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SARS-CoV-2 nsp3 macrodomain expression and purification protocol for crystallization

Forked from Zika NS5 RdRp His-SUMO construct 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-CoV-2 nsp3 macrodomain crystallization construct bearing a N-terminal His-tag at small scale (<6L).

Attachments

PDF

PAGE24-00789 - CVNSP...

493KB

Guidelines

- **Construct / plasmid resource-name:** SARS-CoV-2 nsp3 macrodomain crystallization construct bearing a N-terminal His-tag.

Materials

Plasmid details:

- Vector: pNIC28-Bsa4
- Cell line: E. coli Rosetta strain BL21(DE3)-RR
- Tags and additions: N-terminal His-tag
- Construct protein sequence: MHHHHHHSSGVDLGTENLYFQSMVNSFSGYLKLTDNVYIKNADIVEEAKKVKPTVVVNAANVYLKHGGGVAGALNKATNNAM QVESDDYIATNGPLKVGGSCVLSGHNLAKHCLHVVGPNVNKGEDIQLLKSAYENFNQHEVLLAPLLSAGIFGADPIHSLRVCVDT VRTNVYLAVFDKNLYDKLVSSFL

Expression
TB media,mM IPTG

Purification
Chicken hen egg white lysozyme
Benzonase
Imidazole
Ni Sepharose 6 FF resin
Gravity flow column, 2.5 cm diameter
Centrifugal concentrators, 30 kDa 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)	50 mM
	NaCl	500 mM
	Glycerol	5%
	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 7.4)	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 + 20 mM imidazole
Elution buffer: base buffer, add 500 mM imidazole
Gel filtration buffer: base buffer

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
CVNSP3mac1 - SARS-CoV-2 nsp3 macrodomain

Plasmid Transformation

1d

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

Note

The His-tagged CVNSP3mac1 construct encodes the SARS-CoV-2 nsp3 macrodomain with a N-terminal His 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.

4h

4 Use the 10 mL starter culture to inoculate 1 L TB media (see Materials) supplemented with [M] 50 ug/mL 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} reaches approximately 1.4, reduce temperature to 18 °C and incubate for an additional hour. Add [M] 0.4 millimolar (mM) IPTG. Lower 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-tagged CVNSP3mac1 construct properties
>
> **Before tag cleavage:**
> MW = 20.623 kDa
> E (assume all Cys reduced)= 11920 mM-1cm-1
> PI = 6.70
>
> **After tag cleavage:**
> MW = 18.158 kDa
> E(assume all Cys reduced) = 10430
> PI = 7.30
>
> 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 40% 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 2 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

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

8.4 Wash the column with 10 CV of base buffer, followed by 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 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 10 CV 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

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

10.2 For tag removal, His-TEV was added in 1:20 ratio to the total protein content of the diluted sample, as determined by nanodrop. The mixture was left standing in the cold room at 1d

4 °C Overnight

Note

TEV:total protein ratio increased due to previous difficulty with tag cleavage for this construct. TEV addition at the standard 1:100 ratio left the majority of His-tagged CVNSP3mac1 uncleaved after overnight cold incubation.

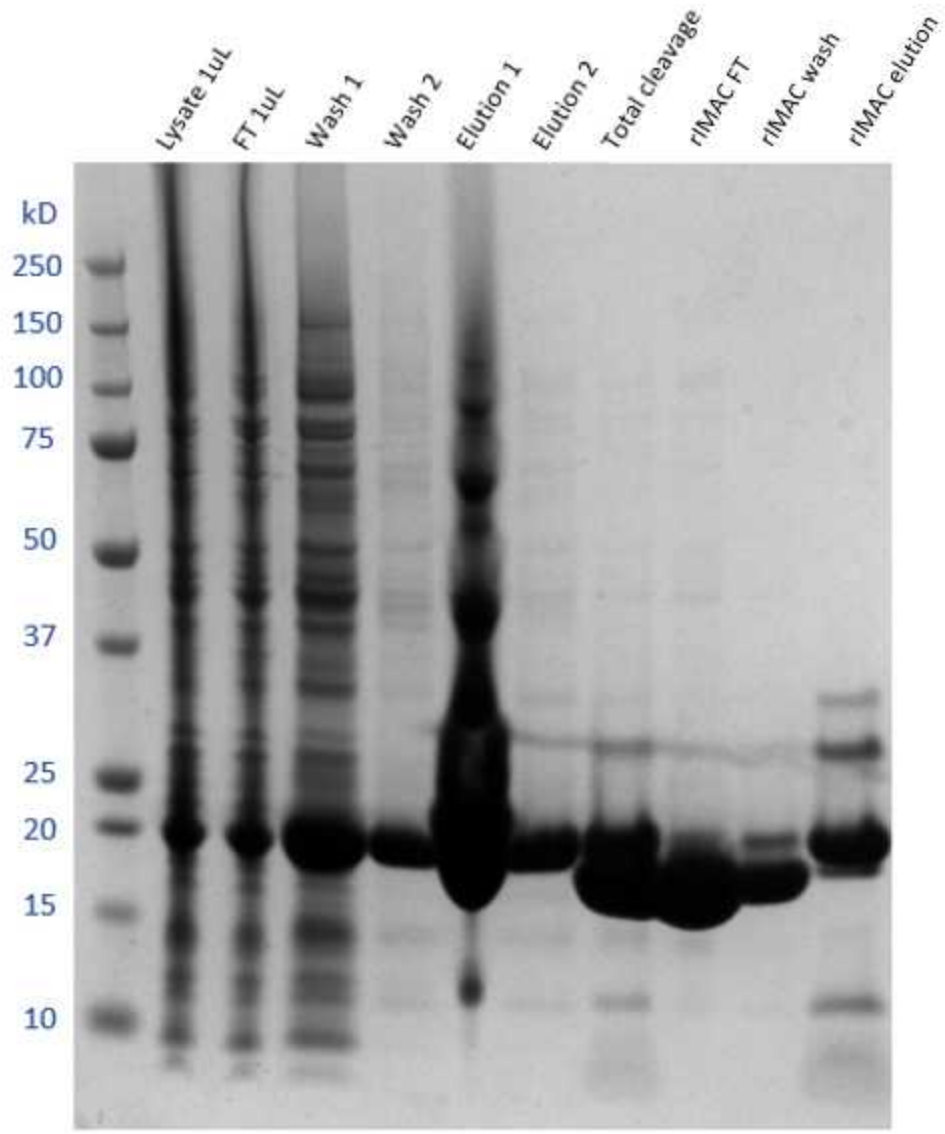
10.3 In morning, pour the cleavage mixture over the washed resin three times and collect final FT. 30m

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.

Previous purification where the cleavage mixture was incubated with the rIMAC resin while rotating for 30mins in cold resulted in protein precipitation. No such issue observed when passing mixture through resin three times.

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 major band in rIMAC FT agrees with the size of the cleaved construct (18.158 kDa)

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

Note

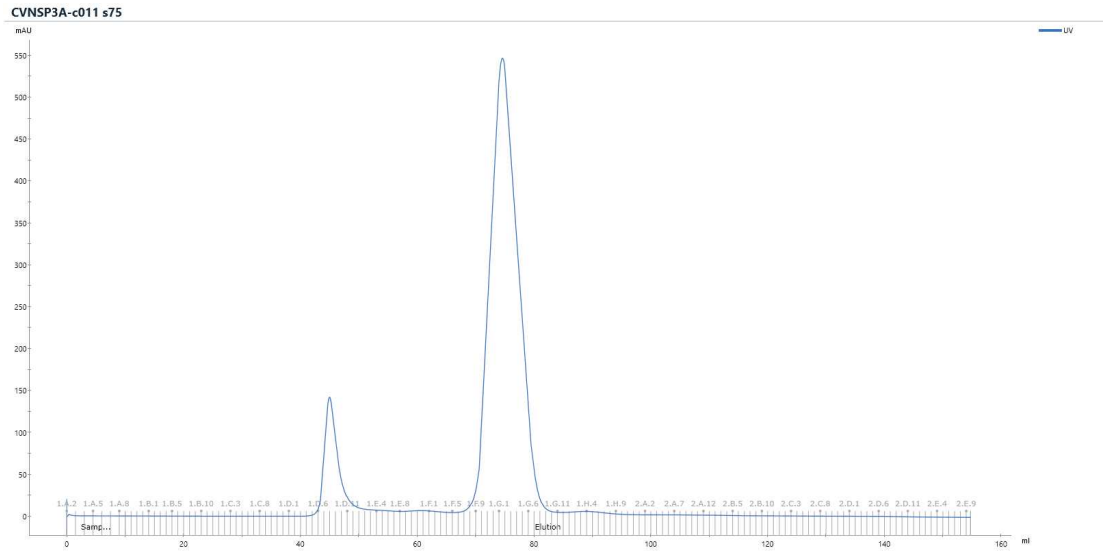
This is to remove as much solid particles from the injection sample as possible, so as to not clog the in-line filter or frit of the column.

12 Using the AKTA Pure system: 2h

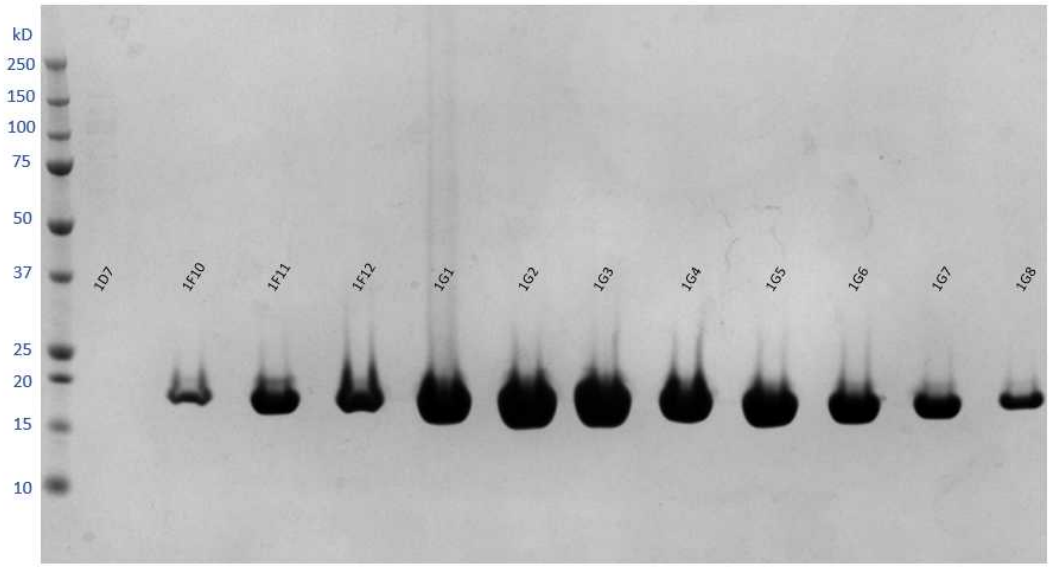
Inject the sample onto a 5 mL sample loop.

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

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



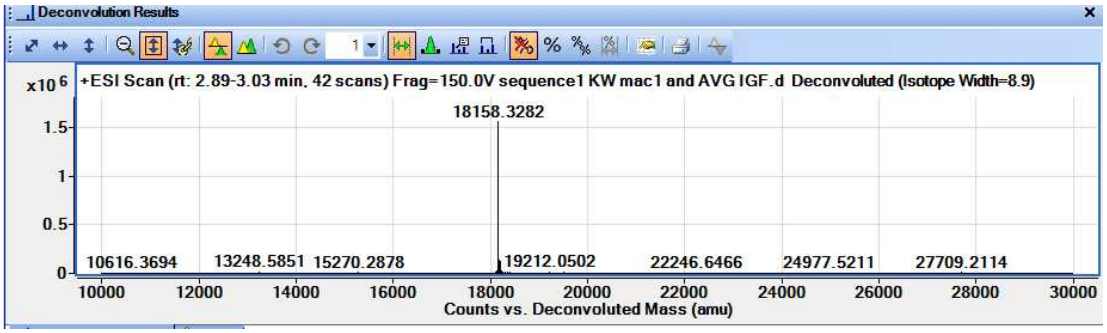
Chromatogram of the CVNSP3mac1 SEC run. Fractions D7 and F10-G8 were analyzed by SDS-PAGE to see which contained the target protein



SDS-PAGE analysis of SEC fraction D7 and F10-G8. Fractions F9-G8 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 F9-G8. Concentrate the final sample in Vivaspin 500 10 kDa MWCO centrifugal concentrator until the concentration reaches > [M] 45 mg/mL . 30m

Take 1 µL of the final sample for SDS-PAGE, which was not carried out here. However, intact MS confirms sample purity.



Intact mass spectroscopy result of the purified CVNSP3mac1 sample. The major peak (18.158 kDa) agrees with the no-tag molecular weight of CVNSP3mac1 (18.158 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. 10m