V.10.0

Ras Activation Assay Biochem Kit™

Cat. # BK008

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Manual Contents

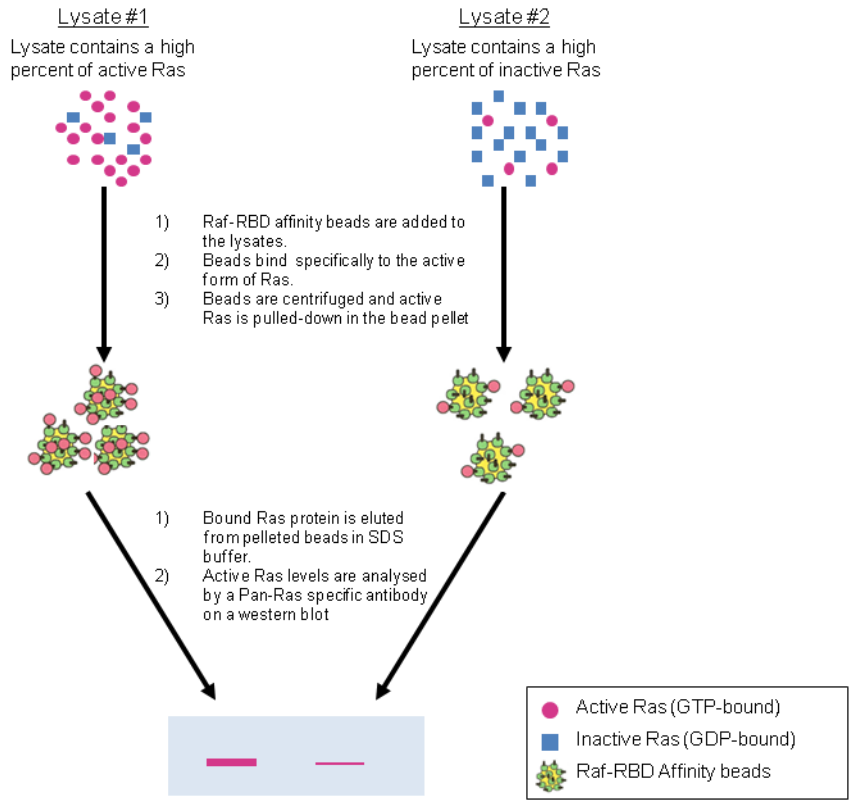
I: Introduction

Background– 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).

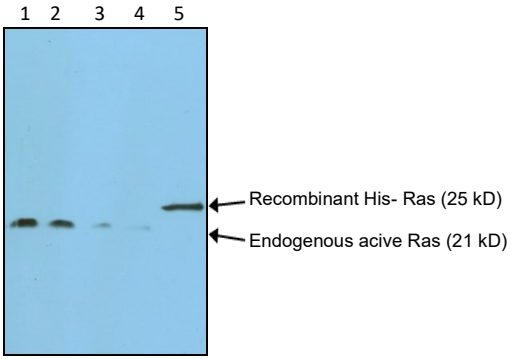
The Ras switch operates by alternating between an active, GTP-bound state and an inactive, GDP-bound state (2-3). Understanding the mechanisms that regulate activation / inactivation of the GTPases is of obvious biological significance and is a subject of intense investigation (4-6). The fact that many 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. Figure 1 shows a schematic representation of a Ras Activation Assay principle.

Figure 1: Schematic of Ras Activation (Pull-Down) Assay



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 Ras pull-down assay using a non-hydrolysable GTP analog (GTPγS) and GDP loaded human platelet extract is shown in Figure 2.

Figure 2: Ras Activation Pull-down Assay



Legend: A. Extract (300 µg) from human platelet cells was loaded with GTPγS (lanes 1 & 2) or 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: Assay Protocol. All bead samples were resuspended in 20 ul of 2x sample buffer and run on a 4-20% SDS gel. Lane 5 shows 20 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.

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 60 pulldown assays.

Table 1: Kit Contents and Storage Upon Arrival

Reagents	Cat. # or Part # *	Quantity	Storage
Raf-RBD beads	Part # RF02	3 tubes, lyophilized; 2 mg of protein per tube bound to colored sepharose beads	Desiccated 4°C
Anti-Pan Ras monoclonal antibody	Cat # AESA02	1 tube, lyophilized, 10 µg protein	Desiccated 4°C
His-H-Ras control protein	Part # RS01-10	1 tube, lyophilized; 10 µg protein (~25 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.**

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 pH 6.8, 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**
Raf-RBD Protein Beads	Reconstitute each tube with 600 µl distilled water. Aliquot into 20 x 30 µl volumes (30 µl of beads = 100 µg of protein, under these conditions one tube of Raf-RBD beads are sufficient for 20 assays. There are 3 tubes per kit = 60 assays). **Snap freeze in liquid nitrogen.**	Store at –70°C.
Anti-Pan Ras monoclonal antibody	Resuspend in 200 µl of PBS. Use at 1:250 dilution. For long term storage, aliquot into 10 µl volumes and store at -20°C.	Store at 4°C or –20°C in small
His-Ras control protein	Reconstitute in 50 µl of distilled water. Aliquot into 10 x 5 µl sizes. **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.

V: Important Technical Notes

A) Notes on Updated Version

The following update should be noted:

1. Incubation temperature has been increased from room temperature to 37°C and time has been increased from 15 to 30 minutes for enhanced GTP loading of positive control proteins (see Control Reactions section).
2. Reconstituted antibody storage has been expanded to include –20°C. NOTE: freeze thaws should be avoided so make small aliquots (Table 2).
3. The Part # on the H-Ras control protein has been changed from RS02 to RS01-10 to avoid confusion with an N-Ras product (Cat# CS-RS02).

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 fibroblast cells such as 3T3 cells should be ready at 50-70% confluency or for non-adherent cells, at approximately 5 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 40-60% 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 Ras activation. Optimal confluency prior to serum starvation and induction should be determined for any given cell line (also see Appendix 1 for cell line specific references).

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. A list of known Ras stimuli are given in Appendix 1. 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 Ras activation studies.

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 must be performed sequentially. This allows rapid processing of each single time point. Once one time point lysate is collected, is 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) Ras is a labile entity and 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

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. You may also want to supplement the Lysis Buffer with phosphatase inhibitors like sodium fluoride (25 mM final concentration) and sodium-vanadate (1 mM final concentration) if you plan to use the cell lysates to probe for downstream signals (see Appendix 4).

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	400
150 mm dish	148	15.0	1200
T-75 Flask	75	10.0	900
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 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 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 67, 100 and 133 µg bead titrations (equivalent to 20, 30 and 40 µl of Raf-RBD beads respectively) .

It is highly recommended that each user titrate the Raf-RBD beads for their particular experiment as cell lysate concentration, G-protein activation efficiency, bead binding and processing times can be variable and must be optimized for reproducible results.

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 100 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 Ras Activation Assay. The following control assays should be performed as an integral part of each experiment:

1. **Total Ras Protein:**

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

2. **Positive Cellular Protein Control**:

   Total cell lysate (300 – 800 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.

   a. Perform GTP loading on 300 – 800 µg of cell lysate 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 Ras protein will load with non-hydrolysable GTPγS and will be "pulled-down" with the Raf-RBD beads in the assay (see Figure 2).

   c. Incubate the control sample at 37°C for 30 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 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 0.2 mg/ml stock solution and stored at -70°C (as 10 x 5 µl aliquots). Storage of the protein at lower concentrations than 0.20 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 Ras family proteins have a molecular weight of between 21 kDa; the His-tagged control protein has a molecular weight of approximately 25 kDa. We recommend that 20 ng of His-Ras control protein be run on the gel as a positive control and as a quantitation estimate for endogenous Ras (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 – 30 µl) 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 Ras activation. See Section V (B): Important Technical Notes.
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 Table 3, Section V: D for recommended volumes. *Note: you may want to supplement the Lysis Buffer with phosphatase inhibitors like sodium fluoride (25 mM final concentration) and sodium-vanadate (1 mM final concentration) if you plan to use the cell lysates to probe for downstream signals (see Appendix 4).*
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 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 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 2):
    - 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:

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

    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.

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 Raf-RBD beads from your bead titration test (see Section V.F.1).

NOTE: In general, a 30 µl bead pull-down will yield optimal results. Under these conditions the 3 x 2 mg of Raf-RBD beads supplied in the kit are sufficient for 60 assays. We do however recommend a bead titration (20, 30 & 40 µl) to determine optimal pull-down conditions.

3. Incubate at 4°C on a rotator 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 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 samples can be centrifuged (14K rpm, 2 minutes, room temperature) to pellet the beads, in this case only the supernatant will be loaded onto the gel. Alternatively, the whole sample including the beads can be loaded onto 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-Ras control protein as a positive control. To do this the protein should be diluted as follows;
   a) Thaw one of the 5 µl aliquots of His-Ras control protein (see Table 2).
   b) Dilute to 4ng/µl by adding 245 µl of Cell Lysis Buffer.
   c) Dilute to 2ng/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.
7. Transfer the 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.
8. Block the membrane surface with 5% nonfat-dry milk in TBST for 30 min at room temperature with constant agitation.
9. Incubate the membrane with a 1:250 dilution of anti-Pan Ras antibody (Part. # AESA02, provided with kit) diluted in TBST (no blocking agent) for 2-3 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 Ras 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 Ras control protein.	1. Storage of the stock control protein at concentrations that are too low (<0.20 mg/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.	1. The kit supplies 10 µg of His-Ras protein, this should be reconstituted to a 0.20 mg/ml stock solution and stored at -70°C (as 10 x 5 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 Ras (for 20 ng of recombinant protein, dilute one 5 µl aliquot of protein stock with 245 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 Ras family proteins have a molecular weight of approximately 21 kDa; the His-tagged control protein has a molecular weight of approximately 25 kDa.
No difference in signal between GTPγS positive control and GDP negative control assay	1. Protein lysate concentrations were not equalized. 2. Titration of Raf-RBD 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 Ras 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 Raf-RBD 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 Ras 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 Ras signal. 2. 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	1. Control reaction not performed for GTPγS. His-Ras control protein not used during Western blot.	1. 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.
	2. Insufficient cell lysate used	2. Titrate the protein amount used in the assay. We recommend 300-800 µg lysate, however, in some cases more lysate may be required.
	3. Lysates not processed rapidly at 4°C	3. 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 .
Ras activation signal does not change upon experimental activation stimulus.	1. Titration of Raf-RBD 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 Ras activation states. This may require titrating bead and / or lysate levels.
	2. Culture conditions have caused cells to become unresponsive to Ras 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. Cells seeded at low densities, grown for 3 days to 50-70% confluency, then serum starved by a serum-step down procedure often respond better than cells grown to higher densities.
	3. Selected Ras activator may not work with your cell line.	3. Use a known Ras activator (eg. EGF) to check the responsiveness of your cell line. A list of some Ras activators are given in Appendix 1. Note that the cell line used for the activation assay is important as response to any given activator can vary considerably between cell lines.
	4. Western blot is overexposed leading to inaccurate readings.	4. As a general guideline, you should expose the film so that the Ras signal gives a grey band rather than a black band. Alternatively, the Ras G-LISA® Activation Assay Kit (Cat. # BK131) can be used to obtain quantitative results within 3 h.

VIII: References

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Appendix 1: Known Ras Activators

Activator	Treatment	Cell line used	Response	Type of Assay Used	Ref.
Epidermal Growth Factor (EGF)	100 ng/ml 5 minutes	HeLa	Dose dependent activation	Raf1-RBD pull-down assay	1
aCD3 + aCD28	5 ng/ml 5 minutes	Jurkat	Dose dependent activation	Raf1-RBD pull-down assay	3
PMA ionomycin	100 ng/ml 500 ng/ml 10 minutes	Jurkat	Dose dependent activation	Raf1-RBD pull-down assay	3
Hepatocyte Growth Factor	100 ng/ml 5 minutes	HeLa	Dose dependent activation	Raf1-RBD pull-down assay	1
IL-3	50 ng/ml 5 minutes	BaF3	14 fold activation after 5 minutes	Raf1-RBD pull-down assay	2

1 Omerovic J. et al. 2008. Ras isoform abundance and signalling in human cancer cell lines. *Oncogene*. **27**, 2754-2762.

2 Satoh T. et al. 1993. Platelet-derived growth factor receptor mediates activation of ras through different signaling pathways in different cell types. *Mol. Cell. Biol*. **13**, 3706-3713.

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Appendix 2: Protein Quantitation (with Precision Red Reagent)

Background

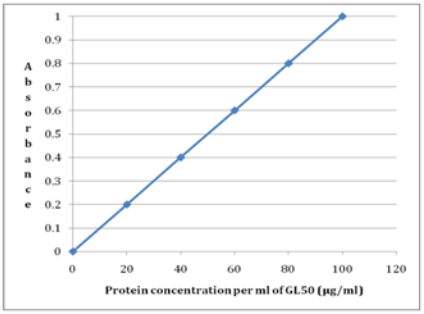
The Precision Red Advanced Protein Assay Reagent is a simple one step procedure that results in a red to purple/blue color change characterized by an increase in absorbance at 600 nm. The reagent is not supplied in this kit, it is sold separately as Cat. # ADV02. Precision Red Advanced Protein Assay 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 eaccuvette.
- 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 x 5

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

Appendix 2: 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 x 3.75

For a 5 µl sample in 295 µl ADV02, the equation becomes C = A x 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 3: 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) Ras 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

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Appendix 4: Evaluating the "controlled" and "responsive" state of the cells

If the G-LISA results suggest that there was no apparent activation of Ras under your assay conditions, it may be worth conducting another experiment to probe the cell lysates for signals downstream of Ras. There are generally two scenarios where this could happen. It should be possible to resolve this issue in both scenarios by probing for downstream signals in the cell lysates while optimizing the conditions of cell growth or treatment. It is important, however, that your cell lysates were prepared using Lysis Buffer that contains both protease inhibitors and phosphatase inhibitors such as NaF (25 mM) and sodium vanadate (1 mM).

Scenario 1: Both the "controlled" state and "responsive" state cell lysates are giving high absorbance readings relative to the background wells and they are not significantly different from each other.

- Typically this means you need to optimize the conditions for your controlled state, which may mean optimizing your serum starvation conditions depending on your experimental design

- Consider running a Western blot to probe for signals downstream of Ras while varying your serum starvation conditions (or other conditions if relevant). When you have found growth conditions that minimize the basal level of Ras pathway activation as determined by the downstream signal, repeat your original experiment.

Scenario 2: Both the "controlled" state and "responsive" state cell lysates are giving roughly equal absorbance readings to the background wells.

- Results of this nature can occur for several reasons, some of which are listed in the Troubleshooting section. Other reasons include…
  1. You've missed the optimal window for Ras activation by your chosen stimulus. Ras activation can be very transient and you may have missed the peak activation timepoint when preparing your cell lysates.
  2. Your Ras activator is not biologically active. The "controlled" state of the cells is excellent, but your cells are not responding to the non-functional Ras activator
  3. Your Ras activator does not work in the cell type you've chosen.

- Consider running a Western blot to probe for signals downstream of Ras while varying your activation conditions. When you have found growth conditions that maximize Ras pathway activation as determined by the downstream signal, repeat your original experiment.

See the next page for antibody recommendations to probe your cell lysates for Ras downstream signaling.

Appendix 4: Evaluating the “controlled” and “responsive” state of the cells

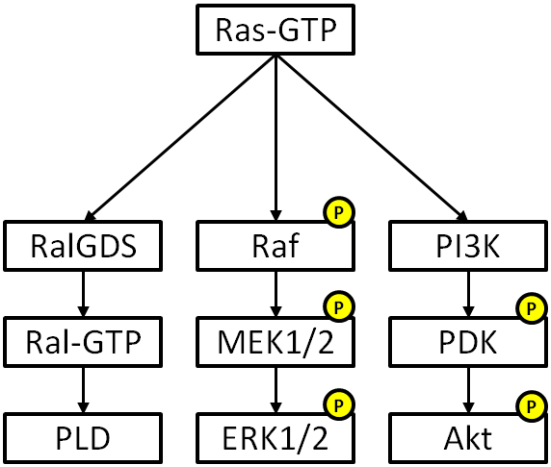

Figure 1. A simplified representation of Ras-GTP signaling in cells. The yellow circles with the letter P inside reflect downstream phosphorylation events that are possible to

Downstream signals to consider: Figure 1 is a highly simplified representation of the 3 main Ras-GTP signaling pathways. High quality antibodies are commercially available for key phosphorylation events that occur downstream of Ras in the Raf / MEK / ERK and PI3K / PDK / Akt pathways, which provide a means of probing your cell lysates for downstream Ras pathway activation by Western blot. The specific phosphorylation events are listed below to help guide your purchase of an appropriate antibody.

- **Phospho-ERK1/2**: The appropriate antibody will recognize ERK1/2 when phosphorylated on Thr202 / Tyr204. These phosphorylation sites are among the most commonly used markers for downstream Ras signaling.
- **Phospho-MEK1/2**: The appropriate antibody will recognize MEK1/2 when phosphorylated on Ser217 / Ser221.
- **Phospho-Raf**: The appropriate antibody will recognize Raf when phosphorylated on one of the several sites that are phosphorylated during Raf activation (e.g. c-Raf: Ser338, Tyr341, Thr491, Ser494, Ser497 and/or Ser499).
- **Phospho-Akt**: The appropriate antibody will recognize Akt when phosphorylated on Thr308.
- **Phospho-PDK**: The appropriate antibody will recognize PDK when phosphorylated on Ser241.

NOTES:

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