Chapter 18

Using Purified Tyrosine Site-Specific Recombinases In Vitro to Rapidly Construct and Diversify Metabolic Pathways

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Abstract

The site-specific recombinase Cre was previously reported to have in vitro activity. Here, we describe the method of purifying two new tyrosine site-specific recombinases VCre and Dre along with Cre by nickel affinity chromatography. We proved the in vitro function of the VCre and Dre on their respective conditional recombination sites. We also developed a methodology to one-step construct and optimize the productivity of a biosynthetic pathway through the combinatorial integration of promoters into a plasmid-encoded pathway by simply incubating a DNA mixture with recombinase system at 37 °C in vitro.

Key words Protein purification, Tyrosine recombinase, DNA recombination, Metabolic engineering, Synthetic biology

1 Introduction

Site-specific recombinases are a class of DNA function enzyme that can recognize specific DNA sequences and drive recombination between the targeted DNA sequence to achieve deletion, inversion, or integration of DNA fragments. They have been discovered and isolated from bacterial and bacteriophage sources and have been engineered as tools for molecular biology in both prokaryotes and eukaryotes for applications as diverse as genome engineering, cell memory devices, cell computational devices, and the Brainbow project [1–7]. While these in vivo applications of site-specific recombination are useful, they have the potential for problems associated with off-target effects in the host genome if they contain intrinsic sequences homologous to the recombination sites. If there are pseudo-sites existing in the host genome, homologous recombination can happen between these pseudo-sites and generate large fragment deletion on the genome; therefore, resulting in toxicity to the host [8, 9]. On one side, the recombinase Cre has been engineered to have lower effects on host genome to improve recombination accuracy [10]; however, it is not a universal way to solve all

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off-target problems to any host of interest. Cell-free synthetic biology is an emerging powerful area that allows independent study of target biological systems by excluding its interaction with the cellular context. Therefore, a cell-free recombinase system is worth exploring to develop more DNA editing tools. In the field of synthetic biology, DNA assembly technology is one of the most important aspects, and site-specific recombinases have also been explored for DNA cloning [11–14]. In this study we add some more recombinases to the family and further developed their in vitro application on metabolic pathway diversification.

Both the Cre and Flp tyrosine recombinases have been shown to function in vitro [15, 16]. However, these published in vitro experiments have mainly been mechanistic and functional studies [16, 17]. To expand the utility of tyrosine recombinases as tools for synthetic biology, we have developed a method for the production of a set of orthogonal tyrosine recombinases and demonstrated their application to optimize a biosynthetic pathway through combinatorial screening of a promoter library. Since a number of orthogonal site-specific recombinases with distinct DNA recognition sequences are available, it is possible to implement multiple enzymes to shuffle DNA in vitro.

Here, we present a method for the purification of a number of orthogonal tyrosine recombinases and a protocol for the combinatorial shuffling of plasmid-carried DNA part libraries in vitro. In recent years, more tyrosine recombinases like Dre, VCre, Vika, and SCre have been identified by genome mining [18–21]. We demonstrated the heterologous production of Cre, Dre, and VCre, which were successfully purified and proved to have in vitro function by DNA electrophoresis (*see* Fig. 1). We further quantified the excision and integration rate by a transformation-based color change method for the three systems (*see* Fig. 2). Excision and integration rate of loxP LE/RE mutant sites of Cre can also be quantified by this method [22, 23]. As a proof of concept demonstration for metabolic engineering, we then used VCre to integrate a standardized yeast constitutive promoter library to diversify β-carotene pathway production (*see* Fig. 3) [24]. Future applications will enable the multiplexing of orthogonal recombinases in concert with cell-free TX-TL systems to build in vitro recombinase devices for synthetic biology [25].

2 Materials

All solutions were prepared from analytical quality reagents with ultrapure deionized water (18.5 MΩ-cm resistivity at 25 °C) and filtered using a 0.22 μm filter prior to use. Cell culture media were autoclaved and cooled to room temperature before use. Local waste disposal regulations were followed diligently throughout the procedures.

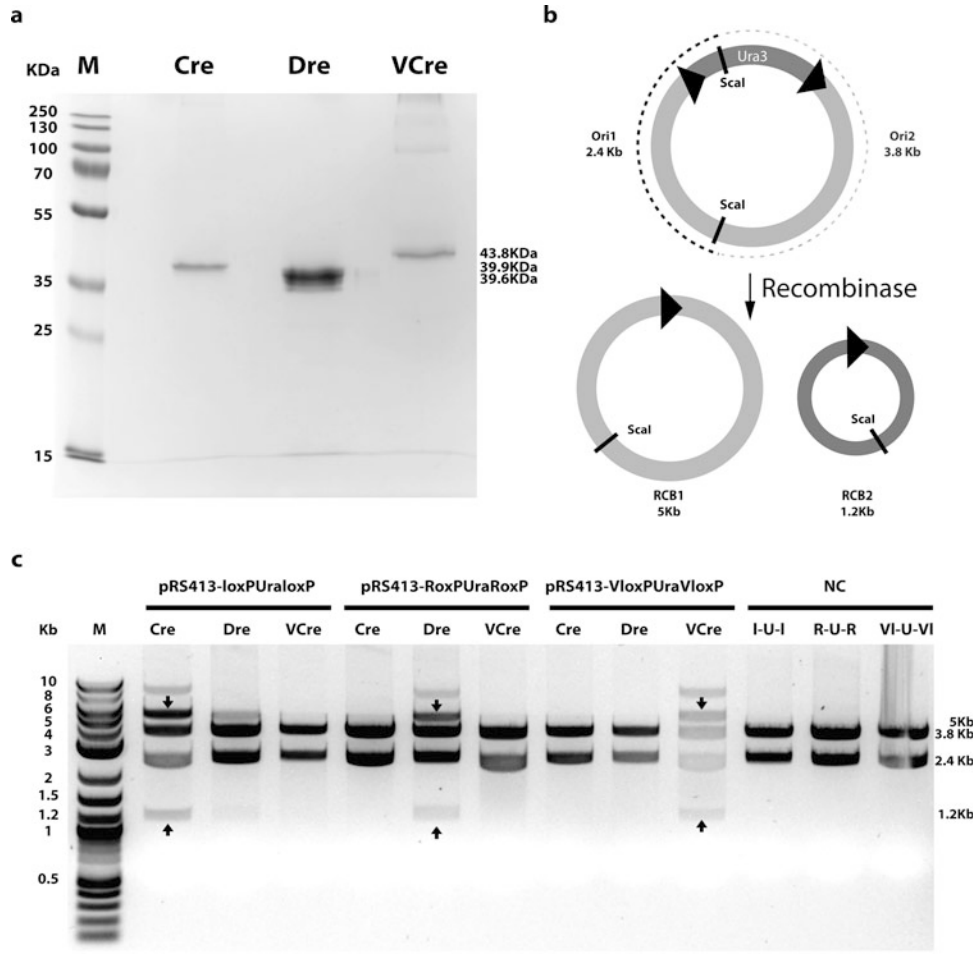

Fig. 1 Purification and in vitro function test of three recombinases. (**a**) SDS-PAGE gel of Cre, Dre, and VCre, the size of which are 39.9 kDa, 39.6 kDa, and 43.8 kDa, respectively. (**b**) Illustration of variation of digestion map after in vitro recombination. Linearization of original plasmid by ScaI will generate a 3.8 kb band and a 2.4 kb band. Digestion of recombined separate plasmids will generate a 5 kb and 1.2 kb band. (**c**) Agarose gel electrophoresis test proves orthogonal function of the three recombinases in vitro. When recombination happens, a 5 kb and a 1.2 kb band will be generated beside the original 3.8 kb and 2.4 kb band. The digestion pattern variation only happens between Cre and loxPUraloxP circuit, Dre and RoxUraRox circuit, and VCre and VloxUraVlox circuit, proving the orthogonal function of the three recombination systems

2.1 DNA Circuit Construction and Molecular Biology

DNA oligonucleotides of fewer than 80 bp length were all purchased from Integrated DNA Technologies (IDT) (25 nmol if below 60 bp and 4 nmol ultrapure DNA if above 60 bp). Double-stranded DNA samples were purchased from either IDT as standard gBlocks or Twist Biosciences as insert in pUC19 vector. All DNA samples and DNA-modifying enzymes should be stored at −20 °C, and DNA purification kits should be stored at room temperature.

1. QIAprep Spin Miniprep Kit (Qiagen).
2. Phusion® High-Fidelity DNA polymerase (NEB).

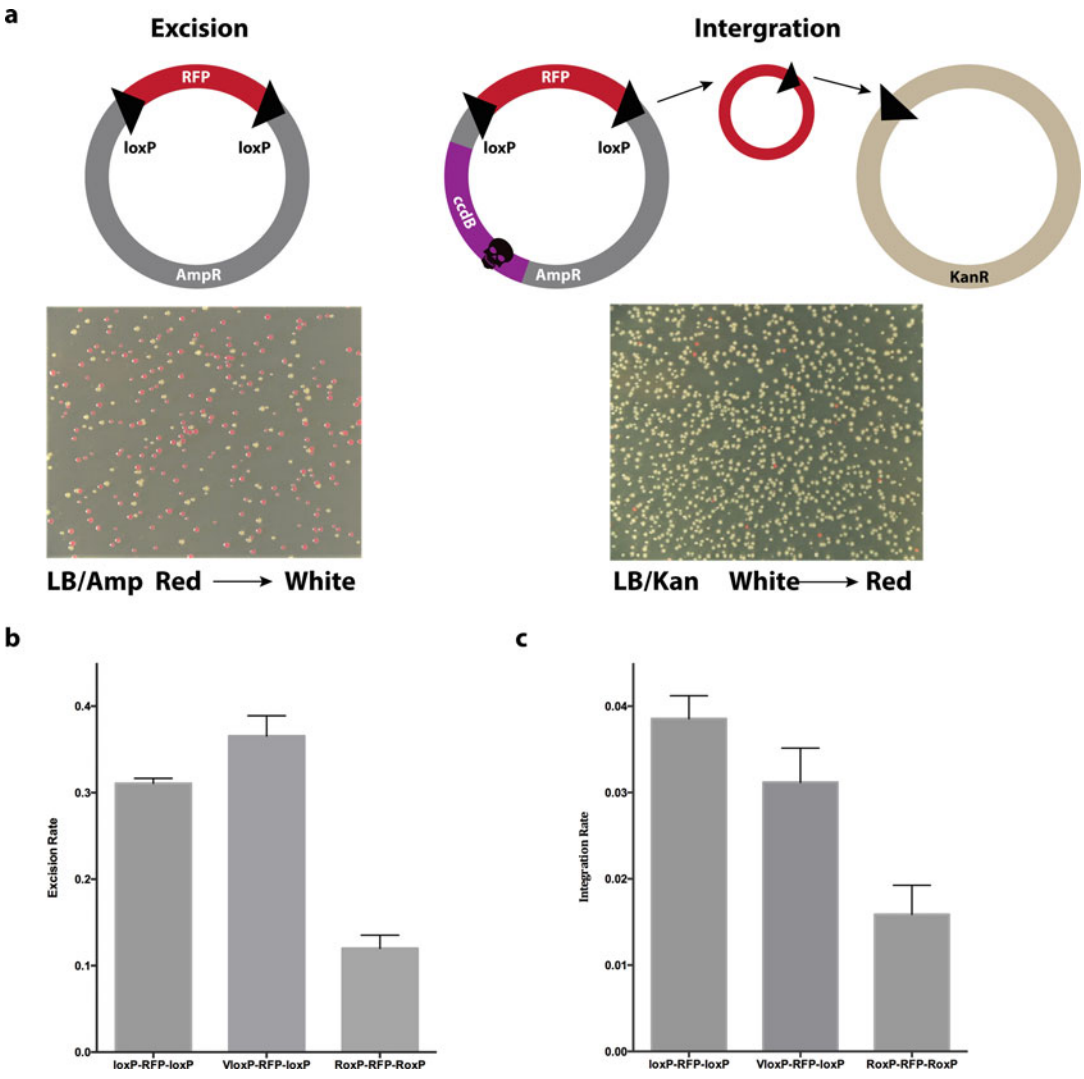

Fig. 2 (**a**) Recombinase function quantification strategy. A transformation-based red-white method was applied to test the in vitro excision and integration rate. When excision happens, the RFP gene is excised, and the colony will turn white with the loss of the RFP gene. When the excised RFP was mixed with KanR vector bearing a single recombinase recognition site, it can integrate into the KanR backbone, turning the color of the colony to red after selection on kanamycin plates. (**b**) Excision rate comparison of Cre/loxP, VCre/Vlox, and Dre/Rox systems. Cre/loxP and VCre/Vlox have around 30% recombination rate after 2 h incubation, while Dre have lower recombination rate around 15%. (**c**) Integration rate comparison of Cre/loxP, VCre/Vlox, and Dre/Rox systems. The relative integration rate of Cre/loxP system is around 4%, that of VCre/Vlox system is lower with 3%, Dre/Rox has lowest integration rate of 1.5% under the condition described here

3. KAPA HiFi PCR Kit (Kapa Biosystems).
4. Restriction enzymes (NEB).
5. T4 DNA ligase (NEB).

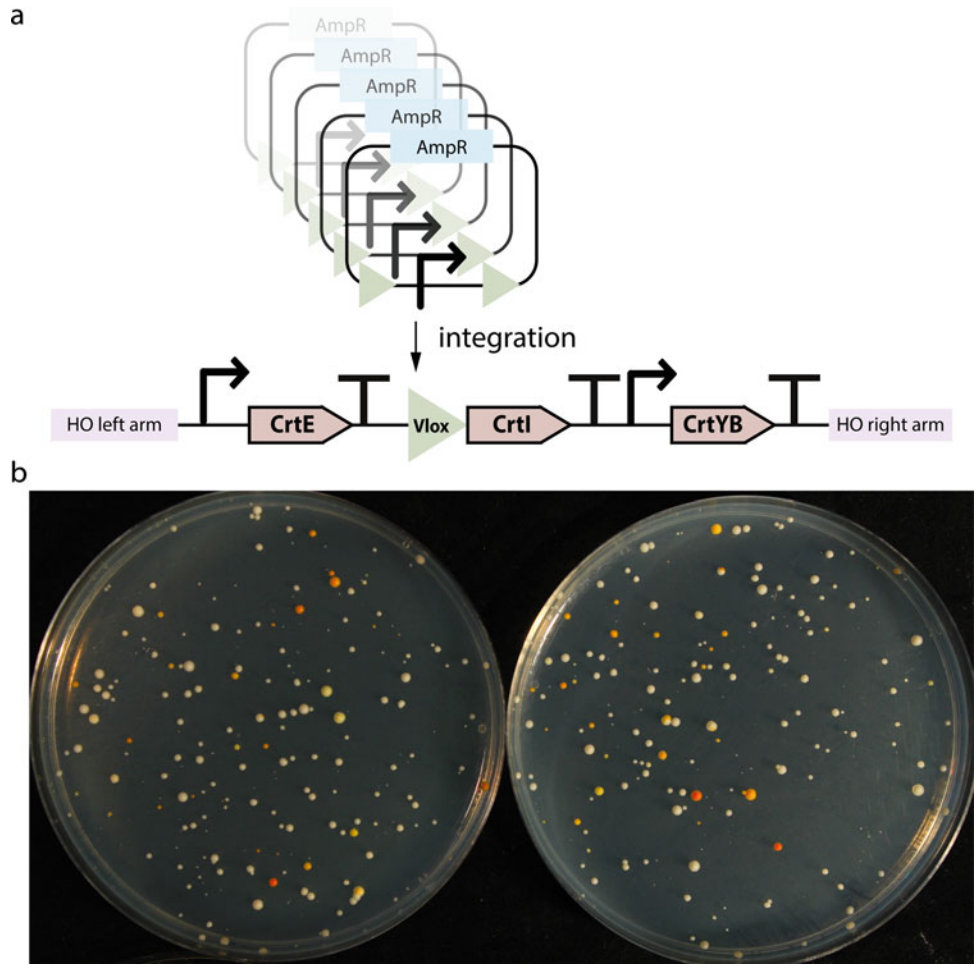

Fig. 3 β-Carotene pathway diversification by VCre recombinase. (**a**) Strategy of promoter integration to gene of interest. Vlox is located upstream of CrtI gene and a YeastFab promoter library with different transcription initiation strengths are chosen for integration. The pathway is flanked by two HO homologous arms and is finally integrated into HO locus on yeast genome for expression. (**b**) Color diversification of yeast containing the promoter integrated pathways. The yeast transformants are selected on SC-His-Ura plates. Color diversity of yeast colonies was generated by in vitro recombinase toolkit and YeastFab promoter library, indicating diversified components and quantity of carotenoid in the cell

6. Golden Gate assembly master mix for a 20 μL reaction: for a final 4.2 μL master mix, add 1 μL BsaI/BsmBI, 1 μL T4 ligase, 2 μL 10× T4 ligase buffer, 0.2 μL 100× BSA.
7. Gibson assembly master mix: for a 15 μL master mix, add 4 μL 5× ISO buffer, 0.16 μL T5 exonuclease (1/20 diluted), 0.25 μL Phusion polymerase, 2 μL 160 μL of 40 (U/μL) Taq Ligase, 8.59 μL H_2O. For a 6 mL 5× Isothermal buffer, add 3 mL 1 M Tris–HCl (pH 7.5), 300 μL 1 M $MgCl_2$, 60 μL 100 mM dGTP, 60 μL 100 mM dATP, 60 μL 100 mM dTTP,

60 μL 100 mM dCTP, 300 μL 1 M DTT, 1.5 g PEG-8000, 300 μL 100 mM NAD.

8. QIAquick Gel Extraction Kit.
9. GoTaq® Green Master Mix (Promega).
10. Zero Blunt® TOPO® (Invitrogen).
11. ProFlex PCR System (Thermo Fisher Scientific).
12. PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific).
13. 2-Log DNA Ladder (0.1–10.0 kb) (NEB).
14. 50× TAE buffer: 2 M Tris base, 57.1 mL glacial acetic acid, 0.05 M EDTA. Dissolve Tris base in around 600 mL ddH_2O, add 57.1 mL glacial acetic acid and 0.05 M EDTA, and mix. Make up to 1 L with ddH_2O. For a 1× working stock, measure out 20 mL of 50× TAE, and make up to 1 L with ddH_2O.
15. TopVision Agarose (Thermo Scientific).
16. 0.8% TAE agarose gel: Weigh 0.8 g of agarose into a 250 mL conical flask. Make up to 100 mL with 1× TAE buffer, and microwave on high for 2.5 min.
17. 6× DNA gel loading buffer (NEB).
18. LB agar plates: 5 g NaCl, 5 g tryptone, 2.5 g yeast extract, 7.5 g Agar. Weigh out reagents into a beaker, make up to 500 mL with ddH_2O, and swirl to mix. Autoclave solution and allow to cool. Once solidified, microwave on low for 20 min until the agar is remelted and allow to cool to 55 °C. Add antibiotic if needed and pour plates.

2.2 Protein Purification

1. PBS buffer: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4. Weigh out the reagents and dissolve in 800 mL ddH_2O. Adjust the pH to 7.4, make up to 1 L with ddH_2O, and then filter sterilize through a 0.22 μm filter.
2. HisTrap FF column (GE Healthcare).
3. ÄKTA chromatography system (GE Healthcare).
4. HisA buffer: 50 mM Tris–HCl pH 8.0, 50 mM imidazole, 500 mM NaCl. Dissolve imidazole in the 50 mM Tris–HCl pH 8.0 and 500 mM NaCl solutions, and make up to 800 mL with ddH_2O. Pour into measuring cylinder, and make up to 1 L with ddH_2O. Filter sterilize with 0.22 μm filter and degas.
5. HisB buffer: 50 mM Tris–HCl pH 8.0, 500 mM imidazole, 500 mM NaCl. As above, noting the difference in imidazole concentration.
6. Resolving gel buffer (16 mL of 15% buffer): 3.7 mL ddH2O, 8 mL 30% acrylamide/bis solution, 4 mL 1.5 M Tris pH 8.8, 160 μL 10% SDS, 160 μL 10% APS, 16 μL TEMED. Add 30%

acrylamide/bis solution to 3.7 mL of ddH2O. Add 4 mL of 1.4 M Tris-HCl pH 8.8 and mix. Add 160 μL of 10% SDS and mix again. Immediately before pouring gel, add 160 μL of APS and 16 μL of TEMED and mix well.

7. Stacking gel buffer (10 mL of 6% buffer): 5.3 mL ddH2O, 2 mL 30% acrylamide/bis solution, 2.5 mL 0.5 M Tris pH 6.8, 100 μL 10% SDS, 100 μL 10% APS, 10 μL TEMED. Add 2 mL of 30% acrylamide/bis solution to 5.3 mL ddH2O. Add 2.5 mL of 0.5 M Tris-HCl pH 6.8 and mix. Add 100 μL of 10% SDS and mix again. Immediately before pouring gel, add 100 μL of APS and 10 μL of TEMED and mix well.
8. 30% acrylamide/bis solution (29.2/0.8%): Severn Biotech Ltd.
9. Ammonium persulfate: 10% solution in water.
10. SDS: 10% solution in water (Sigma-Aldrich).
11. TEMED—Tetramethylethylenediamine (Sigma-Aldrich).
12. 10× SDS-PAGE running buffer: 300 g Tris base, 1440 g glycine, 100 g SDS. Dissolve reagents into 8 L ddH_2O (in fume hood), and check for pH 8.3. Make up to 10 L with ddH_2O. To make 1× running buffer, add 1 L of 10× running buffer to 9 L ddH_2O and mix gently.
13. 5× SDS-PAGE loading buffer (10 mL): 250 mM Tris–HCl pH 6.8, 10% SDS, 30% glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue. Weigh out 1 g SDS powder; dissolve in 2.5 mL of 1 M Tris–HCl pH 6.8, 3 mL 100% glycerol, 500 μL β-mercaptoethanol, 200 μL 1% bromophenol blue solution; and make up to 10 mL with ddH_2O. Make sure to fully dissolve SDS powder.
14. 1% Bromophenol blue solution: Weigh 100 mg bromophenol blue powder into a 15 mL falcon tube, and dissolve in 10 mL of ddH_2O. Mix solution and cover with foil to store.
15. MSE Soniprep 150 Plus Ultrasonic Disintegrator.
16. Protein storage buffer: 15 mM Tris–HCl, 250 mM NaCl, 50% Glycerol, pH 8.0 at 25 °C.
17. 0.45 μm syringe filter (Millipore).
18. Liquid nitrogen.
19. Cellulose dialysis tubing 21.3 mm diameter 8 kDa molecular weight cutoff (Biodesign).
20. Dialysis clips (Clas Ohlson).
21. NanoDrop™ 2000 spectrophotometer (Thermo Fisher).
22. 1 M IPTG: Dissolve 2.38 g IPTG in 8 mL ddH_2O and bring to a final volume of 10 mL. Filter sterilize through a 0.22 μm syringe filter. Store at −20 °C.

23. 50% glycerol solution (Sigma-Aldrich): Autoclave 100% glycerol, and dilute to a 50% solution with ddH_2O. Filter sterilize through a 0.45 μm filter, and store at 4 °C.
24. 50 mL polycarbonate centrifuge tubes (Thermo Fisher Scientific).

2.3 Recombination Reaction

1. 10× recombination buffer: 330 mM NaCl, 500 mM Tris–HCl, 100 mM $MgCl_2$, pH 7.5 at 25 °C. Mix 3.3 mL 1 M NaCl, 5 mL 1 M Tris–HCl, and 1 mL 1 M $MgCl_2$, add ddH_2O to 10 mL, and adjust the pH to 7.5. Aliquot to 1 mL Eppendorf tubes and store in −20 °C for longer use.
2. PureLink® PCR Purification Kit.

2.4 Cell Culture and Transformation

1. DH5α and BL21 (DE3) chemically competent *E. coli* cells (Thermo Fisher Scientific).
2. One Shot® *ccd*B Survival™ 2 $T1^R$ Competent Cells (Thermo Fisher Scientific).
3. BY4741 yeast strain (*MATa, his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*).
4. LB medium: 10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl. Weigh out reagents and dissolve in 800 mL ddH_2O. Adjust the pH to 7.5 and make up to 1 L with ddH_2O. Autoclave and store at room temperature.
5. 1000× ampicillin solution: Weigh out 1 g of ampicillin sodium salt, and make up to 10 mL with ddH_2O. Filter sterilize through a 0.22 μm filter and store in 1 mL aliquots at −20 °C.
6. 1000× kanamycin solution: Weigh out 500 mg of kanamycin powder, and make up to 10 mL with ddH_2O. Filter sterilize through a 0.22 μm filter and store in 1 mL aliquots at −20 °C.
7. Yeast synthetic drop out media:

   Make a synthetic complete -8 (-Ade-Cys-His-Leu-Lys-Met-Trp-Ura) dropout as a master mix. Make 2× SC-8 broth by mixing 8.5 g yeast nitrogen base w/o aa's and amm sulfate (Sigma), 25 g ammonium sulfate (Sigma), and 10 g SC-8-dropout mix and adding distilled water to 2500 mL. Mix well and distribute as 250 mL into ten 500 mL bottles and autoclave for 20 min. To make SC-His-Ura plates, mix 250 mL 2× SC-8 broth, 250 mL 4% autoclaved agar with 25 mL 40% glucose and supplement 5 mL 40 mM tryptophan, 10 mL 100 mM leucine, 5 mL 15 mM adenine, 5 mL 100 mM lysine, and 6 mL 50 mM methionine amino acid solution (all purchased as powders from Sigma).
8. Frozen yeast transformation buffer: for one transformation, mix 260 μL PEG 3350 (50% (w/v)), 36 μL LioAc 1.0 M, and 10 μL single-stranded carrier DNA (10.0 mg/mL), add DNA, and sterile water up to 360 μL.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Construction of the Recombinase Expression Plasmid

1. The desired open reading frames for the tyrosine recombinase sequences can be PCR amplified from genomic or synthetic DNA by Phusion polymerase and subcloned to TOPO blunt end vector by the Zero Blunt® TOPO® Kit (*see* **Note 1**). The fragments are designed such that they are flanked by NcoI and XhoI restriction sites with start and stop codons removed and additional nucleotides added to allow in frame expression from the start codon within the NcoI restriction site and the C-terminal His_6 tag encoded in the pET-28(a) vector. Transform 3 μL of the TOPO cloning reaction to DH5α competent cell, and select on LB agar plates with 50μg/mL kanamycin. Do colony PCR with the amplification primers using the Gotaq® Green kit for screening positive clones, and do plasmid mini-prep and sequencing confirmation afterwards.
2. Digest 1 μg of the sequence confirmed TOPO subcloned recombinase plasmids and the pET-28a for 2 h with NcoI and XhoI restriction enzymes as per the manufacturers' instructions.
3. Prepare a 0.8% TAE agarose gel with sufficient wells for each sample plus size-marker standards, and uncut vector controls. Place the gel in an appropriately sized gel tank, add TAE buffer to cover the gel, and fill any buffer reservoirs. Add 5 μL DNA gel loading buffer to each sample and control, and add to individual wells of the gel. Run the gel at 110 V for 40 min. Visualize the gel with a blue light box and orange-filter glasses. Excise fragments of the correct size for cut pET-28a vector and the recombinase genes with a fresh scalpel, and place in a clean 1.5 mL Eppendorf tube. Weigh each sample, and add an appropriate amount of resuspension buffer from the gel extraction kit. Proceed with the gel extraction as per the manufacturers' instructions; however, at the elution step, add 15 μL of elution buffer to the center of the column membrane, and leave to stand for 2 min before elution. Determine the DNA concentration using a NanoDrop spectrophotometer.
4. Ligate 100 ng purified recombinase gene and pET-28(a) backbone by incubating with 1 μL T4 DNA ligase, 2 μl T4 DNA ligase buffer, and purified water to 20 μL for 2 h at 25 °C or 16 °C overnight. Transform 5 μL of the ligation reaction to DH5α competent cells, and select on LB agar plates supplemented with 50 μg/mL kanamycin. The resulting colonies are screened for the insertion of the recombinase gene by colony

PCR using the GoTaq Green Master Mix. Run the entire PCR reaction on a 0.8% agarose gel with DNA ladder as described before, and correct clones can be identified as those with a band of the appropriate size for the insert DNA.

Colonies identified as containing the correct insertion by colony PCR were cultured in 10 mL LB medium supplemented with 50 μg/mL kanamycin at 37 °C with shaking, 120 rpm, overnight, and plasmid DNA was isolated using a Qiagen Miniprep Kit as per the manufacturers' instructions. The sequence of the plasmid DNA was confirmed by Sanger sequencing using T7 forward and reverse sequencing primers.

3.2 Construction of Recombinase Function Test Plasmid

1. The Ura3 expressing gene was tailed with loxP, Rox, or Vlox sites and 40 bp homologous arms of pRS413 by PCR from template pRS416 using KAPA HiFi Kit. The primers were ordered from IDT as 4 nmol ultrapure DNA. The PCR reaction was set up as the manufacturer's instruction. The program for KAPA HiFi PCR is 95 °C for 3 min, 30 cycles of [98 °C for 20 s, 65 °C for 15 s, 72 °C for 30 s], 72 °C 4 min (*see* **Note 2**). Digest the yeast pRS413 vector by SmaI. Run the recombination site flanking Ura3 PCR reaction and linearized pRS413 on 0.8% agarose gel with DNA ladder, and purify the 1.2 kb Ura3 band and 5 kb pRS413 backbone band by gel extraction. Measure the concentration of purified DNA by NanoDrop spectrophotometer. Mix above 50 ng and equal molar of each of the Ura3 and pRS413 vector after calculation with 15 μL Gibson assembly master mix. Incubate at 50 °C for 30 min, transform 10 μL reaction to DH5α competent cell, and select on LB plate supplemented with 100 μg/mL (*see* **Note 3**). Do colony PCR and sequencing to confirm.

3.3 Construction of Excision and Integration Rate Test Circuit

1. Red fluorescent protein (RFP) was tailed with loxP, loxP mutant sites, Rox, or Vlox sites by PCR using KAPA HiFi Kit. For excision rate test circuit, RFP was further flanked with homologous arms to be assembled with SmaI-linearized pRS415 by Gibson assembly. After colony PCR screening and sequencing verification, digest the RFP-pRS415 plasmid with Eco53kI, and assemble it with PCR-amplified ccdB gene fragment by another round of Gibson assembly. Transform 10 μL of the reaction to ccdB-resistant strain competent cell, and verify by sequencing.
2. For integration rate test circuit, use similar method to assemble the recombinase flanking RFP gene to HCKan vector by Gibson assembly except to digest HCKan vector with BsmBI site. After verification, the plasmid went through a round of in vitro recombination by according recombinase. The recombination condition will be introduced in 3.7.

3.4 Construction of Promoter Loading Vector and Basic Acceptor Pathway

1. For promoter loading vector, BsmBI sites were removed from the pUC19 plasmid by PCR mutagenesis [26]. Three Ura3-BsmBI-RFP-BsmBI cassettes were flanked by recombination sites (loxP, loxJT17, or Vlox) through PCR amplification and further assembled with SmaI-linearized pUC19 by Gibson assembly. Standardized promoters with flanking BsmBI sites from a YeastFab library were loaded in this loading vector by BsmBI Golden Gate assembly.
2. β-Carotene pathway circuit construction includes three rounds of YeastFab standard Golden Gate assembly [24]. In the first round, Promoter PGK1 and ACT1 were cloned into HCKan_P vector by BsaI Golden Gate assembly; RFP gene flanked by loxP, loxJT15, and VloxP pairs were also cloned into HCKan_P vector as the promoters. β-Carotene synthesis gene, CrtI, CrtE, and CrtYB, were cloned into HCKan_O vector. Terminator ACS2, ACS1, and ENO2 were cloned into HCKan_T vector. In the second round, recombination site flanking RFP, CrtI, and terminator ACS2 were assembled to a POT2 vector; promoter PGK1, CrtE gene, and terminator ACS1 were assembled to POT4 vector; promoter ACT1, CrtYB gene, and terminator ENO2 were assembled to POT5 acceptor vector, respectively, by BsmBI Golden Gate assembly. The recombination site RFP containing CrtI unit went through one-step in vitro recombination to remove RFP. In the final round, POT2-CrtI, POT4-CrtE, and POT5-CrtYB were assembled to the HO integration acceptor vector by BsaI Golden Gate assembly.

3.5 Golden Gate Assembly

1. BsaI based Golden Gate assembly: add 4ul master mix, equal molar DNA fragments with a total quantity of 1 μg, and make up to 20 μL with ddH_2O. Incubate in a thermocycler with the following program: 10—30 cycles of 37 °C for 5 min and 16 °C for 10 min, following with 50 °C for 10 min, 80 °C for 5min, finally keep at 4 °C (*see* **Note 4**).
2. BsmBI based Golden Gate assembly: in a 10 μL reaction, mix 1 μL 10× T4 ligase buffer (NEB), 0.2 μL 100× BSA, and 0.5 μL BsmBI, equal molar of DNA fragments with a total quantity of 500 ng, and adjust the volume to 9 μL with dH_2O. Incubate in a thermocycler with the following program: 55 °C for 90 min, add 1 μL T4 ligase, 10—30 cycles of 37 °C for 5 min and 16 °C for 10 min, following with 55 °C for 10 min, 80 °C for 5min, finally keep at 4 °C (*see* **Note 5**).

3.6 Expression, Purification, and Storage of Recombinase

1. Transform the verified pET-28(a) expression plasmids for Cre, Dre, and VCre expression plasmid into chemically competent *E. coli* BL21 (DE3) strain. Transfer a single colony to 100 mL LB media, supplemented with kanamycin 50 μg/mL, and incubate overnight at 37 °C with shaking, 180 rpm.

2. Inoculate 10 mL of the overnight pre-culture into a 2.5 L baffled flask with 1 L of LB medium supplemented with kanamycin 50 μg/mL and incubate at 37 °C with shaking, 180 rpm. Monitor the cell growth by recording the OD_{600} of the culture at regular intervals, and induce recombinant protein production at an OD_{600} 0.6 with the addition of 1 mM IPTG. Reduce the incubation temperature to 18 °C to maximize protein expression and incubate the cells for 15 h (*see* **Note 6**).
3. Pellet the cells from the 1 L cultures by centrifugation at 4000 × *g* for 20 min, 4 °C, and resuspend the cell pellet in tenfold (volume per gram of cell pellet) of PBS. Transfer the resuspended cells to a 50 mL falcon tube to wash cells before a second centrifugation step at 4000 × *g* for 20 min, 4 °C. The cell pellets can be flash cooled in liquid nitrogen and stored at −80 °C until proceeding with the next steps.
4. Resuspend the frozen cell pellets in ten times (volume/cell weight) of HisA buffer, and defrost in a water bath at room temperature until the cell pellet is fully resuspended. Lyse the cells by sonication at 4 °C, with ten 15-second bursts of sonication at 10 μm amplitude followed by 15 s of cooling (*see* **Note 7**).
5. Place the cell lysate into a 50 mL polycarbonate centrifuge tube, clarify the lysate by centrifugation at 35,000 × *g*, 30 min, 4 °C, and filter the resulting cell-free extract with a 0.45 μm syringe filter into a 50 mL falcon tube (*see* **Note 8**).
6. Attach a 5 mL HisTrap FF column to a ÄKTA purification system or suitable pump system, and pre-equilibrate the column with HisA buffer. Load the filtered supernatant onto the column while monitoring the UV absorbance of the flow through at 280 nm. The sample is loaded on the column and washed with HisA buffer until the A_{280nm} has returned to the baseline reading, and the value has been stable for at least two column volumes. Elute the bound His-tagged recombinase proteins with a linear gradient of 0–70% HisB buffer over 20 column volumes, while collecting 3 mL fractions of the elution. As the gradient proceeds, the A_{280} measurement will increase sharply at the point that the recombinase protein elutes to give a symmetrical peak on a trace of the values.
7. Analyze the peak fractions for the presence of protein at the correct molecular weight by subjecting the samples to SDS-PAGE. Take 10 μL of each peak fraction, and add 5 μL SDS-loading buffer in a 1.5 mL Eppendorf tube. Boil the samples in a heat block at 98 °C for 5 min to fully denature any protein in the sample. Set up a 15% SDS-PAGE gel in an appropriate gel tank, and top up the anode and cathode chamber with SDS-running buffer. Ensure no bubbles are present in the sample

wells. Add 5 μL of the prestained ladder to the first well, and then slowly add the boiled samples to the wells. Run the gel at 150 V for 1 h. Monitor the movement of the bromophenol blue loading dye and prestained ladder, and adjust the gel running time to ensure adequate separation of the bands on the ladder. Disconnect the gel from the power supply and drain the SDS-running buffer. Remove the gel from the tank and carefully remove the gel from the gel plates. Place the gel in a plastic container and rinse five times with deionized water. Stain the gel with a Coomassie-based stain, and determine which fractions contain the protein of interest.

8. Pool the fractions from the HiTrap purification, and place in a reconstituted dialysis bag sealed with dialysis clips or sandwich clips. Dialyze against 2000 times volume of protein storage buffer overnight at 4 °C with stirring (*see* **Note 9**).
9. Using the calculated molar extinction coefficient of each protein, estimate the concentration by measuring the A_{280} with a NanoDrop 2000 spectrophotometer (*see* **Note 10**).
10. For storage, transfer 50 μL aliquots of protein at 20 μM mixed with an equal volume of 100% glycerol in thin-walled PCR tubes. Flash cool with liquid nitrogen and store at −80 °C (*see* **Note 11**).

3.7 In Vitro Function Assay

1. DNA recombination test by digestion and gel electrophoresis: Mix purified recombinase to a final concentration of 100 nM, add 1 μg DNA, 10 μL 10× recombination buffer, make up to 100 μL with ddH_2O, and incubate at 37 °C for 2 h. Purify the recombination reaction using PureLink® PCR Purification Kit, and use 23 μL ddH_2O to elute the DNA. Digest the eluted sample by adding 2.5 μL 10× CutSmart buffer and 0.5 μL ScaI-HF restriction enzyme, and incubate at 37 °C for an hour. Load the digestion reaction to a diagnostic 1% agarose gel at 110 V for 40 min.
2. Excision rate quantification of the recombinase: add 250 ng RFP-AmpR plasmid, 1 μL recombinase to final concentration of 100 nM for Cre, 50 nM for Dre, 30 nM for VCre, and 5 μL 10× recombination buffer in a 50 μL reaction. Incubate the reaction at 37 °C for 16 h, and deactivate the recombinase at 75 °C for 15 min. Transform 10 μL of the reaction mixture into *E. coli* DH5α competent cells, and plate the transformation to LB ampicillin plates. Perform the reactions and transformation in triplicate for statistical analysis.
3. Integration rate quantification of the recombinase: add 400 ng RFP-ccdB-AmpR plasmid, 100 ng KanR plasmid, 1 μL recombinase to final concentration of 100 nM for Cre, 50 nM for Dre, 30 nM for VCre, and 5 μL 10× recombination buffer in a

50 μL reaction. Incubate at 37 °C for 16 h, and deactivate the recombinase at 75 °C for 15 min. Transform 12 μL of the reaction mixture to *E. coli* DH5α competent cells, and plate the transformation to LB Kanamycin plates (*see* **Note 12**). Perform the reactions and transformation in triplicate for statistical analysis.

4. Use ImageJ/Fiji to facilitate the counting of white and red colonies after transformation (*see* **Note 13**). Image each transformation plate using a Canon digital SLR camera and light box. Load each colony image into ImageJ, use the "Oval" selection tool to select the extent of the plate, and select the "clear outside" function in the edit menu to crop out the background and the edge of the plate. To analyze red colonies in a white background, use "Subtract Background" function in the process menu; select light background and separate color with a rolling bar radius of 10–30 pixels depending on picture quality. To analyze white colonies on a dark background, use "Invert" in the edit menu to change it to a light background, adjust "Brightness/Contrast" to lower the noise first, and then execute "Subtract Background" as for the red colonies. Change the bit depth of the image to 16 bit and adjust the "Threshold" to make clear black spots in a white background. Use the "Watershed" function in Binary menu to detach any merged colonies if necessary. Remove any additional noise by paintbrush tool. Finally use "Analyze Particles" function in analyze menu to calculate the colony number (*see* **Note 14**).

3.8 Promoter Integration for the Tuning of β-Carotene Pathway

1. To assess the utility of using this system for pathway optimization, mix 25 promoters from the YeastFab library with the Vlox-promoter loading vector by BsmBI Golden Gate assembly. The concentration of the promoters were standardized to 50 ng/μL and mixed together for BsmBI Golden Gate assembly. Add 2 μL promoter mix to the 20 μL Golden Gate reaction. Transform 10 μL reaction to DH5α competent cell, and select on LB plates supplemented with ampicillin. Take off red colonies from the plate by sterile scalpel or gel extraction tip, wash all white colonies on the plate by 200 μL distilled water, and transfer to fresh LB liquid media with ampicillin for overnight culture. Perform plasmid miniprep from this mixture of clones and perform a test digestion by BamHI and SacI. Run this on a 1% agarose gel to verify assembly efficiency by estimating the percentage of target bands. Repeat the process if the percentage of correct digestion map is below 50%.
2. Set up the recombination reaction by adding 1500 ng promoter mixture, 800 ng HO-Vlox-CrtI Carotene circuit, 10 μL 10× recombination buffer, and 100 nM VCre, and add ddH_2O up to 100 μL (*see* **Note 15**). Incubate at in 37 °C for 16 h.

3. Purify the recombination reaction by PureLink® PCR Purification Kit. Linearize the reaction by BsmBI digestion, and transform it into BY4741 by frozen yeast transformation method [27].
4. Culture on SC-His-Ura media for 3–4 days for transformants to grow.
5. Image the yeast transformants and compare the color with negative control strain. Reinoculate yeast colonies of different degree of orange color to fresh SC-His culturing media for further genotype analysis such as promoter integration sequencing confirmation and phenotype analysis, including growth curve comparison and HPLC quantification of lycopene and beta-carotene production.

4 Notes

1. Sometimes TOPO cloning for certain genes fails because that gene fragment is in frame with the ccdB gene and will result in toxicity to host cell. If this happens, try another blunt end cloning kit such as CloneJET PCR Cloning Kit from Thermo Fisher, or add homologous tails for Gibson assembly.
2. Since the recombinase recognition site contains palindromic sequences, it is easy to form primer dimers or mis-annealing may happen during PCR reaction. The Phusion polymerase does not amplify all fragments well, but the KAPA polymerase gives a higher performance. However, if still the fragment can't be amplified, standard PCR optimization techniques such as the addition of betaine or DMSO can be used.
3. Time for incubation can vary from 30 to 60 min. To assemble multiple fragments by Gibson assembly, extend the incubation time to 60 min.
4. Normally for a three part Golden Gate assembly reaction, 10 cycles of 37 °C and 16 is sufficient for assembly. For more and longer fragment ligation, increase the cycle number to 30 cycles. Also enlarge the reaction volume and add more BsaI and T4 DNA ligase for more parts assembly. Extend the 50 °C incubation step to remove uncut fragments to lower background if necessary.
5. The process for BsmBI type Golden Gate assembly can also be simplified using an alternative enzyme Esp3I from Thermo Fisher. Since Esp3I have the same recognition site and cutting site as BsmBI with an optimal digestion temperature of 37 °C, it can use the same assembly protocol as BsaI avoiding two step incubation. Though BsmBI Golden Gate assembly can also be

operated with one-step incubation, it needs a longer time or more cycles to achieve good assembly efficiency.

6. Follow the OD_{600} of the culture closely. It usually takes around 2.5 h to reach OD_{600} 0.6. The OD_{600} shouldn't exceed 0.8 to ensure a good protein expression. An alternative induction method can be culture at 37 °C for 4 h. The efficiency of the two methods varies for different proteins.
7. During sonication, keep the cells on ice to avoid overheating and protein loss.
8. Take samples from both the supernatant and the pellet. Run both fractions on the SDS-PAGE gel to determine the level of protein in the soluble and insoluble fraction to assess protein solubility.
9. Dre may precipitate during dialysis. Remove protein precipitate by centrifugation of the protein solution at 4000 × *g*, 30 min, 4 °C. Clarified Dre should be stored at −80 °C as soon as possible after dialysis. The yield of VCre is much lower than the other two recombinases and may require concentration prior to use using a centrifugal concentrator.
10. Choose "Other protein (E & MW)" sample type for protein concentration test. Get the Extinction efficient and molecular weight from ExPASy database for the recombinases.
11. Don't freeze protein directly at −80 °C. The activity of VCre can be severely compromised by slow cooling. To use recombinase from −80 °C stock, thaw the recombinase at 37 °C for 10 s. Aliquot in small volume to avoid repeated freeze and defrost cycles.
12. Make sure the density of the colonies is appropriate. Too crowded a colony pattern will result in inaccurate counting of cell numbers; too loose colony pattern can't represent the integration rate properly since the integration rate is not high enough.
13. When taking pictures of the excision rate test plates, use white background for imaging red colonies; use a dark pink background for imaging white colonies. When taking pictures of the integration rate test plates, use a white background for red colonies; but use black background for white colonies.
14. Adjust the size option and shape option to further get rid of any non-colony noise in the graph. Show "Outline" to monitor the counting of the colony.
15. Linearizing the promoter mixture first can improve integration efficiency. Increase the quantity of VCre recombinase by protein concentration if necessary to improve promoter integration efficiency.

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