DNA and RNA: isolation and purification

I. DNA
   a. Genomic DNA
   b. Plasmid DNA
   c. Removal of contaminating proteins
   d. Concentrating dilute samples of nucleic acids
   e. Old/ancient DNA

II. RNA
   a. What kind of RNA?
   b. Special problems with RNA
   c. Battling RNAse

Thought experiment: devise separation strategy for these items, based on each item's unique properties:

- 18 basketballs
- 350 ping pong balls
- 15000 metal ball bearings
- 40 metal cannon balls
- 300 plastic balls (same size and density as wooden)
- 300 wooden balls
- 10 golf balls

References:

1) MC4 DNA Purification
   - Introduction to DNA purification, and kits (p. 2-5)
   - Phenol extraction of proteins (p. 44-46)
   - Ethanol precipitation of DNA (p. 21-25)
   - Isopropanol precipitation of DNA (p. 26-27)
   - Concentration of DNA (p. 28-30)

2) MC4 RNA Purification
   - Overview and introduction to monophasic lysis reagents (p. 346-350)
   - RNA quantification and storage (p. 365-371)
   - Oligo dT beads for mRNA isolation (p. 377-380
   - Controlling RNAses; DEPC (p. 450-453)

3) The Kit Generation: know how the kits work!

4) Friedrich Miescher: the first isolation of DNA
5) Ancient DNA 2001: challenges of old DNA analysis

Pure DNA is essential

- Detect and clone genes
- Identify organisms/viruses
- Sequence DNA regions
- Create new DNA constructions (recombinant DNA)

Pure RNA is also essential

- Identify transcribed genes, exons
- Determine transcription levels
- Clone transcribed genes
- control gene expression (RNAi)

Basic principles of biomolecular separation

You break open cells to release molecules: but then what?
how do you isolate the macromolecule you want?

- **Unique chemistry** of the biomolecule
  - hydrophobicity/hydrophilicity
  - surface charge

- **Size** of the biomolecule

- **Topological state** of the biomolecule

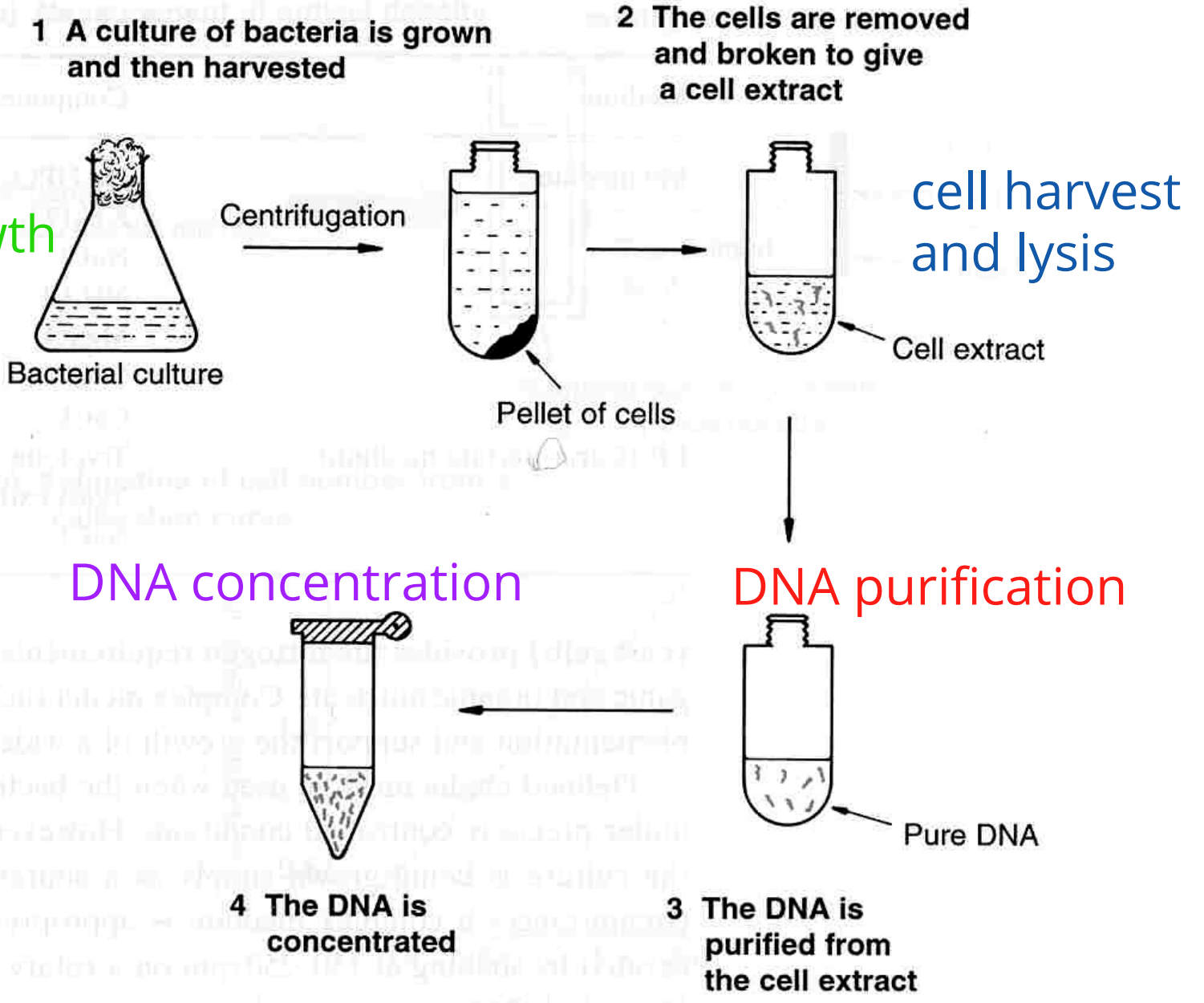
- Susceptibility/resistance to **enzyme treatment**

- **Interactions** with other biomolecules

Thought experiment: devise separation strategy for these items, based on each item's unique properties:

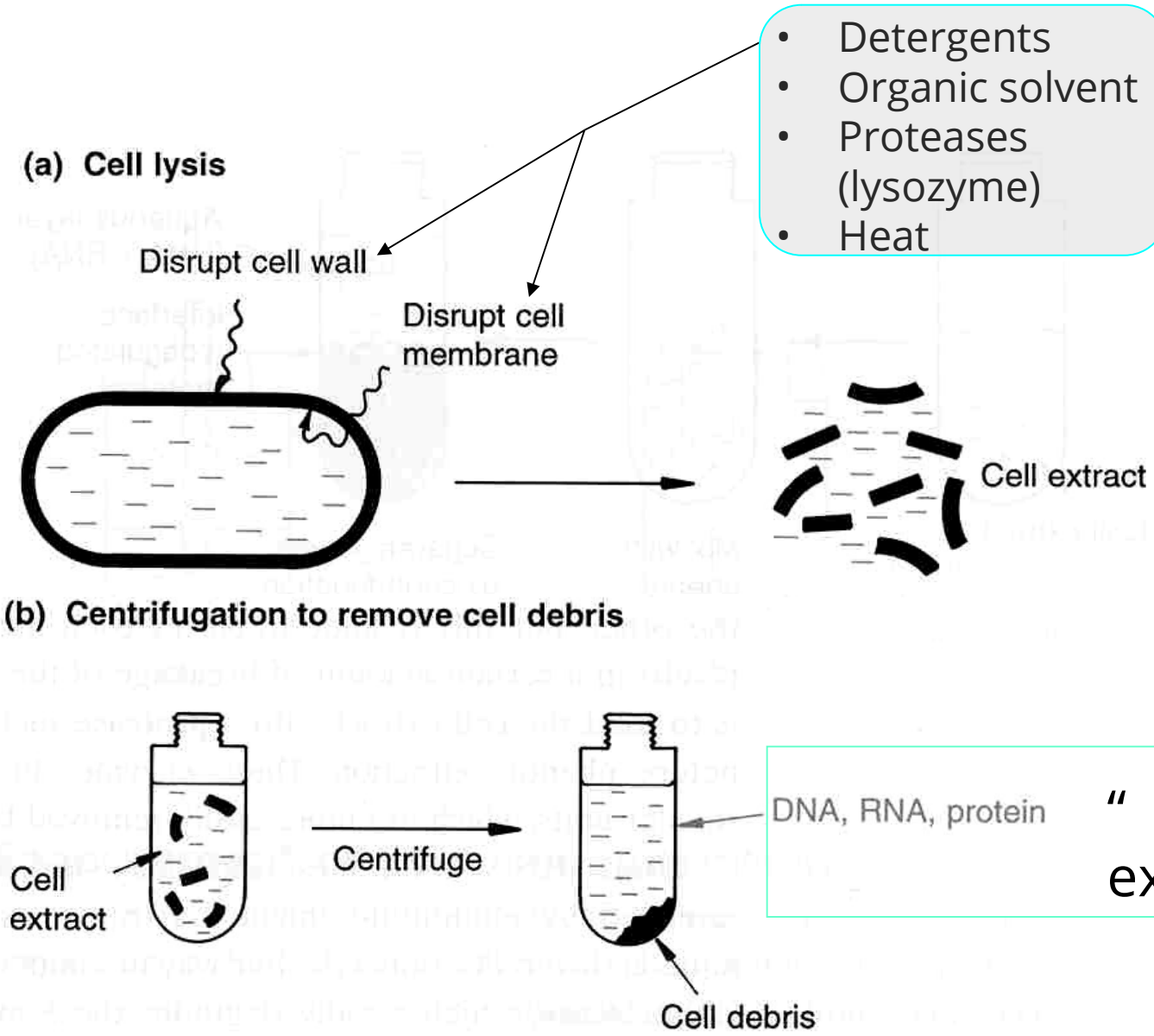
- Protein
- RNA
- genomic DNA
- Small DNA molecules (like plasmids)
- Phospholipids
- Small molecules/ions (nucleotides, Mg++ or Ca++ ions, etc.)

Isolating DNA: overview for bacterial cells



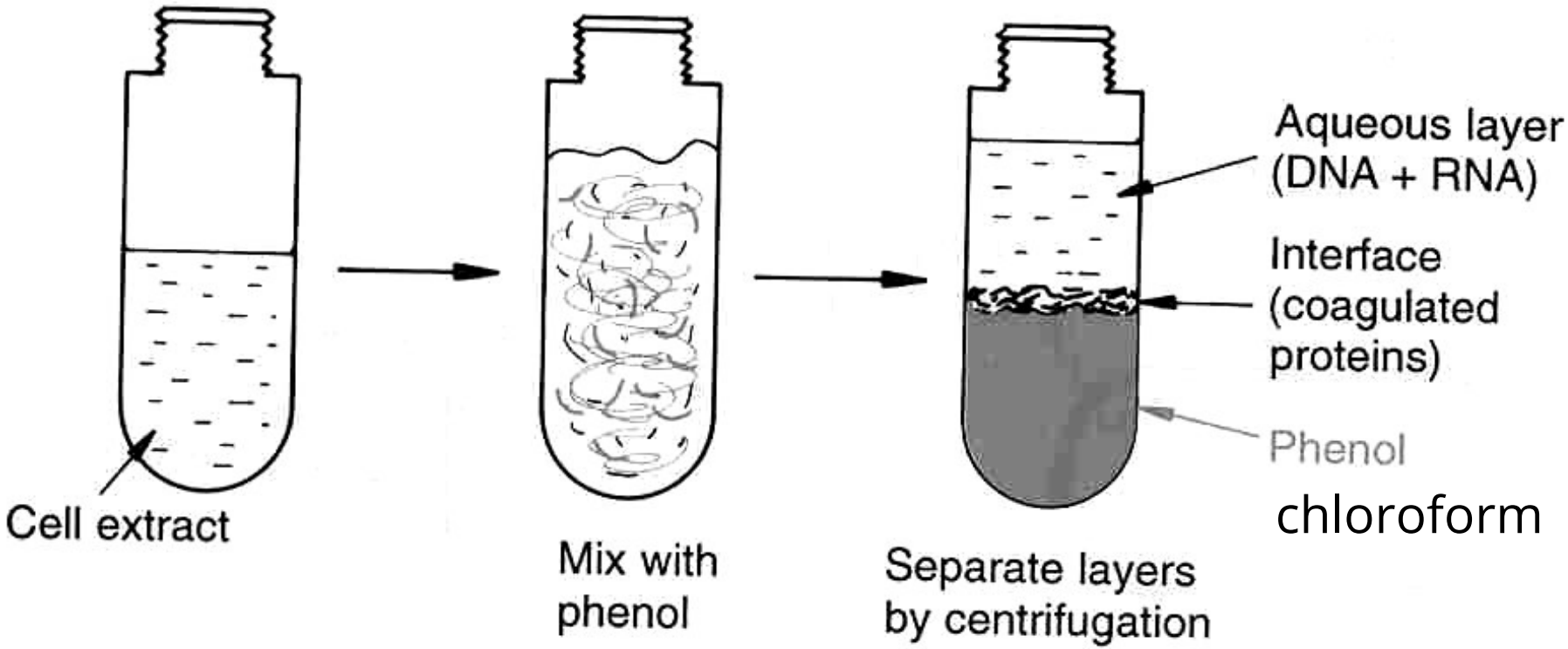
Bacterial genomic DNA: cell extract

- Detergents
- Organic solvent
- Proteases (lysozyme)
- Heat



" soluble cell extract"

Genomic DNA: remove proteins and RNA

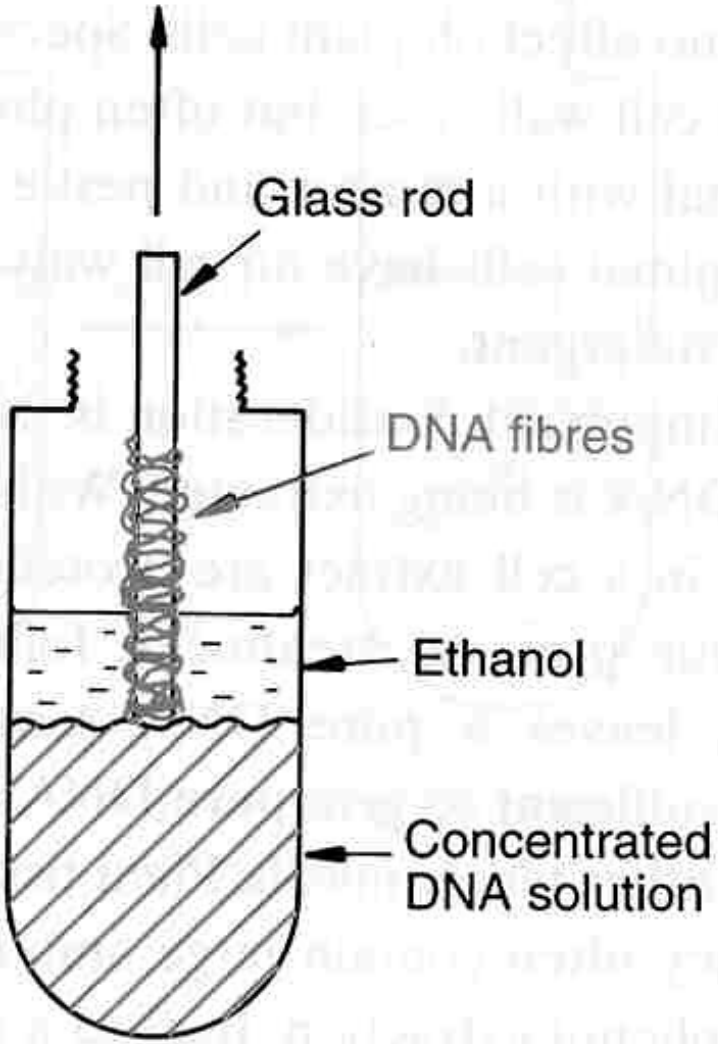


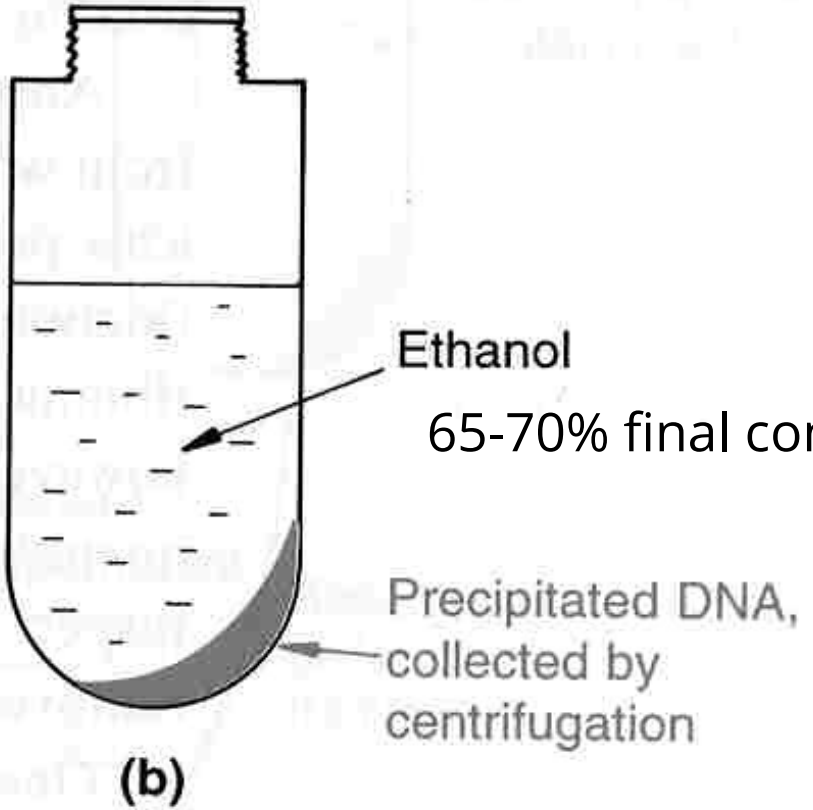
Mix gently (to avoid shearing breakage of the genomic DNA)

DNA and RNA are recovered in aqueous layer

Add the enzyme RNase to remove the RNA

Recover the genomic DNA as a solid precipitate





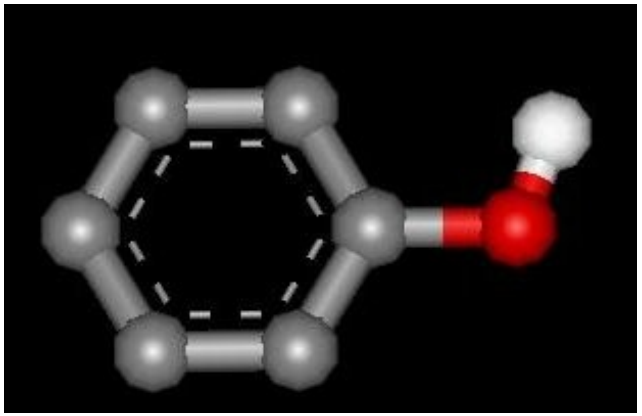
" spooling"

Ethanol precipitation

Separating nucleic acids from protein: phenol extraction

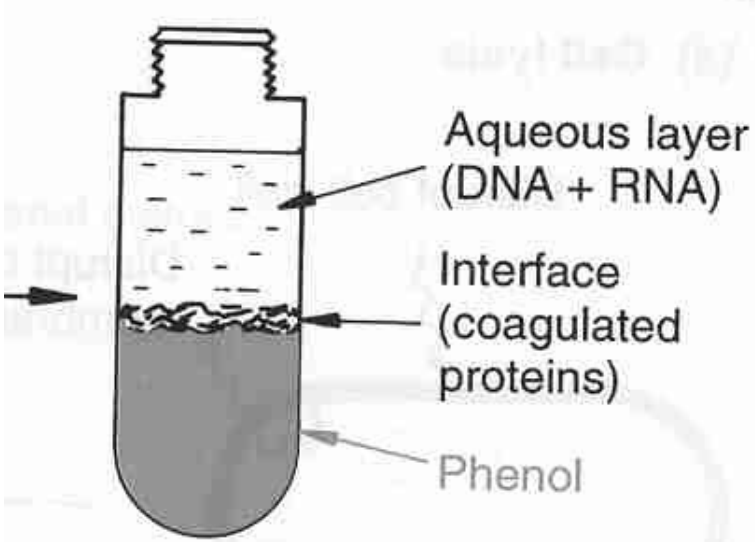
Phenol

- Organic liquid, forms a separate phase from water
- Amphipathic molecule that unfolds proteins by disrupting hydrophobic core
- Insoluble proteins collect at the interface between water and phenol layers



Phenol extraction to remove proteins

1. Aqueous volume (at least 200 microliters)
2. Add 2 volumes of phenol:chloroform, mix well
3. Spin in centrifuge, save aqueous phase in a new tube, avoiding the protein at the interface
4. Repeat steps 2 and 3 until there is no precipitate at phase interface
5. Extract aqueous layer with 2 volumes of chloroform to remove traces of phenol



DNA concentration by ethanol precipitation

Ethanol depletes the hydration shell surrounding DNA...

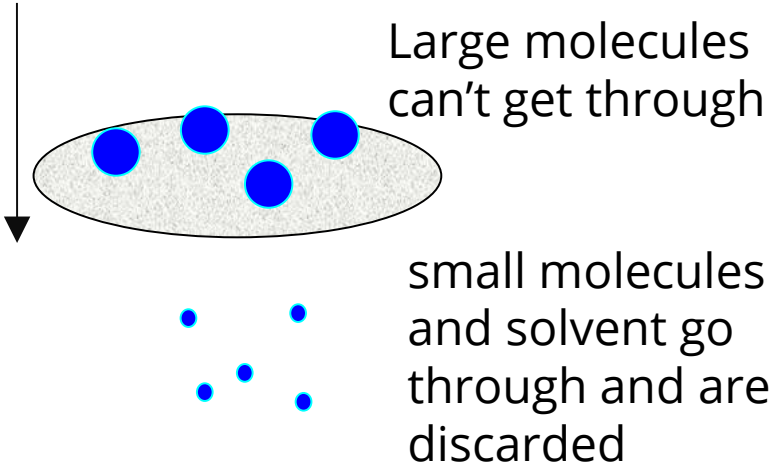
- Allowing cations from added salt to interact with the DNA phosphates
- Reducing repulsive forces between DNA strands
- Causing aggregation and precipitation of DNA

- Aqueous volume of dilute DNA: 180 microliters
  -- add 20 microliters sodium acetate 3M pH 5.2
  -- add 1 microliter of glycogen (gives a visible pellet)
  -- add 2x volumes (400 microliters) 100% ethanol
  -- mix well, centrifuge at high speed, decant liquid
  -- wash DNA pellet (70% ethanol), dry, dissolve in small vol. H_2O, determine DNA concentration)

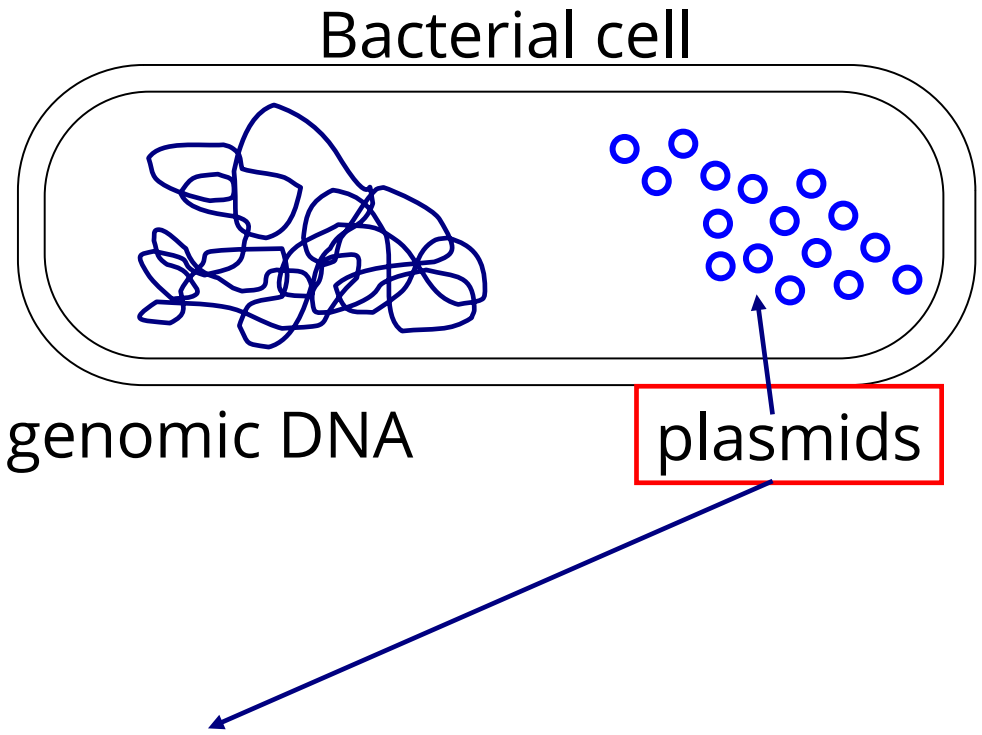
Another way to concentrate DNA solutions (or other large biomolecules)

Molecular concentrators

- Filter with defined pore size (MWCO, molecular weight cutoff)
- Spin in centrifuge to increase rate of passage through the filter
- Water, salts and other small molecules pass through the filter
- DNA (and anything else larger than the cutoff) does not pass through filter



Plasmids: essential for recombinant DNA work

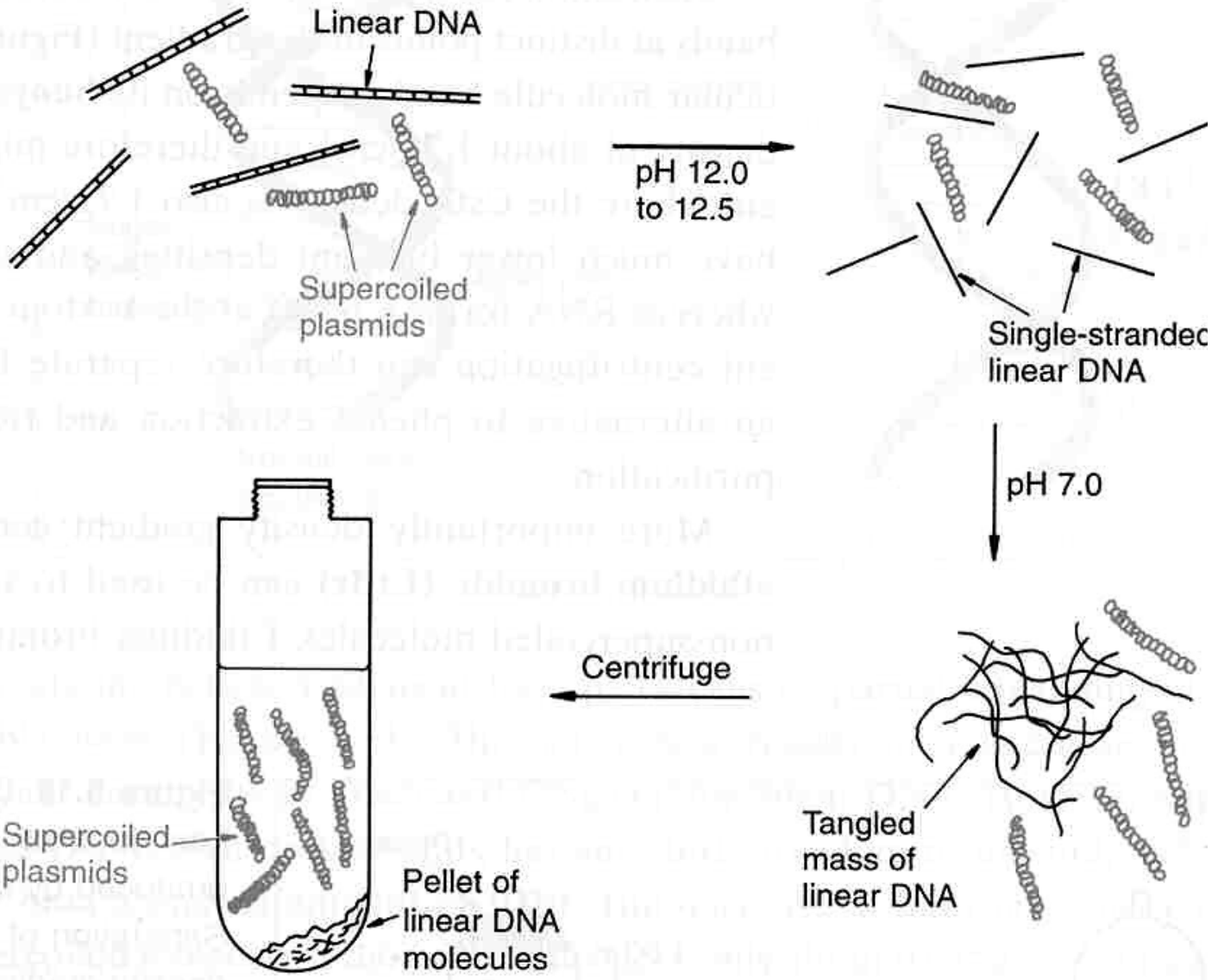


Small pieces of circular DNA that replicate independently of the chromosome

- Many copies per cell
- Easy to isolate and manipulate
- Easy to put back in cells

Plasmid purification: “alkaline lysis”

Add RNAse & break open cells with SDS



Alkaline conditions denature DNA

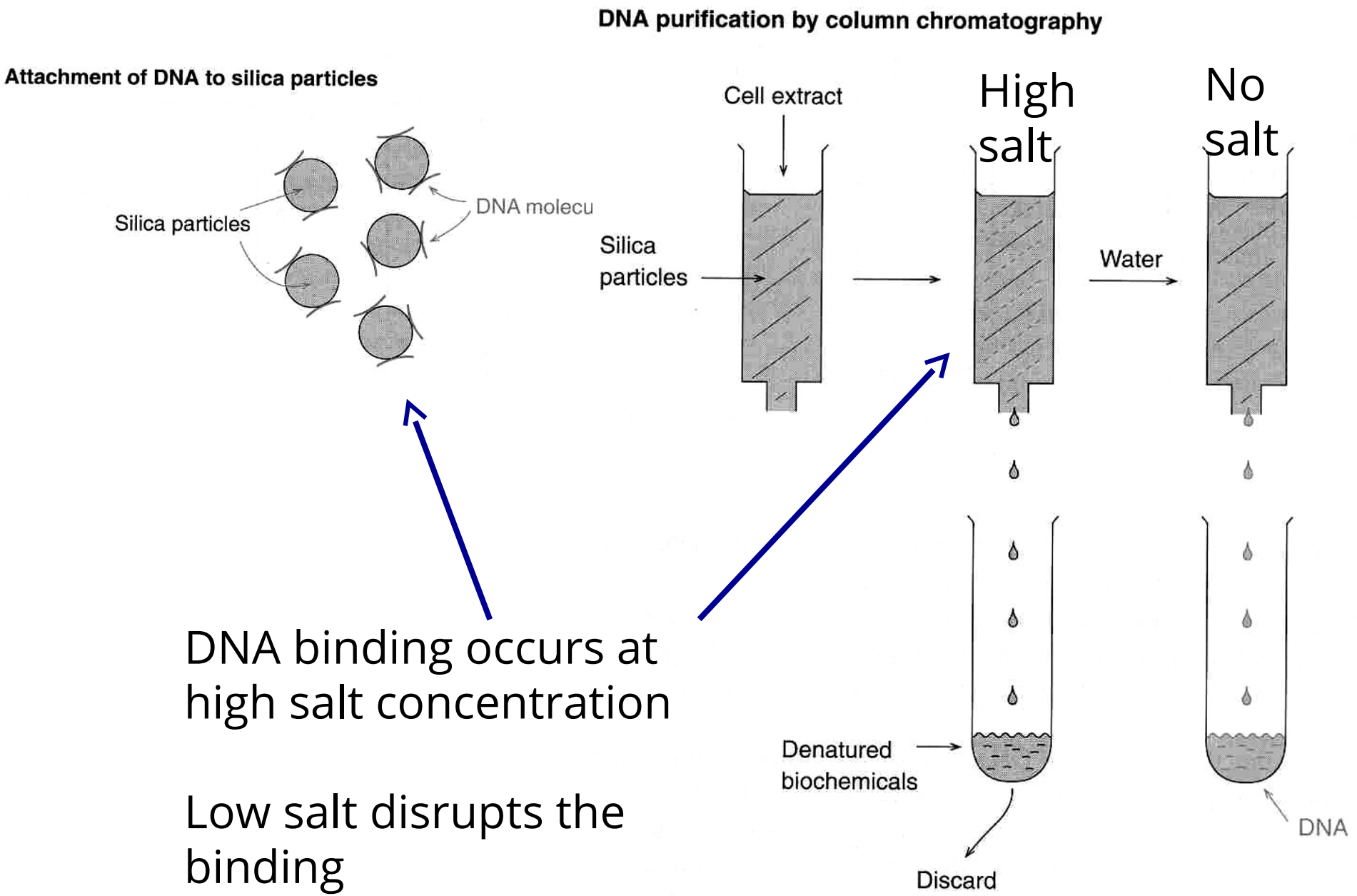
Neutralize: genomic DNA can’ t renature

(plasmids CAN because they never fully separate)

Kits for DNA purification: high reproducibility, saves time

- Guanidinium salts disrupt hydration shell around nucleic acids
- Cations in solution form salt bridges between negative charges of DNA and silica or some other charged resin
- Ethanol (50%) washes away proteins and RNA, but leaves DNA
- Silica/resin can be in form of beads, column matrix, membranes, etc.

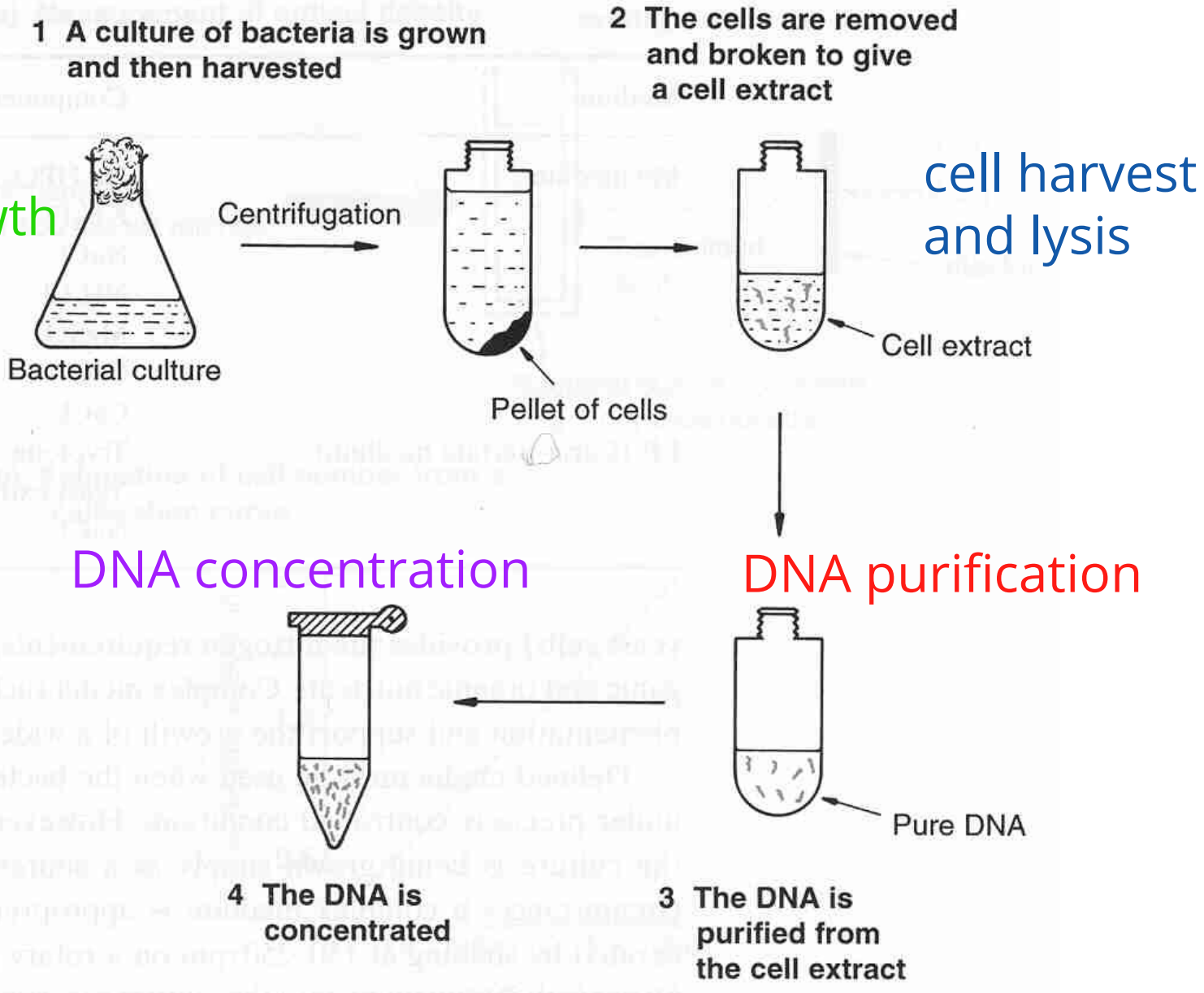
DNA purification: silica binding



DNA binding occurs at high salt concentration

Low salt disrupts the binding

DNA purification: overview



What is aDNA, and what can it tell us?

- aDNA is isolated from archaeological, paleontological remains, museum specimens, etc.

- aDNA provides information for molecular evolution studies
  - Compare DNA sequences of modern organisms to ancestral organisms, trace speciation at the molecular level – example: human evolution

- aDNA can be used to define animal diets, which gives ecological and behavioral information

- aDNA can give information about ancient disease

DNA sequences from extinct animals: snapshots of genetic information from the past

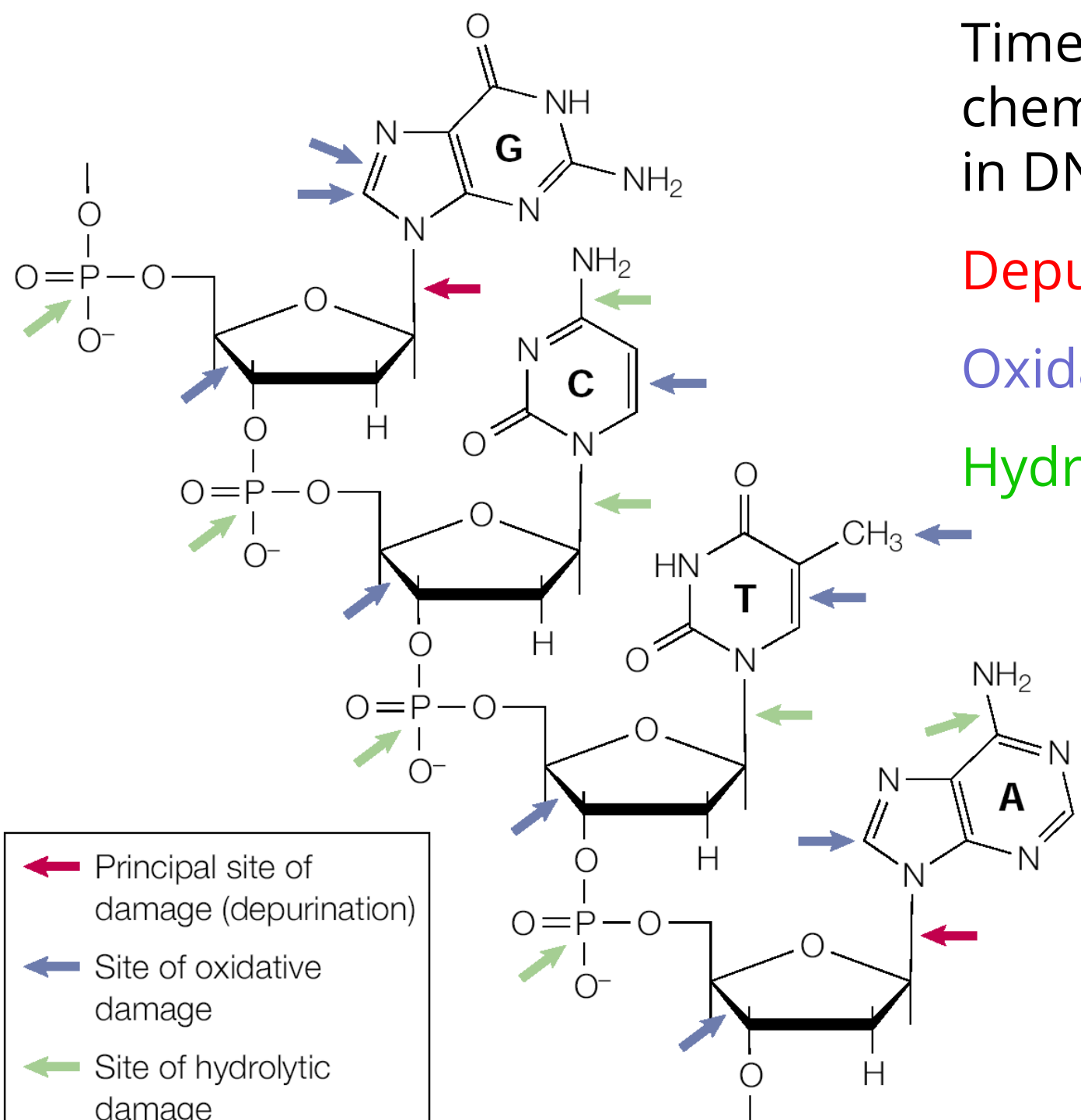
quagga, marsupial wolf, sabre-toothed cat, moa, mammoth, cave bear, blue antelope, giant ground sloth, Aurochs, mastodon, New Zealand coot, South Island piopio, Steller's sea cow, Neanderthal, Aptornis defossor, Shasta ground sloth, pig-footed bandicoot, moa-nalo and Myotragus balearicus

What happens to nucleic acids following death?

- Rapid decay from the action of nucleases, microbial decomposition

- Occasionally DNA is spared this fate:
  - Rapid dessication
  - Low temperatures
  - High salinity

- However, spontaneous, slow decay is inevitable
  - Depurination (loss of A and G bases)
  - Oxidative damage
  - Hydrolytic damage



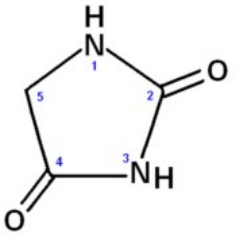
Time-dependent chemical changes in DNA:

Depurination

Oxidative damage

Hydrolytic damage

Effects of DNA damage



- Backbone breakage -- fragmentation
- C and T residues oxidize to make hydantoins, blocking DNA polymerases (PCR)
- Deamination of C causes wrong base to be added during PCR--false mutations

- Increasing time, increasing degradation, decreasing utility
- 100,000 to 1,000,000 years is the approximate age limit for DNA to yield useful sequences

PCR is good, but not perfect for ancient DNA isolation

- Need very little template DNA
- sequence PCR products directly (no need for cloning)
- Specific genes or DNA regions can be targeted
  - Mitochondrial DNA is typical target in aDNA PCR isolations
  - Copy number of mitochondria is high relative to nuclear DNA

However

- Generally only short pieces of ancient DNA can be amplified, because of damage
- PCR artifacts may cause sequence misreads

First retrievals of old DNA

- Quagga (extinct relative of the zebra) DNA isolated from museum specimen (Higuchi et al. 1984)

- 2430 year-old Mummy DNA cloned (Paabo 1985)
  1) Isolated DNA (20 micrograms/gram mummy tissue
  2) Treated with Klenow enzyme (DNA polymerase) to make DNA fragments blunt ended
  3) Cloned into alkaline phosphatase treated pUC8 (pMUM plasmids)

***Cloning presents problems, eg. repair of mutagenized DNA following transformation, which gives false sequence

DNA ----------> mRNA ----------> protein

<u>Information from mRNA:</u>

When is a gene expressed?

How is the mRNA spliced?

What is the timing of gene expression?

What is the level of gene expression?

Isolation of RNA – Molecular Cloning reading

RNA in a typical eukaryotic cell:

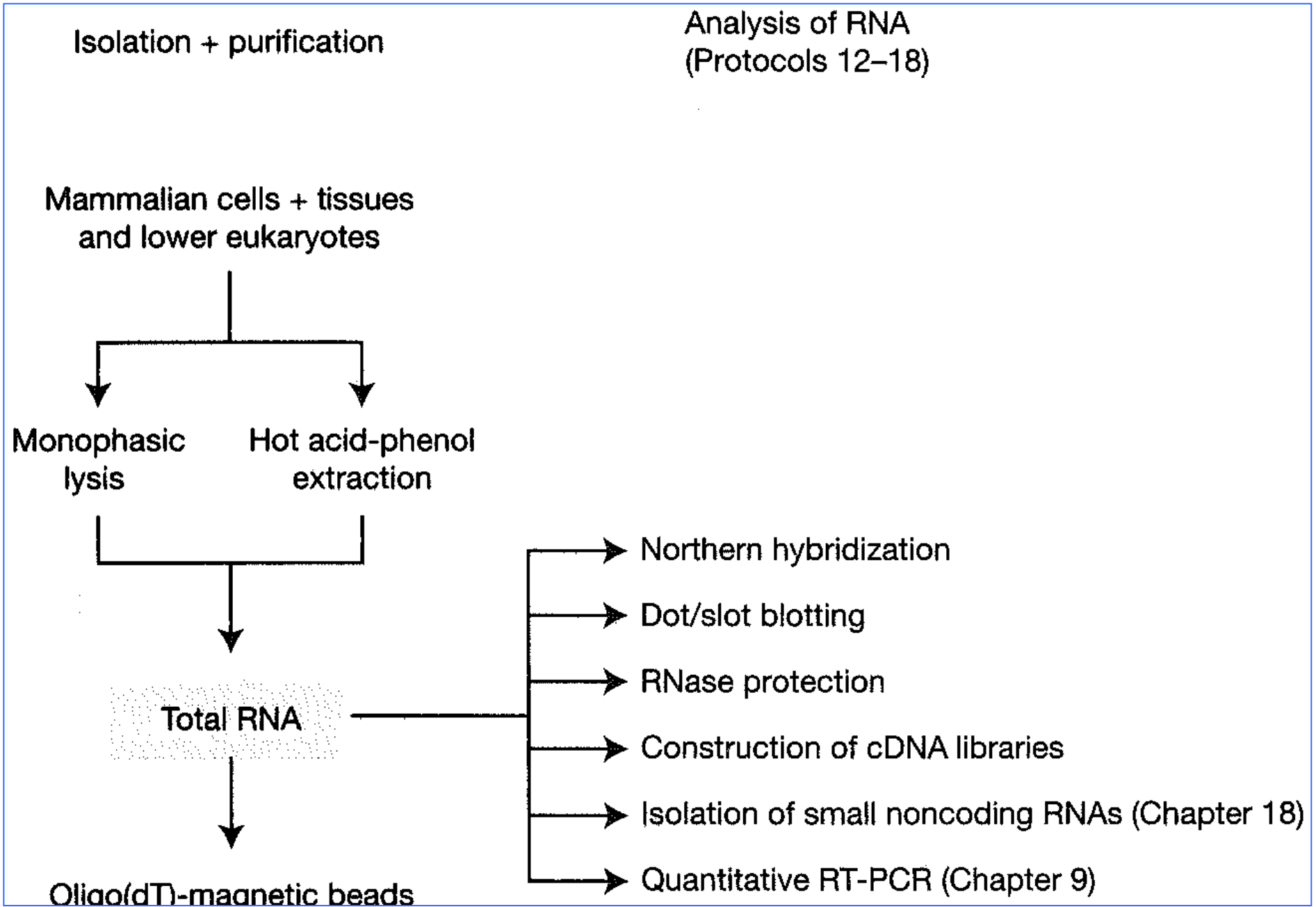
10^{-5} micrograms RNA

80-85% is ribosomal RNA
15-20% is small RNA (tRNA, small nuclear RNAs)

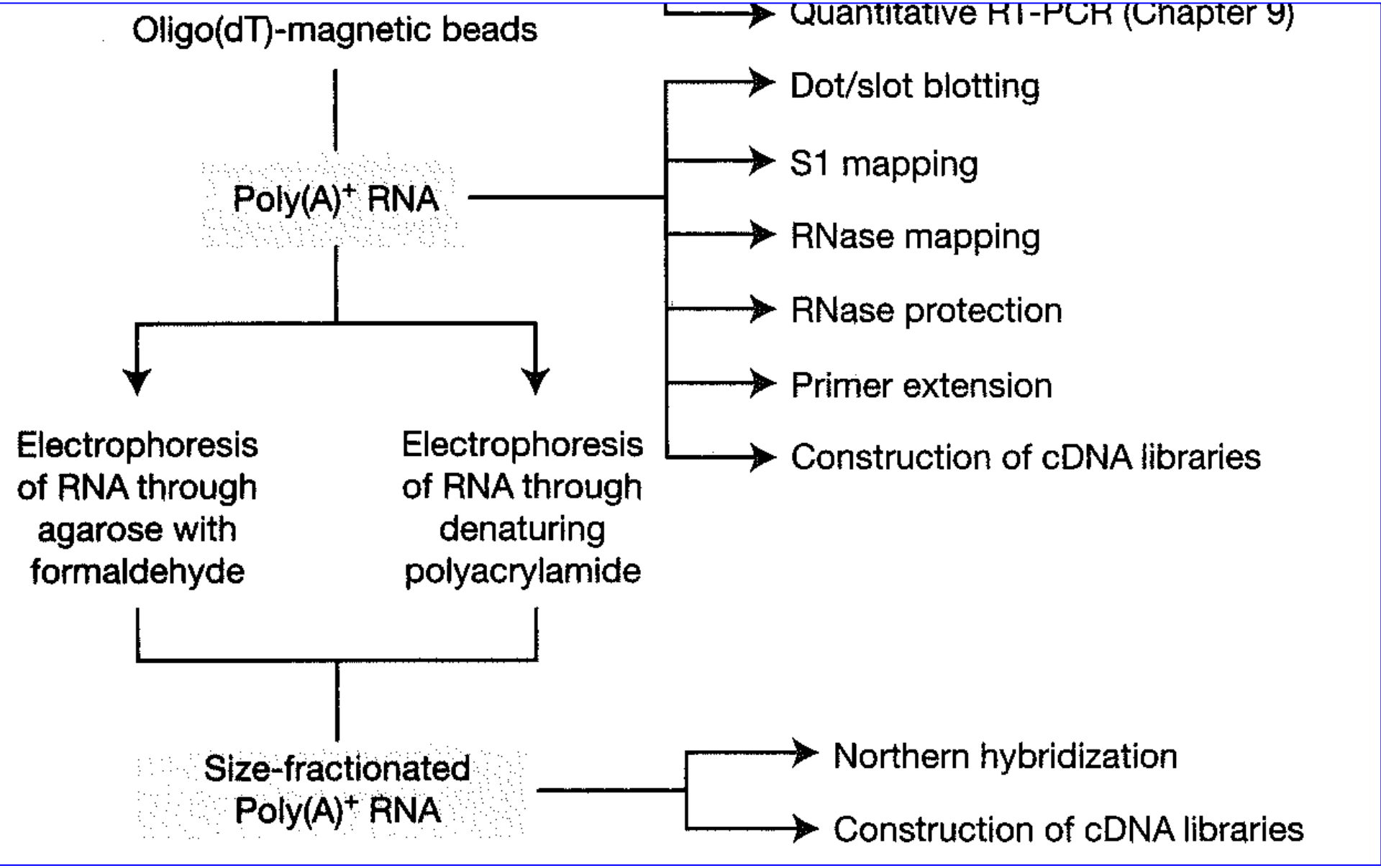
About 1-5% is mRNA

-- variable in size
-- usually contains 3’ polyadenylation

Making and using mRNA (1)



Making and using mRNA (2)



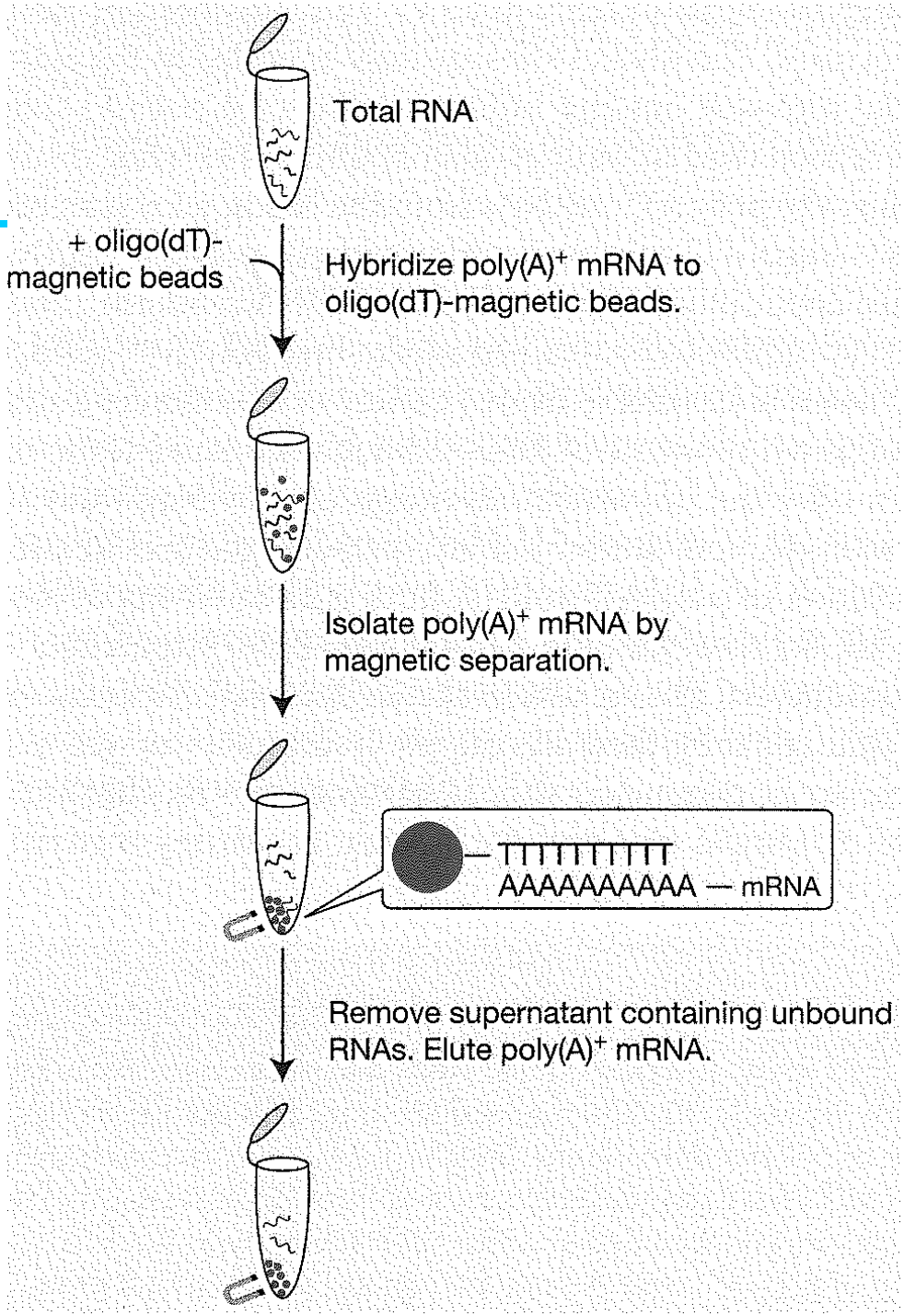
Selective capture of mRNA: oligo dT-cellulose

Oligo dT is linked to magnetic beads

RNA is mixed with beads at high salt concentration

Non-polyadenylated RNAs do not hybridize to beads

polyA RNA can be removed under low salt conditions



The problem(s) with RNA:

RNA is chemically unstable

spontaneous cleavage of phosphodiester backbone via intramolecular transesterification

RNA is susceptible to nearly ubiquitous RNA-degrading enzymes (RNases)

RNases are released upon cell lysis
RNases are present on the skin
RNases are very difficult to inactivate
-- Very stable (disulfide bridges)
-- Divalent cations not needed for activity

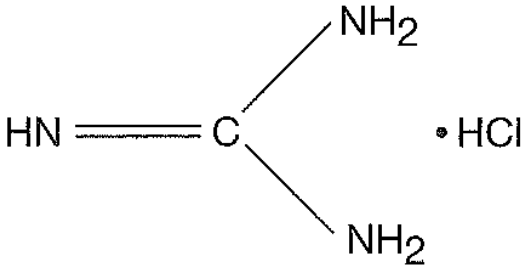
Purifying RNA: the need for speed

Break the cells/solubilize components/inactivate RNAses by the addition of guanidinium salts (very powerful protein denaturant)

Extract RNA using phenol/chloroform (at low pH)

OR use a monophasic lysis reagent (trizol):

- guanidinium salt
- acidified phenol
- phenol solubilizer (e.g. glycerol)



Guanidinium hydrochloride

Precipitate the RNA using ethanol/LiCl

Store RNA:

in DEPC-treated H_20 (-80°C)

in formamide (deionized) at -20°C

Storage of RNA: need to prevent degradation

In solution:
• pH 7 to 7.6
• SDS (sodium dodecyl sulfate), a detergent that inhibits RNAses
• EDTA (Ethylene Diamine Tetra Acetate) captures (chelates) metal ions (e.g. Mg^{+2}) that can degrade RNA
• Store at very low temperatures (-80°C)

Long term: store in ethanol precipitation conditions at -80°C. The low temp and high alcohol concentration reduces RNAse activity

Inhibitors of Rnase

DEPC: diethylpyrocarbonate

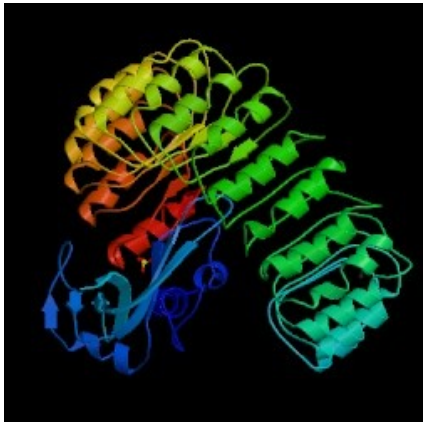
- alkylating agent, modifies and inactivates enzymes, including RNAses

Modified ribonucleoside complexes

- competitive inhibitors that bind to RNAse enzyme active site

Protein inhibitors of RNAse

- horseshoe-shaped, leucine rich protein, found in cytoplasm of most mammalian tissues



10 common sources of RNAse contamination

1) Ungloved hands
2) Tips and tubes
3) Water and buffers
4) Lab surfaces
5) Endogenous cellular RNAses
6) RNA samples
7) Plasmid preps
8) RNA storage (slow action of small amounts of RNAse
9) Chemical nuclease action (Mg^{+2}, Ca^{+2} at 80°C for 5’ +)
10) Impure enzyme preparations

Common sources of RNase and how to avoid them

Contaminated solutions/buffers

Use good sterile technique
Treat solutions with DEPC (when possible)
Make small batches of solutions, and don't reuse

Contaminated equipment

Use "RNA only" pipets, glassware, etc
Bake glassware, 300°C, 4 hours, to 'kill' RNAses
USE " RNase-free" pipet tips
Treat equipment with DEPC

DNA and RNA isolation and purification

I. How are biomolecular separations accomplished in general?

II. How is genomic DNA prepared?

II. How is plasmid DNA prepared?

III. How can DNA be separated from other cell components?

IV. How can RNA be isolated successfully?