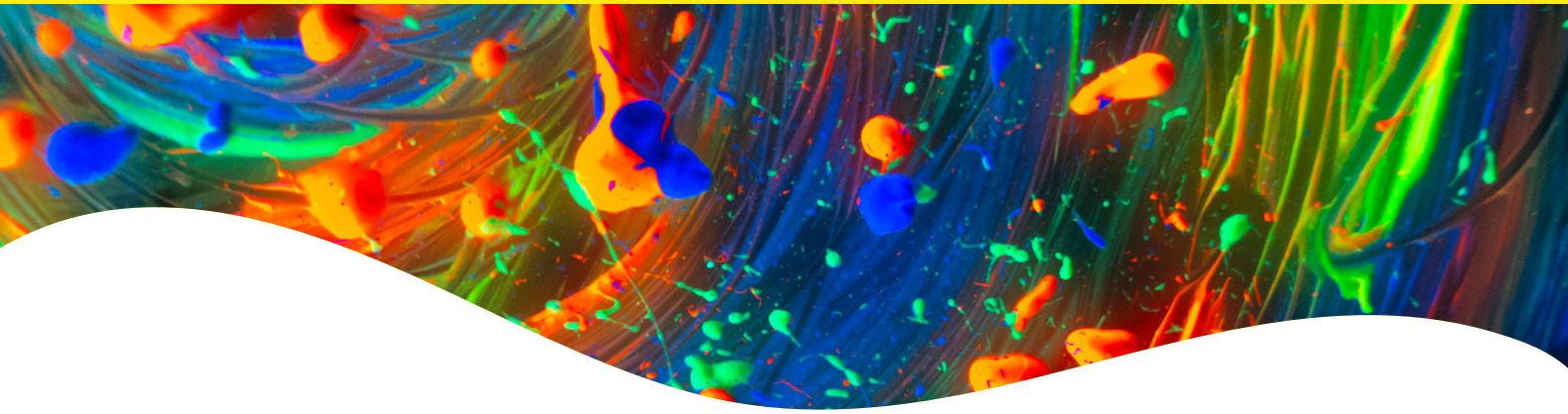
PhenoVue Human IgG Antibody Internalization Kit (Fluor 647, pH sensitive)
(2 x 96-well and 10 x 96-well plates)



Overview

Antibody-mediated internalization is increasingly studied, particularly in the field of immuno-oncology. When an antibody binds to its target receptor, the antibody–receptor complex is endocytosed into the cell and trafficked through acidic vesicles. This complex may then either recycle back to the plasma membrane or undergo lysosomal degradation. Such mechanisms are central to the mode of action of antibody–drug conjugates (ADCs), a promising class of biopharmaceuticals that combine a monoclonal antibody with a cytotoxic payload via specialized linkers. Upon internalization and lysosomal trafficking, the payload is released and induces cell death through mechanisms such as DNA damage or microtubule disruption. Therefore, the ability to accurately and efficiently evaluate antibody internalization is critical in the development of ADCs and related biologics.

The PhenoVue™ human IgG antibody internalization kit has been developed to offer a robust, ready-to-use, and reproducible method for studying antibody internalization. The kit contains PhenoVue Fluor 647 pH-sensitive Fab anti-human IgG, which binds with high affinity to the Fc region of antibodies, whether unconjugated or conjugated (including ADCs). This fluorescent Fab remains weakly fluorescent at neutral extracellular pH (≥7), but its signal increases markedly upon entry into acidic intracellular compartments such as early and late endosomes and lysosomes. This pH-responsive behavior enhances the signal-to-background ratio, enabling clear and reliable visualization of internalization events.

When combined with PhenoVue 488 lysosomal stain and PhenoVue Hoechst 33342 nuclear stain, the kit enables no-wash live-cell, time-lapse monitoring of pH sensitive Fab conjugate–antibody complex trafficking into lysosomes.

Importantly, the probes included in the PhenoVue human IgG antibody internalization kit (Fluor 647, pH-sensitive) are compatible with standard cell culture conditions and have been optimized to exert minimal impact on cell viability and proliferation, ensuring reliable results even during extended live-cell imaging studies.

Product information

Product name	Part no.	Number of vials per unit	Shipping conditions
PhenoVue human IgG antibody internalization kit (Fluor 647, pH sensitive) - 2 x 96 wells*	PHAINTDR14	3	Dry ice
PhenoVue human IgG antibody internalization kit (Fluor 647, pH sensitive) – 10 x 96 wells**	PHAINTDR15	11	Dry ice

* PhenoVue human IgG antibody internalization kit (Fluor 647, pH sensitive) PHAINTDR14 provides sufficient reagents to stain 2 x 96-well plates or 1 x 384-well plate, following the recommended concentrations and volumes.
** PhenoVue human IgG antibody internalization kit (Fluor 647, pH sensitive) PHAINTDR15 provides sufficient reagents to stain 10 x 96-well plates or 5 x 384-well plates, following the recommended concentrations and volumes.

Part no.	Kit contents (For 2 x 96-well plates)	Format	Packaging	Storage
PHAINTDR14	PhenoVue Hoechst 33342 nuclear stain (also available as spare part CP71)	Liquid (H_2O)	1 vial (70 µL, 10000x)	2-8 °C or below. Protect from light
	PhenoVue 488 lysosomal stain (also available as spare part CP104881)	Liquid (DMSO)	1 vial (25 µL, 1000x)	-16 °C or below. Protect from light.
	PhenoVue Fluor 647 pH sensitive Fab anti-human IgG (also available as spare part 2HFAB647PH1)	Liquid (PBS)	1 vial (100 µL, 18 µM)	-16 °C or below. Protect from light.

Part no.	Kit contents (For 10 x 96-well plates)	Format	Packaging	Storage
PHAINTDR15	PhenoVue Hoechst 33342 nuclear stain (also available as spare part CP71)	Liquid (H_2O)	1 vial (70 µL, 10000x)	2-8 °C or below. Protect from light
	PhenoVue 488 lysosomal stain (also available as spare part CP104881)	Liquid (DMSO)	5 vials (25 µL, 1000x)	-16 °C or below. Protect from light.
	PhenoVue Fluor 647 pH sensitive Fab anti-human IgG (also available as spare part 2HFAB647PH1)	Liquid (PBS)	5 vials (100 µL, 18 µM)	-16 °C or below. Protect from light.

Storage and stability

- For convenience, store the kit at ≤ -16 °C. However, each reagent can be stored separately between ≤ -16 °C and 2-8 °C, as indicated in the table above. Avoid repeated freeze-thaw cycles. After reconstitution, aliquoted reagents must be stored at -16 °C or below.
- 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.
- 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.

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.

Other materials and reagents not provided

Reagents or consumables	Usage
Complete cell culture medium	Diluent for PhenoVue stains and compounds.
IgG isotype controls	Available as spare parts PAHIGG1CT1 or PAHIGG4CT1
PhenoPlate™ 96 or 384-well microplates	Cell plating, stimulation, staining and imaging

Live- and fixed-cell compatibility

Product name	Live cell staining	Fixation/permeabilization steps post live-cell staining	Fixed-cell staining
PhenoVue human IgG antibody internalization kit (Fluor 647, pH sensitive)	Yes	No*	No

* PhenoVue Fluor 647 pH sensitive Fab anti-human IgG can be fixed, while the PhenoVue 488 lysosomal stain cannot.

Spectral and pH-sensitive properties of the PhenoVue human IgG antibody internalization kit

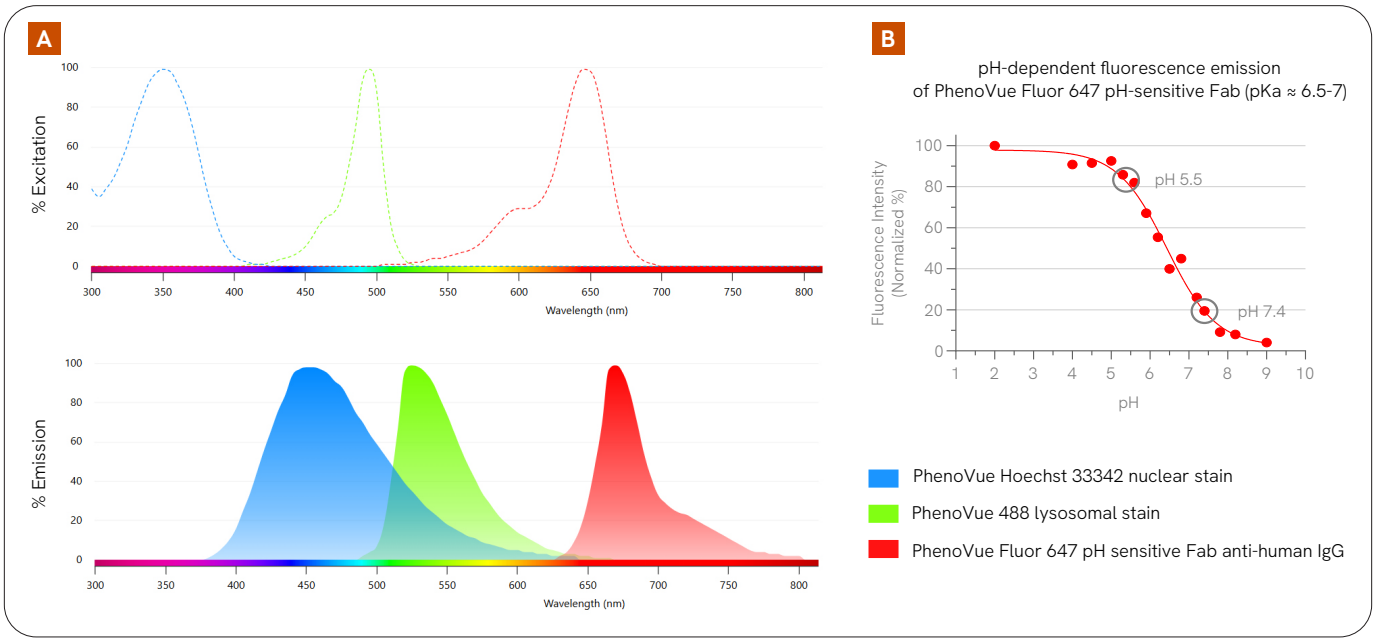


Figure 1: Spectral and pH-sensitive properties of the PhenoVue human IgG antibody internalization kit (Fluor 647, pH-sensitive).
(A) Excitation and emission spectra of the kit components: PhenoVue Hoechst 33342 nuclear stain, PhenoVue 488 lysosomal stain, and PhenoVue Fluor 647 pH-sensitive Fab anti-human IgG. (B) pKa curve of the PhenoVue Fluor 647 pH-sensitive Fab anti-human IgG, showing a pKa between 6.5 and 7, with strong fluorescence at acidic pH (e.g. pH 5.5) and reduced signal at neutral pH (e.g. pH 7.4).

Reagent reconstitution and preparation of staining solution

1. Ready-to-use stock solutions

Reagents	PhenoVue human IgG antibody internalization kit 2 x 96-well / 10 x 96-well
PhenoVue Hoechst 33342 nuclear stain	Ready-to-use stock solution at 1 mg/mL (10,000x)
PhenoVue 488 lysosomal stain	Ready-to-use stock solution (1,000x)
PhenoVue Fluor 647 pH-sensitive Fab anti-human IgG	Ready-to-use stock solution (18 µM)

Aliquot stock solutions and store at **–20 °C for up to 6 months.** Avoid repeated freeze–thaw cycles.

2. Preparation of staining solutions

Reagents	Preparation of staining solutions (100 µL/well)	Example volumes
Staining Solution 1	Prepare in **24.725 mL cell culture medium**	**25 mL total**
PhenoVue Hoechst 33342 nuclear stain	Step 1: Dilute stock solution **1:100** in culture medium (intermediate solution). Step 2: Dilute this intermediate solution **1:100** in culture medium (final 1x solution).	Add **250 µL** from Step 2
PhenoVue 488 lysosomal stain	Dilute stock solution **1:1000** in culture medium (final 1x solution).	**25 µL**
Staining Solution 2	Prepare in **10 mL of Staining Solution 1**	**10 mL total**
Human IgG antibody or human IgG isotype controls *(not provided)*	Dilute in Staining Solution 1 to obtain a final concentration of **60 nM* (2x).**	Adjust volume depending on antibody concentration
PhenoVue Fluor 647 pH-sensitive Fab anti-human IgG	Dilute in Staining Solution 1 to obtain a final concentration of **180 nM (2x).**	**100 µL**

*The recommended 60 nM antibody concentration is provided as a guideline and may be adjusted according to experimental needs. At this concentration, the PhenoVue Fluor 647 pH-sensitive Fab anti-human IgG provides sufficient reagent to stain either two 96-well plates or one 384-well plate

Important: Discard staining solutions after use. Do not store or reuse.

Example preparation of staining solution

The following example describes the preparation of 25mL staining solution 1 and 10mL staining solution 2 - sufficient for 2 x 96-well plates.

Staining solution 1 uses cell culture medium and contains:
- PhenoVue Hoechst 33342 nuclear stain
- PhenoVue 488 lysosomal stain

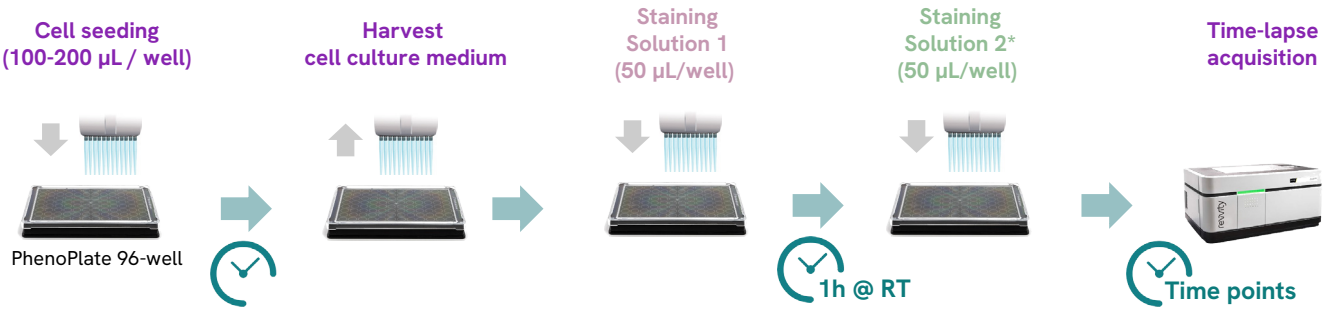
25 mL Staining solution 1
To 24.725 mL of **complete culture medium** :
- Add 250 µL of pre-diluted PhenoVue **Hoechst 33342**
- Add 25 µL of PhenoVue **488 lysosomal** stain

Staining solution 2 uses Staining solution 1 and contains:
- Your Human IgG antibody
- PhenoVue Fluor 647 pH sensitive Fab anti-human IgG
- PhenoVue Hoechst 33342 nuclear stain
- PhenoVue 488 lysosomal stain

10 mL Staining solution 2
Start with **9.9 mL – Vx** of **Staining solution 1**.
- Add a volume **Vx** of your human IgG antibody to obtain a final concentration of **60 nM**.
- Add 100 µL of **PhenoVue Fluor 647 pH-sensitive Fab anti-human IgG** to reach a final concentration of **180 nM**.

Incubate for 30 minutes at RT.

Experimental workflow



*__Prior to use__, the prepared **staining solution 2** should be **pre-incubated for 30 minutes at RT**.

Protocol for 96-well imaging plate

Cell culture

Seed cells in PhenoPlate 96-well imaging microplates* or any other suitable cell culture vessel. Incubate under appropriate conditions (typically 37 °C, 5% CO_2) until cells reach 50–70% confluency.

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

Live cell staining protocol

1. Cell preparation
Carefully remove the culture medium by gentle aspiration.

2. Add 50 µL of Staining Solution 1 per well.
Incubate for 1 hour at 37 °C, 5% CO_2.

3. Preparation of Staining Solution 2
During the pre-staining step, prepare Staining Solution 2 and allow it to equilibrate for 30–90 minutes at room temperature (RT). Maintain an antibody:Fab molar ratio of 1:3.

4. Add 50 µL of Staining Solution 2 per well.
Incubate at 37 °C, 5% CO_2.

Note: If additional compounds are used (e.g. Bafilomycin A), adapt the protocol accordingly. Ensure that the final concentration of staining solutions and the Ab:Fab ratio remain consistent with the recommended conditions.

5. Dose-response assays (optional)
For dose-response experiments, serially dilute the pre-incubated PhenoVue Fluor 647 pH-sensitive Fab–antibody complex in cell culture medium to cover the desired concentration range.

Include a **Fab-only control** (PhenoVue Fluor 647 pH-sensitive Fab anti-human IgG without therapeutic antibody) at the final concentrations tested. This control enables calculation of the **specific internalization signal:**

- **Mean Signal (Therapeutic antibody) – Mean Signal (Fab-only control).**
- This correction is particularly important for accurate **EC_{50} determination.**

Image acquisition

- Transfer the microplates to the imaging system and begin acquisition (e.g. for time-lapse imaging).
- An **optional acquisition point** can be included between steps 2 and 3.
- Refer to the following section for recommended acquisition settings.

Image analysis

For image analysis using Revvity high-content imaging instruments, refer to the dedicated section below.

Tips

- **No-wash protocol:** Do not remove culture medium containing PhenoVue stains, as washing steps reduce signal intensity.
- **Live-cell only:** The PhenoVue human IgG antibody internalization kit (Fluor 647, pH-sensitive) is designed for **live-cell imaging only**. The detected compartments are not preserved after fixation.
- **Optimized conditions:** Pre-staining reagent concentrations are optimized for reliable signals over 24 h of live-cell imaging. For longer experiments, adjust concentrations or acquisition settings, particularly for the **PhenoVue 488 lysosomal stain.**
- **Cytotoxicity caution:** Concentrations are optimized for high signal while minimizing cytotoxicity. Exceeding recommended concentrations may impair cell viability.
- **Nuclear stain:** PhenoVue Hoechst 33342 is used at a low concentration (100 ng/mL) to limit effects on proliferation. While non-toxic, it may slightly slow growth depending on the cell type.
- **Controls:** Signal dynamics vary by cell type. Include an **IgG isotype control** in your experiment.
- **Acquisition mode:** Confocal acquisition is recommended for higher signal-to-background ratios compared to widefield mode.
- **Minimize phototoxicity and photobleaching:** The brightness of the dyes means that unnecessarily high exposure times or laser powers can be avoided. Limit timepoints to **4–5 per experiment** in time-lapse mode to reduce risk of phototoxicity or photobleaching. Alternatively, change acquisition fields to reduce repeated exposure of the same cells.

Recommendations for acquisition settings

HCS instruments		PhenoVue Hoechst 33342	PhenoVue 488	PhenoVue 555 (free channel)	PhenoVue 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

The PhenoVue human IgG antibody internalization kit (Fluor 647, pH-sensitive) enables simultaneous multiplexing of three fluorescent channels. To achieve optimal signal intensity and image quality on Revvity high-content screening systems, we recommend the following:

Channel configuration :

- Three channels are dedicated to the kit reagents.
- An additional free channel may be used for another compatible marker. For best spectral separation, we recommend selecting a fluorescent dye with properties similar to PhenoVue Fluor 555.

Imaging mode :

- Use confocal mode to obtain superior signal-to-background ratios and sharper localization of intracellular structures.

Z-stacks :

- Acquire 2–3 z-planes per field and apply a maximum intensity projection to capture internalization events while minimizing acquisition time.

Live-cell considerations :

- Thanks to the high brightness of PhenoVue dyes, high exposure times or laser powers can be avoided.
- Limit the number of timepoints in time-lapse experiments to a maximum of 4–5 per acquisition series. This minimizes the risk of phototoxicity or photodamage, particularly in short acquisition cycles. Under these conditions, reduced cell stress and optimal image quality have been observed.

Recommendations for image analysis using Revvity's Harmony™ or Signals Image Artist™

The following analysis workflow can be considered a basic protocol for quantifying antibody internalization intensity within acidic compartments.

Flexibility of the method: depending on the cell type used, the method and specific building block parameters may require adjustments to optimize segmentation and measurement accuracy.

1. Input Image

Acquire 2 or 3 z-planes and apply a "maximum projection" to better account for cellular variability and morphology over time.

Set Flatfield correction to "Basic" or "Advanced".

2. Find Nuclei

Use the PhenoVue Hoechst channel for nuclei segmentation.

3. Filter Image

Use the PhenoVue 488 lysosomal channel to create a new and smoothed PV488 lysosomal image.

4. Find Cytoplasm

Apply the "Find Cytoplasm" building block on smoothed PV488 lysosomal image (channel) and the nuclei selected (with removed border objects).

5. Select Population to remove border cells

Apply the "Select Population" building block on the nuclei population with common filters method after selection of "Cell" Region to keep only whole cells.

6. Find Spot

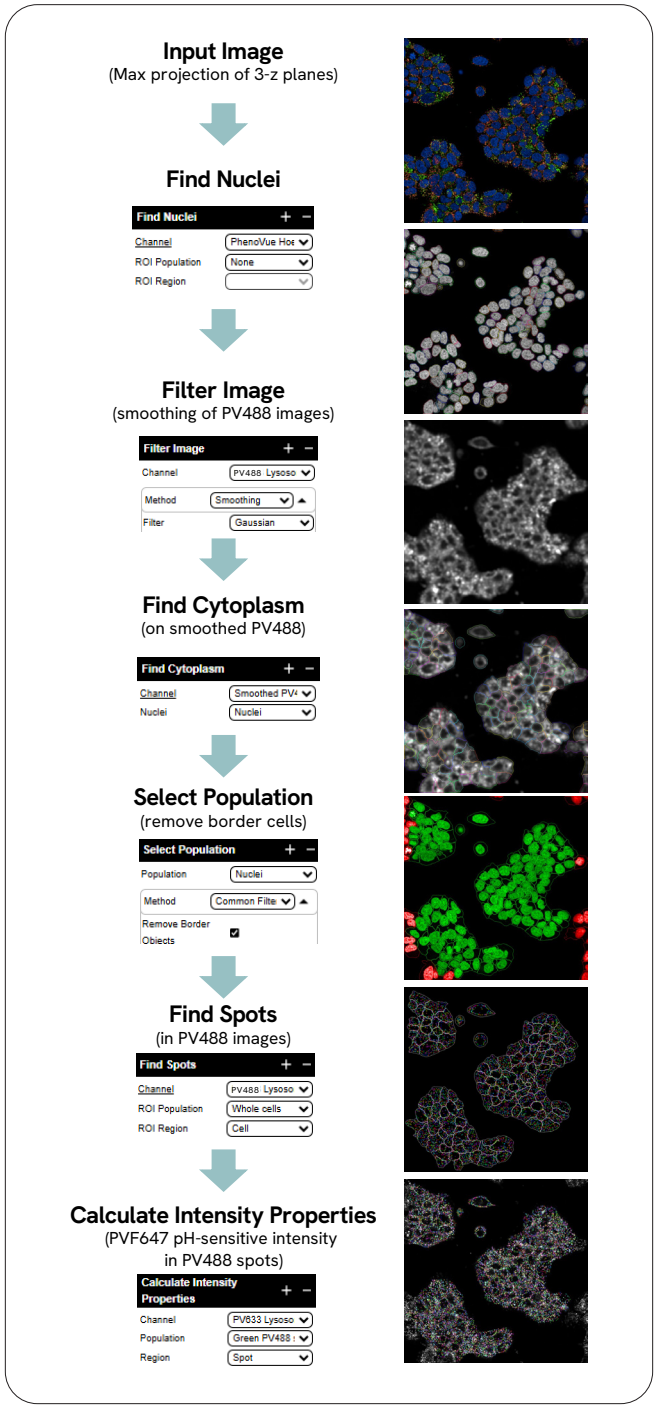
Apply the "Find Spots" building block on PV488 lysosomal channel and select the appropriate method to detect the PV488 spots corresponding to acid pH vesicles.

7. Calculate Intensity Properties

Apply the "Calculate Intensity Properties" building block to quantify the PVF647-pH sensitive intensity in the PV488 spots.

Configurable: additional building blocks can be included if further parameters need to be evaluated, such as number of spots, spot size, degree of overlap, or co-localization metrics.

This modular approach allows the analysis pipeline to be tailored to experimental needs while maintaining a reproducible basis for quantifying internalization.



Assay validation

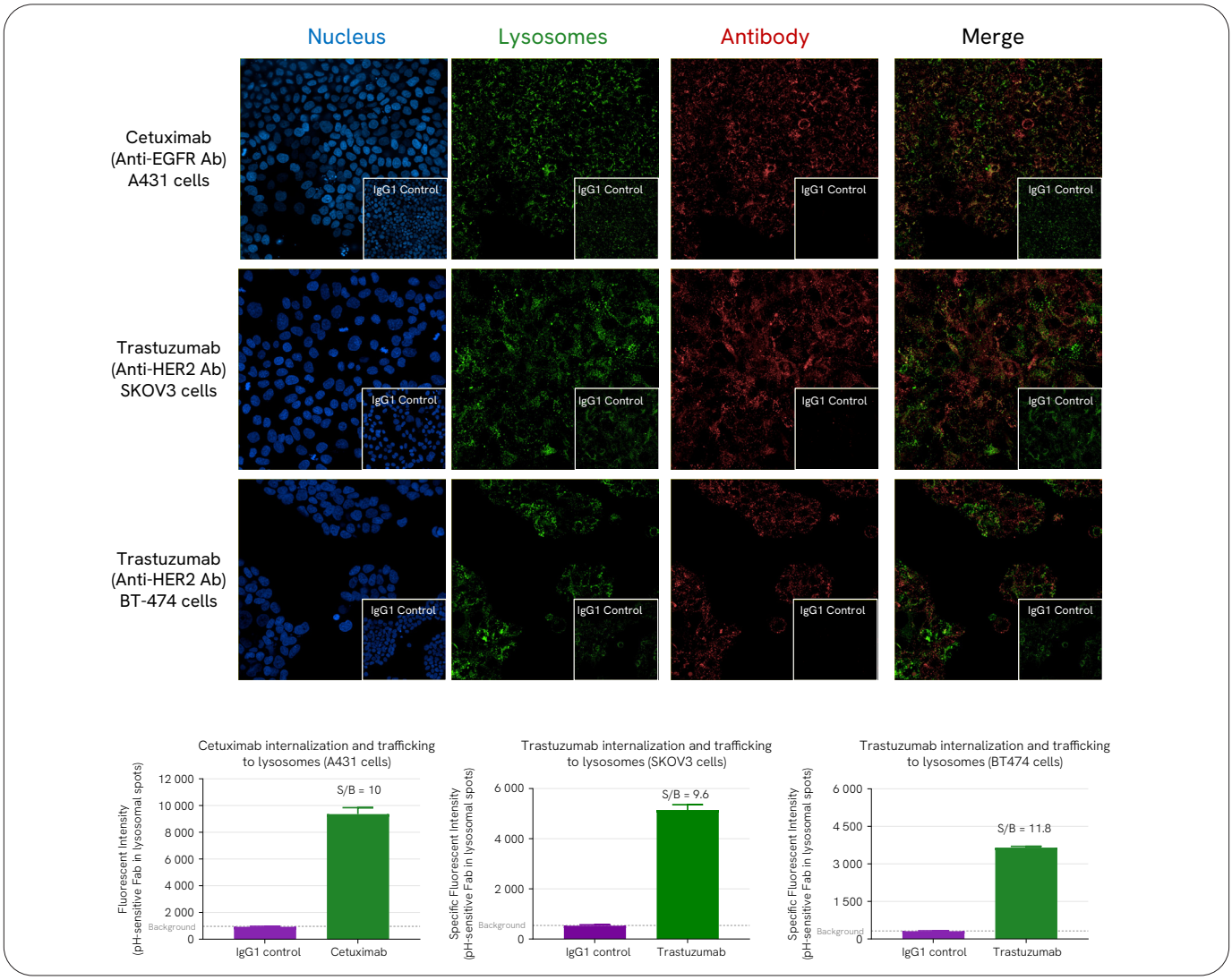


Figure 2: Antibody internalization monitored with the PhenoVue human IgG antibody internalization kit (Fluor 647, pH-sensitive) in different cell models.

Figure 2 shows A431, SKOV3, and BT-474 cells seeded in PhenoPlate 96-well microplates and incubated overnight at 37 °C, 5% CO_2. Cells were then treated in their respective complete culture medium with test human antibodies (anti-EGFR/Cetuximab or anti-HER2/Trastuzumab) or with the PhenoVue human IgG1 isotype control (PAHIGG1CT1), in combination with the PhenoVue human IgG antibody internalization kit (Fluor 647, pH-sensitive) stains. Test antibodies were applied at 4 nM with an antibody:Fab molar ratio of 1:3. In parallel, Fab-only controls (same Fab concentration without therapeutic antibody) were included to define the background signal.

Time-lapse imaging was performed over a 24-hour period using the Operetta CLS high-content imaging system for A431 cells and the Opera Phenix Plus high-content screening system for SKOV3 and BT-474 cells, both with a 40x water-immersion objective in confocal mode. Representative images after 24 h are shown.

Robust internalization of Cetuximab (anti-EGFR) in A431 cells and Trastuzumab (anti-HER2) in SKOV3 and BT-474 cells was observed, with a strong pH-dependent fluorescent signal in lysosomes. In contrast, the IgG1 isotype and Fab-only controls showed minimal background signal. These results demonstrate the kit's ability to specifically quantify antibody uptake and trafficking to acidic lysosomal compartment and discriminate true internalization events from non-specific background.

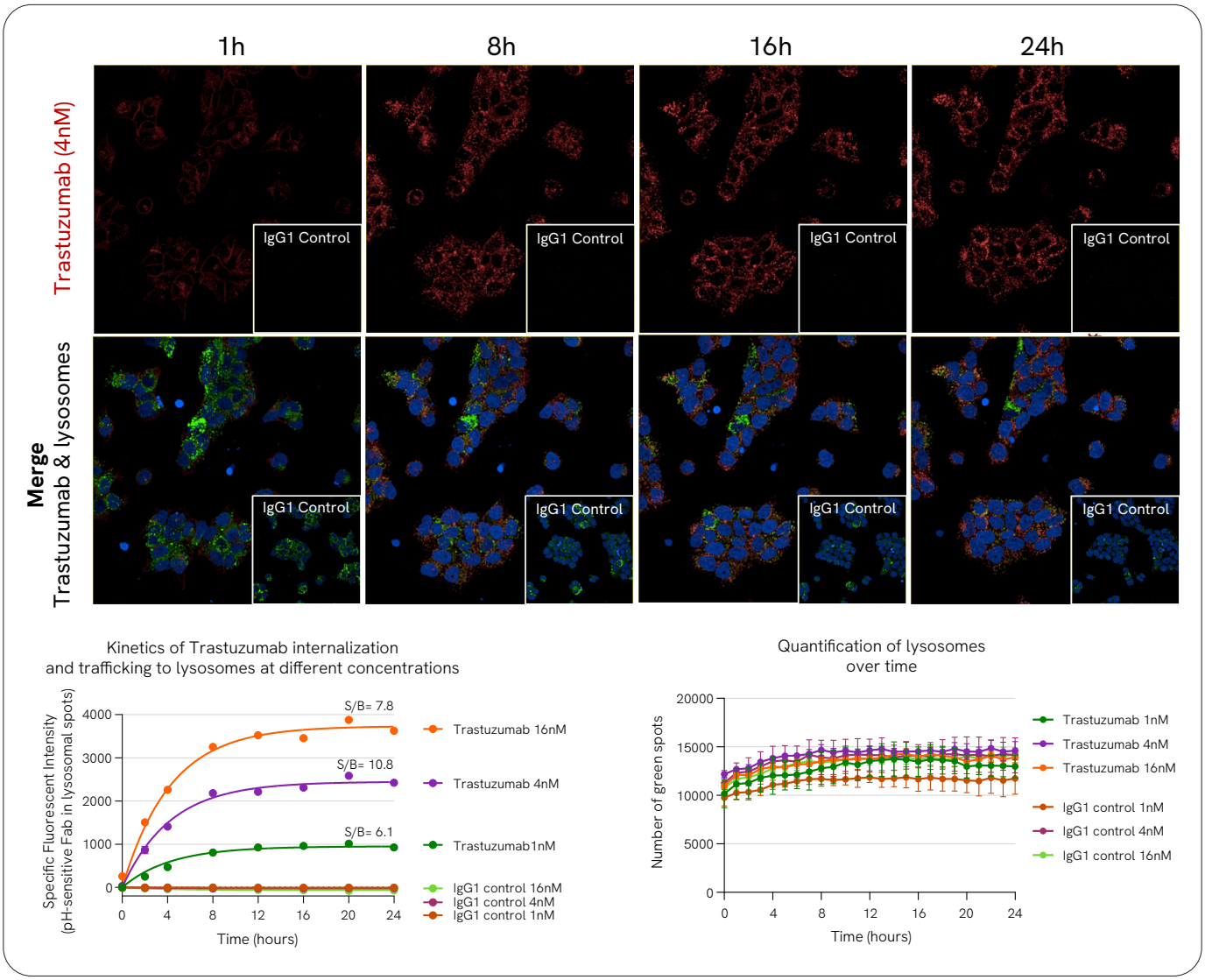


Figure 3: 24-hour kinetics of Trastuzumab internalization in BT-474 cells monitored with the PhenoVue human IgG antibody internalization kit (Fluor 647, pH-sensitive).

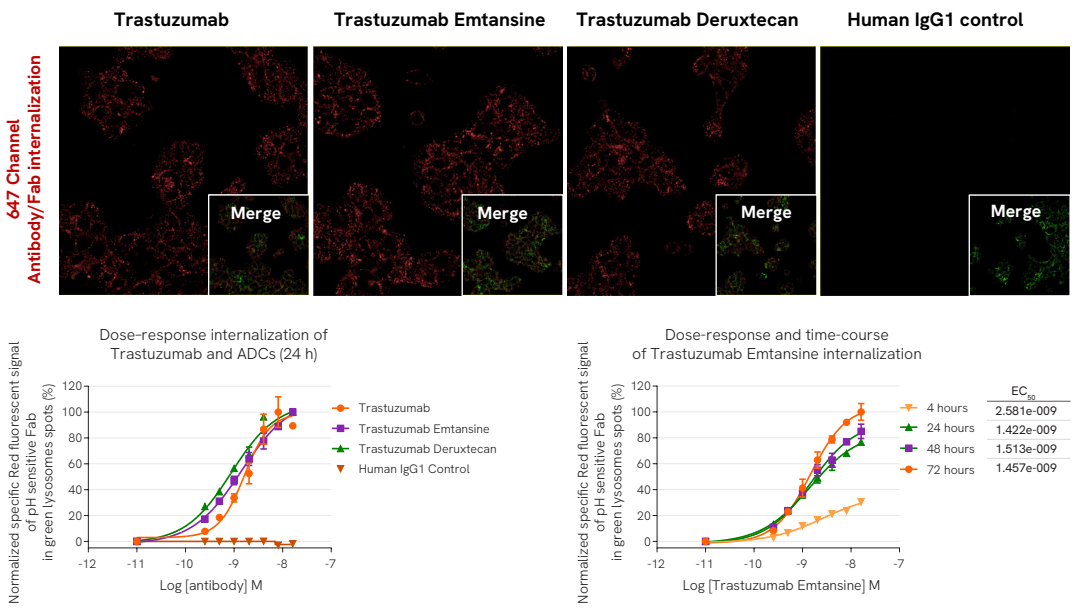
Figure 3 shows BT-474 cells seeded in PhenoPlate 96-well microplates at a density of 20,000 cells per well and incubated overnight at 37 °C, 5% CO_2. Cells were then stained with the PhenoVue human IgG antibody internalization kit (Fluor 647, pH-sensitive) following the recommended live-cell protocol. Therapeutic antibody (Trastuzumab, anti-HER2) and the PhenoVue human IgG1 isotype control were applied at 1, 4, and 16 nM, with an antibody:Fab molar ratio of 1:3. In parallel, Fab-only controls (same Fab concentrations without therapeutic antibody) were included to define background signal.

Time-lapse imaging was performed every hour for 24 h on the Opera Phenix Plus high-content screening system using a 40x water-immersion objective in confocal mode. To minimize phototoxicity and photobleaching during repeated acquisitions, low-exposure settings were applied.

Representative images are shown at 1 h, 8 h, 16 h, and 24 h.

Specific internalization was quantified as: Mean signal (Therapeutic antibody) – Mean signal (Fab-only control).

Our results demonstrate a dose-dependent internalization of Trastuzumab over time, while the number of lysosomes remained stable.



EC_{50} (nM)	Trastuzumab	Trastuzumab Emtansine	Trastuzumab Deruxtecan
4h	1.90 nM	2.58 nM	0.93 nM
24h	1.64 nM	1.43 nM	0.87 nM
48h	1.58 nM	1.51 nM	0.91 nM
72h	1.79 nM	1.46 nM	1.13 nM

Figure 4: Long-term dose-response kinetics of Trastuzumab and associated ADCs monitored with the PhenoVue human IgG antibody Internalization kit (Fluor 647, pH-sensitive) in a 384-well format.

Figure 4 shows BT-474 cells seeded in PhenoPlate 384-well microplates at a density of 7,000 cells per well and incubated overnight at 37 °C, 5% CO_2. Cells were then stained with the PhenoVue human IgG antibody internalization kit (Fluor 647, pH-sensitive) in complete culture medium, following the recommended protocol. Antibodies included Trastuzumab, Trastuzumab Emtansine, Trastuzumab Deruxtecan and the PhenoVue human IgG1 isotype control. Dose-response experiments were performed over a concentration range of 0.25–16 nM, with an antibody:Fab molar ratio of 1:3 maintained throughout. Parallel Fab-only controls (same Fab concentrations without IgG antibody) were included to assess background signal.

Time-lapse imaging was carried out at 4 h, 24 h, 48 h, and 72 h using the Opera Phenix Plus high-content screening system with a 40x water-immersion objective in confocal mode, without any washing steps. Representative images at 24 h are displayed.

Normalized specific internalization was calculated as: (Mean signal therapeutic antibody – Mean signal Fab-only control) / maximum signal across dose-response and kinetics for each antibody. For the isotype control, normalization was performed using the maximum signal obtained with Trastuzumab.

The results show that the PhenoVue human IgG antibody internalization kit (Fluor 647, pH-sensitive) enables accurate determination of EC_{50} values for both naked antibodies and ADCs in a miniaturized 384-well format, with reliable performance over 72 hours of culture without adverse effects on cell health (data not shown).

Copyright ©2025, Revvity, Inc. All rights reserved.
Revvity is a trademark of Revvity, Inc.
All other trademarks are the property of their respective owners.

For research use only. Not for use in diagnostic procedures.