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MERS-CoV Mpro large scale purification protocol

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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We use this protocol and it's working

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Abstract

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

Attachments

PAGE24-00563 - MVMPR...
324KB

Guidelines

- **Construct / plasmid resource-name: MERS**-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: `
  MHHHHHHGSGDQEAKPSTEDLGDKKEGEYIKLKVIGQDSSEIHFKVKMTTHLKKLKESYCQRQGVPMNSLRFLFEGQRIADNHT
  PKELGMEEEDVIEVYQEQTGG/////SGLVKMSHPSGDVEACMVQVTCGSMTLNGLWLDNTVWCPRHVMCPADQLSDPNYDALLI
  SMTNHSFSVQKHIGAPANLRVVGHAMQGTLLKLTVDVANPSTPAYTFTTVKPGAAFSVLACYNGRPTGTFTVVMRPNYTIKGSF
  LCGSCGSVGYTKEGSVINFCYMHQMELANGTHTGSAFDGTMYGAFMDKQVHQVQLTDKYCSVNVVAWLYAAILNGCAWFVKP
  NRTSVVSFNEWALANQFTEFVGTQSVDMLAVKTGVAIEQLLYAIQQLYTGFQGKQILGSTMLEDEFTPEDVNMQIMGVVMQ

Purification

Chicken hen egg white lysozyme
Benzonase
Imidazole
Ni Sepharose 6 FF resin
Gravity flow column, 2.5cm diameter
Centrifugal concentrators, 10kDa MWCO

On an FPLC system:
SEPAX SEC SRT-100 or Cytiva HiLoad 16/600 Superdex 200 pg
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
	Lysozyme	0.5 mg/mL
	Benzonase	0.05 mg/mL

Prepare 100L per 1L E.coli expression

Base buffer:

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

Prepare 2L per 6L 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: base buffer, but HEPES concentration reduced to 10mM

SDS-PAGE gel: NuPage 4-12%, Bis-Tris protein gel, 27 well.
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 MERS Mpro N-terminal His-SUMO tagged construct was inoculated from its BL21(DE3)-RR glycerol stock.

> **Note**
>
> MERS Mpro plasmid was transformed into BL21(DE3)-RR strain along other constructs, and stored at -80 °C .

Protein Expression

3 See (Nathan's protocol DOI) for MERS-MPro large scale expression protocol

Protein Purifcation

2d

4 **Lyse cell pellet**

2h 30m

4.1

1h

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

Note

Before tag cleavage:
MW=45.382 kDa
E (assume all Cys reduced)=48360 mM-1cm-1
PI=5.92

After tag cleavage:
MW=33.330 kDa
E (assume all Cys reduced)=43890 mM-1cm-1
PI=5.86

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.

4.2 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 pellet is 0 °C during sonication to prevent overheating. 7m 16s

4.3 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

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

5.1 Dispense 5 mL Nickle affinity resin Ni Sepharose 6 FF - Cytiva into a gravity flow column. Equilibrate resin by first rinsing with ~ 10 CV distilled water, then ~ 10 CV binding buffer to remove the storage solution. 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 FT 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 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

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

5.6 Repeat step 5.5 once more, collecting a total of 2 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.

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

6 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.

7 Elution de-salting, tag cleavage and reverse IMAC 1d

7.1 The three elutions are pooled and desalted using HiPrep 26/10 deasalting columns, run on AKTA pure at the maximum flow rate of 10mL/min. 30m

Note

This is to reduce imidazole concentration in the sample. High concentration of imidazole will inhibit protease activity during tag cleavage and removal.

7.2 For tag removal, His-SENP1 is added in 1:100 ratio to the total protein content of the desalted sample, as determined by nanodrop. The mixture is left at 4 °C Overnight 1d

7.3 In morning, pour the cleavage mixture over the washed resin and collect FT. 30m

Note

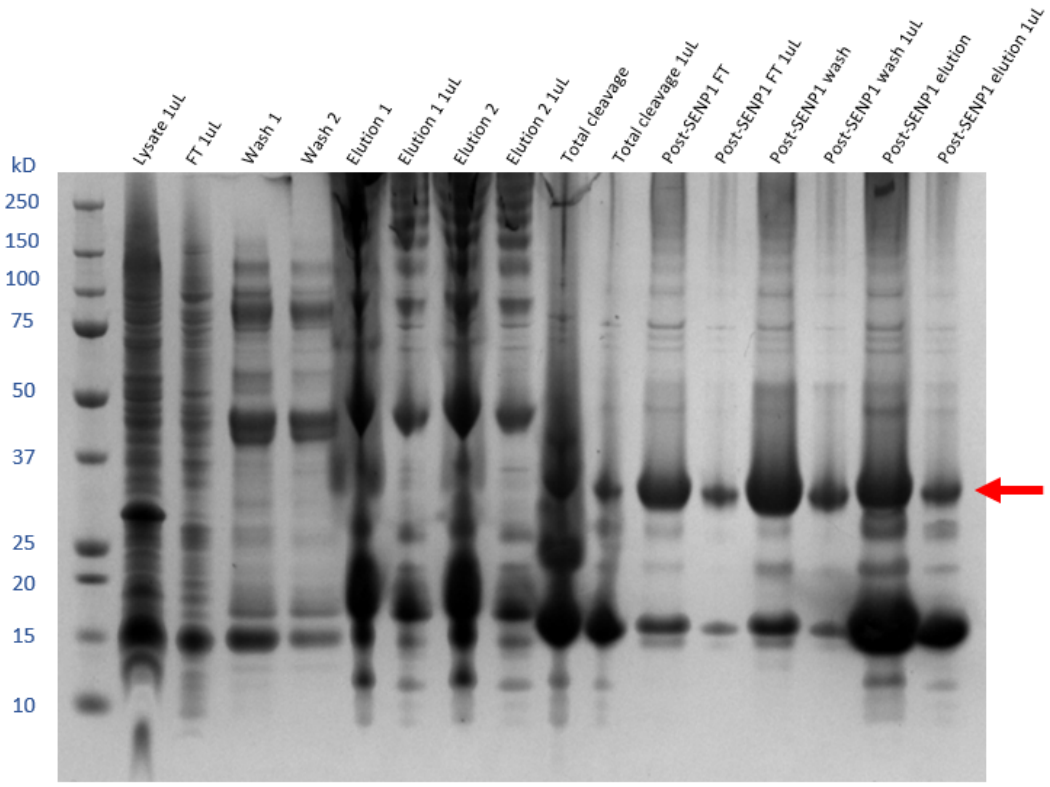
This step will remove the cleaved tag and any uncleaved target from the sample. If the protease used is His-tagged, then the protease is removed from sample too.

7.4 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

7.5 (Optional) elute rIMAC resin with 2 CV elution buffer to confirm if the protein shows non-specific binding to the resin used.

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.



SDS-PAGE analysis of IMAC and cleavage fractions. The higher molecular weight band, highlighted with red arrow, agrees with the size of cleaved target protein, while the lower band corresponds to the size of the cleaved SUMO tag (12.372 kDa but band appears at ~15kDa on the SDS-PAGE gels used)

8 Purify sample further by size exclusion chromatography.

6h

8.1 Concentrate all fractions of the rIMAC containing the target protein in spin concentrators of the appropriate MWCO, to a final volume of under 5 mL .

1h

8.2 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

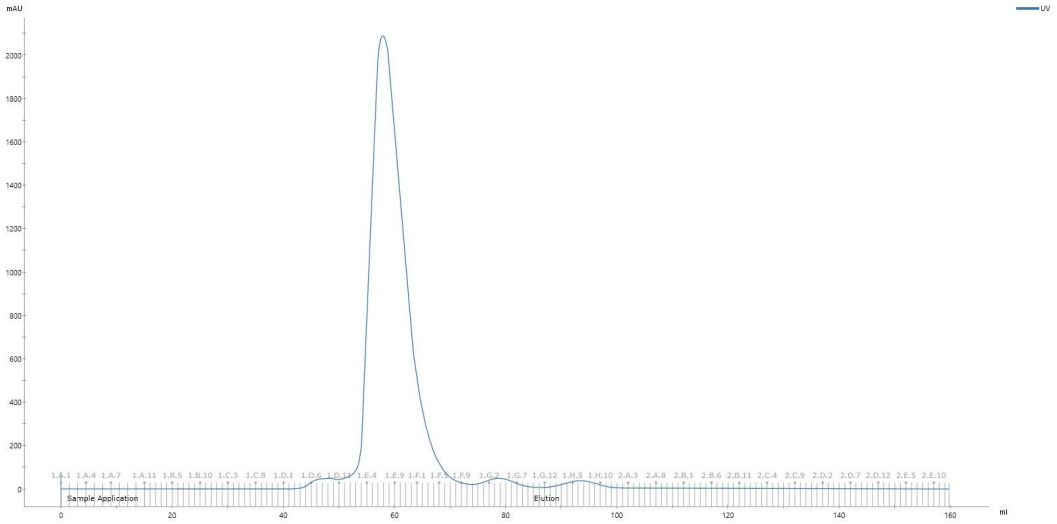
This is to remove as much solid particles from the injection sample as possible, so as to not clog the in-line filter or frit of the column.

9 Using the AKTA Pure system: 2h

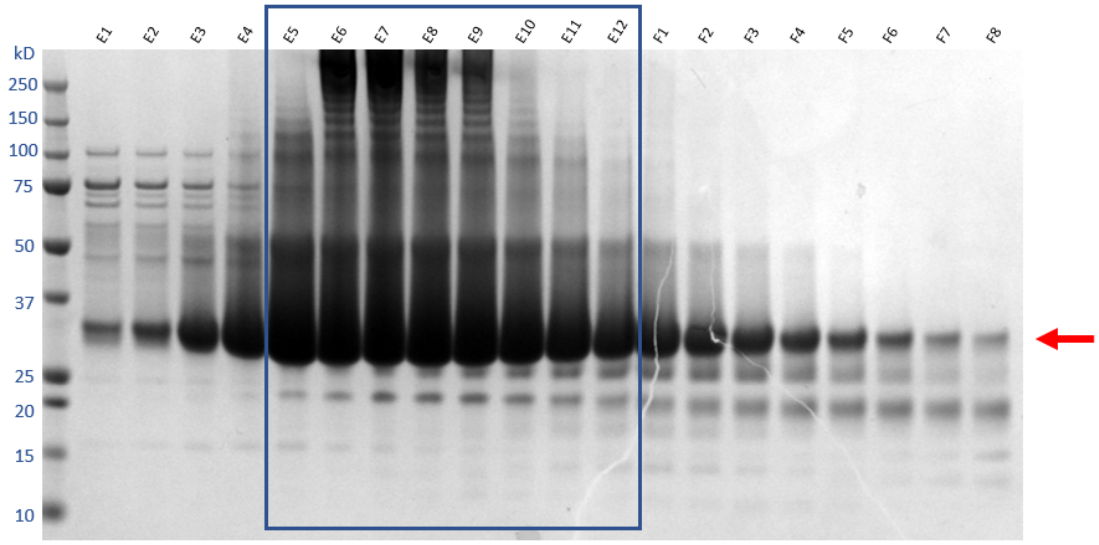
Inject the sample onto a 5mL sample loop.

Run the sample down HiLoad 16/60 Superdex 200 pg gel filtration column at 1mL/min in gel filtration buffer, collecting 1mL aliquots.

10 From the chromatogram, fraction E1-F8 analyse by SDS-PAGE. 30m



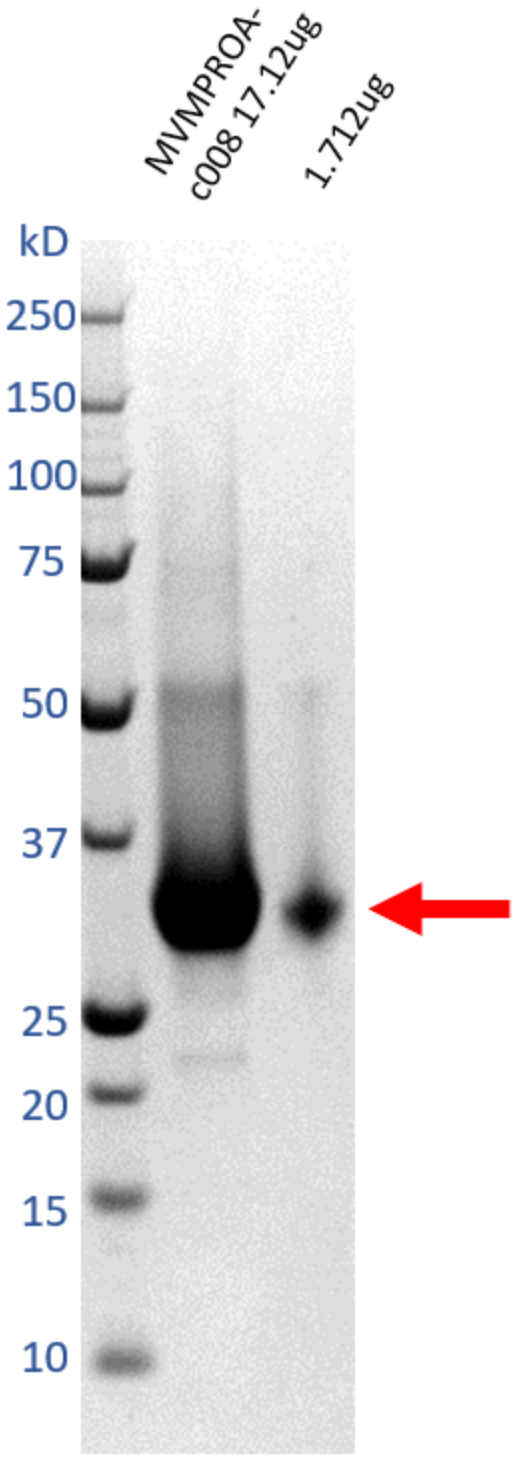
Chromatogram for MERS-Mpro SEC



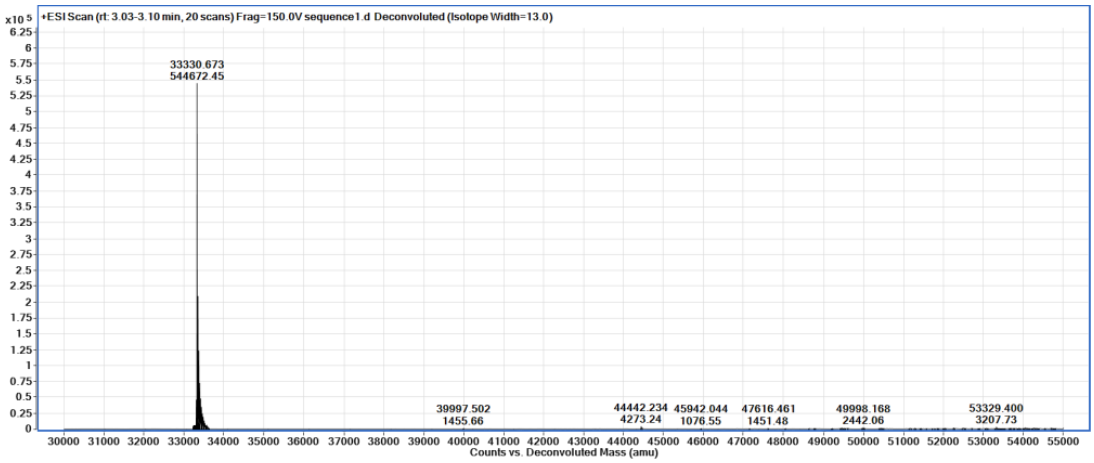
SDS-PAGE analysis of SEC fraction E1-F8. Fractions E5-E12 were pooled as they contain majority target protein in comparison to contaminants.
Red arrow indicated the position of bands corresponding to the target protein.

10.1 Take the fractions that contain the target protein, which in this case are fraction E1-F6. Concentrate the final sample in Vivaspin 6 10kda MWCO centrifugal concentrator until the concentration reaches > [M] 33 mg/mL or [M] 1 millimolar (mM) . 30m

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



SDS-PAGE of the final purified MERS-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.



Intact MS result of the final sample. Major peak molecular weight (33330.673 Da) agrees with the expected weight (33330.29 Da), confirming the purity of the sample.

10.2 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