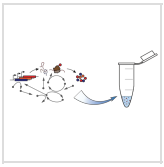

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# Cell-free lysate (E. coli) preparation with sonication

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1 Works for me dx.doi.org/10.17504/protocols.io.8nthven

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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

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

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

MATERIALS TEXT

- 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



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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.

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.

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.

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



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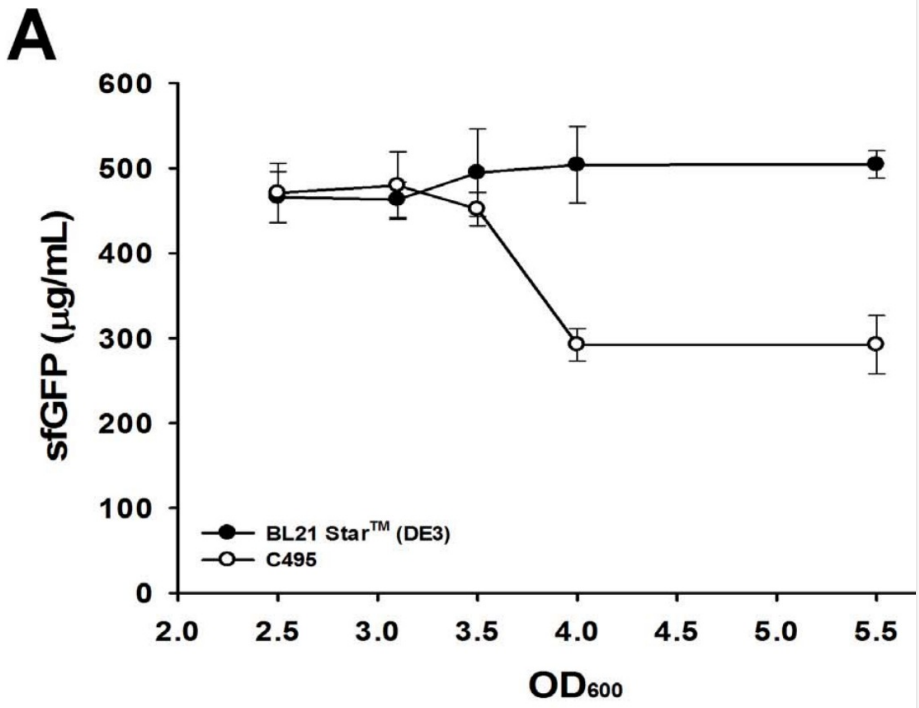
3.2 Add **1 ml** of overnight culture to **200 ml** of LB medium in a 500 mL Erlenmeyer flask.

4h

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

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 K 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.



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

Centrifugation and cleaning

20m

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.

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.

10m

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** .

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** .

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



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to ensure reproducible protocol.

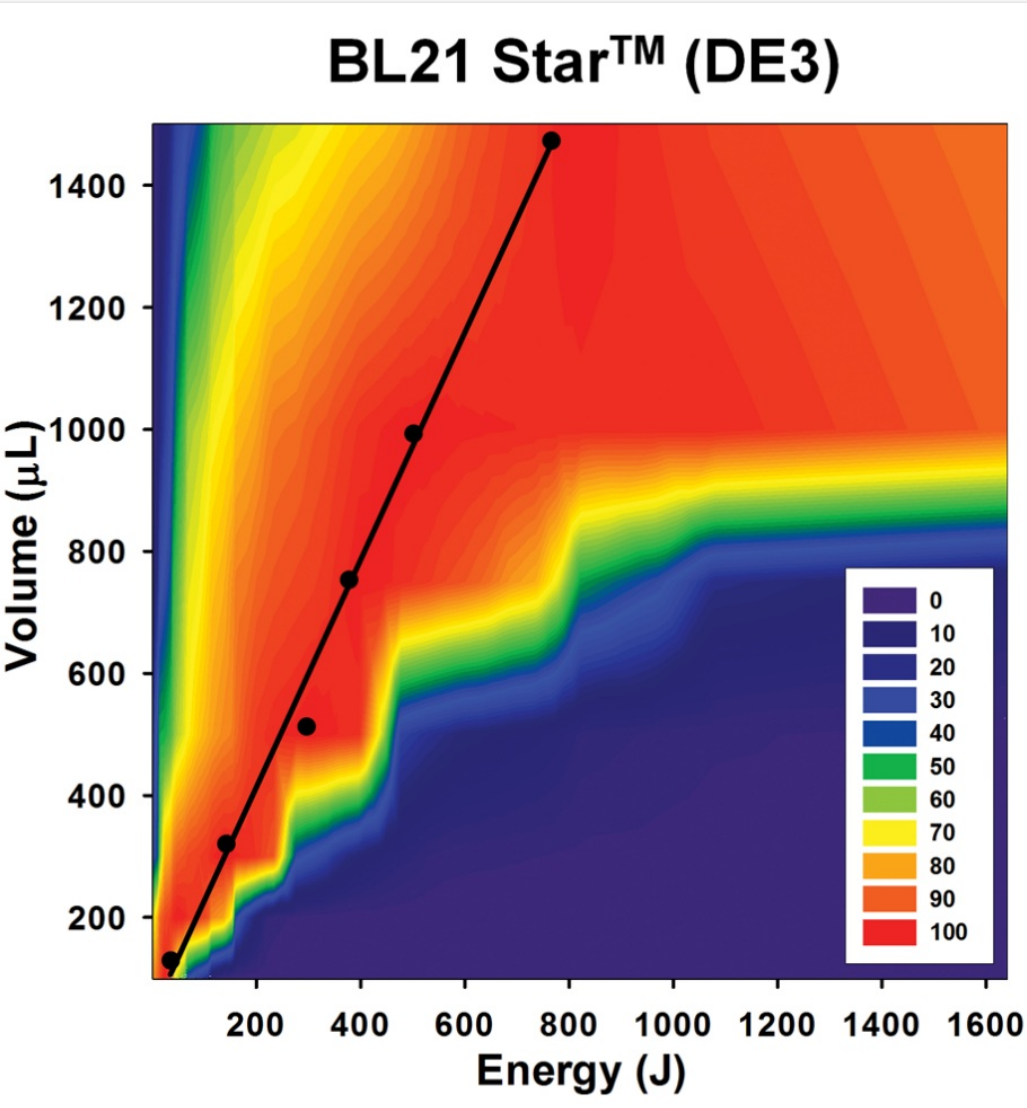
Sonication

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.

The larger the volume the more robust the lysis:



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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

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**



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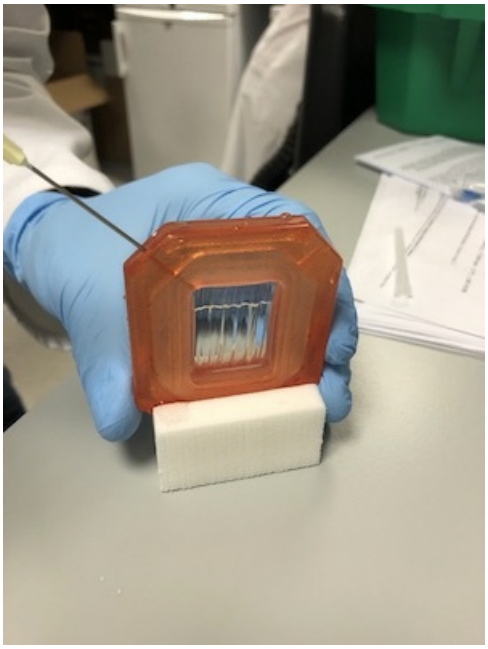
26.1 Prepare **Buffer A + DTT** and add **900 ml** to a 1 L beaker at **4 °C** . Add a magnetic stir bar.

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 3h **03:00:00** **4 °C** with stirring.



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.



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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**



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