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ZIKV NS2B-NS3 protease dose response screening assay



Forked from [ZIKV NS2B-NS3 fluorescence single point screening assay](#).

DOI

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Protocol status: Working

We use this protocol and it's working

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Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Abstract

Purpose: performing dose-response test of selected compounds from screening experiments against ZIKV NS2B-NS3 to confirm efficacy

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 and some hits demonstrated dose-response



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 °C

Additional Reagents:

[M] 84090 nanomolar (nM) **ZIKV 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)
- Plate containing inhibitors

Troubleshooting

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



ZIKV NS2B/NS3 expression and purification

- 1 **The protein used in this assay was expressed and purified and received in batch QQ01ZVNS2B -c001 -p006 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
	ZIKV-NS2B/NS3	84090	1000	500	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

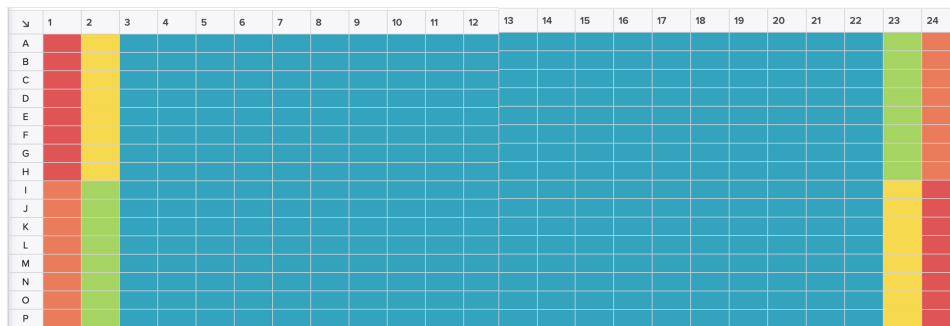
- 6 **READ** and **RECORD** the plate Relative fluorescence units (RFU) using Cytation 3 Multi-Mode Reader (BioTek, Winooski, VT)

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:

$$Z' = 1 - 3 * (\text{Std.Dev of positive} + \text{Std.Dev of negative}) / |(\text{Average of positive} - \text{Average of negative})|$$

Calculate the percentage of inhibition:

$$\text{Inhibition \%} = ([\text{RFU no inhibitor}] - [\text{RFU with compounds}]) / (\text{RFU no inhibitor}) * 100\%$$

PLOT DATA

In Prism software, plot percentage of inhibition with corresponding inhibitor concentrations of compounds

Result

8 Exemplar results are shown

