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WNV NS2B-NS3 fluorescence single point

Forked from ZIKV NS2B-NS3 fluorescence single point screening assay

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ASAP Discovery

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Protocol status: Working
We use this protocol and it's working

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Disclaimer

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Abstract

Purpose: performing single point test of selected compounds from screening experiment libraries containing serine-targeting fragments and serine protease inhibitors against WNV NS2B/NS3 to find warheads and candidates for medicinal optimization
General description: This protocol details the fluorescent assays for WNV NS2B/NS3 cleavage of substrate Bz-Nle-KRR-AMC peptide. This method measures the fluorescence from the released AMC product as a result of the enzymatic reaction. When hydrolyzed, AMC is liberated from the peptide substrate. Excitation of AMC at 350 nm emits a resonant energy of 450 nm. When the enzyme is inhibited, the fluorescence signal will decrease as a result of lower enzyme activity. The screening method is validated by calculating the Z prime number of each plate, and the percentage of inhibition is calculated and then evaluated for inhibitory efficacy.
Outcome: hits were selected from screening assays with more than 50% inhibition

Materials

Assay Buffer Reagents (Concentration listed are from Stock Solutions)

1. Tris-HCl (Fisher Scientific). Dissolving Tris powder in MilliQ water and adjust pH to 8.6 for a final concentration of 1 M. Filter with 0.2 um filter
2. Triton (Sigma Aldrich) dissolving one part of Triton in 4 part of MilliQ water for a final concentration of 20 %

Additional Reagents:

[M] 84090 nanomolar (nM) **WNV NS2B/NS3 Enzyme**

- The Enzyme stock was originally [M] 242140 nanomolar (nM) and was diluted to [M] 10000 nanomolar (nM) before every experiment in **freshly made Assay Buffer**. The final assay concentration is [M] 12.5 nanomolar (nM)

[M] 20000000 nanomolar (nM) **Substrate Bz-Nle-KRR-AMC**

- Substrate stock was dissolved in DMSO to the stock concentration. Before each experiment, the substrate stock was diluted to [M] 10000 nanomolar (nM) in freshly made Assay Buffer.The final assay concentration is [M] 500 nanomolar (nM)

Safety warnings

Please be sure to wear proper Personal Protective Equipment (PPE) while performing this experiment.

Before start

Thaw TCEP solution on ice to make sure it is fresh

WNV NS2B/NS3 expression and purification

1 **The protein used in this assay was expressed and purified and received from** QQ01WNVNS2B -c003 -p005 from Centre for Medicines Discovery

Prepare Reagents

2 **PREPARE** all of the reagents/buffers required for this experiment.

Assay Buffer

	A	B	C	D	E
	Reagent	**Stock**	**Final**	**Units**	**Note**
	Tris pH 8.6	1000	10	mM	
	Triton	20	0.01	%	
	glycerol	100	20	% v/v	

Reagents (dilute reagents in assay buffer for required volume)

	A	B	C	D	E
	Reagent	**Stock**	**Prep**	**Final in assay plate**	**Units**
	WNV-NS2B/NS3	1735000	83.3	50	nM
	Substrate (Bz-Nle-KRR-AMC)	20000000	100000	20000	nM

Prepare 384-well Plate

16m

3 **DILUTE** Dilute protein and substrate using the assay buffer

- Protein dilution: 150 µL protein stock solution is added into 3480 µL Assay Buffer
- Substrate dilution: 1 µL x 20 mM substrate is added into 2 mL Assay buffer

4 **MIX** Add 10 µL enzyme stock solution into 384-well plates containing inhibitor stocks (column 2-22)
add 20 µL reaction buffer to A1-H1, I24-P24 (blank control)
add 10 µL reaction buffer to I1-P1, A24-H24 (substrate control)
MIX 180 µL diluted enzyme solution with 3.6 µL x 10 mM DCI (3,4-Dichloroisocoumarin) and aliquot 10 µL into I2-P2, A23-H23 (positive control inhibitor)
MIX 180 µL diluted enzyme solution with 3.6 µL DMSO and aliquot 10 µL into A2-H2, I23-P23 (no inhibitor control)

Incubate at room temperature for 1 hour

5 **REACT** Add 10 µL substrate solution into enzyme solution in the plate and mix well.

Read Plate Fluorescence

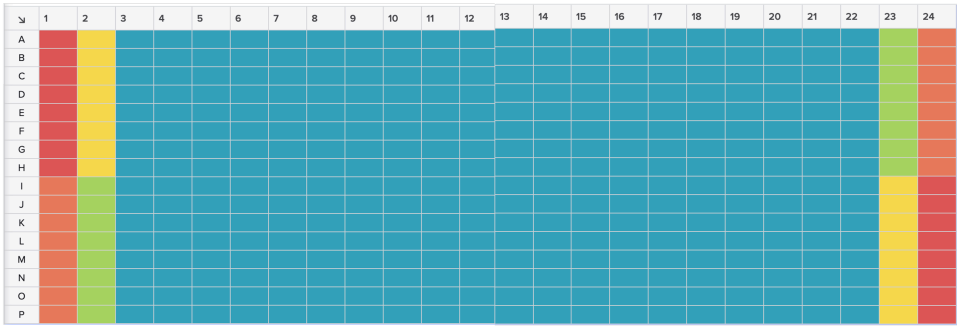
6 **READ** and **RECORD** the plate Relative fluorescence units (RFU) using Cytation 3 Multi-Mode Reader (BioTek, Winooski, VT)

Expected result

AMC product will give RFU signal at ex 350/em 450

Experimental Design

7 Plate Layout



- Buffer
- Substrate
- Enzyme + Substrate (negative control)
- Enzyme + Substrate + Inhibitor (positive control)
- Enzyme + Substrate + Library compounds

DATA PROCESSING

Calculate the Z prime number of the screening plates to validate the screening:
Z′ = 1 − 3 ∗(Std.Dev of positive + Std.Dev of negative)/ |(Average of positive − Average of negative)|

Calculate the percentage of inhibition:
Inhibition % = ([RFU no inhibitor]-[RFU with compounds])/(RFU no inhibitor) * 100%

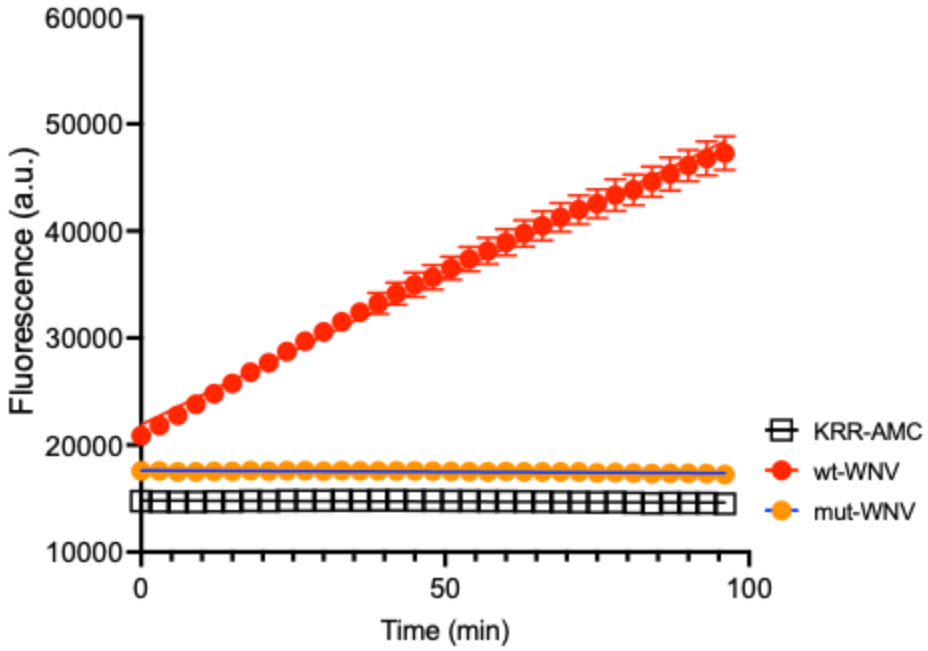
PLOT DATA

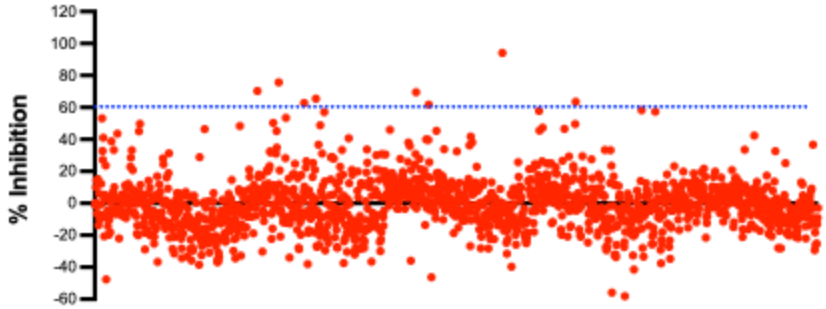
In Prism software, plot percentage of inhibition with corresponding number of compounds

Result

8

Results shown are exemplar


60000
50000
40000
30000
20000
10000
Fluorescence (a.u.)
0
50
100
Time (min)
KRR-AMC
wt-WNV
mut-WNV


120
100
80
60
40
20
0
-20
-40
-60
% Inhibition