Product Manual

ROCK Activity Immunoblot Kit

Catalog Number

STA-415 **20 assays**

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

CELL BIOLABS, INC.
Creating Solutions for Life Science Research

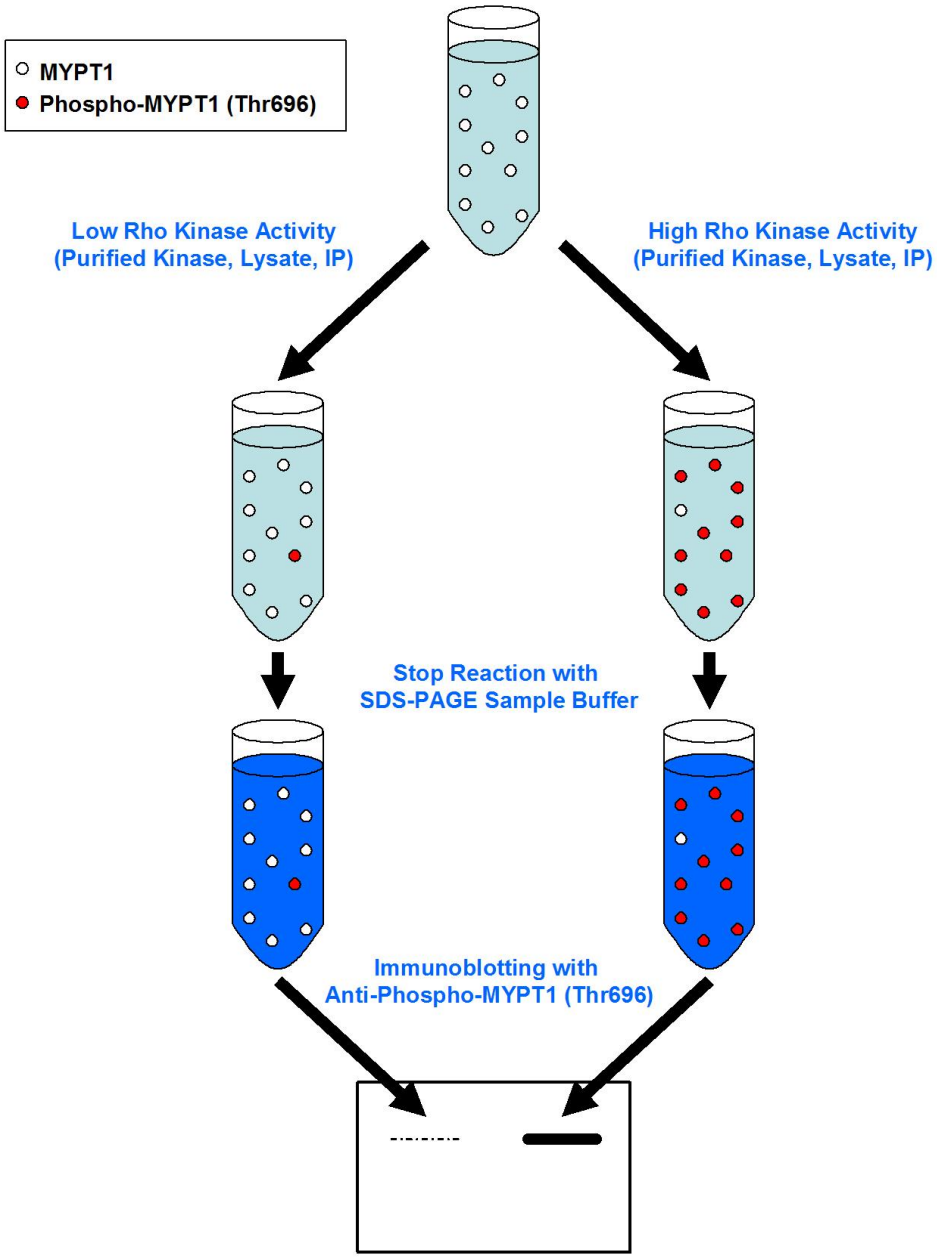
Introduction

Members of the Rho family are essential regulatory components of the signaling pathway that direct cell motility, adhesion, and cytokinesis through reorganization of actin cytoskeleton. Rho is activated by extracellular signals such as lysophosphatidic acid (LPA). The actions of Rho are mediated by downstream Rho effectors. One of these effectors is Rho-associated kinase (ROCK). Two ROCK isoforms have been identified: ROCK-I (also known as ROKβ) and ROCK-II (also known as Rho Kinase and ROKα). ROCK mediates Rho signaling and reorganizes actin cytoskeleton through phosphorylation of several substrates that contribute to the assembly of actin filaments and contractility. For example, ROCK inactivates myosin phosphatase through the specific phosphorylation of myosin phosphatase target subunit 1 (MYPT1) at Thr^{696}, which results in an increase in the phosphorylated content of the 20-kDa myosin light chain (MLC20).

Cell Biolabs' ROCK Activity Immunoblot Kit utilizes recombinant MYPT1 as ROCK substrate. After incubating the substrate with ROCK samples (such as purified kinase, cell lysate or immunoprecipitate), the phosphorylated MYPT1 is detected by western blot analysis using an anti-phospho-MYPT1 (Thr^{696}) (Figure 1).

Cell Biolabs' ROCK Activity Immunoblot Kit provides a simple and fast tool to monitor ROCK activity using its physiological substrate. The kit also includes active ROCK-II as a positive control. Each kit provides sufficient quantities to perform 20 assays.

Assay Principle



Related Products

1. STA-416: 96-well ROCK Activity Assay Kit
2. STA-400: Ras Activation Assay Kit
3. STA-405: Rho/Rac/Cdc42 Activation Assay Combo Kit
4. STA-411: Raf1 PBD Agarose Beads
5. STA-412: Rhotekin PBD Agarose Beads

Kit Components

1. ROCK Substrate (Part No 241501): One 40 µL vial containing 0.25 mg/mL recombinant MYPT1

2. 10X Kinase Buffer (Part No. 241502): Three 1.0 mL vials of 250 mM Tris, pH 7.5, 100 mM $MgCl_2$, 50 mM Glycerol-2-Phsophate, 1 mM Na_3VO_4

3. ATP Solution (Part No. 241503): One 400 µL vial of 10 mM ATP

4. Anti-phospho-MYPT1 (Thr^{696}) (Part No. 241504): One 50 µL vial

5. Secondary Antibody, HRP-conjugate (Part No. 230805): One 100 µL vial

6. Active ROCK-II (Part No. 241505): One 20 µL vial containing 10 ng active ROCK-II in 25 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM Glycerol-2-Phosphate, 0.1 mM Na_3VO_4, 10% Glycerol, 0.1% BSA

Materials Not Supplied

1. ROCK sample (purified kinase, cell lysate or immunoprecipitate)
2. DTT
3. 30°C incubator or water bath
4. 4X SDS-PAGE sample buffer
5. Electrophoresis and immunoblotting systems
6. Immunoblotting wash buffer such as TBST (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20)
7. Immunoblotting blocking buffer (TBST containing 5% Non-fat Dry Milk)
8. PVDF or nitrocellulose membrane
9. ECL Detection Reagents

Storage

Store active ROCK-II at -80ºC and all other kit components at -20ºC. The 10X Kinase Buffer may be stored at 4ºC, or for convenience it may be frozen at -20ºC to keep it with the other kit components. Avoid multiple freeze/thaw cycles.

Preparation of Reagents

- 1X Kinase Buffer: Dilute to 10X Kinase Buffer to 1X in deionized water. 1X Kinase Buffer may be stored at 4ºC for short term (1-2 weeks). Just prior to usage, add DTT to a final concentration of 1 mM.

- 1X Kinase/ATP/Substrate Solution: For each kinase assay, freshly prepare 50 µL of 1X Kinase/ATP/Substrate Solution by adding 1 µL of 10 mM ATP solution, 2 µL of ROCK substrate to 47 µL of 1X Kinase Buffer containing DTT.

Assay Protocol

I. Kinase Reaction

1a. For Immunoprecipitations with anti-ROCK antibody: ROCK is first immunoprecipitated from cell or tissue lysate with anti-ROCK antibody and Protein A/G bead slurry. Immediately before kinase assay, wash bead slurry once with 1X Kinase Buffer, remove all supernatant, assay immediately by adding 50 µL of 1X Kinase/ATP/Substrate Solution directly to the beads and mixing well.

1b. Purified Kinase or Cell Lysate: Purified kinase or cell lysate sample can be used directly in the kinase assay or further diluted with 1X Kinase Buffer. Add 25 µL of ROCK sample to a microcentrifuge tube, and initiate kinase reaction by adding 50 µL of 1X Kinase/ATP/Substrate Solution to the ROCK sample.

2. (optional) Add 2 µL of the provided active ROCK-II and 23 µL of 1X Kinase Buffer to a microcentrifuge tube, initiate kinase reaction by adding 50 µL of 1X Kinase/ATP/Substrate Solution.
3. Incubate the tubes at 30°C for 30-60 minutes with gentle agitation.
4. Stop kinase reaction by adding 25 µL of 4X reducing SDS-PAGE sample buffer.
5. Boil each sample for 5 minutes.
6. Centrifuge each sample for 10 seconds at 12,000 x g.

II. Electrophoresis and Transfer

1. Load 20 µL of supernatant to a polyacrylamide gel. Also, it's recommended to include a pre-stained MW standard (as an indicator of a successful transfer in step 3).
2. Perform SDS-PAGE as per the manufacturer's instructions.
3. Transfer the gel proteins to a PVDF or nitrocellulose membrane as per the manufacturer's instructions.

III. Immunoblotting and Detection (all steps are at room temperature, with agitation)

1. Following the electroblotting step, immerse the PVDF membrane in 100% Methanol for 15 seconds, and then allow it to dry at room temperature for 5 minutes.

   Note: If Nitrocellulose is used instead of PVDF, this step should be skipped.
2. Block the membrane with 5% non-fat dry milk in TBST for 1 hr at room temperature with constant agitation.
3. Incubate the membrane with the anti-phospho-MYPT1 (Thr^{696}) antibody, freshly diluted 1:1000 in 5% non-fat dry milk/TBST, for 2 hr at room temperature with constant agitation.

   Note: To conserve antibody, incubations should be performed in a plastic bag.
4. Wash the blotted membrane three times with TBST, 5 minutes each time.
5. Incubate the membrane with the secondary antibody, HRP-conjugated, freshly diluted in 1:1000 in 5% non-fat dry milk/TBST, for 1 hr at room temperature with constant agitation.

6. Wash the blotted membrane three times with TBST, 5 minutes each time.
7. Use the detection method of your choice. We recommend enhanced chemiluminescence reagents from Pierce.

Example of Results

The following figure demonstrates typical results seen with Cell Biolabs ROCK Activity Immunoblot Kit. One should use the data below for reference only.

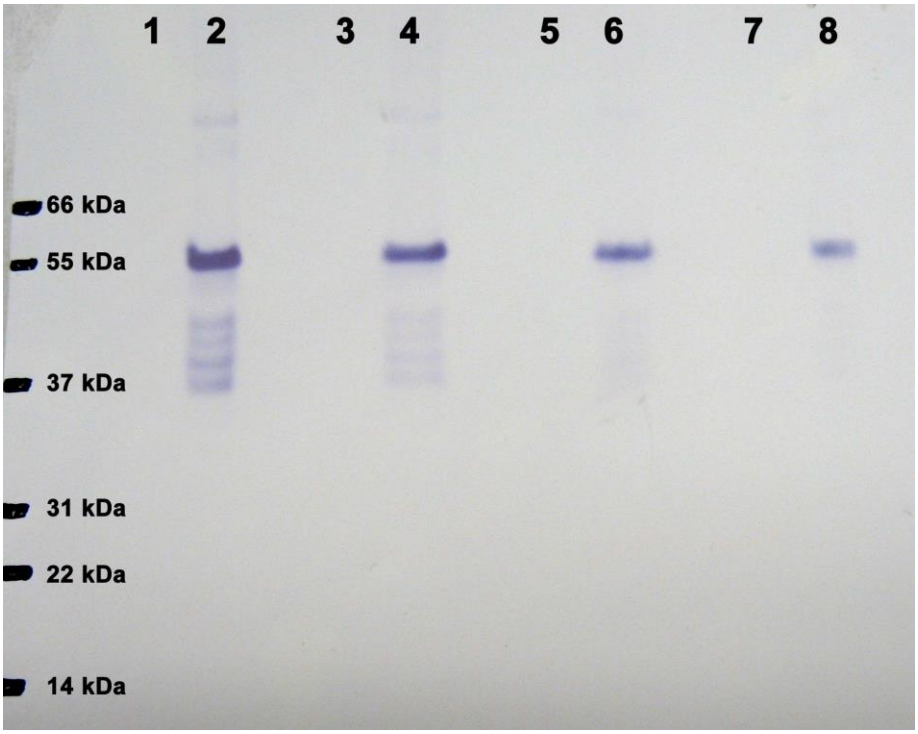


Figure 1: ROCK-II Activity Immunoblot Assay. 25 µL of 1X Kinase Buffer containing 10 ng of active ROCK-II was incubated with 50 µL of 1X Kinase Buffer containing 0.2 mM ATP and 500 ng of recombinant MYPT1 for 30 minutes at 30°C. Kinase reaction was stopped by adding 25 µL of 4X SDS-PAGE Sample Buffer. Lane 1, 3, 5, 7: Without kinase (negative control); Lane 2, 4, 6, 8: with kinase. 200 ng (Lane 1 and 2), 100 ng (Lane 3 and 4), 50 ng (Lane 5 and 6) or 25 ng (Lane 7 and 8) of recombinant MYPT1 substrate were loaded onto SDS-PAGE. Phosphorylation of MYPT1 substrate was detected by anti-phospho-MYPT1 (Thr^{696}) antibody as described in Assay Protocol.

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Recent Product Citations

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