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Automated screening potential Thrombin Inhibitors using the ep*Motion*® 5075

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Abstract

A team at CSIRO® has developed a method to screen candidate molecules for serpin-mediated inhibition of thrombin using the ep*Motion* 5075 automated liquid handling system. The mammalian glycosaminoglycan heparin is an efficacious antithrombotic agent. However, there is an ongoing need to research and identify heparin alternatives due to the risk of serious side-effects associated with its use.

To aid in this search, an automated screening method in 384-well plate format has been developed. This assay has been used to measure heparin cofactor 2 and antithrombin-III mediated thrombin inhibition. It is conceivable that it could also be adapted to measure inhibition mediated by the other two serpins that act on thrombin, protein C inhibitor (PCI) and protease nexin-1 (PN1).

Introduction

Thrombin is a serine protease active in thrombosis. It plays a critical role in both hemostasis and clot formation [1]. Inhibitors can either act directly on thrombin or indirectly through the potentiation of one of four serpins being antithrombin-III, heparin cofactor 2 (HCII), protein C inhibitor and protease nexin-1 [2]. Heparin is one such indirect thrombin inhibitor.

Heparin is a sulphated polysaccharide belonging to the family of molecules known as glycosaminoglycans and has been in widespread use since the 1930's because of its efficacy, short half-life and cost effectiveness [3]. However, heparin treatment requires frequent monitoring to ensure correct dosing [4] and it can also have side effects including bleeding, heparin induced thrombocytopenia [5] and hypoaldosteronism [6]. These complications highlight the need to discover and develop alternatives to heparin without its serious side effects.

To assist in these clinical research discoveries, we have developed an automated 384-well plate high-throughput assay to screen for inhibitors of thrombin. This assay is based on a previously reported method [7,8] and employs the chromogenic substrate Chromozym® TH to measure the activity of thrombin and its inhibition. -Thrombin cleaves Chromozym TH to produce 4-nitraniline which can be measured on a spectrophotometer at 405 nm. Heparin and other anionic carbohydrates can inhibit the action of thrombin but only in the presence of a serpin, with complete inhibition leading to no production of 4-nitranaline. The assay can be used to identify candidate heparin alternatives that can range from other known mammalian glycosaminoglycans, like dermatan sulphate [8], to uncharacterized anionic carbohydrates from other sources including marine [9] and terrestrial organisms [10,11].

The application Heparin_Standard has a runtime of approximately 11 minutes and the application Thrombin_Inhibition has a runtime of approximately 30 minutes.

Material and Methods

Required Labware

ep*Motion* 5075l/ ep*Motion* 5075t equipped with:
> Thermal module on position C2
> Dispensing Tools TS 50, TS 300, TM50-8, TM300-8
> Reservoir rack
> Reservoirs: 30 mL
> Reservoir adapter 30 mL
> Reservoir 400 mL
> Reservoir rack module for Safe-Lock Tubes, for 4 x 0.5/1.5/2 mL tubes
> Thermorack for 24 x 1.5 mL Safe-Lock Tubes
> Thermoblock for 96 well PCR Plates

Spectrophotometer capable of 384-well plate kinetic reads with a 405 nm filter

Required Consumables

> epT.I.P.S.® Motion 50 µL and 300 µL, filtertips
> Eppendorf Safe-Lock Tubes 1.5 mL
> Eppendorf twin.tec® PCR Plate 96
> Perkin-Elmer® SpectraPlate™ 384 TC, clear, tissue culture treated sterile, with lid

Required Reagents

> Heparin sodium salt from porcine intestinal mucosa
> Heparin Cofactor 2, Human
> Human Antithrombin-III
> Thrombin, alpha, Human
> Chromozym TH
> Tris PEG buffer - Trizma® hydrochloride (pH7.4) (0.02 M)
> Sodium chloride (NaCl) (0.15 M); PEG 6000 (1 mg/mL).
> Tris/glycerol buffer - Trizma hydrochloride (pH7.4) (0.02 M); NaCl (0.05 M) Glycerol (50% v/v)
> Heparin stock solution 1.25 mg/mL

The assay measures the ability of samples to inhibit thrombin compared to the effects of heparin standards. The assay identifies if a sample is inhibiting thrombin as well as the mechanism behind it. This is useful as inhibitors have different modes of action. For example, dermatan sulfate inhibits thrombin in the presence of heparin cofactor II, but not with other serpins [1]. This contrasts with heparin which inhibits thrombin in the presence of all serpins in the coagulation cascade [2]. Knowing the mechanism of inhibition is important as it enables more specific targeting of the coagulation cascade.
The protocol is designed to assay 40 samples and 8 Heparin standards per 384-well plate.

Reagent Preparation

Heparin Standards

The Heparin standards are a 1:2 dilution series created into the first column a 96-well twin.tec plate. Well A1 will contain only water, with the other wells having dilutions ranging from 31.25 (B1) to 0.488 µg/mL (H1). These figures equate to a range of 0.520 to 0.008 µg/mL in the final 384-well assay plate.
For the application Heparin_Standard the setup of the worktable is described in Figure 1 and Table 1.

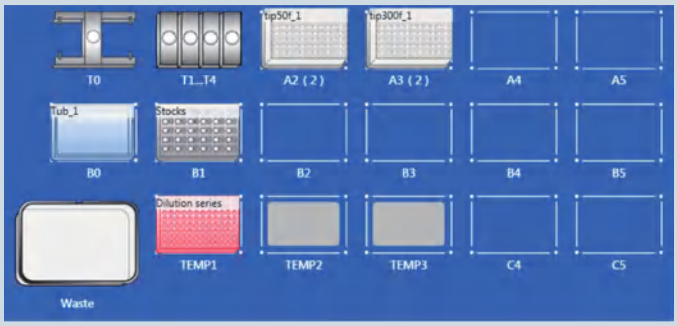

Figure 1: Screenshot of the worktable layout for the file Heparin_Standard.

Worktable Position	Labware
A2	50 µL epT.I.P.S., Filtertips
A3	300 µL epT.I.P.S., Filtertips
B0	Liquid Waste Tub (400 mL tub)
B1	Thermorack for 24 x 1.5 mL Safe-Lock Tubes Position 1: 1 mL of water Position 2: 50 µL of 1.25 mg/mL heparin
C1	Thermoblock with 96-Well Eppendorf twin.tec® PCR plate, empty
T1	TS 50, 50 µL single-channel dispensing tool
T2	TS 300, 300 µL single-channel dispensing tool

The final volume for each well will be 80 µL, sufficient for multiple assays.

Sample Preparation

Manually aliquot a maximum of 40 samples into a twin.tec 96 well PCR plate using wells A1 to H5. The minimum volume required per well for the program is 15 µL and the maximum volume is 180 µL. Greater volume enables repeat runs if required. Keep samples on ice until required.

Program a spectrophotometer with the following settings: read type kinetic, wavelength 405 nm, period 40 minutes, interval 2 minutes, oven temperature 37°C, plate type 384-well. Turn the oven on to pre-heat.

Warm some Tris-PEG buffer to use as a diluent for Chromozym TH. Chromozym TH is the final addition in the reaction series and the epMotion file has been programmed to keep this reagent at 37 °C. The other reagents should be prepared with room temperature Tris-PEG buffer, as strong enzymatic activity is not desired until all reagents have been combined. Prepare each reagent as described in Table 2.

Table 2: Reagent preparation Note:* reagents diluted in Tris-PEG buffer

Reagent	Stock concentration	Volume (µL) for use with the ep*Motion*	Amount per well (µL)	Final concentration	Comments
Tris-PEG Buffer		6,000	Variable. Either 0, 9, 20 or 29 µL depending on the addition of other ingredients		
Heparin Cofactor II*	2.55 µg/mL	4,120	20	0.85 µg /mL	
Thrombin*	0.11 µg/mL	2,870	9	0.0165 µg/mL	
Chromozym TH*	166 µM	13,000	30	83 µM	Make with warm (37°C) Tris-PEG buffer.

Open the file Thrombin_Inhibition and set up the worktable as described in Figure 2 and table 3. The reservoir rack is similar to the reservoir rack for Heparin run.

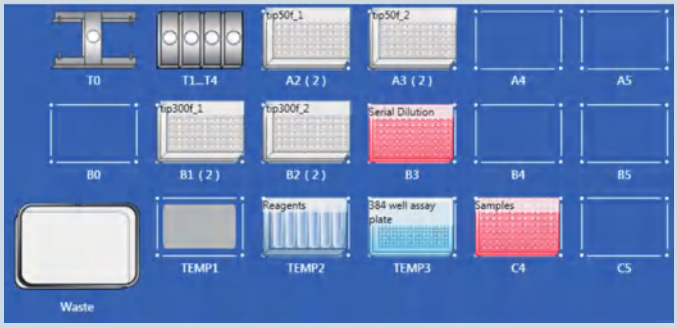

Figure 2: Screenshot of the worktable layout for the file Thrombin_inhibition.

Please note that Tris-PEG buffer is only added to control wells to make up the final volume. Upon completion of the robot run, immediately transfer the 384-well plate to the spectrometer for reading.

Table 3: ep*Motion* worktable allocation for the file Thrombin_inhibition.

Worktable Position	Labware
A2	50 µL epT.I.P.S., Filtertips
A3	50 µL epT.I.P.S., Filtertips
B1	300 µL epT.I.P.S., Filtertips
B2	300 µL epT.I.P.S., Filtertips
B3	Thermoblock with 96-Well Eppendorf twin.tec PCR plate, containing the serial dilution of heparin.
C2 (TEMP2)	> Reservoir Rack with 3 Reagent Reservoirs 30 mL > Position 1: Tris-PEG Buffer - 6 mL > Position 2: Heparin cofactor 2 (or antithrombin III) – 4,120 µL > Position 3: Thrombin – 2,870 µL > Position 7 with Reservoir Adapter 30 mL: Chromozym TH – 13 mL
C3	384 well plate, empty
C4	Thermoblock with 96-Well Eppendorf twin.tec PCR plate, containing samples.
T1	TM 50, 50 µL 8-channel dispensing tool
T2	TM 300, 300 µL 8-channel dispensing tool

Results and Discussion

Figure 4 shows a typical 384-well assay result. Each sample or standard is added to eight wells in a 2x4 grid (e.g. wells A1 to B4). The rows alternating from A (i.e. A, C, E etc.) contains no serpin, whilst those alternating from B (i.e. B, D, F etc.) contain serpin (either heparin cofactor 2 or antithrombin-III). For the standards, which are found in the first 4 columns, heparin only inhibits thrombin in the presence of a serpin. Wells without a serpin will show normal thrombin activity, leading to an accumulation of 4-nitraniline and hence increasing absorbance at 405 nm. In contrast, the wells containing heparin cofactor 2 or antithrombin-III will have a reduced signal depending on the amount of heparin added, thus contributing to a standard curve.

An additional set of controls has been designed into the assay. The first column of the group (e.g. columns 1, 5, 9 etc.) contains no thrombin (no thrombin control) and hence no cleavage of Chromozym TH is expected to occur in these wells, with no accumulation of 4-nitranaline. This control is necessary to ensure that Chromozym TH is not being cleaved by another enzyme or chemical that may be present in the sample.

The samples are assayed in columns 5-24, in this case being a variety of marine extracts. The sample assayed in wells M9 to N12 (outlined in orange) shows typical antithrombin-III-mediated inhibition of thrombin, with strong inhibition of the reaction on the 'B' line but not on the 'A'. Other examples also occur in the plate.

We have used the assay to test for HCII and antithrombin-III mediated inhibition and believe that it could be suitable for the other two serpins that act on thrombin (i.e. protein C inhibitor (PCI) and protease nexin-1 (PN1)) [2].

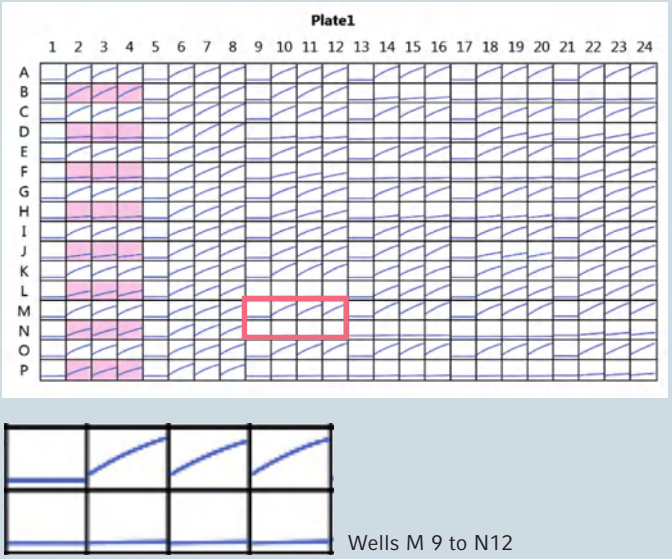

Figure 4: A screen capture from SoftMax®Pro (SoftMax Pro 6.2.1. Molecular Devices®, Sunnyvale, USA) showing an assay result. The sample assayed in well M9 to N12 (outlined in orange and enlarged) shows typical antithrombin-II mediated inhibition of thrombin, with a flat line on the "B" row not on the "A". Other examples are also present. For further details please refer to the text.

Conclusion

The method described here provides an automated assay to efficiently screen large numbers of samples for anti-thrombin activity. Molecules that inhibit thrombin can be identified as well as the role of individual serpins as co-factors in this inhibition.

References

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Ordering information

Description	Order no. international
ep*Motion*® 5075l	5075 000.301
ep*Motion*® 5075t	5075 000.302
Thermal module	5075 757.001
Dispensing Tool TS 50	5280 000.010
Dispensing Tool TS 300	5280 000.037
Dispensing Tool TM 50-8	5280 000.215
Dispensing Tool TM 300-8	5280 000.231
Reservoir Rack	5075 754.002
Reservoir Rack Module TC for reservoir 30 mL	5075 799.146
Reservoir 30 mL	0030 126.505
Reservoir Rack Module TC for Safe-Lock tubes	5075 799.103
Thermorack 24 tubes	5075 771.004
Thermoblock for 96 PCR plate	5075 766.000
Eppendorf twin.tec® 96 real-time PCR Plates, skirted	0030 132.513
Reservoir 400 mL	5075 751.364
epT.I.P.S.® Motion filter 300 µL	0030 014.456
epT.I.P.S.® Motion Filter 50 µL	0030 015.413
Eppendorf Safe-Lock Tubes 1.5 mL	0030 120.086