western blot

WHAT IS IT AND WHAT IS IT USED FOR

Western Blot - Theory and method

Western blotting technique: principle, procedure and application

DROP

S - SOUTHERN - DNA - D

N - NORTHERN - RNA - R

O - OOOOOOO - OOOO - O

W - WESTERN - PROTEIN - P

Blotting techniques mnemonic

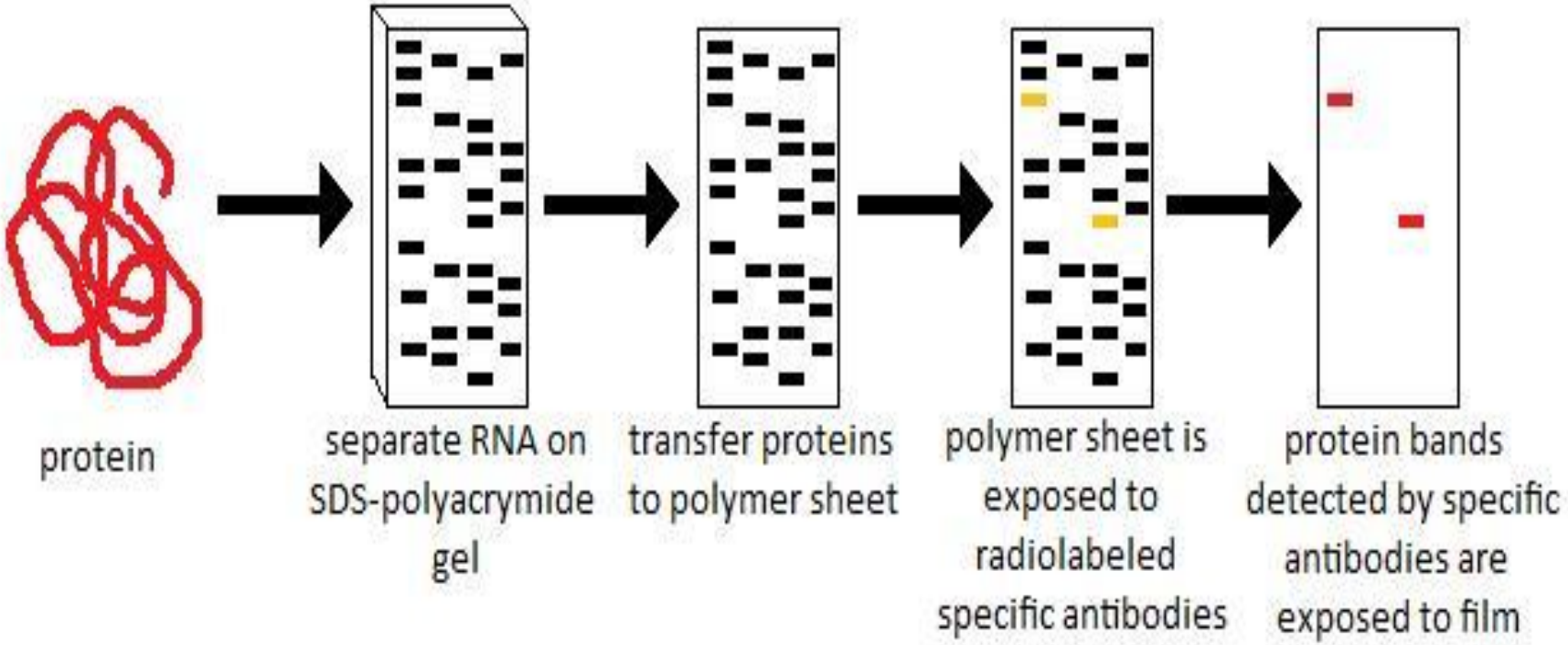
The term “blotting” refers to the transfer of **biological samples from a gel to a membrane** and their subsequent detection on the surface of the membrane. **Southern blot is used for transferring DNA, Northern blot for RNA and Western blot for Pro**tein.

Western blotting (also called **immunoblotting,** because an **antibody is used to specifically detect its antigen**) was introduced by Towbin, et al. in 1979 and is now a routine technique for protein analysis.

Western blotting can produce **qualitative and semi-quantitative data about the protein of interest**. It is an important technique used in cell and molecular biology. It enables the researchers to **identify the specific protein** from mixture of proteins extracted from cells as well as evaluation of their **size and amount**. The **SDS PAGE technique is prerequisite for western blotting**.

Principle

Western blotting (protein blotting or immunoblotting) is a


protein
separate RNA on SDS-polyacrymide gel
transfer proteins to polymer sheet
polymer sheet is exposed to radiolabeled specific antibodies
protein bands detected by specific antibodies are exposed to film

Procedure

Western blotting procedures includes following steps:

Tissue Preparation *(preparation of sample lysate)*:ake the sample, add ice cold PBS and lysis buffer such as RIPA buffer which is a commonly used buffer for maximum protein yield. *(The choice of lysis buffer largely depends on localization of the protein of interest, solubilization of **membrane bound proteins** require stronger extraction detergents compared with **isolated cytoplasmic proteins**).* Always use freshly prepared protease inhibitors, keep samples on ice and work quickly.

Lysis buffer should **contain protease inhibitors to prevent the degradation of the protein of interest**. **Cells are lysed by incubating on ice** and later applying shear pressure **using pipette**. The cell mixture is centrifuged and **pellet is discarded**. The supernatant is the lysate which we will use for further processing.

Western blots are typically performed under reduced and **denatured conditions**. These conditions will allow **proteins to be separated by their molecular weight rather than their native conformational shape or charge**.

To reduce and denature samples dilute each in **a loading buffer such as laemmli sample buffer**. This buffer contains

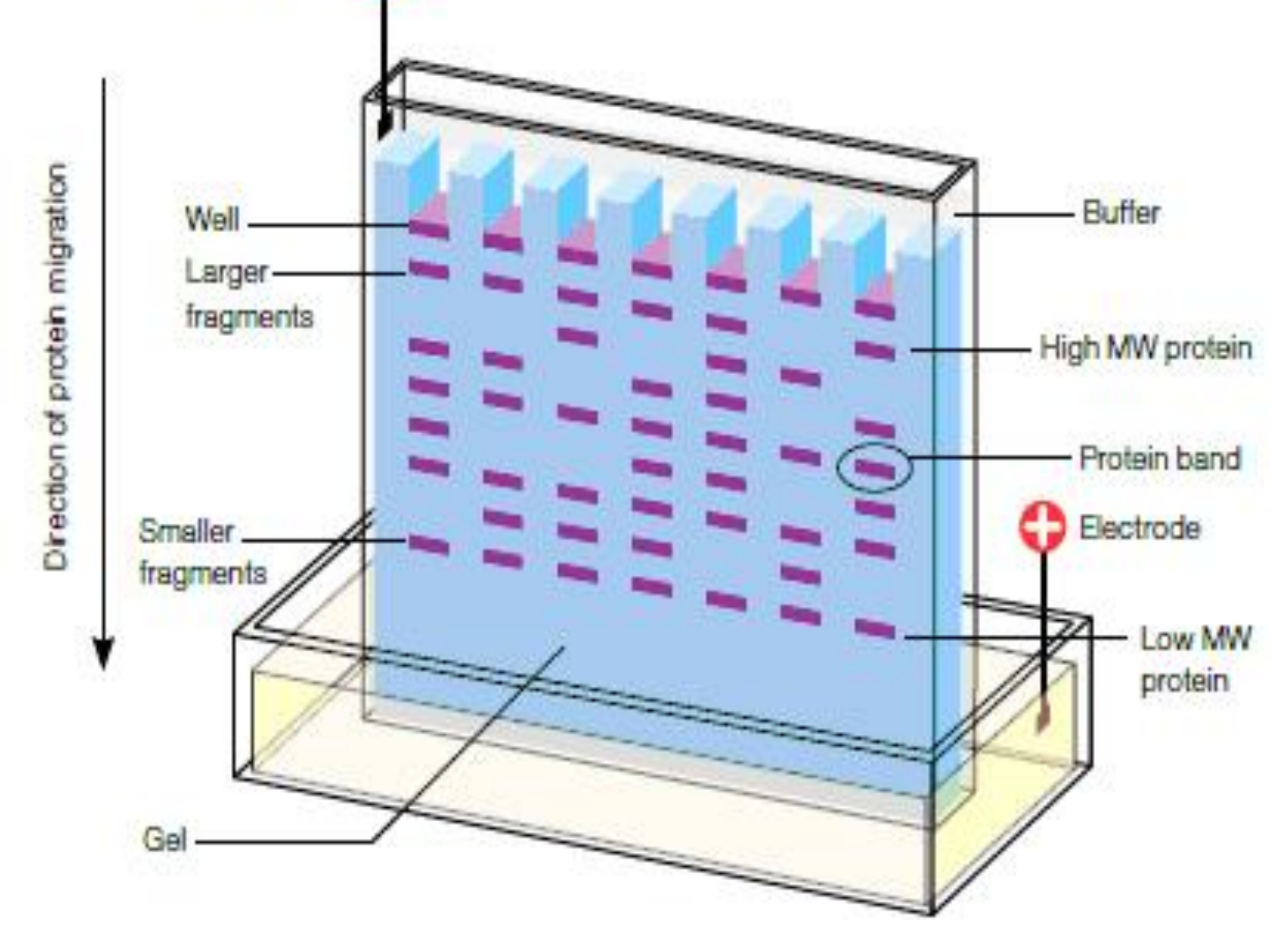
beta-mercaptoethanol, or DTT, to reduce **disulfide ridges between cysteines**, **SDS** to assist **denaturing and to provide a net negative charge** to the protein, **glycerol** to allow the **samples to sink** into each well, **bromophenol blue** to visualize the lysate and an ionic buffer.

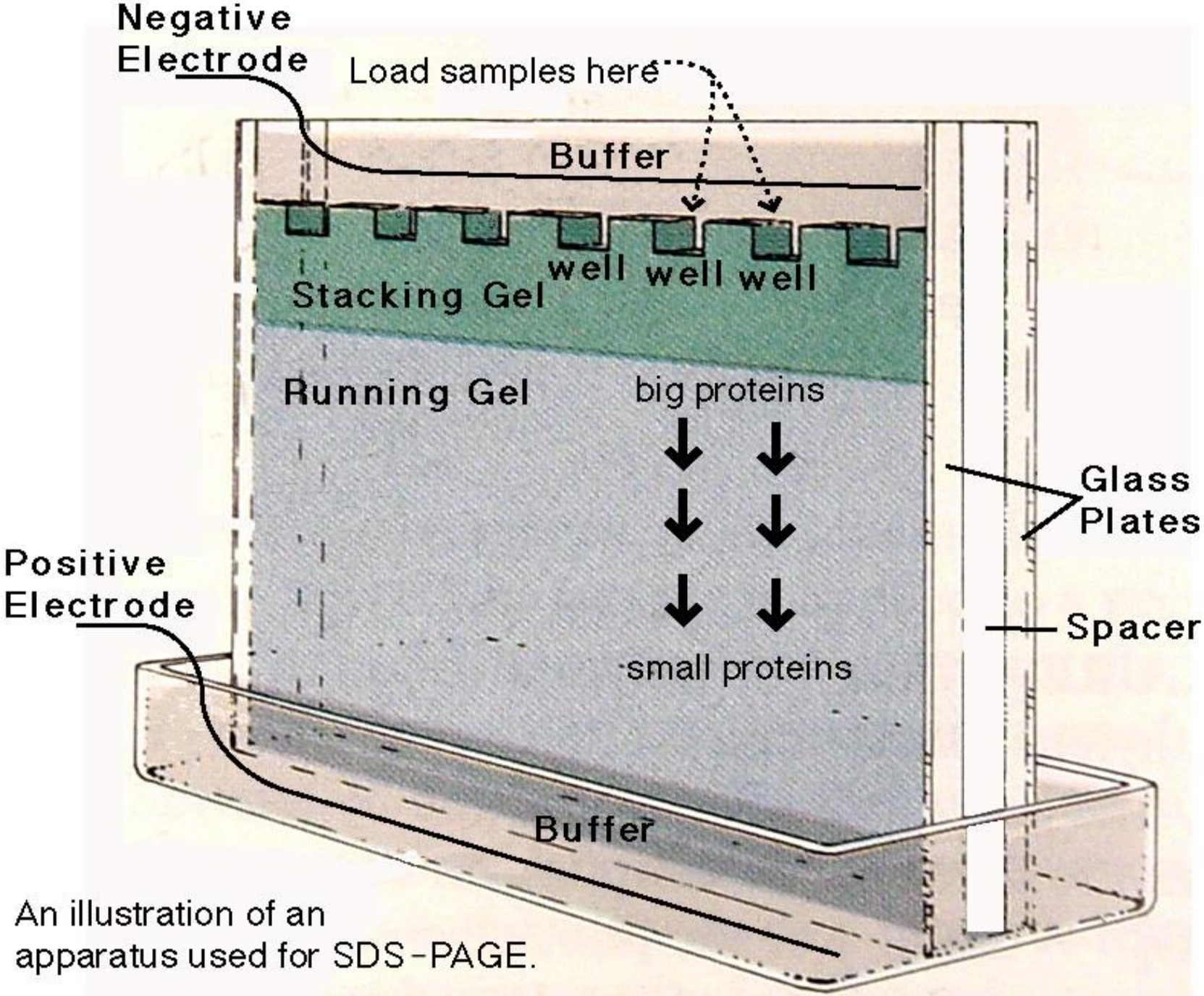
Vortex each sample and **incubate at 95 degrees Celsius for five minutes** to **completely denature the proteins.** Now the sample is ready to **load into an SDS page gel.**

Gel Electrophoresis:

In this step, we will separate the individual proteins in our sample lysate **based upon their molecular weight** using a positive electrode to attract **a negatively charged protein.** To do this, we load our previously prepared protein samples into a commercially **available polyacrylamide gel**.

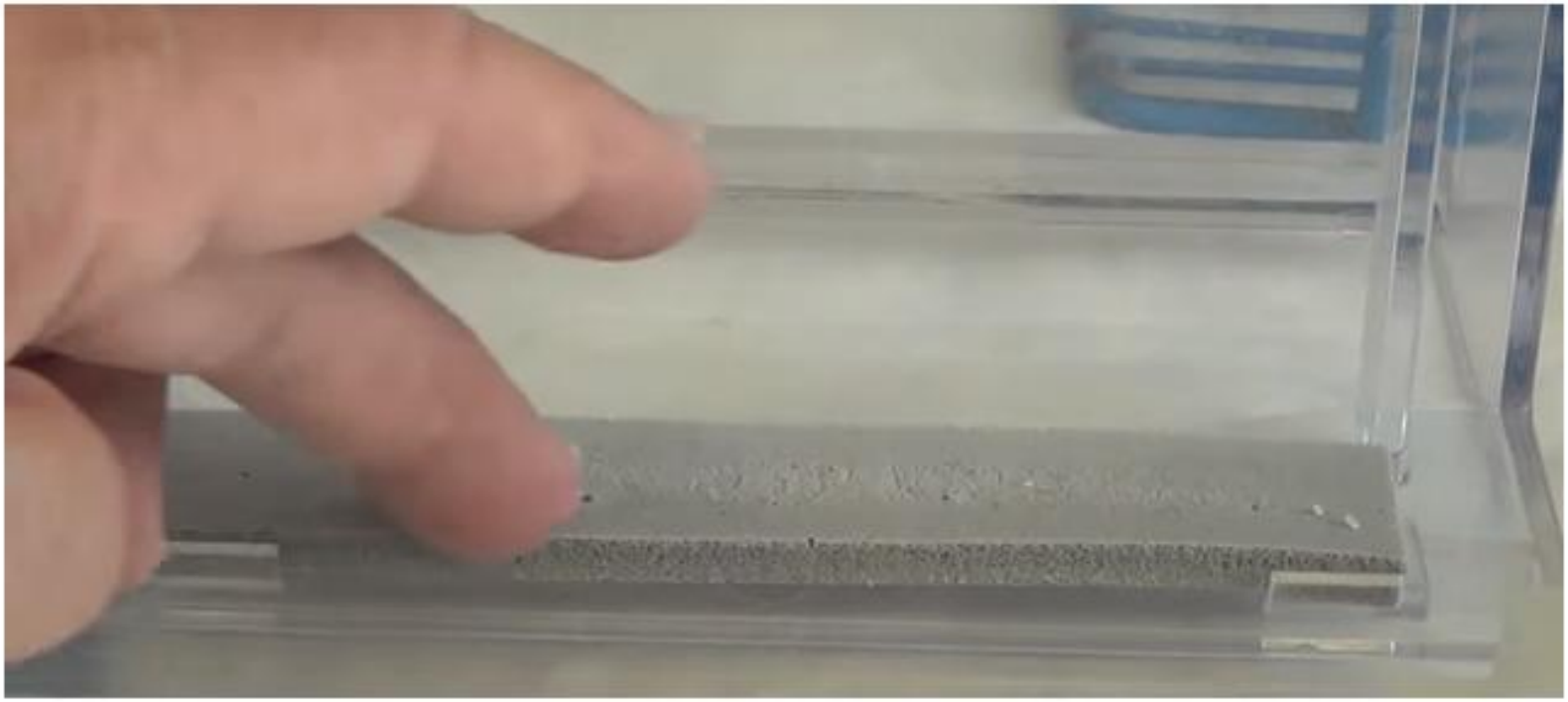
Gels are available in fixed percentages or gradients of acrylamide. **The higher the acrylamide percentage** the **smaller** the **pore size** of the gel matrix.
better for **low molecular weight proteins**, **low percentage of gel** are useful for **large proteins** and gradient gels can be used for proteins of all sizes due to their varying range in pore size.


Direction of protein migration
Well
Larger fragments
Smaller fragments
Gel
Buffer
High MW protein
Protein band
Electrode
Low MW protein



An illustration of an apparatus used for SDS-PAGE.

Preventig SDS-PAGE Gels From Leaking Using Stacking Ge



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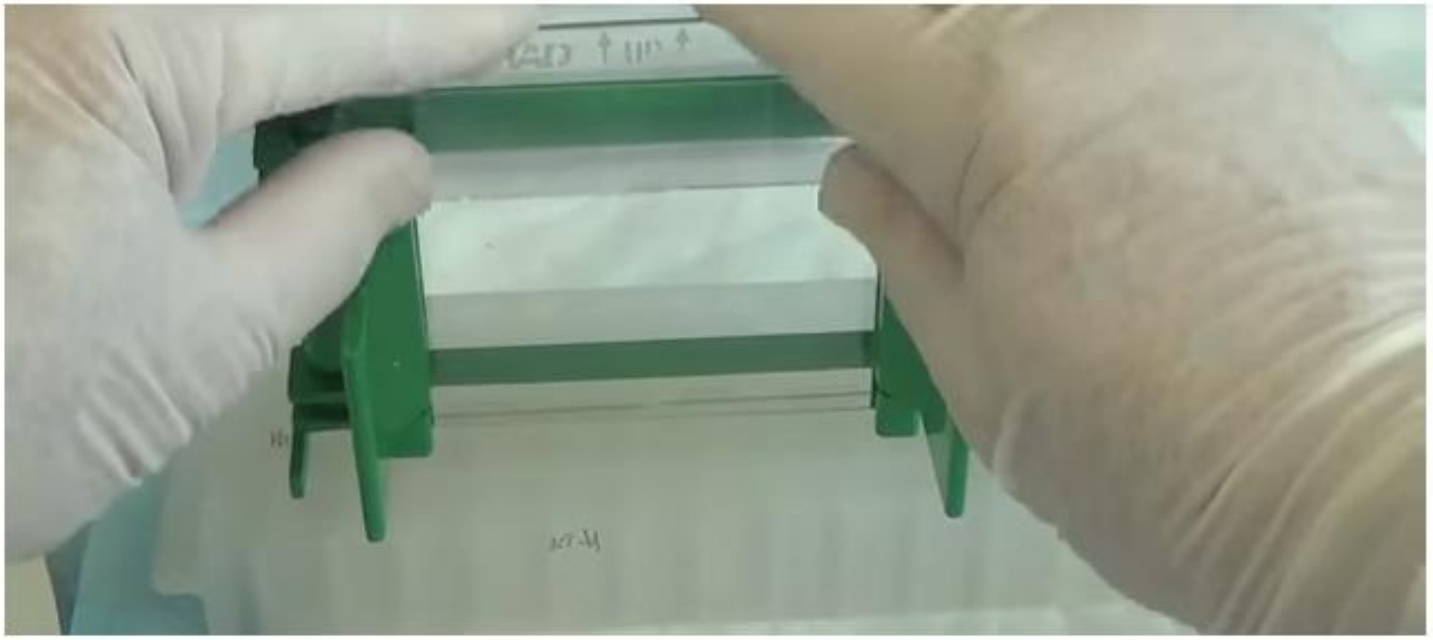

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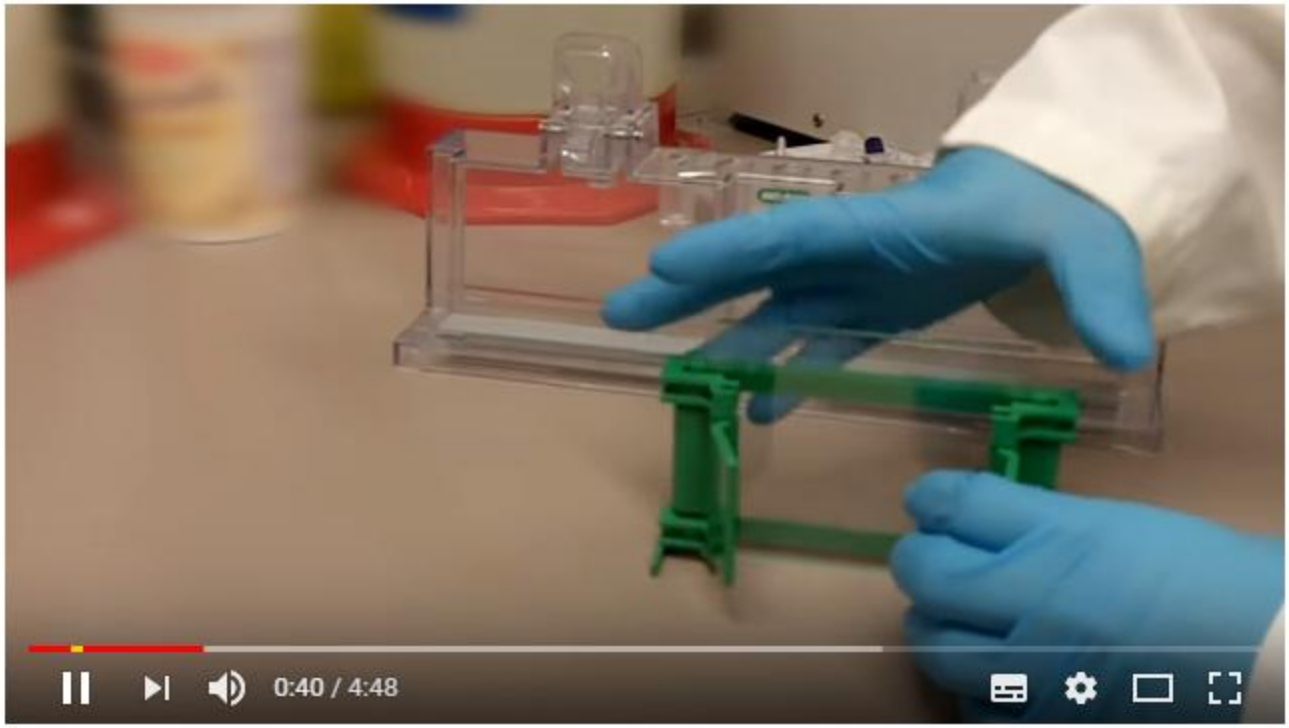
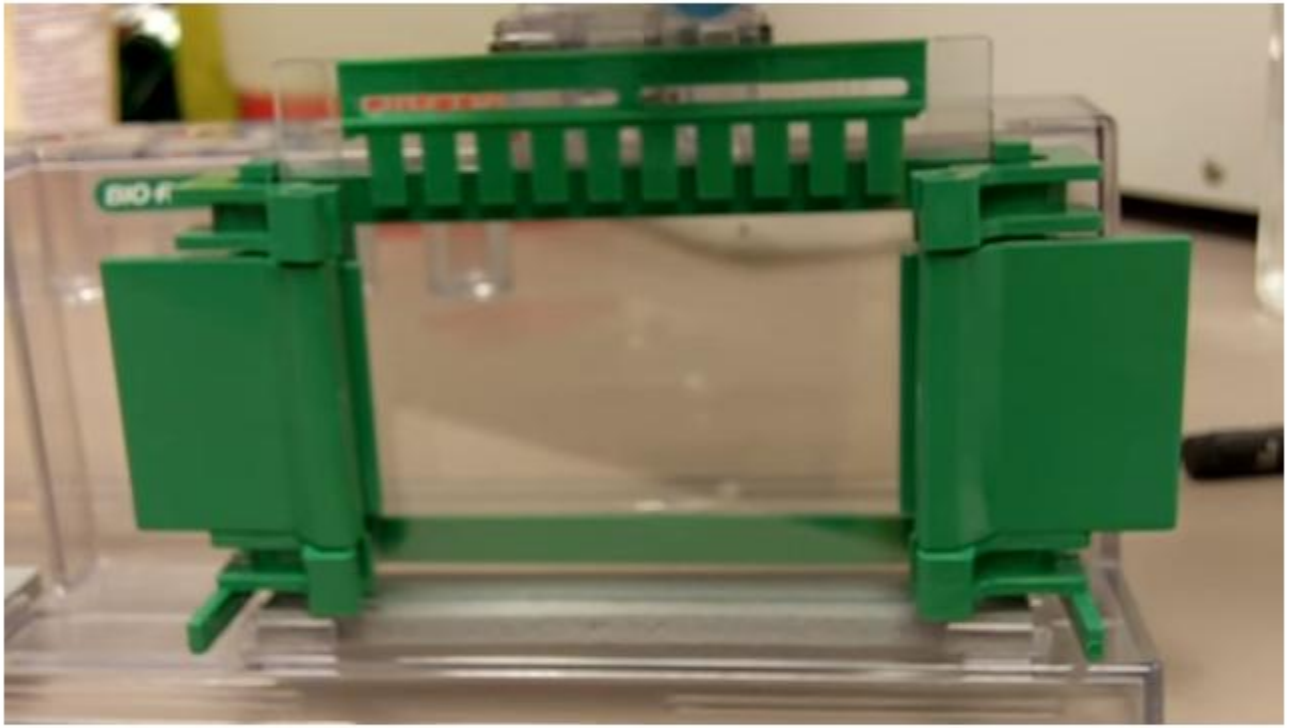


How to Make an SDS-PAGE gel



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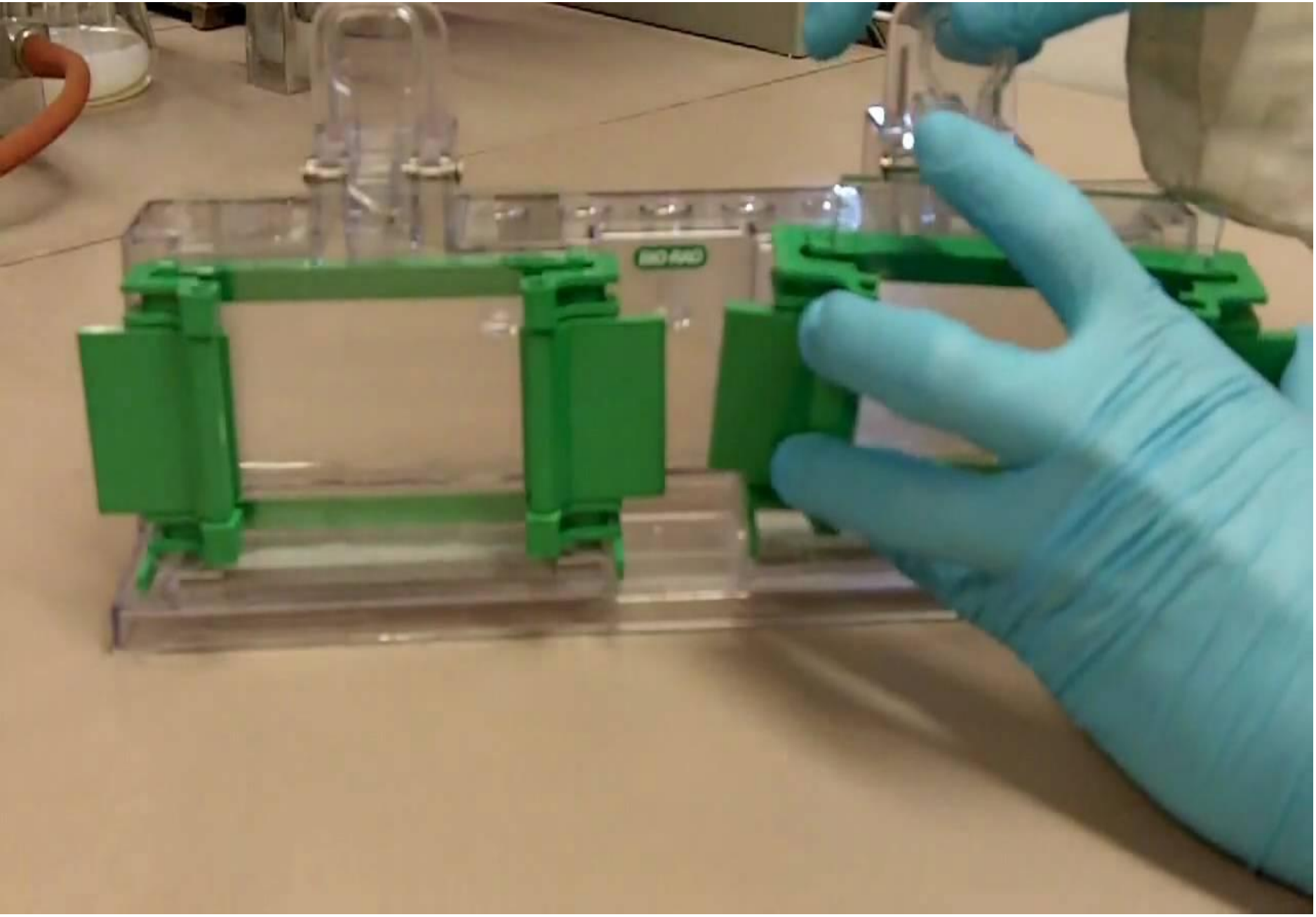
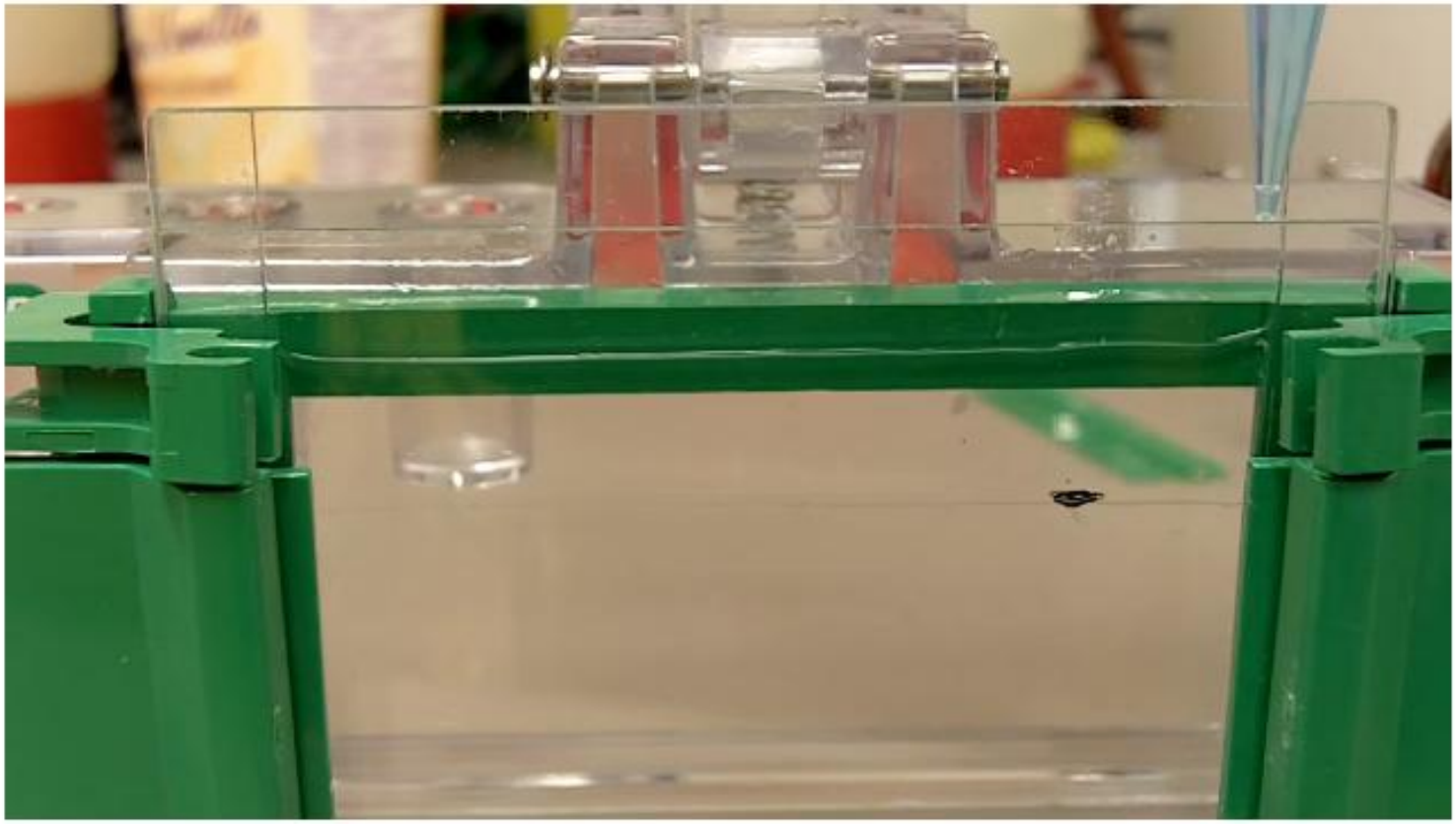


Resolving Gel:

- Acrylamide
- 1.5M Tris, pH 8.8
- SDS
- dH_2O
- Ammonium Persulphate
- TEMED — add this LAST!

*recipes may vary, but ingredients are the same


4:17 / 4:48
HD



How to Make an SDS-PAGE gel

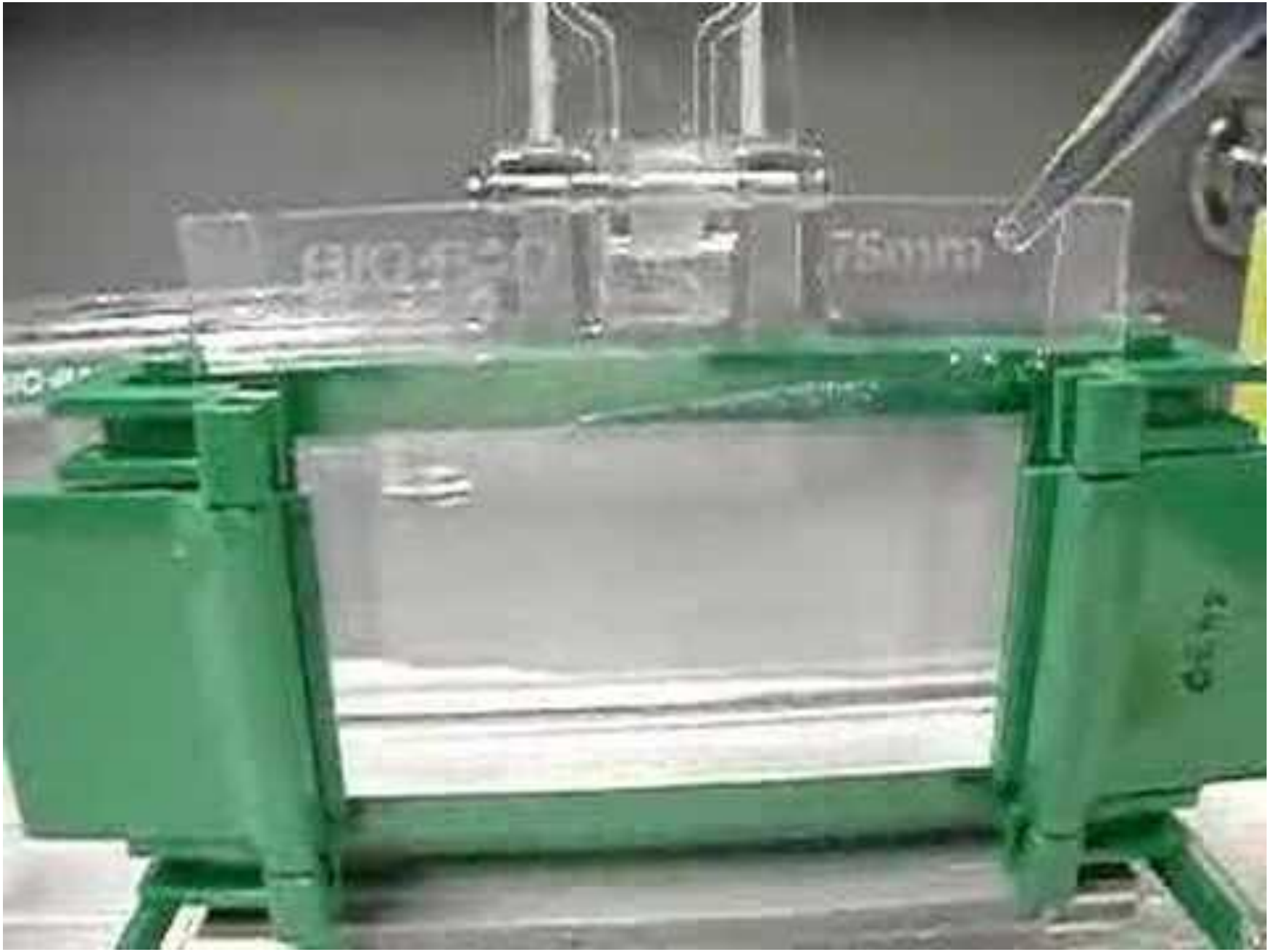
Stacking Gel:

- Acrylamide
- 0.5M Tris, pH 6.8
- SDS
- dH_2O
- Ammonium Persulphate
- TEMED

This is the difference:
Used 1.5M Tris, pH 8.8
for resolving gel

again, add this LAST!

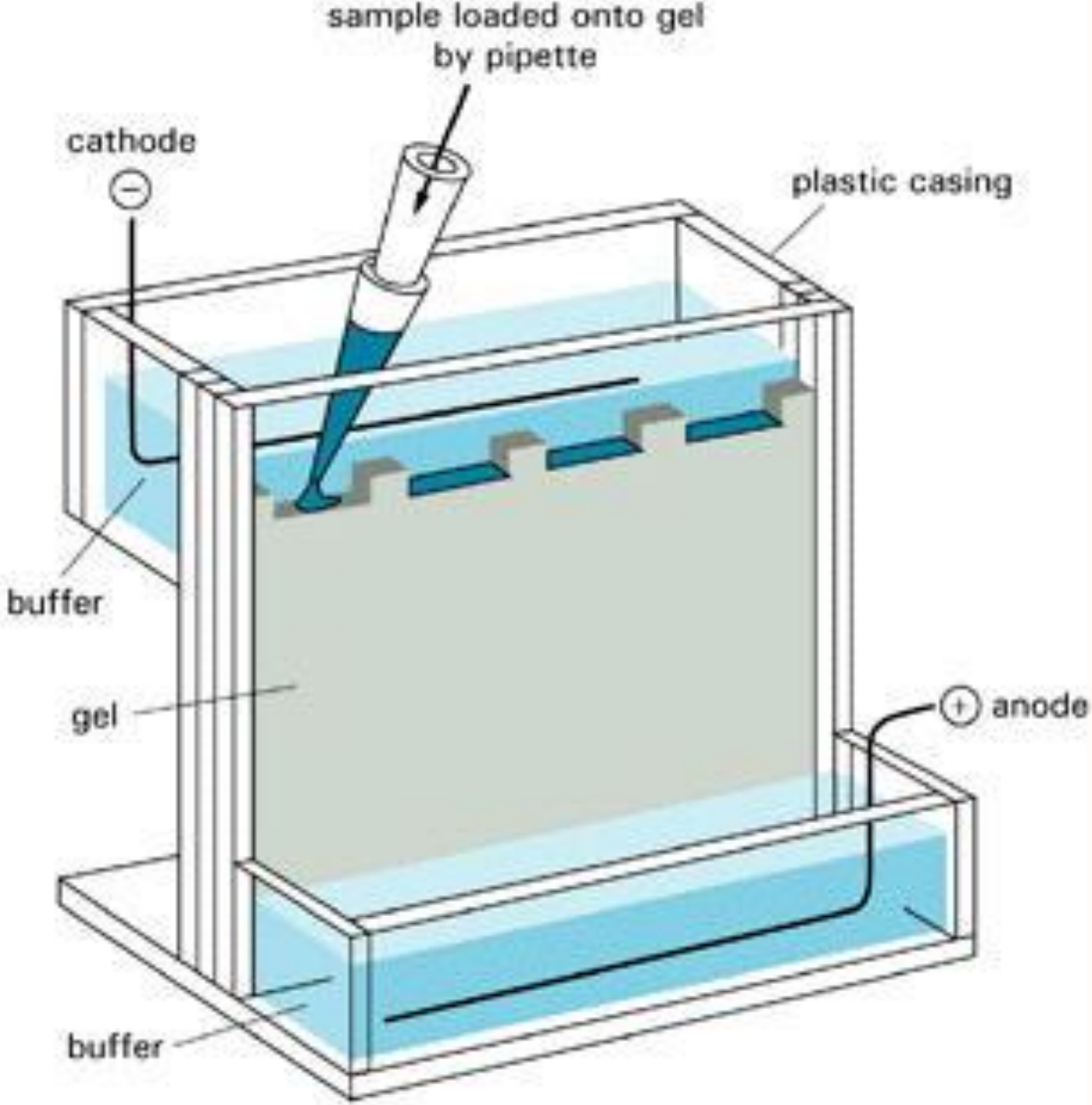
*recipes may vary, but ingredients are the same

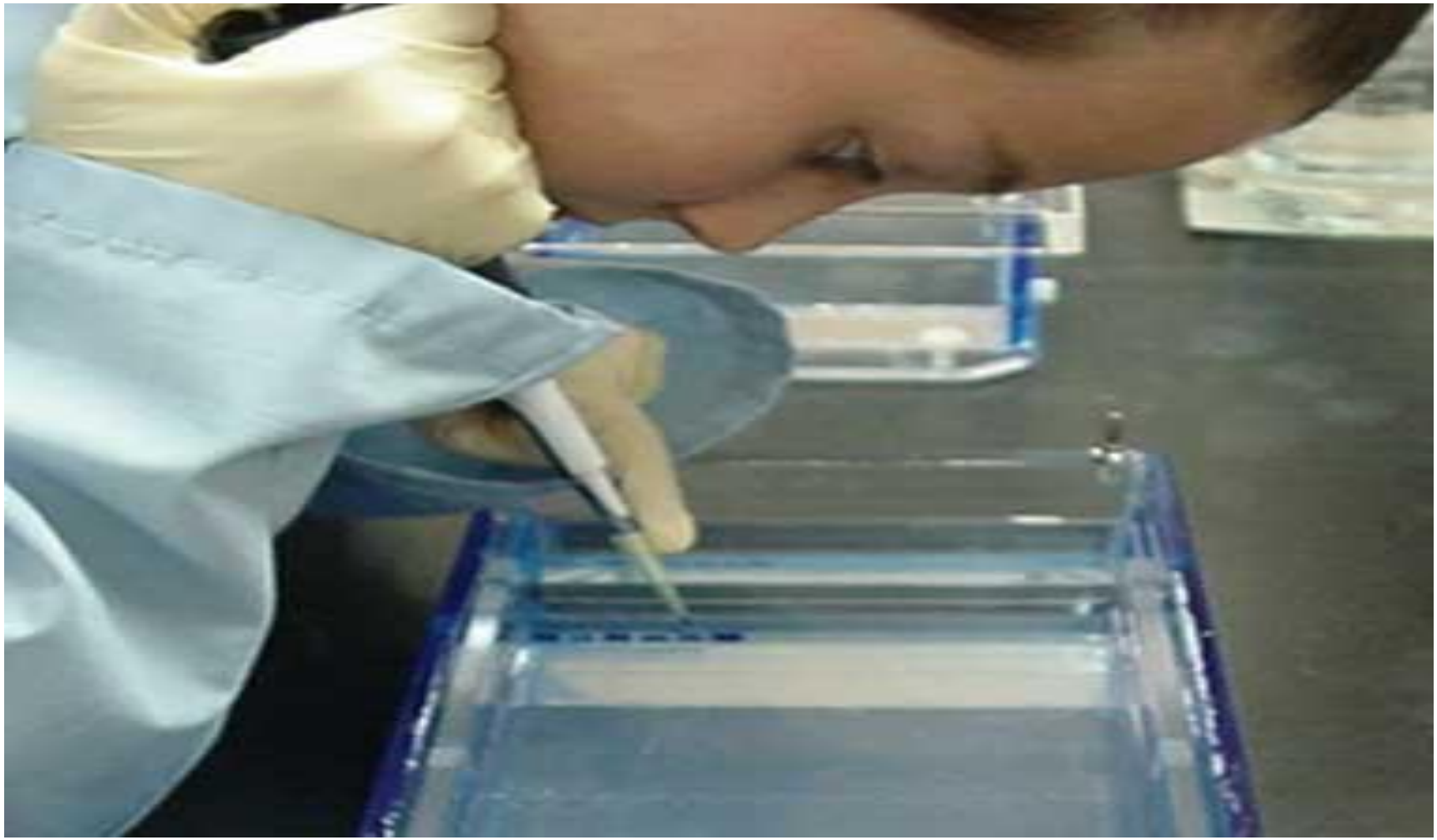

75mm

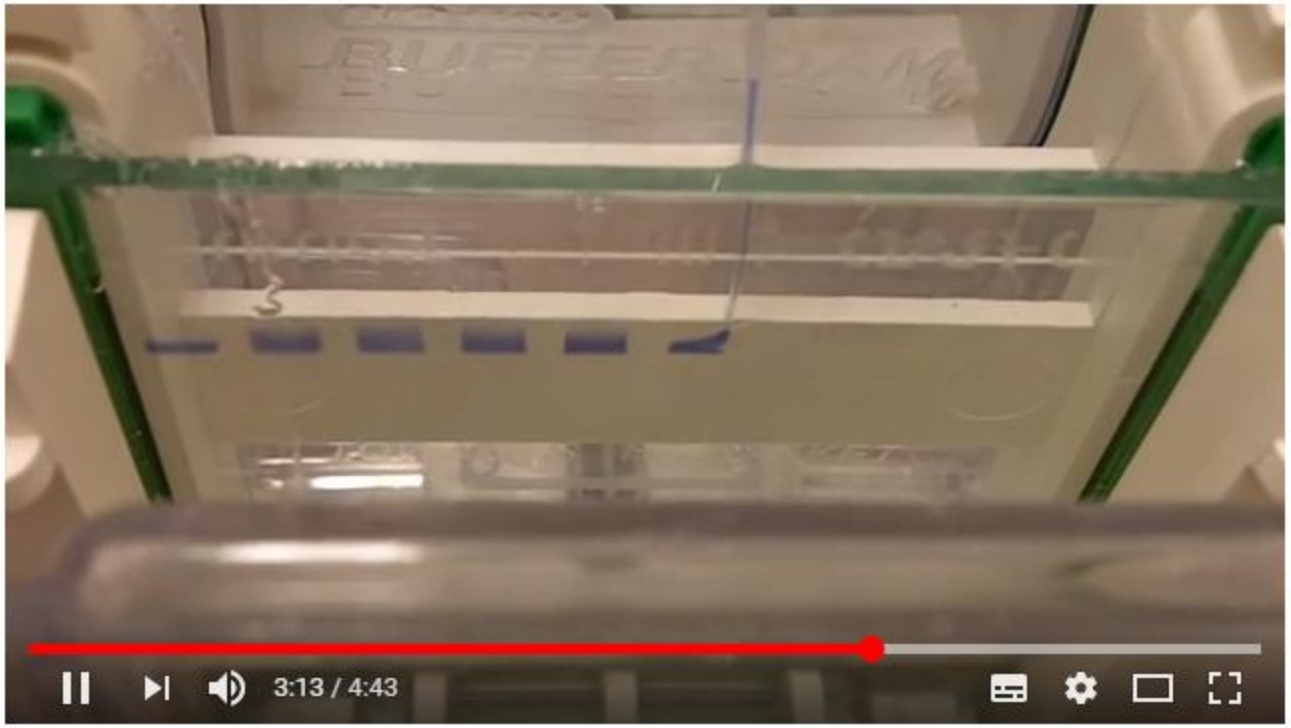
How to Make an SDS-PAGE gel

How to Run an SDS-PAGE gel

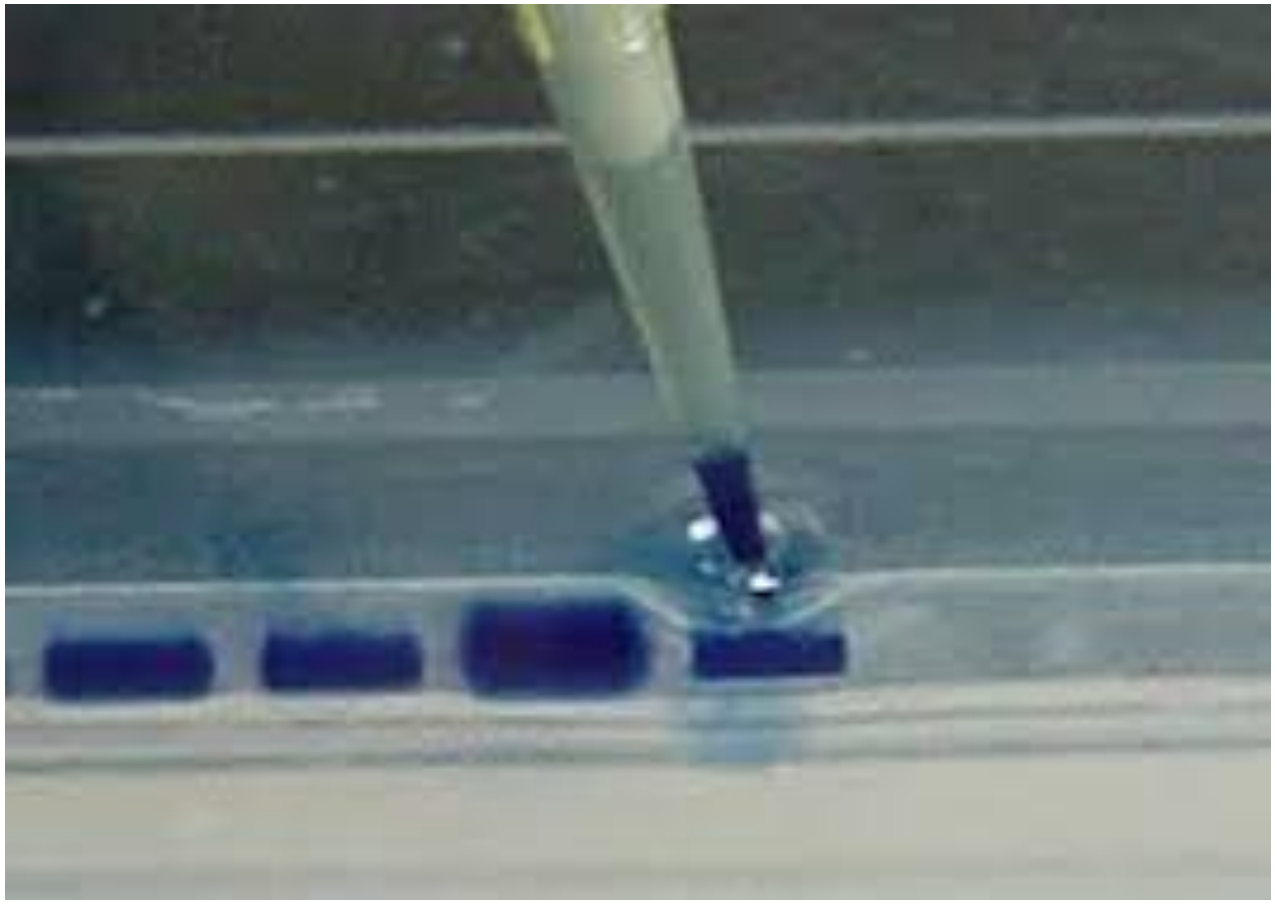
Blotting
1
2
3
4
5
6
7
8
9
10

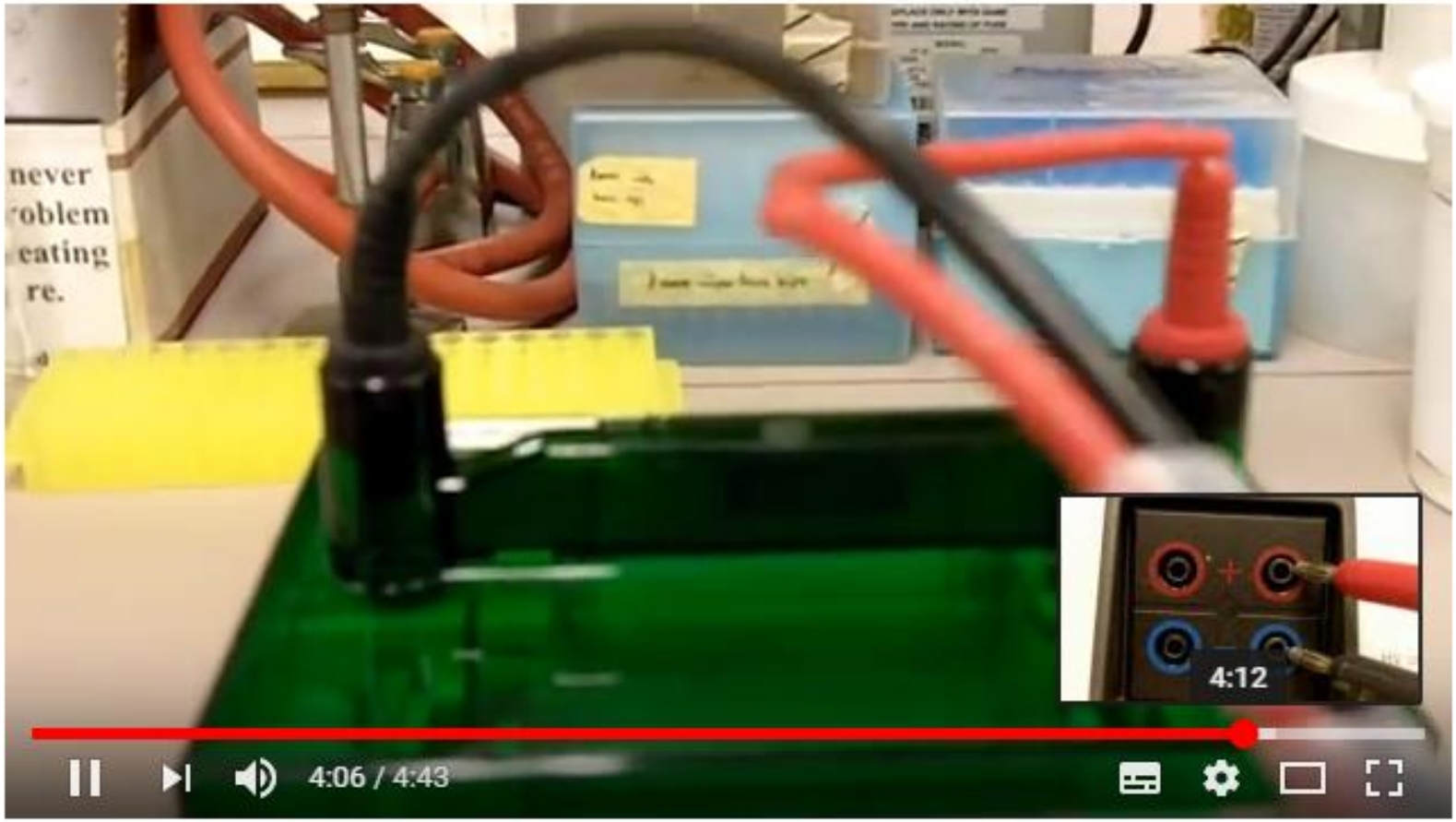

sample loaded onto gel
by pipette
cathode
plastic casing
buffer
gel
anode
buffer





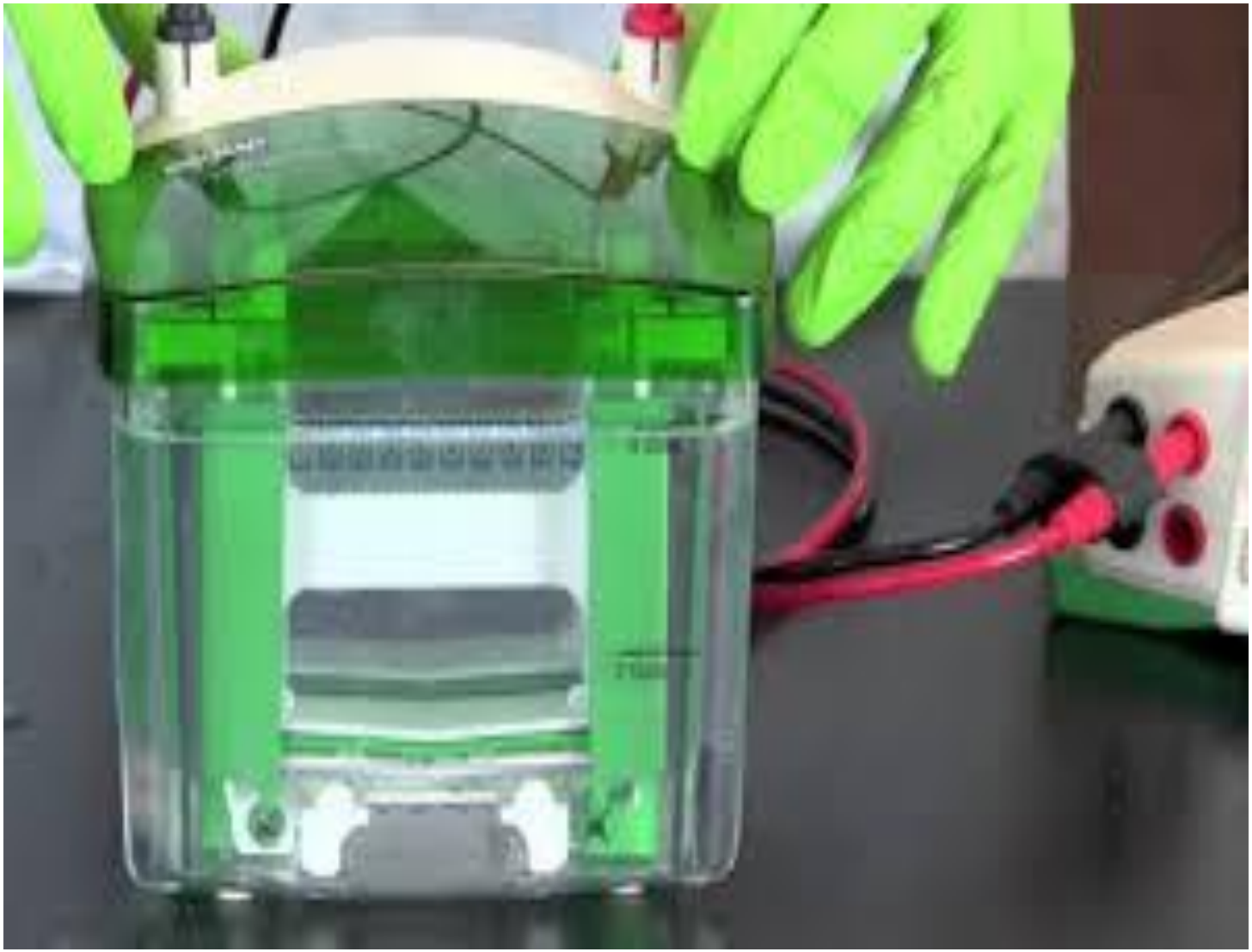
How to Run an SDS-PAGE gel

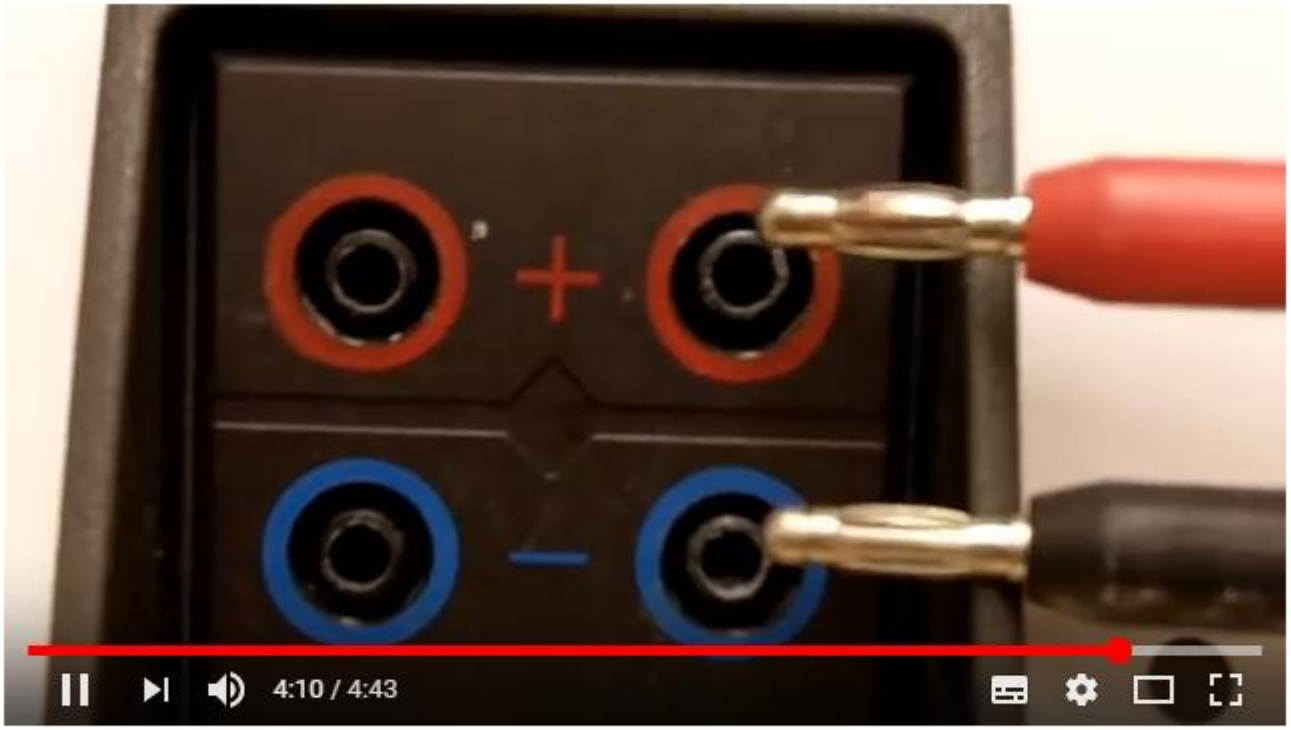




How to Run an SDS-PAGE gel

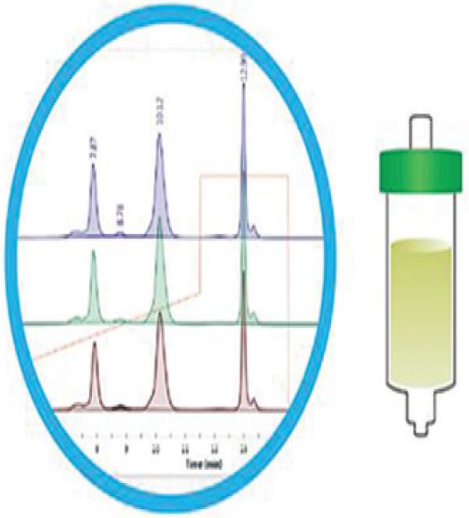

350
300
250
200
150
100
50
Kimberly-Clark
7. 16. 2004





How to Run an SDS-PAGE gel

Simplified Protein Purification



Chromatography

To separate complex biological mixtures within minutes

Fast Run Times

Gel Electrophoresis

Run gels in as little as 20 min

Available in handcast and precast gel formats

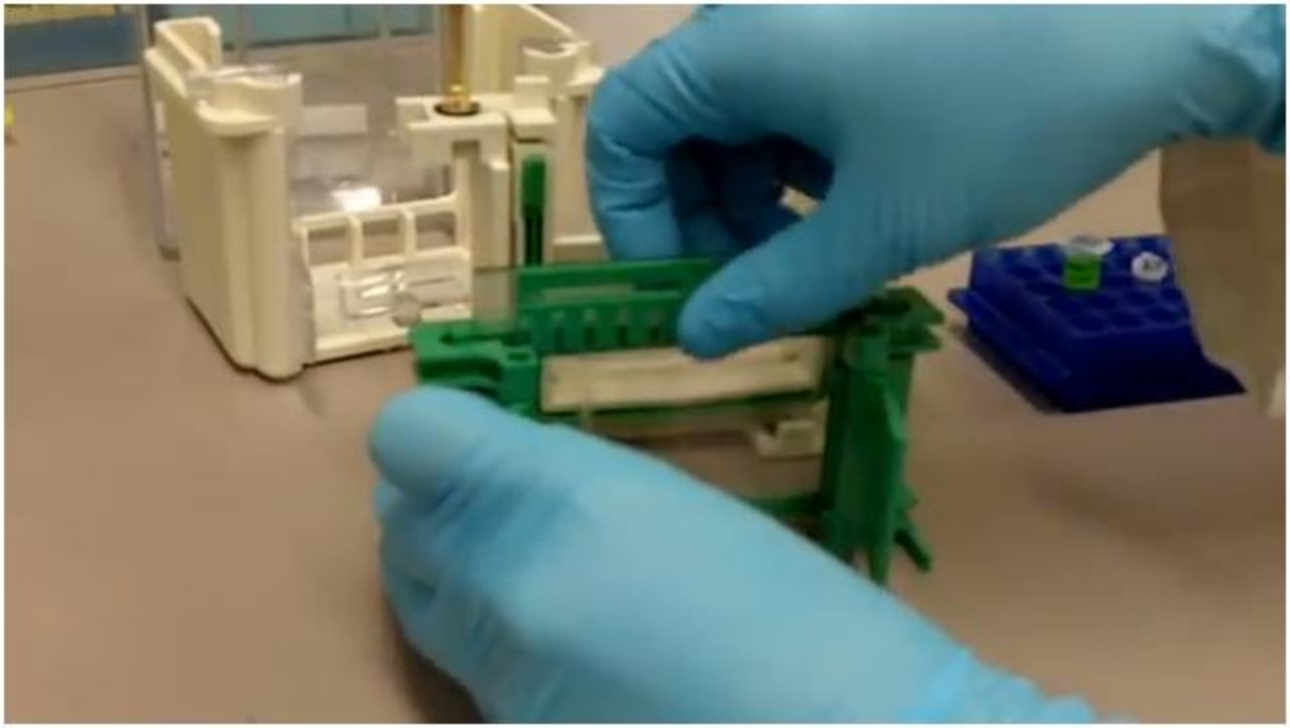
Quick Protein Visualization

Gel Imaging

Visualize proteins in 5 min with any stain-free–enabled imager

Eliminates all staining and

How to Run an SDS-PAGE gel



How to Run an SDS-PAGE gel

Prepare **your gel by inserting** it into the **electrophoresis apparatus** and filling with **running buffer** that is appropriate for your gel chemistry. Rinse the **wells of the gel** with running buffer and add buffer to the chambers.

Load your samples into the wells **and load a pre-stained molecular weight ladder into one well**. The ladder will allow you to **monitor protein separation during electrophoresis** and subsequently **verify protein weight** in your sample during **later analysis.**

Close the **electrophoresis unit and connect it to a power supply.** Most units typically run 45-60 minutes at **200 volts or** until the **loading buffer reaches the bottom** of the gel. During this time the **negatively** charged proteins in each sample will migrate toward the **positively charged** electrode making their way through the polyacrylamide gel matrix.

Transfer:

In this next step, we will **transfer separated proteins** out of the gel into a **solid membrane** or blot. This is based upon the same principal as the previous step in which an electric field is charged to move the **negative proteins towards a positive electrode**. Transfer can occur under **wet or semi-dry conditions**.

The steps of traditional wet transfer method are as follows:

Start by removing the **gel from its cassette cutting the top portion containing the wells**. Notch the **top left corner** to indicate **gel orientation**. Float **the gel in transfer buffer** while preparing the **transfer sandwich**. To make the transfer sandwich, a cassette,sponges, filter paper, the gel and PVDF or nitrocellulose membrane paper is needed. Notch the top left corner of blotting paper to indicate blot orientation and **incubate membranes in transfer buffer for 10**

Create a stack by placing the following components from the black negative cathode to red positive anode:**sponge, filter paper, gel, membrane, filter paper and sponge** (Be careful not to touch the **gel or membrane with your bare hands** and use clean tweezers or spatula instead. Touching the membrane during any phase can contaminate the blot and lead to **excessive background signal**).

Use a clean roller with each layer to gently roll out any bubbles that may be present since bubbles will inhibit efficient protein transfer. Lock the cassette and place it in the transfer apparatus containing cold transfer buffer ensuring that the cassette is **properly positioned from negative to positive**.

In order to prevent heat buildup, it is beneficial to transfer with a cold pack in the apparatus or in a cold room with the spinner bar placed at the bottom of the chamber.

Close the chamber and **connect to a power supply.**

Perform the transfer according to the manufacturer's instructions which is **normally a 100 volts for third to 120 minutes.**

Western Blot - Theory and method

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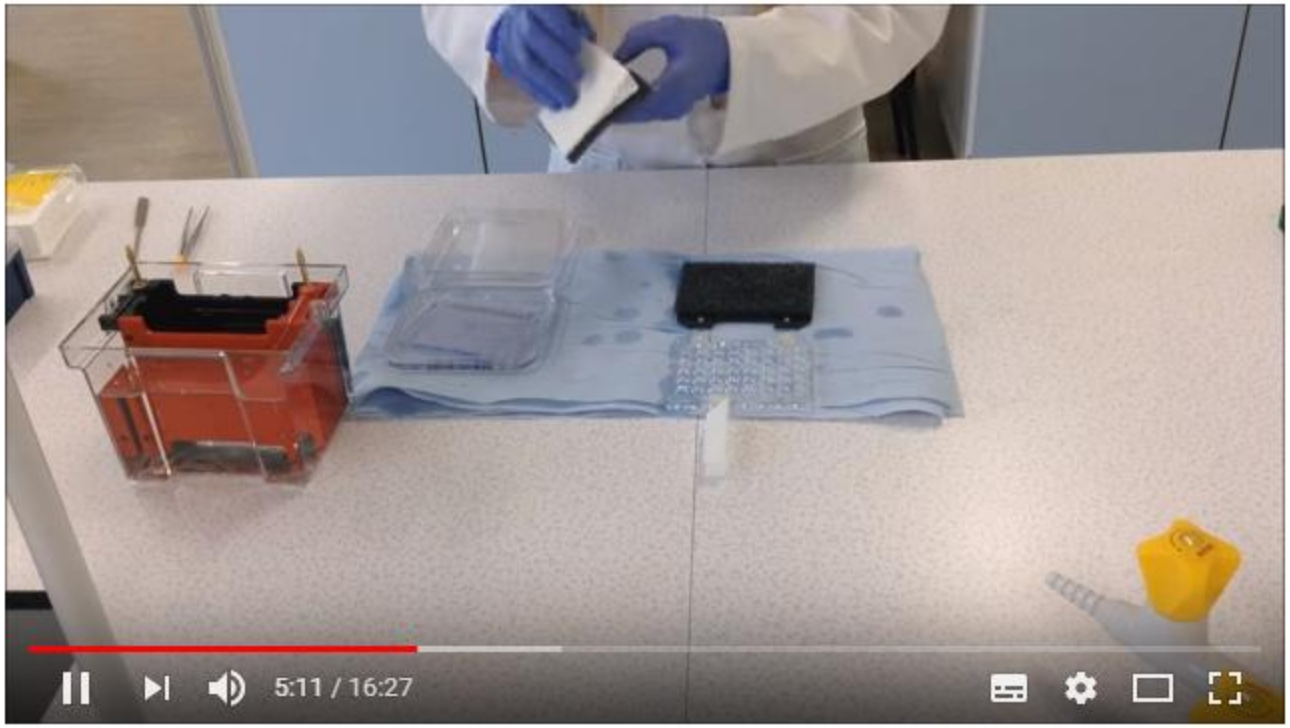

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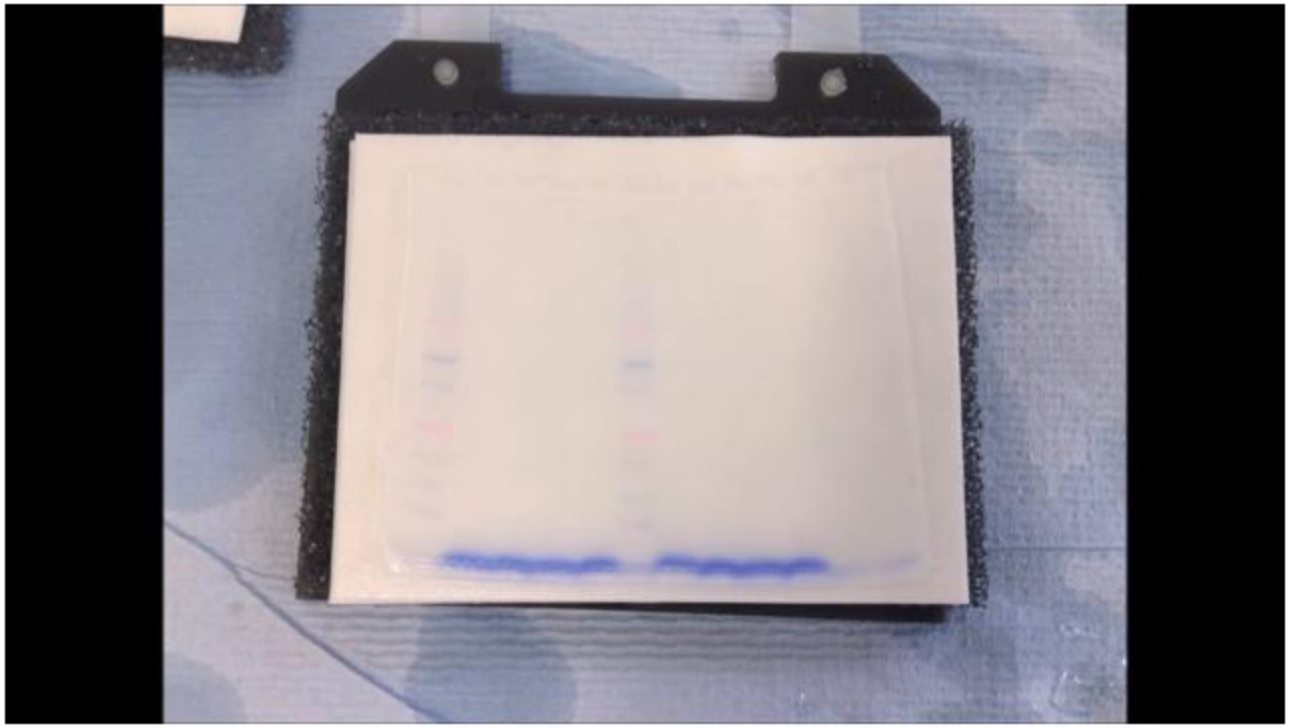

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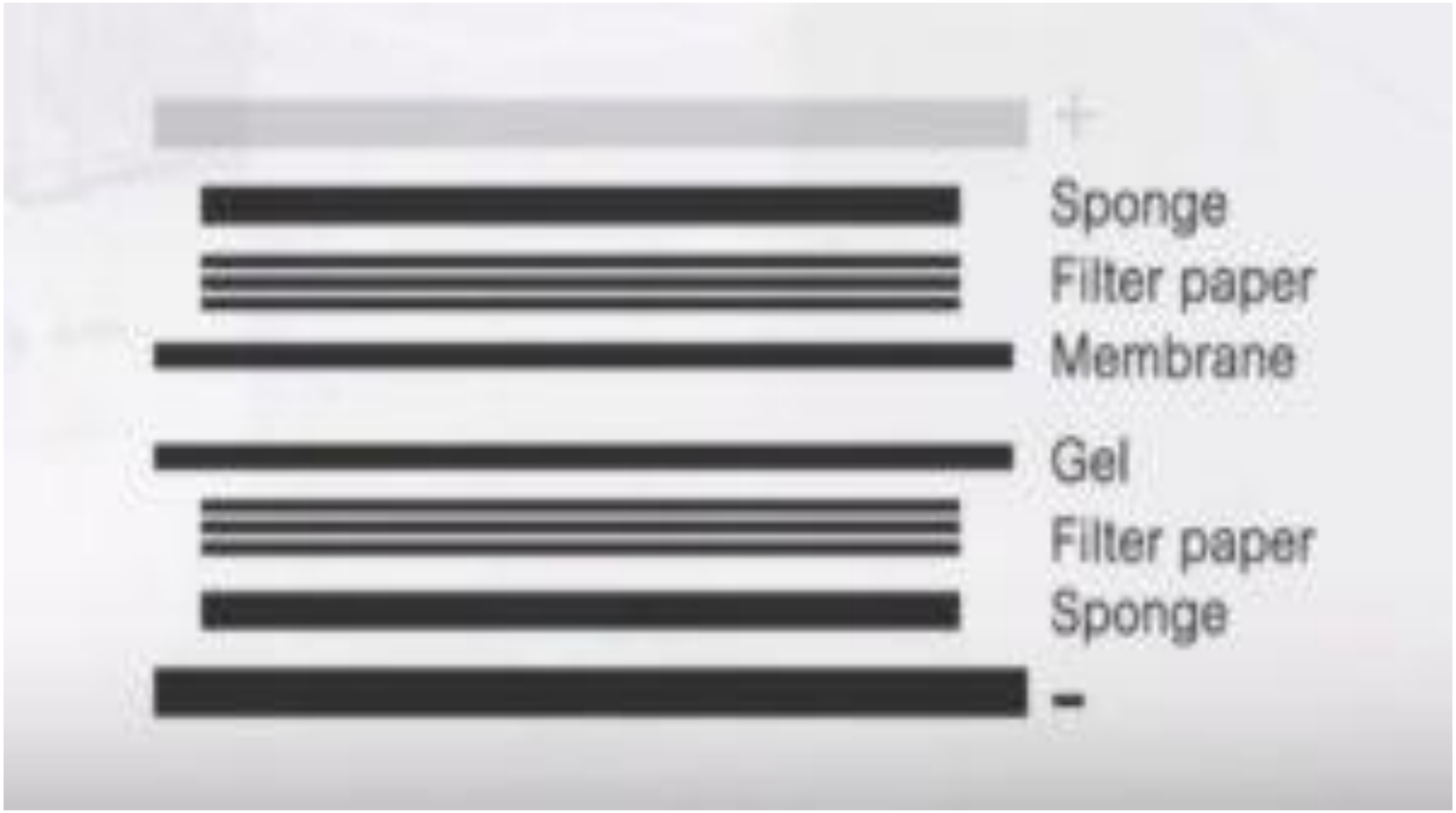
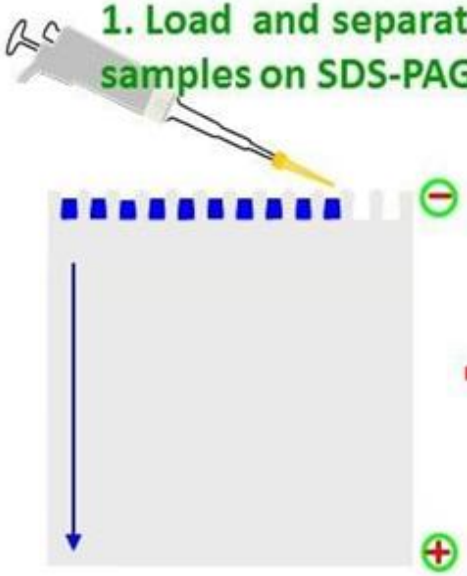
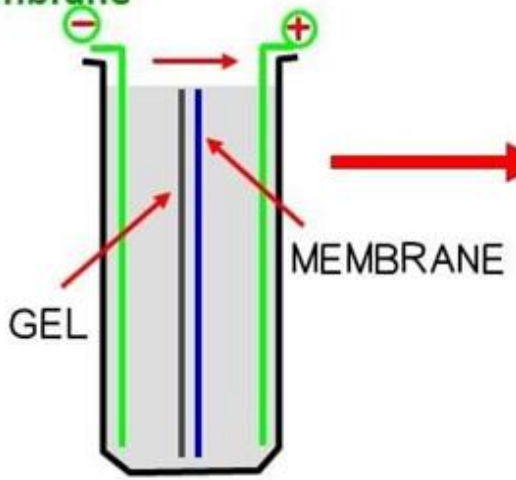

+
Sponge
Filter paper
Membrane
Gel
Filter paper
Sponge
-

Western Blotting Procedure

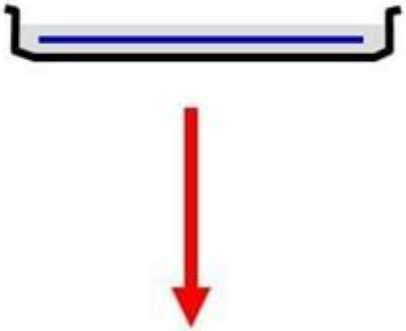
1. Load and separate protein samples on SDS-PAGE



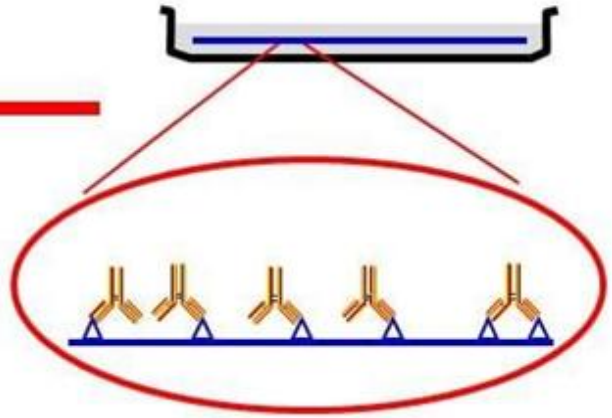
2. Electrophoretically transfer fractionated proteins onto PVDF membrane



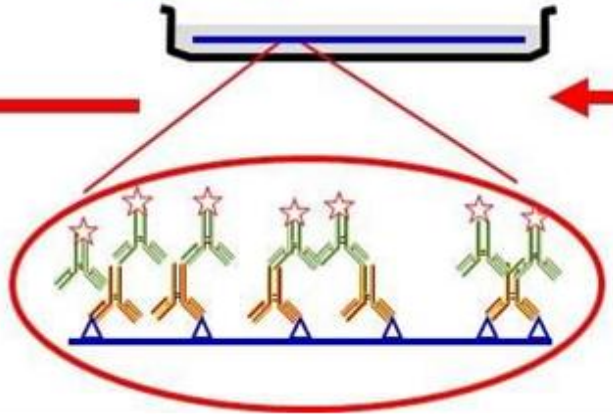
3. Block the membrane with neutral protein (BSA or milk casein)



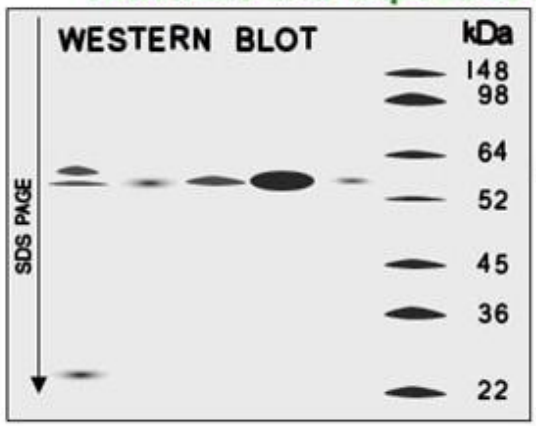
4. Incubate the membrane with primary antibody specific to target protein



5. Incubate the membrane with HRP-labeled secondary antibody specific to primary antibody



6. Incubate the blot with chemiluminescent HRP substrate and expose to film



•**Immunobloting:**

After electrotransfer of protein to a membrane, Start by **removing the membrane from the cassette** and rinsing three times in water.

we will **now block the blot,** applying a primary antibody specific for our protein of interest and then a secondary antibody which will recognize the primary antibody.

Steps:

•**As an optional step**,: we can verify the proteins were **transferred successfully by staining the membrane** with ponceau red. Incubate the membrane **for five minutes and wash with water until the bands are clear**.

After verification the blot can then **be de-stained by continuing to wash with water or TBS tween** until the dye is completely removed.

We need **to block all areas of the blot which do not already contain protein.** This will prevent **non-specific binding of the antibody** and **reduce overall background** signal. Common blocking buffers include **5% non-fat dry milk or BSA in a TBS-Tween** solution. However, do not use a milk solution when probing with **phosphor-specific antibodies** as it can cause **high background from its endogenous phosophoprotein,** casein.

Incubate the membrane with **blocking solution for one hour at room temperature** under **slight agitation.**

Decant the blocking solution and **wash with TBS tween for five minutes.**

We are not ready to add our primary antibody.

Dilute the primary antibody in a blocking buffer at the **concentration recommended on the datasheet** and incubate **overnight at 4 degrees Celsius with gentle shaking.**

A recommended optional step is to also use **a positive loading control antibody** which allows the user to verify equal amounts of total protein were loaded into each well and aides in troubleshooting by removing any uncertainties with the Western Blot procedure.

Next day: decant **off the primary antibody** and **wash the membrane with large volumes or TBS tween** and **vigorous agitation five times for five minutes each**.These stringent washes are extremely important for **removing non-specific background signals.**

After washing, **dilute the secondary antibody in blocking solution** and **incubate the membrane for one hour** at room temperature at the **concentration recommended on** the datasheet.In our example, the secondary antibody is also conjugated to HRP for later detection.

Decant membrane and **wash the membrane with large volumes of TBS tween with vigorous agitation five times** for **five minutes each**.

You are **now ready for the detection phase.**

Detection:

In this final phase, we will demonstrate **signal development** using:

the most common, **most sensitive** and most inexpensive detection method the **electrochemiluminescence** or ECL reaction.

This method utilizes the **HRP enzyme** which was conjugated to the secondary to catalyze the ECL reaction and produce light. **A light is then gathered onto x-ray film** and developed or digitized with the aid of a **specialized camera sensitive** enough for this application.

Detection can be done by other methods such as:

Colorimetric detection:

It depends on **incubation of the western blot with a substrate** that reacts with **the reporter enzyme** (such **as peroxidase**) that is bound to the **secondary antibody.**

This converts the **soluble dye into an insoluble form** of a **different color** that precipitates next to the enzyme and thereby **stains the membrane.**

Development of the blot is **then stopped by washing away the soluble dye**. Protein levels are evaluated **through spectrophotometry.**

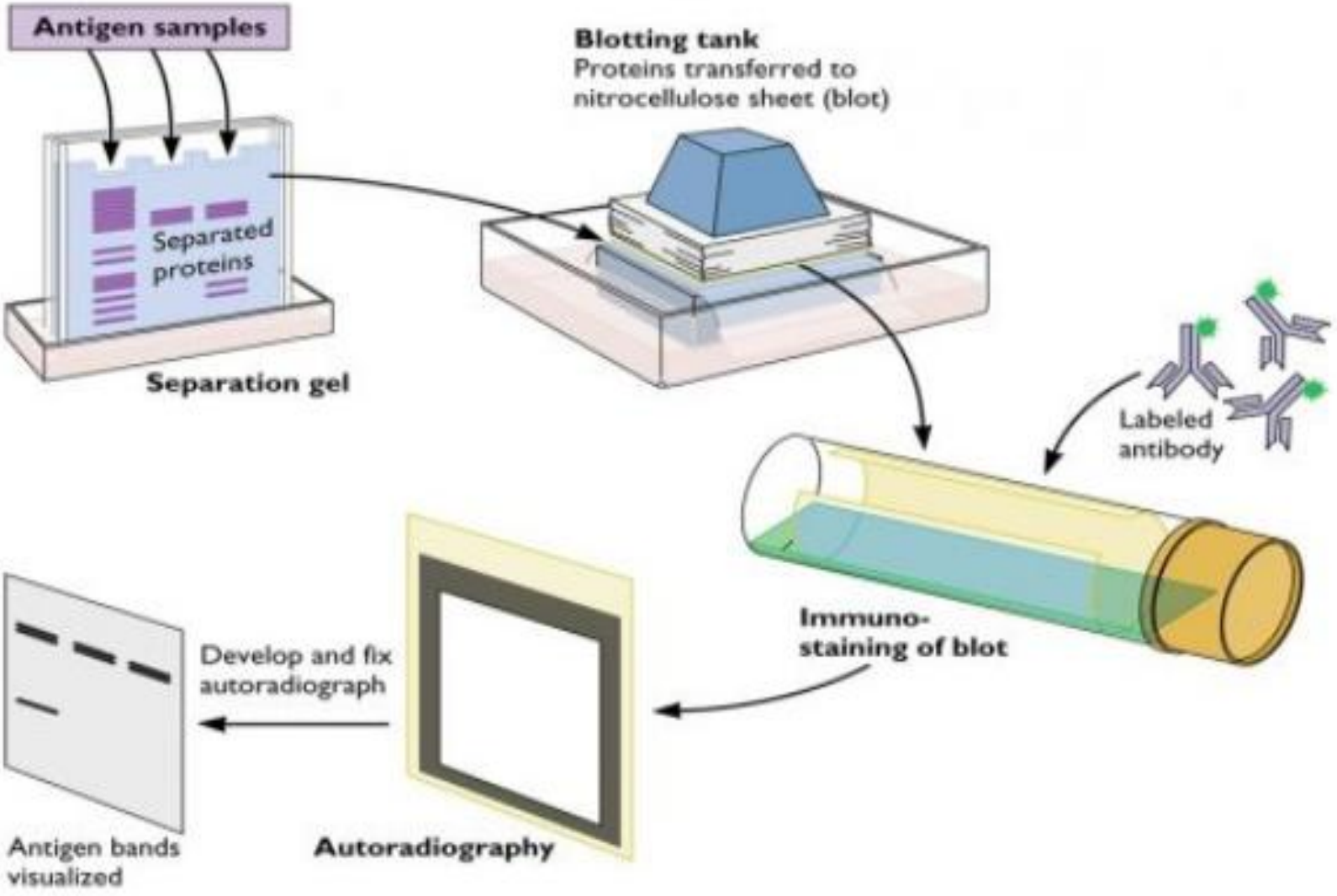
Uses

It is most sensitive and specific test for determining size and amount of protein present in any material.

The **confirmatory HIV test** employs a western blot to detect **anti-HIV antibody** in a human serum sample.

A western blot is also used as the definitive test for Creutzfeldt-Jakob Disease, Lyme disease, **Hepatitis B infection and HSV-2 (Herpes Type 2) infection.**

Western Blotting Technique



Western Blotting Procedure

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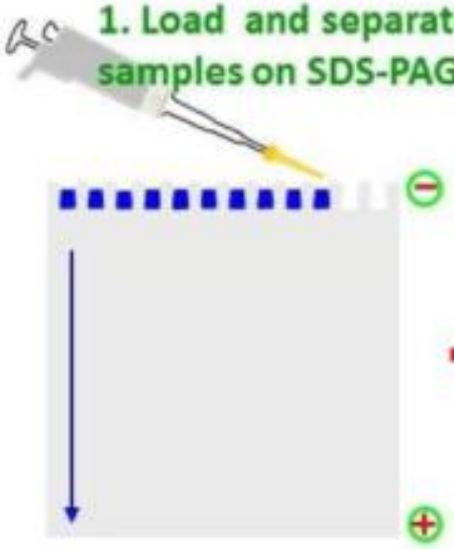

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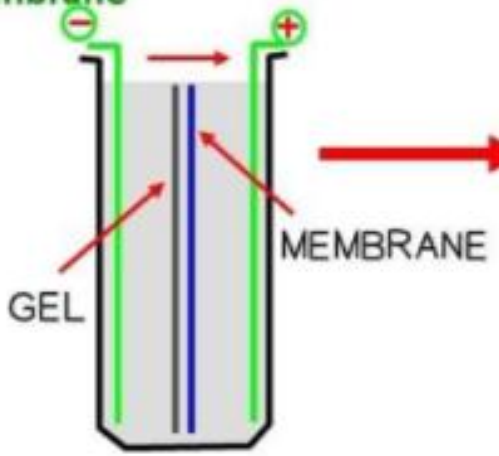

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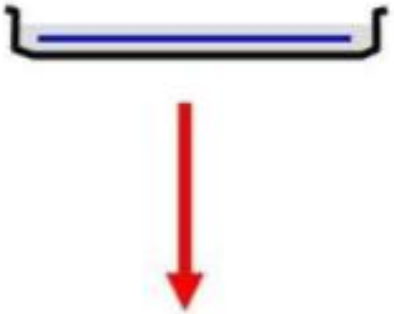

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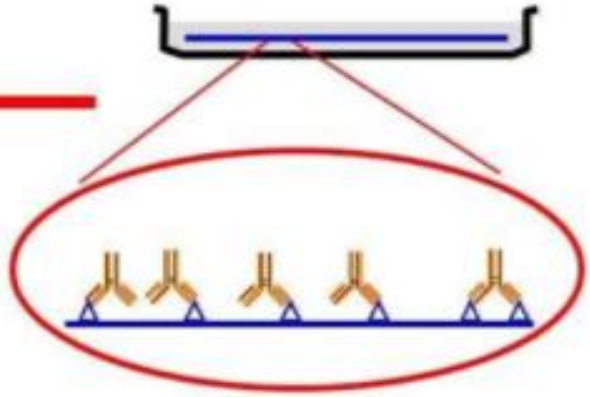

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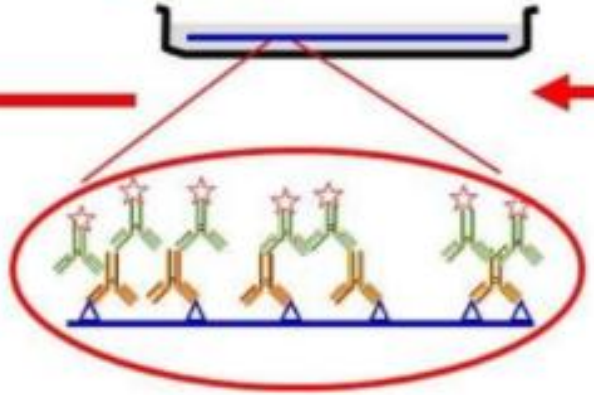

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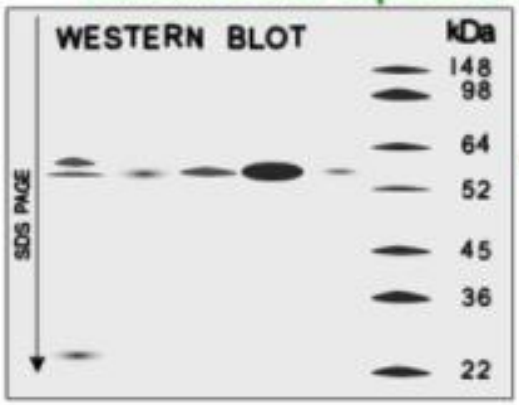


Step I: Extraction of Protein

Cell lysate is most common sample for western blotting.

Protein is extracted from cell by m**echanical or chemical lysis of cell.** This step is also known as tissue preparation.

To prevent **denaturing of protein protease inhibitor** is used.

The concentration of protein is **determined by spectroscopy**.

When sufficient amount of protein sample is obtained, it is diluted in **loading buffer containing glycerol** which helps **to sink the sample in well.**

Tracking dye **(bromothymol blue) is also added in sample** to **monitor the movement of proteins**.

Step II: Gel electrophoresis

The sample is loaded in well of SDS-PAGE Sodium dodecyl sulfate- **poly-acrylamide gel electrophoresis.**

The proteins are separated on the basis of **electric charge, isoelectric point, molecular weight, or combination of these all.**

The **small size** protein moves **faster than large size protein.**

Protein are negatively charged, so they move toward **positive (anode) pole** as electric current is applied

Step III: Blotting

The nitrocellulose membrane is placed on the gel. The separated protein from gel get transferred to nitrocellulose paper by capillary action. This type of blotting is time consuming and may **take 1-2 days**

For fast and more efficient transfer of desired protein from the gel to nitrocellulose paper electro-blotting can be used.

In electro-blotting nitrocellulose membrane is sandwich between gel and cassette of filter paper and then electric current is passed through the gel causing transfer of protein to the membrane.

Step IV: Blocking

Blocking is very important step in western blotting.

Antibodies are also protein so they are likely to bind the nitrocellulose paper. So before adding the primary antibody the membrane is non-specifically saturated or masked by using casein or Bovine serum albumin (BSA).

Step V: Treatment with Primary Antibody

The primary antibody (1° Ab) is specific to desired protein so it form Ag-Ab complex

Step VI: Treatment with secondary antibody

The secondary antibody is enzyme labelled. For eg. **alkaline phosphatase or Horseradish peroxidase (HRP) is labelled with secondary antibody**.

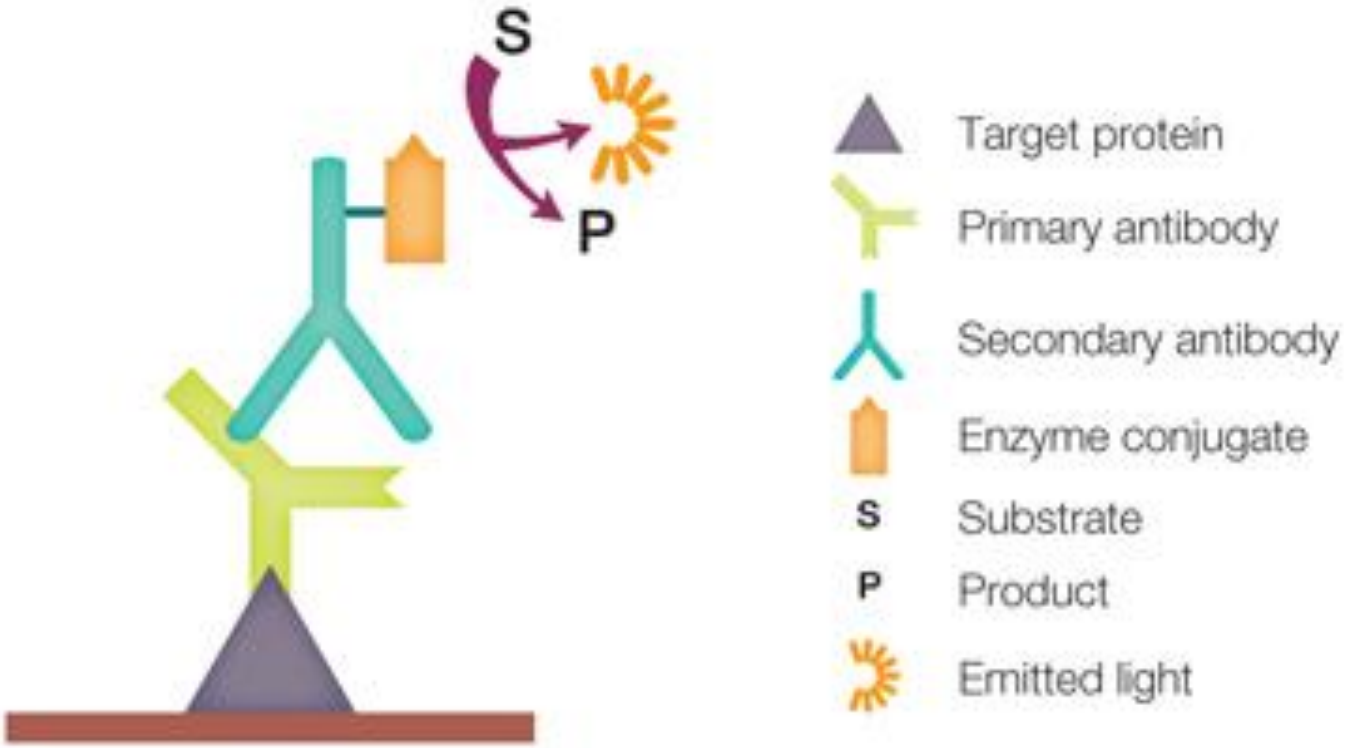
Secondary antibody (2° Ab) is antibody against primary antibody (anti-antibody) so it can bind with Ag-Ab complex.

Step VII: Treatment with suitable substrate

To visualize the **enzyme action, the** reaction mixture is **incubated with specific substrate.**

The enzyme convert the substrate to give **visible colored product, so band of color can be visualized in the membrane.**

Western blotting is also a **quantitative test to determine the amount of protein in sample.**


S
P
Target protein
Primary antibody
Secondary antibody
Enzyme conjugate
S
Substrate
P
Product
Emitted light

Application:

To determine the size and amount of protein in given sample.

Disease diagnosis: detects **antibody against virus or bacteria in serum.**

Western blotting technique is **the confirmatory test for HIV. It detects anti HIV antibody in patient's serum.**

Useful to detect defective proteins. For eg Prions disease.

Definitive test for Creutzfeldt-Jacob disease, Lyme disease, **Hepatitis B and Herpes.**