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DENV NS2B-NS3 fluorescence single point screening assay

Forked from SARS-CoV-2 nsp3 macrodomain Time-Resolved FRET peptide displacement assay

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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 libraries containing serine-targeting fragments and serine protease inhibitors against DENV NS2B-NS3 to find warheads and candidates for medicinal optimization
General description: This protocol details the fluorescent assays for ZIKV 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. HEPES(Fisher Scientific). Dissolving HEPES powder in MilliQ water and adjust pH to 8.5 for a final concentration of 0.5 M. Filter with 0.2 um filter
2. Sodium chloride (sigma aldrich). Dissolving crystal into MilliQ water for a final concentration of 5 M. Filter with 0.2 um filter
3. glycerol (Sigma Aldrich)
4. Igepal (Sigma Aldrich) dissolving one part of Igepal in 200 part of MilliQ water for a final concentration of 0.5 %
5. TCEP (GoldBio). Dissolving in MilliQ water into final concentration of 1 M. Store in -20 ºC

Additional Reagents:

[M] 242140 nanomolar (nM) **DENV 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

DENV NS2B-NS3 expression and purification

1 **The protein used in this assay was expressed and purified and received from batch** QQ01DVNS2B -c001 -p007 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**
	HEPES pH 8.5	500	10	mM	
	NaCl	5000	50	mM	
	glycerol	100	5	% v/v	
	Igepal	0.5	0.05	%	
	TCEP	1000	0.5	mM	add freshly

Reagents (dilute reagents in assay buffer for required volume)

	A	B	C	D	E
	Reagent	**Stock**	**Prep (2x)**	**Final in assay plate**	**Units**
	DENV-NS2B/NS3	242140	10000	5000	nM
	Substrate (Bz-Nle-KRR-AMC)	20000000	10000	5000	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

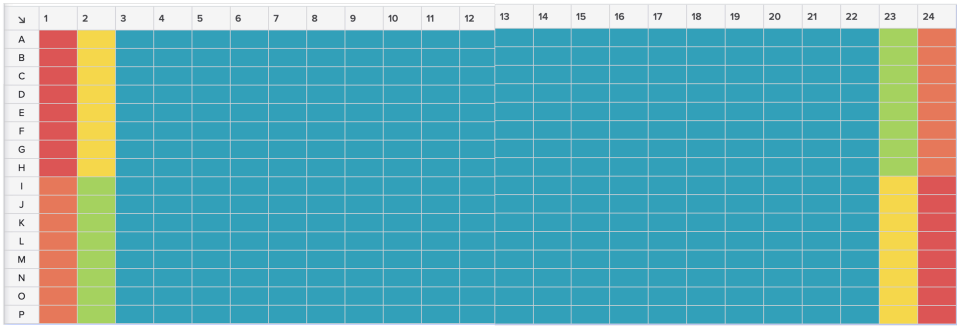
6 **READ** and **RECORD** the plate Relative fluorescence units (RFU) using Cytation 3 Multi-Mode Reader (BioTek, Winooski, VT)

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)|

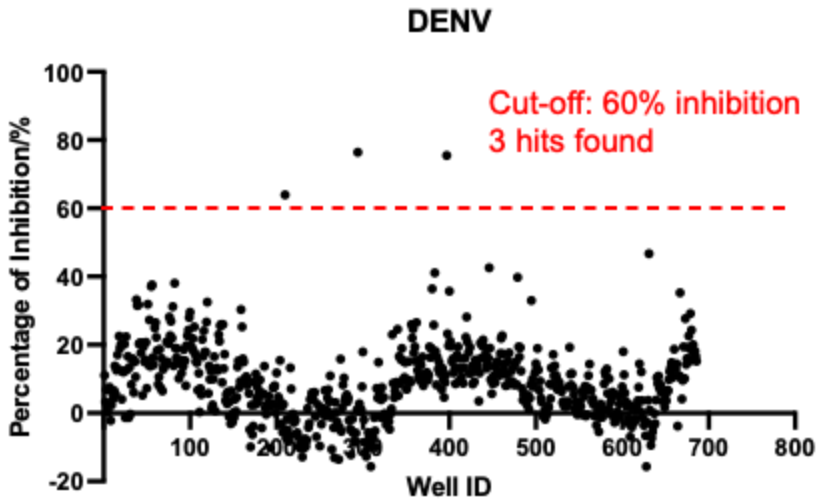
Calculate the percentage of inhibition:
Inhibition % = ([RFU no inhibitor]-[RFU with compounds])/(RFU no inhibitor) * 100%

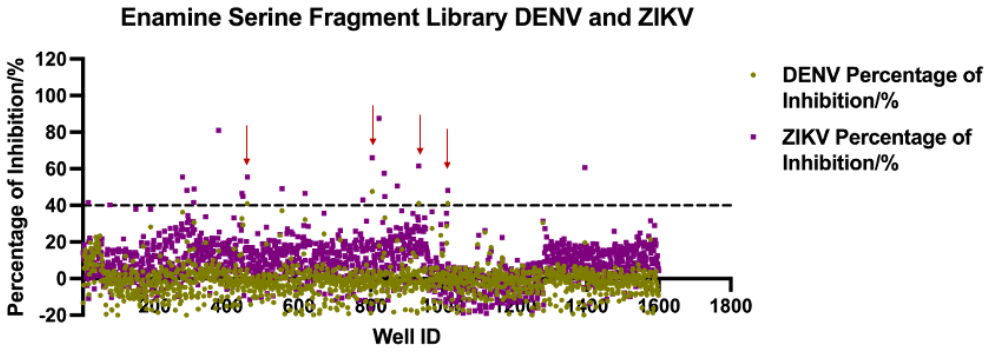
PLOT DATA

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

Result

8 Exemplar results are shown





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