LenioBio®
Applied Nature for Better Science

ALiCE®
Protein Expression Made Easy

Instruction Manual for Cell-Free Protein Expression with ALiCE® for Research

Product numbers AL00000001 (ALiCE® for Research Mini Kit)
AL00000002 (ALiCE® for Research Midi Kit)
AL00000003 (ALiCE® for Research Maxi Kit)

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Disclaimer

We assume no liability for any direct or indirect damages or loss arising from the use, misuse, or results of use of this kit.

1 Introduction

Cell-free protein expression (CFPE) systems derived from crude cell extracts have been used for decades in research. LenioBio has leveraged plant cell biology to introduce a novel eukaryotic cell-free expression system called ALiCE®, enabling scientists and researchers to produce complex proteins requiring post-translational modifications (PTMs) such as, but not limited to, *N*-glycosylation and disulfide bonds. In a single reaction, ALiCE® overcomes the limitations imposed by cell-based systems on the overexpression of challenging proteins while exceeding the yield expectations of other eukaryotic cell-free systems.

ALiCE® is based on a plant cell lysate of the *Nicotiana tabacum* strain BY-2 containing all the necessary factors for in vitro transcription (RNA polymerase, NTPs, etc.) and translation reactions (ribosomes, translation initiation/elongation factors, tRNA, etc.). The expression reaction is initiated by introducing template DNA to ALiCE®, followed by a reaction duration of 24–48 hours for maximum protein yields without additional user intervention (Figure 1).

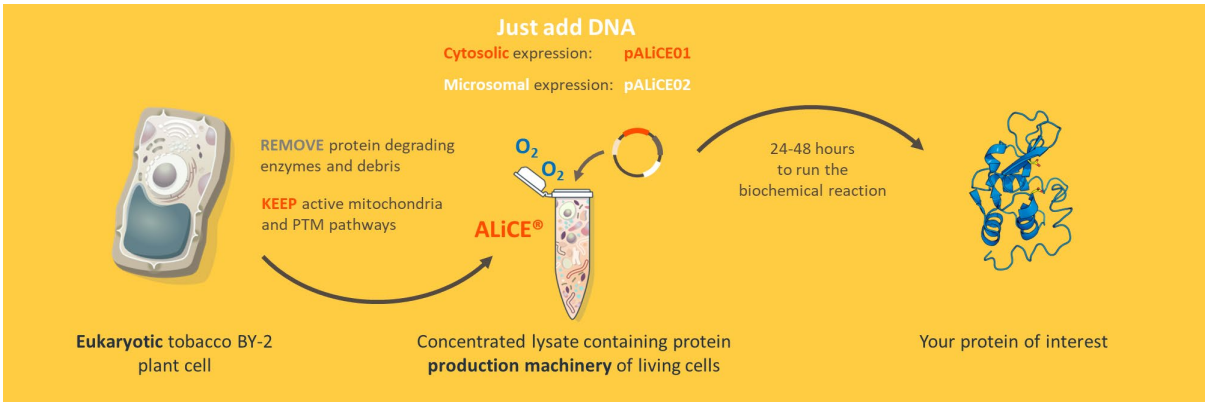

Figure 1. Overview of the ALiCE® technology.

Additionally, ALiCE® contains native membrane structures called microsomes, derived from the plant cell endoplasmic reticulum (ER) and Golgi apparatus. Without the addition of any co-factors or other reaction elements, microsomes enable post-translational modifications including disulfide bonds and *N*-glycosylation, making ALiCE® highly effective in producing functional complex eukaryotic proteins.

ALiCE® for Research kits contain two expression vectors, pALiCE01 and pALiCE02. These vectors allow the expression of your protein-of-interest in different cellular compartments, providing expression of simple, complex membrane and PTM-containing proteins. pALiCE02 contains melittin signal peptide, which targets proteins to the microsomes (the signal peptide is cleaved by the system after expression). For simple proteins expressed using pALiCE01 requiring no ER/Golgi-specific post-translational modifications, protein yields of up to 2 mg/mL before purification can be expected. For more complex proteins or membrane proteins expressed using pALiCE02, yields are routinely between 0.05 and 0.5 mg/mL before purification.

A full overview of the ALiCE® for Research expression workflow is provided in Figure 2.

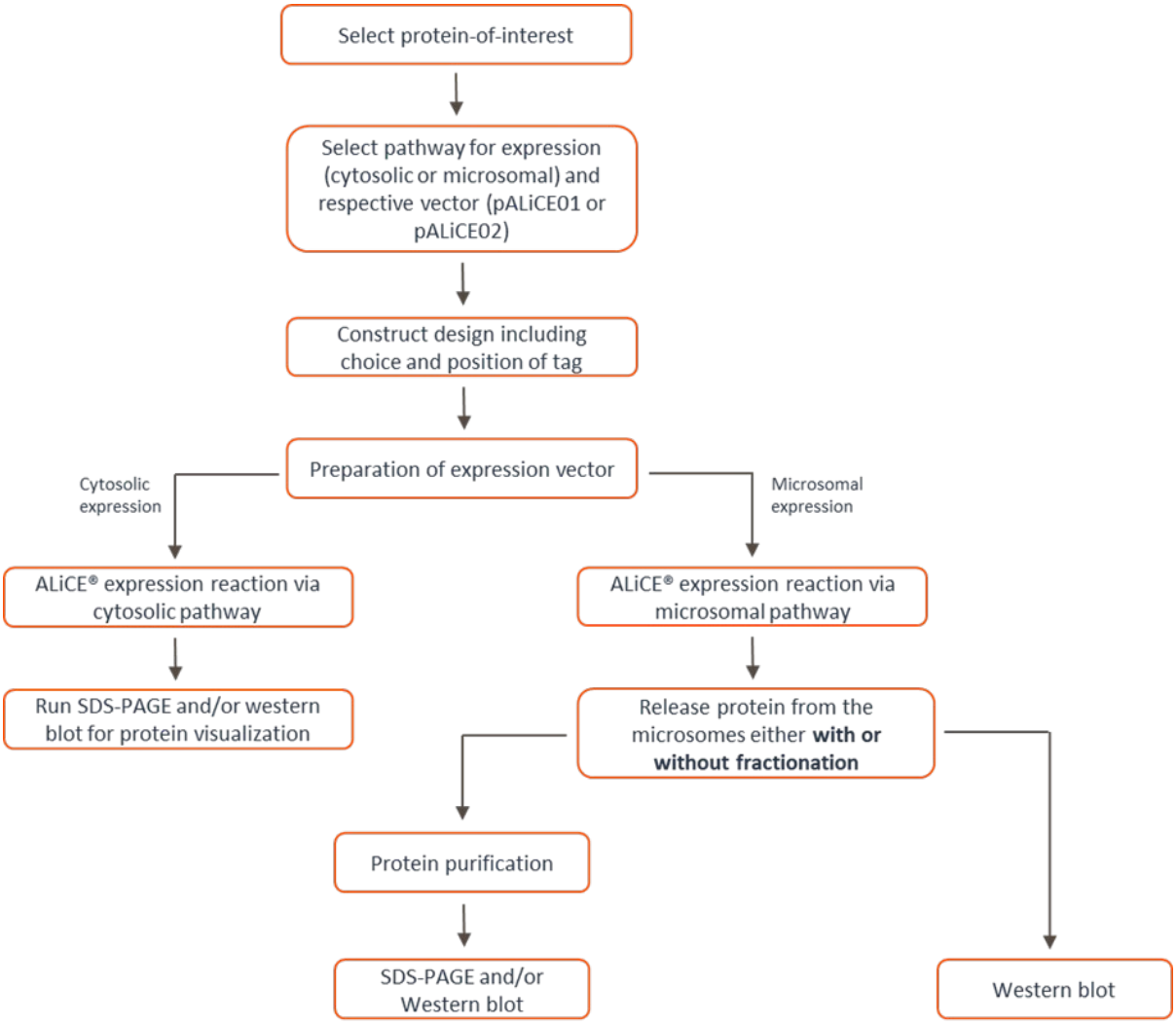

Figure 2. ALiCE® workflow from protein selection to analysis.

2 Product information

2.1 Shipping and storage

ALiCE® kits are shipped on dry ice. Upon arrival, immediately store the components as indicated in the table in “Kit contents”, page 8. ALiCE® reaction mix is stable for at least 12 months when properly stored. Avoid more than one freeze-thaw cycle. ALiCE® reaction mix should be aliquoted according to your needs.

2.2 Product use limitations

This kit is for research purposes only. It is not to be used for diagnostic or preventive action or treatment of a disease, nor to be administered to humans. Please refer to the Limited Use Label License (LULL) on our website: www.leniobio.com/lull

2.3 Product warranty

The kit is shipped frozen on dry ice. If there is no dry ice remaining in the package upon delivery, or if the package is damaged, the quality of the kit may be compromised. Contact us immediately at support@leniobio.com if any delivery issues occurred. The warranty remains in effect up to the expiration date indicated on the product label.

2.4 Safety

The user should observe all applicable regulations for handling chemicals and recombinant DNA. Lab coat, safety glasses and gloves should be worn when handling kit components. When handling ultra-cold material, wear additional protective gloves to avoid frostbite. Used components are to be disposed of in accordance with local regulations.

2.5 Kit contents

The ALiCE® reaction mix is provided in 2 mL screw cap micro tubes.

ALiCE® for Research Cell-Free Protein Expression Mini-Kit (6 x 50 µL ALiCE®)

Component	Quantity	Concentration	Volume	Storage
ALiCE® reaction mix	6	n/a	50 µL	−80 °C
Vector pALiCE01	1	250 ng/µL	15 µL	At or below −20 °C
Vector pALiCE02	1	250 ng/µL	15 µL	At or below −20 °C
ALiCE® tube caps, perforated	6	n/a	n/a	Room temperature

n/a: not applicable

ALiCE® for Research Cell-Free Protein Expression Midi-Kit (6 x 200 µL ALiCE®)

Component	Quantity	Concentration	Volume	Storage
ALiCE® reaction mix	6	n/a	200 µL	−80 °C
Vector pALiCE01	1	250 ng/µL	50 µL	At or below −20 °C
Vector pALiCE02	1	250 ng/µL	50 µL	At or below −20 °C
ALiCE® tube set, 12 pcs.	2	n/a	n/a	Room temperature

n/a: not applicable

ALiCE® for Research Cell-Free Protein Expression Maxi-Kit (6 x 500 µL ALiCE®)

Component	Quantity	Concentration	Volume	Storage
ALiCE® reaction mix	6	n/a	500 µL	−80 °C
Vector pALiCE01	1	250 ng/µL	50 µL	At or below −20 °C
Vector pALiCE02	1	250 ng/µL	50 µL	At or below −20 °C
ALiCE® tube set, 12 pcs.	5	n/a	n/a	Room temperature

n/a: not applicable

2.6 Equipment and materials provided by user

- Gloves
- Pipette filter tips (RNase-free)
- RNase-free water
- 1X PBS (phosphate-buffered saline)
- Microcentrifuge capable of 16,000 x *g* at 4 °C, with 1.5 ml microcentrifuge tube rotor
- Equipment and materials for performing SDS-PAGE and/or Western blot analysis
- Plasmid purification kit for isolating RNase-free, high-quality transfection-grade plasmid DNA using anion exchange chromatography (recommended kit: Macherey-Nagel® NucleoBond® Xtra kit).
- For reactions in tubes: heated table-top shaker (e.g., Eppendorf® ThermoMixer® series or similar with an equivalent shaking diameter of 3 mm).
- For reactions in 96-well half-area microplates:
    - 96-well half-area microplates
    - Humidified shaking incubator (e.g., Kuhner LT-X or ISF1-Z, Adolf Kuhner AG)
    - We recommend using the Sandwich Cover System (#CR1801) from EnzyScreen BV in combination with their universal clamp holder system
    - For other recommended plate sealing methods and recommendations if no humidity control is available, see “Instructions for incubation of ALiCE® reactions”, page 11

Materials needed for protein expression using the microsomal pathway

- n-dodecyl-β-D-maltoside (DDM) for protein release from microsomes, or n-decyl-β-maltoside (DM) if a higher critical micelle concentration (CMC) is required for downstream detergent removal by dialysis
- Magnetic stand for magnetic bead separation in 1.5 mL microcentrifuge tubes
- For protein purification: magnetic beads (e.g., Thermo Fisher Scientific® Dynabeads™ Magnetic Beads)

3 Before starting

3.1 General ALiCE® reaction recommendations and control guidelines

Important notices

RNase contamination leads to reduced or no protein yields.
Only use RNase-free filter-tips and wear gloves at all times!

ALiCE® requires oxygen during the entire reaction time for a successful reaction.
Do not seal the reaction vessels with air-tight materials!

Avoid vortexing the reaction mix, as excessive mechanical stress will lead to destruction of organelles and expression reaction failure.

High-quality transfection grade template DNA must be used in ALiCE® procedures.
Guidance for template preparation outlined in "Preparing pALiCE expression constructs for ALiCE® reaction", page 13 must be followed.

Positive control

The supplied pALiCE01 plasmid can be used directly for a positive control reaction. pALiCE01 expresses the fluorescent protein eYFP (with Strep-tag® II) via the cytosolic expression pathway. Successful expression serves as the positive control for ALiCE® system function and successful reaction setup. Follow "Instructions for incubation of ALiCE® reactions", page 11, and "Protein expression via the cytosolic pathway", page 18 for instructions on how to run a cytosolic pathway expression including the pALiCE01 positive control.

Note: The supplied pALiCE02 plasmid does not serve as a specific microsomal expression pathway control. Please contact us at support@leniobio.com if you need specific microsomal pathway control recommendations.

3.2 Instructions for incubation of ALiCE® reactions

IMPORTANT: The ALiCE® cell-free expression system requires oxygenation due to the presence of mitochondria. Gas exchange between the reaction mix and the environment is necessary. Air-tight sealing of the reactions must be avoided.

Reactions can be performed in the provided reaction tubes or in 96-well half-area microplates. To ensure sufficient oxygen supply while preventing excessive evaporation, the following conditions need to be considered:

- **Reactions in ALiCE® reaction tubes** must be sealed with the provided punctured screw cap lids and can be run in a bench-top thermal controlled shaking incubator (e.g., Eppendorf ThermoMixer series or similar with an equivalent shaking diameter of 3 mm) at an environment relative humidity of >55 % with 700 rpm shaking.
- **Reactions in 96-well half-area microplates** can be incubated in a shaker system with humidity control above 70 %, shaking frequency of 500 rpm with a shaking diameter ≥12.5 mm (e.g., Kuhner LT-X or ISF1-Z, Adolf Kuhner AG).

  For plate sealing, we recommend using the Sandwich Cover System from EnzyScreen BV in combination with their universal clamp holder system.

  Plates can also be sealed with semi-permeable membranes, preferably polyolefin sealing foil (HJ-Bioanalytik, part no. 900371), which have been found to provide the best balance between aeration and evaporation. BREATHseal (Greiner Bio-One) and Adhesive Seal Gas Perm Woven (Porvair Sciences, cat. no. SF32080TS) are also recommended, although evaporation rates may be elevated.

  If humidity control above 70 % is not available, as an alternative option, reactions in plates can still be performed, but higher than optimal evaporation rates will occur. At 55 % relative humidity, 10–15 µL evaporation per well, per 24 hours can be expected when using the provided sealing membranes.

3.3 Selecting the right ALiCE® expression pathway for your protein

ALiCE® for Research can be used to express very simple to highly complex proteins. This is achieved by providing two different pathways for expression, each requiring cloning of your gene of interest into a pathway-specific vector. Proteins without ER/Golgi-specific post-translational modifications (e.g., N-glycosylation or disulfide bonds) can be expressed using the cytosolic protein expression pathway using pALiCE01. More complex proteins (e.g., those containing ER/Golgi-specific post-translational modifications, or membrane proteins) can be produced using the microsomal expression pathway using pALiCE02, which introduces a signal peptide for protein targeting. Selecting the correct expression pathway is very important for achieving success. The decision tree for pathway selection (Figure 3) can be used to determine which pathway is best suited for your protein(s) of interest. For any questions, contact us at support@leniobio.com and we can help you select the appropriate expression pathway.

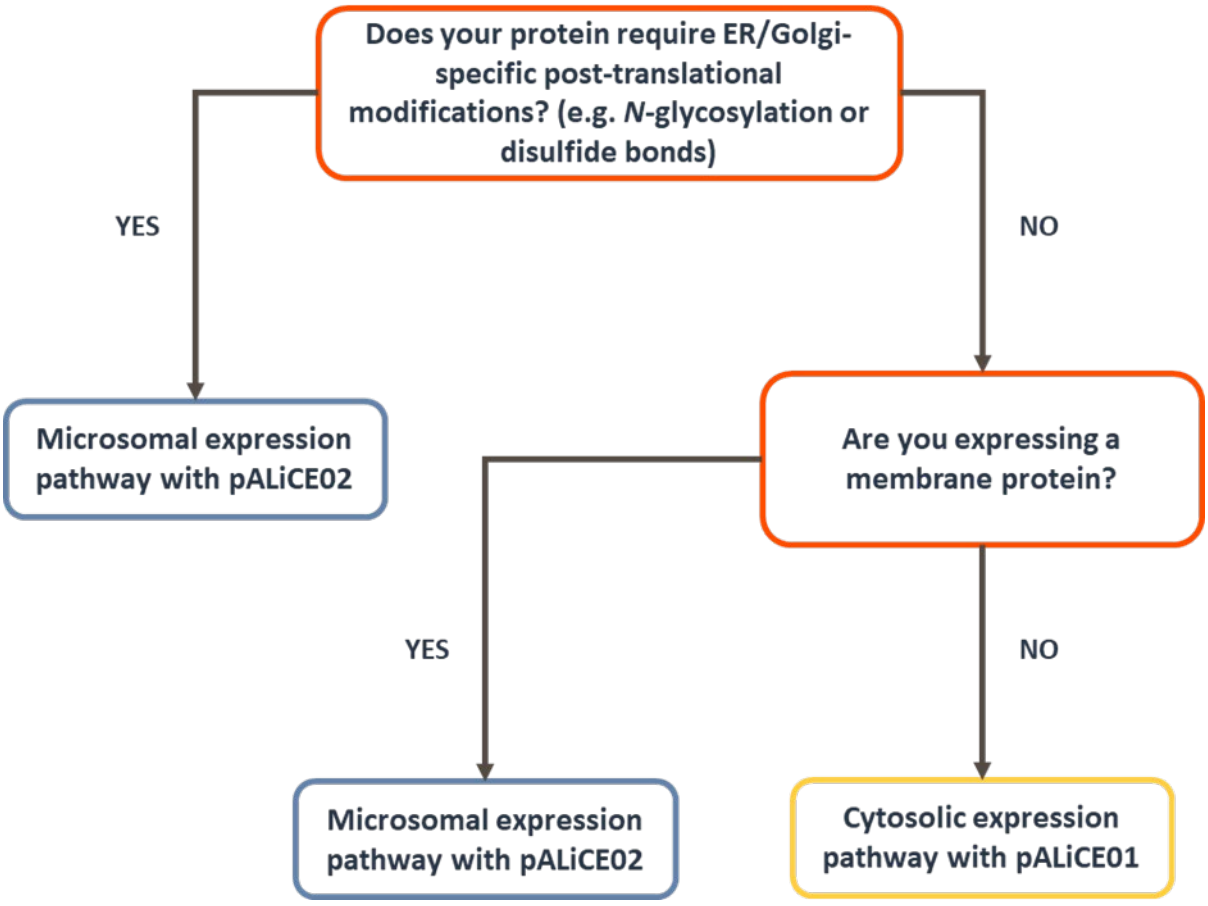

Figure 3. Decision tree for selecting the ALiCE® expression pathway for optimal expression of functional protein.

4 Preparing pALiCE expression constructs for ALiCE® reaction

4.1 pALiCE vectors overview

Full sequence information, including vector maps, is provided in “Appendix 2: Sequences details of plasmids provided with ALiCE® for Research”, page 37.

ALiCE® for Research is provided with a set of two vectors, pALiCE01 and pALiCE02 (Figure 4). Each vector serves different needs depending on whether your protein of interest (POI) requires expression via the cytosolic or microsomal pathway (see “Selecting the right ALiCE® expression pathway for your protein”, page 12).

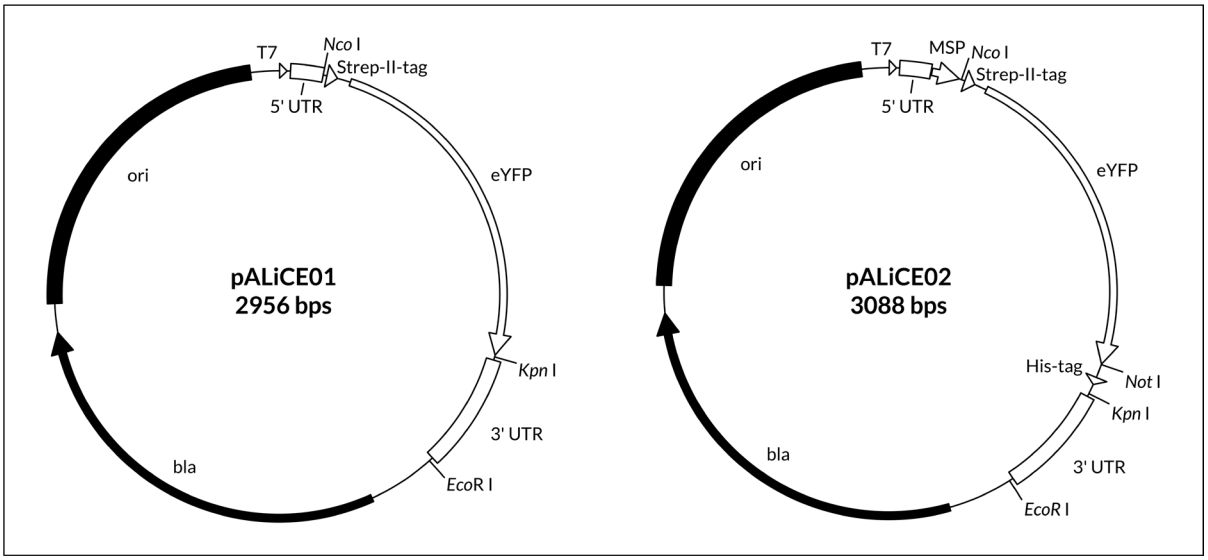

Figure 4. Plasmid maps for pALiCE01 and pALiCE02, including sequence features, vector size and select restriction sites.

The pALiCE vector encodes a defined expression cassette encoding several features essential for successful protein expression in ALiCE® (Figure 5):

- A T7 promoter for transcription driven by the T7 RNA polymerase.
- Two plant translational enhancer elements from the tobacco mosaic virus (TMV).
- **pALiCE02 vector only**: A 20-residue honeybee melittin signal peptide (MSP), which is introduced at the N-terminus of your POI (cleaved by the system after expression). This directs expression in the microsomes (organelles that possess the machinery for introducing post-translational modifications).

Given the critical nature of these expression cassette features that have been optimized for ALiCE® expression, we strongly recommend using pALiCE vectors for your expressions. Other vectors for mammalian and bacterial systems do not contain the necessary expression cassette elements required for use in ALiCE®.

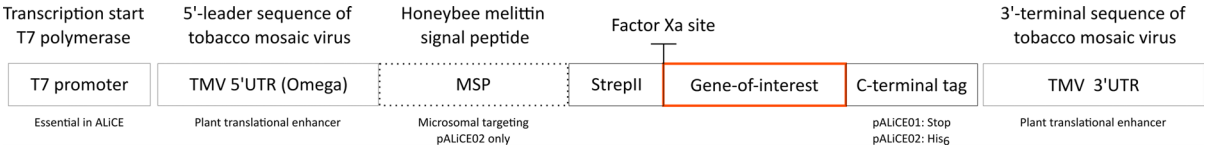

Figure 5. General sequence features of the pALiCE vector suite. The MSP tag is unique to pALiCE02 for expression via the microsomal pathway.

IMPORTANT: The distance of the TMV 5' untranslated region (UTR), also referred to as Omega, to the start codon (ATG) in pALiCE vectors must not be modified.

pALiCE expression vectors include a gene encoding a Strep-tag II enhanced yellow fluorescent protein (eYFP) sequence (Figure 6). The supplied pALiCE01 vector can be used directly as a general expression control when included in the ALiCE® reaction mix.

Figure 6. Sequence features of pALiCE01 encoding Strep-tag II eYFP.

IMPORTANT: The Strep-tag II eYFP gene is removed during the cloning of your gene of interest when using restriction cloning or when ordering constructs from our third-party suppliers. Therefore, please ensure that any desired purification or solubility tags are encoded in your POI gene. We recommend the use of a Strep-tag II for quick, one-step purification. Due to current processing requirements, a His_6-tagged T7 polymerase is present in the ALiCE® reaction mix at low concentrations, which may impact downstream detection and purification efforts when a His_6-tag is used.

All pALiCE vectors also encode a β-lactamase (*bla*) gene, which provides resistance against ampicillin for microbiological selection.

4.2 Cloning information: producing ALiCE®-ready pALiCE constructs

Expressing your protein of interest (POI) using ALiCE® requires inserting the gene sequence into the pALiCE expression cassette at predefined locations in the appropriate pALiCE vector.

The cytosolic expression pathway routinely produces protein concentrations up to 2 mg/mL before purification. Produced POIs can be visualized by SDS-PAGE. When purifying cytosolic POI after expression, make sure to include an appropriate tag.

The microsomal expression pathway routinely produces protein concentrations between 0.05–0.5 mg/mL before purification. Therefore, we recommend either a purification step prior to SDS-PAGE analysis OR performing a Western blot without prior protein purification for POI visualization. For both options, make sure to include an appropriate tag.

There are three methods for producing ALiCE®-ready expression constructs:

- Restriction-free cloning (page 15)
- Restriction enzyme cloning (page 16)
- Third-party supplier services (page 16)

4.2.1 Restriction-free cloning

We recommend using Gibson Assembly as a seamless (e.g., no scars or other cloning artifacts) cloning strategy to introduce your POI gene into pALiCE expression vectors. The Strep-tag II eYFP start and stop codons provide guidance for designing appropriate sequence overlaps. If overlaps are designed accordingly, Gibson Assembly also allows the possibility of retaining the Strep-tag II and/or Factor Xa protease cleavage site encoded in the pALiCE vector (Figure 7).

Figure 7. Sequence features of pALiCE01 encoding Strep-tag II eYFP and Factor Xa.

We recommend NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs) for restriction-free cloning. Comparable products that support Gibson Assembly may also be used. If additional guidance is needed, contact us at support@leniobio.com for suggested primers with appropriate sequence overlaps for pALiCE construct Gibson Assembly.

4.2.2 Restriction enzyme cloning

The *Nco*I and *Kpn*I restriction sites can be used to introduce POI genes into pALiCE vectors.

IMPORTANT: Make sure to include two additional nucleotides after the *Nco*I recognition site in your gene of interest fragment to avoid a frame shift. Failure to do so will result in unsuccessful expression of your protein (Figure 8). A stop codon must also be included in your gene of interest.

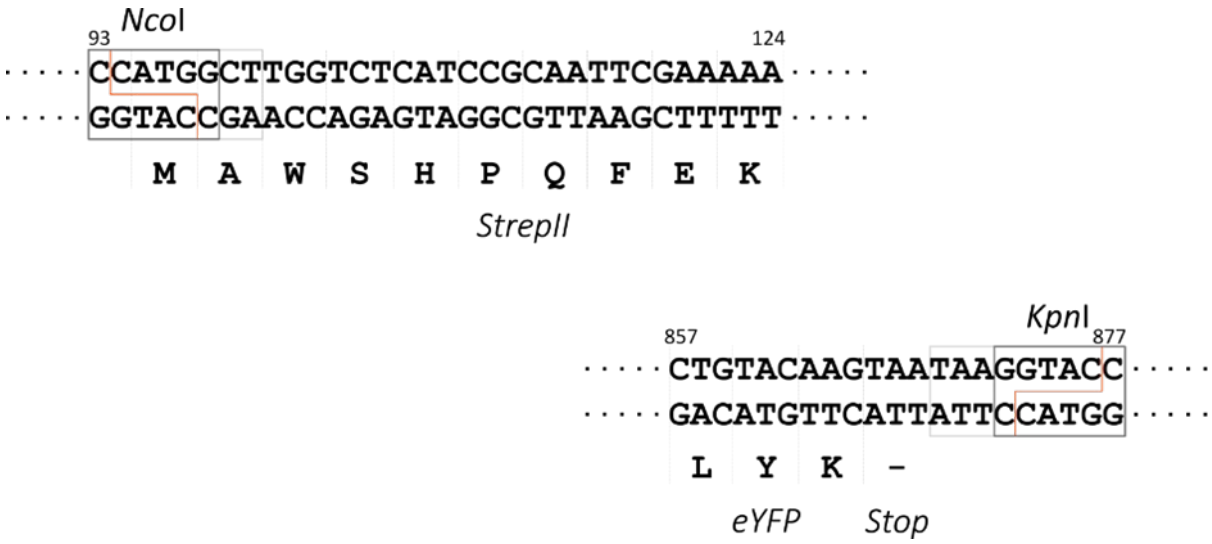

Figure 8. *Nco*I and *Kpn*I restriction enzyme recognition sites in pALiCE vectors for insertion of POI genes. Light grey box near *Nco*I indicates the extra codons that should be included in the primer design to avoid a frame shift. A stop codon needs to be included on the 3' end of your amplified gene segment before *Kpn*I.

While we do not recommend the usage of a His-tag for purification of proteins expressed using the cytosolic expression pathway, *Xho*I or *Not*I can be used in place of *Kpn*I in pALiCE02 to introduce a C-terminal His_6-tag. If a C-terminal His_6-tag is used, a stop codon should be omitted from your gene of interest and an additional 7 or 10 amino acids will be inserted between your protein and the His_6-tag for *Xho*I and *Not*I, respectively.

Make sure that your POI gene encodes any purification or solubility tags necessary for your downstream application.

4.2.3 Third-party supplier services

For your convenience, pALiCE vectors are available from third party suppliers that can synthesize your protein of interest (POI) gene and perform all cloning steps, including plasmid amplification.

Make sure that your POI gene encodes any purification or solubility tags necessary for your downstream application.

Contact us at support@leniobio.com for further information on established pALiCE providers and details on ALiCE®-ready construct design.

4.3 Preparation of plasmid DNA for expression in ALiCE®

After your protein of interest (POI) gene is cloned into one of our pALiCE vectors, transform a competent *E. coli* DH5α® strain for template amplification.

IMPORTANT: Using non-DH5α *E. coli* strains may diminish plasmid DNA quality and reduce target protein yield.

pALiCE vectors encode a β-lactamase (*bla*) gene that provides resistance against ampicillin for microbiological selection.

To facilitate an efficient transcription-translation reaction, plasmid DNA with at least high-quality transfection-grade purity should be used in ALiCE®. It is strongly recommended to use a plasmid preparation procedure based on anion exchange chromatography (e.g., NucleoBond Xtra Maxi kit for high-quality transfection-grade plasmid DNA, Macherey-Nagel).

IMPORTANT: Silica-based plasmid purification methods are not recommended. Silica-based plasmid preparations may contain impurities that prevent successful protein expression.

We recommend adjusting your final plasmid DNA stock concentration to 250–1000 ng/µL with nuclease-free distilled water or 5 mM Tris-HCl buffer, pH 8.5.

IMPORTANT: Do not use EDTA as it may interfere with the expression reaction.

IMPORTANT: DNA concentrations <250 ng/µL will dilute the reaction mixture and may impact the reproducibility of the expression reaction.

5 Protocols

5.1 Protein expression via the cytosolic pathway

5.1.1 Checklist for using the cytosolic pathway for protein expression

- All "General ALiCE® reaction recommendations and control guidelines" (page 10) have been followed.
- The ALiCE® reaction incubation method (i.e., reaction tubes or 96-well half-area microplates) has been prepared according to "Instructions for incubation of ALiCE® reactions", page 11.
- Your protein of interest is a non-membrane protein that does not require ER/Golgi-specific post-translational modifications (see "Selecting the right ALiCE® expression pathway for your protein", page 12).
- Your protein of interest has been cloned into the pALiCE01 vector and (optionally) includes any tags necessary for purification or analysis (see "Preparing pALiCE expression constructs for ALiCE® reaction", page 13).
- Plasmid DNA has been prepared for expression in ALiCE® as instructed in "Preparation of plasmid DNA for expression in ALiCE®", page 17.

IMPORTANT: Do not proceed with protein expression via the cytosolic pathway unless all the conditions in the checklist have been completed. For any questions, contact us at support@leniobio.com.

5.1.2 Positive control setup

For the positive control (PC) reaction using pALiCE01, which is provided with each kit (encoding Strep-tag II eYFP for cytosolic pathway expression), follow the instructions below:

- Use 2 µL of provided pALiCE01 plasmid DNA (equivalent of 5 nM final concentration) in a 50 µL total reaction volume.
- Incubate for 24–48 hours.

Successful expression can be observed visually as an intense yellow color or measured by fluorescence readout (excitation peak at 514 nm, emission peak at 530 nm). Additionally, the Strep-tag II eYFP can be visualized using SDS-PAGE or Western blot analysis (see "Protein purification and protein analysis (SDS-PAGE)", page 21).

5.1.3 Non-template control setup

If you plan to analyze your protein via SDS-PAGE, we recommend running a non-template control reaction (NTC) as a background reference for the analysis (incubate 50 µL of reaction mix without DNA alongside the ALiCE® reaction).

5.1.4 Run the ALiCE® expression reaction using the cytosolic pathway

Before starting the procedure, be aware that ALiCE® reactions must be started within 30 minutes of thawing the reaction mix.

1. Remove ALiCE® reaction mix from storage and thaw in a water bath at room temperature (20–25 °C) until the reaction mix is fully thawed (approximately 10 minutes). **Do not exceed recommended temperature!** Thaw enough ALiCE® reaction mixes for each sample, positive control (PC) and non-template control (NTC) reaction.
2. Mix thoroughly by inverting the tubes. **Do not vortex!**
3. Pulse spin the reaction tubes at 100 x *g* for a maximum of 5 seconds to collect all liquid.
4. Place the tubes on ice immediately.
5. Assemble the sample, positive control and non-template control reactions in ALiCE® reaction tubes or a 96-well half-area microplate:

Component	Volume
ALiCE® reaction mix	48 µL
DNA template	X µL
Nuclease free H_2O	X µL
Total	**50 µL**

   For Mini Kits, simply add the appropriate volume of your DNA template directly to the provided ALiCE® reaction mix tube.

   For calculation of the required sample DNA template volume, see “Appendix 1: DNA concentration calculation”, page 36.

   For the positive control reaction, use 2 µL of provided pALiCE01 plasmid DNA directly (encoding Strep-tag II eYFP for cytosolic pathway expression).

   For the non-template control reaction, do not add any DNA template.

   Remaining reaction mix can be refrozen by directly returning aliquots to a −80 °C freezer. **Do not flash freeze using liquid nitrogen!**

6. Mix reactions gently by pipetting up and down slowly.
7. Seal the reactions:
   - ALiCE® reactions performed in tubes: Seal the reaction tubes with the provided perforated lids.

     IMPORTANT: Make sure the perforated lids are used to provide oxygenation for the reaction!
   - ALiCE® reactions performed in 96-well half-area microplates: Either place in the Sandwich Cover System from EnzyScreen BV, or seal with semi-permeable foil (see “Instructions for incubation of ALiCE® reactions”, page 11).
8. Incubate at 25 °C for 24–48 hours at 700 rpm for ALiCE® tubes (Eppendorf ThermoMixer series or equivalent shaking diameter) or 500 rpm for 96-well half-area microplates. **Ensure incubation is performed at 25 °C!**

9. Stop reactions by placing the tubes or 96-well half-area microplates on ice. Finished reactions can be stored overnight at 4 °C, or frozen at −20 °C before further processing.

   Depending on your protein of interest, prolonged storage or freezing can have a detrimental effect on protein quality. **We recommend processing the expression reaction immediately after completion.**

5.1.5 Protein purification and protein analysis (SDS-PAGE)

Protein expression via the cytosolic pathway usually results in sufficient protein concentration for direct analysis via SDS-PAGE without need for prior purification. If the protein has an appropriate tag, purification (using affinity chromatography with column or magnetic beads) and/or Western blot protein analysis can be performed. To perform magnetic bead purification, see “Magnetic bead purification followed by SDS-PAGE analysis”, page 27 and use the obtained supernatant S1 from step 4 in this procedure.

For SDS-PAGE analysis, to obtain a background reference, perform the steps below using the non-template control (NTC) reaction and use its supernatant as background reference. Label the sample “S1BR” after completing step 5.

Materials needed for procedure

- 1.5 mL microcentrifuge tubes
- 1X PBS

1. Transfer the completed 50 µL expression reaction into a 1.5 mL microcentrifuge tube.
2. Transfer a 2 µL sample from the completed reaction into a new tube for analysis. Label the sample “whole lysate”.
3. Centrifuge the remaining lysate at 4 °C at 16,000 x *g* for 20 minutes.
4. Avoiding the pellet, carefully transfer the supernatant (approximately 40–45 µL) into a new tube while leaving the pellet intact. **Your soluble protein of interest is expected to be in this supernatant.**
5. Transfer a 2 µL sample of supernatant to a new tube for analysis. Label the sample “S1”.
6. Fully resuspend the pellet in 50 µL 1X PBS by pipetting up and down.
7. Transfer a 2 µL sample into a new tube for analysis. Label the sample “P1”.
8. Use 0.5 µL of samples “whole lysate”, “S1”, “P1” and background reference (“S1BR” from NTC) for SDS-PAGE analysis.

   Note: Functional protein may be visible in analysis of sample “P1” due to carryover during liquid handling.

For the positive control reaction using pALiCE01, the expected outcome is shown in Figure 9.

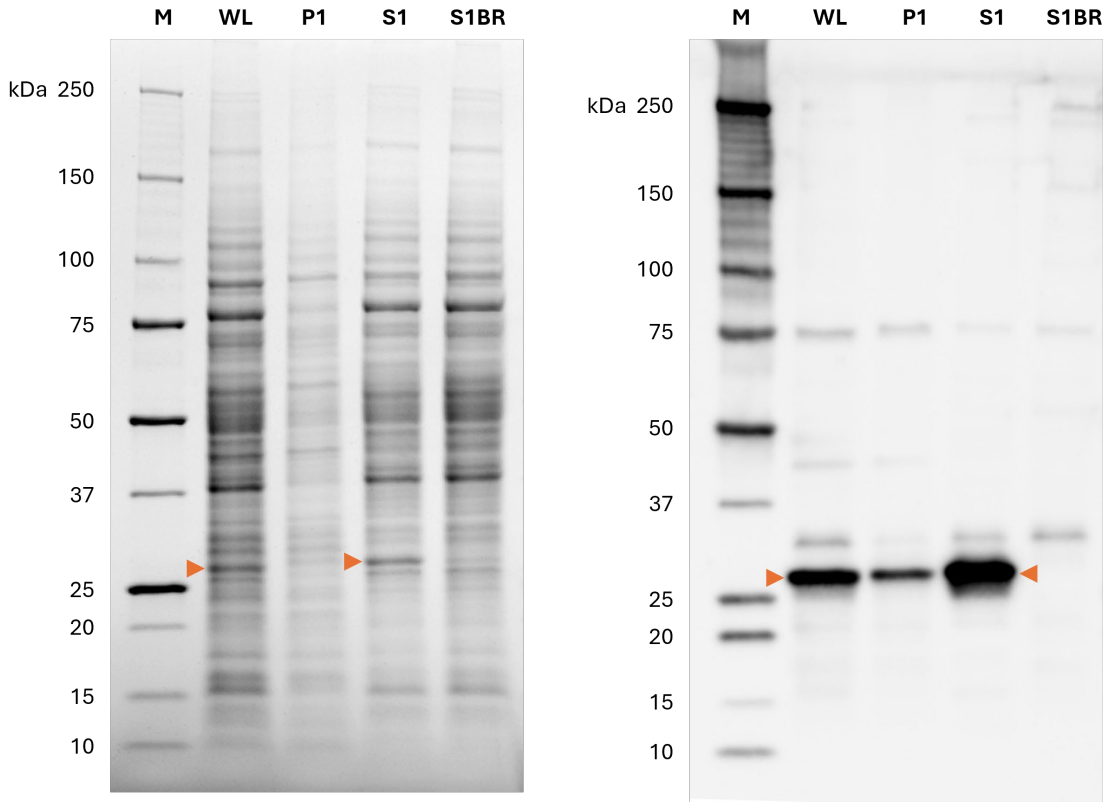

Figure 9. Reducing SDS-PAGE (left) and Western blot (right) analysis of pALiCE01 control expression reaction. Conditions: 50 µL expression (96-well half-area microplate), using 5 nM DNA concentration and 24-hour expression duration. Orange arrows indicate Strep-tag II-eYFP control protein (Exp: 29 kDa). M = Precision Plus Protein™ Unstained Protein Standard, *Strep*-tagged recombinant (Bio-Rad® #1610363); WL = whole lysate; P1 = Pellet 1; S1 = Supernatant 1; S1BR = Supernatant 1 background reference (NTC S1). Western blot performed using HRP-conjugated antibody against Strep-tag II (Bio-Rad #1610381).

5.2 Protein expression via the microsomal pathway

5.2.1 Checklist for using the microsomal pathway for protein expression

- All "General ALiCE® reaction recommendations and control guidelines" (page 10) have been followed.
- The ALiCE® reaction incubation method (i.e., reaction tubes or 96-well half-area microplates) has been prepared according to "Instructions for incubation of ALiCE® reactions", page 11.
- Your protein of interest is a protein that contains ER/Golgi-specific post-translational modifications, and/or is a membrane protein (see "Selecting the right ALiCE® expression pathway for your protein", page 12).
- Your protein of interest has been cloned into the pALiCE02 vector, including the melittin signal peptide (MSP) and a tag that allows purification and/or Western blot analysis (recommended) (see "Preparing pALiCE expression constructs for ALiCE® reaction", page 13).
- Plasmid DNA has been prepared for expression in ALiCE® as instructed in "Preparation of plasmid DNA for expression in ALiCE®", page 17.

IMPORTANT: Do not proceed with protein expression via the microsomal pathway unless all the conditions in the checklist have been completed. For any questions, contact us at support@leniobio.com.

5.2.2 Non-template control setup

If you plan to analyze your protein via SDS-PAGE, we recommend running a non-template control reaction (NTC) as a background reference for the analysis (incubate 50 µL of reaction mix without DNA alongside the ALiCE® reaction).

5.2.3 Run the ALiCE® expression reaction using the microsomal pathway

Before starting the procedure, be aware that ALiCE® reactions must be started within 30 minutes of thawing the reaction mix.

1. Remove ALiCE® reaction mix from storage and thaw in a water bath at room temperature (20–25 °C) until the reaction mix is fully thawed (approximately 10 minutes). **Do not exceed recommended temperature!** Thaw enough ALiCE® reaction mixes for each sample, positive control (PC) and non-template control (NTC) reaction.
2. Mix thoroughly by inverting the tubes. **Do not vortex!**
3. Pulse spin the reaction tubes at 100 x *g* for a maximum of 5 seconds to collect all liquid.
4. Place the tubes on ice immediately.
5. Assemble the reactions in ALiCE® reaction tubes or a 96-well half-area microplate:

Component	Volume
ALiCE® reaction mix	48 µL
DNA template	X µL
Nuclease free H_2O	X µL
Total	**50 µL**

   For Mini Kits, simply add the appropriate volume of your DNA template directly to the provided ALiCE® reaction mix tube.

   For calculation of the required DNA template volume, see “Appendix 1: DNA concentration calculation”, page 36.

   For the non-template control reaction, do not add any DNA template.

   Remaining reaction mix can be refrozen by directly returning aliquots to a −80 °C freezer. **Do not flash freeze using liquid nitrogen!**

6. Mix reactions gently by pipetting up and down slowly.
7. Seal the reactions:
   - ALiCE® reactions performed in tubes: Seal the reaction tubes with the provided perforated lids.

     IMPORTANT: Make sure the perforated lids are used to provide oxygenation for the reaction!
   - ALiCE® reactions performed in 96-well half-area microplates: Either place in the Sandwich Cover System from EnzyScreen BV, or seal with semi-permeable foil (see “Instructions for incubation of ALiCE® reactions”, page 11).
8. Incubate at 25 °C for 24–48 hours at 700 rpm for ALiCE® tubes (Eppendorf ThermoMixer series or equivalent shaking diameter) or 500 rpm for 96-well half-area microplates. **Ensure incubation is performed at 25 °C!**
9. Stop reactions by placing the tubes or 96-well half-area microplates on ice. Finished reactions can be stored overnight at 4 °C, or frozen at −20 °C before further processing.

   Depending on your protein of interest, prolonged storage or freezing can have a detrimental effect on protein quality. **We recommend processing the expression reaction immediately after completion.**

5.2.4 Considerations for protein purification and analysis

Proteins expressed via the microsomal pathway are directed to the microsomes for folding and post-translational modification (PTM). To obtain your protein, it is essential to release it from the microsomes using a detergent treatment.

There are two methods to consider for protein purification and analysis:

- Obtaining your protein with fractionation
- Obtaining your protein without fractionation (one-step workflow)

Obtaining your protein with fractionation

The protocol "Obtaining your protein using fractionation" provides the highest ratio of fully processed protein (containing desired PTMs, and the correct structure and function) vs. non-processed, partially-processed or aggregated proteins that may also be present in the expression reaction.

The microsomal pathway routinely produces expressed protein concentrations between 0.05–0.5 mg/mL, which can make direct visualization on an SDS-PAGE gel difficult without prior purification. Therefore, we recommend the following options for visualizing proteins expressed in the microsome following microsomal release (fractionation):

- Magnetic bead purification followed by SDS-PAGE analysis
- Western blot analysis (purification not required)

To use this method for protein purification and analysis, proceed to "Obtaining your protein using fractionation", page 26.

Obtaining your protein without fractionation (one-step workflow)

Alternatively, for a faster and simpler way to access your protein, fractionation and centrifugation can be skipped. The protocol "Obtaining your protein without using fractionation (one-step workflow)" provides processing of the completed expression reaction in a single step.

Important: This procedure may result in obtaining a reduced ratio of fully processed protein (non-processed, partially-processed or aggregated proteins may also be present in the expression reaction). If you plan on performing protein analyses where the highest ratio of correctly processed protein could be important (e.g., mass spectrometry, NMR, cryo-EM, detailed activity studies, etc.), or just want to maximize the ratio of fully processed protein, we recommend obtaining your protein using fractionation. Magnetic bead purification followed by SDS-PAGE and Western blot analysis may be performed for protein visualization.

To use this method for protein purification and analysis, proceed to "Obtaining your protein without fractionation (one-step workflow)", page 25.

5.2.5 Non-template controls for protocols with and without fractionation

For SDS-PAGE analysis using either protocol (with or without fractionation), if a background reference is desired, process a non-template control (NTC) in parallel using the selected protocol. For the fractionation protocol, use the supernatant after disruption of the microsomes as background reference and label the sample "S2BR" after completing step 8). For the protocol without fractionation, use the sample from the completed reaction and label the sample "whole lysate BR" after completing step 1.

5.2.6 Obtaining your protein using fractionation

Materials needed for procedure

- 1.5 mL microcentrifuge tubes
- 1X PBS
- 1X PBS containing 0.5 % DDM

1. Transfer the completed 50 µL expression reaction into a 1.5 mL microcentrifuge tube.
2. Transfer a 2 µL sample from the completed reaction into a new tube for analysis. Label the sample "whole lysate".
3. Centrifuge the remaining lysate at 4 °C at 16,000 x *g* for 20 minutes.
4. Avoiding the pellet (P1), carefully transfer the supernatant (approximately 40–45 µL) into a new tube while leaving the pellet intact.
5. Transfer a 2 µL sample of supernatant into a new tube for analysis. Label the sample "S1".
6. Fully resuspend the pellet (P1) in 50 µL 1X PBS containing 0.5 % DDM by pipetting up and down. Incubate at room temperature using a tabletop shaker at 800 rpm for 30 minutes.

   IMPORTANT: Make sure the entire pellet (P1) is fully dispersed during resuspension before proceeding with Step 7.

7. After incubation, centrifuge at 4 °C at 16,000 x *g* for 30 minutes to pellet organelles and aggregated protein.
8. Avoiding the pellet (P2), carefully transfer the supernatant (S2) into a new tube. **Your soluble protein of interest is expected to be in this supernatant.**
9. Transfer a 2 µL sample of supernatant to a new tube for analysis. Label the sample "S2".
10. Fully resuspend the pellet in 50 µL 1X PBS by pipetting up and down.
11. Transfer a 2 µL sample into a new tube for analysis. Label the sample "P2".

To visualize your proteins, proceed to either "Magnetic bead purification followed by SDS-PAGE analysis", page 27 or "Western blot analysis", page 28.

5.2.6.1 Magnetic bead purification followed by SDS-PAGE analysis

This protocol uses Thermo Fisher Scientific Dynabeads Magnetic Beads, but it can be adapted for use with magnetic beads from other suppliers, or for other affinity tags. If alternative products are used, make sure to follow the manufacturer's instructions for successful purification.

Note: Protein A magnetic beads are not suitable for purification using Strep-tag II. Use corresponding beads instead.

Materials needed for procedure

- 1.5 mL microcentrifuge tubes
- 1x PBS containing 0.05 % Tween® 20
- 50 mM glycine, pH 2.8
- Thermo Fisher Scientific Dynabeads Magnetic Beads

1. Pipette 50 µL (i.e., 0.50 mg) of Thermo Fisher Scientific Dynabeads Magnetic Beads into a 1.5 mL microcentrifuge tube.
2. Add 150 µL of Binding/Wash Buffer to the beads. Gently vortex to mix.
3. Place the tube in a magnetic stand to collect the beads against the side of the tube. Using a pipette, remove and discard the supernatant.
4. Transfer reaction supernatant 2 (S2) to the magnetic beads and add 200 µL 1x PBS containing 0.05 % Tween 20. Incubate at 25 °C using a tabletop shaker at 800 rpm for 10 minutes.
5. Place the tube in a magnetic stand to collect the beads against the side of the tube. Using a pipette, remove and discard the supernatant.
6. Wash the magnetic beads by adding 200 µL 1x PBS containing 0.05 % Tween 20 to the magnetic beads and mix by pipetting up and down.
7. Place the tube in a magnetic stand to collect the beads against the side of the tube. Using a pipette, remove and discard the supernatant.
8. To elute the protein, resuspend the magnetic beads in 20 µL 50 mM glycine, pH 2.8. Mix by flicking the tube and incubate at room temperature for 2 minutes.
9. Place the tube in a magnetic stand to collect the beads against the side of the tube. Using a pipette, remove the eluate and transfer to a new tube. Label the sample "BP-S2". **Your purified protein of interest is expected to be in this eluate**.
10. Analyze via SDS-PAGE using 0.5 µL of samples "whole lysate", "S1", "S2" and "P2" from "Obtaining your protein using fractionation", page 26 and 1 µL of "BP-S2". Include 0.5 µL of non-template control (NTC) background reference if needed.

Note: Functional protein from the microsome pellet may be visible in analysis of samples "S1" and "P2" due to carryover during liquid handling.

Note: Specific optimization may be required for certain proteins, especially membrane-bound proteins. A detergent with a higher critical micelle concentration may be used for convenient detergent removal by dialysis (not possible with DDM). For this we recommend n-decyl-β-maltoside (DM).

5.2.6.2 Western blot analysis

Use 0.5 µL of samples “whole lysate”, “S1”, “S2” and “P2” from “Obtaining your protein using fractionation”, page 26 to perform Western blot analysis with the antibody of your choice.

Note: Functional protein from the microsome pellet may be visible in analysis of samples “S1” and “P2” due to carryover during liquid handling.

5.2.7 Obtaining your protein without using fractionation (one-step workflow)

IMPORTANT: Protein purification without using fractionation may result in obtaining a reduced ratio of fully processed (non-processed, partially-processed or aggregated proteins may also be present in the expression reaction). See "Considerations for protein purification and analysis", page 25 for more information.

To visualize your proteins without using fractionation, proceed to either "One-step magnetic bead purification followed by SDS-PAGE analysis", page 29 or "One-step protein release followed by Western blot analysis", page 31.

5.2.7.1 One-step magnetic bead purification followed by SDS-PAGE analysis

This protocol uses Thermo Fisher Scientific Dynabeads Magnetic Beads, but it can be adapted for use with magnetic beads from other suppliers, or for other affinity tags. If alternative products are used, make sure to follow the manufacturer's instructions for successful purification.

Note: Protein A magnetic beads are not suitable for purification using Strep-tag II. Use corresponding beads instead.

Materials needed for procedure

- 1.5 mL microcentrifuge tubes
- 1X PBS containing 0.05 % Tween 20
- 1X PBS containing 0.5 % DDM and 0.05 % Tween 20
- 50 mM glycine, pH 2.8
- Thermo Fisher Scientific Dynabeads Magnetic Beads

1. Transfer a 2 µL sample from the completed 50 µL expression reaction into a new tube for analysis. Label the sample "whole lysate".
2. Add 200 µL 1X PBS containing 0.5 % DDM and 0.05 % Tween 20 to the remaining expression reaction. Incubate at 25 °C using a tabletop shaker at 800 rpm for 20 minutes.
3. Pipette 50 µL (i.e., 0.50 mg) of Thermo Fisher Scientific Dynabeads Magnetic Beads into a 1.5 mL microcentrifuge tube.
4. Add 150 µL of Binding/Wash Buffer to the beads. Gently vortex to mix.
5. Place the tube in a magnetic stand to collect the beads against the side of the tube. Using a pipette, remove and discard the supernatant.
6. Add the PBS/expression reaction from step 2 to the magnetic beads. Incubate at 25 °C using a tabletop shaker at 800 rpm for 10 minutes.
7. Place the tube in a magnetic stand to collect the beads against the side of the tube. Using a pipette, remove and discard the supernatant.
8. Add 200 µL 1X PBS containing 0.05 % Tween 20 to the magnetic beads and mix by pipetting up and down.
9. Place the tube in a magnetic stand to collect the beads against the side of the tube. Using a pipette, remove and discard the supernatant.
10. To elute the protein, resuspend the magnetic beads in 20 µL 50 mM glycine, pH 2.8. Mix by flicking the tube and incubate for at room temperature for 2 minutes.

11. Place the tube in a magnetic stand to collect the beads against the side of the tube. Using a pipette, remove the eluate and transfer to a new tube. Label the sample “BP-WL”. **Your purified protein of interest is expected to be in this eluate**.
12. Analyze via SDS-PAGE using 0.5 µL of sample “whole lysate” and 1 µL of “BP-WL”). Include 0.5 µL of non-template control (NTC) background reference if needed.

5.2.7.2 One-step protein release followed by Western blot analysis

If Western blotting is performed, microsomes will need to be disrupted before analyzing the sample.

Materials needed for procedure

- 1.5 mL microcentrifuge tubes
- 1X PBS
- 1X PBS containing 0.5 % DDM

1. Transfer a 2 µL sample from the completed 50 µL expression reaction (from step 9 in "Run the ALiCE® expression reaction using the microsomal pathway"), page 24) into a new tube for analysis. Label the sample "whole lysate".
2. Add 200 µL 1X PBS containing 0.5 % DDM to the remaining expression reaction. Incubate at 25 °C using a tabletop shaker at 800 rpm for 20 minutes.
3. Centrifuge at 4 °C at 16,000 x *g* for 20 minutes.
4. Avoiding the pellet, carefully transfer the supernatant (approximately 240–245 µL) into a new tube while leaving the pellet intact.
5. Transfer a 2 µL sample of supernatant into a new tube for analysis. Label the sample "S1".
6. Fully resuspend the pellet in 50 µL 1X PBS by pipetting up and down.
7. Transfer a 2 µL sample into a new tube for analysis. Label the sample "P1".
8. Use 0.5 µL of samples "whole lysate", "S1", and "P1" and perform Western blot analysis with the antibody of your choice.

6 Troubleshooting

If your protein of interest is not produced, or reduced yields of protein are produced, refer to the troubleshooting list of common issues and solutions. Contact support@leniobio.com for questions and assistance with troubleshooting.

Problem	Possible cause	Recommended action
Failure or low yield of positive control	Kit component deterioration	Make sure all the components have been stored at the appropriate temperature and the expiration date on the label is not exceeded.
	Poor oxygenation	Ensure proper shaking of the samples. Do not seal the plate or the vial with air-tight tape, film or air-tight tube lids.
	Temperature fluctuation	Maintain a stable production temperature over the complete reaction cycle. Lower temperature can lead to lower protein yield. Higher temperatures may possibly damage ALiCE®.
	Mechanical damage	Do not vortex the reaction mix; pipette carefully.

Continued on next page.

Problem	Possible cause	Recommended action
Low yield or no target protein	Poor plasmid DNA quality	Make sure to follow important DNA preparation instruction as outlined in manual. If you used silica-based DNA purification methods, switch to anion exchange chromatography resin. Avoid EDTA. Make sure to avoid heat-treating the purified template plasmids prior to the protein expression reaction.
	Reaction duration too short	Let reaction run for at least 40 hours to maximize protein yield
	Reaction duration too long / protein degradation or precipitation	Sample the reaction in regular intervals (such as every 8 or 12 hours) for presence and yield of the target protein
	Suboptimal plasmid DNA concentration	For some templates, lower or higher plasmid DNA concentration influences the level of target protein yield. Cytosolic expression pathway: re-run expression using 2.5 and 10 nM plasmid concentration. Microsomal expression pathway: re-run expression using 7.5 and 10 nM plasmid concentration.
	Target protein visualization failed	Protein might be aggregated: make sure to check pellet fractions for protein presence to detect protein if aggregated (P1 in case of cytosolic pathway, P2 in case of microsomal pathway). Protein might not be visible in SDS-PAGE analysis due to background: either purify protein before SDS-PAGE, and/or use Western blot to detect your target protein and avoid background signal.

Continued on next page.

Problem	Possible cause	Recommended action
Evaporation of sample during the reaction	Incorrect reaction vessel	When running expression reaction in tubes, use ALiCE® tubes with perforated caps provided by LenioBio. When running expression reactions in 96-well half-area microplates, use a recommended cover system (e.g., the Enzyscreen Sandwich Cover) and a controlled humidity of >70 %.
	Environmental temperature too high	Make sure the environmental temperature does not exceed 25 °C.
	Environmental humidity too low	When using 96-well half-area microplates, maintain an optimal humidity level of 70 %. When using ALiCE® tubes with a tabletop shaker, maintain the room humidity level above 50 %.
	Air circulation	Do not place ALiCE® next to a ventilator or a machine that produces exhaust.

7 FAQ

A current list of our frequently asked questions can be found at www.leniobio.com.

8 Further reading

1) Buntru, M., Vogel, S., Stoff, K., Spiegel, H., Schillberg, S. 2015. A Versatile Coupled Cell-Free Transcription-Translation System Based on Tobacco BY-2 Cell Lysates. Biotechnology & Bioengineering, 112(5):867-78.
2) Das Gupta, M. et. al. 2023. Scaling Eukaryotic Cell-Free Protein Synthesis Achieved with the Versatile and High-Yielding Tobacco BY-2 Cell Lysate. Biotechnology & Bioengineering, 120(10):2890-2906

9 Contact

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10 Appendices

Appendix 1: DNA concentration calculation

Each protein expressed in ALiCE® has a unique plasmid DNA concentration optimum. This depends on the accuracy of the DNA concentration determination, the size of your insert and the overall efficiency of the transcription/translation reaction observed for the lysate. To allow a vector-independent comparison of DNA concentration, a conversion from mass per volume (here in ng/µL) to a molar DNA concentration (here nmol/L = nM) is needed.

The calculation for determining optimal DNA concentration requires the following input:

1) The full length of your plasmid (with insert).
2) The desired reaction volume (usually 50 µL).
3) The desired final molar concentration in the reaction mix (start with 5 nM).
4) The DNA mass concentration of your plasmid stock (>250 ng/µL).
5) A conversion factor to immediately receive a convenient volume output in µL.

The formula below (Figure 10) provides a volume output in µL of the plasmid stock you need to add to your expression reaction mix. It assumes an average molecular weight of a double-stranded DNA (dsDNA) base pair (bp) of 618 g/mol for simplicity. An example calculation for a 3000 bp vector is provided for reference.

$$V\,[\mu L] = \overbrace{\text{Plasmid length [bp]} \times 618\ \text{g/mol/bp}}^{\text{Average dsDNA molecular weight}} \times \text{Reaction volume [µL]} \times \frac{\text{Final concentration [nM]}}{\text{Plasmid stock [ng/µL]}} \times \overbrace{10^{-6}}^{\text{Conversion factor}}$$

Calculation example:

Plasmid length = 3000 bp
Reaction volume = 50 µL
Final concentration = 5 nM
Plasmid stock = 1000 ng/µL

$$V\,[\mu L] = 3000\ \text{bp} \times 618\ \text{g/mol/bp} \times 50\ \mu L \times 5\ \text{nM} / 1000\ \text{ng/}\mu L \times 10^{-6} = 0.5\ \mu L$$

Result: Add 0.5 µL of DNA to 50 µL of ALiCE reaction mix

Figure 10. Formula and example calculation for determining the required volume of plasmid DNA for addition to ALiCE® reaction mix.

Appendix 2: Sequences details of plasmids provided with ALiCE® for Research

pALiCE01 plasmid details

Table 1. Reference elements of the pALiCE01 vector. Locations of elements are shown in relation to the T7 promoter.

Reference elements	Features	pALiCE01
T7 promoter	Transcription	1–17
TMV Omega 5' UTR	Translation enhancer	24–93
*Nco*I (contains start codon ATG)	Restriction enzyme site	93
StrepII-tag	Protein purification	101–124
Factor Xa recognition site	Tag cleavage	140–151
eYFP from *Aequorea victoria*	Reporter	152–868
*Kpn*I	Restriction enzyme site	876
TMV 3' UTR	Translation enhancer	879–1124
*Eco*RI	Restriction enzyme site	1133
AmpR	Selection marker	1172–2137
Origin of replication (Ori)	Plasmid maintenance	2308–2896

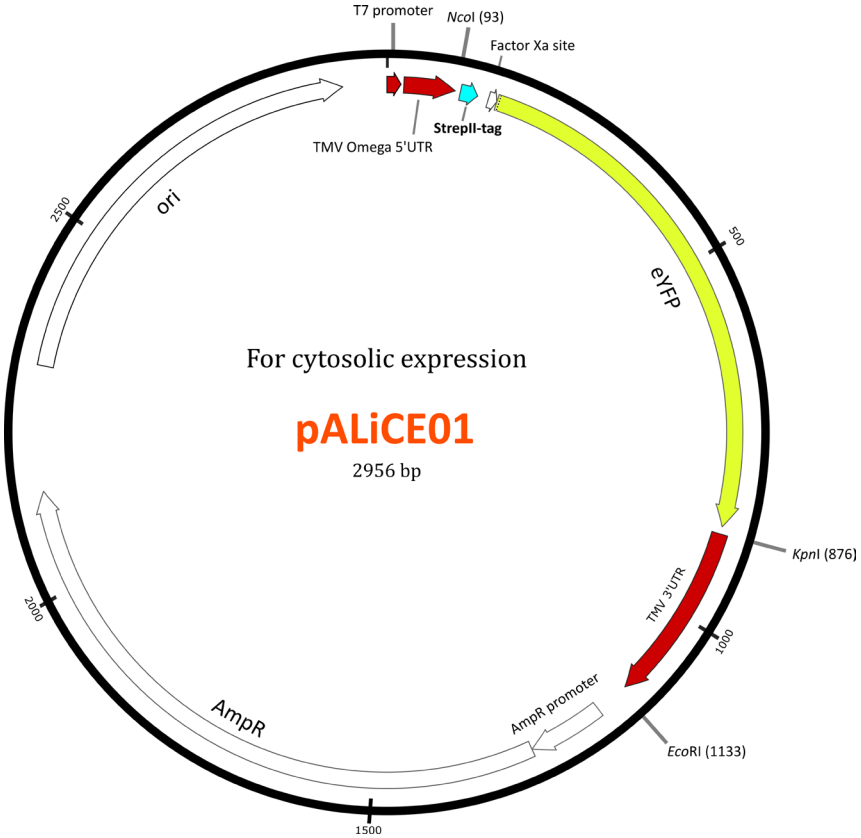

Figure 11. Plasmid map of pALiCE01.

Full sequence pALiCE01

TAATACGACTCACTATAGGGAGAGTATTTTTACAACAATTACCAACAACAACAACAAACAACAACAACATTACAT
TTTACATTCTACAACTACCATGGCTTGGTCTCATCCGCAATTCGAAAAAAGCGCTGAAAACCTGATCGAAGGCCG
TGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCA
CAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCAC
CGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCTTCGGCTACGGCCTGCAGTGCTTCGCCCGCTACCC
CGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTT
CAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCT
GAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGT
CTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAG
CGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTA
CCTGAGCTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGAC
CGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAATAAGGTACCAAGCTCTTCTGGTTTGGTTTGGA
CCTCTGGTCCTGCAACTTGAGGTAGTCAAGATGCATAATAAATAACGGATTGTGTCCGTAATCACACGTGGTGCG
TACGATAACGCATAGTGTTTTTCCCTCCACTTAAATCGAAGGGTTGTGTCTTGGATCGCGCGGGTCAAATGTATA
TGGTTCATATACATCCGCAGGCACGTAATAAAGCGAGGGGTTCGAATCCCCCCGTTACCCCCGGTAGGGGCCCAT
TATAGCCGAATTCGGCGCGCCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTA
AATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAG
TATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCC
AGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAA
CAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATG
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TTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTA
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GCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACC
AACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTT
AGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGC
CAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTG
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ATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGA
GCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGA
GCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTT
CCTGGCCTTTTGCTGGCCTTTTGCTCACATG

pALiCE02 plasmid details

Table 2. Reference elements of the pALiCE02 vector. Locations of elements are shown in relation to the T7 promoter.

Reference elements	Feature	pALiCE02
T7 promoter	Transcription	1–19
TMV Omega 5' UTR	Translation enhancer	24–93
Melittin signal peptide (MSP)	Protein targeting	95–157
*Nco*I (contains start codon ATG)	Restriction enzyme site	162
StrepII-tag	Protein purification	170–193
Factor Xa recognition site	Tag cleavage	209–220
eYFP from *Aequorea victoria*	Reporter	221–934
*Not*I	Restriction enzyme site	936
*Xho*I	Restriction enzyme site	944
His_6-tag	Protein purification	965–982
*Bam*HI	Restriction enzyme site	992
*Xba*I	Restriction enzyme site	998
*Kpn*I	Restriction enzyme site	1004
TMV 3' UTR	Translation enhancer	1011–1256
*Eco*RI	Restriction enzyme site	1265
AmpR	Selection marker	1304–2269
Origin of replication (Ori)	Plasmid maintenance	2440–3028

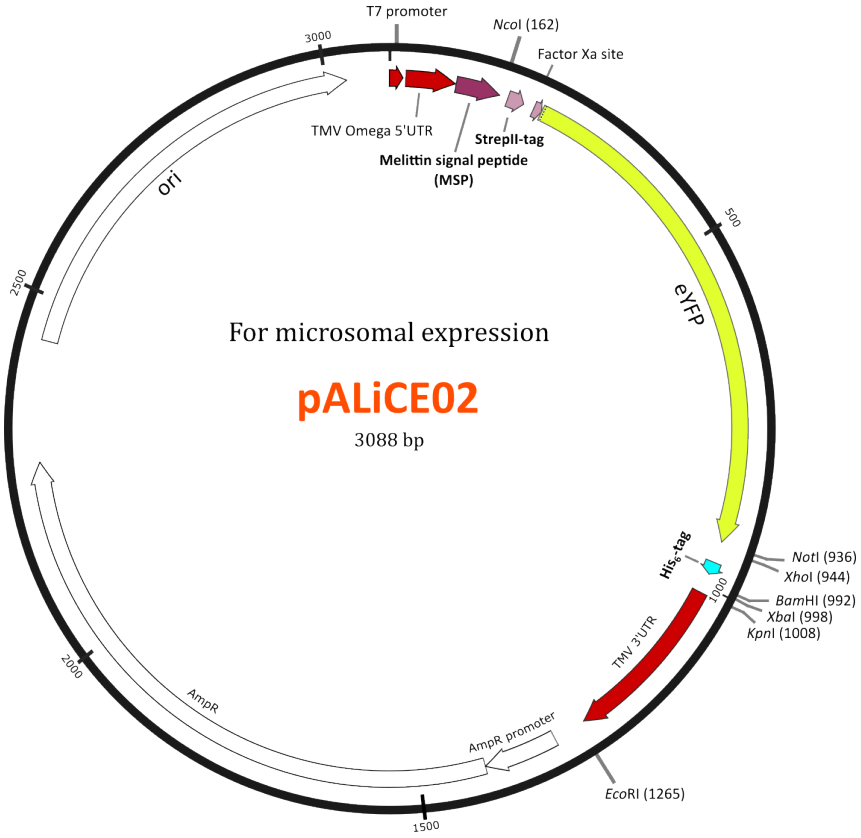

Figure 12. Plasmid map of pALiCE02.

Full sequence pALiCE02

TAATACGACTCACTATAGGGAGAGTATTTTTACAACAATTACCAACAACAACAACAAACAACAACAACATTACAT
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CAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTT
CAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAA
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CATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGA
CGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGG
CATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATAT
CATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCA
GCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAG
CTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGC
CGGGATCACTCTCGGCATGGACGAGCTGTACAAGGCGGCCGCTCTCGAGCCCGGGGGGGGTTCTCATCATCATCA
TCATCATTAGTAATAAGGATCCTCTAGAGGTACCAAGCTCTTCTGGTTTGGTTTGGACCTCTGGTCCTGCAACTT
GAGGTAGTCAAGATGCATAATAAATAACGGATTGTGTCCGTAATCACACGTGGTGCGTACGATAACGCATAGTGT
TTTTCCCTCCACTTAAATCGAAGGGTTGTGTCTTGGATCGCGCGGGTCAAATGTATATGGTTCATATACATCCGC
AGGCACGTAATAAAGCGAGGGGTTCGAATCCCCCCGTTACCCCCGGTAGGGGCCCATTATAGCCGAATTCGGCGC
GCCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTA
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CCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGT
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GAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCG
TATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGT
CACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACAC
TGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCA
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TCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCC
AGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATG
CTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCC
TTTTGCTCACATG

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