# ELISA PRODUCT INFORMATION & MANUAL

## p62/SQSTM

## NBP2-61300

Enzyme-linked Immunosorbent Assay for quantitative detection of Human, Mouse, Rat p62/SQSTM1.
For research use only. Not for diagnostic or therapeutic procedures.

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Reagents require separate storage conditions.

Check our website for additional protocols, technical notes and FAQs.

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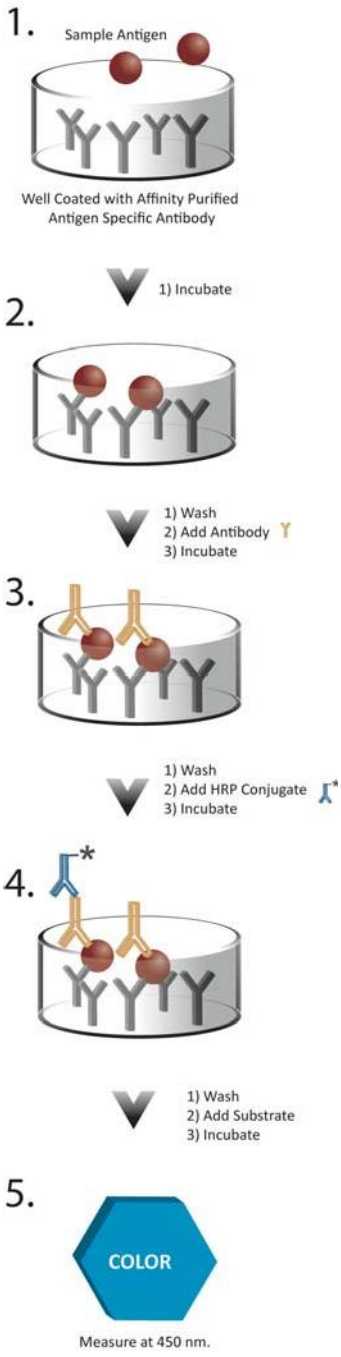



## Introduction

The Novus Biologicals p62 Enzyme-Linked Immunosorbent Assay (ELISA) kit is a complete kit for the quantitative determination of p62 in cell lysates of human, mouse and rat origin. Please read the complete kit insert before performing this assay.

The generic term ''autophagy'' comprises several processes by which the lysosome acquires cytosolic cargo, with three types of autophagy being discerned in the literature: (1) macroautophagy, characterized by the formation of a crescent-shaped structure (the phagophore) that expands to form the double-membrane autophagosome, capable of fusion with the lysosome; (2) microautophagy, in which lysosomes invaginate and directly sequester cytosolic components; and (3) chaperone-mediated autophagy (CMA), which involves translocation of unfolded proteins across the lysosomal membrane[1-3].

Upregulation of autophagy pathways occurs in response to extra- or intracellular stress and signals such as starvation, growth factor deprivation, ER stress and pathogen infection[3]. Malfunction of these pathways is linked to various human pathologies including cancer, neurodegeneration and infectious diseases[2-4].

Selective *macro*autophagy describes the pathway of self-degradation of whole cellular components, protein aggregates or unusually long-lived proteins; in which double-membrane autophagosomes sequester organelles, ubiquitinylated proteins or ubiquitinylated protein aggregates and subsequently fuse with lysosomes for breakdown by resident hydrolases[1,3,4]. Autophagic clearance of protein aggregates requires the ubiquitin-binding receptors p62 and NBR1[3,4].

The p62 protein, also known as sequestosome 1 (SQSTM1), has a dual functionality as both a scaffold protein and aiding in trafficking for protein degradation. It can polymerize and bind to NBR-1 via a PB1 (Phox and Bem1) domain[1], interact with ubiquitinated proteins linking them to the autophagic machinery via a UBA (ubiquitin-associated) domain[5] and bind to the LC3II protein of the autophagy pathway through an LIR (LC3 interacting region) motif[6]. p62 provides a key link between the ubiquitin-proteasome system (UPS) and autophagy by facilitating autophagic degradation of ubiqutinated proteins, decreasing aggregation of misfolded and non-functional proteins within cells, resulting in enhanced cellular survival characteristics[7]. Because p62 accumulates when autophagy is inhibited, and decreased levels can be observed when autophagy is induced[5,8], p62 may be used as a biomarker to study autophagic flux. Also, p62 has been implemented in neurodegenerative diseases such as Parkinson's[9] and Alzheimer's[10] and in the skeletal disorder Paget's disease of bone[11], establishing the p62 protein as a potential therapeutic target.

## Principle

1. Samples and standards are added to wells coated with a monoclonal antibody specific for p62. The plate is then incubated.
2. The plate is washed, leaving only bound p62 on the plate. A yellow solution of rabbit polyclonal antibody to p62 is then added. This binds to the p62 captured on the plate. The plate is then incubated.
3. The plate is washed to remove excess antibody. A blue solution of horseradish peroxidase (HRP) conjugate is added to each well that binds to the rabbit polyclonal p62 antibody. The plate is again incubated.
4. The plate is washed to remove excess HRP conjugate. TMB Substrate solution is added. An HRP-catalyzed reaction generates a blue color in the solution.
5. Stop solution is added to stop the substrate reaction. The resulting yellow color is read at 450 nm. The amount of signal is directly proportional to the level of p62 in the sample.

Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.

## Materials Supplied

1. **Assay Buffer 13**
   100 mL
   Tris buffered saline containing BSA and detergents

2. **p62 Standard**
   0.05 mL
   1 vial containing 2 µg/mL of recombinant p62

3. **p62 Clear Microtiter Plate**
   One plate of 96 wells
   A clear plate of break-apart strips coated with a monoclonal antibody specific for p62

4. **p62 Antibody**
   10 mL
   A yellow solution of rabbit polyclonal antibody to p62

Activity of conjugate is affected by nucleophiles such as azide, cyanide, and hydroxylamine.

5. **p62 Conjugate**
   10 mL
   A blue solution of donkey anti-rabbit IgG conjugated to horseradish peroxidase.

Protect substrate from prolonged exposure to light.

6. **Wash Buffer Concentrate**
   100 mL
   Tris buffered saline containing detergents

Stop solution is caustic. Keep tightly capped.

7. **RIPA Cell Lysis Buffer 2**
   100mL
   50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS, pH 7.4.

8. **TMB Substrate**
   10 mL
   A solution of 3,3’,5,5’ tetramethylbenzidine (TMB) and hydrogen peroxide

9. **Stop Solution 2**
   10 mL
   A 1N solution of hydrochloric acid in water

10. **p62 Assay Layout Sheet**
    1 each

11. **Plate Sealer**
    2 each

Reagents require separate storage conditions.

## Storage

All components of this kit, except the Standard, are stable at 4°C until the kit's expiration date. The standard must be stored at or below -70°C upon receipt.

## Materials Needed but Not Supplied

1. Deionized or distilled water
2. Phenylmethylsulfonyl fluoride (PMSF)
3. Protease Inhibitor Cocktail (PIC)
4. DNase
5. Precision pipettes for volumes between 5 µL and 1,000 µL
6. Repeater pipette for dispensing 100 µL
7. Disposable polypropylene test tubes for dilution of samples and standards
8. Disposable beakers for diluting buffer concentrates
9. Graduated cylinders
10. A microplate shaker
11. Lint-free paper for blotting
12. Microplate reader capable of reading 450 nm
13. Software for extrapolating sample values from optical density readings utilizing a four parameter logistic curve fit.

## Reagent Preparation

If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

**1. Wash Buffer**

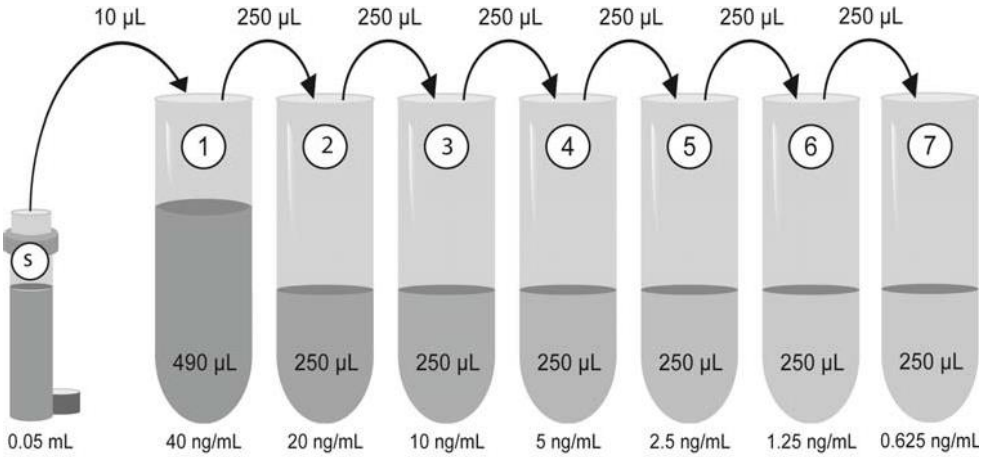
Prepare the wash buffer by diluting 30 mL of the supplied Wash Buffer Concentrate with 570 mL of deionized water. This can be stored at room temperature until the kit's expiration date, or for 3 months, whichever comes first.

**2. p62 Standards**

Allow the 2 µg/mL recombinant p62 standard to warm to room temperature. Label eight disposable 12 x 75 mm polypropylene tubes #1 through #7. Pipette 490 µL assay buffer into tube #1. Pipette 250 µL assay buffer into tubes #2 through #7. Add 10 µL of the p62 standard to tube #1 and vortex thoroughly. Remove 250 µL from tube #1 and add to tube #2. Vortex thoroughly. Continue this for tubes #3 through #7.

The standard should be handled with care due to the known and unknown effects of the antigen.

The concentration of recombinant p62 in tubes #1 through #7 will be 40, 20, 10, 5, 2.5,1.25 and 0.625 ng/mL respectively (see image below). Standards should be used within 60 minutes of preparation.




**Diluted standards should be used within 1 hour of preparation.** The concentrations of the standards are labeled above.

Samples must be stored frozen at or below -20° to avoid loss of bioactive analyte. Repeated freeze/thaw cycles should be avoided.

## Sample Handling

The p62 ELISA kit is compatible with human, mouse and rat cell lysates for the determination of p62 protein levels. Samples diluted sufficiently into the assay buffer can be read directly from a standard curve. Samples containing a visible precipitate must be clarified prior to use in the assay. A minimum dilution of 1:16 is recommended for measuring p62 in cell lysates In order to remove matrix/buffer interference in the assay. It is up to the end user to validate the use of any lysis buffer other than that provided in the p62 kit.

Note: Experimentally observed concentrations of p62 protein in cell lysates may vary due to cell culture/treatment conditions and/or alterations of the lysis procedure. Variations may be caused by, but are not limited to one or more of the following: cell type/species, frequency of media changes, concentration of chemical treatment, treatment duration, media supplements and cell confluency. Therefore, it is very important for each end user to optimize sample handling/dilution for their unique samples in order to obtain the best possible results for their experiment, and interpretation of experimental data should include considerations of these sources of variability.

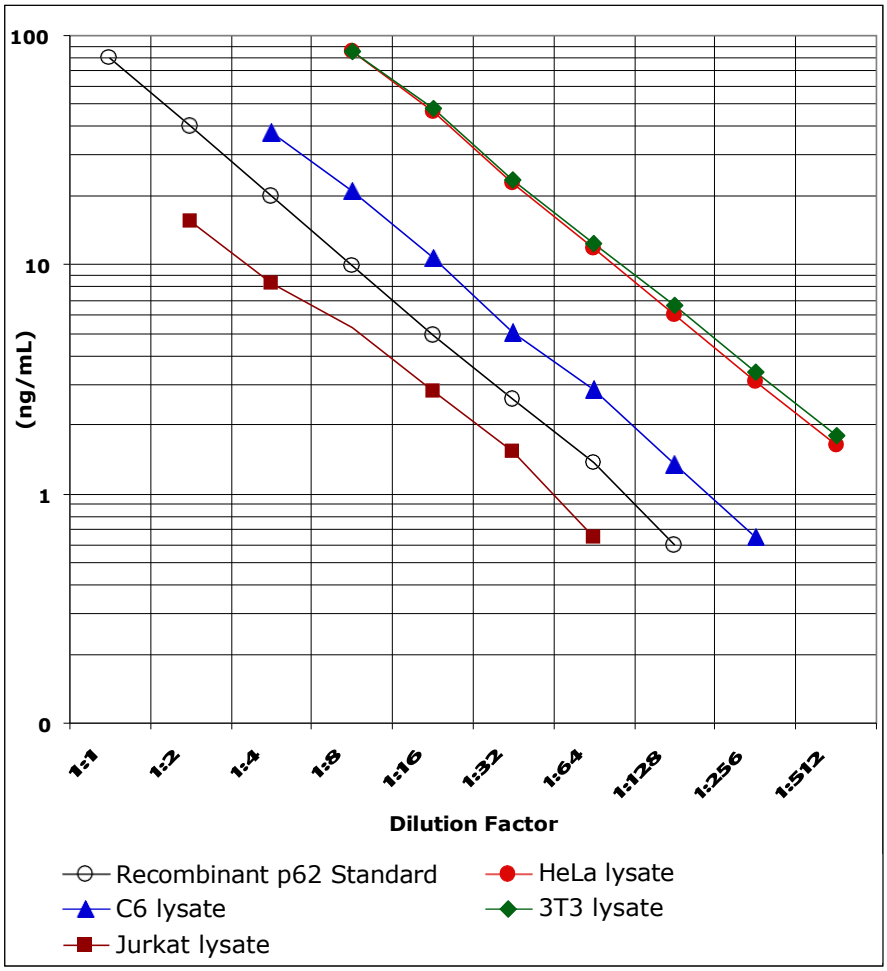
### Dilutional Linearity

The minimum required dilution for lysates of several cell lines was determined by serially diluting samples into the assay buffer and identifying the dilutions at which linearity was observed. Cell lysates were serially diluted in assay buffer and values were calculated from a standard curve also prepared in assay buffer in order to determine linearity of the lysates.

| | % Dilutional Linearity | | | |
|---|---|---|---|---|
| **DF** | HeLa | Jurkat | C6 | 3T3 |
| 1:2 | --- | 63 | --- | --- |
| 1:4 | --- | 67 | 92 | --- |
| 1:8 | 85 | 86 | 102 | 77 |
| 1:16 | 94 | 90 | 104 | 88 |
| 1:32 | 91 | 100 | 99 | 86 |
| 1:64 | 95 | --- | 110 | 91 |
| 1:128 | 97 | --- | 105 | 97 |
| 1:256 | 100 | --- | 100 | 100 |

## Parallelism

Parallelism experiments were carried out to determine if the recombinant p62 standard accurately determines p62 concentrations in biological matrices. Cell lysates from Hela, Jurkat, 3T3 and C6 cell lines prepared with RIPA Cell Lysis Buffer 2 were used to assess parallelism. Values were obtained using the cell lysates serially diluted in assay buffer and assessed from a standard curve using four parameter logistic curve fitting. The observed values were plotted against the dilution factors. Parallelism of the curves demonstrates that the antigen binding characteristics are similar enough to allow the accurate determination of native analyte levels in diluted samples from cell lines of human, mouse and rat origin.



## Spike and Recovery

After diluting each lysate to its minimum required dilution, recombinant human p62 was spiked at high, medium, and low concentrations and assay buffer was spiked in a separate lysate sample as a control for endogenous p62 levels. The control (endogenous) levels of p62 were subtracted from the spiked samples and the recovery of the spiked p62 standard in the diluted cell lysates was compared to the recovery of identical spikes in assay buffer. The percent recovery at the three spike concentrations tested, are indicated below for each lysate.

| Sample Matrix | Minimum Required Dilution | Spike Concentration (pg/mL) | Recovery of Spike |
|---|---|---|---|
| HeLa lysate | 1:16 | 30 | 95% |
| | | 7.5 | 111% |
| | | 3.75 | 125% |
| Jurkat lysate | 1:16 | 30 | 96% |
| | | 7.5 | 97% |
| | | 3.75 | 85% |
| 3T3 lysate | 1:16 | 30 | 80% |
| | | 7.5 | 87% |
| | | 3.75 | 95% |
| C6 lysate | 1:16 | 30 | 89% |
| | | 7.5 | 86% |
| | | 3.75 | 76% |

## Cell Lysate Preparation

1. Add protease inhibitors to RIPA Cell Lysis Buffer 2. Add 0.5uL of PIC per mL of lysis buffer and add PMSF to a final concentration of 1mM. Also add DNase to a final concentration of 20ug/mL. Inhibitors must be added fresh just prior to lysis. RIPA 2 lysis buffer containing inhibitors can not be stored for later use.
2. Resuspend cell pellet in lysis buffer with inhibitors and DNase and incubate on ice for 30 minutes. Vortex occasionally.
3. Pellet cellular debris via centrifugation at 10,000 x g for 10 minutes.
4. Divide the lysates into aliquots and store at or below -20˚C, or use immediately in the assay.
5. Refer to Sample Handling section for minimum required dilution.

## Assay Procedure

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

Pipet the standards and samples to the bottom of the wells.

Pipet the reagents to the sides of the wells to avoid possible contamination.

Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells/strips back into the foil pouch with the desiccant and seal the ziploc. Store unused wells at 4°C. Bring all reagents (except standard) to room temperature for at least 30 minutes prior to opening.

1. Pipet 100 µL of Assay Buffer 13 into the Std0 (0 pg/mL standard) wells.
2. Pipet 100 µL of Standards #1 through #7 into the appropriate wells.
3. Pipet 100 µL of the Samples into the appropriate wells.
4. Seal the plate and incubate at room temperature (RT) on a plate shaker for 1 hour at ~500 rpm.
5. Empty the contents of the wells and wash by adding a full well volume (~300 µL) of 1x wash buffer to every well. Repeat the wash 2 more times for a total of **3 washes**. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
6. Pipet 100 µL of the yellow p62 Antibody into each well, except blank and NSB.
7. Seal the plate and incubate at RT on a plate shaker for 1 hour at ~500 rpm.
8. Wash as above (Step 5).
9. Pipet 100 µL of the blue p62 Conjugate into each well except blank.
10. Seal the plate and incubate at RT on a plate shaker for 30 minutes at ~500 rpm.
11. Wash as above (Step 5).
12. Pipet 100 µL of TMB Substrate into each well.
13. Seal the plate and incubate at RT on a plate shaker for 30 minutes at ~500 rpm.
14. Pipet 100 µL of Stop Solution 2 into each well.
15. Zero the plate reader against the Blank wells and read the optical density (OD) at 450 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all of the readings during data analysis .

## Calculation of Results

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

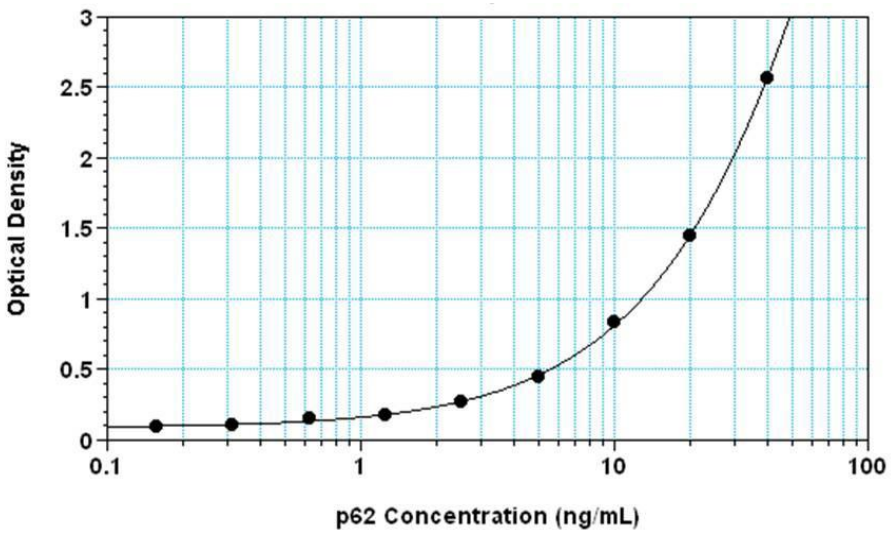
1. Several options are available for the calculation of the concentration of p62 protein in the samples. We recommend that the data be handled by an immunoassay software package utilizing a four parameter logistic curve fitting program. Such software is often supplied by plate reader manufacturers.

Samples with concentrations outside of the standard curve range will need to be reanalyzed using a different dilution.

## Typical Results

The results shown below are for illustration only and should not be used to calculate results from another assay.

| Sample | Net OD | p62 (ng/mL) |
|---|---|---|
| S0 | 0.058 | 0 |
| S1 | 2.570 | 40 |
| S2 | 1.451 | 20 |
| S3 | 0.832 | 10 |
| S4 | 0.446 | 5 |
| S5 | 0.270 | 2.5 |
| S6 | 0.177 | 1.25 |
| S7 | 0.148 | 0.625 |



## Performance Characteristics

### Specificity

The cross reactivities for a number of related compounds were determined by diluting cross reactants in the assay buffer from a starting concentration of 8,000-40,000 pg/mL. These samples were then measured in the assay.

| Analyte | Cross Reactivity |
|---|---|
| LC3A | not detected |
| LC3B | not detected |
| LC3B2 | not detected |
| NBR-1 | not detected |
| NUP54 | not detected |
| NUPL1 | not detected |
| NTF2 | not detected |
| PKCι | not detected |
| PKCζ | not detected |

### Sensitivity

The sensitivity or limit of detection of the assay is 100 pg/mL. The sensitivity was determined by interpolation at 2 standard deviations above the mean signal at background (0 ng/mL) using data from at least 7 standard curves.

### Precision

**Intra-assay precision** was determined by assaying 20 replicates of two buffer controls containing p62 in a single assay.

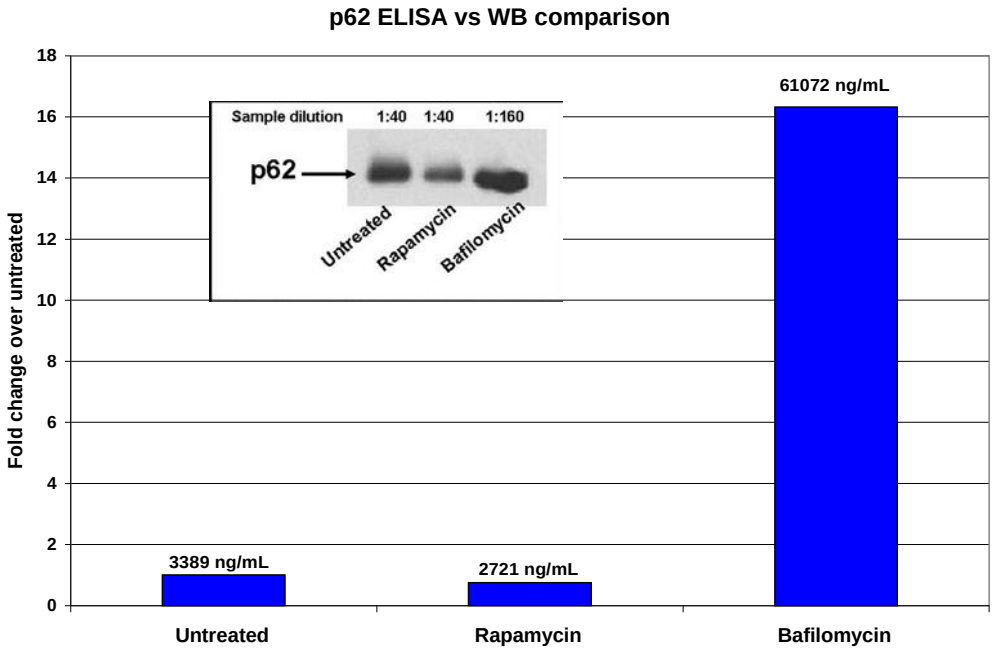
| ng/mL | %CV |
|---|---|
| 18.05 | 3.31 |
| 2.04 | 4.46 |

**Inter-assay precision** was determined by measuring buffer controls of varying p62 concentrations in multiple assays over several days.

| ng/mL | %CV |
|---|---|
| 13.75 | 5.46 |
| 1.26 | 11.27 |

## Stimulation Experiments

This experiment was adapted from a protocol in reference 12. Human HeLa cells were treated for 24 hours with 800nM Rapamycin, an inducer of autophagy, or 800nM Bafilomycin A1, an inhibitor of vacuolar ATPase which leads to an accumulation of autophagosomal structures. Cells were then harvested, washed and lysed at $5x10^6$ per mL RIPA Cell Lysis Buffer 2 following the procedure in the Cell Lysate Preparation section. These samples were diluted and resolved on an 8-12% Tris-glycine gel, transferred to nitrocellulose membrane and probed for p62. The same cell lysates were also diluted in assay buffer and run in this kit.




The Western Blot provided a visual for both the decrease and increase in p62 levels after treatment of cells in culture. The p62 ELISA kit was able to assign values to the amounts of p62 protein (numbers above each bar) thus allowing a quantitative comparison of the decrease with Rapamycin treatment (~25%) and the increase with Bafilomycin A1 treatment (~1800%). It is also worth noting that the minimal required dilutions needed for the samples in the ELISA kit were 1:40, 1:80 and 1:640 for data acquisition amongst the linear portion of each treatment curve.

## References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## Notes

## Limited Warranty

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