Preparation of a cell-free expression system from Escherichia coli

Renuka Kudva[1]

[1]Stockholm University

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Renuka Kudva
Stockholm University

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ABSTRACT

This protocol describes the procedure make an S30 derived E.coli cell lysate that lacks membrane components. Modified from a protocol published in https://doi.org/10.1091/mbc.e11-07-0590
The procedure takes three days in total, including preparation time.

GUIDELINES

Start up ultra-centrifuges 30 min prior to use to stabilise vacuum. Work on ice. Plot growth curves for the strain used in the medium used for preparation (see materials.)

MATERIALS

NAME	CATALOG #	VENDOR
Magnesium acetate		
Sodium Hydroxide	BP359500	Fisher Scientific
L-aminoacids	LAA21−1KT	Sigma Aldrich
DTT	D0632	Sigma Aldrich
NaCl	53014	Sigma Aldrich
Tris acetate	TD0101.SIZE.500g	Bio Basic Inc.
BD Bacto™ Yeast Extract	212750	BD Biosciences
Sucrose	S7903	Sigma Aldrich
Potassium acetate	1.04820.1000	Merck Millipore
Tryptone (pancreatic digest of casein)	T9410	Sigma – Aldrich
Phospho(enol)pyruvic acid mono potassium salt	860077	Sigma Aldrich
Adenosine 5'-triphosphate (ATP) disodium salt hydrate	A2383	Sigma Aldrich
Pyruvate kinase from rabbit muscle	83330	

MATERIALS TEXT

Cells can be cultured in LB medium (buffered with phosphates), TB or S30 medium. Prepare 4 litres i.e. 1 litre each in 4 5 litre erlenmeyer flasks. Autoclave.

S30 medium
9 g/l bacto-tryptone
0.8 g/l yeast extract
5.6 g/l NaCl
1 mM NaOH

Prepare a mix of the 20 amino acids 1 mM each. Aliquot and store at -20°C.
Prepare stock solutions of the acetates: 1M Tris Acetate at pH 7.5, 1M Magnesium acetate at pH 7.5, 4 M Potassium acetate at pH 7.5

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Prepare stock solutions: 0.25 M ATP pH neutralised with KOH, 0.2 M PEP. Make aliquots and store at -20°C.

Strains tested: E.coli KC6, MRE600, MC4100.

If using a rich medium, do a growth curve for the strain being used under those growth conditions, and harvest at early exponential phase.

S30 buffer (pH 7.5)
10 mM Tris-acetate
14 mM magnesium acetate
60 mM potassium acetate
1 mM DTT

Sucrose cushion (pH 7.5)
10 mM Tris acetate
14 mM Magnesium acetate
60 mM potassium acetate
1 mM DTT
1.44 M sucrose

SAFETY WARNINGS

Use ultracentrifuges carefully, ensure to fill tubes up to requisite volumes. Handle chemicals according to local safety requirements. Wear eye-goggles and a mask while weighing yeast extract and peptone. Use fume hoods where indicated.
Handle sharps with care. Dispose off responsibly.
Handle liquid nitrogen with cryo-protective gear including eye goggles.
Handle autoclaves carefully.

BEFORE STARTING

Wash all flasks and bottles with milliQ water. Prepare all buffers in milliQ water.

1 Day 1: Make medium and autoclave 12:00:00

1. Wash all flasks with milliQ water.

2. Prepare medium as described, autoclave. 4 5 L erlenmeyer flasks, and one 200 ml flask with 50 ml medium for overnight culture. (This protocol uses S30 medium, see materials).

3. Autoclave milliQ water to make the buffers.

4. Prepare stock solutions of all the buffers, filter sterilise. Prepare stocks of amino acids, PEP, Pyruvate Kinase, and ATP, and freeze at -20 °C

5. Prepare S30 buffer (4 L and store at 4 °C).

6. Set up pre-culture of E.coli cells (Top10 and MRE600 used in manuscript, protocol can be used for other strains too!). Inoculate either from a plate or from a glycerol stock of a clone which gave a functional cell extract.
Grow pre-culture at 37 °C shaking at 200 rpm.

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2 Day 2: Ribosome isolation

1. Set up main cultures (1 L) from the pre-cultures to an A600 of 0.1.

2. Grow at 37 °C to an A600 of 1.0-1.2. (check growth every 20 min).

3. Cool down cells on ice for 00:30:00

4. Harvest cells by centrifugation at 4000 x g for 00:15:00 in a JLA-8.1000 rotor (Beckman-Coulter).

5. Resuspend cell pellet in 50 ml cold S30 buffer. Centrifuge again at 3500 x g . Repeat step 5 thrice.

6. Determine pellet mass. Resuspend in 1.5 ml S30 buffer/g cell mass. Plunge-freeze half of the suspension in liq Nitrogen and store at -80 °C .

7. Supplement the other half of the cell suspension with 5 mM PMSF/1% Roche cOmplete protease inhibitor cocktail.

8. Lyse cells either through a french press or an emulsifex at 8000 psi. Three passes.

9. Clarify lysate by ultra-centrifugation. Twice for 00:25:00 45000 x g in a Ti70 rotor (Beckman Coulter).

10. Distribute clarified lysate into clean Ti70 polycarbonate centrifuge tubes to half its volume (around 14 ml), and underlie with 14 ml sucrose cushion with a needle and syringe. (Use a 20G needle because the solution is viscous.)

11. Centrifuge at 33000 rpm for 17:00:00 in an ultra-centrifuge (rotor Ti 70).

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3 Day 3: Cell extract

1. After 17 hours, decant the supernatant obtained after ultra-centrifugation and rinse the 70S pellet with **500 µl** S30 buffer. Store on ice. Shake occasionally by hand to resuspend gently.

2. Thaw the cells stored the previous day at **-80 °C** in an ice-water bath.

3. Lyse with a french press or emulsifex similarly to what was done for the ribosome cell pellet.

4. Clarify lysate at **48000 rpm** in TLA 100.3 tubes (Beckman Coulter) for **02:30:00** .

5. Transfer the lysate onto the ribosome pellet and homogenise with a dounce homogeniser (Wheaton, loose piston.)

6. Transfer the cell extract to a **15 ml** tube and treat the following way.
   For each ml of lysate, add the following:

60 ul Tris Acetate pH 7.5
0.6 ul 1 M DTT
1.6 ul 1 M Magnesium acetate
6 ul amino acid mix
2 ul 0.25 M ATP (pH neutralised)
27 ul 0.2 M phosphoenol pyruvate
2.4 ul 2mg/ml pyruvate kinase

Volumes indicated are per ml of lysate. Simply multiply by the volume you have.

Shake well (by hand) and incubate at 37°C for 1 hour.

7. Prepare a dialysis tube (pore diameter 14,000 to 16,000) by placing in beaker full of water and slow stirring for 30 min.

8. Transfer the cell extract into the dialysis tube and dialyse against 1 l S30 buffer thrice at 4°C. The third dialysis step can be carried out overnight. (This step can be adapted for use with PEG to concentrate the cell lysate).

9. Aliquot the extract and flash freeze in liq Nitrogen. Store at -80°C long-term.

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