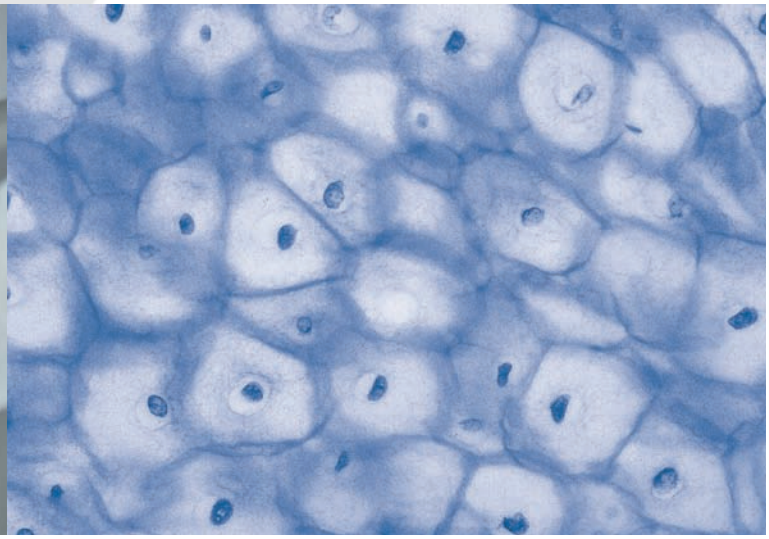

A fluorogenic assay for identification of coagulation factor IIa inhibitors- screening for stroke treatment

Easy automation of a biochemical assay on the Fluent™ laboratory automation solution

Introduction

The protease, thrombin (factor IIa) is an important drug discovery target, playing a central role in the blood coagulation and wound healing processes. It is generated by proteolytic activation of prothrombin, and is commonly recognized as the enzyme responsible for the conversion of fibrinogen to fibrin. The central role of thrombin in the coagulation process is established by the fact that any perturbation within the blood coagulation system resulting in significant amplification, impairment, acceleration or delay in thrombin generation leads to clinically relevant hemorrhagic or thrombotic events. There are many ongoing efforts in the pharmaceutical industry to identify compounds which can inhibit thrombin activity, and recent studies have also focused on the identification of dual inhibitors which act on Factor Xa, another key component in the coagulation process, as well as thrombin.

European ScreeningPort GmbH (ESP) is a public-private partnership which receives project-based funding from the national governments, industry partners and academic institutions. ESP offers industrial scale small molecule hit-finding capabilities to academic organizations as well as collaborating closely with the pharmaceutical industry. Approximately half of the Company's hit finding projects involve cell-based assays, and ESP provides screening services to multiple academic partners from across the globe, screening in excess of fifteenthousand 15,000 384-well plates per annum. To support these projects, ESP operates comprehensive assay development, cell culture, compound logistics and screening platforms. This is combined with industry standard informatics systems for the recording of experimental results and the analysis of screening data.

Tecan has re-invented automation with Fluent, a unique instrumentation concept built around the application-specific needs of cell biology laboratories. Fluent breaks new ground, delivering greater capacity and increased speed; the platform provides superior throughput and walkaway time, making it easier to get more done, more effectively. Tecan and ESP have developed protocols that demonstrate the power of this *in-vitro* assay solution for biochemical assays, combining expertise in automated liquid handling, detection and biochemical assays to offer a new dimension in fast, reliable, completely automated assays without the need for expert automation personnel.

Materials and methods

Figure 1: The Fluent assay workstation. A Fluent 780 is shown, equipped with an eight-channel Flexible Channel Arm, a Multiple Channel Arm 384 and a Robotic Gripper Arm. A Carousel is integrated onto the right hand side of the instrument, along with the latest generation of CO2 incubator. An Infinite® M1000 PRO microplate reader is located below the Dynamic Deck. The dimensions of the compact system are indicated.

Fluent laboratory automation solution

Fluent is the latest in Tecan's successful family of liquid handling automations platforms. The Fluent offers rapid, high definition pipetting for both the eight-channel Flexible Channel Arm (FCA) and the Multiple Channel Arm 384 (MCA 384). Its patented Dynamic Deck increases the worktable capacity and boosts productivity by allowing intergration of a wide range of Tecan modules – including a Carousel™, for storage of various consumables; a HydroSpeed™ plate washer (not required for this assay); an Infinite M1000 PRO plate reader; and carriers for troughs, stacked disposable tips and microplates (up to six deep on the worktable, Figure 2) – as well as a third-party high capacity CO_2 incubator.

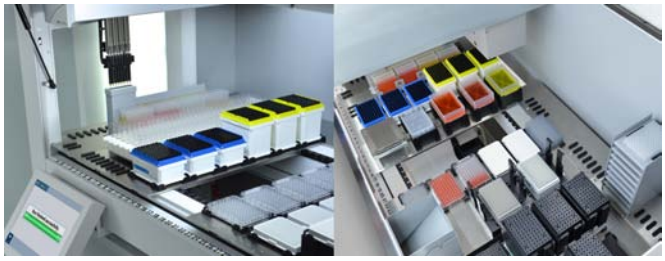

Figure 2: Dynamic Deck with six ANSI/SLAS positions in the depth, equipped with six boxes for disposable tips for the Flexible Channel Arm on the left. Blue colour code for 200µl and yellow colour code for 1000µl disposable tips. Top view of the Dynamic Deck on the right showing nested disposable tips, positions for troughs, active carrier for the Multiple Channel Arm 384 and a hotel in the back.

The Multiple Channel Arm 384 uses tip adapters which can be automatically exchanged during a run, allowing it to act as either a 384- or a 96-channel arm within the same protocol. Fully independent, task-specific arms allow parallel processing and coordinated scheduling, ensuring the runs are completed faster and more efficiently. Each Fluent cell-based assay solution is equipped with:

- Flexible Channel Arm – fitted with eight pipetting channels using disposable tips that can individually access any well or tube, perfect for explicit sample and control distribution or serial dilutions.
- Multi Channel Arm – instantly swaps between 96- and 384- channel adapters during a run, offering outstanding capabilities for reagent distribution or plate replication.
- Robotic Gripper Arm (RGA) – quickly and smoothly transfers plates and consumables between storage modules, integrated devices and the worktable without interrupting pipetting.

Thrombin assay

Principle of the assay technology

The aim of this study was to test the reproducibility of the Fluent laboratory automation system using an assay monitoring thrombin activity. The assay was run in 384-well format against compounds selected from the ESP, screening each compound in duplicate. Twenty assay plates comprising two copies of 10 compound plates were run.

The assay used Boc-VPR-AMC subtrate, a specific, highly fluorogenic substrate for thrombin and the thrombin-staphylocoagulase complex. Trypsin, tryptase from rat mast cells, and two trypsin-like enzymes from sperm of the ascidian Halocynthia roretzi, acrosin and spermosin, also hydrolyze this substrate.

Automation process

Overview

The thrombin assay was run in 384-well low volume plates with test compounds from the ESP library in duplicates. The compounds and controls were pre-plated offline and the plates stored in the Carousel until needed. The thrombin as well as the substrate were prepared offline in the required concentrations and manually placed onto the Dynamic Deck prior to starting the process. Assay plates were automatically retrieved from the Carousel and thrombin was added. After a 10 minute incubation step at room temperature in the Carousel, the substrate was added and, the plate incubated at room temperature for further 30 minutes. Finally, the fluorescence signal in the wells was

detected using the fully integrated Infinite M1000 PRO reader and the percentage of thrombin inhibition as well as Z' were determined.

Detailed automation protocol

Lidded 384-well low volume plates containing 20 nl per well of compounds or controls were loaded into the Carousel: two equal sets of ten plates were prepared resulting in a total of twenty assay plates. Forty boxes of 15 µl Disposable Tips, two boxes per assay plate, for enzyme and substrate addition were also loaded into the Carousel. Thrombin was prepared at a concentration of 3 ng/ml in assay buffer and placed on the Dynamic Deck along with the AMC substrate solution at a concentration of 80 µM in assay buffer.

The twenty assay plates were processed in five batches of four plates. An assay plate was retrieved from the Carousel and 5 µl of thrombin solution was added by the Multiple Channel Arm 384 to give a final enzyme concentration of 1.5 ng/ml. The plate was then brought back to the Carousel incubating the compound and enzyme for a 10 min period. During this incubation assay plates 2, 3, and 4 were prepared in a similar manner. Thereafter, subsequently 5 µl of substrate solution was added to assay plates 1, 2, 3, and 4 using the Multiple Channel Arm to give a final substrate concentration of 40 µM. After a 30 min incubation at room temperature in the Carousel the fluorescence signal (excitation at 355 nm, emission at 460 nm) in the plate was detected using the Infinite M1000 PRO reader. Finally, the plate was transferred from the reader to the Carousel, ready for disposal. This process was automatically repeated five times in a loop processing all 20 plates within 4.5 h (Figure 3).

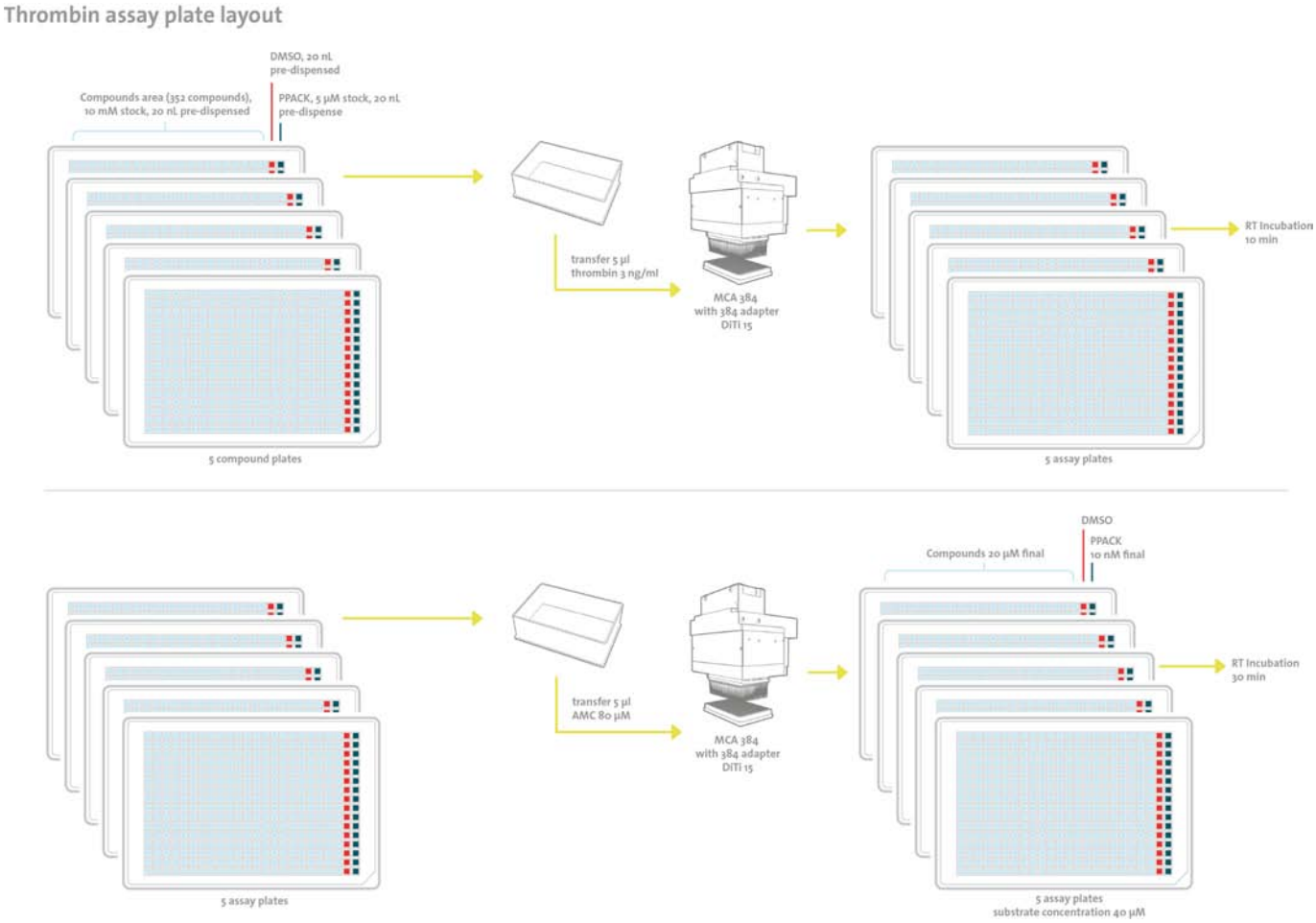

Figure 3: Pipetting schemes and plate layouts of thrombin assay.

Lids were automatically removed and replaced by the Robotic Gripper Arm, controlled by FluentControl™ software, to minimize evaporation. Delivery of plates and disposable tips from the Carousel was optimized using the system's unique Path Finder™ technology, enabling high speed, coordinated movement of all arms in parallel.

Compound well responses were normalized with respect to controls and data from the two repetitions were then plotted against each other. Additionally, Z' values were determined for each assay plate.

Results

The data was presented as heat maps, and 384-well plate-to-plate comparison show a very good correlation, see figure 4. The results and controls were also plotted as the percentage inhibition for plate 1 against the percentage inhibition for its duplicate plate. The data showed a very good correlation for both controls (Figure 5) and test compounds (Figure 6) with R^2=0.98.

Figure 4: Typical heat maps of the first plate (left) and its duplicate (right).

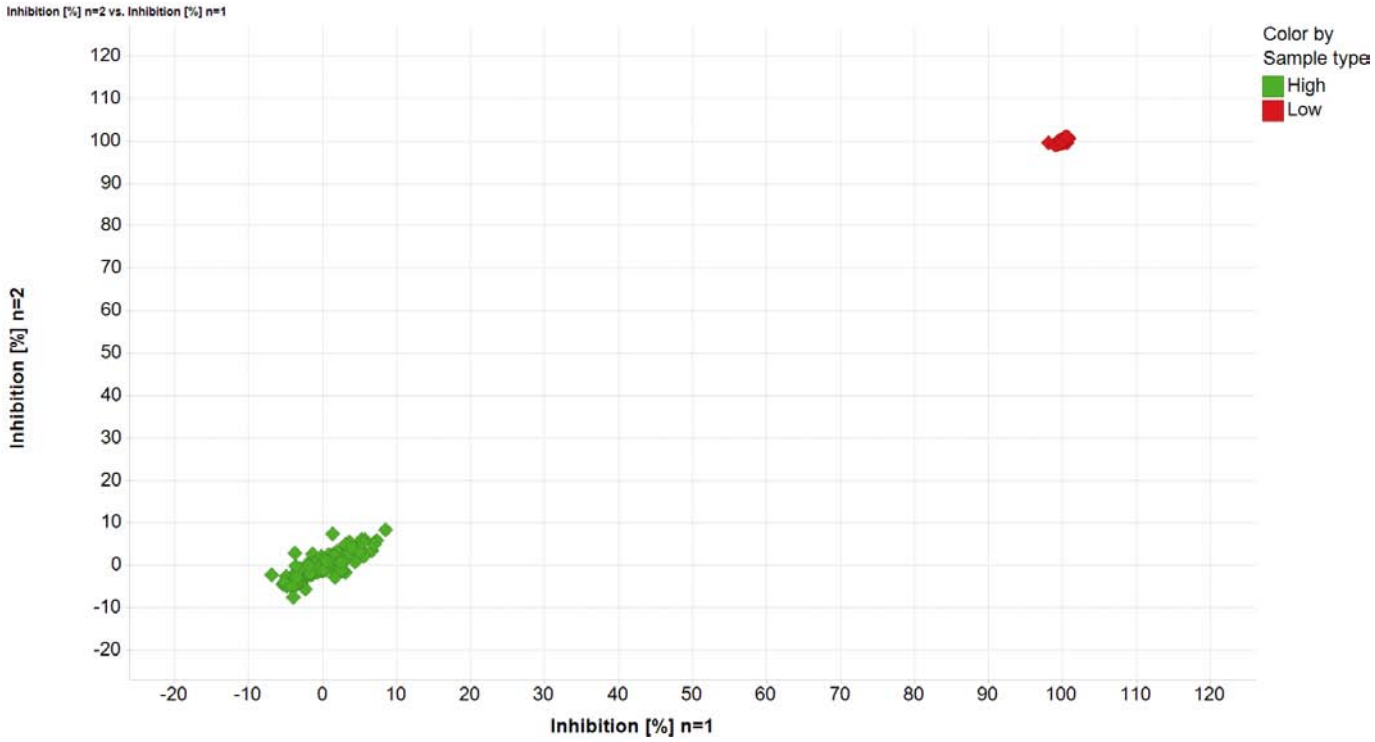

Figure 5: Inhibition % (repeat 1) versus Inhibition %(repeat 2), data shows high (0 inhibition) and low control (100% inhibition) control performance across 20 plates.

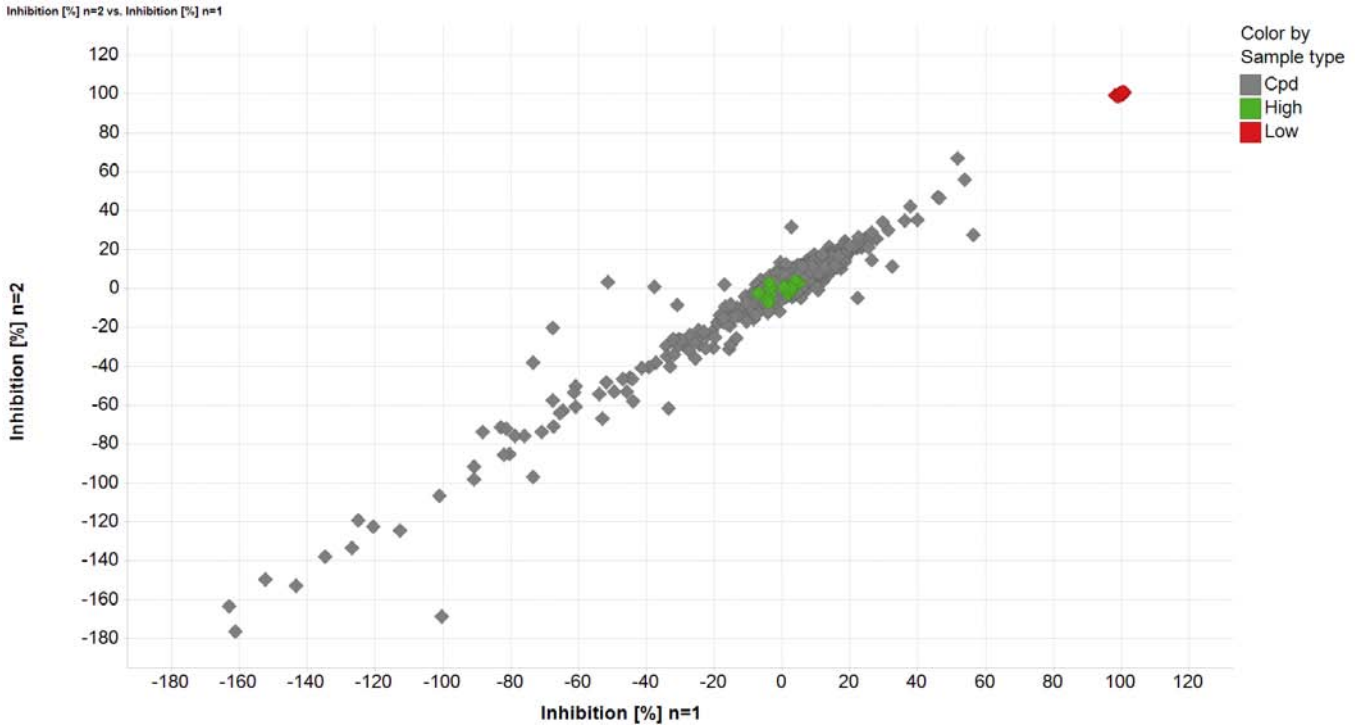

Figure 6: Inhibition % (repeat 1) versus Inhibition % (repeat 2), data shows compound and wells control from all 20 plates (R^2=0.98)

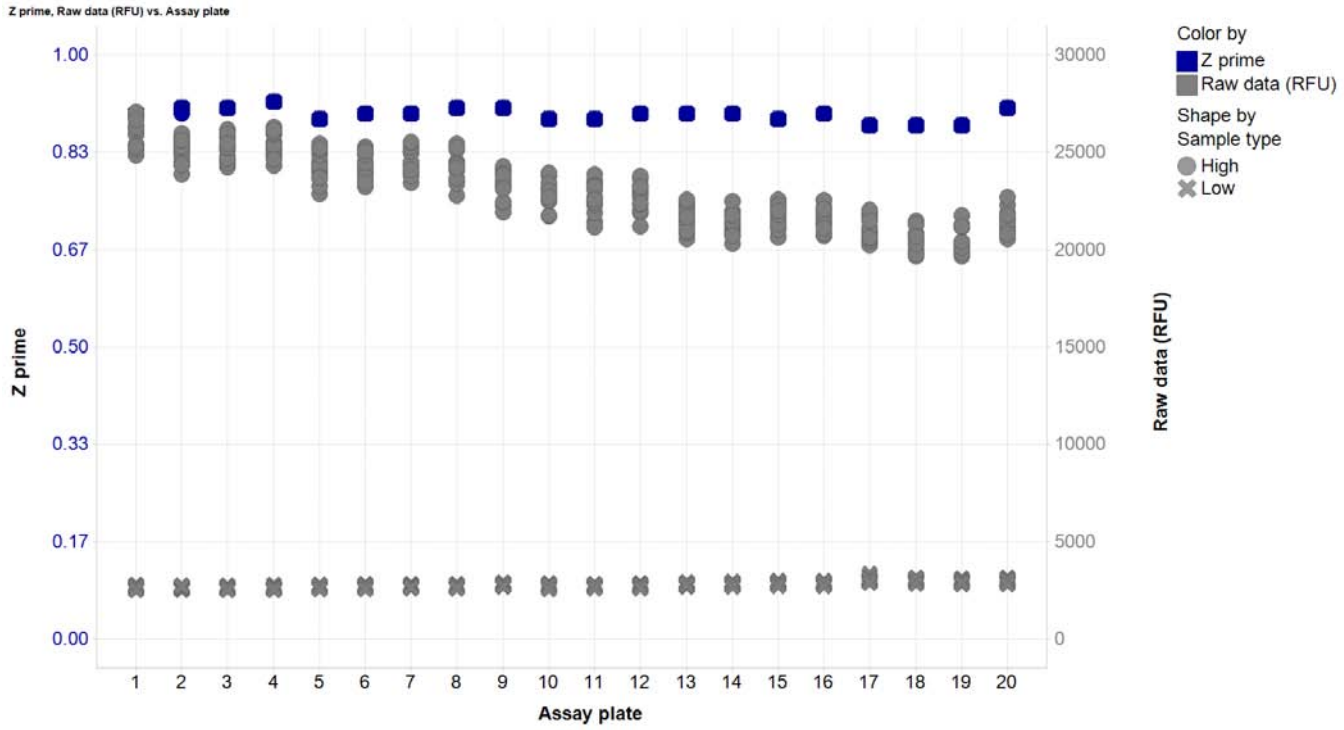

Figure 7: Intensity and Z' values as a single plot over 20 plates.

Summary

This application note demonstrates that automation of biochemical assays such as the thrombin assay, in 384-well format be performed in a can straightforwardly manner using the Tecan's, Fluent laboratory automation solution. The 20 plate assay was complete in 4.5 hours, generating 7680 individual data points. The large deck capacity and the multi-tasking of the eight-channel Flexible Channel Arm, the Multiple Channel Arm and the Robotic Gripper Arm allow the full assay to be completed in such a short time and without human intervention.

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