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Heterologous protein expression in E. coli V.2

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ABSTRACT

Protocol for recombinant protein expression in E. coli for protein purification for 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. It can be tricky to organise, practice and familiarity are the best tools. 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 with the protocol. 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 800mL culture in 2L flasks, 1.3L culture in 3L flasks and 2L culture in 5L flasks), depending on the desired downstream application and expected protein yield (e.g. trialling expression vs bulk production for crystallography). 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		
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
Heat transfer block	Z3271	Promega
IPTG	IB0168.SIZE.100g	Bio Basic Inc.
Coomassie Blue Staining & Destaining Solution	AR0140	Boster Bio
BL21(DE3) or BL21-Star(DE3) or Rosetta2(DE3) or etc for protein purification		
Magnesium chloride hexahydrate	M2670	Sigma Aldrich

NAME	CATALOG #	VENDOR
Electroporation Cuvette 1mm	1652089	BioRad Sciences
Falcon® Conical Tubes, 50 mL 500 Tubes	38010	Stemcell Technologies
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		
UV/Vis spectrophotometer	View	
GelCode™ Blue Stain Reagent	24590	Thermo Fisher Scientific

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 (ideally).

Prepare buffers

1 **10X PBS:**

- Dissolve the following in 800 mL H_2O:
- 80 g of NaCl
- 2.0 g of KCl
- 14.4 g of Na_2HPO_4
- 2.4 g of KH_2PO_4

- 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.

Denaturing buffer:

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

Resuspension buffer:

- 50 mM Tris-HCl pH 8
- 2 mM EDTA

Transformation

14h

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).

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.

1h

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.

Protein expression

1h

6 Innoculate bacterial colony from selective media into LB + antibiotic of choice. 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 rpm shaking.

1m

7 Innoculate larger culture using the starter culture generated from Step 3 at 1:100 dilution (e.g. 0.5 mL in 50 mL LB + antibiotic). Larger culture volume will depend on what you plan to do with the protein. For example, for large-scale protein production you may use 1 - 2 L cultures - ensure you use an appropriate vessel to allow appropriate aeration (see suggestions in guidelines). For smaller scale tests for protein induction and solubility, you may prefer 10 - 15 mL cultures in 50 mL falcon tubes.

3h

8 Grow larger culture at 37 ºC and check OD_{600} after 2.5 - 3 hours (times may vary depending on total culture volume and quality of cells).

1m

9 When OD_{600} is between 0.6 - 0.8, take an aliquot of culture (up to 15 mL) as non-induced control. To the remaining culture, add induction media. We typically use BL21 (DE3) derived strains and, thus, add 100 mM IPTG to achieve $[IPTG]_{final}$ = 1 mM (i.e. 1:100 dilution, 100 µL IPTG to 10 mL culture).

10 Grow cultures for approx. 5 hours, then check OD for difference between induced vs non-induced. Non-induced should be higher by at least 0.1.

QC protein induction

2m

11 If you are running small scale test induction (e.g. testing constructs, induction, protein solubility), we can run SDS-page gels on the crude lysate (~1-2 mL culture) before trying larger cultures. With larger cultures, you may want to take an aliquot of the culture to check induction before proceeding with purification. If you are confident, proceed to step 21 for washing and storage.

12 Spin down culture (~1 - 3 mL) at max speed for 3 minutes and remove supernatant. This pellet can be stored O/N @ -20 ºC until you are ready to proceed with checking induction of your expressed recombinant protein.

1m

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

1m

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

µL to load = [180/CF]/OD

15 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.

1m

16 Heat sample @ 72 ºC for 10 minutes on heat transfer block.

2m

17 Place samples on ice for 5 minutes and spin for 15 minutes at max speed.

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

19 Run supernatant on SDS-PAGE gel (160 V, 40-45 minutes).

20 Stain gel with Coomasie or Gel Code Blue (or other stain of choice). Alternatively, perform Western blot if antibodies are available or recombinant proteins are epitope tagged (e.g. MYC).

21 The above will demonstrate if recombinant protein has been expressed, however, it is still unknown whether this is soluble protein. To isolate the soluble fraction and run on gel: treat the PBS suspension with lysozyme (1 mg/mL) and incubate for 30 minutes on ice.

22 Sonicate 3 x 1 minutes to lyse the cells (water bath sonicator). Cool on ice (1 minute) between sonications.

[or 3 x 20 s if using a dry sonicator filled with ice]

23 Spin at max speed for 20 minutes and recover the supernatant to a new tube. Retain the pellet at -20 °C to isolate the insoluble fraction of proteins.

24 Run the same amount of supernatant per sample on SDS-PAGE as before. This will indicate how much soluble recombinant protein is present.

25 To isolate the insoluble fraction and run on gel: with the remaining pellet after sonication, resuspend in a *denaturing buffer* (same volume as initial 1X PBS e.g 100 µL PBS = 100 µL denaturing buffer).

26 Repeat the sonication plus centrifugation. Run samples on SDS-PAGE as per the soluble protein fraction.

Store cells for purification

1m

27 If you are confident with your protein induction (see QC) and want to preserve the cell culture for protein purification, spin down cells at 4 ºC (~7000 rcf for 5 minutes with gentle/no brake). Remove supernatant.

28 Wash cells in resuspension buffer, spin down, and remove supernatant.

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

Likely, this will be done pre-emptively until results from the QC are obtained, then cells can be discarded or retained accordingly.