ELISA Kit for Measuring Human/Mouse p62 in Cell Lysate

CycLex Total p62 ELISA Kit

Cat# CY-7055

Intended Use

The MBL Research Product **CycLex Total p62 ELISA Kit** is used for the quantitative measurement of human or mouse p62 protein in cell lysate.

Individual users should determine appropriate conditions when using other types of samples.

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.
- Do not expose reagents to excessive light.

Introduction

The sequstosome1, p62/SQSTM1, hereafter referred as p62, is initially identified as a phosphotyrosine-independent ligand of the SH2 domain of p56lck (1). p62 is composed of multiple domain, which is Phox/Bem domain1, ZZ-type zinc finger domain, TRAF6 binding domain, LC3-interacting region (LIR) motif, Keap1-interacting region (KIR), and ubiquitin-associated domain (UBA). Thus p62 plays a multifunctional scaffold protein that organizes signal transduction and protein traffic (2, 3).

Additionally, binding to the autophagy regulator Atg8/LC3 through LIR motif and to the polyubiquitinated proteins through UBA domain, p62 is an important mediator for targeting polyubiquitinated proteins to the autophagic degradation system and p62 has a crucial role in the removal of aggregated proteins that are poorly degraded by proteasomes. (4-11). The protein aggregates are characteristic features of a variety of chronic toxic and degenerative diseases, such as Mallory bodies in hepatocytes in alcoholic and non-alcoholic steatohepatitis, intracytoplasmic hyaline bodies in hepatocellular carcinoma, neurofibrillary tangles in neurons in Alzheimer's disease, Lewy bodies in Parkinson's disease, and Rosenthal fibers (12-14). Indeed, p62 is detected as a common component of these protein aggregates (15, 16).

The phosphorylation of p62 modifies the affinity of domain for target protein. The phosphorylation of p62 on serine 403 (human) / serine 405 (mouse) in UBA domain, which is phosphorylated by casein kinase 2 and/or TANK-binding kinase 1, promotes its translocation to ubiquitinated proteins, and which progresses selective autophagic clearance of ubiquitinated proteins aggregates (17, 18).

On the other hand, phosphorylation of p62 on serine 349 (human) / serine 351 (mouse) in KIR motif, which is phosphorylated by mTORC1 *in vitro*, enhances its affinity for Keap1, and which follows selective autophagy of Keap1, leading to Nrf2 activation (19, 20). mTORC1 interacts with p62 via raptor and phosphorylates serine 349 (human) / serine 351 (mouse) of p62, which is required for mTORC1 activation in response to amino acids (21) and is shown to be a nice marker of the activation of mTORC1 in autophagy pathways (19).

Principle of the Assay

The MBL Research Product **CycLex Total p62 ELISA Kit** employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for p62 is pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any p62 protein present. After washing away any unbound substances, an HRP conjugated antibody specific for p62 is added to the wells. Following a wash to remove any unbound antibody HRP conjugate, the remaining conjugate is allowed to react with the substrate H_2O_2-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is proportional to the concentration of p62. A standard curve is constructed by plotting absorbance values versus p62 concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

Summary of Procedure

Culture cells in plate or dish at 50-70 % confluency
↓ Incubate in CO_2 incubator at 37°C overnight
Add appropriate amount of compounds for induction of p62 expression
↓ Incubate for appropriate time in CO_2 incubator at 37°C
Harvest the cells by scraping and centrifugation
↓
Make cell lysates by adding 1st Lysis Buffer and Complete 2nd Lysis Buffer
↓ Mix for 60 minutes at 4°C
Centrifuge and collect the clear lysates
↓
Dilute the lysates with Dilution Buffer
↓
Add 100 µL of diluted lysates to each well of the microplate
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 100 µL of HRP conjugated anti-p62 antibody to each well
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 100 µL of Substrate Reagent
↓ Incubate for 5-15 minutes at room temp.
Add 100 µL of Stop Solution
↓
Measure absorbance at 450 nm

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 pre-coated with anti-p62 antibody as a capture antibody.

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

50X Phosphatase Inhibitor: One vial of lyophilized phosphatase inhibitor mix.

1st Lysis Buffer: One bottle containing 12 mL of 1X buffer.

2nd Lysis Buffer: One bottle containing 50 mL of 1X buffer.

p62 Standard: One vial containing X* ng of lyophilized p62.
***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**
Dilution Buffer: One bottle containing 50 mL of 1X buffer; use for standard and sample dilution. Ready to use.

HRP conjugated Detection Antibody: One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-p62 antibody. Ready to use.

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

Stop Solution: One bottle containing 20 mL of 1 N H_2SO_4. Ready to use.

Materials Required but not Provided

- **Protease Inhibitor:** e.g. available from SIGMA, Protease Inhibitor Cocktail Tablets, Cat# 11697498001 or GE Healthcare, Protease Inhibitor Mix, Cat# 80650123.

- **Orbital microplate shaker**

- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.

- **Precision repeating pipettor**

- **Microcentrifuge tubes** for sample preparation.

- **Vortex mixer**

- **Tube rotator**

- **(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**

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.**

Detailed Protocol

The MBL Research Product **CycLex Total p62 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 p62 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 use.

1. Prepare a working solution of Wash Buffer by adding **100 mL** of **10X Wash Buffer** (provided) to **900 mL** of deionized (distilled) water (**ddH_2O**). Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Reconstitute a vial of **50X Phosphatase Inhibitor** (provided, lyophilized) with **1.0 mL** of **ddH_2O**. Mix gently until dissolved. Store the **50X Phosphatase Inhibitor Solution** in small aliquots (e.g. 200 µL) at -20°C.

3. Prepare **Complete 2nd Lysis Buffer** by adding **100 µL** of **50X Phosphatase Inhibitor Solution** and appropriate amount of **Protease Inhibitor** (not provided)* to **4.5 mL** of **2nd Lysis Buffer** (provided). Bring the final volume up to **5.0 mL** with **ddH_2O** and mix well.

   * See the section of "Materials Required but not Provided" above.

4. Prepare **Standard Reconstitution Buffer** by adding **150 µL** of **1st Lysis Buffer** (provided) to **1.35 mL** of **2nd Lysis Buffer** (provided).

5. Reconstitute **p62 Standard** (provided, lyophilized) with X* **mL** of **Standard Reconstitution Buffer** by gently mixing. After reconstitution, immediately dispense it in small aliquots (e.g. 100 µL) to plastic micro-centrifuge tubes and store below -70°C to avoid non-specific adsorption to glass surface and multiple freeze-thaw cycles. The concentration of the p62 in vial should be **960 ng/mL**, which is referred to as the **Master Standard** of p62.
   ***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. **Std.1 (96 ng/mL)** serves as the highest standard. The **Dilution Buffer** serves as the zero standard (Blank).

	Volume of Standard	Dilution Buffer	Concentration
Std.1	50 µL of Master Standard (960 ng/mL)	450 µL	96 ng/mL
Std.2	250 µL of Std. 1 (96 ng/mL)	250 µL	48 ng /mL
Std.3	250 µL of Std. 2 (48 ng/mL)	250 µL	24 ng/mL
Std.4	250 µL of Std. 3 (24 ng/mL)	250 µL	12 ng/mL
Std.5	250 µL of Std. 4 (12 ng/mL)	250 µL	6 ng/mL
Std.6	250 µL of Std. 5 (6 ng/mL)	250 µL	3 ng/mL
Std.7	250 µL of Std. 6 (3 ng/mL)	250 µL	1.5 ng/mL
Blank	-	250 µL	0 ng/mL

Note: Do not use a Repeating pipette. Change tips for every dilution. Wet tip with Dilution Buffer before dispensing. Discard any unused Standard Solutions after use.

Assay Procedure

I. Preparation of Cell Lysates

Note: This protocol has been successfully applied to several cell lines. The optimal experimental conditions will vary depending on the parameters, e.g. cell lines. Users should optimize this protocol for their own experimental conditions.

1. Plate cells* in 1 mL of culture medium to each well in a 12-well plate at 50-70 % confluency.

   * Aseptically coat surface of wells with 1 mL of poly-L-lysine (10 µg/mL, in PBS). Rock gently to ensure even coating of the wells surface. After 5 minutes, remove poly-L-lysine solution by aspiration and plate cells.
   * For HeLa cells: Plate cells at a density of 1.5-3.0 × 10^5 cells/mL.
   * For MEF cells: Plate cells at a density of 2.5-5.0 × 10^5 cells/mL.

2. Incubate the 12-well plate in CO_2 incubator at 37°C overnight.

3. Remove media and add 1 mL of culture medium containing appropriate amount of test compounds to each well.

4. Incubate the 12-well plate for appropriate time in CO_2 incubator at 37°C.

5. Remove media and gently wash the surface of cells with cold PBS twice*.

   * Remove PBS by aspiration completely.
   * At this point the cells on the plate can be frozen below -70°C for making cell lysates later.

6. Add **50 µL** of **1st Lysis Buffer*** from pipette drop by drop to different areas of the well surface.

   * Containing SDS. Please dissolve precipitates completely before use, as by heating in water bath at 25-37°C.

7. Mix the solution with the tip of a disposable tip to lyse the cells completely at room temperature for several minutes.

8. Add **450 µL** of **Complete 2nd Lysis Buffer** to each well and mix gently by pipetting.

9. Transfer the lysates to microcentrifuge tubes and mix well by vortexing.

10. Mix the tubes at 10-20 rpm vertically on a tube rotator for 60 minutes at 4°C.

11. Centrifuge the tubes at 13,000 rpm for 5 minutes at 4°C.

12. Aliquot the clear lysates to new microcentrifuge tubes. These samples are ready for assay. The lysates can be stored below -70°C. Avoid multiple freeze/thaw cycles.

II. 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 samples with **Dilution Buffer**.*

   * Lysates prepared in the preceding paragraph must be diluted 1:5 (e.g. 60 µL of lysates + 240 µL Dilution Buffer) or higher. In some types of samples, higher dilution may be more favorable. The dilution ratio should be optimized by the individual user.
   * Dilutions of lower than 1:5 should not be used.

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

4. Incubate the plate **at room temperature (ca. 25°C) for 60 minutes**, 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 60 minutes**, 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** to each well and incubate **at room temperature (ca.25°C) for 5–15 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker.

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

11. 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 0.2 units for the blank (zero concentration), or 2.5 units for the highest standard concentration. The plate should be monitored at 5-minute intervals for approximately 30 minutes.

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 the concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

Calculations

Average the duplicate readings for each standard, control and sample, and subtract the optical density of the average zero standard. Plot the optical density versus the concentration of standards and draw the best curve. Most microtiter plate readers perform automatic calculations of analyte concentration. The standard curve fits best to a sigmoidal four-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a four-parameter logistic function.

A standard curve is also to be constructed by plotting the absorbance (Y) versus log of the known concentration (X) of standards, using a cubic function. Alternatively, the logit log function can be used to linearize the standard curve (i.e. logit of optical density (Y) is plotted versus log of the known concentration (X) of standards). To determine the concentration of each sample, first find the optical density 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 concentration.

If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Measurement Range

The measurement range is 1.5 ng/mL to 96 ng/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 concentration of the sample.

Troubleshooting

1. All samples and controls 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. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in the MBL Research Product **CycLex Total p62 ELISA Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date.

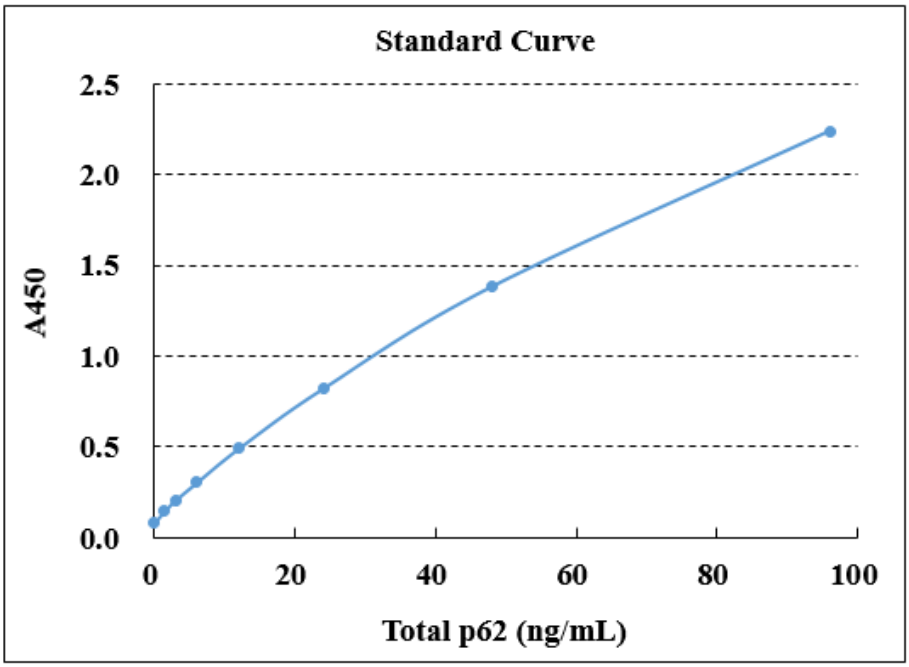
Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of p62 giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 0.154 ng/mL of sample.

* Dilution Buffer was pipetted into blank wells.

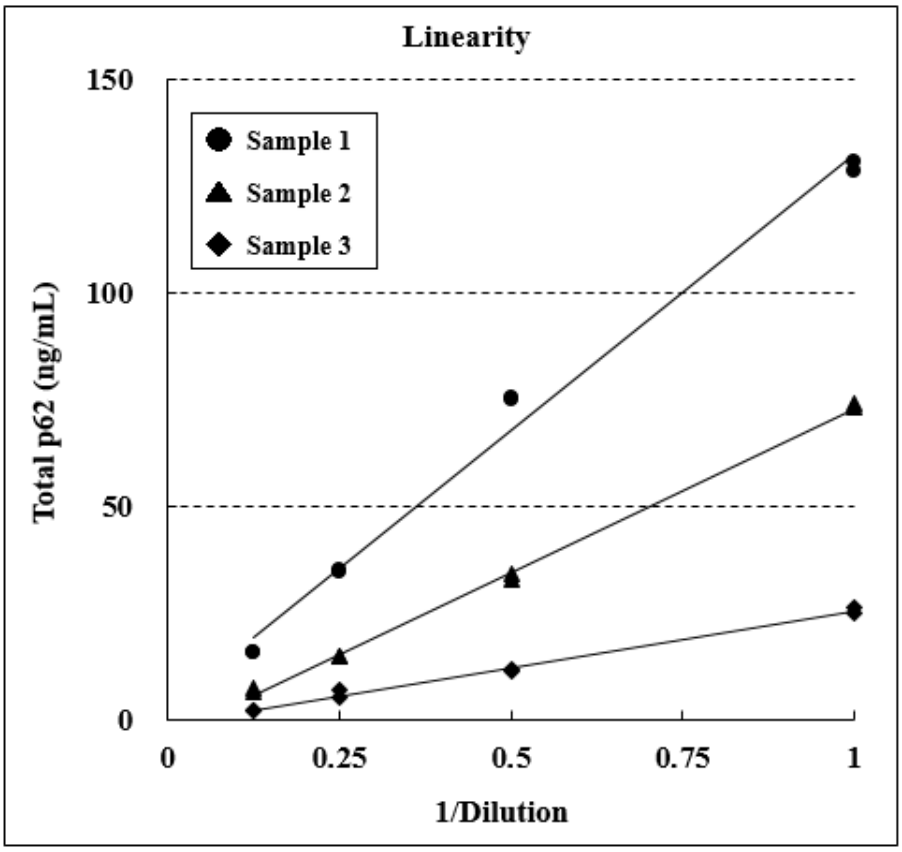
Typical Standard Curve



2. Linearity

Three samples* were diluted with Dilution Buffer and assayed after dilution. The neat sample was set to 1.

* Samples: Human cell lysates



Example of Test Results

Fig.1 Measurement of total p62 (by Cat#CY-7055), phospho-p62 Ser349 (by Cat#CY-7056), and Ser403 (by Cat#CY-7057) in cell lysates of HeLa cells treated with 0-25 nM Bafilomycin A1 and/or 12.5-100 nM Rapamycin for 17 hours.

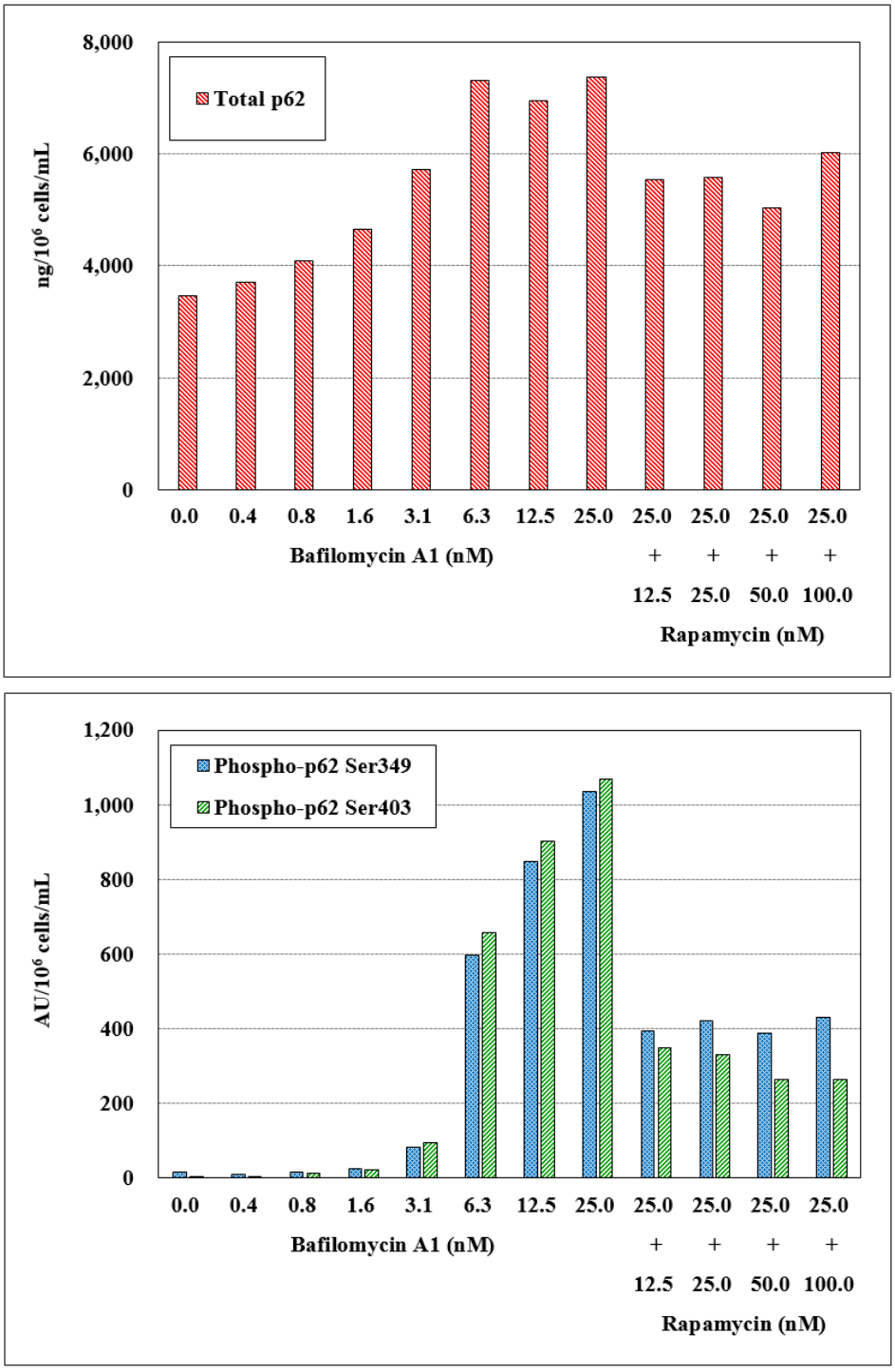
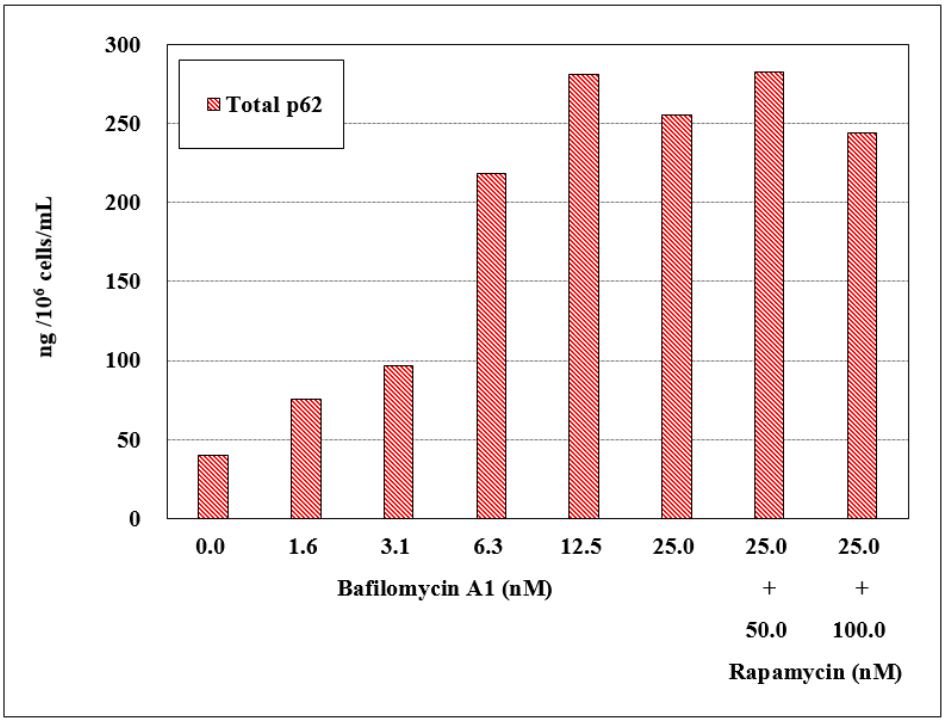
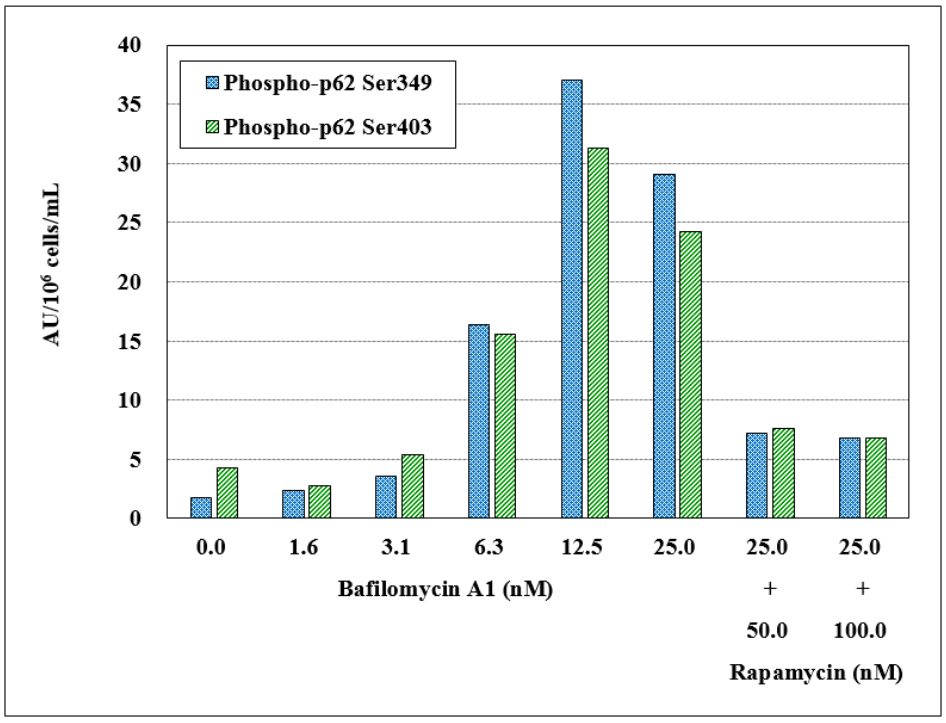


Fig.2 Measurement of total p62 (by Cat#CY-7055), phospho-p62 Ser349 (by Cat#CY-7056), and Ser403 (by Cat#CY-7057) in cell lysates of MEF (mouse embryonic fibroblast) treated with 0-25 nM Bafilomycin A1 and/or 50-100 nM Rapamycin and/or for 17 hours.





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