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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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Protocol status: Working
We use this protocol and it's working

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Disclaimer

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The pOPINS-ZVRdRP construct cannot be distributed due to intellectual property restrictions on the vector. We are currently working on re-cloning the sequence into an open access vector, to be available for Addgene orders later.

Abstract

This protocol details the co-expression and purification of Zika NS5 NS5 RNA-dependent RNA polymerase bearing a N-terminal His-SUMO tag at small scale (<6L).

Attachments

PDF

PAGE24-00544 - New Z...

369KB

Guidelines

- **Construct / plasmid resource-name:** Zika NS5 NS5 RNA-dependent RNA polymerase bearing a N-terminal His-SUMO tag.

Materials

Plasmid details:

- Vector: pOPINS-DLS
- Cell line: E. coli SixPack strain
- Tags and additions: N-terminal His-SUMO tag
- Construct protein sequence:
  MGSSHHHHHHGSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQAD
  QTPEDLDMEDNDIIEAHREQIGGYHGSYEAPTQGSASSLVNGVVRLLSKPWDVVTGVTGIAMTDTTPYGQQRVFKEKVDTRVPD
  PQEGTRQVMNIVSSWLWKELGKRKRPRVCTKEEFINKVRSNAALGAIFEEEKEWKTAVEAVNDPRFWALVDREREHHLRGECHS
  CVYNMMGKREKKQGEFGKAKGSRAIWYMWLGARFLEFEALGFLNEDHWMGRENSGGGVEGLGLQRLGYILEEMNRAPGGKMY
  ADDTAGWDTRISKFDLENEALITNQMEEGHRTLALAVIKYTYQNKVVKVLRPAEGGKTVMDIISRQDQRGSGQVVTYALNTFTNLV
  VQLIRNMEAEEVLEMQDLWLLRKPEKVTRWLQSNGWDRLKRMAVSGDDCVVKPIDDRFAHALRFLNDMGKVRKDTQEWKPST
  GWSNWEEVPFCSHHFNKLYLKDGRSIVVPCRHQDELIGRARVSPGAGWSIRETACLAKSYAQMWQLLYFHRRDLRLMANAICSA
  VPVDWVPTGRTTWSIHGKGEWMTTEDMLMVWNRVWIEENDHMEDKTPVTKWTDIPYLGKREDLWCGSLIGHRPRTTWAENIK
  DTVNMVRRIIGDEEKYMDYLSTQVRYLGEEGSTPGVL

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 200 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

	A	B
	Glycerol	5%
	TCEP	1 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	50 mM
	Glycerol	5%
	TCEP	1 mM

Prepare 2L per 6L E.coli expression. Used to prepare the following buffers

Binding buffer: base buffer + 20mM imidazole
Wash buffer: base buffer + 30mM imidazole
Elution buffer: base buffer, add 300mM imidazole
Gel filtration buffer: base buffe

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
LB- Lysogeny Borth

Plasmid Transformation

1d

2 ZVRdRp N-terminal 6His-SUMO tagged co-expression construct was inoculated from its SixPack glycerol stock.

Note

The ZVRdRp construct encodes the NS5 RdRp with a N-terminal His-SUMO tag fusion on a kanamycin resistant plasmid backbone with a T7 promoter.

see disclaimer section regarding pOPINS 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 auto-induction media (see Materials) supplemented with [M] 50 ug/mL carbenicillin in a baffled flask. 200 rpm, 37°C

6h

5 When the OD_{600} reaches approximately 2.0, lower the temperature and shaker speed to 180 rpm, 18°C and incubate Overnight . Harvest in late afternoon on the second day.

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.

Thaw and resuspend the pellet in ~7mL of lysis buffer per gram of cell 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 00:00:04 On 00:00:12 Off for a total 'on' time of 00:03:00 at 40% amplitude to fully rupture the cells. Ensure pellet is 0 °C during sonication to prevent overheating of the sample. 3m 16s

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 1 mL Nickel affinity resin Ni Sepharose 6 FF (Cytiva) into a empty gravity flow column. Equilibrate resin by first rinsing with ~ 10 CV distilled water, then ~ 10 CV binding buffer. 10m

8.2 Resuspend the equilibrated resin with some binding buffer and add to the supernatant bottle. Incubate the resin with the supernatant for 00:10:00 while rotating or otherwise mixing gently at 4 °C 10m

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 and weak binding contaminant proteins from the resin for a cleaner elution.
Collect all the washes separately for SDS-PAGE analysis. 30m

8.5 Elute the protein with 5 CV 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, as well as removing remaining contaminants for later reverse IMAC. 20m

Measure the absorbance (A280) of the elution fractions using using Nanodrop and estimate the total protein concentration. Although still a mixture of proteins, A280 value can give an estimate of the protein content which will help to determine the amount of protease required to remove the affinity tag.

8.7 Wash used IMAC resin with 10CV of base buffer, and leave in the column stored with a small amount of the same buffer such that the resin is kept moist.
This washed IMAC resin will later be reused for reverse IMAC (rIMAC)

9 Run SDS-PAGE for all samples from total lysis supernatant to final elution. Stain gel with protein staining solution Coomasssie Blue and determine the fractions that contain the protein of interest, by finding the band corresponding to the expected protein 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 and dilute the two elutions with base buffer to 50mL (1:4) , lowering the total imidazole concentration to 60mM. 30m

Note

High concentration of imidazole will inhibit protease activity during tag cleavage.

Alternatively, dialysis or desalting using pre-packed columns are also acceptable methods of lowering sample imidazole concentration.

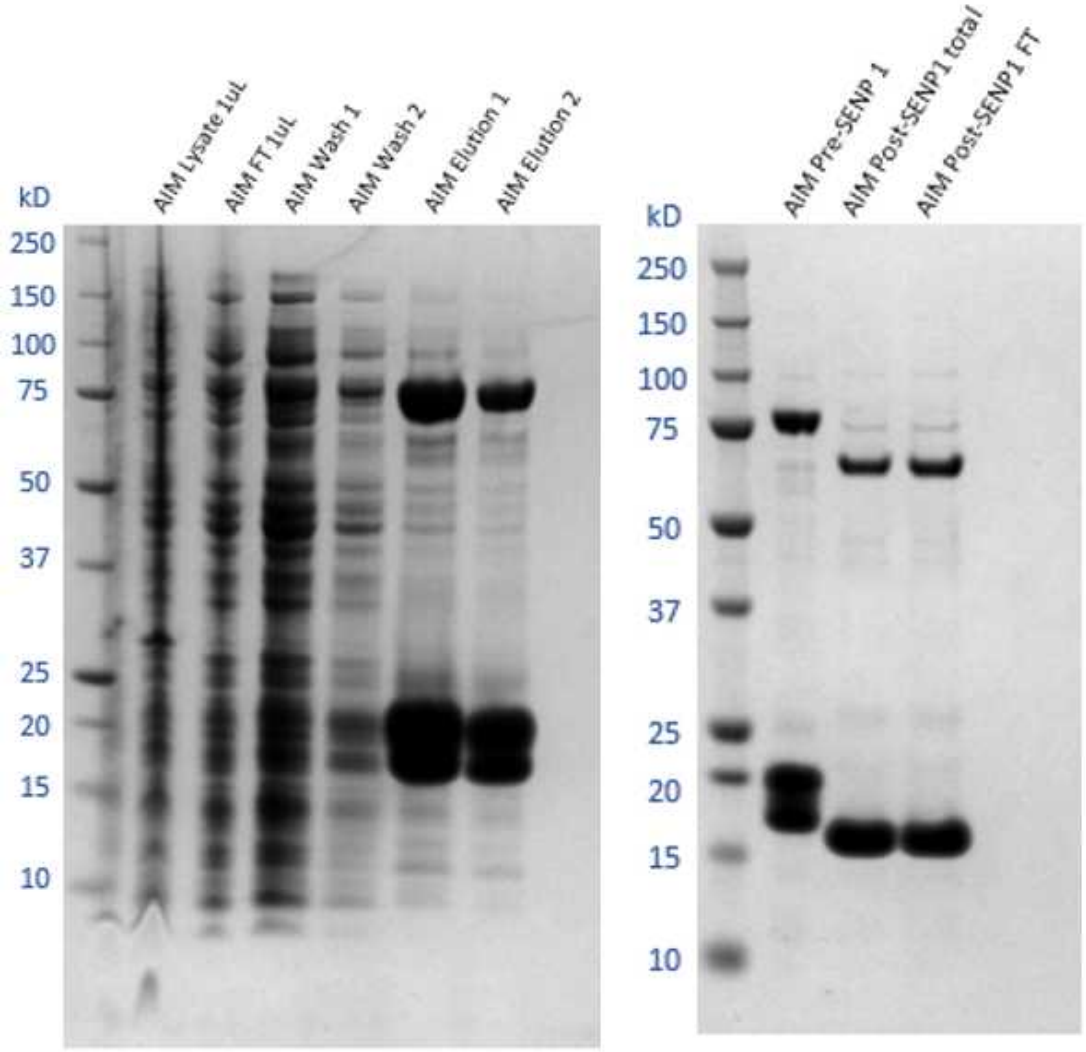
10.2 For tag removal, add His-SENP1 in 1:300 ratio to the total protein content of the diluted sample, as determined by nanodrop. Keep the mixture in the cold room at 4 °C Overnight 1d

10.3 Next day, pass the cleavage mixture over the washed resin (mentioned in step 8.7) three times and collect the 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 also removed from this sample.

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



SDS-PAGE analysis of IMAC and cleavage fractions. The lower prominent band in rIMAC FT agrees with the size of the cleaved construct (68.921 kDa), confirming successful tag cleavage.

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 30,000 MWCO spin concentrators, concentrate the fractions, from the rIMAC step, that contain target protein to a maximum final volume of 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

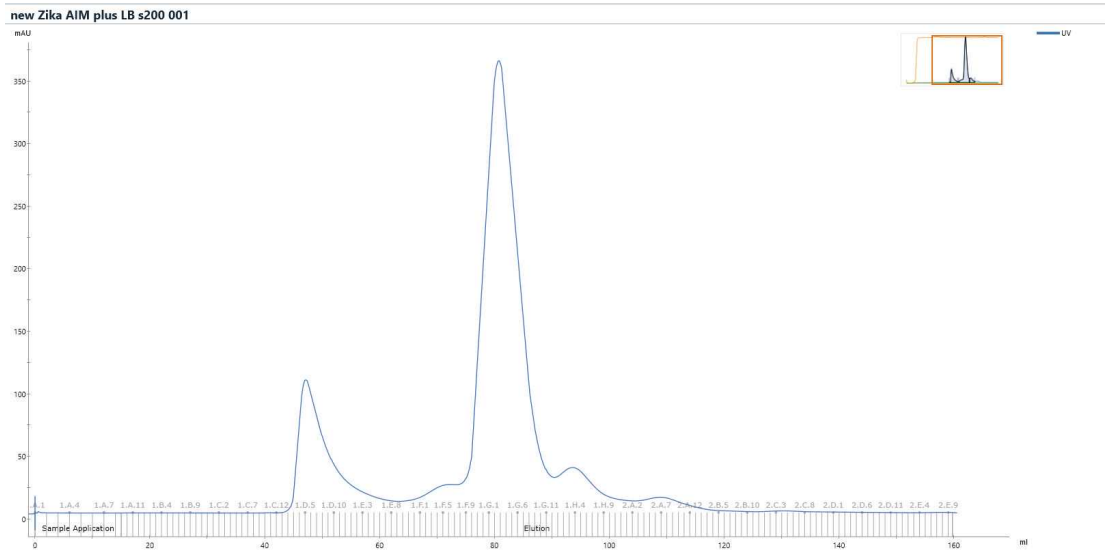
This is to remove as much solid particles from the injection sample as possible, so as to not clog the in-line filter or frit of the column.

12 Using the AKTA Pure system: 2h

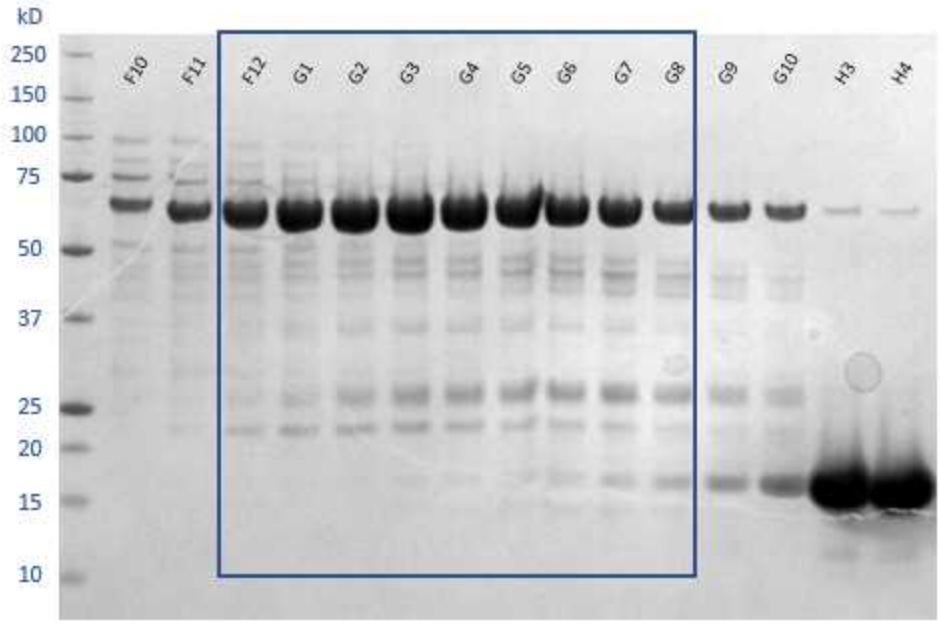
Inject the sample onto a 5mL sample loop.

Run the sample down HiLoad 16/60 Superdex 200 pg gel filtration column at 1mL/min in gel filtration buffer, collecting 1mL fractions.

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



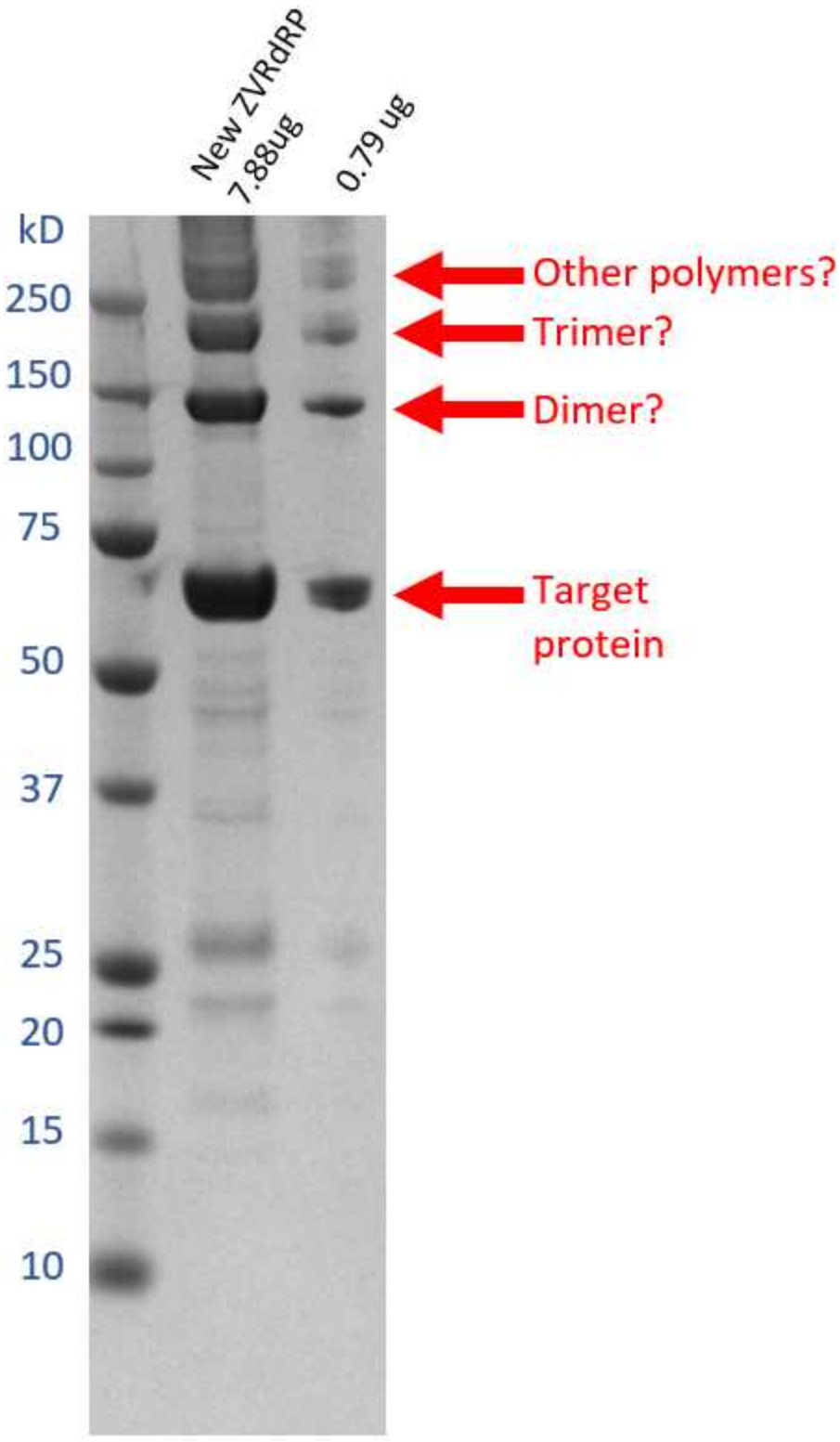
Chromatogram of the ZVRdRp SEC run. Fractions F10-H4 were analyzed by SDS-PAGE to dentify the fractions that contained the target protein



SDS-PAGE analysis of SEC fraction F10-H4. Fractions F12-G8 were pooled as they contain of target protein in comparison to contaminants.

13.1 Pool the fractions that contain the target protein, which in this case includes fractions from F12 to G8. Concentrate the sample in Vivaspin 500 30kDa MWCO centrifugal concentrator until the protein concentration reaches [M] 4.8 mg/mL . 30m

Take 1 µL of the final sample for SDS-PAGE.



SDS-PAGE of the final purified ZVRdRp construct. The higher molecular weight bands are likely polymeric forms of the target that failed to be monomerized during SDS-PAGE sample preparation.

Another 1 µL can be taken for mass spectroscopy (MS) analysis, which was not carried out here.

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