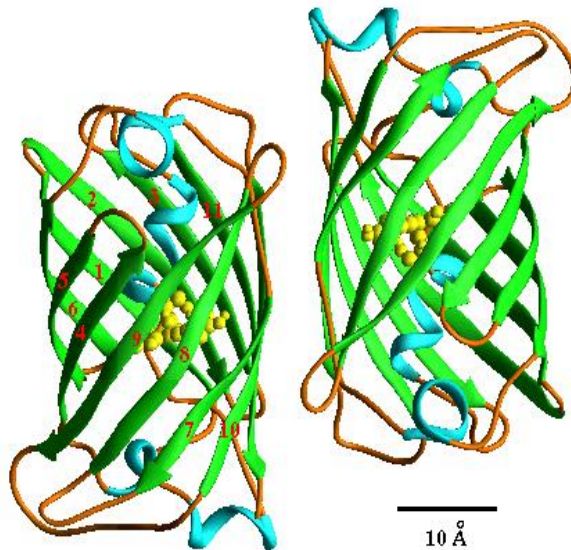


COC Biotechnology Program

Green Fluorescent Protein (GFP) Western Blotting



Macromolecular model of two GFP proteins
Version 10-26-05

- The GFP you purified in the Amgen lab series will be detected using Western blot technology.
- A vertical PAGE gel will be used to purify/isolate the GFP, and then the proteins will be transferred to a membrane and probed with a series of antibodies.
- The final antibody has been cross-linked with an enzyme that catalyzes a color change, making it possible to reveal the presence of the GFP.
- Blotting technology like this is used in forensics, research labs and in disease diagnosis technology in labs all over the world.

For more information on the College of the Canyons Biotechnology program contact Jim Wolf, Professor of Biology/Biotechnology at (661)362-3092 or Jim.Wolf@canyons.edu

I. Objectives:

- To become familiar with the basic theory and rationale of blotting procedures.
- To explain the rationale for the various reagents used in visualizing a blotted protein.
- To characterize the molecular mass of GFP using standards and interpolation of a graph.
- To refine basic lab skills including gel electrophoresis, blotting and handling a membrane during analysis.

II. Background:

Blotting techniques allow the researcher to transfer protein or nucleic acids to a membrane that is more stable than the gel in which they were initially separated. The transfer technique may use a form of simple capillary action or as in our lab, a blotting device that uses an electrical current to transfer the proteins. The term Western is a tongue in cheek homage to the first researcher to employ the technique of blotting. His last name was Southern and his original work was done with DNA. So RNA blotting became known as Northern and protein blotting became known as Western. Nucleic acids are usually treated in such a way as to help it to bind to the membrane (heating, UV light or certain chemicals can all help to bind the target molecule to the membrane). Proteins tend to hold strongly to the membrane because they have been treated with SDS (sodium dodecyl sulfate) a strong, negatively charged detergent) which holds the protein to the positively charged membrane. Heating or UV light will severely damage proteins and as a result is not used. Once the target is firmly attached to the membrane, a blocking agent is used to cover any portion of the membrane not covered already in the transfer. In this experiment, the proteins in dry milk will ensure that subsequent proteins used in probing do not just stick anywhere on the membrane, but instead bind antibodies to the target protein.

A crude GFP extract (similar to your earlier Amgen samples) will be added to a vertical gel rig along with some molecular weight standards. Prior to addition, the GFP (and standards) are all treated with SDS (in the sample (or loading) buffer. The SDS is a very powerful detergent. The non-polar tails stick in the non-polar pockets on the protein. The negatively charged heads all face outward, imparting a negative charge to the protein. As a result, it will migrate in an electrical field towards the positive pole according to its molecular weight. The transfer to the membrane takes place in an electrical blotting device and a series of chemicals are then added to probe for and ultimately visualize the protein. Many of the procedures relating to the vertical gel rig are explained in the protein diversity lab FYI.

III. SOP/Lab Activities:

Day One: Sample Preparation

- 1.1 From the provided stock GFP tubes (your saved GFP from the Amgen lab), add 25 ul of GFP to a labeled 1.5 ml microfuge tube. Add 10 ul of sample buffer (this has SDS in it).
- 1.2 Create a standard by aliquoting 10 uls of Kaledoscope standard to a 1.5 ml microfuge tube. Add 5 ul of sample buffer.
- 1.3 Heat the samples at 90 °C for 5 minutes.

Day One: Vertical Gel Rig Loading

Note: two - three teams of students should use one vertical gel rig

- 2.1 Attach a PAGE gel to box (remember to add a small film of silicone to the rubber seal on the gel rig prior to inserting the gel and remove the tape), fill with SDS running buffer and remove comb and tape.
- 2.2 Remove the comb and rinse all of the wells three times with 150 µl of SDS running buffer using a micropipette (it is not necessary to change the tip between samples).
- 2.3 Load the GFP samples (15 µl per lane) and the 2 protein standard samples (7.5 per lane). Remember to make a legend of the gel lanes so you can identify what was added to each lane and that two-three students teams should use one gel. The gel should have at least two (and preferably three) sets of 2 GFP lanes with two standards (on either side of the GFP lane. If possible, leave one lane between each set empty to allow for space.
- 2.4 Run the gel @ 40 milliamps. The required amperage will slowly change overtime, so check the scale periodically (~ every 15 minutes) and increase as needed. The gel will need to run for about 2 hours or until the marker dye has reached 1 cm from the bottom of the gel. During this time, prepare the needed reagents for the Western blot. Read part 3 for details.
- 2.5 Shut off the power and remove the electrophoresis buffer from the bottom chamber with a 10 ml transfer pipet and pipet pump.
- 2.5 Pour the buffer from the top chamber (using a funnel) directly into the electrophoresis buffer container. Once most of the buffer has been removed, remove the gel from the rig.
- 2.6 Clean the rig using soapy water first and then distilled water, invert it to allow it to dry.
- 2.7 With the instructor's help, remove the gel from the casing and nick the lower right corner (to help identify the gel lane's orientation as noted in your gel diagram).

Day One: Western Blotting of Gel

Part 3 Transfer of proteins from gel to membrane

Note: either PVDF membrane or nitrocellulose membrane can be used. PVDF requires pre-wetting with methanol. Nitrocellulose MUST NOT be wet with methanol. PVDF membrane is probably preferable to nitrocellulose because it is sturdier. Proteins are stable on this membrane for months after transfer.

Materials

- Forceps
- Gloves

- nitrocellulose cut to the size of the gel (1 piece per gel). Cut a small notch into one corner. Note: the Biorad nitrocellulose is exactly one-half the size of the gel
- Filter paper cut to the size of the gel (2 pieces per gel)
- Blotting pads

Transfer buffer

Trays

Power supply

XCell II Blot module including positioning wedge

Glass stirring rod or small piece of a plastic pipet.

It is very important to assemble your gel/membrane sandwich in the correct orientation. Otherwise proteins will go from the gel into the transfer buffer instead of onto your membrane. It is also important to wear gloves to avoid contaminating the gel or membrane with proteins from your hands and to protect your skin from methanol.

3.1 As the gel front approaches the bottom of the gel, be sure to have all of the materials you need for transfer.

3.2 Soak the filter paper, blot pads, and nitrocellulose membrane in transfer buffer.

Notch the membrane in a similar manner to the notch in the gel. Remember, do not touch the membrane with ungloved hands!

3.3 Slide the gel onto a pre-cut piece of filter paper. If it is not easily removed, it may be helpful to try this while immersing the gel in transfer buffer.

3.4 Set up your transfer as follows: Place two soaked blotting pads onto the cathode (black or negative) core of the blot module. Lift the filter paper with the gel and place it above the pads so that the gel faces up. Pour a little transfer buffer over the gel, and use a pipette (or glass stirring rod), to roll away any bubbles between the paper and the gel. Orient the membrane directly over the gel so that the notch matches that on the gel. Pour a little buffer onto the membrane, and use a broken plastic pipette to gently roll away any bubbles between the gel and the membrane. Cover the membrane with the other filter paper. Then put the two blotting pads on top.

3.5 If two gels are being transferred in the same unit, only put one blotting pad on top of the filter paper. Then the other group will place their sandwich directly on top of the first one. First the filter paper and gel are piled on top of the blotting pad. Pour a little transfer buffer over the gel, and use a pipette, to roll away any bubbles between the paper and the gel. Orient the membrane directly over the gel so that the notch matches that on the gel. Pour a little buffer onto the membrane, and use a pipette to gently roll away any bubbles between the gel and the membrane. Cover the membrane with the other filter paper. Then put the one blotting pads on top.

3.6 Add enough pre-soaked blotting pads to rise 0.5 cm over the rim of the cathode core. Place the anode (red or positive) core on top of the pads. The gels/membrane sandwich(s) should be held securely between the two halves of the blot module ensuring complete contact of all components.

3.7 Position the gel membrane sandwich and blotting pads into the cathode core of the blot module to fit horizontally across the bottom of the unit. There should be a gap of ~1 cm at the top of the electrodes when the pads and assembly are in place.

- 3.8 Hold the blot module together and slide it into the lower buffer chamber. (The plus sign should be seen in the upper left hand corner of the blot module). Insert the positioning wedge.
- 3.9 Fill the blot module with transfer buffer until the sandwich is covered with transfer buffer. Do not fill the unit all the way to the top.
- 3.10 Place the lid on top of the unit.
- 3.11 Plug the red and black leads into the power supply. Transfer at 35 V for at least one hour.
- 3.12 Following transfer, disassemble the rig and clean all of the related components by first rinsing in water, then in de-ionized water and placing onto the drying tray so that all items will dry completely (do not stack items too densely). the membranes can be wrapped in saran wrap and stored at 4°C or the membranes can be immediately blocked and probed. Be sure to label completely for later detection.

Day Two: Probing the membrane: (Glove should be used at all times)

Materials Required:

- Primary antibody solution: Anti-GFP diluted 1:1000 in 1 % milk in TBST
- Alkaline Phosphatase –conjugated anti-mouse IgG antibody diluted 1:3000 in 1 % milk in TBST
- NBT/BCIP detection reagent (0.46mM NBT, 0.43 mM BCIP in 0.1 M Tris, 0.1 M NaCl) pH 9.5. 200 ul of stock solution NBT/BCIP to 10 ml of detection buffer is correct amount of each coloring reagent.
- Blocking Solution: 5% (w/v) nonfat dry milk, 0.1% Tween 20
- TBS (Tris Buffered Saline): 50 mM Tris, 0.15 M NaCl, pH 8.0
- TBST: TBS with, 0.1% Tween 20
- Detection buffer: 0.1 Tris, 0.1 M NaCl, , pH 9.5
- 20 mM EDTA solution
- Blot transfer membranes

Review protocol and ensure that all reagents are added as noted.

The amount of each solution is about 10 mls, or enough to cover the membrane completely in the tray.

- 3.1 At this time, the membrane should be cut to allow each team to probe them independently. Be sure you note the membrane orientation and to cut the lower right edge to help identify this orientation.
- 3.2 Mark the location of the standards using a alcohol proof marking pen. Also mark the location of the gel wells across the top of the membrane.
- 3.3 Add Blocking Solution and incubate for at least 30 minutes at room temperature with mild agitation.
- 3.4 Pour off blocking solution (can be reused). Add 10 mL of primary antibody solution.
- 3.5 Incubate for at least 1 hour at room temperature with mild agitation. After incubation wash the blot three times for 5 minutes each in TBST.
- 3.6 Add the alkaline phosphatase-conjugated secondary antibody, diluted in blocking buffer. Use just enough to cover the membranes (about 10 mls)

- 3.7 Incubate for at least 30 minutes at room temperature with agitation. After incubation, wash the blot two times for 5 minutes in TBST and one time for five minutes in TBS.
- 3.8 Rinse the blot briefly using detection buffer.
- 3.9 Immerse the blot in the NBT/BCIP detection reagent. Stir once to ensure membrane is covered. Place tray aside and cover to keep membrane out of the light.
- 3.10 Continue development until visibly dark bands appear (this could take several minutes). Stop the reaction by immersing the blot in 20 mM EDTA solution.

Determination of GFP molecular mass. Using the membrane and gloved hands record the distance migrated of the standards and GFP. Remember to measure from the bottom of the gel well to the middle of the gel band. Use millimeter demarcations and provided graph paper and standard molecular weights to determine GFP's mass. Include this graph and a copy of the Western blot in the results section of your notebook.

IV. Post-Lab Questions/Activities The following post lab questions are for your benefit. The questions will help you to address a range of topics relating to the lab activity. Along with the post lab handouts, these questions will help to ensure that you have both correct information regarding the lab data and crucial lab processes. Complete the post lab questions at the end of the lab and post lab handouts (keys for both of these are available from your instructor) before making any lab-notebook entries.

1. Diagram a cross section through your blot showing the membrane, GFP, primary and secondary antibodies. For a hint, review your ELISA lab.
2. How could there be a false positive? Hint, consider the blocking agent role in your answer.
3. GFP has about 240 amino acids in it. Does this agree with your calculated molecular mass?

IV. Notebook Entries

Data from the lab should be the focus of this section and if there are any incorrect results, you should discuss this as well as expected results. The final section will contain both your results and discussion. Your data should drive the discussion. An informed discussion is dependent on understanding the post lab questions/activities.

Your intro should:

- Process of blotting, general steps and reasons (3-4 sentences only).
- Role of antibodies in process of identifying GFP location and visualization of GFP.
- Molecular weight idea and overall rationale for process.

Results should be:

- Western blot image with location of GFP and standard protein high-lighted. Do not forget to include the molecular weight of the standards.

- Graph of MW verses distance migrated for the standards and use interpolation to show the estimated weight of GFP. Be sure to label all axis and record the GFP weight.

Discussion should consider the following:

- What the process successful? If not, provide possible reasons.
- Conclude with a few sentences stating the usefulness of this technology.

The previous lab protocol can be reproduced for educational purposes only. It has been developed by Dr. Beth Miller and Professor Jim Wolf. and/or those individuals or agencies mentioned in the references.

References:

Professor Becky Green-Marcellin, Valley College, San Fernando, CA

Invitrogen life technologies instruction manual. X-Cell 2 Blot Module: Catalog no. EI9051. Version 1, 9, Dec, 2003 iM-9051

DIG Nucleic Acid Detection Kit. Cat # 175-041. Instructional Manual Version 2, Feb 2001.