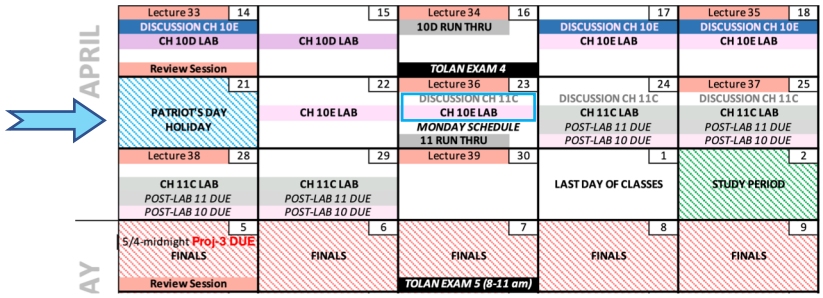
Announcements

- Laboratory Report Template for Chapter 10 was posted Thr 4/17 10 AM
- Chapter 10 Laboratory Report is due week of Apr 24 – 29
- If you need Acquastain and/or PAS-stained TF gels, email or Slack Dr. Ho and he will send via Slack.
- Next week Monday meets on Wednesday; Monday Discussion for Ch11 and B1 will meet at their normal times that day.



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Chapter 10E: Lipids and Membranes

Objectives

- To probe for the presence of a glucose transporter 1 (GLUT1) in prepared fractions using a GLUT1 specific antibody (Ab)
- Assess if GLUT1 is an integral or peripheral membrane protein
- To probe for the presence of different concentrations of His-tagged KHK using a His-tag antibody

Procedures

- To complete two **immunoblot assay** using either a glucose transporter 1 (GLUT1) specific or His-tag ***primary antibody***
- Visualize location of GLUT1 and his-tagged KHK with a **horseradish peroxidase conjugated secondary antibody** and **luminol with enhancers (ECL substrate)**

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Membrane fractions isolated from RBCs in Ch 10

In which fraction(s) would you "expect" to observe the integral membrane protein GLUT1?

- **Unwashed membrane** ☑
  - ***Integral and peripheral proteins***
  - Membrane is intact. All membrane associated proteins are present in this fraction
- **Washed membrane** ☑
  - ***Integral proteins***
  - Peripheral proteins removed with high salt membrane wash buffer
- **Washed supernatant** ☒
  - ***Peripheral proteins***
  - Removed from membrane using high salt membrane wash buffer

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Immunoblotting

- *Western Blotting – Used to identify a particular protein in a complex mixture*

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

☑ • **2nd Step:** Transfer proteins to a membrane in same position as SDS-PAGE separation

☑ • **3rd Step:** Incubate membrane with blocking buffer to prevent the non-specific binding of the antibodies (TFs will do it for you)

- **4th Step:** Incubate membrane, with attached proteins, to an antibody specific for a particular protein or proteins of interest
  - Often called the **primary antibody**
- **5th Step:** Detect binding of the primary antibody using a colored or visual reaction
  - Usually requires a **secondary antibody**

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Immunoblotting: detection of a target protein

Recap of what you did…

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

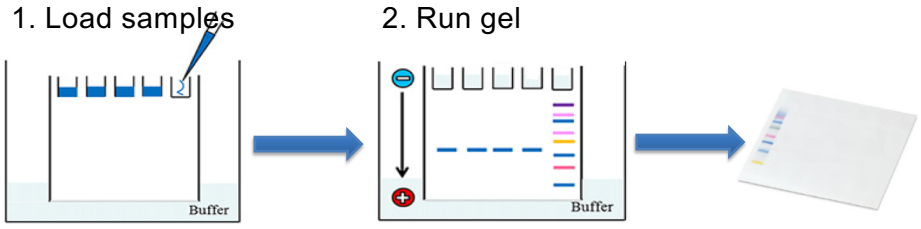
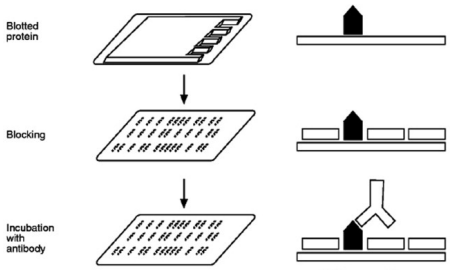


Image: www.sigmaaldrich.com

Immunoblotting: detection of a target protein

- **3rd Step:** Blocking of membrane by incubation with blocking buffer
- Prevent or reduce nonspecific binding of the primary and secondary antibodies to proteins on the membrane or to the membrane itself
- Reduce background and improve the signal-to-noise ratio



Typically, non-fat dry milk, gelatin, or BSA are frequently used as blocking agents

Immunoblotting: detection of a target protein

- **4th Step:** Probe for the protein of interest using a primary antibody (1˚ Ab). The major serum antibodies are made as immunoglobulins (Ig) class-G, or IgGs.
  - For this lab, will use an antibody raised against **GLUT1*** and a **His-tag antibody**

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

*This means that GLUT1 was injected into a host animal, usually rabbits or goats, and after a period of time, these animals have an immune response to the injected foreign protein. The animals produce antibodies that will bind to that foreign protein. These "anti-foreign-protein bodies" or antibodies are in the blood of the host animals.

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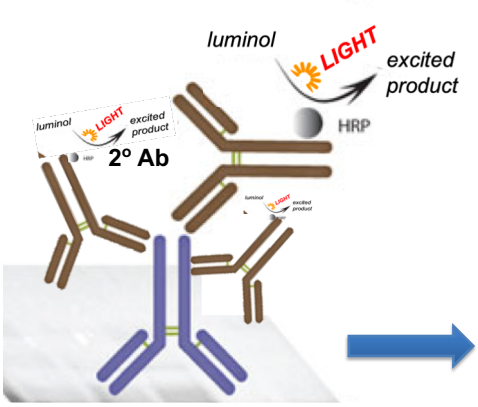
Immunoblotting: detection of a target protein

- **5th Step:** Detect 1˚ Ab using a 2˚ Ab to visualize where target protein appeared in the original gel



- Immunoglobulin recognizes and binds to 1˚ Ab
- Amplify signal of 1˚ Ab
- Allows visualization of protein of interest through reporter molecule

IB after development*:

*Composite

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

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

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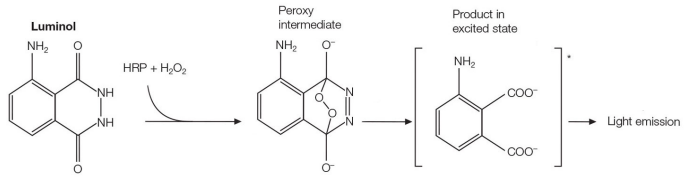
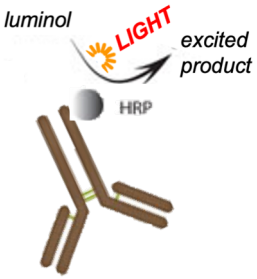
Immunoblotting: detection of a target protein

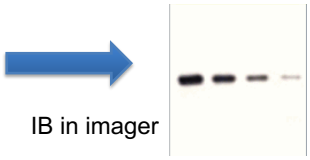
- **5th Step (continued):** visualization of protein of interest (chemiluminescence method)



Light signal can be captured on X-ray film or by a digital image



- However, luminol emits light only weakly, so enhancers are added to the reaction to increase the signal.
- Enhancement of a luminol-based signal is commonly referred to as enhanced chemiluminescence (ECL)



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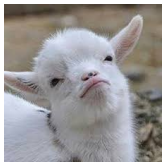
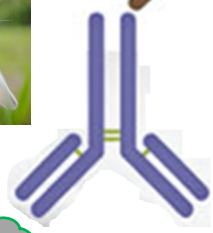
How are these reagents created?



HRP Gt IgG α Rb IgG 2° Ab



Rb IgG α GLUT1 1° Ab

From your samples

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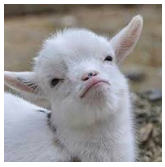
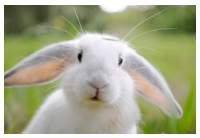
How are these reagents created?



HRP

HRP Gt IgG α Rb IgG 2° Ab



Rb IgG α His 1° Ab

KHK

From your samples

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Procedure: Chapter 10E

- **Probing of Immunoblots:**
  - You will find both of your membranes pre-blocked in blocking buffer (5% milk in 1X Tris-Buffered Saline (**TBS***)) in two separate containers**. Discard blocking buffer.

**

OR, each pair of groups, doing 2 blots, could each develop one of them for both groups

  - Begin with the **primary antibody incubation** step.
  - Use these nice plastic trays** for incubations

*50 mM NaCl, 20 mM Tris-HCl, pH 7.5

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Procedure: Chapter 10E

- **Probing of Immunoblots:**
  - Apply 15 mL primary antibody solution. We are using anti-GLUT1 antibodies made in rabbit (**Rb** α-**GLUT1**) for your membrane fractions immunoblot and 6x-His Tag Recombinant antibodies made in rabbit (**Rb** α-**His**) for the KHK immunoblot. The 1˚ Abs are in 5% milk/1X **TBS**.
  - Incubate membranes for 50 – 55 min at room temperature on shaker.
  - ***Decant your primary antibodies (separately!!) back into Falcon tubes and give the primary antibody solution back to your TFs after using it!***
  - Wash membrane with 15 mL 1X Tween-20 Tris-Buffered Saline (**TTBS)*** for 5 min on shaker (*repeat two more times*)

*0.05% (v/v) Tween-20, 50 mM NaCl, 20 mM Tris-HCl, pH 7.5

Procedure: Chapter 10E

- **Staining of Immunoblots:**
  - Incubate membranes with ~15 mL ***secondary antibody solution*** (2° Ab) for 50 – 55 min on shaker at room temperature. We are using anti-rabbit IgG antibodies made in goats with the enzyme, horseradish peroxidase attached covalently (**HRP Goat-IgG** α-**Rb-IgG** 2˚Ab). The 2˚ Abs are in 5% milk/1X **TBS**.
  - Wash membrane twice with 15 mL 1X **TTBS** for 5 min with shaking
  - Wash membrane twice with 15 mL 1X **TBS** for 5 min with shaking to remove residual detergent

Procedure: Chapter 10E

- **Visualization of Immunoblots:**
  - ➢ You will be using the Pierce™ ECL Western Blotting Substrate.
  - ➢ TFs will prepare ~12 mL substrate working solution by mixing 6 mL Detection Reagent 1 and 6 mL Detection Reagent 2*.
  - ➢ **Follow your TF up to the 3rd floor! One group at a time as you are ready.*
  - ➢ On the 3rd floor, you will incubate your blot with the substrate working solution for 2-5 minutes when you are at the chemiluminescent imager.
    - Remove the immunoblot from the working solution and place it on the transilluminator. Use a Kimwipe to remove excess liquid. (You may apply a clear plastic wrap on it if desired)
    - Visualize blot using the ChemiDoc™ XRS+

*This working solution is stable and can be re-used to visualize multiple blots within the hour. (***So, this 12 mL can be shared and re-used by the entire class, ask TFs***)

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Hazards

- Separate waste bottle for liquid western blot slutions

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CH10E Tips

- Primary antibodies is expensive! Please do not throw it away. RETURN TO YOUR TFS.
- Save the plastic trays after your western blot so that the next section can use it.
- The ECL substrate to visualize your blot will be shared with all the groups.

Chapter 10E

- Use of ECL substrate; we will NOT use the chloronapthol described on pages 352-353 (parts E.g and E.h). Instead, see slide 21.
- Use of His6 primary Ab in addition to the GLUT1 Ab

Chapter 10E

Before the lab period, you should have:

- ✓ Completed your Pre-lab Write-up and submit on Gradescope
  - ✓ Title, purpose and procedures
  - ✓ Staining reagent recipe
  - ✓ Flow chart of process

At the end of lab, you should have:

- ✓ Imaged both of your immunoblots (upload as part of post-lab)

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Questions?

Discussion Quiz