

Reversal of NAD(P)H Cofactor Dependence by Protein Engineering

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Abstract

There is increasing interest in utilization of engineered microorganisms for the production of renewable chemicals and next-generation biofuels. However, imbalances between the cofactor consumption of the engineered production pathway and the reducing equivalents provided by the cell have been shown to limit yields. This imbalance can be overcome by adjusting the cofactor dependencies of the pathway enzymes to match the available cofactors in the cell. We show how cofactor preference can be reversed by structure-guided directed evolution of the target enzyme.

Key words: Cofactor switch, Enzyme engineering, Homology modeling, Nicotine amide dinucleotide phosphate, Nicotine amide dinucleotide, Directed evolution

1. Introduction

Recent advances in biotechnology have made it possible to construct microorganisms harboring engineered metabolic pathways that convert renewable sugars into a wide array of chemicals (1, 2). However, the sugar-to-product yields of these engineered strains can be limited by imbalances between the specific (nicotine amide dinucleotide phosphate (NADPH) or nicotine amide dinucleotide (NADH)) cofactor requirements of the pathway enzymes and the form in which the reducing equivalents are provided by the cells. Reduced yields can pose a major obstacle to commercialization and economic viability of a microbial process for production of chemicals and fuels.

One solution to balancing NAD(P)H cofactor utilization in *Escherichia coli* is to overexpress a transhydrogenase, such as *E. coli* PntAB (3), which catalyzes the transfer of a hydride ion between NADP^+ and NADH resulting in the simultaneous regeneration of

NADPH and NAD⁺. However, applicability of this approach to yeast or other industrial microorganisms is questionable, since the transhydrogenase may not always shift the hydride ion in the preferred direction (4). A more general solution is to adjust the cofactor specificity of pathway enzymes to match the available reducing equivalents. Such an approach has improved ethanol yields of xylose fermentations (5, 6) and recently led to anaerobic production of isobutanol from glucose at theoretical yield (7).

Early strategies for engineering an enzyme's cofactor specificity in the absence of structural information involved disrupting or constructing salt bridges to the NADPH 2' phosphate group. While this is sometimes sufficient to switch cofactor specificity, it is often accompanied by loss of catalytic activity (8, 9). Engineering an enzyme whose catalytic efficiency with its new cofactor is comparable to that of the native enzyme with its native cofactor requires more intricate remodeling of the cofactor-binding site. An accumulated wealth of structural information (10), software for homology modeling when structural information is not available, and established methods of directed enzyme evolution (11) supply the tools to successfully reverse cofactor dependence.

The following protocol was tailored for switching the cofactor preference of wild-type ketol-acid reductoisomerase (IlvC) from *E. coli* from NADPH to NADH (7). The overall strategy was to identify target residues based on their potential to affect cofactor recognition and use site-saturation mutagenesis and screening to discover beneficial mutations at those sites. Individual beneficial mutations were recombined to generate the cofactor-switched enzyme. This protocol is appropriate for a recombinant enzyme expressed in *E. coli* and can be adapted with appropriate adjustments in heterologous expression conditions, buffers and storage, substrates, etc.

2. Materials

2.1. Cell Culture and Lysis

1. Luria-Bertani (LB) broth supplemented with 100 µg/mL of ampicillin (Note 1).
2. Aqueous solution of 100 mg/mL ampicillin (=1,000× stock solution).
3. Aqueous solution of 0.5 M isopropyl β-D-1-thiogalactopyranoside (IPTG).
4. Electro-competent *E. coli* strain BL21(DE3) (Lucigen Corp., Middleton, WI, USA), stored at -80°C.
5. Lysis buffer for high-throughput lysis: 250 mM potassium phosphate buffer, pH 7.0, 750 mg/L lysozyme, 0.5 U/mg DNaseI.

6. Lysis buffer for purification: Buffer A (see Subheading 2.2).
7. Lysis buffer for assays in crude lysate generated by sonication: 250 mM potassium phosphate, pH 7.0.
8. Humidified plate shaker (e.g., Infors (Switzerland)).

2.2. Purification

1. Buffer A: 20 mM Tris pH 7.4, 20 mM imidazole, 100 mM NaCl, and 10 mM MgCl₂.
2. Buffer B: 20 mM Tris pH 7.4, 300 mM imidazole, 100 mM NaCl, and 10 mM MgCl₂.
3. 1 or 5 mL Histrap High Performance (HP) columns pre-charged with nickel (GE Healthcare, Waukesha, WI, USA).
4. Sonicator (Misonix, Inc., Farmingdale, NY, USA).
5. FPLC system such as an AKTA purifier (GE Healthcare, Waukesha, WI, USA).

2.3. Activity Assay

1. Substrate precursor ethyl 2-acetoxy-2-methylacetoacetate (EAMAA) (Sigma, St. Louis, MO), stored at room temperature.
2. 2 M NaOH solution, stored at room temperature.
3. Cofactors NADPH and NADH (Codexis, Inc., Redwood City, CA, USA), 10 mM, dissolved in water, stored in 1-mL aliquots at -20°C.
4. 2 M stock solution of MgCl₂, dissolved in water, stored at room temperature.
5. 100 mM stock solution of dithiothreitol (DTT), dissolved in water, stored in 1-mL aliquots at -20°C.
6. Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA), stored at 4°C.
7. UV-vis spectrophotometer (Varian, Inc., Palo Alto, CA, USA).
8. Transparent flat-bottom assay plates (Evergreen Scientific, Los Angeles, USA).
9. Liquid handling robot (e.g., Beckman Coulter, Inc., Brea, CA, USA).
10. Plate reader (e.g., TECAN Group Ltd., Switzerland).

2.4. Construction of Enzyme Mutant Libraries by Site-Saturation Mutagenesis

1. PyMOL software (Molecular Graphics System, Version 1.3, Schrödinger, LLC., San Diego, USA). For questions and assistance using PyMOL, refer to <http://www.pymolwiki.org/>.
2. SWISS-Model: <http://swissmodel.expasy.org/> (University of Basel, Switzerland).
3. Thermomixer (e.g., Eppendorf, Germany).
4. Thermocycler (e.g., Eppendorf, Germany).

5. Colony picking robot (e.g., Genetix, San Jose, CA, USA).
6. Design primers with Clone Manager or similar software, or use online tools such as <http://arep.med.harvard.edu/labgc/adnan/projects/Utilities/revcomp.html>.
7. Dissolve lyophilized primers in PCR-grade water to obtain 100-mM stock solutions. For the working solutions, prepare a 1:10 dilution in PCR-grade water. Primer stocks and working solutions are stored at -20°C .
8. NEB PhusionTM polymerase (New England Biolabs, Ipswich, MA, USA), stored at -20°C .
9. Aqueous stock solutions of deoxynucleotide triphosphates (dNTPs) (10 mM each), stored at -20°C .
10. *DpnI* (Roche, Indianapolis, IN, USA), stored at -20°C .
11. Restriction enzymes *NdeI* and *XhoI* (New England Biolabs, Ipswich, MA, USA), stored at -20°C .
12. Expression vector pET22b(+) (EMD Chemicals Group, Darmstadt, Germany), stored at -20°C .
13. T4 ligase (New England Biolabs, Ipswich, MA, USA), stored at -20°C .
14. DNA sequencing may be performed by Laragen (Los Angeles, CA, USA) or other companies providing sequencing service.
15. Freeze “n” Squeeze tubes (Bio-Rad, Hercules, CA, USA), stored at room temperature.
16. Pellet paint kit (Merck, Germany), stored at -20°C .
17. Zymo DNA clean & concentratorTM-25 kit (Zymo Research Corporation, Irvine, CA, USA), stored at room temperature.

3. Methods

The methods described herein are in order of the workflow. For purification purposes, an N- or a C-terminal his-tag is required (Note 2).

3.1. Structure Alignment and Choice of Target Residues

1. Assess cofactor–side chain interactions with PyMOL.
2. Target mutation sites can be selected following inspection of the cofactor-binding site in PyMOL, if the structure of choice has the native cofactor co-crystallized. Otherwise prepare a structural alignment of the X-ray crystal structures of homologs that contain the cofactor.
3. If the parent enzyme’s structure is not available, computational models based on the structures of homologs and the amino acid sequence of the target enzyme (e.g., with SWISS-Model) will be very helpful in choosing the target amino acid residues (12).

3.2. Preparation of Site-Saturation Libraries

4. When examining the cofactor-binding site for residues that when mutated could disrupt the salt bridges to the 2' phosphate of NADPH, one should consider not only the positively charged residues such as arginines and lysines, but also residues that can impact the general positioning of the cofactor orientation.
1. Flanking primers may bind up to 50 bp upstream and downstream of the gene (see Note 3).
 2. Design mutagenesis primers for targeted positions with NNK (N stands for A, C, T, G; K stands for T, G; reverse complement MNN) in lieu of the triplet encoding the targeted amino acid residue. Adding 12–15 base pairs upstream and downstream of the NNK site will result in fragments with sufficiently long overlapping sequences for successful assembly PCR (Fig. 1) (see Note 4).
 3. Use flanking primer and mutagenesis primer pairs to generate fragments with Phusion polymerase: PCR cyclor program for Phusion: 98°C for 30 s initial denaturation; 98°C for 10 s, 55°C for 10 s, 72°C for 30 s/kb (25 cycles); 10 min at 72°C, 4°C hold step (not required) (see Note 5).
 4. PCR mix for generation of fragments: The numbers in parentheses are the concentrations of the stock solutions: 10 µL HF buffer (5×), 1 µL dNTPs (10 µM each), 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 0.5 µL plasmid template (100 ng/µL), 0.5 µL Phusion polymerase, and 36 µL PCR-grade water (see Note 6).
 5. Check for correctly sized PCR products on Tris-acetic acid-EDTA (TAE) agarose gel.
 6. *DpnI* digest template DNA for 1 h at 37°C: Add 1 µL of *DpnI* to 50 µL of PCR mixture and mix gently by snapping it with your fingers rather than pipetting up and down. No desalting

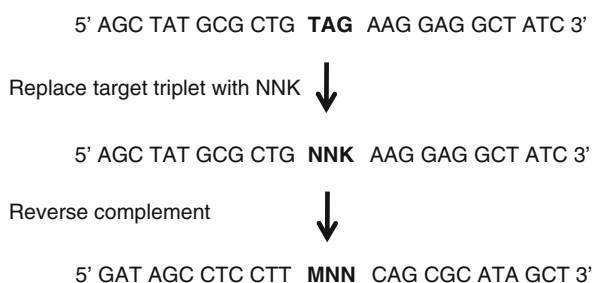


Fig. 1. Primer design for site-saturation libraries: replace target triplet TAG (in **bold**) with NNK to obtain forward primer. Then, reverse complement NNK forward primer to obtain reverse primer. N stands for A, T, G, and C; K stands for T and G; M is the reverse complement of K.

or any other kind of PCR purification is required prior to addition of the enzyme.

7. Separate fragments on preparative TAE agarose gel, excise on UV table, transfer gel fragments into Freeze “n” Squeeze tubes, and then freeze for at least 10 min at -20°C (see Note 7).
8. Spin for 10 min at room temperature at $10,000\times g$. For larger gel fragments, longer spin times may be necessary.
9. Precipitate DNA contained in the eluate of the Freeze “n” Squeeze tubes using the pellet paint kit (see steps 10–16) (see Note 8).
10. For sample volumes smaller than 500 μL , add 2 μL of co-precipitant and vortex. For larger sample volumes, split samples in half and add pellet paint co-precipitant.
11. Add 1/10 volume of 3 M sodium acetate, pH 5, provided in the kit, and vortex.
12. Add 2.5 \times volume of 100% ethanol and vortex.
13. Incubate on ice for 2 min and centrifuge for 5 min at $16,000\times g$ and 4°C .
14. Remove supernatant with pipette; add 200 μL 70% ethanol, vortex, and spin again as described above.
15. Remove supernatant with pipette; add 200 μL 100% ethanol, vortex, and spin as described above.
16. Remove supernatant with pipette, dry DNA in thermomixer at 55°C for 10 min or until pellet is dry by visual inspection, and resuspend pellets in 10 μL of PCR-grade water.
17. Use fragments as templates for assembly PCR with flanking primers, the numbers in parentheses are the concentrations of the stock solutions: 10 μL HF buffer (5 \times), 1 μL dNTPs (10 mM each), 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), 0.5 μL fragment a and fragment b each (~ 50 ng/ μL each), 0.5 μL Phusion polymerase, and 35.5 μL PCR-grade water.
18. Program for assembly PCR with Phusion: 98°C for 30 s initial denaturation; 98°C for 10 s, 55°C for 10 s, 72°C for 30 s/kb (25 cycles); 10 min at 72°C , 4°C hold step (not required).
19. Check for successful assembly PCR on TAE agarose gel.
20. Zymo purification according to modified manufacturer’s instructions and elute with 20 μL PCR-grade water (see Note 9).
21. Restriction digest insert and vector with *NdeI* overnight at 37°C : Use the entire 20 μL of the purified insert, and add 5 μL NEB buffer 4, 23 μL PCR-grade water, and 1 μL *NdeI*; mix gently and spin briefly. On the next morning, add 1 μL *XhoI*, mix gently, spin briefly, and incubate for another 2 h at 37°C .

Use 10 μL of vector (100 ng/ μL stock solution) and treat the same as insert, but adjust volume of PCR-grade water so that the total volume is 50 μL (see Note 10).

22. Gel extract as described above (steps 7–16, this Section).
23. Ligation: Use T4 ligase from NEB. Prepare the following mixture: molar ratio of 3–5:1 for insert:vector, 3 μL ligation buffer (10 \times), 2 μL ligase, add PCR-grade water to a total reaction volume of 30 μL . Incubate at 16°C overnight.
24. Zymo purify ligation mixture as described above (step 20), but elute in 10 μL PCR-grade water.
25. Use appropriate volumes of desalted ligation mixture for electroporation of *E. coli* BL21(DE3) cells.
26. Plate on LB_{amp} plates to obtain single colonies and incubate at 37°C overnight.

The entire process described in this section is summarized in Fig. 2.

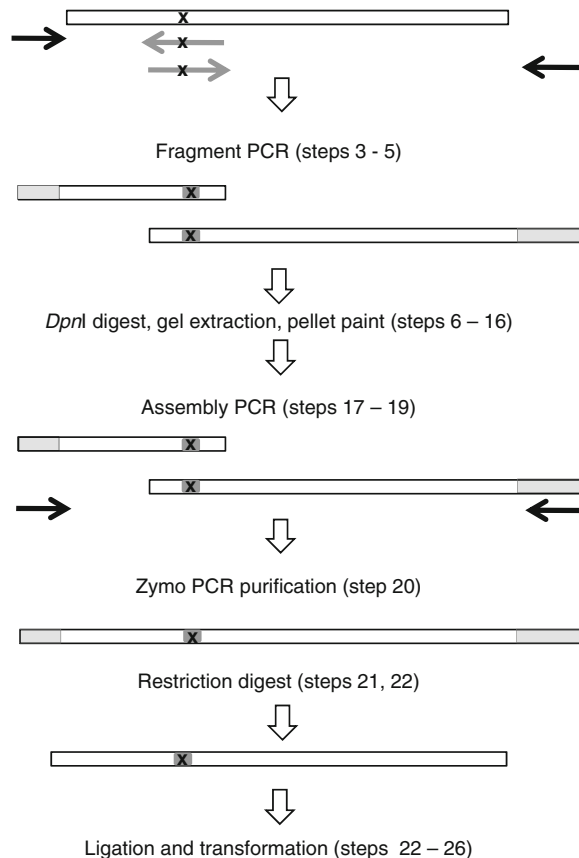


Fig. 2. Schematic for preparation of site-saturation mutagenesis libraries, based on SOE PCR (13). *Black arrows*: flanking primers; *gray arrows*: mutagenesis primers; *black x*: targeted site.

3.3. Protein Expression for Library Screening

1. A picking robot may be used to transfer single *E. coli* colonies into shallow 96-well plates filled with 300 μL LB_{amp}. As an alternative, single colonies can be picked manually using sterile toothpicks.
2. The 96-well plates should contain several controls: parent or wild-type clones, BL21(DE3) colonies carrying pET22b(+) only to control for background reactions in cell lysates, and a well filled with medium only, which serves as sterility control (see Note 11).
3. Cover plates with either air pore tapes or plastic lids and let grow overnight at 37°C with shaking at 210 rpm at 80% humidity (see Note 12).
4. The next morning, fill 96-well deep-well plates with 600 μL of LB_{amp} per well and inoculate with 75 μL of the overnight cultures.
5. Let cultures grow at 37°C and 210 rpm for 4 h in a humidified plate shaker with 80% humidity. Then, reduce the temperature of the incubator to 25°C, let cultures cool for 1 h and induce with IPTG at a final concentration of 0.5 mM (see Note 13).
6. After induction, growth and expression continue for up to 24 h at 25°C and 210 rpm.
7. Centrifuge the cells at 5,300 $\times g$ and 4°C for 5 min, pour out supernatant, tap plates onto paper towels, and store upside down at -20°C for a minimum of 2 h (see Note 14).

3.4. Screening of Libraries

1. Prepare the substrate (*R/S*)-2-acetolactate fresh for each assay (see Note 15): Mix 50 μL ethylethyl-2-acetoxy-2-methylacetate (EAMAA) with 990 μL of water. Then, gradually add 260 μL of 2 M NaOH in 10- μL increments. Each addition is followed by a 15-second vortexing step. After the entire amount of 2 M NaOH is added, mix the solution on a shaker at 130 rpm for 20 min at room temperature. Store substrate on ice until further use (14).
2. Thaw 96-well deep well plates containing the libraries at room temperature for 20 min upside down with open lids on paper towels (see Note 16).
3. Add lysis buffer (see Subheading 2.1, step 5 and see Note 17) using a multichannel pipette and vortex vigorously until pellets are resuspended. Then, agitate plates at 37°C and 130 rpm for 1 h.
4. Dilute lysate appropriately using 250 mM potassium phosphate buffer, pH 7.0 and centrifuge for 10 min at 5,000 $\times g$ and 4°C (see Note 18).
5. Transfer appropriate amount of lysate (see Notes 19 and 20) into transparent flat-bottom assay plates using a liquid handling robot.

6. Start reaction by addition of assay buffer (250 mM potassium phosphate pH 7, 10 mM MgCl₂, 1 mM DTT, 4 mM acetolactate, and 200 mM NADH or NADPH).
7. Monitor the depletion of cofactor at 340 nm in a plate reader over 1 min at 25°C.

3.5. Rescreening and Recombination Library

1. Calculate the ratio of NADH/NADPH consumed during the screening assay.
2. Choose variants that exhibit favorable activity with the new cofactor, but are only moderately active or inactive with the native cofactor.
3. Streak selected variants on LB_{amp} plates and pick at least in triplicate for rescreening in 96-well plates. This is necessary to ascertain that monoclonal variants are being rescreened.
4. Rescreen as described above and sequence improved variants.
5. Design primers for recombination of beneficial mutations: (1) design one complementary primer pair for each targeted site, (2) ensure that the primers encode all beneficial mutations found at the targeted site, (3) include the wild-type sequence, (4) use the IUPAC nomenclature to find the correct degenerate codon triplet.
6. If the IUPAC degeneration covers the desired mutations, but also adds unwanted amino acid changes (which enlarge the library size and increase the screening effort), design primers individually for each beneficial mutation and prepare a mixture of equimolar concentrations. Include a primer pair encoding the wild-type sequence (see Note 21).
7. Generate the recombination library using the protocol described above (see Subheading 3.2) with the difference that the recombination library contains several targeted sites in the gene rather than just one as in the example above.
8. Create all fragments individually (Fig. 3) and treat as described above.
9. Use up to five fragments for one assembly PCR, and then continue as described above or refer to Note 22 for solutions to encountered difficulties.
10. Proceed with screening and rescreening of recombination library and sequence the best hits (see Subheadings 3.3 and 3.4).

3.6. Protein Expression on 25-mL Scale

1. Inoculate 5 mL Luria-Bertani (LB) broth supplemented with 100 µg/mL of ampicillin with an *E. coli* BL21(DE3) transformant carrying the pET22b(+) expression plasmid. Grow overnight at 37°C with shaking at 250 rpm.

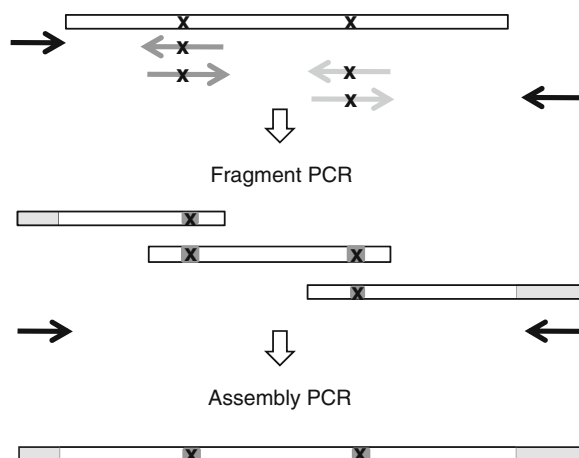


Fig. 3. Schematic for preparation of the fragments and the assembly of the fragments of a recombination library. *Black arrows*: flanking primers; *dark gray* and *light gray arrows*: mutagenesis primers; *black x*: targeted sites. For the entire process, refer to Fig. 2.

2. Use overnight culture to inoculate a 25-mL LB_{amp} expression culture supplemented with 100 µg/mL of ampicillin to an initial optical density (OD₆₀₀) of 0.1.
3. Grow expression culture at 250 rpm and 37°C until an OD₆₀₀ of 1 is reached.
4. Reduce temperature to 25°C and let culture cool for 30 min at 250 rpm.
5. Induce expression by the addition of IPTG to a final concentration of 0.5 mM.
6. After incubation for 24 h at 250 rpm and 25°C, centrifuge cells at 5,300×g and 4°C for 10 min, discard supernatant and freeze pelleted cells at −20°C.

3.7. Protein Purification

1. For additional information on purification, refer to Note 23.
2. Thaw *E. coli* cell pellets at room temperature for 20 min.
3. Resuspend cell pellets at a ratio of 0.25 g wet weight/mL in Buffer A.
4. Lyse the resuspended cells by sonication for 1 min with a 50% duty cycle.
5. Centrifuge at 11,000×g and 4°C for 15 min and then filter through a 0.45-µm filter.
6. Perform the following purification steps at 4°C.
7. Use 1 mL/min flow rate for 1-mL histrap columns and 5 mL/min for 5-mL histrap columns.
8. Check binding capacity of columns.

9. Purification method: (1) 4-column volume (cv) equilibration step with buffer A, (2) injection of sample, (3) washout unbound sample step with buffer A for 2 cv, (4) 5-cv wash step with 10% elution buffer B + 90% buffer A, (5) elution at 40% buffer B + 60% buffer A, (6) clean after elution at 100% B, (7) re-equilibration with buffer A.
10. IlvC and its variants were stored at 4°C in 40% B as eluted. A desalting step may be added, if the enzyme does not store well in high imidazole concentrations.

3.8. Characterization of Enzyme Variants

1. As a preliminary assessment of enzyme variants, the activity ratios (in U/mg) in the presence of the new and the native cofactor can be determined in *E. coli* lysates. Interesting variants should be purified using a highly reproducible method (as described in Subheading 3.7) for ease of comparison.
2. IlvC activity was assayed by monitoring the linear decrease in NAD(P)H concentration at 340 nm over 1 min at 25°C on a UV-vis spectrophotometer.
3. Assay buffers: 250 mM potassium phosphate pH 7, 1 mM DTT, 10 mM (R/S)-2-acetolactate, and 10 mM MgCl₂. Prepare one batch of assay buffer containing 200 μM NADPH and a second batch containing 200 μM NADH final concentration.
4. Place lysate or purified enzyme (sample volume 100 μL) into a cuvette (see Note 24), and initiate the reaction by addition of 900 μL of the assay buffer containing either NADPH or NADH, thereby diluting the assay buffer by 10%.
5. Use Bio-Rad Protein assay or similar assays by other suppliers to determine the concentration of total protein in lysates and in purified samples.
6. Desired properties: After first round of NNK libraries, the variants should have gained activity on the new cofactor while maintaining or losing activity on the native one. After the recombination round, the preferred variants should have native-like activity with the new cofactor while exhibiting no or very low activity with the native cofactor.

4. Notes

1. Unless mentioned otherwise, all solutions should be prepared in water that has a resistivity of 18.2 MΩ-cm and a total organic content of less than five parts per billion. The standard referred to as “PCR-grade water” for molecular biology applications was autoclaved.

2. This protocol can be adapted for other proteins that are expressed in *E. coli*.
3. We found using flanking primers, which bind ~50 bp upstream and downstream of the insert, to be beneficial. These 50-bp fragments will be visible on an agarose gel after restriction digest and will be an indicator of digest completion.
4. NNK primers:
 - (a) For DNA degeneracies, see IUPAC nomenclature <http://www.chem.qmul.ac.uk/iupac/>.
 - (b) NNK (32 codons, 1 stop codon, 20 amino acids, 94 colonies to screen for threefold oversampling) is preferred over NNN (64 codons, 3 stops, 20 amino acids, 190 colonies to screen for threefold oversampling).
5. We found Phusion polymerase worked very well for generating fragments and assembly PCR products. Also, it was beneficial for the incorporation of the mutations to reduce the annealing temperature to 55°C in cases where the recommended annealing temperature was higher.
6. General advice for assembly PCRs: Always perform a positive control PCR with the flanking primers using the plasmid as template. The length of this PCR product will serve as comparison to identify correctly assembled products on the agarose gel.
7. Freeze “n” Squeeze tubes with DNA containing gel fragments may be frozen longer than 10 min, even overnight, but then spinning at lower speeds than 10,000×g is recommended to avoid breaking the tubes in the centrifuge.
8. Pellet paint recommendations:
 - (a) The manual recommends precipitating for 2 min at room temperature. For optimal results, precipitate on ice or in the freezer (−20°C). Also, the centrifugation steps are better performed at 4°C instead of the suggested room temperature spin step.
 - (b) The pink pellets are fairly loose. It is best to discard the supernatant carefully with a pipette rather than pouring it out.
 - (c) Pellets containing ethanol have a dark pink appearance. As soon as they are dry, the color is very light pink.
9. Zymo PCR purification kit modifications and recommendations:
 - (a) For optimal results, add a spin step (13,000×g, 4°C, 1 min) to remove residual ethanol after washing twice with ethanol and before eluting the DNA in PCR-grade water. Be sure to empty collecting tube before spinning to dry.

- (b) For elution of a total volume of 20 μL , place 10 μL on top of the spin columns, let sit for at least 1 min, centrifuge, add the remaining 10 μL , let sit again for 1 min, and finally elute by centrifugation. This increases the yield.
- 10. Even though fast digest enzymes from NEB were used, we obtained better results with longer incubation. We digested with *NdeI* overnight and then the next morning added *XhoI* for 2 h instead of the recommended 10–30 min. This was not detrimental to the inserts.
- 11. High-throughput expression for screening: it is crucial to place parent and negative control on each plate, since plate to plate expression and activity variations may be observed.
- 12. Cultivation conditions for high-throughput expression: when growing small culture volumes at 37°C, prevent uneven evaporation of cultures across the plates by increasing the shaker's humidity to 80%.
- 13. Induction with 0.5 mM IPTG worked very well for the expression of wild-type IlvC and its variants. However, the IPTG concentration should be adjusted in cases where inclusion bodies are observed.
- 14. Freezing the 96-well plates containing the cell pellets is essential for a successful lysis step. The plates should be frozen for a minimum of 2 h, preferably overnight.
- 15. In the case of acetolactate, it was important to make the substrate fresh as required. The substrate cannot be stored in the freezer or fridge. This may not be true for other substrates.
- 16. Thawing the cells at room temperature is important to assist the resuspension step. If the cell pellets do not resuspend despite vigorous vortexing, it is best to resuspend individual wells by pipetting up and down with a P200 or larger.
- 17. It is crucial to add DNaseI to the lysis buffer of the high-throughput screen. Without the addition of DNaseI, the lysate will turn out too viscous for accurate transfer with either a liquid handling robot or a multichannel pipette.
- 18. Dilution of the lysate should be done prior to centrifugation. The amount of buffer added depends on how active an enzyme is. This should be tested prior to the actual screening in a screening validation experiment using an entire 96-well plate containing parent with three wells carrying the vector only to account for background activity.
- 19. When establishing a high-throughput assay, we found that transferring larger lysate volumes (>20 μL) reduces the pipetting error.

20. Lysate transfer with the liquid handling robot usually generates bubbles, which will disturb activity measurements in a plate reader. A simple, but efficient, way to remove them is to centrifuge the assay plates briefly (e.g., 1 min at $4,000 \times g$).
21. When using an equimolar solution of mixed primers in the recombination library, it is useful to sequence randomly chosen clones to control for potential nucleotide biases.
22. Solutions for difficulties encountered with recombination libraries:
 - (a) More than five fragments to assemble: Assemble four or fewer fragments, Zymo purify the resulting products, and then use these products as templates for a final assembly PCR step.
 - (b) Two targeted sites are so close to each other that the resulting fragment would be <100 bp, but the sites cannot be covered with one primer: Recombine all sites except one of the two, then use the full-length assembly product as template to introduce the last site following the site-saturation mutagenesis protocol.
 - (c) Less than five fragments, but one fragment does not assemble: Assemble stepwise. If necessary, elongate the primer to generate a longer overlap sequence. This problem can occur with very short fragments (<100 bp) at the 5' or the 3' end.
23. General notes on purification:
 - (a) In general, all purification steps, from sonication to elution, should be performed at 4°C to minimize potential protease activities and to ascertain protein stability.
 - (b) The histrap column manuals contain information on storage, flow rate, pressure limits, and binding capacity.
 - (c) For optimal purification results, it is beneficial to determine the amount of his-tagged protein in the crude extract and then choose a column size (1 or 5 mL) that will allow for loading at or even slightly exceed the capacity limit.
 - (d) When working in Tris buffer and at 4°C , it is important to readjust the pH after the buffers have cooled down. The pH of Tris is temperature-sensitive.
 - (e) Determine the point of elution of your protein via a linear gradient. Then, establish a step gradient such as the method described for IlvC.
24. We determined the Michaelis–Menten constants for the cofactors in a spectrometer rather than using a plate reader. The plate reader did not have the required sensitivity to accurately measure K_M values.

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