Please cite this article as: Zaoui and Duhamel, (2020). Colorimetric RhoB GTPase Activity Assay,*Bio-protocol* 10 (9): e3609. DOI: 10.21769/BioProtoc.3609.

bio-protocol www.bio-protocol.org/e3609 Bio-protocol 10(09): e3609. DOI:10.21769/BioProtoc.3609

Colorimetric RhoB GTPase Activity Assay

Kossay Zaoui[1, 2, *] and Stephanie Duhamel[2]

[1]Department of Biochemistry, McGill University, Montreal, Quebec, Canada; [2]Rosalind and Morris Goodman Cancer Centre, McGill University, Montreal, Quebec, Canada

*For correspondence: Kossay.zaoui@mail.mcgill.ca

[Abstract] The Ras homologous protein (Rho) GTPase subfamily, including RhoA, RhoB, and RhoC are small molecules (~21 kDa) that act as molecular switches in a wide range of signaling pathways to orchestrate biological processes associated with both physiological and tumorigenic cellular states. The Rho GTPases are crucial regulators of actin cytoskeleton rearrangements and FA dynamics and are required for effective cell migration and invasion, as well as cell cycle progression and apoptosis. The Rho GTPases activity is regulated by conformational switching between GTP-bound (active) and GDP-bound (inactive) states. This GTP/GDP cycling is tightly controlled by the guanine nucleotide exchange factors (GEFs), which function as activators by catalyzing the exchange of GDP for GTP and by the GTPase-activating proteins (GAPs), which enable hydrolysis of GTP leading to the Rho GTPase inactivation. Here, we describe a detailed protocol to perform a RhoB G-LISA activation assay to detect the level of GTP-loaded RhoB *in vitro*. This is the first colorimetric assay designed to specifically measure RhoB activation. This method was developed by adapting the RhoA G-LISA Activation Assay Kit (Cytoskeleton, Inc.) and allow the precise measurement of RhoB activity in less than 3 hours. This rapid methodology can be broadly used to assess the level of GTP-loaded RhoB in any kind of cellular models, to appreciate either the role RhoB activation in physiological processes, diseases, oncogenic transformation or for drug discovery in high throughput screens.

Keywords: Rho GTPases activity, *In vitro* colorimetric G-LISA activation assay, RhoB, GTP-loaded, GTP/GDP cycling

[Background] Although the Rho GTPases RhoA, RhoB, and RhoC share more than 85% amino acid sequence identity, they play distinct roles in tumorigenesis by interacting with different signaling pathways. While the contributions of RhoA and RhoC in tumor development have been detailed in numerous studies, the role of RhoB in cancer progression remains unclear. RhoB controls several fundamental processes such as cell morphology, motility, adhesion, intracellular transport, as well as cell proliferation and mitosis (Spiering and Hodgson, 2011; Ridley *et al.*, 2003; Ju and Gilkes, 2018; Zaoui *et al.*, 2019; Svensmark and Brakebusch, 2019). The role of RhoB in tumorigenesis appears to be complex, as it is likely that RhoB functions in a contextual manner, responding to specific signals in the tumor microenvironment (Mazieres *et al.*, 2004). In addition, RhoB possesses several features that are not found in other Rho GTPases. While the small Rho GTPases are generally localized in the cytoplasm and in response to stimuli, translocate to the plasma membrane, RhoB localizes not only at the plasma membrane and the cytoplasm, but also on endosomes and multivesicular bodies (Wheeler

Please cite this article as: Zaoui and Duhamel, (2020). Colorimetric RhoB GTPase Activity Assay,*Bio-protocol* 10 (9): e3609. DOI: 10.21769/BioProtoc.3609.

and Ridley, 2004; Vega and Ridley, 2018; Zaoui *et al.*, 2019). In addition, RhoB has a “GCI” (glycine, cysteine, and isoleucine) tripeptide, localized in the hypervariable C-terminus domain (residues 188-190) (Wang and Sebti, 2005). Palmitoylation of cysteine in this tripeptide regulates RhoB stability and RhoB subcellular localization (Perez-Sala *et al.*, 2009). These unique features contribute to the diversity and potentially opposing functions of RhoB in an oncogenic context. *In vitro*, GTPase assays are extensively used to study general Rho GTPases properties, cycling between active and inactive states, as well as protein expression, and stability. Our studies aim at understanding the role of RhoB GTPase activity on cancer cell signaling regulating cytoskeletal rearrangement, cell migration, and invasion. The Colorimetric RhoB GTPase Activity Assay described herein presents a method to study activation of the RhoB GTPases in these multistep processes, crucial for cancer cell dissemination and metastatic colonization. This methodology may have substantial value in monitoring the level of GTP-loaded RhoB in various contexts such as fundamental research on cell motility as well as therapeutic compounds development.

Materials and Reagents

1. Paper towel (Staples MMP®, catalog number: DUSPA350RKL)
2. 6-well plate (Nunc®, catalog number: 140675)
3. Microcentrifuge tubes: 1.5 ml (Axygen Corning®, catalog number: MCT-150-C)
4. HeLa cell line (ATCC® CCL-2)
5. Hepatocyte growth factor, HGF (Genentech, Inc.)
6. G-LISA® RhoA Activation Assay (cytoskeleton, Inc., catalog number: BK124) (Figure 1)
7. DMEM–Dulbecco's Modified Eagle Medium (Gibco®, catalog number: 11966025)
8. Rat tail collagen I (Roche®, catalog number: 11179179001)
9. Foetal Bovine Serum (FBS) (Gibco®, catalog number: 26140079)
10. RhoB-specific antibody (Santa Cruz Biotechnology, sc-8048)
11. Bradford Protein Assay (Bio-Rad®, catalog number: 5000006)
12. Triton X-100 (Sigma-Aldrich®, catalog number: 93443)
13. Tris (Wisent Inc., catalog number: 600-125-IK)
14. HCl (Sigma-Aldrich®, catalog number: 320331)
15. NaCl (Wisent Inc., catalog number: 600-082-IK)
16. $MgCl_2$ (Sigma-Aldrich®, catalog number: M1028)
17. Deoxycholate (Sigma-Aldrich®, catalog number: D6750)
18. SDS (Sodium dodecyl sulfate) (Wisent Inc., catalog number: 800-100-LG)
19. Glycerol (Sigma-Aldrich®, catalog number: G7893)
20. Dithiothreitol (DTT) (Bio-Rad®, catalog number: 1610611)
21. Protease inhibitors cocktail (Roche cOmplete®, catalog number: 11697498001)
22. Phosphate-buffered saline (PBS) (Wisent Inc., catalog number: 311-012-LL)
23. Lysis buffer (see Recipes)

Please cite this article as: Zaoui and Duhamel, (2020). Colorimetric RhoB GTPase Activity Assay,*Bio-protocol* 10 (9): e3609. DOI: 10.21769/BioProtoc.3609.

Figure 1. Reagent for the colorimetric RhoB GTPase activity assay. Wash buffer, Binding buffer, Antigen presenting buffer, RhoB primary antibody, HRP-labeled secondary antibody, Rho-GTP affinity wells (strips and strip holder), Rho control and BK 124 component from G-LISA® RhoA Activation Assay (Cytoskeleton, Inc.).

Equipment

1. Strip holder (Cytoskeleton®, catalog number: BK124)
2. Cell scraper (Sarstedt®, catalog number: 83.1832)
3. Cell incubator Steri-Cycle CO_2 (Thermo Fisher Scientific®, catalog number: 51030303)
4. Varioskan Flash Spectral Scanning Multimode reader at 490 nm (Thermo Fisher Scientific®, catalog number: 5250030)
5. Centrifuge (Eppendorf®, catalog number: 5424/5424R)
6. Orbital Shaker (Labline®, model: Labline 3508 Dual Action Shaker)
7. GENESYS™ 10S Vis Spectrophotometer at 595 nm (Thermo Fisher Scientific®, catalog number: 840-208200)
8. Multi-channel pipettor (Eppendorf®, catalog number: 3122000060)

Software

1. Microsoft Excel®

Procedure

A. Collagen I coating and cell seeding
   1. Coat the 6-well plate with 1 ml rat-tail collagen I diluted in PBS (25 µg/ml).
   2. Incubate the 6-well plate coated with 1 ml collagen I at 37 °C, for 1 h.
   3. Wash the 6-well plate twice with 1 ml PBS and aspirate off PBS buffer.

Please cite this article as: Zaoui and Duhamel, (2020). Colorimetric RhoB GTPase Activity Assay,*Bio-protocol* 10 (9): e3609. DOI: 10.21769/BioProtoc.3609.

4. Put 1 x 10^6 HeLa cells/well in 3 ml DMEM with 10% FBS and Incubate the 6-well plate at 37 °C for at least 24 h.
5. Wash twice with 1 ml PBS, aspirate off and add 3 ml media (0% serum), then incubate overnight at 37 °C.
6. Add HGF at 0.5 nM (or any other growth factor) to stimulate the cells at different times and to measure the kinetics of activation of RhoB.

Note: Collagen I and HGF must be kept on ice. Transfected cells with constitutively active form of RhoB (RhoB^{V14}) could be used as positive control and an unstimulated condition for each analytical experiment is needed as a negative control.

B. Cell washing
1. Retrieve culture plate from the incubator, immediately place on ice, aspirate off media, and wash twice cells each with 1 ml ice-cold PBS.
2. Aspirate off all PBS buffer.

C. Cell Lysis
1. Lyse cells for 15 min in ice-cold cell lysis buffer, 50 µl per well.
2. Harvest cell lysates with a cell scraper.
3. Transfer lysates into pre-chilled 1.5 ml microfuge tubes on ice.
4. Centrifuge cell lysates at 10,000 *x g*, 4 °C for 10 min.
5. Transfer supernatant (cell lysates fraction) into a new pre-labeled and pre-chilled 1.5 ml microfuge tubes on ice.

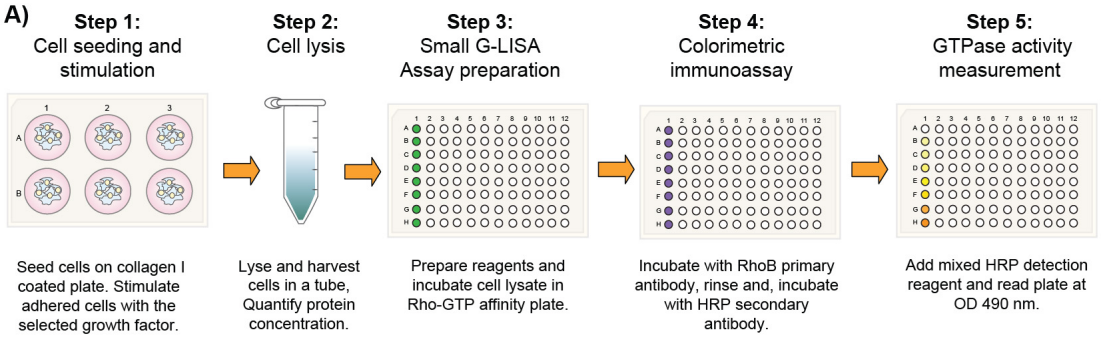
Note: All these steps must be performed on ice or in a 4 °C cold room.

D. Quantification of total protein concentration
1. Perform a Bradford protein assay by diluting 2 µl of the cell lysate, the undiluted lysates we obtained by this protocol are typically around 5 µg/µl. Protein solutions are normally assayed in duplicate.
2. Measure absorbance at 595 nm.

E. Small G-LISA Assay preparation
1. Reconstitute all lyophilized components provided in the kit of Small G-LISA Assay, as indicated in Table 2 of the G-LISA Activation Assay Technical Guide.
2. Vortex 15 s for mixing Binding Buffer solutions. Keep on ice.

F. Small G-LISA Assay Protocol (Figure 2)

Please cite this article as: Zaoui and Duhamel, (2020). Colorimetric RhoB GTPase Activity Assay,*Bio-protocol* 10 (9): e3609. DOI: 10.21769/BioProtoc.3609.



B)

Samples	Replicate 1	Replicate 2	Replicate 3	Mean minus blanck	SEM
HGF - 0 min	1.00	1.00	1.00	0.84	0
HGF - 1 min	1.28	1.19	1.45	1.31	0.06
HGF - 3 min	1.43	1.34	1.57	1.45	0.05
HGF - 5 min	0.74	0.7	0.82	0.75	0.03
HGF - 10 min	1.15	1.12	1.22	1.16	0.02
HGF - 15 min	1.72	1.69	1.99	1.8	0.07
HGF - 30 min	1.64	1.56	1.75	1.65	0.04
HGF - 60 min	1.39	1.22	1.51	1.37	0.06
Buffer Blank	0.14	0.15	0.19	0.16	0.01

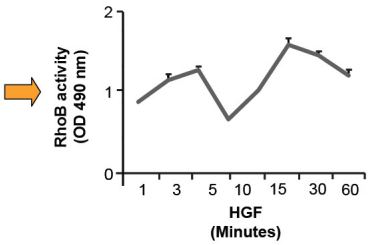


Figure 2. Colorimetric RhoB GTPase activity assay overview. A. Experimental procedure. Step 1: Seed cells in collagen I coated 6-well plate. Stimulate cells with HGF (or any other growth factors) at different time points. Step 2: Lyse cells with lysis buffer, harvest cell lysates with a cell scraper and transfer cell lysates into pre-chilled microfuge tubes. Quantify the total protein concentration. Step 3: Prepare reagents and incubate cell lysates in a Rho-GTP affinity plate. Step 4: For the colorimetric immunodetection, add RhoB primary antibody, rinse, and add HRP secondary antibody, and rinse. Step 5: To measure the GTPase activity, add HRP detection reagent and read the signal by measuring absorbance at 490 nm using the microplate spectrophotometer. B. Data analyses. Use the Lysis Buffer wells as reference blank. Three replicates of each sample should be made. Read the signal by measuring absorbance at 490 nm using the microplate spectrophotometer. When the data are “Lysis Buffer subtracted”, import them into a simple graph software such as Excel.

1. Prepare the blank buffer by mixing 60 µl lysis buffer with 60 µl ice-cold Binding Buffer and put on ice.
2. Take a number of strips required from Rho plate and place in a strip holder, and then put on ice.
3. Keep the plate on ice and add 100 µl ice-cold water in each well to dissolve the powder coat, covering the bottom of the well. Tap pellets to the bottom of the wells prior to resuspension.
4. Aliquot cell lysate for duplicate (60 µl) or triplicate (90 µl) assays into fresh ice-cold microcentrifuge tubes.
5. Add an equal volume of ice-cold Binding Buffer to each tube. Vortex each tube for 3-5 s on a high setting and return tubes to ice.
6. Remove completely the water from the microplate wells by flicking it vigorously, followed by a series of 5 pats on paper towels.
7. Return the plate to ice. Immediately add 50 µl of equalized cell lysate to wells.
8. Pipette 50 µl of buffer blank control into duplicate wells.

Please cite this article as: Zaoui and Duhamel, (2020). Colorimetric RhoB GTPase Activity Assay,*Bio-protocol* 10 (9): e3609. DOI: 10.21769/BioProtoc.3609.

9. Pipette 50 µl of RhoA positive control into duplicate wells.
10. Immediately place the plate on an orbital microplate shaker (250 rpm) at 4 °C for exactly, 30 min.
11. Dilute anti-RhoB primary antibody to 1/100 in Antibody Dilution Buffer.
12. After 30 min, remove the solution from the wells and wash twice each with 200 µl Wash Buffer at room temperature using a multi-channel pipette. Do not leave the plate unattended at this time. Remove the Wash Buffer completely after each wash as described in Step F6.
13. Pipette immediately 200 µl of room temperature Antigen Presenting Buffer into each well using a multi-channel pipettor and incubate at room temperature for exactly 2 min.
14. Flick out vigorously the Antigen Presenting Buffer and wash the wells three times each with 200 µl of room temperature Wash Buffer as described in Step F6 (Figure 3).

Figure 3. Flicking procedure. As described in Step F6, the plate should be vigorously flicked on a paper towel to completely remove the solution in the wells. The flicking should be repeated 5 times.

15. Add 50 µl of diluted anti-RhoB primary antibody to each well and leave the plate on the orbital microplate shaker (250 rpm) at room temperature for 45 min.
16. Dilute the secondary HRP labeled antibody to 1/62.5 in Antibody Dilution Buffer.
17. Vigorously flick out the anti-RhoB primary antibody as described in Step F6.
18. Wash the wells three times each with 200 µl of room temperature Wash Buffer as described in Step F6.
19. Add 50 µl of diluted secondary antibody to each well and leave the plate on a microplate shaker (250 rpm) at room temperature for 45 min.
20. Prepare an aliquot of HRP detection reagents A and B. Do not mix.
21. Flick out the secondary antibody and wash the wells three times each with 200 µl of room temperature Wash Buffer as described in Step F6.
22. Pipette 50 µl of the mixed HRP detection reagent into each well and incubate at 37 °C for 10 min.
23. Add 50 µl of HRP Stop Buffer to each well and check that the wells are free of bubbles.
24. Read the signal by measuring absorbance at 490 nm using the microplate spectrophotometer. At least three readings are recommended to capture the maximum signal of absorbance.

bio-protocol www.bio-protocol.org/e3609 Bio-protocol 10(09): e3609. DOI:10.21769/BioProtoc.3609

Data analysis

1. Transfer the measurements to Microsoft Excel® software.
2. Subtract the average absorbance obtained with the blank samples from the absorbance obtained for each measured sample to obtain the correct value. Cells stimulated with HGF (or any other growth factors) can be used as a positive control.

Recipes

1. Lysis buffer (can be made in advance and stored at 4 °C)
   Triton X-100, 1%
   Tris-HCl, pH 7.5, 50 mM
   NaCl, 150 mM
   $MgCl_2$, 10 mM
   Deoxycholate, 0.5%
   SDS, 0.1%
   Glycerol, 10%
   Dithiothreitol, 2 mM (can be made in advance and store at -20 °C), must be added freshly to lysis buffer
   Protease inhibitors cocktail (stock solution 25x in 2 ml water), can be stored at -20 °C for up to 4 months as recommended by the manufacturer
   Note: Lysis buffer should be made fresh prior to use.

Acknowledgments

This work was performed as a part of the publication of Zaoui *et al.* (2019) and was supported by a Susan G. Komen for the Cure Postdoctoral fellowship (KG111467) to K. Zaoui.

Competing interests

The authors declare no competing financial interests.

References

1. Ju, J. A. and Gilkes, D. M. (2018). RhoB: team oncogene or team tumor suppressor? *Genes (Basel)* 9(2).
2. Mazieres, J., Antonia, T., Daste, G., Muro-Cacho, C., Berchery, D., Tillement, V., Pradines, A., Sebti, S. and Favre, G. (2004). Loss of RhoB expression in human lung cancer progression. *Clin Cancer Res* 10(8): 2742-2750.

3. Perez-Sala, D., Boya, P., Ramos, I., Herrera, M. and Stamatakis, K. (2009). The C-terminal sequence of RhoB directs protein degradation through an endo-lysosomal pathway. *PLoS One* 4(12): e8117.
4. Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T. and Horwitz, A. R. (2003). Cell migration: integrating signals from front to back. *Science* 302(5651): 1704-1709.
5. Spiering, D. and Hodgson, L. (2011). Dynamics of the Rho-family small GTPases in actin regulation and motility. *Cell Adh Migr* 5(2): 170-180.
6. Svensmark, J. H. and Brakebusch, C. (2019). Rho GTPases in cancer: friend or foe? *Oncogene.*
7. Vega, F. M. and Ridley, A. J. (2018). The RhoB small GTPase in physiology and disease. *Small GTPases* 9(5): 384-393.
8. Wang, D. A. and Sebti, S. M. (2005). Palmitoylated cysteine 192 is required for RhoB tumor-suppressive and apoptotic activities. *J Biol Chem* 280(19): 19243-19249.
9. Wheeler, A. P. and Ridley, A. J. (2004). Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility. *Exp Cell Res* 301(1): 43-49.
10. Zaoui, K., Rajadurai, C. V., Duhamel, S. and Park, M. (2019). Arf6 regulates RhoB subcellular localization to control cancer cell invasion. *J Cell Biol* 218(11):3812-3826.

