Using the PURExpress® *In Vitro* Protein Synthesis Kit for Heterologous *In Vitro* Expression and Functional Screening of FMN-dependent Oxidoreductase Variants

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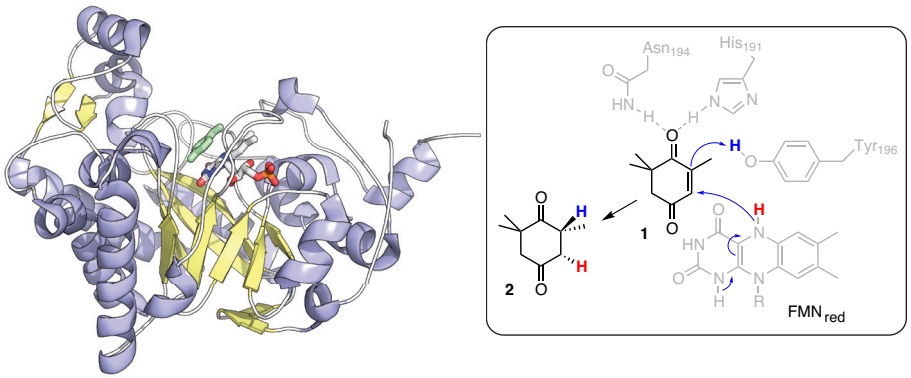
Introduction

Cell-free *in vitro* transcription/translation (IVTT) is an attractive and promising alternative to traditional methods for heterologous *in vivo* expression of proteins, such as in the case of small-scale protein synthesis for functional screening of large combinatorial libraries. Firstly, IVTT offers substantial time savings over host-based expression systems. In IVTT, protein synthesis of a linear or plasmid-based target gene is typically completed within 2-4 hours. In contrast, gene transformation into a host strain, followed by culture growth and induction of protein expression routinely requires at least 24 hours. Secondly, cell-free systems are highly scalable, allowing for synthesis of one protein variant in an individual reaction tube or parallel protein expression of entire libraries in 96 or 384-well microtiter plates with minimal change in protocol or equipment. Finally, functional screening of protein libraries often requires only small quantities of individual protein variants, thereby eliminating potential benefits of *in vivo* systems, which allow for high cell densities and culture volumes to increase protein yields. Among the various commercially available IVTT systems, PURExpress offers additional benefits over regular cell lysate-based systems. The purification of each individual component in PURExpress eliminates all unnecessary cellular contamination which can reduce background activity, and improve signal-to-noise for a targeted enzymatic function (1).

In this application note, we explore the use of PURExpress for the high-throughput screening of a combinatorial library of Old Yellow Enzyme (OYE) variants. The OYE from *Saccharomyces pastorianus* (OYE1) is a flavin mononucleotide (FMN) containing NAD(P)H-dependent oxidoreductase that catalyzes the highly stereoselective reduction of (α),(β)-unsaturated ketones, aldehydes, carboxylates, nitriles and nitroalkenes (Figure 1) (3–5). Despite their appeal to biotechnological and pharmaceutical applications, the tailoring of OYEs, for specific industrial processes by combinatorial protein engineering and directed evolution, has been hampered by challenges during OYE library analysis. Expression of OYEs in cell-based systems typically requires an enzyme purification step prior to activity measurements to eliminate high background activity by endogeous reductases.

Figure 1: Molecular structure of Old Yellow Enzyme (OYE).



Structure of Old Yellow Enzyme with bound flavin mononucleotide (FMN; shown in grey stick) and substrate analog (green) (PDB access code: 1K03)(12). OYE catalyzes the asymmetric reduction of (α)/(β)-unsaturated ketones such as ketoisophorone (structure 1) to R-levodione (structure 2).

Materials

- PURExpress *In Vitro* Protein Synthesis Kit (NEB #E6800)
- Template DNA
- OYE variant (cloned in pET-14b)
- RNase Inhibitor, Murine (NEB # M0314)
- *E. coli* BL21(DE3) pLysS
- LB medium
- 2xYT medium
- Selective antibiotics
- IPTG
- Buffers A, B & C
- Assay solution
- Lysis buffer
- Protease inhibitor
- Benzonase
- Anion exchange column
- Coy's anaerobic chamber

In PURExpress, contaminating reductase activity is greatly diminished due to purification of all components in the reaction system (Figure 2).

For OYE library preparation, we employed Circular Permutation (CP) for generating a set of ~400 OYE1 variants. Conceptually, CP covalently links the native amino- and carboxyl-termini of a protein via a short peptide sequence and new termini are introduced through cleavage of one of the existing peptide bonds in the polypeptide sequence. As such, CP does not change the amino acid composition but simply reorganizes the primary protein sequence (6). Previous studies have shown that such relocation of termini can translate into significant functional improvements due to changes in protein flexibility and active site accessibility (7–11). In practice, the CP library of OYE1 (cpOYE) was prepared by whole gene synthesis and cloned into a DNA plasmid (pET-14b). Individual library members were stored in separate wells of 96-well microtiter plates. For functional evaluation of enzyme variants encoded by the gene library, plasmid aliquots were used as template DNA which were added to the PURExpress system for protein synthesis and subsequent activity assay.

General Protocol

For smaller scale (ng amounts) OYE expression, follow the protocol for *in vitro* expression. For larger scale (µg amounts) OYE expression, for detailed kinetic characterization studies, follow the protocol for *in vivo* expression.

In Vitro Expression:

1. Thaw PURExpress solutions A and B on ice.
2. Prepare reaction mixtures in either nuclease-free 0.5 ml Eppendorf tubes or a 96-well microtiter plate.
3. Add template DNA.
4. Incubate samples at 37°C for 2.5 hours to allow for transcription and translation.
5. Stop the reaction by cooling to 4°C, on ice.

REACTION	OYE (+ FMN)	OYE (– FMN)	DHFR (+ FMN)	Comments
PURExpress Solution A	4.0 µl	4.0 µl	4.0 µl	NEB
PURExpress Solution B	3.0 µl	3.0 µl	3.0 µl	NEB
FMN (2 mM stock)	0.5 µl	–	0.5 µl	$[FMN]_{final}$ = 100 µM
RNase Inhibitor	0.25 µl	0.25 µl	0.25 µl	NEB; 10 units/rxn
H_2O	1.25 µl	1.75 µl	1.25 µl	nuclease-free
DNA (template)	1.0 µl	1.0 µl	1.0 µl	DNA = 100 ng/rxn
Total	10.0 µl	10.0 µl	10.0 µl	

In Vivo Expression:

1. Transform the corresponding gene of the OYE variant (cloned in pET-14b) into *E. coli* BL21(DE3) pLysS for heterologous expression.
2. Spread the transformation reaction onto antibiotic selection plates.
3. Incubate the plates at 37°C overnight.
4. Pick a single colony and add it to 2 ml of LB medium containing antibiotics.
5. Incubate the culture at 37°C in a shaking incubator overnight.
6. In a 1 liter baffled flask, combine 250 µl of overnight culture with 250 ml of 2 x YT medium containing antibiotics.
7. Shake the culture at 37°C until an O.D. (600 nm) reading of approximately 0.5.
8. Induce protein expression by adding IPTG to a final concentration of 0.4 mM.
9. Incubate the induced culture at 20°C for 18 hours.
10. Centrifuge the culture at 10,000 rpm and 4°C for 20 minutes.
11. Carefully decant the supernatant and proceed with the protocol or store at –20°C until needed.

12. Resuspend the cell pellet in 6 ml of lysis buffer (40 mM Tris-HCl pH 8.0, 20 mM NaCl).
13. Add 75 µl of protease inhibitor and 7.5 µl of benzonase, and incubate on ice for 30 minutes.
14. Sonicate.
15. Centrifuge the mixture at 10,000 rpm and 4°C for 30 minutes.
16. Apply the supernatant to an anion exchange column.
17. Follow with a linear gradient of Buffer B (40 mM Tris-HCl pH 8.0, 1000 mM NaCl) and monitor elution at 460 nm (flavin absorbance).
18. Combine and concentrate product fractions.
19. Equilibrate a size selection column with Buffer C (40 mM Tris-HCl pH 8.0, 300 mM NaCl) and load the fractions.
20. Quantify protein by absorbance at 280/460 nm, and assess purity by SDS-PAGE analysis.

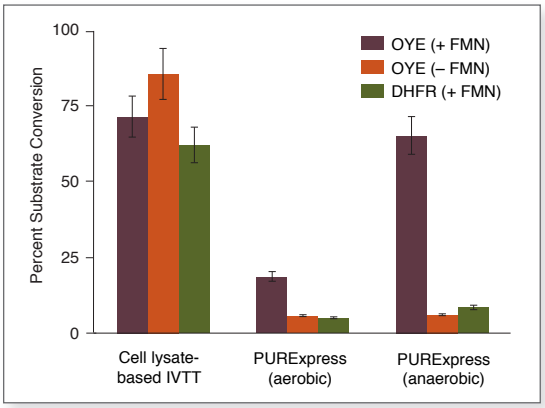
Ene-Reductase Assay:

1. In a Coy anaerobic chamber, combine 10 µl of the IVTT reaction mixture and 20 µl of assay solution (200 µM ketoisophorone, 200 µM NADP+, 2 Units GDH (*Thermoplasma acidophilum*) and 100 mM glucose in Buffer C).
2. Separately, combine and mix 2.4 µg of purified OYE from *in vivo* expression with 400 µl of assay solution and dH_2O, to a final volume of 500 µl.
3. Incubate the assay reaction for 2.5 hours at room temperature.
4. Quench the reaction with ethyl acetate containing 1 mM cyclohexanone as an internal standard.
5. Analyze a sample of the organic phase with a chiral column (Agilent 6850 w/ FID detector; CycloSil-B column).
6. Generate standard curves with known amounts of ketoisophorone and R-levodione and calculate the percent substrate conversion and enantiomeric excess.

Results

The PURExpress *In Vitro* Synthesis Kit from New England Biolabs was successfully employed for the expression of active OYE1 from *Saccharomyces pastorianus*, as well as CP variants derived from OYE1. Protein yields obtained from 10 µl-scale reactions were sufficient to accurately monitor catalytic performance for candidates with as low as 10% of wild type reductase activity. More importantly, protein synthesis by PURExpress dramatically lowers the background signal due to contaminating reductases in cell-based *in vitro* and *in vivo* expression systems (Figure 2).

Figure 2: Effect of FMN and DHFR on ketoisophorone conversion.

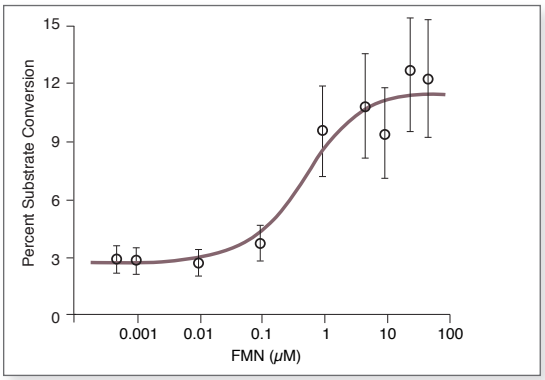


Percent conversion of ketoisophorone to R-levodione by OYE in the presence or absence of FMN and DHFR (– control) synthesized by either cell-lysate based IVTT or PURExpress. Standard error ± 10%.

The reduced background improved signal-to-noise for OYE expression which allowed for direct functional analysis of native and engineered enzymes. By performing the assay under anaerobic instead of aerobic conditions, sensitivity was further increased as futile reaction of reduced FMN with molecular oxygen was eliminated. Critical for the production of functional enzyme was supplementation of the IVTT reaction mixture with FMN.

As shown in Figure 3, maximum enzyme activity is reached at (FMN) >1 µM which is approximately 100-fold above the reported dissociation constant for FMN in OYE1 (12). The FMN concentration in our experimental protocol was chosen at 100 µM to ensure saturation of enzyme with cofactor, even for engineered OYEs with potentially lower cofactor binding affinity.

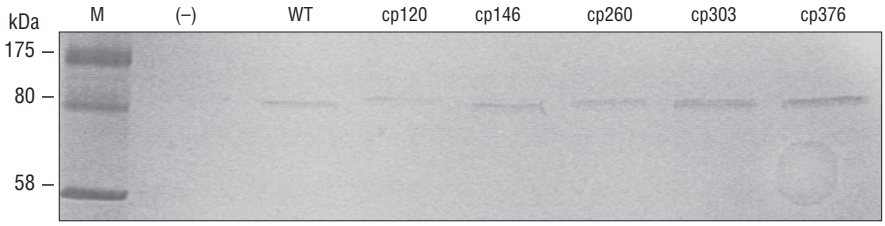
Figure 3: Effect of FMN concentration on ketoisophorone conversion.



Percent conversion of ketoisophorone by wild type OYE at (FMN) ranging from 0.5 nM to 100 µM.

The expression levels of individual members of the protein library is another major factor influencing the enzyme activity assay. SDS-PAGE analysis of wild type OYE and five randomly selected members of the OYE library indicates that protein yields vary by ~2-fold (based on densitometry analysis of gel image) with the exception of cp120 which suffers from poor protein folding and stability (Figure 4).

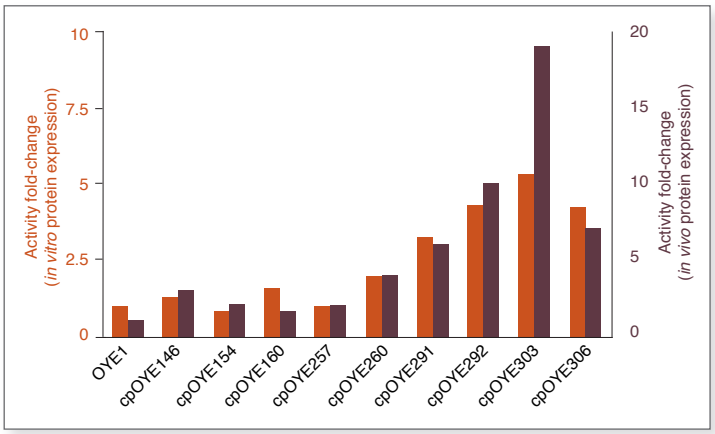
Figure 4: Relative protein yields from five randomly selected members of the cpOYE library.



SDS-PAGE analysis of protein yields from 25 µl PURExpress reactions using wild type OYE1 (~48 kDa) and five randomly selected members of the cpOYE library. M: molecular weight marker; (–) negative control with empty DNA plasmid.

The relatively consistent yields from IVTT were also reflected in the good correlation in activity data from functional assays with IVTT products and results with purified cpOYE variants after *in vivo* expression (Figure 5).

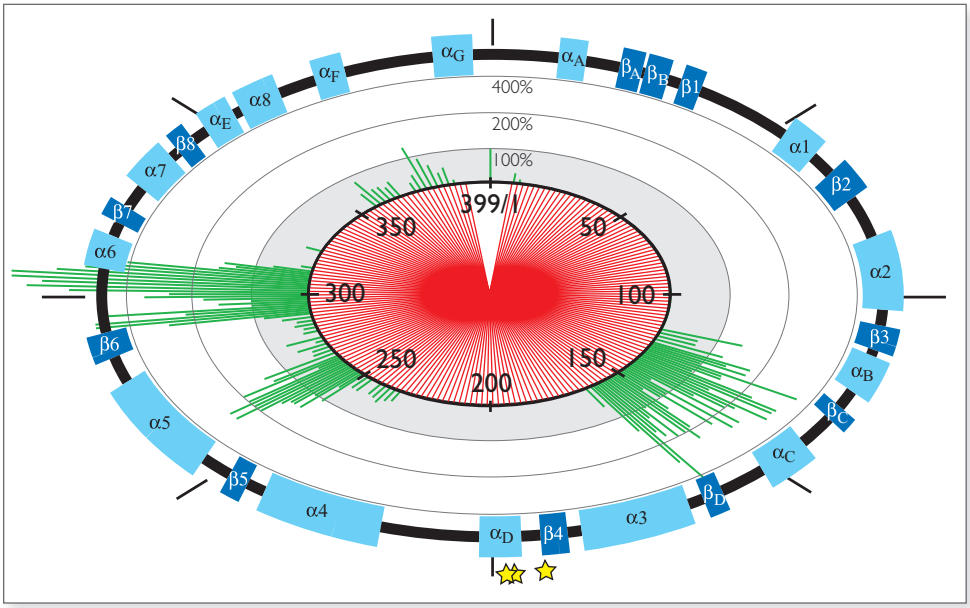
Figure 5: Comparison of *in vitro* and *in vivo* protein expression.



Summary of catalytic activities measured with wild type OYE1 and selected cpOYE library members from in vitro and in vivo protein expression.

An example for the functional analysis of the entire circular permutation library for OYE1 is shown in Figure 6. Protein expression by PURExpress and activity assays – going from gene sequence to enzyme activity – could be performed on the entire OYE1 library (399 individual gene constructs) in a 96-well microtiter plate format in a few hours. The screening of the library identified several cpOYE variants with enhanced catalytic activity. The functional performance of individual cpOYE library members with improved catalytic activity in the PURExpress screen was confirmed with purified enzyme after *in vivo* expression. A comparison of the activity data from *in vitro* and *in vivo* experiments showed that the PURExpress system give a good estimate of catalytic turnover and accurately identifies the top-performing candidates in the protein library (Figure 5).

Figure 6: Functional analysis of cpOYE library for reduction of ketoisophorone to levodione (9).



Whole-gene synthesis created a cpOYE library with perfect distribution (red lines, inner circle). Each line points at the location of its new N and C-termini in the OYE1 sequence (outer-most circle). Based on IVTT and activity assay, the catalytic performance of individual library members is reflected in the length of the green lines. For reference, wild type activity is marked by the grey-shaded area. The outer-most circle marks the polypeptide sequence of OYE1, placing the native N-terminus (position 1) and C-terminus (position 399) at 12 o'clock. The locations of secondary structure elements (shades of blue) and active site residues (yellow stars) are marked.

Summary

The PURExpress *In Vitro* Protein Synthesis Kit OYE1 from *Saccharomyces pastorianus* is an FMN containing NAD(P)H-dependent oxidoreductase that catalyzes the asymmetric reduction of activated alkenes, making it a promising biocatalyst for industrial applications. Protein engineering of OYE1 offers a strategy to further improve catalytic efficiency and tailor the enzyme to specific substrates and reaction conditions. The PURExpress *In Vitro* Protein Synthesis kit was successfully used as protein expression platform for a combinatorial library of OYE1 variants. The short time required to go from genes to enzyme activity, as well as the simplicity and scalability of the IVTT process makes PURExpress a very powerful tool for protein engineering.

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