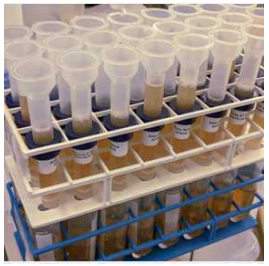
Oct 30, 2024 Version 2

Dengue virus serotype 3 NS2B-NS3 protease fusion construct using parallel rapid protein expression and purification: 100 mL cultures V.2



Version 1 is forked from Parallel rapid expression and purification of proteins for crystallography (PREPX): 48× 100 mL cultures

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ASAP Discovery

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CMD

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We use this protocol and it's working

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Abstract

This protocol details the small scale (100mL) expression and purification of Dengue virus serotype 3 NS2B-NS3 protease fusion constructs using the parallel rapid protein expression and purification (PREPX) method. Recombinant proteins are expressed in *Escherichia coli* using the autoinduction method and then purified in parallel using a IMAC, desalt, tag cleavage, reverse IMAC and gel filtration work flow.

This protocol is adapted from the original PREPX protocol, which is suitable for expressing and purifying multiple constructs in parallel. The protocol can also be adapted for single construct.

Attachments

PDF

PAGE24-00103 - AVIDD...

560KB

Guidelines

Method overview

Standard workflow is expression via autoinduction followed by purification using IMAC/PD-10/revIMAC and serial gel filtration

Materials

Plasmid details:
Vector: pNIC

- Cell line: E. coli Rosetta strain BL21(DE3)-RR
- Tags and additions: N-terminal His-Tandem Streptag II - GG
- Construct protein sequence: `
  MHHHHHHSSGASWSHPQFEKGGGSGGGSGGSAWSHPQFEKGSGVDLGTENLYFQSMADLTVEKAADVTWEEEAEQ
  TGVSHNLMITVDDDGTMRIKDDETENILGGGGSGGGGSGVLWDVPSPPETQKAELEEGVYRIKQQGIFGKTQVGVGVQK
  EGVFHTMWHVTRGAVLTHNGKRLEPNWASVKKDLISYGGGWRLSAQWQKGEEVQVIAVEPGKNPKNFQTMPGIFQTT
  TGEIGAIALDFKPGTSGSPIINREGKVVGLYGNGVVTKNGGYVSGIAQTNAEPDGPTPELEEEFRK

- His Gravitrap columns (Cytiva) supplier item 11003399 - for capture and purification of histidine tagged proteins, maximum binding capacity of 40 mg of tagged protein per column
  His GraviTrap | Cytiva (cytivalifesciences.com)

- PD-10 buffer reservoir (Cytiva) supplier item 18321603 - increases the His Gravitrap and PD-10 desalting column load volumes to 40mL
  Buffer Reservoir | Cytiva (cytivalifesciences.com)

- PD-10 column spin adapters (Cytiva) supplier item 28923245 - used to prevent wobbling of columns in nalgene racks
  Spin Adapters for Gravity Columns | Cytiva (cytivalifesciences.com)

- PD-10 desalting columns (Cytiva) supplier item 17085101 - used for rapid buffer exchange
  PD-10 desalting columns packed with Sephadex G-25 resin | Cytiva (cytivalifesciences.com)

- Nalgene™ Unwire™ Test Tube Racks: Resmer™ Manufacturing Technology, for 30mm tubes, white **Thermo Fisher Catalog #**5970-0030
- AIM – Terrific Broth Base including Trace elements **Formedium Catalog #**AIMTB0210
- Ultra Yield 2.5L Flask, Sterile **Generon Catalog #**931136-B

Optional but useful

- BENCHMIXER™ XL MULTI-TUBE VORTEXER **Benchmark Scientific Catalog #**BV1010

Materials (1 L cultures) for Expression:

- LB-agar+antibiotics plate
- 100 mL of autoclaved autoinduction TB + 20 g/L glycerol + antibiotics
- 0.01 mL of 10 % Antifoam 204 (Sigma) in ethanol
- 1x 250 mL Corning baffled flasks (fitted with loose foil cover**)

Materials for Purification:

- **5 L of Base Buffer**

	A	B
	HEPES	10 mM
	Glycerol	5%
	NaCl	500 mM
	TCEP, pH 7.5	0.5 mM

- 100 mL of [M] 3 Mass Percent imidazole pH 7.5.
- 100 mL of 10 % Triton X-100 in water.
- 50 µL Lysozyme solution (100 x).
- 1 µL homemade benzonase (1000x). Maybe susbtituted for 10 mg/mL of commercial DNase I
- 1 His GraviTrap columns to be purified (Cytiva) fitted with LabMate extender (Cytiva) and PD-10 spin adapter (Cytiva) in 24 place Nalgene rack.
- 1 PD-10 desalting column to be purified fitted with LabMate extender and PD-10 spin adapter in 24 place Nalgene rack.
- 1 × 50 mL centrifuge tubes per litre of culture to be purified in a 24 place Nalgene rack.

Safety warnings

Triton x-100 is currently restricted for use in the EU and cannot be used without an exemption certificate REACH Annex XIV (Jan 2021). It can be readily subsituted with IGEPAL CA-630 (which is likely to be subject to the same restrictions in the near future). Alternatives that also maybe used are Tergitol 15-S-9 or Tween-20 or octyl glucoside.

Expression

12h 20m

1 Either transform *BL21 (DE3)* with the appropriate plasmid OR streak from glycerol stock onto 24 well agar plate and incubate Overnight 37 °C *. 4h

Note

* Freshly transformed or re-streaked cells always give better yields than growing overnights directly from frozen glycerol stocks.

2 Grow 1.5 mL Overnight in suitable container (2 mL 96-well plate, 15 ml faclon) of each clone in superbroth + 1 % glucose + the appropriate antibiotics. 4h

3 Use 1 mL to inoculate 100 mL AIM-TB (+ Antibiotics + Antifoam 204) in a baffled flask.

4 Grow 250 rpm, 37°C, 04:00:00 shaking using loose foil cover**. 4h

AERATION IS ESSENTIAL!.

Note

**An upturned 50 mL plastic beaker with a 1.5 mL microcentrifuge tube taped to the side of the flask to act as a spacer can also be used.
**A breathable membrane such as an AirOtop enhanced flask seal may also be used.

5 Grow 40-48 h 250 rpm, 18°C shaking.

6 Harvest at 4000 x g, 4°C, 00:20:00 in 50 ml falcon tubes and discard supernatant. 20m

7 Harvest remainder of culture in same tube at 4000 x g, 4°C, 00:20:00 , discard supernatant and freeze pellet in falcon tube at -80 °C . 20m

Note

Final wet cell weight is typically 5.5 g of culture

Cell lysis

3h 30m

8 Thaw pellets.

9 Add 20 mL Base Buffer to each tube containing pellet ([M] 10 millimolar (mM) HEPES, [M] 500 millimolar (mM) NaCl, 5 % Glycerol, [M] 0.5 millimolar (mM) TCEP, pH 7.5) + 0.5 µL Lysozyme, 1 µL Benzonase or 10 µL DNase I, [M] 20 millimolar (mM) imidazole.

10 Vortex to dissolve pellet and leave 00:30:00 Room temperature . 30m

11 Add 4 mL 10 % Triton X-100*** to each tube and bring volume to 40 mL using Base Buffer

Note

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12 Freeze -80 °C 1-2 h or overnight if preferred.

13 Thaw in Room temperature water bath 01:00:00 and mix. 1h

14 Centrifuge 4000 x g, 4°C, 01:00:00 . 1h

> Note
>
> Higher speed centrifugation can also be performed if desired, e.g. 20,000 g but transfer to suitable centrifuge tubes will be necessary.

Purification 3h 30m

15 Apply supernatant from a single tube to 1 mL His GraviTrap column (Cytiva) fitted with LabMate extender.

> Note
>
> Imidazole concentration can be increase to 40 mM in most cases, but may affect yield.

16 Wash 10 mL Base Buffer + [M] 20 millimolar (mM) Imidazole**.

> Note
>
> **10 mL of a 40 mM or 70 mM imidazole wash can also be done, but this is very target dependent and may lead to significant reduction in final yield BUT can also increase purity substantially, worth trying if your purity is poor.

17 Slot His GraviTrap column into PD10 column (Cytiva) fitted with LabMate extender (pre-equilibrated in Base Buffer + [M] 20 millimolar (mM) Imidazole).

18 Elute protein with 2.5 mL of Base Buffer + [M] 500 millimolar (mM) Imidazole directly onto PD10 column.

19 Remove His GraviTrap column.

20 Place PD10 into 50 mL falcoln tube and add 3.5 mL Base Buffer + [M] 20 millimolar (mM) Imidazole and collect.

21 Measure A280.

22 Add protease 1 OD unit TEV for every 10 OD units target and incubate Overnight 1h
4 °C .

Note

Some targets exhibit significant affinity for IMAC columns even after TEV cleavage try increasing the imidazole concentration to 40 or 70 mM or use an MBP-TEV construct so that the protease can be removed using an amylose column rather than reverse IMAC.

23 Run back over His GraviTrap column equilibrated in Base Buffer + [M] 20 millimolar (mM) Imidazole.

24 Wash column 2.5 mL [M] 20 millimolar (mM) Imidazole.

25 Check purity of 6 mL pool.

26 Concentrate to 1 mL ish.

27 Transfer to 1.6 mL glass autosampler vial ensure at least 1.1 mL in vial!.

28 Run through serial gel filtration system injecting 1 mL .

29 Take peak fraction(s) only (1-2 mL) and concentrated to 10-20 mg/mL if possible.

Column regeneration: PD-10

30 Wash PD-10 columns with 50 mL - 100 mL of Milli-Q water.

31 Store all columns in water at 4 °C. For long term storage use 20 % Ethanol

Column regeneration: His GraviTrap

32 Wash IMAC columns 40 mL Milli-Q.

33 Wash IMAC columns 10 mL 20 % Ethanol + [M] 0.1 Mass Percent EDTA*.

*PUT NICKEL WASTE IN APPROPRIATE CONTAINER FOR DISPOSAL!

34 Wash IMAC columns 40 mL Milli-Q.

35 Wash IMAC columns 10 mL [M] 1 Mass Percent NaOH.

36 Wash IMAC columns 40 mL Milli-Q.

37 Wash IMAC columns 10 mL [M] 1 Mass Percent Acetic Acid + 1 % Triton X-100.

38 Wash IMAC columns 40 mL Milli-Q.

39 Wash IMAC columns 0.5 mL [M] 100 millimolar (mM) Nickel Sulfate + [M] 20 millimolar (mM) Tris.HCl pH 8*.

Note

*PUT NICKEL WASTE IN APPROPRIATE CONTAINER FOR DISPOSAL!

40 Wash IMAC colums 40 mL Milli-Q.

41 Store all columns in water at 4 °C. For long term storage use 20 % Ethanol