNA DEGRADATION

↓

RNAses

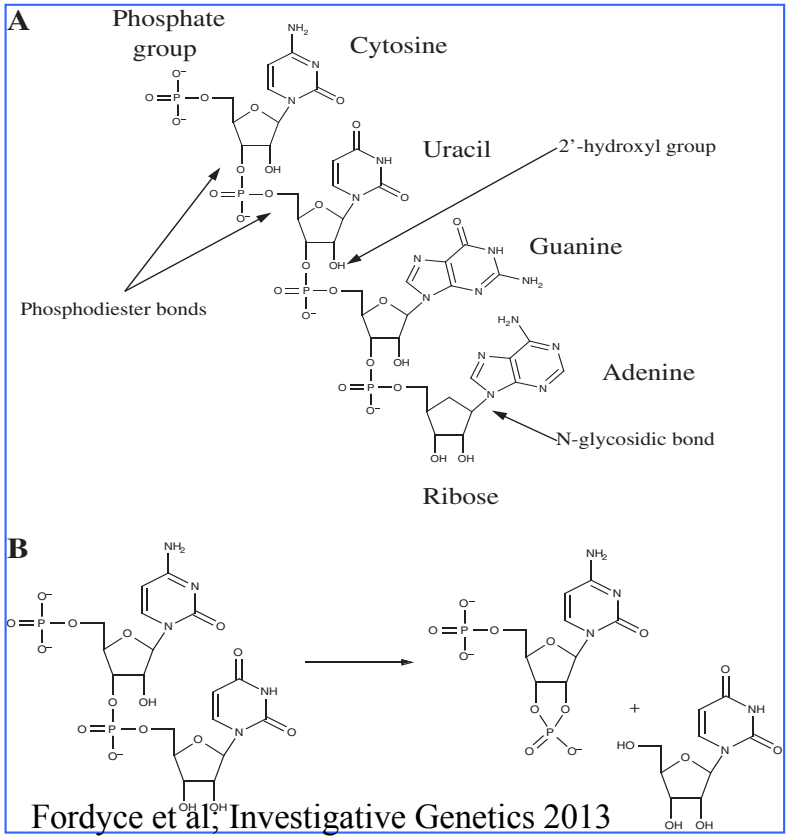
↓

Temperature

↓

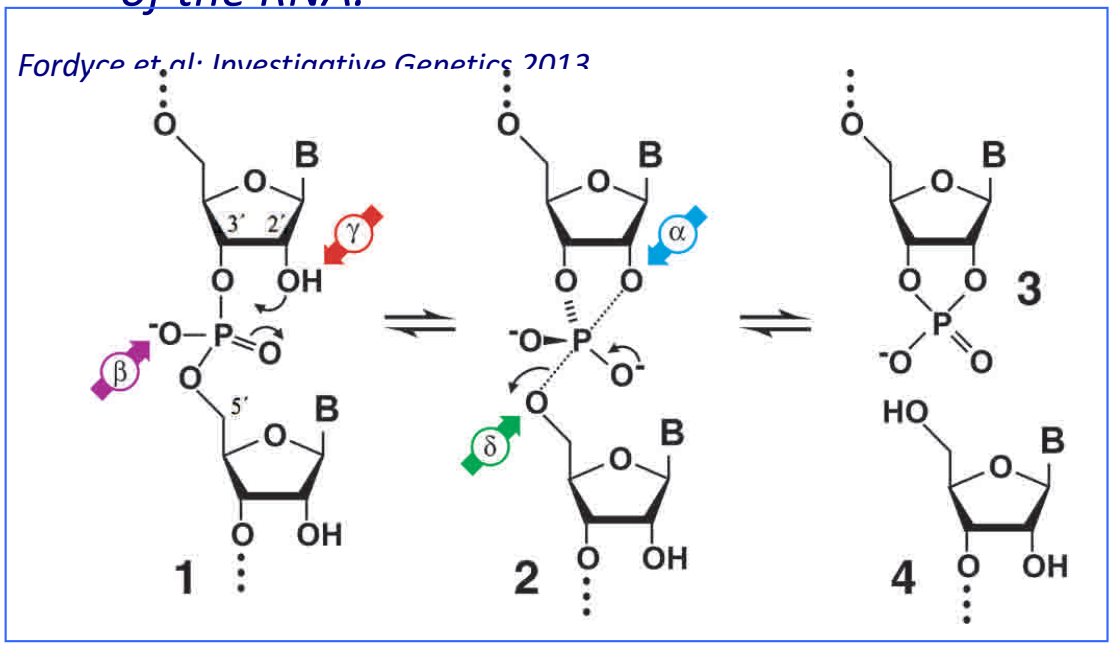
Alkaline environment

RNA degrades differently from DNA



Fordyce et al; Investigative Genetics 2013

- *The 2'-OH allows the RNA molecule to be more easily degraded via hydrolysis than DNA.*
- *The phosphodiester bond in RNA can be broken during hydrolysis.*
- *The N-glycosidic bond is stronger in RNA than DNA*
- *The chemical process of hydrolysis, where the 2'-hydroxyl group has attacked the adjacent phosphodiester bond, cleaving the backbone of the RNA.*



RNAse A increases the rate of RNA cleavage by internal phosphoester transfer

3-RNA extraction from fresh tissues

§-Tissue homogenization (or cells washing)

§-Cell lysis

§-Phenol/Chloroform extraction

§-RNA precipitation

§-RNA pellet solubilization

* Chomczynski P; Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem, 162: 156-159; 1987

3-RNA extraction from fresh tissues

§-Tissue homogenization (or cells washing)

-For tissues homogenization in liquid nitrogen / wash the cells with PBS (keep always in ice and use ice cold solution).

§-Cell lysis

-Put it in D solution* (1 ml for 20 million cells) with ß-mercapto-ethanol (0.36 ml for 5 ml solution) (1 ml per 2.0 10^7 cells) for 5' in ice.

**D solution: 250 g guanidinium thiocyanate + 293 ml of DEPC-H_2O + 17.6 ml of sodium citrate 0.75 M pH 7.0 + 26.4 ml sarcosyl 10% at 65°C.*

§-Phenol/Chloroform extraction

-Add 1/10 volume of Sodium Acetate 2M with pH 4.0-4.5. (acid pH is necessary to obtain a good separation at the interface) and extract w/ 1 volume of phenol/H_2O and chloroform (7:3 ratio). Keep in ice for 20' and centrifuge for 20'.

§-RNA precipitation

-Add 1 volume of isopropanol, ON at -20^0C -centrifuge -Wash w/ chilled 75% EtOH.

§-RNA pellet solubilization

-Resuspend in DEPC H_2O.

RNA extraction from fresh tissues

§-*Tissue homogenization (or cells washing)*

-For tissues homogenization in liquid nitrogen / wash the cells with PBS (keep always in ice and use ice cold solution).

§-*Cell lysis*

-Put it in D solution (1 ml for 20 million cells) with ß-mercapto-ethanol (0.36 ml for 5 ml solution) (1 ml per 2.0 10^7 cells) for 5' in ice.*

**D solution: 250 g guanidinium thiocyanate + 293 ml of DEPC-H_2O + 17.6 ml of sodium citrate 0.75 M pH 7.0 + 26.4 ml sarcosyl 10% at 65° C.*

§-*Phenol/Chloroform extraction*

-Add 1/10 volume of Sodium Acetate 2M with pH 4.0-4.5. (acid pH is necessary to obtain a good separation at the interface) and extract w/ 1 volume of phenol/H_2O and chloroform (7:3 ratio). Keep in ice for 20' and centrifuge for 20'.

§-*RNA precipitation*

-Add 1 volume of isopropanol, ON at -20^0C -centrifuge -Wash w/ chilled 75% EtOH.

§-*RNA pellet solubilization*

-*Resuspend in DEPC H_2O*.