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RADOM, an Efficient *In Vivo* Method for Assembling Designed DNA Fragments up to 10 kb Long in *Saccharomyces cerevisiae*

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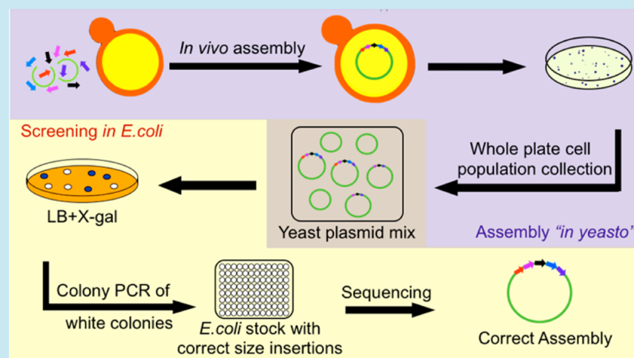
Supporting Information

ABSTRACT: We describe rapid assembly of DNA overlapping multifragments (RADOM), an improved assembly method *via* homologous recombination in *Saccharomyces cerevisiae*, which combines assembly *in yeasto* with blue/white screening in *Escherichia coli*. We show that RADOM can successfully assemble ∼3 and ∼10 kb DNA fragments that are highly similar to the yeast genome rapidly and accurately. This method was tested in the Build-A-Genome course by undergraduate students, where 125 ∼3 kb "minichunks" from the synthetic yeast genome project Sc2.0 were assembled. Here, 122 out of 125 minichunks achieved insertions with correct sizes, and 102 minichunks were sequenced verified. As this method reduces the time-consuming and labor-intensive efforts of yeast assembly by improving the screening efficiency for correct assemblies, it may find routine applications in the construction of DNA fragments, especially in hierarchical assembly projects.



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With the availability of whole-genome sequence data and the development of fast and cost-effective methods to chemically synthesize DNA, the assembly of DNA fragments into large constructs, chromosomes and even genomes is becoming increasingly commonplace. Restriction digestion and ligation has long been the universal method in fundamental research and metabolic engineering.[1] However, the sequence dependency of traditional cloning approaches inherently relies on identifying useful pre-existing sites or introducing new recognition sequences that leave scars, which can often be problematic for large synthesis projects.[2]

Sequence-independent cloning relies on terminal sequence homology of adjacent fragments in assembly reactions and includes methods such as ligation-independent cloning (LIC),[3] sequence and ligation-independent cloning (SLIC),[4] circular polymerase extension cloning (CPEC)[5] and Gibson isothermal assembly.[6] These methods enable efficient assembly of as many as six (or possibly more) overlapping DNA fragments into a plasmid. Although these methods all have their own special characteristics and advantages, many take additional preparation steps and all of them require the use of enzyme systems to generate complementary single-stranded overhangs for assembly. Moreover, the termini sequence of DNA fragments to be assembled using SLIC/CPEC/Gibson methods should not contain stable single stranded DNA secondary structures, such as hairpins or stem loops, as these would compete with the desired single stranded annealing of adjacent assembly fragments.[7] The emergence of the field of synthetic biology has put an ever-increasing demand on developing more accurate, efficient, convenient and economical cloning technologies for purposes such as building synthetic genomes.

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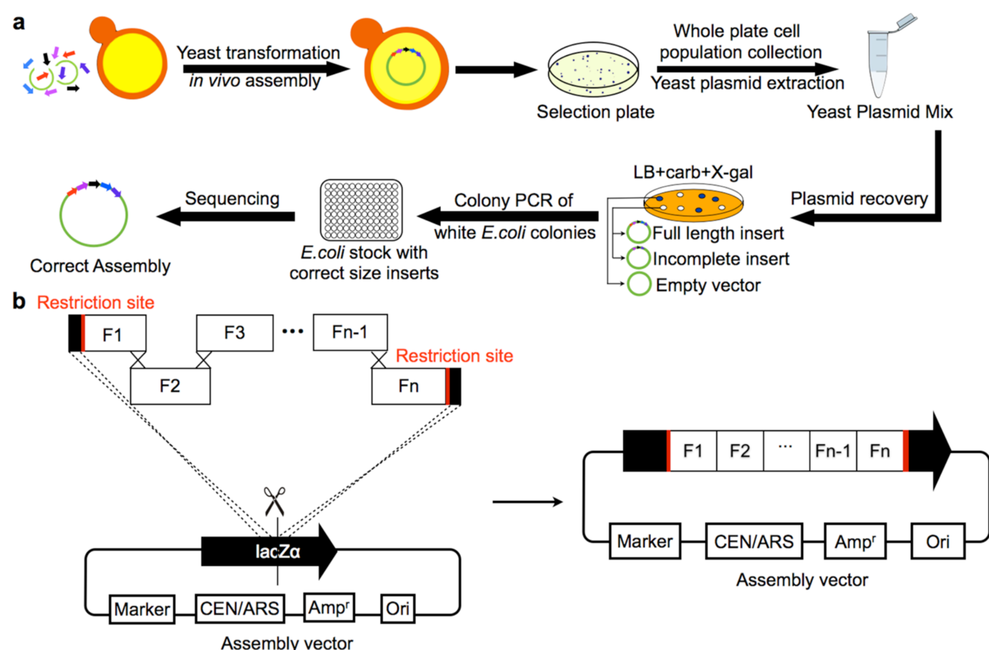

Figure 1. RADOM schematic. (a) RADOM workflow. DNA fragments that share at least 40 bp terminal homology with each other and a linearized assembly vector are cotransformed into yeast for assembly by homologous recombination. Yeast transformants are combined into a single pool for plasmid recovery into *E. coli*, where blue/white screening distinguishes empty vector from clones in which inserts assembled. Colony PCR identifies clones encoding full-length inserts that can be sent for sequencing. (b) Vector and DNA fragment details. F_1 to F_n represents DNA fragments that encode internal homology with each other and terminal homology to the backbone (black). The assembly vector must be linearized in the lacZα gene to enable downstream blue/white screening. Assembled constructs can be released from the assembly vector by digestion using restriction enzymes encoded in the design. The assembly vector must encode selection and replication genetic information for both yeast (marker, CEN/ARS) and *E. coli* (Amp, Ori).

The synthetic yeast genome project, Sc2.0, is the first eukaryotic genome project and aims to construct a modified version of the 12 Mb *Saccharomcyes cerevisiae* genome (www.syntheticyeast.org). Recently, the first synthetic eukaryotic chromosome *synIII*, has been successfully synthesized and shown to be functional in *S. cerevisiae* in the absence of the corresponding wild type version.[8,9] Now, the remaining chromosomes, ranging in size from 200 kb to greater than 1 Mb, are being constructed individually using a "bottom-up" hierarchical assembly approach. In brief, synthetic chromosomes are constructed first as ~3 kb DNA fragments (arbitrarily defined as "minichunks") that may be further assembled into ~10 kb DNA fragments (arbitrarily defined as "chunks") prior to incorporation into yeast.[8,10] To meet the needs of a genome synthesis project like Sc2.0, an ideal assembly method should be sequence independent, fast, accurate, and adaptable to work at any scale. Additionally, for such high-throughput cloning, higher efficiency and lower cost are paramount.

The natural capacity of *S. cerevisiae* to perform homologous recombination meets many of the requirements described above and was discovered nearly three decades ago.[11] More recently, exploiting the capacity of *S. cerevisiae* for homologous recombination is fast becoming an important tool in laboratory practice ranging from simple plasmid construction to building entire genomes. The great potential of cloning in yeast was demonstrated when the J. Craig Venter Institute assembled the first synthetic bacterial genome using yeast homologous recombination and successfully assembled 38 single stranded 200 bp oligonucleotides with 20 bp overlaps into a linearized plasmid.[12,13] Additionally, yeast represents a versatile platform for biochemical pathway assembly, as demonstrated by the similar one-step "DNA Assembler" method developed by Shao *et al.*[14,15] Despite its advantages, using yeast as a cloning tool can suffer from a high false positive transformant rate due to recircularization of an empty assembly plasmid by the nonhomologous end joining machinery in yeast.[16,17] This problem coupled with the laborious effort of plasmid recovery from yeast into *Escherichia coli* can severely compromise parallelization of assembly and hence the applicability of yeast cloning for large synthesis projects. To address these shortcomings, the vector backbone may be transformed as multiple overlapping fragments encoding unique genetic elements[18] and selection procedures based on counter-selection and dual markers have been developed.[19] However, these solutions introduce additional complexity to the assembly procedure and do not always adequately deal with the problems.

Here we present a method termed rapid assembly of DNA overlapping multifragments (RADOM), based on homologous recombination in yeast and relies on DNA fragments with homologous overhang regions of at least 40 bp.[20] Distinct from yeast assembly methods described above, RADOM specifies the extraction of plasmids from the entire population of yeast transformants to ensure that all variations of *in yeasto* plasmid assemblies are extracted as a complex pool that is subsequently transformed *en masse* into *E. coli* (Figure 1a). Blue-white screening is then used to distinguish bacterial clones carrying uncut and self-ligated empty vector backbones from assembled constructs, and colony PCR is used to identify full length inserts. The vector used in this method must be linearized within the lacZα gene to enable blue/white screening by disruption of the α-complementation process (Figure 1b).[21] The assembly accuracy may then be analyzed by PCR from

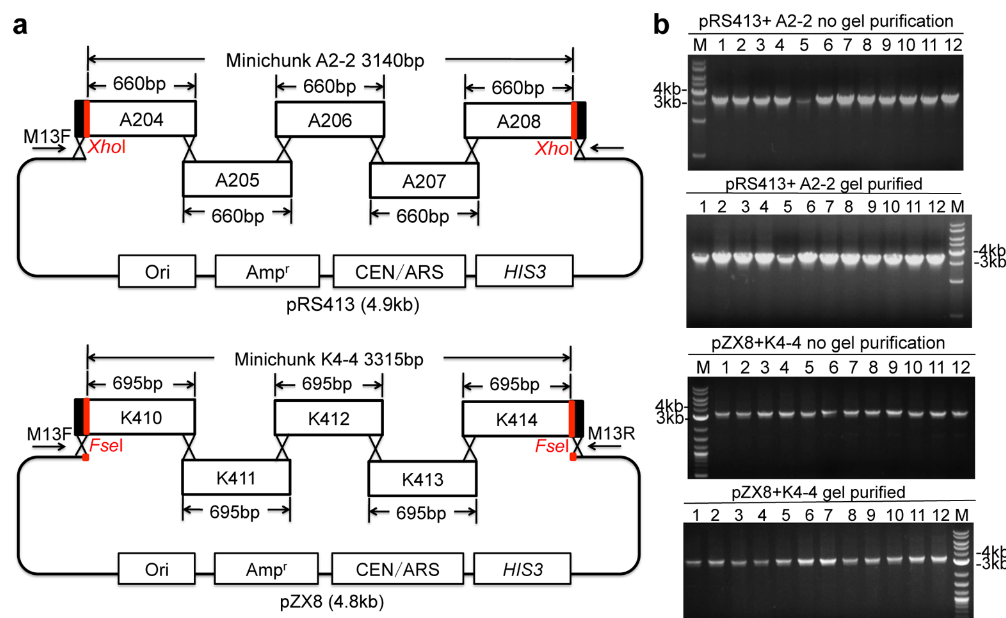

Figure 2. Construction of ∼3 kb minichunks. (a) Assembly scheme of minichunk A2−2 and K4−4 from 5 building blocks with overlapping regions of 40 bp. Restriction sites *Xho*I and *Fse*I marked in red are used to release minichunks from assembly backbones. (b) Bacterial colony PCR analysis of 12 white clones of A2−2 and K2−2 minichunk assembly. M represents DNA ladder.

Table 1. Assembly of Minichunk A2-2 and K4-4[a]

	assembly length (bp)	colonies on SC-His[b]	blue colonies on X-gal	white colonies on X-gal	% blue colonies	csPCR correct rate	sequence correct rate
L-pRS413	–	280	–	–	–	–	–
A2−2-P	3140	517	406	302	57	12/12	2/2
A2−2-NP	3140	880	205	169	54	12/12	2/2
L-pZX8	–	0	–	–	–	–	–
K4−4-P	3315	164	3	62	5	12/12	2/2
K4−4-NP	3315	264	2	51	4	12/12	2/2

[a]L-pRS413 and L-pZX8 represent transformation with 200 ng linearized pRS413 and pZX8 vector alone. A2−2 P and K4−4 P represent assembly using the 200 ng gel purified fragments. A2−2 NP and K4−4 NP directly used the 5 μL PCR fragments without purification for assembly. [b]Number of colonies was calculated from the low density plating on SC−His plates (50 μL out of 400 μL of resuspended cell mixture). "L", "P", "NP" are short for "linearized", "gel purified", and "no gel purification", respectively.

individual white *E. coli* colonies, and clones with the expected insert size are sequenced and/or stored for future analysis (Figure 1a). As a proof of principle, RADOM was used to assemble ∼3 and ∼10 kb synthetic DNA constructs as part of the Sc2.0 project. We also demonstrated the scalability and ease-of-use of RADOM in the Build-A-Genome course,[22,23] where undergraduates applied the method to rapidly assemble ∼3 kb DNA minichunks.

We began with minichunks A2−2 and K4−4 from synthetic chromosomes V (*synV*) and VIII (*synVIII*), each consisting of five building blocks,[24] to assemble into vectors pRS413 and pZX8 (see Supporting Information Figure S1), respectively (Figure 2a). In order to test the assembly capacity and system versatility, BB PCR products (primers listed in Supporting Information Table S1), with and without gel purification, were used in independent assembly reactions with the appropriate linearized vector. Homologous recombination between BB PCR products relied on pre-existing 40 bp overlaps that were incorporated during the design stage.[8] During the PCR stage of the right- and leftmost BBs in each assembly, vector homology was introduced to enable homologous recombination of a vector containing unique restriction enzyme sites (*Xho*I and *Fse*I) for future higher-order chunk assemblies (Figure 2a). Linearized pRS413 and pZX8, each encoding a *HIS3* selectable marker, were transformed in the absence of insert DNA as controls to estimate the frequency of false-positive transformants resulting from reclosure of linearized backbones in yeast or uncut plasmid. After transformation, 50 and 350 out of 400 μL of resuspended cell mixture were spread onto synthetic complete medium lacking histidine (SC−His) and incubated at 30 °C. The higher density plating was always used for whole plate cell population collection, while the numbers of transformants (Table 1) were calculated from the lower density plating.

Plasmids from pooled yeast transformants for each assembly reaction were recovered into *E. coli*. The universal primers M13F and M13R, which are encoded on both assembly vectors pZX8 and pRS413 (Figure 2a), were used for *E. coli* colony PCR reactions to test the inset sizes of 12 randomly selected white colonies from the X-gal plate for each assembly reaction, with and without gel purification of BB PCR products. If assembled correctly, we expected to generate 3.3 and 3.5 kb amplicons for A2−2 and K4−4 respectively. As shown in Figure 2b, 100% of clones in all assembly experiments yielded amplicons of correct size. Given that no difference was observed with and without gel purification of DNA fragments

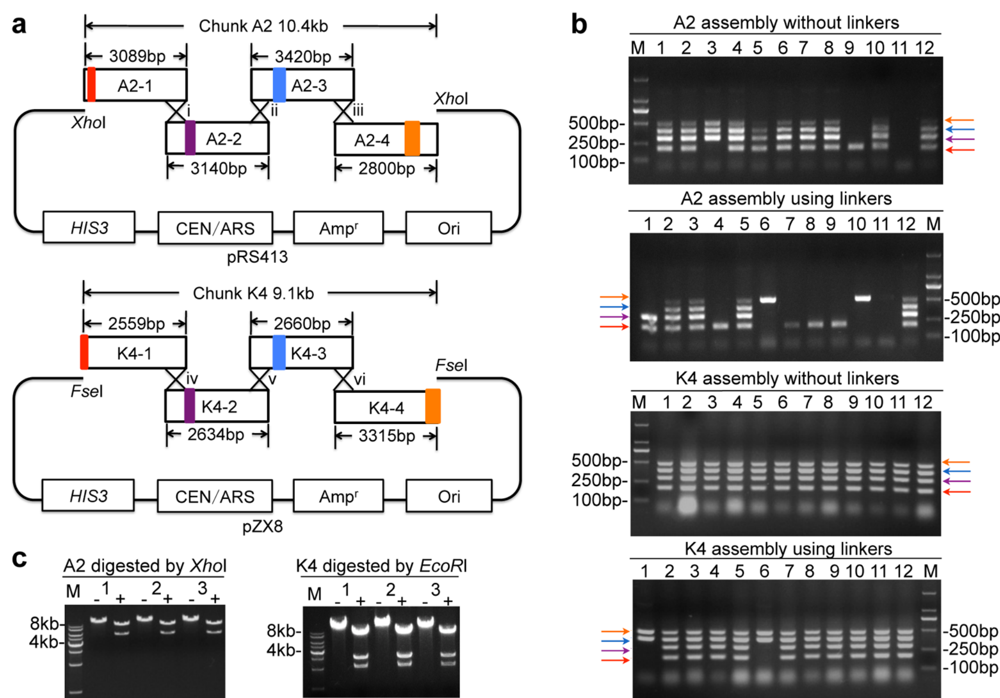

Figure 3. Construction of ∼10 kb chunks. (a) Assembly scheme of chunk A2 and K4. Chunk A2 (10.4 kb) and K4 (9.1 kb) were assembled onto pRS413 and pZX8, respectively. Restriction sites *Xho*I and *Fse*I were used to digest chunks from their backbones. Colored rectangles (red (200 bp), purple (300 bp), blue (400 bp), orange (500 bp)) represent the location of amplicons generated by multiplex colony PCR (MCPCR) primers. Roman numerals i−vi represent overlap region between minichunks. (i) 660 bp, (ii) 660 bp, (iii) 730 bp, (iv) 670 bp, (v) 695 bp, (vi) 695 bp. (b) MCPCR analysis of 12 white clones of A2 and K4 chunk assembly. (c) *Xho*I and *EcoR*I digestion profile for three clones of chunk A2 or K4. Chunks before digestion (−) and after digestion (+) were run on the same agarose gel.

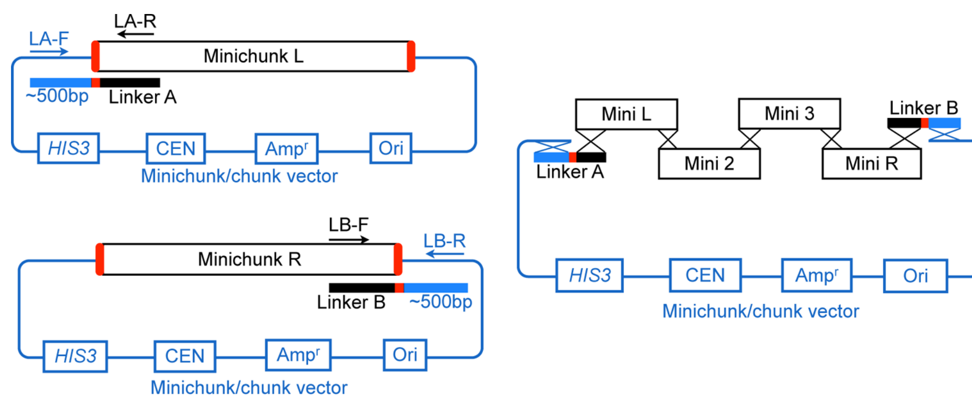

Figure 4. Linker construction. Minichunk L and minichunk R represent the left- and rightmost minichunk in each chunk assembly. Linkers A and B were PCR amplified from their minichunk constructs and consisted of ∼500 bp homologous to backbones and the rest homologous to the terminal minichunks. The forward primer to amplify linker A and the reverse primer to amplify linker B are universal primers that anneal to the backbones (shown in blue). The resulting PCR fragments of linkers provide sequence overlaps between minichunks and vectors, as well as restriction sites to digest chunks from the vector. Highlighted in red are restriction sites introduced from minichunk. "L" represents left; "R" represents right; "Mini" represents minichunk.

(Table 1), we conclude that gel purification provides no major advantage in minichunk assembly. As all fragments are highly similar to native yeast chromosome with only minor modifications,[8,10] two clones from each assembly were sequenced to determine whether this method can achieve high fidelity without interference from the native genome. Sequencing results revealed that in all four cases the assembled minichunks perfectly matched the expected sequence.

We then applied RADOM to construct 10 kb chunks starting from minichunks. Here, adjacent minichunks overlapped by a single BB to provide the required homology for *in yeasto* assembly. Notably, the individual sequence verified minichunks had previously been constructed using RADOM. Chunk A2 (10.4 kb) from *synV*, consisting of minichunks A2−1, A2−2, A2−3 and A2−4, was assembled into pRS413, while chunk K4 (9.8 kb) from *synVIII*, consisting of minichunks K4−1, K4−2, K4−3 and K4−4, was assembled into the pZX8 vector (Figure 3a). In this study, two variant approaches were used to address the lack of homology between terminal minichunks and the assembly vector. The first introduced overhangs by PCR amplification across terminal minichunks. More specifically, 40 bp of sequence overlapping with the terminal ends of the assembly vector were introduced by PCR to the right- and leftmost minichunks in each assembly. In the second approach,

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Table 2. Assembly of Chunk A2 and K4[a]

	assembly length (kb)	colonies on SC-His[b]	blue colonies on X-gal	white colonies on X-gal	% blue colonies	csPCR correct rate	sequence correct rate
L-pRS413	–	305	–	–	–	–	–
A2-Linker	10.4	530	494	356	58	4/12	2/2
A2-nolinker	10.4	660	348	368	49	9/12	2/2
L-pZX8	–	0	–	–	–	–	–
K4-Linker	9.1	253	5	73	6	10/12	1/2
K4-nolinker	9.1	238	4	68	6	12/12	2/2

[a]L-pRS413 and L-pZX8 represent transformation with 200 ng linearized pRS413 and pZX8 vector alone. A2-Linker and K4-Linker represent chunk assembly using linkers with length of ~1 kb to join the unrelated DNA fragments to the backbones. In A2-nolinker and K4-nolinker assembly, 40 bp homology with backbones were introduced by PCR. [b]Number of colonies was calculated from the low density plating on SC−His plates (50 μL out of 400 μL of resuspended cell mixture). "L" is short for "linearized".

we exploited the fact that the same assembly vector was used for both minichunk and chunk assemblies. Here, ~1 kb synthetic linkers spanning the terminal minichunk/assembly vector junction were generated by PCR using a vector specific primer (~500 bp upstream of the assembly junction) and a minichunk specific primer (~500 bp internal to the minichunk sequence) (Figure 4). In the second approach, all minichunks were released from their respective backbones by restriction digestion followed by cotransformation with linearized vector plus PCR-generated linkers.

Following recovery of assembled plasmids into *E. coli*, 12 white colonies were randomly picked and analyzed by multiplex colony PCR, which was designed to produce four unique amplicons each corresponding to a unique minichunk (Figure 3a). Incomplete patterns of multiplex PCR were observed in some cases (Figure 3b), indicating partial and/or incorrect chunk assembly. However, in all cases at least four clones yielded all the expected multiplex PCR amplicons, and in one case 100% of assembled chunks were correct (Figure 3b, K4 without linkers). In general, assemblies with terminal overhangs of 40 bp to the corresponding vector backbones were more efficient than linker based assemblies (Table 2). This may be the result of the increased number of fragments required to engage in homologous recombination when linkers were used.

To ensure that correct multiplex colony PCR profiles indeed reflected the desired assembly, 3 single clones were randomly selected for each of chunk A2 and K4. Digestion profiles of the plasmids isolated from the above clones were consistent with correct assembly of the chunks (Figure 3c), thereby supporting the utility of multiplex colony PCR to identify correctly assembled constructs. Sequence analysis revealed perfect sequence in 7 of 8 clones, where the single mutation was found in K4 at the junction of two minichunks. Here, it is possible that the cohesive ends of the *Fse*I site introduced the mutation. In summary, these results demonstrate the high fidelity of *in yeasto* assembly to yield perfect assemblies that can be rapidly identified *via* our streamlined RADOM protocol.

In this work we observed that recovery of blue colonies can vary between assembly vectors and the restriction site/enzyme used for linearization both in the chunk and minichunk assembly (Tables 1 and 2). Specifically, *Eco*RI-digested pRS413 typically yielded ~50% blue colonies following recovery of yeast assemblies into *E. coli*, consistent with the observation that linearized pRS413 transformed into yeast in the absence of insert yielded ~200−300 yeast transformants in our hands (Tables 1 and 2); this was also observed in earlier studies.[19] In contrast, *Bsa*I-digested pZX8 rarely yielded colonies when transformed into yeast without insert DNA and recovery of blue colonies was relatively low (~5 *versus* ~50%) for both minichunk and chunk assembly (Tables 1 and 2). This could result from increased efficiency of digestion by *Bsa*I and/or lower rates of nonhomologous end-joining in yeast of the pZX8 vector. (The two *Bsa*I sites in pZX8 expose incompatible 4-nt overhangs designed to reduce self-ligation.) However, even with the dramatically higher rate of empty pRS413 vector as compared to pZX8, blue/white screening in *E. coli* effectively mitigated this problem enabling immediate identification of clones encoding inserts. Together the combination of blue/white screening and colony PCR efficiently streamlined process of colony screening and demonstrated the utility of RADOM to circumvent the problem of empty vector assemblies.

To date, RADOM has been used extensively by undergraduate students of the Build-A-Genome (BAG) course taught at both Tianjin University in China and at The Johns Hopkins University in the USA to assemble synthetic minichunks. This application demonstrates robustness and high efficiency of RADOM, as BAG students typically have little to no experience in molecular biology. In the 2013 school year BAG students at the Johns Hopkins University were assigned assembly 125 minichunks corresponding to synthetic chromosome VIII (*synVIII*) into the pZX8 vector. Building blocks were amplified from subcloned, sequence verified constructs[24] and used without gel purification for assembly reactions in frozen yeast competent cells.[25] Here, in 122 out of 125 cases constructs encoding inserts of the correct size were identified by colony PCR. The success rate in screening white colonies for correctly sized inserts ranged from 2 to 100% depending on the minichunk, indicating that not all minichunks assembled with equal efficiency (Supporting Information Table S3). Two clones for each of the 122 minichunks that passed were initially selected for sequencing, yielding 102 sequence verified minichunks. Of the 20 that failed, in addition to sequence variations in homopolymeric runs and other repetitive sequences likely introduced by PCR, two common sequence errors were the absence of either a synthetic PCRTag sequence or a loxPsym recombination site, two features introduced to Sc2.0 synthetic chromosomes during design.[8,10] It is likely that patchwork homologous recombination events between the native chromosome and the synthetic minichunk are the source of these assembly errors. Additional clones that were kept in reserve following primary colony PCR screening are now being sequenced to identify correct clones for the remaining 20 minichunks.

RADOM is particularly amenable to projects like Sc2.0 that employ a hierarchical assembly strategy whereby increasingly large constructs are built from smaller ones. It is imperative that

design at each assembly level incorporates terminal homology into the ends of adjacent fragments. For instance, here we assembled segments of *synV* and *synVIII* as part of the Sc2.0 project. Minichunks were built from ~750 bp BBs encoding 40 bp overlaps with one another; subsequently chunks were assembled from minichunks encoding single BB overlaps. One major consideration is the requirement to incorporate vector homology into each terminal fragment for assembly at any stage of the workflow. We present two efficient solutions: PCR to introduce 40 bp vector sequence for minichunk and chunk assembly, as well as PCR generated linkers used exclusively here for chunk assembly. The application of linkers promotes recombination-dependent joining of unrelated DNA fragments. While linkers proved slightly less efficient in assembly (Figure 3b and Table 2), likely due to the increased number of fragments undergoing homologous recombination, they avoid the use of PCR and minimize the potential of PCR-induced mutations.

Both minichunk and chunk assembly using RADOM showed high efficiency in our hands. We also report here that using RADOM JHU undergraduates have built ~300 kb of synthetic DNA in the form of minichunks corresponding to *synVIII* as part of the Sc2.0 project. This highlights both the ease of manipulation and scalability of our method. We have found that assembly efficiency can vary, which likely depends on the sequence being assembled as well as the experimental proficiency of the undergraduate student. However, in almost all cases we have identified full-length clones, many sequence verified, despite the high sequence similarity between the native yeast genome and DNA being assembled. Technically, the workflow of RADOM can be streamlined by using frozen yeast competent cells,[25] as was done in the BAG class at JHU, although in our hands the efficiency of transformation using frozen yeast competent cells is roughly an order of magnitude lower than freshly made competent cells. The most difficult step in RADOM is the recovery and transfer of plasmids from yeast to *E. coli.* Among different methods tried, column purification prior to *E. coli* transformation improved our success at this step. Additionally, we recommend transforming into *E. coli* cells with a competency of 10^8 cfu/μg DNA or higher to yield a sufficient number of transformants for downstream screening.

RADOM has many other general applications. It has already been extensively used in minichunk construction for synthetic yeast chromosome V and chromosome VIII from the synthetic yeast genome project Sc2.0. The use of our method facilitates the simultaneously assembly of several genetic blocks. Meanwhile, we also used this method in our laboratory to construct metabolic pathways like violacein pathway of *E. coli and* beta carotene pathway of *S. cerevisiae* (Data not shown). The protocol has proved to be very robust and reliable. We anticipate that RADOM will be widely used in construction of synthetic biological systems in a rapid and effective manner.

■ METHODS AND MATERIALS

Strains and Media. *S. cerevisiae* strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was used as the chassis for all DNA assembly in this study. YPD medium containing yeast extract (10 g/L), peptone (20 g/L) and dextrose (20 g/L) was used to culture the BY4741 strain. Synthetic complete medium lacking histidine (SC−His) was used to select transformants. *Escherichia coli (E. coli)* strain DH5α was used for yeast plasmid recovery. LB media containing tryptone (10 g/L), yeast extract (5 g/L), NaCl (10 g/L), and Carbenicillin (100 μg/mL) was used to culture transformed *E. coli*. LB X-gal agar plates containing LB media Carbenicillin (100 μg/mL), 1.5% agar and 0.5% X-gal were used for blue-white screening.

Vector Construction and Assembly Preparation. The yeast/*E. coli* shuttle vector pRS413[26] was linearized using *Eco*RI. The pZX8 vector was constructed using multichange isothermal mutagenesis[27] by replacing the multiple cloning site of pRS413 with abutting *Fse*I-*Bsa*I-*Bsa*I-*Fse*I recognition sequences and removing *Bsa*I site in the beta lactamase (*bla*) gene (Supporting Information Figure S1). Note that the two *Bsa*I sites are inverted relative to each other in a "divergent" orientation and the outer *Fse*I sites are regenerated during the subcloning step. Specifically, pRS413 was PCR amplified through primer pairs that produce two PCR products with homologous ends. After gel purification, the two DNA fragments were assembled using a one-step isothermal reaction.[6] The resulting pZX8 plasmid (Supporting Information Figure S1) was linearized by *Bsa*I digestion to generate intentionally incompatible 4-nt 5′ overhangs on the two ends. *Fse*I restriction sites on pZX8 were used in subsequent steps to release assembled minichunks from the pZX8 vector. For plasmid linearization, a solution of 50 μL of each plasmid (2 μg) was digested with 2 units of *Fse*I for 4 h at 37 °C and inactivated for 10 min at 80 °C. After digestion, assembled inserts were gel purified using TIANquick Midi Purification Kit (TIANGEN Biotech, DP204) according to the manufacturer's instructions. *Fse*I was stored at −80 °C between experiments to better preserve its activity. The linearized pZX8 used in the Build-A-Genome course was purified using Zymoclean Gel DNA Recovery Kit (Zymo Research, D4002) and stored at −20 °C. Restriction enzymes were purchased from New England Biolabs.

Primer Design and DNA Fragments Preparation. The plasmids and primers used in this study are listed in Supporting Information Table 1. For primers used to amplify the leftmost and rightmost DNA fragments of each assembly, 40 nucleotides homologous to the ends of the *Eco*RI or *Bsa*I-linearized assembly backbone (pRS413 or pZX8) were introduced by PCR. The extension overlap sequences between pRS413 and DNA fragments were 5′-CCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTC-3′ and 5′-CCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTC-3′, whereas the overhang sequences for pZX8 were designed to be 5′- CCAGTGAGCGCGCGTAATACGACTCACTATAGGGCCGGCC-3′ and 5′- CAATTAACCCTCACTAAAGGGAACAAAAGCTGGGGCCGGCC-3′. The 8 base pair *Fse*I restriction site was chosen because it is absent from synthetic chromosome VIII (*synVIII*), the only focus of the 125 minichunk assemblies in Build-A-Genome course showed in this study, as well as the K4−2 minichunk and K4 chunk assembly. *Xho*I was chosen for A2−2 minichunk and A2 chunk assembly from synthetic chromosome V (*synV*) because it is a noncutter for A2−2 and A2, thus can be used to digest inserts from the backbones. Primers were designed to consist of an annealing sequence (20−30 nt, $T_m \approx 55$ °C), 40 nucleotides corresponding to extension overhang sequences with restriction sites to release insertions.

PCR reactions were prepared as follows: 5−10 ng template DNA, 0.2 mM of each dNTP (Takara, 4030), 0.2 μM of each primer, 1× Phusion HF buffer, 0.02 U/μL Phusion DNA polymerase in a final volume of 50 μL. PCR products were verified on a 1% agarose gel followed by gel purification using

the TIANgel Midi Purification (TIANGEN Biotech, DP209) or directly used for cotransformation into yeast.

Yeast Transformation. The detailed protocol for yeast transformation using the LiAc/SS carrier is described elsewhere.[28] Before transformation, 5 μL of each PCR product fragment used directly or alternatively 200 ng of each gel purified fragments were mixed together with 200 ng of linearized vector. The molar ratio of each fragment to vector in minichunk assembly is around 7:1 while in chunk assembly is around 2:1. Transformations with 200 ng of linearized vector alone were used as control to indicate quality of linearization. Following transformation, yeast colonies were selected on synthetic medium lacking histidine (SC−His) agar plates for 3 days at 30 °C.

Yeast Whole Plate Cell Population Collection and Plasmid Recovery. From each assembly transformation experiment the entire population of yeast colonies growing on the SC−His selection plate was collected into a single pool. To achieve this, 3 mL of sterile water was added to each transformation plate to collect all the colonies. The above mixture was then transformed into an Eppendorf tube with a final pellet volume around 100 μL to ensure successful plasmid extraction. After the collection step, plasmids were extracted from the mixture using the TIANprep yeast plasmid DNA kit (TIANGEN, DP112) according to manufacturer's instruction. Alternatively, in the Build-A-Genome course plasmids were recovered using standard alkaline lysis preparations followed by column purification. Briefly, cells were resuspended into 200 μL P1 buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0 with 100 μg/mL RNaseA), lysed by adding 200 μL of solution 2 (200 mM NaOH 1% SDS), and finally neutralized with 200 μL solution 3 (3 M KOAc pH 5.5). Following centrifugation at room temperature for 5 min at 14 000 rpm, the supernatant was applied to a Zymo spin column from the Zyppy Plasmid Miniprep Kit (Zymo research, D4037) and subsequently purified according to the manufacturer's instruction. 10 μL of the resulting yeast plasmid was used for *E. coli* transformation. To achieve sufficient numbers of transformants, the competency of *E. coli* cells should be higher than 10^7 cfu/μg DNA.

Blue/White Screening and PCR Analysis of *E. coli* Colonies. *E. coli* transformations were plated on LB-carb (100 μg/mL) with X-gal (0.5%), and white colonies were selected for further analysis by colony PCR using M13F/R primers. Generally, colony PCR was carried out in a final volume of 12 μL consisting of 0.5 pmol of each primer, 0.2 mM concentration of each dNTP (Takara, 4030) and 1.2 unit *TransFast* Taq DNA Polymerase (Transgene Biotech CO.LTD AP101−01). For chunk assembly, as the insets of ∼8−10 kb were too large for amplification in a single reaction, multiplex colony PCR (MCPCR) was performed. Several groups of MCPCR primers were designed to generate four amplicons with sizes of ∼200 to ∼500 bp for each chunk. Primers were designed with a T_m range from 59 to 62 °C. MCPCR was carried out in 20 μL of PCR reaction containing 2 pmol of each primer, 0.6 μL of DMSO, 0.2 mM of each dNTP (Takara, 4030) and 2 units of *TransFast* Taq DNA Polymerase (Transgene Biotech CO.LTD AP101−01). PCR cycles consisted of 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at experimentally a T_m of 62 °C, and 30 s at 72 °C, and a final extension for 5 min at 72 °C. Of each reaction, 10 μL PCR products were loaded on a 2% agarose gel, which produced the optimal separation.

DNA Sequences of the Synthesized Fragments. Assembled synthetic DNA fragments were sequence verified by Genewiz, Inc., using the oligonucleotides listed in Supporting Information Table 2.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1 and S2 and Tables S1−S3. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Author Contributions

Q.L. and B.J. are co-first authors of this work and designed the experiments; Q.L., B.J., J.L., and W.Z. performed the research; Q.L., L.A.M., and B.J. wrote the manuscript; Z.X. designed and constructed the pZX8 plasmid; J.L. and K.Y. summarized the BAG results; K.I.Z. and L.A.M. supervised undergraduate students in the BAG course; K.Y., G.S., J.S.B., and J.D.B designed BioStudioDB software to record experimental data from the BAG course; the BAG course was performed at Johns Hopkins University under J.D.B.'s supervision; the remaining work described here was performed in the lab of Y.Y.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

RADOM, rapid assembly of DNA overlapping multifragments; BAG, Build-A-Genome; BB, building block; PCR, polymerase chain reaction; bp, base pair; kb, kilobase pair; Mb, megabase pair; *synV*, synthetic chromosome V; *synVIII*, synthetic chromosome VIII; nt, nucleotide

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