


Aug 09, 2024

SARS-CoV-2 nsp3 macrodomain His-tagged expression and purification protocol for assay

 Forked from [SARS-CoV-2 nsp3 macrodomain His-SUMO tagged expression and purification protocol for crystallization \(c004\)](#).

DOI

dx.doi.org/10.17504/protocols.io.4r3l2qb9jl1y/v1

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ASAP Discovery



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CMD

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DOI: dx.doi.org/10.17504/protocols.io.4r3l2qb9jl1y/v1

Protocol Citation: Korvus Wang, michael fairhead, Eleanor Williams 2024. SARS-CoV-2 nsp3 macrodomain His-tagged expression and purification protocol for assay. [protocols.io](https://dx.doi.org/10.17504/protocols.io.4r3l2qb9jl1y/v1) <https://dx.doi.org/10.17504/protocols.io.4r3l2qb9jl1y/v1>

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Protocol status: Working

We use this protocol and it's working

Created: August 09, 2024

Last Modified: August 09, 2024

Protocol Integer ID: 105043

Keywords: expression, purification, ASAP, CMD, AViDD, SARS-CoV-2, SARS-CoV-2 nsp3, SARS-CoV-2 nsp3 macrodomain, SARS-CoV-2 macrodomain, nsp3 macrodomain, #nsp3, macrodomain, mac1, his-tag



Funders Acknowledgement:

National Institutes of
Health/National Institute Of
Allergy and Infectious
Diseases (NIH/NIAID)
Grant ID: U19AI171399

Disclaimer

Research was supported in part by NIAID of the U.S National Institutes of Health under award number U19AI171399. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Abstract

This protocol details the expression and purification of SARS-CoV-2 nsp3 macrodomain assay construct bearing a N-terminal His tag at small scale (<6L).

Attachments



PAGE22-01678 - SARS-...

264KB

Guidelines

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

Materials

Plasmid details:

- Vector: pDEST?
- Cell line: E. coli Rosetta strain BL21(DE3)-RR

Tags and additions: N-terminal His tag.

- Construct protein sequence:
MSYYHHHHHHLESTSLYKKAGFLEVLFGQPEVNSFSGYLKLTDNVYIKNADIVEEAKKVKPTVVVNAANVYLKHGGGVAGALNK
ATNNAMQVESDDYIATNGPLKVGGSCVLSGHNLAHCLHVVGPNNVKGEDIQLLKSAZENFNQHEVLLAPLLSAGIFGADPIHSL
RVCVDTVRTNVYLA VFDKNLYDKLVSSFLEMKSEK

Expression

TB media, 1mM IPTG

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

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 + 500 mM imidazole + 50 mM L-Arginine + 50 mM L-Glutamine

Gel filtration buffer: base buffer

SDS-PAGE gel: NuPage 4-12%, Bis-Tris protein gel, 27 well.

Run in MES buffer, 200V 35 mins.



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 assay construct was inoculated from its BL21(DE3)-RR glycerol stock.

Note




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

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 carbenicillin. 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 ug/mL carbenicillin in a baffled flask.
 200 rpm, 37°C

6h



Note

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

- 5 When the OD₆₀₀ approximately 4.0, lower the temperature and shaker speed to  180 rpm, 16°C . Add IPTG to final concentration of [M] 1 millimolar (mM) . 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 Purification

2d

7 Lyse cell pellet

2h 30m

7.1

1h

Note

See Materials tab for buffer compositions.

Note

His-CVNSP3mac1 properties

MW = 22.378 kDa

E (assume all Cys reduced)= 14900 mM-1cm-1

PI = 7.00

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 00:15:00 at 35% amplitude to fully rupture the cells. Ensure pellet is 0 °C during sonication to prevent overheating.






15m 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

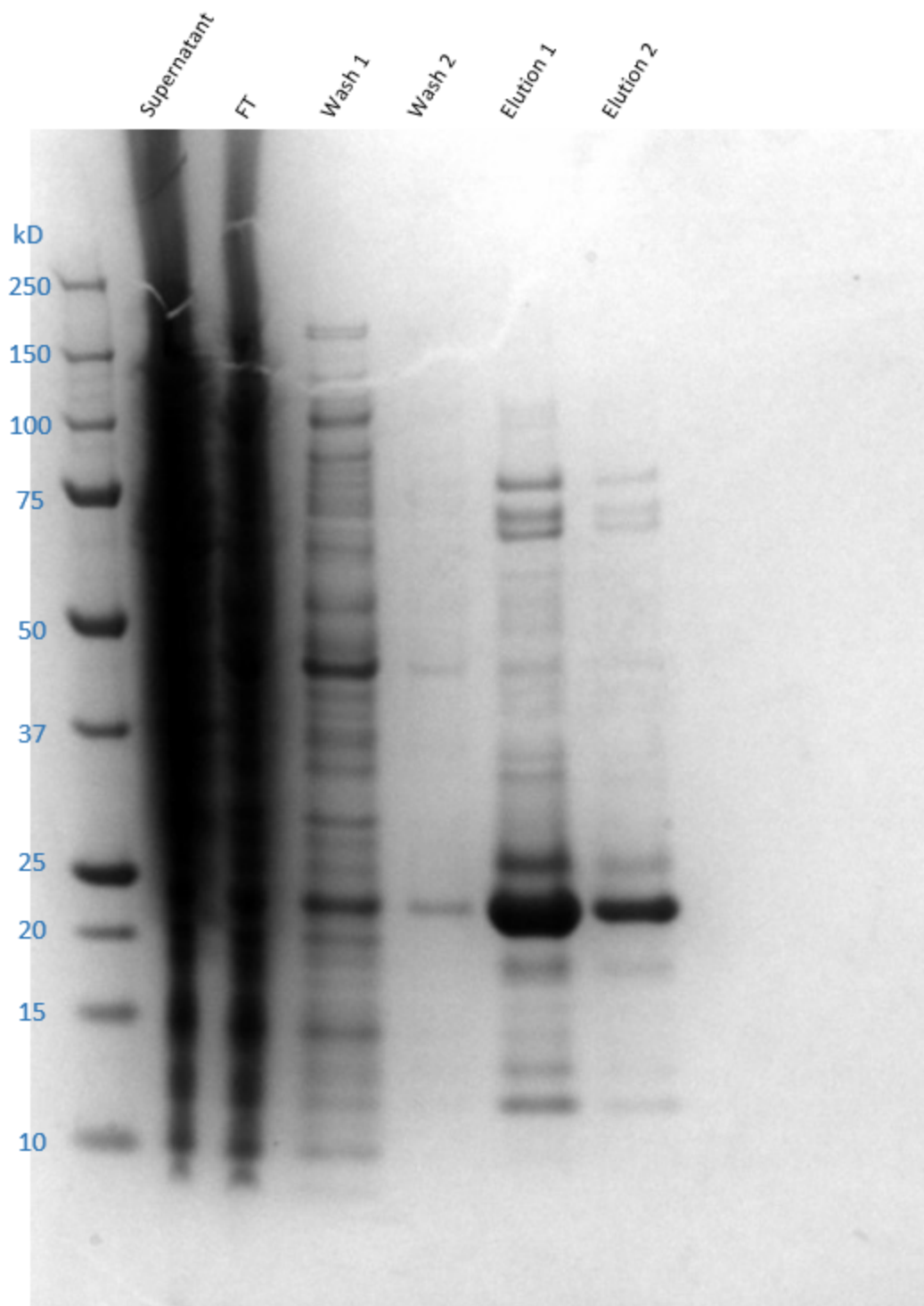
1h

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  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 15uL sample with 5uL 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  15 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.
- 9 Run SDS-PAGE of all samples from total lysis supernatant to final elution. Stain gel with protein staining solution Coomassie Blue and determine which fractions contain the target protein by finding the band corresponding to the target molecular weight. 40m




SDS-PAGE analysis of IMAC fractions. The prominent band in both IMAC elutions corresponds to the correct size of the cleaved target protein (22.378 kDa)

**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 Purify sample further by size exclusion chromatography.

6h

10.1 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

10.2 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.

11 Using the AKTA Pure system:

2h

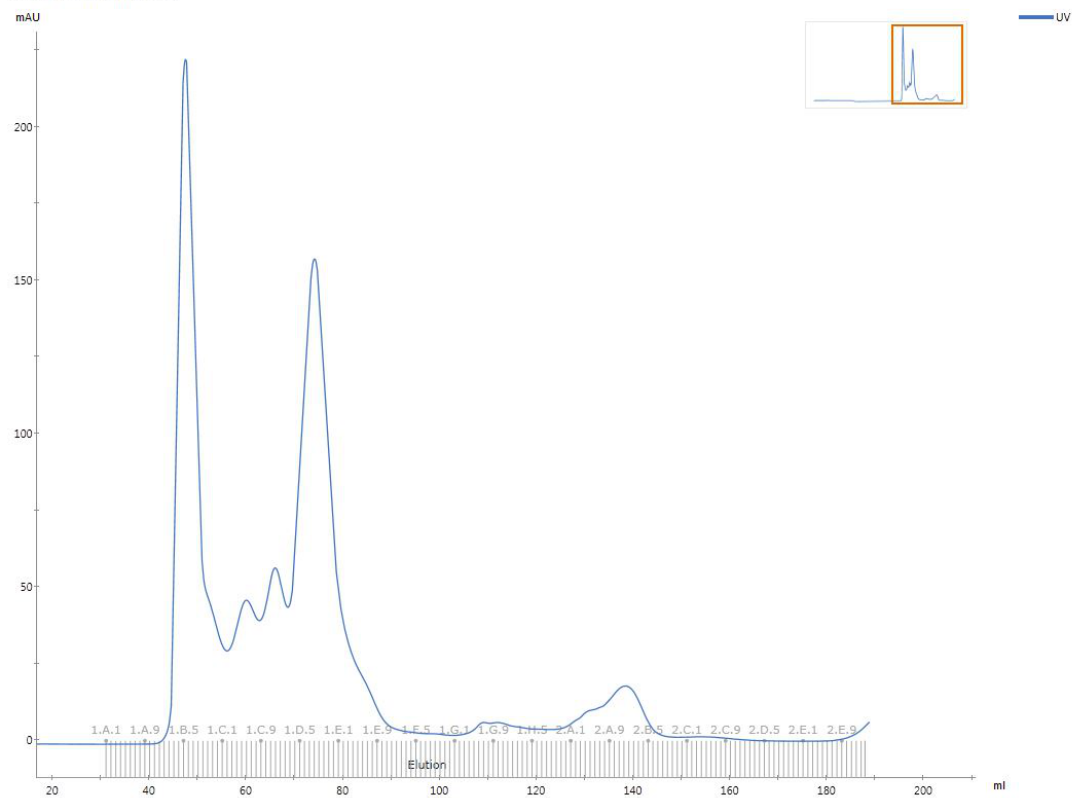
Inject the sample onto a 5mL sample loop.

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

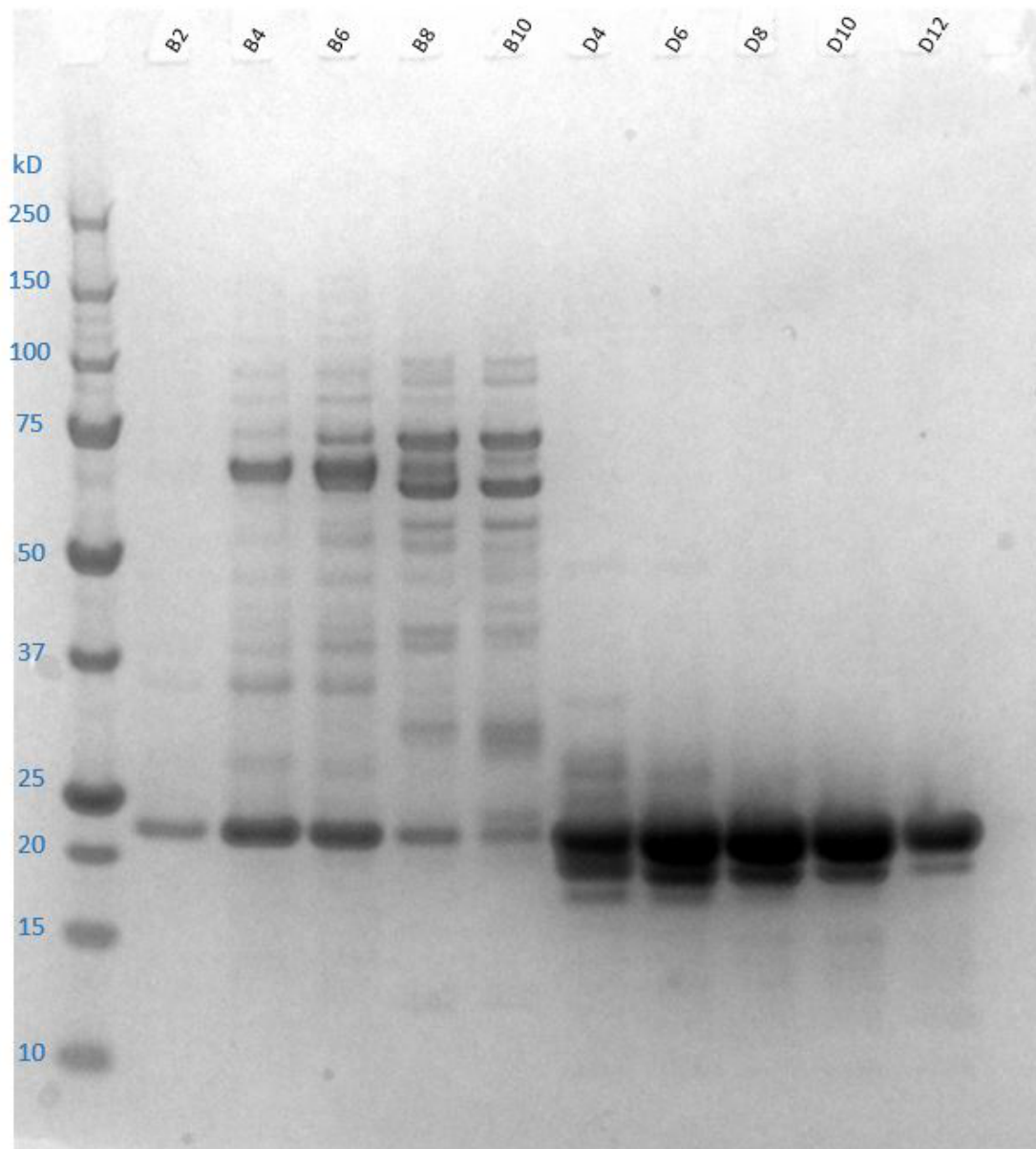
12 From the chromatogram, fraction F9-H8 analyse by SDS-PAGE.

1h

Mac1 6L round 2 001




Chromatogram of the cleaved His-CVNSP3mac1 SEC run. Fractions B2-B10 and D4-D12 were analysed by SDS-PAGE to see which contained the target protein

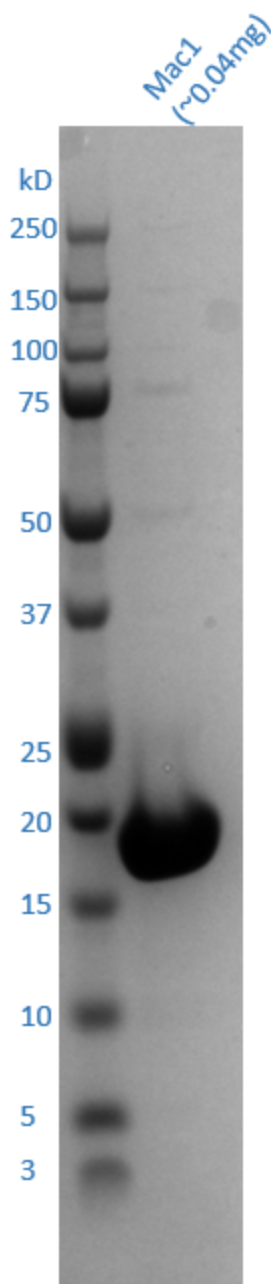


SDS-PAGE analysis of SEC fraction B2-B10 and D4-D12. Fractions D4-D11 were pooled as they contain majority target protein in comparison to contaminants.

- 12.1 Take the fractions that contain the target protein, which in this case are fraction D4-D11. Concentrate the final sample in Vivaspın 500 10kda MWCO centrifugal concentrator until the concentration reaches > [M] 6 mg/mL .

30m

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

- 12.2 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