Kit for Enrichment and Activity Assay of Proteasome

CycLex Proteasome Enrichment & Activity Assay Kit

20 Assays

Cat# CY-7002

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Intended Use

The CycLex Research Product **Proteasome Enrichment & Activity Assay Kit** is designed to enrich proteasome from cell lysate and measure its protease activity. Since the amino acid sequence of ubiquitin-like domain in hHR23B is well conserved among mammalian, this kit can be used for all mammalian cells.

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.

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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 (1-3), in addition to degrading abnormal proteins in the cytosol and nucleus. Protein substrates are marked with a poly-ubiquitin chain (4) and then degraded to peptides and free ubiquitin by a large multicatalytic complex, the proteasome, which exists within all eukaryotic cells (1-3). 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 (5-7). By controlling the intracellular levels of such proteins, the activity of the ubiquitin-proteasome pathway might also be linked to apoptosis.

A class of proteins containing an N-terminal ubiquitin-like (UbL) domain has been implicated in the regulation of proteolysis (8-13). A member of this superfamily, Saccharomyces cerevisiae Rad23 was first identified for its role in DNA repair (14). For the human homolog of Rad23, two proteins hHR23A (363 residues) and hHR23B (409 residues) exist, which share an identity of 59 % on the amino acid level. Differences in function between the two homologs are unclear (15). hHR23A and hHR23B also contain N-terminal 80 residues, UbL domain that have been shown to interact with the 26S proteasome. Both UbL domain fused to GST was shown to pull down 20S proteasome subunits containing chymotrypsin-like proteolytic activity.

Principle of the Assay

The CycLex Research **Proteasome Enrichment & Activity Assay Kit** is for the isolation and study of intracellular proteasome proteins. Through the use of a hHR23B ubiquitin-like domain resin (UbL-Resin), 20S proteasome are isolated from cell or tissue lysate. The bound 20S proteasome protease activity can be measured by fluorogenic Proteasome Substrate (Suc-LLVY-AMC). In addition, this kit can be used to study 20S proteasome function and interactions with other proteins. The proteasome subunits can be identified by loading the UbL-Resin directly onto an SDS-PAGE gel and immunoblotting with subunit specific antibodies. This kit contains all the necessary materials for 20S proteasome enrichment from cell or tissue lysates and for detection of proteolytic activity using proteasome substrate.

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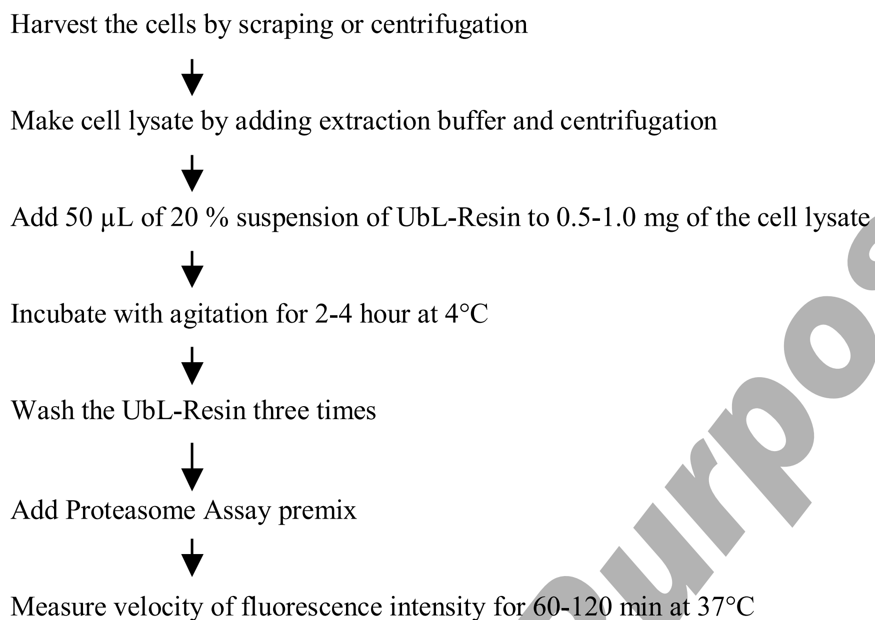
Summary of Procedure



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Materials Provided

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

① **UbL-Resin:** One vial containing 1 mL of 20 % suspension of GST-hHR23BUbL-Resin in PBS containing 10 % glycerol and 0.05% NaN_3. Store at 4°C.

② **Control-Resin:** One vial containing 0.5 mL of 20 % suspension of GST-Resin in PBS containing 10 % glycerol and 0.05% NaN3. Store at 4°C.

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

④ **Proteasome Substrate (Suc-LLVY-AMC):** One vial containing 2 mM peptide substrate in DMSO. Store at -20°C for long period.

⑤ **10X Assay Buffer:** One vial containing 2 mL of 250mM HEPES, pH 7.5, 5mM EDTA, 0.5% NP-40, and 0.01% SDS (w/v).

Materials Required but not Provided

- **Microplate for fluorometer**
- **Microplate reading fluorometer** capable of excitation at a wavelength in the range 350-380 nm and detection of emitted light in the range 440-460 nm.
- **Wash Buffer:** 1X TBSTE (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA 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.
- **DMSO:** Dimethyl Sulfoxide (Sigma: Cat# D2438)
- **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 pipettors with disposable tips.
- **Vortex mixer**
- **SDS-PAGE apparatus**
- **Blotting apparatus**
- **Rocking platform**
- **X-ray film or CCD imaging instrument**.

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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 (ddH_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.**

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Detailed Protocol

The CycLex Research Product **Proteasome Enrichment & Activity Assay Kit** is provided with 10 times concentrated reagents except UbL-Resin and Control-Resin.

Since experimental conditions may vary, a Control-Resin within the kit, should be included in each experiment as a negative 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 UbL-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. Prepare a working solution of Assay Buffer by adding 1 mL of ⑤10X Assay Buffer to 9 mL of ddH_2O. *Store at -20°C.*

Preparation of Other Reagents, not provided in this kit

1. Wash Buffer: 1X TBSTE (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA containing 0.1 % Tween-20 ®)

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

3. 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%).

4. 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

5. 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. 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.

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3. Remove and discard the supernatant and collect the cell pellet.
   At this point the cell pellet can be frozen at -80°C and lysed at a later date.

4. Lyse the cell pellet in **0.5 mL*** of **#1. 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 -80°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.

B. Binding of Proteasome

1. Resuspend the ①**UbL-Resin** and ②**Control-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 50 µL of the ① **UbL-Resin** suspension and the ② **Control-Resin** suspension to different microcentrifuge tubes.

3. Add the 0.5-1.0 mg of the cell lysate prepared above at a concentration of ~2 mg/mL to each microcentrifuge tube containing the UbL-Resin and the Control-Resin.

4. Incubate at 4°C for 2-4 h with constant mixing to keep the UbL-Resin and the Control-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.

C. Proteasome activity Assay

1. Suspend the UbL-Resin and the Control-Resin in 40 µL of **#2. Assay Buffer**. Transfer the UbL-Resin suspension and the Control-Resin suspension to different well of microplate for fluorometer.

2. Make an Assay Premix by mixing 50 µL of **#2. Assay Buffer** and 2.5 µL of ④ **Proteasome Substrate** per one assay.

3. Add 52.5 µL of Assay Premix to each well containing the UbL-Resin or the Control-Resin in the microplate for fluorometer.

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4. Incubate samples at 37°C. Read fluorescence intensity using a 380/460 nm filter set in a fluorometer for 1-2 hours at 5 minutes interval.

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'. SDS-PAGE and Western Blotting

1'. Suspend the the UbL-Resin and the Control-Resin in 20 µL of 2X SDS Sample Buffer and boil for 5 min.

2'. 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.

3'. Transfer the resolved proteins to PVDF membrane.

4'. Rinse the PVDF membrane with 50 mL of Wash Buffer.

5'. Block the PVDF membrane for 1 hr to O/N on a rocking platform with Blocking Buffer at room temperature.

6'. Incubate the PVDF membrane for 60 minutes on a rocking platform with 5 mL of primary antibody of your interest.

7'. Wash the PVDF membrane 4 times with 100 mL of Wash Buffer for 10 min each on a rocking platform.

8'. Incubate with 5 mL of HRP conjugated anti-mouse IgG/ anti-rabbit IgG or appropriate enzyme conjugated secondary antibody for 60 min on a rocking platform.

9'. Wash the PVDF membrane 4 times with 100 mL of Wash Buffer for 10 min each on a rocking platform.

10'. Develop with enhanced chemiluminescence to maximize detection.

11'. Detect emitted chemiluminescent signal by film or CCD imaging instrument.

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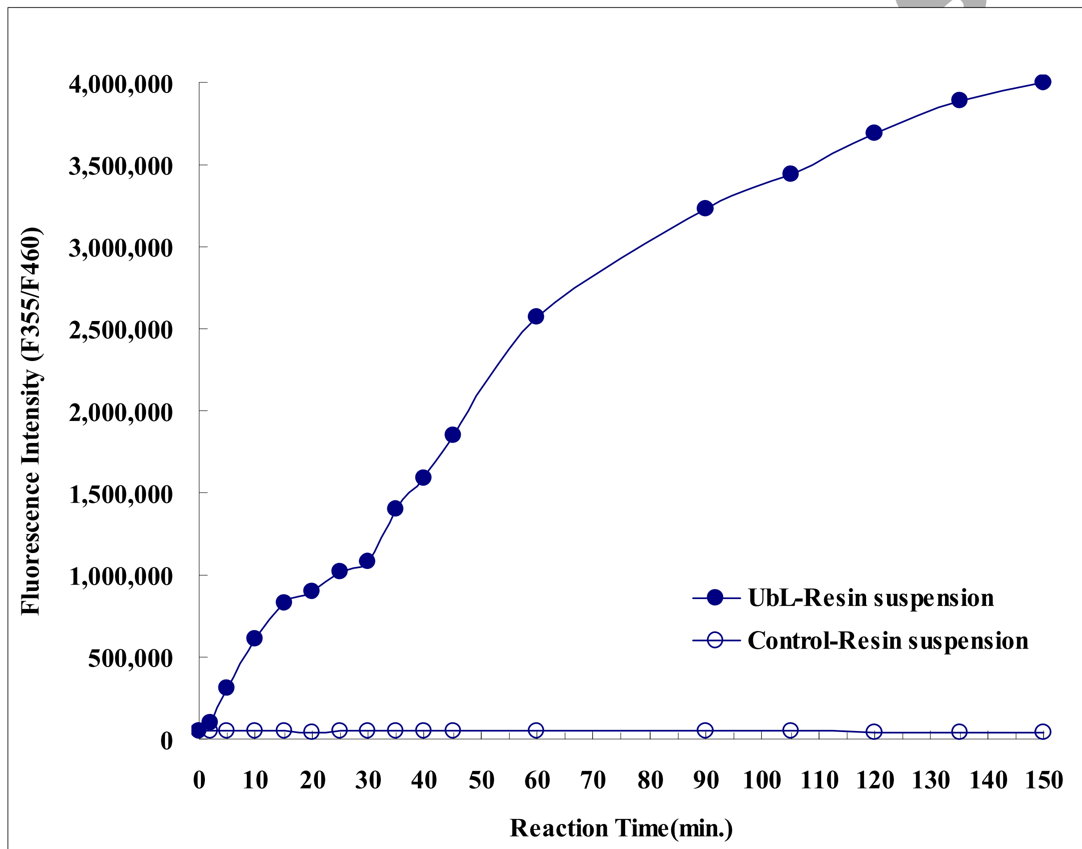
Reagent Stability

All of the reagents included in the CycLex Research Product **Proteasome Enrichment & Activity Assay 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.

Example of Test Results

Fig.1 Proteolytic activity of proteasome, isolated form breast cancer cell line, MCF-7 using UbL-Resin



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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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