

SUPPLEMENTARY MATERIALS

RAPID PARALLEL PROTEIN EVALUATOR (RAPPER), FROM GENE TO ENZYME FUNCTION IN 24 HOURS.

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Materials and Methods

Materials: PURExpress kits were purchased from New England Biolabs (Ipswich, MA). *Pfu* DNA polymerase (Stratagene, La Jolla, CA) in combination with the manufacturer's buffer was used for PCR amplification. All other enzymes were purchased from New England Biolabs. Oligonucleotide primers were ordered from Integrated DNA Technologies (Coralville, IA). Reagents, substrates, and reference materials were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Preparation of linear DNA templates: Linear DNA sequences as templates for in vitro transcription/translation were prepared by PCR amplification using site-specific oligonucleotide primers (listed below) and pET14b-OYE1 [ref] as template.

res. 2424-2424: for: 5'-GCTGCATGTGTCAGAGGTTTTTCAC-3'
 rev: 5'-GGAGACGGTCACAGCTTGTCTGT-3'

res. 3086-1984: for: 5'-ATGCAGATCCGGAACATAATGGTGCAG-3'
 rev: 5'-AGCTGGGCTGTGTGCACGAACC-3'

res. 3748-1544: for: 5'-GCGGGGAGGCAGACAAGGTATAGGG-3'
 rev: 5'-CCACGCTCACCGGCTCCAGATTTA-3'

res. 4409-1104: for: 5'-GCCGAAAATGACCCAGAGCGCTG-3'
 rev: 5'-GGCAAAATGCCGCAAAAAGGGAAT-3'

res. 401-678: for: 5'-CTCGATCCCGCGAAATTAATACGACT-3' (T7 promoter)
 rev: 5'-CAGCAAAAACCCCTCAAGACCCG-3' (T7 terminator)

Mutagenesis by primer overlap extension PCR: Mutations in specific locations of *oye1* were introduced by primer overlap extension PCR, using either pET14b-OYE1 or pET14b-cpOYE303 (5) as templates. To facilitate PCR product purification without agarose gel electrophoresis, the flanking T7 promoter and T7 terminator primers (see sequence above) were modified at the 5'-end by a desthiobiotin moiety on a PEG linker. These tagged primers were used in combination with the appropriate mutagenic primers listed below for the preparation of individual DNA fragments by PCR amplification (Fig. 1).

OYE_W116I:	for: 5'-GGGTTTCAGTTAATAGTTTTGGGTTG-3'
	rev: 5'-CAACCCAAAACCTATTAACCTGAACCC-3'
OYE_F250Y:	for: 5'-CCCCATACGGTGTTTACAACAGTATGTCTG-3'
	rev: 5'-CAGACATACTGTTGTAAACACCGTATGGGG-3'
OYE_P295A:	for: 5'-CGTGTAACCTAACGCATTCTTGACTGAAG-3'
	rev: 5'-CTTCAGTCAAGAATGCGTTAGTTACACG-3'
OYE_Y375F:	for: 5'-GACAGAGATACTTTCTTCCAGATGTCTGCTC-3'
	rev: 5'-GAGCAGACATCTGGAAGAAAGTATCTCTGTC-3'

For DNA fragment purification, the 50 µl-PCR reactions were diluted with 50 µl water and mixed with high-capacity streptavidin agarose beads (Pierce, Rockford, IL) in binding buffer (5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 1 mM NaCl). After incubation on a rocking platform at 21°C for 15 min, the suspension was centrifuged (8000g, 2 min) and the supernatant discarded. The streptavidin beads were washed with 3 x 100 µL of binding buffer, followed by incubation with 50 µL elution buffer (4 mM biotin, 10 mM Tris-HCl (pH 8.5)) at 21°C for 10 min. Purified DNA fragments were recovered in clear supernatant after sample centrifugation, quantified spectrophotometrically by absorbance at 260 nm and stored at 4°C. For recombination of DNA fragments into the full-length *oye1* genes, approximately 40 ng of each fragment were used as templates for PCR amplification with standard T7 forward and reverse primers. PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA).

Cloning of *oye1* variants: As part of the RAPPER validation experiments, a separate aliquot of linear DNA products used with PURExpress was also cloned into pET-14b for DNA sequencing and traditional heterologous protein expression in an *E. coli* host organism. Briefly, full-length *oye1* genes obtained by primer overlap extension PCR were digested with *Xho*I and *Xba*I, followed by T4 DNA ligase catalyzed insertion into pET-14b, linearized with the same two restriction endonucleases. Ligation products were transformed into *E. coli* host and processed for protein overexpression and purification as previously described (5).

PURExpress In Vitro Transcription Translation: Full-length *oye1* genes were used as linear DNA templates for in vitro transcription/translation (IVTT). Briefly, IVTT reactions were assembled using the PURExpress *in vitro* protein synthesis kit (PURE) following manufactures protocol with a few adjustments (see below)

optimized for our application. Reactions were assembled on a 10- μ L scale containing PURExpress solution A, PURExpress solution B, 100 μ M FMN, 10 units Murine RNase Inhibitor (NEB), 100 ng DNA template, and nuclease-free H₂O. All reactions were run simultaneously with native OYE1 (positive control) and dihydrofolate reductase (DHFR; negative control; provided by NEB) under the same experimental conditions. The reaction mixtures were incubated at 37°C for 2.5 hours to allow for protein synthesis, followed by cooling to 4°C.

Enzyme activity assay: The ene-reductase activity of enzymes produced by IVTT or traditional heterologous expression/purification (referred to as “purified enzyme”) was measured under anaerobic conditions (Coy Laboratory Products, Grass Lake, MI) at ambient temperature using glucose dehydrogenase (GDH) from *Thermoplasma acidophilum* for NADPH regeneration. A reaction stock solution was prepared, containing 10 mM (*S*)-carvone (**1**), 200 μ M NADP⁺, GDH (2 or 5 units for IVTT or purified enzyme reactions, respectively), 100 mM glucose in 50 mM Tris-HCl (pH 7.5).

For activity assays of IVTT reactions, 20 μ L of reaction stock was added directly to the IVTT reaction mixture (10 μ L). To assay purified enzyme, the OYE1 variant (final concentration: 250 nM) was added to 500 μ L of reaction stock solution. After 4 h reaction time, a 30- μ L aliquot of the assay solution was quenched by mixing thoroughly with 30 μ L of ethyl acetate containing 1 mM cyclohexanone as internal standard. A sample of the organic phase was injected onto an Agilent Technologies 6850 GC instrument equipped with a chiral CycloSil-B column (30 m x 0.32 mm / 0.25 μ m, Agilent, Santa Clara, CA) using hydrogen as a carrier gas (flow rate 1.8 mL/min) and an FID detector (detector temperature 200°C, split ratio 25:1). The temperature program for the GC was as follows: 90°C, hold 5 min, then 1°C/min to 120°C (retention time: **1** = 27.35 min, (*1R,4S*)-**2** = 22.9 min, (*1S,4S*)-**2** = 22.6 min). The percent conversions and diastereomeric excess was calculated from substrate and product integration areas and were quantified using standard curves generated using known amounts of the substrate and product.