Aug 04, 2025

Assay for screening of compounds that inhibit enzymatic activity of DENV-2 NS2B-NS3 protease

DOI
https://dx.doi.org/10.17504/protocols.io.kqdg32m87v25/v1

Kateryna Horbatok[1,2], Oleksandr Zhadovets[1,2]

[1]Enamine Ltd; [2]ASAP Discovery Consortium

ASAP Discovery

Nick Lynch
Curlew Research, ASAP Discovery Consortium

DOI: https://dx.doi.org/10.17504/protocols.io.kqdg32m87v25/v1

Protocol Citation: Kateryna Horbatok, Oleksandr Zhadovets 2025. Assay for screening of compounds that inhibit enzymatic activity of DENV-2 NS2B-NS3 protease. **protocols.io** https://dx.doi.org/10.17504/protocols.io.kqdg32m87v25/v1

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

Created: October 07, 2024

Last Modified: August 04, 2025

Protocol Integer ID: 109692

Keywords: Fluorescence (RFU), Robust Coefficient of Variation (RCV), Protease, DENV-2, enzymatic, NS2B-NS3 protease, inhibitors, enzymatic activity of the dengue virus protease, dengue virus protease, enzymatic assay, direct quantification of enzyme activity, quantification of enzyme activity, enzyme activity, enzymatic activity, cleavage by the enzyme, ns3 protease, enzyme, assay, screening of compound, ns3 protease this protocol, fluorescence detection

Funders Acknowledgements:
National Institutes of Health/National Institute Of Allergy and Infectious Diseases (NIH/NIAID)
Grant ID: U19AI171399

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 protocol describes the enzymatic assay used to identify compounds that inhibit enzymatic activity of the dengue virus protease (DENV-2 NS2B-NS3 protease). Enzymatic assay is based on the conversion of substrate into product, which is used for quantification of enzyme activity. In case of using fluorogenic substrates, cleavage by the enzyme releases a fluorescent fragment, enabling the direct quantification of enzyme activity via fluorescence detection. This assay is run as single point and dose response.

Guidelines

Introduction:

	A	B
	Target name	**DENV-2 NS2B-NS3**
	Project Aim	To identify compounds that inhibit enzymatic activity of the dengue virus protease DENV-2 NS2B-NS3.
	Assay Principle	Under standard conditions the enzyme cleaves its substrate (Bz-Nle-Lys-Lys-Arg-AMC) with the release of a fluorogenic moiety 7-AMC, that was quenched before. In the presence of compounds that inhibit activity of the enzyme, substrate is not cleaved which results in a low fluorescent signal.
	Assay readout	Fluorescence (RFU)

Summary of Assay Conditions:

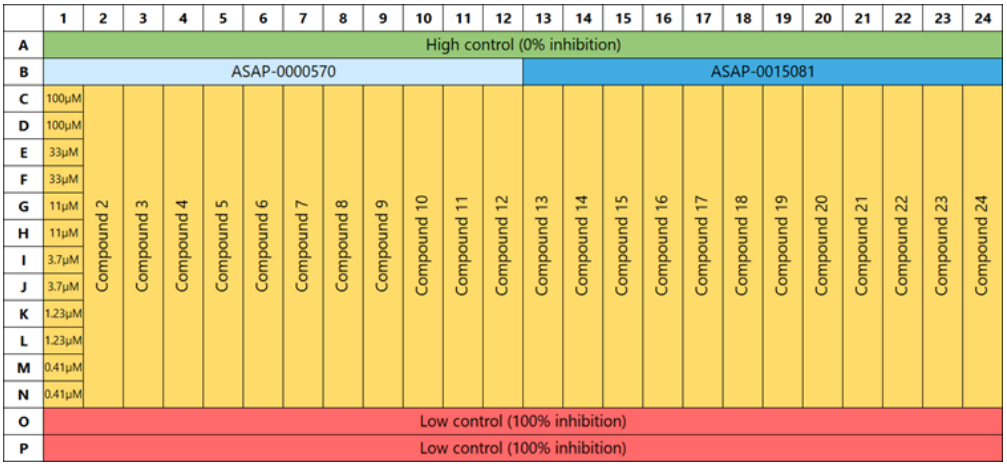

Figure 1. Plate loading for dose response format

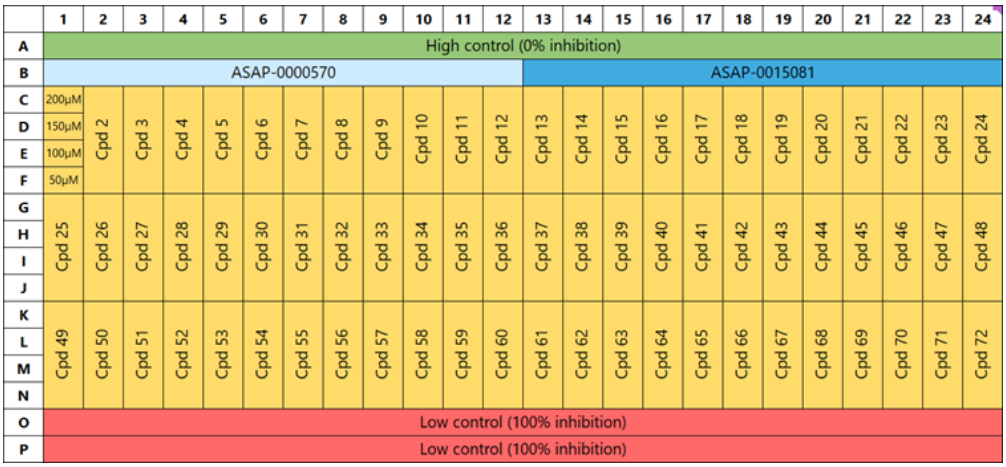

Figure 2. Plate loading for single point format

	A	B
	Description	**Reaction volume composition*, 10 µL (before 10 µL of stop-reaction reagent is added)**
	Negative control (100% of inhibition)	0.5% of DMSO, 5 µM of Substrate
	Positive control (0% of inhibition)	0.5% of DMSO, 25 nM of DENV-2, 5 µM of Substrate
	Reference 1 control	ASAP-0000570, 3-fold dilution, 25 – 0.1 µM, 6 points (n = 2), 25 nM of DENV-2, 5 µM of Substrate
	Reference 2 control	ASAP-0015081, 3-fold dilution, 25 – 0.1 µM, 6 points (n = 2), 25 nM of DENV-2, 5 µM of Substrate
	Compounds	Compounds in dose response format, 3-fold dilution, 100 – 0.4 µM, 6 points (n = 2), 25 nM of DENV-2, 5 µM of Substrate Compounds in single point format, at 4 concentrations 200, 150, 100, 50 µM (n = 1), 25 nM of DENV-2, 5 µM of Substrate

*The final concentrations of compounds, enzyme and substrate are calculated considering that enzymatic reaction volume is 10 µl, while total volume is 20 µl after the stop-reagent is added.

Materials

Tools/Equipment required:

	A	B	C
	№	**Tool Name**	**Tool Source**
	1.	Manual single channel pipettes Finnpipette 1-10 µL Proline® Plus 10-100 µL MLine® 20-200 µL IKA PETTE 100-1000 µL	Thermo Scientific Sartorius Sartorius IKA
	2.	Multichannel electronic pipettes (E1-ClipTip) 1-30 µL 2-125 µL	ThermoFisher
	3.	Multidrop Combi	ThermoFisher
	4.	DLAB Levo plus (for stripettes)	DLAB
	5.	Biomek Span-8	Beckman Coulter
	6.	Labcyte Echo 650	Beckman Coulter
	7.	SpectraMax Paradigm	Molecular Devices
	8.	ASSAB incubator	Assab
	9.	accuSpin 3 centrifuge	Fisher Scientific

Consumables:

	A	B	C	D
	№	**Disposable Name**	**Disposable Source**	**Catalogue Number**
	1.	384-well, round bottom, small volume, non-binding, black plate	Corning	4514
	2.	Multichannel Pipette tips (F1-ClipTip) 30 µL 125 µL	Thermo Scientific	94420103 94420153

	A	B	C	D
	3.	Stripettes 5 mL 10 mL 25 mL 50 mL	Thermo Scientific	11829660 11839660 11517752 11537752
	4.	15 mL Polypropylene falcon tubes	Sente-Lab	SL50352/SG
	5.	50 mL Polypropylene falcon tubes	Sente-Lab	SL65351/SG
	6.	Manual Pipette tips 30 µL 200 µL 1000 µL	Matrix Biosigma Sente-Lab	7611 597799 SL96153
	7.	Small tube metal tip dispensing cassette SN 837823692	Thermo Scientific	24073295
	8.	Silverseal Sealer, Aluminium	Greiner	676090
	9.	SealPlate film	Excel Scientific	Z369659-100EA
	10.	Axygen® Ultra-Clear, Pressure-Sensitive Sealing Film for Real-Time PCR	Corning	UC-500

384-well Low Volume Black Round Bottom Polystyrene NBS Microplate, 10 per Bag, without Lid, Nonsteri **Corning Catalog #**4514

ClipTip™ Filtered 384-Format Pipette Tips **Thermo Fisher Scientific Catalog #**94420103

ClipTip™ Filtered 384-Format Pipette Tips **Thermo Fisher Scientific Catalog #**94420153

Small tube metal tip dispensing cassette **Thermo Fisher Scientific Catalog #**24073295

SILVERSEAL SEALER, ALUMINIUM **greiner bio-one Catalog #**676090

Axygen® 70 µm Ultra Clear Pressure Sensitive Sealing Film for Real Time PCR, Nonsterile **Corning Catalog #**UC-500

Reagents:

	A	B	C	D	E	F
	№	**Reagent Name**	**Stock Concentration**	**Reagent Source**	**Catalogue No.**	**Storage Conditions**

	A	B	C	D	E	F
	1.	DENV-2 NS2B-NS3 (DENV-2)	217 µM	Client Supplied	-	-80°C
	2.	Bz-Nle-Lys-Lys-Arg-AMC (Substrate)	-	Cayman Chemicals	27710	-20°C
	3.	ASAP-0000570 (Reference 1)	20 mM	Client Supplied	-	-80°C
	4.	ASAP-0015081 (Reference 2)	20 mM	Client Supplied	-	-80°C
	5.	Tris hydrochloride (Tris-HCl)	-	Bio Basic	TB0103	RT
	6.	Glycerol	100%	ALLHIM	2905450000	RT
	7.	Triton	100%	Sigma-Aldrich	93443-100ML	RT
	8.	Dimethyl sulfoxide (DMSO)	100%	Honeywell	34869-2.5L	RT
	9.	Trisodium citrate dihydrate (Stop-reaction reagent)	-	Klebrig	1361407143	RT
	10.	Tween 20	100%	PanReac AppliChem	142312.2	RT

Bz-Nle-KRR-AMC (hydrochloride) **Cayman Chemical Company** **Catalog #**27710

Tris hydrochloride **Bio Basic Inc.** **Catalog #**TB0103.SIZE.250G

Triton X-100 Solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**93443-100ML

Dimethyl sulfoxide **Honeywell Fluka** **Catalog #**34869

Tween ® 20 **Panreac AppliChem** **Catalog #**142312.1611

Standard Reagent Formulations:

	A	B	C	D	E	F
	№	**Prepared Reagent Name**	**Component Name**	**Prepared Reagent Volume**	**Component Quantity**	**Storage**
	1.	1 M Tris-HCl (adjusted to pH 8.5)	Tris hydrochloride MQ H2O	10 mL	10 g 10 mL	4°C
	2.	10 mM Substrate	Bz-Nle-Lys-Lys-Arg-AMC	40×15 µL	5 mg 600 µL	-20°C

	A	B	C	D	E	F
			DMSO			
	3.	1 M Trisodium citrate dihydrate pH 4.0	Trisodium citrate dihydrate HCl MQ H2O	20 mL	5.88 g 3 mL 17 mL	RT
	4.	1% Tween 20	100% Tween 20 MQ H2O	2 L	20 mL 1.98 L	RT
	5.	10% Triton	100% Triton MQ H2O	10 ml	1 ml 9 ml	RT

Daily Reagent Formulations*:

	A	B	C	D	E	F
	№	Prepared Reagent Name	Components	Component Quantity	Final Concentration in solution	Prepared Reagent Volume
	1.	Assay Buffer	1 M Tris-HCl pH 8.5 100% Glycerol 10% Triton MQ H2O	800 µL 4 mL 40 µL 35.16 mL	20 mM 10% 0.01% -	40 mL
	2.	21.7 µM DENV-2	217 µM DENV-2 Assay Buffer	3 µL 27 µL	21.7 µM (Intermediate stock)	30 µL
	3.	50 nM DENV-2 (2x solution)	21.7 µM DENV-2 Assay Buffer	29 µL 12.557 mL	50 nM -	12.586 mL
	4.	10 µM Substrate (2x solution)	10 mM Substrate Assay Buffer	13.5 µL 13.486 mL	10 µM -	13.5 mL
	5.	500 mM Stop-reaction reagent (2x solution)	1 M Trisodium citrate dihydrate solution MQ H2O	11 mL 11 mL	500 mM -	22 mL
		*All calculations shown here are for a 4-plate HTS run. This consists of 4 assay plates with a dummy plate at the beginning of the dispense and dead volume of dispensing equipment.				

Safety warnings

Always wear appropriate PPE for this protocol
Refer to Material Safety Data Sheets for additional safety and handling information.

Assay plate preparation

1 **Plate loading for dose response format.**

- Prepare DMSO, tested compounds (a total of 24 compounds per plate) and reference compounds by Biomek Span-8 and printed onto assay-ready plates (assay plate — Corning #4514) using the Labcyte Echo 650.
- Reference controls are formatted in row B in the following order:
- **Reference compound 1** – B1-B12 – 50 nL of ASAP-0000570 in dose response format, 3-fold dilution, [M] 5 millimolar (mM) – [M] 0.02 millimolar (mM), 6 points (n = 2).
- **Reference compound 2** – B13-B24 – 50 nL of ASAP-0015081 in dose response format, 3-fold dilution, [M] 5 millimolar (mM) – [M] 0.02 millimolar (mM), 6 points (n = 2).
- **DMSO** is formatted in rows A, O and P – 50 nL in each well.
- **Compounds** are formatted in rows C-N – 50 nL in each well in dose response format, 3-fold dilution, [M] 20 millimolar (mM) – [M] 0.08 millimolar (mM), 6 points (n = 2).

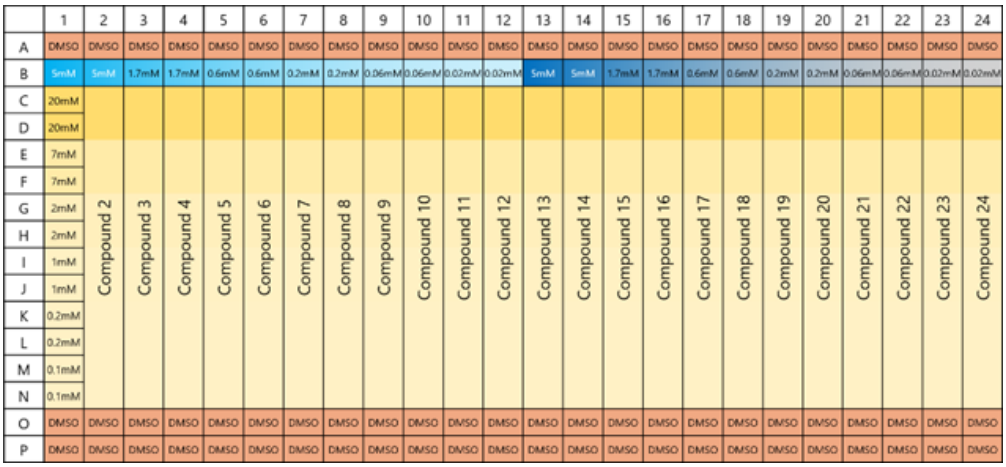

Figure 1. Plate map for dose-response scheme prepared by liquid handling department.

Protein expression and purification

2 The assays performed here used the following protein.

Assay plate preparation

3 **Plate loading for single point format.**

- Prepare DMSO, tested compounds (a total of 72 compounds per plate) and reference controls by Biomek Span-8 and printed onto assay-ready plates (assay plate — Corning #4514) using the Labcyte Echo 650.
- Reference controls are formatted in row B in the following order:
- **Reference compound 1** – B1-B12 – 50 nL of ASAP-0000570 in dose response format, 3-fold dilution, [M] 5 millimolar (mM) – [M] 0.02 millimolar (mM), 6 points (n = 2).
- **Reference compound 2** – B13-B24 – 50 nL of ASAP-0015081 in dose response format, 3-fold dilution, [M] 5 millimolar (mM) – [M] 0.02 millimolar (mM), 6 points (n = 2).
- **DMSO** is formatted in rows A, O and P – 50 nL in each well.

3.1 **Format the compounds in rows C-N in the following order:**

- Compounds 1-24 – C1-F24 – 50 nL in each well at 4 concentrations 40, 30, 20, 10 mM (n = 1).
- Compounds 25-48 – G1-J24 – 50 nL in each well at 4 concentrations 40, 30, 20, 10 mM (n = 1).
- Compounds 49-72 – K1-N24 – 50 nL in each well at 4 concentrations 40, 30, 20, 10 mM (n = 1).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
B	5mM	5mM	1.7mM	1.7mM	0.6mM	0.6mM	0.2mM	0.2mM	0.06mM	0.06mM	0.02mM	0.02mM	5mM	5mM	1.7mM	1.7mM	0.6mM	0.6mM	0.2mM	0.2mM	0.06mM	0.06mM	0.02mM	0.02mM
C	40mM	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6	Compound 7	Compound 8	Compound 9	Compound 10	Compound 11	Compound 12	Compound 13	Compound 14	Compound 15	Compound 16	Compound 17	Compound 18	Compound 19	Compound 20	Compound 21	Compound 22	Compound 23	Compound 24
D	30mM																							
E	20mM																							
F	10mM																							
G	Compound 25	Compound 26	Compound 27	Compound 28	Compound 29	Compound 30	Compound 31	Compound 32	Compound 33	Compound 34	Compound 35	Compound 36	Compound 37	Compound 38	Compound 39	Compound 40	Compound 41	Compound 42	Compound 43	Compound 44	Compound 45	Compound 46	Compound 47	Compound 48
H																								
I																								
J																								
K	Compound 49	Compound 50	Compound 51	Compound 52	Compound 53	Compound 54	Compound 55	Compound 56	Compound 57	Compound 58	Compound 59	Compound 60	Compound 61	Compound 62	Compound 63	Compound 64	Compound 65	Compound 66	Compound 67	Compound 68	Compound 69	Compound 70	Compound 71	Compound 72
L																								
M																								
N																								
O	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO
P	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO

Figure 2. Plate map for single point scheme prepared by liquid handling department.

4 Seal the plates with Silverseal Sealer, Aluminium (no heating is applied) and store at -20 °C. On the day of the assay, remove the assay plates from the -20 °C

freezer and left to warm up to Room temperature .

5 After liquid handling dispensing and before the start of assay procedures plates must be centrifuged at 1000 rpm, 00:01:00 . 1m

6 Further assay procedures are identical for both screening formats: dose-response and single point, and summarized in "Assay performance" section.

Assay performance 2h 43m

7 **Multidrop Combi settings:**

- Plate: 384 standard (15 mm)
- Cassette type: Small tube dispensing cassette
- Dispense volume: 5 µL / 10 µL
- Predispense volume: 50 µL
- Dispense speed: medium
- Dispense direction: by rows

> Note
>
> Multidrop Combi small tube dispensing cassette should be checked before screening procedures using gravimetric accuracy verification and photometric precision verification, in accordance with the Thermo Scientific Multidrop Combi User Manual. Multidrop Combi cassette must have dispensing accuracy ± 5% & RCV ≤ 3% for each channel, and no obvious dispensing patterns.

8 **Multidrop Combi dispense #1.** 2h 5m

- Fill the tubes with assay buffer, preincubate for 00:02:00 , then empty the tubes.
- Fill tubes A-G with DENV-2 2x solution and tube H with assay buffer, prime ~ 50 µL , preincubate the solutions in the tubes for 00:02:00 . Then empty the tubes and mix the solution in the falcon gently.
- Fill the tubes with the working solution another time, prime ~ 50 µL . Then dispense 5 µL to half of the dummy plate, followed by the assay plates (fig 3.).
- Seal the plates with SealPlate film and centrifuge at 1000 rpm, 00:01:00 and then left to incubate for 02:00:00 at 25 °C in the incubator, while Multidrop Combi is flushed through with 1% Tween 20 and MQ H2O before the next dispense.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
B	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
C	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
D	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
E	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
F	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
G	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
H	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
I	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
J	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
K	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
L	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
M	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
N	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
O	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
P	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL

Figure 3. Multidrop Combi dispense #1. Rows A-N are dispensed with 5 µL of 50 nM DENV-2, Rows O-P are dispensed with 5 µL of assay buffer.

9 **Multidrop Combi dispense #2.**

35m

- Fill the tubes with assay buffer, preincubate for 00:02:00 then empty the tubes.
- Fill the tubes with Substrate 2x solution, prime ~ 50 µL, preincubate the Substrate in the tubes for 00:02:00. Then empty the tubes and mix the solution in the falcon gently.
- Fill the tubes with the working solution another time, prime ~ 50 µL. Then dispense 5 µL to half of the dummy plate, followed by the assay plates (fig 4.).
- Seal the plates with SealPlate film and centrifuge at 1000 rpm, 00:01:00 and then left to incubate for 00:30:00 at 25 °C in the incubator in the dark, while Multidrop Combi is flushed through with 1% Tween 20 and MQ H2O before the next dispense.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
B	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
C	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
D	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
E	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
F	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
G	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
H	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
I	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
J	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
K	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
L	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
M	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
N	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
O	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL
P	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL	5uL

Figure 4. Multidrop Combi dispense #2. Full plate is dispensed with 5 µL of Substrate.

10 **Multidrop Combi dispense #3.**

3m

- Fill the tubes with Stop-reaction reagent 2x solution, prime ~ 50 µL , preincubate the solution in the tubes for 00:02:00 . Then empty the tubes and mix gently the solution in the falcon.
- Fill the tubes with the working solution another time, prime ~ 50 µL . Then dispense 10 µL to half of the dummy plate, followed by the assay plates (fig 5.).
- Seal the plates with Axygen films and centrifuge at 1000 rpm, 00:01:00 , while Multidrop Combi is flushed through with 1% Tween 20 and MQ H2O before shutdown.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL
B	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL
C	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL
D	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL
E	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL
F	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL
G	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL
H	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL
I	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL
J	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL
K	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL
L	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL
M	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL
N	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL
O	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL
P	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL	10uL

Figure 5. Multidrop Combi dispense #3. Full plate is dispensed with 10 µL of 500 mM Stop-reaction reagent.

Read the plates using SpectraMax Paradigm.

11 **Reader settings:**

- Mode: FL
- Ex./Em.: 360/460
- Integration Time: 70 ms
- Read Order: by columns
- Read Temperature: Room temperature

Data analysis

12 Export the raw data in .xls files as RFU signals (in plate format).

13 The data is analyzed using specifically designed scripts in Python.

14 Parameters for QC:

- Robust Signal to Background (RS/B) = median of high control (0% of inhibition) / median of low control (100% inhibition).
- Plate Robust Z′-factor (RZ′) = 1 – (3*(RSD of high control (0% of inhibition) + RSD of low control (100% inhibition))/(median of high control (0% of inhibition) – median of low control (100% inhibition)).
- Robust Coefficient of Variation (RCV) = RSD of data / median of data*100%.

Note

NB: Plate must pass criteria:
RZ’ ≥ 0.5
RS/B ≥ 2
RCV < 20%
No obvious liquid handling-related patterns

15 Report the compound response as % inhibition based on 0% and 100% inhibition controls:

- Compound response (% inhibition by median) = (median of high control (0% of inhibition) - data point median)/(median of high control (0% of inhibition) – median of negative control (100% of inhibition)) * 100.

Acknowledgements

Grateful for Haim Barr and Noa Lahav for sharing their protocol developed at Weizmann Institute of Science as part of ASAP Discovery Consortium