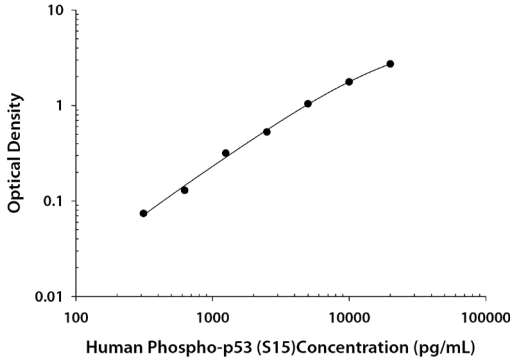
CALCULATION OF RESULTS

Average the duplicate readings for each standard and sample, then subtract the average zero standard optical density (O.D.). Results may be normalized to total protein or cell number.

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 (S15) 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 (S15) 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 (S15) DuoSet IC ELISA is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-p53 (S15) produced at R&D Systems®. Samples containing natural phospho-p53 (S15) showed linear dilution parallel to the standard curve obtained using the Human Phospho-p53 (S15) Standard. These results indicate that O.D. values from this DuoSet IC ELISA can be used to determine the relative concentration of phospho-p53 (S15) in natural samples.

SPECIFICITY

The Human Phospho-p53 (S15) DuoSet IC ELISA specifically recognizes p53 phosphorylated at S15. The p53 protein is not present in untreated MCF-7 cells. Following camptothecin (CPT) treatment, total p53 protein is induced and phosphorylated at S15. To demonstrate the phospho-specificity of this DuoSet® IC ELISA, CPT-treated MCF-7 cellular extract was subsequently treated with λ-phosphatase (CPT, λ-PPase) prior to sample evaluation in the ELISA. The λ-Pase-treated sample indicates phospho-specificity as total p53 is detected while phospho-p53 (S15) is not.

TECHNICAL HINTS & 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 samples and standard reflect the environment of the samples being measured. The diluents 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.

PRECAUTIONS

The Stop Solution recommended for use with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B recommended for use with this kit may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

DuoSet™ IC ELISA
INTRACELLULAR

Human Phospho-p53 (S15)

Catalog Number: DYC1839-2 (2 plates)
DYC1839-5 (5 plates)
DYC1839E (15 plates)

INTENDED USE

For the development of sandwich ELISAs to measure human p53 phosphorylated at S15 in cell lysates.

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 S15 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 S15 is used to detect phosphorylated protein, utilizing a standard Streptavidin-HRP format.

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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MATERIALS PROVIDED & STORAGE CONDITIONS

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

DESCRIPTION	PART #	CATALOG # DYC1839-2	CATALOG # DYC1839-5	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Phospho-p53 (S15) Capture Antibody	841496	1 vial	2 vials	Store for up to 1 month at 2-8 °C or aliquot and store at ≤ -20 °C for up to 3 months in a manual defrost freezer.*
Human Phospho-p53 (S15) Detection Antibody	841497	1 vial	2 vials	
Streptavidin-HRP A	890803	1 vial	1 vial	Store for up to 3 months at 2-8 °C.* **DO NOT FREEZE.**
Human Phospho-p53 (S15) Standard	841498	3 vials	5 vials	**Use within one hour of reconstitution. Use a fresh standard for each assay.**

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

DYC1839-2 contains sufficient materials to run ELISAs on at least two 96 well plates.†
DYC1839-5 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 # DYC1839E). Economy Packs contain sufficient materials to run ELISAs on 15 microplates.† Specific vial counts of each component may vary. 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.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

OTHER MATERIALS REQUIRED

- Aprotinin (Tocris™ # 4139)
- Leupeptin (Tocris # 1167)
- Pepstatin (Tocris # 1190)
- β-Glycerophosphate (Sigma # G6251)
- Sodium Azide (NaN_3) (Sigma # S2002)
- Sodium Fluoride (NaF) (Sigma # S6521)
- Triton™ X-100 (Sigma # T9284)
- Pipettes and pipette tips
- Deionized or distilled water
- 96 well microplates (R&D Systems, Catalog # DY990).
- 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 Na_2HPO_4, 1.5 mM KH_2PO_4, 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% NaN_3 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 96-well plate.*

Lysis Buffer #13** - 1 mM EDTA, 0.5% Triton X-100, 10 mM NaF, 150 mM NaCl, 20 mM β-Glycerophosphate, 25 µg/mL Leupeptin, 25 µg/mL Pepstatin, 3.0 µg/mL Aprotinin in PBS, pH 7.2-7.4.

Substrate Solution - 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems, Catalog # DY999).

Stop Solution - 2 N H_2SO_4 (R&D Systems, Catalog # DY994).

*The use of R&D Systems Reagent Diluent Concentrate 2 (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.
**Alternatively, use Sample Diluent Concentrate 1 (5X) (R&D Systems, Catalog # DYC001), prepared as described in the DYC001 package insert.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human Phospho-p53 (S15) Capture Antibody (Part 841496) - Each vial contains 360 µg/mL of mouse anti-human p53 antibody when reconstituted with 200 µL of PBS.

Human Phospho-p53 (S15) Detection Antibody (Part 841497) Each vial contains 14.4 µg/mL of biotinylated rabbit anti-human phospho-p53 (S15) antibody when reconstituted with 1.0 mL of IC Diluent #1. Immediately before use, dilute the detection antibody to a working concentration of 400 ng/mL in IC Diluent #1. Prepare only as much detection antibody as required to run each assay.

Human Phospho-p53 (S15) Standard (Part 841498) - **Reconstitute with a recommended volume of 500 µL of IC Diluent #4 to produce a stock solution. Refer to the vial label for the concentration of recombinant human phospho-p53.** A seven point standard curve using 2-fold serial dilutions and a high standard of 20,000 pg/mL is recommended.

Streptavidin-HRP A (Part 890803) - 1.0 mL of streptavidin conjugated to horseradish-peroxidase. Immediately before use, dilute the Streptavidin-HRP A to the working concentration specified on the vial label using IC Diluent #1.

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 x 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.

GENERAL ELISA PROTOCOL

Plate Preparation

1. Dilute the capture antibody to the working concentration of 2.0 µg/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 20,000 pg/mL is recommended.*
2. Repeat the aspiration/wash as in step 2 of the Plate Preparation.
3. 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 the Plate Preparation.
5. Add 100 µL of the diluted Streptavidin-HRP A 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.