Revision 2

Store at +4C

#28384

PathScan® Phospho-Ubiquitin (Ser65) Sandwich ELISA Kit

Cell Signaling TECHNOLOGY®

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1 Kit (96 assays)

Species Cross Reactivity: H

UniProt ID: #P62987, #P0CG48, #P0CG47, #P62979

Entrez-Gene Id: #7311, #7316, #7314, #6233

For Research Use Only. Not for Use in Diagnostic Procedures.

Product Includes	Product #	Quantity	Color	Storage Temp
Phospho-Ubiquitin (Ser65) Rabbit mAb Coated Microwells	42815	96 tests		+4C
Ubiquitin Mouse Detection mAb	60737	1 ea	Green (Lyophilized)	+4C
Anti-mouse IgG, HRP-linked Antibody (ELISA Formulated)	13304	1 ea	Red (Lyophilized)	+4C
Detection Antibody Diluent	13339	11 ml	Green	+4C
HRP Diluent	13515	11 ml	Red	+4C
TMB Substrate	7004	11 ml		+4C
STOP Solution	7002	11 ml		+4C
Sealing Tape	54503	2 ea		+4C
ELISA Wash Buffer (20X)	9801	25 ml		+4C
ELISA Sample Diluent	11083	25 ml	Blue	+4C
Cell Lysis Buffer (10X)	9803	15 ml		-20C

Kit contents scale proportionally with size, except sealing tape.
Example: The V1 kit contains 5X the listed quantities above, but will exclude the sealing tape.

The microwell plate is supplied as 12 8-well modules - Each module is designed to break apart for 8 tests.

Description

The PathScan® Phospho-Ubiquitin (Ser65) Sandwich ELISA Kit is a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) that detects endogenous levels of ubiquitin protein phosphorylated at Ser65. A phospho-ubiquitin (Ser65) rabbit antibody has been coated onto the microwells. After incubation with cell lysates, phospho-ubiquitin (Ser65) protein is captured by the coated antibody. Following extensive washing, a ubiquitin mouse detection antibody is added to detect the captured phospho-ubiquitin (Ser65) protein. Anti-mouse IgG, HRP-linked Antibody is then used to recognize the bound detection antibody. HRP substrate, TMB, is added to develop color. The magnitude of the absorbance for this developed color is proportional to the quantity of ubiquitin protein phosphorylated at Ser65.

*Antibodies in this kit are custom formulations specific to kit.

Specificity/Sensitivity

The PathScan® Phospho-Ubiquitin (Ser65) Sandwich ELISA Kit detects endogenous levels of ubiquitin protein phosphorylated at Ser65. The kit sensitivity is shown in Figure 1. This kit detects proteins from the indicated species, as determined through in-house testing, but may also detect homologous proteins from other species.

Background

Ubiquitin is a conserved polypeptide unit that plays an important role in the ubiquitin-proteasome pathway. Ubiquitin can be covalently linked to many cellular proteins by the ubiquitination process, which targets proteins for degradation by the 26S proteasome. Three components are involved in the target protein-ubiquitin conjugation process. Ubiquitin is first activated by forming a thiolester complex with the activation component E1; the activated ubiquitin is subsequently transferred to the ubiquitin-carrier protein E2, then from E2 to ubiquitin ligase E3 for final delivery to the epsilon-NH_2 of the target protein lysine residue (1-3). The ubiquitin-proteasome pathway has been implicated in a wide range of normal biological processes and in disease-related abnormalities. Several proteins such as IκB, p53, cdc25A, and Bcl-2 have been shown to be targets for the ubiquitin-proteasome process as part of regulation of cell cycle progression, differentiation, cell stress response, and apoptosis (4-7).

Ubiquitin is phosphorylated at Ser65 by PINK1, leading to activation of the E3 ubiquitin ligase Parkin (8,9). PINK1 accumulates on depolarized mitochondria, resulting in phosphorylation of ubiquitin and activation of Parkin, which then triggers the mitophagy pathway to clear damaged mitochondria. Loss-of-function mutations in PINK1 and Parkin result in early onset Parkinson's disease (10,11).

Background References

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2. Hochstrasser, M. (2000) *Nat Cell Biol* 2, E153-7.
3. Hochstrasser, M. (2000) *Science* 289, 563-4.
4. Bernardi, R. et al. (2000) *Oncogene* 19, 2447-54.
5. Aberle, H. et al. (1997) *EMBO J* 16, 3797-804.
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7. Jesenberger, V. and Jentsch, S. (2002) *Nat Rev Mol Cell Biol* 3, 112-21.
8. Kane, L.A. et al. (2014) *J Cell Biol* 205, 143-53.
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Trademarks and Patents

Limited Uses

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PathScan® Phospho-Ubiquitin (Ser65) Sandwich ELISA Kit

ELISA Colorimetric (Lyophilized)

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **Microwell strips**: Bring all to room temperature before use.
2. **Detection Antibody**: Supplied lyophilized as a green colored cake or powder. Add 1.0 ml of Detection Antibody Diluent (green solution) to yield a concentrated stock solution. Incubate at room temperature for 5 min with occasional gentle mixing to fully reconstitute. To make the final working solution, add the 1.0 ml volume of reconstituted Detection Antibody to 10.0 ml of Detection Antibody Diluent in a clean tube and gently mix. Unused working solution may be stored for 4 weeks at 4°C.
3. **HRP-Linked Antibody***: Supplied lyophilized as a red colored cake or powder. Add 1.0 ml of HRP Diluent (red solution) to yield a concentrated stock solution. Incubate at room temperature for 5 min with occasional gentle mixing to fully reconstitute. To make the final working solution, add the 1.0 ml volume of reconstituted HRP-Linked Antibody to 10.0 ml of HRP Diluent in a clean tube and gently mix. Unused working solution may be stored for 4 weeks at 4°C.
4. **Detection Antibody Diluent**: Green colored diluent for reconstitution and dilution of the detection antibody.
5. **HRP Diluent**: Red colored diluent for reconstitution and dilution of the HRP-Linked Antibody.
6. **Sample Diluent**: Blue colored diluent provided for dilution of cell lysates.
7. **1X Wash Buffer**: Prepare by diluting 20X Wash Buffer (included in each PathScan® Sandwich ELISA Kit) in purified water.
8. **Cell Lysis Buffer**: 10X Cell Lysis Buffer #9803: This buffer can be stored at 4°C for short-term use (1–2 weeks). Recommended: Add 1 mM phenylmethylsulfonyl fluoride (PMSF) immediately before use.
9. **TMB Substrate** (#7004).
10. **STOP Solution** (#7002).

***NOTE**: Some PathScan® ELISA Kits may include HRP-Linked Streptavidin in place of HRP-Linked Antibody.

B. Preparing Cell Lysates

For adherent cells.

1. Aspirate media when the culture reaches 80–90% confluence. Treat cells by adding fresh media containing regulator for desired time.
2. Remove media and rinse cells once with ice-cold 1X PBS.
3. Remove PBS and add 0.5 ml ice-cold 1X Cell Lysis Buffer plus 1 mM PMSF to each plate (10 cm diameter) and incubate the plate on ice for 5 min.
4. Scrape cells off the plate and transfer to an appropriate tube. Keep on ice.
5. Sonicate lysates on ice.
6. Microcentrifuge for 10 min (x14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at −80°C in single-use aliquots.

For suspension cells

1. Remove media by low speed centrifugation (~1200 rpm) when the culture reaches 0.5–1.0 x 10^6 viable cells/ml. Treat cells by adding fresh media containing regulator for desired time.
2. Collect cells by low speed centrifugation (~1200 rpm) and wash once with 5–10 ml ice-cold 1X PBS.
3. Cells harvested from 50 ml of growth media can be lysed in 2.0 ml of 1X Cell Lysis Buffer plus 1 mM PMSF.
4. Sonicate lysates on ice.
5. Microcentrifuge for 10 min (x14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at −80°C in single-use aliquots.

C. Test Procedure

1. After the microwell strips have reached room temperature, break off the required number of microwells. Place the microwells in the strip holder. Unused microwells must be resealed and stored at 4°C immediately.
2. Cell lysates can be undiluted or diluted with Sample Diluent (supplied in each PathScan® Sandwich ELISA Kit, blue color). Individual datasheets for each kit provide a sensitivity curve that serves as a reference for selection of an appropriate starting lysate concentration. The sensitivity curve shows typical kit assay results across a range of lysate concentration points.
3. Add 100 µl of each undiluted or diluted cell lysate to the appropriate well. Seal with tape and press firmly onto top of microwells. Incubate the plate for 2 hr at 37°C. Alternatively, the plate can be incubated overnight at 4°C.

4. Gently remove the tape and wash wells:
    1. Discard plate contents into a receptacle.
    2. Wash 4 times with 1X Wash Buffer, 200 µl each time for each well.
    3. For each wash, strike plates on fresh towels hard enough to remove the residual solution in each well, but do not allow wells to completely dry at any time.
    4. Clean the underside of all wells with a lint-free tissue.
5. Add 100 µl of reconstituted Detection Antibody (green color) to each well (refer to Section A, Step 2). Seal with tape and incubate the plate at 37°C for 1 hr.
6. Repeat wash procedure (Section C, Step 4).
7. Add 100 µl of reconstituted HRP-Linked secondary antibody (red color) to each well (refer to Section A, Step 3). Seal with tape and incubate the plate for 30 min at 37°C.
8. Repeat wash procedure (Section C, Step 4).
9. Add 100 µl of TMB Substrate to each well. Seal with tape and incubate the plate for 10 min at 37°C or 30 min at 25°C.
10. Add 100 µl of STOP Solution to each well. Shake gently for a few seconds.

NOTE: Initial color of positive reaction is blue, which changes to yellow upon addition of STOP Solution.

11. Read results.
    1. Visual Determination: Read within 30 min after adding STOP Solution.
    2. Spectrophotometric Determination: Wipe underside of wells with a lint-free tissue. Read absorbance at 450 nm within 30 min after adding STOP Solution.

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