ELISA Kit for Measuring Human Phospho-PKR Thr451

CycLex Phospho-PKR Thr451 ELISA Kit

Cat# CY-7054

Intended Use

The CycLex Research Product **CycLex Phospho-PKR Thr451 ELISA Kit** is designed to detect and quantify the level of human PKR protein phosphorylated at threonine 451 (phospho-PKR Thr451). Since the amino acid sequence surrounding threonine 451 is not conserved in mouse and rat PKR, this ELISA kit can not be used for mouse and rat cells.

This assay is intended for the measurement of phospho-PKR Thr451 in lysates from human cells.

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 double-stranded RNA-activated protein kinase, PKR, is a ubiquitously expressed serine/threonine protein kinase that plays a key role in the innate immunity response to viral infection in higher eukaryotes and has also been implicated in several cellular signal transduction pathways (1-4).

The dsRNAs-mediated activation leads to autophosphorylation of PKR and allows the kinase to phosphorylate its natural substrate, the α subunit of initiation factor eIF2, resulting in rapid inhibition of translation and suppression of virus spread (5, 6). PKR also has been implicated in regulating other cellular functions such as differentiation (7), transcription (8, 9), signal transduction (10), cell growth (11, 12) and apoptosis in the event of virus infection and other forms of cellular stress (13-15).

It was reported that the activation of PKR in adipose and liver tissue is caused by obesity (16). In the absence of PKR, metabolic deterioration due to excess energy or nutrition is alleviated. These findings demonstrate that PKR is an important component of inflammation complex that responds to nutrients and organelle dysfunction.

The phosphorylation status of PKR at threonine 451 is shown to be a nice marker of the activation of PKR in vitro as well as in vivo.

Principle of the Assay

The CycLex Research CycLex Phospho-PKR Thr451 ELISA Kit is a solid phase sandwich ELISA. An antibody specific for human PKR has been coated onto the wells of the microtiter strips provided. Samples and phospho-PKR Thr451 Standards are pipetted into these wells. During the first incubation, PKR protein binds to the capture antibody on the well. After washing away any unbound substances, an HRP conjugated antibody specific for phospho-PKR Thr451 is added to the wells. During the second incubation, this antibody serves as a detector by binding to the immobilized phospho-PKR Thr451 captured during the first incubation. Following a wash to remove any unbound HRP conjugate antibody, the remaining conjugate is allowed to react with the substrate H_2O_2-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is proportional to the concentration of phospho-PKR Thr451. A standard curve is constructed by plotting absorbance values versus phospho-PKR Thr451 concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

Summary of Procedure

Culture cells in culture flask or dish at 50-70 % confluency

↓ Incubate O/N at 37°C in CO_2 incubator

Add appropriate amount of the drug for induction of PKR Thr451 phosphorylation

↓ Incubate for appropriate time at 37°C in CO_2 incubator

Harvest the cells by scraping and centrifugation

↓

Make cell lysate by extraction with cell extraction buffer and centrifugation

↓

Add 100 µL of diluted cell lysate to the wells

↓ Incubate for 1 hour at room temp.

Wash the wells

↓

Add 100 µL of HRP conjugated anti-phospho-PKR Thr451 antibody

↓ Incubate for 1hour at room temp.

Wash the wells

↓

Add 100 µL of Substrate Reagent

↓ Incubate for 5-10 minutes at room temp.

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 microplate 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 anti-human PKR antibody as a capture antibody.

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

Cell Extraction Buffer: One bottle containing 20 mL of buffer

Dilution Buffer: One bottle containing 50 mL of 1X buffer; use for sample and standard dilution. Ready to use.

Phospho-PKR Thr451 Standard: One vial containing 250 units of lyophilized phospho-PKR Thr451.

50X Phosphatase Inhibitors: One vial of lyophilized phosphatase inhibitor mix.

HRP conjugated Detection Antibody: One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-phospho-PKR Thr451 antibody. Ready to use.

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

Stop Solution: One bottle supplied ready to use, containing 20 mL of 1 N H_2SO_4.

Materials Required but not Provided

- **(Optional) Calyculin A:** An inhibitor of protein phosphatases, e.g. available from Cell Signaling, Cat# 9902S.
- **(Optional) Polyinosinic:polycytidylic acid (poly I:C):** A viral mimic immunostimulant, e.g. available from SIGMA, Cat# P1530.
- **Orbital microplate shaker**
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1000 µL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **(Optional) Microplate washer:** Manual washing is possible but not preferable
- **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.
- **(Optional) Software package facilitating data generation and analysis**
- **500 or 1000 mL graduated cylinder**
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

Precautions and Recommendations

- 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 pipette 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 the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.
- Wear gloves and eye protection when handling immunodiagnostic materials and samples of human origin, and these reagents. 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 Phospho-PKR Thr451 ELISA 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 Phospho-PKR Standard within the kit, should be included in each assay as a calibrator. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Working Solutions

All reagents need to be brought to room temperature prior to the assay. Assay reagents are supplied ready-to-use, with the exception of **10X Wash Buffer**, **50X Phosphatase Inhibitors, Cell Extraction Buffer** and **Phospho-PKR Thr451 Standard**.

1. Prepare a working solution of Wash Buffer by adding 100 mL of the **10X Wash Buffer** to 900 mL of deionized (distilled) water (ddH_2O). Mix well.

2. Reconstitute **50X Phosphatase Inhibitor Solution** by adding **1.0 mL** of ddH_2O to the vial of **50X Phosphatase Inhibitors** (provided, lyophilized). Mix well until dissolved. Store the solution in small aliquots (e.g. 200 µL) at -20°C.

3. Prepare **Complete Cell Extraction Buffer**, which contains phosphatase inhibitors, by adding **100 µL** of **50X Phosphatase Inhibitor Solution** to **4.9 mL** of **Cell Extraction Buffer**. Mix well.

4. Reconstitute **Phospho-PKR Thr451 Standard** with **0.5 mL** of **ddH_2O**. The concentration of the phospho-PKR Thr451 in vial should be **500 units/mL**, which is referred as a **Master Standard** of phospho-PKR Thr451.

Prepare Standard Solutions as follows:

Use the **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 100 units/mL standard (Std.1) serves as the highest standard. The **Dilution Buffer** serves as the zero standard (Blank).

	Volume of Standard	Dilution Buffer	Concentration
Std.1	100 µL of Master Standard (500 units/mL)	400 µL	100 units/mL
Std.2	250 µL of Std. 1 (100 units/mL)	250 µL	50 units/mL
Std.3	250 µL of Std. 2 (50 units/mL)	250 µL	25 units/mL
Std.4	250 µL of Std. 3 (25 units/mL)	250 µL	12.5 units/mL
Std.5	250 µL of Std. 4 (12.5 units/mL)	250 µL	6.25 units/mL
Std.6	250 µL of Std. 5 (6.25 units/mL)	250 µL	3.13 units/mL
Std.7	250 µL of Std. 6 (3.13 units/mL)	250 µL	1.56 units/mL
Blank	-	250 µL	0 units/mL

Note: Do not use a Repeating pipette. Change tips for every dilution. Wet tip with Dilution Buffer before dispensing. Unused portions of Standards should be aliquoted and stored at below -70°C immediately. Avoid multiple freeze and thaw cycles.

Assay Procedure

A. Treatment of Cells with Compounds

1. Plate adherent cells or non-adherent cells in culture flasks at 50-70 % confluency.

2. Incubate the culture flasks at 37°C over night in CO_2 incubator.

3. Add appropriate amount of test compounds to each flask.

4. Incubate the culture flasks at 37°C for appropriate time.

B. Cell Extraction

Note: This protocol has been successfully applied to several cell lines. Users should optimize the cell extraction procedure for their own applications.

1. Collect cells by centrifugation (non-adherent cells) or scraping from culture flasks (adherent cells).

2. Wash cells twice with cold PBS.

3. Remove and discard the supernatant and collect the cell pellet. At this point the cell pellet can be frozen at below -70°C and lyse at a later date.

4. Lyse the cell pellet in **Complete Cell Extraction Buffer*** with vortexing at 10-minute intervals for 150 minutes at 4°C.

   * *The volume of Complete Cell Extraction Buffer depends on the cell number in cell pellet and phosphorylation level of PKR. For example, when 1.0 x 10^7 of HepG2 cells (in 10 cm dish) is extracted in 1.5 mL of Complete Cell Extraction Buffer, the protein concentration will be 2-4 mg/mL.*

5. Transfer the lysates to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4°C.

6. Aliquot the clear lysate to clean microfuge tubes. These samples are ready for assay. The lysates can be stored at below -70°C. Avoid multiple freeze/thaw cycles.

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.

C. ELISA

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. Dilute samples* with **Dilution Buffer**.

   * *Lysates prepared in Complete Cell Extraction Buffer must be diluted 1:5 (e.g. 60 µL sample + 240 µL Dilution Buffer) or greater with Dilution Buffer. While a 1:5 lysate dilution has been found to be satisfactory, higher dilutions such as 1:10 or 1:20 may be optimal. The dilution chosen should be optimized for each experimental system.*

3. Pipette **100 µL** of **Standard Solutions (Std1-Std7, Blank)** and the **diluted lysate** in duplicates, into the appropriate wells.

4. Incubate the plate **at room temperature (ca. 25°C) for 1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.

5. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

6. Add **100 µL** of **HRP conjugated Detection Antibody** into each well.

7. Incubate the plate **at room temperature (ca. 25°C) for 1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.

8. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

9. Add **100 µL** of **Substrate Reagent** to each well and incubate **at room temperature (ca.25°C) for 5–15 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker.

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 microplate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the microplate 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 standard curves are obtained when either O.D. values do not exceed 0.2 units for the blank (zero concentration), or 2.5 units for the highest standard concentration. The plate should be monitored at 5-minute intervals for approximately 30 minutes.

    Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine Phospho-PKR Thr451 concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

Calculations

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation. To determine the phospho-PKR Thr451 concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding phospho-PKR Thr451 concentration. If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

1. The dose-response curve of this assay fits best to a sigmoidal 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 5-parameter logistic function. It is important to make an appropriate mathematical adjustment to accommodate for the dilution factor.

2. Most microtiter plate readers perform automatic calculations of analyte concentration. The calibration curve is constructed by plotting the absorbance (Y) of calibrators versus log of the known concentration (X) of calibrators, using the four-parameter function. Alternatively, the logit log function can be used to linearize the calibration curve (i.e. logit of absorbance (Y) is plotted versus log of the known concentration (X) of calibrators).

Measurement Range

The measurement range is 1.56 units/mL to 100 units/mL. Any sample reading higher than the highest standard should be diluted with Dilution Buffer to higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the phospho-PKR Thr451 concentration.

Troubleshooting

1. All samples and controls should be assayed in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.

2. 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.

3. 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 Phospho-PKR Thr451 ELISA 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 reconstituted Human Phospho-PKR Thr451 Standard must be stored at below -70°C. Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of phospho-PKR Thr451 giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 0.505 units/mL of sample.

** Dilution Buffer is pipetted into blank wells.*

2. Specificity

The HRP conjugated Detection Antibody is highly specific of human phospho-PKR Thr451, with no detectable cross-reactivity to mouse or rat phospho-PKR.

3. Linearity

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of phospho-PKR Thr451 were serially diluted with the Dilution Buffer to produce samples with values within the dynamic range of the assay.

Example of Test Results

Fig.1 Typical curve of phospho-PKR Thr451 standard

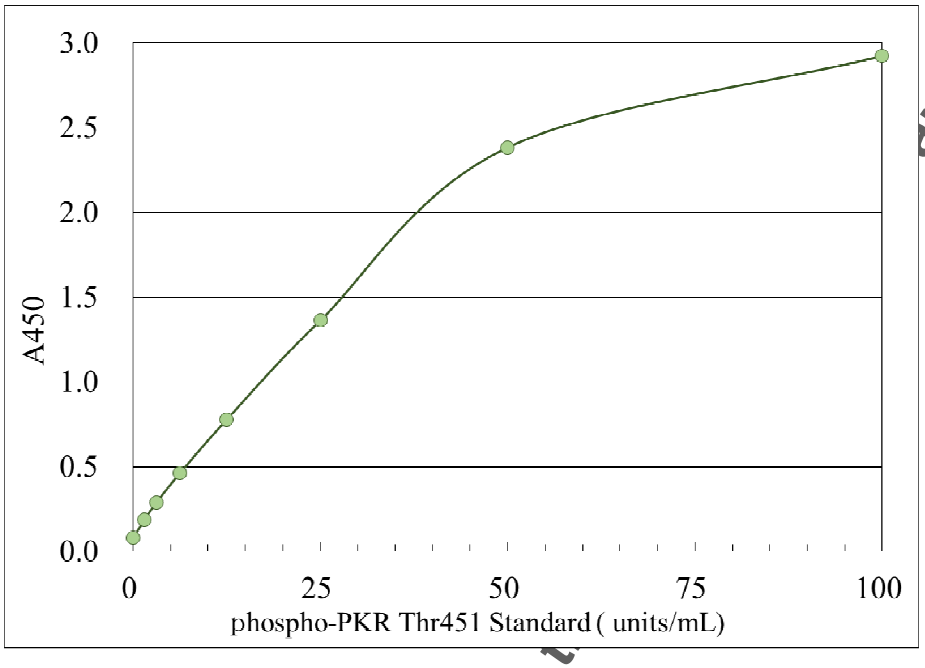
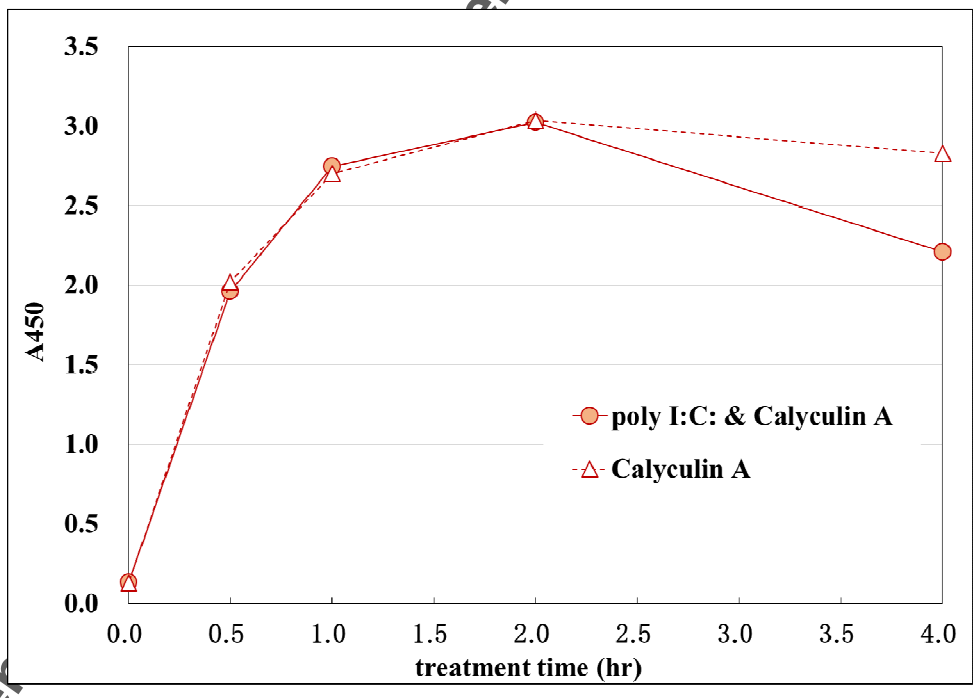


Fig.2 Measurement of phospho-PKR Thr451in HepG2 cell lysates treated with 10 µg/mL poly I:C & 100 nM Calyculin A or 100 nM Calyculin A only for indicated time



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Related Products

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*** CycLex Phospho-PKR Thr451 ELISA Kit: Cat#CY-7054**
*** Anti-Human PKR/EIF2AK2 Polyclonal Antibody: Cat#CY-P1043**
*** PKR/EIF2AK2 Positive Control (Full length): Cat#CY-E1184-1**
*** PKR/EIF2AK2 (Human), Active: Cat#CY-SPP80**

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