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Heterologous protein expression in E. coli V.5

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Works for me

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ABSTRACT

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

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KEYWORDS

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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 °C 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 | | P212121 |
| Petri Dish | LI-PD01100 | P212121 |

| NAME | CATALOG # | VENDOR |
|--|------------------|----------------------------|
| 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 |
| 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 | | |
| GelCode™ Blue Stain Reagent | 24590 | Thermo Fisher Scientific |
| Q125 Sonicator | Part #Q125 | |

SAFETY WARNINGS

Ensure use of appropriate aseptic 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. 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 KH₂PO₄ (2.99 g/L)
- 42 mM Na₂HPO₄ (5.96 g/L)
- Supplement fresh: 0.6% glycerol, 0.05% glucose, 0.2% lactose

10X PBS

Dissolve the following in 800 mL H₂O:

- 80 g of NaCl (1.37 M)
- 2.0 g of KCl (27 mM)
- 14.4 g of Na₂HPO₄ (100 mM)
- 2.4 g of KH₂PO₄ (18 mM)

Adjust pH to 7.4.

- Add H₂O 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 NaH₂PO₄ 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 °C 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 °C
- 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 °C 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 °C.

12h

Adjust volume as needed in order to obtain single colonies that can be picked for subsequent inoculation.

- 4 Pick single bacterial colony and inoculate culture (3-5 mL LB or TB medium) supplemented with the appropriate antibiotic. Grow O/N @ 37 °C 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 °C until OD₆₀₀ = 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 improve protein folding and solubility e.g. 18-25 °C). If desired, Measure OD₆₀₀ 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 OD₆₀₀ = 2. Repeated sampling over time can be performed to ensure induction of expected protein fragment.
- 7 Pellet cells by centrifugation at 4 °C (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 °C. 5m
- 8 Remove supernatant and wash cells in *re-suspension buffer*. Pellet as above and discard supernatant. Snap-freeze pellet in LN₂ and store at -80 °C.

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. [MgCl₂]_{final} = 100 mM.
- These should be calculated first, and a mastermix of LDS, DTT and MgCl₂ can be prepared then added to each sample accordingly.
- 13 Calculate how much crude lysate to load, based on OD₆₀₀ 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 °C 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, 3x30 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 2h

concentrations to normalise input volumes for each sample to a desired loading amount (e.g. 5 µg).

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

3h