Chapter 2

Preparation and Testing of *E. coli* S30 In Vitro Transcription Translation Extracts

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Abstract

Crude cell-free extracts are useful tools for investigating biochemical phenomena and exploiting complex enzymatic processes such as protein synthesis. Extracts derived from *E. coli* have been used for over 50 years to study the mechanism of protein synthesis. In addition, these S30 extracts are commonly used as a laboratory tool for protein production. The preparation of S30 extract has been streamlined over the years and now it is a relatively simple process. The procedure described here includes some suggestions for extracts to be used for ribosome display.

Key words: *Escherichia coli*, S30 extract, Cell-free protein synthesis, In vitro transcription translation

1. Introduction

Ribosome display is a powerful and useful technique for protein engineering. One key component of the method is the translation machinery (ribosomes, tRNAs, translation factors, etc.) that must be supplied. These numerous and complex biochemical catalysts can be obtained as a crude mixture extracted from various biomass sources or as a reconstituted mixture of individually purified components. Several vendors sell *E. coli*-derived crude cell-free extracts for translation (Promega, Invitrogen, 5 PRIME, Qiagen, etc.), and a purified system is available from New England Biolabs. In addition to *E. coli* extracts, several other cell-free extracts are commercially available including wheat germ, insect cell, and rabbit reticulocyte-based systems. While buying a kit is the fastest and easiest way to obtain extract, it is difficult or impossible to customize the extract to a specific application. For example, in some cases one may wish to delete a gene in the source organism to reduce the background

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signal in an assay or to remove a detrimental protease. In addition, the cost of kits may be prohibitive if many experiments are needed. Purifying all the required components for translation provides the greatest control of the system composition; however, it is very labor intensive and time-consuming. Preparation of crude cell-free extracts is relatively quick, simple, and inexpensive. This chapter describes the preparation of *E. coli* crude cell-free extract suitable for in vitro combined transcription and translation reactions.

The goal of extract preparation is to maximize the translational capacity of the final extract, so it is important to start with cells having a high concentration of the translation machinery. This means the cells must be harvested during rapid exponential growth since the intracellular content of ribosomes and other translation components is positively regulated by growth rate (1). After growth, the cells are harvested and washed. The washed cells are processed into extract by lysis, clarification, and pre-incubation.

2. Materials

The amounts here are based on 1 L of culture which can be grown in a single Tunair 2.5-L shake flask (IBI Scientific). Using 2YTPG medium and the A19 strain, each liter of culture should produce about 8 g wet cell weight (harvested at 3 OD) which will yield about 10 mL of extract. The amounts can be scaled up if more extract is needed. In that case, it may be more convenient to grow the culture in a fermenter or bioreactor.

2.1. Cell Growth and Harvest

1. *E. coli* strain A19 (see Note 1).
2. 2.5-L Tunair FUL-BAF flask with a Dri-Gauze filter lining in the cap (see Note 2).
3. 2YT medium for inoculum, 50 mL: 16 g/L tryptone, 10 g/L yeast extract, 5 g/L sodium chloride (see Note 3).
4. 2YTPG medium (2), 1 L: 16 g/L tryptone, 10 g/L yeast extract, 5 g/L sodium chloride, 22 mM sodium phosphate monobasic, 40 mM sodium phosphate dibasic, 100 mM glucose, 100 μL antifoam 204 (Sigma-Aldrich), optional (see Note 4).
5. 1 M potassium, sodium, or ammonium hydroxide for pH control, optional (see Note 5).
6. S30 buffer, approximately 50 mL (about 150 mL total needed for the complete process): 10 mM Tris acetate, 14 mM magnesium acetate, 60 mM potassium acetate (see Note 6).

2.2. Biomass Processing

1. S30 buffer, approximately 100 mL: 10 mM Tris acetate, 14 mM magnesium acetate, 60 mM potassium acetate (see Note 6).
2. Avestin Emulsiflex homogenizer.
3. Pre-incubation mix, about 3 mL: 370 mM Tris acetate pH 8.2, 11.1 mM magnesium acetate, 16.5 mM ATP, 50 μM each of the 20 amino acids, 105 mM phosphoenol pyruvate (PEP), 8.4 U/mL pyruvate kinase (Sigma-Aldrich #P7768).

2.3. Extract Testing

1. 10× salt solution: 1.3 M potassium glutamate, 100 mM ammonium glutamate, 80 mM magnesium glutamate (see Note 7).
2. 10× master mix: 12 mM ATP, 8.5 mM GTP, 8.5 mM CTP, 8.5 mM UTP, 340 μg/mL folinic acid, 1.7 mg/mL *E. coli* total tRNA (Roche).
3. AA mix: 50 mM each of the 20 amino acids (see Note 8).
4. 1 M sodium pyruvate.
5. 1 M sodium oxalate.
6. 200 mM putrescine.
7. 200 mM spermidine.
8. 20 mM coenzyme A (CoA).
9. 100 mM nicotinamide adenine dinucleotide (NAD).
10. L-[^{14}C(U)]-leucine (PerkinElmer).
11. T7 RNA polymerase (see Note 9).
12. Plasmid DNA (see Note 10).
13. Whatman 3MM chromatography paper.
14. Straight pins.
15. 5% (w/v) trichloroacetic acid (TCA).
16. Ethanol (95–100%).
17. Scintillation cocktail.
18. Liquid scintillation counter.

3. Methods

The extract procedure described here is based on the method of Liu et al. (3) which was developed for bulk protein production. Some modifications to this procedure may provide advantages for ribosome display as suggested by Hanes et al. (4). See Notes 13 and 17 for comments on the differences in the procedures.

3.1. Cell Growth and Harvest

1. Inoculate 50 mL of 2YT medium in a 250-mL baffled flask with at least 10 μL from a thawed glycerol stock of *E. coli* A19.

Alternatively, inoculate from a colony on an agar plate or a tube culture. Incubate the culture overnight at 37°C with vigorous shaking (~250–280 rpm).

2. In the morning, transfer the entire 50 mL culture into 1 L of 2YTPG medium in a 2.5-L Tunair FUL-BAF flask with a Dri-Gauze filter lining in the cap (see Note 2). Incubate at 37°C vigorous shaking (~250–280 rpm) and monitor growth by OD at 600 nm.
3. Harvest the culture during exponential phase before the growth rate drops during the transition to stationary phase (see Note 11). The culture should be chilled as quickly as possible either by adding ice directly to the culture or by passing it through a heat exchanger or cooling coil in ice water.
4. Once the culture is chilled, collect the cells by centrifugation at 8,000 × *g* for 20–30 min. If the entire culture volume cannot be centrifuged at once, pour the supernatant off the cell pellets and add more culture on top the pellet for a second centrifugation. Approximately 2.5 g/L/OD wet cells should be collected (~8 g from 1 L at 3 OD).
5. After all the culture is harvested, resuspend the cell pellet in at least 5 mL of S30 buffer for each gram of wet cell weight.
6. Centrifuge the cell suspension at 8,000 × *g* for 20–30 min.
7. Discard the supernatant and freeze the washed cell pellet at −80°C (see Note 12).

3.2. Biomass Processing

1. Break the frozen cell paste into small pieces and thaw in 1 mL of room temperature S30 buffer per gram cell paste (see Note 13).
2. Shake and stir the cell suspension periodically until it is well mixed. Once thawed, keep the cell suspension on ice if the lysis step cannot be done immediately. Rinse the homogenizer with S30 buffer before processing the cell suspension.
3. Lyse the cells with a single pass through an Avestin Emulsiflex high pressure homogenizer at 17,500 psi (see Note 14). The lysate should be cooled as quickly as possible after exiting the homogenizer, preferably through a cooling coil or heat exchanger. Keep the lysate on ice until it is all collected.
4. Centrifuge the lysate at 30,000 × *g* for 30 min at 4°C (see Note 15).
5. Transfer the supernatant to a clean tube and repeat the centrifugation (see Note 16).
6. Add 2 mL of pre-incubation mix for each 10 mL of clarified lysate and place in a closed tube or bottle in a 37°C shaker with gentle shaking (~100 rpm) for 80 min (see Notes 17 and 18).

7. Centrifuge the extract at 4,000 × *g* for 10 min.
8. Aliquot the supernatant into tubes and freeze in liquid nitrogen before storing at –80°C (see Note 19).

3.3. Extract Testing

Testing an extract consists of running a cell-free transcription/translation reaction to produce a model protein. The procedure is described below for the Cytomim system (5) (see Note 20) using chloramphenicol acetyl transferase (CAT) as a model protein. Ideally the model product would be similar to the candidate protein to be used in experiments. Modifications to the reaction conditions may be needed for disulfide bond formation in the product (6–8).

The procedure below uses radiolabeling to quantify the product protein. There is also an enzymatic assay for CAT activity (9, 10). Alternative model proteins can be used to enable other detection methods (i.e. fluorescence for GFP production or luminescence for luciferase production).

The exact optimal expression conditions vary slightly for each batch of extract and potentially for each product, with magnesium concentration being the most sensitive. If the standard conditions do not provide acceptable activity, the magnesium concentration in the cell-free reaction should be varied about ±4 mM in 1 or 2 mM increments to identify the optimum conditions. Optimizing plasmid, T7 RNA polymerase, potassium and extract concentrations can also help improve activity.

1. Thaw an aliquot of extract and all the cell-free reagents in Table 1.
2. Mix reagents in the order listed in Table 1, adding the plasmid and extract last (see Note 21).
3. Incubate at 37°C for 3–5 h (see Note 22).
4. While the reactions are running, cut two small slips of Whatman 3MM filter paper for each reaction and label them with pencil. Pierce each strip with a straight pin and stick the pin in a sheet of Styrofoam covered in aluminum foil (shipping cooler lids work well). Make sure the filter paper is held above the foil by the pin so that the sample will not spread from the filter to the foil.
5. At the desired endpoint, for each reaction, spot 5 μL of cell-free reaction each on two separate filter paper slips.
6. Dry the slips for 15 min under an incandescent lamp or overnight on the bench.
7. Place one set of slips (with the pins still in them) in a small beaker and wash three times with cold 5% (w/v) trichloroacetic acid (TCA) for 10 min. Wash once with ethanol (see Note 23), then return to the Styrofoam sheet. Dry under a lamp for

Table 1
Recipe for a 15 μL cell-free transcription-translation reaction

Reagent	Stock concentration	Reaction concentration	Volume (μL)
Milli-Q water			5.5525
10× salt solution	10×	1×	1.5
10× master mix	10×	1×	1.5
AA mix	50 mM each	2 mM	0.6
Pyruvate	1 M	33 mM	0.5
Oxalate	1 M	4 mM	0.06
Putrescine	200 mM	1 mM	0.075
Spermidine	200 mM	1.5 mM	0.1125
CoA	20 mM	0.27 mM	0.2
NAD	100 mM	0.33 mM	0.05
^{14}C Leu	0.1 mCi/mL	1.7 μCi/mL	0.25
T7 RNA polymerase	0.8 mg/mL	33 μg/mL	0.6
Plasmid	0.5 mg/mL	13.3 μg/mL	0.4
Extract		24% (v/v)	3.6

15 min or overnight on the bench. Ensure that the washed slips can be differentiated from the unwashed slips.

8. Remove the pins from the filters and place each slip into a separate scintillation vial, add cocktail, and count in a liquid scintillation counter.
9. Calculate the protein produced with the following formula:

$$[\text{Product}](\text{mg/L}) = \text{Counts}(\text{washed}) / \text{Counts}(\text{unwashed}) \times [\text{Leucine}] \times \Upsilon / X,$$

where [Leucine] is the total concentration of leucine (2 mM in this case), Υ is the molecular mass of the product, and X is the number of leucine residues in the product.

4. Notes

1. Other strains can be used (MRE600, BL21, etc.). A strain with reduced RNase activity (i.e. *rna* and/or *rne* mutations) may give better results. Make sure the growth medium contains any required nutritional supplements if an auxotrophic strain is used.

2. Tunair flasks are available from IBI Scientific and several distributors. The flasks have a higher oxygen transfer rate than standard baffled glass flasks, so they support higher growth rates and cell densities. Alternatively, the culture volume can be split between multiple glass flasks (typically no more than 400 mL in a 2-L glass flask).
3. The specific medium used for the inoculum is not critical, but preferably it would be the same as the main culture medium. However, in my experience, 2YT works better for overnight inoculum cultures than 2YTPG. If 2YTPG is used for the inoculum, the lag phase may be longer than with a 2YT inoculum.
4. Other media such as LB, 2YT, or a defined medium can be used, but 2YTPG provides a good balance of cost, simplicity, and reasonable cell density (which affects the yield of extract). Also, 2YTPG will produce better extract than 2YT for longer cell-free reactions (2). The 2YTPG medium should be sterile filtered instead of autoclaved to avoid carmelization of the glucose. Alternatively, a separate 50% (v/v) glucose stock can be made and added to the other medium components after autoclaving. The growth rate of the culture determines the ribosomal content of the extract (11), so a medium which supports rapid growth ($\mu > 0.5$ h^{-1}) should be used for best results. If antifoam 204 is used, samples should be chilled to ≤25°C before measuring the OD to eliminate the cloudiness of the antifoam. Placing a 1 mL sample on ice for a couple minutes works well as long as the sample is mixed before OD measurement.
5. The optimum pH for *E. coli* is typically about 7.2–7.4, but can vary depending on the strain and medium used. Metabolic waste products generated from the glucose in the 2YTPG medium will cause the culture to become acidic as the cell density increases, so pH control can help sustain rapid growth to higher cell densities. If pH control is not possible, high quality extract can still be produced, but the culture may need to be harvested at a lower cell density. In addition to pH control, fed-batch fermentation methods can be used to reach higher cell densities (thus producing more extract per batch), but such methods are more complicated to set up and operate. There are many published strategies for fed-batch fermentations and a detailed description is beyond the scope of this chapter. If a fed-batch method is employed, it is important that it maintains a rapid growth rate to produce the highest ribosome content in the extract (11).
6. S30 buffer is typically prepared as needed from purified water and three separate 100× concentrates (1 M Tris acetate pH 8.2, 1.4 M magnesium acetate, and 6 M potassium acetate) which are sterile filtered for increased stability and stored at room temperature. The pH of the 1 M Tris solution is adjusted to 8.2

with glacial acetic acid. The pH of the final S30 buffer usually is not checked or adjusted. A large container of distilled water can be chilled and used for making cold S30 buffer as needed. If desired, the water can be sterilized prior to chilling.

7. It is convenient to prepare small amounts of the salt solution with varying magnesium concentrations for testing the magnesium optimum of each batch of extract. Typically, 8 mM magnesium in the cell-free reaction (equivalent to 80 mM in the 10× salt solution) gives acceptable performance, but the optimum may vary from about 4 to 12 mM. All reagents for extract testing should be divided into small aliquots, frozen in liquid nitrogen, and stored at −80°C for the greatest stability.
8. Tyrosine is not soluble in the amino acid mixture and other amino acids may precipitate over time. This does not affect the performance as long as the mixture is thoroughly mixed immediately before use.
9. T7 RNA polymerase can be purchased from a number of suppliers. Kigawa recommended Ambion, Takara, and Promega products (12). Alternatively, the polymerase can be prepared in-house as a His-tagged protein and purified with a standard IMAC procedure (13) followed by dialysis or ultrafiltration to remove the imidazole. As another option, Nevin and Pratt (14) as well as Kim et al. (15) have expressed T7 RNA polymerase directly in the source cells used for extract preparation so that the extract already contains the T7 RNA polymerase.
10. Any vector designed for T7-driven expression should work in a cell-free system, but the specific vector design can affect productivity. Invitrogen sells vectors for cell-free expression that should be suitable for this system. Standard plasmid purification kits produce suitable quality plasmid for cell-free reactions.
11. The maximum OD before stationary phase will depend on the strain, medium, and culture vessel used. Virtually any system should support at least 1 OD, so harvesting at this point will likely produce good extract (but less volume of extract). The A19 strain grown in 2YTPG in a Tunair flask with 280 rpm shaking can reach 5–8 OD in exponential phase if the pH is controlled. Without pH control, growth may start to slow around 3 OD as the pH drops. Usually, the culture will have a 1–2 h lag phase followed by 2–3 h of exponential growth. The density and age of the overnight culture will affect the lag time and duration of the growth phase. Checking the OD every 30 min once growth starts will provide good data for growth rate calculation and determination of the maximum OD with rapid growth.
12. It is most convenient to scrape the cell paste into a plastic sample bag (VWR #89004-424) and flatten to a thin sheet

before freezing. This takes up less freezer space than a centrifuge bottle and the frozen sheet can be broken up into smaller pieces to speed thawing. Also, if a large batch of cells is prepared, it can be split into portions for multiple extract preparations, for example, to test variations on the extract procedure. Frozen cells can be stored at −80°C for several months. If the rest of the extract preparation procedure will be done immediately, there is no need to freeze the cells.

13. Hanes et al. (4) used 4 mL of buffer per gram cell paste. Using more buffer will result in a more dilute cell lysate which will improve clarification. However, it also results in a larger volume of cell suspension to be lysed and clarified. Most likely, the buffer volume used does not have a strong impact on the total amount of extract activity produced. So the volume can be chosen to make processing most convenient for the lysis method used and the available equipment. The optimum volume of extract used for transcription/translation reactions will depend on the cell dilution factor. If more buffer is used to resuspend the cells, more extract will be needed in the transcription/translation reaction.
14. Avoid getting any air bubbles or foam in the homogenizer, otherwise the extract activity may be reduced. Other cell lysis methods (bead milling, French press, etc.) have been used for extract preparation. To my knowledge, there is no published study comparing the various lysis techniques, but Kigawa recommended glass bead milling and discouraged sonication (12).
15. The centrifugation can be done at a lower *g*-force (15). If bottles larger than 50 mL are used, extending the centrifugation time 10–20 min will help improve the clarification.
16. The pellet material is very detrimental to extract performance, and the pellet is usually somewhat loose and fluid. So, the supernatant should be transferred to a clean tube as soon as possible, preferably by using a pipette and leaving the bottom 10% or so on the pellet to avoid transferring any pellet material.
17. This pre-incubation step dissociates the polysomes and ribosomes in the extract into 30S and 50S ribosomal subunits (3). Liu and co-workers showed that omitting the reagent additions to the pre-incubation did not affect bulk protein synthesis by the finished extract. Leaving out the additions simplifies the process and greatly reduces the cost. However, without the reagent additions, the 70S ribosomes do not dissociate into subunits. Since some ribosome display translation reactions last only a few minutes, it may be important to have the ribosomes already dissociated in the extract for the best activity. Also, Hanes et al. (4) suggest better performance is achieved with a pre-incubation of 60 min at 25°C. In addition, their

pre-incubation mix has slightly different concentrations of the components. A few experiments can determine the effects of these variations in any specific application.

18. Most extract preparation procedures include extensive dialysis after the pre-incubation. Typically this is done at 4°C for four 45 min periods versus 20 volumes of S30 buffer (changed every 45 min) using 6–8,000 Da molecular weight cut-off dialysis tubing. The work of Liu et al. (3) showed little if any benefit of the dialysis step for bulk transcription/translation reactions, so it was omitted from the procedure here.
19. Extract is usually stable for at least three freeze-thaw cycles. Frozen extract stored at −80°C is stable for at least a year. If a large batch of extract is made, it is convenient to make a few dozen single-use aliquots and then divide the rest of the extract into larger portions. When needed, a large aliquot can be thawed and split into another set of single-use aliquots. All extract samples should be frozen in liquid nitrogen and stored at −80°C. Slow freezing or storage at higher temperatures can reduce the extract activity.
20. Other energy generation systems have been developed for use in cell-free reactions. For more information, see the review by Calhoun and Swartz (16). The Cytomim system requires inverted membrane vesicles to perform oxidative phosphorylation. These vesicles are formed during the high pressure homogenization lysis step and remain in the final extract as prepared with this procedure. If a different cell lysis method is used, it may be necessary to use a different energy generation system in the cell-free reaction to achieve good performance.
21. If only a few reactions are planned, the recipe should be multiplied to ensure all the required volumes are reasonable to pipette accurately. The standard 15 μL reactions work well in 1.5-mL microcentrifuge tubes. If larger reactions are required, the reaction vessel may need to be changed. Voloshin and Swartz have investigated the effects of volume and reactor geometry (17).
22. Some proteins express better at lower temperatures. If desired, a range of temperatures can be tested for each new product to find the optimum (typically 30°C–37°C). Protein synthesis may continue for 5 h or more (5), so a time course should be run if maximum product titer is important.
23. The ethanol wash speeds up the drying process. It usually does not affect the results if it is omitted.

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