Announcements

- Mid-term feedback and comportment grades will be up on website by next week
- Chapter 9 Laboratory Report Template will be posted by Wednesday (3/26)
- Chapter 9 Raw Data file and Laboratory Report due April 3 – 8 (>1 week; ~2 weeks from when you finish the lab)

Chapter 9 Calculations: Comparing two methods of protein labeling

- Figure 1: BCA standard curve
* • Table 1: Protein Concentration (μM) for both **DTNB** and **F5M** reactions
- Figure 2: Effect of **DTNB** on KHK rates.
* • Table 2: Effect of **DTNB** on KHK specific activity
- Figure 3: Effect on Coupled assay of [PK].
- Figure 4: Plot of all **DTNB** reactions.
* • Table 3: Calculate μM Cys from **DTNB** data.
- Figure 5: **F5M** standard curve
* • Table 4: Calculate μM Cys from **F5M** data
* • Table 5: Calculate the number of Cys per KHK molecule by these two different labeling methods
- Table 6: Show calculation of error propagation

Error Propagation in Chapter 9

Purpose:

- To account for uncertainty in measured data which will carry over into subsequent steps and assays
- Often denoted as $X \pm x$ where,
  - X = data measurements
  - x = error values
  - $(x/X)100$ = (fractional error) percent error

You will need to take error propagation into account for ALL steps in:

Chapter 9A

- Measuring the effect of DTNB on KHK activity (Tbl2)
- Measuring the amount of DTNB that reacted with Cys residues in KHK (Tbl3)

Chapter 9BE

- Measuring RFUs vs. µM (or moles) of F5M in standard curve (Fig5)
- Measuring µM (or moles) of F5M labeled on KHK (Tbl4)
- Measuring µM (or moles) of KHK in DTNB & F5M reactions from BCA assay (Tbl1)
- Measuring A_{562} vs. µM (or moles) BSA in BCA assay in standard curve (Fig1)
- Calculate number of Cys by two methods (Tbl 5&6)

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Overview of Chapter 9 Experiment

Table 3
Table 4
Table 5

Determination of number of Cys residues in KHK

DTNB:

$$\frac{\frac{A_{412}\ \text{X}\ 0.00025\ \text{L}}{(\text{“14,000”}\ \text{M}^{-1}\text{cm}^{-1})(\text{”x cm”})}}{\frac{\text{mg of KHK protein in the 250 µL reaction x 0.001 g/mg}}{\text{MW of KHK subunit “34,000” g/mole}}} = \frac{\text{number of moles Cys in KHK in the 250 µL reaction (D and F)}}{\text{moles KHK}} = \frac{\text{moles Cys}}{\text{moles KHK}}$$

Can be simplified if use µM as the reaction volumes cancel out

F5M:

Need to account for the various dilutions you subjected your samples to, e.g., 10x dilution

$$\frac{\text{Concentration of F5M labeled to KHK X}\sim 0.000035\ \text{L}}{\frac{\text{mg of KHK protein in the eluted ~35 µL reaction x 0.001 g/mg}}{\text{MW of KHK subunit “34,000” g/mole}}} = \frac{\text{number of moles Cys in KHK in the 35 µL reaction (A and B)}}{\text{moles KHK}} = \frac{\text{moles Cys}}{\text{moles KHK}}$$

- Compare the two numbers and determine if they agree with each other or not
- Are the values statistically different? – *Important to do error propagation!*

Table 6

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Error Propagation formulas

- Follow these formulas for propagating data measurements with error

Addition: $(X \pm x) + (Y \pm y) = (X + Y) \pm \sqrt{x^2 + y^2}$

Subtraction: $(X \pm x) - (Y \pm y) = (X - Y) \pm \sqrt{x^2 + y^2}$

Multiplication: $(X \pm x) \times (Y \pm y) = (X \times Y) \pm (X \times Y)\sqrt{\left(\frac{x}{X}\right)^2 + \left(\frac{y}{Y}\right)^2}$

Division: $(X \pm x) \div (Y \pm y) = (X \div Y) \pm (X \div Y)\sqrt{\left(\frac{x}{X}\right)^2 + \left(\frac{y}{Y}\right)^2}$

Calculated result

Calculated error

Make your own error propagation calculator using excel!

Error Propagation Calculator

Values below are entered as:
(A ± a) <function> (B ± b) = (C ± c)

A		a
10.00000	±	0.01000

B		b
5.00000	±	0.05000

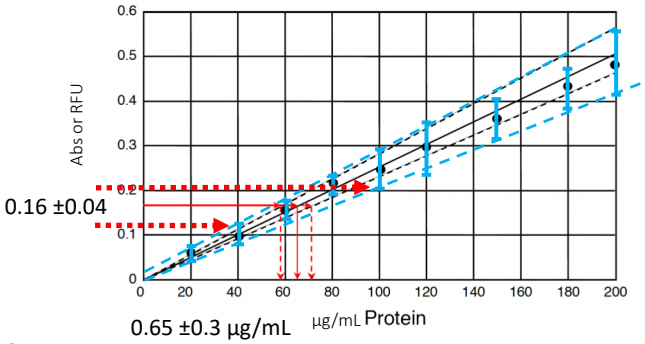
	C		c
Addition	15.00000	±	0.0509902
Subtraction	5.00000	±	0.0509902
Multiplication	50.00000	±	0.5024938
Division	2.00000	±	0.0200998

Error Propagation from standard curves that are not absolutely linear: BCA & F5M

Fig. 1
Table 1
Fig. 5
Table 4

Method 1:

- Repeat assay on several identical aliquots of your proteins. Use average and standard deviation of measured values from standard curve. *(This is what you will be doing in 9C!)*



Method 2:

- ❖ Use LINEST function in Excel based on your standard curve

https://www.youtube.com/watch?v=BvQ11wtKeCg

Error Propagation from BCA assay to determine protein concentration

To calculate moles of KHK in your DTNB and F5M reactions:

- Find KHK concentration of DTNB assay / F5M labeling reaction by making use of the BSA standard curve you created and measured triplicate absorbance values. Take the average and standard deviation in your raw data workbook.* Use to get concentration in the 25 µL of sample you measured in µg/mL.
- Since we have the same volumes from the assay as in the standard curve, and they are directly from the reaction without dilution, we can convert µg/mL to µM using the molecular weight of KHK (**34,000 g/mol**; the error in MW is so low (<0.01%), it can be ignored)

  ➢ Example: [(275 ± 25 µg/mL)(10^{-6} g/µg)(10^{+3}mL/L)] ÷ [**34000 g/mol**] = 8.1 ± 0.7 x10^{-6} mol/L x 10^{+6} µmol/mol = 8.1 ± 0.7 µM

  ➢ [The easy calculation is µg/mL ÷ **34** = µM]

* STDEV uses the following formula:

$\sqrt{\frac{\sum(x-\bar{x})^2}{(n-1)}}$ where x is the sample mean AVERAGE(number1,number2,...) and n is the sample size.

How to convert DTNB signal to moles of Cysteine residues?

Fig. 1
Table 1
Fig. 5
Table 4

To calculate μM –SH groups from DTNB modification:

- Calculate the average **net** absorbance by subtracting the background
  - Propagate the error by using subtraction equation

Subtraction: $(X \pm x) - (Y \pm y) = (X - Y) \pm \sqrt{x^2 + y^2}$

- Use **Beer's Law -> $A = \varepsilon lc$**
  - End-point average net absorbance = extinction coefficient x path length x concentration
  - Extinction coefficient @ 412nm for DTNB = 14,000 $M^{-1}cm^{-1}$ **(or 0.014 $\mu M^{-1}cm^{-1}$)**
  - Path length of 250 μL sample in 96-well plate is 1 cm
  - Solve for concentration **in μM** by taking the highest absorbance reading of your sample after 1 h run time, or the last from your plot

- Calculate Error in your determination
  - Propagating the error by using the same fractional error (since $\varepsilon \& l$ have negligible errors, OR
  - Repeat entire calculation of 3 trials and calculate the standard deviation

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How to convert F5M RFU signal to moles of Cysteine residues?

Fig. 5
Table 4

To calculate μM –SH groups from F5M reactions:

- Use **standard curve** which relates concentration of F5M with RFU
  - Calculate **net** RFU and error for each sample
  - Propagate the error by using subtraction equation

Subtraction: $(X \pm x) - (Y \pm y) = (X - Y) \pm \sqrt{x^2 + y^2}$

  - Use graph to find concentration and error in μM from your sample **net** average RFU values and standard deviations

Chapter 10

LIPIDS AND MEMBRANES

Chapter 10: Overview

- **Week 1 – 10A:**
  - Isolate and wash erythrocytes and plasma membrane fractions (washing & centrifugation)
- **Week 2 – 10BC:**
  - Determine yield of membrane, lipids, and composition of lipids (TLC & cholesterol assay)
- **Week 3 – 10D:**
  - Separation of proteins by SDS-PAGE and immunoblotting
- **Week 4 – 10E:**
  - Visualization of SDS-PAGE and immunoblot results (*i.e.* Western Blot analysis)

Chapter 10A: Lipids and Membranes

Objectives

- Understand lipid membrane composition, structure, and function
- To isolate red blood cells from bovine (*Bos taurus*) blood
- Prepare washed and unwashed membrane fractions

Procedures

- **Isolate erythrocytes** from fresh bovine blood by **centrifugation**
- **Lyse erythrocytes** via **osmotic shock using low-salt buffer**
- To fractionate membrane preparations containing peripheral bound proteins away from integral membrane proteins
- **Lyophilize** (freeze dry) membrane preparations for next week's lipid extraction

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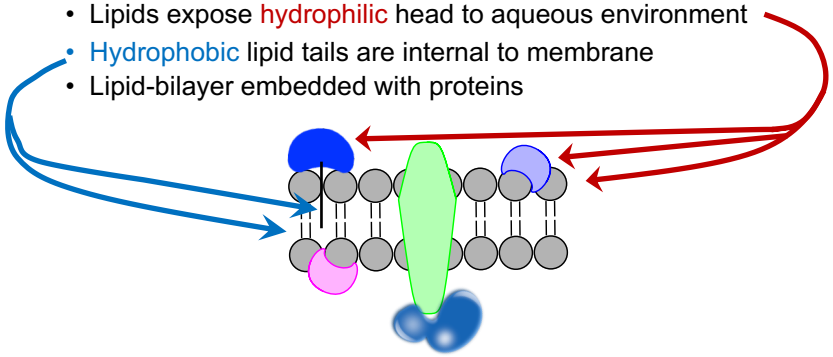
Plasma Membrane

- Physical barrier between cell and external environment
- Separates organelles and subcellular structures
- Composed of lipids, proteins, and carbohydrates
- Exists in a ***fluid-mosaic model***
  - Lipids expose hydrophilic head to aqueous environment
  - Hydrophobic lipid tails are internal to membrane
  - Lipid-bilayer embedded with proteins



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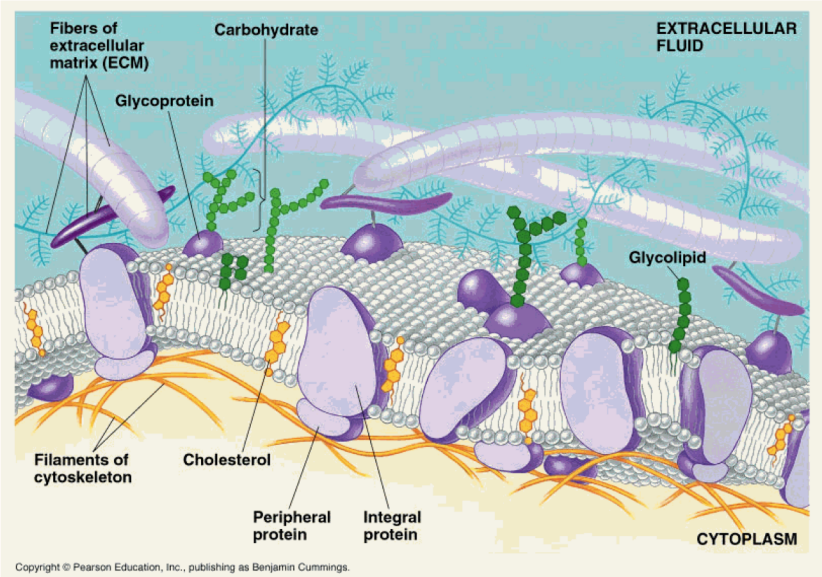
Plasma Membrane



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Erythrocyte Membranes

- **Red Blood Cells – Erythrocytes**
- Human Erythrocyte Membrane:
  - 50% protein
  - 40% lipid
  - 10% carbohydrate

- Isolated as “ghosts” –remains after removing hemoglobin and intracellular components
- Isolated from whole blood extract through ***cell lysis and centrifugation***
- Do not contain other organelles – easier sample preparation

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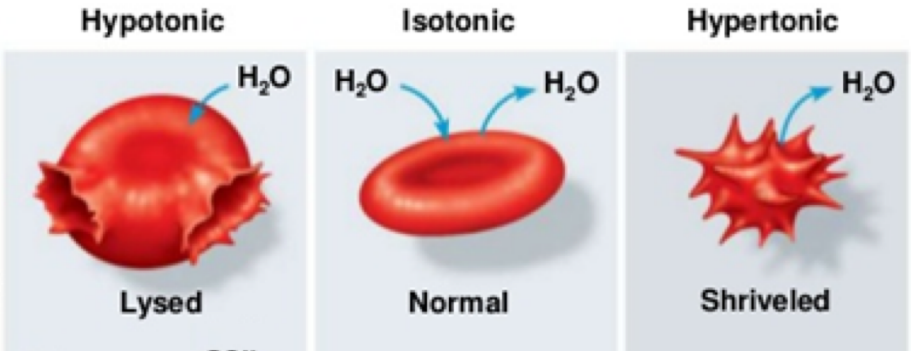
Isolation of Membranes

- **Red Blood Cells ISOLATED from blood with low-speed centrifugation using Phosphate Buffered Saline (PBS)**
  - Platelets and lymphocytes (white blood cells) float to top, RBCs to bottom
- **RBCs are LYSED using Hypotonic Phosphate Buffered Saline (HPBS)**
  - Low osmotic pressure in HPBS buffer – cells swell and rupture due to hypotonic solution
  - Only plasma membrane remains, because no organelles in RBCs

How does HPBS rupture erythrocytes?

Osmolysis

If EXTRACELLULAR ENVIRONMENT is:

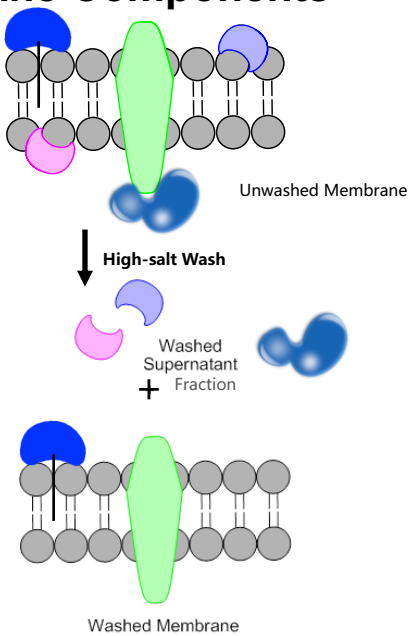


Isolation of Membrane Components

- Membrane components can be dissolved and separated by various solvents:
  - **Lipids** – organic solvent mixtures
  - **Carbohydrates** – typically found associated with proteins
  - **Proteins**
    - ***Integral*** – treating with detergents
    - ***Peripheral*** – washing with a high-salt buffer

Isolation of Membrane Components

- **3 Fractions:**
- Unwashed Membrane
  - Peripheral and Integral
- Washed Membrane
  - Integral
- Washed Supernatant Fraction
  - Peripheral



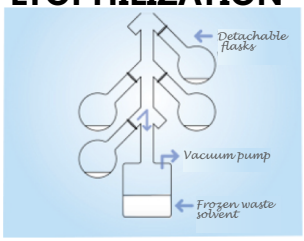
Isolation of Membrane Components

- **3 Fractions:**
- Unwashed Membrane
  - Peripheral and Integral
- Washed Membrane
  - Integral
- Washed Supernatant Fraction
  - Peripheral

– **Lyophilization**
- Freeze drying of membrane preparations
- Removes water by sublimation
- Leaves all solids

– **Solids will be used to extract lipids with organic solvents**

LYOPHILIZATION



Freeze dried raspberries

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Part I: Isolating erythrocytes

Obtain bovine blood

60 mL blood + 20 mL PBS
Split into 2 conical tubes

Centrifuge for 10 min (1000 x *g*)

Discard supernatant fraction (serum)

Keep pellet (= RBCs)

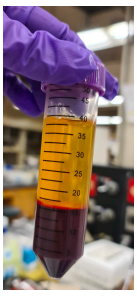
Resuspend in 20 mL PBS for each tube

Centrifuge for 10 min (1000 x *g*)

Discard supernatant fraction

Keep pellet (RBCs) for next step

Keep everything on ice!



- Use swinging-bucket centrifuge rotors for 50 ml conical tubes
- **Make sure to balance tubes!**
- **Make sure to use ONLY PBS – NOT HPBS!!!** *(Remember why??)*
- **Some steps may be done in advance by TFs**

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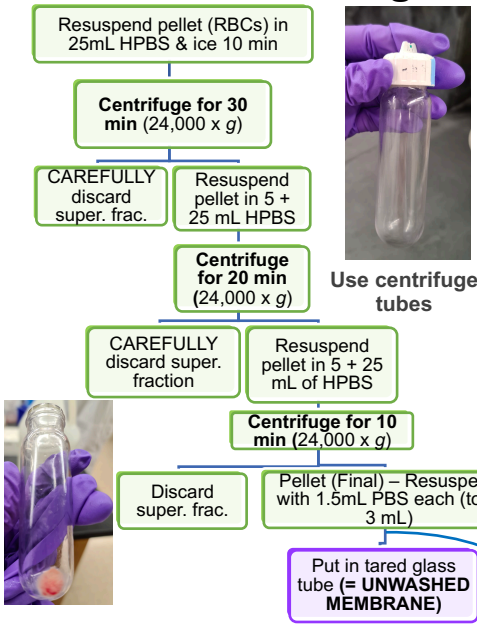
Part II: Isolating the plasma membrane



- HPBS addition with gentle mixing to lyse cells
- Do not shake vigorously!
- **Balance centrifuge tubes!**
- Resuspend pellet with 5 ml HPBS & vortex, then add 25 ml more
- When resuspending membrane pellets, start with 0.75 mL of solution using a cut P1000 pipet tip
- Make sure you have empty weight of glass tube + label + cap on ***analytical balance!***
  - Will need to measure change in mass of tube next week
- Final fraction is **UNWASHED MEMBRANE**

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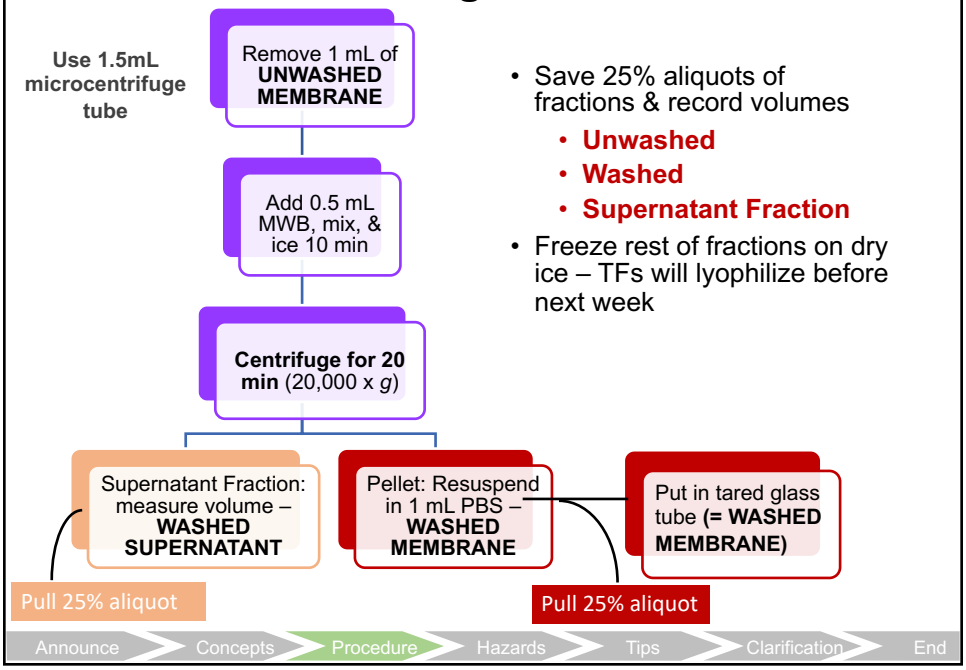
Part III: Washing the membrane



- Save 25% aliquots of fractions & record volumes
  - **Unwashed**
  - **Washed**
  - **Supernatant Fraction**
- Freeze rest of fractions on dry ice – TFs will lyophilize before next week

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CH 10A waste disposal

- Blood waste can go into a **separated** nearby collection carboy in the fume hood
- All other reagents are safe to dispose into the main steam waste carboy

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Chapter 10a Lab Tips

Work efficiently between centrifugation steps! (there are 1 h 40 min of spin time)

Reminders for accurate taring

1. Pre-tape and write **ALL** information on tubes ***and*** caps ***before*** taring
2. Stick to one analytical balance for the remainder of Ch 10
3. Do not shake or move benches when weighing out tubes
4. Wait 5-10 seconds after shutting glass screen to record mass

At the end of lab you will turn in the following:

In tared labeled glass tubes (+caps) --

1. 75% unwashed membrane
2. 75% washed membrane

In 1.5 mL eppendorf tubes --

3. 25% aliquot of unwashed membrane
4. 25% aliquot of washed membrane
5. 75% aliquot of washed supernatant fraction
6. 25% aliquot of washed supernatant fraction

Don't forget to ALWAYS record your measurements of mass and volume when directed to do so in procedures

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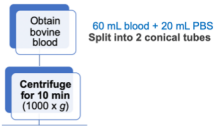
Chapter 10A clarification

- First step involves preparing 2 tubes of 30 mL bovine blood + 10 mL PBS. Subsequent volume mentioned in the manual refers to each of the tubes



- Combine the two tubes of 1.5 mL each of the final pellet → UNWASHED membrane fraction

- For the step involving removal of 1.0 mL of unwashed membrane preparation to 1.5 mL microcentrifuge tube, use the P1000 pipets. It should fit.
- Remember to balance centrifuge tubes for ALL centrifugation steps

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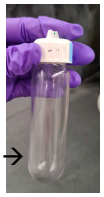
Chapter 10A

Before the lab period, you should have:

- ✓ Completed your Pre-lab Write-up and submit on Gradescope
  - ✓ Title, purpose and procedures
  - ✓ Remember to include:
    - ✓ Flowchart for your membrane isolation procedure
    - ✓ Note masses to record
      - ✓ Masses for **tared** Pyrex tubes & caps for 75% washed & 75% unwashed membrane
    - ✓ Note **volumes** for all six fractions
      - ✓ (25% & 75%) x (Unwashed membrane, washed membrane, and washed supernatant fraction)

At the end of lab, you should have:

- ✓ Turned in your **six** aliquots to your TFs
  - ✓ Unwashed and washed membrane glass tubes, "parafilmed"
  - ✓ Four eppendorf tubes and glass tube caps in a conical tube
- ✓ Washed your high-speed centrifuge tubes → Clean →
  (do not throw them away in the trash)



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Questions?

Activity & Discussion Quiz