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DENV2 NS2B-NS3 protease co-expression construct small scale expression and purification protocol

Forked from Enterovirus coxsackievirus A16 2A protease small scale expression and purification protocol

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ASAP Discovery

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CMD

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We use this protocol and it's working

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Abstract

This protocol details the co-expression and purification of DENV2 NS2B-NS3 protease construct bearing a N-terminal His-GST tag at small scale (<6L).

Attachments

PDF

PAGE23-01623 - SRF-B...

573KB

Guidelines

- **Construct / plasmid resource-name:** A71EV2A protease construct bearing a N-terminal SUMO-His tag.
- **Construct Addgene ID**

Materials

Plasmid details:

- Vector: pNIC
- Cell line: E. coli Rosetta strain BL21(DE3)-RR
- Tags and additions: N-terminal GST-His tag
- Construct protein sequence: `
  MHHHHHHSSMSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPYYIDGDVKLTQSMAIIRYIADK
  HNMLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVL
  YMDPMCLDAFPKLVCFKKRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPKSGGGSENLYFQSMGKSVDMYIERAGDITWE
  KDAEVTGNSPRLDVALDESGDFSLVEE

Expression
TB media, 1mM IPTG

Purification
Chicken hen egg white lysozyme
Benzonase
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

SDS-PAGE sample buffer, gel, and gel tank

Lysis buffer:

	A	B
	Hepes (pH 7.5)	50 mM
	NaCl	500 mM
	Glycerol	5%
	TCEP	1 mM
	Lysozyme	0.5 mg/mL
	Benzonase	0.05 mg/mL
	TX-100	1%

Prepare 100L per 1L E.coli expression

Base buffer:

	A	B
	Hepes (pH 7.5)	50 mM
	NaCl	50 mM
	Glycerol	5%
	TCEP	1 mM

Prepare 2L per 6L E.coli expression. Used to prepare the following buffers

Binding buffer: base buffer + 20mM imidazole
Wash buffer 1: base buffer + 30mM imidazole
Wash buffer 2: base buffer + 50mM imidazole
Elution buffer: base buffer, add 500mM imidazole
Gel filtration buffer: base buffer + 20mM imidazole

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

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 DVNS2B3 N-terminal His-GST tagged co-expression construct was inoculated from its BL21(DE3)-RR glycerol stock.

Note

The DENV2 NS2B-NS3 co-expression construct encodes the NS2B and NS3 protease with a N-terminal His6-GST 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 a 50 mL falcon tube containing 10 mL of LB supplemented with [M] 50 ug/mL 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 Sample (see Materials) supplemented with [M] 50 ug/mL kanamycin in a baffled flask. 200 rpm, 37°C

6h

Note

For this protocol 2L of pellet was grown for purification

Note

Media and expression condition are especially important for the production of this construct, as it is prone to auto-cleavage of the affinity tag during expression.

5 When the OD_{600} approximately 1.8, add 1mM 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

Note

For reference: total pellet weight from 2L TB media was 33g

Protein Purifcation 2d

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

7.1 1h

Note

See Materials tab for buffer compositions.

Note

DENV2 NS2B-NS3 His6-GST fusion protein properties

Before tag cleavage:
MW = 32.812 kDa
E (assume all Cys reduced)= 51340 mM-1cm-1
PI = 5.47

After tag cleavage:
NS2B
MW = 5.0635 kDa
E(assume all Cys reduced) = 6990
PI = 3.98

NS3
MW = 18.096 kDa
E(assume all Cys reduced) = 30940
PI = 6.73

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 00:00:04 On 00:00:12 Off for a total 'on' time of 00:07:00 at 50% amplitude to fully rupture the cells. Ensure pellet is 0 °C during sonication to prevent overheating. 7m 16s

7.3 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 3 mL Nickle affinity resin Ni Sepharose 6 FF - Cytiva into a gravity flow column. Equilibrate resin by first rinsing with ~ 10 CV distilled water, then ~ 10 CV binding buffer to remove the storage solution. 10m

8.2 Resuspend the equilibrated resin with some binding buffer and add to the supernatant bottle. Incubate the resin with the supernatant for 00:30:00 while rotating or otherwise mixing 30m

gently at 4 °C

8.3 Load the resin/supernatant mix back onto the gravity flow column, retaining the FT separately for SDS-PAGE analysis. 30m

Note

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

8.4 Wash the column with 10 CV of base buffer, followed by 10 CV of wash buffer 1 and 2. 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. 30m
Collect washes separately for SDS-PAGE analysis.

8.5 Elute the protein with 2.5 CV of elution buffer. 20m

8.6 Repeat step 8.5 one more time, collecting a total of 2 separate elution fractions. This is to ensure maximum retrieval of protein from the resin. 20m

Measure the total protein concentration of the elutions by Nanodrop. Although still a mixture, A280 value can give an estimate of the protein content, which will determine how much protease need to be added to remove the affinity tag.

For example, E1 = 18.28 mg/mL, E2 = 13.02 mg/mL at 1 A280 unit = 1 mg/mL

8.7 Wash used IMAC resin with 10CV of base buffer, and leave in the column submerged in a small amount of base buffer such that the resin is kept moist.
This washed IMAC resin will later be reused for reverse IMAC (rIMAC)

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

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 Elution de-salting, tag cleavage and reverse IMAC

1d

10.1 Pool and desalt the two elutions using HiPrep 26/10 deasalting columns, run on AKTA pure at the maximum flow rate of 10mL/min. 30m

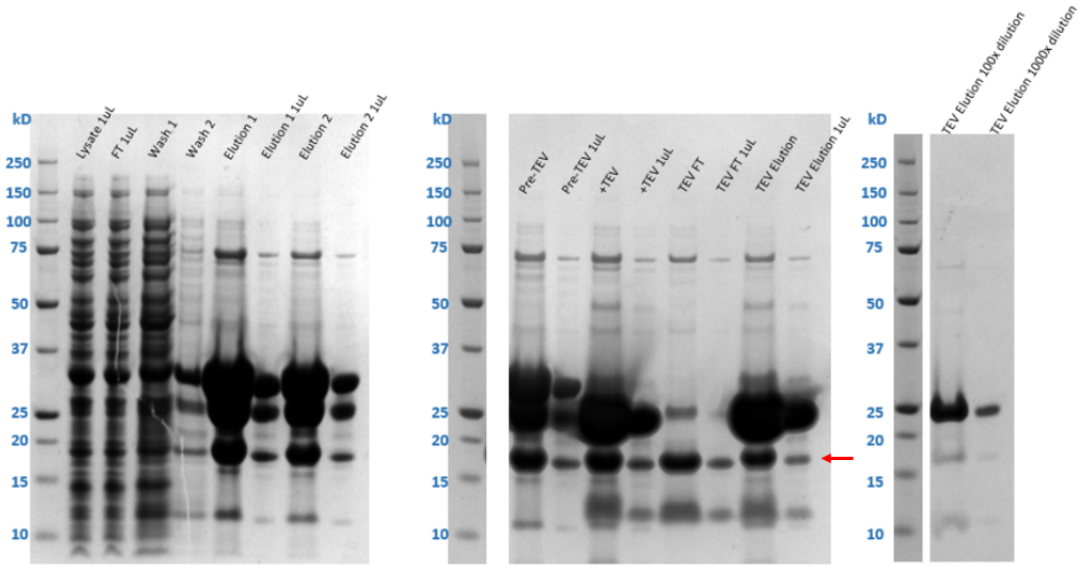
> **Note**
>
> This is to reduce imidazole concentration in the sample. High concentration of imidazole will inhibit protease activity during tag cleavage and removal.

10.2 For tag removal, His-TEV was added in 1:100 ratio to the total protein content of the desalted sample, as determined by nanodrop. The mixture was left in the cold room at 4 °C Overnight 1d

10.3 In morning, pour the cleavage mixture over the washed resin three times and collect final FT. 30m

> **Note**
>
> This step will remove the cleaved tag and any uncleaved target from the sample. If the protease used is His-tagged, then the protease is removed from sample too.

10.4 Wash rIMAC resin with 2 CV wash buffer 1 and 2 to remove any target protein still bound to the resin. 30m
Take samples of the FT and wash, characterise content by SDS-PAGE



SDS-PAGE analysis of IMAC and cleavage fractions. The band highlighted by red arrow agrees with the size of the cleaved NS3 construct (18.095 kDa)

10.5 (Optional) elute rIMAC resin with 2 CV elution buffer to confirm if the protein shows non-specific binding to the resin used. 5m

> **Note**
>
> This will help determine if the protein is "sticky" to the Ni resin matrix material, and help in further troubleshooting if the final yield is lower than expected.

11 Purify sample further by size exclusion chromatography. 6h

11.1 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

11.2 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

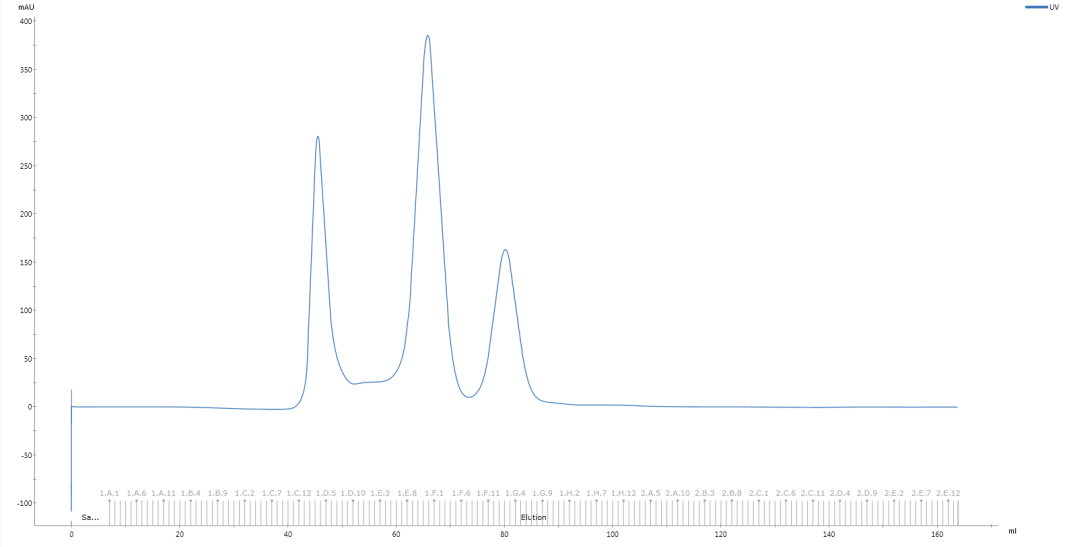
This is to remove as much solid particles from the injection sample as possible, so as to not clog the in-line filter or frit of the column.

12 Using the AKTA Pure system: 2h

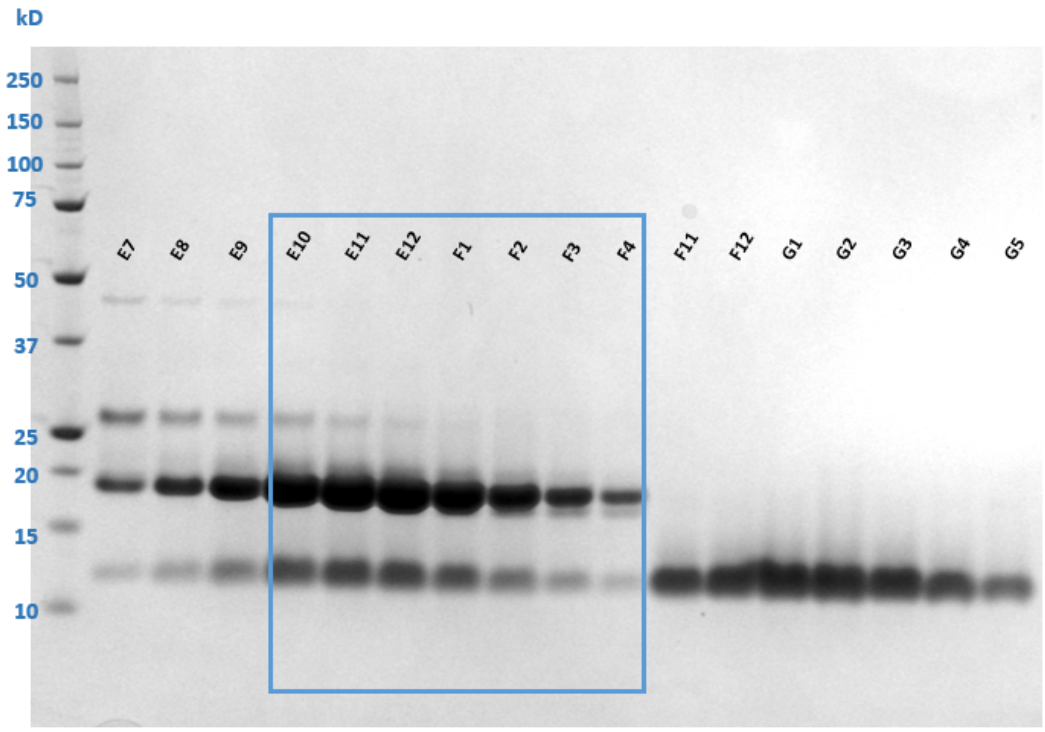
Inject the sample onto a 5mL sample loop.

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

13 From the chromatogram, fraction F9-H8 analyse by SDS-PAGE. 1h



Chromatogram of the DVNS2B3 SEC run. Fractions E7-G5 were analyzed by SDS-PAGE to see which contained the target protein



SDS-PAGE analysis of SEC fraction E7-G5. Fractions E10-F4 were pooled as they contain majority target protein in comparison to contaminants.
Red arrow indicated the position of bands corresponding to the target protein.

13.1 Take the fractions that contain the target protein, which in this case are fraction E10-F4. Concentrate the final sample in Vivaspin 500 10kda MWCO centrifugal concentrator until the concentration reaches > [M] 23 mg/mL or [M] 1 millimolar (mM) . 30m

Take 1 µL of the final sample for SDS-PAGE, and another for mass spectroscopy (MS).

13.2 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