

Phospho-p53 (pSer¹⁵)(pSer¹⁵) ELISA, HumanProduct Number **CS0040**
Storage Temperature 2-8 °C**Product Information****Technical Bulletin****Product Description**

Phospho-p53 (pSer¹⁵) ELISA, Human is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) designed to detect and quantify the level of human p53 protein that is phosphorylated at serine 15. A monoclonal antibody specific for p53 regardless of phosphorylation state is coated onto the multiwell plate provided. Standard dilutions, controls and unknown samples containing p53 (pSer¹⁵) are pipetted into these wells. During the first incubation, the p53 antigen binds to the immobilized (capture) antibody. After incubation, excess reagents are washed away and a rabbit antibody specific for p53 (pSer¹⁵) is added. During the second incubation, this antibody serves as a detection antibody by binding to the immobilized p53 protein captured during the first incubation. After removal of excess detection antibody, a horseradish peroxidase-labeled anti-rabbit IgG (Anti-Rabbit IgG-HRP) is added. It binds to the detection antibody to complete the four-member sandwich. After a third incubation and washing to remove excess Anti-Rabbit IgG-HRP, substrate solution is added, which is acted upon by the bound enzyme to produce yellow color. The intensity of this colored product is directly proportional to the concentration of p53 (pSer¹⁵) present in the original specimen. The optical density measured at 450 nm in the multiwell plate reader is used to calculate the concentration of p53 (pSer¹⁵). For the evaluation of total p53 content of the samples, use Sigma p53 ELISA, Human (Prod. No. CS0070).

Phospho-p53 (pSer¹⁵) ELISA is intended for the detection of p53 (pSer¹⁵) in human cell lysates. The assay does not detect phosphorylated p53 in mouse or rat lysates.

p53 gene, located on chromosome 17p, is the most commonly mutated gene in human cancer and more than 500 gene mutations have been described. These mutations are found in various types of malignancies, hematological as well as solid tumors. However, all the

mutants are not necessarily equivalent in terms of biological activity. p53 protein is highly conserved and expressed in normal tissues. Wild-type p53 is shown to be a sequence-specific transcription factor, directly interacting with various cellular and viral proteins. Intact p53 function is essential for the maintenance of the non-tumorigenic phenotype of cells. Thus, p53 plays a vital role in suppressing the development of cancer. The p53 tumor suppressor protein is important in the cellular response to DNA damage and other genomic aberrations. Cells exposed to DNA-damaging agents such as ionizing radiation, UV radiation, and chemical agents initiate a complex response that includes the inhibition of cell cycle progression until damage is repaired. If the DNA damage is beyond repair, cells may enter a prolonged state of arrest or undergo a programmed cell death known as apoptosis, thereby maintaining genetic stability in the organism. In response to DNA damage, p53 is phosphorylated at multiple sites by several protein kinases. Phosphorylation of p53 at serine 15 by ATM, ATR, and DNAPK leads to a reduced interaction with its negative regulator, MDM2, and accumulation of p53 protein. Chk2 and Chk1 can phosphorylate p53 at serine 20, enhancing its activity, tetramerization and stability. Elevation of p53 protein induces the transcriptional activation of multiple genes, including p21^{waf1}. p21^{waf1} interacts directly with cyclin-dependent kinases, important for cell cycle progression, thereby inhibiting their activity and resulting in cell cycle arrest.

Reagents

- **Phospho-p53 (pSer¹⁵) Standard, 2 vials, Product No. P 5620**- lyophilized Actinomycin D - treated human CCRF-CEM cell extract –Refer to vial label for quantity and reconstitution volume.
- **Standard Diluent Buffer, 25 mL, Product No. S 3943** - contains BSA and sodium azide as a preservative.

- **Monoclonal Anti-Human p53 Antibody-coated 96-well plate, 1 plate, Product No. P 5245-** A plate using break-apart strips coated with monoclonal antibody specific for full-length p53 (regardless of phosphorylation state).
- **Anti-Human Phospho-p53 (pSer¹⁵), 11 mL, Product No. P 5495** – A detection antibody, produced in rabbit. Contains sodium azide and BSA. Ready to use.
- **Anti-Rabbit IgG-Horseradish Peroxidase Concentrate, 100X, 1 vial, Product No. I 3283** – contains 3.3 mM thymol and 50% glycerol, viscous. *See Reagent Preparation for handling, dilution and storage instructions.*
- **HRP Diluent, 25 mL, Product No. H 8912** – contains 3.3 mM thymol and BSA. Ready to use.
- **Wash Buffer Concentrate 25X, 100 mL, Product No. W 2639** - *See Reagent Preparation for handling, dilution and storage instructions.*
- **Stabilized Chromogen, Tetramethylbenzidine (TMB), 25 mL, Product No. S 3318** –Avoid prolonged exposure to light. Avoid exposure to metal. Ready to use.
- **Stop Solution, 25 mL, Product No. S 2818** – Ready to use.
- **Plate Covers, Adhesive strips, 3 each, Product No. P 4870**

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 450 nm.
- Calibrated adjustable precision pipettes for volumes between 5 μ L and 1,000 μ L.
- Cell extraction buffer (see recommended extraction procedure).
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard.
- Absorbent paper towels to blot the plate.
- Calibrated beakers and graduated cylinders in various sizes.
- Vortex mixer.
- Graph paper: linear, log-log, or semi-log, as desired.
- Reagent Name
- Equipment Name

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- Samples of choice – extracts of cell lysates
- Samples should be frozen if not analyzed shortly after collection.
- Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
- Cell Extraction Buffer
 10 mM Tris, pH 7.4
 100 mM NaCl
 1 mM EDTA
 1 mM EGTA
 1 mM NaF
 20 mM Na₄P₂O₇
 2 mM Na₃VO₄
 1% Triton X-100
 10% Glycerol
 0.1% SDS
 0.5% Deoxycholate
 1 mM PMSF (stock is 0.3 M in DMSO).
PMSF is very unstable and must be added prior to use, even if added previously.
 Protease inhibitor cocktail (Product. No. P 2714).
 Add 250 μ L of reconstituted cocktail per 5 mL of Cell Extraction Buffer.

Stable for 2-3 weeks at 4 °C or for up to 6 months when aliquoted (without protease inhibitors and PMSF added) and stored at –20 °C.

Thaw on ice. Add the protease inhibitors just before use.

Procedure for Extraction of Proteins from Cells

The recommended Cell Extraction Buffer and procedure are optimized to achieve effective protein phosphorylation. Researchers may use the procedures that work best in their hands. They will have to assay their lysates for the satisfactory extraction and/or phosphorylation.

Protocol for Cell Extraction

1. Collect cells in PBS by centrifugation (non-adherent) or scraping from culture flasks (adherent).
2. Wash 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 -70°C and lysed at a later date).
4. Lyse the cell pellet in Cell Extraction Buffer for 30 minutes on ice with vortexing at 10-minute intervals. The volume of Cell Extraction Buffer depends on the cell number in cell pellet and expression of p53 (pSer¹⁵).
5. For example, 10^7 JCCRF/CEM cells grown in RPMI plus 10% FBS and treated with Actinomycin D for 20 hours can be extracted in 1 mL of Extraction Buffer.
6. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4°C .
7. Aliquot the clear lysate to clean microcentrifuge tubes.

Reagent Preparation

Phospho-p53 (pSer¹⁵) Standard

Phospho-p53 (pSer¹⁵) standard is lyophilized cell extract from Actinomycin D treated CCRF/CEM cells. It is designated in Units/mL. **One unit** is equivalent to the amount of p53 (pSer¹⁵) derived from approximately 11,000 CCRF/CEM cells treated with Actinomycin D for 20 hours.

1. Reconstitute one vial of Standard with *Standard Diluent Buffer* according to label directions.
2. Mix gently and wait 10 minutes to ensure complete reconstitution.
3. Label as **100 Units/mL p53 (pSer¹⁵)**
4. Prepare serial standard dilutions as follows:

Tube#	Standard Buffer	Standard from tube #:	Final concentration U/mL
1	Reconstitute according to label instructions		8000 Units/mL
2	0.25 mL	0.25 mL (1)	4,000
3	0.25 mL	0.25 mL (2)	2,000
4	0.25 mL	0.25 mL (3)	1,000
5	0.25 mL	0.25 mL (4)	500
6	0.25 mL	0.25 mL (5)	250
7	0.25 mL	0.25 mL (6)	125
8	0.5 mL	-	0

Mix thoroughly between each dilution.
Use within 1 hour of reconstitution.

Anti-Rabbit IgG Horseradish Peroxidase (HRP), 100x Concentrate

Solution contains 50% glycerol and is very viscous. To ensure accurate dilution follow instructions below:

1. Equilibrate to room temperature, mix gently, pipette slowly.
2. Remove excess concentrate solution from pipette tip with clean absorbent paper.
3. Mix: 10 μL IgG-HRP concentrate with 1 mL HRP Diluent (sufficient for one 8-well strip, prepare more if needed)
4. Label as **Anti-Rabbit IgG-HRP Working Solution**.
5. Return the unused Anti-Rabbit IgG-HRP concentrate to the refrigerator.

Wash Buffer

1. Equilibrate Wash Buffer Concentrate (25X) to room temperature and mix to redissolve any precipitated salts.
2. Mix 1 volume Wash Buffer Concentrate (25X) with 24 volumes of deionized water
3. Label as **Working Wash Buffer**.
5. Store both the concentrate and the Working Wash Buffer in the refrigerator. Use within 14 days

Storage/Stability

All components of this kit are stable at 2 to 8°C . Any unused reconstituted standard should be discarded or frozen at -70°C . Standard can be frozen and thawed one time only without loss of immunoreactivity.

Refer to the Certificate of Analysis for kit expiration date. To obtain C of A go to www.sigma-aldrich.com

Procedure

Precautions

- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15 - 30°C).
- Use only the coated 96 well capture plate provided with the kit.
- Multiwell plate: equilibrate to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2 - 8°C to maintain plate integrity.
- When not in use all kit components should be refrigerated.
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay.
- Maintain a consistent order of components and reagents addition from well to well. This ensures equal incubation times for all wells.

- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents after the kit expiration date.
- Standards and samples can be made up in either glass or plastic tubes.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbances within 2 hours of assay completion.
- Stabilized Chromogen (TMB) is light sensitive. Avoid prolonged exposure to light. Avoid contact with metal, it may cause color development.

Washing directions

- The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
- Incomplete washing will adversely affect the assay and render false results.
- Use only *Wash Buffer* provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Each wash cycle is repeated four times. Blot as dry as possible after the 4th wash.
- When washing manually, fill wells forcefully with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
- It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

Assay Procedure

- Determine the number of wells for the assay run, including 2 zero wells, 2 chromogen blank wells, 14 standard dilutions wells and 2 wells for each sample to be assayed.
- Remove appropriate number of multiwell plate strips and return the unused strips to the pouch. Reseal pouch.

Assay Summary for Phospho-p53 (pSer15)(pSer¹⁵) ELISA, Human

- 1) **Incubate 100 μ L of Standards and Samples (diluted >1:10) for 2 hours at RT. (Optional: Incubate overnight at 4°C)**



aspirate and wash 4x

- 2) **Incubate 100 μ L of Detection Antibody for 2 hours at RT.**



aspirate and wash 4x

- 3) **Incubate 100 μ L of HRP Anti-Rabbit IgG for 2 hours at RT.**



aspirate and wash 5x

- 4) **Incubate 100 μ L of stabilized Chromogen for 30 minutes at RT.**



- 5) **Add 100 μ L of Stop Solution and read at 450nm.**

Total Time 4 hours

1st incubation

- a. Add 100 μ L Standard Diluent to zero wells.
- b. Add 100 μ L standards, samples or controls to the appropriate wells. Note: *Samples lysed in Cell Extraction Buffer must be diluted 1:10 or greater in Standard Diluent Buffer to maintain matrix compatibility. Higher dilutions (1:25 – 1:50) may be necessary. The dilutions should be optimized for each assay.*
- c. Tap gently on the plate to mix, cover with Plate Cover and incubate 2 hours at room temperature. *Alternatively, plate may be incubated overnight at 2 to 8 °C.*
- d. Wash wells 4 times following washing instructions.
- e. After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

2nd incubation

- a. Add 100 μ L Anti-Phospho-p53 (pSer15) detection antibody to all wells (except chromogen blanks).
- b. Tap gently on the plate to mix, cover with Plate Cover and incubate 1 hour at room temperature.
- c. Wash wells 4 times following washing instructions
- d. After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

3rd incubation

- a. Add 100 μ L Anti-Rabbit IgG-HRP Working Solution to each well except the chromogen blanks.
- b. Cover with Plate Cover and incubate 30 minutes at room temperature.
- c. Wash wells for a total 4 times following washing instructions.
- d. After the final wash blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate incubation

- a. Add 100 μ L of Stabilized Chromogen into all wells. *The liquid in the wells will begin to turn blue.*

- b. Do not cover the plate
- c. Incubate approximately 30 minutes at room temperature in the dark (place plate in a drawer or cabinet).

Note: The substrate incubation time may vary depending on the plate reader used. If your plate reader does not register optical density (OD) above 2.0, shorten the incubation time to 20-25 minutes.

Stop reaction

- a. Add 100 µL of Stop Solution to each well. This stops the reaction
- b. Tap gently to mix. *The solution will turn yellow.*

Absorbance reading

- a. Any commercially available multiwell plate reader capable of reading at OD 450 nm may be used.
- b. Blank the plate reader against the *Chromogen Blank* wells (contain Chromogen and *Stop Solution*).
- c. Read the absorbance of the entire plate at 450 nm within 2 hours after addition of *Stop Solution*.

Results

1. The results may be calculated using any immunoassay software package.
2. The four-parameter algorithm provides the best curve fit.
3. If the software program is not readily available, the concentrations of p53 (pSer¹⁵) may be calculated as follows:
4. Average Net OD (nm) =
Average Bound OD (nm) – Average Chromogen Blank OD (nm)
5. On graph paper plot the Average Net OD (nm) of standard dilutions against the concentrations (pg/mL) of p53 (pSer¹⁵) for the standards. Draw the best smooth curve through these points to construct the standard curve.
6. The p53 (pSer¹⁵) concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
7. Multiply value(s) obtained for sample(s) by dilution factor to correct for the dilution.
8. Samples producing signals higher than the highest standard (100 U/mL) should be further diluted in *Standard Diluent Buffer* and re-analyzed, multiplying the concentration by the appropriate dilution factor.

Product Profile

Typical Results

The standard curve below is for illustration purposes only (see Limitations)

p53 (pSer ¹⁵) Standard U/mL	OD 450 nm
0	0.156
1.6	0.265
3.12	0.336
6.25	0.433
12.5	0.625
25	0.993
50	1.57
100	2.72

Limitations:

- Do not extrapolate the standard curve beyond the 100 U/mL standard point.
- The dose response is non-linear in this region and accuracy is compromised.
- Dilute samples >100 U/mL with *Standard Diluent Buffer*; re-analyze and multiply results by the appropriate dilution factor.
- Other buffers and matrices have not been investigated.
- Although the rate of degradation of native p53 (pSer¹⁵) in the *Cell Extraction Buffer* described in this protocol has not been seen to date, the possibility of this occurrence cannot be excluded.

Performance Characteristics

Specificity

The Phospho-p53 (pSer¹⁵) ELISA specifically measures human p53 (pSer¹⁵). The specificity of this assay was confirmed by peptide competition. The data presented in Fig. 1 show that the peptide containing phosphorylated serine 15 blocks the ELISA signal.

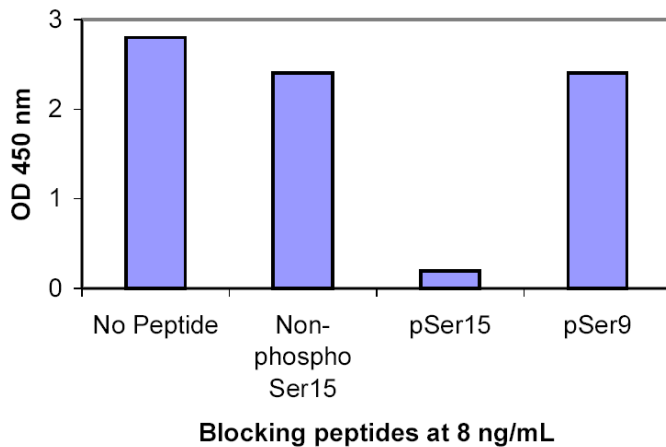


Figure 1 Peptide Competition

Sensitivity

The analytical sensitivity of this assay is <0.9 U/mL of human p53 (pSer¹⁵). This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times. The sensitivity of this ELISA was compared to immunoblotting using known quantities of p53 (pSer¹⁵). The data presented in Figure 2 show that the sensitivity of the ELISA is approximately 8 times greater than that of immunoblot. The bands shown in the immunoblot data were developed using rabbit anti- p53 (pSer¹⁵) and an alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

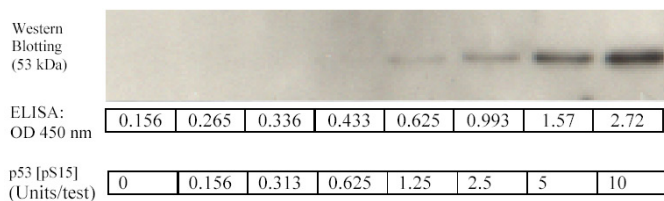


Fig. 2 Detection of p53 (pSer¹⁵) by ELISA vs. immunoblot

Precision

1. Intra-Assay Precision

Samples of known p53 (pSer¹⁵) concentration were assayed in replicates of 12 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean U/mL	93.9	28.5	9.9
SD	3.4	1.1	0.6
%CV	3.6	3.7	6.2

2. Inter-Assay Precision

Samples were assayed 36 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean U/mL	94.4	27.9	9.4
SD	6.6	2.3	0.99
%CV	7.0	8.1	10

SD=Standard Deviation

%CV=Coefficient of variation

Sample Recovery

The recovery of p53 (pSer¹⁵) added to p53 deficient Jurkat cell line (adjusted to 10 µg/mL total protein) averaged 102% when diluted in *Standard Diluent*

Parallelism

Natural p53 (pSer¹⁵) from human CCRF/CEM cell lysate was serially diluted in *Standard Diluent Buffer*. The optical density of each dilution was plotted against the p53 (pSer¹⁵) standard curve. Parallelism was demonstrated by the figure 3 below and indicated that the standard accurately reflects p53 (pSer¹⁵) content in samples.

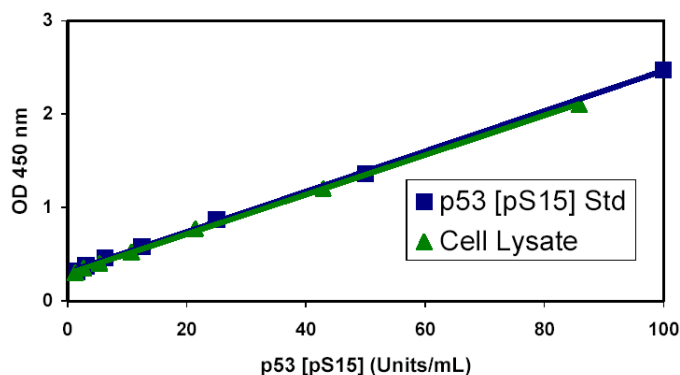


Fig. 3 Human p53 (pSer¹⁵): Parallelism

Linearity of Dilution

CCRF/CEM cells were grown in tissue culture medium containing 10% fetal calf serum, treated with 5 nM actinomycin D for 20 hours and lysed with *Cell Extraction Buffer*. This lysate was diluted in *Standard Diluent Buffer* over the range of the assay and measured for p53 (pSer¹⁵) content. Linear regression analysis of samples versus the expected concentration yielded a correlation coefficient of 0.99.

Cell Lysate			
Dilution	Measured (U/mL)	Expected (U/mL)	% Expected
Neat	85.8	85.8	100
1:2	43.1	42.9	100
1:4	21.4	21.5	99.7
1:8	10.1	10.7	94
1:16	4.7	5.4	88
1:32	2.8	2.7	104

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