Kit for Enrichment and Detection of Poly-Ubiquitinated Proteins

# CycLex Poly-Ubiquitinated Protein Enrichment & Detection Kit

20 Assays

Cat# CY-7001



## Intended Use

The CycLex Research Product **Poly-Ubiquitinated Protein Enrichment & Detection Kit** is designed to enrich and detect total poly-ubiquitinated proteins in cell lysate. Since the amino acid sequence of ubiquitin is well conserved among eukaryotes, this kit can be used for all eukaryote cells including yeast. This kit is intended for the enrichment and detection of poly-ubiquitinated proteins in cell lysate.

**This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.**

## Storage

- Upon receipt, store the kit at 4°C.
- Don't expose reagents to excessive light.

## Introduction

The ubiquitin-proteasome pathway is the principle pathway of proteolysis in eukaryotic cells and may contribute to controlling the intracellular levels of a variety of short-lived proteins, in addition to degrading abnormal proteins in the cytosol and nucleus. Protein substrates are marked with a poly-ubiquitin chain and then degraded to peptides and free ubiquitin by a large multicatalytic complex, the proteasome, which exists within all eukaryotic cells. Numerous examples of regulatory proteins have been found to undergo ubiquitin-dependent proteolysis.

Protein substrates of the ubiquitin-proteasome pathway include a number of cell regulatory molecules, such as cyclins, the Myc oncogene protein, and p53, and the regulated degradation of these molecules has been linked to the control of cell proliferation and cell cycle progression. By controlling the intracellular levels of such proteins, the activity of the ubiquitin-proteasome pathway might also be linked to apoptosis.

## Principle of the Assay

The CycLex Research **Poly-Ubiquitinated Protein Enrichment & Detection Kit** is for the isolation and study of intracellular poly-ubiquitin-modified proteins. Through the use of a Poly-Ubiquitin Affinity Resin, poly-ubiquitinated proteins are isolated from cell or tissue lysates. The bound proteins can then be eluted from the affinity resin and analyzed using the anti-poly-ubiquitin monoclonal antibody. This kit contains all the necessary materials for poly-ubiquitinated protein enrichment from cell or tissue lysates and for detection of poly-ubiquitinated protein by means of western blotting. The poly-ubiquitinated protein control allows for evaluating performance of resin and antibody.

*The Poly-Ubiquitin Affinity Resin binds polymers of ubiquitin containing four or more ubiquitin subunits. Mono-ubiquitinated proteins and short chain polymers are recovered in the flow-through.*

## Summary of Procedure

Culture cells in culture flask or dish at 50-70 % confluency

↓ Incubate O/N at 37°C in a $CO_2$ incubator

Add appropriate amount of the drug or equivalent for induction of poly-ubiquitination

↓ Incubate appropriate time at 37°C in a $CO_2$ incubator

Harvest the cells by scraping or centrifugation

↓

Make cell lysate by adding extraction buffer and centrifugation

↓

Add 20 µL of Poly-Ubiquitin Affinity Resin to 0.5-1.0 mg of the cell lysate

↓ Incubate for 2-4 hour at 4°C

Wash the resin three times

↓

Add 20 µL of 2X SDS-PAGE sample buffer

↓ Boil the sample for 5 minutes

Perform SDS-PAGE

↓

Perform Western blotting

↓

Detect poly-ubiquitinated proteins using Anti-Poly-Ubiquitin Monoclonal Antibody or protein-specific antibody

## Materials Provided

The following components are supplied and are sufficient for 20 samples of cell lysate.

1. **Poly-Ubiquitin Affinity Resin:** One vial containing 1 mL of 25 % suspension of affinity resin in PBS containing 10 % glycerol and 0.05% $NaN_3$. Store at 4°C.

2. **10X Poly-Ubiquitinated Protein Control:** One vial containing of lyophilized 10X Poly-Ubiquitinated Protein. Reconstitute with 500 µL of $ddH_2O$ just prior to use. Store at below -70°C after reconstitute.

3. **10X Cell Extraction Buffer:** Two vials containing 2 mL each of 10X Cell Extraction Buffer. Store at 4°C.

4. **10X Anti-Poly-Ubiquitin Monoclonal Antibody:** One vial containing 2 mL of 10X Anti-poly-ubiquitin Monoclonal Antibody. Store at 4°C.

5. **10X HRP conjugated Anti-Mouse IgG:** One vial containing 2 mL of 10X HRP conjugated Anti-Mouse IgG. Store at 4°C.

6. **10X Antibody Dilution Buffer:** Two vials containing 2 mL each of 10X Antibody Dilution Buffer. Store at 4°C.

## Materials Required but not Provided

- **Wash Buffer:** 1X TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl containing 0.1 % Tween-20 ®)
- **Protease inhibitor cocktail:** ex. Sigma Cat# P-2714 (reconstituted according to manufacturer's guideline). Add 250 µL per 5 mL Cell Extraction Buffer.
- **2X SDS Sample Buffer:** 125 mM Tris-HCl (pH 6.8 at 25°C), 4% w/v SDS, 10%, glycerol, 100 mM DTT, 0.02% w/v bromophenol blue
- **12.5-7.5 % SDS- polyacrylamide gel**
- **SDS-PAGE Running Buffer:** 25 mM Tris, 92 mM glycine, 0.1% w/v SDS, pH 8.3
- **Blotting Membrane:** PVDF membrane (Milipore)
- **Enhanced chemiluminescence reagent:** ECL™ chemiluminescent reagent (GE Biosciences)
- **Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
- **Blocking Buffer:** 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk.
- **Deionized water of the highest quality ($ddH_2O$)**
- **Microcentrifuge and tubes**
- **Microcentrifuge tube Rotator (End-over-end rotator)**
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1000 µL precision pipetors with disposable tips.
- **Vortex mixer**
- **SDS-PAGE apparatus**
- **Blotting apparatus**
- **Rocking platform**
- **X-ray film or CCD imaging instrument**.

## Precautions and Recommendations

- Do not use kit components beyond the indicated kit expiration date.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality (dd$H_2O$).
- Do not mix reagents from different kits.
- The buffers and reagents used in this kit contain $NaN_3$ as preservatives. Care should be taken to avoid direct contact with these reagents.
- Do not mouth pipette or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**

## Detailed Protocol

The CycLex Research Product **Poly-Ubiquitinated Protein Enrichment & Detection Kit** is provided with 10 times concentrated reagents except Poly-Ubiquitin Affinity Resin. Since experimental conditions may vary, an aliquot of the Poly-Ubiquitinated Protein Control within the kit, should be included in each experiment as a positive control. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

### Preparation of Working Solutions

All reagents, with the exception of Poly-Ubiquitin Affinity Resin, are supplied as 10X concentrate, therefore those should be diluted 10 times prior to the experiment.

1. **Prepare a working solution of Cell Extraction Buffer** by adding 250 µL of Protease inhibitor cocktail (Sigma Cat. # P-2714) to 5 mL of 1X Cell Extraction Buffer (10 times diluted ③10X Cell Extraction Buffer that is provided in the kit). Mix well. Unused buffer should be stored at -20°C.

2. **Reconstitute 10X Poly-Ubiquitinated Protein Control** with 0.5 mL of $ddH_2O$. Dilute the reconstituted ②10X Poly-Ubiquitinated Protein Control 1:10 with Cell Extraction Buffer prepared above.

   *Unused portions of ②10X Poly-Ubiquitinated Protein Control should be aliquoted and stored at below -70 °C immediately. Avoid multiple freeze and thaw cycles.*

3. **Prepare a working solution of Antibody Dilution Buffer** by adding 1 mL of ⑥10X Antibody Dilution Buffer to 9 mL of $ddH_2O$. Store at 4°C.

4. **Prepare a working solution of Anti-Poly-Ubiquitin Monoclonal Antibody** by adding 0.5 mL of ④ 10X Anti-Poly-Ubiquitin Monoclonal Antibody to 4.5 mL of Antibody Dilution Buffer prepared above.

   *Store at 4°C. Working solution of Anti-Poly-Ubiquitin Monoclonal Antibody should be used within a week.*

5. **Prepare a working solution of HRP conjugated Anti-Mouse IgG** by adding 0.5 mL ⑤**10X HRP conjugated Anti-Mouse IgG** to 4.5 mL of Antibody Dilution Buffer prepared above.

   *Store at 4°C. Working solution HRP conjugated Anti-Mouse IgG should be used within a day.*

## Preparation of Other Reagents not provided in this kit

1. 10X TBS (Tris-buffered saline): For 1 liter of 10X TBS, use 24.2 g Tris base and 80 g NaCl. Adjust pH to 7.5 with HCl (use at 1X).

2. Wash Buffer: 1X TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl containing 0.1 % Tween-20 ®)

3. Transfer Buffer: 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)

4. Blocking Buffer: 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk. For 150 ml, add 15 ml 10X TBS to 135 ml water, mix. Add 7.5 g nonfat dry milk and mix well. While stirring, add 0.15 ml Tween-20 (100%).

5. 2X SDS Sample Buffer: 125 mM Tris-HCl (pH 6.8 at 25°C), 4% w/v SDS, 10%, glycerol, 100 mM DTT, 0.02% w/v bromophenol blue

6. 10X SDS-PAGE Running Buffer: 38.28 g of Tris, 144.11 g of glycine, 10 g of SDS, fill up to 1 L with $ddH_2O$, pH 8.3

## Procedure

### A. Treatment of Cells

1. Plate adherent cells or non-adherent cells in culture dish or flasks at 50-70 % confluency.
2. Incubate the culture dish or flasks at 37°C over night in $CO_2$ incubator.
3. Add appropriate amount of test compound or equivalent to each dish or flask.
4. Incubate the culture flasks at 37°C for appropriate time.

### B. Cell Extraction

***Note:*** *This protocol has been successfully applied to several cell lines. Users should optimize the cell extraction procedure for their own applications.*

1. Collect cells in PBS by centrifugation (non-adherent cells) or scraping from culture flasks (adherent cells).
2. Wash cells twice with cold PBS.
3. Remove and discard the supernatant and collect the cell pellet.
   *At this point the cell pellet can be frozen at below -70°C and lysed at a later date.*
4. Lyse the cell pellet in **0.5 mL*** of Cell Extraction Buffer for 30 minutes, on ice, with vortexing at 10-minute intervals.

   * *To get a rough idea you could adjust the cell concentration to around 2 x $10^7$ cells/mL. Resulting protein concentration of the cell lysate should be 2-4 mg/mL using this Cell Extraction Buffer.*

   * *The volume of Cell Extraction Buffer depends on the cell line, the cell number in cell pellet and the amount of poly-ubiquitinated protein. For example, 1 x $10^7$ MCF-7 cells can be extracted in 0.5 mL of Cell Extraction Buffer.*

5. Transfer the lysate to microcentrifuge tubes and centrifuge at 15,000 rpm for 15 minutes at 4°C.
6. Aliquot the clear extract to clean microcentrifuge tubes. These cell lysates are ready for assay. The cell lysate can be stored at below -70°C. Avoid multiple freeze/thaw cycles. After thaw the cell lysate, Centrifuge at 15,000 rpm for 15 minutes at 4°C again since the cell lysate should be clear of any sediments or particulate matter.

**NOTE: THE ABOVE PROCEDURES ARE INTENDED ONLY AS A GUIDELINE. THE OPTIMAL EXPERIMENTAL CONDITIONS WILL VARY DEPENDING ON THE PARAMETERS BEING INVESTIGATED, AND MUST BE DETERMINED BY THE INDIVIDUAL USER. NO WARRANTY OR GUARANTEE OF PERFORMANCE USING THESE PROCEDURES IS MADE OR IMPLIED.**

**C. Binding of Poly-Ubiquitinated Proteins**

1. Resuspend the ①**Poly-Ubiquitin Affinity Resin** by gentle inversion, until the beads are completely unpacked.
2. Use a wide-bore or cut pipette tip (by cutting off the terminal 3 mm with a razor blade) to transfer 40 µL of the ①**Poly-Ubiquitin Affinity Resin** suspension to the microcentrifuge tubes.
3. Add the 0.5-1.0 mg of **the cell lysate** or 0.5 mL of **Poly-Ubiquitinated Protein Control**, at a concentration of ~2 mg/mL to the microcentrifuge tube containing the Poly-Ubiquitin Affinity Resin.
4. Incubate at 4°C for 2-4 h with constant mixing to keep the Poly-Ubiquitin Affinity Resin well suspended using an End-over-end rotator. Avoid aeration or vigorous mixing.
5. Carefully remove the supernatant after centrifugation for 5 seconds at 4°C in a microcentrifuge (~1,000 X g), and resuspend in 1 ml of pre-cooled Wash buffer. Repeat three more times.
6. Suspend the affinity matrix in 20 µL of 2X SDS Sample Buffer and boil for 5 min.

**D. SDS-PAGE and Western Blotting**

1. Centrifuge the sample for 1 min at full speed in a microcentrifuge, and apply 10 µL of the supernatant to an 8-12% SDS-PAGE.
2. Transfer the resolved proteins to PVDF membrane.
3. Rinse the PVDF membrane with 50 mL of Wash Buffer.
4. Block the PVDF membrane for 1 hr to O/N on a rocking platform with Blocking Buffer at room temperature.
5. Incubate the PVDF membrane for 60 minutes on a rocking platform with 5 mL of **Anti-Poly-Ubiquitin Monoclonal Antibody** or primary antibody of your interest.
6. Wash the PVDF membrane 4 times with 100 mL of Wash Buffer for 10 min each on a rocking platform.
7. Incubate with 5 mL of **HRP conjugated Anti-Mouse IgG** or appropriate HRP conjugated secondary antibody for 60 min on a rocking platform.
8. Wash the PVDF membrane 4 times with 100 mL of Wash Buffer for 10 min each on a rocking platform.
9. Develop with enhanced chemiluminescence to maximize detection.
10. Detect emitted chemiluminescent signal by film or CCD imaging instrument.

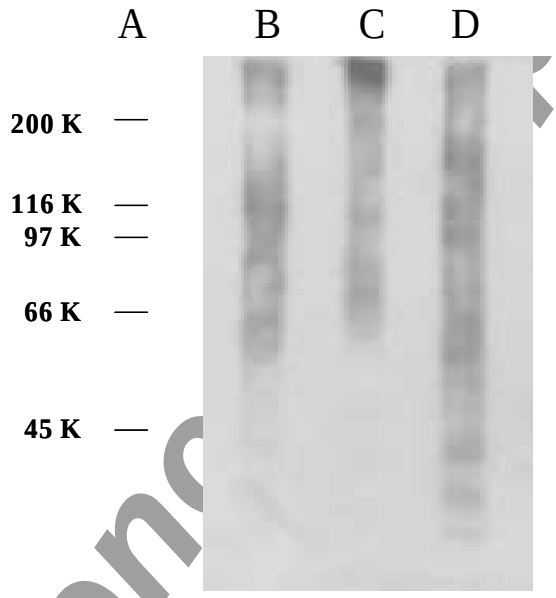
**Note:** A western blot of polyubiquitin typically appears as a high molecular-weight smear caused by heterogeneity of the modified proteins

## Reagent Stability

All of the reagents included in the CycLex Research Product **Poly-Ubiquitinated Protein Enrichment & Detection Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, kit reagents should be stored at 4°C, except the reconstituted Poly-Ubiquitinated Protein Standard must be stored at below -70°C.

## Example of Test Results

Fig.1 Western Blot of Poly Ubiquitinated Protein: Breast cancer cell line, MCF-7 was treated with 10 μM MG132 for 8 hours. Poly-ubiquitinated proteins were detected by performing a Western blot on the total lysate (lanes D) and the flowthrough and elution (lanes B and C, respectively) from the Poly-Ubiquitin Affinity Resin. Lane A is the molecular weight marker.



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## Related Products

*** CycLex Poly-Ubiquitinated Protein Enrichment & Detection Kit: Cat# CY-7001**
*** CycLex Proteasome Enrichment & Activity Assay Kit: Cat# CY-7002**
*** CycLex Poly-Ubiquitinated Protein ELISA Kit: Cat# CY-7053**

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