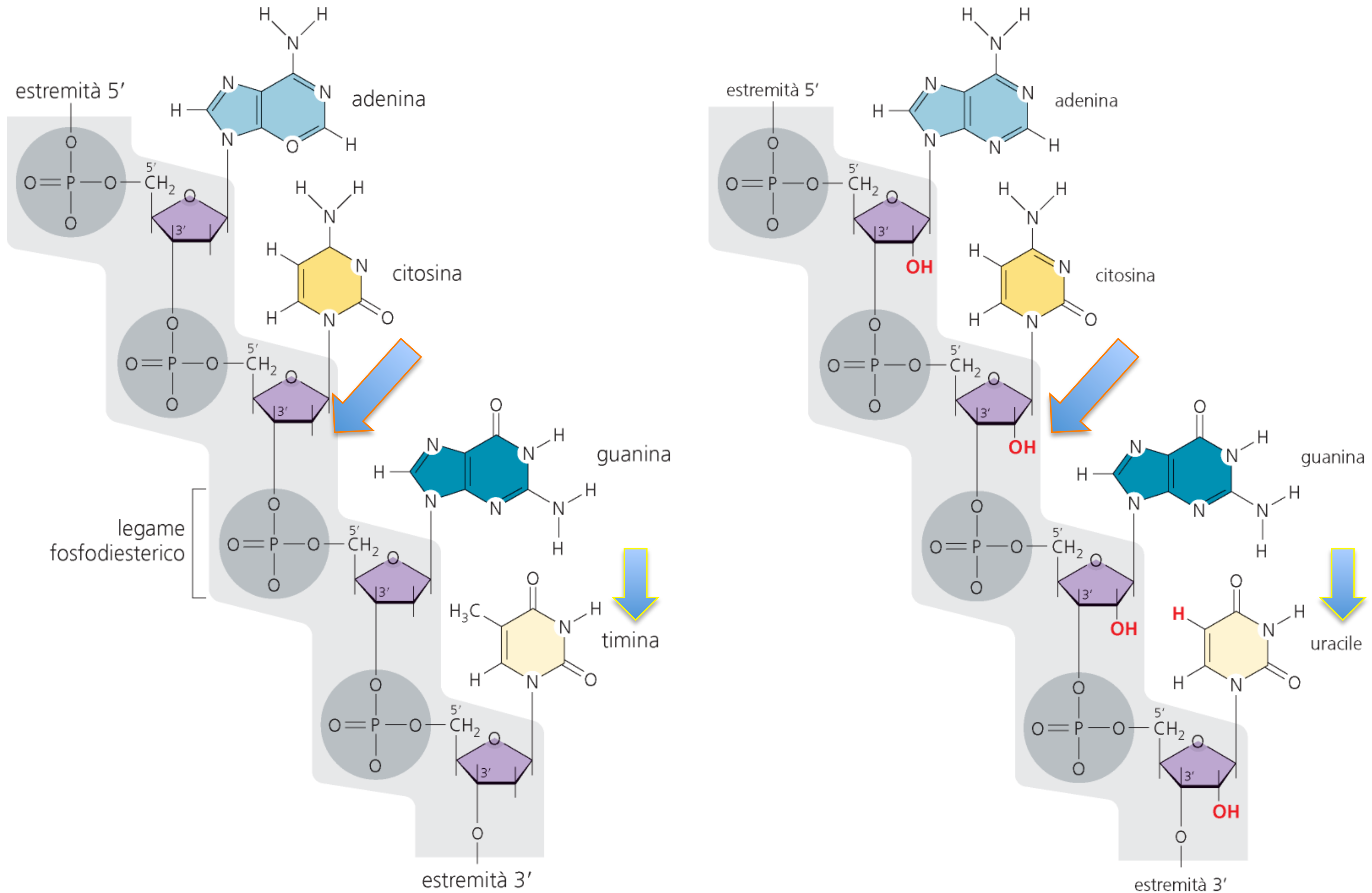
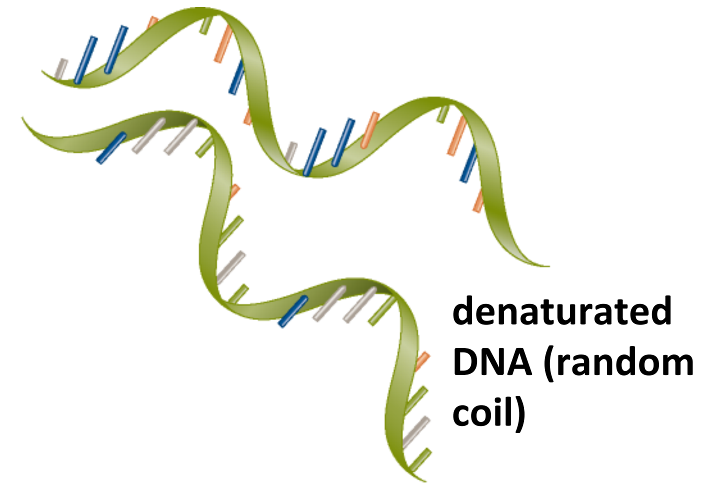
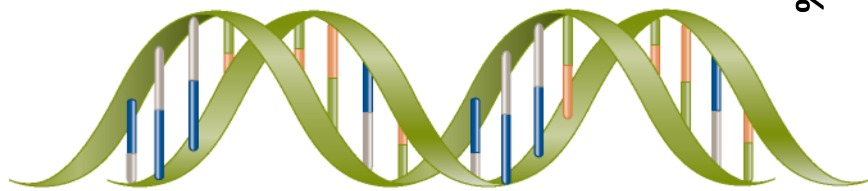
Methods for the study of nucleic acids (Southern blot, Northen blot and PCR)

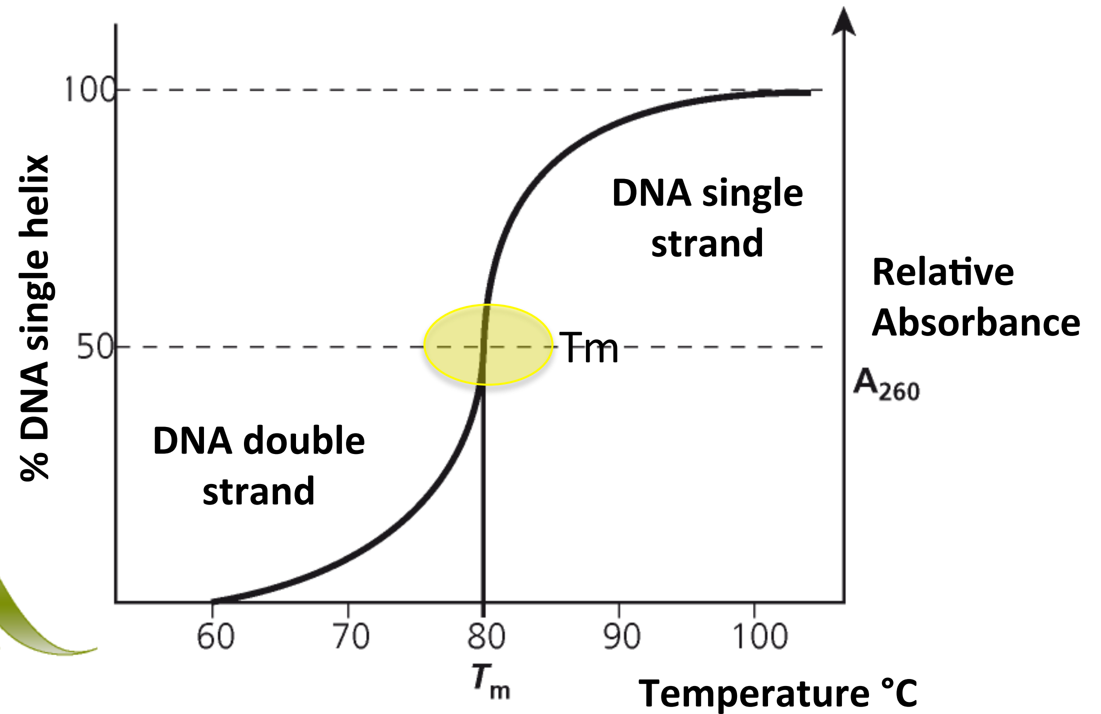
The nucleic acids: DNA and RNA

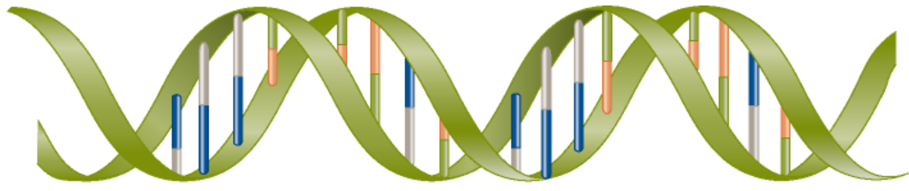


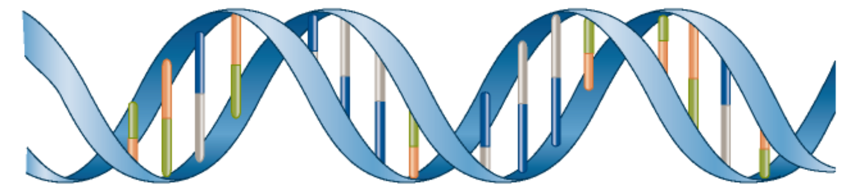
DNA can be denaturated and renaturated





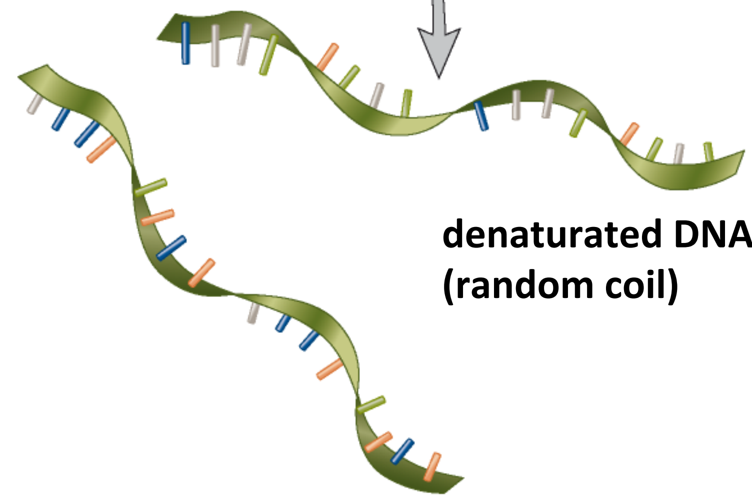






Native DNA (double helix)

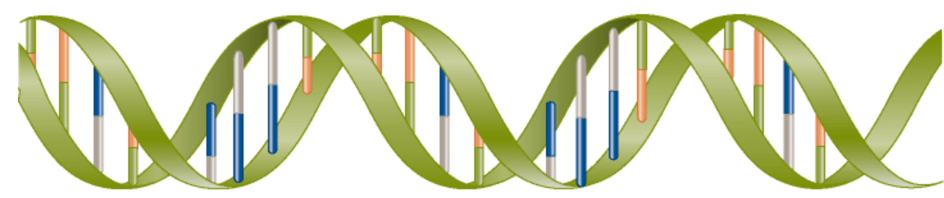
Temperature, OH^-, formamide

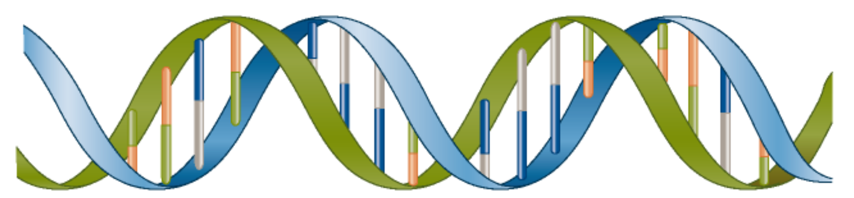


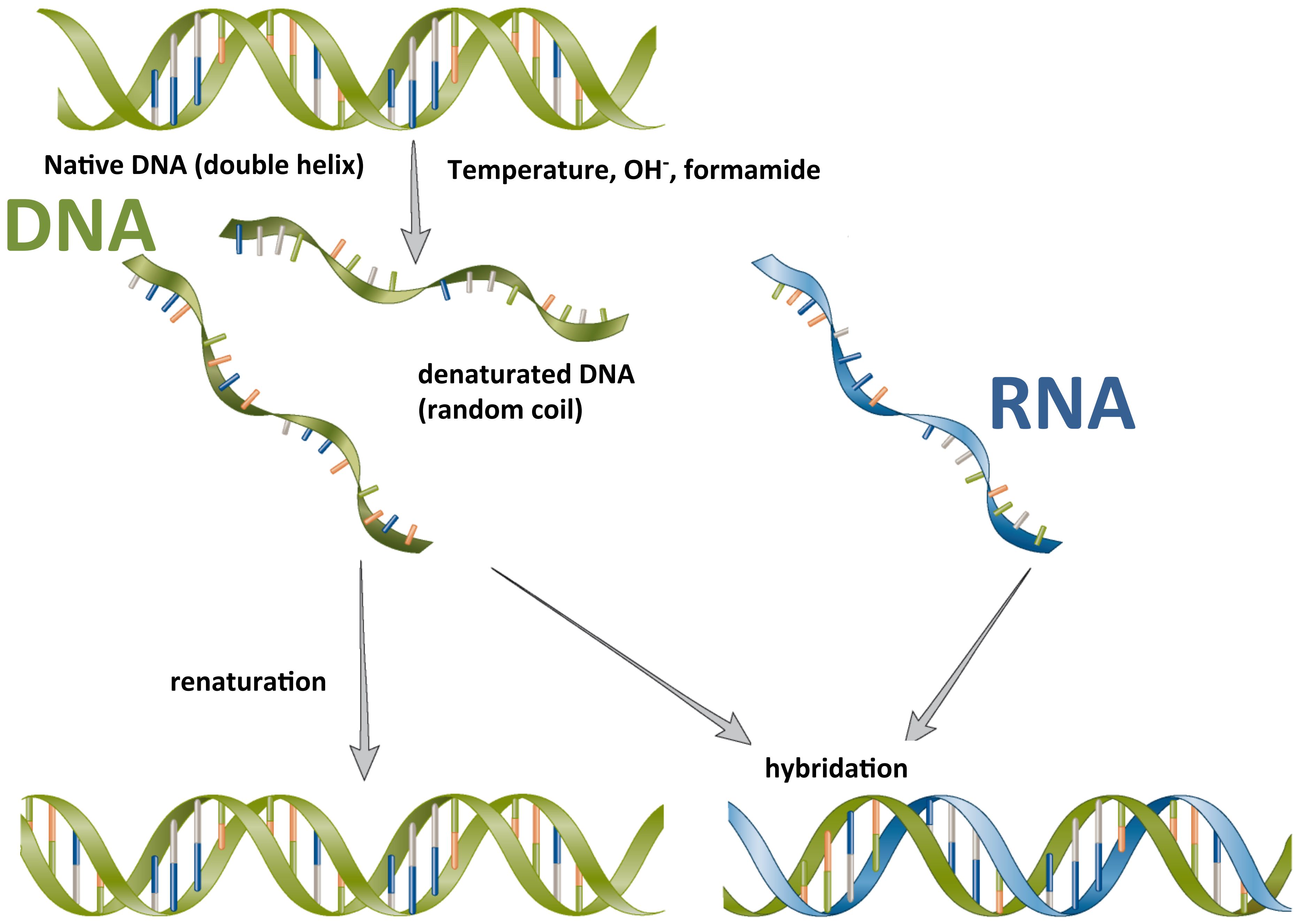
denaturated DNA
(random coil)

renaturation

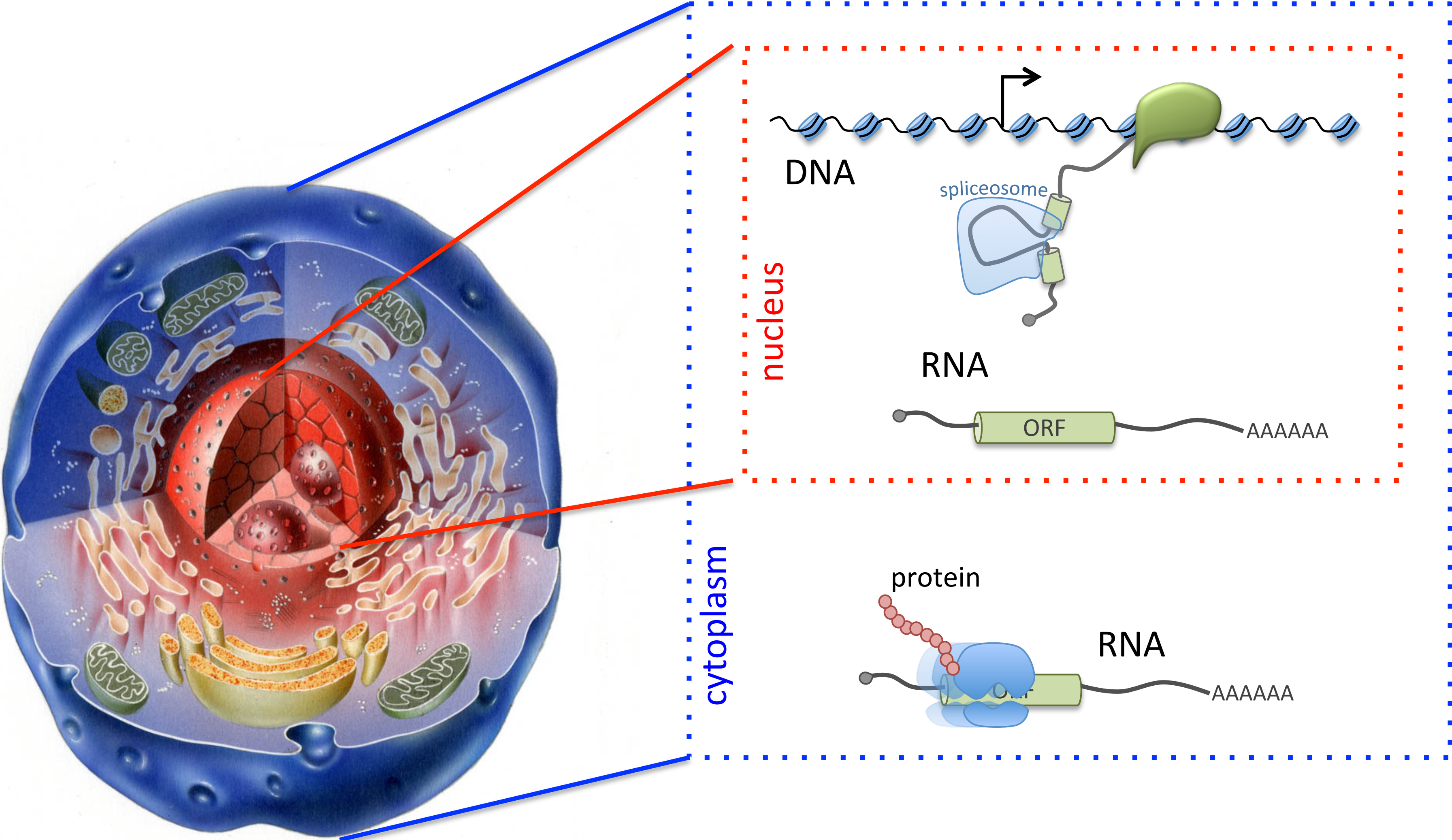
hybridation






Native DNA (double helix)
Temperature, OH-, formamide
DNA
denaturated DNA
(random coil)
RNA
renaturation
hybridation

Nucleic acid subcellular localization



Eukaryotic cell

Genomic DNA isolation

Organic extraction methods

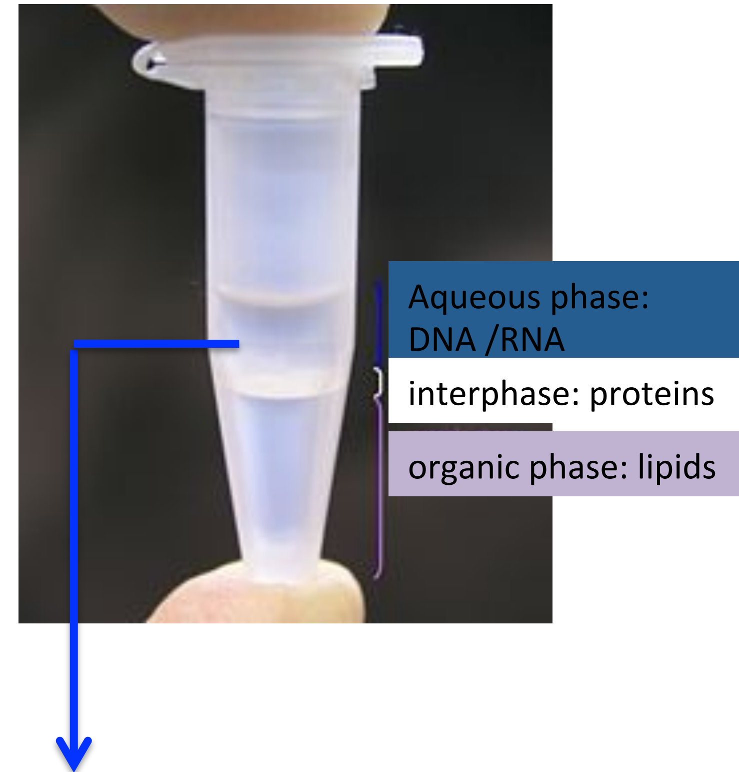
The cells are lysed using a detergent, and then mixed with phenol, chloroform, and isoamyl alcohol.

Invert several times to mix

Centrifuge

Transfer as much of each aqueous layer as possible to new tubes

Residual protein and RNA contamination can be eliminated by proteinase K and Rnase treatment



Precipitation by NaAC and Ethanol addition

Note:

- The pH of the mixture determines which nucleic acids get purified. Under neutral conditions (pH 7-8), both DNA and RNA partition into the aqueous phase.

RNA isolation

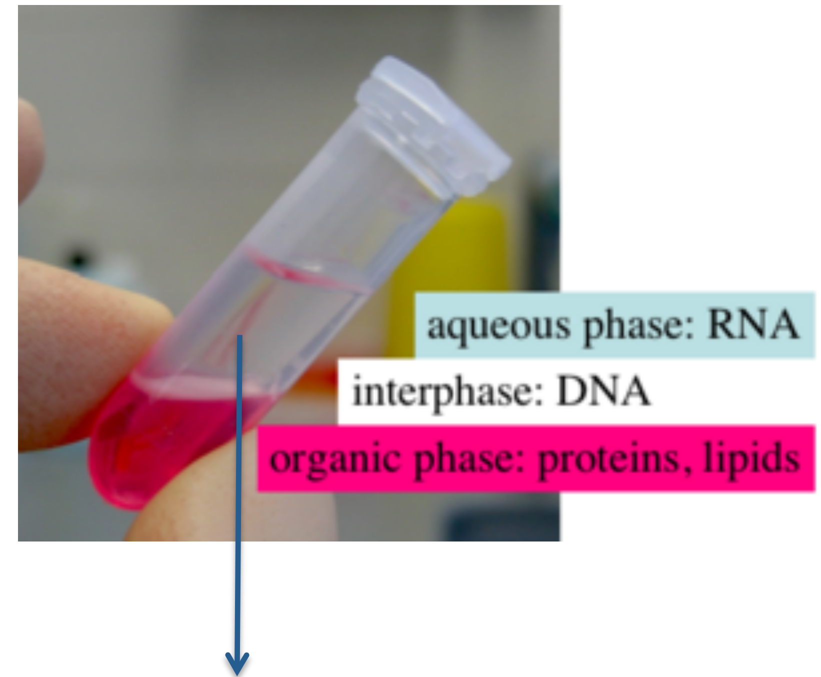
Phenol/Chloroform Extraction
or
Guanidinium thiocyanate-phenol-chloroform extraction

Add an equal volume (0.5 mL) of water-saturated phenol:chloroform:isolamyl alcohol (25:24:1)

Invert several times to mix

Centrifuge

Transfer as much of each aqueous layer as possible to new tubes



Precipitation by LiCl and Ethanol addition

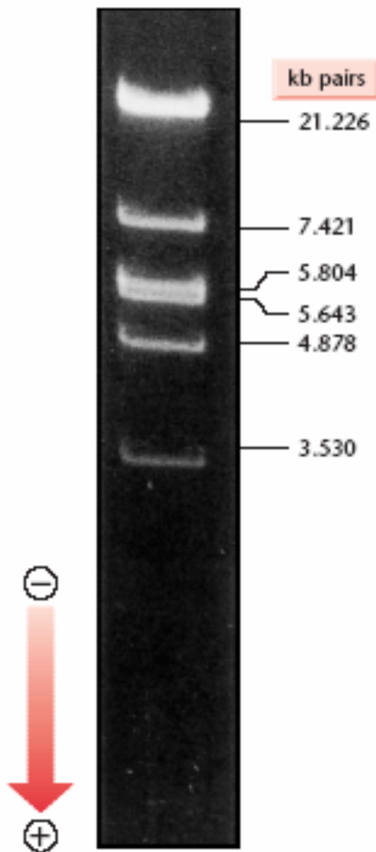
Note:

- The pH of the mixture determines which nucleic acids get purified. Under acidic conditions (pH 4-6), DNA partitions into the organic phase while RNA remains in the aqueous phase.
- Guanidinium thiocyanate denatures proteins, including RNases
- LiCl is preferred to NaAc becouse it precipitates DNA ineficiently leading to more pure RNA pellet

Electrophoresis of nucleic acids

Electrophoresis of nucleic acids

Electrophoresis is a biochemical technique which allows to separate molecules with charge based on their different molecular weight. The electrophoretic separations is carryed out through a gel.

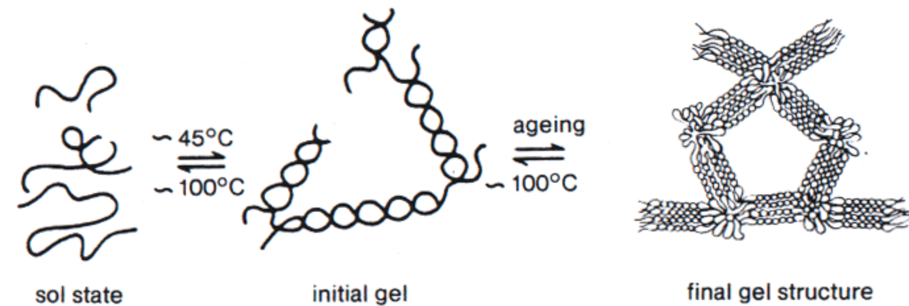


The gel can be made by:

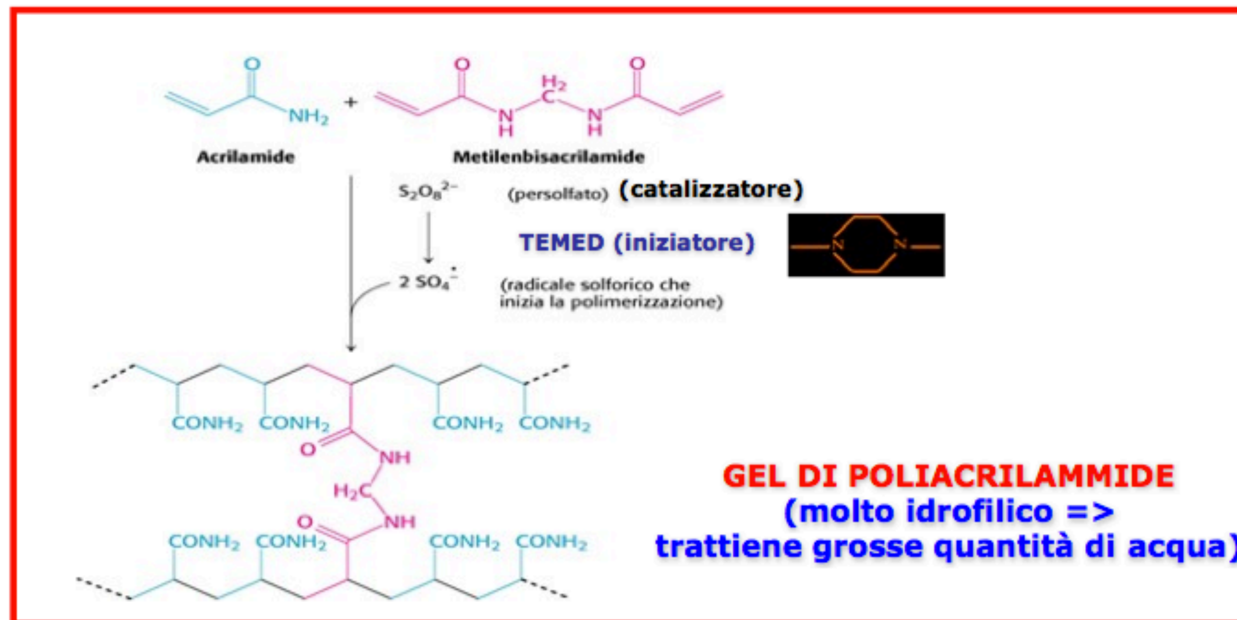
✔**agarose** (separation range : 0.5-20 kb)

✔**polyacrylamide** (separation range 10-500 bp)

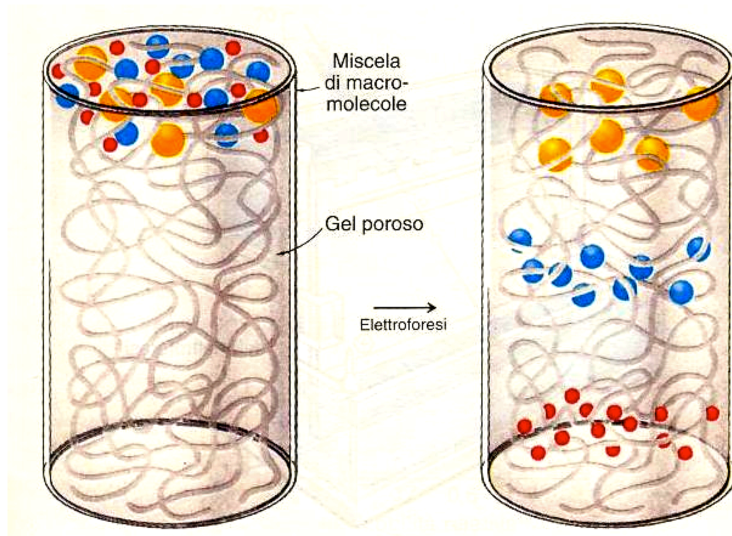
The agarose is a polysaccharide composed of D-galactose and 3,6-anhydro-L-galactose. residues

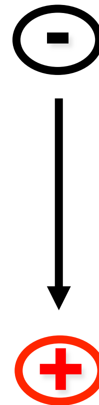


The polyacrylamide gel is formed by the copolymerization of acrylamide and of an agent which forms crosslinks (usually N, N'-methylene bisacrilamide) to form a three-dimensional lattice



Sieve effect





For linear fragments of DNA and/or RNA the migration distance is inversely proportional to the size of the molecule (that corresponds to its length in bases)

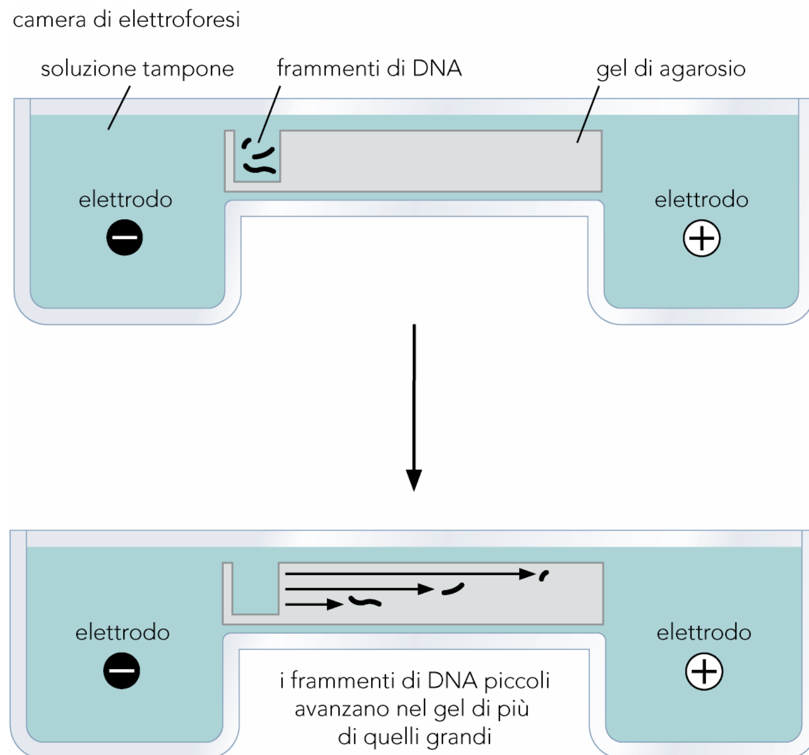
Electrophoresis

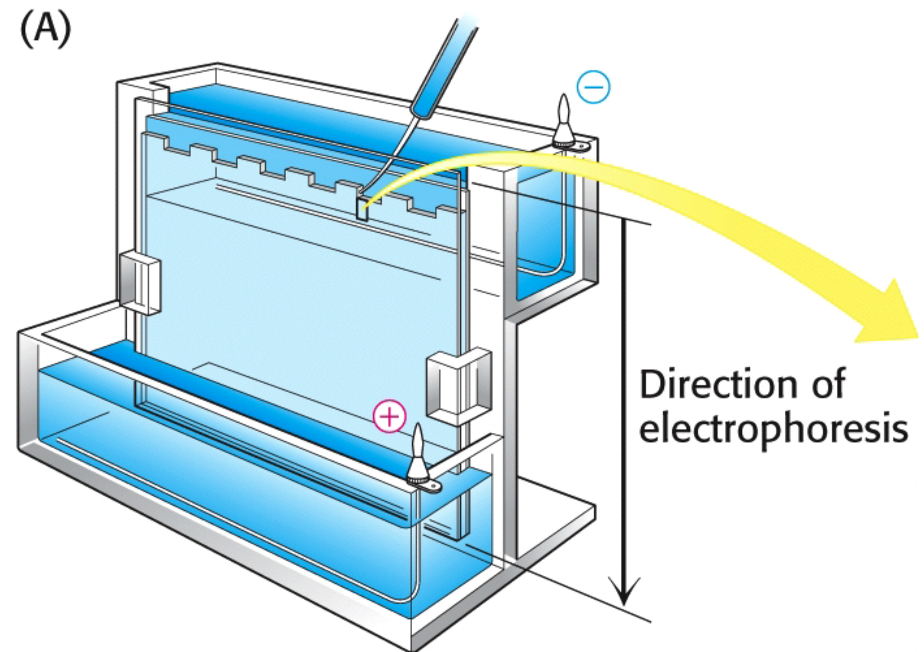
✔ horizzontal

agarose

✔ vertical

acrylamide

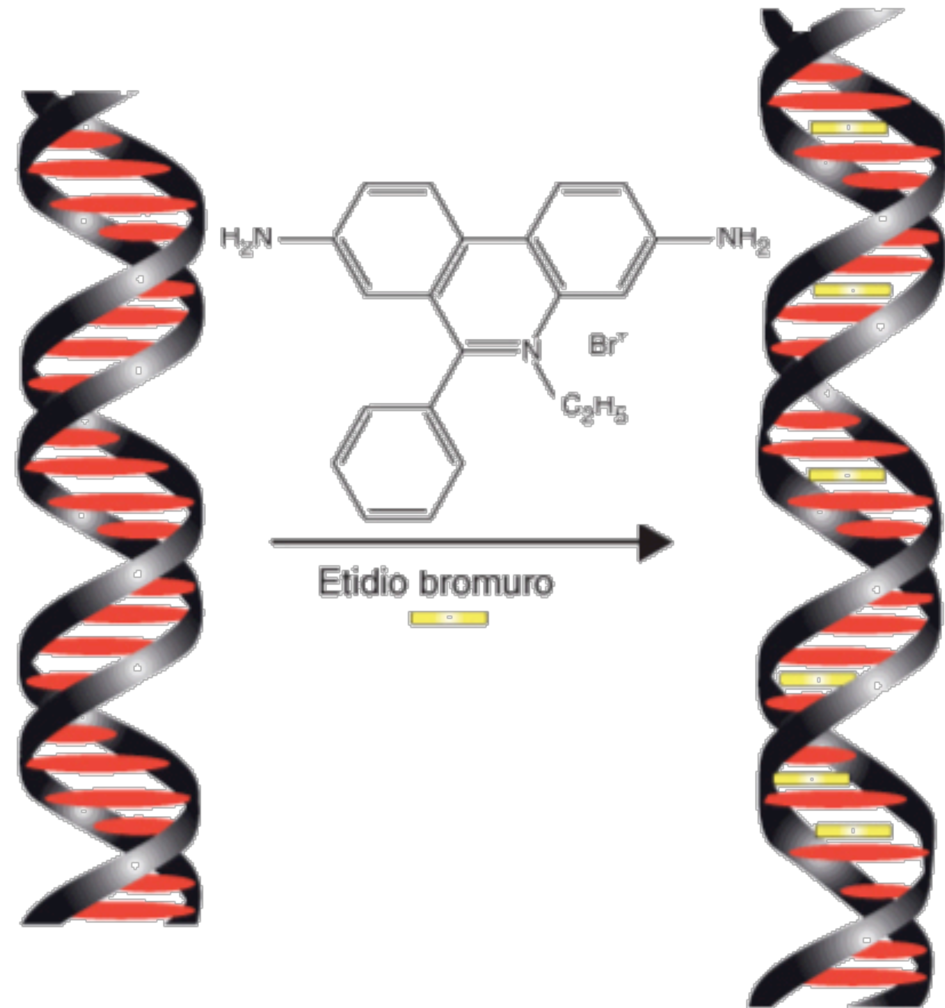




ethidium bromide (EtBr):

fluorescent dye that absorbs UV light at 300 nm resulting yellow-orange

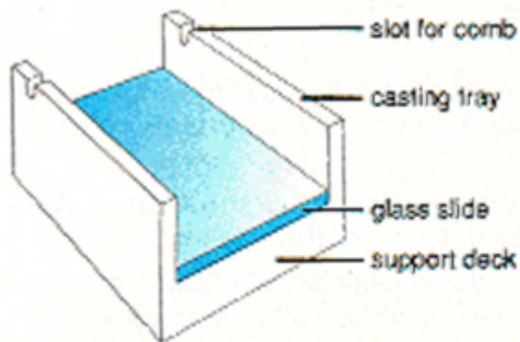
Useful both for visualise and quantify the sample: the intensity of the fluorescence is, in fact, proportional to the amount of the sample.



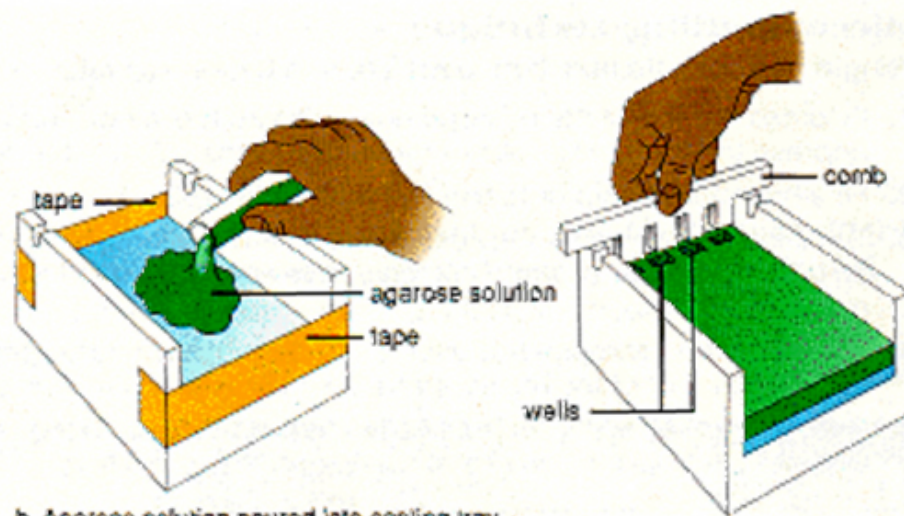
How to prepare agarose gel:

dissolve the agarose powder in a buffer solution at 100°C

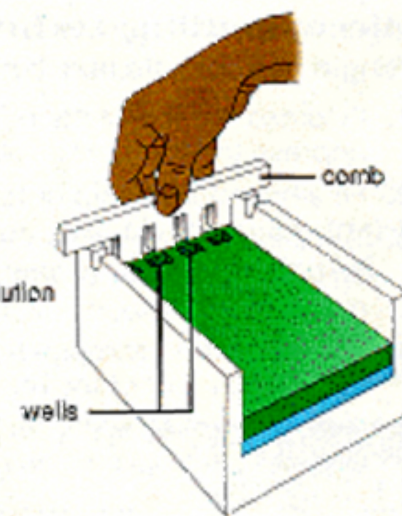
add ethidium bromide



a. Casting tray for making gel slab

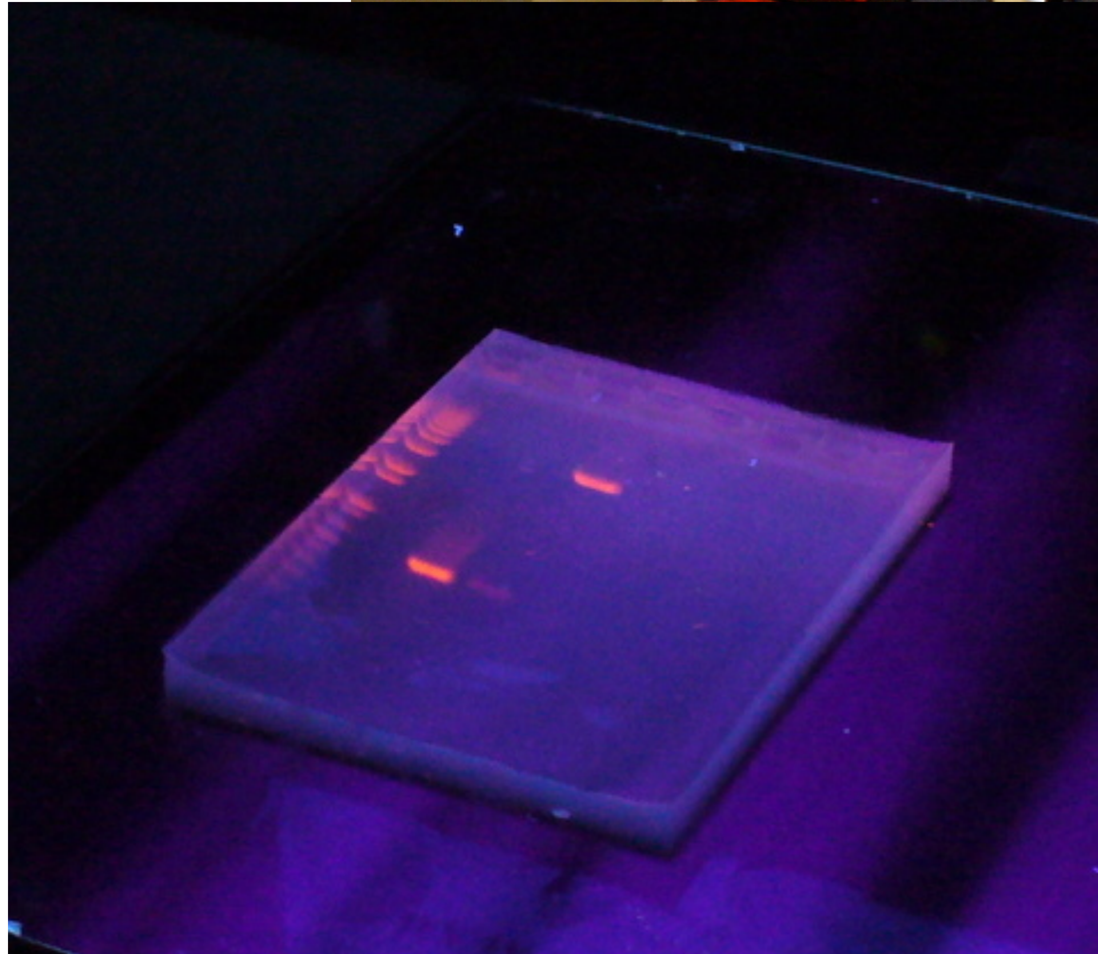


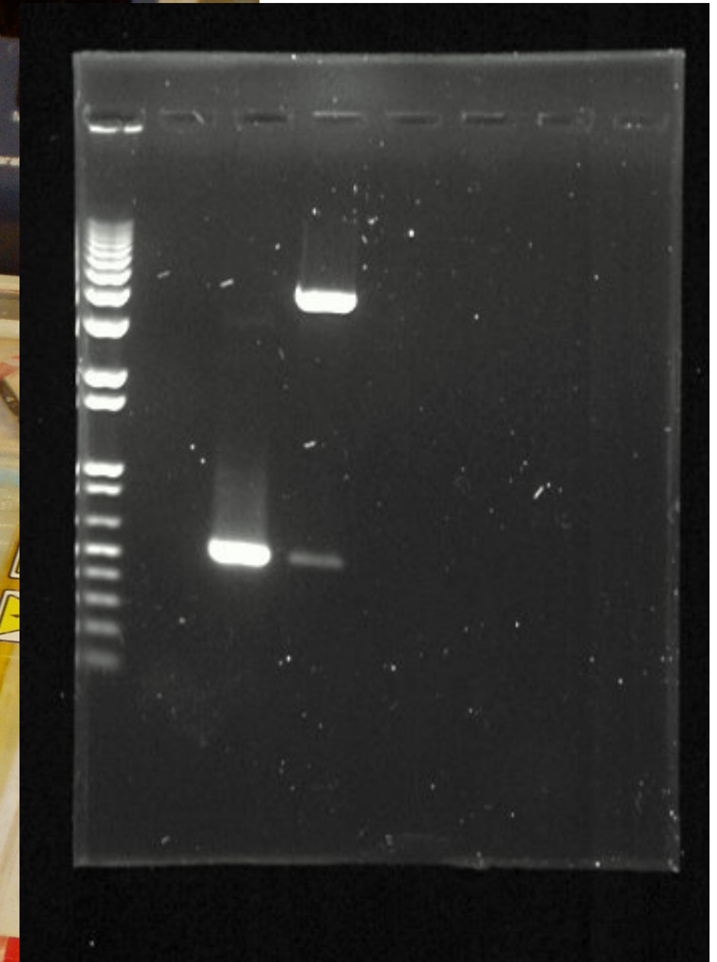
b. Agarose solution poured into casting tray

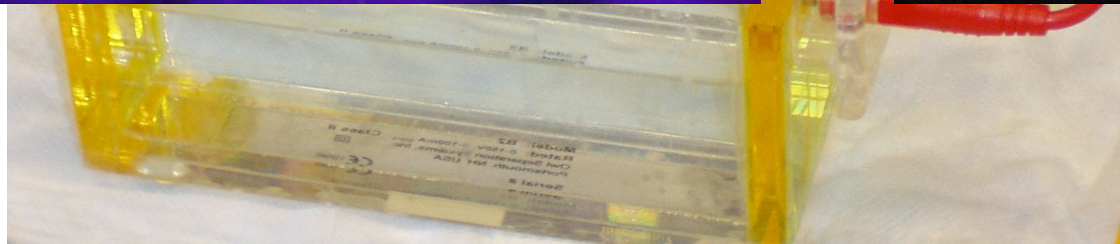


c. Comb that forms

EC 105
Check
Fuse
WARNING

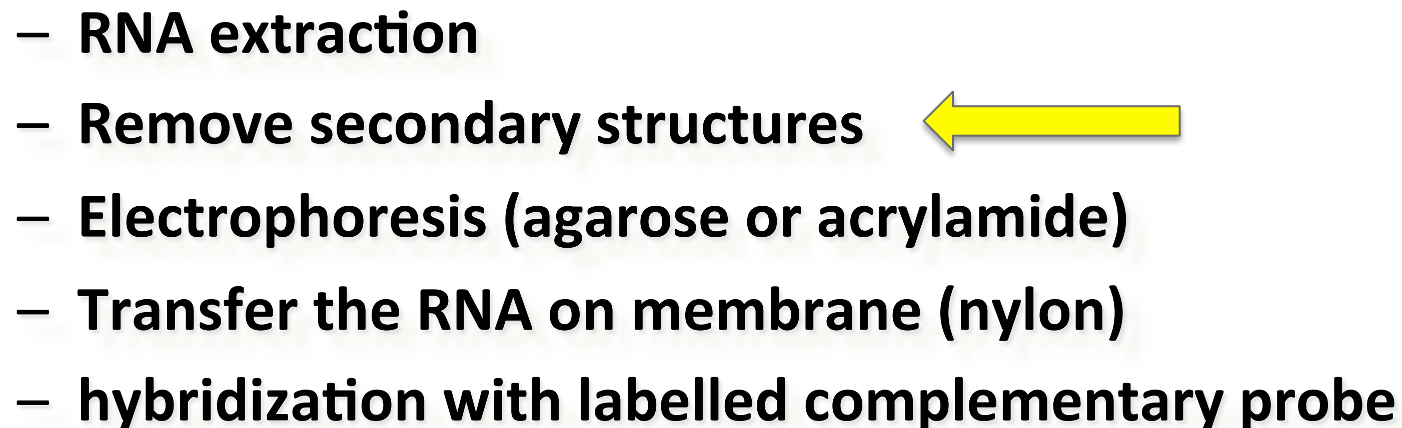




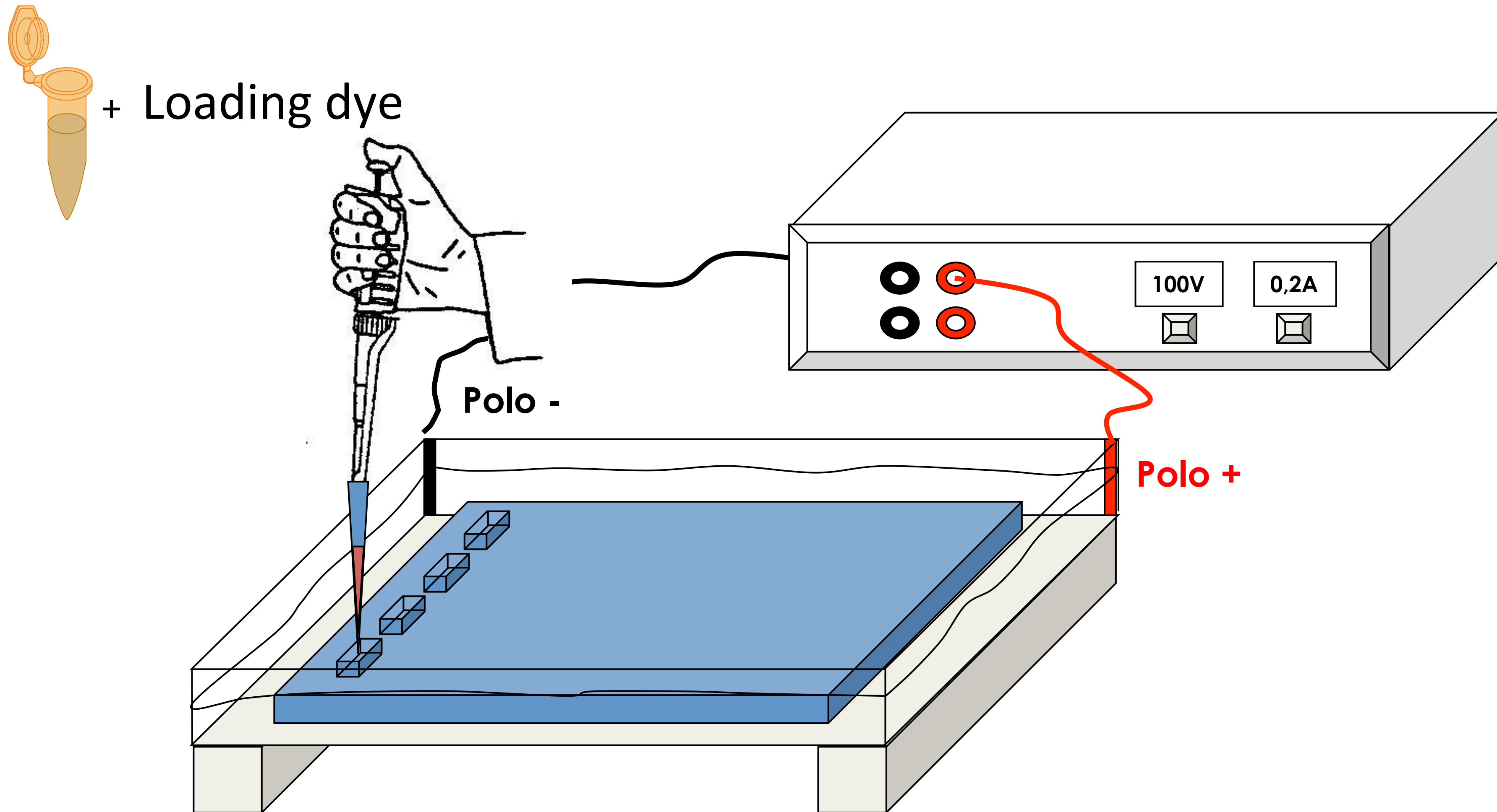


Northern blot

- **Procedure**

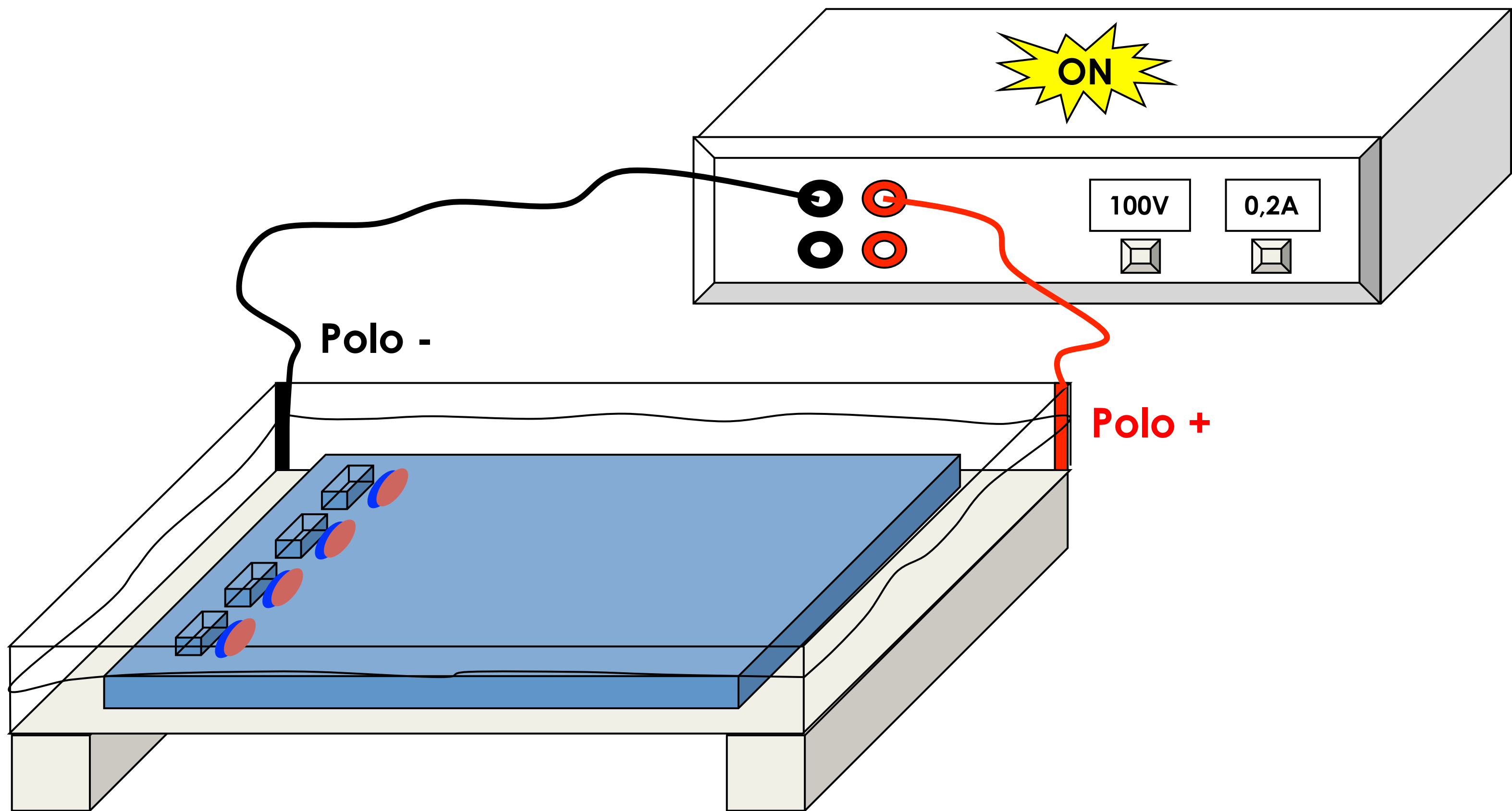


  - RNA extraction
  - Remove secondary structures
  - Electrophoresis (agarose or acrylamide)
  - Transfer the RNA on membrane (nylon)
  - hybridization with labelled complementary probe

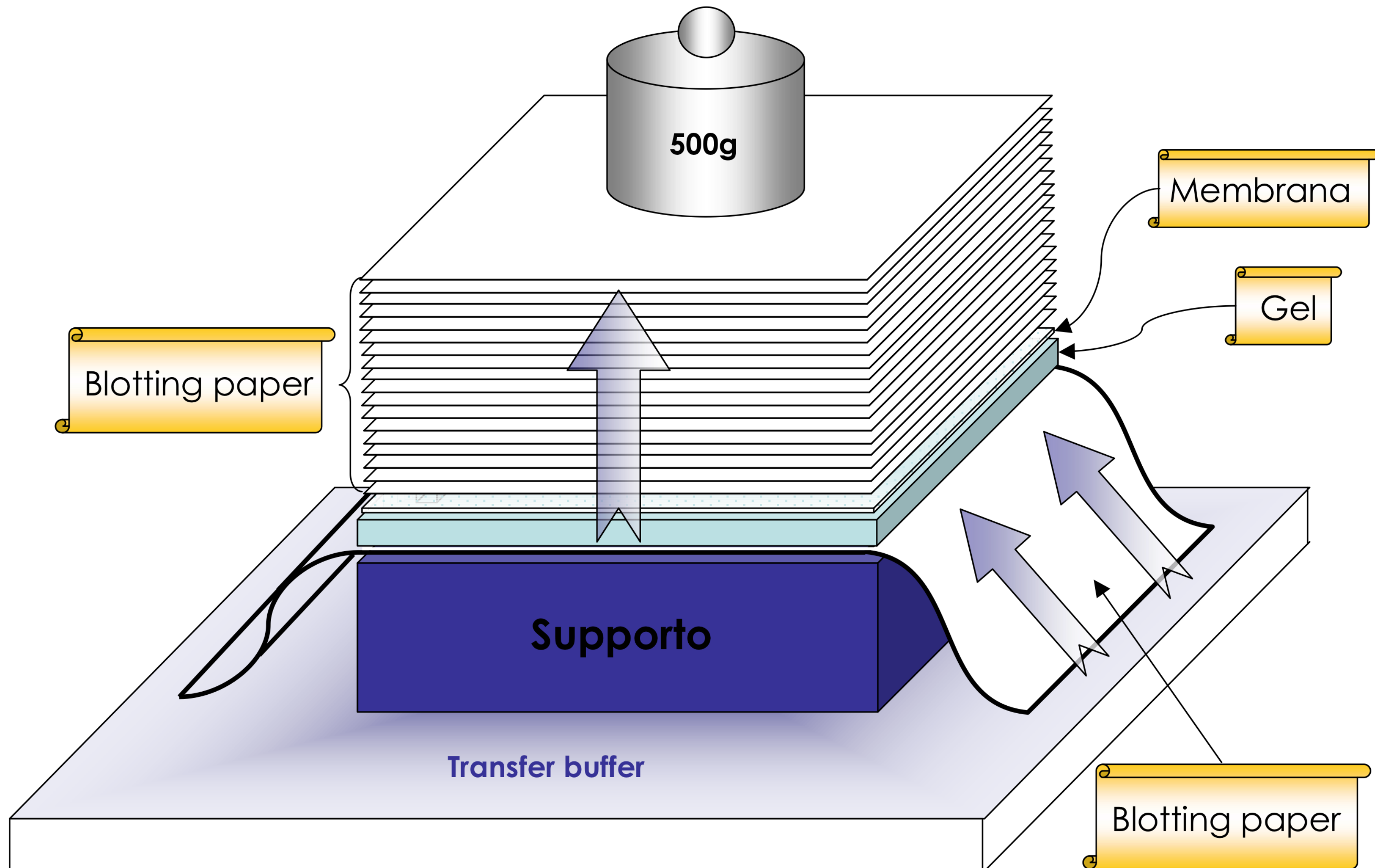


Loading dye helps the sample loading in the well

It contains glycerol, bromophenol Blu and xilencianol Blu that migrate in the gel at different speeds.


ON
100V
0,2A
Polo -
Polo +

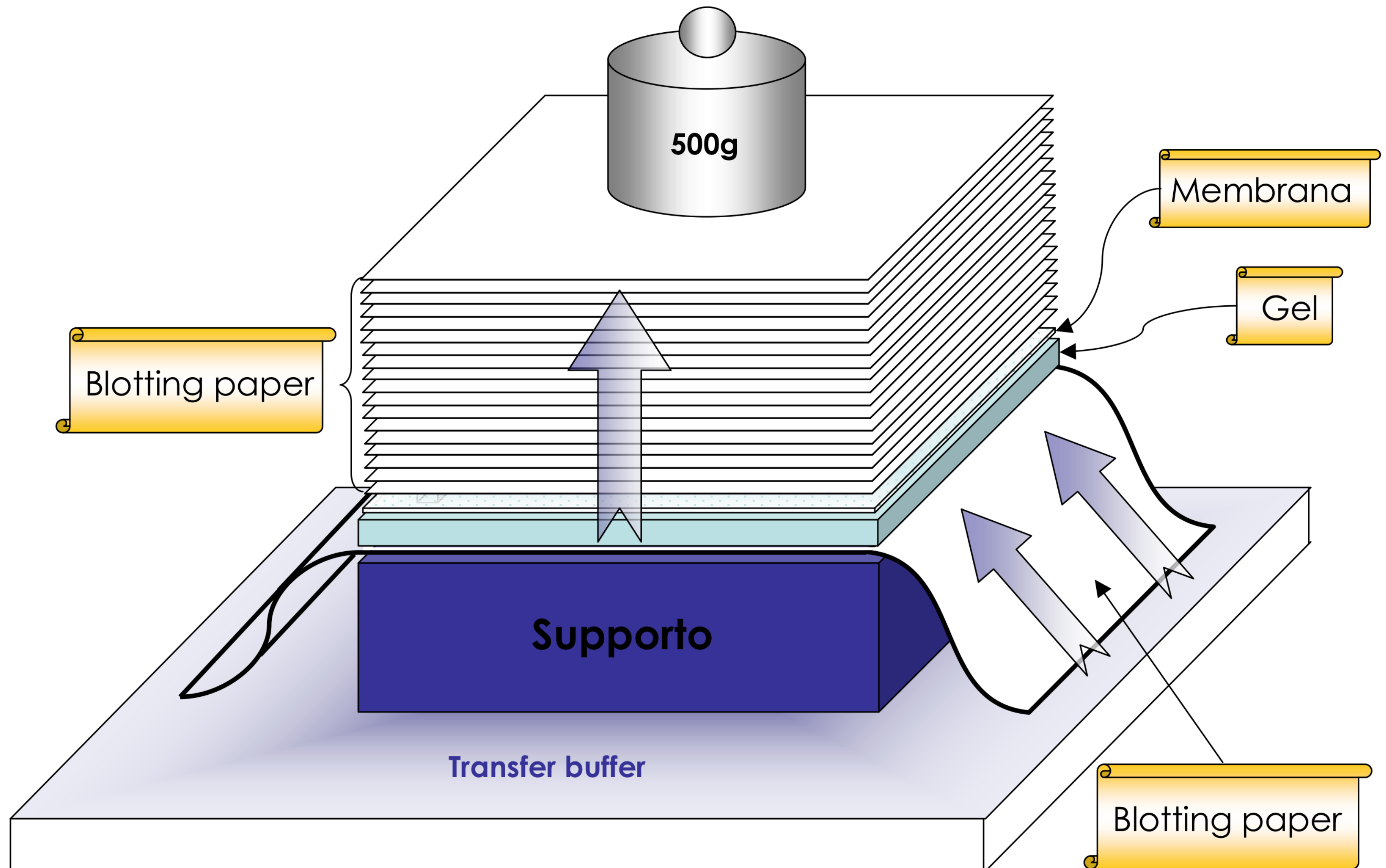
BLOT: transfer the nuclei acids from the gel to the membrane by diffusion (for agarose gel)



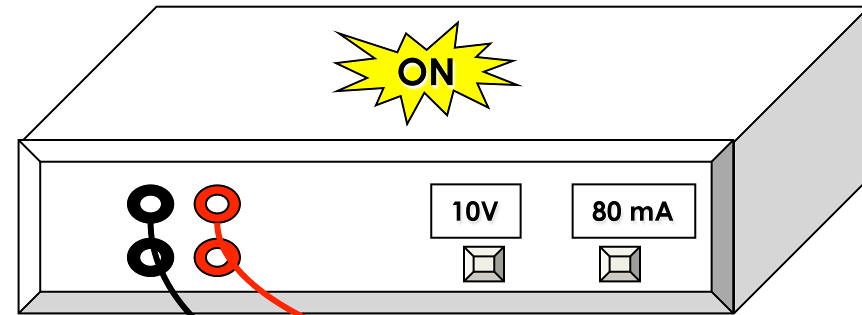
The nucleic acids will be fixed to the membrane by:

-UV irradiation for nylon membrane

-boiling for 2hrs at 80°C for cellulose nitrate membrane



Elettroblot
(for acrylamide gel)



Membrana (nylon)

gel

Buffer
TB 0.5X

blotting paper

Polo -

blotting paper

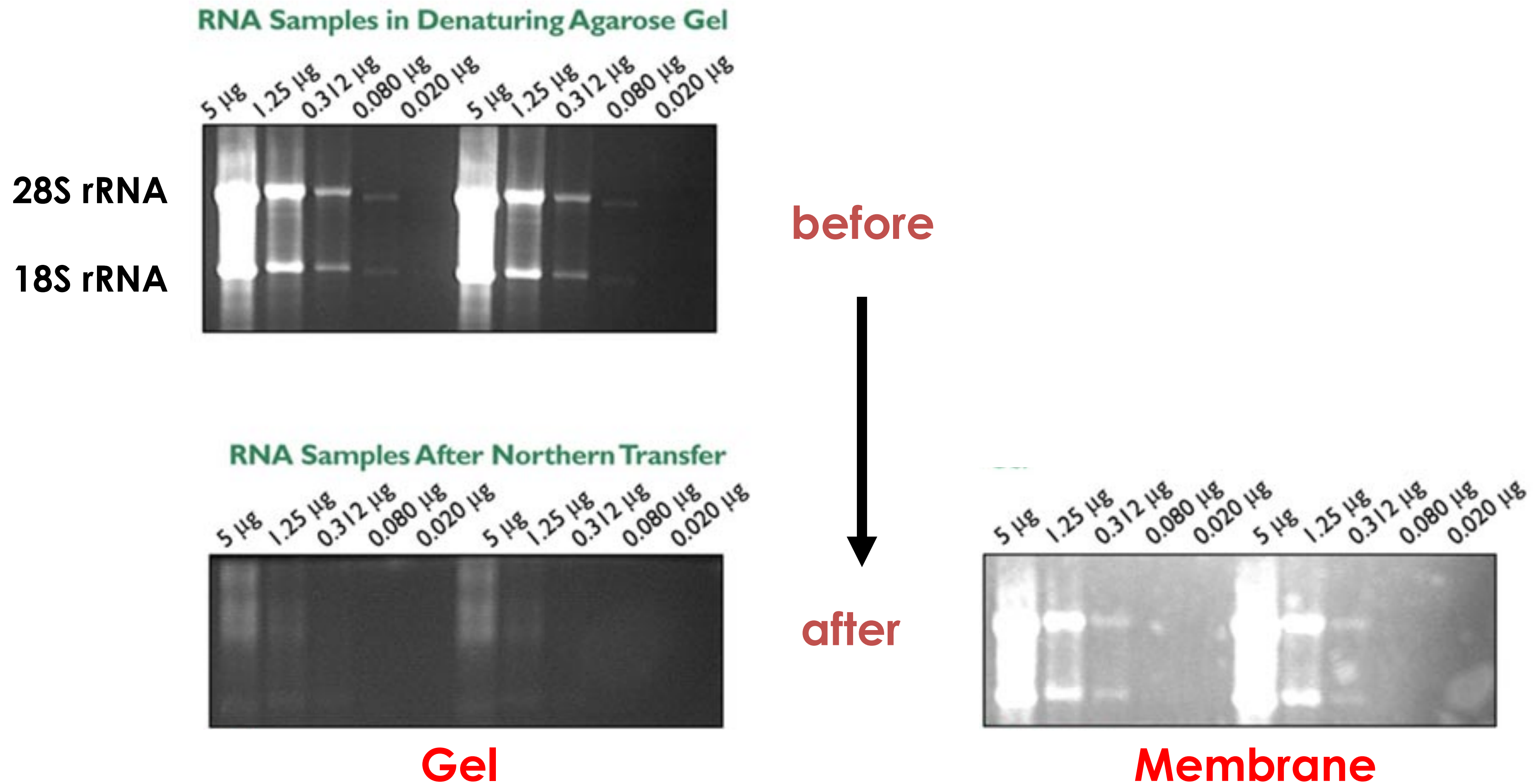
Polo +

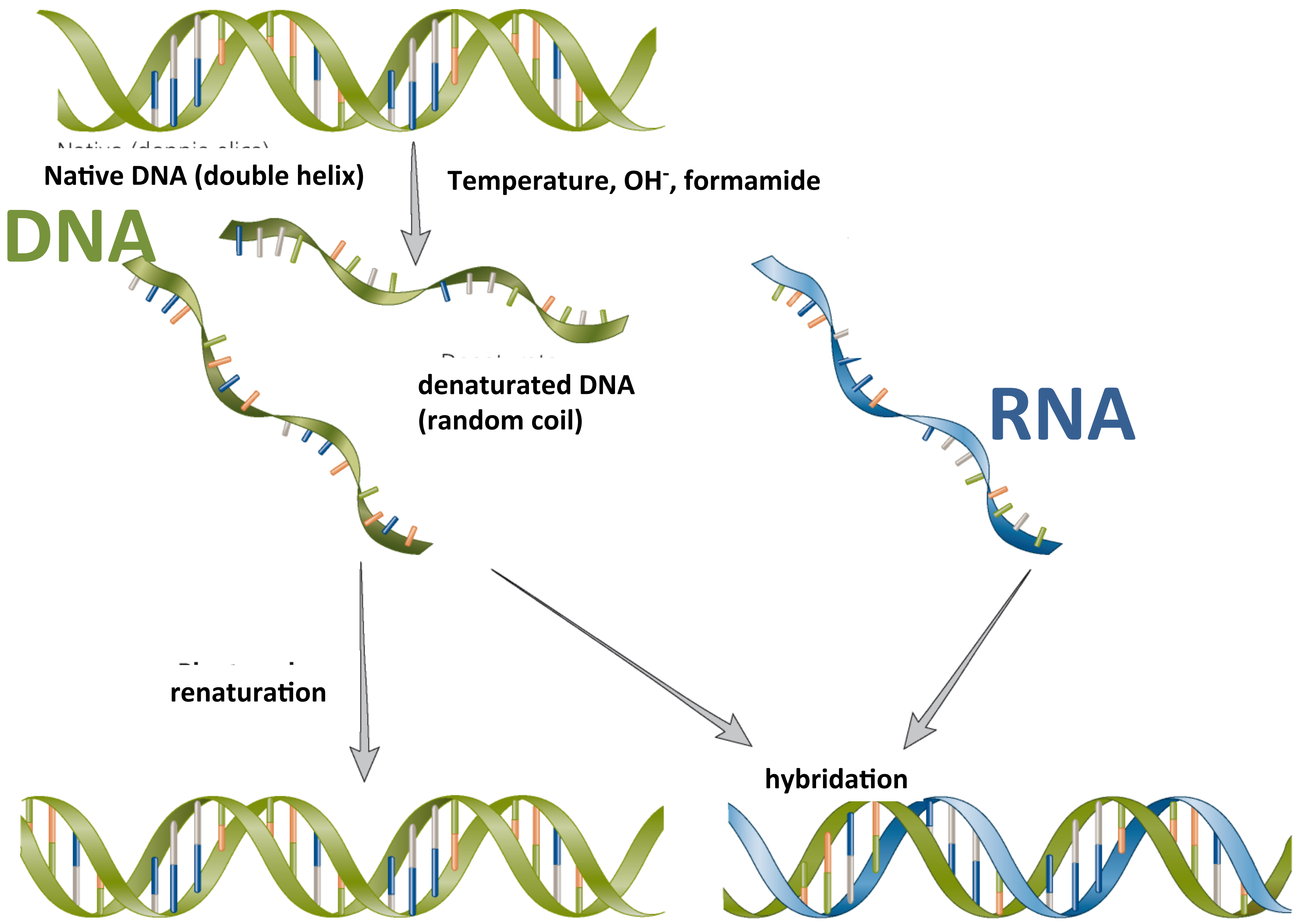
Membrana
(nylon)

gel
(acrilammide)

blotting paper

Ethidium bromide staining helps to check the proper RNA transfer on the membrane





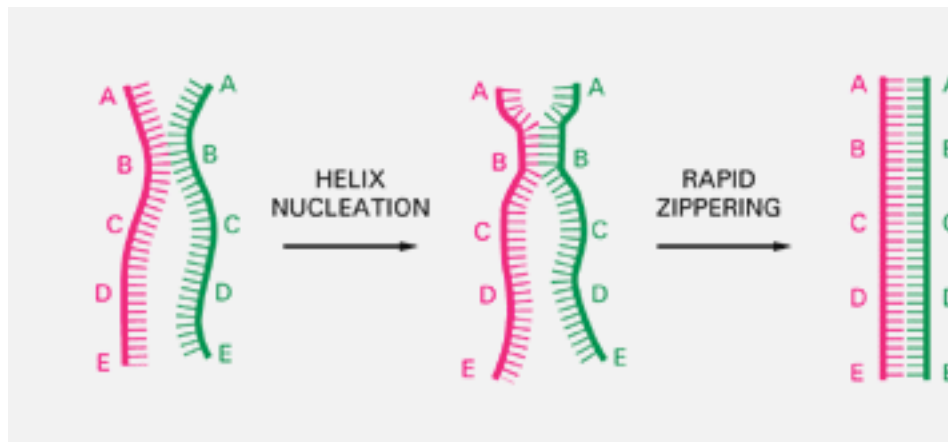
Native DNA (double helix)
Temperature, OH^-, formamide
DNA
denaturated DNA
(random coil)
RNA
renaturation
hybridation

How to generate labelled probes

Labelled probes are used to determine the presence and the amount of nucleic acids, they consists in a specific sequence complenentary to the DNA or RNA target in the biological sample analyzed.

The hybridisation consists in a pairing of single stranded molecules coming from two different sources:

- **DNA or RNA** targets (fixed on a membrane)
- **Probe** (labelled DNA o RNA with a specific sequence)

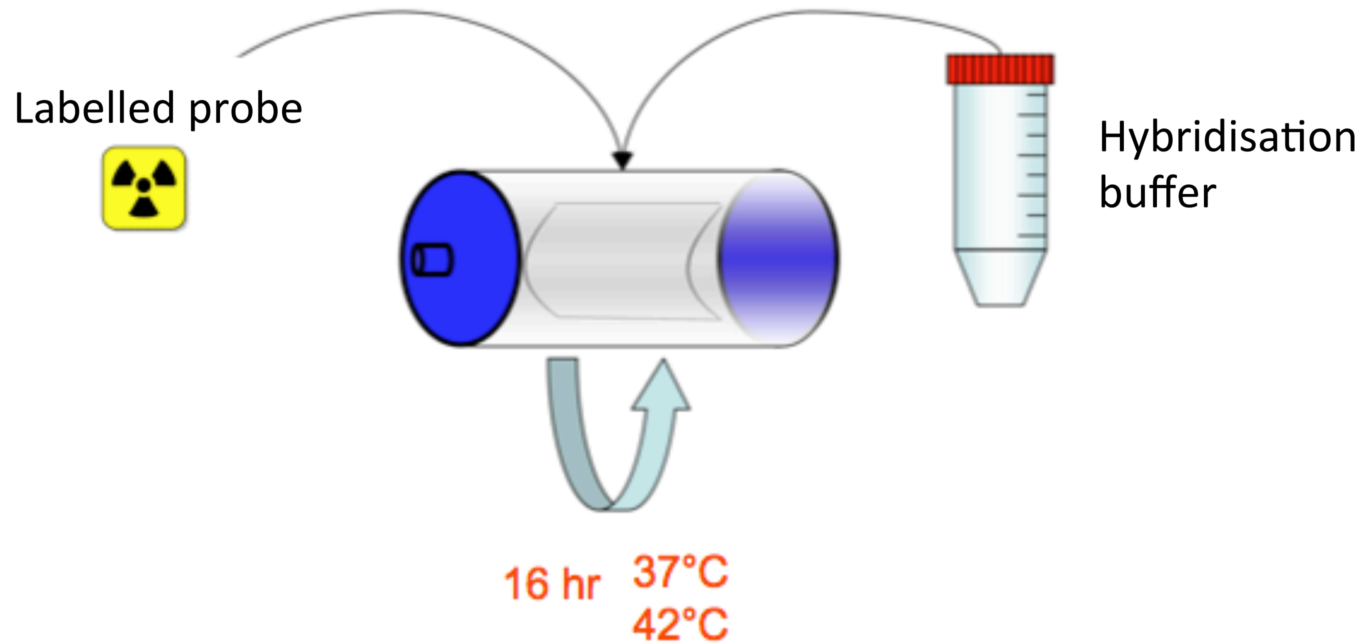


By hybridisation DNA-DNA, DNA-RNA o RNA-RNA double strands can be formed.

The hybridisation is an extremely specific molecular recognition strategy.

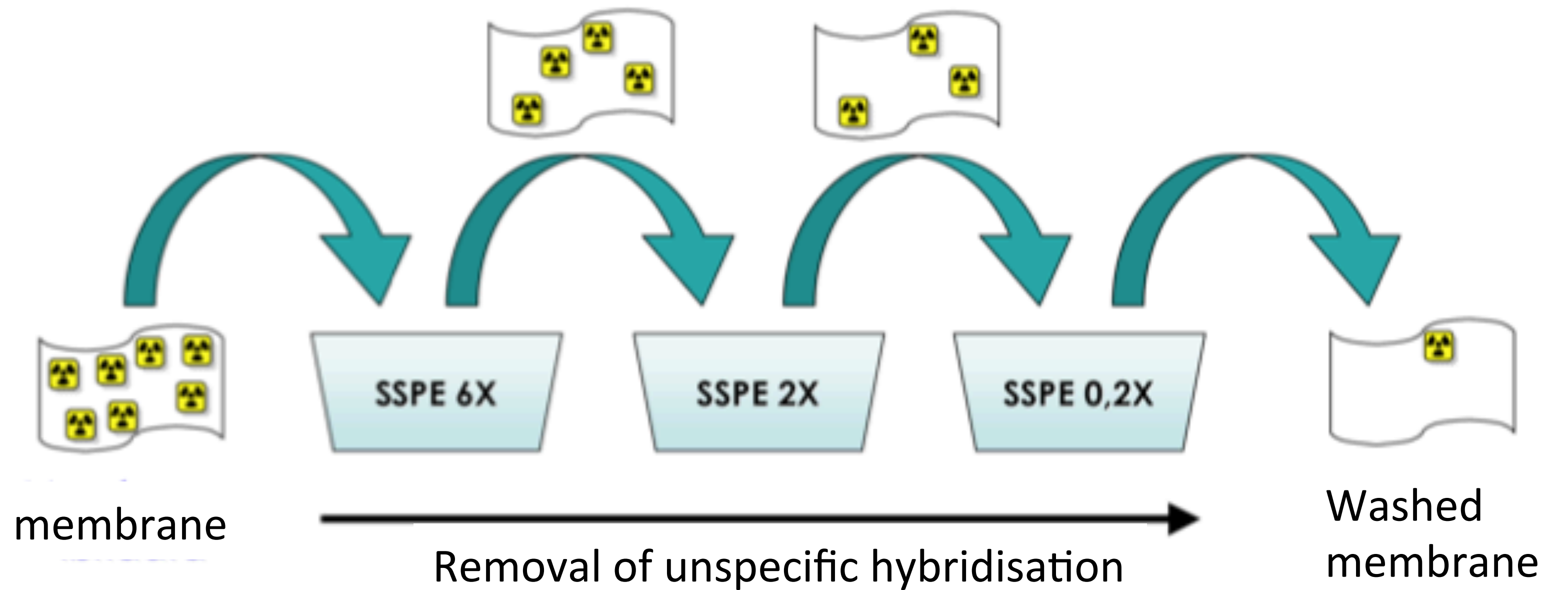
Hybridisation

A specific labelled probe is added to the membrane in the hybridisation tube for 16 hrs at 37-42 °C in the hybridisation buffer

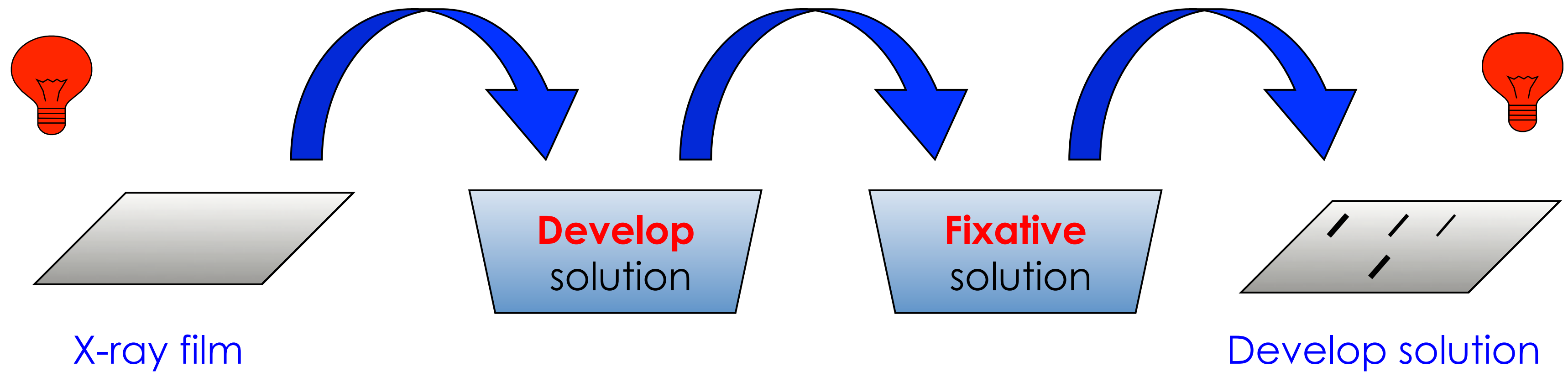


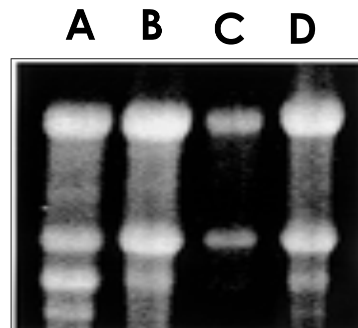
Washes

Several washes are needed in order to wash out the unspecific binding to the membrane. The unspecific hybridisation is not facilitated by high temperature and low salt. In this way will be removed the molecules weakly bound to the membrane

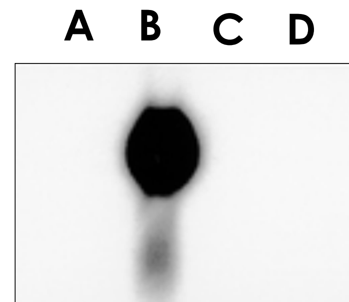


Detection of the signal





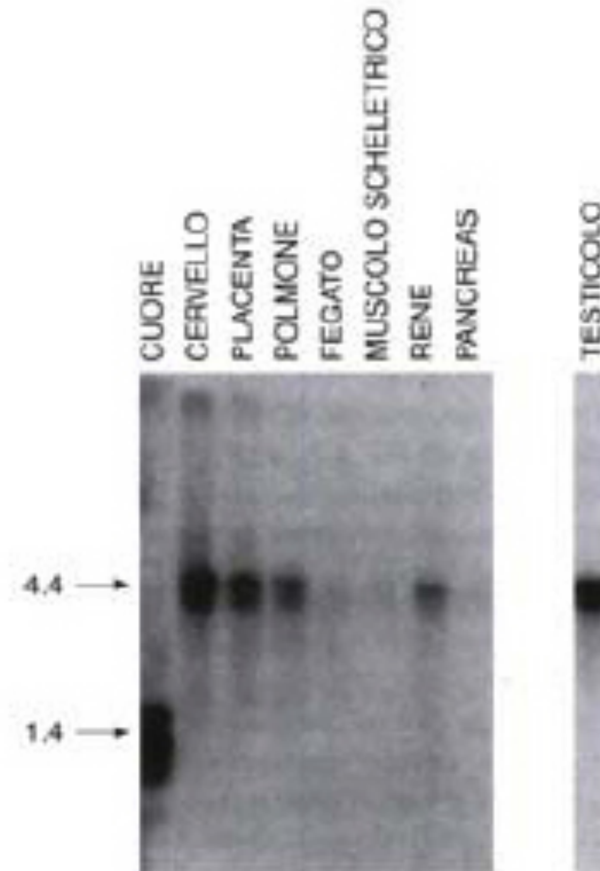
Gel



X-ray

Applications

1. In which tissue is the RNA expressed?



Many information will be obtained:

1- expression levels in many tissues
2- lenght of produced RNA

Southern blot

- **Procedure**

- Genomic DNA isolation

- Digestion with restriction enzymes for generating short fragments (from hundreds to some Kilo bases)

- Electrophoresis of DNA fragments on agarose gel

- Denaturation of complementary DNA filaments

- Transfer the DNA from the gel to the membrane

- Hybridation with specific labelled probe

Southern blot

Target GENE

Genomic DNA

Restriction Endonuclease

DNA Fragments

Gel electrophoresis

Long fragments

Short fragments

Direction of migration

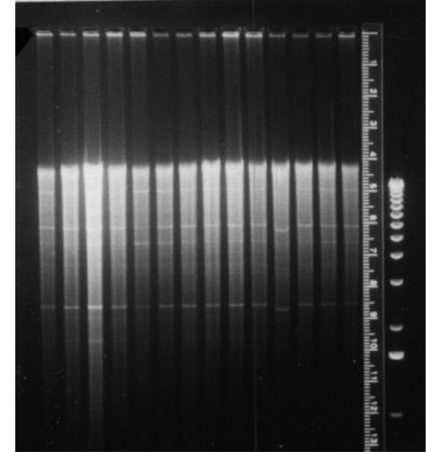
- denature in alkali
- transfer to nitrocellulose
- bake

SS DNA fragments

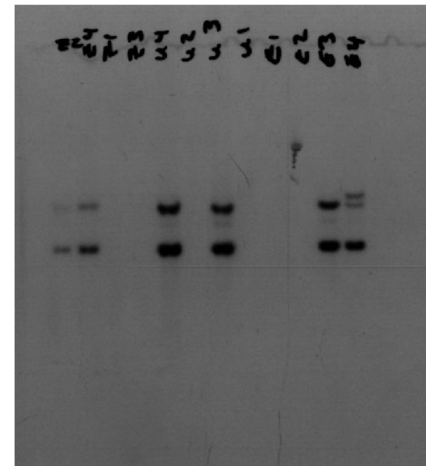
- hybridize with radiolabelled probe
- wash

Autoradiogram

Gel



X-ray film



The labelling is a basic technique of molecular biology, represents a preliminary stage for applications related to the study of *gene expression*.

The labelling allows to determine the position of a particular nucleic acid molecule on a membrane or on a *gel*, on a chromosome, within a tissue or in a cell.

The labelling produces a signal that can be appropriately detected and allows the viewing of the specific nucleic acid molecule

Making labelled probes

- ✔ Radioactive tracer
- ✔non radioactive tracer
  (Fluorescence, Chemioluminescence)

- ✔ Terminal labelling
- ✔ internal labelling

- ✔ DNA probe
- ✔ RNA probe

✔ Radioactive tracer

benefits: high sensitivity

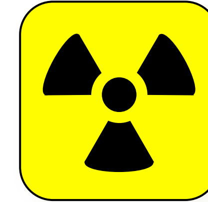
disadvantages: dangerous because mutagenic
short life

✔ non radioactive tracer

benefits: safety
long life

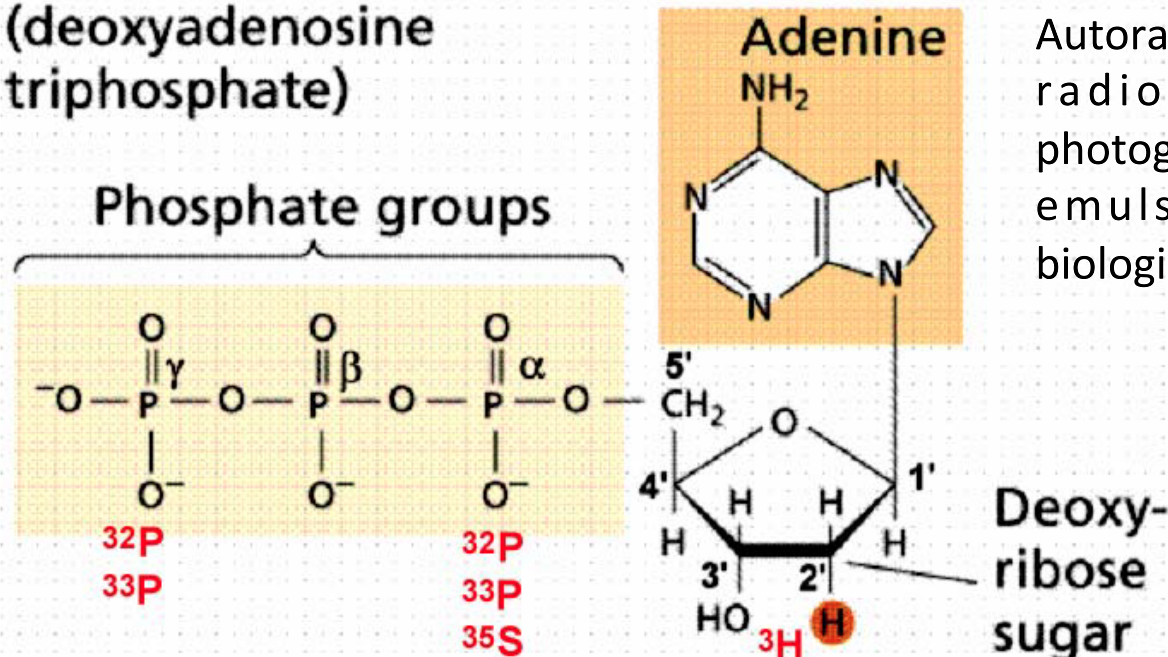
disadvantages: lower sensitivity

Labelling nucleotides with radioisotopes



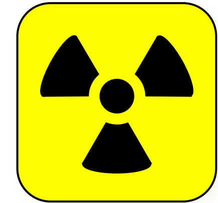
To label the DNA are used radioisotopes that become part of the nucleotides.
It is possible to synthesize nucleotides in which:

- A phosphorus atom is replaced with ^{32}P or ^{33}P
- An oxygen atom of the phosphate group is replaced with ^{35}S
- One or more H atoms are replaced with ^{3}H



Autoradiography is a means of detecting radioactive compounds with a photographic emulsion. The form of emulsion favored by molecular biologists is a piece of x-ray film.

Nucleotides Labelled with radioisotopes



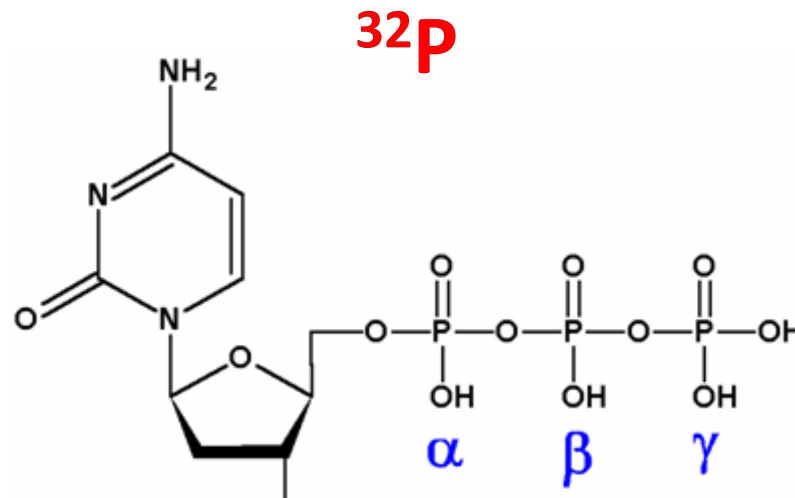
The radioactive atom in a labelled nucleotides or the can be:

- Added to one end of the probe (external labelling)

or

- Inserted into the nucleic acid chain (internal labelling).

^{32}P



dNTP labelled at position α (dATP e dCTP)

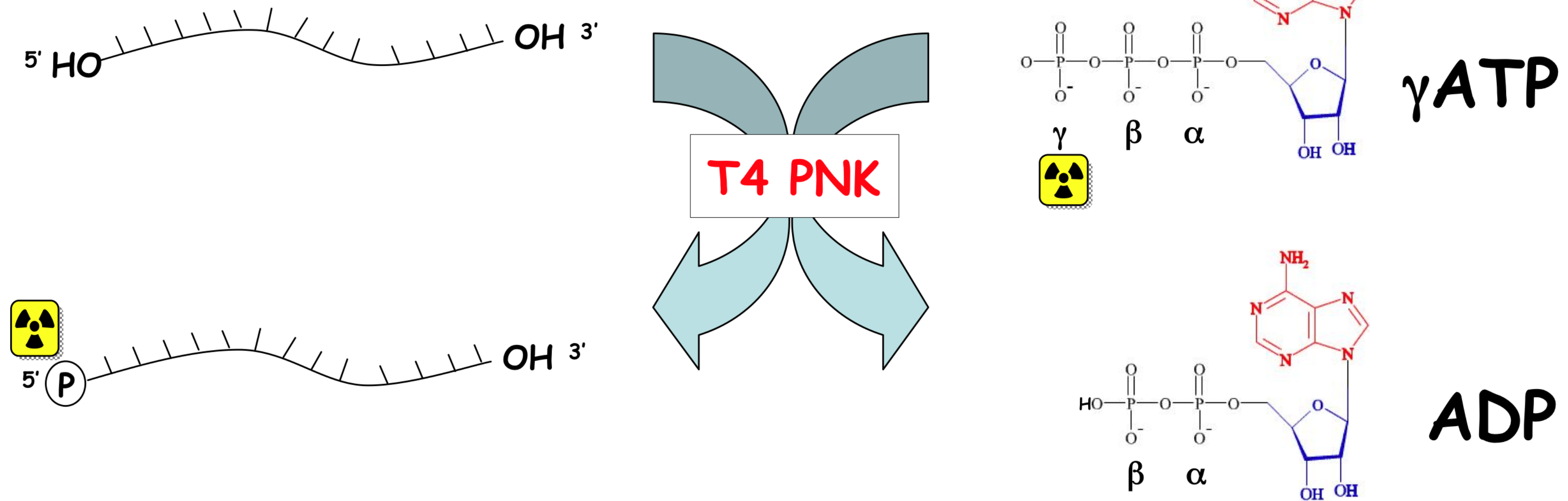
NTP labelled at position γ (ATP) e α (ATP, UTP, CTP e GTP)

DNA/RNA Oligonucleotides probes can be terminally labelled with radioactive tracer

Double stranded DNA probes can be internally labelled with radioactive tracer

RNA probes can be in vitro transcribed and internally labelled with radioactive tracer

How to label DNA/RNA oligonucleotides



T4 Kinase enzyme is employed to transfer a radioactive phosphorus atom from γ ATP to the 5' end of the DNA/RNA oligonucleotides

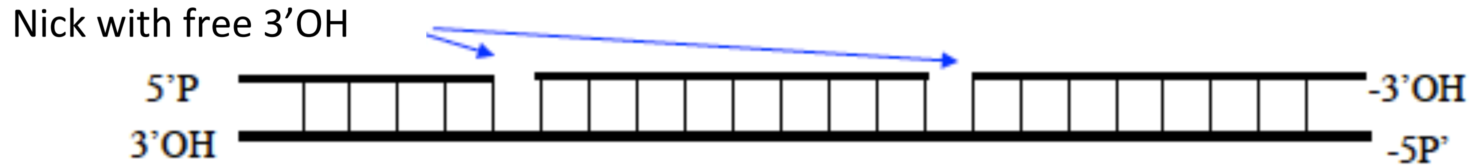
How to label DNA molecules:

Nick translation

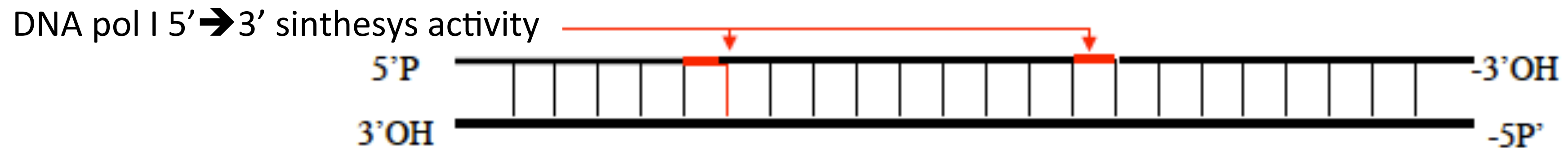
Random priming

Nick translation

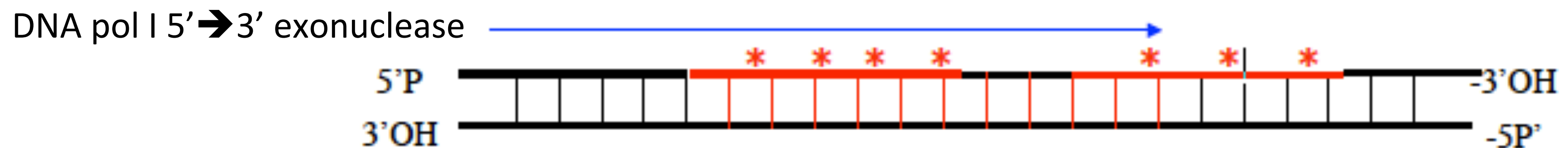
DNasi I breaks the phosphodiester bond and creates a nick on one strand.



DNA polymerase I synthetises a new labelled DNA strand starting from the free 3'OH



DNA polymerase I exonuclease activity removes nucleotides from the 5' toward the 3' end

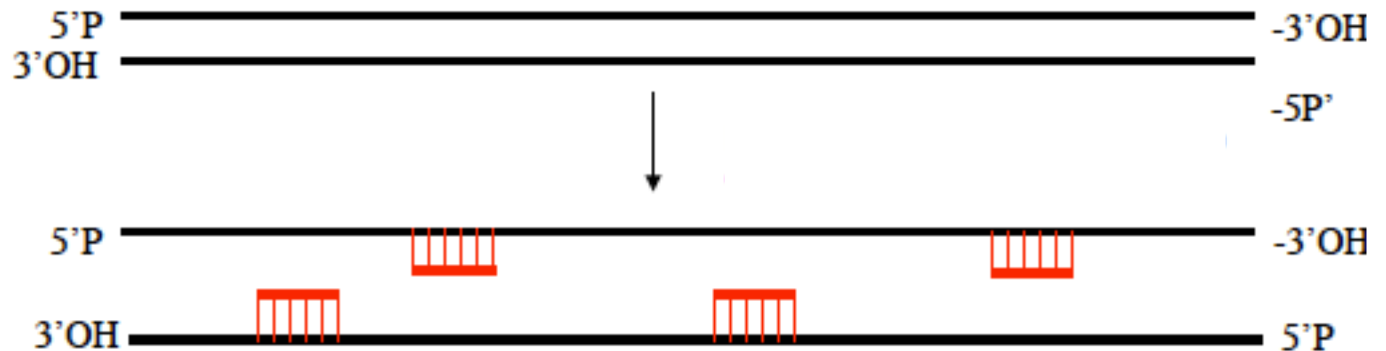


The result of these activities in presence of lablelled nucleotides is the generation of labelled DNA fragments.

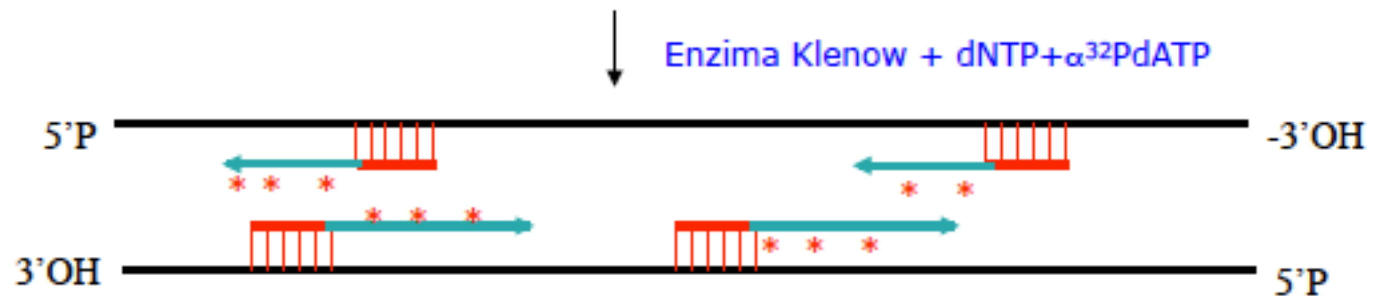
Random priming

This technique is based on the random hybridization of a mix of short primers (6-12 nt) to a DNA template.

DNA denaturation and primer hybridization



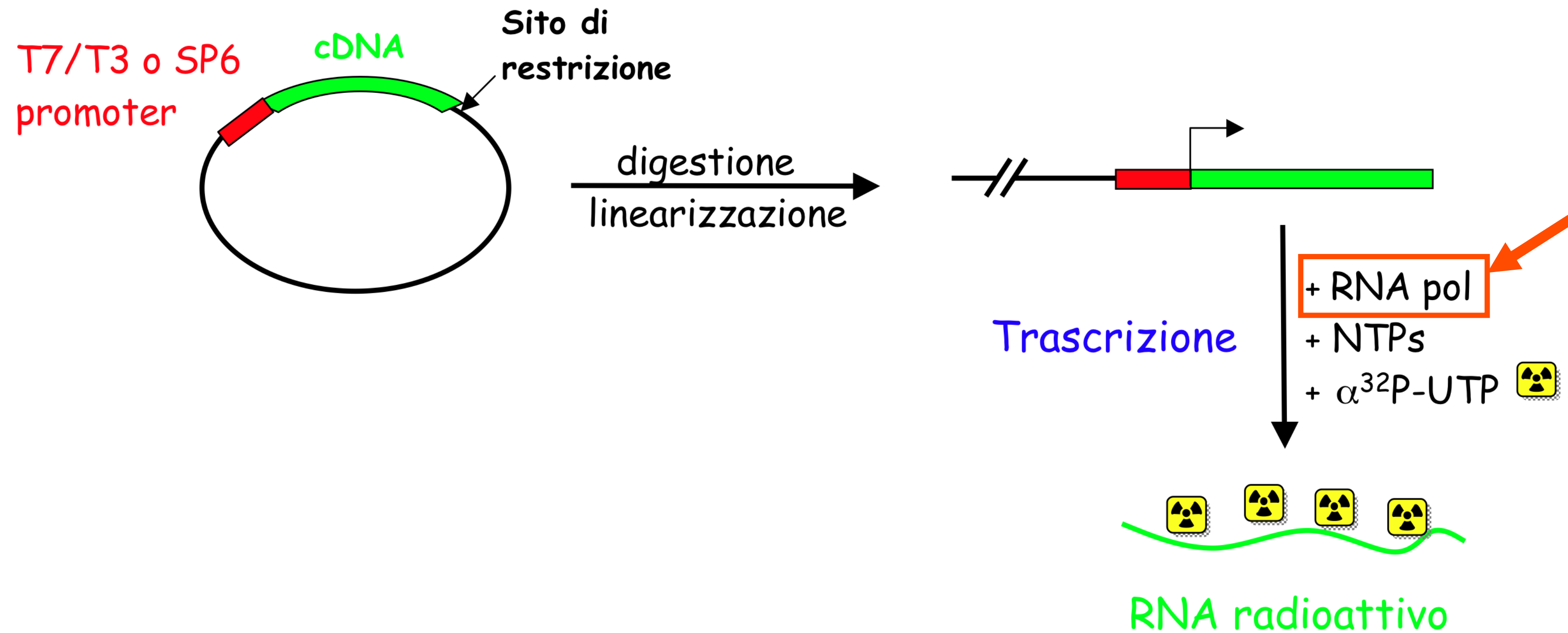
The Klenow polymerase sinthetyses a DNA strand starting from the primers. It is used in this assay beacause it lacks the 5'-3' exonuclease activity avoiding the degradation of the primers.



By adding α^{32}PdNTP to this reaction the new sinthetised DNA filament will result labelled.

How to generate RNA probes :

In vitro transcription:



Non radioactive tracers

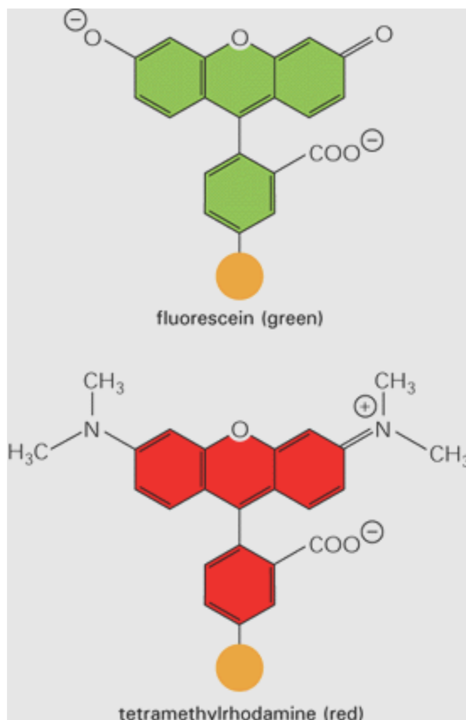
- ✔ fluorochromes (direct labelling)

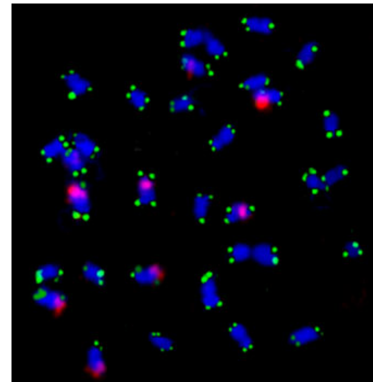
- ✔ digoxigenin or Biotin (undirect labelling)

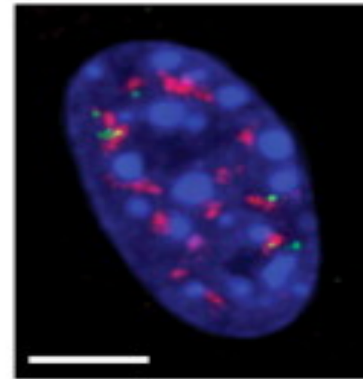
Oligonucleotides labelled with non radioactive tracers

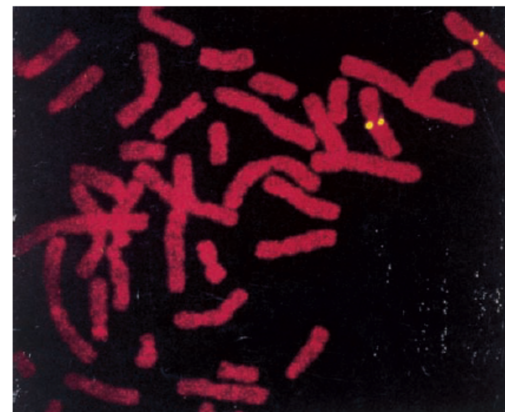
Fluorescence

It works with nucleotides linked to fluorochromes (Fluorescein, rhodamine) that can be detected a fluorescence microscope or other fluorescence detectors



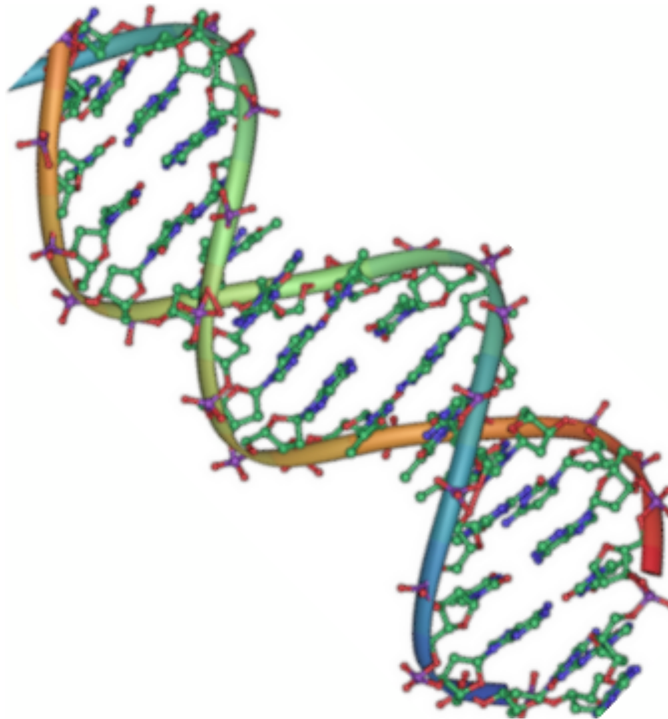






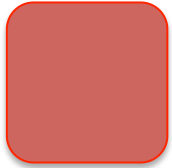
Polymerase Chain Reaction (PCR)

Enzymatic method which allows the in vitro amplification of specific DNA sequences



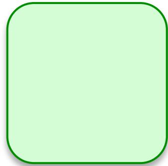
What's needed?

DNA template

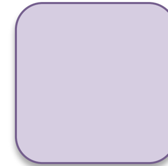


DNA polymerase

primers

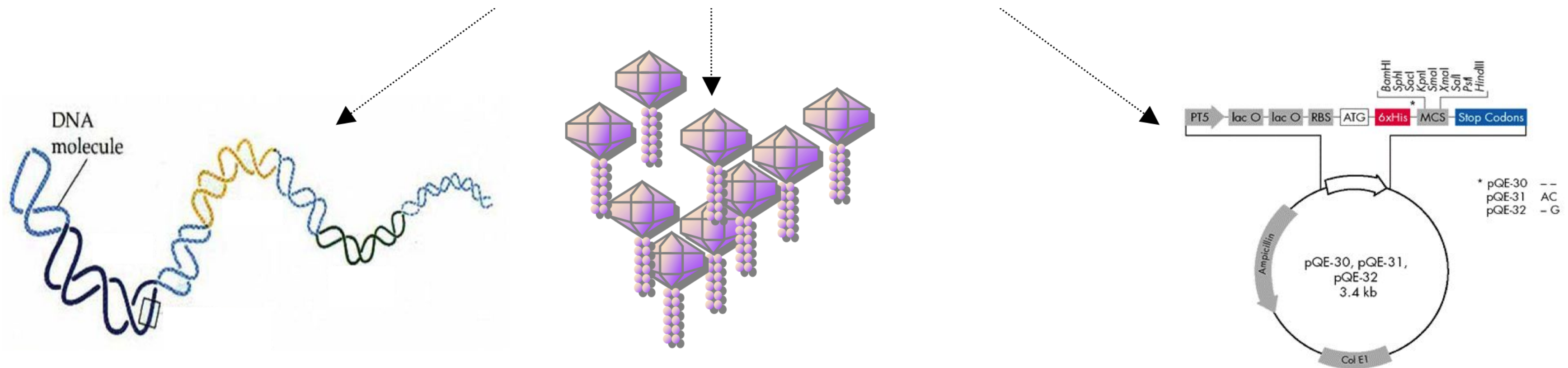


dNTPs



DNA template

genomic, viral, plasmid DNA



>YLR175W Chr 12

ATGTCAAAGGAGGATTTCGTTATTAAGCCTGAAGCTGCAGGTGCTTCCACTGACACTAGT

GAATGGCCATTACTTTTGAAGAATTTTGATAAGTTATTAGTCAGAAGTGGTCATTATACC

CCTATCCCAGCTGGTAGTTCACCACTAAAGAGAGACCTGAAATCATATATCAGCTCTGGT

GTCATTAATCTAGATAAACCTTCCAACCCATCATCGCACGAAGTTGTAGCTTGGATCAAA

GTCGAAACTGAGAAGGAAGAAGTGAAAGAGGATGACAGCAAAAAGGAAAAGAAAGAGAAG

AAGGACAAGAAGGAAAAGAAGGAAAAGAAAGAAAAGAAGGACAAGAAGGAAAAGAAAGAG

AAAAAGGAGAAGAAAAGAAAGTCTGAAGACGGTGATTCTGAGGAAAAGAAATCTAAGAAA

TCTAAGAAATGA

Primers

Primers are short custom-designed DNA oligonucleotides that are sigle stranded and 18-25 nt long. They target a specific DNA sequence and one is complementary to the 3′ end of one strand of the DNA to be amplified, and the other is complementary to the 3′ end of the opposite strand

5’-ATGTCAAAGGAGGATTTCGTTATTAAGCCTGAAGCTGCAGGTGCTTCCACTGACACTA-3’
3’-TACAGTTTCCTCCTAAAGCAATAATTCGGACTTCGACGTCCACGAAGGTGACTGTGAT-5’ dsDNA

CACGAAGGTGACTGTGAT
5’-ATGTCAAAGGAGGATTTCGTTATTAAGCCTGAAGCTGCAGGTGCTTCCACTGACACTA-3’

ATGTCAAAGGAGGATTTC
3’-TACAGTTTCCTCCTAAAGCAATAATTCGGACTTCGACGTCCACGAAGGTGACTGTGAT-5’

Important parameters:

-The lenght (18-25nt)

-The GC percentage (55-60%)

-The appropriate melting temperature (Tm); Wallace methods : $T_m = 2(A + T) + 4(G + C)$.

thermostable DNA polymerase

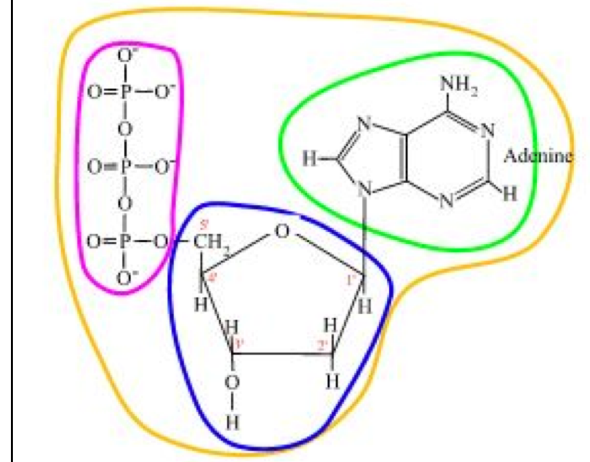
DNA polymerase	Error rate	Activities	Extension times
PfuTurbo	1.3×10^{-6}	5'-3' polymerase activity 3'-5' proofreading activity	1kb/min
Taq	8×10^{-6}	5'-3' polymerase activity	1kb/min

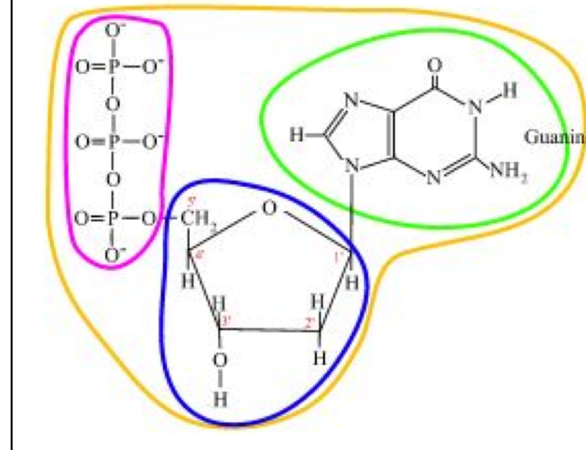
The enzyme to be used will be selected based on the type of experimets performed:

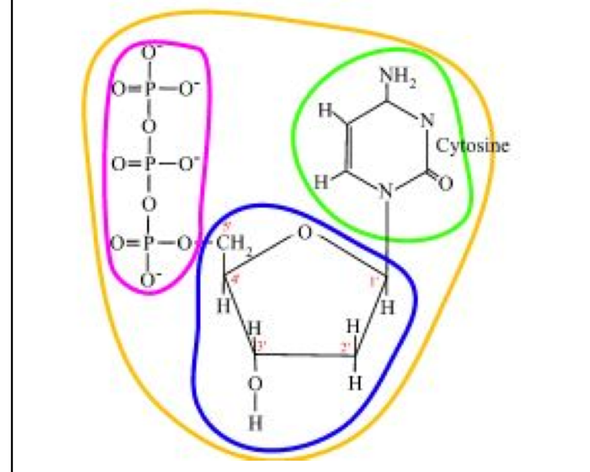
- A proof-reading polymerase will be used if the resulted DNA fragment has to be cloned
- A cheaper polymerase will be used if the experiment does not required that the resulted DNA is identical in sequence to the starting template.

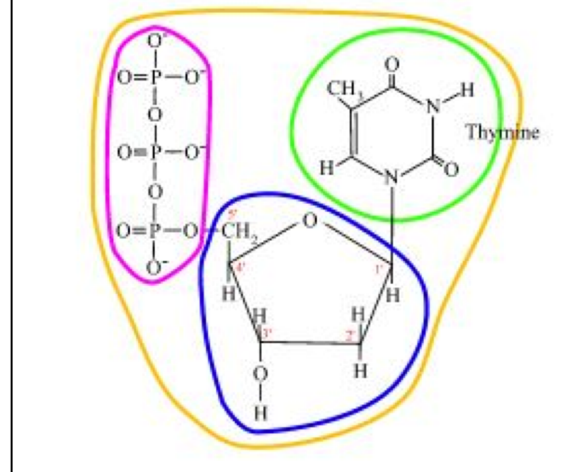
dNTP

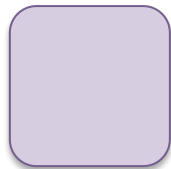
Nucleotide = base + sugar + phosphate







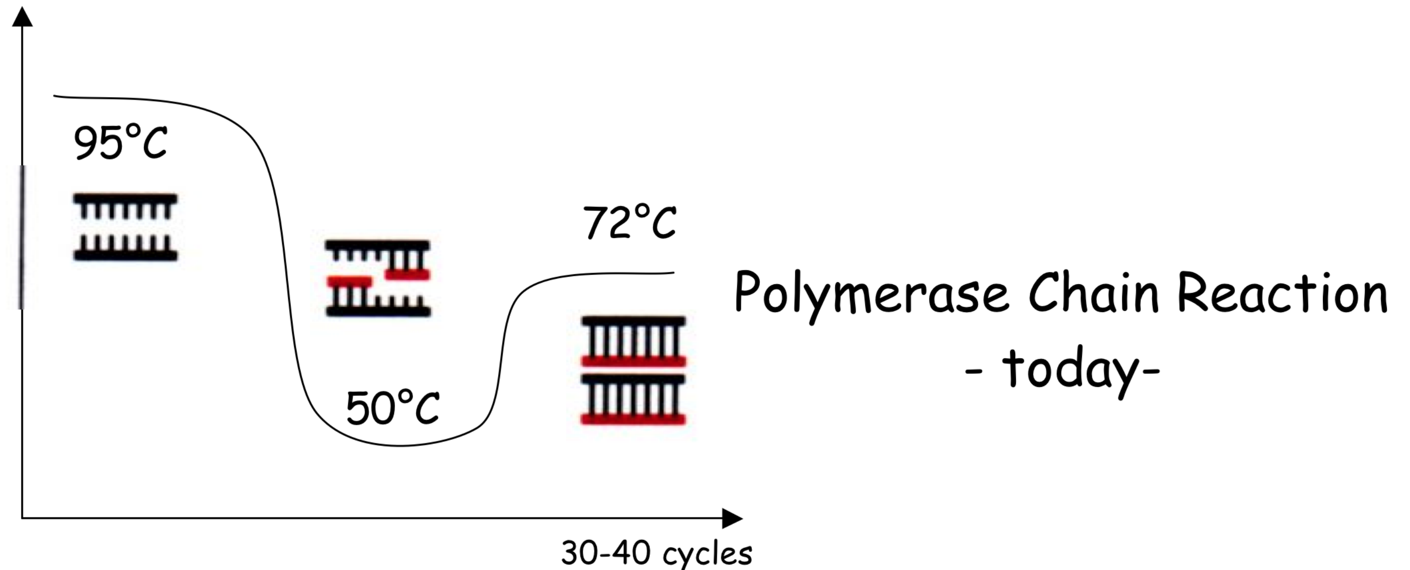




How we proceed???

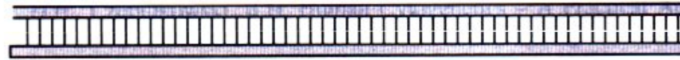
All the components will be mixed together as follow:

Buffer 10X	1X (10 µl)
dNTP 2.5mM	0,25 mM (10 µl)
$MgCl_2$ 50mM	≅2mM (2 µl)
primer F (10mM)	1mM (10 µl)
primer R (10mM)	1mM (10 µl)
Polymerase	x
template	y (ng)
H_2O	up to final volume (100 µl)

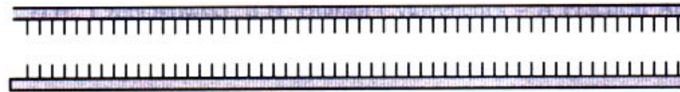


Polymerase Chain Reaction
- today-

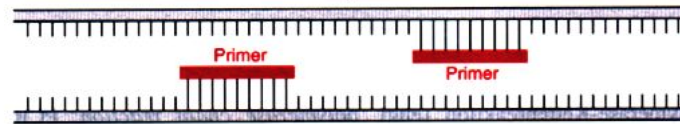
I cycle...



dsDNA



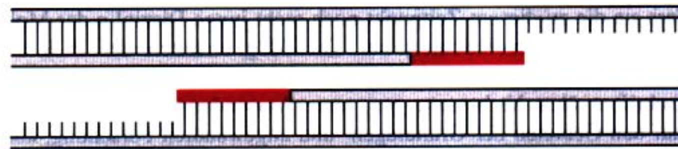
DENATURATION



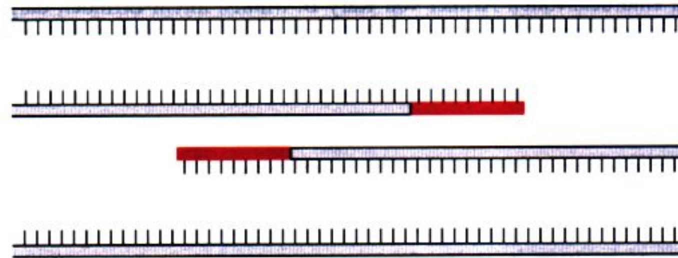
ANNEALING

SYNTHESIS

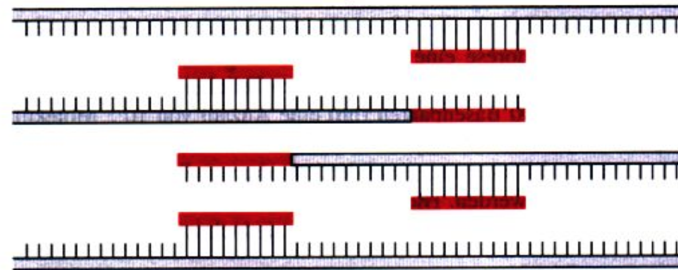
..II cycle



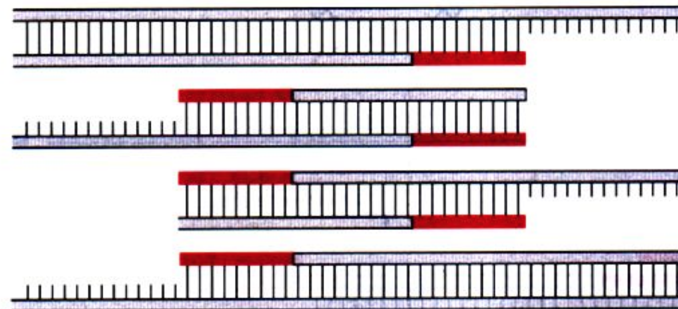
dsDNA



DENATURATION

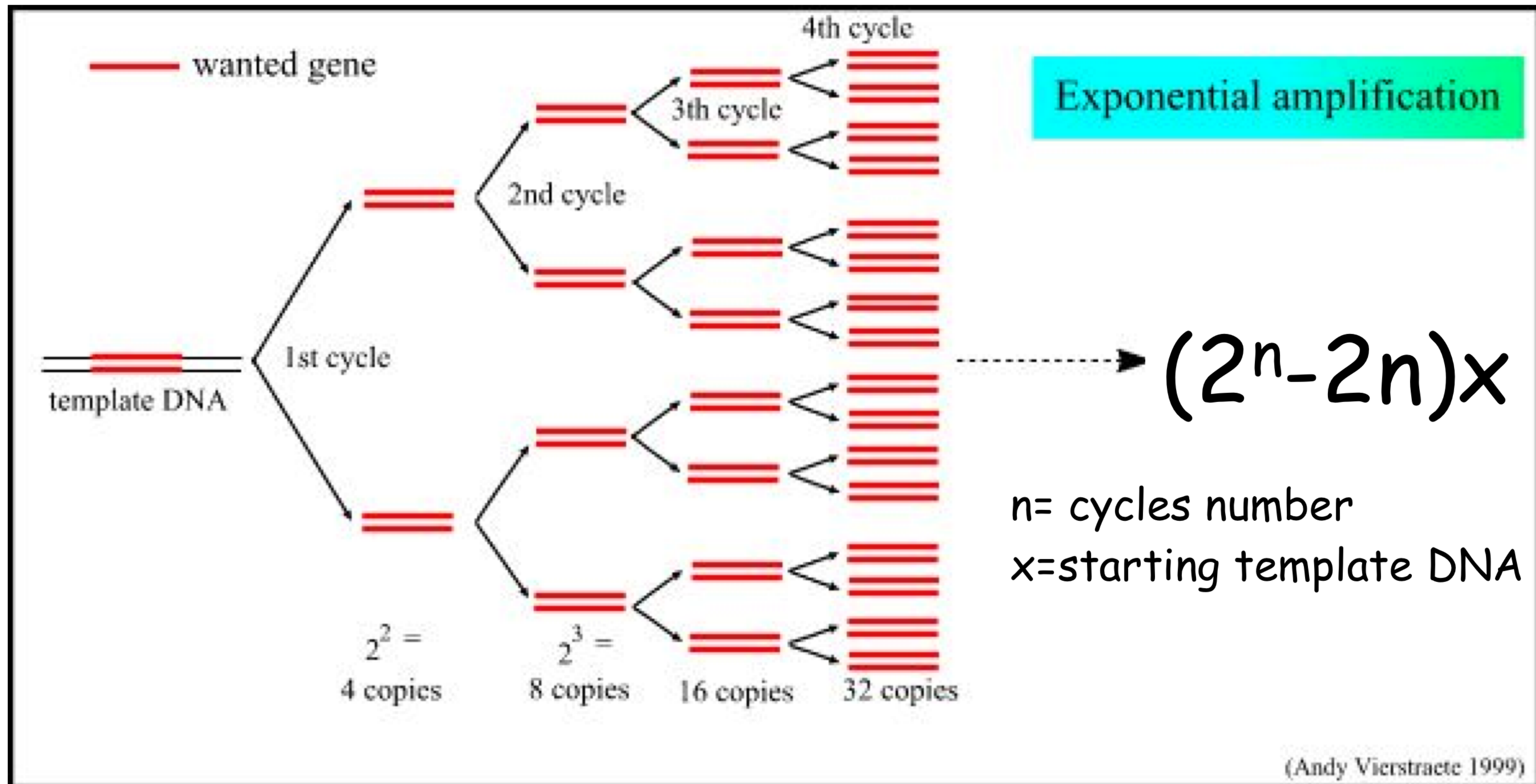


ANNEALING



SYNTHESIS

…additional cycles

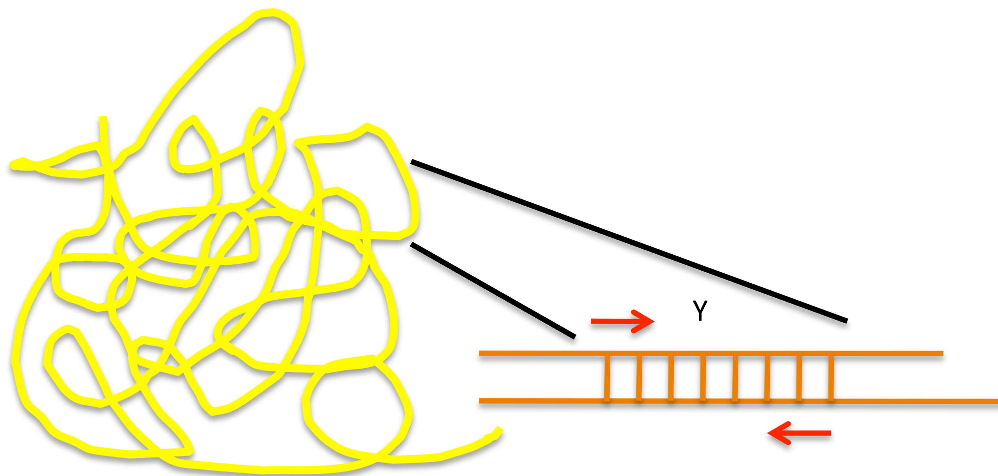


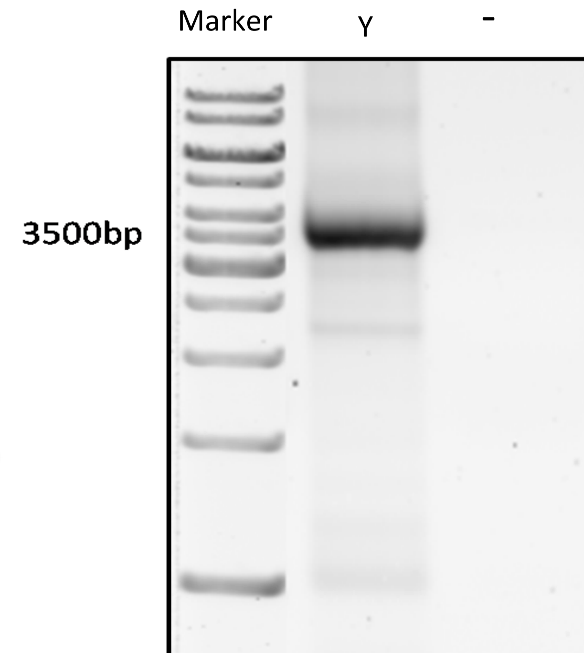
Benefits:

It can be performed in vitro
It allows to obtain high amount of DNA
Very easy to perfomed

Disadvantage:

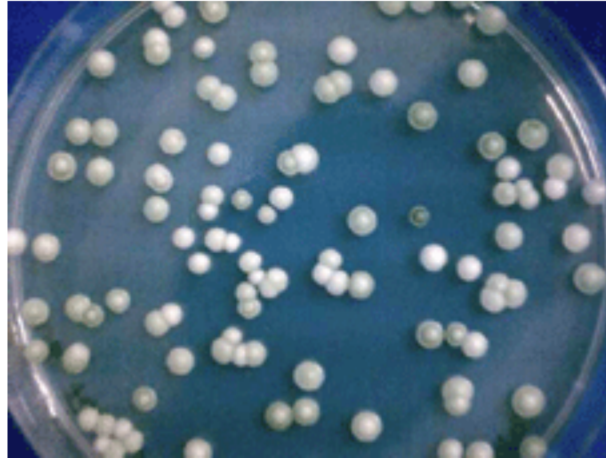
Because of the high sensitivity of the technique contamination are very frequent and the appearance of unspecific bands. For this reason it is better to run in parallel a PCR reaction without DNA template (negative control).





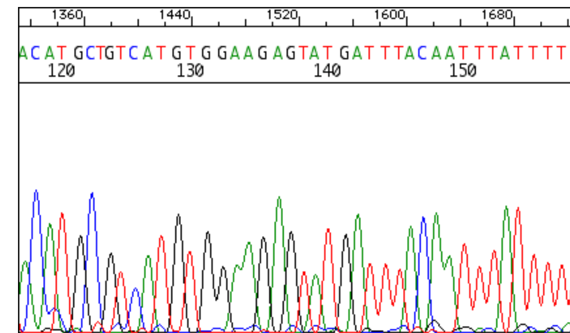
Colony Screening

GFP cloning

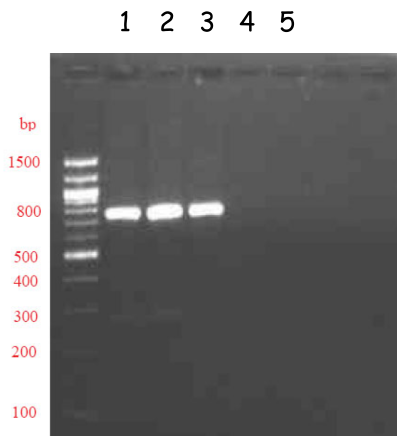


Selection of prositive clones

Sequencing



PCR



Detection of the presence of infectious diseases: latent or active virus/bacteria

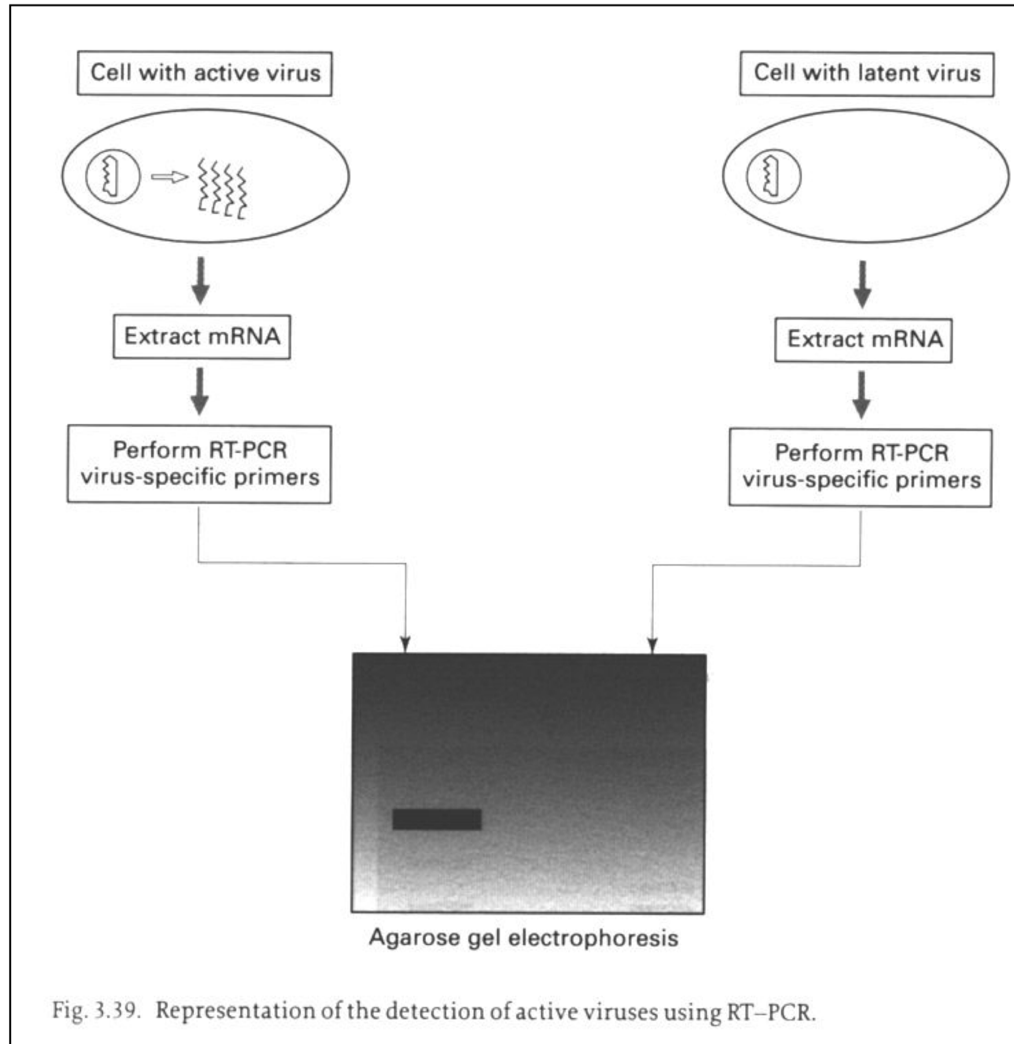
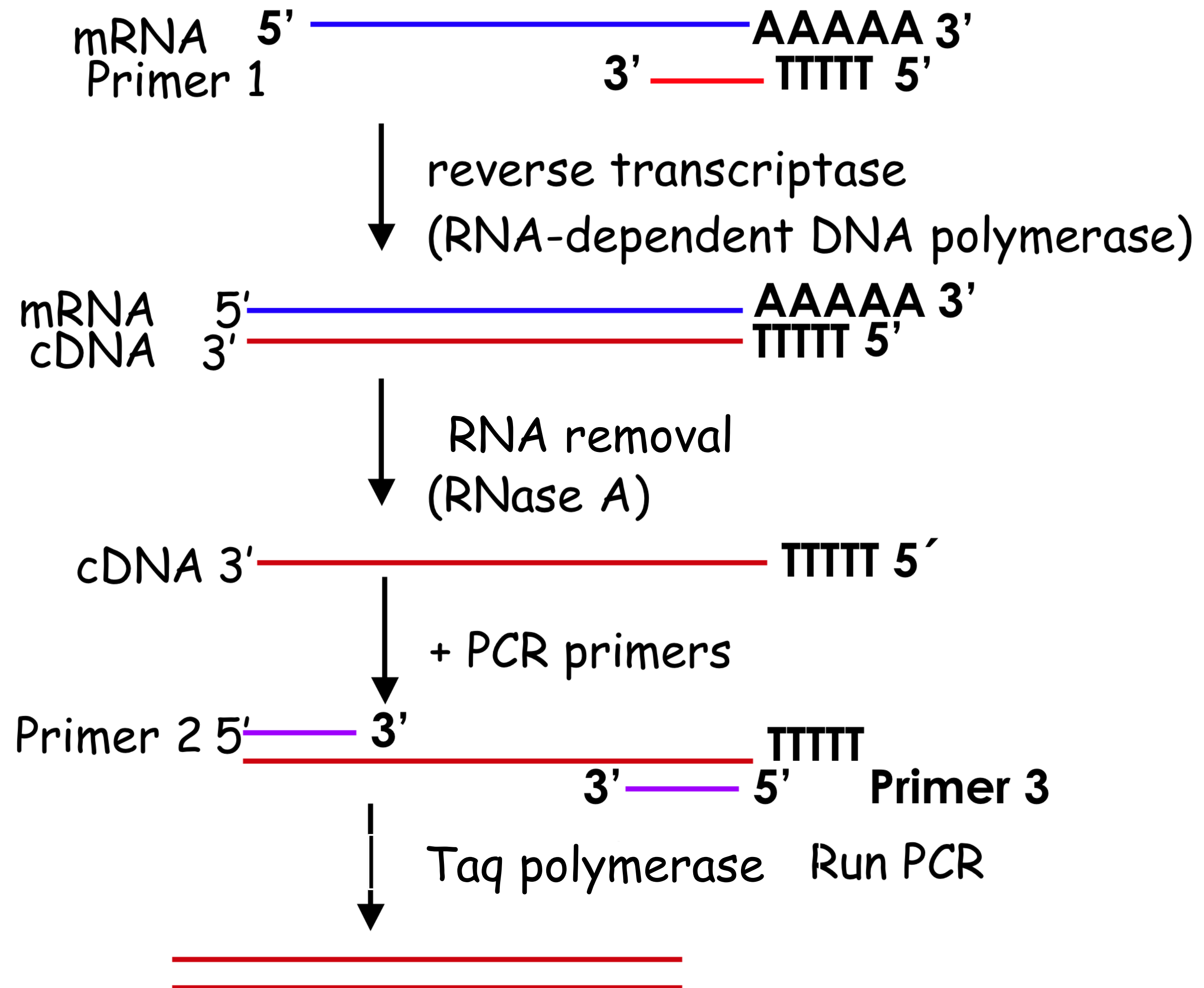


Fig. 3.39. Representation of the detection of active viruses using RT–PCR.

By applying the PCR assay it has been possible to detect the presence of few active viral/ bacterial particles allowing the development of new diagnostic technique. Some examples are the detection of several viruses and bacteria such as HCV, HBV,HIV, Chlamydia, Mycobacterium, Neisseria and Salmonella.

RT PCR: Reverse Transcription PCR to study gene expression

Allows to detect very rare mRNAs



Analysis of gene expression in different experimental condition