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Ribosome Purification for OnePot PURE cell-free system V.1

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

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Abstract

In this protocol we explain the procedure of ribosomes purification with the method of hydrophobic interaction chromatography (HIC) so they can be used for a PURE cell-free system.

Materials

Material/Consumables:

- Liquid LB medium (pH 7) Autoclaved
- Ethanol 70%
- A19 E. Coli strain
- Glycerol stock 40%
- Suspension buffer
- β-Mercaptoethanol
- Milli-Q water
- Ice
- 0.22μm PES membrane filter
- 20% Ethanol
- Buffer C
- Buffer B
- NaOH (1M)
- Acetic Acid (0.1M)
- Cushion buffer
- Ribosomes buffer

Equipment:

- Flame
- 1l Erlenmeyer Flask narrow mouth with baffles Autoclaved
- Incubator
- 2 * 5l Erlenmeyer Flask narrow mouth with baffles Autoclaved
- OD600 Spectrophotometer
- Centrifuge
- Sonicator
- 3*250ml Beaker
- Syringe
- 2*5ml HiTrap Butyl HP Column
- Ultracentrifuge
- Magnetic stirrer

Safety warnings

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

Before start

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

Protocol

NAME

Buffer preparation for OnePot PURE cell-free system

CREATED BY

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PREVIEW

Small cell culture

1

Note

Work under flame
Sterilize the bench with 70% Ethanol

Add 100ml of LB medium to the 1L Erlenmeyer flask

1 Inoculate the LB with 10µl of A19 E. coli strain kept in glycerol stock

2 Incubate overnight (at least 12 hours) at 37^{o}C rotating at 250rmp

Large cell culture

3

Note

Work under flame
Sterilize the bench with 70% Ethanol

Add 2l of LB medium to each of the two 5l flasks

4 Inoculate the flasks with 30ml of the A19 E. Coli culture each

Note

To store some of the remaining bacteria:
Add to the desired tubes same quantity of the bacterial sample and 40% Glycerol stock and mix.
Then you may store them at -80^{o}C for future use.

5 Incubate the flasks for 2 hours at 37^{o}C rotating at 180rpm

6 Test the bacterial sample with OD600 Spectrophotometer

6.1 Add 1ml of the sample to a cuvette and measure it

6.2 If the measurement is lower than 1.0 continue incubating and after the appropriate time repeat the measurement

Note

Take into account that E.coli has a doubling time of about 20-30 minutes

Cell suspension and lysis

7

Note

Precool the centrifuge to 4^{O}C before using as the sample is quit sensitive

Centrifuge the bacterial sample for 20min with 6000 RCF at 4^{O}C

8 Remove the supernatant from the tubes .The cells should be shaped into a pellet on the wall of the tube

9 Add 25µl of β-Mercaptoethanol to 50ml of Suspension buffer

Note

The final concentration of β-Mercaptoethanol in the buffers should always be 7mM before each use

10 Use 40ml of the buffer to resuspend the cells and then with the another 10ml wash the tubes to collect any cells left attached to the tubes

11 Gather the sample into two tubes (appr. 25-35ml liquid in each)

Note

At this point we can store the samples at -80^{O}C overnight

12

Note

Clean the sonicator by applying one operation cycle to milli-Q water

Sonicate each tube on ice with 20s on 20s off for an active time of 4min at 70% of total power

Note

It is very important to keep the cells on ice to avoid damaging the proteins as they are very sensitive to temperature

13

Note

Precool centrifuge at 4^{O}C

Centrifuge the samples for 20min with 20000RCF at 4^{O}C

Note

Debris are gathered on the walls in pellet shape

14 Collect the sample into a single beaker and measure the total volume (e.g. 60ml)

15 Prepare the same quantity of High salt suspension buffer by adding 2000x concentration of β-Mercaptoethanol (e.g. add 30µl of β-Mercaptoethanol to 60ml of High salt suspension buffer) and mix the solutions.

16

Note

Precool centrifuge at 4^{o}C

Centrifuge the samples for 20min with 20000RCF at 4^{o}C

Note

Due to the use of the High salt buffer the unwanted debris will be precipitated

17 Filter the supernatant through a syringe filter with a 0.22µm PES membrane and gather the sample to a single Beaker

Ribosome purification

18 Preparation of the purifier: Clean the input filter of the AKTA Purifier
Set the ambient temperature to 4^{o}C
Connect three HiTrap Butyl HP column (5ml) in series to the purifier
Preparation of the buffers: Add 75µl of β-Mercaptoethanol to 150ml of Buffer C
Add 60µl of β-Mercaptoethanol to 120ml of Buffer D

19 Remove the ethanol in the columns with 45ml of Milli-Q water

Note

Set up the flow rate as 4 ml/min (Monitor the pressure while running the column and do not exceed the Max limit of pressure of columns.)

20 Equilibrate columns with 60 ml buffer C

21 Add approximately 90% of the lysate sample to the system

22 Wash the column with 45mL of wash buffer 1 (100% buffer C)

23 Wash the column with 75mL of wash buffer 2 (80 buffer C, 20% buffer D)

24 Elute the ribosomes with 60ml of elution buffer 1 (50% buffer C, 50% buffer D) and then with 60ml of elution buffer 2 (100% buffer D)

25 The eluted ribosomes are collected into tubes of 4.5ml

26 Pick the tubes that have the biggest absorbance in wave length 280nm

Column recovery

27 Wash with 45ml of milli-Q water

28 Wash with 45ml of NaOH (1M)

29 Wash with 45ml of milli-Q water

30 Wash with 45ml of Acetic Acid (0.1 M)

31 Wash with 45ml of milli-Q water

32 Equilibrate with 45ml of Ethanol 20%

Note

After the cleaning the column is stored at 4^{o}C in 20% ethanol and can be reused

Ribosome collection

33

Note

We will use two tubes for Ultracentrifuge

Add 14µl of β-Mercaethanol to 28ml of Cushion buffer and mix

34 Place 14ml of Cushion Buffer to each of the Ultracentrifuge tubes

35 Carefully place the ribosomes solution on top of the cushion buffer and make sure that we still see the separation layer

36 Ultracentrifuge for 16 hours with 24000RPM (100000RCF) at 4^{O}C

37 Discard the liquid

Note

The pellet of ribosomes might not be visible at this point

38 Add 2µl of β-Mercaethanol to 4ml of Ribosome Buffer and mix

39 Add 500µl of Ribosome buffer to the wall of the tube and slightly spin to wash the cushion buffer on each tube and then discard the liquid

40 Repeat step No 39

41 Add 100µl of Ribosome buffer to each tube and resuspend the cells with a magnetic stirrer on ice for 10min at 200rpm

42 Collect the samples to a single tube (Tube No1)

43 Wash the centrifuge tubes with 100µl of Ribosome Buffer and add that to another tube (Tube No2)

Measurements and concentration

44 Load the solution to a 0.5mL Amicon Ultra filter with 3kDa molecular weight cutoff and centrifuge at 14000RCF for 10min at 4

Note

Use two separate filter for Tube No1 and No2

45 Dilute 1µl of the concentrated solution to 99µl of ribosome buffer (or milli-Q water?) and measure the absorbance at 260nm

46 Optionally , if the absorbance of Tube No2 is more than 10% of the one measured for the Tube No1 you can mix the two samples and repeat steps 44 and 45

47

Note

Absorbance of 10 corresponds to 23uM concentration

Adjust the final concentration 10uM by dilution with ribosome buffer

Storage

48 Aliquote the final solution according to your needs (0.9µl are needed for one 5µl reaction) and store at -80^{o}C