Immunoassays for the quantitation of two key autophagy biomarkers p62 and NBR1

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ABSTRACT

p62 and NBR1 share similar functions within the autophagy pathway. NBR1 and p62 aid in protein trafficking and degradation. Although the proteins differ in size and sequence, both proteins contain N-terminal Phox domains and Bem1 (PB1) domains, LC3-interacting region (LIR) motifs, and C-terminal ubiquitin-associated (UBA) domains. Although NBR1 and p62 interact and form oligomers, they can also function independently.

Immunoassay kits using a colorimetric 96-well microtiter plate format ELISA have been developed for the detection and quantitation of p62 and NBR1. The sensitivities of the individual kits were determined to be 100 pg/mL and 66 pg/mL, respectively. Both assays demonstrated parallel dose response curves between the recombinant standard and cell lysates from human, mouse and rat origin. Distinct concentrations of recombinant p62 were spiked into three cell lysates, diluted in assay buffer and run in the assay. Similarly, recombinant NBR1 concentrations were spiked into lysis buffer, diluted in assay buffer and run in the assay. The recovery of the spiked p62 was 88, 95 and 99% following a 1:16 dilution. The recovery of the spiked NBR1 was 89, 88 and 106% following a 1:8 dilution. The p62 ELISA intra-assay precision ranged from 3.31 to 4.46% CV and inter-assay precision ranged from 5.5 to 11.27% CV. The NBR1 ELISA intra-assay precision ranged from 3.7 to 7.8% CV and inter-assay precision ranged from 15.6 to 21.8% CV.

The immunoassays were used to measure autophagic flux by detecting changes in p62 and NBR1 levels. This was shown in a time course study in which MDA-MB-231 human breast cancer cells were treated with an autophagy inducing drug withaferin A (WA). Both p62 and NBR1 levels were reduced in response to drug treatment at 6 hour and 12 hour time points. Interestingly, both protein levels increased at the 24 hour time point. p62 ELISA results correlated with western blot (NBR1 not tested). WA treatment of cells correlated with induction of autophagy as confirmed by LC3-II levels in western blot and puncta formation in immunofluorescence staining. The accumulation of p62 and NBR1 at the 24 hour time point contrasted with elevated LC3-II possibly indicating the beginning of autophagy resolution. In a second experiment, HeLa cells were treated with Bafilomycin A1 or Rapamycin. After Bafilomycin A1 treatment, both p62 and NBR1 protein levels showed significant increases, 18-fold and 1.8-fold, respectively. In the Rapamycin cell treatment, p62 protein level showed a modest decrease of 25%, whereas no decrease in NBR1 protein level was noted. ELISA results for both p62 and NBR1 correlated with western blot results.

PERFORMANCE CHARACTERISTICS

p62 and NBR1 Standard Curves

Recombinant p62 or NBR1 were serially diluted in assay buffer and run in their respective ELISA. Sensitivity of the p62 and NBR1 assay, defined as the concentration of analyte measured at 2 standard deviations above background, was determined to be 100 and 66 pg/mL, respectively.

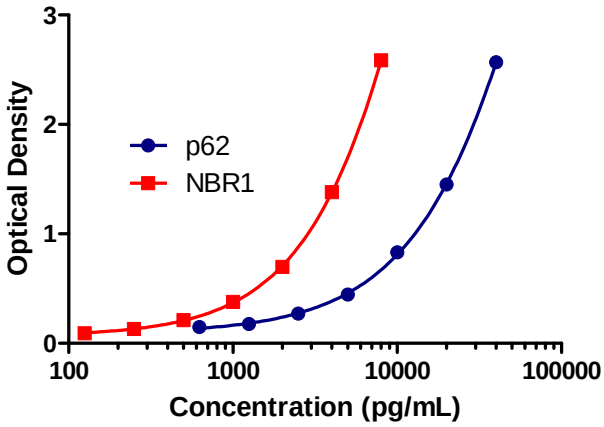


Figure 1: Recombinant human p62 and NBR1 standard curves

ASSAY VALIDATION

Dilutional Linearity of cell lysates in p62 and NBR1 ELISA

Cell lysates were prepared in RIPA cell lysis buffer 2 containing protease inhibitors and DNase. Clarified lysates were serially diluted in assay buffer and run in either p62 (Table 1A) or NBR1 (Table 1B) immunoassays. Values were calculated from each immunoassay's respective protein standard. Dilutional linearity was determined by dividing the assigned concentration at dilution over the assigned concentration of the most dilute sample that read within the dynamic range of the assay and multiplying by 100. Dilutional linearity of cell lysates was generally achieved at 1:8 dilution in the p62 ELISA and 1:4 in the NBR1 ELISA.

Table 1: Dilutional linearity of cell lysates from human, mouse and rat origin in p62 (A) and NBR1 immunoassay (B)

A

Dilution Factor	HeLa	C6	3T3
1:2	---	---	---
1:4	---	92	---
1:8	85	102	77
1:16	94	104	88
1:32	91	99	86
1:64	95	110	91
1:128	97	105	97
1:256	100	100	100

B

Dilution Factor	HeLa	C6	3T3
1:4	90	103	99
1:8	104	103	106
1:16	116	110	113
1:32	112	100	100
1:64	100	---	

p62 and NBR1 spike and recovery in diluted cell lysates

Recombinant p62 was spiked into 1:16 diluted cell lysate or assay buffer at three concentrations. Recombinant NBR1 was spiked into neat cell lysate or assay buffer at three concentrations and subsequently diluted 1:8 in assay buffer. The spiked samples were analyzed in the assay alongside the standard for assigning concentrations. The recovery of three spiked concentrations in the p62 (Table 2A) and in the NBR1 immunoassays (Table 2B), are represented as percentage by calculating the ratio of spiked lysate to the spiked assay buffer control, and multiplying by 100.

Table 2: Spike and Recovery experiments of recombinant antigen spiked in cell lysate matrix

A

p62 Spike	Recovery of Spike
High	91%
Medium	90%
Low	95%

B

NBR1 Spike	Recovery of Spike
High	89%
Medium	88%
Low	106%

Inter- and intra-assay reproducibility of p62 and NBR1 immunoassays

Interassay precision was determined by measuring buffer controls of varying recombinant antigen concentrations in multiple assays over several days. Intraassay precision was determined by assaying 20 replicates of two (p62) or three (NBR1) buffer controls containing recombinant antigen in a single assay. Reproducibility data for p62 and NBR1 is shown in Tables 3A and 3B, respectively.

Table 3: p62 and NBR1 inter- and intra-assay reproducibility

A

Interassay		Intraassay	
ng/mL	%CV	ng/mL	%CV
13.75	5.46	18.05	3.31
1.26	11.27	3.31	4.46

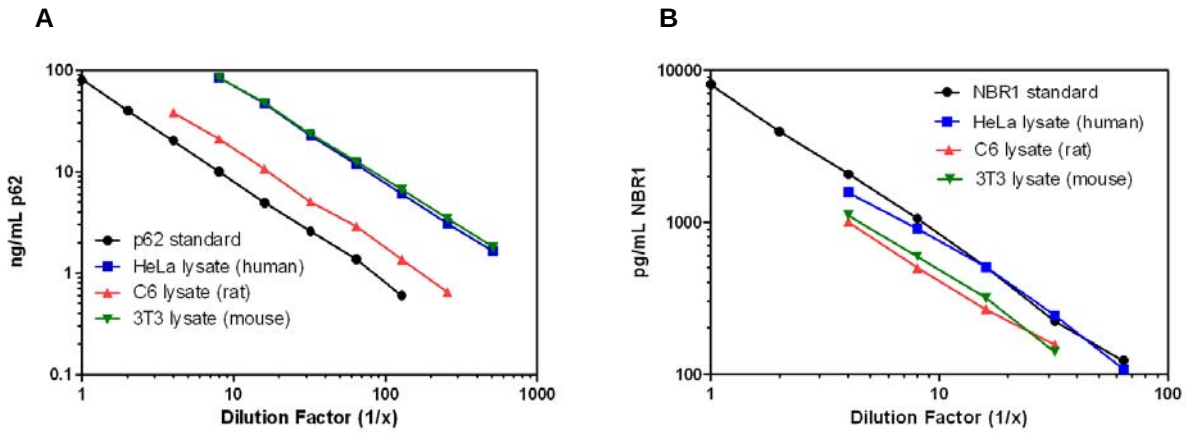
B

Interassay		Intraassay	
pg/mL	%CV	pg/mL	%CV
8000	15.6	5000	3.7
1000	17.2	1250	4.4
125	21.8	312.5	7.8

Parallelism studies for p62 and NBR1 immunoassays

HeLa, C6 and 3T3 cell lines were lysed in RIPA cell lysis buffer 2 containing protease inhibitors and DNase. Clarified cell lysates were serially diluted in assay buffer and run in either the p62 (Figure 2A) or NBR1 (Figure 2B) ELISA with the respective standards. p62 or NBR1 concentrations were assigned to each dilution and plotted as a function of dilution factor. 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, rat and mouse origin.

Figure 2: Dose response curves of recombinant standard and native antigen

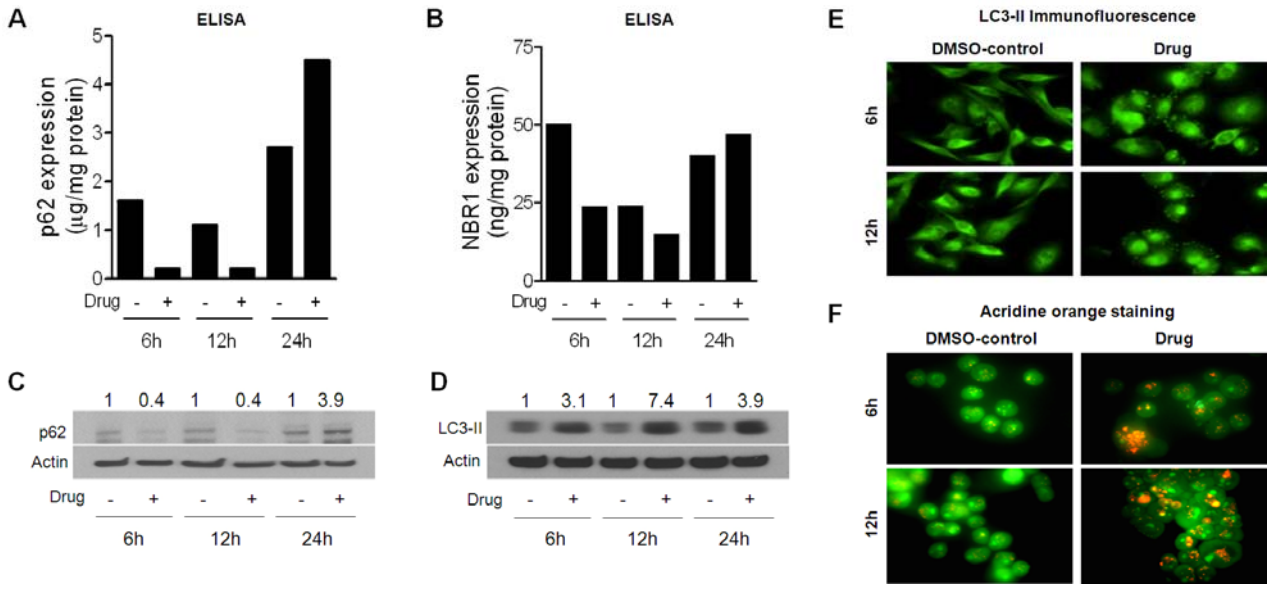


Immunoassay Correlation

Correlation of p62 and NBR1 immunoassay to autophagy induction

MDA-MB-231 human breast cancer cells were treated with 2 µM of withaferin A (WA), an autophagy inducing drug. Cells were harvested at 6, 12 and 24 hours post-treatment and lysed in RIPA cell lysis buffer 2 containing protease inhibitors and DNase. Cell lysates were clarified by centrifugation and analyzed in p62 assay (Figure 3A) and NBR1 assay (Figure 3B). Concentration of antigen was normalized to total cellular protein. Cell lysates were resolved by SDS-PAGE and analyzed by western blot using anti-p62 antibody from Cell Signaling, CN #5114 (Figure 3C) or anti-LC3 antibody from Cell Signaling, CN #2775 (Figure 3D). Signal intensity in both western blots were compared to actin levels and numbers above band represent changes in protein levels relative to corresponding DMSO-treated control. LC3-II immunofluorescence and acridine orange staining indicating puncta formation and colocalization with autolysosomes demonstrated autophagy induction post WA treatment (Figure 3E and 3F).

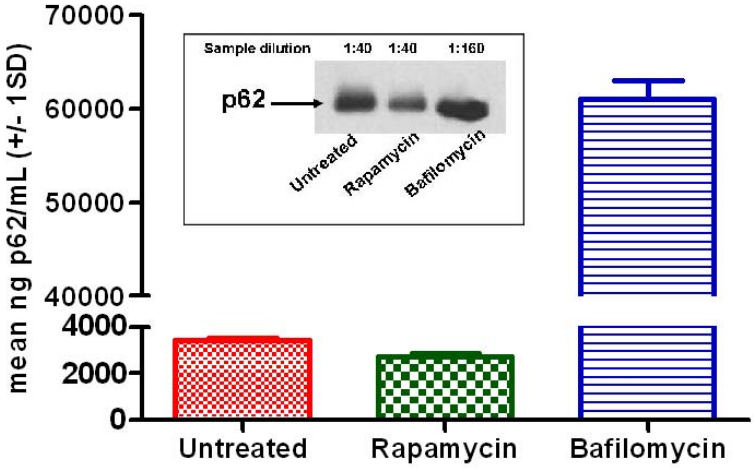
Figure 3: ELISA results correlated to western blot and LC3-II immunofluorescence



p62 ELISA Correlation to Western Blot

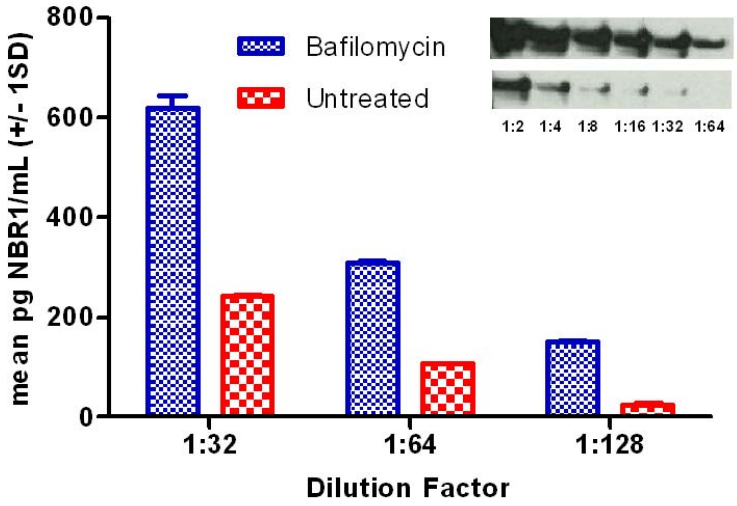
HeLa cells were treated with DMSO, 800nM Rapamycin (inducer of autophagy) or 800nM Bafilomycin A1 (inhibitor of autolysosome formation). After treatment for 24 hours, cells were collected and lysed with RIPA cell lysis buffer 2 containing protease inhibitors and DNase. Cell lysates were clarified by centrifugation and analyzed in the p62 assay. Rapamycin treatment showed a slight decrease in p62 levels, whereas Bafilomycin A1 treatment showed a large increase in p62 levels in comparison to the control. ELISA results correlated to western blot.

Figure 4: HeLa cell lysates analyzed by ELISA and western blot



NBR1 ELISA Correlation to Western Blot

Figure 5: HeLa cell lysates analyzed by ELISA and western blot



HeLa cells were treated with DMSO (control) or 800nM Bafimomycin (inhibitor of autolysosome formation) for 24 hours. Cells were harvested and lysed in RIPA cell lysis buffer 2 containing protease inhibitors and DNase. After centrifugation, the supernatant was collected, diluted in assay buffer and analyzed in the NBR1 assay. Bafilomycin treatment showed a 2x accumulation of NBR1. ELISA results correlated to western blot.

CONCLUSIONS

- Immunoassays for the detection of p62 and NBR1 have been successfully developed with each having a dynamic range of 100 – 40,000 and 66 – 8,000 pg/mL, respectively.
- The assays have been validated for use with cell lysates of human, mouse and rat origin. Dilutional linearity of a cell lysate matrix is achieved with a 1:8 dilution in assay buffer for the p62 assay and a 1:4 dilution in assay buffer for the NBR1 assay.
- Cell lysate dose response curves are parallel to the recombinant standard for both p62 and NBR1 immunoassays, indicating antibody-binding characteristics of the native and standard proteins are similar, allowing for accurate determination of these analytes.
- Immunoassays correlate to standard western blot and immunofluorescence techniques while providing improved sensitivity.
- p62 and NBR1 biomarkers may be early indicators of autophagy, resolving after cell treatment with withaferin A (inducer of autophagy).

Acknowledgments:

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