Dec 20, 2019

Heterologous protein expression in E. coli V.4

Diep R. Ganguly[1], Timothy Rhodes[1], Nay Chi Khin[1], Estee E Tee[1], Kai Xun Chan[1]

[1]The Australian National University

In Development dx.doi.org/10.17504/protocols.io.95qh85w

Pogson Genomics Group

Diep R. Ganguly
The Australian National University

ABSTRACT

Protocol for recombinant protein expression in E. coli for protein purification and subsequent enzyme assays, protein crystallography etc.

GUIDELINES

This protocol will take a few days so be sure to have all buffers, cell strains, and plasmids on hand. Different sections do not need to be performed immediately after each other - there are various safe stopping steps where cells can be stored at -20/-80 ºC until you are ready to continue. However, for convenience, the entire protocol is described here.

Adjust volumes, taking care to ensure appropriate vessels are used to allow proper aeration (e.g. grow 800 mL culture in 2 L flasks or 2 L culture in 5 L flasks), depending on the desired downstream application and expected protein yield. We commonly use BL21 (DE3) strains for T7 expression (i.e. IPTG induction).

MATERIALS

NAME	CATALOG #	VENDOR
Potassium chloride	View	P212121
Petri Dish	LI-PD01100	P212121
Lysozyme from chicken egg white	L6876	Sigma Aldrich
Luria-Bertani (LB) broth, makes 1L	K488	Amresco
EDTA		
cOmplete™, EDTA-free Protease Inhibitor Cocktail	05056489001	Sigma Aldrich
1.5 mL Eppendorf tubes		
Electroporation System Gene Pulser XCell		Bio-rad Laboratories
37˚C Incubator		
DTT	D0632	Sigma Aldrich
14ml Polystyrene Cell Culture Tubes	CT5250	Alkali Scientific
4X Bolt LDS Sample Buffer	B0007	Invitrogen - Thermo Fisher
NaCl	53014	Sigma Aldrich
IPTG	IB0168.SIZE.100g	Bio Basic Inc.
BL21(DE3) or BL21-Star(DE3) or Rosetta2(DE3) or etc for protein purification		
Magnesium chloride hexahydrate	M2670	Sigma Aldrich
Electroporation Cuvette 1mm	1652089	BioRad Sciences
Falcon® Conical Tubes, 50 mL 500 Tubes	38010	Stemcell Technologies

NAME	CATALOG #	VENDOR
Tris-HCl	AM9855	Life Technologies
28°C incubator without CO2		Thermo Fisher Scientific
Disodium phosphate	S7907	Sigma Aldrich
Monopotassium phosphate	P9791	Sigma Aldrich
42°C water bath		
Imidazole	I5513	Sigma Aldrich
UV/Vis spectrophotometer	View	
GelCode™ Blue Stain Reagent	24590	Thermo Fisher Scientific
Q125 Sonicator	Part #Q125	

SAFETY WARNINGS

Ensure use of appropriate asceptic technique. Use caution if using a bunsen burner and ethanol.

BEFORE STARTING

Make sure you have your verified plasmid transformed into your desired E. coli strain for protein expression e.g. BL21 Star (DE3). These should be plated on selective LB media to produce positive colonies for starter cultures. Prepare all the buffers described in Step 1, except make fresh IPTG stocks.

Prepare buffers

1 Prepare the following buffers:

10X PBS
Dissolve the following in 800 mL H_2O:

- 80 g of NaCl (1.37 M)
- 2.0 g of KCl (27 mM)
- 14.4 g of Na_2HPO_4 (100 mM)
- 2.4 g of KH_2PO_4 (18 mM)

- Adjust pH to 7.4.
- Add H_2O to 1L.
- Autoclave

Store 10X stock at 4 °C from which you can dilute 1:10 to make 1X working stock to keep at room temp.

Re-suspension buffer
- 50 mM Tris-HCl pH 8
- 2 mM EDTA

Lysis buffer
- 50 mM sodium phosphate pH 7.5 (adjust to ~1-2 units away from pI of expressed protein)
- 300 mM sodium chloride
- 10 mM imidazole
- 5% glycerol
- 10 mM 2-Mercaptoethanol or 1 mM DTT (if disulfide-bonds are a problem)
- 1x Protease-inhibitor cocktail

Denaturing buffer
- 6 M Urea
- 4% CHAPS
- 35 mM Tris-HCl pH 8

Transformation

2 Transform desired E. coli cell strain with plasmid to be expressed using desired method (e.g. heat shock or electroporation depending on type of competency). 14h

3 For electrocompetent cells:
- Add 0.5 - 1 µL purified plasmid to 50 µL cells (thawing on ice, 15 minutes)
- Gently flick with finger to mix
- Transfer mixer to chilled electroporation cuvette ensuring there are no bubbles. Keep on ice until ready to electroporate
- Set machine to 1.8 kV, 25 µF, 200-400 Ω
- Dry the outside of the cuvette and place into electroporation chamber.
- Prepare p1000 and p200 pipettes to be ready
- Close chamber and electroporate
- Immediately remove cuvette and add 1 mL LB. Transfer contents to microfuge tube using both p1000 and p200.
- Let cells recover at 37 ºC with ~200 rpm shaking for > 1 hour.

4 For chemically competent cells:
- Add 0.5 - 1 µL purified plasmid to 50 µL cells (thawing on ice, 15 minutes)
- Gently flick with finger to mix
- Sit on ice for 30 minutes, set water bath to heat to 42 ºC
- Depending on cells, incubate in water bath for 30 - 90 seconds.
- Return to ice for 5 minutes
- Add 1 mL LB and let cells recover at 37 ºC with ~200 rpm shaking for > 1 hour.

5 Plate recovered transformed cells (~100 µL of transformed cells) onto selective LB media and grow O/N @ 37 ºC. Adjust volume as needed in order to obtain single colonies that can be picked for subsequent innoculation. 1h

Protein expression

6 Use single bacterial colony to inoculate into LB + appropriate antibiotic (if possible, make multiple inoculations with separate single colonies). 1h

Use p100-200 pipette tip to scrape colony and drop into 3-5 mL broth in 10 mL culture tube. Grow O/N @ 37 ºC with ~200-250 rpm shaking.

7 Inoculate larger culture using the starter culture generated from Step 3 at 1:50 dilution (e.g. 0.5 mL in 25 mL LB + antibiotic). 1m

Final culture volume will depend on downstream applications. Ensure use of an appropriate vessel to allow effective aeration (see suggestions in guidelines).

8 Grow larger culture at 37 ºC and check OD_{600} after 2.5 - 3 hours (time will vary depending on dilution, total culture volume, cell strain and quality, and aeration). 3h

9 When OD_{600} is between 0.6 - 0.8, take an aliquot of culture (up to 15 mL) as a non-induced control. 1m

To the remaining culture, add induction media. We typically use BL21 (DE3) derived strains and, thus, add IPTG to achieve $[IPTG]_{final}$ = 0.4 mM. You may need to find an optimal concentration of IPTG to achieve effective induction of protein expression vs toxicity to bacterial cells.

10 Grow cultures overnight (ideally at 18-25 ºC). Measure OD_{600} for difference between induced vs non-induced. Non-induced should be higher by at least 0.1 (minimum difference). 12h

11 Once OD_{600} difference of 0.1 is achieved, move cultures to ice and prepare chilled centrifuge. 1m

12 Pellet cells at 4 ºC (~7,000 rcf for 5 minutes with gentle stop).

13 Remove supernatant and wash cells in *re-suspension buffer* (practical volume to resuspend cells). Pellet as above and discard supernatant.

14 Snap-freeze pellet in LN_2 and store at -80 ºC.

QC protein expression

15 It may be worthwhile testing for successful induction of recombinant protein expression on an aliquot (>1 mL) of induced culture. 2m

16 Thaw cells on ice and aliquot appropriate volume of culture to microcentrifuge tube (can resuspend in small volume of *re-suspension buffer*). Store remaining cells at -80 ºC (avoid repeated freeze-thaws).

17 Spin down culture at max speed for 3 minutes and remove supernatant.

Note, this pellet can be stored @ -20 ºC.

18 Resuspend cells in 100 µL 1X PBS (per 1 mL culture). Store the resuspended crude lysate at -20 °C when not in use. 1m

19 To these volumes, add the appropriate amount of 4X LDS (or required SDS-PAGE sample buffer), $[DTT]_{final}$ = 50 mM, and $[MgCl_2]_{final}$ = 100 mM. These should be calculated first, and a mastermix of LDS, DTT and $MgCl_2$ can be prepared and added to samples accordingly.

20 Calculate how much crude lysate to load onto gel based on OD and concentration factor (CF): 1m
CF = volume of culture / volume of resuspension (e.g. CF = 1 mL culture / 100 µL 1X PBS = 10x)

µL to load = [180/CF]/OD

21 Heat sample @ 72 ºC for 10 minutes in water bath. 1m

22 Place samples on ice for 5 minutes then spin for 15 minutes at max speed. 2m

23 Transfer supernatant to new tubes, taking care to avoid any "sticky" DNA coating the tube.

24 Run supernatant on SDS-PAGE gel and perform Coomassie staining. Alternatively, transfer to PVDF membrane and perform Western blot analysis (ideally, recombinant protein contains epitope tag).

Solubilisation

25 To isolate soluble protein, re-suspend frozen, pelleted cells in an adequate volume of *lysis buffer* (alternatively, trial 1x PBS + 1 mg/mL lysosyme, however, proteins are likely to be unstable in this buffer).

Note: ensure pH of *lysis buffer* is >1 pH units away from the isoelectric point of your expressed protein.

Keep samples on ice at all times.

26 Transfer cell re-suspension to clean microfuge tube (or appropriate vessel for probe sonication). Retain any remaining cell suspension at -20 °C.

27 Sonicate samples with the Q125 ultrasonicator (125 watt, 20 kHz, 60% amp, 30 seconds).

Keep samples on ice during and between rounds of sonication.

28 Spin at 16,000g for 20 minutes at 4 °C.

29 Recover supernatant in a clean tube. Retain the pellet at -20 °C to for insoluble protein fraction.

30 To isolate the insoluble fraction; resuspend the remaining pellet in *denaturing buffer* (same volume as *lysis buffer*).

Repeat the sonication and centrifugation steps as per the soluble fraction. Recover supernatant in a clean tube.

31 Perform a Bradford assay to determine protein concentration in the soluble and insoluble fractions. Use these concentrations to normalise input volumes for each sample to a desired loading amount (e.g. 3 µg).

32 Run samples on SDS-PAGE and stain gel with Coomassie or perform Western blot to verify expression.