RhoGAP Assay Biochem Kit

Cat. # BK105

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Manual Contents

Section 1: Introduction

The small GTPases are molecular switches which control cellular processes such as cytoskeletal re-organization, axonal guidance, vesicle trafficking, budding in yeast, gene expression and cell motility. The balance of the GTP to GDP bound state underlies the switch mechanism as they turn from an activated (GTP form) to inactive state (GDP form). The balance of GTP to GDP bound states is controlled by catalytic proteins that either increase the rate of exchange of GDP for GTP (GEFs), increase the GTPase activity (GAPs), or prevent the exchange of GDP (GDIs) (see **Figure 1**). Recently it has been shown that the GAP family of proteins is large (70 members) and potentially important for changing a cell from a normal to disease status (see review in ref. 1).

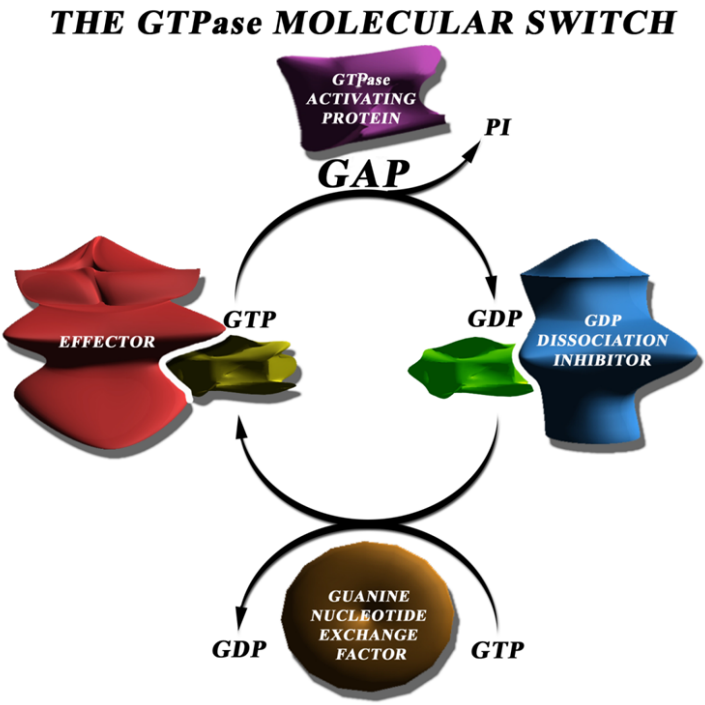

Figure 1: The GTPase Molecular Switch.

GAP activity can be reduced by deletion or mutation in cells; in this case the small GTPase targeted by the GAP has an extended time in the active GTP state, in essence creating a permanently active state. For example the ability of Rho GTPase to control axonal guidance is exemplified in the finding that mutations of a Rho-GAP can lead to mental retardation (refs. 2 and 3). In cancer, mutated Ras-GAPs (NF1 gene) have been shown to cause neurofibromatosis (ref. 4), and mutated Rheb-GAP has been shown to cause tuberous sclerosis complex (ref. 5).

Although some GAPs are known to have GAP activity, the majority of assumed GAP proteins have only been implicated by homology to contain GAP activity. Cytoskeleton Inc. is facilitating the exploration of this field by introducing the GAP Assay Kit (Cat. # BK105). Several small GTPase proteins (Ras, RhoA, Cdc42 and Rac1) are included such that the researcher can screen the small G-proteins for GAP-like activity which is usually small G-protein specific i.e. RhoGAP or Ras GAP. It is likely that new domain information will identify other small G-protein GAPs which have not been apparent. In addition the new assay kits are easily adapted for High-Throughput Screen format which allows development of ligands for pharmaceutical studies. Please inquire for bulk reagent quotes if you are considering a screening application (tservice@cytoskeleton.com).

The reagents in this kit have been optimized to enable high activity from your GAP protein such that you can detect the enhanced GTPase of a small G-protein through a simple absorbance-based detection method, as shown in **Figure 2**. The small G-protein is incubated in the presence of GAP protein (RhoGAP is the control protein included in this kit), GTP and the optimized buffer. The overall GTPase activity of small G-proteins is composed of two components which limit the activity; these are a) endogenous GTPase activity and b) GDP dissociation rate. The endogenous activity can be enhanced by the addition of a suitable GAP protein, whereas the dissociation rate can be enhanced by buffer optimization.

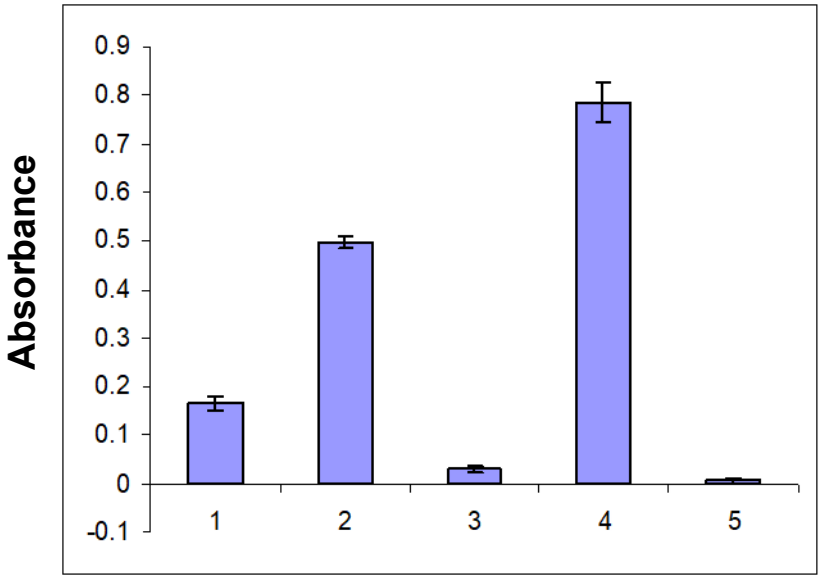

Figure 2: p50 RhoGAP activity measured by GTP hydrolysis by RhoA and Cdc42 protein. *Method: The small GTPases RhoA (Cat. # RH01), Cdc42 (Cat. # CD01) and the human p50 RhoGAP protein (Cat. # GAS01) were expressed and purified from E. coli and are available as separate items from Cytoskeleton Inc. The reactions were conducted in a half area clear 96 well plate (Corning Cat. # 3696) format (40 µl reaction volumes). Each reaction contains +/- 5 µg RhoA or +/- Cdc42, +/- 8 µg p50 RhoGAP domain and 200 µM GTP in Reaction Buffer. Sample 1 contained Cdc42 only, sample 2 contained Cdc42 and p50 RhoGAP, sample 3 contained RhoA only, sample 4 contained RhoA and p50 RhoGAP, and sample 5 contained p50 RhoGAP alone. Reactions were incubated at 37ºC for 20 min followed by the addition 120 µl of CytoPhos reagent for 10 min to determine the phosphate generated by the hydrolysis of GTP.*

II: Important Technical Notes

The following technical notes should be carefully read prior to beginning the assay.

GAP assay reagents

1. All protein samples and buffers must be free from phosphate prior to beginning the assays. If your proteins are in PBS buffer then they must be dialyzed twice in 1000 volumes of 50 mM PIPES pH 7.0 or 50 mM Tris pH 7.5 based buffer to reduce the phosphate content to non detectable levels.
2. This kit contains sufficient purified GTPases to perform 20 reactions (40 µl volume for 96-well plate) for each GTPase. This corresponds to 80 GAP assays for all four GTPases (Ras, RhoA, Rac1 and Cdc42) using the half area 96-well plate (Corning plate Cat. # 3696). Up to 40 reactions per GTPase can be achieved if a 384-well plate is used.
3. The positive control protein, Rho GAP domain of human p50 RhoGAP (Cat. # GAS01), contained in the kit provides sufficient reagent for at least 12 control assays in a half area 96-well plate format and 24 control assays for a 384-well plate. Human p50 RhoGAP is predominantly a GAP of Rho sub-family members and it is less active at promoting Ras sub-family members GTPase activity.
4. Four well characterized small GTPases Ras, RhoA, Rac1 and Cdc42 are contained in this kit. If you need to examine more GTPases, a wide selection of small GTPases (including Ran and RhoC) are available from Cytoskeleton Inc. and can be purchased separately (see Section VII).
5. Some of the reagents in this kit require reconstitution and division into convenient experiment sized aliquots. It is important to carry out the aliquoting step as multiple freeze/thaw cycles of some reagents (for example the purified proteins) will result in the inactivation of the reagents.

Assay Optimization

The GAP assay kit has been developed to provide good general conditions for a broad range of GTPase activating proteins (GAPs). Using this kit as outlined in the introduction (Section I) will result in an enhanced GTPase activity for RhoA of approximately 10 fold over the endogenous rate which is consistent with published data. **The GAP assay should be performed at 37°C.** It should be noted however, that optimization of the GAP assay may be needed for any given GAP (see next). And please refer to the trouble shooting guide at the end of the manual for other possible improvements in assay design.

There are several parameters that may particularly affect GAP protein activity:

1. **Temperature.** An incubation temperature of 37°C is recommended. Different GAPs may require a different optimal temperature for their normal *in vitro* GAP activity so it is a good idea to test 20 and 30°C. The lower temperatures may help situations where proteases are present and they are denaturing proteins at 37ºC.
2. **Protein concentration.** A titration of the GAP of interest should be performed to achieve optimal results.
3. **Reaction buffer conditions**. It may be necessary to optimize the assay for a particular GAP by adjusting the salt concentration (25-500 mM), the pH (6.0-8.5) using appropriate buffers such as MES, PIPES and Tris, and the $MgCl_2$ concentration (0.01, 0.1, 1 and 10 mM).
4. **Control reactions.** It is important to include control reactions in the assay, particularly if your GAP of interest is in an impure state. Control reactions are discussed in Section V, and should be small G-protein only and GAP protein only.

Instrumentation

This absorbance-based assay is based upon a wavelength of 640 to 660 nm with an optimal of 650 nm. Wavelengths outside of this range are not recommended.

The majority of the work in the design of this assay has been based on the SpectroMax250 from Molecular Devices Inc. This instrument is a monochromatic prism based machine but filter based machines can be used without a problem. The parameters of a Protocol file in this scenario are:

Parameters	Character	Contents
Measurement	End Point	One reading after 10 min color development.
Absorbance wavelength	Monochromatic or Filter-based	650 nm +/- 10 nm 650 nm +/- 20 nm
Temperature	Celsius	37°C (possibly 20 or 30 for other GAPs)
Shaking	Medium, orbital	5 s at 200 rpm
Plate foot print type	Standard 96-well	Corning Cat. # 3696

Kit Uses

1. Determination of the activity of uncharacterized GAPs.
2. Biochemical characterization of small GTPases and their associated GAPs.
3. Examination of the regulation of GAP activity by different cofactors or protein domains.
4. Screen the mutant protein of either GAPs or GTPases for activity and substrate specificity.
5. Identification of GAP inhibitors in HTS (high throughput screen) format. Please inquire for significant discounts on large quantities of any reagents in this kit.

III: Kit Contents

This kit contains sufficient reagents for approximately 20 assays with each small G-protein, and 12 reactions with the positive control GAP protein. The assay can be adapted to a 384 well format which will allow approximately three fold the number of assays. Upon receipt the kit can be stored at 4°C. See **Table 2 (Section VI)** for storage of components after reconstitution.

Table 1: Kit Contents and Storage Upon Arrival

Kit Component	Cat # or Part #	Quantity	Storage Conditions
Reaction Buffer (2X)	RXB01	One bottle, lyophilized. Contains 10 ml of buffer.	Store at 4°C, desiccated
p50 RhoGAP domain	GAS01	Two tubes, lyophilized. Contains 50 µg each of purified p50 RhoGAP domain protein.	Store at 4°C, desiccated
His-RhoA protein	RH01	One tube, lyophilized. Contains 100 µg of purified His tagged RhoA protein.	Store at 4°C, desiccated
His-Rac1 protein	RC01	One tube, lyophilized. Contains 100 µg of purified	Store at 4°C, desiccated
His-Cdc42 protein	CD01	One tube, lyophilized. Contains 100 µg of purified	Store at 4°C, desiccated
His-Ras p21 protein	RS01	One tube, lyophilized. Contains 100 µg of purified His tagged Ras p21 protein.	Store at 4°C, desiccated
GTP stock	BST06-001	One tube, lyophilized. Contains 100 µl of 100 mM GTP.	Store at 4°C, desiccated
CytoPhos Reagent	CYPH	One bottle, liquid. Contains 70 ml of reagent.	Store at 4°C.
96-well plate			Store at room temperature.

IV: Things to do Prior to Beginning the Assay

Prior to beginning the assay you will need to reconstitute several components as detailed in **Table 2**. When stored and reconstituted as described, reagents are guaranteed to be stable for a minimum of 6 months.

When working with the proteins described below it is essential to work on ice at all times.

Table 2: Component Reconstitution and Storage

Kit Component	Cat # or Part #	Reconstitution	Storage Conditions
Reaction Buffer (2X)	RXB01	1) Dissolve the powder in 10 ml Milli-Q water. 2) Store at 4°C.	Store at 4°C.
p50 RhoGAP domain	GAS01	1) Reconstitute each vial in 20 µl ice cold Milli-Q water to give a 2.5 mg/ml (43 µM) solution. Pool the protein into one tube. 2) Aliquot into 4 x 9 µl sizes. 3) Snap freeze in liquid nitrogen.	Store at –70°C.
His-RhoA protein	RH01	1) Reconstitute in 20 µl ice cold Milli-Q water to give a 5 mg/ml (250 µM) solution. 2) Aliquot into 4 x 4.5 µl sizes. 3) Snap freeze in liquid nitrogen.	Store at –70°C.
His-Rac1 protein	RC01	1) Reconstitute in 20 µl ice cold Milli-Q water to give a 5 mg/ml (250 µM) solution. 2) Aliquot into 4 x 4.5 µl sizes. 3) Snap freeze in liquid nitrogen.	Store at –70°C.
His-Cdc42 protein	CD01	1) Reconstitute in 20 µl ice cold Milli-Q water to give a 5 mg/ml (250 µM) solution. 2) Aliquot into 4 x 4.5 µl sizes. 3) Snap freeze in liquid nitrogen.	Store at –70°C.
His-Ras p21 protein	RS01	1) Reconstitute in 20 µl ice cold Milli-Q water to give a 5 mg/ml (250 µM) solution. 2) Aliquot into 4 x 4.5 µl sizes. 3) Snap freeze in liquid nitrogen.	Store at –70°C.
GTP stock	BST06-001	1) Reconstitute the vial in 100 µl of ice cold Milli-Q water to give a 100 mM stock solution. Add an additional 900 µl of cold Milli-Q water to dilute to a 10 mM stock. 2) Aliquot into 10 x 100 µl sizes. 3) Immediately store at -20°C or –70°C.	Store at -20°C or -70°C.
CytoPhos Reagent	CYPH	None required.	Store at 4°C and warm to room temperature for use.
96-well plate		None required.	Store at room temperature.

V: Assay Protocol

The following protocols are for a 96-well plate format. The assay can be adapted to a 384 well format, it is recommended to use 1/3rd the volume of reaction mix per well for the 384 format. The GAP reaction is started with all of the reaction components, minus GTP. For drug screening applications drugs may be added to these mixtures. Finally, GTP is added to initiate the reaction.

Instrumentation Settings and Microtiter Plates

The plate reader should be set to 650 nm.

Protein and compound preparation immediately prior to assay:

1. Prepare all the protein solutions on ice to avoid denaturation. Place the Reaction Buffer on ice to keep it cold.

2. For drug screening, prepare 8x concentrations of compound in Milli-Q water or for low aqueous soluble compounds use Milli-Q water and 10% DMSO. We recommend a final drug concentration of 30 µM – 100 µM for initial screening. Add 5 µl aliquots of 8x concentrated compounds to individual wells prior to adding the GAP mixture and GTP activator.

3. Defrost the small GTPase and GAP proteins by placing the tube in a room temperature water bath for 1 min, then place on ice. These are stable for 4 h on ice.

4. Dilute the 9 µl p50 RhoGAP aliquots prepared from this kit with 24 µl of ice cold Milli-Q water, pipette up and down, and place on ice. Protein concentration is now 0.68 µg/µl.

5. Dilute the 4.5 µl small GTPase aliquots prepared from this kit with 25 µl of ice cold Milli-Q water and pipette up and down and place back on ice. Protein concentration is now 0.78 µg/µl.

Reaction mixture preparation:

Prepare enough reaction mix to cover the intended experiment. For each replicate reaction prepare the following amounts of mixture on ice:

Reaction Mix	Half-area 96-well plate
Reaction Buffer	16 µl
Small GTPase protein	5.0 µl
GAP protein	9.0 µl

1. For the "**small GTPase only**" reactions replace the GAP protein addition with Milli-Q water of the same volume.

2. For the "**GAP protein only**" reactions replace the small GTPase protein addition with Milli-Q water of the same volume.

3. Immediately prior to initiating the reaction, defrost 100 µl of 10 mM GTP stock and place on ice. Add 1.15 ml of Milli-Q water and pipette up and down. Final concentration is 800 µM which will be diluted to 200 µM in the reaction.

Reaction initiation:

1. If necessary pipette 5 µl of 8x concentrated drug compound into the appropriate wells and 5 µl of 1x Reaction Buffer into control wells.

2. Pipette 30 µl of 1x Reaction Buffer in to control wells. These are used for background subtraction as described below.

3. Pipette 30 µl of reaction mixtures into individual wells.

4. Pipette 10 µl of an 800 µM GTP solution into each well and shake for 5 s at 200 rpm, then incubate for 20 min at 37ºC.

Reaction termination and signal development:

1. Immediately after the 20 min incubation, pipette 120 µl of CytoPhos reagent into each well.

2. Develop green color, indicating phosphate, by incubating for room temperature for exactly 10 min; do not incubate longer than 10 min because the acid environment of the CytoPhos reagent will hydrolyze any remaining GTP which will increase the background signal. Incubations in CytoPhos can be less than 10 min if the signal develops too quickly, read ODs at any stage when the darkest sample is green colored.

3. Using the buffer blank as the background subtraction, read absorbance at 650 nm.

Note: Specific GAP activity in nmoles/min/mg of small G-protein can be determined by using a phosphate standard of 0.1, 0.2. 0.5 and 2 nmoles of phosphate (KH_2PO_4) per well. Prepare a 0.1 mM KH_2PO_4 solution in Milli-Q water and add 1, 2, 5 and 20 µl of this standard to individual wells. Bring the volume to 30 µl with Milli-Q water. Add 120 µl of CytoPhos reagent into each well and proceed with color development as described above. E-mail technical service if you need more help with this (tservice@cytoskeleton.com).

VI: References

1. Bernards A. and Settleman J. 2004. GAP control: regulating the regulators of small GTPases. *Trends Cell Biol.* 14, (7), 377-385.

2. Billuart P. et al. 1998. Oligophrenin-1 encodes a rhoGAP protein involved in X-linked mental retardation. *Nature*, 392, 923-926.

3. Faucherre A. et al. 2003. Lowe syndrome protein OCRL1 interacts with Rac GTPase in the trans-Golgi network. *Hum. Mol. Genet*. 12, 2449-2456.

4. Cichowski K. and Jacks T. 2001. NF1 tumor suppressor gene function: narrowing the GAP. *Cell*, 104, 593-604.

5. Li Y. et al. 2004. TSC2: filling the GAP in the mTOR signaling pathway. *Trends Biochem. Sci.* 29, 32-38.

Appendix: Troubleshooting

Observation	Possible cause	Remedy
1. No difference in GAP + small GTPase reactions versus GAP only and Rho only reactions.	1. Incorrect wavelength chosen 2. Incorrect labeling of tubes. 3. Inactive proteins.	1. Check wavelength 2. Repeat the experiment. 3. Follow correct storage procedure.
2. No green color developed.	1. GTP not added. 2. GTPase not active or concentration is too low.	1. Repeat experiment. 2. Check nativity of GTPase with a GEF assay (e.g. BK100).
3. All GAP or all small G- protein samples contain intense green color.	1. Protein sample contains phosphate, probably as PBS buffer.	1. Dialyze protein from PBS to a PIPES or Tris based buffer twice in 1000x the volume.
4. Assay is not reproducible.	1. Inconsistent preparation of reaction mixture. 2. GAP protein is not stable due to poor purity.	1. More consistent technique, increasing pipetting accuracy, or test machine for signal stability using buffer alone. 2. Increase the purity of the protein by optimizing the purifying process, e.g. reducing the proteases.
5. Signals are too low in GAP containing samples.	1. Concentration of GAP too low 2. Buffer needs to be optimized	1. Increase GAP concentration. 2. Read Section II for optimization parameters to test.

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