Izolace a purifikace nukleových kyselin

METODY MOLEKULÁRNÍ BIOLOGIE

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**,...

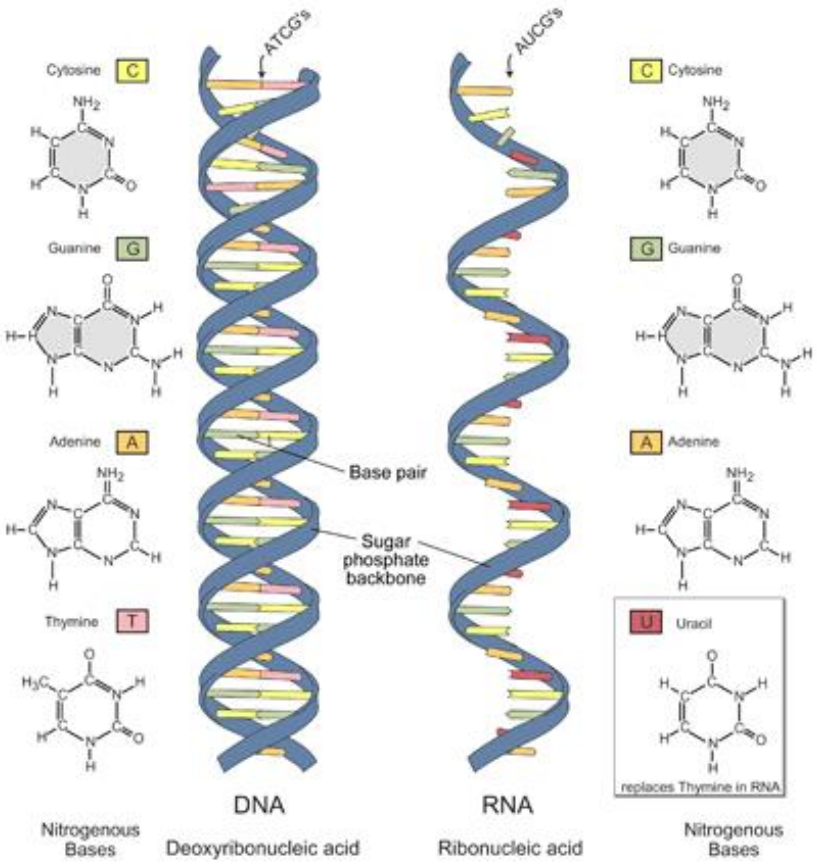


Image adapted from: National Human Genome Research Institute.

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 třeba NK oddělit od ostatních buněčných komponent, které by mohly bránit jejich dalšímu využití a analýze

porušení buněčných stěn – lyze buněk, homogenizace vzorků

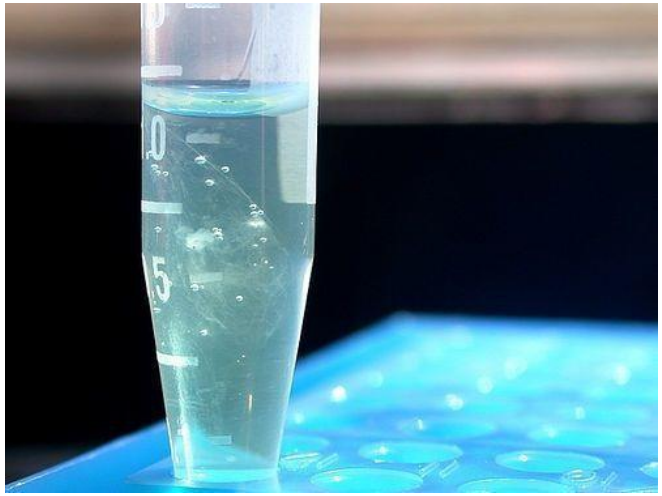
použití enzymů – proteináza K, RNáza, DNáza

centrifugace

adsorpce NK na silikát (SiO_2)

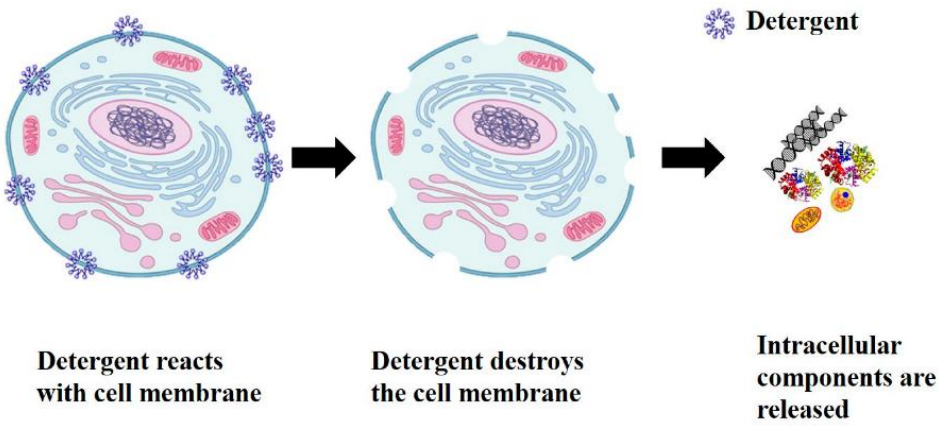
fenol-chloroformová extrakce

precipitace (srážení) NK



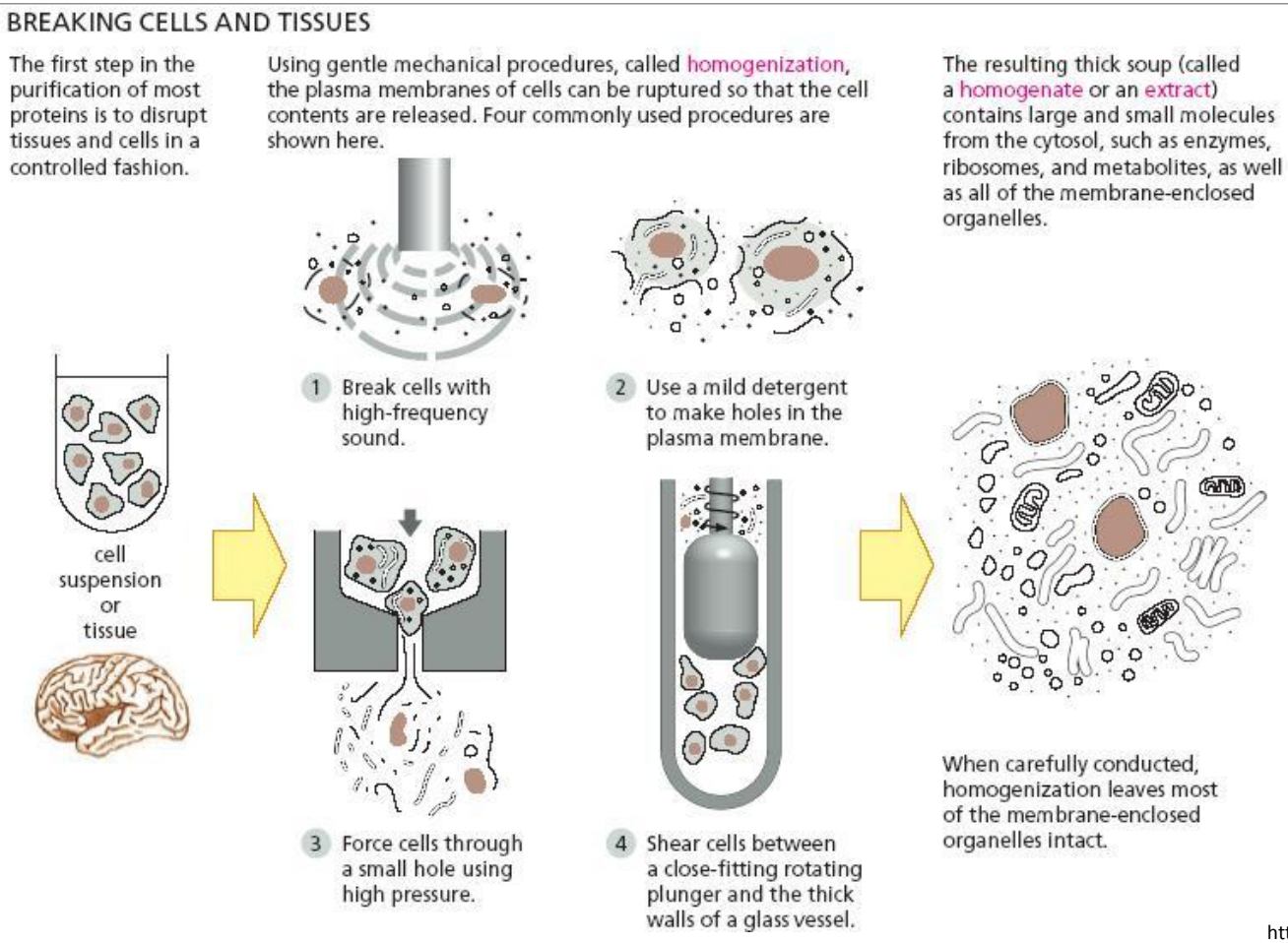
Izolace NK – lyze buněk

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



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

Homogenizace vzorků

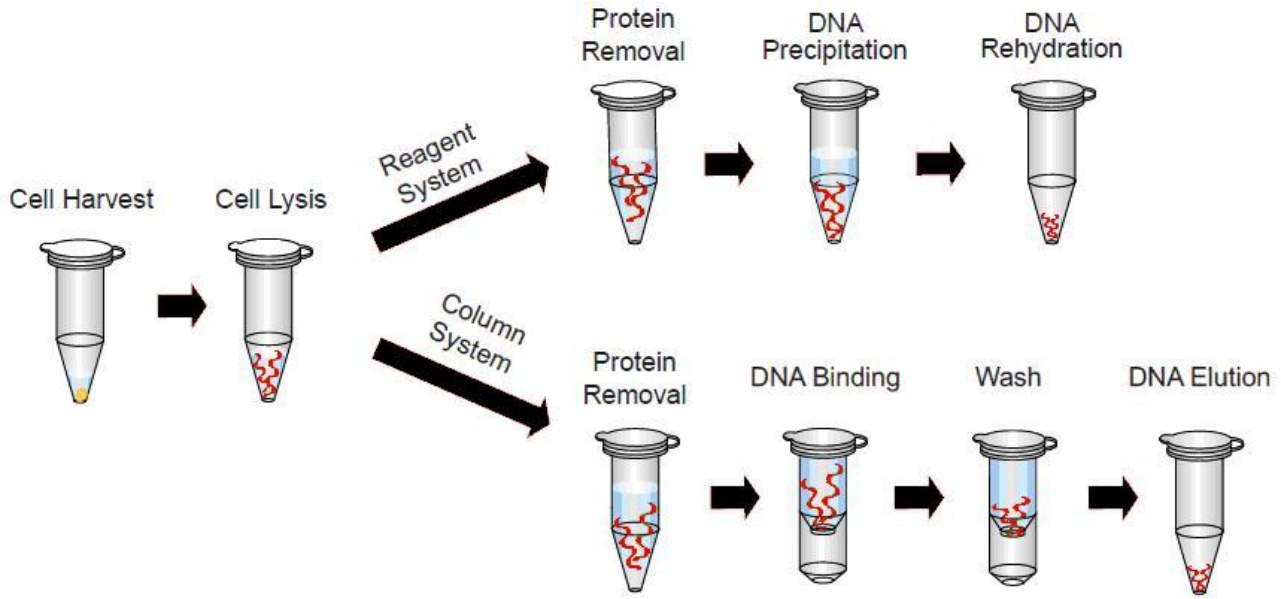


http://cellbiologyolm.stevegallik.org/node/74

Metody izolace NK

1. Využití vazby NK na silikátovou kolonu (SiO_2)

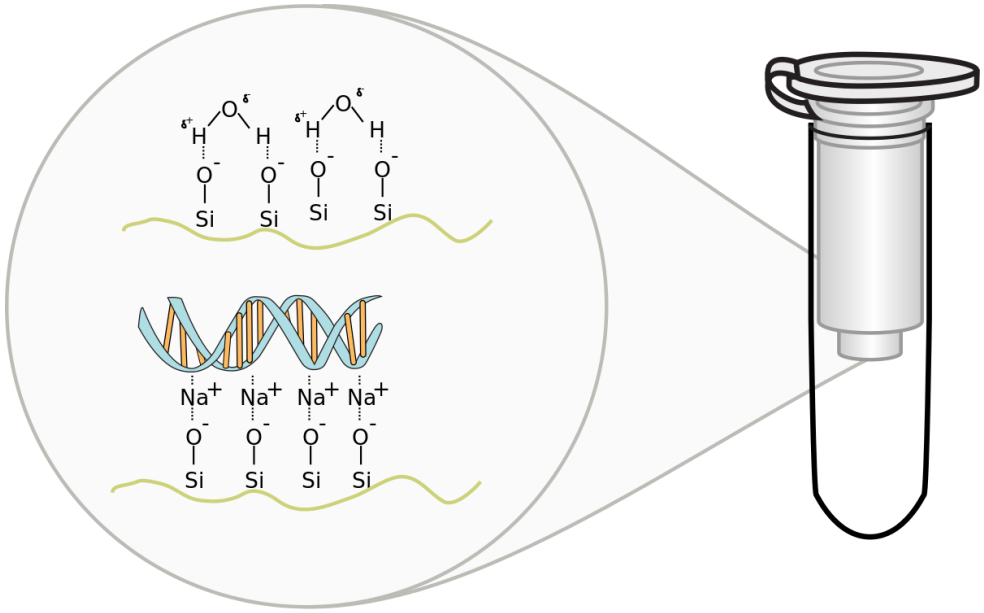
2. Extrakce a precipitace NK



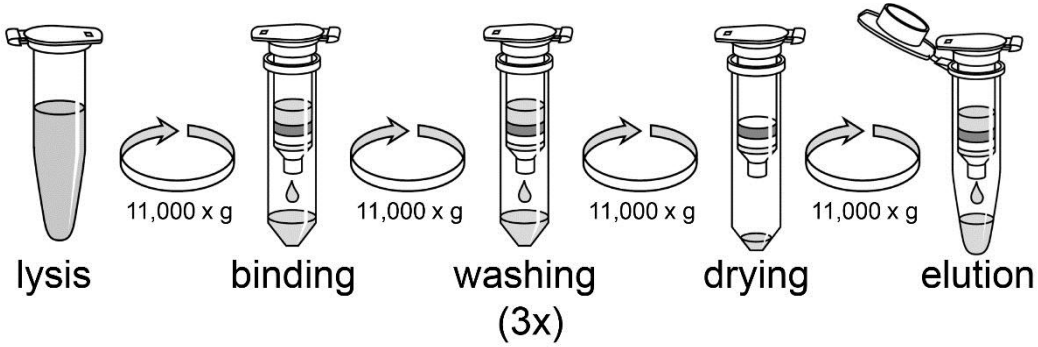
Metody izolace DNA

1. Využití vazby DNA na silikátovou kolonu

v přítomnosti chaotropních solí (zvyšujících iontovou sílu, např. NaI) se DNA váže na silikát (SiO_2)

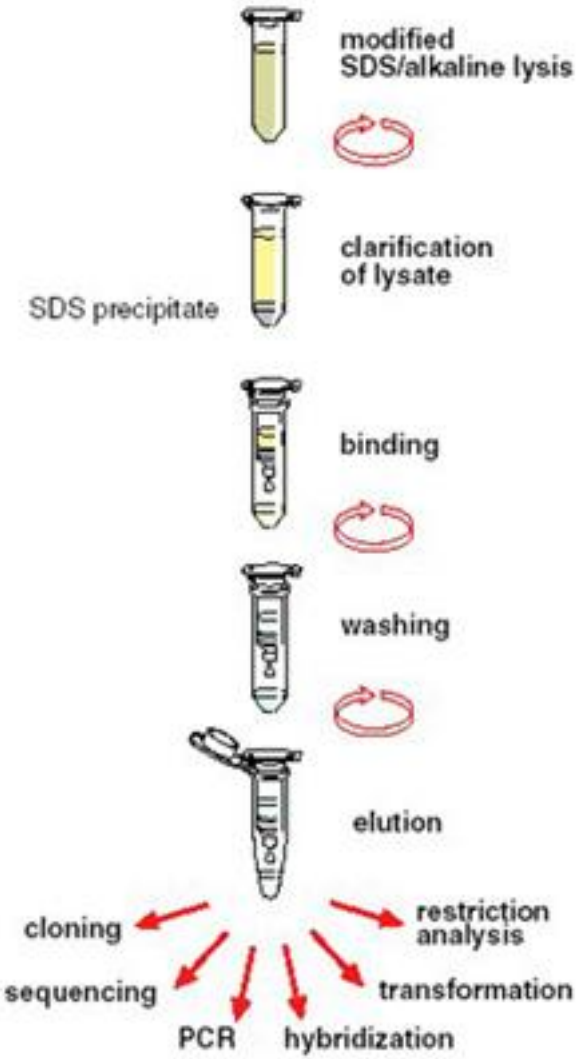


Izolace DNA na koloně



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 - KAc

promývací pufr - ethanol

eluční pufr – nízká IS

		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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Fenol-chloroformová extrakce

fenol:chloroform:isoamylalkohol (25:24:1) **pH 8,0**

EDTA – chelatační činidlo

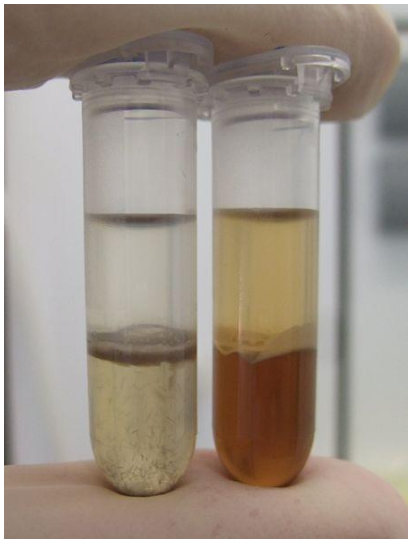
fenol – rozpouštědlo, chloroform – denaturuje proteiny

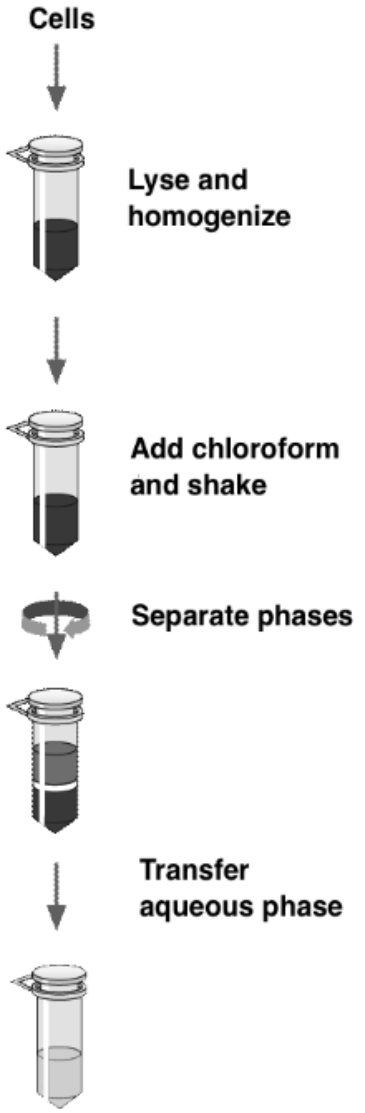
třepáním a centrifugací vznikne fázové rozhraní

spodní část (organická fáze, lipidy)

rozhraní (vysrážené proteiny)

horní část (vodná fáze, polární látky, NK)



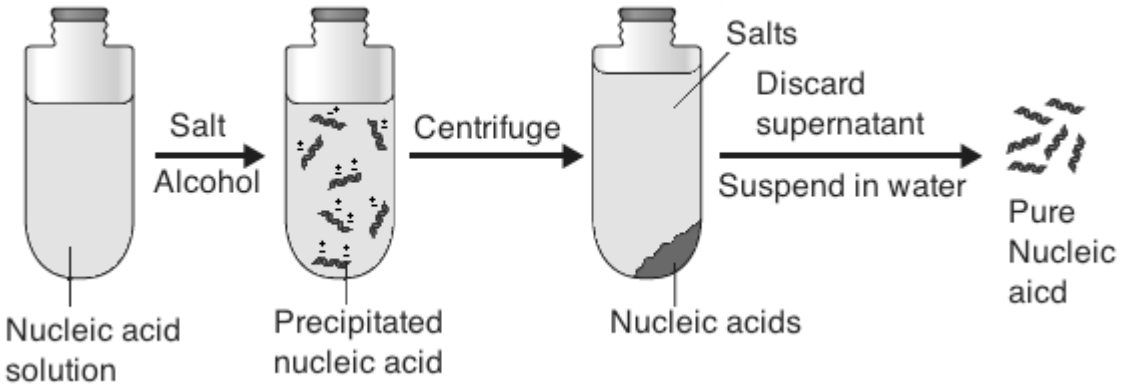


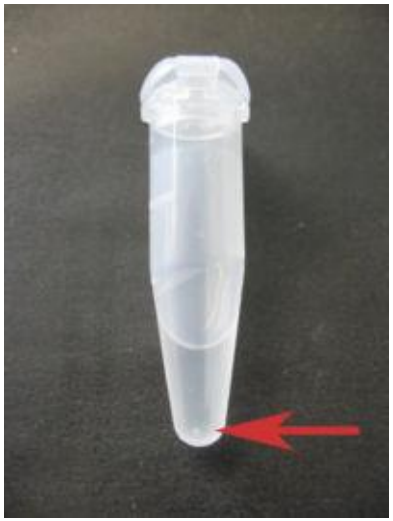
http://physiology.med.cornell.edu/faculty/mason/lab/zumbo/files/PHENOL-CHLOROFORM.pdf

Precipitace 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) – 2,5x objem – zmrazit – **precipitace DNA**
3. centrifugace (11 000 g)
4. odebrat supernatant, přidat 80% ethanol





Precipitace DNA

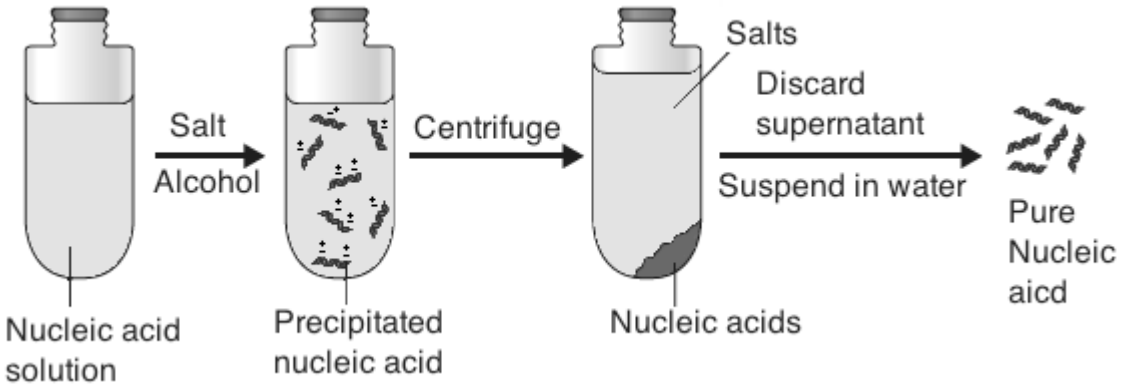
5. centrifugace

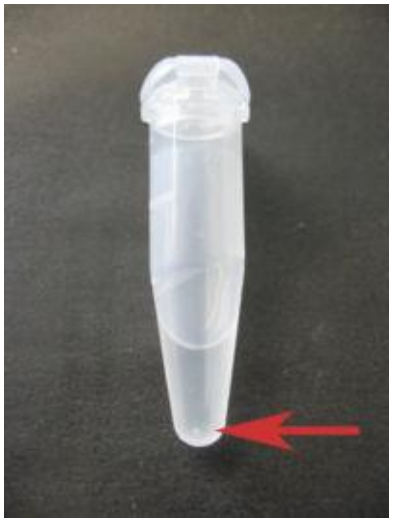
6. odebrat supernatant, přidat 80% ethanol

7. centrifugace

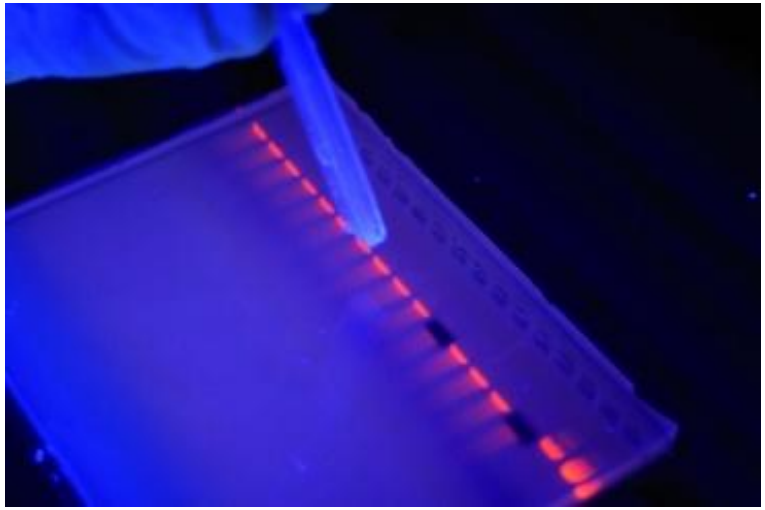
8. odebrat supernatant, vysušit, rozpustit ve vodě nebo TE pufru

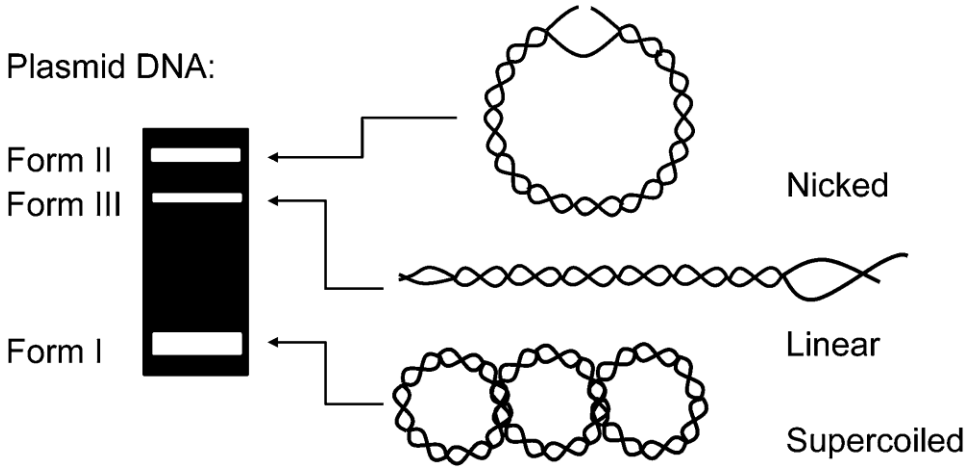
(pro lepší výtěžek lze přidat nosič – tRNA, glykogen)





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

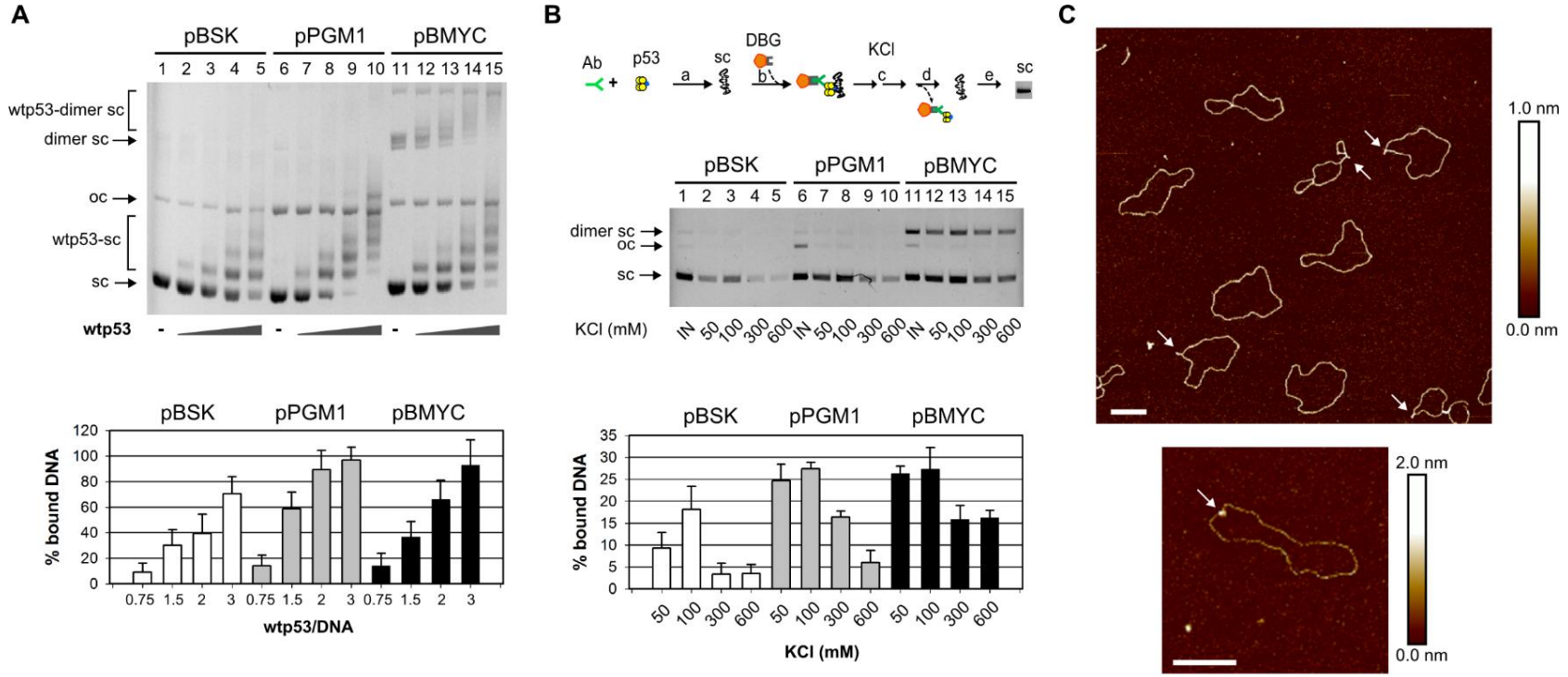


Figure 4

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

Izolace RNA

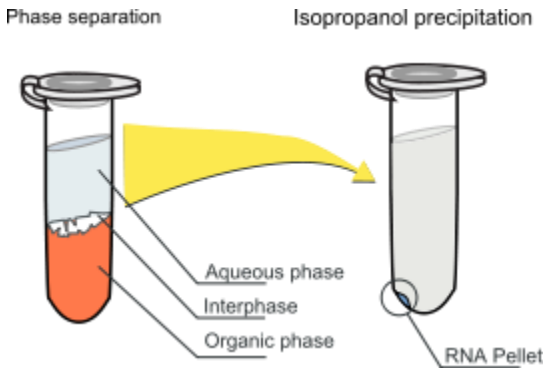
http://physiology.med.cornell.edu/faculty/mason/lab/zumbo/files/PHENOL-CHLOROFORM.pdf

guanidium thiokyanát-

fenol-chloroformová extrakce

při pH 4,5

!ochrana proti působení RNáz!



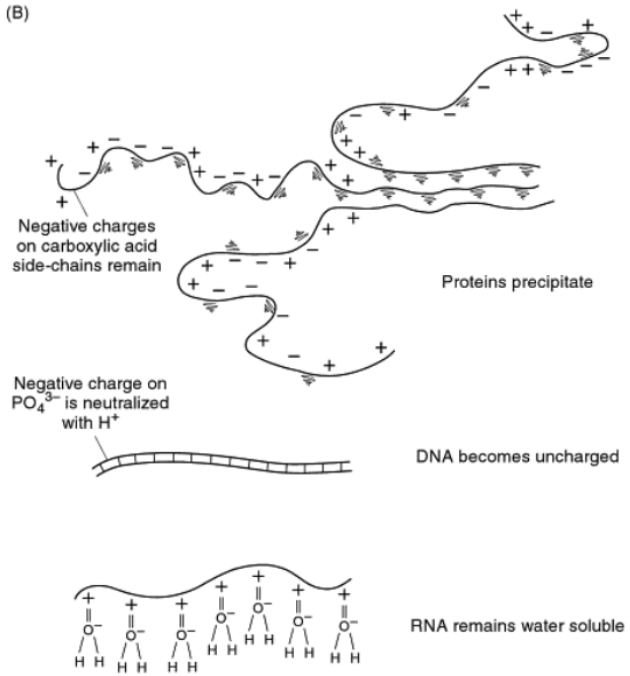
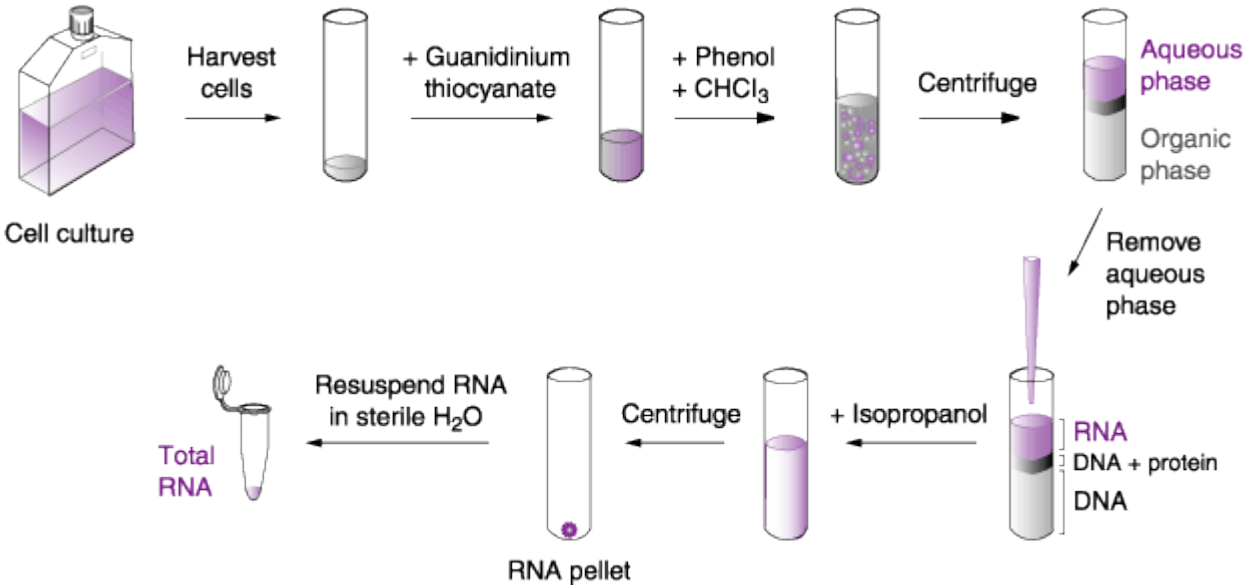


FIGURE 2. Acid phenol specifically leaves RNA in the aqueous phase. As the pH decreases, the concentration of protons increases. DNA carries a negative charge because of the phosphate groups in its sugar-phosphate backbone, which are neutralized in acid by protonation. In this case, DNA dissolves in 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)

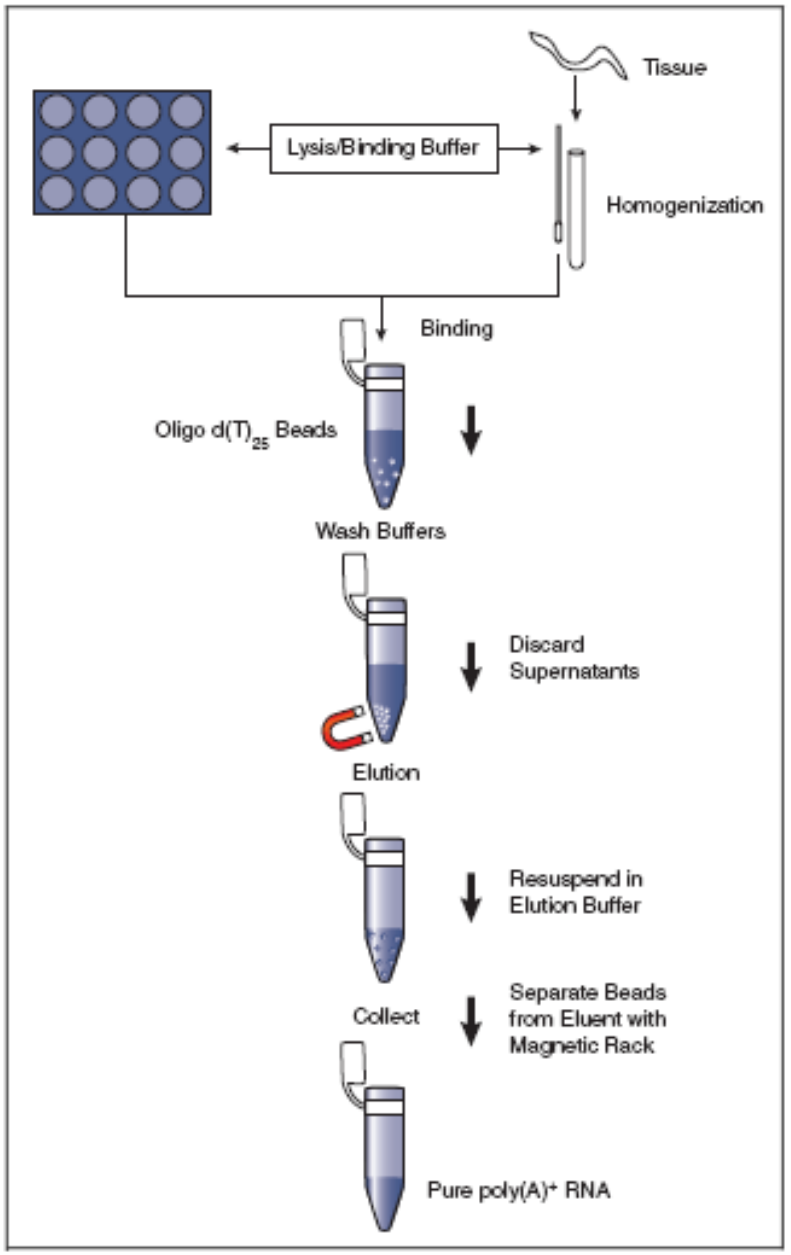
Izolace RNA

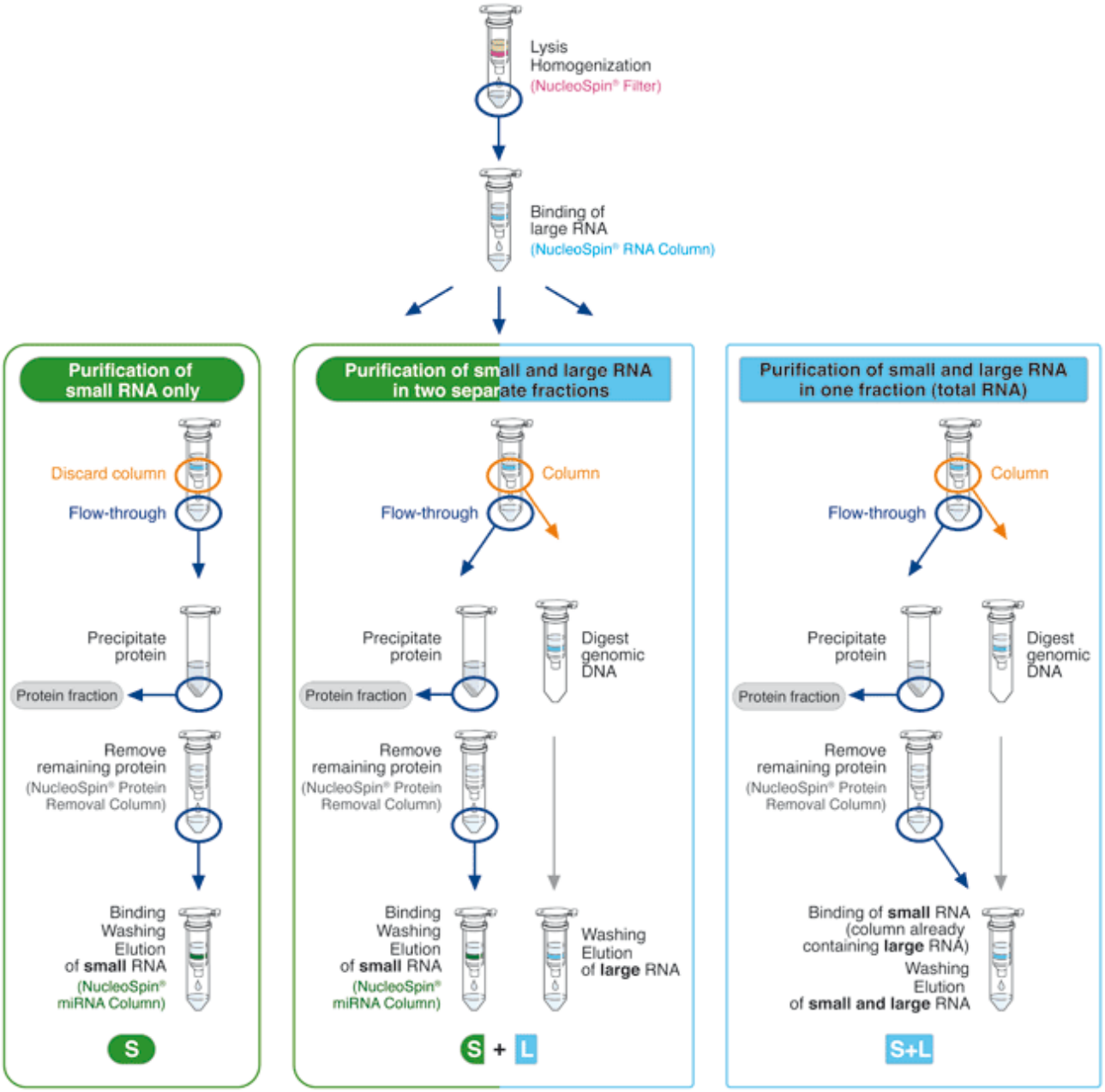
guanidium thiokyanát-fenol-chloroformová extrakce



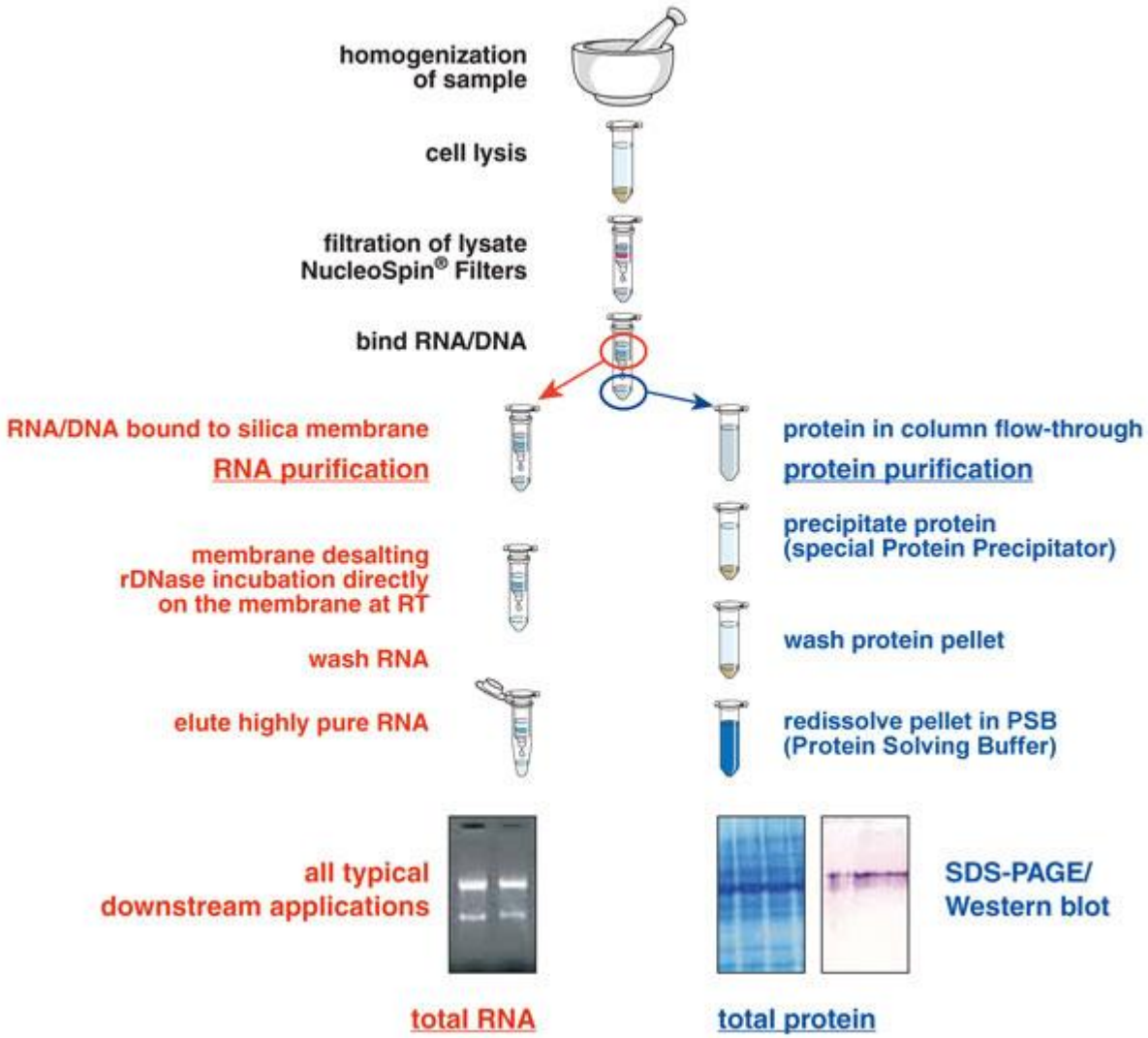
Izolace mRNA

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

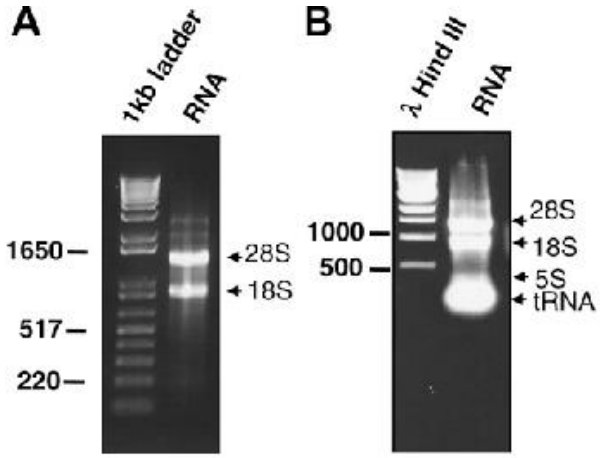



Lysis
Homogenization
(NucleoSpin® Filter)
Binding of
large RNA
(NucleoSpin® RNA Column)
Purification of small RNA only
Discard column
Flow-through
Precipitate protein
Protein fraction
Remove remaining protein
(NucleoSpin® Protein Removal Column)
Binding
Washing
Elution
of small RNA
(NucleoSpin® miRNA 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 RNA/Protein



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. 10.1016/j.ab.2011.02.029.