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Chemically Inducible Chromosomal Evolution (CIChE) for Multicopy Metabolic Pathway Engineering

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Abstract

Chemically inducible chromosomal evolution (CIChE) was developed for stable multicopy chromosomal integration of heterologous genes. In this technique, flanking an antibiotic selection marker and a gene of interest with identical regions of homology permits gene duplication via *recA* mediated homologous recombination. A strong selective pressure for gene duplication can be applied by increasing antibiotic concentration, and in a week's time one can create a set of strains with a wide range of cassette copy numbers (upward of 20×), which can be made stable by deletion of *recA*. Herein, we describe a generalized workflow for this methodology.

Keywords

Chromosomal integration; Molecular cloning; Metabolic engineering; Synthetic biology; *Escherichia coli*; Tools

1 Introduction

In metabolic engineering, tuning the expression of pathway enzymes is often necessary to optimize the production of a molecule of interest [1]. Typically, this is accomplished by varying promoter strength and copy number using plasmid-based systems, due to their ease of manipulation. However, plasmid-based systems are known to possess limitations, including poor long-term stability, especially under heavy metabolic burden [2]. Although this is generally tolerable during initial strain construction and screening, industrial production strains ought to be more robust. One common solution is to chromosomally integrate a pathway, which forces ordered inheritance. While methods continue to be developed to this end, they generally remain more laborious than plasmid-based systems, restricting the user to only a handful of integrations. This may preclude achieving optimal expression levels.

Chemically inducible chromosomal evolution (CIChE), in contrast, provides a facile and rapid (just over a week) approach for preparing a strain with a wide range of chromosomal copy numbers (upward of 20×). This is accomplished by first integrating a cassette ready for tandem gene duplication (TGD), which consists of a gene of interest and antibiotic selection marker that are flanked by identical homologous regions. Once the cassette is integrated, the strain is subcultured into media with increasing concentrations of antibiotic, thus increasing the selective pressure for gene duplication. This process proceeds through *recA* mediated homologous recombination to produce head-to-tail uneven tandem gene duplication events

[3, 4] (Fig. 1). Eventually, CICHÉ duplicates the desired cassette to a copy number that is proportional to the strength of the selective pressure applied [2]. Strains with a range of copy numbers can then be “fixed” by deletion of *recA*. Because of the ubiquitous nature of *recA*, this process is theoretically applicable in numerous host organisms. Constructed pathways can then be assayed for productivity to identify the most optimal construction, with the best strain carried forward for long-term selection-free fermentation.

2 Materials

Be sure to follow all waste disposal procedures for your institution.

2.1 Construction of Plasmids for Initial Integration

1. Plasmid pTrcHis2B (available from ThermoFisher).
2. Plasmid pAC-LYC (AddGene: Plasmid # 53270) [5] (*see* Note 1).
3. *Synechocystis* PCC 6803 (available from ATCC).
4. An instrument for measuring DNA concentration such as a NanoDrop 1000 spectrophotometer.
5. Restriction enzymes: SphI, ClaI, MluI, BamHI, SacI, EcoRI, XbaI.
6. CutSmart® Buffer.
7. Mung bean nuclease.
8. Primers (*see* Table 1).
9. Materials to run a 1% agarose gel, including 1× TAE (Tris–acetate–EDTA) buffer, agarose, 6× DNA gel loading dye, 1 kb DNA ladder, a nucleic acid gel stain such as SYBR Gold or SYBR safe, a gel station, gel imaging system, and a scalpel or razor to excise DNA fragments.
10. Qiagen QIAquick or similar gel extraction kit.
11. Wizard Genomic DNA Purification Kit.
12. PCR Reagent materials, including dNTPs, and high-fidelity polymerase such as PrimeSTAR HS, Q5 or iProof and accompanying buffers.
13. (Optional) Luria broth, agar, plates, ampicillin, and *E. coli* cloning strain DH5α (Invitrogen Corp.).

2.2 Integration of CICHÉ pTGD Construct into Chromosome

1. *E. coli* strain K12MG1655 (ATCC #700926).
2. Lysogens and transfer strains (From Dr. Dana Boyd and Dr. Jon Beckwith) [6].
3. λ InCh lysates.
4. Phage transduction donor strain: BW26547 *recA::kan* Lambda *recA+* (CGSC *E. coli* Genetic Stock Center, Yale University).

5. *E. coli* DHB6521 (λ InCh strain).

2.3 CICH Gene Duplication Process

1. Luria–Bertani (LB) broth.
2. 15 mL Falcon culture tubes.
3. Chloramphenicol.
4. Stock of 60% glycerol and cryotube vials.
5. Shaker incubator set at 37 °C.

2.4 *recA* Deletion Validation by UV Sensitivity

1. BW26547 P1 phage lysate.
2. Stratalinker UV cross-linker model 1800.
3. LB agar plates.
4. Sodium citrate.
5. CrimsonTaq DNA polymerase.

3 Methods

3.1 Construction of Plasmids for Initial Integration (See Note 2)

1. Digest pTrcHis2B with SphI and ClaI, using standard restriction enzyme reaction conditions as dictated by the supplier (1 μ g of template DNA, 37 °C, 1 h, using CutSmart® Buffer).
2. Blunt the digested backbone with mung bean nuclease, following manufacturer recommended reaction conditions (0.1 μ g/ μ L DNA template, 30 °C, 30 min, in CutSmart® Buffer). Deactivate by either phenol–chloroform extraction or by the addition of SDS to 0.01%. Purify by ethanol precipitation.
3. Run a 1% agarose gel to separate digested plasmid fragments and extract the 2.8 kb band containing the pBR322 origin of replication and the β -lactamase (ampicillin resistance) gene (*see* Note 3).
4. Self-ligate this blunted 2.8 kb fragment using T4 DNA ligase (*see* Note 4)
5. Amplify a ~1 kb region of ChlB from *Synechocystis* PCC 6803 using IntP1 and IntP2 primers (*see* Table 1) (*see* Note 5).
6. Amplify chloramphenicol acetyltransferase gene (CAT) from pAC-LYC using primers IntP3 and IntP4 (*see* Table 1) using a high-fidelity DNA polymerase. This will produce a 777 bp fragment (*see* Note 6); about any titratable marker will do.
7. Purify products by 1% agarose gel electrophoresis and use a gel purification kit to isolate the DNA fragments.

8. Use overlap PCR to connect the 1 kb region of chlB1 to the CAT.
9. Amplify the constructed chlB1-CAT fragment with primers LamP1 and LamP4 (see Table 1) to introduce BamHI and SacI/SphI sites.
10. Cut the truncated pTrcHis2B and the chlB-CAT fragment with BamHI and SacI.
11. Ligate these two fragments together.
12. Amplify a second copy of chlB using LamP7 and LamP8 to introduce EcoRI/MluI and XbaI sites.
13. Cut the resulting chlB fragment and modified pTrcHis2B with EcoRI/XbaI, then ligate together to create pTGD.
14. Insert gene of interest into KpnI/MluI site of this newly created pTGD plasmid (see Note 7).

3.2 Transformation of ClChE pTGD Construct into Chromosome (See Note 8)

1. Transfer TGD DNA constructs from the TGD platform plasmid to the genome of K12 *E. coli* using the λ InCh protocol (Into the Chromosome), which consists of three steps: (see Note 9).
 - a. Homologous recombination between the pTGD plasmid and a λ phage.
 - b. Integration of the phage-pTGD DNA into the att site of your *E. coli* strain.
 - c. Removal of phage DNA by looping out.

A thorough λ InCh protocol can be found at: http://beck2.med.harvard.edu/resources/InCh/download/Lambda_InCh_Manual.pdf.

3.3 ClChE Gene Duplication Process (See Note 10)

1. Grow cells in 5 mL of LB using 14 mL culture tubes overnight at 37 °C with 13.6 μ g/mL of chloramphenicol added to the solution until stationary phase.
2. The following day, transfer 50 μ L of the cell culture to 5 mL of fresh LB media (1:100) with 27.2 μ g/mL chloramphenicol concentration and grow overnight at 37 °C to stationary phase.
3. Repeat **step 12** for several days, each time subculturing the cells into fresh LB with double the concentration of antibiotic until the desired concentration is reached (~1500 μ g/mL) (see Note 11).
4. At each concentration of chloramphenicol, aliquot a portion of the stationary phase cell culture for *recA* deletion to stabilize the tandem repeats. *RecA* deletion is readily accomplished by P1 phage transduction of the *recA::kan* allele from BW26547 to the TGD strain using the open access protocol reported from Sauer lab (http://openwetware.org/wiki/Sauer:P1_vir_phage_transduction) (see Note 12; Gene copy stability).

3.4 *recA* Deletion Validation by UV Sensitivity

1. Each population of cells with a *recA*-genotype must be routinely validated using a UV sensitivity assay. To do so, inoculate 10 mL of LB broth with 100 µL of an overnight culture of a *recA*-CICHe strain and grow for 2 h at 37 °C.
2. Measure the concentration of cells by OD₆₀₀, then centrifuge and wash with 10 mL of M9 minimal medium.
3. Dilute the cells with M9 to a density of ~100 colonies per sample and place on two LB agar plates.
4. Expose one of the two plates to UV using a device, such as a Stratalinker UV cross-linker model 1800, in a darkened room at 3000 µJ energy, and set the second aside to serve as a control.
5. Incubate both plates overnight at 37 °C in the dark and the following day, determine the number of colonies on each plate to calculate the corresponding cell survival. *recA*- strains are very sensitive to UV exposure, and UV exposed *recA*- strains should have substantially reduced colonies compared to WT.
6. *recA*- strains can then be grown and cryostored for further use.
7. Verify copy number quantification can be accomplished using qRT-PCR on the amplified genomic DNA TGD cassette relative to a reference gene by growing post CICHe *recA*- variants in LB overnight at 37 °C, then use the Promega Wizard Genomic DNA purification kit to isolate genomic DNA containing the amplified cassette (see Note 13).
8. Use a standard qRT-PCR SYBR green method to quantify the CAT gene in the amplified cassette DNA relative to the *E. coli* genomic DNA gene *BioA*, with gene specific primers (see Table 1).

4 Notes

1. This plasmid is used here for its chloramphenicol resistance marker. Other sources of the CAT gene are sufficient substitutes, although this would likely alter the primers necessary for the protocol.
2. There are multiple options for constructing the cassette. Theoretically, much of the tandem gene duplication cassette could be directly synthesized or assembled with other methods than the one outlined. However, due to the homologous flanking regions, some level of restriction digestion and ligation is necessary. In addition, it should be ensured there are no other tandem repeats in the cassette that may result in duplication of the antibiotic resistance marker without duplicating the entire desired region inside the two homologous repeats. This is critical to the experiment's success.
3. Anecdotally, we have observed that freezing excised gel fragments overnight at 20 °C has produced better gel extraction yields. If the gel extraction product is giving poor OD 230/260 readings, consider allowing wash buffers to sit on the

column 2–5 min before spin down. In addition, consider adding a second loading buffer wash in addition to the ethanol washes.

4. You may have a low yield of this ligated product or simply want more of this product to use for different steps downstream. If so, at this step you could transform this product and plate on ampicillin, pick colonies, grow them in LB with ampicillin, and perform a plasmid miniprep to get more of this product.
5. The regions of homology dictated here have been validated for use in *E. coli*. Certainly, other ~1 kb segments could be sufficient. We provide this segment of DNA as an example. In addition, in lieu of obtaining this entire strain, just the ChlB gene could be directly synthesized and carried forward for subsequent steps.
6. As mentioned, other sources of CAT are possible. In fact, other selective markers are also usable as long as their effect is titratable [7].
7. Remember to include any necessary regulatory elements along with your gene of interest in your cassette design, including a promoter.
8. Be certain the strain you are transforming into is recA+, otherwise recombination will not occur. Also, be sure your background strain can tolerate high expression of your gene.
9. Other chromosomal integration techniques are possible, including that described by Datsenko and Wanner [8].
10. Once this CICE process has begun, it will take several days of maintenance.
11. If cells are unable to grow to stationary phase overnight, allow to continue growing for an additional 24 h and/or subculture 50 μ L into a fresh 5 mL of the same concentration of chloramphenicol supplemented LB. At higher antibiotic concentrations, it is recommended to subculture the evolving population into both double and 1.5 $\times$, antibiotic concentration with respect to the previous culture.
12. Gene copies are not stable during amplification. A mutation in one copy can propagate quickly causing many copies to be inactive. Make freezer stocks of strains after each subculture as backups, and only do the minimum number of cell doublings required to achieve the copy number you desire.
13. Select several colonies from each population for qPCR characterization, as not all variants within a given antibiotic concentration culture will have the same copy number.

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Fig. 1.

Design and process of chemically inducible chromosomal evolution (CICHe). **(a)** Schematic for duplication cassette. Each cassette contains two identical ~1 kb flanking regions with little homology to the host organism. Inside the flanking region are both the gene of interest and a selection marker (i.e., antibiotic resistance gene). **(b)** Here we see that the duplication event occurs as homologous regions are recombined mediated by *recA*. This process can result in both increases and decreases in the copy number of the gene of interest. **(c)** Maintaining selection pressure ensures that only cells with the gene of interest and selection marker survive. By subculturing cells in increasing antibiotic concentration, as depicted by the darker shades of green, one can screen for cells with higher gene copy number

Table 1

Primers for PCR (IDT, Coralville, IA, USA)

Primer	Purpose	Sequence (5'-3')
Int P1	Amplification of chlB1 (Sense)	GCACCCCTACGCATCGCCAGTTCTT
Int P2	Amplification of chlB1 (Antisense)	CGCTCTCGGGCAAACTTTTCTGTGTT
Int P3	Amplification of CAT (Sense)	CGTTGATCGGCACGTAAAGAGGTTCC
Int P4	Amplification of CAT (Antisense)	CCTTAAAAAAATTACGCCGCCGCC
Lam P1 (Sense)	Introduction of BamHI to chlB1-CAT (Sense)	TATCGGATCCCCAGTTCTTCAAAAAACGTCCACGCC
Lam P4 (Antisense)	Introduction of SacI/SphI to chlB1-CAT (Antisense)	GGGAGCTCAGCATGCCCTTAAAAAAATTACGCCGCCGCC
Lam P7 (Sense)	Introduction of EcoRI/MluI to chlB copy #2	GGAAATTCAACGCGTCCAGTTCTTTCAAAAAACGTCCACGCC
Lam P8 (Antisense)	Introduction of XbaI to chlB copy #2	CGTCTAGAGCCCCCGTTGTCTTCACTGATCAACACT
Cm qPCR1	Measure genomic CAT copy number (Sense)	CGCCTGATGAATGCTCATCC
Cm qPCR2	Measure genomic CAT copy number (antisense)	AGGTTTTCACCGTAACACGC
BioA qPCR1	qPCR Reference gene to normalize cat copy number to number genomic copy (Sense)	GTGATGCCGAAATGGTTGCC
BioA qPCR2	qPCR Reference gene to normalize cat copy number to number genomic copy (antisense)	GCGGTCAGACGCTGCAACTG