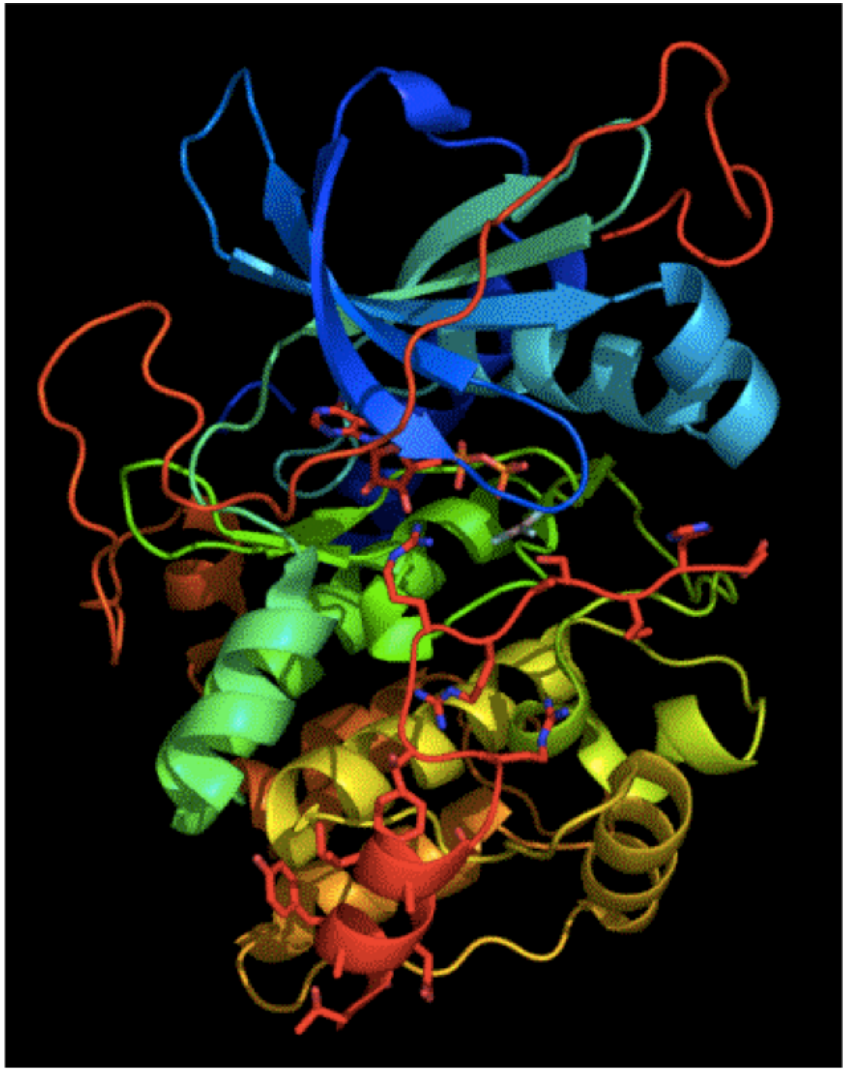
Module 2: Western Blot

BMES Cell Team

Fall 2020

Outline

- Protein Basics
- What is a Western Blot?
- Western Blot Protocol
    - BCA Assay
    - SDS Gel Electrophoresis
    - Immunoblotting
- Western Blot Video
- Pipetting basics



http://young.biochem.med.umich.edu/Courses/biochem516/Pymol/index.htm

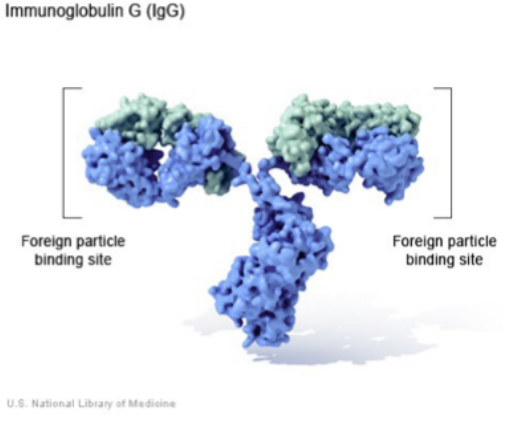
Proteins

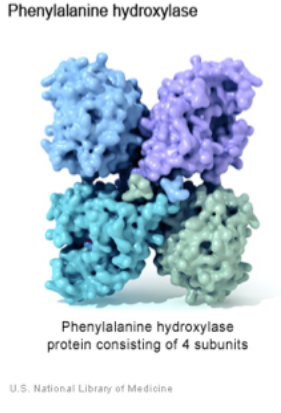
- **Definition:** Proteins are macromolecules made of amino acids.

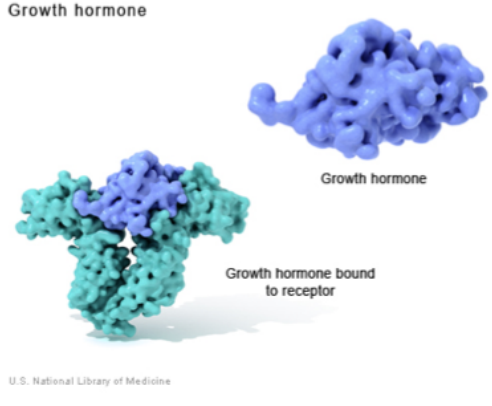
→ Chains of amino acids make up proteins

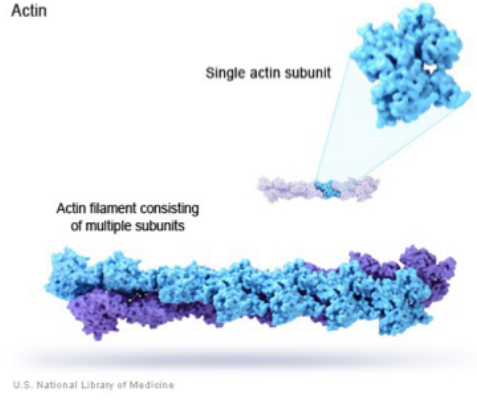
→ Proteins have a wide structural range → large functional range

→ Key players in organism's metabolic & regulatory activity









https://ghr.nlm.nih.gov/primer/howgeneswork/protein

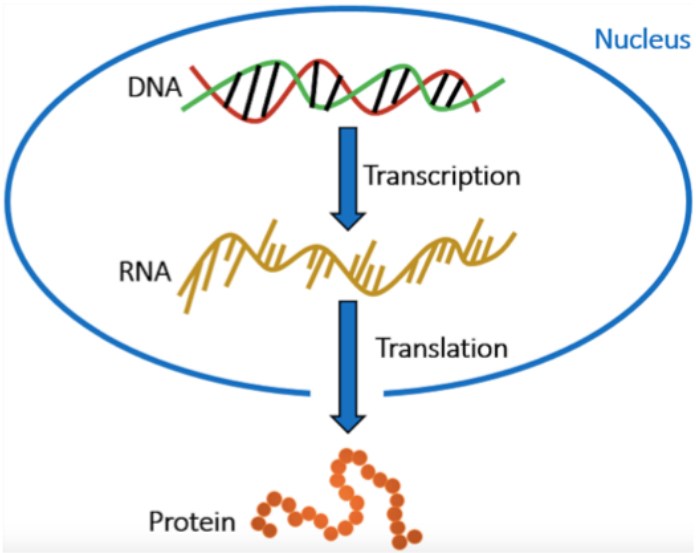
What is Western Blotting?

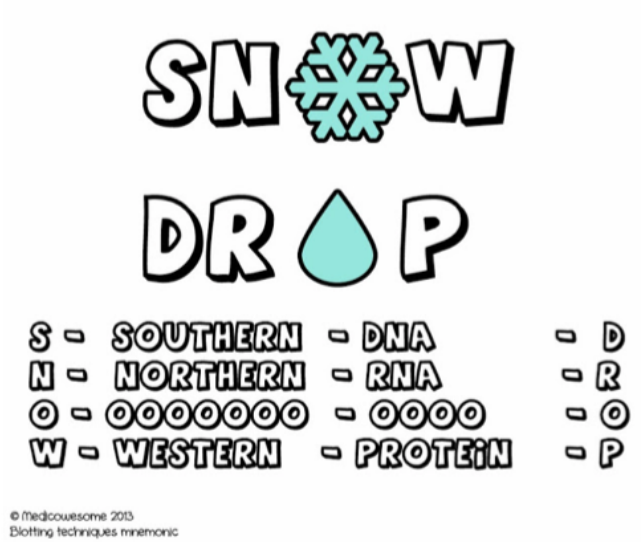
- **Definition:** A Western Blot separates and identifies target proteins.

→ Two stage procedure confirms protein presence and quantifies target

→ Proteins separated by size through gel electrophoresis

→ Target proteins visualized through immunostaining



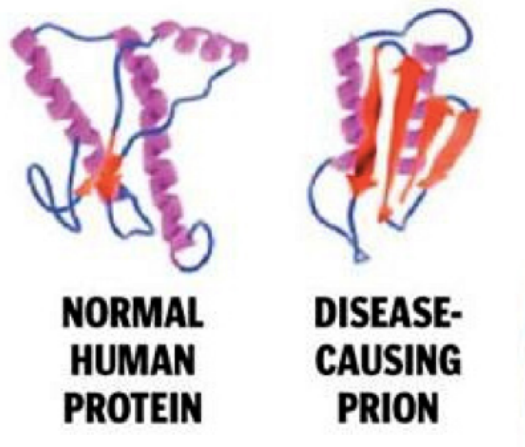


https://www.researchgate.net/profile/Radin_Tahvildari/publication/313798200/figure/fig1/AS:462661172371457@1487318427401/1-Overview-of-the-central-dogma-shows-the-flow-of-genetic-information-inside-a.png

Western Blot Utility

- Understand mechanisms of cell behavior
    - Protein's primary function in organism

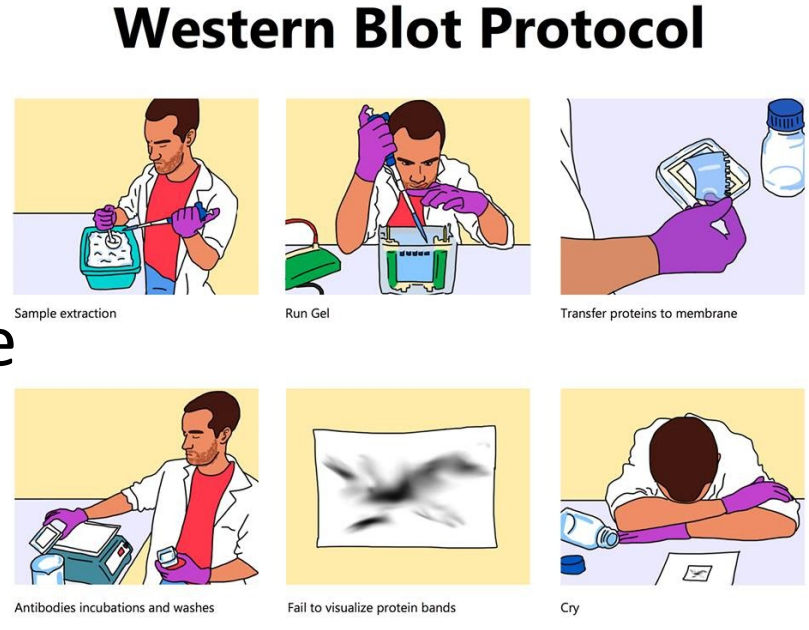
- Probe for a specific disease
    - **Viral:**
        - HIV
    - **Bacterial:**
        - Meliodosis
    - **Prion:**
        - Creutzfelt-Jakob disease



https://www.labroots.com/trending/microbiology/2234/melioidosis-a-global-health-threat http://microbe-canvas.com/uploads/image/diseases/creutzfeldt-jakob-disease/01_cid_niet-ry.jpg

Basic Protocol for a Western Blot

1. Lyse cells and collect proteins
2. BCA Assay
    - Calculate sample protein concentration
3. SDS Gel Electrophoresis
    - Separate proteins by size
4. Transfer proteins to membrane
5. Immunoblot target proteins
    - Antibody Binding
6. Image membrane



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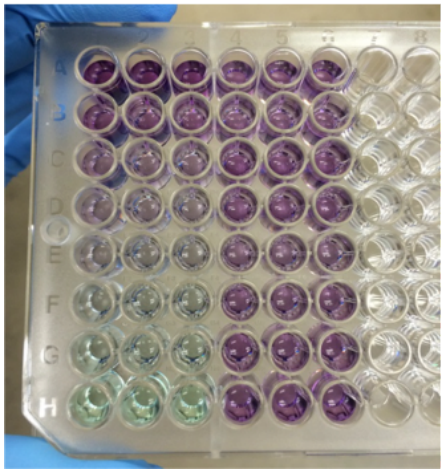
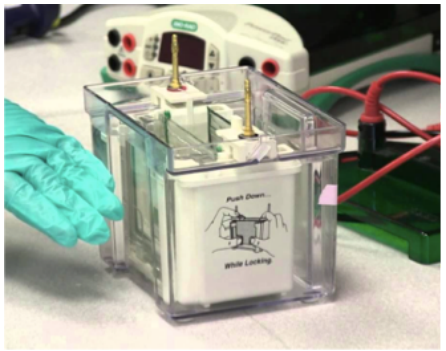
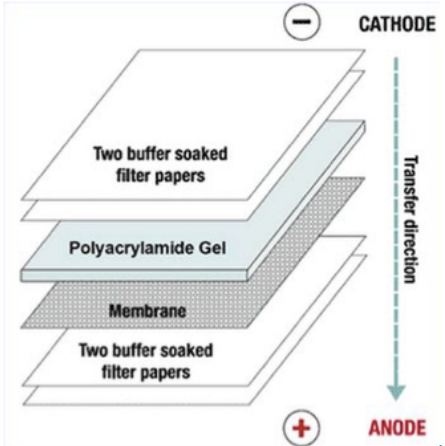






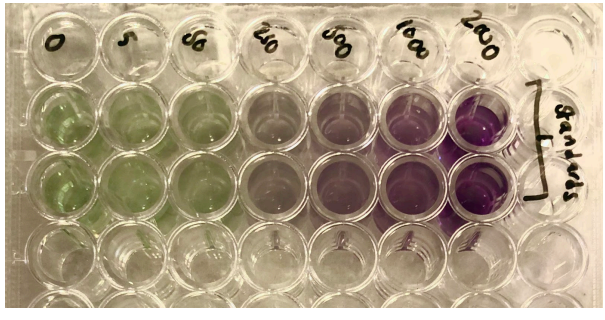
https://www.youtube.com/watch?v=JYY6bfvvw7M

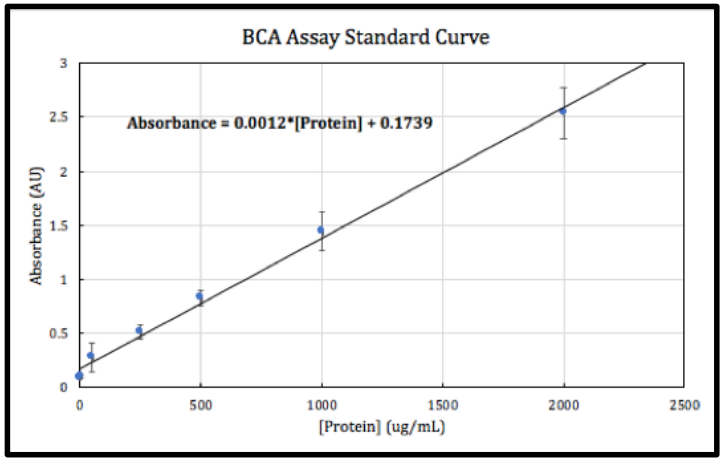
https://www.youtube.com/watch?v=fTsDHDIc89k https://www.biocompare.com/Product-Reviews/177742-Pierce-BCA-Protein-Assay-Kit-from-Thermo-Scientific/

BCA Assay

- **Definition:** A bicinchoninic acid assay (BCA Assay) uses absorbance readings to determine total protein concentration in a sample.

- First, create a **standard curve**
  - Concentration of protein in each well is known
- Then, add experimental samples
  - Concentration of protein is unknown
- Using standard curve absorbance readings, correlate protein concentration to absorbance
- Use standard curve to calculate experimental protein concentration

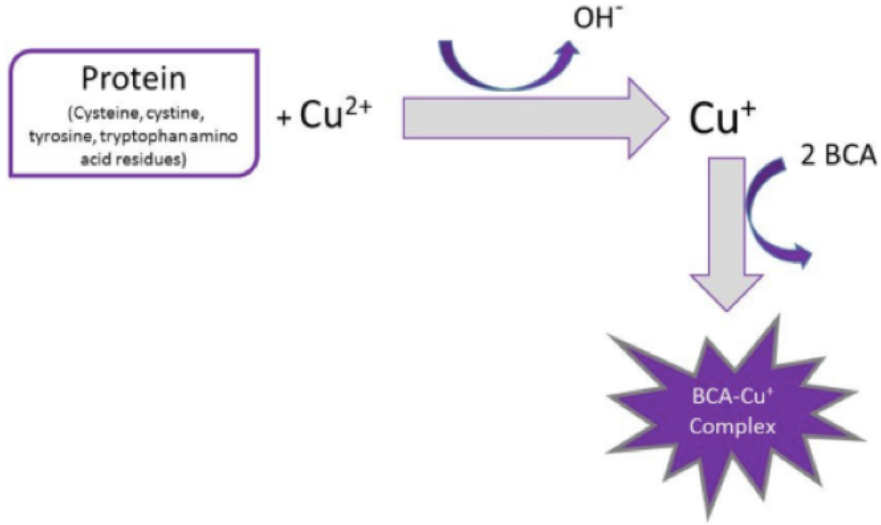




https://bioquochem.com/product/bca-protein-quantification-assay-kit-kb-03-005/

How does a BCA Assay work?

- Step 1: Biuret Reaction
  - Green cupric Cu^{2+} in BCA reagent binds to sample protein
    → reduction to cuprous Cu^{1+}
- Step 2: BCA and Copper Chelation
  - 2 BCA molecules bind to Cu^{1+}
    → purple chelated complex
- Step 3: Measure Absorbance
  - Purple complex absorbs maximally at 562 nm
  - Absorbance ∝ # purple complexes ∝ # peptides



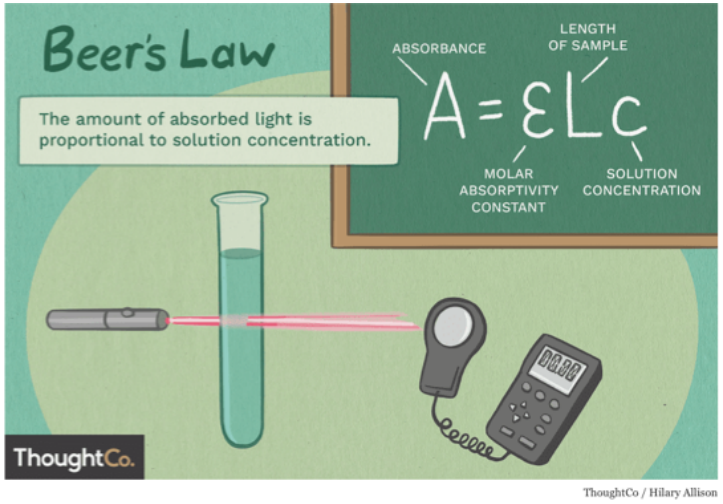
https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/chemistry-protein-assays.html

Beer's Law

- **Definition:** Beer's Law relates a sample's absorbance reading to total protein concentration.

$$\boldsymbol{A = \varepsilon \cdot L \cdot C}$$

- $\boldsymbol{A}$ = absorbance reading from plate reader
- $\boldsymbol{\varepsilon}$ = molar absorptivity constant
- $\boldsymbol{L}$ = path length
- $\boldsymbol{C}$ = protein concentration



ThoughtCo / Hilary Allison

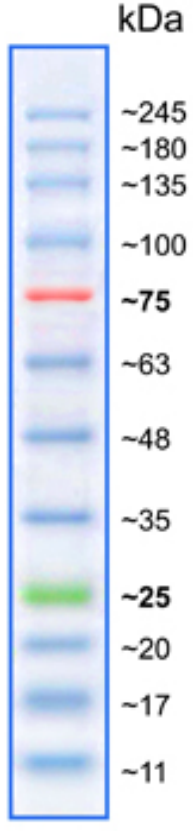
- As ε and L are constant, there is a linear relationship between absorbance and protein concentration ($A \propto C$)

https://www.thoughtco.com/beers-law-definition-and-equation-608172

SDS-PAGE

- **Definition:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins by size.

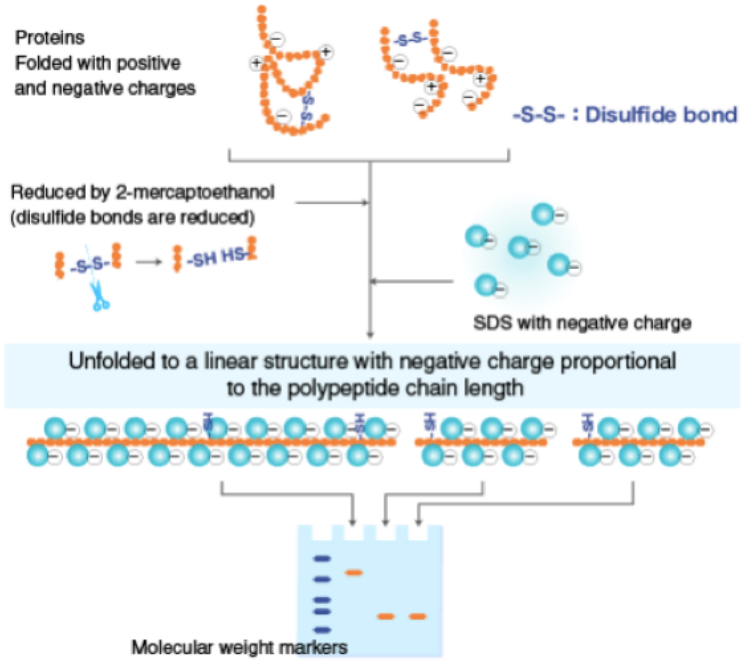
- First, load the protein ladder at the two ends of the well
  - Set of standards that allow us to estimate protein size
  - Dalton (Da) = atomic mass unit
  - kDa = 1000 Da
- Then, load your sample into the central wells
- Run the gel and use the ladder to estimate protein size



How does SDS-PAGE work?

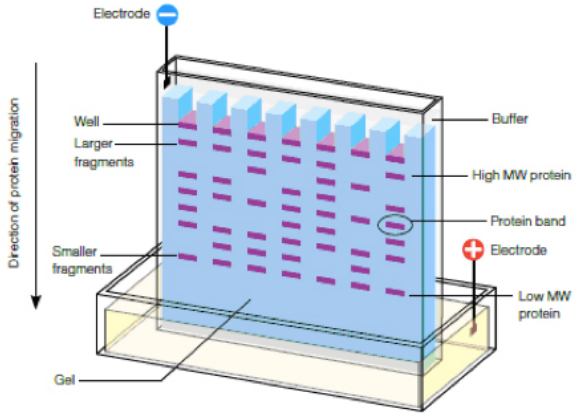
Key Reagents

- Beta-mercaptoethanol (βME)
    - Reduces disulfide bonds in protein → disruption covalent bonds
    - Protein linearization
- Sodium Dodecyl Sulfate (SDS)
    - Anionic detergent that binds to protein side chains → disruption noncovalent bonds → protein denaturation
    - Coats denatured protein in uniform negative charge
    - Charge of protein ∝ length of protein



https://ruo.mbl.co.jp/bio/e/support/method/sds-page.html

How does SDS-PAGE work?

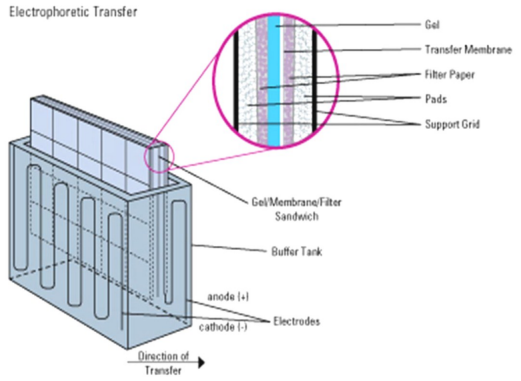


- Polyacrylamide (PA)
  - Water soluble polymer
  - 3D networks of polyacrylamide → porous gel
  - Smaller proteins can travel faster through the porous gel
- Gel Electrophoresis (GE)
  - When placed in an electric field, the negatively charged proteins will migrate toward the positive electrode
  - Since smaller proteins can travel faster through a porous gel, loading protein samples into a gel then creating an electric field around the gel **separates proteins by size**

https://www.bio-rad.com/en-us/applications-technologies/protein-electrophoresis-methods?ID=LUSQW4GRI

Protein Transfer

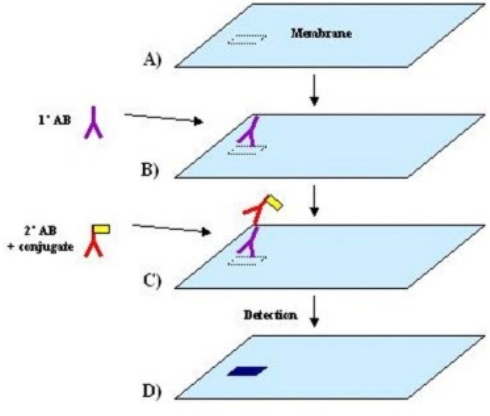
- Polyacrylamide gel → nitrocellulose membrane
  - Antibodies cannot bind to proteins when they are on the gel
  - Must transfer proteins onto a nitrocellulose membrane and retain the gel electrophoresis size sorting
- Transfer proteins using electroblotting
  - Align the gel and the membrane
  - Use an electric current to pull negatively charged proteins toward a positively charged anode and onto the membrane



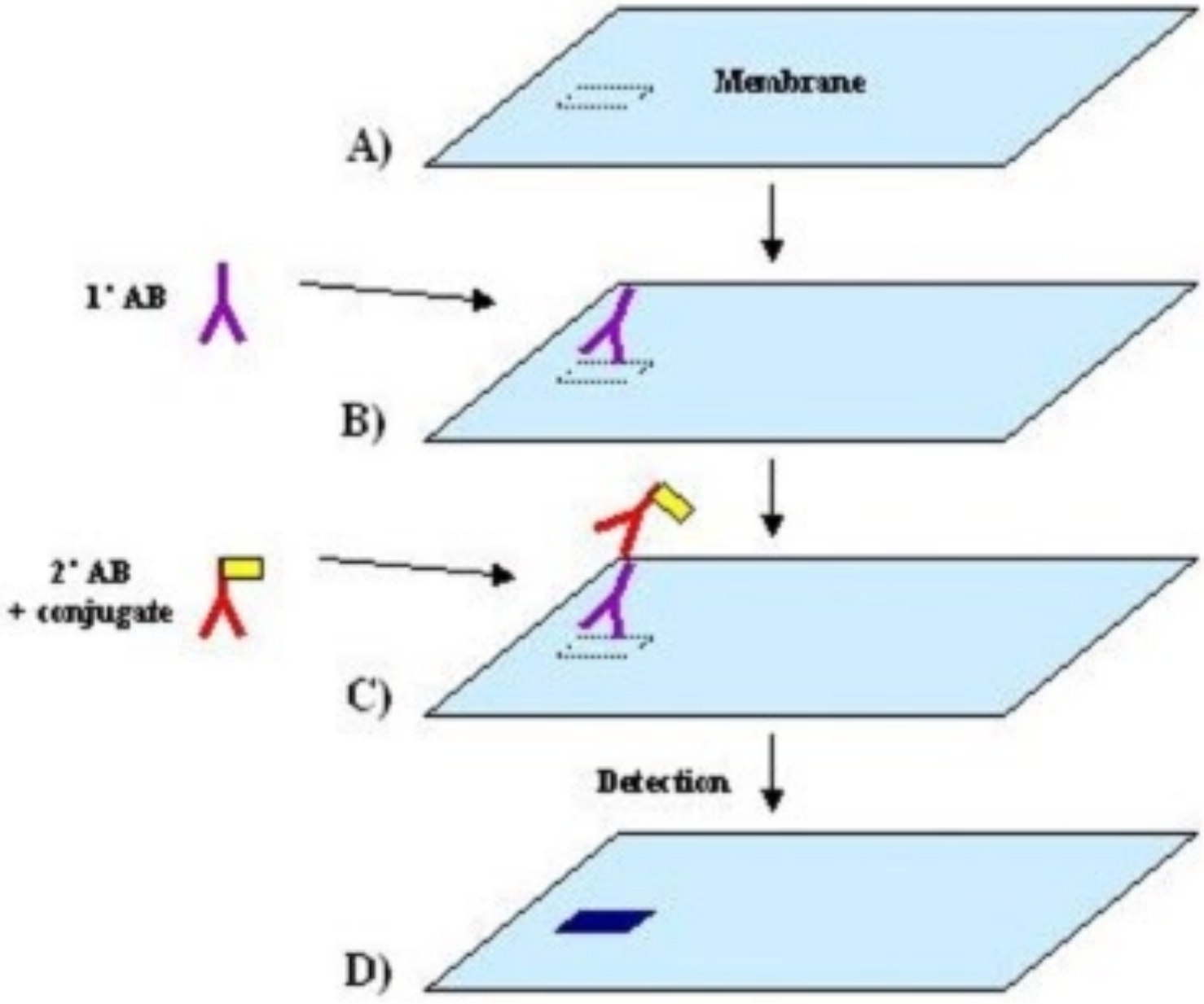
Immunoblotting (What Makes it a Western Blot!)

- **Definition:** Immunoblotting uses antibodies to identify proteins.

- Antibodies are proteins in the immune system that target specific antigens
- Primary Antibody: binds to target protein
    - Loading Control: Actin (constitutively expressed in all cells)
- Secondary antibody: binds to primary antibody and amplifies the signal
    - Primary and secondary antibodies must be from a different species than the target protein
        - If not, will have non-specific binding

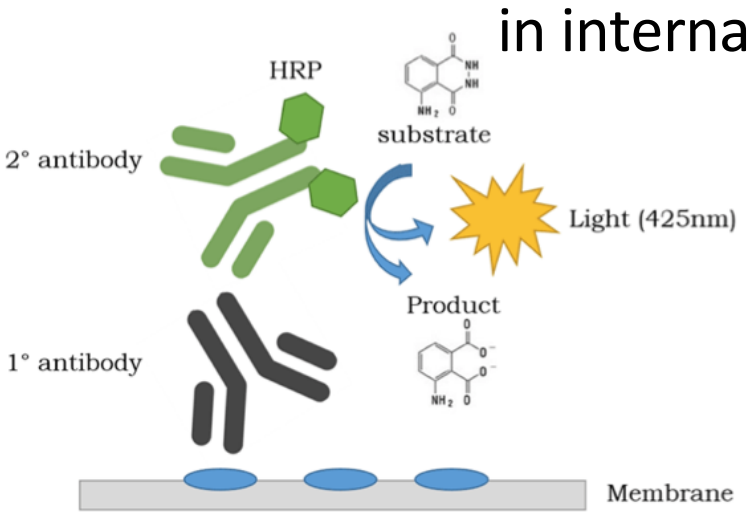


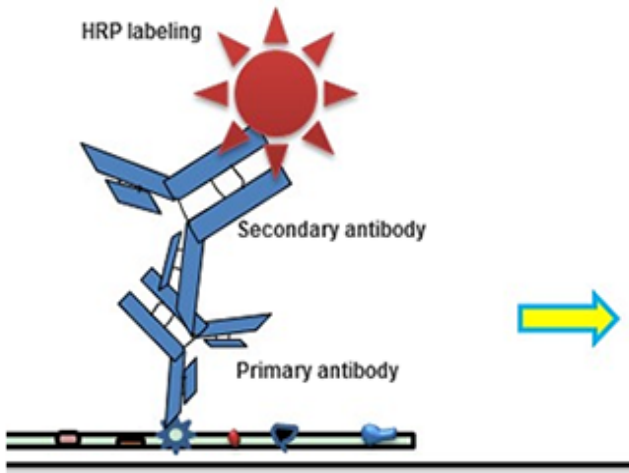
How does Immunoblotting work?

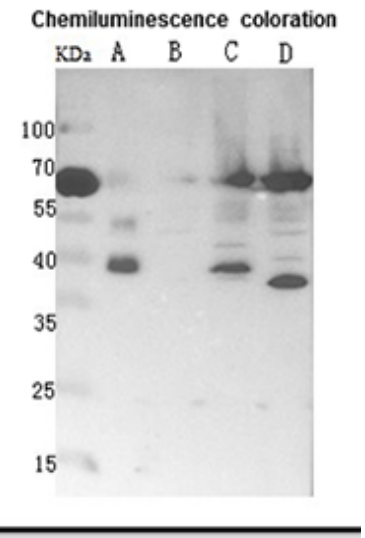


Imaging

- Chemiluminescence
  - Chemical reaction between enhanced chemiluminescence (ECL) substrate and horseradish peroxidase (HRP) enzyme conjugated to the secondary antibody
    - Releases energy as light
    - One of the easiest ways to examine proteins involved in internal cell behavior and controls





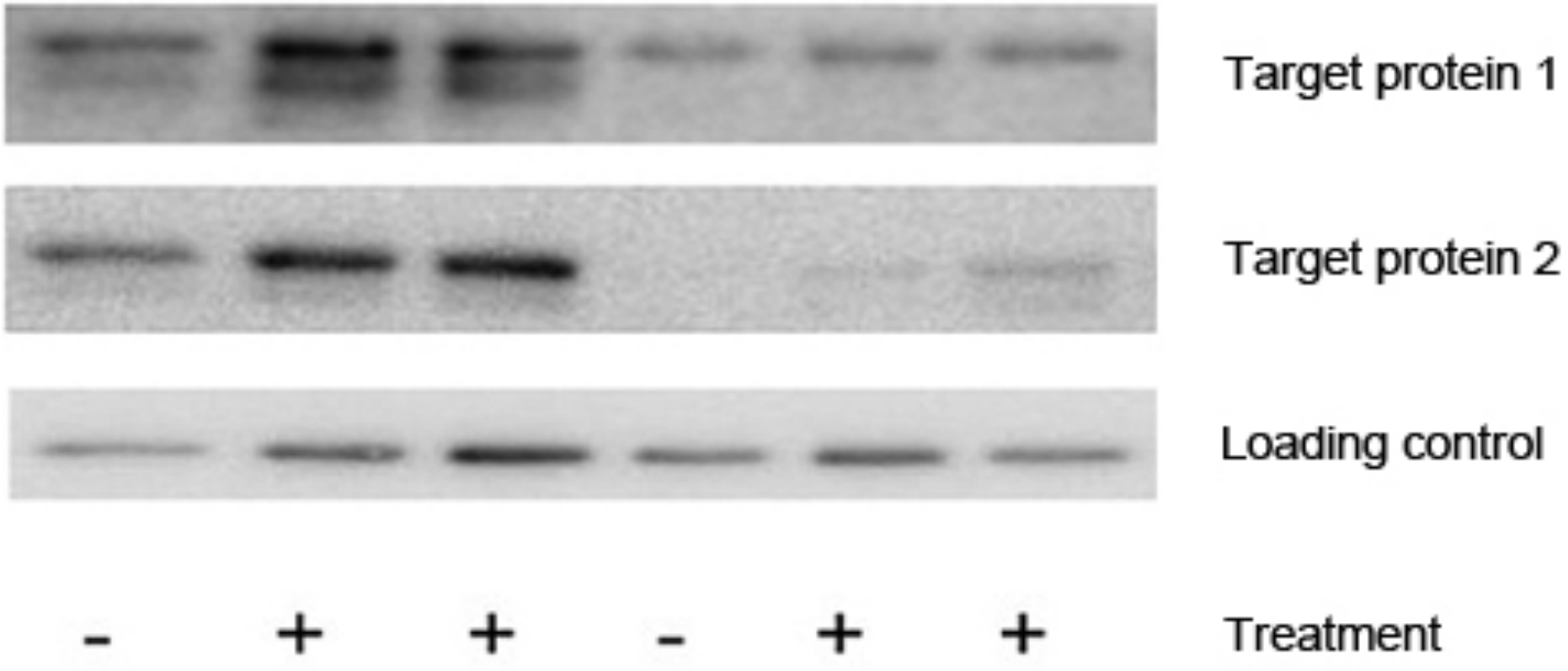


https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/chemiluminescent-western-blotting.html

Interpreting a Western Blot

- Loading control band:
    - should be the same in all samples
    - If loading control is not the same, result is invalid
- Band position on gel:
    - Different sized proteins show up at different heights
    - Larger proteins show up closer to the original well position
- Band intensity:
    - The darker the band, the more protein is present

Interpreting a Western Blot



- Loading control band is the same in all trials
- Greater target protein intensity in wells 2 and 3

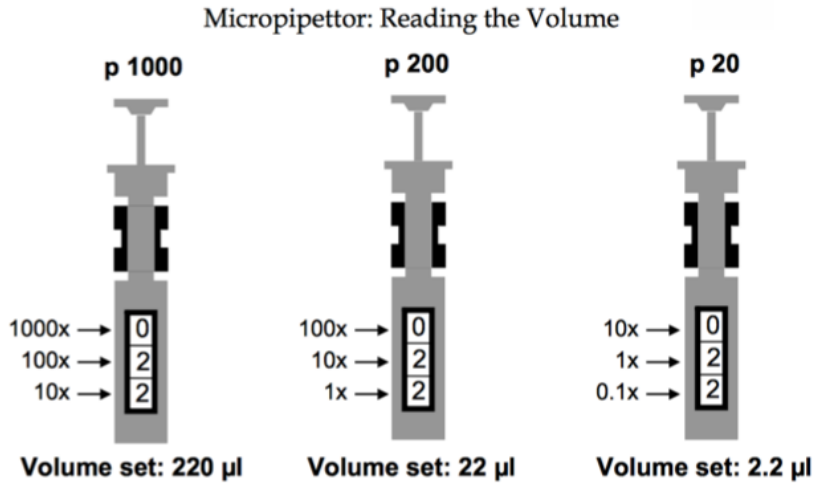
Western Blot Video

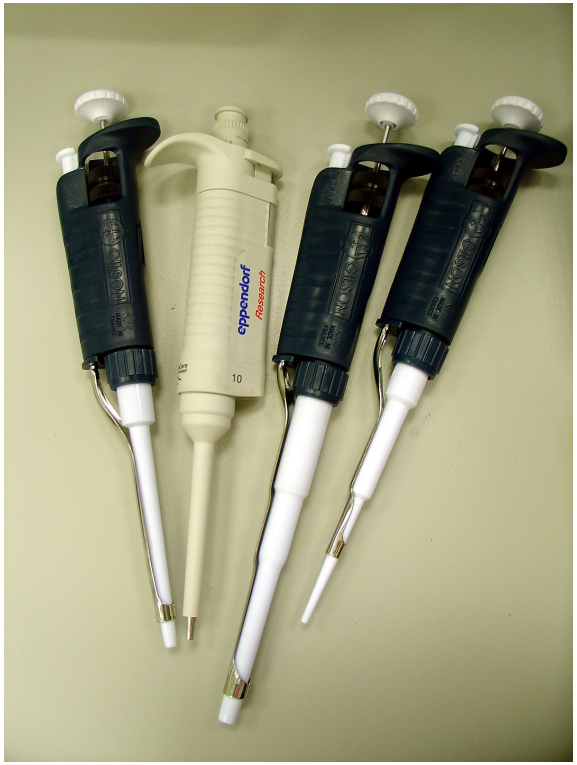
https://youtu.be/yUstng0npaY

Micropipettes

- **Definition:** A micropipette is a laboratory instrument used to measure small volumes (on the order of microliters).

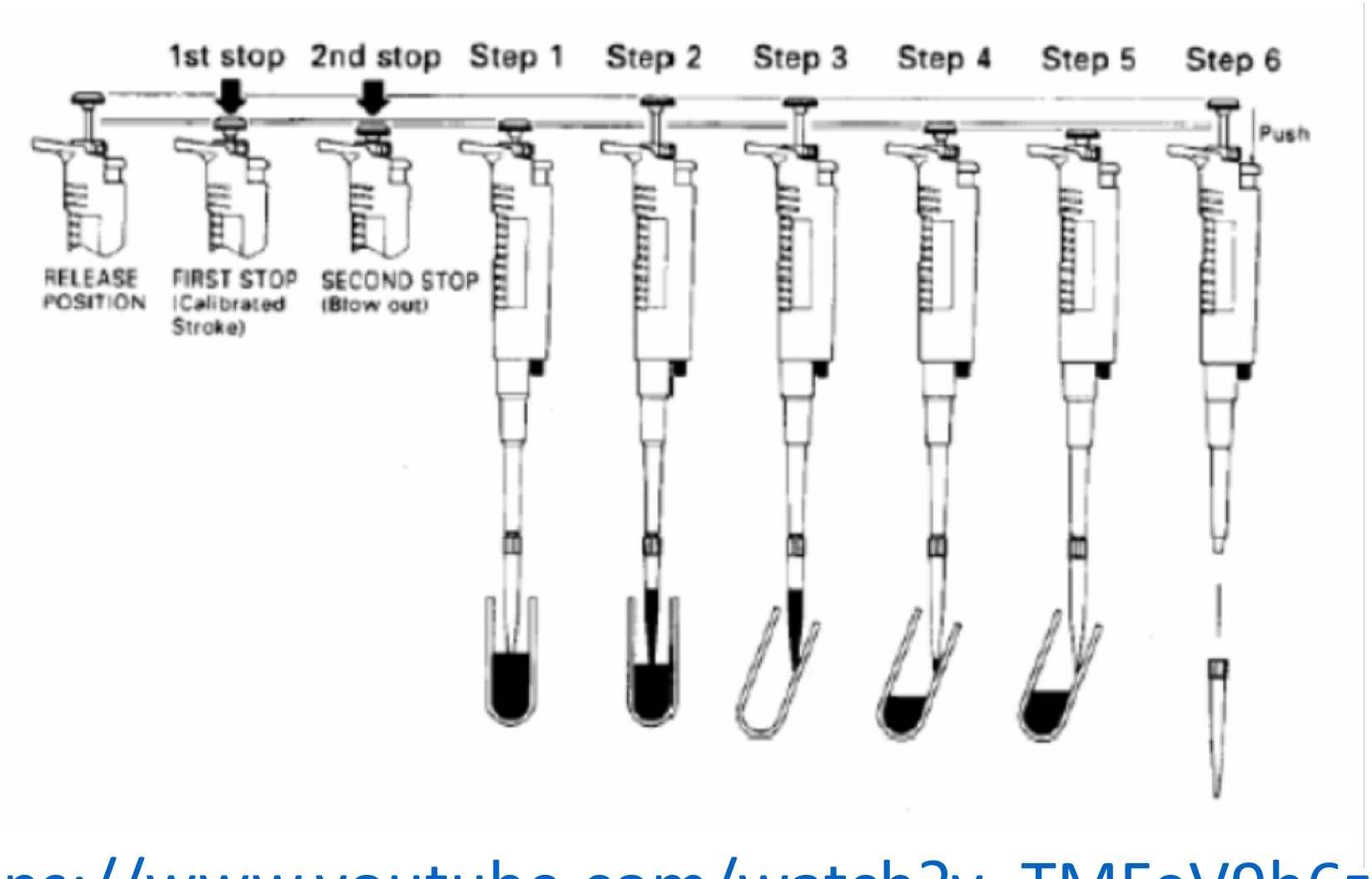
- Micropipette sizes
  - P20: 2 – 20 uL
  - P200: 20-200 uL
  - P1000: 100-1000 uL
- Be mindful of which size you are using





http://faculty.buffalostate.edu/wadswogj/courses/bio211%20page/resources/micropipetting%20lab.pdf

How to use a Micropipette



https://www.youtube.com/watch?v=TMFeV9h6zEA

https://www.youtube.com/watch?v=uEv_NGDfo_8