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Zika NS2B-3-4A Protein Construct cleavage pattern assay

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Adeeba Dhalech[1,2]

[1]Stanford University; [2]ASAP Discovery Consortium

ASAP Discovery

Nick Lynch
Curlew Research, ASAP Discovery Consortium

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

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Disclaimer

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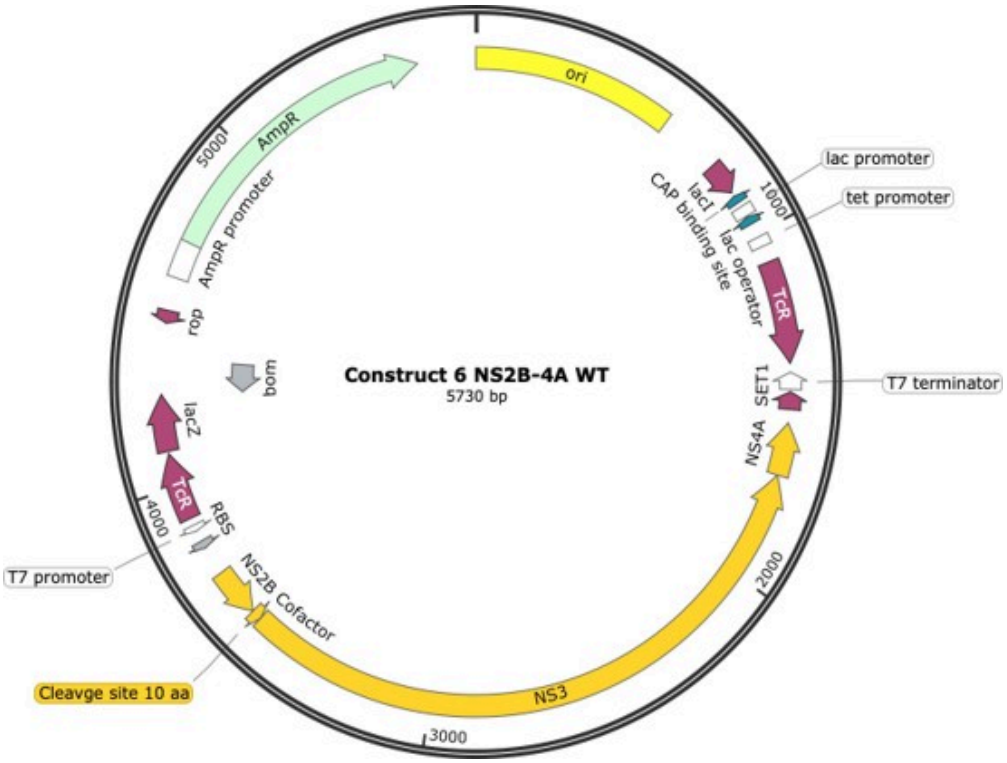
Abstract

Cis cleavage pattern by viral protease is analyzed using an expression plasmid pT7CFE1-NHA-Chis-target of interest. For flaviviruses (ZIka/DENV) the plasmid has been designed to include the cofactor domain of NS2B, NS2B-3 cleavage site, protease and helicase domains of NS3, and first 49 residues of NS4A to include the NS3-4A cleavage site (1). The plasmid lacks transmembrane domains. For enteroviruses (EVD68/EVA71), the plasmid includes the cleavage of structural protein VP1 from VP12A by the enteroviral 2A protease (2). Protein synthesis is carried out using a Rabbit reticulocyte lysate (TNT® Quick Coupled Transcription/Translation Systems; Promega) and 1 µg/50 µL of plasmid template via a coupled transcription/translation system in presence of S-35 labeled L-Methionine (EasyTag; PerkinElmer) (3) and output is quantified using SDS gels.



Zika plasmid used for IVT assays

1. D.A. Constant, R. Mateo, C.M. Nagamine, & K. Kirkegaard, Targeting intramolecular proteinase NS2B/3 cleavages for trans-dominant inhibition of dengue virus, Proc. Natl. Acad. Sci. U.S.A. 115 (40) 10136-10141, https://doi.org/10.1073/pnas.1805195115 (2018).
2. Doherty JS, Kirkegaard K. 2023. Differential inhibition of intra- and inter-molecular protease cleavages by antiviral compounds. J Virol 97:e00928-23.https://doi.org/10.1128/jvi.00928-23
3. https://www.promega.com/products/protein-expression/cell-free-protein-expression/tnt-quick-coupled-transcription_translation-system/?catNum=L1170#protocols

Attachments

ASAP-0035345-001.png
89KB

ASAP-0035345-001plot...
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Guidelines

ASAP Compounds are diluted in DMSO and adjusted to obtain the desired final concentration in the assay.

	Compound	Stock Conc (0.5 uL)	Final Conc (in 10 uL)
	ASAP-00XXXXX	200 nM	10 nM
	600 nM	30 nM	
	2 uM	100 nM	
	6 uM	300 nM	
	20 uM	1 uM	
	60 uM	3 uM	
	200 uM	10 uM	
	600 uM	30 uM	
	2 mM	100 uM	

Materials

TNT T7 Quick Master Mix, [35S]methionine (1,000 Ci/mmol at 10 mCi/ml), Plasmid DNA Template (0.5 µg)/Controls, PCR Enhancer, Nuclease-Free Water, Compound Stock Conc (0.5 µL), Final Conc (in 10 µL)

Safety warnings

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

Assay Prep

1 Prepare Master Mix for the number of reactions based on Assay Reagents.

Assay Plate Setup

2 Have 0.5 ml tubes/96 well plates ready for each drug and desired concentration. Add equal amount of DMSO to control tubes.

3

	Assay Reagent	Standard Reaction (uL)	Scaled Down Reaction (uL)
	TNT T7 Quick Master Mix	25	8
	[35S]methionine (1,000Ci/mmol at 10mCi/ml)	2	0.4
	Plasmid DNA Template (0.5µg)/Controls	2	0.4
	PCR Enhancer	1	0.2
	Nuclease-Free Water to a final volume of	47.5	9.5

Assay Setup

4 Aliquot 9.5 µL of the Master mix to each tube and pipet gently to mix.

5 Incubate the reaction

For enteroviruses (EVD68 and EVA 71) we run the assay for 30 mins at 30 °C.

For flaviviruses (Zika and Dengue), we run it for 60 mins at 30 °C.

6 Remove from heat and add 10 µL (or equal volume) of 2X Sample buffer to each tube to stop the reaction.

7 Heat at 65°C for 5 mins.

8 Run the samples on 12 or 15% SDS Page gel.

8.1 30 mins at 70 volts followed by 100 V until the dye front reaches the bottom of the gel.

9 Remove gels from cassette and transfer to blotting paper.

10 Cover with Serum wrap and expose the gel overnight on phosphor screen.

11

Template	ASAP Compound								
DNA Template	DMSO	0.01	0.03	0.1	0.3	1	3	10	
Catalytically Inactive Protease	DMSO	0.01	0.03	0.1	0.3	1	3	10	
TNT Assay Control	DMSO								
NTC	DMSO								

In the table, for zika/dengue we use pT7NS2B-3-4A DNA template, and study 3 to 4 cleavage patterns. NS3 S135A is the catalytically inactive protease. For EVD68 and EVA71, we use pT7VP1-2A as the enterovirus template and focus on one cleavage between Vp1 and 2A. C107R is the EVD68 catalytically inactive protease and C110A mutation makes the EVA71 protease catalytically inactive.

Results

12 Read the exposed gel using a Typhoon Biomolecular Imager (or any machine that reads autoradiography). We use the Typhoon FLA 9500 Biomolecular Imager by GE and read gels at 200 and 50 uM.

Analysis

13 Gel analysis is carried using LI-COR Studio to optimize visualization of protein bands.

14 Quantify band intensity using ImageJ. This is the protocol we follow to quantify the bands:

14.1 1)Open the file in ImageJ as16-bit

2)Each lane is individually boxed to encompass the entire lane using the rectangle tool.

3)Plot lanes based on rectangular selection using Gel Analyser.

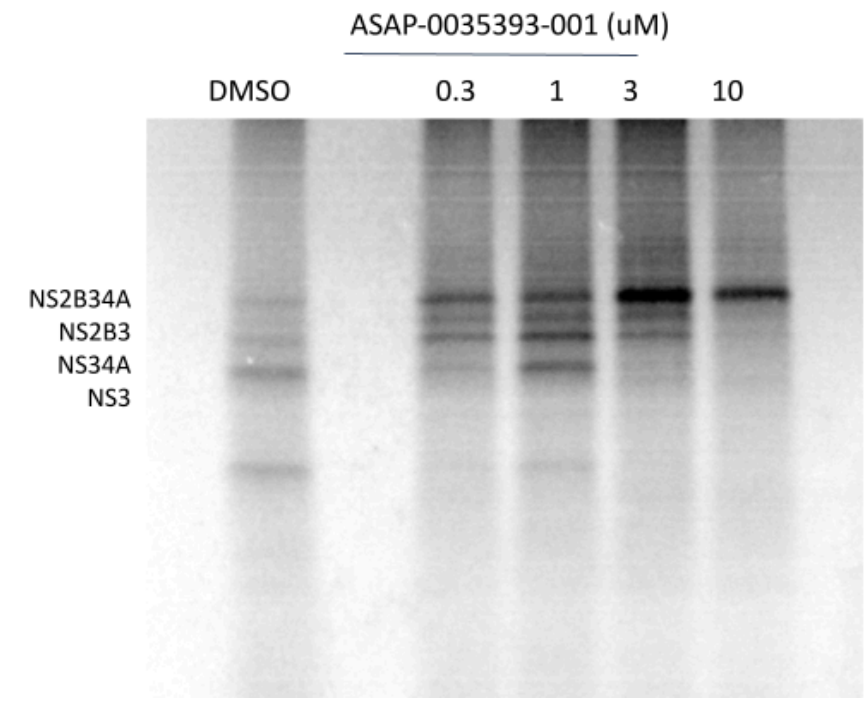
4)Normalize the peak density of each band against background.

5)Record the area of peak intensity of each band using the wand tool.

6)Plot data depending on output on Prism.

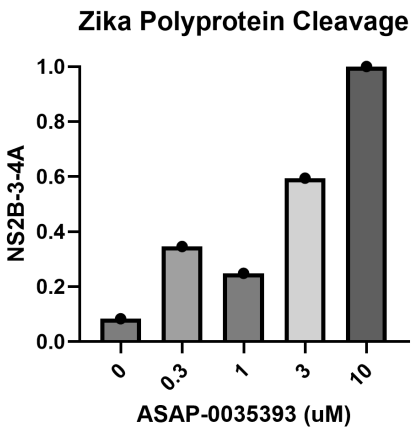
15 Plot data on Graphpad Prism.

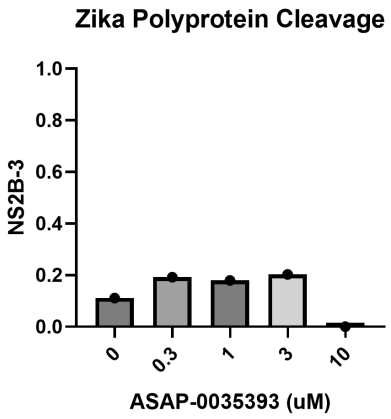
15.1

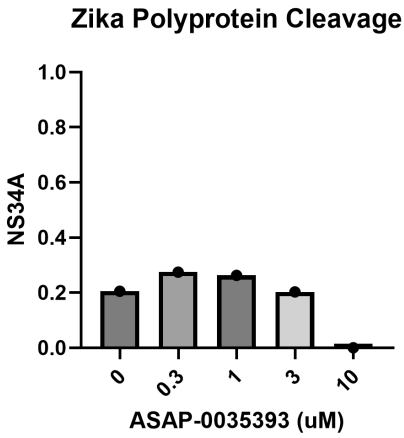


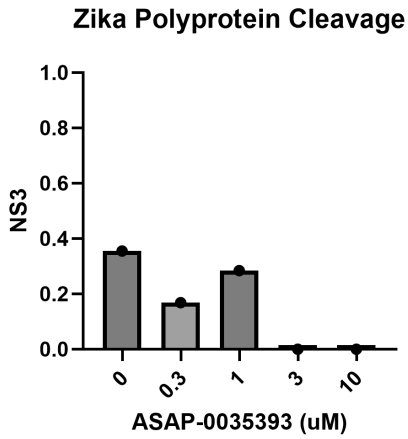
Gel Output

15.2







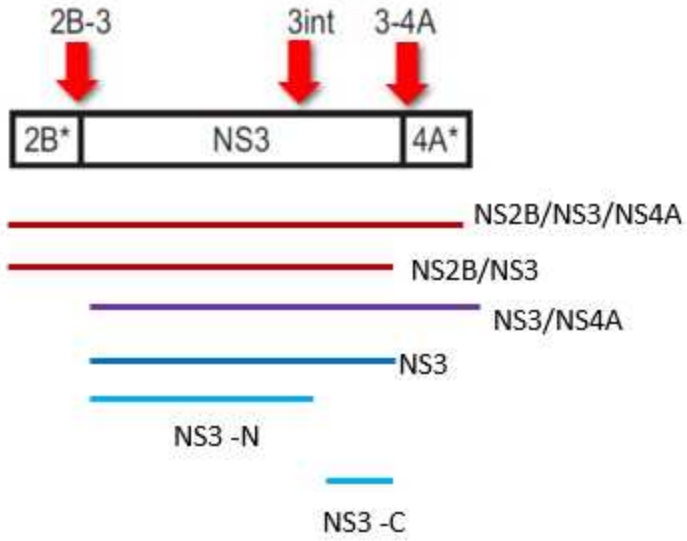


Data from above Gel

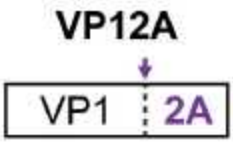
Template showing cleavage patterns

16

16.1 Flavivirus (Dengue/Zika) NS3 Protease cleavage and products.



16.2 Enterovirus (EVA-71/EVD-68) 2A Protease cleavage and products.



Protocol references

1. D.A. Constant, R. Mateo, C.M. Nagamine, & K. Kirkegaard, Targeting intramolecular proteinase NS2B/3 cleavages for trans-dominant inhibition of dengue virus, Proc. Natl. Acad. Sci. U.S.A. 115 (40) 10136-10141, https://doi.org/10.1073/pnas.1805195115 (2018).
2. Doherty JS, Kirkegaard K. 2023. Differential inhibition of intra- and inter-molecular protease cleavages by antiviral compounds. J Virol 97:e00928-23.https://doi.org/10.1128/jvi.00928-23
3. https://www.promega.com/products/protein-expression/cell-free-protein-expression/tnt-quick-coupled-transcription_translation-system/?catNum=L1170#protocols