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We use this protocol and it's working

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Flaviviruses (West Nile, Zika, Dengue) NS2B/NS3 Fluorescence Dose Response V.2

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ASAP Discovery

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ABSTRACT

This is a **functional, biochemical assay** used to identify treatments for viral infectious diseases related to viral **Flaviviridae infection**, (specifically **West Nile, Zika, and Dengue**) and targets the conserved **NS2B/NS3 protein**.

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.

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

Experiment Concentrations (From Stock to Assay)

	A	B	C	D	E

	A	B	C	D	E
	Reagent	Stock	Concentration Loaded into GNF	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	D	E
	Reagent	Stock	Concentration Loaded into GNF	Final Concentration in Assay Plate	Units
	HEPES (pH 7.3)	20	10	10	mM
	NaCl	100	50	50	mM
	Glycerol	50	5	5	%
	Igepal	10	0.05	0.05	%
	TCEP	1000	1	1	mM

Compound Plate Design for Dose Response:

Total assay volume: 20 µL
Compounds top assay concentration: 60 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: 16 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] 20 millimolar (mM)

HEPES 1M Solution pH 7.3 **Fisher Scientific Catalog #AAJ16924K2**

2. [M] 100 millimolar (mM)
   Sodium Chloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S9888**
3. [M] 50 % volume
   Glycerol - for molecular biology, ≥99% **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G5516**
4. [M] 10 Mass Percent
   IGEPAL-CA630 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #I3021 SIGMA-ALDRICH**
5. [M] 1000 millimolar (mM)
   Tris(2-carboxyethyl)phosphine hydrochloride **Merck MilliporeSigma (Sigma-Aldrich) Catalog #75259**

*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/NS3was 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 in **DMSO** to create [M] 10 millimolar (mM) Substrate Stock Before each experiment, the Substrate Stock was diluted again to be [M] 10 micromolar (µM) Substrate before every experiment with freshly made **Assay Buffer**

Zika (ZIKV) Virus Reagents:

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

- WNV NS2B/NS3was 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 in **DMSO** to create [M] 10 millimolar (mM) Substrate Stock Before each experiment, the **Substrate Stock** was diluted again to be [M] 10 micromolar (µM) Substrate before every experiment with freshly made **Assay Buffer**

Dengue (DENV) Reagents:

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

- WNV 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) WNV Enzyme Substrate

- **Enzyme Substrate** was Bz-Nle-KRR-AMC (hydrochloride) **Cayman Chemical Company Catalog #27710**
- **Substrate Stock** was created by dissolving the substrate in **DMSO** to create [M] 10 millimolar (mM) Substrate Stock Before each experiment, the **Substrate Stock** was diluted again to be [M] 10 micromolar (µM) Substrate before every experiment with freshly made **Assay Buffer**

SAFETY WARNINGS

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

BEFORE START INSTRUCTIONS

Note: Inhibitor compounds stock concentration is 20 mM. Compounds are pre-dispensed into 384 plates and stored at -200˚C until use.

Determine which Flavivirus is needed and prepare solutions

1 Determine which Flavivirus is needed and prepare solutions based on the materials section.

	A	B	C	D	E

	A	B	C	D	E
	Reagent	**Stock**	**Loaded into GNF**	**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				
	HEPES pH=7.3	20	10	10	mM
	NaCl	100	50	50	mM
	Glycerol	50	5	5	%
	Igepal	10	0.05	0.05	%
	TCEP	1000	1	1	mM

Prepare 384-well Plate for experiment

2h 31m

2 **OPEN** the EQUIcon Software and **SELECT** the "**Flavivirus dispense 7,8 C**" Program

3 **PRIME** the GNF Washer/Dispenser II (GNF) with 3 mL Ehtanol and 3 mL Dionized Water

4 **CONFIRM** that the GNF had accurately dispensed Ethanol and Water

4.1 **WEIGH** the plate and **RECORD**

4.2 **DISPENSE** 3 mL Ehtanol and 3 mL Dionized Water into a plate

4.3 **WEIGH** the plate and **RECORD**. Determine if the GNF Washer/Dispenser II had accurately dispensed 3 g Dionized Water and 2.367 g Ethanol

5 **CONNECT Assay Buffer** to 7C and your **Flavivirus NS2B/NS3** to position 8C of the GNF Washer/Dispenser II.

5.1 **PRIME** the GNF with 300 µL Assay Buffer and with 300 µL [M] 200 nanomolar (nM) Flavivirus NS2B/NS3 respectively.

6 **DISPENSE** 10 µL Assay Buffer to columns **1 and 23** using the 7C position of the GNF

- **Note:** These columns will be the inhibitor control columns (Containing: substrate + assay buffer + DMSO, no compounds)

7 **DISPENSE** 10 µL [M] 200 nanomolar (nM) Flavivirus NS2B/NS3 to columns 2 through 22 and column 24 using the 8C position of the GNF.

- **Note:** [M] 200 nanomolar (nM) Flavivirus NS2B/NS3 is two times the assay concentration. The final concentration of the Flavivirus NS2B/NS3 is [M] 100 nanomolar (nM) during the assay.
- Columns 2 and 24 are ***neutral control columns*** (Contain: Enzyme + substrate + DMSO, no compounds)

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

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

⚠ Make sure the plate is protected from light!

During Incubation: PREPARE the GNF to dispense the Flavivirus Substrate

9.1 **EMPTY** 7C of the GNF.

9.2 WASH 7C tubing in **Assay Buffer.** Discard used Assay buffer

9.3 **PRIME** 7C of the GNF with 300 µL [M] 10 micromolar (µM) Flavivirus Substrate

10 **DISPENSE** 10 µL [M] 10 nanomolar (nM) Flavivirus Substrate to Columns 1 through 23 (the full plate)

- **Note:** [M] 10 nanomolar (nM) Flavivirus Substrate is two times the assay concentration. The final concentration of the Flavivirus Substrate is [M] 5 nanomolar (nM) during the assay.

11 **CENTRIFUGE** 1500 rpm, Room temperature, 00:01:00 plate to remove bubbles

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

⚠ Make sure the plate is protected from light!

Recommended: Clean GNF during incubation

Read Plate Flourescence

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

Expected result

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

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