

DuoSet[®] IC

Human Phospho-p53 (S46)

Catalog Number DYC1489-2

DYC1489-5

DYC1489E

For the development of sandwich ELISAs to measure p53 phosphorylated at S46 in cell lysates.

This package insert must be read in its entirety before using this product.

**FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

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PRINCIPLE OF THE ASSAY

This DuoSet IC ELISA contains the basic components required for the development of sandwich ELISAs to measure human p53 phosphorylated at S46 in cell lysates. An immobilized capture antibody specific for human p53 binds both phosphorylated and unphosphorylated p53. After washing away unbound material, a biotinylated detection antibody specific for human p53 phosphorylated at S46 is used to detect phosphorylated protein, utilizing a standard Streptavidin-HRP format.

MATERIALS PROVIDED

Store the unopened kit at 2-8° C. Do not use past kit expiration date.

Description	Part #	Storage Conditions	Vials Provided	
			Cat. # DYC1489-2	Cat. # DYC1489-5
Human Phospho-p53 (S46) Capture Antibody	841533	2-8° C	1	2
Human Phospho-p53 (S46) Detection Antibody	841534	2-8° C	1	2
Human Phospho-p53 (S46) Standard	841535	2-8° C	3	5
Streptavidin-HRP	890803	2-8° C	1	1

DYC1489-2 contains sufficient materials to run ELISAs on at least two 96 well plates.*
DYC1489-5 contains sufficient materials to run ELISAs on at least five 96 well plates.*

This kit is also available in an Economy Pack (R&D Systems, Catalog # DYC1489E). Economy Packs contain sufficient materials to run ELISAs on 15 microplates.* Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

*Provided the following conditions are met:

- The reagents are prepared as described in this package insert.
- The assay is run as described in the General ELISA Protocol on page 6.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

OTHER MATERIALS REQUIRED

- Aprotinin (Sigma # A6279)
- Leupeptin (Tocris # 1167)
- Pepstatin (Tocris # 1190)
- Sodium fluoride (NaF) (Sigma # 201154)
- Sodium azide (NaN₃) (Sigma # S2002)
- β-Glycerophosphate (Sigma # G6251)
- Triton™ X-100 (Sigma # T9284)
- Pipettes and pipette tips
- Deionized or distilled water
- 96 well microplates [Costar EIA Plates (Catalog # 2592 or R&D Systems Catalog # DY990) are suggested]
- Plate sealers (R&D Systems, Catalog # DY992)
- Squirt bottle, manifold dispenser, or automated microplate washer

SOLUTIONS REQUIRED

PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2-7.4, 0.2 μm filtered (R&D Systems, Catalog # DY006).

Wash Buffer - 0.05% Tween[®] 20 in PBS, pH 7.2-7.4 (R&D Systems, Catalog # WA126).

Block Buffer - 1% BSA,* 0.05% NaN₃, in PBS, pH 7.2-7.4.

IC Diluent #1 - 1% BSA* in PBS, pH 7.2-7.4, 0.2 μm filtered.

IC Diluent #4** - 1 mM EDTA, 0.5% Triton X-100 in PBS, pH 7.2-7.4

Note: *Approximately 50 mL of this diluent is required to run the assay on one plate.*

Lysis Buffer #13** - 1 mM EDTA, 0.5% Triton X-100, 10 μg/mL Leupeptin, 10 μg/mL Pepstatin, 3 μg/mL Aprotinin, 150 mM NaCl, 10 mM NaF, 20 mM β-glycerophosphate in PBS, pH 7.2-7.4.

Substrate Solution - 1:1 mixture of Color Reagent A (H₂O₂) and Color Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

Stop Solution - 2 N H₂SO₄ (R&D Systems, Catalog # DY994).

*The use of R&D Systems Reagent Diluent Concentrate 2 (R&D Systems, Catalog # DY995) or Millipore Bovine Serum Albumin, Fraction V, Protease free (Catalog # 82-045) is recommended. All buffers containing BSA must be stored at 2-8° C.

**Sample Diluent Concentrate 1 (5X) (R&D Systems, Catalog # DYC001), prepared as described in the DYC001 insert.

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REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human Phospho-p53 (S46) Capture Antibody (Part 841533) - Each vial contains 1440 µg/mL of mouse anti-human p53 antibody when reconstituted with 200 µL of PBS. After reconstitution, store at 2-8° C for up to 30 days or aliquot and store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.*

Human Phospho-p53 (S46) Detection Antibody (Part 841534) - Each vial contains 54 µg/mL of biotinylated rabbit anti-human phospho-p53 (S46) antibody when reconstituted with 1.0 mL of IC Diluent #1. After reconstitution, store at 2-8° C for up to 30 days or aliquot and store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.*

Human Phospho-p53 (S46) Standard (Part 841535) - Each vial contains 250 ng/mL of recombinant human phospho-p53 (S46) when reconstituted with 500 µL of IC Diluent #4. **Use within one hour of reconstitution. A fresh standard should be used for each assay.** A seven point curve using 2-fold serial dilutions in IC Diluent #4 and a high standard of 40,000 pg/mL is recommended.

Streptavidin-HRP (Part 890803) - 1.0 mL of streptavidin conjugated to horseradish-peroxidase. Store at 2-8° C. **DO NOT FREEZE.**

*Provided this is within the expiration date of the kit.

PREPARATION OF SAMPLES

Cell Lysates - Rinse cells two times with PBS, making sure to remove any remaining PBS after the second rinse. Solubilize cells at 1×10^7 cells/mL in Lysis Buffer #13 and allow samples to sit on ice for 15 minutes. Assay immediately or store at ≤ -70° C. Before use, centrifuge samples at 2000 x g for 5 minutes and transfer the supernate to a clean test tube. Sample protein concentration may be quantified using a total protein assay. If needed, further dilutions should be made in IC Diluent #4.

PRECAUTION

The Stop Solution suggested for use with this kit is an acidic solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

TECHNICAL HINTS AND LIMITATIONS

- This DuoSet IC ELISA should not be used beyond the expiration date on the kit label.
- Individual results may vary due to differences in technique, plasticware and water sources.
- It is important that the diluents selected for reconstitution and for dilution of the standard reflect the environment of the samples being measured. The diluent suggested in this protocol should be suitable for most cell lysates.
- The type of enzyme and substrate and the concentrations of capture/detection antibodies used can be varied to create an immunoassay with a different sensitivity and dynamic range. A basic understanding of immunoassay development is required for the successful use of these reagents in immunoassays.
- A thorough and consistent wash technique is essential for proper assay performance. Wash Buffer should be dispensed forcefully and removed completely from the wells by aspiration or decanting. Remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- Use a fresh reagent reservoir and pipette tips for each step.
- It is recommended that all standards and samples be assayed in duplicate.
- Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing protein should be made under aseptic conditions and stored at 2-8° C or be prepared fresh daily.

GENERAL ELISA PROTOCOL

A plate layout is provided to record standards and samples assayed.

Plate Preparation

1. Dilute the Capture Antibody to a working concentration of 8.0 $\mu\text{g/mL}$ in PBS, without carrier protein. Immediately coat a 96 well microplate with 100 μL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.
2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of 3 washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
3. Block plates by adding 300 μL of Block Buffer to each well. Incubate at room temperature for 1-2 hours.
4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

Assay Procedure

1. Add 100 μL of sample or standards in IC Diluent #4 per well. Use IC Diluent #4 as the zero standard. Cover with a plate sealer and incubate 2 hours at room temperature.
Note: *A seven point standard curve using 2-fold serial dilutions and a high standard of 40,000 pg/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of Plate Preparation.
3. Dilute the Detection Antibody to a working concentration of 1.5 $\mu\text{g/mL}$ in IC Diluent #1 before use. Add 100 μL of the diluted Detection Antibody to each well. Cover with a new plate sealer and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Immediately before use, dilute the Streptavidin-HRP to the working concentration specified on the vial label using IC Diluent #1. Add 100 μL of the diluted Streptavidin-HRP to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
6. Repeat the aspiration/wash as in step 2 of the Plate Preparation.
7. Add 100 μL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.
8. Add 50 μL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

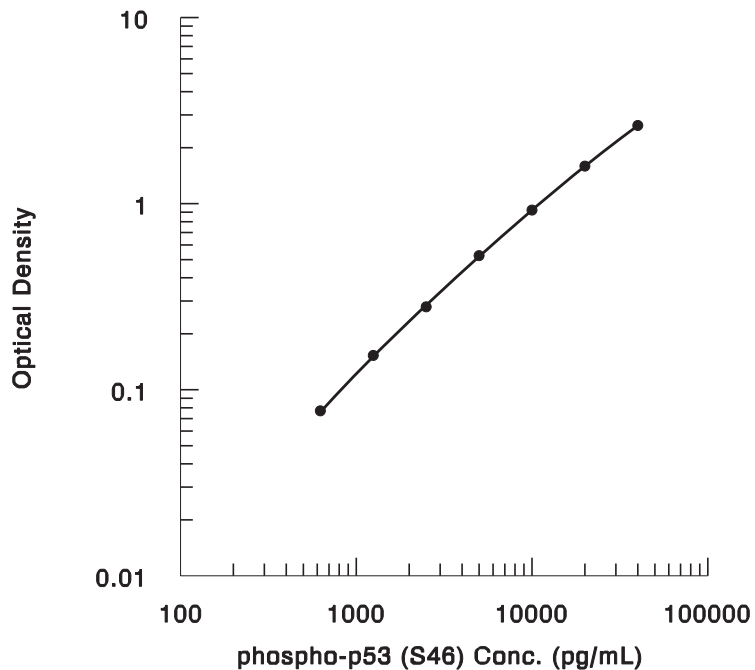
CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human phospho-p53 (S46) concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

TYPICAL DATA

A standard curve should be generated for each set of samples assayed. The graph below represents typical data generated when using this Human Phospho-p53 (S46) DuoSet IC ELISA. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is for demonstration purposes only.



CALIBRATION

The Human Phospho-p53 (S46) DuoSet IC ELISA is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-p53 (S46) produced at R&D Systems. Samples containing natural phospho-p53 (S46) showed linear dilution parallel to the standard curve obtained using the Phospho-p53 (S46) Standard. These results indicate that O.D. values from this DuoSet IC ELISA can be used to determine the relative concentrations of human phospho-p53 (S46) in natural samples.

SPECIFICITY

The Human Phospho-p53 (S46) DuoSet IC ELISA specifically recognizes human p53 phosphorylated at S46. Specificity was determined by Western blot analysis of the protein bound by the capture antibody supplied in the kit.

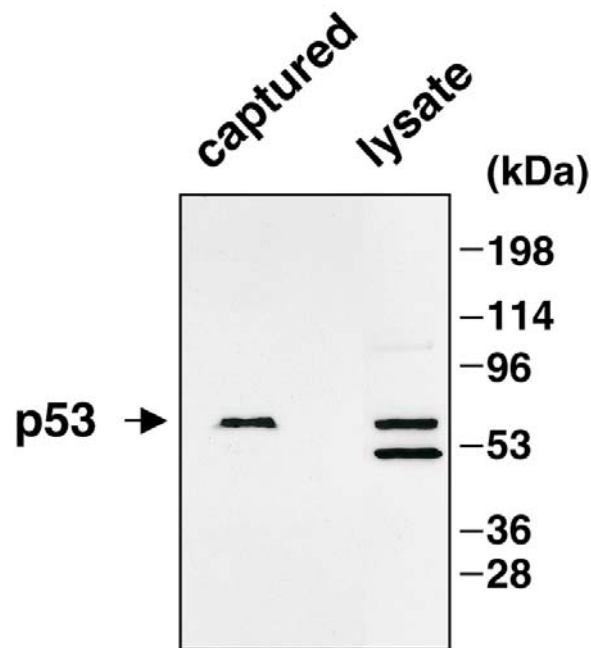


Figure 1: Lysates prepared from MCF-7 human breast cancer cells treated with 1 μ M camptothecin (CPT) were incubated in wells coated with Human Phospho-p53 (S46) Capture Antibody. Unbound material was removed by washing and bound material was solubilized in SDS gel sample buffer. The same lysate and captured proteins were resolved by SDS-PAGE, transferred to a PVDF membrane and immunoblotted with Human Phospho-p53 (S46) Detection Antibody. A single band corresponding to human phospho-p53 (S46) was detected in the captured sample.

The specificity of this kit was also demonstrated using peptide competition. Only the phosphopeptide containing the p53 S46 phosphorylation site blocked the signal, indicating that the Duoset IC ELISA is phosphospecific for p53 phosphorylation at S46 versus the phosphorylation of p53 at S15.

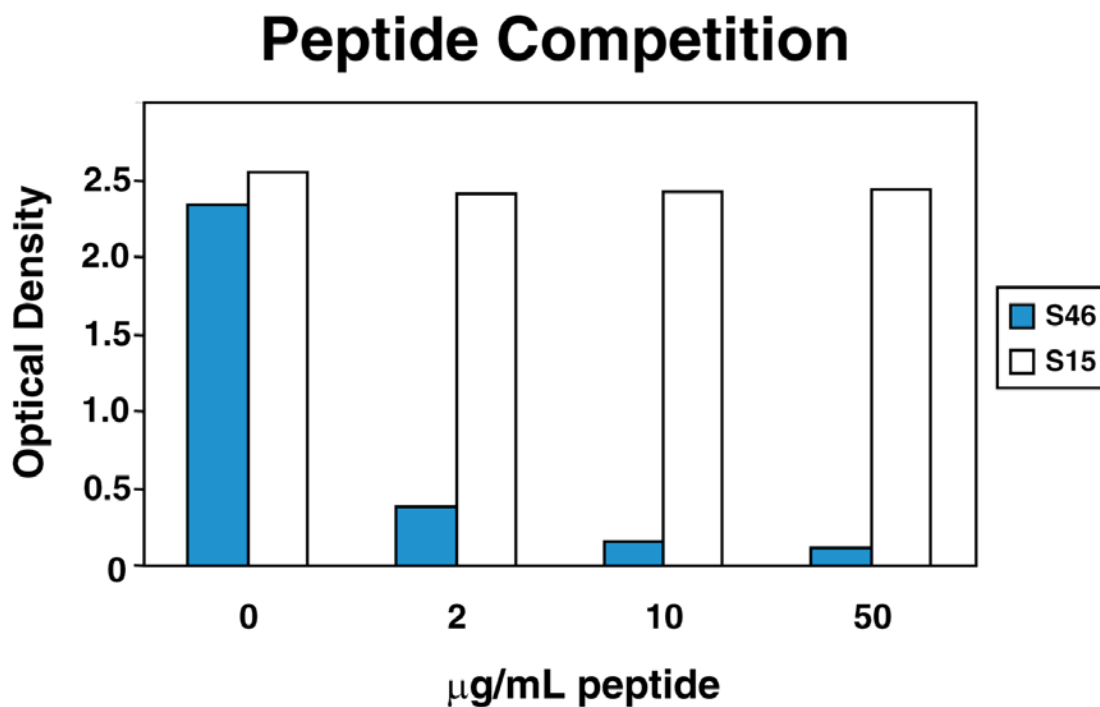


Figure 2: 40 ng/mL of the Human Phospho-p53 (S46) Standard was analyzed in this Human Phospho-p53 (S46) Duoset IC ELISA. The Detection Antibody was either untreated (0) or preincubated with specific phosphopeptides containing the p53 (S46) phosphorylation site (solid bars) or the p53 (S15) phosphorylation site (open bars). Peptides were used at 2, 10, or 50 µg/mL.

QUANTIFICATION

Amounts of human p53 phosphorylated at S46, as quantified by this Human Phospho-p53 (S46) DuoSet IC ELISA, are consistent with the amounts of phospho-p53 (S46) determined by qualitative Western blot analysis. The p53 protein is not present in the untreated MCF-7 cells. Following camptothecin (CPT) treatment, total p53 protein levels rise and p53 is phosphorylated at S46. CPT-treated MCF-7 lysates were treated with λ -phosphatase (λ PPase) prior to sample evaluation in the assay. The λ -PPase treated sample indicates phospho-specificity as total p53 is still detected.

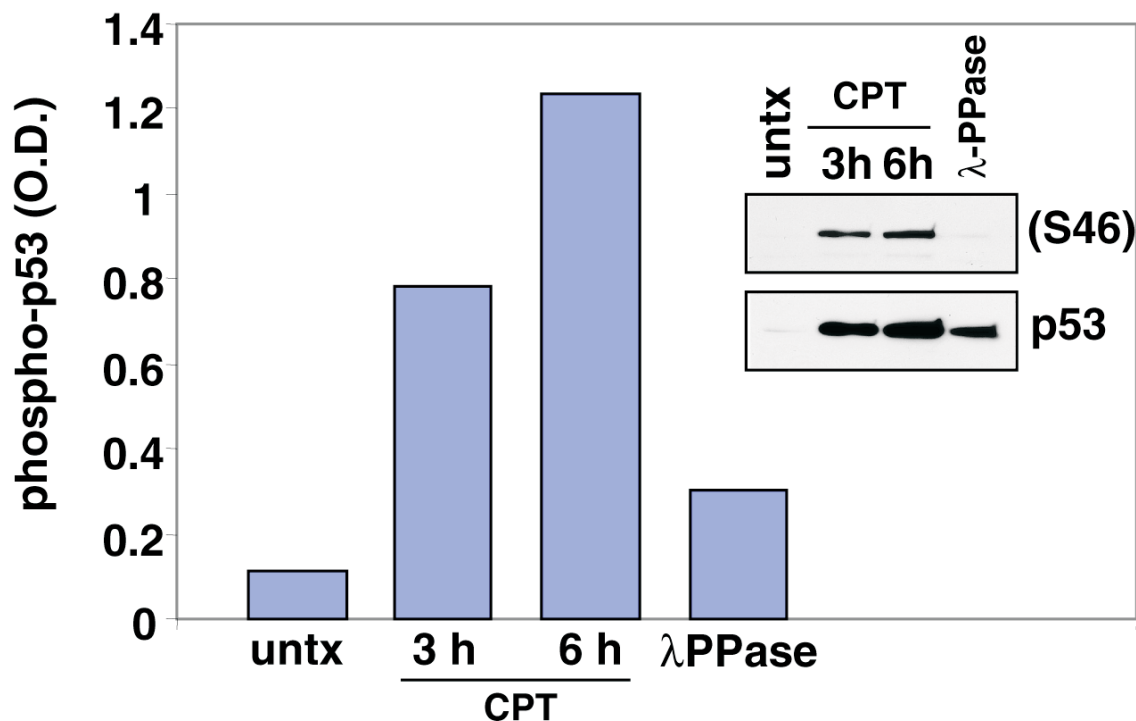


Figure 3: MCF-7 human breast cancer cells were left untreated (untx) or treated with 1 μ M CPT. Cell lysates were prepared after 3 or 6 hours, and a portion of the 6 hour sample was subsequently treated with λ -phosphatase (λ -PPase). Phosphorylated p53 was quantified with this DuoSet IC ELISA, and the same lysates were immunoblotted (inset) with anti-phospho-p53 (S46) (R&D Systems, Catalog # AF1489) or anti-p53-HRP (R&D Systems, Catalog # HAF1355) antibodies. The DuoSet IC ELISA results correlate well with the amounts of human phospho-p53 (S46) detected by Western blot.

PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.

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