Expression and Purification of Zika NS5 RDRP Trial 3

PAGE23-00163

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Background

Zika NS5 RDRP protein purification has been consistently problematic with final protein yields exhibiting contaminant bands. Previous discussions with Andre confirmed that LB media may be a better option for expression, with overnight dialysis for TEV cleavage as an additional step to optimise the process. This trial will take onboard these suggestions to see if they improve the purity of the target protein.

Expression of Zika NS5 RDRP in LB Media

Siyi Wang inoculated a 2X LB media 100 mL starter culture on the evening of January 18th 2023, using a scraping from a previously prepared glycerol stock. Grown at 37 °C at 250 RPM.

The following morning (0900 hours), the starter culture did not look very turbid and the measured OD_{600} was 0.1. The starter culture was left to grow throughout the afternoon to check for viability (they reached OD_{600} > 2 by 1200 hours).

Three 1 L flasks of fresh autoclaved 1X LB (Millller formulation: 10 g/L Peptone, 10 g/L Sodium Chloride and 5 g/L Yeast Extract) media (Melford, #L24400) were inoculated with 10 mL of the starter culture (1020 hours) and the cultures were incubated at 37 °C at 220 RPM until they reached OD_{600} 0.5 (1500 hours). Cultures were cooled to 18 °C and protein expression was induced with 500 µM Isopropyl β-d-1 thiogalactopyranoside (IPTG). The cultures were left to express protein overnight at 18 °C at 120 RPM.

The following morning (0900 hours - 18 hours total expression time) the cells (now at OD_{600} 3.0) were harvested via centrifugation at 4000 x *g* using a JLA 8.1000 (Beckman Coulter, #363688). The resulting pellets (16 g combined wet pellet mass) were scrapped into a plastic beaker, covered with foil and left to freeze at - 80 °C.

List of Buffers

Lysis Buffer

50 mM HEPES pH 7.5
500 mM NaCl
30 mM Imidazole
5 % (v/v) Glycerol
4 mM Dithiothreitol (DTT)

Supplemented with: 0.5 mg/mL Lysozyme, 1 X cOmplete™ EDTA free Protease Inhibitor, 2 mM $MgCl_2$ and Benzonase (1:4000 dilution).

Wash Buffer

50 mM HEPES pH 7.5
500 mM NaCl
30 mM Imidazole
5 % (v/v) Glycerol
4 mM Dithiothreitol (DTT)

Elution Buffer

50 mM HEPES pH 7.5
500 mM NaCl
500 mM Imidazole
5 % (v/v) Glycerol
4 mM Dithiothreitol (DTT)

Gel Filtration Buffer (Based on Andre's Protocol)

20 mM HEPES pH 7.5
200 mM NaCl
5 % (v/v) Glycerol
4 mM Dithiothreitol (DTT)

Cell Lysis, Clarification and IMAC Chromatography

Cell pellet (~ 16 g) was defrosted from - 80 °C in Lysis Buffer (200 mL final volume) and left on a magnetic stirrer in the cold room (4 °C) until fully homogenised.

Sonication was carried out on ice:
0.75 inch sonicator probe tip
40 % amplitude
2 seconds on, 4 seconds off
10 minutes total sonication on time

The sonication was very effective and the lysis solution was smooth with minimal viscosity.

Lysis mixture was clarified via centrifugation using the JA 25.50 rotor at 50000 *x g* for 40 minutes at 4 °C. The resulting supernatant was transferred to a 250 mL bottle.

1 mL of equilibrated (with Purification Buffer) of Nickel Sepharose 6 Fast Flow Resin (Cytiva, #17531804) was added to the supernatant and the sample was left to mix and incubate on the rotator for 1 hour in the cold room (4 °C).

Supernatant was then poured onto a clean gravity flow column, washed twice (1-2 Column Volumes) throughly with Wash Buffer.

Bound protein was eluted with 4 x 2.5 mL of Elution Buffer (incubated for 10 minutes per elution).

Protein concetration after IMAC = ~ 30 mg total
TEV Protease was added at a 1:20 (w/w) ratio to protein sample:
TEV concentration = 4.56 mg/mL
1.50 mg of TEV (330 µL) (4 x 100 µL aliquots used) was added to the protein sample.

All of the elutions were pooled and TEV protease was added (1:20 (w/w) ratio to protein sample) and injected into an equilibrated Slide-a-Lyzer 3.5 MWCO dialysis casette (Thermo Scientific, #66130) with equal volume of Gel Filtration Buffer. The sample was left to cleave during dialysis, overnight in 2 L of gel filtration buffer in the cold room (4 °C).

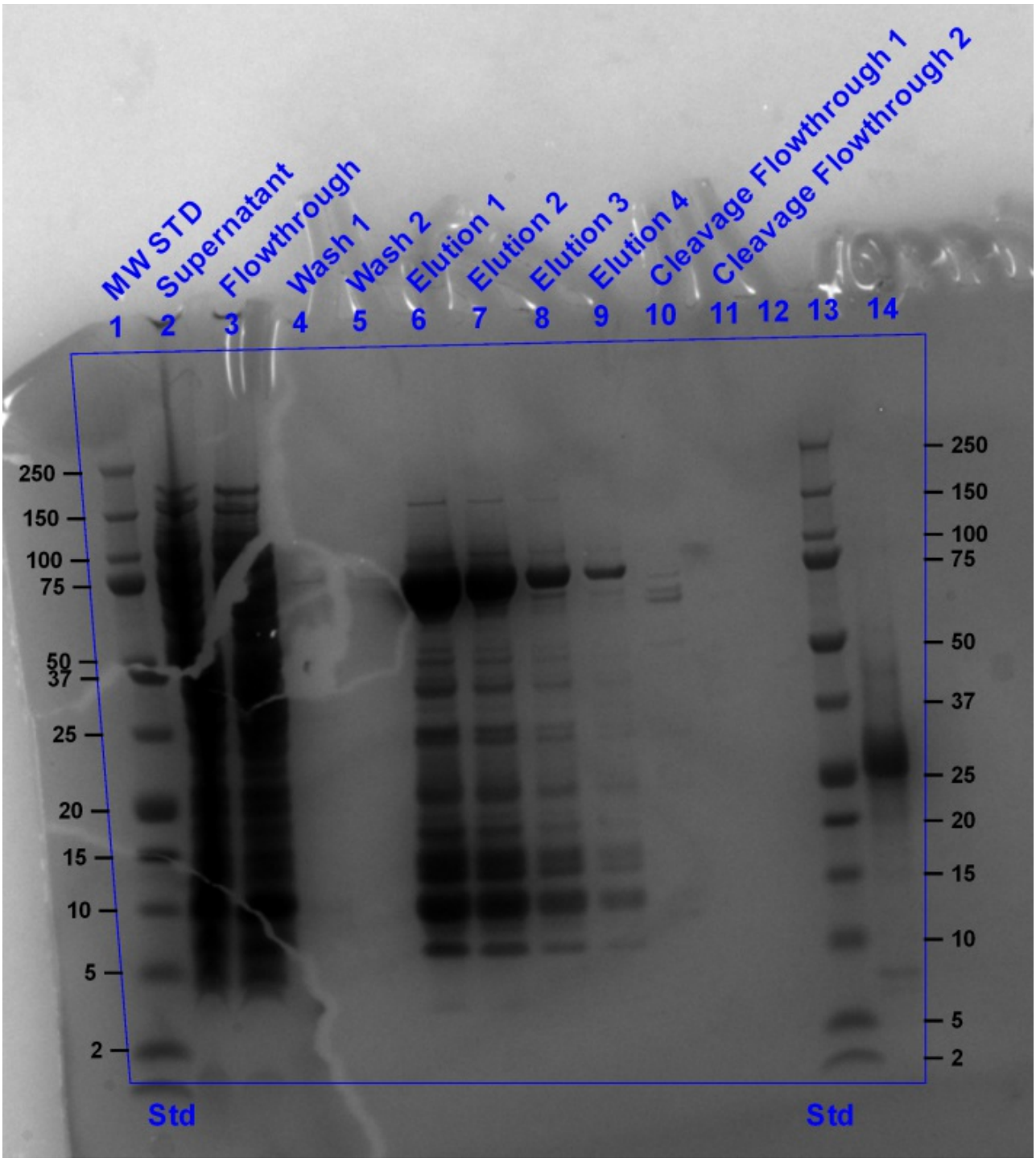
The following morning, the protein sample was removed the dialysis casette and 1 mL of fresh equilibrated (with Purification Buffer) of Nickel Sepharose 6 Fast Flow Resin (Cytiva, #17531804) was added to the sample and left to incubate on the rotator for 1 hour in the cold room (4 °C).

After incubation with Nickel Resin, the protein sample revealed signs of precipitation - indicating that the resin may have crashed the sample - possibly due to interacting with the metal binding regions on the protein?

The sample was poured into a gravity flow column and the flowthrough was collected.

All samples were run on SDS-PAGE (NuPAGE 4-12 % Bis-Tris) (Invitrogen, #WG1403) for analysis.

SDS-PAGE of IMAC and TEV Cleavage



TEV Cleavage Repeat

Overall the IMAC purification was good and the elutions were relatively clean due to the imidazole washes. However, the TEV cleavage appeared unsuccessful, with very little cleaved protein after the overnight incubation (Lanes 10 and 11).

This TEV protease batch may not be very active at 4 °C.

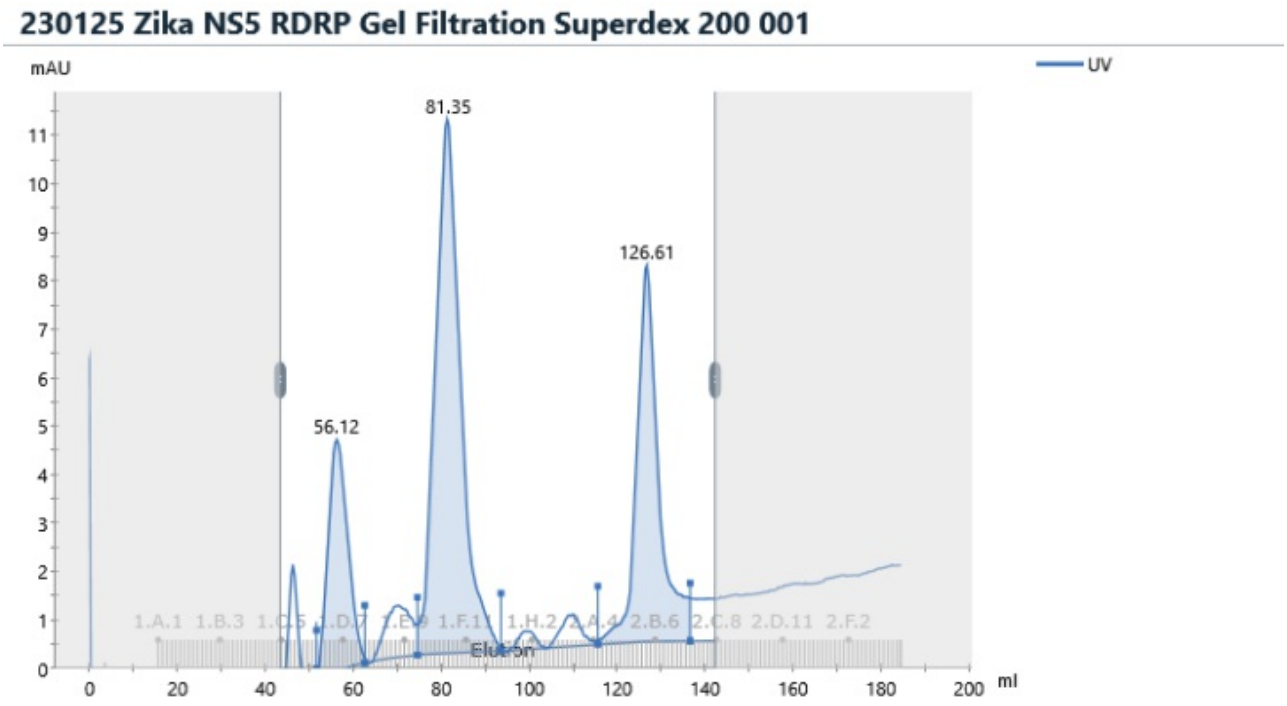
Lanes 13 and 14 are not part of this experiment and can be ignored.

The samples were concentrated to 1mL and run through a HiLoad 16/60 Superdex-200 Prep Grade column for Gel Filtration, to check for any pure cleaved protein. Additionally, the uncleaved protein was eluted off the gravity flow column with Elution Buffer (4 x 5 mL elutions).

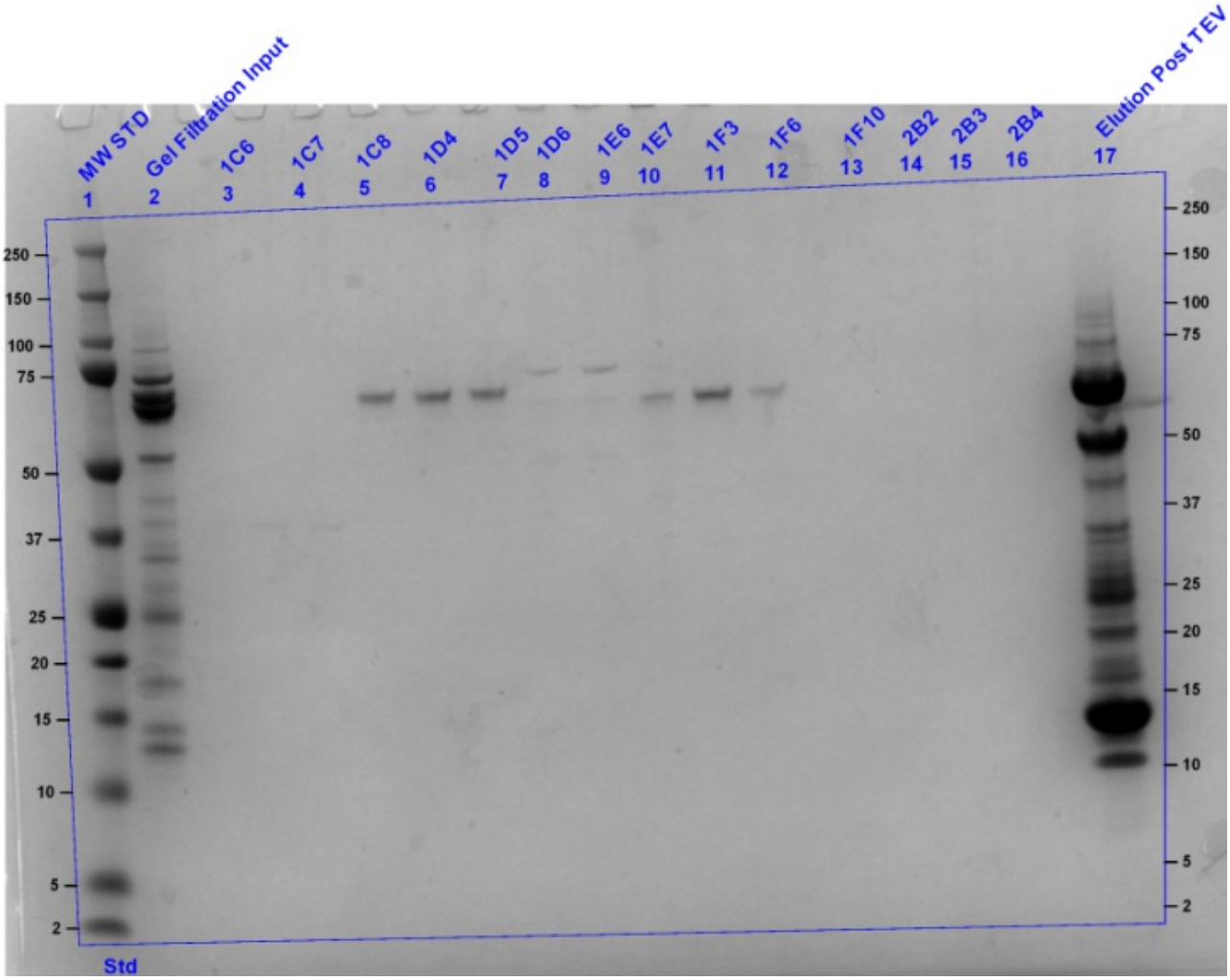
Overall, there was very little protein recovered after the Gel Filtration, but the fractions appeared pure (Lanes 5-7 and 10-12). Elution from the gravity column revealed a significant amount (of possibly cleaved?) protein left on the resin. This protein was buffer exchanged into Gel Filtration buffer using a HiPrep 26/10 Desalting column (chromatogram below).

The TEV cleavage was repeated using the same amount of TEV (new batch) as previously described, but the reaction was left for 1 hour at room temperature (20 °C), with gentle agitation. 1 mL of fresh equilibrated (with Purification Buffer) of Nickel Sepharose 6 Fast Flow Resin (Cytiva, #17531804) was added to the sample at room temperature (20 °C) and left to incubate for 5 minutes, with gentle agitation. Protein was poured into a fresh gravity flow column and the flowthrough was collected (concentrated to 1 mL) and injected onto a HiLoad 16/60 Superdex-200 Prep Grade column for Gel Filtration.

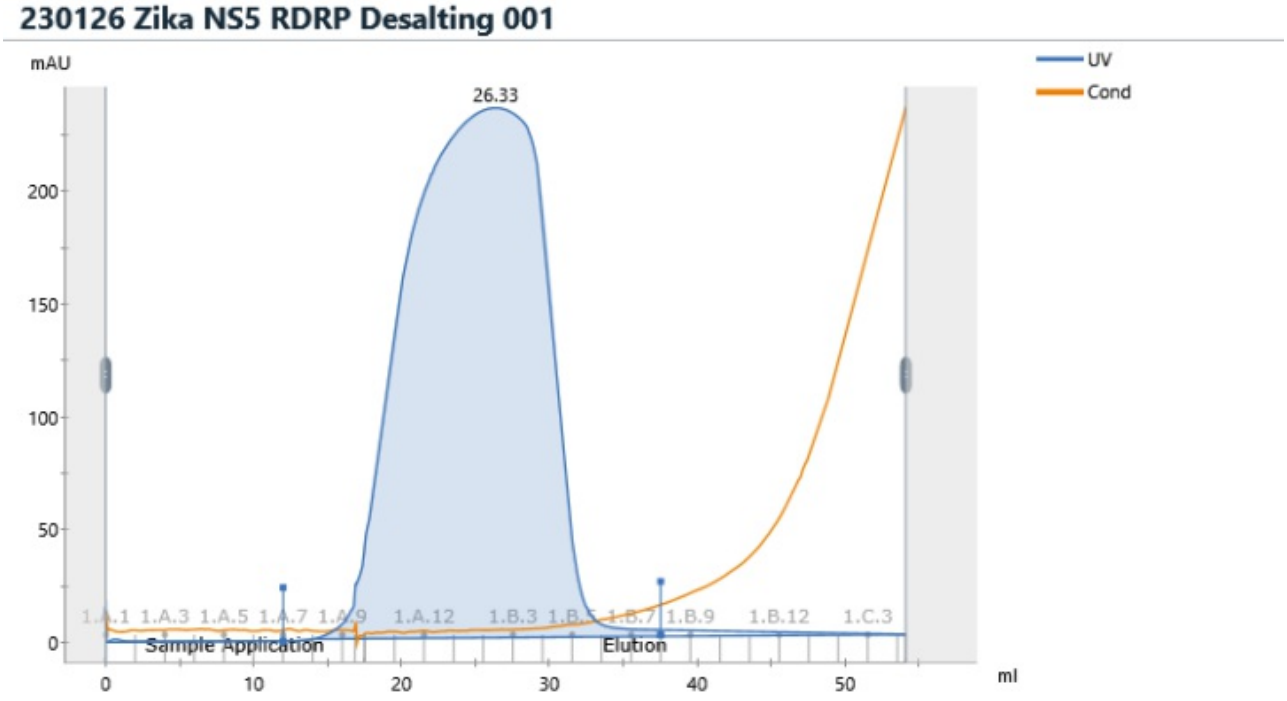
Gel Filtration of 1st Cleavage Attempt - HiLoad 16/60 Superdex-200 Prep Grade



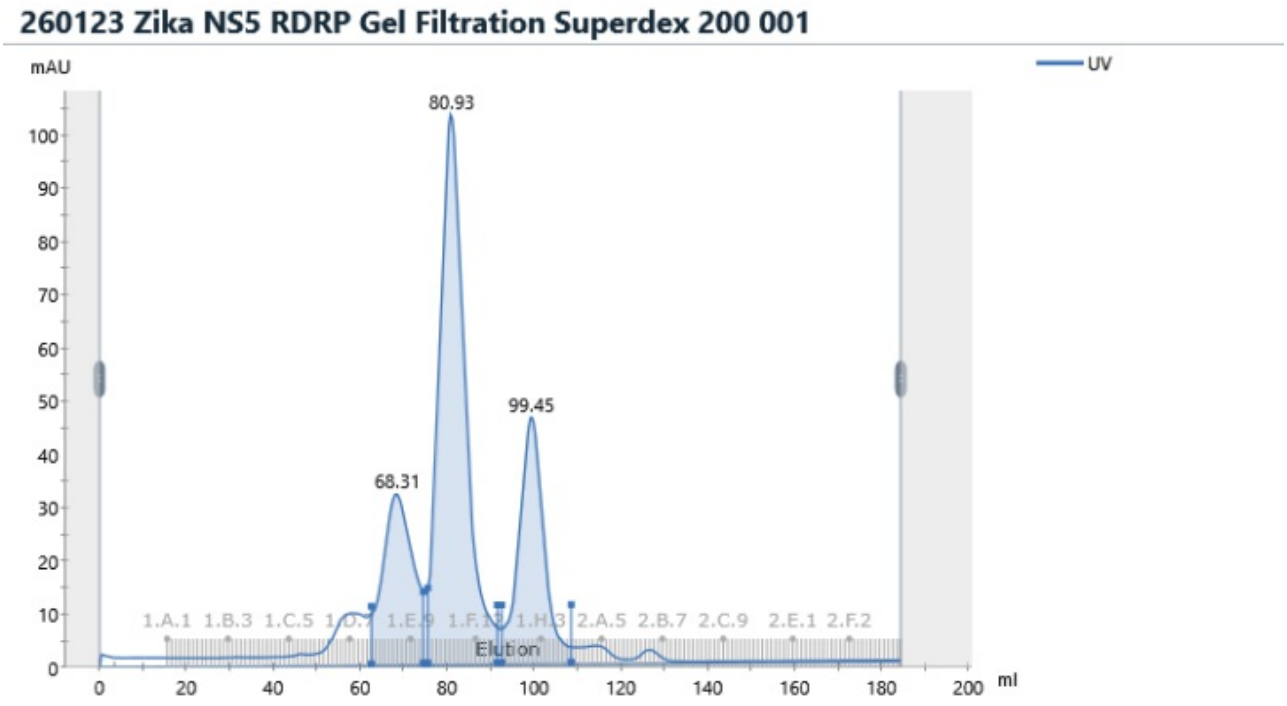
SDS-PAGE Gel Filtration Fractions 1st Attempt



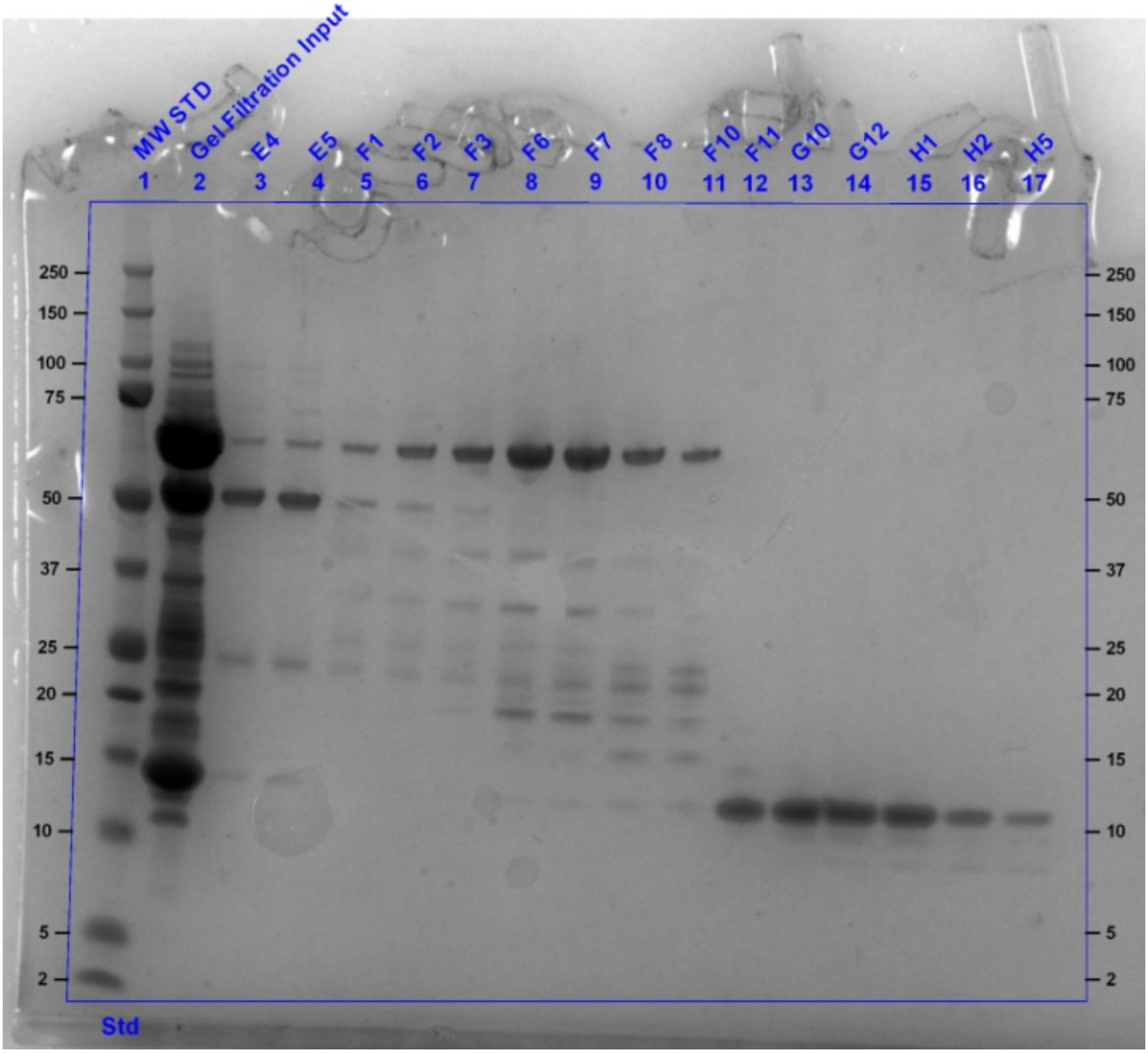
Buffer Exchange for 2nd Cleavage Attempt - HiPrep 26/10 Desalting



Gel Filtration of 2nd Cleavage Attempt - HiLoad 16/60 Superdex-200 Prep Grade



SDS-PAGE Gel Filtration Fractions 2nd Attempt



Conclusion and Final Thoughts

Overall, the second cleavage attempt produced a much greater amount of target protein, although the multiple cleavage attempts and previous precipitation during the Nickel resin binding has affected the purity (Lanes 5-11). The fractions were cleaner than previous attempts and it looks like the newer protocol is an overall improvement.

Final yield after concentration = 0.549 mg (3 x 30 µL at 6.1 mg/mL).

The final yield was disappointing, however the TEV cleavage step and nickel resin associated precipitation likely contributed to a significant loss of protein. Future attempts should make sure the cleavage reaction is carried out at room temperature (20 °C) with the new batch of TEV protease and ensure the reverse IMAC incubation is short (5 minutes). Cytiva troubleshooting suggest incubation at room temperature can reduce the chance of precipitation upon binding with nickel resin. Additionally, the addition of Magnesium may stabilise the metal binding domains within the protein during this stage. TCEP-HCl is likely fine for future purifications over DTT and the overnight dialysis is not required. Quick and efficient protocols would be preferred, as the initial cleavage (although low in yield) clearly exhibited higher purity.

Update

Ran a 10 µg sample of the 6.1 mg/mL aliquot for QC testing. Protein is showing clear signs of degradation over time. It appears the protein is naturally unstable and the TEV issues + extended protocol is not optimal.

SDS-PAGE Zika NS5 RDRP QC Test