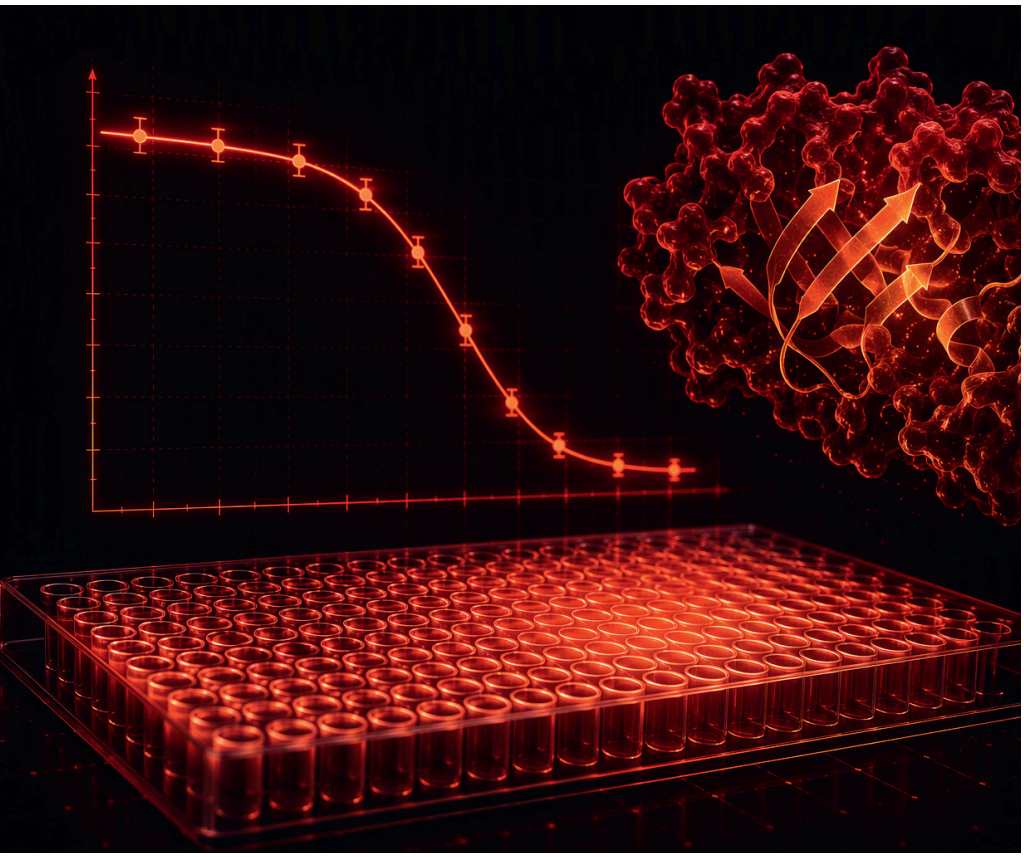

Challenge
Reliable IC_{50} dose-response curves demand precise inhibitor titrations with minimal sample and reagent consumption.

Solution
PULSEspencer R enables automated, non-contact direct titration in miniaturized volumes, bypassing error-prone serial dilutions. With qTOWERiris 384, it provides continuous, high-res real-time fluorescence detection.

Intended audience
Researchers in drug discovery, assay development, biochemical characterization, or high-throughput screening and enzyme kinetic studies.

Unlock high-quality dose-response and IC_{50} data at minimal volumes with PULSEspencer

Introduction

Enzyme kinetic studies form the analytical backbone of modern drug discovery and biochemical characterization. A central parameter for evaluating inhibitor potency is the IC_{50} (half-maximal inhibitory concentration) value. It represents the inhibitor concentration required to reduce enzymatic activity by 50%. To generate reliable and reproducible dose-response curves, these assays require highly precise titrations of the inhibitor across a broad concentration range.

Traditionally, these inhibitor gradients are generated through manual or semi-automated serial dilutions, an approach that entails significant methodological limitations. The sequential nature of serial dilutions creates an inherent inter-well dependency, meaning that a single pipetting error early in the process propagates through the entire concentration series. This cumulative error can severely distort the actual compound concentrations and skew the resulting IC_{50} calculations. Furthermore, conventional dilution schemes typically operate in larger reaction volumes, tying up substantial amounts of expensive enzymes, specialized substrates, and precious compound libraries. Additionally, planning and executing these complex matrices consumes valuable laboratory time. Furthermore, simple titration patterns may introduce spatial edge effects or temporal gradients across the microplate, potentially affecting assay performance.
To address these challenges, digital dispensing technology offers a promising alternative. By enabling non-contact fluid transfer in the pico- to microliter volume range, the PULSEspencer series provides the capability to bypass serial dilutions entirely through automated direct titration. This application note describes the implementation of a highly miniaturized continuous fluorometric protease assay to evaluate inhibitor dose-response. This study demonstrates how replacing traditional liquid handling workflows with digital dispensing enables the rapid generation of precise inhibitor gradients.

To provide a robust proof of concept for IC50 determination, a well-characterized model system comprising trypsin as the target enzyme, FITC-casein as the fluorogenic substrate, and benzamidine as the competitive inhibitor was utilized. The underlying biochemical mechanism of this assay is illustrated in Figure 1. In its intact state, the heavily labeled FITC-casein exhibits strong intra-molecular self-quenching. Upon proteolytic cleavage by trypsin, the fluorophores are spatially separated, leading to a measurable, time-dependent increase in fluorescence. Benzamidine acts as a competitive inhibitor by binding to the active site of trypsin, thereby preventing substrate cleavage and suppressing the fluorescence increase. By analyzing the kinetic responses and resulting IC_{50} profiles, this study demonstrates how digital dispensing streamlines assay development through intuitive software guidance and automated calculations. Furthermore, it minimizes error propagation associated with serial dilutions while significantly reducing reagent consumption.

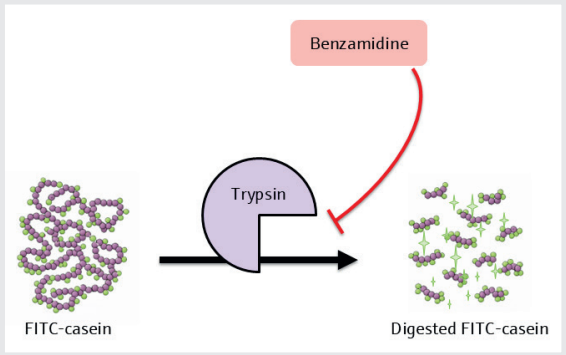

Figure 1: Schematic principle of the continuous fluorometric protease assay. Intact FITC-casein exhibits low baseline fluorescence due to strong intra-molecular self-quenching. Active trypsin cleaves the substrate into smaller peptide fragments, releasing the quenching effect and generating a strong fluorescence signal over time. Benzamidine acts as a competitive inhibitor by binding to the active site of trypsin, thus preventing substrate degradation and resulting in a dose-dependent reduction of the fluorescence signal.

Materials and Methods

Reagents and consumables

- ddH_2O
- PCR-grade water
- NaOH (1 M)
- HCl (1 mM)
- HEPES (H4034-500G, Sigma-Aldrich)
- $CaCl_2$ (CN92, Carl Roth GmbH + Co. KG)
- Trypsin from porcine pancreas (T4799-5G, Sigma-Aldrich)
- Benzamidine hydrochloride hydrate (8201220002, Sigma-Aldrich)
- Casein fluorescein isothiocyanate from bovine milk Type III (C0528-10MG, Sigma-Aldrich)
- 1 x RR8 PULSEspencer Cassette (875-300725, Analytik Jena)
- 1 x RR8s PULSEspencer Cassette (875-300720, Analytik Jena)
- 384 well PCR plate, full-skirted, white (844-70039-0, Analytik Jena)

Hardware

- PULSEspencer R (875-300010, Analytik Jena)
- qTOWERiris 384, 230V, incl. color module 1 (844-00858-2, Analytik Jena)
- CyBio SELMA 384/25 µL (OL7001-26-216, Analytik Jena)
- CyBio TipTray 384/25 µL (OL3800-25-513-N, Analytik Jena)
- Microplate Adapter; Soft Touch (30-7215-790-24, Analytik Jena)
- Adapter 384, passive cooling function (844-00141-0, Analytik Jena)

Preparation of reagents

The reaction buffer was prepared by dissolving HEPES (final concentration 50 mM) and $CaCl_2$ (final concentration 10 mM) in deionized water. The pH was adjusted to 8.0 by adding NaOH. For the enzyme solution, trypsin powder was dissolved in 1 mM HCl to reach a final concentration of 0.1 mg/mL and for the inhibitor benzamidine, a 200 mM stock solution was prepared by dissolving the powder in 100% DMSO. The substrate, FITC-casein (10 mg), was reconstituted directly in its original glass vial with 2 mL of PCR-grade water. This solution was divided into 100 µL aliquots, wrapped in aluminum foil, and stored at -20 °C. Immediately prior to the experiment, an aliquot was thawed and diluted to a 1 mg/mL working solution using PCR-grade water.

Prefilling the buffer into a 384-well plate

Prior to the addition of the assay components, the 384 well PCR plate was prefilled with 5 µL per well of reaction buffer (50 mM HEPES, 10 mM $CaCl_2$, pH 8.0) using the CyBio SELMA 384/25 µL semi-automated pipettor. To ensure high volumetric accuracy, a reverse pipetting protocol was employed. The buffer was aspirated from a one-column reservoir located at the lower deck position and dispensed into all 384 wells of the PCR plate simultaneously at the upper position. Because the buffer was dispensed into an initially dry plate, a soft-touch adapter was utilized beneath the destination plate. This setup ensured uniform and gentle contact between the pipette tips and the well bottoms across the entire plate, facilitating a clean liquid release. The residual overstroke volume was subsequently returned to the

reservoir to minimize reagent waste. Immediately following the prefilling step, the plate was transferred onto a passive cooling adapter (pre-chilled to -20 °C).

Preliminary assay optimization

Prior to the main IC_{50} determination, an initial assay optimization was performed to identify the ideal stoichiometric ratio of trypsin to FITC-casein. To streamline this assay development process, the *Synergy* wizard feature within the Analytik Jena dispenser software was utilized. This tool enabled the rapid and intuitive design of a two-dimensional concentration matrix (checkerboard titration), automatically calculating and dispensing the precise fluid volumes across the previously with reaction buffer prefilled plate (Figure 2). Upon monitoring the kinetic reactions, the resulting fluorescence curves were evaluated based on their signal-to-background ratio and linearity. The specific concentration combination that yielded the most robust and linear signal increase without reaching premature substrate depletion was selected and established as the standard baseline parameter for all subsequent benzamidine inhibition experiments.

Dispensing the inhibitor titration

Directly after prefilling a new PCR 384 well plate with reaction buffer using CyBio SELMA, the reagents were dispensed with the PULSEspencer R. In the fluid list of the Analytik Jena dispenser software, the reagents were created with the appropriate fluid class and stock concentration (Figure 3).
The *Set Value* function of the dispenser software was used to add enzyme and substrate, trypsin and FITC-casein.

For benzamidine, the *Titration* wizard was applied to generate a logarithmic titration pattern across the wells with concentrations from 2 mM down to 0.520 µM with 3 replicates for every concentration. Furthermore, appropriate positive and negative controls were included to validate the uninhibited kinetic signal and to determine the inherent background fluorescence of the assay components. Due to different added volumes of DMSO by titrating benzamidine, the *Normalization* wizard was applied to achieve the same DMSO concentration in every well for comparability. In addition to this, for the titration pattern, the *Randomization* wizard was applied to achieve randomized positions of the different concentrations and therefore statistically exclude the dispensing order as well as the well position (Figure 4).

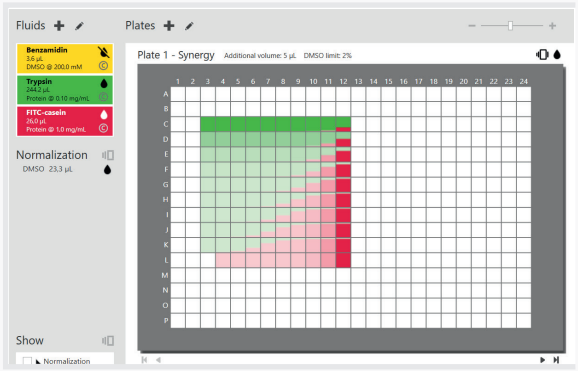

Figure 2: Experimental design for assay optimization. Screenshot of the *Synergy* wizard within the Analytik Jena dispenser software, illustrating the setup of a two-dimensional concentration matrix (checkerboard titration) to determine the optimal stoichiometric ratio of trypsin and FITC-casein.

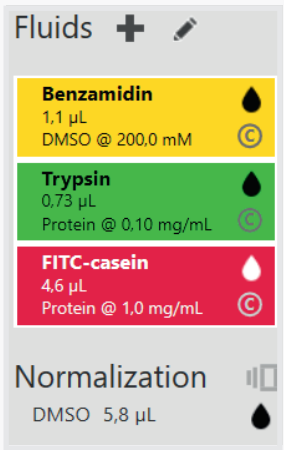

Figure 3: Reagent configuration in the Analytik Jena dispenser software. The fluid list overview details all required assay components, specifying their respective fluid classes, stock concentrations, and dispense volumes.

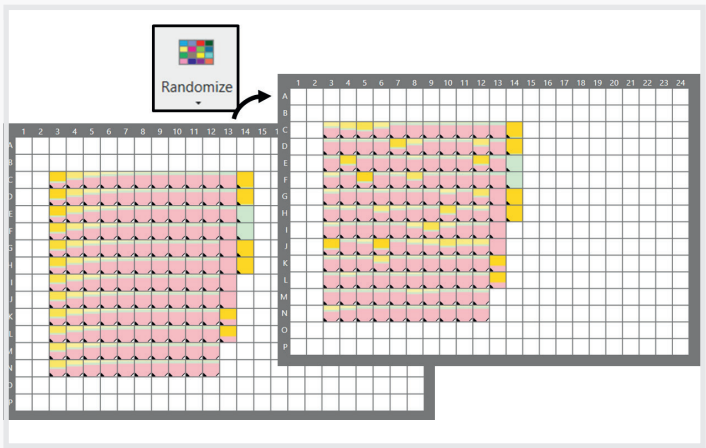

Figure 4: Automated plate layout randomization. The initial logarithmic titration pattern (left) is seamlessly rearranged across the 384 well plate (right) using the *Randomization* wizard.

Importantly, for better homogenization of the reagents *Shake during dispense* was turned on in the settings for the protocol. After preparation of this protocol in the Software and preparation of all components, the dispensing was performed on the PULSEspencer R. For this, a 384 well PCR plate was inserted into the destination plate holder of the dispenser. The instructions of cassette loading and pipetting of fluids of the Analytik Jena dispenser software were followed and all fluids were dispensed into the respective wells. After the dispensing was completed, the plate was sealed with a PCR sealing foil and briefly centrifuged for subsequent analysis with the qTOWERiris 384.

Continuous detection using qTOWERiris 384

Immediately after sealing, the 384 well plate was transferred to the qTOWERiris 384 for continuous real-time fluorescence monitoring. To prevent condensation and ensure stable reaction volumes during the assay, the instrument's heated lid was set to 50 °C. The thermal protocol was initiated with a 5-minute equilibration step at 5 °C to establish a uniform thermal baseline across the entire plate and prevent premature enzymatic activity. Subsequently, the temperature was raised to the physiological reaction temperature of 37 °C (temperature ramp: 2 °C/s). The kinetic progression of the substrate cleavage was monitored continuously over a period of 60 minutes. Fluorescence excitation and emission were recorded using color module 1 (FAM channel; excitation: 455 ± 15 nm, emission: 515 ± 10 nm), matching the spectral properties of the FITC fluorophore. Scans were acquired every 30 seconds (totaling 120 measurement cycles), with three measurement repeats per scan to maximize the signal-to-noise ratio and precision. Upon completion of the thermal protocol, the raw fluorescence data was exported for subsequent data analysis and IC_{50} determination.

Results and discussion

The real-time monitoring of the trypsin-catalyzed cleavage of FITC-casein yielded high-resolution progress curves across the reactions. A strong continuous increase of fluorescence was observed for the positive controls (0 µM benzamidine) over all 120 measuring points during the 60 min of incubation. With rising concentrations of benzamidine, the gradients of the kinetic curves get lower down to completely flat lines in the highest benzamidine concentrations as well as the negative controls without enzyme addition. The controls without substrate also show no increase and are significantly lower in absolute fluorescence values.

To translate the high-resolution kinetic progress curves into quantitative inhibition profile, the activity of the enzymatic reaction was determined for each well, this was achieved by applying linear regression to the linear phase of the fluorescence increase. The resulting slopes (representing change in fluorescence units per minute) directly correlate with the residual trypsin activity at each respective benzamidine concentration. To calculate the relative inhibition, the measured slopes were normalized using the upper and lower asymptotes derived directly from the standard four-parameter logistic (4PL) regression. These fitted curve boundaries were defined as the 100% and 0% enzymatic activity limits, respectively. The normalized enzyme activity was plotted against the logarithmic concentration of benzamidine to generate dose-response curves (Figure 5).

As illustrated in Figure 5 (A), the data points of one representative replica show a tight alignment with the sigmoidal fit line. The small error bars of the individual data points, representing the standard deviation of the three technical replicates per concentration, underline the high technical precision of the non-contact fluid transfer. The slightly larger error bars observed at lower inhibitor concentrations are a common kinetic phenomenon; higher enzymatic activity in these wells leads to faster initial reaction rates, where even minor variations in substrate turnover are proportionally amplified. To evaluate the overall robustness and run-to-run consistency of the assay setup, Figure 5 (B) displays an overlay of the sigmoidal fits from three independent experimental replicates. The three regression curves show nearby perfect congruence, with similar slopes and nearly overlapping inflection points. The calculated IC_{50} value from all three replicates shown in Figure 5 (B) for benzamidine was determined to be 20.19 ± 1.62, which perfectly aligns with the established literature values for competitive trypsin inhibition under similar conditions.

A major advantage of implementing PULSEspencer technology is the significant reduction in reagent and sample consumption, enabling high-density data generation with minimal material input. By bypassing manual serial dilutions and dispensing directly into a highly miniaturized assay volume of just 5.1 µL per well, the assay maximizes the output of valuable and often expensive biochemical components. To illustrate the efficiency of this digital dispensing workflow, the consumption per individual

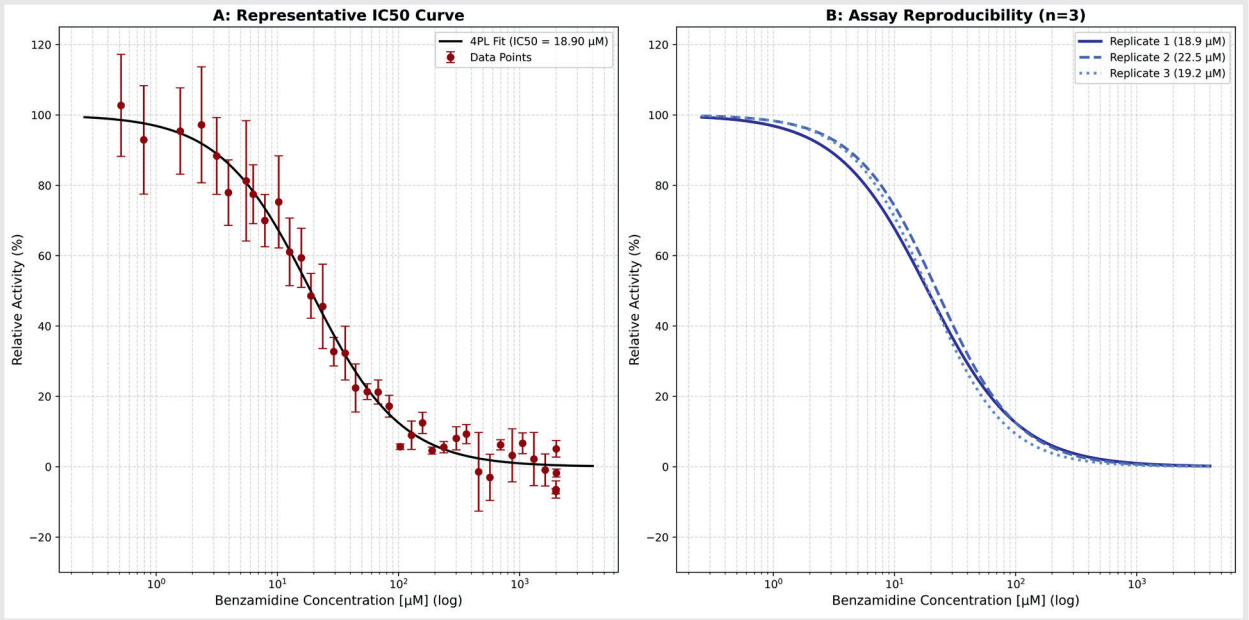

Figure 5: Dose-response analysis of trypsin inhibition by benzamidine. (A) Representative IC_{50} curve generated via automated direct titration. Data points represent the normalized mean enzymatic activity of three technical replicates, with error bars indicating the standard deviation. The data is fitted using a four-parameter logistic (4PL) regression, yielding an IC_{50} of 20.19 µM. (B) Overlay of 4PL regression fits from three independent experimental replicates. The nearly perfect congruence of the curves and the minimal variance in calculated IC_{50} values (18.9 µM to 22.5 µM) highlight the excellent reproducibility and precision of the digital dispensing workflow.

reaction (well) and for one dose-response experiment (including controls and replicates) is detailed below (Table 1). A fixed volume of 5 µL HEPES + $CaCl_2$ reaction buffer in every well was added prior to the valuable assay components.

The immense resource efficiency of the PULSEspencer series becomes particularly evident when compared to traditional manual assay protocols in 96 well formats. While conventional methods typically require total reaction volumes of up to 200 µL and consume approximately 1 µg of fluorogenic substrate and 50 ng of enzyme per single data point, the described digital dispensing workflow cuts material input drastically. By miniaturizing the assay to a 5.1 µL format, substrate and enzyme consumption per well are reduced by over 96% and 98%, respectively. Consequently, to achieve equivalent inhibitor concentrations in a standard 200 µL reaction volume, nearly 40 times the amount of benzamidine would be required for each data point across the titration gradient. This extraordinary conservation of material underscores the system's value for screening of rare or expensive compound libraries and hard-to-source biological targets such as purified enzymes.

Table 1: Reagent consumption of enzyme, substrate and inhibitor for one dose-response setup in 136 wells of 384 well plate with 5.1 µL reaction volumes.

Reagent	Mass per single well	Dispensed volume per single well	Total mass per experiment (including dead volumes)
Trypsin	0.57 ng	5.7 nL	0.4 µg
FITC-casein	35.6 ng	35.6 nL	7.8 µg
Benzamidine	0-1.23 µg	0-50.9 nL	48.06 µg

In summary, the highly reproducible IC_{50} values and the successful miniaturization highlight several key advantages of the digital dispensing workflow:

1. **Elimination of pipetting drift:** Bypassing manual serial dilutions via direct titration successfully prevented the propagation of systematic volume errors across the concentration range.
2. **Effective matrix equalization:** The automated normalization of the DMSO concentration across all wells ensured that solvent-induced effects on enzyme kinetics were completely uniform, eliminating a common source of assay interference.
3. **Mitigation of edge effects:** The application of the *Randomization* wizard statistically neutralized any potential spatial artifacts across the 384 well plate, such as thermal gradients during the incubation in the thermal cycler as well as time-dependent variations linked to the dispensing order.
4. **Low sample consumption:** Non-contact nanoliter dispensing drastically reduces the required amounts of expensive enzymes, substrates, and precious compound libraries compared to traditional 20–50 µL formats.
5. **Increased data density:** The ability to easily generate complex concentration gradients enables a seamless transition to 384 well formats, significantly increasing data points and assay resolution without inflating reagent costs.
6. **Rapid assay preparation:** The entire automated dispensing protocol, including the manual loading of reagents and subsequent cassette exchanges, was completed in approximately 8 minutes, significantly minimizing bench time.
7. **Intuitive design of experiment:** The Analytik Jena dispenser software simplifies complex assay setups. Dedicated features like *Titration*, *Normalization*, and *Randomization* wizards reduce manual calculation and planning time while minimizing the risk of human error.

Summary

The combination of the PULSEspencer R, CyBio SELMA and the qTOWERiris 384 offers a highly efficient and reliable workflow for IC_{50} determination. The prefilling of buffer with the CyBio SELMA ensures rapid and uniform plate preparation, while the subsequent non-contact dispensing of enzymes and direct titration of inhibitors on the PULSEspencer R eliminates the need for manual serial dilutions. By downsizing the reaction to a highly miniaturized 5.1 µL assay volume, this workflow drastically reduces reagent consumption and maximizes data density without compromising precision. Continuous fluorescence detection with the qTOWERiris 384 delivers high-resolution kinetic data, ensuring highly reproducible and robust curve fitting. Although the precise dispensing of assay components and inhibitor gradients was demonstrated on the PULSEspencer R, this automated dispensing process can be seamlessly transferred to the PULSEspencer RC, making it a robust solution for both instruments within the series.

Figure 6: CyBio SELMA (left) and PULSEspencer R (right)

Recommended device configuration

Table 2: Devices, accessories and consumables used for the dose-response assay.

Article	Article number	Description
PULSEspencer R	875-300010	PULSEspencer R brings pL-level dispensing to your lab, automating workflows with unmatched accuracy.
PULSEspencer RC[1]	875-150010	PULSEspencer RC takes pL-level dispensing further, enabling advanced applications like single-cell omics.
RR8 PULSEspencer Cassettes	875-300725	Dispensing of surfactant-free reagents, 8 dispenseheads, 8 pL – 20 µL
RR8s PULSEspencer Cassettes	875-300720	Dispensing reagents with surfactants, 8 dispenseheads 11 pL – 20 µL
CRL PULSEspencer Cassettes[2]	875-150725	Dispensing of surfactant-free reagents, 1 dispensehead, 8 pL – 20 µL
CRLs PULSEspencer Cassettes[2]	875-150720	Dispensing reagents with surfactants, 1 dispensehead, 11 pL – 20 µL
qTOWERiris 384	844-00858-2	Real-time PCR thermal cycler, 230V, incl. color module 1
CyBio SELMA 384/25 µL	OL7001-26-216	Semi-automated pipetting station including a 384 channel pipetting head from 0.5 till 25 µl
CyBio TipTray 384/25 µL	OL3800-25-513-N	384 tips 25 µL per TipTray, 24 individually packaged TipTrays per case
Microplate Adapter; Soft Touch	30-7215-790-24	Spring-loaded adapter for precise low-volume transfers
Adapter 384, passive cooling function	844-00141-0	Adapter for 384 well non-skirted, semi-skirted or full-skirted microplates with passive cooling function.

[1] The assay described in this application note was performed using the PULSEspencer R; however, the PULSEspencer RC can be used for the same workflow.

[2] Required for workflows performed with the PULSEspencer RC.

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