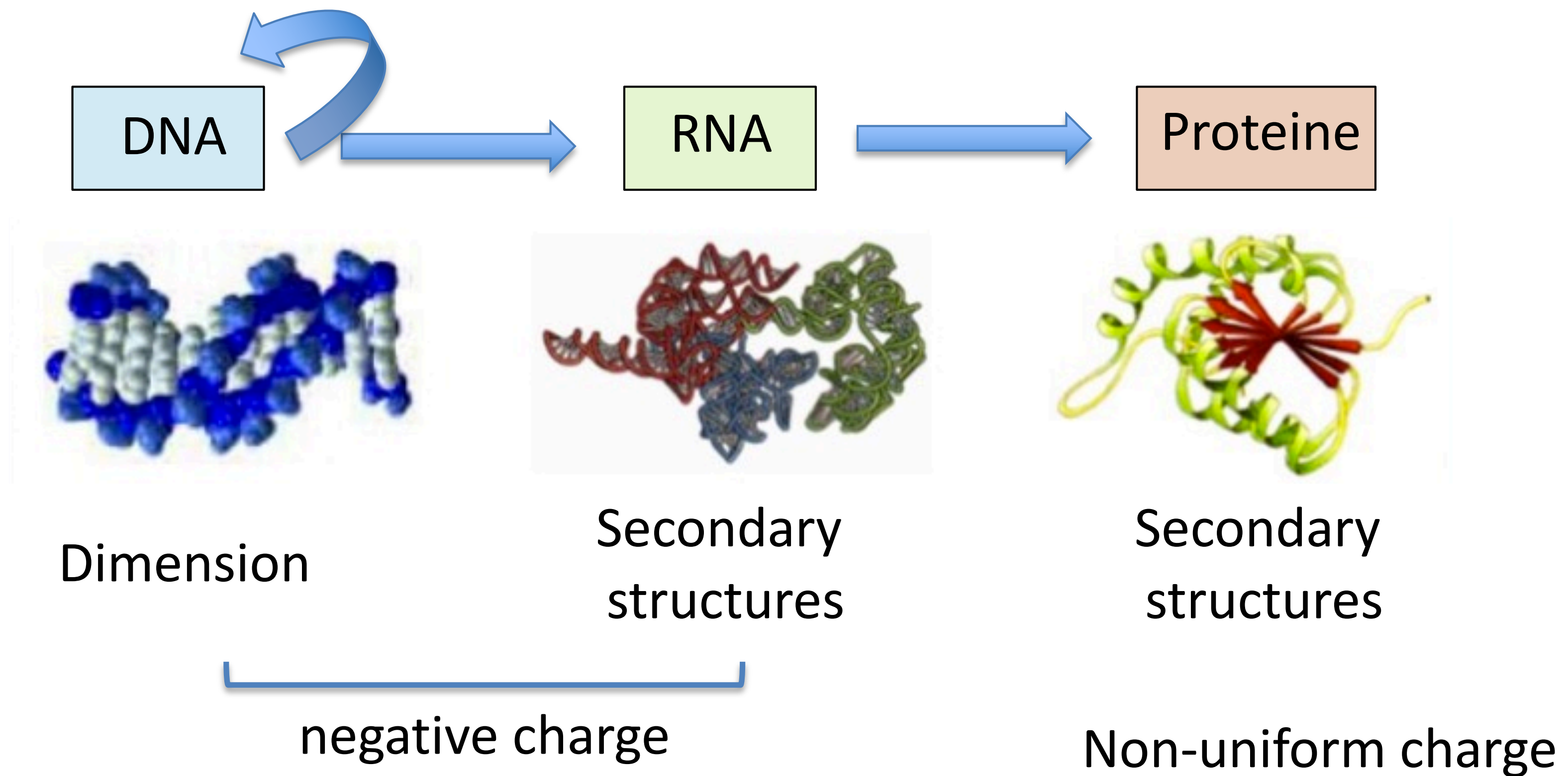
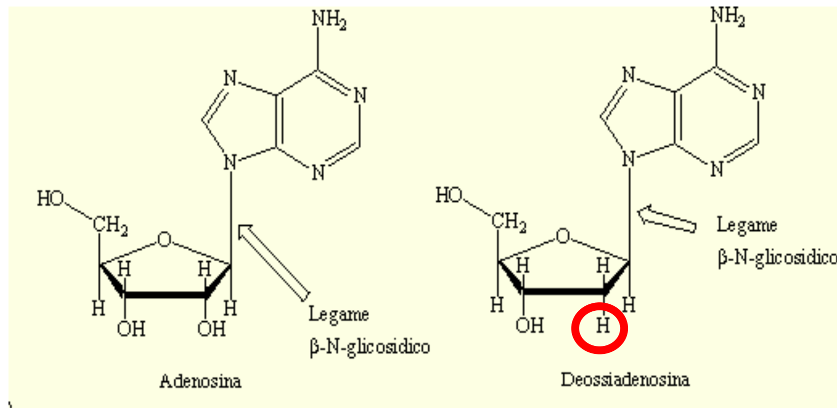
Nucleic acid labelling, Northern Blot and RNA-DNA/protein interaction

The Macromolecules and the Central Dogma of Molecular Biology

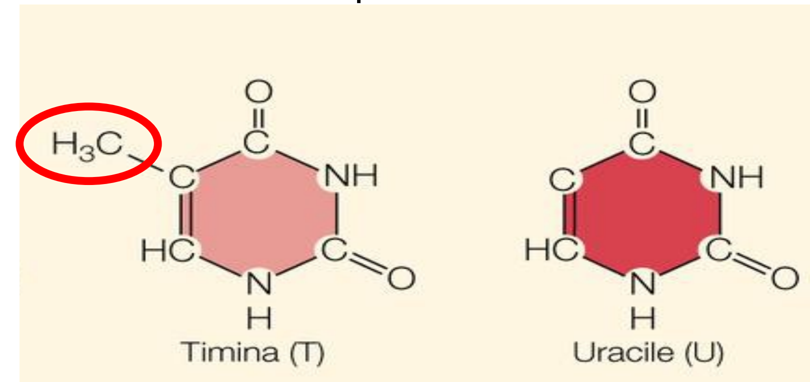


The nucleic acids: RNA and DNA

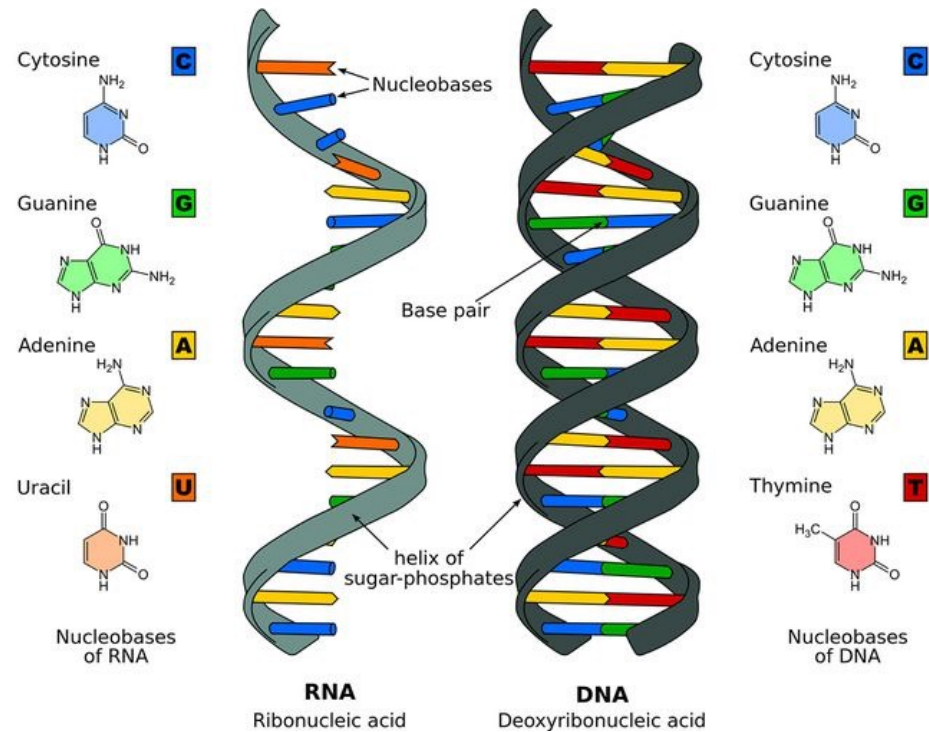
Ribose vs Desossiribose



Uracile in place of Timine



Single strand vs double strand



How to get your Nucleic Acids

Organic Nucleic Acid extraction

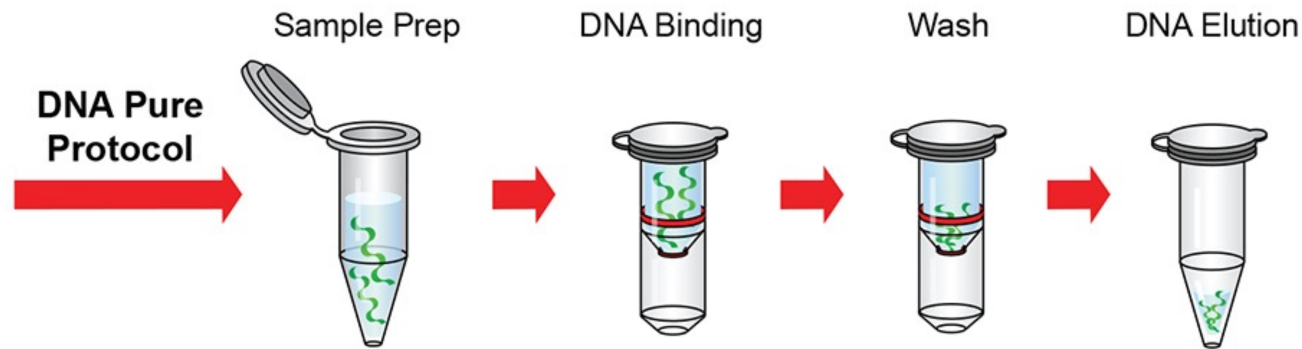
1) Cell Lysis (using detergents)

2) Protein extraction and separation from the nucleic acid phase (e.g. using Phenol/chloroform)

3) Precipitation of nucleic acids (using Ethanol of isopropanol)

4) The purified RNA or DNA has to be stored at -20/-80 °C

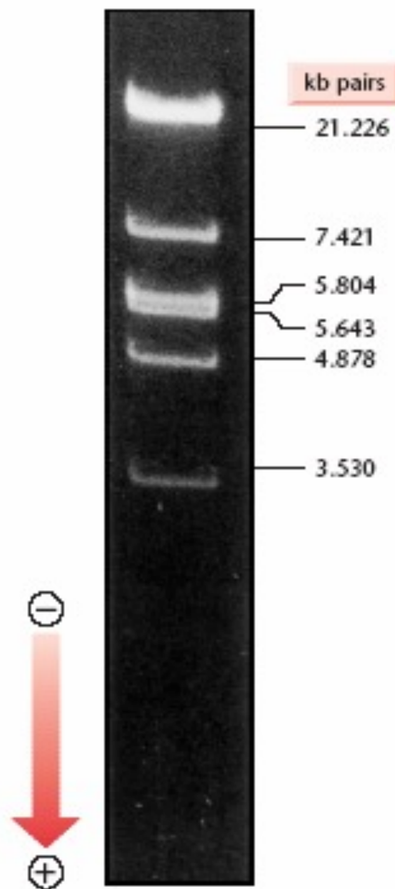
Nucleic Acid extraction Kits



How to see your Nucleic Acids

Electrophoresis of nucleic acids

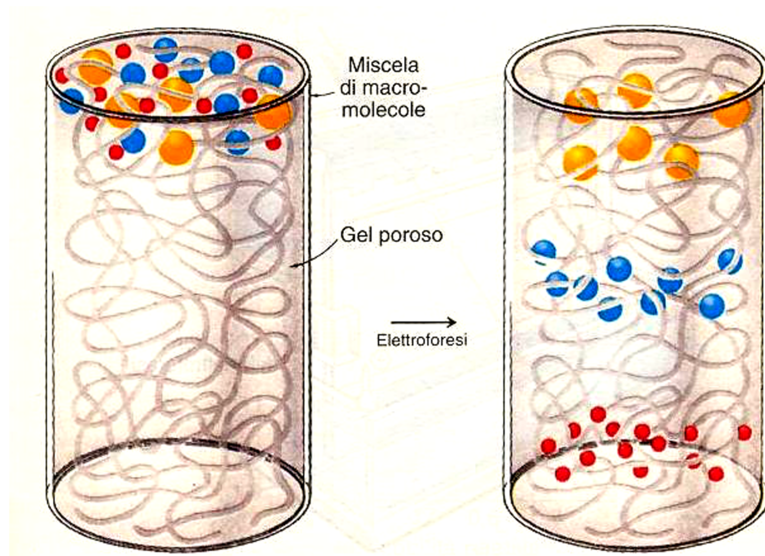
Electrophoresis is a biochemical technique which allows to separate molecules with charge based on their different molecular weight. The electrophoretic separations is carryed out through a gel.

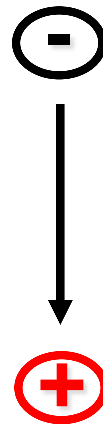


The gel can be made by:

- ✓**agarose** (separation range : 0.5-20 kb)
- ✓**polyacrylamide** (separation range 10-500 bp)

How to see your Nucleic Acids



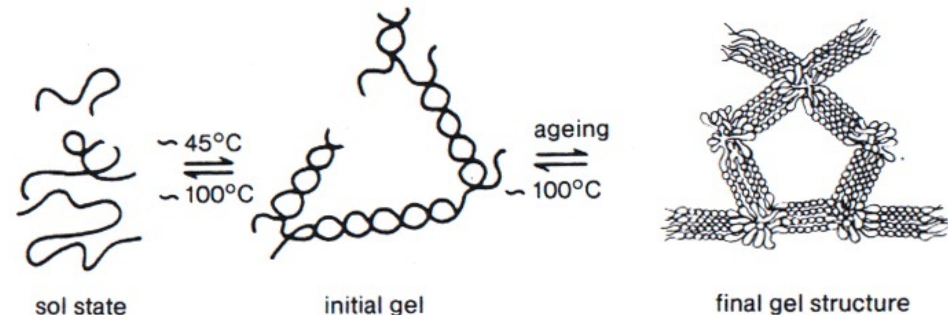


Sieve effect

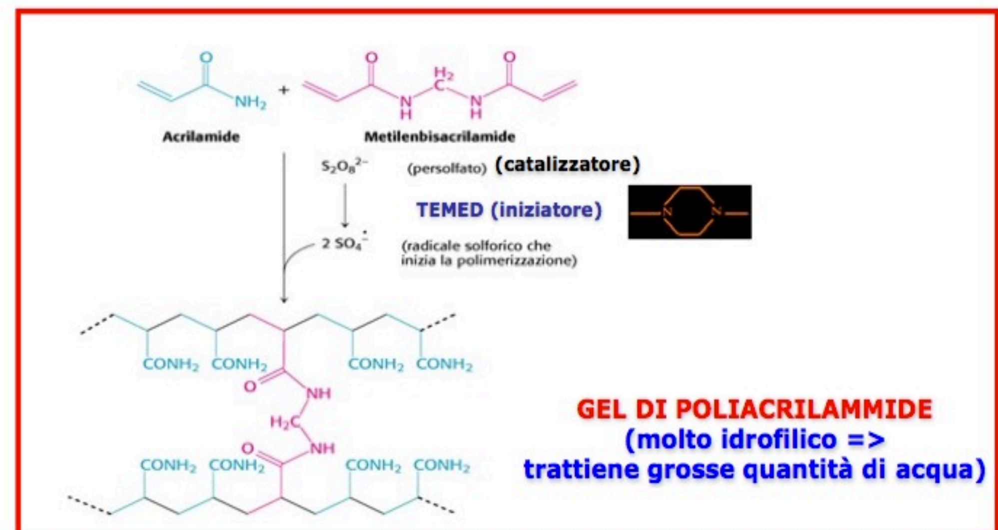
For linear fragments of DNA and/or RNA the migration distance is inversely proportional to the size of the molecule (that corresponds to its length in bases)

How to see your Nucleic Acids

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



How to see your Nucleic Acids

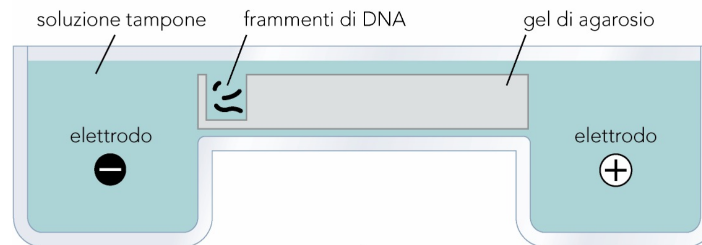
Electrophoresis

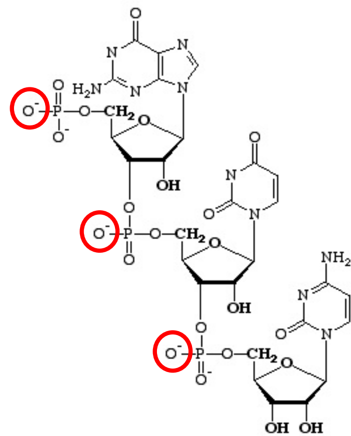
✓ horizzontal

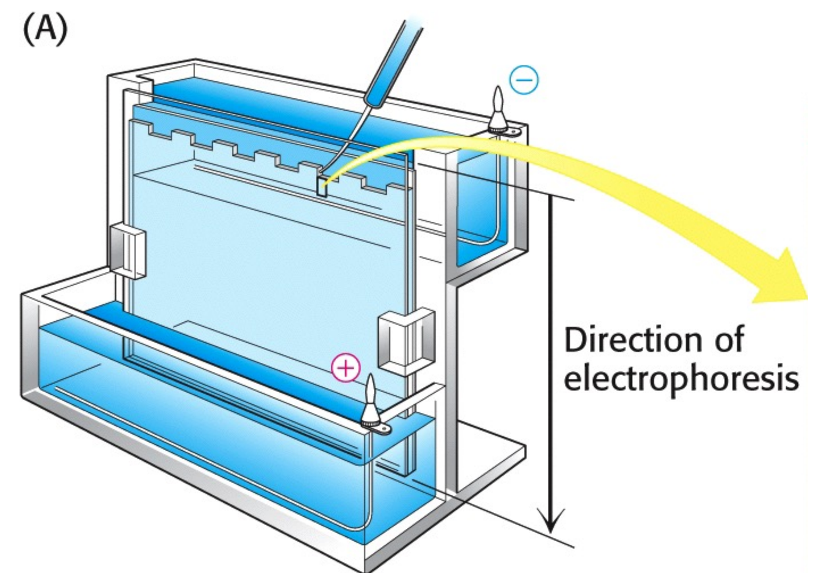
✓ vertical

agarose

acrylamide

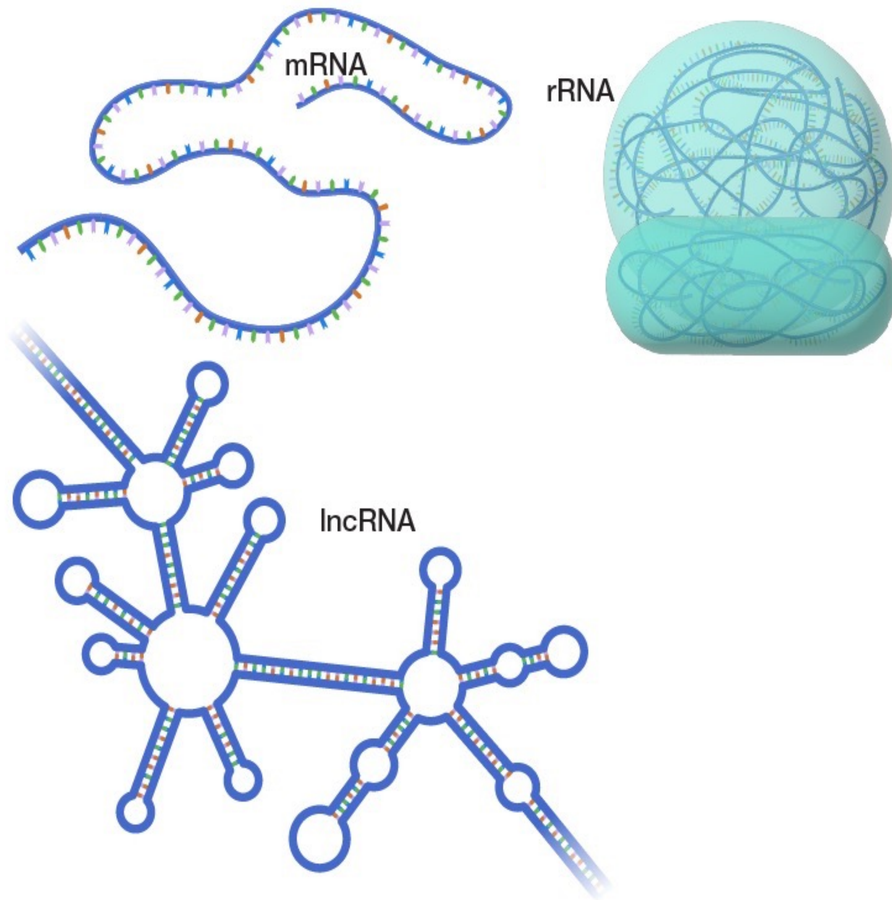






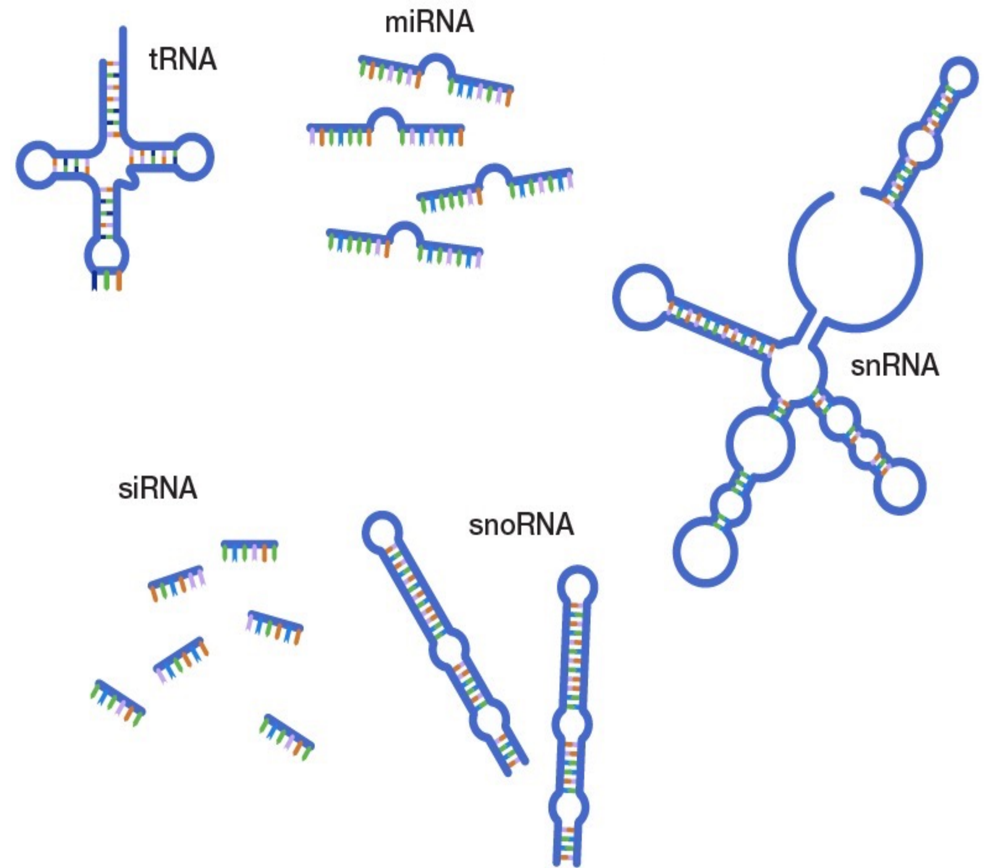
Cellular RNAs have different size

Agarose



A thousand nts

Acrylamide



A hundred nts

Cellular RNAs are expressed at different levels

Characterisation of the transcriptome

RNA sub-classes in a mammalian cell:

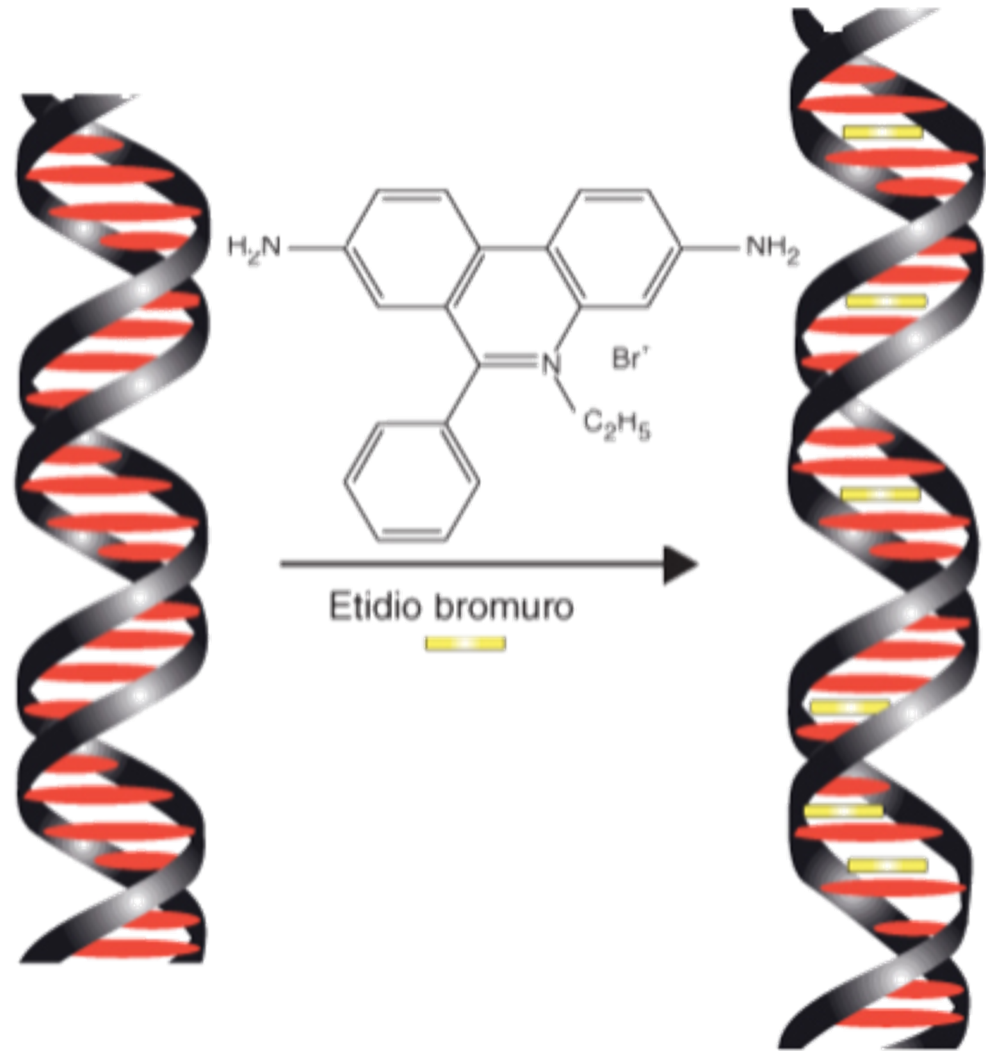
ribosomal RNA	rRNA	80-85%	*(5S, 18S und 28S)*
transfer RNA	tRNA	10-15%	
messenger RNA	**mRNA**	**1-5%**	
average length	1930 bases		
high abundant	<10 genes	10-20000 copies/cell	>1%
intermediate abundant	~500 genes	200-400 copies/cell	0,1%
low abundant	>10000 genes	<20 copies/cell	0.004%

How to see your Nucleic Acids

ethidium bromide (EtBr):

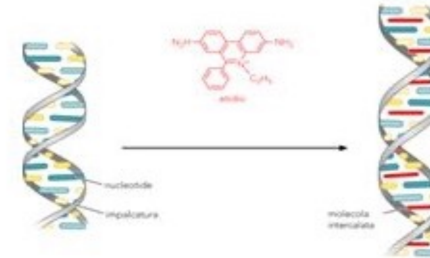
Intercalating agent, fluorescent dye that absorbs UV light at 300 nm resulting yellow-orange

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



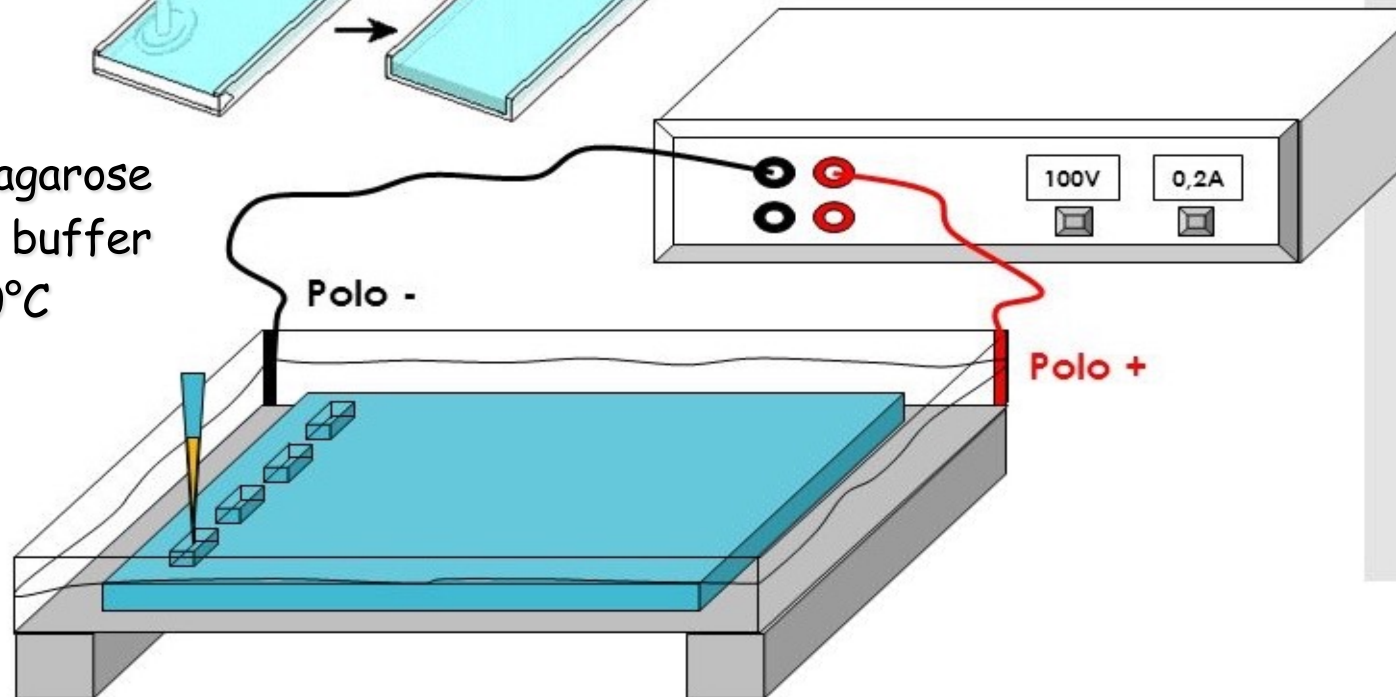
How to prepare agarose gel:

- 1% agarose gel in 1X TAE electroforesis buffer
- Run Time: 40 min.
- Voltage: 80 V
- Samples: 1/20 final Volume (5 μl) + Loading buffer

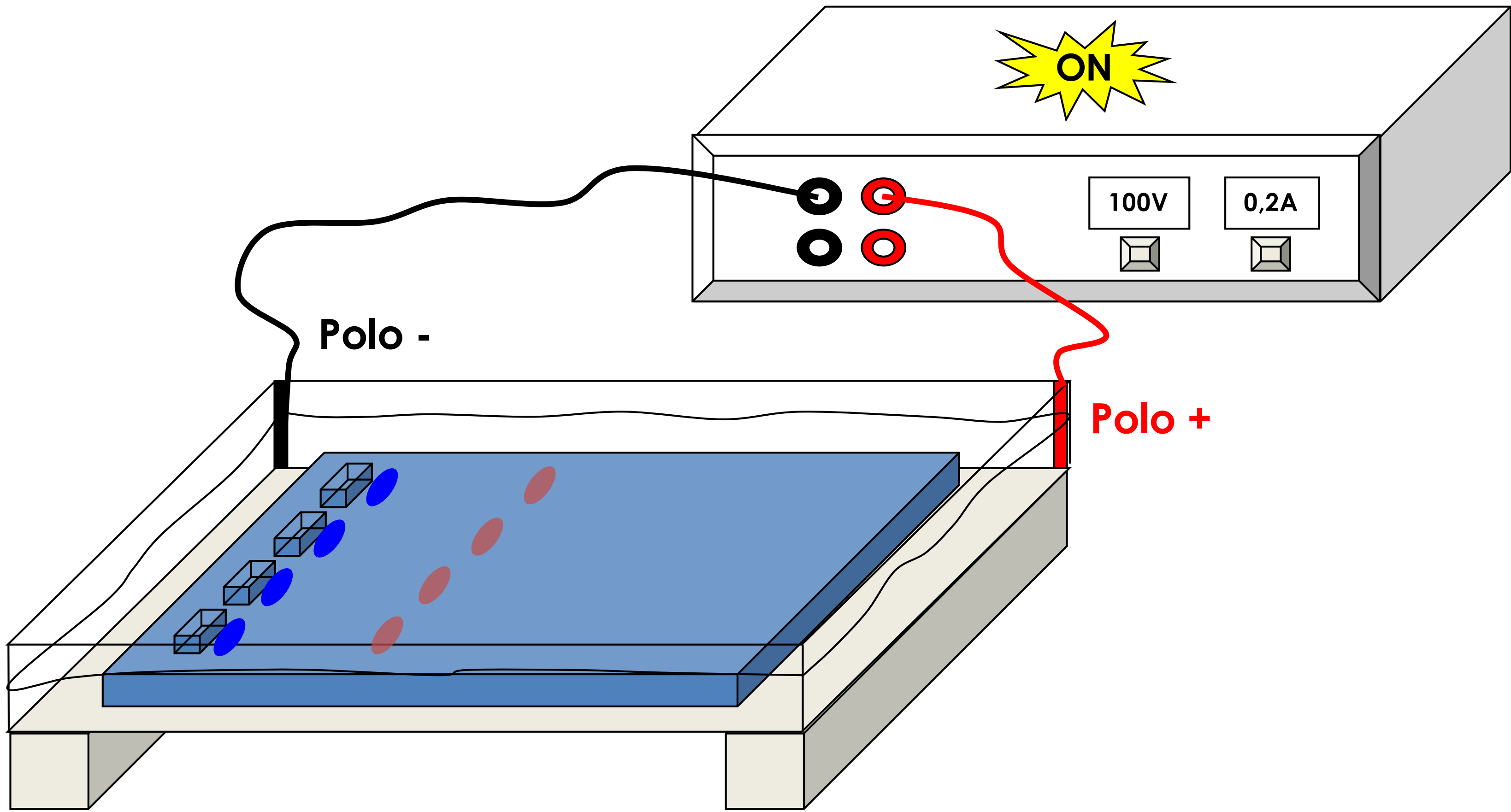


add ethidium bromide

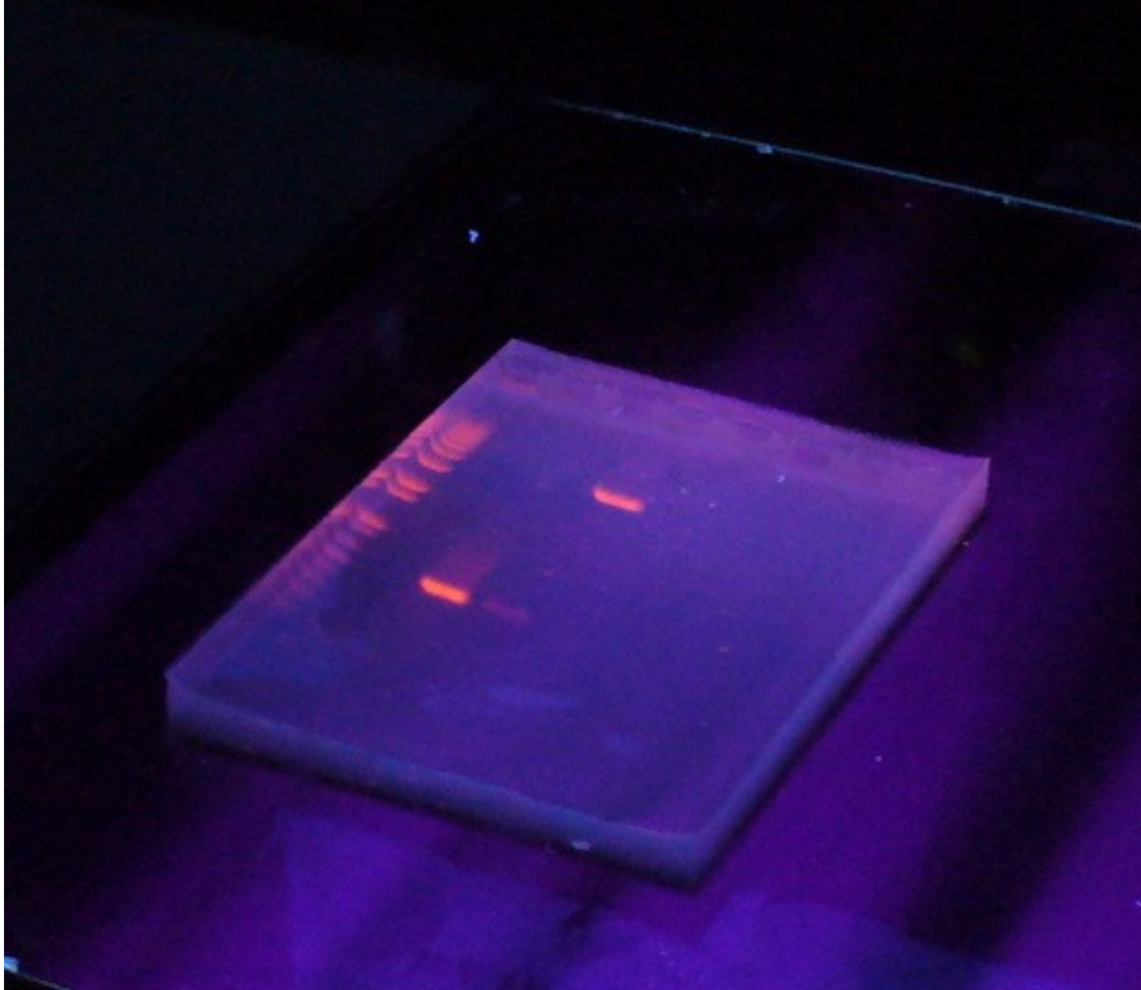
dissolve the agarose powder in a buffer solution at 100°C

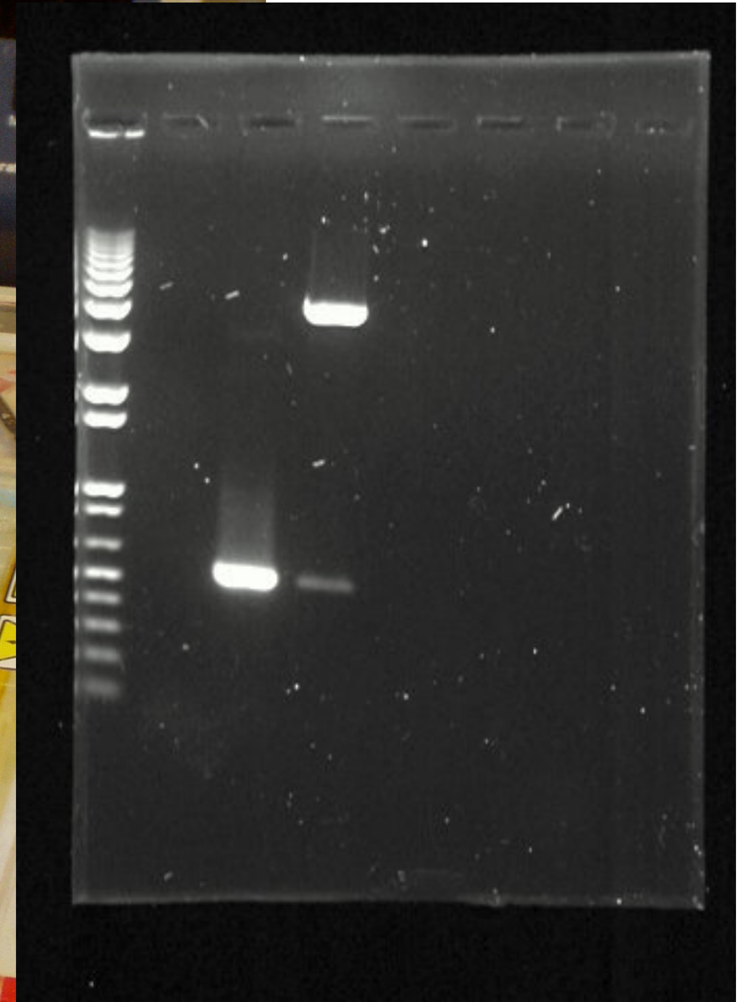


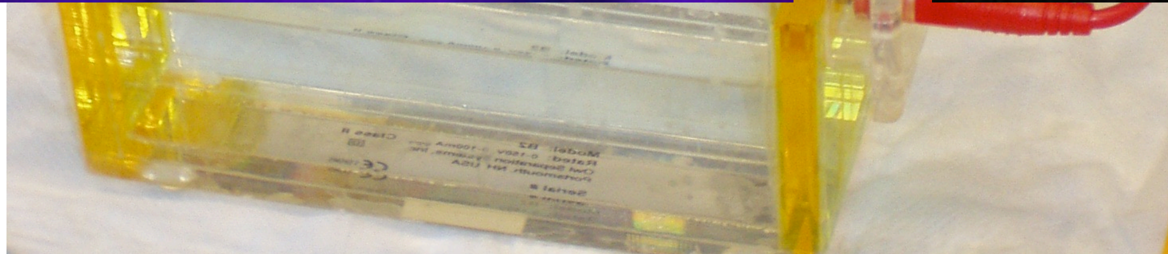
https://www.youtube.com/watch?v=wXiiTW3pflM
https://www.youtube.com/watch?v=U2-5ukpKg_Q

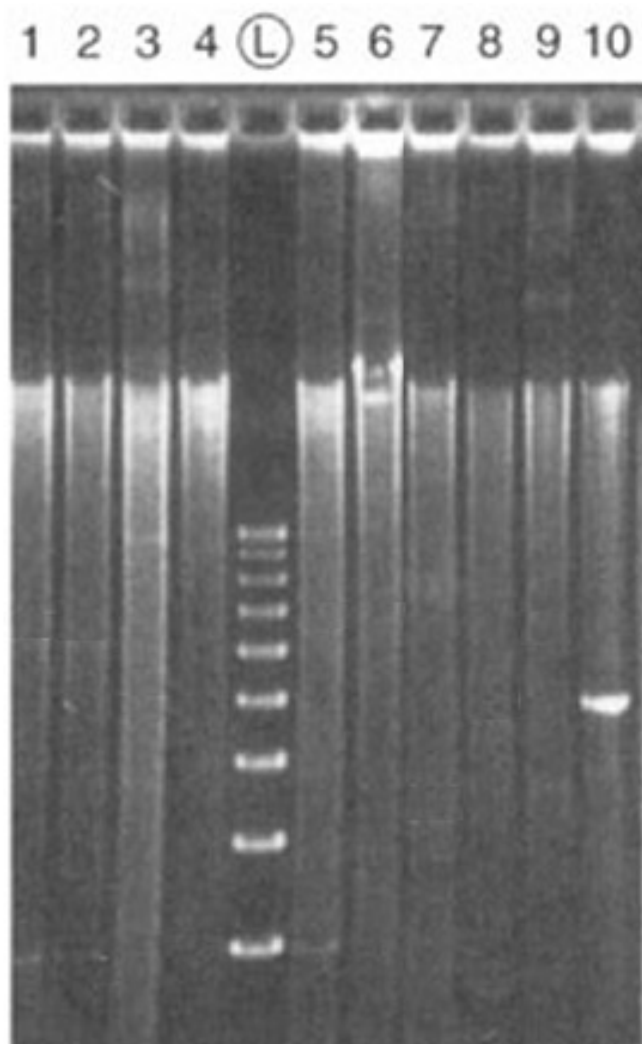

ON
100V
0,2A
Polo -
Polo +

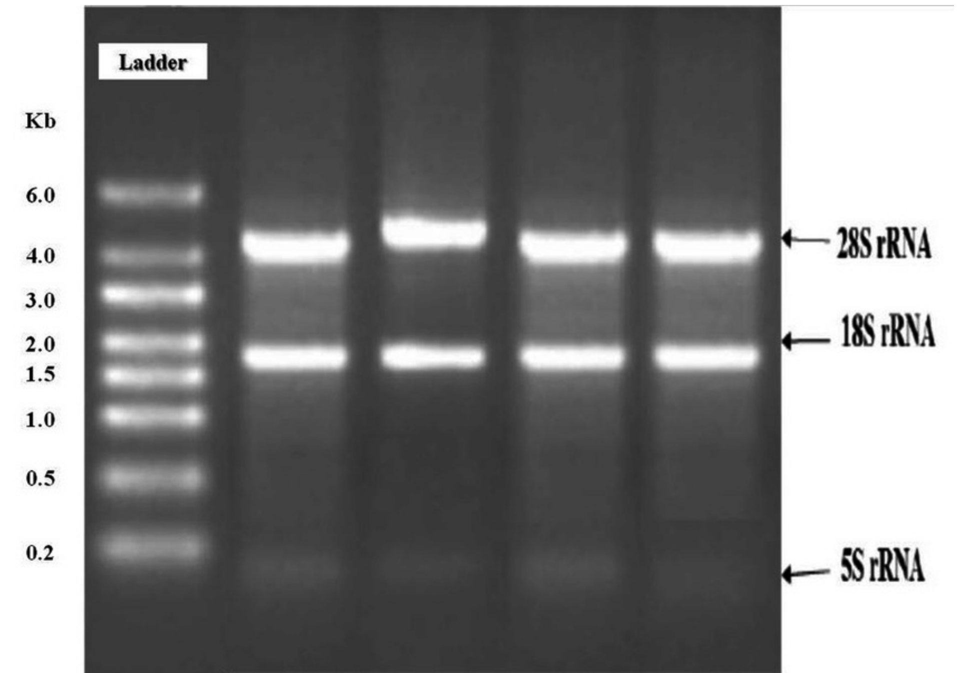
EC 105
Check
Fuse








1 2 3 4 L 5 6 7 8 9 10

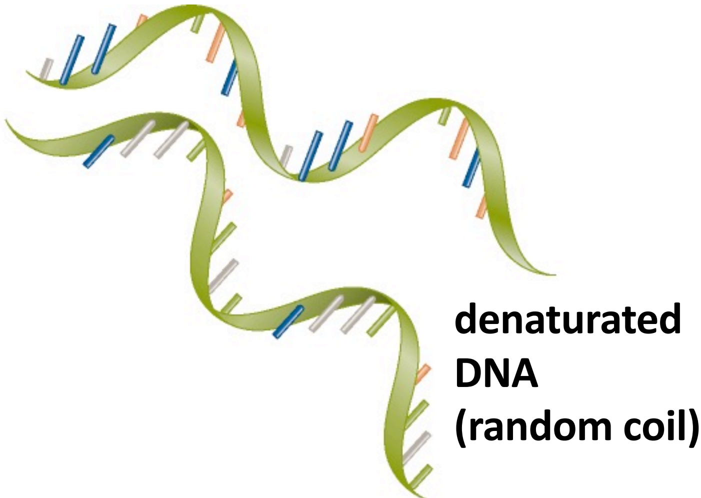

Ladder
Kb
6.0
4.0
3.0
2.0
1.5
1.0
0.5
0.2
28S rRNA
18S rRNA
5S rRNA

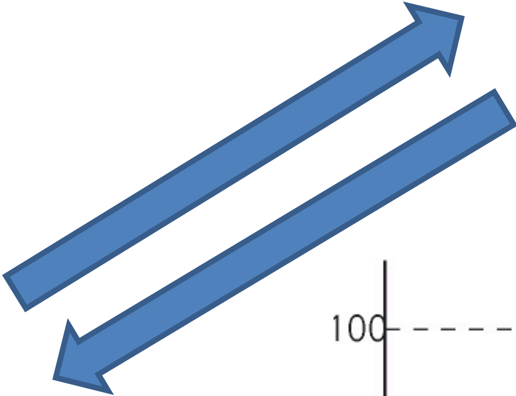
How to see Nucleic Acids

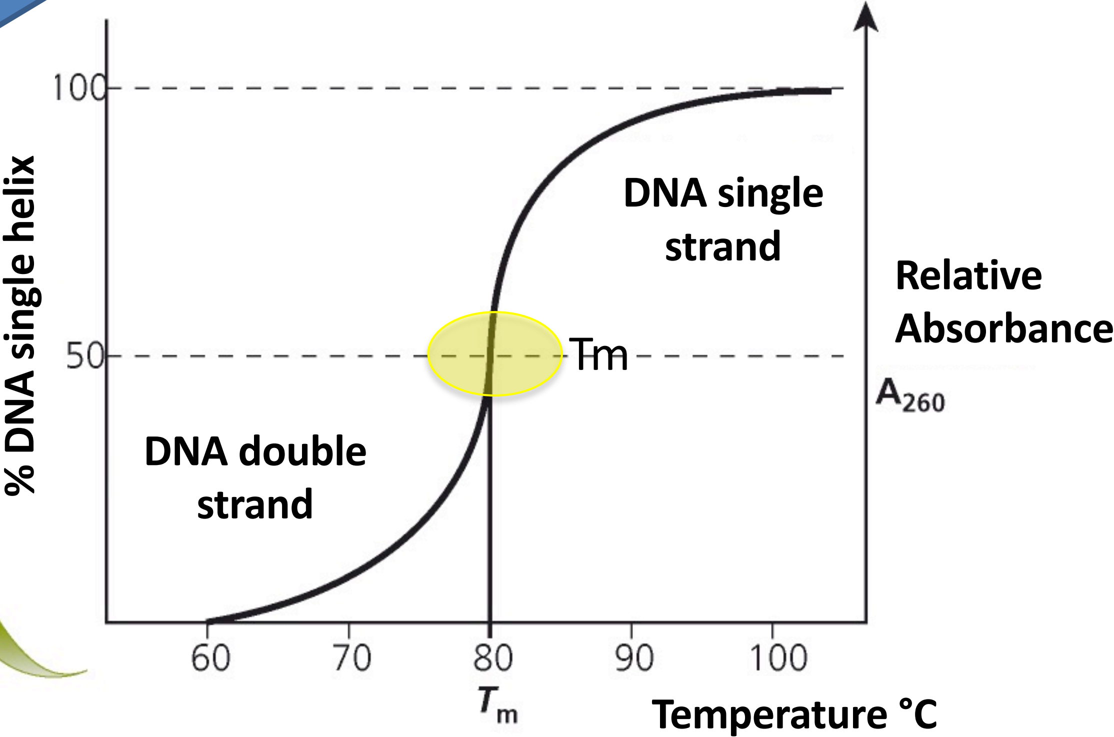
Visualizing and quintifying nucleic acids:

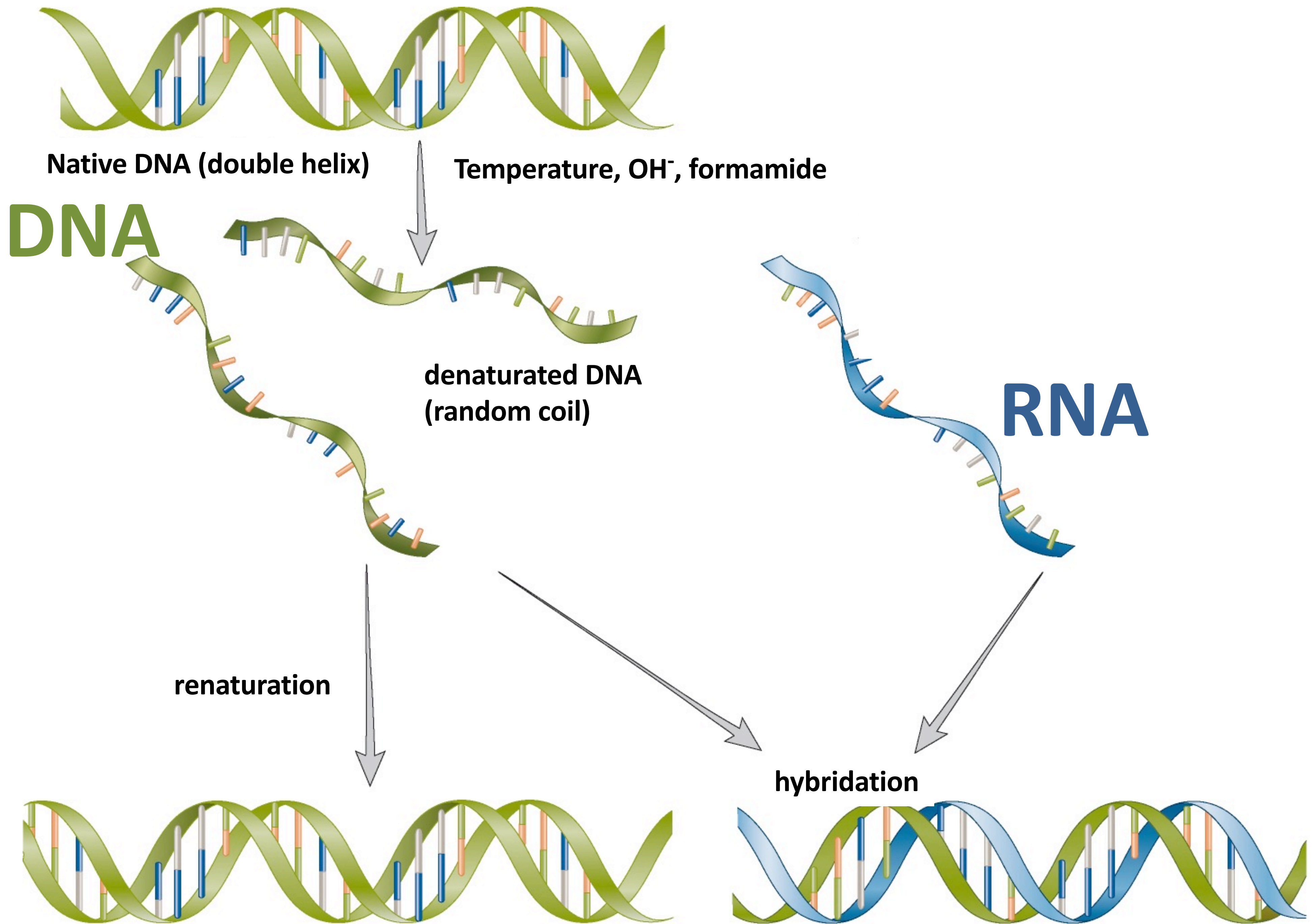
- In situ hybridization
- Southern/Northern blot
- In vitro transcription
- Realtime PCR

Preliminary: Nucleic acid can be denaturated and renaturated








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

How to see your Nucleic Acids

Labelled probes

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

How to see your "specific" Nucleic Acids

Making labelled probes

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

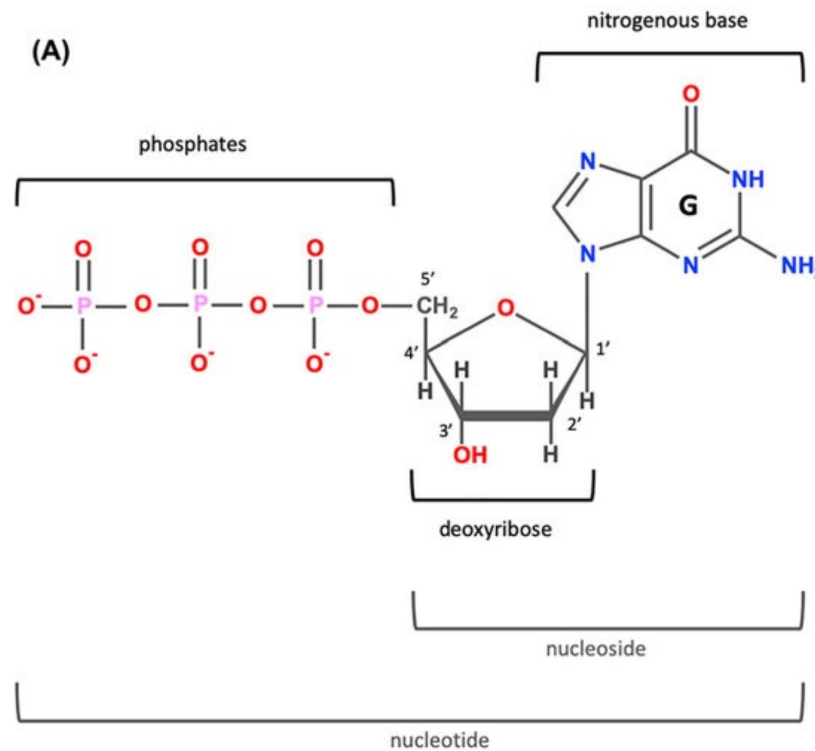
- ✓ Terminal labelling
- internal labelling

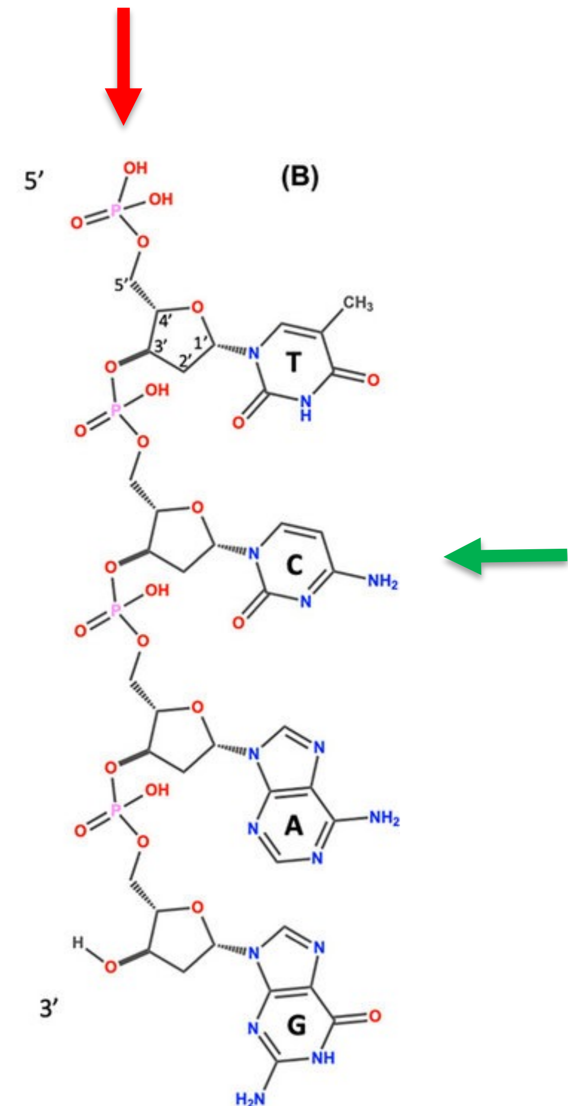
- ✓ DNA probe
- RNA probe

How to see your "specific" Nucleic Acids

Making labelled probes

- ✓ Terminal labelling
- internal labelling





How to see your "specific" Nucleic Acids

✓ **Radioactive tracer**

benefits: high sensitivity

disadvantages: dangerous because mutagenic
short life

non radioactive tracer

benefits: safety
long life

disadvantages: lower sensitivity

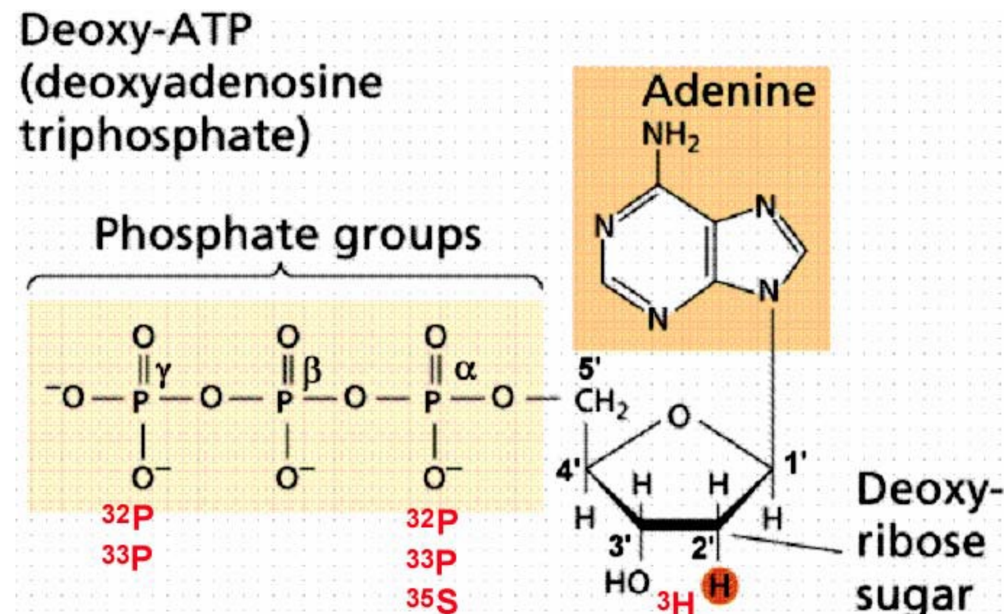
Labelling nucleotides with radioisotopes

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

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

or

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



dNTP labelled at position α (dATP e dCTP) for internal labelling

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

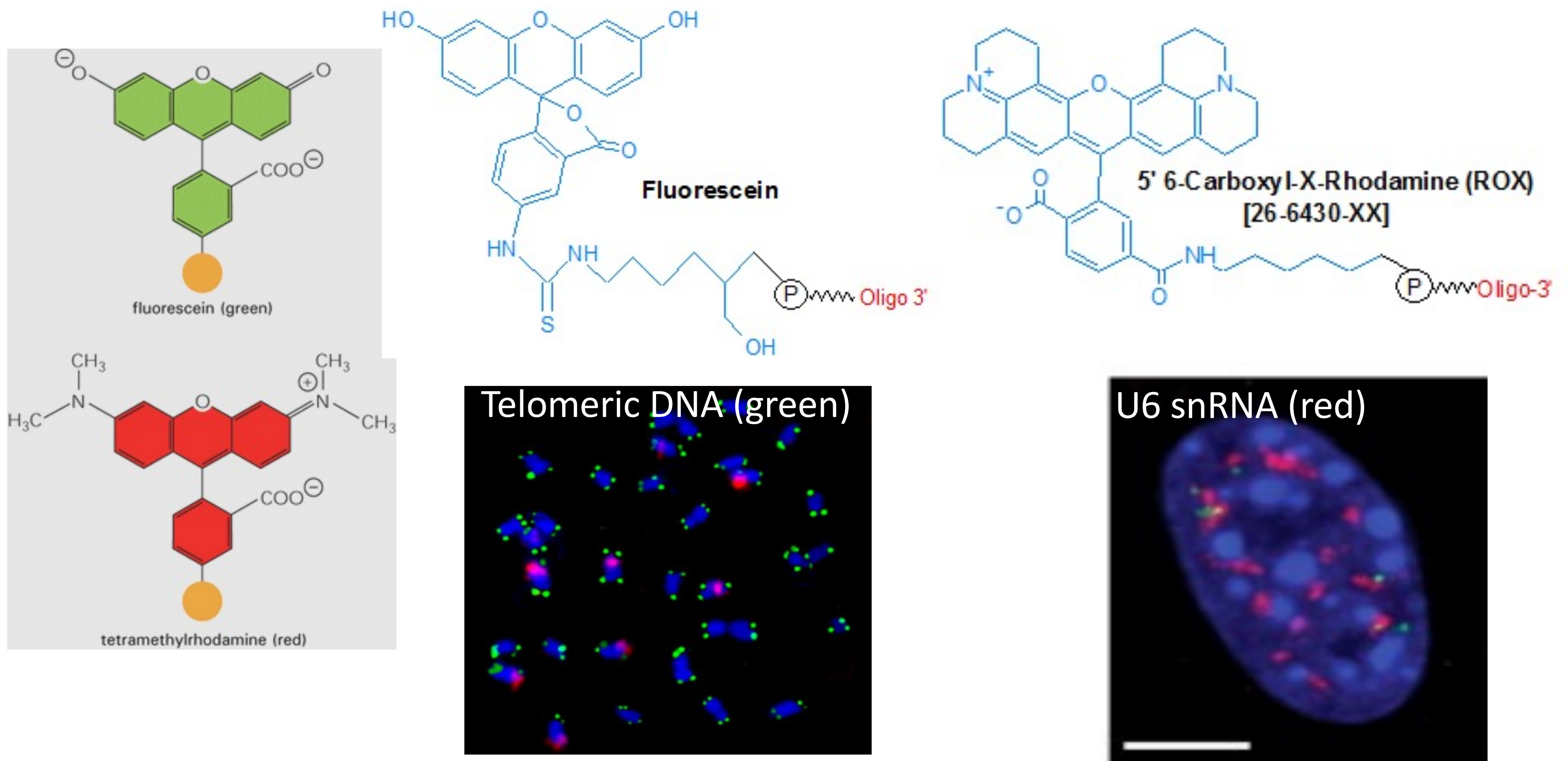
Non radioactive tracers

- ✓ fluorochromes (direct labelling)
- ✓ digoxigenin or Biotin (undirect labelling)

Non radioactive tracers

Fluorescence

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



Non radioactive tracers

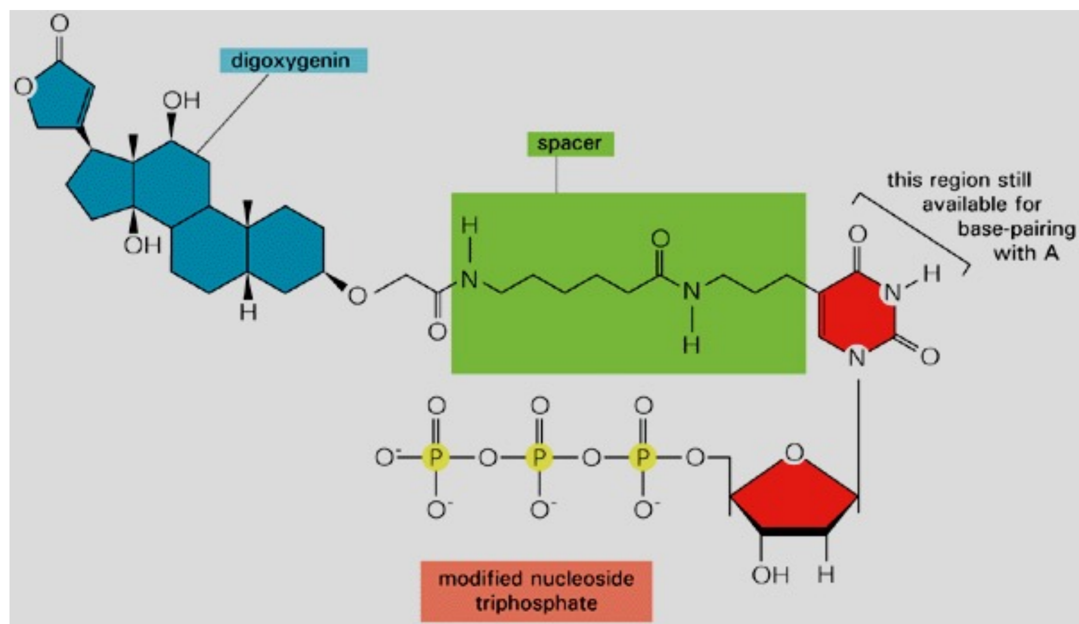
Chemiluminescence

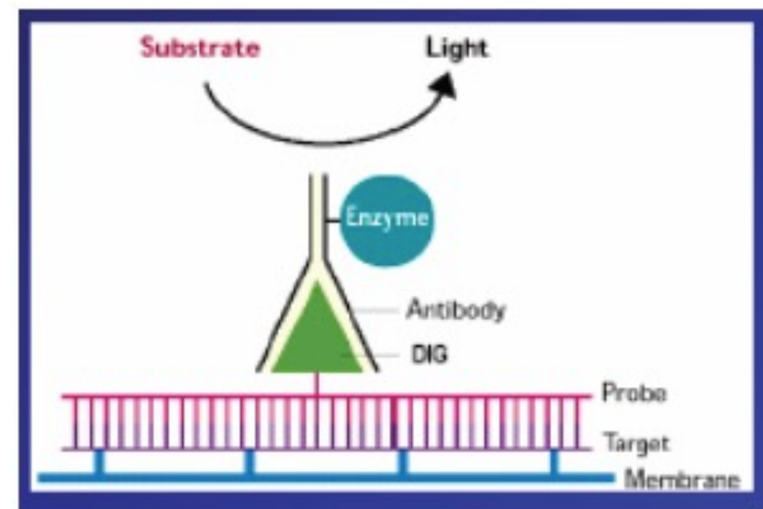
Nucleotides digoxigenin marked:

Digoxigenin is a cardiotonic steroid isolated from the Digitalis purpurea.

The probe is bound and recognized by an immuno-enzimatic system that uses an antibody against the digoxigenin (anti-DIG). The antibody is ligated to an alkaline phosphatase. The presence of such a complex is reveiled by adding a chromogenic or luminogenic substrate that only "emits" when is processed by the alkaline phosphatase.

The high specificity of the antibody ensures the high specificity of the detection.



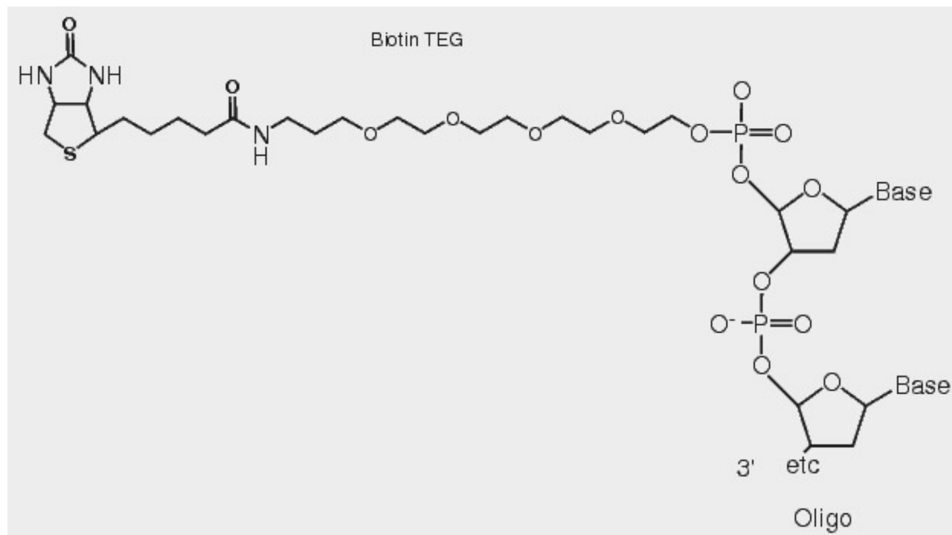


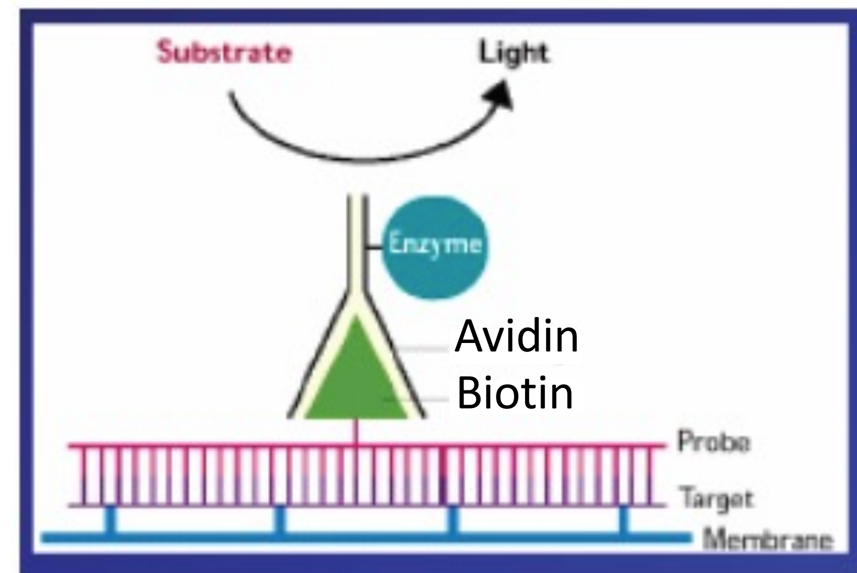
Non radioactive tracers

Biotinilated nucleotides

Biotinilated nucleitides can be incorporated in the DNA both terminally and internally.

The Biotin is specifically bound by avidin or streptavidin. Therefore it is possible to use avidin or spreptavidin marked with fluorophores or linked to enzymes (phosphatases or peroxidases). Providing chromogenic or fluorogenic substrates for these enzymes you allow the production of a measurable signal.





Labelled Nucleic Acids

DNA/RNA Oligonucleotides probes can be **terminally** labelled

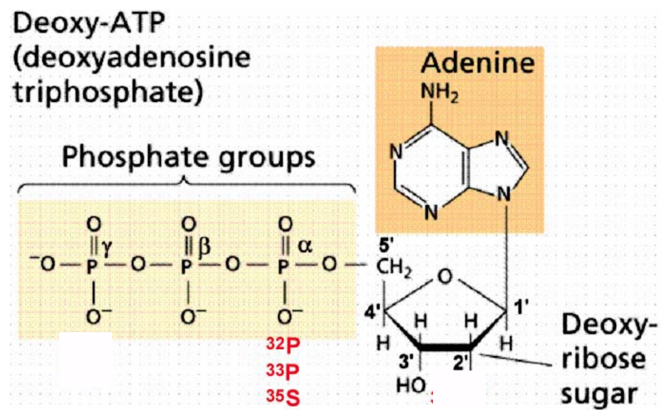
Double stranded DNA probes can be **internally** labelled by nick translation or random rpiming

RNA probes can be in vitro transcribed and **internally** labelled

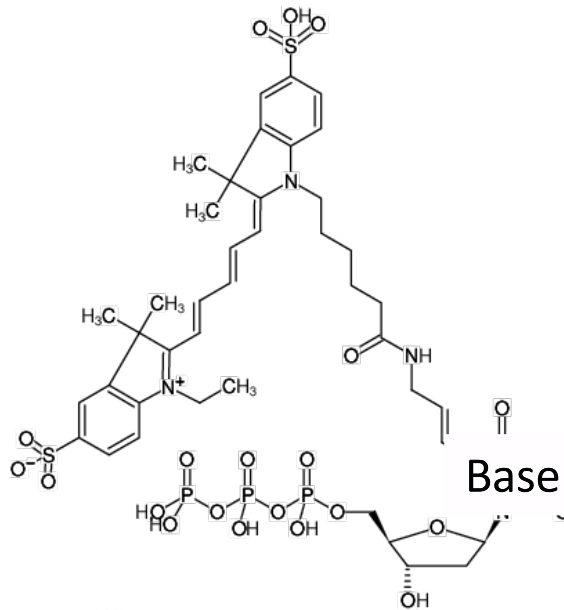
How to label DNA

Nick translation

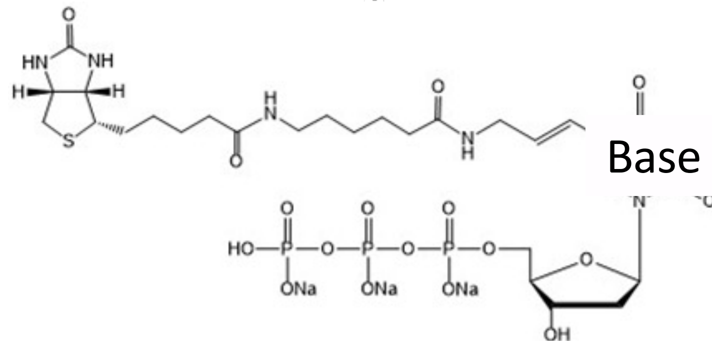
Random priming



Structural formula of α–dATP



Structural formula of dNTP-Cy5

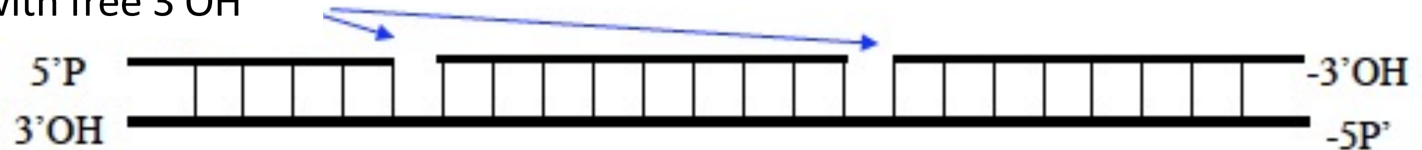


Structural formula of dNTP-Biotin

Nick translation

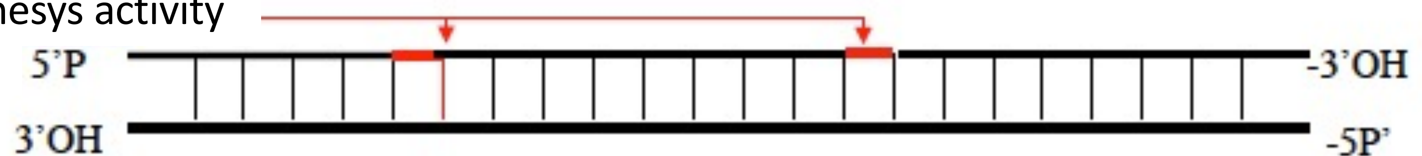
DNasi I breaks the phosphodiester bond and creates a nick on one strand.

Nick with free 3’OH



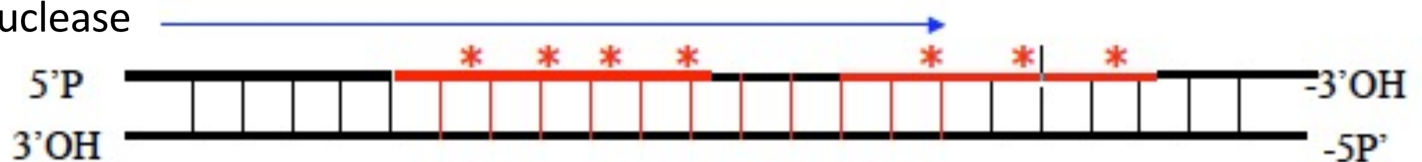
DNA polymerase I synthetises a new labelled DNA strand starting from the free 3’OH

DNA pol I 5’→3’ sinthesys activity



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

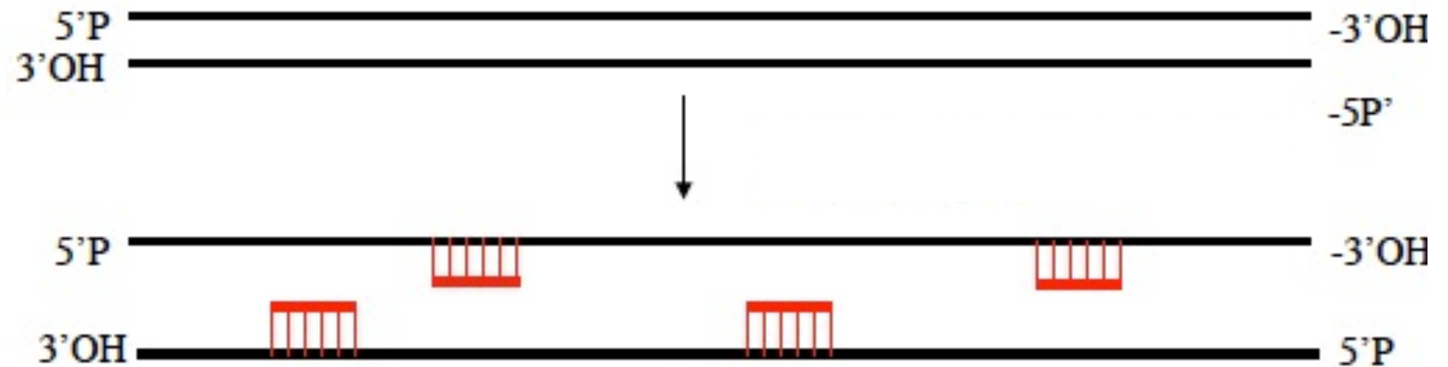
DNA pol I 5’→3’ exonuclease



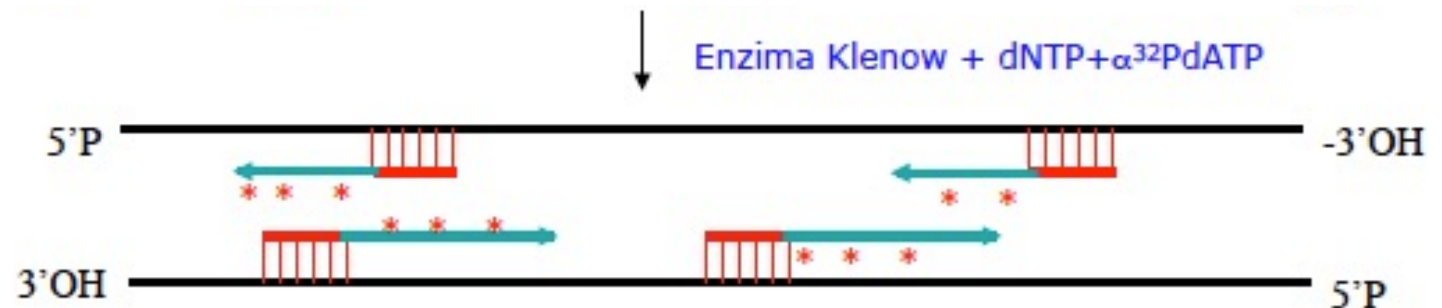
The result of these activities in presence of lablelled nucleotides is the generation of labelled DNA fragments.

Random priming

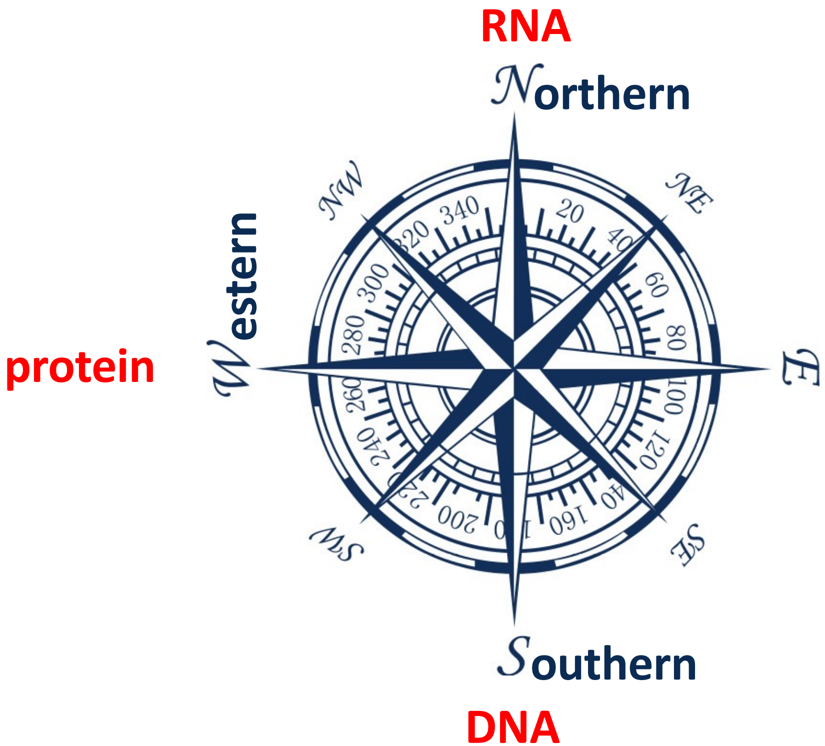
DNA denaturation and primer hybridization



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

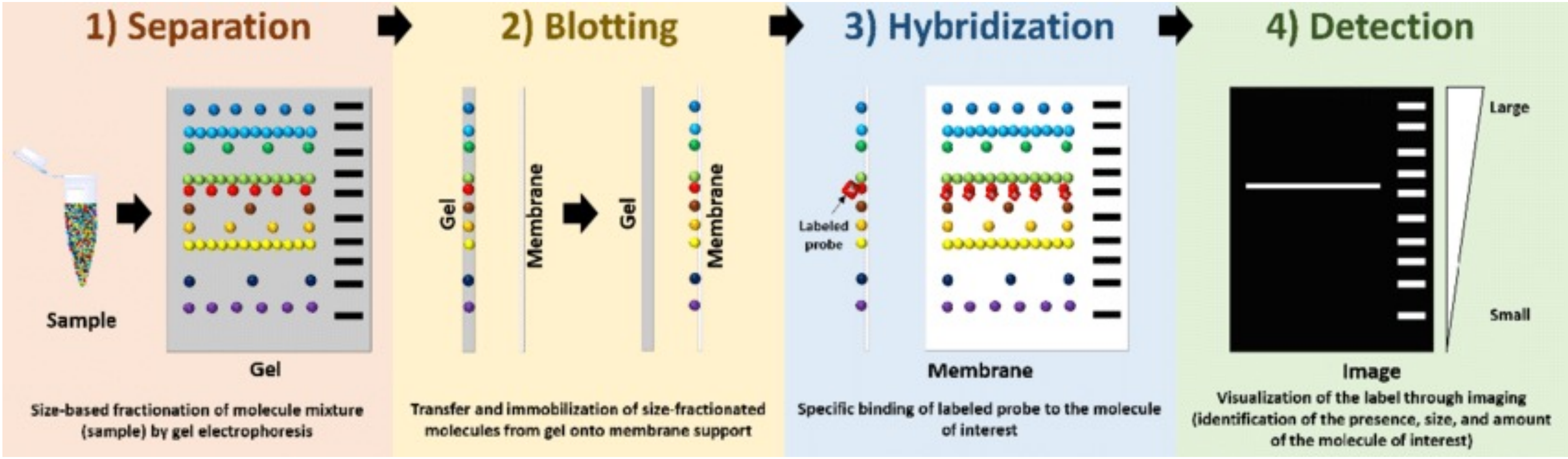


By adding α^{32}PdNTP to this reaction the new sinthetised DNA filament will result labelled.



Blotting compass

DNA/RNA probe
And/or
Antibodies



Southern Blot

- **Procedure**

- Genomic DNA isolation

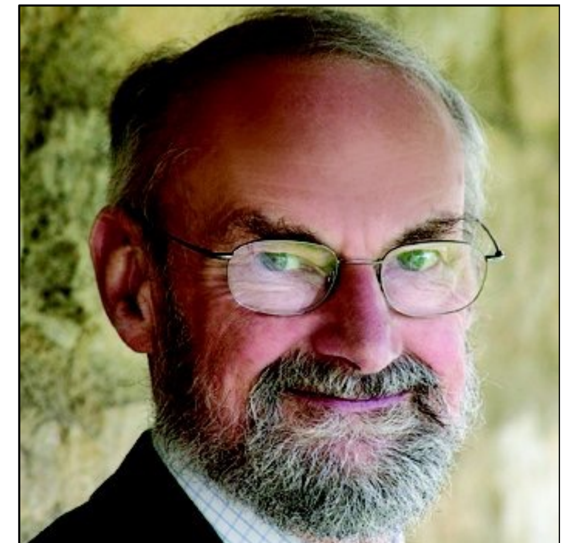
- Digestion with restriction enzymes for generating short fragments (from hundreds to some Kilo bases)

- 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



Sir Edwin Southern

Southern Blot

Target GENE

Genomic DNA

Restriction Endonuclease

DNA Fragments

Gel electrophoresis

Long fragments

Short fragments

Direction of migration

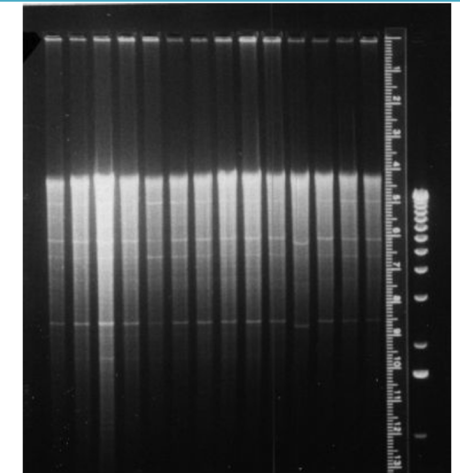
- denature in alkali
- transfer to nitrocellulose
- bake

SS DNA fragments

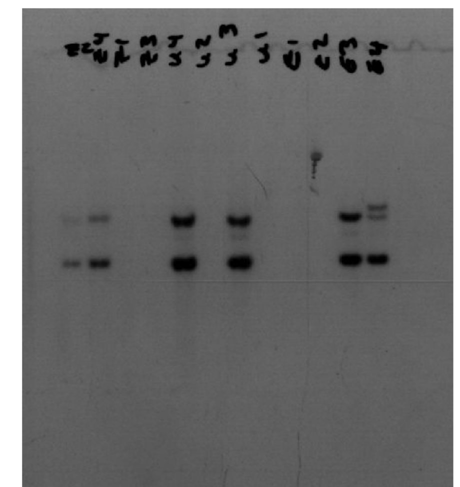
- hybridize with radiolabelled probe
- wash

Autoradiogram

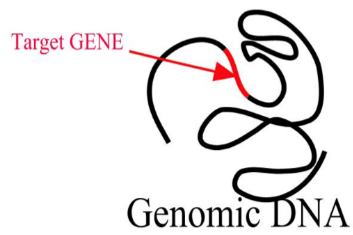
Gel



X-ray film



Southern Blot



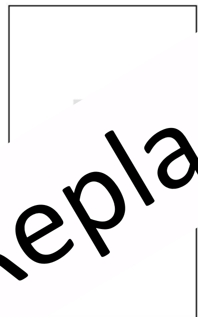
Restriction Endonuclease

Gel electrophoresis

Long fragments

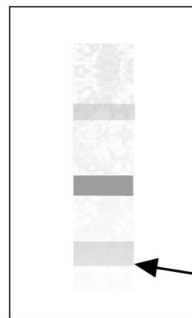
Short fragments

Directic



wash

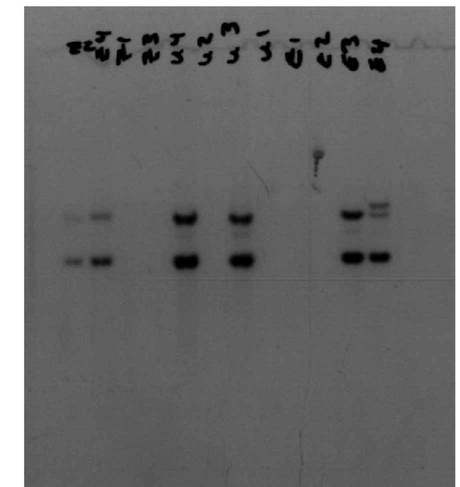
Autoradiogram



- denature in alkali
- transfer to nitrocellulose
- bake

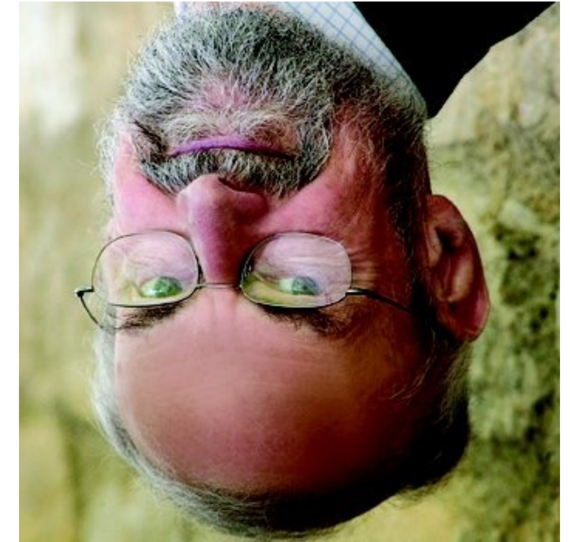
SS DNA fragments

X-ray film

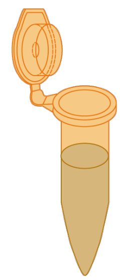


Replaced by PCR or Genomic sequencing

Northern blot



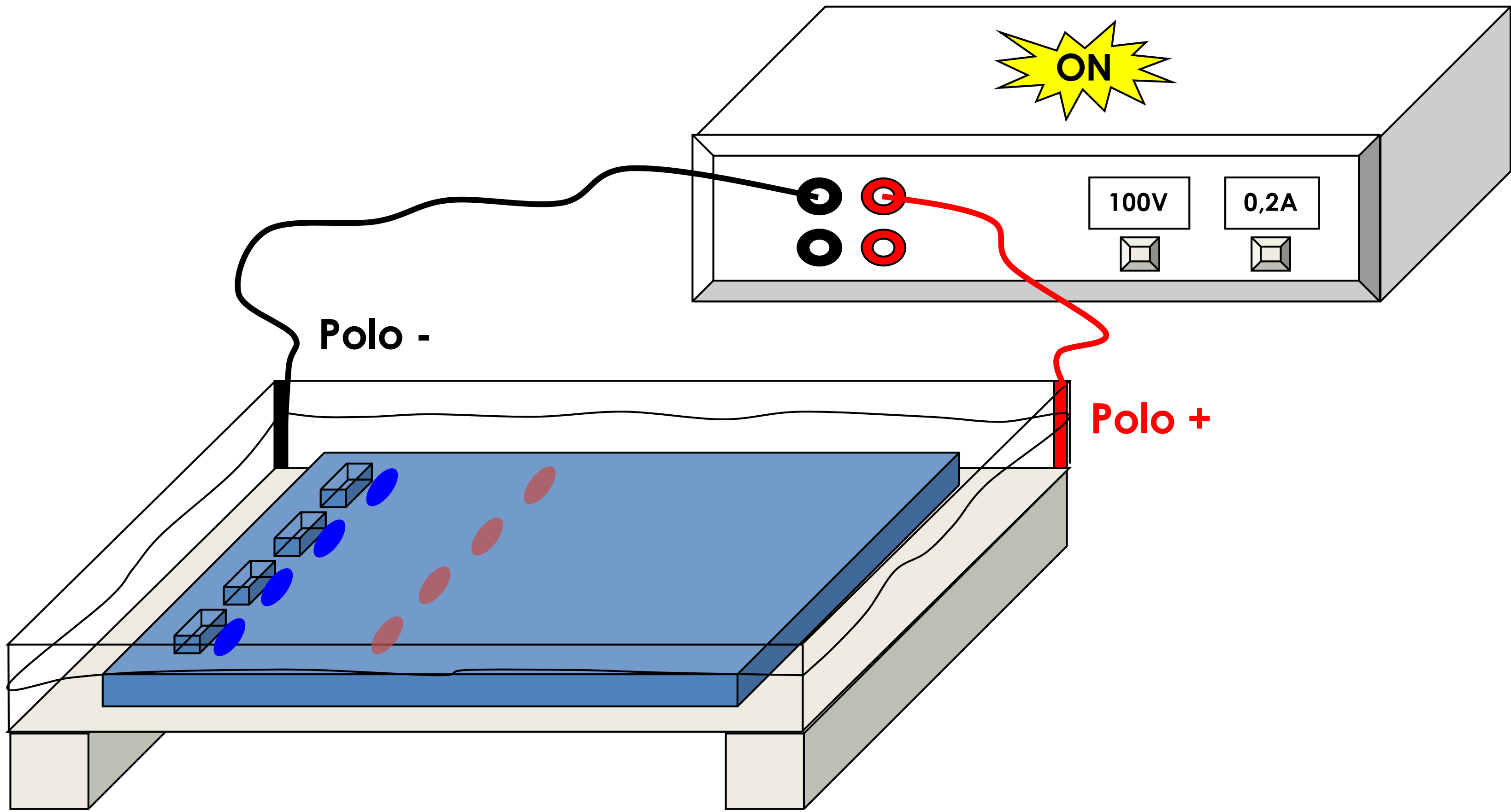
- Procedure
  - RNA extraction
  - Remove secondary structures
  - Electrophoresis (agarose or acrylamide)
  - Transfer the RNA on membrane (nylon)
  - hybridization with labelled complementary probe



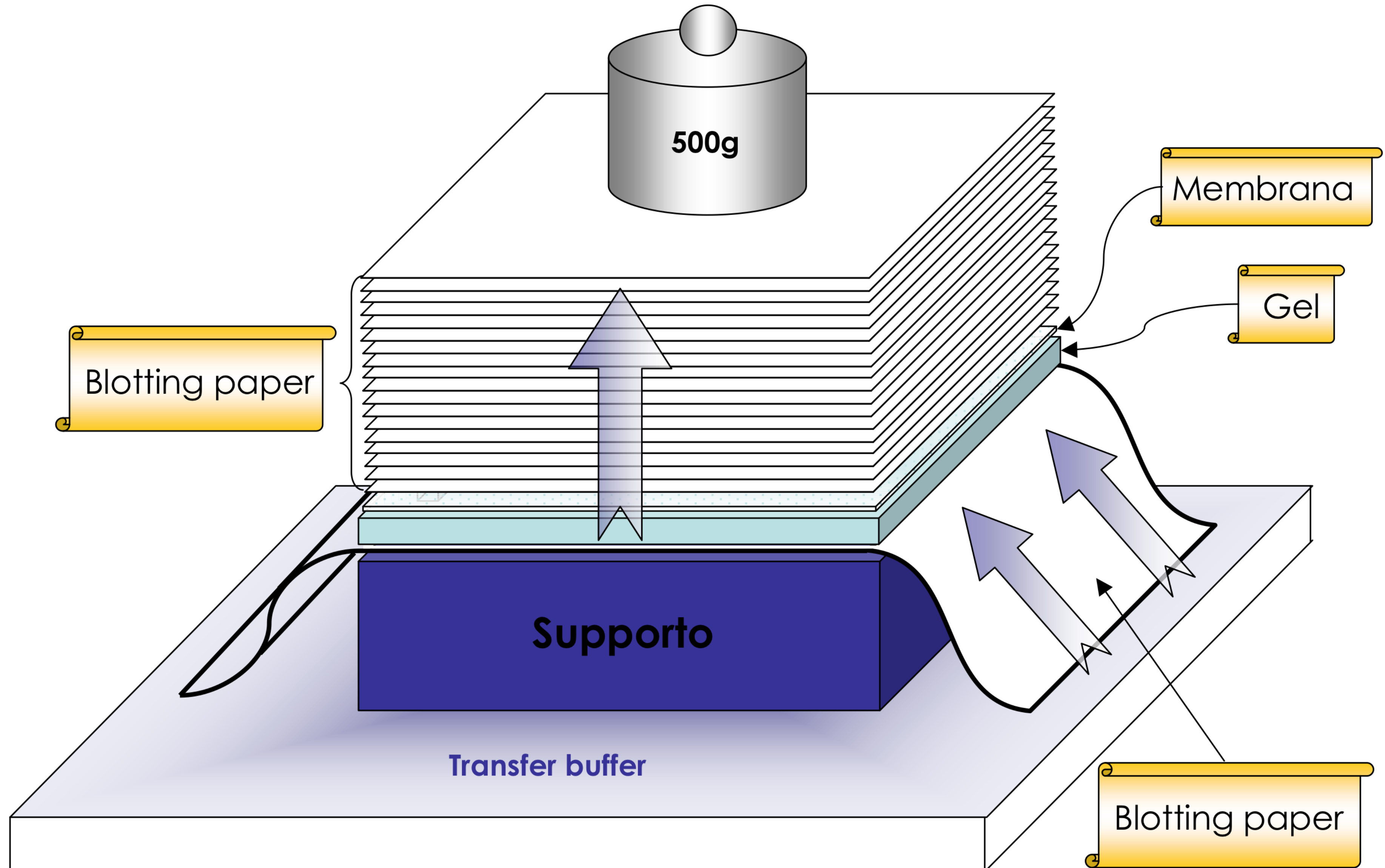
+ Loading dye

Loading dye helps the sample loading in the well

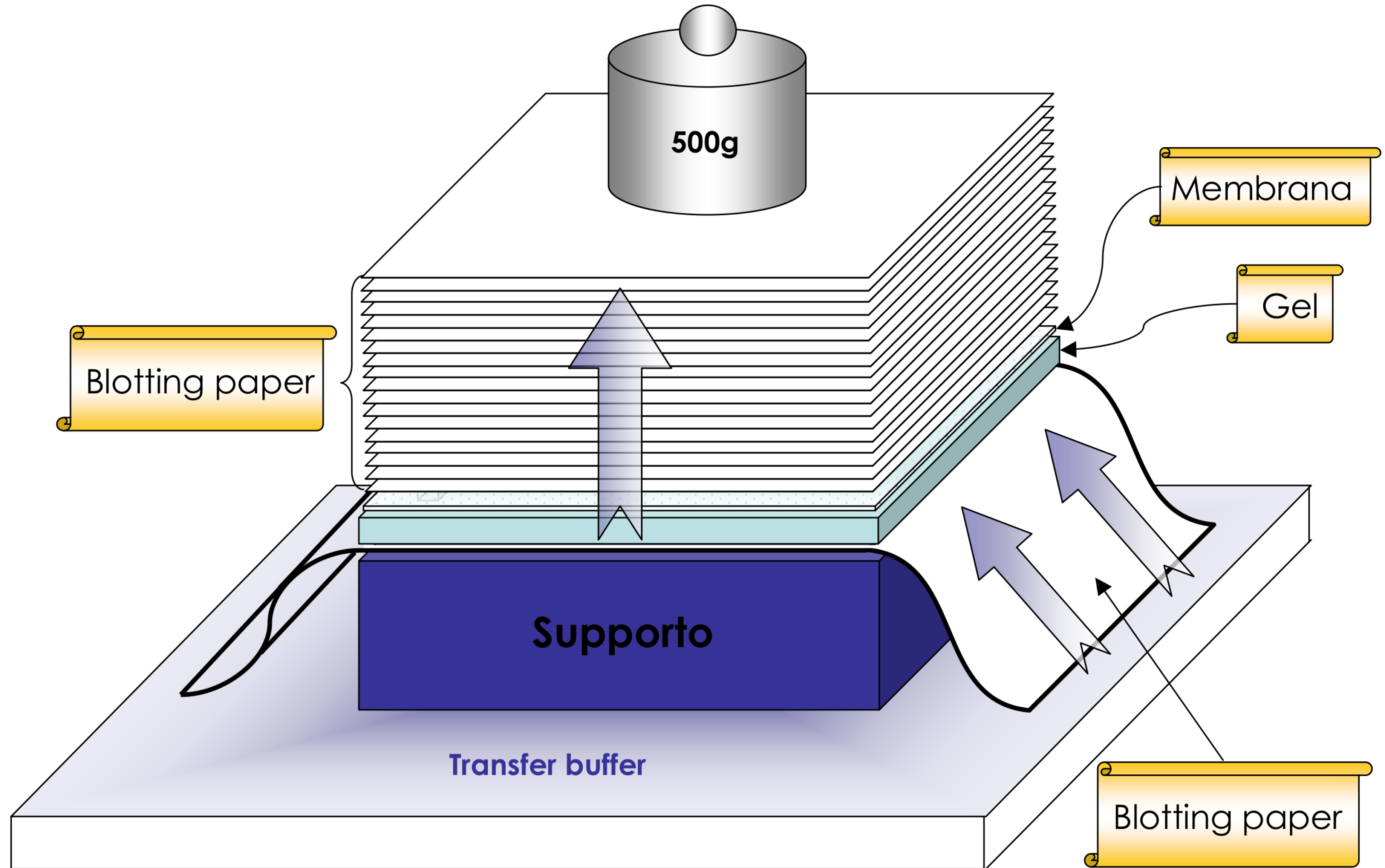
It contains glycerol, bromophenol Blu and xilencianol Blu that migrate in the gel at different speeds.


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)

ON

10V

80 mA

Membrana (nylon)

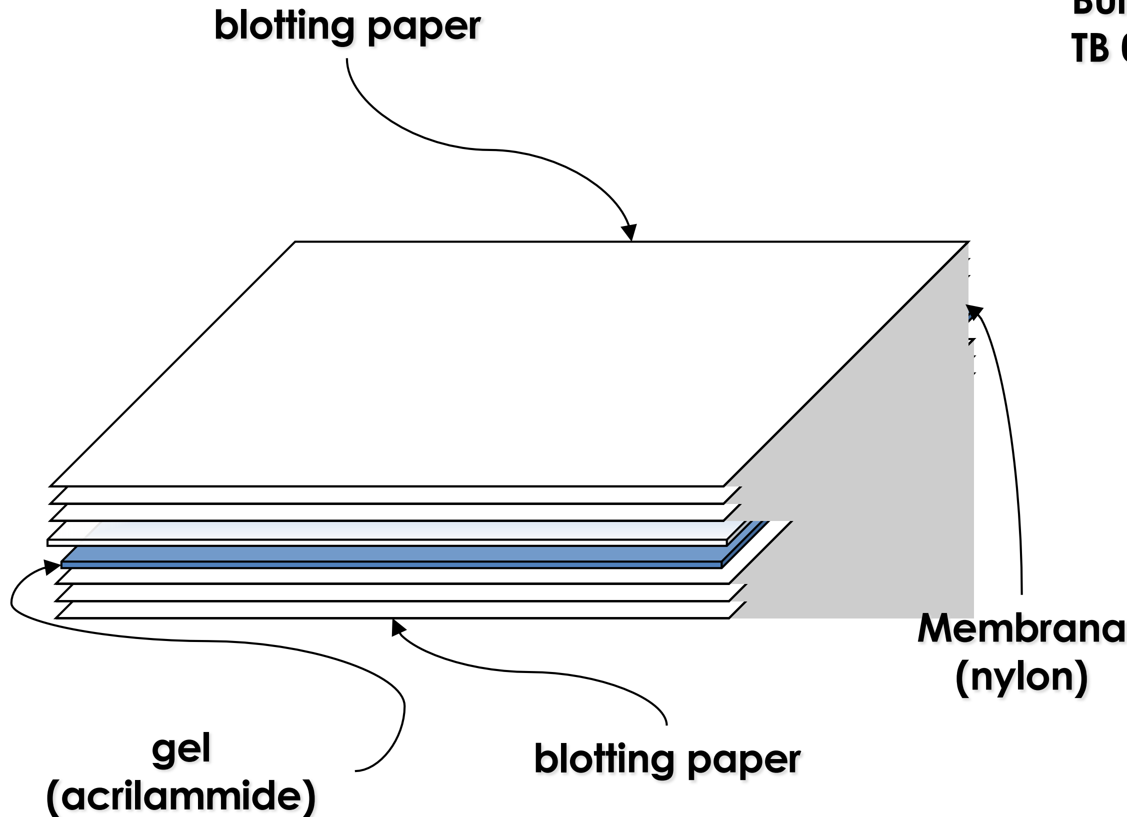
gel

Buffer
TB 0.5X

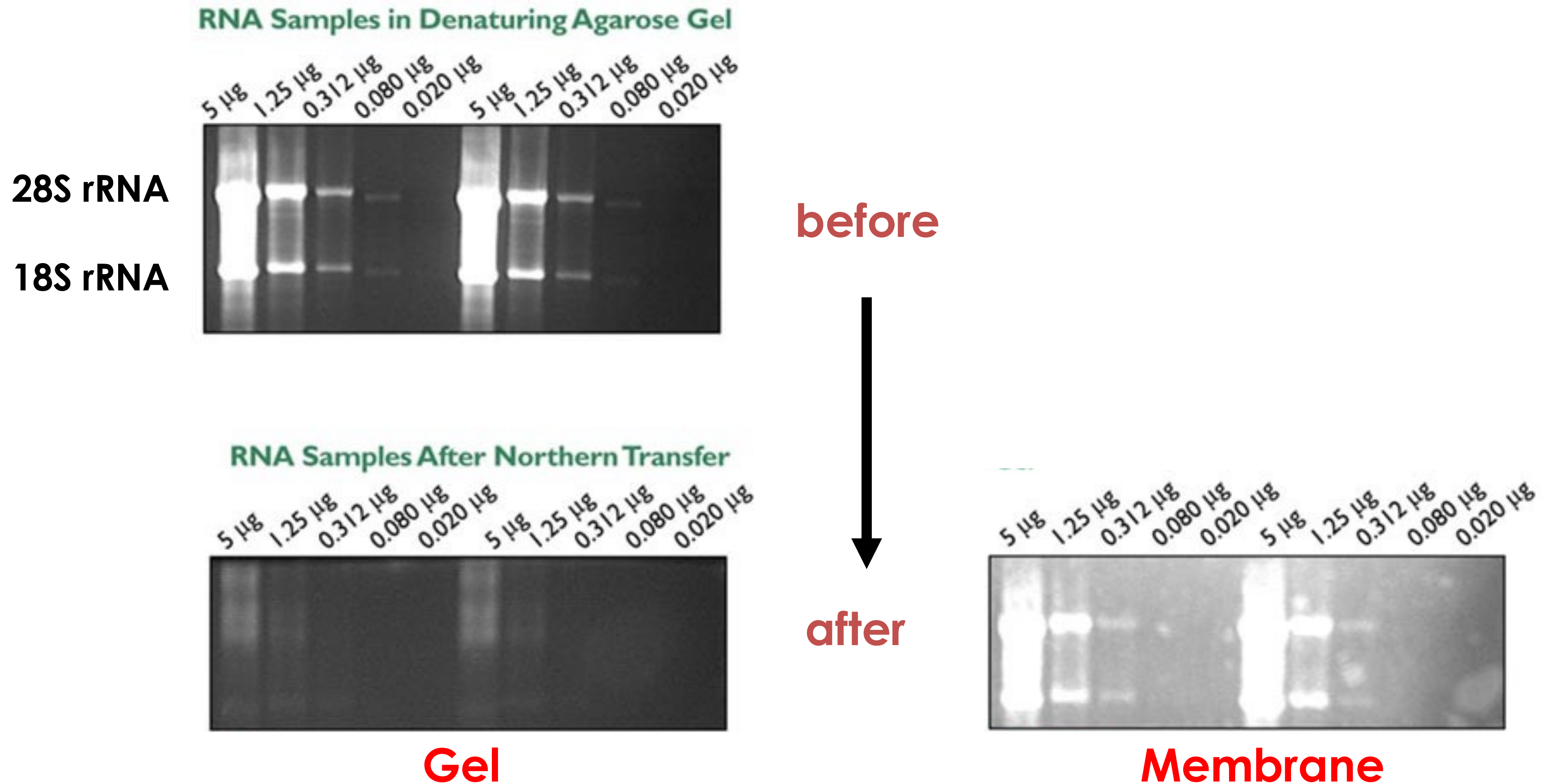
Polo -

blotting paper

Polo +

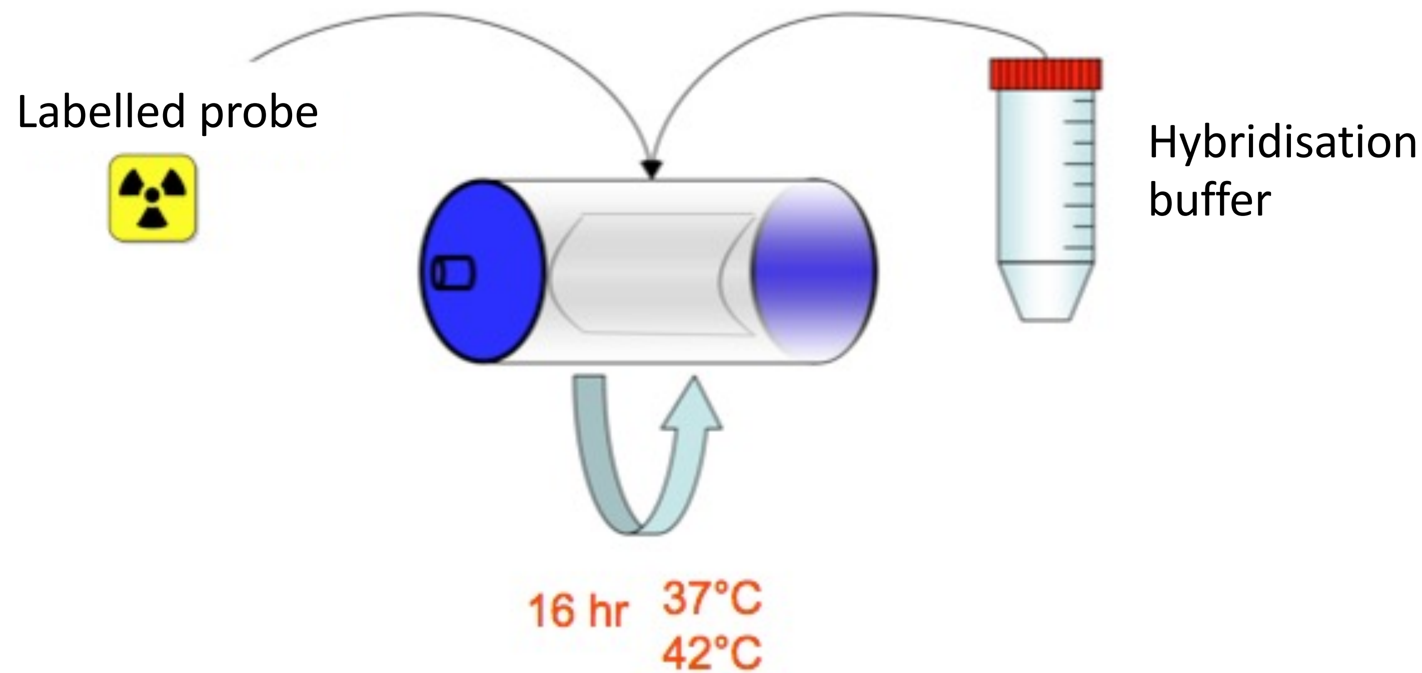


Ethidium bromide staining helps to check the proper RNA transfer on the membrane

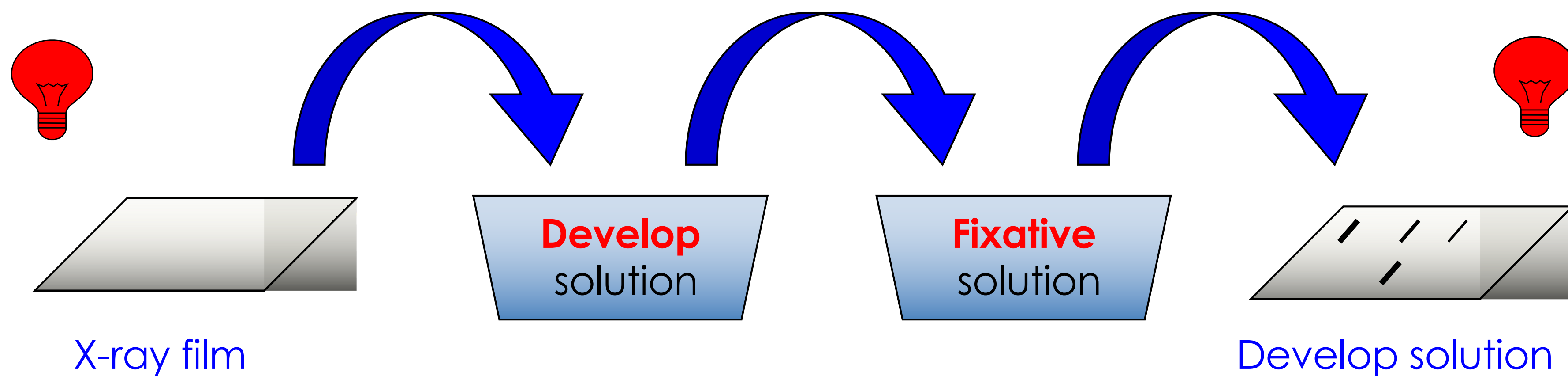


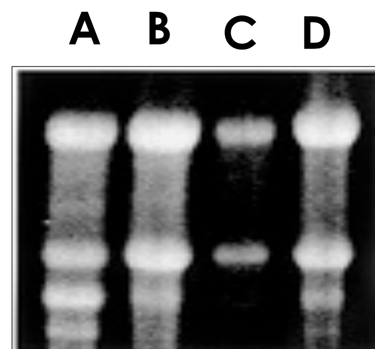
Hybridisation

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



Detection of the signal



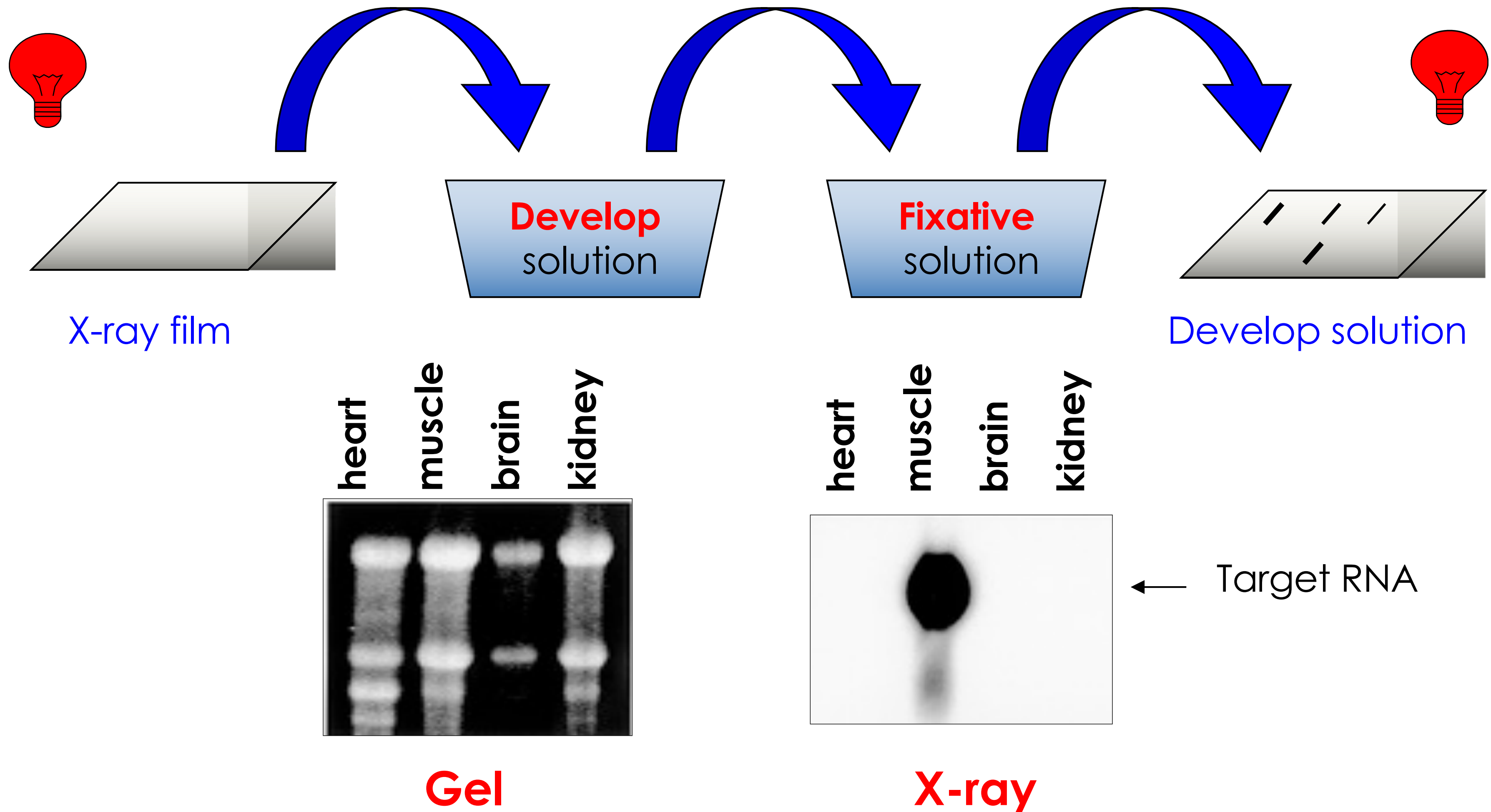


Gel

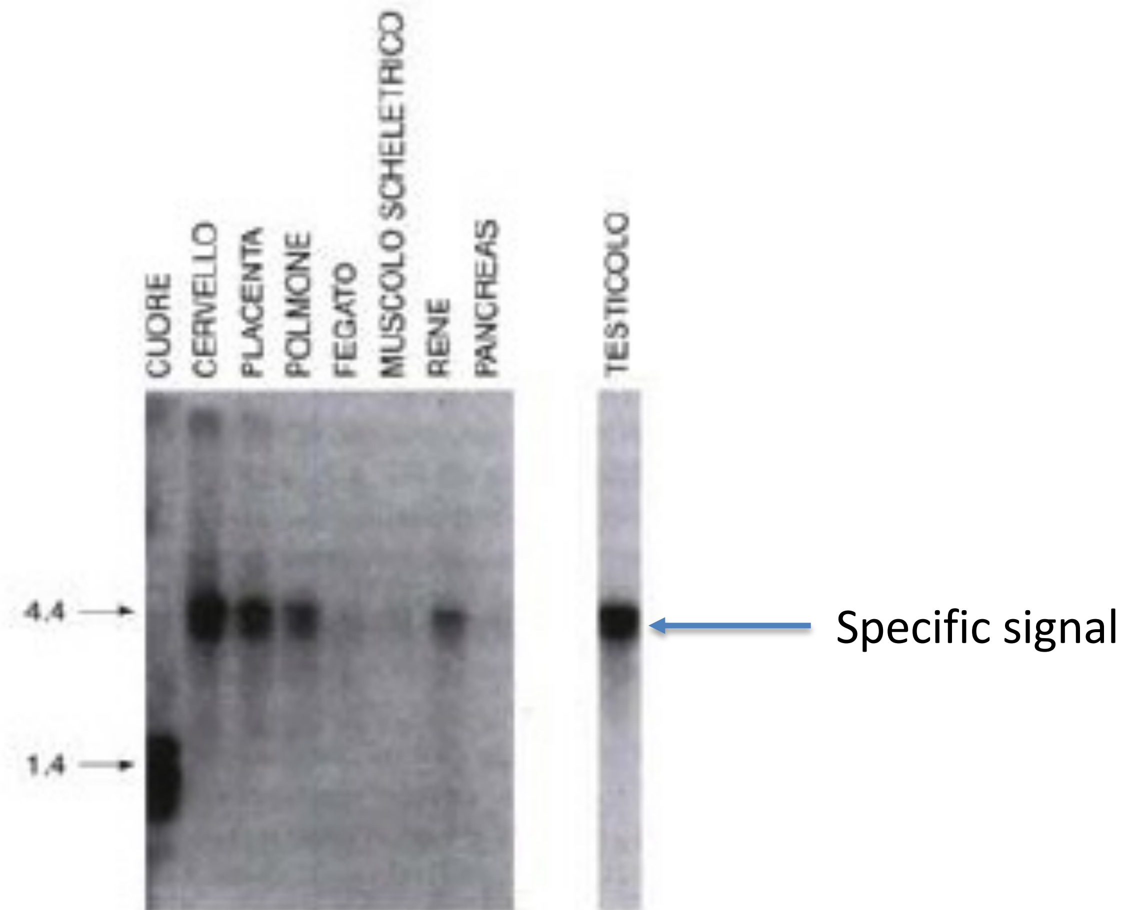
X-ray

Detection of the signal

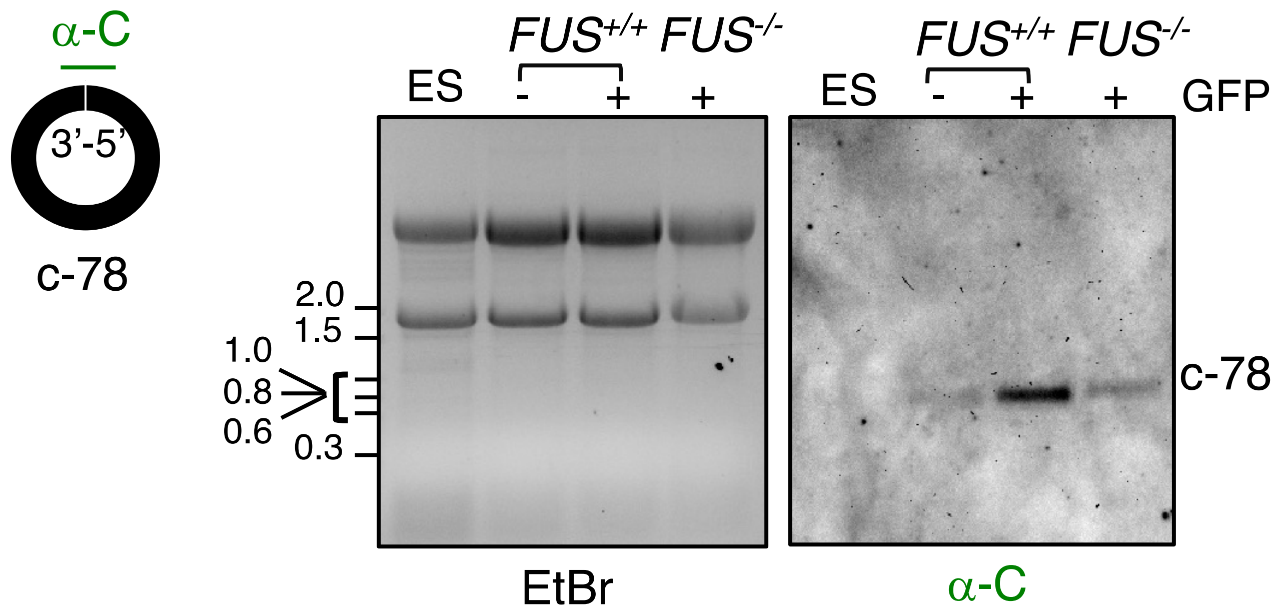
In which tissue is the RNA expressed?



Northern blot to study gene expression

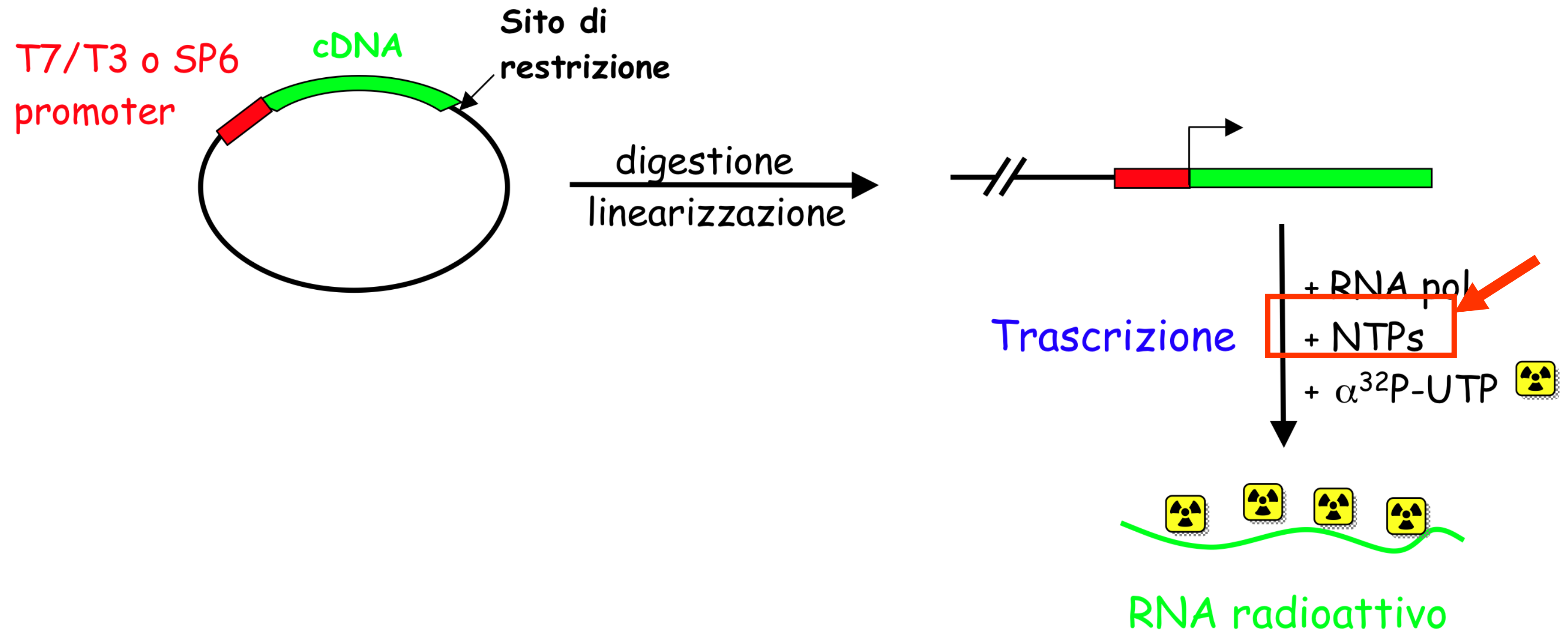


Northern blot to study circRNA expression



How to label RNA

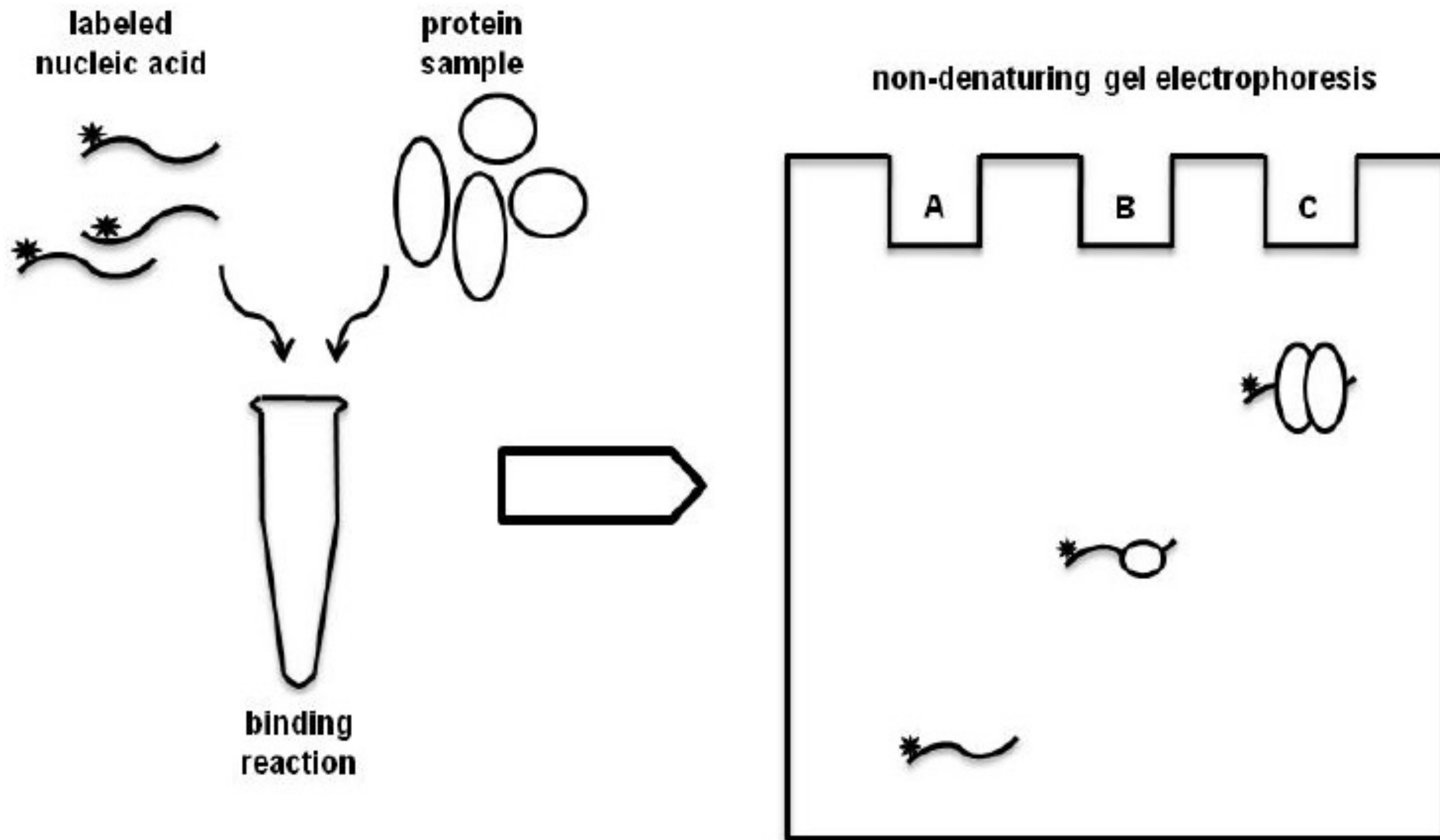
In vitro transcription:



https://www.youtube.com/watch?v=ClhpfLq2RIc

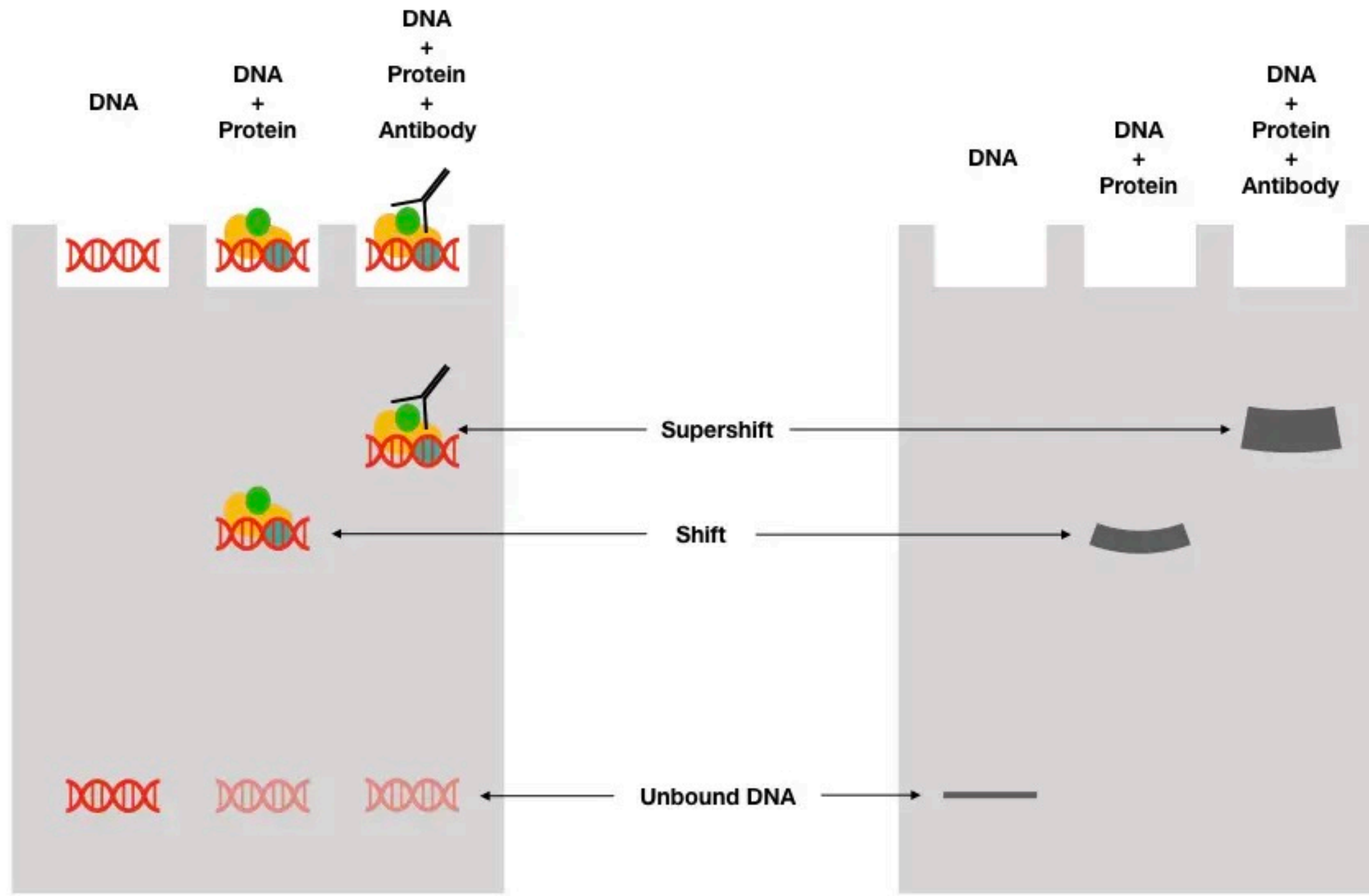
Electrophoretic mobility shift assay - Band shift assay

Does this protein bind this RNA/DNA?

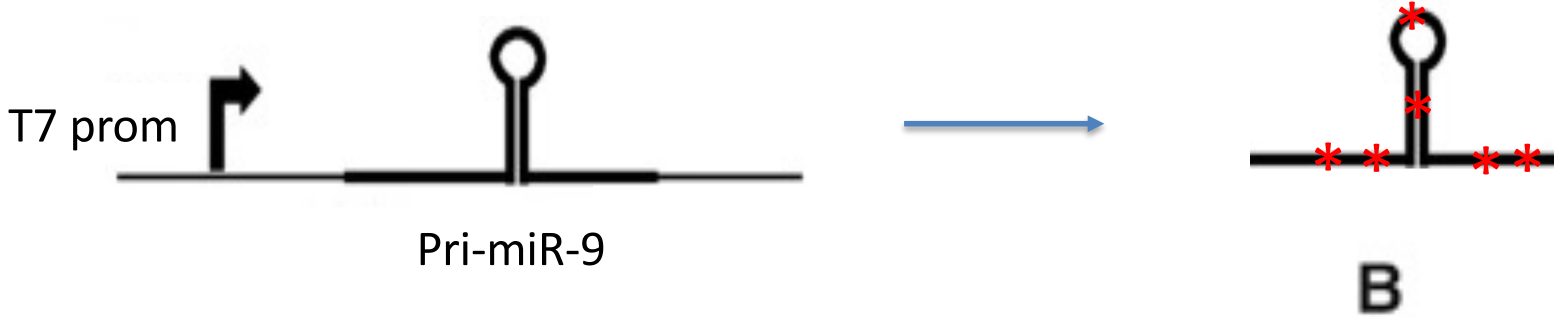


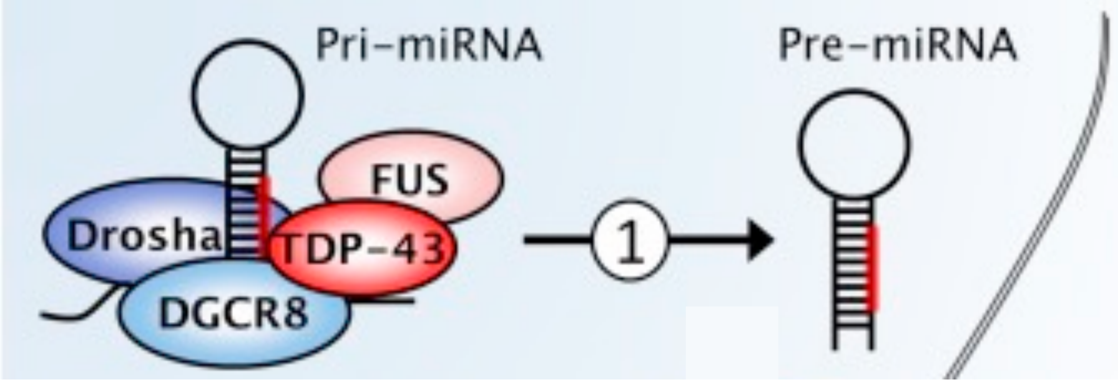
Electrophoretic mobility shift assay - Band shift assay

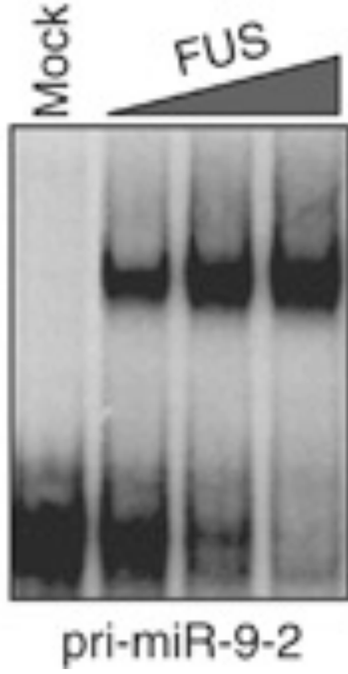
Is the shift specific?



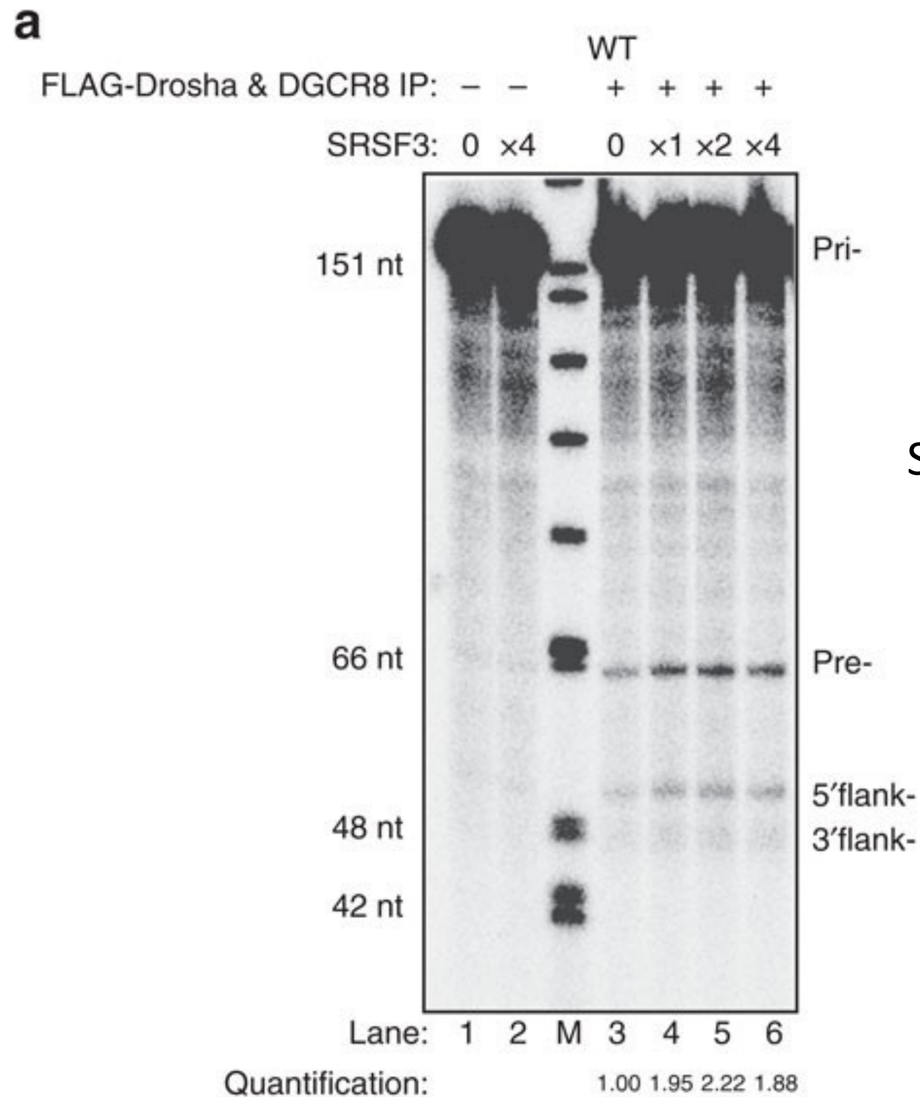
FUS binds pri-miRNA and stimulate their processing







pri-miRNA in vitro processing



SRSF3 stimulates pri-miRNA processing

How to study splicing vitro