Non-Radioisotopic Kit for Measuring IKK activity

CycLex IKK α and β Assay/Inhibitor Screening Kit

Cat# CY-1178

Intended Use

The CycLex Research Product **CycLex IKK α and β Assay/Inhibitor Screening Kit** designed to measure the activities of purified IKKα and IKKβ for the rapid and sensitive evaluation of inhibitors or activators. The sequence-specific phosphoserine monoclonal antibody used in this assay kit has been demonstrated to recognize the phospho-serine 32 in recombinant IκBα, which is efficiently phosphorylated by IKKs *in vitro* and *in vivo*.

Applications of this kit include:
1) Screening inhibitors or activators of IKKα and IKKβ.
2) Detecting the effects of pharmacological agents on IKKα and IKKβ activity.

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

Although a number of recent studies suggest that IκB degradation and nuclear translocation of NF-κB may not be the sole regulatory events in the transcription of NF-κB-dependent genes (1–3), it has been proposed that there is the central dogma of NF-κB activation, which suggests that NF-κB is sequestered in the cytoplasm in resting cells by the inhibitory IκB proteins (4–7). In response to a variety of agonists, IκB is rapidly phosphorylated, ubiquitinated, and degraded, thus releasing NF-κB for translocation into the nucleus to initiate gene transcription (9–11). IκB kinase (IKK) is the convergence point in most signaling pathways activated by many stimuli leading to the inducible phosphorylation and degradation of IκB. IKK is a multisubunit complex that contains two catalytic subunits, IKKα and IKKβ, and the regulatory subunit IKKγ (8, 12-17). Gene knock out studies have clearly demonstrated that IKKβ and IKKγ subunits of the IKK complex are required for NF-κB activation by all known pro-inflammatory stimuli including lipopolysaccharide (LPS), TNF, and IL-1 (18, 19). Thus a selective inhibitor of IKKβ would not only be of great interest as a potential anti-inflammatory agent but also as a valuable tool to understand the mechanisms regulating NF-κB activation by these inflammatory agonists.

Peters et al. (2000) reported the identification of a novel PMA-inducible IκB kinase complex. They characterized one kinase from this complex, which they designated IKKε (20). The IKKε protein shows 33% and 31% amino acid identity with IKKα and IKKβ, respectively, within the kinase domain and 27% amino acid identity with each throughout the entire sequence. Although recombinant IKKε directly phosphorylates only Ser36 of IκBα, the PMA-activated endogenous IKK complex phosphorylates both critical serine (Ser32 and Ser36) residues. Remarkably, this activity appears to be due to the presence of a distinct kinase in this complex. A dominant-negative mutant of IKKε (Lys38 to Ala) blocks induction of NF-κB by both PMA and activation of the T-cell receptor but has no effect on the activation of NF-κB by TNF or IL1. These observations indicate that the activation of NF-κB requires multiple distinct IκB complexes that respond to both overlapping and discrete signaling pathways.

Measurement of IKK activity

The protocol generally regarded as most sensitive for the quantitative measurement of IKKs including IKKα, IKKβ and IKKε activity involves incubation of the IKK sample with substrate, either a natural or synthetic polypeptide (such as IκBα-S32/S36 peptide; KKKERLLDDRHDSGLDSMKDEEYE), in the presence of Mg^{2+} and ^{32}P-labeled ATP. The reaction is terminated by "spotting" a sample onto a phosphocellulose P81 filter paper disc, followed by washing extensively to remove unincorporated radiolabel and the incorporated radioactivity on P81 filter 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 IKK α and β Assay/Inhibitor Screening Kit** uses a peroxidase coupled anti-phospho- IκBα S32 monoclonal antibody as a reporter molecule in a 96-well ELISA format. This assay provides a non-isotopic, sensitive and specific method to detect IKK activity.

Principle of the Assay

The CycLex Research Product **CycLex IKK α and β Assay/Inhibitor Screening Kit** is a single-site, semi-quantitative immunoassay for activities of IKKα and IKKβ. Plates are pre-coated with a substrate corresponding to recombinant IκBα, which contains two serine residues that are phosphorylated by IKKα and IKKβ (IκB kinases).

The detector antibody specifically detects only the phosphorylated form of IκBα. The CycLex Research Product **CycLex IKK α and β Assay/Inhibitor Screening Kit** can be used to study the kinetics of a purified or partially purified IKK as well as to screening these kinases 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 AS-2E8, an anti-phospho- IκBα S32 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 IKK activity in the sample. For kinetic analysis, the sample containing IKK 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 IKK α and β Assay/Inhibitor Screening Kit** is designed to accurately determine the presence and relative amount of IKKα and β activities to determine non-isotopic kinetic analysis of IKKα and β activities.

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 Anti-phospho- IκBα serine32 monoclonal antibody (AS-2E8).
↓ Incubate for 30 min at room temp.
Wash the wells
↓
Add 100 µL of HRP conjugated anti-mouse IgG
↓ 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 IκBα as substrate of IKK.

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: Lyophilized ATP Na_2 salt.

Anti-Phospho-IκBα S32 Monoclonal Antibody (AS-2E8): One vial containing 12 mL of anti-phospho-IκBα S32 monoclonal antibody (AS-2E8). Ready to use.

HRP conjugated Anti-mouse IgG: One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-mouse IgG. 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

- **IKK positive control:** Available from CycLex (IKKβ positive control: Cat # CY-E1178-2), One vial contains 2 units/400 µL IKKβ enzyme. Positive control should be added to the first well at 10 m units/well. For instance, diluted positive control 1:5, use 10 µL for 1 assay. (Unused IKKβ enzyme should be stored in aliquots at below -70°C.)
- **10X K252a (100 µM):** K252a is available from Wako, Cat#. 1683. 10 mM stock solution (DMSO) diluted 1:100 in Kinase Buffer.
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1000 µL precision pipettors with disposable tips.
- **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 used in this kit contain Kathon-CG as preservatives. 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 Research Product **CycLex IKK α and β Assay/Inhibitor Screening 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 IKKβ (Cat # CY-E1176-2) 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 **1.6 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 **1.25 mM**. Store the solution in small aliquots (e.g. 100 µL) at -20°C.

3. Prepare **Kinase Reaction Buffer (ATP plus)** by mixing following reagents.

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

You will need 80-90 µL of Kinase Reaction Buffer (ATP plus) per assay well. Mix well. Discard any unused Kinase Reaction Buffer (ATP plus) 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 partially purified recombinant IKK, add **10 µL** of **each fraction** to the wells of the assay plate on ice. Duplicate wells containing 10 m units/10 µL IKKβ positive control (Cat # CY-E1176-2) 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 four 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 **Anti-Phospho-IκBα S32 Monoclonal Antibody** AS-2E8 into each well, cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 30 minutes**. Discard any

unused antibody after use.

7. Wash wells five times as same as in step 5.

8. Pipette **100 µL** of **HRP-conjugated Anti-mouse IgG** into each well, cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 30 minutes**. Discard any unused conjugate after use.

9. Wash wells five times as same as in step 5.

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

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

12. 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 IKK positive control.

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the IKKβ positive control, perform a second reading at 405 nm. A new O.D. values, measured at 405 nm, is used to determine IKK 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 partially purified recombinant IKKβ, add **10 µL** of **each fraction** to the wells of the assay plate on ice. Duplicate wells containing 10 m units/10 µL IKKβ positive control (Cat # CY-E1176-2) 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 **Anti-Phospho-IκBα S32 Monoclonal Antibody** AS-2E8 into each well, cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 30 minutes**. Discard any unused antibody after use.

8. Wash wells five times as same as in step 6.

9. Pipette **100 µL of HRP-conjugated Anti-mouse IgG** into each well, cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 30 minutes**. Discard any unused conjugate after use.

10. Wash wells five times as same as in step 6.

11. Add **100 µL** of **Substrate Reagent** to each well and incubate **at room temperature for 10-15 minutes**.

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

13. 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 or inhibitors

In order to estimate the inhibitory effect on IKK 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 IKK activity, the level of A450 is weakened as compared with "Solvent control".

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 K252a (100 µM)*	**-**	**-**	**10 µL**
IKK Positive Control (1 m unit/µL) or your enzyme fraction**	**10 µL**	**10 µL**	**10 µL**

* 10X K252a (100 µM): See page 4, section "Materials Required but not Provided"
** Cat # CY-E1176-2: 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 IKK positive control" 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 5-12, page 6-7.

Special considerations when measuring precise IKK activity

In order to measure the activity of IKK 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 IKK 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 (ATP minus)	**-**	**-**	**90 µL**	**-**	**-**
10X K252a (100 µM)*	**-**	**10 µL**	**-**	**-**	**-**
Your enzyme fraction	**10 µL**	**10 µL**	**10 µL**	**-**	**-**
IKK Positive Control (1 m unit/µL)**	**-**	**-**	**-**	**10 µL**	**-**
Buffer	**-**	**-**	**-**	**-**	**10 µL**

* 10X K252a (100 µM): See page 4, section "Materials Required but not Provided"
** Cat # CY-E1176-2: 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 5-12, page 6-7.

Evaluation of Results

1. Average the absorbance values for the IKK sample duplicates (positive control) and all experimental sample duplicate values (when applicable). When the IKK 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 0.2.

2. For screening of purification/chromatography fractions of recombinant IKK, 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 IKK.

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 **CycLex IKK α and β Assay/Inhibitor Screening Kit** has been shown to detect the activity of IKK in column fractions of recombinant IKK. The assay shows good linearity of sample response. The assay may be used to follow the purification of recombinant IKK.

Troubleshooting

1. The IKK 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 **CycLex IKK α and β Assay/Inhibitor Screening 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

Example of Test Results

Fig.1 Dose dependency of recombinant IKKβ enzyme reaction

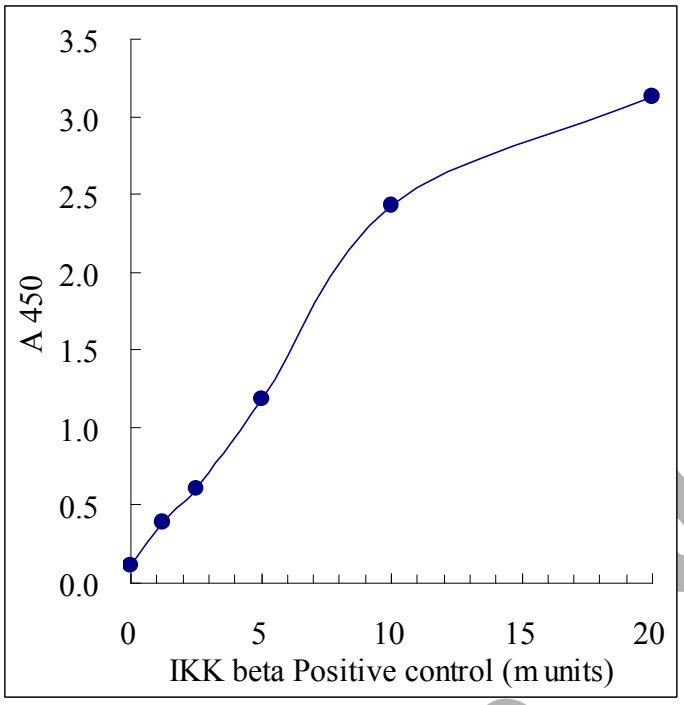


Fig.2 Time course of recombinant IKKβ enzyme reaction

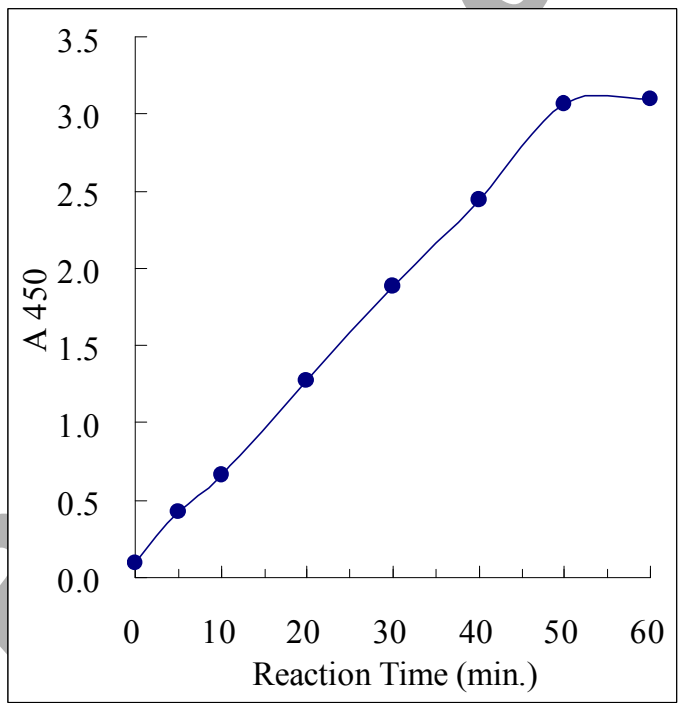


Fig.3 Dose dependency of ATP

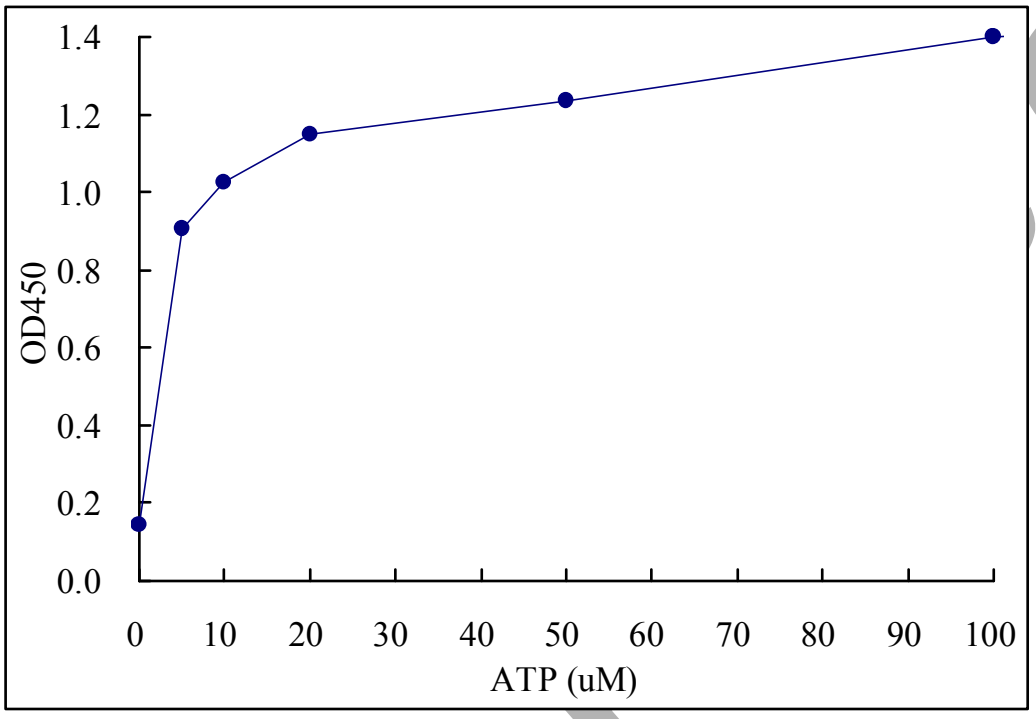


Fig.4 Km for ATP

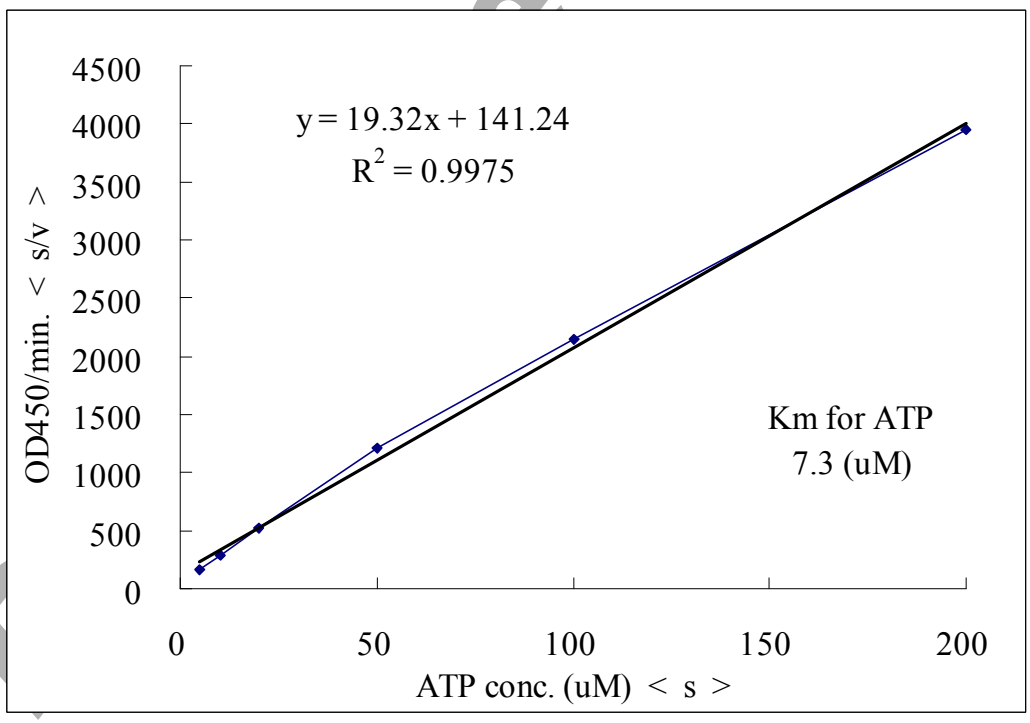
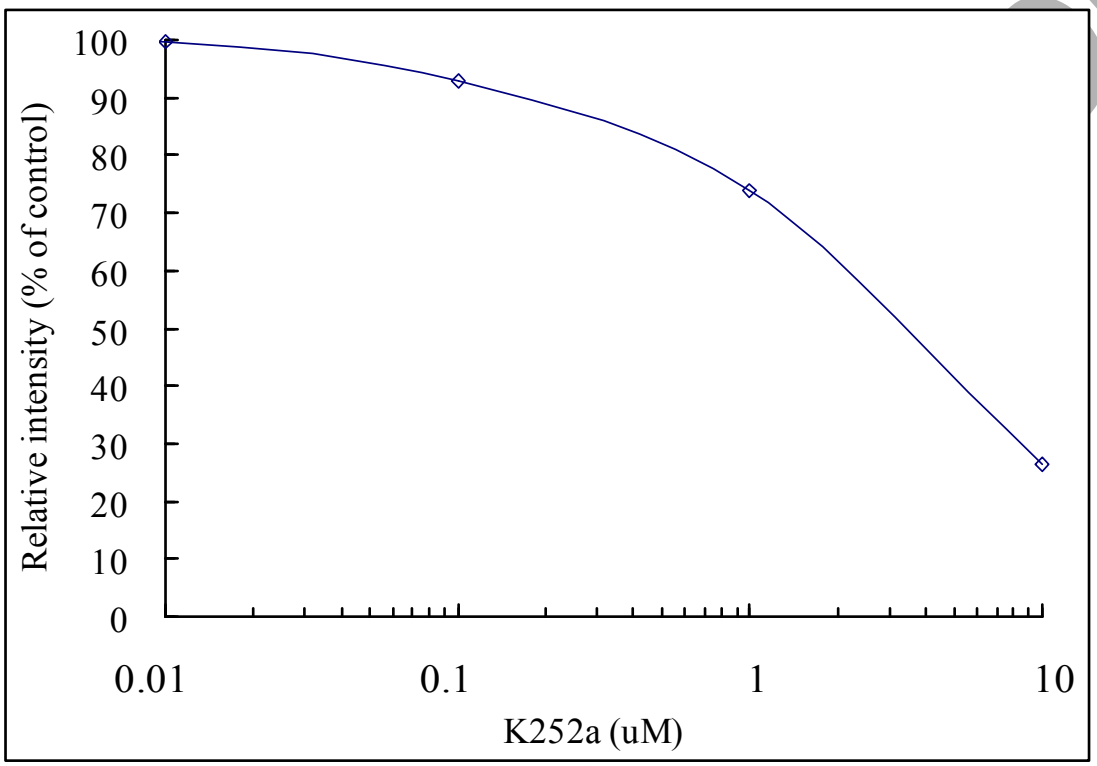


Fig.5 Effect of broad-spectrum kinase inhibitor, K252a on activity of recombinant IKKβ



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Related Products

***IKKβ Positive control: Cat# CY-E1178-2**

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