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Detection of Retinoblastoma Protein Phosphorylation by Immunoblot Analysis

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Abstract

The retinoblastoma tumor suppressor protein (pRb) is a preeminent tumor suppressor that acts as a cell cycle repressor, specifically as an inhibitor of the G1-S transition of the cell cycle. pRb is a phosphor-protein whose function is repressed by extensive phosphorylation in several key residues, and therefore, pRb phosphorylation status has become a surrogate for pRb activity. In particular, hyperphosphorylation of pRb has been associated with pathological states such as cancer, and therefore, assessing pRb phosphorylation status is increasingly gaining diagnostic and prognostic value, may be used to inform therapeutic decisions, and is also an important tool for the cancer biologists seeking an understanding of the molecular etiology of cancer. In this chapter, we discuss an immunoblot protocol to detect pRb phosphorylation in two residues, serine 612 and threonine 821, in protein extracts from cancer cells.

Keywords

Retinoblastoma protein; immunoblot; phosphorylation; cell cycle control; oncogenesis; lung adenocarcinoma

1. Introduction

The retinoblastoma tumor suppressor protein (pRb) is a preeminent tumor suppressor that acts as a cell cycle repressor, specifically as an inhibitor of the G1-S transition of the cell cycle (1, 2). Given pRb role as a cell cycle repressor, it is expected that pRb function is impaired in most human cancers. A mechanism of loss of pRb function may be genetic mutations in the *RB1* locus encoding the pRb protein. However, this mechanism of pRb inactivation is seen only in a small subset of human cancers, primordially in small cell lung carcinomas, osteosarcomas, and in retinoblastomas (3). Instead, the vast majority of human tumors experience loss of pRb function via a physiologically common mechanism of pRb inactivation consisting on pRb hyperphosphorylation by Cyclin-dependent kinases (Cdks)

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4. Notes

27. If there are unstained “white spots” on the membrane seen after Ponceau-S staining, this may have been caused by air bubbles trapped between the gel and the membrane. Make sure to remove all the bubbles when preparing the transfer cassette. An air bubble does not necessarily ruin an experiment, it depends on its size and on the molecular weight range in which it formed. If your protein of interest is not in this range, you may choose to proceed with the subsequent steps.

(4). It is a common occurrence in human cancers to have over-activated Cdks, either by the genetic amplification of the genes coding for them or for their cyclin activating subunits, or by the repression of the activity of Cdk inhibitors (4). Therefore, in a cancer context, pRb is usually hyperphosphorylated and consequently impaired from its cell cycle repressive function.

pRb phosphorylation state has become a surrogate for pRb function, pRb hyperphosphorylation strongly indicating functional inactivation. Particularly telling about pRb function is the phosphorylation of serine and threonine residues in pRb central pocket domain and its C-terminal. These phosphorylations are particularly disruptive of pRb function as a cell cycle repressor, since they disrupt pRb interaction with members of the E2F transcription factor family, an interaction that is central for pRb tumor suppressive role (1, 2). pRb phosphorylation in the pocket and C-terminal domains is mechanistically associated with over-proliferation and with the molecular etiology of cancer (5, 6, 7). Therefore, assessing pRb phosphorylation state, specially phosphorylations occurring in the pocket and C-terminal domains, has become extremely informative regarding the oncogenic proclivity of a cell, and, when conducted in human tumor samples, it can provide valuable diagnostic, prognostic and therapy responsiveness information.

In this chapter, we describe a procedure for detecting hyperphosphorylation in two key pRb residues mediating its function as a cell cycle repressor, namely, phosphorylation of serine 612 (in the pocket domain), and of threonine 821 (C-terminal). These two phosphorylations have been mechanistically proven to strongly affect pRb-E2F interactions and are therefore strongly implicated in abrogating pRb tumor suppressive capacity as well as in oncogenicity (8–13). This protocol describes the detection of such phosphorylations by immunoblot analysis using protein extracts from lung cancer cell lines. Therefore, this protocol will definitively be of great value for molecular cancer biologists seeking to pursue *in vitro* studies of the molecular aspects associated to the loss of cell cycle control commonly observed in human cancers.

2. Materials

Prepare all solutions using distilled water and analytical grade reagents. Unless otherwise noted, the reagents and solutions below can be stored at room temperature. Follow all waste disposal regulations when disposing waste materials.

2.1 Cell lysis and protein extraction

1. 1X Phosphate-Buffered Saline (PBS). For convenience, many vendors provide pre-made PBS either in the form of a solution, or as pre-mixed components ready to be diluted in water. If these are not available, the standard recipe for 1X PBS should work well. To prepare 1X PBS, dissolve 8 g NaCl, 0.2 g KCl, 1.44 g $\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$ and 0.24 g of KH_2PO_4 in 800 mL of water. Adjust the pH to 7.2 with HCL, and add distilled water to complete the volume to 1 L.
2. Cell scrapers or Trypsin-EDTA solution (*see* Note 1).

3. RIPA buffer. For convenience, we use the pre-mixed 10X RIPA buffer from Cell Signaling (Cat. No. 9806). However, the standard recipe can also be used, which consists of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% (v/v) NP-40 (or 1% Triton X-100, if NP-40 is not available), 0.5% (v/v) sodium deoxycholate, 0.1% (v/v) SDS, and 150 mM NaCl.
4. Protease and phosphatase inhibitor cocktails, use according to manufacturer's specifications (*see* Note 2)

2.2 SDS polyacrylamide gel electrophoresis

1. 1.5 M Tris-HCl, pH 8.8. To prepare, dissolve 181.7 g of Tris base in 800 ml H₂O. Adjust pH to 8.8 with concentrated HCl (*see* Note 3). Add H₂O to complete the volume to 1 L. Store at 4 °C.
2. 0.5 M Tris-HCl, pH 6.8. To prepare, dissolve 60.6 g of Tris base in 800 ml H₂O. Adjust pH to 6.8 with concentrated HCl. Add H₂O to complete the volume to 1 L. Store at 4 °C. (*see* Note 4).
3. 30% acrylamide/Bis-acrylamide solution (*see* Note 5). Please be aware that polyacrylamide is toxic. Carefully read the accompanying Materials Safety Data Sheet for specific instructions on how to handle and dispose polyacrylamide solutions.
4. Ammonium persulfate (APS): 10% (w/v) APS solution in water. Dissolve 0.5 g ammonium persulfate in 5 ml of dH₂O, make aliquots and store at -20°C. Avoid repeated freezing and thawing cycles. It is recommended that the aliquots are of a small amount. Avoid reusing left overs.
5. Tetramethylethylenediamine (TEMED)
6. 2X SDS gel-loading buffer: 100 mM Tris-HCl pH 6.8, 4% (w/v) sodium dodecyl sulfate (or SDS, electrophoresis grade), 0.2% bromophenol blue, 20% (v/v) glycerol, 200 mM dithiothreitol (DTT). *See* Note 6.

¹-Given that pRb is an intra-nuclear antigen, detaching the cells with trypsin is acceptable. There are many suppliers for trypsin-EDTA solutions which are sold as a 10X stock that require a 1:10 dilution before using. Ready-to-use trypsin-EDTA solutions are also available. The important aspect to consider is that you should aim for a final concentration of 0.05% trypsin and 0.02% EDTA. Also, if adding the trypsin solution, you need to wash the culture plate with PBS before the trypsin step to ensure that you completely remove the culture medium which may contain trypsin inhibitors.

²-Protease inhibitors in the cell lysis buffer minimize protein degradation during extraction. We use Protease Inhibitor Cocktail from Sigma-Aldrich (Cat. No. P8340-5ML). Phosphatase inhibitors are particularly important in this protocol; keep in mind that you want to assess pRb phosphorylation status, therefore inhibition of the endogenous phosphatases is of paramount importance. If you use the 10X RIPA buffer from Cell Signaling (Cat. No. 9806), it already includes the phosphatase inhibitors 2.5 mM sodium pyrophosphate and 1 mM sodium orthovanadate (Na₃VO₄). If you do not use the Cell Signaling pre-mixed RIPA buffer, you can purchase these phosphatase inhibitors separately and add them to the RIPA buffer at the moment of use, making sure you use them at the final concentrations indicated above.

³-When adjusting pH with HCl, you can start with concentrated HCl (e. g., 12 M), but then change to a more diluted acid (e. g., 1 M) as you get closer to the desired pH.

⁴-You can also prepare this solution by diluting the 1.5 M Tris-HCl stock and adjusting the pH.

⁵-We use the pre-mixed BioRad (Cat. No. 161-0158, keep stored at 4°C), but you can try other options.

⁶-You can prepare this solution from the Tris-HCl pH 6.8 stock and a 10% SDS stock. 200 mM beta-mercaptoethanol (BME) can be used in place of DTT as a reducing agent to break disulfide bonds. Without the reducing agent (DTT or BME), this buffer can be stored at room temperature. Add the DTT from a 1 M stock, and use the solution right after adding the DTT. If pipetting concentrated glycerol becomes difficult, you can cut the pipette tip.

7. 10X SDS PAGE running buffer: Dissolve 30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS in 1 L of H₂O. This solution should have a pH of 8.3, but is expected that no pH adjustment will be required. Store the running buffer at room temperature and dilute to 1X before use. *See Note 7.*
8. Ethanol 70% (for cleaning the glass plates)
9. 10% SDS: dissolve 10 g of SDS in 80 ml H₂O. Complete volume to 100 ml. This solution can be kept at room temperature for up to 6 months.

2.3 Transfer

1. Nitrocellulose blotting membranes. We use 0.45 µm pore size for immunoblotting pRb, but a smaller pore size may be recommended should you want to adapt this protocol for low molecular weight proteins.
2. 1X transfer buffer: Dissolve 3.03 g Tris base and 14.4 g glycine in 500 ml H₂O. Add 200 ml methanol, and complete to a final volume of 1 L with dH₂O.
3. Tris-buffered saline with Tween-20 (TBST): First prepare a 10X TBS stock by dissolving 24.2 g of Tris base and 87.6 g NaCl in 800 ml H₂O, adjust to pH 7.6 with 1 M HCL, and complete to final volume of 1 L. To prepare the TBST, add 1 ml of Tween-20 to 1 L of 1X TBS.
4. Ponceau-S membrane staining solution: 0.5% (w/v) Ponceau-S in 1% acetic acid

2.4. Immunoblotting solutions and antibodies

The protocols described in this chapter were optimized specifically for the antibodies described below and for several lung cancer cell lines.

1. Blocking solution: Dissolve 0.5 g of bovine serum albumin (BSA) in 10 ml of 1X TBST.
2. Primary antibody against phosphorylated serine 612 in pRb (anti-Rb Phospho-Ser612). We purchased this rabbit polyclonal antibody from Gene Tex (Cat. No. GTX24777), and use it at a dilution of 1:1000 in TBST. We usually prepare primary antibody solutions in TBST that can be stored for several months at 4°C. To prepare such antibody solutions, we dissolve 0.5 g of BSA in 10 ml of TBST, and add 30 µl of a 20% sodium azide stock. Sodium azide is used as a preservative to prevent bacterial growth in the solution. Sodium azide is toxic and a potential carcinogen, therefore, read carefully its accompanying Material Safety Data Sheet for information regarding proper handling, storage and disposal. Add 10 µl of the antibody to this solution. Mix and store at 4 °C. You can use this solution for several months, but you need to be attentive for signs of contamination such as a strong odor or cloudiness in the solution. In such case, discard and prepare a fresh solution. Use of contaminated antibody solution

⁷For convenience, BioRad has a pre-made 1X SDS PAGE running buffer (Cat. No. 161–0732), but preparing this buffer from common laboratory reagents should work well.

usually yields high background, meaning that it is time to replace the solution with a fresh one.

3. Primary antibody against phosphorylated threonine 821 (T821) in pRb (also rabbit monoclonal, Abcam Cat. No. ab32015). We prepare an antibody solution exactly as described above for the antibody against phosphor-S612, except that for this antibody we use a dilution of 1:500. For this, we add 20 μ l of antibody to the 10 ml TBST antibody solution including sodium azide as well.
4. Primary antibody against total pRb (mouse monoclonal 4H1, Cell Signaling Cat. No. 9309). We use at a dilution of 1:1000 in TBST, and we prepare it exactly as described above for the antibody against phosphor-S612. It is important to blot for total pRb, as the extent of pRb phosphorylation is assessed and reported as the ratio of phosphorylated pRb to total pRb protein.
5. Secondary antibodies: we use horse-radish peroxidase (HRP)-conjugated secondary antibodies. For mouse monoclonal primary antibodies, we use an HRP-conjugated, affinity-purified horse anti-mouse IgG (Cell Signaling, Cat. No. 7076S). For rabbit polyclonal primary antibodies, we use we use an HRP-conjugated, affinity-purified goat anti-rabbit IgG (Cell Signaling, Cat. No. 7074S). For both of these secondary antibodies, we prepare a TBST solution of the antibody exactly as described above for primary antibodies (except that we omit the sodium azide since we prepare fresh for each use), with the antibody diluted 1:5,000.
6. Supersignal West Pico PlusTM Chemiluminescent Kit (Thermo Scientific Cat. No. 34078). This kit is compatible with HRP-conjugated secondary antibodies. The selection of the kit to develop the chemiluminescent signal is dictated by the enzyme conjugated to the secondary antibody (HRP, versus alkaline phosphatase, for example).
7. ChemiDoc imaging system and software, or other equivalent imaging system compatible with chemiluminescent signals.

2.5. Additional equipment and plastic-ware

1. Tabletop centrifuge with capacity for 15 ml tubes, preferably refrigerated
2. 15 and 50 ml conical tubes
3. Culture plates or bottles (we culture cells in T75 culture bottles)
4. Gel Electrophoresis system: parts and assembly as per manufacture s instructions.
5. Transfer system: parts and assembly as per manufacture s instructions.
6. 1.5 ml microcentrifuge tubes
7. Refrigerated centrifuge for microcentrifuge tubes

3. Methods

This protocol focuses on the immunoblot technique, and therefore it assumes that the user has cell cultures ready for protein extraction. We optimized the procedure described below using a variety of lung cancer cell lines, but this protocol is applicable to any pRb-expressing cell line in which pRb's phosphorylation status needs to be studied. It is important that you grow your cell cultures in the appropriate medium such that they are at approximately 90% confluence at the moment of protein extraction. This will ensure a good protein yield per plate, especially if you use large culture vessels such as T75 culture bottles.

3.1 Cell lysis and preparation of protein extracts

1. Collect cells by scrapping them from the culture plate in 2–3 ml of 1X PBS. We culture cells in T75 culture bottles, you should adjust the volume of PBS depending on your culture plate, but it is important to use the minimum volume of PBS that covers the entire culture surface. Alternatively, detach cells from the plate by incubating in trypsin-EDTA solution at 37°C for 5 minutes (*see Note 8*).
2. Transfer the cell suspension to a 15 ml conical tube and pellet cells by low speed centrifugation (5 min at 400x g). If you detach the cells using the trypsin EDTA solution, you need to dilute it 1:10 with culture medium to ensure inactivation of trypsin (before the centrifugation step). Remove the supernatant after the centrifugation.
3. Lyse cells by resuspending the cell pellet in RIPA buffer supplemented with proteases and phosphatase inhibitor cocktails (*see Note 9*). Transfer the cell suspension to a 1.5 ml microcentrifuge tube.
4. Incubate at 4°C for 30 min to allow lysis to proceed (*see Note 10*).
5. Centrifuge tube for 10 min at 1,400x g at 4°C. Transfer supernatant to a fresh microcentrifuge tube.
6. Quantify the protein in your cell lysate using your method of choice (*see Note 11*)

3.2 SDS Polyacrylamide Gel Electrophoresis

1. Assemble your gel electrophoresis apparatus following manufacturer's instructions (*see Note 12*). At this point you will only need to assemble the gel

⁸. Avoid over-trypsinization as it may kill cells. Fresh trypsin solution should detach cells in under 5 minutes. If you find that 5 minutes under trypsin-EDTA are not enough to detach cells from the plate, it is better to get a fresh trypsin-EDTA batch than prolonging the trypsinization time.

⁹. It is recommended that you obtain an estimate of the number of cells per plate (using any common procedure such as trypan blue exclusion staining) before extracting proteins from each plate. This information is useful since we recommend that you lyse cells in 200 µL of RIPA buffer per 10⁶ cells. To minimize protein degradation, lysis should be conducted using cold solutions, and keeping cells on ice as much as possible during the process.

¹⁰. During those 30 minutes, you can enhance the amount of proteins released by cells by passing the lysate 3–5 times through a syringe with a 27.5-gauge needle. While this significantly increases protein yield, it could also produce foaming, which should be avoided to prevent protein loss.

¹¹. We perform a standard protein quantification assay using Bio-Rad's Protein Assay (Cat. No. 500–0006), following its instructions, and using BSA as a quantification standard. Other substitute assays can be used. After quantification, protein lysates that will not be used immediately can be stored at –20°C for up to 3 months (protein integrity cannot be ensured beyond that).

casting system needed to pour the gels. We use a standard Bio rad gel electrophoresis apparatus with its accompanying gel casting system. Be sure to clean thoroughly all glass plates with 70% ethanol. This will ensure a smooth finger-print free glass surface, which will translate into decreased risk of forming bubbles while pouring the gel in between the glass plates.

2. Prepare the separating gel as follows: In a 50 ml conical tube, mix 7.9 ml of distilled water, 7 mL of the 30% acrylamide, bis-acrylamide mix, 5.0 ml of 1.5 M Tris HCl pH 8.8, 0.2 mL of 10% SDS. Add then 200 μ l of 10% APS and 8 μ l of TEMED. Gently mix avoiding as much as possible the formation of bubbles. Keep in mind that the gel will start rapidly polymerizing after the addition of APS and TEMED, therefore these two reagents should be the very last to be added to the mix, and the gel should be poured immediately after their addition. Maintaining the mix on ice will also retard the polymerization. *See Notes 13 and 14.*
3. Allow the gel to completely polymerize for 30–60 minutes at room temperature (*see Note 15*). Be sure to always use freshly prepared or thawed APS (avoid re-freezing of left-overs). Loss of APS activity is manifested in abnormally long polymerization times.
4. Prepare the upper stacking gel as follows: in a 15 ml conical tube, mix 4.1 ml of distilled H₂O, 1.0 mL of 30% acrylamide/bis-acrylamide mix, 750 μ l of 0.5 M Tris-HCl pH 6.8, and 60 μ l of 10% SDS. Mix gently avoiding foam and bubbles. When ready to pour, add 60 μ l of 10% APS and 6 μ l of TEMED. Mix and add it to the glass plates (*see Note 16*).
5. Immediately after pouring the stacking gel, insert comb being careful not to form any bubbles at the base of the wells. Allow the stacking gel to polymerize for 30–60 minutes at room temperature.
6. Carefully remove the comb and the bottom spacer. Please note that we do not recommend that you remove the comb straight out of the dry gel. Rather, we recommend that first you assemble the whole electrophoresis apparatus,

¹²Choose the number of wells according to your number of samples and experimental design. Regarding the thickness of the gel (this is determined by the thickness of the spacers that you use), a thicker gel will allow you to load a larger sample volume, which is a necessity if your protein sample is not very concentrated. This, however, can adversely affect the efficiency of the transfer, high molecular weight proteins being particularly affected. For that reason, aim to get as concentrated as possible protein samples, in order to be able to load smaller volumes on the gel. Be aware then, that thinner gels require extra caution when handling, as they are prone to break apart during handling.

¹³It is recommended that you add a thin layer of isopropanol on top of the separating gel solution immediately after pouring it. Isopropanol is not miscible in water, thus it will form a distinct layer. Adding isopropanol eliminates any bubbles on the surface of the separating gel solution and will produce a smooth surface. As an alternative, we use the pre-mixed gel system 10% SDS-PAGE FastCast acrylamide starter kit (Cat. No. 1610172) from Bio-Rad. This kit contains the buffers for the separating and stacking gels, and has a considerably reduced time for polymerization.

¹⁴For pRb, which has a molecular weight of approximately 110 kDa, we use a 10% polyacrylamide separating gel. The % of polyacrylamide you will choose depends on the molecular weight range in which you want to have good resolution. Take this into consideration if you wish to adapt this protocol to other phosphor-proteins.

¹⁵After 45 minutes, you can verify if the gel has polymerized by *gently* tilting the casting apparatus sideways. Only the isopropanol layer should move while the underlying separating gel should be static if it has polymerized.

¹⁶To save time, you can start preparing the stacking gel while the separating gel is polymerizing. However, **do not add the TEMED and the APS** until immediately before adding the stacking gel to the casting apparatus. The stacking gel without APS and TEMED can be kept on ice until pouring. The % of acrylamide of the stacking gel is usually smaller (4–6%) than that of the separating gel.

including inserting the gel inside it, fill the liquid reservoir with running buffer, and then remove the comb. We use a standard protein gel electrophoresis apparatus from BioRad. Dilute the 10X SDS PAGE running buffer to 1X with water and fill the assembled apparatus with it until you cover the gel. Only then we recommend that you slowly and carefully remove the comb (*see* Note 17). Using a glass or plastic Pasteur pipette, rinse the wells with running buffer to remove excess acrylamide.

7. Prepare the protein samples for loading into the gel. Mix enough volume of protein extract to contain 30–50 µg of total protein with an equal volume of 2X gel-loading buffer. Denature proteins by boiling the mixture at 95–100°C for 5 minutes, or heating at 70°C for 30 minutes.
8. Load the proteins into the wells, being careful not to over flood the wells (if you allow this to happen, you risk having a protein sample over flooding into an adjacent lane). Remember also to load a suitable protein ladder (*see* Note 18).
9. Place the lid on the electrophoresis apparatus, connect to a power supply, and run the gel at 160 V for 60 min (do not set a limit for Amperes). Monitor the run by following the bromophenol blue dye front. Stop the run when the dye front reaches about two-thirds of the length of the frontal glass plate (*see* Note 19).

3.3 Transfer of proteins to nitrocellulose membranes

1. Disassemble the electrophoresis apparatus and remove the gel assembly. Very gently and carefully separate the glass plates from the gel carefully inserting a fine spatula in between the glass plates, and slowly twisting the spatula until the plates start to separate from the gel. Use a razor blade to carefully remove the stacking gel without damaging the separating gel. Rinse the gel with transfer buffer. *See* Note 20.
2. Cut a piece of nitrocellulose membrane of the approximate size of the gel (a little bit larger so you can handle it by the edges without touching the gel) and immerse in cold transfer buffer for 2–5 minutes. *See* Note 21.
3. Pre-wet several pieces of filter paper (cut in a size similar to the gel) in cold transfer buffer by submerging one side of the paper first and then slowly lowering it into the buffer.

¹⁷If you slowly remove the comb having the gel submerged in running buffer, you will notice that as you remove the comb the empty well space is immediately filled with buffer. This will avoid the collapse of the well that is experienced if you remove the comb out of the dry gel, as a vacuum is formed inside the well as the comb is retrieved.

¹⁸When blotting for pRb, we use BioRad Precision Plus Protein Kaleidoscope (Cat. No. 1610–375). You can use any other protein ladder provided it has sufficient markers in the molecular weight range of your protein of interest, in this case, around 110 kDa, which is the approximate molecular weight of pRb.

¹⁹Running time depends on gel thickness. Here we used 1.5 mm gels. If you notice a distortion of band shapes (“smiley bands”), decrease the voltage. Be careful to stop the run when the tracking dye has reached two-thirds of the way, otherwise you risk losing low molecular weight proteins. This may not be an issue with pRb, but is an issue to consider if you want to adapt this protocol to study other proteins.

²⁰This is a very sensitive step; if not done carefully, you risk breaking the gel apart. Remember that the thinner the gel, the higher the risk of this occurring. Never let the gel get dry. Keep it soaked from here on in transfer buffer.

²¹Always handle the membrane with gloves or with tweezers. Never touch the membrane with bare hands, this may leave fingerprints oils on the membrane and this in turn will prevent even wetting of the membrane. This usually results in areas in the membrane where transfer of proteins is impaired.

4. Assemble the gel electro-transfer cassette following the manufacturer instructions (*see* Notes 22 and 23).
5. Insert the transfer cassette into the electro-transfer unit. It is usual for electro-transfer units to have a special compartment for an ice block or any other cooling device. As the transfer process generates heat and the transfer buffer can get warm (or even hot), it is recommended that the ice block/cooling devices are used. *See* Note 24.
6. Transfer for 60 min at 100V (do not set a limit for Amperes).
7. After the transfer is completed, disassemble the unit. Wash the membrane in TBST to remove residual SDS and potential gel fragments.
8. Check the efficiency of transfer by staining the membrane in Ponceau-S solution for 5 min at room temperature. Ponceau-stained membranes can be seen in Fig. 1A–B (*see* Note 25). Record an image of the Ponceau-stained membrane using a document scanner or camera. Never let membranes to get dry. Keep membranes moist during the documentation process by keeping them wrapped in plastic wrap after soaking in transfer buffer. *See* Notes 26 to 28 for transfer troubleshooting tips.

3.4. Immunoblotting, image development and capture

1. Remove the Ponceau-S staining from the membrane by incubating in TBST containing 5% milk. You will notice the milk solution turning red. Discard and rinse the membrane with TBST (no milk). Repeat until no trace of the red Ponceau-S stain remains in the membrane. At this point you should only see the rainbow-colored protein markers. Repeat a final rinse with TBST (*see* Note 29).

²²It is difficult on this step to elaborate on the specifics of the assembly of the electro-transfer transfer cassette and unit as this depends on the specific model and vendor. However, regardless of the apparatus that you use, it is very important that you are mindful of the direction in which the proteins will flow. Due to the SDS in the sample buffer, proteins will be negatively charged, and therefore they will move towards the positive pole in an electrical field. Therefore, the membrane must be positioned between the gel and the positive pole. This will make the proteins become attached to the membrane as they transit to the positive pole.

²³When assembling the transfer unit, it is extremely important that you avoid at all costs the formation of bubbles between the gel and the membrane. A place where a bubble is formed is a place where transfer of proteins will be affected.

²⁴The transfer can be done in a cold room, or the whole transfer apparatus can be inserted in a tray and surrounded with ice during the transfer process.

²⁵We strongly recommend that you stain the membrane after transfer with Ponceau-S stain. This procedure is highly informative, as it will allow you to assess whether you loaded approximately equal amounts of total protein in all wells, and if you obtained an even and efficient transfer of proteins (Fig. 1A–B). Usually places where bubbles formed (or transfer did not occur due to any other reason) can be seen in the stained membrane as protein-free clear areas. The decision of whether it is worth proceeding to subsequent steps can usually be done after assessing the quality of transfer in a Ponceau-S stained membrane. Poor transfer is usually the culprit of most problems commonly encountered in this technique.

²⁶Do not exceed the transfer time as this leads to gel shrinkage and distortion of the membrane. In case of poor transfer efficiency, opt for making thinner gels, rather than prolonging transfer time.

²⁸Ponceau-S staining may also help you to spot degraded proteins, which are appreciated as a diffuse smear in the lower half on the membrane. In this case, ensure that you are taking all the precautions necessary to deal with protein degradation, such as not using protein samples that have been stored for prolonged times (or repeatedly frozen-thawed), ensuring that cell lysis was done on ice and the samples were kept cold or refrigerated all the times, and that you added protease inhibitors to the RIPA buffer.

²⁹When doing a western blot using antibodies against phosphorylated residues, it is important that you thoroughly remove any traces on milk from the membrane. Casein (milk protein) is heavily phosphorylated and any traces of milk in the membrane can lead to high background due to non-specific antibody binding. For the same reason, the blocking solution must not contain milk. The blocking step is usually one of the steps in which you can make adjustments in case you experience high background levels.

2. Block membranes in TBST with BSA (*see* Note 29). You can block for 2 hours at room temperature or overnight at 4°C. Use constant agitation to ensure that the membrane is constantly bathed by the solution.
3. Incubate membranes with the primary antibody. Like the blocking step, primary antibody incubation can be done either 2 hours at room temperature, or overnight at 4°C. Use constant agitation during this step as well. *See* Note 30.
4. Wash three times in TBST.
5. Incubate with secondary antibody for 1 hour at room temperature while agitating. *See* Note 31.
6. For development of the membrane chemiluminescence, we use the Supersignal West Pico Plus™ Chemiluminescent Kit (Thermo Scientific Cat. No. 34580), following the procedures exactly as described by the manufacturer. We add 1 ml of substrate solution per membrane. Do not leave it for more than 3 minutes. *See* Note 32.
7. Capture the chemiluminescent signal using a ChemiDoc imaging system or its equivalent (use ChemiDoc XRS+), using the accompanying image software for image capturing and quantification of signal intensity. A typical immunoblot result using this protocol is shown in Fig. 2A. Notice that you need to run in parallel immunoblots using the phosphor-specific antibodies as well as antibodies recognizing total pRb protein. You need to assess pRb phosphorylation in the residues of your interest relative to total pRb levels, in other words, as the ratio of phosphorylated pRb to total pRb (Fig. 2A–B) (*see* Note 33).

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³⁰Incubating in primary antibody for 2 hours versus overnight must be empirically determined. Longer incubation periods are recommended if you are having trouble obtaining strong signals. However, be aware that longer incubation times also increase the likelihood of obtaining a strong background. The length of the incubation time (with primary antibody) is one of the factors that affect signal strength. Dilution of antibody also usually affects background noise. If you are obtaining too much background, in addition to extending blocking time, you can try diluting the primary antibody. Conversely, try concentrating the primary antibody if you obtain weak signals.

³¹Ensure that the secondary antibody is properly chosen to match the primary antibody. For example, if you use a mouse primary antibody, the secondary antibody must be a goat-anti-mouse or donkey-anti-mouse antibody. Incompatibility between primary and secondary antibody is a usual source or mistakes in this technique.

³²Other commercially available kits can be acceptable substitutes, as long as they are compatible with the enzyme that is conjugated to the secondary antibody. The signal development step also has a major impact on signal intensity and background. This step can be performed for 30 seconds to 3 minutes. If you are experiencing weak signals, in addition to using more concentrated primary antibody and/or increasing incubation time, you can try developing the membrane for longer (but do not exceed 3 minutes, as this may blacken the membrane). Some antibodies give a very strong signal and in such cases, 30 seconds to 1 minute is sufficient.

³³As part of the optimization of phosphor-specific antibodies, it is important that you ensure that they are indeed detecting the phosphorylated residues of interest. A simple way to verify this is to conduct initial experiments in which you treat protein extracts with calf intestinal phosphatase (treat with 50 units of phosphatase per 50 µg of total protein, 37°C overnight). This treatment should eliminate immunoreactivity if the antibody is indeed recognizing a phosphorylated form of the protein of interest. Remember to include controls treated with phosphatase buffer alone, as well as a control with phosphatase in the presence of phosphatase inhibitors.

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Figure 1.

Representative membranes stained with Ponceau-S stain. A. This membrane shows unequal loading of the lanes, as it can be appreciated that the middle lane has a higher amount of protein compared to the lanes at the sides. A clear protein-less spot can be seen in the bottom part of the middle lane (arrow). This effect was likely generated by a bubble being trapped between the gel and the membrane during the transfer process. B. This membrane shows similar protein loading in the lanes, no air bubble marks, and no signs of protein degradation, which are usually as a low molecular weight diffuse smear in the lower half of the membrane.

Figure 2.

Typical immunoblot analysis of different lung cancer cell lines (lanes 1–6) using antibodies against phosphorylated pRb residues S162 and T821, as well as against total pRb. A. Except for cell lines in lanes 4 and 5, all other cell lines express endogenous pRb. Notice that cell lines in lanes 1–3 and 6 express total pRb as well as showing pRb phosphorylation in serine 612. On the other hand, of these 4 cell lines, the one in lane 6 is the only one showing hyperphosphorylation in threonine 821. B. After densitometric analysis of band intensity for signal quantification, we determined the ratio of signal intensity of pRb phosphor-T821 to total pRb levels. The graph shows the phosphorylation in T821 as a fraction of total pRb for cell lines in lanes 1 and 6. Serine 821 is hyperphosphorylated in 6 relative to 1 in a statistically significant manner (p value= 0.007).