LenioBio

A cell-free future for protein production

How cell-free protein synthesis is challenging current paradigms in biomanufacturing.

- A new generation of cell-free systems
- Versatility to express simple to challenging protein classes
- Reducing complexity and timelines without compromising on yield
- Producing proteins with complex PTMs, painlessly
- Scaling protein production with demand

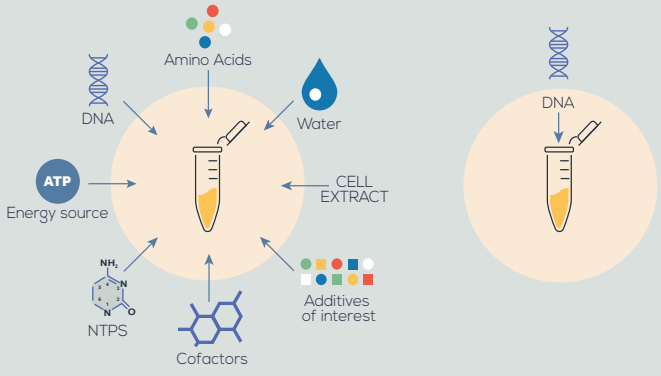
INTRODUCTION

With more than 15,000 protein therapeutics currently under development and the Global Protein Therapeutics Market expected to reach $490.2 billion by 2028 (1), we truly are in the age of proteins. Yet, protein production still relies on laborious and contamination-prone cell-based expression of recombinant proteins. Slow development timelines and high attrition rates of protein drug candidates (2), particularly therapeutically promising but 'difficult-to-express' proteins, should incentivize drug developers to adopt more efficient methods of protein production. When so many technological advancements have been made in other sectors, why are we still using antiquated methods for potentially life-saving therapeutics?.

Cell-free protein synthesis (CFPS) systems have for many years promised a faster and easier means of protein production. Yet, while various cell-free systems have been developed, they have remained firmly in the domain of small-scale research laboratories, blighted by reputation for lack of reproducibility, low protein yields, inability to correctly implement post translational modifications (PTMs), and lack of scalability. In this White Paper, we challenge the current protein production status quo and share data on a new generation of cell-free systems that will change the face of protein production.

A new generation of cell-free systems

CFPS enables protein production without the constraints imposed by a living cell. It harnesses the cell's protein synthesis machinery for in vitro expression of proteins under diverse conditions. In addition to a cellular lysate, typical components include protein-encoding DNA and other mandatory substrates such as energy sources, nucleotides, amino acids, salts, and cofactors (1). ALiCE differs from this in that all of these components are already included in the system (2).



The benefits offered by CFPS systems are manifold. They enable proteins to be produced in a controlled environment, can be used to express proteins that are typically difficult-to-express or toxic to cells, and more easily incorporate novel PTMs. Perhaps most striking, is the unprecedented speed of these systems CFPS eliminates the need for time-intensive cell culture processes, condensing the DNA to protein timeline into hours (5).

Here we explore recent developments in CFPS and introduce a scalable and versatile eukaryotic CFPS system derived from Nicotiana tabacum BY-2 cells, commercialized as ALiCE® (standing for Almost Living Cell-Free Expression). Setting it apart from other recent newcomers to the CFPS market is its ability to reproducibly **produce a wide range of proteins at unusually high yields**, **express complex eukaryotic PTMs**, **and produce proteins at scale**.

Versatility to express simple to challenging protein classes

Cell-free systems differ in their ability to express different protein types. For instance, cell-free systems based on E. coli can express soluble, disulfide-bond containing, and membrane proteins but not glycoproteins. Many eukaryotic cell-free systems require supplementation to perform the more complex post-translational modifications. The ALiCE system is distinguished from other CFPS systems by its ability to express a wide range of proteins, including difficult-to-express classes like membrane proteins (see Table 1). This is made possible through two protein production pathways – one cytosolic for expressing unmodified proteins and one microsomal for expressing proteins requiring PTMs. The presence of native, actively-translocating microsome vesicles gives ALiCE an important edge over other cell-free systems in performing PTMs. Examples of the diverse range of complex proteins that have been successfully expressed in ALiCE are listed in Figure 1, Table 2.

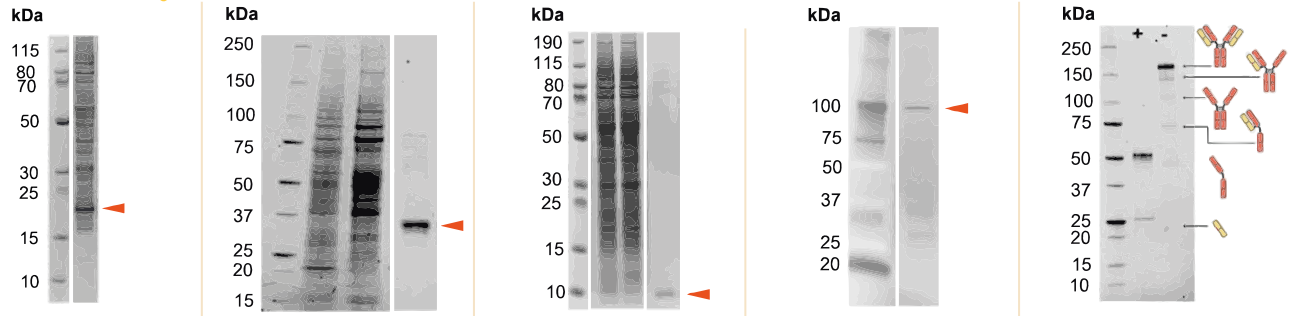
Table 1. ALiCE is superior in producing varied protein classes compared to other CFPS

	Cytosolic proteins	Disulfide proteins	Membrane proteins	Glycoproteins	Costs per gram protein
ALiCE®	Yes	Yes	Yes	Yes	Low
Archaea/Protozoa	Yes	No	No	No	High
E. coli	Yes	Possible	Possible	No	Medium
Wheat Germ	Yes	Possible	Possible	No	High
Yeast	Yes	No	No	Possible	High
Rabbit Reticulocyte	Yes	Possible	Possible	Possible	High
CHO/Human	Yes	Possible	Yes	Yes	High
Insect	Yes	Possible	Yes	Yes	High

Table 2. ALiCE has been used to produce varied proteins.

Image in Figure 1	Protein class	Protein	Features
A	Virus-like particle	Hepatitis B Core (HBc) virus-like particle subunit	21 kDa monomer which multimerizes to form virus-like particle structures.
B	Antigen	SARS-CoV-2 receptor-binding domain (RBD)	25 kDa protein with four intramolecular disulfide bonds and two N-glycosylation sites.
C	Cytokine (growth factor)	Human Epidermal Growth Factor (hEGF)	53 amino acid, 6 kDa peptide possessing three intramolecular disulfide bonds.
D	G-coupled protein receptor (GPCR) membrane protein	Human Cannabinoid Receptor (hCB-2)	44 kDa protein with additional N-terminal maltose-binding protein (MBP), StrepII-tag and His-tag. Contains seven transmembrane spanning domains, and two N-glycosylation sites.
E	Antibody	Adalimumab	Complex heterotetramer of 150 kDa comprised of two heavy and two light chains, together containing two N-glycosylation sites, 12 intramolecular and four intermolecular disulfide bonds.

Composite gel images showing varied proteins expressed using the ALiCE cell-free protein expression system. Full gel images can be viewed in our recent publication on BioRxiv



A SDS-PAGE of the Hepatitis B core (HBc) virus-like particle monomer in the ALiCE lysate.

B SDS-PAGE of the SARS-CoV-2 Receptor Binding Domain (RBD). Shown are: P-insoluble pellet fraction; S - soluble fraction; E – elution fraction.

C SDS-PAGE of the human Epidermal Growth Factor (hEGF). Shown are: P- insoluble pellet fraction; S - soluble fraction; E – elution fraction.

D Anti-Strep-II Western blot of the human Cannabinoid Receptor. Larger band size is due to inclusion of N-terminal maltose binding protein (MBP) tag..

E Anti-IgG Western blot of the monoclonal antibody Adalimumab under reducing (+) and non-reducing (-) conditions.

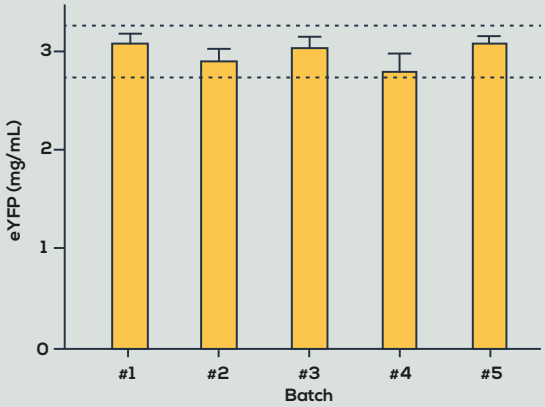
CTA: For full data on proteins displayed here, read our papar on BioRxiv

Reducing complexity and timelines without compromising on yield

Cell-based manufacturing of recombinant proteins entails complex and elaborate infrastructure, with many steps requiring a highly sterile environment. Cell-free systems do not need much of this infrastructure, since sterile maintenance of recombinant cells is not required. For instance, the average laboratory can run an expression in ALiCE with existing lab equipment – protein synthesis is initiated simply by adding DNA, as the lysate comes with all other necessary components. This simplicity makes it a responsive and flexible system, amenable to rapid screening of multiple constructs – a critical feature when rapid response is warranted, such as for pandemic preparedness platforms.

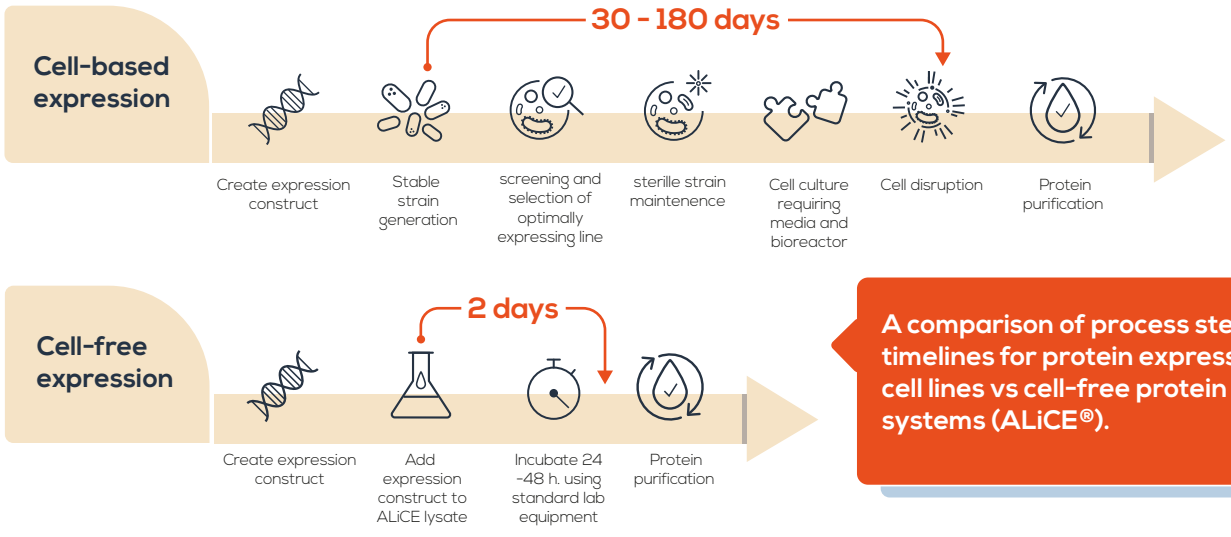
Does this mean we need to compromise on yield? It's true that established CFPS systems lack the ability to obtain high yields in batch mode, and therefore, require a switch to expensive continuous-exchange formats to make such reactions feasible (6). With the ALiCE cell-free system, such a compromise becomes unnecessary. The system is optimized to produce exceptionally high-protein yields in batch mode, reliably producing up to 3 mg/ml protein in under 48 hours (see Figure 2). These yields may not at first glance appear impressive compared to high-yielding cell-based systems. However, when the production time is taken into account (48 hours from introduction of DNA to collecting protein in the cell-free system vs weeks or even months for stably expressing cell-based systems), the true advantage of the system becomes apparent (see Figure 3).

Figure 2. ALiCE produces consistently high protein yields



eYFP expression after 48 hours in commercial batches of ALiCE consistently produces up to 3 mg/ml reporter protein. The fluorescence of lysate samples was compared against eYFP standards to calculate yields.

Figure 3. Cell-free systems simplify and speed up time to protein production



A comparison of process steps and timelines for protein expression in stable cell lines vs cell-free protein expression systems (ALiCE®).

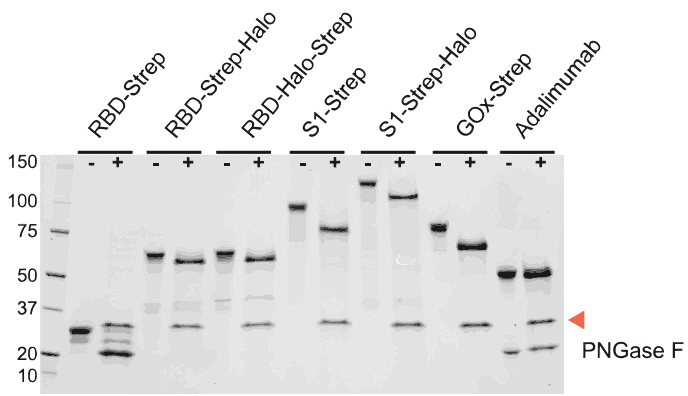
Producing proteins with complex PTMs, painlessly

Glycosylation and disulfide bond formation are highly sought-after PTMs, without which many proteins are rendered inactive. Reliably achieving these PTMs has often been cited as a barrier to the widespread adoption of CFPS systems. Essential features such as N-linked glycosylation are not possible in prokaryotic CFPS without extensive engineering, and for the majority of eukaryotic CFPS, additional supplementation is also required.

The ALiCE® lysate contains native, actively-translocating microsome vesicles derived from the endoplasmic reticulum and Golgi apparatus. Targeting of the protein to these endogenous microsomes enables addition of complex PTMs, without additional supplementation of the lysate (7). Using ALiCE, we were able to show the glycosylation of a panel of proteins (see Figure 4). Glycan analysis (not shown) revealed a strong prevalence of complex high-mannose N-glycan structures. Disulfide bond formation can also be seen in the formation of fully assembled Adalimumab antibody (see Figure 1).

Figure 4. Proteins produced by ALiCE contain expected N-glycosylation

SDS-PAGE mobility shift after glycosidase treatment demonstrating successful glycosylation of a panel of proteins. PNGase F – Peptide:N-glycosidase F calculate yields.



Scaling protein production with demand

The inability to scale reactions to commercial levels has been an inherent limitation of cell-free systems so far. Although cell-free reactions have been successfully scaled using E. coli lysates (8), eukaryotic systems have lagged behind. Bucking this trend, **ALiCE became the first – truly scalable – eukaryotic CFPS platform** that can produce complex, functional proteins in reaction volumes of up to 10 liters. Reporter proteins produced via both the cytosolic pathway (eYFP reporter protein) and the microsomal pathway (multi-domain glycoprotein, glucose oxidase (GOx) were expressed in ALiCE lysate volumes from 0.1 to 100 ml, using standard laboratory equipment (microtiter plates and Erlenmeyer flasks). Results showed no significant loss of protein yield at the reaction endpoint, regardless of expression pathway or protein, across the scaling factor of 1000x (Figure 5). Achieving this level of scalability removes a major hurdle to adoption of CFPS for commercial protein manufacture and opens the door to future applications of ALiCE beyond research labs. Further scaling of both the lysate production and cell-free reaction are ongoing.

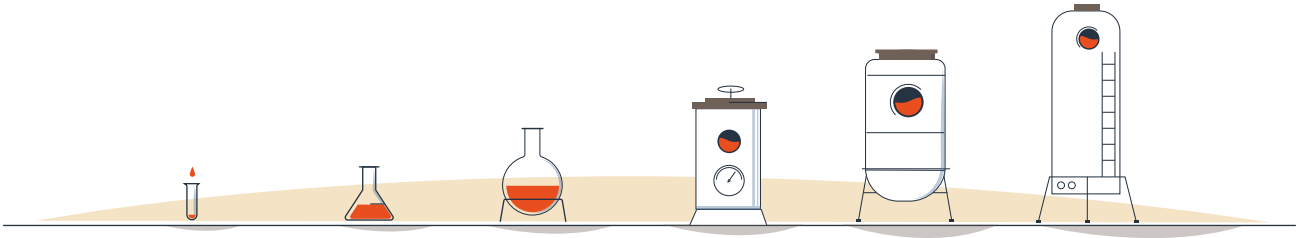
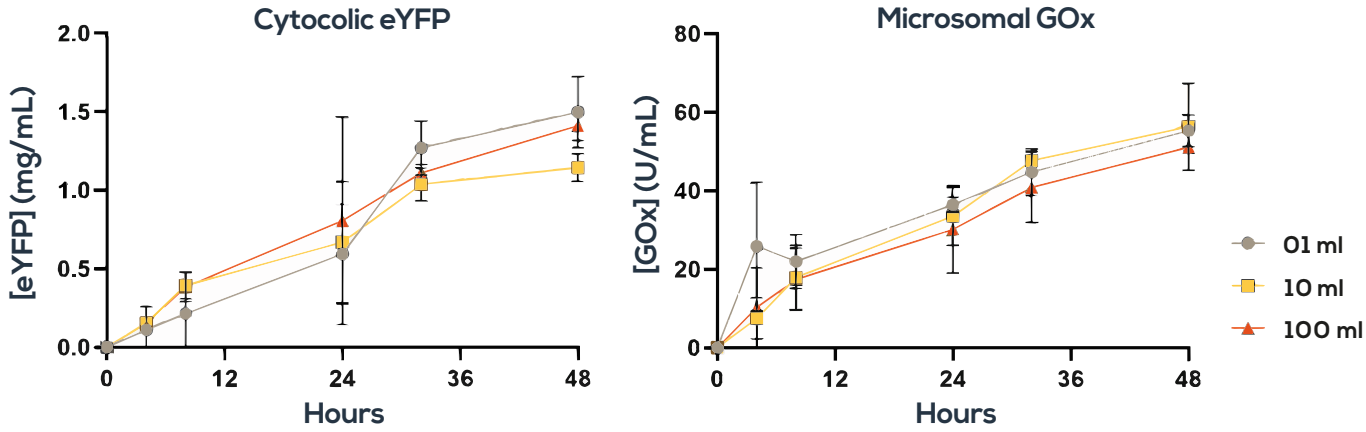


Figure 5.



Protein expressions across a 48-hour period for eYFP and GOx produced using 0.1 ml, 10 ml, and 100 ml ALiCE reaction volumes showed no noticeable loss of protein yield at the reaction endpoint. Reactions were performed in either microtiter plates or shake flasks for 48 hours at 25°C.

CTA: For full results of scaling experiments, see pre-print

CONCLUSION

Although cell-free systems have been around since the discovery of the genetic code (9), their low yields, inability to correctly implement PTMs, and lack of scalability have limited their use mainly to research laboratories. By overcoming these critical barriers to widespread adoption, versatile cell-free systems like ALiCE are beginning to shift current paradigms in protein production. Achieving scalability was a significant step forward toward this goal. Rapid synthesis capabilities, together with their ability to express a broad array of functional proteins at scale, cell-free systems are set to redefine biomanufacturing.

ALiCE® is available to purchase as a kit, a service or in larger volumes.

As a kit | As a service | As bulk

Contact us: lb_sales@leniobio.com

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