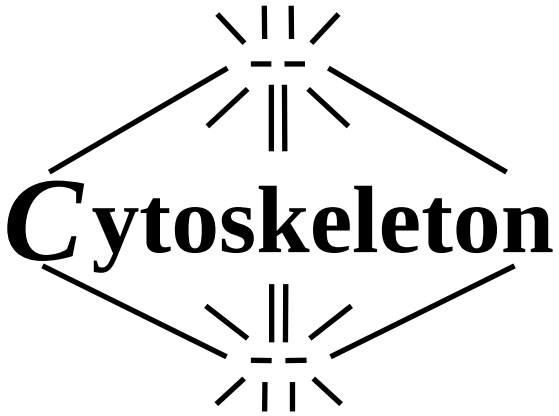


RhoGEF EXCHANGE ASSAY BIOCHEM KIT

BK100

ORDERING INFORMATION

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

MANUAL CONTENTS

Section I: Introduction

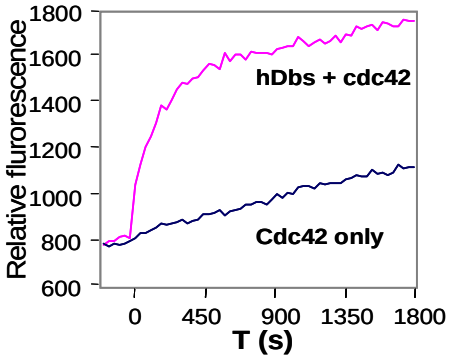
The Ras superfamily of small GTPases consist of more than 150 members, which based on their sequence homology, are divided into several subfamilies such as Rho, Ras, Ran, Rab, Arf and Rem/Rad families. This group of small GTPases serve as binary switches cycling between GDP-bound inactive and GTP-bound active state (1,2). The regulatory proteins for this switch include guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (1,2).

GEFs catalyze the exchange of GDP for GTP to generate the active state of small GTPases in response to extracellular signals. In order to facilitate the exchange, the GEFs must bind to the GDP bound GTPases, destabilize the GDP-GTPase complex, and then stabilize a nucleotide free reaction intermediate. Because of the high intracellular ratio of GTP to GDP, the released GDP is replaced with GTP, leading to release of GEFs from the complex and activation of the GTPase (1,2). Many GEF proteins have been identified as oncogenes and are involved in human disease such as cancer. Interestingly, the expression of GEF protein is tissue or cell type specific, providing a therapeutic potential for cancer treatment (2, 3).

Recently developed fluorescence analogs of guanine nucleotides have greatly improved the technical ability to define the real time exchange reaction of GEFs, including kinetic and thermodynamic properties, eliminating the need for traditional radioactive labeling method (4, 5). This fluorescence based assay takes advantage of the spectroscopic difference between bound and unbound fluorescent analogs to guanine nucleotides and thus is able to monitor the states of small GTPases (4, 5). One of most widely used fluorescent analogs is mant (N-methylanthraniloyl) fluorophores. Excitation of mant flurophore at 360nm (+/-10nm) gives a fluorescence emission at 440nm (+/-20nm). Once bound to GTPases, the fluorophore emission intensity increases dramatically approximately 2 fold. Therefore, the enhancement of fluorescent intensity in the presence of small GTPases and GEFs will reflect the respective GEF activities of known or unknown proteins.

Cytoskeleton Inc. has developed a mant fluorophore based GEF assay suitable for both 96-well and 384-well format. This assay can be applied to multiple research purposes such as characterizing the GEFs and identifying GEF inhibitors in a high throughput screen format. This kit contains human Cdc42, Rac1 and RhoA proteins and the GEF domain of Dbs as a positive control GEF for Cdc42 (fig. 1) and RhoA. Dbs shows extremely low GEF activity for Rac1 (see Fig.2 and ref. 4). Interestingly, it was reported that human Dbs can activate Rac1 in a FRET based assay (6).

Figure 1. Cdc42 exchange assay



Method: The small GTPases Cdc42 (Cat# CD01) and the human Dbs protein (Cat# GE01) were expressed as His-tagged proteins in E. coli and purified on a nickel affinity column. The reactions were conducted in a 96 well black flat bottom half area plate (Corning Cat# 3686) format (150 µl reaction volumes). Each reaction contains 1 µM GTPases, 50 µg/ml bovine serum albumin (BSA), 20 mM Tris pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, and 0.8 µM mant-GTP with or without the presence of 0.5 µM human Dbs(DH/PH) protein. Reactions were measured in a Tecan Spectrofluor plus fluorimeter(λ_{ex}=360nm, λ_{em}=460nm). Readings were taken at 20°C every 30 seconds for a total reaction time of 30 minutes.

Section II: Important Technical Notes

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

Exchange assay reagents

1) This kit contains sufficient purified GTPases and mant-GTP to carry out more than 20 reactions (100 µl volume for 96-well plate) for each GTPase. This corresponds to 60 GEF assays for all three GTPases (Cdc42, RhoA, Rac1) of 100 µl volume using a black flat-bottom 96-well half area plate (Corning plate Cat# 3686). Up to 130 reactions per GTPase can be achieved if a 384-well black round bottom plate (Corning Cat# 3676) is used. This type of 384 well plate is strongly recommended due to the greater signal/noise ratio. DO NOT USE CLEAR PLATES SINCE THAT WILL GIVE YOU SIGNIFICANT BACKGROUND NOISE.
2) The positive control protein, DH/PH domain of human Dbs (Cat # GE01), contained in the kit provides sufficient reagent for at least 20 control assays in a half area 96-well plate format and 130 control assays for a 384-well black round bottom plate format in this assay. Human Dbs is a promiscuous exchange factor for Cdc42 and RhoA, and a weak exchanger on Rac1 (4, 7).
3) The three best characterized small GTPases RhoA, Rac1 and Cdc42 are contained in this kit. If you need to examine more GTPases, a wide selection of small GTPases (Ras, Ran, RhoC and a growing number of GEFs) are available from Cytoskeleton Inc. and can be purchased separately (see Section VII).
4) Many 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 exchange buffer and purified protein) may result in the inactivation of the reagents. It is strongly recommended to store the exchange buffer without exposing to light for long period of time.

Assay Optimization

The exchange assay kit has been developed to provide a good general substrate for a broad range of research for characterization and examination of guanine nucleotide exchange factors (GEFs). For example, using this kit as outlined in the introduction (Section I) will result in a V_{max} value of above 1.8 x 10^{-3} mol mant-GTP loaded per mol Cdc42 per second, which is consistent with published data. **The exchange assay should be performed at 20°C. A higher temperature may cause a higher intrinsic rate therefore influencing signal to noise ratio significantly.** It should be noted, however, that optimization of the exchange assay may be needed for any given GEFs. Please refer to **Appendix I** for troubleshooting.

There are several parameters that may particularly affect GEF protein activity:
1) **Temperature.** An exchange reaction at 20°C is required. Besides the fact that lower or higher temperature may cause significant change on signal to noise ratio, different GEFs may require a different optimal temperature for their normal in vitro exchange activity.
2) **Protein concentration.** A titration of the GEF of interest should be performed to achieve optimal results.
3) **Exchange buffer conditions.** Although the condition of reaction buffer in this kit has been optimized, it could be necessary to optimize a particular GEF assay by adjusting the salt concentrations (25-500 mM) and the pH (6.0-8.3) using appropriate buffers such as MES, PIPES and Tris.
4) **Control reactions.** It is important to include control reactions in the assay, particularly if your GEF of interest is in an impure state. Control reactions are discussed in Section V.

Instrumentation

The fluorescence reaction is based upon an excitation at 360 nm and an emission at 440 nm. Your fluorimeter should therefore be set at an excitation filter wavelength at 360 nm and emission filter wavelength at 440 nm for readings. The bandwidth of the filter should be no more than 20 nm or you may experience significant background noise and reduced sensitivity of the assay. The fluorimeter should be at 20°C and set on kinetic mode, it is recommended to take a reading once every 30 seconds for at least 60 cycles (30 minutes total). There is no need to elect a blank well, as the reaction without GEFs will serve as a background control.

The majority of the work in the design of this assay has been based on the Tecan GmbH machine called SpectroFluor Plus. This instrument is filter based and is one of the most sensitive machines on the market (pmoles of fluorescein can be detected), the Perkin-Elmer LS spectrometer is also recommended. The parameters of a Protocol file for the Tecan instrument is given below:

Parameters	Character	Contents
Measurement type	Kinetic	60 cycles of one reading per 30 seconds.
Fluorescence wavelengths	Excitation	360 nm +/- 10 nm
	Emission	440 nm +/- 20 nm
Gain	80	On a scale of 0-120, where 120 is the highest
Reads per well (flashes)	5	
Fluorescence reading	From top	
Integration		0-40 µs (microseconds)
Shaking	Medium, orbital	5 seconds
Plate type	Greiner	GRE384fb or GRE96fb (Flat, black) Standard template on Tecan
Reading times	Seconds	Read plate once every 30 seconds
Vmax	AFU/second	8 readings

Uses

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

Section III: Kit Contents

This kit contains sufficient reagents for approximately 20 assays of 100 µl volume (96-well plate) and 130 assays of 15 µl volume (384-well plate) for each GTPase.

KIT COMPONENT	DESCRIPTION
Exchange Buffer (2x) (Part# EB01)	Two bottles, lyophilized. When reconstituted: 40 mM Tris pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$, 1.5 µM mant-GTP.
Cdc42-His protein (Cat# CD01)	One tube, lyophilized. Contains 100 µg of purified His tagged Cdc42 protein.
RhoA-His protein (Cat# RH01)	One tube, lyophilized. Contains 100 µg of purified His tagged RhoA protein
Rac1-His protein (Cat# RC01)	One tube, lyophilized. Contains 100 µg of purified His tagged Rac1 protein.
hDbs-His protein (Cat# GE01)	One tube, lyophilized. Contains 50 µg of purified His tagged human Dbs protein (DH/PH domain).
384 well plate	One 384 well black half area round bottom plate (Corning Cat# 3676)
96 well plate	One 96 well black half area flat bottom plate (Corning Cat# 3686)

Section IV: Things to do Prior to Beginning the Assay

Prior to beginning the assay you will need to reconstitute several components as follows:

Kit Component	Reconstitution	Storage Conditions
Exchange Buffer (2x)	1) Dissolve the powder in each bottle with 5 ml of distilled water. 2) Aliquot into 10 x 0.5 ml sizes (per bottle).	Store at -70°C. Stable for six months under these conditions.
Cdc42-His protein	1) Briefly centrifuge the tube to make sure all the white protein powder is at the bottom of the tube. 2) Check that you can see the white powder pellet. 3) Reconstitute in 20 µl of distilled water to give a 200 µM (5 mg/ml) solution. 4) Aliquot into 5 x 3.5 µl sizes. 5) Snap freeze in liquid nitrogen.	Same as above.
RhoA-His protein	Same operation as Cdc42-His protein.	Same as above.
Rac1-His protein	Same operation as Cdc42-His protein.	Same as above.
hDbs-His protein	1) Briefly centrifuge the tube to make sure all the white protein powder is at the bottom of the tube. 2) Check that you can see the white powder pellet. 3) Reconstitute in 25 µl of distilled water to give a 50 µM (2 mg/ml) solution. 4) Aliquot into 5 x 4.5 µl sizes. Snap freeze in liquid nitrogen.	Same as above.

Section V: Protocol

The following protocols are for either a 96-well plate format or 384-well plate format (Corning Cat# 3686 and 3676 respectively). We highly recommend 384 well plate we provide in this kit. You can adjust the volume by ratio if you are using different volume plates. The exchange reaction is started with all of the reaction components, minus GEFs. GEF is added after 5-10 readings to examine its exchange activity.

Reaction Protocol for 96 well plate

1) Set up and test the plate reader and kinetic parameters, and prepare protein or other samples prior to start.
2) Thaw out an aliquot of 2x exchange buffer by placing in a room temperature water bath for 1 minute. Keep at ROOM TEMPERATURE and protect from light (foil wrap works well for this).
3) Thaw out one 3.5 µl aliquot of respective GTPase stock (200 µM) depending which GTPase you want to test (Please refer to section VII for more GTPase choices), by placing in a room temperature water bath for 1 minute. Dilute to 50 µM by addition of 12 µl of milli-Q water and place on ice.
4) Thaw out one 4.5 µl aliquot of positive control protein human Dbs or your own GEF by placing them in a room temperature water bath for 1 minute. Dilute to 8 µM by addition of 25 µl milli-Q water. Place on ice.

   PLEASE ADJUST THE ALIQUOTED VOLUME BASED ON HOW MANY REACTIONS YOU NEED AND WHICH KIND OF PLATE YOU ARE GOING TO USE. The recommended working concentration of GEF is 0.2 – 2 µM (Consider the percent purity of your GEF protein in this calculation). NOTE: we use 0.8 µM GEF in the standard hDbs assay (see Figs. 1 – 3), you may want to titrate the concentration of GEF in your particular experimental set up.

5) Add the following components together at ROOM TEMPERATURE and mix well by pipetting or gentle vortex:

Exchange reaction mix	One 96 well reaction
2x Exchange Reaction Buffer	50 µl
Respective GTPase (50 µM)	4 µl
Distilled water	36 µl

   PLEASE ADJUST YOUR VOLUME BASED ON THE PLATE YOUR ARE USING. THE RECOMMENDED VOLUMES HERE ARE BASED UPON TOTAL VOLUME OF 100 µl (96-WELL HALF AREA PLATE, CORNING CAT# 3686)

6) The reaction mix is now ready for the fluorescence reading (λ_{ex}=360nm, λ_{em}=440nm). Aliquot to the assigned well and place the plate in the fluorimeter.

   WE STRONGLY RECOMMEND THE OPERATION TEMPERATURE FOR THE FLUORIMETER SHOULD BE 18-22°C. A HIGHER TEMPERATURE MAY CAUSE HIGHER INTRINSIC RATE AND THUS INFLUENCE THE SIGNAL/NOISE RATIO SIGNIFICANTLY.

7) After 5 readings (150 seconds), pipette 10 µl of either your GEF, hDbs protein (8 µM) or distilled water (intrinsic control) in respective wells and immediately pipette up and down twice and start reading.

   IT IS RECOMMENDED TO ADD GEF PROTEINS (OR WATER) USING A MULTICHANNEL PIPET. In this way all the reactions begin simultaneously. IT IS IMPORTANT TO KEEP THIS PROCESS AS SHORT AS YOU CAN TO GET A SMOOTH CURVE. Keep reading under the same conditions.

8) Save the readings after the kinetic protocols are finished. The exchange rate can be calculated by reducing the data to Vmax with the software that accompanies the plate reader. The exchange curve can be achieved by exporting raw data to Microsoft Excel. Examples using positive control hDbs are shown in Figure 2. To calculate the specific activity please read Appendix II.

Figure 2. Dbs exchange activity for Cdc42, RhoA and Rac1 in 96-well half area plate format

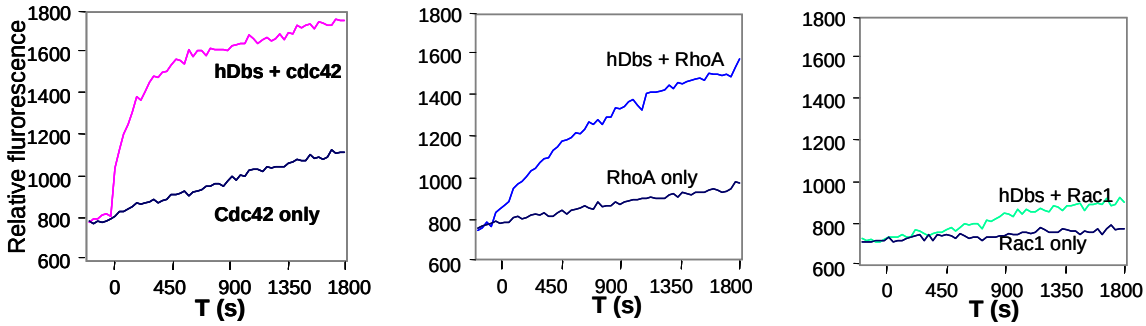
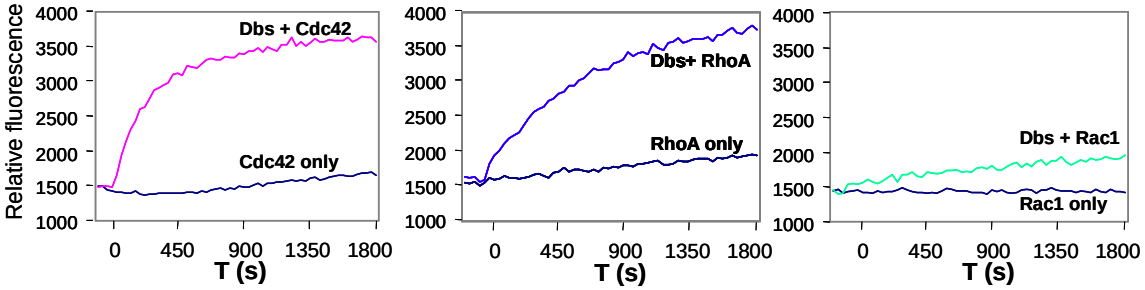


Figure 3. Dbs exchange activity for Cdc42, RhoA and Rac1 in 384-well half area plate format

See protocol on following page



Reaction protocol for 384 well plates (Corning Cat# 3676) - Highly recommended, especially for high throughput screen

1) Set up and test the plate reader and kinetic parameters, and prepare protein samples or compounds prior to start.
2) Thaw out relative amount of respective GTPase and GEF, drug solution (for high throughput screen of GEF inhibitors only) by placing in a room temperature water bath for 1 minute. Place on ice.
3) Thaw out 2x exchange buffer by placing in a room temperature water bath and leave at room temperature, protected from light.
4) Dilute your GEF or positive control hDbs to 2.5 µM with milli-Q water (this is 95 µl of water for your 4.5 µl stock of 50 µM Dbs).
5) Dilute the GTPase to 15 µM with milli-Q water (this is 49 µl of water for your 3.5 µl 200 µM stock of GTPase). If you are using 384-well black round bottom half area plate (Corning Cat# 3676), the required volumes of 2x exchange buffer, GTPase and GEF are (Nx7.5) µl, (Nx2) µl, and (Nx3) µl respectively (N = number of wells). For each reaction, add the following components together and mix well by pipetting or gentle vortex:

Exchange reaction mix	Standard Assay	Assay plus compound
Exchange Reaction Buffer	7.5 µl	7.5 µl
Respective GTPase (15 µM)	2 µl	2 µl
Drug / protein solution		1 µl
Water	2.5 µl	1.5 µl

PLEASE ADJUST YOUR VOLUME BASED ON THE PLATE YOUR ARE USING. The recommended volume here is based upon total volume of 15 µl (384-well black round bottom half area plate, corning Cat# 3676). The recommended working concentration of GEF is 0.2 – 1 µM (Consider the percent purity of your GEF protein in this calculation). NOTE: we use 0.5 µM GEF in the standard Dbs assay (see Figs. 1 – 3), you may want to titrate the concentration of GEF in your experimental set up.

For HTS only: THE FINAL CONCENTRATION OF DRUG SOLUTION WE RECOMMEND IS 10 µM – 0.5 mM. We recommend you titrate your drug solution for optimal results. Generally a concentration at 30 µM – 50 µM is good for initial screening.

6) The reaction mix is now ready for the fluorescence reading (λ_{ex}=360nm, λ_{em}=440nm). Aliquot to the assigned wells and place the 384-well round bottom black plate in the fluorimeter.

WE STRONGLY RECOMMEND THE OPERATION TEMPERATURE FOR THE FLUORIMETER SHOULD BE AROUND 20°C. A HIGHER TEMPERATURE MAY CAUSE HIGHER INTRINSIC RATE AND THUS INFLUENCE THE SIGNAL/NOISE RATIO SIGNIFICANTLY.

7) After 5 readings (150 seconds), add 3 µl of the 2.5 µM GEF (or hDbs) protein or dH_2O to respective wells and immediately pipette up and down twice and resume reading.

IT IS HIGHLY RECOMMENDED TO ADD GEF (OR WATER) USING A MULTICHANNEL PIPET. In this way all the reactions begins simultaneously. IT IS IMPORTANT TO KEEP THIS PROCESS AS SHORT AS YOU CAN TO GET A SMOOTH CURVE. Keep reading under the same conditions.

8) Save the readings after the kinetic protocols are finished. The exchange rate can be calculated by reducing the data to Vmax with the software that accompanies the plate reader. The exchange curve can be achieved by exporting raw data to Microsoft Excel. Examples using positive control Dbs are shown in Figure 3 (page 9). To calculate the specific activity please read Appendix II.

Section VI: References

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Citations for BK100

1) Wang, S. et al. PLC-g1 and Rac1 coregulate EGF-induced cytoskeleton remodeling & cell migration. *Mol. Endocrinology* **23**: 901-913 (2009).
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Section VII: Related Products

ITEM CAT#	GTPases	Ras subfamily	QUANTITY
CD01	Cdc42 protein (His-tagged)	Rho	1 x 100 µg
RH01	RhoA protein (His-tagged)	Rho	1 x 100 µg
RC01	Rac1 protein (His-tagged)	Rho	1 x 100 µg
RN01	Ran protein (His-tagged)	Ran	1 x 10 µg
RS01	Ras protein (His-tagged)	Ras	8 x 50 µg
RC03	RhoC protein (His-tagged)	Rho	1 x 10 µg
GE01-A GE01-C	Human Dbs protein (His-tagged)	Rho GEF family	1 x 50 µg 3 x 50 µg
BK008	Ras activation assay biochem kit	Ras	25 assays
BK034	Cdc42 activation assay biochem kit	Rho	25 assays
BK035	Rac1 activation assay biochem kit	Rho	25 assays
BK036	RhoA activation assay biochem kit	Rho	25 assays

APPENDIX I: Troubleshooting

Observation	Possible cause	Remedy
1. No increase in fluorescence after adding positive control Dbs.	1. Sensitivity of fluorescence spectrophotometer set too low. 2. Incorrect labeling of tubes. 3. Inactive proteins.	1. Increase sensitivity by: increasing emission gain or increase intensity of excitation. 2. Repeat the experiment. 3. Follow correct storage procedure.
2a. During the assay, increase in fluorescence is too slow for tested GEF protein.	1. GEF concentration is too low or GEF is a weak exchanger for this GTPase. 2. Excitation light is too intense. 3. Purity of GEF protein is too low. 4. GTPase concentration is too low.	1. Increase GEF concentration or titrate the GEF concentration. 2. Reduce light intensity. 3. Increase GEF purity. 4. Increase GTPase concentration.
2b. During the assay, increase in fluorescence is too slow for positive control Dbs.	1 Excitation light is too intense. 2. GTPase concentration is too low.	1. Reduce light intensity. 2. Increase GTPase concentration.
3. During the assay, increase in fluorescence is too quick.	1. GEF concentration is too high. 2. GTPase concentration is too high. 3. GEF is a strong exchanger for specific GTPase.	1. Decrease GEF concentration. 2. Decrease GTPase concentration.
4. During the assay increase in fluorescence is not reproducible.	1. Inconsistent preparation of reaction mixture. 2. GEF protein is not stable due to poor purity.	1. More consistent technique, increase 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. During GEF assay increase in fluorescence is low.	1. Concentration of GEF too low 2. The GEF is a weak exchanger for a specific GTPase.	1. Increase GEF concentration. 2. Perform the experiment together with positive control GEF.
6. Buffer components of the reaction interfere with the activity of the test protein or compound.	1. Tris-HCl 2. pH 3. $MgCl_2$ 4. NaCl	1. Tris-HCl: Make new GEF Buffer with a different “GOOD” Buffer. 2. pH: Generally GEF can function at pH6.0-8.5. 3. $MgCl_2$: Lower the concentration to 5 mM. 4. NaCl: Adjust the concentration to 20 mM.
7. The intrinsic exchange rate is too high.	1. The reaction temperature is too high.	1. Cool down the instrument to 20°C.
8. Test GEF protein has no exchange activity.	1. Test GEF does not exchange the specific GTPase. 2. The optimized condition is not good for testing this GEF protein.	1. Try different GTPases. 2. Titrate pH etc. for optimal results.

APPENDIX II: Specific Activity Calculation

Two steps to calculate specific exchange rate:

1. [V_{max} (AFU/sec)] / [Basal mant-GTP AFU* x N**] = A (µM/sec)

2. Exchange rate = A / 0.75*** (µmol mant-GTP/µmol GTPase/sec)

* Basal mant-GTP level is the start point level of fluorescence units of 0.75 µM mant-GTP without GEF under our condition.

** N values (Ref. 8 and our unpublished observations):
For Cdc42, N=2
For RhoA, N=1.8
For Rac1, N=1.3

*** This number is based upon fully mant-GTP loaded GTPases.

Example:

This example calculation uses data derived from Fig 1 in this manual (Page 3):

1. [V_{max} (AFU/sec)] / [Basal mant-GTP AFU* x N**] = 2.0 (AFU/sec) / 750 AFU x 2

$= 1.33 \times 10^{-3}$ (µM/sec)

2. Therefore, Exchange rate $= 1.33 \times 10^{-3}$ (µM/sec) / 0.75 µM

Volumes cancel out,

$= 1.8 \times 10^{-3}$ (µmol mant-GTP/µmol Cdc42/sec)