MIROSYNTH™ CELL-FREE DNA · PROTEIN ENGINEERING

One-Shot Protein Engineering with Ribbon's MiroSynth™ DNA in a Rapid and Integrated Cell-free Workflow

AUTHORS

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ABSTRACT

Protein engineering has rapidly evolved from a niche discipline into a central driver of innovation in various fields of the life sciences and biopharma. One central bottleneck of protein engineering is the availability of high-accuracy synthetic DNA templates and the integration of the required workflow steps to rapidly progress from a protein target to engineered proteins with improved properties. Conventional protein engineering workflows include a Design-Build-Test-Learn flow that arrives across multiple engineering cycles at protein designs meeting desired properties.

In this whitepaper we describe a rapid and simplified fully cell-free protein engineering workflow integrating (1) one-shot computational protein design (Scala Biodesign), (2) fully cell-free production of high-accuracy MiroSynth™ Cell-Free Linear DNA (Ribbon Bio), (3) digital microfluidics-based multiplex screening of designed constructs against cell-free protein expression and purification conditions, and (4) upscaled protein production of the optimized construct and condition pair for biochemical and biophysical characterization of engineered protein variants. We applied this workflow to the engineering of a DNA endonuclease that proved difficult to express and purify in a cell-based workflow. By eliminating the risk of DNA toxicity, protein toxicity to host cells, and tedious optimization requirements in cell-based workflows we obtained an engineered protein variant with significantly improved protein stability, as well as high cell-free expression yield. The data presented in this whitepaper highlight the potential of the described approach to radically simplify protein engineering workflows and accelerate the discovery of proteins and enzymes with desirable properties.

Introduction

The revolution in the field of protein engineering is in large part driven by the progress in computational and AI-driven protein design and modelling. Traditionally, protein engineering relied on random mutagenesis covering a small fraction of the protein sequence space. Advances in protein structure prediction and protein modelling now allow for the highly targeted exploration of a fraction of the total sequence space with highly encouraging results. These novel tools are reducing the number of required designs for lab testing by orders of magnitude. This is especially relevant for proteins that do not lend themselves to high-throughput *in vivo* screening (10^4–10^{10} variants) due to protein properties that require purified proteins for *in vitro* testing.

Once protein variants are designed, appropriate DNA expression constructs are required. Traditionally, expression plasmids are produced that represent the gold standard in terms of sequence accuracy. Since expression plasmids are produced mostly in *E. coli*, their payload must be compatible with *E. coli*. Proteins that are toxic to *E. coli*, require tailored approaches to enable DNA production. Additionally, certain classes of proteins represent biosafety risks, requiring substantial efforts to satisfy safety requirements. Furthermore, DNA sequence complexities can be incompatible with plasmid production. Cell-free DNA production is therefore highly desirable to mitigate these risks but has been fraught with limited sequence accuracy in the past.

Expression conditions in bacterial hosts often need optimization of yield and purity for compatibility with protein characterization. This can be both, time consuming and costly and might even lead to project failure. Cell-free protein expression is mitigating several of these risks, but optimization of conditions might still be required.

To address these challenges, we sought to integrate the three workflow components – protein design, generation of template DNA, protein expression/purification optimization – in an integrated, rapid, and fully-cell free workflow to obtain recombinant proteins for a biophysical and biochemical characterization. To accelerate our integrated workflow, we employed ScalaOS (Scala Biodesign) for one-shot protein engineering without iterative design rounds. DNA templates were produced with Ribbon Bio's MiroSynth™ Cell-Free DNA technology to obtain linear DNA with highest sequence accuracy. Protein expression and purification screening was conducted with Nuclera's eProtein Discovery™ instrument. Results were compared with plasmid DNA as DNA template source (gold standard) and a competitor product for linear DNA. A subset of the designs with superior purification yields, was produced by cell-free protein expression for biophysical and biochemical testing. Furthermore, the top performing design was tested for expression in *E. coli* to test for compatibility of the improved engineered variant with a scalable protein production workflow.

Results discussed below describe the successful implementation of this fully cell-free one-shot protein engineering workflow to obtain a significantly improved DNA endonuclease.

Results

To develop a one-shot protein engineering workflow, we selected a DNA modifying enzyme (DNA endonuclease) difficult to obtain by traditional protein expression and purification in *E. coli* with various host strains and expression conditions. Observed protein expression levels were low, and purification did not yield amounts and protein purity required for further biochemical testing (Fig. 1).

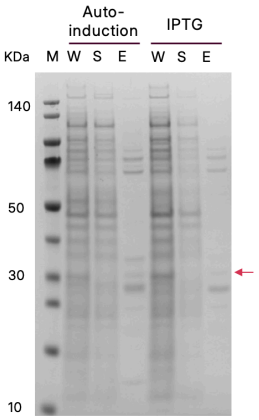
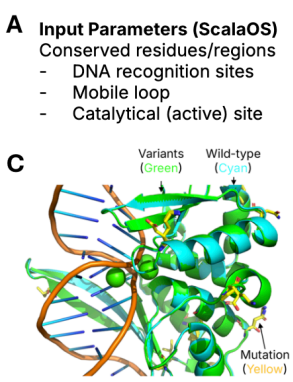


Figure 1. Production of DNA endonuclease in *E. coli*. Endonuclease was cloned into pET29b(+) and overexpressed in *E. coli* BL21 (DE3). SDS-PAGE analysis of fractions tested for overexpression and protein purity after purification. Target protein marked with a red arrow. M, protein marker; W, whole cell extract; S, Supernatant; E, Eluate; IPTG, isopropyl β-D-thiogalactopyranoside)

This protein was therefore selected for protein engineering. To increase protein expression and yield ScalaOS (Scala Biodesign) was applied to obtain protein variants with increased stability and expression. ScalaOS applies evolutionary analysis, machine learning models, computational stability, and fitness prediction for sequence optimization. We selected the stability module within the enzyme platform to design 23 protein variants (Fig. 2).



B Protein Variants

Name	# of mut.	% of mut.
#1	9	3.8%
#2	5	2.1
#3	5	2.1
#4	5	2.1
#5	6	2.5
#6	5	2.1
#7	7	2.9
#8	5	2.1
#9	5	2.1
#10	5	2.1
#11	5	2.1
#12	13	5.5
#13	5	2.1
#14	5	2.1
#15	5	2.1
#16	8	3.4
#17	5	2.1
#18	5	2.1
#19	11	4.6
#20	8	3.4
#21	5	2.1
#22	5	2.1
#23	15	6.4

Figure 2. Input parameters, predicted structural impact, and design output of ScalaOS. **A.** ScalaOS input parameters **B.** characteristics of the 23 variants generated. **C.** selection of the protein's structure in complex with dsDNA. Yellow (sticks and balls) depicts select mutated residues.

For lab testing of these variants, codon-optimized DNA templates were designed with the eProtein Discovery™ Cloud Software and were produced as MiroSynth™ Cell-Free DNA. MiroSynth™ DNA exhibited high length purity, as well as high sequence accuracy (Fig. 3).

Nuclera's eProtein Discovery™ Instrument requires proprietary 3′ and 5′ DNA sequence tags introduced via PCR. MiroSynth™ DNA molecules were used as templates and yielded high length purity and yield uniformity of PCR products (Fig. 3C).

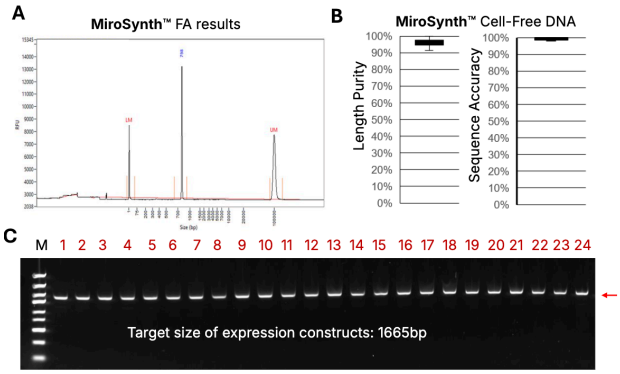


Figure 3. QC of MiroSynth™ DNA. **A.** representative Fragment Analyzer result testing DNA length purity. **B.** Average length purity and sequence accuracy (ONT sequencing; mean ± standard deviation for 24 DNA molecules shown). **C.** Agarose Gel Electrophoresis results of PCR products containing Nuclera tag sequences.

For rapid expression and purification testing Nuclera's eProtein Discovery™ Instrument was used. This digital microfluidics instrument enables parallel evaluation of multiple expression conditions to identify conditions that yield high expression. Expression screening is coupled to purification assessment for a selected subset of conditions to determine purifiability and predicted yields. After generating the required template DNA input material for the endonuclease variants, samples were loaded onto the eProtein Discovery™ Instrument, which evaluates 8 expression conditions for each of the 24 DNA constructs (wild-type and 23 protein variants) resulting in a total of 192 conditions. Robust expression of almost all variants was observed with several engineered variants outperforming the parental wild-type protein (Fig. 4).

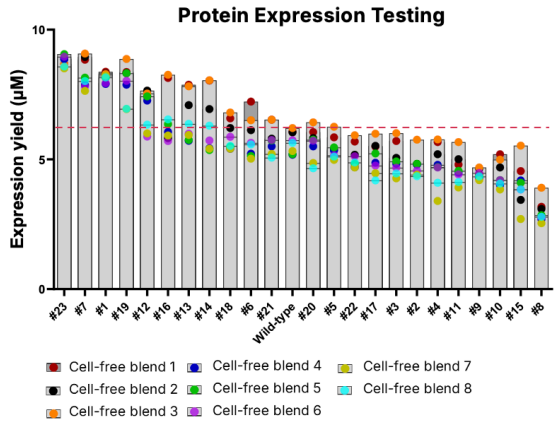


Figure 4. Expression of MiroSynth™ templates on Nuclera's eProtein Discovery™ instrument. Ranked expression levels for each variant and parental wild-type protein. Bars represent highest observed expression levels; coloured dots: expression levels for each of the 8 different blends.

Plasmid DNA is the current gold standard for template DNA, and plasmid production represents a central bottleneck in protein engineering workflows. The high sequence accuracy of MiroSynth™ DNA and its fully cell-free production workflow offer an attractive alternative to plasmid DNA. To assess the performance of plasmids versus MiroSynth™ linear DNA, we produced sequence-verified expression plasmids for the protein variants. In addition, linear DNA molecules from a competitor were obtained to benchmark the performance of these three DNA template sources. MiroSynth™ Cell-Free DNA performs comparable to plasmid DNA, the gold standard in sequence accuracy. MiroSynth™ DNA can therefore replace plasmid DNA without performance loss, enabling full replacement of bacterial DNA production workflows. Additionally, MiroSynth™ DNA outperformed linear DNA from a competitor, yielding higher average expression yields over the tested conditions (Fig. 5).

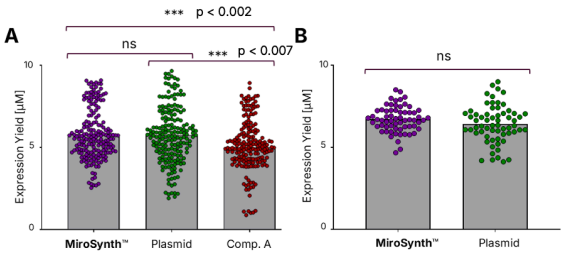


Figure 5. MiroSynth™ DNA performance vs plasmid DNA vs competitor linear DNA. Bar diagram depicts mean expression levels observed for variants, dots represent observed expression levels for the various engineered variants for select expression condition.

Nuclera's eProtein Discovery™ Instrument allows testing of both expression levels and purification yield. Based upon the expression levels observed with the cell-free blends tested (Figure 4), a total of 30 purification tests were conducted (1–2 tests per protein) (Fig. 6).

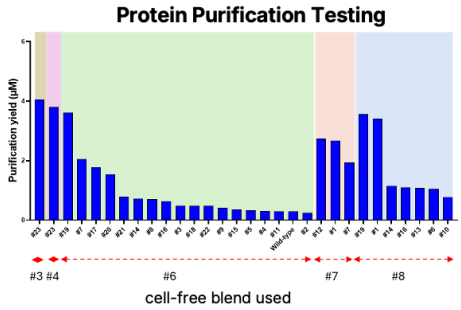


Figure 6. Purification testing. Bar diagram depicts purification yields for each protein variant. X-axis indicates expression blends chosen for purification testing.

Our results indicate that successful expression does not necessarily translate into the production of properly folded protein at levels sufficient for high yield purification (Fig. 6). While differences in expression across tested variants were relatively small, purification yields varied by up to nine-fold difference between the highest- and lowest yielding variants.

It is noteworthy that the wild-type protein exhibited the lowest purification yield among all proteins evaluated. In alignment with cell-based testing results (Fig. 1), the parental wild-type protein did not yield sufficient protein during purification testing to be considered for further purification. The intrinsic protein properties of this protein likely interfere with the expression of properly folded proteins, suitable for purification at scale.

Several of the engineered endonuclease variants expressed at high levels and achieved purification yields up to nine-fold higher than the parental wild-type protein. Based upon expression yields observed (Fig. 4), variants #1, #7, #12, #19, and #23 were selected for production at a 200 µl scale with Nuclera's cell-free blend #5.

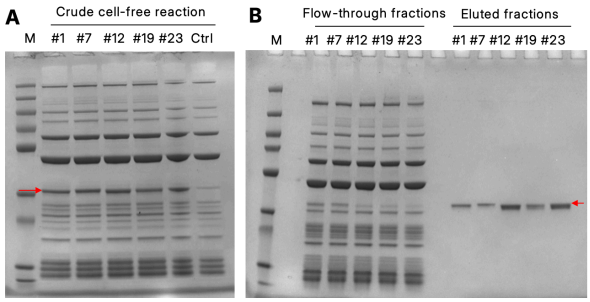


Figure 7. Cell-free production of selected variants. Left panel: SDS-PAGE analysis of crude lysates. Red arrow indicates height of target protein band showing high levels of protein expression. Right panel: SDS-PAGE analysis results from bead-based protein purification. M, protein size marker; ctrl, control reaction without DNA template.

The successful cell-free production of the selected variants resulted in 20–44 µg of highly pure protein (>95% purity; Fig. 7). In contrast to the parental wild-type protein, engineered proteins gave sufficient yield for biophysical and biochemical testing, as demonstrated below (Fig. 8, 9 and 10).

The wild-type version of the target protein is available from a commercial source and is known to be sensitive with regards to stability and storage conditions. The protein expression and purification results shown above have established that several of the engineered protein variants have superior protein properties. We therefore tested the impact of protein engineering on enzymatic activity. Since the wild-type protein performed poorly in our cell-based and cell-free expression experiments, we compared the 5 top performing variants produced at a larger scale to the commercially available wild-type protein.

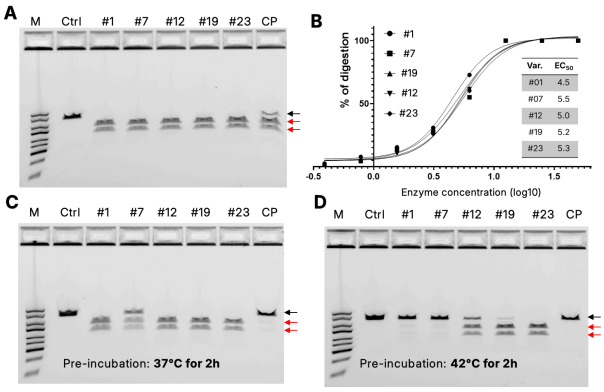


Figure 8. Activity and thermal stability of engineered proteins. Substrate DNA was digested at 37 °C for 15 min. Upper left panel depicts efficient cleavage by the engineered and commercially available protein. Arrows indicate substrate (black) and product bands (red arrows). Upper right panel: Titration experiments to assess EC50 of the engineered variants. Lower left and lower right: digestion activity after 2 h pre-incubation at 37 °C or 42 °C. M, DNA size marker, Ctrl, no enzyme added; CP, commercial product.

Engineered endonuclease variants exhibited enzymatic activity comparable to the commercially available product in our cleavage assay (Fig. 8). Importantly, these variants demonstrated substantially higher thermal stability. Pre-incubation at 37 °C completely abolished the activity of the commercially available enzyme, while four of the five engineered variants did not show signs of activity loss. Pre-incubation at 42 °C for 2 h resulted in near-complete loss of activity for two of the five protein variants (#1 and #7), with partial loss observed for two additional variants (#12 and #19). Remarkably, no activity loss was observed for variant #23, whose activity was indistinguishable from non-pre-incubation conditions. These results indicate that variant #23 is a highly active and stable endonuclease with properties superior to the parental wild-type protein.

We conducted biophysical measurements to further characterize thermal stability. Prolonged exposure to higher temperatures leads to protein unfolding and a concomitant loss of enzymatic activity. Changes in protein structure can be measured by tracking temperature dependent changes in the intrinsic fluorescence of aromatic residues using nano differential scanning fluorimetry (nanoDSF). NanoDSF enables the measurement of unfolding transitions, as well as the melting temperature (T_m) of a protein (T_m defined as the temperature at which 50% of a protein population is unfolded).

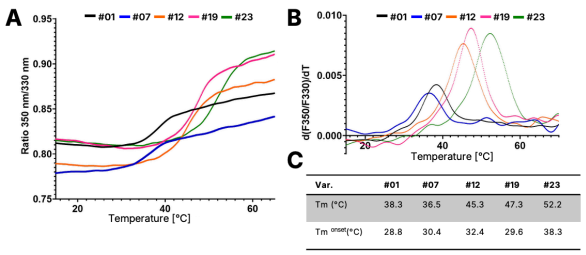


Figure 9. Thermal stability assessed with NanoDSF. **A.** unfolding curves and **B.** derivatives of the unfolding curves with the apparent melting temperatures at which 50% of proteins are unfolded. **C.** Summary table for recorded temperature (inflection point and T_m)

We observed an increase in thermal stability for several variants, which was especially pronounced for variant #23, exhibiting a 14 °C elevation in melting temperature relative to the wild-type protein (Fig. 9). This result agrees with maintained enzymatic activity after thermal stress (pre-incubation). The observed increase in thermal stability did not come at the expense of enzymatic activity (Fig. 8), validating the strategy chosen for protein engineering with ScalaOS, that focused on maintaining key residues and protein regions required for enzymatic activity.

Protein engineering workflows as illustrated in this whitepaper aim at providing a favourable throughput and timeline for protein engineering projects. Once protein variants with stability and expression parameters meet the pre-defined requirements, production at scale is required as a next step. Upscaling of production needs to be tailored to protein amounts required and both cell-free production (<1 mg) and cell-based production (>>1 mg) are possible. While << 1 mg scale production is sufficient for an initial characterization as described above, a more in-depth characterization requires more material.

To test for the scalability of variant #23 production (cell-free), we increased the volume of cell-free expression to 1 mL which resulted in an upscaled cell-free production with a total yield of ~200 µg (Fig. 10A). Additionally, we addressed the question, whether the engineered protein variant #23 would be a suitable candidate for cell-based expression in *E. coli*. If the increase in intrinsic protein stability was the main cause for the initial failure, the much more stable variant #23 could be compatible with cell-based expression enabling scaling by orders of magnitude, which is required for many commercial applications. We therefore generated expression constructs for variant #23 (pET 29b (+) expression vector) with an affinity tag for protein purification and overexpression in *E. coli* (Fig. 10B). Variant #23 showed high expression and could be purified at mg scale from 1 L culture volume and with significantly higher purity than could be achieved with the parental wild-type protein. Furthermore, enzyme activity testing showed that the purified protein was active under the conditions described above.

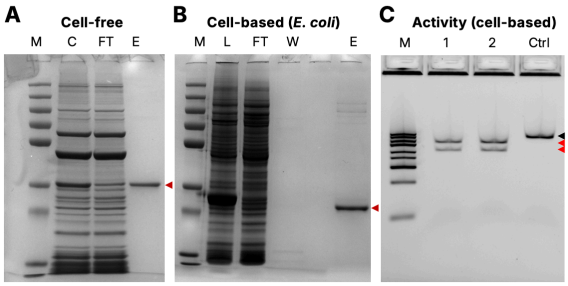


Figure 10. Cell-free and cell-based production scale-up of variant #23. **A.** SDS-PAGE analysis of cell-free expression/purification of variant #23 at 1 mL scale (data from one of two 500 µl batches shown). **B.** SDS-PAGE analysis of expression and single-step purification of variant #23 in *E. coli*. M, protein marker; C, crude lysate; L, Lysate; FT, flow-through; W, Wash fraction; E, elution fraction. **C.** Activity of variant #23 produced cell-free or from *E. coli*. Substrate DNA was digested at 37 °C for 15 min. Arrowheads indicate substrate bands (black) and product bands (red). M, DNA ladder; 1, Cell-free protein; 2, *E. coli* produced protein; Ctrl, no enzyme added.

These results demonstrate that the engineered endonuclease outperformed the parental wild-type both in activity upon thermal stress and is suitable for upscaled expression both in cell-free and cell-based expression systems.

Discussion

This whitepaper describes a workflow for successful one-shot and entirely cell-free protein engineering with Ribbon Bio's MiroSynth™ Cell-Free DNA. With just 23 variant designs produced and tested, a superior version of an endonuclease could be produced that outperforms its parental wild-type protein in several tested metrics. Importantly, this high-performance protein can be produced in both cell-free and cell-based workflows to yield highly pure, active, and thermostable enzyme.

For one-shot engineering, we applied ScalaOS (Scala Biodesign) to obtain 23 variant proteins with 5–15 mutations per variant. This high number of mutations enabled us to limit the number variants to be screened, when compared to alternative platforms that oftentimes require iterative rounds of screening and potentially hundreds of protein designs.

One central bottleneck in screening engineered proteins is the speed of DNA manufacturing and the quality of DNA templates. Historically, DNA has been obtained as expression plasmids, requiring both time consuming production workflows, as well as host cell compatibility. Ribbon's MiroSynth™ DNA solves this challenge and relieves this bottleneck by providing linear DNA produced entirely cell-free and with industry leading sequence accuracy. Benchmarking the performance of MiroSynth™ and plasmid DNA in this whitepaper demonstrates the excellent performance of Ribbon's product. MiroSynth™ DNA matched the performance of plasmid DNA, the current gold standard, in the experiments discussed above. Furthermore, MiroSynth™ Cell-Free DNA enables high-sequence complexity targets that are both beyond standard synthesis capabilities of competitors and mitigates the risk of such complex DNA being incompatible with bacterial hosts for plasmid production.

To establish a rapid and standardized protein expression and purification workflow, we applied Nuclera's eProtein Discovery™ Instrument, a digital microfluidics, protein multiplex screening system optimized for rapid screening of expression and purification conditions. This enabled the successful identification of top performing engineered variants for production at a scale, allowing for biochemical and biophysical testing. The top performing engineered variant described herein did outperform the parental wild-type protein in both expression and purification yield and outperformed the wild-type protein sourced from a commercial supplier in thermal stability, a key attribute for many downstream applications.

Importantly, the top performing variant could be expressed in *E. coli* at high yields, indicative of protein properties, not cellular toxicity, being responsible for the low expression and purification yields of the parental protein. This enables the production of the engineered variant at industrial scale, if desired.

In summary, the one-shot cell-free protein engineering workflow presented in this whitepaper describes a rapid approach to obtain superior engineered proteins with dramatically shortened timelines, reduced development costs and risks and can serve as a protein engineering blueprint for projects with similar requirements.

Trademarks & acknowledgements

MiroSynth is a registered trademark by Ribbon Bio GmbH.

eProtein Discovery is a registered trademark by Nuclera Ltd.

About Ribbon Bio

Ribbon Bio develops fully cell-free DNA manufacturing technology that delivers plasmid-grade sequence accuracy without the bottleneck of bacterial production. MiroSynth™ Cell-Free DNA is built for protein engineering, cell-free expression, and high-complexity sequence targets that conventional plasmid workflows cannot handle.

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