Application Note

LenioBio

A eukaryotic cell-free platform for reliable expression of functional membrane proteins

Introduction

Membrane proteins – owing to their cell surface accessibility and vital roles in cellular regulation – are well-established drug targets, contributing to around 55% of the biologics market (1). Various applications, including drug discovery, require purified membrane proteins, which are often challenging to obtain. Their aggregation-prone nature results in low expression and purification yields, necessitating specialist know-how and equipment (such as ultracentrifuges) to optimize expression and purification. A simplified workflow that eliminates the need for such dedicated resources would facilitate fast screening of drug targets. Here, we describe the successful expression of two human G-protein coupled receptor (GPCR) membrane proteins – β2-adrenergic receptor (ADRB2) and cannabinoid receptor 2 (CB_2) – in the eukaryotic cell-free platform ALiCE®. ALiCE is particularly suited for membrane protein expression as it contains native microsomes (derived from the endoplasmic reticulum and Golgi), forming a scaffold into which membrane proteins can insert themselves. And unlike cell-based systems, no cell-lysis is required for further downstream processing. In addition to successful expression, we also showed that the activity profile of cell-free produced CB_2 is comparable to CB_2 expressed from cell-based systems.

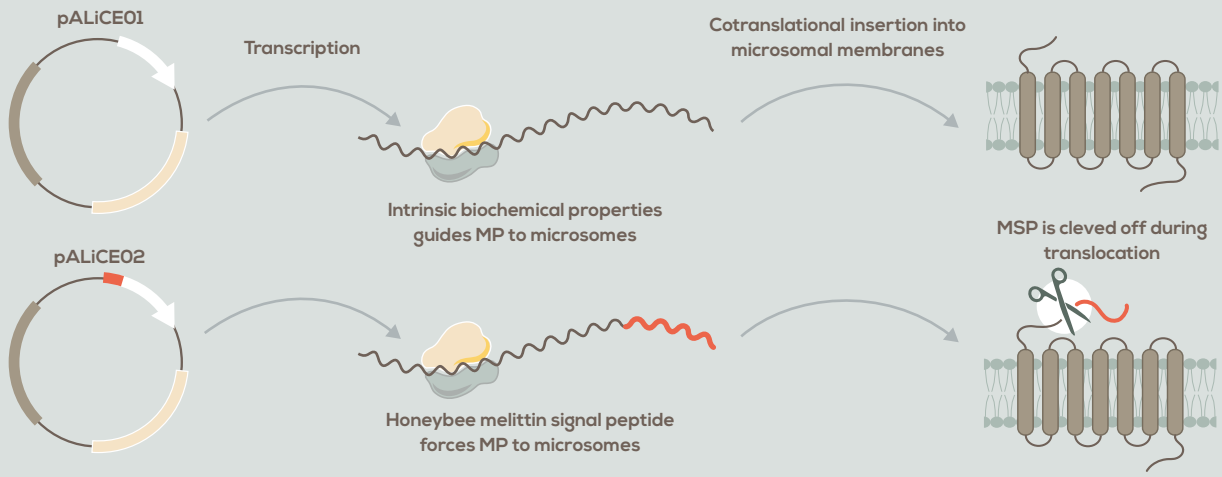
ALiCE is a scalable eukaryotic cell-free protein expression system capable of producing even the most complex proteins in under 48 hours. The proprietary cell-free lysate derived from *Nicotiana tabacum* c.v. BY-2 cells contains all of the machinery necessary to implement eukaryotic post-translational modifications, without specific optimizations. Learn more about ALiCE at www.leniobio.com/technology

Materials and Methods

Creation of constructs for membrane protein expression

The ALiCE protein expression kit comes with two vectors: pALiCE01, which contains all essential elements for cytosolic protein expression, and pALiCE02, which adds an N-terminal melittin signal peptide (MSP) to actively target the protein-of-interest to the microsomes. We recommend starting your expression experiment using pALiCE01, as many membrane proteins contain structural elements or native signal peptides that naturally direct them to membranes and therefore do not require the melittin signal peptide for microsomal targeting. If expression using pALiCE01 is not successful, or protein yield optimization is desired, we suggest cloning into pALiCE02 (Figure 1). For this application note, the genes encoding ADRB2 and CB_2 proteins were cloned into both vectors to assess performance. The ADRB2 gene was cloned with a C-terminal mOxVenus gene encoding a green/yellow fluorescent protein for easy readout of protein expression yield. The CB_2 gene was cloned with an N-terminal Maltose Binding Protein (MBP) tag, a commonly-used tag to increase the solubility of its fusion partner, thereby increasing expression levels.

Figure 1 : **Schematic overview comparing membrane protein expression using the two ALiCE vectors – pALiCE01 and pALiCE02**



Cell-free expression reaction

Small-scale ALiCE reactions of 50 µl were performed as suggested in the ALiCE user manual. The lysate was thawed on ice, and expression plasmids were added to a final concentration of 5 nM for CB_2 and 10 nM for ARDB2 (1). Reaction mixtures were aliquoted into half-well 96-well plates and incubated for 48 h in a KuhnerShakerX at 25°C with 75% humidity and 500 rpm with a 12.5 mm shaking diameter (2). The Duetz lid system (Prod. ref. CR1296, EnzyScreen BV) was used to reduce fluid loss and ensure even evaporation rates across the plate. The same results should be obtainable using the tubes provided in the ALiCE kits and a non-humidified incubator as per the ALiCE user manual.

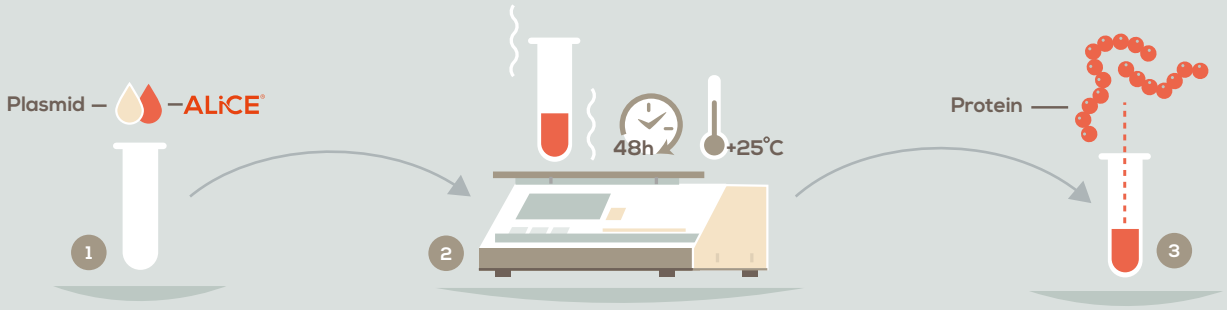


Figure 2 : Microsomal recovery of membrane proteins

Microsomal release of proteins

Release of the expressed membrane protein from the microsomes for quantification was performed as per the ALiCE user manual (Figure 2, Step 3). In brief, the crude lysate was centrifuged for 20 min. (4°C at 16,000 x g) and the supernatant (sample S1) was removed. Next, the pellet which contains the microsomes was re-suspended in 2x PBS and an equal volume of a 1% w/v stock of the detergent n-dodecyl-β-maltoside (DDM) and incubated at room temperature for 30 min. During this time, the detergent solubilizes the membrane protein from the microsomal membrane by embedding it in a DDM micelle. Following a final centrifugation step (16,000 x g for 30 min at 4°C), the solubilized membrane protein was collected in the supernatant (sample S2).

Protein quantification and Western blot analysis

1 µL of lysate was used for SDS-PAGE analysis using 4–15% Mini-PROTEAN® TGX™ Precast Protein Gels, 15-well (Bio-Rad), followed by Western Blot analysis with HRP conjugated anti-StrepII antibody (StrepMAB-Classic, IBA® Lifesciences) for protein visualization.

Additionally, for ADRB2, the fluorescence of mOxVenus was measured in black 96-well plates using Infinite® M1000 (Tecan®) at a wavelength of 485/528 nm (excitation/emission). Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific™, Cat No.: 23225). Fluorescence was normalized to protein concentration to compare the target protein amount per total protein amount (fluorescence intensity/amount of total protein). The mOxVenus fluorescent tag also allows for convenient in-gel visualization and estimation of protein concentration.

CB_2 activity assay

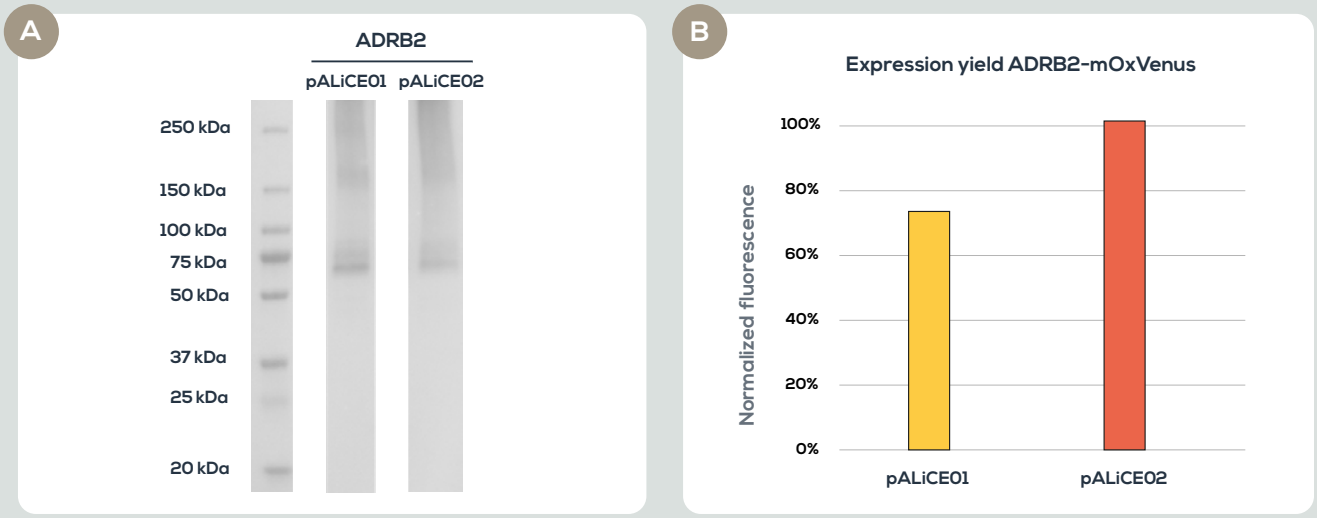
After 48 h, the crude lysate from the CB_2 expression reaction was used the GPCR agonist 10 µM CP-55,940 (Cayman Chemical), ^{35}S-γ-GTP, Gαi1 (100 nM) and Gβ1γ2 (500 nM). Activity was estimated via scintillation counting to compare ALiCE-expressed MBP-CB_2 with MBP-CB_2 expressed from eukaryotic Expi293™ cells (Thermo Fisher Scientific™).

Results and Discussion

Expression of membrane proteins is possible from both pALiCE01 and pALiCE02

Successful expression of ADRB2 and CB_2 from both pALiCE01 and pALiCE02 was demonstrated by Western Blot analysis (Figure 3A and Figure 4A). Additionally, for ADRB2, expression yields were compared using fluorescence signal detection (Figure 3B). Levels of pALiCE02-expressed ADRB2 were about 25% higher than of pALiCE01. This suggests a more targeted expression of ADRB2 in the microsomes due to the presence of MSP at the N-terminus of the pALiCE02 construct in this instance.

Figure 3 : Expression yield comparison of ADRB2-mOxVenus in pALiCE vectors.



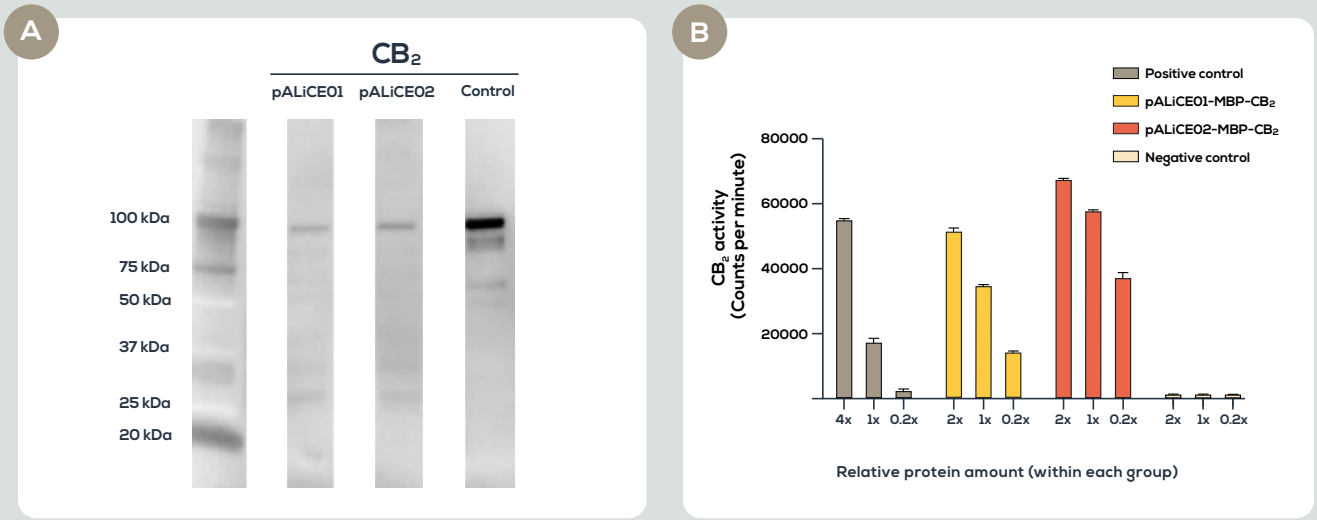
A Anti-StrepII Western blot of supernatant S2 fraction of pALiCE-ADRB2 expression after DDM treatment.

B Relative fluorescence of S2 fractions of pALiCE-ADRB2 expression normalized to protein concentration. The maximum fluorescence value of pALiCE02 was set to 100% for comparison.

The activity of cannabinoid receptor CB_2 expressed in ALiCE is comparable to cell-based expression

Successful expression of membrane proteins becomes void if the expressed proteins fail to retain their activities. We, therefore, compared the activity of ALiCE-expressed CB_2 (crude lysate) to that of purified CB_2 expressed in the eukaryotic Expi293 cell line (positive control). As shown in Figure 4B, the activity of CB_2 expressed from both vectors – pALiCE01 and pALiCE02 – was comparable to the positive control representing cell-based expression. Remarkably, CB_2 expressed in ALiCE showed a favorable activity profile even though the assay was performed using crude lysate. This highlights the unique advantage of using ALiCE for rapid drug screening without the need for additional downstream processing for GPCR activation assays.

Figure 4 : Expression profile and activity assay of CB_2 expressed in ALiCE.



A Anti-StrepII Western blot of supernatant S2 fraction of pALiCE-MBP-CB_2. Positive control of MBP-CB_2 standard (200 ng) was included for reference.

B CB_2 activity assay comparing the activity of CB_2 expressed from pALiCE01 and pALiCE02 to the positive control, purified CB_2 expressed in Expi293 cell line. pALiCE01-eYFP was included as negative control.

Conclusion

Membrane proteins have always been challenging to work with. Yet, as high-value targets for drug discovery, they hold significant potential for growth in the future. Classic cell-based expression and purification of membrane proteins in their native lipid environment typically yield low protein amounts (2). Even cell-free expression of membrane proteins like GPCRs has only achieved limited success despite decades-long efforts (3).

With cell-free expression using ALiCE, we successfully expressed two challenging GPCRs – ADRB2 and CB_2 – with a remarkable yield of up to 200 µg/mL of lysate (4) without extensive optimization. Furthermore, we showed that the activity of ALiCE-expressed cannabinoid receptor CB_2 is comparable to more native CB_2 expressed in a cell-based system. The ability of ALiCE to reliably produce 'difficult-to-express' membrane proteins while retaining their activity makes it a useful alternative to cell-based expression for fast screening of functional molecules.

References

1. Sood, V. D. & Gross, A. W. "Membrane proteins as targets for biological drugs".Advances in membrane proteins: building, signaling and malfunction (2019): 49–65.
2. Bernaudat F., et al. "Heterologous expression of membrane proteins: choosing the appropriate host". *PLoS One* 2011;6(12):e29191.
3. Orbán, E. et al. "Cell-free expression of G-protein-coupled receptors". Methods Mol Biol. 2015;1261:171–195.
4. "ALiCE®: A versatile, high yielding and scalable eukaryotic cell-free protein synthesis (CFPS) system" (in pre-print at www.biorxiv.org/content/10.1101/2022.11.10.515920v1.full)

Ordering information

Product	Contents	Catalog number
ALiCE® for Research - Cell-Free Protein Expression Mini Kit	6 x 50 µl ALiCE lysate, pALiCE01 and pALiCE02 vectors	AL00000001
ALiCE® for Research - Cell-Free Protein Expression Midi Kit	6x200 µl ALiCE lysate, pALiCE01 and pALiCE02 vectors	AL00000002
ALiCE® for Research - Cell-Free Protein Expression Maxi Kit	6 x 500 µl ALiCE lysate, pALiCE01 and pALiCE02 vectors	AL00000003

Can't wait to put ALiCE® to work on your protein-of-interest?

- www.leniobio.com/webshop

Get in touch to learn more about our contract research Protein Services options.

- connect@leniobio.com

LenioBio GmbH,
Erkrather Straße 401
40231 Düsseldorf
Germany

www.leniobio.com