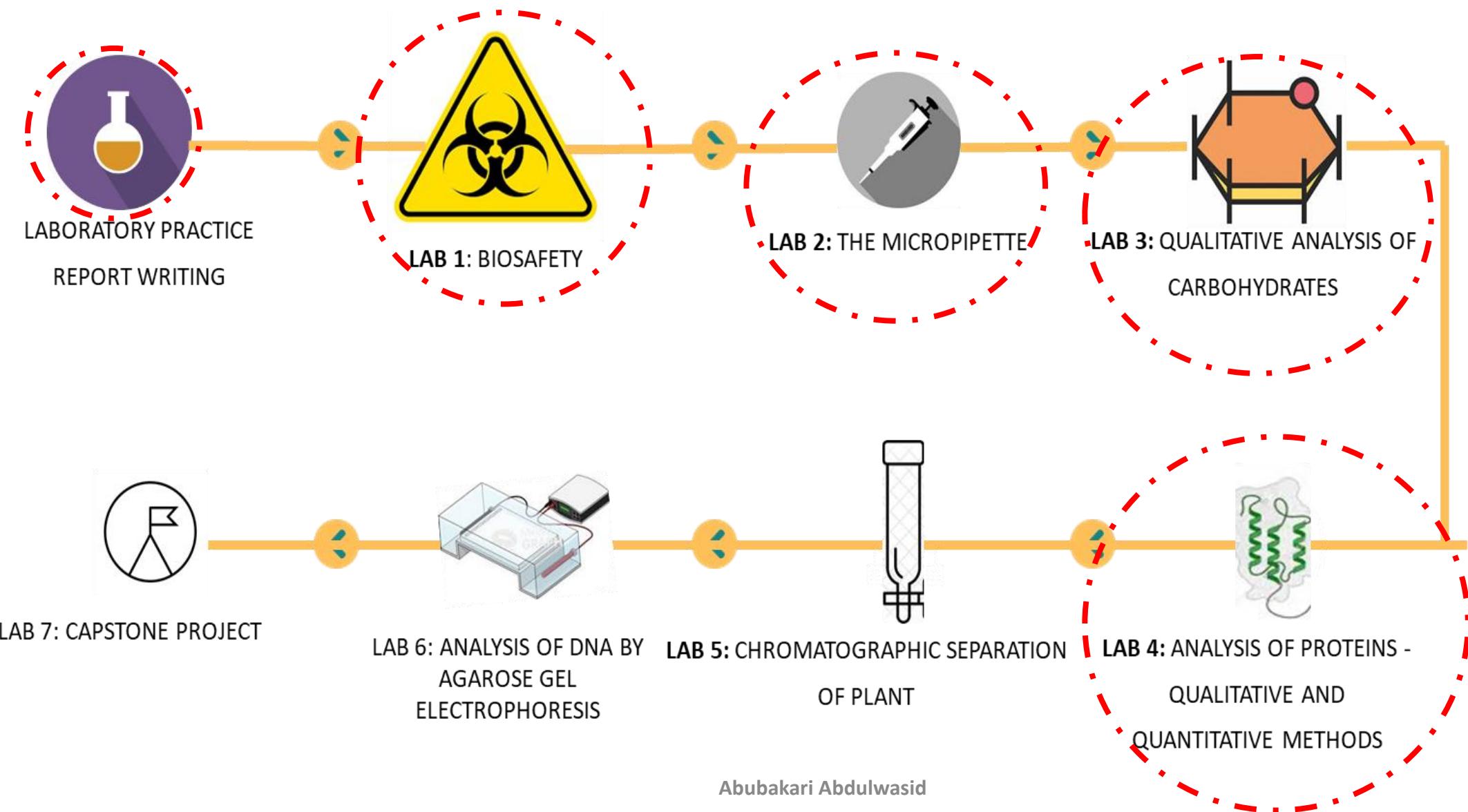


# Lecture 3



# Table of content for the Course

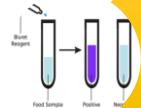
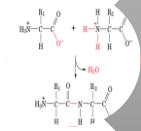


# This week Lecture

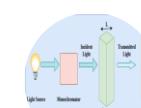


**What are proteins and why are they important?**

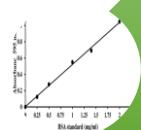
**Describe the chemical principles of the Biuret and Bradford tests**



**Outline the procedure for each assay**



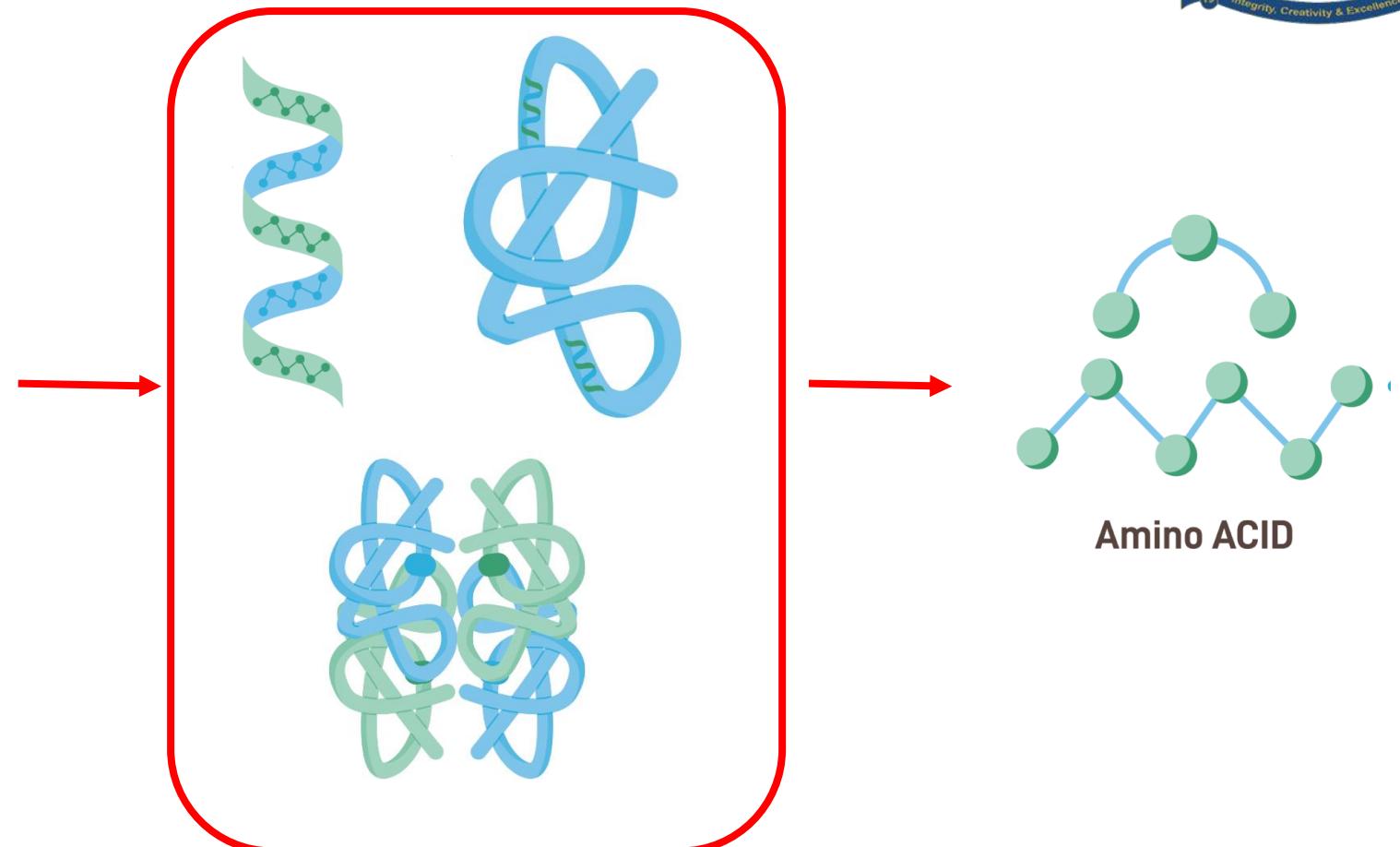
**Correctly interpret qualitative and quantitative results**



**Construct and use a standard curve to determine an unknown protein concentration**



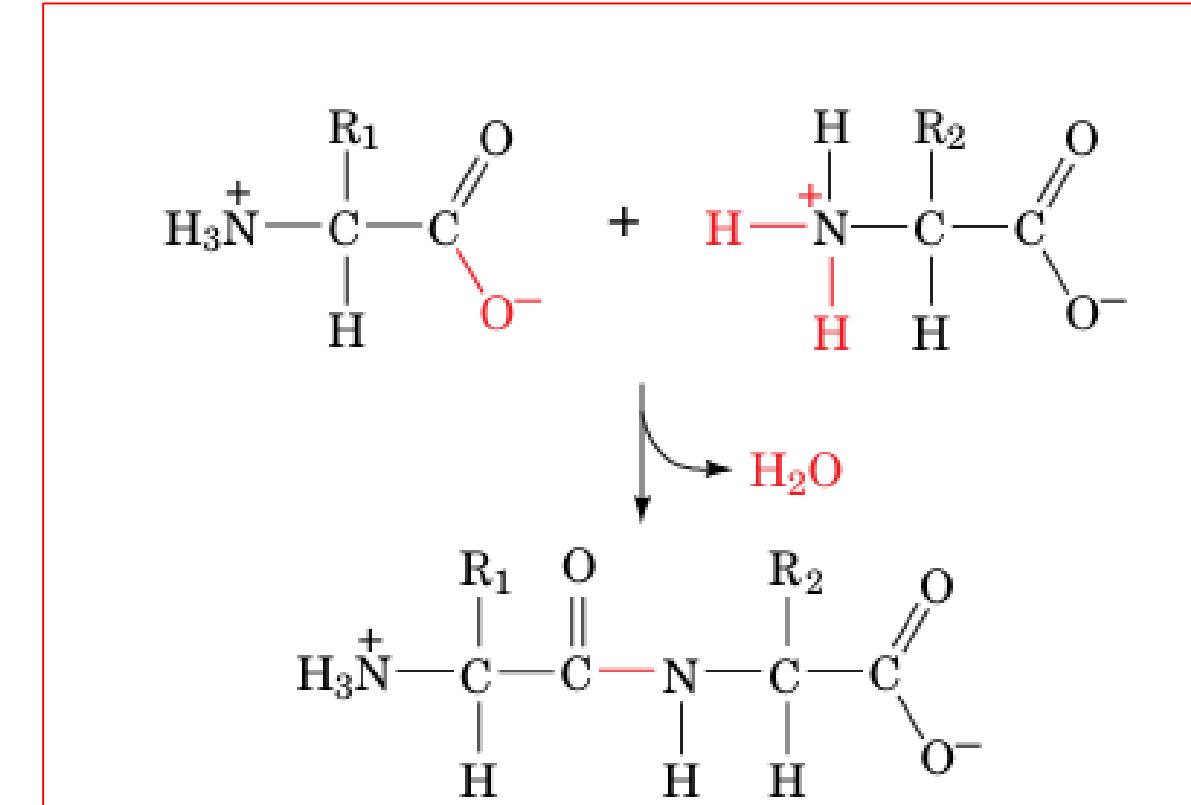
# LAB 4: ANALYSIS OF PROTEINS - QUALITATIVE AND QUANTITATIVE METHODS



# Understanding Proteins

## What are Proteins?

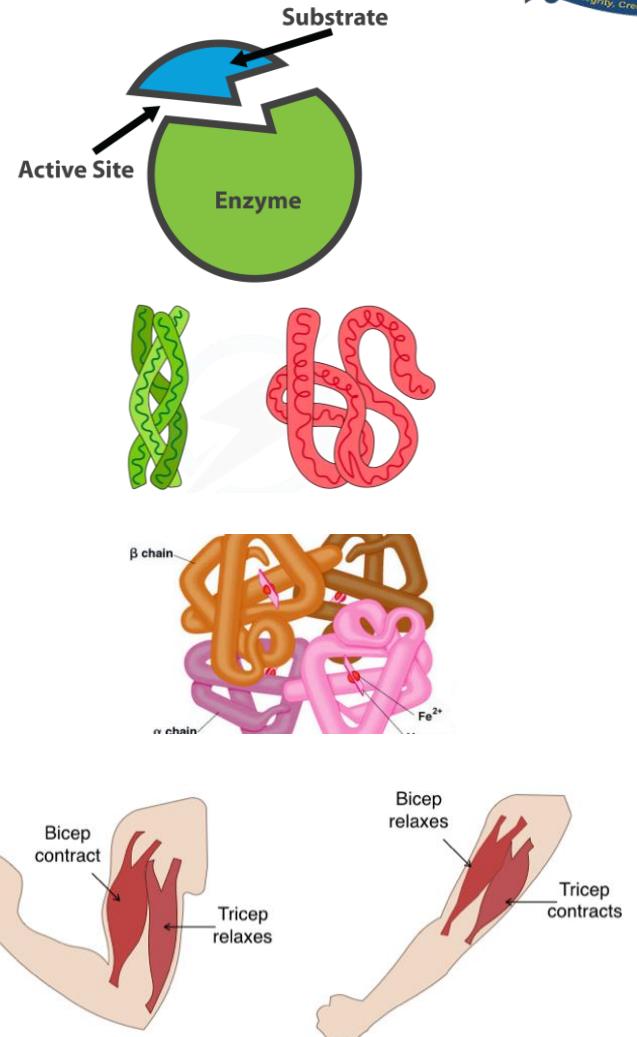
- ❖ The most **abundant** and **functionally diverse** class of organic macromolecules.
- ❖ Polymers of **amino acids** linked by **peptide bonds**.
- ❖ Composed of **Carbon, Hydrogen, Oxygen, and Nitrogen (N)**.
- ❖ Essential for nearly all biological processes.



# Why Proteins are Critical

## Proteins perform countless functions:

- ❖ Enzymes: Catalyze biochemical reactions (e.g., amylase).
- ❖ Structure: Provide support and shape (e.g., collagen, keratin).
- ❖ Transport: Carry substances (e.g., hemoglobin).
- ❖ Movement: Enable muscle contraction (e.g., actin, myosin).
- ❖ Signaling: Transmit signals (e.g., insulin).



# The Four Levels of Protein Structure

- ❖ **Primary Structure:** The linear sequence of amino acids.
- ❖ **Secondary Structure:** Coiling ( $\alpha$ -helix) or folding ( $\beta$ -sheet) of the polypeptide chain.
- ❖ **Tertiary Structure:** The overall 3D shape of a single polypeptide.
- ❖ **Quaternary Structure:** The arrangement of multiple polypeptide subunits. Structure dictates function.

– Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly – Ala –

– Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly – Ala –

– Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly – Ala –

– Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly – Ala –

– Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly – Ala –

– Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly – Ala –

– Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly – Ala –

– Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly – Ala –

– Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly – Ala –

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– Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly – Ala –

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– Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly – Ala –

– Lys – Ala – His – Gly – Lys – Lys – Val – Leu – Gly – Ala –



# The Central Question?

**If given known & unknown samples:**

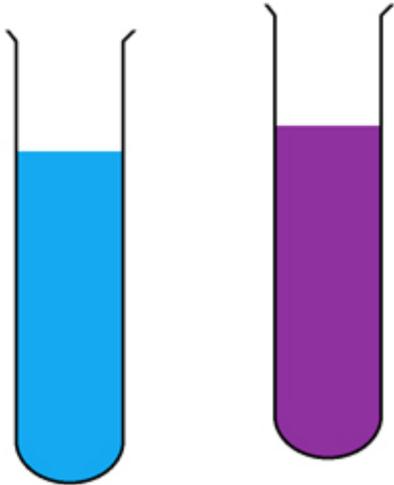
- ❖ **Qualitative:** Is protein present in our sample at all?
- ❖ **Quantitative:** If so, precisely how much protein is there?

# The Toolkit - Our Chemical Assays

## Our Two-Part Investigative Process:

We will analyze our unknown using a logical sequence:

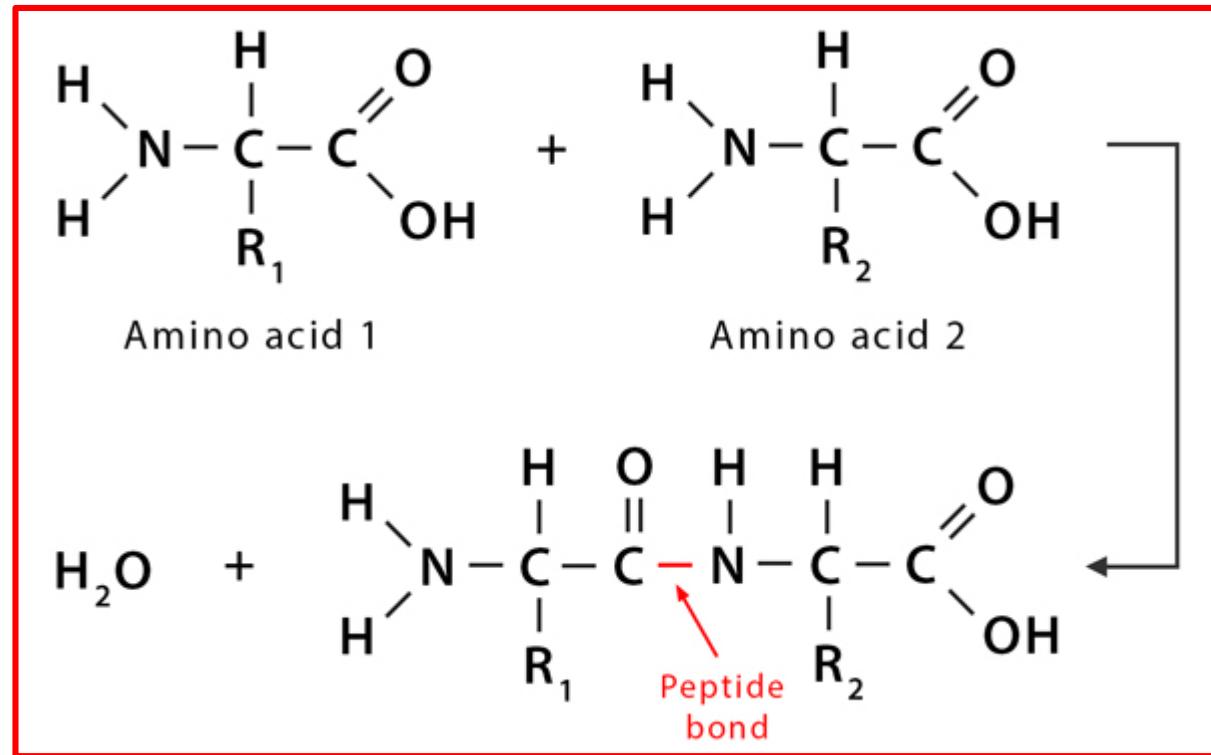
- ❖ **Biuret Test (Qualitative):** Is there protein in my sample? Detects peptide bonds.
- ❖ **Bradford Assay (Quantitative):** How much protein is in my sample? Measures concentration.



# Test 1: Biuret Test - The Qualitative Screen

**Purpose:** To detect the presence of peptide bonds, and therefore, confirm the presence of protein.

- ❖ It is the **first test** you should perform to see if your sample contains protein.
- ❖ Reagent: **Biuret Reagent** (contains copper(II) sulfate in a strong alkaline solution).



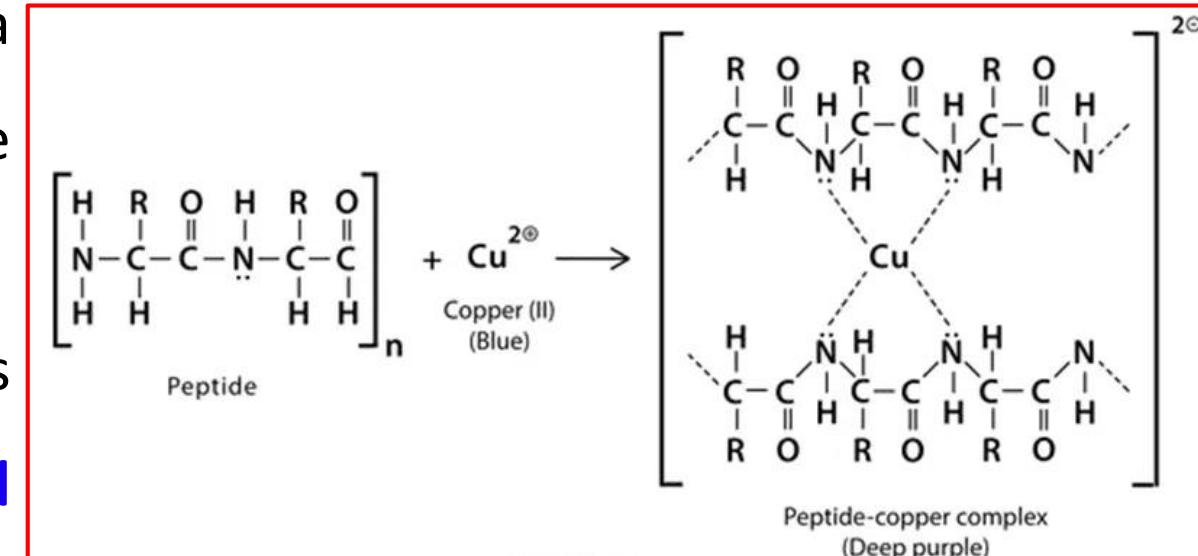
# The Principle of the Biuret Test

## A two-step, base-catalyzed reaction:

❖ **Alkaline Environment:** In the presence of a **strong base (like NaOH)**, the acidic proton on the peptide nitrogen is removed.

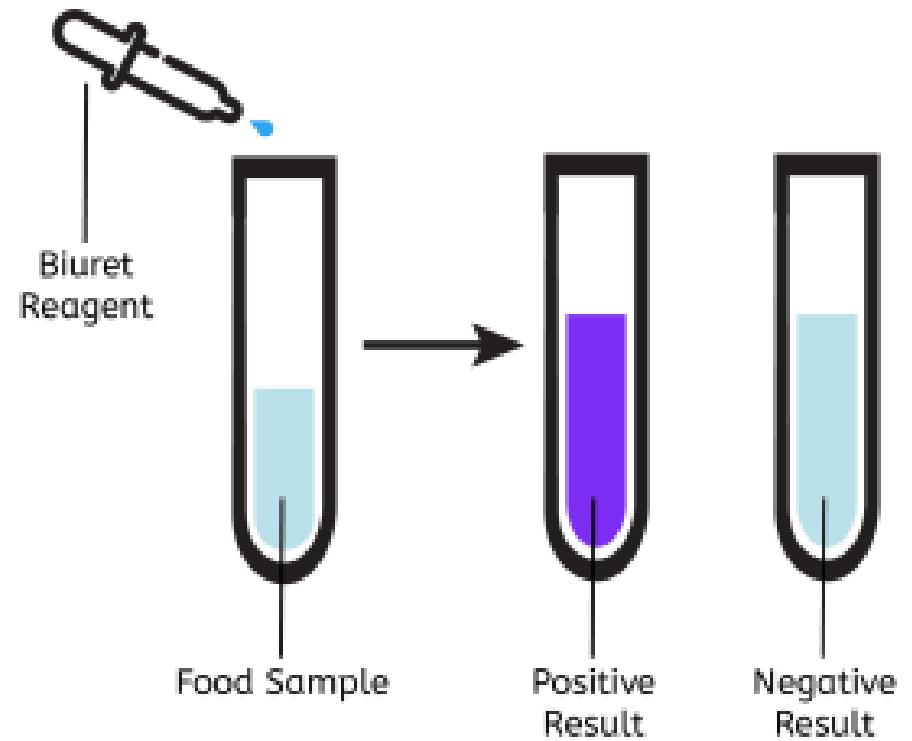
❖ **Coordination:** The nitrogen atoms then act as electron donors, coordinating with a **central copper(II) ion ( $\text{Cu}^{2+}$ )** to form a vivid purple-colored coordination complex.

❖ A minimum of **two peptide bonds** is required for the reaction.



# Procedure for Biuret Test

- ❖ Pipette 1 mL of each sample into its respective labeled test tube.
- ❖ Add 1 mL of Biuret Reagent to every tube.
- ❖ Mix the contents of the tubes thoroughly by swirling.
- ❖ Allow the tubes to stand at room temperature for 5 minutes.
- ❖ Observe the final color. A change from blue to violet/purple indicates the presence of protein.



# Biuret Test: A Positive Result

## What to expect:

- ❖ **Positive Test:** A change from **blue** to **violet/purple** indicates the **presence** of **protein**.
- ❖ **Negative Test:** The solution **remains blue**. This indicates the absence of protein (or the presence of only single amino acids/dipeptides).



# Biuret Test: SAFETY

## EXTREME CAUTION

- ❖ Biuret reagent contains Sodium Hydroxide (NaOH), a strong base that is highly corrosive and can cause severe burns.
- ❖ Always wear your safety goggles and gloves.
- ❖ Handle with extreme care. Report any spills immediately.

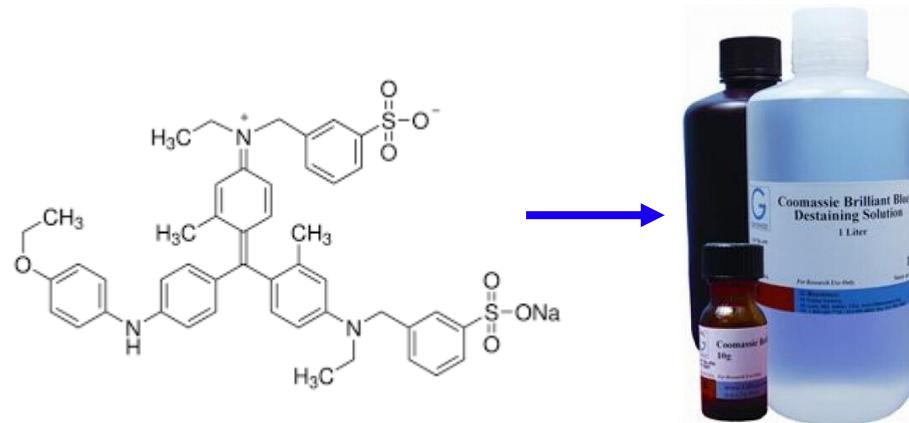


# Test 2: Bradford Assay - The Quantitative Method



Purpose: To quantify the concentration of protein in a sample?

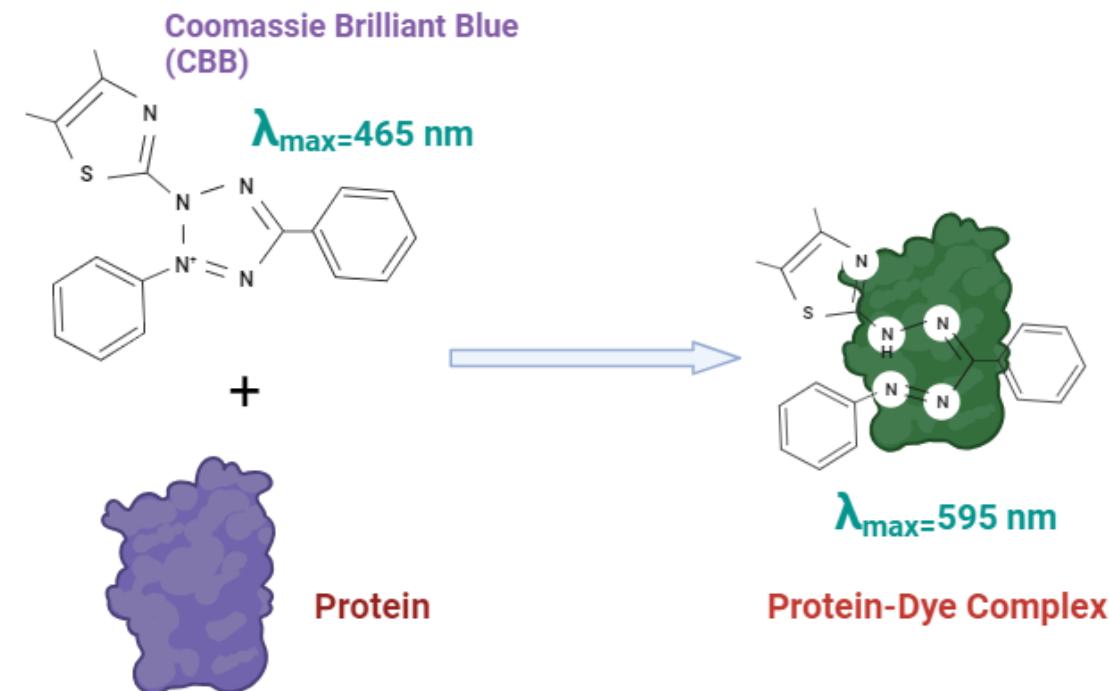
- ❖ This is a highly sensitive colorimetric assay.
- ❖ It relies on the binding of a specific dye, Coomassie Brilliant Blue G-250, to protein molecules.



# The Principle of the Bradford Assay

## The Dye: Coomassie Brilliant Blue G-250:

- ❖ In the **acidic Bradford reagent**, the dye exists in a reddish-brown, cationic state (absorbance max at 465 nm).
- ❖ When the dye **binds to protein** (primarily basic amino acid residues like Arginine), it converts to a **stable, blue, anionic state**.
- ❖ This blue form has a new **absorbance maximum at 595 nm**.



# The Bradford Dye Shift

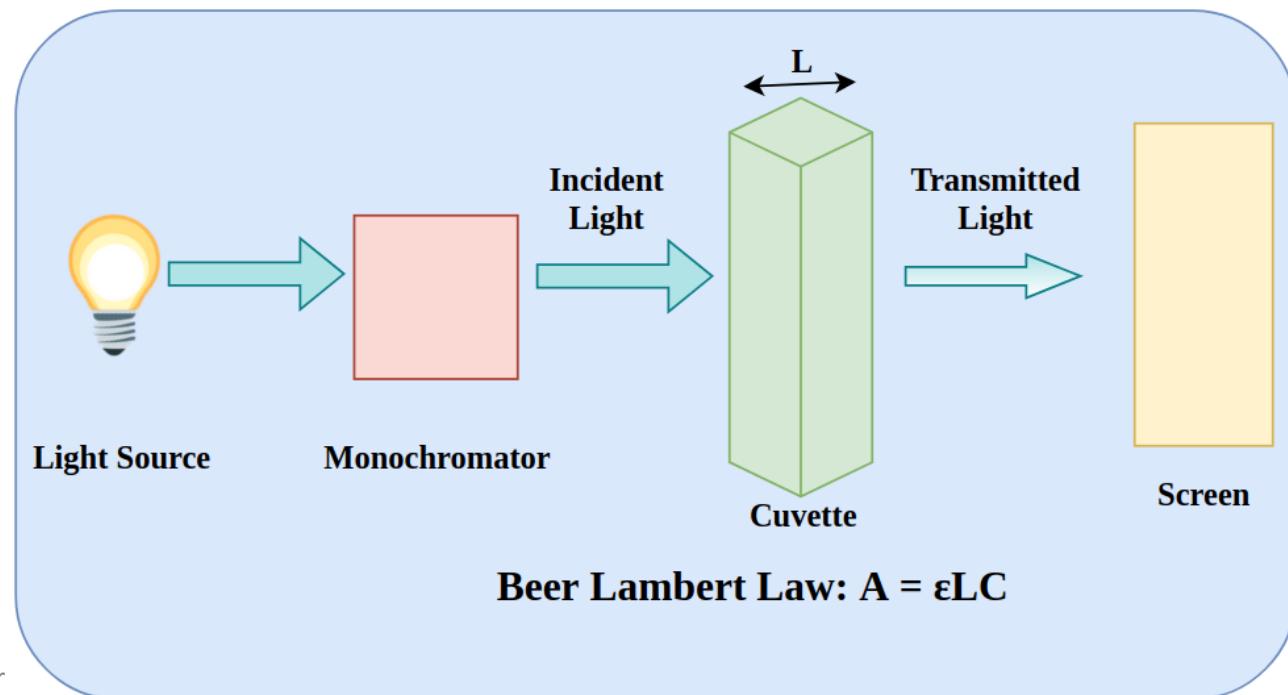


- ❖  $[\text{Dye-H}]^+$  (Cationic, Red-Brown,  $\lambda_{\max}=465\text{nm}$ ) + Protein  $\rightleftharpoons$   $[\text{Dye-Protein}]^-$  (Anionic, Blue,  $\lambda_{\max}=595\text{nm}$ ) +  $\text{H}^+$
- ❖ The intensity of the blue color, measured at 595 nm, is directly **proportional** to the protein concentration.

# Measuring Color: Spectrophotometry



- ❖ We use a **spectrophotometer** to measure the intensity of the blue color.
- ❖ It measures **Absorbance (A)** at the wavelength where the blue dye absorbs most **strongly: 595 nm**.
- ❖ According to the **Beer-Lambert Law (A = εbc)**, absorbance is directly proportional to concentration.



# The Challenge with Beer's Law

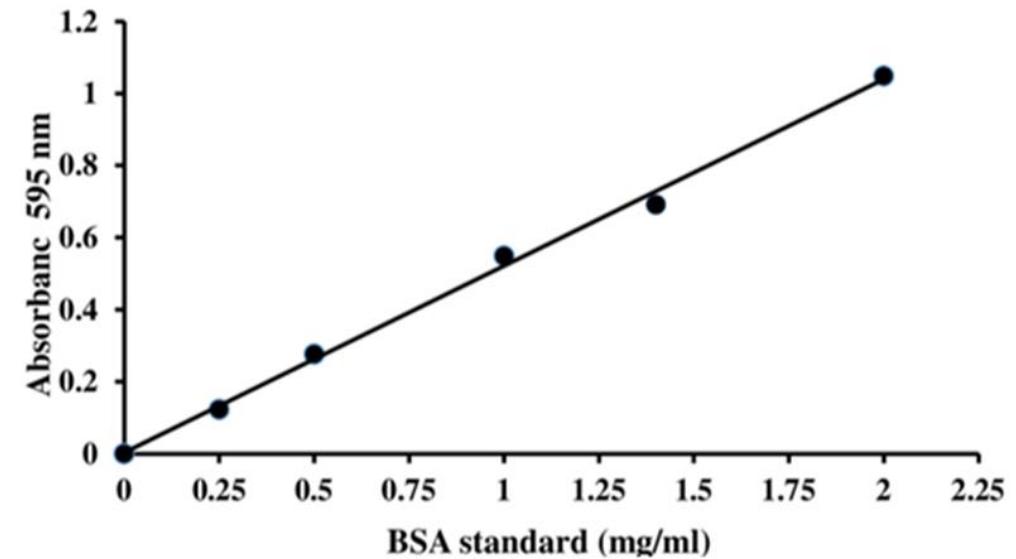
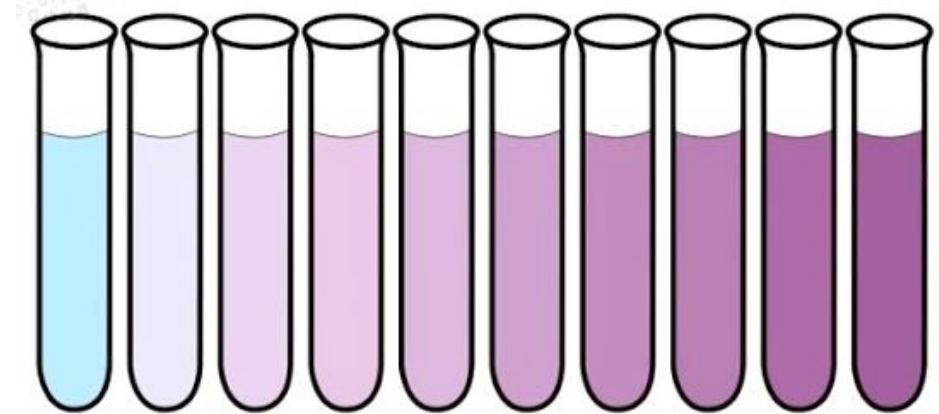
Why can't we just use the formula  $A = \epsilon bc$  directly?

- ❖ The **extinction coefficient ( $\epsilon$ )** for the protein-dye complex is:
- ❖ **Unknown** for our specific protein.
- ❖ **Variable** from one protein to another.
- ❖ Therefore, we cannot **directly convert Absorbance to Concentration.**

# The Solution: The Standard Curve

**The Standard Curve is our calibration tool.**

- ❖ It's a graph that relates a **known property (concentration)** to a measured property **(absorbance)**.
- ❖ We create it by measuring the **absorbance for a series of solutions** with **known protein concentrations**.
- ❖ This creates **a "ruler"** we can use to measure our unknown.



# Step 1: Building the Standard Curve (Preparation)

## Prepare Protein Standards (Serial Dilution).

- ❖ Label a set of test tubes 1-6.
- ❖ Prepare them according to the table in your manual to create a range of known concentrations from a stock solution (e.g., 100 µg/mL BSA).
- ❖ Tube 1 (0 µg/mL) will be your Blank.

Tube #	Vol. of Stock BSA (e.g., 100 µg/mL)	Vol. of dH <sub>2</sub> O	Final Protein Conc. (µg/mL)
1	0 µL (Blank)	100 µL	0
2	20 µL	80 µL	20
3	40 µL	60 µL	40
4	60 µL	40 µL	60
5	80 µL	20 µL	80
6	100 µL	0 µL	100

# Step 2: Building the Standard Curve (Assay)

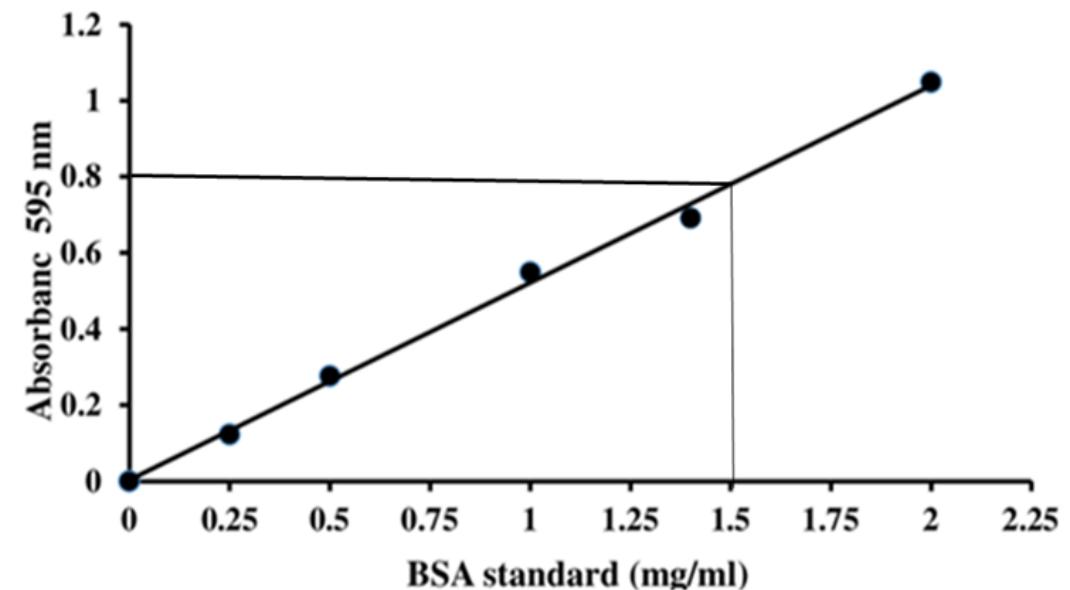
- ❖ Add 2.0 mL of Bradford Reagent to all standards and your unknown.
- ❖ Mix and incubate for 5 minutes.
- ❖ Set the spectrophotometer to 595 nm.
- ❖ Use the Blank (Tube 1) to zero the instrument.
- ❖ Measure and record the absorbance for all other tubes.



# Building the Standard Curve (Plotting)

Plot your data:

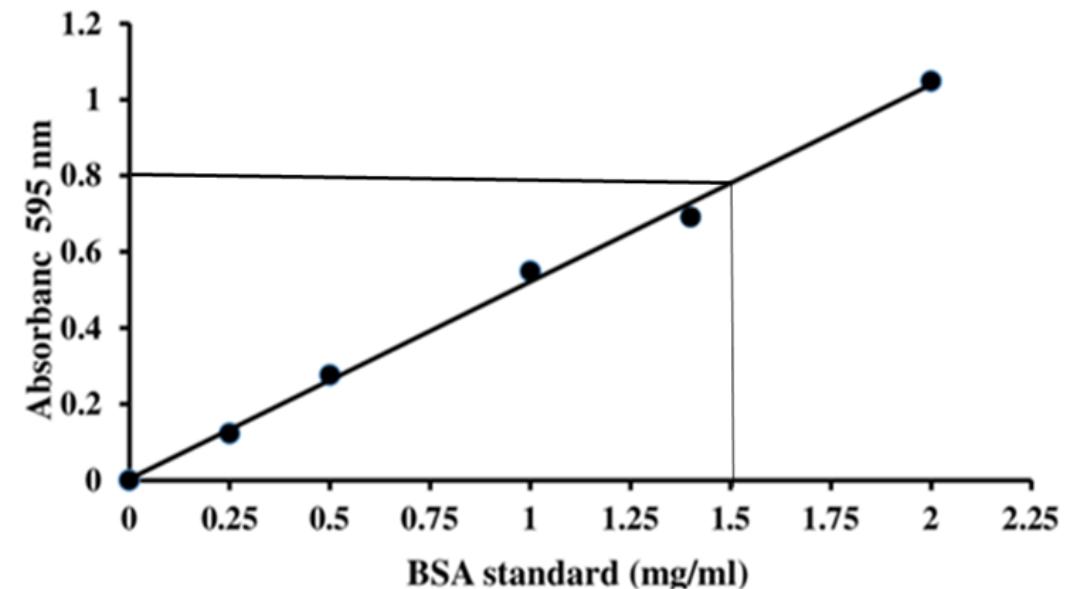
- ❖ **Y-Axis:** Absorbance at 595 nm
- ❖ **X-Axis:** Known Protein Concentration ( $\mu\text{g/mL}$ )
- ❖ **Draw a line of best fit through your data points.**
- ❖ The **line should be straight**. Check the  **$R^2$  value** (a measure of linearity). A value  $> 0.990$  is excellent.



# Using Your Standard Curve to Find the Unknown



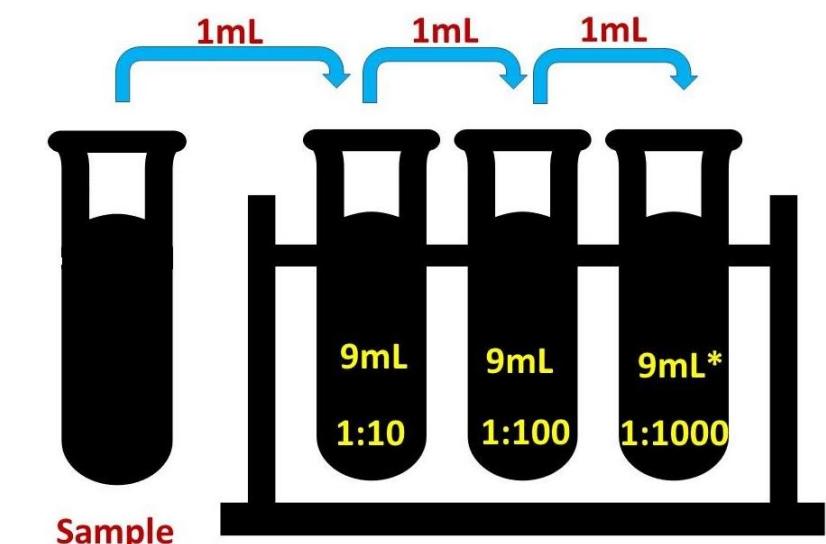
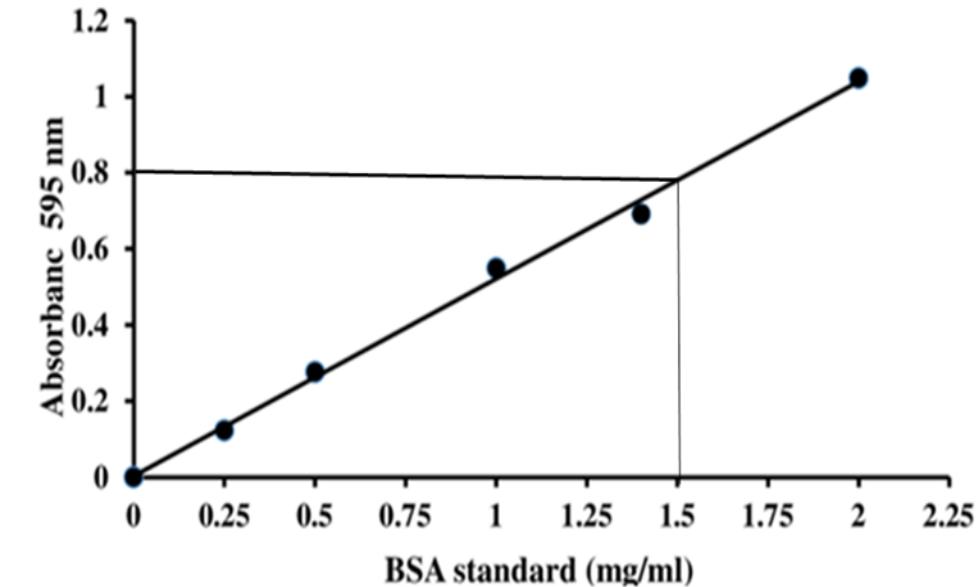
- ❖ Measure the **absorbance** of your Unknown sample.
- ❖ Find this value on the **y-axis**.
- ❖ Trace a horizontal line over to your line of best fit.
- ❖ Drop a vertical line down to **the x-axis**.
- ❖ The **value on the x-axis** is the **concentration of your unknown!**



# An Important Note: Working within the Range

What if my unknown's absorbance is higher than my highest standard?

- ❖ The **result is unreliable**. The standard curve is only accurate within its **measured range**.
- ❖ **Solution:** You must perform **a precise dilution of your unknown sample** (e.g., 1:10, 1:100) and **rerun the assay**.
- ❖ **Crucial:** Remember to **multiply your final calculated concentration by the dilution factor!**  
(e.g.,  $5 \mu\text{g/mL} \times 10 = 50 \mu\text{g/mL}$ ).



# Bradford Assay: SAFETY

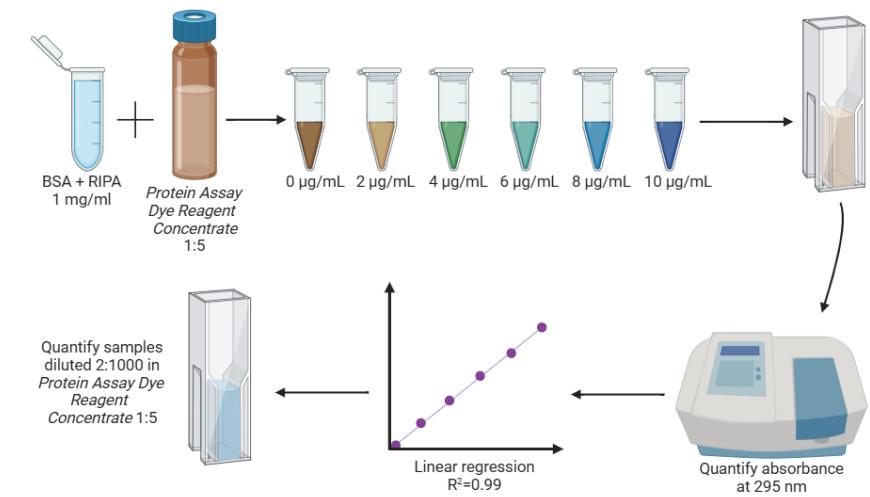
## EXTREME CAUTION.

- ❖ Bradford Reagent contains phosphoric acid, which is corrosive and can cause burns.
- ❖ Always wear your safety goggles and gloves.
- ❖ Report any spills to your instructor immediately.



# Lab Workflow: A Summary

- ❖ Start with the **Unknown Sample**.
- ❖ Perform **Biuret Test**.
- ❖ If **Negative (Blue)**: Stop. Conclude "No protein detected."
- ❖ If **Positive (Purple)**: Proceed to the next step.
- ❖ Perform **Bradford Assay** (including standards).
- ❖ Plot **Standard Curve and calculate R<sup>2</sup>**.
- ❖ Determine **Unknown Concentration** from the curve.
- ❖ Conclude with the **final concentration value**.

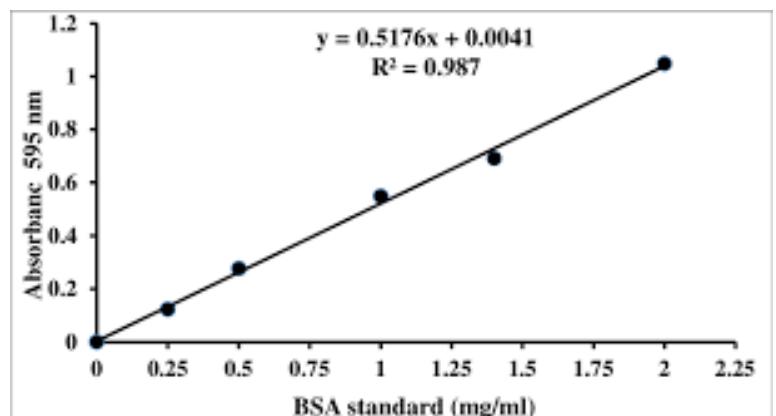


# Thinking Like a Scientist

## Scenario A

Your unknown gives the following results:

- ❖ **Biuret Test:** Positive (purple color)
- ❖ **Bradford Assay:** Absorbance = 0.450
- ❖ From **Standard Curve:** Concentration = 55  $\mu\text{g/mL}$
- ❖ **Conclusion:** The unknown is a protein solution with a concentration of 55  $\mu\text{g/mL}$ .

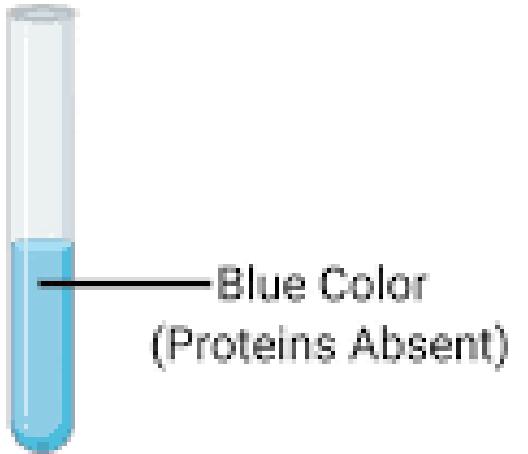


# Thinking Like a Scientist

## Scenario B

Your unknown gives the following results:

- ❖ **Biuret Test:** Negative (remains blue)
- ❖ **Bradford Assay:** Not performed (no protein detected)
- ❖ **Conclusion:** The unknown sample does not contain protein.

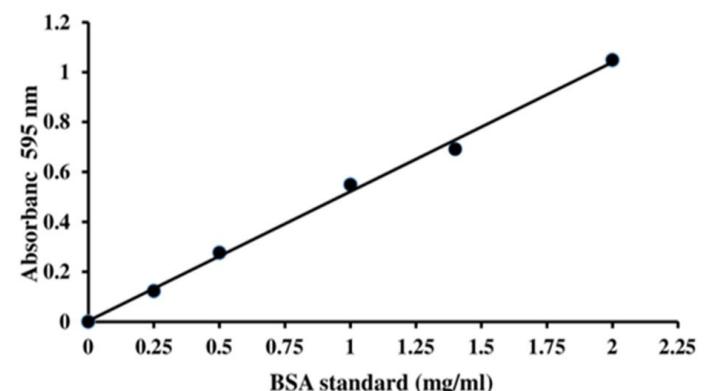


# Thinking Like a Scientist

## Scenario C

Your unknown gives the following results:

- ❖ **Biuret Test:** Positive (purple)
- ❖ **Bradford Assay:** Absorbance is too high (O.D. > 2.0), outside the range of your standards.
- ❖ **Conclusion:** The unknown is a highly concentrated protein. You must perform a precise dilution (e.g., 1:10) and re-run the assay. Remember to multiply your final result by the dilution factor!



# Application: The "High Protein" Shake

- ❖ **From your Study Questions:** A company claims its new fitness shake contains "50 grams of protein per serving". The serving size is 500 mL.
- ❖ **Required concentration:**  $50 \text{ g} / 500 \text{ mL} = 100 \text{ g/L} = 100 \text{ mg/mL}$
- ❖ How would you test this claim using only our two tests?



# Case Study Analysis

## How to Test the Claim:

- ❖ **Biuret Test:** Should be strongly positive.
- ❖ **Bradford Assay:** The target concentration (100 mg/mL) is far too high for our standard curve (which is in  $\mu\text{g/mL}$ ).
- ❖ **Action:** A significant, multi-step dilution is required.
- ❖ **Example:**
  - ❖ Dilute 1:100 (to get 1 mg/mL)
  - ❖ Dilute that another 1:10 (to get 100  $\mu\text{g/mL}$ , which is on our curve).
  - ❖ Total Dilution Factor =  $100 * 10 = 1000$ .
  - ❖ Measure the diluted sample and multiply the result by 1000.

# A Note on Interference

Could other substances cause a false positive?

- ❖ **Biuret Test:** Unlikely. Very specific for the peptide bond complex.
- ❖ **Bradford Test:** Possible. Strong detergents can interfere with dye-binding. The protein's own amino acid composition can also affect accuracy (e.g., a low number of arginine residues will lead to an underestimation).
- ❖ Understanding the limitations of a test is key to good science.

# Housekeeping & Waste Disposal

- ❖ **Biuret Waste:** Contains copper and strong base. Dispose of in the "Corrosive/Heavy Metal" waste container.
- ❖ **Bradford Waste:** Contains acid. Dispose of in the "Aqueous Acidic Waste" container.
- ❖ Rinse all glassware thoroughly when you are finished.

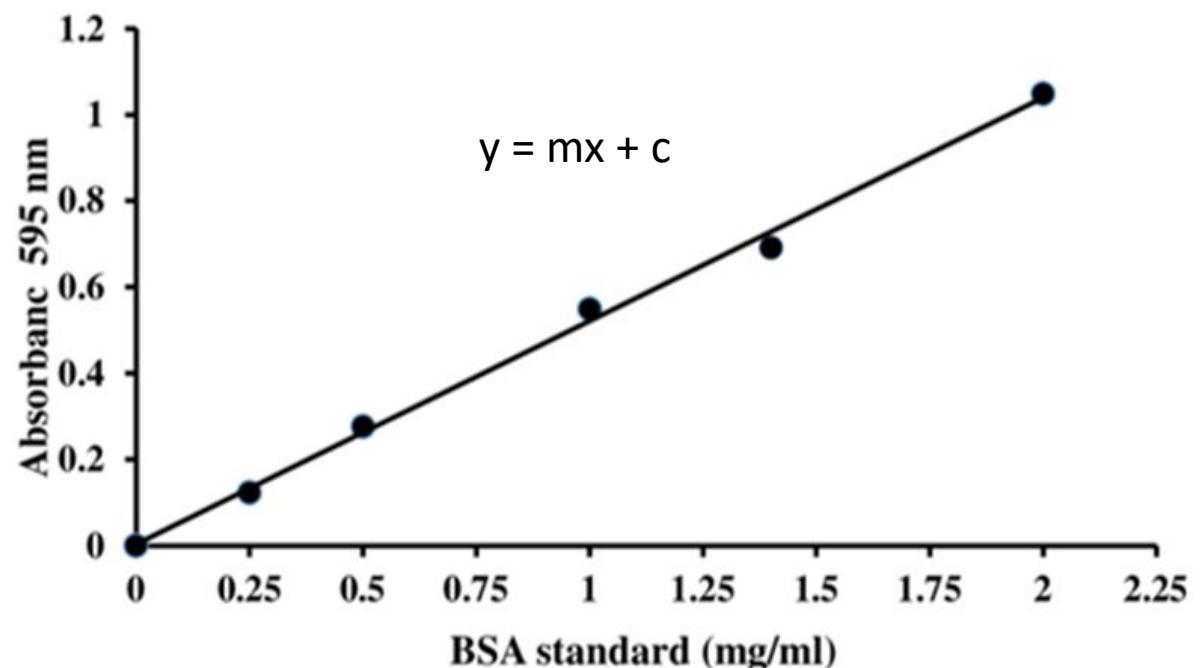


# Key Takeaways

- ❖ Proteins are the functional molecules of life.
- ❖ The **Biuret Test** qualitatively answers "**Is it there?**"
- ❖ The **Bradford Assay** quantitatively answers "**How much is there?**"
- ❖ A **Standard Curve** is essential for converting absorbance to concentration when  $\epsilon$  is unknown.
- ❖ Safety and proper waste disposal are non-negotiable.

# Practice Question

You are a quality control analyst testing a purified enzyme solution. You perform a Bradford Assay and generate the standard curve shown below.



You have two samples to analyze:

**Sample A** gives an Absorbance at 595 nm of **0.60**.

**Sample B** gives an Absorbance at 595 nm of **1.40**.

- a) Using the **standard curve**, what is the concentration of protein in **Sample A**? (Be sure to include the correct units).
- b) The Bradford Assay relies on a color change when the dye binds to protein. What color is the dye reagent before it binds to protein, and what color does it become after binding? What property of the protein is primarily responsible for this binding?
- c) Your colleague suggests confirming the presence of protein with the Biuret test. Explain why this test would be positive for your enzyme solution but **negative** for a solution containing only free Arginine, even though Arginine is the primary amino acid the Bradford dye binds to.



# Questions?

Abubakari Abdulwasid