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# Enterovirus D68 3C protease large scale purification protocol

Forked from Enterovirus D68 3C protease small scale expression and purification protocol

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ASAP Discovery

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CMD

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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

This protocol details the expression and purification of enterovirus D68 3C protease construct bearing a C-terminal His-tag at large scale (>6L)

## Attachments

nyixb5id7 (1).docx 23KB

PAGE24-00037 - D68EV... 478KB

## 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: ` MGPGFDFAQAIMKKNTVIARTEKGEFTMLGVYDRVAVIPTHASVGEIIYINDVETRVLDACALRDLTDTNLEITIVKLDRNQKFRDIR HFLPRCEDDYNDAVLSVHTSKFPNMYIPVGQVTNYGFLNLGGTPTHRILMYNFPTRAGQCGGVVTTTGKVIGIHVGGNGAQGFA AMLLHSYFTDTQKHHHHHH

**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)
Vivaflow 50 (10kDa MWCO) tangential flow concentrators (Sartorius, VF05H0)

On an FPLC system:
XK 50/100 Superdex 200 pg gel filtration column (Cytiva, 90100045)

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 |
| | Lysozyme | 1 mg/mL |
| | Benzonase | 0.05 mg/mL |
| | MgCl2 | 2mM |

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 20mM imidazole
**Wash buffer 1:** base buffer. reduce NaCl to 100mM, +0.1 mg/mL benzonase, 2mM MgCl2
**Wash buffer 2:** 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.

## Abbreviations

1 CV - column volume, total volume of resin in a column
IMAC - immobilised metal affinity chromatography

## 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 See (Nathan's protocol DOI) for D68EV3C large scale expression protocol

## Protein Purifcation

2d

4 **Lyse cell pellet**

2h 30m

4.1

1h

**Note**

See Materials tab for buffer compositions.
In the below examples 244 g of cell pellet was processed

**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 ~8mL 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.

4.2 Lyse cells 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

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

## 5 Perform IMAC to extract target protein from the lysed cell mixture

5.1 Dispense 30 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

5.2 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 gently at 4 °C 30m

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

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

5.4 Wash the column with 10 CV of wash buffer 1, then 10 CV wash buffer 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.
Collect washes separately for SDS-PAGE analysis. 30m

5.5 Elute the protein with 1 CV of elution buffer. 20m

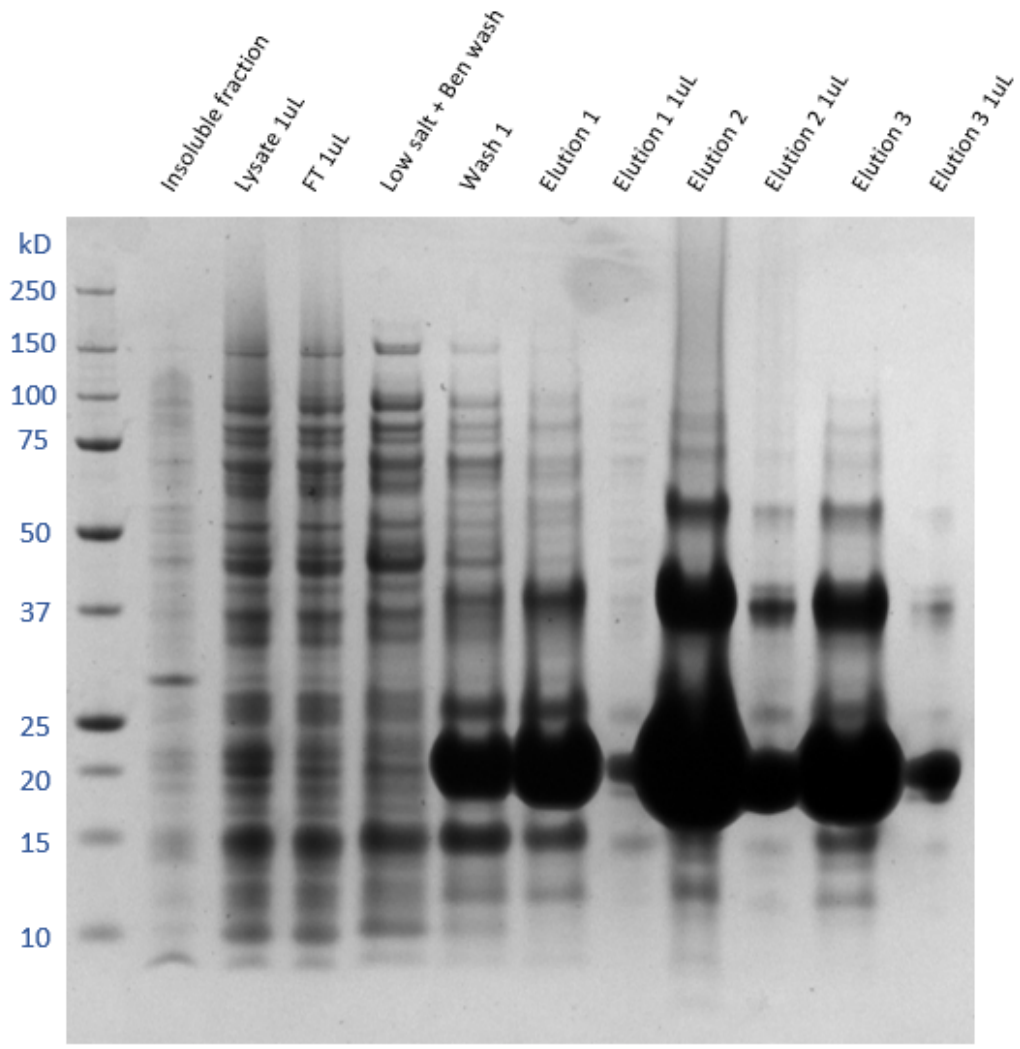
5.6 Repeat step 5.5 a further 2 times, collecting a total of 3 separate elution fractions. This is to ensure maximum retrieval of protein from the resin. 20m
Measured the A280 values of the elution fractions to estimate the protein content

For example:
E1: A280=1.16
E2: A280=6.22
E3: A280=3.24

6 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.

## 7 Purify sample further by size exclusion chromatography.

7.1 Pool and dilute the elutions with base buffer to reduce the sample imidazole concentration to [M] 100 millimolar (mM) .

7.2 Concentrate the diluted sample with Vivaspin 50 (10kDa MWCO) tangential flow concentrators connected to a peristaltic pump, to a final volume of under 30 mL . 2h

Note

Tangential flow concentrators should be placed on ice to keep the sample cool. Peristatic pumps generate heat during operation which may denature the target protein.

Note

If the final concentration resulted in sample more than 30mL, gel filtration may need to be carried out in multiple batches.

7.3 Remove any solid aggregates from the sample by centrifugation at 20000 x g, 4°C, 00:10:00 , then immediately draw up the supernatant with a 50mL 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.

8 Using the AKTA Pure system:

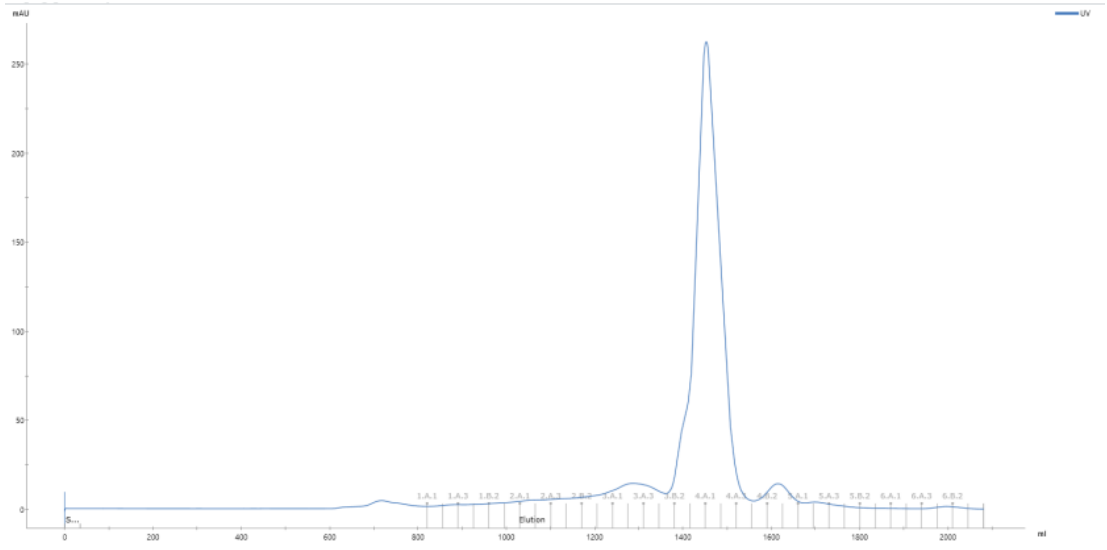
Sample injected onto 50mL Superloop (Cytiva) through a 0.2uM syringe filter to further remove any aggregates in the sample.

Run the sample down XK 50/100 Superdex 200 pg gel filtration column at 6mL/min in gel filtration buffer, collecting 35mL aliquots in 50mL falcon tubes. Collection started after 0.4CV into elution, as the fraction collector can only hold 6x6 falcon tubes. Fraction collection parameters can be adjusted to individual FPLC system specs.

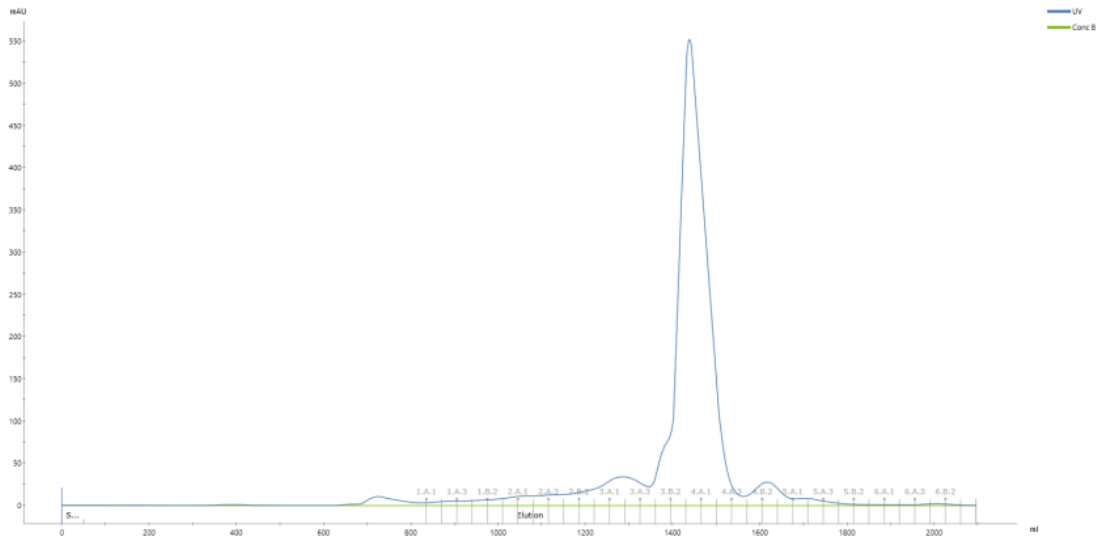
The column should be pre-equilibrated in SEC buffer.

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

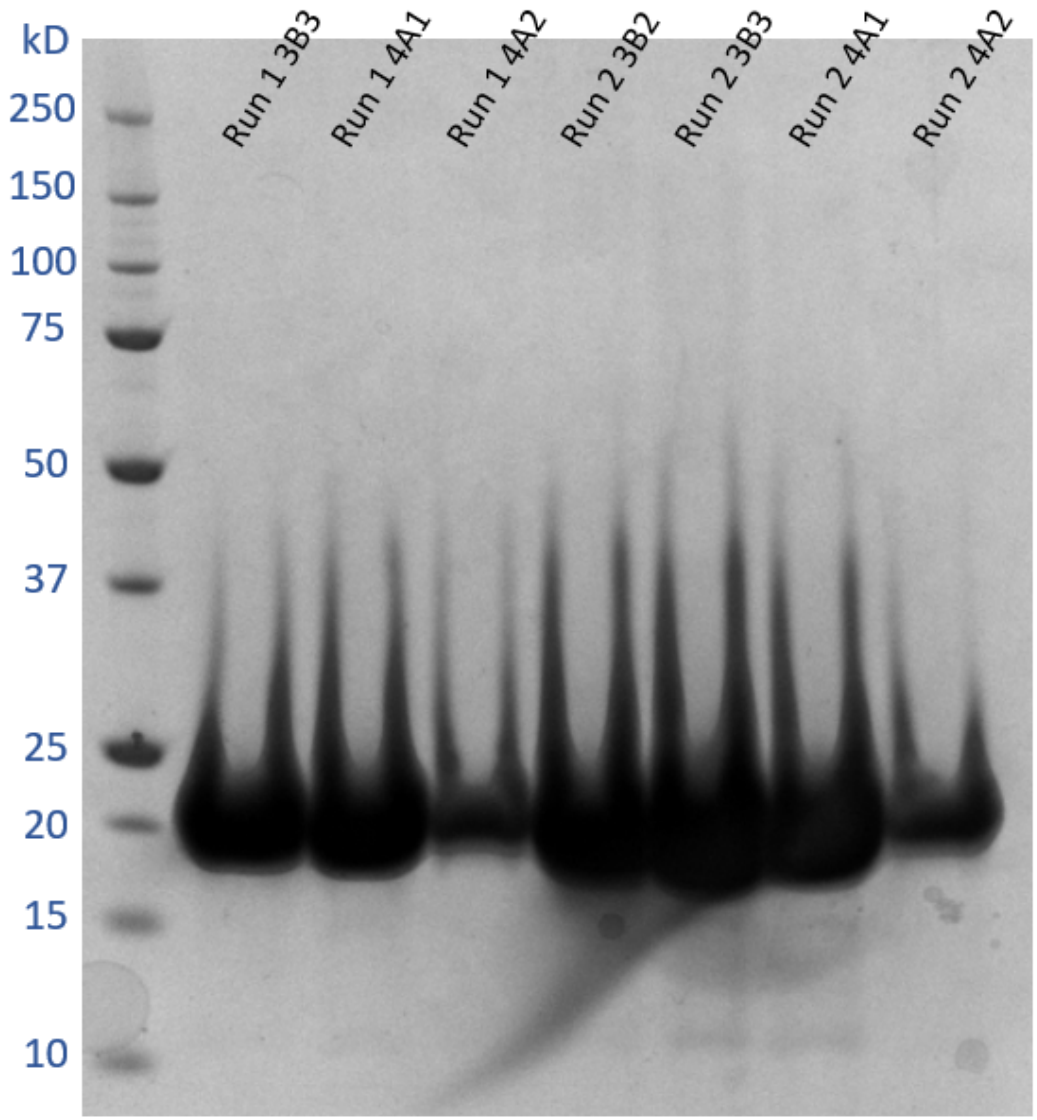
For example:
Below two SEC runs were carried out due to large volume of sample.




**Chromatogram of SEC run 1:** fractions 3B3, 4A1 and 4A2 were analysed by SDS-PAGE.



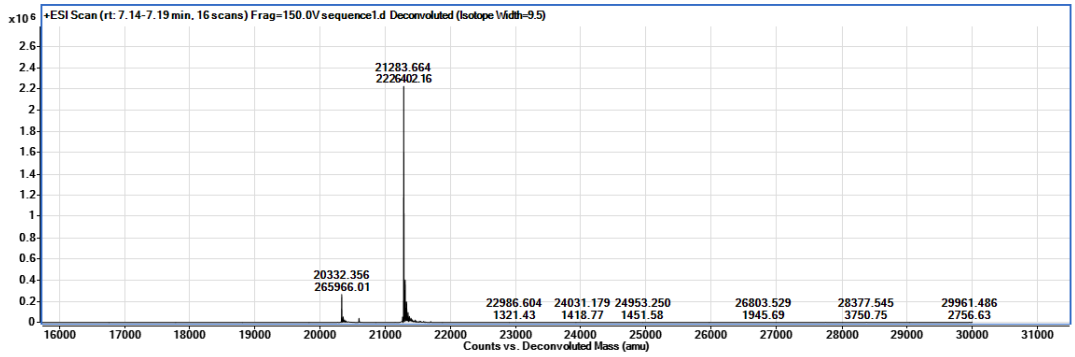

**Chromatogram of SEC run 2:** fractions 3B2, 3B3, 4A1 and 4A2 were analysed by SDS-PAGE




**SDS-PAGE analysis of SEC fractions: Samples from both the above SEC runs are shown,** band observed at 21.3 kDa agrees with the calculated molecular weight of D68EV3C protease.

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

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




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

10.1 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 243 g of cell pellet was 543 mg of pure D68 EV 3C protease