V.5.0

Rac1 Activation Assay Biochem Kit™

(50 Assays)

Cat. # BK035

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Manual Contents

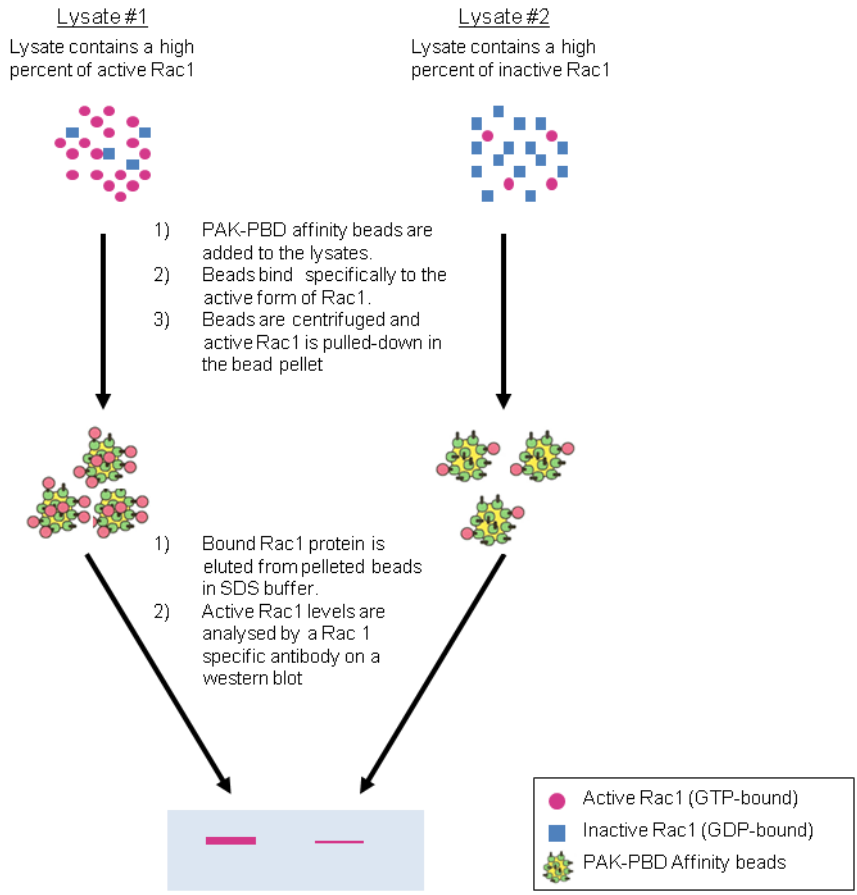
I: Introduction

Background– Rac1 Activation Assay

The Rho family of small GTPases consists of at least 20 members, the most extensively characterized of which are the Rac1, RhoA and Cdc42 proteins (1-4). In common with all other small G-proteins, the Rho family proteins act as molecular switches that transmit cellular signals through an array of effector proteins. This family mediates a diverse number of cellular responses including cytoskeletal reorganization (1-4), regulation of transcription (5), DNA synthesis, membrane trafficking and apoptosis (6-9).

The Rho family switch operates by alternating between an active, GTP-bound state and an inactive, GDP-bound state (10-12). Understanding the mechanisms that regulate activation / inactivation of the GTPases is of obvious biological significance and is a subject of intense investigation. The fact that Rho family effector proteins will specifically recognize the GTP bound form of the protein (13) has been exploited experimentally to develop a powerful affinity purification assay that monitors Rac1 and Cdc42 protein activation (14). Figure 1 gives a schematic representation of the Rac1 Activation Assay principle.

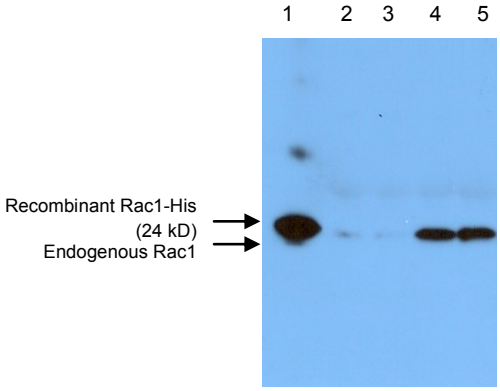
Figure 1: Schematic of Rac1 Activation (Pull-down) Assay



I: Introduction (cont.)

The Rac1 Activation assay uses the Cdc42/Rac Interactive Binding (CRIB) region (also called the p21 Binding Domain, PBD) of the Cdc42/Rac effector protein, p21 activated kinase I (PAK). The CRIB/PBD protein motif has been shown to bind specifically to the GTP-bound form of Rac and/or Cdc42 proteins (15). The fact that the PBD region of PAK has a high affinity for both GTP -Rac and GTP-Cdc42 and that PAK binding results in a significantly reduced intrinsic and catalytic rate of hydrolysis of both Rac and Cdc42 make it an ideal tool for affinity purification of GTP-Rac and GTP-Cdc42 from cell lysates (16). The PAK-PBD protein supplied in this kit contains amino acids 67-160, this includes the highly conserved CRIB region (aa 74-88) plus sequences required for the high affinity interaction with GTP-Rac and GTP-Cdc42 (17). The PAK-PBD is also in the form of a GST fusion protein which allows one to "pull-down" the PAK-PBD/GTP-Rac (or GTP-Cdc42) complex with glutathione affinity beads. The assay therefore provides a simple means of quantitating Rac/Cdc42 activation in cells. The amount of activated Rac1 is determined by a Western blot using a Rac1 specific antibody. Figure 2 shows typical Rac1 Activation Assay results from serum starved and EGF treated Swiss 3T3 cells. Serum starvation greatly reduces the amount of active Rac1 in cells while EGF is a potent activator of Rac1.

Figure 2: Rac Activation by EGF



Swiss 3T3 cells were serum starved for approx. 40 h; after this some cells were treated with 10 ng/ml of EGF for 2 min. (Lanes 4 & 5), others were not treated and remained serum starved (Lanes 2 & 3). Rac1 activation was measured using the Rac1 Activation pull-down assay. 500 µg of lysate were assayed with 10 µg of PAK-PBD beads (Lanes 2-5). Lane 1 shows 20 ng of recombinant Rac1-His protein run as a western blot standard. Endogenous Rac1 runs at 21 kD, His-Rac1 runs at approx. 24 kD.

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III: Kit Contents

This kit contains enough reagents for approximately 50 pull-down assays. There is sufficient Rac1 antibody for 100 ml working strength primary antibody solution.

Table 1: Kit Contents and Storage Upon Arrival

Reagents	Cat. # or Part # *	Quantity	Storage
PAK-PBD beads	Part # PAK02	1tube, lyophilized; 500 µg of protein per tube bound to colored sepharose beads	Desiccated 4°C
Anti-Rac1 monoclonal antibody	Cat # ARC03	2 tubes, lyophilized, 50 µg protein per tube	Desiccated 4°C
His-Rac1 control protein	Part # RCWT	1 tube, lyophilized; 10 µg protein (~24 kDa) as a Western Blot standard.	Desiccated 4°C
Cell Lysis Buffer	Part # CLB01	1 bottle, lyophilized; 50mM Tris pH 7.5, 10mM $MgCl_2$, 0.5M NaCl, and 2% Igepal when reconstituted	Desiccated 4°C
Wash Buffer	Part # WB01	1 bottle, lyophilized; 25 mM Tris pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl when reconstituted	Desiccated 4°C
Loading Buffer	Part # LB01	1 tube, 1 ml; 150 mM EDTA solution	4°C
STOP Buffer	Part # STP01	1 tube, 1 ml; 600 mM $MgCl_2$ solution	4°C
GTPγS stock: (non-hydrolysable GTP analog)	Cat # BS01	1 tube, lyophilized; 20 mM solution when reconstituted	Desiccated 4°C
GDP stock	Part # GDP01	1 tube, lyophilized; 100 mM solution when reconstituted	Desiccated 4°C
Protease Inhibitor Cocktail	Cat. # PIC02	1 tube, lyophilized; 100X solution: 62 µg/ml Leupeptin, 62 Ég/ml Pepstatin A, 14 mg/ml Benzamidine and 12 mg/ml tosyl arginine methyl ester when reconstituted	Desiccated 4°C
DMSO	Part # DMSO	1 tube, 1.5ml. Solvent for protease inhibitor cocktail	4° (will freeze at 4°C)

*** Items with part numbers (Part #) are not sold separately and available only in kit format. Items with catalog numbers (Cat. #) are available separately.**

III: Kit Contents (Continued)

The reagents and equipment that you will require but are not supplied:

- Cell lysate (see Section V: B-D and Section VI: Step 2)
- 2X Laemmli sample buffer (125mM Tris pH6.8, 20% glycerol, 4% SDS, 0.005% Bromophenol blue, 5% beta-mercaptoethanol)
- Polyacrylamide gels (12% or 4-20% gradient gels)
- SDS-PAGE buffers
- Western blot buffers (see Section VI: Step 4)
- Protein transfer membrane (PVDF or Nitrocellulose)
- Secondary antibody (e.g. Goat anti-mouse HRP conjugated IgG, Jackson Labs. Cat# 115-035-068)
- Chemiluminescence based detection system (e.g. SuperSignal West Dura Extended Duration Substrate; ThermoFisher)
- Cell scrapers
- Liquid nitrogen for snap freezing cell lysates

IV: Reconstitution and Storage of Components

Many of the components of this kit have been provided in lyophilized form. Prior to beginning the assay you will need to reconstitute several components as detailed in Table 2. When properly stored and reconstituted, components are guaranteed stable for 6 months.

Table 2: Component Storage and Reconstitution

Kit Component	**Reconstitution**	**Storage Conditions**
PAK-PBD Protein Beads	Reconstitute in 500 µl distilled water. Aliquot into 50 x 10 µl volumes (10 µl of beads = 10 µg of protein, under these conditions 500 µl is sufficient for 50 assays). **Snap freeze in liquid nitrogen.**	Store at –70°C
Anti-Rac1 monoclonal antibody	Resuspend each tube in 100 µl of PBS. Use at 1:500 dilution.	Store at 4°C **DO NOT FREEZE**
His-Rac1 control protein	Reconstitute in 30 µl of distilled water. Aliquot into 10 x 3 µl sizes and snap freeze in liquid nitrogen.	Store at –70°C
Cell Lysis Buffer	Reconstitute in 100 ml of sterile distilled water. This solution may take 5-10 min to resuspend. Use a 10 ml pipette to thoroughly resuspend the buffer.	Store at 4°C
Wash Buffer	Reconstitute in 100 ml of sterile distilled water.	Store at 4°C
Loading Buffer	No reconstitution necessary.	Store at 4°C
STOP Buffer	No reconstitution necessary.	Store at 4°C
GTPγS stock (non-hydrolysable GTP analog)	Reconstitute in 50 µl of sterile distilled water. Aliquot into 5 x 10 µl volumes, snap freeze in liquid nitrogen.	Store at –70°C
GDP Stock	Reconstitute in 50 µl of sterile distilled water. Aliquot into 5 x 10 µl volumes, snap freeze in liquid nitrogen.	Store at –70°C
Protease Inhibitor Cocktail	Reconstitute in 1 ml of dimethyl sulfoxide (DMSO) for 100x stock.	Store at –20°C

A) **Notes on Updated Version**

1. Version 4.4 updated to fit new 5.5 x 8.5 format.

2. Version 5.0 updated to correct recipe for 2x Laemmli buffer (Section VII: Troubleshooting)

B) **Growth and Treatment of Cell Lines**

The health and responsiveness of your cell line is the single most important parameter for the success and reproducibility of Rac1 activation assays. Time should be taken to read this section and to carefully maintain cell lines in accordance with the guidelines given below.

Adherent fibroblast cells such as 3T3 cells should be ready at 30% confluency or for non-adherent cells, at approximately 3 x 10^5 cells per ml. Briefly, cells are seeded at 5 x 10^4 cells per ml and grown for 3-5 days. Serum starvation (see below) or other treatment should be performed when cells are approximately 30% confluent. It has been found that cells cultured for several days (3-5 days) prior to treatment are significantly more responsive than cells that have been cultured for a shorter period of time. Other cell types may require a different optimal level of confluency to show maximum responsiveness to Rac1 activation. Optimal confluency prior to serum starvation and induction should be determined for any given cell line (also see Appendix 2 for cell line specific references).

When possible, the untreated samples should have cellular levels of Rac1 activity in a "controlled state". For example, when looking for Rac1 activation, the "controlled state" cells could be serum starved. Serum starvation will inactivate cellular Rac1 and lead to a much greater response to a given Rac1 activator. A detailed method for serum starvation is given in Appendix 1.

Cells should also be checked for their responsiveness ("responsive state") to a known stimulus. A list of known Rac1 stimuli are given in Appendix 2. In many cases poor culturing technique can result in essentially non-responsive cells. An example of poor culturing technique includes the sub-culture of cells that have previously been allowed to become overgrown. For example, Swiss 3T3 cells grown to >70% confluency should not be used for Rac1 activation studies.

To confirm the "controlled state" and "responsive state" of your cells, it is a good idea to include a small coverslip in your experimental tissue culture vessels and analyze the "controlled state" cells versus the "responsive state" cells by rhodamine phalloidin staining of actin filaments. A detailed method for actin staining is given in Appendix 1. Rac1 activation causes the formation of characteristic actin-rich lamelliopdia and membrane ruffles.

If you are having difficulty determining a "controlled state" for your experiment then contact technical assistance at 303-322-2254 or email tservice@cytoskeleton.com.

C) **Timing and Intensity of Rac1 Activation**

Upon stimulation, Rac1 proteins are generally activated very rapidly and transiently. Maximal activation ranges from 30 s to 30 min and declines thereafter to basal levels. Examples of known Rac1 activators are given in Appendix 2. For potent activators such as EGF, the intensity of maximal Rac1 activation over "control state" (serum starved) cells is generally in the order of 2-5 fold (see Appendix 2). However, using a single time point you are more likely to miss this maximum. It is therefore critical to take timed samples for at least the first experiment with an unknown activating entity. Recommended time points are 0, 1, 3, 6, 12 and 30 minutes (a time course is also recommended for Rac1 inactivation studies).

In practical terms the timed experiment must be performed sequentially. This allows rapid processing of each single time point. Once one time point lysate is collected, it should be snap frozen in "experiment sized" aliquots immediately and kept in -70°C. The Activation Assay uses approximately 300-800 µg of total protein per assay; this translates to 600-1600 µl of a 0.5 mg/ml cell lysate. We recommend duplicate samples per time-point or condition, therefore 1.2– 3.2 ml aliquots are recommended for snap freezing.

D) **Rapid processing of cells**

GTP bound (active) Rac1 is a labile entity and the bound GTP is susceptible to hydrolysis by Rac-GAPs during and after cell lysis, resulting in Rac1 inactivation. Rapid processing at 4°C is essential for accurate and reproducible results. The following guidelines are useful for rapid washing of cells.

Washing

a. Retrieve culture dish from incubator, immediately aspirate out all of the media and place firmly on ice.
b. Immediately rinse cells with an appropriate volume of ice cold PBS to remove serum proteins (see Table 3 for recommended wash volumes).
c. Aspirate off all residual PBS buffer. This is essential so that the Lysis Buffer is not diluted. Correct aspiration requires that the culture dish is placed at a steep angle on ice for 1 min to allow excess PBS to collect in the vessel for complete removal.

Cell Lysis

To avoid making too dilute or too concentrated lysate samples (<0.25 or >2.0 mg/ml), it is recommended to adjust the amount of Cell Lysis Buffer depending on your cell type and plate type. Table 3 gives guidelines for suitable lysis volumes for 3T3 cells which tend to give low protein yields. The exact lysis volumes for any given cell line will have to be determined empirically. NOTE: Cell Lysis Buffer should contain 1X Protease Inhibitor Cocktail.

Table 3: Recommended Wash and Lysis Volumes for 3T3 Cell Culture

Culture Vessel	Vessel surface area (cm^2)	Volume of PBS wash (ml)	Volume of Lysis Buffer (µl)
100 mm dish	56	10.0	250
150 mm dish	148	15.0	700
T-75 Flask	75	10.0	500
T-150 Flask	150	15.0	700

The time period between cell lysis and addition of lysates to the PAK-PBD beads is critically important. Take the following precautions:

1. Work quickly.

2. Keep solutions and lysates embedded in ice so that the temperature is below 4°C. This helps to minimize changes in signal over time. The Assay Protocol (Section VI) gives very specific instructions regarding temperature and must be strictly adhered to for successful results.

3. We strongly recommend that cell lysates be immediately frozen after harvest and clarification. A sample of at least 20 µl should be kept on ice for protein concentration measurement. A 20-50 µg sample should also be kept for Western blot quantitation of total Rac1 per sample. The lysates **must** be snap frozen in liquid nitrogen and stored at -70°C. Lystaes can be stored at -70°C for several months.

4. Thawing of cell lysates prior to use in the pull-down assay should be in a room temperature water bath, followed by rapid transfer to ice and immediate use in the assay.

E) **Protein Concentration Equivalence**

Equal protein concentration in all samples is a prerequisite for accurate comparison between samples in Rac1 activation assays. Cell extracts should be equalized with ice cold Cell Lysis Buffer to give identical protein concentrations. For example, cell lysates of protein concentrations ranging from 0.5–1.3 mg/ml would all need to be diluted to 0.5 mg/ml. It is not necessary to equalize protein concentrations if the variation between them is less than 10%.

F) **Assay Linearity**

There are several factors to consider when performing the Rac1 activation assays:

1) **Bead Titration:** PAK-PBD will bind to Rac1-GDP with a much lower affinity than Rac1-GTP. If too many PAK-PBD beads are added to the pull-down assay there will be significant binding to inactive (GDP-bound) Rac1. The result of this will be an underestimate of Rac1 activation. For this reason we highly recommend performing a bead titration to determine optimal conditions for any given Rac1 activation or inactivation assay. Once optimal conditions have been established, bead titrations should no longer be necessary. We recommend 10, 15 and 20 µg bead titrations.

2) **Strictly Maintain Experimental Conditions:** Once assay conditions are established one should strictly maintain experimental conditions. For example, lysate concentrations should be consistent between experiments. Thus, if 10 µg of beads are used to assay 400 µg of lysate at 0.5 mg/ml protein concentration, it is recommended to keep subsequent assays at 0.5 mg/ml lysate rather than using half the volume of a 1 mg/ml lysate to give 400 µg total protein. As a further example, the growth and treatment of cell lines should be consistent between experiments; this point can not be over-emphasized and is discussed in detail in Section V: B.

3) **Densitometric Quantitation:** The linear range of X-ray film is very narrow. Multiple exposures of the western blot may be required to analyze data in the linear range of the film. As a general guideline, protein bands that appear grey rather than black will be within the linear range of the film.

STEP 1: Control Reactions

The correct control reactions are key components of the Rac1 Activation Assay. The following control assays should be performed as an integral part of each experiment:

1. **Total Rac1 Protein:**

   Total Rac1 present in each sample should be determined by Western quantitation. Usually 20-50 µg of cell lysate will result in a good signal. Normalization of active Rac1 against total Rac1 is an important parameter in understanding the mechanisms underlying Rac1 activity.

2. **Positive Cellular Protein Control**:

   Total cell lysate (300 – 800 µg) should be loaded with GTPγS as a positive control for the pull-down assay. The following reaction details how to load endogenous Rac1 with the non-hydrolysable GTP analog (GTPγS), this is an excellent substrate for PAK -PBD beads and should result in a strong positive signal in a pull-.down assay. (See Figure 3, Lane 3).

   a. Perform GTP loading on 300 – 800 µg of cell lysate (0.5 mg/ml protein concentration) by adding 1/10th volume of Loading Buffer.

   b. Immediately add 1/100th volume of GTPγS (10 µl GTPγS per 990 µl of lysate) to give a 200 µM final GTPγS concentration. Under these conditions 5 - 10% of the Rac1 protein will load with non-hydrolysable GTPγS and will be "pulled-down" with the PAK-PBD beads in the assay.

   c. Incubate the control sample at room temperature for 15 min with gentle rotation.

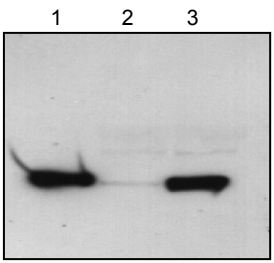
   d. Stop the reaction by transferring the tube to 4°C and adding 1/10th volume of STOP Buffer (100 µl STOP Buffer per 900 µl of lysate) .

   e. Use this sample immediately in a pull-down assay as detailed in STEP 3.

3. **Negative Cellular Protein Control:**

   This reaction should be performed in an identical manner to the Positive Control reaction except that 1/100th volume of GDP (1 mM final concentration) should be added to the reaction in place of the GTPγS. Loading endogenous Rac1 with GDP will inactivate Rac1 and this will bind very poorly to PAK-PBD beads (see Figure 3, Lane 2).

Figure 3: Control Assay Results for Rac1 Activation Assay



Legend: Control assays using HT1080 cells (500 µg per sample) were performed according to the above protocol. Lane designations are as follows: Lane 1, 20 ng of Rac1-His protein; Lane 2, negative control; Lane 3, positive control. The recombinant His-Rac1 protein (24 kD) runs slightly higher than the endogenous Rac1 (21 kD) due to the presence of the His tag.

4. **His-Rac1 Protein Control:**

   The kit supplies 10 µg of His-Rac1 control protein (part# RCWT); this will be reconstituted to a 0.33 mg/ml stock solution and stored at -70°C (as 10 x 3 µl aliquots). Storage of the protein at lower concentrations than 0.33 mg/ml or freeze/ thaw cycles will result in denaturation, precipitation of the protein and incorrect quantitations or no signal in the western blot. The endogenous Rac1 protein has a molecular weight of 21 kDa; the His-tagged control protein has a molecular weight of approximately 24 kDa (see Figure 3, Lane 1). We recommend that 20 ng of His-Rac1 control protein be run on the gel as a positive control and as a quantitation estimate for endogenous Rac1 (see STEP 4).

STEP 2: Lysate Collection

We strongly recommend that you snap freeze your cell lysates in liquid nitrogen right after you harvest and clarify. This is especially necessary if you have many samples. It is recommended to freeze lysates in 1-3 ml aliquots and to save a small amount of each lysate (approximately 20 - 50 µg) for protein quantitation. Details of lysates processing are given below:

Cells Grown in Tissue Culture Vessels as Monolayers

1. Grow cells in appropriate culture conditions. It is important to keep cells in a "controlled state" prior to Rac1 activation. See Section V (B): Important Technical Notes.
2. Treat cells with Rac1 activator, e.g. EGF (or inactivator) as your experiment requires.
3. After treatment, place culture vessel on ice, aspirate media, wash with ice cold PBS. See Table 3, Section V: D for recommended volumes.
4. Aspirate off PBS. Tilt plates on ice for an additional 1 min to remove all remnants of PBS. Residual PBS will adversely affect the assay.
5. Lyse cells in an appropriate volume of ice-cold Cell Lysis Buffer (Lysis Buffer should be supplemented with 1X Protease Inhibitor Cocktail). See Table 3, Section V: D for recommended volumes.
6. Harvest cell lysates with a cell scraper. It is useful to incline the culture plate for this method because the Lysis Buffer is spread thinly on the surface.
7. Transfer lysates into the pre-labeled sample tubes on ice.
8. Immediately clarify by centrifugation at 10,000 x g, 4°C for 1 min.
9. At this point each lysate volume should not exceed 130% of the original Cell Lysis Buffer volume.
10. Save at least 20 µl of lysate for protein quantitation and 20 - 50 µg of lysate for Western blot quantitation of total Rac1.
11. Aliquot and snap freeze the remaining cell lysates in liquid nitrogen. Store at -70°C for future use. It is recommended to aliquot into 1-3 ml of lysate per tube (This should be sufficient for duplicate assays of 300-800 µg per assay).
12. Measure lysate protein concentrations. We recommend using Precision Red Advanced Protein Assay (Cat. # ADV02) for quantitations (see Appendix 3):
    - Add 20 µl of each lysate or Lysis Buffer into disposable 1 ml cuvettes.
    - Add 1 ml of Precision Red™ Advanced Protein Assay Reagent (Cat # ADV02) to each cuvette.
    - Incubate for 1 min at room temperature.
    - Blank spectrophotometer with the Cell Lysis Buffer at 600 nm.
    - Read absorbance of lysates samples.
    - Multiply the absorbance by 5 to obtain the protein concentration in mg/ml.

13. Calculate how to equalize the cell extracts with ice cold Lysis Buffer to give identical protein concentrations. It is essential to have equal protein concentration in each sample for a successful assay. It is also important that the equalized protein concentration is not higher than 2.0 mg/ml or below 0.25 mg/ml. It is not necessary to equalize protein concentration if the sample variation is less than 10%.

    The volume of cold cell lysis buffer to be added to the more concentrated samples can be calculated as follows:

    $$\frac{A - B}{B} \text{ x (volume of A)} = \_\_\_\_\_ \mu l$$

    Where A is the higher concentration lysates (mg/ml) and B is the concentration of the most dilute sample (mg/ml)

NOTE: You can dilute the lysates to a given concentration (e.g. 0.5 mg/ml) prior to snap freezing aliquots. This makes subsequent pulldown assays simpler. Be aware of the length of time cell lysates stay on ice (should not exceed 10 min), since Rac1-GTP hydrolysis will occur.

STEP 3: Pull-down Assay

1. If using freshly prepared cell lysates, use as soon as possible after lysis and protein equalization and always maintain samples at 4°C. If using frozen lysates (recommended), thaw in a room temperature water bath and remove immediately to ice upon thawing. Use immediately.

2. Add equivalent protein amounts of lysate (300 – 800 µg total cell protein) to a pre-determined amount of PAK-PBD beads from your bead titration test (see Section V.F.1).

NOTE: In general, 10 µg (10 µl) of PAK-PBD bead pull-down will yield optimal results. Under these conditions the 500 µg of PAK-PBD beads supplied in the kit are sufficient for 50 assays. We do however recommend a bead titration (10, 15 & 20 µg) to determine optimal pull-down conditions.

3. Incubate at 4°C on a rotator for 1 h.

4. Pellet the PAK-PBD beads by centrifugation at 3-5,000 x g at 4°C for 1 min.

5. Very carefully remove 90% of the supernatant. Do not disturb the bead pellet. If you do disturb the pellet simply re-centrifuge the sample as in step 4.

6. Wash the beads once with 500 µl each of Wash Buffer. **NOTE**: Add the buffer to the bead pellet in a manner that completely resuspends the beads. **DO NOT invert the tube as the beads will disperse over the surface of the tube and protein will be lost. This step should take less than 1 min to perform.**

7. Pellet the PAK-PBD beads by centrifugation at 3-5,000 x g at 4°C for 3 min.

8. Very carefully remove the supernatant. Do not disturb the bead pellet. If you do disturb the pellet simply re-centrifuge the sample as in step 7.

9. Add 10-20 µl of 2x Laemmli sample buffer to each tube and thoroughly resuspend the beads. Boil the bead samples for 2 min.

10. The samples are now ready to be analyzed by SDS-PAGE and Western blot analysis (see STEP 4).

NOTE: The whole sample including the beads can be loaded onto the SDS gel (do not use a gel loading tip for this method), alternatively the beads can be pelleted in a microcentrifuge tube and the total supernatant can be run on the gel. It is recommended that the necessary control samples be run on each gel.

STEP 4: Western Blot Protocol

1. Run the test protein samples and controls on a 4-20% or 12% SDS gel until the dye front reaches the bottom of the gel.
2. We recommend running a lane containing 20 ng of His-Rac1 control protein as a positive control. To do this the protein should be diluted as follows;
   a) Thaw one of the 3 µl aliquots of His-Rac1 control protein (see Table 2).
   b) Dilute to 4 ng/µl by adding 247 µl of Cell Lysis Buffer.
   c) Dilute to 2 ng/µl by adding 250 µl of 2X Laemmli sample buffer (125mM Tris pH6.8, 20% glycerol, 4% SDS, 0.005% Bromophenol blue, 5% beta-mercaptoethanol).
   d) Load 10 µl (20ng).
   e) Discard any unused control protein as it will "crash out" during storage at 4°C or frozen.
3. Equilibrate the gel in Western blot buffer (See recipe below) for 15 min at room temperature prior to electro-blotting.
4. Transfer the protein to a PVDF membrane for 45 minutes at 75V.
5. Wash the membrane once with TBS (10 mM Tris-HCl pH 8.0, 150 mM NaCl).
6. Allow the membrane to air dry for 20-30 minutes at room temperature.
7. Transfer membrane to TBST (10 mM Tris-HCl ph 8.0, 150 mM NaCl, 0.05% Tween 20) at room temperature for 15 minutes to rehydrate the membrane. It is convenient, at this point, to leave the membrane in TBST overnight at 4°C.
8. Block the membrane surface with 5% nonfat-dry milk in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 30 min at room temperature with constant agitation.
9. Incubate the membrane with a 1:500 dilution of anti-Rac1 antibody (Cat. # ARC03, provided with kit) diluted in TBST (no blocking agent) for 1-2 h at room temperature or overnight at 4°C with constant agitation.
10. Rinse the membrane in 50 ml TBST for 1 min.
11. Incubate the membrane with an appropriate dilution (eg. 1:20,000) of anti-mouse secondary antibody (eg. goat anti-mouse HRP conjugated IgG from Jackson Labs., Cat. # 115-035-068) in TBST for 30 min-1 h at room temperature with constant agitation.
12. Wash the membrane 5 times in TBST for 10 min each.
13. Use an enhanced chemiluminescence detection method to detect the Rac1 signal (eg. SuperSignal West Dura Extended Duration Substrate; ThermoFisher).

Recipe for Western Blot Buffer (1 L)

1 M Tris pH 8.3	25 ml	(25 mM final)
Glycine	14.4 g	(192 mM final)
Methanol	150 ml	(15% final)
Distilled water to 1 L		

VII: Troubleshooting

Observation	Possible cause	Possible Remedy
No signal from the His-tagged Rac1 control protein.	1. Storage of the stock control protein at concentrations that are too low (<0.33mg/ml). 2. Repeated freeze/thaw cycles of the reconstituted positive control stock protein. 3. Attempts to store the diluted stock at 4°C or frozen for future use. 4. Failure to follow the western blot protocol detailed in section VI: STEP 4.	1. The kit supplies 10 µg of His-Rac1 protein, this should be reconstituted to a 0.33 mg/ml stock solution and stored at -70°C (as 10 x 3 µl aliquots, see Table 2). Storage of the protein at lower concentrations will result in denaturation and precipitation of the protein and incorrect quantitations or no signal at all. 2. The stock protein must be aliquoted as described in Table 2. Repeated freeze thaws of the stock will result in denaturation and precipitation. 3. We recommend loading 20 ng of the positive control on the gel as a positive control and quantitation estimate for endogenous Rac1 (for 20 ng of recombinant protein, dilute one 3 µl aliquot of protein stock with 247 µl of Cell Lysis Buffer and then 250 µl of 2x Laemmli sample buffer; load 10 µl of this on the SDS gel). The diluted protein is unstable and will precipitate. Unused protein must be discarded. The Rho family proteins have a molecular weight of between 20-25 kDa; the His-tagged control protein has a molecular weight of approximately 24 kDa. 4. Follow the western blot protocol in this manual. It has been optimized for small G-proteins.
No difference in signal between GTPγS positive control and GDP negative control assay	1. Protein lysate concentrations were not equalized. 2. Titration of PAK-PBD Beads not performed. 3. GDP requirements are higher for your cell line.	1. The absolute amount of protein in lysates can have a dramatic effect upon Rac1 signal. It is therefore very important to have equal amounts of cell lysate protein in each reaction. See section V (E). 2. Perform bead titration per section V (F). In cases where there is a high signal in both GTPγS and GDP lanes, using half the amount of PAK-PBD beads will often result in a better differential signal. 3. Some cell lines have very high levels of endogenous GTP and exchange of GDP requires addition of greater than the 1 mM GDP outlined in this manual. We recommend trying 10 mM GDP in these cases.
No detectable Rac1 activation in the positive control (GTPγS) assay	1. STOP buffer not added to the reactions. 2. Leaving the lysates for >10 minutes before use.	1. Follow the instructions carefully, for example, STOP buffer must be added to the reaction or you will not get a Rac1 signal. 2. GTPγS AND GDP loaded lysates should be used within 2-3 minutes after STOP buffer has been added.

Observation	Possible cause	Remedy
No detectable signal in the experimental samples	1. Control reaction not performed for GTPγS. His-Rac1 control protein not used during Western blot.	1. Always run a GTPγS control to make sure the PAK-PBD beads are working and always run the recombinant His-Rac1 control protein to make sure that the Western blot / Rac1 antibody is working correctly. Once these controls are working you can go on to determine the likely cause of a lack of signal or a lack of activation in the experimental samples.
	2. Insufficient cell lysate used	2. Titrate the protein amount used in the assay. We recommend 300-800 µg lysate, howver, in some cases more lysate may be required.
	3. Lysates not processed rapidly at 4°C	3. Rac1 is still able to hydrolyze GTP during lysate PAK-PBD beads are bound to Rac1-GTP. The temperature and speed of lysate preparation are therefore very important parameters in this assay .
Rac1 activation signal does not change upon experimental activation stimulus.	1. Titration of PAK-PBD Beads not performed.	1. Make sure that your control GDP and GTPγS lanes give a clear positive and negative response; this indicates that the bead and cell lysate levels are in the correct linear range to detect differential Rac1 activation states. This may require titrating bead and / or lysate levels.
	2. Culture conditions have caused cells to become unresponsive to Rac1 activators.	2. Continuous overgrowing of a cell line can result in unresponsive cells. Swiss 3T3 cells should only be used for 10 passages and then discarded as their properties change if they are passaged longer than this (17). Cells seeded at low densities, grown for 3 days to 30- 40% confluency, then serum starved by a serum-step down procedure often respond better than cells grown to higher densities. See Appendix 1 for a cell culture protocol.
	3. Selected Rac1 activator may not work with your cell line.	3. Use a known Rac1 activator (eg. EGF) to check the responsiveness of your cell line. A list of some Rac1 activators are given in Appendix 2. Note that the cell line used for the activation assay is important as response to any given activator can vary considerably between cell lines. It may also be useful to examine actin morphology via rhodamine-phalloidin labeling of cells. (See Appendix 1). The serum starved cells should have very few actin stress fibers while stimulated cells should have a large number of actin rich lamellipodia and membrane ruffles (see Appendix 1).
	4. Western blot is overexposed leading to inaccurate readings.	4. As a general guideline, you should expose the film so that the Rac1 signal gives a grey band rather than a black band. Alternatively, the Rac1 G-LISA® Activation Assay Kit (Cat. # BK128) can be used to obtain quantitative results within 3 h.

VII: Troubleshooting (cont.)

Observation	Possible cause	Remedy
Rac1 signal appears as doublet	1. Samples in sample loading buffer stored at –20°C.	1. Rac1 can form irreversible doublets during processing. The most sensitive point for doublet formation is after resusepending beads in sample buffer. Run samples on SDS-PAGE immediately after resuspension of the beads in sample buffer. Samples stored at –20°C in sample buffer may have enhanced doublet formation.
	2. Sample loading buffer is not fresh.	2. If doublet formation is a problem, try preparing fresh 2X Laemmli sample buffer (125 mM Tris pH 6.8, 4% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.005% bromophenol blue) and boil samples before loading onto SDS-PAGE.

Section VII: References

1. Ridley, A.J. & Hall, A. 1992. The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell.* **70**, 389-399.
2. Ridley, A.J. et al. 1992. The small GTP-binding protein Rac regulates growth factor-induced membrane ruffling. *Cell.* **70**, 401-410.
3. Nobes, C.D. et al. 1995. Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell.* **81**, 53-62.
4. Waterman-Storer, C.M. et al. 1999. Microtubule growth activates Rac1 to promote lamellipodial protrusion in fibroblasts. *Nature Cell Biol.* **1**, 45-50.
5. Hill, C.S. et al. 1995. The Rho family GTPases RhoA, Rac1, and Cdc42Hs regulate transcriptional activation by SRF. *Cell.* **81**, 1159-1170.
6. Seasholtz, T.M. et al. 1999. Rho and Rho Kinase Mediate Thrombin-Stimulated Vascular Smooth Muscle Cell DNA Synthesis and Migration. *Circulation Res.*. **84**, 1186-1193.
7. Gasman, S. 1999. Involvement of Rho GTPases in calcium-regulated exocytosis from adrenal chromaffin cells. *J. Cell Sci.* **112**, 4763-4771.
8. Hotchin, N.A. et al. 2000. Cell Vacuolation Induced by the VacA Cytotoxin of *Helicobacter pylori* is Regulated by the Rac1 GTPase. *J. Biological Chem.* **275**, 14009-14012.
9. Subauste, M.C. et al. 2000. Rho Family Proteins Modulate Rapid Apoptosis Induced by Cytotoxic T Lymphocytes and Fas. *J. Biological Chem.* **275**, 9725-9733.
10. Lamarche, N. and Hall, A. 1994. GAPs for Rho-related GTPases. *Trends in Genetics.* **10**, 436-440.
11. Zhou, K. et al. 1998. Guanine Nucleotide Exchange Factors Regulate Specificity of Downstream Signaling from Rac and Cdc42. *J. Biological Chem.* **273**, 16782-16786.
12. Hall, A. 1999. Rho GTPases and the Actin Cytoskeleton. *Science.* **279**, 509-514.
13. Aspenstrom, P. 1999. Effectors for the Rho GTPases. *Curr. Opin. In Cell Biol.* **11**, 95-102.
14. Benard, V. et al. 1999. Characterization of Rac and Cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. J. Biol. Chem. 274: 13198-13204.
15. Burbelo, P et al. 1995. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. J. Biol. Chem. 270: 29071-29074.
16. Zhang, B., et al. 1998. Interaction of Rac1 with GTPase-activating proteins and putative effectors. J. Biol. Chem. 273: 8776-8782.
17. Leonard, D.A, et al. 1997. Biochem. 36: 1173-1180.

Appendix 1: Observation of Actin Morphology By Rhodamine-Phalloidin Staining

Reagents needed

- Control state and Responsive state cells (e.g. serum starved cells and EGF treated cells)
- Suitable growth media
- EGF stock solution (20 mg/ml in PBS)
- PBS solution pH 7.4 (150 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2PO_4, 1.47 mM KH_2PO_4)
- Rhodamine-phalloidin stock (14 mM in methanol, Cat. # PHDR1)

Method

Serum starvation for Swiss 3T3 cells and addition of growth factors

1. Swiss 3T3 cells are seeded at low density of 3 – 5 x 10^4 cells in DMEM plus 10% FCS on a 10 cm diameter plate containing two 13 mm diameter glass coverslips.
2. Once cells are 30-40% confluent (usually 3 days) the media is replaced with DMEM plus 1% FCS and cultured for 15-18 h.
3. The media is again replaced with DMEM without FCS and the cells are incubated for 24 h.
4. After serum starvation remove one coverslip and process for actin staining as described below.
5. Add fresh EGF to the remaining cells to 10 ng/ml for 2 min.
6. Remove the coverslip and process for actin staining as described below.

Actin Staining

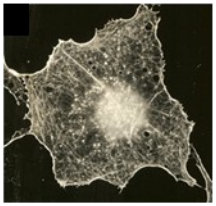
1. Wash the cells once with PBS and fix for 20 min at room temperature in 3% paraformaldehyde diluted in PBS.
2. Wash the cells once for 30 s with PBS to remove excess fixative.
3. Incubate the cells with 0.2% Triton-X 100 in PBS for 5 min at room temperature to permeabilize cells.
4. Wash twice in PBS for 30 s each.
5. Incubate with 200 µl of 0.1 µg/ml Rhodamine-phalloidin for 30 min at room temperature in the dark.
6. Wash five times with PBS for 30 s each.
7. Invert the cells into mounting medium (eg. Polyvinyl alcohol mounting medium, Fluka Chemie) and allow the coverslip to set for 30 min.
8. View actin filaments with a 63 – 100X oil immersion objective.
9. Examples of serum starved and EGF treated cells are shown in Figure 1.

NOTE: All the required reagents for fixing cells and staining F-actin can be found in the F -actin Visualization Kit (Cat. # BK005).

Appendix 1: Observation of Actin Morphology By Rhodamine-Phalloidin Staining (cont.)

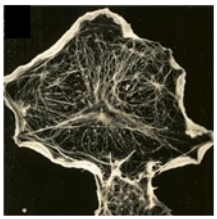
Figure 1: Rhodamine-Phalloidin Staining of the Actin Cytoskeleton in Serum Starved and Rac1 Activated 3T3 Cells

A



Serum Starved Actin Morphology:
Swiss 3T3 cells serum starved for 16h prior to actin filament staining with rhodamine-phalloidin. In the absence of Rho family activation there is a notable paucity of actin filaments visible in the cell.

B



Rac Activated Actin Morphology:
Cells treated for 2 min with 10 ng/ml EGF after serum starvation and subsequently stained with rhodamine phalloidin. Rac induced actin-rich lamellipodia and membrane ruffles are clearly visible.

Appendix 2: Known Rac Activators

Activator*	Treatment	Cell line used	Response	Type of Assay Used	Ref.
Epidermal Growth Factor	50 ng/ml	US7MG human glioblastoma	1.5 fold activation after 5 minutes with 2D cultures. 1.3 fold activation in 3D cultures.	Rac G-LISA®	1
MCP-1	10 ng/ml	Murine alveolar macrophages	Maximal activation at 4h.	Rac G-LISA®	2
Heregulin beta1	0-30 ng/ml	Breast cancer cell lines	Dose dependent activation	PAK-PBD pulldown	3
Interleukin-3	5 µg/ml for 5 min	Primary bone marrow derived mast cells	2 fold increase over control	PAK-PBD pulldown	4

1 Kim H.D. et al. 2008. Epidermal growth factor-induced enhancement of glioblastoma cell migration in 3D arises from an intrinsic increase in speed but an extrinsic matrix- and proteolysis-dependent increase in persistence. Mol Bio Cell. 19, 4249-4259.

2 Tanaka T. et al. 2010. Monocyte chemoattractant protein-1/CC chemokine ligand 2 enhances apoptotic cell removal by macrophages through Rac1 activation. Biochem. Biophys. Res. Commun. 399, 677-682.

3 Yang C. et al. 2006. Essential role for Rac in heregulin beta1 mitogenic signaling: a mechanism that involves epidermal growth factor receptor and is independent of ErbB4. Mol. Cell. Biol. 26,831-842.

4 Grill B. and Schrader J.W. 2002. Activation of Rac-1, Rac-2, and Cdc42 by hemopoietic growth factors or cross-linking of the B-lymphocyte receptor for antigen. Blood. 100, 3183-3192.

Appendix 3: Protein Quantitation (with Precision Red Reagent)

Background

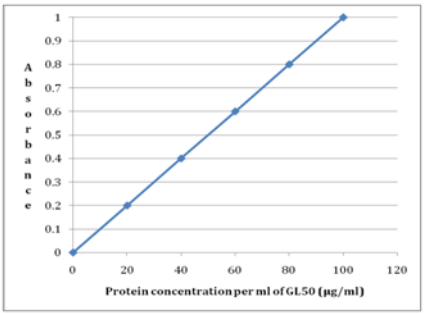
The Precision Red Advanced Protein Assay Reagent is a simple one step procedure that results in a red to purple/blue color change characterized by an increase in absorbance at 600 nm. The reagent is not supplied in this kit, it is sold separately as Cat. # ADV02. Precision Red Advanced Protein Assay Reagent is supplied in the G-LISA activation assays (Part# GL50).

The assay exhibits low variance in readings between different proteins of the same concentration and high reproducibility of the colorimetric response. This allows one to utilize a generally applicable standard curve (Fig. 1) for protein quantitation. The assay can also be performed in approximately 1-2 minutes. These properties are particularly valuable when applied to the labile lysates required for activation assays.

Quick Protein Concentration Method for 1 ml Cuvette (recommended)

- Add 20 µl of each lysate or Lysis Buffer into disposable 1 ml cuvettes.
- Add 1 ml of Precision Red™ Advanced Protein Assay Reagent (Cat# ADV02) to eac- cuvette.
- Incubate for 1 min at room temperature.
- Blank spectrophotometer with 1 ml of ADV02 plus 20 µl of Lysis Buffer at 600 nm.
- Read absorbance of lysate samples.
- Multiply the absorbance by 5 to obtain the protein concentration in mg/ml.

Fig. 1: Standard Curve for Protein Quantitation in a 1ml Cuvette



Legend: The standard curve shown in Fig. 1 represents the average absorbance reading of several common proteins (e.g., actin, BSA, casein) measured in a 1 ml cuvette format using 1 ml of ADV02 reagent. The protein reading pathlength for a cuvette is 1 cm. Linear range of this assay is 0.05 - 0.6.

Example Calculation

Assume a 20 µl sample of cell lysate added to 1 ml of ADV02 gives an absorbance reading of 0.1.

$$C = \frac{A}{\varepsilon l} = \frac{0.1}{10 \times 1} \times 50 = 0.5 \text{ mg/ml}$$

Where c = protein concentration (mg/ml), A = absorbance reading, l = pathlength (cm), ε = extinction coefficient ($[mg/ml]^{-1}$ cm^{-1}) and the multiplier of 50 is the dilution factor for the lysate in ADV02 (20 µl lysate in 1 ml ADV02).

Thus, for a 20 µl sample in 1 ml ADV02, the equation becomes $C = A \times 5$

For a 10 µl sample in 1 ml ADV02, the equation becomes $C = A \times 10$

Appendix 3: Protein Quantitation (with Precision Red Reagent)

Quick Protein Concentration Method for 96 Well Plate

- Add 10 µl of each lysate or Lysis Buffer into the well of a 96 well plate.
- Add 290 µl of Precision Red™ Advanced Protein Assay Reagent to each well.
- Incubate for 1 min at room temperature.
- Blank spectrophotometer with 290 µl of ADV02 plus 10 µl of Lysis Buffer at 600 nm.
- Read absorbance of lysate samples.
- Multiply the absorbance by 3.75 to obtain the protein concentration in mg/ml

96 Well Plate Method

The linear range of this assay is 0.05 - 0.4 and is recommended when lysates are below the linear range of the 1 ml cuvette method. The pathlength for 96 well plate readings is 0.8 cm, hence the equation is modified as shown in the example below:

Example Calculation for 96 Well Plate Measurement

Assume a 10 µl sample of cell lysate added to 290 µl of ADV02 gives an absorbance reading of 0.1

$$C = \frac{A}{\varepsilon l} = \frac{0.1}{10 \times 0.8} \times 30 = 0.375 \text{ mg/ml}$$

Where c = protein concentration (mg/ml), A = absorbance reading, l = pathlength (cm), ε = extinction coefficient ($[mg/ml]^{-1}$ cm^{-1}) and the multiplier of 30 is the dilution factor for the lysate in ADV02 (10 µl lysate in 290 µl ADV02).

Thus, for a 10 µl sample in 290 µl ADV02, the equation becomes $C = A \times 3.75$

For a 5 µl sample in 295 µl ADV02, the equation becomes $C = A \times 7.5$

NOTE: The protein concentrations generated by using the standardized protein curve (Fig.1) will generate approximate lysate concentrations. Data will be highly reproducible from lysate to lysate and will generate excellent values for relative concentrations of a series of lysates. It should be noted for activation assays, the relative protein concentration between experimental extracts is far more important than the absolute protein quantitation. However, if desired, one can create a standard curve using BSA or IgG protein standards for each experiment. The standard curve should be performed prior to lysate preparations due to the labile nature of the lysates.

Appendix 4: Processing Tissue Samples for Pull-Down Assays

Tissue lysates can be used in pull-down assays (1). Recommendations regarding tissue lysates are given below;

1) Rho family GTPases are labile proteins that will hydrolyze bound GTP during sample handling. Tissues should therefore be processed quickly and at 4°C if possible. Tissues should be processed immediately using 4°C buffers or cut into small chunks (3-5 mm diameter), snap frozen in liquid nitrogen and stored at –70°C for later processing.
2) Tissues can be extracted using a micro-pestel on ice. Homogenates should be clarified by a 1 minute centrifugation at 4°C. Lysates can be used immediately in an activation assay or snap frozen in "experiment-sized" volumes. The Activation Assay uses approximately 300-800 µg of total protein per assay; this translates to 600-1600 µl of a 0.5 mg/ml cell lysate. We recommend duplicate samples per time-point or condition, therefore 1.2– 3.2 ml aliquots are recommended for snap freezing.
3) When possible tissues should be extracted in Cell Lysis Buffer (Part# CLB) as this is the recommended buffer for pull-down assays.
4) It is recommended that lysis buffer be supplemented with protease inhibitors and phosphatase inhibitors. Recommended inhibitors include; Cytoskeleton protease inhibitor cocktail (Cat# PIC02), sodium fluoride (50 mM final), sodium pyrophosphate (20 mM final), p-Nitrophenyl phosphate (1 mM final) and microcystin LR (1 µM final).
5) A final lysate protein concentration of 0.5 mg/ml is recommended.

Reference

1) ElAli, A. and Hermann, D. 2012. Liver X receptor activation enhances blood-brain barrier integrity in the ischemic brain and increases the abundance of ATP-binding cassette transporters ABCB1 and ABCC1 on brain capillary cells. *Brain Pathology* **22**, 175-187.

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