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Protein Purification for OnePot PURE cell-free system

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

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ABSTRACT

In this protocol we explain the procedure of protein purification with a single coculture and a single Ni-NTA affinity His-tag purification to produce the protein solution needed for a PURE cell-free system.

EXTERNAL LINK

https://pubs.acs.org/doi/10.1021/acssynbio.8b00427

MATERIALS TEXT

Material/Consumables:

- Liquid LB medium (pH 7) Autoclaved
- Ethanol 70%
- 36 PURE Bacteria in glycerol stock
- Ampicillin
- Aluminum sealing
- 14ml culture tubes
- Breath-Easy sealing membrane
- Isopropyl β- d-1-thiogalactopyranoside (*IPTG*)
- Glycerol stock 40%
- β-Mercaptoethanol
- Milli-Q water
- Ice
- Buffer A
- Buffer B
- HT Buffer
- Stock Buffer B
- IMAC Sepharose 6 FF (GE Healthcare)
- Econo-Pac chromatography columns (Bio-Rad)
- Nickel sulfate solution
- DEMI water
- EDTA
- NaCl solution
- 15 mL Amicon Ultra filter (3 kDa cutoff)
- 0.5 mL Amicon Ultra filter (3 kDa cutoff)

Equipment:

- Flame
- 96-well plate (for storage)
- 96 deep-well plate (1.5 mL void, for culture)
- 96 well replicator

- Incubator
- 1l Erlenmeyer Flask narrow mouth with baffles Autoclaved
- Centrifuge
- Sonicator (Vibra cell 75186)
- Beakers
- Nanodrop

SAFETY WARNINGS

When handling β-Mercaptoethanol the researcher should work in a chemical hood and wear protective glasses.

BEFORE STARTING

The recipes of the buffers used in this protocol can be found here:

Buffer preparation for OnePot PURE cell-free system
by Konstantinos Ragios
PREVIEW RUN

As it is stated in the buffers' protocol you need to add β-Mercaptoethanol each time before the use of the buffer.

The list of the bacteria you will need for the protein solution is described in the following paper :

Lavickova B, Maerkl SJ (2019). A Simple, Robust, and Low-Cost Method To Produce the PURE Cell-Free System.. ACS synthetic biology.
https://doi.org/10.1021/acssynbio.8b00427

one of the depositing labs is :

https://www.addgene.org/Sebastian_Maerkl/

Small cell culture

1

Work under flame
Sterilize the bench with 70% Ethanol

Prepare 10ml of autoclaved LB medium and supplement it with 100μg/ml Ampicillin

2

We assumed that the 35 different bacterial strains are stored as a glycerol stock in a 96-well plate and the bacteria that express the EF-tu are also stored as a glycerol stock

Fill 35 of the wells of a 96 deep-well plate with 0.3 ml of the LB medium according to the pattern of the stored strains.

2.1 Use a 96-well replicator to innoculate the deep-well plate. Disinfect the replicator very well, with ethanol and flame and then dip it first in the bacterial stock and then into the 96 deep-well plate.

If you are performing this experiment for the first time and you don't have a well plate with stored bacteria, add the strains you acquired to their respective well,add an arbitrary volume in a way that you don't alter the final volume of 0.3ml significantly but making sure that at least some bacteria are transferred to the new solution, and after you culture them (step x) you can use the overnight culture to create your stock solution by adding to them 40% glycerol stock with a ratio of 1:1 and store at -80^{o}C.

2.2 Cover the plate with a Breath-Easy sealing membrane to ensure no sample is spilled but also maintaining aerobic culture conditions.

3 Add 3ml of the LB medium to a standard 14ml culture tube (needed for the EF-Tu culture)

If you plan to do 2 different OnePot Protein purification batches you need to prepare one more tube for the EF-Tu culture

3.1 Add an arbitrary volume of stored strain with EF-Tu encoding plasmid. 20-50µl should be sufficient.

4 Incubate overnight (12-16 hours) at 37^{o}C rotating at 260 rpm

Large Cell culture

5

It is advised to produce two batches of protein solution at the same time. All you need to do is repeat all the following steps for the second batch. However, even if the procedure for both of them will be the same, using the same stock buffers, you should not mix the two solutions at any point of the purification. In that way if there is any problem with one of them, it will not affect to the other one.

6

Work under flame
Sterilize the bench with 70% Ethanol

Add 500ml of autoclaved LB medium into a 1L baffled flask and supplement it with 100µg/ml Ampicillin

7 Inoculate the the LB with 1675µl from the overnight culture of the EF-Tu strain and 55µl from each of the rest 35 the cultures.

8 Incubate for 2hours at 37^{O}C rotating at 260 rpm

9 Induct with 0.1mM IPTG and continue incubating for 3 more hours

Cell suspension and lysis

10

Precool the centrifuge at 4^{O}C

Harvest the cells in two tubes by centrifugation at 4000rpm for 10minutes in 4^{O}C

At this point you can store the tubes at -80^{O}C overnight

11 Prepare 7.5 ml of Buffer A, by adding 7mM β-Mercaptethanol (usually the stock solution is 2000x)

12 Resuspend the cells

12.1 First use 5ml to resuspend the cells in the first tube, then transfer the solution to the second tube and resusend the cell. Finally use the other 2.5 ml of buffer to wash the first tube for any cells left behind and move the solution to the second tube.

13

Clean the sonicator by applying a working cycle to a tube with milli-Q water

Sonicate the solution with probe tip of 6mm with 4 cycles of a 20s on - 20s off pulse at 70% amplitude.

Keep the tube in ice to avoid heating up the solution during the sonication process.

14

Precool the centrifuge at 4^{O} C

Centrifuge the lysate at 15000rpm for 20minutes at 4^{O} C. The debris form a pellet on the wall of the tube.

Ni-NTA Resin Preparation

15 Pipette 2 ml of IMAC Sepharose 6 FF into an Econo-Pac chromatography column

16 Charge with 15ml of 100mM Nickel sulfate solution

Instead of the IMAC Sepharose you may work with a ready-to-use Ni-NTA resin. In this case you need to skip this step.

17 Wash with 50ml of DEMI Water

Do not let the resin dry out entirely at any point of the experiment.

18 Equilibrate with 35ml of Buffer A (don't forget to add the β-Mercaptoethanol).

Protein Purification

19 Put the supernatant of step 14 into the column with the resin and incubate at $4^{o}C$ rotating slowly for 3 hours.

20 Let the unbound lysate to flow through the column.

21 Wash with 25ml of wash buffer consisting of 95% Buffer A and 5% Buffer B

22 Elute the proteins using 5m of Elution buffer (10% Buffer A and 90% Buffer B) and collect the solution.

Resin regeneration

23 Regenerate the resin with a 10ml mixture of 0.2M EDTA and 0.5M NaCl

24 Wash with 30ml of 0.5M NaCL

25 Wash with 30ml of DEMI water

26 Store in 20% Ethanol at 4^{O} C.

The resin can be reused. Before the next experiment you need to let ethanol flow through and then wash with DEMI water before starting the new preparation.

Buffer exchange and concentration

27 Mix the 5ml of elution solution with 25ml of HT Buffer.

28 Load half the new solution to a 15ml Amicon Ultra filter (3 kDa) and centrifuge for 60minutes at 4000 rpm at 4^{O} C.

29 After that, load the other half of the solution to the same filter and centrifuge for one more hour.

The expected concentrated quantity left at this point is around 1ml

30 Dilute the concentrated sample with 10 ml of HT Buffer and concentrate to 1.5ml (another 1 hour of centrifuge)

31 Measure the exact quantity you have left and add Stock Buffer B with a ratio of 1:1

32 Concentrate the solution with a 0.5ml Amicon Ultra filter (3 kDA) by centrifugation at 14000rpm for 30min at 4^{O} C.

Measurements and Storage

33 Mix 24µl of water with 1µl of the concentrated solution and vortex it.

34 Perform a nanodrop measurement and calculate the sample's concentration: Multiply by 25 and divide by 0.5 (Extinction coefficient)

35 The final concentration must be 12.25mg/mL
If it is lower concentrate for more minutes and if it is higher dilute with Stock Buffer B

36 The Protein solution is stored at -80^{O} C