ZJUTiGEM

Date: Oct. 17 2021

CELL-FREE SYSTEM HANDBOOK

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CHAPER1
CELL-FREE SYSTEM OF ZJUT-China

1.1 cell-free system

1.1.1 Introduction and principle of cell-free system

Cell-free System (CFS) is a System that utilizes Cell extracts for in vitro transcription and translation. Since cell extracts retain the cell's own transcription and translation mechanisms, protein synthesis can be initiated by adding exogenous resources, including amino acids, nucleotides and secondary energy sources, to CFS [4]. Compared with conventional cell systems, cell-free systems remove cell membranes and natural genomic DNA, eliminate unnecessary gene regulation, eliminate the need for cell growth, avoid competition between cell growth and cell products, and maximize the efficiency and benefit of synthesis. And the cell-free system has an open operating system that allows experimenter to directly manipulate the system, providing unprecedented control over the molecular environment of gene expression and metabolism [1]. SCell-free systems allow rapid and cost-effective production of recombinant proteins, enhancing control over efficient substrate processing, and conveniently promotes precise real-time reaction monitoring and direct process optimization, all while by-passing time-consuming procedures such as bacterial transformation, clone selection and cell lysis. Their extremely user-friendly and safe handling makes them similarly well-suited for cutting-edge research in academia and biotech industry as well as student

education [2].

1.1.2 The composition of cell-free system

The cell-free system mainly consists of three parts: cell extract, reaction mixture, and nucleic acid template.

Cellular extract A substance extracted from a cell that contains ribosomes, RNA polymerases, other transcription and translation proteins (such as σ factors, initiation factors, and elongation factors), and enzymes and cofactors used in energy metabolism. Different cell-free products can be developed according to different cells, such as Escherichia coli and rabbit reticulocytes.

Reaction mix: ATP, nucleotides, amino acids, metabolic cofactors, buffers and other raw materials required for the synthesis of proteins.

Template DNA: circular plasmid or linear DNA.

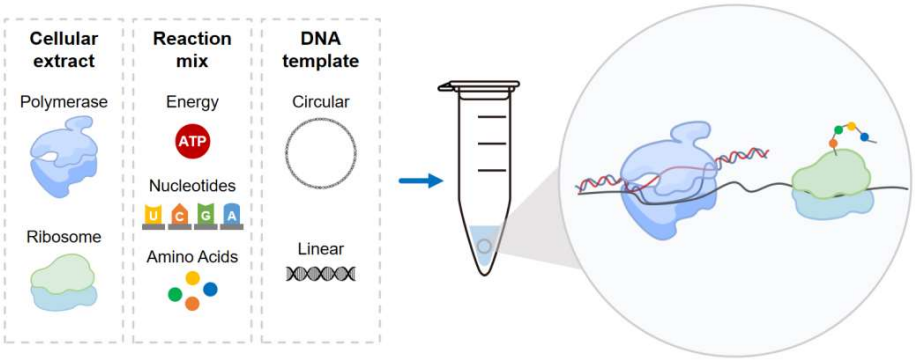

Figure 1. Schematic diagram of cell-free system composition

1.1.3 Cell-free system reagent:

Zjut-china uses Arbor's myTXTL ® series myTXTL SIGma70 Mix, a

perfect choice for high-yield production of soluble and membrane proteins, rapid prototyping and execution of complex multi-gene networks using plasmid DNA and RNA, the construction of synthetic minimal cells and to study cellular biology. It was developed by Prof. Vincent Noireaux at the University of Minnesota (USA) and has been demonstrated to be valuable for various applications in protein engineering and synthetic biology [4][5]. It entirely relies on the endogenous TXTL machinery of E. coli employing the core RNA polymerase and E. coli transcription factor sigma 70 (s70) (Figure 2). Therefore, any endogenous E. coli promoter – either constitutive or inducible – is suitable for in vitro gene expression in myTXTL.

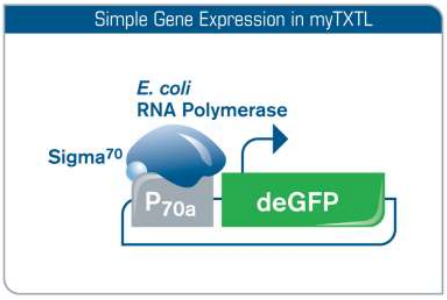

Figure 2. Gene expression in myTXTL driven by its core RNA polymerase and transcription factor sigma 70.

σ factor acts as a cofactor of all RNA polymerases. It is an inherent component of DNA-dependent RNA polymerases and can recognize the common sequence of promoters and bind to RNA polymerase to transform into polymerase holoenzyme, thus making RNA polymerase active.

In E.coli, the main σ factor is σ70, which can identify all the constituent expression promoters in E.coli and promote the initiation of transcription of core enzymes. MyTXTL sigma70 MIX takes advantage of this feature and starts the expression of complex gene lines from the promoters regulated by σ70.

1.2 cell-free system experiment

Materials：

• Nuclease-free, barrier tips and pipets capable of pipetting 0–100 µL

• Sterile and nuclease-free 1.5 or 2.0-mL Eppendorf tubes, PCR tubes or multi-well plates

• Nuclease-free, molecular biology-grade water

• Incubator, thermo block or water bath

• Table-top microcentrifuge

• Vortex mixer

• Fluorescence plate reader (e.g. Tecan Genios)

Procedure：

The following steps describe setting up myTXTL reactions with the positive control plasmid P70a(2)-deGFP that is supplied with the myTXTL Sigma 70 Master Mix Kit.

1. Preheat incubator (or thermo block or water bath) to 29 °C.

2. Completely thaw the myTXTL Sigma 70 Master Mix and the positive control plasmid on ice. Keep reagents on ice till use. Note: To minimize freezing and thawing cycles, only thaw the number of reagent tubes required to set up the desired number of myTXTL reactions.
3. Directly before use, vortex the myTXTL Sigma 70 Master Mix for 2-3 seconds and briefly spin down. If any precipitate is visible hereafter, gently resuspend master mix solution about 10 times to ensure homogeneity. Avoid formation of bubbles and foam.
4. Setting up a myTXTL reaction. The recommended total volume of a myTXTL reaction is 12μL.

   A).Make stocks which are 10× of desired final reaction concentrations.

   B).Assemble a reaction (either prepackaged or multi-

   C)component),completing with water to 80% volume
5. Vortex gently to mix and split the reaction into each tube evenly.
6. Add 1.2 μL of respective plasmid encoding sigma factors under P70a promoter to each tube. Add 1.2 μL of the corresponding deGFP plasmid to each tube to complete to 12 μL. To the background tube, add water to 12 μL. Gently mix all the tubes.
7. Pipette 5 μL from each reaction into two wells in the well plate.

8. Seal the wells with caps, place the well plate into the plate reader, and begin measurement.
9. When kinetics are done, use the GFP calibration (488/535)to calibrate the raw data.
10. Subtract the background reaction from all other reactions for all time points.
11. Compare the slope of the deGFP kinetics between each transcription cascade
12. Repeat as necessary for error bars.

1.3 Fluorescence-based Analysis

Materials：

• Fluorescence plate reader (e.g. Tecan Genios)

• Black, optical-bottom 384-well plate (e.g. Nunc)

• Phosphate-buffered saline (1x PBS)

• Recombinant eGFP (Cell Biolab, # STA-201)

Procedure:

(A) Qualitative (visual) Analysis

Compare the intensity of (green) color in your myTXTL control reaction to the following standard eGFP color strip (Figure 3) to assess deGFP produced in your tube.

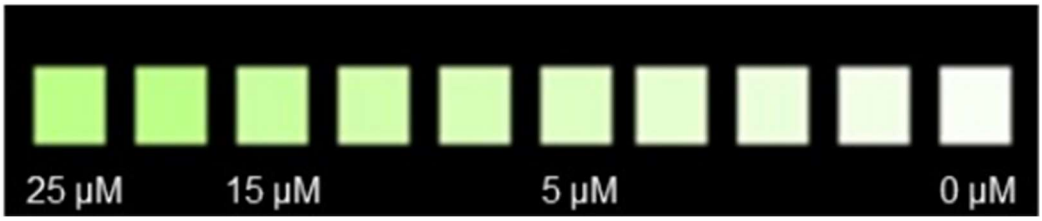

Figure 3. Color strip for the qualitative analysis of deGFP production

(B) Quantitative Analysis(*Endpoints*)

1.3.1 Prepare an eGFP standard curve (0-5 μM)

• Thaw and keep the recombinant eGFP standard (Cell Biolabs, # STA-201) on ice. Determine the molar concentration of your protein solution.

• Prepare an eGFP stock solution of 5 μM in PBS (V = 70 μL) in a 1.5 mL reaction tube. Example: If your eGFP standard has a concentration of 30 μM, transfer 11.7 μL of the 30 μM eGFP protein solution to 58.3 μL PBS, mix thoroughly and collect mixture on the tube bottom by a short centrifugation step.

• Prepare a 2-fold dilution series of eGFP in the concentration range of 0-5 μM in 1.5 mL reaction tubes (5, 2.5, 1.25, 0.63 and 0.31 μM). Example: To prepare a 2.5 μM eGFP solution, transfer 35 μL of the 5 μM eGFP solution to 35μL PBS and mix thoroughly. Then take 35 μL of the 2.5 μM eGFP solution to prepare the next dilution step. Proceed to 0.31μM eGFP (five dilution steps).

• For each dilution, transfer 10 μL/well in triplicate into a black,

optical-bottom 384-well plate. Also include a Blank measurement in triplicate using PBS only.

1.3.2 Dilute the myTXTL control reaction

• Prepare a 10-fold dilution of the centrifuged myTXTL control reaction in PBS. In a 1.5 mL reaction tube, add 4 µL myTXTL control reaction to 36 µL PBS, mix thoroughly and collect mixture on the tube bottom by a short centrifugation step.

• Transfer 10 µL/well of this diluted sample in triplicate to the same 384-well plate as the eGFP standard dilution series (see above).

1.3.3 Perform fluorescence measurement using a plate reader

• Before the fluorescence measurement, carefully tap or briefly spin down the 384-well plate to remove any air bubbles and to equally distribute each sample in the well.

• Fluorescence reader setting: Choose an excitation and emission wavelength appropriate for eGFP measurement (e.g. excitation: 488 nm, emission: 535 nm).

1.3.4 Calculate the deGFP concentration using a calibration curve (linear regression)

• Subtract the fluorescence values of the Blank (PBS only) from that of each standard protein and myTXTL control reaction.

• Plot the Blank subtracted fluorescence values of the eGFP standard

(Y-axis) against their respective protein concentration (X-axis) and fit the curve to the linear regression formula (y = m*x) to determine the deGFP concentration in the myTXTL control reaction (Figure 4).

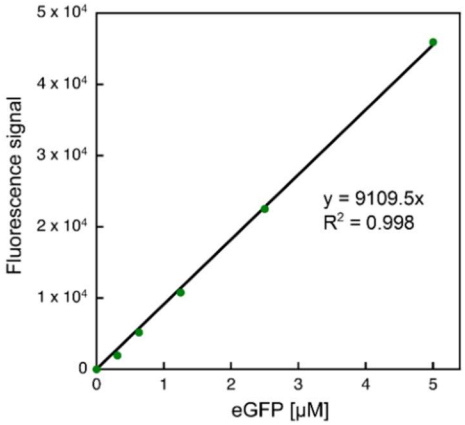

Figure 4. eGFP standard curve to evaluate protein production efficiency in myTXTL

(C) Quantitative Analysis(*Kinetics*)

Immediately after a cell-free reaction is assembled, the fluorescence kinetics can be measured. Set up the plate reader kinetics settings before assembling the cell-free reaction, to minimize the time between finishing assembling the reaction and the first measurement (time zero point) on the plate reader.

1. Set up the kinetics protocol on the plate reader. For example, measure for 16 h with a 3-min increment, at gain 60.
2. Preincubate the well plate to be used at 29 °C (or whatever temperature to be used during kinetic). Plates should preincubate at

least 30 min before reactions are added into the plate.

3. Assemble the cell-free reaction.
4. Pipette reactions into the now-warm well plate. For example, pipette 5 μL from each reaction into a 96 V-bottom well plate. With standard 12 μL reactions, a replicate can be made, so that there are two wells for each reaction.
5. Place the plate in the plate reader and start the measurement.
6. When the measurement is complete, the data can be analyzed.
7. Using the plate reader calibration, calculate the molar concentration of fluorescent protein for all the reactions, including the background reaction, and for all timepoints. There should now be a table, where each column is one reaction kinetic, and each row is an increasing time point.
8. Subtract the background reaction concentration from all other reactions, for all time points.
9. Take the average and standard deviation (for error bars) of each set of repeat reactions (if multiple repeats were done), for all time points.

1.4 Analysis of Protein Synthesis in myTXTL Using SDS-PAGE

In case of the target protein lacking any fluorescent properties, an SDS-PAGE analysis of myTXTL reactions is an alternative for a visual evaluation of its in vitro production. The 15 % SDS-PAGE gel in Figure 3

demonstrates the kinetic analysis of protein production in myTXTL over an incubation period of 16 h. Typically, only a small volume (1 – 3 µL) of the myTXTL reaction is loaded onto the SDS-PAGE gel. As myTXTL Master Mixes are derived from a E. coli whole cell extract, the lanes show also bacterial host cell proteins. In case the protein of interest has a molecular weight similar to a host cell protein or its in vitro production is less optimized, a polypeptide tag such as a His6-tag, Strep-tag® or FLAG-tag added to the N- or C-terminus of the target protein would allow protein detection via a Western Blot and/or isolation of the target protein using suitable affinity purification resins.

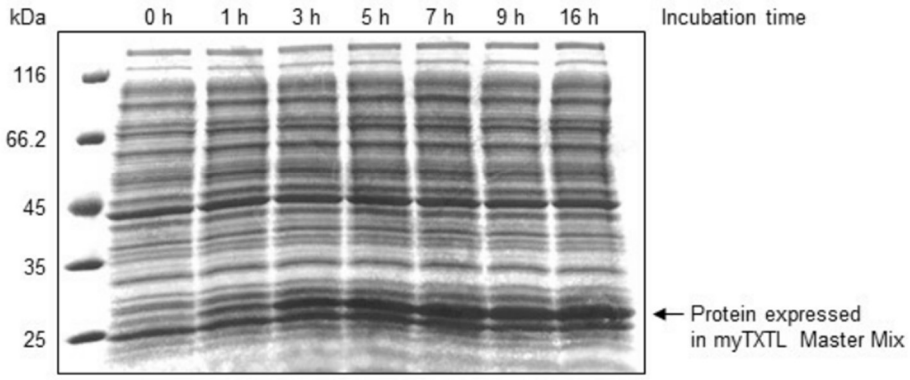

Figure 5. Kinetic analysis of deGFP expression in myTXTL Master Mix using SDS-PAGE. The gel was stained with a standard Coomassie® procedure.

1.5 Reference

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[4]Marshall, W. N. et al. The Dependence of Cell- Free Protein Synthesis in E. coli upon Naturally Occurring or Synthetic Polyribonucleotides[J]. Proceedings of the National Academy of Sciences of the United States of America,1961,47(10):

[5]Nirenberg M. et al. RNA codewords and protein synthesis. the effect of trinucleotides upon the binding of sRNA to ribosomes.[J]. Science (New York, N.Y.),1964,145(3639):

CHAPER 2
CELL-FREE SYSTEM OF XJTLU-CHINA

2.1 PREMEGA Principle

The Escherichia coli S30 circular DNA extraction system simplifies transcription/translation of cloned DNA sequences Provides a powerful tool for identifying and characterizing peptides in plasmids or lambda vectors. Researchers need only need to provide cloned DNA with the appropriate prokaryotic promoter and ribosome binding site. By improving the method described by Zubay from an escherichia coli strain B deficient in protease and lon protease activity in OmpT, this escherichia coli S30 circular DNA extraction system was prepared. The results showed that the expressed protein had greater stability than otherwise if in vivo.

The S30 in vitro system also allows higher expression of proteins that are normally low in expression Levels of host-encoded inhibitors in vivo. The S30 system for circulating DNA contains an S30 premix that contains no amino acids and is optimized for each lot extract and contain all other essential ingredients including rNTPs, tRNAs, ATP regeneration systems, IPTG and appropriate salt. Amino acid mixtures lacking cysteine, methionine, or leucine are provided for facilitation Translate radioactive labels for products.

2.2 PREMEGA Protocol

1) Mix the reagents in the following manner:

S30 Premix	20μL

Amino Acid minus Met	2μL	
Amino Acid minus Leu	2μL	
Amino Acid minus Cys	2μL	
S30 Cell Extract	15μL	
Plasmid	10μL	
DD water	to 20μL	(50μL in total)

2) Briefly vortex and centrifugate the mixture.
3) Transfer the sample into a well on a 96-well plate.
4) Incubate the plate inside the plate reader at 37°C with the plate lidded, with or without shaking.
5) The result could be read by microplate reader.

Note: *All the reagents should be put on the ice during the experiments. The kit provides 3 types of amino acid mixture without one kind of amino acids respectively. Therefore, for the common use, it is required to add all three types of amino acid mixture.*

2.3 PREMEGA Further advise

1. It is recommended to mix the reagents except the plasmid in advance.
2. The reaction volume could be adjust to 60μL.
3. Some regents such as imidazole may negatively affect the cell free system.

CHAPER 3
CELL-FREE SYSTEM COMPARE

3.1NEB

Principle

The NEBExpress Cell-free Escherichia coli protein Synthesis system is a coupled transcription/translation system designed to synthesize proteins encoded by DNA templates controlled by T7 RNA polymerase promoters.

protocol

1) Mix the reagents in the following manner:

Synthesis buffer	25μL	
Cell Extract	12μL	
AHL solution (0.5mg/mL)	0.5μL	
Plasmid sample	8μL	
RNase Inhibitor	1μL	
Plasmid	2.5μL	(49μL in total)

2) Briefly vortex and centrifugate the mixture.
3) Incubate the mixture in 37°C water bath for 5 minutes.
4) Add 1μL of Sigma 70 RNA polymerase into the tube.
5) Shake the tube at 37°C, 200 RPM, for 4 hours.

3.2Promega

Principle

The most common application of Promega S30 extracts is the synthesis of

small amounts of radio-labeled proteins. Synthesizing proteins of the correct size is a useful means of verifying gene products of a particular DNA sequence.

Additional information

The Promega regents requires a premix of the reagents before use, which lead that the amount of any regents will affect the result of the experiment. Therefore, it is recommended to process a series of qualitative test but not quantitative test. Meanwhile, the Promega in vitro expression system have a larger reaction volume, which means that it has more material such as dNTPs and amino acids for protein expression. Moreover, the reaction system could be set in several types of 96-wells plate including U-shaped and V-shaped plates.

Thanks to the large reaction volume od Promega, we could relatively free to change the components of reaction system, which provided more chances to test and rebuild our systems.

3.3Arbor

Principle

Cell-free gene expression platforms allow rapid and inexpensive production of recombinant proteins, providing a high degree of versatility and flexibility due to their open reaction conditions.

This allows for increased control over efficient substrate processing,

conveniently facilitating accurate real-time response monitoring and direct process optimization.

Additional information

According to the manual of the Arbor cell free system, it is recommended to use 384-well plate or V-shaped 96-wells plates to process the in vitro protein expression. Though the reaction system is smaller than any other cell free system at all, it performs a high efficiency of protein expression. Furthermore, the whole 12μL reaction system could be separate into 2 part which means there was a natural control experiment.

As the in vitro expression system kit is master mix kit, which means it is not required to mix the cell extract with other reagents including dNTPs, ATP and RNase inhibitors. That help to control the errors cause by operation.