



Cell-Free Protein Synthesis Course

Manual for

Protein Expression Reagent Kit

(Catalog No. CFS-EDU)

and

Laboratory Instrument Set

(Catalog No. CFS-EDU-E)

**Store Protein Expression Reagent Box at -80°C upon arrival.
Avoid unnecessary freeze/thawing of reagents.**

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Table of Content

1. INTRODUCTION.....	2
2. EXPERIMENTAL OUTLINE.....	4
3. REAGENTS AND MATERIALS	5
3.1 Content of “Protein Expression Reagent Kit”	5
3.2 Content of “Laboratory Instrument Set”	7
3.3 Other instruments required for conducting experiment	8
3.4 Other consumables required for conducting experiment	8
4. PROTOCOL	9
4.1 Preparation of reagents and distribution to groups	9
4.2 Experiment I: Transcription Step.....	10
4.2 Experiment II: Translation Step.....	12
4.3 Experiment III: RNA Detection	14
4.4 Experiment IV: Visualization of GFP Protein.....	15
4.5 Confirmation of experimental Data	16
5. TROUBLESHOOTING	17
6. REFERENCES AND FURTHER READING	18
7. LABEL LICENSE POLICY.....	21
Contact Information	22

1. INTRODUCTION

Protein biosynthesis is a fundamental process for maintaining life. The genetic information defining any living organism is commonly stored in genomic DNA and has to be translated into proteins for “use” for conducting most processes in a cell and multicellular organisms. Hence a wide range of proteins are essential components of every organism and have to perform many distinct functions, for examples as enzymes in metabolic processes or as regulatory switches in cell signaling.

Conversion of genetic information into proteins is in principle a two-step process, where in the first step genetic information encoding for a given protein is “transcribed” from a DNA template into a messenger RNA molecule, the mRNA. This mRNA is required to transport the genetic information from its place of storage (the genomic DNA) to the place of protein synthesis (at the ribosomes). In the second step, the ribosome functions as the cellular machinery where the genetic information contained in the mRNA is “translated” into a protein during protein biosynthesis. Protein expression is a tightly controlled biological process, and the flow of information from DNA into making functional proteins had often been referred to as one of the “central dogmas of life science”.

Proteins are often prepared for conducting research, are used in many industrial processes, and are very valuable therapeutic drugs. Therefore, many different protein expression systems have been developed with different preferences for certain groups of proteins and/or protein applications. Besides the expression of proteins in living cells (“*in vivo*”), both steps of protein expression can also be performed outside of a living cell using an “*in vitro*” or “cell-free” protein expression system. For cell-free protein expression, a purified bacterial RNA polymerase (here a SP6 RNA polymerase from *Salmonella typhimurium*) is used in the first step for mRNA production, whereas the ribosomes and all other necessary components for protein synthesis during the second step are provided by a cellular extract (here obtained

from wheat germ). Cell-free protein expression systems have proven very useful for many applications, and are routinely used in research and applied sciences.

During this “Cell-Free Protein Synthesis Course”, experiments are performed to demonstrate both steps of protein biosynthesis: Transcription of an mRNA from a DNA template and translation of the mRNA into a functional protein. The kit provides a DNA template for the expression of a Green Fluorescent Protein, or “GFP”, that can easily be detected by exhibiting bright green light upon exposure to blue or ultraviolet light. Visibility of the green light proves that a functional protein had been prepared during the “Cell-Free Protein Synthesis Course”.

2. EXPERIMENTAL OUTLINE

The “Protein Expression Reagent Kit” provides sufficient reagents to conduct one “Cell-Free Protein Synthesis Course” for 20 students. In the experimental outline, it is assumed that two students perform the experiments together, and therefore reagents are provided for 10 groups/sets of experiments. Experiment specific instrumentation for conducting the experiments can separately be obtained from CellFree Sciences as a “Laboratory Instrument Set”.

Before starting the “Cell-Free Protein Synthesis Course” for the students, reagents and consumables have to be prepared by the teaching staff as outlined in Section 4.1 of this manual.

The students will perform four experiments during the course as outline in Figure 1 given below. All experiments can be done within one day, where the timetable provides for extra time for a lecture or discussion during the incubation time of Experiment I (Transcription Step). Additional time may be required at the end of the course if the students have to prepare a report on their classes.

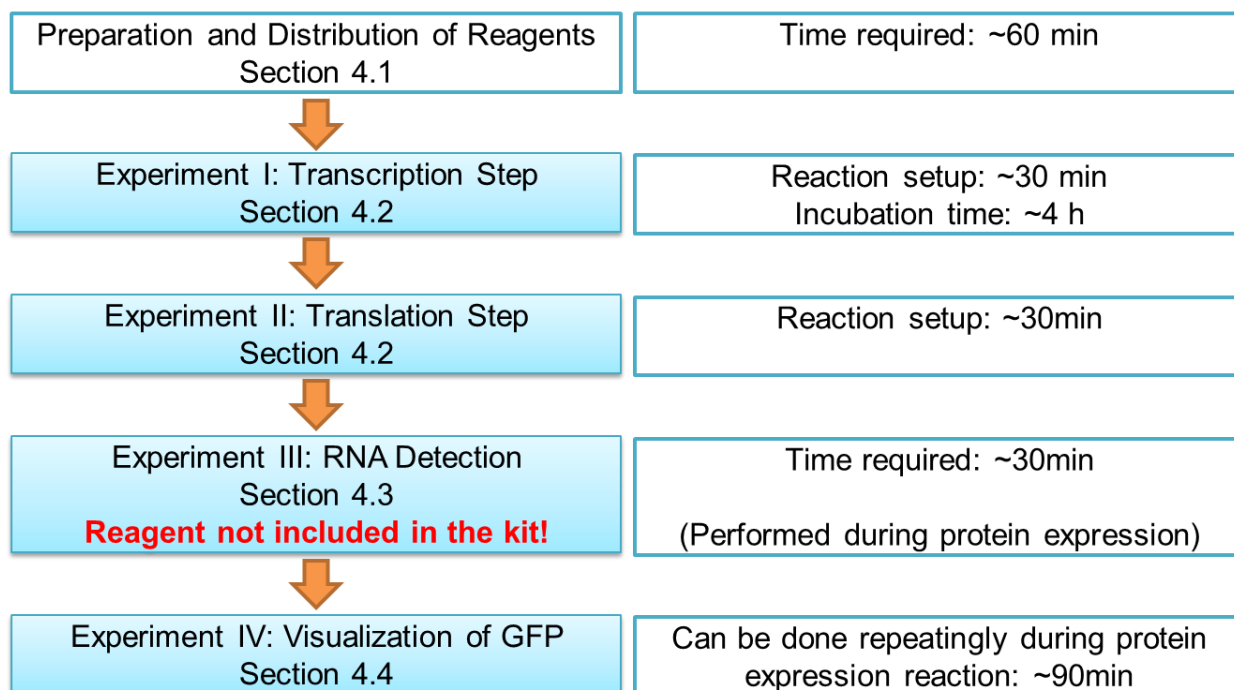


Figure 1: Outline of the “Cell-Free Protein Synthesis Course”

3. REAGENTS AND MATERIALS

3.1 Content of “Protein Expression Reagent Kit”

Reagents provided with the kit:

Reagent name	Figure	Qty	Volume	Description	Experiment No.
① Distilled water	Microtube	1	1,200 µL	To dilute reagents	I to III
② Transcription buffer	Microtube	1	200 µL	To use in transcription	I
③ Ribonucleotides	Microtube	1	100 µL	Substrate for mRNA synthesis	I
④ 10mg/mL RNase	Microtube	1	35 µL	Enzyme to degrade RNA	III
⑤ 10,000 unit/mL RNase inhibitor	Microtube	1	68 µL	Inhibitor of RNase Activity	I
⑥ 10,000 unit/mL RNA Polymerase	Microtube	1	99 µL	Enzyme for synthesizing RNA	I
⑦ 1 mg/mL Plasmid DNA	Microtube	1	66 µL	Template DNA with GFP gene	I

⑧ Fluorescent detection reagent Not including in Kit*	Microtube	1	1,050 μ L	To visualize RNA	III
⑨ Wheat germ extract	Microtube	1	330 μ L	Lysate for synthesizing protein from mRNA	II
⑩ Amino acids	Microtube	5	1,050 μ L	Substrate for protein synthesis	II

All reagents can be clearly identified by the corresponding number given in the table above and on top of each microtube.


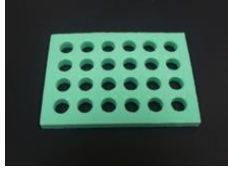





* Please note that CellFree Sciences did not obtain the resale rights for sales of the Quant-iT™ RiboGreen® RNA Reagent outside of Japan. Therefore, please purchase the Quant-iT™ RiboGreen® RNA Reagent (Catalog number: R11491) locally from Invitrogen. Note, the Quant-iT™ RiboGreen® RNA Reagent from Invitrogen must be diluted by 1 to 1000 using water before making the aliquots for the students. Do not use the original solution provided by the maker for the experiment.

Store all reagents upon arrival at -80°C until use in the teaching class. Keep reagents on ice at all times when preparing the class. We recommend to use all reagents once they have been thawed. Avoid repeated freeze/thawing of the wheat germ extract! The wheat germ extract is unstable when stored at higher temperatures and may lose some of its protein synthesis activity.

3.2 Content of “Laboratory Instrument Set”

(Sold separately from Protein Expression Reagent Kit.)

Item	Quantity	Description		Ex. No.
Tube rack	11	To keep tubes and vial with reagents		I to III
Floater	2	Using water-bath as incubator, to float microtubes with reagent on the water		I
Orange filter	11	Confirmation of RNA expression		III
Blue LED light	11	Confirmation of RNA expression		III
UV – LED light	11	To confirm fluorescence if GFP was synthesized		IV




The parts of the Laboratory Instrument Kit can be reused for multiple classes.

3.3 Other instruments required for conducting experiment

Item	Quantity	Description	Ex. No.
Micropipettes	10 units	To prepare the reagents. Both 200 μ L scale and 20 μ L scale type are needed.	I to III
Incubator	1 unit	To incubate reactions of Experiment I at 37°C.	I
Ice buckets	10 units	To keep reagents and to setup reactions.	I to III

These items are not included in the kits.

3.4 Other consumables required for conducting experiment

Item	Quantity	Description	Ex. No.
Microtube (1.5 mL)	170		I to III
Tip (for 2-200 μ L)	4 x 96 tips boxes		I to III
Vial (clear transparent tubes to see light reaction)	22		II

These items are not included in the kits.

4. PROTOCOL

For your safety:

Do not drink or eat in the laboratory. Wearing lab coat is desirable. Wash hands before and after doing an experiment. If you have reagent in your eyes or reagent is attached to your skin, wash eyes or skin immediately with water although this kit does not contain any hazardous reagents.

Read this manual carefully before starting the experiment. Contact CellFree Sciences for further support and advice if you have any questions on the experiments and materials provided with the kits.

4.1 Preparation of reagents and distribution to groups

After thawing the reagents, mix the reagents in the tube by pipetting slowly up and down. Take special care for pipetting especially reagents ⑤, ⑥ and ⑨ because of their high viscosity. Before dispensing reagents, write in advance the corresponding number on each empty microtube or vial before adding the reagent. After the dispensing, the tubes and vials should be stored on ice or in a refrigerator until use.

Follow the directions in the table below to aliquot the reagents and provide the indicated aliquots to each group or for two groups to share. You may use the tube racks from the Laboratory Instrument Set to provide all necessary reagents for each group in a tube rack.

These steps should be conducted by the teaching staff before the course starts.

Reagents	Dispensing
① Distilled water	Dispense 100 μL to 10 microtubes each, and distribute 1 microtube per 1 group.
② Transcription buffer	Dispense 15 μL to 10 microtubes each, and distribute 1 microtube per 1 group.
③ Ribonucleotides	Dispense 15 μL to 5 microtubes each, and distribute 1 microtube per 2 groups.
④ 10mg/mL RNase	Dispense 5 μL to 5 microtubes each, and distribute 1 microtube per 2 groups.
⑤ RNase inhibitor (10,000 unit/mL)	Dispense 10 μL to 5 microtubes each, and distribute 1 microtube per 2 groups.
⑥ RNA polymerase (10,000 unit/mL)	Dispense 15 μL to 5 microtubes each, and distribute 1 microtube per 2 groups.
⑦ Plasmid DNA (1 mg/mL)	Dispense 10 μL to 5 microtubes each, and distribute 1 microtube per 2 groups.
⑧ Fluorescent detection reagent Not including in Kit*	Dispense 198 μL to 30 microtubes each, and distribute 3 microtubes per group.
⑨ Wheat germ extract	Dispense 25 μL to 10 microtubes each, and distribute 1 microtube per 1 group.
⑩ Amino acids	Dispense 200 μL to 20 vials each, and distribute 2 vials per 1 group.

4.2 Experiment I: Transcription Step

Each group of two students should perform the following experiments together. During Experiment I, an mRNA transcript is prepared from a DNA template containing the GFP gene using an RNA polymerase. The reaction requires ribonucleotides for building the RNA molecules. To avoid degradation of the synthesized RNA, an inhibitor of RNA-degrading enzymes (so-called RNases) is added to the reaction. This RNase inhibitor is not required for RNA synthesis, but is a precaution to assure the success of the experiment.

For the experiment, three RNA synthesis reactions are conducted: In experiment "A" the DNA

template is added, whereas in experiment “B” the DNA template is omitted. Accordingly, experiment “A” should in the end yield the active GFP protein, while experiment “B” is a negative control where no GFP protein should be detected. Tube “C” contains in addition an RNase that will destroy the RNA prepared during the transcription experiment. Therefore tube “C” should not contain any detectable RNA in Experiment III, when we will confirm the RNA synthesis. - Each student may set up at least one reaction.

Setup experiment:

- 1) Prepare 3 empty microtubes and label the first tube [A], the second tube [B], and the third tube [C].
- 2) Place tubes on ice.
- 3) Setup the three reactions by adding the reagents in the order indicated in the table below.
- 4) Setup reaction [C] last after you have completed pipetting reactions [A] and [B].
- 5) Pipette reagents slowly, avoid air bubbles, mix by pipetting up and down.
- 6) Use a new pipette tip for each pipetting step – do NOT use the same pipette tip twice; do NOT use the same pipette tip to pipette different reagents.
- 7) Open the tube with the RNase last only after the other reaction setups are completed.

Reagents	Tube A	Tube B	Tube C
① Distilled water	8 μ L	10 μ L	8 μ L
② Transcription buffer	4 μ L	4 μ L	4 μ L
③ Ribonucleotides	2 μ L	2 μ L	2 μ L
④ 10mg/mL RNase	0 μ L	0 μ L	2 μ L
⑤ RNase inhibitor (10,000 unit/mL)	2 μ L	2 μ L	0 μ L
⑥ RNA polymerase (10,000 unit/mL)	2 μ L	2 μ L	2 μ L
⑦ Plasmid DNA (1 mg/mL)	2 μ L	0 μ L	2 μ L
Total	20 μ L	20 μ L	20 μ L

Operate micropipette slowly. Please pipette gently dispensing and mixing the reagents. 2 μ L solution for setting up reactions A to C is a very small volume.

Dispense and mix the reagents by pipetting slowly without making bubbles; take special care for reagents ⑤ and ⑥ because of their high viscosity and small volumes.

- 8) Incubate reactions for 4 h at 37°C in an incubator or water bath. The RNA expression reactions may be maintained overnight. Place reaction tubes on ice once the RNA expression reaction is completed.

The Laboratory Instrument Kit provides floaters in case a water bath is used for the incubations. Students should make sure to mark their own tubes before they are incubated together with tubes from other groups in the same water bath or incubator.

4.2 Experiment II: Translation Step

During the RNA expression step three independent experiments were conducted by each group. Keep experiments "A" and "B" separate as each student will keep on working on his/her sample in the following Experiment II. Tube "C" is not required for this experiment and will only be used as a control in Experiment III, the RNA detection step. Keep tube "C" aside while setting up the protein expression reactions.

In Experiment II, the RNA obtained from Experiment I will be used as a template for protein synthesis during the translation step. Note, that an aliquot of each RNA expression reaction will be needed for RNA detection in Experiment III. – Do not use the entire RNA expression reaction in the protein synthesis reaction.

The protein expression reaction requires ribosomes and multiple other factors contained in the wheat germ extract. In addition, amino acids that function as the building blocks for

protein synthesis, have to be added to the reactions. Protein expression reactions are preferably setup in a so-called “bilayer” reaction that nicely demonstrates how protein expression depends on reagent supply. Alternatively, the protein expression reactions may be set up in batch by mixing all reagents. Batch reactions may start faster than bilayer reactions, but provide overall lower protein yields. In the following we describe only the setup of the bilayer reaction for conducting the protein expression reaction.

Setup experiment:

- 9) Prepare 2 empty microtubes and label the first tube [A'] and the second tube [B'].
- 10) Place tubes on ice.
- 11) Add 10 μ L of reagent ⑨ (wheat germ extract) into the tubes labeled [A'] and [B'].
- 12) Add 10 μ L of the reactions [A] from Experiment I into the newly labeled [A'] and mix by pipetting slowly up and down.
- 13) Add 10 μ L of the reactions [B] from Experiment I into the newly labeled [B'] and mix by pipetting slowly up and down.
- 14) Prepare 2 empty vials (not microtubes) and label the first tube [A''] and the second tube [B'']. Those vials should already contain 200 μ L of the amino acids solution (reagent ⑩).
- 15) Carefully transfer the whole mixture (20 μ L) from the tubes labeled [A'] to the bottom of the vial labeled [A''] and [B'] to the bottom of the vial labeled [B''], respectively, to form the bilayer reaction. See the instructions in Figure 2 on how to place the wheat germ extract having a higher density below the amino acid solution. Because of the higher density of the wheat germ extract compared to the amino acid solution, the wheat germ extract with the RNA will “naturally” sink to the bottom of the vial. **DO NOT MIX THE SOLUTION IN THE VIAL BY PIPETTING OR ANY OTHER MEANS!**
- 16) Close the lid of the vials without disturbing the bilayer and incubate them at room temperature for 1 to 2 hours. An overnight incubation is possible. Make sure the vials

are standing safely and do not fall or tilt.

Do not touch with the end of the pipette tip the bottom of the vial when discharging the solution. Discharge the solution slowly by spending approximately 10 seconds. It naturally makes a bilayer. After discharging, remove the pipet tip slowly without mixing the solution in the vial.

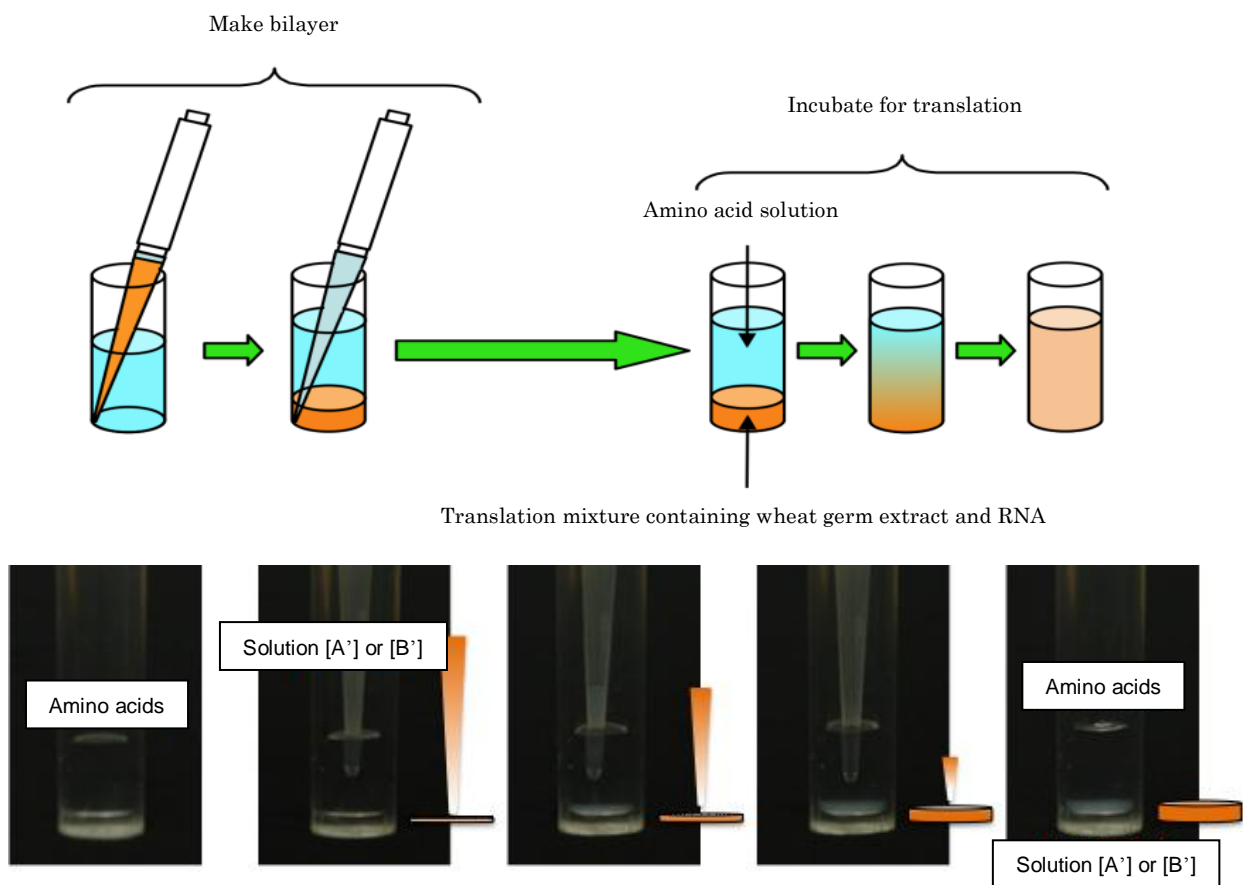


Figure 2: Setup of bilayer reaction

4.3 Experiment III: RNA Detection

While the protein synthesis reactions run, you have time to confirm that in Experiment I indeed RNA had been prepared. The RNA is detected by a special dye that binds specifically to RNA and emits a strong fluorescent light upon RNA binding. The dye does not bind to DNA

and proteins, and therefore the RNA can be detected directly in the reaction mixture from Experiment I without interference of the template DNA.

Setup experiment:

- 17) Prepare 3 microtubes containing the RNA-specific dye and label the first tube [A'''], the second tube [B'''], and the third tube [C'''].
- 18) Each tube should already contain 198 μL RNA fluorescent reagent (8).
- 19) Add 2 μL of the reactions [A] from Experiment I into the newly labeled [A'''] and mix well by pipetting slowly up and down.
- 20) Add 2 μL of the reactions [B] from Experiment I into the newly labeled [B'''] and mix well by pipetting slowly up and down.
- 21) Add 2 μL of the reactions [C] from Experiment I into the newly labeled [C'''] and mix well by pipetting slowly up and down.
- 22) In a dark surrounding, use light from the blue LED lamp provided with the Laboratory Instrument Set to illuminate the tubes A''', B''', and C'''. Use the orange filter to observe the emitted fluorescent signal.
- 23) Note in which tubes you detected the RNA and in which ones you did not detect RNA.

Operate micropipette slowly. Please pipette gently dispensing and mixing the reagents. 2 μL reaction solution from reactions A to C is a very small volume.

4.4 Experiment IV: Visualization of GFP Protein

As indicated above, GFP is very easy to detect because it exhibiting bright green light upon exposure to blue or ultraviolet light. Use the white UV – LED lamp provided with the Laboratory Instrument Set to induce the fluorescence of GFP and observe the green light. Do not disturb the bilayer when checking the protein expression.

Check your two reactions from time to time to see the progress of the protein synthesis. There should be no protein synthesized in vial [B’] (the negative control), because no DNA template had been added in Experiment I (RNA synthesis). If the bilayer reaction had been setup correctly, you will see over time how division between the two layers moves the reaction forward.

Refer to Figure 3 for an example on how the bilayer progresses.

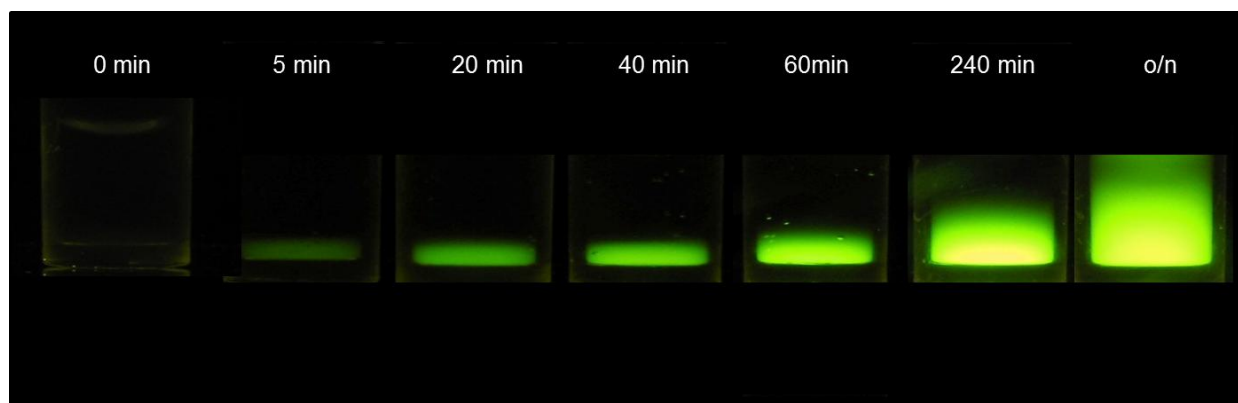


Figure 3: Progress of bilayer reaction over time

4.5 Confirmation of experimental Data

If your group has conducted all experiments correctly, you should have obtained the following results:

Step	Tube A	Tube B	Tube C
Experiment III: RNA Detection	Yes	No	No
Experiment IV: Protein Detection	Yes	No	-

Successful protein synthesis requires an intact DNA template to prepare the RNA transcript, and an intact RNA to synthesize the protein.

5. TROUBLESHOOTING

The experiments require correct and accurate pipetting during reaction setup. Any mistakes in the volumes added to the reactions, or even mixing the reagents, or forgetting any of the reagents will lead to wrong results. Therefore carefully check the number on each reagent and reaction tube prior to starting the pipetting step.

Mark in your protocol each pipetting step you have completed.

Change the pipetting tip after each pipetting step. Do not use the same pipetting tip to pipette different reagents or reaction mixtures.

Leaving out the plasmid template (⑦) will always yield negative results.

Be particularly careful when using the RNase (④)! The RNase will destroy your RNA template and no protein synthesis will occur. This applies also to reaction mixture C containing RNase. Do not spill any RNase and reaction mixture C.

6. REFERENCES AND FURTHER READING

Cell-free protein expression is an important method and commonly used in protein and molecular biology research. Since cell-free protein expression does not require any manipulation of a living cell, no other method allows to prepare more quickly protein from a DNA template for a first analysis of a cloned cDNA or to test protein expression. While the proteins prepared by any expression system (including cell-free methods) always require further analysis on whether or not they are fully functional, cell-free protein expression systems offer additional advantages over *in vivo* protein expression, because of the open nature of the reactions happening just in a reaction tube. Hence proteins can be prepared without being concerned about enzymatic activities within cells that may modify or even truncate the expressed protein. In addition, cell-free systems allow the preparation of proteins that are toxic to living cells. Moreover, the open nature of cell-free reactions offers many more options to modify the conditions of the expression reactions including the addition unnatural components to prepare for example labeled proteins. It is envisioned that cell-free protein expression systems will play an important role in future developments in synthetic biology, where unnatural amino acids are used to design and prepare proteins having entirely new features and functions.

Selected references on the wheat germ cell-free protein expression system used for Cell-Free Protein Synthesis Course:

Wheat germ systems for cell-free protein expression.

Harbers M.

Abstract

Cell-free protein expression plays an important role in biochemical research. However, only recent developments led to new methods to rapidly synthesize preparative amounts of protein that make cell-free protein expression an attractive alternative to cell-based methods.

In particular the wheat germ system provides the highest translation efficiency among eukaryotic cell-free protein expression approaches and has a very high success rate for the expression of soluble proteins of good quality. As an open in vitro method, the wheat germ system is a preferable choice for many applications in protein research including options for protein labeling and the expression of difficult-to-express proteins like membrane proteins and multiple protein complexes. Here I describe wheat germ cell-free protein expression systems and give examples how they have been used in genome-wide expression studies, preparation of labeled proteins for structural genomics and protein mass spectroscopy, automated protein synthesis, and screening of enzymatic activities. Future directions for the use of cell-free expression methods are discussed.

FEBS Lett. 2014 Aug 25;588(17):2762-73. doi: 10.1016/j.febslet.2014.05.061. Epub 2014 Jun 12.

PMID: 24931374

Wheat germ cell-free protein production system for post-genomic research.

Madono M, Sawasaki T, Morishita R, Endo Y.

Abstract

Genomic information becomes useful knowledge only when the structures and functions of gene products are understood. In spite of a vast array of analytical tools developed for biological studies in recent years, producing proteins at will is still a bottleneck in post-genomic studies. The cell-free protein production system we developed using wheat embryos has enabled us to produce high quality proteins for genome-wide functional and structural analyses and at the same time circumvent almost all the limitations, such as biohazards and costs, that have hampered conventional cell-free protein synthesis systems. In the present article, we introduce examples of our new wheat germ cell-free protein production system and its application to functional and structural analyses, with the focus on the former.

N Biotechnol. 2011 Apr 30;28(3):211-7. doi: 10.1016/j.nbt.2010.08.009. Epub 2010 Aug 26.

PMID: 20800705

The cell-free protein synthesis system from wheat germ.

Takai K, Endo Y.

Abstract

The wheat-germ cell-free protein synthesis system had been one of the most efficient eukaryotic cell-free systems since it was first developed in 1964. However, radio-labeled amino acids had long been essential for detection of the products. Since the discovery of a method for prevention of the contamination by a protein synthesis inhibitor originated from endosperm, the wheat cell-free system has found a wide variety of applications in postgenomic high-throughput screening, structural biology, medicine, and so on. In this chapter, we describe a method for preparation of the cell-free extract and a standard protein

synthesis method, as the methods for the applications are found in later chapters.

Methods Mol Biol. 2010;607:23-30. doi: 10.1007/978-1-60327-331-2_3.

PMID: 20204845

Practical cell-free protein synthesis system using purified wheat embryos.

Takai K, Sawasaki T, Endo Y.

Abstract

Biochemical characterization of each gene product encoded in the genome is essential to understand how cells are regulated. The bottleneck has been and still is in how the gene products can be obtained. The wheat cell-free protein synthesis system we have developed is a powerful method for preparation of many different proteins at a time and also for preparation of large amounts of specific proteins for biochemical and structural analyses. Here, we show a method for preparation of the wheat embryo extract useful for the cell-free reactions, by which 5 ml of a high-activity extract is obtained in 4-5 d. We also describe the methods for small- and large-scale protein synthesis by hands-down operations with the use of mRNAs prepared by transcription of PCR products and pEU plasmids harboring the target cDNAs, which need 2-4 d excepting the time required for plasmid preparation.

Nat Protoc. 2010 Feb;5(2):227-38. doi: 10.1038/nprot.2009.207. Epub 2010 Jan 21.

PMID: 20134421

7. LABEL LICENSE POLICY

By opening the cap of any of the reagents listed in the above Section 3.1, the buyer of this kit is agreeing to be bound by the terms of the following Label License Policy.

<< Label License Policy>>

This product is covered by US Patent Nos. 6905843, 6869774 and 7919597, and other pending or equivalent patents.

The purchase of the products conveys to the buyer the non-transferable right to use the purchased products and components of the products in education conducted by the buyer. The buyer cannot sell or otherwise transfer (a) the products, (b) their components or (c) materials made using the products or their components to a third party or otherwise use the products or their components or materials made using the products or their components for commercial purposes.

Quant-iT™ and RiboGreen® are registered product names owned by LifeTechnologies.

This product is for teaching purpose only and shall not be used for any medical application.

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