Chapter 5

High-Throughput DNA Assembly Using Yeast Homologous Recombination

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Abstract

Yeast homologous recombination is a reliable, low-cost, and efficient method for DNA assembly. Using homology regions as short as 24 base pairs, constructs of up to 12 unique parts can be assembled into a diverse range of vectors. The simplicity and robustness of this protocol make it amenable to laboratory automation and high-throughput operations. Here we describe a high-throughput protocol to generate DNA parts through PCR, assemble them into a vector via yeast transformation, and "shuttle" the resulting plasmid constructs into *E. coli* for storage and propagation. Though this protocol is intended for high-throughput workflows, it can be easily adapted for bench-scale DNA assembly.

Key words DNA assembly, Yeast homologous recombination, High-throughput, Automation, Synthetic biology

1 Introduction

Homologous recombination is a natural phenomenon in eukaryotes used to repair broken DNA at overlapping common sequences [1]. In synthetic biology, homologous recombination has been co-opted for DNA assembly and multi-part plasmid construction. *Saccharomyces cerevisiae* is typically the host of choice for this method due to its unmatched ability to facilitate homologous recombination. The only requirement for this method is sequence homology between adjacent pieces of DNA: when flanked with 20 to 40 base pairs of homologous sequences, DNA parts can be assembled in a linearized vector backbone with high efficiency (Fig. 1) [2]. The simplicity and flexibility of this protocol make it an attractive "BioFoundry" platform. With minor adjustments, this protocol can accommodate standardization (e.g., standardized homologous "linkers") and other application-specific modifications. For example, a commonly used variation of this protocol employs flanking restriction sites in the vector (e.g., PmeI) to liberate an assembled "payload" for homology-mediated genome

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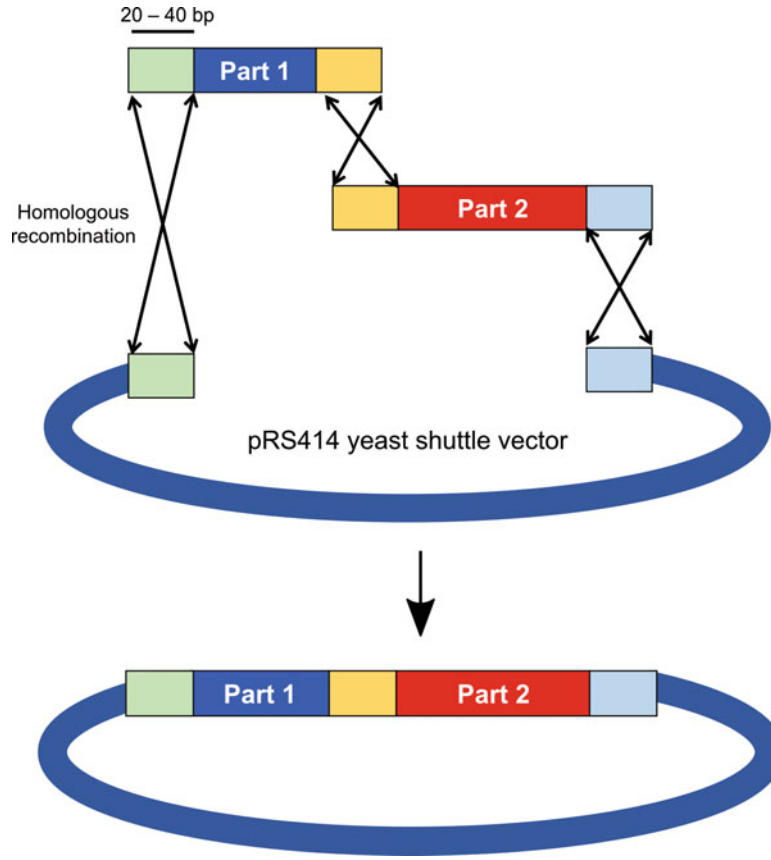

Fig. 1 Homologous recombination of a two-part assembly. (**a**) Parts flanked with 20–40 bp homology regions to adjacent DNA parts are generated by PCR. DNA parts are combined with a linearized yeast shuttle vector such as pRS414. (**b**) Yeast successfully assembles the DNA construct through homologous recombination. Assembled construct can be liberated from the vector via restriction digestion

integration. Similar modifications can be applied depending on the desired application.

The efficiency of homologous recombination in *Saccharomyces cerevisiae* makes it highly scalable for high-throughput, automated platforms. To maximize flexibility, the protocol described here uses PCR to generate compatible DNA parts. Homologous sequence "overhangs" of 20–40 bp are embedded in primer tails, effectively generating assembly-ready DNA in high throughput PCR reactions. Following verification and optional purification, these parts are transformed into *Saccharomyces cerevisiae* to be assembled in a "shuttle" vector containing selectable markers and replication sequences compatible with both yeast and *E. coli* [3]. After a 36-h "outgrowth" in selective media, assembled plasmids are isolated and transformed into *E. coli* that is subsequently plated on solid

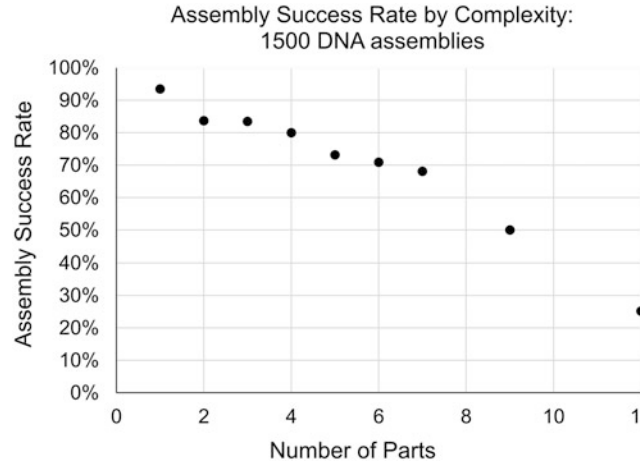

Fig. 2 Observed assembly rates for 1500 constructs of varying complexity, achieved with high-throughput operations. These assembly rates reflect individual replicates, i.e., assume only 1 colony is screened for each DNA construct. Screening 4 or more colonies for each construct results in success rates >90% for most complexities. The assemblies shown here used 24 bp homology sequences between compatible DNA parts. Note that DNA part concentrations in each assembly reaction were *not* normalized to facilitate high-throughput operations. If higher success rates are required, users can increase the homology length between adjacent DNA pieces and carefully normalize each reaction.

media. These resulting *E. coli* colonies are then screened via sequencing or restriction analysis for successful assemblies. Positive colonies can be banked as glycerol stocks or used for high copy plasmid isolation.

This protocol has been validated for the simultaneous assembly of 1500 DNA constructs of up to 12 parts each. A sample of observed success rates is shown in (Fig. 2). While this protocol is written primarily in 384- or 96-well format and is intended to be used with modern liquid handlers, volumes can be scaled up to perform homologous recombination at the bench-scale.

2 Materials

2.1 DNA Part Generation

1. 50 μM synthetic DNA oligonucleotide primers.
2. Synthetic DNA fragments or genomic DNA templates 10–100 ng/μL.
3. Phusion Hot-Start Flex DNA Polymerase with 5X HF buffer.
4. dNTP Solution Mix, 10 mM per nucleotide.
5. DpnI, 20,000 units/mL.
6. Molecular Grade or sterile water.

7. Capillary electrophoresis reagents and equipment, or agarose gel, loading dye, and ladder.
8. 96-well PCR plates.
9. 384-well PCR plates (optional).
10. PCR plate seals.
11. Liquid handling robot (e.g., Biomek FX).
12. Labcyte Echo 525 acoustic liquid handler and compatible 384-well plates (optional).
13. Thermocycler.
14. Fragment Analyzer or Zero Agarose Gel (ZAG) DNA Analyzer (Agilent) capillary electrophoresis system (optional).

2.2 Prepare Competent Yeast Cells

1. Yeast host strain: *Saccharomyces cerevisiae* strain CEN.PK2-1c, tryptophan auxotroph (*MATa ura3-52 trp1-289 leu2-3112 his3Δ1 MAL2-8C SUC2*) (*see* **Note 1**).
2. 100 mM Lithium Acetate (LiAC): Make 1 M LiAC by dissolving 102.02 g of Lithium Acetate dehydrate in 1 L of deionized water. Filter-sterilize with a 0.22 μm filter. Dilute 1 M LiAC with deionized water.
3. YPD + Kan: 1% yeast extract, 2% peptone, 2% dextrose, 50 mg kanamycin. Dissolve 10 g yeast extract and 20 g peptone into 700 mL deionized water. Raise volume to 900 mL with deionized water. Autoclave for 20 min at 15 psi on liquid cycle. Cool to 50 °C. Add 100 mL of 20% glucose and 1 mL of 50 mg/mL Kanamycin (*see* **Note 2**).
4. Molecular grade or sterile water.
5. Centrifuge with compatible rotor and bottles.
6. 15 mL culture tube.
7. Shaker flasks.
8. 30 °C shakers.

2.3 Yeast Transformation

1. pRS414 Yeast shuttle entry vector (*see* **Note 3**).
2. Salmon sperm DNA (ssDNA), 10 mg/L.
3. 50% PEG Solution: Weigh out 50% by volume of PEG and add to deionized water. Filter-sterilize.
4. 1 M Lithium Acetate (LiAC): Dissolve 102.02 g of lithium acetate dehydrate in 1 L of deionized water. Filter-sterilize with a 0.22 μm filter.
5. CSM -W (complete yeast synthetic medium without tryptophan): To a 2 L beaker add 700 mL of dH_2O and a stir bar. Add 0.74 g of CSM without tryptophan (114511012 MP Biomedicals or similar) and 43.7 g DOB (114025012 MP Biomedicals or similar) and stir until dissolved. Transfer the solution to

a glass media bottle. Loosely cap and sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle (*see* **Note 4**).

6. Molecular grade or sterile water.
7. 96-well PCR plates.
8. PCR plate seals.
9. 2.2 mL 96-well culture plates.
10. Gas-permeable plate seals.
11. Thermocycler.
12. Liquid handling robot (e.g., Biomek FX).
13. Labcyte Echo 525 acoustic liquid handler and compatible 384-well plates (optional).
14. Spectrophotometer.
15. Centrifuge with compatible 96-well plate rotor.
16. 30 °C shakers.

2.4 E. coli *Shuttling*

1. Column-based 96-well yeast miniprep kit (e.g., Zymoprep-96 Yeast Plasmid Miniprep).
2. 1× TE buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
3. Competent *E. coli* (e.g., DH5α).
4. SOC Medium: 0.5% yeast extract, 2% tryptone, 10 mM NaCl, 20 mM glucose. Dissolve 20 g of Tryptone, 5 g of Yeast Extract, and 0.5 g of NaCl in 0.5 L of deionized water. Raise volume up to 1 L. Autoclave for 20 min at 15 psi on liquid cycle. Cool to 50 °C. Add 20 mL of sterile 1 M Glucose and mix by swirling.
5. LB + Carb broth: Dissolve 25 g of powdered LB Broth into 900 mL of deionized water. Raise volume to 1 L. Autoclave for 30 min at 15 psi on liquid cycle. Cool to 50 °C. Add 1 mL of 50 mg/mL of carbenicillin.
6. LB + Carb agar plates: Dissolve 25 g of powdered LB Broth into 900 mL of deionized water. Raise volume to 1 L. Add 15 g of agar. Autoclave for 30 min at 15 psi on liquid cycle. Cool to 50 °C. Add 1 mL of 50 mg/mL of carbenicillin. Pour into plates. Allow to solidify and dry overnight. To use liquid handlers for plating, use compatible plate types such as Qtrays (Molecular Devices).
7. 96-well PCR plates.
8. Liquid handling robot (e.g., Biomek FX).
9. Centrifuge with compatible 96-well plate rotor.
10. 37 °C incubator.
11. Thermocycler.

3 Methods

3.1 DNA Part Generation

Unless otherwise stated, perform all liquid transfers above 1 μL on a traditional liquid handler (e.g. Biomek FX), and all transfers 1 μL or below on an acoustic liquid handler (e.g., Labcyte Echo 525). If only using a traditional liquid handler, adjust volumes and concentrations to minimize transfer error (*see* **Note 5**).

1. Design primers to amplify each DNA part. Primers should be both specific to the part and flanked with desired homology. Include 20–30 bp that anneal to the DNA part (T_m ~ 60 °C) and 20–40 bp that anneal to the adjacent DNA part. Note that the first and last DNA part will require homology to the vector backbone. Order these sequences using synthetic DNA ordering service at 25 nmol, desalted, and at a final concentration of 50 μM. For high throughput set-ups, it may be preferable to place order in 96-well or 384-well plates (some vendors will also deliver primers directly in 384-well Echo source plates, facilitating reaction setup).
2. Set up the PCR reactions. Rearray 100 nL of each primer at 50 μM and 10–100 ng of template DNA into each well on a 96- or 384-well PCR plate (*see* **Notes 6** and **7**).
3. Prepare a PCR master mix of 5× HF buffer, dNTPs, molecular grade water, and Phusion DNA polymerase by hand on ice. Each well should contain: 6.67 μL of 5X HF Buffer, 22 μL of water, 0.67 μL of dNTPs, and 0.67 μL of Phusion polymerase. Use a liquid handler to dispense and mix 30 μL of premade master mix into each well containing re-arrayed primers and templates. Table 1 shows suggested recipes for each plate format (*see* **Note 7**).
4. Calculate PCR extension time based on the largest amplicon on a given plate. In general, 15–30 s/kilobase is used, with a minimum extension time of 16 s.

Table 1
Sample master mix volumes with safety factor implemented

Reagent	96-well plate (mL)	384-well plate (mL)
Molecular grade water	7.60	10.14
5X HF buffer	2.304	3.072
dNTPS (10 mM/nt)	0.2304	0.3072
Phusion DNA polymerase	0.2304	0.3072
Total	10.4	13.8

5. Place plate in the thermocycler and run the following PCR program: Initial denaturation at 98 °C for 2 min; 10 cycles of 98 °C for 12 s, 60 °C for 30 s, and 72 °C for calculated extension time; 30 cycles of 98 °C for 12 s, 55 °C for 30 s, and 72 °C for calculated extension time; final extension of 72 °C for 5 min.
6. Verify amplicons by running a small volume on an agarose gel or use capillary electrophoresis (*see* **Note 8**).
7. Reactions containing templates with plasmid backbones need to be digested before assembly. Add 20 U of DpnI to each reaction. Incubate at 37 °C for 30 min, 80 °C for 20 min to inactivate (*see* **Note 9**).

3.2 Prepare Competent Yeast Cells

1. Inoculate a yeast starter culture from frozen glycerol stock or streaked colony in 5 mL of YPD + Kan in a 15 mL culture tube. Grow overnight, shaking at 30 °C.
2. Check the OD600 of the starter culture (*see* **Note 10**).
3. Inoculate a flask with appropriate amount of starter into 0.5–1 L of YPD + Kan and grow overnight to OD of 0.7 (*see* **Note 11**).
4. Transfer culture to centrifuge bottle. Pellet the cells by centrifuging for 4000 × *g* for 4 min. Decant supernatant.
5. Completely re-suspend pellet with water. Use at least half of the original volume of culture to ensure thorough washing. Centrifuge at 4000 × *g* for 4 min. Decant supernatant.
6. Repeat **step 5** of this section with 100 mM LiAC.
7. After decanting, re-suspend the cell pellet with residual LiAC. Measure the volume. Add 100 mM LiAC to reach final desired OD-mL/well of 1.5 (*see* **Note 12**).

3.3 Yeast Transformation

1. Re-array 1 μL of each DNA part into 96-well PCR plates. Each well on the final assembly plate should represent a single assembly (*see* **Note 13**).
2. Prepare a mixture of 50% PEG, 1 M LiAC, and salmon sperm DNA (ssDNA) by hand. ssDNA should be boiled on a heat block at 95 °C for 10 min before use. Each transformation well should contain 42 μL of 50% PEG, 6.3 μL of 1 M LiAC, and 1.7 μL of ssDNA.
3. To the mixture prepared in the last step, add cells and entry vector. Each transformation should contain 10 μL of yeast cells and 2 ng of entry vector.
4. Transfer 62 μL of the final mixture into each well of 96-well plates containing mixed DNA parts from **step 1** of this section. Swirl the mixture before pouring into a reservoir. This will

homogenize the solution and prevent it from leaking out of the pipette tips during transfer.

5. Heat-shock using the thermocycler at 42 °C for 45 min.
6. Fill 2.2 mL 96-well culture plates with 1 mL of Molecular Grade or sterile water per well.
7. After heat shock is complete, transfer the entire transformation mixture to the pre-filled 2.2 mL plates. Wash by aspirating and dispensing several times.
8. Centrifuge to pellet the cells at 4000 × *g* for 3 min. Remove supernatant by inverting and flicking each plate over a waste container.
9. Resuspend pellets in 1 mL per well of CSM -W medium.
10. Seal each plate with a gas-permeable seal. Outgrow plate at 30 °C, shaking, for at least 36 h and up to 60 h.

3.4 E. coli *Shuttling*

1. Miniprep DNA from yeast using a column-based yeast miniprep kit following manufacturer's protocol. Elute in 30 μL of 1× TE buffer.
2. Thaw frozen competent *E. coli* cells on ice. Aliquot 50 μL per well into a 96-well plate.
3. Prepare for plating by drying LB + Carb agar plates under laminar flow hood until surface looks opaque. Do not over-dry.
4. Transfer and mix 10 μL of eluted DNA to 96-well plates containing 50 μL of competent cells. Work quickly to keep cells cold.
5. Keep plates containing cells with DNA on ice for 20 min.
6. Heat-shock plate at 42 °C for 45 s. This can be done by placing the plate, lightly covered, on a thermocycler set to 42 °C with the lid open.
7. Remove and immediately return to ice for at least 5 min.
8. Dilute cells in SOC (*see* **Note 14**).
9. Plate diluted cells on pre-dried agar plates. If using a liquid handler, calibrate the dispense height with the agar level. If plating multiple transformations on one plate, use appropriate droplet sizes to leave adequate spacing between wells to prevent contamination.
10. Let plates dry before handling. Cover, place upside down in an incubator, and grow overnight at 37 °C for about 18 h to obtain single isolate colonies.
11. Pick colonies into 1 mL/well of LB + Carb broth (*see* **Note 15**). After overnight incubation at 37 °C, isolate the plasmids via standard miniprep protocols and analyze via sequencing or restriction digest analysis. Alternatively, rolling circle amplification (RCA) may be used to generate sequence-ready DNA directly from boiled *E. coli* culture [4].

4 Notes

1. The protocol provided here is written for a tryptophan auxotroph. Auxotrophy can be achieved through point mutations or a complete knockout of *TRP1*. Other auxotrophic yeast strains or selectable markers can be used—users will need to adjust vector choice and growth media (i.e., replace CSM -W) accordingly.
2. Kanamycin is added to YPD to inhibit bacterial contamination that may occur during high-throughput operations.
3. pRS414 is an example shuttle vector compatible with this protocol. The key features of this shuttle vector are (1) low copy CEN/ARS sequence for replication in yeast, (2) higher copy pMB1 ori for replication in *E. coli*, (3) *TRP1* marker for auxotrophic selection in yeast, and (4) ampicillin resistance marker for selection in *E. coli*. The shuttle entry vector should be provided as a linearized DNA product.
4. Media used for yeast outgrowth should be compatible with the selectable marker of the shuttle vector. In this case, a shuttle vector containing the *TRP1* marker is paired with selection media without tryptophan.
5. In a high throughput setting, it is recommended to avoid liquid transfers <2 μL on standard liquid handling robotics such as the Biomek FX. Users can adjust volumes and stock concentrations accordingly to avoid low volume transfers.
6. Template DNA is frequently miniprepped plasmid DNA, synthetic DNA (e.g., gene fragments), or purified genomic DNA. If using an acoustic liquid handler, transfer of genomic DNA can be problematic. Users should dilute their genomic DNA stock to about 5 ng/μL and visually confirm transfer before proceeding.
7. When using liquid handlers to dispense reagents, the dead volume of the instrument should always be considered. Typically, a 10–20 mL dead volume should be added to all bulk-reagent transfers on the Biomek. To ease calculations for master mix preparation, a safety factor of 1.3×–1.5× can be multiplied for each reagent. On the Labcyte Echo, the dead volume of a polypropylene 384 square-well plate is 20 μL, with a maximum volume of 60 μL. Users should consult manufacturing instructions when changing labware.
8. For high-throughput verification via capillary electrophoresis, each DNA fragment should be within 10% of its expected size with a concentration greater than 5 ng/μL. We have found that as little as 5 ng of a DNA part can successfully assemble, though higher concentrations are preferable. Importantly, the molar

purity must be >50%, with limited nonspecific amplification. Primer dimers are often the most common nonspecific products, particularly for large amplicons. If primer dimers become problematic, users should consider decreasing the primer concentration by two-fold or purifying the PCR products via column or bead-based methods (e.g., AMPure XP).

9. Plasmid DNA from *E. coli* contains methylated DNA. DpnI cleaves methylated DNA and prevents any residual template from being carried over in the recombination process. Residual plasmid template due to ineffective DpnI digestion (typically due to a liquid transfer error) is a common failure mode in this process, though it is easily identified (i.e., picked *E. coli* colonies contain plasmid template rather than assembled DNA) and remedied.
10. To take an accurate OD of the starter culture, perform a 20-fold dilution before reading. For example, combine 50 μL of starter with 950 μL of media in a spectrophotometer cuvette. Then, multiply the reading by 20 to get the OD of the starter culture. Typically, a 5 mL overnight starter culture should reach an OD of 7 after 24 h.
11. Calculate the number of doublings by using this equation. 95 min is the expected doubling time. Total incubation time is calculated based on time between inoculation and harvest.

$$\text{num.of doublings} = \frac{\text{Total Incubation Time}}{95\ \text{min}}$$

Calculate the start inoculation OD by using this equation:

$$\text{Start inocul.OD} = \frac{0.7}{(\text{num.of doublings})^2}$$

Finally, calculate the start inoculation volume by using this equation. The total volume to be inoculated is based on how many transformations are needed. Typically, a single 1000 mL flask is enough for perform five 96-well plates of transformations (with dead volume considered).

$$\text{Vol of starter to inoculate} = \frac{\text{Start inocul.OD}}{\text{OD of starter}} \times (\text{total vol to be inoculated})$$

12. Each transformation well should contain 10 μL of harvested cells at an OD-mL per well of 1.5.
13. For maximum assembly efficiencies, users should normalize the molar concentrations of each DNA part. The recommended composition of each assembly reaction is 150 fmol of each DNA part and 5 fmol of shuttle vector. For high-throughput workflows, simply adding 1 μL of each DNA part (provided it

meets the QC metrics described in **Note 8**) is sufficient, though the minor decrease in assembly success rate may necessitate picking additional colonies. Note that the assembly efficiency metrics provided in Fig. 2 were generated *without* normalization.

14. A dilution factor of 16 yields pickable colonies for most transformations. A more concentrated dilution can be plated alongside within the same Q-tray well to ensure pickable colonies. Colony densities and growth time will differ depending on the agar plate dimensions. Plating dilution and method should be optimized for each plate-type.
15. Picking four colonies per assembly typically results in at least one positive construct for assemblies up to 12 parts. Users should pick additional replicates if more complex assemblies are attempted.

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