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Heterologous protein expression in E. coli V.3

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Protocol status: In development
We are still developing and optimizing this protocol

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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

MATERIALS

- Potassium chloride **P212121**
- Petri Dish **P212121 Catalog #**LI-PD01100
- Lysozyme from chicken egg white **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**L6876
- Luria-Bertani (LB) broth, makes 1L **Amresco Catalog #**K488
- EDTA
- cOmplete™, EDTA-free Protease Inhibitor Cocktail **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**05056489001
- 1.5 mL Eppendorf tubes
- Electroporation System Gene Pulser XCell **Bio-Rad Laboratories**
- 37˚C Incubator
- DTT **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D0632
- 14ml Polystyrene Cell Culture Tubes **Alkali Scientific Catalog #**CT5250
- 4X Bolt LDS Sample Buffer **Invitrogen - Thermo Fisher Catalog #**B0007
- NaCl **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**53014
- IPTG **Bio Basic Inc. Catalog #**IB0168.SIZE.100g
- BL21(DE3) or BL21-Star(DE3) or Rosetta2(DE3) or etc for protein purification
- Magnesium chloride hexahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M2670
- Electroporation Cuvette 1mm **Bio-Rad Laboratories Catalog #**1652089
- Falcon® Conical Tubes, 50 mL 500 Tubes **STEMCELL Technologies Inc. Catalog #**38010
- Tris-HCl **Life Technologies Catalog #**AM9855
- 28°C incubator without CO2 **Thermo Fisher Scientific**
- Disodium phosphate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S7907
- Monopotassium phosphate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9791
- 42°C water bath
- Imidazole **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**I5513
- UV/Vis spectrophotometer
- GelCode™ Blue Stain Reagent **Thermo Fisher Scientific Catalog #**24590
- Q125 Sonicator **Catalog #**Part #Q125

Safety warnings

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

Before start

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 **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.

Lysis buffer

- 50 mM sodium phosphate pH 7.5 (make sure ~1-2 pH 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

Re-suspension buffer:

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

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 (either at 18, 25, or 37 ºC - we typically induce at 18 or 25 ºC). Measure OD_{600} for difference between induced vs non-induced. Non-induced should be higher by at least 0.1.

QC protein expression 1

11 It is often worthwhile to test for protein expression and solubility on an aliquot (>1 mL) of culture with SDS-PAGE prior to protein purification. 2m

12 Spin down culture (>1 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.

13 Re-suspend cells in 100 µL 1X PBS (per 1 mL culture). Store the re-suspended 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 1m

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.

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

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

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

19 Run supernatant on SDS-PAGE gel as appropriate.

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).

QC protein expression 2 - soluble protein with PBS

21 The above will demonstrate protein expression, 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 with the Q125 ultrasonicator (125 watt, 20 kHz, 60% amp, 1× 30 seconds). Keep samples cool during and between sonication.

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

24 To isolate the insoluble fraction; resuspend the remaining pellet in *denaturing buffer* (same volume as initial 1X PBS e.g 100 µL PBS = 100 µL denaturing buffer). Repeat the sonication and centrifugation steps.

25 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).

26 Run samples on SDS-PAGE and stain gel with Coomassie or perform Western blot.

QC protein expression 3 - soluble protein fraction with *lysis buffer*

27 1x PBS may not be the best buffer for maintaining protein stability particularly at higher concentrations. Instead, one can use a *lysis buffer* containing imidazole (described in step 1) for cell lysis and protein purification, which may improve protein solubility.

28 Spin down an aliquot of (or entire) induced culture (and non-induced control) and concentrate by removing the *majority* of supernatant (but not all). Keep samples on ice.

29 Sonicate with the Q125 ultrasonicator (125 watt, 20 kHz, 60% amp, 1× 30 seconds) and immediately add *lysis buffer* (same volume as calculated for 1x PBS). Keep on ice.

30 Spin at max speed for 20 minutes and recover the supernatant. Retain pellet (can keep at -20 °C)

31 To the pellet, add *denaturing buffer* (same volume as 1x PBS) and repeat the sonication and centrifugation steps.

32 Perform a Bradford assay to determine protein concentration in the soluble and insoluble fractions.

33 Run samples on SDS-PAGE using determined concentrations to load a normalized amount of protein (e.g. 3 µg). Stain gel with Coomassie or perform Western blot.

Store cells for purification

34 To preserve the majority of your cell culture for protein purification, spin down cells at 4 ºC (~7000 rcf for 5 minutes with gentle/no brake). 1m

35 Remove supernatant and wash cells in *Re-suspension buffer.* Spin down and remove supernatant as above.

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