Surveyor™ IC

Human Phospho-TOR (S2448) Immunoassay

Catalog Number SUV1665

For the quantitative determination of Target of Rapamycin (TOR) phosphorylated at S2448 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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INTRODUCTION

The mammalian Target of Rapamycin (TOR) is a serine/threonine kinase that is a member of the PI 3-kinase-related kinase (PIKK) family. The TOR kinase, a downstream target of the PI 3-kinase/Akt signal transduction pathway, controls cell growth, size, proliferation, and survival (1-4). TOR is the protein target of rapamycin.

TOR is phosphorylated at S2448 in a wortmannin-sensitive manner, and the region of TOR surrounding S2448 has been shown to be part of a repressor domain. Incubation with an anti-TOR antibody against this region or deletion of this region of TOR enhances kinase activity. Recent evidence suggests that TOR is phosphorylated at S2448 by the S6 protein kinase 1 (S6K1) (5, 6), rather than a direct phosphorylation by the Akt protein kinase. This S2448 phosphorylation activates TOR to effect the downstream control of cell growth and survival.

PRINCIPLE OF THE ASSAY

This Surveyor IC Immunoassay employs a two-site sandwich ELISA to quantitate TOR phosphorylated at S2448 in cell lysates. An antibody specific for human TOR, binding both phosphorylated and unphosphorylated protein, has been pre-coated onto a microplate. Standards and samples are added and any TOR present is bound by the immobilized antibody. After washing away unbound material, a biotinylated detection antibody recognizing human TOR phosphorylated at S2448 is used to detect only phosphorylated protein utilizing a standard streptavidin-HRP format. Substrate Solution is added to the wells and color develops in proportion to the amount of TOR phosphorylated at S2448 bound in the initial step. The color development is stopped, and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- This Surveyor IC Immunoassay should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Sample Diluent (diluted 1:5) and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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 remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

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

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

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

MATERIALS PROVIDED & STORAGE CONDITIONS

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

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Phospho-TOR (S2448) Microplate	841953	96 well polystyrene microplate (12 strips of 8 wells) coated with a goat polyclonal antibody against TOR.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Phospho-TOR (S2448) Standard	841742	2 vials (210 ng/vial) of recombinant human phospho-TOR in a buffered protein base with preservatives; lyophilized	Use within 1 hour of reconstitution. Use a fresh standard for each assay.
Phospho-TOR (S2448) Detection Antibody	841954	15 µg of a biotinylated rabbit anti-phospho-TOR (S2448) polyclonal antibody; lyophilized.	May be stored for up to 1 month at 2-8 °C.*
Lysis Buffer 13	895899	21 mL of a cell lysing buffer with phosphatase inhibitors and preservatives.	
Sample Diluent Concentrate 1 (5X)	895562	21 mL of a concentrated buffer with preservatives. *Used diluted 1:5 in this assay.*	
Reagent Diluent Concentrate 2 (10X)	841380	21 mL of a 10-fold concentrated solution of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Streptavidin-HRP	890803	1.0 mL of streptavidin conjugated to horseradish-peroxidase.	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

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

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- 100 mL and 500 mL graduated cylinders.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Phosphate-buffered saline (PBS).
- Phenylmethylsulfonylfluoride (PMSF) (optional; Sigma, Catalog # P7626).
- Protease Inhibitor Cocktail (optional; Sigma, Catalog # P2714).
- **Polypropylene** test tubes for diution of standards and samples.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Sample Diluent 1 (diluted 1:5) - Add 20 mL of Sample Diluent Concentrate 1 (5X) to deionized or distilled water to prepare 100 mL of Sample Diluent 1 (diluted 1:5). Prepare only enough diluent to run the assay.

Reagent Diluent 2 - Add 5 mL of Reagent Diluent Concentrate 2 (10X) to deionized or distilled water to prepare 50 mL of Reagent Diluent 2.

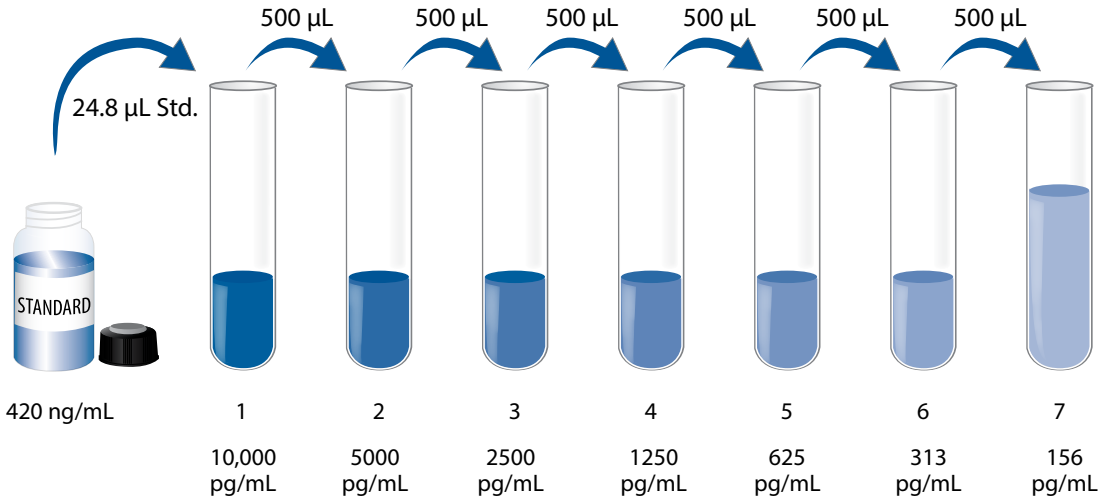
Phospho-TOR (S2448) Detection Antibody - Reconstitute the Phospho-TOR (S2448) Detection Antibody with 1.0 mL Reagent Diluent 2. This reconstitution produces a stock solution of 15 µg/mL. Immediately before use, dilute the Detection Antibody to a working concentration of 1.0 µg/mL in Reagent Diluent 2.

Streptavidin-HRP - Immediately before use, dilute Streptavidin-HRP to the working concentration specified on the vial label using Reagent Diluent 2.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Prepare only enough Substrate Solution as needed to run the assay. Protect from light. 100 µL of the resultant mixture is required per well.

Phospho-TOR (S2448) Standard - Reconstitute the Phospho-TOR (S2448) Standard with 0.5 mL of Sample Diluent 1 (diluted 1:5). This reconstitution produces a stock solution of 420 ng/mL. Mix the standard to ensure complete reconstitution. **Allow the standard to sit for a minimum of 15 minutes.**

Label seven **polypropylene tubes** 1 through 7. Add 976.2 µL of Sample Diluent 1 (diluted 1:5) into tube 1 and 500 µL of Assay Diluent into tubes 2 through 7. Add 24.8 µL of the 420 ng/mL Standard to tube 1. Mix thoroughly and continue to prepare a seven point standard curve using 2-fold serial dilutions by transferring 500 µL from tube 1 into tube 2 and subsequent 500 µL transfers as shown below. Use Sample Diluent 1 (diluted 1:5) as the zero standard. **Use a fresh standard for each assay. Use within 1 hour of preparation.**



CELL LYSIS PROCEDURE

Note: *It is recommended to supplement Lysis Buffer 13 with PMSF and the Protease Inhibitor Cocktail prior to use. PMSF and Protease Inhibitor Cocktail supplements should be used according to the manufacturer's instructions.*

1. Using PBS, collect non-adherent cells by centrifugation or adherent cells by scraping the culture flask.
2. Rinse cells two times with PBS, making sure to remove any remaining PBS after the second rinse.
3. Solubilize cells at 1 x 10^7 cells/mL in Lysis Buffer 13.
4. Incubate on ice for 15 minutes. Centrifuge at 2000 x g for 5 minutes, and transfer the supernates into a clean test tube. Assay samples immediately or aliquot and store at ≤ -20 °C in a manual defrost freezer. Sample protein concentration may be quantified using a total protein assay.
5. If needed, further dilute samples in Sample Diluent 1 (diluted 1:5).

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Standard or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
4. Aspirate each well and wash, repeating the process two times for a total of three 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 to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Immediately before use, prepare the Phospho-TOR (S2448) Detection Antibody. Add 100 µL of diluted Detection Antibody to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
6. Repeat the aspiration/wash as in step 4.
7. Immediately before use, prepare the Streptavidin-HRP. Add 100 µL of the diluted Streptavidin-HRP to each well. Incubate for 20 minutes at room temperature.
8. Repeat the aspiration/wash as in step 4.
9. Immediately before use, prepare the Substrate Solution. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. **Avoid placing the plate in direct light.**
10. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 30 minutes, 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

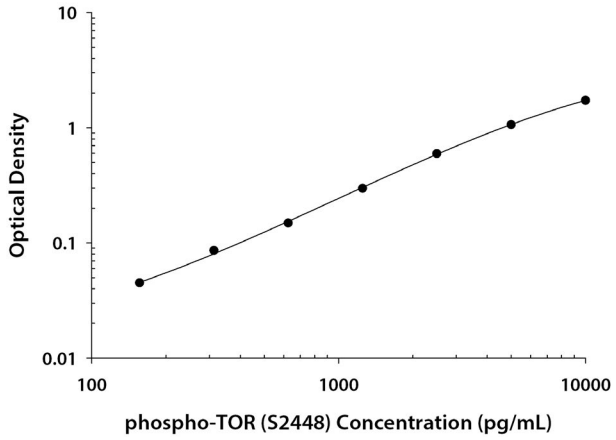
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.). Results may be normalized to total protein or cell number.

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-TOR (S2448) 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.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

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-TOR (S2448) Surveyor IC Immunoassay. The standard curve was calculated using a computer generated 4-PL curve-fit. This standard curve is provided for demonstration purposes only.



(pg/mL)	O.D.	Corrected	Average
0	0.090 0.090	—	—
156	0.154 0.160	0.064 0.070	0.067
313	0.200 0.203	0.110 0.113	0.112
625	0.303 0.325	0.213 0.235	0.224
1250	0.483 0.499	0.393 0.409	0.401
2500	0.810 0.817	0.720 0.727	0.724
5000	1.295 1.369	1.205 1.279	1.242
10,000	1.990 2.138	1.900 2.048	1.974

CALIBRATION

The Human Phospho-TOR (S2448) Surveyor IC Immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human phospho-TOR (S2448) produced at R&D Systems. Samples containing natural phospho-TOR (S2448) showed linear dilution parallel to the standard curve obtained using the Phospho-TOR (S2448) Standard. These results indicate that O.D. values from this Surveyor IC Immunoassay can be used to determine the concentration of human phospho-TOR (S2448) in natural samples.

SPECIFICITY

The Human Phospho-TOR (S2448) Surveyor IC Immunoassay specifically recognizes human TOR phosphorylated at S2448. Specificity was demonstrated by Western blot analysis of the protein bound by the capture antibody supplied in the ELISA.

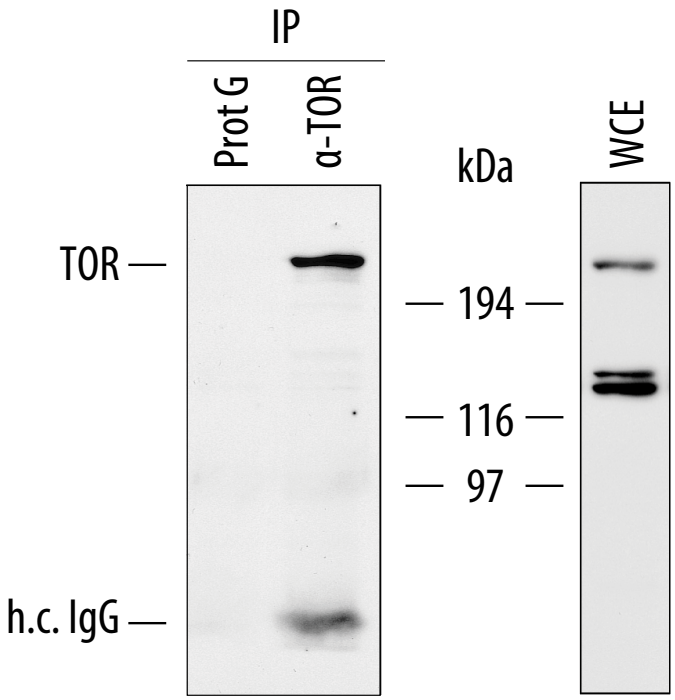


Figure 1: The Phospho-TOR (S2448) Surveyor IC Capture Antibody bound to Protein G agarose was used to immunoprecipitate TOR from cell lysates of MCF-7 cells (α-TOR). As a control, Protein G agarose (Prot G) was incubated with the same MCF-7 lysate. The agarose beads were washed and bound material was solubilized in SDS gel sample buffer. The same lysate (WCE) and immunoprecipitated proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with Phospho-TOR (S2448) Detection Antibody. A single band corresponding to phospho-TOR (S2448) was detected in the captured sample.

QUANTIFICATION

Amounts of human phosphorylated TOR (S2448), as quantified by the Human Phospho-TOR (S2448) Surveyor IC Immunoassay, are consistent with the relative amounts of phosphorylated TOR determined by qualitative Western blot analysis.

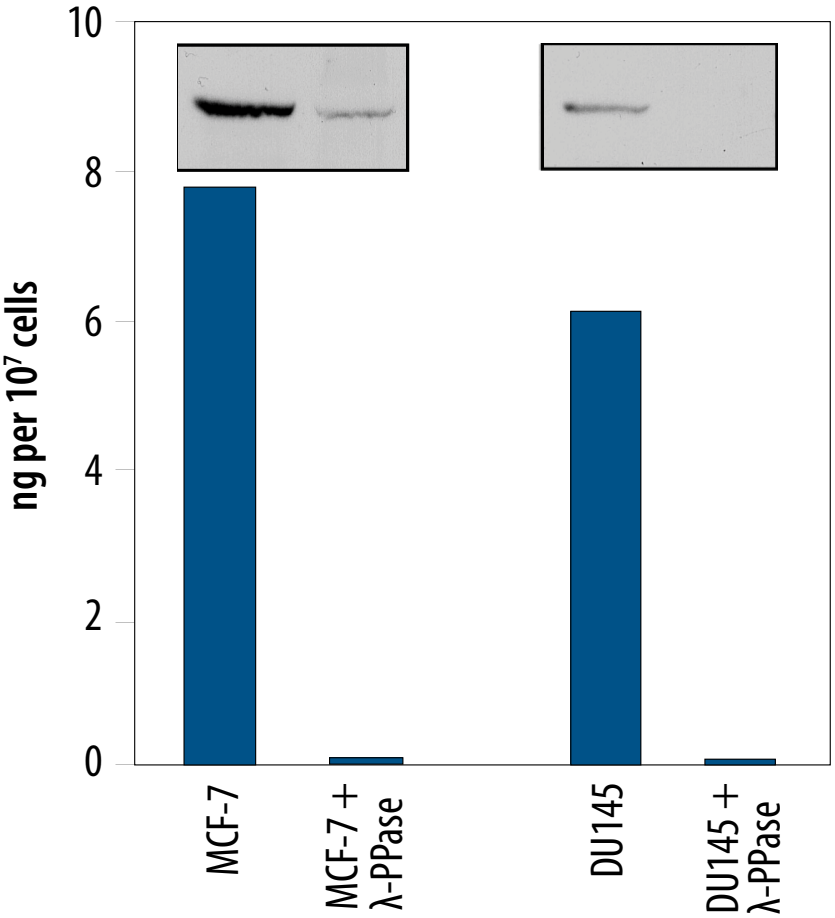


Figure 2: Exponentially growing MCF-7 and DU145 cells were harvested and cell lysates were prepared. The indicated samples were treated with λ-phosphatase (λ-PPase) prior to analysis. Phosphorylated human TOR was quantified with this Surveyor IC Immunoassay, and the same cell lysates were immunoblotted (inset) with anti-phospho-TOR (S2448) (R&D Systems, Catalog # AF1665). The Surveyor IC Immunoassay results correlate well with the relative amounts of human phospho-TOR (S2448) detected by Western blot.

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