Quantification of GTPase cycling rates of GTPases and GTPase : effector mixtures using GTPase Glo™ assays

Sophie Tschirpke[†]*, **Werner K-G. Daalman**[†]*, **Liedewij Laan**[†]**

[†] Bionanoscience Department, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands
* equally contributing authors
** corresponding author; L.Laan@tudelft.nl

Abstract

In different cellular activities like signal transduction, cell division, and intracellular transportation, small GTPases take on a vital role. Their functioning involves hydrolysing guanosine triphosphate (GTP) to guanosine diphosphate (GDP). In this article we explain the application of a commercially accessible GTPase assay, known as the GTPase Glo™ assay by Promega, for the quantitative investigation of GTPase - effector interactions and the interplay between effectors.
Basic Protocol: Conducting GTPase assays with GTPase : effector protein mixtures using the GTPase Glo™ assay (Promega).
Supporting Protocol 1: Analysing GTPase assays to correlate the assay readout (luminescence) to amount of remaining GTP.
Supporting Protocol 2: Fitting GTPase assay data to obtain GTPase cycling rates.

Keywords

Cdc42, GTPase activity, GTPase assay, GTPase effectors, GTPase cycling rates;

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 GTPase 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) boost step 2 and GDP/GTP exchange factors (GEFs) enhance step 3 [Cherfils and Zeghouf, 2013, Bos et al., 2009, Vetter and Wittinghofer, 2001]. Several assays exist that examine single GTPase cycle steps, which help us understand the mechanistic details of these cycle steps and their regulation. At the same time, they do not allow studying the interplay of effectors acting on different GTPase cycle steps. Furthermore, approaches that reconstitute the complex cellular functions of GTPases *in vitro* [Vendel et al., 2019, Loose et al., 2008, Bezeljak et al., 2020, Kohyama et al., 2022] 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 (GTPase Glo™ assay (Promega), from here on referred to as 'GTPase assay') can be used to quantitatively study GTPase effector interactions and effector interplay and to easily activity test GTPase and effector purification batches. In the GTPase assay the proteins of interest are incubated with GTP for a certain amount of time for GTPase cycling to occur. Then the reaction is stopped and the amount of remaining GTP, measured as luminescence signal, is determined. GTPase cycling rates can be obtained from this data through 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. We like to note that we do not advice to use this protocol if single GTPase steps are to be examined in-depth, but if effects of a protein on the entire GTPase cycle are of interest. This method is exemplified for a basic GTpase assay using the human Ras (GTPase) (example 1) and for an elaborate GTpase assay set using *S. cerevisae* Cdc42 (GTPase), Cdc24 (GEF), and Rga2 (GAP) [Tschirpke et al., 2023a] (example 2). Tab. 1 summarises how these examples are used to illustrated the protocols described herein. It can also be applied to other GTPases and their effectors. In the Basic Protocol we outline the workflow for conducting GTPase assays using the GTPase Glo™ assay (Promega). In Supporting Protocol 1 we describe how the GTPase assays data (luminescence) can be converted to amounts of remaining GTP. In Supporting Protocol 2 we describe how data obtained by Supporting Protocol 1

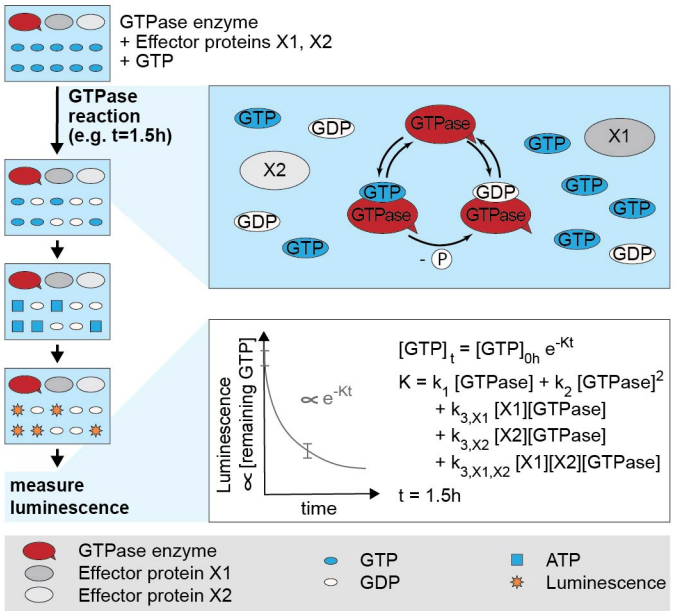

Figure 1. Schematic illustration of the assay steps and GTPase cycling reaction in the GTPase Glo™ assay (Promega). A GTPase, alone or in combination with effector proteins, is incubated with GTP at for a certain time (e.g 1.5 h) in which GTPase cycling occurs. After two processing steps, the amount of remaining GTP, measured as luminescence, is assessed. GTP hydrolysis cycling rates can be extracted by fitting the data with an exponential model.

Abbreviations:

AA	amino acid
GAP	GTPase activating protein
GEF	GDP/GTP exchange factor

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can be fitted with a GTPase cycling model (and the by us developed, openly available Matlab code) to obtain GTPase cycling rates.

	Example 1: **A basic GTPase assay**	**Example 2:** **An elaborate GTPase assay set**
Experimental procedure		
Basic Protocol	described using this example (+ see S2)	-
Strategic planning	-	features consideration absolutely necessary for this assays set (esp. 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 the of amount of remaining GTP from luminescence values	described using this example → input data: 'example1.xlsx' (tab: 'E1', 'E2') → output data: same file as input data → re-formatting of SP 1 output data to generate SP 2 input data: see S9	-
Support Protocol 2: Fitting GTPase cycling rates using an exponential model (S8)	described using this example → input: 'example1.xlsx' (tab: 'matlab'), 'Assaylist-example1.xlsx' → output in folder 'example1 matlab output': 'Data_summary.xlsx', 'Data_assays.mat', figure folder → fitting parameters: k_1, k_2	described using this example → input: 'example2-matlab.xlxs', 'Assaylist-example2.xlsx' → output in folder 'example2 matlab output': 'Data_summary.xlsx', 'Data_assays.mat', figure folder → fitting parameters: k_1, k_2, $k_{3,Cdc24}$, $k_{3,Rga2}$, $k_{3,Cdc24,Rga2}$, c_{corr}
Understanding Results		
Explanation of ...	Luminescence k_1, k_2 output file structure pooling of rates k	c_{corr} $k_{3,Cdc24}$, $k_{3,Rga2}$, $k_{3,Cdc24,Rga2}$ Comparison of rates k

Table 1. Summary of how the examples are used throughout the protocols. Data files are available at data.4tu.nl. More information can be found in the section: Data Sharing and Data Availability.

Strategic Planning

If the GTPase assay is done for the first time, we advice to first practice pipetting of the required small volumes into the wells using coloured water. We advice to conduct smaller assays using a larger number of replicas per sample (e.g. 8 samples rows per assay with 5 replicas each, as described in the basic protocol) first, before moving on to larger assay sets with more proteins.

Once you feel more routined with the assay, larger assay sets can be conducted. For example, assays in which the interaction between a GTPase and one or several effectors is studied. The following section applies predominantly (but not solely!) to such larger assays sets involving multiple GTPase enzymes and/or effector proteins (example 2). We advice to determine before starting which assays with how many proteins will need to be carried out. Specifically, we advice to:

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 luminescence (e.g. fluorescent tags, see S3) and can alter it are therefore not suitable for this assay.

Buffers containing ADP, ATP, GDP, GTP, and guanosine phosphate analogues are also not suitable for the GPase assay, as they interfere with other GTPase assay reactions.

We advice to consider the buffer components and verify that used effector proteins (proteins that are not GTPase enzymes) show no seemingly GTPase activity.

Verify that the used incubation times lay in the exponential decline regime of remaining GTP

In the GTPase assay the proteins of interest are incubated with GTP for a certain amount of time, in which GTPase cycling can occur. Then the reaction is stopped and the amount of remaining GTP, measured as luminescence signal, is determined. GTPase cycling rates can be obtained from this data through using a coarse-grained exponential fitting model (Fig. 1, S8). The model is based on our observation that the amount of remaining GTP declines exponentially with time (S4). It is still advisable to verified that this is also true for the proteins (GTPase enzymes and GTPase:effector mixtures) and incubation times intended to be used.

To obtain such data, prepare one batch of serial dilutions of the to-be-used proteins. Use exactly these dilutions to conduct several assays using different incubation times. Check if the amount of remaining GTP of these assays follows an exponential decline. Further, analyse each assay individually using our GTPase cycling model. Verify that different incubation times (= different assays) yield the same rates (e.g. S4).

Dialyse all to-be-used proteins into the same buffer

All proteins should ideally be in the same buffer. In the assay the protein activity is determined by normalising the assay readout (luminescence) of that protein well to that of 'buffer only'. Differences between those two (in ion concentrations, detergents, or other additives (such as glycerol)) can lead to differences in luminescence. This is easiest avoided if all proteins used in one assay set are in the same buffer. We advice 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, and other guoanosine analogues, ADP, ATP, and other nucleotide triphosphates, EDTA or other chelating agents that can complex magnesium. If a GTPase GEF interaction ought to be investigated, the buffer usually requires magnesium salts (e.g. 10 mM $MgCl_2$).

Many proteins require glycerol in the buffer for storage. We advice to keep the protein at a high concentration 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 a buffer of the same composition *without* glycerol (e.g. 50 mM Tris-HCl (pH=7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol). 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 sample in the GTPase reaction will be negligible.

If it's not possible to transfer all proteins into the same buffer, it needs to be verified that the differences in buffer composition do not affect luminescence intensities. A GTPase assay containing only the different buffer mixtures can elucidate

this: If all buffer mixtures exhibit the same luminescence values then the difference in buffer composition do not affect luminescence. However, it is still possible that the buffer components affect protein behaviour!

Use the same serial dilution for all assays:

The assay is sensitive to concentration changes (especially of highly active GPases and of effectors strongly boosting GTPase activity), and thus sensitive to any pipetting errors when preparing the protein dilutions. We advice to prepare serial dilutions of every protein, and use exactly the same serial dilution for the entire assay series. Different serial dilutions of the same protein can exhibit slightly different rates. For example, if the effect of effector protein X on GTPase A and GTPase B ought to be compared, the same serial dilution of effector X ought to be used for both GTPases.

Include a reference sample if data from different assays should be compared

GTPase assays can be sensitive to GTPase activity changes, which can be due to small pipetting errors or condition changes (incl. temperature, shaker speed, ...). This can lead to slight changes in protein activity between separate assays. It is therefore best to include all samples that ought to be compared into the same assay.
However, this is not always possible. If the strength of several effectors on one GTPase enzyme ought to be compared, we advice to always include one sample containing only the GTPase into each assay (see Fig. 2). Then the assay-specific activity of the GTPase sample can be used to account for any assay-specific variability (through fitting the correction factor c_{corr}, as will be explained in later sections and in S8 Eq. 9).
(Usually these assay-variations are very small ($c_{corr} \approx 1.0$) [Tschirpke et al., 2023b, Tschirpke et al., 2023a]. We advice to exclude assays with a big or very small c_{corr}, as these indicate that the GTPase behaviour/assay conditions are unusual.)

Determine useful concentration ranges before conducting an assay series

In our experience a good readout regime is between 5-90% remaining GTP. Higher values (90-100% remaining GTP) have a bigger error (due to the normalisation), and in lower regimes (0-5% remaining GTP) saturation effect due to little remaining GTP might take place. In this 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.

Make an assay plan

If multiple protein interactions ought to be quantified and compared, the same serial dilutions of these proteins ought to be used. Given that we advice against the use of a glycerol-containing buffer, that many proteins require glycerol when being frozen, and that many proteins are not stable for weeks at room temperature or 4°C, all required assays need to be conducted within a rather short time period to obtain reliable and comparable results. We therefore strongly recommend to carefully plan which assays with which proteins need to be conducted, and calculate the required volumes of protein needed.
We will explain our recommended procedure on the example of a GTPase assay series that investigates the GTPase cycling of a GTPase, and the effect of both a GEF and a GAP, alone and in combination, on it (example 2, Fig. 2) [Tschirpke et al., 2023a]:
Considerations (as we described earlier)**:** (1) Different serial dilutions of the same protein might exhibit slightly different rates (due to small pipetting errors when making the dilution). Hence, the same dilution should be used for the entire assay set. (2) For larger assays it is advisable to already know which concentration ranges give good signal to reduce the time the proteins need to be stored. (3) Include a reference sample (here: sample of the GTPase enzyme alone in the concentration used in the particular assay). (4) The incubation times used are in the exponential regime. (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 readout regimes.
Protein dilutions: To conduct the assay, several protein solutions need to be made: Protein dilutions of GTPase, GEF,

and GAP that will be used to conduct the entire assay set (Fig. 2 left column). These starting dilutions will be used to create protein mixtures, that will be used to for one GTPase assay (Fig. 2 middle column).

Which concentrations should these starting dilutions have? To initiate the GTPase reaction, a 2× GTP solution is mixed in a 1:1 volume ratio with the protein mixtures. Therefore, the protein mixture needs to be 2× of the final concentration in the assay. Further, in the final assay the protein mixture needs to contain the GTPase, GEF, and GAP. It is easiest to make this mixture if all proteins can be mixed at 1:1:1 volume ratios. Hence, the initial starting protein serial dilutions, which will be used for all assays of the set, should be 6× stocks of the final protein concentrations. (Similarly, if an assay set will contain at maximum a mixture of 4 (5) proteins, the initial starting protein serial dilutions need to be 8× (10×) stocks of the final protein concentrations.

Procedure: In which order should the assays be conducted? First, a GTPase assay with a serial dilution of only the GTPase enzyme is conducted (Fig. 2 top row). Pick one or two GTPase concentrations to conduct all following assays with. In this case the following assays will involve a GEF and a GAP, both of which boost the activity of the GTPase. It is therefore wise to choose a GTPase concentration that leads to a high amount of remaining GTP (e.g. 90-80% remaining GTP). Next, a GTPase assay with the GTPase and several GEF concentrations (obtained from serial dilutions) is performed. Use the same GTPase concentration for all samples; samples of GTPase:GEF mixtures, and a sample containing only the GTPase (Fig. 2 second row). The same procedure is applied to the GAP: Conduct a GTPase assay with the GTPase and several GAP concentrations (obtained from serial dilutions). Use the same GTPase concentration for all samples; samples of GTPase:GAP mixtures, and a sample containing only the GTPase (Fig. 2 third row). Lastly, perform GTPase assays in which all proteins are present (GTPase alone, GTPase:GEF mixtures, GTPase:GAP mixtures, and GTPase:GEF:GAP mixtures, all using the same GTPase concentration). Choose GEF and GAP concentrations that resulted in a medium amount of remaining GTP (e.g. 60-70% remaining GTP), 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).

Control for non-canonical effects through adding an inert protein

We observed that the non-canonical effects can take place during GTPase cycling in the GTPase assay. These effects are generally small, and can be accounted for: For example, we observed that both Bovine serum albumin (BSA) and Casein slightly boost GTPase activity of both Ras and Cdc42. BSA and Casein are considered inert and have no known interaction with neither Ras or Cdc42. We suspect that they seemingly boost the GTPase's activity through increasing the effective GTPase concentration: A few GTPase molecules might stick to the well wall in each reaction chamber, being rendered inactive. Through adding another protein the chamber wall will be covered with both some GTPase molecules and some molecules of 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 advice to additionally conduct assays with the GTPase and an inert protein (e.g. BSA or Casein). The effect of the to-be-studied protein will need to exceed that of a equimolar concentration of the inert protein to be of non-canonical origin.

Both BSA and Casein are considered inert proteins. However, BSA is strongly negatively charged, which could lead to non-specific protein interactions. Therefore Casein, or another supposedly inert protein, might be more suitable as a control.

For the previously used example 2 (an assay series including a GTPase, GEF, and GAP, Fig. 2), control assays for non-canonical effects include: (1) GTPase:Casein serial dilution, (2) GTPase:GEF:Casein, (3) GTPase:GAP:Casein.

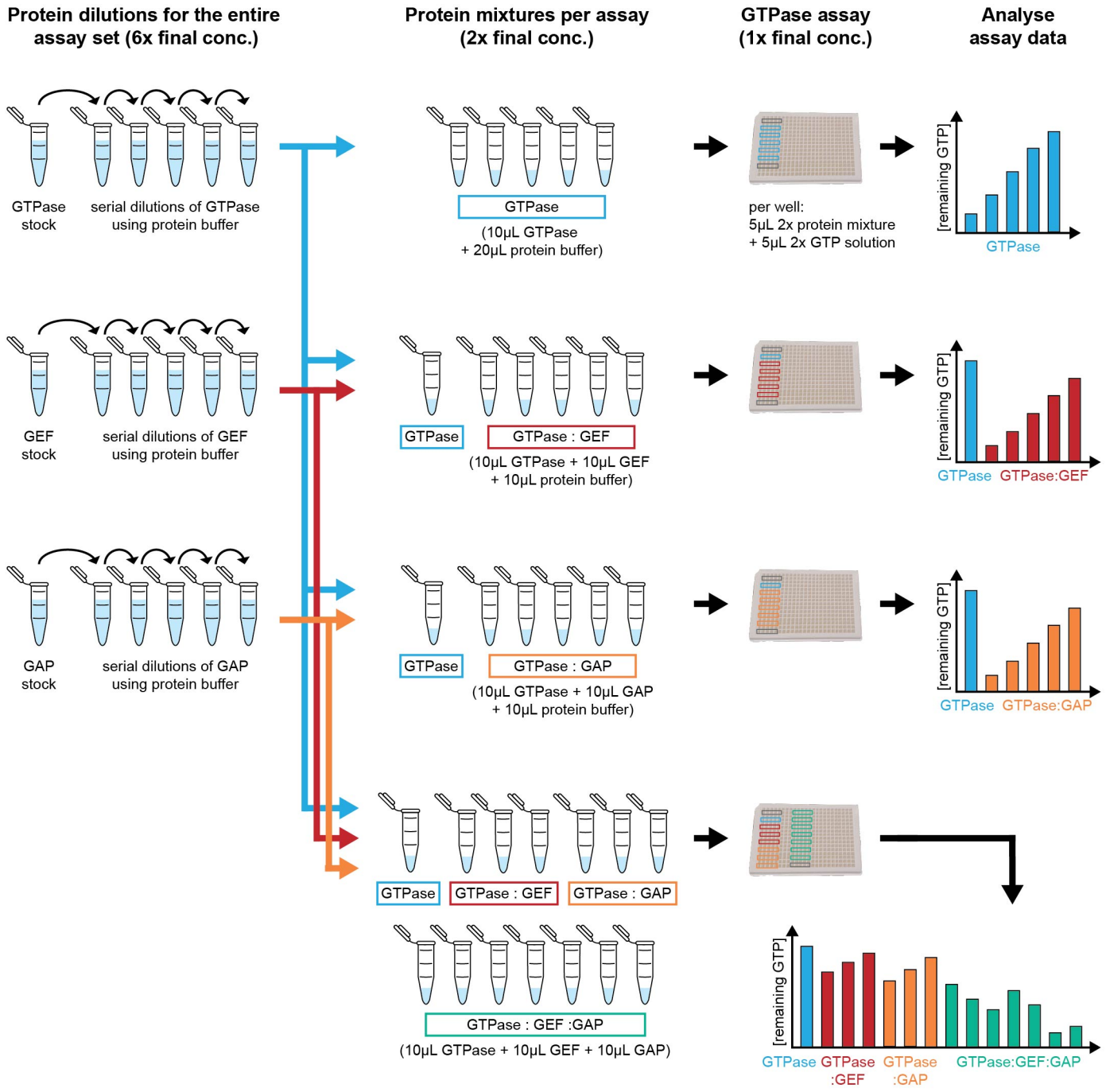


Figure 2. Schematic illustrating the assays and protein dilutions required for conducting a GTPase assay set investigating GTPase, GEF, and GAP actitvity and GEF-GAP interaction (example 2).

Basic Protocol: Conducing GTPase assays

In the GTPase assay the proteins of interest (GTPase enzymes with or without effectors) are incubated with GTP for a certain amount of time for GTPase cycling to occur. Then the reaction is stopped. The remaining GTP is, through two follow up reactions, translated into luminescence signal, which is measured (Fig. 1).

The following protocol steps are, with only minor modifications, those described in the assay manual. The volumes are for assays conducted in 384-well microplates. Assays using larger volumes can be conduced using different plates (see GTPase Glo™ assay manual). In addition to following the steps described here we advice to carefully read the assay manual [Promega Corporation, 2015].

The following protocol describes the steps to conduct a GTPase assay using 6 concentrations of Ras GTPase (example 1) (Fig. 3). An illustration of required assay planning steps is shown in S2. The assay results in data found in 'example1.xlsx' (tab: 'E1', 'E2').

Materials

- GTPase Glo™ assay (Promega, cat. no. V7681 (1'000 reactions) or V7682 (10'000 reactions))
- proteins of interest: a GTPase enzyme (and optionally effector proteins). As a test GTPase human Ras (EMD Millipore, cat. no. 553325) can be used.
- buffer in which the proteins of interest are stored ('protein buffer')
- optional: Casein (Sigma-Aldrich, cat. no. C7078) or Bovine serum albumin (BSA) (Thermo Scientific, cat. no. 23209)
- 1.5 mL and 15 mL reaction tubes
- 384-well white flat bottom microplates (Corning, cat. no. 3572)
- parafilm (Bemis Company Inc., cat. no. PM996)

Equipment

- pipettes: 0.5 µL, 10 µL, 20 µL, 100 or 200 µL, 1 mL
- vortex mixer (e.g. Thermo Scientific, cat. no. 88882011)
- Mini centrifuge (e.g. Thermo Scientific, cat. no.75004061)
- orbital shaker (e.g. Innova 2300 platform shaker (New Brunswick Scientific))
- optional: temperature-controlled incubator
- a plate reader that can measure luminescence (e.g. Synergy HTX plate reader from BioTek)

Preparation of proteins

1. Dialyse the GTPase enzyme into a suitable buffer ('protein buffer').

   E.g. 50 mM Tris-HCl (pH=7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol. More considerations on the buffer are described in the strategic planning section.

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

Before starting the assay

2. 'Mise-en-place': Decide on the assay scope and prepare all assay materials needed. Templates can be found in S1. An example for an assay of Ras GTPase is shown in S2 and Fig. 3.

   (a) Decide which samples and how many replica wells per sample you want to use.

   We recommend 3-5 replicas per sample. In the beginning In the beginning we recommend using one GTPase enzyme per assay and 5 replicas. We suggest using 8 samples per assay: 6 concentrations of the GTPase enzyme (here: Ras GTPase) + 2 protein buffer samples (for normalisation) (Fig. 3).

   (b) Calculate which volumes of protein dilutions, 2× GTP solution, Glo solution, and detection reagent are needed to conduct one assay (example calculation are given in S2).

Vol.(protein dilution) = 5μL × number of replicas

Vol.(2× GTP solution) = 5μL × number of replicas per sample × number of samples

Vol.(Glo solution) = 2 × Vol.(2× GTP solution)

Vol.(detection reagent) = 4 × Vol.(2× GTP solution)

We recommend to always prepare a bit more of the solutions that needed.

We recommend preparing 5μL more of each protein dilution than calculated, to ensure that there is also sufficient solution for the last well. E.g. for 5 replicas per sample, for each dilution prepare 5μL × 5 + 5μL = 30μL.

(c) Calculate the dilution series steps for proteins used. Note that the solutions prepared need to be 2× of the final concentration in the assay (example calculation are given in S2).

(d) Prepare the plate: seal the assay wells that will not be used with parafilm to prevent contamination with dirt, and label/mark the wells where sample will be added.

Leave one empty row between all sample rows to avoid any spill-over of luminescence signal between samples (see Fig. 3 and S5).

(e) Cut 4 pieces of parafilm that are sufficiently big to cover the area where samples will be added.

Conducting GTPase Glo^{TM} assays

3. Thaw protein samples and assay solutions on ice.
4. Serial dilute the Ras GTPase enzyme with protein buffer (according to your calculation) to obtain 2× GTPase enzyme dilutions (S2 and Fig. 3a).

   Vortex for proper mixing, then spin down mildly to collect all volume in the bottom of the tube.
5. Prepare a 2× GTP solution. A pipetting scheme is given in Tab. 2.

   The 2× GTP solution should always be freshly prepared immediately before starting the assay. Vortex the GTP and DTT before use. Vortex the 2× GTP solution for proper mixing. The 2× GTP solution can only be used freshly and should not be stored/frozen (S6). We advice to aliquot GTP and DTT to reduce the number of freeze-thaw cycles, in our experience up to 3 freeze-thaw cycles do not impact assay performance.
6. Add 5μL 2× GTP solution to all wells (Fig. 3b).
7. Add 5μL protein buffer and 2× Ras GTPase dilutions to designated wells (Fig. 3c).

   We advice to have one row containing protein buffer on top of the assay and one row in the bottom. In rare cases the luminescence values slightly drift towards lower values throughout the assay. Placement of a buffer row as a first and last sample row ensures that this drift can be (1) detected and (2) accounted for in the analysis.
8. Seal the wells with two sheets of parafilm and incubate for a designated time on an orbital shaker.

   We recommend to seal the wells through placing one sheet of parafilm on top of the plate (without stretching it) and firmly pressing it into to plate with a rounded surface (such as sissor handles). Then repeat the same process with the second sheet of parafilm. This ensures that the wells are tightly closed. Using only one sheet of parafilm can result insufficient sealing (small holes in the parafilm from the too much pressure).

   The incubation time and temperature depends on the protein of interest and used concentrations. We recommend choosing a temperature in accordance to the protein's environment in vivo. We recommend incubation times of 60-90 min.
9. Prepare the Glo solution a few minutes before the incubation time is reached. A pipetting scheme is given in Tab. 3. Then add 10μL Glo solution to all wells (Fig. 3e).

   Preparation of the Glo solution:

   (a) Vortex the Glo buffer and 10 mM ADP before use. Do NOT vortex the GTPase-Glo reagent. Instead, gently tap it for proper mixing.

   (b) To use the pipetting scheme provided in Tab. 3, 10 mM ADP provided in the assay kit needs to be diluted to 1 mM ADP using ultra pure water!

   (c) We advice to aliquot 10 mM ADP, Glo buffer, and Glo reagent to reduce the number of freeze-thaw cycles. These aliqouts can be re-used (S6).

(d) *Both 1 mM ADP and Glo solution can only be used freshly and should not be stored/frozen. Vortex both for proper mixing.*

(e) *The Glo reagent is the reagent that usually runs out first. To increase the number of assay runs per kit, prepare only the volume needed.*

10. Seal the wells with two sheets of parafilm and incubate for 30 min on an orbital shaker.
11. Add 20µL detection reagent to all wells (Fig. 3g).

    We advice to aliquote 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. In some cases we observed that two seperately stored aliquots from the same batch can lead to distinct luminescence values (S7). To avoid such a shift within an assay, pull a sufficient volume into one tube (e.g. pull several aliquots) before adding the detection reagent to the wells. Vortex for proper mixing.
12. Measure the luminescence of each well using a plate reader for 20 min.

    Activate orbital shaking between measurements to ensure proper mixing. If the plate reader does not have a shaking option, incubate the plate for 5 min on an orbital shaker before measuring luminescence. Then measure for 15 min.

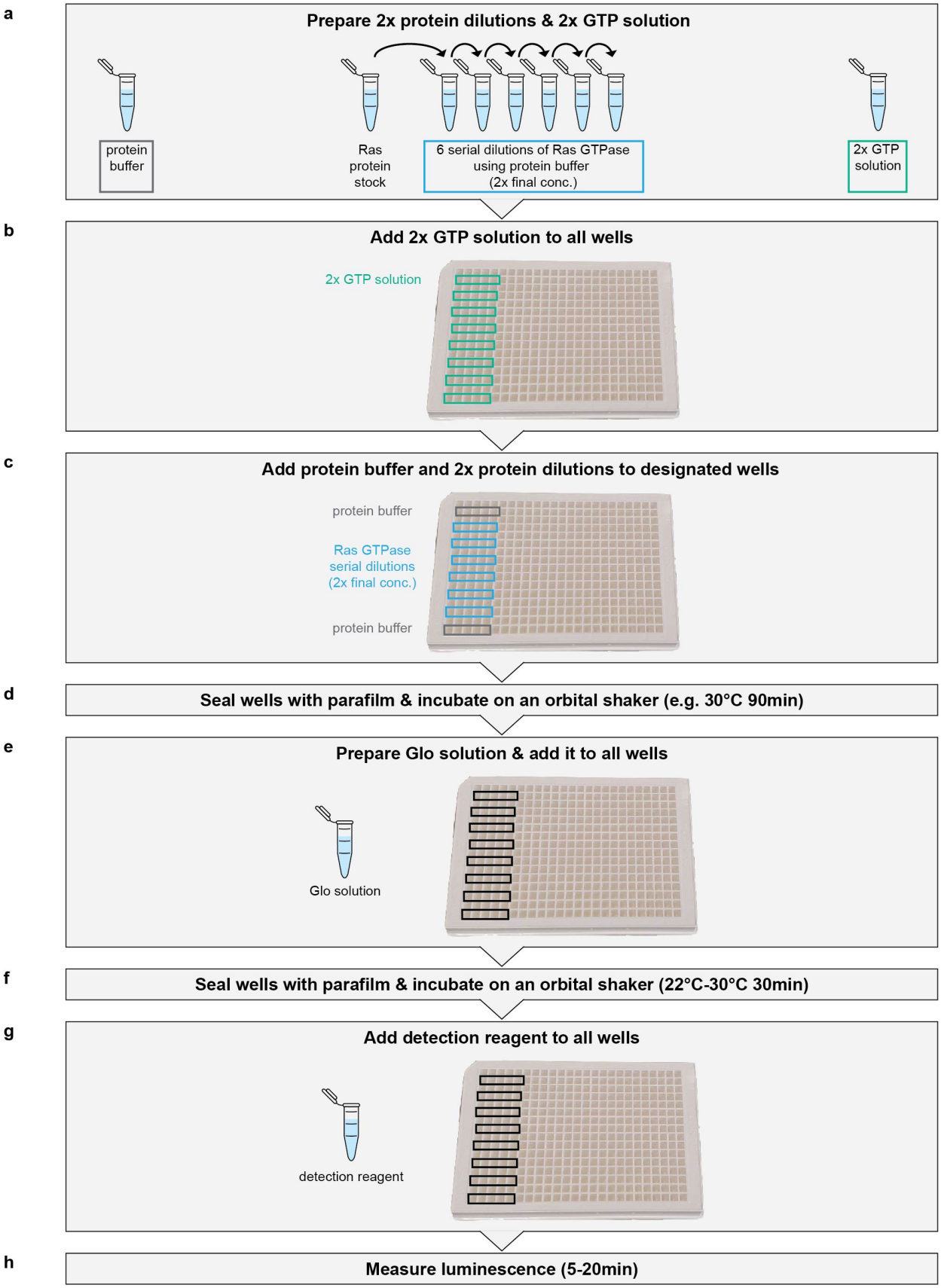


Figure 3. Schematic illustration the steps necessary to conduct a GTPase assay using a serial diluton of 6 Ras GTPase concentrations. The preparatory steps to conduct this assay are illustrated in S2.

Support Protocol 1: Basic analysis of GTPase assay data

This protocol describes basic analysis steps to calculate from the GTPase assay readout (luminescence) the amount of remaining GTP. The amount of remaining GTP is a simple measure used to compare the GTPase activity of different GTPases (of the same concentration, incubated with GTP for the same amount of time).

The protocol illustrates the steps for GTPase data from example 1: an assay including 6 Ras GTPase serial dilutions and two buffer reference samples, with each 3 replicas per sample (see basic protocol and S2). This protocol uses *and* generates data in 'example1.xlsx', tab: 'E1', 'E2'.

Necessary resources

- A spreadsheet editor or other analysis software (e.g. python script)

Protocol steps

1. Calculate the average luminescence for time points 5-20 min per well (= replica) (Fig. 4)
2. Calculate the luminescence average for each sample: Average between wells (=replicas) of the same sample (Fig. 5a).

   Combine the wells/replicas of the first and last buffer row. Calculate the average using all values. I.e. do not distinguish between the first and last buffer row (see Fig. 3, S2).
3. Calculate the standard error of the mean for each sample (Fig. 5b).
4. Use the average luminescence values (Fig. 5a) to translate luminescence into amount of remaining GTP using Eq. 1 (Fig. 5c): The luminescence values of the buffer samples correspond to 100% remaining GTP. The amount of remaining GTP of each protein sample corresponds to the luminescence of this sample divided by the luminescence of the buffer.

   This step normalises the luminescence values of the protein samples to 100% remaining GTP (luminescence of the buffer). In principle, the protein samples also need to be normalised to 0% GTP. Given that we observed basically no luminescence in the blank samples (S5), this step is not necessary.

$$\text{remaining GTP} = \frac{\text{Luminescence sample}}{\text{Luminescence buffer}} \times 100\% \tag{1}$$

5. Calculate the error ('Δ remaining GTP') using error propagation (Eq. 2, Fig. 5d): 'Lum.' is the average luminescence and 'ΔLum.' refers to the standard error of the mean.

$$\Delta \text{ remaining GTP} = \sqrt{\left(\frac{\Delta\text{Lum. sample}}{\text{Lum. sample}}\right)^2 + \left(\frac{\Delta\text{Lum. buffer}}{\text{Lum. buffer}}\right)^2} \times \frac{\text{Lum. sample}}{\text{Lum. buffer}} \times 100\% \tag{2}$$

The following step is only required if the data analysis will be continued using Supporting Protocol 2:

6. Re-format the data in a spreadsheet to follow the formatting shown in Fig. 6. This can be done using the python script provided in S9. In short, the data must be organised into the following columns with specific headers:
   - 'Run': state the assay number. The assay number must start with an 'E' followed by a number, which can be followed by a letter (and underscore letter). Eg. E1, E2, E100, E1a, E100abc, E1_a, E100_abc, E1a_abc, E100f_abc
   - 'Time': incubation time, stated in hours
   - 'GTP_remaining': amount of remaining GTP, normalised to 1
   - 'Error': error values, normalised to 1
   - 'Buffer_error': error of the buffer, normalised to 1
   - '[GTPase]_conc': The header must contain the name of the GTPase, followed by '_conc' (e.g. Ras_conc, Fig. 6). The concentration must be stated in μM
   - optional: '[Effector]_conc': The header must contain the name of the effector, followed by '_conc'. The concentration must be stated in μM. This column should only be there if it contains more than one unique value per assay.

We advice to group assays of the same type (i.e. containing the same proteins and dilution types) into one speadsheet tab. They must follow the same structure. The header can only be included once. E.g. An assay set examines the GTPase Cdc42 and its GEF Cdc24. Two types of assays were conducted: (A) GTPase assays using only serial dilutions of the GTPase Cdc42. (B) GTPase assays using a constant Cdc42 concentration and serial dilutions of the GEF Cdc24. We advice to group all assay data of (A) into one tab and all assay data of (B) into one tab.
More examples are given in Support Protocol 2.

		buffer			5uM			4uM		
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 GTPase assay described in the basic protocol (Fig. 3 and S2): Time-averaging luminescence values. (Data is taken from 'example1.xlsx', tab: 'E1'.)

				Ras					
	Well	**buffer**	**buffer**	**5uM**	**4uM**	**3uM**	**2uM**	**1uM**	**0.5uM**
	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 GTPase assay described in the basic protocol (Fig. 3 and S2): calculating the amount of remaining GTP from luminescence values. (Data is taken from 'example1.xlsx', tab: 'E1'.)

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 Fig. 5 (e.g. using a python script provided in S9). (Data is taken from 'example1.xlsx', tab: 'E1'.)

Support Protocol 2: Fitting GTPase cycling rates

This protocol describes how the data, pre-processed using the steps described in Support Protocol 1, can be fitted using a GTPase activity model (S8) to obtain GTPase cycling rates. It can be used to fit data of GTPases and GTPase - effector X mixtures (up to 2 effectors).

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

$$\begin{aligned} [\text{GTP}]_t &= [\text{GTP}]_{0h} \exp(-Kt) \\ \text{using } [\text{GTP}]_{0h} &= 1 \\ \text{and } K &= K_1^* + K_2^* = k_1[\text{GTPase}] + k_2[\text{GTPase}]^2 \end{aligned} \tag{3}$$

Here 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 an cooperative contribution by two GTPase molecules (K_2^*).

Then, mixtures of one GTPase and one effector X_1 are fitted with

$$\begin{aligned} K &= K_1 + K_2 + K_{3,X_1} \\ &= k_1 c_{corr}[\text{GTPase}] + k_2(c_{corr}[\text{GTPase}])^2 + k_{3,X_1} c_{corr}[\text{GTPase}][X_1]^n \end{aligned} \tag{4}$$

and mixtures of one GTPase and two effectors (X_1, X_2) are fitted with

$$\begin{aligned} K &= K_1 + K_2 + K_{3,X_1} + K_{3,X_2} + K_{3,X_1,X_2} \\ &= k_1 c_{corr}[\text{GTPase}] + k_2(c_{corr}[\text{GTPase}])^2 + k_{3,X_1} c_{corr}[\text{GTPase}][X_1]^n + k_{3,X_2} c_{corr}[\text{GTPase}][X_2]^m + k_{3,X_1,X_2} c_{corr}[\text{GTPase}][X_1]^n[X_2]^m \end{aligned} \tag{5}$$

using n and m either 1 or 2. This fits yield correction factors c_{corr} and cycling rates k_{3,X_1} (and k_{3,X_2}, k_{3,X_1,X_2}).
c_{corr} is dimensionless and accounts for variability between assays. It ought to be close to 1.0 (S8).
K_1 and K_2 represent corrected version of K_1^* and K_2^* obtained from the GTPase serial dilutions ($K_1 = c_{corr}K_1^*$, $K_2 = c_{corr}K_2^*$).
K_{3,X_1}, K_{3,X_2}, and $K_{3,X_1,X2}$ represents 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, in order to analyse GTPase - effector mixtures, k_1 and k_2 must be known. It is thus required to fit data of GTPase (serial) dilutions first. The model allows to fit GTPase - effecotr mixtures of up to two effectors present, 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 advice to either only include the linear/quadratic regimes into the analysis or extend our 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, Eq. 4) (example 2)
- GTPase + effector $X1$ serial dilutions + effector $X2$ serial dilutons (Eq. 5, Fig. 1) (example 2)

The model is based on our observation that the amount of remaining GTP declines exponentially with time (S4). It is still advisable to verified that this is also true for the proteins (GTPase enzymes and GTPase:effector mixtures) and incubation times intended to be used.

The model code analyses data of each GTPase assay individually and then generates pooled rate values . The analysis code allows to exclude GTPase assays from pooling if c_{corr} values or standard errors of k are out of range. Decision criteria are set in the code using two parameters (see steps below):

1. `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 in particular 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 this assay.

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

   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 domineered by k_2 (and has $k_1 \approx 0$ and thus std.err(k_1)≈0), this parameter needs to be set to 'false' to still allow for pooling of k. In many cases this can not be known before an initial analysis. If chosen inappropriately, the parameter simply needs to be changed and the code needs to be re-run.

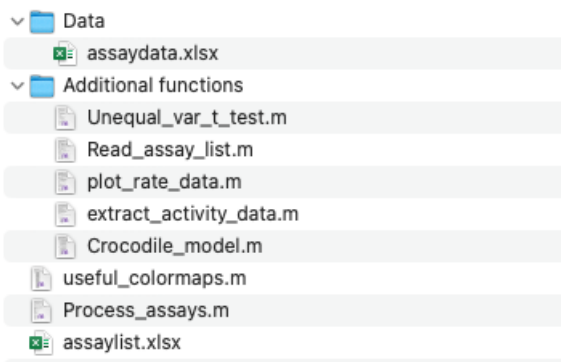
In the following we first describe the general protocol steps, which we will specify for both data of example 1 and example 2 thereafter.

Necessary resources

- spreadsheet editor
- Matlab software license
- Matlab analysis scripts and example data files encompassing this protocol

General protocol steps

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



   Assay data ('assaydata.xlsx') must follow the formatting described at the end of Support Protocol 1 (Fig. 6).
   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 colourmap from [Smith et al., 2015]). When needed, an unequal variance t-test (*`Unequal_var_t_test.m`*) [Ruxton, 2006], 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 these are built up. The data is then read using* `extract_activity_data.m` *and passed to* `Crocodile_model.m`*.*

2. Define the processing of the data in the 'assaylist.xlsx' speadsheet. Follow the formatting provided in the example files (discussed in the next sections). The following sections are required:

   - Assay name: Gives an internal assay name. This name will be used in the output to refer to this data.
   - Proteins: These are the names of proteins whose concentration varies within the assay. State each protein in a separate column. At maximum 2 proteins can be varied per assay.
     GTPase ref.: State the assay name where the GTPae protein used in this assay was varied. Ensure that this assay is processed already (i.e. 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.

3. 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 (1e-10 or lower) on the k values.
% If nothing is provided for a GTPase, default is true (i.e. runs with low
% standard errors will be excluded).
```

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

Protocol steps: Simple assay (example 1: Ras GTPase)

We here show the specific protocol steps to run data of example 1: For this example a GTPase assay containing only serial dilutions Ras GTPase was conducted twice. Assay steps were described in the basic protocol and S2. Basic analysis of the assay data was described in Support Protocol 1 (and S9), resulting in data shown in 'example1.xlsx' tab: 'matlab'.
The data of each individual assay will be fitted with the model:

$$[\text{GTP}]_t = [\text{GTP}]_{0h} \exp(-Kt)$$

$$\text{using } [\text{GTP}]_{0h} = 1$$

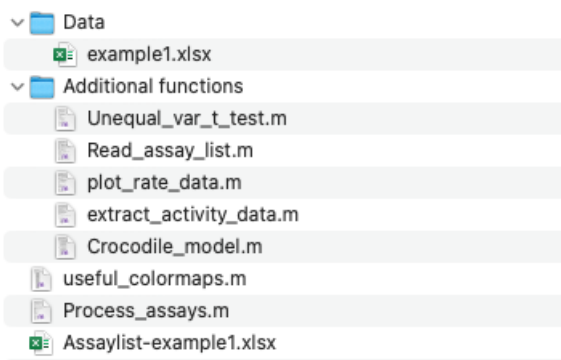
$$\text{and } K = K_1 + K_2$$

$$K = k_1 c_{corr}[\text{Ras}] + k_2 (c_{corr}[\text{Ras}])^2$$

resulting one k_1 and $k2$ each. A pooled estimate of k_1 and $k2$ will be generated.

Steps:

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



2. Define the processing of the data in the 'assaylist-example1.xlsx' speadsheet.

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' in tab 'matlab'. Because the concentration of Ras is varied in each assay, 'Proteins' states 'Ras'.

3. 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;
```

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

Protocol steps: Elaborate assay set (example 2: GTPase effector mixtures)

We here show the specific protocol steps to run data of example 2: Example 2 entails GTPase assay data ('example2.xlsx') of several protein mixtures (also see Fig. 2):
(A) GTPase assays in which the GTPase Cdc42 is varied (all in tab: 'dCdc42').
(B) GTPase assays using a constant Cdc42 concentration and serial dilutions of the GEF Cdc24 (all in tab: 'Cdc42-dCdc24').
(C) GTPase assays using a constant Cdc42 concentration and serial dilutions of the GAP Rga2 (all in tab: 'Cdc42-dRga2'). (D) GTPase assays using a constant Cdc42 concentration and serial dilutions of both GEF Cdc24 and GAP Rga2 (all in tab: 'Cdc42-dCdc24-dRga2').

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

$$[\text{GTP}]_t = [\text{GTP}]_{0h} \exp(-Kt)$$

$$\text{using } [\text{GTP}]_{0h} = 1$$

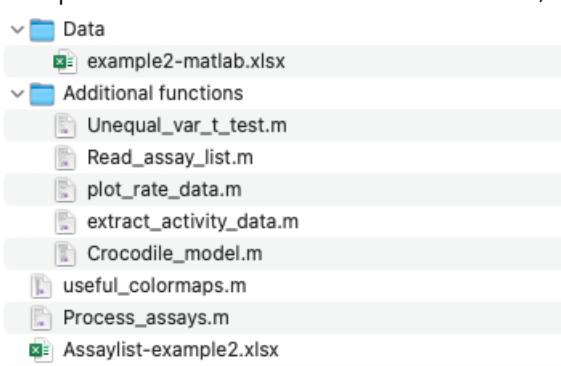
$$\text{and } K = K_1 + K_2 + K_{3,X1Cdc24} + K_{3,Rga2} + K_{3,Cdc24,Rga2}$$

$$K = k_1 c_{corr}[\text{Cdc42}] + k_2(c_{corr}[\text{Cdc42}])^2 + k_{3,Cdc24} c_{corr}[\text{Cdc24}]^2[\text{Cdc42}] + k_{3,Rga2} c_{corr}[\text{Rga2}][\text{Cdc42}] + k_{3,Cdc24,Rga2} c_{corr}[\text{Cdc24}]^2[\text{Rga2}][\text{Cdc42}]$$

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

Steps:

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



2. Define the processing of the data in the 'assaylist-example2.xlsx' speadsheet.

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 analyse this assay set, first all data with varied Cdc42 needs to be fitted. We give these assays the name 'C42'. Because Cdc42 is varied, 'Proteins' states 'Cdc42'.

Then, Cdc42-effector mixtures can be analysed. We call assays with Cdc42-Cdc24 mixtures 'C42-C24'. They include a constant concentration of Cdc42 and varied concentrations of Cdc24. Hence, 'Proteins' states 'Cdc24' (it does not state 'Cdc42' as well because the Cdc42 concentration is constant in these assays). To fit these assay data we want to use the rates of Cdc42 obtained in the line above, so we need to refer to 'C42' in 'GTPase ref.' (also see Eq. 3,4).
We apply the same logic to Cdc42-Rga2 assay data, which we call 'C42-R2'.
We call assays in which both Cdc24 and Rga2 is varied 'C42-C24-R2'. Here 'Proteins' states 'Cdc24' and 'Rga2' (because both proteins are varied), and 'GTPase ref.' states 'C42' to refer to the Cdc42 assay analysis in the first line.

3. 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;
```

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

Protocol steps: Combined analysis of several assays

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

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

2. Define the processing of the data in the 'assaylist-example1and2.xlsx' speadsheet.

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

3. 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;
```

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

Reagents and solutions

Table 2. Pipetting scheme for the 2× GTP solution.

Starting solution	final concentration	500 µL	750 µL	1 mL
protein buffer	-	494 µL	742 µL	989 µL
DTT (100 mM)	1 mM	5 µL	7.5 µL	10 µL
rGTP (10 mM)	10 µM	0.5 µL	0.75 µL	1 µL

Table 3. Pipetting scheme for the Glo solution. Please note, to follow this pipetting scheme the ADP solution provided in the kit (ADP, 10 mM) has to be diluted to 1 mM (e.g. mix 2 µL ADP (10 mM) with 18 µL of ultra pure water)!

Starting solution	final concentration	500 µL	750 µL	1 mL
GTPase-Glo™ buffer	-	496 µL	745 µL	993 µL
ADP (1 mM)	5 µM	2.5 µL	3.75 µL	5 µL
GTPase-Glo™ reagent (500×)	1×	1 µL	1.5 µL	2 µL

Commentary

Background Information

In the GTPase assay the proteins of interest (GTPase enzymes with or without effectors) are incubated with GTP for a certain amount of time for GTPase cycling to occur, hydrolysing GTP to GDP and free phoshate. Then the reaction is stopped through 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, which is measured using a plate reader in luminescence mode (Fig. 1) [Promega Corporation, 2015]. The luminescence signal correlates with the amount of remaining GTP (in inversly correlates with the activity of the GTPase): the higher the luminescence values, the more GTP remainded in soluion (and thus the less active the GTPase is). Low or no luminescence corresponds to very little or no remaining GTP and a higher 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. The obtained rates might be more comparable to those observed *in vivo* (in contrast to other *in vitro* assays that only examine one specific step of the GTPase cycle), as GTPase cycling constantly occurs *in vivo*. Further, it enables comparison of the strength of effectors that act on different steps of the GTPase cycle, such as GEFs and GAPs. Also, effector interplay can be studied. The origin of these advantages is also at the centre of the assay's disadvantage: Because GTPase cycling (multiple completions of the entire GTPase cycle) is studied, the origin of observed effects remain elusive. It is less suitable if detailed mechanistic features of a GTPase cycle step ought to be investigated. For this, other GTPase assasy, such as the MESG/phosphorylase system [Zhang et al., 1997] or N-methylanthraniloyl-GTP/GDP [Rapali et al., 2017] system, which examine only the GTP hydrolysis or GDP release step.

Critical Parameters

The GTPase assay is sensitive to small pipetting errors, 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 S1) and the materials are prepared before the assay is stared. This greatly reduce the likelihood of errors due to inattentiveness, hurry/time stress, and chaos. Accurate pipetting is essential for this assay!

Further, we advice to

- aliquot and vortex all components before use (except the Glo reagent)
- dialyse all proteins into the same buffer
- include control samples such as a buffer row in the beginning and end of the plate, a positive control (Ras GTPase enzyme), and potentially inert proteins to account for non-canonical effects
- always prepare the 2× GTP solution, 1 mM ADP, and Glo solution freshly and to never re-use/store it

If executed properly, the assay leads to reliable and reproducible results (e.g. see [Tschirpke et al., 2023b, Tschirpke et al., 2023a]).

Troubleshooting

For a detailed troubleshooting on basic problems such as

- no change in luminescence with increasing or decreasing concentration of GTPase, GAP or GEF
- low signal-to-background ratio
- high luminescent signal
- low luminescent signal

please consult the user manual of GTPase Glo™ assay kits [Promega Corporation, 2015], we here (Tab. 4) only discuss these issues briefly.

Problem	Possible Cause	Solution
No or low luminescence	sample/ buffer components interfere with assay steps (e.g. fluorescent tags (S3))	dialyse sample into a new buffer (e.g. 50 mM Tris-HCl (pH=7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol), also see assay manual
	one of the assay solutions is no longer active	use fresh aliquots of GTP, DTT, ADP, Glo reagnet, and detection reagent
High luminescence	contamination with ATP, GTP, or other nucleotide triphosphates	dialyse the sample into a buffer, also see assay manual
Luminescence of first and last buffer row differs a lot	two different detection reagent aliquots were used for the same assay (S7)	calculate how much detection reagent every assay needs and prepare a sufficient volume through mixing several detection reagent aliquots
Large error bars	2× GTP solution is not fresh/ was re-used (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	dialyse sample into a new 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 the GEF or GTPase
	GEF conc. 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 $MgCl_2$
	the buffer contains chelating agents such as EDTA that complex Mg^{2+}	use a buffer that does not contain chelating agents

Table 4. Troubleshooting guide for GTPase Glo™ assays.

Understanding Results

The GTPase assay yields luminescence values, which are translated into amount of remaining GTP. These values can be fitted with GTPase cycling rates k. Using 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 example 1 GTPase assay data for 5 serial dilutions of Ras GTPase were collected. The basic assay steps are described in the Basic Protocol (and S2), and their subsequent analysis is outlined in Support Protocol 1 and Support Protocol 2.

Luminescence: Basic protocol yields luminescence values, from which the amount of remaining GTP is calculated (Support Protocol 1). The luminescence values of the buffer rows should always be quite similar, although a small shift can be observed (Fig. 7 (top)). If this shifts gets big, the error bars of the entire assay will increase (because the buffer wells are used for normalisation). Dilutions of Ras need to show lower luminescence values than the buffer (because Ras is a GTPase and hydrolyses GTP, which correlates with luminescence (Fig. 1)). With increasing Ras concentration, the luminescence should drop. Replicas/wells containing the same Ras concentration should have the same luminescence (Fig. 7 (bottom)). The bigger their spread, the bigger the error will be. In some cases we observe a small decrease of the luminescence over time (Fig. 7). As long as this decrease is not drastic and occurs in all wells to roughly the same extend, it will not impact the analysis. Assays in which the luminescence decreases drastically within 20 min should be discarded from further analysis, as well as wells that behave differently than the majority.
According to the assay manual, the luminescence should be measured after 5 min. We average the luminescence values from 5-20 min for every assay, to smoothen out possible small deviations in the luminescence curve. The exact time window (as long as it begins after 5 min) is less important than choosing exactly the same time window for every assay.
The luminescence values are used to calculate the amount of remaining GTP (Fig. 5). It is possible to use these values to compare the activity of different GTPases, the activity of two different effectors on a 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 in the same concentration.

GTPase cycling rates k_1, k_2: To increase comparability of GTPase and effector activities, we developed a GTPase cycling model (S8). Support Protocol 2 describes how the model can be used to fit data of Ras. In short, we fit an exponential to the amount of remaining GTP (S8):

$$[\text{GTP}]_t = [\text{GTP}]_{0h} \exp(-Kt)$$
$$\text{using } [\text{GTP}]_{0h} = 1$$
$$\text{and } K = K_1 + K_2 = k_1[\text{Ras}] + k_2[\text{Ras}]^2$$

Here K refers to the 'overall GTP hydrolysis rate' and depends on the concentration of the GTPase 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 (Eq. 3). K_1 accounts for the linear contribution of the GTPase ($K_1 = k_1$ [GTPase]). K_2 accounts for the quadratic contribution ($K_2 = k_2$ [GTPase]^2) which can be due to dimerisation 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 non-linear term. This can suggest that the GTPase forms a dimer that is more active than the monomer. However, additional studies examining GTPase di-/oligomerisation are required before making such a deduction. The data indicates that some effect leads to a non-linear concentration dependence of the rate K. It does not reveal its origin. Ras does not show a linear concentration dependence (Fig. 8) and is domineered by k_2 (Fig. 9: $k_2 >>> k_1$). In this case this is likely due to dimerisation - Ras GTPases are known to dimerise.

Output file structure and pooling of rates k: Estimates of Ras GTPase cycling rates k_1 and k_2 are summarised in 'Data_summay.xlsx' (Fig. 9). The file is structured in the following way: The column 'Run' shows the assay number, followed by two 'varied protein'

colmuns stating which protein(s) were varied in the assay (i.e. for which proteins several dilutions were used). If the assay contains only one varied protein, the second column is empty (as it is the case for example 1: here only Ras was used). Then Parameter 1 is named, and its values, 95% upper and lower bound, and standard error is shown. The same is repeated for possible parameters 2 and 3. The last column show adjusted R^2 of the fit and a Boolean if the experiment is used for pooling ('Used for pooling').

For each assay (row, 'Run': E1, E2) the rate value estimates are shown. Below, in the row which contains 'pooled' in the 'Run' column, the pooled values of k estimates are stated . The column 'varied protein' states which proteins were varied in the assay (i.e. for which proteins several dilutions were used). 'Used for pooling' indicates if experiment is used for pooling.

An experiment can be excluded from pooling if c_{corr} or standard errors of k are out of range. Decision criteria are set before analysing the data using two parameters (see Support Protocol 2): (1) `conc_corr_bounds` states which range of c_{corr} allows assays to be included in the pooling. (2) `k_low_err_filt.Ras` states if assays with low standard errors (1e-10 or lower) on the k 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 analysing Ras data. `conc_corr_bounds` states which range of c_{corr} allows assays to be included in the pooling. It does not influence pooling of k_1 and k_2, as c_{corr} only is used for fitting $k_{3,X}$ (Eq. 4,5). We set `k_low_err_filt.Ras` to 'false' - 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 has $k_1 \approx 0$ (and thus k_1 errors are close to 0).

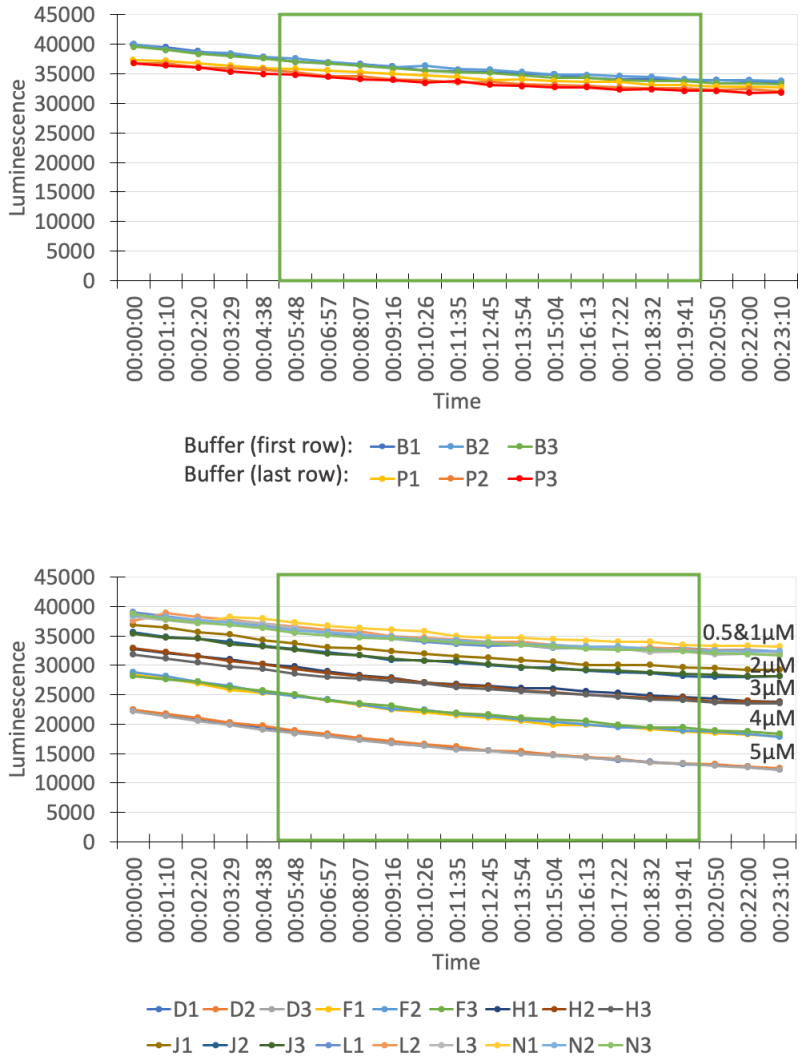


Figure 7. Example 1: Measured luminescence values of buffer wells (top) and Ras GTPase dilutions (bottom) over the time course of the measurement. The green box indicates values that are used for averaging.

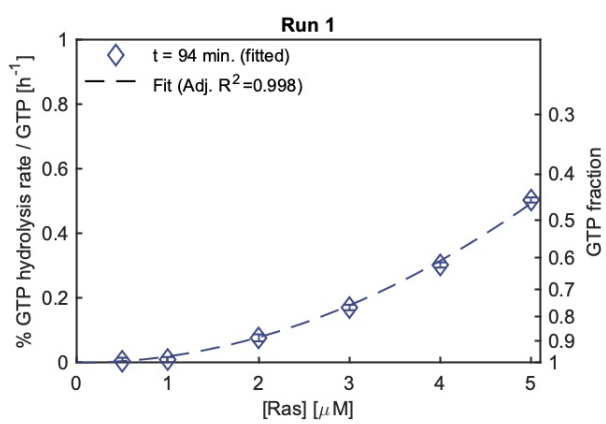


Figure 8. Example 1: The overall GTP hydrolysis rate K does not scale linearly with Ras concentration. (The figure is automatically generated when running the matlab code (using 'plot_fits = true(1)').)

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 9. Example 1: GTPase cycling rates k_1 and k_2 for Ras.

Example 2

For this example data of a set of GTPase assays is provided, including GTPase assays of

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

The analysis is described in Support Protocol 2. In short, a GTPase cycling model (S8) is fitted:

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

Here K refers to the 'overall GTP hydrolysis rate' and depends on the concentration of the GTPase (and effector X).
First data of Cdc42 serial dilutions (data (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 isngle pooled estimate for k_1 and k_2. Then, Cdc42 - Cdc24, Cdc42 - Rga2, and Cdc42 - Cdc24 - Rga2 mixtures (data (B), (C), (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 get single estimate for the k_3's (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 small concentration differences introduced though pipetting of small volumes (as are required for this assay), temperature and shaker speed fluctuations during the incubation step, small changes in effective GTPase concentration through sequestration of GTPases in complexes with effectors, and/or intrinsic changes in the 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 the estimates of cycling rates from the GTPase serial dilution assay and $\hat{K}_{mix}$ are the estimated median of the overall hydrolysis rate fit at zero effector concentration value (i.e. $\hat{K}_{mix} = \hat{K}_{mix}([X] = 0)$). By using random draws of the fitted overall hydrolysis rate distribution, we can also obtain a distribution for c_{corr} as well. Consequently, $c_{corr} = 1.0$ means that relative to the serial dilution assay with the same GTPase (= reference assay), the GTPase in this assay has the same intrinsic activity, which is optimal. This is optimal. $c_{corr} = 0.5$ means that relative to the reference assay, the GTPase in this assay is 50% less active, and $c_{corr} = 1.5$ means that that the GTPase in this assay is 50% more active. Very low correction values indicate that in the assay the GTPase is behaving differently than before (possibly because the conditions are different) or that a fundamental error occurred (e.g. large pipetting error). To ensure consistency within an assay set we advice to disregard that assay from further analysis.
To implement this recommendation, we used `conc_corr_bounds = [0.5 1.5]` for analysing the data set in Support Protocol 2 (example 1). This means that assays yielding c_{corr} values lower than 0.5 and larger than 1.5 will be excluded from pooling. Fig. 10 shows a histogram of all correction factors. Apart from one assay, which yielded $c_{corr} \approx 0.1$, all c_{corr} values are within range, with most values being close to 1.

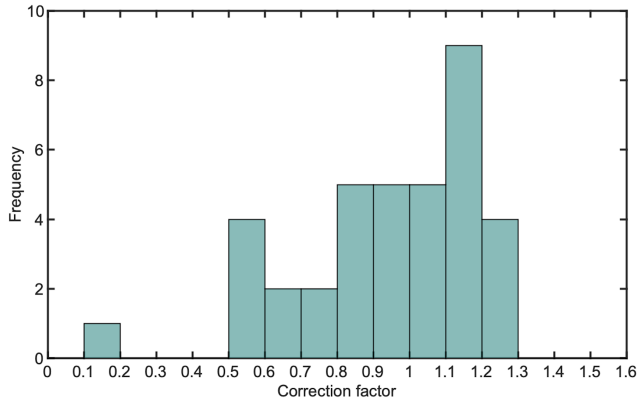


Figure 10. Example 2: Histogram plot of c_{corr} using a bin size of 0.1. Generated for all c_{corr} values of example 2 (using 'Plot_c_corr_histogram.m' (S10) and 'example2-histogram.xlsx' in the folder 'example2 matlab output').

'Data_summay.xlsx' states correction factors as 'parameter 1' for assays in which either Cdc24 or Rga2 or both effectors are varied ('Varied protein 1', 'varied protein 2').

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 an effector X affects the GTPase cycle, but do not reveal which step of the GTPase cycle the effector is acting upon or how strongly the effector is effecting this step (or if the effector acts on multiple steps). To investigate these mechanism, other GTPase assays (e.g. those mentioned in the background information section), need to be conducted.

Assays of group (B) are used to fit cycling rates $k_{3,Cdc24}$ which describe the effect of the GEF Cdc24 on Cdc42's overall GTPase cycle. We choose a quadratic fit ($K_{3,Cdc24} = k_{3,Cdc24}[Cdc42][Cdc24]^2$) because the data showed a non-linear dependence of the overall rate K on the Cdc24 concentration (Fig. 11). The fit is a phenomenological description of the data and does not reveal where the non-linearity originates from. One possibility could be dimerisation, with Cdc24 dimers exhibiting an increased activity [Tschirpke et al., 2023a]. $k_{3,Cdc24}$ values are shown 'Data_summay.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 the GAP Rga2 on Cdc42's overall GTPase cycle. A linear fit ($K_{3,Rga2} = k_{3,Rga2}[Cdc42][Rga2]$) was used to describe the data.

Data of group (D) contains Cdc42 - Cdc24, Cdc42 - Rga2, and Cdc42 - Cdc24 - Rga2 mixtures. In these assays both Cdc24 and Rga2 are varied ('varied protein 1', 'varied protein 2'). The data is fitted with 3 rate parameters (Fig. 12): $k_{3,Cdc24}$ (= 'parameter 2'), $k_{3,Rga2}$ (= 'parameter 3'), $k_{3,Cdc24,Rga2}$ (= 'parameter 4'). $k_{3,Cdc24}$ describes the effect of Cdc24 on the entire GTPase cycle and $k_{3,Rga2}$ describes the effect of Rga2. These rates should be close to those obtained in assays including only Cdc42 - Cdc24 (or Cdc42-Rga2 mixtures) (Fig. 13). 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 both proteins. $k_{3,Cdc24,Rga2} = 0$ means that there is no interaction between both proteins. $k_{3,Cdc24,Rga2} < 0$ suggests that the proteins antagonise/ inhibit each other. $k_{3,Cdc24,Rga2} > 0$ suggests that there is synergy. The term states if there is an interaction between both protein, i.e. if the combined effect of both proteins is bigger than the sum of their individual contributions to the *overall* rate. (The significance of the deviation from zero is 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 sub-field 'k_tot', under 'Interaction_p'.) While a positive $k_{3,Cdc24,Rga2}$ indicates that there is some synergy when both Cdc24 and Rga2 are added to Cdc42, it does not reveal the origin of the synergy. The origin could be due to physical protein-protein interactions or due to rate-limiting steps in the cycle, which are relieved once both proteins are added [Tschirpke et al., 2023a]. One has to thus be careful in the interpretation of this rate.

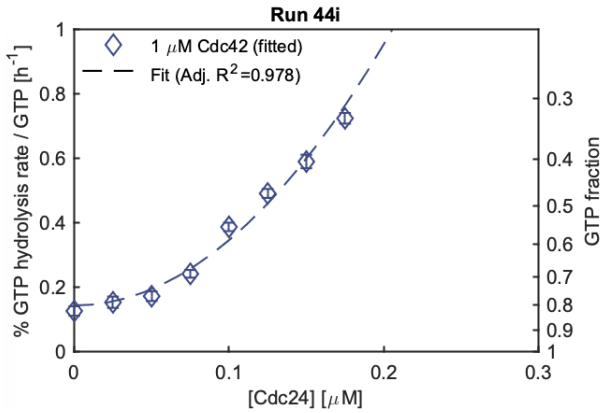


Figure 11. Example 2: The overall GTP hydrolysis rate K scales non-linearly with Cdc24 concentration. (The figure is automatically generated when running the matlab code (using 'plot_fits = true(1)').)

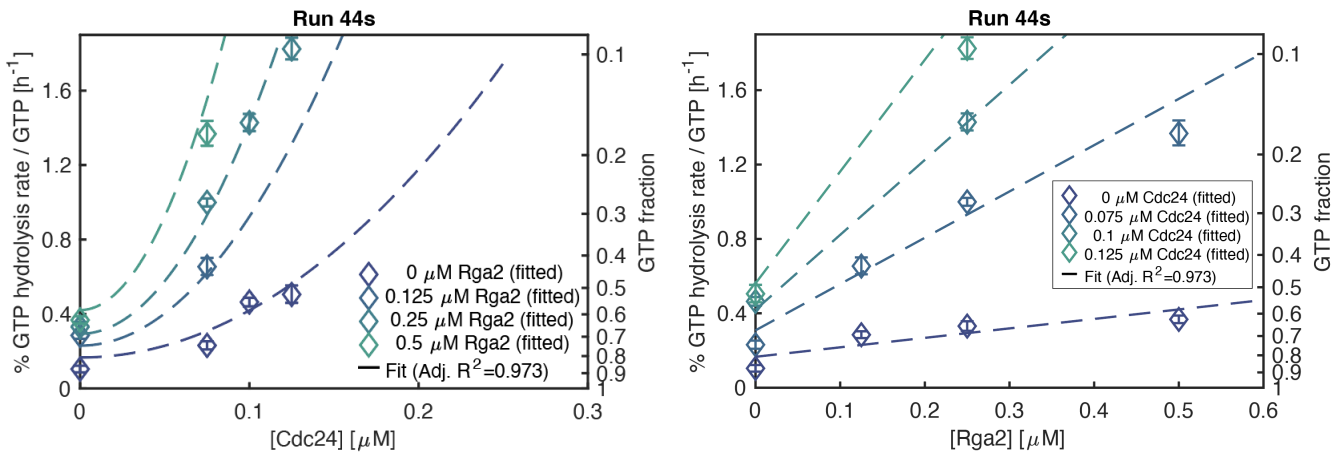


Figure 12. Example 2: Fit of Cdc42-Cdc24-Rga2 mixtures. (The figures are automatically generated when running the matlab code (using 'plot_fits = true(1)').)

Comparison of cycling rates *k*: Overall GTP hydrolysis rates K are concentration dependent and have the unit h^{-1}. Cycling rates k are concentration-independent, but have different units and are therefor difficult to compare: k_1 : [μM$^{-1}h^{-1}$], k_2 : [μM$^{-2}h^{-1}$], $k_{3,Cdc24}$: [μM$^{-3}h^{-1}$], $k_{3,Rga2}$: [μM$^{-2}h^{-1}$], $k_{3,Cdc24,Rga2}$: [μM$^{-4}h^{-1}$]. 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. 13). However, one has to consider the different concentration-dependencies when interpreting/comparing the rates K. A rate that scales quadratically with protein concentration will be twice as big as a rate that scales linearly when doubling the protein concentration. Some rates might also just be valid for the regime in which they were fitted in (they might show saturation in higher concentration regimes). Thus, one has consider which protein concentrations one uses to calculate K (in order to make cycling rates k comparable).

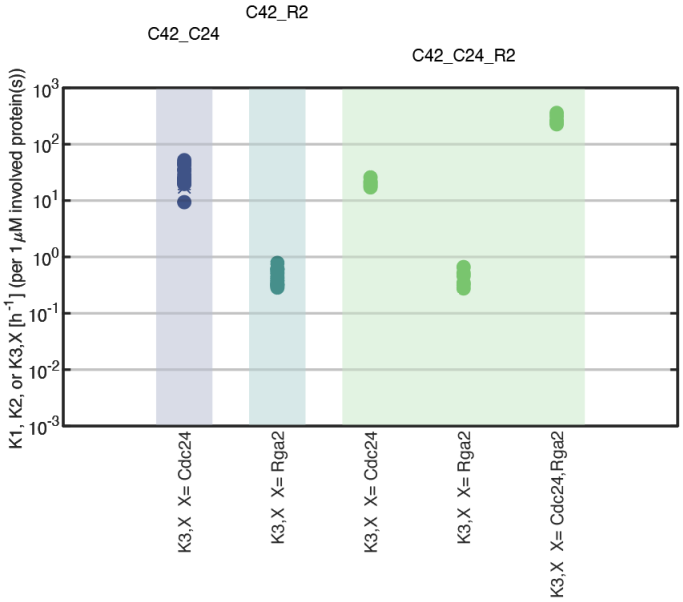


Figure 13. Example 2: Plot of rates K_3, X for Cdc42-Cdc24 (left: 'C42-C24'), Cdc42-Rga2 (middle: 'C42-R2'), and Cdc42-Cdc24-Rga2 (right: 'C42-C24-R2') assays. Rate values of individual experiments are shown as filled dots. The average is shown as a cross and error bars represent the standard error. Generated for assay names 'C42-C24', 'C42-R2', and 'C42-C24-R2' (using 'Plot_pooled_values_plot_std_err.m' (S10) and 'Data_assays.mat' in the folder 'example2 matlab output').

Time Considerations

GTPase assays usually take a few hours, of which time is consumed by incubation steps. For an assay involving 8 samples, including one Ras postive control and 5 serial dilutions of the GTPase of interest (Fig. 3), using an incubation time of 1.5 h, we estimate 0.5 h for preparation of materials and solutions, 2 h for conducting the assay, and another 0.5 h for the luminescence readout. This amounts to a total of 3 h, of which 2 h account for incubation/measurement steps.
Assays encompassing more samples may involve longer preparation times (as more protein dilutions need to be prepared), and longer incubation times subsequently also prolong the total amount of time spent.
If proteins need to be dialysed into a suitable buffer first, an additional overnight step of dialysis is required.

The analysis time depends on the level of automatisation and can range from only a few to 30-40 min. If the first step is done manually in Excel, we estimate 30 min for this step. The following analysis in Matlab requires only a few minutes of runtime.

Conflict of interest statement

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 Sharing and Data Availability

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.

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Contributions

S. Tschirpke: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration. **W. K.-G. Daalman:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Software. **L. Laan:** Writing - Review & Editing, Project administration, Funding acquisition, Supervision.

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Supplement S1

The following pages include preparation sheets for conducting GTPase assays with 1, 2, or 3 sample rows.

Assay number: Name: Date:
Incubation:

	5 µL 2xGTP solution	5 µL protein	10 µL Glo solution	20 µL Det. reagent
A				
B				
C				
D				
E				
F				
G				
H				
I				
J				
K				
L				
M				
N				
O				
P				

Assay preparation/ protein dilutions:

2x GTP solution:			Glo solution:				Det. Reag.:
500 µL	1000 µL		500 µL	750 µL	1000 µL		
494 µL	989 µL	protein buffer	496 µL	745 µL	993 µL	Glo buffer	
5 µL	10 µL	DTT	2.5 µL	3.75 µL	5 µL	**1mM** ADP (2µL **10mM** ADP + 18µL mQ)	
0.5 µL	1 µL	GTP	1 µL	1.5 µL	2 µL	Glo-reagent	

Assay number: Name: Date:
Incubation:

	5µL 2xGTP	5µL protein	10µL Glo	20µL Det reag		5µL 2xGTP	5µL protein	10µL Glo	20µL Det reag
A					A				
B					B				
C					C				
D					D				
E					E				
F					F				
G					G				
H					H				
I					I				
J					J				
K					K				
L					L				
M					M				
N					N				
O					O				
P					P				

Assay preparation/ protein dilutions:

2x GTP solution:			Glo solution:				Det. Reag.:
500 µL	1000 µL		500 µL	750 µL	1000 µL		
494 µL	989 µL	protein buffer	496 µL	745 µL	993 µL	Glo buffer	
5 µL	10 µL	DTT	2.5 µL	3.75 µL	5 µL	**1mM** ADP (2µL **10mM** ADP + 18µL mQ)	
0.5 µL	1 µL	GTP	1 µL	1.5 µL	2 µL	Glo-reagent	

Assay number: Name: Date:
Incubation:

	5	5	10	20		5	5	10	20		5	5	10	20
A					A					A				
B					B					B				
C					C					C				
D					D					D				
E					E					E				
F					F					F				
G					G					G				
H					H					H				
I					I					I				
J					J					J				
K					K					K				
L					L					L				
M					M					M				
N					N					N				
O					O					O				
P					P					P				

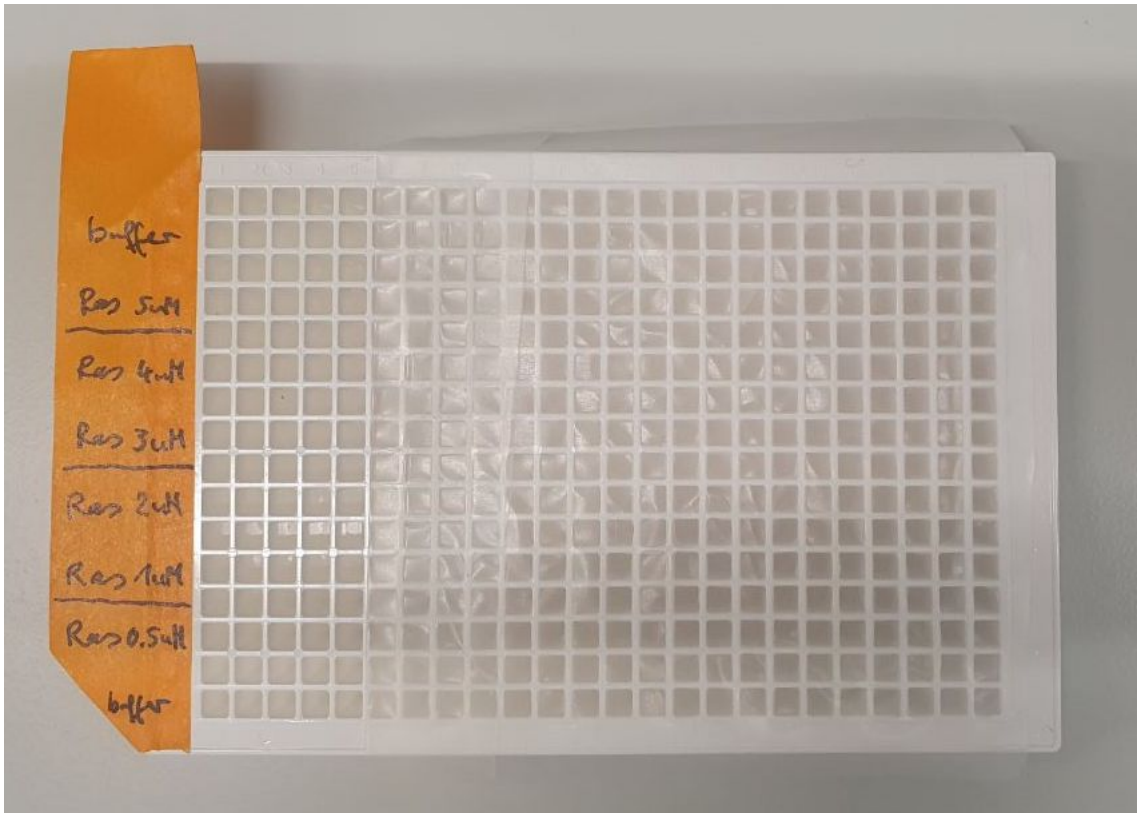
Assay preparation/ protein dilutions:

2x GTP solution:			Glo solution:				Det. Reag.:
500 µL	1000 µL		500 µL	750 µL	1000 µL		
494 µL	989 µL	protein buffer	496 µL	745 µL	993 µL	Glo buffer	
5 µL	10 µL	DTT	2.5 µL	3.75 µL	5 µL	**1mM** ADP (2µL **10mM** ADP + 18µL mQ)	
0.5 µL	1 µL	GTP	1 µL	1.5 µL	2 µL	Glo-reagent	

Supplement S2

This supplement shows a prepared plate and preparatory sheet for a GTPase assay of 6 Ras GTPase serial dilutions (example 1) (see basic protocol).

It leads to data that can be found in 'example1.xlsx', tab: 'E1', 'E2'.



S2 Figure 1. Preparation of a plate for a GTPase assay containing 6 Ras GTPase serial dilutions (example 1).

Assay number: 1 Name: ST Date: 2023-03-30

Incubation: 94min 30°C

Wells 1-5	5 µL 2xGTP solution	5 µL protein	10 µL Glo solution	20 µL Det. reagent
A				
B buffer				
C				
D Ras 5µM				
E				
F Ras 4µM				
G				
H Ras 3µM				
I				
J Ras 2µM				
K				
L Ras 1µM				
M				
N Ras 0.5µM				
O				
P buffer				

Assay preparation/ protein dilutions:

Ras stock ~160µM

Serial dilutions:

- per dilution: 5 x 5µL + 5µL = 30µL
- dilutions:

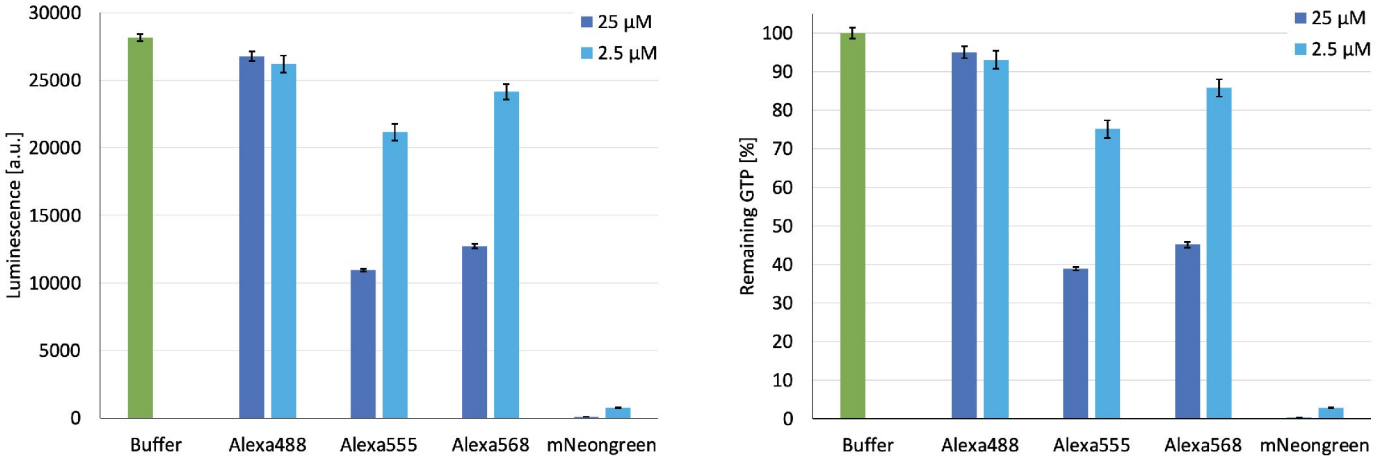
	2x5µM = 10µM	2x4µM = 8µM	2x3µM =6µM	2x2µM =4µM	2x1µM =2µM	2x0.5µM =1µM
Ras [µL]	6.5 (of 160µM)	68 (of 10µM)	51 (of 8µM)	36 (of 6µM)	23 (of 4µM)	15 (of 2µM)
Protein buffer [µL]	97.5	17	17	18	23	15
Total vol. [µL]	104-68 = 36	85-51 = 34	68-36 = 32	54-23 =31	46-15 = 31	30

2x GTP solution: 8x5x5µL=200µL			Glo solution: 400µL				Det. Reag.: 800µL Prepare 1mL
500 µL	1000 µL		500 µL	750 µL	1000 µL		
494 µL	989 µL	protein buffer	496 µL	745 µL	993 µL	Glo buffer	
5 µL	10 µL	DTT	2.5 µL	3.75 µL	5 µL	**1mM** ADP (2µL **10mM** ADP + 18µL mQ)	
0.5 µL	1 µL	GTP	1 µL	1.5 µL	2 µL	Glo-reagent	

S2 Figure 2. Preparation of a GTPase assay of 6 Ras GTPase serial dilutions (example 1) (made using templates provided in S1).

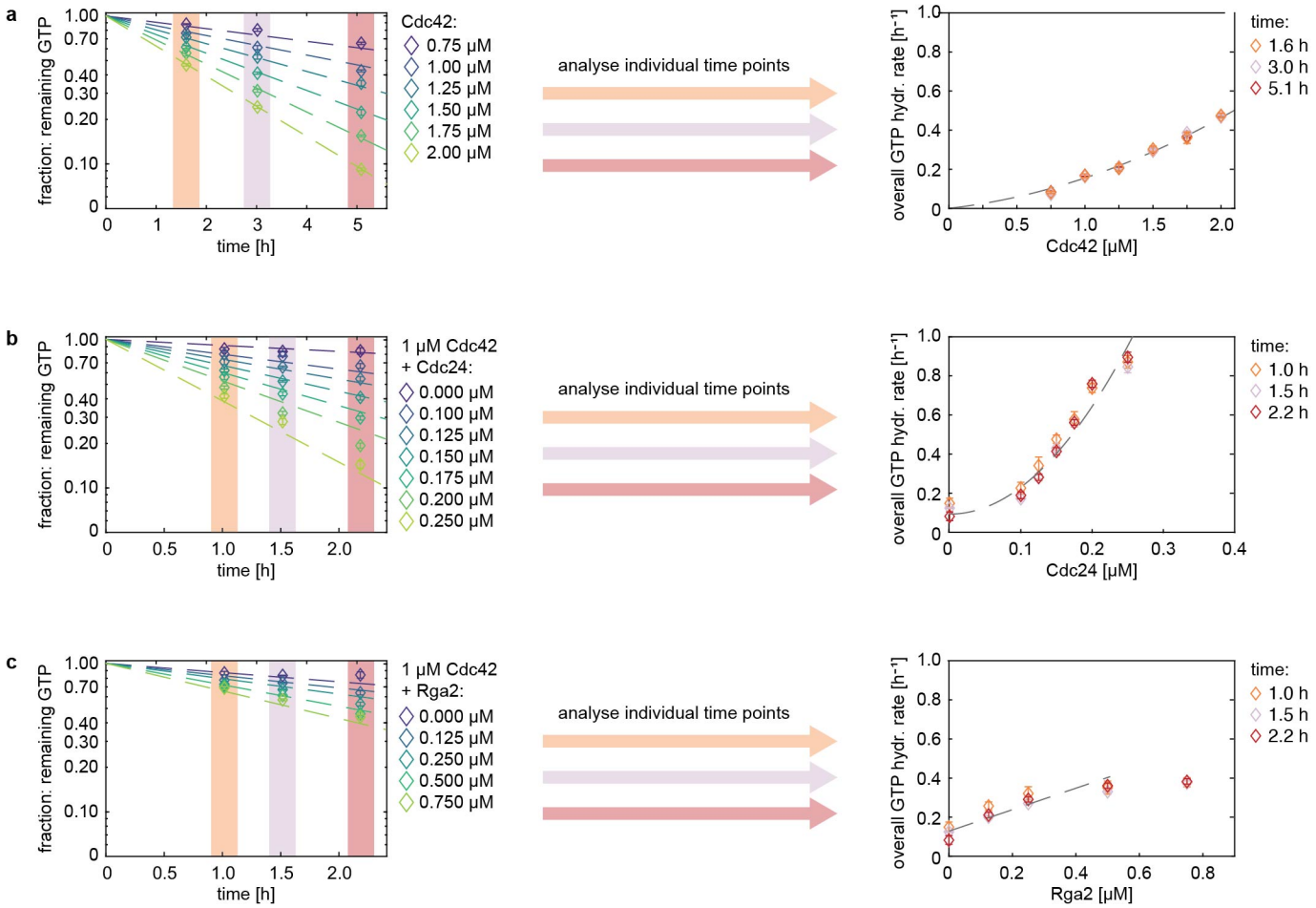
Supplement S3

Before including fluorescently labelled proteins into the GTPase assay, assess if the fluorescent tags interfere with the assay readout (= luminescence). Some fluorescent tags (e.g. Alexa488) reduce the luminescence almost completely (e.g. mNeongreen), while others only affect it mildly (e.g. Alexa488) (S3 Fig. 1).



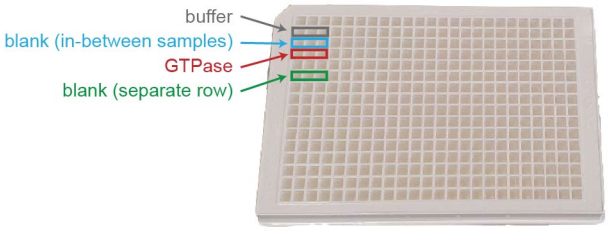
S3 Figure 1. Fluorescent tags can interfere with the GTPase assay readout: Luminescence (left) and perceived amount of remaining GTP (right) of four fluorophores (normalised to buffer). Fluorophores are not GTPase enzymes and are not expected to hydrolyse GTP. A drop in luminescence signal (and thus a decrease in the perceived amount of remaining GTP) is likely due to absorption of some of the luminescence signal by the fluorophores.

Supplement S4



S4 Figure 1. The GTP concentration declines exponentially with time in GTPase reactions. Amount of remaining GTP for (a) Cdc42 concentrations (b) Cdc42 Cdc24 mixtures, and (c) Cdc42 Rga2 mixtures, each for three time points (measured as one individual assay per time point). The remaining GTP content declines exponentially with time (left). Data of each individual time point shows the same overall GTP hydrolysis cycling rate for each GTPase - effector mixture. Thus, only one time point per assay condition is needed, to fit the data (right).

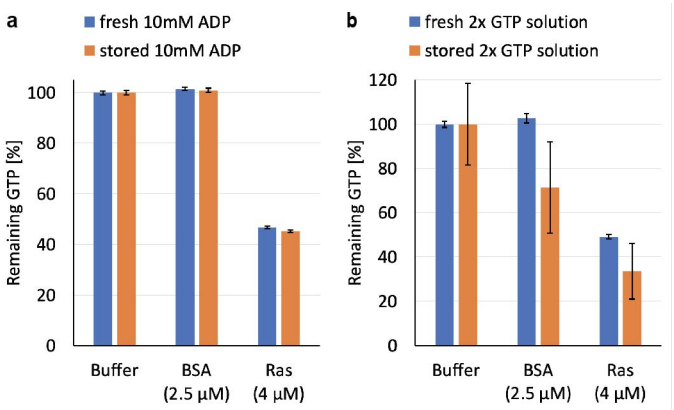
Supplement S5



Well	buffer	GTPase	blank in-between samples	blank separate row
1	30376	22725	373	30
2	31316	22891	451	31
3	30448	22553	425	31
Average (Luminescence)	30713	22723	416	30
Std. err. mean (Lum.)	302	98	23	0
Remaining GTP [%]	100,0	74,0	1,4	0,1
Error [%]	1,4	0,8	0,1	0,0

S5 Figure 1. Leave one empty row between all sample rows to avoid any spill-over of luminescence signal between samples. The row 'blank (in-between samples)' (light blue) does not contain any solution, it is empty. It is placed between a row that contains buffer and a row with a GTPase sample, both of which have a strong luminescence signal (as is expected). This leads to a small, but detectable luminescence signal in this row. In comparison, a similar blank/empty row that is not placed next to a sample row (green), exhibits a 10× reduced background luminescence. This spill-over of luminescence signal in in-between sample rows (light blue) translates to an 1% increase in remaining GTP - a small but unnecessary error to the assay's accuracy.

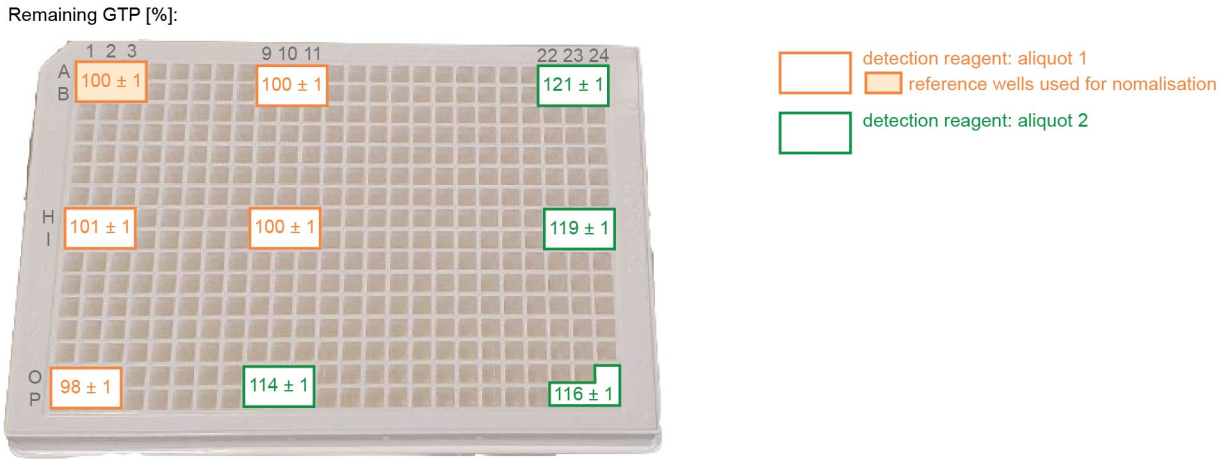
Supplement S6



S6 Figure 1. In GTPase assays the by the kit provided 10 mM ADP solution can be re-used (here: 3 freeze/thaw cycles) (a). In contrast, the 2× GTP solution can not be stored (b)! The graphs show the amount of remaining GTP for buffer (used for normalisation), BSA, and Ras GTPase. BSA is not a GTPase and does not change the GTP content. Ras is a GTPase and hydrolyses GTP, decreasing the amount of remaining GTP. Re-using 2× GTP solution results in huge variations between replicas of the same sample, leading to large error bars.

Supplement S7

We advice to aliquote the detection reagent to reduce the number of freeze-thaw cycles and decrease the time required for thawing. Before using the detection reagent in GTPase assays, prepare a sufficient volume (e.g. through mixing of several aliquots) and vortex for proper mixing. We strongly advice against using separate detection reagent aliquots in one assay. *In some cases* this results in a large shift in luminescence, negating assay reliability (S7 Fig. 1).



Well	A1-A3 B1-B3	A9-A11 B9-B11	H1-H3 I1-I3	H9-H11 I9-I11	O1-O3 P1-P3	A22-A24 B22-B24	H22-H24 I22-I24	O9-O11 P9-P11	O24 P22-P24
1	34293	34202	34944	34710	34271	41728	41384	39597	
2	33683	33608	34298	33252	33075	41069	41524	38479	
3	34268	34596	34876	34442	33015	40874	40438	37845	39692
4	35463	33775	35026	35490	33531	42263	41625	40377	40512
5	34616	35616	35646	34335	34150	42724	40857	40479	39558
6	34704	35814	35265	35350	35035	42505	41042	40395	41698
Average (Luminescence)	34465	34359	34958	34446	33608	41731	41165	39355	39921
Std. err. mean (Lum.)	291	358	215	360	262	349	225	520	298
Remaining GTP [%]	100	100	101	100	98	121	119	114	116
Error [%]	1	1	1	1	1	1	1	2	1

S7 Figure 1. Luminescence (and resulting remaining GTP) values of a GTPase assay where buffer was added to all wells. In wells marked in orange one detection reagent aliquot was used and in wells marked in green a separate detection reagent aliquot was used. The use of distinct aliquots resulted in this case in a large difference in luminescence between both groups, propagating to an up to 20% difference in the perceived amount of remaining GTP.

Supplement S8

We developed a GTPase activity model for determining the GTPase cycling rates k. It is briefly described in the following.

A GTPase cycling model

GTPase cycling involves three steps: (1) A GTP molecule from solution binds to the GTPase. (2) The GTPase hydrolyses GTP. (3) The GTPase releases GDP.

$$[\mathrm{GTP}] + [\mathrm{GTPase}] \rightleftharpoons [\mathrm{GTPase{-}GTP}]$$

$$[\mathrm{GTPase{-}GTP}] \longrightarrow [\mathrm{GTPase{-}GDP}]$$

$$[\mathrm{GTPase{-}GDP}] \rightleftharpoons [\mathrm{GTPase}] + [\mathrm{GDP}]$$

The activity of some GTPases can further be upregulated by effector proteins: GAPs have been shown to enhance GTP hydrolysis by the GTPase (step 2), GEFs enhance the release of GDP from the GTPase (step 3) [Bos et al., 2009, Vetter and Wittinghofer, 2001, Cherfils and Zeghouf, 2013].

To quantitatively describe the GTPase reaction cycle, we coarse-grained the GTPase reaction steps with

$$[\mathrm{GTP}] + n\,[\mathrm{GTPase}] \longrightarrow [\mathrm{GDP}] + n\,[\mathrm{GTPase}]$$

To account for possible GTPase dimerisation and cooperativity, we included the following reactions into the model:
(1) Some GTPase enzymes can dimerise [Zhang and Zheng, 1998, Zhang et al., 1999, Zhang et al., 2001, Kang et al., 2010]:

$$2\,[\mathrm{m\ GTPase}] \rightleftharpoons [\mathrm{d\ GTPase}]$$

and both monomeric and dimeric forms of the GTPase can contribute to the overall GTP hydrolysis with different rates:

$$[\mathrm{GTP}] + [\mathrm{m\ GTPase}] \xrightarrow{k_1'} [\mathrm{GDP}] + [\mathrm{m\ GTPase}]$$

$$[\mathrm{GTP}] + [\mathrm{d\ GTPase}] \xrightarrow{k_2'} [\mathrm{GDP}] + [\mathrm{d\ GTPase}]$$

Assuming that the majority of the GTPase enzyme is in its monomeric form ([m GTPase] $< C_d$, with C_d as the concentration at which half of the total GTPase is dimeric), we can approximate

$$\begin{aligned} [\text{d GTPase}] &= \frac{[\text{m GTPase}]^2}{2C_d} \\ [\text{m GTPase}] &\approx [\text{GTPase}] - \frac{[\text{GTPase}]^2}{C_d} \end{aligned} \tag{6}$$

(2) Next to cooperativity from dimerisation, cooperativity can also emerge when GTPase proteins come in close contact with each other - they can affect each other's behaviour without forming a stable homodimer, effectively functioning as an effector protein for themselves:

$$[\mathrm{GTP}] + 2\,[\mathrm{m\ GTPase}] \xrightarrow{k_3'} [\mathrm{GDP}] + 2\,[\mathrm{m\ GTPase}]$$

$$[\mathrm{GTP}] + [\mathrm{m\ GTPase}] + [\mathrm{d\ GTPase}] \xrightarrow{k_4'} [\mathrm{GDP}] + [\mathrm{m\ GTPase}] + [\mathrm{d\ GTPase}]$$

(3) Effector proteins, such as GAPs and GEFs, affect the speed of the GTP hydrolysis cycle:

$$[\mathrm{GTP}] + [\mathrm{GTPase}]\,\mathrm{n[X]} \xrightarrow{k_5'} [\mathrm{GDP}] + [\mathrm{GTPase}]\,\mathrm{n[X]}$$

Here X is an effector protein and $n \in \mathbb{N}$.

Our data showed that the amount of remaining GTP follows an exponential decline over time (S4):

$$[\text{GTP}]_t = [\text{GTP}]_{t_0} \exp(-Kt)\text{, using } [\text{GTP}]_{t_0} = 1 \tag{7}$$

Considering reactions (1) - (3), we can thus define K in Eq. 7 as

$$K = k_1'[\text{m GTPase}] + k_2'[\text{d GTPase}] + k_3'[\text{m GTPase}]^2 + k_4'[\text{m GTPase}][\text{d GTPase}] + k_5'[\text{GTPase}][X]^n$$

Using Eq. 6, and considering only up to second-order terms, results in

$$\begin{aligned} K &= k_1'[\text{GTPase}] + \left(\frac{k_2'}{2C_d} + k_3' - \frac{k_1'}{C_d}\right)[\text{GTPase}]^2 + k_5'[\text{GTPase}][X]^n \\ &= k_1[\text{GTPase}] + k_2[\text{GTPase}]^2 + k_{3,X}[\text{GTPase}][X]^n \end{aligned} \tag{8}$$

where k_1 refers to GTP hydrolysis cycling rates of monomeric GTPase, k_2 includes effects of cooperativity and dimerisation and k_3 represents the rate of GTPase - effector interaction. We refer to K as 'overall GTP hydrolysis rate'.

Variability between assays

Eq. 8 with [X]=0 can be used to determine the rates of the GTPase alone. Then assays with the GTPase and an effector protein can be conducted to determine k_3. While doing so one needs to account for assay variability, i.e. for the observation that the rates for the GTPase can vary between assays. Possible reasons for this include small concentration differences introduced though pipetting of small volumes (as are required for this assay), temperature and shaker speed fluctuations during the incubation step, and/or intrinsic changes in the protein activities due to other external conditions. To account for this variance, we introduced the parameter c_{corr}. It maps all factors that lead to variations between assays onto the GTPase concentration.

The assay data, including samples containing only GTPase and GTPase - (effector X) mixtures, are fitted with

$$K = k_1 c_{corr}[\text{GTPase}] + k_2(c_{corr}([\text{GTPase}])^2 + k_{3,X} c_{corr}[\text{GTPase}][X]^n \tag{9}$$

to determine c_{corr} and $k_{3,X}$ (using k_1 and k_2 determined earlier) (with n either 1 or 2).

c_{corr} values are usually close to 1.0 (e.g. [Tschirpke et al., 2023b, Tschirpke et al., 2023a]), showing that the variation between assays is small. We advice to exclude assays with a big or very small c_{corr}, as these indicate that the GTPase behaviour/assay conditions are unusual.

GTPase - effector interactions

The accompanying matlab code allows to fit GTPase - effector mixtures that depend either linearly ($n = 1$) or quadratically ($n = 2$) on the effector concentration $[X]$ (Eq. 9). If the effectors show neither a linear nor a quadratic concentration-dependence (e.g. due to saturation), we advice to either only include the linear/quadratic regimes into the analysis or extend our fitting model to match the specific case.

The model allows to fit GTPase - effector mixtures with up to two effectors present:

$$K = k_1 c_{corr}[\text{GTPase}] + k_2(c_{corr}[\text{GTPase}])^2 + k_{3,X_1} c_{corr}[\text{GTPase}][X_1]^n + k_{3,X_2} c_{corr}[\text{GTPase}][X_2]^m + k_{3,X_1,X_2} c_{corr}[\text{GTPase}][X_1]^n[X_2]^m \tag{10}$$

with n and m either 1 or 2.

Supplement S9

This supplement describes a simple python script that reads in GTPase data analysed in a spreadsheet editor (Support Protocol 1) and re-formats it into the input required for Support Protocol 2.
The script is illustrated using data of example 1 (Fig. 5).

Necessary resources

- Python script file: 'Ras_example.ipynb'
- Data file: 'example1.xlsx'
- a spreadsheet editor
- software to run a python script

Steps

1. Open 'Ras_example.ipynb', state the input data and relevant tab names:

```
datafilename = 'example1.xlsx'
tabnamelist = ['E1', 'E2']
```

2. Run the python script. It will generate two excel sheeets: 'E1.xlsx' and 'E2.xlsx'.
3. Copy data of both outputs into one excel sheet, but only include one header (S9 Fig. 1)! This will be the input for the matlab script used in Support Protocol 2.
4. Use the find/replace option of the spreadsheet editor to replace '.' (a point) with ',' (a comma).

   The python script generates numbers of the format '1.00' while the matlab script requires the format '1,00'.

General considerations on how the script operates

The spreadsheet data needs to conform to the following formatting to be processed by the python script (S9 Fig. 2):

- The script processes values in the spreadsheet area A80-Z89.

  This area can only contain relevant numbers. If comments are placed in this area, the script will given an error.
- The incubation time, stated in hours, must be stated in cell C82 (S9 Fig. 2 blue box).
- The error of the buffer must be stated in cell E81 (S9 Fig. 2 blue box).
- Remaining GTP values must be stated in cells F80-Z80. Cells not in use must remain empty (S9 Fig. 2 red box).
- Remaining GTP error values must be stated in cells F81-Z81. Cells not in use must remain empty (S9 Fig. 2 red box).
- Protein names must follow the formatting `'ProteinName_conc'` and be stated in A83-A89. Cells not in use must remain empty (S9 Fig. 2 orange box).
- Protein concentration values must be stated in the area B83-Z89. It is important to state the concentration of each protein listed in the protein name section here. (I.e. if a protein is not part of a sample, its concentration is 0.) Cells not in use must remain empty (Fig. 2 orange box).

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
E2	1,75	0,378457167	0,002579438	0,008967404	5
E2	1,75	0,557180709	0,008588182	0,008967404	4
E2	1,75	0,739454841	0,006747585	0,008967404	3
E2	1,75	0,878599651	0,013720581	0,008967404	2
E2	1,75	0,95195911	0,008710646	0,008967404	1
E2	1,75	0,977303953	0,00689024	0,008967404	0,5

S9 Figure 1. Required input format for the matlab scirpt (Support Protocol 2).

	A	B	C	D	E	F	G	H	I	J	K
69						Ras					
70	**Well**			**buffer**	**buffer**	**5uM**	**4uM**	**3uM**	**2uM**	**1uM**	**0.5uM**
71	**1**			35330	34324	15662	21294	26750	31392	33846	35117
72	**2**			35668	33599	15829	21478	26466	30209	34289	34076
73	**3**			35151	33313	15579	21739	26105	30286	33948	33736
74	**4**										
75	**5**										
76											
77	**Average**				34564	15690	21503	26440	30629	34027	34310
78	**Std. err. mean**				396	74	129	187	382	134	415
79											
80	**Remaining GTP [%]**				**100,0**	**45,4**	**62,2**	**76,5**	**88,6**	**98,4**	**99,3**
81	**Error [%]**				1,6	0,6	0,8	1,0	1,5	1,2	1,7
82	**time [min; h]**	94	1,567								
83	**Ras_conc**					5	4	3	2	1	0,5
84											
85											
86											
87											
88											
89											
90											

E1 E2 matlab +

S9 Figure 2. Required input format for the python scirpt.

Supplement S10

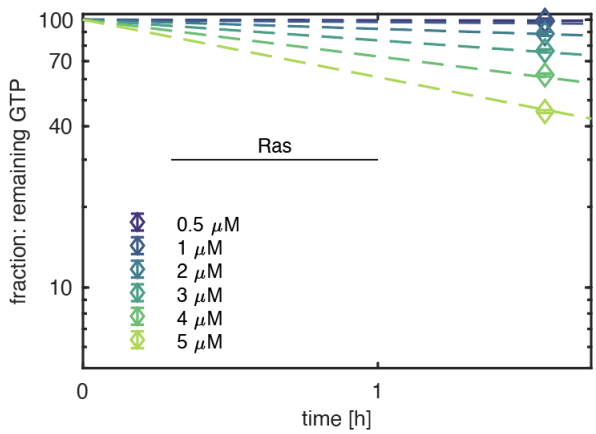
To use the plotting scripts, a 'Data_assays.mat' file (output of Support Protocol 2) is required. All scripts automatically save the plot as '.tif' and '.pdf'.

Plot_Semilog_GTP_time.m

This script produces plots showing of the amount of GTP over time fitted with an exponential, as shown in S4. It can be used for assays of GTPases and GTPase effector mixtures with one effector.

1. Run 'Plot_Semilog_GTP_time.m'.
2. Select a 'Data_assays.mat' file.
3. Choose an assay name, as stated in the previously used 'assaylist.xlsx' file. Then choose which assay (shown by assay number) should be plotted.
   An example plot is given in S10 Fig. 1.

   This script is especially useful to assess/ verify that the amount of GTP declines exponentially. To do so, several assays (e.g. 3) of different incubation times using the same protein concentrations need to be conducted. To plot all (3) assays in the same plot, give these assays ***the same*** *assay number before analysing them using 'Process_assays.m'.*

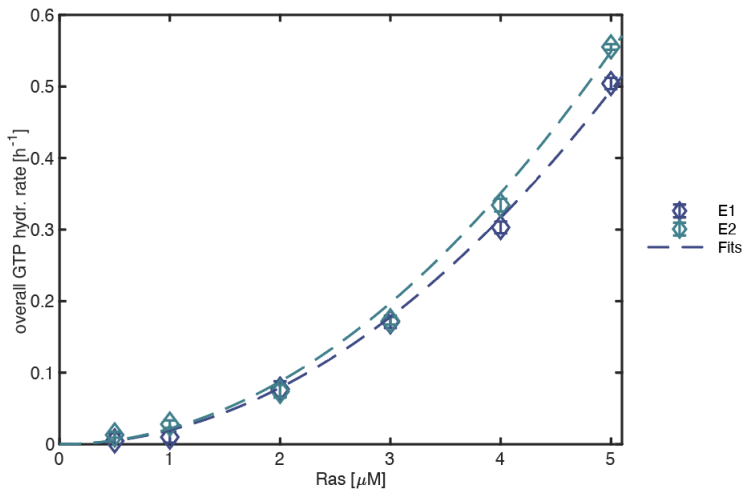


S10 Figure 1. Exponential fit for the amount of amount of GTP over time for Ras concentrations. Generated for assay name 'Ras' and experiment number 'E1' (using 'Plot_Semilog_GTP_time.m' and 'Data_assays.mat' in the folder 'example1and2 matlab output').

Plot_rate_concentration.m

This script produces allows to plot of the overall GTP hydrolysis rate for several assays in one figure. It can be used for assays of GTPases and GTPase effector mixtures with one effector.

1. Run 'Plot_rate_concentration.m'.
2. Select a 'Data_assays.mat' file.
3. Choose an assay name, as stated in the previously used 'assaylist.xlsx' file.
   An example plot is given in S10 Fig. 2.

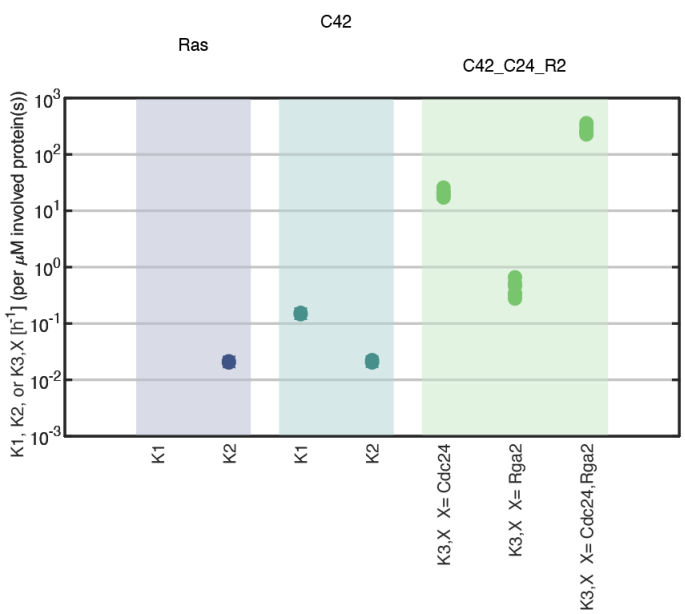


S10 Figure 2. Overall GTP hydrolysis rate over Ras concentration for assays 'E1' and 'E2'. Generated for assay name 'Ras' (using 'Plot_rate_concentration.m' and 'Data_assays.mat' in the folder 'example1and2 matlab output').

Plot_pooled_values_plot_std_err.m

This script produces plots of the pooled rates k_1, k_2 k_3 (that are also shown in the 'Data_summary.xlsx' file).

1. Run 'Plot_pooled_values_plot_std_err.m'.
   The parameter 'y_limits' in the code can be used to modify the limits of the y-axis. It is currently set to [1e-3 1e3].
2. Select a 'Data_assays.mat' file.
3. Choose an assay name, as stated in the previously used 'assaylist.xlsx' file.
   An example plot is given in S10 Fig. 3.



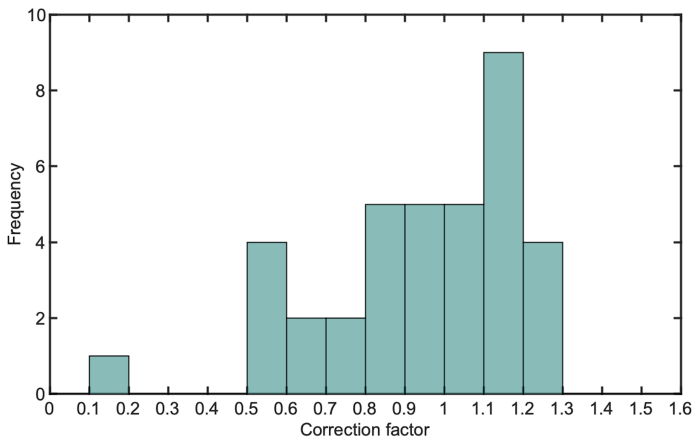
S10 Figure 3. Plot of rates K_1, K_2 for Ras (left), K_1, K_2 for Cdc42 (middle), and K_3, X for Cdc42-Cdc24-Rga2 assays. The assay name is stated on top. Rate values of individual experiments are shown as filled dots. The average is shown as a cross and error bars represent the standard error. Generated for assay names 'Ras', 'C42', and 'C42-C24-R2' (using 'Plot_pooled_values_plot_std_err.m' and 'Data_assays.mat' in the folder 'example1and2 matlab output').

Plot_c_corr_histogram.m

This script produces a histogram plot of c_{corr} values. Before running the script, first copy/paste all c_{corr} values that should be plotted into a spreadsheet file.

1. Copy/paste all c_{corr} values that should be plotted from 'Data_summary.xlsx' into the first column of a spreadsheet file. All values should be in column A. Change the number formatting from ',' to '.' (i.e. change the number formatting from '0,863' to '0.863'.
2. Run 'Plot_c_corr_histogram.m'.
   An example plot is given in S10 Fig. 4.

   The parameter 'bin_size' , 'x_start' , and 'x_end' in the code can be used to modify the plot. 'bin_size' states the bin size for the histogram segmentation, and 'x_start' and 'x_end' define the x-axis limits (i.e. minimum and maximum c_{corr} values that will be plotted).



S10 Figure 4. Histogram plot of c_{corr} using a bin size of 0.1. Generated for all c_{corr} values of example 2 (using 'Plot_c_corr_histogram.m' and 'example2-histogram.xlsx' in the folder 'example2 matlab output').