Product Manual

96-well ROCK Activity Assay Kit

Catalog Number

STA-416	96 assays
STA-416-5	5 x 96 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

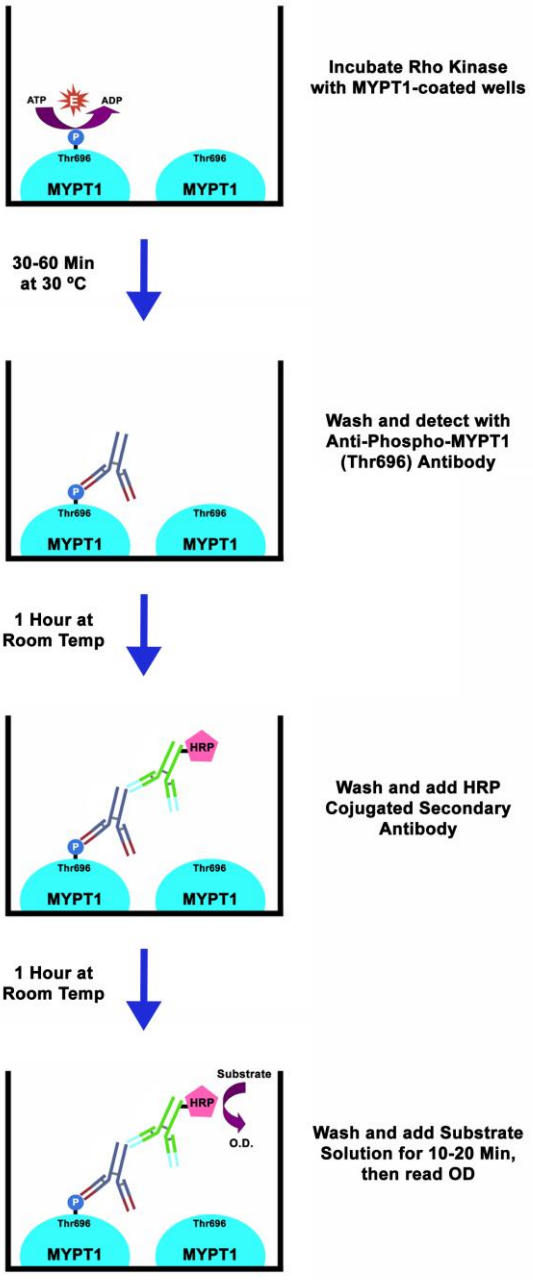
Introduction

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' 96-well ROCK Activity Assay Kit is an enzyme immunoassay developed for detection of the specific phosphorylation of MYPT1 at Thr^{696} by ROCK. A strip well microtiter plate is precoated with a recombinant MYPT1. After incubating the substrate wells with ROCK samples (such as purified kinase, cell or tissue lysate) the phosphorylated MYPT1 is detected by an anti-phospho-MYPT1 (Thr^{696}) antibody (Figure 1).

Cell Biolabs' 96-well ROCK Activity Assay Kit provides a non-isotopic, sensitive and specific method to monitor ROCK activity using its physiological substrate; it can also be used in screening ROCK inhibitors. The kit has detection sensitivity limit of 200 pg of active ROCK-II. A recombinant active ROCK-II is also provided as a positive control. Each kit provides sufficient quantities to perform up to 96 assays.

Assay Principle



Related Products

1. STA-415: ROCK Activity Immunoblot Kit
2. STA-400: Ras Activation Assay Kit
3. STA-404: Rac/Cdc42 Activation Assay Combo Kit
4. STA-405: Rho/Rac/Cdc42 Activation Assay Combo Kit

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5. STA-410: PAK1 PBD Agarose Beads

Kit Components

Box 1 (shipped at room temperature)

1. ROCK Substrate Coated Plate (Part No 241601): One strip well 96-well plate precoated with recombinant MYPT1
2. 10X Kinase Buffer (Part No. 241602): One 20 mL bottle of 250 mM Tris, pH 7.5, 100 mM MgCl2, 50 mM Glycerol-2-Phsophate, 1 mM Na3VO4
3. ATP Solution (Part No. 241604): One 100 µL vial Of 100 mM ATP
4. Anti-phospho-MYPT1 (Thr696) (Part No. 241603): One 20 µL vial
5. Secondary Antibody, HRP Conjugate (Part No. 231009): One 20 µL vial
6. Assay Diluent (Part No. 310804): One 50 mL bottle.
7. 10X Wash Buffer (Part No. 310806): One 100 mL bottle
8. Substrate Solution (Part No. 310807): One 12 mL amber bottle
9. Stop Solution (Part. No. 310808): One 12 mL bottle

Box 2 (shipped on blue ice packs)

1. 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 or tissue lysate)
2. Lysis Buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM 2-glycerophosphate, 1 % Triton X-100 or 1 % Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4 and Proteinase inhibitors.
3. DTT
4. 0.5 M EDTA
5. 30°C incubator or water bath
6. Microplate reader capable of reading at 450 nm (620 nm as optional reference wave length)

Storage

Store active ROCK-II at -80ºC, ATP Solution at -20ºC and all other kit components at 4ºC. Avoid multiple freeze/thaw cycles.

Preparation of Reagents

- 10X Kinase Reaction Buffer containing DTT and ATP: Just prior to usage, add DTT to a final concentration of 10 mM and ATP to a final concentration of 2 mM to the 10X Kinase Buffer. For

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Example, add 10 µL of 1M DTT (not provided) and 20 µL of 100 mM ATP solution to 970 uL of 10X Kinase Buffer. 10X Kinase Reaction Buffer containing DTT and ATP may be stored at 4°C for short term (1-2 weeks).

- Diluted Active ROCK-II Positive Control: Just prior to usage, dilute the provided active ROCK-II (0.5 µg/mL) to 0.02 µg/mL with 1X Kinase Buffer. For example, add 8 µL of the active ROCK-II and 20 µL of 10X Kinase Buffer to 172 µL deionized water.
- 1X Wash Buffer: Dilute the 10X Wash Buffer Concentrate to 1X with deionized water. Stir to homogeneity.
- Anti-Phospho-MYPT1 (Thr^{696}) Antibody and HRP-Conjugated Secondary Antibody: Immediately before use dilute the anti-phospho-MYPT1 (Thr^{696}) antibody 1:1000 and HRP-conjugated secondary antibody 1:1000 with Assay Diluent. Do not store diluted solutions.

Assay Protocol

1. Purified kinase or cell lysate sample can be used directly in the kinase assay or further diluted with 1X Kinase Buffer. Each sample should be assayed in duplicate.
2. Add 90 µL of the diluted active ROCK-II positive control or unknown ROCK samples to the wells of the substrate plate.
3. Initiate the kinase reaction by adding 10 µL of the 10X Kinase Reaction Buffer containing DTT and ATP. Mix well.
4. Cover with a plate cover and incubate the wells at 30°C for 30-60 minutes with gentle agitation.
5. Stop kinase reaction by flicking out the content or by adding 50 µL of 0.5 M EDTA, pH 8.0, to each well.
6. Remove plate cover and empty wells. Wash microwell strips 3 times with 250 µL 1X Wash Buffer per well with thorough aspiration between each wash. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess 1X Wash Buffer.
7. Add 100 µL of the diluted anti-phospho-MYPT1 (Thr^{696}) antibody to each well.
8. Cover with a plate cover and incubate at room temperature for 1 hour on an orbital shaker.
9. Remove plate cover and empty wells. Wash the strip wells 3 times according to step 6 above.
10. Add 100 µL of the diluted HRP-conjugated secondary antibody to each well.
11. Cover with a plate cover and incubate at room temperature for 1 hour on an orbital shaker.
12. Remove plate cover and empty wells. Wash microwell strips 3 times according to step 6 above. Proceed immediately to the next step.
13. Warm Substrate Solution to room temperature. Add 100 µL of Substrate Solution to each well, including the blank wells. Incubate at room temperature for 5-20 minutes on an orbital shaker.
14. Stop the enzyme reaction by adding 100 µL of Stop Solution into each well, including the blank wells. Results should be read immediately (color will fade over time).
15. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length.

Example of Results

The following figure demonstrates typical results seen with Cell Biolabs' 96-well ROCK Activity Assay Kit. One should use the data below for reference only.

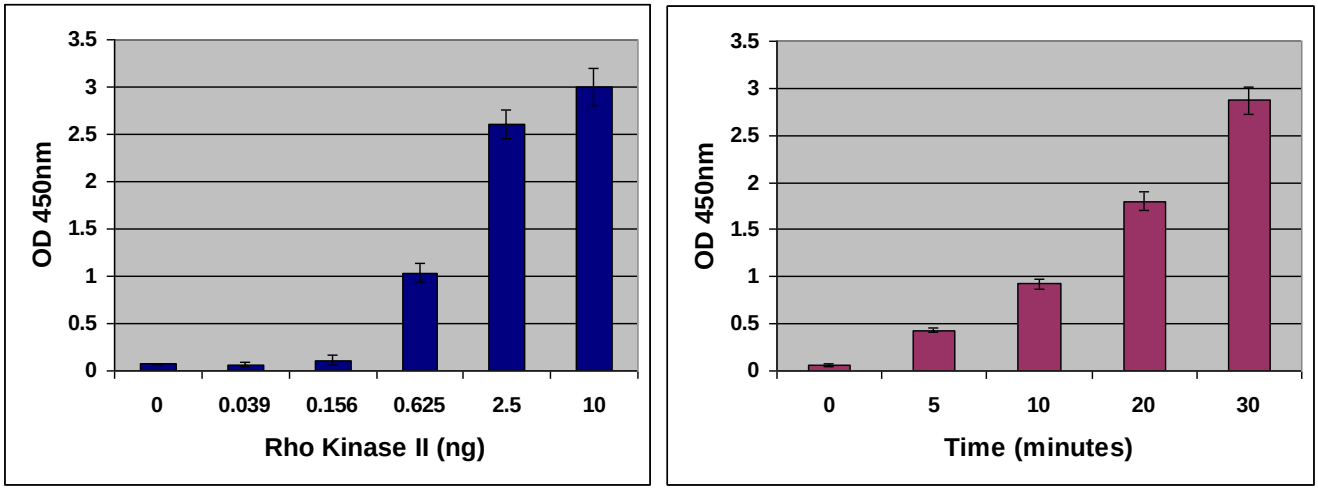

Figure 1: ROCK-II Activity Assay. Left: Active ROCK-II was incubated for 60 minutes at 30°C. Right: 2.5 ng of active ROCK-II was incubated at 30°C for times as shown. Phosphorylation of MYPT1 substrate was detected by anti-phospho-MYPT1 (Thr^{696}) antibody as described in Assay Protocol.

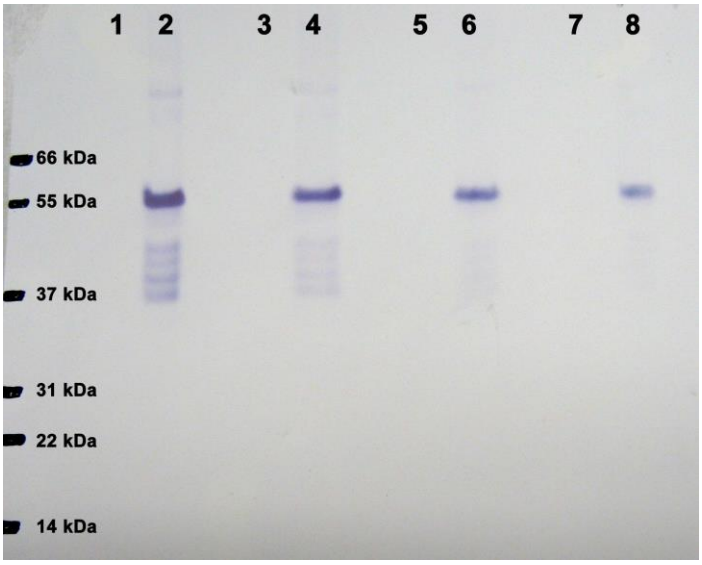

Figure 2: ROCK-II Activity Immunoblot Assay. 25 µL of 1X Kinase Buffer containing 10 ng of active ROCK-II was incubated with 50 µL of 1X Kinase Buffer containing 0.2 mM ATP and 500 ng of recombinant MYPT1 for 30 minutes at 30°C. Kinase reaction was stopped by adding 25 µL of 4X SDS-PAGE Sample Buffer. 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 the Assay Protocol for Cat. # STA-415.

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Recent Product Citations

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Contact Information

Cell Biolabs, Inc.
7758 Arjons Drive
San Diego, CA 92126
Worldwide: +1 858-271-6500
USA Toll-Free: 1-888-CBL-0505
E-mail: tech@cellbiolabs.com
www.cellbiolabs.com

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