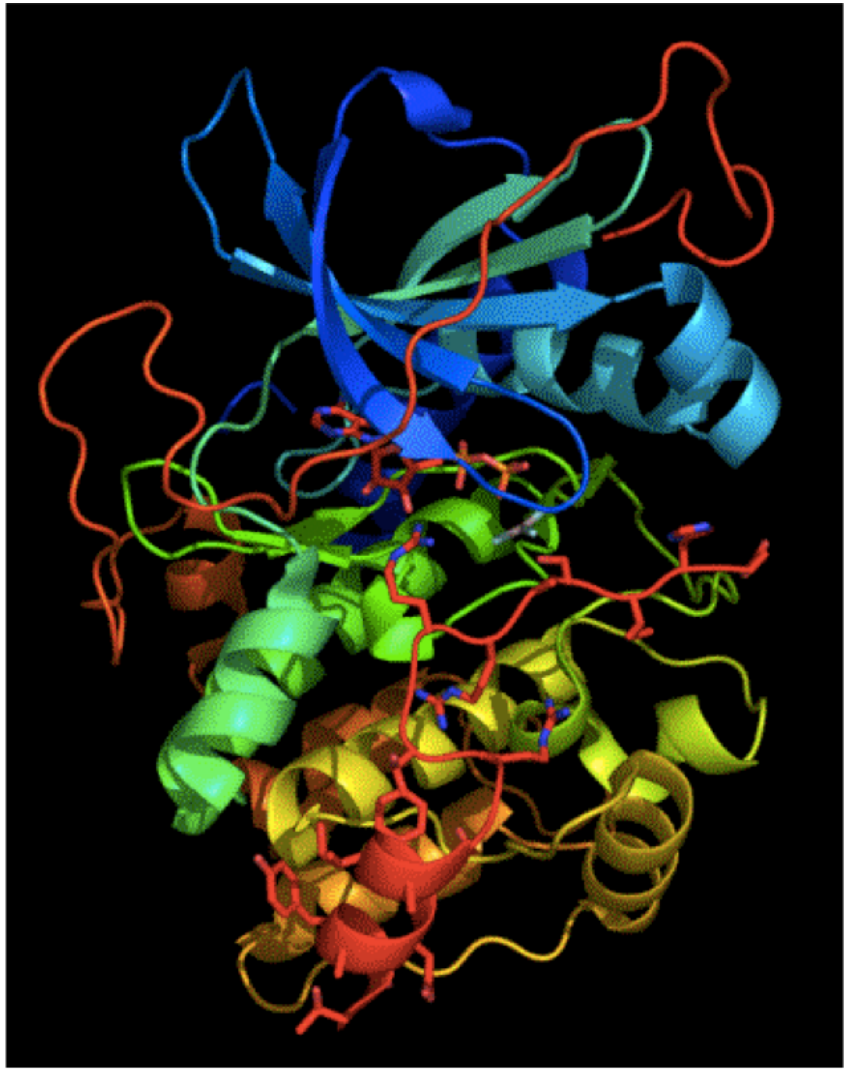
Module 2: Western Blot

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

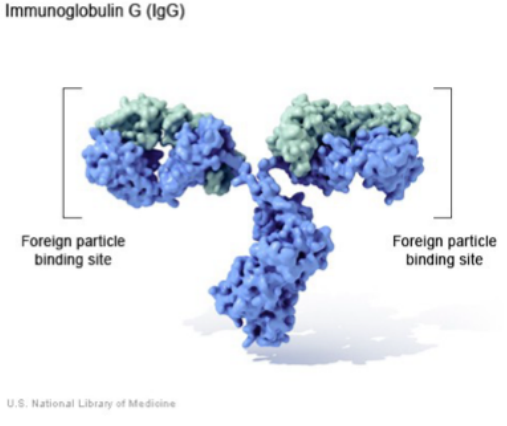
Proteins

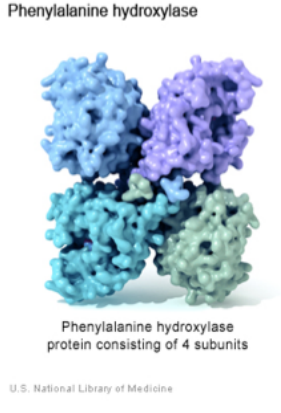
- **Definition:** Proteins are macromolecules made of amino acids.

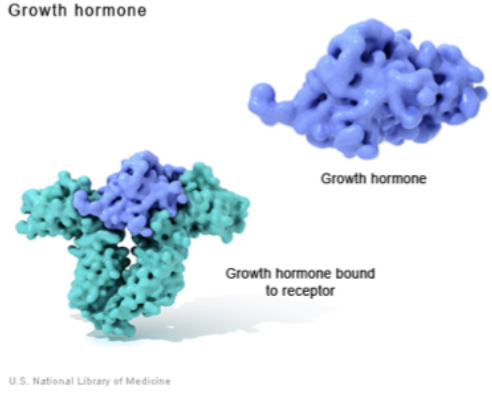
→ Chains of amino acids make up proteins

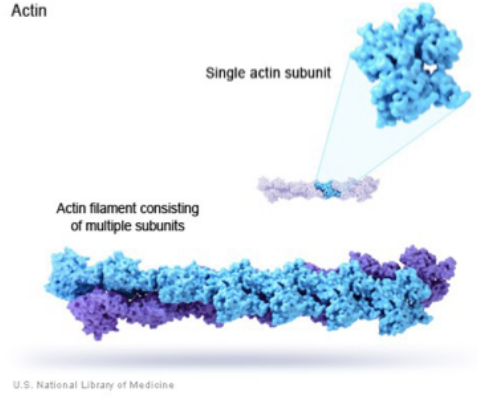
→ Proteins have a wide structural range → large functional range

→ Key players in organism's metabolic & regulatory activity









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

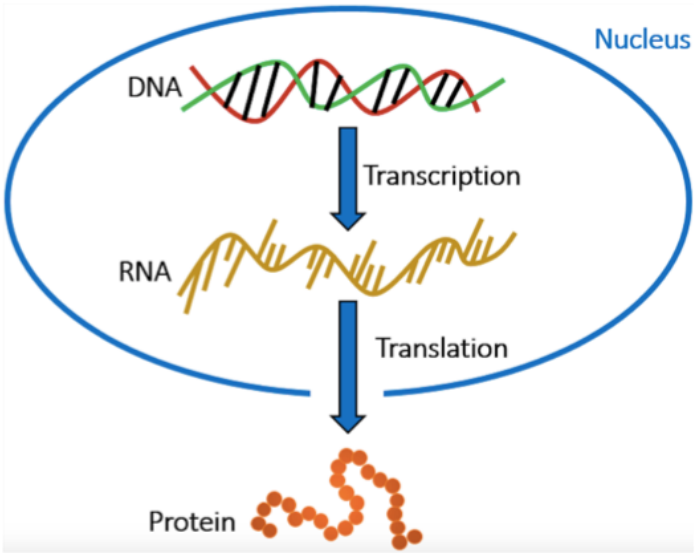
What is Western Blotting?

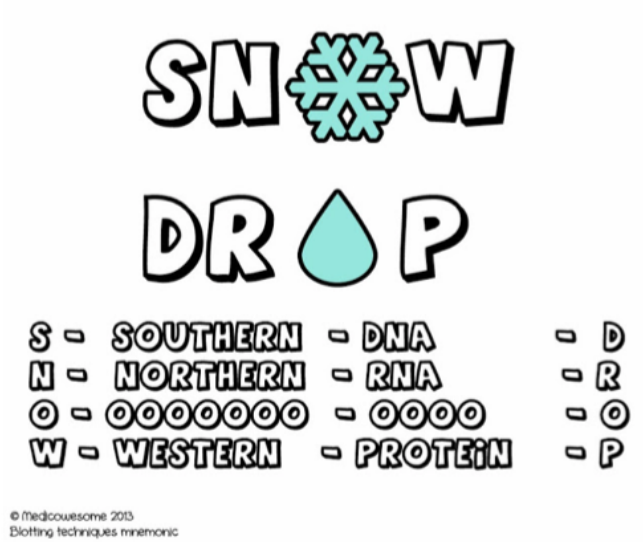
- **Definition:** A Western Blot separates and identifies target proteins.

→ Two stage procedure confirms protein presence and quantifies target

→ Proteins separated by size through gel electrophoresis

→ Target proteins visualized through immunostaining



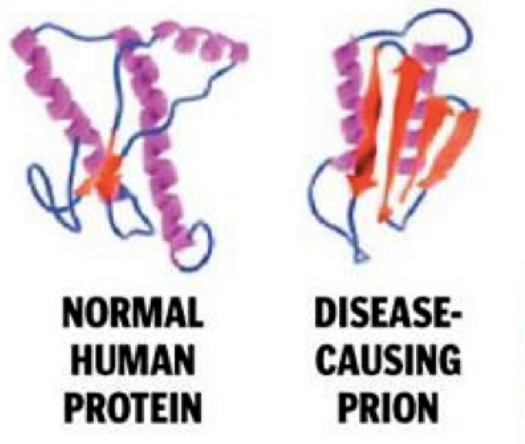


https://www.researchgate.net/profile/Radin_Tahvildari/publication/313798200/figure/fig1/AS:462661172371457@1487318427401/1-Overview-of-the-central-dogma-shows-the-flow-of-genetic-information-inside-a.png

Western Blot Utility

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

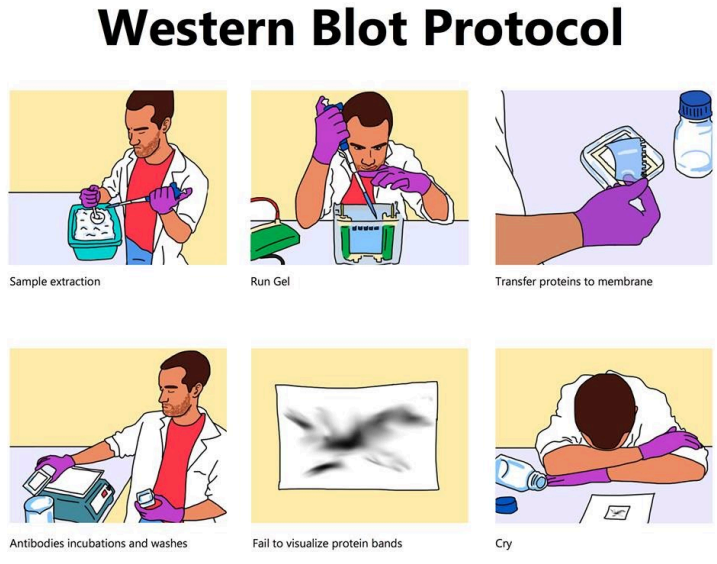
- Probe for a specific disease
    - **Viral:**
        - HIV
    - **Bacterial:**
        - Meliodosis
    - **Prion:**
        - Creutzfelt-Jakob disease



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



Basic Protocol for a Western Blot

1. Lyse cells and collect proteins
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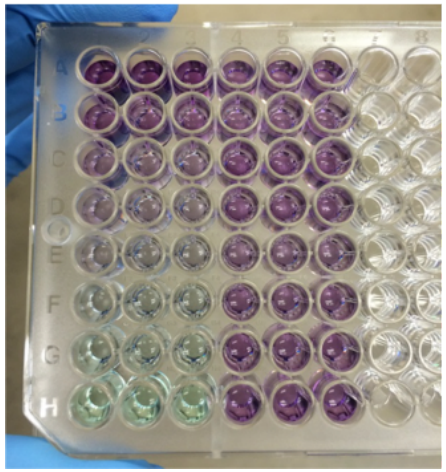
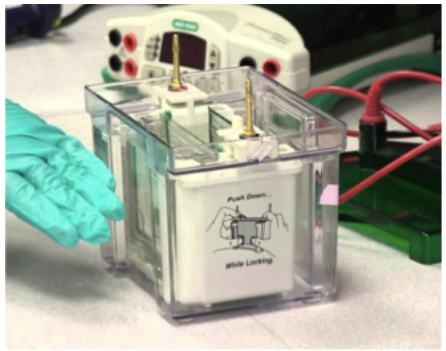
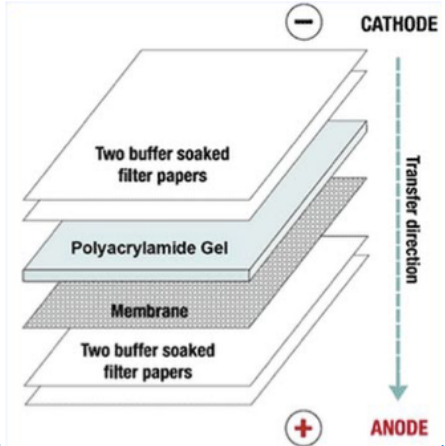






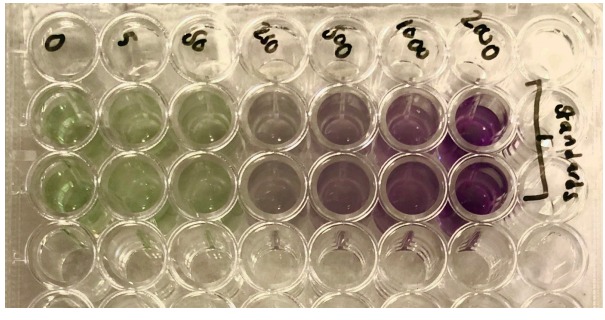
https://www.youtube.com/watch?v=JYY6bfvvw7M

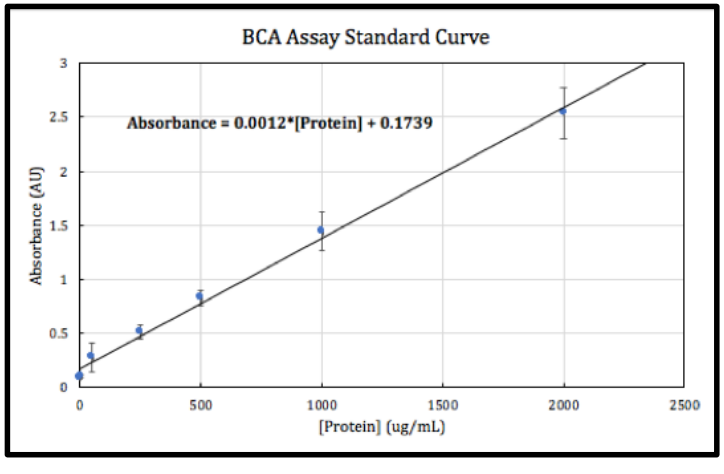
https://www.youtube.com/watch?v=fTsDHDIc89k https://www.biocompare.com/Product-Reviews/177742-Pierce-BCA-Protein-Assay-Kit-from-Thermo-Scientific/

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
- Use standard curve absorbance readings to correlate protein concentration to absorbance
- Use standard curve to calculate experimental protein concentration



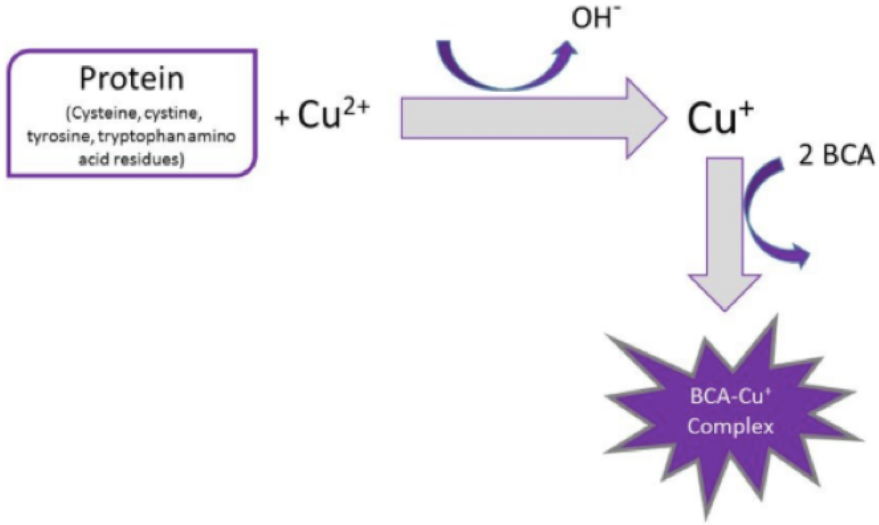


https://bioquochem.com/product/bca-protein-quantification-assay-kit-kb-03-005/

How does a BCA Assay work?

- Step 1: Biuret Reaction
    - Green Cu^{2+} in BCA reagent binds to sample protein

      → reduction of 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 ∝ peptide

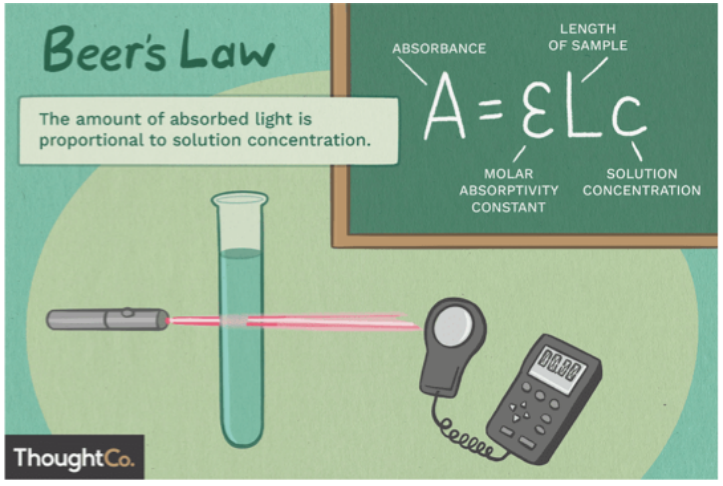
https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/chemistry-protein-assays.html

Beer's Law

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

$$A = \varepsilon \cdot L \cdot C$$

- A = absorbance reading from plate reader
- ε = molar absorptivity constant
- L = path length
- C = protein concentration
  - As ε and L are constant, there is a linear relationship between absorbance and protein concentration



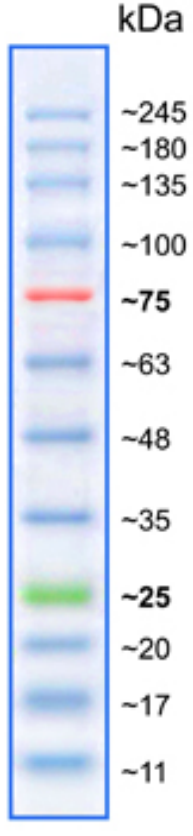
ThoughtCo / Hilary Allison

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.

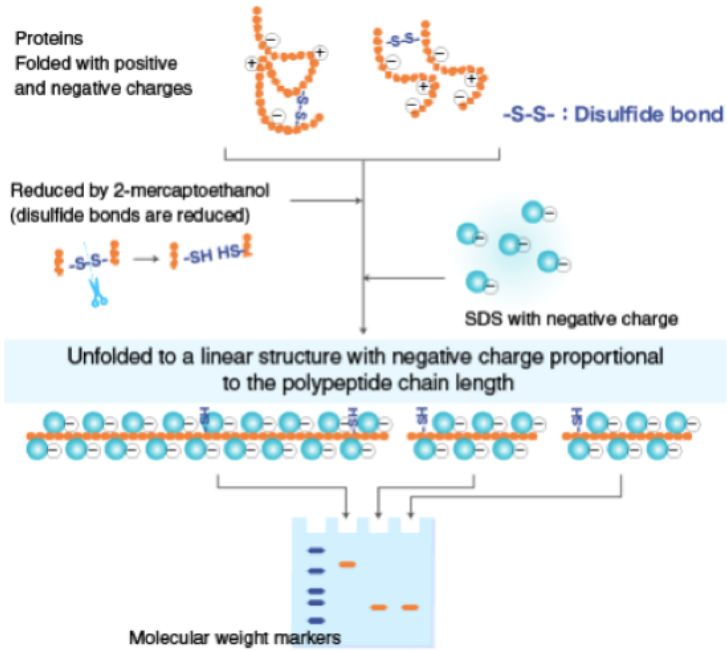
- First, load the protein ladder at the two ends of the well
  - Set of standards that allow us to estimate protein size
  - Dalton (Da) = atomic mass unit
  - kDa = 1000 Da
- Then, load your sample into the central wells
- Run the gel and use the ladder to estimate protein size



How does SDS-PAGE work?

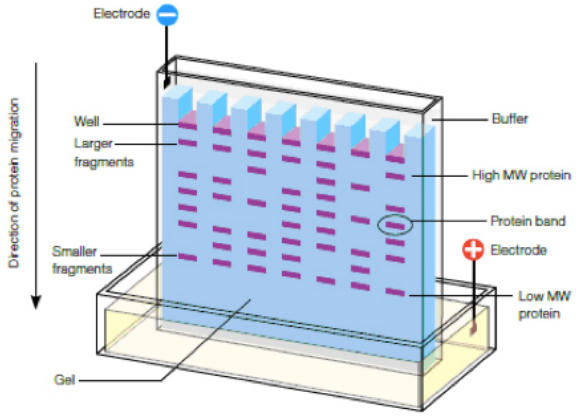
Key Reagents

- 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
- Beta-mercaptoethanol (βME)
    - Reduces disulfide bonds in protein → disruption covalent bonds → protein linearization



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

How does SDS-PAGE work?

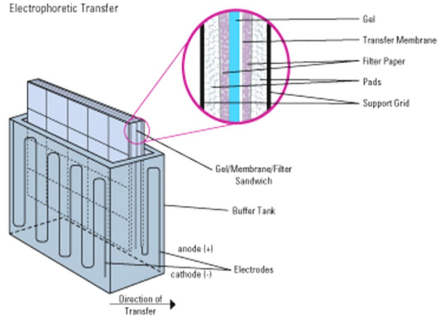


- Polyacrylamide (PA)
  - Water soluble polymer
  - 3D networks of polyacrylamide → porous gel
  - Smaller proteins can travel faster through the porous gel
- Gel Electrophoresis (GE)
  - When placed in an electric field, the negatively charged proteins will migrate toward the positive electrode
  - 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

- Need to transfer proteins from gel to 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

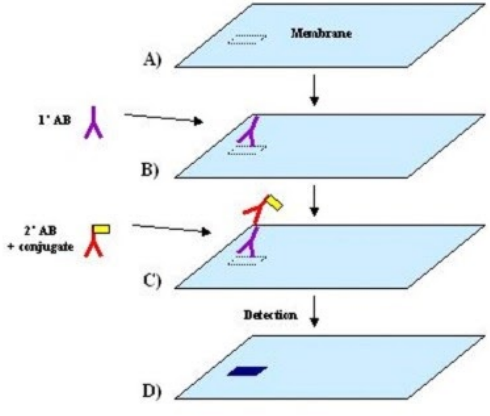


- Transfer proteins using electroblotting
  - Align the gel and the membrane
  - Use an electric current to pull negatively charged proteins toward a positively charged anode and onto the membrane

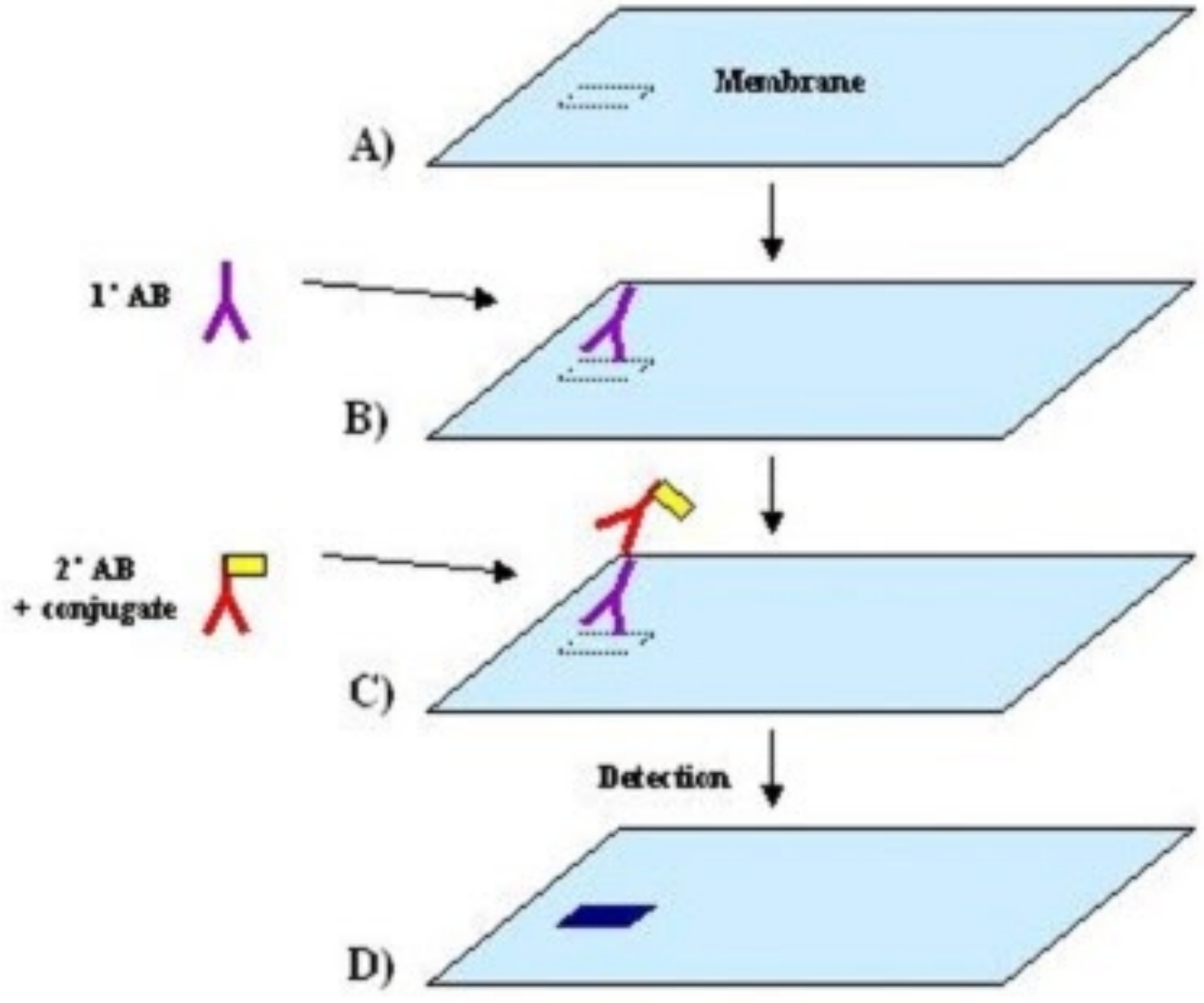
Immunoblotting (What Makes it a Western Blot!)

- **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

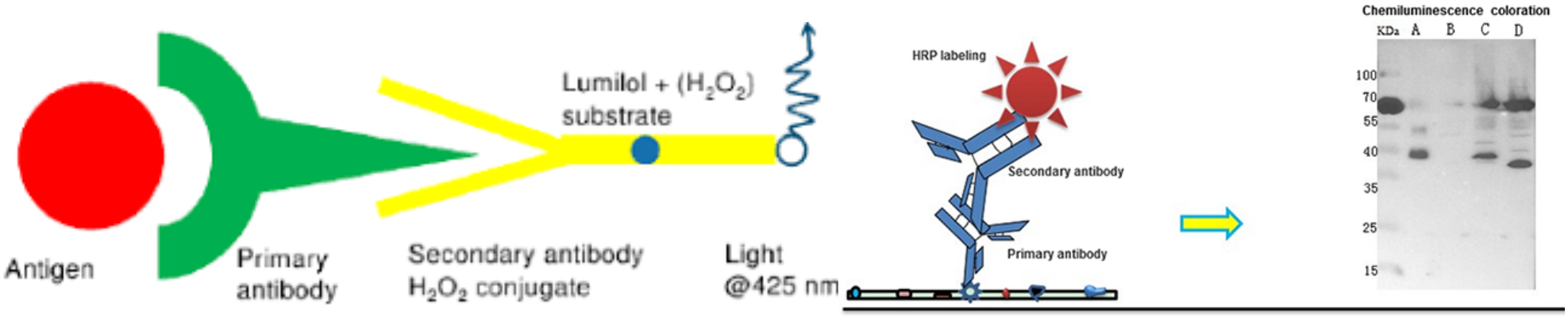


How does Immunoblotting work?



Imaging

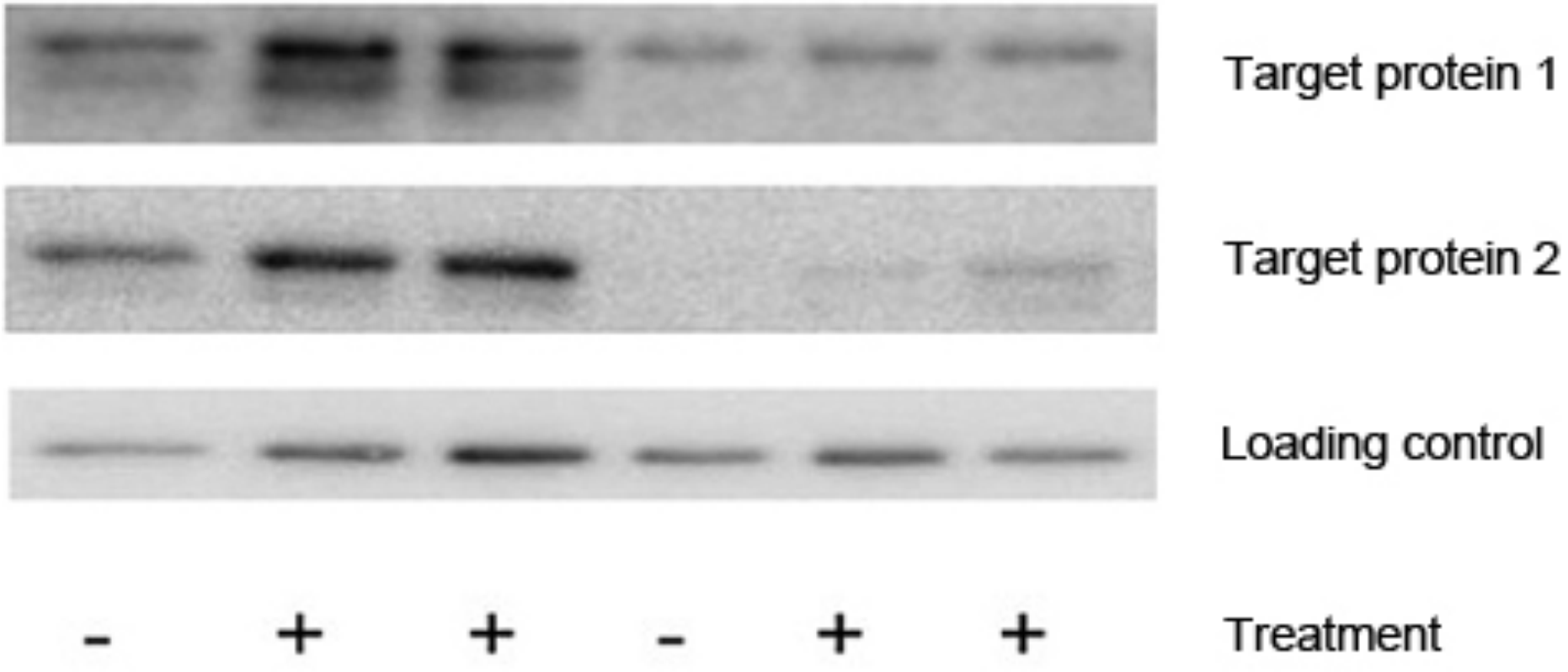
- Chemiluminescence
  - Chemical reaction between ECL substrate and HRP enzyme in secondary antibody
    - Releases energy as light
    - One of the easiest ways to understand internal cell behavior and controls



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

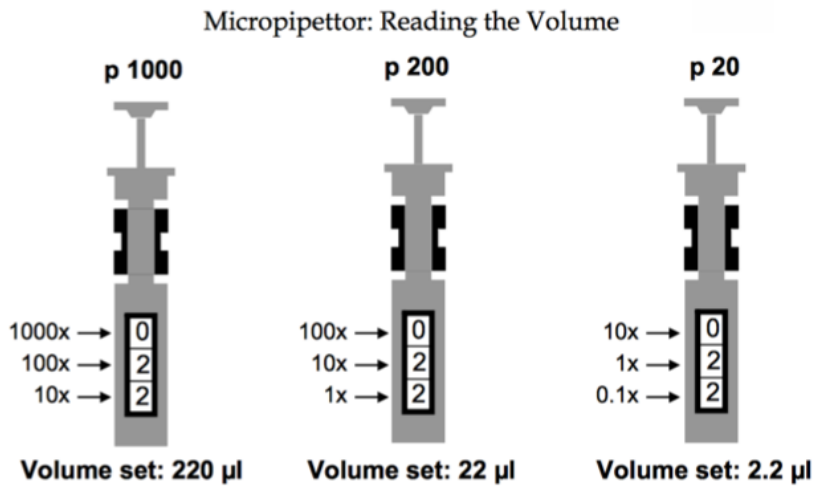
Western Blot Video

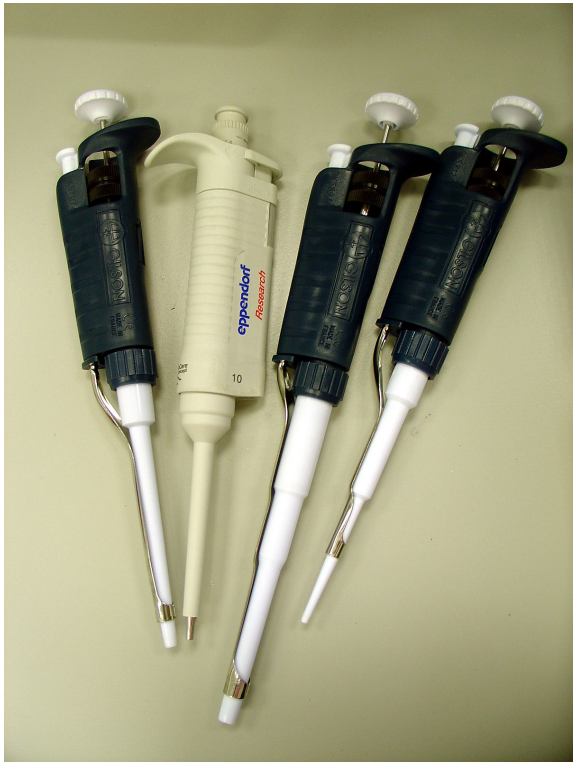
https://youtu.be/yUstng0npaY

Micropipettes

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

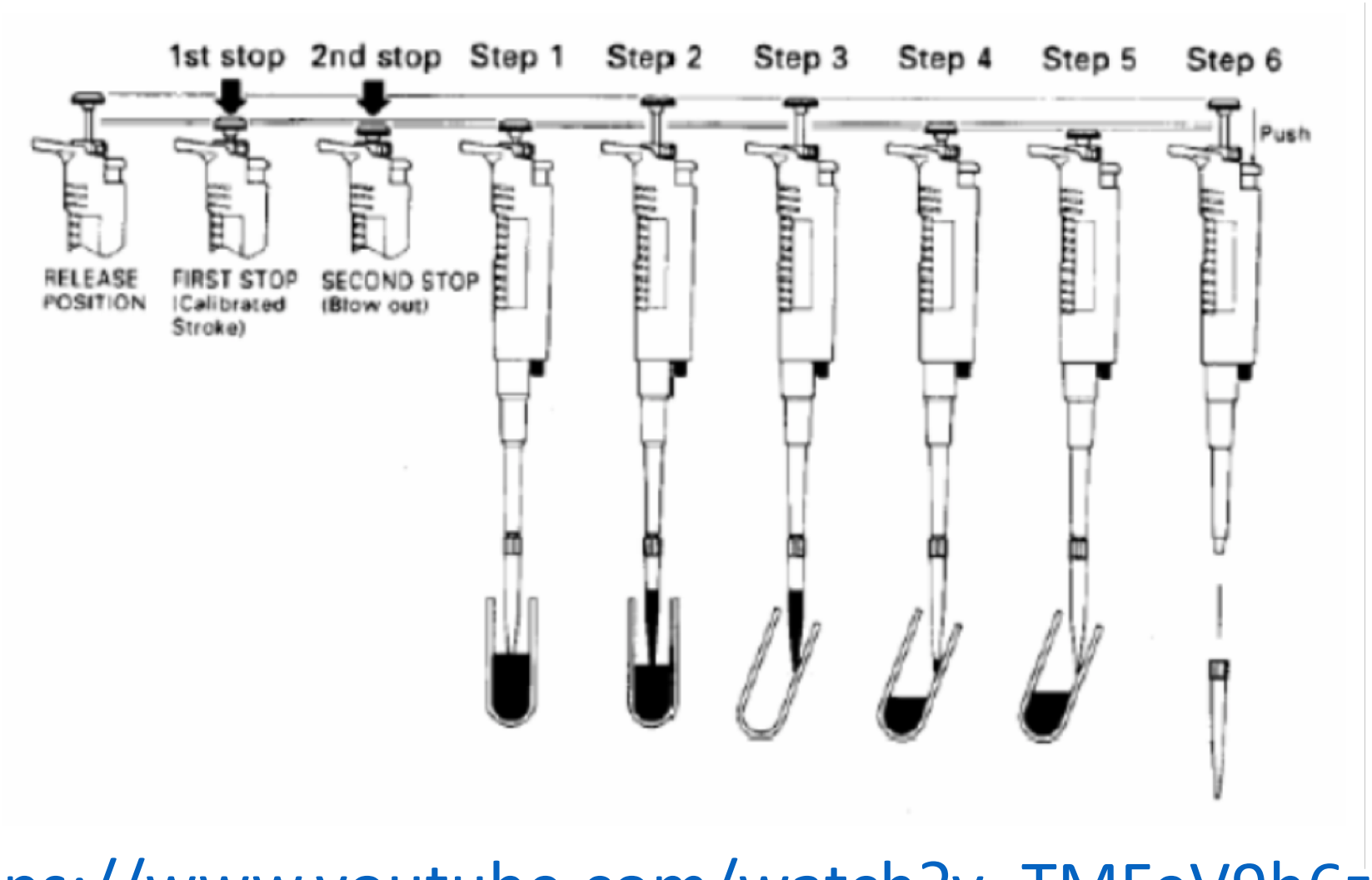
- Micropipette sizes
  - P20: 2 – 20 uL
  - P200: 20-200 uL
  - P1000: 100-1000 uL
- Be mindful of which size you are using





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

Module 2 Worksheet

BMES Cell Team

Fall 2020

Overview of Module 2

1. Name one protein function:

_____

2. What is the purpose of a Western blot?

_____

3. What is the purpose of a BCA Assay? Which metal is present in BCA reagent?

_____

4. What is the purpose of SDS-PAGE?

_____

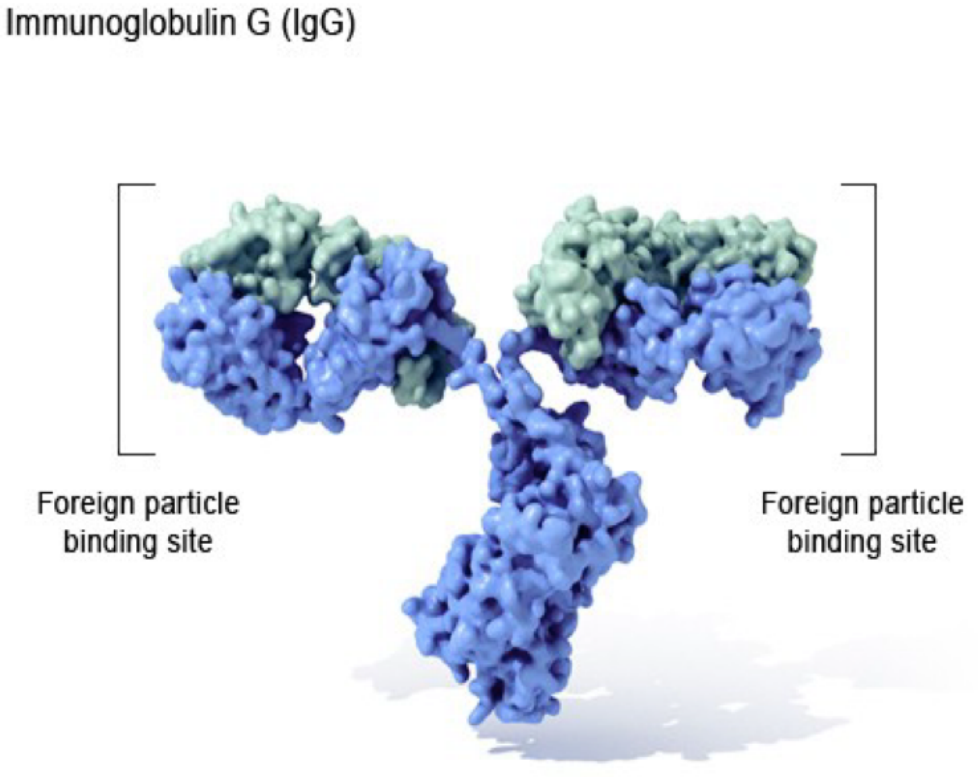
5. Why do we need to transfer proteins from a gel to a membrane?

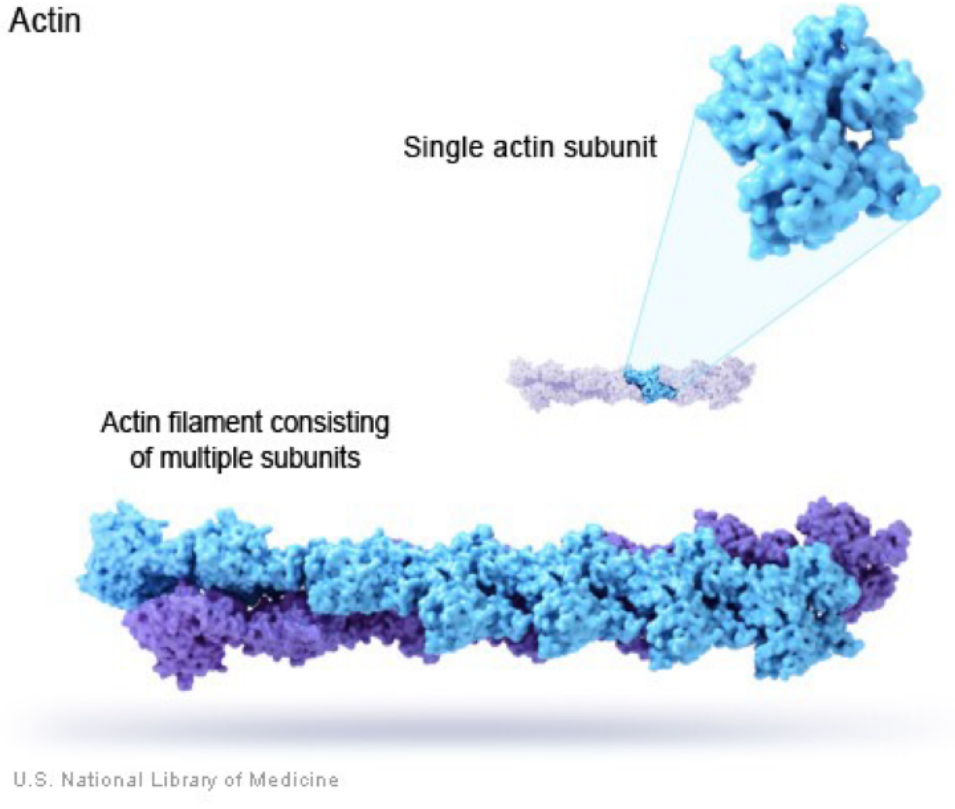
_____

Overview of Module 2

1. Name one protein function:

antibody, structural component, messenger, enzyme, regulatory

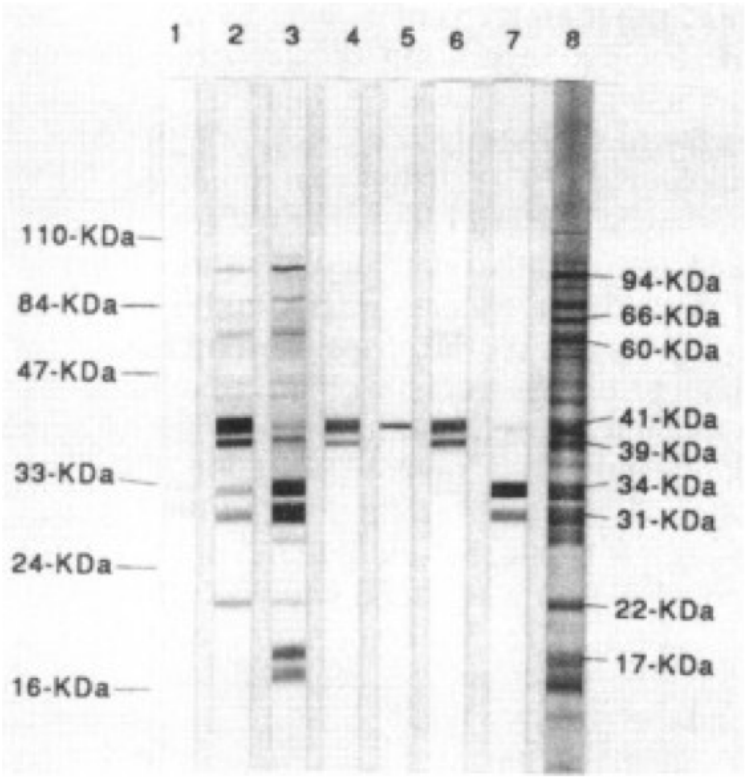


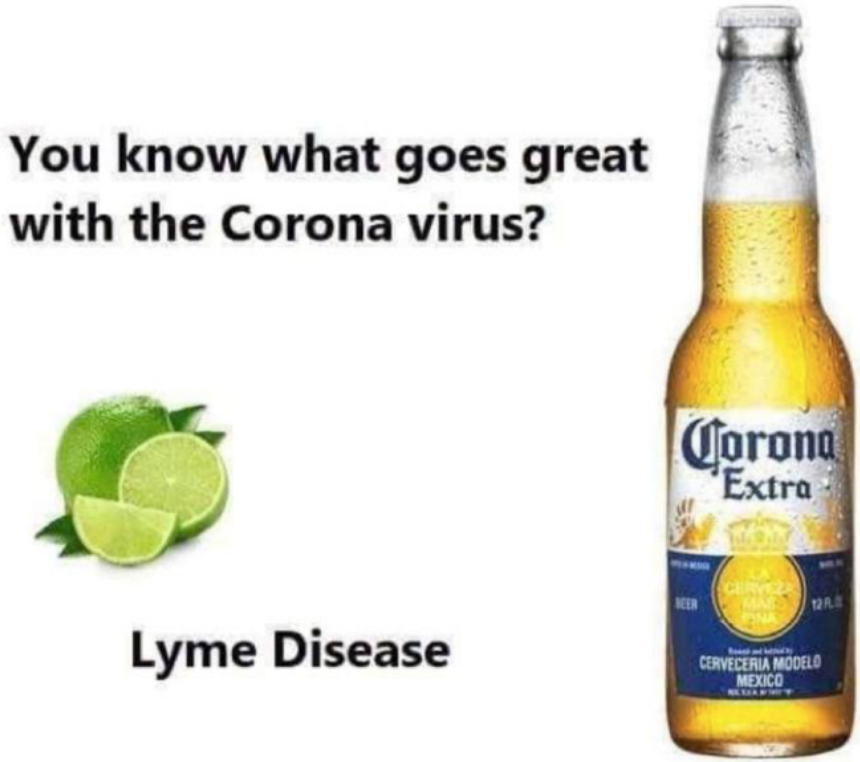


Overview of Module 2

2. What is the purpose of a Western blot?

separate and identify target protein to better understand the protein's role and how protein mutations can result in disease state





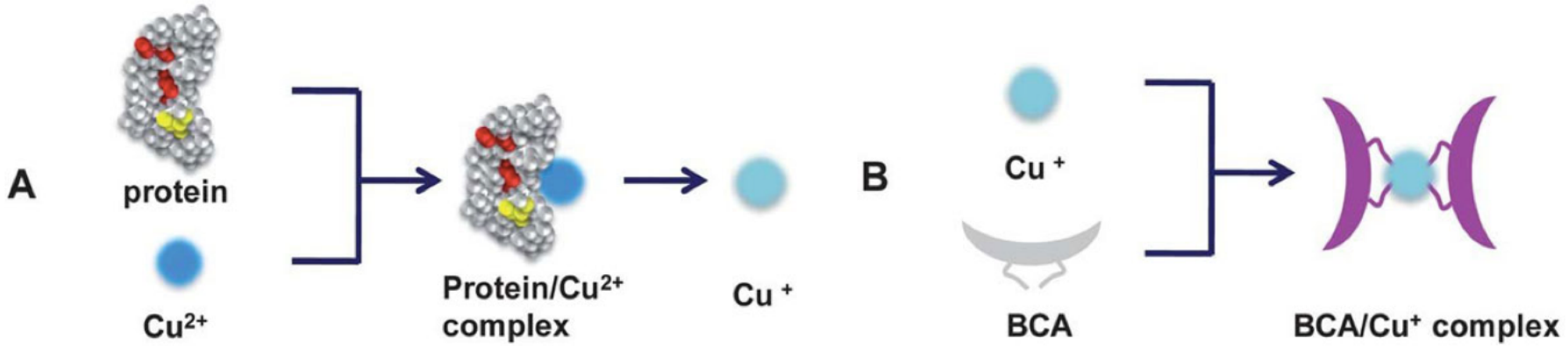
https://www.reddit.com/r/EmKay/comments/evc1j3/a_corona_virus_gets_it_lyme_disease/ https://www.lymedisease.org/lyme-disease-test/

Overview of Module 2

3. What is the purpose of a BCA Assay? Which metal is present in BCA reagent?

A BCA Assay allows us to calculate sample protein concentration using absorbance readings.

The copper ion (Cu^{2+}) is reduced during the Biuret Reaction in a BCA Assay

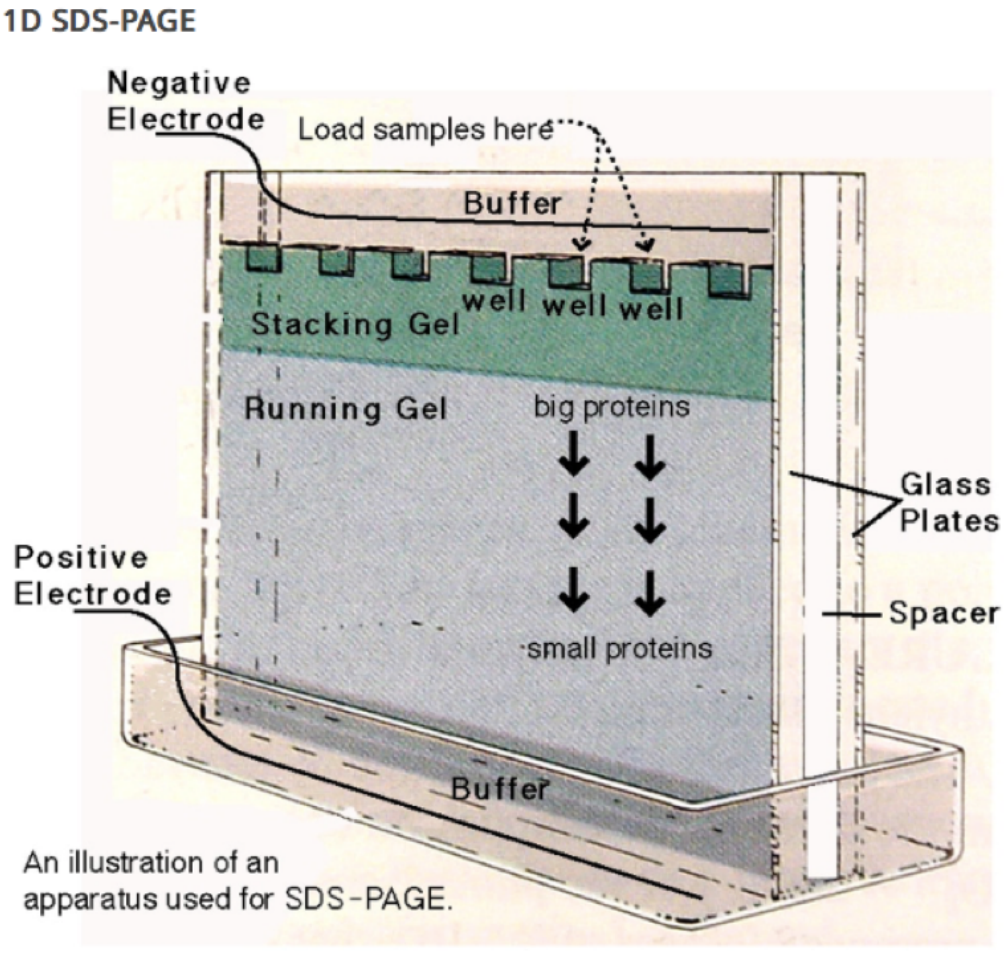


https://onelab.andrewalliance.com/library/bca-colorimetric-protein-assay-X5nPg0gP

Overview of Module 2

4. What is the purpose of SDS-PAGE?

Gel electrophoresis separates protein fragments by size.



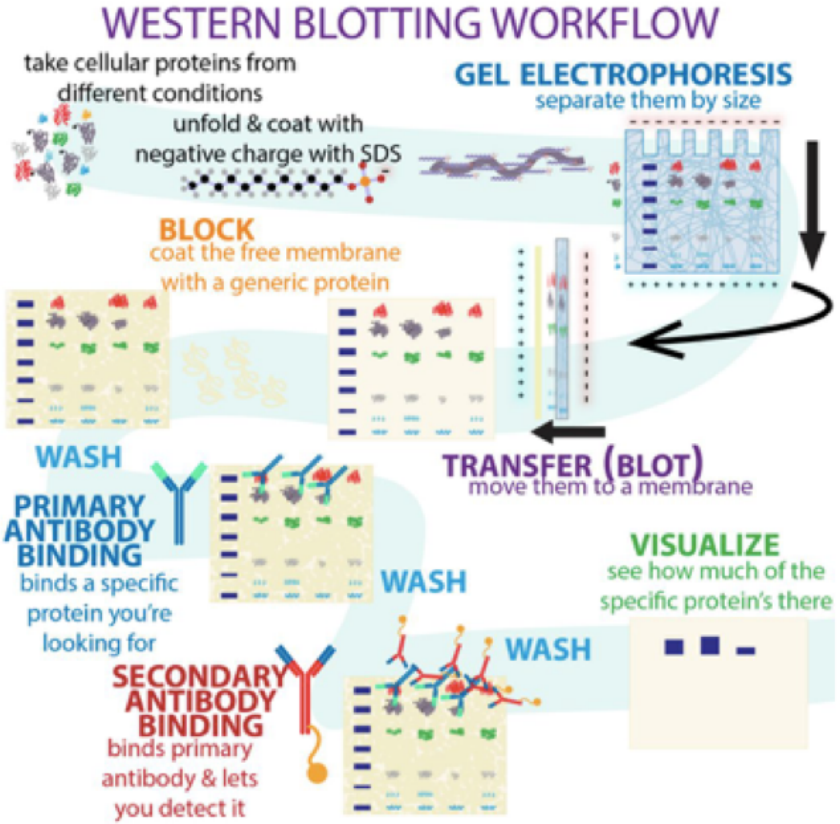
An illustration of an apparatus used for SDS-PAGE.

https://www.creative-proteomics.com/services/1d-sds-page-ief.htm

Overview of Module 2

5. Why do we need to transfer proteins from a gel to a membrane?

The antibodies utilized in immunoblotting cannot bind to the protein when the protein is on the gel.



https://thebumblingbiochemist.com/365-days-of-science/western-blots/

Practice Preparing a BCA Assay

1. **Calculate serial dilution volumes.**

We will start with a 20 ug/mL BSA solution.

a. Need 25 uL of standard for each well x 3 replicates = 75 uL per standard
b. Always make a little extra: _____ uL per standard

BSA Concentration (ug/mL)	BSA Solution Added (uL)	Water Added (uL)	Total Volume (uL)	Final Volume (uL)
2000 ug/mL	160 uL of 2000 ug/mL	0	160	160 - 80 = 80
1000 ug/mL	80 uL of 2000 ug/mL	80	160	160 - ___ = ___
500 ug/mL	___ uL of 1000 ug/mL	___	160	160 - ___ = ___
250 ug/mL	___ uL of 500 ug/mL	___	160	160 - ___ = ___
50 ug/mL	___ uL of 250 ug/mL	___	160	160 - ___ = ___
5 ug/mL	___ uL of 50 ug/mL	___	160	160 - ___ = ___
0 ug/mL	0	160	160	160

Practice Preparing a BCA Assay

1. **Calculate serial dilution volumes.**

We will start with a 20 ug/mL BSA solution.

a. Need 25 uL of standard for each well x 3 replicates = 75 uL per standard

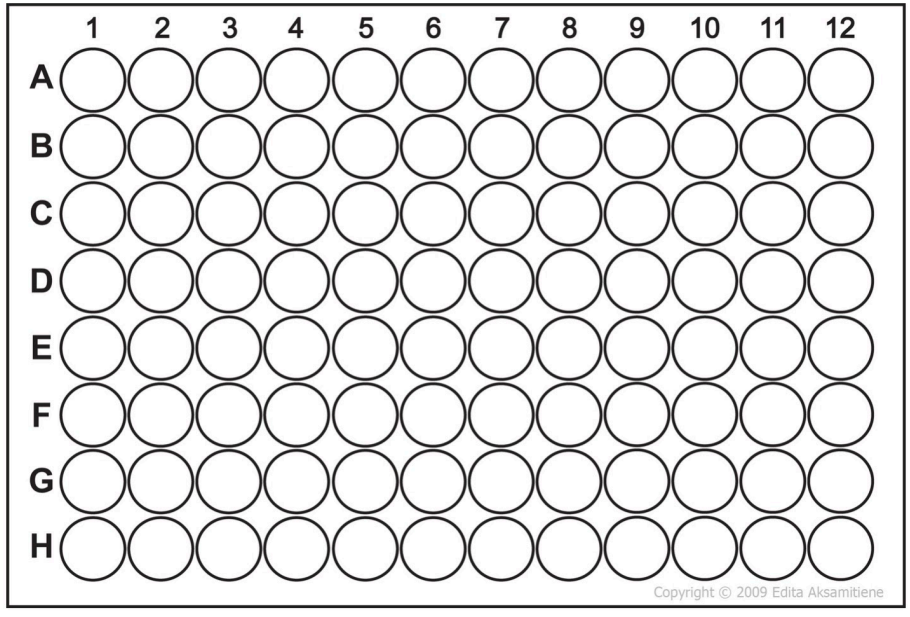
b. Always make a little extra: 80 uL per standard

BSA Concentration (ug/mL)	BSA Solution Added (uL)	Water Added (uL)	Total Volume (uL)	Final Volume (uL)
2000 ug/mL	160 uL of 2000 ug/mL	0	160	160 - 80 = 80
1000 ug/mL	80 uL of 2000 ug/mL	80	160	160 - 80 = 80
500 ug/mL	80 uL of 1000 ug/mL	80	160	160 - 80 = 80
250 ug/mL	80 uL of 500 ug/mL	80	160	160 - 80 = 80
50 ug/mL	32 uL of 250 ug/mL	128	160	160 - 16 = 144
5 ug/mL	16 uL of 50 ug/mL	144	160	160 - 0 = 160
0 ug/mL	0	160	160	160

Practice Preparing a BCA Assay

2. Design layout for 96 well plate:

a. Number of standards _____ x Number of repeats _____ = _____ wells
b. Number of samples__2__ x Number of repeats __2__ = __4__ wells
c. Total number of wells = Standard wells _____ + Sample wells __4__ = ___ wells
d. Label the well plate below with standard and sample placement



Practice Preparing a BCA Assay

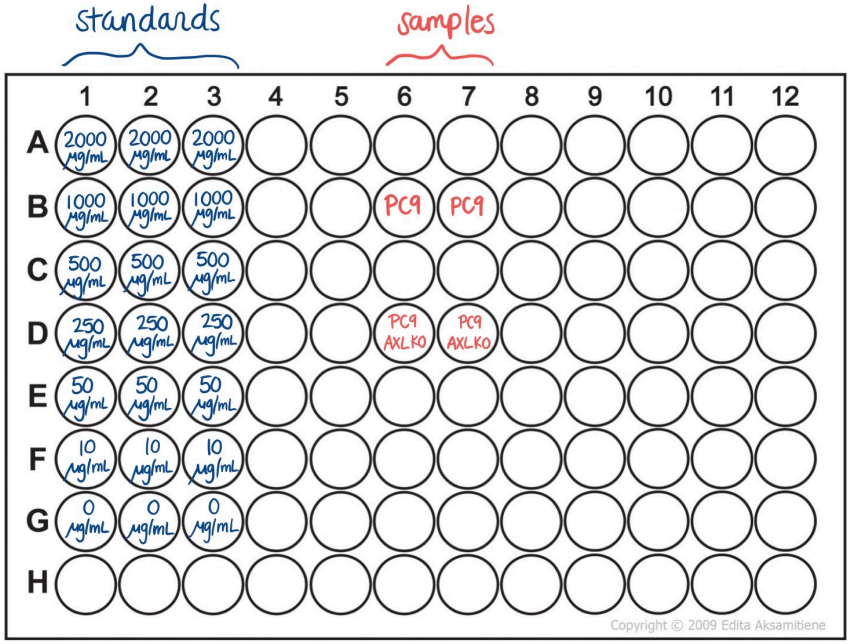
2. Design layout for 96 well plate:

a. Number of standards 7 x Number of repeats 3 = 21 wells

b. Number of samples 2 x Number of repeats 2 = 4 wells

c. Total number of wells = Standard wells 21 + Sample wells = 4 = 25 wells

d. Label the well plate below with standard and sample placement:



Practice Preparing a BCA Assay

3. Prepare reaction buffer:

a. Total number of wells ____ x 200 uL/well = _____ uL reaction buffer

b. Always make a little extra: _____ uL

c. Combine reagents A, B, and C in ratio 25:24:1 to make reaction buffer

i. Reagent A = (total volume of buffer ____ uL)(25/50) = _____ uL

ii. Reagent B = (total volume of buffer ____ uL)(24/50) = _____ uL

iii. Reagent C =(total volume of buffer ____ uL)(1/50) = _____ uL

Practice Preparing a BCA Assay

3. Prepare reaction buffer:

a. Total number of wells 25 x 200 uL/well = 5000 uL reaction buffer

b. Always make a little extra: 5500 uL

c. Combine reagents A, B, and C in ratio 25:24:1 to make reaction buffer

i. Reagent A = (total volume of buffer 5500 uL)(25/50) = 2750 uL

ii. Reagent B = (total volume of buffer 5500 uL)(24/50) = 2640 uL

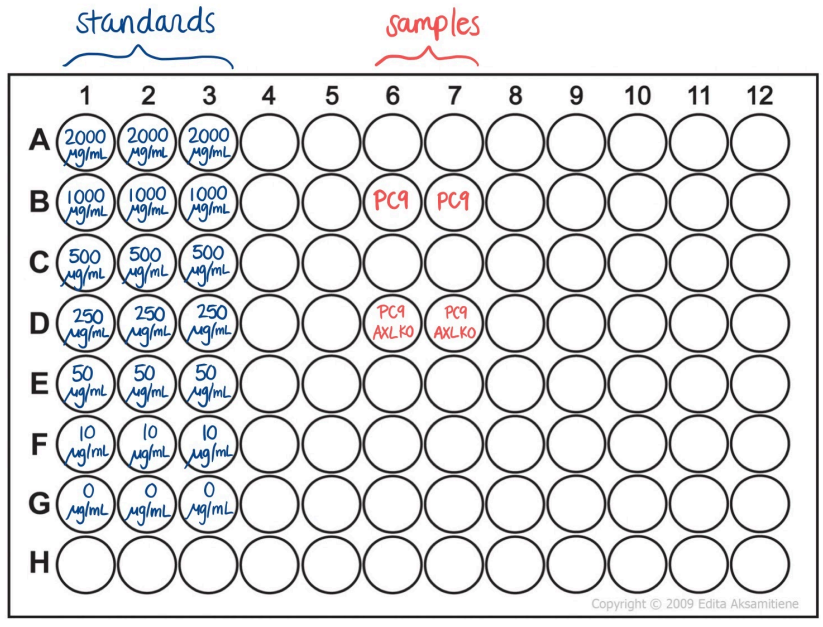
iii. Reagent C =(total volume of buffer 5500 uL)(1/50) = 110 uL

Practice Making a Standard Curve

→ Consider: You run this plate …
and get the below absorbance readings

→ Create a standard curve

→ Use the standard curve to determine the sample concentration of protein



1	2	3	4	5	6	7
A	2.799	2.482	2.343	0.047	0.049	0.048
B	1.644	1.313	1.368	0.048	0.253	0.246
C	0.904	0.806	0.774	0.049	0.047	0.049
D	0.566	0.529	0.452	0.047	0.305	0.26
E	0.206	0.201	0.438	0.048	0.048	0.047
F	0.106	0.119	0.101	0.047	0.048	0.048
G	0.097	0.096	0.096	0.048	0.048	0.048

Practice Making a Standard Curve

Step 1: Determine the average and standard deviation of replicates

1	2	3	4	5	6	7
A	2.799	2.482	2.343	0.047	0.049	0.048
B	1.644	1.313	1.368	0.048	0.253	0.246
C	0.904	0.806	0.774	0.049	0.047	0.049
D	0.566	0.529	0.452	0.047	0.305	0.26
E	0.206	0.201	0.438	0.048	0.048	0.047
F	0.106	0.119	0.101	0.047	0.048	0.048
G	0.097	0.096	0.096	0.048	0.048	0.048

[Protein] (ug/mL)	AVG Absorbance (AU)	STD Absorbance (AU)
2000	2.541333333	0.233718492
1000	1.441666667	0.17737061
500	0.828	0.067734777
250	0.515666667	0.058157831
50	0.281666667	0.135411718
5	0.108666667	0.009291573
0	0.096333333	0.00057735

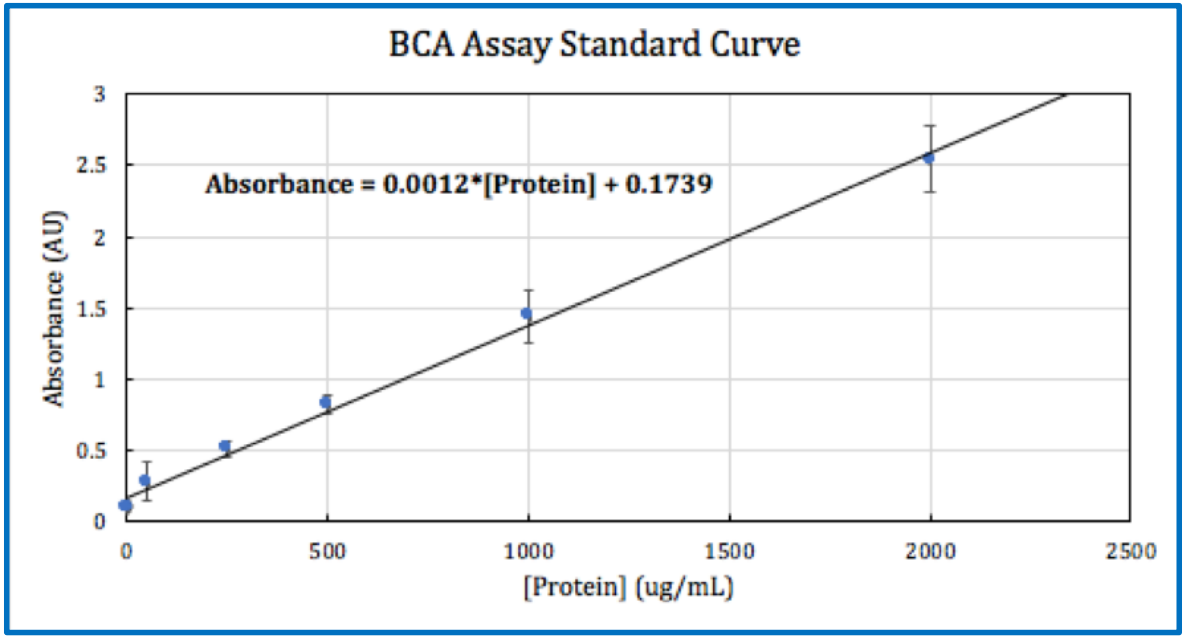
Practice Making a Standard Curve

Step 2: Plot the average and standard deviation of replicates

[Protein] (ug/mL)	AVG Absorbance (AU)	STD Absorbance (AU)
2000	2.541333333	0.233718492
1000	1.441666667	0.17737061
500	0.828	0.067734777
250	0.515666667	0.058157831
50	0.281666667	0.135411718
5	0.108666667	0.009291573
0	0.096333333	0.00057735

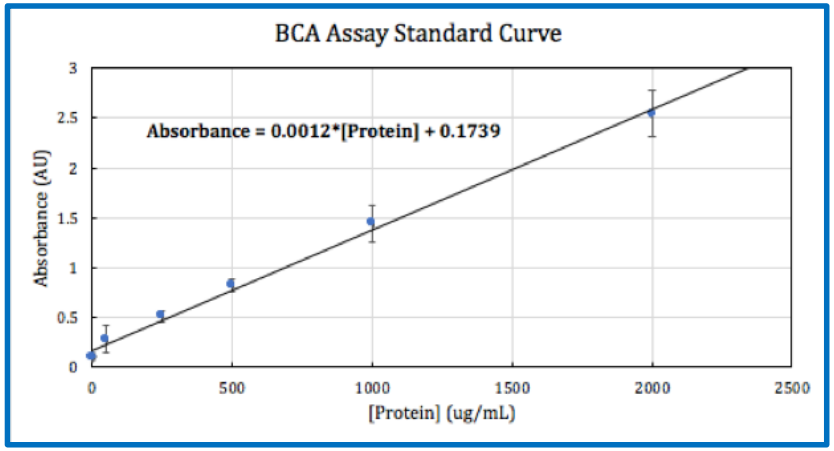
→ Display equation of trendline

→ Note y-intercept



Practice Making a Standard Curve

Step 3: Use standard curve equation and absorbance data to calculate sample protein concentration



1	2	3	4	5	6	7
A	2.799	2.482	2.343	0.047	0.049	0.048
B	1.644	1.313	1.368	0.048	0.253	0.246
C	0.904	0.806	0.774	0.049	0.047	0.049
D	0.566	0.529	0.452	0.047	0.305	0.26
E	0.206	0.201	0.438	0.048	0.048	0.047
F	0.106	0.119	0.101	0.047	0.048	0.048
G	0.097	0.096	0.096	0.048	0.048	0.048

$$Absorbance = 0.0012 * [Protein] + 0.1739 \rightarrow [Protein] = \frac{Absorbance - 0.1739}{0.0012}$$

Sample	Absorbance 1	Absorbance 2
PC9	0.253	0.246
PC9 AXO KO	0.305	0.26

Sample	[Protein] 1	[Protein] 2
PC9	65.91666667	60.08333333
PC9 AXO KO	109.25	71.75

Sample	AVG [Protein] (ug/mL)	STD [Protein] (ug/mL)
PC9	63	4.124789557
PC9 AXO KO	90.5	26.51650429