"Western Blot"

- تكملة لموضوع الـ electrophoresis و الـ purification و الـ isolation للبروتين ...

- إذا مرتفت بروتين داخل M0 ، كيف بدي أتأكد إنه بروتيني مش بروتين M0 ؟!

- الـ "Western Blot" هو لي بدي بيه أعرف الـ indication

Two Main Types of Westerns

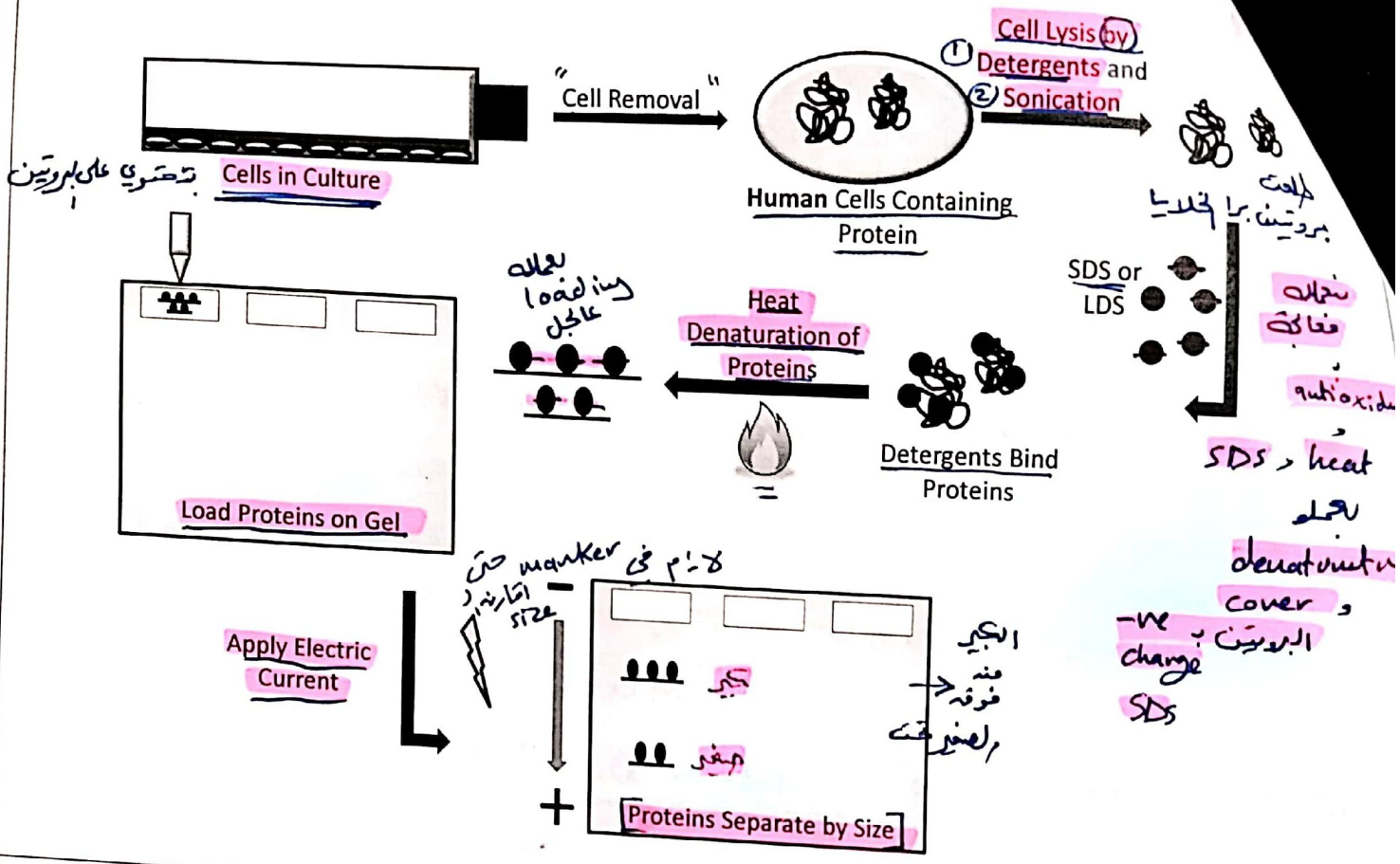
1. **Denaturing (Most Commonly Used)** الأكثر استخداماً .

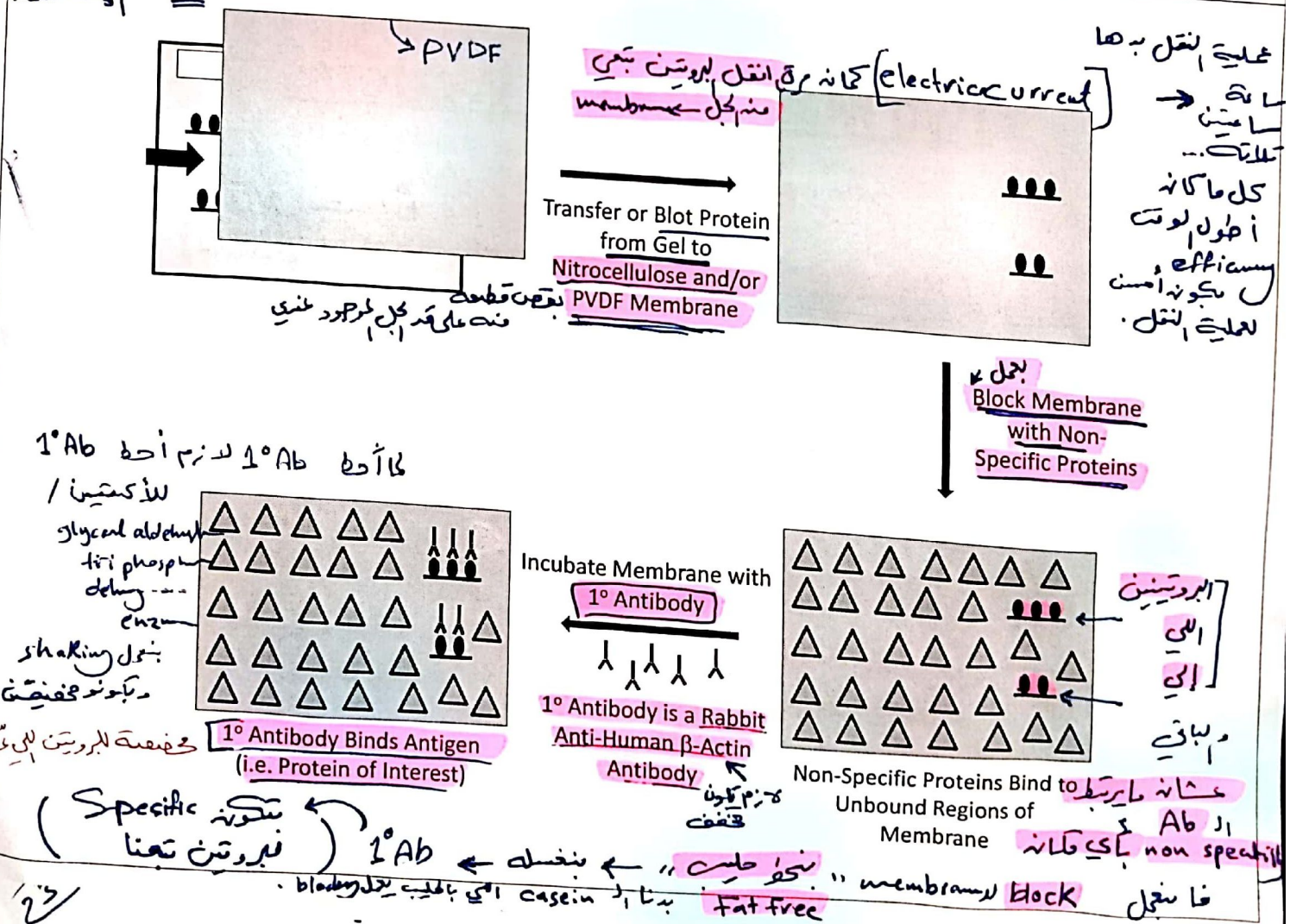
   - SDS-PAGE

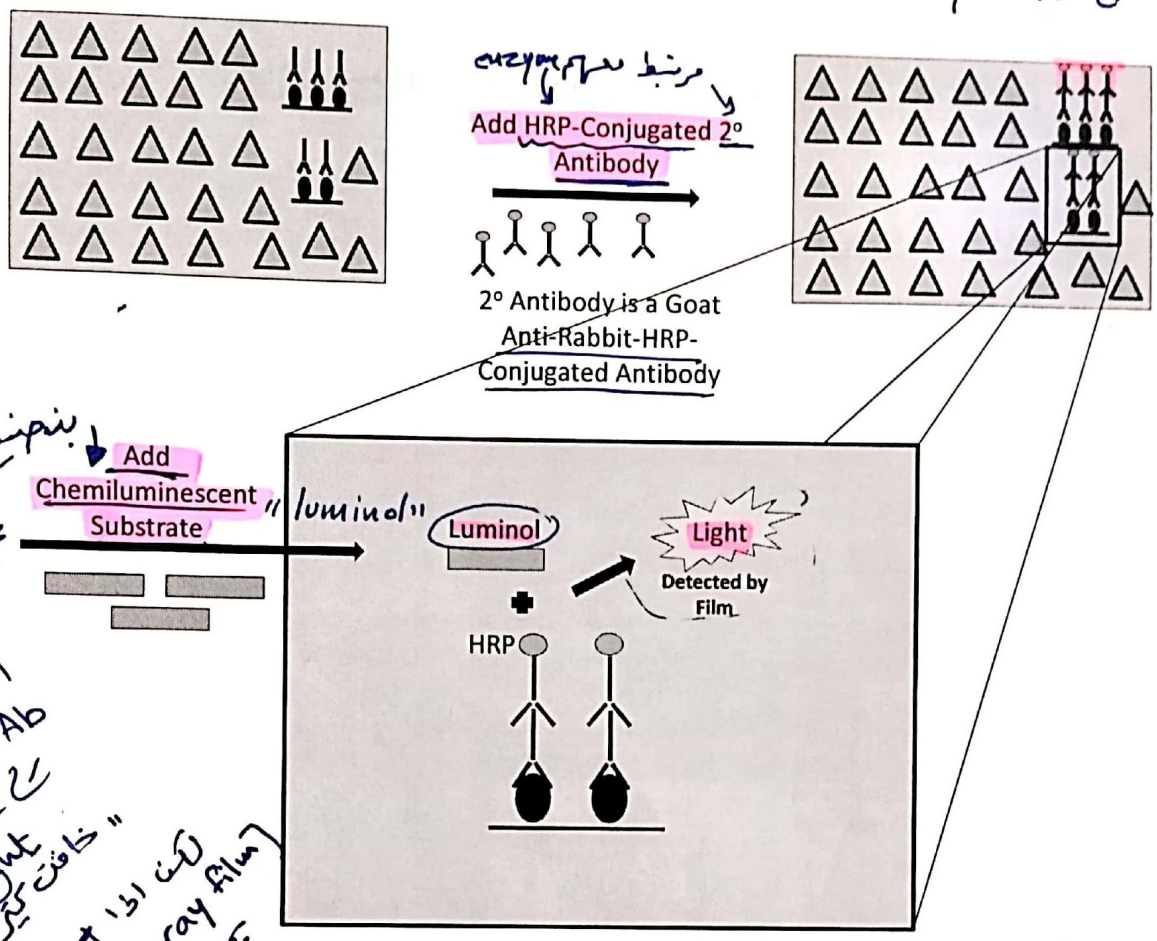
2. **Non-Denaturing**

   - Native PAGE

SDS-PAGE Western Blot Method







Advantages and disadvantages of western blot

Advantages:

1. Verify the expression of a protein ~~with high sensitivity and specificity~~
2. Determine the relative amount of a protein present in different samples "Semi-quantitative method for the protein present in the sample"
3. Analyze protein-protein interactions

Disadvantages:

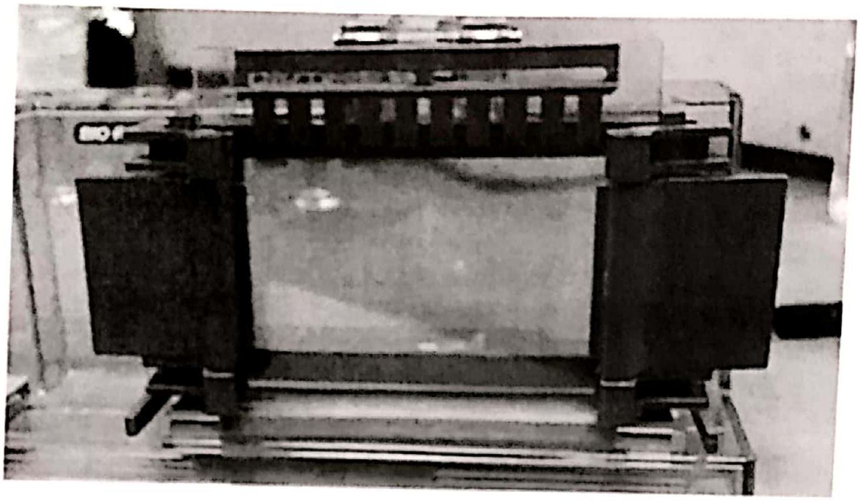
1. Many steps where errors can occur
2. Accurate quantitation is very difficult
3. Time consuming protocol
4. High cost
5. Large amount of sample needed (5-50 mg).

* بستخدم 12% gel ← 45 KDa 6 وإذا حجمه أكبر بنزل لـ 10%

* بقدر أعمل detection لـ another protein ← بستخدم ② 1° Ab
in same time
كل واحد برتبط مع ا antigen شكل

← بعد هيك بضيف ا 2°Ab ...

← بيبين معي 2 bands على الجل



Volumes for 1X 1.0μm Space Resolving Gels

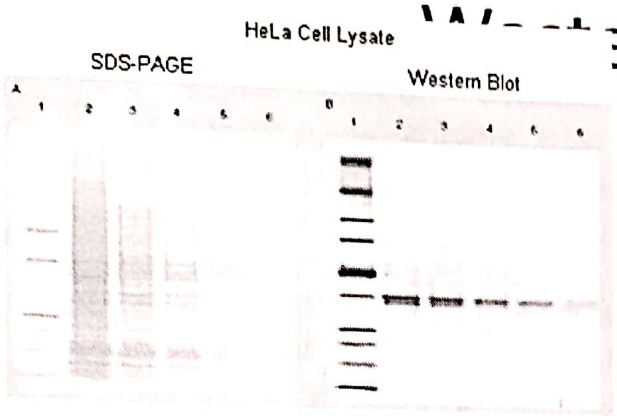
(5 ml total)

	7%	8%	9%	10%	12.5%	15%
30% Acrylamide	1.16 ml	1.33 ml	1.5 ml	1.66 ml	2.08 ml	2.5 ml
H2O	2.48 ml	2.32 ml	2.15 ml	1.98 ml	1.57 ml	1.15 ml
1.5M Tris (pH 8.8)	1.25 ml	1.25 ml	1.25 ml	1.25 ml	1.25 ml	1.25 ml
10% SDS	50 μl	50 μl	50 μl	50 μl	50 μl	50 μl
10% APS	50 μl	50 μl	50 μl	50 μl	50 μl	50 μl
TEMED	5 μl	5 μl	5 μl	5 μl	5 μl	5 μl

Volumes for 1.0μm Stacking (4%)

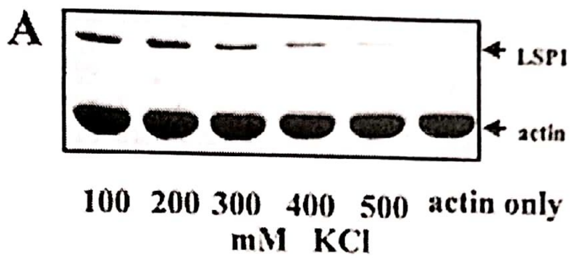
	1 Stack (2ml)	2 Stacks (4ml)	3 Stacks (6ml)	4 Stacks (8ml)
30% Acrylamide	340 μl	680 μl	1020 μl	1.36 ml
H2O	1.36 ml	2.72 ml	4.08 ml	5.44 ml
1M Tris (pH 6.8)	250 μl	500 μl	750 μl	1 ml
10% SDS	20 μl	40 μl	60 μl	80 μl
10% APS	20 μl	40 μl	60 μl	80 μl
TEMED	2 μl	4 μl	6 μl	8 μl

Western blot

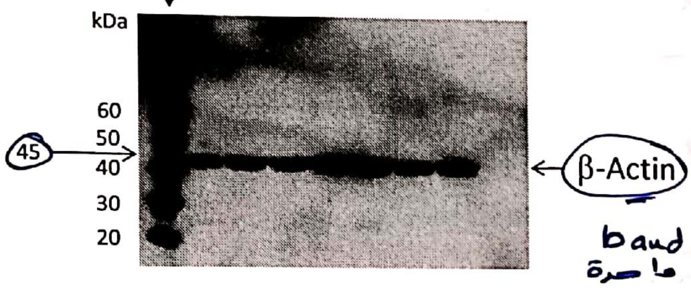


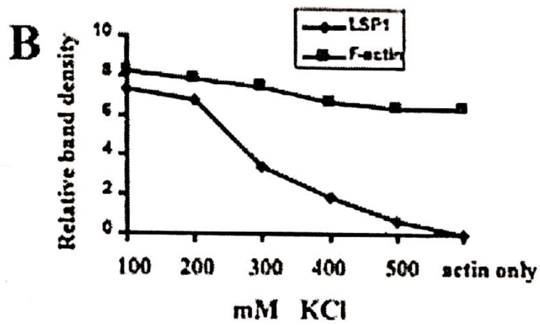
Chemiluminescent Detection of CDK7
BioRad Bulletin 2032

Lymphocyte specific protein 1



Magic Mark XP
Western Protein
Standard





Western Blot Protocol

1. Sample Preparation :-

A) Add 10 mg of protein to 5 ml of 4X LDS Loading Buffer plus 2.5 ml of 10X Reducing Agent. Then add purified water to a total volume of 25 ml.

anti oxidant — water ← 25 بكمل

For example: If your total protein concentration is 2.0 mg/ml, you would need 5 Ml ~~ml~~ of total protein to equal 10 Mg ~~mg~~. So you would mix:

5 Ml of protein
5 Ml of 4X LDS Loading Buffer
2.5 Ml 10X Reducing Agent
12.5 Ml purified water.

B) Heat sample mixture at 70°C for 10 minutes. → حرك عملت للبروتين Denaturation

أو 95°C for 5 minutes.

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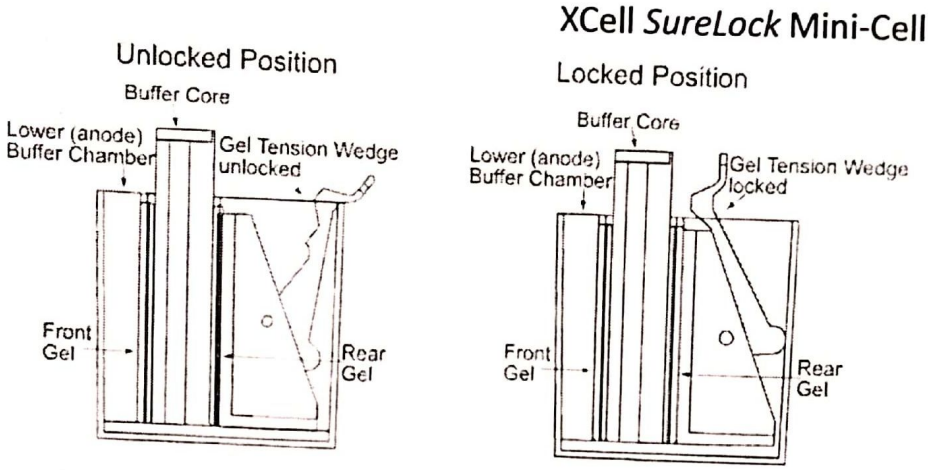
CamScanner المسوحة ضوئيا بـ

2. Electrophoresis

A) While protein samples are heating, assemble electrophoresis unit.

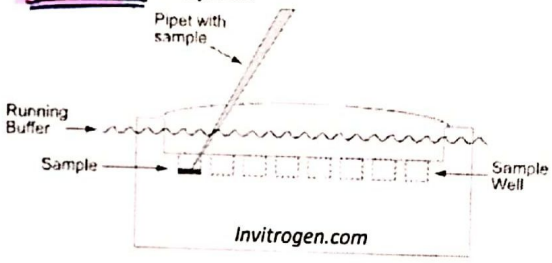
Demonstration



B) Load Gel

-Molecular weight marker and protein samples

Demonstration



C) Add 500 µl Antioxidant to top chamber to maintain proteins in a reduced state and ensure optimal band sharpness.

D) Run gel at 180V for 45 minutes

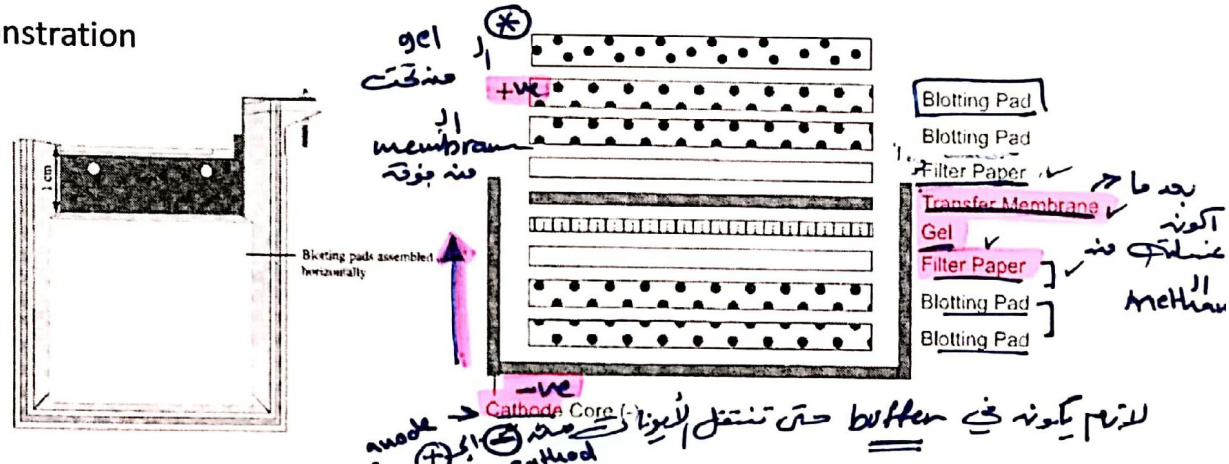
3. Transfer

A) Soak marked (for orientation) nitrocellulose (or PVDF) membrane in transfer buffer containing 10% Methanol at least 10 minutes prior to transfer.

B) When gel run is complete, turn off power source, remove gel from pre-cast plates, place transfer buffer-soaked filter paper sheet on top of gel, remove gel from plate, and place on top of membrane blotting pads that have been removed of bubbles.

C) Place membrane on top of gel and cover with another transfer buffer-soaked filter paper sheet and blotting pads to fill the transfer chamber.

Demonstration



D) Add 500 µl Antioxidant and run transfer at 30V for 1 hour.

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4. Blocking by milk

A) Remove membrane from transfer chamber and incubate in 5% Blotto [5% powdered milk in TBS-Tw (1X TBS, 0.1% Tween 20)] at room temperature for 30 minutes with slow shaking.

Fill up the space on the membrane to prevent non-specific antibody binding

Recommended to block for >1 hour

Milk	BSA
Strong blocking agent	High signal
Less signal, less background	High background!
Not-recommended for phospho-proteins	
Cheap!	

BSA: Bovine serum albumin

Diluted in same Buffer use for washing (PBST/TBST)

5. Primary Antibody Incubation ← 1° Ab

A) Prepare a 1:1000 dilution of primary antibody (Rabbit Anti-Human b-Actin) in 5% Blotto. ← TBS-Tween 20

B) Incubate membrane in primary antibody solution overnight at 4°C with gentle rocking.

6. Membrane Washing

A) Wash membrane 3 x 5 minutes each in TBS-Tw with gentle shaking at room temperature.

7. Secondary Antibody Incubation

A) Prepare a 1:5000 dilution of secondary antibody (Goat Anti-Rabbit IgG-HRP) in 5% Blotto.

B) Incubate membrane in secondary antibody solution for 30 minutes at room temperature with gentle shaking.

8. Repeat Membrane Washing

9. Visualization of Protein of Interest

A) Place membrane protein side up on saran wrap on a flat surface.

B) Quickly add 50 ml of ECL solution B to 2 ml of ECL solution A, mix, and add directly to membrane.

C) Incubate in the dark for 3-5 minutes, remove excess solution, and place membrane protein side down onto a new piece of saran wrap.

D) Close saran wrap around membrane, tape to film cassette and expose film in the dark room for 30 seconds to 1 minute.

E) Develop film & identify protein of interest.

Detection

- Colorimetric – less sensitive
- Radioactive label
- Fluorescently labelled secondary antibody – highly quantitative
- Chemiluminescent – HRP or AP labelled secondary antibody - very sensitive!

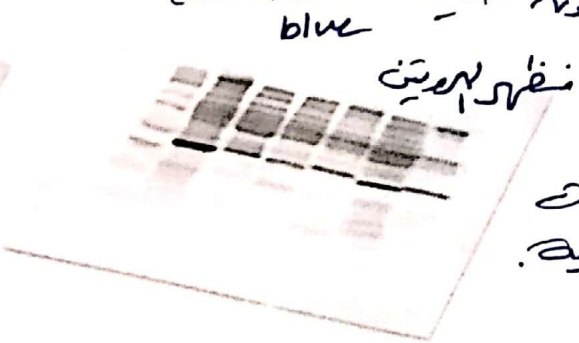
- Rabbit primary antibodies
  - Natural diversity ✓
  - High affinity and specificity ✓
  - Novel epitope recognition

- Why goat anti-rabbit secondary antibodies
  - To increase the intensity of the signal

Result of Western Blot

band البروتين واضح عبارة عن
1°,2° واحدة تفاعل مع
Ab

لونتها بـ
commassie blue



Blot from unstained Gel

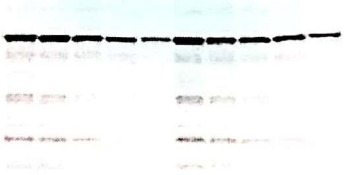
Blot from stained Gel

Confirm HIV virus

- The confirmatory HIV test employs a western blot to detect anti-HIV antibody in a human serum sample.
- Proteins from known HIV-infected cells are separated and blotted on a membrane as above.
- Then, the serum to be tested is applied in the primary antibody incubation step; free antibody is washed away, and a secondary anti-human antibody linked to an enzyme signal is added.
- The stained bands then indicate the proteins to which the patient's serum contains antibody.
- Western blot can also be used as a confirmatory test for Hepatitis B infection

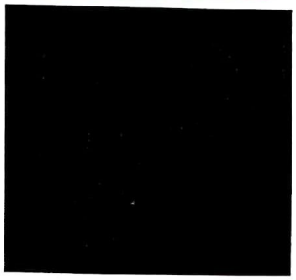
Common problems

1. Non specific bands



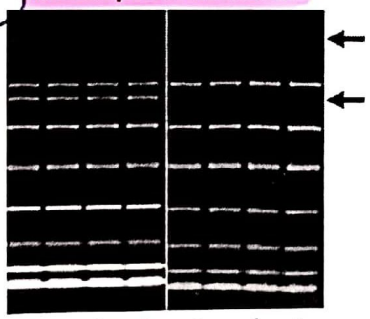
Probably too much antibody
Or insufficient blocking

2. High background



Probably too much antibody
Or insufficient blocking
Or insufficient washing

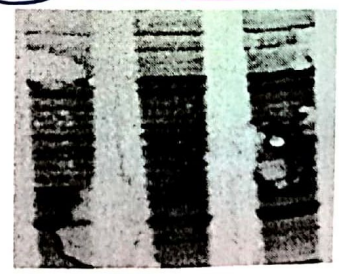
3. Incomplete transfer



Transfer time too short
Transfer current too low

4. Blotchy transfer

↗ air bubbles between



Air bubbles between gel and membrane

Other types of blotting

- Southern blotting is used to detect specific sequences of DNA in DNA samples.
- Northern blotting for RNA
- Eastern for post-translational protein modifications
- South-western for DNA-protein interactions blotting.