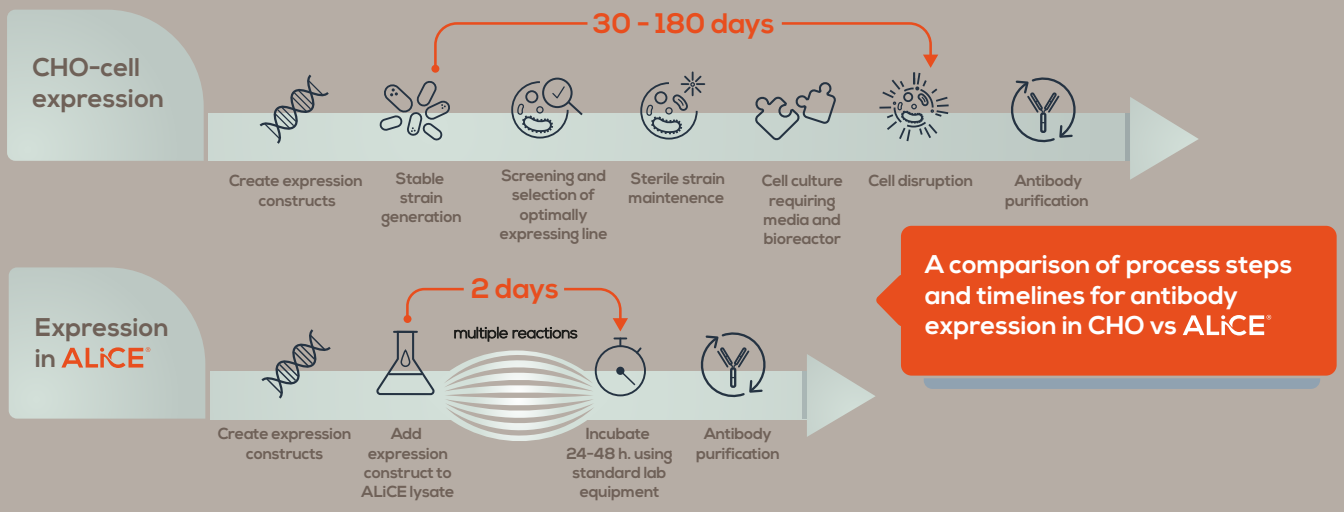
LenioBio

Expression of a functional therapeutic antibody in the ALiCE cell-free protein expression system

Introduction

Monoclonal antibodies (mAbs) and other therapeutic antibody variants such as antibody-drug conjugates (ADCs), bi-specifics, and antibody fragments fuel the treatment of cancer and autoimmune diseases. Despite their widespread use, early screening to developability assessment still relies on material obtained through labor- and time-intensive cell-based expression in higher eukaryotic systems. Can we accelerate the time-to-approval of antibody candidates by reliably producing them in useful quantities in a cell-free system? In this Application Note, we demonstrate the successful production of the blockbuster mAb adalimumab (sold under the name Humira®) [1] in the eukaryotic cell-free platform ALiCE®. Adalimumab is a human monoclonal IgG1 antibody that binds tumor necrosis factor α (TNF-α) and contains 12 intra- and 4 intermolecular disulfide bonds and 2 glycosylation sites [2]. Here we demonstrate the dual expression of adalimumab heavy and light chains in a single cell-free reaction, together with in-lysate assembly of the functional, tetrameric antibody [3]. Cell-free expression can accelerate antibody screening while retaining functionality (Figure 1.)

Figure 1: Comparison of cell-based and cell-free protein expression systems for antibody production.



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 mAb expression constructs

Heavy and light chain sequences of adalimumab were cloned into the pALiCE02 vector. This vector contains the honeybee melittin signal sequence for microsomal protein targeting. The two constructs were added to the reaction mix in different heavy:light chain ratios (2:1, 1:1, 1:2) to assess which would give the most fully assembled antibody.

Cell-free mAb expression

Small-scale BYL CFPS reactions of 50 µl were performed as described in the ALiCE user manual to test ideal ratios and plasmid concentrations. The lysate was thawed on ice, and expression plasmids were added to a final concentration of 5, 7.5, 10, and 15 nM (1). Reaction mixtures were aliquoted into half-well 96-well plates and incubated for 48 h in a KuhnerShaker LT-X 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 loss of liquid and to ensure even evaporation rates across the plate. After 48 hours, expressed protein was ready for collection and downstream analysis (3).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.

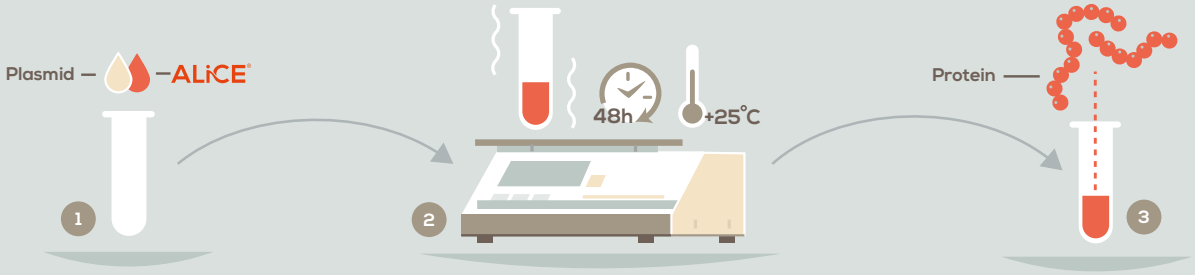


Figure 2 : Steps involved in cell-free mAb expression using ALiCE

Scale up and mAb purification

Reaction volumes of 50 µl are sufficient for heavy:light chain optimization, Western blot and some downstream analysis. However, larger volumes were needed to obtain sufficient antibody for the comprehensive functional analysis we planned in this proof of concept study. Therefore, cell-free reactions were scaled to 10 ml using 250 ml shaker flasks with shaking speed of 105 rpm. A LT-X shaker incubator (Kuhner) with on-line respiration monitoring and humidity control (TOM system, Kuhner) was used. mAb purification was carried out using a protein A affinity column following a standard protocol (Figure 2, Step 3).

mAb functionality analysis

Protein A-purified mAb was used to assess mAb functionality. ELISA assay was performed using TNF-α as the ligand. To assess glycosylation, N-glycopeptide analysis was performed on trypsin-digested peptides via mass spectrometry analysis [4]. To assess antibody purity and stability, SDS-PAGE was performed under reducing (R, +DTT) and non-reducing (NR, -DTT) conditions followed by Western blot analysis using anti-IgG antibody [4]. Lastly, to compare and quantify binding affinities of adalimumab expressed in ALiCE and mammalian CHO (commercially available chinese hamster ovary) cells to the ligand TNF-α (Abcam), surface plasmon resonance (SPR) analysis was performed [4]. Contact us for detailed information on SPR analysis.

Results and Discussion

Correct assembly of mAb heavy and light chains in ALiCE

The correct assembly of heavy and light chains is essential for the maturation of antibodies into fully functional proteins. In particular, the success of an antibody expression system relies heavily on the correct formation of intra- and intermolecular disulfide bonds.

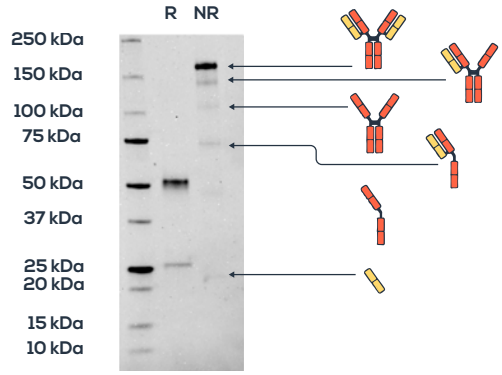


Figure 3. Western blot analysis, demonstrates a high proportion of fullly-formed, tetrameric Adalimumab.

SDS-PAGE was performed under reducing (R, +DTT) and non-reducing (NR, -DTT) conditions to visualize full-size antibody versus split heavy and light chain. We show that under non-reducing (NR) conditions, a large fraction of the full-sized antibody is present.

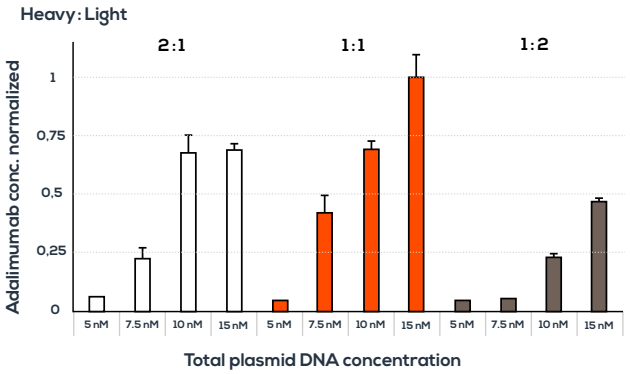
Optimizing the heavy-to-light chain ratio yields improved mAb assembly

ALiCE, compared to cell-based systems, offers a significant advantage by facilitating fast and easy expression of multiple proteins simultaneously. This is achieved by simply adding different plasmids to the ALiCE reaction mix in a molar ratio that reflects the theoretical stoichiometry of the protein complex. In this case, two plasmids expressing heavy and light chain sequences of adalimumab were combined in a 1-step cell-free reaction to obtain fully assembled, functional anitibodies.

To maximize the proportion of fully assembled molecules, different molar ratios should be tested for each new protein complex, as illustrated here for adalimumab. Different heavy-to-light chain ratios were tested at different total plasmid DNA concentrations. The expected stochiometric ratio of 1:1 at a total plasmid DNA concentration of 15 nM (7.5 nM for each chain fragment) gave the highest expression of fully-assembled antibodies. The total amount of adalimumab in the lysate was quantified using an ELISA assay (Figure 4).

Figure 4. An optimal ratio of 1:1 heavy-to-light chain constructs was identified using ALiCE.

The bar chart shows total amount of adalimumab in the lysate, quantified using an ELISA assay. Results are shown for different ratios of heavy and light chain, and different total plasmid DNA concentrations.

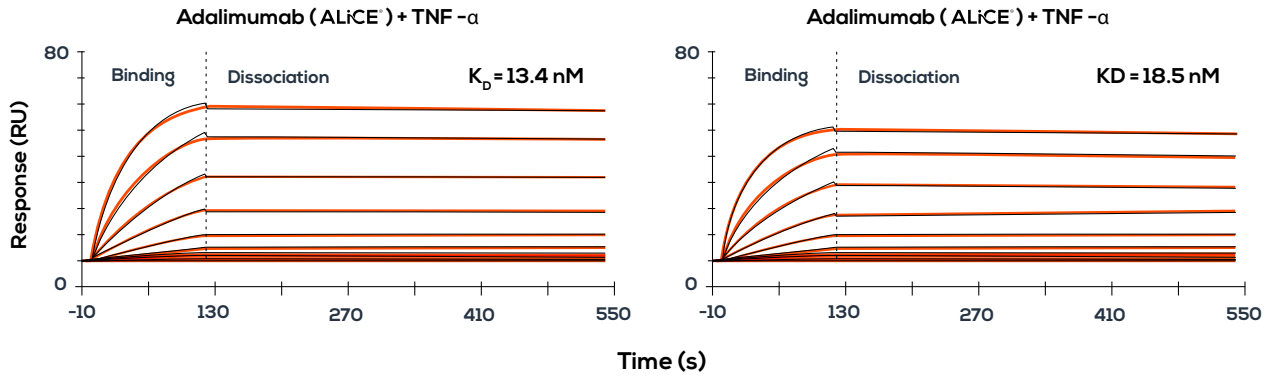


ALiCE-expressed adalimumab is functional and comparable to the commercially available version

How does adalimumab expressed in ALiCE compare with commercially available adalimumab expressed in CHO cells, a commonly used expression system for complex eukaryotic proteins? Adalimumab binds TNF-α to inhibit TNF-α/TNFR complex formation reducing pro-inflammatory signal cascades [2]. A surface plasmon resonance (SPR) analysis was carried out measuring the dissociation constant of adalimumab with TNF-α. Both adalimumab samples from ALiCE and CHO cells showed similar dissociation constants in the double-digit nM range (Figure 5).

Figure 5. Comparable dissociation constants for TNF-α interaction with adalimumab, expressed in both ALiCE and CHO cells, were measured using SPR analysis.

SPR analysis of adalimumab and its binding partners. Immobilized antibody molecules produced from either ALiCE or CHO cells were proved with recombinant TNF-α to show ligand and receptor binding respectively.



Glycan analysis (data not shown) also demonstrated a high degree of N-glycosylation occupancy. See our article for full details of glycan analysis.

Conclusion

mAbs have emerged as an important modality in treating cancer and other diseases. Using the ALiCE cell-free system, we successfully expressed a clinically relevant antibody, adalimumab, at a pace that challenges established cell-based systems. As demonstrated, optimization of plasmid ratios for correct tetramer formation can be performed very rapidly in this system. We further showed that ALiCE-expressed adalimumab is functionally identical to a reference expressed in a cell-based system. These results highlight the advantages of ALiCE over cell-based systems to quickly test and validate parameters for optimal mAb expression.

Antibodies expressed in ALiCE can be conveniently used for in vitro studies, especially when small changes are desired in the antibody construct, circumventing the need to spend weeks in cell culture. Furthermore, the flexibility of the ALiCE expression system allows easy adaptation to express other antibody derivatives or fragment variations. Cell-free expression is emerging as a promising alternative to eukaryotic cell-based systems for faster screening of therapeutic antibodies.

References

1. Patient information webpage: www.humira.com
2. Bellinvia S. et al. Adalimumab Biosimilars in Europe: An Overview of the Clinical Evidence. BioDrugs. 2019;33(3):241–253.
3. Das Gupta, M., et al. 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)
4. Palladino MA, Bahjat FR, Theodorakis EA, Moldawer LL. Anti-TNF-alpha therapies: the next generation. *LTttOazt ÐpuetÐerXlzo* 2003;2(9):736–746.

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

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