Izolace a purifikace nukleových kyselin

Metody molekulární biologie

19.9.2019

Nukleové kyseliny

- DNA - jaderná, mitochondriální, chloroplastová, plasmidová
- vodíkové vazby v DNA jsou stabilní v rozmezí pH 4-9

- RNA – rRNA (80 %), tRNA (10-20 %), mRNA (5 %), miRNA,...

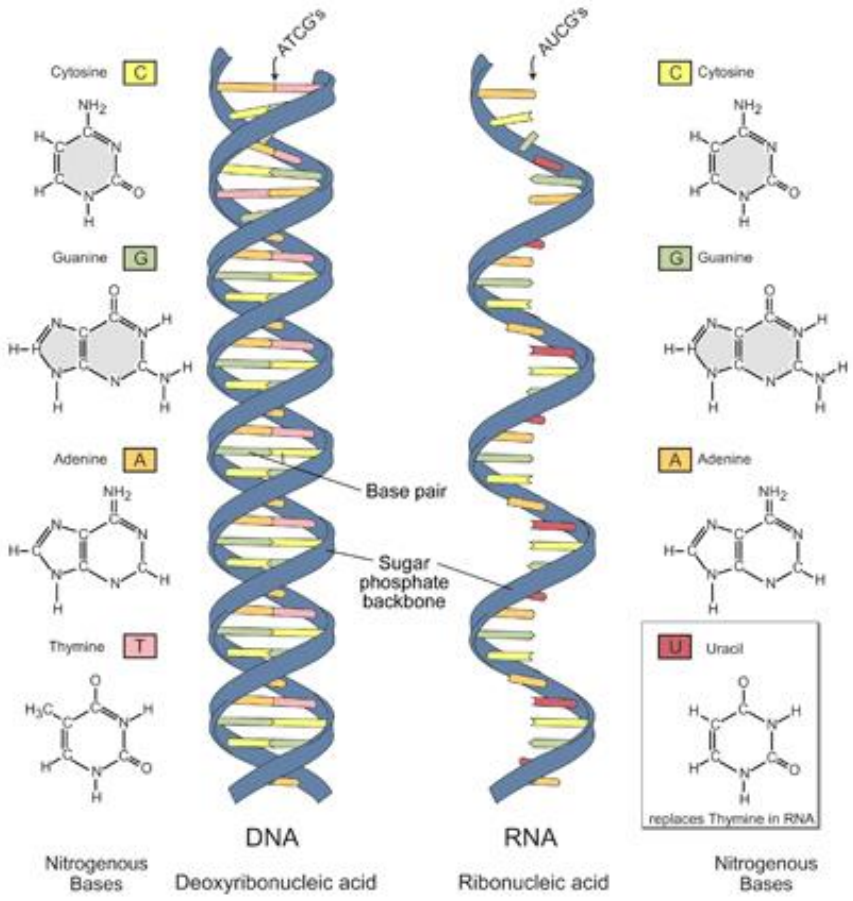


Image adapted from: National Human Genome Research Institute.

Zdroje NK pro izolaci

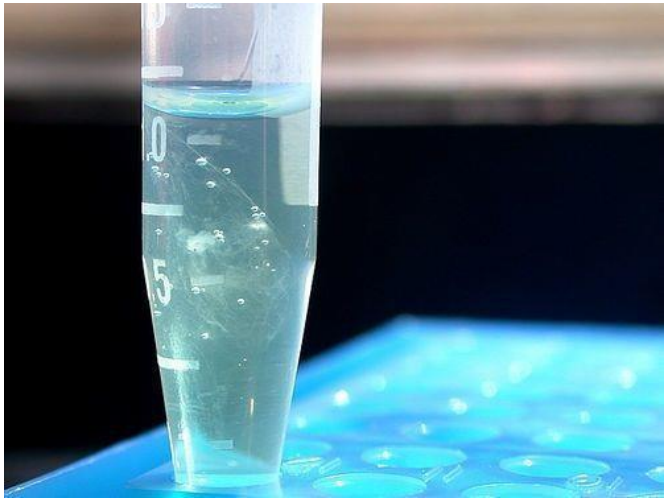
- buňky – bakterie (genomová nebo plasmidová DNA), kvasinky
- virové částice
- produkty PCR, DNA v gelu po elektroforéze
- buněčné kultury – izolace genomové DNA, mRNA
- tkáně a orgány eukaryot

- klinické laboratoře - leukocyty, amniocyty, buňky z bukální sliznice, buňky z cerebrospinálního moku, vlasové kořínky, bioptický materiál
- forenzní genetika – tělní tekutiny, vlasy, části tkání nebo orgánů

Izolace nukleových kyselin

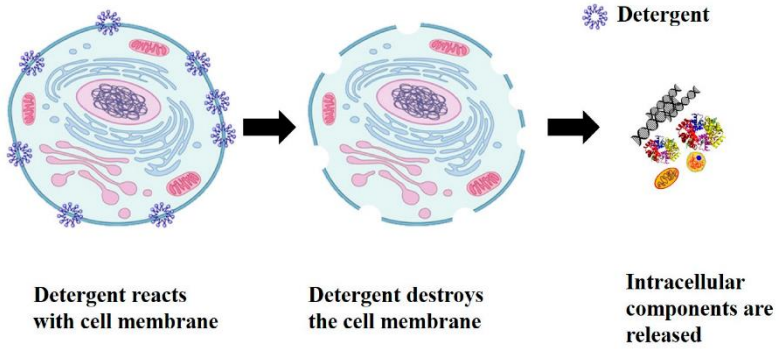
- **je potřeba oddělit NK od ostatních buněčných komponent, které by mohly bránit jejich dalšímu využití a analýze**

- porušení buněčných stěn – lýza buněk, homogenizace vzorků
- použití enzymů – proteináza K, RNáza, DNáza
- centrifugace
- adsorpce NK na silikát (SiO_2)
- fenol-chloroformová extrakce
- precipitace (srážení) NK



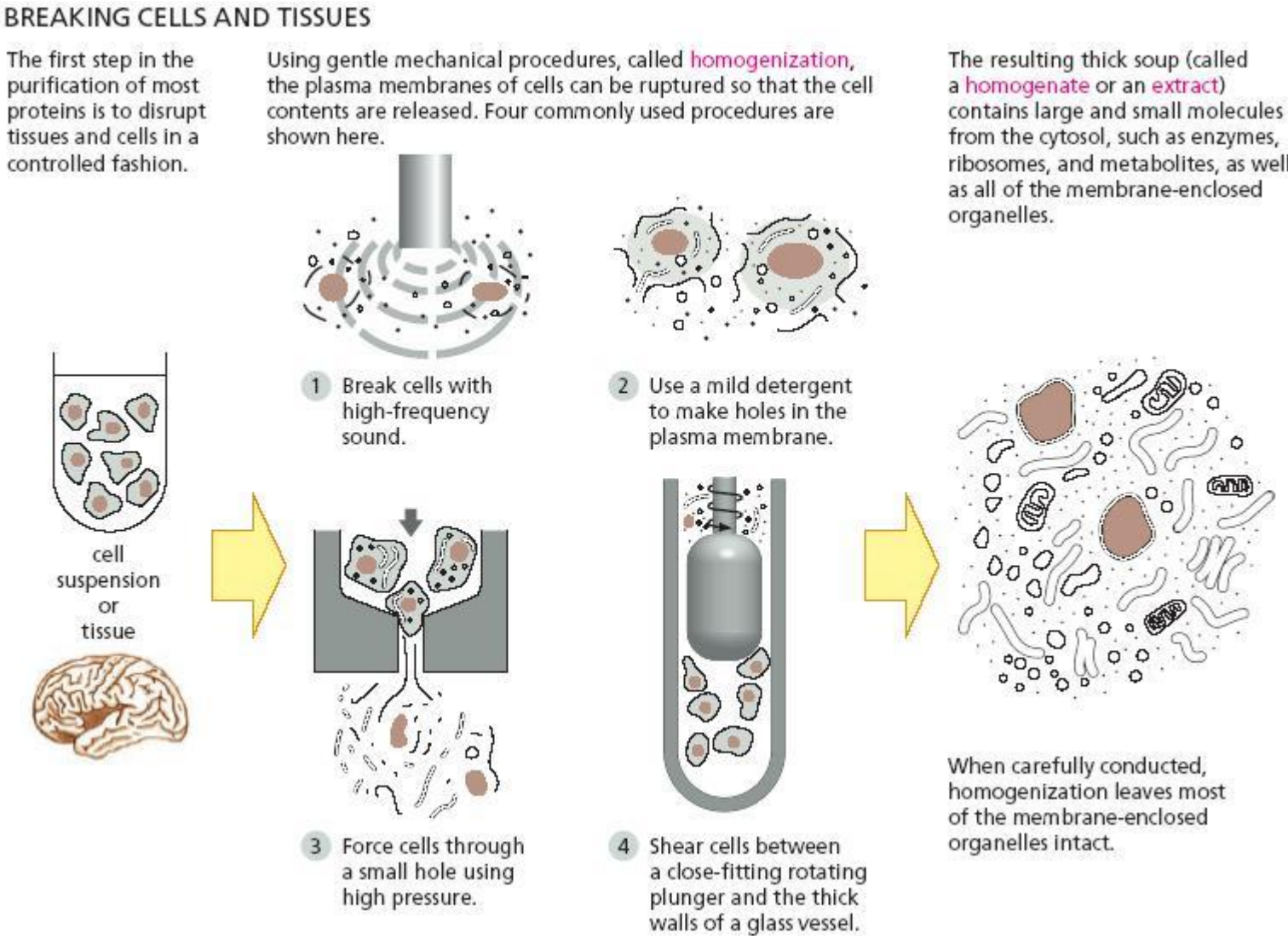
Izolace NK – lýza buněk

degradace buněčných stěn : enzymatická (lysozym), chemická (detergenty – SDS, Triton X-100) nebo fyzikální (mechanické tření, opakované mražení a rozmrazování, ultrazvuk)



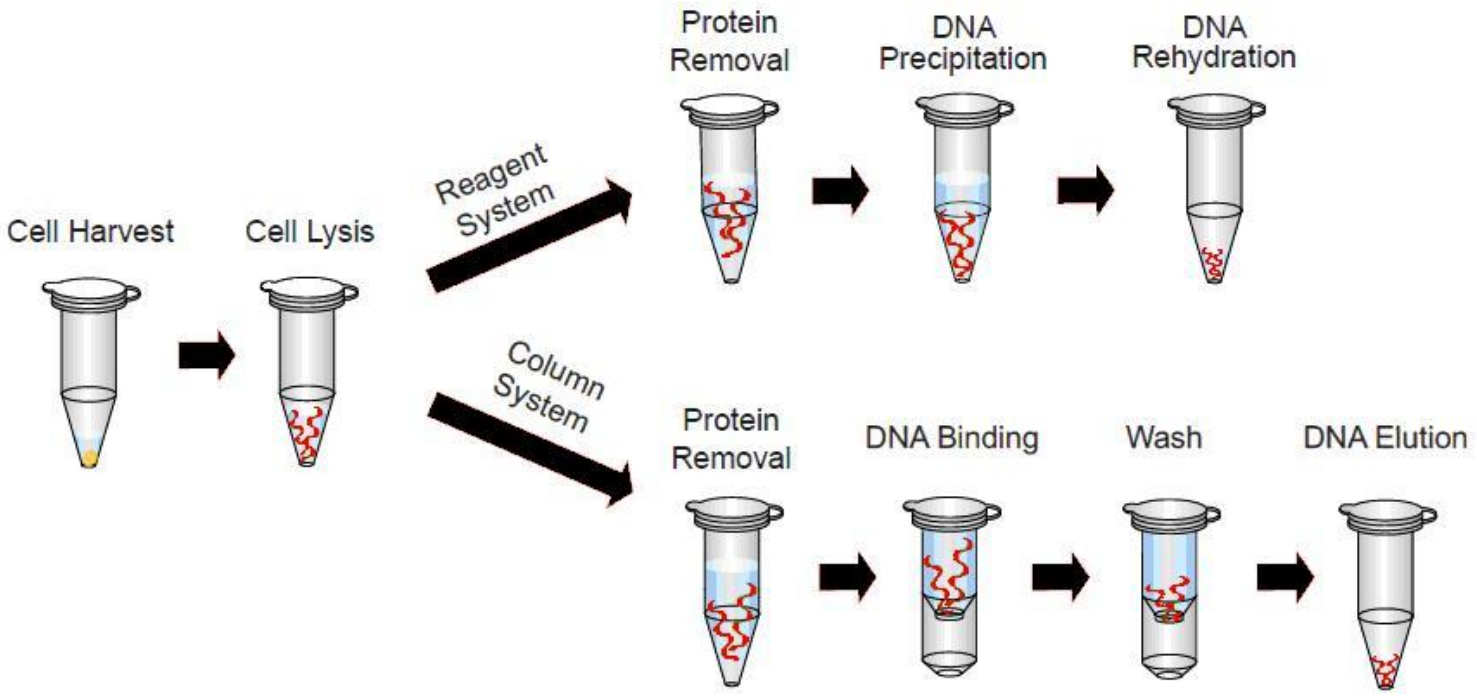
http://www.mdpi.com/2072-666X/8/3/83

Homogenizace vzorků



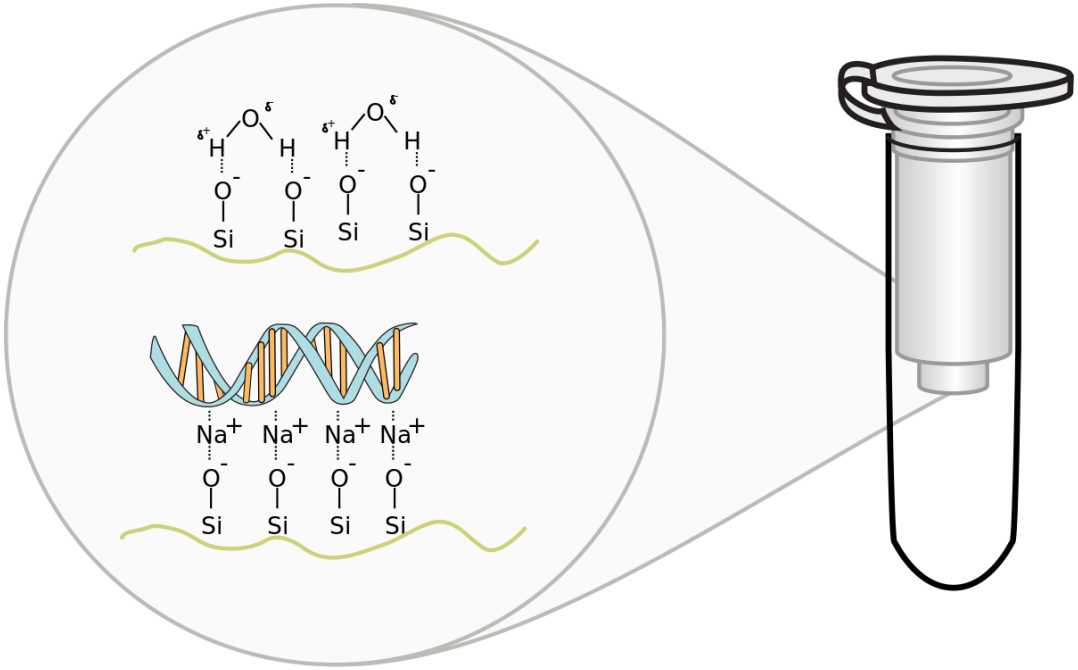
Metody izolace NK

1) Využití vazby NK na silikátovou kolonu (SiO_2)
2) Extrakce a precipitace NK

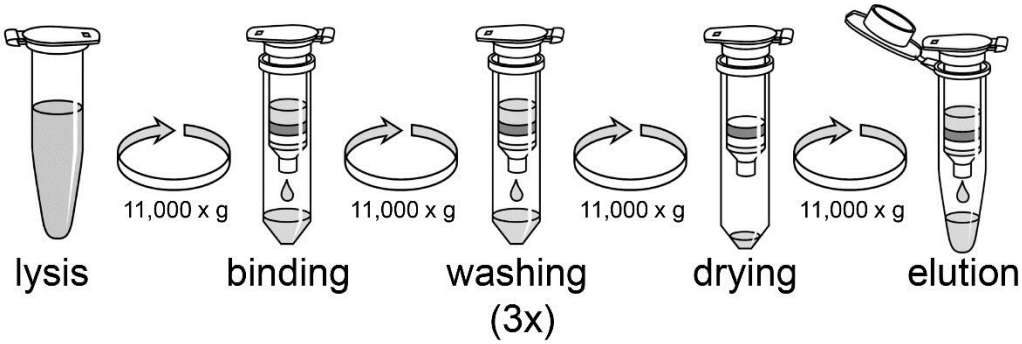


Metody izolace DNA

1) Vazba DNA na silikátovou kolonu (SiO_2) v přítomnosti chaotropních solí (zvyšujících iontovou sílu, např. NaI)

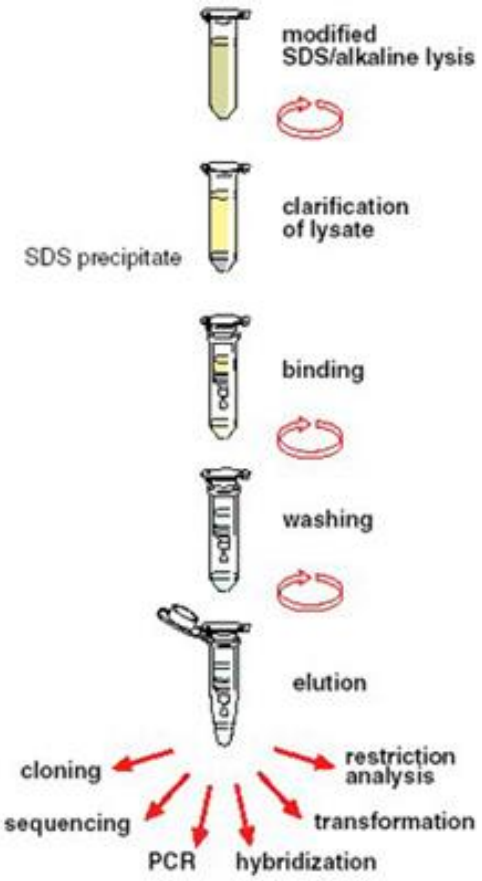


Izolace DNA na koloně



komerčně dostupné kity pro různé druhy a objemy vzorků:

rostlinné vzorky, plná krev, plasmidová DNA, celková RNA, mRNA, miRNA, izolace DNA z gelu, přečistění PCR produktů



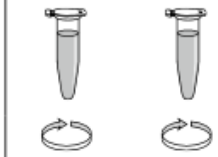
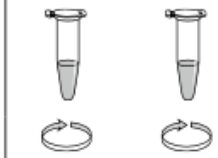
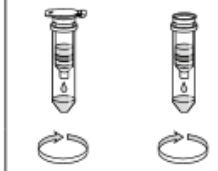
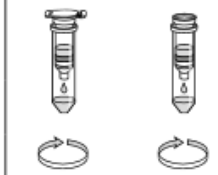
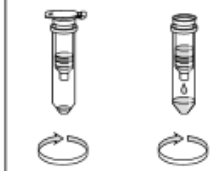
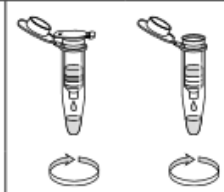
resuspendační pufr

lyzační pufr – SDS, NaOH

neutralizační pufr – CH_3COOK

promývací pufr – ethanol

eluční pufr – nízká iontová síla

		Plasmid	Plasmid (NoLid)	Plasmid QuickPure
1	Cultivate and harvest bacterial cells		11,000 x *g*, 30 s	 11,000 x *g*, 30 s
2	Cell lysis		250 µL Buffer A1 250 µL Buffer A2 RT, up to 5 min 300 µL Buffer A3	 250 µL Buffer A1 250 µL Buffer A2 RT, up to 5 min 300 µL Buffer A3
3	Clarification of the lysate		11,000 x *g*, 5–10 min	 11,000 x *g*, 5 min
4	Bind DNA		Load supernatant 11,000 x *g*, 1 min	 Load supernatant 11,000 x *g*, 1 min
5	Wash silica membrane		*(Optional: 500 µL Buffer AW: RT or 50 °C)* 600 µL Buffer A4 11,000 x *g*, 1 min	 450 µL Buffer AQ 11,000 x *g*, 3 min
6	Dry silica membrane		11,000 x *g*, 2 min	Drying is performed during centrifugation of the single washing step
7	Elute DNA		50 µL Buffer AE RT, 1 min 11,000 x *g*, 1 min	 50 µL Buffer AE RT, 1 min 11,000 x *g*, 1 min

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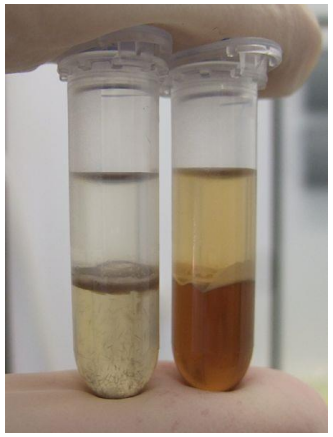
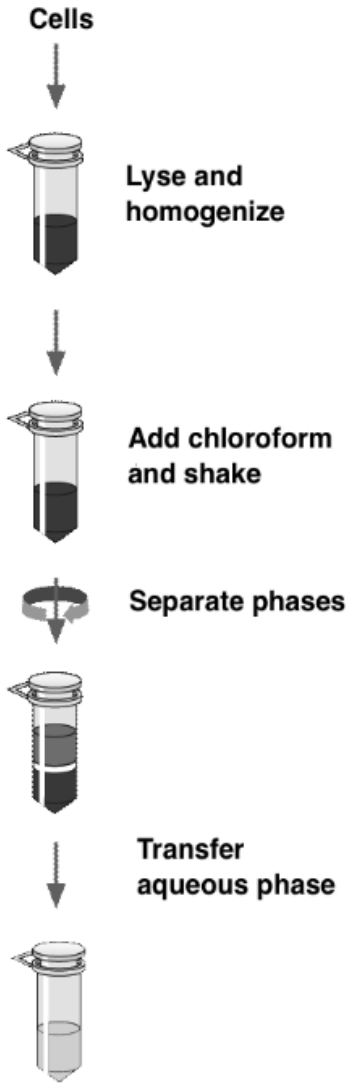
Tel.: +49 24 21 969-270 · Fax: +49 24 21 969-199 · tech-bio@mn-net.com · ***www.mn-net.com***

Fenol-chloroformová extrakce

- fenol:chloroform:isoamylalkohol (25:24:1) **pH 8,0**
- EDTA – chelatační činidlo
- fenol – rozpouštědlo, chloroform – denaturuje proteiny
- třepáním a centrifugací vznikne fázové rozhraní

- spodní část (organická fáze, lipidy)
- rozhraní (vysrážené proteiny)
- horní část (vodná fáze, polární látky, NK)

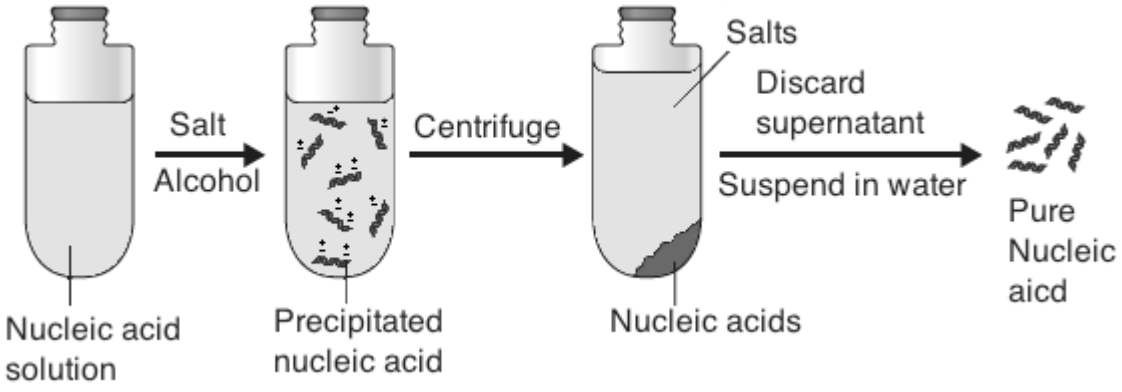


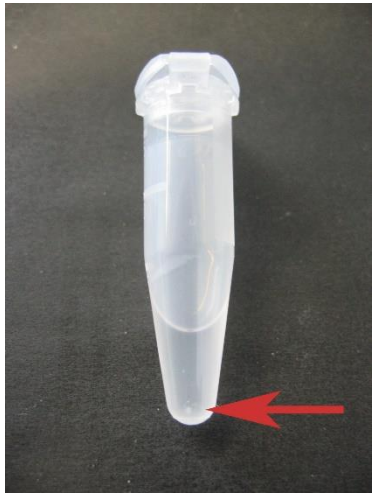


Precipitace (srážení) DNA

přečištění DNA např. po fenol-chloroformové extrakci

1. přidat 3M octan sodný (pH 5,2) do 1/10 celkového objemu
2. přidat 100% ethanol (vychlazený) – 2,5× objem – zmrazit – **precipitace DNA**
3. centrifugace
4. odebrat supernatant, přidat 80% ethanol

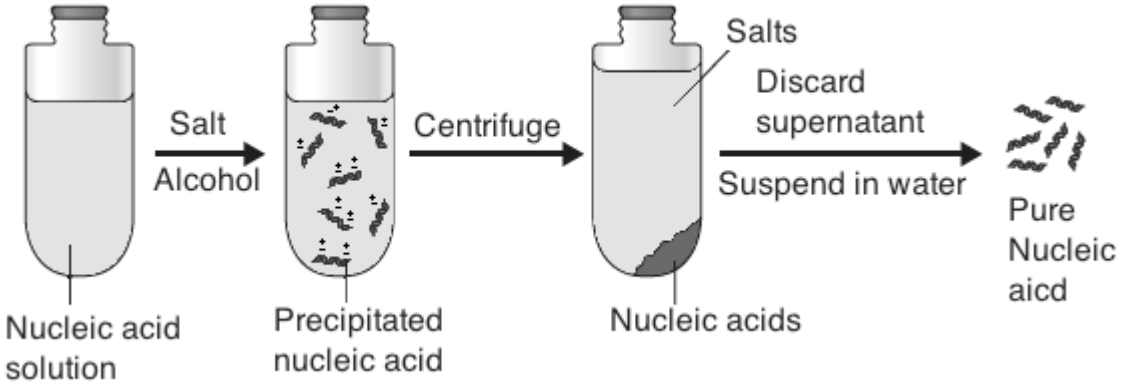


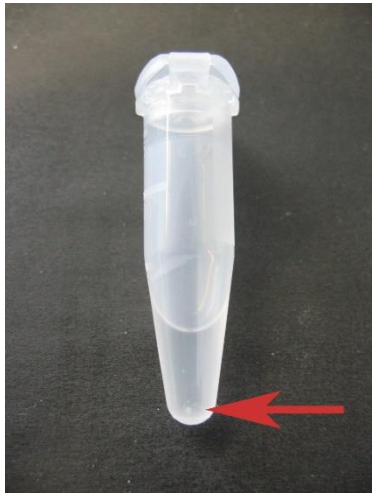


Precipitace (srážení) DNA

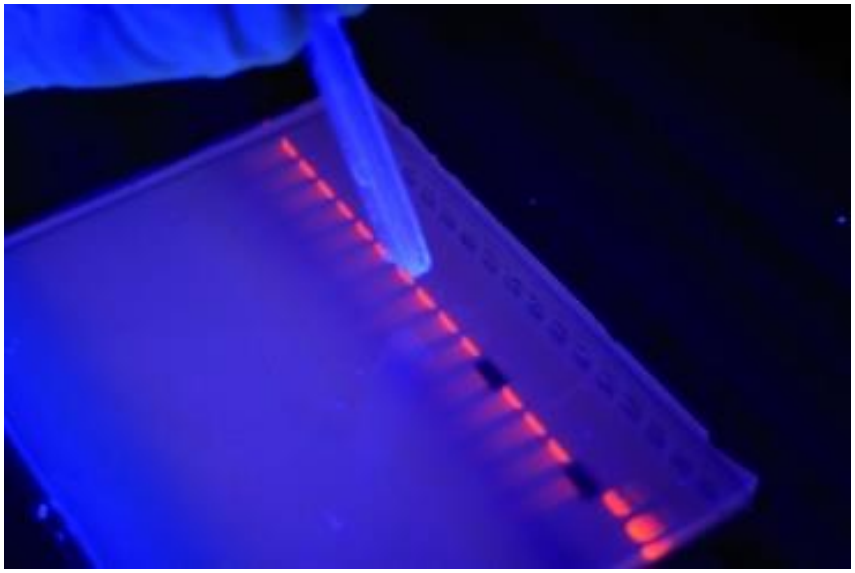
5. centrifugace
6. odebrat supernatant, přidat 80% ethanol
7. centrifugace
8. odebrat supernatant, vysušit, rozpustit ve vodě nebo TE pufru

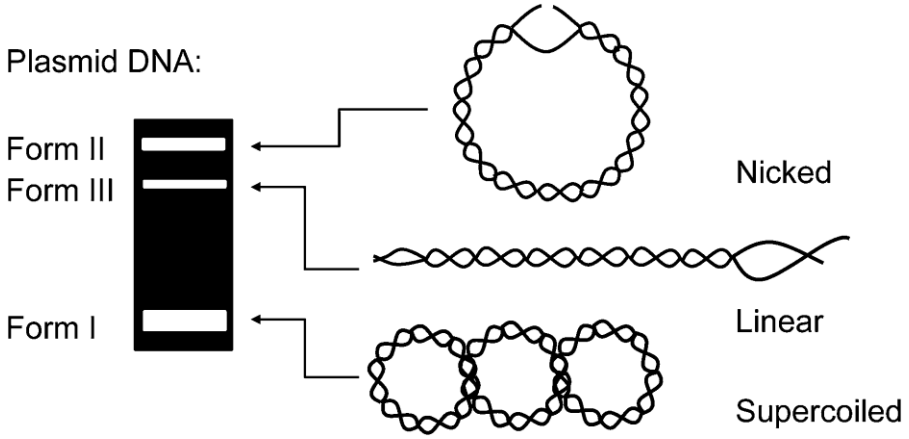
(pro lepší výtěžek lze přidat nosič – tRNA, glykogen)





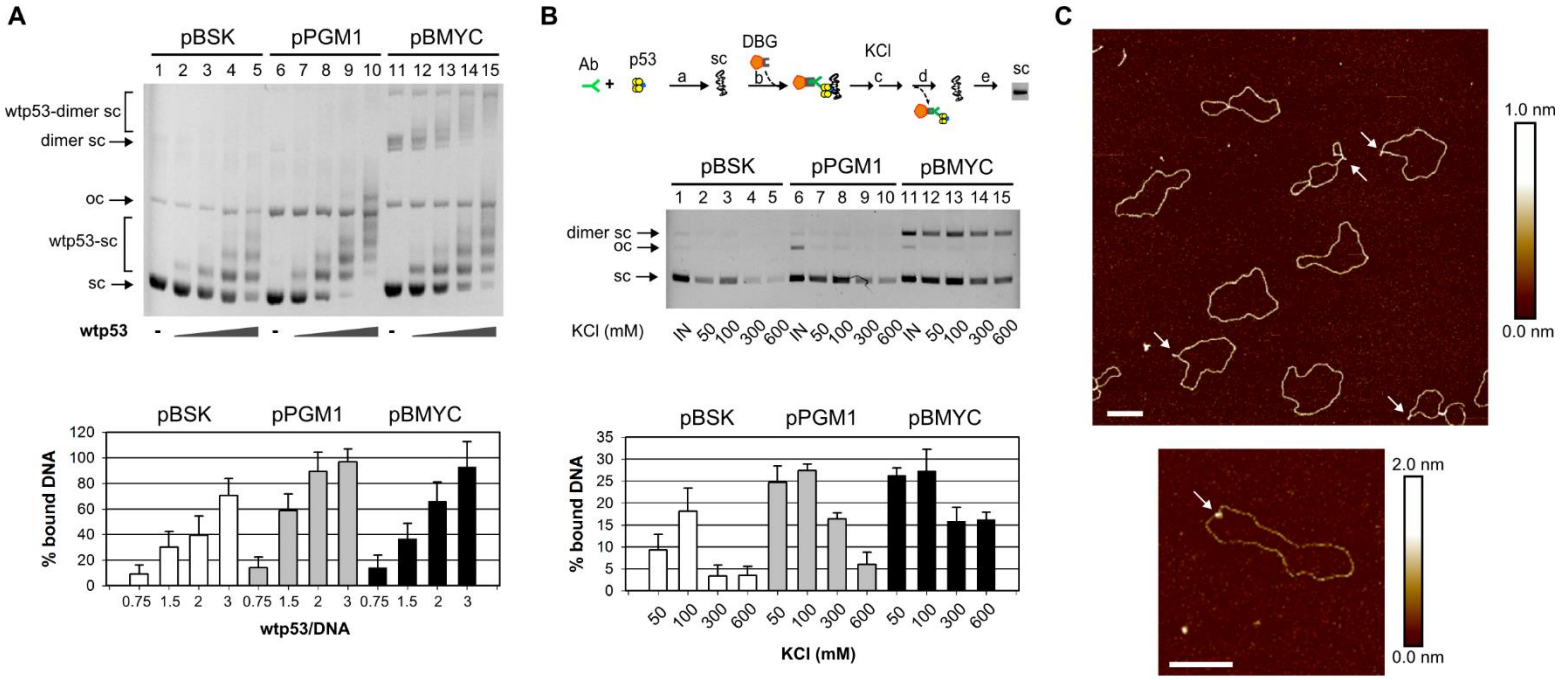
Analýza plasmidové DNA





DNA photo-cleaving agents in the far-red to near-infrared range – a review. RCS Advances DOI: 10.1039/C5RA28102D

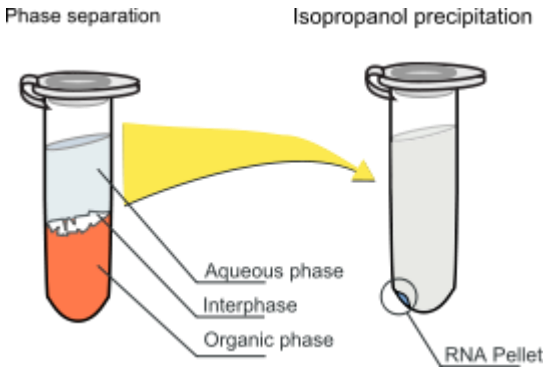
Analýza plasmidové DNA



Petr et al., *Bioscience Reports* (2016) 36, e00397, doi:10.1042/BSR20160232

Izolace RNA

guanidium thiokyanát-
fenol-chloroformová extrakce
při pH 4,5
!ochrana proti působení RNáz!



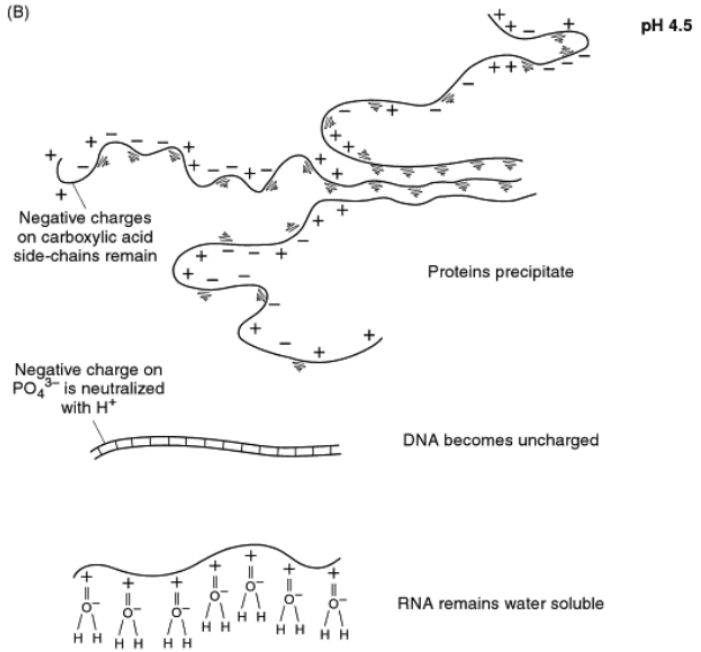
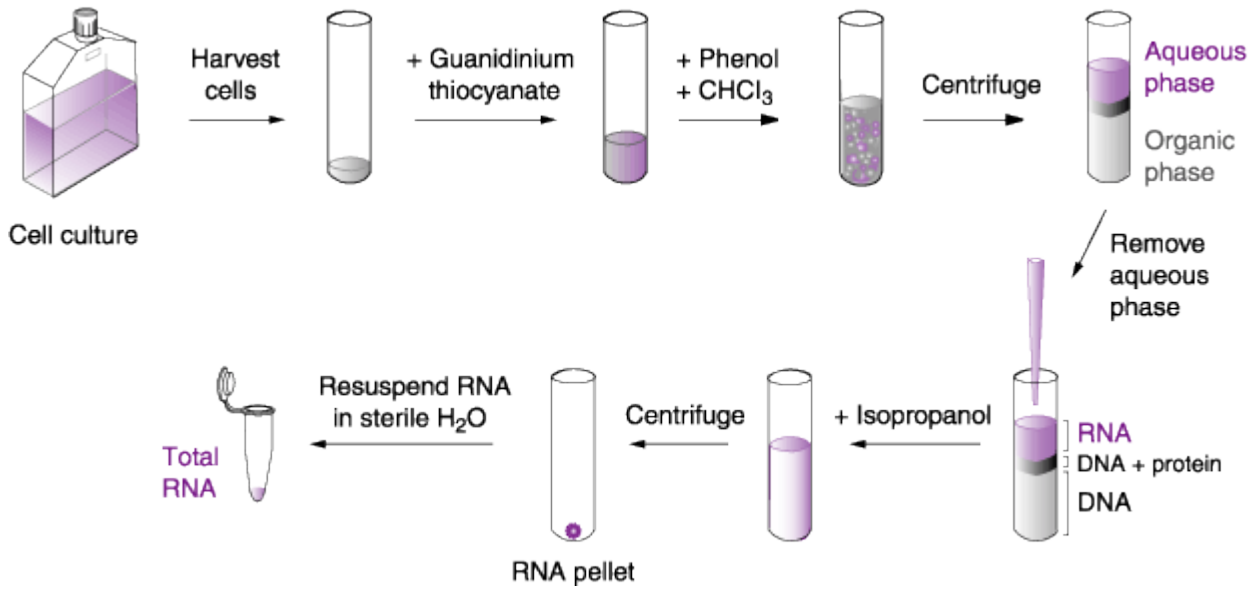


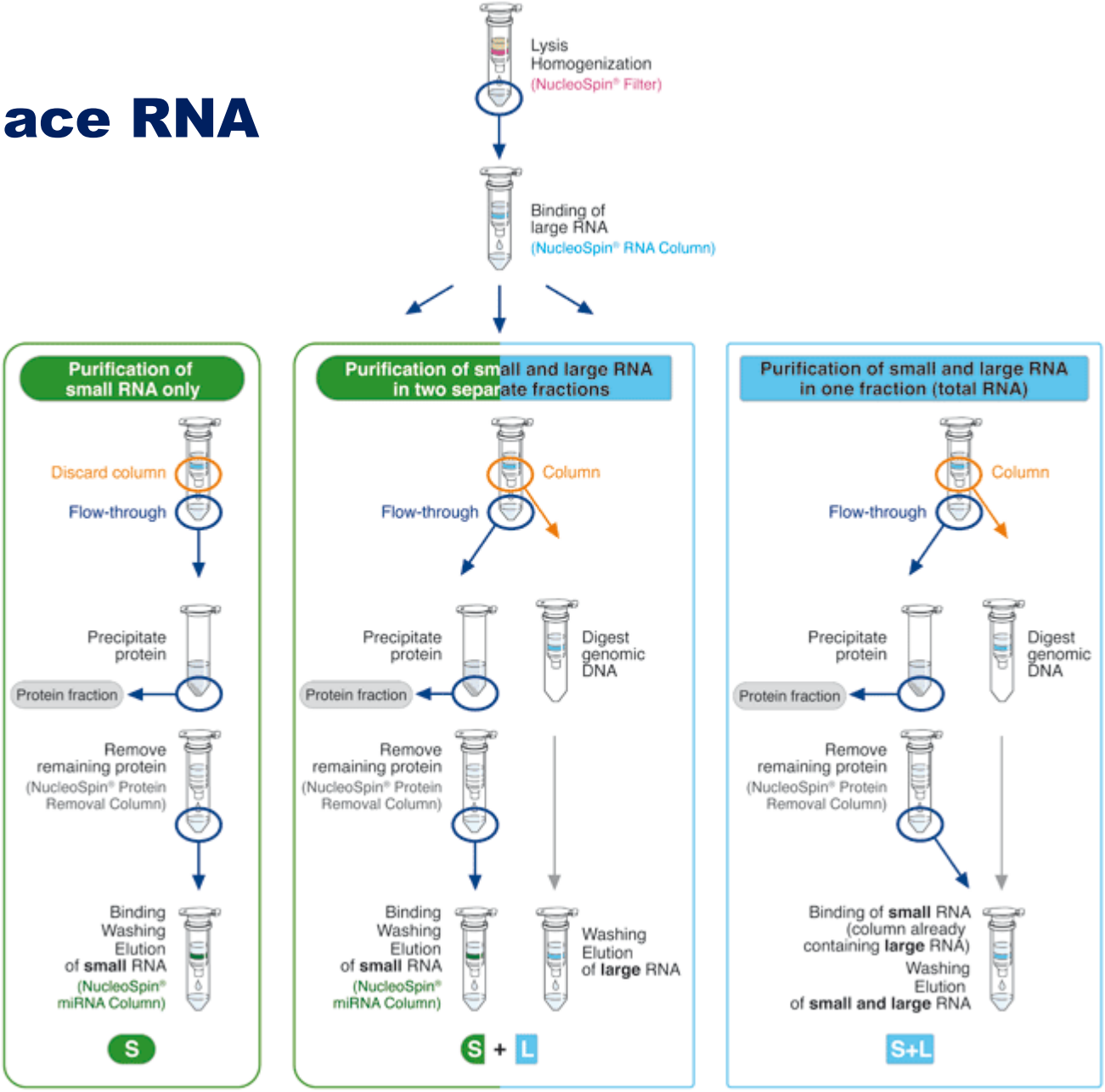
FIGURE 2. Acid phenol specifically leaves RNA in the aqueous phase. As the pH decreases, the concentration of protons increases. DNA carries a negative charge because of the phosphate groups in its sugar-phosphate backbone, which are neutralized in acid by protonation. In this case, DNA dissolves in the organic phase (*like dissolves like*). RNA, on the other hand, is not neutralized in acid because, even though it also has a negative charge, it has exposed nitrogenous bases (it is single-stranded), which can form hydrogen bonds with water, keeping it in the aqueous phase. (3)

Izolace RNA

guanidium thiokyanát-fenol-chloroformová extrakce



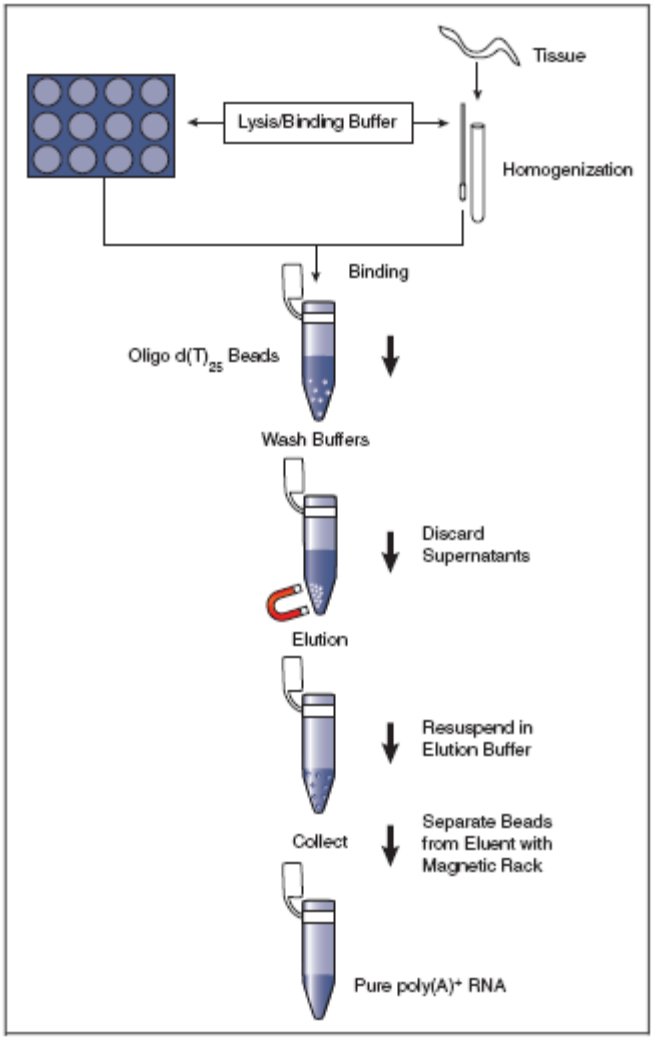
Izolace RNA


Lysis
Homogenization
(NucleoSpin® Filter)
Binding of
large RNA
(NucleoSpin® RNA Column)
Purification of
small RNA only
Discard column
Flow-through
Precipitate
protein
Protein fraction
Remove
remaining protein
(NucleoSpin® Protein
Removal Column)
Binding
Washing
Elution
of small RNA
(NucleoSpin®
miRNA Column)
S
Purification of small and large RNA
in two separate fractions
Column
Flow-through
Precipitate
protein
Protein fraction
Digest
genomic
DNA
Remove
remaining protein
(NucleoSpin® Protein
Removal Column)
Binding
Washing
Elution
of small RNA
(NucleoSpin®
miRNA Column)
Washing
Elution
of large RNA
S + L
Purification of small and large RNA
in one fraction (total RNA)
Column
Flow-through
Precipitate
protein
Protein fraction
Digest
genomic
DNA
Remove
remaining protein
(NucleoSpin® Protein
Removal Column)
Binding of small RNA
(column already
containing large RNA)
Washing
Elution
of small and large RNA
S+L

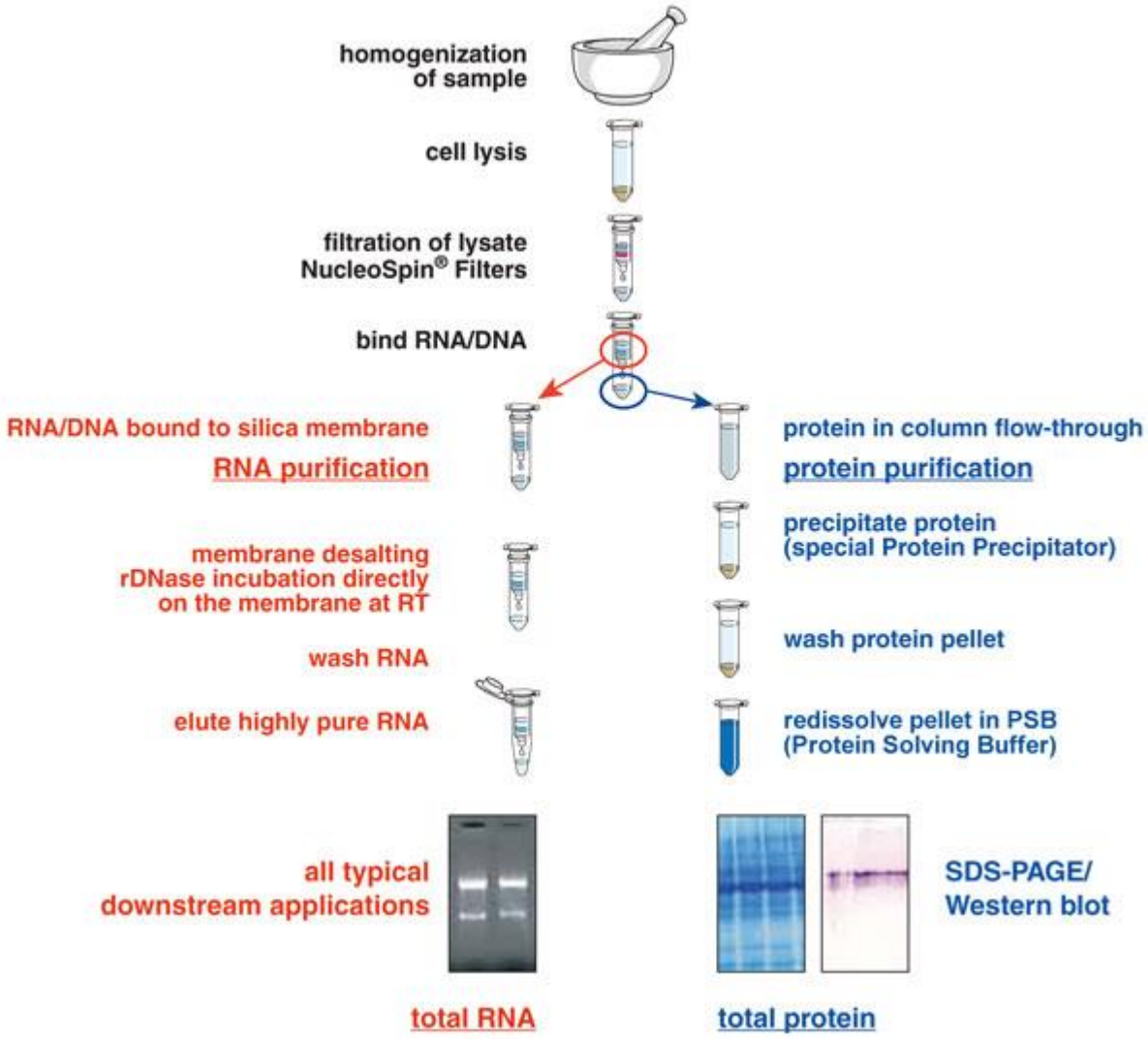
Izolace mRNA

- mRNA obsahuje poly(A) sekvenci na 3' konci

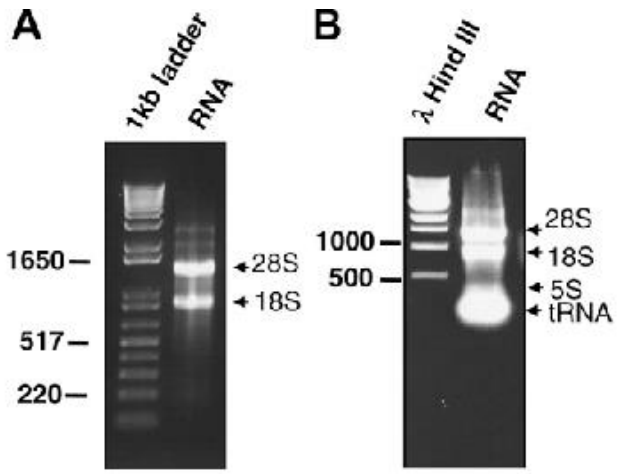
- magnetické částice s vázaným oligo $d(T)_{25}$



Izolace RNA a proteinů



Integrita RNA



RNA integrity following Balch homogenization. Two methods were employed to extract RNA from C. elegans following sample disruption with the Balch homogenizer. (A) Guanidinium isothiocyanate method employing a NucleoSpin RNA Isolation Kit. (B) Trizol (phenol-based) method. In both instances, worm samples were homogenized before molecular disruptants were added. No obvious compromise in RNA integrity was observed in either method. Listed in each panel are DNA marker sizes (bp) and RNA species (28S, 18S, and 5S ribosomal RNA and transfer RNA [tRNA]).

Bhaskaran, Shylesh et al.(2011). Breaking Caenorhabditis elegans The Easy Way Using The Balch Homogenizer – An Old Tool For a New Application. Analytical biochemistry. 413. 123-32. doi: 10.1016/j.ab.2011.02.029.