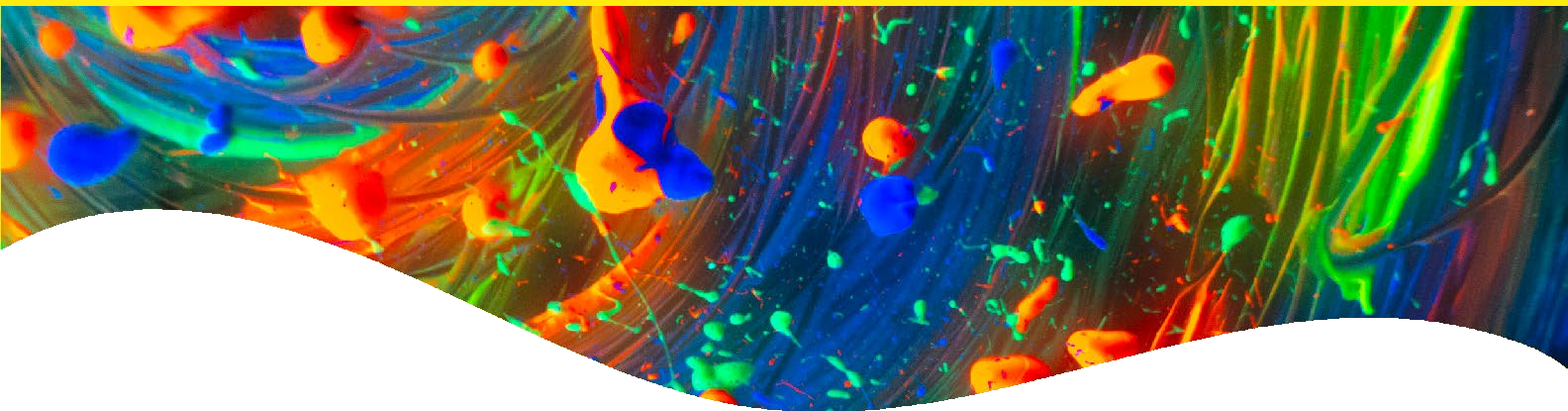




PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody (2 x 96-wells)



Overview

Antibody-mediated internalization is increasingly studied, particularly in immuno-oncology. When an antibody binds to its target receptor, the antibody-receptor complex is endocytosed and trafficked through acidic vesicles. Depending on the receptor pathway, the complex may recycle to the plasma membrane or progress toward lysosomal degradation. These mechanisms are central to the mode of action of antibody-drug conjugates (ADCs), where internalization and lysosomal trafficking support payload release. Accurate and efficient evaluation of antibody internalization is therefore critical for the development of ADCs and related biologics.

The PhenoVue™ Fluor 515 pH-sensitive Fab anti-human IgG antibody is a fluorescent probe developed for no-wash live-cell monitoring of antibody and ADC internalization. This Fab fragment binds with high affinity to the Fc region of human IgG antibodies, whether unconjugated or conjugated, including ADCs. Its fluorescence remains low at neutral extracellular pH but increases as the Fab-antibody complex traffics into acidic intracellular compartments. PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody combines high photostability with a lower pKa (~5.7), helping to reduce fluorescence at neutral and mildly acidic pH and preferentially report antibody trafficking into strongly acidic intracellular compartments. This profile makes it particularly well suited to repeated time-lapse acquisitions and kinetic profiling of deep internalization.

This product is supplied as an individual reagent and is also a key component of the PhenoVue Human IgG Antibody Internalization Kit (Fluor 515, pH-sensitive; part no. PHAINTG14). In both formats, the probe is compatible with standard cell culture conditions and supports robust and reliable live-cell internalization measurements.

For low-target-expression models, weak internalization signals, or imaging systems not optimized for Fluor 515 detection, PhenoVue Fluor 647 pH-sensitive Fab anti-human IgG antibody (part no. 2HFAB647PH1) provides a complementary far-red detection option with broad instrument compatibility. It is also available in the PhenoVue Human IgG Antibody Internalization Kit (Fluor 647, pH-sensitive; part no. PHAINTDR14).

Product information

Product name	PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody - 2 x 96*
Part number	2HFAB515PH1
Packaging	1 vial of 90µg
Concentration	18 µM (0.9 mg/mL) after reconstitution with 100µL H ₂ O
Molecular Weight	50 kDa
Vial format	Lyophilized
Antibody format	Fab fragment
Host	Goat
Specificity	Human IgG antibody, Fc region
Human isotype cross-reactivity	Recognizes human IgG1, IgG2, IgG3 and IgG4 antibody isotypes
Purification	Affinity chromatography
Formulation	200 mM PO4 pH7 + 1% BSA
Applications	High content analysis (immunofluorescence, imaging, microscopy)
Shipping conditions	RT
Storage conditions	2-8°C. Protect from light

* PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody provides sufficient reagents to stain 2 x 96-well plates or 1 x 384-well plate, following the suggested concentrations and volumes.

Storage and stability

- Avoid repeated freeze-thaw cycles. After reconstitution with 100 µL dH₂O, aliquot the reagent and store at -16 °C or below.
- Allow the reagent to warm to room temperature for 30 minutes before opening the vial and reconstitution. After reconstitution, aliquoted reagent stored at -16 °C or below is stable for up to 6 months. Protect from light.

- The stability of these products is supported until the expiration date provided in the certificate of analysis, when stored as provided 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.

Spectral and pH-sensitive properties

Product name	Maximum excitation wavelength* (nm)	Maximum emission wavelength* (nm)	Common filter set	Quantum yield (Φ)	Epsilon* (E in M ⁻¹ .cm ⁻¹ at λ max)	Brightness (Φ x ε)	pKa
PhenoVue Fluor 515 pH-sensitive	515	532	FITC	Nd**	75000	Nd**	≈5.7

* Properties measured corresponding to the fluorescent form of the dye and mainly presents in acidic pH

** Nd = not determined

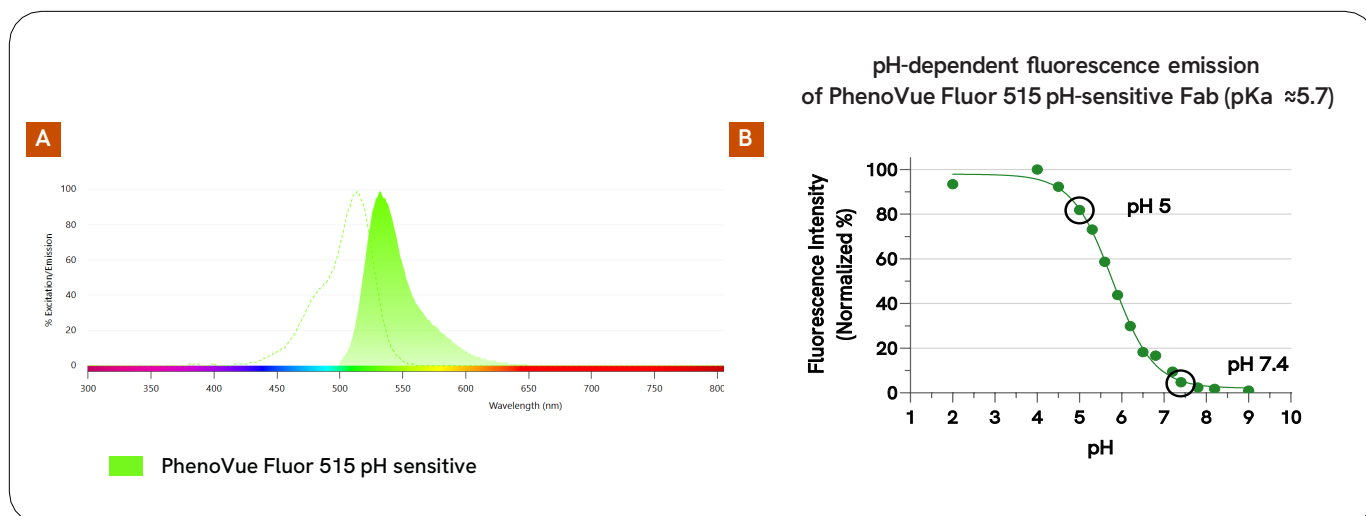


Figure 1: Spectral and pH-sensitive properties of the PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody (A) Excitation and emission spectra. (B) pKa curve of the PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody, showing a pKa around 5.7, with strong fluorescence at acidic pH (e.g. pH 5) and reduced signal at neutral pH (e.g. pH 7.4).

Reagent reconstitution and preparation of staining solution

1. Preparation of reagent stock solution

Reagents	
PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody	Reconstitution using 100 μ L dH ₂ O gives a stock concentration of 18 μ M

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

Preparation of staining solution

Reagents	Antibody/Fab Staining Solution (1:3 Ratio, 100 μ L/well)
Diluent	Cell culture medium
Human IgG antibody or Human IgG antibody isotype controls (available as spare parts PAHIGG1CT1 or PAHIGG4CT1)	Dilute to obtain a final concentration of 60* nM (2x).
PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody	Dilute to obtain a final concentration of 180 nM (2x).

*The suggested 60 nM antibody concentration is provided as a guideline and may be adjusted according to experimental needs. At this concentration, the PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody 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 antibody/Fab staining solution

Antibody/Fab staining solution (1:3 ratio)

Your human IgG antibody (or human IgG antibody isotype controls)

PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody



10 mL cell culture medium

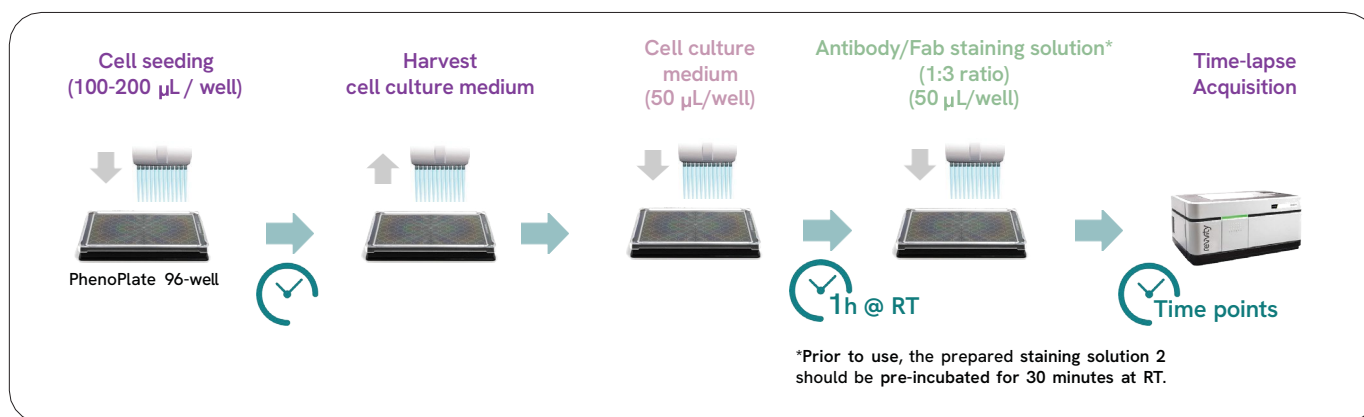
Start with 9.9 mL – Vx of cell culture medium.

- Add a volume Vx of your human IgG antibody to obtain a final concentration of 60 nM.

- Add 100µL of PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody to reach a final concentration of 180 nM.

Incubate for 30 minutes at RT.

Experimental workflow



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% CO₂) 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 cell culture medium per well.

Incubate for 1 hour at 37 °C, 5% CO₂.

3. Preparation of antibody/Fab staining solution

Prepare the staining solution as described above and allow it to equilibrate for 30 minutes at room temperature (RT). Maintain an antibody:Fab molar ratio of 1:3.

Optional: for image segmentation, PhenoVue Hoechst 33342 nuclear stain may be added at 100 ng/mL. This low concentration helps reduce potential effects on cell proliferation while enabling robust nuclear segmentation. If the nuclear signal is too weak, first optimize exposure time; if needed, the Hoechst concentration can also be adjusted for the cell model.

3. Add 50 µL of antibody/Fab staining solution per well. Incubate at 37 °C, 5% CO₂.

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 suggested conditions.

5. Dose-response assays (optional)

For dose-response experiments, serially dilute the pre-incubated PhenoVue Fluor 515 pH-sensitive Fab-antibody complex in cell culture medium to cover the desired concentration range.

Include a Fab-only control (PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody without test antibody) at the final concentrations tested.

Calculation of the specific internalization signal:

Mean Signal (Test Antibody) – Mean Signal (Fab-only control).

This correction is particularly important for accurate EC₅₀ determination.

Image acquisition

- Transfer the microplates to the imaging system and begin acquisition (e.g. for time-lapse imaging).
- For image acquisition settings with Revvity high-content imaging instruments, please refer to the PhenoVue human IgG antibody internalization kit (Fluor 515, pH-sensitive) product information sheet (part no. PHAINTG14)

Image analysis

For image analysis using Revvity high-content imaging instruments, refer to the dedicated image-analysis workflow in the PhenoVue Human IgG Antibody Internalization Kit (Fluor 515, pH-sensitive) product information sheet (part no. PHAINTG14).

Tips

• Ab:Fab ratio optimization:

A 1:3 antibody:Fab molar ratio is a good starting condition. Because PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody has a relatively low pK_a (~5.7), resulting in lower background, performance may be further improved by increasing the antibody:Fab ratio and testing a broader range of antibody concentrations. For challenging cell models, such as those with low receptor expression or high background, both parameters should be optimized.

• No-wash protocol:

Do not remove the medium containing the antibody/Fab complex, as washing reduces signal intensity.

• Live-cell imaging:

PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody is intended primarily for live-cell internalization measurements. For workflows involving fixation or additional compartment markers, refer to the compatibility guidance of the complete PhenoVue Human IgG Antibody Internalization Kit (Fluor 515, pH-sensitive).

• Controls:

Always include a Fab-only control and an appropriate human IgG antibody isotype control to enable reliable interpretation and background correction.

• Acquisition mode:

Confocal acquisition is suggested for higher signal-to-background ratios compared with widefield mode.

• Multiplexing and crosstalk:

PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody is compatible with standard FITC-channel configurations. Potential crosstalk into the 555 channel should be considered during multiplex design, particularly on imaging systems where spectral separation is less favorable. This crosstalk is negligible on Opera Phenix™ imagers but may be more significant on Operetta™ CLS imagers.

• Phototoxicity/photobleaching:

Thanks to the high brightness of PhenoVue dyes, avoid unnecessarily high exposure times or laser powers during time-lapse experiments. Optimize acquisition frequency and, when appropriate, alternate imaging fields to reduce repeated exposure of the same cells.

Assay validation

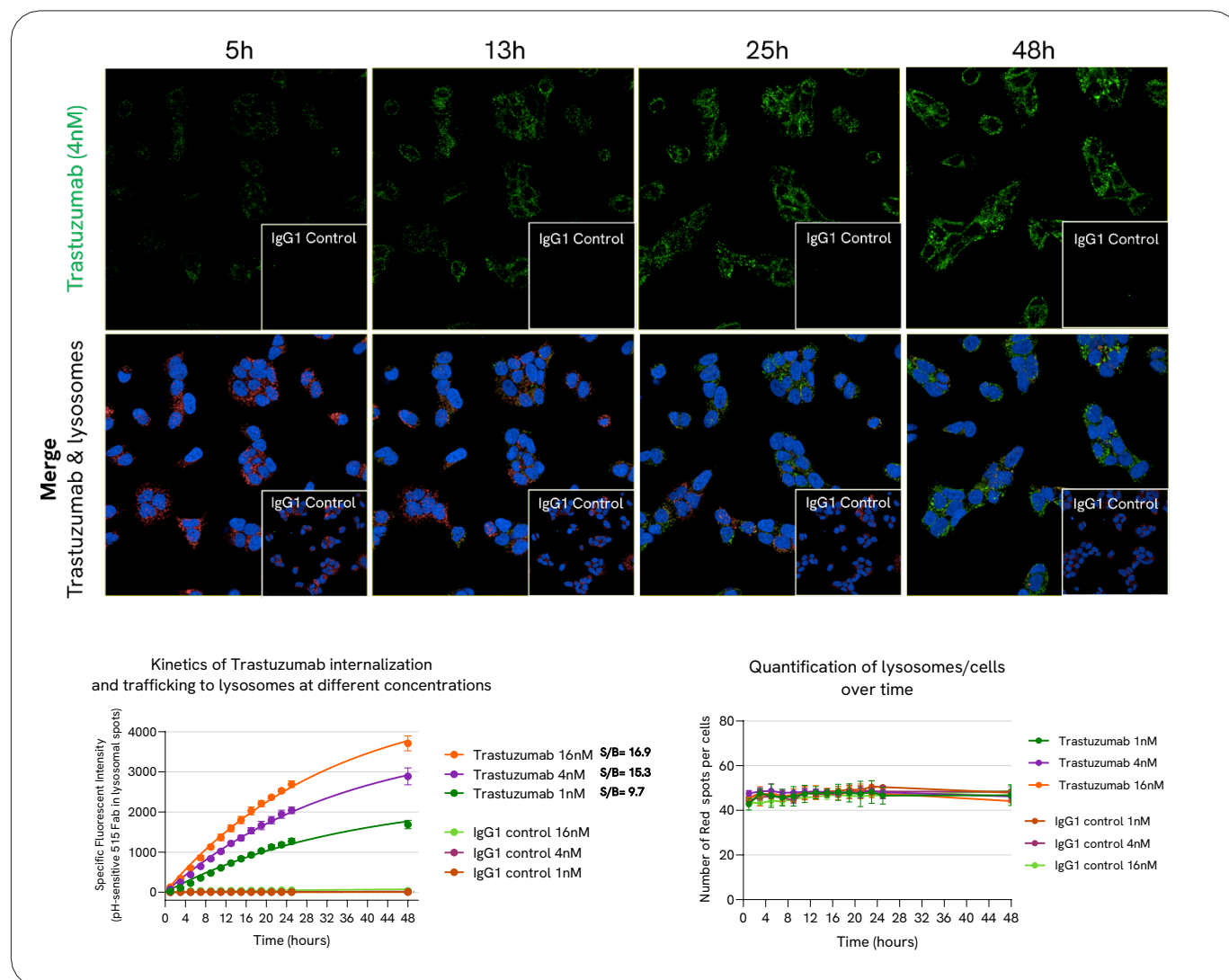


Figure 2. Long-term kinetics of Trastuzumab internalization and trafficking to lysosomes in BT-474 cells monitored with the PhenoVue Human IgG Antibody Internalization Kit (Fluor 515, pH-sensitive) in a 384-well format.

BT-474 cells were seeded in PhenoPlate 384-well microplates and incubated overnight at 37 °C, 5% CO₂. Cells were stained following the suggested live-cell protocol and treated with Trastuzumab (anti-HER2) or the PhenoVue Human IgG1 antibody isotype control at 1, 4, and 16 nM, maintaining an antibody:Fab molar ratio of 1:3. Fab-only controls at the corresponding concentrations were included to define background signal.

Time-lapse imaging was performed for up to 48 h. Representative images obtained with 4 nM Trastuzumab are shown at 5, 13, 25, and 48 h. Specific internalization was quantified as the PhenoVue Fluor 515 pH-sensitive Fab intensity within PhenoVue 633-positive lysosomal spots, after subtraction of the corresponding Fab-only background.

Trastuzumab showed a clear concentration- and time-dependent increase in internalization, reaching S/B values of approximately 9.7, 15.3, and 16.9 at 48 h for 1, 4, and 16 nM, respectively. In parallel, the number of lysosomal spots per cell remained stable over time, supporting that the increase in Fluor 515 signal reflects progressive antibody internalization and trafficking rather than changes in lysosome abundance.

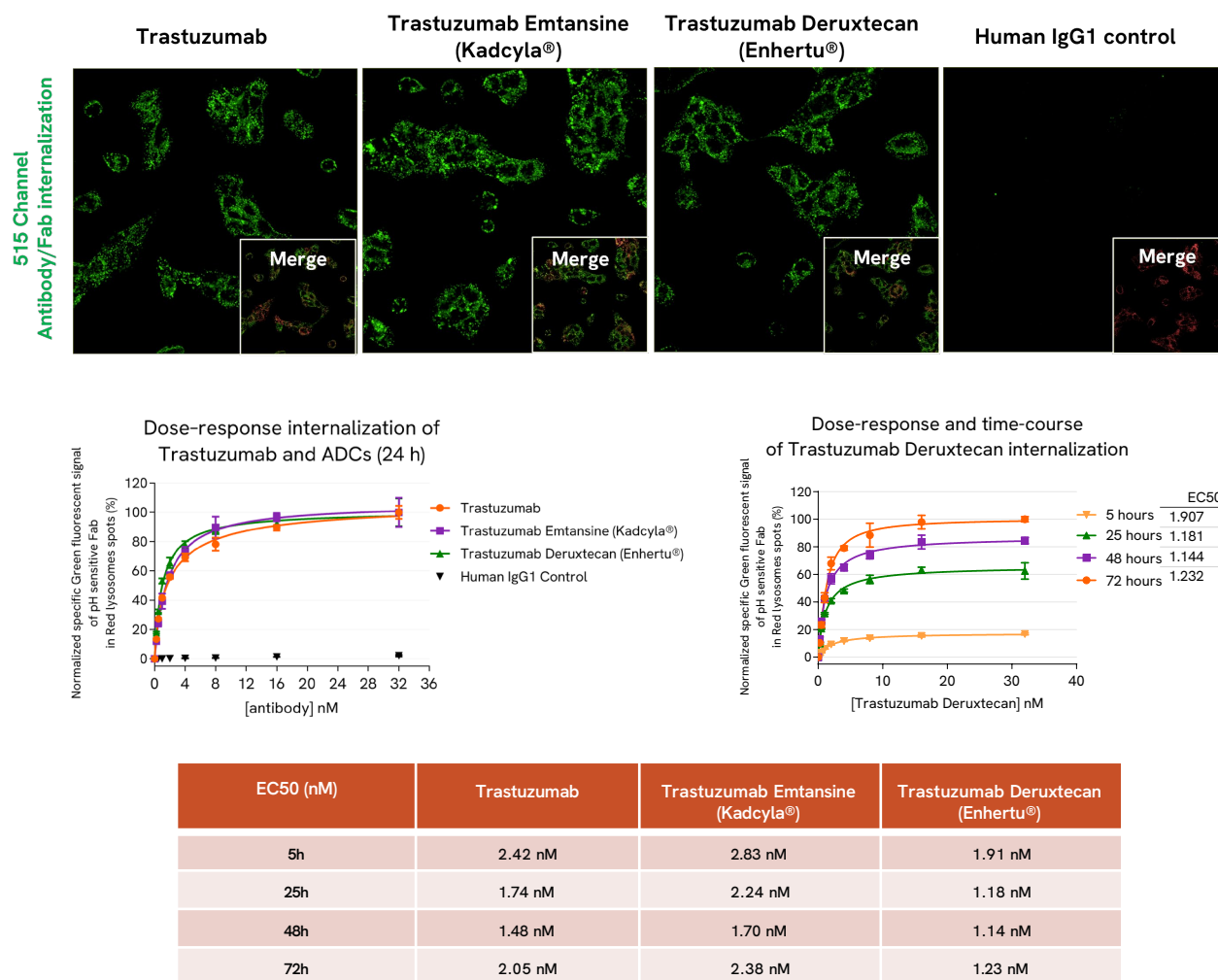


Figure 3. Long-term dose-response internalization of Trastuzumab and associated ADCs monitored with the PhenoVue Human IgG Antibody Internalization Kit (Fluor 515, pH-sensitive) in a 384-well format.

BT-474 cells were seeded in PhenoPlate 384-well microplates and incubated overnight at 37 °C, 5% CO₂. Trastuzumab, Trastuzumab Emtansine (Kadcyla®), Trastuzumab Deruxtecan (Enhertu®), and the PhenoVue Human IgG1 antibody isotype control were evaluated over a concentration range of 0.25–16 nM, with an antibody:Fab molar ratio of 1:3. Parallel Fab-only controls were included to determine background signal.

Internalization was monitored for up to 72 h without washing. Representative images show strong Fluor 515 pH-sensitive signals for Trastuzumab and both ADCs, with minimal signal from the IgG1 antibody isotype control. Dose-response analysis generated robust concentration-dependent internalization curves and reproducible EC₅₀ values over time.

EC₅₀ values ranged from 1.48–2.42 nM for Trastuzumab, 1.70–2.83 nM for Trastuzumab Emtansine, and 1.14–1.91 nM for Trastuzumab Deruxtecan between 5 and 72 h. The stability of these values demonstrates that the Fluor 515 pH-sensitive format supports reliable quantitative comparison of naked antibodies and ADCs during extended live-cell experiments.

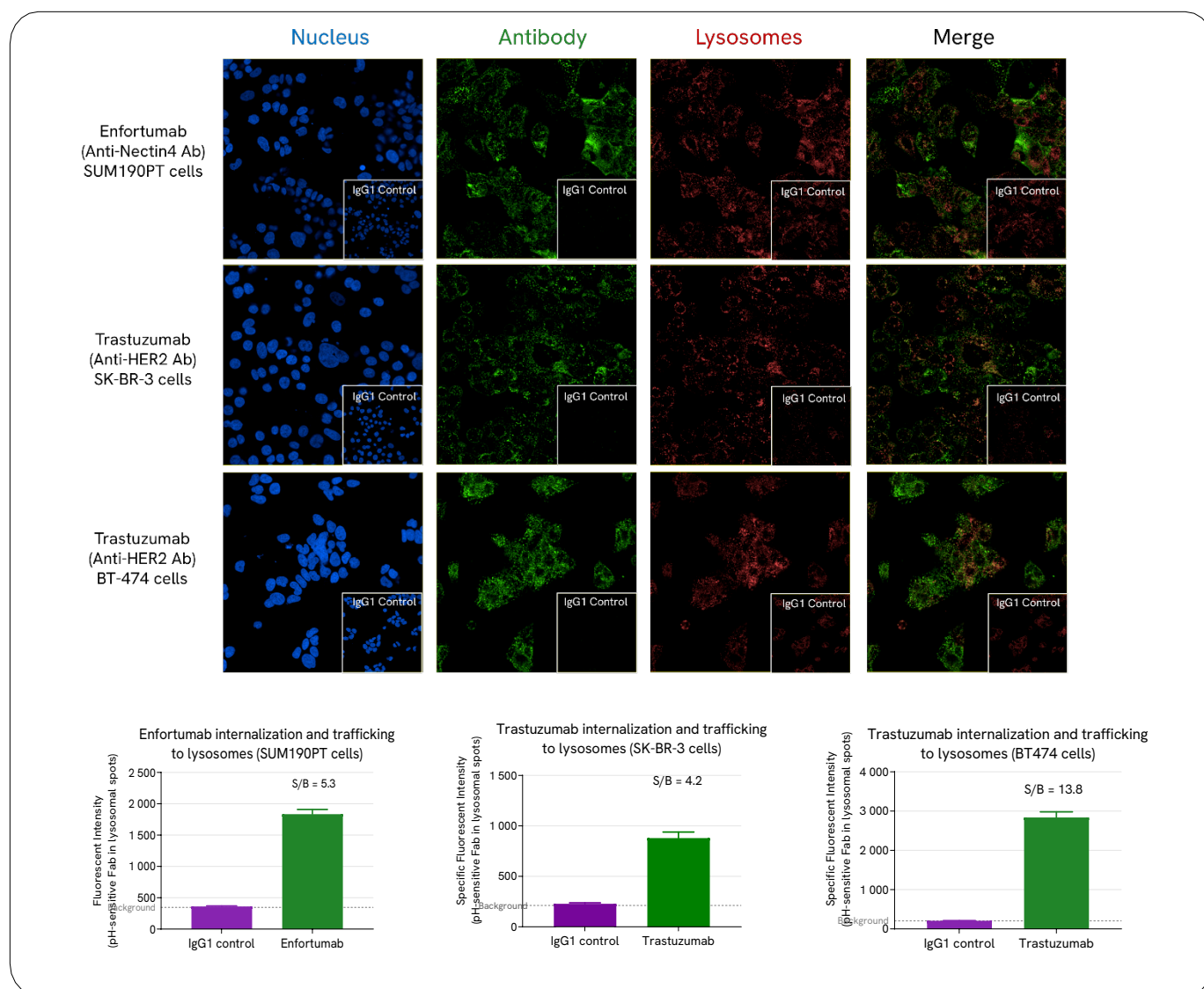
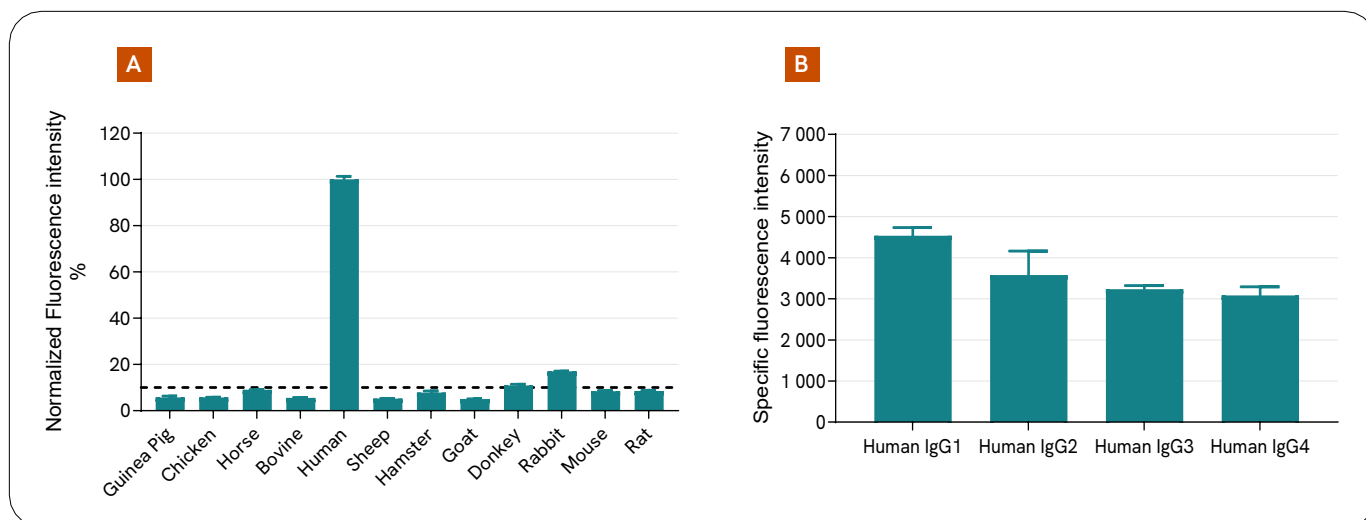


Figure 4. Antibody internalization monitored with the PhenoVue Human IgG Antibody Internalization Kit (Fluor 515, pH-sensitive) across different antibody targets and cell models.

The performance of the Fluor 515 pH-sensitive internalization assay was evaluated across three receptor-positive cell models: Enfortumab (anti-Nectin-4) in SUM190PT cells and Trastuzumab (anti-HER2) in SK-BR-3 and BT-474 cells. The PhenoVue Human IgG1 antibody isotype control was included in parallel to assess non-specific background.

In all three models, antibody treatment produced a strong PhenoVue Fluor 515 pH-sensitive signal associated with PhenoVue 633-positive lysosomal compartments, whereas IgG1 antibody control samples showed low background.

Quantification demonstrated clear discrimination between specific antibody internalization and the isotype control, with S/B values of 5.3 for Enfortumab in SUM190PT cells, 4.2 for Trastuzumab in SK-BR-3 cells, and 13.8 for Trastuzumab in BT-474 cells. These results demonstrate robust detection and quantification of antibody internalization across different therapeutic targets, antibodies, and cellular backgrounds, supporting the broad applicability of the Fluor 515 pH-sensitive assay.



| Figure 5. F-LISA experiments.

Different IgG antibody species and (B) human IgG antibody isotypes coated onto a 96-well microplate and incubated with PhenoVue Fluor 647 pH-sensitive Fab anti-human IgG antibody (5 µg/mL). Fluorescence intensity was measured at pH 4 buffer using an EnVision™ multimode plate reader, leveraging the pH-sensitive properties of the probe. The results demonstrate that the PhenoVue Fluor pH-sensitive Fab anti-human IgG antibody is highly specific for human IgG antibody, with only weak cross-reactivity observed with rabbit IgG antibody. The Fab recognizes all four human IgG antibody isotypes (IgG1, IgG2, IgG3, and IgG4).

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