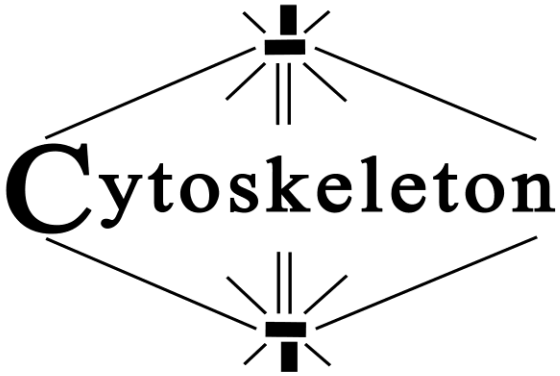

Ras ACTIVATION ASSAY BIOCHEM KIT

Cat. # BK008

ORDERING INFORMATION

To order by phone: (303) - 322 - 2254
To order by Fax: (303) - 322 - 2257
Technical assistance: (303) - 322 - 2254
World Wide Web: www.cytoskeleton.com
Write to us: Cytoskeleton, Inc., 1830 S. Acoma Street, Denver, CO 80223. U.S.A.

MANUAL CONTENTS

Section I: Introduction

Ras Activation Assay:

The Ras small G-proteins act as molecular switches that transmit cellular signals through an array of effector proteins. Ras plays an important role in many cellular functions including the control of cell proliferation and differentiation (1).

The Ras switch operates by alternating between an active, GTP-bound state and an inactive, GDP-bound state (2-3). Understanding the mechanisms that regulate activation / inactivation of the GTPases is of obvious biological significance and is a subject of intense investigation (4-6). The fact that many Ras effector proteins will specifically recognize the GTP bound form of the protein has been exploited experimentally to develop a powerful affinity purification assay that monitors Ras protein activation (7-9). The assay uses the Ras Binding Domain (RBD) region of the Ras effector protein, Raf kinase. The RBD protein motif has been shown to bind specifically to the GTP-bound form of Ras proteins (9,10). The fact that the RBD region of Raf kinase has a high affinity for all isotypes of GTP-Ras and that Raf-RBD binding results in a significantly reduced intrinsic and catalytic rate of hydrolysis of Ras make it an ideal tool for affinity purification of GTP-Ras from cell lysates. The Raf-RBD protein supplied in this kit contains amino acids 51-149 of the human Raf1 protein fused to GST and bound to colored glutathione sepharose beads. This allows one to "pull-down" the Raf-RBD/GTP-Ras complex in a single step. The assay therefore provides a simple means of quantitating Ras activation in cells. The amount of activated Ras is determined by a quantitative western blot using a Ras Pan specific antibody (supplied in this kit). If you wish to detect activation of a specific Ras isotype then you should use an isotype specific antibody (not supplied in this kit). A typical Rho pulldown assay using GTP and GDP loaded human platelet extract is shown in Figure 1.

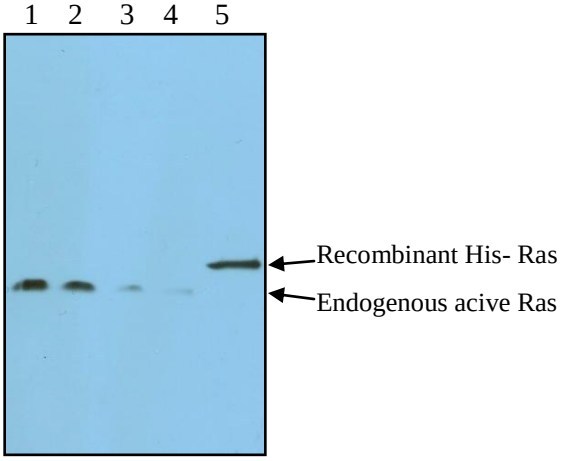

Figure 1. Raf-RBD bead pulldown Assays. *A. Extract (300 μg) from human platelet cells was loaded with GTPγS (GTP lanes 1 & 2) or GDP (GDP lanes 3 & 4) using the method described in Section VI: Control Reactions. Extracts were incubated with 30 μl of Raf-RBD beads and processed as described in Section VI: Pull-down Assay. All bead samples were resuspended in 20 ul of 2x sample buffer and run on a 4-20% SDS gel. Lane 5 shows 30 ng of recombinant His-Ras control protein. Protein was transferred to PVDF, probed with a 1:250 dilution of anti-Pan Ras and processed for chemiluminescent detection as described in Section VI: STEP 4.*

Section 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 can not 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 for a full refund.

Section III: Kit Contents

This kit contains enough reagents for approximately 50-60 pull-down assays. There is sufficient anti-Ras antibody for 50 ml working strength primary antibody solution.

Table 1: Kit Contents

Reagent	Cat. # Part #	Quantity	Description
Raf RBD beads	Cat. # RF02	3 tubes	Lyophilized. 2 mg of protein per tubes bound to colored sepharose beads.
Anti-Pan Ras monoclonal antibody	Part # AESA02	1 tube	Lyophilized. 10 µg antibody protein.
His-Ras control protein	Part # RS02	1 tube	Lyophilized. 10 µg of protein (~25 kDa) as a Western blot standard.
Cell Lysis Buffer	Part # CLB01	1 bottle	Lyophilized. 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.5 M NaCl, and 2% Igepal when reconstituted.
Wash Buffer	Part # WB01	1 bottle	Lyophilized. 25 mM Tris pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl when reconstituted.
Loading Buffer	Part # LB01	1 tube	Liquid. 150 mM EDTA solution.
STOP Buffer	Part # STP01	1 tube	Liquid. 600 mM $MgCl_2$ solution.
GTPγS stock: (non-hydrolysable GTP analog)	Cat. # BS01	1 tube	Lyophilized. 20 mM solution, when reconstituted.
GDP stock	Part # GDP01	1 tube	Lyophilized. 100 mM solution, when reconstituted.
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.
Anhydrous DMSO	Part # DMSO	1 tube	Solvent for protease inhibitor cocktail.

Required reagents/components that are not supplied:

Laemmli sample buffer.
Polyacrylamide gels (12% or 4-20% gradient gels).
SDS-PAGE buffers.
Western blot buffers.
Protein transfer membrane (PVDF or Nitrocellulose).
Secondary antibody (eg. Goat **anti-mouse** HRP conjugated IgG, Jackson Labs. Cat. # 115-035-068).
Chemiluminescence based detection system. (eg. ECL Advanced Western Blotting Detection Kit GE Healthcare)

Section 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 follows:

Table 2: Reconstitution and Storage of Components

Component	**Reconstitution**	**Storage**
Raf-RBD protein beads	Reconstitute each tube in 600 µl distilled water. Aliquot into 20 x 30 µl volumes (30 µl of beads is sufficient for one assay). **Snap freeze in liquid nitrogen.**	Store lyophilized protein **desiccated** at 4°C. Stable for 6 months. Store resuspended protein frozen at -70°C. Stable for 1 year.
Anti-Pan Ras monoclonal antibody	Resuspend in 200 µl of PBS. Use at 1:250 dilution.	Store at 4°C. Stable for 6 months. For long term storage, aliquot into 10 µl volumes and store at -20°C.
His-Ras control protein	Reconstitute in 55 µl of distilled water. Aliquot into 10 x 5 µl sizes and snap freeze in liquid nitrogen.	Store lyophilized protein desiccated at 4°C. Stable for 6 months. Store resuspended protein at -70°C. Stable for 6 months.
Cell Lysis Buffer	Reconstitute in 100 ml of sterile distilled water and store at 4°C. **NOTE**: This solution may take 5-10 min to resuspend, use a 10 ml pipette to thoroughly resuspend the buffer.	Store lyophilized product desiccated at 4°C. Stable for 6 months. Store resuspended solution at 4°C. Stable for 12 months.
Wash Buffer	Reconstitute each in 100 ml of sterile distilled water and store at 4°C.	Store lyophilized product desiccated at 4°C. Stable for 6 months. Store resuspended solution at 4°C. Stable for 12 months.
Loading Buffer	None required.	Store at 4°C.
STOP Buffer	None required.	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 lyophilized product desiccated at 4°C. Stable for 6 months. Store resuspended solution at -70°C. Stable for 6 months.
GDP stock	Reconstitute in 50 µl of sterile distilled water. Aliquot into 5 x 10 µl volumes, snap freeze in liquid nitrogen.	Store lyophilized product desiccated at 4°C. Stable for 6 months. Store resuspended solution at -70°C. Stable for 6 months.
Protease inhibitor cocktail	Reconstitute in 1 ml of DMSO (100x solution).	Store lyophilized product desiccated at 4°C. Stable for 6 months. Store resuspended solution at -20°C. Stable for 6 months.

Section V: Important Technical Notes

A. Updated Manual (Version 7.0) Review

The following updates from the previous Version should be noted:

1) As a result of in house RAF02 bead titration studies, Version 7.0 recommends 30 µl of RAF02 beads per assay rather than 35 µl. NOTE: To determine the optimal bead volume per assay it is still highly recommended to titrate beads for any given set of experimental conditions (see Section V:F).
2) The BK008 manual has been updated to give more extensive details on how to process cell lysates prior to performing the pull-down. The new manual reflects a strong preference towards freezing "experiment-sized" aliquots of lysates prior to performing the assay. Snap freezing aliquots of lysate in liquid nitrogen has many advantages:
   i) The level of small G-protein activation is identical between fresh, rapidly processed lysates and lysates that have been snap frozen in liquid nitrogen and stored at -70°C prior to thawing and use in an activation assay. By freezing multiple aliquots of any given lysate one can perform multiple assays on a given lysate to compare results from different experiments.
   ii) Rapid processing and freezing of lysate allows more time to quantitate protein concentration in a given lysate without allowing the whole lysate sample to sit on ice for excessively long periods of time. Remember that lysates are relatively unstable prior to addition of the beads and excessive processing time will result in GTP hydrolysis by the small G-protein and consequent loss of signal.
   iii) Time-courses can be performed sequentially and each time-point can be snap frozen prior to performing the second time-point experiment. This makes the taking of time-points a very simple process and does not result in excessive processing time for early time-point lysates.

   NOTE: Lysate samples MUST be snap frozen in liquid nitrogen to maintain the correct activation level of small G-protein. Slower freezing methods are not acceptable.

3) The Western blot procedure for small-G protein transfer has been optimized (see Section VI: STEP 4). This protocol optimizes the transfer of small G-proteins and recommends the use of the anti-Pan Ras antibody at a 1:250 dilution.

4) Protease Inhibitor Cocktail should be added to the Cell Lysis Buffer (1x final conc.) immediately prior to use.

Section V: Important Technical Notes, continued

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 Ras activation assays. Time should be taken to read this section and to carefully maintain cell lines in accordance with the guidelines given below.

Adherent cells should be ready at 40 – 60% confluence or for non-adherent cells at approximately 3×10^5 cells per ml. Briefly, cells are seeded at 0.5×10^4 per ml (approximately 5×10^4 cells per 10 cm dish) and grown for 3 days. Serum starvation or other treatment will be performed when they are 40 - 50% confluent.

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

Cells should also be checked for their responsiveness ("responsive state") to a known stimulus. 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. In general, cells grown to >80% confluence should not be used for Ras activation studies.

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

C. Timing and Intensity of Ras Activation

Ras activation is a transient event, therefore time-points should be taken when characterizing a potential Ras activator. For potent activators such as EGF (100 ng/ml final concentration), the intensity of maximal Ras activation is generally 35 – 50% of total cellular Ras after 10 minutes treatment, while untreated serum starved "control state" cells generally have negligible activation. Recommended time points are 0, 3, 10, 30, 60 minutes and 3hours.
In practical terms the timed experiment should 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 – 400 µg of total protein per assay; this translates to 600 – 800 µl of a 0.5 mg/ml cell lysate. We recommend duplicate samples per time-point or condition, therefore 1.2 – 1.6 ml aliquots are recommended for snap freezing.

Section V: Important Technical Notes, continued

D. Rapid processing of cells

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

Washing

1) Retrieve culture dish from incubator, immediately aspirate out all of the media and place firmly on ice.
2) Immediately rinse cells with an appropriate volume of ice cold PBS to remove serum proteins (see Table 3 for recommended wash volumes).
3) Aspirate off all residual PBS buffer. This is essential so that the Cell Lysis Buffer is not diluted. Correct aspiration requires that the culture dish is placed at an 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 Cultures

Culture Vessel	Vessel surface area (cm^2)	Volume of PBS wash (ml)	Volume of Lysis Buffer (µl)
35 mm dish	8	2.0	100
60 mm dish	21	3.0	150
100 mm dish	56	10.0	400
150 mm dish	148	15.0	1200
6-well cluster plate	9.5 / well	3.0	100
12-well cluster plate	4 / well	1.5	60
T-25 Flask	25	4.0	160
T-75 Flask	75	10.0	800
T-150 Flask	150	15.0	1200

The time period between cell lysis and addition of lysates to the Raf-RBD 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 Ras per sample. The lysates **must** be snap frozen in liquid nitrogen and stored at -70°C. Lysates should be stored at -70°C for no longer than 30 days.
4) Thawing of cell lysates prior to the 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.

Section V: Important Technical Notes, continued

E. Equalization of Protein Concentration:

Equal protein concentration in all samples is a prerequisite for accurate comparison between samples in Ras 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 Ras activation assays:

1) **Bead Titration:** Raf-RBD will bind to Ras-GDP with a much lower affinity than Ras-GTP. If too many Raf-RBD beads are added to the pull-down assay there will be significant binding to inactive (GDP-bound) Ras. The result of this will be an underestimate of Ras activation. For this reason we highly recommend performing a bead titration to determine optimal conditions for any given Ras activation or inactivation assay. Once optimal conditions have been established, bead titrations should no longer be necessary. We recommend 20, 40 and 60 µl 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 40 µl 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.

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Section VI: Assay Protocol

STEP 1: Control Reactions

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

1. **Total Ras Protein Quantitation:**
   Total cellular Ras protein present in each sample should be determined by Western quantitation. Usually 20 – 50 µg of cell lysate will result in a good signal.

2. **Positive Cellular Protein Control:**
   Total cell lysate (200 – 500 ug) should be loaded with GTPγS as a positive control for the pull-down assay. The following reaction details how to load endogenous Ras with the non-hydrolysable GTP analog (GTPγS), this is an excellent substrate for Raf-RBD beads and should result in a strong positive signal in a pull-down assay.

   Perform GTP loading on 200 – 500 µg of cell lysate that is at a protein concentration between 0.4 – 2.0 mg / ml as follows;
   a. Add 1/10th volume of Loading Buffer.
   b. Immediately add 1/100th volume of GTPγS (200 µM final concentration). Under these conditions 5 - 10% of the Ras protein will load with non-hydrolysable GTPγS and will be "pulled-down" with the Raf-RBD beads in the assay (see Figure 1).
   c. Incubate the control sample at 37°C for 30 minutes with gentle rotation.
   d. Stop the reaction by transferring the tube to 4°C and adding 1/10th volume of STOP Buffer.
   e. Use this sample in a pull-down assay immediately 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 Ras with GDP will inactivate Ras and this will bind very poorly to Raf-RBD beads.

4. **His-Ras Protein Control:**
   The kit supplies 10 µg of His-Ras control protein; this will be reconstituted to a 0.18 mg/ml stock solution and stored at -70°C (as 10 x 5 µl aliquots). Storage of the protein at lower concentrations than 0.18 mg/ml will result in denaturation, precipitation of the protein and incorrect quantitations or no signal in the western blot. We recommend that 30 - 40 ng of His-Ras control protein be run on the gel as a positive control and as a quantitation estimate for endogenous Ras. The Ras family proteins have a molecular weight of approximately 21 kDa; the His-tagged control protein has a molecular weight of approximately 25 kDa.

Section VI: Assay Protocol, Continued

STEP 2: Lysate Collection

We strongly recommend that you snap freeze the 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 – 2 ml aliquots and to save a small amount of each lysate (approximately 20 – 30 µl) for protein quantitation. Details of lysate processing are given below:

1. Grow cells in appropriate culture conditions. It is important to keep cells in a "controlled state" prior to Ras activation, see Section V: B.

2. Treat cells with Ras activator (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 Section V: D Table 3, 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 rpm, 4°C for 2 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 quantitation of total Ras.

11. Aliquot and snap freeze the remaining cell lysates in liquid nitrogen. Store at -70°C for future use. It is recommended to aliquot sufficient lysate for duplicate assays of 300 - 400 µg lysate per assay. For a 0.5 mg/ml lysate this would be 1.2 – 1.6 ml lysate.

12. Measure lysate protein concentrations. We recommend using Precision Red Advanced Protein Assay (Cat. # ADV02) for quantitations:
    a. Add 1 ml of Precision Red Advanced Protein Assay Reagent (ADV02) to each disposable 1 ml cuvette
    b. Add 20 µl of each lysate or lysis buffer into cuvettes.
    c. Incubate for 1 min at room temperature.
    d. Blank spectrophotometer with the Cell Lysis Buffer at 600 nm.
    e. Read absorbance of lysates samples.
    f. Multiply the absorbance by 5 to obtain the protein concentration in mg/ml.

Section VI: Ras Activation Assay Protocol, Continued

13. Calculate how to equalize the cell extracts with ice cold Cell 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 be 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 Ras-GTP hydrolysis will occur.

Section VI: Ras Activation Assay Protocol, Continued

STEP 3: Pulldown 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 (200 – 500 µg total cell protein) to a pre-determined amount of Raf-RBD beads from your bead titration test (see Section V.F).

   NOTE: In general, a 30 µl bead pull-down will yield optimal results. Under these conditions the 6 mg of Raf-RBD beads supplied in the kit are sufficient for approximately 50-60 assays. We do however recommend a bead titration (20, 30 & 60 µl) to determine optimal pull-down conditions.

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

4. Pellet the Raf-RBD 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 Raf-RBD 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 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 if necessary. It is recommended that the necessary control samples be run on each gel. Samples may be boiled prior to loading onto gel (optional).

Section VI: Ras Activation 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. Equilibrate the gel in Western blot buffer (25 mM Tris, 192 mM glycine, 15% methanol, pH 8.3) for 15 min at room temperature prior to electro-blotting.

3. Transfer the protein to a PVDF membrane for 45 minutes at 75V.

4. Wash the membrane once with TBS (20 mM Tris-HCl pH 8.0, 50 mM NaCl).

5. Air dry membrane for 20 minutes room temperature (optional). After 20 minutes the membrane should be placed in TBST for 10 minutes to re-wet membrane before placing in block solution.

6. Block the membrane surface with 5% nonfat-dry milk in TBST (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.05% Tween 20) for 30 min at room temperature with constant agitation.

7. Incubate the membrane with a 1: 250 dilution of anti-Pan Ras antibody (Part # AES02, provided with kit) diluted in TBST (no blocking agent) for 1 h at room temperature or overnight at 4°C with constant agitation.

8. Wash the membrane three times in TBST for 10 min each.

9. 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.

10. Wash the membrane 5 times in TBST for 10 min each.

11. Use an enhanced chemiluminescence detection method to detect the Ras signal (eg. ECL Advanced Western Blotting Detection Kit GE Healthcare).

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	

Section VII: Troubleshooting

1. No signal from the His-tagged Ras control protein.

The kit supplies 10 µg of His-Ras protein, this should be reconstituted to a 0.18 mg/ml stock solution and stored at -70°C (as 10 x 5 µl aliquots). Storage of the protein at lower concentrations will result in denaturation and precipitation of the protein and incorrect quantitations or no signal at all. We recommend loading 20 ng of this positive control on the gel as a quantitation estimate for endogenous Ras (for 20 ng of recombinant protein, dilute one 5 µl aliquot of protein stock with 495 µl of Laemmli sample buffer; load 11 µl of this on the SDS gel). The Ras family proteins have a molecular weight of 21 kDa; the His-tagged control protein has a molecular weight of approximately 25 kDa.

2. No difference in signal between GTPγS positive control and GDP negative control assay.

a) Equalize the protein lysate concentration.

The absolute amount of protein in lysates can have a dramatic effect upon Ras signal. It is therefore very important to have equal amounts of cell lysate protein in each reaction. Any difference in total protein concentration (>10% between samples) will result in unreliable data. Use the Precision Red Protein Assay reagent (Cat. # ADV02) from Cytoskeleton Inc. for a 1 min protein concentration assay. Also using too much lysate can cause a reduction in signal differentiation between activated and non-activated Ras. A lysate range of 300-500 ug is recommended, if signals look equivalent try using less lysate.

b) Titration of Raf-RBD beads. The amount of Raf-RBD protein beads that will result in a good differential between GTP and GDP-bound Ras must be determined from a bead titration experiment. In cases where there is a high signal in both GTPγS and GDP lanes, using half the amount of Raf-RBD beads will often result in a better differential signal.

c) Titrate Nucleotide addition

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.

d) Lysis Buffer Composition

Addition of 0.5% Sodium deoxycholate, 200 mM NaCl and 0.1% SDS to 1X Cell Lysis Binding buffer can give a better differential signal between GDP and GTPγS samples, probably due to an increased stringency of Raf-RBD binding. In some cell lines these detergents cause nuclear lysis resulting in viscous cell extracts and difficulty in handling the samples.

3. No detectable Ras activation in the positive control (GTPγS) assay.

Follow the instructions carefully, for example, STOP buffer must be added to the reaction or you will not get a Ras signal.

4. No detectable signal in the experimental samples.

a) Always run a GTPγS control to make sure the Raf-RBD beads are working and always run the recombinant His-Ras control protein to make sure that the Western blot / Ras 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.

Section VII: Troubleshooting, continued

b) A lack of signal may be due to insufficient cell lysate being used. Titrate the protein amount used in the assay.

c) Ras is still able to hydrolyze GTP during lysate preparation; hydrolysis is stopped only when the Raf-RBD beads are bound to Ras-GTP. The temperature and speed of lysate preparation are therefore very important parameters in this assay. ALWAYS process lysates at 4ºC and proceed as rapidly as possible.

5. Ras activation signal does not change upon experimental activation stimulus

a) 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 Ras activation states. This may require titrating bead and / or lysate levels.

b) In some cases culture conditions have caused the cells to become unresponsive to Ras activators, this can be a major obstacle to obtaining meaningful results. For example, continuous overgrowing of a cell line can result in unresponsive cells. It has been reported that 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). It is the experience of scientists at Cytoskeleton Inc. that 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.

   i. It is always a good idea to use a known Ras activator (eg. PDGF at 100 ng/ml for 10 minutes, EGF at 200 ng/ml for 10 minutes) or inhibitor to check the responsiveness of your cell line.

c) The linear range of X-ray film is fairly narrow and overexposure of blots can result in inaccurate readings. As a general guideline, you should expose the film so that the Ras signal gives a grey band rather than a black band.

Section VIII: References

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