Chapter 14

Gene Assembly and Combinatorial Libraries in *S. cerevisiae* via Reiterative Recombination

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Abstract

While mutagenesis of single genes is now common practice in molecular biology, engineering multiple target genes still requires complex cloning techniques and thus is limited to expert laboratories. Here, we describe "Reiterative Recombination," a user-friendly DNA assembly technique in *Saccharomyces cerevisiae* for the integration of an indefinite number of DNA fragments sequentially into the yeast genome. The high efficiency of chromosomal integration can further be utilized for the assembly of large combinatorial libraries for metabolic engineering.

Key words: DNA assembly, Homologous recombination, Combinatorial libraries, Biosynthetic pathways, Homing endonuclease, *Saccharomyces cerevisiae*, Cell engineering

1. Introduction

Here we describe "Reiterative Recombination," a technically simple, robust method for in vivo DNA assembly on the chromosome of *Saccharomyces cerevisiae* (1). Harnessing the endogenous homologous recombination process in yeast, Reiterative Recombination offers high-efficiency DNA integration, making it especially attractive for nonexpert investigators in the field of yeast genetics. The protocol described below enables (1) cloning of multiple DNA fragments in yeast, with indefinite potential for elongation of the same construct (10s–100s of kbs), (2) construction of stable strains by integration of all DNA constructs (e.g., entire multicomponent pathways) into the yeast chromosome at a predefined location, and (3) cloning of large combinatorial DNA libraries.

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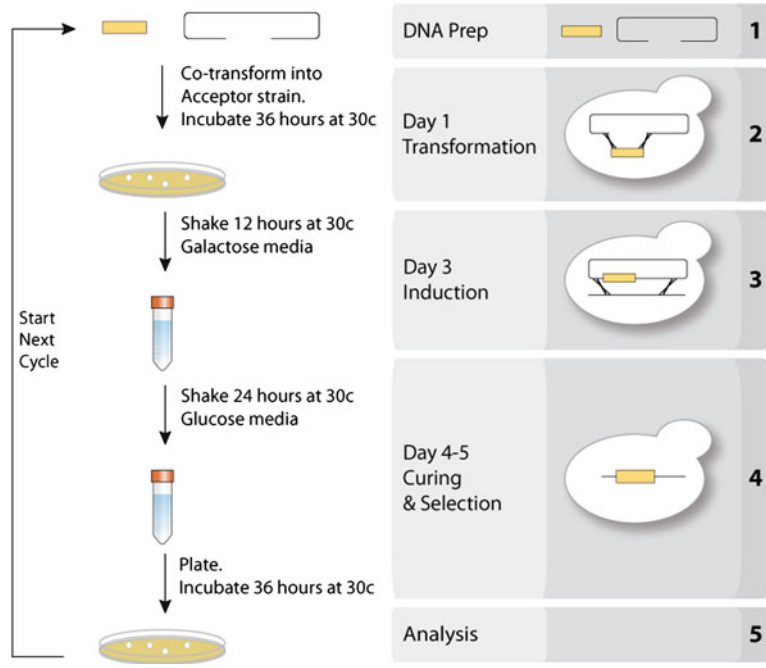

Fig. 1. Reiterative Recombination protocol overview. The DNA of interest (*yellow*) is incorporated into the chromosome using a donor plasmid in a 5-day cycle. Homologous recombination between DNA fragment, donor plasmid, and genomic DNA occurs entirely in vivo, requiring only the exchange of selective media.

Reiterative Recombination utilizes pairs of alternating, orthogonal endonucleases and selectable markers as well as the efficient process of in vivo endonuclease-stimulated homologous recombination to integrate and elongate a construct of interest. The system consists of two modules: a "donor" plasmid carrying the DNA to be assembled and an "acceptor" strain carrying a predefined target locus for assembly. As shown in Fig. 1, each cycle consists of four steps: (1) *DNA preparation*—the DNA fragment to be assembled is PCR amplified to add homology regions on both ends. (2) *Transformation*—co-transformation of the PCR product and a linearized donor plasmid into an acceptor strain. (3) *Induction*—galactose-induced expression of an endonuclease triggers homologous recombination, resulting in integration of the PCR product and an auxotrophic marker into the acceptor strain chromosome. (4) *Curing and selection*—cells in which successful integration events occurred are isolated by selecting for the new auxotrophic marker. The acceptor strain is cured from excess donor plasmid by counter selection, and recombinants containing the correct construct are used as acceptor strain for the next cycle of elongation. A single cycle takes 5 days to complete. Importantly, the use of only two recyclable auxotrophic selective markers (histidine and leucine metabolic genes, Fig. 2) provides a generic selection independent of the nature of DNA that is being assembled. At each cycle of assembly, one or

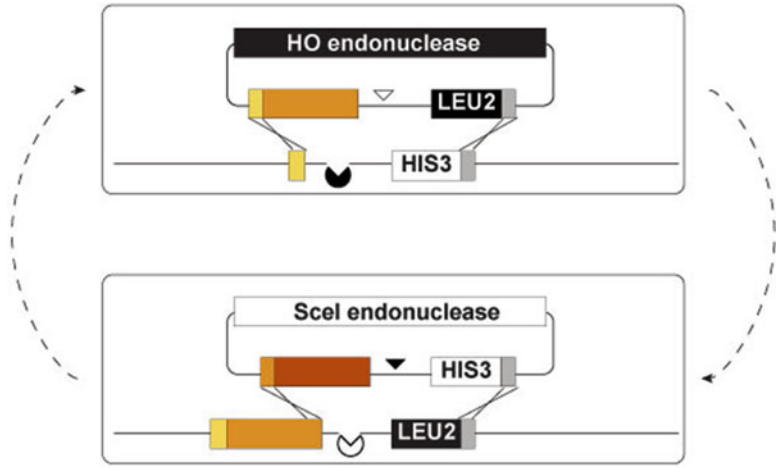

Fig. 2. General scheme of Reiterative Recombination, showing endonucleases and selective markers for each cycle of assembly. Assembled DNA fragments are in (*orange*) and (*brown*). *Triangles* indicate endonuclease cleavage site. Generic homology sequences are indicated in gray.

more DNA fragments can be integrated, and the order of fragment assembly is determined by the design of appropriate homology on both ends of the fragments that is incorporated (see Fig. 3).

Each cycle of Reiterative Recombination assembly yields ~10^5/mL recombinant colonies. Considering yeast transformation (Fig. 1, step 2 and methods section 3.2 below) should yield ~10^6–10^8 cells, the efficiency of DNA integration with Reiterative Recombination is estimated to be 1–10% of all transformed, induced cells. It is important to note, however, that the number of recombinant colonies drops significantly if one attempts to co-transform multiple DNA fragments in a single cycle.

Significantly, all that is required to adapt Reiterative Recombination as a tool for library mutagenesis is to use a pool of mutagenized DNA fragments as opposed to a single DNA sequence during the transformation step (figure 1, step 2). At the end of the cycle each of the ~10^5/mL cells will contain a different DNA mutant integrated at the acceptor site in the chromosome. The mutant displaying the desired phenotype can be isolated using a suitable screen or selection.

While generic Reiterative Recombination acceptor strains are readily available (1), any strain can be converted to be an acceptor for DNA assembly. Two cloning steps are necessary, both performed using classical integration vectors (see Subheading 3 below): (1) knock out of the endogenous HO endonuclease cleavage site on the acceptor strain, to avoid unwanted double-strand breaks during assembly and (2) integration of the "acceptor module" at the HO endonuclease gene locus of the strain. It is important to note that the acceptor strain must have *his3, trp1, ura3,* and *leu2* auxotrophies (Table 1).

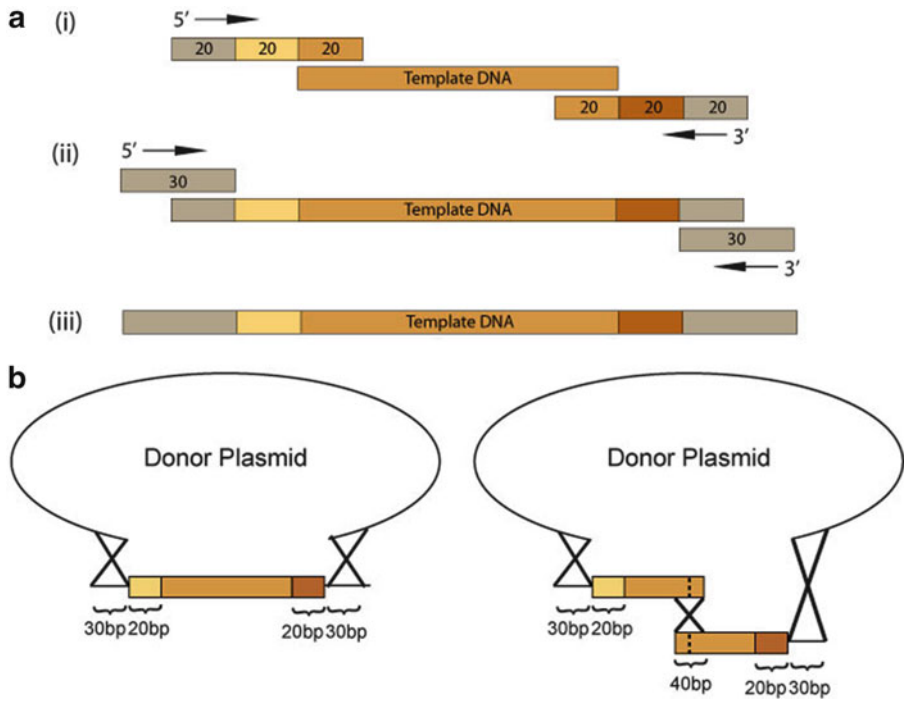

Fig. 3. Primer design. (**a**) PCR overview. Each DNA fragment is added with homology to allow its recombination with the donor plasmid and other fragments previously assembled on the chromosome. (i) Primers are designed to add homology to preceding assembly fragments (*yellow*), next assembly fragment (*brown*), and donor plasmid (*gray*) see 'PCR1' in Table 4 and Note 1–2. (ii) Generic primers extend the homology to donor plasmid and make the final fragment (iii) see 'PCR2' in Table 4, Note 1–3. (**b**) Assembly of DNA fragments with a donor plasmid. Single (*left*) or multiple (*right*) DNA fragments can be used at each Reiterative Recombination cycle, provided each is added with 30–40 bp of overlapping sequence.

Table 1
Plasmid list

Plasmid	Description
pLMW2594	Donor plasmid—cycle 1 (integrating selective marker: leucine)
pLMW2593	Donor plasmid—even cycles (integrating selective marker: histidine)
pLMW2592	Donor plasmid—odd cycles (integrating selective marker: leucine)
pLMW2588	Integration plasmid containing non-cleavable allele "*MATa-inc*" to be used in 'a' acceptor strain
pLMW2586	Integration plasmid containing non-cleavable allele "*MATα-inc*" to be used in 'alpha' acceptor strain
pLMW2590	Integration plasmid for acceptor module

2. Materials

All solutions should be prepared using deionized water and sterile techniques. All reagents should be prepared and stored at room temperature (unless indicated otherwise).

2.1. Yeast Media Components

1. Amino acid mix (500 mL, not including histidine, tryptophan, leucine, and uracil): Weigh and combine the amino acids listed in Table 2 (except for aspartic acid and threonine) with 500 mL water in a 1 L flask. Autoclave 15 min. When cooled to about 50°C, add aspartic acid and threonine. Using sterile technique, filter sterilize into autoclaved bottles.
2. 40% Glucose (500 mL): Autoclave 300 mL water in a 1 L flask. When cooled to about 50°C, add 200 g glucose and shake or stir until fully dissolved (keep sterile). Add autoclaved water to reach 500 mL total volume. Filter sterilize into autoclaved bottles.
3. 30% Galactose (500 mL): Same protocol as glucose, using 150 g galactose.
4. 20% Raffinose (500 mL): Same protocol as glucose, using 100 g raffinose.

Table 2
Amino acid mix (no histidine, tryptophan, uracil, leucine)

Compound name	Amount for 500 mL (g)
Adenine sulfate	0.2
Arginine HCl	0.2
*Aspartic acid	1.0
Glutamic acid	1.0
Isoleucine	0.3
Lysine HCl	0.3
Methionine	0.2
Phenylalanine	0.5
Serine	4.0
*Threonine	2.0
Tyrosine	0.3
Valine	1.5

*Amino Acid added after autoclave. See section 2.1

Table 3
SC (synthetic complement) media[b]

Component	For 250 mL media (mL)
SD (synthetic-defined) media	125
40% glucose[a]	12.5
Amino acid mix (HUTL-)	12.5
1% Histidine	0.5
1% Tryptophan	0.5
0.2% Uracil	2.5
1% Leucine	2.25
Sterile H_2O (for liquid) or 4% agar (for plates)	250

[a]Glucose is replaced with galactose and raffinose for SC–galactose media (see 2.1.9 below)
[b]For counter selection (SC-FOA) plates see section 2.18

5. Amino acids: For the following amino acid solutions, add the indicated amino acid into 200 mL water in 250 mL bottle and autoclave. 1% tryptophan, 2 g tryptophan; 0.2% uracil, 0.4 g uracil; 1% histidine, 2 g histidine; 1% leucine, 2 g leucine.
6. 4% Agarose: Mix 8 g Bacto-agar with 200 mL water. Autoclave. Microwave before use.
7. SD (synthetic-defined) media (YNB w/ammonium sulfate, 500 mL)*:* Dissolve 6.7 g in 500 mL water. Autoclave.
8. Yeast SC-glucose (synthetic complete) dropout liquid media: Mix components as described in Table 3 using histidine/tryptophan/leucine/uracil, as required. Use autoclaved water in the last step. For 0.1% FOA plates, add 0.1% g/volume 5-FOA (5-fluoroorotic acid) to media components; shake at 37°C until fully dissolved. Filter sterilize before use.
9. Yeast SC–galactose dropout liquid media: Mix components as described in Table 3 replacing the 40% glucose solution with 16.6 mL 30% galactose and 25 mL 20% raffinose. Add sterile water to 250 mL.
10. Yeast SC-glucose (synthetic complete) dropout plates: Same as dropout liquid media above, using 4% agar in the last step. (Dissolve in microwave if solid).
11. 15 mL culture tubes.
12. Sterile inoculation loops.
13. QIAquick Gel Extraction Kit.
14. ZymoPrep Genomic DNA Extraction Kit.

2.2. Yeast Transformation Components

1. E-buffer (250 mL, keep at 4°C): Weigh 46.1 g sucrose and 0.78 g Tris-HCl. Add to 500 mL sterile water and stir to dissolve. Adjust pH to 7.5. Add $MgCl_2$ to final concentration 1 mM and filter sterilize before use. (Final concentration: 10 mM Tris-HCl pH 7.5, 270 mM sucrose, 1 mM $MgCl_2$).
2. DTT (1,4-dithiothreitol) solution: Prepare 1 M Tris-HCl, pH 8. Immediately before use, dissolve DTT to a final concentration of 2.5 M, vortex, and filter sterilize.
3. Yeast complete media (YPD): Weigh 10 g Bacto-Yeast extract and 20 g Bacto-peptone. Add 500 mL water in 1 L bottle. Autoclave. When cooled, add 25 mL of 40% glucose solution (*see* Subheading 2.1 above).
4. Pellet Paint co-precipitant—for concentrating DNA samples (Novagen).
5. Autoclaved 250 mL flask.
6. Electroporation cuvettes.

3. Methods

Carry out all procedure using sterile technique (2, 3). Fig. 1 depicts each step.

3.1. Preparation of DNA for Assembly

It is critical to follow the primer design instructions for PCR1 (see Note 1 and Fig. 3a).

3.1.1. PCR of DNA Fragments for Assembly

1. Prepare PCR1 mix (see Note 1 and Fig. 3): Add 0.5–1 μL template DNA (containing your fragment of interest), 0.2 μL 5′-primer (100 μM stock), 0.2 μL 3′-primer (100 μM stock), 0.5 μL VENT polymerase, 0.5 μL dNTP mix, and 5 μL ThermoPol buffer, and add sterile water to final volume of 50 μL. PCR amplify (see Note 2). Run the PCR product on agarose gel; verify the expected fragment size, and purify the fragment from gel.
2. Prepare PCR2 mix (see Fig. 3a and Note 3): 1 μL of purified PCR1 template, 0.2 μL of 5′-PCR2 primer (100 μM stock), 0.2 μL of 3′-PCR2 primer (100 μM stock), 0.5 μL VENT polymerase (NEB), 0.5 μL dNTP mix (NEB), and 5 μL ThermoPol buffer (NEB) and add sterile water to final volume of 50 μL. PCR amplify and purify the resulting fragment from gel.
3. Measure the DNA concentration of all purified PCR products.

3.1.2. Linearization of Donor Plasmid

The donor plasmid for the first cycle, pLW2594, is used below. For each assembly cycle, choose the suitable donor plasmid and its respective restriction enzyme using Table 4. Note that odd and even donor plasmids are alternated for all cycles except for the first cycle.

Table 4
Cycle-specific DNA preparation

	PCR1		PCR2			
Cycle	Add sequence to 5′ end of 5′-primer	Add sequence to 5′ end of 3′-primer	5′-primer	3′-primer	Donor plasmid	Choose restriction enzyme
Cycle 1	5′-AAAATTGTGCCTTTGG ACTTAAAATGGCGT	5′-CTTAGGGATAA CAGGGTAAT	No PCR2 primers required. (Use PCR1 primer)	LW367	pLW2594	*Sma*I, *Xma*I, *Tsp*MI, *Bso*BI, *Ava*I. (1,500 bp KanMX gene will be lost upon digestion)
Even cycle	5′-GGACGCTCGAAGGCTTT	5′-CTGTTGCGGAA AGCTGAAA	LW374	LW375	pLW2593	*Sph*I, *Sal*I, *Tsp*MI, *Xma*I, *Sma*I, *Eco*53kI, *Sac*I, *Eco*RI
Odd cycle	5′- GGACGCTCGAAGGCTTT	5′-CTTAGGGATAA CAGGGTAAT	LW374	LW367	pLW2592	*Hin*dIII, *Bsa*BI, *Not*I, *Eag*I, *Ale*I, *Eco*53kI, *Sac*I

1. Mix 1–2 μg donor plasmid pLW2594, 2 μL of restriction enzyme SmaI, 5 μL of NEB buffer 4, and sterile water to final volume of 50 μL in a microcentrifuge tube. Incubate at 25°C for 6 h.
2. Run the products on agarose gel (compare to a non-digested plasmid sample control). Purify the linearized plasmid DNA from gel.
3. Measure the DNA concentration of the purified linear plasmid.

3.2. Yeast Transformation

We use a high-efficiency yeast electroporation protocol with slight modifications (4). The media described below is specific for the first cycle of DNA assembly in the parent acceptor strain (1). Ensure that the appropriate media is used for the particular cycle of assembly (see Table 5). The resulting colonies after this step will be referred to as "transformants."

1. Day before transformation: Use a patch of the acceptor strain on SC-glucose(H-) to inoculate a 5 mL SC-glucose(H-) liquid culture in 15 mL culture tube. Shake at 30°C overnight.
2. Day of transformation: Measure OD_{600} of the acceptor strain culture.
3. Inoculate 100 mL YPD media using the overnight culture to reach final $OD_{600} = 0.1$. Grow cells in 30°C shaker until $OD_{600} \approx 0.8$ (approx. ~7 h). While waiting, prepare DNA mix to be transformed (see Note 4) and concentrate DNA mix to 4 μL using Pellet Paint co-precipitant (see Subheading 2).
4. When culture reaches $OD_{600} \approx 0.8$, add 1 mL filter-sterilized DTT solution (see Subheading 2) and incubate for another 20 min in 30°C shaker.
5. *From now on, try to keep the cells on ice and work quickly to maintain the cell competence.* Harvest the cells (5 min, 2,000×*g*, 4°C, in two 50 mL Falcon tube swinging bucket centrifuge). Discard the supernatant and resuspend cells using 25 mL cold E-buffer (see Subheading 2) by pipetting. Harvest the cells again.
6. Discard the supernatant and resuspend cells in 1 mL of cold E-buffer and transfer them to a sterile 1.5 mL microcentrifuge tube. Harvest the cells (1 min, 20,000×*g*).
7. Discard the supernatant and resuspend the cells using 40 μL cold E-buffer (see Note 5). Aliquot 60 μL of cells into sterile 1.5 mL microcentrifuge tubes containing the DNA and donor plasmid for assembly. Mix by gently pipetting, and incubate for 10 min on ice.

Table 5
Cycle-specific media

	Transformation (overnight culture)	Transformation media	After electroporation	Induction media	Curing		Inoculate next cycle
	(liquid)	(liquid)	(plates)	(liquid)[a]	(liquid)	(plates)	(liquid)
Cycle 1	[b]SC-glucose/H-	SC-glucose/H-	SC-glucose/HU-	SC–galactose/U-	SC-glucose/L-	SC-glucose/L- 0.1% FOA	SC-glucose/L-
Even cycle	SC-glucose/L-	SC-glucose/L-	SC-glucose/LU-	SC–galactose/U-	SC-glucose/H-	SC-glucose/H-0.1% FOA	SC-glucose/H-
Odd cycle	SC-glucose/H-	SC-glucose/H-	SC-glucose/HU-	SC–galactose/U-	SC-glucose/L-	SC-glucose/L-0.1% FOA	SC-glucose/L-

[a]It is highly recommended to perform a negative control by culturing in SC–glucose (noninductive), to determine the induction efficiency with galactose.
[b]"SC–glucose" media refers to glucose media, unless indicated otherwise

8. Set the gene pulser to 25 μF, 540 V, 0 Ω. Transfer the cells to a 0.2 cm electroporation cuvette and pulse (see Note 6). Immediately after electroporation, add 1 mL warm YPD and let shake at 30°C for 1 h.
9. Plate 1:10 and 1:10^3 dilutions (see Note 7) on selective plates SC-glucose(HU-). Incubate plates at 30°C for 2 days or until colonies are visible (see Note 8).

3.3. Induction of DNA Integration into the Chromosome

The resulting colonies after this step are referred to as "induced recombinants."

1. Using a sterile inoculation loop, pick 5–10 transformant colonies from SC-glucose(HU-) plate and resuspend them in 1 mL sterile water in 1.5 mL microcentrifuge tube (see Note 9). Spin down in tabletop centrifuge (5 min at 20,000×*g*) and carefully discard the supernatant (a cell pellet should be clearly visible, otherwise repeat this step by picking more colonies).
2. Resuspend cell pellet in 1 mL sterile water; aliquot 500 μL of cell suspension into two sterile microcentrifuge tubes. One tube is used as a negative control and labeled "Glucose (control)" and the other for induction, labeled "Galactose." Spin down both samples (5 min at 20,000×*g*) and carefully discard the supernatant. Cell pellet should be visible.
3. Resuspend the "Glucose" control sample with 1 mL of SC–glucose(U-) and the "Galactose" sample with SC–Galactose(U-). Transfer each 1 mL sample into a 15 mL culture tubes and incubate in 30°C shaker for 12 h (see Note 10).
4. Optional: To evaluate the efficiency of the induction process, plate 100 μL of each cell culture immediately after 12 h induction on SC-glucose(L-) plates, incubate at 30°C, and compare the number of colonies (see Note 11). Otherwise, skip straight to curing step.

3.4. Curing and Selection for Recombinants

The resulting colonies after this step are referred to as "cured recombinants."

1. To cure cell from donor plasmid, take 100 μL of induced cell culture from SC–galactose(U-) and add 900 μL SC-glucose(L-) media in a 15 mL culture tube. Incubate the samples for 24 h in 30°C shaker (see Note 12).
2. After 24 h, take 100 μL of the cured sample into 900 μL sterile water, mix, and plate 100 μL on SC-glucose(L-/0.1% FOA) selective plates. It is recommended to plate several dilutions, as necessary (see Notes 13 and 8). Incubate at 30°C for 2–3 days or until colonies are visible.

Table 6
Primer sequences

Primer name	Priming location	Sequence
VC1052	Reverse primer, CYC terminator	GGGACCTAGACTTCAGGTTG
VM172	Reverse primer, end of HO gene	TTAGCAGATGCGCGCAC
LMW274	Reverse Primer, 76 bp after end of MAT locus	CATTTGTCATCCGTCCCGTATA
LMW308	Downstream of assembly construct	CAGCCGAACGACCGAGCGCAGCG AGTCAGTGATCTAGAATGTC TAAAGGTGAAGAATTAT
LMW309	Downstream of assembly construct	GACAACACCAGTGAATAATTCTTCAC CTTTAGACATTGTGATGATGTTTTA
LMW317	Forward primer, upstream of HO gene (1820bp prior to start codon) HO gene	CTTTGGACTTAAAATGGCGT
LMW318	Reverse primer, 1200bp into HO gene ORF HO gene	GTGAAGTTGTTCCCCCAG
LMW319	Internal MATa primer	TTAGAAGAAAGCAAAGCCTTA
LMW320	Internal MATα primer	CCTGTTCCTTCCTCTCGA
LMW367	Reverse primer, Downstream of integrated fragment for odd cycles	TCAGTACAATCTTAGGGATAACA GGGTAAT
LMW374	Forward Primer, Upstream of integrated fragments for even and odd cycles	TGAGAAGGTTTTGGGACG CTCGAAGGCTTT
LMW375	Reverse Primer, Downstream of integrated fragment for even cycles	GCACAGTTATACTGTTGCGGAAAG CTGAAA

3.5. Analysis of Assembly Products

1. Patch a few cured recombinant colonies on SC-glucose(L-/0.1% FOA) plate and incubate plates 2–3 days in 30°C incubator (to produce more cells for analysis).
2. Perform colony PCR (see step 5 of Subheading 3.7.1 for protocol and Table 6 for primers. see Note 14)
3. Analyze the resulting DNA fragment on an agarose gel to verify the correct assembly of DNA.

3.6. Starting the Next Cycle of Assembly

1. Pick a single colony from the previous cycle (confirm by PCR or sequencing) and inoculate 5 mL SC-glucose(L-) media in a 15 mL culture tube. Incubate in 30°C shaker overnight, and use as acceptor strain for the next cycle of assembly (starting Subheading 3.1 above). (For libraries assembly, see Note 15).

3.7. Construction of Acceptor Strain (This Step Is Optional, See Note 16)

3.7.1. For MATa Strains: Replace MATa Locus with Non-cleavable Allele "MATa-inc"

1. Mix 1–2 µg plasmid pLW2588, 2 µL NEB Buffer 3 and 2 µL restriction enzyme BglII (total volume of 20 µL). Incubate the reaction at 37°C for 3 h.
2. Purify the resulting linear plasmid from agarose gel (see Subheading 2) and determine DNA concentration.
3. Plasmid "pop-in"—transform >500 ng of purified linear plasmid (see Subheading 3.2 above). Plate the transformants on SC-glucose(U-) plates and incubate at 30°C for 2 days.
4. Plasmid "pop-out"—use one or more "pop-in" colonies colony to inoculate 5 mL YPD media, shake at 30°C for 1 day. Dilute the culture (1:100–1:1,000) and plate on SC-glucose(0.1% FOA) plates. Incubate at 30°C for 2 days (see Note 17).
5. Plasmid "pop-out" analysis by *colony PCR*—patch a few of the colonies and perform colony PCR to confirm correct DNA integration: Using an inoculation loop or sterile tip, pick some of the patch cells and suspend them in 30 µL 0.2% SDS solution in a PCR tube. Boil the sample for 5 min at 95°C in a PCR block. Spin down the sample 10 min at 2,000×*g* (swinging bucket centrifuge) and incubate 30 min at RT.
6. Perform PCR: Mix 1–2 µL colony/SDS supernatant, 0.2 µL primer LMW274 (100 µM), 0.2 µL primer LMW319 (100 µM), 0.2 µL VENT polymerase, 0.2 µL dNTPs, 4 µL polymerase buffer, and 13.4 µL sterile water. Expected band size is 650 bp (see Table 6 for primer sequences).
7. Digest PCR product: Mix 10 µL PCR product with 1.5 µL Buffer 3, 1 µL restriction enzyme AciI, and 2.5 µL sterile water. Incubate at 37°C for 3 h. Run on agarose gel. Correct integration of *MATa-inc* allele results in digestion product 357 and 293 bp. Wild-type *MATa* results in digestion products 293, 225, and 132 bp.

3.7.2. For MATα Strains: Replace MATα Locus with Non-cleavable Allele "MATα-inc"

1. Mix 1–2 µg plasmid pLW2586, 2 µL NEB buffer 2, 2 µL BSAx 10, and 2 µL restriction enzyme NheI (total volume of 20 µL). Incubate the reaction at 37°C for 3 h.
2. Purify the resulting linear plasmid from agarose gel by kit (see Subheading 2) and determine DNA concentration.
3. Plasmid "pop-in"—transform >500 ng of purified linear plasmid into the strain (see Subheading 3.2 above). Plate the transformants on SC-glucose(U-) plates and incubate at 30°C for 2 days.
4. Plasmid "pop-out"—use one colony to inoculate 5 mL YPD media, shake at 30°C for 1 day. Dilute the culture (1:100–1:1,000) and plate on SC-glucose(0.1% FOA) plates. Incubate at 30°C for 2 days (see Note 17).
5. Plasmid "pop-out" analysis—patch a few of the colonies and perform *colony PCR* primers LMW274 and LMW320 to

confirm correct DNA integration (see step 5 of Subheading 3.7.1 for colony PCR protocol). Expected band size is 675 bp (see Table 6 for primer sequences).

6. Digest PCR product: Mix 10 μL PCR product with 1.5 μL buffer 4, 1.5 μL BSA buffer 10 (NEB), 1 μL restriction enzyme HhaI, and 1 μL sterile water (total volume 15 μL). Incubate at 37°C for 3 h. Run on agarose gel. Correct integration of *MATα-inc* allele results in 675 bp (PCR is not digested). Wild-type *MATα* results in digestion products 423 and 252 bp.

3.7.3. Integration of Acceptor Module at HO Gene Locus

1. Mix 1–2 μg plasmid pLW2590, 3 μL NEB buffer 4, and 2 μL restriction enzyme SpeI (total volume 20 μL). Incubate the reaction at 37°C for 3 h.
2. Purify the resulting linear fragment from agarose gel and determine DNA concentration.
3. Transform 2 μg of purified linear plasmid into the acceptor strain (see Subheading 3.2). Plate the transformants on SC-glucose(H-) plates, and incubate at 30°C for 2 days.
4. Plasmid integration analysis: patch a few of the colonies and perform colony PCR (see step 5 of protocol section 3.7.1 above) using primers LMW309 and LMW318. Expected band for successful integration is 913 bp. No amplification if wild type (i.e., if integration unsuccessful). For further verification, purify the genomic DNA (using kit, see Subheading 2) and PCR amplify using the following primers: (1) primers LMW309 and LMW319, expected band for successful integration is 913 bp. No amplification if unsuccessful. (2) Primers LMW309 and VM172, expected band for successful integration is 1,458 bp. No amplification for unsuccessful. (3) Primers LMW317 and LMW308, expected band for successful integration is 1,710 bp. No amplification if unsuccessful.
5. After acceptor module integration was confirmed by restriction analysis (preferably by sequencing as well), inoculate the confirmed strain for use in the first cycle of DNA assembly.

4. Notes

1. Primer design is critical for successful DNA assembly, as the integration of new fragments is guided in vivo solely by homologous sequences. Each DNA fragment is amplified using primers that add short regions of homology (30–40 bp) on both ends (a) to the preceding and next piece of the growing assembly and (b) to the donor plasmid.

To design primers for PCR1, start by determining the sequences for PCR amplification of your template of interest (~20 bp). Then, as shown in Fig. 3a(i), add the 5′ end of your 5′-primer with 20 bp of overlap with the preceding DNA assembly fragment (i.e., last 20 bp of the last fragment assembled in the previous cycle of assembly). Similarly, add the 5′ end of your 3′-primer with 20 bp of overlap with the next DNA assembly fragment (i.e., the first 20 bp of the next fragment to be inserted in the next cycle of assembly). Lastly, to add donor plasmid homology ("gray" sequences in Fig. 3a), add the sequences specified in Table 4 at the respective ends. Perform PCR1. PCR1 products are then purified and used as template for PCR2, to add further homology to the donor plasmid. The primers for PCR2 are generic and do not require any adjustments, and they are specified in Table 4. *We want to emphasize that each cycle requires specific pairs of PCR1 primers unique to the specific pathway you desire to integrate into the yeast chromosome and that PCR1 primers change at every cycle for each new DNA fragment.*

2. Both annealing temperature and elongation time should be adjusted according to the Tm of each primer pair and the length of the template DNA, respectively. (Suggested PCR cycle—using the following cycle: 95°C for 5 min, 95°C for 30 s, 50°C for 30 s,72°C for 2 min, repeat steps 2–4 29 times, 72°C for 10 min). In case multiple fragments are assembled in a single cycle, PCR1 should be performed to add homology to each fragment.
3. PCR2 primers add homology to the donor plasmid. Therefore, in case multiple DNA fragments are assembled in a single cycle (see Fig. 3b), PCR2 should only be performed on the outermost 5′ and 3′ fragments of PCR1.
4. We recommend using a molar ratio of 1:100 plasmid: PCR product for yeast transformation. In case the volume of total DNA to be transformed (donor plasmid + PCR fragments) is higher than 10 μL—pellet the DNA using Pellet Paint co-precipitant and resuspend pellet in 4 μL (see Subheading 2).
5. This last resuspension step aims to form a paste of cells, rather than a liquid cell culture, for electroporation. Thus it might be necessary to use less E-buffer at this stage to achieve the required consistency, depending on the amount of cells.
6. Electroporation time constant should be ~18 ms or lower.
7. Dilution 1:10 means plating 100 μL of 1 mL YPD culture. $1:10^2$ means plating 100 μL of a 1:10 diluted 1 mL culture, etc.
8. Cultures can be kept at 4°C for up to 2 days and replated, in case a different dilution is required.

9. The number of colonies used for induction depends on the size of the colonies; therefore in the case of very small colonies, it is recommended to use more colonies or wait 1–2 more days of plate incubation. Cell pellet should be visible after spinning down.
10. In our hands, Induction works better if it is initialized at exponential phase. Therefore, if the induction culture seems to have a high OD (visibly white and cloudy)—it is recommended to dilute the sample 1:10 (100 μL sample + 900 μL appropriate media to final OD_{600} = ~0.1–0.2) to improve recombination efficiency.
11. For control purposes, we recommend plating a sample of the induced culture immediately after 12 h of induction on selective plates with the opposite marker (histidine/leucine) and with *no FOA*. This allows better observation of the difference between induced (galactose) and uninduced (glucose) samples than if plated after the curing step.
12. In our hands, a 1-day period was found to be sufficient for curing a significant number of cells of their donor plasmid. However, the curing period can be extended if necessary.
13. In order to get single colonies of cured recombinants, it might be necessary to plate higher dilutions. We recommend plating 1:10, 1:10^3, 1:10^5 dilutions the first time and adjust as necessary.
14. In case colony PCR does not work, especially for long DNA fragments (above 2 kb), purify genomic DNA (see Subheading 2) and use it as template for PCR analysis.
15. *Assembly of DNA Libraries*: In the case of DNA libraries, a pool of excess mutant DNA is co-transformed with the donor plasmid, and each recombinant cell encodes a unique DNA mutant at the end of the assembly cycle:
    - Mutagenesis technique—any technique can be used to construct the library, as long as all DNA variants carry homology on both sides to enable assembly by Reiterative Recombination, as described for standard non-library assembly. Common methods for DNA mutagenesis include error-prone PCR and DNA shuffling.
    - Tips for improved transformation—since library experiments require a large number of cells (i.e., a large number of transformant colonies) to cover the total number of variants, it is critical to carefully execute the yeast transformation step in order to maintain high number of transformants: Use fresh patch to inoculate the cultures, prewarm YPD media to 30°C prior to sample inoculation,

and perform the protocol quickly, keeping the cells on ice as much as possible.

- Recover more recombinants—in order to carry all library variants to the next cycle of assembly, use a large (150 mm) plate in the last curing step to recover more recombinant colonies. Then, scrape the entire plate by spreading ~10 mL sterile water and gently lifting all the colonies into a micro-centrifuge tube. Use the entire pool of colonies as an acceptor strain for the next cycle of assembly.

16. The integration of acceptor module is optional, since a genetic acceptor strain is available (1). Integration of acceptor module should be performed in cases where a specific host strain is required. The generic acceptor strain carries the acceptor module at the HO gene locus, knocking out the function of the endogenous HO endonuclease gene. The acceptor strain also carries non-cleavable allele at the MATa locus.
17. It is highly recommended to perform the pop-out step for multiple colonies of the previous step, to increase the chances of isolating a successful integration event.

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