Quantification of GTPase Cycling Rates of GTPases and GTPase:Effector Mixtures Using GTPase Glo Assays

Tschirpke, Sophie; Daalman, Werner K.G.; Laan, Liedewij

DOI
10.1002/cpz1.1000

Publication date
2024

Document Version
Final published version

Published in
Current Protocols

Citation (APA)
Tschirpke, S., Daalman, W. K. G., & Laan, L. (2024). Quantification of GTPase Cycling Rates of GTPases and GTPase:Effector Mixtures Using GTPase Glo Assays. *Current Protocols*, *4*(4), Article e1000. https://doi.org/10.1002/cpz1.1000

Quantification of GTPase Cycling Rates of GTPases and GTPase:Effector Mixtures Using GTPase Glo Assays

Sophie Tschirpke,[1,2] Werner K.-G. Daalman,[1,2] and Liedewij Laan[1,3]

[1]Bionanoscience Department, Delft University of Technology, Delft, The Netherlands
[2]These authors contributed equally to this work
[3]Corresponding author: *L.Laan@tudelft.nl*

Published in the Molecular Biology section

In different cellular activities such as signal transduction, cell division, and intracellular transportation, small guanosine triphosphatases (GTPases) take on a vital role. Their function involves hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP). In this article, we explain the application of a commercially available GTPase assay—the GTPase Glo assay by Promega—for investigation of GTPase-effector interactions. We provide experimental protocols together with an analysis model and software to obtain GTPase cycling rates of GTPases and GTPase:effector mixtures. GTPase cycling rates refer to the rates by which a GTPase completes an entire GTPase cycle. These rates enable quantification of the strength of GTPase effectors in a concentration-dependent fashion, as well as quantification of the combined effect of two effectors, independent of which GTPase cycle step they are affecting. © 2024 The Authors. Current Protocols published by Wiley Periodicals LLC.

Basic Protocol: Conducting GTPase Glo assays
Support Protocol 1: Analyzing GTPase assays to correlate luminescence with remaining GTP
Support Protocol 2: Fitting GTPase assay data to obtain GTPase cycling rates

Keywords: Cdc42 • GTPase activity • GTPase assay • GTPase cycling rates • GTPase effectors

How to cite this article:
Tschirpke, S., Daalman, W. K.-G., & Laan, L. (2024). Quantification of GTPase cycling rates of GTPases and GTPase:effector mixtures using GTPase Glo assays. *Current Protocols*, *4,* e1000. doi: 10.1002/cpz1.1000

INTRODUCTION

Small guanosine triphosphatases (GTPases) are a class of enzymes that play a fundamental role in various cellular processes, including signal transduction, cell division, and intracellular transport. GTPases regulate the hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP). Mechanistically, this activity involves three steps (Fig. 1): (1) binding of GTP to the GTPase, (2) hydrolysis of GTP to GDP and free phosphate, and (3) release of GDP from the GTPase. GTPase activity is often regulated by effector proteins: GTPase activating proteins (GAPs) that boost step 2 and GDP/GTP exchange factors (GEFs) that enhance step 3 (Bos et al., 2009; Cherfils & Zeghouf, 2013; Vetter & Wittinghofer, 2001). Several assays exist that examine single GTPase cycle

CURRENT PROTOCOLS
A Wiley Brand

Current Protocols e1000, Volume 4
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpz1.1000

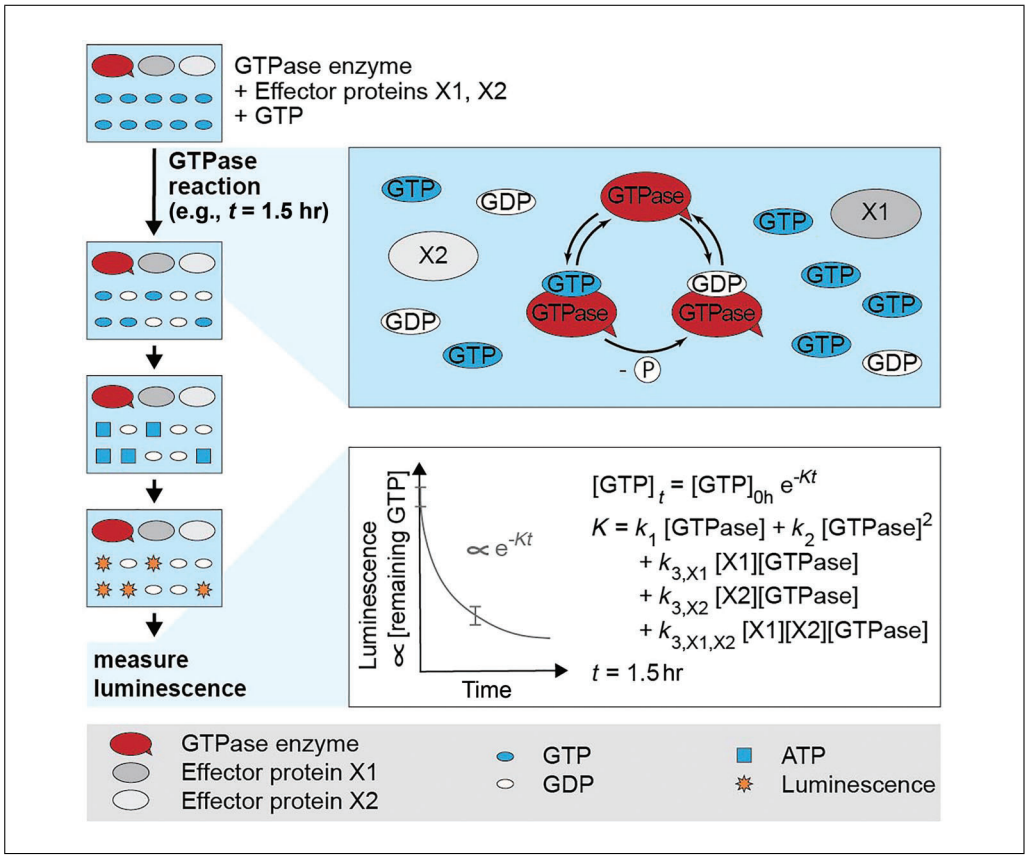


Figure 1 Schematic illustration of assay steps and the GTPase cycling reaction in the GTPase Glo assay (Promega). A GTPase, alone or in combination with effector proteins, is incubated with GTP for a specified time (e.g., 1.5 hr) during which GTPase cycling occurs. This is followed by two processing steps, in which GTP (blue ovals) is first converted to ATP (blue squares) and then made luminescent (orange stars) using an ATP-specific detection reagent. Finally, luminescence, reflecting the remaining GTP, is measured. GTP hydrolysis cycling rates can be extracted by fitting the data with an exponential model.

steps, which helps us understand the mechanistic details of these cycle steps and their regulation. On the other hand, they do not allow study of the interplay of effectors acting on different GTPase cycle steps. Furthermore, approaches that reconstitute the complex cellular functions of GTPases *in vitro* (Bezeljak et al., 2020; Kohyama et al., 2022; Loose et al., 2008; Vendel et al., 2019) are sensitive to variations in protein batch activities and benefit from easy and accessible assays assessing protein purification batches activities.

We here describe how a commercially available GTPase assay (the Promega GTPase Glo assay, henceforth referred to as the GTPase assay) can be used to quantitatively study GTPase-effector interactions and effector interplay and to easily test activity of GTPase and effector purification batches. In the GTPase assay, proteins of interest are incubated with GTP for a specified amount of time for GTPase cycling to occur. The reaction is then stopped and the amount of remaining GTP is determined as luminescence signal. GTPase cycling rates can be obtained from the resulting data using a coarse-grained exponential fitting model (Fig. 1). This article describes the workflow for conducting GTPase assays using GTPase:effector protein mixtures, and provides analysis software to obtain GTPase cycling rates. GTPase cycling rates describe the rate by which the GTPase completes *entire* GTPase cycles. Our protocols enable a quantitative analysis of the single and combined effect of one or two effectors on the entire GTPase cycle in a concentration-depended fashion, thus allowing the study of effectors (e.g., GEFs and GAPs) acting together on different steps of the GTPase cycle. We do not advise using this protocol if single GTPase steps are to be examined in-depth, but rather when the effects of a

Table 1 Summary of How Example Assays 1 and 2 are Used throughout the Protocols[a]

	Example 1: Basic GTPase assay	**Example 2: Elaborate GTPase assay set**
Experimental procedure		
Basic Protocol	Based on this example	—
Strategic Planning	—	Features considerations absolutely necessary for this assays set (especially Fig. 2)
Proteins	Ras (GTPase)	Cdc42 (GTPase), Cdc24 (GEF), Rga2 (GAP)
Number of assays	2	23
Raw data	`example1.xlsx` (tab: `'E1', 'E2'`)	—
Data processing		
Support Protocol 1: Calculating remaining GTP from luminescence values	Based on this example	—
	Input: `example1.xlsx` (tab: `'E1', 'E2'`) Output: same file as input	—
	Reformatting of Support Protocol 1 output data to generate Support Protocol 2 input data (see Supporting Information S9)	—
Support Protocol 2: Fitting GTPase cycling rates using an exponential model (see Supporting Information S8)	Based on this example	Based on this example

	Input:
	`example1.xlsx` (tab: `'matlab'`)
	`Assaylist-example1.xlsx`
		Input:
		`example2-matlab.xlxs`
		`Assaylist-example2.xlsx`

	Output in `example1 matlab output` folder:
	`Data_summary.xlsx`
	`Data_assays.mat`
	Figure folder
		Output in `example2 matlab output` folder:
		`Data_summary.xlsx`
		`Data_assays.mat`
		Figure folder

	Fitting parameters: k_1, k_2	Fitting parameters: k_1, k_2, $k_{3,\mathrm{Cdc24}}$, $k_{3,\mathrm{Rga2}}$, $k_{3,\mathrm{Cdc24,Rga2}}$, c_{corr}
Understanding Results		
Explanation of:	Luminescence k_1, k_2 Output file structure pooling of rates k	c_{corr} $k_{3,\mathrm{Cdc24}}$, $k_{3,\mathrm{Rga2}}$, $k_{3,\mathrm{Cdc24,Rga2}}$ Comparison of rates

[a] Data files available at *data.4tu.nl* (see Data Availability).

protein on the entire GTPase cycle are of interest. This method is illustrated using a basic GTPase assay with human Ras (GTPase) (example 1) and an elaborate GTPase assay using *Saccharomyces cerevisae* Cdc42 (GTPase), Cdc24 (GEF), and Rga2 (GAP) (example 2) (Tschirpke, Daalman et al., 2023). Table 1 summarizes how these examples are used to illustrate the protocols described. The protocols can be applied to other GTPases and effectors. In the Basic Protocol, we outline the workflow for conducting GTPase Glo assays. In Support Protocol 1, we describe how the GTPase assay data (luminescence) are converted to amounts of remaining GTP. In Support Protocol 2, we describe how

data obtained by Support Protocol 1 can be fitted with a GTPase cycling model (and the openly available Matlab code developed by us) to obtain GTPase cycling rates.

STRATEGIC PLANNING

If the GTPase assay is being performed for the first time, we advise first practicing the pipetting of the small volumes required into the wells using colored water. It is best to conduct smaller assays using a larger number of replicates per sample (e.g., eight sample rows per assay with five replicates each, as described in the Basic Protocol) before moving on to larger assay sets with more proteins.

Once you feel more comfortable with the assay, larger assay sets can be conducted, for example, to look at the interaction between a GTPase and one or several effectors (e.g., GTPase-GEF, GTPase-GEF-GAP). The following section applies predominantly (but not solely) to such larger assays sets involving multiple GTPases and/or effector proteins (example 2). We advise users to determine how many proteins will be assayed well before starting the assays. Specifically, we recommend the following steps.

1. Verify that the proteins and protein buffers do not interfere with the assay signal. The GTPase assay measures luminescence, which correlates with the amount of remaining GTP. Proteins that interact with and can alter luminescence (e.g., fluorescent tags, see Supporting Information S3) are therefore not suitable for this assay. Buffers containing ADP, ATP, GDP, GTP, and guanosine phosphate analogs are also not suitable, as they interfere with GTPase assay step reactions. Consider the buffer components carefully and verify that the effector proteins used do not exhibit GTPase activity.

2. Verify that incubation times lie in the exponential decline region of the remaining GTP. In the GTPase assay, the proteins of interest are incubated with GTP for a specified amount of time during which GTPase cycling can occur. The reaction is then stopped and the amount of remaining GTP is determined as a luminescence signal. GTPase cycling rates can be obtained from these data through a coarse-grained exponential fitting model (Fig. 1 and Supporting Information S8). The model is based on our observation that the amount of remaining GTP declines exponentially with time (Supporting Information S4). It is advisable to verify that this is true for the proteins being studied (GTPase enzymes and GTPase:effector mixtures) and incubation times to be used. To obtain such data, prepare one batch of serial dilutions of the proteins you will use. Use exactly these dilutions to conduct several assays with different incubation times. Check whether the amount of remaining GTP in these assays follows an exponential decline. Further, analyze each assay individually using our GTPase cycling model. Verify that different incubation times (different assays) yield the same rates (e.g., Supporting Information S4).

3. Dialyze all proteins in same buffer. In the assay, protein activity is determined by normalizing the assay readout (luminescence) to a well containing buffer only. Thus, differences in ion concentrations, detergents, or other additives (such as glycerol) can lead to differences in luminescence. This is easily avoided if all proteins used in an assay set are prepared in the same buffer. We advise against using a buffer containing glycerol, as the increased viscosity may affect protein activities (unless protein activities in a denser environment are of interest). Further, buffers should not contain DTT; GDP, GTP, or other guanosine analogs; ADP, ATP, and other nucleotide triphosphates; or EDTA or other chelating agents that can complex magnesium. If a GTPase-GEF interaction is to be investigated, the buffer usually requires magnesium salts (e.g., 10 mM $MgCl_2$).

Many proteins require glycerol for storage. In this case, keep the protein at a high concentration (at least six times of the concentration that ought to be used in the assay) in a buffer containing glycerol (e.g., 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10% glycerol) and then dilute it into the same buffer without

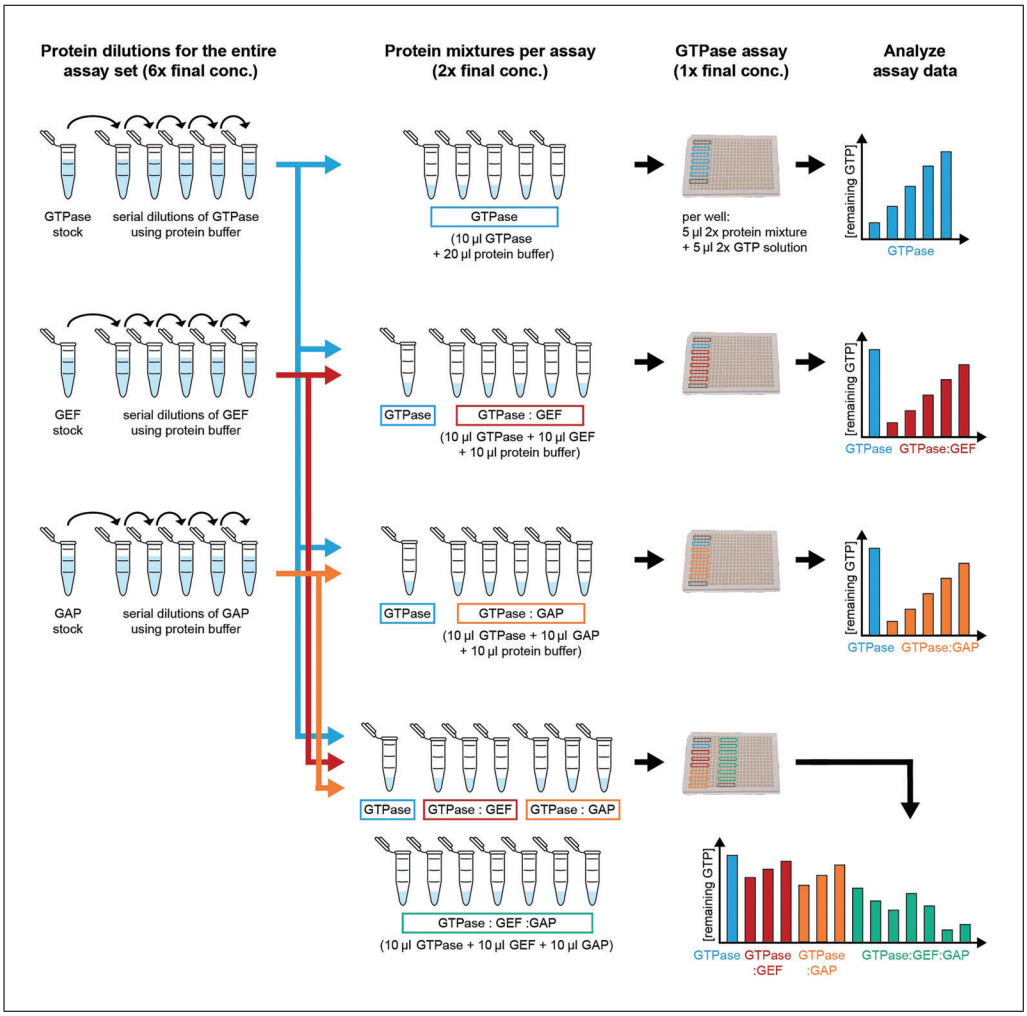


Figure 2 Schematic of protein dilutions required to conduct a GTPase assay set investigating GTPase, GEF, and GAP activity and interactions (example 2).

glycerol. GTPase assays usually require small concentrations of proteins (in our experience, 0.5-5 µM). If the protein is stored at a high concentration, the amount of glycerol in the final reaction will be negligible.

If it is not possible to prepare all proteins in the same buffer, one must verify that the differences in buffer composition do not affect luminescence intensities. A GTPase assay containing only the different buffers can elucidate this. If all buffer mixtures exhibit the same luminescence values, then the differences in buffer composition do not affect luminescence. It should be noted that it is still possible that the buffer components affect protein behavior.

4. Use the same serial dilution for all assays. The assay is sensitive to concentration changes, especially for highly active GTPases and effectors that strongly boost GTPase activity, and thus is also sensitive to pipetting errors when preparing protein dilutions. Prepare serial dilutions of every protein and use exactly the same serial dilution for the entire assay series (Fig. 2). Different serial dilutions of the same protein can exhibit slightly different rates. For example, when comparing the effect of effector protein X on GTPase A and GTPase B, the same serial dilution of effector X must be used for both GTPases.

5. Include a reference sample if data from different assays will be compared. GTPase assays can be sensitive to GTPase activity changes resulting, for example, from small pipetting errors or changes in conditions (e.g., temperature, shaker speed). As this

can lead to slight changes in protein activity between assays, it is best to include all samples to be compared in the same assay. This is not always possible. If the strength of several effectors on one GTPase will be compared, we recommend always including one sample containing only the GTPase in each assay (Fig. 2). The assay-specific activity of the GTPase sample can then be used to account for any assay-specific variability (through fitting the correction factor c_{corr}, as will be explained in Support Protocol 2 and in Eqn. 9 of Supporting Information S8). Assay variations are typically very small (reflected as $c_{corr} \approx 1.0$) (Tschirpke, Daalman et al., 2023; Tschirpke, van Opstal et al., 2023). We recommend excluding assays that show vastly different protein activities (reflected in a c_{corr} much larger or much smaller than 1), as these indicate that the GTPase behavior/assay conditions are unusual.

6. Determine useful concentration ranges before conducting an assay series. In our experience, a good readout regime is 5%-90% remaining GTP. Higher values (>90% remaining GTP) have a larger error (due to normalization) and lower values (<5% remaining GTP) may show a saturation effect due to the small amount of remaining GTP. In the recommended regime, a saturation of the fitted cycling rates may occur. If an assay with multiple proteins (e.g., a GTPase and two effector proteins) is conducted, it is useful to determine beforehand which concentration ranges of each protein lead to optimal readouts to reduce the time it takes to conduct the entire assay series. It is also advisable to stick to similar incubation times for all assays, as this streamlines the selection of protein concentrations (for assays containing protein mixtures) that yield suitable readout regimes.

7. Make an assay plan. Use the same serial dilutions when quantifying and comparing multiple protein interactions. Since many proteins are not stable for weeks at room temperature or 4°C and require glycerol for cryopreservation (which is not recommended), all required assays must be conducted within a rather short time period to obtain reliable and comparable results. We strongly recommend careful planning of which assays will be conducted using which proteins, and calculating the required volumes of protein needed. Our recommended procedure is explained below using as an example a GTPase assay series investigating the cycling of a GTPase and the effects of both a GEF and a GAP, alone and in combination (example 2; Fig. 2; Tschirpke, Daalman et al., 2023).

Considerations (as described earlier): (1) Different serial dilutions of the same protein may exhibit slightly different rates due to small pipetting errors. Hence, the same dilution should be used for the entire assay set. (2) For larger assays, it is advisable to know in advance which concentration ranges give good signal to reduce the time the proteins need to be stored. (3) A reference sample should be included (a sample of the GTPase alone, at the concentration used in the particular assay). (4) The incubation times used should be in the exponential region. (5) It is advisable to use similar incubation times for all assays, as this will make it easier to choose protein concentrations for assays containing protein mixtures that will result in suitable readouts.

Protein dilutions: To conduct the assay, protein dilutions are needed for the three proteins used in the entire assay set (GTPase, GEF, and GAP; Fig. 2, left column). These dilutions will be used to create the protein mixtures for one GTPase assay (Fig. 2, middle column). The concentration of the dilutions depends on two factors: (1) The number of protein species in the assay (N). For a mixture containing a GTPase, GEF, and GAP, N is 3. As it is easiest to prepare the various mixtures by combining proteins in 1:1:1 volume ratios, each protein will be diluted by a factor of three in setting up the assay. (2) To initiate the GTPase reaction, a 2× GTP solution is mixed in a 1:1 volume ratio with each protein mixture. Together, the protein dilutions (left column) should be prepared at a $2\times N$-fold higher concentration than the final concentration in the assay (right column). Thus, for an assay set with a GTPase, GEF, and GAP, all protein dilutions should be prepared at

6× concentration. For assay sets investigating mixtures of four or five proteins, *N* will be 4 or 5 and the dilutions must be 8× or 10×, respectively.

Procedure: First, an assay is conducted with a serial dilution of only the GTPase (Fig. 2, top row). From this assay, pick one or two GTPase concentrations to conduct all subsequent assays. Because the subsequent assays involve a GEF and a GAP, both of which boost GTPase activity, it is wise to choose a GTPase concentration that leads to a high amount of remaining GTP (e.g., 80%-90%). Next, an assay is conducted with the chosen concentration of GTPase and a serial dilution of the GEF (Fig. 2, second row). A similar assay is conducted with GTPase and the GAP (Fig. 2, third row). Finally, an assay is conducted with all three proteins in multiple combinations (GTPase alone, GTPase:GEF, GTPase:GAP, and GTPase:GEF:GAP) all using the same GTPase concentration. For this final assay, choose GEF and GAP concentrations that resulted in a medium amount of remaining GTP (e.g., 60%-70%) to leave room for even lower values when both proteins are combined (Fig. 2, bottom row). Ideally, additional assays will be added to control for potential non-canonical effects (see below).

8. Control for non-canonical effects by adding an inert protein. We have observed several potential artefacts during GTPase cycling in this assay. These effects are generally small and can be accounted for. For example, we observed that both bovine serum albumin (BSA) and casein cause a slight boost in GTPase activity of both Ras and Cdc42. BSA and casein are considered inert and have no known interaction with either Ras or Cdc42. We suspect that they seemingly boost GTPase activity by increasing the effective GTPase concentration. A few GTPase molecules might stick to the well in each reaction chamber, being rendered inactive. When another protein is added, the chamber wall will be covered with some molecules of both GTPase and the other protein, thereby increasing the effective GTPase concentration. To ensure that the effect of a protein on GTPase cycling is not only due to these non-canonical effects, we advise conducting additional assays with the GTPase and an inert protein (e.g., BSA or casein). The effect of the protein of interest will need to exceed that of an equimolar concentration of the inert protein to be of non-canonical origin. It should be noted that BSA is strongly negatively charged, which could lead to non-specific protein interactions. Therefore, casein might be more suitable as a control. For example 2 with a GTPase, GEF, and GAP (Fig. 2), control assays for non-canonical effects include: (1) GTPase:casein serial dilution, (2) GTPase:GEF:casein, and (3) GTPase:GAP:casein.

CONDUCTING GTPase Glo ASSAYS

BASIC PROTOCOL

In the GTPase Glo assay, proteins of interest (GTPase enzymes with or without effectors) are incubated with GTP for a specified amount of time for GTPase cycling to occur (Fig. 1). Once the reaction is stopped, the remaining GTP is translated into a luminescence signal and measured. The following steps are from the assay manual with only minor modifications. The volumes provided are for assays in 384-well microplates. Assays using larger volumes can be conducted using different plates (see the assay manual). In addition to following the steps here, we recommend carefully reading the assay manual.

The following steps are for a GTPase assay with a serial dilution for six concentrations of Ras GTPase (example 1; Fig. 3). An illustration of the required assay planning steps is shown in Supporting Information S2. The assay results in data that are found in `example1.xlsx`, tab: `'E1', 'E2'`.

Materials

Proteins of interest:
GTPase (e.g., human Ras, EMD Millipore, cat. no. 553325)
Optional effector proteins (GEP, GAP)

Protein buffer: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol

Inert protein (*optional*): casein (Sigma-Aldrich, cat. no. C7078) or bovine serum albumin (BSA; Thermo Scientific, cat. no. 23209)

GTPase Glo assay (Promega, cat. no. V7681 or V7682 for 1,000 or 10,000 reactions)

384-well white, flat-bottom microplates (Corning, cat. no. 3572)

Parafilm

1.5- and 15-ml reaction tubes

Orbital shaker (e.g., Innova 2300 platform shaker, New Brunswick Scientific)

Temperature-controlled incubator (*optional*)

Plate reader to measure luminescence (e.g., Synergy HTX, BioTek)

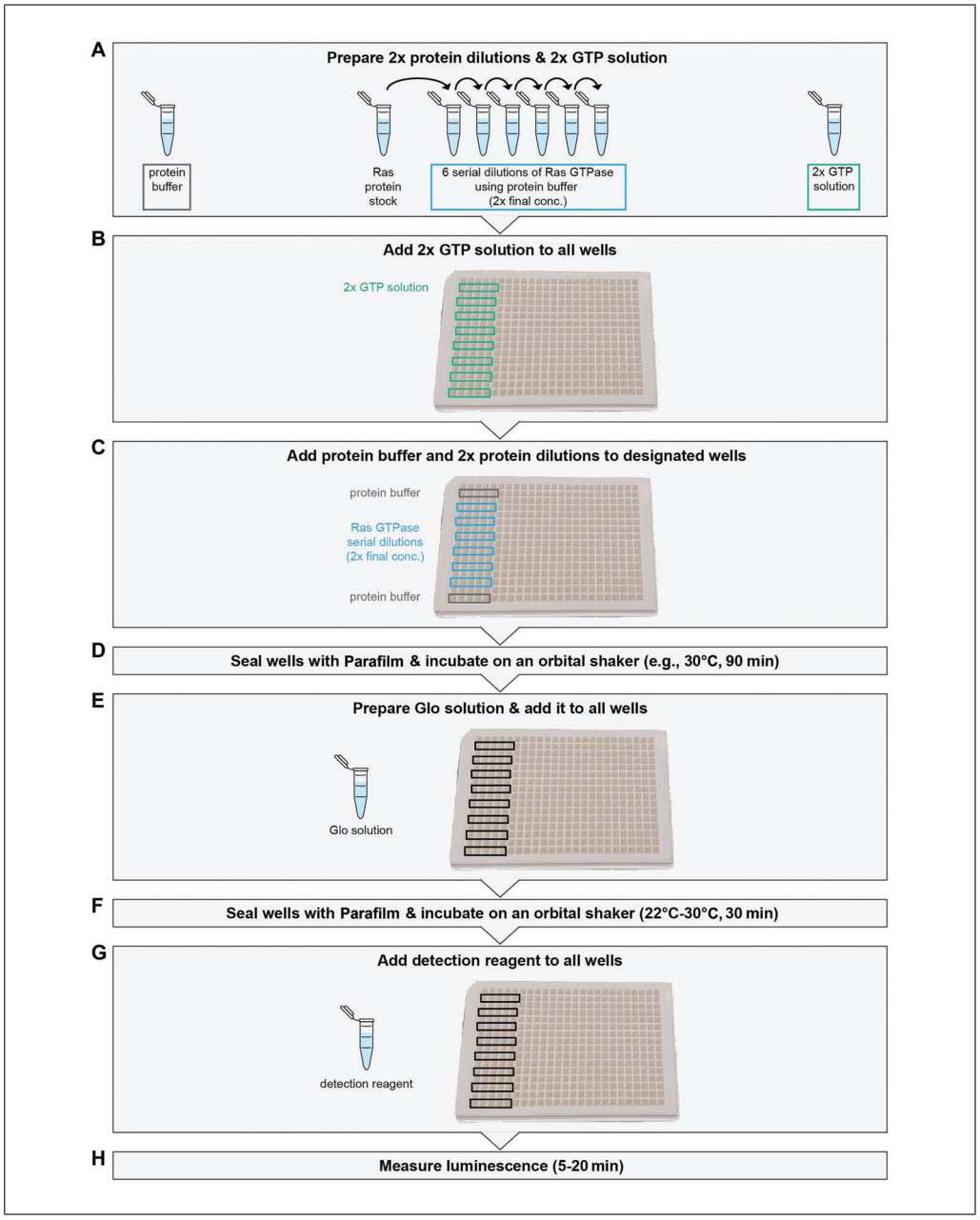


Figure 3 Schematic of steps for a GTPase assay using a serial dilution of six Ras GTPase concentrations. The preparatory steps are further illustrated in Supporting Information S2.

Prepare protein

1. Dialyze GTPase in protein buffer following the instructions accompanying the chosen dialysis device.

   If the GTPase is at a high concentration (e.g., 50-100 μM) and is stored in buffer that does not differ significantly from protein buffer, it can be sufficient to dilute the protein using protein buffer.

   For more details on buffer considerations, see Strategic Planning.

   In general, shorter dialysis times at lower temperatures (e.g., 4 °C) are favorable for most proteins.

Prepare for assay (mise-en-place)

Before beginning, decide on the assay scope and prepare all materials needed. For templates, see Supporting Information S1; for an example assay using Ras GTPase, see Supporting Information S2 and Figure 3.

2. Decide which samples and how many replicates are needed.

   We recommend three to five replicates per sample. In the beginning, we recommend one GTPase per assay and five replicates of each GTPase concentration. We suggest a total of eight samples per assay, including six GTPase concentrations plus two protein buffer samples for normalization.

3. Calculate the volumes (V) of protein dilutions, 2× GTP solution, Glo solution, and detection reagent needed to conduct the assay (for example calculations, see Supporting Information S2).

   $V_{\text{prot}} = 5\ \mu\text{l} \times \text{number of replicates}$
   $V_{\text{GTP}} = 5\ \mu\text{l} \times \text{number of replicates per sample} \times \text{number of samples}$
   $V_{\text{Glo}} = 2 \times V_{\text{GTP}}$
   $V_{\text{det}} = 4 \times V_{\text{GTP}}$

   We recommend preparing a small excess of each solution. In particular, we recommend preparing 5 μl extra of each protein dilution to ensure that there is sufficient solution for the last well. For five replicates per sample, prepare 5 μl × 5 + 5 μl = 30 μl per dilution.

   When calculating V_{GTP}, the number of samples includes buffer samples as well. For the example shown in Fig. 3, the number of samples = 6 dilutions + 2 buffer = 8.

4. Calculate the dilution series steps for the GTPase, keeping in mind that the solutions must be 2× the final concentration in the assay (for example calculations, see Supporting Information S2).

5. Set up the 384-well assay plate as in Figure 3C. Label wells that will be used in the assay and seal wells that will not be used with Parafilm to prevent contamination with dirt.

   a. For one assay, designate five columns of a 96-well plate to give five replicates per sample.
   b. Arrange the two protein buffer samples in the top and bottom rows of the plate.

      In rare cases, luminescence values drift slightly towards lower values throughout the assay. Placement of a buffer row as the first and last rows ensures that this drift can be detected and be accounted for in the analysis.

   c. Arrange the six GTPase dilutions between the buffer rows.
   d. Leave an empty row between all buffer/sample rows.

      This will avoid spill-over of luminescence signal between samples (see Supporting Information S5).

6. Cut four pieces of Parafilm that are big enough to cover the area used for the assay.

Table 2 Pipetting Scheme for 2× GTP Solution

Reagent	Final concentration	Volume		
		For 500 µl	For 750 µl	For 1 ml
Protein buffer	—	494 µl	742 µl	989 µl
100 mM DTT	1 mM	5 µl	7.5 µl	10 µl
10 mM rGTP	10 µM	0.5 µl	0.75 µl	1 µl

Table 3 Pipetting Scheme for Glo Solutions

Reagent	Final concentration	Volume		
		For 500 µl	For 750 µl	For 1 ml
GTPase-Glo buffer	—	496 µl	745 µl	993 µl
1 mM ADP[a]	5 µM	2.5 µl	3.75 µl	5 µl
500× GTPase-Glo reagent	1×	1 µl	1.5 µl	2 µl

[a]The ADP solution provided in the kit must first be diluted from 10 mM to 1 mM (e.g., mix 2 µl of 10 mM ADP with 18 µl ultrapure water).

Conduct assay

7. Thaw protein samples and assay solutions on ice.
8. Make six 2× serial dilutions of GTPase with protein buffer according to your calculations (Fig. 3A). Vortex to mix and spin down gently to collect all volume at the bottom of the tubes.
9. Prepare the 2× GTP solution (Fig. 3A). Vortex the GTP and DTT stock solutions, then combine as then combine with protein buffer according to Table 2 and vortex again.

   2× GTP should always be prepared immediately before starting the assay and should never be stored or frozen (see Supporting Information S6). We also advise aliquoting the GTP and DTT stocks to reduce the number of freeze-thaw cycles. In our experience, up to three freeze-thaw cycles will not impact assay performance.

10. Add 5 µl of 2× GTP solution to all wells (Fig. 3B).
11. Add 5 µl protein buffer and 2× GTPase dilutions to the designated wells (Fig. 3C).
12. Seal wells with two sheets of Parafilm and incubate for the designated time on an orbital shaker.

    We recommend sealing the wells by placing one sheet of Parafilm on top of the plate (without stretching it), pressing it firmly into the plate with a rounded implement (e.g., scissor handles), and then repeating the process with the second sheet of Parafilm. This ensures that the wells are tightly closed. Using only one sheet can result in insufficient sealing (i.e., small holes form in the Parafilm from too much pressure).

    The incubation time and temperature depend on the protein of interest and concentrations used. We recommend choosing a temperature in accordance with the protein's environment in vivo. For incubation times, we recommend 60-90 min.

13. Several minutes before the incubation is finished, prepare the Glo solution (Fig. 3E).
    a. Vortex Glo buffer and 10 mM ADP to mix.
    b. Tap the tube of Glo reagent to mix (do *not* vortex).
    c. Dilute 10 mM ADP to 1 mM using ultrapure water. Vortex to mix.
    d. Combine reagents according to Table 3 and vortex to mix.

We advise aliquoting the 10 mM ADP, Glo buffer, and Glo reagent for future use to reduce the number of freeze-thaw cycles. The aliquots can be reused (see Supporting Information S6). The 1 mM ADP and the prepared Glo solution can only be used fresh and should not be stored or frozen.

The Glo reagent usually runs out first. To increase the number of assay runs per kit, it is important to prepare only the volume needed.

14. At the designated time, add 10 µl Glo solution to all wells (Fig. 3E), seal with two sheets of Parafilm (as above), and incubate 30 min at room temperature on an orbital shaker.

    If an incubation at room temperature is not possible, this incubation step can, in our experience, also be conducted at temperatures of up to 30°C.

15. Add 20 µl detection reagent to all wells (Fig. 3G).

    We advise aliquoting the detection reagent to reduce the number of freeze-thaw cycles and decrease the time required for thawing.

    Calculate how much detection reagent is required per assay run. We have observed cases where two separately stored aliquots from the same batch lead to distinct luminescence values (see Supporting Information S7). To avoid such a shift within an assay, place a sufficient volume in one tube (e.g., take several aliquots) and vortex to mix before adding the reagent to the wells.

16. Measure luminescence of each well using a plate reader for 20 min.

ANALYZING GTPase ASSAYS TO CORRELATE LUMINESCENCE WITH REMAINING GTP

SUPPORT PROTOCOL 1

This protocol describes basic analysis steps to calculate the amount of remaining GTP from the GTPase assay readout (luminescence). The amount of remaining GTP is a simple measure used to compare the activity of different GTPases (of the same concentration, incubated with GTP for the same amount of time). The protocol illustrates steps for example 1: an assay including six Ras GTPase serial dilutions and two buffer reference samples (see Basic Protocol and Supporting Information S2), with three replicates each. The protocol uses and generates data in `example1.xlsx`, tab: `'E1', 'E2'`. A spreadsheet editor or other analysis software is needed. The provided Python script (see Supporting Information S9) is needed if cycling rates will also be determined.

Calculate remaining GTP and its error

1. Calculate the average luminescence for all time points from 5 to 20 min for each well (Fig. 4).
2. Calculate the average luminescence for each sample (Lum_s) by averaging the wells (replicates) for that sample (Fig. 5, row a).

   Each sample refers to a different dilution of GTPase.

3. Calculate the average luminescence for protein buffer (Lum_b) by averaging the wells/replicates from *both* buffer rows in a single calculation (Fig. 5, row a). Do not distinguish between the first and last buffer row.
4. Calculate the standard error of the mean for each sample and buffer (ΔLum_s and ΔLum_b) (Fig. 5, row b).
5. Use the average luminescence values to calculate the amount of remaining GTP (Fig. 5, row c) using the equation:

$$\%\ \text{remaining GTP} = \left(\frac{Lum_s}{Lum_b}\right) \times 100\%$$

		buffer			5 µM			4 µM		
Time	T° Lum	B1	B2	B3	D1	D2	D3	F1	F2	F3
00:00:00	24,3	39877	40039	39616	22419	22382	22161	28359	28846	28165
00:01:10	24,3	39497	39184	39033	21630	21800	21307	27699	28196	27610
00:02:20	24,3	38825	38658	38396	20976	21123	20512	26958	27199	27169
00:03:29	24,3	38170	38535	38051	20153	20266	19856	25830	26559	26284
00:04:38	24,3	37777	37883	37648	19402	19658	18985	25249	25450	25645
00:05:48	24,3	37042	37597	36982	18749	18947	18413	24819	24806	25017
00:06:57	24,3	36743	37039	36790	18147	18302	17946	24006	24186	24104
00:08:07	24,4	36651	36638	36415	17455	17674	17214	23236	23401	23478
00:09:16	24,4	36248	36152	35900	16675	17141	16767	22427	22520	23084
00:10:26	24,4	35606	36403	35491	16360	16521	16322	22053	22428	22321
00:11:35	24,4	35359	35792	35296	15938	16177	15652	21428	21748	21830
00:12:45	24,4	35224	35713	35194	15514	15545	15475	21062	21284	21610
00:13:54	24,4	35006	35301	34685	15107	15287	15005	20486	20802	21043
00:15:04	24,4	34846	34939	34341	14683	14856	14636	19895	20444	20857
00:16:13	24,4	34375	34852	34385	14365	14408	14237	19839	19960	20532
00:17:22	24,4	34123	34658	33916	13833	14080	14017	19625	19400	19850
00:18:32	24,4	34235	34479	33779	13611	13480	13502	19184	19385	19456
00:19:41	24,4	33832	34122	33786	13168	13365	13346	18756	18849	19423
00:20:50	24,4	33899	33917	33404	12975	13195	12884	18514	18760	18912
00:22:00	24,4	33787	33973	33360	12798	12795	12567	18225	18313	18733
00:23:10	24,5	33558	33802	33296	12256	12560	12289	17975	17807	18340
Time average (5-20min)		35330	35668	35151	15662	15829	15579	21294	21478	21739

Figure 4 Data analysis part 1 for the assay in the Basic Protocol: time-averaging of the luminescence values highlighted in green. Data from `example1.xlsx`, tab: `'E1'`.

				Ras					
	Well	buffer	buffer	5 µM	4 µM	3 µM	2 µM	1 µM	0.5 µM
	1	35330	34324	15662	21294	26750	31392	33846	35117
	2	35668	33599	15829	21478	26466	30209	34289	34076
	3	35151	33313	15579	21739	26105	30286	33948	33736
	4								
	5								
a	Average		34564	15690	21503	26440	30629	34027	34310
b	Std. err. mean		396	74	129	187	382	134	415
c	Remaining GTP [%]		100,0	45,4	62,2	76,5	88,6	98,4	99,3
d	Error [%]		1,6	0,6	0,8	1,0	1,5	1,2	1,7

Figure 5 Data analysis part 2 for the assay in the Basic Protocol: calculating the amount of remaining GTP from luminescence values. Data from `example1.xlsx`, tab: `'E1'`.

The luminescence value of buffer corresponds to 100% remaining GTP. The amount of remaining GTP for each protein sample is determined as a percent of the buffer value and thus normalizes the protein samples. In principle, the protein samples also need to be normalized to 0% GTP. Given that we observed basically no luminescence in the blank samples (see Supporting Information S5), this step is not necessary.

6. Calculate the error (Δ remaining GTP; Fig. 5, row d) by error propagation using the equation:

$$\Delta \text{ remaining GTP} = \sqrt{\left(\frac{\Delta \text{Lum}_s}{\text{Lum}_s}\right)^2 + \left(\frac{\Delta \text{Lum}_b}{\text{Lum}_b}\right)^2} \times \left(\frac{\text{Lum}_s}{\text{Lum}_b}\right) \times 100\%$$

Format data for subsequent analysis (optional)

This process is only required if the data analysis will be continued to determine GTPase cycling rates in Support Protocol 2. It involves organizing the data in a spreadsheet with a specific format (Fig. 6) and the following column headers:

Run = assay number. This must consist of an E followed by a number, which can be followed by letter(s) with or without an underscore. For example: E1, E2, E100, E1a, E100abc, E1_a, E100_abc, E1a_abc, E100f_abc
Time = incubation time. Values must be in hours.
GTP_remaining = amount of remaining GTP normalized to 1

Run	Time	GTP_remaining	Error	Buffer_error	Ras_conc
E1	1,566666667	0,453944399	0,005618298	0,016198535	5
E1	1,566666667	0,622129048	0,008046552	0,016198535	4
E1	1,566666667	0,764956994	0,010290555	0,016198535	3
E1	1,566666667	0,886149288	0,015009141	0,016198535	2
E1	1,566666667	0,984468547	0,01192282	0,016198535	1
E1	1,566666667	0,992636905	0,01654503	0,016198535	0,5

Figure 6 Reformatting of data from Figure 5 using the Python script provided in Supporting Information S9. Data taken from `example1.xlsx`, tab: `'E1'`.

Error = error values normalized to 1
Buffer_error: error of buffer normalized to 1
[GTPase]_conc = name of the GTPase followed by '_conc' (e.g., Ras_conc in Fig. 6). Values must be in μM.
[Effector]_conc (*optional*) = name of the effector followed by '_conc'. Values must be in μM. This column should only be present if it contains more than one unique value per assay.

Reformatting the data can be done using the Python script `Ras_example.ipynb` (see Supporting Information S9). For example 1, the steps are:

7. Open `Ras_example.ipynb` and state the input data and relevant tab names:
```
datafilename = 'example1.xlsx'
tabnamelist = ['E1','E2']
```
8. Run the Python script to generate two Excel sheets: `'E1.xlsx'` and `'E2.xlsx'`.
9. Copy data from both outputs into a single Excel sheet, but include only one header (see first figure in Supporting Information S9). This will be the input for the Matlab script used in Support Protocol 2.
10. Use the find/replace option of the spreadsheet editor to replace all points (.) with commas (,).

The Python script generates numbers in the format 1.00, but the Matlab script requires the format 1,00.

We advise grouping only assays of the same type (i.e., containing the same proteins and dilutions) in one spreadsheet tab, as they must follow the same structure. For example, for an assay set examining the GTPase Cdc42 and its GEF Cdc24, two types of assays were conducted: (1) assays using only serial dilutions of the GTPase and (2) assays using a constant GTPase concentration and serial dilutions of the GEF. All assay data from (1) should be grouped in one tab and all assay data from (2) should be grouped in a second tab.

Additional examples are given in Support Protocol 2.

FITTING GTPASE ASSAY DATA TO OBTAIN GTPASE CYCLING RATES

SUPPORT PROTOCOL 2

This protocol describes how the data (preprocessed in Support Protocol 1) can be fitted using a GTPase activity model (see Supporting Information S8) to obtain GTPase cycling rates k, which describe the rate with which a GTPase completes GTPase cycles. It can be used to fit data of GTPases and GTPase:effector mixtures (with up to two effectors, X1 and X2).

In short, data from the GTPase serial dilutions are first fitted with an exponential to obtain GTPase cycling rates k_1 and k_2:

$$[\mathrm{GTP}]_t = [\mathrm{GTP}]_{0\mathrm{h}} \exp(-Kt)$$
$$\text{using } [\mathrm{GTP}]_{0\mathrm{h}} = 1 \text{ and}$$
$$K = K^*_1 + K^*_2 = k_1[\mathrm{GTPase}] + k_2[\mathrm{GTPase}]^2$$

Equation 1

where K refers to the overall GTP hydrolysis rate and depends on the concentration of the GTPase. We call the concentration-independent rates (k) GTPase cycling rates, referring to the fact that they describe GTPase cycling and not specific GTPase cycle steps. The overall hydrolysis rate K is composed of an unaided hydrolysis contribution by an individual GTPase (K^*_1) and a cooperative contribution by two GTPase molecules (K^*_2).

Next, mixtures of one GTPase and one effector (X1) are fitted with:

$$K = K_1 + K_2 + K_{3,\mathrm{X1}}$$
$$K = k_1 c_{\mathrm{corr}}[\mathrm{GTPase}] + k_2(c_{\mathrm{corr}}[\mathrm{GTPase}])^2 + k_{3,\mathrm{X1}} c_{\mathrm{corr}}[\mathrm{GTPase}][\mathrm{X1}]^n$$

Equation 2

and mixtures of one GTPase and two effectors (X1, X2) are fitted with:

$$K = K_1 + K_2 + K_{3,\mathrm{X1}} + K_{3,\mathrm{X2}} + K_{3,\mathrm{X1,X2}}$$
$$k = k_1 c_{\mathrm{corr}}[\mathrm{GTPase}] + k_2(c_{\mathrm{corr}}[\mathrm{GTPase}])^2 + k_{3,\ \mathrm{X1}} c_{\mathrm{corr}}[\mathrm{GTPase}][\mathrm{X1}]^n + k_{3,\ \mathrm{X2}} c_{\mathrm{corr}}[\mathrm{GTPase}][\mathrm{X2}]^m + k_{3,\ \mathrm{X1,\ X2}} c_{\mathrm{corr}}\,[\mathrm{GTPase}]\,[\mathrm{X1}]^n[\mathrm{X2}]^m$$

Equation 3

using values of 1 or 2 for the exponents n and m (i.e., $n = 1$ and $m = 1$, $n = 1$ and $m = 2$, $n = 2$ and $m = 1$, or $n = 2$ and $m = 2$). This fits yield correction factors (c_{corr}) and cycling rates ($k_{3,\mathrm{X1}}$, $k_{3,\mathrm{X2}}$, $k_{3,\mathrm{X1,X2}}$). c_{corr} is dimensionless and accounts for variability between assays. It should be close to 1.0 (see Supporting Information S8). K_1 and K_2 represent corrected versions of K^*_1 and K^*_2 obtained from the GTPase serial dilutions ($K_1 = c_{\mathrm{corr}}K^*_1$, $K_2 = c_{\mathrm{corr}}K^*_2$). $K_{3,\mathrm{X1}}$, $K_{3,\mathrm{X2}}$, and $K_{3,\mathrm{X1,X2}}$ represent the overall hydrolysis rate contribution connected to effectors. They depend linearly on the corrected GTPase concentration, but also on the respective effector concentrations, either linearly or quadratically.

Note that, in order to analyze GTPase:effector mixtures, k_1 and k_2 must be known. It is thus required to fit the data of GTPase (serial) dilutions first. The model allows one to fit GTPase:effector mixtures with up to two effectors, with each effector showing either a linear or quadratic concentration dependence. If the effectors show neither of these concentration dependencies (e.g., due to saturation), we advise either including only the linear/quadratic regimes into the analysis or extending the fitting model to match the specific case. With the provided software, it thus is possible to fit the following protein (mixtures):

GTPase serial dilution (example 1)
GTPase + effector serial dilution (linear or quadratic conc. dependence, Eqn. 2) (example 2)
GTPase + effector X1 serial dilutions + effector X2 serial dilutions (Eqn. 3) (example 2, Fig. 2)

The model is based on our observation that the amount of remaining GTP declines exponentially with time (see Supporting Information S4). It is advisable to verify that this is true for the proteins (GTPase and effectors) and incubation times to be used.

The model code analyzes data of each GTPase assay individually and then generates pooled rate values (for details on weighting of k for pooling and error propagation, see Supporting Information S11). The analysis code allows one to exclude GTPase assays

from pooling if c_{corr} values or standard errors of are out of range. Decision criteria are set in the code using two parameters:

`conc_corr_bounds` states which range of c_corr allows assays to be included in the pooling.

As c_{corr} is expected to be close to 1.0, it is generally advisable to set the lower bound not too close to 0, as such low values are indicative that the inferred activity of the GTPase is unusually low in the assay.

`k_low_err_filt.'GTPase-name'` states whether assays containing the particular GTPase that have low standard errors (1^{-10} or lower) for k values are excluded from pooling. This parameter must be declared individually for each GTPase.

In general, it is advisable to set this parameter to 'true' (i.e., assays with low standard errors will be excluded). If, however, a GTPase is dominated by k_2 (and has $k_1 \approx 0$ and thus std.err.(k_1) ≈ 0), this parameter must be set to 'false' to allow for pooling of k. In many cases this cannot be known before an initial analysis. If chosen inappropriately, the parameter simply needs to be changed and the code re-run.

Below, we first describe the general protocol steps (case 1), which we then apply to example 1 (a simple assay containing one GTPase) (case 2) and example 2 (an elaborate assay set containing assay data for the GTPase alone and GTPase:GEF, GTPase:GAP, and GTPase:GEF:GAP mixtures) (case 3). Finally, we provide analysis steps for the combined analysis of example 1 and example 2 data (case 4).

Necessary resources

Spreadsheet editor
Matlab software license, analysis scripts, and example data files encompassing this protocol

Case 1: General procedure

1a. Set up the Matlab environment. To run the code, ensure that all required files are present:

The assay data (`assaydata.xlsx`) must follow the formatting described in Support Protocol 1 and Figure 6.

The core of the fitting of the model occurs in the function `Crocodile_model.m`. This function determines which model scenario of effectors applies and then generates fit results. To plot these, `plot_rate_data.m` is required, which uses colormaps from `useful_colormaps.m` (containing a colormap from Smith et al., 2015). When needed, an unequal variance t test (`Unequal_var_t_test.m` ; Ruxton, 2006) is used to test for non-zero interactions between effectors. From the main function `Process_assay.m`, a predefined spreadsheet list is read using `Read_assay_list.m` to determine which data sets to use and how they are built up. The data are then read using `extract_activity_data.m` and passed to `Crocodile_model.m`.

2a. Define the processing of the data in the `assaylist.xlsx` spreadsheet. Follow the formatting provided in the example files (discussed below in the steps of cases 2-4). The following sections are required:

Assay name: State an internal assay name that will be used in the output to refer to these data.
Proteins: State the names of proteins whose concentration varies within the assay (maximum of two proteins per assay). State each protein in a separate column.
GTPase ref.: State the assay name where the GTPase was varied. Ensure that this assay has already been processed (i.e., is earlier in the list).
Data file location: State the relevant file location.
Relevant tab names: State which tab of the data file should be used.

3a. Open `Process_assays.m`. Define the input parameters and processing options:

```
%% Input parameters

AssayListName = ' .xlsx';
% State the assaylist file name

GTPases = {' '};
% state proteins (that are stated in the assaylist
  file) that are GTPases

LinFitEffector = {' '};
% state proteins X (that are stated in the assaylist
  file) that should be
% fitted with a linear fit: k_3,X [GTPase] [X]

QuadFitEffector = {' '};
% state proteins X (that are stated in the assaylist
  file) that should be
% fitted with a quadratic fit : k_3,X [GTPase] [X]^2
act_corr = true(1);
% Logical to determine whether terms in the rate
  equation that depend on
% GTPase] are corrected for run-specific GTPase
  activity/concentration
% differences

num_draws = 1e5;
% Number of random draws from distribution of rate
  parameters k

plot_fits = true(1);
% Logical indicating whether fits should be outputted
  in .pdf and .tiff
% (false is faster)

print_fits = true(1);
% Logical indicating whether fit results should be
  printed in the command window

GTP_filt = true(1);
```

```
% Logical indicating whether data points 0.00 – 0.05
  % GTP remaining
% should be disregarded for the fits

conc_corr_bounds = [0.5 1.5];
% Two–element vector that states a c_corr lower and
  upper bound. Assays that
% have c_corr values within this range will be
  included in pooling of
% estimates. [0 Inf] means assays with any c_corr
  value will be included.
% We recommend [0.5 1.5]: assays with c_corr values
  from 0.5 to 1.5 will be
% included.

k_low_err_filt.'GTPase–name' = false;
% Logical that states per GTPase whether to discard
  runs from pooling that
% have very low standard errors (1 e–10 or lower) on
  the k values.
% I f nothing i s provided for a GTPase, default is
  true (i.e. runs with low
% standard errors will be excluded).
```

4a. Run `Process_assays.m`.

Using processing options shown above, the script will generate a `Figures` folder (where all plots are saved), a `Data_summary.xlsx` spreadsheet (summarizing all fitting parameters), and a `Data_assays.mat` file (saved in the `Data` folder). `Data_assays.mat` and plotting functions discussed in Supporting Information S10 can be used to generate plots based on the fits conducted here.

Case 2: Simple assay with Ras GTPase (example 1)

We here show the specific protocol steps to run data for example 1, a GTPase assay containing only serial dilutions of Ras GTPase that was conducted twice. For assay steps, see Basic Protocol and Supporting Information S2. For basic analysis of the assay data, see Support Protocol 1, Supporting Information S9, and the resulting data in `example1.xlsx`, tab: `'matlab'`.

The data for each individual assay will be fitted with the model:

$$[\mathrm{GTP}]_t = [\mathrm{GTP}]_{0\mathrm{h}} \exp(-Kt)$$
using $[\mathrm{GTP}]_{0\mathrm{h}} = 1$ and
$$K = K^*_1 + K^*_2 = k_1 c_{\mathrm{corr}}[\mathrm{Ras}] + k_2 (c_{\mathrm{corr}}[\mathrm{Ras}])^2$$

resulting in k_1 and k_2 for each assay. A pooled estimate of k_1 and k_2 will be generated.

1b. Set up the Matlab environment. To run the code, ensure that all required files are present:

2b. Define the processing of the data in the `assaylist-example1.xlsx` spreadsheet:

Assay name	Proteins		GTPase ref.	Data file location	Relevant tab names
Ras	Ras			./Data/example1.xlsx	matlab

Example 1 entails two assays of Ras serial dilutions, which are stored in `example1.xlsx`, tab: `'matlab'`. Because the concentration of Ras is varied in each assay, the Proteins column states Ras.

3b. Open `Process_assays.m`. Define the input parameters:

```
AssayListName = 'Assaylist-example1.xlsx';
GTPases = {'Ras'};
LinFitEffector = {' '};
QuadFitEffector = {' '};
act_corr = true(1);
num_draws = 1e5;
plot_fits = true(1);
print_fits = true(1);
GTP_filt = true(1);
conc_corr_bounds = [0.5 1.5];
k_low_err_filt.Ras = false;
```

4b. Run `Process_assays.m`.

Using the processing options shown above, the script will generate a `Figures` folder (where all plots are saved), a `Data_summary.xlsx` spreadsheet (summarizing all fitting parameters), and a `Data_assays.mat` file (saved in the `Data` folder). `Data_assays.mat` and plotting functions discussed in Supporting Information S10 can be used to generate plots based on the fits conducted here. The data this script will generate can be found in the `example1 matlab output` folder.

Case 3: Elaborate assay set with GTPase:effector mixtures (example 2)

Here we show the specific protocol steps to run data for example 2, which entails data (`example2.xlsx`) using the following conditions (Fig. 2):

(A) Serial dilutions of the GTPase Cdc42 (in tab `'dCdc42'`).
(B) Constant Cdc42 concentration and serial dilutions of the GEF Cdc24 (in tab `'Cdc42-dCdc24'`).
(C) Constant Cdc42 concentration and serial dilutions of the GAP Rga2 (in tab `'Cdc42-dRga2'`).
(D) Constant Cdc42 concentration and serial dilutions of both Cdc24 and Rga2 (in tab `'Cdc42-dCdc24-dRga2'`).

The data from each individual assay will be fitted with the following model based on Eqn. 3:

$$[\mathrm{GTP}]_t = [\mathrm{GTP}]_{0\mathrm{h}} \exp(-Kt)$$
$$\text{using } [\mathrm{GTP}]_{0\mathrm{h}} = 1 \text{ and}$$
$$K = K_1 + K_2 + K_{3,\mathrm{Cdc24}} + K_{3,\mathrm{Rga2}} + K_{3,\mathrm{Cdc24,Rga2}}$$
$$K = k_1 c_{\mathrm{corr}}[\mathrm{Cdc42}] + k_2 (c_{\mathrm{corr}}[\mathrm{Cdc42}])^2 + k_{3,\mathrm{Cdc24}} c_{\mathrm{corr}}[\mathrm{Cdc24}]^2[\mathrm{Cdc42}] + k_{3,\mathrm{Rga2}} c_{\mathrm{corr}}\left[\mathrm{Rga2}\right][\mathrm{Cdc42}] + k_{3,\mathrm{Cdc24,Rga2}} c_{\mathrm{corr}}[\mathrm{Cdc24}]^2\left[\mathrm{Rga2}\right][\mathrm{Cdc42}]$$

Equation 4

resulting in one set of fitting parameters for each assay. Assays of group (A) yield k_1, k_2; assays of group (B) yield $k_{3,\mathrm{Cdc24}}$, c_{corr}; assays of group (C) yield $k_{3,\mathrm{Rga2}}$, c_{corr}; and assays of group (D) yield $k_{3,\mathrm{Cdc24}}$, $k_{3,\mathrm{Rga2}}$, $k_{3,\mathrm{Cdc24,Rga2}}$, c_{corr}. A pooled estimate of all cycling rates k will be generated.

1c. Set up the Matlab environment. To run the code, ensure that all required files are present:

2c. Define the processing of the data in the `assaylist-example2.xlsx` spreadsheet:

Assay name	Proteins		GTPase ref.	Data file location	Relevant tab names
C42	Cdc42			./Data/example2-matlab.xlsx	dCdc42
C42-C24	Cdc24		C42	./Data/example2-matlab.xlsx	Cdc42-dCdc24
C42-R2	Rga2		C42	./Data/example2-matlab.xlsx	Cdc42-dRga2
C42-C24-R2	Cdc24	Rga2	C42	./Data/example2-matlab.xlsx	Cdc42-dCdc24-dRga2

To analyze this assay set, all data with varied [Cdc42] first need to be fitted. We give these assays the name C42 and list Cdc42 in the first Proteins column.

Next, Cdc42-effector mixtures can be analyzed. We call assays with Cdc42-Cdc24 mixtures C42-C24 and list the varied protein (Cdc24) in the first Proteins column. We do not list Cdc42 because its concentration is constant. To fit these assay data, we want to use the rates of Cdc42 obtained in the line above, so we refer to the assay name C42 in the GTPase ref. column (also see Eqns. 1,2).

We apply the same principles to assays with Cdc42-Rga2 (which we call C42-R2) and also to assays in which the concentration of both effectors is varied (which we call C42-C24-R2). In the latter case, the Proteins columns list both effectors (separately) because the concentration of both is varied.

3c. Open `Process_assays.m`. Define the input parameters:

```
AssayListName = 'Assaylist-example2.xlsx';
GTPases = {'Cdc42'};
LinFitEffector = {'Rga2'};
QuadFitEffector = {'Cdc24'};
act_corr = true(1);

num_draws = 1e5;
plot_fits = true(1);
print_fits = true(1);
GTP_filt = true(1);
conc_corr_bounds = [0.5 1.5];
k_low_err_filt.Cdc42 = true;
```

4c. Run `Process_assays.m`.

Using the processing options shown above, the script will generate a `Figures` folder (where all plots are saved), a `Data_summary.xlsx` spreadsheet (summarizing all fitting parameters), and a `Data_assays.mat` file (saved in the `Data` folder). `Data_assays.mat` and plotting functions discussed in Supporting Information S10 can be used to generate plots based on the fits conducted here. The data this script will generate can be found in the folder `example2 matlab output`.

Case 4: Combined analysis of several assays

It is also possible to analyze assay data of example 1 and example 2 in one go. To do so, simply combine the inputs of the two previous examples.

1d. Set up the Matlab environment. To run the code, ensure that all required files are present:

2d. Define the processing of the data in the `assaylist-example1and2.xlsx` spreadsheet:

Assay name	Proteins		GTPase ref.	Data file location	Relevant tab names
Ras	Ras			./Data/example1.xlsx	matlab
C42	Cdc42			./Data/example2-matlab.xlsx	dCdc42
C42-C24	Cdc24		C42	./Data/example2-matlab.xlsx	Cdc42-dCdc24
C42-R2	Rga2		C42	./Data/example2-matlab.xlsx	Cdc42-dRga2
C42-C24-R2	Cdc24	Rga2	C42	./Data/example2-matlab.xlsx	Cdc42-dCdc24-dRga2

3d. Open `Process_assays.m`. Define the input parameters:

```
AssayListName = 'Assaylist-example1and2.xlsx';
GTPases = {'Ras','Cdc42'};
LinFitEffector = {'Rga2'};
QuadFitEffector = {'Cdc24'};
act_corr = true(1);

num_draws = 1e5;
plot_fits = true(1);
print_fits = true(1);
GTP_filt = true(1);
conc_corr_bounds = [0.5 1.5];
k_low_err_filt.Ras = false;
k_low_err_filt.Cdc42 = true;
```

4d. Run `Process_assays.m`.

Using the processing options shown above, the script will generate a `Figures` folder (where all plots are saved), a `Data_summary.xlsx` spreadsheet (summarizing all fitting parameters), and a `Data_assays.mat` file (saved in the `Data` folder). `Data_assays.mat` and plotting functions discussed in Supporting Information S10 can be used to generate plots based on the fits conducted here. The data this script will generate can be found in the `example1and2 matlab output` folder.

COMMENTARY

Background Information

In the GTPase Glo assay, the proteins of interest (GTPase enzymes with or without effectors) are incubated with GTP for a specified amount of time for GTPase cycling to occur, hydrolyzing GTP to GDP and free phosphate. The reaction is then stopped by addition of Glo solution, which contains a nucleoside-diphosphate kinase and ADP. The kinase converts the remaining GTP to ATP. Addition of the detection reagent, containing a luciferase/luciferin mixture, makes the ATP

luminescent, allowing measurement on a plate reader in luminescence mode (Fig. 1). The luminescence signal correlates with the amount of remaining GTP and thus inversely correlates with GTPase activity. The higher the luminescence values, the more GTP remained in solution and the less activity the GTPase had. Low or no luminescence corresponds to very little or no remaining GTP and a high GTPase activity.

The GTPase Glo assay examines the entire GTPase cycle of the respective GTPase. An assay that examines all steps of the GTPase cycle can be advantageous. For one, it allows investigation of GTPase activities and GTPase-effector interactions. Compared to other *in vitro* assays that examine only one specific step of the GTPase cycle, the rates obtained by this assay may be more comparable to those observed *in vivo*, as GTPase cycling occurs constantly *in vivo*. Further, this assay enables comparison of the strength of effectors that act on different steps of the GTPase cycle, such as GEFs and GAPs. In addition, effector interplay can be studied with this method. The reasons for these advantages are also causes for the assay's main disadvantage. Because GTPase cycling (multiple completions of the entire GTPase cycle) is studied, the origin of observed effects remains elusive. Thus, this method is less suited when detailed mechanistic features of a GTPase cycle step are being investigated. For this purpose, one may need to use other GTPase assays such as the MESG/phosphorylase system (Zhang et al., 1997) or *N*-methylanthraniloyl-GTP/GDP system (Rapali et al., 2017), which examine only the GTP hydrolysis or GDP release step.

Critical Parameters

The GTPase assay is sensitive to small pipetting errors as well as changes in buffer and assay components. We *strongly* recommend a mise-en-place procedure, in which each assay is carefully planned (e.g., use one of the templates provided in Supporting Information S1) and the materials are prepared and in place before the assay is started. This greatly reduces the likelihood of errors. Accurate pipetting is essential.

We further advise:

1. Aliquoting and vortex all components before use (except the Glo reagent).
2. Dialyzing all proteins into the same buffer (lacking glycerol, if possible).
3. Including control samples such as a buffer row at the beginning and end of the plate, a positive control (Ras GTPase), and potentially inert proteins to account for non-canonical effects.
4. Preparing 2× GTP solution, 1 mM ADP, and Glo solution fresh before use (never reuse or store).

If executed properly, the assay leads to reliable and reproducible results (e.g., Tschirpke, Daalman et al., 2023; Tschirpke, van Opstal et al., 2023).

Troubleshooting

For a brief coverage of troubleshooting, see Table 4. Please consult the manufacturer's user manual (Promega) for more detailed discussion of basic problems such as (1) no change in luminescence with increasing/decreasing concentrations of GTPase, GAP, or GEF; (2) low signal-to-background ratio; and (3) high or low luminescence signals.

Understanding Results

The GTPase assay yields luminescence values that are translated into amount of remaining GTP. These values can be fitted with GTPase cycling rates k. The use of example 1 luminescence and cycling rates k_1, k_2 and their pooling will be discussed. Example 2 discusses assay correction factors c_{corr} and cycling rates $k_{3,X}$.

Example 1

In this example, data were collected for five serial dilutions of Ras (spreadsheet: `example1.xlsx` in the `Data` folder; see Data Availability). The assay steps are described in the Basic Protocol and Supporting Information S2, and the analysis is outlined in Support Protocols 1 and 2.

Luminescence. The luminescence values of the buffer rows should always be quite similar (Fig. 7A), although a small shift can be observed. If the shift becomes large, the error bars of the entire assay will increase, because the buffer wells are used for normalization. Addition of Ras should lower luminescence values, as Ras activity reduces GTP concentration (Fig. 7B). With increasing Ras concentrations, the luminescence should drop more. Replicates/wells containing the same Ras concentration should have the same luminescence. The bigger the spread between replicates, the bigger the error will be. In some cases, we observe a small decrease of luminescence over time. As long as this decrease is not drastic and occurs in all wells to roughly the same extent, it will not impact the analysis. Assays in which the luminescence decreases drastically within 20 min should

Table 4 Troubleshooting Guide for GTPase Glo Assays

Problem	Possible cause	Solution
No or low luminescence	Sample/buffer components interfere with assay steps (e.g., fluorescent tags, see Supporting Information S3)	Dialyze sample into fresh buffer (e.g., protein buffer in Basic Protocol); also see assay manual
	One of assay solutions is no longer active	Use fresh aliquots of GTP, DTT, ADP, Glo reagent, and detection reagent
High luminescence	Contamination with ATP, GTP, or other nucleotide triphosphates	Dialyze sample into fresh buffer; also see assay manual
Significant difference in luminescence of the two buffer rows	Two different detection reagent aliquots were used in the same assay (see Supporting Information S7)	Calculate how much detection reagent is needed for the entire assay set and prepare a sufficient volume; if multiple detection reagent aliquots are used, mix well before adding to any assay wells
Large error bars	2× GTP solution was not fresh or was reused (see Supporting Information S6)	Prepare a fresh 2× GTP solution for each assay
Low signal in non-GTPase samples	Contamination with a component that interferes with assay steps or with a GTPase	Dialyze sample into fresh buffer or test a new sample batch
No change in luminescence with increasing GEF concentration	GEF or GTPase batch is not active	Try a new purification batch of GEF or GTPase
	GEF concentration range is too narrow and too low	Try significantly higher GEF concentrations
	Buffer does not contain Mg^{2+}	Add a magnesium salt to the buffer (e.g., 10 mM $MgCl_2$)
	Buffer contains chelating agents (e.g., EDTA) that complex Mg^{2+}	Use a buffer that does not contain chelating agents

be discarded from further analysis, as well as wells that behave differently than the majority.

According to the assay manual, luminescence should be measured after 5 min. We average the luminescence values from 5-20 min for every assay in order to smooth out possible small deviations in the luminescence curve. As long as the time window begins after 5 min, the exact time window is less important than using the same time window for every assay.

The data from this assay can be used to compare the activity of different GTPases, the activity of two different effectors on a single GTPase, or the activity of one effector on different GTPases, as long as all samples are part of the same assay (to avoid small variability between assays) and are used at the same concentration.

GTPase cycling rates k_1, k_2. To increase comparability of GTPase and effector activities, we developed a GTPase cycling model (Supporting Information S8). Support Protocol 2 describes how the model can be used to fit data for Ras. In short, we fit an exponential to the amount of remaining GTP (Supporting Information S8) using Eqn. 1, where [Ras] is used for [GTPase]. Here, K refers to the overall GTP hydrolysis rate and depends on the concentration of Ras. GTPase cycling rates k describe the rate of the *entire* GTPase cycle of Ras. The model fits two cycling rates (k_1 and k_2) for every GTPase. K_1 accounts for the linear contribution of the GTPase ($K_1 = k_1$[GTPase]), while K_2 accounts for the quadratic contribution ($K_2 = k_2$[GTPase]^2), which can be due to dimerization or other cooperative effects increasing the activity of the enzyme (for examples, see Zhang et al., 1999). The bigger the K_2/K_1 ratio, the bigger the contribution of the nonlinear term. This can suggest that the GTPase forms a dimer that is more active than the monomer, but additional studies examining GTPase di- and oligomerization are required before making such a deduction. The data indicate that some effect leads to a nonlinear concentration dependence

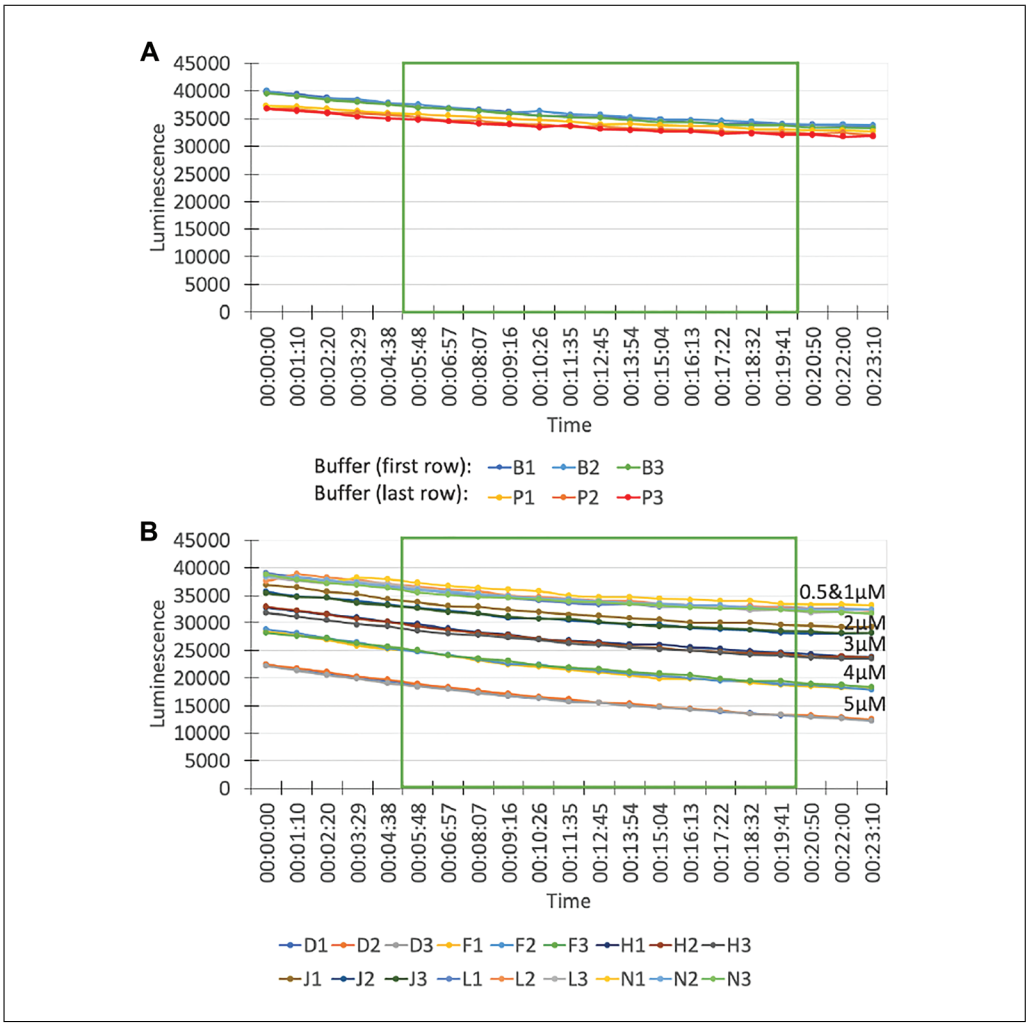
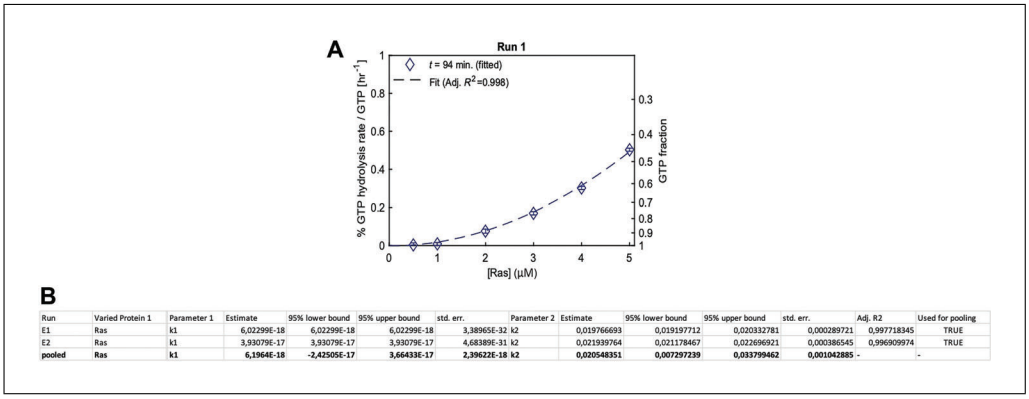


Figure 7 Example 1. Measured luminescence values of buffer wells (**A**) and Ras dilutions (**B**) over the time course of the measurement. The green box indicates values that are used for averaging.



Run	Varied Protein 1	Parameter 1	Estimate	95% lower bound	95% upper bound	std. err.	Parameter 2	Estimate	95% lower bound	95% upper bound	std. err.	Adj. R2	Used for pooling
E1	Ras	k1	6,02299E-18	6,02299E-18	6,02299E-18	3,38965E-32	k2	0,019766693	0,019197712	0,020332781	0,000289721	0,997718345	TRUE
E2	Ras	k1	3,93079E-17	3,93079E-17	3,93079E-17	4,68389E-31	k2	0,021939764	0,021178467	0,022696921	0,000386545	0,996909974	TRUE
pooled	Ras	k1	6,1964E-18	-2,42505E-17	3,66433E-17	2,39622E-18	k2	0,020548351	0,007297239	0,033799462	0,001042885	-	-

Figure 8 Example 1. (**A**) The overall GTP hydrolysis rate K does not scale linearly with Ras concentration. Figure generated automatically when running the Matlab code using `plot_fits = true(1)`. (**B**) GTPase cycling rates k_1 and k_2 for Ras.

of K, but does not reveal its origin. Ras does not show a linear concentration dependence (Fig. 8A) and is dominated by k_2 (Fig. 8B; $k_2 >>> k_1$). In this case, this is likely due to dimerization, as Ras GTPases are known to dimerize.

Output file structure and pooling of k rates. Estimates of Ras cycling rates k_1 and k_2 are summarized in `Data_summary.xlsx` (Fig. 8B). The file is structured in the following way: The Run column shows the assay number and is followed by two Varied Protein

columns stating which proteins were varied in the assay. If the assay contains only one varied protein, the second column is empty (as for this example). Next, Parameter 1 is named along with its values, 95% upper and lower bounds, and standard error. The same is repeated if there are additional parameters (Parameter 2 is shown). The last column lists the adjusted R^2 of the fit and a Boolean ('Used for pooling') if the experiment is used for pooling. For each assay (i.e., each run: E1, E2), the rate value estimates are shown. Below, in the 'pooled' row, the pooled values of k estimates are listed (details on weighting of k for pooling and error propagation are provided in Supporting Information S11).

An experiment can be excluded from pooling if c_{corr} or standard errors of k are out of range. Decision criteria are set before analyzing the data using two parameters (see Support Protocol 2): (1) `conc_corr_bounds` states the range of c_{corr} that allows assays to be included in pooling, and (2) `k_low_err_filt.Ras` states if assays with low standard errors (1^{-10} or lower) on values are excluded from pooling. In Support Protocol 2 (example 1), we used

```
conc_corr_bounds = [0.5 1.5];
k_low_err_filt.Ras = false;
```

for analyzing Ras data. Although `conc_corr_bounds` states the range of c_{corr} that allows assays to be included in the pooling, it does not influence pooling of k_1 and k_2, as c_{corr} is only used for fitting $k_{3,\text{X}}$ (Eqns. 2,3). We set `k_low_err_filt.Ras` to false so experiments with low standard errors on k values are *not* excluded from pooling. This makes sense for Ras, as Ras is dominated by k_2 and has $k_1 \approx 0$ (and thus k_1 errors are close to 0).

Example 2

For this example, data are provided (spreadsheet: `example2-matlab.xlsx` in the `Data` folder; see Data Availability), for the following set of GTPase assays:

(A) Cdc42 (GTPase) dilutions
(B) Cdc24 (GEF) dilutions using a constant Cdc42 concentration
(C) Rga2 (GAP) dilutions using a constant Cdc42 concentration
(D) Cdc24 and Rga2 dilutions using a constant Cdc42 concentration

The analysis is described in Support Protocol 2. In short, a GTPase cycling model (Supporting Information S8) is fitted using Eqn. 4. Here, K refers to the overall GTP hydrolysis rate and depends on the concentrations of Cdc42 and effectors Cdc24 and Rga2. First, the data for Cdc42 serial dilutions (group A) are fitted with an exponential to obtain estimates of GTPase cycling rates k_1 and k_2 for each experiment. These estimates are then bundled to get a single pooled estimate for k_1 and k_2. Next, Cdc42:Cdc24, Cdc42:Rga2, and Cdc42:Cdc24:Rga2 mixtures (groups B, C, and D) are fitted to obtain estimates for the correction factors c_{corr} and cycling rates k_3 (measuring the impact of the effector on GTPase cycling) for every experiment. These latter estimates can then be pooled to obtain a single estimate for the k_3 values (a pooled estimate for c_{corr} has no function here).

Correction factors c_{corr}. The correction factor c_{corr} accounts for variability between assays, i.e., for the observation that the rates for the GTPase can vary between assays. Possible reasons for this include (1) small concentration differences introduced by pipetting of small volumes, (2) temperature and shaker speed fluctuations during incubation, (3) small changes in effective GTPase concentration through sequestration of GTPases in complexes with effectors, and/or (4) intrinsic changes in protein activities due to other external conditions. c_{corr} maps all factors that lead to variations between assays onto the GTPase concentration. The correction factor is estimated for each experiment individually, relative to a predefined GTPase serial dilution assay. This is done by matching the overall hydrolysis rate at zero effector concentration inferred from the fit to the estimated overall hydrolysis rate based on the GTPase serial dilution assay. Concretely, c_{corr} follows from:

$$\hat{k}_1 c_{corr}[\text{GTPase}] + \hat{k}_2 (c_{corr}[\text{GTPase}])^2 = \hat{K}_{mix}$$
$$\text{with } \hat{K}_{mix} = \hat{K}_{mix}([X] = 0)$$

where $\hat{k}_1$ and $\hat{k}_2$ are estimates of cycling rates from the GTPase serial dilution assay and $\hat{K}_{mix}$ is the estimated median of the overall hydrolysis rate fit at zero effector concentration (i.e., $\hat{K}_{mix} = \hat{K}_{mix}([X] = 0)$). By using random draws of the fitted overall hydrolysis rate distribution, we can obtain a distribution for c_{corr} as well. Consequently, $c_{\text{corr}} = 1.0$ means that the GTPase in this assay has the same intrinsic activity relative to the serial dilution assay with the same GTPase (reference assay), which is optimal. Similarly, $c_{\text{corr}} = 0.5$ means that the GTPase in this assay is 50% less active relative to the reference assay, and $c_{\text{corr}} = 1.5$ means

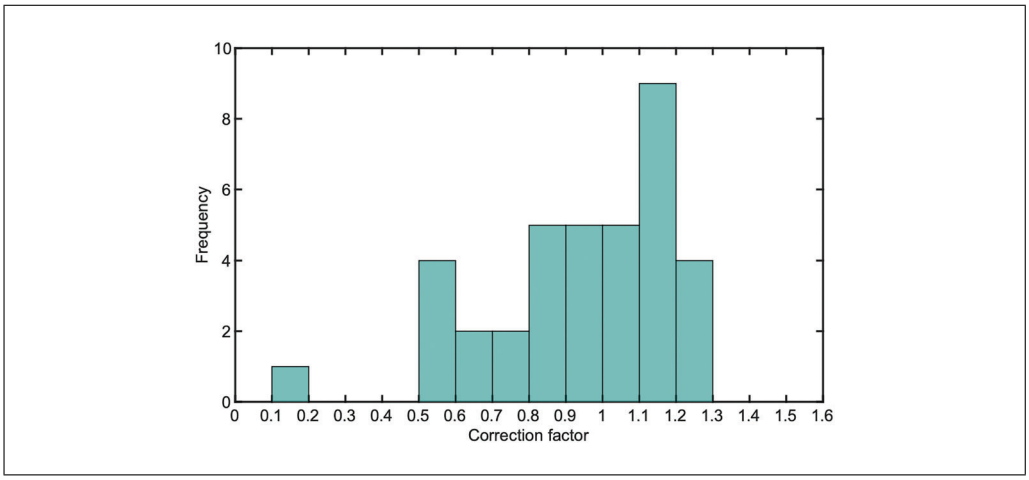


Figure 9 Example 2. Histogram of c_{corr} values using a bin size of 0.1. Generated for all c_{corr} values of example 2 using `Plot_c_corr_histogram.m` (Supporting Information S10) and `example2-histogram.xlsx` in the `example2 matlab output` folder.

that the GTPase in this assay is 50% more active. Very low correction values indicate that the GTPase is behaving differently in the assay than before (possibly because the conditions are different) or that a fundamental error has occurred (e.g., large pipetting error). To ensure consistency within an assay set, we advise disregarding such assays from further analysis.

To implement this recommendation, we used `conc_corr_bounds = [0.5 1.5]` for analyzing the data set in Support Protocol 2, example 1. This means that assays yielding c_{corr} values <0.5 and >1.5 will be excluded from pooling. Figure 9 shows a histogram of all correction factors. Apart from one assay that yielded $c_{corr} \approx 0.1$, all c_{corr} values were within range, with most values being close to 1.

`Data_summary.xlsx` states correction factors as Parameter 1 for assays in which Cdc24 and/or Rga2 effectors are varied (Varied Proteins 1 and 2). Correction factor errors are discussed in Supporting Information S11.

GTPase cycling rates k_3. GTPase cycling rates k describe the rate of the *entire* GTPase cycle of the GTPase or the effect of an effector on the overall rate. Cycling rates $k_{3,X}$ can thus describe how strongly effector X affects the GTPase cycle, but do not reveal which step of the GTPase cycle the effector is acting upon, how strongly the effector is effecting this step, or if multiple steps are affected. To investigate these mechanisms, other GTPase assays need to be conducted (see Background Information).

Assays of group B are used to fit cycling rates $k_{3,Cdc24}$, which describe the effect of Cdc24 on the overall GTPase cycle of Cdc42. We use a quadratic fit ($K_{3,Cdc24} = k_{3,Cdc24}[Cdc42][Cdc24]^2$) because the data show a nonlinear dependence of the overall rate K on Cdc24 concentration (Fig. 10). The fit is a phenomenological description of the data and does not reveal the origin of the nonlinearity. One possibility could be dimerization, with Cdc24 dimers exhibiting increased activity (Tschirpke, Daalman et al., 2023). Values of $k_{3,Cdc24}$ are shown in `Data_summary.xlsx` as Parameter 2 for assays in which Cdc24 is varied (Varied Protein 1). In a similar fashion, assays of group C are used to fit cycling rates $k_{3,Rga2}$, which describe the effect of Rga2 on the overall GTPase cycle rate of Cdc42. A linear fit ($K_{3,Rga2} = k_{3,Rga2}[Cdc42][Rga2]$) was used to describe the data.

Data of group D contain mixtures of Cdc42:Cdc24, Cdc42:Rga2, and Cdc42:Cdc24:Rga2. Both Cdc24 and Rga2 are varied (Varied Protein 1 and 2). The data are fitted with three rate parameters (Fig. 11): $k_{3,Cdc24}$ (Parameter 2), $k_{3,Rga2}$ (Parameter 3), and $k_{3,Cdc24,Rga2}$ (Parameter 4). $k_{3,Cdc24}$ and $k_{3,Rga2}$ describe the effects of Cdc24 or Rga2 on the entire GTPase cycle. These rates should be close to those obtained in assays using only Cdc42:Cdc24 or Cdc42:Rga2 (Fig. 12). A large difference can indicate a problem with the fit or the assay. The cycling rate $k_{3,Cdc24,Rga2}$ accounts for any potential synergy between the two effectors. When $k_{3,Cdc24,Rga2} = 0$, there is no interaction between the proteins. When $k_{3,Cdc24,Rga2} < 0$, the proteins antagonize/inhibit each other, and when $k_{3,Cdc24,Rga2} > 0$ there is synergy. Again, any interaction between the proteins refers to their combined effect on the *overall* rate. (The

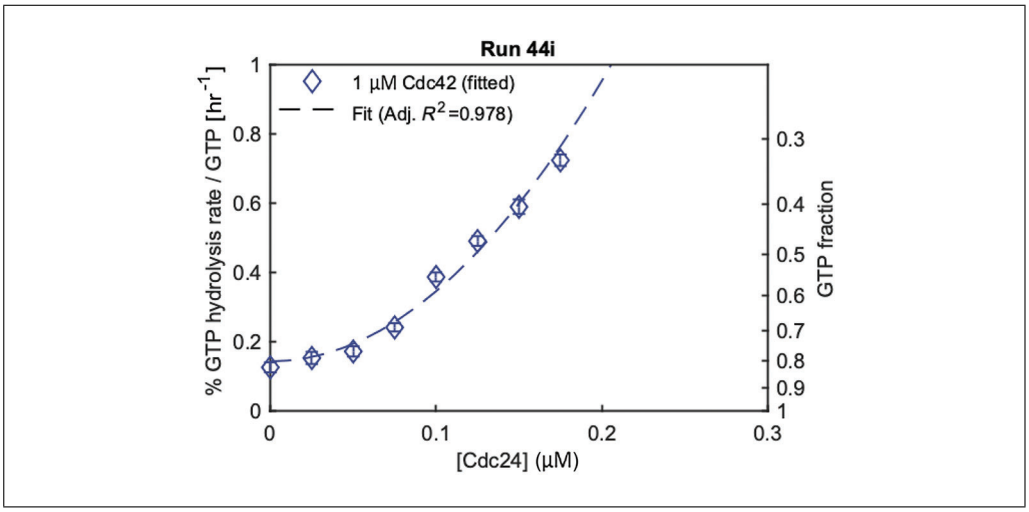


Figure 10 Example 2. The overall GTP hydrolysis rate *K* scales nonlinearly with Cdc24 concentration. Figure generated automatically when running the Matlab code using `plot_fits = true(1)`.

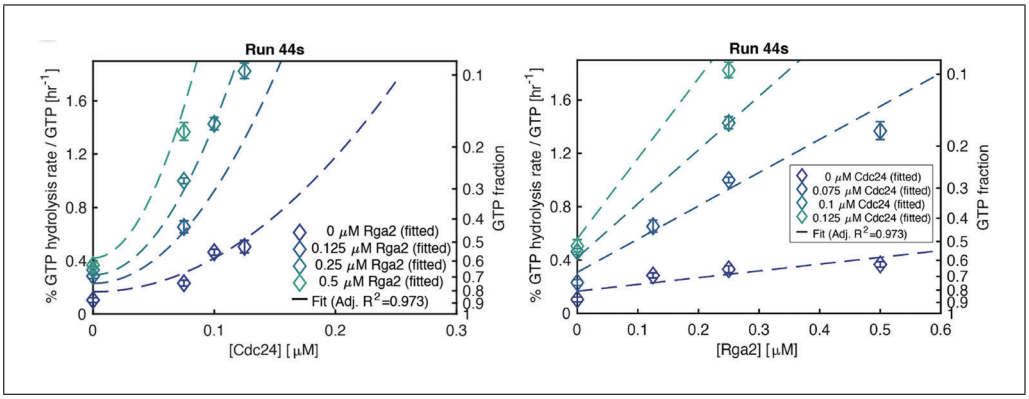


Figure 11 Example 2. Fit of Cdc42:Cdc24:Rga2 mixtures. Figures generated automatically when running the Matlab code using `plot_fits = true(1)`.

significance of the deviation from zero is also tested using Ruxton, 2006). The *p* value for the null hypothesis of zero interaction is stored in the `Data_assays.mat` output file, in the `Assays_processed` variable, in the field of that assay, in the subfield `'k_tot'`, under `'Interaction_p'`. While a positive $k_{3,Cdc24,Rga2}$ indicates that there is some synergy, it does not reveal the origin of the synergy. It could be due to physical protein-protein interactions or to rate-limiting steps in the cycle that are relieved when both proteins are added (Tschirpke, Daalman et al., 2023). One must thus be careful in interpretating this rate.

Comparison of cycling rates k. Overall GTP hydrolysis rates *K* are concentration dependent and have the unit hr^{-1}. Cycling rates *k* are concentration-independent, but the different rates have different units (μM^{-1}hr^{-1} for k_1, μM^{-2}hr^{-1} for k_2, μM^{-3}hr^{-1} for $k_{3,Cdc24}$, μM^{-2}hr^{-1} for $k_{3,Rga2}$, and μM^{-4}hr^{-1} for $k_{3,Cdc24,Rga2}$), making them difficult to compare. The easiest way to represent all rates in one plot is to plot overall GTP hydrolysis rates *K* for 1 μM of each protein (Fig. 12). However, one has to consider the different concentration dependencies when interpreting/comparing *K* rates. A rate that scales quadratically with protein concentration will be twice as big as a rate that scales linearly when the protein concentration is doubled. Some rates may also be valid only for the regime in which they were fitted in (e.g., they may show saturation in higher concentration regimes). Thus, one must consider which protein concentrations are used to calculate *K* in order to make cycling rates *k* comparable.

Time Considerations

GTPase assays usually take a few hours, some of which is consumed by incubation steps. For an assay involving eight samples (including six serial dilutions of GTPase) and using an incubation time of 1.5 hr, we estimate 30 min for preparation of materials and

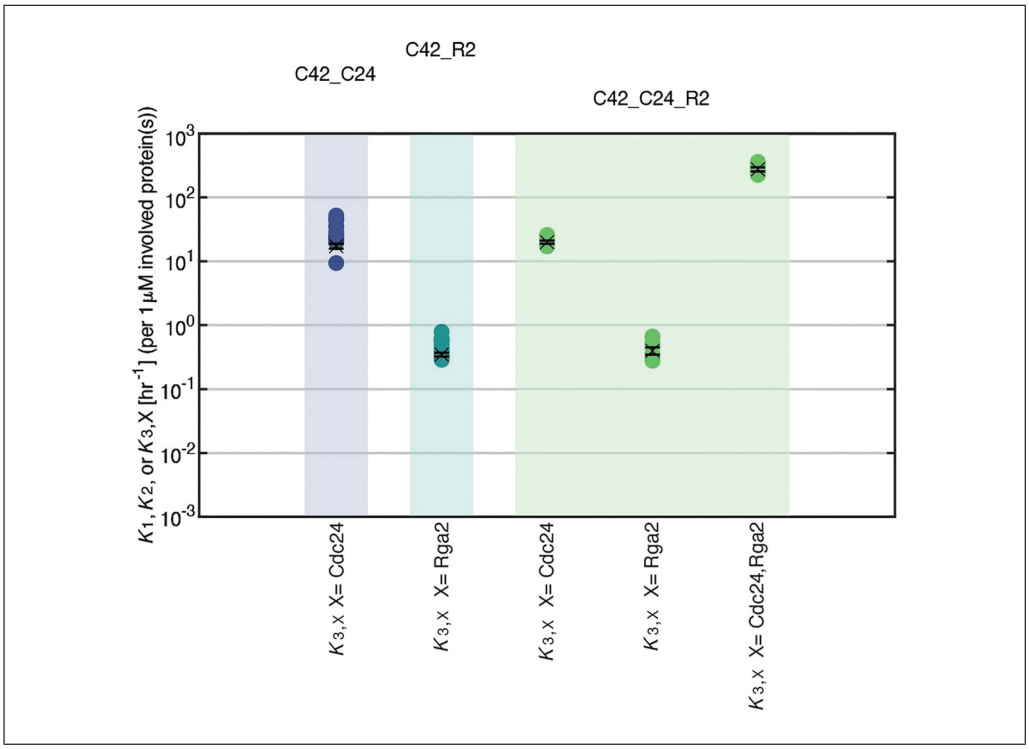


Figure 12 Example 2. Plot of rates $K_{3,X}$ for assays using Cdc42:Cdc24 (purple), Cdc42:Rga2 (blue), and Cdc42:Cdc24:Rga2 (green). Rates of individual experiments are shown as filled dots, the average is shown as a cross, and error bars represent the standard error. Generated for assay names C42-C24, C42-R2, and C42-C24-R2 using `Plot_pooled_values_std_err.m` (see Supporting Information S10) and `Data_assays.mat` in the `example2 matlab output` folder.

solutions, 2 hr for conducting the assay, and another 30 min for the luminescence readout. This amounts to a total of 3 hr, including 2 hr for incubation/measurement steps. Assays encompassing more samples may involve longer preparation times (as more protein dilutions need to be prepared). If proteins need to be dialyzed into a suitable buffer first, an additional dialysis step is required. The analysis time depends on the level of automatization and can range from a few minutes to 30-40 min. If the first step is done manually in a spreadsheet editor, ~30 min are needed for this step. The subsequent analysis in Matlab requires only a few minutes of runtime.

Acknowledgments

We thank D. McCusker (University of Bordeaux) for plasmid pDM272 and N. Dekker (TU Delft) for plasmid pET28a-His-mcm10-Sortase-Flag. L. Laan gratefully acknowledges funding from the European Research Council under the European Union's Horizon 2020 Research and Innovation Programme (grant agreement 758132) and from the Netherlands Organization for Scientific Research (Nederlandse Organisatie voor Wetenschappelijk Onderzoek) through a Vidi grant (016.Vidi.171.060). S. Tschirpke gratefully acknowledges funding from the Kavli Synergy Post-doctoral Fellowship program of the Kavli Institute of Nanoscience Delft.

Author Contributions

Sophie Tschirpke: Conceptualization; formal analysis; investigation; methodology; project administration; validation; visualization; writing—original draft; writing—review and editing. **Werner Daalman:** Conceptualization; formal analysis; investigation; methodology; software; validation; writing—original draft; writing—review and editing. **Liedewij Laan:** Funding acquisition; project administration; supervision; writing—review and editing.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

The data that support and exemplify the protocols are openly available at *https://data.4tu.nl* at *http://doi.org/10.4121/ac196f25-1c20-4c0c-a0b9-f01cd3fadc45*.

Supporting Information

cpz11000-sup-0001-SuppMat.pdf

Supporting Information S1. Preparation sheets for conducting GTPase assays with one, two, or three sample rows.

Supporting Information S2. Prepared plate and preparatory sheet for a GTPase assay of six Ras GTPase serial dilutions (example 1).

Supporting Information S3. Examples of how fluorescently labeled proteins affect the luminescence readout of GTPase assays.

Supporting Information S4. Verification that the GTP concentration in GTPase assays declines exponentially with time.

Supporting Information S5. Luminescence spill-over in directly neighboring plate wells.

Supporting Information S6. Demonstration that 10 mM ADP can be reused, but 2× GTP solution cannot.

Supporting Information S7. Luminescence values can vary between distinct detection reagent batches.

Supporting Information S8. Brief description of the GTPase activity model used to determine GTPase cycling rates.

Supporting Information S9. Description of the Python script used to reformat data in Support Protocol 1.

Supporting Information S10. Description of plotting functions referred to in Support Protocol 2.

Supporting Information S11. Extended description of the GTPase activity model used to determine GTPase cycling rates.

Literature Cited

Bezeljak, U., Loya, H., Kaczmarek, B., Saunders, T. E., & Loose, M. (2020). Stochastic activation and bistability in a Rab GTPase regulatory network. *Proceedings of the National Academy of Sciences of the United States of America*, *117*(12), 6540–6549. https://doi.org/10.1073/pnas.1921027117

Bos, J., Rehmann, H., & Wittinghofer, A. (2009). GEFs and GAPs: Critical elements in the control of small G proteins. *Cell*, *16*(3), 374–383. https://doi.org/10.1016/j.cell.2007.05.018

Cherfils, J., & Zeghouf, M. (2013). Regulation of small GTPases by GEFs, GAPs, and GDIs. *Physiological Reviews*, *93*(1), 269–309. https://doi.org/10.1152/physrev.00003.2012

Kang, P. J., Béven, L., Hariharan, S., & Park, H. O. (2010). The Rsr1/Bud1 GTPase interacts with itself and the Cdc42 GTPase during bud-site selection and polarity establishment in budding yeast. *Molecular Biology of the Cell*, *21*(17), 3007–3016. https://doi.org/10.1091/mbc.e10-03-0232

Kohyama, S., Merino-Salomón, A., & Schwille, P. (2022). *In vitro* assembly, positioning and contraction of a division ring in minimal cells. *Nature Communications*, *13*(1), 6098. https://doi.org/10.1038/s41467-022-33679-x

Loose, M., Fischer-Friedrich, E., Ries, J., Kruse, K., & Schwille, P. (2008). Spatial regulators for bacterial cell division self-organize into surface waves *in vitro*. *Science*, *320*(5877), 789–792. https://doi.org/10.1126/science.1154413

Rapali, P., Mitteau, R., Braun, C., Massoni-Laporte, A., Ünlü, C., Bataille, L., Arramon, F. S., Gygi, S. P., & McCusker, D. (2017). Scaffold-mediated gating of Cdc42 signalling flux. *eLife*, *6*, 1–18. https://doi.org/10.7554/eLife.25257

Ruxton, G. D. (2006). The unequal variance t-test is an underused alternative to Student's t-test and the Mann-Whitney U test. *Behavioral Ecology*, *17*(4), 688–690. https://doi.org/10.1093/beheco/ark016

Smith, N. J., van der Walt, S., & Firing, E. (2015). Magma, inferno, plasma and viridis colormaps. https://github.com/bids/colormap

Tschirpke, S., Daalman, W., & Laan, L. (2023). The GEF Cdc24 and GAP Rga2 synergistically regulate Cdc42 GTPase cycling. *BioRxiv*, https://doi.org/10.1101/2023.06.26.546500

Tschirpke, S., van Opstal, F., van der Valk, R., Daalman, W. K.-G., & Laan, L. (2023). A guide to the *in vitro* reconstitution of Cdc42 activity and its regulation. *BioRxiv*, https://doi.org/10.1101/2023.04.24.538075

Vendel, K. J. A., Tschirpke, S., Shamsi, F., Dogterom, M., & Laan, L. (2019). Minimal *in vitro* systems shed light on cell polarity. *Journal of Cell Science*, *132*(4), 1–21. https://doi.org/10.1242/jcs.217554

Vetter, I. R., & Wittinghofer, A. (2001). The guanine nucleotide-binding switch in three dimensions. *Science*, *294*(5545), 1299–1304. https://doi.org/10.1126/science.1062023

Zhang, B., Gao, Y., Moon, S. Y., Zhang, Y., & Zheng, Y. (2001). Oligomerization of Rac1 GTPase mediated by the carboxyl-terminal polybasic domain. *Journal of Biological Chemistry*, *276*(12), 8958–8967. https://doi.org/10.1074/jbc.M008720200

Zhang, B., Wang, Z. X., & Zheng, Y. (1997). Characterization of the interactions between the small GTPase Cdc42 and its GTPase-activating proteins and putative effectors: Comparison of kinetic properties of Cdc42 binding to the Cdc42-interactive domains. *Journal of Biological Chemistry*, *272*(35), 21999–22007. https://doi.org/10.1074/jbc.272.35.21999

Zhang, B., Zhang, Y., Collins, C. C., Johnson, D. I., & Zheng, Y. (1999). A built-in arginine

finger triggers the self-stimulatory GTPase-activating activity of Rho family GTPases. *Journal of Biological Chemistry*, *274*(5), 2609–2612. https://doi.org/10.1074/jbc.274.5.2609

Zhang, B., & Zheng, Y. (1998). Negative regulation of Rho family GTPases Cdc42 and Rac2 by homodimer formation. *Journal of Biological Chemistry*, *273*(40), 25728–25733. https://doi.org/10.1074/jbc.273.40.25728