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p53 ELISA, Human

ProductInformation

Product Number **CS0070**
Storage Temperature 2-8 °C

Technical Bulletin

Product Description

p53 ELISA, Human is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA) designed to detect and quantify the level of p53 proteins independent of their phosphorylation status. A monoclonal antibody specific for p53 regardless of phosphorylation state is coated onto the multiwell plate provided. p53 standard dilutions, controls and unknown samples 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 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 all 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 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.

The p53 ELISA detects p53 in human cell lysates and does not react with p53 in mouse and rat cell lysates. This ELISA can be used to normalize the p53 content of samples when testing for phosphorylated p53 ($pSer^{15}$) with Sigma Phospho-ELISA (Prod. No. CS0040).

p53 gene, located on chromosome 17p, is the most commonly mutated gene in human cancer and more then 500 gene mutations have been described. These mutations are found in various types of malignancies, hematologic as well as solid tumors. However, all the mutants are not necessarily equivalent in terms of biological activity. The 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-tumorogenic 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 andstability. 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

- **p53 Standard, 2 vials, Product No. P 5370** lyophilized human CCRF/CEM cell extract. *Refer to vial label for quantity and reconstitution volume.*
- **Standard Diluent Buffer**, **25 mL**, **Product No. S 3818** - 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 p53, 11 mL, Product No. P 5120** – A detection antibody, produced in rabbit, which recognizes p53 regardless of phosphorylation state. Contains sodium azide and BSA. Ready to use.
- **Anti-Rabbit IgG-Horseradish Peroxidase Concentrate, 100X, 1 vial, Product No. I 3158** – 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 µL and 1,000 µL.
- Cell extraction buffer (see recommended extraction procedure).
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Graph paper: linear, log-log, or semi-log, as desired.
- 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.

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 (Sigma Cat. No. P 2714)
  Add 250 µL of reconstituted cocktail per 5 mL of Cell Extraction Buffer.

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

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. In such case, they will have to assay 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 ºC 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.
5. For example, 10^7 JCCRF/CEM cells grown in RPMI plus 10% FBS can be extracted in 1 mL of *Extraction Buffer*. Under these conditions 1-10 µL of the clarified cell extract diluted to 100µL/well in *Standard Diluent* Buffer is sufficient for the detection of p53.
6. Transfer extracts to microcentrifuge tubes and centrifuge at 13,000 rpm for 10 minutes at 4 ºC.
7. Aliquot the clear lysate to clean microcentrifuge tubes.

Reagent Preparation

p53 Standard
p53 standard is lyophilized cell extract from CCRF/CEM cells and it is calibrated against the mass of a highly purified, full length, recombinant human p53 protein expressed in *E. coli*.

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 **p53 8000 pg/mL**
4. Prepare serial standard dilutions as follows:

Tube#	Standard Buffer	Standard from tube #:	Final concentration pg/mL
1	Reconstitute according to label instructions		8000 pg/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

5. 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-HR 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**.
4. 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 ºC.
Any unused reconstituted standard should be discarded or frozen at –70 ºC. 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 ºC).
- 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 two zero wells, two chromogen blank wells, 14 standard dilutions wells and two wells for each sample to be assayed.
- Remove appropriate number of multiwell plate strips and return the unused strips to the pouch. Reseal pouch.

Summary of p53 ELISA

1) Incubate 100 µL 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 µL of Detection Antibody for 1 hour at RT.

aspirate and wash 4x

3) Incubate 100 µL of HRP Anti-Rabbit Antibody for 30 minutes at RT.

aspirate and wash 4x

4) Incubate 100 µL of stabilized Chromogen for 30 minutes at RT.

5) Add 100 µL of Stop Solution and read at 450nm.

Total Time 4 hours

1st incubation

a. Add 100 µL Standard Diluent to zero wells.
b. Add 100 µL 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 µL Anti-p53 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 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.

3rd incubation

a Add 100 µL 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 µL 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).*

d. Note: *The substrate incubation time may vary depending on the plate reader used. If your plater 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 may be calculated as follows:
4. Calculate the Average Net OD in nm (average reading of 2 wells)
   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) for the standards.
6. Draw the best smooth curve through these points to construct the standard curve.
7. Multiply value(s) obtained for sample(s) by dilution factor to correct for the dilution factor.
8. Samples producing signals higher than the highest standard (8000 pg/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 Standard Curve

p53 Standard pg/mL	OD 450 nm
0	0.140
125	0.192
250	0.256
500	0.367
1000	0.538
2000	0.865
4000	1.46
8000	2.56

Limitations:

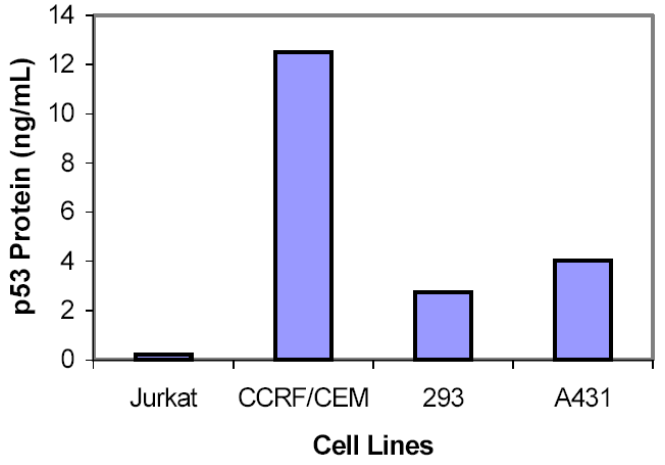
- Do not extrapolate the standard curve beyond the
- 8000 pg/mL standard point.
- The dose response is non-linear in this region and accuracy is compromised.
- Dilute samples >8000 pg/mL with *Standard Diluent Buffer;* re-analyze and multiply results by the appropriate dilution factor.
- Other buffers and matrices have not been investigated.
- Although the rate of degradation of native p53 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

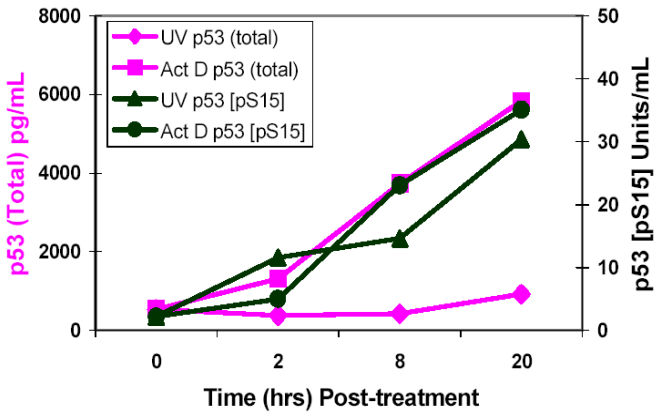
The p53 ELISA is specific for the measurement of human p53. To determine the specificity of this ELISA kit, cell extracts from different cell lines, each at a concentration of 12.5 µg/mL total protein, were analyzed. The data presented in Figure 1 shows that the kit detects p53 protein in cell lysates from CCRF/CEM cells, 293 cells, and A431 cells; however, p53 is not detected in cell lysate from Jurkat cells, a p53-deficient cell line. The levels of p53 protein detected with this ELISA kit are consistent with results obtained by immunoblot analysis (data not shown).

Figure 1 p53 detection in different cell lines



Exponentially growing HT1080 cells were either UV irradiated or treated with 5 nM actinomycinn D for a 20 hour period. Cells were harvested at 2, 8 and 20 hours post-treatment. In Figure 2, the p53 and Phospho-p53 (pSer^{15}) (Product No. CS0040) ELISAs detect a significant increase in total p53 protein and in p53 phosphorylated at serine 15 within 2 hours post-treatment, which remain elevated for the durations of the study.

Figure 2

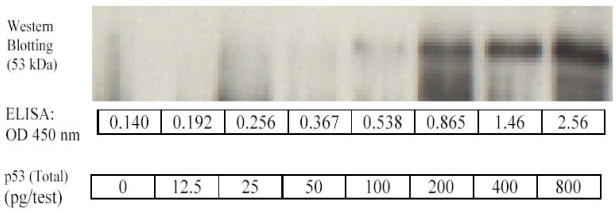


Sensitivity

The analytical sensitivity of this assay is <50 pg/mL of human p53. 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. The data presented in Figure 3 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, and an alkaline phosphatase conjugated anti-rabbit IgG followed by chemiluminescent substrate and autoradiography.

Fig. 3 Detection of p53 by ELISA vs. immunoblot



Precision

1. Intra-Assay Precision

Samples of known concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean pg/mL	6855	2296	769
SD	360	187	44
%CV	5.2	8.2	5.8

2. Inter-Assay Precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean pg/mL	6704	2318	718
SD	531	199	70
%CV	7.9	8.6	9.7

SD=Standard Deviation
%CV=Coefficient of variation

Sample Recovery

The recovery of p53 was evaluated by spiking three levels of p53 into human serum, plasma, and cell lysate of the p53-deficient Jurkat cell line. The recovery in human serum was 78%, while EDTA, heparin and citrate plasma recoveries ranged from 80 to 132%. The recovery of p53 added to Jurkat cells lysate (adjusted to 10 µg/mL total protein), averaged 82%.

Parallelism

Natural p53 from human CCRF/CEM cell lysate was serially diluted in *Standard Diluent Buffer*. The optical density of each dilution was plotted against the p53 standard curve. Parallelism was demonstrated by the figure 4 and indicated that the standard accurately reflects p53 content in samples.

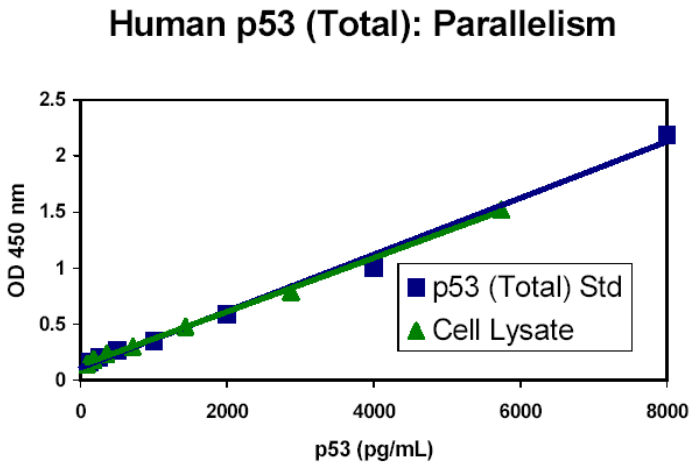


Figure 4

Linearity of Dilution

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

Cell Lysate			
Dilution	**Measured (pg/mL)**	**Expected (pg/mL)**	**% Expected**
Neat	5742	5742	100%
1:2	2858	2871	99.5%
1:4	1686	1436	117%
1:8	820	718	114%
1:16	437	359	122%
1:32	190	179	106%

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