Apr 26, 2024 Version 1

Enterovirus D68 3C protease small scale expression and purification protocol V.1

Forked from a private protocol

DOI
dx.doi.org/10.17504/protocols.io.dm6gpzrkdlzp/v1

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

[1]Center of Medicines Discovery, University of Oxford

ASAP Discovery

Korvus Wang
CMD

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.dm6gpzrkdlzp/v1

Protocol Citation: Korvus Wang, Michael Fairhead, Eleanor Williams 2024. Enterovirus D68 3C protease small scale expression and purification protocol. **protocols.io https://dx.doi.org/10.17504/protocols.io.dm6gpzrkdlzp/v1**

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

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

Created: April 18, 2024

Last Modified: April 26, 2024

Protocol Integer ID: 98385

Keywords: 3C proteases, enterovirusses, IMAC, ASAP, CMD, protein purification, enterovirus d68 3c protease small scale expression, purification of enterovirus d68 3c protease, enterovirus d68 3c protease, enterovirus d68 3c, purification protocol, purification protocol this protocol, purification

Funders Acknowledgements:
National Institutes of Health/National Institute Of Allergy and Infectious Diseases (NIH/NIAID)
Grant ID: U19AI171432

Disclaimer

Research was supported in part by NIAID of the U.S National Institutes of Health under award number U19AI171399. 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 details the expression and purification of enterovirus D68 3C protease construct bearing a C-terminal His-tag at small scale (<6L).

Attachments

DOCX
nyixb5id7 (1).docx
23KB

PDF
PAGE23-01458 - SRF-B...
215KB

Guidelines

- **Construct / plasmid resource-name:** Enterovirus D68 3C protease construct bearing a C-terminal His-tag that crystallised in the space group $P2_12_12_1$

- **Brief description of the plasmid:** This protein yielded highly reproducible crystals upon microseeding, which typically diffracted to 1.7 Å resolution. This crystal system was DMSO tolerant and therefore suitable for our subsequent fragment soaking.

- **Provided Date:** 2022-03-24

Materials

Plasmid details:
Addgene plasmid #204817

- Vector: pNIC
- Cell line: E. coli Rosetta strain BL21(DE3)-RR
- Tags and additions: C-terminal, non-cleavable hexahistidine
- Construct protein sequence: `
  MGPGFDFAQAIMKKNTVIARTEKGEFTMLGVYDRVAVIPTHASVGEIIYINDVETRVLDACALRDLTDTNLEITIVKLDRNQK
  FRDIRHFLPRCEDDYNDAVLSVHTSKFPNMYIPVGQVTNYGFLNLGGTPTHRILMYNFPTRAGQCGGVVTTTGKVIGIHV
  GGNGAQGFAAMLLHSYFTDTQKHHHHHH

Expression
AIM-TB: TB autoinduction media (Formedium AIMTB0210)

Purification
Chicken hen egg white lysozyme (Merck, 62971)
Benzonase (Merck, 1.01654)
Imidazole (Merck, RDD044)
Ni Sepharose 6 FF resin (Cytiva, 17531801)
Gravity flow column, 2.5cm diameter (Bio Rad, 7372532)
Centrifugal concentrators, 10kDa MWCO (Merck, UFC901008)

On an FPLC system:
SEPAX SEC SRT-100 (Sepax Tech, 215100-21230)
or
HiLoad 16/600 Superdex 75 pg (Cytiva, 28989333)

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%
	Imidazole	20 mM
	TCEP	0.5 mM

	A	B
	TX-100	1%
	Lysozyme	0.5 mg/mL
	Benzonase	0.05 mg/mL

Prepare 100 mL per 1 L *E.coli* expression

Base buffer:

	A	B
	Hepes (pH 7.5)	50 mM
	NaCl	500 mM
	Glycerol	5%
	TCEP	0.5 mM

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

Binding buffer: base buffer, add 30mM imidazole
Wash buffer: base buffer, add 30mM imidazole
Elution buffer: base buffer, add 500mM imidazole
Gel filtration buffer: same as base buffer

SDS-PAGE: NuPage 4-12%, Bis-Tris protein gel, 26 well (Thermo-Fisher, WG1403BOX)
Run in MES buffer, 200V 35mins.

Troubleshooting

Abbreviations

1 CV - column volume, total volume of resin in a column
IMAC - immobilised metal affinity chromatography
D68EV3C - Enterovirus D68 3C protease

Plasmid Transformation

1d

2 Transform the D68EV3C construct (Addgene plasmid #204817) into BL21(DE3) and store a glycerol stock of this at -80 °C

Note

The D68EV3C construct encodes the 3C protease with a non-cleavable C-terminal his tag 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 50 ug/mL kanamycin. Grow the starter culture at 37 °C Overnight with 200 rpm shaking.

1d

4 Use the 10 mL starter culture to inoculate 1 L Sample (see Materials) supplemented with 50 ug/mL kanamycin in a baffled flask. 250 rpm, 37°C

6h

Note

For this protocol typically 2 L of culture is grown for each purification

5 When the OD_{600} reaches approximately 4.0, lower the temperature and shaker speed to 200 rpm, 18°C and incubate 24:00:00

1d

6 Harvest the cells by centrifugation at 5000 x g, 4°C, 00:30:00 . Discard supernatant and store pellet at -80 °C .

30m

Protein Purifcation

2d

7 **Lyse cell pellet**

2h 30m

7.1

1h

Note

See Materials tab for buffer compositions.

Note

D68EV3C construct protein properties
MW = 21.283 kDa
Extinction coefficient (assume all Cys reduced)=10430 mM-1cm-1
pI = 7.21

Values determined using Expasy ProtParam

Thaw and resuspend the pellet in ~7mL of lysis buffer per g of pellet. Stir gently with a magnetic stir bar at Room temperature for 00:30:00 to allow lysozyme, benzonase and Triton X-100 to start breaking down cell components.

7.2 Store the homogenised lysate at -80 °C and then thaw in a room temperature water bath to further lyse the cells, freeze-thaw.

7.3 Centrifuge the lysed cells 38000 x g, 4°C, 01:00:00 to remove insoluble cell debris, and collect the supernatant 4 °C

1h

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

8.1 Dispense 10 mL Nickle affinity resin (Ni Sepharose 6 FF, Cytiva) into a gravity flow column.
Wash the resin first with ~ 20 CV distilled water to remove the storage solution and then ~ 20 CV binding buffer to equilibrate

10m

8.2 Pour the clarified supernatant over the equilibrated resin and allow to flow though. This will allow the His-tagged target protein to bind onto the Ni ions in the resin.
Retain the flow through separately for SDS-PAGE analysis.

8.3 Wash the column with 10 CV 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. 30m
Collect washes separately for SDS-PAGE analysis.

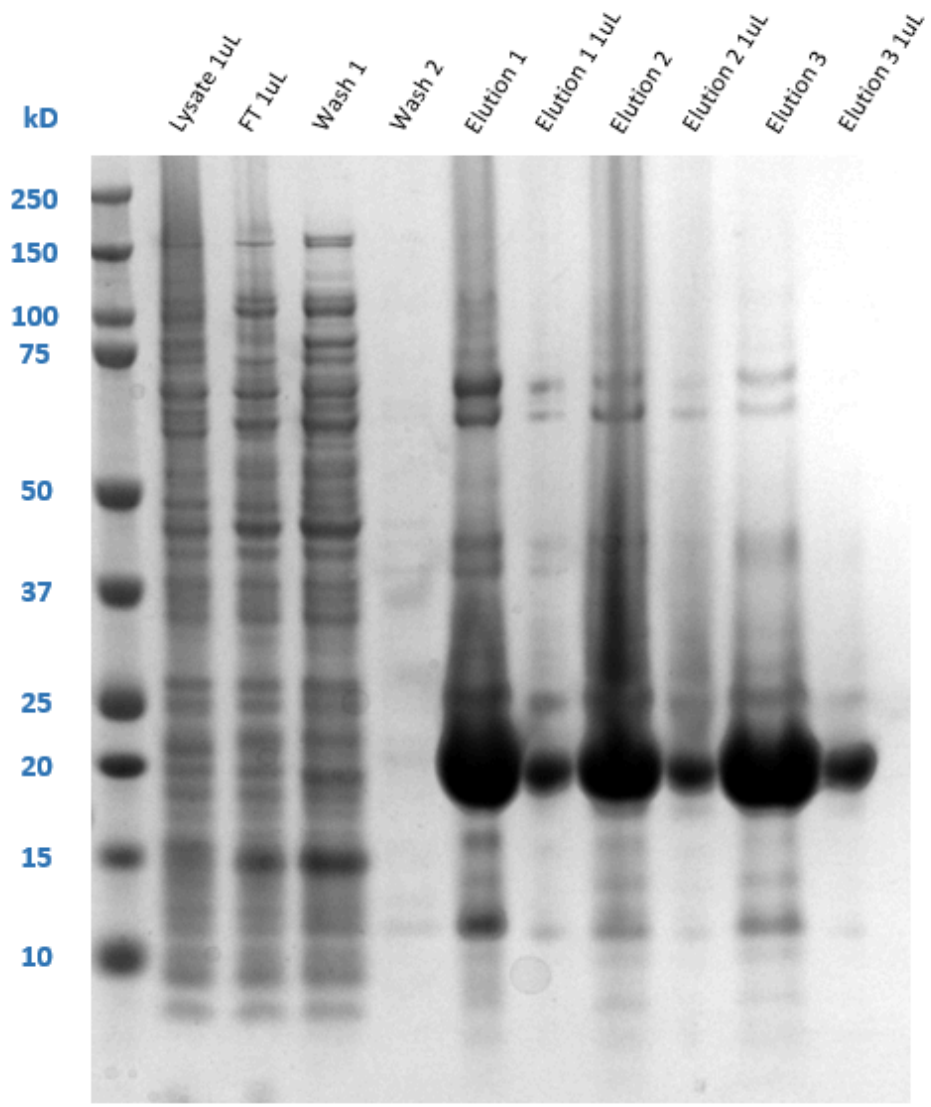
8.4 Elute the protein with 1.5 CV of elution buffer. 20m

8.5 Repeat step 7.5 a further 2 times, collecting a total of 3 separate elution fractions. This is to ensure maximum retrieval of protein from the resin. 20m
Measured the A280 values of the elution fractions to estimate the protein content
For example:
E1: A280=5.69
E2: A280=10.1
E3: A280=4.23

9 Run SDS-PAGE of all samples from total lysis supernatant to final elution. Stain gel with Coomasssie Blue and determine which fractions contain the target protein by finding the band corresponding to the target molecular weight, 21.3 kDa. 40m

Note

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



SDS-PAGE analysis of IMAC fractions. The thick protein band observed in all three elutions agree with the calculated molecular weight of D68EV3C protease, 21.3 kDa.

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

10.1 Pool and concentration all elution fractions to a final volume of under 5 mL using a 10 kDa MWCO centrifugal concentrator

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. 10m

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.

11 Using an AKTA Pure FPLC system or equivalent:

Inject the sample onto a 5mL sample loop.

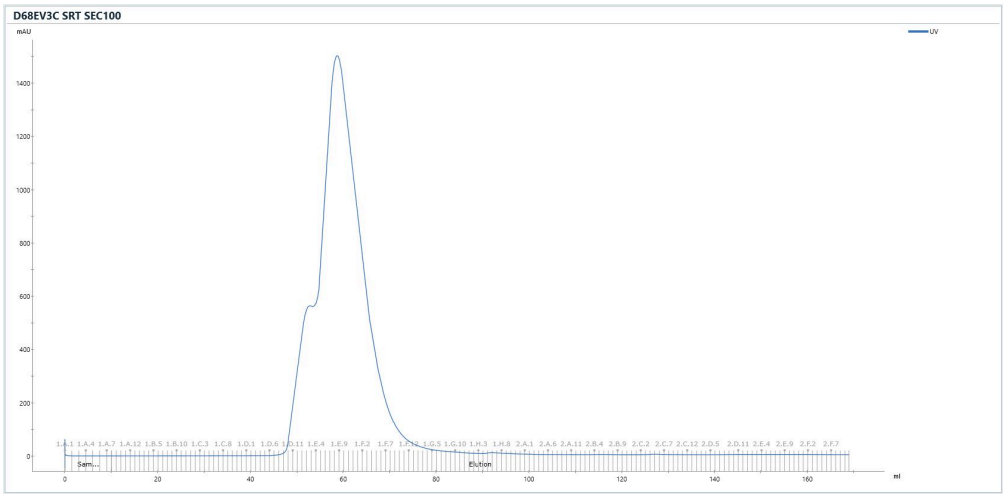
Run the sample down Sepax SRT SEC-100 gel filtration column at 7.5mL/min in gel filtration buffer, collecting 1mL fractions in 96 well deep-well blocks.
The column should be pre-equilibrated in SEC buffer.

Note

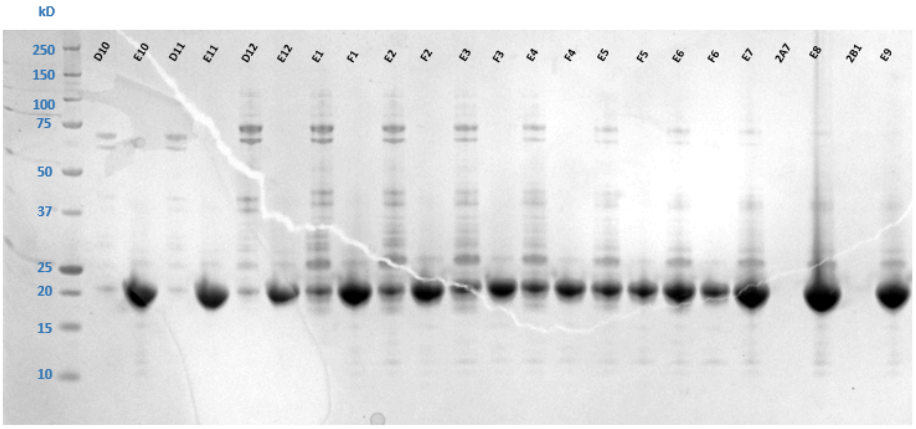
Here a SEPAX SEC SRT-100 column was used due to availability, however other columns would also be suitable such as a Superdex 75 16/60 (Cytiva)

12 Run the peak SEC fractions on SDS PAGE to assess purity. 40m

For example:



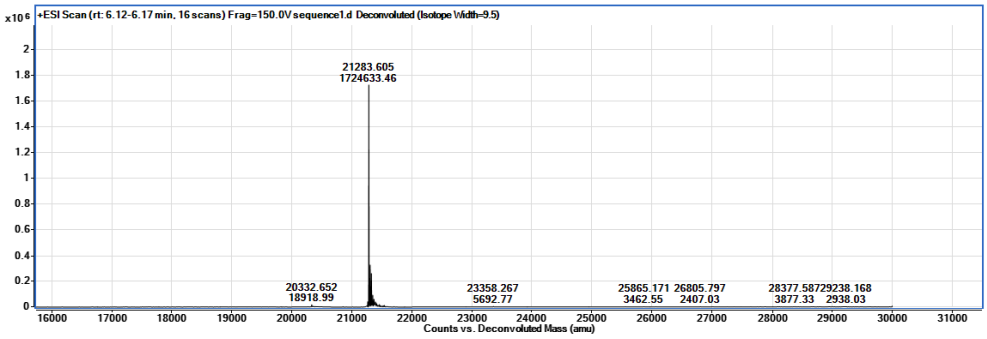
Chromatogram of the SEC run. Fractions D10-F6 were analyse by SDS-PAGE to determine which contained the target protein.



SDS-PAGE analysis of SEC fractions D10-F6. Fractions E6-F6 were pooled as they contain majority target protein in comparison to contaminants.

12.1 Take the fractions that contain the cleanest target protein and concentrate to [M] 21 mg/mL using a 10 kDa MWCO centrifugal concentrator 2h

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



Intact Mass-spectrometry of purified D68EV3C protease sample. Mass spec result showing the purified D68EV3C protease has the expected molecular weight, 21.283 kDa

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.

For example:

The final yield from processing 2 L of cells was 128 mg of pure D68 EV 3C protease