



Cdc42 Activation Assay Biochem Kit[™] (50 Assays)

Cat. # BK034

Manual Contents

Section I: Introduction

Background-Cdc42 Activation Assay	5-6
-----------------------------------	-----

Section II: Purchaser Notification

Section III: Kit Contents

Section IV: Reconstitution and Storage of Components

Section V: Important Technical Notes

A. Notes on Updated Version	11
B. Growth and Treatment of Cell Lines	11
C. Timing and Intensity of Cdc42 Activation	12
D. Rapid Processing of Cells	12-13
E. Protein Concentration Equivalence	13
F. Assay Linearity	13-14

Section VI: Assay Protocol

STEP 1: Control Reactions	15-16
STEP 2: Lysate Collection	17-18
STEP 3: Pull-down Assay	19
STEP 4: Western Blot Protocol	20

Section VII: Troubleshooting

Section VIII: References

APPENDICES

Appendix 1 Serum Starving Cells and F-actin Visualization	25-26
Appendix 2 Table of Known Cdc42 Activators	27
Appendix 3 Protein Quantitation (with Precision Red)	28-29
Appendix 4 Processing tissue samples for pull-down assays	30

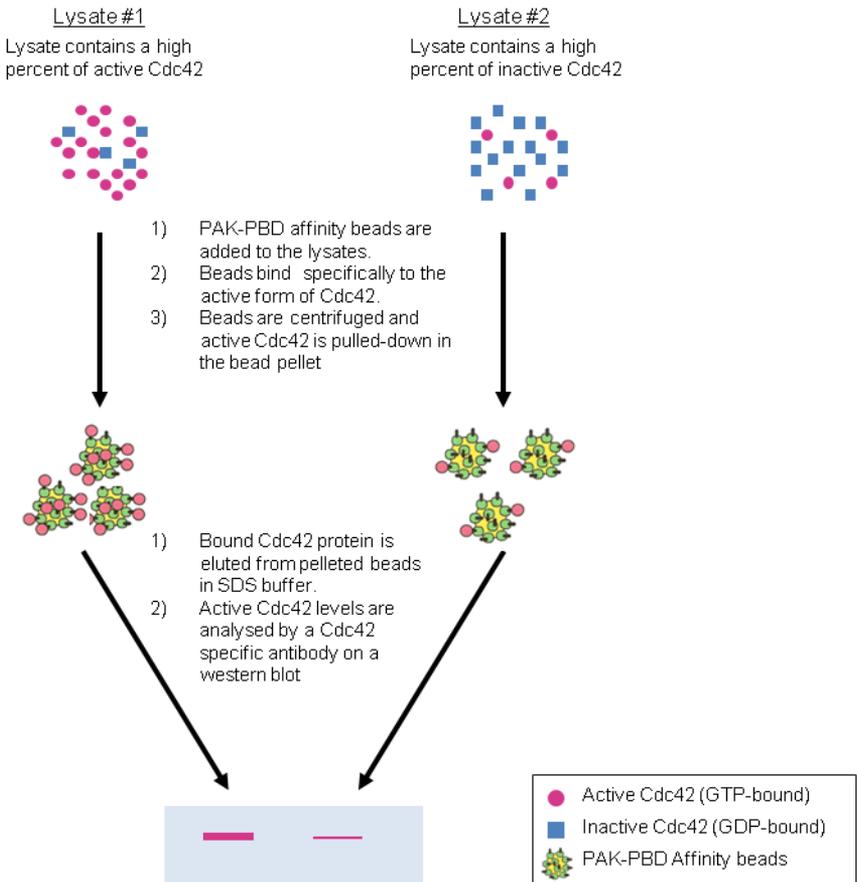
I: Introduction

Background– Cdc42 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 Cdc42 Activation Assay principle.

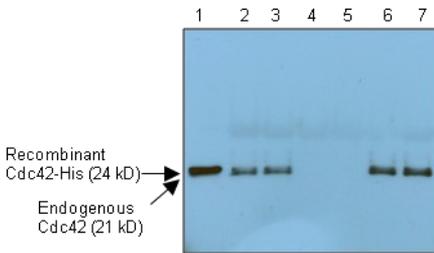
Figure 1: Schematic of Cdc42 Activation (Pull-down) Assay



I: Introduction (cont.)

The Cdc42 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-Cdc42 (or GTP-Rac1) complex with glutathione affinity beads. The assay therefore provides a simple means of quantitating Rac/Cdc42 activation in cells. The amount of activated Cdc42 is determined by a Western blot using a Cdc42 specific antibody. Figure 2 shows typical Cdc42 Activation Assay results from serum starved and EGF treated Swiss 3T3 cells. Serum starvation greatly reduces the amount of active Cdc42 in cells while EGF is a potent activator of Cdc42.

Figure 2: Cdc42 Activation by EGF



Swiss 3T3 cells were serum starved for 40h, after this some cells were treated with 100 ng/ml of EGF for 30 seconds (Lanes 2 & 3) or 2 minutes (Lanes 6 & 7), others were not treated and remained serum starved (Lanes 4 & 5). Cdc42 activation was measured using the Cdc42 Activation pull-down assay, 500 μ g of lysate were assayed with 10 μ g of PAK-PBD beads (Lanes 2-7). Lane 1 shows 20 ng of recombinant Cdc42-His protein run as a western blot standard. Note: the slight shadow signal running at approximately 36 kD in the pull-down lanes is signal from the PAK bead protein.

II: Purchaser Notification

Limited Use Statement

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of product and components of product in research conducted by the buyer. The buyer cannot sell or otherwise transfer this product or any component thereof to a third party or otherwise use this product or its components for commercial purposes. Commercial purposes include, but are not limited to: use of the product or its components in manufacturing; use of the product or its components to provide a service; resale of the product or its components.

The terms of this Limited Use Statement apply to all buyers including academic and for-profit entities. If the purchaser is not willing to accept the conditions of this Limited Use Statement, Cytoskeleton Inc. is willing to accept return of the unused product with a full refund.

III: Kit Contents

This kit contains enough reagents for approximately 50 pull-down assays. There is sufficient Cdc42 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	1 tube, lyophilized; 500 µg of protein per tube bound to colored sepharose beads	Desiccated 4°C
Anti-Cdc42 monoclonal antibody	Cat # ACD03	2 tubes, lyophilized,	Desiccated 4°C
His-Cdc42 control protein	Part # CDWT	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 µg/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 recommended)
- 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 μ l distilled water. Aliquot into 50 x 10 μ l volumes (10 μ l of beads = 10 μ g of protein, under these conditions a 500 μ l aliquot is sufficient for 50 assays). Snap freeze in liquid nitrogen.	Store at -70°C .
Anti-Cdc42 monoclonal antibody	Resuspend each tube in 200 μ l of PBS. Use at 1:250 dilution.	Store at 4°C . DO NOT FREEZE
His-Cdc42 control protein	Reconstitute in 30 μ l of distilled water. Aliquot into 10 x 3 μ l 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
GTPyS stock (non-hydrolysable GTP analog)	Reconstitute in 50 μ l of sterile distilled water. Aliquot into 5 x 10 μ l volumes, snap freeze in liquid nitrogen.	Store at -70°C
GDP Stock	Reconstitute in 50 μ l of sterile distilled water. Aliquot into 5 x 10 μ l 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 .

V: Important Technical Notes

A) **Notes on Updated Version**

1. Version 5.0: Added 0.1% nonfat-dry milk to the primary antibody during incubation in western blot procedure (Section VI: STEP 4). This has been found to reduce background.
2. Version 6.0 corrected 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 Cdc42 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 3×10^5 cells per ml. Briefly, cells are seeded at 5×10^4 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 Cdc42 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 Cdc42 activity in a “controlled state”. For example, when looking for Cdc42 activation, the “controlled state” cells could be serum starved. Serum starvation will inactivate cellular Cdc42 and lead to a much greater response to a given Cdc42 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 Cdc42 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 Cdc42 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. Cdc42 activation causes the formation of characteristic actin-rich filopodia and microspikes.

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.

V: Important Technical Notes (Continued)

C) Timing and Intensity of Cdc42 Activation

Upon stimulation, Cdc42 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 Cdc42 activators are given in Appendix 2. For potent activators such as EGF, the intensity of maximal Cdc42 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 Cdc42 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) Cdc42 is a labile entity and the bound GTP is susceptible to hydrolysis by Cdc42-GAPs during and after cell lysis, resulting in Cdc42 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.

* NOTE: In 3T3 cells, it has been found that omitting the wash step results in a more reproducible Cdc42 activation. However, if the final growth media contains proteins then the wash step cannot be omitted as the protein content of the media will interfere with cell lysate protein quantitations.

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.

V: Important Technical Notes (Continued)

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

* See notes concerning wash step in section D

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 Cdc42 per sample. The lysates must be snap frozen in liquid nitrogen and stored at -70°C. Lysates 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 Cdc42 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 Cdc42 activation assays:

- 1) **Bead Titration:** PAK-PBD will bind to Cdc42-GDP with a much lower affinity than Cdc42-GTP. If too many PAK-PBD beads are added to the pull-down assay there will be significant binding to inactive (GDP-bound) Cdc42. The result of this will be an underestimate of Cdc42 activation. For this reason we highly recommend

V: Important Technical Notes (Continued)

performing a bead titration to determine optimal conditions for any given Cdc42 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.

VI: Assay Protocol

STEP 1: Control Reactions

The correct control reactions are key components of the Cdc42 Activation Assay. The following control assays should be performed as an integral part of each experiment:

1. Total Cdc42 Protein:

Total Cdc42 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 Cdc42 against total Cdc42 is an important parameter in understanding the mechanisms underlying Cdc42 activity.

2. Positive Cellular Protein Control:

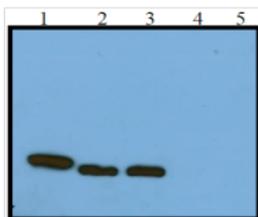
Total cell lysate (300 – 800 μg) should be loaded with GTP γ S as a positive control for the pull-down assay. The following reaction details how to load endogenous Cdc42 with the non-hydrolysable GTP analog (GTP γ S), this is an excellent substrate for PAK-PBD beads and should result in a strong positive signal in a pull-down assay. (See Figure 3, Lane 3).

- Perform GTP loading on 300 – 800 μg of cell lysate (0.5 mg/ml protein concentration) by adding 1/10th volume of Loading Buffer (100 μl Loading buffer per 900 μl lysate).
- Immediately add 1/100th volume of GTP γ S (10 μl GTP γ S per 990 μl of lysate) to give a 200 μM final GTP γ S concentration. Under these conditions 5 - 10% of the Cdc42 protein will load with non-hydrolysable GTP γ S and will be “pulled-down” with the PAK-PBD beads in the assay.
- Incubate the control sample at room temperature for 15 min with gentle rotation.
- 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).
- 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 GTP γ S. Loading endogenous Cdc42 with GDP will inactivate Cdc42 and this will bind very poorly to PAK-PBD beads (see Figure 3, Lanes 4&5).

Figure 3: Control Assay Results for Cdc42 Activation Assay



Control assays using Swiss 3T3 cells (500 μg per sample) were performed according to the above protocol. Lane designations are as follows: Lane 1, 20 ng of Cdc42-His protein; Lanes 2 & 3, positive control; Lanes 4 & 5, negative control. The recombinant Cdc42-His protein (24 kD) runs slightly higher than the endogenous Cdc42 (21 kD) due to the presence of the His tag.

VI: Assay Protocol (Continued)

4. His-Cdc42 Protein Control:

The kit supplies 10 µg of His-Cdc42 control protein (part# CDWT); 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 Cdc42 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-Cdc42 control protein be run on the gel as a positive control and as a quantitation estimate for endogenous Cdc42 (see STEP 4).

VI: Assay Protocol (Continued)

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 Cdc42 activation. See Section V (B): Important Technical Notes.
2. Treat cells with Cdc42 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. NOTE: In some cases, if there are no proteins in the growth media, the wash step can be omitted for Cdc42 activation. (see Section V [B & D]).
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 Cdc42.
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.

VI: Assay Protocol (Continued)

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:

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

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 pull-down assays simpler. Be aware of the length of time cell lysates stay on ice (should not exceed 10 min), since Cdc42-GTP hydrolysis will occur.

VI: Assay Protocol (Continued)

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 or rocker 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.

VI: Assay Protocol (Continued)

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-Cdc42 control protein as a positive control. To do this the protein should be diluted as follows;
 - a) Thaw one of the 3 μ l aliquots of His-Cdc42 control protein (see Table 2).
 - b) Dilute to 4 ng/ μ l by adding 247 μ l of Cell Lysis Buffer.
 - c) Dilute to 2 ng/ μ l by adding 250 μ l of 2X Laemmli sample buffer (125mM Tris pH6.8, 20% glycerol, 4% SDS, 0.005% Bromophenol blue, 5% beta-mercaptoethanol).
 - d) Load 10 μ l (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 1:250 dilution of anti-Cdc42 antibody (Cat. # ACD03, provided with kit) diluted in TBST plus 0.1% nonfat-dry milk 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 Cdc42 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
<p>No signal from the His-tagged Cdc42 control protein.</p>	<ol style="list-style-type: none"> Storage of the stock control protein at concentrations that are too low (<0.33mg/ml). Repeated freeze/thaw cycles of the reconstituted positive control stock protein. Attempts to store the diluted stock at 4°C or frozen for future use. Failure to follow the western blot protocol detailed in section VI: STEP 4. 	<ol style="list-style-type: none"> The kit supplies 10 µg of His-Cdc42 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. The stock protein must be aliquoted as described in Table 2. Repeated freeze thaws of the stock will result in denaturation and precipitation. We recommend loading 20 ng of the positive control on the gel as a positive control and quantitation estimate for endogenous Cdc42 (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. <p>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.</p> <ol style="list-style-type: none"> Follow the western blot protocol in this manual. It has been optimized for small G-proteins.
<p>No difference in signal between GTPγS positive control and GDP negative control assay</p>	<ol style="list-style-type: none"> Protein lysate concentrations were not equalized. Titration of PAK-PBD Beads not performed. GDP requirements are higher for your cell line. 	<ol style="list-style-type: none"> The absolute amount of protein in lysates can have a dramatic effect upon Cdc42 signal. It is therefore very important to have equal amounts of cell lysate protein in each reaction. See section V (E). 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. 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.
<p>No detectable Cdc42 activation in the positive control (GTPγS) assay</p>	<ol style="list-style-type: none"> STOP buffer not added to the reactions. Leaving the lysates for >10 minutes before use. 	<ol style="list-style-type: none"> Follow the instructions carefully, for example, STOP buffer must be added to the reaction or you will not get a Cdc42 signal. GTPγS and GDP loaded lysates should be used within 2-3 minutes after STOP buffer has been added.

VII: Troubleshooting (cont.)

Observation	Possible cause	Remedy
No detectable signal in the experimental samples	<ol style="list-style-type: none"> Control reaction not performed for GTPγS. His-Cdc42 control protein not used during Western blot. Insufficient cell lysate used Lysates not processed rapidly at 4°C 	<ol style="list-style-type: none"> Always run a GTPγS control to make sure the PAK-PBD beads are working and always run the recombinant His-Cdc42 control protein to make sure that the Western blot / Cdc42 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. Titrate the protein amount used in the assay. We recommend 300-800 μg lysate, however, in some cases more lysate may be required. Cdc42 is still able to hydrolyze GTP during lysate PAK-PBD beads are bound to Cdc42-GTP. The temperature and speed of lysate preparation are therefore very important parameters in this assay .
Cdc42 activation signal does not change upon experimental activation stimulus.	<ol style="list-style-type: none"> Titration of PAK-PBD Beads not performed. Culture conditions have caused cells to become unresponsive to Cdc42 activators. Selected Cdc42 activator may not work with your cell line. Western blot is overexposed leading to inaccurate readings. 	<ol style="list-style-type: none"> 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 Cdc42 activation states. This may require titrating bead and / or lysate levels. 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. Use a known Cdc42 activator (eg. EGF) to check the responsiveness of your cell line. A list of some Cdc42 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 filopodia and microspikes (see Appendix 1). As a general guideline, you should expose the film so that the Cdc42 signal gives a grey band rather than a black band. Alternatively, the Cdc42 G-LISA® Activation Assay Kit (Cat. # BK127) can be used to obtain quantitative results within 3 h.

VII: Troubleshooting (cont.)

Observation	Possible cause	Remedy
Cdc42 signal appears as doublet	<ol style="list-style-type: none">1. Samples in sample loading buffer stored at -20°C.2. Sample loading buffer is not fresh.	<ol style="list-style-type: none">1. Cdc42 can form irreversible doublets during processing. The most sensitive point for doublet formation is after resuspending 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. 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 VIII: 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 Na₂PO₄, 1.47 mM KH₂PO₄)
- 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 10⁴ 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 100 ng/ml for 2 min.
6. Remove the coverslip and process for actin staining as described below.

Actin Staining

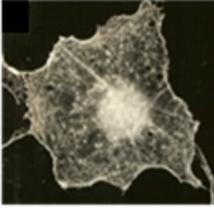
1. Wash the cells once with PBS and fix for 20 min at room temperature in 3% paraformaldehyde diluted in PBS.
2. Wash the cells once for 30 s with PBS to remove excess fixative.
3. Incubate the cells with 0.2% Triton-X 100 in PBS for 5 min at room temperature to permeabilize cells.
4. Wash twice in PBS 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 Cdc42 Activated 3T3 Cells

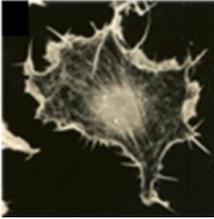
A



Serum Starved Actin Morphology:

Swiss 3T3 cells serum starved, according to the method given in this section, 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



Cdc42 Activated Actin Morphology:

Cells treated for 2min with 100 ng/ml EGF after serum starvation and subsequently stained with rhodamine-phalloidin. Cdc42 induced actin-rich filopodia and microspikes.

Appendix 2: Known Cdc42 Activators

Activator*	Treatment	Cell line used	Response	Type of Assay Used	Ref.
Tumor necrosis factor alpha (TNF α)	20 ng/ml	Swiss 3T3	Cdc42 specific activation after 10 minutes. Longer incubations resulted in Rac and Rho activation.	Actin morphology: filopodia formation	1
Tumor necrosis factor alpha (TNF α)	100 ng/ml	MEF cells	Filopodia formation increased rapidly and was greatest at 10 min after which filopodia decreased. PAK-PBD pull-down assays confirmed maximum Cdc42 activation of 3 fold after 10 minutes. Activation was maintained for several hours.	Actin morphology and PAK-PBD pull-down assay	2
Epidermal Growth Factor (EGF)	50 ng/ml	COS cells	Rapid activation upon exposure to growth factor, reaching a peak at approximately 10 min. Enhanced Cdc42 activation lasted at least 30 min in COS cells.	PAK-PBD pull-down assay	3

1. Puls, A. et al. 1999. Activation of the small GTPase Cdc42 by the inflammatory cytokines TNF α and IL-1, and by the Epstein-Barr virus transforming protein LMP1. *J. Cell Sci.* **112**, 2983-2992.
2. Gadea, G. et al. 2004. TNF α induces sequential activation of Cdc42- and p38/p53-dependent pathways that antagonistically regulate filopodia formation. *J. Cell Sci.* **117**, 6355-6364.
3. Tu, S. et al. 2003. Epidermal growth factor-dependent regulation of Cdc42 is mediated by the Src tyrosine kinase. *J. Biol. Chem.* **278**, 49293-49300.

Appendix 3: Protein Quantitation (with Precision Red Reagent)

Background

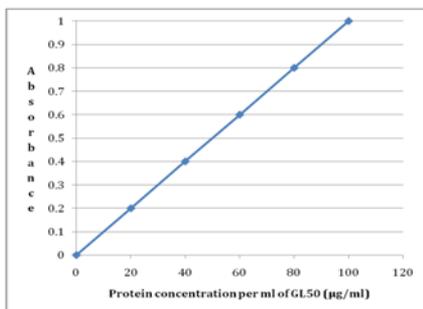
The Precision Red Advanced Protein Assay Reagent is a simple one step procedure that results in a red to purple/blue color change characterized by an increase in absorbance at 600 nm. The reagent is not supplied in this kit, it is sold separately as Cat. # ADV02. Precision Red Advanced Protein Assay 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 μ l 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 1 ml of ADV02 plus 20 μ l 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 μ l sample of cell lysate added to 1 ml of ADV02 gives an absorbance reading of 0.1.

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

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

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

For a 10 μ l 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 μ l of each lysate or Lysis Buffer into the well of a 96 well plate.
- Add 290 μ l of Precision Red™ Advanced Protein Assay Reagent to each well.
- Incubate for 1 min at room temperature.
- Blank spectrophotometer with 290 μ l of ADV02 plus 10 μ l 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 μ l sample of cell lysate added to 290 μ l of ADV02 gives an absorbance reading of 0.1

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

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

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

For a 5 μ l sample in 295 μ l 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.

