StressMarq Biosciences INC.

Discovery through partnership | Excellence through quality

**StressXpress®**

# Autophagy Flux Detection Kit

Catalog# SKT-135 (10 Western Blot Analyses Kit)

Detection of autophagy

# TABLE OF CONTENTS

# GENERAL INFORMATION

## Materials Supplied

| Catalog Number | Reagent | Quantity | Storage |
|---|---|---|---|
| SKC-135A | Bafilomycin A1 MAX (high purity) | 100µg | -20°C |
| SKC-135B | StressXpress® LC3B detection antibody | 20µL | -20°C |
| SKC-135C | StressXpress® p62 detection antibody | 10µL | -20°C |
| SKC-135D | Anti-rabbit IgG antibody (HRP-conjugate) | 20µL | 4°C |
| SKC-135E | StressXpress® β-actin detection antibody | 10µL | -20°C |
| SKC-135F | Anti-mouse IgG antibody (HRP-conjugate) | 10µL | 4°C |

If any of the items listed above are damaged or missing, please contact our Customer Service department at (250) 294-9065. We cannot accept any returns without prior authorization.

**WARNING: Not for human or animal disease diagnosis or therapeutic drug use.**

## Precautions

**Please read these instructions carefully before beginning this assay.**

Kit contains sufficient materials to run 10 Western blot analyses for each marker under specified conditions. Information provided describes application of StressXpress® assay to measure autophagy in cells treated with potential biochemical modulators.

**Note:** These methods can also be directly applied to the measurement of autophagy in cells subject to other interventions such as siRNA gene knockdown and plasmid transfection.

**For research use only. Not for human or diagnostic use.**

## If You Have Problems

**Technical Service Contact Information**


**Phone:** 250-294-9065

**Fax:** 250-294-9025

**E-Mail:** techsupport@stressmarq.com

**Hours:** M-F 9:00 AM to 5:00 PM PST


In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

## Storage and Stability

Kit components should be stored at the stated temperatures to ensure stability and activity. Once dissolved in DMSO aliquot and store bafilomycin A1 MAX solutions at -20ºC for up to 3 months. Avoid multiple freeze-thaws. Diluted antibody solutions can be stored for up to 1 month at 4ºC.

## Materials Needed But Not Supplied

- Reagents for Western blotting
- Image capture equipment / densitometry software (e.g.http://rsbweb.nih.gov/ij/index.html)

# INTRODUCTION

## Background

Autophagy is a lysosomal degradation pathway responsible for the removal of long-lived soluble proteins, misfolded and aggregated proteins, unwanted and damaged organelles (including mitochondria and peroxisomes) and intracellular pathogens (bacteria and viruses) (1,2). It is involved in various physiological or pathological processes, including promoting survival under starvation conditions, development, host defence response and immunity, cancer and neurodegenerative diseases in addition to maintenance of cellular proteostasis (3–5). Autophagy involves concerted action of more than 20 specific autophagy (ATG) proteins that mediate the formation of a double-membrane vesicle, the autophagosome, which engulfs its substrates and delivers them to the lysosome for degradation.

## Autophagy measurement

Autophagy can be accurately assessed by measurement of autophagic flux, the complete process of autophagy from phagophore formation to substrate degradation and release of breakdown products. Changes in the flux or 'turnover' of the autophagy system upon intervention (e.g. drug treatment, gene knockdown, plasmid transfection) provide an excellent indication of its effect on autophagy and offers significant advantages over the static analysis of individual biomarkers levels often employed (6).

LC3-II (lipidated / autophagosome membrane associated form of LC3) and p62 (ubiquitin binding autophagy substrate receptor) proteins can be utilised as markers for measurement of autophagic flux. Differences in marker levels in the presence and absence of lysosomal degradation (inhibited by bafilomycin A1) represent the amount of LC3-II or p62 degraded and are a measure of autophagic flux (6–9).

Use of a combination of autophagy markers to assess autophagic flux helps overcome the limitations of any one single marker in assessing such a complex system, facilitating accurate interpretation of results6. For example LC3-II can associate with non-autophagic membranes and p62 can be degraded by the proteasome.

Measurement of changes in autophagic flux following specific interventions such as drug treatment, gene knockdown or transfection, can be used to assess the effect of the intervention upon autophagy (10).

## About This Assay

A simple, easy-to-use kit for measurement of autophagy

- Measure autophagy using established markers, LC3-II and p62
- Utilise highly characterised antibodies and control inhibitors to assess autophagic flux
- Analyse effect of experiments on autophagy by Western blotting
- Accurately interpret results

The autophagy flux kit facilitates the simple measurement of autophagy in cells. The kit utilises highly characterised antibodies to key autophagy biomarkers, LC3-II and p62, together with application of specific high purity control inhibitor for the accurate assessment of autophagic flux by Western blotting methods. Contains sufficient materials to run 10 Western blot analyses for each marker under specified conditions.

### Use this kit to

1. Determine effect of your experiment on autophagy
2. Identify and characterise autophagy modulators (activators / inhibitors)
3. Assess result of gene knockdown (siRNA / shRNA) on autophagy
4. Evaluate outcome of plasmid transfection / protein expression on autophagy
5. Investigate autophagy in specific cell lines

# PRE-ASSAY PREPARATION

## Overview

- Prepare untreated control and modulator treated cells in the presence and absence of high purity bafilomycin A1 MAX (BafA1)
- Compare, in parallel, LC3-II and p62 (and actin*) levels in resulting cell extracts by Western blotting
- Determine Western blot band intensities by densitometry (normalised to actin*)
- Evaluate autophagic flux for untreated and modulator treated cells
- Assess effect of modulator treatment on autophagy

*Alternative housekeeping proteins such as tubulin can be used instead of actin for normalisation of results.

## Antibodies

| Antibody | Comment |
|---|---|
| StressXpress® LC3B detection antibody (SKC-135B) | LC3B isoform specific, no cross-reactivity with LC3A, LC3C or GABARAP proteins. Detects LC3B-I and LC3-II |
| StressXpress® p62 detection antibody (SKC-135C) | Reactivity with p62 protein from a number of different species including human, rat and mouse. Binds mutated forms of p62 in addition to wild type |

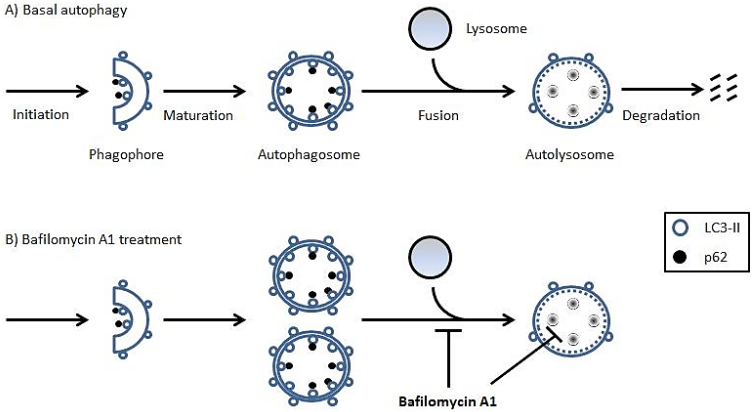


**Figure 1:** Schematic showing basal autophagy in cells and the effect of bafilomycin A1 (BafA1) treatment. Difference between LC3-II or p62 marker levels with and without BafA1 treatment represents the autophagic flux / turnover of the system and can be used to assess changes in autophagy upon experimental investigation

## Assay Preparation

### Bafilomycin A1 (BafA1)

- Dissolve bafilomycin A1 (SKC-135A, 100µg) in DMSO to give stock solution concentration required
- Aliquot and store solutions at -20°C for up to 3 months
- Recommend BafA1 treatment of cells at a final concentration of 100nM for 2 hours as a starting point

### Lysis buffer

- Nature of lysis buffer used can affect the amount of p62 in the soluble and insoluble cell fractions
- To minimise presence of p62 in the insoluble fraction use of RIPA buffer or a similar buffer together with protease inhibitors is recommend

## Assays / controls

| | |
|---|---|
| Standard assay | Untreated control cells +/- BafA1.<br><br>Treated cells +/- BafA1.<br><br>Measure autophagic flux in cells. Compare autophagic flux in untreated and treated cells to assess effect of intervention on autophagy. |
| Optional controls | Rapamycin treatment of cells (+/- BafA1) is a positive control for autophagy induction in cell line of interest and can be used to induce autophagy in cells with low autophagic flux.<br><br>Proteasome inhibitor treatment of cells (+/-BafA1) to assess contribution of proteasome to p62 degradation. Use of epoxomicin or lactacystinrecommended. May induce autophagy. |

## Technical tips

| Component | Comment |
|---|---|
| Time points | LC3-II changes may be rapid, whereas clearance of autophagy substrate p62 may require a longer time. If LC3 levels are tested at 6 or 24 hours after treatment p62 could be tested at these and later time points (24 or 48 hours) to determine maximal impact on p62 clearance. |
| Cell lysis | p62 (and LC3-II) may be found in both soluble and Triton X-100 insoluble fractions following cell lysis. If required, both cell extract fractions can be analysed by Western blotting or lysis buffer containing 1% SDS used to solubilise entire cellular p62 population. |
| Effect on autophagy | Taking additional time points both early and late in the treatment process may assist in the accurate determination of its effect on autophagy, for example a potential modulator might increase autophagic flux initially but inhibit autophagy with prolonged exposure. |
| Low / no autophagic flux in cells | Absence or low levels of autophagic flux in untreated cells of particular interest may be addressed by treatment with an autophagy activator such as rapamycin (50nM-1μM, up to 24 hours) prior to the start of the planned cell treatment process. |
| Bafilomycin A1 treatment | Optimal conditions for treatment of cells with BafA1 will depend on the level of basal autophagy and the specific cell line used. Adjustment of BafA1 concentration and treatment time may therefore be required to optimise assay performance. |

## Assay optimisation

Optimal assay conditions for testing the effect of a particular treatment or intervention on autophagy in a specific cell line must be determined by the user. Adjustment of the following may facilitate this process:

- Modulator concentration
- Modulator treatment time
- Cell lysis buffer
- Number of time points analysed
- Bafilomycin A1 concentration / treatment time (50-400nM, 1-4 hours)
- Western blotting sample loading
- Use of PVDF membrane for Western blotting

## Sample Production

1. Prepare untreated control (UT) and modulator treated (MT) cells using appropriate methods for cell type and treatment / intervention of interest
2. Add bafilomycin A1 to the + BafA1 cells to a final concentration of 100nM 2 hours from end of cell preparation process (Figure 2)
3. Prepare whole cell extracts using preferred lysis buffer (see 'assay preparation' for recommendations)

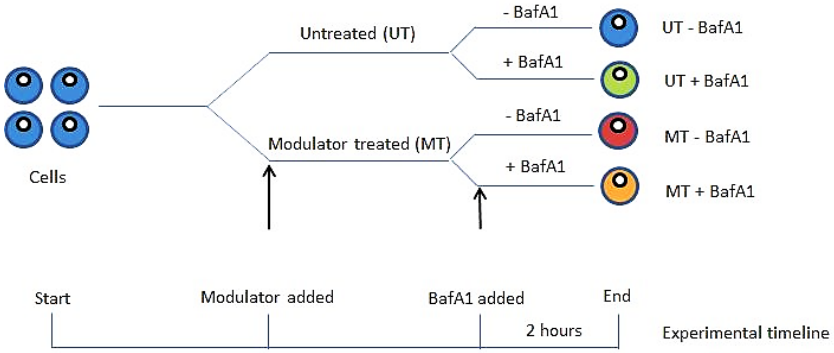


**Figure 2:** Experimental timeline for StressXpress® assay sample production

## Sample analysis

4. Analyse cell extracts by Western blotting
    - StressXpress® Autophagy Flux Kit contains LC3 and p62 rabbit polyclonal detection antibodies and a β-actin mouse monoclonal detection antibody for Western blot analysis
    - Sufficient materials supplied to run at least 10 Western blots analyses for LC3-II, p62 and βactin at recommended dilutions, on the basis of using 2mL of diluted antibody per Western blot
    - Secondary antibody HRP-conjugates provided are optimised for use with StressXpress® detection antibodies
    - Analyse samples for LC3-II and p62 levels alongside β-actin or alternative housekeeping protein levels to enable normalisation of data
    - Use of PVDF membrane recommended over nitrocellulose membrane - improved LC3-II retention
    - Optimal Western blotting sample loading and analysis conditions must be determined by the User

**Note:** Ensure LC3-I and LC3-II are properly resolved by SDS-PAGE

| Variable | Recommendation |
|---|---|
| SDS-PAGE | ≥12% gel |
| Cell lysate (1 mg/mL) sample loading | 10-20µL |
| LC3B detection antibody (SKC-135B) | 1:1000 |
| p62 detection antibody (SKC-135C) | 1:5000 |
| Anti-rabbit IgG antibody (HRP-conjugate) (SKC-135D) | 1:5000 |
| β-actin detection antibody (SKC-135E) | 1:1000 |
| Anti-mouse IgG antibody (HRP-conjugate) (SKC-135F) | 1:5000 |

5. Measure Western blot band intensities using appropriate densitometry software

   (e.g. ImageJ http://rsbweb.nih.gov/ij/index.html)

6. Normalise results relative to β-actin (e.g. divide sample's LC3-II band intensity by β-actin band intensity)

7. Determine autophagic flux (AF) for both LC3-II and p62 markers:

   UT AF = (UT + BafA1) – (UT – BafA1)

   MT AF = (MT + BafA1) – (MT – BafA1)

8. Calculate change in autophagic flux (ΔAF) between untreated control and modulator treated cells:

   ΔAF = MT AF – UT AF

9. Assess change in (UT – BafA1) LC3-II and p62 levels between untreated control and modulator treated cells

10. Compare changes in autophagic flux and in (UT – BafA1) LC3-II / p62 level results with the StressXpress® Interpretation Guide to assess effect of modulator treatment on autophagy

11. Recommend repeating experiment at least twice and determination of standard deviations in order to validate observations

## Example Results

The following results show analysis of starved cells and cells treated with a potential autophagy modulator using the StressXpress® Autophagy Flux Kit in order to elucidate their effect on autophagy.

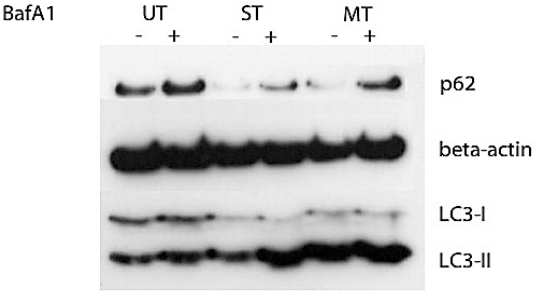


**Figure 3:** Western blot showing detection of LC3-II, p62 and actin in untreated control cells (UT), starved cells (ST) and cells treated with a potential autophagy modulator (MT) and in the absence and presence of bafilomycin A1 (BafA1) using the StressXpress® Autophagy Flux Kit.

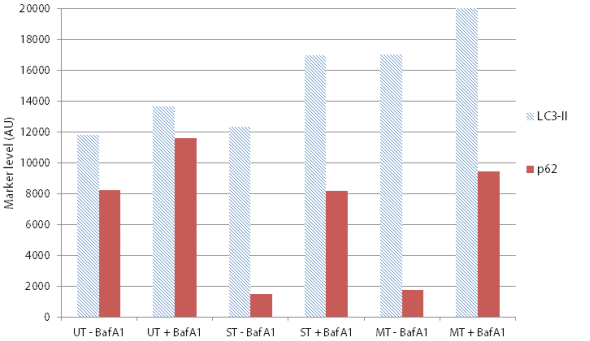


**Figure 4:** Data showing LC3-II and p62 levels (normalised to actin) for untreated control (UT), starved (ST) and modulator treated (MT) cells derived from Western blot band intensities (Figure 3).

| | LC3-II | p62 |
|---|---|---|
| UT–BafA1 | 11793 | 8208 |
| UT+BafA1 | 13627 | 11624 |
| **UT AF** | **1834** | **3416** |
| ST–BafA1 | 12315 | 1472 |
| ST+BafA1 | 16922 | 8174 |
| **ST AF** | **4607** | **6702** |
| **ΔAF (starved cells)** | **2773** | **3286** |
| MT–BafA1 | 17005 | 1730 |
| MT+BafA1 | 20476 | 9422 |
| **MT AF** | **3471** | **7692** |
| **ΔAF (treated cells)** | **1637** | **4276** |

**Table 1:** Data evaluating autophagic flux (AF) and change in autophagic flux (ΔAF) upon starvation (ST) or modulator treatment (MT) of cells using LC3-II and p62 markers.

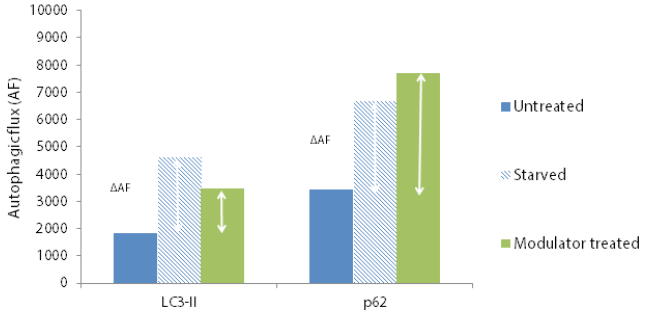


**Figure 5:** Measurement of change in autophagic flux (ΔAF) in cells following starvation or modulator treatment, as determined Table 1.

| | LC3-II | p62 |
|---|---|---|
| (ST–BafA1) – (UT–BafA1) | 522 | -6736 |
| (MT–BafA1) – (UT–BafA1) | 5212 | -6478 |

**Table 2:** Data evaluating change in LC3-II and p62 marker levels upon starvation (ST) or modulator treatment (MT) in the absence of bafilomycin A1 (BafA1).

Both markers exhibit an increase in autophagic flux upon starvation / modulator treatment.

Results show both starvation and treatment of cells with potential autophagy modulator leads to an increase in autophagic flux coupled with an increase in LC3-II levels and a decrease in p62 levels compared to untreated control (-BafA1, figure 4).

Using the StressXpress® interpretation guide it could therefore be concluded that starvation leads to autophagy activation (as expected) and that the potential modulator tested acts as an autophagy activator by increasing autophagosome synthesis and/or turnover.

## Interpretation Guide

Compare data generated using the StressXpress® Autophagy Flux Kit with the following guidelines to facilitate interpretation of results and determination of the outcome of treatment / intervention on autophagy.

| | Outcome | Change in autophagic flux (ΔAF) | | Change in marker level (MT vs. UT, no BafA1) | |
|---|---|---|---|---|---|
| | | LC3-II | p62 | LC3-II – BafA1 | p62 – BafA1 |
| **1** | Activation (induction) | ↑ | ↑ | →/↑ | ↓ |
| **2** | Late stage inhibition | ↓ | ↓ | ↑ | ↑ |
| **3** | Early stage inhibition | ↓ | ↓ | ↓ | ↑ |
| **4** | No effect | → | → | → | → |

**Table 2:** Interpretation of StressXpress® assay results

| Outcome | Effect on autophagy |
|---|---|
| Activation (induction) | Autophagosome synthesis and/or turnover increased |
| Late stage inhibition | Lysosomal degradation and/or autophagosome-lysosome fusion decreased |
| Early stage inhibition | Autophagosome synthesis decreased / autophagy inactivated |
| No effect | No discernible effect on autophagy process |

**Table 3:** Effect of experiment on autophagy

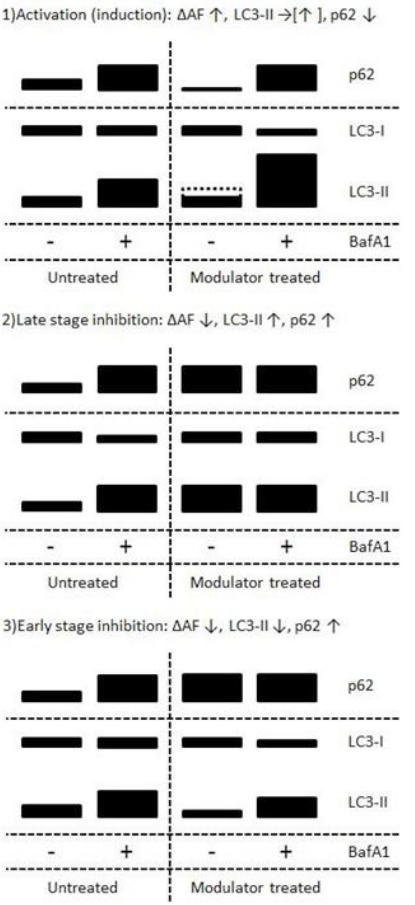


**Figure 6:** Western blot diagram showing comparison of LC3-II and p62 marker levels upon treatment with autophagy modulators resulting in activation (1), late stage inhibition (2) or early stage inhibition (3) of autophagy.

## References


1. Wang, K. & Klionsky, D. J. Autophagy 7, 297–300 (2011). PMID: 21252623
2. Wong, E. & Cuervo, A. M. Cold Spring Harbor perspectives in biology 2, a006734 (2010). PMID: 21068151
3. Rosenfeldt, M. T. & Ryan, K. M. Carcinogenesis 32, 955–63 (2011). PMID: 21317301
4. Galluzzi, L., Kepp, O. & Kroemer, G. The EMBO journal 30, 3213–4 (2011). PMID: 21785408
5. Metcalf, D. J., García-Arencibia, M., Hochfeld, W. E. & Rubinsztein, D. C. Experimental neurology 238, 22–8 (2012). PMID: 21095248
6. Klionsky, D. J. et al. Autophagy 8, 445–544 (2012). PMID: 22966490
7. Mizushima, N., Yoshimori, T. & Levine, B. Cell 140, 313–26 (2010). PMID: 20144757
8. Mizushima, N. & Yoshimori, T. Autophagy 3, 542–5 (2007). PMID: 17611390
9. Rubinsztein, D. C. et al. Autophagy 5, 585–9 (2009). PMID: 19411822
10. Menzies, F. M., Moreau, K., Puri, C., Renna, M. & Rubinsztein, D. C. Current protocols in cell biology / editorial board, Juan S. Bonifacino ... [et al.] Chapter 15, Unit 15.16 (2012). PMID: 22422474

## Warranty and Limitation of Remedy

StressMarq Biosciences Inc. makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. StressMarq **warrants only** to the original customer that the material will meet our specifications at the time of delivery. StressMarq will carry out its delivery obligations with due care and skill. Thus, in no event will StressMarq have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if StressMarq is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of StressMarq, its directors or its employees.

Buyer's **exclusive remedy** and StressMarq's sole liability hereunder shall be limited to a refund of the purchase price, or at StressMarq's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to StressMarq within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

**For further details, please refer to our Warranty and Refund Policy located on our website and in our catalog**.

# NOTES