Announcements

- Chapter 9 Lab Report Due Tuesday midnight
- Chapter 10 post-lab write-up is due week of Apr 24 – 29
- Grading for post-lab chapter 8 have been released; will be posted by the end of the week

Chapter 10D: Lipids and Membranes

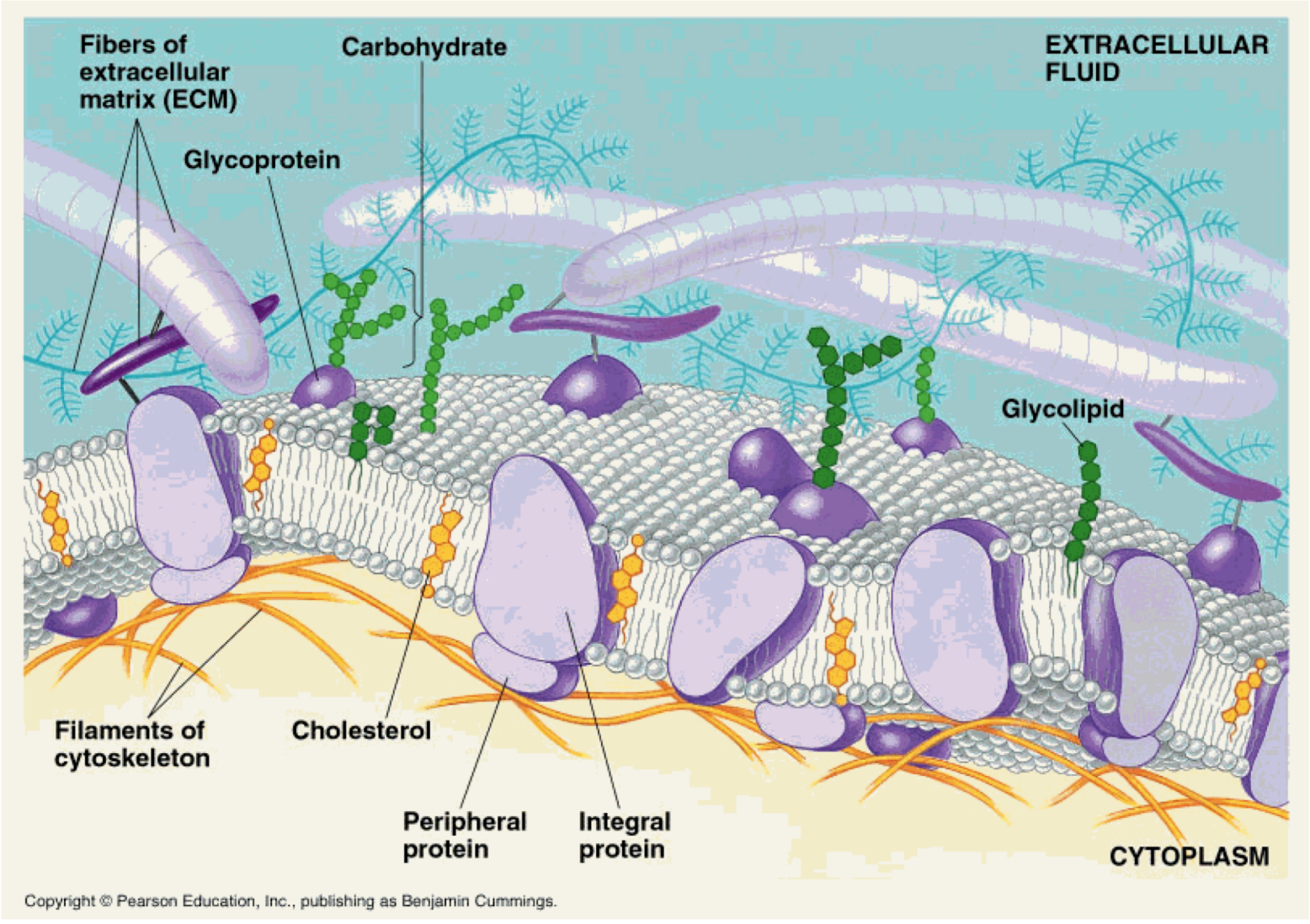
Objectives

- To analyze protein composition in unwashed membrane, washed membrane, and supernatant fraction (Acquastain)
- To qualitatively determine which membrane proteins are glycosylated (PAS stain)
- To prepare immobilized samples on PVDF membrane for probing of proteins (Immunoblot/Western Blot)

Procedures

- To **separate protein preparations** in samples by ***SDS-PAGE***
- To perform SDS-PAGE (3 gels/2 shared)
- To **visualize and stain the SDS-PAGE gel** with ***Acquastain*** and ***PAS stain***
- To **prepare and blot gels** from SDS-PAGE for immunoblotting
- To **cast** two SDS-PAGE gels per pair

Review: Plasma Membrane Composition



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Membrane fractions isolated from RBCs in Ch 10

- **Unwashed membrane (lyophilized 25% sample)**
  - Integral and peripheral proteins
  - *Membrane is intact. All membrane associated proteins are present in this fraction*

- **Washed membrane (lyophilized 25% sample)**
  - Integral proteins
  - *Peripheral proteins removed with high salt membrane wash buffer*

- **Washed supernatant fraction (dialyzed and lyophilized 75% sample)**
  - Peripheral proteins
  - *Removed from membrane using high salt membrane wash buffer*

Lyophilized Samples

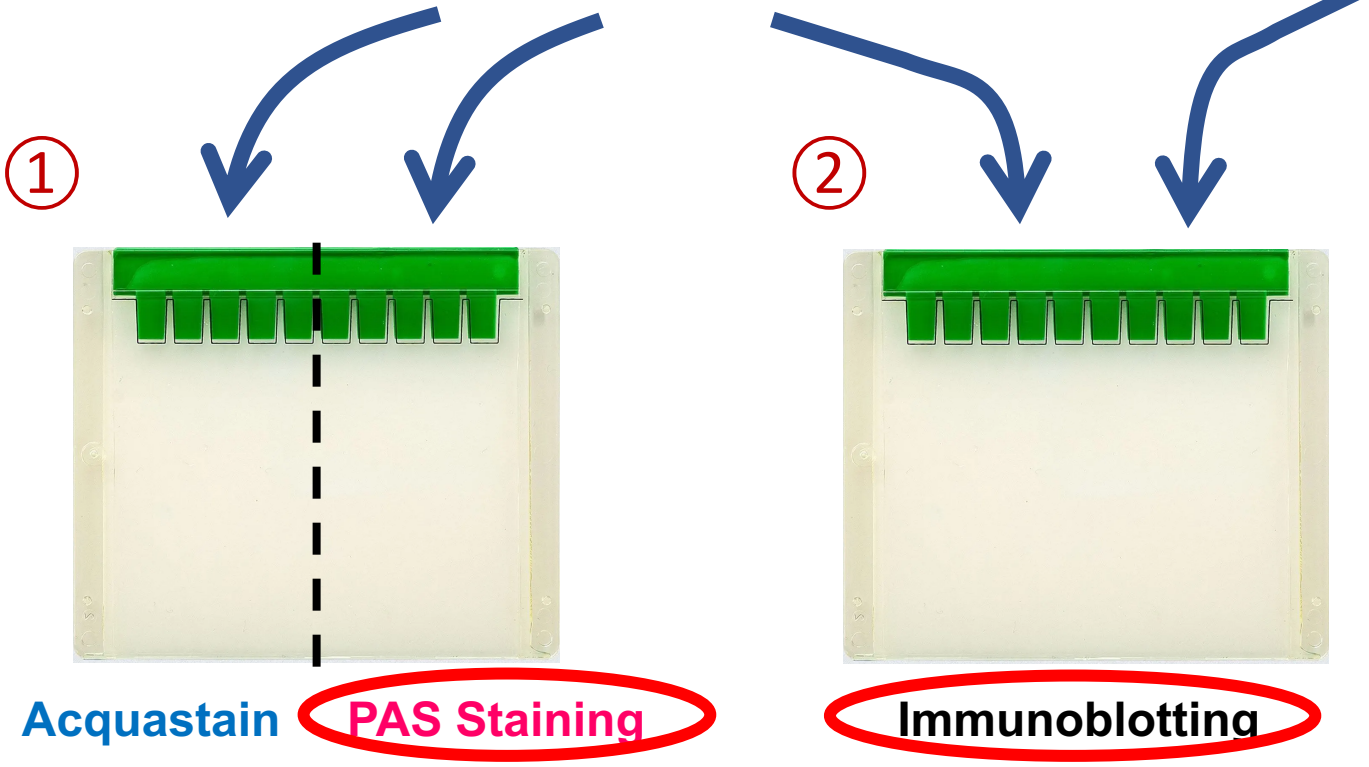
75% Supernatant Dialysate
SF

25% Unwashed Membrane
UWM

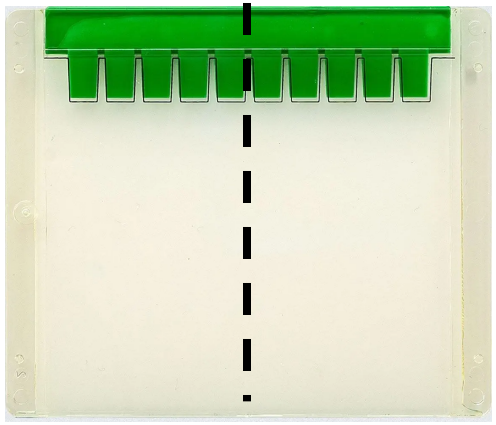
25% Washed Membrane
WM

Prepare samples for SDS-PAGE

Samples from 2nd group

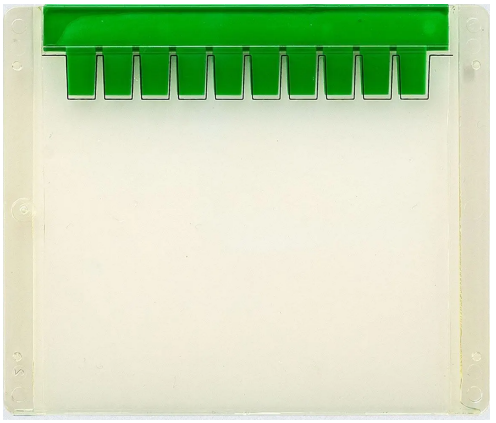


①

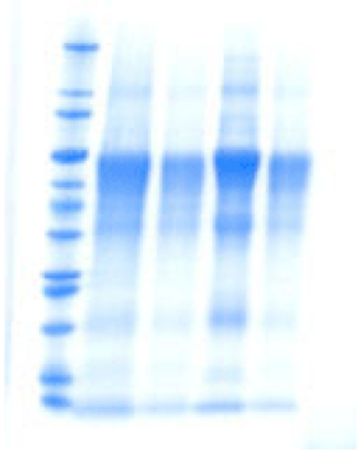


1st gel

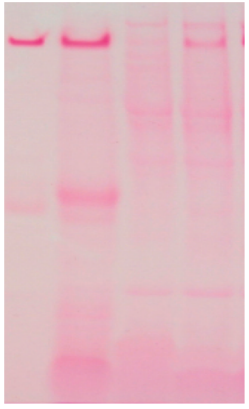
②



2nd gel

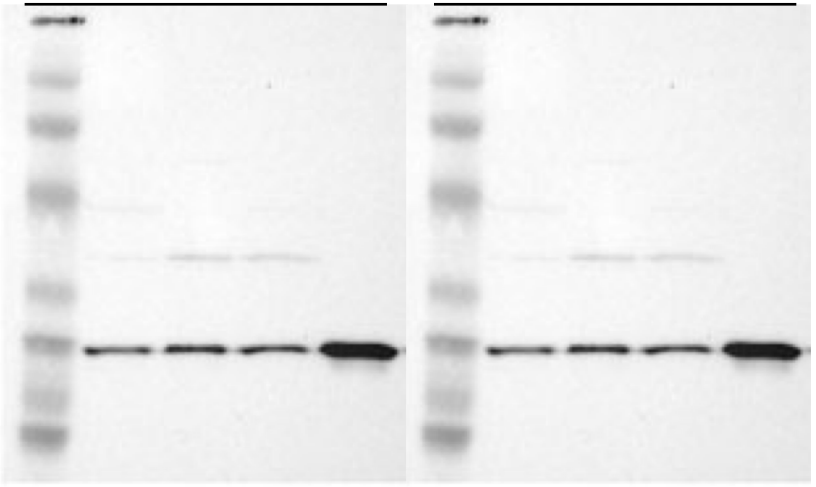


Acquastain



PAS Staining

Your group's samples | The other group



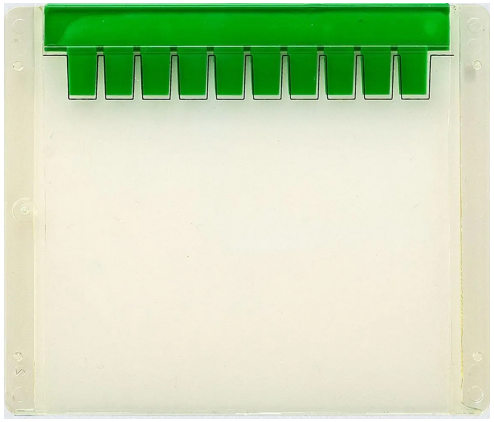
Immunoblotting

Images: E. Cotroneo et al. (2010)
www.sigmaaldrich.com

Note: Images are **examples** only!

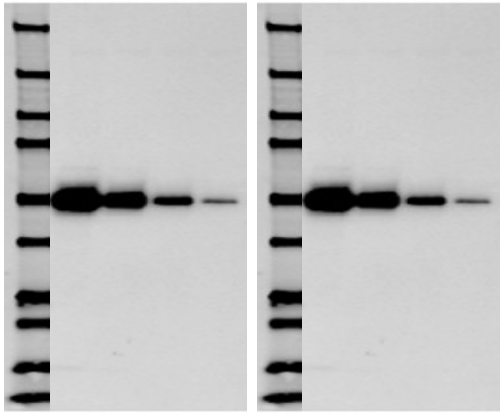
③

Prepare different dilution of a **His-tagged KHK protein** and perform immunoblotting with it



3rd gel

Your group's samples The other group



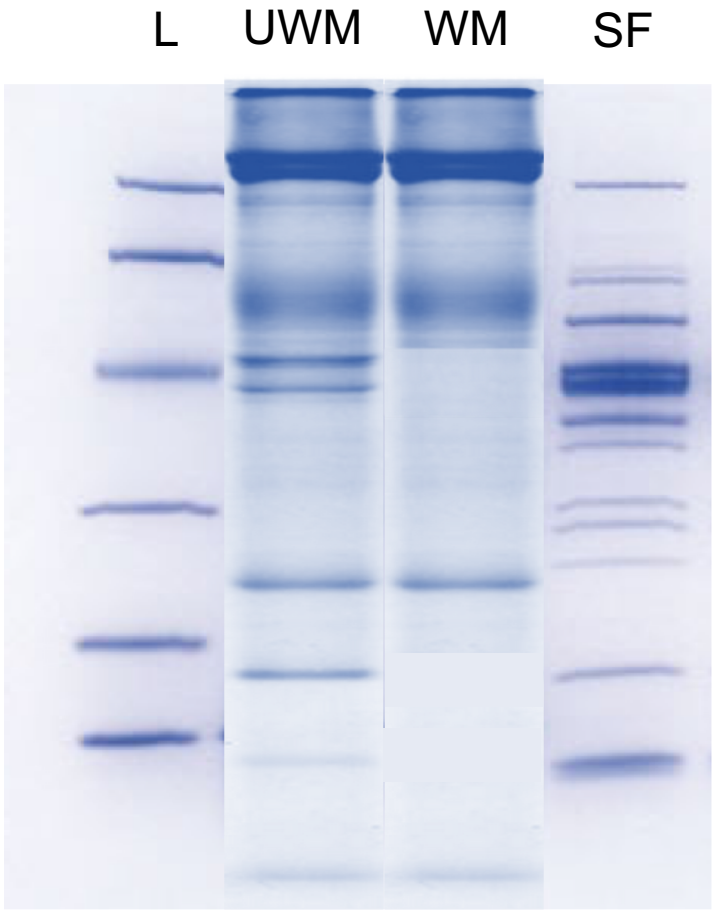
Immunoblotting

Note: Images are **examples** only!

You will be given an aliquot of concentrated KHK and make 10-fold serial dilutions for samples

Acquastain: total protein stain

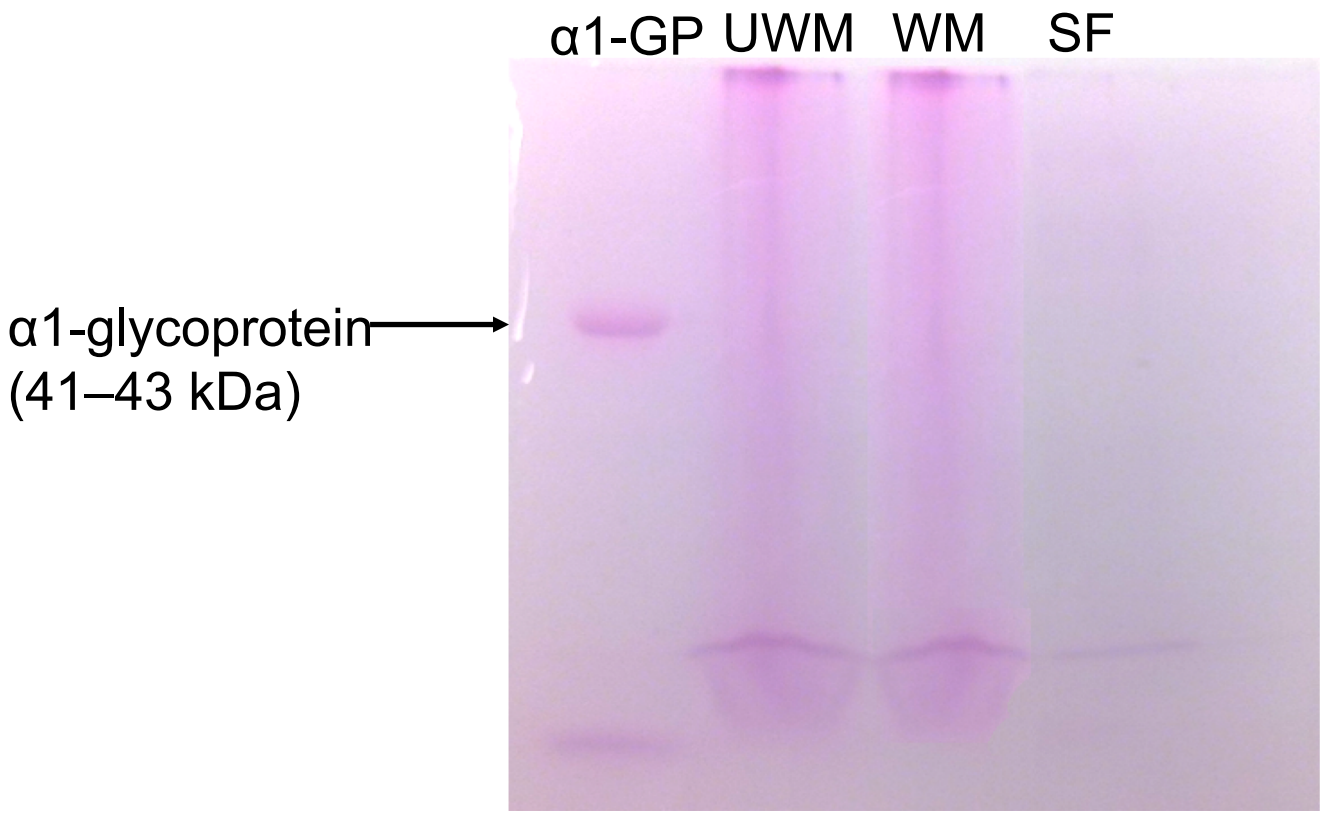
- Used to stain all proteins in a sample



Note: This is an **example** only!

Periodate-Schiff (PAS): glycoprotein stain

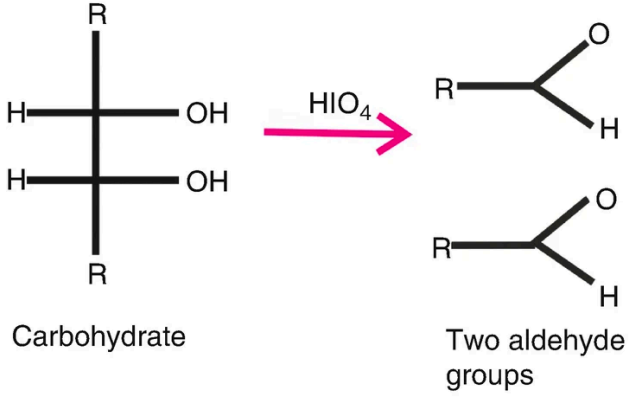
- Compare **glycoprotein stain** to **total protein stain** to determine which proteins are glycosylated

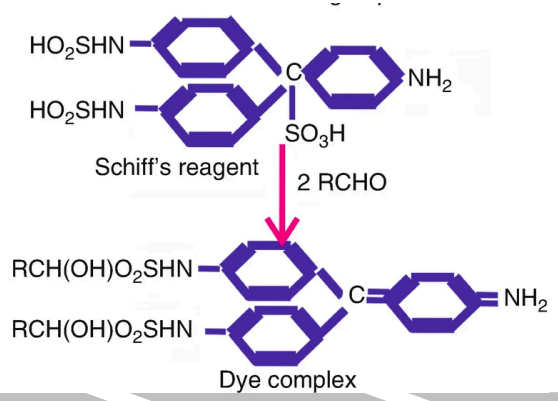


Note: This is an **example** only!

Visualization of glycoproteins via PAS staining

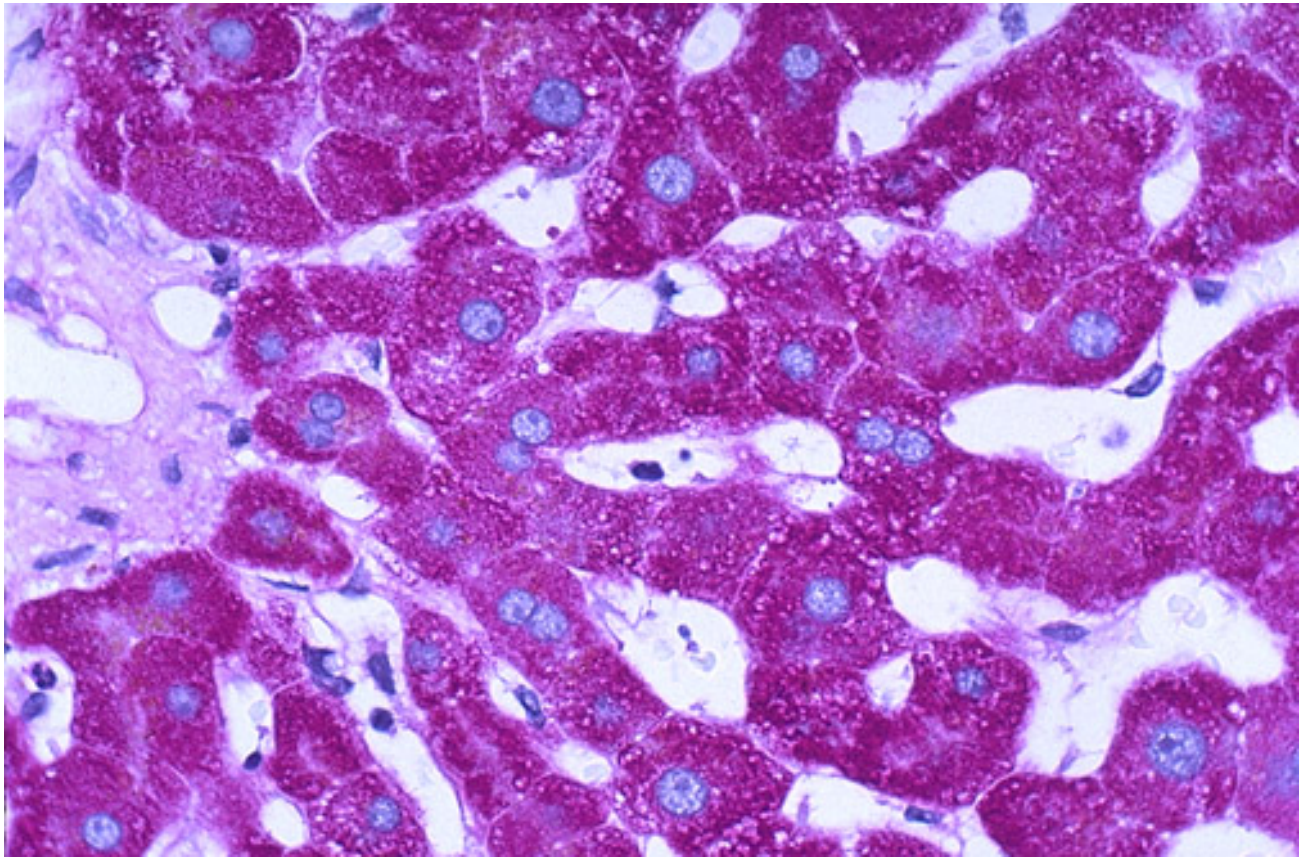
- **Glycoproteins: involved in communication with other cells**
  - Cells recognize glycosylation patterns to transmit cellular information
- **After SDS-PAGE separation, will use different staining technique to observe carbohydrates**
  - **Periodic acid (HIO_4) (pronounced *per-iodic*)** → oxidizes hydroxyl groups on adjacent carbons to aldehydes
  - New dialdehydes react with **Schiff reagent** to form Schiff base and give ***pink*** product
  - Works for all sugars, including those present on glycoproteins





Visualization of glycoproteins via PAS staining

- **The versatile PAS staining technique is a benchmark technique in clinical histology**

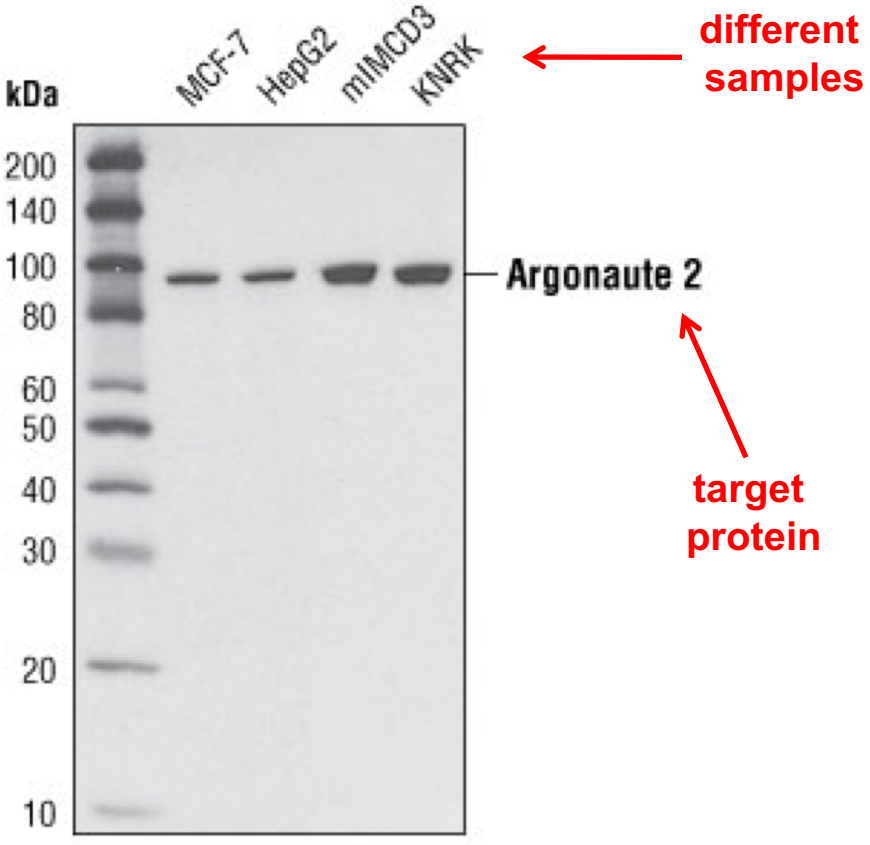


Cytoplasmic (intracellular) glycogen is detected and stained (dark-pink) in hepatocytes (liver cells)

Immunoblotting: detection of a target protein

- *Western Blotting – Used to identify a SPECIFIC protein in a sample*

Example (right): detection of androgen receptor (Argonaute 2) among differing cell lines



Note: This is an **example** only!

Image: www.fantibody.com

Immunoblotting: detection of His-tagged protein

- *Western Blotting – Used to identify His-tagged protein in a sample*

- Affinity tags like 6xHis often added to proteins to facilitate purification and identification; e.g., KHK
- Can be used to quantitatively determine amount of proteins present
- Notice the difference in sensitivity; 0.25 µg would be barely visible with Coomassie Blue

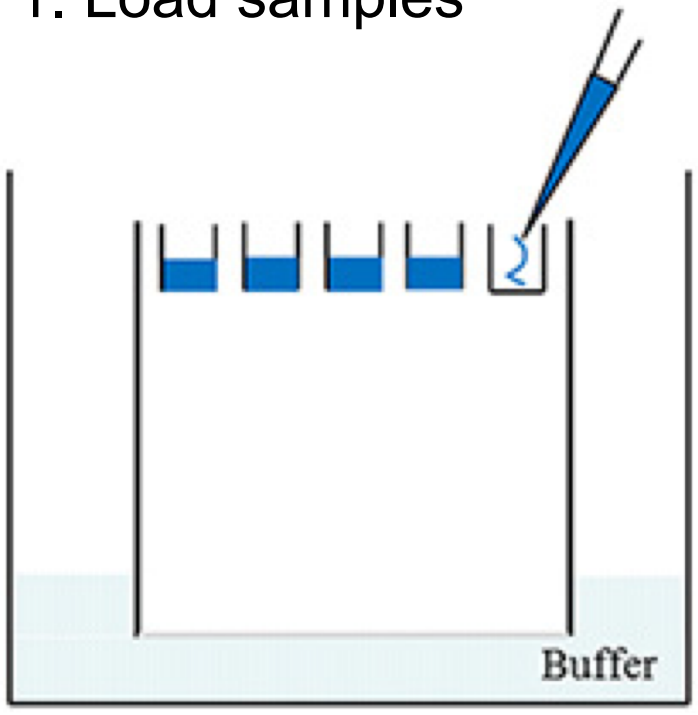
Note: This is an **example** only!

Image: https://www.thermofisher.com/us/en/home/life-science/antibodies/primary-antibodies/epitope-tag-antibodies/his-tag-antibodies.html

Immunoblotting: detection of a target protein

- **1st Step:** Separate proteins by SDS-PAGE

1. Load samples



2. Run gel

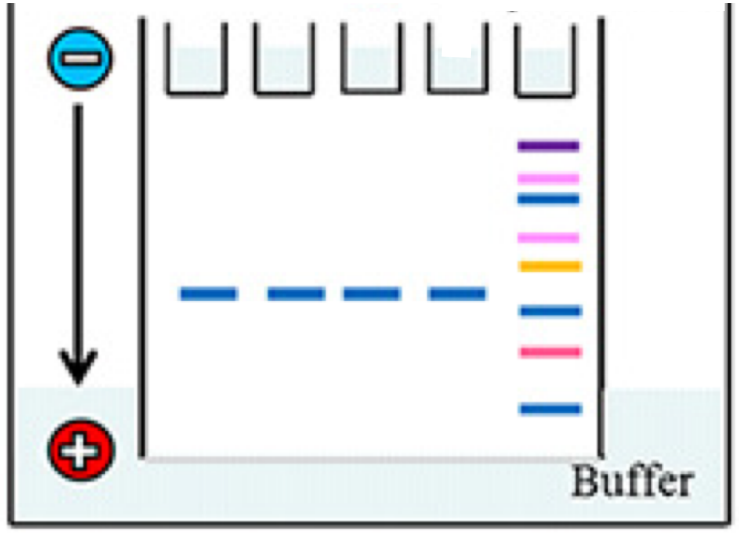


Image: www.sigmaaldrich.com

Immunoblotting: detection of a target protein

- **2nd Step:** Transfer proteins from gel to a membrane (usually nitrocellulose *or* PVDF)

Note: Only the pre-stained ladder is visible at this point!*

Image: www.bio-rad.com

*This year our pre-stained ladder proteins are all **BLUE**

Immunoblotting: detection of a target protein (Next week)

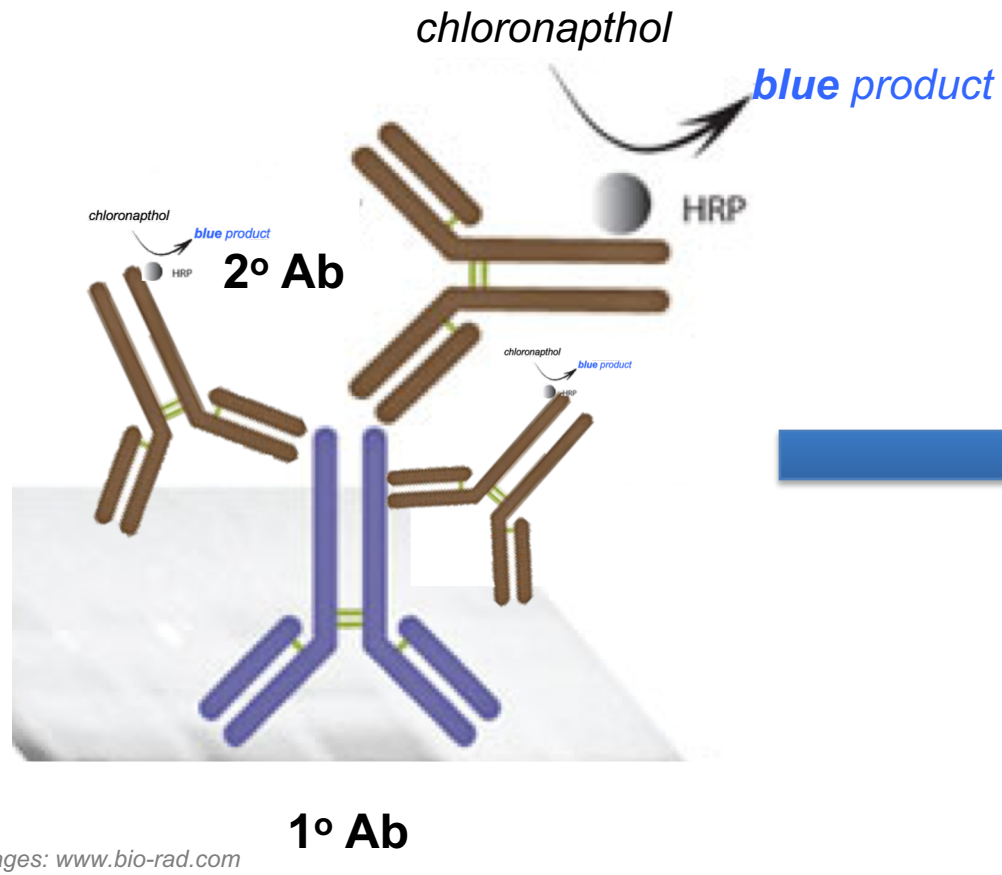
- **3rd Step:** Probe for the protein of interest using a primary antibody (1° Ab)*

Images: www.bio-rad.com
www.sigmaaldrich.com

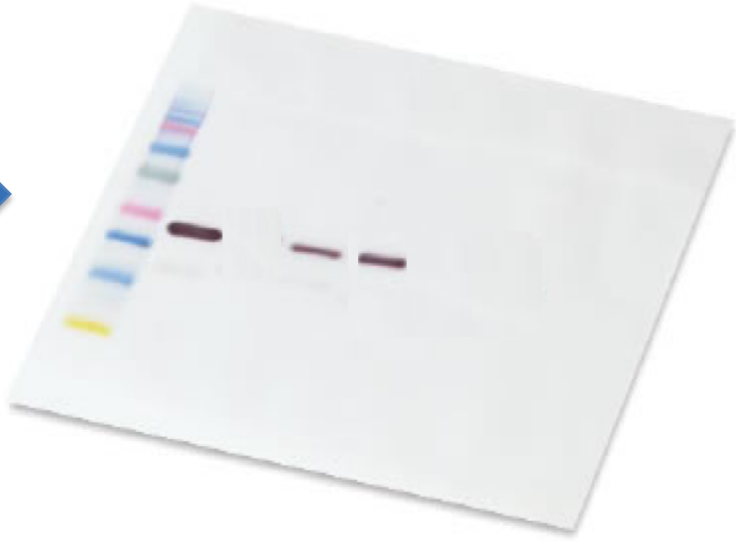
**we will use 2 different primary antibody in Chapter 10E)*

Immunoblotting: detection of a target protein (Next week)

- **4th Step:** Detect 1° Ab using a 2° Ab to visualize where target protein appeared in the original gel



IB after development:

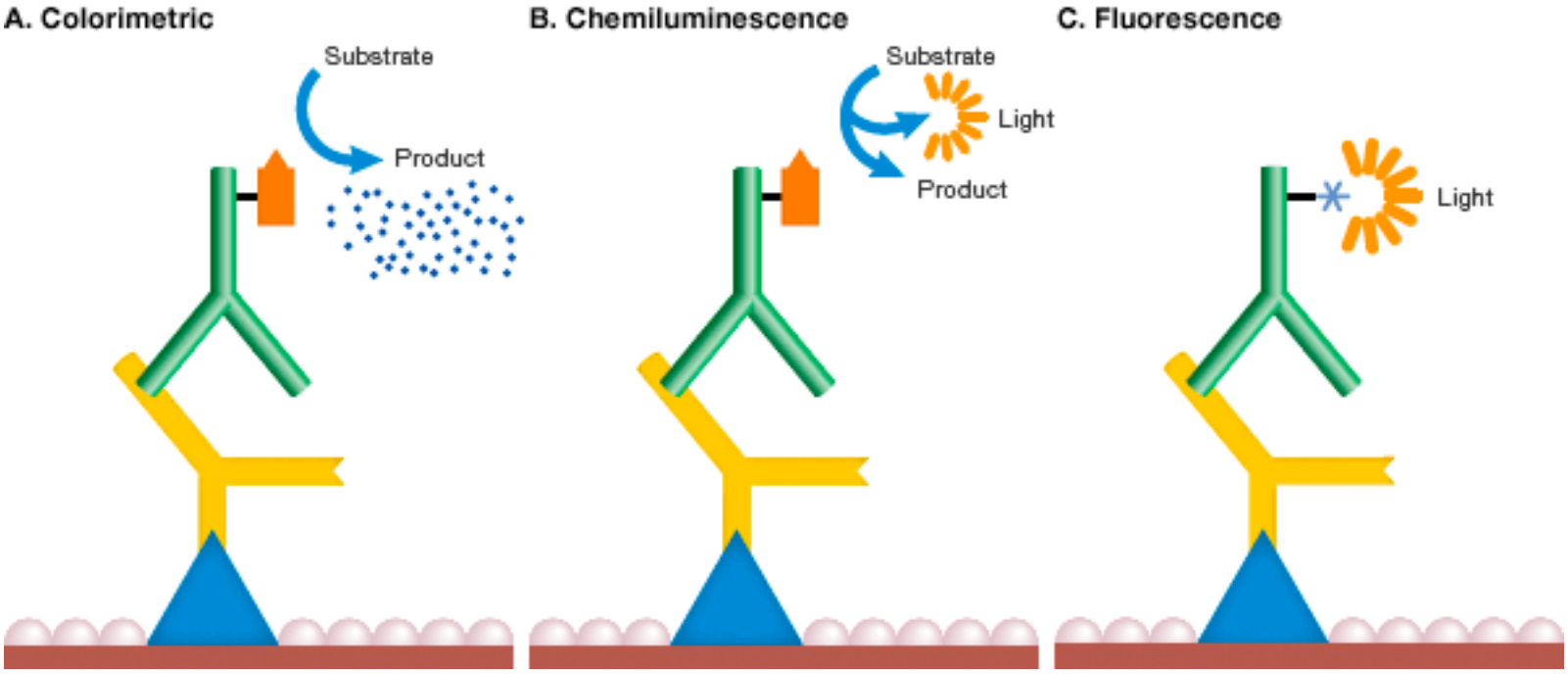


Images: www.bio-rad.com
www.sigmaaldrich.com

Note: This is an **example** only!

Detection methods for western blot

- Main techniques for visualizing are colorimetric, chemiluminescence, and fluorescence.



Mechanism of detection chemistries. In each method of western blot detection, a detectable signal is generated following binding of an antibody specific for the protein of interest. In colorimetric detection (**A**), the signal is a coloured precipitate. In chemiluminescence (**B**), the reaction itself emits light. In fluorescence detection (**C**), the antibody is labelled with a fluorophore.

Image: https://www.bio-rad.com/en-sg/applications-technologies/detection-methods?ID=LUSQ6KKG4

Procedure: Chapter 10D

- **Part I: SDS-PAGE Sample Preparation:**
  - Calculate volume needed to dissolve each sample to appropriate protein concentration
    - Refer to next slide for sample preparation guidance
    - Present in a table
  - Need to prep 3 samples for each gel (total protein, glycoprotein, and one of the immunoblot)
    - **Washed**
    - **Unwashed**
    - **Supernatant fraction**
  - 3 samples x 3 gels = **9 sample lanes**
  - Denature all protein samples at 65 ºC for 15-20 min before loading onto gels

Procedure: Chapter 10D

- **Part I: SDS-PAGE Sample Preparation continued…:**
  - For all of your samples, you will want a **~10 µg/µL concentration** of protein
  - **Calculate how much remaining protein mass you have** in your 25% unwashed membrane, 25% washed membrane, and 75% washed supernatant preps from your dye-binding data.
  - **Calculate volume of 1X loading buffer** needed to resuspend proteins to ~10 µg/µL
  - Resuspended samples must be transferred to a new 1.5 mL Eppendorf tube ***and then*** denatured.

How do you calculate this?
In-class activity

In the Chapter 10BC lab, you did the dye-binding assay to determine the amount of proteins present in your samples and measured how much volume of samples you had left before subjecting it to lyophilization. **Dye-binding data indicates that the protein concentration in the washed supernatant is 2 µg/µL and the sample volume after dialysis for lyophilization is 350 µL.**

Based on this information, calculate the volume of water that is needed to obtain 11.7 µg/µL of protein.

Concentration of protein: 2 µg/µL

Volume of sample: 350 µL

Mass of protein: 2 µg/µL X 350 µL = 700 µg

Volume of water = 700 µg / 11.7 µg/µL = **60 µL**

This 60 µL is 7/8 of your volume, the other 1/8 (8.5 µL) will come from the 8X SDS-sample buffer

You now have 700 µg in 68.5 µL = 10 µg/mL

Procedure: Chapter 10D

- **Part I: SDS-PAGE Sample Loading:**

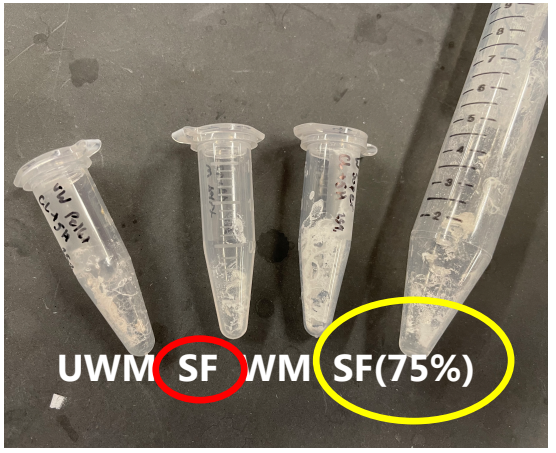
  For each sample in each gel type:

  - **Acquastain** gel: load ~10 µL of each 10 µg/mL sample (~100 µg)
  - **PAS/glycoprotein** gel: load ~15 µL of each 10 µg/mL sample (~150 µg)
  - **Western blot** gel: load ~20 µL of each 10 µg/mL sample (~200 µg)

This means you need *at least 45 µL* in 1X sample buffer to have enough protein for all of your gels! At 10 µg/mL, this is 450 µg of lyophilized protein.

If you determine you have <450 µg:

- First, double check your Bradford and calculations
- Second, if the lyophilized material in your tubes looks like this: then you have plenty to load.
- Third, just add 40 µL H_2O, dissolve by shaking all around, quick spin down, move to a new tube, and add 5.6 µL of 8X SB. This gives you ~45 µL of sample to load on 3 gels.
- Fourth, heat to 65 °C for 10 min
- Fifth, load the 10, 15, and 20 µL for your Aquastain, PAS, and western-blot gels



Do NOT use the 25% aliquot of SF as it has too much salt. But if you use the [protein] of this fraction, and its original volume, your tube of the dialyzed 75% fraction should have 3-times that amount of protein!

Procedure: Chapter 10D

- **Part II: SDS-PAGE Sample Preparation:**
  - To create three 10-fold dilution of **His-tagged KHK protein containing 1X SDS buffer** and load it onto a gel
  - Create a table in your pre-lab showing how you will prepare 50 µL of the protein at varying concentrations by serial dilution
  - Denature all samples at 65 ºC for 15-20 min before loading onto gels

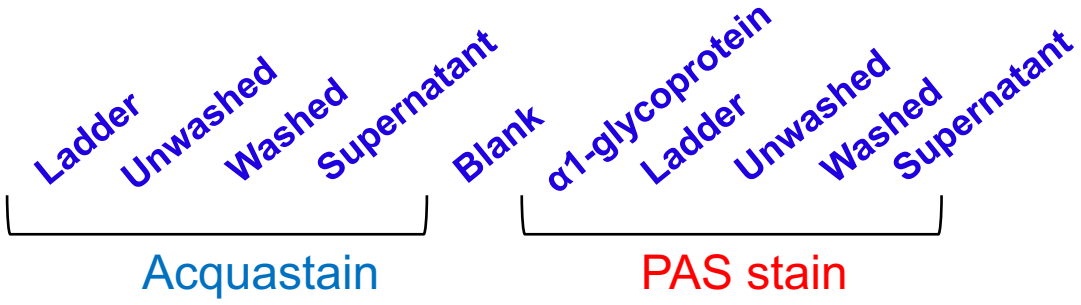
- **Part II: SDS-PAGE Sample Loading:**

  For each sample concentration:
  - Load 20 µL in the well

- Given enough KHK to make 40 µL of 3 mg/mL protein in SDS loading buffer
- Make 10-fold serial dilutions, 50 µL each (combine 5 µL KHK + 45 µL 1X SB)
- Heat 65 °C 15 min
- Load 20 µL on gel

Procedure: Chapter 10D

- **Gel Loading and Running:**
  - Run gels ~80 V thru stacking, ~180 V for resolving
    - Stop gels when dye front is 2-3 cm from bottom
  - 1st gel: Cut in half & stain two ways
    - Acquastain'ing samples on one side, PAS staining samples on other side of SDS-PAGE
    - **Acquastain**:
      - **NEB pre-stained broad range ladder (P7718S) – 3 µL**
        + Unwashed, washed, & supernatant samples – **10 µL each**
    - **PAS staining**:
      - **NEB pre-stained broad range ladder (P7718S) – 3 µL**
      - **α1-glycoprotein standard (unstained) – 5 µL**
        + Unwashed, washed, & supernatant samples – **15 µL each**



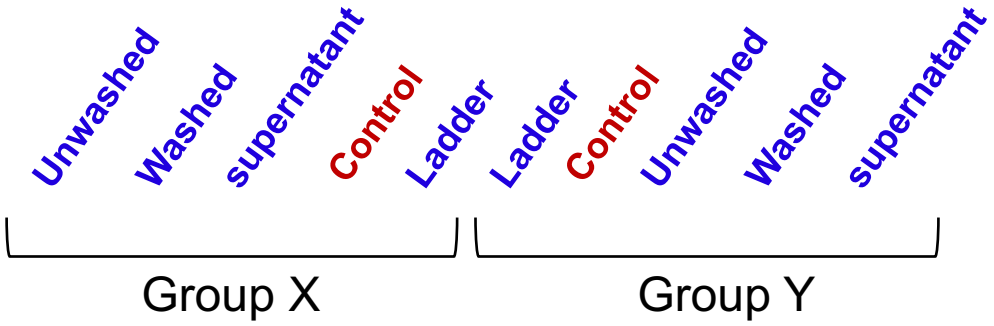
Procedure: 1st gel

- **Acquastain and PAS Visualization:**
  - Remove Acquastain/PAS Staining Gel
    - Cut gel in half and nick a corner of both halves
  - Stain one half with Acquastain
    This half contains your pre-stained NEB ladder
    - Put gel in water (~5min), then stain with Acquastain
    - Destain with water and take a picture of your gel
  - Stain other half with Periodic Acid-Schiff Stain
    This half contains your α1-glycoprotein standard and pre-stained NEB ladder
    - Put gel in 50% methanol (30-60 min), then replace with water (20 min)
    - Replace with 2% Periodate with gentle agitation (15min)
    - Rinse with water (2X 2min)
    - Add Schiff reagent in hood until turns magenta (~15 min)
    - Rinse with water (2X 2min)
    - Replace with 2% sodium metabisulfite
    - Take a picture of your gel

Procedure: Chapter 10D

- **Gel Loading and Running:**
  - Run gels ~80 V thru stacking, ~180 V for resolving
    - Stop gels when dye front is 2-3 cm from bottom
  - 2nd gel: Immunoblot (IB), share half with another group
    - **NEB pre-stained broad range ladder (P7718S) – 5 µL**

    + Unwashed, washed, & supernatant fraction **– 20 µL each**

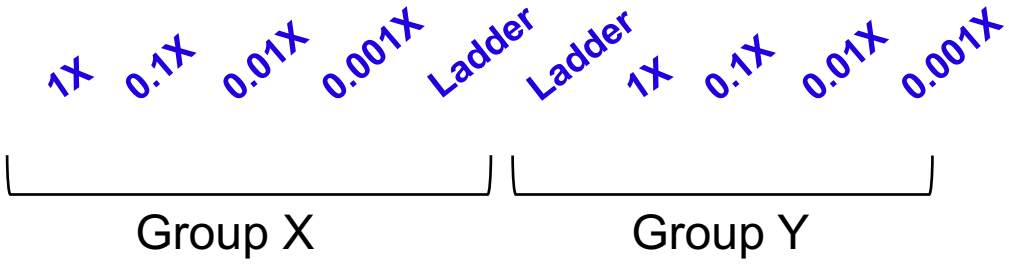


Procedure: Chapter 10D

- **Gel Loading and Running continued…:**
  - Run gels ~80 V thru stacking, ~180 V for resolving
  - 3rd gel: Immunoblot (IB), share half with another group
    - **NEB pre-stained broad range ladder (P7718S) – 5 µL**

    + four different concentration of His-tagged KHK proteins

    – 20 µL each



Immunoblotting: detection of a target protein

- **1st Step:** Separate proteins by SDS-PAGE
- **2nd Step:** Transfer proteins to membrane

10D

- **3rd Step:** Probe for the protein of interest using 1° ab
- **4th Step:** Detect 1° ab using a 2° ab to visualize target protein

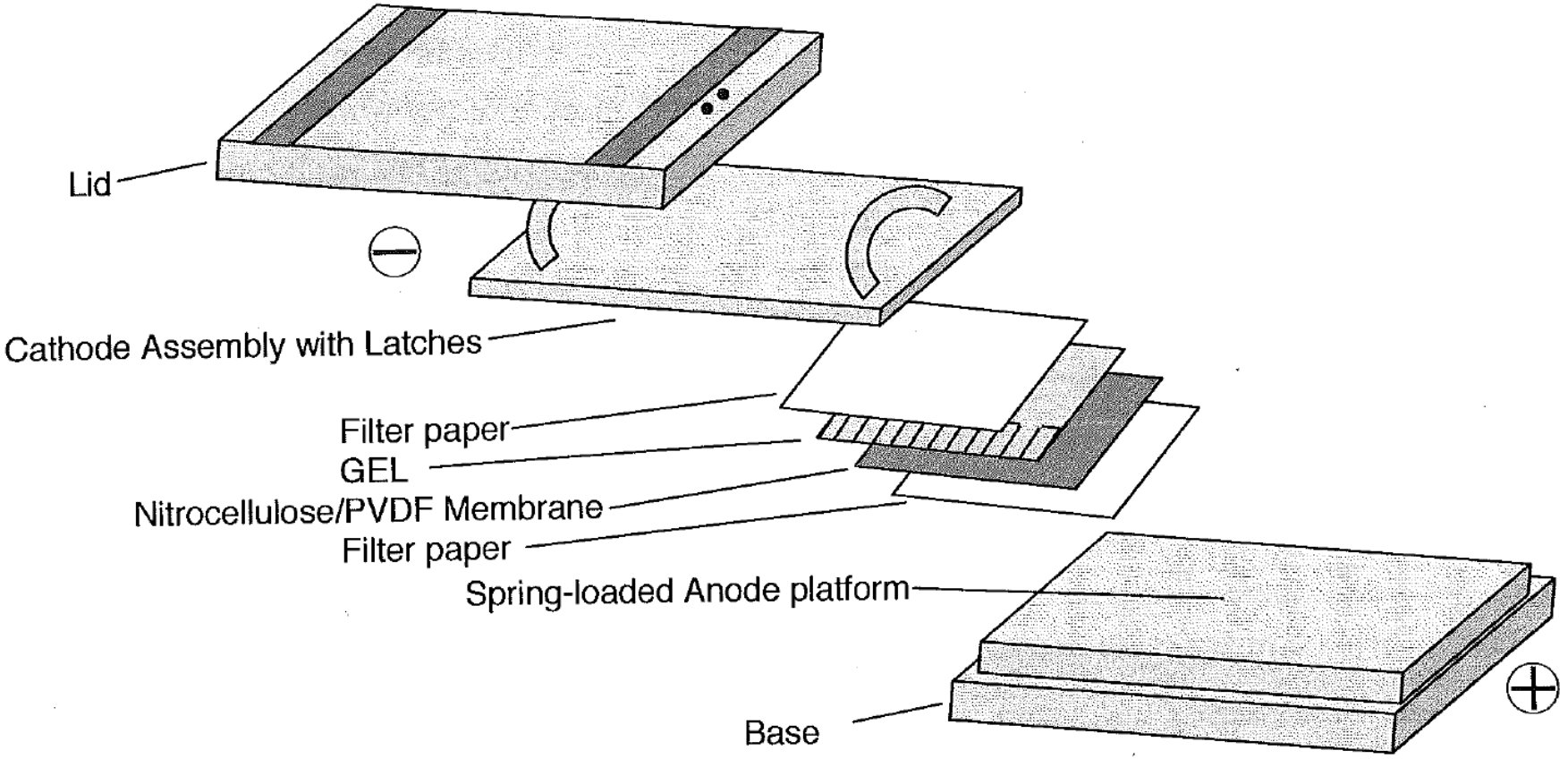
10E

**See updated procedures on Blackboard*

Procedure: 2nd and 3rd gels

- **Preparation of Immunoblot (IB):**
  - Remove Immunoblotting gel from plates
  - Set up for transfer to PVDF membrane
    - TF's will pre-soak filter paper in transfer buffer and PVDF membranes in MeOH, water and transfer buffer
  - Create "transfer sandwich" → gel & PVDF membrane between two pieces of filter paper
  - Get rid of **air bubbles**!
  - Place "sandwich" in **semi-dry transfer apparatus**, and run at 20 V for 30 min.
  - Recover blot membrane and mark orientation
    - You should see pre-stained NEB ladder on membrane!
    - Store membranes for next week to finish IB – Please label a ziplock bag clearly for each immunoblot you have with your section and blot name

Transfer Sandwich Setup



Procedure: Chapter 10D

- **Additional Gel Preparation:**
  - You will cast **new** gels for the next section during downtime
  - Prepare two 12% gels
  - Prepare both running & stacking layers
  - Leave combs in gels
  - **TFs must approve integrity and quality of your gels before you leave!**

Running Gel Solutions

Stock Solution	Volume per gel (mL)
1.0 M Tris-HCl, pH 8.8	1.9
40 % (w/v) acrylamide, 1.5% (w/v) bisacrylamide	1.5
10 % (w/v) SDS	0.05
Water	1.5
Ammonium persulfate, 100 mg/mL (10% w/v)	0.05
TEMED (tetramethylenediamine)	0.0035
TOTAL	5.0

Stacking Gel Solutions

Stock Solution	Volume per gel (mL)
1.0 M Tris-HCl, **pH 6.8**	0.63
40 % (w/v) acrylamide, 1.5% (w/v) bisacrylamide	0.56
10 % (w/v) SDS	0.05
0.5 M EDTA, pH 8.0	0.01
Water	3.695
Ammonium persulfate, 100 mg/mL (10% w/v)	0.05
TEMED (tetramethylenediamine)	0.005
TOTAL	5.00

Hazards

- Non-polymerized acrylamide (if any) must be disposed of special waste in fume hood
- SDS-PAGE running buffer can go down the sinks
- Polymerized gels can go into regular trash after imaging
- PAS staining reagents will ruin the sink traps underneath the benches
- Dispose of all PAS staining reagents and rinsates in a proper waste disposal container.
- Do all PAS stain work in the fume hood. Be careful not to stain your shoes/personal belongings.

Chapter 10D

One person can begin with the PAS staining while the other can do the Acquastaining. *(PAS staining involves lots of incubation time!)*

Help each other prepare samples.

When loading samples for the gel to do immunoblot, ensure that gels are not loaded symmetrically so that you can easily differentiate your samples from the other group.

Chapter 10D

- Additional immuno-blot gel
- His-tagged KHK samples
- Changes in sample preparation: dissolve lyophilized samples in WATER first, NOT 1XSB.
- May have control for GLUT-1?

Chapter 10D

Before the lab period, you should have:

- ✓ Completed your Pre-lab Write-up and submit on Gradescope
  - ✓ Title, purpose and procedures
  - ✓ Remember to include:
    - ✓ Calculations and table showing volume to resuspend lyophilized proteins
    - ✓ Calculations and table showing how to prepare serial-diluted his-tagged KHK samples
    - ✓ Recipes for casting gels
    - ✓ Gel loading schemes

At the end of lab, you should have:

- ✓ Imaged your Acquastain'ed gel
- ✓ Imaged your PAS stained gel
- ✓ Turned in your immunoblots to TFs (will probe in Week 4)
- ✓ Record order of loading for all gels
- ✓ Turned in TWO SDS-PAGE gels casted during lab period

Questions?

Discussion Quiz