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Heterologous protein expression in E. coli V.5

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We use this protocol and it's working

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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

Troubleshooting

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. Add DTT to buffers prior to use.

Prepare buffers

1 **Buffer recipes**

Auto-induction TB medium (alternatively, liquid LB media for IPTG induction)

- 5 g/L yeast extract
- 20 g/L tryptone
- 85.5 mM NaCl (5 g/L)
- 22 mM KH_2PO_4 (2.99 g/L)
- 42 mM Na_2HPO_4 (5.96 g/L)
- Supplement fresh: 0.6% glycerol, 0.05% glucose, 0.2% lactose

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. Make PBS-T by supplementing with 0.05 - 0.1% Tween20.

Can replace with TBS-T
http://cshprotocols.cshlp.org/content/2015/3/pdb.rec085670.full?text_only=true.

Re-suspension buffer

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

Cell lysis buffer

- 50 mM NaH_2PO_4 pH 8 (or ~1-2 pH units away from pI of expressed protein)
- 500 mM sodium chloride
- 10 mM imidazole
- 0.5% Triton X-100
- 10% glycerol
- 2 mM DTT (**add fresh** before use)
- 1x Protease-inhibitor cocktail (**add fresh** before use)

Denaturing buffer

- 8 M Urea
- 4% CHAPS
- 35 mM Tris-HCl pH 8
- Adjust pH to 7.5 if necessary for dissolving urea (or warm to 40 °C).

Transformation

12h

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). 1h

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.

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.

3 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. 12h

4 Pick single bacterial colony and inoculate culture (3-5 mL LB or TB medium) supplmented with the appropriate antibiotic. Grow O/N @ 37 ºC with ~200-250 rpm shaking. 12h

Protein expression

5 Setup desired culture volume for protein expression with auto-induction TB medium (or just LB medium if using standard IPTG induction). An additional control culture can be setup as a control that lacks lactose (or will have *not* have IPTG added). Inoculate larger culture using the starter culture generated from the previous step at 1:50 dilution (e.g. 1 mL in 49 mL culture). Ensure culture is in an appropriate vessel that facilitates effective aeration. 1m

6 Induce protein expression, e.g. using IPTG to induce T7 promoter: 12h
- If using **LB culture** with IPTG induction, grow larger culture at 37 ºC until OD_{600} = 0.8. Then supplement the culture with 0.2 - 0.4 mM IPTG (keep an aliquot of culture without IPTG). Grow cultures overnight at desired temperature (e.g. lower temperature can imrpove protein folding and solubility e.g. 18-25 ºC). If desired, Measure OD_{600} for difference between induced vs non-induced. Non-induced should be higher by at least 0.1 (minimum difference).
- If using **auto-induction TB medium**, grow culture overnight (>12 hrs) or until OD_{600} = 2. Repeated sampling over time can be performed to ensure induction of expected protein fragment.

7 Pellet cells by centrifugation at 4 ºC (7,000 rcf for 10 minutes). If you want to run a quick test for protein expression, remove a 1-3 mL aliquot of culture into a clean microfuge tube and store at -20 ºC. 5m

8 Remove supernatant and wash cells in *re-suspension buffer.* Pellet as above and discard supernatant. Snap-freeze pellet in LN_2 and store at -80 ºC.

QC protein expression

9 It may be worthwhile testing for successful recombinant protein expression on an aliquot of induced culture (Step 7).

10 Pellet culture by centrifugation at max speed for 3 minutes then remove supernatant. 4m

11 Resuspend cells in 100 µL 1X PBS (per 1 mL culture). Store the resuspended crude lysate at -20 °C when not in use. 10m

12 Add the appropriate amount of: 30m
1. 4X LDS (or appropriate SDS-PAGE sample buffer)
2. $[DTT]_{final}$ = 50 mM, and
3. $[MgCl_2]_{final}$ = 100 mM.

These should be calculated first, and a mastermix of LDS, DTT and $MgCl_2$ can be prepared then added to each sample accordingly.

13 Calculate how much crude lysate to load, based on OD_{600} and concentration factor (**CF**): 20m

CF = volume of culture / volume of resuspension

For example, CF for resuspending 1 mL culture in 100 µL 1X PBS = 1,000 / 100 = 10x)

µL to load = [180/CF]/OD

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

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

16 Transfer supernatant to new tubes, taking care to avoid any "sticky" DNA coating the tube. 10m

17 Run supernatant on SDS-PAGE gel and perform Coomassie staining. Alternatively, transfer to PVDF membrane and perform Western blot analysis (ideally, recombinant protein is epitope-tagged). 3h

Solubilisation

18 To isolate soluble protein, re-suspend pelleted cells in *lysis buffer.* 20m

Note: ensure pH of *lysis buffer* is >1 pH units away from the isoelectric point of your expressed protein (calculate: http://isoelectric.org/index.html).

Keep samples on ice at all times.

19 Transfer cell re-suspension to clean microfuge tube (or appropriate vessel for probe sonication). Retain any remaining cell suspension at -20 °C. 10m

20 Sonicate samples with the Q125 ultrasonicator (125 watt, 20 kHz, 60% amp, 3×30 seconds). 30m

Keep samples on ice during and between rounds of sonication.

21 Spin at 16,000g for 30 minutes at 4 °C. 30m

22 Recover supernatant in a clean tube. Retain the pellet at -20 °C to for insoluble protein fraction. 10m

23 To isolate the insoluble fraction; resuspend the remaining pellet in *denaturing buffer* . 1h

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

24 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. 5 µg). 2h

25 Run samples on SDS-PAGE and stain gel with Coomassie or perform Western blot to verify expression. 3h