



## **RaIA ACTIVATION ASSAY BIOCHEM KIT**

**Cat. # BK040**

### **ORDERING INFORMATION**

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## Section I: Introduction

### **RalA Activation Assay:**

The Ras family of small GTPases consists of at least 35 related human proteins that include the oncoproteins HRAS, KRAS and NRAS as the founding members (Colicelli 2004). The Ral proteins, RalA and RalB, share 58% identity to these oncoproteins and 85% identity with each other (Chardin and Tavitian 1986). Ral proteins play an important role in diverse cellular processes including endocytosis, exocytosis, oncogenesis and the regulation of transcription and cell morphology (Feig 2003)

Like other small GTPases, Ral proteins become activated when they switch from the GDP-bound state to the GTP-bound state (Takai, Sasaki and Matozaki 2001), and it is the GTP-bound form that specifically interacts with their downstream effector proteins. The fact that Ral family 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 Ral protein activation (Hofer, Berdeaux and Martin 1998). The assay uses the Ral Binding Domain (RBD) of the effector protein RalBP1. The RBD protein motif has been shown to bind specifically to the GTP-bound form of Ral. The fact that the RBD region of RalBP1 has a high affinity for GTP-Ral makes it an ideal tool for affinity purification of GTP-Ral from cell lysates. The RalBP1-RBD protein supplied in this kit has been expressed as an His-tagged fusion protein in *E. coli*. and bound to colored beads. This allows one to “pulldown” GTP-Ral complexed with Ral-BP1-RBD beads. This assay provides a simple means of analyzing cellular RalA activities in a variety of systems. The amount of activated RalA is determined by a Western blot using a RalA specific antibody. A typical RalA pulldown assay is shown in Figure 1.



**Figure 1. RalBP1-RBD bead pulldown Assays.** *Rat2* cells were grown to 70% confluency in DMEM media supplemented with 10% fetal calf serum (FCS) prior to 48h growth in serum free media. Cell lysates were processed as described in Section VI of this manual. Active RalA was purified from 400  $\mu$ g of cell lysates by incubating lysates with 10  $\mu$ g of RalBP1-RBD beads as described in section VI. All bead samples were resuspended in 10  $\mu$ l of 2x sample buffer and run on a 4-20% SDS gel. Protein was transferred to PVDF, probed with a 1:500 dilution of anti-RalA and processed for chemiluminescent detection of RalA as described in Section VI: STEP 4.

Lane 1: 20 ng recombinant RalA control protein; Lane 2: bead bound RalA from lysates of cells treated for 2 minutes with Epidermal growth factor (100mg/ml); Lane 3: bead bound RalA from lysates of cells grown in serum free media for 48h. Densitometric analysis of chemiluminescent signal showed that RalA activation in EGF treated cells was 2 fold above RalA activation in serum starved cells.

## **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 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 for a full refund.

### Section III: Kit Contents

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

Table 1: Kit Contents

Reagent	Cat. # Part #	Quantity	Description
Active Ral Affinity beads	RL07	2 tubes	Lyophilized. 300 µg of protein per tube bound to colored sepharose beads. Resuspend in 600 µl sterile water.
Anti-RalA monoclonal antibody	ARL01	1 tube	Lyophilized protein. Resuspend in 200 µl PBS.
Ral A constitutively active control protein	RL23	1 tube	Lyophilized. 10 µg of protein (~28 kDa) as a Western blot standard.
Cell Lysis Buffer	CLB01	1 bottle	Lyophilized. 50 mM Tris pH 7.5, 10 mM MgCl <sub>2</sub> , 0.5 M NaCl, and 2% Igepal when reconstituted.
Wash Buffer	WB01	1 bottle	Lyophilized. 25 mM Tris pH 7.5, 30 mM MgCl <sub>2</sub> , 40 mM NaCl when reconstituted.
Loading Buffer	LB01	1 tube	Liquid. 150 mM EDTA solution.
STOP Buffer	STP01	1 tube	Liquid. 600 mM MgCl <sub>2</sub> solution.
GTP $\gamma$ S stock: (non-hydrolysable GTP analog)	BS01	1 tube	Lyophilized. 20 mM solution, when reconstituted.
GDP stock	GDP01	2 tubes	Lyophilized. 100 mM solution, when reconstituted.
Protease inhibitor cocktail	PIC02	1 tube	Lyophilized. 100x solution: 62 µg/ml leupeptin, 62 µg/ml pepstatin A, 14 mg/ml benzamidin and 12 mg/ml tosyl arginine methyl ester when reconstituted.
Anhydrous DMSO	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
RalBP1-RBD protein beads	Reconstitute each tube in 600 $\mu$ l distilled water. Aliquot into 6 x 100 $\mu$ l volumes (20 $\mu$ l of beads = 10 $\mu$ g protein, under these conditions 100 $\mu$ l is sufficient for 5 assays). <b>Snap freeze in liquid nitrogen.</b>	Store lyophilized protein desiccated at 4°C. Stable for 6 months. Store resuspended protein frozen at -70°C. Stable for 1 year.
Anti-RalA monoclonal antibody	Resuspend in 200 $\mu$ l of PBS. Use at 1:500 dilution.	Store at 4°C. Stable for 6 months.
Ral A constitutively active control protein	Reconstitute in 30 $\mu$ l of distilled water. Aliquot into 10 x 3 $\mu$ l sizes and snap freeze in liquid nitrogen.	Store lyophilized protein desiccated at 4°C. Stable for 6 months. Store resuspended protein at -70°C for 6 months.
Cell Lysis Buffer	Reconstitute in 100 ml of sterile distilled water and store at 4°C. <b>NOTE:</b> This solution may take 5-10 min to resuspend, use a 10 ml pipette to thoroughly resuspend the buffer. To keep long term stocks of this buffer; aliquot the solution into 20 x 5 ml volumes and store at -20°C.	Store lyophilized product desiccated at 4°C. Stable for 6 months. Store resuspended solution at 4°C for 3 months.
Wash Buffer	Reconstitute in 100 ml of sterile distilled water and store at 4°C. To keep long term stocks of this buffer; aliquot the solution into 5 x 20 ml volumes and store at -20°C.	Store lyophilized product desiccated at 4°C. Stable for 6 months. Store resuspended solution at 4°C for 3 months.
Loading Buffer	None required.	Store at 4°C.
STOP Buffer	None required.	Store at 4°C.
GTP $\gamma$ S stock (non-hydrolysable GTP analog)	Reconstitute in 50 $\mu$ l of sterile distilled water. Aliquot into 5 x 10 $\mu$ l 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 each tube in 50 $\mu$ l of sterile distilled water. Aliquot into 5 x 20 $\mu$ l 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 Review

The following updates from the previous Version should be noted:

- 1) Loading conditions for control reactions using GTP $\gamma$ S and GDP have been altered to improve in vitro loading of RalA with GTP $\gamma$ S or GDP. Specifically, the incubation temperature and time have been increased and the amount of GTP $\gamma$ S and GDP added to the reactions has been increased.

### 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 RalA 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 70 - 90% confluence or for non-adherent cells at approximately  $3 \times 10^5$  cells per ml. Briefly, cells are seeded at  $0.5 \times 10^4$  per ml (approximately  $5 \times 10^4$  cells per 10 cm dish) and grown for 3-4 days. Serum starvation or other treatment will be performed when they are 60 - 70% confluent.

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

### C. Timing and Intensity of RalA Activation

Upon stimulation, RalA proteins are generally activated very rapidly and transiently, maximal activation ranges from 30 s to 30 min and declines thereafter to basal levels. For potent activators such as epidermal growth factor (EGF), the intensity of maximal RalA activation over “control state” (serum starved) cells is generally in the order of 2 fold. However, using a single time point you are more likely to miss this maximum activation peak. 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 min (this time course is also recommended for RalA inactivation studies).

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  $-70^{\circ}\text{C}$ . The Activation Assay uses approximately 200 – 400  $\mu\text{g}$  of total protein per assay; this translates to 400 – 800  $\mu\text{l}$  of a 0.5 mg/ml cell lysate. We recommend duplicate samples per time-point or condition, therefore 0.8–1.6 ml aliquots are recommended for snap freezing.

### D. Rapid processing of cells

GTP bound (active) RalA is a labile entity, the bound GTP is susceptible to hydrolysis by Ral-GAPs during and after cell lysis, resulting in RalA inactivation. Rapid processing at  $4^{\circ}\text{C}$  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.

**Section V: Important Technical Notes, continued**

- 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 <sup>2</sup> )	Volume of PBS wash (ml)	Volume of Lysis Buffer (µl)
35 mm dish	8	2.0	200
60 mm dish	21	3.0	300
100 mm dish	56	10.0	800
150 mm dish	148	15.0	2400
6-well cluster plate	9.5 / well	3.0	200
12-well cluster plate	4 / well	1.5	120
T-25 Flask	25	4.0	320
T-75 Flask	75	10.0	1600
T-150 Flask	150	15.0	2400

The time period between cell lysis and addition of lysates to the RalBP1-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 RalA 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 the use in the pulldown assay should be in a room temperature water bath, followed by rapid transfer to ice and immediate use in the assay.

**E. Protein Concentration and Quantitation of Total RalA:**

Equal protein concentration in all samples is a prerequisite for accurate comparison between samples in RalA 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%.

To make sure that equal amounts of total RalA are assayed, we recommend including samples of total lysate from experimental samples in the Western blot. Samples of 20 – 50 µg total cell lysate per sample should be sufficient to detect total RalA.

## Section V: Important Technical Notes, continued

### F. Assay Linearity

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

- 1) **Bead Titration:** RalBP1-RBD will bind to RalA-GDP with a much lower affinity than RalA-GTP. If too many RalBP1-RBD beads are added to the pulldown assay there will be significant binding to inactive (GDP-bound) RalA. The result of this will be an underestimate of RalA activation. For this reason we highly recommend performing a bead titration to determine optimal conditions for any given RalA activation or inactivation assay. Once optimal conditions have been established, bead titrations should no longer be necessary. We recommend 5, 10 and 20  $\mu$ g 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  $\mu$ g of beads are used to assay 400  $\mu$ g 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  $\mu$ g total protein. As a further example, the growth and treatment of cell lines should be consistent between experiments; this point cannot 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.

## Section VI: Assay Protocol

### **STEP 1: Control Reactions**

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

1. **Total RalA:**

Total RalA 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 µg) should be loaded with GTPγS as a positive control for the pulldown assay. The following reaction details how to load endogenous RalA with the non-hydrolysable GTP analog (GTPγS), this is an excellent substrate for RalBP1-RBD beads and should result in a strong positive signal in a pulldown assay.

- a. Perform GTP loading on 200 – 500 µg of cell lysate by adding 1/10<sup>th</sup> volume of Loading Buffer.
- b. Immediately add 1/50<sup>th</sup> volume of GTPγS (400 µM final concentration). Under these conditions 1-2% of the RalA protein will load with non-hydrolysable GTPγS and will be “pulled down” with the RalBP1-RBD beads in the assay.
- 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 1/10<sup>th</sup> volume of STOP Buffer.
- e. Use this sample in a pulldown 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 1/25<sup>th</sup> volume of GDP (4 mM final concentration) should be added to the reaction in place of the GTPγS. Loading endogenous RalA with GDP will inactivate RalA and this will bind very poorly to RalBP1-RBD beads.

4. **RalA constitutively active control protein:**

The kit supplies 10 µg of RalA constitutively active control protein; 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 will result in denaturation, precipitation of the protein and incorrect quantitations or no signal in the western blot. We recommend that 1-5 ng of RalA constitutively active control protein be run on the gel as a positive control and as a quantitation estimate for endogenous RalA. The Ral family proteins have a molecular weight of approximately 25 kDa; the His-tag on the recombinant RalA cause it to run slightly higher on PAGE-SDS.

## Section VI: RalA Activation 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 0.8 – 1.6 ml aliquots and to save a small amount of each lysate (approximately 20 – 30  $\mu$ l) 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 RalA activation, see Section V: B.
2. Treat cells with RalA 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  $\mu$ l of lysate for protein quantitation and 20 – 50  $\mu$ g of lysate for Western quantitation of total RalA.
11. Aliquot and snap freeze the remaining cell lysates in liquid nitrogen. Store at -70°C for future use. It is recommended to aliquot 0.8 – 1.6 ml of lysate per tube (this should be sufficient for duplicate assays of 200 – 400  $\mu$ g lysate per assay).
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  $\mu$ l 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: RalA 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:*

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

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

## Section VI: RalA 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 – 400 µg total cell protein) to a pre-determined amount of RalBP1-RBD beads from your bead titration test (see Section V.F.1).

*NOTE: In general, a 10 µg (20 µl) bead pull-down will yield optimal results. Under these conditions the 600 µg of RalBP1-RBD beads supplied in the kit are sufficient for 50 - 60 assays. We do however recommend a bead titration (5, 10 & 20 µg) to determine optimal pulldown conditions.*

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

## Section VI: RalA 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. 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.
6. Incubate the membrane with a 1: 500 dilution of anti-RalA antibody diluted in TBST (no blocking agent) for 1-3 h at room temperature or overnight at 4°C with constant agitation.
7. Wash the membrane three times in TBST for 10 min each.
8. 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.
9. Wash the membrane 5 times in TBST for 10 min each.
10. Use an enhanced chemiluminescence detection method to detect the RalA 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 RalA constitutively active control protein.

The kit supplies 10 µg of RalA constitutively active control protein, this should 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 will result in denaturation and precipitation of the protein and incorrect quantitations or no signal at all. We recommend loading 1-5 ng of this positive control on the gel as a quantitation estimate for endogenous RalA (for 5 ng of recombinant protein, dilute one 3 µl aliquot of protein stock with 1 ml of Milli-Q water and then add 1 ml of 2x Laemmli sample buffer; load 10 µl of this on the SDS gel). The Ral family proteins have a molecular weight of approximately 25 kDa; the RalA constitutively active control protein runs at a slightly higher molecular weight from endogenous RalA.

### 2. No difference in signal between GTP $\gamma$ S 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 RalA 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. Protein quantitations should be carried out prior to bead addition but this should take no longer than 10 min. Use the Precision Red Protein Assay reagent (Cat. # ADV02) from Cytoskeleton Inc. for a 1 min protein concentration assay.

#### b) Titration of RalBP1-RBD beads. The amount of RalBP1-RBD beads that will result in a good differential between GTP and GDP-bound RalA must be determined from a bead titration experiment. Likewise, titration of lysate against a constant bead volume can also help differentiate signals between GTP/GDP signals. For example, if both GTP and GDP samples are giving strong signals then the ratio of bead to lysate may not be optimal, reducing lysate or increasing beads may help.

#### c) Titrate Nucleotide addition

Some cell lines have very high levels of endogenous GTP and exchange of GDP requires addition of greater than the 4 mM GDP outlined in this manual. We recommend trying 10 mM GDP in these cases.

### 3. No detectable RalA activation in the positive control (GTP $\gamma$ S) assay.

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

### 4. No detectable signal in the experimental samples.

#### a) Always run a GTP $\gamma$ S control to make sure the beads are working and always run the recombinant RalA constitutively active control protein to make sure that the Western blot / RalA 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.

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

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

## Section VII: Troubleshooting

### 5. RalA activation signal does not change upon experimental activation stimulus

- a) Make sure that your control GDP and GTP $\gamma$ S 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 RalA 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 RalA activators, this can be a major obstacle to obtaining meaningful results. For example, continuous overgrowing of a cell line can result in unresponsive cells.
  - i. It is always a good idea to use a known RalA activator (eg. EGF) 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. Remember, a good RalA activation signal will be 2 – 3 fold higher than unstimulated RalA signal and care is needed to accurately measure these relatively small increases. As a general guideline, you should expose the film so that the RalA signal gives a grey band rather than a black band.  
Alternatively, the RalA G-LISA™ Activation Assay Kit (Cat. # BK129) can be used to obtain quantitative results within 3 h.

## Section VIII: References

- Chardin, P., and A. Tavittian. "The ral gene: a new ras-related gene isolated by the use of a synthetic probe." *EMBO J* 5 (1986): 2203-2208.
- Colicelli, J. "Human RAS Superfamily Proteins and Related GTPases." *Sci STKE* 250, no. Re13 (2004): 1-53.
- Feig, L.A. "Ral-GTPases: approaching their 15 minutes of fame." *Trends in Cell Biology* 13, no. 8 (2003): 419-425.
- Hofer, F., R. Berdeaux, and G.S. Martin. "Ras-independent activation of Ral by a Ca<sup>2+</sup>-dependent pathway." *Current Biology* 8 (1998): 839-842.
- Takai, Y., T. Sasaki, and T. Matozaki. "Small GTP-binding proteins." *Physiol. Rev.* 81 (2001): 153-208.

**Section IX: Related Products:**

<b>PRODUCT (Cat. #)</b>	<b>DESCRIPTION</b>	<b>QUANTITY</b>
BK129*	G-LISA™ for RalA Activation Assay (Absorbance based)	96 assays
BK124*	G-LISA™ for RhoA Activation Assay (Absorbance based)	96 assays
BK121*	G-LISA™ for RhoA Activation Assay (Luminescence based)	96 assays
BK125*	G-LISA™ for Rac1,2,3 Activation Assay (Absorbance based)	96 assays
BK126*	G-LISA™ for Rac1 Activation Assay (Luminescence based)	96 assays
BK127*	G-LISA™ for Cdc42 Activation Assay (Absorbance based)	96 assays
BK128*	G-LISA™ for Rac1 Specific Activation Assay (Absorbance based)	96 assays
BK008	Ras Activation Assay Biochem Kit™ (Pull down assay)	50 assays
BK034	Cdc42 Activation Assay Biochem™ Kit (Pull down assay)	50 assays
BK035	Rac1 Activation Assay Biochem Kit™ (Pull down assay)	50 assays
BK036	RhoA Activation Assay Biochem Kit™ (Pull down assay)	80 assays
ADV02	Protein quantitation assay kit	1 x 500 ml

\* Technology covered under Patent 7,763,418 B2