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Flaviviruses (West Nile, Zika, Dengue serotypes 1-4) NS2B-NS3 protease fluorescence dose response and single point assay V.4

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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

This is a **functional, biochemical assay** used to identify treatments for viral infectious diseases related to **Flavivirus infection**, (specifically **West Nile, Zika, and Dengue**) and targets the conserved **NS2B-NS3 protein**. In this current version, we added the Addgne id.

Utilizing a direct enzyme activity measurement method, the experiment was performed in a 384-well plate reading the fluorescence intensity. This assay tested the mode of action of inhibition.
Initial screening was conducted with 2 compound concentrations to determine %inhibition, followed by dose response for IC_{50} measurement (as specified below). In each run a reference compound was added to verify the assay reliability and robustness.

Assay was developed at the Weizmann Institute of Science, as a part of the ASAP Discovery Consortium.

Experiment Concentrations (From Stock to Assay)

	A	B	C	D	E
	Reagent	**Stock**	**Concentration Loaded into CERTUS**	**Final Concentration in Assay Plate**	**Units**
	Substrate	10000	10	5	µM
	DENV NS2B-NS3	217000	200	100	nM
	ZIKV NS2B-NS3	225000	200	100	nM
	WNV NS2B-NS3	222000	200	100	nM

Assay Buffer

	A	B	C	E
	Reagent	**Stock**	**Final concentration**	**Units**
	Tris pH=8.5	1000	20	mM
	Glycerol	50	10	%
	Triton	10	0.01	%

Compound Plate Design for Dose Response:

Total assay volume: 20 µL
Compounds top assay concentration: 100 µM
Dilution factor: 2
Dose response points: 12
Number of replicates: 2
Backfill with DMSO: yes

Compounds Plate Design for 2-Point Assay:

Total Assay Volume: 20 µL
Compounds Assay Concentration: 100 µM and 50 µM
Dilution Factor: 2
Dose Response Points: 2
Number of Replicates: 2
Backfill with DMSO: Yes

Materials

Assay Buffer Reagents (Concentration listed are Stock Solution Concentrations)

1. [M] 50 Mass / % volume
   Glycerol - for molecular biology, ≥99% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G5516
2. [M] 1000 millimolar (mM)
   Tris(2-carboxyethyl)phosphine hydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**75259
3. [M] 10 % (v/v) Triton Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T8787

*Note: all components are added fresh to the assay buffer before each experiment

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Additional Reagents:

West Nile Virus (WNV) Reagents:

[M] 222000 nanomolar (nM) WNV NS2B/NS3 Enzyme

- WNV NS2B/NS3 was originally [M] 222000 nanomolar (nM) and was diluted to [M] 200 nanomolar (nM) with freshly made **Assay Buffer** before each experiment

[M] 10000 nanomolar (nM) WNV Enzyme Substrate

- **Enzyme Substrate** was Boc-Gly-Arg-Arg-AMC acetate salt **Biosynth Catalog #**FB110553
- **Substrate stock** was created by dissolving the substrate powder in **DMSO** to create [M] 10 millimolar (mM) Substrate Stock aliquots and stored at -80 °C. Before each experiment, the Substrate Stock was diluted to [M] 10 micromolar (µM) Substrate with freshly made **Assay Buffer.**

Zika (ZIKV) Virus Reagents:

[M] 225000 nanomolar (nM) ZIKV NS2B/NS3 Enzyme

- ZIKV NS2B-NS3 was originally [M] 225000 nanomolar (nM) and was diluted to [M] 200 nanomolar (nM) with freshly made **Assay Buffer** before each experiment

[M] 10000 nanomolar (nM) ZIKV Enzyme Substrate

- **Enzyme Substrate** was Boc-Gly-Arg-Arg-AMC acetate salt **Biosynth Catalog #**FB110553
- **Substrate** stock was created by dissolving the substrate powder in **DMSO** to create [M] 10 millimolar (mM) Substrate Stock aliquots and stored at -80 °C . Before each experiment, the **Substrate Stock** was diluted to [M] 10 micromolar (µM) Substrate with freshly made **Assay Buffer.**

Dengue (DENV) Reagents:

[M] 217000 nanomolar (nM) DENV NS2B/NS3 Enzyme

- DENV NS2B/NS3was originally [M] 217000 nanomolar (nM) and was diluted to [M] 200 nanomolar (nM) with freshly made **Assay Buffer** before each experiment

[M] 10000 nanomolar (nM) DENV Enzyme Substrate

- **Enzyme Substrate** was Bz-Nle-KRR-AMC (hydrochloride) **Cayman Chemical Company Catalog #**27710
- **Substrate Stock** was created by dissolving the substrate powder in **DMSO** to create [M] 10 millimolar (mM) Substrate Stock aliquots and stored at -80 °C. Before each experiment, the **Substrate Stock** was diluted to [M] 10 micromolar (µM) Substrate with freshly made **Assay Buffer.**

Expected result

A literature reference inhibitor (**compound 83**, Journal of Medicinal Chemistry **2015** *58* (23), 9354-9370 https://doi.org/10.1021/acs.jmedchem.5b01441) was added to each run of NS2B-NS3 to give IC50 values of ~2 µM for DENV-2 and WNV and ~3 µM for ZIKA .

Protocol materials

- ZIKV VE_Ganxian2016 NS2B-NS3 protease fusion (non-cleavable linker, N-term Twin-Strep-tag) **addgene Catalog #**228661
- DENV-1 strain US/Hawaii/1944 NS2B-NS3 protease fusion with non cleavable linker **addgene Catalog #**228648
- DENV-2 Thailand/16681/84 NS2B-NS3 protease fusion (non-cleavable linker) **addgene Catalog #**228650
- WNV NY99 NS2B-NS3 protease fusion (non-cleavable linker) K→A mutation to reduce autoproteolysis **addgene Catalog #**228660
- DENV-3 Philippines/H87/1956 NS2B-NS3 protease fusion (non-cleavable linker) **addgene Catalog #**228651
- DENV-4 strain H241 NS2B-NS3 protease fusion (non-cleavable linker) **addgene Catalog #**228652

Safety warnings

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

Before start

Dummy plate: In each step of the protocol, dispense the reagents into an **empty** plate before dispensing them into the assay compounds plate (this will detect and prevent dispensing issues).

Note: Inhibitor compounds stock concentration is 20 mM. Compounds are pre-dispensed into 384 plates and stored at -20˚C until use.

Flavivirus NS2B-NS3 protease expression and purification

1 Zika virus NS2B-NS3 fused protease expression and purification of a construct suitable for biochemical assay. Using the plasmid

ZIKV VE_Ganxian2016 NS2B-NS3 protease fusion (non-cleavable linker, N-term Twin-Strep-tag) **addgene Catalog #**228661

Protocol

NAME

Zika NS2B-NS3 fusion construct for assay small scale expression and purification protocol

CREATED BY

korvus.wang

Preview

2 West Nile virus NS2B-NS3 fused protease expression and purification of the construct suitable for biochemical assay.

WNV NY99 NS2B-NS3 protease fusion (non-cleavable linker) K→A mutation to reduce autoproteolysis **addgene Catalog #**228660

Protocol

NAME

West Nile virus NS2B-NS3 protease fusion construct small scale expression and purification protocol

CREATED BY

korvus.wang

Preview

3 Dengue virus serotype 1 virus NS2B-NS3 fused protease expression and purification of the construct suitable for biochemical assay.

DENV-1 strain US/Hawaii/1944 NS2B-NS3 protease fusion with non cleavable linker **addgene Catalog #**228648

Protocol

NAME

Dengue virus serotype 1 NS2B-NS3 protease fusion construct small scale expression and purification protocol

CREATED BY

korvus.wang

Preview

4 Dengue virus serotype 2 virus NS2B-NS3 fused protease expression and purification of the construct suitable for biochemical assay.

DENV-2 Thailand/16681/84 NS2B-NS3 protease fusion (non-cleavable linker) **addgene Catalog #**228650

Protocol

NAME

Dengue virus serotype 2 NS2B-NS3 protease fusion construct small scale expression and purification protocol

CREATED BY

korvus.wang

Preview

5 Dengue virus serotype 3 virus NS2B-NS3 fused protease expression and purification of a construct suitable for biochemical assay.

DENV-3 Philippines/H87/1956 NS2B-NS3 protease fusion (non-cleavable linker) **addgene Catalog #**228651

Protocol

NAME

Dengue virus serotype 3 NS2B-NS3 protease fusion construct using parallel rapid protein expression and purification: 100 mL cultures

CREATED BY

korvus.wang

Preview

6 Dengue virus serotype 4 virus NS2B-NS3 fused protease expression and purification of a construct suitable for biochemical assay.

DENV-4 strain H241 NS2B-NS3 protease fusion (non-cleavable linker) **addgene Catalog #**228652

Protocol

NAME

Dengue virus serotype 4 NS2B-NS3 protease fusion construct: 100 mL cultures

CREATED BY
korvus.wang

Preview

Determine which Flavivirus is needed and prepare solutions

7 Prepare solutions based on the materials section.

	A	B	C	D	E
	Reagent	**Stock**	**Loaded into CERTUS**	**Final in assay plate**	**units**
	Choice of NS2B-NS3 Enzyme Protein				
	DENV NS2B-NS3	217000	200	100	nM
	ZIKV NS2B-NS3	225000	200	100	nM
	WNV NS2B-NS3	222000	200	100	nM
	Choice of Viral Substrate				
	WNV/ZIKV Substrate	10000	10	5	µM
	DENV Substrate	10000	10	5	µM
	Assay buffer				
	Tris pH=8.5	1000	20	20	mM
	Glycerol	50	10	10	%
	Triton	10	0.01	0.01	%

Prepare 384-well Plate for experiment

2h 31m

8 **PRIME** the dispenser with **Assay Buffer** by selecting the **PRIME** button until the tubes are filled completely.

8.1 **DISPENSE** 10 µL Assay Buffer to Columns **1** and **23** of assay plate

Note: These will represent the ***inhibitor control columns*** (Contain: Substrate + Assay Buffer + DMSO, no enzyme, no compounds)

8.2 **EMPTY** the dispenser tubes.

- Discard Assay Buffer discharged from the dispenser tubes.

9 **PRIME** the dispenser with [M] 200 nanomolar (nM) Flavivirus NS2B/NS3 by selecting the **PRIME** button until the tubes are filled completely.

9.1 **DISPENSE** 10 µL [M] 200 nanomolar (nM) Flavivirus NS2B/NS3 to columns **2 through 22** and column **24.**

Note:

- Be sure to cycle dispensing on an empty plate first, to detect and avoid dispensing issues.
- [M] 200 nanomolar (nM) Flavivirus NS2B/NS3 is two times the assay concentration. The final concentration of the Flavivirus NS2B/NS3 in the assay plate is [M] 100 nanomolar (nM) .
- Columns 2 and 24 are ***neutral control columns*** (Contain: Enzyme + substrate + DMSO, no compounds)

9.2 **EMPTY** the dispenser tubes .

- Discard the [M] 200 nanomolar (nM) Flavivirus NS2B/NS3 discharged from the tubes

10 **CENTRIFUGE** plate 1500 rpm, 00:01:00 to remove bubbles 1m

11 **INCUBATE** plate 02:00:00 at Room temperature 2h

⚠ Make sure the plate is protected from light!

During Incubation: wash and prepare the dispenser to the next step

12 **PRIME** the dispenser with 200 µL [M] 10 micromolar (µM) Flavivirus Substrate

- **Note:** Be sure to cycle dispensing on an empty plate first, to detect and avoid dispensing issues.

13 **DISPENSE** 10 µL of [M] 10 micromolar (µM) Flavivirus Substrate to **Columns 1-24** (the full plate).

- **Note:** [M] 10 micromolar (µM) Flavivirus Substrate is two times the assay concentration. The final concentration of the Flavivirus Substrate in the assay plate is [M] 5 micromolar (µM).

14 **CENTRIFUGE** plate 1500 rpm, Room temperature, 00:01:00 to remove bubbles 1m

15 **INCUBATE** plate for 00:30:00 at Room temperature 30m

⚠ Make sure the plate is protected from light!

Recommended: Clean the dispenser during incubation

Read Plate Fluorescence

16 **READ** and **RECORD** the plate Relative fluorescence units (RFU) via the "**Flavivirus protocol**" on the **PHERAstar FS Control Software.**

- Software is a standard Fluorescence Assay set for Optimal excitation wavelength 360 nm, emission wavelength 470 nm, and a Gain of 200.

Equipment

PHERAstar FS

Microplate reader

BMG LABTECH

0471B0001A

https://www.bmglabtech.com/en/pherastar-fsx/?utm_term=pherastar%20plate%20reader&utm_campaign=usa.roi.products&utm_source
FwOoFR_5EUHUaAIkREALw_wcB

Expected result

gain 300 should yield ~20,000 RFU in full reaction; ~7000 RFU in Buffer control