



ab156027

p53 (pSer15) Human ELISA Kit

Instructions for Use

For the quantitative measurement of phosphorylated Ser15 of p53 protein

This product is for research use only and is not intended for diagnostic use.

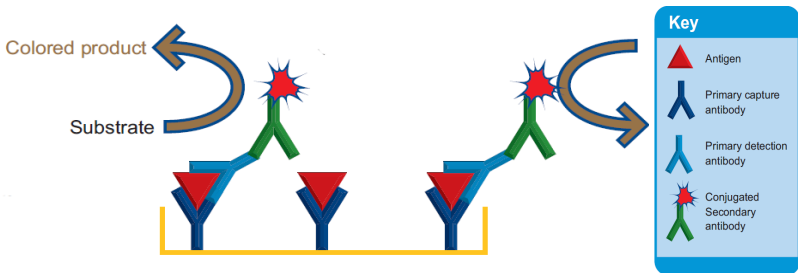
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1. Introduction

Principle: Abcam's p53 (pSer15) Human ELISA kit is an in vitro enzyme-linked immunosorbent assay for the accurate quantitative measurement of phosphorylated Ser15 of p53 protein in human cell and tissue lysates. The assay employs an antibody specific to p53 protein coated onto well plate strips. Standards and samples are pipetted into the wells and p53 present in the sample is bound to the wells by the immobilized antibody. The wells are washed and an anti-p53 phospho Ser15 detector antibody is added. After washing away unbound detector antibody, HRP-conjugated label specific for the detector antibody is pipetted into the wells. The wells are again washed, an HRP substrate solution is added to the wells and blue color develops in proportion to the amount of phosphorylated Ser15 of bound p53. The reaction is stopped by adding hydrochloric acid which changes the color from blue to yellow and the color intensity is measured at 450 nm.



Background: p53 (TP53 gene) acts as a tumor suppressor in many tumor types and induces growth arrest or apoptosis depending on the physiological circumstances and cell type. p53 is involved in cell cycle regulation as a trans-activator that acts to negatively regulate cell division by controlling a set of genes required for this process. One of the activated genes is an inhibitor of cyclin-dependent kinases. p53 mediated apoptosis induction seems to be by stimulation of BAX and FAS antigen expression, or by repression of Bcl-2 expression. p53 is also implicated in Notch signaling cross-over.

The p53 protein is found in increased amounts in a wide variety of transformed cells. p53 is mutated or inactivated in about 60% of cancers. Four types of cancers account for 80% of tumors occurring in TP53 germline mutation carriers: breast cancers, soft tissue and bone sarcomas, brain tumors (astrocytomas) and adrenocortical carcinomas.

p53 levels are kept low through a continuous degradation of p53. Mdm2 binds to p53, preventing its action and transports it from the nucleus to the cytosol. Mdm2 also acts as ubiquitin ligase and covalently attaches ubiquitin to p53 and thus marks p53 for degradation by the proteasome. The ubiquitin can be cleaved by USP7 (or HAUSP), thereby protecting it from this proteasome-dependent degradation. This is one means by which p53 is stabilized in response to oncogenic insults.

Acetylation of the C-terminal end of p53 exposes the DNA binding domain of p53, allowing it to activate or repress specific genes. Deacetylase enzymes, such as Sirt1 and Sirt7, can deacetylate p53, leading to an inhibition of apoptosis.

Phosphorylation of the N-terminal end of p53, and conformational changes to p53, disrupt Mdm2-binding leading to p53 accumulation. Phosphorylation on Ser residues mediates transcriptional activation. Phosphorylation at Ser-9 by HIPK4 increases repression activity on BIRC5 promoter. P53 is phosphorylated on Thr-18 by VRK1. P53 is phosphorylated on Ser-20 by CHEK2 in response to DNA damage, which prevents ubiquitination by MDM2. P53 is phosphorylated on Ser-20 by PLK3 in response to reactive oxygen species (ROS), promoting p53/TP53-mediated apoptosis. P53 is phosphorylated on Thr-55 by TAF1, which promotes MDM2-mediated degradation. P53 is phosphorylated on Ser-33 by CDK7 in a CAK complex in response to DNA damage. P53 is phosphorylated on Ser-46 by HIPK2 upon UV irradiation. Phosphorylation on Ser-46 is required for acetylation by CREBBP. P53 is phosphorylated on Ser-392 following UV but not gamma irradiation. P53 is phosphorylated upon DNA damage, probably by ATM or ATR. P53 is phosphorylated on Ser-15 upon ultraviolet irradiation; which is enhanced by interaction with BANP. P53 is phosphorylated by NUA1 at Ser-15 and Ser-392; was initially thought to be mediated by STK11/LKB1 but it was later shown that it is indirect and that STK11/LKB1-dependent phosphorylation is probably mediated by downstream NUA1. It is

unclear whether AMP directly mediates phosphorylation at Ser-15. P53 is phosphorylated on Thr-18 by isoform 1 and isoform 2 of VRK2. Phosphorylation on Thr-18 by isoform 2 of VRK2 results in a reduction in ubiquitination by MDM2 and an increase in acetylation by EP300. P53 is stabilized by CDK5-mediated phosphorylation in response to genotoxic and oxidative stresses at Ser-15, Ser-33 and Ser-46, leading to accumulation of p53/TP53, particularly in the nucleus, thus inducing the transactivation of p53/TP53 target genes. P53 is phosphorylated at Ser-315 and Ser-392 by CDK2 in response to DNA-damage.

2. Assay Summary

Equilibrate all reagents to room temperature. Prepare all the reagents, samples, and standards as instructed.



Add 50 μ L standard or sample to each well used. Incubate 2 hours at room temperature.



Aspirate and wash each well two times. Add 50 μ L prepared 1X Detector Antibody to each well. Incubate 1 hour at room temperature.



Aspirate and wash each well two times. Add 50 μ L prepared 1X HRP Label. Incubate 1 hour at room temperature.



Aspirate and wash each well three times. Add 100 μ L HRP Development Solution to each well. Add 100 μ L Stop Solution at a user-defined time (15 min) and read at 450 nm.

3. Kit Contents

Items	Quantity
Extraction Buffer	15 mL
10X Buffer	6 mL
10X Blocking Solution	8 mL
10X Wash Buffer	40 mL
p53 Microplate (8 x 12 antibody coated wells)	96 Wells
10X p53 (pSer15) Detector Antibody	700 μ L
10X HRP Label	1 mL
HRP Development Solution	12 mL
Stop Solution	12 mL

4. Storage and Handling

Store all components at 4°C. This kit is stable for 6 months from receipt. Unused microplate strips should be returned to the pouch containing the desiccant and resealed. After opening the unused Incubation Buffer should be stored at -20°C.

5. Additional Materials Required

- Microplate reader capable of measuring absorbance at 450 nm
- Method for determining protein concentration (BCA assay recommended)
- Deionized water
- Multi and single channel pipettes
- PBS (1.4 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 140 mM NaCl, 2.7 mM KCl, pH 7.3)
- Tubes for standard dilution
- Plate shaker for all incubation steps (optional)
- PMSF (or other protease inhibitors) and phosphatase inhibitors (optional)

6. Preparation of Reagents

- 6.1 Equilibrate all reagents and samples to room temperature (18-25°C) before use.
- 6.2 Prepare Incubation Buffer by adding 6 mL 10X Buffer and 6 mL 10X Blocking Solution to 48 mL nanopure water. Mix gently and thoroughly.

- 6.3 Prepare 1X Wash Buffer by adding 40 mL 10X Wash Buffer to 360 mL nanopure water. Mix gently and thoroughly.
- 6.4 Prepare 1X p53 (pSer15) Detector Antibody by diluting the 10X p53 (pSer15) Detector Antibody 10-fold with Incubation Buffer immediately prior to use. Prepare 500 μ L for each 8 well strip used.
- 6.5 Prepare 1X HRP Label by diluting the 10X HRP Label 10-fold with Incubation Buffer immediately prior to use. Prepare 500 μ L for each 8 well strip used.

7. Sample Preparation

Note: Extraction Buffer can be supplemented with PMSF and protease inhibitor cocktail prior to use. Extraction Buffer and Incubation Buffer used for the extract and the detector antibody dilutions should be supplemented with phosphatase inhibitors prior to use. Supplements should be used according to manufacturer's instructions.

7.1 Preparation of extracts from cell pellets

- 7.1.1 Collect non adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 5 minutes at 4°C.

- 7.1.2 Rinse cells twice with PBS.
- 7.1.3 Solubilize cell pellet at 2×10^7 /mL in Extraction Buffer.
- 7.1.4 Incubate on ice for 20 minutes. Centrifuge at 18,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

7.2 Preparation of extracts from adherent cells by direct lysis (alternative protocol)

- 7.2.1 Remove growth media and rinse adherent cells 2 times in PBS.
- 7.2.2 Solubilize the cells by addition of Extraction Buffer directly to the plate (use 0.75 - 1.5 mL Extraction Buffer per confluent 15 cm diameter plate).
- 7.2.3 Scrape the cells into a test tube and incubate the lysate on ice for 15 minutes. Centrifuge at 18,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

7.3 Preparation of extracts from tissue homogenates

- 7.3.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).
- 7.3.2 Suspend the homogenate to a protein concentration of 10 mg/mL in PBS.
- 7.3.3 Solubilize the homogenate by adding 9 volumes of Extraction Buffer to one volume of the homogenate.
- 7.3.4 Incubate on ice for 20 minutes. Centrifuge at 18,000 x g for 20 minutes at 4°C. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

The samples should be diluted to within the working range of the assay in Incubation Buffer. As a guide, typical ranges of sample concentration for commonly used sample types are shown below:

TYPICAL SAMPLE RANGE

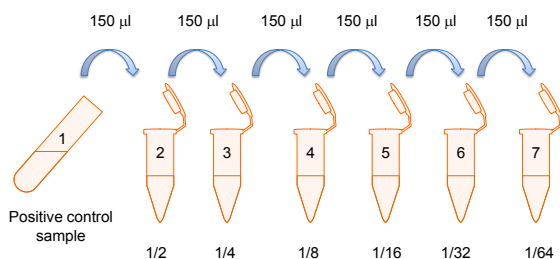
Typical working ranges	
Sample Type	Range
Hek 293T cells (etoposide treated)	16 – 1000 µg/mL
Hek 293T cells (calyculin A + okadaic acid treated)	32 – 2000 µg/mL
MCF7 cells (camptothecin treated)	32 – 2000 µg /mL

1. Preparation of dilution series of positive control sample

Note: It is strongly recommended to prepare a dilution series of a positive control sample. The levels of phosphorylated Ser15 of p53 may be relatively low in normal healthy cells and tissues. Thus it is recommended to treat cells to induce p53 phosphorylation and use this sample to prepare the dilution series. For examples of treatment refer to sections 9 and 10. The relative levels of phosphorylated Ser15 of p53 in other experimental samples can be interpolated from within this positive control sample series.

- 7.4.1 To prepare serially diluted positive control sample, label six tubes #2-7.

- 7.4.2 Prepare a positive control sample lysate or extract as directed in previous sections (7.1, 7.2 or 7.3). Dilute the positive control sample to an upper concentration limit of the working range of the assay in the Incubation Buffer used to dilute other experimental samples. Label this tube #1.
- 7.4.3 Add 150 μ L of the Incubation Buffer to each of tubes #2 through #7.
- 7.4.4 Transfer 150 μ L from tube #1 to tube #2. Mix thoroughly. With a fresh pipette tip transfer 150 μ L from #2 to #3. Mix thoroughly. Repeat for Tubes #4 through #7. Use the diluent as the zero standard tube labeled #8. Use fresh control sample dilution series for each assay.



8. Assay Procedure

Equilibrate all reagents and samples to room temperature before use. It is recommended all samples and standards be assayed in duplicate.

- 8.1 Prepare all reagents, working standards, and samples as directed in the previous sections.
- 8.2 Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
- 8.3 Add 50 μL of each sample per well. It is recommended to include a dilution series of a positive control sample (section 7.4), as well as untreated sample. Also include a no material control as a zero standard.
- 8.4 Cover/seal the plate and incubate for 2 hours at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.
- 8.5 Aspirate each well and wash, repeat this once more for a total of **two** washes. Wash by aspirating or decanting from wells then adding 300 μL 1X Wash Buffer into each well as described above. Complete removal of liquid at each step is essential to good performance. After the last

wash, remove the remaining buffer by aspiration or decanting. Invert the plate and blot it against clean paper towels to remove excess liquid.

- 8.6 Immediately before use prepare sufficient (500 μ L/8 well strip used) 1X p53 (pSer15) Detector Antibody in Incubation Buffer (step 6.4). Add 50 μ L 1X Detector antibody to each well used. Cover/seal the plate and incubate for 1 hour at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.
- 8.7 Repeat the aspirate/wash procedure above.
- 8.8 Immediately before use prepare sufficient (500 μ L/strip used) 1X HRP Label in Incubation Buffer (step 6.5). Add 50 μ L 1X HRP Label to each well used. Cover/seal the plate and incubate for 1 hour at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.
- 8.9 Repeat the aspirate/wash procedure above, however, performing a total of **three** washes.
- 8.10 Add 100 μ L HRP Development Solution to each empty well.

- 8.11 At a user defined time (e. g. 15 min) stop the reaction by adding 100 μ L Stop Solution (1N HCl) to each well and record the OD at 450 nm.
- 8.12 Analyze the data as described below.

9. Data Analysis

Subtract average zero standard reading from all readings. Average the duplicate readings of the positive control dilutions and plot against their concentrations. Draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A four parameter algorithm (4PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Read relative p53 phospho Ser15 concentrations for unknown samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

TYPICAL STANDARD CURVE - For demonstration only.

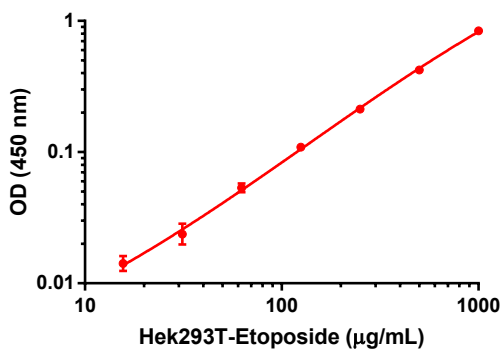


Figure 1. Example positive control sample standard curve. A dilution series of extract in Incubation Buffer in the working range of the assay. The extract was prepared from pellets of Hek293T cells treated with etoposide (ab120227) as described in section 7.1.

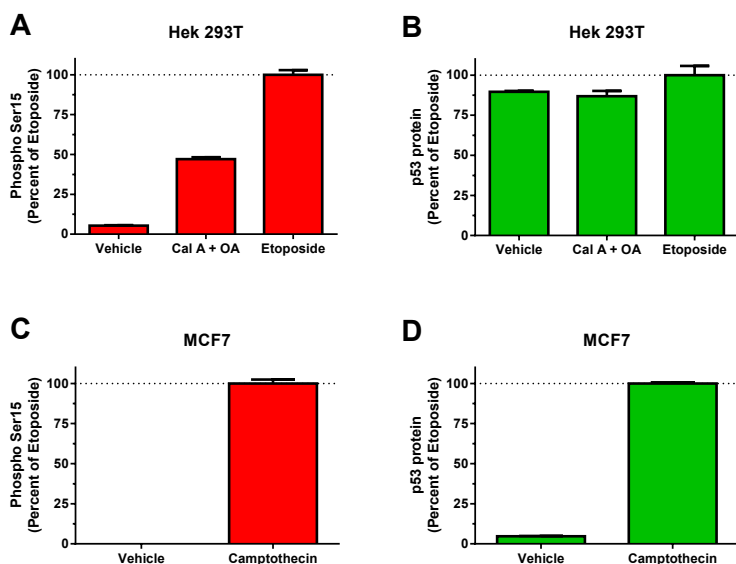


Figure 2. Example experimental analysis of drug treatment of Hek 293T and MCF7 cells. Cells were treated with calyculin A + okadaic acid (Cal A + OA), etoposide (ab120227), camptothecin or drugs' vehicles, as indicated. Diluted cell extracts were analyzed by this kit (A, C) and p53 protein ELISA (B, D), using ab117995. Extract of Hek 293T treated with etoposide was used for positive control sample standard curves. Relative levels interpolated from standard curves and expressed in percent of etoposide- (A, B) and camptothecin- (C, D) treated samples are shown.

SENSITIVITY

Calculated minimum detectable dose = 8 µg/mL (zero dose n=33 + 2 standard deviations) using extract of Hek 293T cells treated with etoposide.

RECOVERY

Sample Type	Average Recovery (%)	Range (%)
50% culture media (10FHGDMEM)	80	78-83
10% goat serum	69	66-71
50% Extraction Buffer	89	88-90

For more accurate comparison dilute all samples in equivalent buffers/media/supplements.

LINEARITY OF DILUTION

Linearity of dilution was determined by comparing dilution series of extracts prepared from camptothecin-treated MCF7 cells (starting protein concentration is 2000 µg/mL) to extracts prepared from etoposide-treated Hek 293T cells.

MCF7-camptothecin (µg/mL)	% Expected Value
2000	100
1000	90
500	77

REPRODUCIBILITY WITHIN WORKING RANGE (using Hek293T cells treated with etoposide (ab120227))

	%CV
Intra (n=3)	8.5
Inter (n=3 days)	14.2

10. Specificity

This kit detects phosphorylated Ser15 of p53 in **human samples only**.

Etoposide treatment of Hek293T cells induced 20-fold increase in phospho Ser15 signal without a significant change in p53 protein level (Fig. 2). These results correlated well with parallel Western blot analysis (Fig. 3). In addition, calyculin A + okadaic acid treatment of Hek293T cells induced 9-fold increase in phospho Ser15 signal without a significant change in p53 protein level (Fig. 2). Taken together, these results indicate that the p53 (pSer15) ELISA Kit specifically measures phosphorylated Ser15 of p53. The specificity of the assay to measure phosphorylated Serine was further demonstrated as the assay's signal being sensitive to λ protein phosphatase treatment of the cell extracts. The relative levels of the phosphorylated Ser15 in extracts decreased to undetectable levels even with the lowest dose of the phosphatase (Fig. 4). This result correlated well with a parallel Western Blot analysis (using the kit's detector antibody) of the λ protein phosphatase-treated extract (Fig. 3). The total p53 protein levels were not sensitive to phosphatase treatment as determined by Western blotting (Fig. 3).

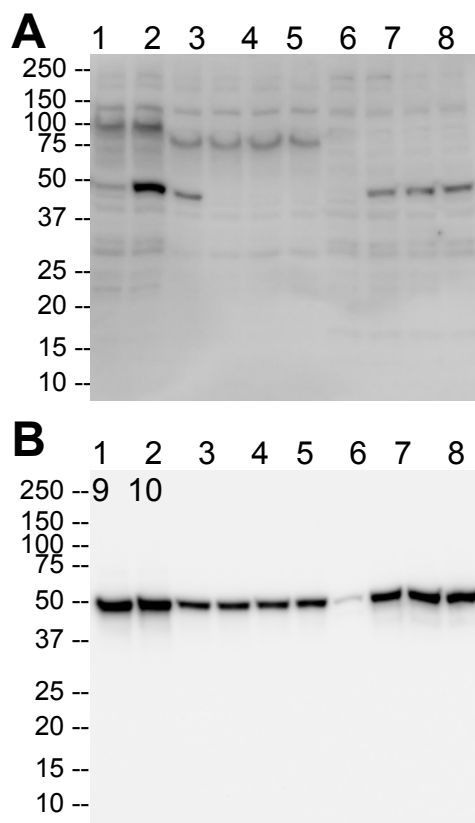


Figure 3. The detector antibody used in this kit specifically detects the phosphorylated p53 as determined by Western blotting. Hek293T cells were treated with vehicle (lane 1) or etoposide (lanes 2-6). MCF7 cells were treated with vehicle (lane 7) or camptothecin for 6

(lane 8), 16 (lane 9) and 24 (lane 10) hours. Extracts of Hek293T cells (induced with etoposide) were treated with increasing concentrations of λ protein phosphatase (lane 4, 400x diluted; lane 5, 100x diluted; lane 6, 25x diluted) or left untreated (lane 3). Samples (lanes 1-2 and 7-10, 20 μ g/lane; lanes 3-6, 8 μ g/lane) were analyzed by Western blotting using the p53 (pSer15) Detector Antibody of this kit (A), or a p53 antibody (ab1101) to detect total p53 protein (B).

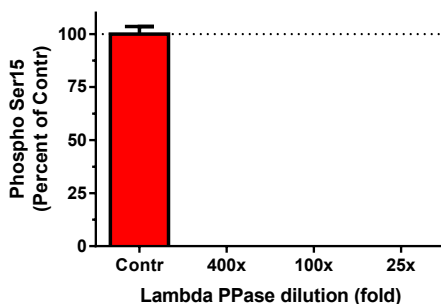


Figure 4. The p53 (pSer15) ELISA specifically measures the phosphorylated Serine. Extracts of Hek 293T cells (induced with etoposide) were treated with increasing concentrations of λ protein phosphatase (400x= 400-times diluted, 100x=100-times diluted, 25x=25-times diluted), or left untreated (Control), and relative phospho Ser15 levels were determined using this kit. Dilutions of extracts of Hek 293T cells treated with etoposide were used to construct the standard curve.

11. Troubleshooting

Problem	Cause	Solution
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Active phosphatase in the extract	Add protein phosphatase inhibitors into the Extraction Buffer and into the Incubation Buffer used for the sample and the detector antibody dilutions
	Incubation times with HRP solution too brief	Ensure sufficient incubation time till blue color develops prior addition of Stop solution
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store the reconstituted standards at -80°C, all other assay components 4°C. Keep substrate solution protected from light

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