



ELISA Kit for Measuring Phosphorylated Human p53 Ser46

CycLex Phospho-p53 Ser46 ELISA Kit

Cat# CY-7050

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Intended Use

The MBL Research Product **CycLex Phospho-p53 Ser46 ELISA Kit** is designed to detect and quantify the level of human p53 protein that is phosphorylated at serine 46. Since the amino acid sequence surrounding serine 46 is not conserved in mouse and rat p53, this ELISA kit can not be used for mouse and rat cells. This assay is intended for the detection of phosphorylated human p53 at serine 46 from cell lysate.

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

p53 is a short-lived, non-abundant protein that regulates the response of cells to DNA damage, in part through transcriptional activation of genes involved in cell cycle control, DNA repair, and apoptosis (1). p53 is a tumor suppressor protein; consequently, mice in which the p53 gene has been disrupted develop tumors with high frequency (2, 3), and deletions or mutations in the p53 gene are prevalent in a majority of human cancers (4, 5). The protein level and activity of p53 are regulated, and this is accomplished by MDM2 (murine double minute 2) (6–10). The MDM2 gene is induced by p53, and MDM2 prevents apoptosis by inhibiting p53 activity and promoting its degradation (6, 9, 11).

In response to DNA damage, the p53 protein is phosphorylated on each of the seven serines and one threonine in the first 50 amino acids of its N-terminal transactivation domain as well as at several sites in its C-terminal tetramerization/regulatory domain (12, 13). As a transcription factor, p53 induces or represses several genes that regulate cell cycle arrest, DNA repair or apoptosis, including p21WAF1, MDM2, GADD45, p53R2, and p53AIP1. Recent studies suggest that specific p53 phosphorylation events are important for the activation or repression of specific promoters (14-17).

It has been shown that the proline-rich domain (residues 64-92) and a transcriptional activation domain (residues 43-63) of p53 is necessary for mediation of apoptosis, because deletion of either of these two domains abolishes this activity. It was found that phosphorylation of Ser46 is induced after DNA damage in vivo. Particularly, phosphorylation of Ser46 was observed only at the late stage after DNA damage by several different agents compared to Ser15 or Ser20. Moreover, upon severe DNA damage by UV, Ser46 on p53 is phosphorylated and apoptosis is induced. In addition, substitution of Ser46 to Alanine inhibits the ability of p53 to induce apoptosis but not G1 arrest. These results suggested that phosphorylation of Ser46 is involved in induction of p53-dependent apoptosis.

Principle of the Assay

The MBL Research **CycLex Phospho-p53 Ser46 ELISA Kit** is a solid phase sandwich ELISA. An antibody specific for human p53 has been coated onto the wells of the microtiter strips provided. Samples, including a standard containing phosphorylated form of p53 at Ser46, control specimens, and unknowns, are pipetted into these wells. During the first incubation, p53 protein binds to the capture antibody on the well. After washing, mouse monoclonal antibody, specific for phosphorylated p53 Ser46 as a detection antibody, is added to the wells. During the second incubation, this antibody serves as a detector by binding to the immobilized phosphorylated p53 protein at Ser46 captured during the first incubation. After removal of excess detection antibody, followed by binding with horseradish peroxidase conjugated anti-mouse IgG, 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 quantitated by spectrophotometry and reflects the relative amount of phosphorylated form of p53 at serine Ser46 present in the original specimen.



Summary of Procedure

Culture cells in culture flask or dish at 50-70 % confluency
↓ Incubate O/N at 37°C in CO₂ incubator
Add appropriate amount of the drug for induction of p53 Ser46 phosphorylation
↓ Incubate appropriate time at 37°C in CO₂ incubator
Harvest the cells by scraping and/or centrifugation
↓
Make cell lysate by adding extraction buffer and centrifugation
↓
Add 100 μL of diluted cell lysate to the wells
↓ Incubate for 2 hours at room temp.
Wash the wells
↓
Add 100 μL of Primary Antibody Solution (anti-p53 Ser46 monoclonal antibody)
↓ Incubate for 1 hour at room temp.
Wash the wells
Add 100 μL of Secondary Antibody Solution (HRP conjugated anti-mouse IgG antibody)
↓ Incubate for 1 hour at room temp.
Wash the wells
↓
Add 100 μL of Substrate Reagent
↓ Incubate for 10-20 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 p53 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 1X buffer. For long term storage (over 3 months), store at -20°C.

Dilution Buffer: One bottle containing 50 mL of 1X buffer. Ready to use.

Phospho-p53 Ser46 Standard: One vial containing 200 units of lyophilized phosphorylated p53 Ser46.

Primary Antibody Solution (Anti-Phospho-p53 Ser46 Monoclonal Antibody TK-4D4): One vial containing 12 mL of Anti-Phospho-p53 Ser46 monoclonal antibody. Ready to use.

Secondary Antibody Solution (HRP conjugated Anti-Mouse IgG): One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-mouse IgG 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 supplied ready to use, containing 20 mL of 1 N H₂SO₄.

Materials Required but not Provided

- **Protease inhibitor cocktail:** e.g. available from SIGMA, Cat# P-2714 (reconstituted according to manufacturer's guideline).
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Orbital microplate shaker**
- **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 1,000 mL graduated cylinder.**
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**



Precautions and Recommendations

- **Although we suggest to conduct experiments as outlined below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.**
- 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 MBL Research Product **CycLex Phospho-p53 Ser46 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-p53 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.

I. 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**, **Cell Extraction Buffer** and **Phospho-p53 Ser46 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 (ddH₂O). Mix well. Store at 4°C for two weeks or -20°C for long-term storage.
2. Prepare a working solution of **Complete Cell Extraction Buffer** by adding 250 µL of Protease inhibitor cocktail (see the section “Materials Required but not Provided” above.) to 5 mL of **Cell Extraction Buffer**. Mix well.
3. Reconstitute **Phospho-p53 Standard** with **1 mL** of **ddH₂O**. The concentration of the phospho-p53 in vial should be 200 units/mL, which is referred to as the **Master Standard** of phospho-p53 Ser46.

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	300 µL of Master Standard	300 µL	100 units/mL
Std.2	300 µL of Std. 1 (100 units/mL)	300 µL	50 units/mL
Std.3	300 µL of Std. 2 (50 units/mL)	300 µL	25 units/mL
Std.4	300 µL of Std. 3 (25 units/mL)	300 µL	12.5 units/mL
Std.5	300 µL of Std. 4 (12.5 units/mL)	300 µL	6.25 units/mL
Std.6	300 µL of Std. 5 (6.25 units/mL)	300 µL	3.13 units/mL
Std.7	300 µL of Std. 6 (3.13 units/mL)	300 µL	1.56 units/mL
Blank	-	300 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.



II. 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 CO₂ 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 in PBS 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 0.5 mL* of “**Complete Cell Extraction Buffer**” for 30 minutes, on ice, with vortexing at 10-minute intervals.

** To get a rough idea you could adjust the cell concentration to around 2×10^7 cells/mL. Resulting protein concentration of the cell lysate should be 2-4 mg/mL using “Complete Cell Extraction Buffer”.*

** The volume of “Complete Cell Extraction Buffer” depends on the cell number in cell pellet and phosphorylation level of p53. For example, 1.2×10^7 MCF-7 cells (in 10 cm dish) can be extracted in 0.5 mL of “Complete Cell Extraction Buffer”. Under these conditions, the protein concentration should be 2-4 mg/mL and use of 5-10 μ L of the clarified cell extract diluted to a volume of 100 μ L/well in Dilution Buffer is sufficient for the detection of phospho-p53 Ser46.*

5. Transfer the lysate 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.



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 the lysate **1:10*** with Dilution Buffer (e.g. 30 µL sample + 270 µL Dilution Buffer).

** Lysates prepared in "Complete Cell Extraction Buffer" must be diluted 1:10 or greater in Dilution Buffer. While a 1:10 sample dilution has been found to be satisfactory, higher dilutions such as 1:20 or 1:40 may be optimal. The dilution chosen should be optimized for each experimental system.*

3. Pipette **100 µL** of **Standard Solutions (Std1-Std7, Blank)** and **diluted lysates** in duplicates, into the appropriate wells.
4. Incubate the plate **at room temperature (ca. 25°C) for 2 hours**, 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 **Primary Antibody Solution** 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 **Secondary Antibody Solution** into each well.
10. Incubate the plate **at room temperature (ca. 25°C) for 1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.
11. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.
12. Add **100 µL** of **Substrate Reagent**. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminum foil is recommended. Return Substrate A to 4°C immediately after the necessary volume is removed
13. Incubate the plate **at room temperature (ca. 25°C) for 10-20 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker. The incubation time may be extended up to 30 minutes if the reaction temperature is below than 20°C.
14. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.
15. 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-p53 Ser46 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-p53 Ser46 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-p53 Ser46 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 in higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the phospho-p53 Ser46 concentration.



Troubleshooting

1. All samples and standards 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 MBL Research Product **CycLex Phospho-p53 Ser46 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-p53 Ser46 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-p53 Ser46 giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 1.450 units/ml of sample.

* *Dilution Buffer was pipetted into blank wells.*

Eighty assays were evaluated and the minimum detectable dose (MDD) of Phospho-p53 Ser46 ranged from 0.894-1.785 units/mL. The mean MDD was 1.450 units /mL. The MDD was determined by adding three standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

2. Specificity

The antibody in the Phospho-p53 Ser46 ELISA kit is highly specific of human Phospho-p53 Ser46, with no detectable cross-reactivity to mouse and rat Phospho-p53.

3. Linearity

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Phospho-p53 Ser46 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 standard of phospho-p53 Ser46

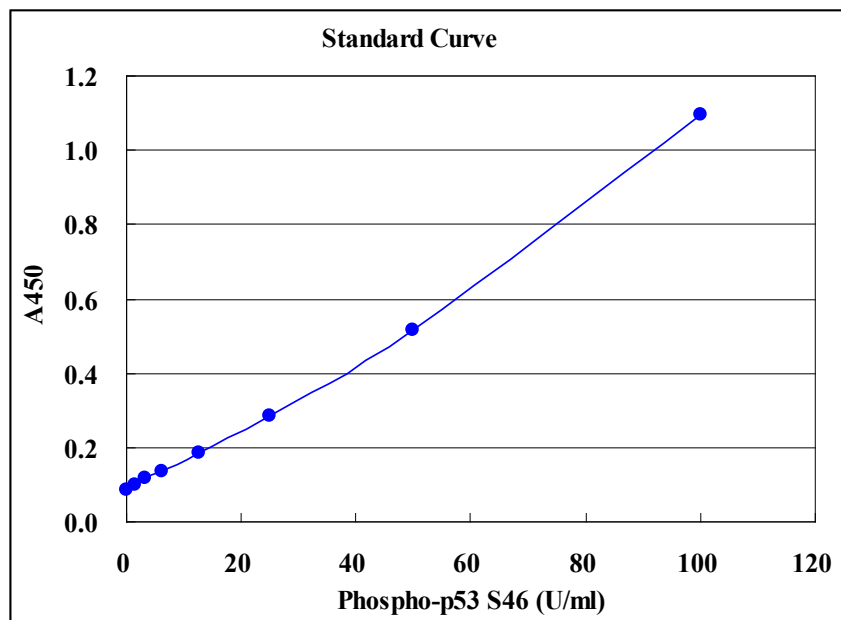
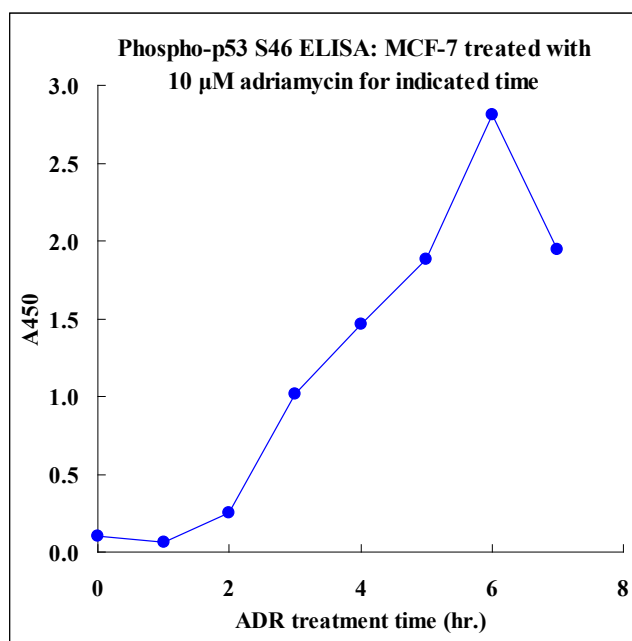


Fig.2 Phospho-p53 Ser46 ELISA using breast cancer cell line MCF-7 that expresses wild type p53, which have been treated with 10 μ M adriamycin for indicated times





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