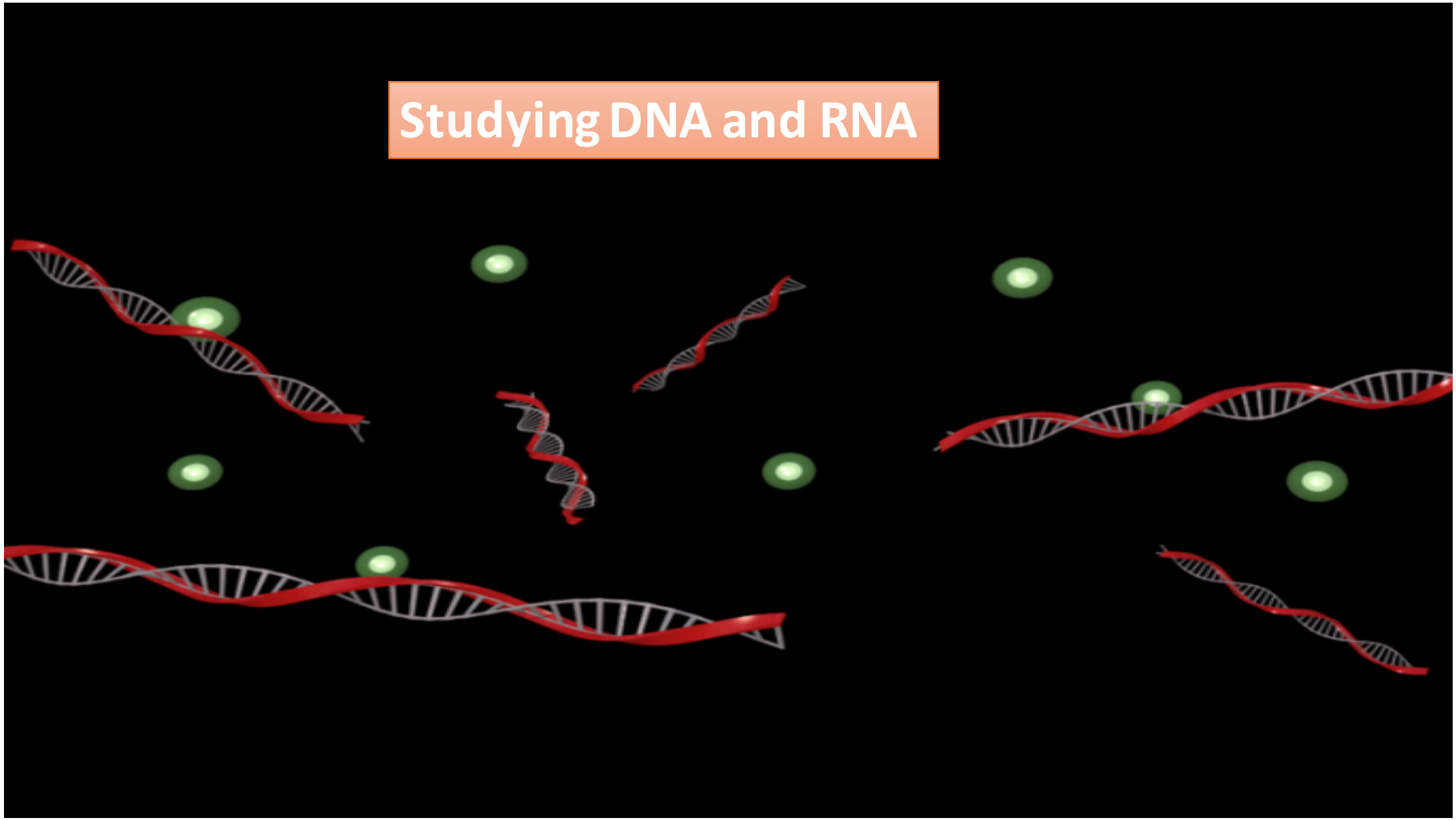
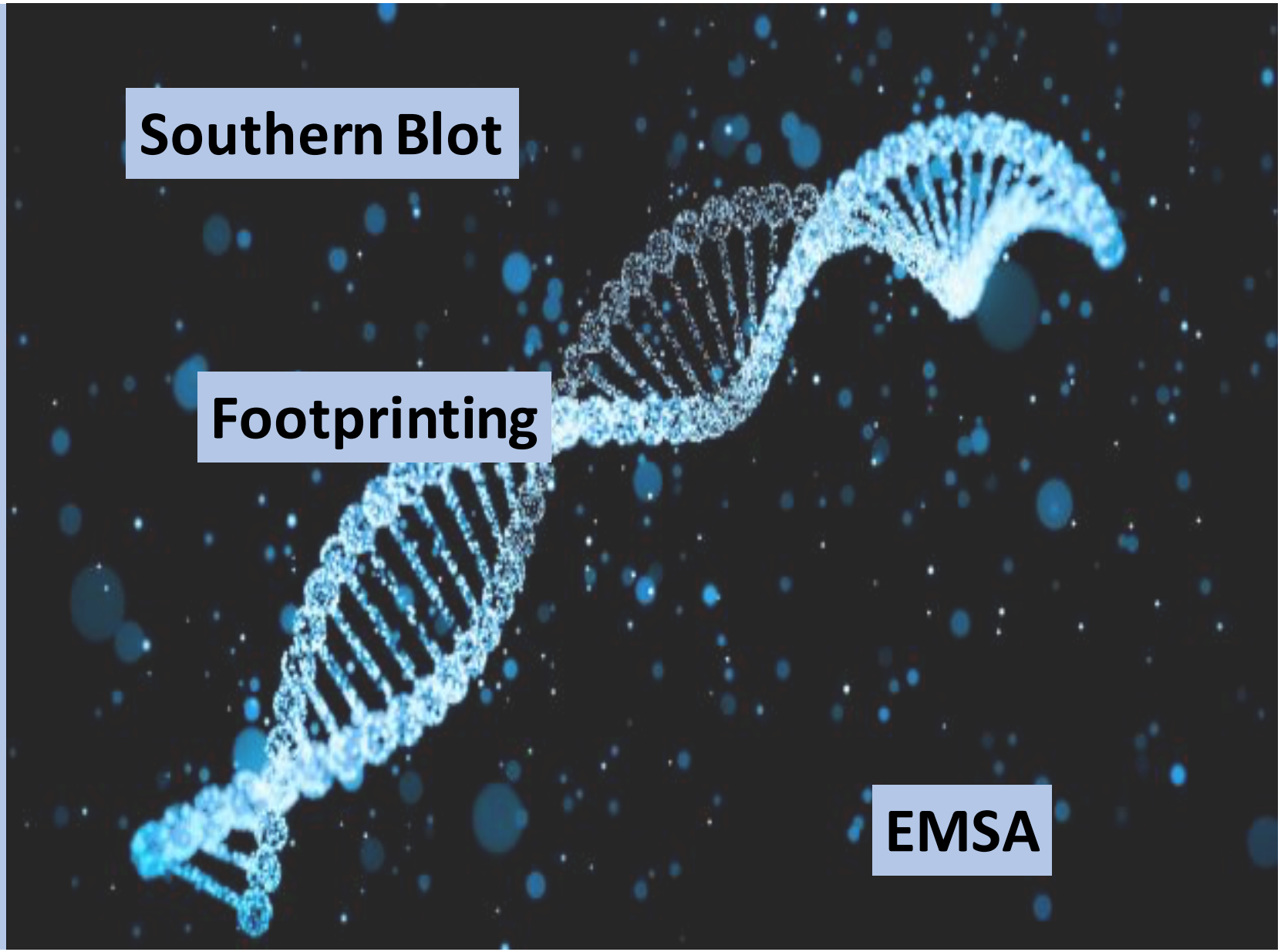
Studying DNA and RNA



Focus on

DNA



Focus on

DNA

Southern blot - Introduction

Blots are techniques for transferring **DNA** ,**RNA** and **proteins** onto a carrier so they can be separated, and often follows the use of a gel electrophoresis.

The **Southern blot** is used for transferring DNA, the **Northern blot** for RNA and the **western blot** for PROTEIN.

Southern blotting

In the 1970s **Edwin Southern** of Oxford University invented a revolutionary DNA blotting technique.

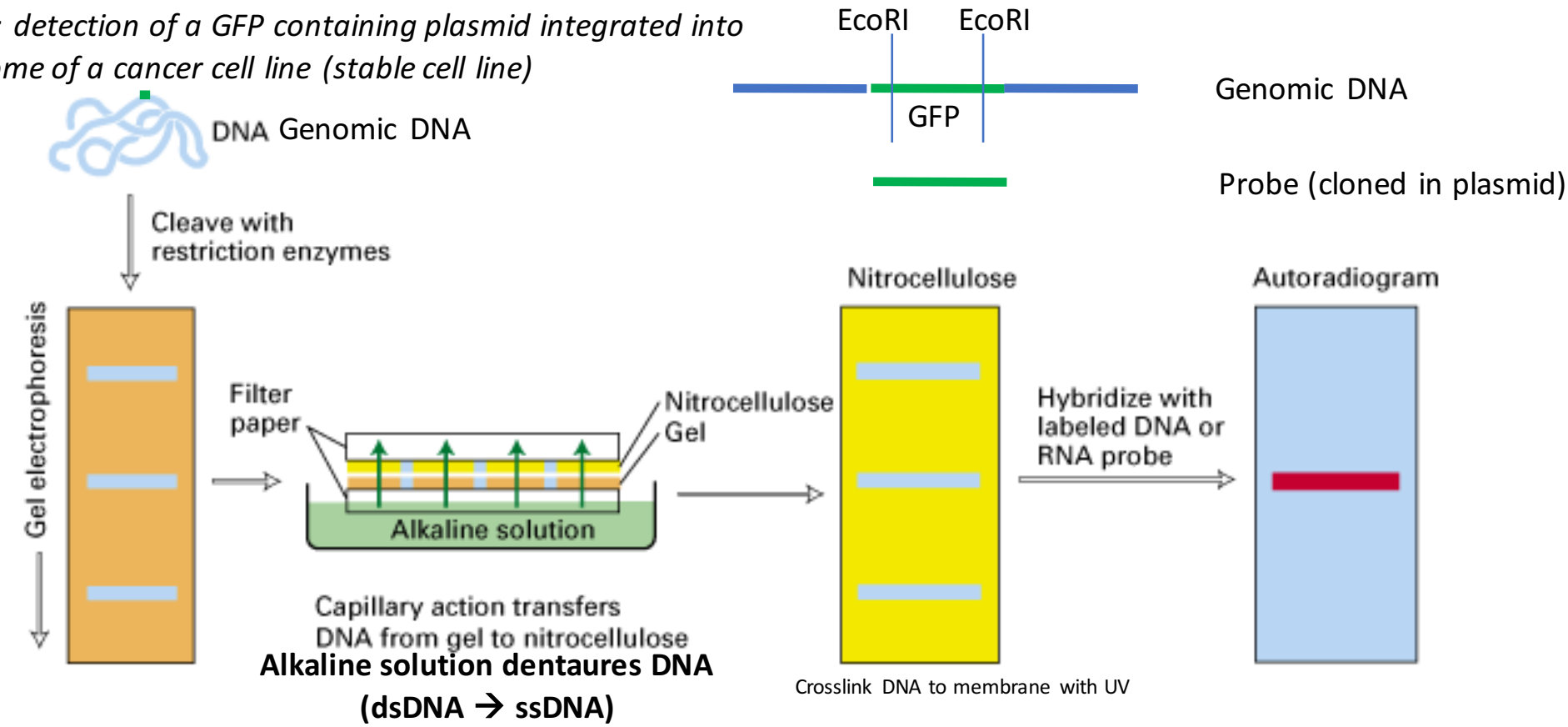
The Southern Blot allows the visualization of one DNA fragment from a whole genome DNA extract.

Southern Blotting

Identifying a fragment of DNA in a complex DNA sample (genomic DNA) that contains a sequence (for example a (fragment of a gene)) of interest

Required: a portion of the desired seqeunce must have been cloned first –> for example: an exon (ca 200-500nt)

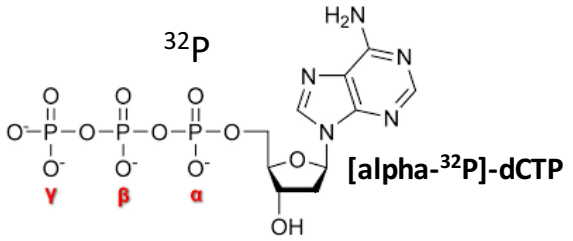
Example: detection of a GFP containing plasmid integrated into the genome of a cancer cell line (stable cell line)



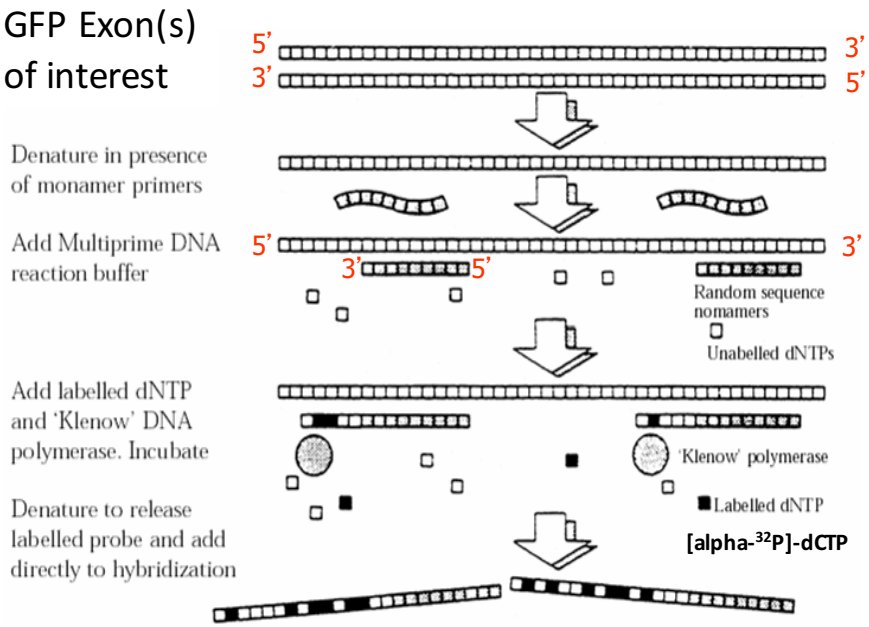
Probe labelling

LABELLING OF A PROBE

1. Labelling by incorporation of [alpha-^{32}P]-dCTP by klenow fragement (exo$^-$) PROBE SIZE 200 – 1000nt



Step 1: Get dsDNA for making the probe:
Fragment (for example 1 exon) of gene cloned into plasmid MCS
Step 2: Use restriction enzymes to cut out and gel-purify your fragment of interest
Step 3: denature dsDNA; add non-labelled mix of dNTPs and random 9-mer primers and buffer
Step 4: reanneal random primers to your DNA of interest and add [alpha-32P]-dCTP and the Klenow fragment. A fraction of primers that anneal perfectly will prime DNA synthesis by Klenow → radioactive [alpha-32P]-dCTP will be incorporated
Step 5: purify labelled fragments using silca column (remove non-incorporated [alpha-32P]-dCTP and other dNTPs, primers are remove
Step 6: Denature probe and mix with hybridization buffer -→ ready for hybridization

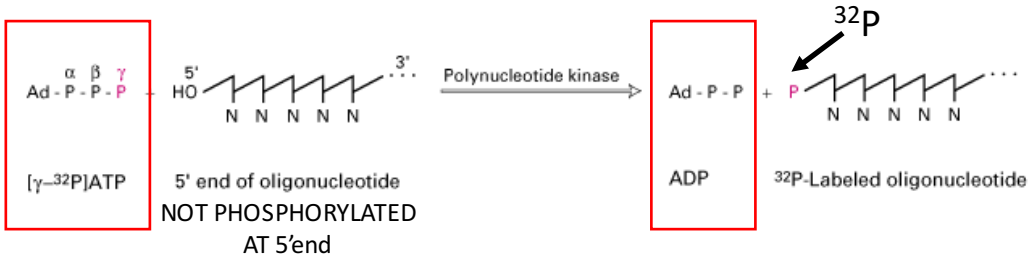


^{32}P is a high energy beta emitter and and decays into sulfur-32 by beta decay
half-life: 14,3 days
Protection required
A beta particle, also called beta ray or beta radiation, is a high-energy, high-speed electron (or positron) emitted by the radioactive decay of an atomic nucleus during the process of beta decay.

Probe labelling

LABELLING OF A PROBE

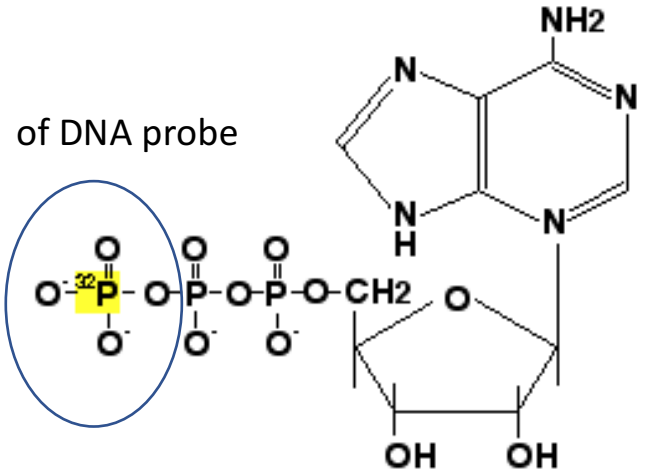
2. Labelling of terminus of OLIGONUCLEOTIDE using Polynucleotide Kinase (PNK) and [gamma-^{32}P]-ATP → size ca. 20 – 60 nt



Step 1: Get our oligonucleotide of interest (single stranded DNA; >20mer; must be highly specific!!!
Step 2: Mix oligonucleotide with PNK and [gamma-32P]-ATP, buffer = PNK reaction
Step 3: purify labelled oligonucleotides with special slica column; remove non-incorporated [gamma-32P]-ATP and other dNTPs
Step 5: Add hybridization buffer → ready for hybridization

REMEMBER THE DIFFERENCE

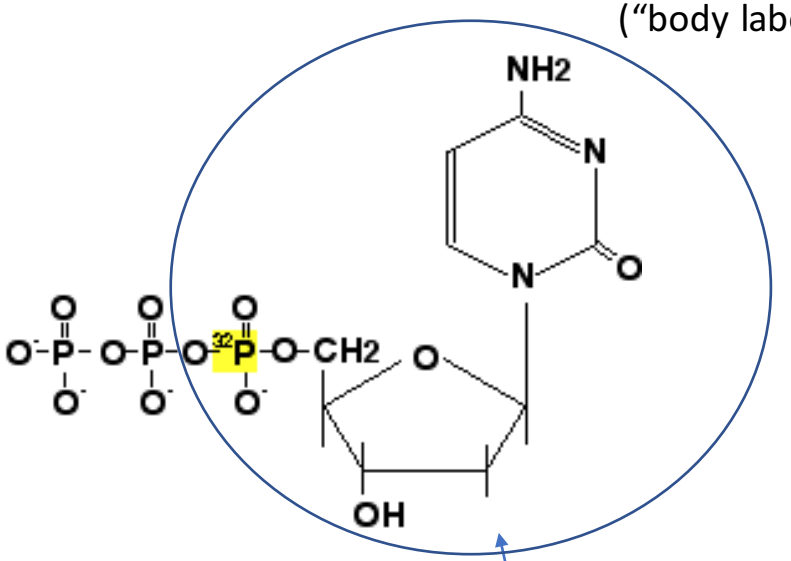
Transfered to 5’ end of DNA probe
(“end labelling”)



[γ-^{32}P]ATP

PNK

Incorported into probe
(“body labelling”)



[α-^{32}P]dCTP

KLENOW

Southern Blotting – Steps

Step 1: DNA purification

Isolate the DNA in question from the rest of the cellular material in the nucleus.

Incubate specimen with detergent to promote cell lysis.

Cell Lysis frees cellular proteins and DNA.

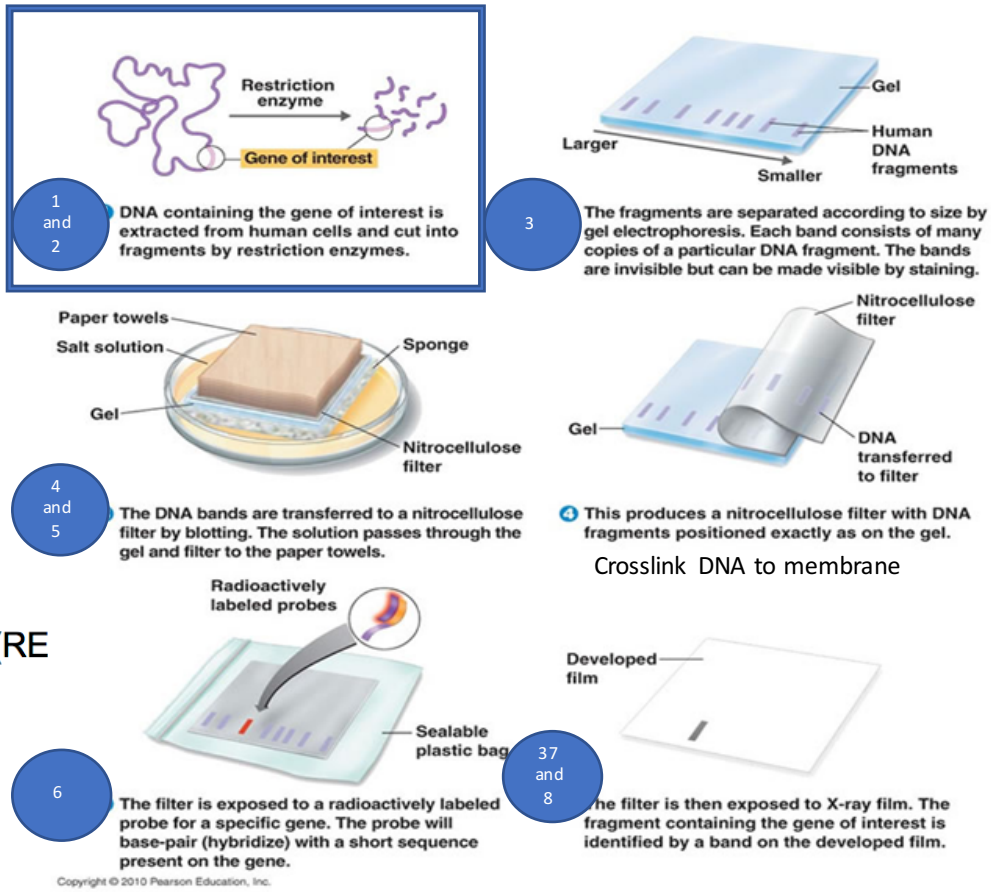
- Proteins are enzymatically degraded by incubation with proteinase.
- DNA is purified from solution by alcohol precipitation.
- Visible DNA fibers are removed and suspended in buffer.

Step 2 : Restriction digestion

Cut the DNA into different sized fragments using restriction endonucleases (RE

By purifying DNA use

- **DNAse free** solutions and reagents
- **RNAse** to avoid RNA contamination
- **ice**

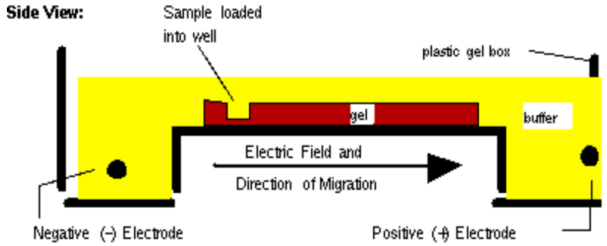


Southern Blotting – Steps

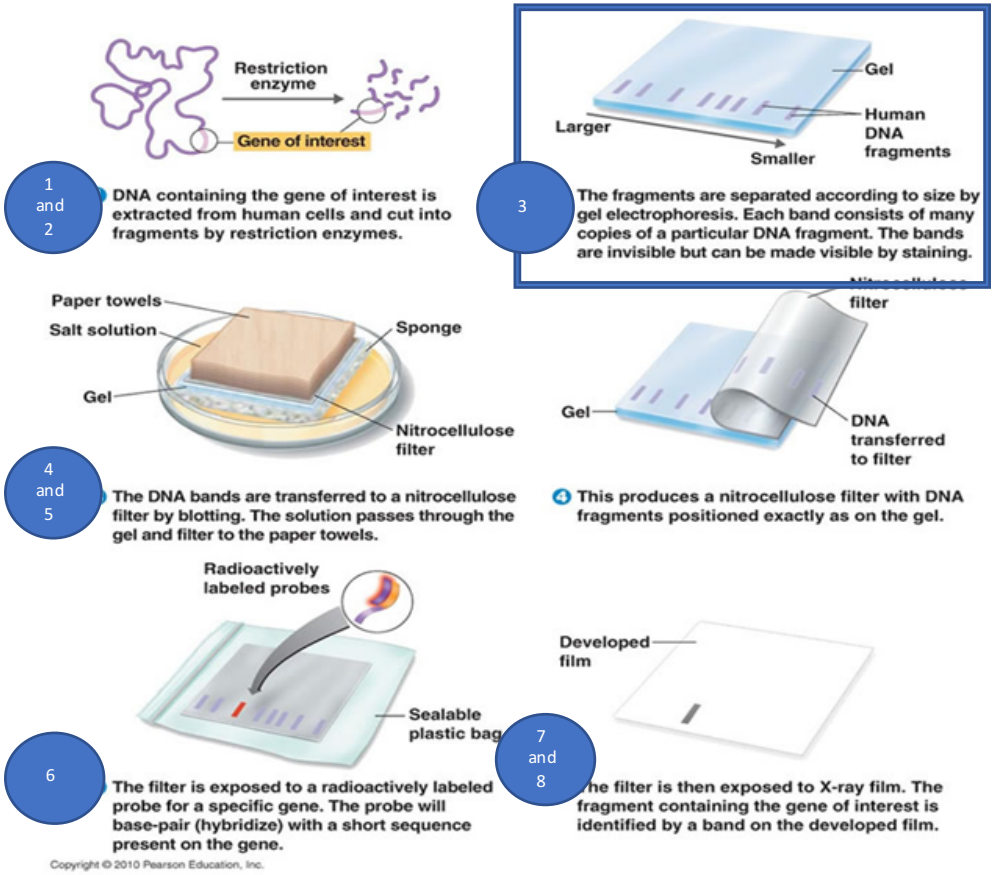
Step 3 : Gel Electrophoresis

Nucleic acids have a net negative charge and will move from the left to the right. The larger molecules are held up while the smaller ones move faster. This results in a separation by size.
Gels are Agarose or polyacrylamide with microscopic pores

Standards should also be run



Gels can be stained with ethidium bromide (EtBr). This causes DNA to fluoresce under UV light which permits photography of the gel.
This will be help us to know the exact migration of DNA standards and the quality of the RE digestion of the test DNA.



Southern Blotting – Steps

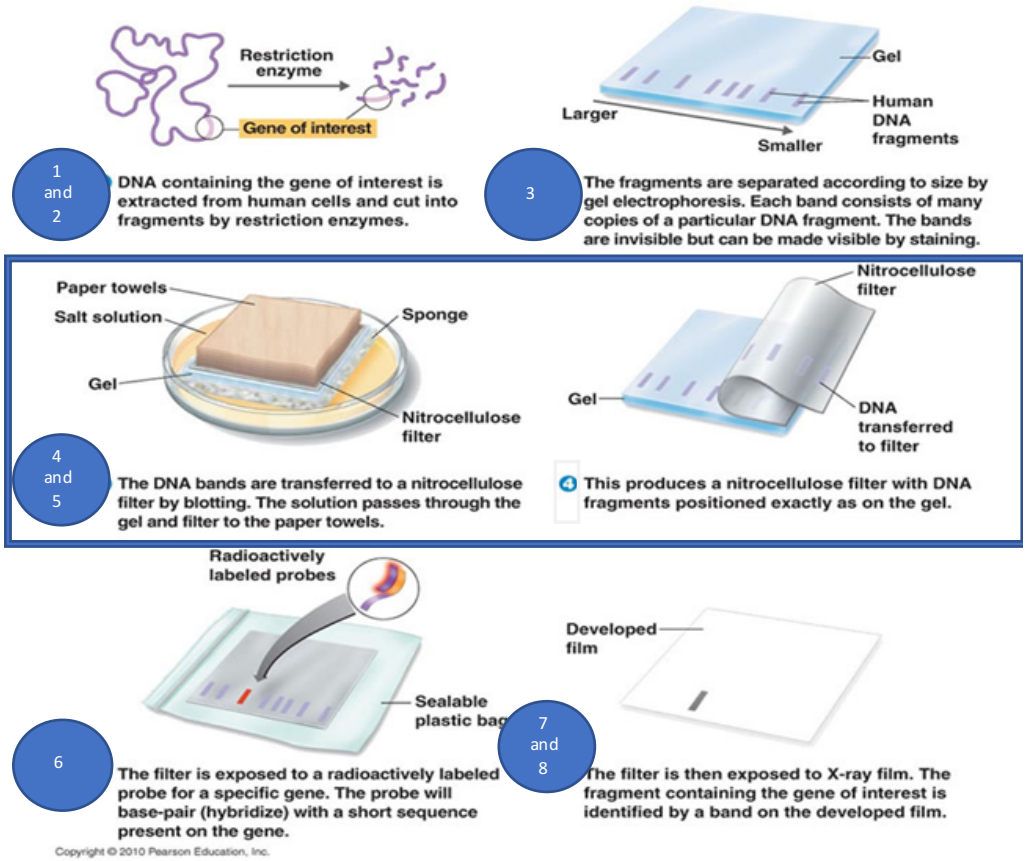
Step 4 & 5 : Denaturation & blotting

DNA is then denatured with an alkaline solution such as NAOH. This causes the double stranded t become single-stranded.

The process of transferring the DNA from the gel to a membrane is called as blotting. The blot is usually done on a sheet of nitrocellulose paper or nylon. DNA is then neutralized with NaCl to prevent re-hybridization before adding the probe. Transferred by either electroblotting or capillary blotting.

The blot is made permanent either by:

- Drying at ~80°C
- Exposing to UV irradiation



Southern Blotting – Steps

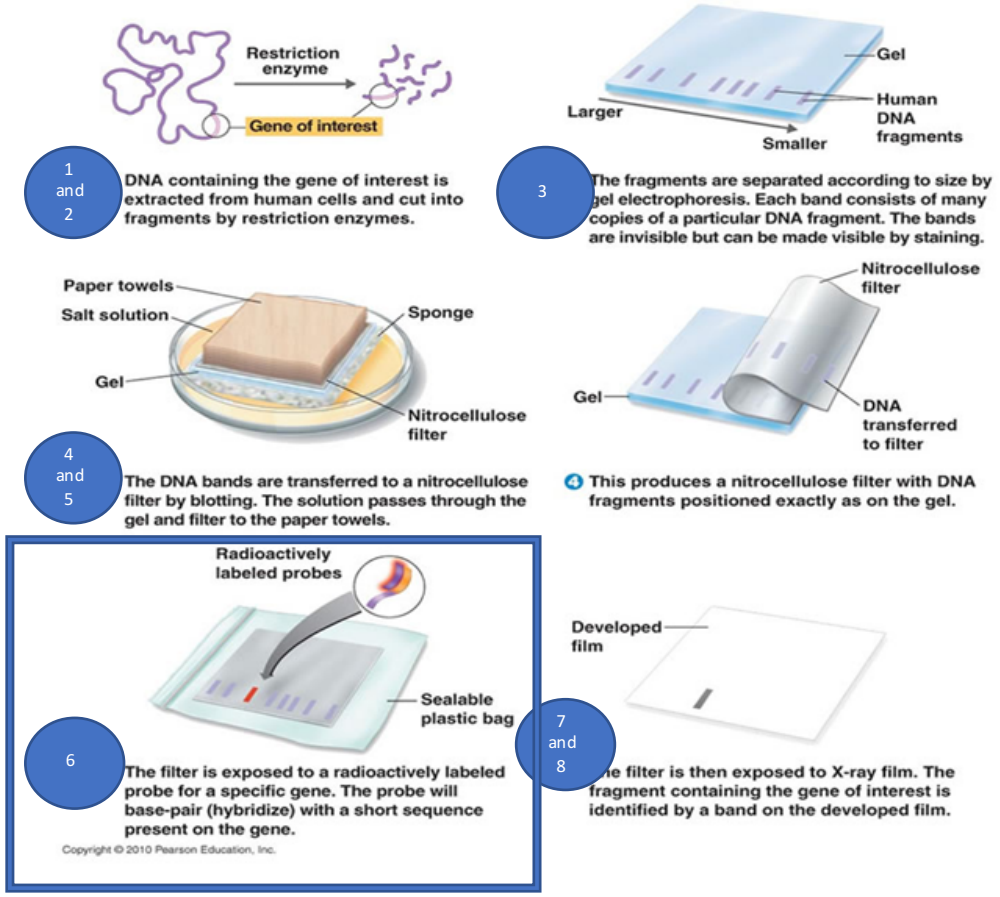
Step 6 : Hybridization

The labelled probe is added to the membrane in buffer and incubated for several hours to allow th probe molecules to find their targets.

Preparing the probe

Small piece of labelled DNA used to find another piece of DNA usually prepared by making a radioactive copy of a DNA fragment.

Probe: fragment of ideally 200 – 1000 bp containing the sequence of interest (present in plasmid; RE; gel-eletrophoresis; purification from gel; alpha-32P-dCTP labelling using Klenow and random oligos)



Southern Blotting – Steps

Step 7 & 8 : Wash and autoradiography

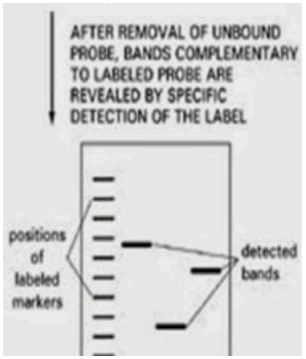
Wash excess probe that are bound non-specifically to the membrane

Blot is incubated with wash buffers containing NaCl and detergent to wash away excess probe ar reduce background.

Detection: Radioactive probes enable autoradiographic detection.

If the probe is radioactive, the particles it emits will expose X-ray film.

By pressing the filter and film, the film will become exposed wherever probe is bound to the filter.



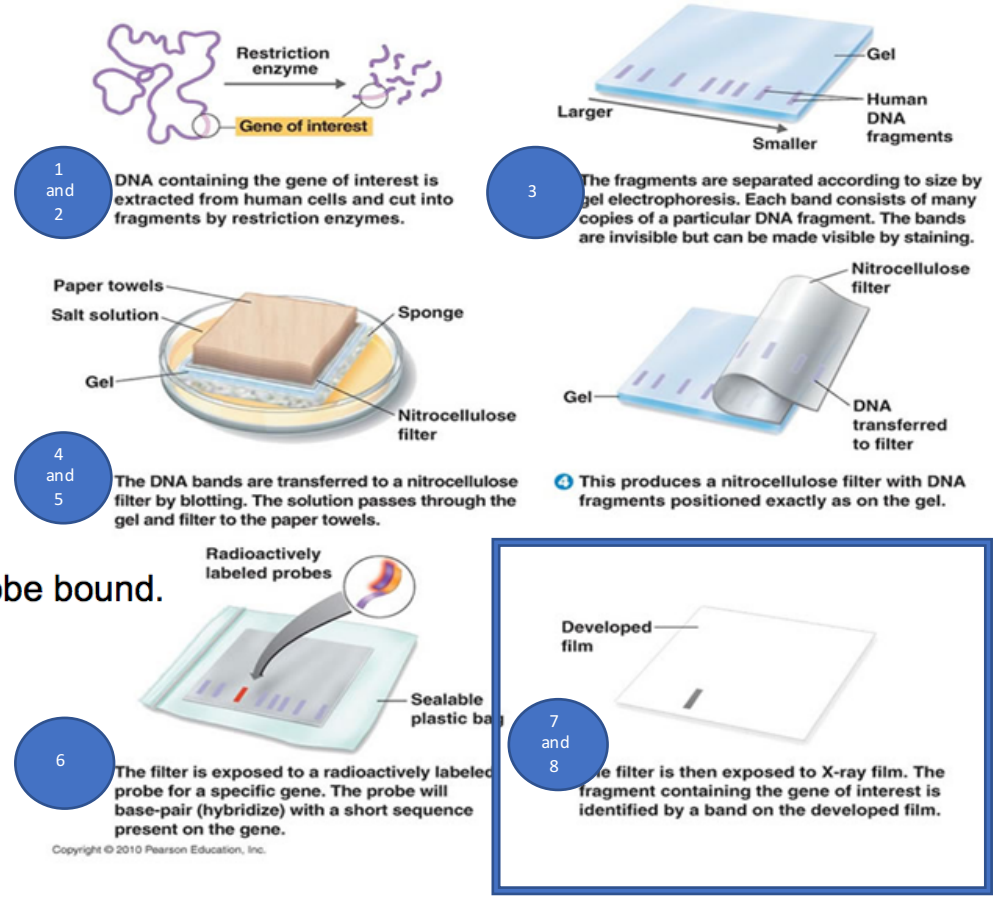
After development, there will be dark spots on the film wherever the probe bound.

Making the probe

Label the probe to be hybridized using radioactive or non-radioactive methods

Non-radioactive methods A) Colorimetric

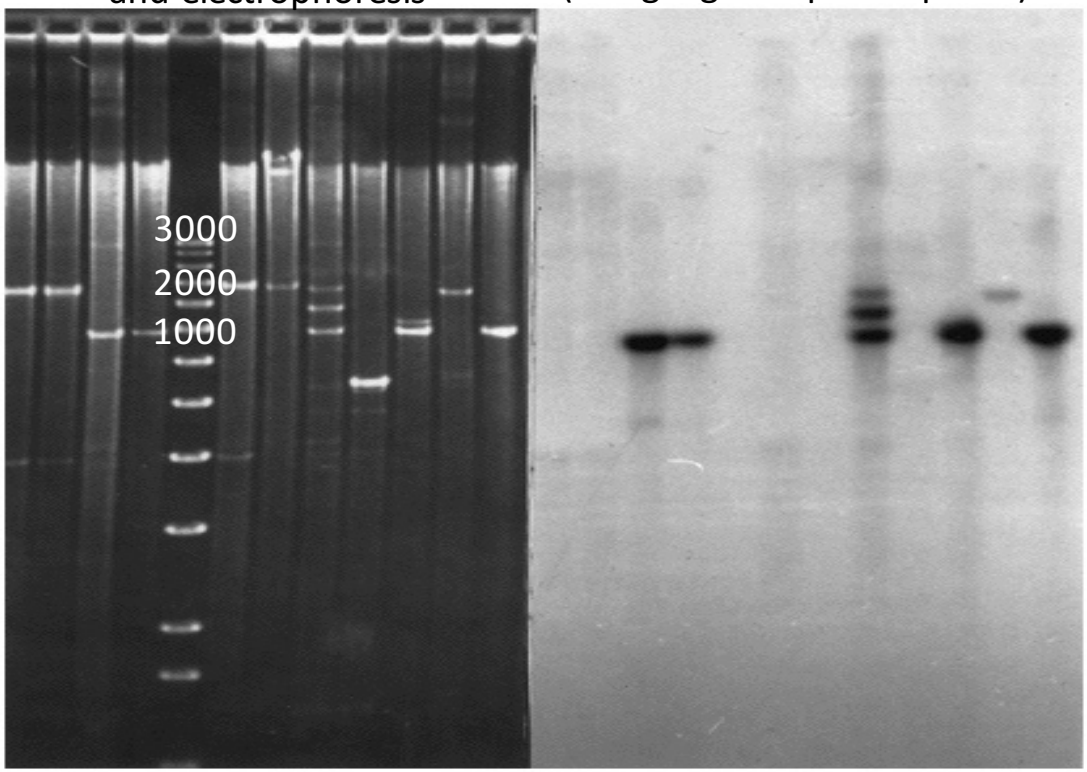
B) Chemiluminescent



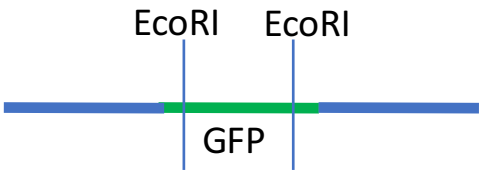
Southern Blotting – Results

Gel after RE digest
and electrophoresis

Hybridized membrane
(using a gene specific probe)



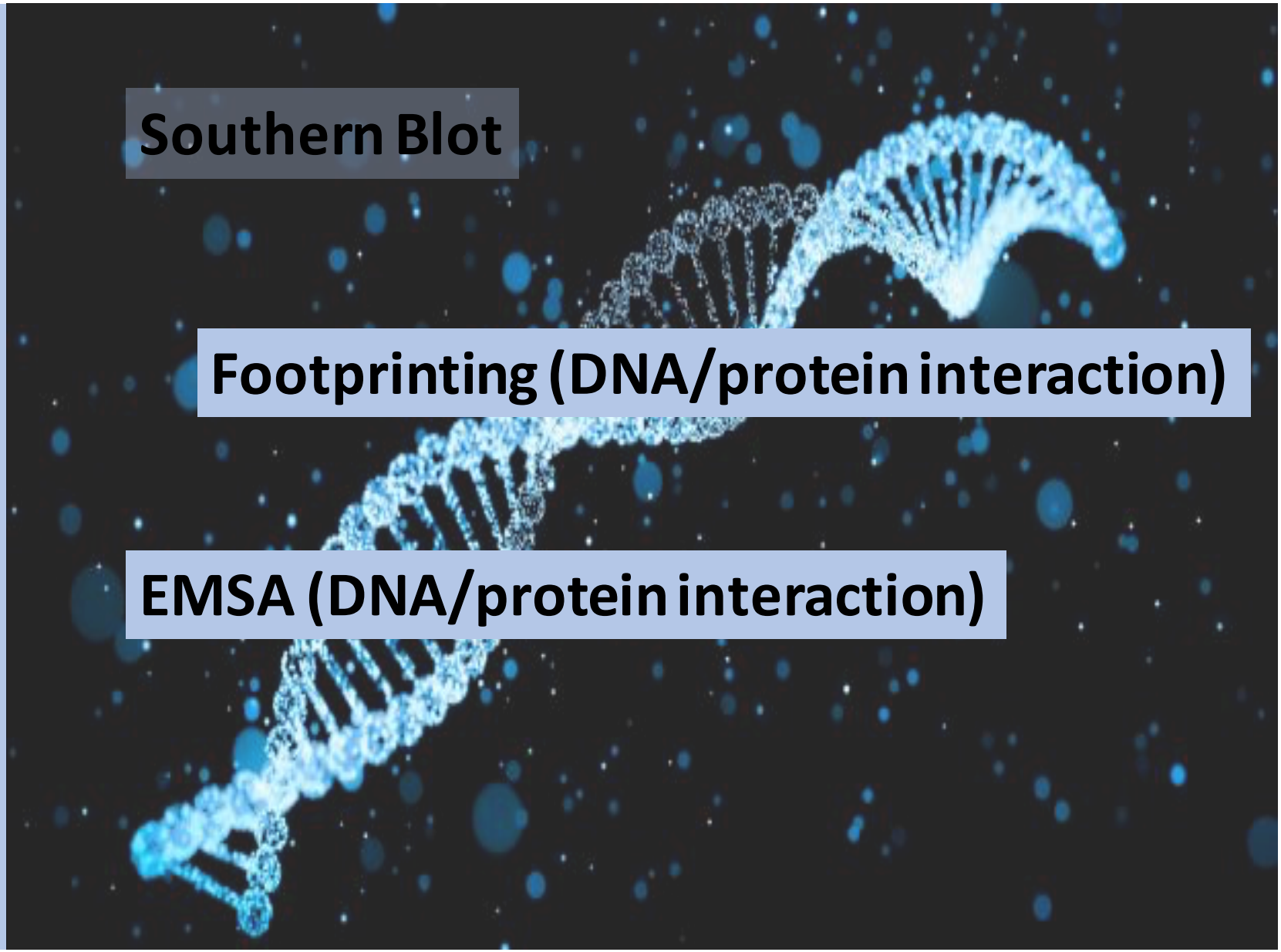
10 different stable cell neomycin resistant lines stably transfected with plasmid encoding GFP and Neo^{R}; preparation of genomic DNA, digest with EcoRI
QUESTION: which of the stable lines contains the GFP gene



GFP
Probe, body labelled

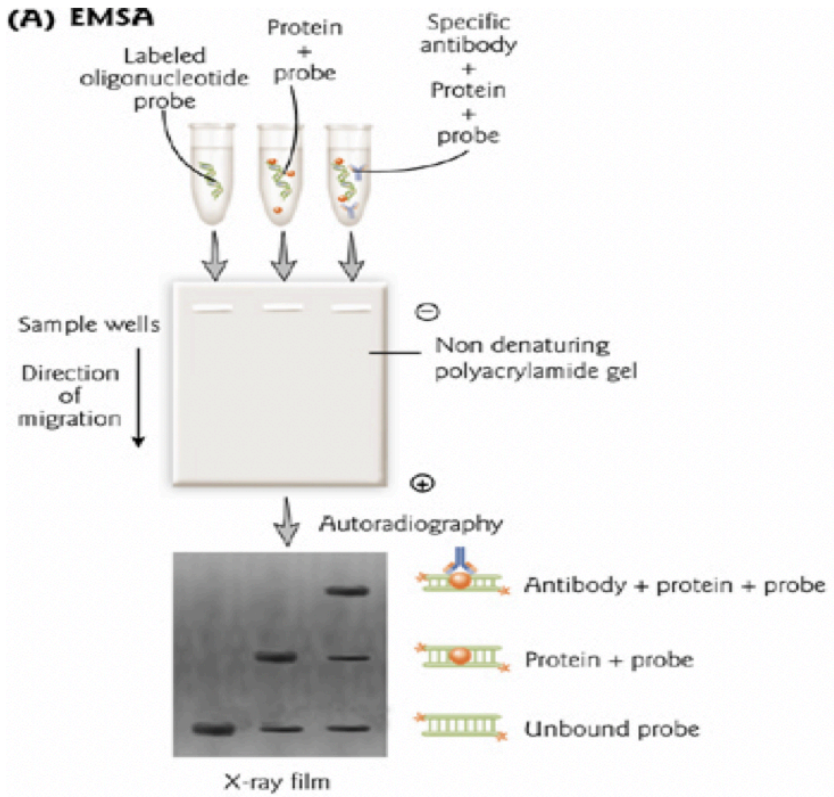
Focus on

DNA


Southern Blot
Footprinting (DNA/protein interaction)
EMSA (DNA/protein interaction)

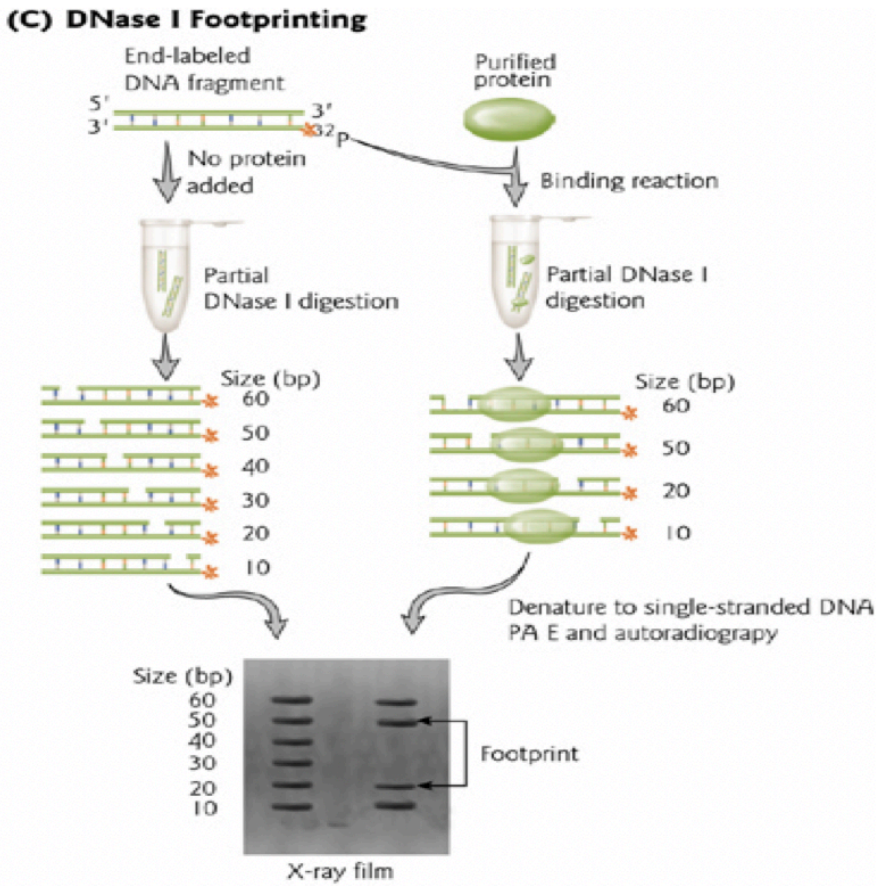
DNA-protein interaction: Footprinting and EMSA

Detecting interactions between DNA and Proteins (for example transcription factor (TF) binding sites)



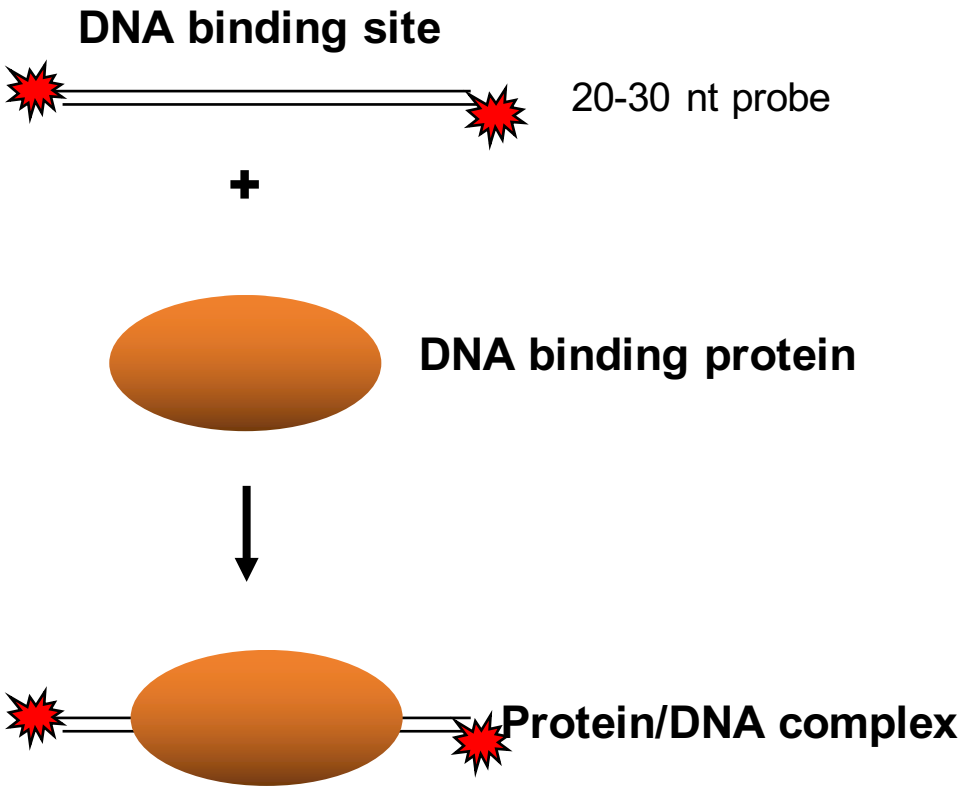
Electophoretic mobility shift assay

DNA: short seqeunce containing putative binding site for TF
Protein: TF, recombinant protein



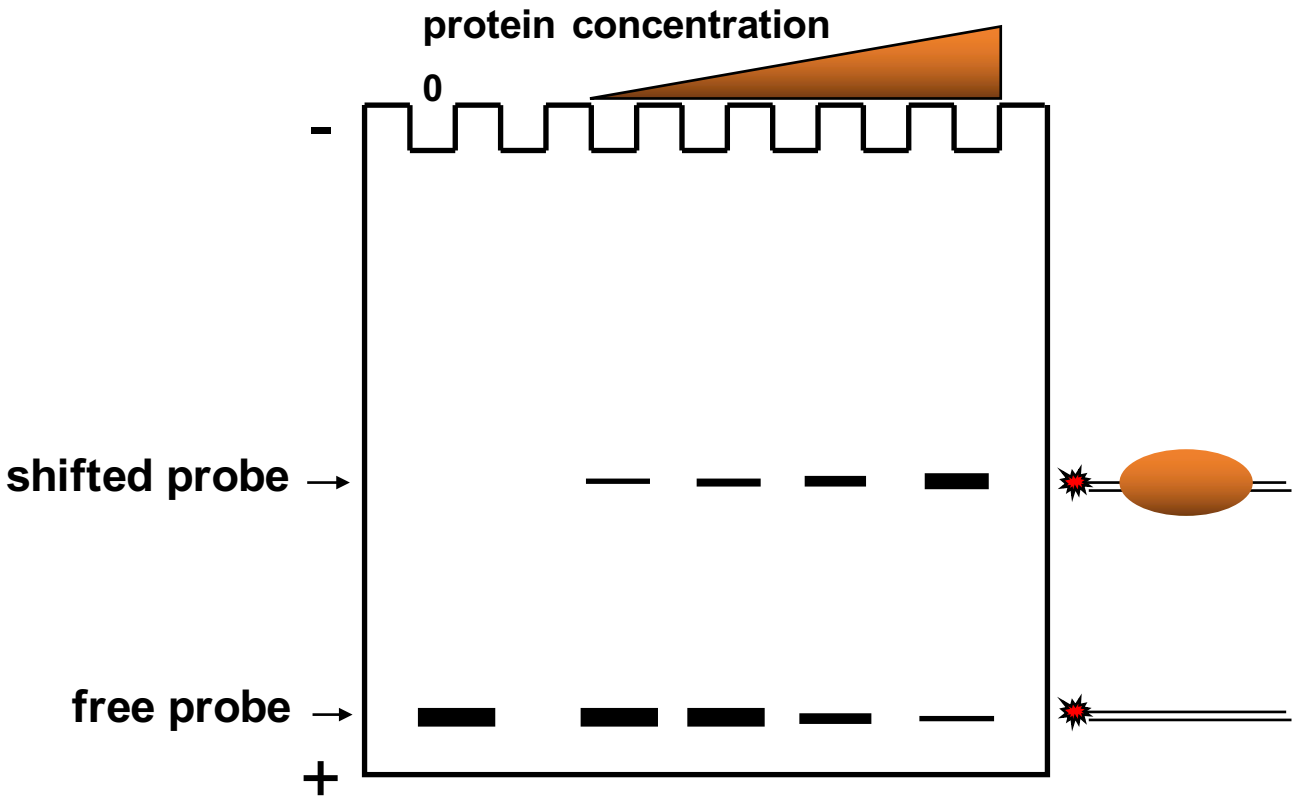
DNA: short seqeunce containing putative binding site for TF
Protein: TF, recombinant protein

Electrophoretic-Mobility Shift Assay (EMSA)



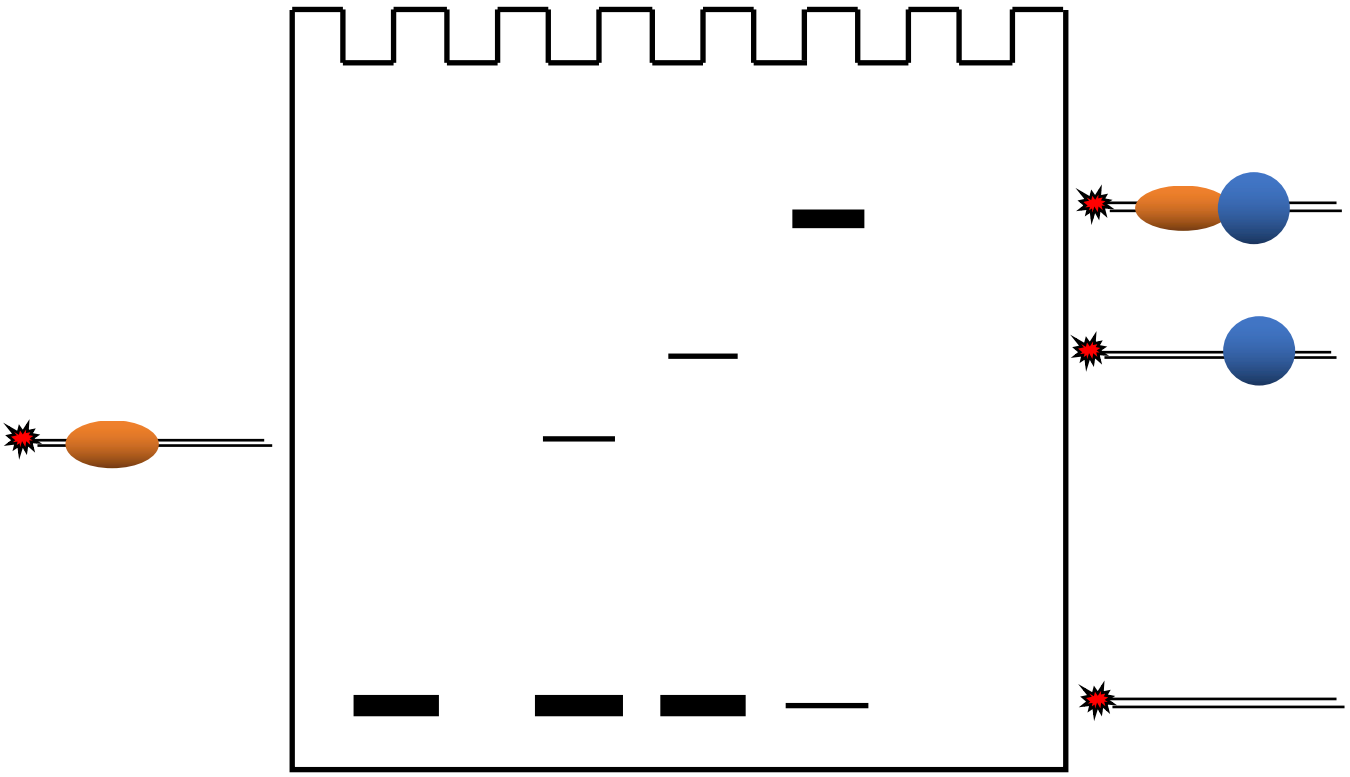
Electrophoretic-Mobility Shift Assay (EMSA)

Incubate protein and DNA probe
Load onto non-denaturing PAGE
Resolve complexes & free probe

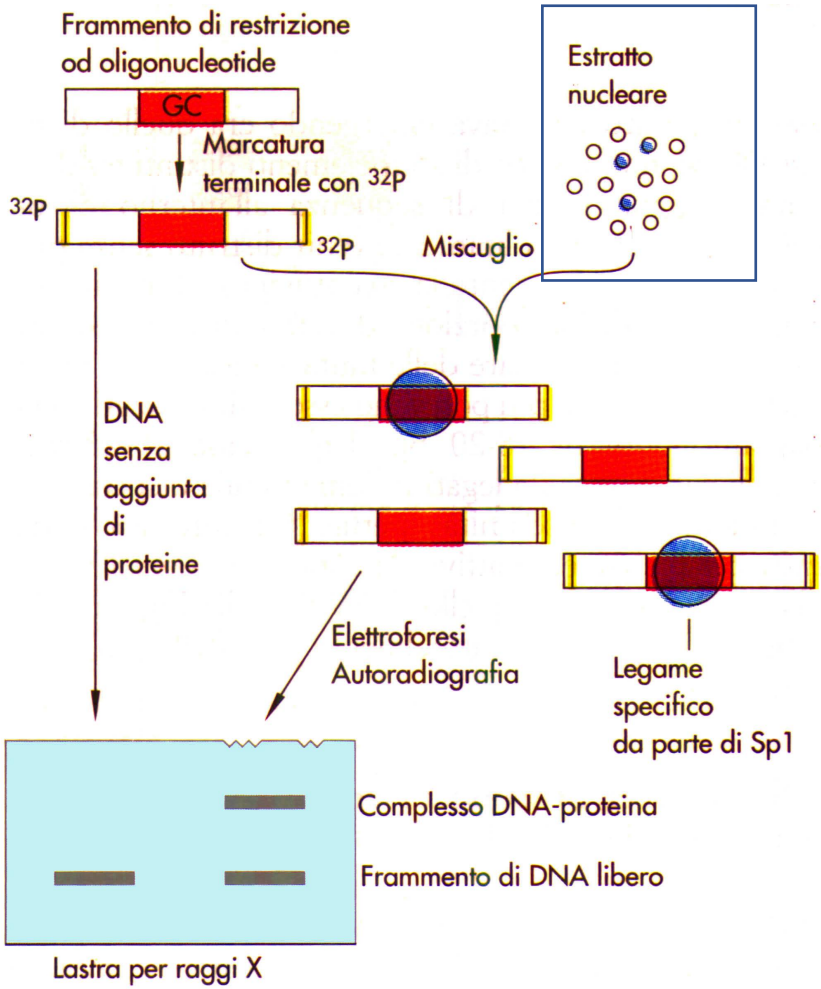


Electrophoretic-Mobility Shift Assay (EMSA)

Resolution of multiple protein/DNA complexes: Proteins sharing a DNA binding element

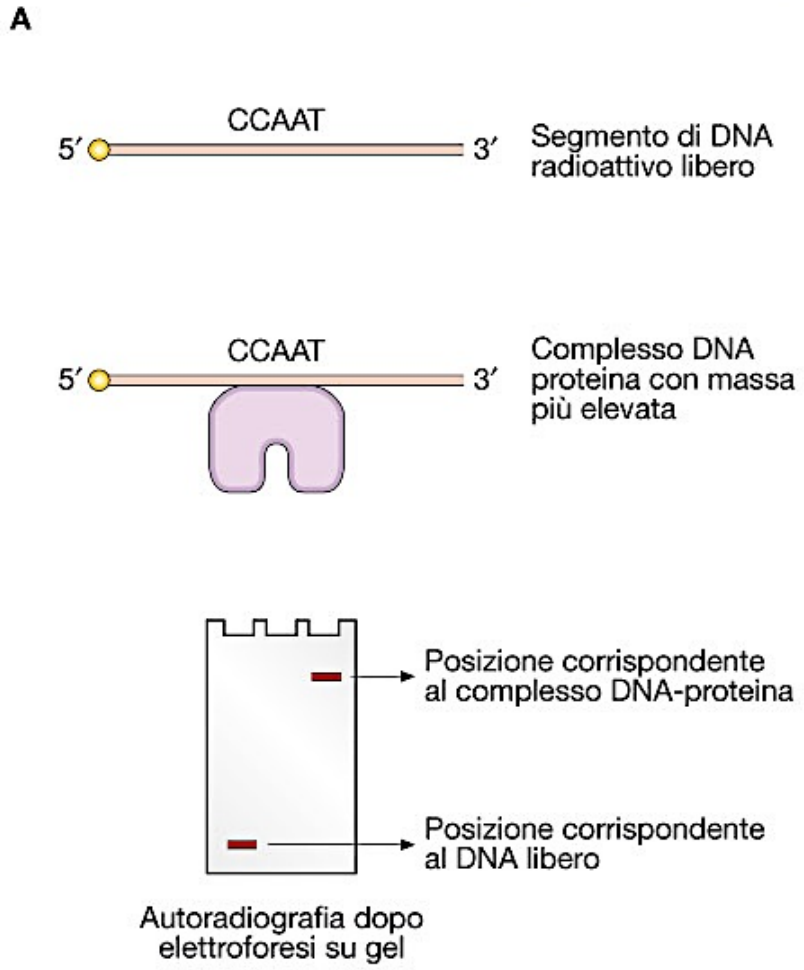


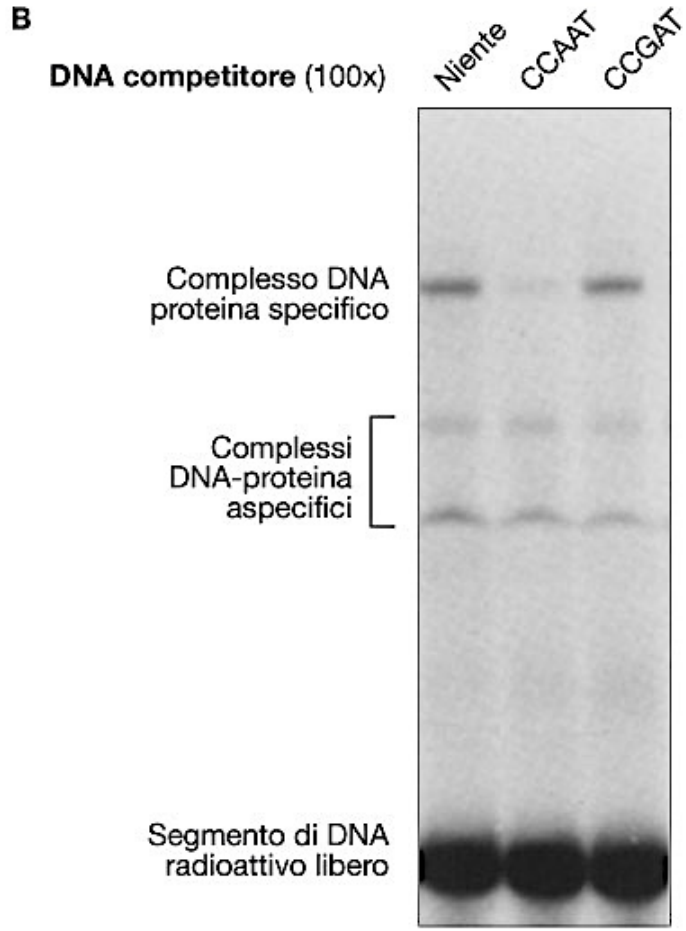
Electrophoretic-Mobility Shift Assay (EMSA)



Electrophoretic-Mobility Shift Assay (EMSA)

Verifying the specificty of DNA-protein interaction





DNAseI- Footprinting

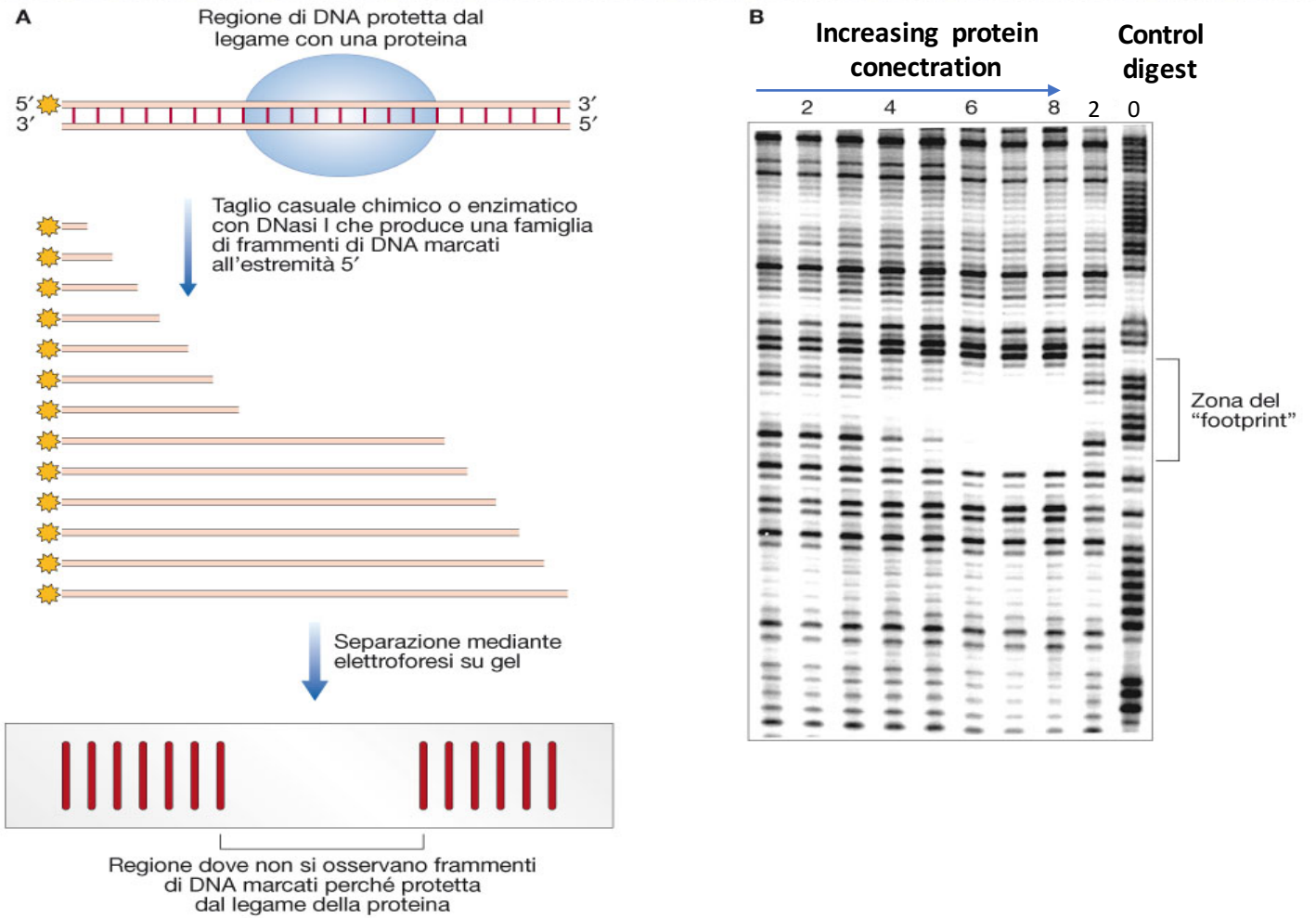
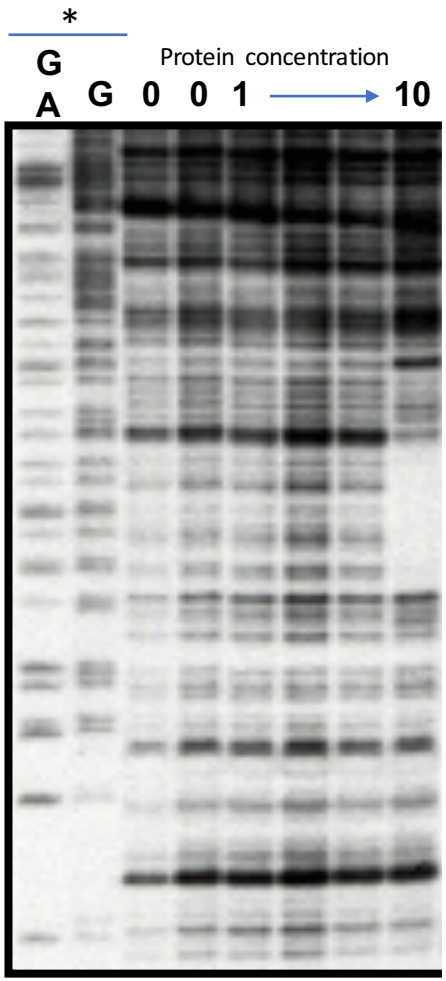
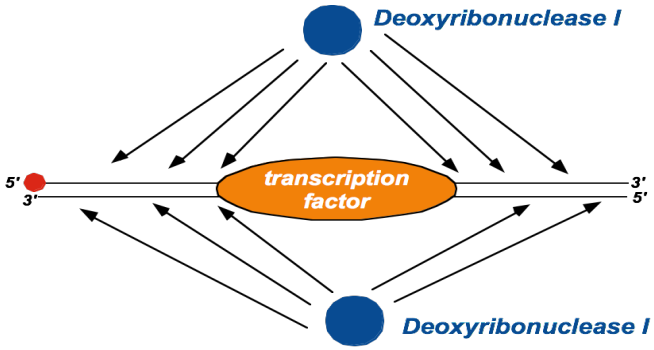


Figura 21.51 Saggio di DNA "footprinting".
(**A**) Schema riassuntivo della procedura. L'asterisco all'estremità 5′ di uno dei due filamenti del frammento di DNA indica un atomo di fosforo radioattivo. Le bande radioattive differiscono una dall'altra per un singolo nucleotide.
(**B**) Esempio di un esperimento di DNA footprinting in cui diverse frazioni di una colonna cromatografica per la purificazione della proteina che si lega al DNA sono state analizzate nelle diverse corsie. La proteina che lega il DNA si trova nelle frazioni 6-8. La posizione dei nucleotidi corrispondenti ai confini della zona di "footprinting" protetta dal legame con la proteina dal taglio con DNasi I può essere definità con estrema precisione.

DNAseI- Footprinting

* Chemical degradation of DNA Maxam-Gilbert sequencing (no protein)





100-300 bp DNA fragment with unique end-label

Incubate with test protein(s)
Digest with DNase I

Run digested DNA on PAGE

FROM

DNA

TO

RNA

Northern Blotting

RNAse Protection Assay

EMSA for RNA (same as DNA)

FROM

DNA

TO

RNA

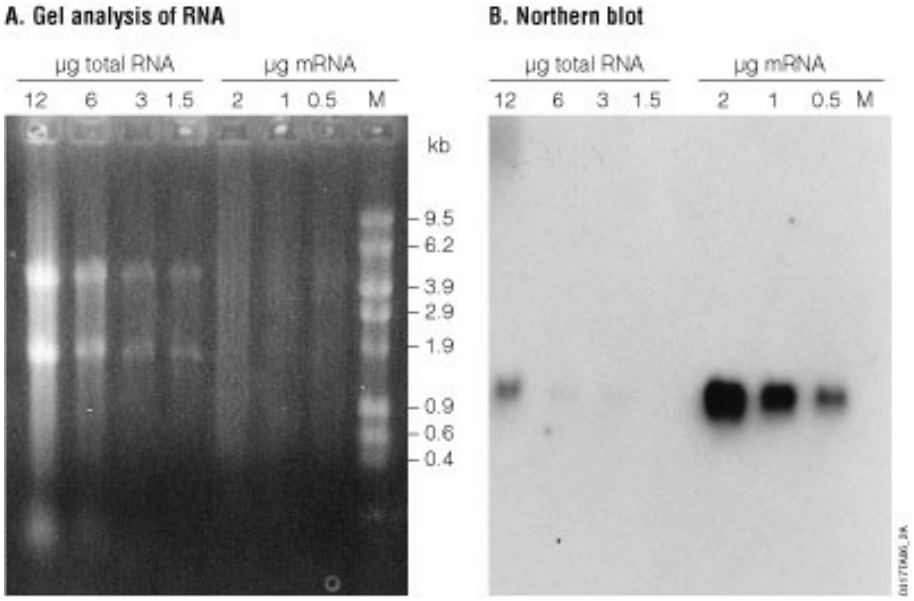
Northern Blotting

RNAse Protection Assay

EMSA for RNA (same as DNA)

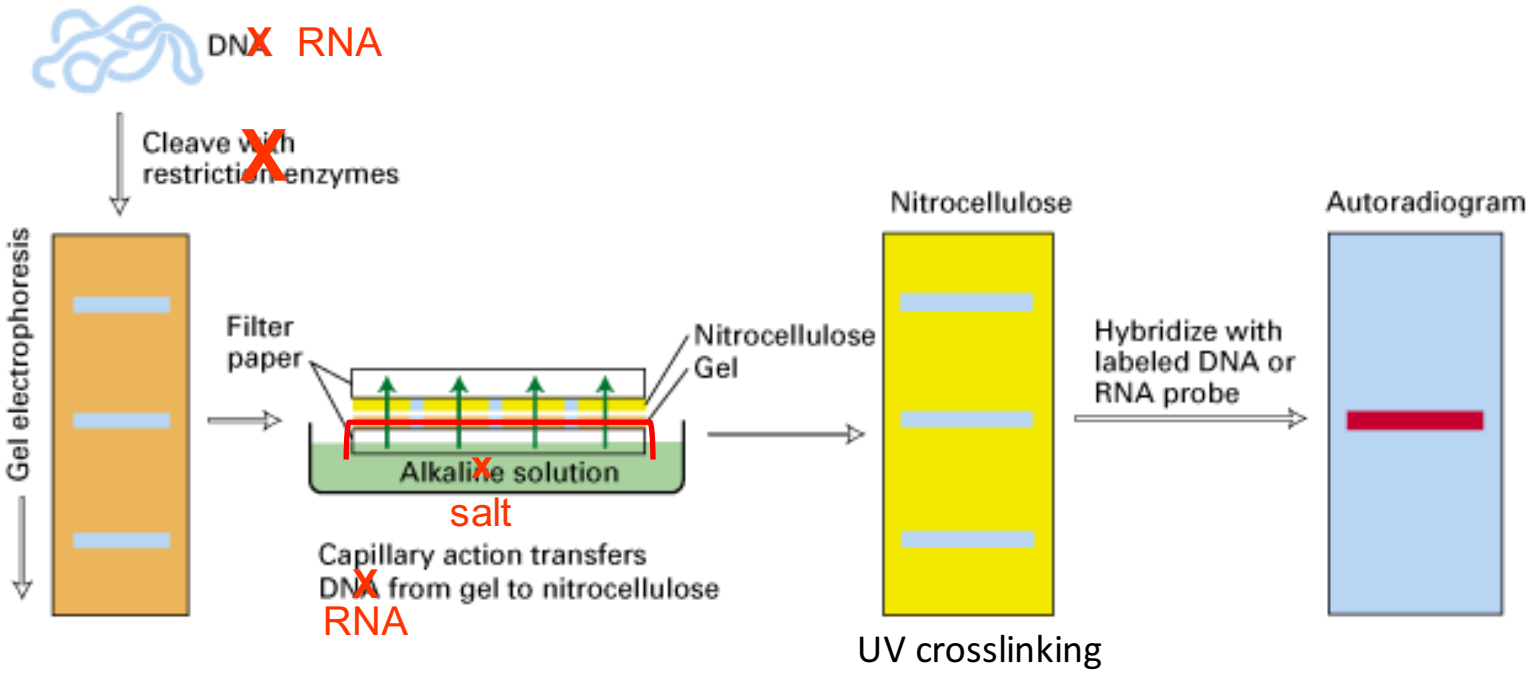
Northern blotting

Northern analysis remains a standard method for **detection and quantitation of mRNA levels** despite the advent of powerful techniques, such as RT-PCR, gene array analysis and nuclease protection assays.
Northern analysis provides a direct relative comparison of message abundance between samples on a single membrane.
It is the preferred method for determining transcript size and for detecting alternatively spliced transcripts

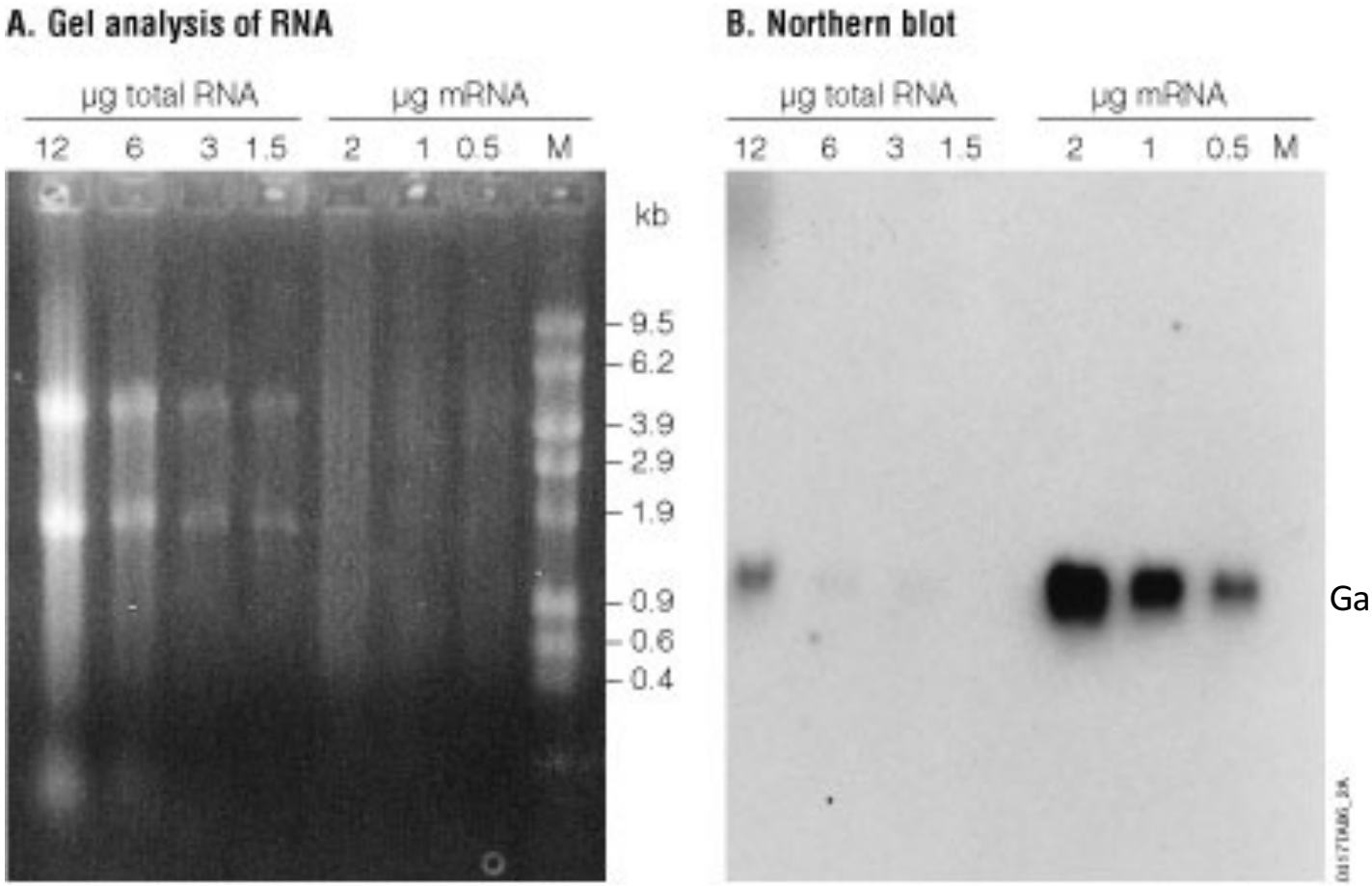


The steps involved in Northern analysis include:
- RNA isolation (total or poly(A) RNA)
- Probe generation
- Denaturing agarose gel electrophoresis
- Transfer to solid support and immobilization
- Prehybridization and hybridization with probe
- Washing
- Detection
- Stripping and reprobing (optional)

Northern blotting vs Southern blotting



-transfer of RNA from a denaturing gel to a membrane (e.g., nitrocellulose, nylon)
-reveals mRNA size (and approximate protein size), tissue- and organ-specific expression, and kinetic patterns of expression



Ribosomal subuntis

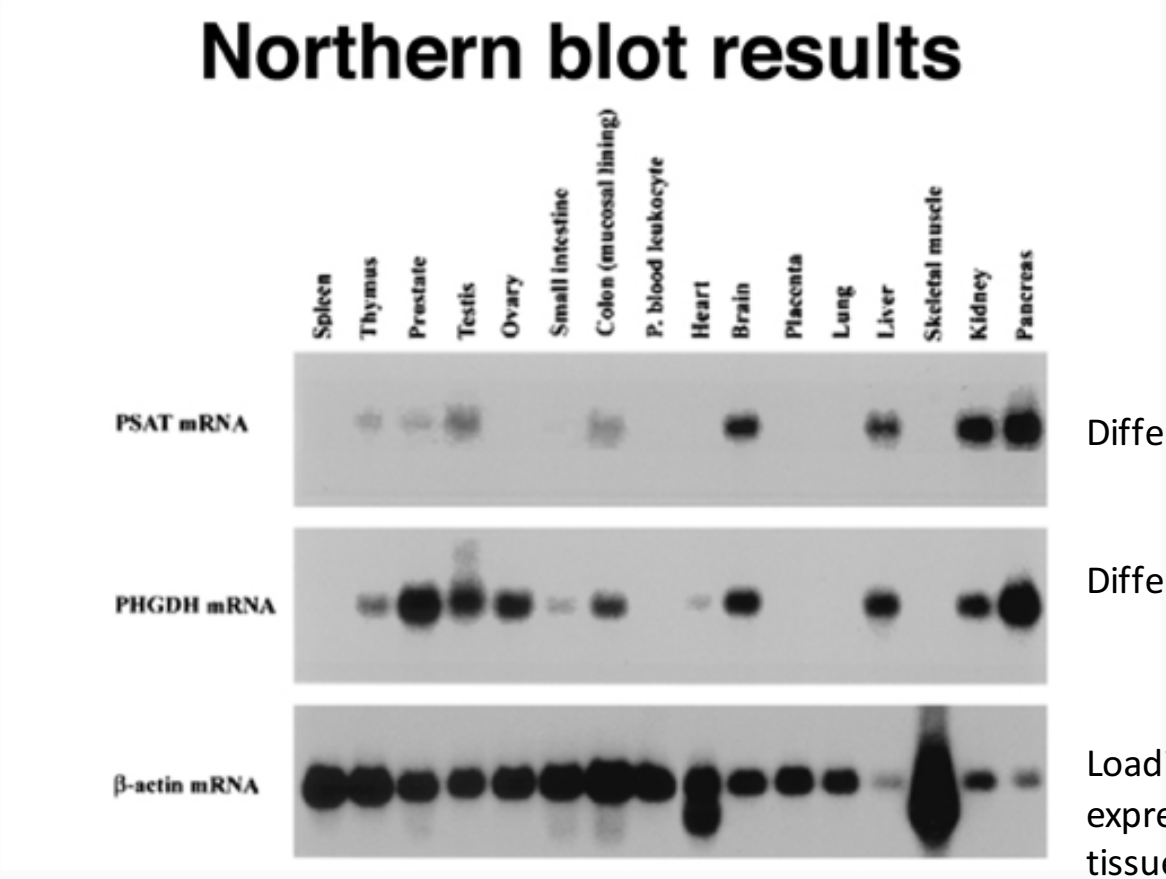
60S

40S

Gapdh mRNA (processed)

Transfer, hybridisation with radioactive gapdh probe

The figure below shows Northern blot analysis of different human tissues. Three probes were used: PSAT, PHGDH and Actin. Actin was used as a loading control, meaning transcription of actin is consistent between human tissues. Which tissue showed the highest level of transcription of the PSAT gene?



Different levels of expression

Different levels of expression

Loading control (actin expressed highly in most tissue)

FROM

DNA

TO

RNA

Northern Blotting

RNAse Protection Assay

EMSA for RNA (same as DNA)

RNAse protection assay

The RNase Protection Assay (RPA) is a specific, sensitive, and qualitative method for the detection, mapping, and **quantitation of specific mRNAs** (detemine gene expression).

The RPA is at least 10-fold more sensitive than Northern blot analysis and is more accurate and direct than RT-PCR analysis.

In addition, RPA provides information that cannot be obtained reliably by other methods:

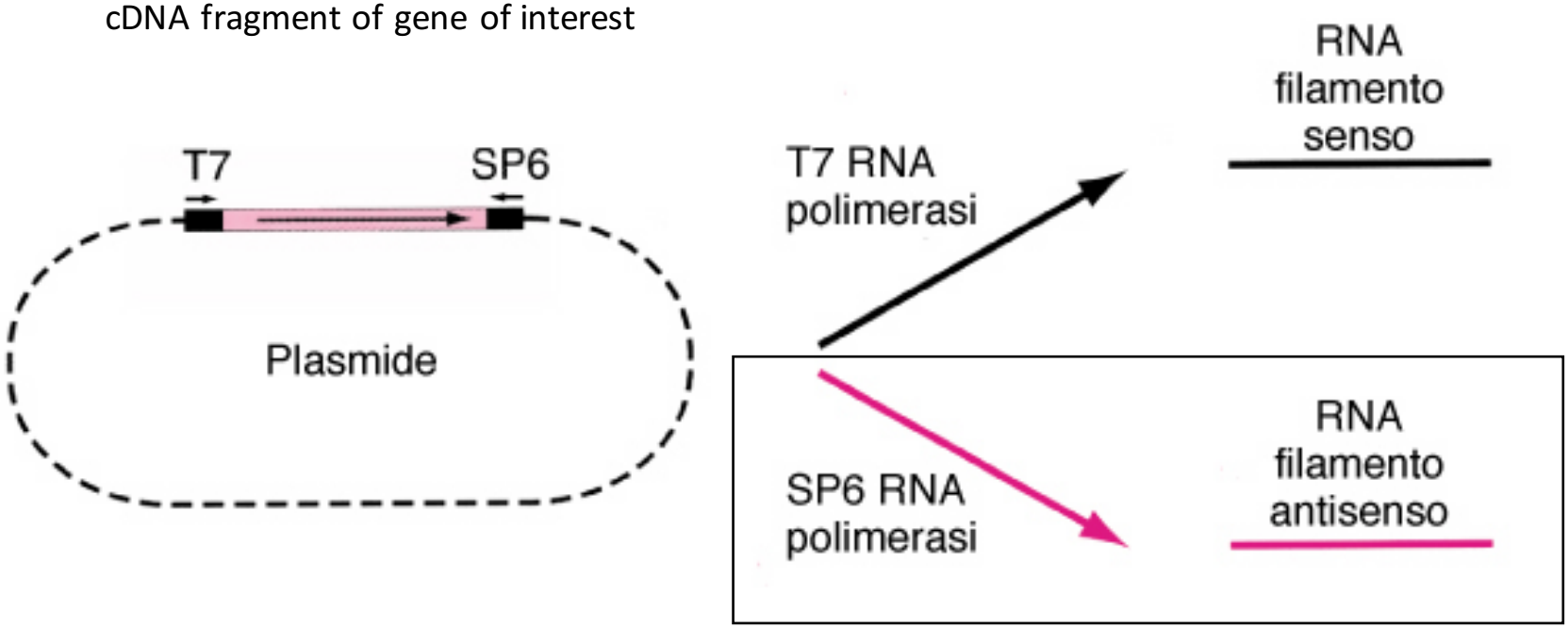
For example, **mapping transcription start sites, studying intron-exon junctions**, and **detecting very small differences in related transcripts** can be achieved with RPA. The RNase protection assay can be performed with either total RNA or poly A+ RNA, and the results are not dependent upon having purified or non-degraded RNA

CONCEPT: a labeled, single-stranded antisense RNA probe is allowed to hybridize to the target RNA. RNA probe molecules and transcripts that do not form hybrids are degraded by a mixture of RNases. The final inactivation of RNases and the precipitation of protected RNA hybrids are performed simultaneously. Electrophoresis followed by autoradiography reveals the presence, size, and relative level of RNA that was protected by the antisense probe.

DISADVANTAGE: DANGEROUSE (high level of radioactivy), EXPENSIVE (waste, radioactivy, developing); TECHNICALLY DEMANDING

Anti-sense RNA preparation

cDNA fragment of gene of interest



- Run on polyacrylamide gel
- Cut out radioactive, in vitro trasncribed RNA
- Purify RNA
- Deterimine concentration by measuring incorporated radioactive nucleotides

Figura 13.2 Produzione di un RNA antisenso

Radioactive alpha^{32}P-rGTP is incorporated during in vitro transcription (body labelling)

RNAse protection

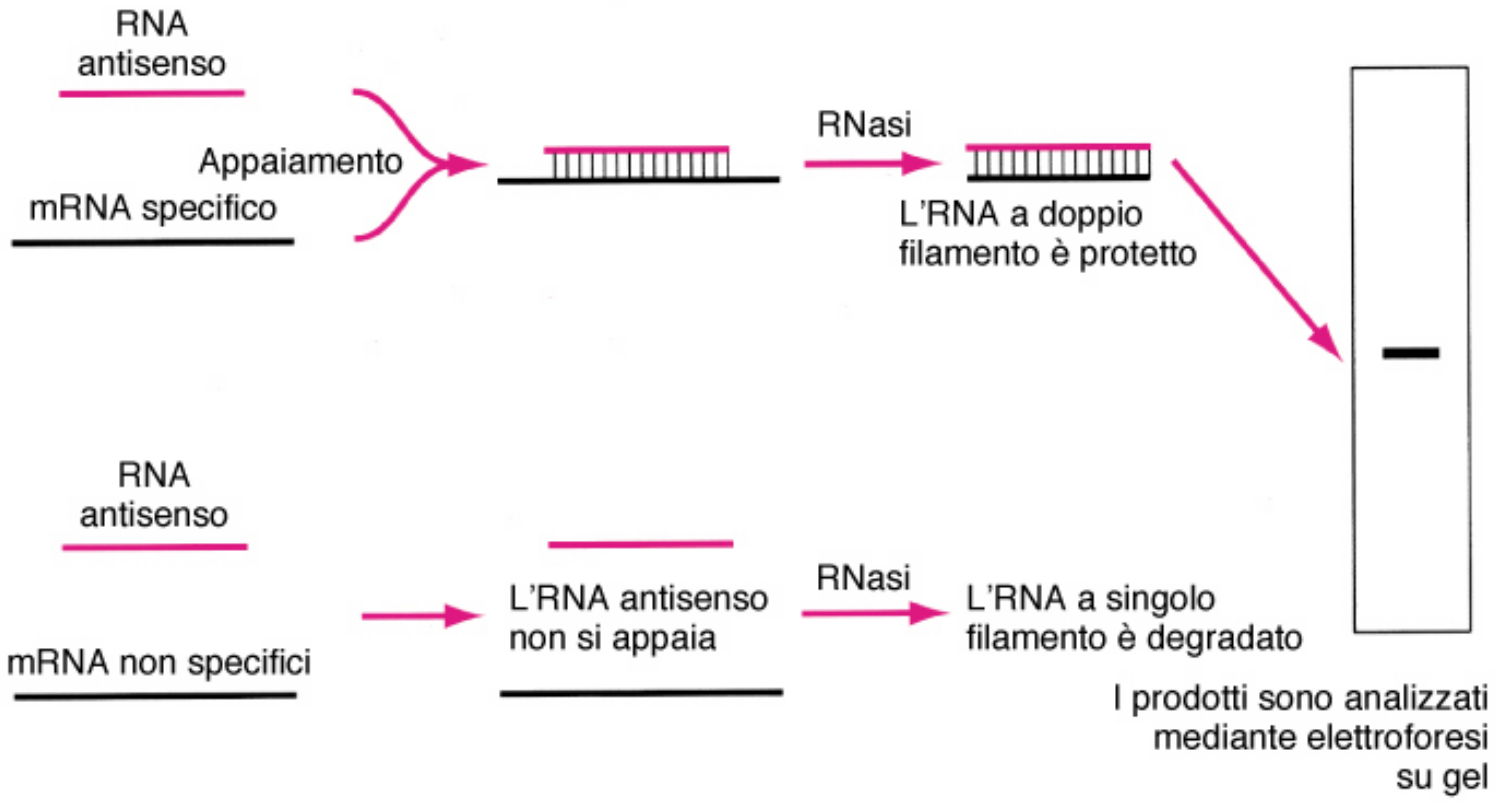
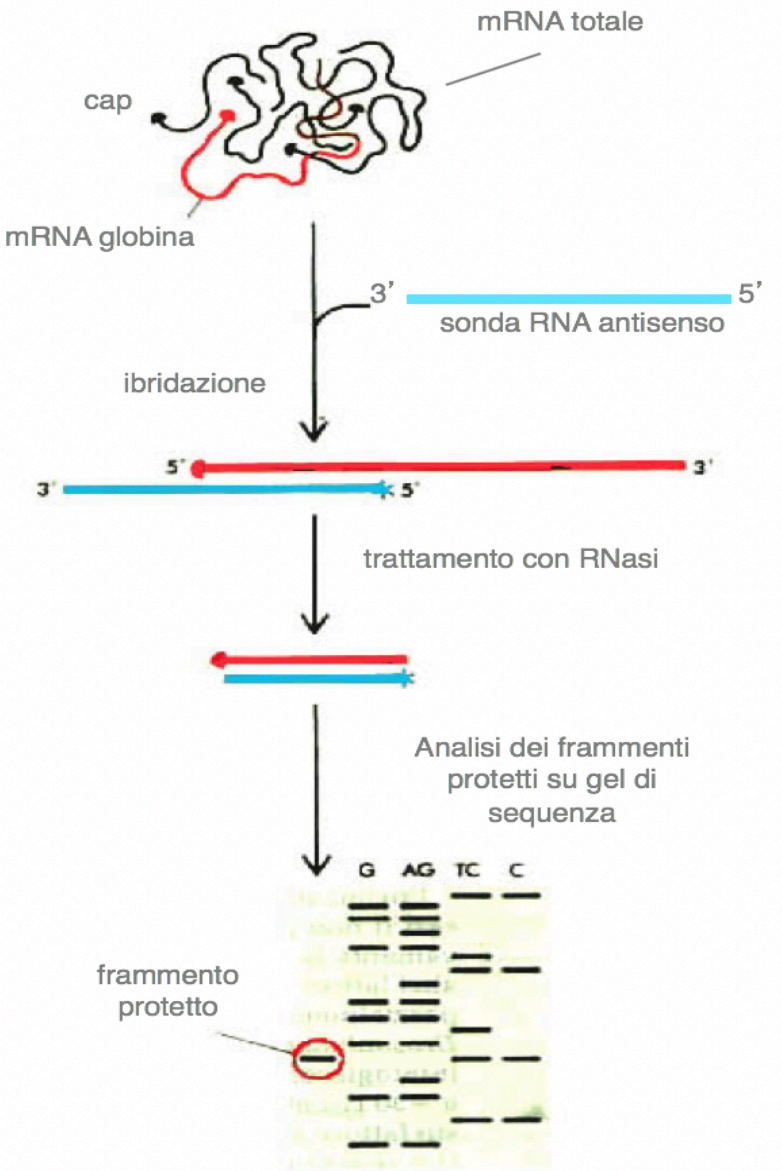


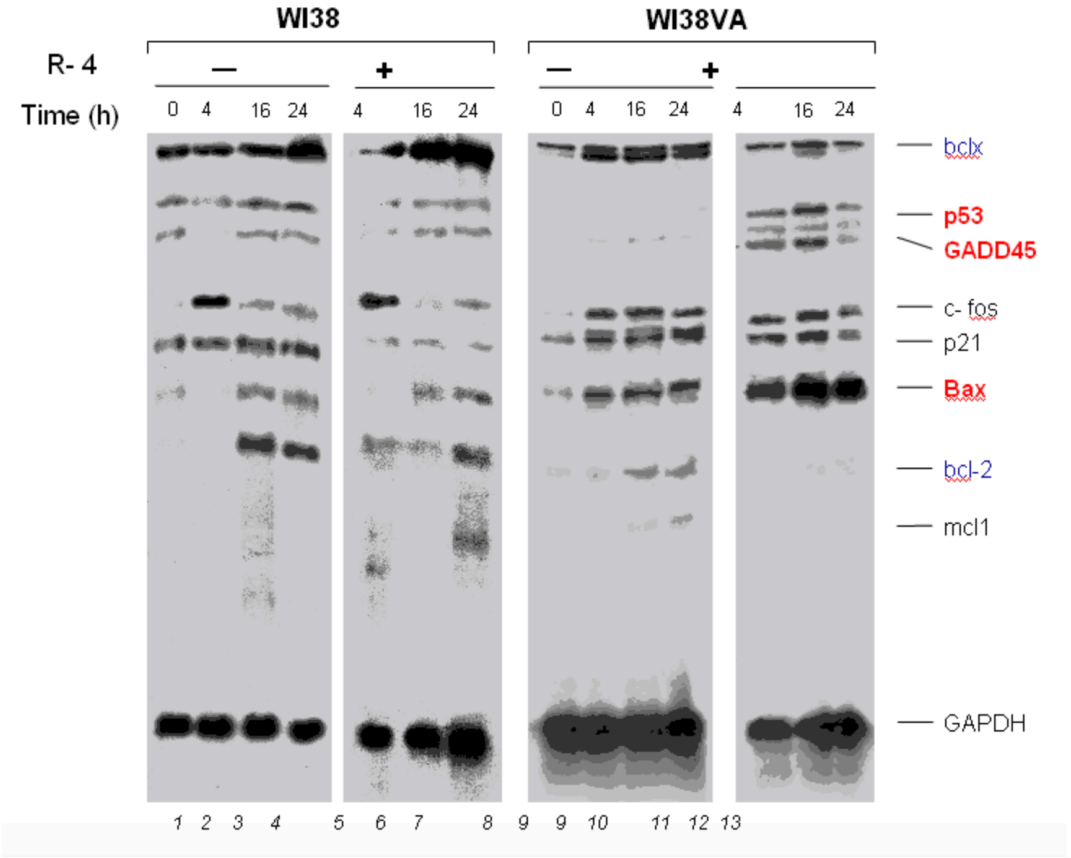
Figura 13.3 Analisi mediante protezione all'RNasi

RNAse protection



RNAse protection

Drug
DNA damage



Multiple probes in parallel

RNAse protection assay protocol

RNase Protection Assay

1) Isolation of RNA sample(s) to be examined for target expression
2) Synthesis of a labeled antisense RNA probe complementary to a several-hundred-base region of the target mRNA,
3) Hybridization of the labeled probe to a total RNA sample,
4) Treatment of the sample with single-strand-specific RNase to degrade unhybridized probe and target
5) Separation of the remaining protected probe::target hybrids on a denaturing polyacrylamide gel
6) Detection/quantitation of the RNase-resistant "protected" probe

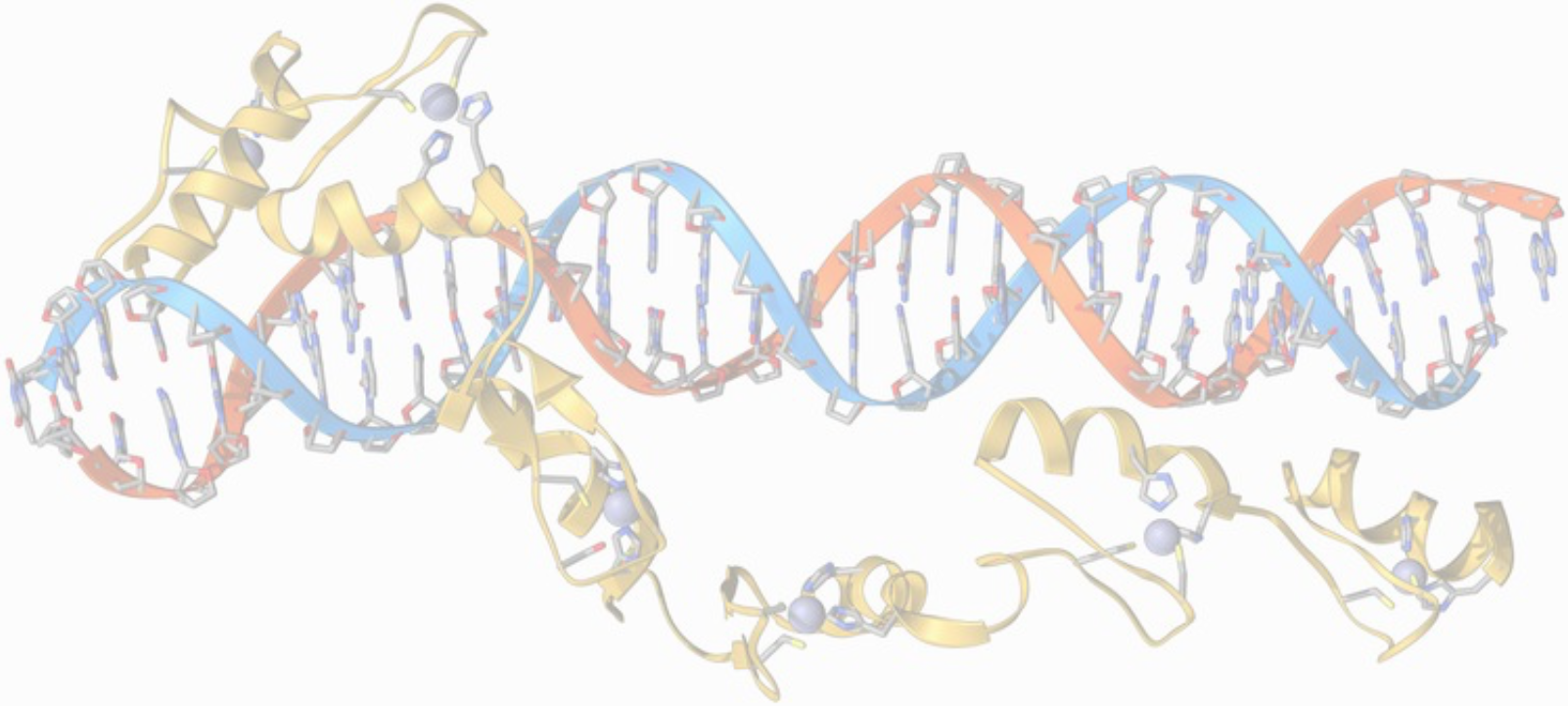
FROM

DNA

TO

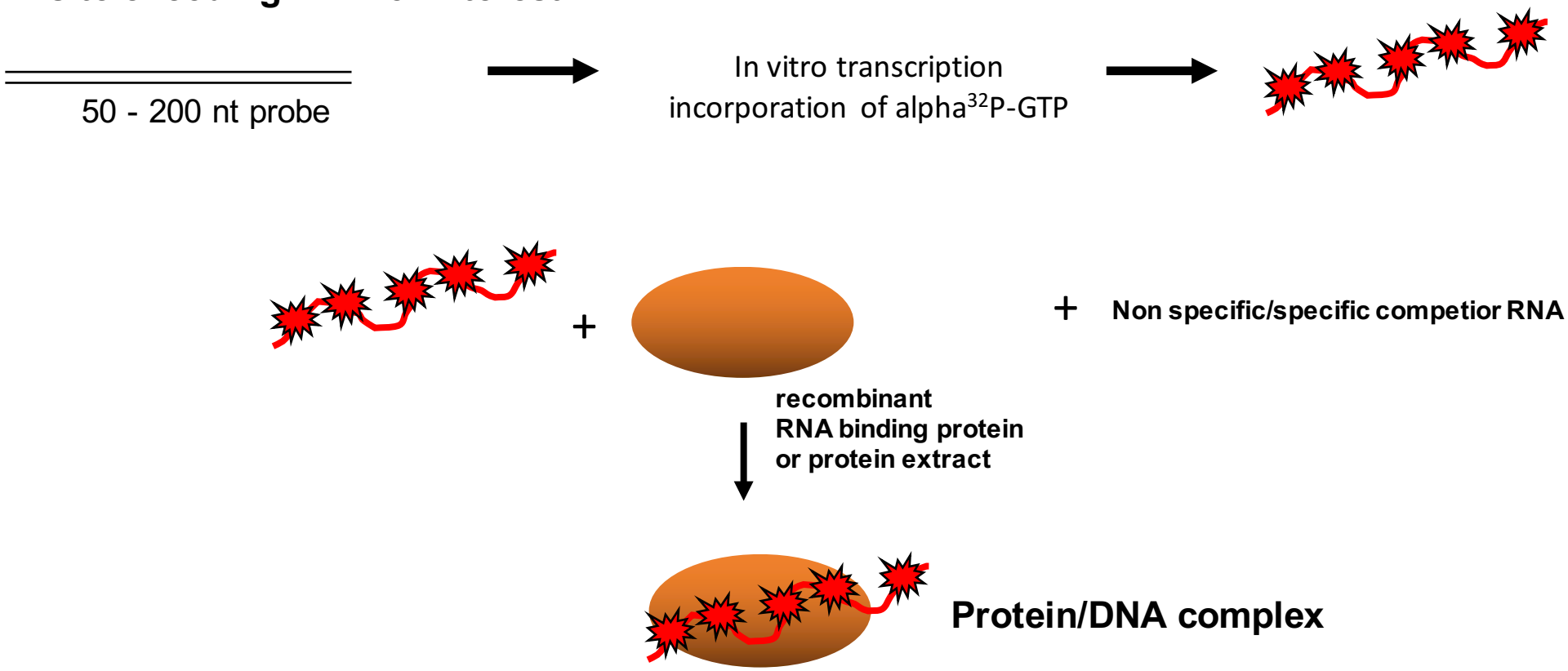
RNA

EMSA for RNA (same strategy as for DNA)



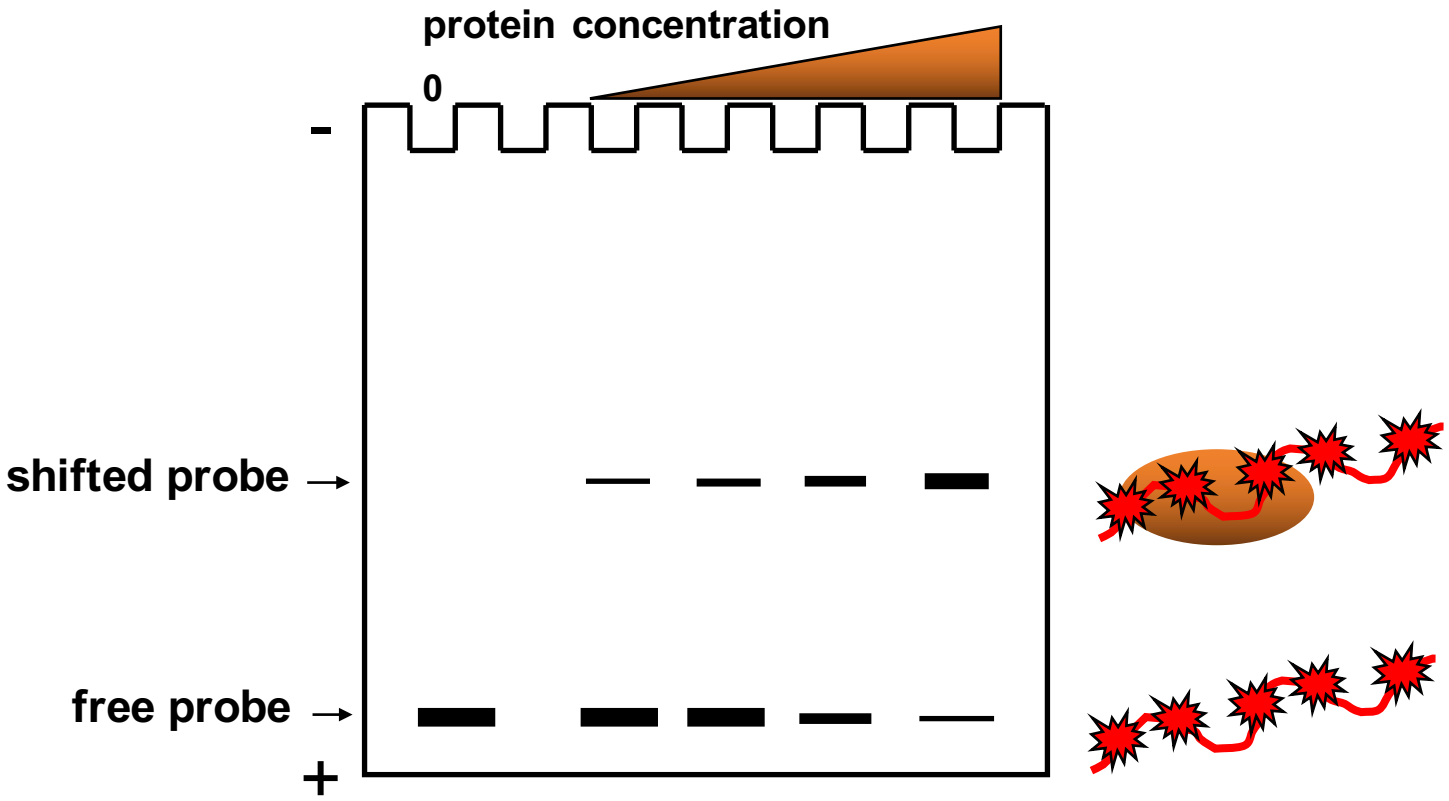
Electrophoretic-Mobility Shift Assay (EMSA)

DNA site encoding RNA of interest



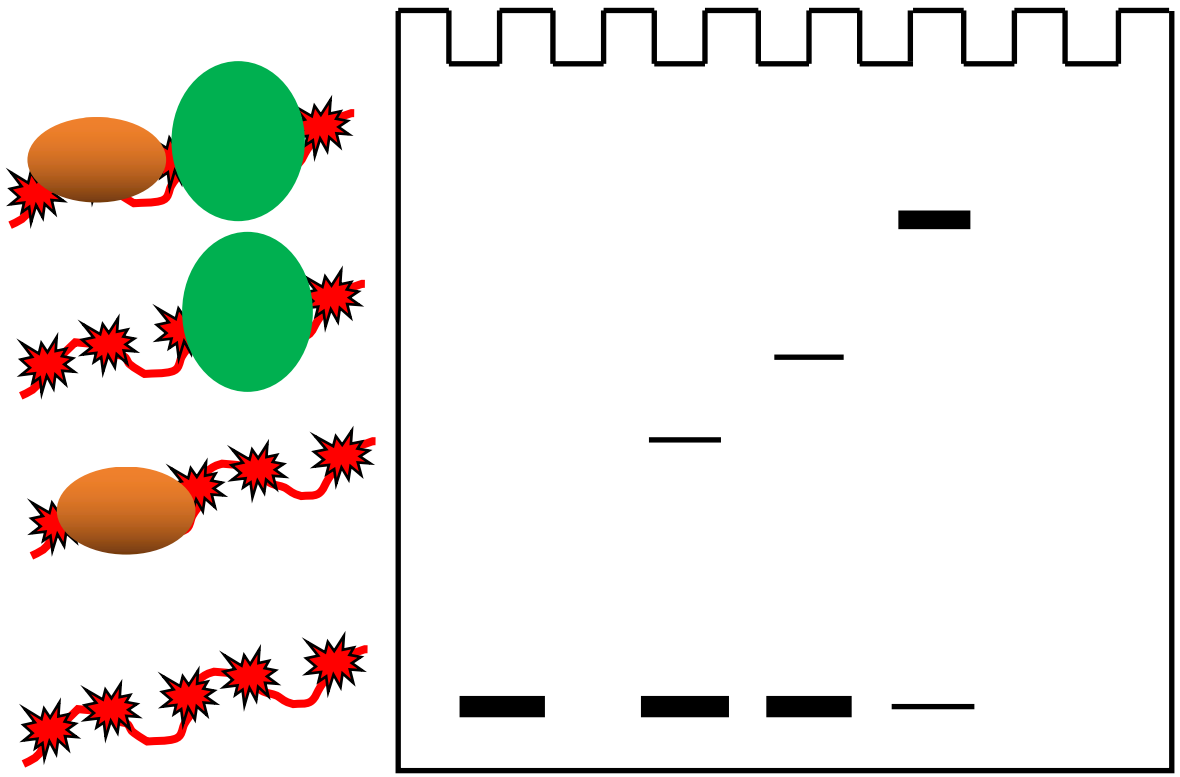
Electrophoretic-Mobility Shift Assay (EMSA)

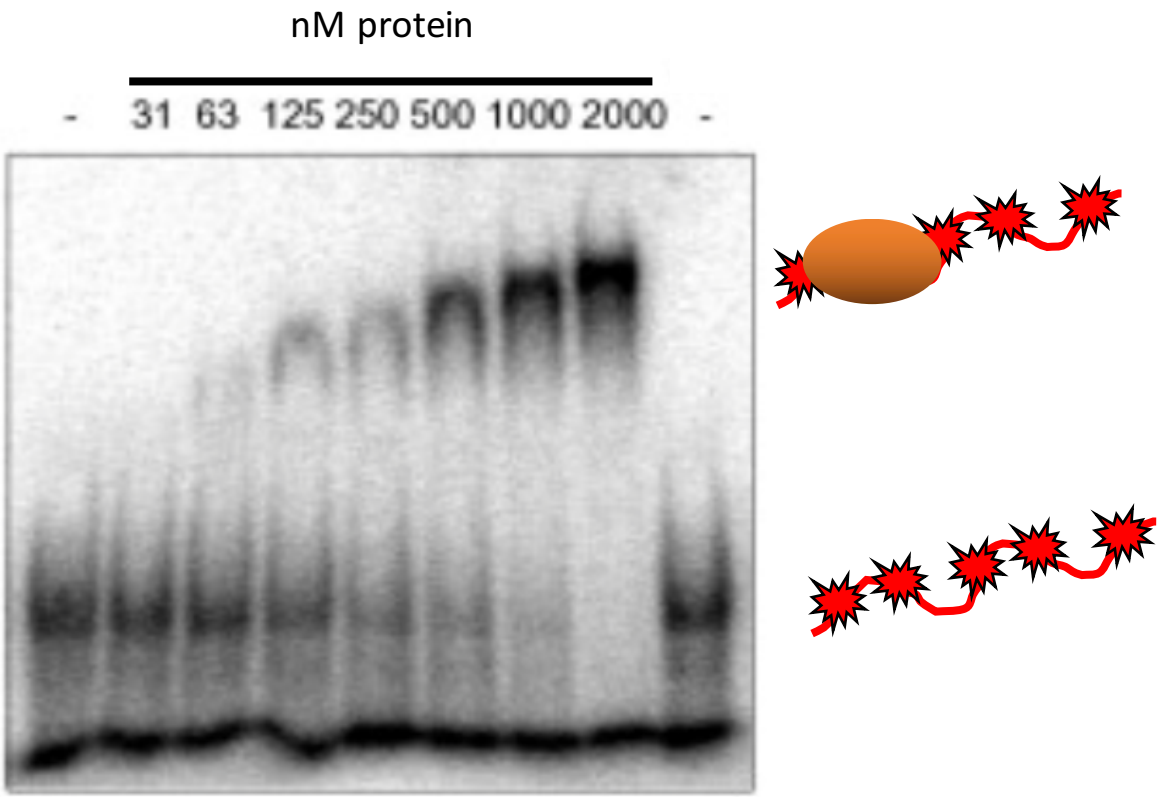
Incubate protein and DNA probe
Load onto non-denaturing PAGE
Resolve complexes & free probe



Electrophoretic-Mobility Shift Assay (EMSA)

Resolution of multiple protein/DNA complexes: Proteins sharing a DNA binding element




nM protein
- 31 63 125 250 500 1000 2000 -