



Cytoskeleton, Inc.

Manual

V 2.5

Total RhoA ELISA Biochem Kit

A Biochem Kit™ product from

Cytoskeleton, Inc.

Cat. # BK150

Manual Contents

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I: Introduction

Background

The Rho family of small GTPases consists of at least 20 members, the most extensively characterized of which are the Rac1, RhoA and Cdc42 proteins (1). In common with all other small GTPases, the Rho proteins act as molecular switches that transmit cellular signals through an array of effector proteins. The Rho switch operates by alternating between an active, GTP-bound state and an inactive, GDP-bound state. Understanding the mechanisms that regulate activation / inactivation of the GTPases is a subject of intense investigation. The Rho family is involved in a wide range of cellular responses, including cytoskeletal reorganization (2-3), regulation of transcription (4), cell migration (5). In addition, there are physiological effects of RhoA levels that have been reported by numerous groups, these include transformation (6), metastasis (6), angiogenesis (7) and apoptosis (8).

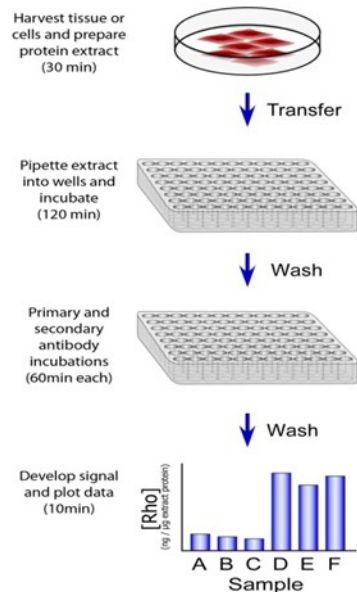
RhoA levels may also change during cell culture so it is critical to measure this protein during studies of Rho activation and transfections which perturb the Rho pathway or its transcription.

Traditionally, this assay has been performed using a Western blotting approach, this method suffers from several drawbacks such as being time consuming, requiring more protein than ELISA based techniques, being limited in the number of samples that can be handled simultaneously and yielding only semi-quantitative results. The Rho ELISA described here results in rapid and accurate measurement without cumbersome techniques.

Assay Principle

The RhoA ELISA kit is designed as a highly selective sandwich ELISA format for RhoA measurement (Figure 1). Each well is pre-coated with anti-Rho IgY antibody which has high affinity for all Rho isoforms. The complementary antibody is a highly selective mouse monoclonal to RhoA. Bound RhoA is subsequently detected with a secondary antibody HRP conjugate and the OPD substrate.

Figure 1: Simple and Quick Protocol



Characteristics of the RhoA ELISA

The RhoA ELISA kit is a sandwich ELISA that is used to determine quantitative measurements of RhoA in cell extracts and tissue samples in less than 5 h. The assay is particularly flexible for different sources of RhoA containing extracts such as mouse, human and porcine tissues, as well as human and mouse tissue culture grown cells (see Appendix A for a list of samples used in development, a current list can be found on line www.cytoskeleton.com). A range of lysis buffers can be used including M-PER and RIPA, urea based or activation assay lysis buffers (pulldowns and GLISAs) but there are a few known interfering substances (see Appendix B & C for a list of compatible buffer components).

I: Introduction (Continued)

The assay is highly efficient at detecting 1-30 ng of RhoA as shown in Figure 2. For measuring RhoA in extracts each assay requires only 3.0 to 30 μ g of total cell or tissue protein, and the assay can detect as little as 0.10ng of RhoA in the high sensitivity format. It is HTS compatible with straightforward ELISA technique. In comparison to the Western blot approach the RhoA ELISA gives very similar but more accurate results (see Figure 3). The coefficient of variation (cv, measure of accuracy) is <3.6% for the ELISA, this is considered very low for an ELISA assay, in comparison to the Western blot approach which has a cv of 10-30%.

The ELISA characteristics and specifications are summarized in Tables 1 and 2 on the next page.

Figure 2: Excellent linearity with high R^2 value

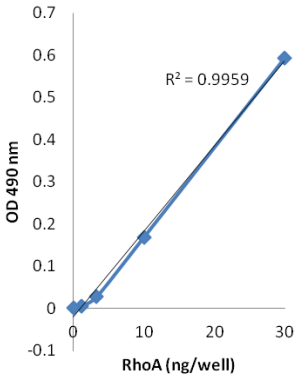
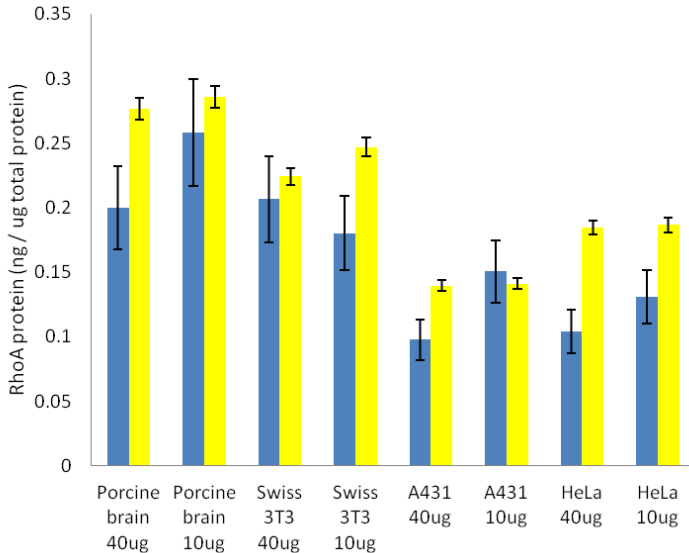


Figure 3: RhoA quantitation in samples run in ELISA and Western blot format



Legend: Yellow bar = ELISA results, Blue bar = Western result

I: Introduction (Continued)

Table 1: Characteristics of the RhoA ELISA

Characteristic	Comparison	
	Western blotting	RhoA ELISA
<i>Assay Time</i>	6-8 h	5 h
<i>Cell material per assay</i>	10-50 µg protein	3 to 30 µg protein
<i>Compatibility with different lysis buffers</i>	Incompatible with high salt buffers	Compatible with most buffers tested*
<i>Sample handling</i>	2 to 20 samples	2 to 96 samples (or more)
<i>Quantitative Data**</i>	Semi	Yes
<i>High throughput compatible</i>	No	Yes

* - see Appendix C.

** - Numerical readouts and fewer sample handling steps make this assay more quantitative.

Table 2: Specifications of the RhoA ELISA

Specification	Comparison	
	Western blotting	RhoA ELISA
<i>Linear range (ng/well or lane)</i>	2-20ng	1 to 30ng
<i>Sensitivity (i.e. +/-ng/well or lane)</i>	5ng	0.5ng
<i>Detection limit (ng/well or lane)</i>	1ng	0.1ng*
<i>Coefficient of variation at OD 0.50 two replicates</i>	10-43%	3.5%
<i>Coefficient of variation at OD 0.50 eight replicates</i>	23%	2.6%
<i>Coefficient of variation at OD 0.50 forty eight replicates</i>	18%	2.3%
<i>Z'-factor**</i>	na	0.73

* - high sensitivity format

** - Z'-factor is calculated as Zhang et al. 1999

II: Purchaser Notification

Limited Use Statement

The RhoA ELISA kit is based on technology developed at Cytoskeleton Inc. and is the subject of patent applications assigned to Cytoskeleton Inc. 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 96 assays. You can assay anywhere from 2 to 96 samples at a time for your own convenience. Kit contents should be kept in the sealed bag prior to resuspending. Table 3 summarizes the kit contents.

Table 3: Kit Contents

Reagents	Cat. #	Quantity	Storage
96 well Total Rho binding plate	Part # PE05	12 strips of 8 wells each	Desiccated 4°C Stable for 6 months
Strip holder for strip	N/A	One holder	Room temperature
Anti-RhoA antibody (mouse monoclonal)	Part # GL09	1 tube, lyophilized, 80 µl after resuspension.	Desiccated 4°C Stable for 6 months
Secondary antibody - horseradish peroxidase conjugate (HRP)	Part # GL02	1 tube, lyophilized, 80 µl after resuspension.	Desiccated 4°C Stable for 6 months
Rho control protein (wild type his-tagged RhoA)	Part # RHWT-10 µg Similar to Cat.# RH01	1 tube, lyophilized, 50 µl after resuspension.	Desiccated 4°C or -70°C Stable for 6 months
Sample Dilution Buffer	Part # PE20	1 bottle, lyophilized, 30 ml after resuspension.	Desiccated 4°C or Room Temperature Stable for 6 months
Wash Buffer (PBST)	Part # PE38	1 tablet for 1 L	Desiccated 4°C Stable for 6 months
Antigen Presenting Buffer	Part # GL39	1 bottle, 30 ml	Room temperature Stable for 6 months
HRP Detection Reagent A	Part # GL43	1 tablet, silver pack	4°C Stable for 6 months
HRP Detection Reagent B	Part # GL44	1 tablet, gold pack	4°C Stable for 6 months
Adhesive film for microplates	N/A	3 films	Room temp or 4°C

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

III: Kit Contents (Continued)

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

- Cold 4°C pure water (30 ml)
- PBS pH 7.4 (10 ml)
- Color development Stop Solution: 1.8 M sulfuric acid (add 3 ml of concentrated sulfuric acid to 27 ml of water, stable for 1 year at room temperature)
- Multi-channel or multi-dispensing pipettor for 25-200 µl range.
- Multi-channel pipettor solution basins (available from VWR Cat. # 21007-970). Used for liquid handling.
- Vortex for mixing cell lysate and Dilution Buffer solutions.
- Microplate spectrophotometer (see Section V: Important Technical Notes for information on settings etc.).
- Liquid nitrogen for snap freezing homogenates.

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 shown in Table 4:

Table 4: Component Storage and Reconstitution

Kit Component	Reconstitution	Storage Conditions
96 well Rho binding plate	It is imperative to keep the plate in the sealed desiccant bag with desiccant at all times. Move to room temperature 30min prior to starting the assay. Reconstitute each well with 100 μ l of Milli-Q water when samples are ready.	Store desiccated at 4°C or room temperature Stable for 6 months
Anti-RhoA antibody	Centrifuge briefly to collect the pellet in the bottom of the tube. Dissolve the powder in 80 μ l of PBS.	Store at 4°C Stable for 6 months
Secondary antibody HRP	Centrifuge briefly to collect the pellet in the bottom of the tube. Dissolve the powder in 80 μ l of PBS. <u>Do not use sodium azide</u> in combination with this antibody as it will inactivate the HRP.	Store at 4°C Stable for 6 months
Rho control protein	Reconstitute with 30 μ l of Milli-Q water. Dilute further with 20 μ l of PBS plus 0.05 % azide and 0.1 mM DTT (or BME). This will give a 200ng/ μ l stock solution.	Store at 4°C Stable for 6 months
Sample Dilution Buffer	Reconstitute in 30 ml of Milli-Q water.	Store at 4°C or room temperature Stable for 6 months
Wash Buffer	Reconstitute in 1 L of Milli-Q water. This solid will take 45-60 min to resuspend. A magnetic stir bar and stir plate can be used to help resuspension.	Store at room temperature Stable for 6 months
Antigen Presenting Buffer	No reconstitution necessary.	Store at room temperature Stable for 6 months
HRP Detection Reagent A	Resuspend tablet in 10 ml sterile distilled water. Aliquot into 10 x 1 ml volumes. Place in -70°C freezer for storage. NOTE -20°C is NOT good for storage.	Store at -70°C Stable for 6 months
HRP Detection Reagent B	Resuspend tablet in 10 ml sterile distilled water. Aliquot into 10 x 1 ml volumes. Place in -70°C freezer for storage. NOTE -20°C is NOT good for storage.	Store at -70°C Stable for 6 months

V: Important Technical Notes

A) Update notes on Version 2.3 and up

Developing time for HRP Detection Reagent has been increased from 10 min to 15 min. See Section VI, Step 3.29.

OD readings for 10 ng samples lowered to improve R² value for assay. See Section V.G. and Section I, Figure 2.

If performing RhoA G-LISA as well as ELISA, use lysate preparation protocol found in RhoA G-LISA instructions (Cat#BK124). See Section V.D.

B) G-LISA samples

Generally G-LISA samples have low protein concentrations e.g. 0.3 to 0.6 mg/ml. In this condition they are at the lower range of detection in the ELISA, therefore we recommend using more lysate and less Sample Dilution Buffer to keep the final concentration higher. In particular, 40 µl of G-LISA extract plus 80 µl of Sample Dilution Buffer is suitable for duplicate ELISA assays (see Assay Protocol for more details).

C) Assay Preparation

It is critical to get the assay components ready before defrosting or preparing cell lysates because proteases can significantly decrease the signals.

Table 5: Assay Preparation

Reagent	Preparation
Total Rho binding 96 well plate	Remove plate from 4°C and keep in its protective bag, place on your bench at room temperature for 30 min. Do not remove the plate (or strips) from the bag until immediately prior to the experiment.
Milli-Q water	30 ml at room temperature.
Sample Dilution Buffer	30 ml bottle at room temperature.
Rho control protein	Place tube on ice.
Wash Buffer	Place on the bench and use at room temperature.
Antigen Presenting Buffer	30ml bottle at room temperature.
PBS	10ml at room temperature (not provided in the kit)
Anti-RhoA antibody	Have primary antibody stock ready on ice. For each assay, you will need to mix 5µl antibody with 10ml Wash Buffer. This dilution step should be performed just prior to use as detailed in assay protocol. These volumes are necessary for high reproducibility and retaining sufficient reagents for future experiments.
Secondary antibody	Have secondary antibody stock ready on ice. For each assay, you will need to mix 5 µl antibody with 1.5 ml Wash Buffer. This dilution step should be performed just prior to use as detailed in assay protocol. These volumes are necessary for high reproducibility and retaining sufficient reagents for future experiments.
HRP Detection Reagents A and B	During the secondary antibody incubation step defrost 1 ml each and bring to room temperature.
HRP Stop Solution	Make 30ml solution of 1.8 M sulfuric acid and store at room temperature. 1.8 M Sulfuric acid is made by adding 3 ml of conc sulfuric acid to 27 ml of pure water. (This reagent is not provided in the kit).

V: Important Technical Notes (Continued)

D) Tissue and cell extracts

NOTE: If performing RhoA G-LISA (Cat# BK124/BK121) in parallel with the Rho ELISA assay, prepare lysates using the method included in the Cat# BK124/BK121 manual rather than the method below.

Because RhoA is a membrane associated protein under some circumstances we recommend using a homogenization / lysis buffer containing 1% Igepal (Sigma Chemical Co.), such as the GLISA lysis buffer (Part # GL36). Homogenize tissues using 4°C buffer and about 5% tissue (v/v) and 95% (v/v) lysis buffer. Be sure to establish that cell disruption is >90% complete to avoid bias toward fragile cell composition and to create high reproducibility between preparations. Lysis buffer volumes for tissue culture can be estimated by using Table 6 below.

Centrifuge homogenates at 30,000xg for 20min at 4°C and remove the supernatant to a fresh tube on ice. Now assay protein using a detergent compatible reagent such as the Precision Red Advanced Protein Assay (Cat. # ADV02), and dilute to 1 to 5mg/ml prior to snap freezing in liquid nitrogen. Higher protein concentrations will lead to protein aggregation and loss of RhoA signal.

Many other lysis buffers can be used without detrimental results. Two known interfering chemicals are >0.1% SDS and reducing agents (DTT, BME) above 10 µM, see Appendices B and C for more information.

Recommended Lysis buffer

50 mM Tris-HCl pH 7.5
10 mM MgCl₂
1% (v/v) Igepal or similar non-ionic detergent
100 mM NaCl
1 µM leupeptin*
1 µM pepstatin*
10 µM aprotinin*
100 µM benzamidine*

* = 1:100 dilution of Protease Inhibitor Cocktail Cat. # PIC02

Notes:

1. Samples need to be assayed for protein concentration prior to the ELISA, a compatible protein assay is the Precision Red Advanced Protein Assay (Cat# ADV02, www.cytoskeleton.com). Samples must be in the range of 1 to 5 mg/ml prior to freezing.
2. Keep solutions and lysates on ice so that the temperature is at or below 4°C. This helps to minimize changes in signal over time.
3. Cell lysates can be stored frozen after harvest and clarification. The lysates **must** be snap frozen in liquid nitrogen and stored at -70°C. Lysates can be stored at -70°C for up to three years.

V: Important Technical Notes (Continued)

4. Thawing of cell lysates prior to the assay should be in a room temperature water bath, followed by rapid transfer to ice and immediate use in the assay.
5. If detergent is not present in a lysis buffer the Rho component may be reduced by 50% or more (see Appendix C).

Table 6: Recommended Lysis Volumes for Cell Cultures

Culture Vessel	Vessel surface area (cm²)	Volume of PBS wash (ml)	Volume of Lysis Buffer (μl)
35 mm dish	8	2.0	70
60 mm dish	21	3.0	100
100 mm dish	56	10.0	250
150 mm dish	148	15.0	700
6-well cluster plate	9.5 / well	3.0	70
12-well cluster plate	4 / well	1.5	35
T-25 Flask	25	4.0	100
T-75 Flask	75	10.0	500
T-150 Flask	150	15.0	700

E) Extracted Protein Concentration Equivalence

Although the assay has a 30 fold linear range between 1 and 30 ng, it is preferable to measure and make equal the protein concentration in all samples. It is also preferable to run two or more protein concentrations of each sample in order to be sure the samples fall in the linear range at the first attempt. Cell extracts should be equalized with ice cold Lysis Buffer to give identical protein concentrations. For example, cell lysates of protein concentrations ranging from 1.0–3.0 mg/ml would all need to be diluted to 1.0 mg/ml. We highly recommend that the final concentration of equalized lysates lies between 1.0–3.0 mg/ml. It is not necessary to equalize protein concentrations if the variation between them is less than 5%.

The volume of 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)

V: Important Technical Notes (Continued)

F) Spectrophotometer Settings

The majority of the work in the design of this assay has been based on the Molecular Devices M2 plate reader. The parameters of a protocol file for the instrument are given below as a reference:

Table 7. Spectrophotometer settings

Parameters	Character	Contents
Wavelength	490 nm	Bandwidth 2 nm (can be \pm 20 nm for filter based machines)
Protocol	End point	Standard end point assay
Shaking	Medium, orbital	5 s
Temperature	22°C	Room temperature

Please inquire to Technical assistance for help in setting up other machines (call 303-322-2254 or e-mail tservice@cytoskeleton.com for assistance within 24 h).

G) ELISA technique tips

I) Removal of solutions from the wells is accomplished by turning the plate upside down and flicking out the well contents into a waste bin. This is followed by hard tapping the plate three times on a paper towel to get rid of residual solution. It has been found that the complete removal of solutions from the well requires a vigorous flick of the plate and a vigorous series of taps onto paper towels. The complete removal of solution from wells between steps is very important as it avoids high background readings in the buffer only wells. The buffer only wells should read between 0.10 – 0.30 at an

absorbance of 490 nm. If background readings are higher than 0.10 – 0.30 then a more vigorous removal of solutions from the well should be practiced. The 10ng of RhoA positive control wells should give a background subtracted read between 0.1 – 0.3 OD_{490nm} for a 15-20min color development time.

II) Pipetting solutions into each well is accomplished by placing the pipette tip in the bottom half of each well, pipetting on the upper half may lead to high backgrounds.

VI: Assay Protocol

STEP 1: Assay Preparation

Prior to beginning the assay, prepare all ELISA assay components as described in Section IV and Section V: Important Technical Notes, Table 5. Use the check-off list below to confirm that the following reagents are ready;

- ◇ Rho binding plate, at room temperature in the desiccant bag
- ◇ Wash Buffer, resuspended at room temperature
- ◇ Sample Dilution Buffer at room temperature
- ◇ Cell or tissue extracts on ice
- ◇ Water, 30 ml, ice cold
- ◇ HRP Stop Solution, at room temperature
- ◇ PBS
- ◇ Ten 1.5 ml microfuge tubes, in a rack at room temperature
- ◇ Rho Control Protein, reconstituted to 50 μ l, on ice

STEP 2: Designing the Assay

Design your experiment as follows:

Choose how many samples you want to measure and design your layout using the following examples. These layouts are designed to optimize well use efficiency and to minimize errors due to edge effects. Note: Use Appendix 3 for a Plate Template.

For 1 to 3 samples:

1	2
R30	R30
R10	R10
R3	R3
R1	R1
Blk	Blk
S 1	S 1
S 2	S 2
S 3	S 3

For 4 to 7 samples:

1	2	3
R30	R30	S 4
R10	R10	S 4
R3	R3	S 5
R1	R1	S 5
Blk	Blk	S 6
S 1	S 1	S 6
S 2	S 2	S 7
S 3	S 3	S 7

For 8 or more samples:

1	2	3	4
R30	R30	S 4	S 4
R10	R10	S 5	S 5
R3	R3	S 6	S 6
R1	R1	S 7	S 7
Blk	Blk	S 8	S 8
S 1	S 1	S 9	S 9
S 2	S 2	S 10	S 10
S 3	S 3	S 12	S 12

R = Rho control protein in nanograms

S = Sample

VI: Assay Protocol (Continued)

Step 3 - Running the assay

1. Label one tube for each sampleⁱ including four tubes (R30, R10, R3.3 and R1.1) for RhoA positive control and place on ice.
2. Make 10 ml of a 1:4 solution of Lysis buffer : Sample Dilution Buffer (SDB) by mixing 2 ml of Lysis buffer with 8ml of SDB.
3. Make R30: Pipette 5 μ l of RHWT (200 ng/ μ l) into R30 tube plus 1650 μ l of Lysis:SDB (1:4) and mix by inverting three times.
4. Make R10: Pipette 80 μ l of R30 into R10 tube plus 160 μ l of Lysis:SDB (1:4) and mix by inverting three times.
5. Make R3.3: Pipette 80 μ l of R10 into R3.3 tube plus 160 μ l of Lysis:SDB (1:4) and mix by inverting three times.
6. Make R1.1: Pipette 80 μ l of R3.3 into R1.1 tube plus 160 μ l of Lysis:SDB (1:4) and mix by inverting three times.
7. Pipette 24 μ l* of Test sample into the pre-labeled sample tubes.
8. Aliquot 96 μ l* of SDB into each sample tube (not Rho control tubes) and mix by inverting three times.

* Note: For GLISA samples with low protein concentration (i.e. <0.6 mg/ml), it is recommended to use 40 μ l of extract and 80 μ l of SDB. In this case, use the same Lysis:SDB ratio for the positive control protein solutions.

9. Remove the required number of strips from the plate and insert them into the provided strip holder. Re-seal the plate bag to retain low humidity.
10. Dissolve powder in each well with 200 μ l Milli-Q water for each well, incubate for 2 min at RT.
11. Flick out the liquid and tap three times on paper towels to remove excess liquid.
12. Pipette* duplicate 50 μ l amounts into the appropriate wells and seal the wells with the micro-plate adhesive tape supplied in the kit.
13. Incubate for 2h at room temperatureⁱⁱ. (Note: do not shake the plate during any step in this protocol)
14. After 1h 50min prepare the following:
 - Wash Buffer, enough for 6 washes of 200 μ l per well plus 20% extra e.g. 16 wells requires 24 ml.
 - Antigen Presenting Buffer, 200 μ l per well plus 20% extra e.g. 16 wells requires 4 ml.
 - GL09 anti-RhoA antibody, pipette 5 μ l into 10 ml of Wash Buffer, mix well by inverting 5 times but do not shake.
15. At 2h, flick out extracts from plate and tap three times hard onto paper towels to remove liquid.

VI: Assay Protocol (Continued)

16. Wash wells three timesⁱⁱⁱ with 200 μ l Wash Buffer each time, between each wash flick out and tap three times on paper towels.
17. Flick out and tap for a final time then pipette 200 μ l of Antigen Presenting Buffer into each well.
18. Incubate at room temperature for 2min.
19. Repeat three washes in Wash Buffer, between each wash flick out and tap three times on paper towels..
20. Flick out and tap three times, then pipette 50 μ l of diluted GL09 into each well.
21. Incubate for 1h at room temperature. (Note: do not shake the plate during any step in this protocol)
22. At 50min prepare the secondary antibody: For each strip, pipette 5 μ l GL02 into 1.5ml of Wash Buffer (three strips), mix well by inverting five times but do not shake.
23. At 1h, wash wells three times with 200 μ l Wash Buffer each time, between each wash flick out and tap three times on paper towels.
24. Flick out and tap three times, then pipette 50 μ l of diluted secondary antibody (GL02) into each well.
25. Incubate for 1h at room temperature. (Note: do not shake the plate during any step in this protocol)
26. At 30min prepare the color development reagent, defrost 1ml of GL43 and GL44 for each strip. At 1min prior to washing the wells mix both components.
27. At 1h, wash wells five times with 200 μ l Wash Buffer each time, between each wash flick out and tap three times on paper towels.
28. Flick out and tap three times on paper towels, then pipette 80 μ l of color development reagent into each well.
29. Incubate for exactly 15 min at room temperature. Note: a 20 min development time can be used for a lower range of detection e.g. 0.1 to 10 RhoA.
30. Pipette 80 μ l of Stop solution (1.8M sulfuric acid) into each well, and read OD 490nm blanking on the Buffer Blank.

Footnotes:

I = For more than 20 samples it is recommended to prepare all samples in a 96-well dilution plate and then transfer 50 μ l for each well into the ELISA plate to start the binding step.

ii = Longer extract incubation times will generate higher signals which plateau after 16h. However, 2h gives the most robust and reproducible results.

iii = Preferable to use a multi-channel multi-dispensing pipettor for wash steps.

VII: Data Analysis

1. It is recommended to use the Lysis Buffer wells as reference blanks in all studies with this kit. Based on the operator designating the appropriate wells, most machines have associated protocols that perform this operation automatically, call Technical Help for the company supplying the plate reader for information on how to perform this function. When the data are “Lysis Buffer subtracted” (Lysis Buffer only samples have been allocated as Blanks in the assay) then you can import them into a simple graph software like Excel or Sigma plot. Alternatively, the Lysis Buffer background can be subtracted manually or in the spreadsheet application.
2. Data should be arranged in columns where the headings are “Sample”, “Mean”, “Standard Deviation”, “rep1”, “rep2”, “rep3” and “rep4” for the number of replicates performed on each sample. E-mail tservice@cytoskeleton.com for a free Excel Template.
3. List your samples under the “Sample” column in the same order that they were assayed in the plate.
4. Enter the following formula into the first sector under “Mean”, “=average(Xn:Yn)” where X = the column designator for “rep1”, Y = column designator for “rep4”, and n= row designator of the row that you are working on. Repeat for each sector under the “Mean” header until there are sufficient rows to cover the number of samples in your experiment.
5. Enter the following formula into the first sector under “Standard deviation”, “=stdev (Xn:Yn)” where X = the column designator for “rep1”, Y = column designator for “rep4”, and n= row designator of the row that you are working on. Repeat for each sector under the “Standard deviation” header until there are sufficient rows to cover the number of samples in your experiment.
6. Enter your replicate data into rep1, rep2 etc. It doesn't matter if you only have duplicates because the program will ignore any sectors that do not contain data. The program will calculate the Mean and Standard deviation of your replicates.
7. When the data has been entered select the Sample, Mean and Standard deviation data sectors by the click and drag method. Then select the chart making function, in Excel this looks like a clickable square with a mini-bar-chart in. This will guide you through the chart making process with the data you have selected. Choose “column chart” initially, designate the Mean numbers for input values. The Standard deviation column for the y-axis error bars needs to be designated after the Mean numbers chart is made. This is achieved by double clicking on the graph bars, and selecting the “Y-axis error” tab, then entering the location of the standard deviation data by clicking the “Custom” option and selecting the area in the worksheet. E-mail tservice@cytoskeleton.com for a free Excel Template. An example of a typical Excel layout and data plot is shown in Figures 6a and b.

VII: Data Analysis (Continued)

Figure 6a: Typical Excel Layout

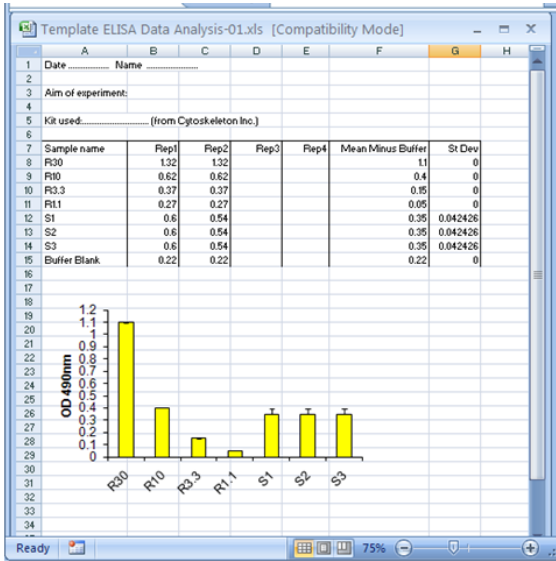
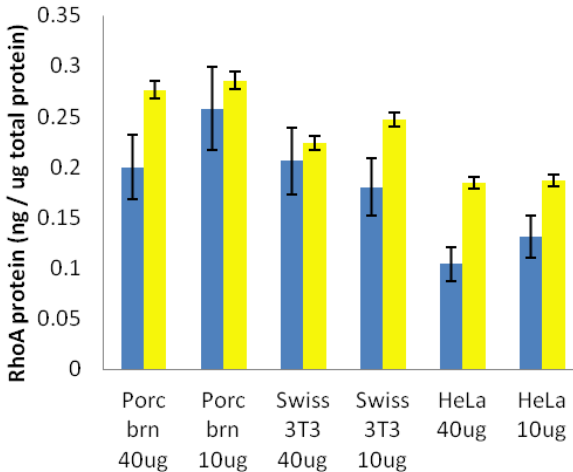


Figure 6b: Typical RhoA ELISA Results



Legend: Absolute RhoA concentrations measured by ELISA and Western blo, using 10 and 40 µg samples of Porcine brain tissue, Swiss 3T3 (mouse) cells and HeLa cells. Signals were read at 490 nm for ELISA and scanned bands for Western blot. Note: similar RhoA concentrations were determined at different sample protein loadings indicating linearity in the assay.

VIII: Troubleshooting

Table 8: Troubleshooting

Observation	Possible cause	Remedy
Weak signal or no signal in all wells	<ol style="list-style-type: none"> 1. The wells were allowed to dry out during the experiment. 2. The plate was allowed to get damp during storage. Well contents will appear sticky and opaque. 3. A step or component of the assay was omitted. 4. The HRP reaction was not developed for long enough 	<ol style="list-style-type: none"> 1. Do not remove the solution in the wells unless the solution of next step is ready. 2. Store the plate in the desiccant bag with the bag securely sealed. If wells appear sticky and opaque the plate can no longer be used. 3. Read instructions carefully. 4. The HRP reaction should be allowed to develop for 10 min at room temperature. HRP Stop Solution should be added prior to reading at 490 nm.
High signal in all wells	<ol style="list-style-type: none"> 1. Concentration of antibodies is too high. 2. Insufficient washes were performed. 	<ol style="list-style-type: none"> 1. Follow the recommended dilution of antibodies in the manual. 2. Follow the instructions for the washing in the manual.
Background readings are high (>0.30)	<ol style="list-style-type: none"> 1. Inefficient removal of solutions from wells during washing and tapping on paper towels. 	<ol style="list-style-type: none"> 1. Background should read between 0.10 – 0.30. Complete removal of solutions from the wells is required to produce a clean assay. Vigorous flicking and patting of the inverted plate is required to completely remove solutions from the wells after each step is complete.
Sample ODs are above 1.50	<ol style="list-style-type: none"> 1. Protein concentration is too high. 	<ol style="list-style-type: none"> 1. Dilute with Lysis buffer five and ten fold and repeat assay.
Random variation between duplicate/triplicate samples.	<ol style="list-style-type: none"> 1. Incorrect volume of solutions for each step added in the wells. 2. Inaccurate pipetting. 3. Flow over of secondary antibody solution during washing steps. 4. The wells were allowed to dry out during the experiment. 	<ol style="list-style-type: none"> 1. Follow the instruction for recommended volume in the manual. 2. A multi-channel pipettor is recommended. 3. Use fresh Wash buffer for the last wash steps 1 and 2, and another fresh Wash buffer for Wash steps 3 to 5. 4. Prepare reagents for each step in the assay in advance, so there is no delay in adding solutions to the empty wells.
Positive control not working	<ol style="list-style-type: none"> 1. Positive control protein contaminated with bacteria. 	<ol style="list-style-type: none"> 1. Use a fresh tube of RhoA positive control protein and be sure to add 0.05% (w/v) sodium azide as a preservative.
Higher values on the plate edges compared to the duplicate.	<ol style="list-style-type: none"> 1. Edge effects caused by drying out, especially in dry climates or air conditioned buildings. 	<ol style="list-style-type: none"> 2. Use micro-plate adhesive tape supplied in the kit to cover wells during incubations.

IX: References

1. Jaffe, AB. & Hall, A. Rho GTPases: Biochemistry and Biology. *Ann. Rev. Cell Dev. Biol.* **21**: 247-269 (2005)
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5. Small, JV., et al. The lamellipodium: where motility begins. *Trends Cell Biol.* **12**: 112-120 (2002)
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8. Turcotte, S. et al. HIF 1-alpha mRNA and protein upregulation involves Rho GTPase expression during hypoxia in renal cell carcinoma. *J. Cell Sci.* **116**, 2247-2260 (2003).

X: ELISA Citation

See website for latest list of citations for this product.

Appendix A

Measurement of Rho concentration in various tissues and cell types.

Table 10: Cell and tissue types extracted with Lysis buffer A* .

Tissue or Cell	RhoA concentration** (ng/μg of total protein)	Species
Swiss 3T3 cells	0.33	Mouse
HeLa cells – human cervical cancer cells	0.28	Human
A431 – human epidermoid carcinoma	0.23	Human
Huvec –primary cells	0.20	Human
Porcine brain (white & grey matter)	0.22 - 0.32	Porcine
Lung tissue	0.31	Mouse
Heart tissue	0.40	Mouse

* - Lysis buffer A is described in the section “Important technical notes” and in Appendix C.

** - Coefficient of variation is +/- 3%.

Interfering substances

Table 11: Interfering substances with quenching reagent

Substance	Interfering concentration	Quenching reagent
Reducing agents e.g. dithiothreitol (DTT) and β -mercaptoethanol (BME)	>10 μ M	2mM potassium dichromate for 5min at room temperature.
Ionic detergents e.g. sodium dodecyl sulphate (SDS)	>0.1%	Not determined, except dilution below 0.05% (w/v).

Appendix C

Lysis buffer and detergent effects.

Recommended Lysis Buffer, Lysis Buffer A:

50 mM Tris-HCl pH 7.5
10 mM MgCl₂
1% (v/v) Igepal
100 mM NaCl
1 μM leupeptin*
1 μM pepstatin*
10 μM aprotinin*
100 μM benzamidine*

* = 1:100 dilution of Protease Inhibitor Cocktail Cat. # PIC02

See Table 12 below for the effects of removing important components from this mixture.

Using the standard procedure, other compatible buffers are: PBS, Pipes, MES, Hepes based. Using a 1:10 or 1:20 ratio of lysis buffer:SDB, heavily detergent loaded buffers such as Ripa and IEF buffers can be used.

Table 12 – Effects of Lysis buffer components

Lysis buffer	1% Igepal	10mM MgCl ₂	Signal (OD490nm)	Measured RhoA concentration (ng RhoA / μg sample protein)
Lysis Buffer A	Yes	Yes	0.88	0.30
Lysis Buffer B	Yes	No	0.93	0.32
Lysis Buffer C	No	Yes	0.51	0.18
Lysis Buffer D	No	No	0.40	0.14

Appendix D: BK150 Experiment Record Sheet

Scientist Name
Contact Tel. #
e-mail
Kit Cat. # / Lot #

<u>STEP</u>	<u>Comments or Changes</u>
-------------	----------------------------

1. Type of cell or tissue?
2. How were the cells/tissue treated prior to homogenization?
..... Time or age
..... Sub-tissue or % confluency
..... Chemical additive
.....mg/ml of protein in lysate
3. Type of Lysis Buffer and homogenization?
.....Lysis Buffer.....equipmentseconds
4. Volume of sample added per well?µl
5. Volume of sample and volume of Sample Dilution Bufferµl Sampleµl SDB
6. Time of sample incubation in well? min
7. Method of plate wash?machine or by hand
8. What volume and for how long was detection reagent was used?
.....µl min

Technical Assistance: call either 303-322-2254 or e-mail tservice@cytoskeleton.com.

Appendix E: Plate Template Record

Operator Name:

Aim of Experiment:

Date of Experiment:

Technical Assistance: call either 303-322-2254 or e-mail tservice@cytoskeleton.com

	H	G	F	E	D	C	B	A	
1									1
2									2
3									3
4									4
5									5
6									6
7									7
8									8
9									9
10									10
11									11
12									12



