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Zika NS5 RdRp Histidine-Thioredoxin tagged construct small scale expression and purification protocol

Forked from Zika NS5 RdRp His-SUMO construct small scale expression and purification protocol

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CMD

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We use this protocol and it's working

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Abstract

We present a comprehensive small-scale (<6L) protocol for the expression and purification of the Zika virus NS5 RNA-dependent RNA polymerase (RdRp) domain with an N-terminal His-Trx (Histidine-Thioredoxin) tag. Using the BL21(DE3)-RR E. coli expression system, the protocol describes bacterial transformation, protein induction with IPTG, and a three-step purification process including immobilized metal affinity chromatography (IMAC), TEV protease-mediated tag cleavage, and size-exclusion chromatography. This method yields purified, functional Zika NS5 RdRp suitable for structural studies and antiviral drug screening. The His-Trx tag enhances protein solubility while allowing efficient separation of the cleaved target protein. Special attention is given to preventing degradation of this inherently unstable protein through optimized buffer conditions and expedited purification at low temperatures. The resulting protein can be concentrated to approximately 7 mg/mL and stored at -80°C in small aliquots to minimize freeze-thaw cycles.

Attachments

PDF

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150KB

Guidelines

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

Materials

Plasmid details:

- Vector: pET
- Cell line: *E. coli* Rosetta strain BL21(DE3)-RR
- Tags and additions: N-terminal His-Trx tag
- Construct protein sequence:
  MKHHHHHHPMSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQNPGTAPKY
  GIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGSENLYFQGAMYHGSYEAPTQGSASSLVNGVVRLLSKP
  WDVVTGVTGIAMTDTTPYGQQRVFKEKVDTRVPDPQEGTRQVMNIVSSWLWKELGKRKRPRVCTKEEFINKVRSNAAL
  GAIFEEEKEWKTAVEAVNDPRFWALVDREREHHLRGECHSCVYNMMGKREKKQGEFGKAKGSRAIWYMWLGARFLEFE
  ALGFLNEDHWMGRENSGGGVEGLGLQRLGYILEEMNRAPGGKMYADDTAGWDTRISKFDLENEALITNQMEEGHRTLA
  LAVIKYTYQNKVVKVLRPAEGGKTVMDIISRQDQRGSGQVVTYALNTFTNLVVQLIRNMEAEEVLEMQDLWLLRKPEKV
  TRWLQSNGWDRLKRMAVSGDDCVVKPIDDRFAHALRFLNDMGKVRKDTQEWKPSTGWSNWEEVPFCSHHFNKLYLK
  DGRSIVVPCRHQDELIGRARVSPGAGWSIRETACLAKSYAQMWQLLYFHRRDLRLMANAICSAVPVDWVPTGRTTWSIH
  GKGEWMTTEDMLMVWNRVWIEENDHMEDKTPVTKWTDIPYLGKREDLWCGSLIGHRPRTTWAENIKDTVNMVRRIIG
  DEEKYMDYLSTQVRYLGEEGSTPGVL

Expression
LB media, 0.5mM 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 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
	MgCl2	2mM
	Lysozyme	0.5 mg/mL
	Benzonase	0.05 mg/mL
	cOmplete EDTA-free PIC tablet	2

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
Wash buffer: base buffer + 25 mM imidazole
Elution buffer: base buffer, add 500mM imidazole
Gel filtration buffer: base buffer

SDS-PAGE gel: NuPage 4-12%, Bis-Tris protein gel, 27 well.
Run in MES buffer, 200V 35mins.

Safety warnings

Always wear appropriate PPE for this protocol
Refer to Material Safety Data Sheets for additional safety and handling information.

Abbreviations

1 CV - column volume, total volume of resin in a column
IMAC - immobilised metal affinity chromatography
FT - flow through
ZIKVRdRp - Zika NS5 RNA-dependent RNA polymerase

Plasmid Transformation

1d

2 Plasmid used: ZIKVRdRp N-terminal His-Trx tagged construct was inoculated from its BL21(DE3)-RR glycerol stock.

Note

The ZIKVRdRp construct encodes the NS5 RdRp 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 LB media (see Materials) supplemented with [M] 50 ug/mL kanamycin in a baffled flask. 250 rpm, 37°C

6h

Note

Previous purifications indicate this target is very unstable and degrades easily throughout expression and purification.
Using LB media for scale-up is essential to minimize degradation during expression.
Carrying out purification steps in cold room and in as few days as possible.

5 When the OD_{600} reaches approximately 0.8, induce expression by adding [M] 0.5 millimolar (mM) IPTG. Lower the temperature and shaker speed to

1d

120 rpm, 18°C and incubate Overnight . Harvest the cells 20hrs post induction.

6 Harvest the cells by centrifugation at 4000 x g, 4°C, 00:30:00 . Discard supernatant, weigh and store the cell 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-Trx-ZIKVRdRp properties

Before tag cleavage:
MW = 83.376 kDa
E (assume all Cys reduced)= 178300 $mM^{-1}cm^{-1}$
PI = 6.74

After tag cleavage:
MW = 69.180 kDa
Exitation coefficient(assume all Cys reduced) = 162830
PI = 8.01

These values are determined by Expasy ProtParam

Thaw the frozen cells and resuspend the cell pellet (around 7mL of lysis buffer per gram of cell pellet). Stir gently with magnetic stir bar at Room temperature for 00:30:00 for lysozyme and bezonase action.

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 and sample denaturation. 3m 16s

7.3 Centrifuge the lysed cells 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) (4mL 50% suspension) 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 collected earlier in the bottle. Incubate the resin with the supernatant for 00:10:00 either on a rotating wheel or by mixing gently at 4 °C 10m

8.3 Load the resin-supernatant mix back onto the gravity flow column. Store the flow through 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 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 the used IMAC resin with 10CV 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 be used later for reverse IMAC (rIMAC)

9 Run SDS-PAGE of all samples from total lysis supernatant to final elution. Stain the gel with protein staining solution, Coomasssie Blue and identify the fractions containing the target protein 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.

10 **Elution de-salting, tag cleavage and reverse IMAC** 1d

10.1 Pool the elutions fractions and desalt using HiPrep 26/10 deasalting columns, into base buffer. 30m

Note

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

Due to target degradation, dialysis is not recommended.

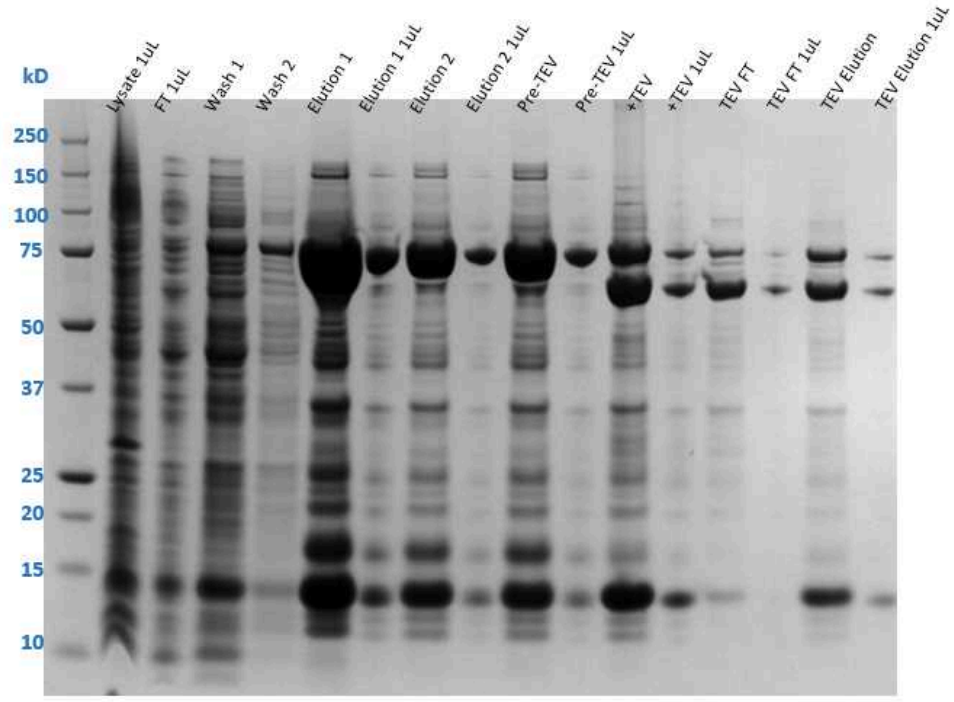
10.2 Measure the protein concentration in the nanodrop. Add TEV protease in the mass ratio 1:50 ratio to the total protein content of the diluted sample, as determined by nanodrop. Supplement the cleavage mixture with [M] 2 millimolar (mM) MgCl2. Keep the cleavage mixture at 4 °C Overnight 1d

10.3 Next day, pour the cleavage mixture over the washed resin (mentioned earlier in the IMAC section) 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 also removed from sample.

10.4 Run an SDS-PAGE to analyse all the samples. 30m



SDS-PAGE analysis of IMAC and cleavage fractions. The lower band in rIMAC FT agrees with the size of the cleaved construct (69.180 kDa)

10.5 (Optional) Elute rIMAC resin with 2 CV elution buffer to identify non-specific binding of the target protein to the resin. 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 flow through from the rIMAC containing the target protein to a final volume of under 5 mL . 1h

11.2 Centrifuge the sample at 17200 x g, 4°C, 00:10:00 to remove any aggregates. 15m

Aspirate the supernatant with a 5mL syringe and a blunt fill needle, taking care not to disturb the pellet.

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.

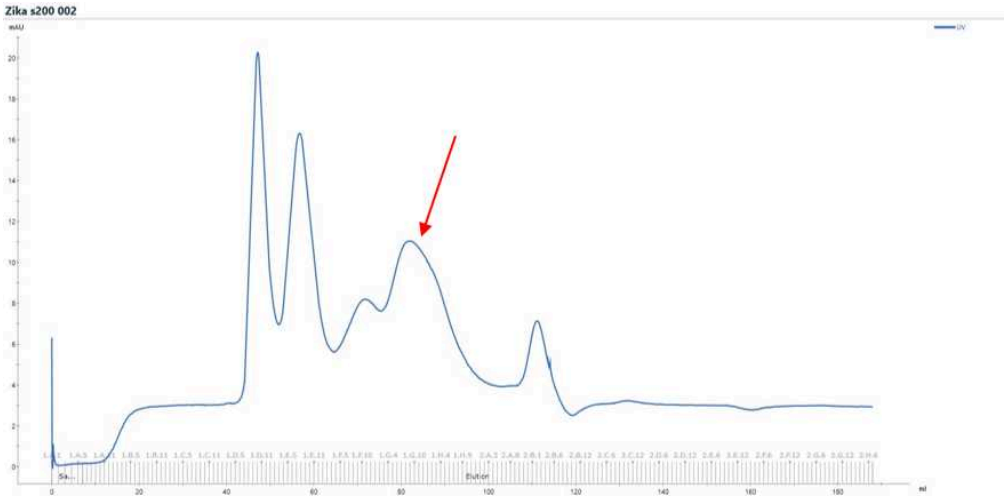
12 The next steps are performed using an AKTA Pure system: 2h

Inject the sample onto a 5mL sample loop.

Equilibrate the column in gel filtration buffer.

Run the sample through a HiLoad 16/60 Superdex 200 pg gel filtration column with a flow rate of 1mL/min, collecting 1mL fractions throughout the run.

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

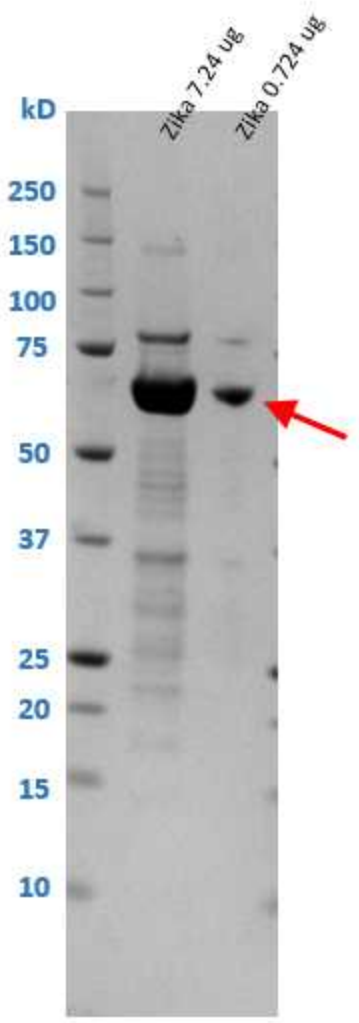


Chromatogram of the ZIKVRdRp SEC run. Based on the result of previous purifications of this construct, it is known that the peak highlighted with red arrow contains the protein of interest. Fractions s G5-H9 were analysed with SDS-PAGE to confirm the presence of the target protein, and were pooled for concentration.

13.1 Pool the fractions that contain the target protein, which in this case are fractions from G5 to H9. Concentrate the protein to around [M] 7 mg/mL using Vivaspin 500 (MWCO: 30m

30kDa) centrifugal concentrator.

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



SDS-PAGE of the final purified ZIKVRdRp construct. Red arrow indicates band that agree with the mass of the target post-cleavage.

Another 1 µL could be taken for mass spectrometry (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