Announcements

- Chapter 9 report due next week!
- Exam next Tuesday
- Ch 10 report template released by end of next week

Chapter 10BC: Lipids and Membranes

Objectives

From your RBC membrane preps you will determine:

- total membrane yield (gravimetric)
- total amount of lipids (gravimetric)
- total cholesterol (standard curve)
- total amount of protein (standard curve)
- types of phospholipids present (thin layer chromatography)

} QUANTITATIVE

} QUALITATIVE

Chapter 10BC: Lipids and Membranes

Objectives

Note that you need to do appropriate back-calculations and account for theoretical masses, that is, "what if you had not washed any portion of the membrane?" for your unwashed fraction and "what if you had processed all of your membrane for washing?" for your supernatant and washed fractions. Think carefully when doing your post-lab write up!

From your RBC membrane preps you will determine:

Weight Data

Fractions	Weight of fractions*	Weight of lipid**	Weight of protein***	Weight of cholesterol****
Unwashed				
Washed				
SF				

* weight of lyophilized preps (week 2) – weight of tared tube (week 1)
** weight of dried extracted lipids (week 2) – weight of tared tube (week 2)
*** conc. of protein in aliquots from standard curve (week 2) x volume of each fraction (week 1)
**** conc. of cholesterol from standard curve x volume of lipids resuspended in chloroform (week 2)

Types of phospholipids present (thin layer chromatography)

Chapter 10BC: Lipids and Membranes

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Fractions	Weight of fractions*	Weight of lipid**	Weight of protein***	Weight of cholesterol****
Unwashed				
Washed				
SF				

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*** conc. of protein in aliquots from standard curve (week 2) x volume of each fraction (week 1)
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From your RBC membrane preps you will determine:

- total membrane yield (gravimetric)
- total amount of lipids (gravimetric)
- total cholesterol (standard curve)
- total amount of protein (standard curve)

QUANTITATIVE

- types of phospholipids present (thin layer chromatography)

QUALITATIVE

Membrane Composition

Component	Unwashed Membrane* (%)	Washed Membrane* (%)
Lipid**		
Cholesterol***		
Protein****		
Peripheral*****		
Integral******		

*Percentage by weight
**weight of total lipid/weight of total membrane fraction
***weight of cholesterol/weight of the total lipid fraction
****weight of the protein/ weight of total membrane fraction
*****weight of the protein in supernatant fraction/weight of total protein. (can be compared to protein in unwashed – washed)
****** Weight of protein in washed membrane/weight of total protein

Overview of procedures for 10BC

LYOPHILIZED:

In tared glass tubes –

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

- **Measure the mass** after lyophilization
- **Lipid extraction**
- **Measure mass of dried lipids**, then **resuspend in chloroform**
- Spot 10 µL on **TLC** plate
- Use remaining in **cholesterol assay**

FROZEN @ -20°C:

In 1.5 mL eppendorf tubes –

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

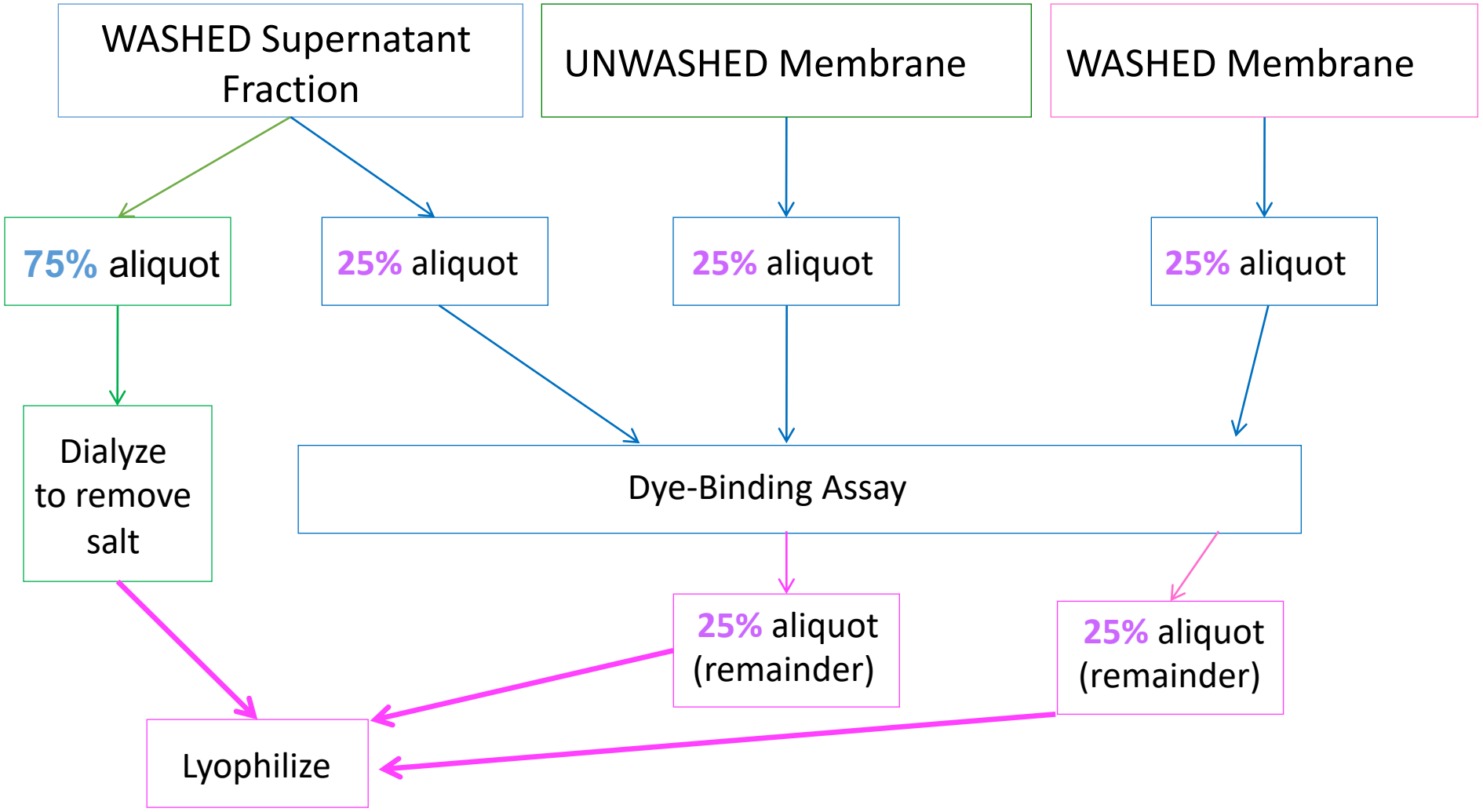
- **Dialyze** over the 4-hr lab period to remove salt

- **Dye-binding assay**

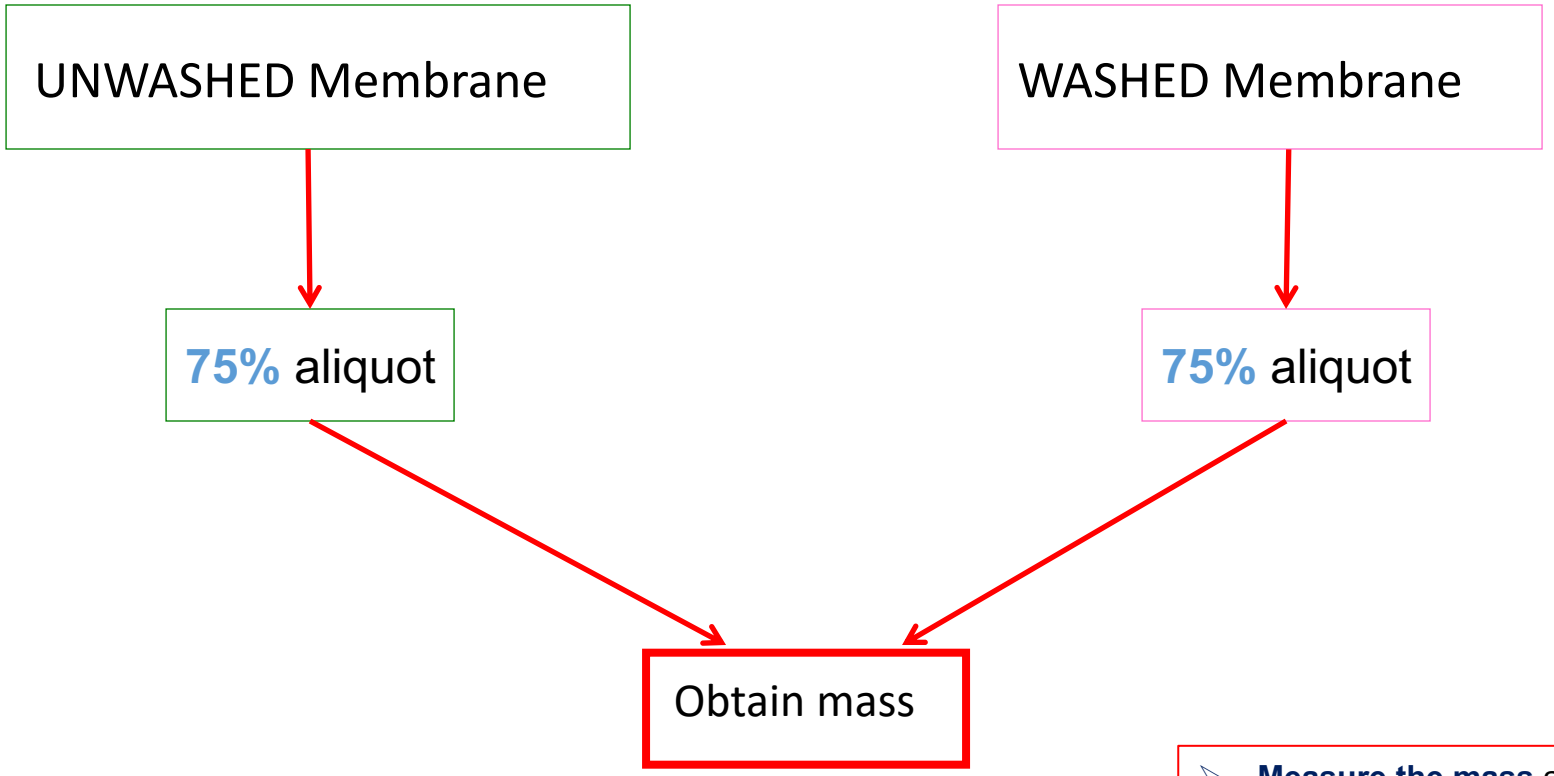
LYOPHILIZE:

- **Measure volume** remaining after dye binding assays of your three 25%-aliquots
- Freeze remaining for lyophilization for next week

Part B. Prep Frozen Aliquots for Analysis



Part C. Characterization of Lyophilized Aliquots



Calculate Total membrane yield

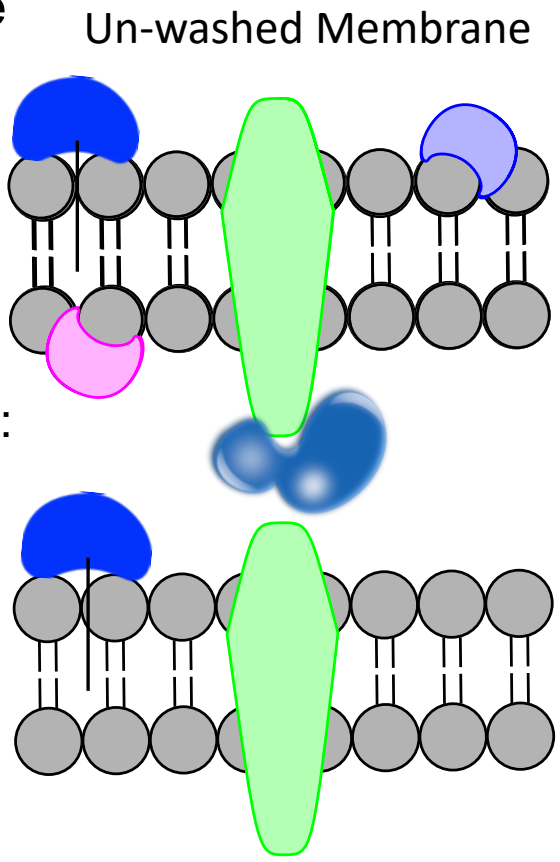
- **Measure the mass** after lyophilization
- **Lipid extraction**
- **Measure mass of dried lipids**, then **resuspend in chloroform**
- Spot 10 µL on **TLC** plate
- Use remaining in **cholesterol assay**

Extraction of Membrane Lipids

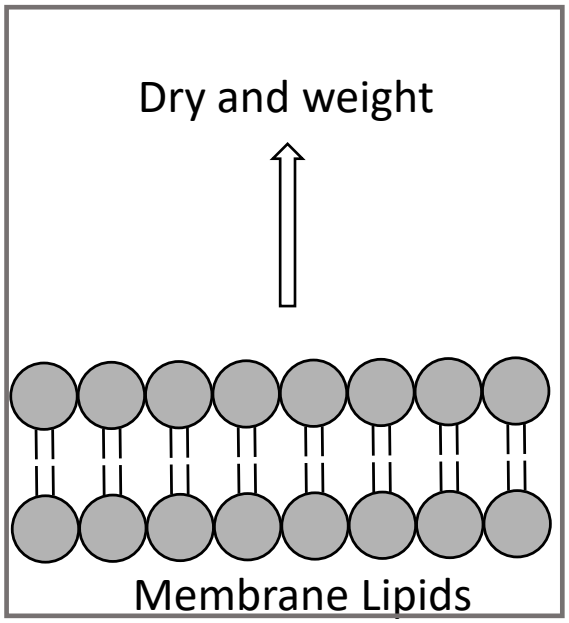
- Can be performed with chloroform:methanol mixture
- Disrupts hydrophobic interactions and denatures membrane proteins
- Separates membrane lipids from other components in the membrane

Major components of membrane lipids:

- **Phospholipids**
- **Cholesterol**



Methanol/Chloroform Extraction



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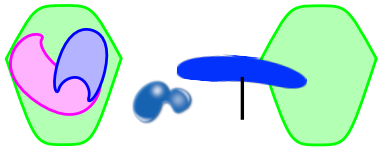
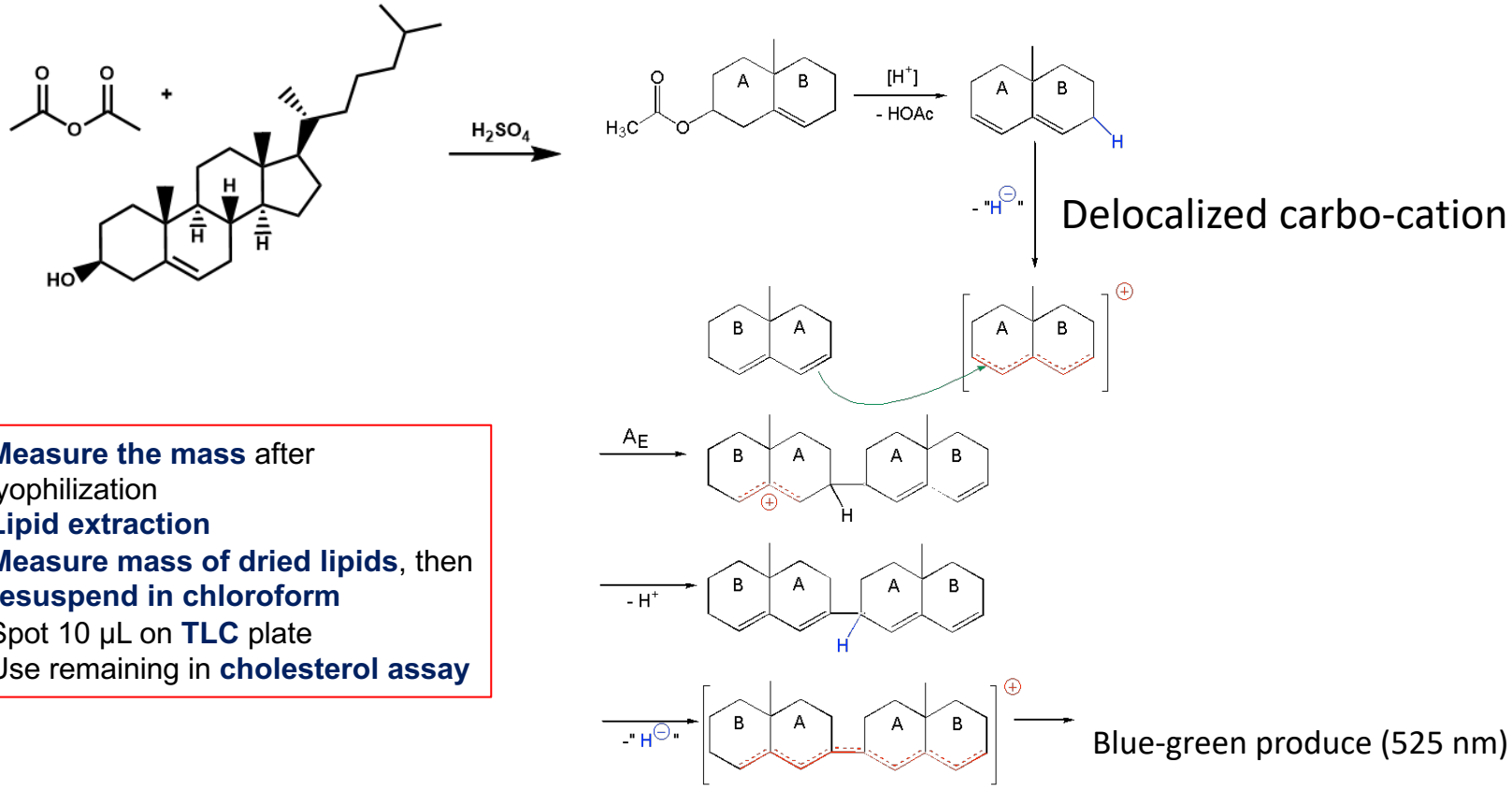
Denatured Proteins



- **Measure the mass** after lyophilization
- **Lipid extraction**
- **Measure mass of dried lipids**, then **resuspend in chloroform**
- Spot 10 µL on **TLC** plate
- Use remaining in **cholesterol assay**

Separation of Membrane Lipids

- Phospholipids will be separated by Thin Layer Chromatography (TLC)
  - **Qualitatively** identify which phospholipids are present
- Cholesterol content determined **quantitatively** by colorimetric assay: **Liebermann-Burchard test**



- **Measure the mass** after lyophilization
- **Lipid extraction**
- **Measure mass of dried lipids**, then **resuspend in chloroform**
- Spot 10 µL on **TLC** plate
- Use remaining in **cholesterol assay**

Procedures: Week 2

LYOPHILIZED:

In tared glass tubes –

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

- **Measure the mass** after lyophilization
- **Lipid extraction**
- **Measure mass of dried lipids**, then **resuspend in chloroform**
- Spot 10 µL on **TLC** plate
- Use remaining in **cholesterol assay**

②

FROZEN @ -20°C:

In 1.5 mL eppendorf tubes –

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

- **Dialyze** over the 4-hr lab period to remove salt

①

- **Dye-binding assay**

③

LYOPHILIZE:

- **Measure volume** remaining after dye binding assays of your three 25%-aliquots
- Freeze remaining for lyophilization for next week

④

Procedure: Chapter 10B

PREPARATION OF MEMBRANES FOR PROTEIN ANALYSIS

- **Dialysis and Lyophilization of 75% Washed Supernatant Fraction:**
  - Thaw Supernatant fraction
  - Dialyze in Slide-A-Lyzer MINI Device
  - Change buffer ~every hour. **TFs will announce time to change.**
  - Measure volume of dialysate, place in 15 mL conical tube, freeze, parafilm, puncture hole and give to TFs
    - TFs will lyophilize samples before next week

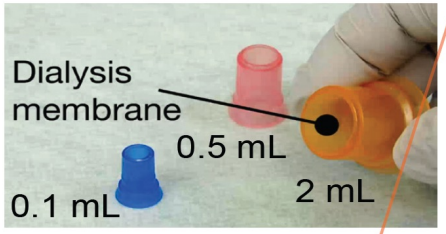
Procedure: Chapter 10B

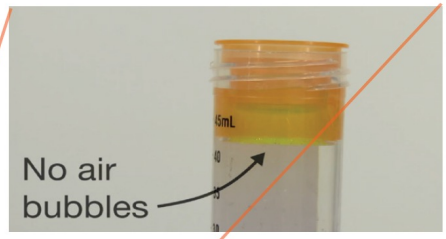
PREPARATION OF MEMBRANES FOR PROTEIN ANALYSIS

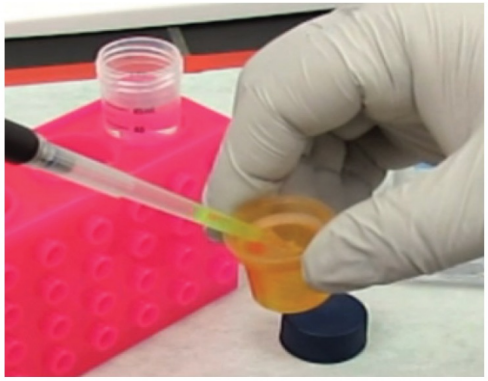
- **Dialysis and Lyophilization of 75% Washed Supernatant Fraction:**
  - Thaw Supernatant fraction

Using A Slide-A-Lyzer™ MINI device

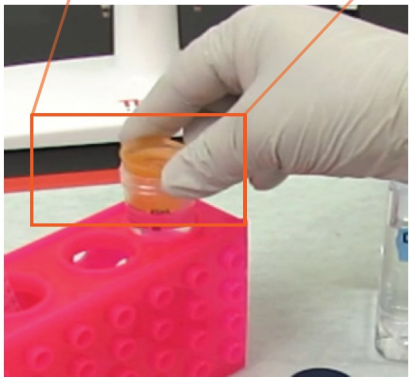
Select size based on maximum volume







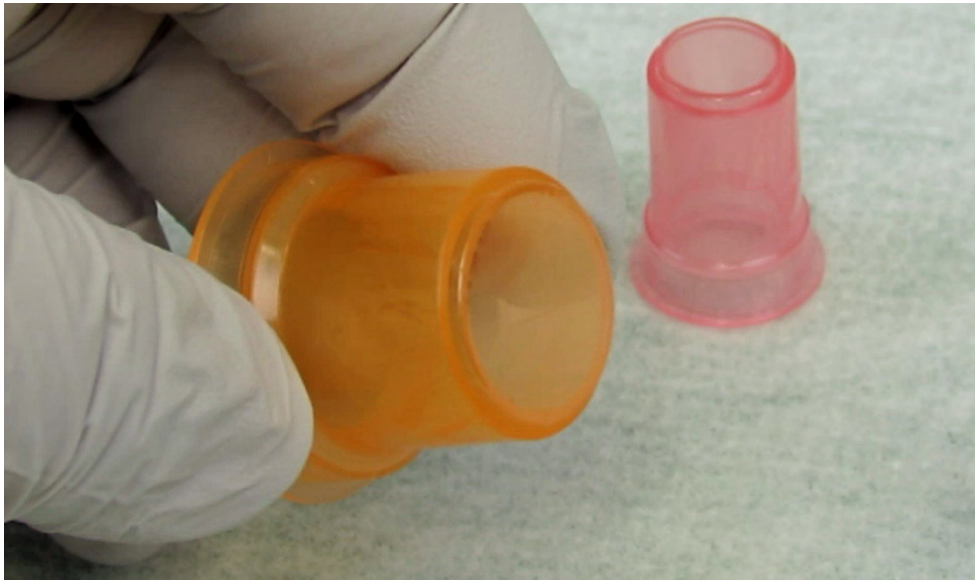
Loading your sample (yellow)



Placing over tube with dialysis buffer

After 3 buffer changes, remove dialyzed sample

Dialysis Setup

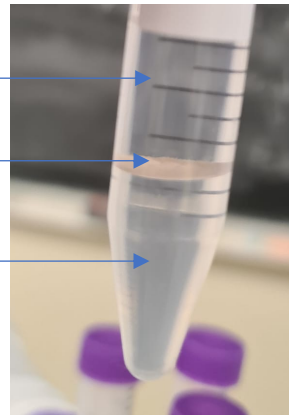


- Fill the 50 mL conical device with cold ammonium bicarbonate
- Pre-wet the membrane for 5 min
- Apply sample onto membrane and dialyze for 50-55 min
- Replace ammonium bicarbonate and repeat the dialysis for an additional three times.
- A total of **four** exchanges over the four-hour lab period

Procedure: Chapter 10C

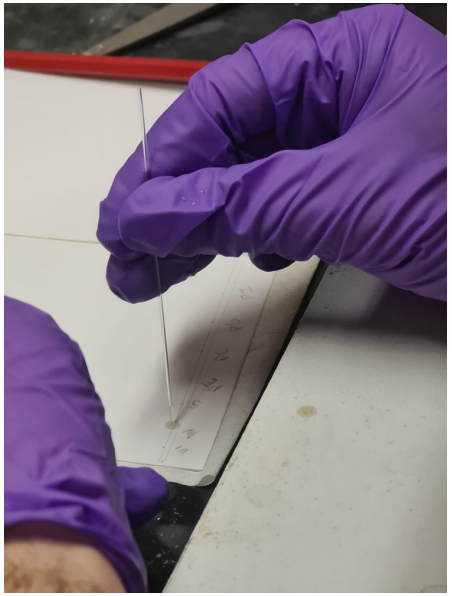
CHARACTERIZATION OF MEMBRANE LIPIDS

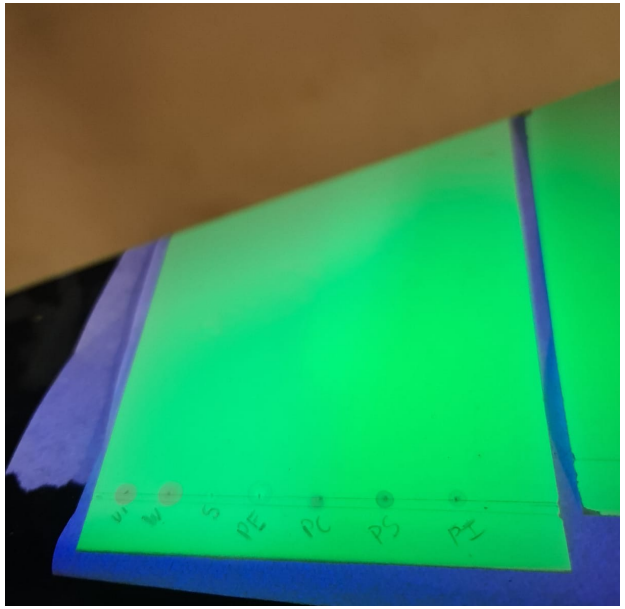
- **Extraction of Lipids from WASHED and UNWASHED membrane preps:**
  - Weigh both tubes and caps from Week 1 on same scale
    - Determine total mass (yield)
  - Resuspend lyophilized membranes in 0.8 mL PBS
    - ***Membrane may not dissolve completely***
  - Add 3 mL chloroform:methanol (1:2)
    - Cap, shake for 1 min, repeat by adding 1 mL
  - Add 1 mL water
    - Cap, shake for 1 min, spin down in centrifuge 1000 x*g* for 5 min
  - Obtain new (clean) glass pyrex tubes
    - ***LABEL & WEIGH*** before use
  - Discard upper aqueous phase
  - Remove lower organic phase using glass pasteur pipet
    - Take liquid *underneath* the interface (denatured proteins)
  - Transfer liquid to **tared** glass tubes & evaporate
    - *TFs will assist with drying using an air stream*
  - Weigh tubes & **record mass** of lipids in each membrane prep



Procedure: Chapter 10C

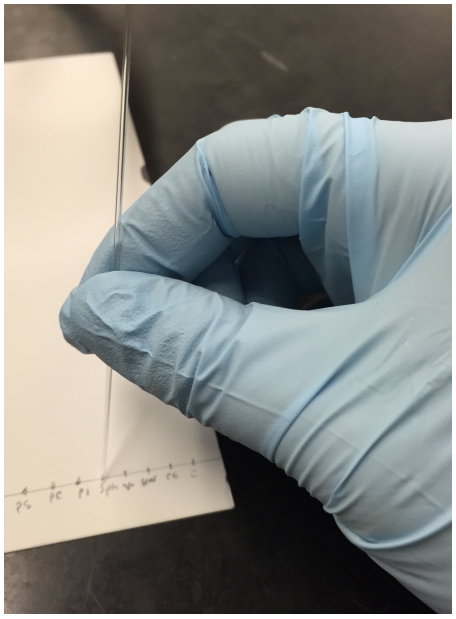
- **Qualitative Analysis of Phospholipids by TLC:**
  - On silica-gel sheet, draw line in **PENCIL** ~1.5-2 cm from bottom
  - Make **7** evenly-spaced marks on penciled line to spot samples
    - *5 standards + 2 lipid preps*
    - *Keep track of sample order!*
  - Dissolve your 2 evaporated lipid preps in chloroform to the correct concentration of 100 µg/µL (***~ 80 µL***)
    - **Chloroform is VERY VOLATILE; KEEP TUBES CAPPED & ON ICE!!**

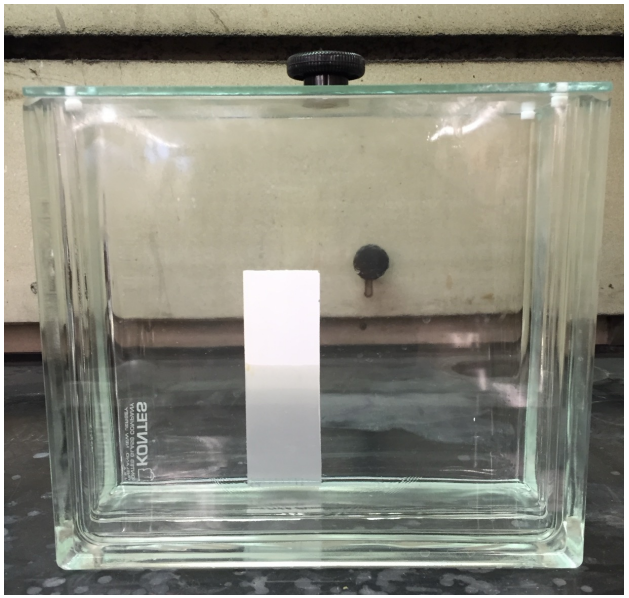


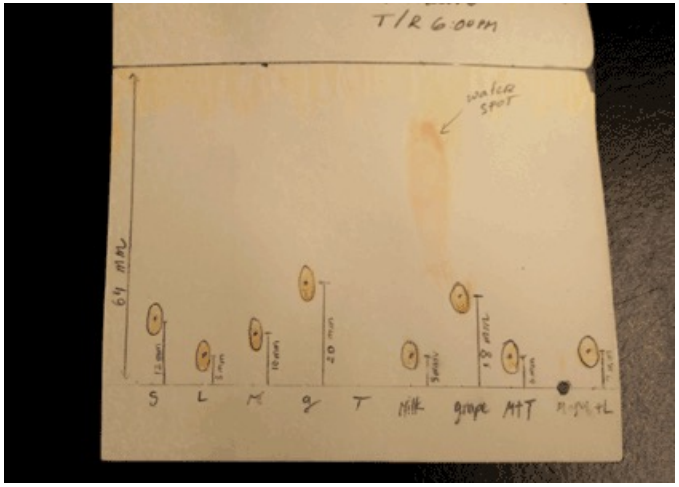


Procedure: Chapter 10C

- **Qualitative Analysis by TLC (continued):**
  - Load & spot samples (5 µL each for standards and 10 µL each for samples) with capillary tubes
  - Perform separation in TLC chamber (1-2 hours, ~12cm)
  - Dry plate & visualize in iodine chamber (2-3 min)
  - Mark & measure solvent distance
  - Circle spots with pencil & measure migration distance
  - Record observations & diagram TLC plate in notebook







Procedure: Chapter 10C

- **Quantitative Determination of Cholesterol:**
  - Transfer ***the remaining amount of lipid prep (~70 µL)*** into two **tared** test tubes & evaporate for unwashed and washed membrane samples
  - **Weigh & record mass** of each tube
  - ~~Prepare known cholesterol solutions for standard curve (0, 0.1, 0.2, 0.4, 0.6 and 0.8 mg)~~
    - Evaporate chloroform from the standards with air stream
    - Be careful with acetic acid & sulfuric acid – ***highly toxic & will cause chemical burns!***
    - **Use these reagents & Liebermann-Burchard reagent in hood!!** *(Liebermann-Burchard **IS** acetic anhydride)*
  - Cover with ***foil*** & incubate in the dark for 30 min
  - Use same time interval for each tube before reading in spec @ A_{525}

Procedure: Chapter 10B

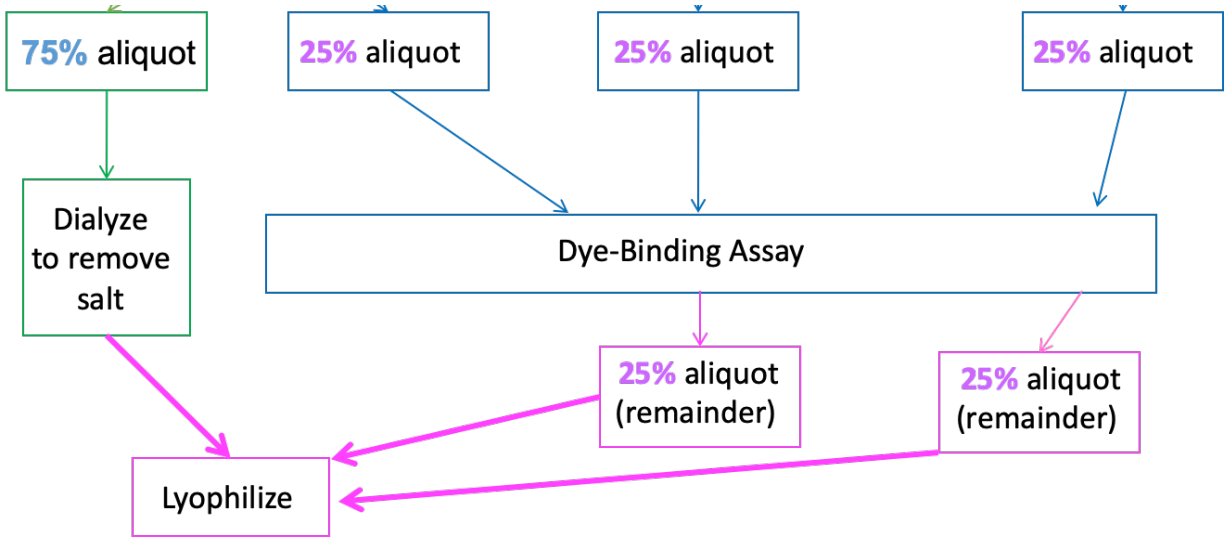
- **Protein Determination: Dye Binding**
  - Make a Bradford standard curve
  - Determine total membrane protein in fractions
    - Start with 20 µL of fraction sample
    - May need to dilute fractions to be in range of standard curve
    - If sample absorbance is too low, simply add more volume to the same tube, mix, wait 2 minutes, and check Absorbance at 595 nm.
      - Keep track of the total volume added in the end!

BSA standard curve

1 mg/mL BSA	di-water	Dye-binding reagent
0 µL	0.5 mL	4.5 mL
10 µL	0.49 mL	4.5 mL
20 µL	0.48 mL	4.5 mL
30 µL	0.47 mL	4.5 mL
40 µL	0.46 mL	4.5 mL
50 µL	0.45 mL	4.5 mL
60 µL	0.44 mL	4.5 mL

- Set-up your other experimental readings at the same time (protein sample volume, adjust to 500 µL with di-water, add 4.5 mL of dye-binding reagent)
- Let tubes sit for 5 minutes at room temperature then read at 595 nm
- Redo any experiments that are outside of standard curve interpolation region (10 µg – 60 µg of BSA) → if absorbance is too low, simply add more samples

Procedure: Chapter 10B



LYOPHILIZE:

- **Measure volume** remaining after dye binding assays of your 3 25%-aliquots
- Freeze remaining for lyophilization for next week

- ***MEASURE THE VOLUME** of remaining 25% aliquots from WASHED & UNWASHED membrane fractions
- MEASURE THE VOLUME of the dialyzed supernatant fraction
- DO THE ABOVE **BEFORE** turning in to TFs for freezing + lyophilization

→ *Need these samples for week 3 SDS-PAGE*

CH 10BC hazards

- Liebermann-Burchard reagents (glacial acetic acid, sulfuric acid and acetic anhydride) are highly toxic, corrosive, flammable and reactive
  - Perform steps in fumehood!
- Ammonium bicarbonate dialysis waste and dye-binding assay waste can go into mainstream waste carboy
- Solvents/acids and cholesterol assay waste goes into large clear glass bottle
- Leftover chloroform goes into small glass bottle

Chapter 10BC Workflow Tips

- One partner starts the dialysis of 75% supernatant **(throughout whole lab)**
- One partner begins the lipid extraction **(~45 min)**
- Help each other to finish the lipid extraction

===

- One partner begins the TLC plate **(1-1.5 h)**
- One partner begins Cholesterol Assay **(~1.5 – 2 h)**

===

- During a long incubation/waiting step, perform the dye-binding assay **(~30 min)**

Important that you start on lipid preparations first and do the dye-binding assay towards the end of lab!

Chapter 10BC

- Note that we are doing 4 rounds of dialysis, with a change in buffer every ~50-55 min
- Final dialyzed sample will be aliquoted into a 15 mL falcon tube for lyophilization
- On silica-gel sheet, draw line in **PENCIL** ~1.5-2 cm from bottom (not 1 cm)
- Dissolve your 2 evaporated lipid preps in chloroform to the correct concentration of 100 µg/µL (***~ 80 µL***)
- Load & spot samples (**5 µL each for standards** and **10 µL each for samples**) with capillary tubes
- After dye-binding assay, the 25% aliquots will be frozen in 1.5 mL microcentrifuge tubes

Chapter 10BC

Before the lab period, you should have:

- ✓ Completed your Pre-lab Write-up and submit on Gradescope
  - ✓ Title, purpose and procedures
  - ✓ Remember to include:
    - ✓ Tables for
      - ✓ Cholesterol determination
      - ✓ Dye-binding assay results
    - ✓ Prepare prompts for when you need to weigh and record the masses of different tubes, volumes (lyophilized samples, lipid extraction steps, and sample prep for cholesterol determination assay, volumes before lyophilization)

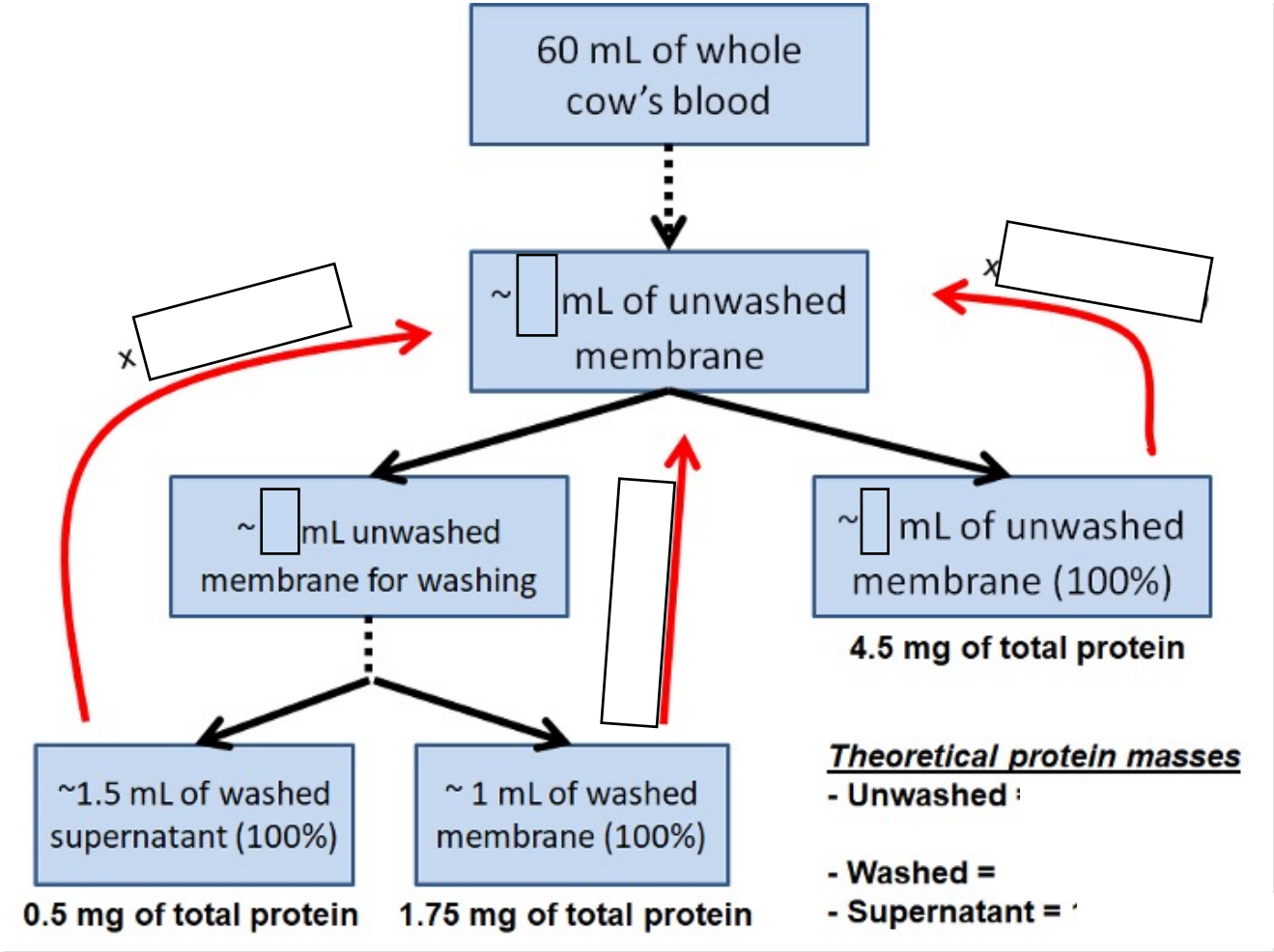
At the end of lab, you should have:

- ✓ Turned in to your TFs
  - ✓ **Dialyzed 75% Washed Supernatant** in 15 mL conical tube
  - ✓ 25% aliquot remainder of **Washed Membrane**
  - ✓ 25% aliquot remainder of **Unwashed Membrane**
  - ✓ 25% aliquot remainder of **Washed Supernatant** (back-up/extra)

TFs will LYOPHILIZE

Questions?

In-class activity
&
Discussion Quiz


60 mL of whole cow's blood
~ mL of unwashed membrane
x
x
~ mL unwashed membrane for washing
~ mL of unwashed membrane (100%)
4.5 mg of total protein
~1.5 mL of washed supernatant (100%)
0.5 mg of total protein
~ 1 mL of washed membrane (100%)
1.75 mg of total protein
Theoretical protein masses
- Unwashed
- Washed =
- Supernatant =