Miniaturization and Optimization of a Fluorescence Polarization Assay to Interrogate a Protein-Peptide Interaction

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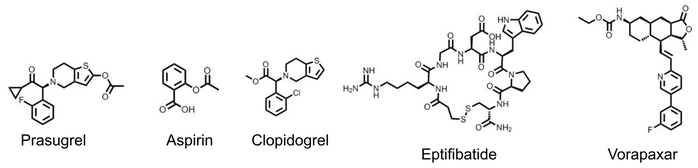
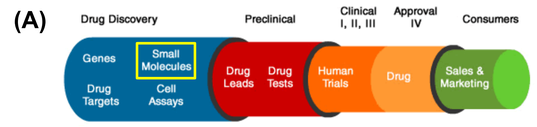
Therapeutic Significance

Cardiovascular diseases are the leading cause of death in America, accounting for 1 in every 4 deaths[1]. Platelet plug formation has been implicated in many of these diseases, such as myocardial infarction and stroke. Current therapies (Prasugrel, Aspirin, Clopidogrel, Eptifibatide, Vorapaxar) are limited by drug side effects and resistance, as well as variability in patient response[2]. Therefore, novel anti-platelet agents are needed. Our therapeutic target is a protein-peptide interaction (PPI) involved in modulating the formation of platelet plugs. Preventing this PPI with a small molecule holds promise as an anti-clotting therapy.

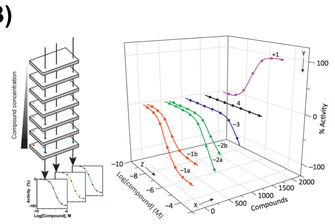
Current FDA Approved Anti-Platelet Drugs



qHTS to Identify a PPI



(B)



(C)

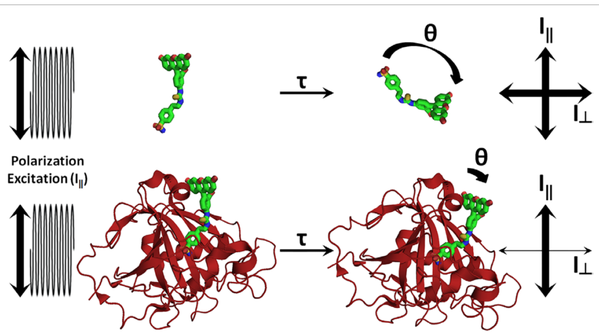
$$\text{Z-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}.$$

Z' factor	Assay Quality
1	Ideal
0.5 - 1	Excellent
0 – 0.5	Double
0	Yes/No Answer
<0	Poor

(A) An overview of the drug discovery pipeline[3]. (B) A typical dose-response curve generated from qHTS[4]. (C) A simple statistical parameter utilized in qHTS for assessing assay quality[5].

PPI Detection Method: Fluorescence Polarization

(A)



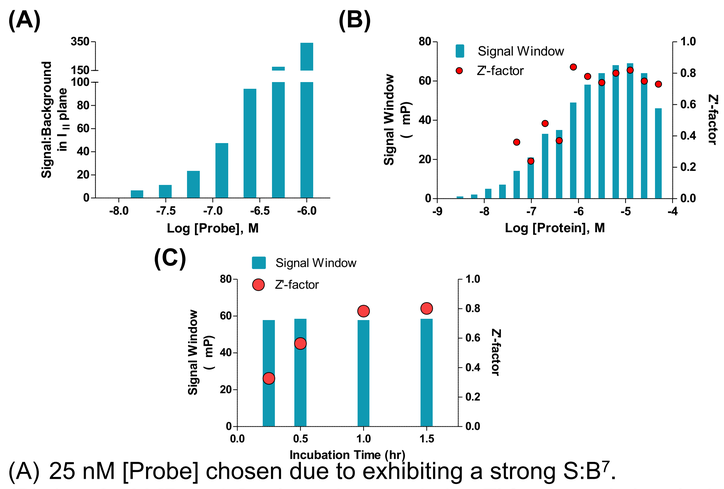
(B)

Molecular Weight of Probe	Rotational Speed of Probe	Predominant Emission Type
High	Low	Polarized
Low	High	Depolarized

(A) Polarized light excites a fluorescent probe whose emission intensity is measured parallel and perpendicular to the excitation plane. The degree of emitted fluorescence remaining polarized is indicative of a binding interaction[6]. (B) The conceptual relationship between the size of the probe complex and emission result.

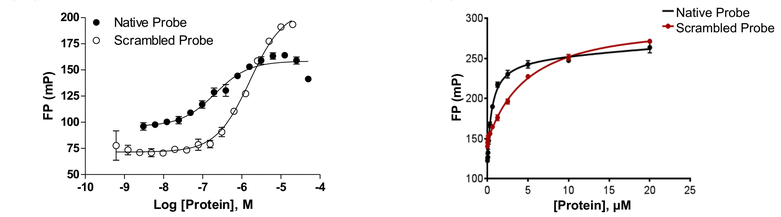
Assay Optimization in 384-well Format

Selection of Optimal Conditions



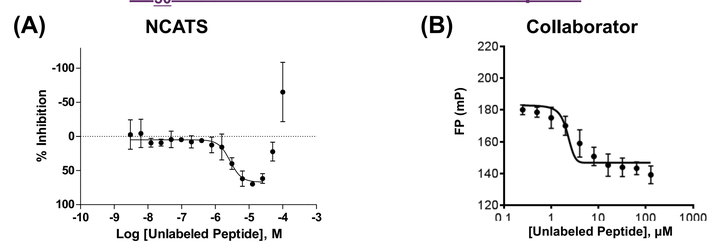
(A) 25 nM [Probe] chosen due to exhibiting a strong S:B[7].
(B) 1 or 2 μM [Protein] selected to maximize Z' factor and signal window.
(C) 1 hour incubation time (RT) selected to maximize Z' factor.

K_D Determination of the Protein-Peptide Interaction



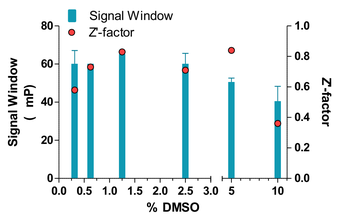
(D) To determine the K_D, protein was titrated against 25 nM probe (n = 3).
(E) Collaborator K_D determination against 25 nM probe (n = 3).

IC_{50} Determination of Unlabeled Peptide



(A) IC_{50} value estimated at 2.7 μM due to artifacts seen at high concentrations (n = 3).
(B) Collaborator IC_{50} determination of 2.2 μM (n = 3).

No DMSO Effect Observed

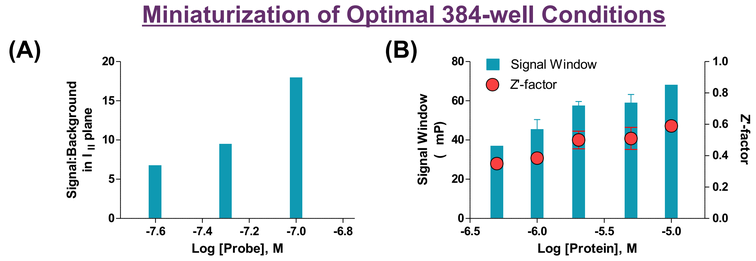


Comparison of Assay Parameters

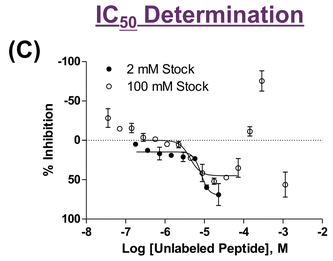
	K_D Determination		Competition Assay	
Parameter	NCATS	Collaborator	NCATS	Collaborator
Assay Volume, μL	20	50	20	50
Incubation Time, hr.	1	0.25	1	0.25
[Probe], nM	25	25	50	100
[Protein], μM	Titration	Titration	1	2.5
[Unlabeled Peptide], μM	-	-	Titration	Titration
[IC_{50} Unlabeled Peptide], μM	-	-	2.72	2.20
[K_D Scrambled Probe], μM	1.63	5.68	-	-
[K_D Native Probe], μM	0.21	0.56	-	-
K_D Fold-Difference	8	10	-	-

Assay Miniaturization to 1536-well Format

Miniaturization of Optimal 384-well Conditions



IC_{50} Determination



Assay Parameters

(D)

Parameter	NCATS
Assay Volume, μL	4
Incubation Time, hr.	1
[Probe], nM	100
[Protein], μM	1.25
[Unlabeled Peptide], μM	Titration
[IC_{50} 2 mM Stock], μM	8.25
[IC_{50} 100 mM Stock], μM	4.55

(A)-(B) Optimal conditions were selected using the same criteria described in 384-well format. (C) A competition assay similar to the experiment described in 384-well format. (D) Conditions of the competition assay.

Discussion and Next Steps

- Achieve full displacement of the probe in the 1536-well format competition assay.
- Perform overnight reagent stability tests for online robotic screen.
- Consider orthogonal methods to interrogate the PPI (e.g. AlphaScreen, Homogenous Time Resolved Fluorescence Assay).
- Screen small molecule libraries to identify small molecule inhibitors the PPI.
  - NCGC Pharmaceuticals Collection (~3,000 FDA approved drugs), Genesis Library (~70,000 compounds), MLSMR Library (~400,000 compounds).
- Utilize physiologically relevant cell-based assays to determine *in vitro* target engagement of small molecule inhibitors (e.g. Cellular Thermal Shift Assay).

References

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