APPLICATION NOTE

Reliable antibody internalization assays using AssayMate system and pHSense Eu probes.

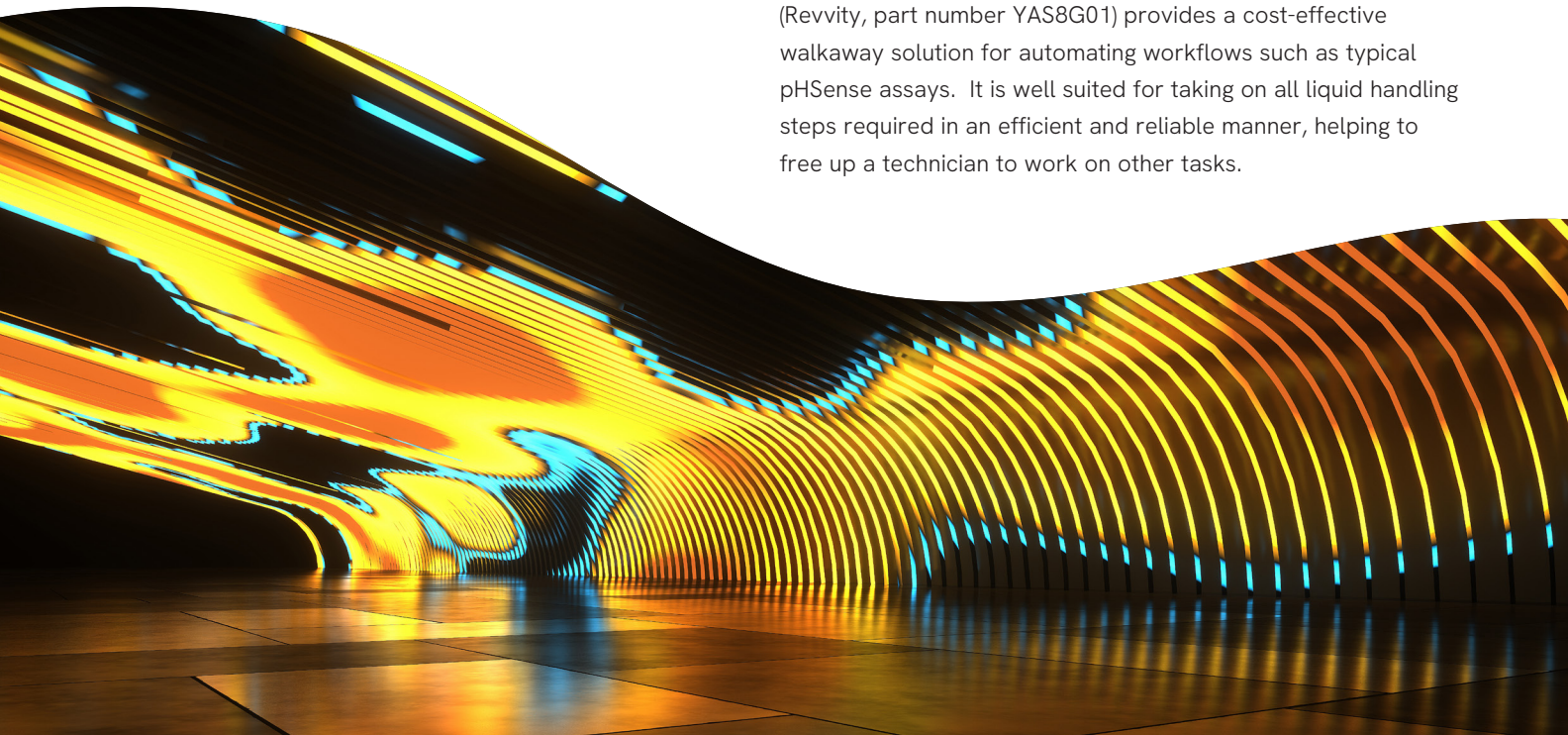
Overview

Cellular uptake studies of antibodies and antibody-drug conjugates (ADCs) have emerged as a critical research focus in recent years. As this therapeutic class continues to advance, there is growing demand for sophisticated analytical methods to assess how effectively these molecules enter cells and exert their therapeutic effects.

The pH-sensitive Europium-labeled reagents, pHSense™ Eu probes offer a distinctive dual-response mechanism: fluorescence intensity and lifetime increase as acidic conditions increase in the environment of the dye. This dual sensitivity, combined with the inherent advantages of Europium's long fluorescence lifetime, enables time-resolved fluorescence detection that helps eliminate short-lived background fluorescence, resulting in dramatically improved signal clarity and detection sensitivity compared to traditional pH-sensitive fluorophores. They allow for an approach which eliminates wash steps while providing flexibility for both real-time kinetic measurements and single time-point analyses but can be time-consuming for a manual user to process at scale.

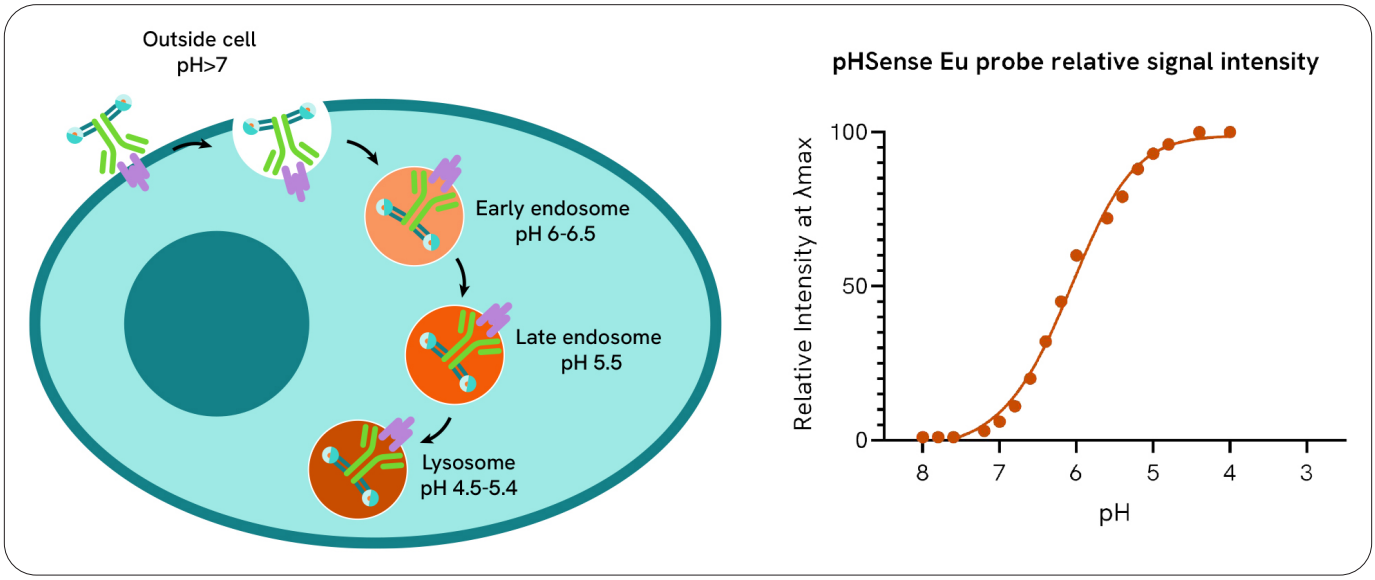
Revvity pHSense Eu probes are important tools in drug discovery and cell biology used to track how cells internalize specific proteins, antibodies, or receptors. They primarily streamline the development of Antibody-Drug Conjugates (ADCs) and G Protein-Coupled Receptor (GPCR) therapeutics.

The AssayMate™ automated liquid handling workstation (Revvity, part number YAS8G01) provides a cost-effective walkaway solution for automating workflows such as typical pHSense assays. It is well suited for taking on all liquid handling steps required in an efficient and reliable manner, helping to free up a technician to work on other tasks.

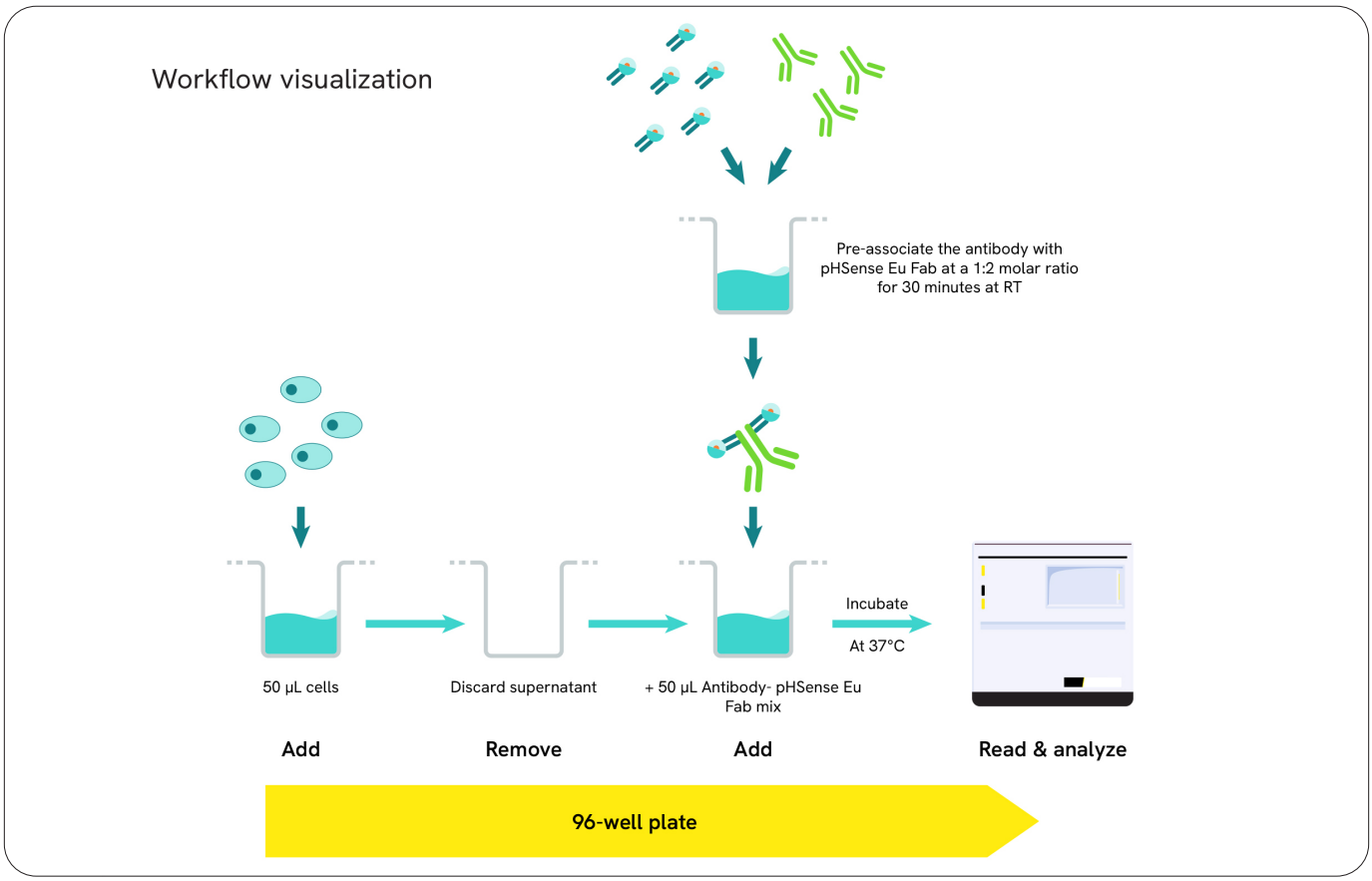


In this study, we compared assay plates processed using the AssayMate system with plates processed manually. The results demonstrate that the AssayMate system can reliably automate pHSense assays, providing reproducible results while reducing hands-on time without compromising assay performance or reliability.

This direct comparison between manual and automated execution shows how automating on the AssayMate system standardizes the pipetting tasks, preserves cell monolayer integrity, and delivers the high-data-fidelity kinetic profiling necessary to help accelerate therapeutic screening campaigns.



Intracellular antibody trafficking and pH-responsive signal activation of pHSense Eu probes



For a detailed experimental protocol, please refer to the user manual.

Materials and methods

Cells and reagents

HER2 positive human cancer cell line BT-474 cells were utilized in these tests. To look at internalization with pHSense reagents, the anti-HER2 internalizing antibody Trastuzumab, conjugated to pHSense Eu Fab anti-Human IgG probes (Revvity, part number 81HUEU1AA) was used.

The pHSense Human IgG1 Isotype Control (Revvity, part number 81HUIGG1NC) was utilized as a negative control, and the nonspecific internalization signal monitored via only the pHSense Eu Fab anti-Human IgG probes.

Internalization assay

24 hours prior to running the experiment, two 96-well white plates were seeded with 20,000 cells/well of BT-474 cells in 50ul of culture media and incubated overnight at 37°C in a humidified CO2 incubator.

1. **Antibody dilution** Trastuzumab and pHSense Human IgG1 Isotype Control were diluted in complete cell culture medium to 30 nM (=2X the desired final starting concentration for serial dilution series). 500 µL of each antibody was prepared.

2. **pHSense Eu Fab anti-Human IgG dilution** The Fab was diluted in cell culture medium to 60 nM (=2X the desired final starting concentration for the serial dilution series). 1400 µL of Fab was prepared.

3. **Complex formation** Equal volumes (1:1) of the 2X test antibody solution and the 2X pHSense Eu Fab reagent solution were mixed.

   - **Mix 1:** 400 µL Trastuzumab at 30 nM + 400 µL pHSense Eu Fab reagent at 60 nM
   - **Mix 2:** 400 µL Human IgG1 Isotype Control at 30 nM + 400 µL pHSense Eu Fab reagent at 60 nM
   - **Mix 3:** 400 µL Cell culture medium + 400 µL pHSense Eu Fab reagent at 60 nM

The mixtures were incubated at room temperature (21 ± 2°C) for 30 minutes to allow complex formation, then split into two equal volume aliquots for each mix, one for use on the AssayMate workstation, the other for manual preparation.

4. **Serial dilution of the 3 mixes** A two-fold, 8-point serial dilution of the 3 mixes was prepared in cell culture medium, both on the AssayMate liquid handler and manually, to achieve the desired concentrations:

Trastuzumab + pHSense Fab anti-Human IgG

	[Trastuzum ab] (nM)	[Fab] (nM)
C1	15	30
C2	7.5	15
C3	3.75	7.5
C4	1.88	3.75
C5	0.94	1.88
C6	0.47	0.94
C7	0.23	0.47
C8	0	0

Human IgG1 negative control + pHSense Fab anti-Human IgG

	[Human IgG1](nM)	[Fab] (nM)
C1	15	30
C2	7.5	15
C3	3.75	7.5
C4	1.88	3.75
C5	0.94	1.88
C6	0.47	0.94
C7	0.23	0.47
C8	0	0

pHSense Fab anti-Human IgG only

	[Fab] (nM)
C1	30
C2	15
C3	7.5
C4	3.75
C5	1.88
C6	0.94
C7	0.47
C8	0

		Trastuzumab + pHSense Fab anti-Human IgG			Human IgG1 negative control + pHSense Fab anti-Human IgG			pHSense Fab anti-Human IgG only					
		1	2	3	4	5	6	7	8	9	10	11	12
C1	A												
C2	B												
C3	C												
C4	D												
C5	E												
C6	F												
C7	G												
C8	H												

5. **Internalization assay** The culture plate was removed from the incubator. The cell supernatant was aspirated on the AssayMate workstation with care taken to not disturb the cell layer. Immediately, 50 µL of mix per well was added according to the plate layout above.

This same process was subsequently repeated by hand on the manual treatment plate.

Both cell plates were then immediately returned to a humidified 37°C CO_2 incubator for 24 hours.

Following this incubation period, fluorescence emission at 620 nM was read on an EnVision Nexus™ multimode plate reader (Revvity, part number HH36000002) using the reader settings specific to pHSense probes (see technical notes).

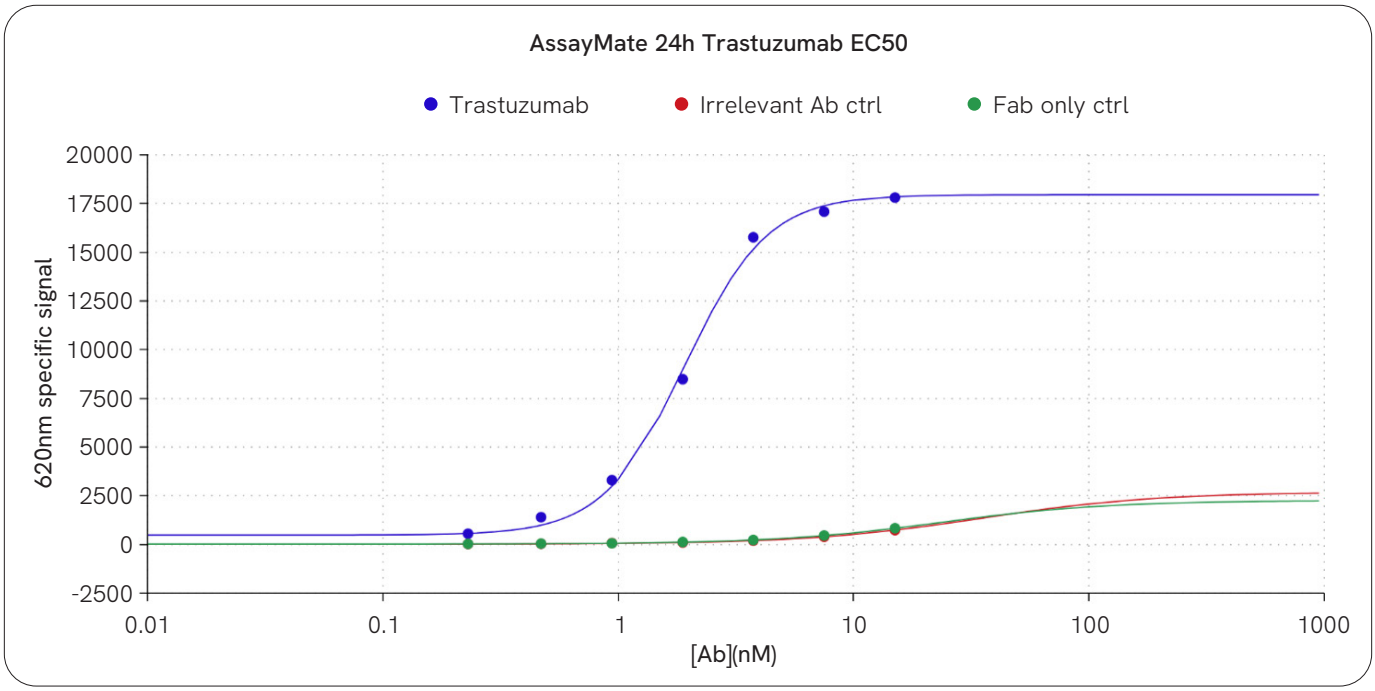
Results

The mean, standard deviation (SD), and coefficient of variation (CV) of all triplicates for the various serial dilution series were determined. The specific signal of internalization was then calculated and EC50 values determined for Trastuzumab (which previously reported data, not shown, found to have been ~2 nM).

BT-474 cell plate processed on AssayMate workstation

Mean & %CV	Trastuzumab + Fab			Irrelevant Ab + Fab			Fab only		
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
A	18641.0	1587.0	8.5	734.0	109.2	14.9	833.3	82.6	9.9
B	17548.5	595.5	3.4	395.7	43.8	11.1	461.3	69.3	15.0
C	15998.0	472.8	3.0	192.7	37.8	19.6	229.0	24.5	10.7
D	8608.7	1907.1	22.2	97.0	20.4	21.0	125.0	20.1	16.1
E	3359.3	405.4	12.1	71.0	5.9	8.3	59.3	5.3	9.0
F	1439.7	319.6	22.2	34.0	1.4	4.2	41.0	4.9	11.9
G	580.3	135.1	23.3	23.3	2.9	12.3	29.3	4.6	15.8
H	20.7	3.4	16.4	16.0	3.6	22.2	23.0	5.4	23.3

Specific signal:	Trastuzumab + Fab	Irrelevant Ab + Fab
A	17808	-99
B	17087	-66
C	15769	-36
D	8484	-28
E	3300	12
F	1399	-7
G	551	-6
H	-2	-7



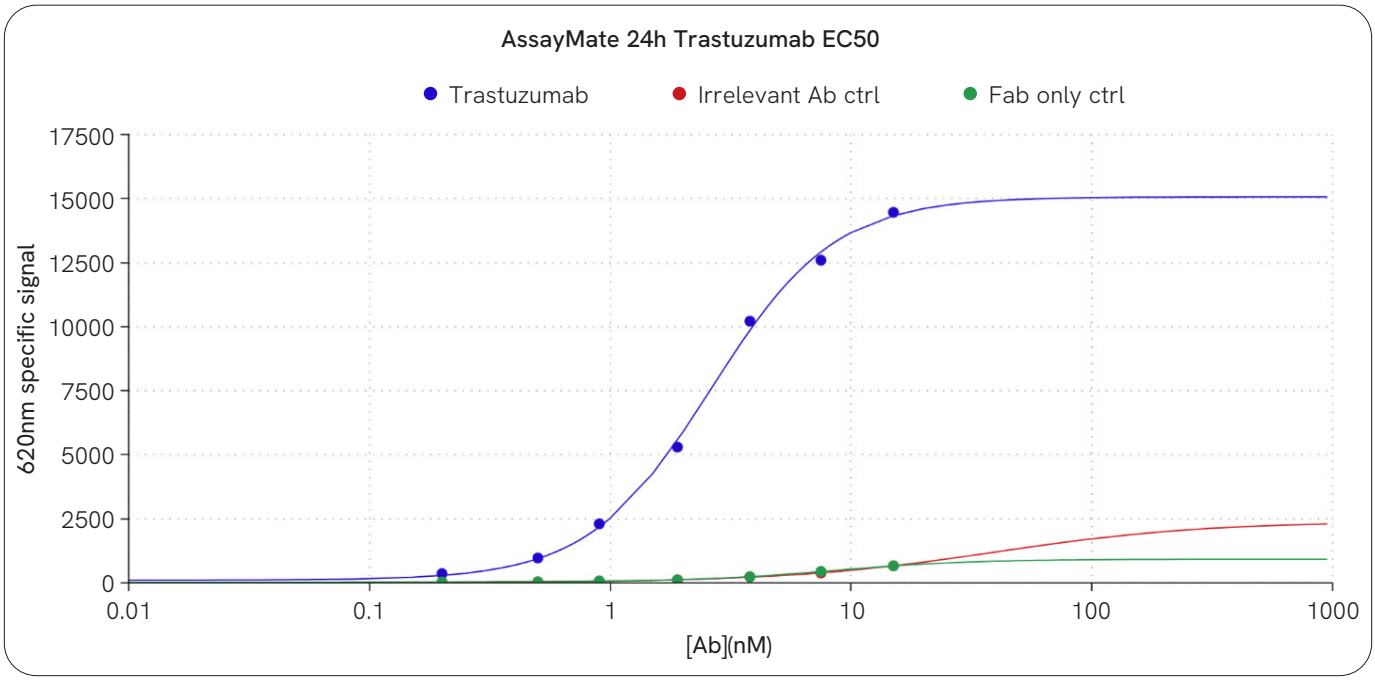
EC50 (nM) from AssayMate: 1.924

BT-474 cell plate processed manually

Trastuzumab + Fab			Irrelevant Ab + Fab			Fab only		
Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
15125.0	1561.8	10.3	666.3	43.3	6.5	660.3	15.1	2.3
13040.3	2507.4	19.2	383.7	20.7	5.4	445.3	34.5	7.7
10456.3	1534.1	14.7	233.3	23.1	9.9	238.3	15.5	6.5
5414.7	645.3	11.9	120.0	7.1	5.9	115.7	5.6	4.8
2376.3	209.7	8.8	65.0	5.7	8.8	73.0	5.0	6.8
1008.3	104.8	10.4	40.3	3.7	9.1	37.7	8.3	22.1
392.7	55.6	14.2	28.3	3.7	13.0	32.7	2.6	8.0
15.0	4.5	30.3	20.3	1.2	6.1	25.7	6.1	23.9

Specific signal:	Trastuzumab + Fab	Irrelevant Ab + Fab
A	14465	6
B	12595	-62
C	10218	-5
D	5299	4
E	2303	-8
F	971	3
G	360	-4
H	-11	-5

Graph update with specific signal



EC50 (nM) from manual process: 2.585

Summary

Our testing has shown pHSense assays processed on the AssayMate automated liquid handling workstation provided results consistent with manual workflow, resulting in EC50 values within the expected experimental range.

Accuracy and precision values were compared to and consistent with the manual process, and no increase in background signal or off-target noise was detected.

This shows the value of the AssayMate workstation in providing researchers with a reliable platform to automate and support repeatability of their workflows and help free up time for technicians to engage in other tasks.

Related application note

For a detailed description of the pHSense technology, assay principle, and biological applications for antibody internalization studies, please refer to the pHSense application note: “pHSense Eu probes for time-resolved fluorescence-based monitoring of antibody and ADC internalization.”