Rho-associated Kinase (ROCK) Activity Assay

Catalog No. CSA001

Sufficient reagents for one 96-well kit

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Introduction

Small GTPase proteins of the Rho family are pivotal regulators of several aspects of cell behavior, such as cell motility, cell proliferation and apoptosis. They have a central role in many motile responses that involve the actin cytoskeleton and/or microtubule network, from neurite extension to phagocytosis and cancer-cell invasion. In addition, they contribute to changes in gene expression that lead to cell-cycle progression or differentiated cell responses. 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 protein 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 the actin cytoskeleton through phosphorylation of several substrates that contribute to the assembly of actin filaments and contractility.

ROCK inactivates myosin phosphatase through the specific phosphorylation of myosin phosphatase target subunit 1 (MYPT1) at threonine residue 696, which results in an increase in the phosphorylated form of the 20 kDa myosin light chain (MLC20). This threonine residue may also be phosphorylated by myotonic dystrophy protein kinase (DMPK) family kinases.

Millipore's Rho-associated Protein Kinase (ROCK) Activity Assay Kit is an enzyme immunoassay for detection of the active ROCK and DMPK family kinases. Plates are pre-coated with recombinant MYPT1, which contains a Thr696 residue that may be phosphorylated upon addition of ROCK-I, ROCK-II, or DMPK. A detection antibody that specifically detects only MYPT1 phosphorylated at Thr696 is then applied. Subsequently, an HRP-conjugated secondary detection antibody is added. The amount of phoshorylated substrate is measured by addition of the chromogenic substrate tetra-methylbenzidine (TMB), which bound HRP converts from a colorless solution to a blue solution (or yellow after the addition of stop solution). The absorbance signal at 450 nm reflects the relative amount of ROCK activity in the sample.

The ROCK assay kit is designed to determine the presence and relative amount of purified ROCK activity as well as for screening inhibitors or activators of ROCK or DMPK family kinases. It can also be used to assess the potency of pharmacological agents on ROCK or DMPK family kinases.

Summary of Procedure

Add 50 µL of sample per well
Incubate for 30 min at 30°C

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Wash the wells

↓

Add 100 µL of anti-phospho-MYPT1 (Thr696) antibody
Incubate for 1 hr at room temp.

↓

Wash the wells

↓

Add 100 µL of HRP conjugated goat anti-rabbit IgG secondary antibody
Incubate for 1 hr at room temp.

↓

Wash the wells

↓

Add 100 µL of Substrate Reagent

↓

Add 100 µL of Stop Solution

↓

Measure absorbance at 450 nm

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Kit Components

Store at 2-8°C

1. **MYPT1 Pre-coated 96-well Multistrip Plate:** (Part No. CS205310) One 96-well multistrip plate pre-coated with MYPT1.
2. **Anti-phospho-MYPT1(Thr696) Antibody:** (Part No. CS205309) One vial containing 10 µg of anti-phospho-MYPT1(Thr696) antibody.
3. **TMB/E Substrate:** (Part No. CS205120) One bottle containing 12 mL TMB/E substrate.
4. **Stop Solution:** (Part No. 2007598) One bottle containing 12 mL stop solution.
5. **TBS, 20X:** (Part No. 20-190) One bottle containing 50 mL 20X TBS.
6. **20% Tween® 20 (v/v):** (Part No. 20-246) One bottle containing 3 mL 20% Tween® 20.
7. **Plate Sealers:** (Part No. 3006581) Two plate covers.

Store at -20°C

8. **Assay Dilution Buffer I:** (Part No. 20-108) One vial containing 1 mL of assay dilution buffer I.
9. **ATP (10mM):** (Part No. CS206509) One vial containing 50 µL of ATP stock at 10 mM.
10. **$MgCl_2$ (1M):** (Part No. CS206511) One vial containing 100 µL of $MgCl_2$ stock at 1 M.
11. **30% BSA:** (Part No. CS205797) One vial containing 1 mL of 30% BSA.
12. **Goat Anti-Rabbit IgG HRP Secondary Antibody:** (Part No. 90276) One vial containing 30 µL goat anti-rabbit IgG HRP-conjugated secondary antibody.
13. **ROCK Inhibitor Y-27632:** (Part No. CS205307) One vial containing 150 µL of ROCK Inhibitor Y-27632 at 5 mM.

Store at -80°C

14. **Active Rho-associated Kinase II (ROCK-II):** (Part No. CSA001-3) One vial containing 10 µg active ROCK-II.

Materials Not Supplied

1. Multi-channel or repeating pipettes
2. Plate shaker (optional)
3. Pipettors and tips capable of accurately measuring 1-1000 μL
4. Graduated serological pipettes
5. 96-well microtiter plate reader with luminescence readout.
6. Graphing software for plotting data or graph paper for manual plotting of data
7. Microcentrifuge tubes for standard and sample dilutions
8. Mechanical vortex
9. 1 liter container
10. Distilled or deionized water
11. DMSO to dissolve inhibitor compounds.
12. (Optional) ROCK-I (Millipore Catalog No. 14-601) or DMPK (Millipore Catalog No. 14-649)

Storage

There are three individual boxes supplied to perform the Rho-associated Kinase (ROCK) Activity Assay. Upon receipt, please store each box at the temperature indicated on the container label. Recommended use is within four months from date of receipt.

CSA001-1: Store at 2-8°C.

CSA001-2: Store at -20°C. Avoid freeze and thaw cycles. Aliquot if necessary.

CSA001-3: Store at -80°C. Avoid freeze and thaw cycles. Aliquot if necessary.

Precautions

- The instructions provided have been designed to optimize the kit's performance. Deviation from the instructions may result in suboptimal performance of the kit and failure to produce accurate data.
- **Safety Warnings and Precautions:** This kit is designed for research use only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

Technical Notes

- All kit reagents should be brought to room temperature (20°C to 25°C) just prior to use.
- Do not use reagents beyond the expiration date of the kit (4 months after receipt).
- Do not mix or interchange reagent from various kit lots.
- Manual Plate Washing: Vigorous washing and complete removal of all liquid by aspiration at the end of each washing step is very important to obtain low background values. Gentle agitation during the wash steps or a 2-3 minutes soak may reduce background values.

Reagent Preparation

1. **200 mL of 1X TBS:** Combine 10 mL 20X TBS with 190 mL Distilled Water.
2. **800 mL of 1X Wash Buffer:** Combine 2 mL 20% Tween® 20, 40 mL 20X TBS, and 758 mL Distilled Water.
3. **30 mL Assay Blocking Buffer:** Combine 1 mL of 30% BSA with 29 mL of 1X wash buffer prepared as above. Store at 4°C.
4. **Anti-phospho-MYPT1(Thr696) Antibody Solution:** Prepare 12 mL per plate of Anti-phospho-MYPT1(Thr696) Antibody Solution by diluting Anti-phospho-MYPT1(Thr696) Antibody to a final concentration of 0.5 µg/mL with assay blocking buffer. Vortex the solution. Discard any unused portion following assay completion. Store at 4°C.
5. **Goat anti-Rabbit IgG HRP Secondary Antibody Solution:** Prepare 12 mL per plate of Secondary Antibody Solution by diluting 6 µL of Goat anti-Rabbit IgG HRP Secondary Antibody in 12 mL assay blocking buffer. Vortex the solution to ensure antibody is evenly diluted in the solution. Discard any unused portion following assay completion. Store at 4°C.
6. **5X $MgCl_2$ Working Solution:** Prepare a 5X $MgCl_2$ working solution by diluting 1 M $MgCl_2$ to 75 mM with distilled water. Store at 4°C.
7. **5X ATP Working Solution:** Prepare a 5X ATP working solution by diluting 10 mM ATP to 0.5 mM with distilled water. Store at 4°C.

   Note: If a final 100 µM ATP will be applied in the enzyme reaction, follow the 5X ATP working solution preparation above. If a different final ATP concentration will used, prepare a corresponding 5X ATP working solution.
8. **Inhibitor (Y-27632) Working Solution:** Prepare a 5X Y-27632 inhibitor working solution by diluting 5 mM Y-27632 inhibitor with distilled water. Store at 4°C.
9. **Compound Working Solutions:** Prepare concentrated stock compound solutions using DMSO. Make further dilution with distilled water to a 5X of desired concentration working solution.

   Note: Final DMSO in the reaction should be less than 2% to minimize any potential effects to the enzyme.
10. **ROCK-II Working Solution:** Just prior to usage, dilute the provided active ROCK-II in distilled water to a proper working solution, using the Units information provided on label. It is recommended that the kinase be titrated by the user. For inhibitor screening, a 1 mUnit/µL working solution is typically used, to obtain 10 mUnits per reaction. Keep the working solution on ice until use. Discard any unused working solution of the kinase.

Optimization of the Kinase Assay

- **Kinase titration**
  Titration of a kinase allows the optimal concentration to be determined, typically where the signal reaches around 80~90% of the maximum. A fixed final concentration of ATP (100 µM) should be tested with a range of kinase concentrations (0-100 mUnits/reaction in a ten-fold serial dilutions). Incubate the enzymatic reaction for 30 minutes at 30°C.

- **Kinetic study**
  Enzyme kinetics are dependent on the kinase and substrate concentrations. A time course is performed using the optimal kinase concentration determined at the kinase titration step and a final concentration of ATP at 100 µM. The reaction is stopped at various end points (2, 5, 10, 15, 30, 60, 90 minutes) by removing enzyme and washing. After the last time point, the standard procedure is continued with all of the wells.
  The optimal reaction time is typically when the signal reaches around 80-90% of the maximum. This optimal reaction time is used for the remaining experiments.

- **ATP titration**
  Titration of ATP allows the determination of the K_m for ATP. Use the optimal kinase concentration to test a range of ATP concentrations (0.0064-100 µM) in five-fold serial dilutions. After incubation at 30°C, the kinase reaction is stopped at 30 minutes.

- **IC_{50} determination of inhibitor compounds**
  The activity of a kinase is tested over a broad range of inhibitor concentrations to generate a dose response curve. The test is generally run using the previously determined optimal assay conditions. A proper ATP concentration (reference the K_m value determined at the ATP titration step) should be considered for ATP-competitive compounds.

Assay Protocol

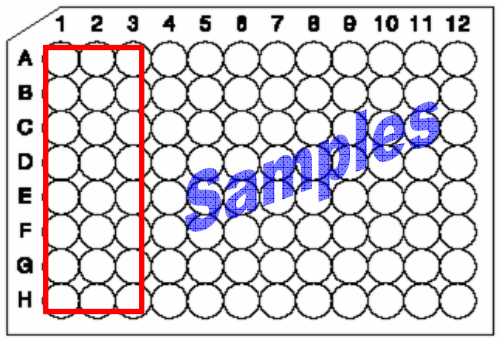
I. Preparation of a standard curve with active recombinant ROCK-II enzyme

1. Refer to the component label for stock concentration of enzyme, and prepare a working solution of active ROCK-II diluted in distilled water in a microfuge tube, just prior to use. The first concentration should be 10 mU/µL in a 50 µL volume. Make a 10-fold series dilution by pipetting 5 µL from the first tube to a second tube containing 45 µL distilled water. Vortex to mix and continue making 1:10 dilutions, up to the 7^{th} tube. Leave no enzyme in the 8^{th} tube as a blank control.

Wells Layout:

STD (mU)

1. 100
2. 10
3. 1
4. 0.1
5. 0.01
6. 0.001
7. 0.0001
8. 0



2. Remove 2-3 strips from the MYPT1-precoated plate to use for the dilution series reactions. Leave the unused strips covered with a plate sealer at 4°C if not in use at the same time.

3. In the MYPT1-precoated wells, assemble the enzyme reaction as indicated in the table below

Components	Standard
ROCK-II Kinase (series points)	10 µL
Assay Dilution Buffer I	10 µL
5X $MgCl_2$ solution	10 µL
5X ATP solution (0.5 mM)	10 µL
Distilled water	10 µL

II. Assay for ROCK activity modulators and/or enzyme provided by user

The following table provides recommended volumes to add for assays employing enzyme and/or compounds provided by the user (Test Enzyme and Test Compounds columns, respectively). Enzyme Control, Buffer Control and Inhibitor Control reactions should be included in duplicate for each experiment.

Note: If a ROCK-II enzyme standard curve will be running at the same time, active ROCK sample signal can be compared to the curve for a relative ROCK activity estimation.

Reaction setup:

Component:	Type of reaction:				
	Enzyme Control	Inhibitor Control	Test Enzyme	Test Compounds	Buffer Control
ROCK-II (1 mU/µL)	10 µL	10 µL	-	10 µL	-
Y-27632 (1 mM)	-	10 µL	-	-	-
5X ATP Solution	10 µL	10 µL	10 µL	10 µL	10 µL
5X $MgCl_2$ Solution	10 µL	10 µL	10 µL	10 µL	10 µL
Assay Dilution Buffer I	10 µL	10 µL	10 µL	10 µL	10 µL
User-supplied enzyme	-	-	10 µL	-	-
Compounds	-	-	-	10 µL	-
Distilled water	10 µL	-	10 µL	-	20 µL

4. Incubate at 30°C for 30 minutes with medium agitation.
5. Stop the reaction by flicking the plate to remove the solution from the wells.
6. Add 200 µL/well 1X washing buffer, agitate, and remove buffer by flicking the plate over a sink. Repeat for a total of 3 times.
7. Add 100 µL/well anti-phospho-MYPT1(Thr696) antibody solution, and incubate at room temperature for 1 hour with medium agitation.
8. Remove the antibody solution by flicking the plate.
9. Add 200 µL/well 1X washing buffer, agitate, and remove buffer by flicking the plate. Repeat for a total of 3 times.
10. Add 100 µL/well goat anti-rabbit IgG HRP secondary antibody solution, and incubate at room temperature for 1 hour with medium agitation. Allow the TMB/E Substrate and Stop Solution to warm to room temperature.
11. Remove the secondary antibody solution by flicking the plate.
12. Add 200 µL/well 1X washing buffer, agitate, and remove buffer by flicking the plate. Repeat for a total of 3 times.
13. Add 200 µL/well 1X TBS, agitate, and remove TBS by flicking the plate. Repeat for a total of 2 times.
14. Add 100 µL/well TMB/E substrate (Catalog CS205120), develop in the dark for 1-15 minutes. Monitor the blue color development. **DO NOT OVERDEVELOP**.
15. Stop the reaction by adding 100 µL of stop solution (Catalog 2007598) to each well. Read absorbance at 450 nm.

ADDITIONAL PROTOCOL NOTES:
A standard curve must be generated with each assay.

For users of automatic plate washers: It is important to ensure that the wash apparatus is properly maintained and operating correctly. Tubing and tips can easily become clogged, leading to incomplete washing and inadequate aspiration of wells. The result may be poor precision and an unsuitable standard curve. For best results, we recommend at least 4 wash cycles.

CAUTION: Bubbles in the wells will cause inaccurate readings. Ensure that all bubbles are removed prior to taking the absorbance reading.

For samples containing very low starting protein levels, wells may be incubated at 37°C during the final incubation to obtain greater sensitivity.

Assay Results

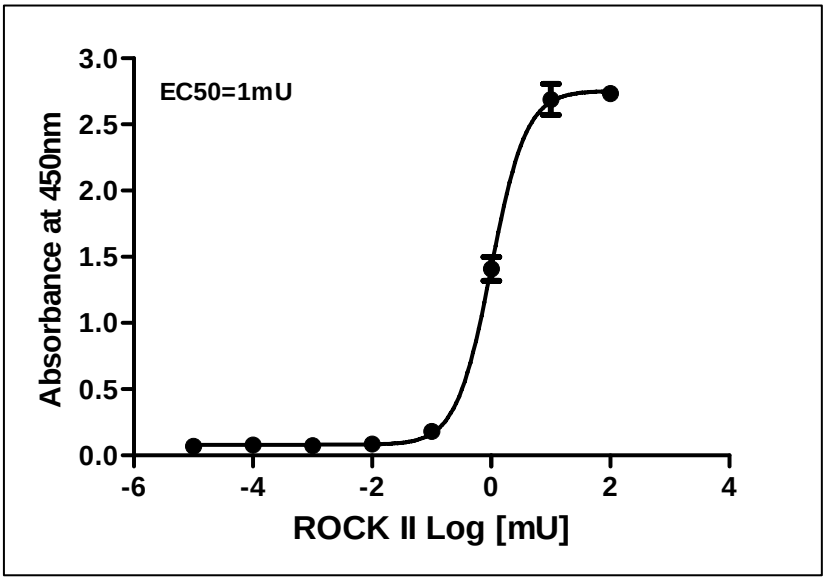


Figure 1. Dose response curve of active ROCK-II enzyme

Active recombinant human ROCK-II (0 ~100 mU) was incubated with MYPT1-coated plate with 100 µM ATP for 30 minutes at 30°C. Absorbance signal was determined at 450 nm, and a non-linear curve fit with sigmoidal dose response was applied.

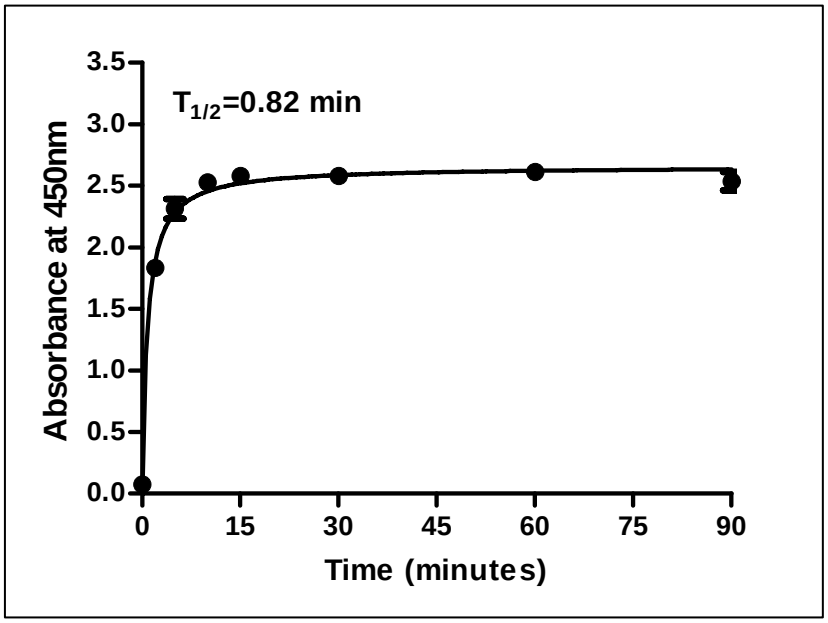


Figure 2. Kinetics of active ROCK-II enzyme

10 mU ROCK-II and 100 µM ATP were utilized for the enzyme reaction. The reaction was complete by 10 minutes at 30°C.

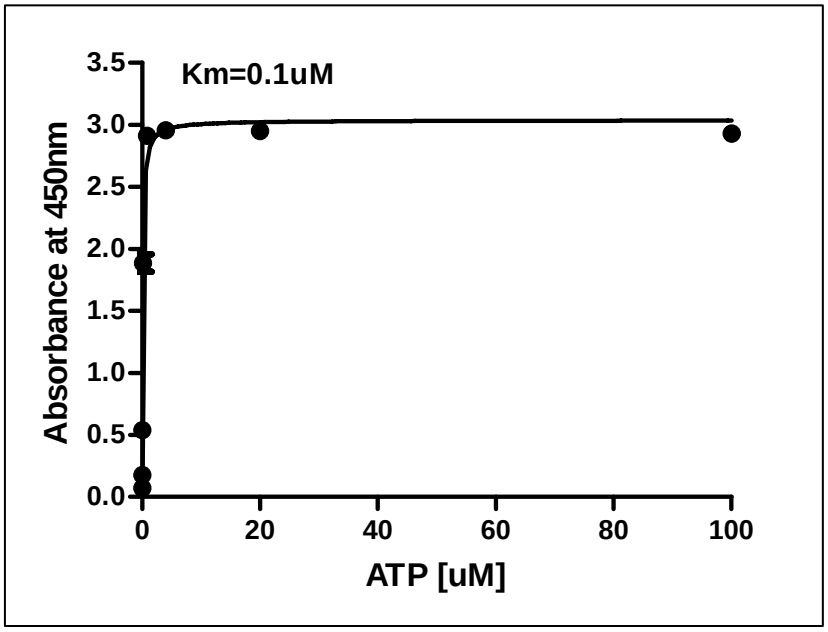


Figure 3. Determination of ATP dependence of active ROCK-II enzyme

10 mU/well active ROCK-II enzyme was incubated in reactions containing 0-100 µM ATP. The K_m value may be used for establishing ATP concentration for screening compounds.

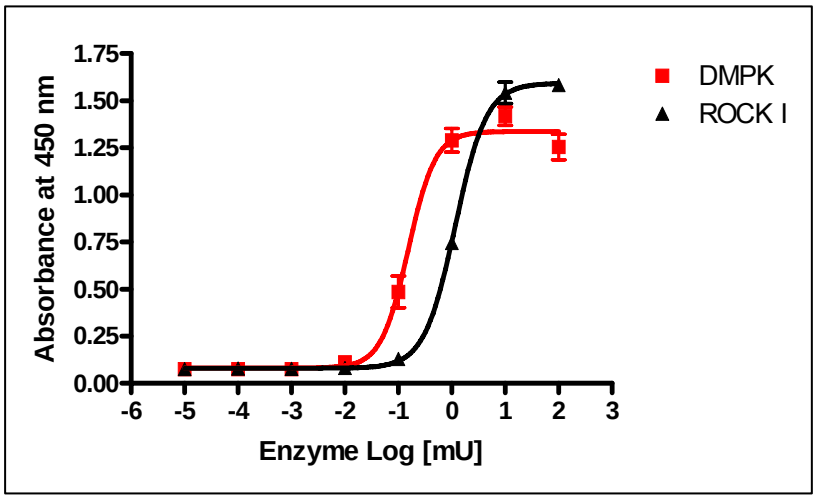


Figure 4. Dose response curve of active ROCK-I and DMPK enzyme

Active recombinant human ROCK-I (Millipore Catalog No. 14-601) and DMPK (Millipore Catalog No. 14-649), each at 0~100 mU/well, were incubated with MYPT1-coated plate with 100 µM ATP for 30 minutes at 30°C. Absorbance signal was determined at 450 nm, and a non-linear curve fit with sigmoidal dose response was applied.

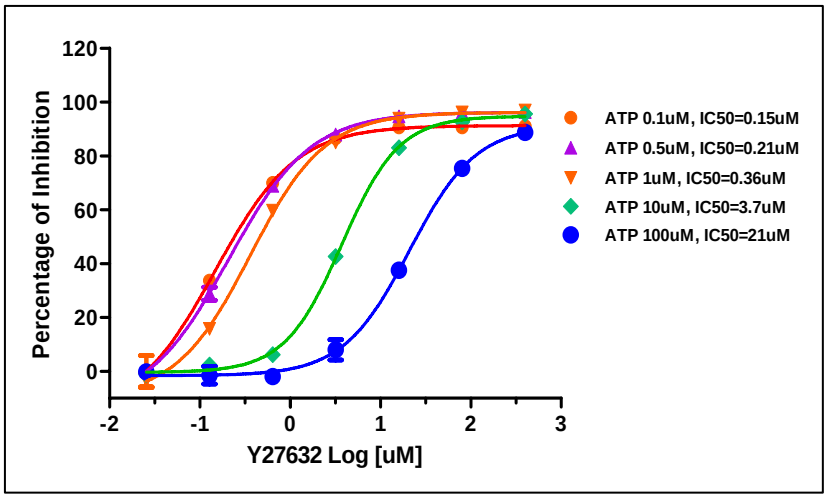


Figure 5. Determination of IC50 values for ROCK specific inhibitor Y-27632 with ROCK-II

Reactions containing 10 mU ROCK-II and ATP concentrations from 1X to 1000X K_m, were incubated for 30 minutes at 30°C. Data obtained was analyzed by non-linear curve fit with sigmoidal dose response with variable slope.

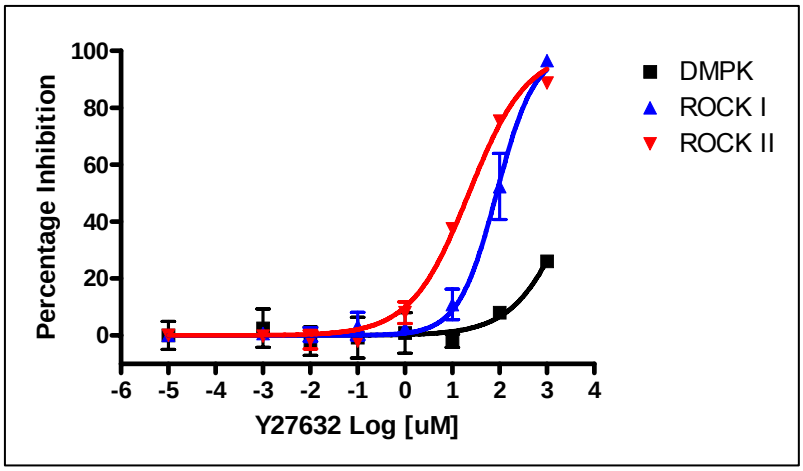


Figure 6. Determination of IC50 values for ROCK specific inhibitor Y-27632 with ROCK-I, ROCK-II and DMPK

Reactions containing 10 mU ROCKI, 10 mU ROCK-II, or 1 mU DMPK with 100µM ATP were incubated for 30 minutes at 30°C. Data obtained was analyzed by non-linear curve fit with sigmoidal dose response with variable slope. Y-27632 selectively inhibited ROCK-I and ROCK-II, with minimal inhibition of DMPK.

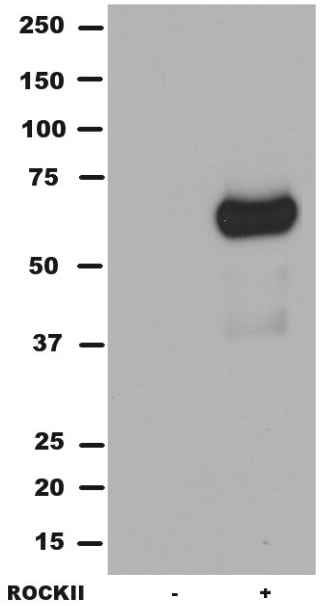


Figure 7. ROCK-II Enzyme Reaction Immunoblot

250 ng of recombinant MYPT1 protein was incubated with (+) and without (-) 20 mU ROCK-II in a total of 250 µL reaction buffer containing 100 µM ATP for 30 minutes at 30°C. 25ng of MYPT1 protein was loaded per lane to SDS-PAGE. Phosphorylation of MYPT1 substrate was detected by anti-phospho-MYPT1(Thr696) antibody.

References

1. Riento K and Ridley A.J., Nat Rev Mol Cell Biol. 2003 Jun; 4 (6):446-56.
2. McKenzie JA, Ridley AJ., J Cell Physiol. 2007 Oct; 213 (1):221-8.
3. Pelosi M. *et al.,* Mol. and Cell. Biol., 2007, p. 6163–6176.
4. Muranyi A. *et al*., FEBS Letters. 2001, 493: 80-84.
5. Bian D. *et al.,* Oncogene, 2006, 25: 2234-2244.
6. Feng J. *et al.,* J. Biol. Chem. 1999, 274 (52): 37385-37390.

Troubleshooting Guide

Problem	Potential Cause	Experimental Suggestions
No signal or weak signal in all wells	Missing components or key steps	Ensure all components are added in the appropriate steps and amounts.
	Active ROCK-II enzyme is no longer active or has reduced activity	Ensure all components are stored at the recommended temperature, with minimal freeze/thaw cycles for repeated use. Make aliquots of components when first thawed if planning more than one assay.
	Plate reader or settings are not optimal	Verify the measurement, read time, and filter on the plate reader.
	Incorrect storage temperatures Incorrect assay temperature	Items are to be stored at the appropriate storage temperatures. Performance can be negatively affected if reagents are not stored and used in the appropriate time period.
No detectable signal in test samples of enzyme	Low ROCK activity level in sample	Make sure to include a positive ROCK-II enzyme control.

Warranty

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