ELISA Kit for Measuring Poly-Ubiquitinated Protein

CycLex Poly-Ubiquitinated Protein ELISA Kit

Cat# CY-7053

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Intended Use

The MBL Research Product **CycLex Poly-Ubiquitinated Protein ELISA Kit** is designed to detect and quantify the level of total poly-ubiquitinated proteins in cell lysate. Since the amino acid sequence of ubiquitin is well conserved among mammal, this ELISA kit can be used for any mammalian cells. This assay is intended for the 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 all components 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, 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.

In a recent decade, a variety of reversible and irreversible inhibitors of the 20S proteasome have been identified that can enter mammalian cells and inhibit degradation of proteins by the ubiquitin-proteasome pathway. One group of such inhibitors is peptide aldehydes, e.g. MG132 which reversibly binds to active sites and inhibits cleavage of hydrophobic or acidic substrates. A more specific inhibitor is the naturally occurring bacterial compound, lactacystin, which covalently modifies threonine residues in the proteasome's active site and does not seem to affect any other known protease. Such agents can inhibit protein degradation and major histocompatibility class I antigen presentation in a variety of mammalian cells and have been widely used to probe the physiological function of the ubiquitin-proteasome pathway.

Principle of the Assay

The MBL Research Product **CycLex Poly-Ubiquitinated Protein ELISA Kit** is a solid phase sandwich ELISA. A monoclonal antibody specific poly-ubiquitinated protein has been coated onto the wells of the microtiter strips provided. Samples, including a standard containing poly-ubiquitinated protein, control specimens, and unknowns, are pipetted into these wells. During the first incubation, poly-ubiquitinated protein binds to the capture antibody on the well. After washing, HRP-conjugated mouse monoclonal antibody, specific poly-ubiquitinated protein as a detection antibody, is added to the wells. During the second incubation, this antibody serves as a detector by binding to the immobilized poly-ubiquitinated protein at captured during the first incubation. After removal of excess detection antibody, followed by which then catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stopping reagent). The color is quantitated by spectrophotometry and reflects the relative amount of poly-ubiquitinated protein present in the original specimen.

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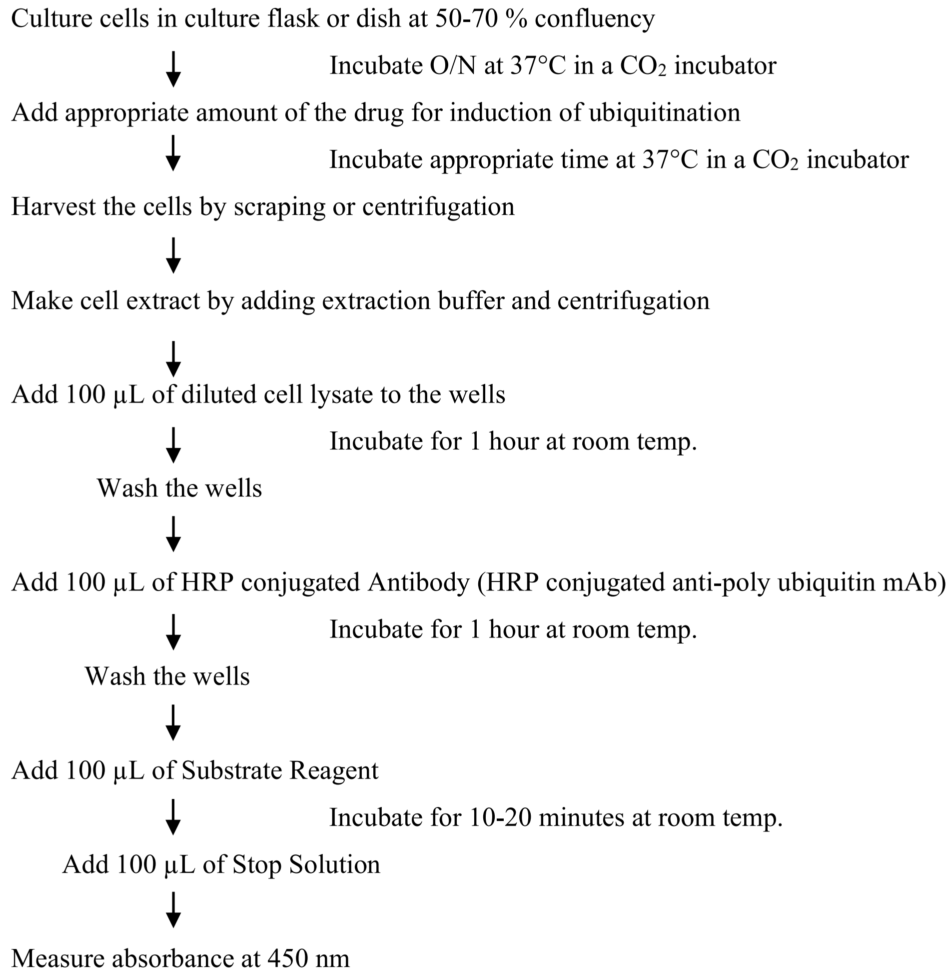
Summary of Procedure



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Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microplate kit.

Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with anti-poly-ubiquitinated protein monoclonal antibody as a capture antibody.

10X Wash Buffer: One 100 mL bottle of 10X buffer containing Tween®-20

Cell Extraction Buffer: One bottle containing 20 mL of 1X buffer.

Dilution Buffer: One bottle containing 50 mL each of 1X buffer; use for standard and sample dilution. Ready to use.

Poly-Ubiquitinated Protein Standard: One vial containing X* units of lyophilized poly-ubiquitinated protein.
***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

20X HRP conjugated Detection Antibody: One vial containing 0.6 mL o HRP (horseradish peroxidase) conjugated anti-poly-ubiquitin monoclonal antibody.

Conjugate Dilution Buffer: One bottle containing 12 mL of Conjugate Dilution Buffer

Substrate Reagent: One bottle containing 20 mL of the chromogenic substrate, tetra methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle supplied ready to use, containing 20 mL of 1 N H_2SO_4.

Materials Required but not Provided

- **Protease inhibitor cocktail:** *e.g.* Sigma Cat#P-2714 (reconstituted according to manufacturer's guideline). Add 250 µL per 5 mL Cell Extraction Buffer.
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Orbital microplate shaker**
- **Microcentrifuge and tubes** for sample preparation.
- **Centrifuge and bucket** for microplate.
- **Vortex mixer**
- **(Optional) Microplate washer:** Manual washing is possible but not preferable.
- **Plate reader** capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.
- **(Optional) Software package facilitating data generation and analysis**
- **500 or 1,000 mL graduated cylinder**.
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

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Precautions and Recommendations

- **Although we suggest to conduct experiments as outlined below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.**

- Allow all the components to come to room temperature before use.

- All microplate strips that are not immediately required should be returned to the zip-lock pouch, which must be carefully resealed to avoid moisture absorption.

- Do not use kit components beyond the indicated kit expiration date.

- Use only the microtiter wells provided with the kit.

- Rinse all detergent residue from glassware.

- Use deionized water of the highest quality.

- Do not mix reagents from different kits.

- The buffers and reagents in this kit may contain preservatives or other chemicals. 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.

- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.

- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.

- Wear gloves and eye protection when handling immunodiagnostic materials and samples of human origin, and these reagents. In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.

- **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.**

- **CAUTION: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

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Detailed Protocol

The MBL Research Product **CycLex Poly-Ubiquitinated Protein ELISA Kit** is provided with removable strips of wells so the assay can be carried out on separate occasions using only the number of strips required for the particular determination. Since experimental conditions may vary, an aliquot of the Poly-Ubiquitinated Protein Standard within the kit, should be included in each assay as a calibrator. 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 need to be brought to room temperature prior to the assay. Assay reagents are supplied ready-to-use, with the exception of **10X Wash Buffer**, **Cell Extraction Buffer** and **Poly-Ubiquitinated Protein Standard**.

1. Prepare a working solution of Wash Buffer by adding 100 mL of the **10X Wash Buffer** to 900 mL of deionized (distilled) water. Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Prepare a working solution of Cell Extraction Buffer by adding 250 µL of Protease inhibitor cocktail (Sigma Cat# P-2714) to 5 mL of **Cell Extraction Buffer**. Mix well.

3. Prepare HRP conjugated Detection Antibody by diluting the **20X HRP-conjugated Detection Antibody** 20-fold with Conjugate Dilution Buffer at the time of assay.
   Prepare appropriate volume for your assay. Discard any unused HRP-conjugated Detection Antibody after diluted.

4. Reconstitute **Poly-Ubiquitinated Protein Standard** with X* **mL** of **Dilution Buffer**. The concentration of the poly-ubiquitinated protein in vial should be **150 units/mL**, which is referred as a **Master Standard**.
   ***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

   Prepare Standard Solutions as follows:
   Use the **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 10 units/mL standard (Std.1) serves as the highest standard. The **Dilution Buffer** serves as the zero standard (Blank)

	Volume of Standard	Dilution Buffer	Concentration
Std.1	40 µL of Master Standard	560 µL	10 units/mL
Std.2	300 µL of Std. 1 (10 units/mL)	300 µL	5 units/mL
Std.3	300 µL of Std. 2 (5 units/mL)	300 µL	2.5 units/mL
Std.4	300 µL of Std. 3 (2.5 units/mL)	300 µL	1.25 units/mL
Std.5	300 µL of Std. 4 (1.25 units/mL)	300 µL	0.625 units/mL
Std.6	300 µL of Std. 5 (0.625 units/mL)	300 µL	0.313 units/mL
Std.7	300 µL of Std. 6 (0.313 units/mL)	300 µL	0.156 units/mL
Blank	-	300 µL	0 units/mL

Note: Do not use a repeating pipette. Change tips for every dilution. Wet tip with Dilution Buffer before dispensing. Unused portions of Master Standard should be aliquoted and stored at below -70°C immediately. Avoid multiple freeze and thaw cycles.

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Assay Procedure-1 (For cells cultured in culture dish or flask)

A. Treatment of Cells with compounds

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 compounds 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. Under these conditions, use of 0.1-0.5 µL of the clarified cell lysate diluted to a volume of 100 µL/well in Dilution Buffer is sufficient for the detection of poly-ubiquitinated protein.*

5. Transfer the lysate to microcentrifuge tubes and centrifuge at 15,000 rpm for 10 minutes at 4°C.

6. Aliquot the clear extract to clean microcentrifuge tubes. These lysates are ready for assay. The cell lysate can be stored at below -70°C. Avoid multiple freeze/thaw cycles.

C. ELISA

1. Remove the appropriate number of microtiter wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.

2. Dilute lysates **1:500*** with **Dilution Buffer** (e.g. 1 µL cell extract + 499 µL Dilution Buffer).

* *Lysates prepared in Cell Extraction Buffer must be diluted 1:200 or greater in Dilution Buffer. While*

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a 1:200 sample dilution has been found to be satisfactory, higher dilutions such as 1:500 or 1:1,000 may be optimal. The dilution chosen should be optimized for each experimental system.

3. Pipette **100 µL** of **Standard Solutions (Std1-Std7, Blank)** and **diluted lysates** in duplicates, into the appropriate wells.

4. Incubate the plate **at room temperature (ca. 25°C) for 1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.

5. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

6. Add **100 µL** of **HRP conjugated Detection Antibody** into each well.

7. Incubate the plate **at room temperature (ca. 25°C) for 1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.

8. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

9. Add **100 µL** of **Substrate Reagent**. Avoid exposing the microtiter plate to direct sunlight covering the plate with e.g. aluminum foil is recommended. Return Substrate A to 4°C immediately after the necessary volume is removed

10. Incubate the plate **at room temperature (ca. 25°C) for 10-20 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker. The incubation time may be extended up to 30 minutes if the reaction temperature is below than 20°C.

11. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

12. Measure absorbance in each well using a spectrophotometric microplate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the microplate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Assay Procedure-2 (For cells cultured in 96 wells culture plate)

A. Treatment of Cells with compounds

1. Plate adherent cells or non-adherent cells in a 96 wells culture plate at 50-70% confluency.

2. Incubate the 96 wells culture plate at 37°C over night in CO_2 incubator.

3. Add appropriate amount of test compounds to each well.

4. Incubate the 96 wells culture plate at 37°C for appropriate time.

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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. Remove the culture medium and wash the cells twice with 200 µL of cold PBS using a centrifuge for microplate.

2. Remove PBS completely. (Tap the 96 wells culture plate on the paper towel in case of the adherent cells. Take PBS away carefully using multi-channel pipette in case of the non- adherent cells.) At this point the 96 wells culture plate can be frozen at below -70°C and lyse at a later date.

3. Add **50 µL*** of Cell Extraction Buffer to each well.

   * *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, 1.2 x 10^4 MCF-7 cells can be extracted in* ***50 µL*** *of Cell Extraction Buffer. Under these conditions, use of 0.5-2 µL of the clarified cell lysate diluted to a volume of 100 µL/well in Dilution Buffer is sufficient for the detection of poly-ubiquitinated protein.*

4. Shake the 96 wells culture plate at ca. 300 rpm on an orbital microplate shaker at 4°C for 1 hr.

5. Centrifuge the 96 wells culture plate at 3,000 rpm at 4°C for 15 min.

6. Carefully transfer the lysates to new a 96 wells microplate.

7. The clear lysates in the 96 wells microplate are ready for assay. These cell lysates can be stored at below -70°C. Avoid multiple freeze/thaw cycles.

C. ELISA

1. Remove the appropriate number of microtiter wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.

2. Dilute the lysates from 96 wells plate **1:50*** with **Dilution Buffer** (e.g. 5 µL cell lysate + 245 µL Dilution Buffer).

   * *Lysates prepared in Cell Extraction Buffer must be diluted 1:50 or greater in Dilution Buffer. While a 1:50 sample dilution has been found to be satisfactory, higher dilutions such as 1:100 or 1:200 may be optimal. The dilution chosen should be optimized for each experimental system.*

3. Pipette **100 µL** of **Standard Solutions (Std1-Std7, Blank)** and **diluted lysates** in duplicates, into the appropriate wells.

4. Incubate the plate **at room temperature (ca. 25°C) for 1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.

5. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

6. Add **100 µL** of **HRP conjugated Detection Antibody** into each well.

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7. Incubate the plate **at room temperature (ca. 25°C) for 1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.

8. Wash 4-times by filling each well with Wash Buffer (350 μL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

9. Add **100 μL** of **Substrate Reagent**. Avoid exposing the microtiter plate to direct sunlight covering the plate with e.g. aluminum foil is recommended. Return Substrate A to 4°C immediately after the necessary volume is removed

10. Incubate the plate **at room temperature (ca. 25°C) for 10-20 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker. The incubation time may be extended up to 30 minutes if the reaction temperature is below than 20°C.

11. Add **100 μL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

12. Measure absorbance in each well using a spectrophotometric microplate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the microplate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Note-1: 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.

Note-2: Reliable standard curves are obtained when either O.D. values do not exceed 0.25 units for the blank (zero concentration), or 2.5 units for the highest standard concentration.

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine poly-ubiquitinated protein concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

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Calculations

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation. To determine the poly-ubiquitinated protein concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding poly-ubiquitinated protein concentration. If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

1. The dose-response curve of this assay fits best to a sigmoidal 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 5-parameter logistic function. It is important to make an appropriate mathematical adjustment to accommodate for the dilution factor.

2. Most microtiter plate readers perform automatic calculations of analyte concentration. The calibration curve is constructed by plotting the absorbance (Y) of calibrators versus log of the known concentration (X) of calibrators, using the four-parameter function. Alternatively, the logit log function can be used to linearize the calibration curve (i.e. logit of absorbance (Y) is plotted versus log of the known concentration (X) of calibrators).

Measurement Range

The measurement range is 0.156 units/mL to 10 units/mL. Any sample reading higher than the highest standard should be diluted with Dilution Buffer in higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the poly-ubiquitinated protein concentration.

Troubleshooting

1. All samples and standards should be assayed in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.

2. Poor duplicates, accompanied by elevated values for wells containing no sample, indicate insufficient washing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for washer maintenance.

3. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. <u>Do not allow the plate to dry out</u>. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in the MBL Research Product **CycLex Poly-Ubiquitinated Protein ELISA 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. Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

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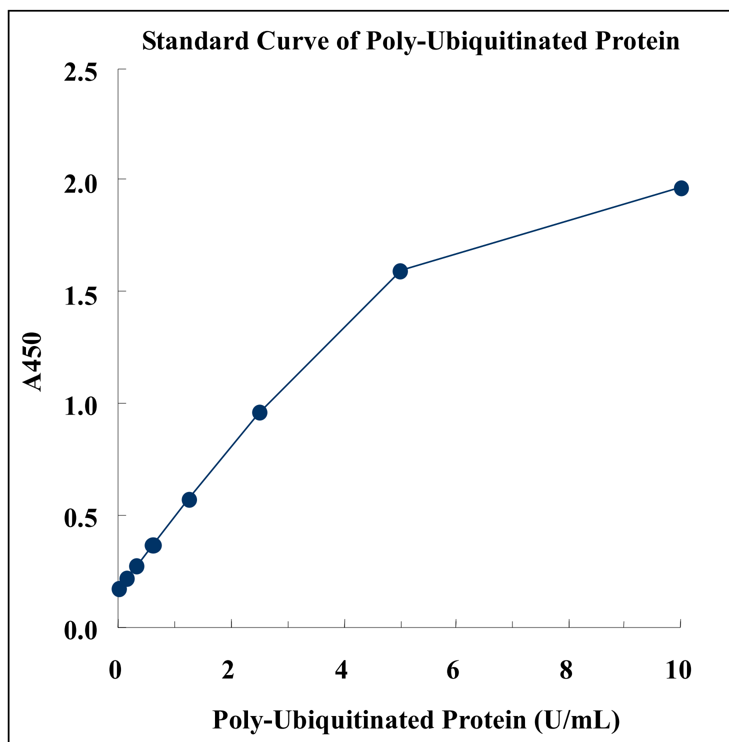
Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of poly-ubiquitinated protein giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 0.08 units/mL of sample.

* Dilution Buffer was pipetted into blank wells.

Typical standard curve



2. Specificity

The antibodies in the CycLex Poly-Ubiquitinated Protein ELISA Kit are highly specific of poly-ubiquitinated protein, with no detectable reactivity to monomer ubiquitin and mono-ubiquitinated proteins

3. Linearity

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of poly-ubiquitinated protein were serially diluted with the Dilution Buffer to produce samples with values within the dynamic range of the assay.

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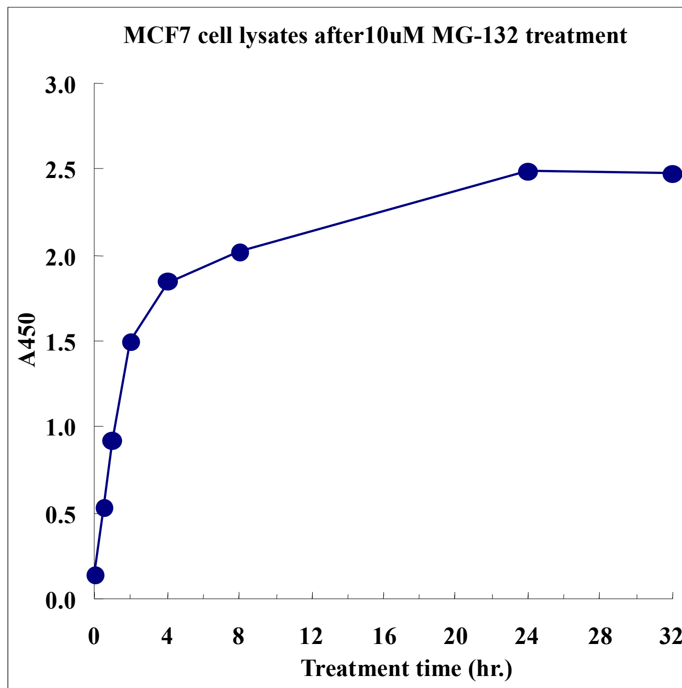
Example of Test Results

Fig. Breast cancer cell line, MCF-7 cells were treated with 10 µM MG132 for indicated time. The lysates of the MCF-7 cells from the indicated time were measured by the CycLex Poly-Ubiquitinated Protein ELISA kit



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