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コスモ・バイオ株式会社

LenioBio®
Applied Nature for Better Science

ALiCE®
Protein Expression Made Easy

Instruction Manual for Protein Expression with ALiCE®

Product numbers AL0103050 (ALiCE® Mini Kit)
AL0103200 (ALiCE® Midi Kit)
AL0103500 (ALiCE® Maxi Kit)

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Contents

Kit Contents

Mini-Kit

Component	Quantity	Concentration	Volume	Storage
ALiCE® Reaction mix	6	n/a	50 µL	-80 °C
Vector pALiCE01	1	250 ng DNA/µL	15 µL	-20 °C to -80 °C
Vector pALiCE02	1	250 ng DNA/µL	15 µL	-20 °C to -80 °C
ALiCE® Tube caps, perforated	6	n/a	n/a	Room temp.

Midi-Kit

Component	Quantity	Concentration	Volume	Storage
ALiCE® Reaction mix	6	n/a	200 µL	-80 °C
Vector pALiCE01	1	250 ng DNA/µL	50 µL	--20 °C to -80 °C
Vector pALiCE02	1	250 ng DNA/µL	50 µL	-20 °C to -80 °C
ALiCE® Tube Set, 12 pcs.	1	n/a	n/a	Room temp.

Maxi-Kit

Component	Quantity	Concentration	Volume	Storage
ALiCE® Reaction mix	6	n/a	500 µL	-80 °C
Vector pALiCE01	1	250 ng DNA/µL	50 µL	-20 °C to -80 °C
Vector pALiCE02	1	250 ng DNA/µL	50 µL	-20 °C to -80 °C
ALiCE® Tube Set, 12 pcs.	1	n/a	n/a	Room temp.

Equipment and material to be provided by user:

- Gloves
- Pipette filter tips (RNase-free)
- RNase-free water (e.g. water from a Millipore-filter unit)
- Orbital shaker
    - When reactions performed in tubes:
      Tabletop shaker (2 mL vessel insert), shaking diameter: 3 mm OR
    - When reactions performed in plates:
      Shaker with controlled humidity (up to 80 %), shaking diameter: 25 mm with plate holder and 96 half well plates (RNase free)

Important Notices

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

ALiCE® requires oxygen during the whole reaction time for a successful reaction. **Do not seal the reaction vessels!**

Shipping and Storage

ALiCE® kits are shipped on dry ice. Upon arrival, immediately store the components as indicated in the table above. The Reaction mix is stable for at least 12 months under these conditions.

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

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 if any issues with the delivery have occurred. The warranty remains in effect up to the expiration date indicated on the product label.

Quality Assurance

High quality chemicals and materials have been used to manufacture the components of this kit. Each lot is carefully tested to ensure that all components meet the stated specifications (see Certificate of Analysis).

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 the kit components. When handling ultra-cold material wear additional protective gloves to avoid frostbite. Used components are to be disposed of in accordance to the local regulations.

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.

Introduction

Cell-free protein expression (CFPE) systems derived from crude cell extracts have been used for decades as a research tool and have introduced many attractive advantages to protein expression technology. However, a critical barrier to the adoption of CFPE systems as alternatives to cell-based approaches has been the problem of relatively low protein yields.

To address this problem, LenioBio has taken advantage of plant cell biology and introduces a novel eukaryotic cell-free expression system: ALiCE®. ALiCE® offers unprecedented yields of up to 3 mg/mL of protein in batch mode. As such, it enables you to overcome the limitations of living host systems and to obtain "difficult to produce" proteins in a matter of hours instead of weeks. Advantages of the systems include among others:

- High-yield: Up to 3 mg/mL expressed protein
- Easy to use: One-step reaction protocol
- Fast: High yield in just 48 hours
- Flexible: Express "difficult to produce" proteins, cytotoxic proteins, etc.

ALiCE® utilizes tobacco cell lysates, depleted of vacuoles. The system contains all factors required for *in vitro* transcription (RNA polymerases, NTPs) and translation reactions (ribosomes, translation initiation/elongation factors, tRNA, etc.). Using a simple protocol, both the RNA transcription and translation take place in a single tube (coupled transcription/translation).

Reactions are started by simply mixing plasmid DNA with the ALiCE® Reaction mix. The outstanding protein yields of ALiCE® are based on highly efficient protein expression over the duration of the reaction. Consequently, we recommend a reaction duration of 48 h.

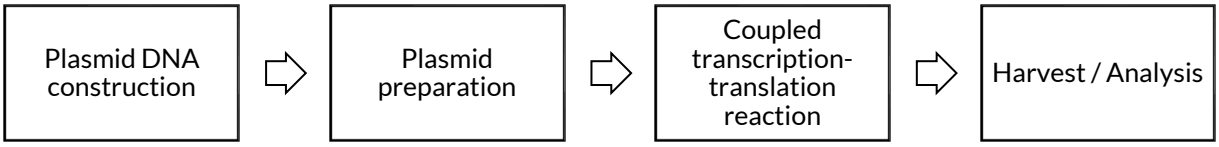
ALiCE® is provided with two expression vectors, pALiCE01 and pALiCE02, which are used according to the targeting of the protein of interest (see Page 5). These have been specially tailored to offer excellent protein yield when used with the ALiCE® expression system. pALiCE01 is used for expression in ALiCE® cytosolic fraction. Targeting the microsomes with pALiCE02 offers the opportunity to express protein with post-translational modifications like disulfide bonds.

Besides screening the ALiCE® system can also be used for larger scale expression of target proteins. Potential additional applications of the ALiCE® system include:

- Target protein characterization
- Protein optimization
- Mutant screening
- Protein-protein interactions
- Expression analysis
- Protein localization analysis
- Protein structure analysis
- Post-translational modification assays
- Metabolomics
- Herbicide screening

Protocols

Every use of the ALiCE® protein expression system follows a simple structure:



ALiCE® expression vectors

ALiCE® is provided with two different expression vectors, pALiCE01 and pALiCE02. pALiCE01 functions as a cytosolic expression vector whereas pALiCE02 uses a signal peptide for microsomal targeting.

Microsomes are organelle-like structures that form from parts of the endoplasmic reticulum during the manufacturing process of the ALiCE® Reaction mix. Using a melittin signal peptide, proteins can be translocated into these organelles (Buntru *et al.*, 2015), where they undergo post-translational modification. Subsequently, the microsomes can be disrupted to recover the expressed proteins (see Page 9).

pALiCE01 and pALiCE02 can also be used as a positive control for expression, expressing eYFP when added to the ALiCE® Reaction mix in the cytosol and the microsomes, respectively.

Template preparation by cloning into pALiCE vectors

A map including the features of the pALiCE01 and pALiCE02 vectors is provided below (Figure 1), as well as expanded views of the cloning sides in 5' and 3' direction of the gene of interest (Figure 2 and Figure 3).

The cloning site allows insertion of the gene of interest behind the T7 promoter and the 5' untranslated region. Cloning your gene of interest can either be done by subcloning from an existing vector or

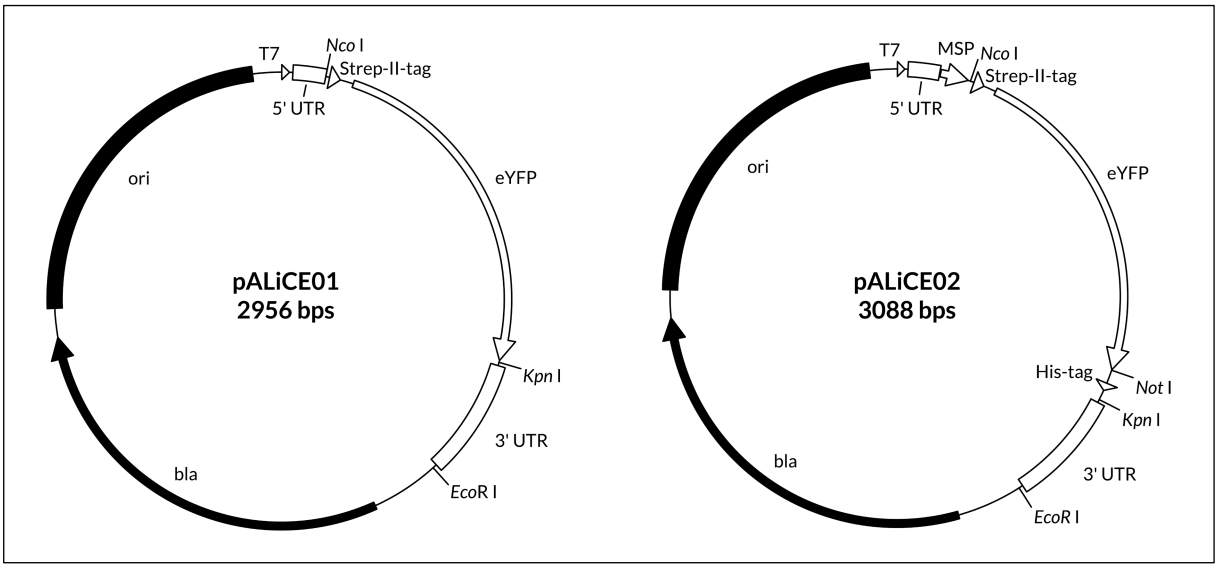


Figure 1: Map of pALiCE01 and pALiCE02 vectors and sequence reference points. Additional description: T7: T7 RNA polymerase promoter; 5' UTR: 5' untranslated region; MSP: melittin signal peptide; 3'UTR: 3' untranslated region; bla: β-lactamase gene (resistant to ampicillin); ori: origin of plasmid replication.

amplification of its coding region by PCR for the introduction of the appropriate restriction enzyme sites. Recommended restriction sites for the cytosolic expression plasmid pALiCE01 are *Nco* I and *Kpn* I while for the microsomal expression plasmid either *Nco* I / *Not* I or *Nco* I / *Kpn* I are recommended. If using *Kpn* I for cloning, please make sure the open reading frame contains a **Stop-codon**.

Table 1: Reference elements of the pALiCE vectors

Reference elements	pALiCE01	pALiCE02
T7	1-17	1-17
5' UTR	24-93	24-93
Nco I	93	162
MSP	-	95-157
Strep-II-tag	101-124	170-193
eYFP	152-868	221-934
Not I	-	935
His-tag	-	965-982
Kpn I	872	1004
3' UTR	879-1124	1011-1256
EcoR I	-	1265
Bla	1277-2137	1409-2269
Ori	2198-2896	2330-3028

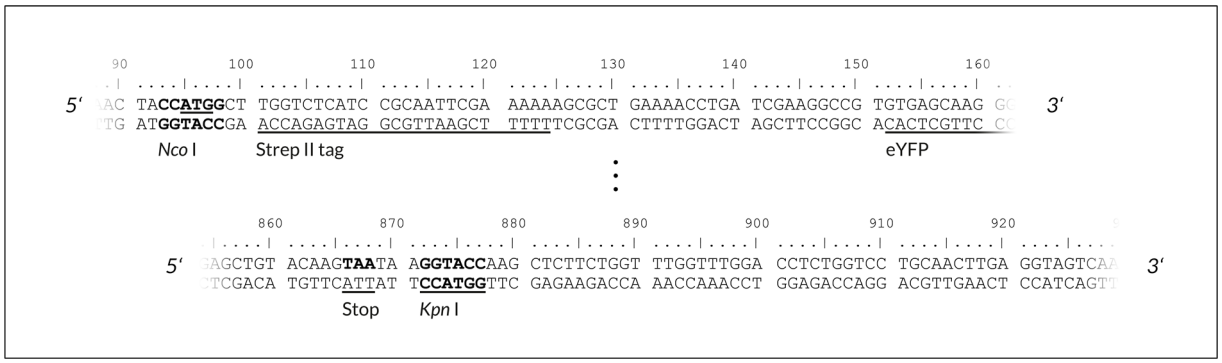


Figure 2: pALiCE01: View of the restriction sites of interest. Upper part: upstream cloning region of the gene of interest (here: eYFP). Lower part: downstream cloning region of the gene of interest. Please note, that the Start-Codon is a part of the restriction side of *Nco* I (underlined).

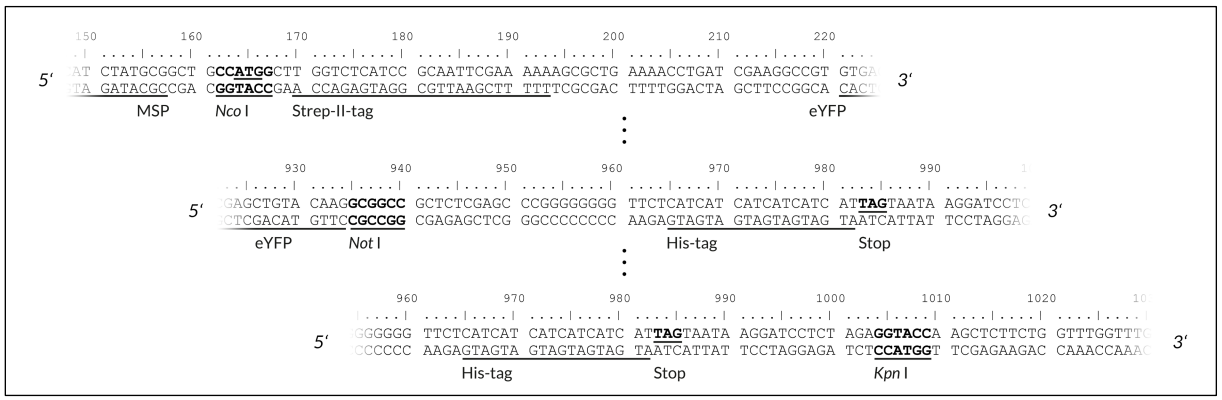


Figure 3: pALiCE02: View of the restriction sites of interest. Upper part: upstream cloning region of the gene of interest (here: eYFP). Lower part: downstream cloning region of the gene of interest. With choosing either *Not* I or *Kpn* I a His6-tag can be added. If using the *Kpn* I restriction site, please add a Stop-Codon to your gene of interest.

After the insertion of the gene of interest by standard cloning techniques (Green & Sambrook, 2012) the plasmid is transferred into competent *E. coli* DH5α-cells. Use of other *E. coli* strains may have an adverse effect on target protein yield due to lower plasmid quality. The vector encodes a β-lactamase (bla) to allow selection on **ampicillin** containing media.

Note: Using other vectors will most probably reduce protein yield and prokaryotic vectors are not applicable in ALiCE®.

LenioBio recommends amplifying the plasmid with your target gene in it through standard cloning while realizing the full potential of ALiCE® in your settings.

To circumvent introducing the plasmid into any cells to save time and labor, various methods are described for different cell-free systems, e.g. Rolling Circle method (Wang *et al.*, 2016, and Kumar & Chernaya, 2009).

Another way for you to circumvent introducing the plasmid into cells is to have your gene synthesized by a 3rd party and have it delivered to you in the LenioBio vector in sufficient amounts to apply directly in the lysate.

These methods have not been validated for high-yield production in ALiCE®

Purification of DNA

To facilitate an efficient transcription-translation reaction, a highly purified plasmid DNA preparation is required. It is therefore highly recommended to use a plasmid preparation procedure based on anion exchange chromatography (e.g. NucleoBond® Xtra Midi from Macherey-Nagel). For plasmid DNA preparation kits based on silica matrices we recommend addition of RNase inhibitor or phenol-chloroform purification of the template prior to the transcription-translation reaction. Adjust the final plasmid DNA to the desired concentration depending on the size of the insert by adding an appropriate volume of 5 mM Tris buffer (pH 8.5). The final concentration in the Reaction mix should be 5 nM. Store in aliquots at -20°C.

Coupled transcription/translation reaction setup

The typical reaction volume for coupled transcription-translation reaction is 50 µL.

1) **Choose the reaction vessel:**
   - ALiCE® tubes = 52 µL per reaction
   - 96 half well plates with lid[1] = 50 µL per reaction

2) **Prepare DNA**
   Remove plasmid DNA template from storage and thaw at room temperature (20 - 25 °C). Mix gently by flipping and briefly spin down to collect the content at the bottom of the tube then place on ice. Dilute or concentrate plasmid, the final concentration in the assembled reaction should be 5 nM. DNA concentration may significantly affect protein yield.

3) **Thaw ALiCE® Reaction mix**
   Remove the ALiCE® Reaction mix from storage and thaw in a water bath at room temperature (20 - 25 °C). Mix gently by inverting the tube. **Do not vortex!** Place on ice directly after thawing. Start the reactions within 30 min after thawing. Freeze remaining lysate directly at -80°C. **Do not use liquid nitrogen!** Avoid more than one freeze-thaw cycle!

[1] For best results, use greiner bio-one, Art. No. 675086

4) Reaction assembly and reaction

a) ALiCE® tubes

- Assemble in ALiCE® tube on ice according to the following table:

Component	Final concentration	Volume per reaction
ALiCE® Reaction mix	n/a	50 µL
pALiCE01 / pALiCE02 vector	5 nM	2 µL*
Total volume		**52 µL**

* When using pALiCE plasmids with different inserts, dilute or concentrate to 5 nM final molarity.

- Only use the supplied punctured caps to close ALiCE® tubes.

- Incubate the ALiCE® tubes in an orbital tabletop shaker at 700 rpm and 25 °C for 48 h. We specify a shaking diameter of 3 mm and the use of a holding block capable of holding 2 mL vessels for optimal results.

Note: Constant oxygen supply is needed throughout the complete reaction cycle. Therefore, the reaction volume in the tubes can be increased up to 200 µL while observing a decreased productivity. For reaction volumes above 200 µL it is recommended to divide the sample into smaller volumes.

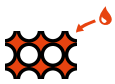
b) 96 half well plates

- Assemble in 96 half well plate on ice according to the following table:

Component	Final concentration	Volume per reaction
ALiCE® Reaction mix	n/a	48 µL
pALiCE01 / pALiCE02 vector	5 nM	2 µL*
Total volume		**50 µL**

* When using pALiCE plasmids with different inserts, dilute or concentrate to 5 nM final molarity.

- Pipette water into the spaces between the reaction compartments of the plate (75 µL each) to maintain a high humidity inside the plate during incubation.



- Incubate the plates in an orbital shaker at 500 rpm and 25 °C for 48 h. We specify a 25 mm shaking diameter and controlled humidity of > 70 % for optimal results.

Note: Evaporation may occur if the reaction humidity is not controlled to > 70 %, also protein expression yield may be diminished.

Do not seal reaction vessels or plates!

Detection of produced proteins

Synthesized target proteins can be detected by SDS-PAGE. In order to ensure a good separation of the expressed protein from background proteins SDS-PAGE requires high resolution and an appropriate gel concentration. We recommend an SDS-PAGE gradient gel and loading of a maximum of 1 µL of sample.

Isolation of produced proteins

Recombinant proteins produced with the ALiCE® system can be purified with conventional chromatography separation methods.

Recovery of proteins produced in the microsomes

Expressing proteins with ALiCE® using the pALiCE02 vector will result in the target protein being produced in the microsomes of the Reaction mix. To release the target protein from the microsomes, the following steps are taken.

1) Add 0.5% n-dodecyl-β-maltoside (DDM) to ALiCE® reaction in order to lyse microsomes harboring the synthesized protein.

2) Incubate for 10 min at room temperature with shaking at 700 rpm. **Do not vortex!**

3) Centrifuge the lysis reaction at 16,000 x g for 10 min at room temperature. Transfer the supernatant containing the synthesized protein to a new reaction tube.

4) Keep in mind that the DDM in the supernatant may affect subsequent steps taken, e.g. activity assays or chromatography purification.

Troubleshooting

If the protein of interest is not produced or only produced in very small quantities, the following issues should be considered:

Problem	Possible cause	Recommended action
Low yield of target protein	Kit component deterioration	Make sure the expiration date on the label is not exceeded. Make sure all the components have been stored at the appropriate temperature.
	Poor plasmid DNA quality	Add RNase inhibitor to the purified DNA. Alternatively, phenol-chloroform purify the template prior to the transcription-translation reaction.
	Inappropriate plasmid DNA concentration	Check concentration of plasmid DNA. Titrate plasmid DNA concentration in a range of 1 to 20 nM in the transcription-translation reaction. For some templates lower or higher plasmid DNA concentration influences the level of protein production.

Problem	Possible cause	Recommended action
Low yield of target protein	Proteolytic degradation or precipitation	Analyze samples after a short incubation time (a few hours) for the presence of the target protein.
	Poor oxygenation	Ensure proper shaking of the samples. Do not seal the plate or the vial with gas-tight tape or film.
	Mechanical damage	Do not vortex the Reaction mix, pipette carefully.
	Temperature fluctuation	Maintain a stable production temperature over the complete reaction cycle – lower temperature can lead to lower productivity and higher temperatures may possibly damage ALiCE®.
Evaporation of sample during the reaction	Wrong reaction vessel	Use ALiCE® Tubes with perforated caps provided by LenioBio.
	Environmental temperature too high	Make sure to control the environmental temperature to no higher than 25 °C.
	Environmental humidity too low	When using 96 half well plates, obtain a reaction environment humidity level of optimally 80 %. When using ALiCE® tubes in a tabletop shaker, control the environment humidity to above 65 %.
	Air circulation	Do not place ALiCE® directly next to a ventilator or a machine with exhaust air.

References

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) Green, M. R., Sambrook, J. 2012. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press.

3) Kumar, G., Chernaya, G. 2009. Cell-free protein synthesis using multiply-primed rolling circle amplification products. Biotechniques, 47(1):637-9.

4) Wang, K., Ma, Q., Jiang, L., Shujuan, L., Lu, X., Hou, Y., Wu, C., Ruan, J. 2016. Ultra-precise detection of mutations by droplet-based amplification of cirucularized DNA. BMC Genomics, 17:214.

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