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Dengue virus serotype 2 NS2B-NS3 protease fusion construct small scale expression and purification for biophysical and biochemical assays protocol V.2

Version 1 is forked from DENV2 NS2B-NS3 protease co-expression construct small scale expression and purification protocol

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Korvus Wang[1,2], Michael Fairhead[1,2], Eleanor Williams[1,2]

[1]Centre for Medicines Discovery, University of Oxford; [2]ASAP Discovery Consortium

ASAP Discovery

korvus.wang
CMD

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

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Abstract

This protocol details the co-expression and purification of Dengue virus serotype 2 NS2B-NS3 protease fusion construct bearing a N-terminal His-StrepII tag at small scale (<6L). The final product does not have its affinity tags removed, and is catalytically active. Final product is suitable for biophysical and biochemical assays. In the current version we added the Addgene id for the plasmid used and corrected the affiliation.

Attachments

PAGE24-01547 - Purif...
322KB

PAGE24-01546 - Expre...
30KB

Guidelines

- **Construct / plasmid resource-name:** DENV-2 NS2B-NS3 protease fusion bearing a TEV-cleavable N-terminal His-StrepII tag.

Materials

Plasmid details:

- Vector: pNIC
- Cell line: E. coli Rosetta strain BL21(DE3)-RR
- Tags and additions: N-terminal His-StrepII tag
- Construct protein sequence: `
  MHHHHHHSSGASWSHPQFEKGGGSGGGSGGSAWSHPQFEKGSGVDLGTENLYFQSMADLELERAADVKWEDQAEIS
  GSSPILSITISEDGSMSIKNEEEEQTLGGGGSGGGGAGVLWDVPSPPPMGKAELEDGAYRIKQKGILGYSQIGAGVYKEGT
  FHTMWHVTRGAVLMHKGKRIEPSWADVKKDLISYGGGWKLEGEWKEGEEVQVLALEPGKNPRAVQTKPGLFKTNAGTI
  GAVSLDFSPGTSGSPIIDKKGKVVGLYGNGVVTRSGAYVSAIAQTEKSIEDNPEIEDDIFRK

Expression
TB media, 0.5mM IPTG

Purification
Chicken hen egg white lysozyme
5% Polyethylenimine (PEI), prepare with distilled water, filter sterilize
Imidazole
Ni Sepharose 6 FF resin
Gravity flow column, 2.5cm diameter
Centrifugal concentrators, 10kDa MWCO

On an FPLC system:
Cytiva HiLoad 16/600 Superdex 75 pg
5mL sample loop
HiPrep 26/10 desalting column

SDS-PAGE sample buffer, gel, and gel tank

Lysis buffer:

	A	B
	Hepes (pH 7.5)	25 mM
	NaCl	150 mM
	Glycerol	5%
	Lysozyme	0.5 mg/mL

Prepare 100L per 1L E.coli expression

Base buffer:

	A	B
	Hepes (pH 7.5)	25 mM
	NaCl	150 mM
	Glycerol	5%

Prepare 2L per 4L E.coli expression. Used to prepare the following buffers

Binding buffer: base buffer + 10mM imidazole
Wash buffer 1: base buffer + 50mM imidazole
Elution buffer: base buffer, add 300mM imidazole

Gel filtration buffer:

	A	B
	Hepes (pH 7.5)	50mM
	NaCl	150mM
	Glycerol	5%
	TCEP	0.5mM

Prepare 500ml per SEC run.

SDS-PAGE gel: NuPage 4-12%, Bis-Tris protein gel, 27 well.
Run in MES buffer, 200V 35mins.

Protocol materials

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

Abbreviations

1 CV - column volume, total volume of resin in a column
IMAC - immobilised metal affinity chromatography
FT - flow through
DVNS2B3 - DENV2 NS2B-NS3 protease

Plasmid Transformation

1d

2 The construct encodes the NS2B and NS3 protease linked with a non-cleavable flexible linker, with a N-terminal His6-StrepII tag fusion. It was inoculated from its BL21(DE3)-RR glycerol stock. Plasmid bellow.

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

Note

The DENV-2 NS2B-NS3 fusion construct encodes the NS2B and NS3 protease with a N-terminal His6-StrepII tag fusion on a kanamycin resistant plasmid backbone with a T7 promoter.

Protein expression

2d 10h

3 Scrape off some of the glycerol stock with a sterile loop and use this to inoculate 2× 50 mL falcon tube containing 20 mL of LB each, supplemented with [M] 50 Mass Percent kanamycin. Grow the starter culture at 37 °C Overnight with 200 rpm shaking.

4h

4 Use 10 mL starter culture to inoculate every 1 L TB media (see Materials) supplemented with [M] 50 Mass Percent kanamycin and 8 mL 50% glycerol in a baffled flask. 200 rpm, 37°C

6h

Note

For this protocol 4L of pellet was grown for purification

5 When the OD_{600} approximately 1.8, add [M] 1 millimolar (mM) IPTG. Lower the temperature and shaker speed to 180 rpm, 18°C . Incubate overnight. 1d

6 Harvest the cell by centrifugation at 4000 x g, 4°C, 00:30:00 . Discard supernatant and store pellet by freezing at -80 °C . 30m

4L of culture was grown for this protocol.

Protein Purifcation 2d

7 **Lyse cell pellet** 2h 30m

7.1 1h

Note

See Materials tab for buffer compositions.

Note

DENV2 NS2B-NS3 His6-StrepII fusion protein properties

Before tag cleavage:
MW = 31543.04 Da
E (assume all Cys reduced)= 54430 mM-1cm-1
PI = 5.49

After tag cleavage:
MW = 25849.03 Da
E (assume all Cys reduced)= 41940 mM-1cm-1
PI = 5.01

These values are determined by Expasy ProtParam

Thaw and resuspend the pellet in ~7mL of lysis buffer per g of pellet. Stir gently with magnetic stir bar at Room temperature for 00:30:00 to allow lysozyme and bezonase to start breaking down cell components.

7.2 Lyse by sonication 00:00:05 On 00:00:10 Off for a total 'on' time of 00:04:00 at 40% amplitude to fully rupture the cells. Ensure pellet is 0 °C during sonication to prevent overheating. 4m 15s

7.3 Add 5% PEI to lysate until final concentration of [M] 0.15 % volume total lysate. This precipitates DNA, which can be removed by centrifugation in the next step.

7.4 Centrifuge the lysed cells for 38000 x g, 4°C, 01:00:00 to remove insoluble cell debris, and collect supernatant in a bottle 4 °C 1h

8 **Perform IMAC to extract target protein from the lysed cell mixture**

8.1 Dispense 2 mL Nickle affinity resin Ni Sepharose 6 FF - Cytiva into a gravity flow column. Equilibrate resin by first rinsing with ~ 10 µL distilled water, then ~ 10 µL binding buffer to remove the storage solution. 10m

8.2 Pour clarified lysate onto the the gravity flow column, and allow it to flow though over the washed resin. Collect FT separately for SDS-PAGE analysis. 30m

Note

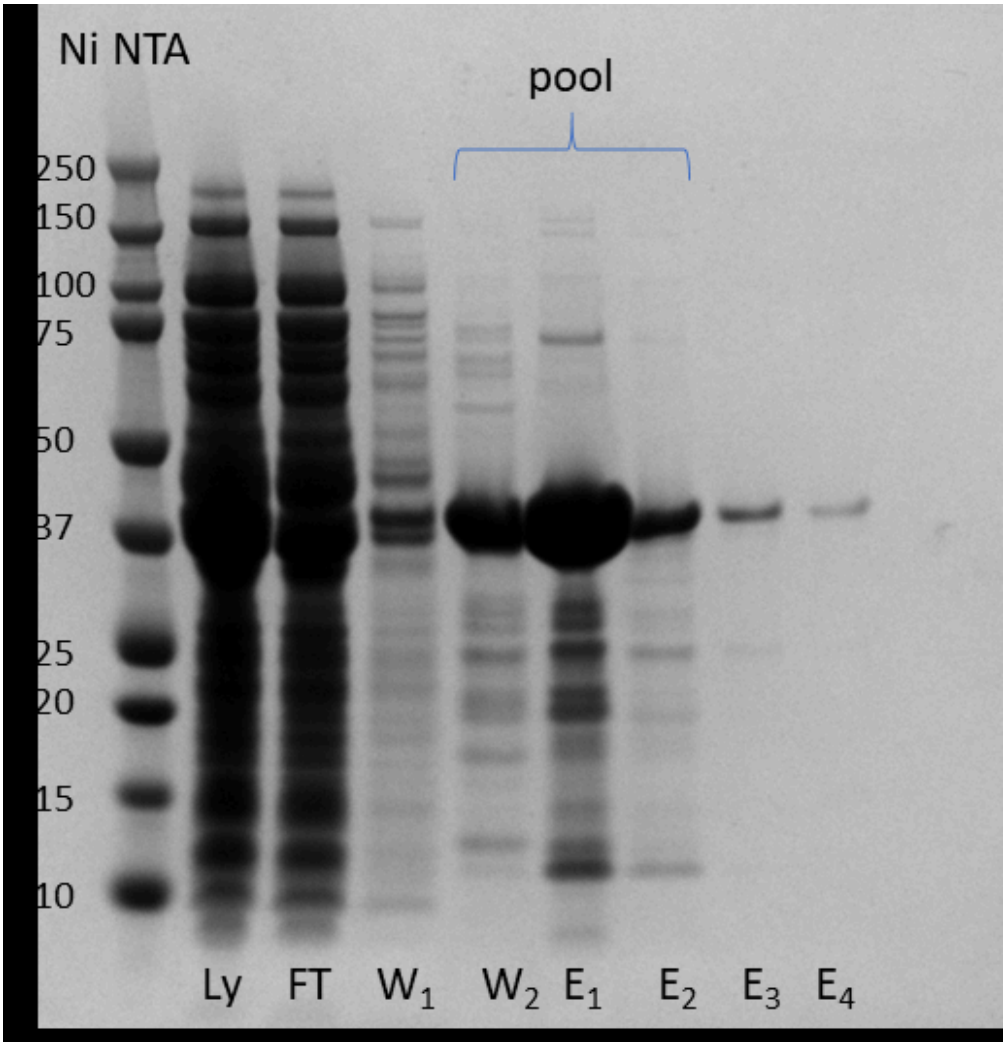
For SDS-PAGE samples, mix 15uL sample with 5uL 4x sample buffer, supplemented with 10mM DTT.

8.3 Wash the column with 10 µL of base buffer, followed by 5 µL of wash buffer twice. Allow wash buffer to pass through completely between washes. This is to remove non-specific, weak binding of contaminant proteins from the resin for a cleaner elution. Collect washes separately for SDS-PAGE analysis. 30m

8.4 Elute the protein with 2.5 µL of elution buffer. 20m

8.5 Repeat step 8.5 three more times, collecting a total of 4 separate elution fractions. This is to ensure maximum retrieval of protein from the resin. 20m

9 Run SDS-PAGE of all samples from total lysis supernatant to final elution. Stain gel with protein staining solution Coomasssie Blue and determine which fractions contain the target protein by finding the band corresponding to the target molecular weight. 40m



SDS-PAGE analysis of IMAC fractions shows target protein as the major species in wash 2 and the first two elution. Significant amount of target is present in wash 2, which suggest that the binding interaction between target and Ni resin is not very strong.

Note

The target protein is expected to be present mostly in the elution samples, although small amounts may be found in the FT and washes.
If that is not the case, then further troubleshooting is required.

10 **Purify sample further by size exclusion chromatography**. 6h

10.1 Using 10,000 MWCO spin concentrators, concentrate the rIMAC step containing fractions of the target protein to a final volume of under 5 mL . 1h

10.2 Remove any solid aggregates from the sample by centrifugation at 17200 x g, 4°C, 00:10:00 , then immediately draw up the supernatant with a 5mL syringe and a blunt-tip fill needle, taking care not to disturb the pellet. 15m

Note

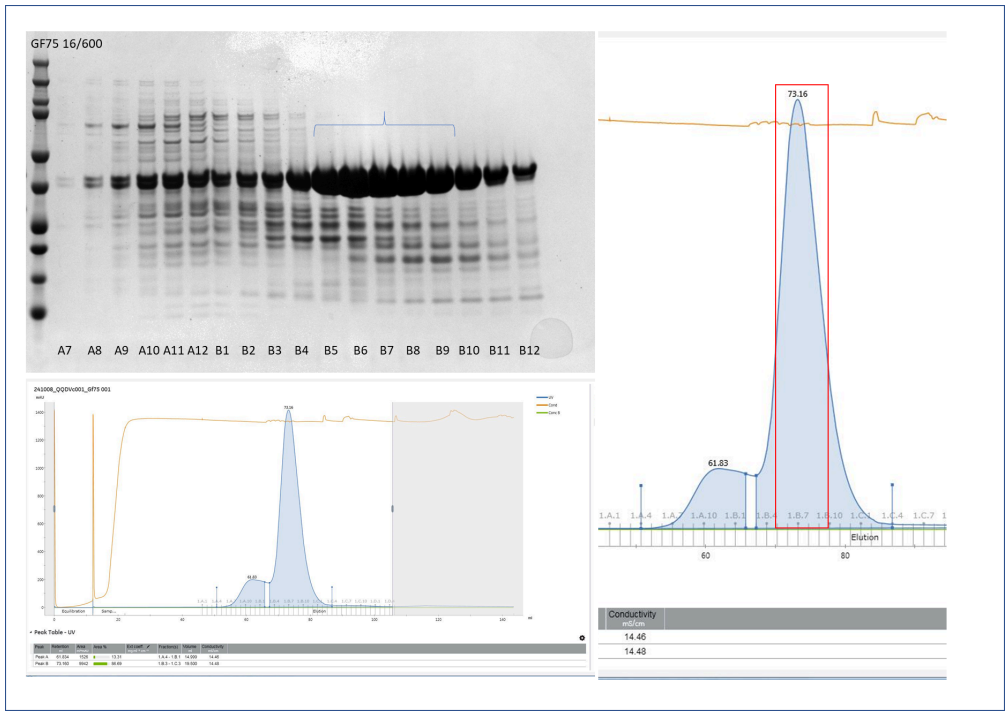
This is to remove as much solid particles from the injection sample as possible, so as to not clog the in-line filter or frit of the column.
This can also be done by passing the sample through a 0.22 micron syringe filter.

11 Using the AKTA Pure system: 2h

Inject the sample onto a 5mL sample loop.

Run the sample down HiLoad 16/60 Superdex 75 pg gel filtration column at 1mL/min in gel filtration buffer, collecting 1mL aliquots.

12 From the chromatogram, fraction A7-B12 were analysed by SDS-PAGE. 1h

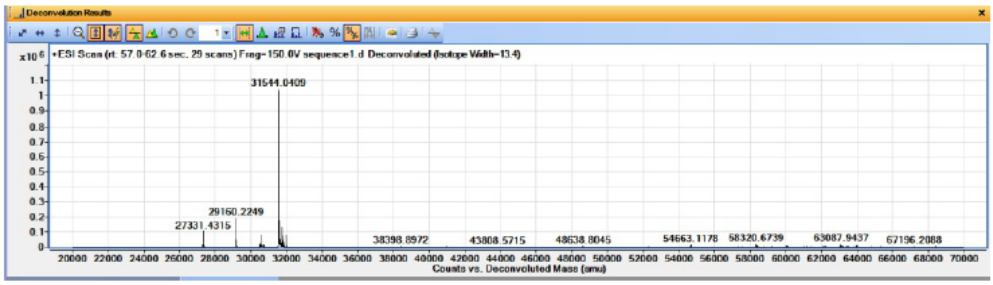


Chromatogram and SEC fraction SDS-PAGE analysis. Fractions A7-B12 were analysed on SDS-PAGE to determine which contained the target protein. Fractions B5-B9 were pooled as they contain mostly target protein in comparison to contaminants.

12.1 Take the fractions that contain the target protein, which in this case are fraction E8-F10. Concentrate the final sample in Vivaspin 15 10kda MWCO centrifugal concentrator until the concentration reaches > [M] 7 mg/mL . 30m

Take 1 µL of the final sample for SDS-PAGE, which is not carried out here.

Another 1 µL can be taken for mass spectroscopy (MS) analysis.



Intact MS of the final sample. This confirms the uncleaved target protein is the major species in the final sample.

12.2 Aliquot into appropriate volumes for future usage to minimise freeze/thaw cycles. Flash-freeze in liquid nitrogen, and store at -80 °C until required. 10m