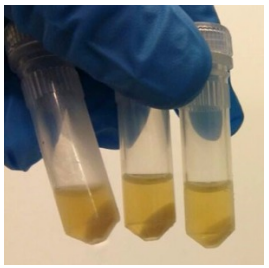
Jun 14, 2017

Step 1: Preparing S12 cell extracts using bead-beater



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Abstract

The protocol described here is an adaptation from this paper:

Sun, Z. Z., Hayes, C. A., Shin, J., Caschera, F., Murray, R. M., Noireaux, V. Protocols for Implementing an *Escherichia coli* Based TX-TL Cell-Free Expression System for Synthetic Biology. *J. Vis. Exp.* (79), e50762, doi:10.3791/50762 (2013).

Please note that some steps were taken literally as they appear in that source, and It is highly recommended to see and study this paper before starting.

Our adaptations include changes in the volumes of cell cultures and buffers, for smaller scale production. Also, it has been eliminated the clarification, ultra-centrifugation, and dialysis steps. Another change is the use of *E.coli* strain BL21 (DE3) STAR, instead of Rosetta 2 cells. All the above modifications allow the preparation of a simpler, and yet very effective cell extract suitable for cell-free RNAPT7 Tx-Tl reactions.

Materials

STEP MATERIALS

- 2XYT Broth **Catalog #**3012-032
- K-glutamate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G1149
- Mg-glutamate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**49605
- Potassium phosphate dibasic solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P8584
- Potassium phosphate monobasic solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P8709
- Micro Bio-Spin Chromatography Columns **Catalog #** 732-6204
- Beads, 0.1mm dia. **Catalog #**11079101
- Bead-beating tubes (polypropylene microvials) **Catalog #**522S
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Prepare all materials needed, media cultures, and buffers

1 Prepare all the needed solutions and materials needed including 2XYT+P media and S30B buffer.

2XYT+P media: Put 62 g 2xYT, into a 3-liter recipient. Add 80 ml of 1M potassium phosphate dibasic solution, and 44 ml of 1M potassium phosphate monobasic solution. Fill with water to 2 Liters. Dissolve and mix well. Aliquot in 1-liter bottles. Autoclave.

S30B: Put 5.44 g of Mg-glutamate and 12.195 g K-glutamate into a 1-liter bottle. Add 800 ml of water and adjust pH to 8.2 using 2M Tris. Finally, fill with water to 1 liter. Autoclave, store at 4 °C. add 1 ml 1 M DTT just before use.

Be sure to have all the things needed in this protocol including the bead beater, 0.1mm diameter beads, 5ml pipet and tips, chromatography columns, autoclaved bead-beater tubes and autoclaved 500ml centrifuges bottles.

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Day One - Prepare mini-culture 1

2 Prepare a bacterial culture tube with 5mL of 2XYT+P media and inoculate it with a glicerol stock of BL21 DE3 STAR. Grow the bacterias overnight at 37°C with agitation.This is the **mini-culture 1**

Day Two - Prepare mini-culture 2

3 Pour 50 mL of 2xYT + P culture medium into a 500mL Erlenmeyer flask and inoculate it with 300 µL of the overnight **mini-culture 1**.
Let it grow for 12 hrs at 37°C with agitation. This is the **mini-culture 2.**

Day Three - Prepare 50ml collection Falcon tube

4 Weigh an empty sterile 50 ml Falcon tube and record its mass. Chill Falcon tube on ice, it will be used later in **step 13.**

Day Three- Prepare cell culture

5 Pour 330 mL of 2xYT + P culture medium into four 2L Erlenmeyer flasks. Preheat the flasks with the medium at 37ºC for 30 min.

6 Inoculate each Erlenmeyer flask with 11mL (~3% of the final volume) of the **mini-culture 2 .** Let the cultures grow at 37°C with agitation.

7 1h 30m later check the OD of the cultures. When the cultures reach OD of ~0.6, add 350 µL of **IPTG 1M** into the Erlenmeyer flask. Let the cultures grow at 37°C with agitation until OD ~1.5-2.0. This OD is normally obtained after 1h 30m of IPTG addition. This whole process should not take longer that 3h 45m.

Day Three- Collect the cells

8 Immediately after growth, transfer all bacterial cultures evenly into 500mL centrifuge bottles and centrifuge them at 5000 x g for 12 min at 4 °C to pellet bacterial cells.

9 While centrifuging, complete S30B buffer by adding 1 ml of 1 M DTT to 1L of previously prepared S30B. Mix and maintain buffer on ice

10 When centrifugation is finished, completely remove supernatant by decanting and blotting the centrifuge bottles on paper towel. Keep pellets and bottles on ice. (Note: Though not recommended, pellets can be stored at 4 ºC overnight to continue with the following steps next day.)

Day Three- Wash cell pellets

11 Add 50 mL of chilled S30B buffer to each of the four centrifuge bottles, and shake the bottles vigorously until pellet is completely solubilized with no remaining clumps. Centrifuge the four bottles at 5,000 g for 12 min at 4 °C.

12 Repeat steps 10 and 11

13 Add 10 ml S30B (with DTT) buffer at 4 °C to each centrifuge bottle. Transfer each pellet dissolved in S30B into the chilled **previously weighted Falcon tube from step 4.**

14 Centrifuge Falcon tube at 2.000g for 8 minutes at 4°C. Remove supernatant by decanting and blotting the centrifuge bottles on paper towel.

15 Re-centrifuge the Falcon tube at 2.000 g for 2 min at 4 °C. Completely remove residual supernatant by pipette and Keep on ice.

Day Three- Calculate the needed amounts of S30B buffer and beads for bead-beater

16 Weigh the 50ml collection Falcon tube with the pellet. Calculate pellet mass, volume of S30B (containing DTT) buffer needed, and mass of beads needed based on the following table:

	Empty Falcon tube mass (g)	*(From step 4)* g
	Falcon tube + Pellet mass (g)	*(This step)* g
	Pellet mass (g)	(Empty Falcon tube mass - (Falcon tube + Pellet mass)) g
	S30B volume (mL)	(Pellet mass x 0.9) mL
	Beads (g)	(Pellet mass x 5.0) g

Day Three- Prepare bead-cell solution

17 Add the amount of S30B buffer needed. Vortex to resuspend the cells. Add 1/3 of the total amount of beads calculated previously and vortex to mix. **Keep on ice** for 30 seconds. Add another load of 1/3 of the total amount of beads. Vortex to mix and repeat this process for the last 1/3 of beads. After the last load of beads is added, vortex to mix and ensure beads are uniformly distributed. A thick paste should be formed.

Note

In order to uniformly distribute the beads with the resuspended cells, the beads are added in three loads, each one containing 1/3 of the total beads needed.

Day Three- prepare 5ml pipette tip

18 Prepare a 5 ml pipette tip by cutting off the end using a sterile razor blade to create a 3-4 mm opening. Dial pipette to 2 ml

Day Three - Fill bead-beating tubes with cell-bead solution

19 Place 5 autoclaved bead-beating tubes on ice. Verify high viscosity of cell-bead solution using modified pipet. It should be viscous to the point of barely exit the pipette tip during ejection. **Two people are required in order to proceed.**

20 Remove the bead-cell solution from the 50ml collection Falcon tube using modified pipet, and transfer into a sterile bead-beating tube, filling it three-quarters full with bead-cell solution. Spin extremely briefly (1s) on a counter mini-centrifuge to remove air bubbles without redistributing beads.

21 Finish adding the bead-cell solution to form a concave meniscus in the falcon tube. Add also, a very small drop of bead-cell solution onto the inside of a bead-beating tube cap and give small taps to it. It is important not to put the mixture on the outside lip of the cap; otherwise, the bead-beating tube will not close sufficiently.

22 Cap the bead-beating tube with the bead-beating cap from the previous step. Hand to assistant for bead beating. If done correctly, the cap should be tightly sealed, no air bubbles should be visible, and little (if any) bead-cell solution should overflow. Redo the loading process if air bubbles are visible or the cap does not fully close.

23 Vortex Falcon tube often while filling beat beatter tubes and caps to ensure even distribution of beads during the whole process.

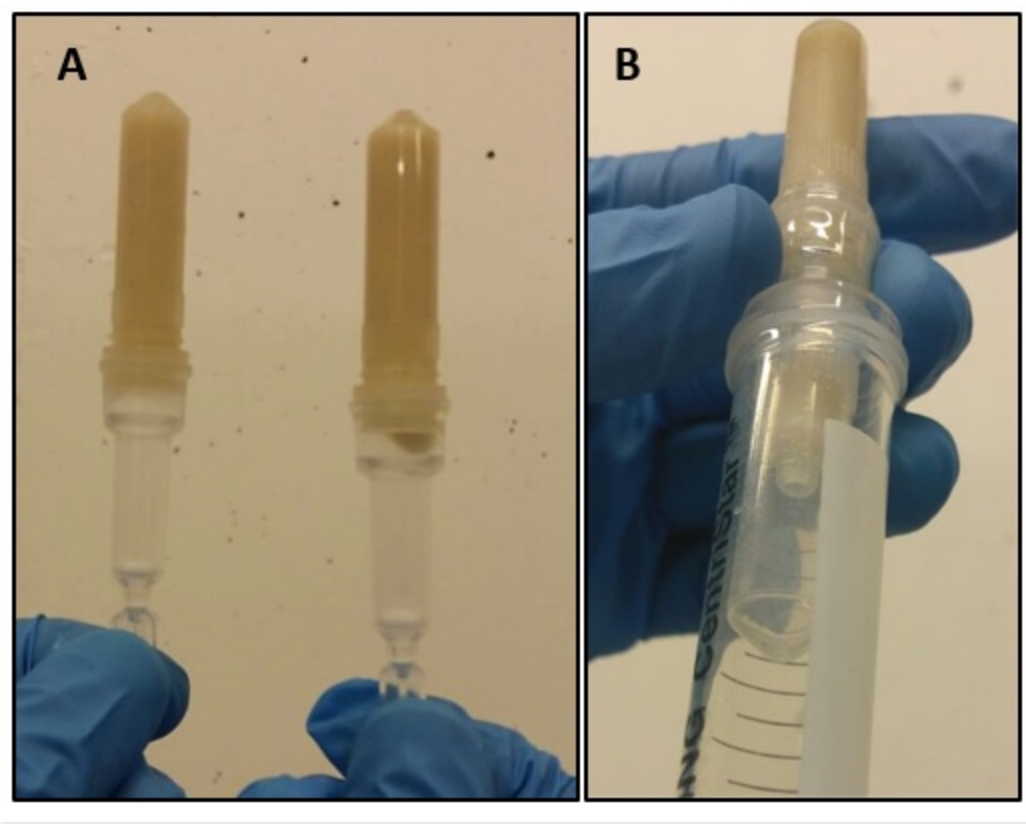
24 Conduct steps 19-23 simultaneously. Have an assistant take filled bead-beating tubes and place on ice. Once two filled bead-beating tubes have been collected and have been on ice for at least one minute, begin bead beating.

Day Three- Bead-beating

25 Beat one tube for 30 sec at 46 rpm. Place upside down on ice for 30 sec while beating the other tube. Come back to the tube put upside down and repeat bead-beating such that each filled bead-beating tube has been beaten for **1 min total**.

Day Three- Filter the beated tubes

26 Once all the bead-cell solution from the Falcon tube had been passed to the bead-beating tubes and beat already.Then, construct filter apparatus from 15 ml Falcon as follows. Add a new bead-beating cap, flat-part face-up, to the bottom of a 15 ml Falcon. Then, remove the cap from processed bead-beating tube and press micro-chromatography column firmly onto the end of processed bead-beating tube until completely sealed (as seen in **Image A**). Snap off elution end of the micro-chromatography column, and place micro-chromatography column, elution end down, into the empty bead-beading tube.Place this complex into 15 mL Falcon (**Image B**). Repeat for all the filled bead-beating tubes; keep on ice when complete.



27 Centrifuge the filter apparatuses, Falcon tube uncapped, at 6,000 g for 5 min at 4 °C to separate extract and pellet from beads.

Day Three- Save and store viable extracts

28 Verify each bead-beating tube has produced viable extract. Properly beaten extracts will not be turbid, and the pellet will have two distinct layers, as seen in those in the **picture bellow**. Discard all turbid tubes, and transfer the supernatant from non-turbid tubes into individual 1.75 ml micro-centrifuge tubes, taking as little pellet as possible.
Those extracts can be stored at -80 for later use.