RhoGTPase Activation Assay

Alan Yiu Wah Lee*

Department of Physiology, Yong Loo Lin School of Medicine, Neurobiology/Ageing Programme, Life Sciences Institute, National University of Singapore (NUS), Singapore, Singapore
*For correspondence: yiu_wah_lee@nuhs.edu.sg

[Abstract] This protocol describes procedures to assay for GTP-bound (active) form of small GTPases of the Rho superfamily in human brain cancer (glioma) cell lines but can also be applied to cells or tissues of other origins. The principle of assay is based on the property of these active GTPases to interact with their specific effectors (*e.g.* Rhotekin for RhoA; p21-activated kinase (PAK) for Rac1 and Cdc42. In essence, Rhotekin or PAK1 are expressed in the form of GST-fusion protein to pull down the corresponding active GTPases.

Materials and Reagents

1. Complete Protease Inhibitor Cocktail, EDTA-free (Roche Diagnostics GmbH, catalog number: 11873580001), prepared as 25x stock according to manufacturer's instructions
2. GST-Rhotekin (Millipore/Upstate, catalog number: 14-662)
3. GST-PAK1 (in-house)
4. anti-RhoA antibody (1:200) (Santa Cruz, catalog number: sc-418)
5. anti-Rac1 antibody (1:200) (Santa Cruz, catalog number: sc-95)
6. anti-Cdc42 antibody (1:500) (Santa Cruz, catalog number: sc-87)
7. anti-GST antibody (1:2,000) (Santa Cruz, catalog number: sc-138)
8. Glutathione (GSH) sepharose 4B (GE Healthcare Life Sciences, catalog number: 17-0756-01)
9. Dithiothreitol (DTT) (Bio-Rad Laboratories, catalog number: 161-0611)
10. Triton X-100 (Bio-Rad Laboratories, catalog number: 161-0407)
11. Phosphate-buffered saline (PBS)
12. Tris-HCl (pH 7.4)
13. NaCl
14. EDTA
15. NP40
16. $Na_2P_2O_7$
17. NaF
18. Na_3VO_4

19. Sample loading buffer
20. 10% or 12% SDS-PAGE gel
21. GST pull-down core buffer (see Recipes)
22. RIPA buffer (see Recipes)

Equipment

1. Centrifuge (Eppendorf, catalog number: 5415R)
2. Nutating mixer (Labnet International)
3. 1.5 ml Eppendorf tubes
4. Heat block

Procedure

1. Lyse cell samples by incubating in RIPA buffer supplemented with 1% Triton X-100 at 4 °C for 2 h. Centrifuge at 18,000 *x g* for 10 min. at 4 °C and collect the cleared crude cell lysate.
2. Transfer 15 µl of GSH-sepharose into 100 µl of GST pull-down core buffer in a 1.5 ml Eppendorf tube. Mix well and spin at 835 *x g* for 1 min. at room temperature. Decant the supernatant and save the washed GSH-sepharose for pre-clearing the samples (step 3).
3. Pre-clear 100 µg of crude cell lysates by adding into 300 µl of GST pull-down buffer (prepared by supplementing core buffer with 0.1% Triton X-100, 1 mM DTT, and 1x protease inhibitors) containing the washed GSH-sepharose (prepared in step 2). Incubate with agitation at 4 °C for 1 h.
   Note: If multiple samples, first bring up 100 µg lysate to equal volume with corresponding lysis buffer to ensure equal input.
4. Centrifuge at 835 *x g* for 1 min at 4 °C. Transfer supernatant to a new 1.5 ml tube without disturbing the sepharose. Save an aliquot of the pre-cleared lysate as “sample input”.
5. Add to the supernatant 30 µl glutathione beads bound with 30 µg GST-Rhotekin (for RhoA-GTP assay) or GST-PAK1 (for Rac1- and Cdc42-GTP assays). A “GST only” control is similarly prepared by adding GST pre-bound sepharose. Mix well and incubate at 4 °C for 2 to 3 h with rocking.
6. Centrifuge at 835 *x g* for 1 min at 4 °C to pellet the sepharose beads.
7. Aspirate the supernatant (*optional:* Save supernatant for quality control of pull-down reaction).
8. Wash the pelleted sepharose beads by adding 300 µl 1x PBS at 4 °C.

9. Spin at 835 *x g* for 1 min at 4 °C and aspirate supernatant (*Optional:* Save supernatant as "first wash" for quality control of washing step).
10. Repeat washing. Save supernatant as "second wash" (Optional).
11. Boil the beads in sample loading buffer for 5 min. and spin to harvest the pull-down proteins.
12. Resolve the eluted proteins and an aliquot of cell/tissue lysates (sample input) by SDS-PAGE (10% or 12% gel). An aliquot of the "supernatant", "first wash" and "second wash" collected above can also be included for quality control.
13. Western blot analysis is then performed using anti-RhoA, Rac1, or Cdc42 antibodies to detect active (pull-down) and total (input) GTPases. The blot is also probed with anti-GST antibody to confirm successful GST pull down at comparable efficiency across samples.
    Note: Both Rhotekin and PAK1 preferentially bind to active (GTP-bound form) RhoA and Rac1/Cdc42, respectively. Quality control of these reagents can be performed by incubating GST-Rhotekin (or GST-PAK1) with recombinant RhoA (or Rac1/Cdc42) that has been preloaded with GDP or nonhydrolyzable GTP-γS.

Recipes

1. GST pull-down core buffer
   20 mM Tris-HCl (pH 7.4)
   150 mM NaCl
   1 mM EDTA
2. RIPA buffer
   50 mM Tris-HCl (pH 7.4)
   150 mM NaCl
   1% NP40
   1 mM $Na_2P_2O_7$
   1 mM NaF
   1 mM EDTA
   2 mM Na_3VO_4

Acknowledgments

This protocol is adapted from Li *et al*. (2012).

References

1. Li, X., Law, J. W. and Lee, A. Y. (2012). Semaphorin 5A and plexin-B3 regulate human glioma cell motility and morphology through Rac1 and the actin cytoskeleton. *Oncogene* 31(5): 595-610.

