ASAP: SARS-CoV2: N-protein: protocol transfer from Jakub Luptak

PAGE22-01018

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Date Started: **2022-May-23**
Experiment Started:
Projects: **Activity Assay**;**Expression**;**Purification**
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Expression and Purification

P1127 – C term domain (248-365) of Sars-CoV-2

Notes

High yield, 1-2 litres enough for 50-100mg

Works with 2XTY and minimal media as well.

Plasmid

pOPTH with C domain 248-365. **Ampicillin resistance**

ProtParam Output

Number of amino acids: 124
Molecular weight: 14038.80
Theoretical pI: 9.60
Ext. coefficient 16960
Abs 0.1% (=1 g/l) 1.208

Sequence

MAHHHHHHMSAAEASKKPRQKRTATKAYNVTQAFGRRGPEQTQGNFGDQELIRQGTDYKHWPQIAQFAPSASAFFGMSRIG
MEVTPSGTWLTYTGAIKLDDKDPNFKDQVILLNKHIDAYKTFP

Expression conditions

Plasmid was transformed into C41 competent *E. coli* and single colony used to inoculate an overnight starter culture (100 mL in LB). Next morning, N x 0.5 L of ZYP-5052 autoinduction media (with 1X Ampicillin) were inoculated 1:100 with the starter culture. Cultures were then incubated for 3 hours at 37 °C after which the cells were cooled down to 25C and left to grow overnight. In the morning cells were pelleted by centrifugation (25 min; 5000rpm any compatible centrifuge). Pellet was snap frozen in liquid nitrogen until processed.

Lysis

Pellet was resuspended in Lysis buffer to achieve a final volume of ~150 mL. To achieve complete lysis, resuspended cells were sonicated on ice at 70% amp, 5s on 10s off for 7 minutes. Soluble protein had been recovered by centrifugation (18 000rpm in JA25.50 rotor for 45 minutes).

Lysis buffer.

1 M NaCl, 10 mM Imidazole, 50 mM Tris pH 8, 2 mM DTT, cOmplete Roche Protease inhibitor tablets, 15% v/v 1XBugBuster+Benzonase

IMAC

Soluble protein was applied onto a gravity flow column, which has been prepared with 10 mL of Ni-NTA Agarose (5 mL of beads) washed in Buffer B**.** The lysate was allowed to drip slowly through the resin and the flow through collected for analysis. Next, the column was washed in 100 mL of Buffer B and collected as Wash. Finally, the protein was eluted with Buffer E in 3 mL fractions (total ~24 ml)

Buffer B.

300 mM NaCl, 10 mM Imidazole, 50 mM Tris pH 8, 1 mM DTT

Buffer E

300 mM NaCl, 400 mM Imidazole, 50 mM Tris pH 8, 1 mM DTT

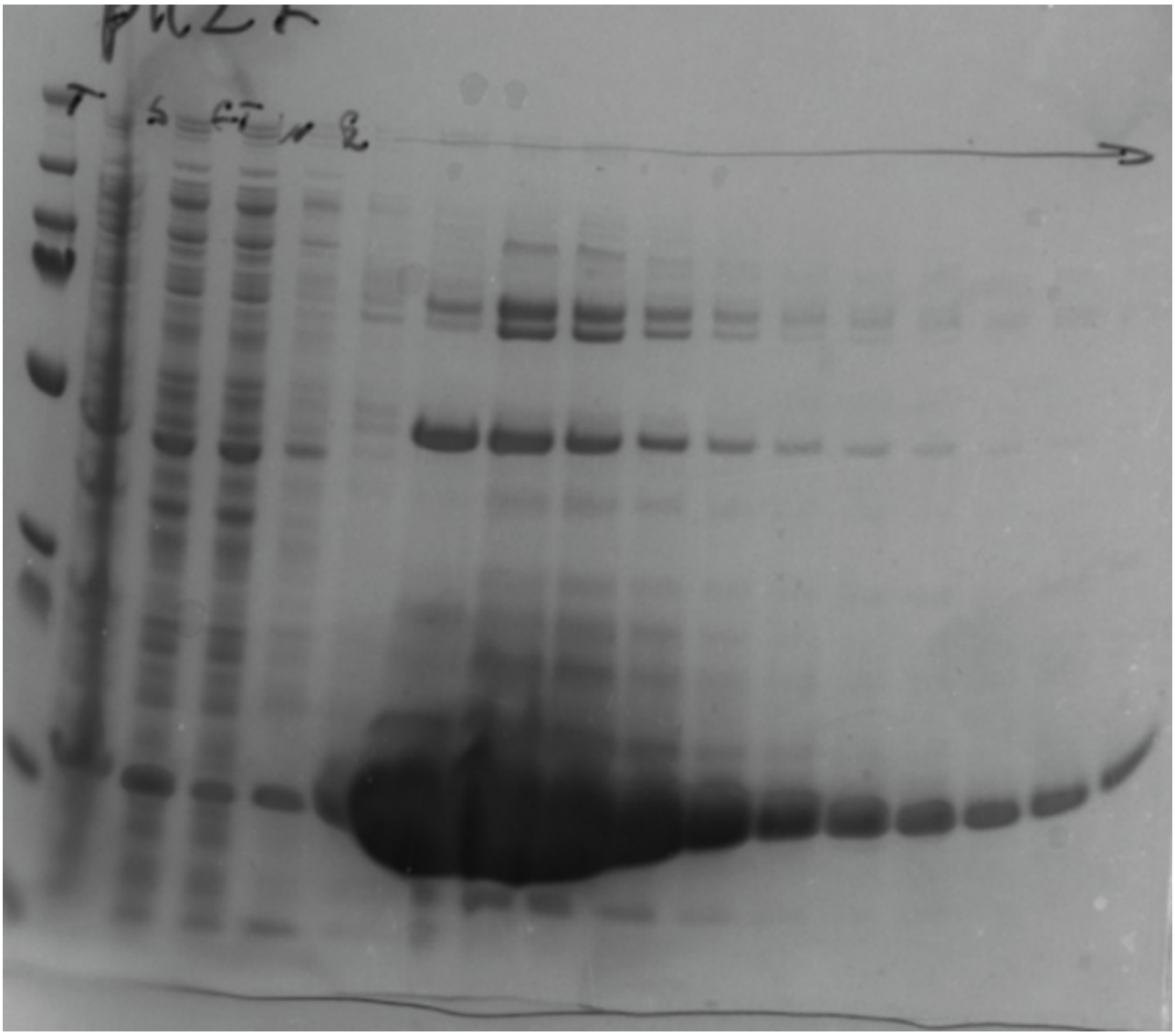
Size exclusion chromatography

HiLoad 26/600 Superdex 75 prep grade used with Buffer SEC with fractions from IMAC.

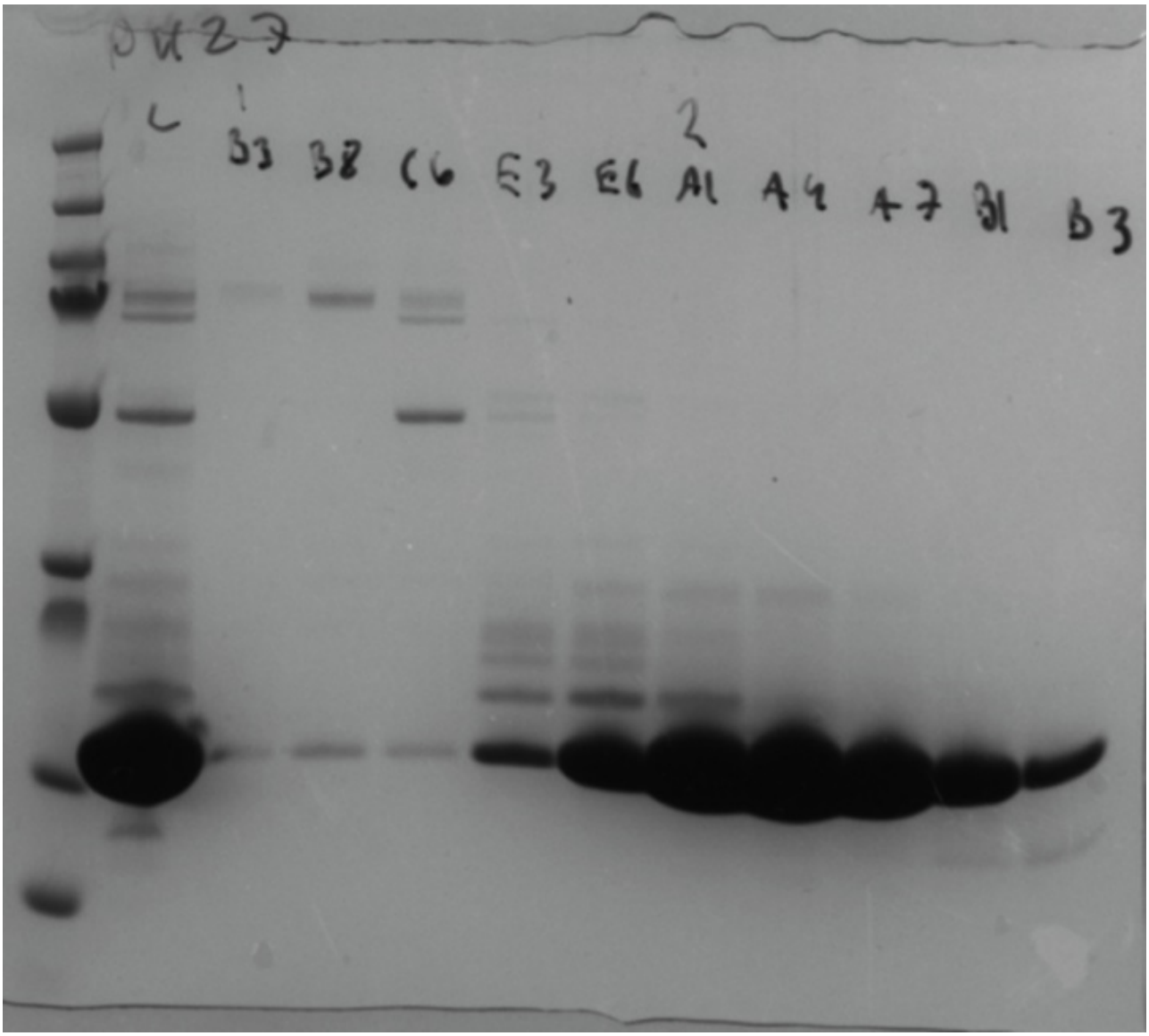
Buffer SEC

300 mM NaCl, 50 mM Tris pH 8, 1 mM DTT

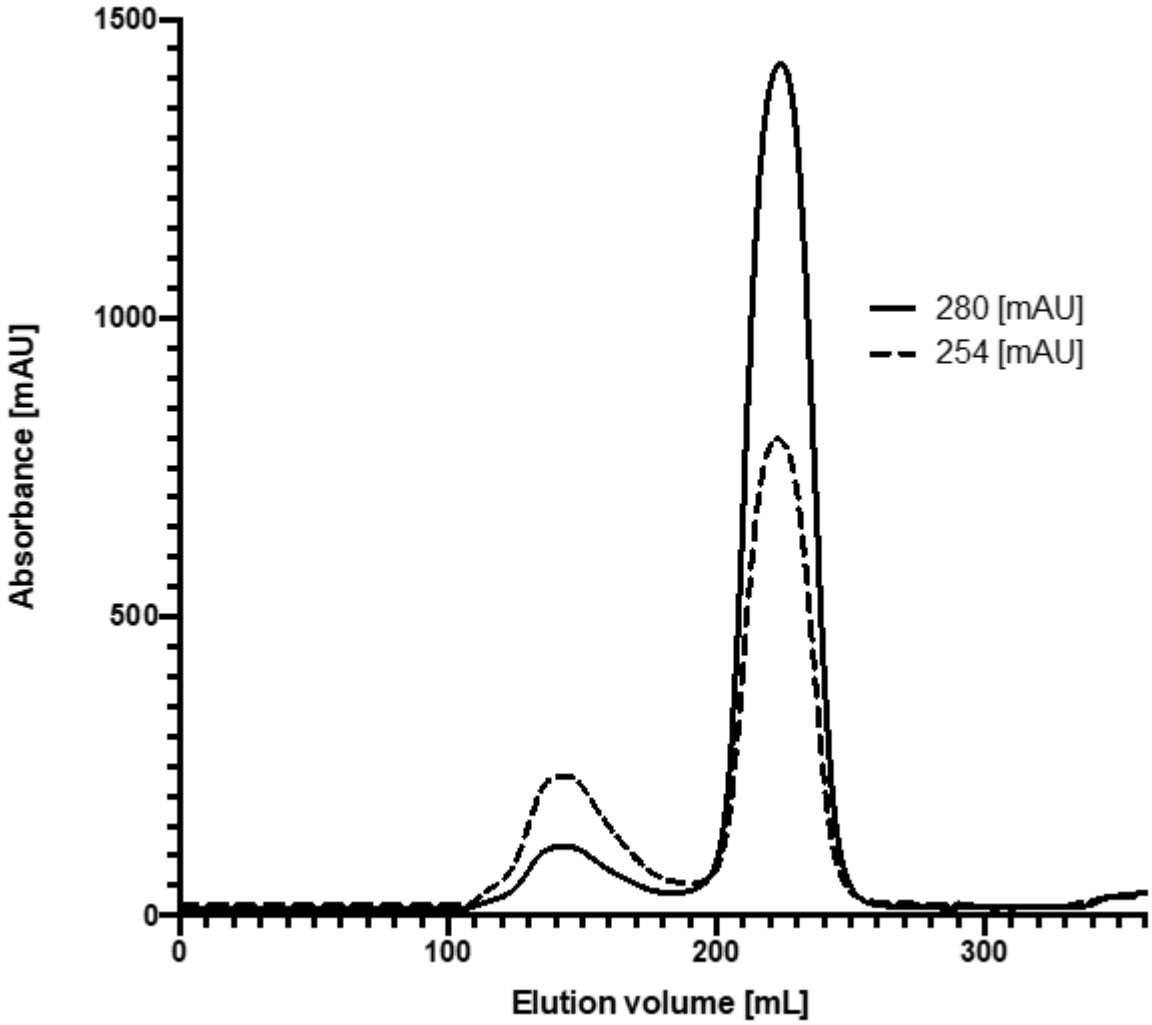
SDS PAGE example gels



SDS PAGE example gels



SEC profile



N-prot W-quench

Set up and reagents

Assay buffer: 150 mM NaCl, 50 mM Tris pH 8, 1 mM DTT(usually prepared as aliquots of 10X, frozen and thawed for use). DMSO added to 1-5% v/v depending on compounds.
Protein: p1127 (nprot) in 300 mM NaCl, 50 mM Tris pH 8, 1 mM DTT
PHERAstar reader
384 well low-binding plate (CLS3575)

Manual protocol

1. Prepare working stock (10X) of p1127; 140 uM in Assay buffer without DMSO

2. Prepare compound dilutions in a 96 well plate, need 45 uL per well. If running a duplicate with blank dilution for compound fluorescence prepare 200 uL of each concentration. Normally I was doing a 1:1.5 dilution series to get a good coverage at the higher concentrations for weak compounds.

3. Pipette 5 uL of 10X protein into 384 well plate for compound titrations

a. (if needed) Pipette 5 uL of assay buffer into 384 well plate for blank titrations

4. Pipette 45 uL of compound dilution on top of the 5 uL of 10X protein, including just assay buffer+DMSO

5. Equilibrate for 10 min
6. Read fluorescence at 350nm
7. If needed subtract compound fluorescence and fit using 4 parameter dose-response in Prism.

Notes

Final concentration of protein is 14 uM (arbitrary, once compounds get more potent this will become limiting).

Note that because of the way the dilutions are done, final compound concentrations are 0.9x of the designed titration.

Notes from Haim on HTS

Regarding the assay:

1. Technically easy and feasible here
2. Highly prone to compound interference, aggregation, false positives
3. Not sure it will give good quantitative data in dose response
4. Would like to have at least another orthogonal assay for this.. maybe DSF What do you think from a structural biology aspect would be the function most likely to give something you could work with? I see Nprotein like to self-associate, bind RNA, gets phosphorylated… https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8227405/