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SARS-CoV-2 nsp3 macrodomain His-SUMO tagged expression and purification protocol for crystallization V.2

Version 1 is forked from SARS-CoV-2 nsp3 macrodomain expression and purification protocol for crystallization

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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 SARS-CoV-2 nsp3 macrodomain crystallization construct bearing a N-terminal His-SUMO tag and truncation of the N-terminal methionine at small scale (<6L). In this version we added the Addgene id.

Attachments

PDF

PAGE23-01317 - SRF-B...

487KB

Guidelines

- **Construct / plasmid resource-name:** SARS-CoV-2 nsp3 macrodomain crystallization construct bearing a N-terminal His-SUMO tag and truncation of the first N-terminal methionine.

Materials

Plasmid details:
Internal ID CVNSP3A-c004

- Vector: pNIC
- Cell line: E. coli Rosetta strain BL21(DE3)-RR
- Tags and additions: N-terminal His-SUMO tag. Removal of the N-terminal methionine
- Construct protein sequence:
  MGSSHHHHHHMASMSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFL
  YDGIRIQADQTPEDLDMEDNDIIEAHREQIGGGEVNSFSGYLKLTDNVYIKNADIVEEAKKVKPTVVVNAANVYLKHGGG
  VAGALNKATNNAMQVESDDYIATNGPLKVGGSCVLSGHNLAKHCLHVVGPNVNKGEDIQLLKSAYENFNQHEVLLAPLL
  SAGIFGADPIHSLRVCVDTVRTNVYLAVFDKNLYDKLVSSFLE

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
Benzonase
Imidazole
Ni Sepharose 6 FF resin
Gravity flow column, 2.5cm diameter
Centrifugal concentrators, 30kDa MWCO

On an FPLC system:
Cytiva HiLoad 16/600 Superdex 75 pg
5mL sample loop
HiPrep 26/10 deasalting column

SDS-PAGE sample buffer, gel, and gel tank

Lysis buffer:

	A	B
	Hepes (pH 7.5)	10 mM
	NaCl	500 mM
	Glycerol	5%
	TCEP	0.5 mM

	A	B
	Lysozyme	0.5 mg/mL
	Benzonase	0.05 mg/mL
	Imidazole	30mM

Prepare 100L per 1L E.coli expression

Base buffer:

	A	B
	Hepes (pH 7.4)	10 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 + 30mM imidazole
Elution buffer: base buffer, add 500mM imidazole
Gel filtration buffer: 20mM Tris-HCl, 150mM NaCl, 5% glycerol, 1mM TCEP, pH8 at RT

SDS-PAGE gel: NuPage 4-12%, Bis-Tris protein gel, 27 well.
Run in MES buffer, 200V 35mins.

Protocol materials

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) isolate:Wuhan-Hu-1 nsp3 macrodomain **addgene Catalog #**228642

Troubleshooting

Abbreviations

1 CV - column volume, total volume of resin in a column
IMAC - immobilised metal affinity chromatography
FT - flow through
CVNSP3mac1 - SARS-CoV-2 nsp3 macrodomain

Plasmid Transformation

1d

2 CVNSP3mac1 N-terminal His-SUMO-tagged construct was inoculated from its BL21(DE3)-RR glycerol stock.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) isolate:Wuhan-Hu-1 nsp3 macrodomain **addgene Catalog #**228642

Note

This CVNSP3mac1 construct encodes the SARS-CoV-2 nsp3 macrodomain with a N-terminal His-SUMO tag fusion, and truncation of the first methionine residue, in pNIC vector.

(See Mike's plasmid design. Removal of the first Met apparently results in better crystallization)

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 Mass Percent kanamycin. Grow the starter culture at 37 °C Overnight with 200 rpm shaking.

4h

4 Use the 10 mL starter culture to inoculate 1 L autoinduction TB media (see Materials) supplemented with [M] 50 Mass Percent kanamycin in a baffled flask.

200 rpm, 37°C

6h

Note

For this protocol 6L of pellet was grown for purification.

5 When the OD_{600} approximately 2.0, lower the temperature and shaker speed to 180 rpm, 18°C . Incubate overnight. 1d

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

Protein Purifcation 2d

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

7.1 1h

Note

See Materials tab for buffer compositions.

Note

His-SUMO-CVNSP3mac1 properties

Before tag cleavage:
MW =30.973 kDa
E (assume all Cys reduced)= 11920 mM-1cm-1
PI = 5.95

After tag cleavage:
MW = 18.254 kDa
E(assume all Cys reduced) = 10430
PI = 6.31

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 00:00:02 On 00:00:04 Off for a total 'on' time of 00:10:00 at 35% amplitude to fully rupture the cells. Ensure pellet is 0 °C during sonication to prevent overheating. 10m 6s

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

8.2 Resuspend the equilibrated resin with some binding buffer and add to the supernatant bottle. Incubate the resin with the supernatant for 01:00:00 while rotating or otherwise mixing gently at 4 °C 1h

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

8.4 Wash the column with 10 µL of base buffer, followed by 10 µL 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 7.5 mL 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. 20m

Measure the total protein concentration of the elutions 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 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)

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

Other desalting methods may be used, such as passing through PD-10 columns, or dialysis.

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.

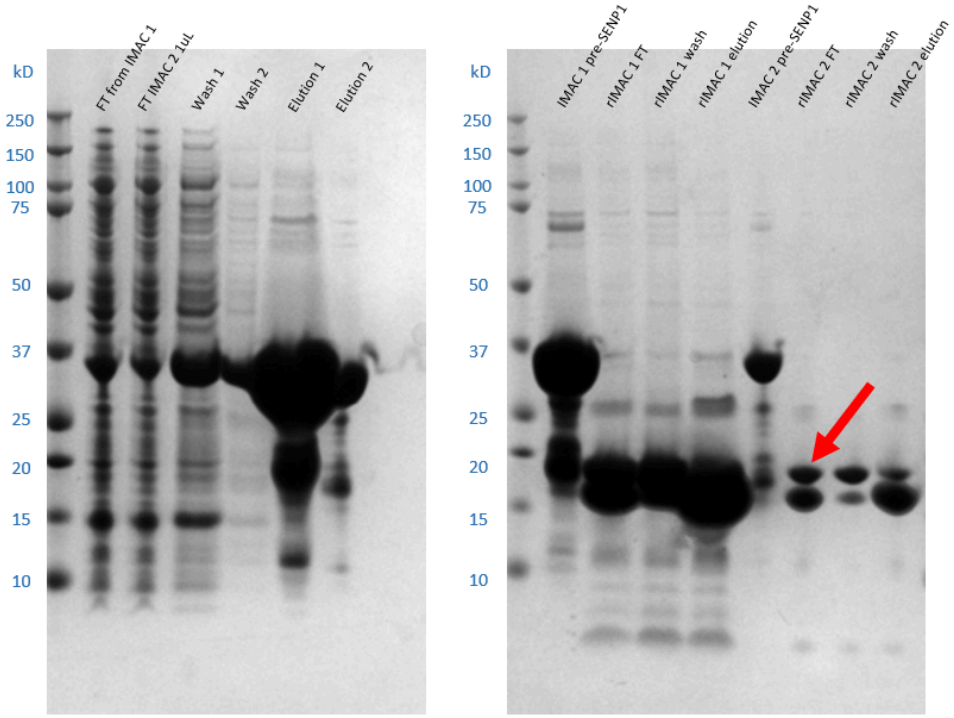
10.2 For tag removal, add His-SENP1 in 1:100 ratio to the total protein content of the diluted sample, as determined by nanodrop. Leave mixture to stand in the cold room at 1d
4 °C Overnight

10.3 In morning, pour the cleavage mixture over the washed resin three times and collect final 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.

10.4 Take samples of the rIMAC FT and wash and characterise content by SDS-PAGE 30m



SDS-PAGE analysis of IMAC and cleavage fractions. The band indicated by red arrow in rIMAC FT corresponds to the correct size of the cleaved target protein (18.254 kDa). The band below is likely to be the cleaved His-SUMO tag.

10.5 (Optional) elute rIMAC resin with 2 µL 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 immediately draw up the supernatant with a 5mL syringe and a blunt-tip fill needle, taking care not to disturb the pellet. 15m

Note

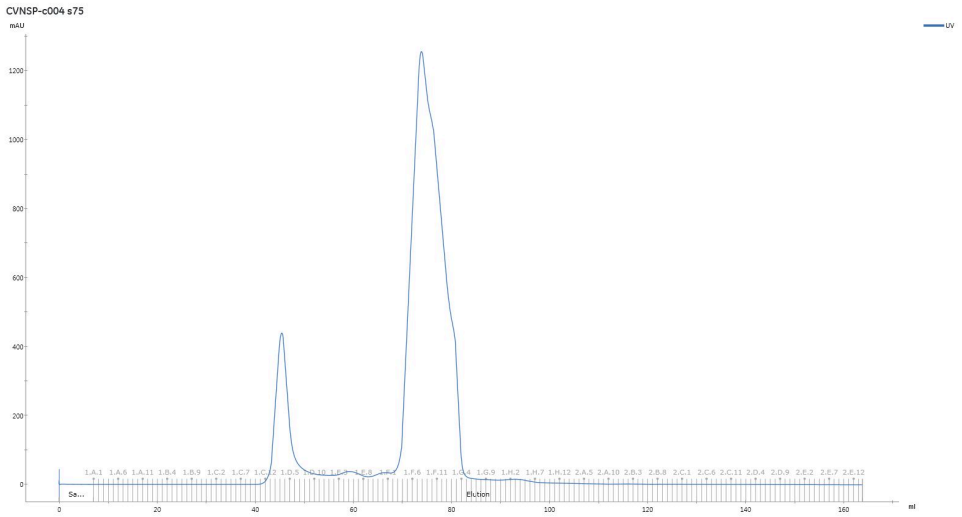
This is to remove as much solid particles from the injection sample as possible, so as to not clog the in-line filter or frit of the column.

12 Using the AKTA Pure system: 2h

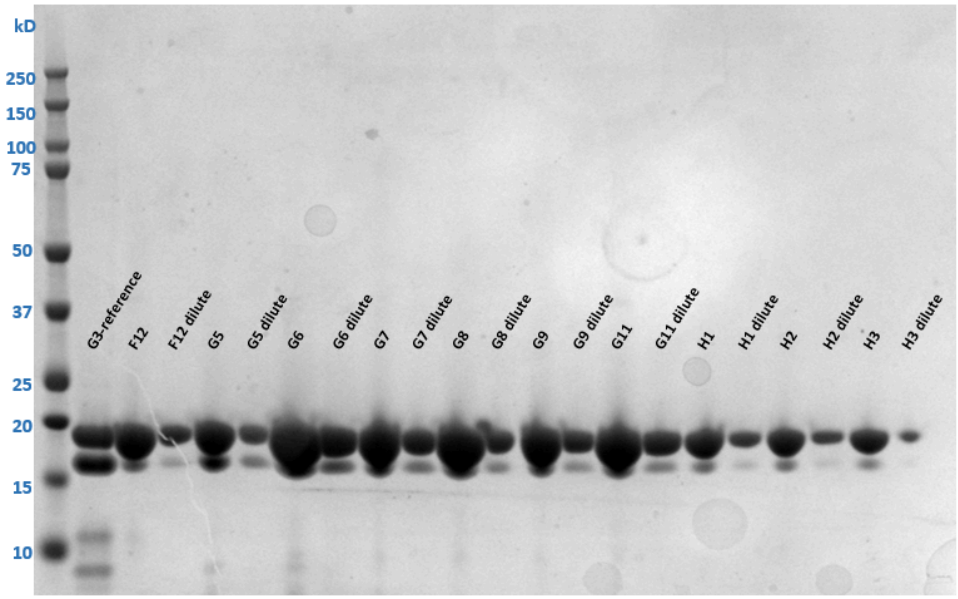
Inject the sample onto a 5mL sample loop.

Run the sample down HiLoad 16/60 Superdex 75 pg gel filtration column at 1mL/min in gel filtration buffer, collecting 1mL aliquots.

13 From the chromatogram, fraction F9-H8 analyse by SDS-PAGE. 1h



Chromatogram of the cleaved His-SUMO CVNSP3mac1 SEC run. Fractions E1-H5 were analysed by SDS-PAGE to see which contained the target protein

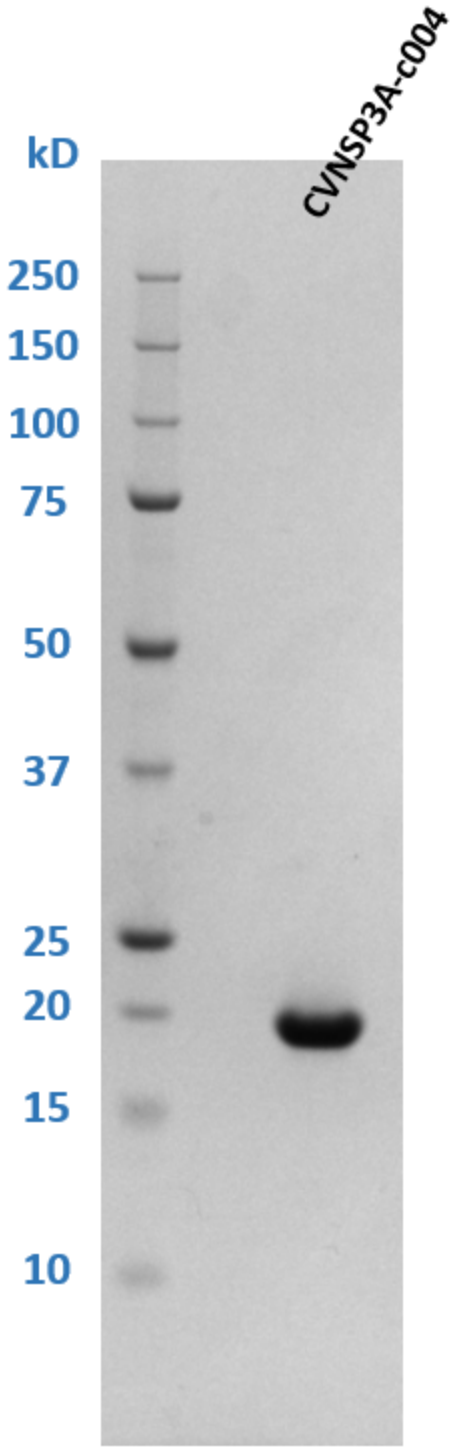


SDS-PAGE analysis of SEC fraction F12 and G3-H3. Please note that due to fraction collector malfunction, this SDS-PAGE result should not be used to reference which fractions may contain the protein. In this instance, fractions F12 and G5-H5 were pooled as they contain majority target protein in comparison to contaminants.

13.1 Take the fractions that contain the target protein, which in this case are fraction F12 and G5-H5. Concentrate the final sample in Vivaspin 500 10kda MWCO centrifugal 30m

concentrator until the concentration reaches > [M] 18 mg/mL , or around [M] 1 millimolar (mM)

Take 1 µL of the final sample for SDS-PAGE. Intact MS can also be carried out to confirm sample purity.



SDS-PAGE analysis of final 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