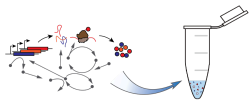
May 13, 2020

Cell-free lysate (E. coli) preparation with sonication

In 1 collection



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Protocol status: Working
We use this protocol and it's working

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Abstract

Production of cell-free lysate from E. coli BL21 Star (DE3) with optional induction of T7 RNAP. Adapted from Kwon and Jewett 2015. Features:

- Variable starting culture sizes (10 mL - 1 L)
- Constant energy sonication
- S12 centrifugation
- Run-off and optional dialysis

Citation

Kwon YC, Jewett MC (2015)
. High-throughput preparation methods of crude extract for robust cell-free protein synthesis. Scientific reports.

https://doi.org/10.1038/srep08663 LINK

Protocol successfully used at the University of Edinburgh by Nadanai Laohakunakorn, and at EPFL by Barbora Lavickova and the 2017 iGEM team.

Materials

- Big centrifuge: Eppendorf 5810R with A-4-62 swing-bucket rotor (holds 50 mL tubes)
- Small centrifuge: Eppendorf 5424R with FA-45-24-11 rotor (holds 2 mL tubes)
- Vibra Cell 75186 sonicator / Qsonica Q125 + CL-18 probe
- spectrophotometer
- incubator with shaker
- autoclave
- magnetic stirrer

- LB/2xYTP/2xYTPG medium autoclaved
- E. coli strain of interest

- Tris base (Sigma T1503-100G)
- magnesium glutamate (L-glutamic acid hemimagnesium salt tetrahydrate) (Sigma T1503-100G)
- potassium glutamate (L-glutamic acid potassium salt monohydrate) (Sigma 49601-500G)

- DTT (1,4-dithiothreitol) (Sigma 10708984001)
- acetic acid
- deionized water

- autoclaved tips and petri dishes
- 500 mL Erlenmeyer flasks sterile
- culture tubes sterile
- 50 mL falcon tubes
- 2 mL eppendorf tubes
- 1 L beakers
- 10k MWCO dialysis cassettes (Slide-A-Lyzer, 3mL, Life Technologies)
- magnetic stir bar
- spectrophotometer cuvettes
- liquid nitrogen, dewar, -80 storage

Bacterial growth

1 **Prepare all materials for bacterial culture**

This protocol will make 200 mL of culture to yield around 1 mL of lysate with a protein concentration of around 40 - 80 mg/mL.

1.1 Reconstitute LB media from premix as per instructions on box.

Note

Protocol has also been successfully carried out with 2x YTP and 2x YTPG (34°C, original). Cells grow slower on YTP media (~5-6h to OD 1.5-1.8) compared to ~4h in LB.

1.2 Autoclave LB media for 00:20:00 at 121 °C along with tips and dishes

2 **Grow overnight mini-culture**

2.1 Add 5 mL of LB medium to a culture tube and inoculate with small amount of bacteria from glycerol stock.

Note

Strains used successfully with this protocol are
- BL21 (DE3) and BL21 star (DE3) (lacking RNaseE), T7 and E. coli RNAP
- Rosetta (DE3) for oscillators (contains rare tRNA encoding plasmid, good for eukaryotic protein expression)
- Top10, Top10-GamS (GamS inhibits RecBCD degradation of linear DNA), E. coli RNAP, alpha-complementation
- M15 (E. coli RNAP, alpha-complementation)
- DH5alpha (E. coli RNAP, alpha-complementation)

2.2 Grow overnight culture in an incubator at 37 °C and 200 RPM. Make sure cap is in loose position to allow for air exchange

3 **Grow lysis culture (the next morning)**

3.1 **Measure and record** OD600 of overnight culture at **10x dilution**: pipette 900 µL LB and 100 µL overnight culture into a cuvette and take absorbance readings at 600 nm.

Expected result

OD600 should be around 4 (0.4 at 10x dilution)

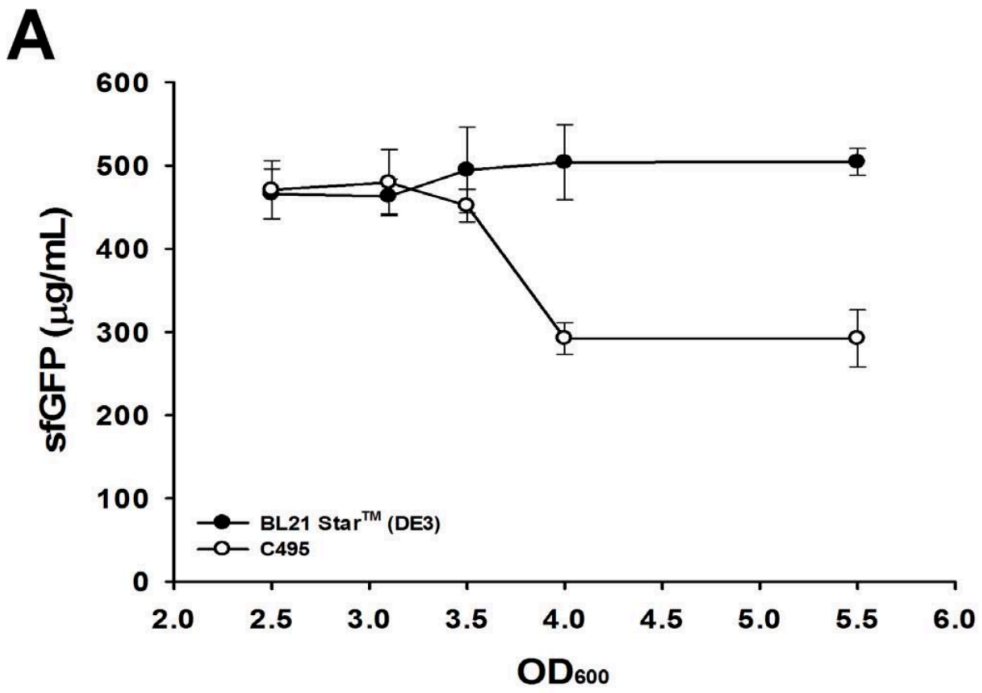
3.2 Add 1 mL of overnight culture to 200 mL of LB medium in a 500 mL Erlenmeyer flask.

3.3 Incubate the culture for 04:00:00 , 37 °C , and 200 RPM.

4h

Expected result

At the end of the step, the OD600 should be ~1.5-2. While Kwon and Jewett grow to OD600 = 3, we grow instead for a fixed time. They observe varying robustness of yield to final OD which is strain dependent. **In the end we should probably grow to fixed OD.**



Activity of cells harvested at different densities, grown in 1L 2xYTPG and sonicated at 1.5 mL/556 J. From Kwon and Jewett 2015, Figure 3, CC-BY license.

3.4 **(If required)** induce after 2 hours with 0.4 mM IPTG (800 µL of 100 mM stock in 200 mL culture) to express T7 polymerase in the BL21 (DE3) strains.

4 A few minutes before incubation step finishes, prepare centrifuge and tubes

4.1 Cool down big centrifuge (Eppendorf 5810R) to 4 °C with fast temp mode; this takes around 10 mins.

4.2 Weigh one 50 mL Falcon tube along with its cap, and record. Clearly label the tube as well as its cap; this tube will be used to spin the final pellet.

5 As soon as the incubation finishes, put the Erlenmeyer flask On ice to arrest growth.

Centrifugation and cleaning

6 Put 50 mL of culture into each of four 50 mL Falcon tubes (one of which has been labelled), and spin at 4000 rpm , = 3220g 4 °C 00:20:00 . Tubes in swing-bucket rotor A-4-62. 20m

7 Put tubes back On ice ; keep bacterial pellet cold as much as possible.

8 Carefully discard supernatant using a pipette, then add 10 mL of **Buffer A (no DTT)** to each pellet. Resuspend by carefully pipetting up and down. Finally, transfer all four parts into the single labelled tube.

9 Balance centrifuge with a second tube containing 40 mL water. Then spin at 4000 rpm , = 3220g 4 °C 00:10:00 . 10m

10 Carefully discard supernatant using a pipette, then add 10 mL of Buffer A to the pellet. Then spin at 4000 rpm , = 3220 g 4 °C 00:10:00 . 10m

11 go to step #10 and repeat spin once more.

12 Finally, carefully discard as much supernatant as possible.

13 Weigh the tube containing the pellet (make sure cap is on), and record:

Final weight of tube + pellet

The **wet mass** of cells is the difference between this weight and the dry weight measured earlier. This is typically around ~1 g.

14 Flash freeze the cells using liquid nitrogen, respecting all safety procedures (wear protective glasses as there is danger of tube explosion etc.). Store the cells at -80 °C .

Note

Flash-freezing is optional (the protocol will work without) but it serves two purposes:
- Convenient pause point
- Frozen cells, once rethawed, lyse slightly more easily than fresh cells. So this step must be kept consistent to ensure reproducible protocol.

Sonication

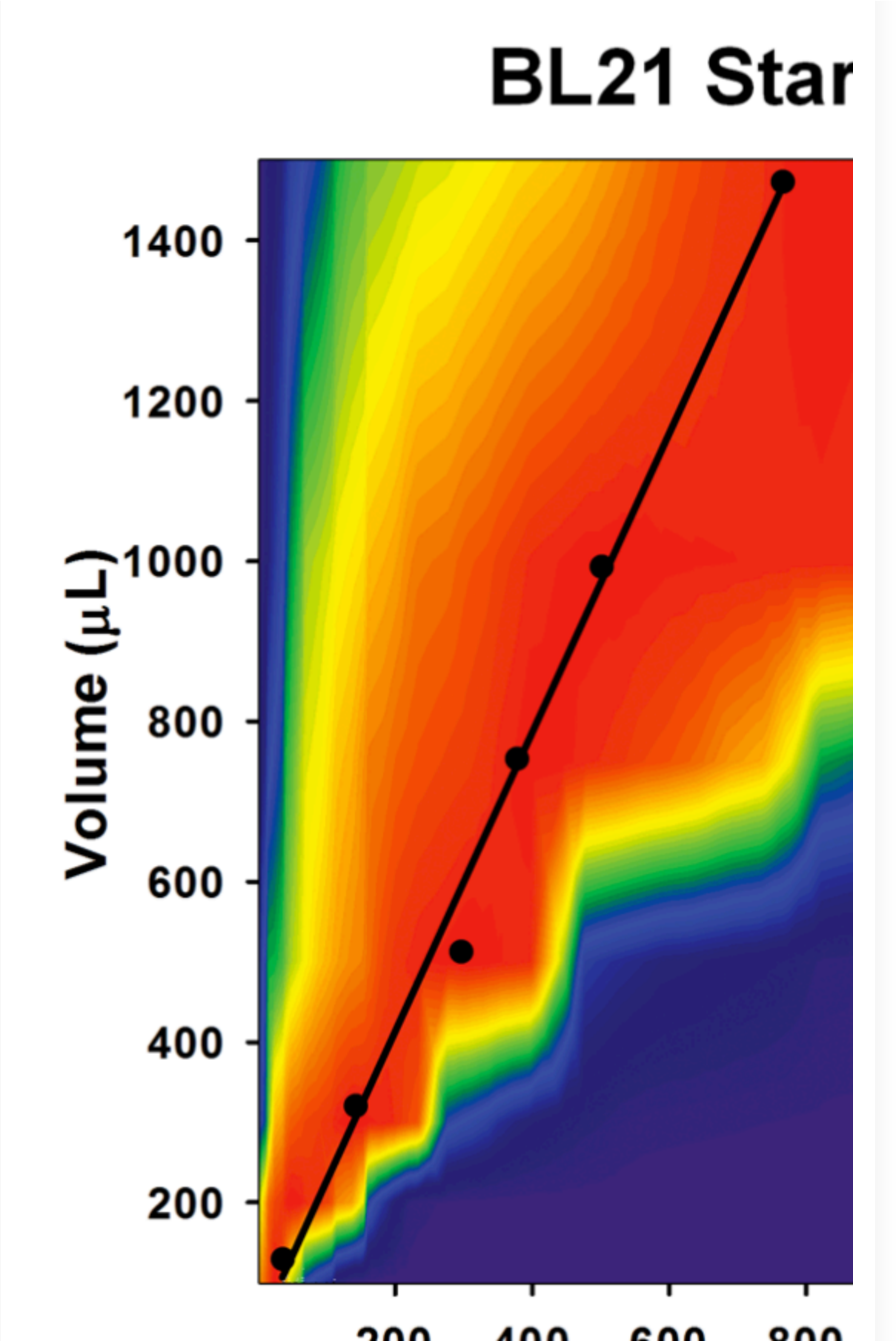
15 Keeping the pellet On ice , add **Buffer A with DTT**: 1 mL buffer per 1 g **wet mass** as determined earlier. **This ratio is critical**. The DTT must be prepared fresh as it will degrade otherwise.

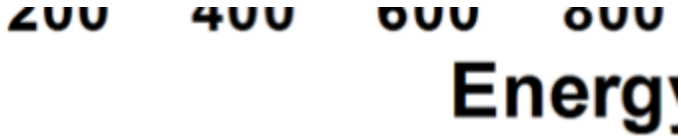
16 Resuspend by vortexing, and put back On ice

17 Put 1 mL **exactly** of resuspended cells into a new 2 mL Eppendorf tube. This volume is critical.

Note

The larger the volume the more robust the lysis:


BL21 Star
Volume (µL)
1400
1200
1000
800
600
400
200



Yield as a function of volume and sonication energy. From Kwon and Jewett Figure 4, CC-BY license.

So if possible, scale up the production to achieve (ideally) at least 5 mL lysis volumes (= 5 g wet mass).

18 Place the tube in an **ice bath** - ice mixed with water - and place the sonicator tip inside the tube so that it is immersed as much as possible but **no part of the tip touches the tube surface**. This is one of the most variable parts of the protocol. A suggested technique is to drop the tip to the bottom of the tube, and then raise it slightly. Holding the tube using a cool rack (e.g. Fisher 15592801) is recommended.

19 Sonicate until **400J** has been achieved, using 50% amplitude and pulses of 10s : 10s (i.e. energy for 10s followed by pause for 10s). This typically takes around 00:01:24 but the time is variable. **The total sonication energy is a critical parameter.**

Separation and Clarification

20 Cool down the small centrifuge (Eppendorf 5424R) to 4 °C using the fast temp mode: this takes around 20 minutes.

21 Balance and centrifuge the lysate at 12000 x g , = 11304 rpm 4 °C 00:10:00 10m

22 Transfer the supernatant to a new tube. To prevent any transfer of bacterial debris, it is critical that you **do not take all the lysate**.

23 Place the lysate in an incubator at 37 °C , 200 RPM for 01:30:00 , to degrade remaining DNA and RNA. This is called the run-off reaction. 1h 30m

Note

Even though Kwon and Jewett observe deterioriation in BL21 production yield with run-off, we do not see the same behaviour. If E. coli RNAP will be used, do dialysis as well as run-off.

24 Making sure the centrifuge is still cool, centrifuge the lysate at 12000 x g , = 11304 rpm 4 °C 00:10:00 10m

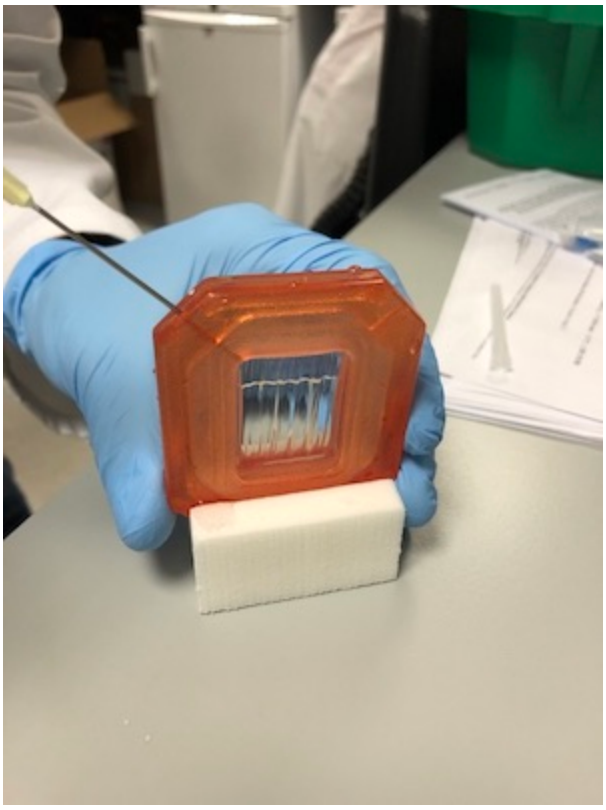
25 Transfer the supernatant to a new tube. Again, **do not take all the lysate**. Aliquot around 3 µL into separate tube for a Bradford assay.

26 **(IF REQUIRED) carry out dialysis**

26.1 Prepare **Buffer A + DTT** and add 900 mL to a 1 L beaker at 4 °C . Add a magnetic stir bar.

26.2 Rehydrate the required number of 10k MCWO cassettes in **Buffer A+DTT** in a beaker at 4 °C for 00:02:00

26.3 Load cassettes with 2.5 mL max (partial loading possible) of extract and dialyze for 03:00:00 4 °C with stirring. 3h



26.4 Finally load extract into centrifuge and centrifuge the lysate at 12000 x g , = 11304 rpm 4 °C 00:10:00

26.5 Transfer the supernatant to a new tube. Again, **do not take all the lysate.** Proceed to step 27.

27 Keeping everything On ice , aliquot the lysate into small tubes as required (around 25 µL per tube is recommended).

28 Flash-freeze the remaining tubes in liquid nitrogen and store at -80 °C

29 To perform the Bradford assay, dilute 1 µL of the lysate in 99 µL of Buffer A. Mix 5 µL of this diluted lysate with 250 µL of Bradford reagent. Incubate for

00:05:00 before measuring the absorbance using a Nanodrop. The final protein concentration is typically 40 - 80 mg/mL.

Buffer A and B preparation

30 **Preparation of 1M stock solutions of tris acetate, magnesium glutamate, and potassium glutamate**

30.1 Weigh 12.11 g of tris base (121.14 g/mol), add it to 100 mL of deionized water, and adjust to pH 8.2 with acetic acid, to make 1M tris acetate stock.

30.2 Weigh 38.86 g of magnesium glutamate (388.61 g/mol), and add it to 100 mL of deionized water, to make 1M magnesium glutamate stock.

30.3 Weigh 20.32 g of potassium glutamate (203.23 g/mol), and add it to 100 mL of deionized water, to make 1M potassium glutamate stock.

31 **Preparation of 1L of buffer A**

31.1 Add 10 mL of 1M tris acetate pH 8.2, 14 mL of 1M magnesium glutamate, and 60 mL of 1M potassium glutamate, and fill to 1 L with deionized water to make buffer A (10 mM tris acetate, 14 mM magnesium glutamate, 60 mM potassium glutamate).

31.2 Store at 4 °C

31.3 Add 2 µL of 1M DTT to 1000 µL Buffer A (final concentration 2 mM) for the lysis solution, or 1.8 mL of 1M DTT to 900 mL of Buffer A for the dialysis solution. This should be made fresh every time but if short-term storage is required, put at -20 °C

Citations

Kwon YC, Jewett MC. High-throughput preparation methods of crude extract for robust cell-free protein synthesis.
https://doi.org/10.1038/srep08663