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Enterovirus coxsackievirus A16 2A protease small scale expression and purification protocol V.2

Version 1 is forked from SARS-CoV-2 Mpro 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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Abstract

This protocol details the expression and purification of coxsackievirus A16 2A protease construct bearing a N-terminal His-SUMO tag at small scale (<6L).

Picornaviridae Enterovirus coxsackievirus A16 (CVA16)

Attachments

PDF

PAGE23-01675 - Enter...

281KB

Guidelines

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

Materials

Plasmid details:

Addgene plasmid processing

- Vector: pNIC
- Cell line: *E. coli* Rosetta strain BL21(DE3)-RR
- Tags and additions: N-terminal His-SUMO tag
- Construct protein sequence:
  MHHHHHHGSGDQEAKPSTEDLGDKKEGEYIKLKVIGQDSSEIHFKVKMTTHLKKLKESYCQRQGVPMNSLRFLFEGQRIADNHT
  PKELGMEEEDVIEVYQEQTGGSGAIYVGNYRVVNRHLATHNDWANLVWEDSSRDLLVSSTTAQGCDTIARCDCQTGVYYCSSRR
  KHYPVSFSKPSLIFVEASEYYPARYQSHLMLAVGHSEPGDCGGILRCQHGVVGIVSTGGNGLVGFADVRDLLWLDEEAMEQ

Expression

AIM-TB: TB autoinduction media (Formedium AIMTB0210, ordered without added glucose and lactose)
After autoclaving, add 20mL of 50x AIM mix (400mL glycerol, 100g lactose, 25g glucose in 1L of ddH2O, filter sterilised) per L of media

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:
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%

	A	B
	TCEP	0.5 mM
	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	50 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
Wash buffer 1: base buffer + 30mM imidazole
Wash buffer 2: base buffer + 50mM imidazole
Elution buffer: base buffer, add 500mM imidazole
Gel filtration buffer:

	A	B
	Hepes (pH 7.5)	25 mM
	NaCl	300 mM
	Glycerol	5%
	TCEP	0.5 mM

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
FT - flow through

Plasmid Transformation

1d

2 Transform the coxsackievirus A16 2A protease plasmid into BL21(DE3) and store a glycerol stock of this at -80 °C
The coxsackievirus A16 2A protease plasmid encodes the 2A protease with an N-terminal His6-SUMO tag on a kanamycin resistant 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.

1d

4 Use the 10 mL starter culture to inoculate 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 typically 6 L of culture is grown for each 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} reaches approximately 3.0, lower the temperature and shaker speed to 180 rpm, 18°C and incubate Overnight

1d

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

> **Note**
>
> For reference: total pellet weight from 6L TB media should be around 90g.

Protein Purifcation 2d

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

7.1 1h

> **Note**
>
> See Materials tab for buffer compositions.

> **Note**
>
> **Coxsackievirus A16 2A protease His6-SUMO fusion protein properties**
>
> Before tag cleavage:
> MW=27.927 kDa
> E (assume all Cys reduced)=32890 mM-1cm-1
> PI=5.81
>
> After tag cleavage:
> MW=15.876 kDa
> E (assume all Cys reduced)=28975 mM-1cm-1
> PI=5.58
>
> These values are determined by Expasy ProtParam

Thaw and resuspend the pellet using ~7mL of lysis buffer per g of pellet. Stir gently at Room temperature for 00:30:00 to allow lysozyme and benzonase to start breaking down

cell components.

7.2 Lyse cells by sonication on ice 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 remains cold 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 the soluble fraction in a bottle 4 °C 1h

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

8.1 Dispense 5 mL of IMAC resin (Ni Sepharose 6 FF, Cytiva) into a gravity flow column. Rinse resin with ~ 10 CV distilled water to remove the storage solution and then ~ 10 CV binding buffer to equilibrate the resin. 10m

8.2 Resuspend the equilibrated resin with 10 mL of binding buffer and add to the supernatant bottle. Incubate the resin with the soluble fraction for 00:30:00 while rotating or otherwise mixing gently at 4 °C 30m

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

> **Note**
>
> For SDS-PAGE samples, mix 15 uL sample with 5 uL 4x sample buffer, supplemented with 10 mM 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. Collect washes separately for SDS-PAGE analysis. 30m

8.5 Elute the protein with 1.5 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. The total protein concentration of the elutions are then measured by Nanodrop. 20m

8.7 Wash used IMAC resin with 10 CV of base buffer, and leave the column submerged in a small amount of base buffer so 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 the elutions and desalt using a HiPrep 26/10 deasalting column, run on an AKTA pure at a maximum flow rate of 10mL/min. 30m

Note

Desalting reduces the concentration of imidazole in the sample which may inhibit SENP1 protease activity during tag cleavage as well as interfering with the reverse IMAC step.

10.2 Add His-SENP1 SUMO protease at a 1:100 ratio to the total protein content of the desalted sample, as determined by nanodrop. Incubate at 4 °C Overnight This cleaves the affinity tag. 1d

10.3 Pour the cleaved Coxsackievirus A16 2A protease, SUMO tag, SENP1 protease mixture over the washed IMAC resin and collect the flow through, rIMAC. 30m

Note

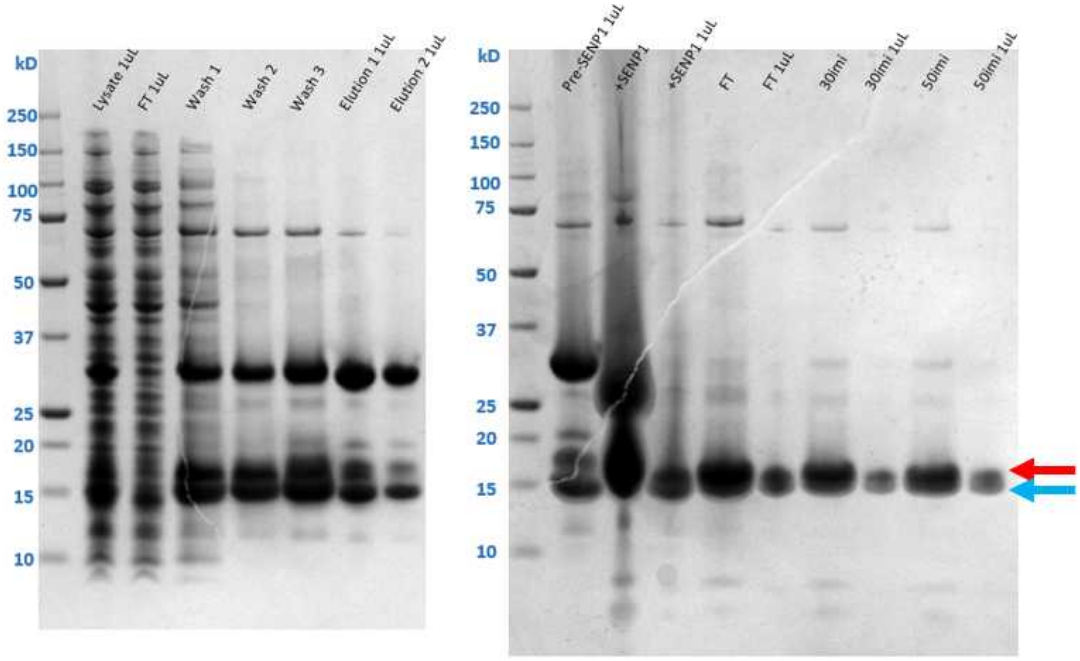
This step will remove the cleaved tag and any un-cleaved target from the sample. As the SENP1 protease used is His-tagged, this is removed from the sample too.

10.4 Wash the IMAC resin with 2 CV wash buffer 1 and 2 to remove any target protein still bound to the resin. Take samples of the FT and washes for SDS-PAGE analysis. 30m

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 IMAC resin matrix material, and help in further troubleshooting if the final yield is lower than expected.
Despite having a His6 on it the cleaved SUMO tag is usually found contaminating the rIMAC fractions containing coxsackievirus A16 2A protease. The tag can however be removed in the subsequent gel filtration step.



SDS-PAGE analysis of IMAC and cleavage fractions. The higher molecular weight band in the right-hand-side gel image, highlighted by red arrow, agrees with the size of cleaved target protein, while the lower band highlighted with blue arrow corresponds to the size of the cleaved SUMO tag (12.372 kDa but band appears at ~15kDa on the SDS-PAGE gels used). Both bands are also observed throughout the IMAC fractions. This is because the coxsackievirus A16 2A protease construct used exhibits auto-cleavage activity during expression and purification.

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

.

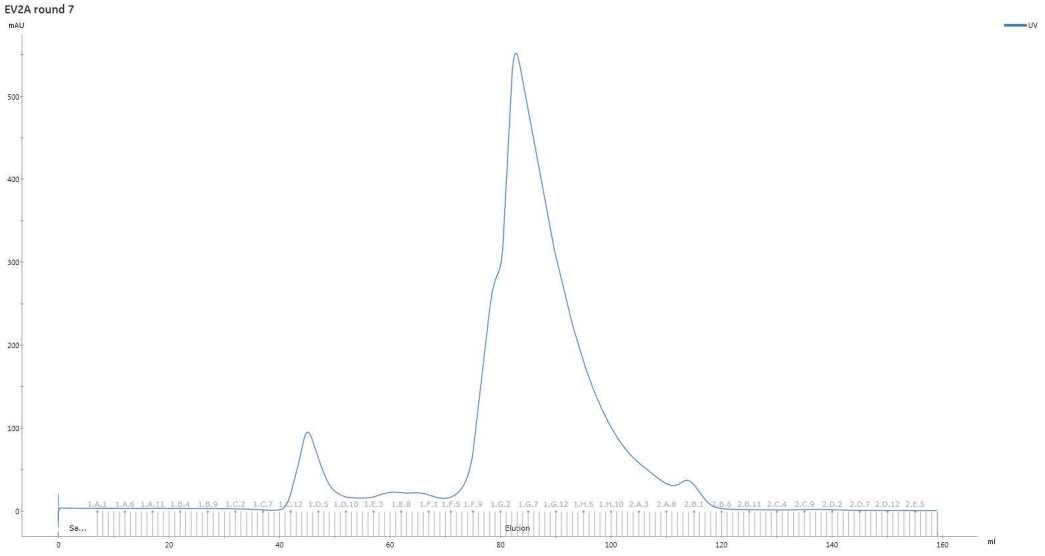
11.2 Remove any solid aggregates from the sample by centrifugation at 17200 x g, 4°C, 00:10:00 , then immediatly draw up the supernatant with a 5mL syringe and a blunt-tip fill needle, taking care not to disturb the pellet. 15m

> 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.

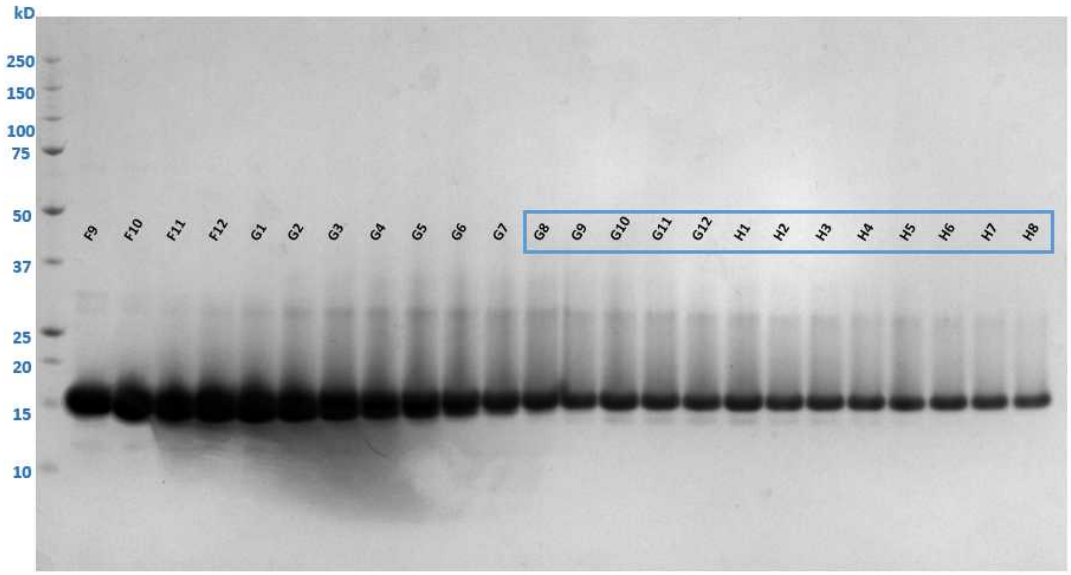
12 Using an AKTA Pure system: 2h

Inject the sample onto a 5mL sample loop and run the sample down HiLoad 16/60 Superdex 75 pg gel filtration column at 1 mL/min using gel filtration buffer as the mobile phase, collect 1mL fractions.

13 Analyze the size exclusion chromatography fractions by SDS-PAGE and pool the fractions with highest amounts of pure 2A protease. 1h



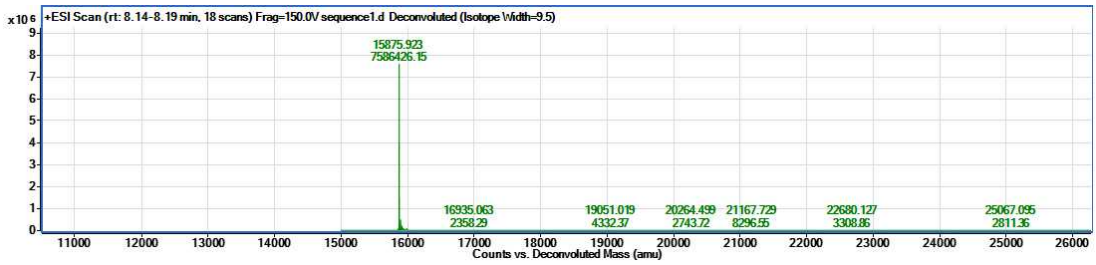
Chromatogram of coxsackievirus A16 2A protease SEC run: Fractions F9-H8 were analyzed by SDS-PAGE to see which contained the target protein



SDS-PAGE analysis of SEC fractions F9-H8. Fractions G8-H8 were pooled as they contain majority coxsackievirus A16 2A protease in comparison to contaminating SUMO.

13.1 Take the fractions that contain the cleanest target protein and concentrate to [M] 15 mg/mL using a 10 kDa MWCO centrifugal concentrator 10m

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



Intact mass spectroscopy result of the purified coxsackievirus A16 2A protease sample.
No mass corresponding to the SUMO tag (12.05 kDa) is observed in the final sample, only that of the untagged coxsackievirus 2A protease, 15.88 kDa.

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. 30m

For example:
The final yield from processing 6 L of cells was 50 mg of pure coxsackievirus A16 2A protease