TECHNICAL MANUAL

GTPase-Glo™ Assay

Instructions for Use of Products
V7681 and V7682

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TM452

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GTPase-Glo™ Assay

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

GTPases are an important part of cell signaling, acting as molecular switches that cycle between an activated GTP-bound state and an inactive GDP-bound state. GTPases have a high affinity for guanine nucleotides GDP or GTP and are slow-acting GTP hydrolases. To mediate the process of shuttling between the active and inactive forms, GTPases need the help of two families of proteins: GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). GEFs replace the GDP bound to inactive GTPase with GTP, making the enzyme active and transducing a wide variety of cellular signals. GTPases then require the help of GAPs to hydrolyze GTP and switch off the signalling cascade. Thus, monitoring the activities of GTPases, GAPs and GEFs is difficult.

The GTPase-Glo™ Assay[a,b,c] assesses the activities of GTPases, GAPs and GEFs, which are components of the GTPase cycle (Figure 1, Panel A), by detecting the amount of GTP remaining after GTP hydrolysis in a GTPase reaction. The remaining GTP is converted to ATP using the GTPase-Glo™ Reagent, and the ATP is then detected using a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) and luciferin substrate to produce bioluminescence (Figure 1, Panel B). The kit contains optimized reaction buffers, GTPase/GAP Buffer and GEF Buffer, for performing GTPase and GAP reactions and GEF reactions, respectively. These two buffers primarily differ in their Mg^{2+} content, which is critical for nucleotide loading and unloading of the GTPase, thereby affecting the GTPase cycle. With the GTPase-Glo™ Assay, you can measure intrinsic GTPase activity, GAP-stimulated GTPase activity, GAP activity and GEF activity. GTPase, GAP and GEF activity is inversely correlated to the amount of light produced. A highly active GTPase hydrolyzes more GTP, reducing the amount of ATP produced from GTP and reducing light output. A less active GTPase hydrolyzes less GTP, leaving a larger amount of GTP to be converted to ATP and producing more light.

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1. Description (continued)

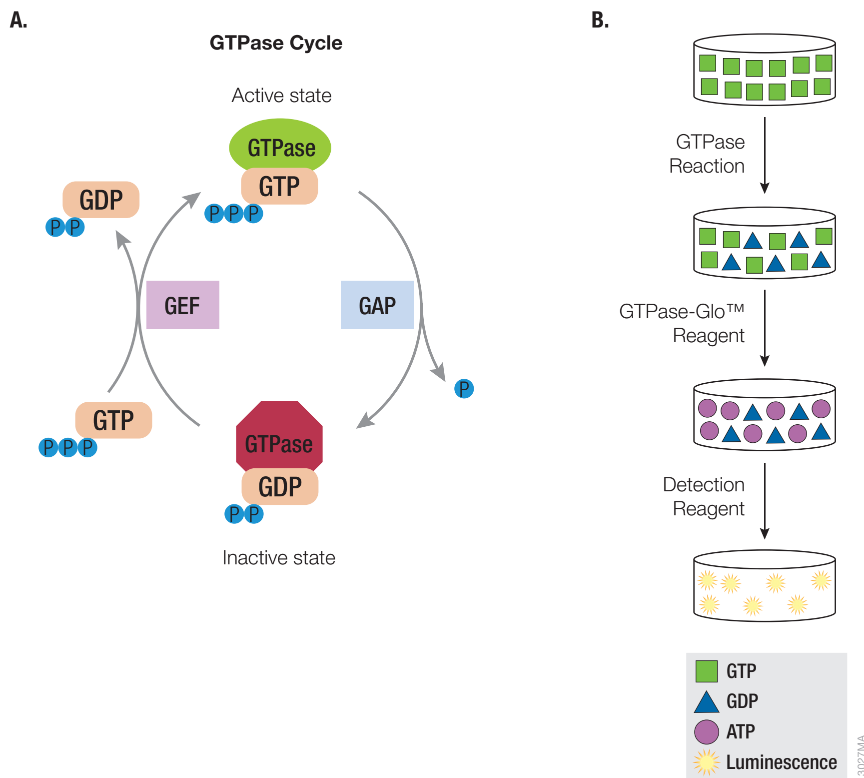

Figure 1. The GTPase cycle and the principle of the GTPase-Glo™ Assay. Panel A. The GTPase cycle. **Panel B.** The principle of the GTPase-Glo™ Assay.

1. Description (continued)

The GTPase-Glo™ Assay contains two optimized reaction buffers:

- **GTPase/GAP Buffer:** To analyze intrinsic GTPase activity, GAP-stimulated GTPase activity or GAP activity, use GTPase/GAP Buffer (Figure 2, upper left). To perform a GAP activity assay, be sure to include the cognate GTPase.
- **GEF Buffer:** The GEF Buffer is used to analyze GEF activity (Figure 2, upper right). To perform a GEF activity assay, be sure to include the cognate GTPase and GAP.

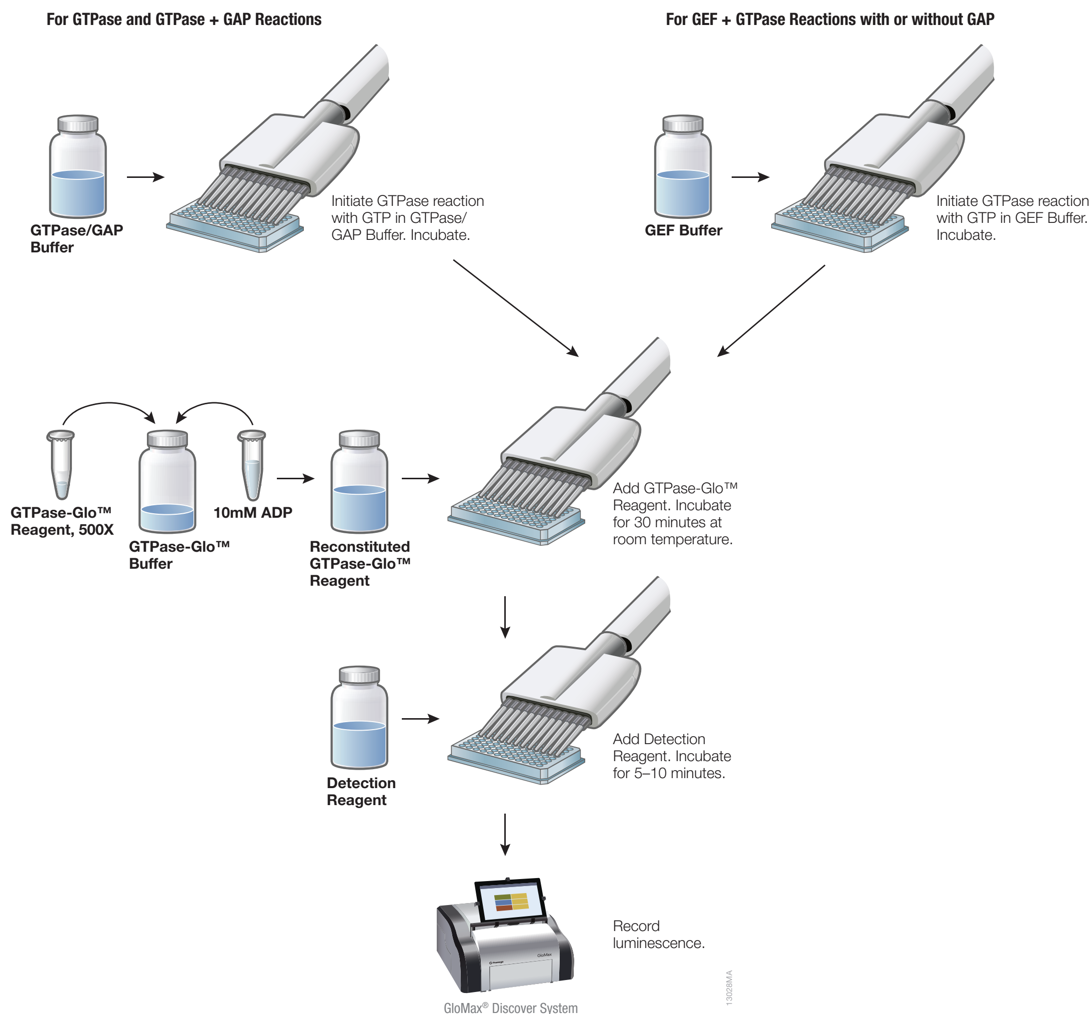

Figure 2. Overview of the GTPase-Glo™ Assay. This diagram shows an overview of the protocols for performing intrinsic GTPase activity, GAP-stimulated GTPase activity and GAP activity assays using GTPase/GAP Buffer and assessing GEF activity using GEF Buffer.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
GTPase-Glo™ Assay	**1,000 reactions**	**V7681**

The system is sufficient for 1,000 assays in 384-well plates using 5µl, 5µl and 10µl of a GTPase reaction, GTPase-Glo™ Reagent and Detection Reagent, respectively, per sample. The system also can be used in 96-well plates using 25µl:25µl:50µl for a total of 200 assays. Includes:

- 5ml GTPase/GAP Buffer
- 5ml GEF Buffer
- 5ml GTPase-Glo™ Buffer
- 15µl GTPase-Glo™ Reagent, 500X
- 0.5ml ADP, 10mM
- 10ml Detection Reagent
- 50µl rGTP, 10mM
- 0.1ml DTT, 100mM

PRODUCT	SIZE	CAT.#
GTPase-Glo™ Assay	**10,000 reactions**	**V7682**

The system is sufficient for 10,000 assays in 384-well plates using 5µl, 5µl and 10µl of a GTPase reaction, GTPase-Glo™ Reagent and Detection Reagent, respectively, per sample. The system also can be used in 96-well plates using 25µl:25µl:50µl for a total of 2,000 assays. Includes:

- 50ml GTPase/GAP Buffer
- 50ml GEF Buffer
- 50ml GTPase-Glo™ Buffer
- 120µl GTPase-Glo™ Reagent, 500X
- 0.5ml ADP, 10mM
- 100ml Detection Reagent
- 500µl rGTP, 10mM
- 1.25ml DTT, 100mM

Storage Conditions: Store the GTPase-Glo™ Assay at –20°C, where it is stable for 6 months. Before use, thaw all components completely at room temperature and mix thoroughly. At first use, dispense the Detection Reagent into single-use aliquots and store at –20°C to minimize freeze-thaw cycles of the reagent.

3. Before You Begin

Prior to assaying GTPase, GAP or GEF activity, we recommend optimizing the GTP concentration to use for each GTPase reaction as shown in Section 7.A. Perform an enzyme titration to determine the optimal amount of enzyme to use per reaction using the protocol in Section 7.B. We also recommend that you determine the optimal reaction time as described in Section 7.C.

Notes:

1. The final GTP concentration in the GTPase reaction should be no more than 5µM. **Do not** exceed 5µM GTP for this assay.
2. The presence of DTT is required for optimal GTPase activity. We recommend adding DTT at a final concentration of 1mM in the GTPase, GAP or GEF reaction (in the appropriate buffer) prior to use.

4. Protocols

Materials to be Supplied by User

- solid white multiwell plate [e.g., Corning Costar Cat.# 3572 (384-well plate), Corning Costar Cat.# 3674 (low-volume 384-well plate), Corning Costar Cat.# 3912 (96-well plate) or Corning Costar Cat.# 3693 (half-area 96-well plate)]
- proteins and enzymes to be analyzed
- test compounds if needed
- pipettes, multichannel pipettes or automated pipetting station
- luminometer with sufficient sensitivity and broad linear range [e.g., GloMax® Discover System (Cat.# GM3000)]

Notes:

1. We recommend adding DTT to a final concentration of 1mM in the GTPase reaction for optimal activity.
2. Maintain a volume ratio of 1:1:2 (GTPase reaction:GTPase-Glo™ Reagent:Detection Reagent) when performing the GTPase-Glo™ Assay in a multiwell plate. Adjust the volumes as needed for your assay, but maintain the same 1:1:2 volume ratio. See Table 1.

Table1. General Assay Format for 96-Well and 384-Well Plates.

	96-Well Plate	384-Well Plate	Low-Volume 384-Well Plate
GTPase Reaction	25µl	10µl	5µl
Reconstituted GTPase-Glo™ Reagent	25µl	10µl	5µl
Detection Reagent	50µl	20µl	10µl

4.A. Intrinsic GTPase Activity Protocol

Intrinsic GTPase activity is extremely slow. When performing intrinsic GTPase activity assays, the amount of GTPase required may be high. Using a cognate GAP may lower the amount of GTPase required.

This protocol is written for 384-well plates. Adjust the volume as needed for 96-well or low-volume 384-well plates to maintain the 1:1:2 volume ratio for the GTPase reaction:GTPase-Glo™ Reagent:Detection Reagent. See Table 1.

1. Prepare a 2X GTP solution containing 10µM GTP and 1mM DTT in GTPase/GAP Buffer.
2. Serially dilute the GTPase to be studied in GTPase/GAP Buffer, and dispense 5µl into each well of a 384-well plate. Alternatively, use a fixed amount of GTPase diluted in GTPase/GAP Buffer.
3. Initiate the GTPase reaction by adding 5µl of the 2X GTP solution prepared in Step 1 to each well. The total GTPase reaction volume is 10µl.
4. Incubate the reaction at room temperature (22–25°C) for the optimal time, generally 60–120 minutes (see Section 7.C).

   Note: During the incubation, thaw the undiluted GTP-Glo™ Reagent until ready to use in Step 5. Calculate the volume of GTPase-Glo™ Reagent, 500X, required for your experiment.
5. Gently mix the thawed GTPase-Glo™ Reagent, 500X, by inversion; do not vortex. Prepare the required volume of reconstituted GTPase-Glo™ Reagent by increasing or decreasing the component volumes provided below.

Reconstituted GTPase-Glo™ Reagent Component	Volume
GTPase-Glo™ Reagent, 500X	2µl
ADP, 10mM	0.5µl
GTPase-Glo™ Buffer	998µl
Total volume	1ml

   Equilibrate the reconstituted GTPase-Glo™ Reagent to room temperature before use.

   Note: Prepare the reconstituted GTPase-Glo™ Reagent immediately before use, and prepare only enough for your experiment. Do not freeze the reconstituted GTPase-Glo™ Reagent.
6. Add 10µl of reconstituted GTPase-Glo™ Reagent to the completed GTPase reaction, mix briefly and incubate with shaking for 30 minutes at room temperature (22–25°C).
7. Add 20µl of Detection Reagent, and incubate the plate for 5–10 minutes at room temperature (22–25°C).
8. Measure luminescence.

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4.B. GAP-Stimulated GTPase Activity Protocol

GTPase-activating proteins (GAP) accelerate the GTP hydrolysis mediated by GTPases by several orders of magnitude. This protocol is written for 384-well plates. Adjust the volume as needed for 96-well or low-volume 384-well plates to maintain the 1:1:2 volume ratio for the GTPase reaction:GTPase-Glo™ Reagent:Detection Reagent. See Table 1.

1. Prepare a 2X GTP-GAP solution containing 10µM GTP, 1mM DTT and the cognate GAP in GTPase/GAP Buffer at twice the desired concentration.
2. Serially dilute the GTPase to be studied in GTPase/GAP Buffer, and dispense 5µl into each well of a 384-well plate. Alternatively, use a fixed amount of GTPase diluted in GTPase/GAP Buffer.
3. Initiate the GTPase reaction by adding 5µl of the 2X GTP-GAP solution prepared in Step 1 to each well. The total GTPase reaction volume is 10µl.
4. Incubate the reaction at room temperature (22–25°C) for the optimal time, generally 60–120 minutes (see Section 7.C).

   Note: During the incubation, thaw the undiluted GTP-Glo™ Reagent until ready to use in Step 5. Calculate the volume of GTPase-Glo™ Reagent, 500X, required for your experiment.
5. Gently mix the thawed GTPase-Glo™ Reagent, 500X, by inversion; do not vortex. Prepare the required volume of reconstituted GTPase-Glo™ Reagent by increasing or decreasing the component volumes provided below.

Reconstituted GTPase-Glo™ Reagent Component	Volume
GTPase-Glo™ Reagent, 500X	2µl
ADP, 10mM	0.5µl
GTPase-Glo™ Buffer	998µl
Total volume	1ml

   Equilibrate the reconstituted GTPase-Glo™ Reagent to room temperature before use.

   Note: Prepare the reconstituted GTPase-Glo™ Reagent immediately before use, and prepare only enough for your experiment. Do not freeze the reconstituted GTPase-Glo™ Reagent.
6. Add 10µl of reconstituted GTPase-Glo™ Reagent to the completed GTPase reaction, mix briefly and incubate with shaking for 30 minutes at room temperature (22–25°C).
7. Add 20µl of Detection Reagent, and incubate the plate for 5–10 minutes at room temperature (22–25°C).
8. Measure luminescence.

4.C. GAP Activity Protocol

This protocol is written for 384-well plates. Adjust the volume as needed for 96-well or low-volume 384-well plates to maintain the 1:1:2 volume ratio for the GTPase reaction:GTPase-Glo™ Reagent:Detection Reagent. See Table 1.

1. Prepare a 2X GTPase solution containing 1mM DTT and GTPase at twice the desired final concentration in GTPase/GAP Buffer.
2. Serially dilute the GAP to be studied in GTPase/GAP Buffer containing 10µM GTP, and dispense 5µl into each well of a 384-well plate. Alternatively, use a fixed amount of GTPase diluted in GTPase/GAP Buffer containing 10µM GTP.
3. Initiate the GTPase reaction by adding 5µl of the 2X GTPase solution prepared in Step 1 to each well. The total GTPase reaction volume is 10µl.
4. Incubate the reaction at room temperature (22–25°C) for the optimal time, generally 60–120 minutes (see Section 7.C).

   Note: During the incubation, thaw the undiluted GTP-Glo™ Reagent until ready to use in Step 5. Calculate the volume of GTPase-Glo™ Reagent, 500X, required for your experiment.
5. Gently mix the thawed GTPase-Glo™ Reagent, 500X, by inversion; do not vortex. Prepare the required volume of reconstituted GTPase-Glo™ Reagent by increasing or decreasing the component volumes provided below.

Reconstituted GTPase-Glo™ Reagent Component	Volume
GTPase-Glo™ Reagent, 500X	2µl
ADP, 10mM	0.5µl
GTPase-Glo™ Buffer	998µl
Total volume	1ml

   Equilibrate the reconstituted GTPase-Glo™ Reagent to room temperature before use.

   Note: Prepare the reconstituted GTPase-Glo™ Reagent immediately before use, and prepare only enough for your experiment. Do not freeze the reconstituted GTPase-Glo™ Reagent.
6. Add 10µl of reconstituted GTPase-Glo™ Reagent to the completed GTPase reaction, mix briefly and incubate with shaking for 30 minutes at room temperature (22–25°C).
7. Add 20µl of Detection Reagent, and incubate the plate for 5–10 minutes at room temperature (22–25°C).
8. Measure luminescence.

4.D. GEF Activity Protocol

GEFs help in loading of GDP-bound inactive GTPases with GTP, thereby rendering them active. Note that the reaction buffer for GEF activities is different from the one used for analyzing intrinsic GTPase and GAP activities. The GEF buffer contains higher amounts of free Mg^{2+}, and as a consequence, the nucleotide loading is catalyzed solely by GEFs.

This protocol is written for 384-well plates. Adjust the volume as needed for 96-well or low-volume 384-well plates to maintain the 1:1:2 volume ratio for the GTPase reaction:GTPase-Glo™ Reagent:Detection Reagent. See Table 1.

1. Prepare 2X GTPase-GAP solution containing 1mM DTT, GTPase and GAP at twice the desired final concentration in GEF Buffer.

   Note: The GEF reaction requires DTT for optimal activity.

2. Serially dilute the GEF to be studied in GEF Buffer containing 10µM GTP, and dispense 5µl into each well of a 384-well plate. Alternatively, use a fixed amount of GEF diluted in GEF Buffer containing 10µM GTP.

3. Initiate the GTPase reaction by adding 5µl of the 2X GTPase-GAP solution prepared in Step 1 to each well. The total GTPase reaction volume is 10µl.

4. Incubate the reaction at room temperature (22–25°C) for the optimal time, generally 60–120 minutes (see Section 7.C).

   Note: During the incubation, thaw the undiluted GTP-Glo™ Reagent until ready to use in Step 5. Calculate the volume of GTPase-Glo™ Reagent, 500X, required for your experiment.

5. Gently mix the thawed GTPase-Glo™ Reagent, 500X, by inversion; do not vortex. Prepare the required volume of reconstituted GTPase-Glo™ Reagent by increasing or decreasing the component volumes provided below.

Reconstituted GTPase-Glo™ Reagent Component	Volume
GTPase-Glo™ Reagent, 500X	2µl
ADP, 10mM	0.5µl
GTPase-Glo™ Buffer	998µl
Total volume	1ml

   Equilibrate the reconstituted GTPase-Glo™ Reagent to room temperature before use.

   Note: Prepare the reconstituted GTPase-Glo™ Reagent immediately before use, and prepare only enough for your experiment. Do not freeze the reconstituted GTPase-Glo™ Reagent.

6. Add 10µl of reconstituted GTPase-Glo™ Reagent to the completed GTPase reaction, mix briefly and incubate with shaking for 30 minutes at room temperature (22–25°C).

7. Add 20µl of Detection Reagent, and incubate the plate for 5–10 minutes at room temperature (22–25°C).

8. Measure luminescence.

5. Representative Data

5.A. Intrinsic GTPase Activity

To study the extremely slow intrinsic GTPase reaction, we used Ras as a representative GTPase. Figure 3 shows representative data.

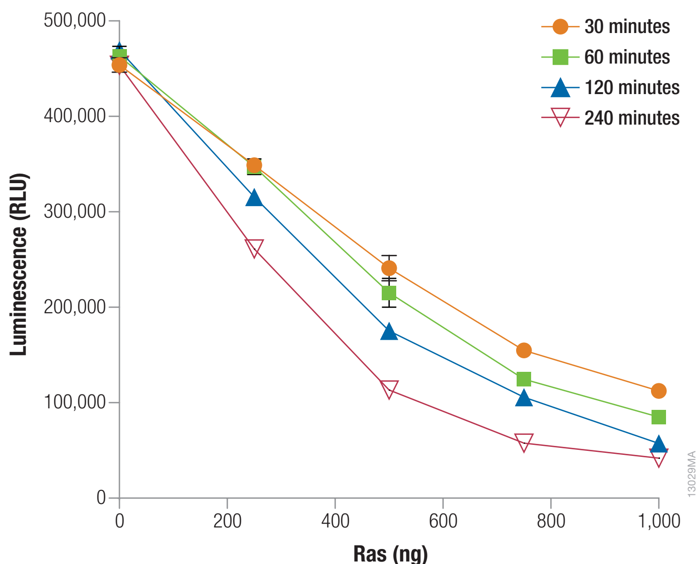

Figure 3. Intrinsic GTPase activity of Ras GTPase. Wildtype Ras was serially diluted in GTPase/GAP Buffer, and 2.5µl was dispensed into wells of a low-volume 384-well plate. A 2X GTP solution containing 10µM GTP and 1mM DTT in GTPase/GAP Buffer was prepared, and 2.5µl was added to each well to initiate the GTPase reaction. The total reaction volume was 5µl. Reactions were incubated for 30 minutes, 60 minutes, 120 minutes and 240 minutes at 23°C. To the completed GTPase reactions, we added 5µl of reconstituted GTPase-Glo™ Reagent to each well and incubated the plate for 30 minutes. Detection Reagent (10µl) was added, reactions were incubated for 5–10 minutes and luminescence was measured using the GloMax®-Multi+ Detection System.

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5.A. Intrinsic GTPase Activity (continued)

We also analyzed the intrinsic GTPase activities of various other small and heterotrimeric GTPases using the method described in Section 4.A (see Figure 4).

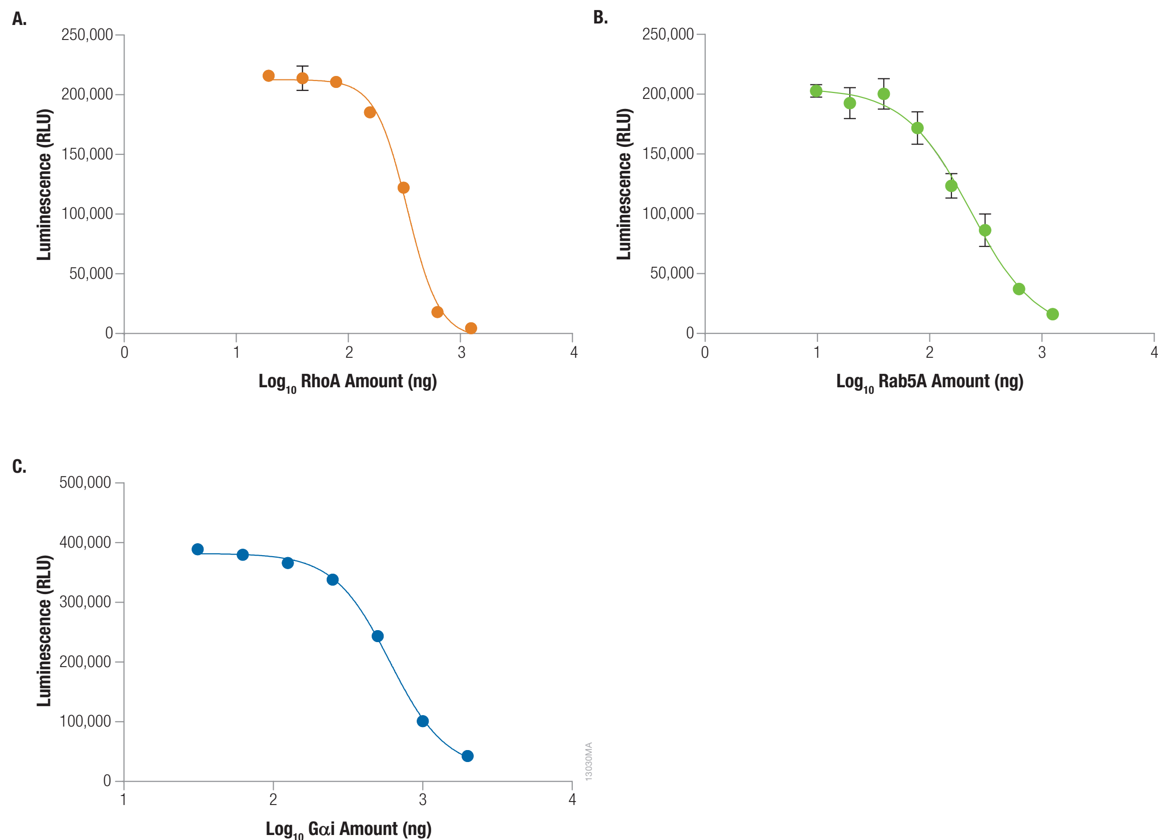

Figure 4. Intrinsic GTPase activity as a function of enzyme concentration. RhoA (**Panel A**), Rab5A (**Panel B**) and Gαi (**Panel C**) were serially diluted in GTPase/GAP Buffer, and 2.5µl was dispensed into each well of a low-volume 384-well plate. A 2X GTP solution containing 10µM GTP and 1mM DTT in GTPase/GAP Buffer was prepared, and 2.5µl was added to each well to initiate the GTPase reaction. The reaction was incubated for 60 minutes at 23°C. To the completed GTPase reaction, we added 5µl of reconstituted GTPase-Glo™ Reagent and incubated reactions for 30 minutes at room temperature. Detection Reagent (10µl) was added to each well, reactions were incubated for 5–10 minutes and luminescence was recorded using the GloMax®-Multi+ Detection System.

5.B. GAP-Stimulated GTPase Activity

To test GAP-stimulated GTPase activities, we assembled two sets of reactions, one with the GTPase Ras and its cognate GAP, NF1-333, and another with the GTPase RAN and its cognate GAP, RanGAP. Constitutively active GTPase mutants Ras^{G12V} and Ran^{E70A}, which cannot hydrolyze GTP, remain bound to GTP and are insensitive to GAP activity, also were included.

In Figure 5, luminescence for the no-enzyme control (buffer only) represents the total amount of input GTP. A decrease in light output, which represents the intrinsic GTPase activity, was observed with Ras and Ran. NF1-333 and RanGAP do not possess any GTPase activity, but in the presence of their cognate GTPase, there was significant GTP hydrolysis and almost all of the input GTP was hydrolyzed. Note that both the constitutively activated form of the GTPases Ras^{G12V} and Ran^{E70A} do not show GAP-stimulated GTPase activities.

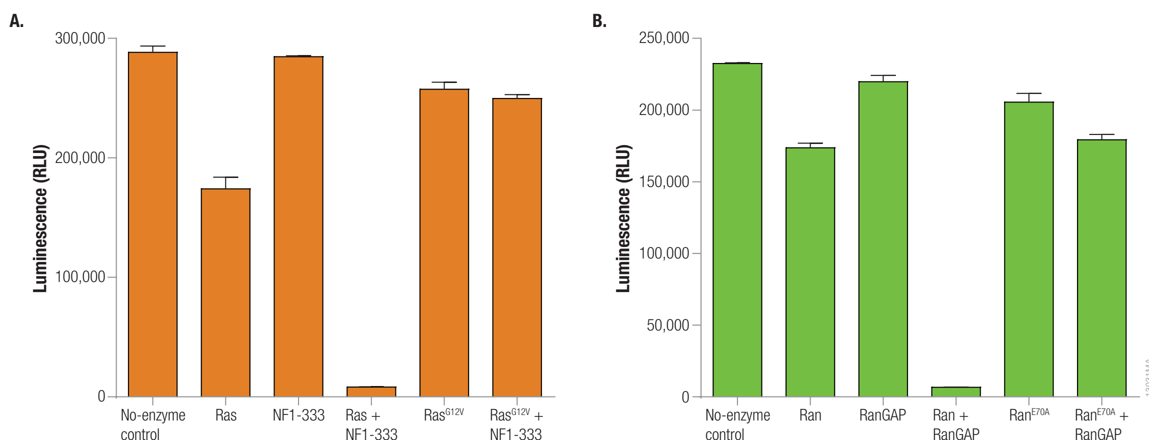

Figure 5. GTP hydrolysis increases with an increasing amount of GTPase. Panel A. Reactions were assembled with 2µM wildtype or mutant Ras and 1µM NF1-333 in GTPase/GAP Buffer. **Panel B.** Reactions were assembled with 2µM wildtype or mutant Ran and 1µM Ran-GAP in GTPase/GAP Buffer. Reactions were initiated by adding 5µl of 10µM GTP in GTPase/GAP Buffer containing 1mM DTT. The final reaction volume was 10µl. Reactions were incubated for 90 minutes at room temperature. To the completed GTPase reactions, we added 10µl of GTPase-Glo™ Reagent and incubated reactions for 30 minutes at room temperature. Twenty microliters of Detection Reagent was added, plates were incubated for 5–10 minutes at room temperature and luminescence was recorded using the GloMax®-Multi+ Detection System.

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5.B. GAP-Stimulated GTPase Activity (continued)

In similar experiments with increasing amounts of GTPase, GTP hydrolysis increased (Figure 6). At equimolar concentrations of GTPase and GAP (in this case 1µM), the GTPase reaction was driven to completion and the GTP was completely exhausted.

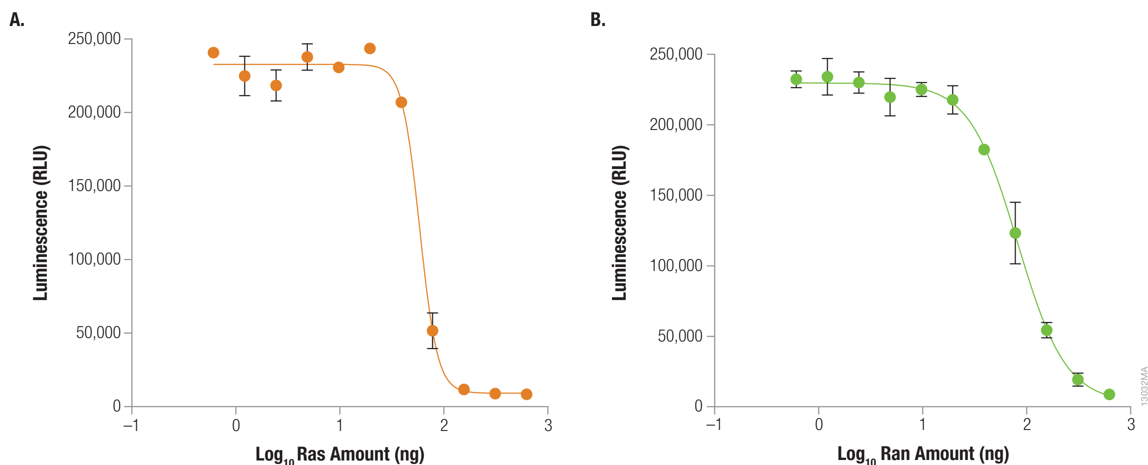

Figure 6. GTP hydrolysis increases with an increasing amount of GTPase. Panel A. Titration of Ras in presence of a fixed concentration of NF1-333. **Panel B.** Titration of Ran in the presence of a fixed concentration of RanGAP. GTPases Ras and Ran were titrated in presence of a fixed concentration of their cognate GAPs. Two 2X GAP-GTP solutions containing 1µM NF1-333 (for Ras) or 1µM RanGAP (for Ran), 10µM GTP and 1mM DTT in GTPase/GAP Buffer were prepared. Five microliters of the appropriate 2X GAP-GTP solution was dispensed to each well. The total reaction volume was 10µl. The GTPase reaction was incubated for 2 hours at room temperature. To each completed GTPase reaction, 10µl of reconstituted GTPase-Glo™ Reagent was added, and reactions were incubated for 30 minutes at room temperature. Twenty microliters of Detection Reagent was added, reactions were incubated for 10 minutes at room temperature and luminescence was recorded.

5.C. GAP Activity

When the amount of GAP increased in the GTPase reaction, the GTP hydrolysis also increased, resulting in lower light output (Figure 7).

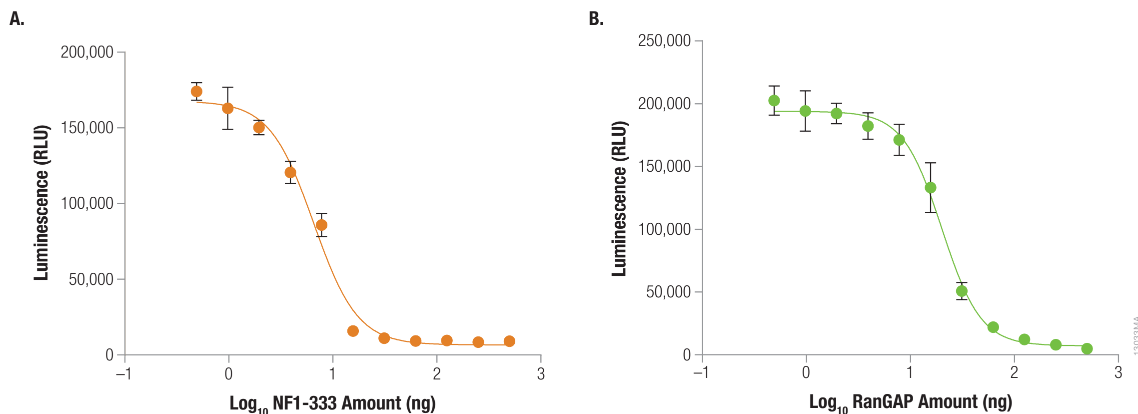

Figure 7. Assessing GAP activities of NF1-333 and RanGAP. NF1-333 (**Panel A**) or RanGAP (**Panel B**) was titrated in presence of a fixed concentration of its cognate GTPase (Ras or Ran, respectively). NF1-333 or RanGAP was serially diluted in GTPase/GAP Buffer containing 10µM GTP, and 5µl was dispensed in each well of a 384-well plate. Five microliters of 2X GTPase solution containing 2µM Ras or 2µM Ran in GTPase/GAP Buffer was added to the dispensed GAPs. GTPase reactions were incubated for 2 hours at room temperature, and then 10µl of reconstituted GTPase-Glo™ Reagent was added. After a 30-minute incubation at room temperature, 20µl of Detection Reagent was added, reactions were incubated for 5 minutes and luminescence was recorded.

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5.D. GEF Activity

We chose RCC1, the GEF for Ran GTPase, to generate representative data for the GEF activity assay (Figure 8). We assembled reactions with wildtype Ran in the presence and absence of RanGAP, both with and without RCC1. Similar reactions were assembled with Ran^{E70A}, a constitutively active Ran mutant. A no-enzyme control (buffer only), which did not contain GTPase, GAP or GEF, was also included. Luminescence for the no-enzyme control represented the total amount of input GTP. Ran exhibited a low level of GTP hydrolysis, which represents the intrinsic GTPase activity when incubated in GEF Buffer. RanGAP and RCC1 did not possess any GTPase activity, but in reactions with Ran, GTP hydrolysis increased with increasing Ran concentration. In the presence of all components required for effective GTPase cycling (Ran, RanGAP and RCC1), all of the input GTP was hydrolyzed, resulting in very low light output. The constitutively active Ran^{E70A} was not able to hydrolyze GTP even in the presence of RCC1 and RanGAP (Figure 8). From these data, we determined that maximal GEF activity was obtained from reactions with GEF and the cognate GAP and GTPase.

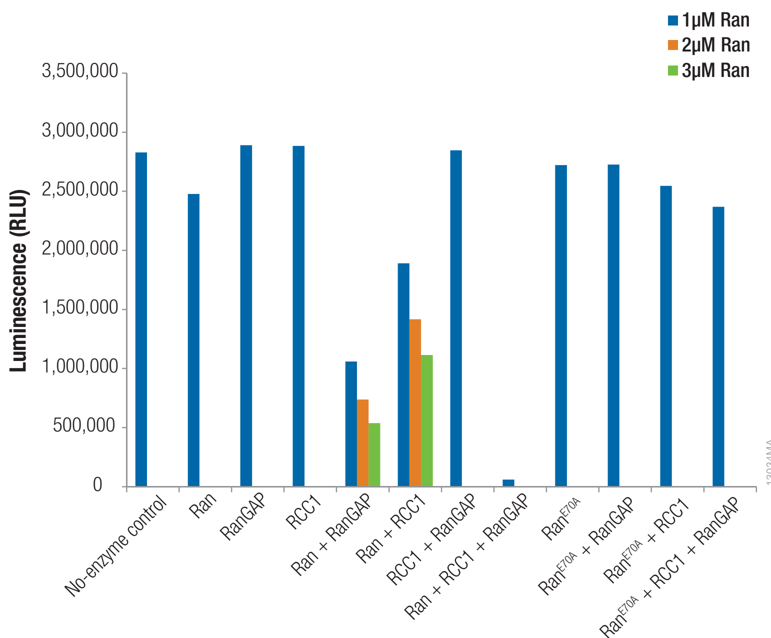

Figure 8. GEF activity of RCC1. GTPase reactions contained 2µM Ran or Ran^{E70A}, 1µM RanGAP and 1µM RCC1 in GEF Buffer. Ran + RanGAP and Ran + RCC1 had higher concentrations of Ran. Reactions were initiated by adding 5µl of 10µM GTP in GEF Buffer containing 1mM DTT. The final reaction volume was 10µl. Reactions were incubated for 90 minutes at room temperature. To the completed GTPase reactions, 10µl of reconstituted GTPase-Glo™ Reagent was added to each well, and plates were incubated for 30 minutes at room temperature. Twenty microliters of Detection Reagent was dispensed into each reaction, plates were incubated for 5–10 minutes at room temperature and luminescence was recorded.

5.D. GEF Activity (continued)

As shown in Figure 9, increasing RCC1 concentration resulted in higher GTP consumption and lower light output.

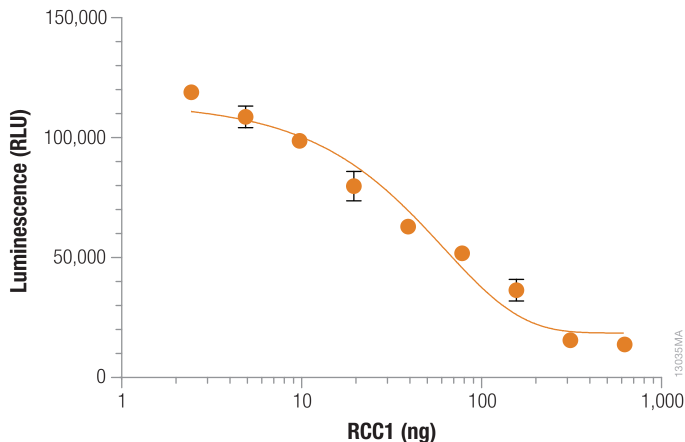

Figure 9. Measuring RCC1 GEF activity by titrating RCC1 in the presence of a fixed concentration of Ran, RanGAP and GTP. RCC1 was titrated in presence of a fixed concentration of its cognate Ran and RanGAP. RCC1 was serially diluted in GEF Buffer containing 10µM GTP in a 384-well plate. An equal volume of 2X GTPase-GAP solution containing 1µM Ran, 0.5µM RanGAP and 1mM DTT in GEF Buffer was added to the RCC1 dilution for a final reaction volume of 10µl. Reactions were incubated for 2 hours at room temperature, and then 10µl of reconstituted GTPase-Glo™ Reagent was added to each well. After a 30-minute incubation at room temperature, 20µl of Detection Reagent was added, reactions were incubated for 10 minutes at room temperature and luminescence was recorded.

6. Troubleshooting

Symptoms	Causes and Comments
No change in luminescence with increasing or decreasing concentration of GTPase, GAP or GEF	GTPase or GAP activity was low in the GTPase/GAP Buffer. Assemble reactions using GEF Buffer instead. Some combinations of GTPase and GAP, such as members of the Arf/Sar family of GTPases, have higher activity in the GEF Buffer.
	The amount of GTPase or GAP was too low. Determine the optimal amount of GTPase, GAP and GEF per reaction, as described in Section 7.B. Test both GTPase/GAP Buffer and GEF Buffer with higher protein concentrations.
	A required cofactor was missing from the GTPase or GAP reaction. Add 10mM calcium chloride ($CaCl_2$) to the GTPase/GAP or GEF Buffer if needed. Some GAPs, such as GAPs for the Arf/Sar family of GTPases, require Ca^{2+} for full activity. The GTPase/GAP and GEF Buffers lack calcium.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
No change in luminescence with increasing or decreasing concentration of GTPase, GAP or GEF (continued)	GTP concentration was not optimized. Optimize GTP concentration as described in Section 7.A. Do **not** use more than 5µM GTP. We recommend using 1µM GTP for intrinsic GTPase activity reactions.
Low signal-to-background ratio	Reaction lacks DTT. Be sure to add DTT to the GTPase/GAP Buffer or GEF Buffer.
High luminescent signal	One or more reaction components were contaminated with GTP or other nucleotide triphosphates. Avoid workspaces and pipettes that are used with nucleotide triphosphate-containing solutions. Use aerosol-resistant pipette tips. Clean work surfaces by wiping with a detergent solution or ethanol and rinsing with clean water. Rinse pipettes and other labware with distilled water at least three times. For automated dispensing systems, replace any components that have been used to dispense nucleotide triphosphate-containing solutions.
	An alternative source of GTP was used to assemble the GTPase reactions, and that source was contaminated with ATP or other nucleotide triphosphates. Use only the GTP provided in the GTPase-Glo™ Luminescent Assay.
	The enzyme was contaminated with ATP or other nucleotide triphosphates. Dialyze the enzyme to remove nucleotides. Be sure to store the enzyme in the proper buffer at the proper temperature.
Low luminescent signal	Incorrect plate was used. Use only solid white plates. Do not use black or clear plates.
	Improper storage of the GTPase-Glo™ reagents. All components should be stored at –20°C. Avoid repeated freeze-thaw cycles.

6. Troubleshooting (continued)

Symptoms	Causes and Comments
Low luminescent signal (continued)	The test compound inhibited a component of the Detection Reagent (e.g., the ATP-generating enzyme). To identify inhibitors, add 10µM GTP to two tubes, and add test compound to one tube but not the other tube. Perform the GTPase-Glo™ Assay. Compare the luminescence from these reactions. A decrease in luminescence in the presence of the test compound indicates inhibition of the GTPase-Glo™ assay.
	The test compound inhibited a component of the Detection Reagent (e.g., luciferase). To identify luciferase inhibitors, add 1µM ATP to two tubes, and add test compound to one tube but not the other tube. Perform the GTPase-Glo™ Assay but omit the GTPase-Glo™ Reagent. Compare the luminescence from these reactions. Low light output in the presence of the test compound indicates luciferase inhibition. **Note:** During a screen of the 1,280 compound LOPAC library (Sigma), we observed less than 0.2% false hits, and only two of the compounds inhibited luciferase.
	The reaction was inhibited by the test compound solvent. Minimize the solvent concentration, or use a different solvent to dissolve the test compound. Perform control reactions that contain solvent but no test compound to determine the effect of the solvent on assay performance. The GTPase-Glo™ Assay is not affected by the presence of up to 2% acetone or 2% DMSO.

7. Appendix

7.A. Optimizing GTP Concentration in GTPase Reactions

For best results, you may need to optimize GTP concentration in GTPase reactions. We recommend the following protocol, which is performed in 384-well plates. Adjust the volume as needed for 96-well or low-volume 384-well plates.

Most of our experiments have been performed using 5µM GTP. We tested the effect of lower GTP concentration in the GTPase reaction for intrinsic GTPase activity and GAP-stimulated GTPase activity. We observed that the percent of GTP hydrolyzed for GAP-stimulated GTPase activity is over 90% for GTP concentrations of 0.5–5µM (Figure 10). Conversely, the percent of GTP hydrolyzed for intrinsic GTPase activity depends on the GTP concentration in the GTPase reaction. We observed that, for 0.5µM GTP in the reaction, 56% of input GTP was hydrolyzed, whereas for 5µM GTP, 16% of the input GTP was hydrolyzed. These results demonstrate the importance of optimizing GTP concentration in the GTPase reaction.

7.A. Optimizing GTP Concentration in GTPase Reactions (continued)

1. Assemble the GTPase or GAP-stimulated GTPase reaction in GTPase/GAP Buffer in a 384-well plate in a volume of 5µl.
2. Prepare a 2X GTP solution containing GTP and 1mM DTT in GTPase/GAP buffer. Vary the GTP concentration, for example, 1µM, 2µM, 4µM, 10µM GTP.
3. Initiate the GTPase reaction by adding 5µl of 2X GTP solution prepared in Step 2 to each well for a total GTPase reaction volume of 10µl. The GTP concentration will now be 0.5µM, 1µM, 2µM or 5µM.
4. Incubate the reaction for 30–60 minutes at room temperature.

   Note: During the incubation, thaw the undiluted GTP-Glo™ Reagent until ready to use in Step 5. Calculate the volume of GTPase-Glo™ Reagent, 500X, required for your experiment.
5. Gently mix the thawed GTPase-Glo™ Reagent, 500X, by inversion; do not vortex. Prepare the required volume of reconstituted GTPase-Glo™ Reagent by increasing or decreasing the component volumes provided below.

Reconstituted GTPase-Glo™ Reagent Component	Volume
GTPase-Glo™ Reagent, 500X	2µl
ADP, 10mM	0.5µl
GTPase-Glo™ Buffer	998µl
Total volume	1ml

   Equilibrate the reconstituted GTPase-Glo™ Reagent to room temperature before use.

   Note: Prepare the reconstituted GTPase-Glo™ Reagent immediately before use, and prepare only enough for your experiment. Do not freeze the reconstituted GTPase-Glo™ Reagent.
6. Add 10µl of reconstituted GTPase-Glo™ Reagent to each completed GTPase reaction, mix briefly and incubate with shaking for 30 minutes at room temperature.
7. Add 20µl of Detection Reagent, and incubate the plate for 5–10 minutes at room temperature.
8. Measure luminescence.

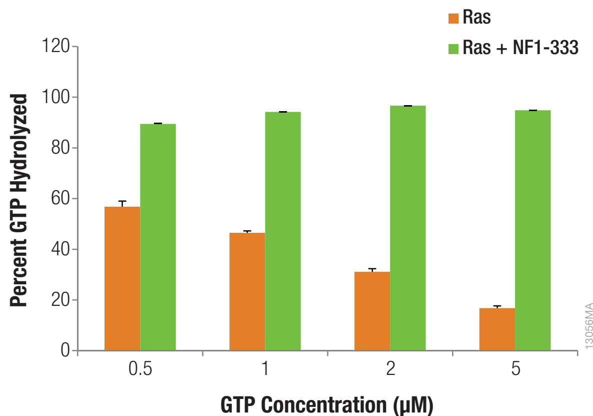

Figure 10. Optimization of GTP concentration in Ras reactions. Intrinsic GTPase reactions containing 1µM Ras or GAP-mediated GTPase reactions containing 1µM Ras and 0.2µM NF1-333 were performed with different concentrations of GTP (0.5µM, 1µM, 2µM and 5µM) in GTPase/GAP Buffer and incubated for 30 minutes at room temperature. Ten microliters of reconstituted GTPase-Glo™ Reagent was added to the completed reactions, and incubated for 30 minutes at room temperature. Detection Reagent (20µl) was added to each reaction, and the plate incubated for 5 minutes at room temperature before detecting luminescence.

7.B. Titrating Enzymes for the GTPase-Glo™ Assay

Some GTPases are slow-acting and may require more enzyme for analyzing intrinsic GTPase activity. To determine the optimal concentration or concentration range of GTPase, GAP or GEF to use in the GTPase-Glo™ Assay, titrate the enzyme as described below.

Use the molecular weight of the GTPase you are studying to determine the molar concentration. If the GTPase is fused to tags (e.g., His_6 or HaloTag® protein), take the molecular weight of the tag into consideration. For example, GTPases in the Ras-superfamily have a molecular weight in the range of 20–25kDa. Therefore, if a GTPase protein is supplied at a concentration of 1mg/ml, the solution is 40–50µM.

1. To determine the optimal GTPase concentration, dilute the GTPase stock solution with GTPase/GAP Buffer to a concentration of 5µM. To determine the optimal GAP concentration, dilute the GAP to 1µM in GTPase/GAP Buffer. To determine the optimal GEF concentration, dilute the GEF to 1µM in GEF Buffer.

7.B. Titrating Enzymes for the GTPase-Glo™ Assay (continued)

2. In a separate 96-well plate, prepare a serial twofold dilution of the enzyme in appropriate reaction buffer as follows:
   a. Add 50µl of appropriate reaction buffer to wells A2 through A12 of a 96-well plate.
   b. Add 100µl of the GTPase, GAP or GEF prepared in Step 1 to well A1.
   c. Perform a serial twofold dilution of enzyme by transferring 50µl from well A1 to well A2, and pipet to mix. Transfer 50µl from well A2 to well A3; mix well. Repeat for wells A3 through A11. See Figure 11. Discard 50µl from well A11. Do not add enzyme to well A12.

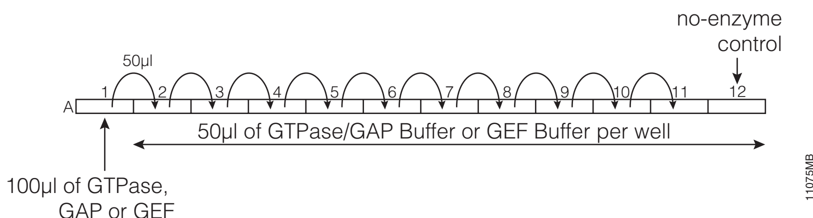

Figure 11. Dilution scheme for enzymes.

3. Dispense 5µl of the enzyme dilution prepared in Step 2 into the wells of a 384-well plate.
4. Initiate reactions as follows:

   For intrinsic GTPase activity, add 5µl of 2X GTP solution containing 10µM GTP and 1mM DTT in GTPase/GAP Buffer.

   For GAP activity, add 5µl of 2X GTP solution containing 10µM GTP, 1mM DTT and GTPase at twice the desired final concentration in GTPase/GAP Buffer.

   For GEF activity, add 5µl of 2X GTP solution containing 10µM GTP, 1mM DTT, GTPase and GAP at twice the desired final concentration in GEF Buffer.

   Note: During incubation, thaw the undiluted GTP-Glo™ Reagent until ready to use in Step 5. Calculate the volume of GTPase-Glo™ Reagent, 500X, required for your experiment.

5. Gently mix the thawed GTPase-Glo™ Reagent, 500X, by inversion; do not vortex. Prepare the required volume of reconstituted GTPase-Glo™ Reagent by increasing or decreasing the component volumes provided below.

Reconstituted GTPase-Glo™ Reagent Component	Volume
GTPase-Glo™ Reagent, 500X	2µl
ADP, 10mM	0.5µl
GTPase-Glo™ Buffer	998µl
Total volume	1ml

Equilibrate the reconstituted GTPase-Glo™ Reagent to room temperature before use.

Note: Prepare the reconstituted GTPase-Glo™ Reagent immediately before use, and prepare only enough for your experiment. Do not freeze the reconstituted GTPase-Glo™ Reagent.

6. Add 10µl of reconstituted GTPase-Glo™ Reagent to each completed GTPase reaction, mix briefly and incubate with shaking for 30 minutes at room temperature.
7. Add 20µl of Detection Reagent to each reaction, and incubate the plate for 5–10 minutes at room temperature.
8. Measure luminescence.

7.C. Optimizing GTPase Reaction Time

GTPases are often slow-acting enzymes. We recommend that you perform preliminary assays to determine the optimal reaction time to detect your enzyme of interest. Use of GAPs and GEFs accelerate the GTPase cycle (i.e., binding GTP and releasing GDP). For GEF reactions, the presence of both GTPase and GAP proteins accelerates the GTPase reaction.

1. To determine the optimal GTPase concentration, dilute the GTPase stock solution with GTPase/GAP Buffer to a concentration of 5µM. To determine the optimal GAP concentration, dilute the GAP to 1µM in GTPase/GAP Buffer. To determine the optimal GEF concentration, dilute the GEF to 1µM in GEF Buffer.
2. In a separate 96-well plate, prepare a serial twofold dilution of the enzyme in appropriate reaction buffer as follows:
   a. Add 50µl of appropriate reaction buffer to wells A2 through A12 of a 96-well plate.
   b. Add 100µl of the GTPase, GAP or GEF prepared in Step 1 to well A1.
   c. Perform a serial twofold dilution of enzyme by transferring 50µl from well A1 to well A2, and pipet to mix. Transfer 50µl from well A2 to well A3; mix well. Repeat for wells A3 through A11. See Figure 11. Discard 50µl from well A11. Do not add enzyme to well A12.
3. Dispense 5µl of the enzyme dilution prepared in Step 2 into the wells of a 384-well plate.

7.C. Optimizing GTPase Reaction Time (continued)

4. Initiate the GTPase reaction by adding 5µl of 10µM GTP in appropriate reaction buffer to the dispensed enzyme at different time points, resulting in several different reaction times across the plate (e.g., 30 minutes, 60 minutes, 90 minutes, 120 minutes and 240 minutes).

   Note: During incubation, thaw the undiluted GTP-Glo™ Reagent until ready to use in Step 6. Calculate the volume of GTPase-Glo™ Reagent, 500X, required for your experiment.

5. Gently mix the thawed GTPase-Glo™ Reagent, 500X, by inversion; do not vortex. Prepare the required volume of reconstituted GTPase-Glo™ Reagent by increasing or decreasing the component volumes provided below.

Reconstituted GTPase-Glo™ Reagent Component	Volume
GTPase-Glo™ Reagent, 500X	2µl
ADP, 10mM	0.5µl
GTPase-Glo™ Buffer	998µl
Total volume	1ml

   Equilibrate the reconstituted GTPase-Glo™ Reagent to room temperature before use.

   Note: Prepare the reconstituted GTPase-Glo™ Reagent immediately before use, and prepare only enough for your experiment. Do not freeze the reconstituted GTPase-Glo™ Reagent.

6. Add 10µl of reconstituted GTPase-Glo™ Reagent to the completed GTPase reaction, mix briefly and incubate with shaking for 30 minutes at room temperature.
7. Add 20µl of Detection Reagent, and incubate the plate for 5–10 minutes at room temperature.
8. Measure luminescence.

7.D. Test Compound Screening Using the GTPase-Glo™ Assay

Below is a general protocol that can be used to screen GTPase, GAP or GEF test compounds. Representative data showing the inhibition of Ras by the nonhydrolyzable GTP analog GMP-PCP are shown in Figure 13. GMP-PCP competes with the natural substrate GTP. Increasing concentrations of GMP-PCP inhibited the GTPase reaction, resulting in higher light output.

1. Assemble the GTPase or GAP-stimulated GTPase reactions in GTPase/GAP Buffer in a 384-well plate as described in Section 4.
2. In a separate 96-well plate, prepare a serial twofold dilution of the test compound in reaction buffer as follows:
   a. Add 50µl of appropriate reaction buffer to wells A2 through A12 of a 96-well plate.
   b. Add 100µl of test compound to well A1.
   c. Transfer 50µl from well A1 to well A2, and pipet to mix. Transfer 50µl to well A3, and mix well. Repeat for wells A3 through A11. See Figure 12. Discard 50µl from well A11. Do not add test compound to well A12, which is the no-test compound control.

7.D. Test Compound Screening Using the GTPase-Glo™ Assay (continued)

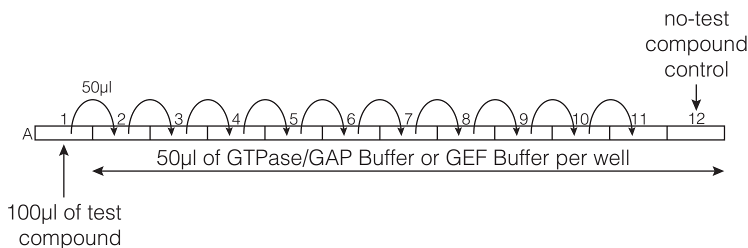

Figure 12. Dilution scheme for test compounds.

3. Dispense 5µl of test compound dilution prepared in Step 2 into wells of a 384-well plate.
4. Prepare a 2X GTP solution containing 10µM GTP and 1mM DTT in GTPase/GAP Buffer.
5. Initiate the GTPase reaction by adding 5µl of 2X GTP solution to each well. The total GTPase reaction volume is 10µl.
6. Incubate the reaction for the optimal reaction time (see Section 7.B) at room temperature.

   Note: During the incubation, thaw the undiluted GTP-Glo™ Reagent until ready to use in Step 8. Calculate the volume of GTPase-Glo™ Reagent, 500X, required for your experiment.
7. Gently mix the thawed GTPase-Glo™ Reagent, 500X, by inversion; do not vortex. Prepare the required volume of reconstituted GTPase-Glo™ Reagent by increasing or decreasing the component volumes provided below.

Reconstituted GTPase-Glo™ Reagent Component	Volume
GTPase-Glo™ Reagent, 500X	2µl
ADP, 10mM	0.5µl
GTPase-Glo™ Buffer	998µl
Total volume	1ml

   Equilibrate the reconstituted GTPase-Glo™ Reagent to room temperature before use.

   Note: Prepare the reconstituted GTPase-Glo™ Reagent immediately before use, and prepare only enough for your experiment. Do not freeze the reconstituted GTPase-Glo™ Reagent.
8. Add 10µl of reconstituted GTPase-Glo™ Reagent to each completed GTPase reaction, briefly centrifuge and incubate with mixing for 30 minutes at room temperature.

   Note: We recommend using a tabletop shaker to mix.
9. Add 20µl of Detection Reagent to each well, and incubate the plate for 5–10 minutes at room temperature.
10. Measure luminescence.

7.D. Test Compound Screening Using the GTPase-Glo™ Assay (continued)

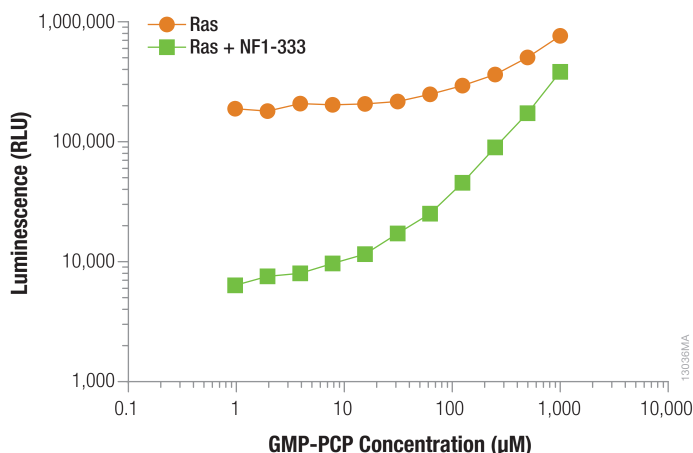

Figure 13. Inhibition of intrinsic GTPase activity and GAP-stimulated GTPase activity by a nonhydrolyzable GTP analog (GMP-PCP). GMP-PCP was titrated into a Ras GTPase reaction or NF1-333-stimulated Ras reaction. The final 10µl reaction contained 1µM Ras, 1µM NF1-333 and 5µM GTP in GTPase/GAP Buffer. After incubating the GTPase reaction for 1 hour at room temperature, 10µl of reconstituted GTPase-Glo™ Reagent was added, and reactions were incubated for 30 minutes at room temperature. Twenty microliters of Detection Reagent was added to each reaction, and the plate was incubated for 5 minutes at room temperature before measuring luminescence.

7.E. GTPase-Glo™ Assay Using Immobilized Proteins from Cell Extracts

The GTPase-Glo™ Assay can be used with immobilized proteins to measure GTPase, GAP or GEF activity of tagged proteins captured from cell extracts by protein pull-down assays (Figure 14, Panel A).

Note: Do not use antibodies to GTPase, GEFs or GAPs for protein pull-down assays. Antibodies may bind to the GTPase active site or switch regions and lead to impaired GTP hydrolysis and nucleotide exchange. Antibodies to GEFs and GAPs also may inhibit the GTPase reaction.

We used the following protocol to measure GTP hydrolysis of wildtype Ras in the presence and absence of immobilized NF1-333 (Figure 14, Panel B). In our experiment, HaloTag® protein was used as the fusion tag, and HaloLink™ Resin was used to immobilized NF1-333; glutathione-S-transferase (GST), which has no GTPase activity, was included in the negative control reactions. Only in the presence of NF1-33, did we observe continuous GTP hydrolysis, resulting in a decrease in light output. No GTP hydrolysis was observed with the constitutively active Ras^{G12V} mutant, resulting in higher light output. Reactions without RAS also showed no GTP hydrolysis.

To show that the GTPase-Glo™ Assay can be used to assess the specificity of GTPases, GAPs and GEFs, we substituted the GTPase RheB for Ras and showed that NF1-333 does not act as a GAP for the Rheb. (The known GAP for Rheb is TSC2).

To measure GTPase, GAP or GEF activity of an immobilized protein, express the protein of interest with a fusion tag that permits capture with the appropriate protein purification substrate. For example, the protein can be expressed using a polyhistidine tag and captured using nickel beads. Purify the captured protein using the protocol that is appropriate for the fusion tag and substrate. Use an aliquot of the resulting immobilized protein in the GTPase reaction as described below.

1. Add 25–30µl of immobilized protein bead bed volume to a 1.5ml tube.
2. To assay GTPase activity, add 50µl of 10µM GTP in GTPase/GAP Buffer to initiate the GTPase reaction. Include the cognate GAP to GTPase reaction if desired. To assay GAP, add 10µM GTP and GTPase at the optimal concentration determined in Section 7.B in a total of 50µl of GTPase/GAP Buffer. To assay GEF activity, add 10µM GTP and the optimal amounts of GAP and GTPase, as determined in Section 7.B, in 50µl of GEF Buffer.
3. Incubate reaction for the optimal reaction time, generally 60–120 minutes (see Section 7.C) at room temperature.

   Note: During the incubation, thaw the undiluted GTP-Glo™ Reagent until ready to use in Step 6. Calculate the volume of GTPase-Glo™ Reagent, 500X, required for your experiment.
4. Briefly centrifuge the completed reactions, and dispense 25µl of each supernatant into two wells of a 96-well plate.

   Note: We recommend performing duplicate reactions with each supernatant.
5. Gently mix the thawed GTPase-Glo™ Reagent, 500X, by inversion; do not vortex. Prepare the required volume of reconstituted GTPase-Glo™ Reagent by increasing or decreasing the component volumes provided below.

Reconstituted GTPase-Glo™ Reagent Component	Volume
GTPase-Glo™ Reagent, 500X	2µl
ADP, 10mM	0.5µl
GTPase-Glo™ Buffer	998µl
Total volume	1ml

   Equilibrate the reconstituted GTPase-Glo™ Reagent to room temperature before use.

   Note: Prepare the reconstituted GTPase-Glo™ Reagent immediately before use, and prepare only enough for your experiment. Do not freeze the reconstituted GTPase-Glo™ Reagent.
6. Add 25µl of reconstituted GTPase-Glo™ Reagent to the complete GTPase reaction, mix briefly and incubate with shaking for 30 minutes at room temperature.
7. Add 50µl of Detection Reagent to each well, and incubate for 10 minutes at room temperature.
8. Measure luminescence.

7.E. GTPase-Glo™ Assay Using Immobilized Proteins from Cell Extracts (continued)

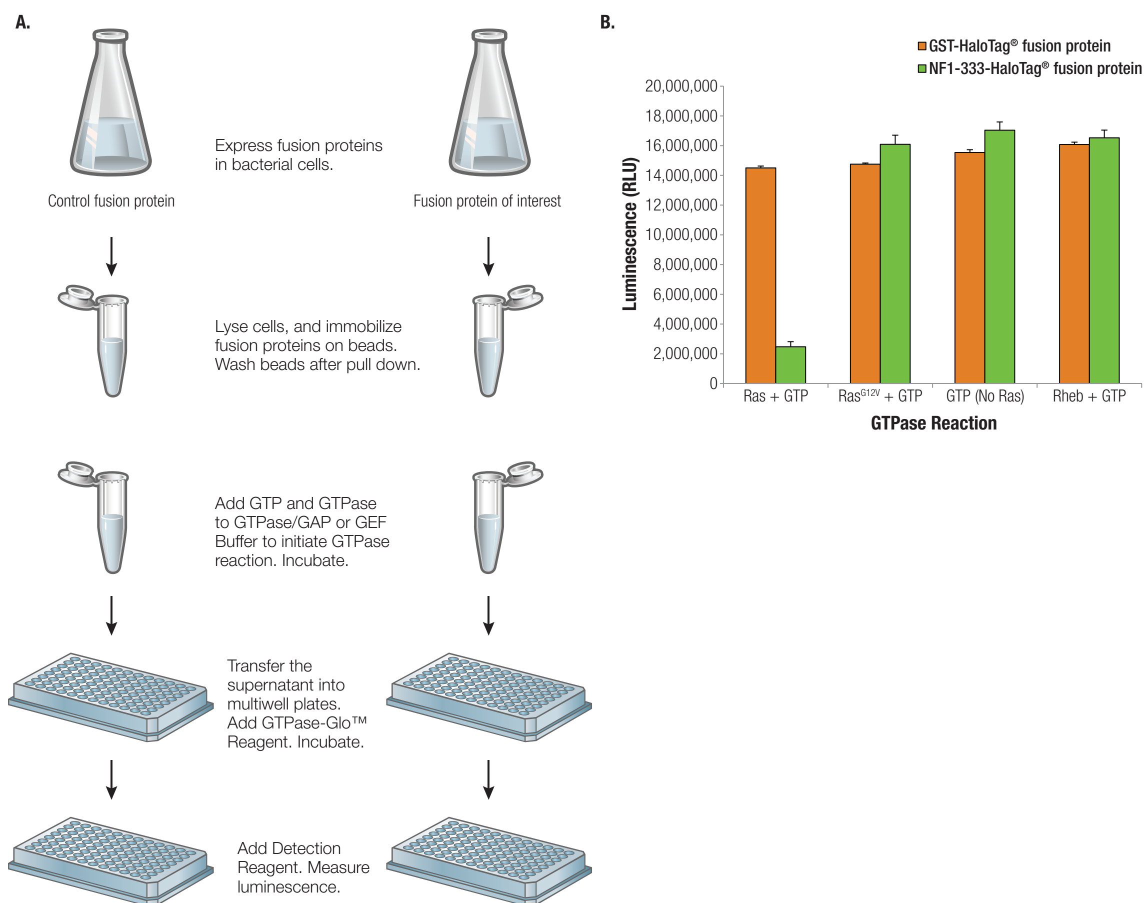

Figure 14. Performing the GTPase-Glo™ Assay with immobilized proteins. Panel A. An overview of the GTPase-Glo™ Assay using immobilized protein from cell extracts. **Panel B.** NF1-333 (GAP for Ras) and GST (negative control) fused to HaloTag® protein were expressed in bacteria and purified using the HaloLink™ Resin. The NF1-333- or GST-coated HaloLink™ Resin (25–30µl) was then incubated with no Ras or wildtype Ras, Ras^{G12V} or RheB in GTPase/GAP Buffer containing 10µM GTP for 1 hour at room temperature. Reactions were centrifuged, and 25µl of supernatant was transferred in duplicate to wells of a 96-well plate. Twenty-five microliters of reconstituted GTPase-Glo™ Reagent was added to each well, and reactions were incubated for 30 minutes at room temperature. Detection Reagent (50µl/well) was added, plates were incubated for 10 minutes at room temperature and luminescence was measured.

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8. Related Products

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000
GloMax® Explorer Fully Loaded Model	1 each	GM3500
GloMax® Explorer with Luminescence and Fluorescence	1 each	GM3510

[a]U.S. Pat. Nos. 7,083,911, 7,452,663 and 7,732,128, European Pat. No. 1383914 and Japanese Pat. Nos. 4125600 and 4275715.

[b]U.S. Pat. Nos. 7,741,067, 8,361,739, 8,603,767, Japanese Pat. No. 4485470 and other patents pending.

[c]U.S. Pat. Nos. 6,602,677, 7,241,584 and 8,030,017, European Pat. No. 1131441, Japanese Pat. Nos. 4537573 and 4520084 and other patents pending.

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