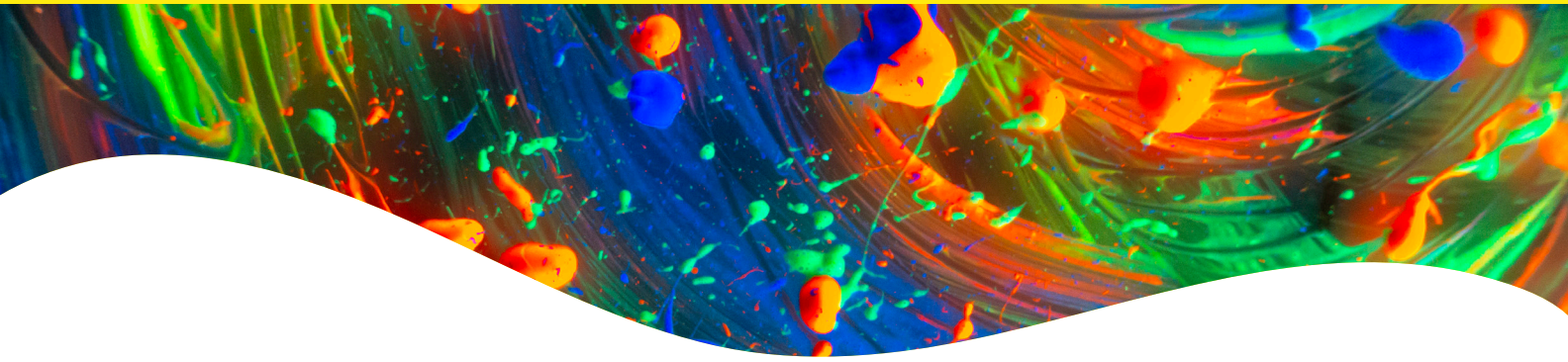
PhenoVue Autophagy Staining Kit (LC3, p62/SQSTM1, LAMP1) for 2 x 96-well plates



Overview

Autophagy is a fundamental catabolic process that enables cells to degrade and recycle damaged organelles, misfolded proteins, and other cytoplasmic constituents via lysosome-mediated degradation. It plays a key role in maintaining cellular homeostasis, adapting to metabolic stress, and regulating inflammation. Autophagy is tightly regulated by multiple signaling pathways and is dynamically modulated in response to environmental conditions such as nutrient deprivation, oxidative stress, or infection.

Dysregulation of autophagy has been implicated in a wide range of pathological conditions including cancer progression and resistance, neurodegenerative disorders (e.g., Alzheimer's, Parkinson's), infectious diseases, and inflammatory syndromes. Therefore, reliable tools to visualize and quantify autophagic activity are essential for both basic research and therapeutic development.

The PhenoVue™ Autophagy staining kit provides a powerful and user-friendly solution for high-content imaging of key autophagy markers in fixed cells. It includes highly characterized, high-specificity antibodies against:

- LC3 (microtubule-associated proteins 1A/1B light chain 3B): involved in autophagosome membrane formation and elongation.
- p62/SQSTM1: a selective cargo adaptor that accumulates when autophagy is impaired, serving as a readout for autophagic degradation.
- LAMP1 (lysosome-associated membrane protein 1): a marker of lysosomes and autolysosomes involved in final cargo degradation.

Also including PhenoVue Fluor secondary antibody conjugates and fixation/permeabilization buffers, the kit enables sensitive and specific multiplexed detection of autophagy-related structures. Together with Revvity's PhenoPlate™ imaging microplates and high content screening instruments, the kit provides both optimal performance and experimental flexibility.

Product information

Product name	Part no.	Number of vials per unit	Shipping conditions
PhenoVue Autophagy staining kit – 2 x 96 well plates	PAUT14	10	Dry ice

Product name	Format	Quantity*	Storage
PhenoVue Hoechst 33342 Nuclear stain	Liquid	1 vial of 70 µL (500x)	2-8 °C or below Protect from light
PhenoVue anti-p62/SQSTM1 mouse IgG1 antibody (100x) Human only	Liquid	1 vial of 100 µL (100x)	-16 °C or below
PhenoVue anti-LAMP1 rat IgG2b antibody (100x) Human only	Liquid	1 vial of 100 µL (100x)	-16 °C or below
PhenoVue anti-LC3 rabbit IgG antibody (100x) Human, mouse and rat	Liquid	1 vial of 100 µL (100x)	-16 °C or below
PhenoVue Fluor 488 - Goat anti-mouse antibody, highly cross-adsorbed (100x)	Liquid	1 vial of 100 µL (100x)	-16 °C or below Protect from light
PhenoVue Fluor 555 - Goat anti-rat antibody, highly cross-adsorbed (100x)	Liquid	1 vial of 100 µL (100x)	-16 °C or below Protect from light
PhenoVue Fluor 647 - Goat anti-rabbit antibody, highly cross-adsorbed (100x)	Liquid	1 vial of 100 µL (100x)	-16 °C or below Protect from light
PhenoVue Paraformaldehyde 4% solution	Liquid	1 vial of 25 mL (1x)	2-8 °C or below Protect from light
PhenoVue Permeabilization 0.5% Triton X-100 solution	Liquid	1 vial of 25 mL (5x)	2-8 °C or below
PhenoVue Dye diluent A (5x)	Liquid	1 vial of 8 mL (5x)	-16 °C or below

*The quantities of reagents provided are sufficient for 2 x 96 or 1 x 384 well microplate using the recommended concentrations.

Storage and stability

- After receiving, store PhenoVue Permeabilization 0.5% Triton X-100 solution and PhenoVue Paraformaldehyde 4% solution at 2–8 °C, protected from light. Other reagents can be stored together at ≤ -16 °C or separately between ≤ -16 °C to 2-8 °C protected from light, as indicated in the table above. Avoid repeated freeze/thaw cycles.
- Allow the reagents to warm up to room temperature for 30 min before opening the vials and reconstitution. Aliquoted reagents must be stored at -16 °C or below and are stable for 6 months.
- After thawing, the PhenoVue Dye diluent A (5x) may contain some aggregates which will not impair the product and image quality. If aggregate removal is preferred, the PhenoVue Dye diluent A (5x) can be filtered (0.22 µm filter) prior to dilution. The diluted PhenoVue Dye diluent A 1x must be stored at 2-8 °C for no more than 2 days.
- The stability of these products is guaranteed until the expiration date provided in the certificate of analysis, when stored as recommended and protected from light.

Spectral and photophysical properties

Product name	Maximum excitation wavelength (nm)	Maximum emission wavelength (nm)	Common filter set	Quantum yield (Φ)	Epsilon* (ε in $M^{-1}.cm^{-1}$ at λ max)	Brightness (Φ x ε)
PhenoVue Hoechst 33342	357**	455**	DAPI	dsDNA: 0.38 ssDNA: 0.22	43,000	dsDNA: 16,340 ssDNA: 9,460
PhenoVue Fluor 488	495	520	FITC	92%	73,000	65,320
PhenoVue Fluor 555	555	570	Cy3	10%	155,000	15,500
PhenoVue Fluor 647	650	670	Cy5	30%	240,000	72,000

*In PBS pH 7.4 ** In methanol with 0.2 M HCl

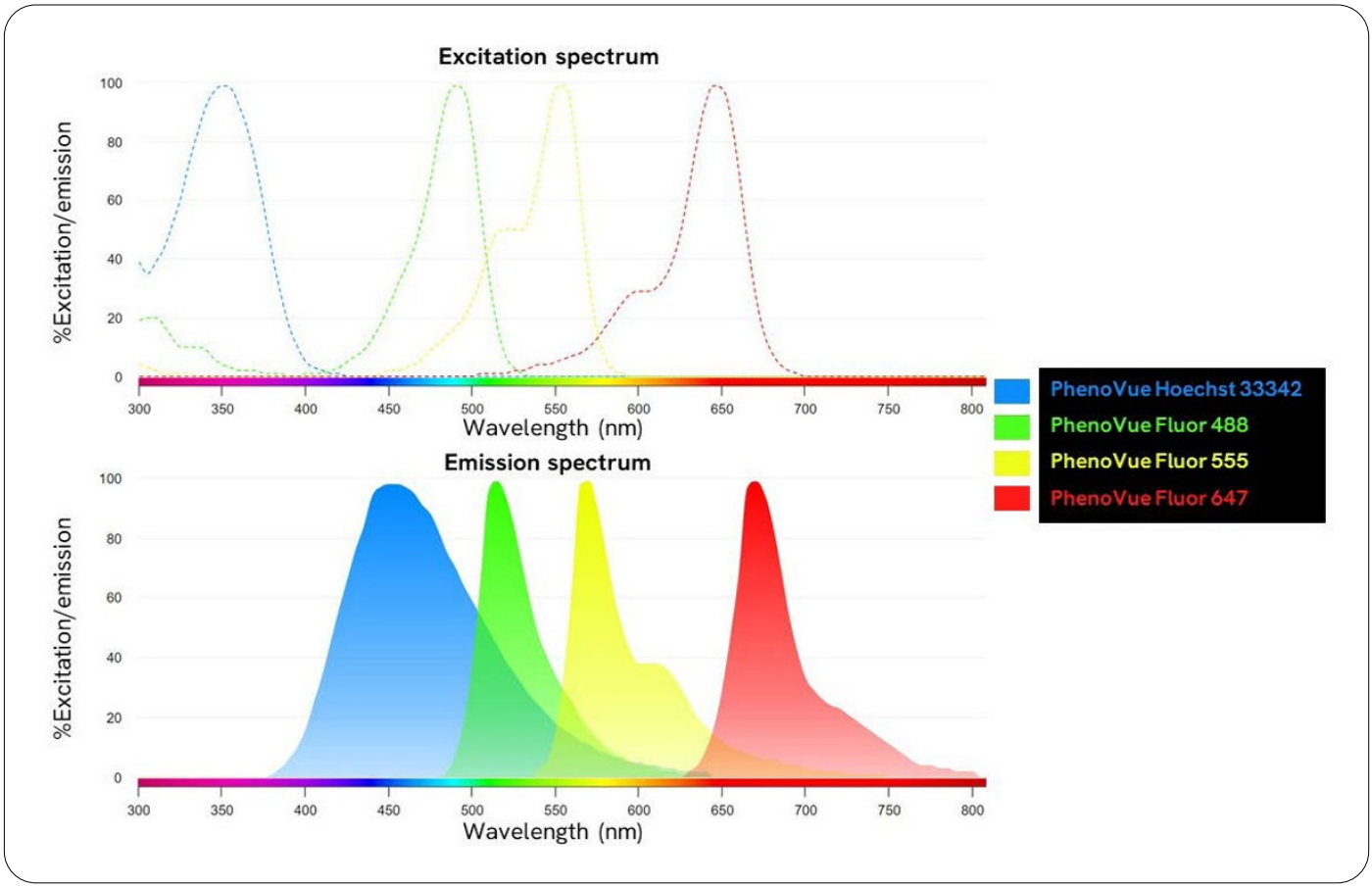


Figure 1: Excitation and emission spectra of the PhenoVue Autophagy staining kit components.

Other materials and reagents required

Reagents or consumables	Usage
PBS	Washing buffer and diluent for PhenoVue Permeabilization 0.5% Triton X-100 solution
Distilled H_2O	Dilution of PhenoVue Dye diluent A (5x)
PhenoPlate™ 96-well microplates*	Cell plating, stimulation, staining and imaging
Aluminum single-tab foil	Plate sealing to protect fluorescent probes from light

*This protocol can be adapted for use with PhenoPlate 384-well microplates. See Protocol Section for details.

Reagent reconstitution and preparation of staining solutions

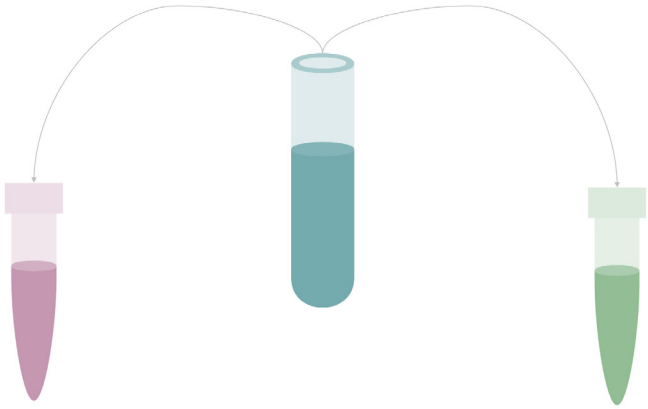
	Reagents	Preparation of staining solution
Buffers	PhenoVue Paraformaldehyde, 4% solution	Ready to use
	PhenoVue Permeabilization 0.5% Triton X-100 solution	Dilute 5 times in PBS
	PhenoVue Dye diluent A (5x) *	Dilute 5 times in distilled H_2O
Staining solution 1	PhenoVue anti-p62/SQSTM1 mouse antibody (100x) Human only	Dilute 100 times in diluent A (1x)
	PhenoVue anti-LAMP1 rat antibody (100x) Human only	Dilute 100 times in diluent A (1x)
	PhenoVue anti-LC3 rabbit antibody (100x) Human, mouse and rat	Dilute 100 times in diluent A (1x)
Staining solution 2	PhenoVue Hoechst 33342 nuclear stain	Dilute 500 times in diluent A (1x)
	PhenoVue Fluor 488 - Goat anti-mouse antibody, highly cross-adsorbed (100x)	Dilute 100 times in diluent A (1x)
	PhenoVue Fluor 555 - Goat anti-rat antibody, highly cross-adsorbed (100x)	Dilute 100 times in diluent A (1x)
	PhenoVue Fluor 647 - Goat anti-rabbit antibody, highly cross-adsorbed (100x)	Dilute 100 times in diluent A (1x)

*PhenoVue dye diluent A 1x is used as staining solution diluent and as saturation buffer.

Example of preparation of staining solutions

The following example describes the preparation of 10 mL staining solution 1 and 10 mL of staining solution 2, sufficient for 2 x 96-well plates (or 1 x 384-well plate).

Prepare **20 mL PhenoVue Dye diluent A (1x)**
To 16 mL ddH_2O, add 4 mL PhenoVue Dye diluent A (5x)



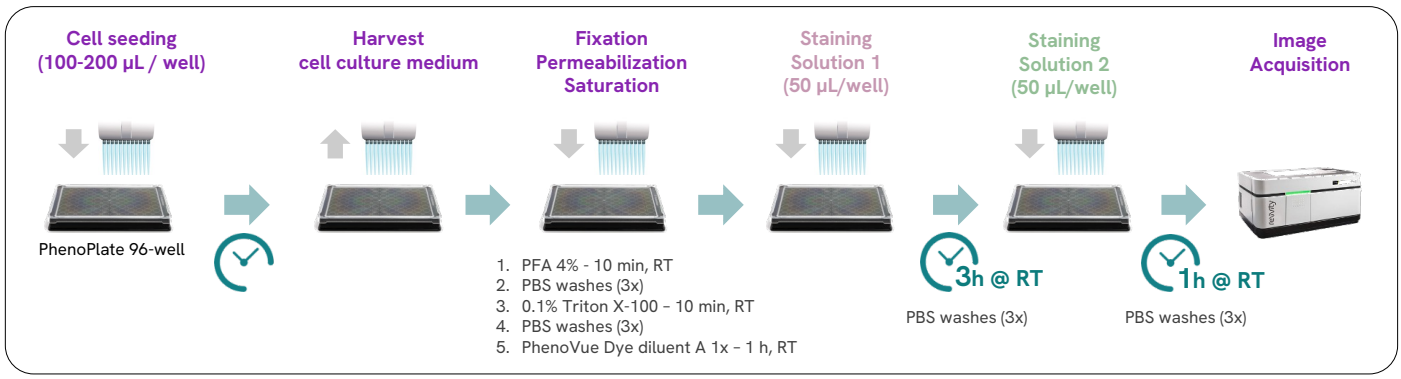
10 mL staining solution 1
To 9.7 mL PhenoVue dye diluent (1x)

- Add 100 µL of PhenoVue anti-p62/SQSTM1 – mouse antibody (100x)
- Add 100 µL of PhenoVue anti LAMP1 - rat antibody (100x)
- Add 100µL of PhenoVue anti LC3 rabbit antibody (100x)

10 mL staining solution 2
To 9.680 mL PhenoVue dye diluent A (1x)

- Add 20 µL of PhenoVue Hoechst 33342 nuclear stain (500x)
- Add 100 µL PhenoVue Fluor 488 - Goat anti-mouse antibody Highly Cross-Adsorbed (100x)
- Add 100 µL PhenoVue Fluor 555 - Goat anti- antibody Highly Cross-Adsorbed (100x)
- Add 100 µL PhenoVue Fluor 647 - Goat anti-rabbit antibody Highly Cross-Adsorbed (100x)

Experimental workflow



Protocol for 96-well imaging plate

A 384-well microplate may also be used; in that case, adjust the cell seeding density appropriately and reduce all reagent volumes two-fold.

Cell culture

Seed cells in PhenoPlate 96-well imaging microplates (or any other convenient cell culture vessels). Incubate in the appropriate cell culture conditions, usually 37 °C, 5% CO_2 until 50-70% confluency.

Fixed-cell imaging

Rinse briefly in phosphate-buffered saline (PBS) then proceed with cell fixation.

1. **Fixation:** Add 50 µL ready-to-use PhenoVue paraformaldehyde 4% solution for 10 min at room temperature.
2. **Washing:** Wash three times with PBS.
3. **Permeabilization:** Add 50 µL PhenoVue permeabilization solution diluted to 0.1% Triton X-100 in PBS for 10 min at room temperature.
4. **Washing:** Wash three times with PBS for 5 min.
5. **Saturation:** Incubate with 50 µL PhenoVue dye diluent A 1x for 1h at room temperature.
6. Remove saturation buffer and **add 50 µL per well of staining solution 1.** Incubate for 3h at room temperature.
7. **Washing:** Wash three times for 5 min with PBS.
8. **Add 50 µL per well of staining solution 2** and incubate for 1h at room temperature protected from light.
9. **Washing:** Wash three times for 5 min with PBS.
10. **Acquire images on an imaging device.**

Tips

- Reagent concentrations have been carefully optimized to limit fluorescence crosstalk on Revvity's high content imaging instruments. Thus, increasing the concentrations may increase crosstalk.
- Staining solution 1 can also be incubated overnight at 4 °C.
- If needed, when using 384-well microplates, centrifuging the plate for 1 min at 500 g and room temperature between each step allows reagents to settle at the bottom of the wells.
- Staining can be performed on human cellular models, as LAMP1 and p62/SQSTM1 are human-specific. Only LC3 antibody recognizes mouse and rat models.

Recommendations for acquisition settings

The PhenoVue Autophagy staining kit allows for multiplexing with 4 colors simultaneously. For optimal fluorescent signal and image quality on Revvity high-content imaging systems, we recommend the following acquisition settings:

HCS instruments		PhenoVue Hoechst 33342	PhenoVue Fluor 488	PhenoVue Fluor 555	PhenoVue Fluor 647
Opera Phenix™ Plus 5 lasers	Excitation laser (nm)	375	488	561	640
	Emission filters (nm)	435-480	500-550	570-630	650-760
Opera Phenix Plus 4 lasers	Excitation laser (nm)	405	488	561	640
	Emission filters (nm)	435-480	500-550	570-630	650-760
Operetta CLS™ 4 or 8 LED	Excitation LED (filters) (nm)	370 (355-385)	475 (460-490)	550 (530-560)	630 (615-645)
	Emission filters (nm)	430-500	500-550	570-650	655-760

Safety information

Chemical reagents are potentially harmful, please refer to the Safety Data Sheet (SDS) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Applications

- High-content analysis / high-content screening
- Imaging microscopy

Assay Validation

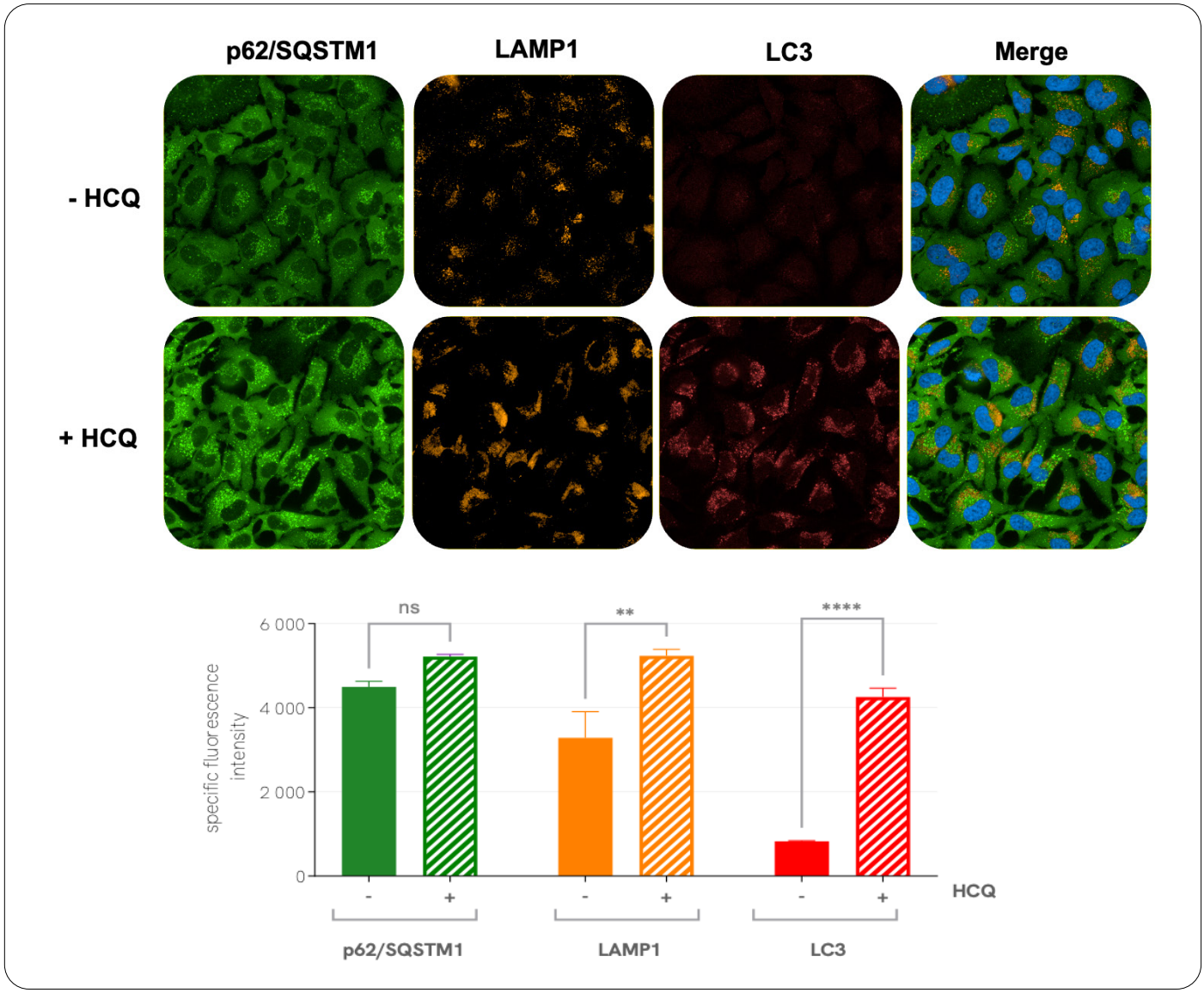


Figure 1: Characterization of autophagic activity in HeLa cells treated with hydroxychloroquine using the PhenoVue autophagy staining kit.

Figure 1 shows HeLa cells seeded in PhenoPlate 96-well microplates at a density of 20,000 cells per well and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Following cell attachment, cells were untreated or treated with 12.5 µM Hydroxychloroquine (HCQ) for 16 hours in complete growth medium. After treatment, cells were fixed, permeabilized, blocked, and stained using the PhenoVue Autophagy staining kit, following the protocol described above. Images were acquired using the Operetta CLS high-content analysis system (8-LED configuration), 63x water immersion objective.

In untreated cells, p62/SQSTM1, LAMP1, and LC3 signals appeared at baseline levels with limited puncta.

Following HCQ treatment, an increase in punctate staining was observed for LAMP1 and LC3, consistent with the accumulation of lysosomes and autophagosomes due to late-stage autophagy inhibition. Quantitative analysis confirmed a significant increase in LAMP1 intensity and a highly significant increase in LC3 intensity. In contrast, p62/SQSTM1 levels remained unchanged, suggesting that HCQ at this dose and timepoint did not lead to measurable accumulation of p62. These results confirm that HCQ disrupts autophagic degradation and validates the sensitivity of the PhenoVue Autophagy staining kit for monitoring autophagy modulation at single cell level

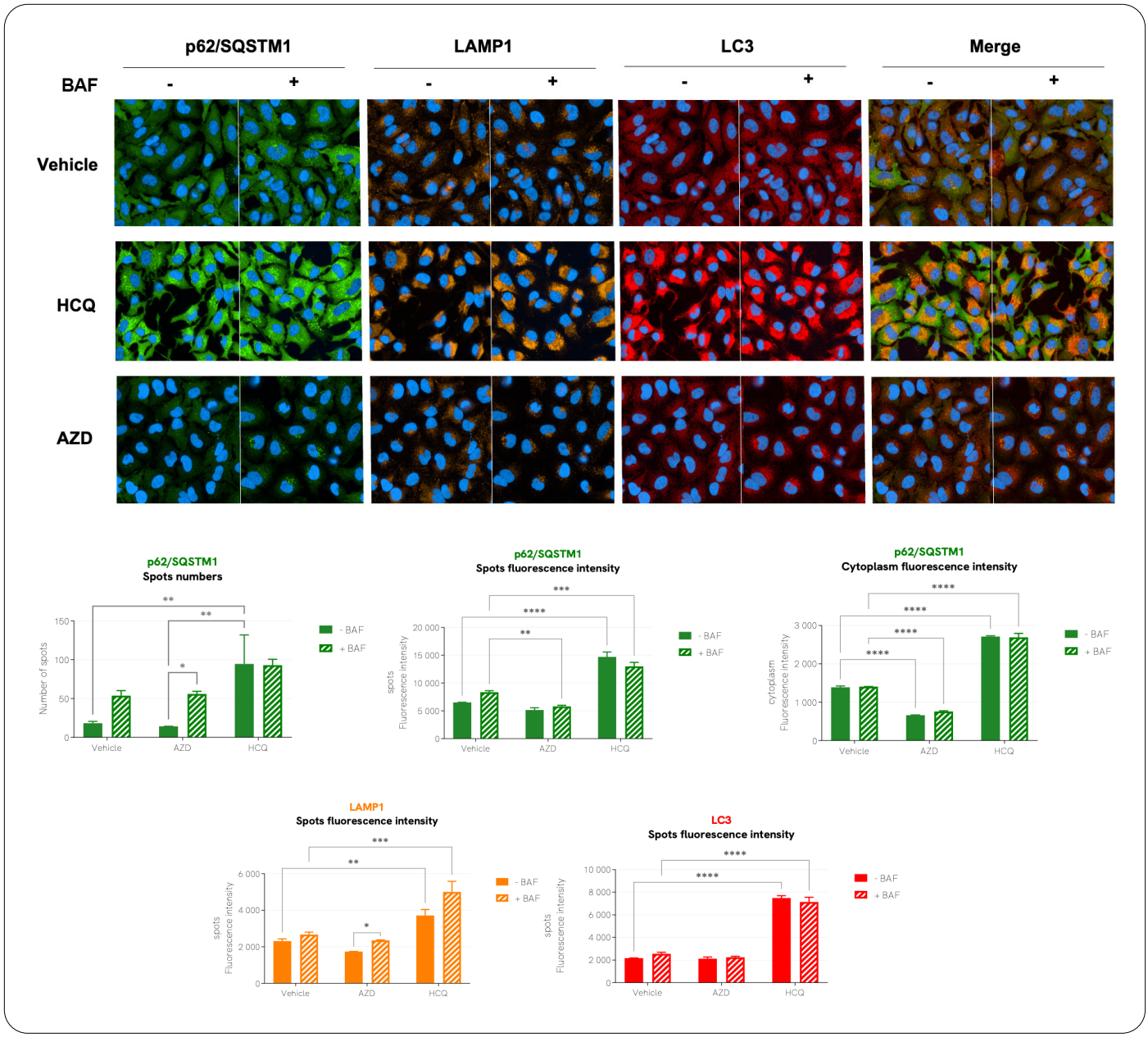


Figure 2: Characterization of autophagic activity in HeLa cells treated with Hydroxychloroquine (HCQ), AZD2014 (an mTOR inhibitor), and/or Bafilomycin A1 (BAF), using the PhenoVue Autophagy staining kit.

Figure 2 shows HeLa cells seeded in PhenoPlate 96-well microplates at a density of 20,000 cells per well and incubated at 37 °C, 5% CO_2. Following cell attachment, cells were treated for 16 hours with either 25 µM Hydroxychloroquine (a late-stage autophagy inhibitor) or 150 nM AZD2014 (Vistusertib), an mTOR inhibitor. For the final 2 hours of incubation, cells were untreated or treated with 200 nM Bafilomycin A1 (BAF), a lysosomal inhibitor that blocks vacuolar-type H+-ATPases (V-ATPases) and prevents lysosomal acidification, thereby impairing the degradation of autophagic cargo. Cells were then stained using the PhenoVue Autophagy staining kit, following the protocol described above. Images were acquired using the Operetta CLS high-content analysis system (8-LED configuration) in confocal mode with a 63x water immersion objective.

The results in figures 1 and 2 offer a comprehensive characterization of autophagic activity in HeLa cells treated with Hydroxychloroquine (HCQ), AZD2014 (an mTOR inhibitor), and/or Bafilomycin A1 (BAF), using the PhenoVue Autophagy staining kit. Quantification was performed at multiple levels: spot-based fluorescence intensity, spot count, and cytoplasmic fluorescence intensity, providing a multi-dimensional view of autophagy dynamics under pharmacological modulation.

Spot-based fluorescence intensity measurements revealed that p62/SQSTM1 levels remained low and unchanged in AZD2014-treated cells, consistent with intact autophagic flux. In contrast, HCQ treatment resulted in a marked increase in p62 spot intensity, further amplified when combined with BAF, indicating impaired degradation and accumulation of autophagosomal cargo. LAMP1, a lysosomal marker, showed significantly increased spot intensity only under HCQ and HCQ + BAF treatment, consistent with lysosomal accumulation resulting from late-stage autophagy blockade. LC3 spot intensity exhibited a substantial increase following HCQ treatment and remained unaffected by additional BAF, suggesting autophagosome accumulation due to impaired autophagosome–lysosome fusion or function.

Spot number analysis for p62/SQSTM1 further confirmed these observations. While AZD2014 alone induced a modest but significant increase in the number of p62-positive structures, this was substantially enhanced when combined with BAF, suggesting that early autophagy induction by mTOR inhibition leads to increased autophagosome formation, but requires lysosomal blockade to reveal accumulation. HCQ alone significantly increased spot number, with BAF co-treatment showing no additional effect, indicating a saturated autophagic block already achieved by HCQ alone.

Cytoplasmic fluorescence intensity of p62/SQSTM1 provided an orthogonal readout of diffuse protein accumulation. While AZD2014 and vehicle treatments maintained low p62 cytoplasmic levels (±BAF), HCQ treatment resulted in a robust increase in diffuse p62 signal, indicative of widespread intracellular accumulation of undegraded protein. BAF co-treatment did not significantly enhance this effect, consistent with complete inhibition of autophagic degradation by HCQ.

Together, these results highlight the sensitivity and versatility of the PhenoVue Autophagy staining kit in monitoring autophagic flux through multiple cellular readouts. They demonstrate that AZD2014 induces early autophagy without impairing degradation, while HCQ and to a similar extent BAF, effectively block autophagic clearance, leading to the accumulation of autophagosomes, lysosomes, and cargo proteins. This multi-parametric approach enables precise dissection of autophagy modulation and offers a robust platform for screening and characterizing autophagy-related drug mechanisms.

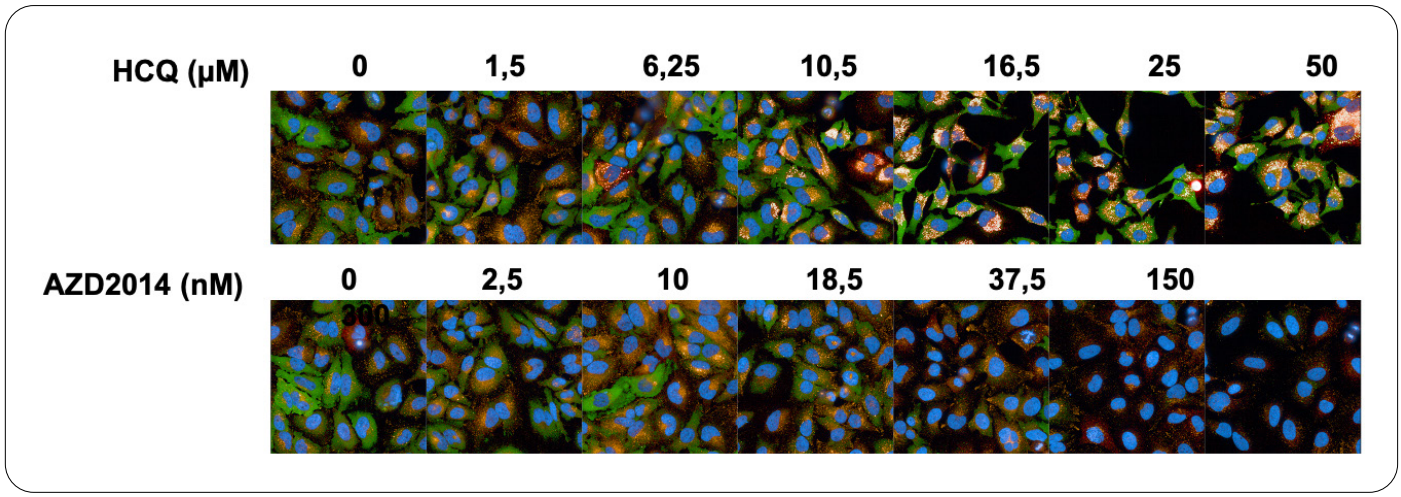


Figure 3: high-content imaging of cells treated with increasing concentrations of either Hydroxychloroquine (a late-stage autophagy inhibitor) or AZD2014 (Vistusertib), an mTOR inhibitor using the PhenoVue Autophagy staining kit.

Figure 3 shows HeLa cells seeded in PhenoPlate 96-well microplates at a density of 20,000 cells per well and incubated at 37 °C, 5% CO_2. Following cell attachment, cells were treated for 16 hours with either increasing concentrations of Hydroxychloroquine (a late-stage autophagy inhibitor) or AZD2014 (Vistusertib), an mTOR inhibitor. Cells were then stained using the PhenoVue Autophagy staining kit, following the protocol described above.

Images were acquired using the Operetta CLS high-content analysis system (8-LED configuration) in confocal mode with a 63x water immersion objective. Multiple parameters were quantified, including cytoplasmic fluorescence intensity, spot fluorescence intensity, spot number, and spot area, using the PhenoVue Autophagy staining kit.

Figure 4: Dose-response curves reflecting the effect of HCQ and AZD on key autophagy markers in HeLa cells, obtained using the PhenoVue Autophagy staining kit.

Figure 4 shows dose response curves that reflect the effect of HCQ and AZD on key autophagy markers in HeLa cells, obtained using the PhenoVue Autophagy staining kit. Multiple phenotypic parameters were measured such as cytoplasmic fluorescence intensity, spot fluorescence intensity, spot number, or spot area for p62/SQSTM1, LAMP1, and LC3, providing a detailed, multiparametric view of autophagic flux modulation.

Figure 4A. p62/SQSTM1 (Green graphs)

HCQ-treated cells exhibited a dose-dependent accumulation of p62 across all readouts:

- Cytoplasmic fluorescence intensity (EC_{50} ≈ 11 µM),
- Spot fluorescence intensity (EC_{50} ≈ 15 µM),
- Spot number (EC_{50} ≈ 9.5 µM).

These results are consistent with blocked autophagic degradation, leading to p62 accumulation. The increased number and intensity of p62-positive spots confirm the accumulation of autophagosomal cargo due to HCQ-induced impairment of lysosomal function.

AZD-treated cells showed the opposite trend:

- A dose-dependent decrease in p62 cytoplasmic intensity (IC_{50} ≈ 46.5 nM),
- A stable or slightly reduced number of p62-positive spots.
- A reduction in spot intensity,

This profile reflects active autophagic degradation following mTOR inhibition. The downregulation of p62 suggests efficient turnover, confirming that AZD2014 enhances flux rather than causing cargo accumulation.

Figure 4B. LAMP1 (Orange graphs)

HCQ induced a clear increase in LAMP1 spot fluorescence intensity and spot area, with an EC_{50} of ~8 µM. These results indicate an accumulation of lysosomal structures, consistent with HCQ's known ability to block lysosomal acidification and fusion events.

AZD, however, had no significant effect on LAMP1 levels across any parameter. This suggests that mTOR inhibition does not alter lysosome abundance or structure.

Figure 4C. LC3 (Red graphs)

HCQ treatment caused a strong increase in LC3 signal:

- Spot number (EC_{50} ≈ 8 µM),
- Spot fluorescence intensity (EC_{50} ≈ 7 µM).

This confirms that HCQ promotes autophagosome accumulation, typical of a late-stage flux block. Elevated LC3 is a hallmark of impaired autophagosome clearance due to defective lysosomal fusion or function.

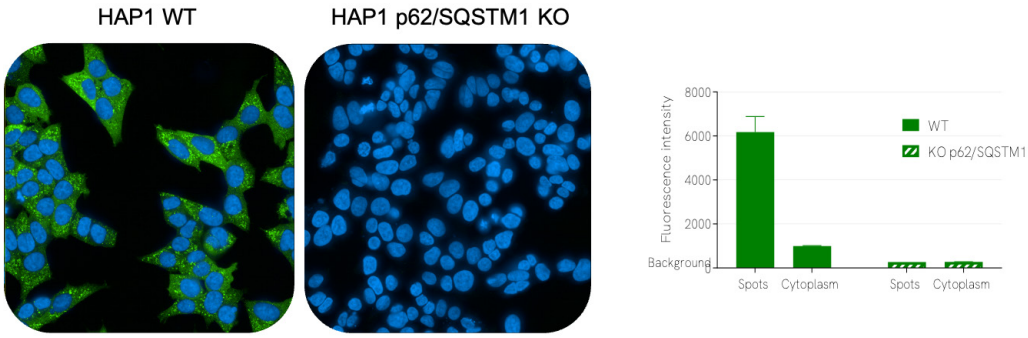
AZD had no measurable impact on LC3 levels, indicating that autophagosomes are formed and cleared efficiently, consistent with intact autophagic flux.

Overall, these results demonstrate the distinct mechanistic profiles of HCQ and AZD2014:

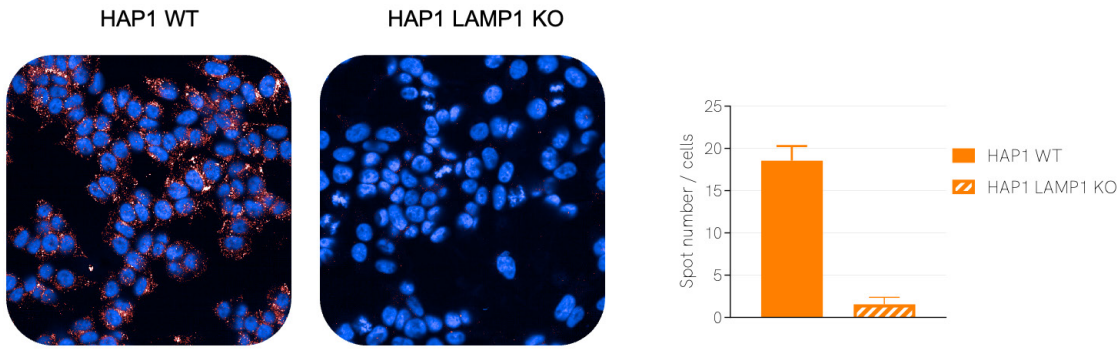
- Hydroxychloroquine acts downstream in the autophagy pathway, blocking degradation, which leads to the accumulation of autophagic cargo (p62), autophagosomes (LC3), and lysosomal structures (LAMP1).
- AZD, by contrast, acts upstream, activating autophagy via mTOR inhibition. This results in efficient degradation of p62 without lysosomal or autophagosome accumulation, validating its role as a flux inducer.

The PhenoVue Autophagy staining kit enables a deep understanding of autophagy modulators' mechanism of action through its ability to simultaneously capture changes in autophagy substrate levels, vesicle accumulation, and lysosomal behavior. The integration of multiple phenotypic parameters ensures a robust and quantitative assessment of autophagy modulation in response to pharmacological agents.

A Anti-p62/SQSTM1 antibody characterization



B Anti LAMP1 antibody characterization



C Anti LC3 antibody characterization

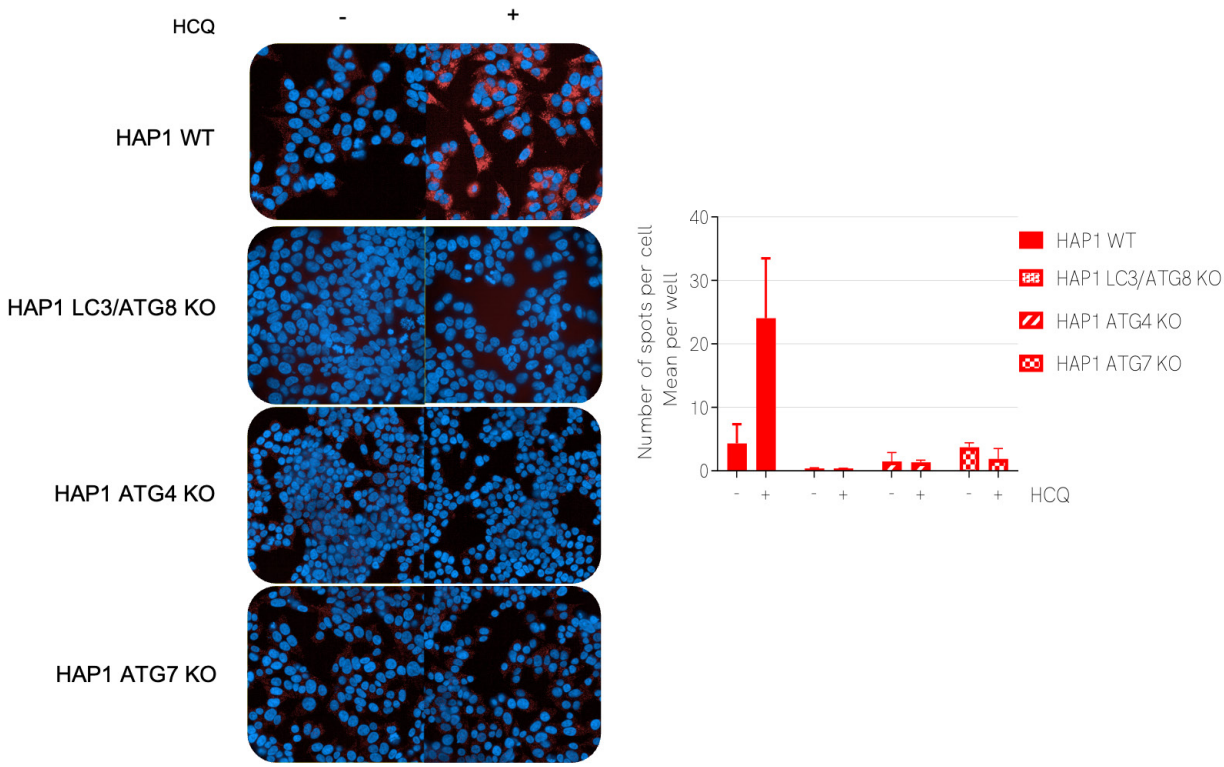


Figure 5: Characterization of anti-p62/SQSTM1, anti-LAMP1 and anti-LC3 antibodies

Figure 5A shows HAP1 wild-type (WT) and p62/SQSTM1 knockout (KO) cells seeded at a density of 15,000 cells per well and treated overnight with 25 µM Hydroxychloroquine. Following treatment, cells were stained using the PhenoVue anti-p62/SQSTM1 primary antibody, followed by the PhenoVue 488 Goat anti-mouse highly cross-adsorbed secondary antibody. Images were acquired using the Operetta CLS high-content analysis system (8-LED configuration) in confocal mode with a 63x water immersion objective.

As expected, no fluorescence signal was detected in the p62 KO cells, confirming the specificity of the PhenoVue anti-p62/SQSTM1 antibody for its target.

Figure 5B shows HAP1 wild-type (WT) and LAMP1 knockout (KO) seeded at a density of 15,000 cells per well and treated overnight with 25 µM Hydroxychloroquine. Following treatment, cells were stained using the PhenoVue anti-LAMP1 primary antibody, followed by the PhenoVue 555 Goat anti-rat highly cross-adsorbed secondary antibody. Images were acquired using the Operetta CLS high-content analysis system (8-LED configuration) in confocal mode with a 63x water immersion objective.

As expected, no fluorescence signal was detected in the LAMP1 KO cells, confirming the specificity of the PhenoVue anti-LAMP1 antibody for its target.

Figure 5C shows HAP1 wild-type (WT) and LAMP1 knockout (KO) cells seeded at a density of 30,000 cells per well and treated overnight with 25 µM Hydroxychloroquine. Following treatment, cells were stained using the PhenoVue anti-LC3 primary antibody, followed by the PhenoVue 647 Goat anti-rabbit highly cross-adsorbed secondary antibody. Images were acquired using the Operetta CLS high-content analysis system (8-LED configuration) in confocal mode with a 63x water immersion objective.

As expected, no LC3 spots were detected in the LC3/ATG8 KO cells, confirming the specificity of the PhenoVue anti-LC3 antibody for its target. Moreover, significant reduction of spots numbers in ATG4 KO and ATG7 KO cells suggest that the anti-LC3 antibody recognizes the LC3-II form

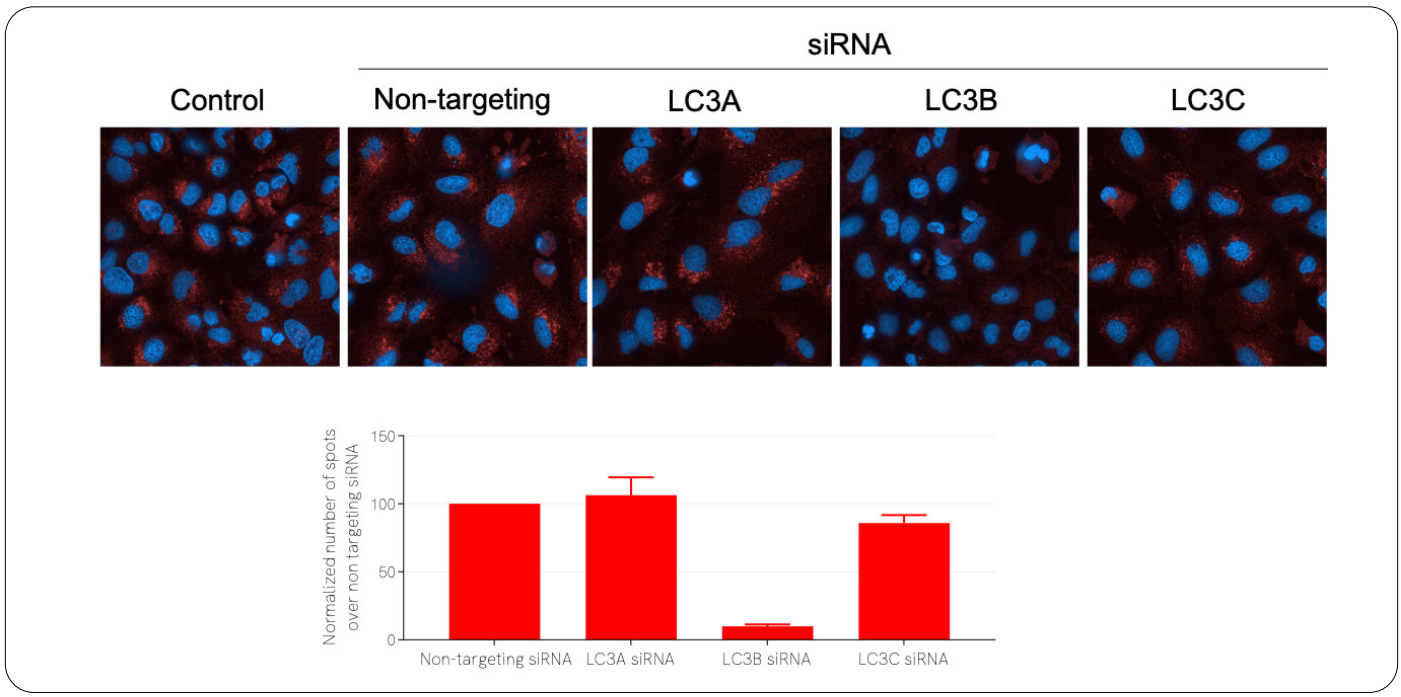


Figure 6: Anti-LC3 antibody characterization - isoform selectivity

Figure 6 shows NCI-H2172 cells seeded in poly-D-lysine-coated PhenoPlate 96-well microplates at a density of 10,000 cells per well and incubated at 37 °C with 5% CO_2. The following day, cells were transfected with 5 µM SMARTPool siRNA for 24 hours. After transfection, cells were treated with 300 µM Hydroxychloroquine for 4 hours in complete medium, then fixed. Immunostaining was performed using the PhenoVue anti-LC3 primary antibody and the PhenoVue Goat anti-rabbit highly cross-adsorbed secondary antibody.

While the number of LC3-positive spots remained unchanged in cells transfected with siRNAs targeting LC3A or LC3C, a marked reduction in LC3 signal was observed following LC3B knockdown. This result indicates that the PhenoVue anti-LC3 antibody predominantly recognizes the LC3B isoform.

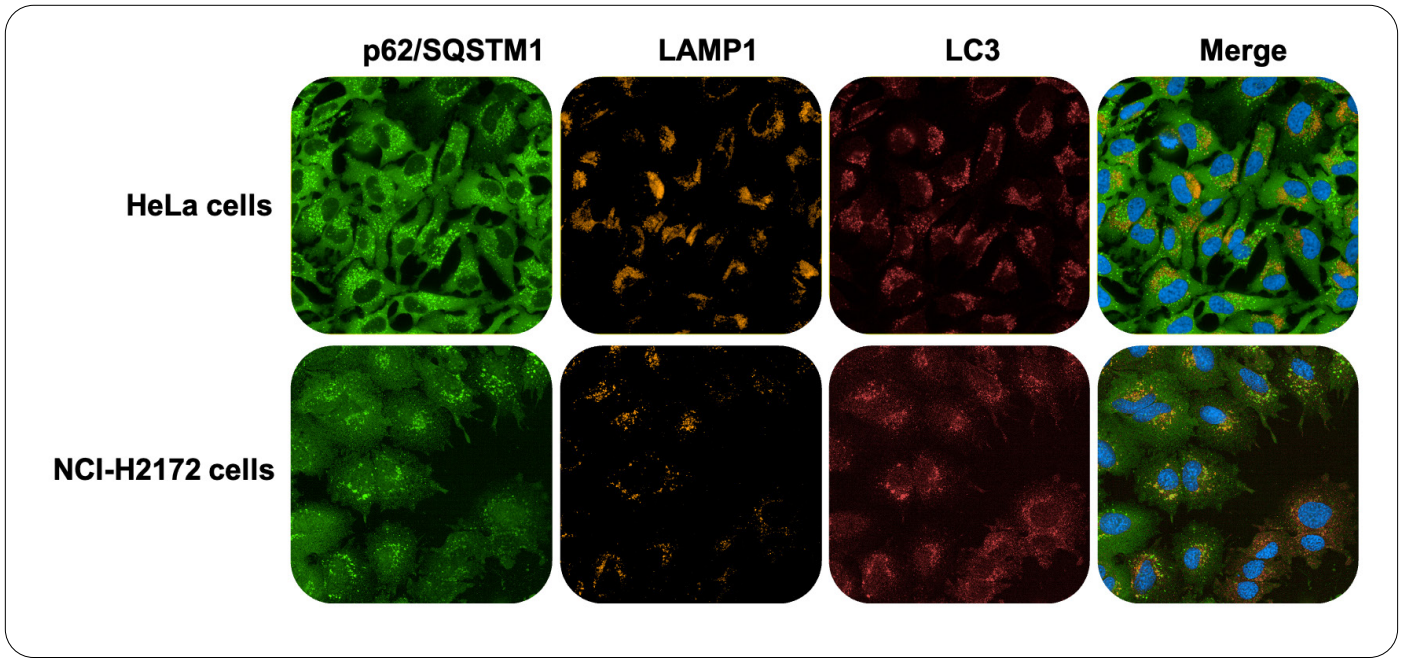


Figure 7: Detection of Hydroxychloroquine-induced autophagosome accumulation in both HeLa and NCI-H2172 cells using the PhenoVue Autophagy staining kit.

Figure 7 shows HeLa cells seeded in PhenoPlate 96-well microplates at a density of 20,000 cells per well and incubated at 37 °C with 5% CO_2. Following cell attachment, cells were treated with 12.5 µM Hydroxychloroquine for 16 hours in complete growth medium. In parallel, NCI-H2172 cells were seeded in poly-D-lysine-coated PhenoPlate 96-well microplates at 10,000 cells per well and incubated under the same conditions. The following day, NCI-H2172 cells were treated with 300 µM Hydroxychloroquine for 4 hours. After treatment, both cell types were fixed, permeabilized, blocked, and stained using the PhenoVue Autophagy staining kit, according to the protocol described above. Imaging was performed using the Operetta CLS high-content analysis system (8-LED configuration) in confocal mode with a 63x water immersion objective. The PhenoVue Autophagy staining kit reliably detected Hydroxychloroquine-induced autophagosome accumulation in both HeLa and NCI-H2172 cells, confirming its suitability for monitoring autophagy across diverse cell types.