

## Purification of 70S Ribosomes from *Bacillus subtilis*

Shota Suzuki<sup>1</sup>, Genki Akanuma<sup>2</sup> and Fujio Kawamura<sup>3\*</sup>

<sup>1</sup>Biotechnology Research Center, The University of Tokyo, Tokyo, Japan; <sup>2</sup>Department of Applied Chemistry, Faculty of Science and Engineering, Chuo University, Tokyo, Japan;

<sup>3</sup>Department of Life Science, College of Science, Rikkyo University, Tokyo, Japan

\*For correspondence: [kawamura@rikkyo.ac.jp](mailto:kawamura@rikkyo.ac.jp)

**[Abstract]** The eubacterial ribosome (70S) is a macromolecular complex that is composed of a small (30S) subunit and a large (50S) subunit. The small subunit comprises the 16S ribosomal RNA (rRNA) and more than 20 ribosomal proteins (r-proteins), whereas the large subunit comprises the 23S and 5S rRNAs and more than 30 r-proteins. *Bacillus subtilis* (*B. subtilis*) has 57 r-ribosomal protein genes and three rRNAs (16S, 23S and 5S rRNAs). Among them, we identified 21 r-proteins of the small subunit and 31 r-proteins of the large subunit in *B. subtilis* (Nanamiya *et al.*, 2004). The functions and roles of individual components of the ribosome have not yet been completely clarified. Herein we describe in detail an ultracentrifugation-based protocol for the preparation of 70S ribosomes from exponentially growing cells of *B. subtilis*.

### Materials and Reagents

1. *Bacillus subtilis* 168
2. NaCl
3. BD Bacto™ tryptone (Difco, catalog number: 211705)
4. BD Bacto™ yeast extract (Difco, catalog number: 212750)
5. Agar (Nissui, catalog number: 05835)
6. Tris (Wako Pure Chemical Industries, catalog number: 512-97505)
7. Magnesium acetate (Wako Pure Chemical Industries, catalog number: 139-15335)
8. Ammonium acetate (Wako Pure Chemical Industries, catalog number: 019-02835)
9. Dithiothreitol (DTT) (Wako Pure Chemical Industries, catalog number: 042-29222)
10. Phenylmethylsulphonyl fluoride (PMSF)
11. Sucrose (Sigma-Aldrich, catalog number: S0389-55G)
12. Diethylpyrocarbonate (DEPC) treated water  
*Note: DEPC was added to distilled water at a final concentration of 1%, and then it stored at room temperature for over two hours. The DEPC-treated distilled water was autoclaved twice (121 °C, 20 min) and stored at room temperature.*
13. LB medium (see Recipes)
14. Buffer I (see Recipes)

15. 10-40% sucrose solutions (see Recipes)

**Equipment**

1. 2 L flasks with cotton plugs
2. Innova 4080 bench top incubator shaker (New Brunswick scientific)
3. French pressure cell press [Aminco International (USA), model: FA-078]
4. Mini cell [Aminco International (USA), model: FA-003]
5. Gradient fractionator (BIOCOMP, catalog number: 152-001)
6. Gradient master [Aminco International (USA), catalog number: 107-201M]
7. Micro collector (ATTO Technology, catalog number: AC-5700P)
8. Nano drop 2000 (Thermo Fisher Scientific)
9. High-speed refrigerated centrifuge (e.g. Hitachi, model: himac CR22GII)
10. Angle rotor R10A3 (fixed angle, max: 10,000 rpm, 18,800 x g, volume: 500 ml x 6)  
(Hitachi)
11. Centrifuge MX-300 (TOMY)
12. TMA-300 rotor (fixed angle, max: 15,000 rpm, 20,380 x g, volume: 2 ml x 24) (TOMY)
13. TMA-27 rotor (fixed angle, max: 15,000 rpm, 21,130 x g, volume: 15 ml x 4, 50 ml x 4)  
(TOMY)
14. Centrifuge tube (Nalgene, catalog number: 3148-0050)
15. Ultra-centrifuge (e.g. Hitachi, model: CP-60E)
16. P55ST2 rotor (swinging bucket rotor, max: 55,000 rpm, 366,000 x g, volume: 5 ml x 6)  
(Hitachi)
17. P28S rotor (swinging bucket rotor, max: 28,000 rpm, 141,000 x g, volume: 40 ml x 6)  
(Hitachi)
18. P40ST rotor (swinging bucket rotor, max: 40,000 rpm, 284,000 x g, volume: 13 ml x 6)  
(Hitachi)
19. Open-top polyclear centrifuge tube for P40ST rotor (Seton Identification Products, part number: 7031)
20. Open-top polyclear centrifuge tube for P28S rotor (Seton Identification Products, part number: 7052)
21. Centrifuge ware for P55ST2 rotor (Hitachi, part number: 332245A)

*Note: These rotors can substitute for other commercial rotors which can be used by the same centrifugal force as described in procedures.*

**Procedure**

A. Crude preparation of 70S ribosomes

1. *B. subtilis* is streaked out for single colonies and pre-incubated on LB agar plates at 30 °C for 14-16 h.
2. Cells are taken from single colonies on the plates and suspended in approximately 10 ml of LB medium with high turbidity. The cell suspension was diluted into 3 L of LB medium at an OD<sub>600</sub> of 0.04, and distributed equally among six 2-liter flasks, each containing 500 ml of the cell culture. The flasks are incubated at 37 °C with shaking at 250 rpm.

*Note: B. subtilis is routinely cultured in volumes that do not exceed one quarter of the volume of the flask used.*

3. Cells are harvested by centrifugation, when the OD<sub>600</sub> of the culture reaches 0.2, using six 500-ml centrifuge tubes (7,000 x g, 5 min, 4 °C). Cell pellets are resuspended with small volume of the culture supernatant, transferred from a 500-ml centrifugation tube to six 15-ml sample tubes and pellets are collected (3,500 x g, 5 min, 4 °C). The cell pellets are frozen by liquid N<sub>2</sub>.
4. Each cell pellet is suspended in 3.5 ml of Buffer I pre-chilled on ice.
5. Cells are disrupted using a French press (8,000 p.s.i.) passing the sample through three times.
6. Cell debris is removed by centrifugation (11,000 x g, 30 min, 4 °C).
7. The supernatant (cell lysate) is centrifuged at 30,000 x g for 30 min at 4 °C, for example in a HITACHI P55ST2 rotor at 18,000 rpm.
8. The resulting supernatant (S30 supernatant) is centrifuged at 200,000 x g for 100 min at 4 °C (e.g. in a HITACHI P55ST2 rotor at 45,000 rpm).
9. Each pellet is resuspended with 200 µl of Buffer I and the suspension stored at -80 °C until required (S100 pellet fraction).

*Note that the S100 fraction can be seen as amber pellet at bottom of the tube. Using a glass rod may be preferable to resuspend the pellet because resuspension of the pellet by pipetting is not easy.*

*Note: Care is taken to avoid generating foam while resuspending the pellet.*

#### B. Purification of 70S ribosomes

10. To further purify the 70S ribosomes, prepare a 10-40% sucrose gradient in SW28 centrifuge tubes using a Gradient Master gradient mixer.
11. 10% and 40% sucrose solutions are prepared in Buffer I.
12. The 10 to 40% sucrose gradient is generated in a SW28 centrifuge tube using a Gradient Master gradient mixer.
13. The S100 pellet fraction is applied on the top of the 10 to 40% sucrose gradient and centrifuged at 67,000 x g for 17 h at 4 °C (e.g. in a HITACHI P28S rotor at 22,500 rpm, using 270 A<sub>260</sub> units per centrifuge tube).

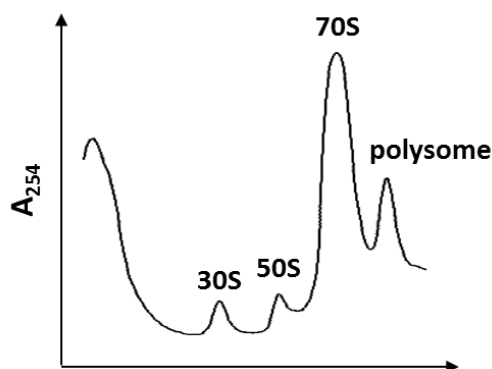
14. Samples are taken by Piston Gradient Fractionator and Micro collector. Absorbance profiles at 260 nm are determined using a Nano drop 2000.

*Note: An example of the ribosome profile from crude cell extracts is shown in Figure 1.*

15. Collected fractions containing 70S ribosomes are identified as a peak with an absorbance at 260 nm.
16. The ribosomes are recovered from the pooled fractions, which are diluted with Buffer I (1:2 dilutions) and centrifuged at 64,000 x g for 17 h at 4 °C. (e.g. in a HITACHI P40ST rotor at 22,500 rpm).
17. The supernatant is removed completely from the centrifuge tubes.
18. The resulting 70S ribosome pellets are covered with 300 µl of Buffer I and left overnight at 4 °C. The pellet is then gently resuspended in Buffer I using a glass rod and stored at -80 °C until required.

*Note: Care is taken to avoid generating foam while resuspending the pellet.*

### Representative data



**Figure 1. Profile of ribosome from crude cell extract by 10-40% sucrose gradient sedimentation.** The crude cell extract prepared from the log phase cells ( $OD_{600} = 0.2$ ) of wild type strain (168 *trpC2*) was applied on the top of the 10 to 40% sucrose gradient and centrifuged at 55,000 x g for 17.5 h at 4 °C (e.g. in a HITACHI P40ST rotor at 21,000 rpm, using 10  $A_{260}$  units per centrifuge tube). The  $A_{260}$  measurement shows as the same profile pattern of  $A_{420}$  measurement, and the peak of 70S ribosome can be determined. When the S100 fraction was subjected to the 10 to 40 % sucrose gradient and centrifugation, the peak smaller than 30S was not observed.

### Recipes

1. LB medium  
0.5% NaCl

- 1% tryptone
  - 0.5% yeast extract
  - 1.5% agar (only use for plates)
  - 2. Buffer I (200 ml is enough, pre-chilled on ice)
    - 20 mM Tris-HCl (pH 7.6)
    - 15 mM magnesium acetate
    - 100 mM ammonium acetate
    - 0.1 mM DTT
    - 2 mM PMSF
- Buffer I is prepared with DEPC treated water.
- Note; Buffer I can be stored without DTT and PMSF. DTT and PMSF are added to the buffer before use. Buffer I should be pre-chilled throughout the entire procedure.*
3. 10-40% sucrose solutions
- 10-40% sucrose solutions are made in Buffer I.

### **Acknowledgement**

This protocol was adapted from Nanamiya *et al.* (2004) and Suzuki *et al.* (2014). This work was supported in part by Grants-in-Aid for Scientific Research (C) (G. A.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

### **References**

1. Nanamiya, H., Akanuma, G., Natori, Y., Murayama, R., Kosono, S., Kudo, T., Kobayashi, K., Ogasawara, N., Park, S. M., Ochi, K. and Kawamura, F. (2004). [Zinc is a key factor in controlling alternation of two types of L31 protein in the \*Bacillus subtilis\* ribosome.](#) *Mol Microbiol* 52(1): 273-283.
2. Nanamiya, H., Kawamura, F. and Kosono, S. (2006). [Proteomic study of the \*Bacillus subtilis\* ribosome: Finding of zinc-dependent replacement for ribosomal protein L31 paralogues.](#) *J Gen Appl Microbiol* 52(5): 249-258.
3. Natori, Y., Nanamiya, H., Akanuma, G., Kosono, S., Kudo, T., Ochi, K. and Kawamura, F. (2007). [A fail-safe system for the ribosome under zinc-limiting conditions in \*Bacillus subtilis\*.](#) *Mol Microbiol* 63(1): 294-307.
4. Suzuki, S., Tanigawa, O., Akanuma, G., Nanamiya, H., Kawamura, F., Tagami, K., Nomura, N., Kawabata, T. and Sekine, Y. (2014). [Enhanced expression of \*Bacillus subtilis\* yaaA can restore both the growth and the sporulation defects caused by mutation of rplB, encoding ribosomal protein L2.](#) *Microbiology* 160(Pt 6): 1040-1053.
5. Tagami, K., Nanamiya, H., Kazo, Y., Maehashi, M., Suzuki, S., Namba, E., Hoshiya, M.,

---

Hanai, R., Tozawa, Y., Morimoto, T., Ogasawara, N., Kageyama, Y., Ara, K., Ozaki, K., Yoshida, M., Kuroiwa, H., Kuroiwa, T., Ohashi, Y. and Kawamura, F. (2012). [Expression of a small \(p\)ppGpp synthetase, YwaC, in the \(p\)ppGpp\(0\) mutant of \*Bacillus subtilis\* triggers YvyD-dependent dimerization of ribosome.](#) *Microbiologyopen* 1(2): 115-134.