Instructions For Use

Syn-Xpress™ Kits

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Instruction Manual 20250925V02

Syn-Xpress™ Kits: Cell-Free Protein Synthesis

Table of contents

This manual applied to the following references:

Syn-Xpress™ Silver kit	SYN90021-A5-01	SYN90021-B1-01	SYN90021-B5-01	SYN90021-P1-01
Syn-Xpress™ Silver Mastermix	5 x 50µL reaction	1 x 1mL reaction	5 x 1mL reaction	96 x 50µL reaction
SynGFP positive control plasmid	1 tube	1 tube	1 tube	1 tube

Syn-Xpress™ Gold VHH kit	SYN90022-A5-01	SYN90022-B1-01	SYN90022-B5-01	SYN90022-P1-01
Syn-Xpress™ Gold VHH Mastermix	5 x 50µL reaction	1 x 1mL reaction	5 x 1mL reaction	96 x 50µL reaction
SynGFP positive control plasmid	1 tube	1 tube	1 tube	1 tube

It will provide step-by-step instructions to maximize the efficiency and versatility of the kit, ensuring reliable results and ease of use in your protein expression experiments.

I. Intended use

Syn-Xpress™ SILVER and GOLD VHH kits are Cell-Free Protein Synthesis (CFPS) kits intended for Research Use Only (RUO) products and should be used exclusively by personnel trained in research laboratory techniques.
Our cell-free expression system based on *E. coli* machinery offers several advantages over traditional cell-based methods. It enables the quick and efficient synthesis of proteins, often within hours, without the complexities associated with cell cultivation, transformation, or the need for cell lysis, offering a streamlined approach for researchers in various fields, including biotechnology, pharmaceuticals, and basic molecular biology. Additionally, this system allows precise control over reaction conditions, making it ideal to produce toxic proteins, membrane proteins, or proteins with complex folding or requiring co-factors that are difficult to express in living cells.

- **Syn-Xpress™ SILVER** is designed to facilitate the **easy and rapid production** of easy to produce recombinant proteins.

- **Syn-Xpress™ GOLD VHH** kit is designed to streamline the production of single-domain antibodies (VHH/nanobodies) and minibinders that require disulfide bond for structural integrity. It is the perfect choice for high-throughput screening and selection of large library of VHH/minbinder variants.

II. Operating principles

The kits contain a pre-assembled reaction mix that includes lysate derived from *E. coli*, as well as essential additives required for protein expression including T7 RNA polymerase for protein expression under T7 promoter (*Figure 1*).

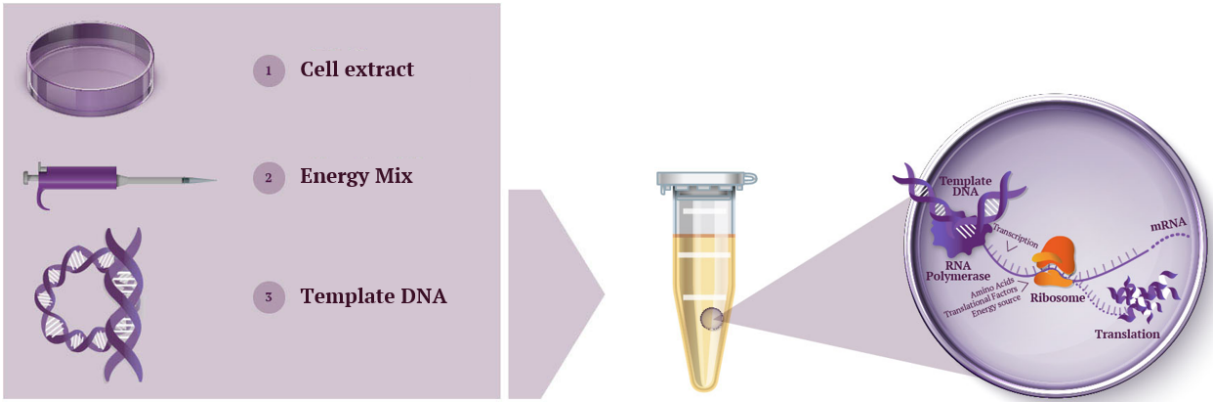

*Figure 1 : principle of **Syn-Xpress™** Cell-Free Protein Synthesis system*

Syn-Xpress™ kits are conceived to work with both circular and linear DNA templates, offering flexibility for a variety of applications. The lysate used in the kits is optimized to efficiently handle both types of DNA ensuring high-level protein expression regardless of the template form. For specific guidelines on using linear DNA, please refer to the details in section III of this manual.

The CFPS system is assembled by adding the DNA template into the pre-assembled reaction mix. Depending on the specific requirements of the protein being expressed, additional additives can be incorporated at this stage. Once the DNA template and any additional additives are added, the reaction is softly mixed to ensure uniform distribution of components.
The assembly is then incubated at an optimal temperature, typically between 18–37°C, allowing both transcription and translation to proceed simultaneously. The reaction typically takes between 2 and 16 hours, depending on the protein's size and complexity, during which time the gene is transcribed by T7 RNA polymerase and translated by the ribosomes present in the lysate.

Note that the protein yield will depend on the DNA quality, the DNA elements, the additional additives and the incubation time. Furthermore, handling the reagents on ice is crucial to preserve their stability and prevent degradation, ensuring optimal results in the experiment.

III. Guidelines for design and preparation of DNA template material

When preparing the DNA template for CFPS reaction, it is important to consider several factors to optimize protein expression and avoid potential issues.

1. Choice of DNA elements

Selecting the appropriate DNA elements is critical for successful CFPS. The principles are the same as for *in cellulo* expression. However, it is not necessary for the genes encoding the protein to be under the control of the lac operon.

The following key DNA components should be carefully considered to optimize expression and ensure efficient protein production:

- **Promoter sequence**: the kits are optimized for use with a T7 promoter, which drives high-level transcription in cell-free systems. The T7 RNA polymerase included in the kit will initiate transcription from this promoter. Ensure that the gene of interest is placed downstream of the T7 promoter.

  Nevertheless, native E. coli promoter can also be used. If you consider using other promoters, please consider customizing the reaction conditions with the addition of the required polymerase.
  For optimal transcription efficiency, the promoter should be placed 20 to 100 bases upstream of the start codon.

- **Terminator sequence**: a strong transcriptional terminator, placed downstream of the gene of interest, will ensure proper termination of transcription and prevent read-through into other regions of the DNA template. If you are using the T7 system, it is recommended to incorporate a T7 terminator downstream of your gene of interest. However, if you choose to use a different RNA polymerase system, it is important to use a corresponding terminator that is compatible with that polymerase.

  For optimal transcription efficiency, the terminator should be placed downstream of the stop codon.

- **Ribosome Binding Site (RBS)**: a functional RBS is essential for efficient translation initiation in prokaryotic systems, such as our CFPS. The strength of the RBS directly influences translation efficiency. A strong RBS typically results in higher translation rates.

  In prokaryotic systems, the start codon (AUG) is preceded by the Shine-Dalgarno (SD) sequence, a purine-rich motif (5'-AGGAGG-3'). This SD sequence binds to the small subunit of the ribosome, promoting the initiation of translation.

  For optimal translation efficiency, the SD sequence should be placed 6–10 bases upstream of the start codon.

- **Gene of Interest**: ensure the gene of interest is in the correct reading frame and is free from any sequence errors that could impair translation. Additionally, consider codon optimization for *E. coli* expression to improve translation efficiency, increase protein yield, and help avoid issues related to rare codon usage.

- **Tags and Fusion Proteins**: are commonly used in CFPS to facilitate protein purification, detection and characterization. Adding a tag to the target protein can improve solubility, increase stability and simplify downstream applications. The position of the tag should be chosen based on the protein's structure and function. Adding a tag to the N- or C-terminus may affect the protein's folding or activity, so it's important to test different configurations when necessary.

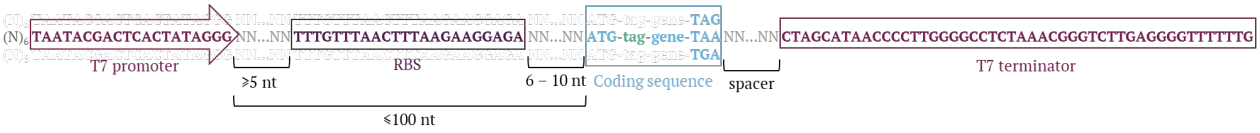

*Figure 2 : scheme of DNA template for **Syn-Xpress™** Cell-Free Protein Synthesis reaction*

In cell-free protein synthesis (CFPS), the choice between circular and linear DNA can significantly impact the efficiency and outcome of the translation process. Both types of DNA are utilized in CFPS systems, with each offering distinct advantages depending on the desired application and system setup.

2. Circular DNA

Plasmid vectors are widely used in cell-free systems due to ease of handling and their stability. They are simple to manipulate and amplify in large quantities within a few days. Their stability ensures consistent performance, leading to reliable and efficient protein synthesis.
Circular DNA is often preferred as it typically yields higher protein production compared to linear DNA, due to its greater resistance to degradation by nucleases.

- **Vector Choice**: it is essential to choose a vector that is compatible with the cell-free reaction conditions and contains the T7 promoter for protein expression.

  For optimal performance, it is recommended to use plasmids that are specifically optimized for CFPS reactions, such as the pIVEX vector, which is designed to work well *in vitro*, providing high yield and stability for protein production.
  However, plasmids that are commonly used for *in-cellulo* protein expression in *E. coli*, like the pET series or pQE-T7, can also be utilized in **Syn-Xpress™** kits. However, it is not necessary for the genes encoding the protein to be under the control of the lac operon.

- **Vector preparation**: proper preparation of the DNA template is crucial for ensuring the success of the CFPS reaction. Detailed instructions can be found in appendix (section IX-1 of this manual).

3. Linear DNA

Linear DNA, such as synthetic DNA or PCR-amplified fragments, can be used effectively in cell-free protein synthesis (CFPS) systems, offering distinct advantages over plasmid DNA. One key benefit is the faster setup, as there is no need for bacterial transformation and plasmid extraction. Additionally, linear DNA is often simpler to engineer, allowing for easy incorporation of modifications, such as tags or mutations, directly into the gene of interest. However, linear DNA can be less stable than plasmid DNA and may be more prone to degradation by nucleases, which can lead to lower yields if not handled properly. While it may offer quicker results and greater flexibility, careful consideration of DNA quality and concentration is critical for ensuring efficient transcription and translation in CFPS.

- **Linear DNA design**: To prepare linear DNA for CFPS, it is important to add nucleotides that protect the DNA extremities. It can be linear extra-nucleotides or stem loop forming sequence of nucleotides These structures help protect both the DNA and the resulting mRNA from exonuclease degradation, ensuring stability during the transcription and translation processes in the CFPS system.

  Table 1 provides an example of primers that can be used to achieve this.

Table 1: suggested extremities for linear DNA synthesis
In turquoise = T7 promoter | turquoise underline = T7 terminator| purple = 5' UTR containing RBS | pink = stem loop

Primer Name	Primer Sequence	Nucleotides
Protect FOR	CGAAACAAGCGCTCATGAGCCCGAAGTGGCGAGCCCGA TCTTCCCCATCGGTGATGTCGGCGATATAGGCGCCAGCA ACCGCACCTGTGGCGCCGGTGATGCCGGCCACGATGCGT CCGGCGTAGAGGATCGAGATCTCGATCCCGCGAAATTAA TACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTA GAAATAATTTTGTTTAACTTTAAGAAGGAGATATACC	231
Protect REV	CTCGAGCGAGCTCTGCAGCCCGGGATCCGGTAACTAACT AAGATCCGGTAAGATCCGGCTGCTAACAAAGCCCGAAA GGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACT AGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGG TTTTTTGCTGAAAGGAGGAACTATATCCGGATATCCACA GGACGGGTGTGGTCGCCATGATCGCGTAGTCGATAGTGG CTCCAAGTAGCGAAGCGAGCAGGACTGGGCGGCGGCCA AAGCGGTCGGACAGTGCTCCGAGAACGGGTGCGCATAG	309
Stem loop FOR	TCGCGGGCCGGCCACGGGTCTTGAGGCCGGCCCGCGAAA TTAATACGACTCACTATAGGGAGACCACAACGGTTTCCC TCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATA CC	119
Stem loop REV	CTCGAGCGAGCTCTGCAGCCCGGGATCCGGTAACTAACT AAGATCCGGTAAGATCCGGCTGCTAACAAAGCCCGAAA GGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACT AGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGG TTTTTTGCTGAAAGGAGGCCGGCCGGCCTCAAGACCCGT TGGCCGGCCGGCCT	208

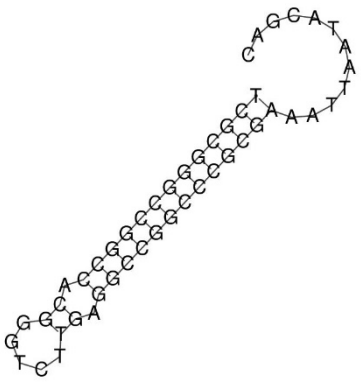

Figure 3: Predicted secondary structure of Stem loop FOR primer

- **Linear DNA preparation**: For CFPS applications, it is crucial to prepare the linear DNA properly, whether you are using synthetic DNA or generating it via PCR. Detailed instructions can be found in appendix (section IX-1 of this manual).

IV. Materials and Methods

The CFPS system uses a reaction mix composed of a cell lysate, key additives to support in vitro transcription and translation, and a DNA template (Figure 1). After assembling and incubating the CFPS reaction, the expressed protein can be analyzed using traditional techniques such as SDS-PAGE and Western blotting, or more rapid methods like bioluminescence assays using a specific tag. The following materials and methods section will provide detailed instructions for achieving successful protein expression using the **Syn-Xpress™** kit.

1. List of Equipment and Reagents

To carry out cell-free reactions, users must provide additional equipment and reagents. Please ensure that all required materials are prepared and available before starting the experiment.

a. Reagents & Kits

❒ Syn-Xpress™ kit
❒ DNA template (circular or linear DNA)
❒ Nuclease-free water

Optional (for protein analysis):

❒ SDS-PAGE kit (gel, Laemmli buffer containing reducing agent, migration system, coloration system and imaging system)
❒ Western blotting kit (membrane, transfer system, antibodies, detection reagents and imaging system)

b. Consumables & Laboratory Equipment

- ❒ Nuclease-free 96 wells plate or microcentrifuge tubes
- ❒ Micropipettes and nuclease free pipette tips
- ❒ Thermomixer or thermal incubator with shaker adjustable to 18-37°C.

Please note that shaking allows better oxygenation of the cell-free reaction and thus, gives higher protein expression yields.

- ❒ Refrigerated centrifuge (for protein solubility assessment)
- ❒ Ice bucket (for keeping reagents cold as needed)

This list outlines the essential materials and equipment for successful protein expression and analysis using the CFPS system. Make sure to follow the specific instructions provided with each reagent and equipment for optimal results.

2. Protocol for CFPS reaction

a. Preparation of the CFPS reaction

Step a.1: Thawing the reagents

Start by thawing all reaction components (**Syn-Xpress™** kit, DNA template, and optional additives) on ice. Ensure that the cell lysate and any other components requiring cold storage are fully thawed before use.
Notes:
- do not vortex the Syn-Xpress™ mastermix
- if additives are required, prepare them using nuclease-free water.

Step a.2: Preheating the incubator

Preheat the incubator to the recommended temperature, typically 25°C, to ensure optimal protein expression during the CFPS reaction.
Note: for best protein yield, the temperature can be adjusted, typically between 18°C and 37°C, based on your specific protein requirements.

Step a.3: Preparing a nuclease-free workspace

Clean the bench surface and all equipment with RNase and DNase-free solutions to ensure the workspace is free from contaminants that could degrade nucleic acids. Always wear gloves to prevent contamination.

b. Assembly of the CFPS reaction on ice

- The **Syn-Xpress™ SILVER** kit is 1.25x concentrated. Each CFPS reaction consists of 80% **Syn-Xpress™ SILVER** reaction mixed by final volume, with the remaining 20% comprising DNA template, additional additives (optional) and nuclease-free water.
- The **Syn-Xpress™ GOLD VHH** kit is 1.1x concentrated. Each CFPS reaction consists of 90% **Syn-Xpress™ GOLD VHH** reaction mixed by final volume, with the remaining

10% comprising DNA template, additional additives (optional) and nuclease-free water.

For example, a 50 µL CFPS reaction is set up as described in Table 2:

Table 2: example of the assembly of 50µL of CFPS reaction

Reagents		Volume Syn-Xpress™ SILVER	Volume Syn-Xpress™ GOLD VHH	Finale Concentration
Syn-Xpress™ Mastermix		40 µL	45 µL	1 X
DNA template	plasmid	X µL	X µL	5 nM
	Linear			10 nM
Additives (optional)		X µL	X µL	
Nuclease-free water		X µL	X µL	
Total volume reaction		**50 µL**	**50 µL**	

Step b.1: Preparing the reaction mix

i. If using **pre-filled kit**, the 1.5 mL tubes or 96-wells plates already contain 40 µL of Syn-Xpress™ Silver mix or 45 µL of Syn-Xpress™ GOLD VHH mix.

- Gently resuspend the content by tapping the bottom of the tubes with your finger several times. For plates, shake slowly in a double orbital motion to ensure homogeneity. Do not vortex.
- Add any additional additives (optional). See section IV-3 below.

Note: avoid the formation of bubbles, as they may negatively impact the CFPS reaction.

ii. If using **bulk kit**: tubes containing the Syn-Xpress™ mix to be distributed into your vessel.

- Choose the appropriate reaction vessel and volume based on the recommendations in the table below to suit your needs.

Table 3: Recommended total CFPS reaction volumes

Vessel Type	Minimum Volume	Maximum Volume
1.5mL Tube	10 µL	150 µL
2mL Tube	10 µL	200 µL
5mL Tube	50 µL	500 µL
50mL Tube	250 µL	5 000 µL
96-wells Plate	50 µL	200 µL
384-wells Plate	15 µL	30 µL

- Gently resuspend the content by inverting the tube up and down several times to ensure homogeneity. Do not vortex.

- Fill your vessel with 80 %, of the final volume, with the 1.25X **Syn-Xpress™ SILVER** reaction or 90 %, of the final volume, with the 1.1X **Syn-Xpress™ GOLD VHH** reaction. The homogeneity of the mix is important. Therefore, ensure that you resuspend the mix frequently during aliquoting by pipetting up and down.
- Add any additional additives (optional). See section IV-3 below.

Note: Avoid the formation of bubbles, as they may negatively impact the CFPS reaction.

Step b.2: Addition of DNA template

Add 5nM (equivalent to 15 ng/µL of a 4kb vector) of plasmid DNA template or 10nM of linear DNA template to the reaction mix.
Note: we recommend to perform a positive control using our synGFP vector, to validate the CFPS efficiency and a negative control with nuclease-free water instead of DNA to evaluate the background protein level.
Bring the total reaction volume to 100 % with nuclease-free water.
Gently mix by pipetting up and down to ensure even distribution of all ingredients.

Step b.3: Sealing the vessel

After adding all the components to the reaction vessel, it is crucial to seal the vessel properly to prevent contamination and ensure the reaction proceeds in a controlled environment.

- For **tubes**: close the tubes while ensuring there is enough oxygen for the reaction. It is generally recommended to use tubes that are 10 to 20 times larger than the reaction volume to allow proper oxygenation.
- For **plates**: the reaction volumes can be larger, but make sure to use a breathable film that will provide adequate oxygenation. However, choose a film that also minimizes evaporation of the reaction.

Ensure that the seal is secure and there are no leaks, which could lead to evaporation or contamination during incubation.

c. Incubation of the CFPS reaction

Step c.1: Incubating the reaction

i. After assembling and sealing the CFPS reaction, transfer the tube or plate into the preheated incubator.

Note: if you are using a porous film that is not very airtight or if the incubation time is long, we recommend keeping the incubator slightly humid to reduce evaporation of the reaction and ensure uniformity between the wells when using plates.

ii. Incubate the CFPS reaction with agitation at 200 rpm.

- For screening purposes, we recommend a short incubation time ranging from 2 to 6 hours.
- For protein production, a longer incubation is advised, as our kit is designed to function for up to 16 hours in batch mode, allowing the reaction to proceed efficiently.

During this period, both transcription and translation will take place, leading to protein expression.

Step c.2: Monitoring of protein expression (optional)

If necessary, monitor the reaction using a reporter gene (such as GFP or luciferase) to track progress. For non-reporter proteins, proceed with post-incubation analysis using SDS-PAGE or Western blot.

d. Analysis of protein expression

After incubation time, recover your reaction and keep it on ice.

To analyze the solubility of your protein we recommend keeping a fraction of the total CFPS reaction on ice, then centrifuge the rest of the CFPS reaction at 20, 000g for 20 minutes at 4°C. This will separate the soluble proteins from the insoluble pellet. Carefully collect the supernatant (soluble fraction) and transfer it to a new, pre-chilled tube. The pellet (insoluble fraction) can also be stored for further analysis if needed.
The success of the reaction can be evaluated by SDS-PAGE and Western-Blot analysis. Detailed instructions can be found in appendix (section IX-2 of this manual).

3. Optimization of CFPS reaction

The yield of protein produced in a CFPS reaction is influenced by several key factors, including the nature of the target protein, the incubation temperature and time, the concentration of the DNA template, and the additives used in the reaction. Each of these elements plays a critical role in determining the efficiency of the translation process, protein folding, and overall yield. Fine-tuning these parameters ensures the highest possible yield and quality of the target protein.

a. Incubation: temperature and time

Temperature and incubation time are two key factors that significantly affect the efficiency of a CFPS reaction. The optimal temperature typically ranges from 18°C to 37°C, with each protein having specific requirements for ideal expression. Lower temperatures, around 18°C, are often chosen for proteins that are sensitive to heat, as they help reduce aggregation and promote proper folding. However, this may result in slower translation rates. Higher temperatures, such as 37°C, can accelerate translation, leading to faster protein production, but they may also increase the risk of protein misfolding or degradation for certain proteins, especially those that require precise structural formation.
In addition to temperature, the incubation time plays a crucial role in determining protein yield. The duration of the CFPS reaction should be optimized based on the desired protein expression level. Shorter incubation times (2-6 hours) may be sufficient for fast-expressing proteins, while longer incubations (up to 16 hours or more) may be needed for slower or more complex proteins. Prolonged incubation beyond the optimal time can result in the accumulation of unwanted by-products or the degradation of the target protein. By systematically adjusting both temperature and incubation time, it is possible to identify the ideal conditions that maximize protein yield while maintaining protein integrity.

b. DNA template concentration

The concentration of the DNA template, whether circular (plasmid) or linear, plays a key role in the efficiency of protein expression in CFPS reactions. Circular DNA templates are more stable and often require lower concentrations for optimal transcription and translation. Linear DNA, while offering higher initial transcription efficiency, may be more prone to degradation, which can limit protein yield over time. The optimal DNA concentration varies depending on the target protein. Typically, concentrations between 2 and 20 nM are used, but fine-tuning is necessary to achieve the best balance between transcription efficiency and protein yield. Adjusting DNA concentration helps maximize protein expression while avoiding excessive by-products or degradation.

c. Protein with complex structures

Proteins with complex structures, such as those requiring precise folding or disulfide bond formation, often need special conditions to achieve proper structure and functionality in CFPS reactions. The addition of molecular chaperones is critical for assisting the protein folding process and preventing misfolding or aggregation, especially for larger or more intricate proteins.

d. Membrane proteins

Membrane proteins, which have hydrophobic regions, present specific challenges in cell-free protein synthesis (CFPS) reactions. These proteins often require a tailored environment to ensure correct folding and insertion into lipid membranes. To optimize expression, it is crucial to include membrane-mimicking systems, such as detergent micelles or lipid nanoparticles, in the CFPS reaction. Detergents like DDM (n-dodecyl-β-D-maltoside) can solubilize membrane proteins, while lipid nanoparticles or bicelles can help mimic the natural membrane environment, promoting proper folding and stability.

Membrane proteins, due to their hydrophobic nature, require special conditions in CFPS reactions to ensure correct folding and integration into membrane-like environments. One effective approach is the use of detergents, liposomes, or nanodiscs, which help to mimic the native membrane environment and stabilize the hydrophobic regions of the protein. Detergents are commonly used to solubilize membrane proteins by surrounding the hydrophobic regions, preventing aggregation. Alternatively, liposomes, which are lipid bilayer vesicles, can be included in the CFPS mix to provide a more native-like environment for membrane protein folding and insertion. Nanodiscs, self-assembled lipid bilayers surrounded by amphipathic proteins, offer another solution, providing a stable platform for membrane proteins while mimicking the natural membrane structure.

Using these systems can enhance the expression and stability of membrane proteins in CFPS, making them suitable for downstream applications such as functional assays or structural studies.

e. Protein requiring specific additives

Certain proteins, especially those involved in enzymatic processes, need the addition of specific additives in CFPS reactions to ensure proper function and activity. These proteins often depend on cofactors and metal ions, which are essential for maintaining their structure and activity.

Enzymes that rely on cofactors, such as NAD+, FAD, or coenzyme A, require the supplementation of these molecules to achieve full functional capacity. These cofactors are involved in key biochemical reactions, including redox reactions and transfer of functional groups, which are

critical for the activity of certain enzymes. The addition of cofactors must be carefully optimized based on the target protein's needs to ensure efficient catalysis and proper protein functionality.
Metal ions also play a crucial role in the structure and function of many proteins, particularly metalloproteins. Ions such as zinc, copper, magnesium, or iron are often required to stabilize the protein structure and enable catalytic activity. The optimal concentration of these metal ions can vary depending on the protein, and they should be included in the CFPS reaction at the correct molar ratio to avoid both deficiency and toxicity.
By carefully optimizing the concentration of cofactors and metal ions in the CFPS system, it is possible to enhance protein expression and ensure that proteins requiring these elements are correctly folded, functional, and suitable for downstream applications.
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V. Storage conditions and stability

Syn-Xpress™ kits are shipped on dry ice to maintain their integrity during transit. **Upon receipt, it is essential to store the kits immediately at -80°C.** When stored under these conditions, the kits remain stable for up to 1 year months.
We recommend avoiding multiple freeze-thaw cycles to ensure optimal performance. However, if the entire reaction mix is not used on the same day, the product can withstand up to 3 cycles of thawing on ice and freezing at -80°C without significant loss of effectiveness.

VI. Product content

Syn-Xpress™ Silver kit	SYN90021-A5-01	SYN90021-B1-01	SYN90021-B5-01	SYN90021-C1-01
Syn-Xpress™ Silver Mastermix	5 x 50µL reaction	1 x 1mL reaction	5 x 1mL reaction	96 x 50µL reaction
SynGFP positive control plasmid	1 tube	1 tube	1 tube	1 tube

Syn-Xpress™ Gold VHH kit	SYN90022-A5-01	SYN90022-B1-01	SYN90022-B5-01	SYN90022-P1-01
Syn-Xpress™ Gold VHH Mastermix	5 x 50µL reaction	1 x 1mL reaction	5 x 1mL reaction	96 x 50µL reaction
SynGFP positive control plasmid	1 tube	1 tube	1 tube	1 tube

VII. Safety information

To be handled only by personnel trained in research laboratory techniques. Suitable protective laboratory equipment (lab coat, disposable gloves, and safety glasses) should be worn when handling **Syn-Xpress™** kit.

Syn-Xpress™ reagents may be harmful if swallowed, may cause skin irritation, may cause serious eye irritation, may cause respiratory irritation.

Disposal should be in accordance with applicable regional, national and local laws regulations.

VIII. Legal

Researchers may use this product for Research Use Only. No commercial use is allowed. Commercial use means any and all uses of this product by a party in exchange for consideration, including, but not limited to (i) use for further product manufacture, (ii) use in provision of services, (iii) resale of product. No other use or transfer of this product is authorized without the prior express consent of Synthelis Biotech.

IX. Appendices

1. Guidelines for preparation of DNA template material

a. Circular DNA preparation

Proper preparation of the DNA template is crucial for ensuring the success of the CFPS reaction. It should be free of nucleases (DNases and RNases) and inhibitors of the transcription-translation machinery (EDTA, SDS, ethidium bromide).
The quality and quantity of the DNA template directly impact the efficiency of transcription and translation, which in turn affects protein yield.
Below are the key steps and considerations for preparing the plasmid DNA template:

i. **Amplification of plasmid**: we recommend transforming the plasmid into DH5α *E. coli* (or equivalent) cells, which are commonly used for cloning due to their efficiency in plasmid uptake and stability. Select bacterial colonies on LB-agar plates containing the appropriate antibiotic for plasmid selection. After colony formation, pick a single colony and inoculate it into LB medium for growth.
ii. **Extraction of plasmid**: use a commercially available plasmid extraction kit (e.g., Midi- or Maxiprep) to isolate the plasmid DNA from the bacterial culture.
iii. **Purification of plasmid**: as DNA purity is a key parameter for CFPS, we recommend performing an additional purification step by precipitating the plasmid DNA with isopropanol or ethanol. The DNA must be resuspended in nuclease-free water. This step helps to remove residual contaminants and ensures that the DNA is concentrated and ready for use in CFPS.
iv. **Analysis of plasmid purity**: It is essential to assess the purity of the extracted plasmid DNA before use. This can be done by measuring the DNA concentration and purity using a spectrophotometer (A260/A280 ratio should be ~1.8–2.0). Additionally, running the DNA on an agarose gel electrophoresis can confirm the integrity and absence of degradation.

b. Linear DNA preparation

For CFPS applications, it is crucial to prepare the linear DNA properly, whether you are using synthetic DNA or generating it via PCR. Below are the steps for preparing linear DNA for use in the CFPS system:

i. **Synthetic DNA**: if you are using synthetic DNA, resuspend the lyophilized DNA in nuclease-free water. The final concentration of the DNA should typically be around 1µg/µL, but this can vary depending on the downstream CFPS reaction requirements. Ensure that the DNA is fully dissolved by gently pipetting up and down or incubating at room temperature for a few minutes. Store the resuspended DNA at -20°C or -80°C for long-term storage.

PCR DNA: perform the PCR amplification according to the manufacturer's protocol for your polymerase. Typically, this involves an initial denaturation step, followed by cycles of denaturation, annealing, and extension, finishing with a final elongation step.

ii. **Purification of linear DNA**: purifying the PCR product is not strictly necessary for CFPS, as primers, dNTPs, or DNA polymerase do not inhibit the reaction. However, if you are not using fluorimetric quantification for your DNA, purification is required for accurate quantification. The most common method is using a PCR cleanup kit, which binds the DNA to a column, removes contaminants, and elutes the purified DNA in nuclease-free water. We do not recommend gel extraction for purification, as traces of ethidium bromide may remain and interfere with the CFPS reaction, unless you need to isolate a specific PCR product and ensure the removal of non-specific fragments.

iii. **Analysis of DNA purity**: after purification, measure the DNA concentration and check purity using a spectrophotometer (A260/A280 ratio should be 1.8-2.0). Finally, run an aliquot on an agarose gel to confirm the correct size and integrity of the DNA. Proper purification ensures high-quality DNA that will perform well in CFPS, free from inhibitory contaminants.

2. SDS-PAGE and Western-blot analysis

Perform SDS-PAGE analysis to validate protein size and quantity.

i. Dilute the total and soluble fractions in Laemmli buffer containing a reducing agent. We recommend heating the sample for 5 minutes at 95°C, or follow the manufacturer's recommendations for sample preparation. This step ensures the denaturation of the proteins and the reduction of disulfide bonds, allowing for accurate size separation.
   - Note: carefully homogenize the cell-free reaction by pipetting up and down multiple times to ensure an even distribution of proteins before taking a sample. This is crucial for obtaining reliable and reproducible results.

ii. Load 0.5 µL of the CFPS reaction (or an appropriate volume depending on the protein concentration) onto the SDS-PAGE gel. Be sure to include both negative and positive controls on the same gel. The negative control will help assess any non-specific binding

or background protein levels, while the positive control will validate that the system is functioning properly.

iii. Run the gel following standard SDS-PAGE protocols, adjusting the voltage and time according to the gel's thickness and the molecular weight range of interest. Once the electrophoresis is complete, stain the gel with Coomassie Brilliant Blue to visualize the expressed protein bands. The Coomassie stain binds to proteins, allowing for visualization of the total protein content and the relative abundance of each protein band.

To specifically identify your target protein, perform a Western blot

iv. First, repeat the SDS-PAGE step with the same settings and transfer the separated proteins from the gel onto a nitrocellulose membrane using a standard transfer method (e.g., semi-dry or wet transfer). This step immobilizes the proteins on the membrane for further analysis.

v. Once the transfer is complete, block the membrane with an appropriate blocking buffer (such as 5% milk or BSA) to prevent non-specific binding of antibodies. Detect your protein of interest by incubating the membrane with the appropriate primary antibody, followed by a secondary antibody conjugated to an enzyme (such as HRP or alkaline phosphatase). The secondary antibody should be chosen based on the species of the primary antibody.

vi. Visualize the protein bands using chemiluminescence or colorimetric detection systems, depending on the secondary antibody used. The signal will allow you to identify your target protein and confirm its size.

This combination of SDS-PAGE and Western blotting offers a powerful method for verifying the expression, size, and identity of proteins produced in a CFPS system.

X. List of symbols used

Manufacturer's name and address

For Research Use Only

Temperature limits between which the product can be safely exposed

Consult the user guide

See accompanying documents