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SARS-CoV-2 Mpro small scale expression and 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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We use this protocol and it's working

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Abstract

This protocol details the expression and purification of SARS Mpro construct bearing a N-terminal His-SUMO tag at small scale (<6L).

Attachments

PAGE24-00585 - CVMPR...
271KB

Guidelines

- **Construct / plasmid resource-name:** SARS-Mpro 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 His-SUMO tag
- Construct protein sequence: `
  MGSSHHHHHHGSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQAD
  QTPEDLDMEDNDIIEAHREQIGGSGFRKMAFPSGKVEGCMVQVTCGTTTLNGLWLDDVVYCPRHVICTSEDMLNPNYEDLLIRKS
  NHNFLVQAGNVQLRVIGHSMQNCVLKLKVDTANPKTPKYKFVRIQPGQTFSVLACYNGSPSGVYQCAMRPNFTIKGSFLNGSCG
  SVGFNIDYDCVSFCYMHHMELPTGVHAGTDLEGNFYGPFVDRQTAQAAGTDTTITVNVLAWLYAAVINGDRWFLNRFTTTLNDF
  NLVAMKYNYEPLTQDHVDILGPLSAQTGIAVLDMCASLKELLQNGMNGRTILGSALLEDEFTPFDVVRQCSGVTFQNTFTNLVVQ
  LIRNMEAEEVLEMQDLWLLRKPEKVTRWLQSNGWDRLKRMAVSGDDCVVKPIDDRFAHALRFLNDMGKVRKDTQEWKPSTGW
  SNWEEVPFCSHHFNKLYLKDGRSIVVPCRHQDELIGRARVSPGAGWSIRETACLAKSYAQMWQLLYFHRRDLRLMANAICSAVP
  VDWVPTGRTTWSIHGKGEWMTTEDMLMVWNRVWIEENDHMEDKTPVTKWTDIPYLGKREDLWCGSLIGHRPRTTWAENIKDT
  VNMVRRIIGDEEKYMDYLSTQVRYLGEEGSTPGVL

Expression
TB media (Invitrogen, 22711022)
1M IPTG stock solution

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 200 pg (Cytiva, 28989335)

5mL sample loop

SDS-PAGE sample buffer, gel, and gel tank

Lysis buffer:

	A	B
	Hepes (pH 7.5)	50 mM
	NaCl	150 mM
	Glycerol	5%
	Imidazole	20 mM
	TCEP	0.5 mM
	Lysozyme	0.5 mg/mL
	Benzonase	0.05 mg/mL

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

Base buffer:

	A	B
	Hepes (pH 7.5)	50 mM
	NaCl	150 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: base buffer

Note

His-SUMO SARS-Mpro has exhibited poor binding interaction with Ni Sepharose FF (Cytiva) in previous purification attempts. Thus no imidazole was included in the wash buffer to reduce product loss.

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
FT - flow through

Plasmid Transformation

1d

2 Transform the SARS-Cov-2 Mpro construct into BL21(DE3) and store a glycerol stock of this at -80 °C

> **Note**
>
> The SARS-CoV-2 Mpro construct encodes the 3C protease with a N-terminal his6-SUMO 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.

1d

4 Use the 10 mL starter culture to inoculate 1 L TB media supplemented with [M] 50 ug/mL kanamycin in a baffled flask. 200 rpm, 37°C

6h

> **Note**
>
> For this protocol 6 L of pellet was grown for purification

5 When the OD_{600} reaches approximately 1.8, add 0.5 mM IPTG. Lower the temperature and shaker speed to 180 rpm, 18°C and incubate Overnight

1d

6 Harvest the cell 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 6 L TB media was 88g.

Protein Purifcation 2d

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

7.1 1h

Note

See Materials tab for buffer compositions.

Note

SARS-CoV-2 Mpro His6-SUMO fusion protein properties

Before tag cleavage:
MW=46.151 kDa
E (assume all Cys reduced)=34380 mM-1cm-1
PI=5.83

After tag cleavage:
MW=33.797 kDa
E (assume all Cys reduced)=32890 mM-1cm-1
PI=5.95

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 sample remains at 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 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 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

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 15 uL sample with 5 uL 4x sample buffer, supplemented with 10 mM DTT.

8.4 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.
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 a further 2 times, collecting a total of 3 separate elution fractions. This is to ensure maximum retrieval of protein from the resin. 20m

The total protein concentration of the elutions are measured 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.

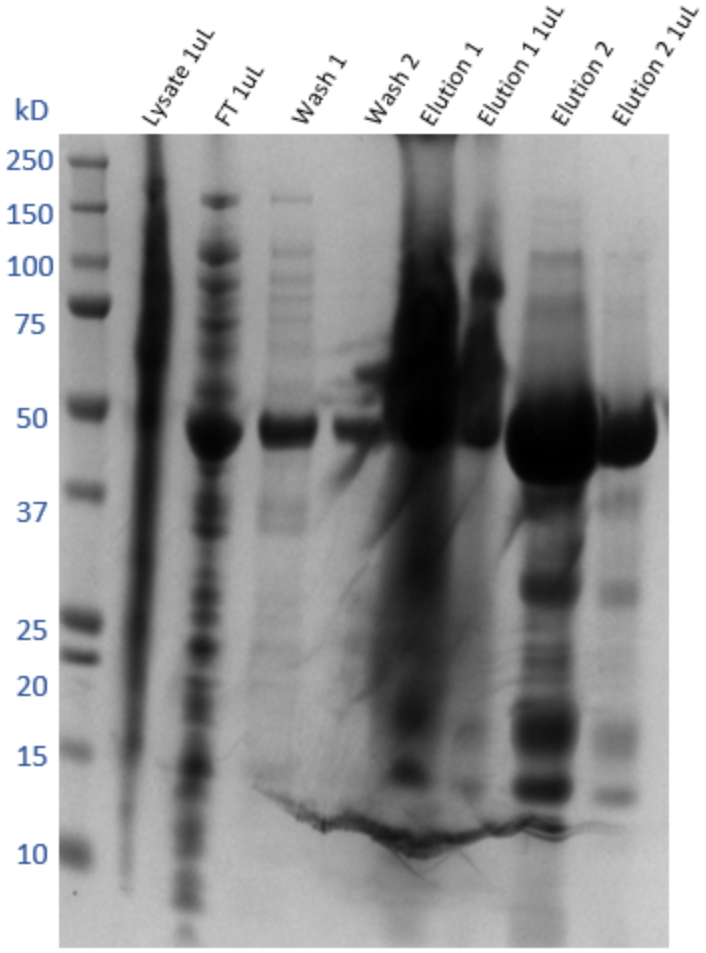
8.7 Wash used IMAC resin with 10 CV of base buffer, and leave the column submerged in a small amount of base buffer so that the resin is kept moist. This washed IMAC resin will later be reused for reverse IMAC (rIMAC)

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.

9.1



SDS-PAGE analysis of IMAC fractions. The thick protein band observed in all three elutions corresponds with the expected molecular weight of the His-SUMO SARS-CoV-2 Mpro fusion protein, 46.1 kDa.

10 Elution de-salting, tag cleavage and reverse IMAC

1d

10.1 Pool and desalt the elutions using HiPrep 26/10 deasalting columns, run on AKTA pure at the 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.

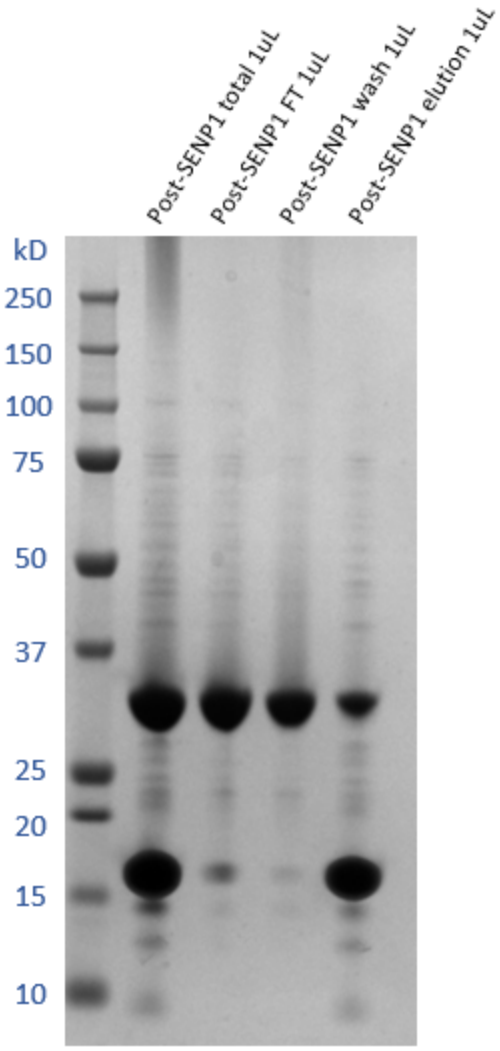
10.2 Add His-SENP1 SUMO protease at a 1:100 ratio to the total protein content of the desalted sample, as determined by nanodrop. Incubate at 4 °C Overnight This cleaves the affinity tag. 1d

10.3 Pour the cleaved SARS-CoV-2 Mpro, 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 rIMAC resin with 2 CV wash buffer 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 cleavage fractions. The higher molecular weight band agrees with the size of SARS-CoV-2 Mpro after SUMO tag cleavage, 33.8 kDa. The lower band corresponds to the size of the cleaved SUMO tag (12.4 kDa but band appears at ~15kDa on the SDS-PAGE gels used)

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 immediatly draw up the supernatant with a 5mL syringe and a blunt-tip fill needle, taking care not to disturb the pellet. 15m

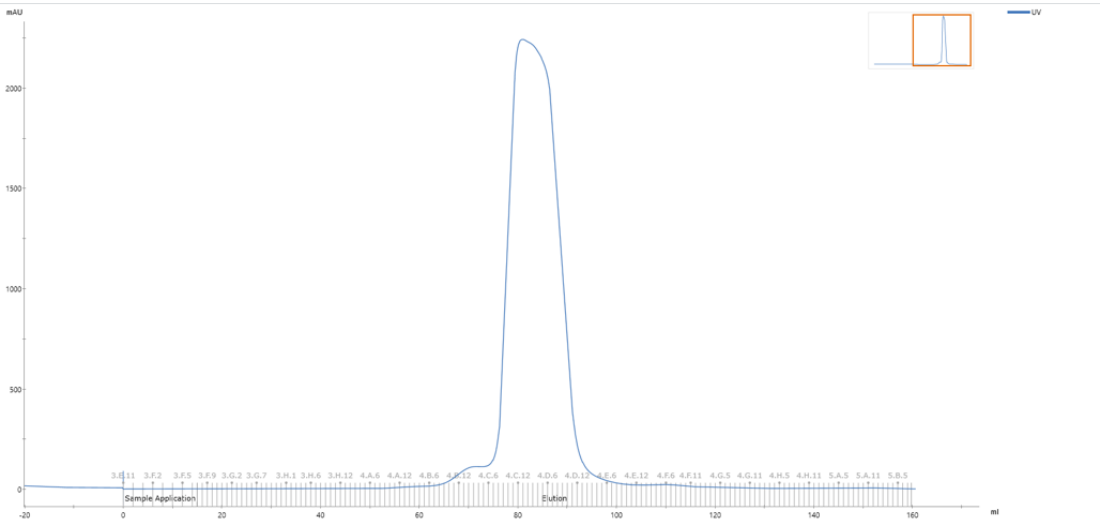
Note

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.

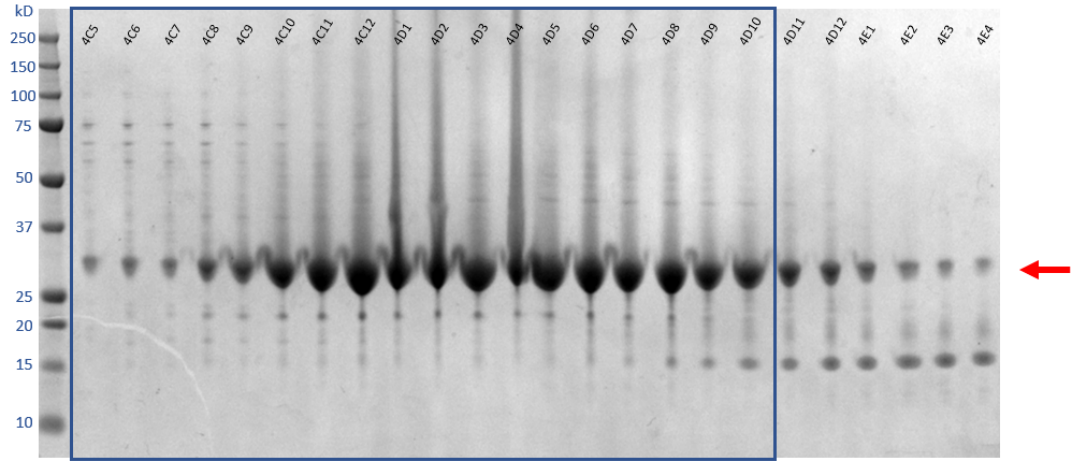
12 Using an AKTA Pure system: 2h

Inject the sample onto a 5mL sample loop and run the sample down HiLoad 16/60 Superdex 200 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 SARS CoV-2 MPro. 1h



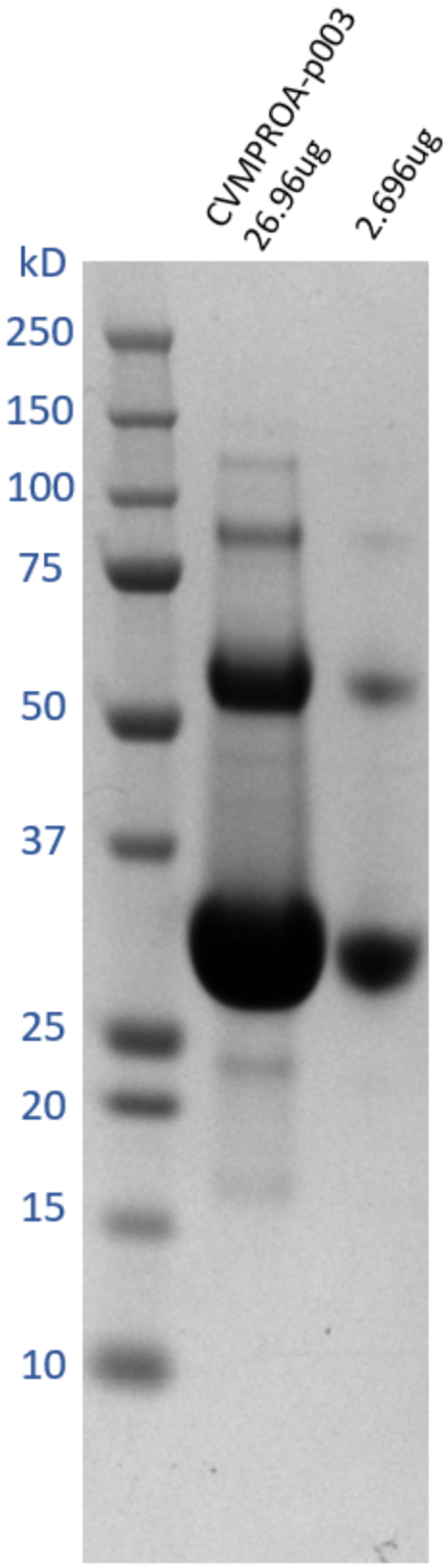
Chromatogram of the SARS-CoV-2 Mpro SEC run. Fractions 4C5-4E2 were analyzed by SDS-PAGE to see which contained the target protein



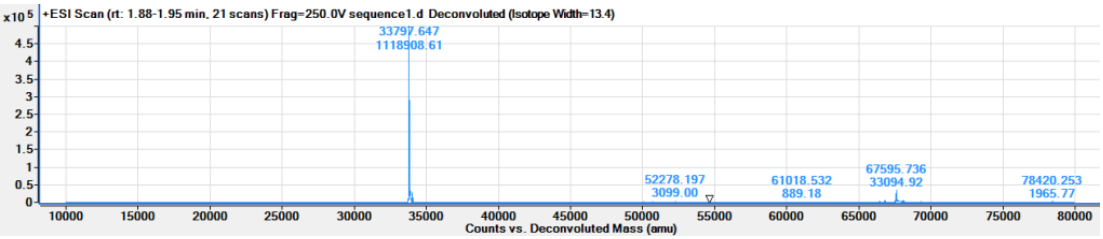
SDS-PAGE analysis of SEC fraction 4C5-4E2. Fractions 4C5-4D10 were pooled as they contain majority target protein in comparison to contaminants.
Red arrow indicated the position of bands corresponding to the target protein.

13.1 Take the fractions that contain the cleanest target protein and concentrate to [M] 33 mg/mL using a 10 kDa MWCO centrifugal concentrator. 30m

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



SDS-PAGE of the final purified SARS-Cov-2 Mpro construct. The higher molecular weight bands are likely polymeric forms of the target that failed to be monomerized during SDS-PAGE sample preparation. MS will confirm the purity of the sample.



IIntact mass spectroscopy result of the purified SARS-Cov-2 Mpro sample. The major peak molecular weight (33797.6 Da) agrees with the expected weight (33797 Da), confirming the purity of the sample.

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

For example:
The final yield from processing 6 L of cells was 128 mg of pure SARS-Cov-2 Mpro