Aug 04, 2025

# Assay for screening of compounds that inhibit enzymatic activity of ZIKV NS2B-NS3 protease

DOI
https://dx.doi.org/10.17504/protocols.io.36wgqn273gk5/v1

Oleksandr Zhadovets[1,2], Maksym Pavliuk[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.36wgqn273gk5/v1

**Protocol Citation:** Oleksandr Zhadovets, Maksym Pavliuk 2025. Assay for screening of compounds that inhibit enzymatic activity of ZIKV NS2B-NS3 protease. **protocols.io** https://dx.doi.org/10.17504/protocols.io.36wgqn273gk5/v1

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

**Created:** October 14, 2024

**Last Modified:** August 04, 2025

**Protocol Integer ID:** 110118

**Keywords:** Inhibitors, Fluorescence (RFU), Gravimetric accuracy, ZIKV, NS2B-NS3 protease, Robust Coefficient of Variation (RCV), enzymatic activity of the dengue virus protease, dengue virus protease, enzymatic activity of zikv ns2b, enzymatic assay, direct quantification of enzyme activity, quantification of enzyme activity, enzyme activity, cleavage by the enzyme, enzyme, ns3 protease, enzymatic activity, dengue, screening of compound, ns3 protease this protocol, fluorescence detection, zikv ns2b, fluorogenic substrate

**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 (ZIKV 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.

## Guidelines

**Introduction:**

| | A | B |
|---|---|---|
| | **Target name** | **ZIKV NS2B-NS3** |
| | Project Aim | To identify the compounds that inhibit enzymatic activity of the ZIKV NS2B-NS3 protease |
| | Assay Principle | 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. In this assay ZIKV NS2B/NS3 cleaves its substrate (Boc-Gly-Arg-Arg-AMC) with the release of a fluorogenic moiety 7-AMC. |
| | Assay readout | Fluorescence (RFU) |

**Summary of Assay Conditions:**

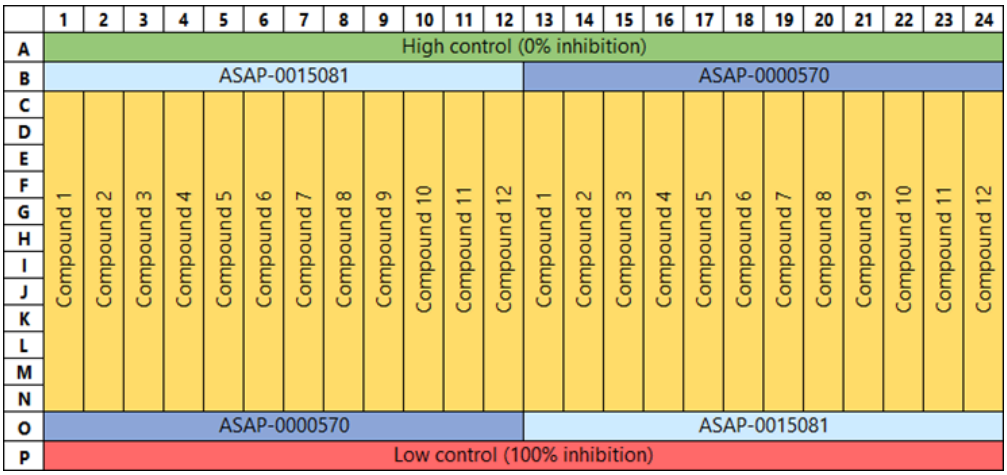


**Figure 1.** Plate loading for dose response format

| | 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, 50 nM of ZIKV NS2B/NS3, 5 µM of Substrate |

| | A | B |
|---|---|---|
| | Reference 1 control | ASAP-0015081, 2-fold dilution, 50-0.02 µM, 12 points (n=2), 50 nM of ZIKV NS2B/NS3, 5 µM of Substrate |
| | Reference 2 control | ASAP-0000570, 2-fold dilution, 100-0.05 µM, 12 points (n=2), 50 nM of ZIKV NS2B/NS3, 5 µM of Substrate |
| | Compounds | Compounds in dose response format, 2-fold dilution, 25 – 0.01 µM, 12 points (n = 2), 50 nM of ZIKV NS2B/NS3, 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-reaction reagent is added.

## Materials

**Tools/Equipment required:**

| | A | B | C |
|---|---|---|---|
| | **№** | **Tool Name** | **Tool Source** |
| | 1. | Manual single channel pipettes<br>Finnpipette 1-10 µL<br>Proline® Plus 10-100 µL<br>MLine® 20-200 µL<br>IKA PETTE 100-1000 µL | <br>Thermo Scientific<br>Sartorius<br>Sartorius<br>IKA |
| | 2. | Multichannel electronic pipettes (E1-ClipTip)<br>1-30 µL<br>2-125 µL | ThermoFisher |
| | 3. | Multidrop Combi nL | 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)<br>30 µL<br>125 µL | Thermo Scientific | <br>94420103<br>94420153 |
| | 3. | Stripettes<br>5 mL | Thermo Scientific | 11829660 |

| | A | B | C | D |
|---|---|---|---|---|
| | | 10 mL<br>25 mL<br>50 mL | | 11839660<br>11517752<br>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<br>30 µL<br>200 µL<br>1000 µL | <br>Matrix<br>Biosigma<br>Sente-Lab | <br>7611<br>597799<br>SL96153 |
| | 7. | Small tube metal tip dispensing cassette<br>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 |
| | 11. | Minisart® NY25 Syringe Filter, 0.2 µm Polyamide (Nylon) | Sartorius | 17845-Q |

- 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
- Minisart® NY25 Syringe Filter, 0.2 µm Polyamide (Nylon) **Sartorius** **Catalog #**17845-Q

**Reagents:**

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | **№** | **Reagent Name** | **Stock Concentration** | **Reagent Source** | **Catalogue No.** | **Storage Conditions** |
| | 1. | ZIKV NS2B/NS3 (ZIKV) | 225 µM | Client Supplied | - | -80°C |
| | 2. | Boc-Gly-Arg-Arg-AMC (Substrate) | - | Biosynth | FB110553 | -20°C |
| | 3. | ASAP-0015081 (Reference 1) | 20 mM | Client Supplied | - | -80°C |
| | 4. | ASAP-0000570 (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. | Sodium acetate (Stop-reaction reagent) | - | Klebrig | 127-09-3 | RT |
| | 10. | Sodium dodecyl sulfate (SDS) | - | Biotech | 151-21-3 | RT |

Boc-Gly-Arg-Arg-AMC acetate salt **Biosynth Catalog #**FB110553

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

**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<br>MQ H2O | 15 mL | 15 g<br>15 mL | 4°C |
| | 2. | 10 mM Substrate | Boc-Gly-Arg-Arg-AMC DMSO | 15×10 µL<br>15×15 µL<br>20×20 µL | 5 mg<br>775 µL | -20°C |

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | 3. | 20% Sodium acetate (adjusted to pH 4.0) | Sodium acetate<br>MQ H2O | 40 mL | 8 g<br>32 mL | RT |
| | 4. | 10% Triton | 100% Triton<br>MQ H2O | 10 ml | 1 ml<br>9 ml | RT |
| | 5. | 1% SDS | Sodium dodecyl sulfate MQ H2O | 2 L | 20 g<br>1.98 L | RT |

**Daily Reagent Formulations*:**

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | **№** | **Prepared Reagent Name** | **Components** | **Component Quantity** | **Concentration in solution** | **Prepared Reagent Volume** |
| | 1. | Assay Buffer | 1 M Tris-HCl pH 8.5<br>100% Glycerol<br>10% Triton<br>MQ H2O | 1.2 mL<br>6 mL<br>60 µL<br>52.74 mL | 20 mM<br>10%<br>0.01%<br>- | 60 mL |
| | 2. | 22.5 µM ZIKV | 225 µM ZIKV<br>Assay Buffer | 6 µL<br>54 µL | 22.5 µM (Intermediate stock) | 60 µL |
| | 3. | 100 nM ZIKV (2x solution) | 22.5 µM ZIKV<br>Assay Buffer | 55 µL<br>12.320 mL | 100 nM<br>- | 12.375 mL |
| | 4. | 10 µM Substrate (2x solution) | 10 mM Substrate<br>Assay Buffer | 12.5 µL<br>12.488 mL | 10 µM<br>- | 12.5 mL |
| | | | | | | |
| | 5. | 10% Stop-reaction reagent (2x solution)<br>MQ H2O | 20% Sodium acetate solution | 11 mL<br>11 mL | 10%<br>- | 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.

## Protein expression and purification

1

## Assay plate preparation

2 **Plate loading for dose response format**

- Prepare DMSO, tested compounds (a total of 12 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 and O in the following order:
- **Reference control 1** – B1-B12, O13-O24 – 50 nL of ASAP-0015081 in dose response format, 2-fold dilution, 10-0.005 mM, 12 points (n=2).
- **Reference control 2** – B13-B24, O1-O12 – 50 nL of ASAP-0000570 in dose response format, 2-fold dilution, 20-0.01 mM, 12 points (n=2).
- **DMSO** is formatted in rows A and P – 50 nL in each well.
- **Compounds** are formatted in rows C-N – 50 nL in each well in dose response format, 2-fold dilution, 5-0.0025 mM, 12 points (n = 2).

| | 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 | 10 mM | 5 mM | 2.5 mM | 1.25 mM | 0.6 mM | 0.3 mM | 0.16 mM | 0.08 mM | 0.04 mM | 0.02 mM | 0.01 mM | 0.005 mM | 20 mM | 10 mM | 5 mM | 2.5 mM | 1.25 mM | 0.6 mM | 0.3 mM | 0.16 mM | 0.08 mM | 0.04 mM | 0.02 mM | 0.01 mM |
| C | 5 mM | Compound 2 | Compound 3 | Compound 4 | Compound 5 | Compound 6 | Compound 7 | Compound 8 | Compound 9 | Compound 10 | Compound 11 | Compound 12 | Compound 1 | Compound 2 | Compound 3 | Compound 4 | Compound 5 | Compound 6 | Compound 7 | Compound 8 | Compound 9 | Compound 10 | Compound 11 | Compound 12 |
| D | 2.5 mM | | | | | | | | | | | | | | | | | | | | | | | |
| E | 1.25 mM | | | | | | | | | | | | | | | | | | | | | | | |
| F | 0.6 mM | | | | | | | | | | | | | | | | | | | | | | | |
| G | 0.3 mM | | | | | | | | | | | | | | | | | | | | | | | |
| H | 0.16 mM | | | | | | | | | | | | | | | | | | | | | | | |
| I | 0.08 mM | | | | | | | | | | | | | | | | | | | | | | | |
| J | 0.04 mM | | | | | | | | | | | | | | | | | | | | | | | |
| K | 0.02 mM | | | | | | | | | | | | | | | | | | | | | | | |
| L | 0.01 mM | | | | | | | | | | | | | | | | | | | | | | | |
| M | 0.005 mM | | | | | | | | | | | | | | | | | | | | | | | |
| N | 0.0025 mM | | | | | | | | | | | | | | | | | | | | | | | |
| O | 20 mM | 10 mM | 5 mM | 2.5 mM | 1.25 mM | 0.6 mM | 0.3 mM | 0.16 mM | 0.08 mM | 0.04 mM | 0.02 mM | 0.01 mM | 10 mM | 5 mM | 2.5 mM | 1.25 mM | 0.6 mM | 0.3 mM | 0.16 mM | 0.08 mM | 0.04 mM | 0.02 mM | 0.01 mM | 0.005 mM |
| 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 1.** Plate map for dose-response scheme prepared by liquid handling department.

3 Seal the plates with Silverseal Sealer, Aluminium (no heating is applied) and store at -20 °C. On the day of the assay, remove assay plates from the -20°C freezer and left to warm up to Room temperature.

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

## Assay performance 2h 49m

5 **Multidrop Combi nL settings:**

- Plate: 384 Corning lvol rnd
- Dispense volume: 5 µL / 10 µL
- Predispense volume: 50 µL
- Dispense direction: by columns
- Speed: 3 (max is 5)
- Correction factor: 1.031

Note

Multidrop Combi nL should be checked before screening procedures using gravimetric accuracy verification and photometric precision verification, in accordance with the Thermo Scientific Multidrop Combi nL User Manual. Multidrop Combi nL must have dispensing accuracy ± 2% & RCV ≤ 4% for each channel, and no obvious dispensing patterns.

6 **Multidrop Combi nL dispense #1.** 5m

- Fill the tubes with freshly prepared filtered assay buffer, prime ~ 50 µL, preincubate for 00:05:00. Dispense 5 µL of assay buffer to half of the dummy plate, followed by the assay plates (fig 2.).
- Cover the plates with plate lids and left at the bench before next dispense.

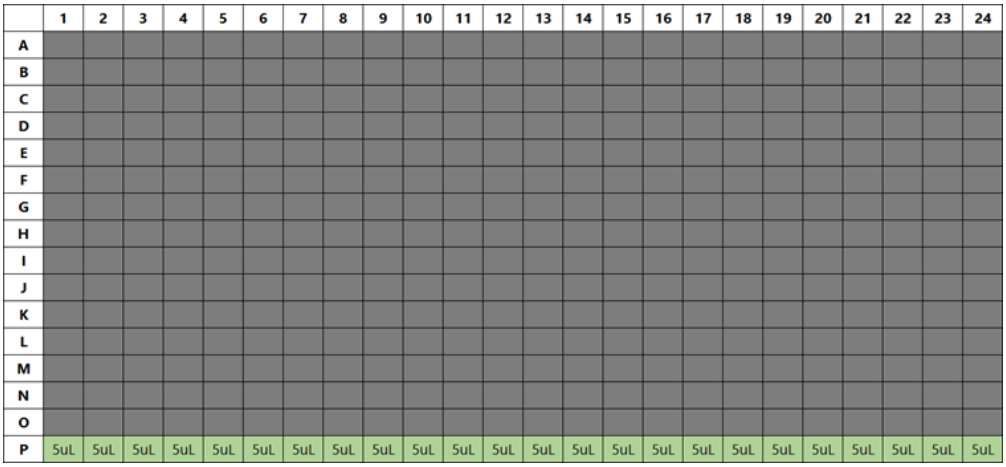


**Figure 2.** Multidrop Combi nL dispense #1. Row P is dispensed with 5 µL of assay buffer.

7 **Multidrop Combi nL dispense #2.**

2h 3m

- Immediately after the first dispense empty the tubes, filled with 100 nM ZIKV (2x solution) and primed ~ 50 µL .
- Preincubate the solution in the tubes for 00:02:00 . Dispense 5 µL to half of the dummy plate, followed by the assay plates (fig 3.).
- Seal the plates with SealPlate film and centrifuged at 1000 rpm, 00:01:00 and then left to preincubate for 02:00:00 at 25 °C in the incubator, while Multidrop Combi nL is flushed through with 45 mL of 1% SDS and 90 mL of MQ $H_2O$ before the next dispense.

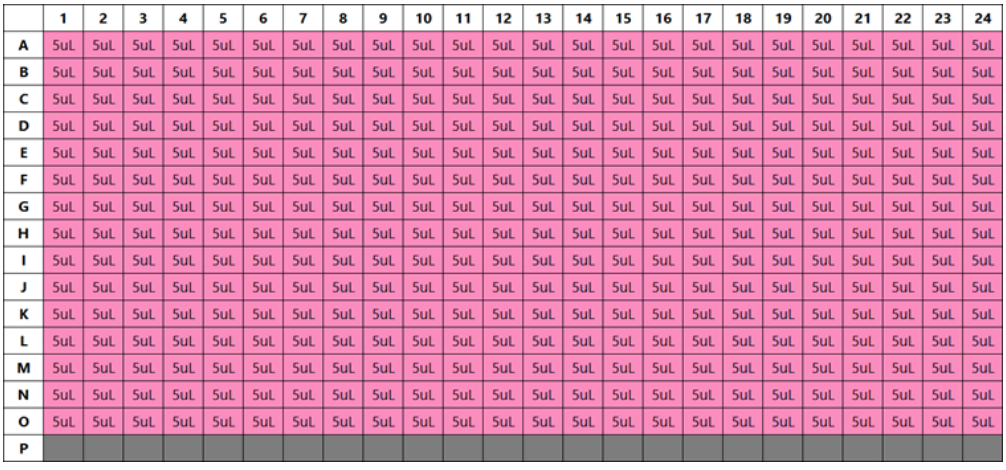


**Figure 3.** Multidrop Combi nL dispense #2. Rows A-O are dispensed with 5 µL of 100 nM ZIKV (2x solution).

8 **Multidrop Combi nL dispense #3.**

38m

- Fill the tubes with assay buffer, preincubate for 00:05:00, then empty the tubes.
- Fill the tubes with [M] 10 micromolar (µM) Substrate (2x solution), prime ~ 50 µL, preincubate the Substrate in the tubes for 00:02:00. Dispense 5 µL to half of the dummy plate, followed by the assay plates (fig 4.).
- Seal the plates with SealPlate film and centrifuged at 1000 rpm, 00:01:00 and then left to incubate for 00:30:00 at 25 °C in the incubator (protected from light), while Multidrop Combi nL is flushed through with 45 mL of 1% SDS and 90 mL of MQ $H_2O$ 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 nL dispense #3. Full plate is dispensed with 5 µL of 10 µM Substrate (2x solution)

9 **Multidrop Combi nL dispense #4.**

3m

- Fill the tubes with 10% Stop-reaction reagent (2x solution), prime ~ 50 µL, preincubate the solution in the tubes for 00:02:00. Dispense 10 µL to half of the dummy plate, followed by the 4 assay plates (fig 5.).
- Seal the plates with Axygen films and centrifuged at 1000 rpm, 00:01:00, while Multidrop Combi nL is flushed through with 45 mL of 1% SDS and 90 mL of MQ $H_2O$.

| | 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 nL dispense #4. Full plate is dispensed with 10 µL of 10% Stop-reaction reagent (2x solution).

Read the plates using SpectraMax Paradigm.

10 **Reader settings:**

- Mode: FL
- Ex./Em.: 360/460
- Integration Time: 140 ms
- Read Order: by columns
- Read Temperature: Room temperature

## Data analysis

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

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

13 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

14 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%.

15 The files with raw and analyzed data are uploaded to CDD Vault Protocol (ZIKV-NS2B-NS3_fluorescence-dose-response_bienta) for further analysis.

## Protocol references

http://dx.doi.org/10.17504/protocols.io.q26g7yebkgwz/v3

## Acknowledgements

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