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Chapter 2

Teaching Western Blots with T Antigen and p53

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Introduction

Western blots are a logical addition to polyacrylamide gel electrophoresis (PAGE) of proteins. PAGE gels stained nonspecifically for all soluble cell proteins reveal a few prominent major structural proteins plus a faint smear of the thousand or so minor proteins. Westerns can reveal minor proteins one at a time using specific antibodies. We demonstrate two examples by blotting gels to nitrocellulose paper that binds all proteins; then we expose the nitrocellulose to monoclonal antibodies to reveal the oncogene SV40 T antigen or the tumor suppressor p53. If a teaching lab is going to do PAGE anyway, the additional work for Western blots would be one overnight blotting step, one additional lab period for the immunochemistry, and a little more time for data analysis.

Despite wide use of Western blots in current research, cell biology texts give scant coverage of them so I recommend instead the Web sites listed below as literature sources. There is some (minimal) discussion in Alberts et al. (2002). [Westerns are discussed on p487; Northerns and Southerns on p498-499; p53 on p344; and SV40 transformation on p347.]

Student Outline

Background

Southern blots

To recapitulate the "geography" of blots, Southern blots ("Southerns") were invented by Edward M. Southern in about 1974 (Southern, 1975) and are named for him. (Also see the following Web site: http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/G/GelBlotting.html.)

Southerns detect restriction enzyme fragments of DNA containing a particular gene's DNA sequence. Restriction fragments are made from whole genomic DNA using one or more restriction enzymes, separated by electrophoresis in an agarose gel, and then transferred out of the gel onto a DNA-binding membrane (the "blot"). The DNA stays attached to the blot but can hybridize in solution by Watson-Crick base pairing to labeled DNA (the "probe") which contains the gene sequence. Probe DNA is usually a cloned plasmid or viral DNA. Probe labeling is done either with a radioisotope or by non-isotopic procedures based on antigenicity, similar to Westerns. The probe hybridizes to one or a few restriction fragments on the blot, identifying the ones containing the gene. Presence of hybridized probe on the blot is detected with x-ray film; labeled probe makes bands on the film. Southerns are sensitive enough to detect single-copy genes in a complex mammalian genome. From measured sizes of the restriction fragments on the gel, Southerns can be used to make gene maps.

Northern blots

Northern blots (Alwine, et al., 1977) were developed later, modeled on Southerns. (See http://www.bio.davidson.edu/courses/genomics/method/Northernblot.html.) Northerns were named facetiously at first because of similarities to Southerns but the name has stuck. Instead of DNA restriction fragments, whole cell RNA, or poly A-containing RNA, is separated on a special denaturing agarose gel to keep RNA intact and single stranded. The rest of the procedure is the same as Southerns. Northerns are also very sensitive and can detect one copy of a particular mRNA per cell.

Western blots

Western blots, ("Westerns") are similar in principle to Southerns and Northerns; but they detect proteins containing particular antigens (epitopes) instead of nucleic acids containing particular sequences. All three methods detect the presence of a particular macromolecule (restriction fragment of DNA, whole mRNA, or protein) by a black spot or "signal" on x-ray film and all three measure the size of a macromolecule by its electrophoretic mobility. The unique features of Westerns are that the gel is polyacrylamide, not agarose, and the probe is an antibody, not labeled DNA. Western blots are used widely in basic research to detect expression of a particular gene by detecting an antigenic determinant (epitope) found only in its particular gene product. Westerns have many practical applications including several in medical diagnostics and forensics. For example, a test for infection by HIV, the AIDS virus, is detection of HIV epitopes in a patient's circulating blood. For an amusing online summary and review of Western blots, go to "Mama Ji's Molecular Kitchen" at http://lsvl.la.asu.edu/resources/mamajis/Western/Western.html.

In our lab, we illustrate Westerns by detecting the oncogene SV40 T antigen and the tumor suppressor p53 in SV40-transformed cell lines. The antibody PAb 122 can detect p53 in most mammalian cells, including human cells.

NOTE: Teaching Westerns starting from scratch can take three or four lab periods of 3-4 hours each. The work is not continuous, so time could be shared with other projects. If the lab is doing polyacrylamide gel electrophoresis (PAGE) of proteins anyway, Westerns would take one extra overnight blotting step, one extra 3-4 hour lab period for detection, and an additional hour or two for data analysis. Here I am assuming we start from scratch with cultured cells.

Outline of first period

We prepare extracts from cell cultures and do a protein assay on part of an extract. We add SDS and mercaptoethanol to the other part of the extract and store that part frozen until the next lab.

Making cell extracts

The cell extracts we use to make Westerns are mixtures of soluble proteins extracted from cell lines grown in culture. Making extracts from monolayer tissue culture cells is particularly easy because the cells are attached to Petri plates and so they can be rinsed by pouring solutions on and off the plates. We rinse the cells to get rid of serum proteins in the growth medium. Then we put on a lysis buffer containing the nonionic detergent Triton X-100 that dissolves all membranes, including organelle membranes. We scrape the lysed cells into a microfuge tube and centrifuge to pellet nuclei and cytoskeletons, which would otherwise make trouble. It is important to avoid using an ionic detergent (instead of a nonionic detergent) at this stage in the procedure because that would dissociate the chromatin structure and release soluble DNA before we could get rid of the nuclei. Soluble high molecular weight DNA is very hard to handle. The extract is a cytoplasmic supernatant, not a nuclear pellet, but it contains the soluble proteins of nuclei because Triton X-100 makes nuclei leaky. The extract therefore contains nearly all proteins of the cell, lacking only very hydrophobic membrane proteins, insoluble cytoskeleton proteins, and some chromatin proteins, particularly histones, which remain with remnants of nuclei. Extracts contain typically 10 major structural proteins and over a thousand different minor proteins, resolvable on two-dimensional gels. We characterize two of the minor proteins without resorting to two-dimensional gels. We could characterize many other proteins if we had the appropriate antibodies.

After getting rid of nuclei we add SDS and beta-mercaptoethanol to the extracts and store them frozen until the next lab period. We also save a little of the extracts without SDS and beta-mercaptoethanol for a protein assay and we do that as soon as possible. The protein assay is a communal lab project with all of the students. We want to load equal amounts of protein in each slot of the gel so we can make comparisons between the different samples on the gel without having to worry about differences in protein concentrations.

Each team gets two plates of cells but keeps plates in the incubator until the very last minute, until everything is ready; because cell cultures will deteriorate if left out of the incubator more than a few minutes. Because the procedure has several steps, students should rehearse the procedure before doing it. Each team of 2-4 students should have:

One ice bucket, with ice, containing

- Tube of 40 mL PBS for rinsing cells free of serum, on ice
- Tube of 20 mL WWB for removing divalent cations and adjusting pH on ice
- Microfuge tube of 1 mL lysis buffer on ice. Contains the nonionic detergent.
- Empty 1.7 mL microfuge tubes on ice, two for each culture, marked with name of cell lines. One will contain the pellet fraction, one the supernatant fraction.
- Microfuge tubes containing 20 µL LSB, one for each cell line, marked accordingly
  (LSB contains SDS, beta-mercaptoethanol, glycerol, and a blue dye.)
- Plate scraper: silicone rubber sheet or piece of old x-ray film,1x3 cm plus clamping forceps
- Large beaker for waste
- Access to microfuge
- 3 Pipetmen with tips, P20, P200, P1000
- Sharpie marker

The class as a whole should have:

- Vortex mixers, one per two or three teams

When all materials are ready, remove one culture from the incubator and immediately dump its medium into the waste beaker. Toss the plate lid. Immediately rinse the plate by pouring on ~5 mL cold PBS. Swirl the PBS around in the plate for about 5 seconds and pour the rinse into the beaker. Repeat the PBS rinse. Rinse once again with ~5 mL WWB. Pour that rinse into the beaker and drain the plate for about 30 seconds by putting it on ice on a slope so the residual WWB runs off to one side. Keep the plate cold by keeping it on ice as much as possible. Using the P1000 remove the puddle of WWB and put that into the waste. Get rid of as much liquid as possible but don't scrape the cells off the plate, yet. Next, make the plate as flat on the ice and add 100 µL lysis buffer. Tip the dish so the lysis buffer spreads over the whole dish, but keep the plate cold. Use the scraper to scrape off the cells to one side of the plate. Try to keep the cells cold. Now pour the scraped cells into an empty microfuge tube by letting the cell suspension run down the scraper from the Petri dish into the microfuge tube held on ice. There should be only 5-10 drops of cell suspension per plate. Keep the suspension on ice. Wait until you have one or more similar tubes from other students to balance the microfuge; then spin in the microfuge for only 5 seconds at 10,000-14,000 rpm. As soon as the rotor stops put the tubes back on ice. Using a P200, remove 80 µL of cytoplasmic supernatant, avoiding the nuclear pellet, and put it into the microfuge tube containing 20 µL LSB. Mix cytoplasmic super with LSB by vortexing for 2 seconds. Save remaining cytoplasmic supernatant on ice (without LSB) over the nuclear pellet for a protein assay. If you cannot do the

assay on the same day, separate the extract from the nuclei and toss the nuclei. The extract with LSB is stable indefinitely and can be stored frozen. The extract without LSB is stable enough for protein assays and can be stored frozen indefinitely or at 4 °C for a week.

Measuring protein concentrations of extracts using the Pierce BCA protein assay.

Here we learn an assay for protein, a standard procedure in biochemistry and cell biology. Students will learn to make precise tiny measurements with a Pipetman and to use a spectrophotometer. We use this particular assay because our extracts have detergent in them and detergent makes a bad background in the more popular Bradford or BioRad assay where proteins are stained with Coomassie Blue dye. The BCA assay is a variation on the classic Lowry protein assay. The method uses the Biuret Reaction which is a reduction of Cu^{++} to Cu^{+} by protein at high pH and then formation of a highly colored (purple) compound by reaction of Cu^{+} with bicinchoninic acid (BCA). The functional protein groups that reduce Cu^{++} are the side chains of cysteine, tryptophan, tyrosine, and to a lesser extent, the peptide bond itself. That is how the amount of protein in the cytoplasmic cell fraction becomes related to purple color. Since color development is strongly dependent on the presence of some particular amino acids, and proteins differ in amino acid composition, a particular protein (here bovine serum albumin, BSA) cannot be a perfect standard for all proteins. We calibrate the assay with a series of BSA concentrations so we should say that the numbers we get are "BSA equivalents." The assay is very sensitive so we usually first make a 1/10 dilution of the cytoplasmic material to get the signal on scale, to be able to bracket the unknowns with standards. If we use straight undiluted cytoplasmic fraction, the signal might be off scale.

Although the BCA assay tolerates Triton X-100, it does not tolerate all reagents, particularly mercaptoethanol. A list of other incompatible reagents is on the Pierce web site **<**http://www.piercenet.com**>.**

Color development, saturation, temperature sensitivity

The BCA reaction color development never really stops; the tubes get darker and darker with time. For that reason it is important to take all readings at about the same time. We should stagger the addition of the chemicals at 30- or 60-second intervals and take readings later at 30- or 60-second intervals; then each tube will have the same time of color development. However, if we use the 37° C protocol below, the time staggering is less critical.

Color development is temperature sensitive. The "standard protocol" for a working range of samples at 20-2000 µg protein/mL is incubation at 37° C for 30 minutes. Above 2000 µg/mL, the assay saturates which means the purple color does not get darker with increasing protein concentrations even though it does get darker with time. The room temperature protocol gives equivalent results but takes longer because of the colder temperature, 2 hours at room temperature or 22° C instead of 30 minutes at 37° C. When we want extra sensitivity (not here), we can choose the "enhanced protocol" where we incubate the BCA reaction at 60° C for 30 minutes; then the working range of the assay is only 5-250 µg protein/mL. The enhanced protocol is 4 times more sensitive but the price we pay for that is that the assay saturates at 250 µg/mL instead of at 2000 µg/mL. We will try the standard protocol first and students are free to experiment with the other protocols.

Add standards and diluted unknowns to dry assay tubes

Add 10 µL of each known BSA concentration in lysis buffer to separate marked glass tubes (the standards). Include a zero tube, with no protein in it, just 10 µL of lysis buffer. In a microfuge tube

make a 1/10 dilution of the cytoplasmic cell fraction in lysis buffer: Mix 5 µL cell extract with 45 µL of lysis buffer. Add 10 µl of diluted cytoplasmic cell fraction to each of two glass assay tubes (duplicates). Measure very carefully because the accuracy of the assay depends on the accuracy of each pipetting step. Make sure you have 10 µL of lysis medium in every tube you use in this assay including the BSA standards and the unknowns, the extracts.

Make working reagent

Count all of the assay tubes to be done, standards and unknowns. Each unknown should have two tubes. Then pretend you have two extra tubes, to be really sure you will have enough WR. The number of tubes (plus 2) is the number of mL of WR you want to make. Each 1 mL of WR consists of 980 µL of Reagent A plus 20 µL of Reagent B. Mix enough A and B together in a 50-mL plastic test tube to do all of your tubes (plus 2). WR is stable at room temperature during class but not overnight.

We assume here that the assay will be done in a standard spectrophotometer with 1-mL plastic cuvettes. If your spectophotometer is a Spectronic 20 which uses an assay tube of 3 or 5 mL capacity, the assay will have to be scaled up in volume. If the lab has enough cuvettes to use a separate cuvettes for each assay, we will not have to rinse and re-use the cuvettes during the assay. In our lab we do soak, rinse, and dry the "disposable" polystyrene 1-mL cuvettes between assays to re-use them many times.

BCR reaction

Using a P1000 add 1000 µL of WR to each assay tube (already containing 10 µL), and vortex. Place the rack of tubes in the 37 °C water bath. After 30 minutes, take the rack out and cool it to room temperature. You should look at the tubes comparing them with each other to see which ones are light and which are dark. You might be able to do a rough assay by eyeball alone, ranking the tubes by darkness of their color and bracketing unknowns between the knowns. But the spectrophotometer is always more quantitative than the eyeball assay.

Make sure the spectrophotometer is on and set at 562 nm. Put deionized water in a cuvette, wipe it clean, and put it in the instrument. Push the zero button. You should get a reading of zero. Now read the A_{562} of your zero tube (no knowns or unknowns, just lysis buffer); it will tell you how much A_{562} (compared to water) is from the lysis buffer and the assay chemicals. Record that number; we hope it is less than 0.15. Now do the rest of the tubes.

If you find that your calibration curve is scattered or if your duplicate samples are not within 15% of each other, you are probably having pipetting difficulties and you might have to repeat the assay. That is why it pays to take the time to be careful with pipetting. If all of your unknown tubes are too dark (darker than all of the standards), you probably forgot the 1/10 dilution of your cytoplasmic fractions in lysis buffer.

Make a calibration curve and calculate concentrations

This part could be done after class, but if you have the time, do it during class in case you have to repeat the assay before you can calculate the amounts to load on the gel. Use Cricket Graph or Delta Graph or Microsoft Excel to graph the standards. (We will show you how if you do not know). Make an equation, a functional relationship between absorbance and BSA equivalent protein concentrations and then use the equation to calculate protein concentrations in your unknowns.

Calculate the protein concentrations of your cytoplasmic fractions, in BSA equivalents. When calculating don't forget the 1/10 dilution.

Now calculate how much of the extracts with LSB to load on the gel. From the dilution of the extracts by adding 5xLSB, calculate the protein concentrations in the tubes containing the LSB addition. (You did the assay on the extracts *without* LSB but you will load the gels using extracts *with* LSB.) Find the most dilute extract in the group you want to load; this will require cooperation between all students using that gel. Plan on loading 12 µL of the most dilute one. Now calculate the volumes to load of all the others; they will all be less than 12 µL to give the same amount of protein.

Second lab period: SDS-PAGE gels and overnight blotting

Students will be making polyacrylamide gels, running one or two SDS-PAGE gels, and beginning overnight blotting. The gel running will take two hours and other projects can be done during that time. Blotting overnight requires that somebody come in the next morning to remove the blots from the gel box and put them in the refrigerator, in transfer buffer. Blots can be stored in the refrigerator for at least a week.

SDS-PAGE

Thaw the extracts containing LSB (with SDS and beta-mercaptoethanol), vortex briefly, and heat the extracts to 85 °C for 1-2 minutes using the Poly Hot Pot. The water in the Poly Hot Pot should be no more than 1/3 full in case the tubes pop their tops during heating. SDS plus heat destroys non-covalent molecular interactions within and between proteins, and beta-mercaptoethanol breaks and blocks covalent disulfide bonds between cysteines. What we have left is linear polypeptides bound to SDS. The SDS gives the polypeptides negative charge so that they move toward the anode (the plus electrode) in polyacrylamide gel electrophoresis (PAGE). We include some pre-stained polypeptides in one of the gel lanes. The stained proteins are colored so we can watch the electrophoresis in progress. After we get Western results we can use the distances migrated of the colored bands to compare with distances migrated of the Western blot antigen proteins to estimate sizes of the antigens. We stop the electrophoresis when the smallest stained polypeptide (which runs the fastest) gets close to the bottom of the gel. We skip gel staining and go directly to overnight blotting. .

Blotting concepts

Gel proteins are next transferred (by blotting) from the polyacrylamide gel to nitrocellulose (NC). NC binds all negatively charged macromolecules in solution including polypeptides conjugated to SDS. NC is the same membrane sometimes used in Northerns and Southerns because it also binds RNA and DNA via their negatively charged phosphate groups. Blotting is necessary because we cannot work with the proteins inside the polyacrylamide gel; antibodies cannot get inside the gel. In Northerns and Southerns we use passive blotting with paper towels which draws liquid and DNA/RNA out of the agarose gel onto the NC. But getting polypeptides out of a polyacrylamide gel is harder than getting DNA and RNA molecules out of an agarose gel so we use electrophoretic blotting ("electroblotting") which is easy to do with the right equipment. We put the gel against a sheet of NC and apply an electric field overnight. As in Southerns and Northerns, NC binds the molecules as they leave the gel. We judge the success of blotting by looking for the pre-stained colored polypeptides we used as size markers in PAGE. The colored markers should have exited the

gel, leaving it colorless, and they should have stuck to the NC making colored bands there. After gel proteins have been blotted to NC, they can be stored stably on the NC at 4 °C in the transfer buffer.

Second period details

Recipe for one 6% resolving gel, for T-antigen. This gel resolves large polypeptides, between 80 kDa and 200 kDa. T antigen should be between 90 and 150 kDa, depending on the cell line.

- Deionized water 2.0 mL
- Acrylamide stock 1.0 mL
- Resolving gel buffer 1.88 mL
- 10% SDS 62 µL
- TEMED 5 µL
- 10% APS (add last) 25 µL

Recipe for one 9% resolving gel, for p53.

- Deionized water 1.5 mL
- Acrylamide stock 1.5 mL
- Resolving gel buffer, pH 9 1.88 mL
- 10% SDS 62 µL
- TEMED 5 µL
- 10% APS (add last) 25 µL

Fill resolving gel to a mark 1 cm below bottom of comb teeth but with comb removed. Overlay resolving gel with water using gravity feed (no plunger) from a syringe and needle to make a flat top on gel. The gel will polymerize 5-10 minutes after adding the APS. Polymerized polyacrylamide forms a sharp interface under the water layer and the layer should not move when the gel box is tipped to one side. After gel is polymerized pour off water and drain the gel upside down until you are ready to pour the stacking gel.

Recipe for two 4% stacking gels

- Deionized water 3.65 mL
- Acrylamide stock 0.67 mL
- Stacking gel buffer, pH 6.8 0.63 mL
- 10% SDS 50 µL
- TEMED 5 µL
- 10% APS (add last) 25 µL

Pour stacking gel with comb in place; fill to overflowing. Leave comb in place until ready to load samples. Pull comb out slowly after running buffer is added to gel box. Running buffer should fill the wells as comb is pulled out; doing it this way avoids rinsing the wells.

After the comb is pulled out, load the samples. Use a P20 with either the standard yellow tips or with special long thin white gel loading tips. The white tips can poke right down into the well while the yellow tips are too fat to go between the glass plates so you must let the sample just fall into the wells from the top of the plate. I can work faster with the yellow tips so I prefer them but beginning students feel safer with gel loading tips. Loading will take practice until you have done it two or three times so maybe practice first with just dilute LSB. After you have confidence, rinse out the

dilute LSB from the wells with a Pasteur pipette or a gel loading tip and then load the real stuff. Load 5 µL of the colored markers first. Then load the calculated volumes of extracts with LSB.

After all of the lanes are loaded on the 6% and 9% gels, fill the buffer reservoirs to the top and turn on the power. Filling the buffer to the max might waste buffer but it minimizes possible leaks and the buffer serves as a cooling sink for the electrophoresis, which generates lots of heat. (Heat is proportional to I^2R, Ohm's law.) Run at 100-120 volts for about 2 hours or until the smallest colored markers reach the bottom of the gel. Look for the marker proteins too small to be resolved; they will run all together in a tight bright band at the solvent front. Then turn off the power and begin blotting. It is possible to use PAGE running buffer 5 times so don't throw it away after your electrophoresis is finished

Blotting details

For each gel we need

- two 8 x 10 cm filter paper pieces
- one 8 x 10 cm sheets of NC
- blotting equipment
    - electrode assembly, contains electrodes and 2 slots for gel clamps
    - one clamp per gel, 2 clamps per electrode assembly
    - two fiber pads per clamp, 4 altogether
- Gel box bottom
- Gel box lid containing 2 wires with jack plugs to power supply
- Power supply capable of producing 15 VDC
- Western transfer buffer
- Small pan containing deionized water
- Scissors

A dry NC sheet is wetted by floating it (not submerging it) on the pan of deionized water. When thoroughly wet, it is finally submerged; then the water is poured off and replaced by Western transfer buffer. This should be done at least a few minutes before the NC is used but it could be done hours before.

After the PAGE is finished, (the smallest colored marker protein has almost reached the bottom of the gel), the power is turned off and the gel plates are pried apart using one of the spacers as a lever. The gel will stick to one plate or the other; it does not matter which. Using one of the spacers as a knife, cut off and discard the stacking gel and unused resolving gel while the gel is still attached to the glass plate. It is advantageous to make the gel and the blot as small as possible.

Put one of the dry pieces of filter paper over the trimmed gel on the glass plate and press it against the gel. Then turn the gel over and looking through the glass make the gel attach to the paper and peel it off the glass with the paper. Place the paper with gel attached on a fiber pad of the blotting apparatus and place the pad on the black perforated side of the clamp. Next drape wet NC over the gel, put another piece of dry filter paper on top of the NC, and then put on the other fiber pad. Close the clamp and slide it into a slot on the blotting gel box. Keep the black side of the clamp towards the black side of the blotting gel box. Repeat for another gel because the Western blot gel transfer box holds two gels. Put the blotting gel box in the gel box and fill the box with Western transfer buffer. Attach the top with wires to the power supply. If you are blotting overnight (recommended), adjust the power to 15 volts. It is possible to blot at 50 volts for 3 hours but then the system heats up so much it needs cooling. It would be best to run it in a cold room at 50 volts.

After blotting overnight turn the power off and recover the NC. While the NC is still attached to filter paper, trim the NC to the smallest possible size by using scissors to cut right through NC and paper. (Don't cut the fiber pads.) There will be an impression of the gel on the NC to show you where to cut with the scissors. You may store the NC blot in transfer buffer in the refrigerator for several days if you cannot proceed to the next step right away. Or you might do the blocking step right away and store the blocked gel in blocking buffer overnight in the refrigerator.

Third lab period: blot blocking, antigen detection

The third period includes blocking of blots, antibody treatment, and antigen detection using enhanced chemiluminecence with x-ray film. Handling x-ray film requires a darkroom with a safelight. Blots are blocked in milk-containing blocking solution for 30 minutes, exposed to the first antibody for 30 minutes, washed for 30 minutes, exposed to the second antibody for 30 minutes, and washed again for 30 minutes. The blots are then bathed in the revealing chemicals for 2 minutes and exposed to x-ray film for 1-5 minutes (usually) or up to 60 minutes for especially low-abundance proteins. Then the film is developed and we allow about 20 minutes for that. The third period therefore takes 3 hours without time for repeating a procedure. There will be some low activity periods and maybe we could use those times for some other activities.

Blocking – concepts

After the polypeptides have been transferred out of the gel and bound to the NC, the NC is "blocked" to make it non-receptive to further protein binding. We do that by bathing the NC in lots of the milk protein casein, which saturates the remaining protein binding sites. We use casein in the form of a 5% solution of nonfat dried milk in TBS (Tris-buffered saline), because it is cheap and because it works. Blocking is important because the next step is bathing the NC in an antibody solution. If NC were not blocked against further protein binding, the antibody (also a protein) would bind mostly to the NC and not just to the polypeptide of interest. If the antibody bound to NC alone we would get terrible background in our Western blots, lots of black marks where we do not want them.

Blocking – details

You should be supplied with blocking buffer, which is a solution of nonfat dried milk in TBS. Put 20 mL of blocking buffer in a small pan and add one or two blots. Notice that the colored markers on the blot are brighter on one side of the NC than on the other; when putting the blot in the pan, leave the brighter side *up*. The brighter side is the side that was closest to the gel and is the side with the most protein on it. Keep the brighter side up throughout all of the procedures today, the blocking, 2 antibody exposures, 2 intervening washing steps, and detection. Shake the pan just hard enough to make the blots move around, without sloshing the blocking buffer out of the pan. If you shake two blots in one pan (possible but tricky), make sure the two move independently and don't stick together. It is easier to shake blots in separate pans. Shake for 30 minutes at room temperature.

Blocking could be instead done in the refrigerator (without shaking) between lab periods. If the blocking is longer than overnight, I would add 1 mM sodium azide (poison!) to the blocking buffer to prevent some kinds of microbial growth in the rich milk medium.

Exposure to first antibody – concepts

The blotted and blocked NC is next treated with an antibody, which should bind to the protein of interest and nowhere else. We have chosen antibodies against two minor cell proteins, but there are of course many other possible choices. The minor proteins we detect are 1) SV40 T-antigen, an oncogene expressed in SV40-transformed human and mouse cells, and 2) p53, a famous anti-oncogene or tumor suppressor gene product expressed by nearly all cells. SV40 is a DNA tumor virus that sometimes integrates its genome in cells it infects; then the infected cell expresses T antigen continuously. The cells with integrated SV40 DNA are called "transformed" because they usually have altered, cancer-like properties compared with uninfected cells. I picked some SV40-transformed cell lines that express different sizes of T-antigen and different levels of p53, which make the Western blots interesting (see Figure 1 below). Detection of T is easier than detection of p53. Comparisons between T and p53 and between the different cell lines will illustrate a wide range of T and p53 levels and a wide range of Western blot sensitivity. The relevance of this work to the real world is that the protein p53 is found to be mutated and non-functional in more than 50% of human cancers so functional p53 is thought to be important in tumor surveillance, in natural protection against cancer.

Our anti-T and anti-p53 antibodies (used as the first antibodies in the Western procedure) are monoclonal antibodies produced by mouse hybridoma cell lines which we can grow ourselves in the lab. Each hybridoma produces one particular IgG antibody and excretes it into its tissue culture medium. The antibody preparation we use is therefore simply hybridoma cell culture medium after the cells have grown in it for a while. The hybridoma cell lines and instructions for their culture are available from the author on request.

Exposure to first antibody – details

Each team gets

- Blocking buffer, 5 mL
- Parafilm for making custom pans for antibody exposure, one sheet of 8x10 cm per blot
- First antibody is supernatant medium from hybridoma cells. Each team will get 1 mL of each kind of antibody. Store in the refrigerator or on ice. There are two kinds of antibody.
    - PAb 108 is for revealing large T antigen
    - PAb 122 is for revealing p53

      Use only one antibody at a time. The class as a whole will use both antibodies on different blots, both antibodies on each cell line.

      Mix 1 mL antibody with 2 mL blocking buffer shortly before use.
- Forceps
- Parafilm sheet, 8 x 10 cm
- 3 mL second antibody diluted 1/1000 in blocking buffer

First washing

After treatment with the first antibody the NC is washed five times for 4-5 min each time in 20 mL tris-buffered saline (TBS) to remove excess weakly-bound first antibody. The tightly bound first antibody remains attached to antigen on the NC; tightly- bound first antibody does not wash off. We want only tightly bound antibody.

Each team should have
- Two plastic (not Parafilm) blot washing pans per blot, four pans for two blots.
- 120 mL TBS

Pick the blot out of the Parafilm pan after 30 min or more of first antibody exposure and put it in a pan containing 20 mL of TBS. Shake the blot pan five times for 4-5 minutes each time. (Longer does not hurt except you might not have time to finish). Switch pans between washes; start each wash with a cleaned pan. Transfer the NC from one pan to the other, also containing 20 mL of fresh TBS, using forceps. Rinse the forceps each time you rinse the pans. While the blot is washing in one pan, rinse the other pan in tap water and shake it nearly dry. After five washes, keep the NC in the last TBS rinse until you are ready for the next step; never let the NC dry out.

Exposure to second antibody

The second antibody is a commercial rabbit or goat serum from an animal immunized to mouse IgG. Since the first antibody is a mouse IgG the second antibody will bind to the first antibody. The epitope on the first antibody to which the second antibody binds is in the constant region of mouse IgG heavy chains. The second antibody is also covalently conjugated to horseradish peroxidase (HRP). HRP is essential in the detection scheme, as described below in "Detection." (A Western blot lab is necessarily a lab in some aspects of immunology. We learn about epitopes, B cells, IgG's, hybridomas, and monoclonal antibodies.)

Each team should have
- Parafilm sheet, 8 x 10 cm
- 3 mL properly diluted (1/1000) second antibody in blocking buffer

Pick the 5x-washed NC blot out of its last wash and place it on a fresh sheet of Parafilm and make another custom pan just like that used for the first antibody. Don't let the blot dry out. Right away add the 3 mL diluted second antibody to it and shake another 30 minutes. Never use the first pan for the second antibody because it will have some first antibody left on it. Always start with a fresh Parafilm pan. Parafilm never has antibodies on it to start with, but re-used pans might. This is one of the precautions we use to avoid background on Western blots.

Second washing

Each team should have
- 120 mL TBS
- 2 well rinsed plastic blot washing pans
- 200 mL bleach plus Joy detergent pan sterilizing solution (slightly hazardous)

After exposure to the second antibody the NC is again washed five times in TBS, to get rid of unbound second antibody. We use the same procedures as in the first washing plus an added step. After we rinse a washing pan 2 or 3 times, we "sterilize" it with 5-10 mL of a mixture of Joy detergent and bleach for about 5 seconds. Then we rinse the pan again 2 or 3 times with tap water to get rid of the detergent and bleach. (Never wash a *blot* with tap water, only TBS.) The sterilization inactivates traces of second antibody stuck to the washing pans; this is an important background-reducing strategy. The bleach and detergent would ruin a blot, so we must get rid of it before using the pan again for rinsing blots. Treat the forceps the same way; rinse, sterilize and rinse when you

do it to the pans. Do not use bleach and treat with detergent after the fifth wash; leave the blots in the pan in TBS until the detection step.

Detection, concepts

After exposure to the first and second antibodies, the washed NC is bathed in a commercial detection mixture using the "enhanced chemiluminescence" procedure first applied and marketed successfully for Westerns by Amersham in about 1990. Since then the procedure has gone through several improvements and is now much more sensitive. The detection mixture is prepared by mixing two expensive reagents purchased from Amersham or Pierce. We can economize by using tiny amounts of reagents, 0.5 mL of each per blot. To use the detection reagents efficiently it helps to have the blot as small as possible with all the excess area trimmed off.

The exact detection mechanism is proprietary but we know approximately how it works. In the presence of hydrogen peroxide, HRP couples the energy of the peroxidase-peroxide reaction to production of free radicals, which in turn activate the heterocyclic molecule Luminol to a high energy state. Activated Luminol is unstable and quickly decays to ground state, emitting blue light as is does. The excitation and decay take only milliseconds. The short lifetimes of free radicals and activated Luminol confine light production effectively to the immediate vicinity of HRP, which is attached indirectly to the primary epitope on the NC.

We use x-ray film to capture a record of blue light emitted by activated Luminol. The x-ray film exposure can be for 1 or 2 seconds, or up to 60 minutes, depending on the intensity of the blue light. We determine the right time of exposure by trial and error. After exposing the x-ray we develop the film like a piece of old-fashioned black and white film using developer, stop bath, and fixer. The results appear as black bands on the developed x-ray film. We save the NC because we need it to know the positions of the colored marker bands, which are still on the NC, so we can relate the size markers on the blot to the bands on the film. We use that information to estimate the sizes of the proteins with the antigens.

Detection – details

Each team should have

- Parafilm sheet, 8x10 cm, one per blot
- Detection reagents, two, 0.5 mL of each per blot
- Cardboard at least 8x10 cm covered smoothly with Saran Wrap
- Roll of Saran Wrap
- P1000 Pipetman and blue tips
- Well rinsed and "sterilized" forceps
- Darkroom available and ready
- Packet of x-ray film in darkroom in light-tight envelope
- Developing chemicals ready in darkroom, developer, stop, fixer

Get the darkroom ready for use with the safelight on and the x-ray film packet ready to use, but you do not have to use the darkroom until you take the x-ray film out of its envelope.

After the fifth blot wash, after the second antibody exposure, make yet another Parafilm pan for the blot, the third one for each blot in this procedure. Mix the two detection reagents, 0.5 mL of each one, in one of the two microfuge tubes that they are in. Use a Pipetman P1000 for this detail. Use the same P1000 tip to apply the reagent mixture to the blot in the Parafilm pan. Shake the blot by hand for about 2 minutes, making sure the reagents cover the blot uniformly. After 2 minutes exposure to the detection chemicals pick the blot out of the Parafilm pan and plop it down on the cardboard covered with Saran Wrap. Cover the blot and the cardboard with a sheet of fresh Saran

Wrap, large enough to wrap around to the back of the card on all 4 sides. Make the covering smooth, with no wrinkles in the Saran Wrap. The Saran Wrap covering the blot should stick to the Saran Wrap on the card. We should now have two layers of Saran Wrap on the card with the blot between the two. The blot should still be wet with detection chemicals. The outside layer of Saran Wrap should be bone dry because it will be against the film and moisture on the outer layer would make terrible background.

Take the wrapped blot into the darkroom and with the white lights out place a piece of x-ray film over the blot for 1 minute. Be careful not to move the film while it is exposing. Then develop the film by pinning it to a wire frame and putting it in the developer for 5 minutes. During this time put the extra, unused film back in its envelope just in case the bright lights go on. After 5 minutes of development slosh the film in stop bath for about 30 seconds and then put it in fixer. As soon as the film is in fixer, it is no longer light sensitive so you can turn the bright lights on, provided that you have put the unused film back in its light-tight envelope. The film should stay in the fixer for 5 minutes, and then be rinsed in running cold tap water for 5 minutes. Then it should be rinsed once in deionized water and dried. It is most convenient to let the films dry overnight.

You will have an hour or two of blue light emission from the blot but after that you will not have another chance to get another exposure of the blot. So shortly after the first film is developed, look at the film carefully and decide if you need another film exposure, longer or shorter. If the bands look overexposed (fat and fuzzy), you need a shorter exposure. If the bands are faint or missing, you need a longer exposure.

A fourth period could be devoted to making calculations, preparation of figures for reports, interpretation, and discussion. Or, fourth period work could be assigned as homework since none of it requires lab facilities. But discussion with other students and TA's is always more productive than working alone so I recommend class time for these activities.

Notes for the Instructor

Safety

The dangerous chemicals are acrylamide monomer, sodium azide, and bleach. Wearing gloves and using pipetting bulbs or pumps should be adequate defenses. The cell lines are not tumorigenic except in Balb/c inbred or immunosuppressed mice. (The mouse lines are all of Balb/c lineage.) The integrated SV40 DNA is either defective in T antigen function (SV80) or else has large deletions in the capsid stuctural proteins (the other three lines).

Cell lines

If you ask me for cell lines, I will send them plus instructions for their culture.

Results and Interpretations

Here are some typical Western blot results with the four cell lines and two primary antibodies used here. Fig. 1 shows the different sizes and relative amounts of T antigen in the four cell lines. Fig. 2 shows the T-antigens(s) of the cell line Y52-8. Notice that the bands are fat and fuzzy. Fig. 3 below was a shorter exposure of Fig. 2. Notice that the major bands are sharper and that the 2 second film exposure was plenty long enough to display the major T antigen which gives a relatively strong signal. The minor bands needed the longer exposure in Fig 3, however, so Figs 2 & 3 are both informative.

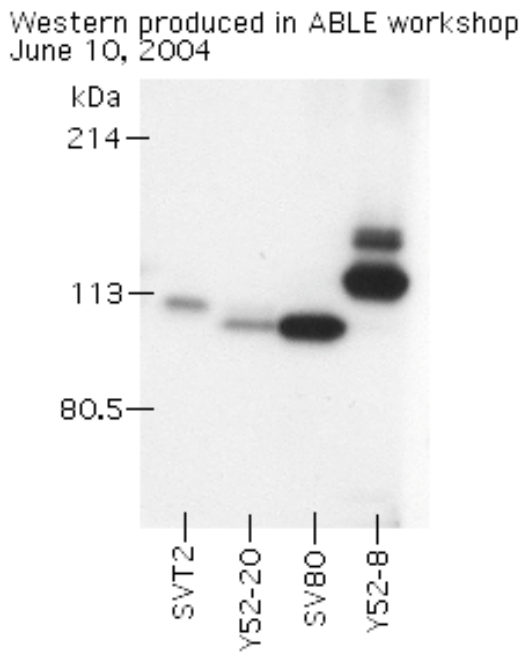

Figure 1. Western blot of 6% gel using PAb 108 on extracts of four cell lines, showing different sizes and relative amounts of SV40 T antigen in the four cell lines. The film exposure was 60 seconds. The positions of size markers were determined by comparing the blot with the film as in Figs. 4 & 5.

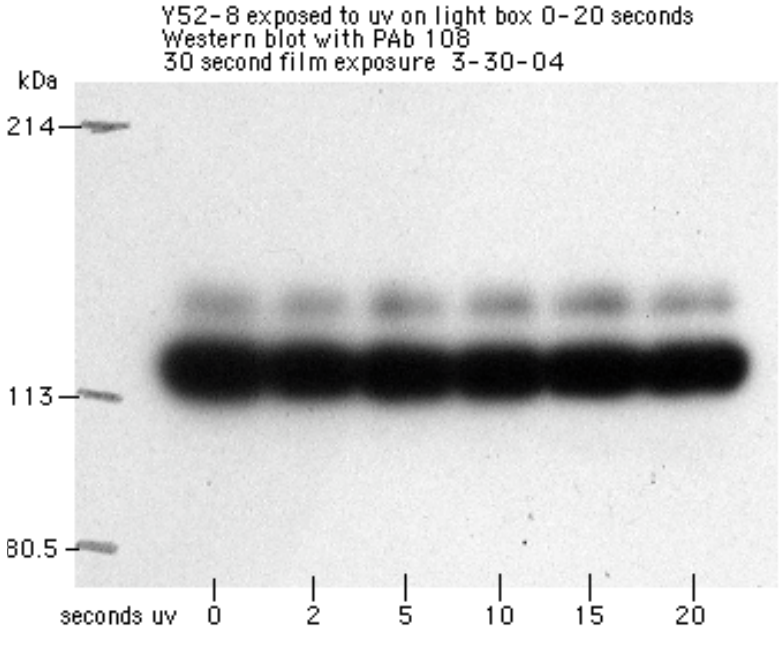

Figure 2. Western blot of 6% gel using PAb 108 on extracts of Y52-8 showing no apparent effect of uv on T antigen expression. Overexposed film. This is a Western blot application to demonstrate p53 induction by ultraviolet light.

Figs 2 & 3 are from an experiment testing the effect of ultraviolet irradiation on T antigen expression and p53 expression in Y52-8. I used a uv transilluminator light box (designed for agarose-ethidium bromide fluorescent DNA gels) as a uv light source. Identical cultures in polystyrene Petri plates were placed on the light box for 0-20 seconds and then incubated for 22 hours before making protein extracts. Then Western blots were prepared. The two films show no effect of ultraviolet light exposure of the cells on the amount of T antigen, under the conditions used.

The size scale of the polypeptides (in kilodaltons) was made by placing the film against the blot, which still had the colored markers on it. Then I made pencil marks on the film to show where the colored markers ran on the gel. See Figs. 4 & 5.

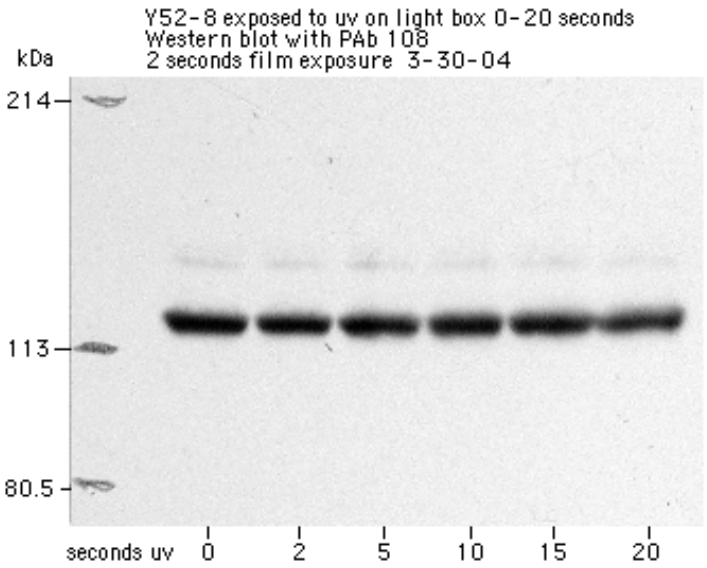

Figure 3. Same Western blot as Fig. 2, but film exposure of 2 seconds instead of 30 seconds.

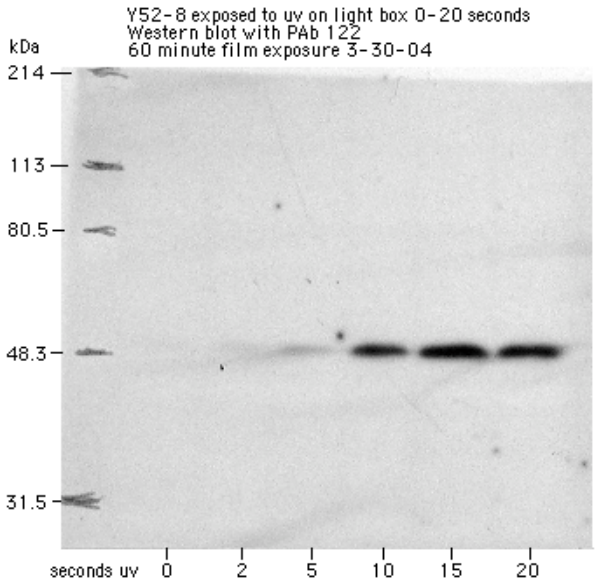

Figure 4. Effect of UV irradiation on p53 expression in Y 52-8. The same extracts were used as in Figs 2 & 3.

The fourth Western (Fig. 4) shows an effect of UV on p53 expression in Y52-8 cells using the same cell extracts as in the Westerns above. The only differences were that the first antibody was PAb122 (anti-p53) instead of PAb108 (anti-T) and in the length of film exposure was longer.

This time there was an effect of UV on amounts of p53, presumably due to induction or stabilization of p53. Notice the different size scale because the T antigen gel was 6% polyacrylamide and the p53 gel was 9% polyacrylamide. Notice also that the film exposure was much longer, 60 minutes instead of 2 seconds. It was very important to reduce the background to be able to use a 60 minute film exposure; washing the blot-rinsing pans in bleach plus detergent was essential. Induction of p53 in Y52-8 by UV would not have been observable without reducing the background.

Figure 5 shows a black and white photograph of the colored markers on the blot used to produce Fig. 4.

The other three cell lines gave different results from Y52-8. There were much stronger p53 signals and there was little or no effect of UV on p53 expression. Fig. 6 shows some results with SV80.

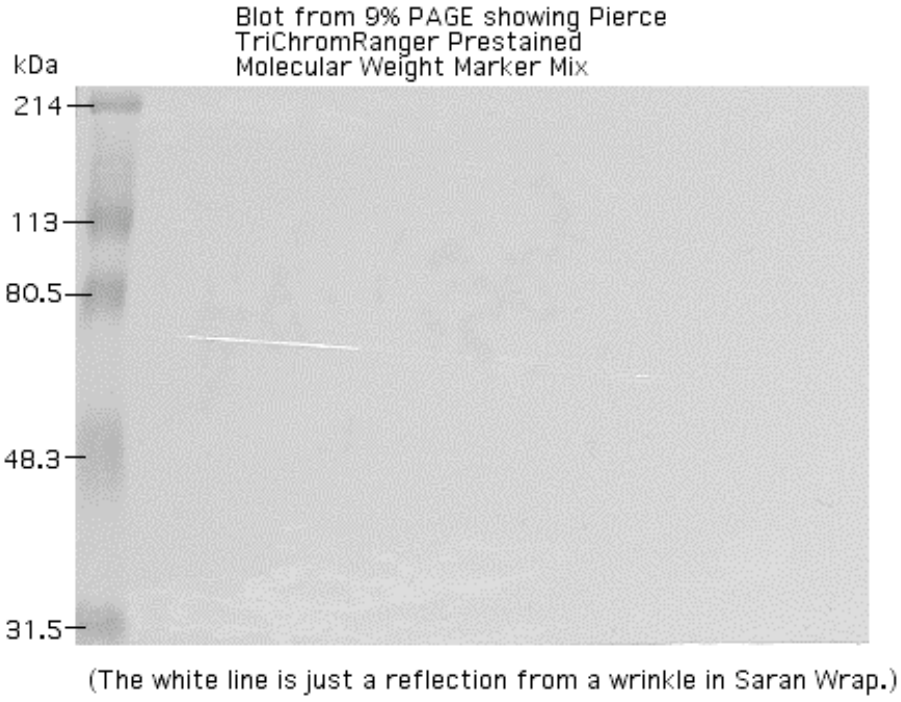

Figure 5. Black and white photograph of colored marker proteins on the blot used to produce the film in Fig 4. The pre-stained marker proteins on the left of the figure transferred from the gel to the blot. This figure shows how the size scale on Fig. 4 was made. The other figures had size scales that were made in the same way.

One interpretation of the results is that in the three cell lines SVT2, SV80, and Y52-20, T antigen and p53 were tightly complexed as has been documented repeatedly. The T-p53 complex inactivates p53 functions but also stabilizes it, protecting it from protein turnover. The complex masks UV-inducibility seen in untransformed cells. By contrast the mutated T antigen of Y52-8 might not form a complex with p53 so p53 would behave as it does in untransformed cells including the re-acquisition of UV inducibility.

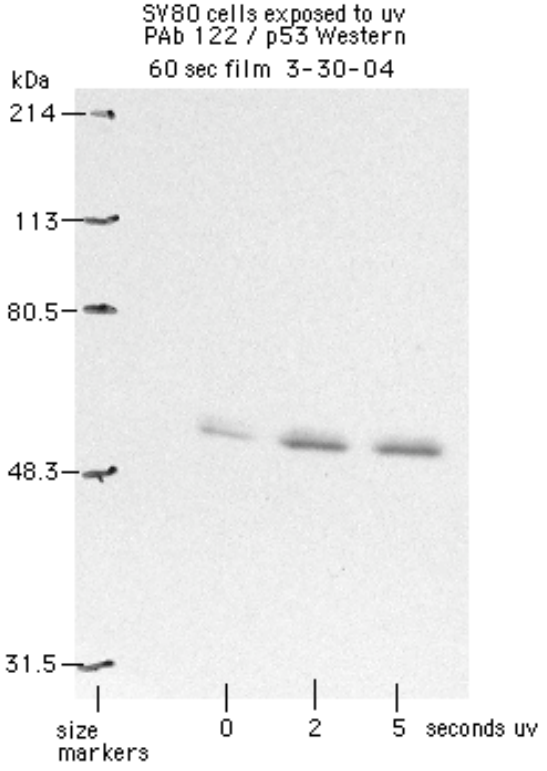

Figure 6 (right). Western of effect of UV on human SV80 cells. Conditions were the same as in Figs 2-4 except that the cell line was SV80 instead of Y52-8 and the film exposure was 60 seconds instead of 60 minutes. Notice that in comparison with Y52-8, SV80 had a stronger p53 signal (much shorter film exposure) and that the effect of UV was much less (some signal at no UV exposure).

Materials

Materials for cell culture

- *Cell lines*: The cell lines SVT2, Y52-8, and Y52-20 (mouse), and SV80 (human) are all SV40 transformed and all grow well in DMEM plus 5% adult bovine serum. Using adult serum instead of fetal serum saves lots of money. The cell lines are available from the author on request.
- *Tissue culture 37 °C humid CO_2 incubator*: Besides the incubator we need a CO_2 tank, a pressure regulator to give 2-5 psi CO_2 and rubber tubing for connections between the regulator and the incubator. CO_2 incubators automatically regulate at 5% CO_2 or else the incubator has a manual CO_2 recharge function to compensate for opening the door. I also install a branch line of tubing from the regulator to the cell culture area to have a low pressure (2-5 psi) source of 100% CO_2 for bubbling medium to adjust the pH.
- *Liquid nitrogen freezer:* (optional) for storing frozen cells: Freezers made by Minnesota Valley Engineering for storing cattle semen work well and are not too expensive. Storing cells frozen eliminates the need for continuous cell culture in the incubator and the concomitant risks of microbial contamination and genetic change with prolonged culture.
- *Petri plates:* tissue culture grade, 60 mm and 100 mm sizes, 1 case of each.
- *DMEM*, Dulbecco's modified Eagle's medium: DMEM is often sold as a sterile pH 7 solution containing sodium bicarbonate. It is both expensive and unstable in that form. To economize, I purchase DMEM in powder form from Sigma, dissolve it in water, and sterilize it myself using a large Millipore filter. I add no neutralizing sodium bicarbonate until just before using it and store it in liquid acid form (yellow in color, pH 5-6). It is stable in acid form for 1-2 years at 4 °C.
- *5% sodium bicarbonate*, sterile, stored at 22 °C, added to DMEM shortly before use.
- *Adult bovine serum*, sterile, stored long term at –20 °C; short term (<6 mo) at 4 °C.
- *Complete growth medium*, made up shortly before use: 180 mL acid DMEM, 10 mL sodium bicarbonate, 10 mL bovine serum in a sterile 500 mL bottle, total 200 mL. The medium is bubbled briefly with CO_2 using the branch line from the CO_2 tank described above, and is stored at +4° C. The color of the phenol red pH indicator in DMEM should change from yellow to red-purple after bicarbonate addition and then back to orange or yellow after CO_2 dissolves in it. In the incubator, the combination of bicarbonate in solution and the controlled atmosphere of 5% CO_2 should buffer the medium to about pH 7, red to red-orange, the correct color and pH for cell growth. Acid medium (orange or yellow, about pH 6) is kinder to cells than basic medium (purple, pH 8-9). Complete medium at pH 7 is stable for about a month in the refrigerator.
- *PBS*: phosphate buffered saline, sterile, stored at 4 °C. PBS is a standard physiological saline solution used in cell culture for washing cells free of medium and serum. Here is a recipe for 2 liters of PBS. It is made by autoclaving 2 solutions separately and mixing the two after thorough cooling. Cooling prevents formation of a calcium phosphate precipitate.

  Solution 1

  16 g NaCl
  0.4 g KCl
  2.3 g Na_2HPO_4
  0.4 g KH_2PO_4

  Dissolve in 2 liters deionized H_2O, distribute in 10 bottles of 200 mL each, and autoclave. Cool overnight to room temperature.

Solution 2

2 g $Mg2Cl_2.6H_2O$
2 g $CaCl_2$

Dissolve in 200 mL deionized H_2O; autoclave at the same time as solution 1. Cool overnight to room temperature. When cool, mix one part Solution 2 with ninety-nine parts Solution 1.

- *Trypsin-EDTA*: 100 µg/mL crystallized trypsin, 3 mM EDTA, in PBS, filter-sterilized, stored at 4° C, 100 mL. A more convenient stock of sterile trypsin at 10 mg/mL in 10^{-3} M HCl can be stored at -20° C and sterile 500 mM Na_3EDTA pH 8 can be stored at room temperature.
- *Hemacytometer* and its special coverslip: just one is needed.
- *Brightfield microscope* with 10x objective for hemacytometer, one.
- *Cultures of 4 cell lines* on 60 mm tissue culture plates: SV80 human cells, SVT2 mouse cells, Y52-8 mouse cells, and Y52-20 mouse cells. Instructors or assistants prepare cultures of each cell line one day before class by seeding 1 x 10^6 cells per 60 mm plate in complete growth medium and incubating plates at 37° C overnight in the CO_2 incubator. Two cultures per team of 2-4 students are enough. We have all four cell lines represented in the class although each team does only one or two.
- *Inverted microscope* (optional), phase contrast: with 10x objective and matching phase ring, one. It is very nice to have for tissue culture because we can record the state of the cultures by photography but it is not really essential.
- *Digital camera* (optional) for photographing cells with the inverted microscope: Cells can be photographed easily through one of the microscope oculars with a hand-held camera.
- *Ice machine* and ice buckets, one ice bucket for each team.

Materials for making cell extracts

- *PBS* Phosphate buffered saline, standard cell washing medium. Store at room temperature but chill some on ice before class. Use it chilled. We need about 40 mL per team.
- *Ice bucket* with ice, one per team.
- *Western washing buffer, (WWB),* non-sterile, store at 4° C, about 20 mL per team.
  125 mM NaCl
  25 mM Tris-HCl, pH 8.0
- *Lysis buffer*, nonsterile, stored at 4° C or on ice. We need 1 mL per team for cell lysis and also about 5 mL per team for diluting extracts for the protein assay.
  125 mM NaCl
  25 mM Tris-HCl, pH 8.0
  5 mM EDTA
  1% (v/v) Triton X-100 (the nonionic detergent)
- *Large plastic beaker* (1 liter) to receive waste rinses, one per team, non-sterile.
- *Pipetmen* (or equivalents). Each team needs three, 1000, 200, and 20 µL sizes. Each team also needs one box of big tips, 200-1000 µL for the P1000, and one box of small tips, 0-200 µL for the other two. Tips need not be sterile.
- *Cell scrapers*, one per team. Each scraper consists of a square of silicone rubber sheet, about 1 inch square, and plastic clamping forceps to hold the rubber sheet. If I have no rubber scrapers I can make scrapers from 1x3 cm pieces of old x-ray film. Rinse in tap water.
- *Microfuge*, one per two teams. It would be nice but not essential to have refrigerated microfuges.
- *Microfuge tubes*, ten per team, non-sterile, 1.7 mL size.
- *Sharpie marker*, one per team.
- *Vortex mixer*, one per 2 teams.

Materials for Pierce BCA protein assay, per team

- *BCA Protein Assay Reagent A*, 5 mL.
- *BCA Protein Assay Reagent B*, 0.5 mL.
- *Lysis buffer* from cell fractionation (same as above) 4 mL

- *Bovine serum albumin*, 10 mg/mL in water plus dilutions made in lysis medium (see above)
  BSA at 10 mg/mL is supplied with the Pierce BCA kit.
  BSA dilutions in lysis buffer 0, 100, 200, 400, 600, 800, 1000, 1500 & 2000 µg/mL
  With careful use these BSA standards can be used many times
- *Spectrophotometer* set on 562 nm, one per class.
  I assume the instrument can use 1 mL cuvettes; if not, scale up volumes accordingly. For example, the Spec 20 needs 3 mL or 5 mL tubes
- *Spectrophotometer cuvettes*, 1 mL, polystyrene, disposable, one box of 100 per class
- *Glass tubes*, 13 x 100 mm, 25 per team, the assay tubes. They can be re-used if washed, rinsed, and dry heat sterilized (>160° C for >5 hr) to get rid of traces of protein.
- *Sharpie* markers to mark the tubes, one per team
- *Test Tube Racks* for assay tubes to put in a 37° C waterbath, one per team
- *Waterbath*, 37° C, covered, and warmed up, share with 2-3 other teams
- *Pipetmen* P1000, P200, and P20 and their tips
- *Pasteur pipettes* (clean!) and bulb for them
- *Microfuge tubes*, 1.7 mL size, 3 per team
- *Plastic centrifuge tubes*, 50 mL two per team
- *Laemmli Sample Buffer*, 5x strength, (5xLSB), 1 mL per team. Stored frozen when not in use but use at room temperature. LSB contains SDS, beta-mercaptoethanol, glycerol for making the solution dense enough to load on a gel, and a blue dye to make the sample visible.
  500 mM Tris-HCL pH 6.8
  500 mM beta-mercaptoethanol
  10% SDS
  10% glycerol
  0.1% bromphenol blue, from a stock solution of 2% bromphenol blue dissolved in formamide, stored frozen

Materials for polyacrylamide gel electrophoresis

- *Gloves and glasses or goggles* for everybody because acrylamide monomer is poisonous.
- *Gel boxes*: BioRad Mini-Protean II dual slab gel electrophoresis kits: one per 4 teams. Each kit includes the gel box top and bottom, a gel casting stand, 4 glass plates (2 of 2 sizes), four 1 mm thick spacers, and two 15 tooth combs. I run communal gels with 2-6 teams on each gel. Comparisons between teams make the results more interesting. Also a full gel reduces the chance of complete failure.
- *Power supply* for electrophoresis: Adjustable up to at least 120 v DC, one per gel box unless the power supply can accommodate two gel boxes.
- *PAGE gel running buffer*, Store at room temperature. Recipe for 4 liters, enough for 4 BioRad gel boxes:
  12 g Sigma 7-9
  57.6 g glycine
  4 g SDS
  add deionized water to 4 liters and stir until dissolved, no need to adjust pH
- *Regal 5 cup Poly Hot Pot* thermostatted electric teapot used for heating gel samples to 85° C plus tube holders, one per 2-4 teams. Fill it no more than 1/3 full of deionized water. Store it dry between uses and use fresh water each time.
- *Metal dial thermometer* should be used for adjusting temperature of the Poly Hot Pot.
- *Stock acrylamide solution*, 2 mL per team or per gel box (Poisonous! Neurotoxin! Do not allow it to contact skin.) Can be stored at room temperature if bottle is tightly sealed.
  30% acrylamide (30 g in 100 mL)

0.8% bis-acrylamide

- *Resolving gel buffer*: 1 M Tris-HCl pH 9.0, 5 mL per gel box. Store at room temperature.
- *Stacking gel buffer*: 1 M Tris-HCl, pH 6.8, 5 mL per gel box. Store at room temperture.
- *10% SDS*, 500 µL per gel box. Store at room temperature. Forms precipitate in refrigerator.
- *10% ammonium persulfate*, (APS) 500 µL per gel box, store frozen but use at room temperature.
- *TEMED*, 50 µL per gel box, store and use at room temperature. Seal tightly. Stench.
- *5 mL syringe* with #20 needle but no plunger, for overlaying gel with water, one per gel box.
- *50 mL conical centrifuge tubes,* plastic disposable, 3 per gel box
- *Pre-stained markers*: Pierce TriChromRanger Prestained Protein molecular weight marker mix, lyophilized, Pierce product number 26691, store dry at room temperature, re-hydrate 1 tube per gel box with 10 µL H_2O right before gel loading. See www.piercenet.com.

Materials for blotting gels to nitrocellulose

- *BioRad electroblotting equipment* for Mini-Protean II gel boxes, one set per gel box. Blotting equipment contains:
  One plastic electrode assembly with electrodes and two slots for gel clamps
  Two plastic gel clamps with holes in them, each with two fiber pads
  The clamps hold a sandwich of fiber pads, gel, and nitrocellulose
- *Nitrocellulose paper (NC):* Schleicher and Schuell BA85 nitrocellulose (or equivalent) cut into 6 x 8 cm sheets; distribute two sheets per gel box, but have several extras available.
- *Filter paper*: Whatman 3MM or (or equivalent) filter paper cut into 6 x 8 cm sheets, four per gel box.
- *Western transfer buffer* (WTB), 4 liters per class or 900 mL per gel box. It can be re-used 4 times. Here is a recipe for 4 liters.
  Three liters deionized water in a 5 liter graduated plastic beaker
  12.1 g Sigma 7-9 powder
  57.7 g glycine
  Stir to dissolve, then add
  800 mL anhydrous methanol
  Fill to 4 liter mark with more deionized water. Store at room temperature in 2 liter bottles, tightly closed.
- *Power supply* for electroblotting, capable of supplying 15 volts overnight.
- *Scissors*, one per team

Materials for processing blots and detection

- *Tris buffered saline (TBS),* 500 mL per team. Here is a recipe for 4 liters.
  Put three liters deionized water in a 5 liter graduated plastic beaker; then add
  36 g NaCl
  12.1 g Sigma 7-9 powder
  Stir to dissolve. Adjust pH to 7.5 with concentrated HCl. Add more deionized water up to the 4 liter mark. Store at room temperature in 2 liter bottles, tightly closed.
- *Forceps*, one per team, good enough to pick up small wet nitrocellulose blots
- *Pans for shaking blots*, about 8x10 cm in size. Pipetman tip box lids will do. 4 per team.
- *Sodium azide* stock, 1M (Poison!), needed only if you block longer than overnight. Make final azide concentration in the blocking buffer 1 mM.
- *Blocking buffer*, 25 mL per team or 150 mL per class.
  7.5 g Carnation nonfat dry milk (stored at room temperature)
  150 mL TBS

Stir to dissolve; it takes about 20 minutes to dissolve milk completely at room temperature. Use up the blocking buffer on the day it is made. Do not try to store it.

- *First antibody* is the supernatant medium from hybridoma cells. Each team will need 1 mL per blot. Store in the refrigerator or on ice. Keep sterile. There are two kinds of antibody available on request.
  PAb 108 is for revealing SV40 large T antigen (Gurney, *et al*, 1986)
  PAb 122 is for revealing p53 (Gurney, *et al*, 1980; Gurney, 1982)
- *Second antibody* is commercial rabbit or goat anti-mouse IgG, HRP conjugated. It is supplied in the Pierce SuperSignal reagent kit (see below). Use it diluted 1/1000 in blocking buffer. Each team will need 3 mL of diluted second antibody.
- *Platform shaker* for shaking blots. Variable speed. One shaker per two or four teams.
- *Parafilm* for making custom pans for antibody exposure, three sheets of 8 x 10 cm per team.
- *Decontaminating solution*: Mixture of 0.5% Joy dishwashing detergent plus 1% chlorine bleach (5-6% sodium hypochlorite, e. g. Clorox), 100 mL per team, for de-contaminating blot-washing pans between washes. Chlorine bleach is a health hazard.
- *Saran Wrap*, one roll per two teams.
- *Kodak XAR x-ray film* cut into 4 pieces of 4 x 8 cm in a light-tight envelope for each team.
- There are more sensitive films made for Westerns and similar autoradiographic applications but XAR is sensitive enough and most importantly it is compatible with ordinary darkroom safelights. It has a shelf life of less than 2 years. Store at +4° C.
- *Light-tight envelopes* to hold x-ray film pieces, one for each team.
- *Stiff cardboard* 8 x 10 cm, wrapped with Saran Wrap, one smooth surface, one per blot.
- *Darkroom* with yellow safelight suitable for handling black and white printing paper.
  If automated film developing is available (X-O-Mat) the last few items are not needed.

- *Developer and fixer*: Kodak x-ray film developer and x-ray film fixer. Sold in 1 pint bottles of 5x concentrate, liquid form. The concentrated developer (5x) has a shelf life of about 1 year; the diluted working strength developer (1x) has a shelf life of 1-2 months. Concentrated (5x) fixer has a shelf life of about 1 year; 1x fixer has a longer shelf life.
- *Stop bath*: 6% acetic acid, 1.8 L or 2.8 L depending on tank size.
- *Tanks*: for 1) 1x Kodak x-ray developer, 2) 6% acetic acid stop bath, and 3) 1x Kodak x-ray fixer
- Use polypropylene cannisters such as Rubbermaid 2.8 liter "Servin' Savers" or GoodCook 1.8 liter oval food storage containers, www.goodcook.com. Both kinds have tight lids to avoid spilling. Try not to get developing chemicals on students' hands.
- *Developing hangers*, stainless steel wire frames for manual film developing, one per team
  The hangers or forceps keep chemicals off hands. The correct terminology is:
  Eastman X-Ray Film Developing Hangers, 5x7 in. They are treasured antiques.
  With the advent of automated x-ray film processing, hangers became scarce.
  A pair of foot-long forceps is a less satisfactory substitute; need one per team
- *Bucket* under the cold water tap in the darkroom is used for rinsing developed film.
- *Pierce SuperSignal* West Dura Extended Duration Substrate 100 mL kit with undiluted anti-mouse and anti-rabbit HRP antibodies. Pierce catalog no. 34075. See www.piercenet.com.
- *50 mL centrifuge tubes* : Two or three per team
- *1.7 mL microfuge tubes*, Two or three per team.

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