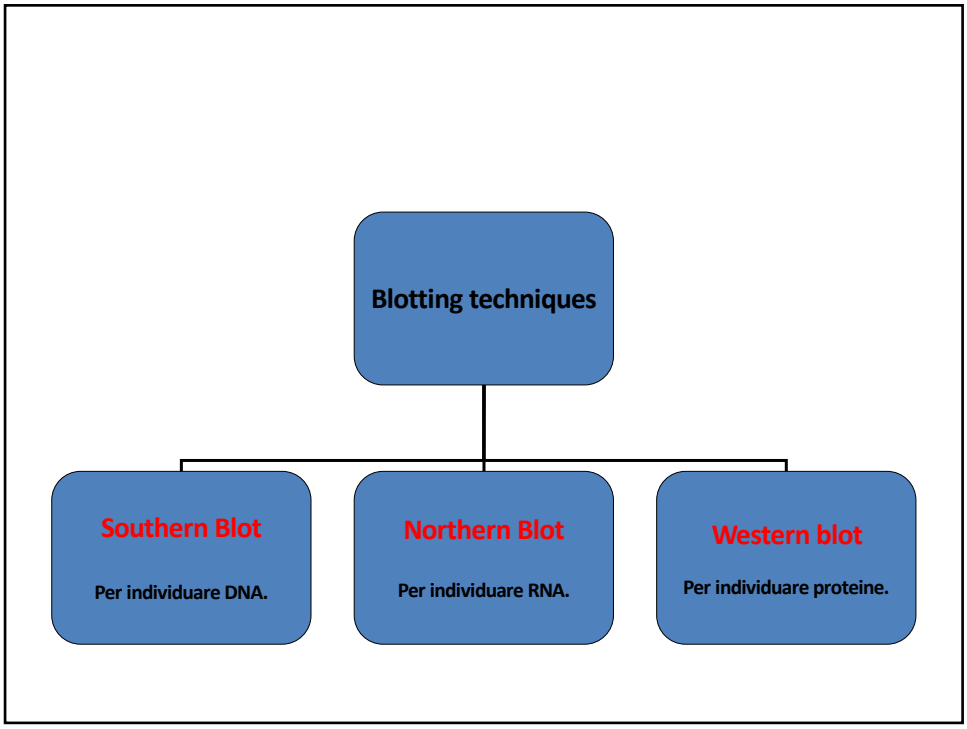
BLOTTING TECHNIQUES

1



2

SOUTHERN BLOTTING

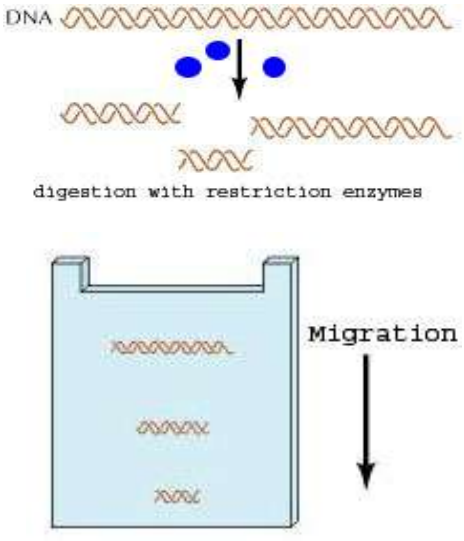
Professor Sir Edwin Southern

3

Steps in southern blotting

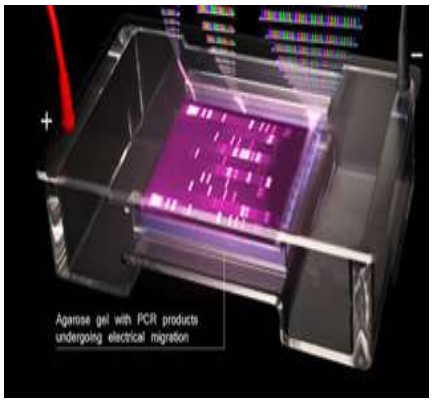
1. **DNA digerito con enzimi di restrizione**

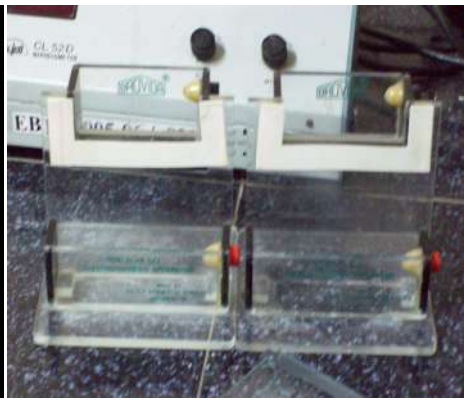
2. **I frammenti sono sottoposti ad elettroforesi per separarli sulla base della grandezza**



4

2. elettroforesi

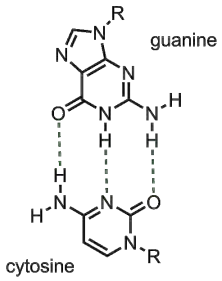


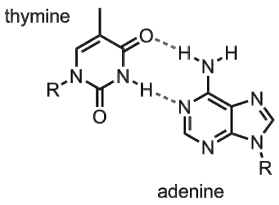


5

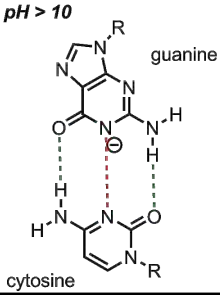
3. Denaturazione (con pH alto)

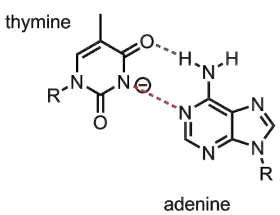
At physiological pH





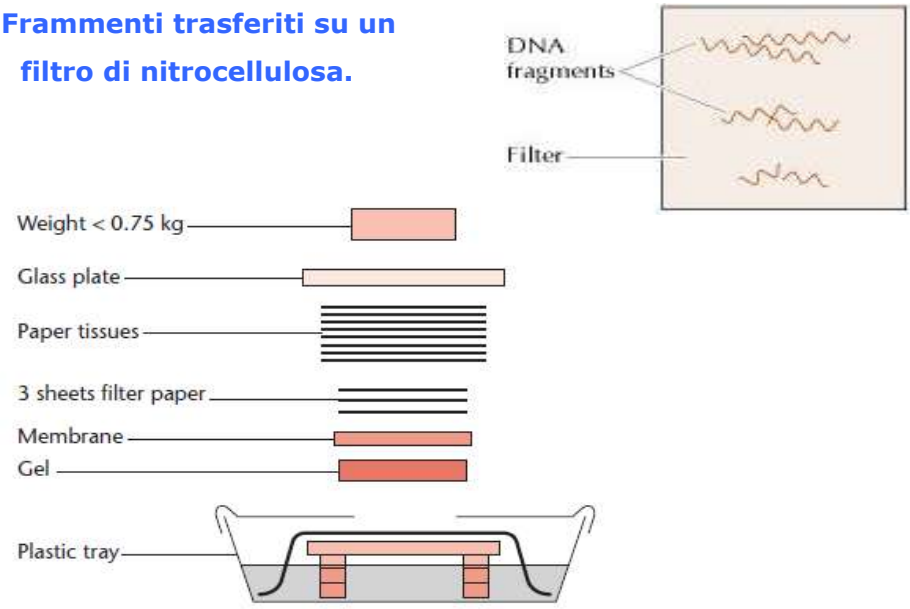
At pH > 10





6

4.Frammenti trasferiti su un filtro di nitrocellulosa.



Capillary plotting apparatus

7

Whatman 3MM paper

membrana di nitrocellulosa

8

SOLID SUPPORT

❖ Nitrocellulose Membrane

-Nucleic acids more than 400 bases are inefficiently bound.
- Attachment by hydrophobic interactions.
-Become brittle while baking in vacuum.
- Care required for storing.

❖ Nylon

-Buffers of low ionic strength can be used.
- Transfer can be carried out electrophoretically.
-Two types a)Neutral b)Positively charged (amines).

9

NITROCELLULOSE MEMBRANE	NYLON MEMBRANE
Hydrophobic binding.	Covalent binding.
Fragile	Durable
>200-300 bp probe length	<200-300 bp probe can be used
Lower background noise	Higher.
Cannot be exposed to basic solution	Can be exposed.
Not easily reprobed	Can be easily reprobed several times.

10

5. Membrana a 80 °C per 2 h o esposta a UV (nylon membrane) per legare in maniera permanente il DNA

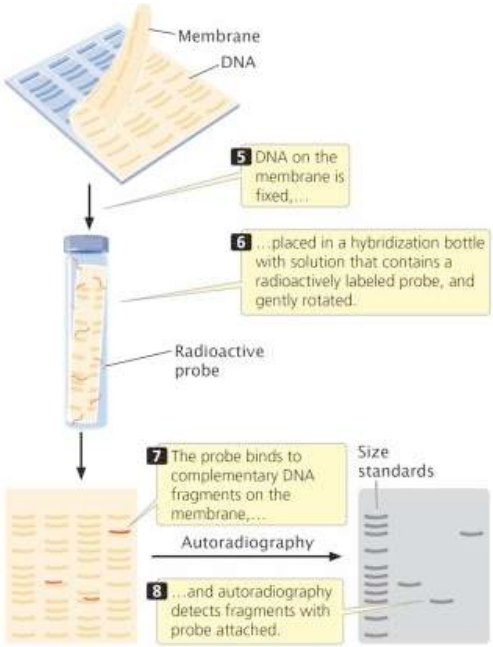
(6. Pre IBRIDIZZAZIONE)

Ficoll or salmon sperm DNA

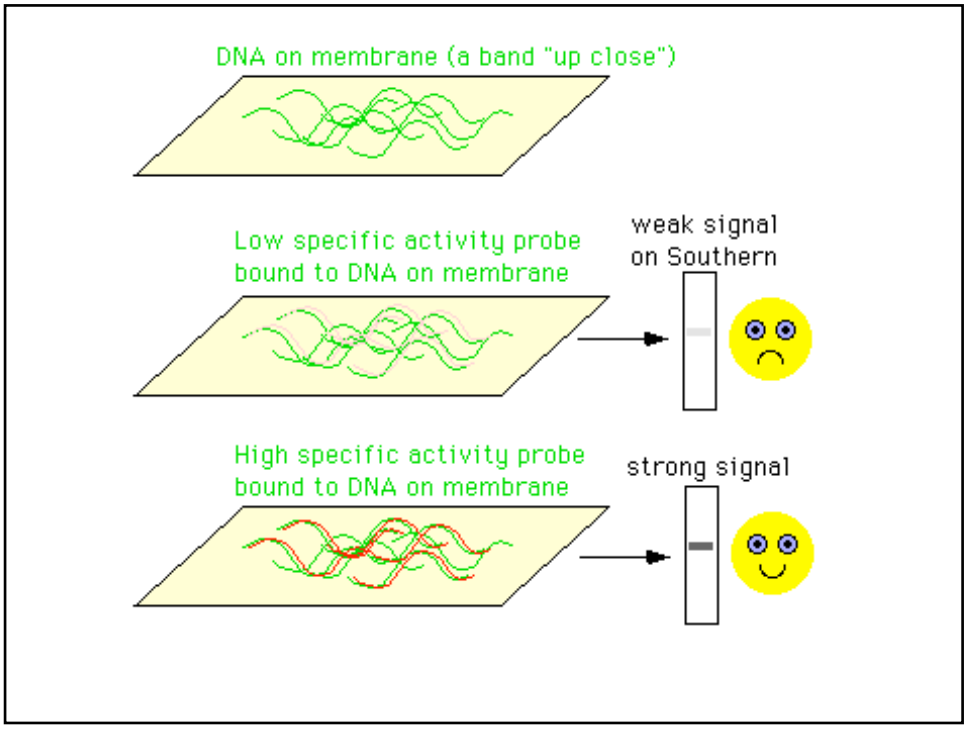
11

7. IBRIDIZZAZIONE: Filtro incubato con una sonda che ibridizza il DNA complementare

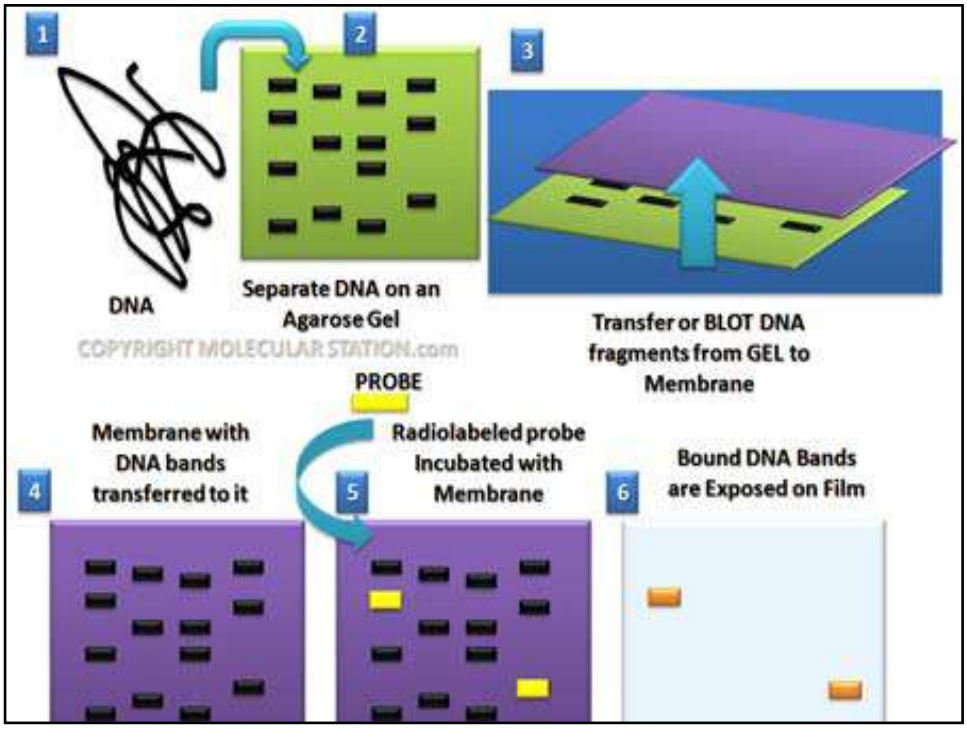
^{32}-P ssDNA



12


DNA on membrane (a band "up close")
Low specific activity probe bound to DNA on membrane
weak signal on Southern
High specific activity probe bound to DNA on membrane
strong signal

13


1
2
3
DNA
Separate DNA on an Agarose Gel
Transfer or BLOT DNA fragments from GEL to Membrane
COPYRIGHT MOLECULAR STATION.com
PROBE
Membrane with DNA bands transferred to it
Radiolabeled probe Incubated with Membrane
Bound DNA Bands are Exposed on Film
4
5
6

14

Alcune APPLICAZIONI

- in gene discovery, mapping, diagnostic e medicina forense and forensics (It is used for DNA fingerprinting, preparation of RFLP maps)
- Per l'identificazione di geni trasferiti in organismi transgenici
- Permette di determinare il peso molecolare di un frammento di restrizione e misurarne quindi la relativa quantità in campioni diversi
- …..

15

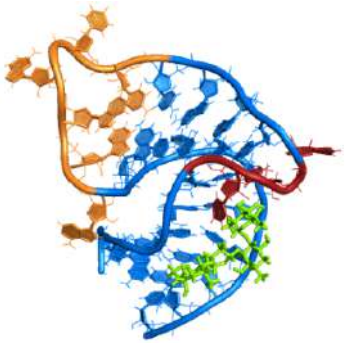
Northern Blotting

16

Steps in Northern blotting

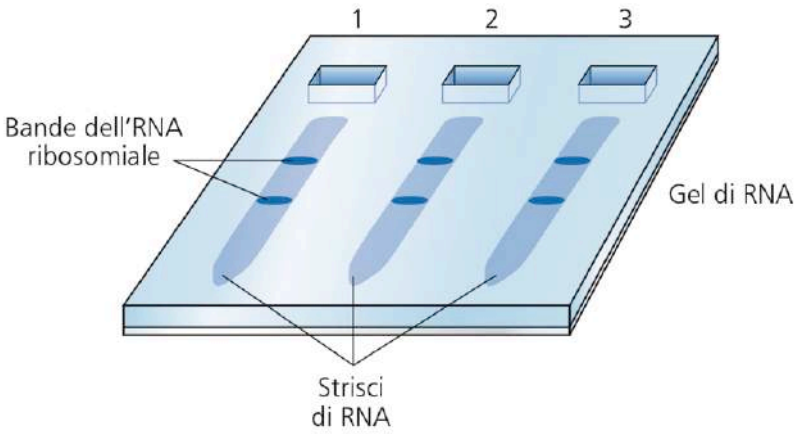
1. **RNA isolato da campioni biologici**

NB RNA è più sensibile alla degradazione rispetto al DNA.

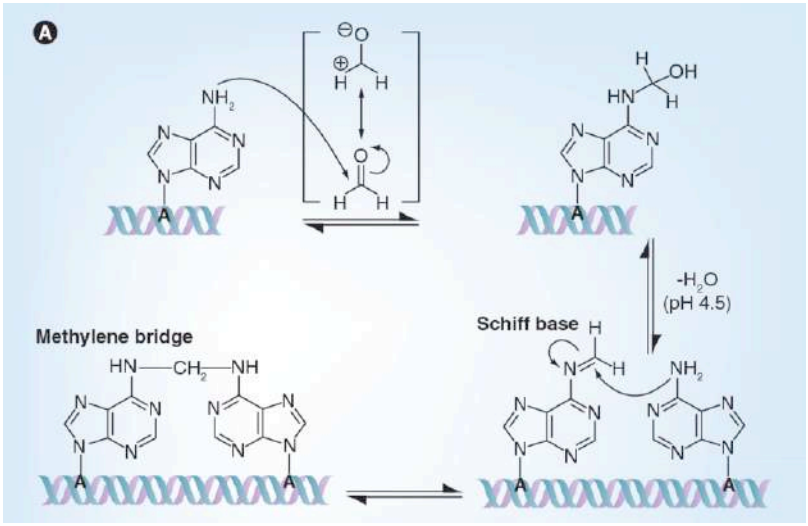


17

2. RNA SOTTOPOSTO AD ELETTROFORESI SU GEL DI AGAROSIO IN CONDIZIONI DENATURANTI (con FORMALDEIDE)

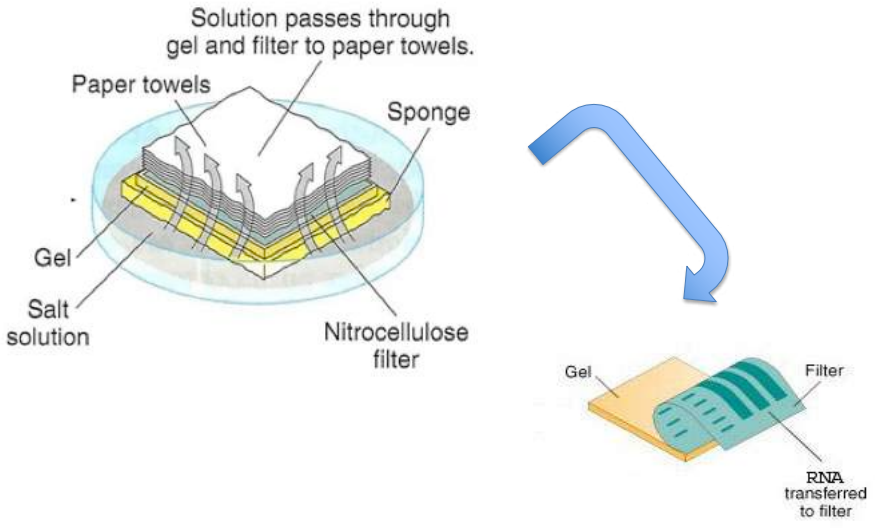


18



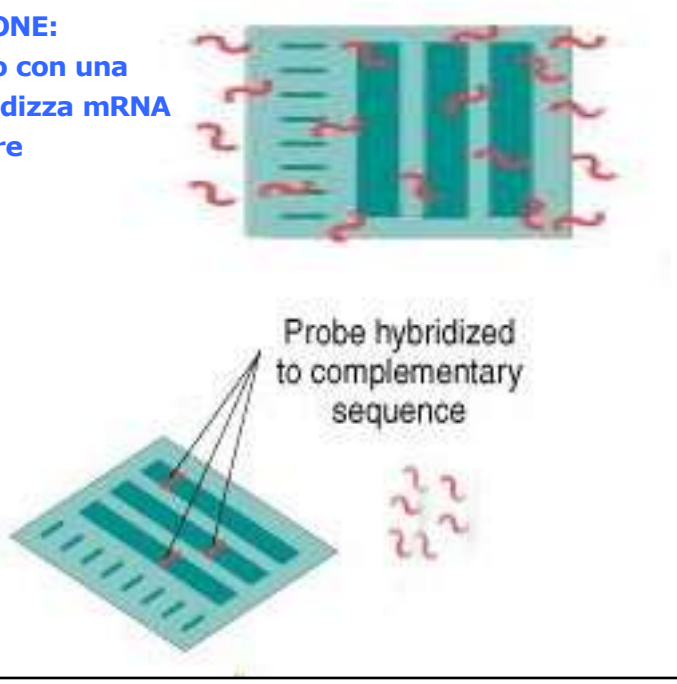
19

3. RNA trasferito su un filtro di nitrocellulosa



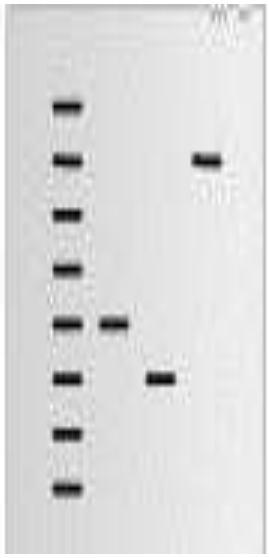
20

4. IBRIDIZZAZIONE:
Filtro incubato con una sonda che ibridizza mRNA complementare



21

5. Rilevamento bande su lastra



22

Alcune APPLICAZIONI

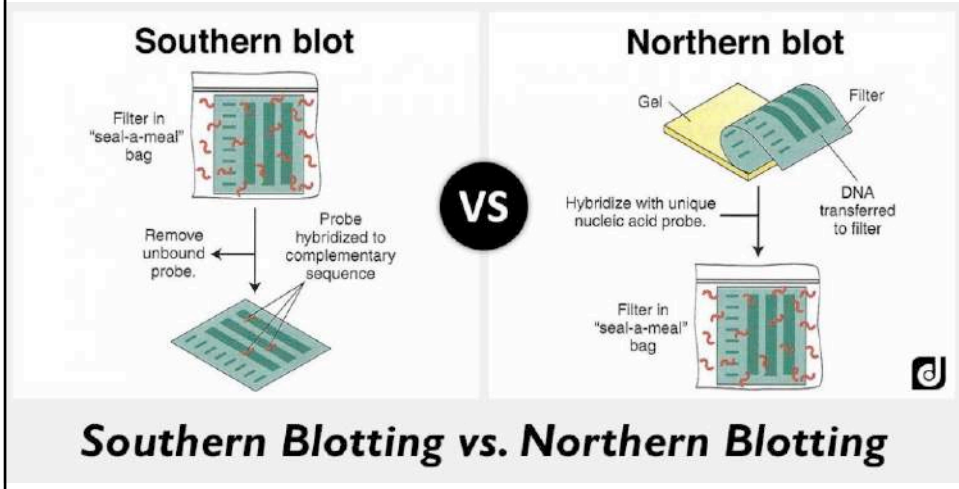
- STUDIARE L'ESPRESSIONE GENICA
- STUDIARE RNA DEGRADATION
- STUDIARE RNA SPLICING
- STUDIARE EMIVITA RNA
- .....

23

SVANTAGGI del Northern blotting

1. *BASSA SENSIBILITÀ RT-PCR*
2. *USO DI PIÙ SONDE È DIFFICOLTOSO*
3. *SE RNA ANCHE SOLO PARZIALMENTE DEGRADATO, LA QUALITÀ DEI DATI È BASSA E LA QUANTIFICAZIONE È DIFFICOLTOSA*

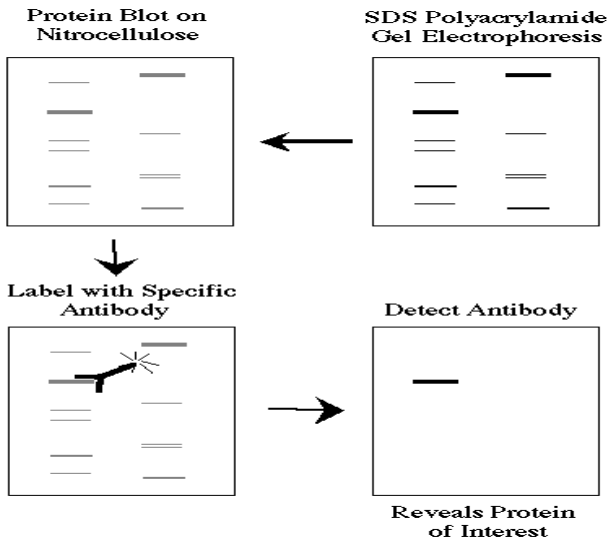
24



25

Western blotting & SDS-PAGE

26



..Western Blot

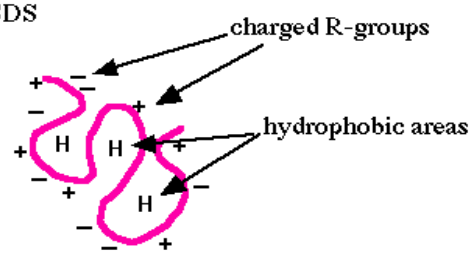
27

SDS-PAGE
(PolyAcrylamide Gel Electrophoresis)

28

SDS

BEFORE SDS



Protein (pink line) incubated with the denaturing detergent SDS showing negative and positive charges due to the charged R-groups in the protein. The large H's represent hydrophobic domains where nonpolar R-groups have collected in an attempt to get away from the polar water that surrounds the protein.

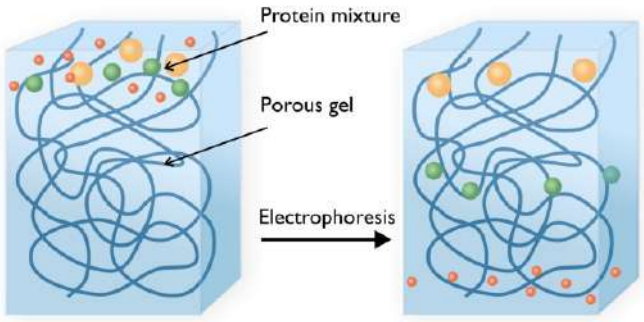
AFTER SDS

SDS disrupt hydrophobic areas (H's) and coat proteins with many negative charges which overwhelms any positive charges the protein had due to positively charged R-groups.

The resulting protein has been denatured by SDS (reduced to its primary structure-aminoacid sequence) and as a result has been linearized.

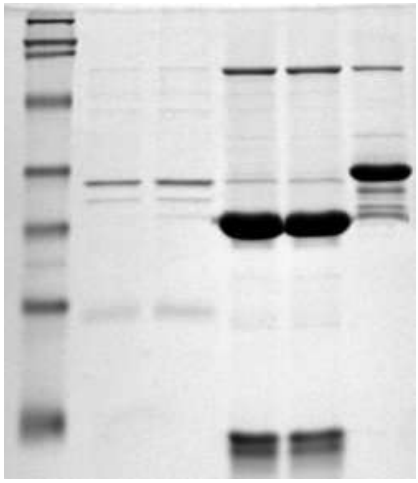
29

…..PAGE



30

Sample of SDS- PAGE



31

What happens after electrophoresis?

1. Fix the proteins in the gel and stain them.
2. Electrophorectic transfer to a membrane and then probe with antibodies- (Western blotting)

32

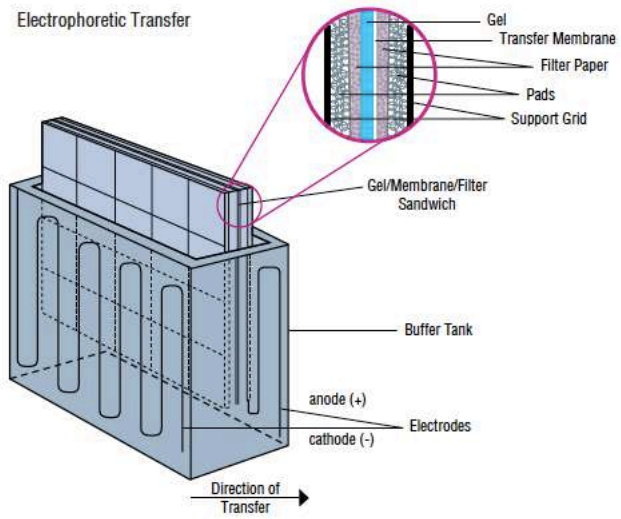
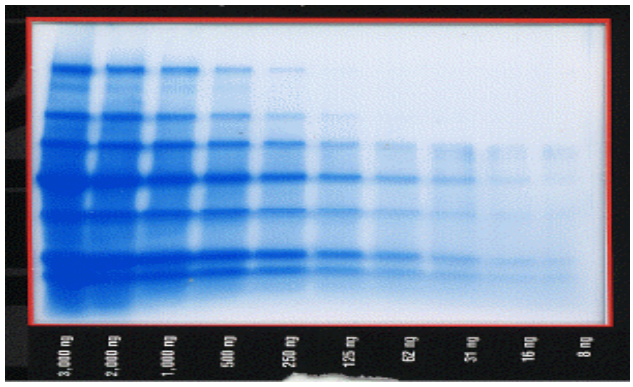


Figure 2. Electrophoretic transfer.

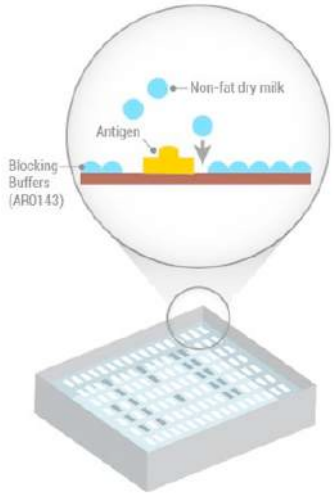
33



Protein gel (SDS-PAGE) that has been stained with Coomassie Blue.

34

Blocking Nonspecific Binding Sites

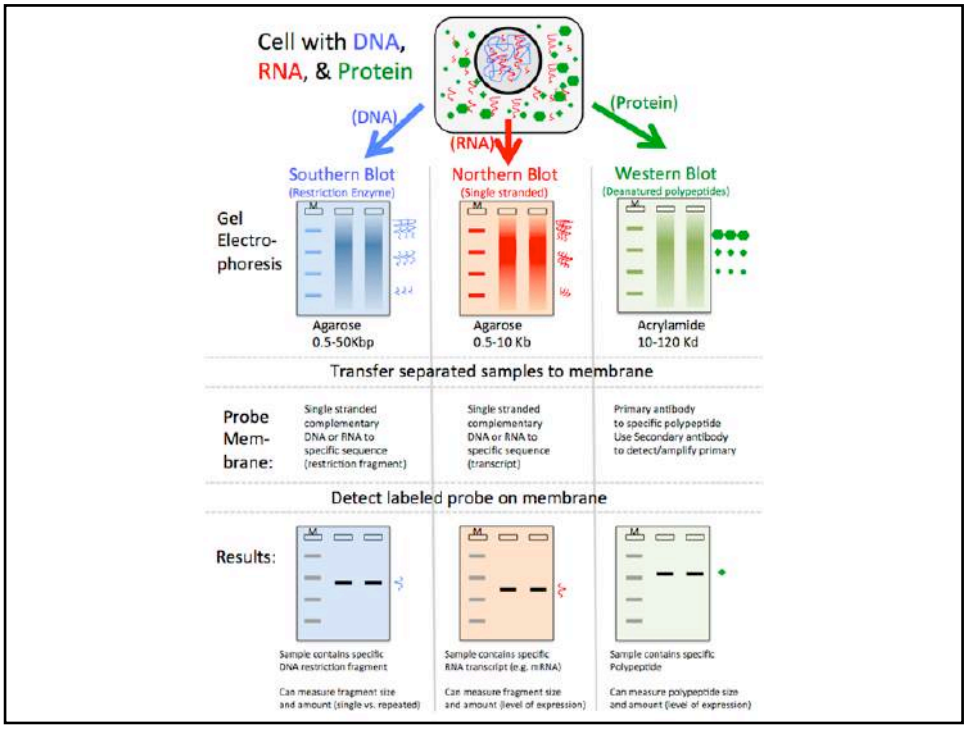


35

..Western blotting

- Western blot analysis can detect **one** protein in a mixture of any number of proteins while giving you information about the size of the protein.
- This method is, however, dependent on the use of a high-quality antibody directed against a desired protein.
- This antibody is used as a probe to detect the protein of interest.

36


Cell with DNA, RNA, & Protein
(DNA)
(RNA)
(Protein)
Southern Blot
(Restriction Enzyme)
Northern Blot
(Single stranded)
Western Blot
(Deanatured polypeptides)
Gel Electro-phoresis
Agarose 0.5-50Kbp
Agarose 0.5-10 Kb
Acrylamide 10-120 Kd
Transfer separated samples to membrane
Probe Mem-brane:
Single stranded complementary DNA or RNA to specific sequence (restriction fragment)
Single stranded complementary DNA or RNA to specific sequence (transcript)
Primary antibody to specific polypeptide Use Secondary antibody to detect/amplify primary
Detect labeled probe on membrane
Results:
Sample contains specific DNA restriction fragment
Can measure fragment size and amount (single vs. repeated)
Sample contains specific RNA transcript (e.g. mRNA)
Can measure fragment size and amount (level of expression)
Sample contains specific Polypeptide
Can measure polypeptide size and amount (level of expression)

37