Non-Radioisotopic Kit for Measuring Rho-kinase and DMPK Activity

CycLex Rho-kinase Assay Kit

Cat# CY-1160

Intended Use

The CycLex Research Product **CycLex Rho-kinase Assay Kit** is primarily designed to measure the activities of purified Rho-kinase or DMPK for the rapid and sensitive evaluation of inhibitors or activators. The phospho-specific monoclonal antibody used in this assay kit has been demonstrated to recognize the phospho-threonine 696 residue in MBS/MYPT1, which is phosphorylated by Rho-kinase or DMPK (Myotonic dystrophy protein kinase) family kinases. Additionally, column fractions of cultured primary cell, cell line, or tissue homogenate can be assayed for Rho-kinase family activity with the CycLex Research Product **CycLex Rho-kinase Assay Kit** if the appropriate dose of Rho-kinase specific inhibitor e.g. Y-27632 or HA-1077 is used.

Applications of this kit include:

1) Monitoring the purification of Rho-kinase or DMPK family kinase.
2) Screening inhibitors or activators of Rho-kinase or DMPK family kinase.
3) Detecting the effects of pharmacological agents on Rho-kinase or DMPK family kinase.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store all components at 4°C.
- Don't expose reagents to excessive light.

Introduction

The small GTPase Rho regulates formation of focal adhesions and stress fibers of fibroblasts, as well as adhesion and aggregation of platelets and lymphocytes by shuttling between the inactive GDP-bound form and the active GTP-bound form. Rho is also essential in cytokinesis and plays a role in transcriptional activation by serum response factor. Ishizaki et al. (1996) identified the protein serine/threonine kinase ROCK1 (Rho-kinase beta), which they called p160-ROCK, which is activated when bound to the GTP-bound form of RhoA. Fujisawa et al. (1996) localized the Rho-binding domain of ROCK1 to a region between residues 934-1015.

ROCK2 (Rho-kinase alpha) is a serine/threonine kinase that regulates cytokinesis, smooth muscle contraction, the formation of actin stress fibers and focal adhesions, and the activation of the c-fos serum response element. ROCK2, which is an isozyme of ROCK1, is a target for the small GTPase Rho. Nakamura et al. (2001) studied the role of Rho in the migration of corneal epithelial cells in rabbit. They detected both ROCK1 and ROCK2 in the corneal epithelium at protein and mRNA levels. They found that exoenzyme C3, a Rho inhibitor, inhibits corneal epithelial migration in a dose-dependent manner and prevents the stimulatory effect of the Rho activator lysophosphatidic acid (LPA). Both cytochalasin B, an inhibitor of actin filament assembly, and ML7, an inhibitor of myosin light chain kinase, also prevent LPA stimulation of epithelial migration. The authors suggested that Rho mediates corneal epithelial migration in response to external stimuli by regulating the organization of the actin cytoskeleton.

Rao *et al*. (2001) investigated the role of Rho-kinase in the modulation of aqueous humor outflow facility. The treatment of human trabecular meshwork and canal of Schlemm cells with a Rho-kinase-specific inhibitor led to significant but reversible changes in cell shape and decreased actin stress fibers, focal adhesions, and protein phosphotyrosine staining. Based on the Rho-kinase inhibitor-induced changes in myosin light chain phosphorylation and actomyosin organization, the authors suggested that cellular relaxation and loss of cell-substratum adhesions in the human trabecular meshwork and canal of Schlemm cells could result in either increased paracellular fluid flow across the canal of Schlemm or altered flow pathway through the juxtacanalicular tissue, thereby lowering resistance to outflow. They suggested Rho-kinase as a potential target for the development of drugs to modulate intraocular pressure in glaucoma patients.

Measurement of Rho-kinase activity

The protocol generally regarded as most sensitive for the quantitative measurement of Rho-kinase activity involves incubation of the Rho-kinase sample with substrate, either a natural or synthetic polypeptide (such as Long S6 Kinase substrate peptide), in the presence of $Mg^{2+}$and ^{32}P-labeled ATP. The reaction is terminated by "spotting" a sample onto a filter paper disc, followed by immersion in acid to precipitate the radiolabeled product. The filter papers are then washed extensively to remove unincorporated radiolabel and the radioactivity is counted. While sensitive, this method is labor-intensive, generates hazardous radioactive waste, and depends on a radioisotope of short half-life. It is particularly unsuitable when kinase assays are only performed on an infrequent basis. The CycLex Research Product **CycLex Rho-kinase Assay Kit** uses a peroxidase coupled anti-phospho-MBS threonine696 monoclonal antibody as a reporter molecule in a 96-well ELISA format. This assay provides a non-isotopic, sensitive and specific method to detect Rho-kinase family activity.

Principle of the Assay

The CycLex Research Product **CycLex Rho-kinase Assay Kit** is a single-site, semi-quantitative immunoassay for Rho-kinase activity. Plates are pre-coated with a substrate corresponding to recombinant the C terminus of MBS (Myosin-Binding Subunit of myosin phosphatase), which contains a threonine residue that may be phosphorylated by DMPK family members, including Rho-kinase (ROCK1 and ROCK2), MRCK (Myotonic Dystrophy kinase-related Cdc42-binding Kinase) and DMPK (Myotonic Dystrophy Protein Kinase). The detector antibody is AF20, an antibody that specifically detects only the phosphorylated form of threonine-696 on MBS. The CycLex Research Product **CycLex Rho-kinase Assay Kit** may be used to determine the presence of Rho-kinase activity in purification column fractions or to follow the kinetics of a purified or partially purified Rho-kinase protein as well as screening Rho-kinase inhibitor. To perform the test, the sample is diluted in Kinase Buffer, pipetted into the wells and allowed to phosphorylate the bound substrate in the presence of Mg^{2+} and ATP. The amount of phosphorylated substrate is measured by binding it with a horseradish peroxidase conjugate of AF20, a anti-phospho-MBS threonine-696 specific antibody, which then catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stopping reagent). The color is quantified by spectrophotometry and reflects the relative amount of Rho-kinase activity in the sample. For kinetic analysis, the sample containing Rho-kinase is added to the wells in a similar fashion and at varying times the reaction is stopped by the addition of a chelator, sodium ethylenediaminetetraacetate (EDTA) and the amount of phosphorylated substrate determined as before.

The CycLex Research Product **CycLex Rho-kinase Assay Kit** is designed to accurately determine the presence and relative amount of Rho-kinase activity in purification column fractions and to determine non-isotopic kinetic analysis of Rho-kinase activity. Careful attention to extraction methods and the assay protocol will provide the investigator with a reliable tool for the evaluation of Rho-kinases.

Summary of Procedure

Add 100 µL of sample to the wells
↓ Incubate for 30 min at 30°C
Wash the wells
↓
Add 100 µL of HRP conjugated anti-phospho-specific antibody
↓ Incubate for 30 min at room temp.
Wash the wells
↓
Add 100 µL of Substrate Reagent
↓
Add 100 µL of Stop Solution
↓
Measure absorbance at 450 nm

Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microtiter plate kit.

Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with recombinant MBS C-terminus (654-880 a.a.) as Rho-kinase substrate.

10X Wash Buffer: One bottle containing 100 mL of 10X buffer containing 2 %Tween®-20

Kinase Buffer: One bottle containing 20 mL of 1X buffer; used for Kinase Reaction Buffer and sample dilution.

20X ATP: One vial of lyophilized ATP Na_2 salt.

HRP conjugated Detection Antibody: One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-phospho-MBS T696 (AF-20) antibody. Ready to use.

Substrate Reagent: One bottle containing 20 mL of the chromogenic substrate, tetra-methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle containing 20 mL of 1 N H_2SO_4. Ready to use.

Materials Required but not Provided

- **Recombinant Rho-kinase-II positive control:** Available from CycLex (Cat # CY-E1160-1): The positive control should be added to the first well at 10 m units/well.
- **Recombinant DMPK positive control:** Available from CycLex (Cat # CY-E1160-2): The positive control should be added to the first well at 10 m units /well.
- **10X Y-27632:** 100 µM Y-27632 (Calbiochem Cat # 688001, 5 mM solution, diluted 1:50 in water)
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1000 µL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Wash bottle or multichannel dispenser** for plate washing.
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **Plate reader** capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.
- **500 or 1000 mL graduated cylinder**
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

Precautions and Recommendations

- Store the ATP at -20°C in aliquots. Store all other components at 4°C. Do not expose reagents to excessive light. Avoid freeze/thaw cycles.
- Allow all the components to come to room temperature before use.
- All microplate strips that are not immediately required should be returned to the zip-lock pouch, which must be carefully resealed to avoid moisture absorption.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents in this kit may contain preservatives or other chemicals. Care should be taken to avoid direct contact with these reagents.
- Do not mouth pipet or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
- Avoid contact with Substrate Solution which contains hydrogen peroxide.
- Avoid contact with Stop Solution which contains Sulfuric Acid.
- In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**
- **CAUTION: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

Detailed Protocol

The **CycLex Rho-kinase Assay Kit** is provided with removable strips of wells so the assay can be carried out on separate occasions using only the number of strips required for the particular determination. Since experimental conditions may vary, an aliquot of the Rho-kinase-II positive control (Cat # CY-E1160-1), available separately from CycLex, should be included in each assay as a positive control. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Working Solution

1. Prepare a working solution of **Wash Buffer** by adding 100 mL of the **10X Wash Buffer** (provided) to 900 mL of ddH_2O. Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Prepare **20X ATP Solution** by adding **0.8 mL** of ddH_2O to the vial of **20X ATP** (provided, lyophilized). Mix gently until dissolved. The final concentration of the **20X ATP Solution** should be **2.5 mM**. Store the solution in small aliquots (e.g. 100 µL) at -20°C.

3. Prepare **Kinase Reaction Buffer** by mixing following reagents.

	96 assays	**10 assays**	**1 assay**
Kinase Buffer (provided)	**9.5 mL**	**950 µL**	**95 µL**
20X ATP Solution	**0.5 mL**	**50 µL**	**5 µL**
Total	**10 mL**	**1000 µL**	**100 µL**

**You will need 80-90 µL of Kinase Reaction Buffer per assay well. Mix well. Discard any unused Kinase Reaction Buffer after use.*

Standard Assay

1. Remove the appropriate number of microtiter wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.

2. Prepare all samples (diluted with Kinase Buffer as needed). All samples should be assayed in duplicate.

3. To assay individual column fractions, add **10 µL** of **each fraction** to the wells of the assay plate on ice. Crude lysates or cell extracts should be added to wells either neat or diluted as describe above with Kinase Buffer if necessary. (Suggested starting dilutions are 1:5, 1:10 and 1:20.) Duplicate wells containing 10 mUnits/10 µL Rho-kinase-II positive control (Cat # CY-E1160-1) should be included in each assay as a positive control for phosphorylation.

4. Begin the kinase reaction by addition of **90 µL Kinase Reaction buffer** per well, cover with plate sealer, and incubate **at 30°C for 30 minutes**.

5. Wash wells five times with Wash Buffer making sure each well is filled completely. Remove residual Wash Buffer by gentle tapping or aspiration.

6. Pipette **100 µL** of **HRP conjugated Detection Antibody** into each well, cover with a plate sealer and incubate **at room temperature (ca.25°C) for 30 minutes**. Discard any unused conjugate.

7. Wash wells five times as same as in step5.

8. Add **100 µL** of **Substrate Reagent** to each well and incubate **at room temperature (ca.25°C) for 5–15 minutes**.

9. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

10. Measure absorbance in each well using a spectrophotometric plate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the plate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Note-1: Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

Note-2: Reliable signals are obtained when either O.D. values do not exceed 0.25 units for the blank (no enzyme control), or 2.5 units for the Rho-kinase positive control.

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the Rho-kinase-II positive control, perform a second reading at 405 nm. A new O.D. values, measured at 405 nm, is used to determine Rho-kinase activity of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

Kinetic Assay

1. Remove the appropriate number of microtiter wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.

2. Prepare all samples (diluted with Kinase Buffer as needed). All samples should be assayed in duplicate.

3. To assay individual column fractions, add **10 µL** of **each fraction** or Rho-kinase II positive control (Cat # CY-E1160-1) to the wells of the assay plate. Duplicate wells containing 10 mUnits/10 µL Rho-kinase-II positive control (Cat # CY-E1160-1) should be included in each assay as a positive control for phosphorylation

4. Begin kinase reaction by addition of **90 µL Kinase Reaction Buffer** in duplicate per well in timed intervals (suggested interval is 5 minutes but should be individually determined for each system). After the final addition, incubate **at 30°C for 20 minutes**.

5. Stop the reaction by flicking out the contents. (Alternatively, the reaction may be terminated by the addition of 150 µL 0.1 M Na EDTA, pH 8.0 to each well).

6. Wash wells five times with Wash Buffer making sure each well is filled completely. Remove residual Wash Buffer by gentle tapping or aspiration.

7. Pipette **100 µL** of **HRP conjugated Detection Antibody** into each well, cover with a plate sealer and incubate **at room temperature (ca.25°C) for 30 minutes**. Discard any unused conjugate after use.

8. Wash wells as same as in Step 6.

9. Add **100 µL** of **Substrate Reagent** to each well and incubate **at room temperature (ca.25°C) for 10-15 minutes**.

10 add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

11. Measure absorbance in each well using a spectrophotometric plate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the plate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Recommendations

Special considerations when screening activators and inhibitors

In order to estimate the inhibitory effect on Rho-kinase family activity in the test chemicals correctly, it is necessary to conduct the control experiment of "Solvent control" at least once for every experiment and "Inhibitor control" at least once for the first experiment, in addition to "Test sample", as indicated in the following table. When test chemicals cause an inhibitory effect on Rho-kinase activity, the level of A450 is weakened as compared with "Solvent control". The high level of A450 is not observed in "Inhibitor control" (usually A450<0.2).

Assay reagents	Test sample	Solvent control	Inhibitor control
Kinase Reaction Buffer	**80 µL**	**80 µL**	**80 µL**
10X Inhibitor or equivalent	**10 µL**	**-**	**-**
Solvent for Inhibitor	**-**	**10 µL**	**-**
10X Y-27632 (100 µM)*	**-**	**-**	**10 µL**
CycLex Rho-kinase Positive Control (1 m unit/µL) or your enzyme fraction**	**10 µL**	**10 µL**	**10 µL**

* 10X Y-27632: See page 4, section "Materials Required but not Provided"
** Cat # CY-E1160-1: See page 4, section "Materials Required but not Provided"

1. Following the above table, add the Reagents to each well of the microplate. Finally, initiate reaction by adding 10 µL of "Diluted CycLex Rho-kinase" to each well and mixing thoroughly at room temperature. Cover with plate sealer. Incubate **at 30°C for 30 minutes**.

2. Follow the **Standard Assay**, steps 5-10, page 6-7.

Special considerations when measuring precise Rho-kinase activity

In order to measure the activity of Rho-kinase family correctly, it is necessary to conduct the control experiment of "Inhibitor control" at least once for every experiment and "ATP minus control" at least once for the first experiment, in addition to "No enzyme control" as indicated in the following table. Although the level of A450 increases in "Test sample" when Rho-kinase family enzyme activity is in the sample, the high level of A450 is not observed in "Inhibitor control", "ATP minus control" and "No enzyme control".

Assay reagents	Test Sample	Inhibitor control	ATP minus control	Positive control	No enzyme control
Kinase Reaction Buffer	**90 µL**	**80 µL**	**-**	**90 µL**	**90 µL**
Kinase Buffer (provided)	**-**	**-**	**90 µL**	**-**	**-**
10X Y-27632 (100 µM)*	**-**	**10 µL**	**-**	**-**	**-**
Your enzyme fraction	**10 µL**	**10 µL**	**10 µL**	**-**	**-**
CycLex Rho-kinase Positive Control (1 m unit/µL)**	**-**	**-**	**-**	**10 µL**	**-**
Buffer	**-**	**-**	**-**	**-**	**10 µL**

* See page 4, section "Materials Required but not Provided"
** Cat # CY-E1160-1: See page 4, section "Materials Required but not Provided"

1. Following the above table, add the Reagents to each well of the microplate. Finally, initiate the reaction by adding 10 µL of "Your enzyme fraction" or "Buffer" to each well and mixing thoroughly at room temperature. Cover with plate sealer. Incubate **at 30°C for 30 minutes**.

2. Follow the **Standard Assay**, steps 5-10, page 6-7.

Evaluation of Results

1. Average the absorbance values for the Rho-kinase sample duplicates (positive control) and all experimental sample duplicate values (when applicable). When the Rho-kinase-II positive control (10 m units/assay) is included as an internal control for the phosphorylation reaction, the absorbance value should be greater than 1.0 with a background less than 0.15.

2. For screening of purification/chromatography fractions, on graph paper, plot the mean absorbance values for each of the samples on the Y-axis versus the fraction number on the X-axis to determine the location of the eluted, purified Rho-kinase.

3. For kinetic analysis, on graph paper, plot the mean absorbance values for each of the time points on the Y-axis versus the time of each reaction (minutes) on the X-axis.

Assay Characteristics

The CycLex Research Product **Rho-kinase Assay Kit** has been shown to detect the activity of Rho-kinase family in column fractions. The assay shows good linearity of sample response. The assay may be used to follow the purification of Rho-kinases or may be used to detect the presence of Rho-kinases in cell lysates.

It should be noted that this assay kit detects not only Rho-kinase activity but also other protein kinase activities, e.g. ZIP kinase, DMK and ILK, in crude extract and column sample in the absence of Rho-kinase inhibitor.

Troubleshooting

1. The Rho-kinase positive control should be run in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.

2. The reaction curve is nearly a straight line if the kinetics of the assay is of the first order. Variations in the protocol can lead to non-linearity of the curve, as can assay kinetics that are other than first order. For a non-linear curve, point to point or quadratic curve fit methods should be used.

3. Poor duplicates, accompanied by elevated values for wells containing no sample, indicate insufficient washing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for washer maintenance.

4. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in the CycLex Research Product **Rho-kinase Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, kit reagents should be stored at 4°C, except the ATP must be stored at -20°C. Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

For research use only, not for use in diagnostic or therapeutic procedures

Sample Preparation

Numerous extraction and purification methods can be used to isolate Rho-kinases. The following protocols have been shown to work with a number of different tissues and enzyme sources and are provided as examples of suitable methods. Crude samples can frequently be used without dilution while more concentrated or highly purified Rho-kinases should be diluted. It is strongly advised that the user always perform an initial experiment to determine the proper dilution to be used in subsequent experiments. This need not be any more than a single time point assay using serial dilutions of the crude extract, cell lysate or sample fraction taken prior to a purification step. One eight well strip of the substrate plate should be sufficient for this initial experiment. All sample preparation should be performed at 4°C and recovered fractions should be kept at 4°C to prevent loss of enzymatic activity.

CAUSION: It should be noted that this assay kit detects not only Rho-kinase activity but also other protein kinases e.g. ZIP kinase, DMK and ILK, in crude extract and column sample. You should trace Rho-kinase protein level by western blotting in column fractions.

Column Purification Fractions

1. Homogenize 5-8 g of fresh tissues (brain, kidney, platelet, etc.) in four volumes of an appropriate extraction buffer (for example; 50 mM Tris-HCl, pH 8.0, 0.1 % Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 1 µg/mL pepstatin, 0.5 µg/mL leupeptin, 10 mM NaF, 2 mM Na_3VO_4, 10 mM beta-mercaptoethanol) in a Potter-Elvehjem tissue grinder.

2. Centrifuge the homogenate for 30 min. at 30,000 x g to pellet the insoluble membrane/organelle fraction.

3. Apply the supernatant fraction to a 1 x 8 cm column of Q-Sepharose (Amersham Pharmacia Biotech) that has been equilibrated with Q-buffer (20 mM Tris-HCl, pH 8.0, 0.1 % Triton X-100, 0.5 mM EDTA, 1 mM EGTA, 1 µg/mL pepstatin, 0.5 µg/mL leupeptin, 5 mM beta-glycerophosphate, 2 mM NaF, 2 mM Na_3VO_4, 5 mM beta-mercaptoethanol) containing 50 mM NaCl.
.
4. Wash the column with five column volumes of Q-buffer containing 50 mM NaCl.

5. Elute the protein with a linear gradient of NaCl (0.05-0.6 M) in Q-buffer collecting 1-2 mL fractions. These samples are now ready for analysis according to the instructions provided in the **Detailed Protocol**. Collect the Rho-kinase containing fraction. Usually, Rho-kinase-activity is enriched in the 0.3 M NaCl eluent.

6. Dilute the pooled Rho-kinase containing fraction with three volumes of S-buffer (25 mM MES-NaOH, pH 6.0, 0.05 % Triton X-100, 0.5 mM EDTA, 1 mM EGTA, 1 µg/mL pepstatin, 0.5 µg/mL leupeptin, 2 mM NaF, 2 mM Na_3VO_4, 5 mM beta-mercaptoethanol, 5% glycerol).

7. Apply the diluted sample to a 1 x 2 cm column of S-Sepharose (Amersham Pharmacia Biotech) that has been equilibrated with S-buffer containing 50 mM NaCl.

8. Wash the column with ten column volumes of S-buffer containing 50 mM NaCl.

9. Elute the protein with a linear gradient of NaCl (0.05-0.6 M) in S-buffer collecting 1 mL fractions. These samples are now ready for analysis according to the instructions provided in the **Detailed Protocol**. Collect the Rho-kinase containing fraction. Usually, Rho-kinase-activity is enriched in the 0.2 M NaCl eluent.

Cell Culture Lysates

1. Harvest and pellet cells by centrifugation using standard methods.

2. Resuspend the cell pellet with an appropriate extraction buffer (for example; 50 mM Tris-HCl, pH 8.0, 0.1 % Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 1 µg/mL pepstatin, 0.5 µg/mL leupeptin, 2 mM NaF, 2 mM Na_3VO_4, 10 mM beta-mercaptoethanol) and lyse the resuspended cells using either a Dounce Homogenizer, sonication, or three cycles of freezing and thawing.

3. Transfer extracts to microcentrifuge tubes and centrifuge for 5 minutes.

4. Aliquot cleared lysate to a clean microfuge tube. These samples are now ready for analysis according to the instructions provided in the **Detailed Protocol**.

NOTE: THE ABOVE PROCEDURES ARE INTENDED ONLY AS A GUIDELINE. THE OPTIMAL EXPERIMENTAL CONDITIONS WILL VARY DEPENDING ON THE PARAMETERS BEING INVESTIGATED, AND MUST BE DETERMINED BY THE INDIVIDUAL USER. NO WARRANTY OR GUARANTEE OF PERFORMANCE USING THESE PROCEDURES IS MADE OR IMPLIED.

Example of Test Results

Fig.1-1 Dose dependency of Rho kinase-II catalytic domain enzyme reaction

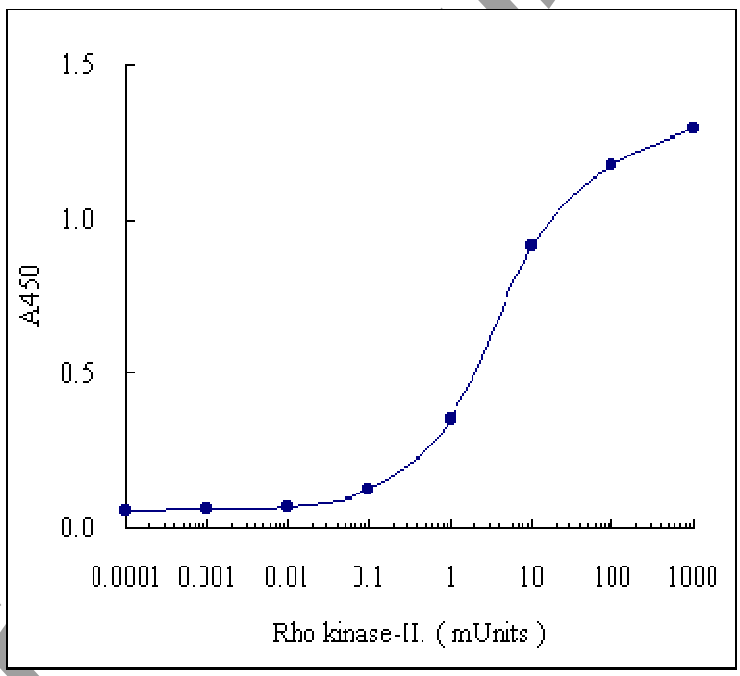


Fig.1-2 Dose dependency of recombinant DMPK enzyme reaction

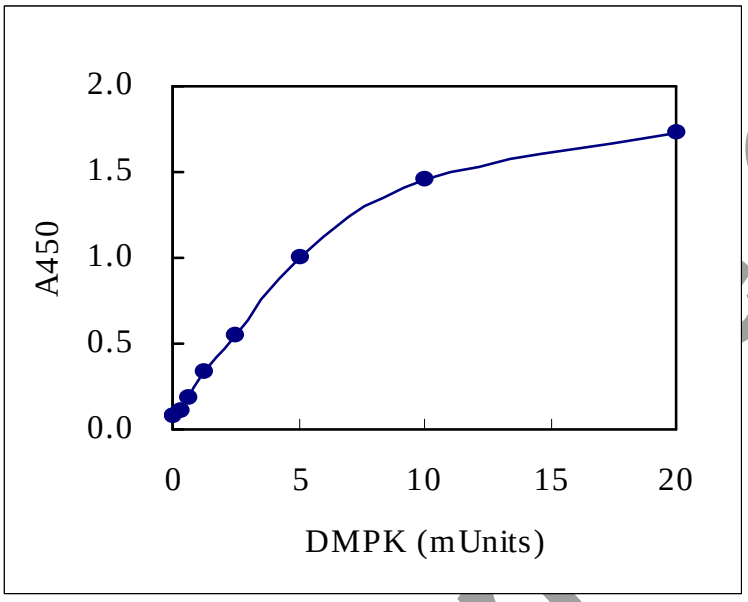


Fig.2-1 Time course of recombinant Rho kinase-II catalytic domain enzyme reaction

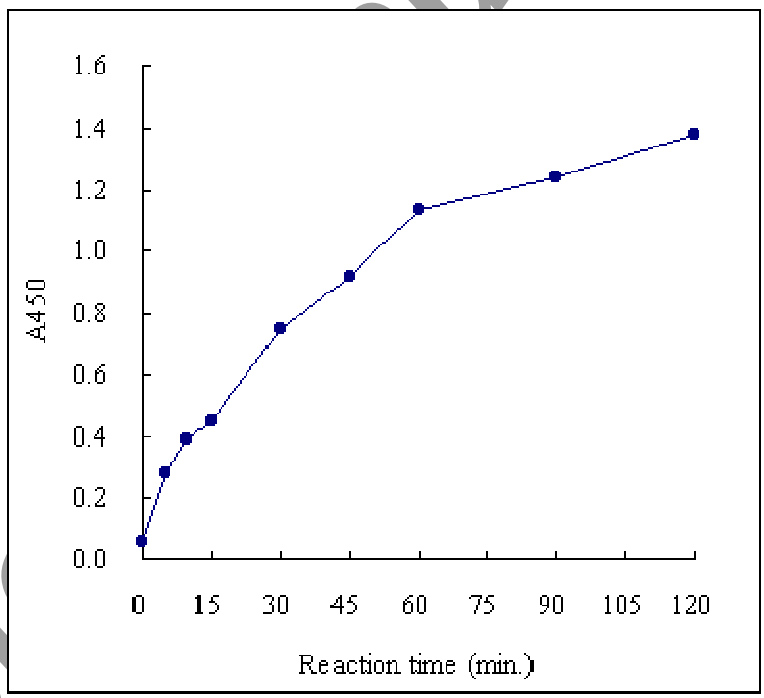


Fig.2-2 Time course of recombinant DMPK enzyme reaction

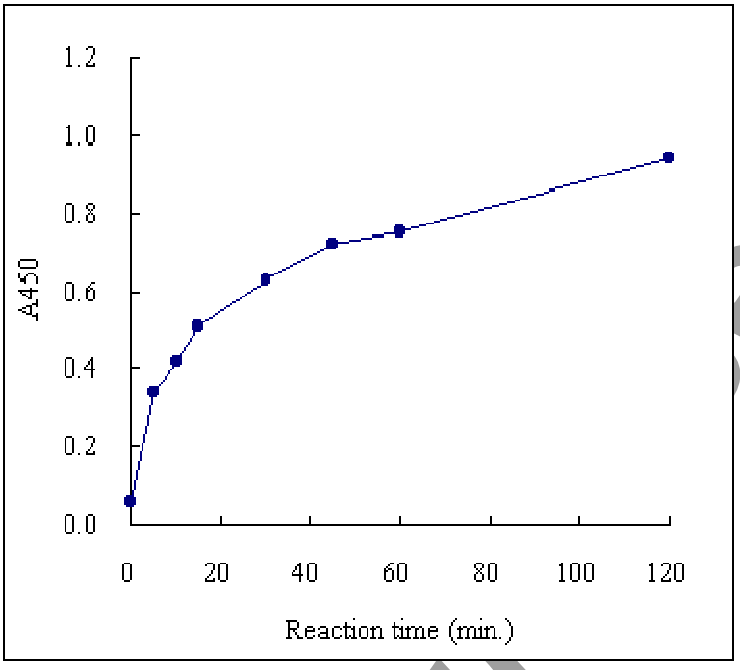


Fig.3-1 Km for ATP (recombinant Rho kinase-II catalytic domain)

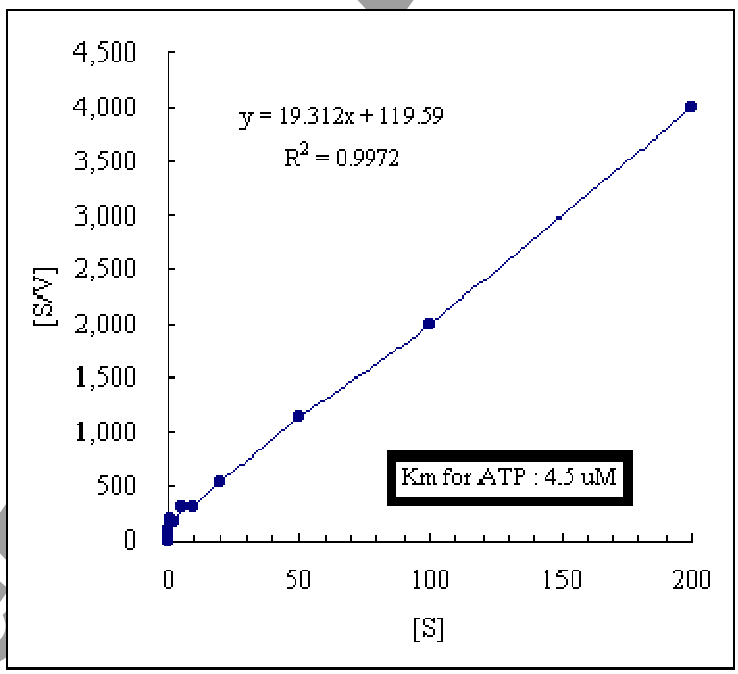


Fig.3-2 Km for ATP (recombinant DMPK)

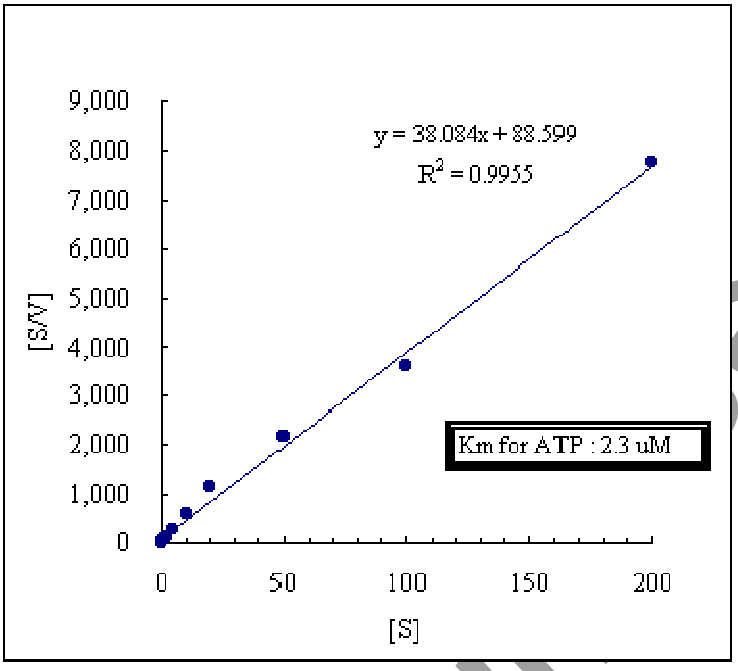


Fig.4-1 Effect of Rho-kinase specific inhibitor Y27632 on activity of recombinant ROCK II catalytic domain

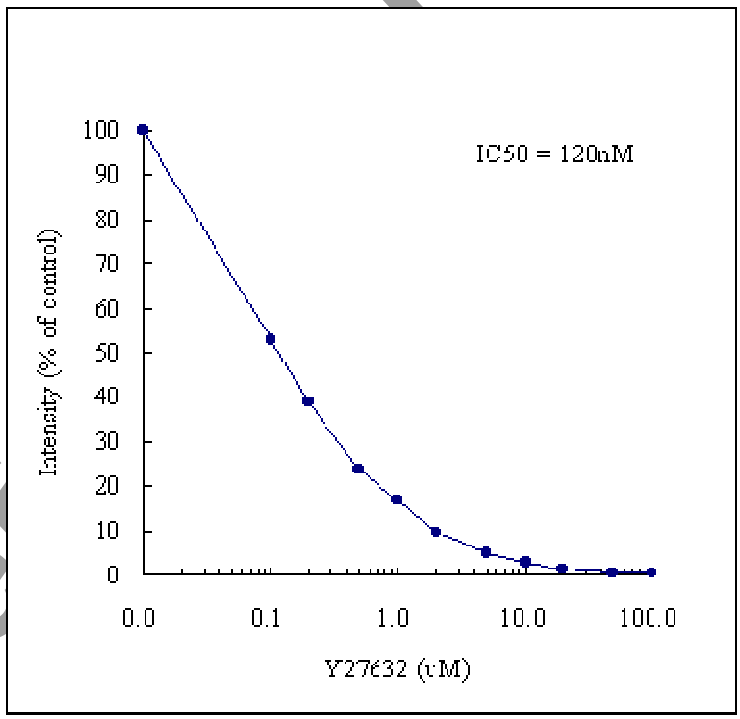


Fig.4-2 Effect of Rho-kinase specific inhibitor Y27632 on activity of recombinant DMPK

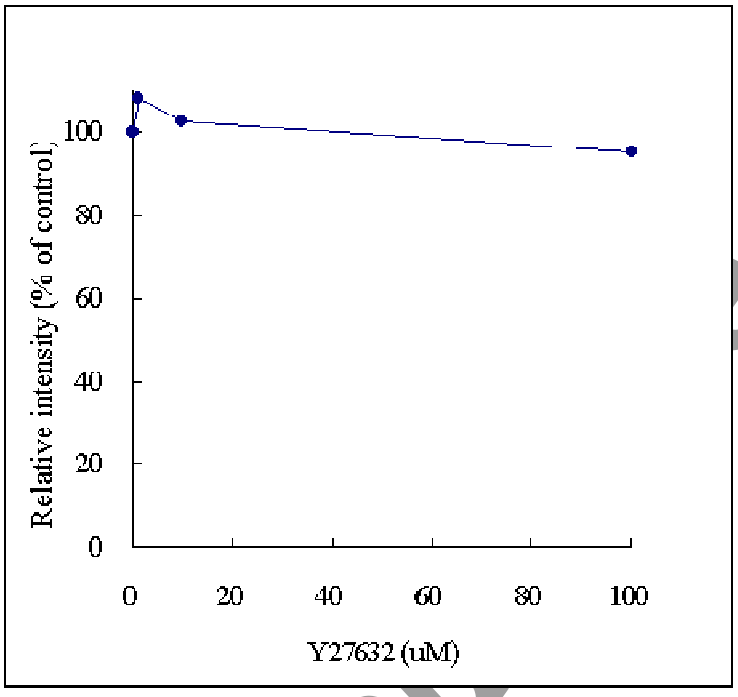


Fig.5 RESOURCE Q column elution profile of Rho-kinase activity (Rabbit Brain crude extract)

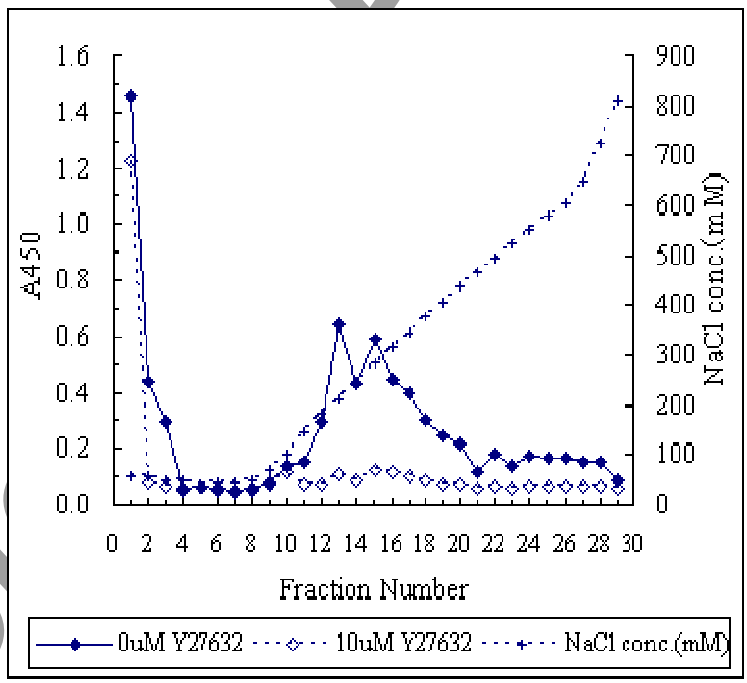
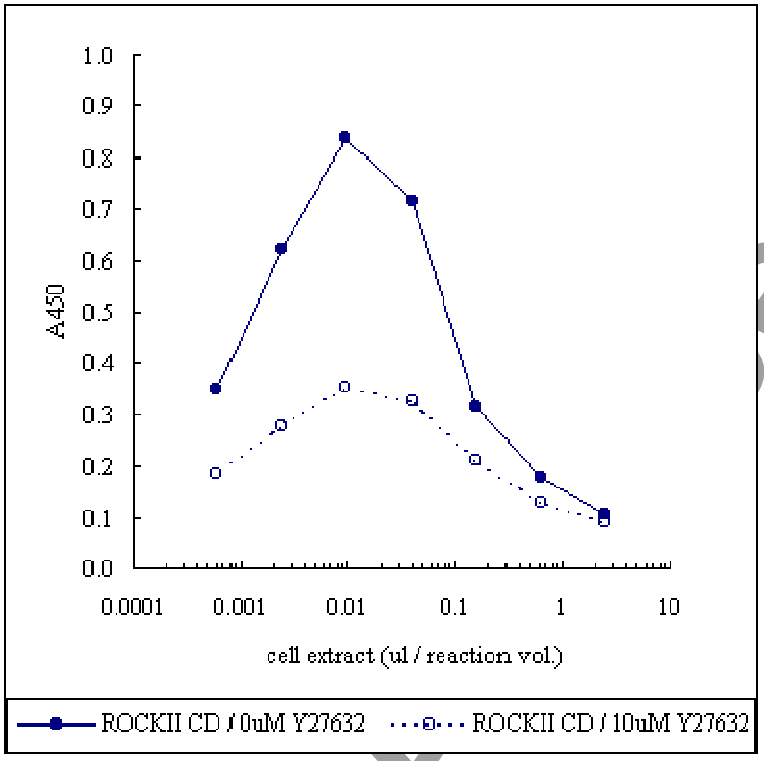


Fig.6 Measurment of Rho-kinase II activity in cell extract of 293T which had been transfected with Rho-kinase-II catalytic domain expression plasmid DNA.



References

1. Ishizaki, T., Mackawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakisuka, A., Morii, N. and Narumiya, S. *EMBO J.* **15**: 1885-1893, 1996.

2. Fujisawa, K., Fujita, A., Ishizaki, T., Saito, Y. and Narumiya, S. *J. Biol. Chem.* **271**: 23022-23028, 1996.

3. Nakamura, M., Nagano, T., Chikama, T. and Nishida, T. Invest. Ophthal. *Vis. Sci.* **42**: 941-947, 2001.

4. Rao, P. V., Deng, P.-F., Kumar, J. and Epstein, D. L. Invest. Ophthal. *Vis. Sci.* **42**: 1029-1037, 2001.

5. Muranyi, A, Zhang R, Liu F, Hirano K, Ito M, Epstein HF, Hartshorne DJ. Myotonic dystrophy protein kinase phosphorylates the myosin phosphatase targeting subunit and inhibits myosin phosphatase activity. *FEBS Lett.* **493**(2-3):80-4, 2001.

6. Koyama M, Ito M, Feng J, Seko T, Shiraki K, Takase K, Hartshorne DJ, Nakano T. Phosphorylation of CPI-17, an inhibitory phosphoprotein of smooth muscle myosin phosphatase, by Rho-kinase. *FEBS Lett.* **475**(3):197-200, 2000.

7. Hamaguchi T, Ito M, Feng J, Seko T, Koyama M, Machida H, Takase K, Amano M, Kaibuchi K, Hartshorne DJ, Nakano T. Phosphorylation of CPI-17, an inhibitor of myosin phosphatase, by protein

kinase N. Biochem. *Biophys. Res. Commun.* 274(3):825-30, 2000.

8. MacDonald JA, Borman MA, Muranyi A, Somlyo AV, Hartshorne DJ, Haystead TA. Identification of the endogenous smooth muscle myosin phosphatase-associated kinase. *Proc. Natl. Acad. Sci. USA.* **98**(5):2419-24, 2001.

9. Niiro N and Ikebe M. Zipper-interacting protein kinase induces Ca(2+)-free smooth muscle contraction via myosin light chain phosphorylation. *J Biol Chem.* **276**(31):29567-74, 2001.

10. Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase) *Science.* **273**(5272):245-8, 1996.

11. Kawano Y, Fukata Y, Oshiro N, Amano M, Nakamura T, Ito M, Matsumura F, Inagaki M, Kaibuchi K. Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase in vivo. *J Cell Biol.* **147**(5):1023-38, 1999.

12. Nagumo H, Sasaki Y, Ono Y, Okamoto H, Seto M, Takuwa Y. Rho kinase inhibitor HA-1077 prevents Rho-mediated myosin phosphatase inhibition in smooth muscle cells. *Am. J. Physiol. Cell Physiol.* **278**(1):C57-65, 2000.

13. Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J.* **15**(9):2208-16, 1996.

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