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Expression and purification of truncated Zika virus NS5 RNA-Dependent RNA Polymerase for structural studies

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Anu V. Chandran[1,2,3]

[1]Diamond Light Source; [2]Research Complex at Harwell; [3]ASAP Discovery Consortium

ASAP Discovery

Mary-Ann Xavier
Diamond Light Source, Centre for Medicines Discovery, ASAP D...

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We use this protocol and it's working

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Abstract

We present a detailed protocol for the expression and purification of a truncated form of Zika virus (ZIKV) NS5 RNA-dependent RNA polymerase (RdRp), comprising residues 306-903. The construct contains an N-terminal hexahistidine-SUMO tag and was expressed in E. coli Lemo21 cells. The protein was purified through nickel affinity chromatography, SUMO protease treatment, reverse affinity chromatography, and size exclusion chromatography. The final purified protein showed a single monodisperse peak by size exclusion chromatography and was successfully used for crystallization experiments.

Guidelines

Lab coat, safety specs and gloves should be worn always.

Materials

Plasmid : 2A1

Truncated ZIKV NS5 RdRp (306-903) cloned into pOPPINS-DLS vector (proprietary vector). This construct has an N-terminal 6 Histidine and a sumo tag in that order.

Expressed protein:

MGSSHHHHHHGSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQ
ADQTPEDLDMEDNDIIEAHREQIGGYHGSYEAPTQGSASSLVNGVVRLLSKPWDVVTGVTGIAMTDTTPYGQQRVFKEKVD
TRVPDPQEGTRQVMNIVSSWLWKELGKRKRPRVCTKEEFINKVRSNAALGAIFEEEKEWKTAVEAVNDPRFWALVDREREH
HLRGECHSCVYNMMGKREKKQGEFGKAKGSRAIWYMWLGARFLEFEALGFLNEDHWMGRENSGGGVEGLGLQRLGYILE
EMNRAPGGKMYADDTAGWDTRISKFDLENEALITNQMEEGHRTLALAVIKYTYQNKVVKVLRPAEGGKTVMDIISRQDQRG
SGQVVTYALNTFTNLVVQLIRNMEAEEVLEMQDLWLLRKPEKVTRWLQSNGWDRLKRMAVSGDDCVVKPIDDRFAHALRF
LNDMGKVRKDTQEWKPSTGWSNWEEVPFCSHHFNKLYLKDGRSIVVPCRHQDELIGRARVSPGAGWSIRETACLAKSYAQ
MWQLLYFHRRDLRLMANAICSAVPVDWVPTGRTTWSIHGKGEWMTTEDMLMVWNRVWIEENDHMEDKTPVTKWTDIPY
LGKREDLWCGSLIGHRPRTTWAENIKDTVNMVRRIIGDEEKYMDYLSTQVRYLGEEGSTPGVL-

Protparam values (https://web.expasy.org/protparam/):

Number of amino acids: 706
Molecular weight: 81276.36 Da
Theoretical pI: 6.65
Ext. coefficient 164320
Abs 0.1% (=1 g/l) 2.022, assuming all Cys residues are reduced

Final Protein after tag cleavage:

YHGSYEAPTQGSASSLVNGVVRLLSKPWDVVTGVTGIAMTDTTPYGQQRVFKEKVDTRVPDPQEGTRQVMNIVSSWLWK
ELGKRKRPRVCTKEEFINKVRSNAALGAIFEEEKEWKTAVEAVNDPRFWALVDREREHHLRGECHSCVYNMMGKREKKQGE
FGKAKGSRAIWYMWLGARFLEFEALGFLNEDHWMGRENSGGGVEGLGLQRLGYILEEMNRAPGGKMYADDTAGWDTRIS
KFDLENEALITNQMEEGHRTLALAVIKYTYQNKVVKVLRPAEGGKTVMDIISRQDQRGSGQVVTYALNTFTNLVVQLIRNME
AEEVLEMQDLWLLRKPEKVTRWLQSNGWDRLKRMAVSGDDCVVKPIDDRFAHALRFLNDMGKVRKDTQEWKPSTGWSN
WEEVPFCSHHFNKLYLKDGRSIVVPCRHQDELIGRARVSPGAGWSIRETACLAKSYAQMWQLLYFHRRDLRLMANAICSAV
PVDWVPTGRTTWSIHGKGEWMTTEDMLMVWNRVWIEENDHMEDKTPVTKWTDIPYLGKREDLWCGSLIGHRPRTTWAE
NIKDTVNMVRRIIGDEEKYMDYLSTQVRYLGEEGSTPGVL

Protparam values (https://web.expasy.org/protparam/):

Number of amino acids: 598
Molecular weight: 68921.59 Da
Theoretical pI: 8.01
Ext. coefficient 162830
Abs 0.1% (=1 g/l) 2.363, assuming all Cys residues are reduced

For protein expression:

AIM -Terrific Broth Base including Trace elements (Formedium)

Carbenicillin (100mg/mL stock)
Chloramphenicol (34mg/mL stock)
L-rhamnose (10% w/v stock)
SOC media

For protein purification:
His60 Ni Superflow resin (Takara Bio)
HisTrap FF 5mL Catridges (Cytiva)
Protease Inhibitor Tablets without EDTA (Roche)
Bio-Rad Econo column
0.22µm sterile syringe filter (Millipore)
Centripure P100 desalting column (emp BIOTECH)
Centrifugal filters with a 10kDa molecular weight cut off (Millipore)
NuPAGE Bis-Tris 4-12% polyacrylamide gels

Stock solutions required for protein purification :
1M HEPES pH 7.5, 5 M NaCl, 50% Glycerol, 1 M Imidazole pH 8.0, 0.5 M TCEP, 1 M $ZnCl_2$, 1 M $MgCl_2$

Cell Lysis Buffer:
50 mM HEPES pH 7.5, 500 mM NaCl, 10% Glycerol, 10 mM Imidazole, 10 µM $ZnCl_2$, 1 mM $MgCl_2$, 1 mM TCEP

Add directly to lysate:
Lysozyme - 0.1 mg per litre of cell culture
Benzonase (0.4 mg/mL) - 5 µL per litre of cell culture

Protease inhibitor tablets: dissolve in 1 mL of lysis buffer and add directly to lysate. One tablet per 50 mL of resuspended cells.

Column Equilibration Buffer:
50 mM HEPES pH 7.5, 500 mM NaCl, 10% Glycerol

Wash Buffer:
50 mM HEPES pH 7.5, 500 mM NaCl, 10% Glycerol, 40 mM Imidazole pH 8.0, 0.5 mM TCEP

Elution Buffer 1:
50 mM HEPES pH 7.5, 500 mM NaCl, 10% Glycerol, 300 mM Imidazole pH 8.0, 0.5 mM TCEP

Desalting buffer:
50 mM HEPES pH 7.5, 500 mM NaCl, 10% Glycerol, 1 mM TCEP

Reverse affinity column equilibration buffer:
50 mM HEPES pH 7.5, 500 mM NaCl, 10% Glycerol, 0.5 mM TCEP, 5 mM Imidazole

Reverse Affinity wash buffer 1: 50 mM HEPES pH 7.5, 500 mM NaCl, 10% Glycerol, 0.5 mM TCEP, 10 mM Imidazole
Reverse Affinity wash buffer 2: 50 mM HEPES pH 7.5, 500 mM NaCl, 10% Glycerol, 0.5 mM TCEP, 20 mM Imidazole

Size Exclusion Chromatography (SEC) Buffer:
20 mM HEPES pH 7.5, 500 mM NaCl, 2.5% Glycerol, 1 mM TCEP
Crystallisation buffer:
20mM HEPES pH 7.5, 300 mM NaCl, 2.5% Glycerol, 10 µM $ZnCl_2$, 2 mM TCEP

ZIKV RdRp: Cloning of the truncated construct (residues 306-903) in to pOPPINS-DLS vector

30m

1

Note

GeneStrands encoding for the residues 306-903 of Zika NS5 RdRp (codon optimised for E. coli expression) were purchased from Eurofins with the necessary overhangs for ligation independent cloning (LIC) cloning.
The Clon-Express II One Step Cloning kit was employed for inserting the GeneStrands into KpnI/HindIII-digested pOPINS-DLS vector (proprietary vector).

Prepare the LIC reaction by combining reaction buffer, gene strands, vector and Exnase II following manufacturers instructions.

2 Incubate the reaction mix at 37 °C C for 00:30:00 . 30m

3 Transfer the reaction mix to ice.

4 Transform the reaction mix to *E. coli* Mach1 competent cells.

5 Screen multiple colonies and send the resultant plasmid from these colonies for sequencing to confirm the gene sequence.

Protein expression and purification

5d 22h 20m

6 Transform the plasmid containing gene of interest (2A1) to Lemo21 competent cells.

Note

Lemo21 cells are designed for tunable expression of challenging proteins like RNA dependent RNA polymerases. This fine tuning is achieved by varying the concentration of L-rhamnose in the culture medium.

7 Inoculate a few colonies to 1 mL of Super optimal broth (SOC) media in a 1.5mL eppendorf tube containing 100 µg/mL Carbenicillin, 34 µg/mL Chloramphenicol, [M] 0.5 millimolar (mM) L-rhamnose.

8 Grow this culture at 37 °C with shaking at 200 rpm .

9 After 01:00:00 , transfer the 1 mL culture to 10 mL SOC media containing 100 µg/mL Carbenicillin, 34 µg/mL Chloramphenicol, [M] 0.5 millimolar (mM) L-rhamnose and grow the cells again at 37 °C for 04:00:00 with shaking at 200 rpm . 5h

10 After 04:00:00 , transfer the 10 mL culture to 1 L pre-warmed AIMTB (Formedium) containing 100 µg/mL Carbenicillin and grow this culture at 37 °C with shaking at 200 rpm . 4h

11 After 05:00:00 , drop the temperature to 18 °C and continue the growth further with shaking at 200 rpm . 2d 21h

12 After 64:00:00 , harvest the cells by centrifugation at 7550 x g for 00:20:00 in Beckman High-Capacity Centrifuge at 4 °C . 2d 16h 20m

> Note
>
> After reducing the temperature, the culture is grown for a total of 64 hours

13 Resuspend the cell pellets in protein lysis buffer containing protease inhibitor tablets (Complete protease inhibitor tablet without EDTA, Roche). Aprox. 30 mL lysis buffer is used per 10 g of cell pellet. The resuspended cells can be either directly used for the protein preparation or flash frozen and stored at -80 °C .

Purification of 2A1 from 2L culture

3h 30m

14 Thaw the frozen cell suspension by keeping at Room temperature for 00:15:00 and transfer it to a precooled beaker. Keep it on a stirrer until it is completely thawed. Add Benzonase and lysozyme directly to the resuspended cells.

> Note
>
> Please check the materials section for Benzonase and lysozyme concentrations for cell lysis

15 Lyse the cells using a cell disrupter (Constant Systems Ltd). Pass the sample three times at 30 kPsi at 4 °C .

16 Spin at samples at 195426 x g, 4°C for 01:00:00 at 4 °C in a Beckman ultracentrifuge using 45 Ti rotor. This step could also be done on a high-capacity Beckman centrifuge at 48380 x g for 01:30:00 at 4 °C . 2h 30m

17 Equilibrate 20 mL of Nickel resin (50% His60 Ni Superflow resin, Takara Bio) by washing using 2 column volumes of water and 2 column volumes of column equilibration buffer. (10 mL 50% resin per litre of the culture i.e. around 5 mL bead volume per litre of cells).

18 Filter the supernatant from step 3 using 0.22 µm pore size filter and mix with the equilibrated Nickel beads.

19 Keep the sample-bead mixture in a roller for 1 hour 01:00:00 at 4 °C . 1h

20 Pass the sample from step 6 through an empty Bio-Rad Econo-column. Discard the flow through.

21 Wash the resin with 25 column volumes of wash buffer.

22 Elute the protein as 5 fractions, 10 mL each, using the elution buffer, collecting each in a separate 50 mL falcon tube.

23 Pool the fractions that contain the protein of interest as identified using an SDS-PAGE gel.

24 Concentrate the protein using a 10 kDa centricon from Millipore to 20mL and desalt using a Centripure P100 desalting column (emp BIOTECH) following the instructions given in the product manual.

Note

The Centripure P100 desalting column can handle up to 10mL of protein per run. If the protein solution is concentrated to 20mL, it could be split into two 10mL batches for desalting. Alternatively, multiple runs could be performed without concentrating the protein, ensuring each run stays within the 10mL limit. Wash the column according to the product manual after each run to maintain its performance and longevity. This will help ensure consistent results across all the batches.

25 Measure the concentration of the protein, add Ulp1 (SUMO protease) at a mass ratio 1:50 and place on a rotating wheel in the cold room overnight for tag cleavage.

Note

Dialysis is not suitable for desalting and tag cleavage as this could result in protein aggregation.

26 Pass the sample after tag cleavage through two 5 mL HisTrapFF column (Cytiva) connected in tandem, equilibrated with reverse affinity column equilibration buffer and collect the flow through from the column. This step is carried out using a peristaltic pump.

27 Wash the column with reverse affinity wash buffer. Collect 11 fractions, 10 mL each. (Optional) wash the column using RA wash buffer 2 and collect 4 fractions 10 mL.

28 Concentrate as before, the flow through from the column and the washes from the reverse affinity step separately, to a final volume of 5 mL each. Size exclusion chromatography (SEC) is carried out separately for the flow through and the reverse affinity wash. The reverse affinity wash is always better than flow through in terms of protein yield and quality.

29 Equilibrate a HiLoad S200 16/60 column, connected to an AKTA purifier maintained at 4 °C , with SEC buffer.

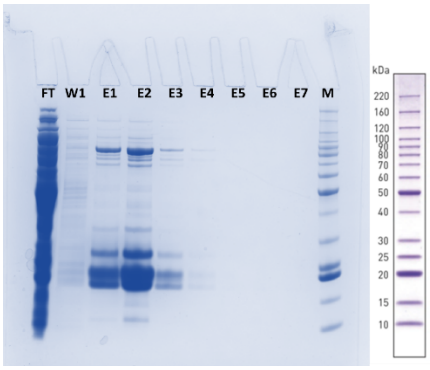
30 Inject the sample via a 5 mL loop. Run the SEC at a flow rate of 1.2 mL/min, with 2 mL fractions collected in a 96-well block.

31 Pool the fractions with the protein of interest and buffer exchange to crystallisation buffer if setting up crystallisation. Otherwise, concentrate the protein using a 10kDa Centricon from Millipore to [M] 5 mg/mL , make 50 µL aliquots, flash freeze in Liquid Nitrogen and store at -80°C freezer.

Results

32

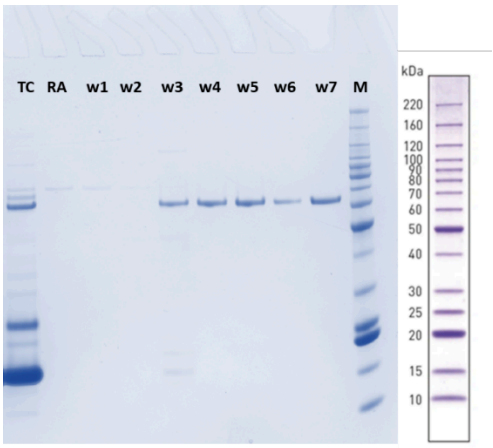
NuPAGE Bis-Tris 4-12% polyacrylamide gels were used for all SDS-PAGE in this purification. SDS-PAGE was run using 1X MES running buffer for 45min at 200V.



Picture 1: Flow through, wash and the elution from the Ni affinity step

Samples:
FT: Flow through from His60 affinity column
W1 : Wash
E1 : Elution 1
E2 : Elution 2
E3 : Elution 3
E4 : Elution 4
E5 : Elution 5
E6 : Elution 6
E7 : Elution 7
M : Marker (Invitrogen Bench Mark Protein ladder)

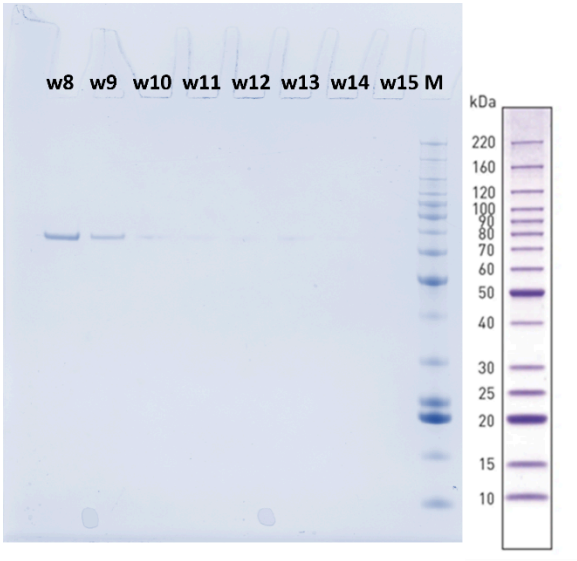
32.1



Picture 2: Protein after tag-cleavage, RA flow through and fractions (1-7) from the reverse affinity wash using RA wash buffer 1

Samples:
TC : after o/n tag cleavage
RA : Reverse affinity flow through
W1 : RA wash 1
W2 : RA wash 2
W3 : RA wash 3
W4 : RA wash 4
W5 : RA wash 5
W6 : RA wash 6
W7 : RA wash 7
M : Protein Marker

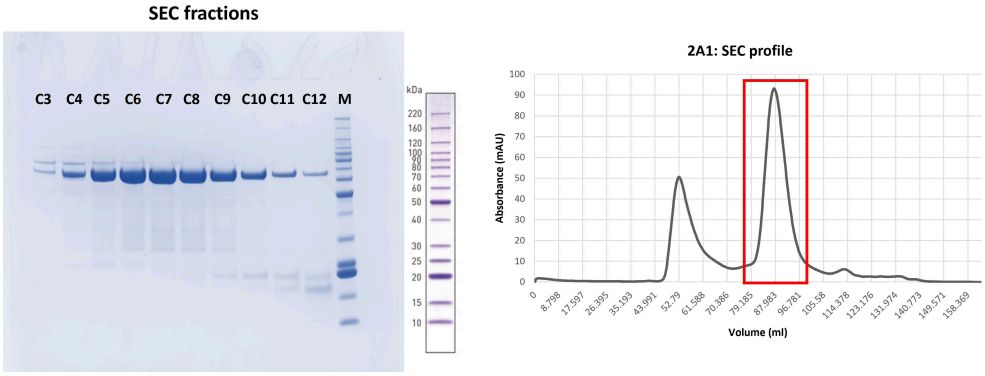
32.2



Picture 3: Fractions (8-11) from the reverse affinity wash using buffer 1 and those from the reverse affinity buffer 2 wash

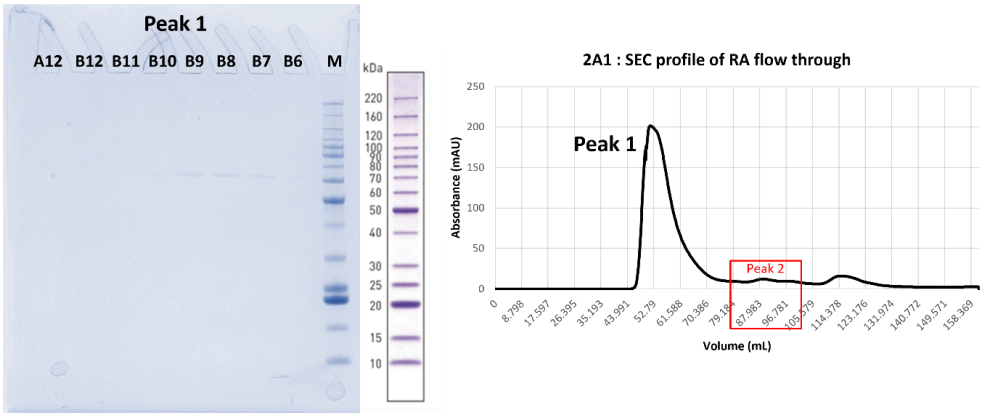
Samples:
W8 :RA wash 8
W9 :RA wash 9
W10 :RA wash 10
W11 :RA wash 11
W12 :RA wash using second buffer 1
W13 :RA wash using second buffer 2
W14 :RA wash using second buffer 3
W15 :RA wash using second buffer 4
M: Protein Marker

32.3 **SEC fractions: C3-C12 corresponding to the peak 2. This is the protein sample that has been used for crystallisation.**



Picture 4: SDS-PAGE (of peak 2) and the SEC profile of the protein from the reverse affinity washes are shown here. The fractions corresponding to peak 2 (highlighted in red box) are shown in the SDS-PAGE

32.4



Picture 5: SDS-PAGE (of peak 1) and SEC profile of the protein from the reverse affinity flow through. As this protein showed aggregation during concentration, protein from this run was not used for any experiment.

32.5

Protocol references

ASAP Discovery Consortium