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Enterovirus A71 3C protease large scale purification protocol

Forked from Enterovirus D68 3C protease large scale purification protocol

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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 expression and purification of enterovirus A71 3C protease construct bearing a C-terminal His-tag at large scale (>6L)

Attachments

DOCX
nyixb5id7 (1).docx
23KB

PDF
PAGE24-00037 - D68EV...
478KB

Guidelines

- **Construct / plasmid resource-name:** Enterovirus A71 3C protease construct bearing a C-terminal His-tag.

Materials

Plasmid details:
Addgene plasmid #204816

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

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
	Bicine (pH 8.5)	10 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
	Bicine (pH 8.5)	10 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.

Protocol materials

Enterovirus A71 3C protease **addgene Catalog #**204816

Safety warnings

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

Abbreviations

1 CV - column volume, total volume of resin in a column
IMAC - immobilised metal affinity chromatography
A71EV3C - Enterovirus A71 3C protease

Plasmid Transformation

1d

2 Transform Enterovirus A71 3C protease **addgene Catalog #**204816 into BL21(DE3) and store a glycerol stock of this at -80 °C

Note

The A71EV3C 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 Large scale expression of EV-A71 3C protease using single use column reactors

Protocol

NAME

Setup and Operation of Single Use (SUB) Bubble Column Reactors (BCR) for Recombinant Proteins: Litre-Scale Expression of enterovirus 3C protease for Structural Biology and Drug Design (SBDD)

CREATED BY
Mary-Ann Xavier

Preview

Protein Purifcation

2d

4 **Lyse cell pellet**

2h 30m

4.1 1h

Note

See Materials tab for buffer compositions.

Note

A71EV3C construct protein properties
MW = 21.332 kDa
Extinction coefficient (assume all Cys reduced)=9970 mM-1cm-1
pI = 7.22

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

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 Add 10 CV of wash buffer 1 to the column. Replace cap on the column and resuspend resin. Incubate at 4 °C while gently rotating for 30mins. This is to remove any DNA contaminants bound to the resin. 30m

5.5 Drain wash buffer 1, then wash the resin with 10 CV of wash buffer 2. Allow wash buffer to pass through completely. 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.

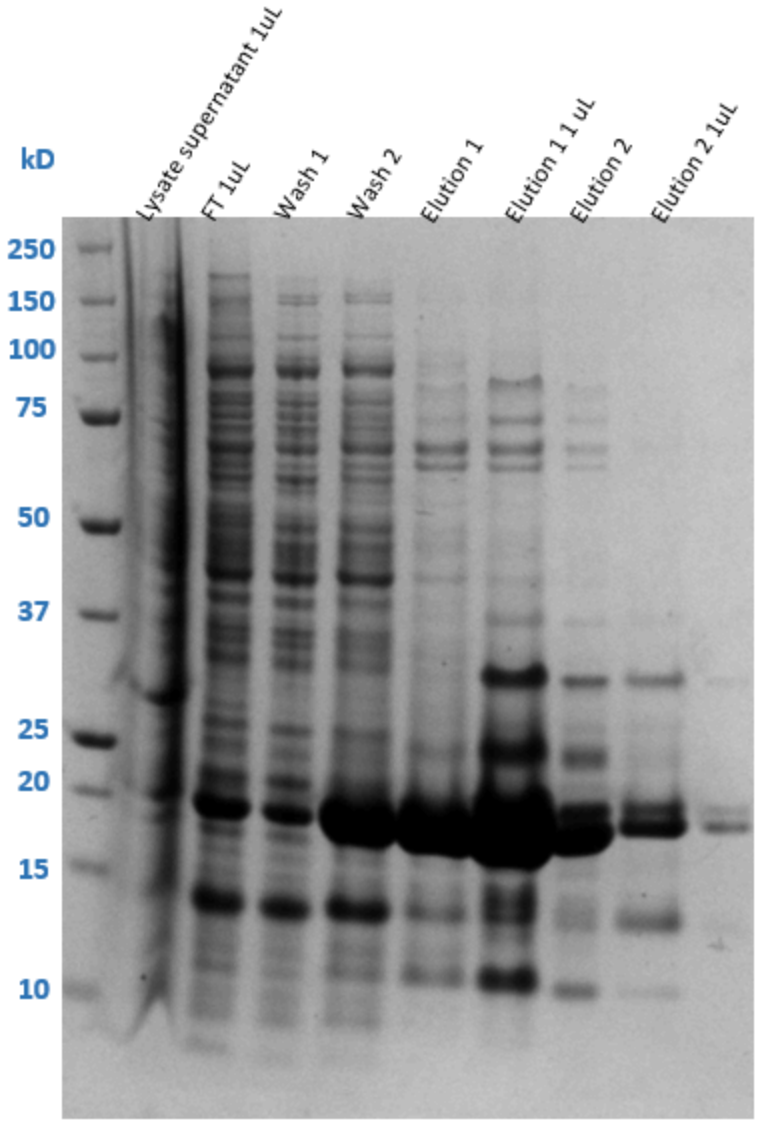
5.6 Elute the protein with 1 CV of elution buffer, 10 min incubation. 20m

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

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 both elutions agree with the calculated molecular weight of A71EV3C 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:

Inject the sample onto a 5mL sample loop. Multiple 5mL loops were attached to individual positions on the loop valve for multiple runs, due to large sample volume.

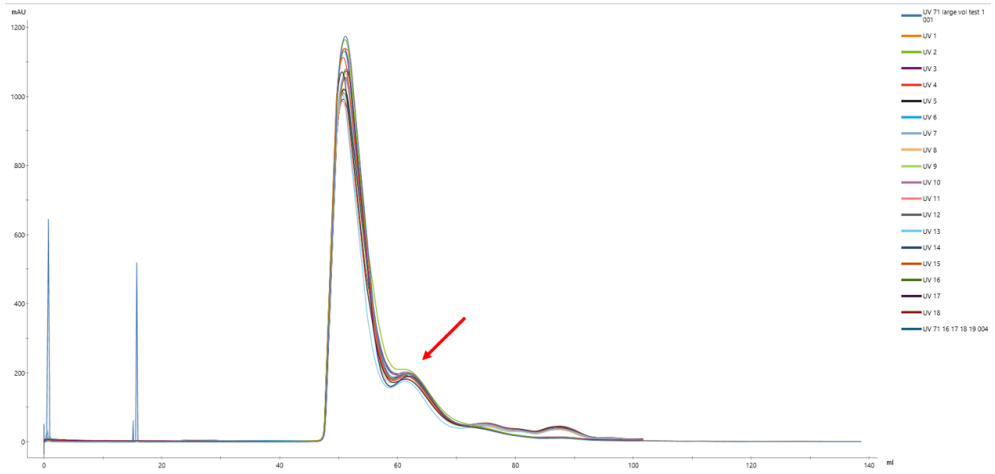
Run the sample down Sepax SRT SEC-100 gel filtration column at 7.5mL/min in gel filtration buffer, collecting 1mL fractions in 96 well deep-well blocks.
The column should be pre-equilibrated in SEC buffer.

Note

For alternative gel filtration protocol using Superloop (Cytiva) and XK 50/100 Superdex 200 pg gel filtration column, see D68EV3C large scale purification protocol (dx.doi.org/10.17504/protocols.io.n92ld8yd7v5b/v1).

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

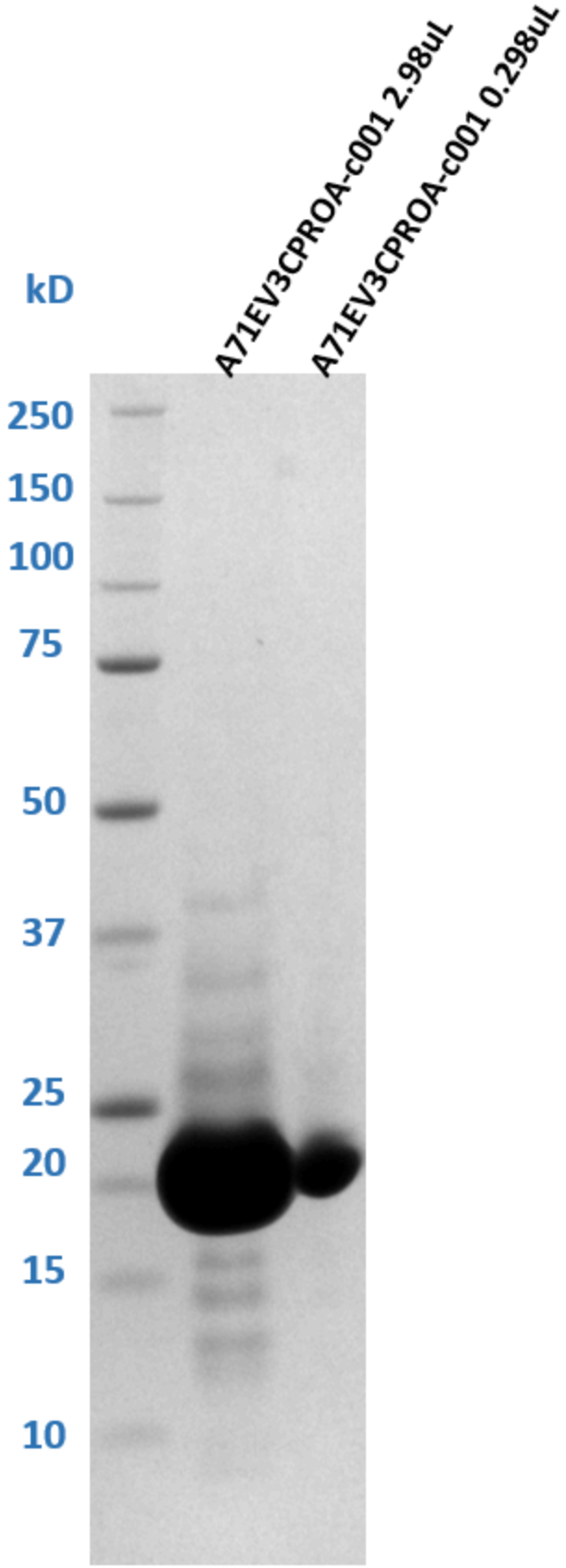
For example:



Overlay of 19 SEC runs of A71EV3C large scale expression. No fractions were taken for analysis, as it was determined from previous small scale A71EV3C purifications, that the peak indicated by red arrow contained the target protein (See "Enterovirus A71 3C protease small scale expression and purification protocol" for details). Due to the large number of SEC runs for one batch of expression, it was impractical to analyse fractions from individual runs.

10 Pool the fractions that contain the peak indicated by red arrow in the figure above. Concentrate to around [M] 30 mg/mL using a 10 kDa MWCO centrifugal concentrator.

Take 1 µL of the final sample for SDS-PAGE, and another for mass spectroscopy (result not shown here).


A71EV3CPROA-c001 2.98uL
A71EV3CPROA-c001 0.298uL
kD
250
150
100
75
50
37
25
20
15
10

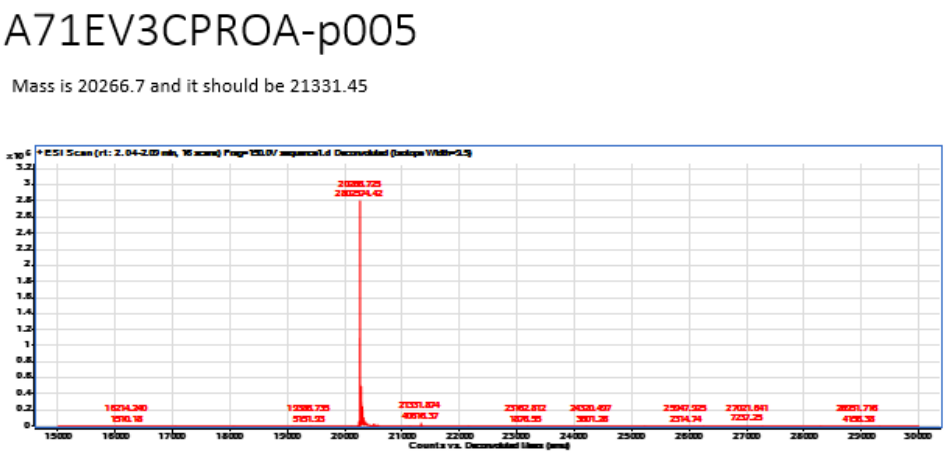
SDS-PAGE of the final purified A71EV3CPROA.

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 10L of culture was 264 mg of pure A71 EV 3C protease

NOTE: A71EV3C sample self-cleavage in storage

11 This A71EV3C construct exhibits self-cleavage of its C-terminal sequence: LEHHHHHH in -80 storage. This is caused by the presence of a 3C cleavage motif near the C-terminal His-tag, and the fact that this is an active construct.



Intact Mass Spectroscopy of sample after around 3 months of -80 storage. Mass shift is observed from the expected 21.331 kDa to 20.267 kDa.

A71EV3C

[1-192] mass = 21331.4
mass to match = 20266.0 ± 2.0

M[1-184]Q = 20266.3

1	M	G	P	S	L	D	F	A	L	S	L	L	R	R	N	I	R	Q	V	Q	T	D	Q	G	H	F	T	M	L	G	30
31	V	R	D	R	L	A	V	L	P	R	H	S	Q	P	G	K	T	I	W	V	E	H	K	L	I	N	I	L	D	A	60
61	V	E	L	V	D	E	Q	G	V	N	L	E	L	T	L	V	T	L	D	T	N	E	K	F	R	D	I	T	K	F	90
91	I	P	E	N	I	S	A	A	S	D	A	T	L	V	I	N	T	E	H	M	P	S	M	F	V	P	V	G	D	V	120
121	V	Q	Y	G	F	L	N	L	S	G	K	P	T	H	R	T	M	M	Y	N	F	P	T	K	A	G	Q	C	G	G	150
151	V	V	T	S	V	G	K	V	I	G	I	H	I	G	G	N	G	R	Q	G	F	C	A	G	L	K	R	S	Y	F	180
181	A	S	E	Q	L	E	H	H	H	H	H	H																			192

PAWS truncation analysis showing cleavage site