

Guanine Nucleotide Exchange Assay Using Fluorescent MANT-GDP

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[Abstract] GTPases are molecular switches that cycle between the inactive GDP-bound state and the active GTP-bound state. GTPases exchange nucleotides either by its intrinsic nucleotide exchange or by interaction with guanine nucleotide exchange factors (GEFs). Monitoring the nucleotide exchange *in vitro*, together with reconstitution of direct interactions with regulatory proteins, provides key insights into how a GTPase is activated. In this protocol, we describe core methods to monitor nucleotide exchange using fluorescent N-Methylanthraniloyl (MANT)-guanine nucleotide.

Keywords: GTPase, N-Methylanthraniloyl (MANT), Nucleotide exchange, GEF assay, *in vitro*, Fluorescence, Fluorescent nucleotides, Fluorescent GDP

[Background] GTPases are guanine nucleotide binding proteins that regulate a breadth of cellular processes, ranging from protein biosynthesis to cell-cycle progression and from cytoskeletal reorganization to membrane trafficking. GTPases can be thought of as molecular switches that cycle between a GDP-bound 'off' state and a GTP-bound 'on' state; upon GTP-binding via nucleotide exchange of GDP for GTP, GTPases become active and will bind to down-stream effector proteins to recruit and activate the biological function of these effectors. GTPases bind the γ -phosphate of GTP via interactions with a highly conserved threonine of the switch I loop (G2 domain) and a glycine within a DxxG motif of the switch II loop (G3 domain). Upon GTP hydrolysis, the loss of the interaction with the γ -phosphate causes a dynamic conformational change that turns the GTPase into an off state (Vetter and Wittinghofer, 2001). Typically, small GTPases have a very high affinity for guanine nucleotides, with dissociation constants in the nanomolar to picomolar range (Bos *et al.*, 2007), and therefore require guanine nucleotide exchange factors (GEFs) to lower their nucleotide affinity to allow for rapid activation. Notable exceptions include several highly conserved centrosomal/ciliary small GTPases, such as Rab12 (Kanie *et al.*, 2017), ARL13B (Ivanova *et al.*, 2017), Ift27/Rab14 (Bhogaraju *et al.*, 2011), and Arl6 (Price *et al.*, 2012), and many large GTPases, such as the dynamin family (Gasper *et al.*, 2009), which have lower affinities for GDP/GTP, with dissociation constants in the micromolar range. In this configuration, these GTPases can become activated without the use of GEFs via their intrinsic nucleotide exchange. Although small GTPases that show spontaneous exchange may have additional regulatory proteins, finding that a small GTPase has a micromolar affinity for GDP/GTP and is capable of spontaneous exchange may suggest the absence of a traditional GEF and suggest looking for other forms of GTPase regulatory factors (see Kanie *et al.* [2017] for a notable example).

Fluorescently-labeled guanine nucleotides are more suitable for monitoring nucleotide exchange than radioactive GDP/GTP, as they are safer and allow continuous spectroscopic monitoring and thus provide a more detailed analysis of kinetics. N-Methylanthraniloyl (MANT) is the most widely used fluorescent analog to label guanine nucleotides because it is smaller than most fluorophores and unlikely to cause major perturbations of protein-nucleotide interactions (Hiratsuka, 1983). Another attractive feature of this fluorescent nucleotide is that the emitted fluorescent signal increases dramatically upon binding to a GTPase (typically twice as high as the signal of unbound MANT-guanine nucleotide) (John *et al.*, 1990), allowing one to directly monitor the association and dissociation of guanine nucleotides from GTPases. The most commonly used method to monitor nucleotide exchange is tracking the decrease in the fluorescence of protein-bound MANT-GDP upon addition of an excess amount of GppNHp, a non-hydrolyzable GTP analog. MANT-GDP-bound GTPase can be prepared by the incubation of nucleotide free GTPase with 1.5 fold excess of MANT-GDP (John *et al.*, 1990; Eberth and Ahmadian, 2009). Although this protocol is described in great detail and allows us to save expensive MANT-GDP, it is time-consuming and limited by the accessibility to high performance liquid chromatography. Alternatively, we describe here a simpler protocol where MANT-GDP is loaded onto GTPase by incubating GTPase with 20-fold molar excess of MANT-GDP in the presence of ethylenediaminetetraacetic acid (EDTA). EDTA chelates magnesium ions, which form coordination bonds with the β and γ phosphates of GTP (or β phosphate of GDP) and with the GTPase (Pai *et al.*, 1990; Tong *et al.*, 1991). EDTA significantly lowers the affinity of the GTPase for guanine nucleotide. The loading reaction is stopped by the addition of excess magnesium chloride. Unbound MANT-GDP is removed by gel filtration using a NAP-5 prepacked column and the nucleotide exchange reaction is initiated by the addition of 100-fold molar excess of GppNHp. Exchange is monitored by a decrease in the fluorescent signal as recorded by a spectrometer with excitation wavelength at 360 nm and emission at 440 nm.

Variations on this method are readily accomplished by addition of biochemically pure nucleotide variants, drugs, or purified protein regulatory factors, allowing for a range of mechanistic experiments and definitive tests of biochemical mechanisms.

Materials and Reagents

1. Microcentrifuge tube (Corning, Costar[®], catalog number: 3621)
2. 50 ml conical tube (Thermo Fisher Scientific, Thermo Scientific[™], catalog number: 339652)
3. Oak ridge tube (Thermo Fisher Scientific, Thermo Scientific[™], catalog number: 3119-0050)
4. 15 ml conical tube (Thermo Fisher Scientific, Thermo Scientific[™], catalog number: 339650)
5. (Optional) Amicon ultra concentrator (Merck, catalog number: UFC901024)
6. Dialysis tubing (Thermo Fisher Scientific, Thermo Scientific[™], catalog number: 88242)
7. NAP-5 columns (GE Healthcare, catalog number: 17085301)
8. Aluminum foil
9. 384-well microplate (Greiner Bio One International, catalog number: 784076)
10. (Optional) 96-well microplate (Corning, catalog number: 3686)

11. 0.2 μm disposable bottle top filter (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 597-4520)
12. 0.22 μm syringe filter (Merck, catalog number: SLGV033RS)
13. Disposable cuvettes (Fisher Scientific, catalog number: 14-955-127)
14. Multichannel pipette tip (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 7421)
15. Rosetta2 bacterial cells (Merck, EMD Millipore, catalog number: 71403)–Store at $-80\text{ }^{\circ}\text{C}$
16. Recombinant GTPase
Note: Ideally, the concentration should be 100 μM or greater. See the protocol below for the expression and purification of recombinant GTPases. Store at $-80\text{ }^{\circ}\text{C}$.
17. Gateway-cloning compatible pGEX6p vector
18. 4x LDS sample buffer (Thermo Fisher Scientific, Invitrogen™, catalog number: NP0008)
19. 2-Mercaptoethanol (Sigma-Aldrich, catalog number: M3148)–Store at $4\text{ }^{\circ}\text{C}$
20. GppNHp (Abcam, catalog number: ab146659)
Note: Dissolve in Milli-Q water to prepare a 50 mM stock solution. Store at $-20\text{ }^{\circ}\text{C}$.
21. Nu-PAGE gel (Thermo Fisher Scientific, Invitrogen™, catalog number: NP0321BOX)
22. Coomassie Brilliant Blue R-250 (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 20278)
23. (Optional) Classical Laemmli sodium dodecyl sulfate (SDS) sample buffer/SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel
24. Glutathione Sepharose 4B media (GE Healthcare, catalog number: 17075605)–Store at $4\text{ }^{\circ}\text{C}$
25. GST-PreScission (10 mg/ml)
Note: We prepare GST tagged PreScission by ourselves. 1 μl of our protease cleaves approximately 1 mg of GST-ARL3, a test protein. Alternatively, you can purchase the protease from GE Healthcare, catalog number: 27084301. Store at $-80\text{ }^{\circ}\text{C}$.
26. Bradford Reagent Concentrate (Bio-Rad Laboratories, catalog number: 5000006)–Store at $4\text{ }^{\circ}\text{C}$
27. Liquid nitrogen
28. BSA standard, 2 mg/ml (Thermo Fisher Scientific, catalog number: 23209)–Store at $4\text{ }^{\circ}\text{C}$
29. TritonX-100 (Acros Organics, catalog number: 215682500)
30. Milli-Q water
31. Protease inhibitor tablet (Thermo Fisher Scientific, Thermo Scientific™, catalog number: A32965)–Store at $4\text{ }^{\circ}\text{C}$
32. Glycerol (Sigma-Aldrich, catalog number: G5516)
33. MANT-GDP triethylammonium salt solution (Sigma-Aldrich, catalog number: 69244)–Store at $-20\text{ }^{\circ}\text{C}$ protected from light
34. Trizma base (Sigma-Aldrich, catalog number: T6066)
35. Concentrated HCl (Aqua Solutions, catalog number: H2505-500ML)
36. Glacial acetic acid (Fisher Scientific, catalog number: A490-212)
37. Methanol (Fisher Scientific, catalog number: A412-4)
38. HEPES (Sigma-Aldrich, catalog number: H3375)

39. Sodium hydroxide (Fisher Scientific, catalog number: BP359-500)
40. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S3014-500G)
41. Magnesium chloride hexahydrate (MgCl₂·6H₂O) (Sigma-Aldrich, catalog number: M2670)
42. Dithiothreitol (DTT) (Promega, catalog number: V3155)
43. Ethylenediaminetetraacetic acid (EDTA) (Fisher Scientific, catalog number: S311)
44. LB broth (Fisher Scientific, catalog number: BP9723)
45. LB agar (Thermo Fisher Scientific, Invitrogen™, catalog number: 22700)
46. Terrific broth (Fisher Scientific, catalog number: BP24682)
47. Carbenicillin (Sigma-Aldrich, catalog number: C3416)
48. Ampicillin (Fisher Scientific, catalog number: BP1760)
49. Chloramphenicol (Sigma-Aldrich, catalog number: C0378)
50. IPTG (Thermo Fisher Scientific, Invitrogen™, catalog number: 15529019)–Store at -20 °C
51. LB media (see Recipes)
52. TB media (see Recipes)
53. 100 mg/ml ampicillin (see Recipes)
54. 50 mg/ml carbenicillin (see Recipes)
55. 34 mg/ml chloramphenicol (see Recipes)
56. LB agar plate containing 50 µg/ml carbenicillin and 34 µg/ml chloramphenicol (see Recipes)
57. 1 M IPTG (see Recipes)
58. 1 M Tris-HCl (pH 7.5) (see Recipes)
59. 1 M HEPES-NaOH (pH 7.5) (see Recipes)
60. 5 M NaCl (see Recipes)
61. 1 M MgCl₂ (see Recipes)
62. 1 M DTT (see Recipes)
63. 0.25 M EDTA (pH 8) (see Recipes)
64. 10 M NaOH (see Recipes)
65. Coomassie Brilliant Blue staining solution (see Recipes)
66. Coomassie Brilliant Blue destaining solution (see Recipes)
67. Lysis buffer (see Recipes)
68. PreScission cleavage buffer (see Recipes)
69. Storage buffer (see Recipes)
70. Low magnesium buffer (see Recipes)
71. 2x MANT-GDP loading buffer (see Recipes)
72. Nucleotide exchange buffer (see Recipes)

Note: Unless otherwise noted, materials are stored at room temperature. Frozen proteins and nucleotides can be maintained for at least a year if pure.

Equipment

1. Milli-Q generator (Merck, model: Milli-Q® Advantage A10, catalog number: Z00Q0V0WW)
2. 250 ml culture flask (Corning, PYREX®, catalog number: 4980-250)
3. 2,800 ml culture flask (Corning, PYREX®, catalog number: 4424-2XL)
4. Incubator shaker (Eppendorf, New Brunswick™, model: Excella® E25, catalog number: M1353-0002)
5. Refrigerated centrifuge (Eppendorf, model: 5424 R)
6. 500 ml centrifuge bottle (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 3141-0500)
7. JA-10 rotor (Beckman Coulter, model: JA-10, catalog number: 369687)
8. Avanti J-25I refrigerated centrifuge (Beckman Coulter, model: Avanti J-25I, catalog number: 363106)
9. JA-17 rotor (Beckman Coulter, model: JA-17, catalog number: 369691)
10. Allegra X-15R refrigerated centrifuge (Beckman Coulter, model: Allegra® X-15R, catalog number: 392932)
11. BioSpectrometer (Eppendorf, catalog number: 6136000010)
12. Thermomixer (Fisher Scientific, catalog number: 05-412-401)

Note: This equipment has been discontinued. Thermomixer C (Fisher Scientific, catalog number: 05-412-503; Manufacturer: Eppendorf, catalog number: 5382000023) is available as an alternative. Any type of temperature-controlled mixer with cooling function should work for this experiment.

13. TECAN Infinite M1000 Microplate reader (TECAN, part number: 30034301)

Note: There are a number of plates or cuvette-based fluorimeters that can be adapted to this procedure. Those include FluoroMax-4 (Horiba) (Price et al., 2012), Synergy H4 Hybrid Microplate reader (BioTek) (Ivanova et al., 2017), and Envision (Perkin-Elmer) (Maurer et al., 2012).

14. Branson Digital Sonifier (Branson)

Note: This equipment has been discontinued. Branson Ultrasonics Sonifier SFX250/SFX550 (Fisher Scientific, catalog number: 15-345-141; Manufacturer: EMERSON, Branson, catalog number: 101063969R) is available as an alternative.

15. 360° vertical rotator (Grant Instruments, model: PTR-30)

Note: This equipment has been discontinued. Grant bio 360° vertical rotator PTR-35 (Grant Instruments, model: PTR-35) is available as an alternative.

16. Lab stand (Humboldt, catalog number: H-21207)
17. Vortex mixer (Scientific Industries, model: Vortex-Genie 2, catalog number: SI-0236)
18. Multichannel electronic pipette (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 2069MTRX)

Note: This equipment has been discontinued. A newer model (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 4671020BT) is available as an alternative.

19. Autoclave

Software

1. Microsoft Excel (Microsoft)
2. GraphPad Prism 7 software (GraphPad Software)

Procedure

A. Expression and purification of recombinant GTPases from *E. coli*

Recombinant GTPases can be produced and purified from *Escherichia coli*, since the nucleotide exchange assay does not generally require post-translational modifications of the GTPase. Experimental conditions, such as temperature and culture time for inducing protein expression and Isopropyl β -D-1-thiogalactopyranoside (IPTG) concentration, need to be determined for each GTPase to obtain the maximal yield of soluble recombinant proteins. Conditions can be optimized in small scale (we typically perform a pilot experiment with 2 ml of LB media). We typically induce the protein expression with 0.1 mM IPTG at 18 °C overnight. A typical yield from 1 L culture is 10 mg of protein.

1. Transform Rosetta2 bacteria cells with a vector containing gene of interest. Plate the cells onto an LB agar plate containing 50 μ g/ml carbenicillin (or 100 μ g/ml ampicillin) and 34 μ g/ml chloramphenicol. Incubate the plate at 37 °C overnight.

Note: We typically use Gateway-cloning compatible pGEX6p vector (Wright et al., 2011), which produces an N-terminally Glutathione S-transferase (GST) tagged protein with PreScission protease recognition site between GST and protein of interest. The vectors containing CDC42, a GEF-dependent GTPase, and IIGP1 and CrRab12, GEF-independent GTPases, work as positive controls for the experiment and are provided by the authors upon request. Chloramphenicol is added into an agar plate because tRNAs for rare codons are maintained by the chloramphenicol-resistant plasmid in the Rosetta2 bacteria cells. Other antibiotics might have to be used depending on bacteria cells used.

2. Inoculate 50 ml of LB media containing 100 μ g/ml ampicillin and 34 μ g/ml chloramphenicol with a single colony of the transformed cells in a 250 ml flask. Grow the starter culture at 37 °C at 250 rpm overnight.
3. Pre-warm 1 L of autoclaved TB media without antibiotics in 2,800 ml flask at 37 °C overnight in an incubator shaker, before starting a large-scale culture.
4. Inoculate 1 L of pre-warmed TB media with 25 ml of the starter culture. Shake the flask at 37 °C at 250 rpm until OD₆₀₀ reaches to 0.6-0.7. It takes approximately 2 h.
5. As soon as OD₆₀₀ reaches to 0.6-0.7, set the flask on ice and chill culture for 15 min.

6. Add 100 μ l of 1 M IPTG (the final concentration is 0.1 mM) to induce protein expression.
7. Shake the flask at 18 °C at 250 rpm in an incubator shaker overnight.
8. Place the flask on ice before harvesting the bacteria cells.
9. To confirm the expression level and the solubility of the protein, transfer 1 ml of the bacterial suspension into a microcentrifuge tube. Collect the cell pellet by centrifugation at 8,000 rpm (6,010 \times g) at 4 °C for 1 min using a refrigerated centrifuge. Re-suspend the cell pellet in 300 μ l of pre-chilled lysis buffer and lyse the cells by sonication for 1 min on ice (0.6 sec/0.4 sec on/off cycle at 30% amplitude), followed by centrifugation at 15,000 rpm (21,130 \times g) at 4 °C for 10 min. Transfer the supernatant into a microcentrifuge tube and add 100 μ l of 4x LDS sample buffer and 10 μ l of 2-mercaptoethanol to prepare the soluble fraction. To prepare the insoluble fraction, add 290 μ l of the lysis buffer, 100 μ l of 4x LDS sample buffer and 10 μ l of 2-mercaptoethanol to the pellet. Incubate the samples at 70 °C for 10 min and run them on Nu-PAGE gel at 200 V until the dye front reaches to the bottom of the gel. Stain the gel in Coomassie Brilliant Blue staining solution for 1 h at room temperature with gentle agitation, followed by destaining in Coomassie Brilliant Blue destaining solution overnight at room temperature with gentle agitation. Your protein of interest should appear in the soluble fraction.

Note: Classical Laemmli sodium dodecyl sulfate (SDS) sample buffer/SDS-polyacrylamide gel electrophoresis (SDS-PAGE) can be used instead of 4x LDS sample buffer/Nu-PAGE system.

10. Harvest bacteria by centrifugation in four 500 ml centrifuge bottles at 6,000 rpm (6,371 \times g) in JA-10 rotor using Avanti J-25I refrigerated centrifuge at 4 °C for 10 min.

Note: Put a maximum of 300 ml in each tube, to prevent spilling during centrifugation.

11. Discard supernatant and freeze the cell pellet at -20 °C until you confirm the expression and the solubility of the protein in Step A9.

Note: Bacterial pellet can be stored at -20 °C for at least one week. Alternatively, you may keep the bacteria pellet on ice for same day confirmation of the expression/solubility then proceed immediately to the next step.

12. Re-suspend the 1 L cell pellet in 30 ml of pre-chilled lysis buffer and transfer suspension to a 50 ml conical tube.
13. Sonicate the cells on ice until the suspension becomes watery (approximately 2 min; 0.6 sec/0.4 sec on/off cycle at 80% amplitude).
14. Transfer the lysate into an oak ridge tube and clarify the lysate by centrifugation at 15,000 rpm (30,996 \times g) at 4 °C in the JA-17 rotor using the Avanti J-25I refrigerated centrifuge for 30 min.
15. During centrifugation, wash 2 ml bed volume of glutathione Sepharose 4B media twice in 20 ml of ice-cold lysis buffer in a 50 ml conical tube. To wash the beads, pellet the beads by centrifugation at 1,466 rpm (500 \times g) at 4 °C for 5 min using the Allegra X-15R refrigerated centrifuge and aspirate the supernatant. Add 20 ml of the lysis buffer and mix by inverting the tube. All washes in the following steps will be done in the same manner.

Note: 1 ml bed volume of beads bind approximately 5 mg of GST.

16. Add clarified lysate into the washed glutathione Sepharose beads and rotate the 50 ml conical tube on a 360° vertical rotator at 4 °C for 2 h.
17. Wash the beads three times with 40 ml (20 resin volumes) of lysis buffer without protease inhibitor.
18. Wash the beads twice with 40 ml (20 resin volumes) of PreScission cleavage buffer.
19. Add 4 ml (2 resin volumes) of PreScission cleavage buffer and transfer them into a 15 ml conical tube.
20. Add 10 µl of 10 mg/ml GST-PreScission to elute the protein. Rotate the 15 ml conical tube on a 360° vertical rotator at 4 °C for 16 h.
Note: 10 µl of 10 mg/ml GST-PreScission is added, because we expect the final yield 10 mg and 1 µl of our GST-PreScission cleaves 1 mg of GST tagged protein. The amount of GST-PreScission that should be added depends on the expression level of the protein and/or how much protein is desired. The amount of proteins contained in the lysate can be estimated based on the amount of protein in the test sample taken at Step A9.
21. Pellet the beads by centrifugation at 1,466 rpm (500 x g) at 4 °C for 5 min using the Allegra X-15R refrigerated centrifuge and collect supernatant into a 15 ml conical tube.
22. Wash the beads with 2 ml (one resin volume) of PreScission cleavage buffer and collect supernatant following centrifugation at 1,466 rpm (500 x g) at 4 °C for 5 min. Repeat wash one more time and combine with the previous supernatant.
23. Measure the concentration of the protein using the Bradford assay (see Step B8 for detail).
Note: If the concentration is less than 100 µM, concentrate the eluted protein using the Amicon ultra concentrator (Merck, catalog number: UFC901024).
24. Check the quality of the protein by Nu-PAGE and Coomassie Brilliant Blue staining.
Note: Classical Laemmli SDS sample buffer/SDS-PAGE can be used instead of 4x LDS sample buffer/Nu-PAGE system.
25. Dialyze the protein using dialysis tubing in at least 100-fold protein volumes of storage buffer at 4 °C overnight.
26. Prepare 200 µl aliquots of the purified protein (the concentration should be 100 µM or greater) and snap freeze the aliquots in liquid nitrogen. Store at -80 °C.

B. Buffer exchange to low magnesium buffer

Loading MANT-GDP on GTPase is performed in the buffer containing 0.5 mM MgCl₂ in the presence of 5 mM EDTA. Since our storage buffer contains 5 mM MgCl₂ (see Recipes) for the stability of GTPase, we first exchange the storage buffer for the low magnesium buffer (see Recipes) using a gel filtration column.

1. Thaw 200 µl of purified GTPase (100 µM or greater) on ice.
Note: 100 µM of GTPase is generally a good starting point.
2. Clamp a NAP-5 column onto a lab stand.

3. Remove the top and bottom cap from the NAP-5 column to allow the excess manufacturer storage buffer to flow through.
4. Equilibrate the NAP-5 gel filtration column with low magnesium buffer by three complete refills of the column. The volume of buffer used for equilibration is approximately 10 ml in total.
5. Apply 200 μ l of purified GTPase onto the center of the equilibrated NAP-5 column and allow the sample to enter the gel bed completely by gravity flow.
6. Apply 500 μ l of low magnesium buffer to the column and collect the flow through in a microcentrifuge tube.

Note: This fraction should not contain any proteins.

7. Apply 1 ml of low magnesium buffer to elute the protein. Collect eluate 100 μ l at a time in microcentrifuge tubes.

Note: Most of the protein should be eluted in the first 4 fractions.

8. Measure the concentration of each fraction including the flow through by the Bradford assay.
 - a. Briefly, dilute one part of Bradford Reagent Concentrate with 4 parts of Milli-Q water, and add 1 ml of diluted Bradford reagent to disposable cuvettes. Add 10 μ l of Milli-Q water or BSA standards (at 0.1, 0.2, 0.4, and 0.8 mg/ml), or 1-2 μ l of each fraction of buffer exchanged GTPase into each cuvette. Mix the cuvettes by vortexing and incubate at room temperature for 5 min. Measure the concentration of each fraction on the BioSpectrometer.
 - b. Calculate the molar concentration from the measured concentration. Use the fraction containing the highest concentration of protein for further experiment. The protein concentration following buffer exchange is generally half as much as the starting concentration.

Note: A detailed instruction manual from Bio-Rad can be found online. There are different methods available to measure a protein concentration, including the Bradford assay, bicinchoninic acid (BCA) assay, and spectrophotometric methods. We prefer the Bradford assay, because it is a fast and easy method and all of the buffer components used in this protocol are compatible with the Bradford reagent (reagent compatibility chart from Bio-Rad can be found online). BCA assay might be affected by a reducing reagent contained in the nucleotide exchange buffer. We do not recommend a spectrophotometric method because GTPases are often purified with guanine nucleotides, which absorb at the wavelength used for the measurement. We typically get more than half of the starting concentration in a peak fraction.

C. Loading MANT-GDP on GTPase

1. Mix 100 μ l of buffer exchanged GTPase with 100 μ l of 2x MANT-GDP loading buffer (see Recipes) in microcentrifuge tubes. The final reaction contains 20 mM HEPES-NaOH (pH 7.5), 50 mM NaCl, 0.5 mM MgCl₂, 5 mM EDTA, 1 mM dithiothreitol (DTT), GTPase, and 20-fold molar excess of MANT-GDP.

Note: The concentration of MANT-GDP depends on the protein concentration and must be calculated in every experiment.

2. Incubate the reaction at 20 °C in a thermomixer without shaking for 90 min. Protect the reaction from the light with aluminum foil.
3. Stop the reaction by adding 2 µl of 1 M MgCl₂ (the final concentration is 10 mM). Mix the reaction by inverting the tubes, followed by centrifugation then incubate the tubes at 20 °C for 30 min.
4. During the incubation, equilibrate a NAP-5 gel filtration column with three complete refills of nucleotide exchange buffer (see Recipes). The volume of buffer used for equilibration is approximately 10 ml in total.
5. To remove unbound MANT-GDP, apply the entire volume (200 µl) of MANT-GDP loaded GTPase onto the NAP-5 column. Allow the sample to enter the gel bed completely by gravity flow.
6. Apply 500 µl of nucleotide exchange buffer and collect flow through.
Note: This fraction should not contain any proteins.
7. Apply 1 ml of nucleotide exchange buffer to elute the protein. Collect 100 µl at a time in microcentrifuge tubes. Keep MANT-GDP loaded GTPases on ice, protected from light (by aluminum foil).
Note: Most of the protein should be eluted in the first 4 fractions.
8. Measure the concentration of each fraction by the Bradford assay as described above.

D. Measure the loading efficiency on the fluorimeter

1. Prepare MANT-GDP standards by making two-fold serial dilutions of free MANT-GDP from 40 µM to 0.15625 µM in nucleotide exchange buffer.
Note: The minimal concentration of free MANT-GDP that can be detected on TECAN Infinite M1000 Microplate reader is around 0.1 µM.
2. Pipette 15 µl of nucleotide exchange buffer, free MANT-GDP standards, or MANT-GDP loaded GTPase into 384-well microplate.
Note: This measurement also works with 96-well format. If you use 96-well format, pipette 50 µl of each sample into a 96-well microplate (Corning).
3. Read the fluorescent signal every 15 sec, 10 times in total, at room temperature (25 °C). The parameters used for the detection are listed (Table 1).

Table 1. The parameters used for the detection of MANT fluorescence

Categories	Parameters	Values
Plate	Plate definition	[GRE384sb]-Greiner 384 Flat Black
Kinetic cycles	Number of cycles	10 (Nucleotide loading assay) or 2,000 (Nucleotide exchange assay)
	Kinetic interval	15 sec
Shaking	Duration	5 sec
	Mode	Orbital
	Amplitude	3 mm
	Frequency	217.3 rpm
Fluorescence Intensity	Excitation wavelength	360 ± 10 nm
	Emission wavelength	440 ± 20 nm
	Detection from	Top
	Gain (0-255)	80
	The number of Flash (400 Hz)	8
	Z-position	24,012 μm

- To calculate the loading efficiency, average the fluorescence intensity for each sample from all 10 time points, then calculate the normalized average by subtracting the background fluorescence value measured for the buffer-only control.
- Plot the free MANT-GDP concentration vs. measured fluorescence and fit the standard curve to a linear function using Microsoft Excel. Use the standard curve to determine the concentration of the MANT-GDP-loaded GTPase from its measured fluorescence; since MANT-GDP signal typically increases approximately two-fold upon binding to protein, first divide the fluorescent signal by two then estimate the concentration of loaded GTPase using the standard curve function. A representative example of this data (Table 2) and the standard curve of free MANT-GDP created from the data (Figure 1) are shown.

Note: The loading efficiency calculated here might not be accurate, as the increase in the signal intensity of MANT-GDP upon binding to GTPases varies among GTPases. Examples of the loading efficiency can be found in the original paper. (Figure S4E in Kanie et al., 2017).

Table 2. An example of the data obtained from loading of MANT-GDP on GTPase

	0 sec	15 sec	30 sec	45 sec	60 sec	75 sec	90 sec	105 sec	120 sec	135 sec	Average	Normalized
Chlamydomonas Rabi2 (11.4 μ M)	2,792	2,729	2,760	2,828	2,757	2,709	2,705	2,720	2,745	2,766	2,751.1	2,685.5
MANT-GDP (40 μ M)	52,067	51,551	51,727	51,208	51,800	51,723	51,333	51,236	51,764	51,289	51,569.8	51,504.2
MANT-GDP (20 μ M)	24,902	24,844	24,600	24,682	24,824	24,822	24,435	25,051	24,705	24,351	24,721.6	24,656
MANT-GDP (10 μ M)	12,562	12,763	12,738	12,763	12,891	12,741	12,762	12,712	12,869	12,693	12,749.4	12,683.8
MANT-GDP (5 μ M)	5,952	5,964	5,929	5,935	5,995	6,050	6,029	6,037	5,969	5,942	5,980.2	5,914.6
MANT-GDP (2.5 μ M)	2,930	2,922	2,890	2,985	2,905	2,971	2,943	2,906	2,891	2,901	2,924.4	2,858.8
MANT-GDP (1.25 μ M)	1,533	1,537	1,553	1,528	1,517	1,557	1,522	1,524	1,512	1,521	1,530.4	1,464.8
MANT-GDP (0.625 μ M)	822	813	813	817	807	809	812	799	808	826	812.6	747
MANT-GDP (0.3125 μ M)	428	423	421	419	421	424	420	420	422	419	421.7	356.1
MANT-GDP (0.15625 μ M)	241	236	239	235	238	234	233	232	230	231	234.9	169.3
reaction buffer	67	64	67	66	66	64	68	65	65	64	65.6	0
The signal corresponds to free MANT-GDP	2.336											
Estimated MANT-GDP concentration (μ M)	1.168											
Mol concentration of protein (μ M)	11.4											
Loading efficiency (%)	10.25											

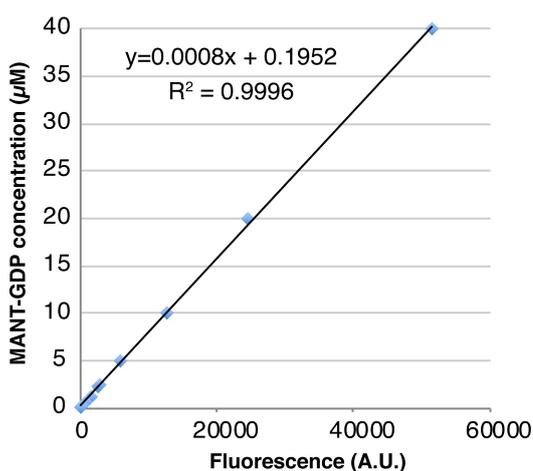


Figure 1. A standard curve of free MANT-GDP created using the data shown in Table 2

E. Nucleotide exchange assay

1. Add 14 µl of nucleotide exchange buffer or MANT-GDP loaded GTPase into an appropriate number (the number of conditions x 4 technical replicates) of wells of a 384-well microplate.
Note: The concentration of protein that is added to the well depends on the loading efficiency calculated above, since the starting fluorescence intensity of the sample should be high enough to detect dynamic changes in the fluorescence. If the loading efficiency is high (more than 50%), 1 µM or less GTPase can be used. If the loading efficiency is low (less than 10%), the highest concentration of GTPase should be added.
2. Before measuring the fluorescence, prepare a working solution of GppNHp, which has 15 x 100 times higher concentration than that of the GTPase, with nucleotide exchange buffer. If you use 1 µM of GTPase, prepare 1.5 mM GppNHp.
3. Read the fluorescence for 150 sec at room temperature (25 °C) and confirm the fluorescent signal is stable.
Note: The same detection parameters used in the Step D3 (see Table 1) can be used in the nucleotide exchange assay.
4. Press the 'Pause' button on the display to eject the plate, and then add 1 µl of nucleotide exchange buffer or GppNHp working solution (the final concentration is 100 times higher than that of GTPase) to respective wells. If GEF mediated nucleotide exchange is to be measured, add purified GEF in addition to GppNHp.
Note: A multichannel pipet should be used to simultaneously add buffer or GppNHp to start all reactions simultaneously. To ensure rapid addition of the buffer or GppNHp, we pre-fill a multichannel pipette with 1 µl of the buffer/GppNHp solution from another 384-well plate before ejecting the assay plate, and then pipette reagent into the assay plate immediately after ejection of the plate. To avoid making any bubble, we set the fill volume to 1.5 µl and the dispense volume to 1 µl.
5. Press the 'Continue' button to insert the plate and continue reading fluorescence every 15 sec at room temperature (25 °C) for at least 30 min.

Data analysis

1. Export the fluorimeter data to a Microsoft Excel spreadsheet.
2. Subtract the background intensity of the nucleotide exchange buffer from each fluorescence intensity.
3. Set the fluorescence intensity at the first time point after the addition of either buffer or GppNHp to 1 and calculate the relative fluorescence of each of the later time points.
4. A pseudo first order rate constant (observed rate constant or K_{obs}) can be calculated from the data, since the reaction contains a large excess of GppNHp over GTPase (100 times or more). Because the fluorescence of MANT-bound GTPase decreases exponentially with time, software programs that are capable of fitting data with nonlinear regression can be used for calculating K_{obs} . Those programs include, but are not limited to, GraphPad Prism, KaleidaGraph and Grafit. We describe here how to do the analysis by using GraphPad Prism 7 as an example.
 - a. Open GraphPad Prism 7 and create a new XY table.
 - b. Enter the values into each column, click the 'Analyze' button and choose Nonlinear regression from the list of XY analyses.
 - c. Select 'Dissociation-One phase exponential decay' to obtain the K_{obs} value.

Note: The data should be confirmed by at least three independent experiments.

Notes

In our hand, the loading efficiency of GTPases with low guanine nucleotide affinity was much lower than that of typical small GTPases, which have a high affinity for guanine nucleotides. Presumably that reflects the higher dynamics of the low affinity GTPase. In the former case, the fluorescence signal is largely affected by dilution of the protein, probably because dilution itself changes the equilibrium.

Recipes

1. LB media (1 L)

Dissolve 25 g of LB broth in 900 ml of Milli-Q water
Adjust pH to 7.2 with 10 M NaOH and bring volume with Milli-Q water to 1 L
Sterilize by autoclave and store at room temperature
2. TB media (1 L)

Dissolve 50.8 g of terrific broth in 900 ml of Milli-Q water and bring volume with Milli-Q water to 1 L
Transfer into a 2.8 L flask and rotate the flask at 250 rpm at 28 °C in an incubator shaker until the powder is completely dissolved

Note: It may take less than 30 min.

- Sterilize by autoclave
3. 100 mg/ml ampicillin (10 ml)

Dissolve 1 g of ampicillin in 8 ml of Milli-Q water and bring volume with Milli-Q water to 10 ml

Sterilize with a 0.22 μ m syringe filter

Dispense into 1 ml aliquots, and store at -20 °C
 4. 50 mg/ml carbenicillin (10 ml)

Dissolve 500 mg of carbenicillin in 8 ml of Milli-Q water and bring volume with Milli-Q water to 10 ml

Sterilize with a 0.22 μ m syringe filter

Dispense into 1 ml aliquots

Store at -20 °C
 5. 34 mg/ml chloramphenicol (10 ml)

Dissolve 340 mg of chloramphenicol in 8 ml of 100% ethanol and bring volume with 100% ethanol to 10 ml

Sterilize with a 0.22 μ m syringe filter

Dispense into 1 ml aliquots

Store at -20 °C
 6. LB agar plate containing 50 μ g/ml carbenicillin and 34 μ g/ml chloramphenicol

Dissolve 32 g of LB agar in 900 ml of Milli-Q water and bring volume with Milli-Q water to 1 L

Sterilize by autoclave and cool down to 50 °C

Add 1 ml of 50 mg/ml carbenicillin and 1 ml of 34 mg/ml chloramphenicol

Pour into plates and store at 4 °C
 7. 1 M IPTG (10 ml)

Dissolve 2.383 g of IPTG in 8 ml of Milli-Q water and bring volume with Milli-Q water to 10 ml

Sterilize with a 0.22 μ m syringe filter

Dispense into 1 ml aliquots

Store at -20 °C
 8. 1 M Tris-HCl (pH 7.5, 1 L)

Dissolve Trizma base in 800 ml Milli-Q water

Adjust pH with concentrated HCl to 7.5 and bring volume with Milli-Q water to 1 L

Autoclave or filter to sterilize
 9. 1 M HEPES-NaOH (pH 7.5, 1 L)

Dissolve 238.30 g of HEPES base in 800 ml of Milli-Q water

Adjust pH to 7.5 with 10 M NaOH and bring volume with Milli-Q water to 1 L

Filter sterilize through a 0.2 μ m disposable bottle top filter

Store at 4 °C
 10. 5 M NaCl (1 L)

Dissolve 292.2 g of NaCl in 700 ml of Milli-Q water and bring volume with Milli-Q water to 1 L

Sterilize by autoclave and store at room temperature

11. 1 M MgCl₂ (100 ml)
 Dissolve 20.33 g of MgCl₂·6H₂O in 70 ml of Milli-Q water and bring volume with Milli-Q water to 100 ml
 Sterilize by autoclave and store at room temperature
12. 1 M DTT (30 ml)
 Dissolve 4.63 g of DTT in 20 ml of Milli-Q and bring volume with Milli-Q water to 30 ml
 Dispense into 1 ml aliquots
 Store at -20 °C
13. 0.25 M EDTA (pH 8.0, 1 L)
 Dissolve 93.06 g of EDTA·2H₂O in 800 ml of Milli-Q water
 Adjust pH to 8.0 with NaOH
Note: EDTA will not dissolve unless pH is ~8.
 Bring volume with Milli-Q water to 1 L
 Sterilize by autoclave and store at room temperature
14. 10 M NaOH (500 ml)
 Slowly add 200 g of sodium hydroxide into 400 ml of Milli-Q water while stirring continuously
 Bring volume with Milli-Q water to 500 ml
15. Coomassie Brilliant Blue staining solution (1 L)
 Dissolve 2.5 g Coomassie Brilliant Blue R-250 in 500 ml methanol
 Add 400 ml of Milli-Q water and 100 ml of glacial acetic acid
 Filter through a 0.2 µm disposable bottle top filter
16. Coomassie Brilliant Blue destaining solution (1 L)
 Add 500 ml Milli-Q H₂O and 400 ml methanol to a 1 L bottle
 Add 100 ml glacial acetic acid slowly to solution
17. Lysis buffer (500 ml)

	Volume	Final concentration
1 M Tris-HCl (pH 7.5)	12.5 ml	25 mM
5 M NaCl	50 ml	500 mM
1 M MgCl ₂	2.5 ml	5 mM
1M DTT	0.5 ml	1 mM
Triton X-100	5 ml	1%
Milli-Q water	429.5 ml	

One tablet of a protease inhibitor is added in 50 ml of lysis buffer immediately before use
 Make fresh each time

18. PreScission cleavage buffer

1 M Tris-HCl (pH 7.5)	12.5 ml	25 mM
5 M NaCl	15 ml	150 mM
1 M MgCl ₂	2.5 ml	5 mM
1M DTT	0.5 ml	1 mM
Triton X-100	0.5 ml	0.1%
0.25 M EDTA	2 ml	1 mM
Milli-Q water	467 ml	

Make fresh each time

19. Storage buffer (500 ml)

	Volume	Final concentration
1 M HEPES-NaOH (pH 7.5)	12.5 ml	25 mM
5 M NaCl	5 ml	50 mM
1 M MgCl ₂	2.5 ml	5 mM
1M DTT	0.5 ml	1 mM
Glycerol	50 ml	10%
Milli-Q water	429.5 ml	

Make fresh each time

20. Low magnesium buffer (200 ml)

	Volume	Final concentration
1 M HEPES-NaOH (pH 7.5)	4 ml	20 mM
5 M NaCl	2 ml	50 mM
1 M MgCl ₂	100 μ l	0.5 mM
Milli-Q water	193.9 ml	

Store at room temperature

 21. 2x MANT-GDP loading buffer (100 μ l)

	Volume	Final concentration
1 M HEPES-NaOH (pH 7.5)	2 μ l	20 mM
5 M NaCl	1 μ l	50 mM
100 mM MgCl ₂	0.5 μ l	0.5 mM
0.25 M EDTA	4 μ l	10 mM
100 mM DTT	2 μ l	2 mM
MANT-GDP (10 mM)	X μ l	20-fold excess
Milli-Q water	90.5-X μ l	

Make fresh each time. The concentration of MANT-GDP should be calculated from the GTPase concentration

22. Nucleotide exchange buffer (100 ml)

	Volume	Final concentration
1 M HEPES-NaOH (pH7.5)	4 ml	40 mM
5 M NaCl	1 ml	50 mM
1 M MgCl ₂	1 ml	10 mM
1 M DTT	200 µl	2 mM
Milli-Q water	93.8 ml	

Make fresh each time

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