INSTRUCTIONS

Ubiquitin Enrichment Kit

PIERCE
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89899

1734.0

Number	Description
89899	**Ubiquitin Enrichment Kit,** contains sufficient materials for enriching up to 15 lysate samples containing ~0.15 mg total protein per sample

Kit Contents:

Pack 1

Polyubiquitin Positive Control (1,000X), 50 µl, 2 mg/ml

Anti-ubiquitin Antibody, 50 µl rabbit antiserum

Pack 2

Polyubiquitin Affinity Resin, 300 µl, supplied as a 25% slurry

Binding Capacity: ~1 µg per 20 µl of slurry

BupH™ Tris Buffered Saline Pack, 1 each, results in 0.025 M Tris, 0.15 M NaCl; pH 7.2 when reconstituted with 500 ml of ultrapure water

Spin Columns and Accessories, includes 18 columns, top and bottom caps

Storage: Upon receipt store Pack 1 at -20°C and Pack 2 at 4°C. Do not freeze the Polyubiquitin Affinity Resin. Pack 1 is shipped with dry ice and Pack 2 is shipped with an ice pack.

Introduction

The Ubiquitin Enrichment Kit is for the isolation and study of intracellular polyubiquitin-modified proteins. Through the use of a high-binding affinity resin, polyubiquitinated proteins are isolated from cell or tissue lysates. The bound proteins can then be eluted from the affinity resin and analyzed using the anti-ubiquitin antibody.

Cells degrade proteins by first covalently tagging lysine residues on the protein substrate with ubiquitin, a small highly conserved protein. Additional ubiquitin molecules are successively added forming a polyubiquitin chain. Proteins containing these polyubiquitinated chains are recognized by the 26S proteosome and undergo simultaneous proteolytic degradation and recycling of ubiquitin monomers by deubiquitinating enzymes.

The ubiquitin proteosome pathway is the principal mechanism for protein catabolism. This pathway is significantly involved in a variety of cellular processes including: DNA repair, signal transduction, cell metabolism and growth, mutated or post-translationally damaged proteins, and the processing of MHC class I antigens. Differences in total ubiquitination or the ubiquitination of specific proteins affect numerous pathological conditions, including malignancies, certain genetic diseases and neurodegenerative diseases.[1]

This kit contains all the necessary materials for polyubiquitinated protein enrichment from cell or tissue lysates. The polyubiquitin affinity resin has superior binding characteristics compared to other commercially available resins and is compatible with Pierce Poppers Cell Lysis Reagents (see the Related Pierce Products Section) and standard formulations of RIPA buffer. The positive control allows for determining compatibility with other lysis buffers, as well as evaluating resin and antibody performance.

Note: The Polyubiquitin Affinity Resin binds polymers of ubiquitin containing four or more ubiquitin subunits. Mono-ubiquitinated proteins and short chain polymers (< 4 ubiquitin monomers) are recovered in the flow-through.

Procedure for Enrichment of Polyubiquitinated Protein Conjugates

A. Additional Materials Required

- Cell or tissue lysate containing ~0.15 mg total protein per sample
- Lysis buffer (~1.0 ml) – use the same lysis buffer that was used to prepare the lysate samples
- End-over-end rotator
- Microcentrifuge and 1.5 ml tubes
- SDS-PAGE sample loading buffer (e.g., ImmunoPure® Lane Marker Reducing Sample Buffer, Product No. 39000)

B. Material Preparation

Tris Buffered Saline (TBS)	Dissolve the dry-blend buffer with 500 ml of ultrapure water. For long-term storage of excess buffer, sterile-filter the solution and store at 4°C. If desired, add a final concentration of 0.02% sodium azide to the buffer as a preservative.
Wash Buffer	Combine one part lysis buffer with nine parts TBS (e.g., 0.1 ml lysis buffer added to 0.9 ml TBS). Prepare 1.0 ml of Wash Buffer for each enrichment procedure.
Sample	For best results, use a sample containing at least 0.1 mg total protein. In a spin column, dilute sample 1:1 with TBS. Use a total sample volume (i.e., lysate with TBS) of ≥ 200 µl. Samples must be diluted at least 50% with TBS and not more than 90%. **Note:** The spin columns accommodate up to 500 µl of sample. If the sample is greater than 500 µl, perform resin incubation in separate tube(s) then transfer to spin columns.
Polyubiquitin Positive Control	Thaw the Polyubiquitin Positive Control. Mix 500 µl of TBS and 500 µl of lysis buffer in a 1.5 ml microcentrifuge tube. For best results use the identical lysis buffer for the positive control as was used for the sample. Briefly vortex the polyubiquitin tube and add 1 µl to the 1.0 ml TBS/lysis buffer solution. Pipette 300 µl of this solution into a spin column. Store excess control frozen at -20°C.

C. Binding of Polyubiquitinated Proteins

1. To obtain a homogeneous suspension, gently mix the tube of Polyubiquitin Affinity Resin end over end several times.
2. Use a wide-bore or cut pipette tip to transfer 20 µl of slurry to the spin column containing the sample or positive control.
3. Cap the spin column securely with the screw cap and incubate at 4°C for 2 hours to overnight on an end-over-end rotator.

D. Elution of Polyubiquitinated Proteins

1. Remove column plug by twisting it off the column. Place column in a 1.5 ml microcentrifuge tube and centrifuge at 5,000 × *g* for 15 seconds.
2. Remove column from the tube, mark tube as flow-through and save for analysis, if desired.
3. Place bottom cap on the column and remove top cap. Add 300 µl of Wash Buffer to the column, replace top cap and invert column several times. Remove bottom cap, place column in a new tube and centrifuge at 5,000 × *g* for 15 seconds. Repeat this step two additional times and save each wash in a separate tube.
4. Place bottom cap on the column and add 50-75 µl of SDS-PAGE sample loading buffer or IEF sample buffer.
5. Replace top cap and secure tightly. Vortex column for 2-3 seconds.
6. Place the capped column in a tube and loosen the top screw cap. In a heat block or water bath, warm samples according to the manufacturer's instructions for the sample loading buffer.
7. Remove the bottom cap. Centrifuge column and tube assembly at 5,000 × *g* for 30 seconds. Eluate contains ubiquitin-enriched fraction ready for Western blotting analysis or isoelectric focusing.

Example Procedure for Western Blotting

This protocol has been optimized using the indicated materials. Using materials other than those listed may require additional optimization.

A. Materials Required

- Precise™ Protein Gel, 4-20% (Product No. 25244)
- PVDF Transfer Membrane (Product No. 88518 or 88585)
- PBS-T: Phosphate Buffered Saline (Product No. 28374) containing 0.05% Tween®-20 (Product No. 28320)
- Blocking buffer: StartingBlock™ T20 (PBS) Blocking Buffer (Product No. 37539)
- ImmunoPure® Peroxidase Conjugated Goat Anti-rabbit IgG (Product No. 31460)
- SuperSignal® West Dura Chemiluminescent Substrate (Product No. 34075)

B. Method

1. Dilute the Polyubiquitin Positive Control 1:1,000. Apply protein to the gel, electrophorese and transfer protein to a membrane according to standard procedures.
2. Remove membrane from the transfer unit and block membrane with blocking buffer for 15 minutes.
3. Dilute the anti-ubiquitin antibody in blocking buffer 1:7,500-1:10,000.
4. Remove the blocking buffer and apply the diluted antibody to the membrane. Incubate for 2 hours at room temperature or overnight at 4°C.
5. Remove primary antibody solution and rinse membrane once with PBS-T. Wash membrane four times for 10 minutes each with PBS-T on a rocking platform.
6. Dilute the anti-rabbit HRP conjugate 1:100,000-1:200,000 in blocking buffer.
7. Apply the diluted HRP conjugate to the membrane and incubate at room temperature on a rocking platform for 1 hour.
8. Empty the HRP conjugate and rinse membrane once with PBS-T. Wash membrane four times for 10 minutes each with PBS-T on a rocking platform.
9. Add the SuperSignal® West Dura Substrate Working Solution to the membrane and incubate for 5 minutes.
10. Detect emitted chemiluminescent signal by film or CCD imaging instrument.

Note: A Western blot of polyubiquitin typically appears as a high molecular-weight smear caused by heterogeneity of the modified proteins (Figure 1). When probing for a specific ubiquitinated protein, note that a polyubiquitinated protein has an added mass of at least 32 kDa compared to the mass of the non-modified protein.

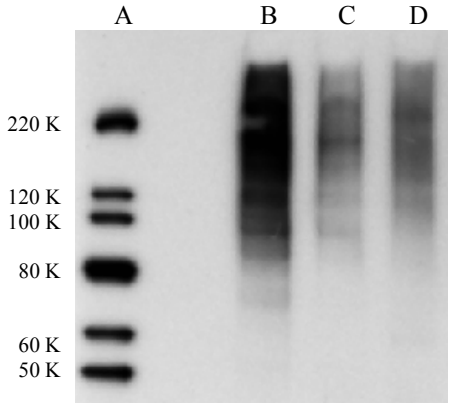

Figure 1. Western blot of polyubiquitin. Epoxomicin-treated HeLa cells were lysed with M-PER® Reagent (Product No. 78501) containing Halt™ Protease Inhibitors (Product No. 78415). Polyubiquitin was detected by performing a Western blot on the total lysate (lane B) and the flow-through and elution (lanes C and D, respectively) from the Polyubiquitin Affinity Resin. Lane A is the molecular weight marker.

Troubleshooting

Problem	Possible Cause	Solution
Cannot detect ubiquitin signal by Western blot	Polyubiquitinated proteins are in low abundance	Increase sample amount added to the resin – if necessary, incubate sample in a larger tube, and then transfer to column
	Improperly performed Western blot	Test polyubiquitin controls in the Western blot
	Incompatible lysis buffer	Determine lysis buffer compatibility by using the polyubiquitin positive control in the enrichment procedure
	Detection system is not functioning properly or requires optimization	Consult instructions for the detection system being used
Excessive amount of ubiquitinated proteins in the flow-through	Overloaded resin	Decrease sample amount while maintaining minimum volume of 200 µl
	Improperly diluted sample	Ensure sample is diluted at least 50% with TBS

Related Pierce Products

78248 **B-PER® Bacterial Protein Extraction Reagent,** 500 ml

78266 **B-PER® Bacterial Protein Extraction Reagent (in phosphate buffer),** 500 ml

78990 **Y-PER® Yeast Protein Extraction Reagent,** 500 ml

78501 **M-PER® Mammalian Protein Extraction Reagent**, 250 ml

78510 **T-PER® Tissue Protein Extraction Reagent,** 500 ml

78415 **Halt™ Protease Inhibitor Cocktail, EDTA-Free,** 1 ml

24585 **MemCode™ Reversible Protein Stain Kit – for PVDF Membranes,** sufficient material for 10 (8 cm × 8 cm) PVDF membranes

88600 **Western Blotting Filter Paper, 8 cm × 10.5 cm,** 100 sheets

21059 **Restore™ Western Blot Stripping Buffer,** 500 ml

34090 **CL-XPosure™ Film (5" × 7"),** 100 sheets

34091 **CL-XPosure™ Film (8"× 10"),** 100 sheets

34089 **CL-XPosure™ Film (18 × 24 cm),** 100 sheets

21065 **Erase-It® Background Eliminator Kit,** for eliminating background from X-ray film

Reference

1. Ciechanover A. (1998). The ubiquitin-proteosome pathway: on protein death and cell life. *EMBO J*. **17(24):**7151-60.

The most current versions of all product instructions are available at *www.piercenet.com.* For a faxed copy, contact customer service (in the USA call 800-874-3723) or your local distributor.