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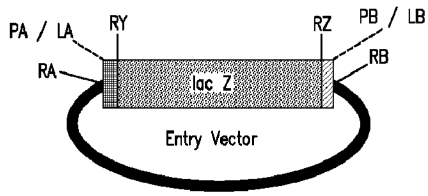
(54) **Titre : COMPOSITIONS ET PROCEDES POUR L'ASSEMBLAGE DE POLYNUCLEOTIDES**
(54) **Title: COMPOSITIONS AND METHODS FOR THE ASSEMBLY OF POLYNUCLEOTIDES**



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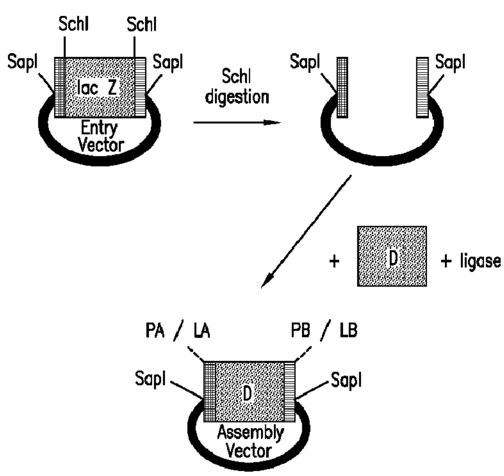

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(57) **Abrégé/Abstract:**
The present invention provides compositions and methods for rapid assembly of one or more assembled polynucleotides from a plurality of component polynucleotides. The methods of the invention utilize circular nucleic acid vectors that comprise a DNA

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(57) **Abrégé(suite)/Abstract(continued):**
segment D flanked by an annealable linker sequence, annealable linker sequence pairs LA and LB, or annealable linker sequence / primer binding segment pairs LA and PB or PA and LB. Restriction endonuclease digestion of a plurality of vectors containing the DNA segments to be assembled generates a plurality of DNA fragments comprising the elements PA-D-LB, LA-D-LB, and LA-D-PB or D-LB, LA-D-LB, and LA-D. The sequences of annealable linker sequences LA and LB provide complementary termini to the DNA fragments, which are utilized in host cell mediated homologous recombination or together with promer binding segments PA and PB in a polymerase cycling assembly reaction for the ordered assembly of the various DNA segments into one or more assembled polynucleotides.

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(54) **Title**: COMPOSITIONS AND METHODS FOR THE ASSEMBLY OF POLYNUCLEOTIDES

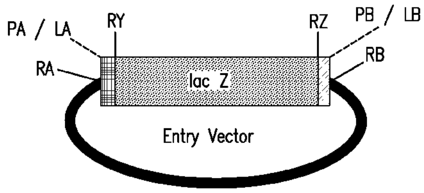

FIG.1A

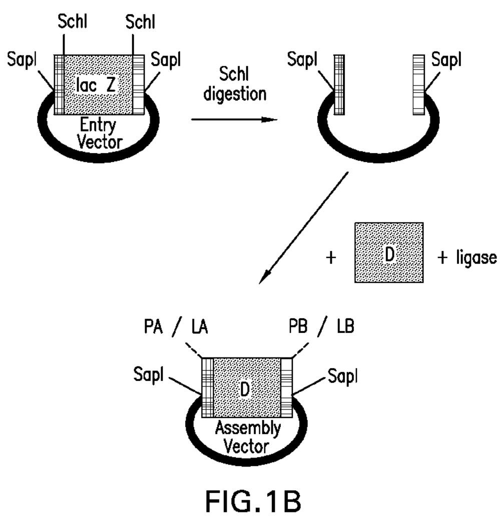

FIG.1B

(57) **Abstract**: The present invention provides compositions and methods for rapid assembly of one or more assembled polynucleotides from a plurality of component polynucleotides. The methods of the invention utilize circular nucleic acid vectors that comprise a DNA segment D flanked by an annealable linker sequence, annealable linker sequence pairs LA and LB, or annealable linker sequence / primer binding segment pairs LA and PB or PA and LB. Restriction endonuclease digestion of a plurality of vectors containing the DNA segments to be assembled generates a plurality of DNA fragments comprising the elements PA-D-LB, LA-D-LB, and LA-D-PB or D-LB, LA-D-LB, and LA-D. The sequences of annealable linker sequences LA and LB provide complementary termini to the DNA fragments, which are utilized in host cell mediated homologous recombination or together with promer binding segments PA and PB in a polymerase cycling assembly reaction for the ordered assembly of the various DNA segments into one or more assembled polynucleotides.

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COMPOSITIONS AND METHODS FOR THE ASSEMBLY OF POLYNUCLEOTIDES

[0001] This application claims benefit of priority of U.S. Provisional Application No. 61/116,109, filed on November 19, 2008, and U.S. Provisional Application No. 61/162,230, filed on March 23, 2009.

1. FIELD OF THE INVENTION

[0002] The present invention relates generally to the field of recombinant DNA technology and, more particularly, to improved methods for the ordered assembly of a plurality of DNA segments into an assembled polynucleotide.

2. BACKGROUND OF THE INVENTION

[0003] Recombination of polynucleotides can be carried out using many methods known in the art. Traditional techniques for recombining nucleic acids have utilized restriction enzymes and ligating enzymes for the creation of novel nucleic acid molecules. Recombinant molecules such as cloning and expression vectors can be utilized to integrate a nucleic acid sequence of interest into the genome of a host cell, and/or drive the expression of one or more genes of interest. Utilization of a vector to drive expression of a gene of interest in the cell, for example a yeast cell, requires that the vector contain requisite genetic elements that enable replication and expression of the gene of interest. These elements may include, for example, the gene or genes of interest, a promoter sequence, a terminator sequence, selectable markers, integration loci, and the like.

[0004] Assembly of elements into a single vector using traditional restriction and ligation enzyme-based methods can be time-consuming and laborious. Each sub-cloning step, *i.e.*, the introduction of a new nucleic acid fragment into an existing polynucleotide, can require that the resulting clone be screened and characterized before the introduction of additional fragments. Clones produced by blunt end ligation require confirmation that the fragment was introduced in the proper orientation. On the other hand, sticky-end ligation requires that the restriction sites utilized to produce the sticky ends on the acceptor fragment also be present in the donor fragment, but not at a site that would interrupt the sequence of interest within the donor fragment. Thus, the selection of workable restriction sites depends entirely on the compositions of the pieces being joined and must be carefully considered in each case. In addition, these methods often introduce extraneous nucleic acid sequences to the resulting clone that can interfere with the structure and function of the desired gene products. Further limiting the efficiency of restriction-enzyme based cloning methods is the intrinsic limitation on the number of nucleic acid molecules that can be ligated together in a single reaction.

[0005] The polymerase chain reaction (PCR) is a powerful technique by which specific polynucleotide sequences, including genomic DNA, cDNA and mRNA, are amplified *in vitro*. PCR typically comprises contacting separate complementary strands of a target nucleic acid with two oligonucleotide primers under conditions that allow for the formation of complementary primer extension products on both strands. These strands act as templates for the synthesis of copies of the desired nucleic acid sequences. By repeating the separation and synthesis steps in an automated system, exponential duplication of the target sequences can be achieved.

[0006] One method of PCR, termed "splicing by overlap extension" ("SOE"; *see, e.g.,* U.S. Patent No. 5,023,171), facilitates the assembly of DNA molecules at precise junctions without the use of restriction enzymes or ligase. Component fragments to be recombined are generated in separate polymerase chain reactions using uniquely designed primers which produce amplicons having complementary termini to one another. Upon mixing and denaturation of these amplicons, strands having complementary sequences at their 3' ends overlap and act as primers for each other. Extension of this overlap by DNA polymerase produces a nucleic acid molecule in which the original sequences are "spliced" together. Subsequent rounds of PCR amplify the resulting spliced polynucleotide.

[0007] SOE, while more efficient than traditional ligation enzyme-based methods for combining a plurality of nucleic acid fragments, does require time to optimize primer sequences and amplification conditions to produce desired products. Each junction between the fragments to be spliced together must be individually considered, and a pair of primers must be designed for each fragment in order to make the ends compatible. Traditional considerations for the design of PCR primers, *e.g.,* melting temperature, G-C content, avoidance of hairpin and dimer formation, and stringency for false priming sites, must be considered even more carefully as the number of fragments to be spliced in the SOE reaction increases.

[0008] Thus, despite advances in recombinant DNA technology, there exists a need for improved methods that provide for the rapid and ordered assembly of polynucleotides. Particularly needed are methods which can facilitate the assembly of a number of polynucleotides with minimal manipulation and characterization of intermediate products, and without the need for primer optimization steps. These and other needs can be met by compositions and methods of the present invention.

3. SUMMARY OF THE INVENTION

[0009] The compositions and methods provided herein allow for rapid and ordered assembly, or "stitching," of component polynucleotides into assembled polynucleotides. In some embodiments, the methods provided herein utilize circular nucleic acid assembly vectors. In certain embodiments, an assembly vector comprises a component polynucleotide wherein the component polynucleotide comprises a DNA segment flanked by: (i) an annealable linker on the 3' end; (ii) a primer binding segment on the 5' end and an annealable linker on the 3' end; (iii) an annealable linker on both the 3' end and on the 5' end; (vi) an annealable linker on the 5' end and primer binding segment on the 3' end; or (v) an annealable linker on the 5'end.

[0010] In some embodiments, a plurality of component polynucleotides can be stitched together by providing a plurality of assembly vectors in a single reaction vessel. In certain embodiments, component polynucleotides can be excised from their assembly vectors within the reaction vessel. In some embodiments, the component polynucleotides can then be denatured, annealable linker sequences can be annealed to complementary strands on an adjacent component polynucleotide, and the component polynucleotides can be stitched together into an assembled polynucleotide by splicing by overlap extension (SOE) followed by PCR. In other embodiments, component polynucleotides excised from assembly vectors can be assembled into an assembled polynucleotide *in vivo* by homologous recombination within a host cell transformed with the component polynucleotides. Assembled polynucleotides can be further combined *in vivo* by host cell mediated homologous recombination.

[0011] The efficiency of polynucleotide assembly can be enhanced by the provision of a standard set of annealable linker sequences that are used within the assembly vector, for example, those described herein as SEQ ID NOS: 1 to 23. The annealable linker sequences provide sequence overlap between adjacent component polynucleotides in the assembly reaction. Ideally, the annealable linker sequences lack appreciable secondary structure both at the RNA and at the DNA level, do not cross react in an undesirable manner with one another, and have relatively high melting temperatures (T_m). Consequently, a number of component polynucleotides can be stitched together without the need for designing unique primers for each component polynucleotide, thereby saving time and labor. Compositions and methods provided herein can be used to assemble many types of polynucleotides, including synthetic genes, constructs, cloning vectors, expression vectors, chromosomes, genomes, peptide libraries, and the like.

[0012] In one aspect, provided herein is a vector, *i.e.*, an assembly vector, that can be used in the assembly of one or more assembled polynucleotides from a plurality of component polynucleotides.

[0013] In some embodiments, the assembly vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a DNA segment D, an annealable linker sequence LB, and a restriction site RB (*i.e.*, 5'-RA-LA-D-LB-RB-3'). In some embodiments, the assembly vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a DNA segment D, an annealable linker sequence LB, and a restriction site RB (*i.e.*, 5'-RA-D-LB-RB-3'). In some embodiments, the assembly vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a DNA segment D, and a restriction site RB (*i.e.*, 5'- RA-LA-D-RB-3'). In some embodiments, the assembly vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a primer binding segment PA, a DNA segment D, an annealable linker sequence LB, and a restriction site RB (*i.e.*, 5'-RA-PA-D-LB-RB-3'). In some embodiments, the assembly vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a DNA segment D, a primer binding segment PB, and a restriction site RB (*i.e.*, 5'- RA-LA-D-PB-RB-3'). In some embodiments, the assembly vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a DNA segment D, an annealable linker sequence LB, and a restriction site RB (*i.e.*, 5'-RA-LA-D-LB-RB-3'). Exemplary assembly vectors are provided in FIG. 1B and FIG. 2.

[0014] In some embodiments, a primer binding segment (*i.e.*, PA or PB) can be any nucleotide sequence that is not complementary with any of the annealable linker sequences that are used to make an assembled polynucleotide. In some embodiments, a primer binding segment includes a restriction endonuclease recognition site and/or cleavage site. In some embodiments, a primer binding segment comprises a nucleic acid sequence of one of the available linker sequences (*e.g.*, one of SEQ ID NOS: 1 to 23), or complements thereof, not being used in the particular assembly reaction. In some embodiments, the nucleic acid sequence of primer binding segment PA is selected from the group consisting of SEQ ID NOS: 24, 25, and complements thereof. In some embodiments, the nucleic acid sequence of primer binding segment PB is selected from the group consisting of SEQ ID NOS: 24, 25, and complements thereof. In preferable embodiments, primer binding segment PA and primer binding segment PB are not identical in sequence.

[0015] In some embodiments, the two or more annealable linker sequences are at least 24 nucleotides in length and have a T_m of at least 60 °C.

[0016] In some embodiments, two or more annealable linker sequences have a G-C content of at least 70% and a T_m of at least 70 °C, and do not form appreciable secondary DNA structures. In some embodiments, the nucleic sequence of annealable linker sequence LA is selected from the group consisting of SEQ ID NOS: 1 to 8, and complements thereof. In some embodiments, the nucleic sequence of annealable linker sequence LB is selected from the group consisting of SEQ ID NOS: 1 to 8, and complements thereof. In some embodiments, the nucleic sequences of annealable linker sequence LA and annealable linker sequence LB are selected from the group consisting of SEQ ID NOS: 1 to 8, and complements thereof.

[0017] In some embodiments, two or more annealable linker sequences have an A-T content of at least 30% and a T_m of at least 65 °C, and do not form appreciable secondary DNA or RNA structures. In some embodiments, two or more annealable linker sequences have a low G-C content and a T_m of at least 65 °C, and comprise the sequence motif 5'-ANNNNNNNNANNNAANTANNTTNANA-3', wherein A stands for adenine, N for any nucleotide, and T for thymine. In some embodiments, the nucleic sequence of annealable linker sequence LA is selected from the group consisting of SEQ ID NOS: 9 to 23, and complements thereof. In some embodiments, the nucleic sequence of annealable linker sequence LB is selected from the group consisting of SEQ ID NOS: 9 to 23, and complements thereof. In some embodiments, the nucleic sequences of annealable linker sequence LA and annealable linker sequence LB are selected from the group consisting of SEQ ID NOS: 9 to 23, and complements thereof.

[0018] The ordered assembly of the plurality of component polynucleotides can be controlled by the selection of annealable linker sequences that flank a DNA segment within the assembly vector. Accordingly, in some embodiments, to ensure that component polynucleotides can be assembled in an ordered fashion, the sequences of an annealable linker sequence/annealable linker sequence pair within a particular assembly vector are not complementary. Similarly, in some embodiments, the sequences of a primer binding segment/annealable linker sequence pair within a particular assembly vector are not complementary.

[0019] In a particular embodiment, restriction sites RA and RB are cleavable by the same restriction endonuclease so as to facilitate the excision of the component polynucleotide from the assembly vector. In some embodiments, restriction site RA or RB is cleavable by a restriction endonuclease that leaves a 5' or 3' overhang. In other embodiments, restriction site RA or RB is cleavable by a restriction endonuclease that leaves a blunt end. In some embodiments, restriction sites RA and RB are cleavable by the same restriction endonuclease. In still other embodiments, the restriction sites RA and RB are cleavable by a Type IIS restriction endonuclease. In some embodiments, the restriction sites RA and RB are cleavable by the same Type IIS restriction endonuclease. In a particular embodiment, restriction sites RA and RB are cleavable by SapI or LguI restriction endonucleases.

[0020] In another aspect, the invention provides an entry vector useful in the preparation of an assembly vector comprising a DNA segment.

[0021] In some embodiments, the entry vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a restriction site RY, a DNA segment D, a restriction site RZ, an annealable linker sequence LB, and a restriction site RB (*i.e.*, 5'-RA-RY-D-RZ-LB-RB-3'). In some embodiments, the entry vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a restriction site RY, a DNA segment D, a restriction site RZ, and a restriction site RB (*i.e.*, 5'-RA-LA-RY-D-RZ-RB-3'). In some embodiments, the entry vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a restriction site RY, a DNA segment D, a restriction site RZ, an annealable linker sequence LB, and a restriction site RB (*i.e.*, 5'-RA-LA-RY-D-RZ-LB-RB-3'). In some embodiments, the entry vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a primer binding segment PA, a restriction site RY, a DNA segment D, a restriction site RZ, an annealable linker sequence LB, and a restriction site RB (*i.e.*, 5'-RA-PA-RY-D-RZ-LB-RB-3'). In some embodiments, the entry vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a restriction site RY, a DNA segment D, a restriction site RZ, a primer binding segment PB, and a restriction site RB (*i.e.*, 5'-RA-LA-RY-D-RZ-PB-RB-3'). An exemplary entry vector is provided in FIG. 1A.

[0022] Digestion of an entry vector with one or more restriction endonucleases capable of cleaving RY and RZ can create a linearized vector capable of acceptance of a DNA segment. The DNA segment can be ligated into RY and RZ sites using standard cloning techniques to generate an assembly vector of the invention. In some embodiments, restriction sites RY and RZ of the entry vector are cleavable by the same restriction endonuclease. In some embodiments, restriction sites RY and RZ of the entry vector are cleavable by a Type IIS restriction endonuclease. In some embodiments, restriction sites RY and RZ of the entry vector are cleavable by the same Type IIS restriction endonuclease. In particular embodiments, the Type IIS restriction endonuclease is SchI or MlyI.

[0023] In some embodiments, restriction sites RA and RB of the entry vector are cleavable by the same restriction endonuclease. In some embodiments, restriction sites RA and RB of the entry vector are cleavable by a Type IIS restriction endonuclease. In some embodiments, restriction sites RA and RB of the entry vector are cleavable by the same Type IIS restriction endonuclease. In particular embodiments, the Type IIS restriction endonuclease is SapI or LguI.

[0024] In another aspect, the invention provides an assembly composition comprising a plurality of assembly vectors for use in the assembly of one or more assembled polynucleotides from a plurality of component polynucleotides. In some embodiments, the assembly composition comprises:

(a) one or more first nucleic acid molecules, wherein each first nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_0, any DNA segment selected from the group D_0, an annealable linker sequence LB_0, and a second restriction site RB_0;

(b) one or more intermediate nucleic acid molecules wherein each intermediate nucleic acid molecule n is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_n, a first annealable linker sequence LA_n, any DNA segment selected from the group D_n, a second annealable linker sequence LB_n, and a second restriction site RB_n, and wherein n represents an integer from one to the number of intermediate nucleic acid molecules; and

(c) one or more last nucleic acid molecules, wherein each last nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_m, an annealable linker sequence LA_m, any DNA segment selected from the group D_m, a second restriction site RB_m wherein m represents an integer one greater than the number of intermediate nucleic acid molecules;

whereupon cleavage of restriction sites RA_0 through RB_m and denaturation of the resulting linear nucleic acid molecules, each annealable linker sequence $LB_{(p-1)}$ is capable of hybridizing to the complement of annealable linker sequence LA_p, wherein n is an integer that varies from 1 to (m-1), wherein p represents an integer from 1 to m, and wherein each group D_0,... D_n,...and D_m consists of one or more DNA segments.

[0025] In certain embodiments, one or more first nucleic acid molecules further comprises a primer binding segment PA positioned 5' to the DNA segment selected from the group D_0. In certain embodiments, one or more last nucleic acid molecules further comprises a primer binding segment PB positioned 3' to the DNA segment selected from the group D_m.

[0026] In certain embodiments, the assembly composition comprises two or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises three or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises four or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises five or more intermediate nucleic acid molecules. In certain assembly embodiments, the composition comprises six or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises seven or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises eight or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises nine or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises ten or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises fifteen or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises twenty or more intermediate nucleic acid molecules.

[0027] In certain embodiments, m is equal to 1. In certain embodiments, m is equal to 2. In certain embodiments, m is equal to 3. In certain embodiments, m is equal to 4. In certain embodiments, m is equal to 5. In certain embodiments, m is equal to 6. In certain embodiments, m is equal to 7. In certain embodiments, m is equal to 8. In certain embodiments, m is equal to 9. In certain embodiments, m is equal to 10. In certain embodiments, m is equal to or greater than 10.

[0028] In some embodiments, upon cleavage of restriction sites RA_0 through RB_m and denaturation of the resulting linear nucleic acid molecules, each annealable linker sequence $LB_{(p-1)}$ is capable of selectively hybridizing to the complement of annealable linker sequence LA_p compared to the other annealable linker sequneces, or their complements, in the assembly composition. In some embodiments, each annealable linker sequence $L_{(p-1)}$ is identical in sequence to annealable linker sequence LA_p.

[0029] In a particular embodiment, the restriction sites RA_0 through RB_m are cleavable by the same restriction endonuclease so as to facilitate excision of the component polynucleotides from the assembly vectors. In some embodiments, the restrictions sites RA_0 through RB_m are cleavable by SapI and/or LguI restriction endonucleases.

[0030] In another aspect, the invention provides a components composition comprising a plurality of linear nucleic acid molecules wherein the linear nucleic acid molecules can be formed by digesting an assembly composition with one or more restriction endonucleases capable of cleaving restriction sites RA_0 through RB_m wherein the assembly composition comprises:

(a) one or more first nucleic acid molecules, wherein each first nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_0, any DNA segment selected from the group D_0, an annealable linker sequence LB_0, and a second restriction site RB_0;

(b) one or more intermediate nucleic acid molecules wherein each intermediate nucleic acid molecule n is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_n, a first annealable linker sequence LA_n, any DNA segment selected from the group D_n, a second annealable linker sequence LB_n, and a second restriction site RB_n, and wherein n represents an integer from one to the number of intermediate nucleic acid molecules; and

(c) one or more last nucleic acid molecules, wherein each last nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_m, an annealable linker sequence LA_m, any DNA segment selected from the group D_m, a second restriction site RB_m wherein m represents an integer one greater than the number of intermediate nucleic acid molecules;

whereupon cleavage of restriction sites RA_0 through RB_m and denaturation of the resulting linear nucleic acid molecules, each annealable linker sequence $LB_{(p-1)}$ is capable of hybridizing to the complement of annealable linker sequence LA_p, wherein n is an integer that varies from 1 to (m-1), wherein p represents an integer from 1 to m, and wherein each group D_0,... D_n,...and D_m consists of one or more DNA segments.

[0031] In certain embodiments, one or more first nucleic acid molecules further comprises a primer binding segment PA positioned 5' to the DNA segment selected from the group D_0. In certain embodiments, one or more last nucleic acid molecules further comprises a primer binding segment PB positioned 3' to the DNA segment selected from the group D_m.

[0032] In certain embodiments, the components composition comprises two or more intermediate nucleic acid molecules. In certain embodiments, the components components composition comprises three or more intermediate nucleic acid molecules. In certain embodiments, the components composition comprises four or more intermediate nucleic acid molecules. In certain embodiments, the components composition comprises five or more intermediate nucleic acid molecules. In certain embodiments, the components composition comprises six or more intermediate nucleic acid molecules. In certain embodiments, the components composition comprises seven or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises eight or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises nine or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises ten or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises fifteen or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises twenty or more intermediate nucleic acid molecules.

[0033] In certain embodiments, m is equal to 1. In certain embodiments, m is equal to 2. In certain embodiments, m is equal to 3. In certain embodiments, m is equal to 4. In certain embodiments, m is equal to 5. In certain embodiments, m is equal to 6. In certain embodiments, m is equal to 7. In certain embodiments, m is equal to 8. In certain embodiments, m is equal to 9. In certain embodiments, m is equal to 10. In certain embodiments, m is equal to or greater than 10.

[0034] In another aspect, provided herein is a kit useful for assembling a plurality of polynucleotides in accordance with the methods provided herein. In some embodiments, the kit comprises: (a) one or more entry vectors described herein; (b) one or more restriction endonucleases capable of cleaving restriction sites RA and RB of the entry vectors; and (c) one or more restriction endonucleases capable of cleaving restriction sites RY and RZ of the entry vectors.

[0035] In another aspect, the invention provides a library of nucleic acid molecules. In some embodiments, a nucleic acid molecule of the library comprises a first restriction site RA, a DNA segment D, an annealable linker sequence LB, and a second restriction site RB. In some embodiments, a nucleic acid molecule of the library comprises a first restriction site RA, a primer binding segment PA, a DNA segment D, an annealable linker sequence LB, and a second restriction site RB. In some embodiments, a nucleic acid molecule of the library comprises a first restriction site RA, an annealable linker sequence LA, a DNA segment D, an annealable linker sequence LB, and a second restriction site RB. In some embodiments, a nucleic acid molecule of the library comprises a first restriction site RA, an annealable linker sequence LA, a DNA segment D, and a second restriction site RB. In some embodiments, a nucleic acid molecule of the library comprises a first restriction site RA, an annealable linker sequence LA, a DNA segment D, a primer binding segment PB, and a second restriction site RB.

[0036] In some embodiments, the library comprises at least one of each of the following vectors:

(a) a vector that consists of a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a DNA segment D, an annealable linker sequence LB, and a restriction site RB;

(b) a vector that consists of a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a DNA segment D, an annealable linker sequence LB, and a restriction site RB; and

(c) a vector that consists of a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a DNA segment D, and a restriction site RB_0.

[0037] In some embodiments, the library comprises at least one of each of the following vectors:

(a) a vector that consists of a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a primer binding segment PA, a DNA segment D, an annealable linker sequence LB, and a restriction site RB;

(b) a vector that consists of a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a DNA segment D, an annealable linker sequence LB, and a restriction site RB; and

(c) a vector that consists of a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a DNA segment D, a primer binding segment PB, and a restriction site RB_0.

[0038] In some embodiments, the DNA segment D comprises a nucleic sequence selected from the group consisting of a selectable marker, a promoter, genomic targeting sequence, a nucleic acid sequence encoding an epitope tag, and a nucleic acid sequence encoding a gene of interest, a nucleic acid sequence encoding a termination codon and lacZ.

[0039] In some embodiments, the library comprises at least one of each of the following nucleic acid molecules:

(a) a first nucleic acid molecule wherein the first nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_0, a DNA segment D_0, an annealable linker sequence LB_0, and a second restriction site RB_0;

(b) an intermediate nucleic acid molecule wherein the intermediate nucleic acid molecule n is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_n, a first annealable linker sequence LA_n, a DNA segment D_n, a second annealable linker sequence LB_n, and a second restriction site RB_n, and wherein n represents an integer from one to the number of intermediate nucleic acid molecules; and

(c) a last nucleic acid molecule wherein the last nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_m, an annealable linker sequence LA_m, a DNA segment D_m, a second restriction site RB_m wherein m represents an integer one greater than the number of intermediate nucleic acid molecules;

whereupon cleavage of restriction sites RA_0 through RB_m and denaturation of the resulting linear nucleic acid molecules, each annealable linker sequence $LB_{(p-1)}$ is capable of hybridizing to the complement of annealable linker sequence LA_p wherein p represents the integers from 1 to m. In some embodiments, a first nucleic acid molecule further comprises a primer binding segment PA positioned 5' to the DNA segment selected from the group D_0. In some embodiments, a last nucleic acid molecules further comprises a primer binding segment PB positioned 3' to the DNA segment selected from the group D_m.

[0040] In certain embodiments, the library comprises two or more intermediate nucleic acid molecules. In certain embodiments, the library comprises three or more intermediate nucleic acid molecules. In certain embodiments, the library comprises four or more intermediate nucleic acid molecules. In certain embodiments, the library comprises five or more intermediate nucleic acid molecules. In certain embodiments, the library comprises six or more intermediate nucleic acid molecules. In certain embodiments, the library comprises seven or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises eight or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises nine or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises ten or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises fifteen or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises twenty or more intermediate nucleic acid molecules.

[0041] In certain embodiments, m is equal to 1. In certain embodiments, m is equal to 2. In certain embodiments, m is equal to 3. In certain embodiments, m is equal to 4. In certain embodiments, m is equal to 5. In certain embodiments, m is equal to 6. In certain embodiments, m is equal to 7. In certain embodiments, m is equal to 8. In certain embodiments, m is equal to 9. In certain embodiments, m is equal to 10. In certain embodiments, m is equal to or greater than 10.

[0042] In another aspect, provided herein are methods of assembling one or more assembled polynucleotides from a plurality of component polynucleotides, comprising the steps of:

(a) digesting an assembly composition with one or more restriction endonucleases to generate a components composition, the assembly composition comprising:

(i) one or more first nucleic acid molecules, wherein each first nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_0, any primer binding segment selected from the group PA, any DNA segment selected from the group D_0, an annealable linker sequence LB_0, and a second restriction site RB_0;

(ii) one or more intermediate nucleic acid molecules wherein each intermediate nucleic acid molecule n is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_n, a first annealable linker sequence LA_n, any DNA segment seleted from the group D_n, a second annealable linker sequence LB_n, and a second restriction site RB_n, and wherein n represents an integer from one to the number of intermediate nucleic acid molecules; and

(iii) one or more last nucleic acid molecules, wherein each last nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_m, an annealable linker sequence LA_m, a DNA segment selected from the group D_m, any primer binding segment selected from the group PB, a second restriction site RB_m wherein m represents an integer one greater than the number of intermediate nucleic acid molecules; whereupon cleavage of restriction sites RA_0 through RB_m and denaturation of the resulting linear nucleic acid molecules, each annealable linker sequence $LB_{(p-1)}$ is capable of hybridizing to the complement of annealable linker sequence LA_p, wherein n is an integer that varies from 1 to (m-1), wherein p represents an integer from 1 to m, and wherein each group D_0,... D_n,...and D_m consists of one or more DNA segments;

wherein the one or more restriction endonucleases are capable of cleaving the restriction sites RA_0 through RB_m; and

(b) contacting the components composition with DNA polymerase, deoxyribonucleoside triphosphates and one or more first primers and one or more second primers, under conditions suitable for denaturation of the nucleic acid molecules, annealing of annealable linker sequence $LB_{(p-1)}$ to annealable linker sequence LA_p, and extension therefrom; wherein each said first primer is capable of hybridizing to one of said primer binding segments selected from the group PA and each said second primer is capable of hybridizing to one of said primer binding segments selected from the group PB; and subjecting the components composition to polymerase chain reaction,

wherein a polynucleotide is assembled which comprises, in a 5' to 3' orientation, one DNA segment selected from each of the groups D_0,... D_n,...and D_m. In the method, p represents the integers from 1 to m.

[0043] In certain embodiments, the assembly composition comprises two or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises three or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises four or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises five or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises six or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises seven or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises eight or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises nine or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises ten or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises fifteen or more intermediate nucleic acid molecules. In certain embodiments, the assembly composition comprises twenty or more intermediate nucleic acid molecules.

[0044] In certain embodiments, m is equal to 1. In certain embodiments, m is equal to 2. In certain embodiments, m is equal to 3. In certain embodiments, m is equal to 4. In certain embodiments, m is equal to 5. In certain embodiments, m is equal to 6. In certain embodiments, m is equal to 7. In certain embodiments, m is equal to 8. In certain embodiments, m is equal to 9. In certain embodiments, m is equal to 10. In certain embodiments, m is equal to or greater than 10.

[0045] In some embodiments, the assembly composition comprises one first nucleic acid molecule and one last nucleic acid molecule. In other embodiments, the assembly composition comprises more than one first nucleic acid molecule and more than one last nucleic acid molecule, and the assembly methods provide for the ordered assembly of multiple component polynucleotides into a plurality of assembled polynucleotides in a combinatorial fashion. In certain embodiments, the assembly composition comprises comprises at least two nucleic acid molecules that comprise the same annealable linker sequence LA or LB, or the same primer binding segment PA or PB, or the same pair of annealable linker sequences LA and LB, or the same pair of annealable linker sequence / primer binding segment LA and PB, or LB and PA.

[0046] In another aspect, provided herein are methods for generating host cells comprising assembled polynucleotides. In some embodiments, the methods comprise transforming a host cell with an assembled polynucleotide generated by the methods of polynucleotide assembly described herein. In other embodiments, the methods comprise transforming a host cell with a plurality of assembled polynucleotides generated by the methods of polynucleotide assembly described herein. In a particular embodiment, the host cell combines two or more assembled polynucleotides into one or more combined polynucleotide by homologous recombination. In yet other embodiments, the methods comprise transforming a host cell with a plurality of component polynucleotides and allowing the host cell to generate one or more assembled or combined polynucleotides by homologous recombination.

[0047] In another aspect, the present invention provides methods for generating a plurality of host cells comprising a plurality of assembled polynucleotides. In some embodiments, the plurality of host cells are generated by transforming host cells with a composition comprising a plurality of assembled polynucleotides generated by combinatorial assembly of component polynucleotides. In other embodiments, the plurality of host cells are generated by transforming host cells with a composition comprising a plurality of assembled polynucleotides of which at least two assembled polynucleotides comprise non-functional segments of a selectable marker that upon host cell mediated homologous recombination generate a functional selectable marker, and by selecting host cells comprising a combined polynucleotide. In yet other embodiments, the plurality of host cells are generated by combinatorial methods by transforming host cells with a component composition comprising multiple component polynucleotides of which at least two component polynucleotides comprise the same annealable linker sequence LA or LB or the same pair of annealable linker sequences LA and LB, and by selecting host cells comprising an assembled polynucleotide.

[0048] In another aspect, provided herein is a polynucleotide having a sequence selected from the group consisting of SEQ ID NOS: 1 to 25.

[0049] In another aspect, provided herein is a polynucleotide comprising one or more sequences selected from the group consisting of SEQ ID NOS: 1to 25.

4. BRIEF DESCRIPTION OF THE FIGURES

[0050] FIG. 1A provides a schematic of an entry vector useful for the preparation of an assembly vector of the invention. The vector contains a restriction site RA_0, a primer binding segment PA or an annealable linker sequence LA, a restriction site RY, a DNA segment D, a restriction site RZ, a primer binding segment PB or an annealable linker sequence LB, and a restriction site RB.

[0051] FIG. 1B provides an exemplary method of preparing an entry vector for acceptance of a DNA segment to form an assembly vector. In the exemplary, RY= RZ=SchI. Digestion with SchI, a Type IIS restriction endonuclease that is capable of producing blunt ends allows for isolation of the vector with the linker sites open to be fused to the DNA segment (D). Blunt-end ligation of D into the entry vector can be performed by traditional methods using, *e.g.*, T4 DNA ligase.

[0052] FIG. 2 presents a schematic of an assembly composition comprising a plurality of assembly vectors (first, intermediate, and last), each comprising a DNA segment of interest (D_0, D_n, D_m). The first nucleic acid molecule comprises a first restriction site RA_0, a primer binding segment PA, a DNA segment D_0, an annealable linker sequence LB_0, and a second restriction site RB_0. The one or more intermediate nucleic acid molecules comprise a first restriction site RA_n, a first annealable linker sequence LA_n, a DNA segment D_n, a second annealable linker sequence LB_n, and a second restriction site RB_n wherein n represents an integer from one to the number of intermediate nucleic acid molecules; and the last nucleic acid molecule comprises a first restriction site RA_m, an annealable linker sequence LA_m, a DNA segment D_m, a primer binding segment PB, a second restriction site RB_m wherein m represents an integer one greater than the number of intermediate nucleic acid molecules.

[0053] FIG. 3 presents an exemplary method of assembling, *i.e.*, "stitching" a assembled polynucleotide from four (4) component polynucleotides. Assembly vectors comprising DNA segments to be assembled are pooled in a single tube and digested with SapI to release component polynucleotide fragments from the assembly vector backbones. Following heat inactivation of SapI, the component polynucleotide fragments are subjected to denaturing conditions, followed by annealing conditions sufficient for hybridization of the complementary annealable linker pairs. Following primer extension in the presence of DNA polymerase and dNTPs, primers complementary to PA and PB are added, followed by traditional PCR amplification. An assembled polynucleotide comprising component polynucleotides D_0, D_1, D_2, and D_3 assembled in a 5' to 3' direction is produced as a result of the assembling reaction.

[0054] FIG. 4 shows a map of the pRYSE vector.

[0055] FIG. 5 shows assembled polynucleotides obtained by assembling 2 to 4 component polynucleotides (Assemblies 1 through 6 in Table 7) using different methods for removing the SapI restriction endonuclease (column purification or heat inactivation), different assembly vector DNA concentrations (5 ng (low DNA concentration) or 50 ng (high DNA concentration) of smallest fragment with equal molar concentration of all other fragments, and different annealing temperatures for PCR amplification (54 °C and 72 °C).

[0056] FIG. 6 shows assembled polynucleotides obtained by assembling 6 or 9 component polynucleotides (Assemblies 7, and 13 through 16 in Table 7) using different DNA polymerases (Phusion (New England Biolabs, Ipswich, MA) and PfuUltraII (Stratagene/Agilent, La Jolla, CA)).

[0057] FIG. 7 shows a map of the pMULE vector. The pMULE entry vector differs from the pRYSE entry vector in that it lacks a primer binding segments or annealable linker sequences.

[0058] FIG. 8 present an exemplary method of combining assembled polynucleotides into a combined polynucleotide by host cell mediated homologous recombination, and integrating the combined polynucleotide into a chromosome of the host cell. Assembled polynucleotide A comprises a DNA segment D_{m1} encoding a first non-functional segment of a selectable marker and a DNA segment D_{01} encoding an upstream genomic targeting sequence. Assembled polynucleotide B comprises a DNA segment D_{m2} encoding a second non-functional segment of the selectable marker and a DNA segment D_{02} encoding a downstream genomic targeting sequence. The host cell recombines assembled polynucleotide A and assembled polynucleotide B at the region of homology in DNA segments D_{m1} and D_{m2} to form a combined polynucleotide comprising a functional selectable marker, and uses the genomic targeting sequences encoded by DNA segments D_{01} and D_{02} to insert the combined polynucleotide by homologous recombination into its chromosome.

[0059] FIG. 9 presents an exemplary method of generating an assembled polynucleotide by homologous recombination in a host cell and integration of the assembled polynucleotide into the chromosome of the host cell. In the first step, an assembly composition comprising assembly vectors is digested with a restriction endonuclease, resulting in the excision of component polynucleotides from the assembly vector backbones. In the second step, the component polynucleotides are introduced into a host cell where they are recombined at the regions of homology in the annealable linker sequences to form an assembled polynucleotide, and the assembled polynucleotide is integrated into the chromosome of the host cell.

[0060] FIG. 10 presents an exemplary method of assembling a plurality of assembled polynucleotide from seven (7) component polynucleotides in the same reaction. Assembly vectors comprising DNA segments to be assembled are pooled in a single tube and digested with SapI to release component polynucleotides from the assembly vector backbones. Following heat inactivation of SapI the component polynucleotide fragments are subjected to denaturing conditions, followed by annealing conditions sufficient for hybridization of the complementary annealable linker pairs. Following primer extension in the presence of DNA polymerase and dNTPs, primers complementary to PA and PB are added, followed by traditional PCR amplification. The assembly reaction results in the production of an assembled polynucleotide comprising component polynucleotides $D_{01/02}$, $D_{1/2}$, D_3, and $D_{41/42}$ assembled in a 5' to 3' direction.

[0061] FIG. 11 presents an exemplary method of generating a plurality of host cells comprising combinatorially combined polynucleotides. Assembled polynucleotides A1 and A2, each comprising the same upstream genomic targeting sequence and the same first non-functional portion of a selectable marker, and assembled polynucleotides B1 and B2, each comprising the same downstream genomic targeting sequence and the same second non-functional portion of a selectable marker, are combinatorially combined by host cell mediated homologous recombination to generate four different combined polynucleotides, A1/B1, A1/B2, A2/B1, and A2/B2, each comprising a functional selectable marker, that can be inserted into a chromosome to generate four different host cells.

[0062] FIG. 12A shows the component polynucleotides used in Example 10 for the high-throughput generation of combinatorially assembled polynucleotides and yeast cells comprising combinatorially assembled and combined polynucleotides, and the expected assembled and combined polynucleotides. US = upstream genomic targeting sequence, DS = downstream genomic targeting sequence, P = various promoter sequences, G = various protein coding sequences, URA = 5` segment of selectable marker, RA3 = 3' segment of selectable marker, PA = primer binding segment PmeI-5', PB = primer binding segment PmeI-3', LB_0 = annealable linker sequence RYSE 2, LA_{n1} = annealable linker sequence

RYSE 2, LB_{n1} = annealable linker sequence RYSE 15, LA_{n2} = annealable linker sequence RYSE 3, LB_{n2} = annealable linker sequence RYSE16, LA_{n3} = annealable linker sequence RYSE 15, LB_{n3} = annealable linker sequence RYSE 3, LA_{n4} = annealable linker sequence RYSE 16, LB_{n4} = annealable linker sequence RYSE 4, LA_{m1} = annealable linker sequence RYSE 3, LA_{m2} = annealable linker sequence RYSE 4, LA_{m3} = annealable linker sequence RYSE 3.

[0063] FIG. 12B shows exemplary assembled polynucleotides (boxed) generated as described in Example 10 and resolved on a 1% agarose gel.

[0064] FIG. 12C shows restriction analysis for exemplary cell colonies obtained as described in Example 10.

[0065] FIG. 13A shows the assembled polynucleotide and component polynucleotides used in Example 11, and the expected chromosomal locus obtained upon assembly and chromosomal integration by the host cells.

[0066] FIG. 13B shows cPCR analysis results obtained for yeast cell transformants generated in Example 11 that comprise chromosomally integrated assembled polynucleotides.

[0067] FIG. 14 shows the component polynucleotides used in Example 12 for the high-throughput generation of yeast cells comprising chromosomally integrated combinatorially assembled and combinatorially combined polynucleotides, and the expected combined polynucleotides obtained upon assembly and combination by host cell mediated homologous recombination. US = upstream genomic targeting sequence, DS = downstream genomic targeting sequence, P = various promoter sequences, G = various protein coding sequences, URA = 5' segment of selectable marker, RA3 = 3' segment of selectable marker, PA = primer binding segment PmeI-5', PB = primer binding segment PmeI-3', LB_0 = annealable linker sequence RYSE 2, LA_{n1} = annealable linker sequence RYSE 2, LB_{n1} = annealable linker sequence RYSE 15, LA_{n2} = annealable linker sequence RYSE 3, LB_{n2} = annealable linker sequence RYSE16, LA_{n3} = annealable linker sequence RYSE 15, LB_{n3} = annealable linker sequence RYSE 3, LA_{n4} = annealable linker sequence RYSE 16, LB_{n4} = annealable linker sequence RYSE 4, LA_{m1} = annealable linker sequence RYSE 3, LA_{m2} = annealable linker sequence RYSE 4, LA_{m3} = annealable linker sequence RYSE 3.

5. DETAILED DESCRIPTION OF THE EMBODIMENTS

5.1 Definitions

[0068] As used herein, the term "polynucleotide" refers to a polymer composed of nucleotide units as would be understood by one of skill in the art. Preferred nucleotide units include but are not limited to those comprising adenine (A), guanine (G), cytosine (C), thymine (T), and uracil (U). Useful modified nucleotide units include but are not limited to those comprising 4-acetylcytidine, 5-(carboxyhydroxylmethyl)uridine, 2-O-methylcytidine, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylamino-methyluridine, dihydrouridine, 2-O-methylpseudouridine, 2-O-methylguanosine, inosine, N6-isopentyladenosine, 1-methyladenosine, 1-methylpseudouridine, 1-methylguanosine, 1-methylinosine, 2,2-dimethylguanosine, 2-methyladenosine, 2-methylguanosine, 3-methylcytidine, 5-methylcytidine, N6-methyladenosine, 7-methylguanosine, 5-methylaminomethyluridine, 5-methoxyaminomethyl-2-thiouridine, 5-methoxyuridine, 5-methoxycarbonylmethyl-2-thiouridine, 5-methoxycarbonylmethyluridine, 2-methylthio-N6-isopentyladenosine, uridine-5-oxyacetic acid-methylester, uridine-5-oxyacetic acid, wybutoxosine, wybutosine, pseudouridine, queuosine, 2-thiocytidine, 5-methyl-2-thiouridine, 2-thiouridine, 4-thiouridine, 5-methyluridine, 2-O-methyl-5-methyluridine, 2-O-methyluridine, and the like. Polynucleotides include naturally occurring nucleic acids, such as deoxyribonucleic acid ("DNA") and ribonucleic acid ("RNA"), as well as nucleic acid analogs. Nucleic acid analogs include those that include non-naturally occurring bases, nucleotides that engage in linkages with other nucleotides other than the naturally occurring phosphodiester bond or that include bases attached through linkages other than phosphodiester bonds. Thus, nucleotide analogs include, for example and without limitation, phosphorothioates, phosphorodithioates, phosphorotriesters, phosphoramidates, boranophosphates, methylphosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs), and the like.

[0069] As used herein, a "component polynucleotide" refers to a polynucleotide sequence that can be assembled together to form a "assembled polynucleotide" using the methods of polynucleotide assembly described herein. When a plurality of assembly vectors are digested with one or more restriction endonucleases capable of excising the component polynucleotides from the assembly vectors, the resulting population of component polynucleotides can comprise the totality of DNA segments to be assembled into a assembled polynucleotide.

[0070] As used herein, an "assembled polynucleotide" refers to a polynucleotide produced by the methods of polynucleotide assembly described herein. The assembled polynucleotide can be comprised of the two or more component polynucleotides. In some embodiments, the assembled polynucleotide comprises 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more component polynucleotides. Assembled polynucleotide length can range from about 100 to about 20,000 nucleotides, or more. In some embodiments, the assembled polynucleotide length ranges from about 200 to about 10,000, about 200 to about 8000, about 200 to about 5000, about 200 to about 3000, or about 200 to about 1000 nucleotides. In other embodiments, the assembled polynucleotide length can range from about 200 to about 2000, about 2000 to about 5000, about 5000 to about 10,000, about 10,000 to about 20,000, or greater than 20,000 nucleotides.

[0071] Conventional notation is used herein to describe polynucleotide sequences: the left-hand end of a single-stranded polynucleotide sequence is the 5'-end; the left-hand direction of a double-stranded polynucleotide sequence is referred to as the 5'-direction.

[0072] As used herein, the term "DNA segment," alternately referred to as "Bits" in the examples below, refers to any isolated or isolatable molecule of DNA. Useful examples include but are not limited to a protein-coding sequence, reporter gene, fluorescent marker coding sequence, promoter, enhancer, terminator, intron, exon, poly-A tail, multiple cloning site, nuclear localization signal, mRNA stabilization signal, selectable marker, integration loci, epitope tag coding sequence, degradation signal, or any other naturally occurring or synthetic DNA molecule. In some embodiments, the DNA segment can be of natural origin. Alternatively, a DNA segment can be completely of synthetic origin, produced *in vitro*. Furthermore, a DNA segment can comprise any combination of isolated naturally occurring DNA molecules, or any combination of an isolated naturally occurring DNA molecule and a synthetic DNA molecule. For example, a DNA segment may comprise a heterologous promoter operably linked to a protein coding sequence, a protein coding sequence linked to a poly-A tail, a protein coding sequence linked in-frame with a epitope tag coding sequence, and the like.

[0073] "Complementary" refers to the topological compatibility or matching together of interacting surfaces of two polynucleotides as understood by those of skill in the art. Thus, two sequences are "complementary" to one another if they are capable of hybridizing to one another to form a stable anti-parallel, double-stranded nucleic acid structure. A first polynucleotide is complementary to a second polynucleotide if the nucleotide sequence of the first polynucleotide is substantially identical to the nucleotide sequence of the polynucleotide binding partner of the second polynucleotide, or if the first polynucleotide can hybridize to the second polynucleotide under stringent hybridization conditions. Thus, the polynucleotide whose sequence 5'-TATAC-3' is complementary to a polynucleotide whose sequence is 5'-GTATA-3'.

[0074] "Primer" refers to a polynucleotide sequence that is capable of specifically hybridizing to a polynucleotide template sequence, *e.g.*, a primer binding segment, and is capable of providing a point of initiation for synthesis of a complementary polynucleotide under conditions suitable for synthesis, *i.e.*, in the presence of nucleotides and an agent that catalyzes the synthesis reaction (*e.g.*, a DNA polymerase). The primer is complementary to the polynucleotide template sequence, but it need not be an exact complement of the polynucleotide template sequence. For example, a primer can be at least about 80, 85, 90, 95, 96, 97, 98, or 99% identical to the complement of the polynucleotide template sequence. A primer can be of variable length but generally is at least 15 bases. In some embodiments, the primer is between 15 and 35 bases long. In some embodiments, the primer is more than 35 bases long. In other embodiments, the primer has a melting temperature (T_m), *i.e.*, the temperature at which one half of the DNA duplex will dissociate to become single stranded, of at least 50°C. In other embodiments, the primer has a T_m between about 50°C and 70°C. In still other embodiments, the primer does not form appreciable DNA or RNA secondary structures so as to not impact the efficiency of hybridization to the polynucleotide template sequence.

[0075] As used herein, the term "primer binding segment" is a polynucleotide sequence that binds to a primer so as to provide a point of initiation for synthesis of a complementary polynucleotide under conditions suitable for synthesis. In some embodiments, the primer binding sequence is one of the annealable linkers of the present invention. A sequence is a primer binding sequence instead of an annealable linker by the absence of a complementary linker within a given set of assembly vectors or component polynucleotides within an assembly composition. In some embodiments, the primer binding segment can function as a genomic targeting sequence, *e.g.*, an upstream or downstream genomic targeting sequence.

[0076] As used herein, the term "linker sequence" and "annealable linker sequence" are used interchangeably and refer to a polynucleotide sequence contained within an entry vector and assembly vector described herein. In particular, an annealable linker sequence flanks a DNA segment within an entry vector or assembly vector. Upon excision of a component polynucleotide from an assembly vector, and denaturation of the component polynucleotide, an annealable linker is capable of specifically hybridizing to a complementary annealable linker sequence of an adjacent component polynucleotide in a polynucleotide assembly reaction, as described herein. An annealable linker, upon annealing with a complementary linker strand, can provide a point of initiation for synthesis of a complementary polynucleotide.

[0077] As used herein, the term "vector" is used in reference to extrachromosomal nucleic acid molecules capable of replication in a cell and to which an insert sequence can be operatively linked so as to bring about replication of the insert sequence. Useful examples include but are not limited to circular DNA molecules such as plasmid constructs, phage constructs, cosmid vectors, *etc.*, as well as linear nucleic acid constructs (*e.g.*, lambda phage constructs, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), *etc.*). A vector may include expression signals such as a promoter and/or a terminator, a selectable marker such as a gene conferring resistance to an antibiotic, and one or more restriction sites into which insert sequences can be cloned. Vectors can have other unique features (such as the size of DNA insert they can accommodate).

[0078] As used herein, the term "entry vector" refers to a cloning vector plasmid that can serve as a parental vector for the preparation of an assembly vector to be used in the polynucleotide assembly methods provided herein. An entry vector comprises two annealable linker sequences, or an annealable linker sequence and a primer binding segment, which flank restriction sites that can be utilized for the introduction of a DNA segment to form an assembly vector. As used herein, an "assembly vector" refers to an entry vector to which a DNA segment has been introduced. An assembly vector can be used in the polynucleotide assembly methods described herein to provide a component polynucleotide to be assembled into a assembled polynucleotide.

[0079] As used herein, the term "assembly vector" refers to a vector comprising one annealable linker sequence, two annealable linker sequences, or an annealable linker sequence and a primer binding segment, and a DNA segment.

[0080] As used herein, the term "restriction enzyme" or "restriction endonuclease" refers to a member or members of a classification of catalytic molecules that bind a cognate sequence of DNA and cleave the DNA molecule at a precise location within that sequence. Restriction endonucleases include Type IIS restriction endonucleases. This class of enzymes differs from other restriction endonucleases in that the recognition sequence is separate from the site of cleavage. Some examples of Type IIS restriction enzymes include AlwI, BsaI, BbsI, BbuI, BsmAI, BsrI, BsmI, BspMI, EarI, Esp3I, FokI, HgaI, HphI, LguI, MboII, MnlI, PleI, SapI, SchI, SfaNi, and the like. Many of these restriction endonucleases are available commercially and are well known to those skilled in the art.

[0081] As used herein, the term "annealable linker sequence duplex" refers to one annealable linker sequence strand aligned with a substantially complementary annealable linker sequence strand in antiparallel association. Complementarity need not be perfect; annealable linker sequence duplexes may contain mismatched base pairs or unmatched bases, although in particular embodiments, the annealable linker sequence duplex comprises two annealable linker sequence strands having perfect complementarity.

[0082] As used herein, the term "genomic targeting sequence" refers to a nucleotide sequence that is present in the genome of a host cell at a site at which a polynucleotide of the invention is to be inserted by host cell mediated homologous recombination. The terms "upstream genomic targeting sequence" and "downstream genomic targeting sequence" refer to genomic targeting sequences that are located upstream and downstream of each other in the genome of a host cell.

[0083] As used herein, the term "chromosomal targeting sequence" refers to a nucleotide sequence that is present in a chromosome of a host cell at a site at which a polynucleotide of the invention is to be inserted by host cell mediated homologous recombination. The terms "upstream chromosomal targeting sequence" and "downstream chromosomal targeting sequence" refer to chromosomal targeting sequences that are located upstream and downstream of each other in a chromosome of a host cell.

5.2 Methods of Polynucleotide Assembly

[0084] In one aspect, the present invention provides rapid, robust, and high-throughput methods for the ordered assembly of a plurality of component polynucleotides into one or more assembled polynucleotides. The methods of the invention utilize circular nucleic acid vectors, termed assembly vectors, that each comprise a DNA segment, D, flanked by an annealable linker sequence (*i.e.*, LA or LB), a pair of annealable linker sequences (*i.e.*, LA and LB), or an annealable linker sequence and a primer binding segment (*i.e.*, LA and PB or LB and PA), and a pair of restriction sites, RA and RB (FIG. 1B). Restriction endonuclease digestion of a plurality of assembly vectors at restriction sites RA and RB generates a plurality of component polynucleotides comprising the elements 5'-LA-D-3', 5'-D-LB-3', 5'-LA-D-LB-3', 5'-LA-D-PB-3', or 5'-LB-D-PA-3' (FIG. 3). In the methods of the invention annealable linker sequences LA and LB provide the component polynucleotides with complementary termini that are utilized in a splice overlap extension assembly reaction followed by polymerase chain reaction (SOE/PCR) to assemble the component polynucleotides into an assembled polynucleotide with an ordered sequence.

[0085] In particular, the methods can provide for assembly into a single assembled polynucleotide of a number of functional DNA elements, including but not limited to protein-coding sequences, reporter genes, fluorescent marker coding sequences, promoters, enhancers, terminators, introns, exons, poly-A tails, multiple cloning sites, nuclear localization signals, mRNA stabilization signals, selectable markers, integration loci, epitope tag coding sequences, and degradation signals. The methods can be used for the assembly of any type of assembled polynucleotide, including but not limited to synthetic genes, constructs, cloning vectors, expression vectors, chromosomes, genomic integration constructs, genomes, and DNA libraries. Furthermore, the methods can be used to assemble DNA segments in a single reaction without need for manipulation and characterization of intermediate products.

[0086] In some embodiments, the methods can also provide for the assembly of an assembled polynucleotide from a plurality of component polynucleotides not originating from an assembly vector (*i.e.*, DNA segments obtained by standard procedures known in the art, such as for example, PCR amplification, chemical synthesis, and the like, that are flanked by one or two annealable linker sequences, LA and/or LB, or by an annealable linker sequence and a primer binding segment (*i.e.*, LA and PB or LB and PA). The component polynucleotides not originating from an assembly vector may be added to the assembly reaction at any stage prior to the SOE/PCR reaction or host cell mediated homologous recombination for assembly into the assembled polynucleotide. Thus, in some embodiments, the assembly methods can be used to assemble:(1) component polynucleotides derived from assembly vectors comprising one or two annealable linker sequences, or an annealable linker sequence and a primer binding segment, and generated by digestion of the assembly vectors; (2) vectorless DNA fragments flanked by one or two annealable linker sequences, or by an annealable linker sequence and a primer binding segment ; and (3) combinations thereof.

[0087] In some embodiments, provided herein are methods of assembling a plurality of component polynucleotides into one or more assembled polynucleotides, comprising the steps of:

(a) digesting an assembly composition with one or more restriction endonucleases to generate a components composition, the assembly composition comprising:

(i) one or more first nucleic acid molecules, wherein each first nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_0, any primer binding segment selected from the group PA, any DNA segment selected from the group D_0, an annealable linker sequence LB_0, and a second restriction site RB_0;

(ii) one or more intermediate nucleic acid molecules wherein each intermediate nucleic acid molecule n is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_n, a first annealable linker sequence LA_n, any DNA segment seleted from the group D_n, a second annealable linker sequence LB_n, and a second restriction site RB_n, and wherein n represents an integer from one to the number of intermediate nucleic acid molecules; and

(iii) one or more last nucleic acid molecules, wherein each last nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_m, an annealable linker sequence LA_m, a DNA segment selected from the group D_m, any primer binding segment selected from the group PB, a second restriction site RB_m wherein m represents an integer one greater than the number of intermediate nucleic acid molecules; whereupon cleavage of restriction sites RA_0 through RB_m and denaturation of the resulting linear nucleic acid molecules, each annealable linker sequence $LB_{(p-1)}$ is capable of hybridizing to the complement of annealable linker sequence LA_p, wherein n is an integer that varies from 1 to (m-1), wherein p represents an integer from 1 to m, and wherein each group D_0,... D_n,...and D_m consists of one or more DNA segments;

wherein the one or more restriction endonucleases are capable of cleaving the restriction sites RA_0 through RB_m; and

(b) contacting the components composition with DNA polymerase, deoxyribonucleoside triphosphates and one or more first primers and one or more second primers, under conditions suitable for denaturation of the nucleic acid molecules, annealing of annealable linker sequence $LB_{(p-1)}$ to annealable linker sequence LA_p, and extension therefrom; wherein each said first primer is capable of hybridizing to one of said primer binding segments selected from the group PA and each said second primer is capable of hybridizing to one of said primer binding segments selected from the group PB; and subjecting the components composition to polymerase chain reaction,

wherein a polynucleotide is assembled which comprises, in a 5' to 3' orientation, one DNA segment selected from each of the groups D_0,... D_n,...and D_m. In the method, p represents the integers from 1 to m.

[0088] FIG. 3 depicts one embodiment of the assembly methods of the invention for illustrative purposes. In this example, a total of four component polynucleotides are assembled to yield an assembled polynucleotide. However, the assembly methods provided herein can be used to assemble any number of component polynucleotides into one or more assembled polynucleotides. In some embodiments, the methods provided herein result in the assembly of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more component polynucleotides into one or more assembled polynucleotides.

[0089] In the example illustrated in FIG. 3, the assembly composition from which the assembled polynucleotide is generated comprises four input assembly vectors, denoted "first," "intermediate 1 (int_1)," "intermediate 2 (int_2)," and "last." Each assembly vector comprises a DNA segment flanked either by an annealable linker sequence and a primer binding segment, or by two annealable linker sequences. Specifically, DNA segment D_0 is flanked by 5' primer binding segment PA and 3' annealable linker sequence LB_0. DNA segment D_1 is flanked by 5' and 3' annealable linker sequences LA_1 and LB_1, and DNA segment D_2 is flanked by 5' and 3' annealable linker sequences LA_2 and LB_2. DNA segment D_3 is flanked by 3' primer binding segment PB and 5' annealable linker sequence LA_3. The 5'-PA-D-LB-3', 5'-LA-D-LB-3', or 5'-LA-D-PB-3' elements in the assembly vectors are further flanked by SapI restriction endonuclease sites.

[0090] In the first step of the assembly reaction shown in FIG. 3, the assembly composition is digested with SapI, resulting in the excision of component polynucleotides, comprising the elements 5'-PA-D-LB-3' , 5'LA-D-LB-3', or 5'-LA-D-PB-3', from the assembly vector backbones into a components composition. Because Sap I is a Type IIS restriction endonuclease, its recognition site is distal to its cleavage site, and cleavage occurs outside of its recognition sequence. This property makes Type IIS restriction endonucleases particularly useful in the assembly of a polynucleotide according to the methods provided herein, since polynucleotides can be assembled which do not comprise a restriction-site scar, which may otherwise result from cleavage of restriction sites RA and RB with a non-TypeIIS restriction endonuclease. Referring to Figure 2, the Type IIS recognition site is 5' of the corresponding cleavage site for each of RA_0, RA_n, and RA_m, and 3' of its cleavage site RB_0, RA_n, and RA_m. Thus, restriction sites RA_0 through RB_m are oriented so that cleavage by one or more Type IIS restriction endonucleases capable of cleaving RA_0 through RB_m results in separation of RA_0 from D_0, LB_0 from RB_0, RA_n, from LA_n, LB_n from RB_n, RA_m from LA_m, and D_m from RB_m, wherein resultant linearized nucleic acid molecules comprising D_0, LB_0, RA_n, LB_n, LA_m or D_m do not comprise any of RA_0 through RB_m. As a consequence, the resulting component polynucleotides do not include any trace of either the restriction enzyme's recognition or cleavage sites. As a result, the inventive methods of polynucleotide assembly can be used to transform host cells multiple times without the introduction of sequence repeats which may cause genetic instability.

[0091] Subsequently, the restriction endonuclease is optionally inactivated. If inactivation is desired, any method known in the art for inactivating endonuclease enzyme activity may be employed, including column or gel-based purification methods. One convenient method is heat inactivation, *e.g.*, at 65° for 20 minutes, which requires little or no manipulation of the components composition outside of the reaction tube.

[0092] Assembly of the component polynucleotides into an assembled polynucleotide is enabled by sequence duplexes formed by overlapping strands of complementary termini among the component polynucleotides. Specifically, the annealable linker sequences are designed such that annealable linker sequence LB_0 can hybridize to the complement of annealable linker sequence LA_1, annealable linker sequence LB_1 can hybridize to the complement of annealable linker sequence LA_2, and annealable linker sequence LB_2 can hybridize to the complement of annealable linker sequence LA_3. Thus, in the second step of the assembly reaction, the component polynucleotides are subjected to denaturing conditions (*e.g.*, heat) to generate single-stranded component polynucleotides, which concomitant with or subsequent to the denaturation step of the assembly reaction are contacted with a thermostable DNA polymerase and deoxyribonucleoside triphosphates.

[0093] The thermostable DNA polymerase can be any thermostable DNA polymerase deemed suitable by those of skill in the art. Thermostable DNA polymerases suitable for use in the present methods include but are not limited to *Thermus thermophilus* (Tth) DNA polymerase, *Thermus aquaticus* (Taq) DNA polymerase, *Thermotoga neopolitana* (Tne) DNA polymerase, *Thermotoga maritima* (Tma) DNA polymerase, *Therm°C°Ccus litoralis* (Tli or VENT™) DNA polymerase, *Pyr°C°Ccus furiosus* (Pfu or DEEPVENT™) DNA polymerase, *Pyr°C°Ccus woosii* (Pwo) DNA polymerase, *Bacillus sterothermophilus* (Bst) DNA polymerase, *Sulfolobus acid°Caldarius* (SAC) DNA polymerase, *Thermoplasma acidophilum* (Tac) DNA polymerase, *Thermus flavus* (Tfl/Tub) DNA polymerase, *Thermus ruber* (Tru) DNA polymerase, *Thermus br°Ckianus* (DYNAZYME™) DNA polymerase, *Methanobacterium thermoautotrophicum* (Mth) DNA polymerase, and mutants, variants, and derivatives thereof. Thermostable DNA polymerases having high fidelity (*i.e.*, proofreading properties) and low error rates are preferred. In certain embodiments, the DNA polymerase is Phusion™ DNA Polymerase (New England Biolabs, Ipswich, MA). In other embodiments, the DNA Polymerase is PfuUltra™ II Fusion DNA Polymerase (Strategene / Agilent, La Jolla, CA).

[0094] The assembly reaction is then subjected to conditions that allow for strand elongation from the 3'-hydroxyl portions of the overlapping annealable linker sequences, during which the thermostable DNA polymerase fills in the portion between the overlapping annealable linker sequences. The assembly reaction is subjected to a limited number of repeating cycles of denaturation / annealing / extension (*e.g.*, for 5-15 cycles) during which a substantial amount of double-stranded assembled polynucleotides are formed. During this cycling, the component polynucleotides act as both primers and template to generate a full length template for the assembled polynucleotide. In certain embodiments, the annealing and extension steps of the PCR can both be performed at 72 °C.

[0095] In contrast to the annealable linker sequences LA and LB, the primer binding segments PA and PB are designed to not overlap with each other or any of the annealable linker sequences or DNA segments, but rather serve as binding sites for primers used to amplify the full length assembled polynucleotide. Thus, in steps 4 and 5 of the assembly reaction, primers complementary to primer binding segments PA and PB are added, and the composition is subjected to traditional PCR amplification conditions. The PCR amplification conditions can be any PCR amplification conditions deemed suitable by those of skill in the art, including those described in *PCR Technology: Principles and Applications for DNA Amplification*, ed. HA Erlich, Stockton Press, New York, N.Y. (1989); *PCR Prot°Cols: A Guide to Methods and Applications*, eds. Innis, Gelfland, Snisky, and White, Academic Press, San Diego, Calif. (1990); Mattila *et al.* (1991) *Nucleic Acids Res.* 19: 4967; Eckert, K. A. and Kunkel, T. A. (1991) PCR Methods and Applications 1: 17; and U.S. Pat. Nos. 4,683,202 and 4,965,188. In certain embodiments, the PCR step of the assembly reaction comprises about 35 cycles of denaturation, annealing, and extension in the presence of primers complementary to primer binding segments PA and PB. In certain embodiments, the annealing and extension steps of the PCR can both be performed at 72°. However, one of skill in the art will understand that optimal conditions for successful amplification will depend on the thermostable DNA polymerase and the annealable linker sequences utilized, and these conditions may be adjusted accordingly.

[0096] Optionally, the assembled polynucleotide can be purified by any technique apparent to one of skill in the art, *e.g.*, gel electrophoresis purification methods and used for a variety of purposes. For example, the assembled polynucleotide can be inserted into an expression vector backbone for sequence verification.

5.3 Methods of Generating Host Cells Comprising Assembled Polynucleotides

[0097] In another aspect, the present invention provides methods for generating host cells comprising assembled polynucleotides. In some embodiments, the assembled polynucleotide is at least 3 kb in size. In other embodiments, the assembled polynucleotide is at least 5 kb in size. In still other embodiments, the assembled polynucleotide is at least 6, 7, 8, 9, or 10 kb in size. In still other embodiments, the assembled polynucleotide is greater than 10 kb in size. In still other embodiments, the assembled polynucleotide is greater than 15 kb in size. In still other embodiments, the assembled polynucleotide is greater than 20 kb in size.

[0098] In some embodiments, methods are provided that comprise transforming a host cell with an assembled polynucleotide generated by the methods of polynucleotide assembly described herein. The assembled polynucleotide can be circularized prior to transformation or can be transformed as a linear molecule. The assembled polynucleotide can be maintained in the host cell as an extrachromosomal polynucleotide. Alternatively, the assembled polynucleotide can be integrated into the genome of the host cell, *e.g.*, by host cell mediated homologous recombination. To integrate an assembled polynucleotide into the genome by homologous recombination, the assembled polynucleotide must comprise at one terminus a nucleic acid sequence comprising an upstream genomic targeting sequence and at the other terminus a nucleic acid sequence comprising a downstream genomic targeting sequence. Accordingly, an assembled polynucleotide that is to be integrated into a chromosome of a host cell is generated from an assembly composition comprising a first nucleic acid molecule comprising an upstream chromosomal targeting sequence and a last nucleic acid molecule comprising a downstream chromosomal targeting sequence, each chromosomal targeting sequence being of sufficient length to initiate homologous recombination by the host cell with its chromosome.

[0099] In other embodiments, the methods comprise transforming a host cell with a plurality of assembled polynucleotides generated by the methods of polynucleotide assembly described herein. In a particular embodiment, the host cell combines two or more assembled polynucleotides into a single combined polynucleotide by homologous recombination. Host cell transformants comprising the combined polynucleotides are selected by virtue of expressing a selectable marker that is generated in the process of combining the assembled polynucleotides. The method is particularly useful for inserting relatively large pieces of polynucleotide into a target polynucleotide by homologous recombination. For chromosomal integration to occur, the combined polynucleotide must comprise an upstream genomic targeting sequence located 5' or 3' of the coding sequence of the selectable marker and a downstream genomic targeting sequence located 3' or 5' of the coding sequence of the selectable marker, respectively. Genomic integration as used herein includes chromosomal integration, *i.e.*, integration of a polynucleotide into a chromosome of a host cell. Suitable chromosomal integration sites in *Saccharomyces cerevisiae* include but are not limited to the *NDT80*, *HO*, *GAL2*, and *GAL1-GAL10-GAL7* locus. The method can also be useful for generating host cells comprising an extrachromosomally maintained polynucleotide, *e.g.*, vectors and expression plasmids. The stability of either a chromosomally integrated or an extrachromosomally maintained combined polynucleotide is increased when the combined polynucleotide does not comprise identical annealable linker sequences or DNA segments arranged as direct repeats that can otherwise initiate additional homologous recombination events resulting in the excision of segments of the component polynucleotide. Therefore, in some embodiments, the assembled polynucleotides comprise unique annealable linker sequences and DNA segments. In other embodiments, the assembled polynucleotides contain one or more identical annealable linker sequences or DNA segments that upon combination of the assembled polynucleotides are arranged as inverted repeats in the combined polynucleotide.

[00100] The generation of an exemplary combined polynucleotide and integration of the combined polynucleotide into a chromosome of the host cell by homologous recombination is illustrated in FIG. 8. Two assembled polynucleotides A and B are taken up by a host cell that is capable of homologous recombination. Each assembled polynucleotide comprises a DNA segment D_m that encodes a segment of a selectable marker, wherein DNA segment D_{m1} of assembled polynucleotide A encodes a first segment of a selectable marker and DNA segment D_{m2} of assembled polynucleotide B encodes a second segment of the selectable marker, wherein DNA segment D_{m1} and DNA segment D_{m2} comprise a region of homology sufficient to initiate host cell mediated homologous recombination, and wherein neither DNA segment D_{m1} nor DNA segment D_{m2} produces a functional selectable marker, but whereupon homologous recombination by the host cell a functional selectable marker is generated. Each assembled polynucleotide further comprises a DNA segment D_0 encoding a chromosomal targeting sequence of sufficient length to initiate host mediated homologous recombination, wherein DNA segment D_{01} of assembled polynucleotide A encodes an upstream chromosomal targeting sequence and DNA segment D_{02} of assembled polynucleotide B encodes a downstream chromosomal targeting sequence. Once inside the cell, the host cell recombines assembled polynucleotide A and assembled polynucleotide B at the region of homology in DNA segments D_{m1} and D_{m2} to form a combined polynucleotide. Moreover, the host cell uses the chromosomal targeting sequences encoded by DNA segments D_{01} and D_{02} to insert the combined polynucleotide by homologous recombination into its chromosome. Host cells comprising the combined polynucleotide can be readily identified based on the functional selectable marker generated.

[00101] In yet other embodiments, the methods comprise transforming a host cell with a plurality of component polynucleotides and allowing the host cell to generate one or more assembled polynucleotides by homologous recombination. The assembled polynucleotide can be extrachromosomally maintained in the host cell or integrated into the chromosome of the host cell. The generation of an exemplary assembled polynucleotide by homologous recombination in a host cell and integration of the assembled polynucleotide into the chromosome of the host cell is illustrated in FIG. 9. In the first step, an assembly composition comprising assembly vectors is digested with a Type IIS restriction endonuclease such as SapI or LguI, resulting in the excision from the assembly vector backbones of component polynucleotides. In this embodiment, D_0 and D_3 can be the upstream and downstream chromosomal targeting sequence, in which case the presence of a primer binding segment in the first and last assembly vectors is optional. Alternatively, the two primer binding segments could function as the upstream and downstream genomic targeting sequences.

[00102] Once excised, each excised component polynucleotide comprises an annealable linker sequence LB that is homologous to an annealable linker sequence LA of another component polynucleotide and that is of sufficient length to initiate host mediated homologous recombination. The component polynucleotide excised from the first assembly vector further comprises an upstream chromosomal targeting sequence, and the component polynucleotide excised from the last assembly vector further comprises a downstream chromosomal targeting sequence, wherein both chromosomal targeting sequences are of sufficient length to initiate host mediated homologous recombination with a chromosome of the host cell. The restriction endonuclease can subsequently be inactivated. In the second step of the method, the components composition is introduced into a host cell capable of homologous recombination. Once inside the cell, the host cell recombines the component polynucleotides at the regions of homology between the annealable linker sequences to form an assembled polynucleotide, and the assembled polynucleotide is integrated into the chromosome. Host cells comprising the assembled polynucleotide can be readily identified based on a selectable marker encoded by a DNA segment of the assembled polynucleotide.

[00103] Any host cell can be used in the methods describe herein. In particular embodiments, suitable host cells are host cells that are capable of recombining polynucleotides based on complementary sequence stretches such as provided by the selectable marker segments, genomic targeting sequences, and annealable linker sequences provided herein. Illustrative examples of such host cells include but are not limited to *Saccharomyces cerevisiae*. Conditions suitable for uptake of DNA by such host cells are well known in the art.

[00104] Host cell transformants comprising an assembled or combined polynucleotide can be readily identified by virtue of expressing a selectable marker encoded by the assembled polynucleotide or by the combined polynucleotide that permits selection for or against the growth of the cells. The selectable marker may be encoded by a single DNA segment present in an assembly vector of an assembly composition. Alternatively, non-functional segments of the selectable marker may be encoded by DNA segments present in multiple assembly vectors of an assembly composition or in multiple assembled polynucleotides such that a functional selectable marker is generated only upon generation of an assembled polynucleotide or upon generation of a combined polynucleotide, respectively.

[00105] A wide variety of selectable markers are known in the art (*see,* for example, Kaufinan, *Meth. Enzymol.*, 185:487 (1990); Kaufman, *Meth. Enzymol.*, 185:537 (1990); Srivastava and Schlessinger, *Gene,* 103:53 (1991); Romanos *et al.*, in DNA Cloning 2: Expression Systems, 2nd Edition, pages 123-167 (IRL Press 1995); Markie, *Methods Mol. Biol.*, 54:359 (1996); Pfeifer *et al.*, *Gene,* 188:183 (1997); Tucker and Burke, *Gene,* 199:25 (1997); Hashida-Okado *et al.*, *FEBS Letters,* 425:117 (1998)). In some embodiments, the selectable marker is a drug resistant marker. A drug resistant marker enables cells to detoxify an exogenous drug that would otherwise kill the cell. Illustrative examples of drug resistant markers include but are not limited to those which confer resistance to antibiotics such as ampicillin, tetracycline, kanamycin, bleomycin, streptomycin, hygromycin, neomycin, Zeocin™, and the like. In other embodiments, the selectable marker is an auxotrophic marker. An auxotrophic marker allows cells to synthesize an essential component (usually an amino acid) while grown in media that lacks that essential component. Selectable auxotrophic gene sequences include, for example, hisD, which allows growth in histidine free media in the presence of histidinol. Other selectable markers include a bleomycin-resistance gene, a metallothionein gene, a hygromycin B-phosphotransferase gene, the AURI gene, an adenosine deaminase gene, an aminoglycoside phosphotransferase

gene, a dihydrofolate reductase gene, a thymidine kinase gene, a xanthine-guanine phosphoribosyltransferase gene, and the like.

[00106] Auxotrophy can also be used to identify host cell transformants comprising a chromosomally integrated assembled or combined polynucleotide when the integration of the assembled or combined polynucleotide results in the disruption of a gene that the host cell requires to synthesize a component essential for cell growth, thus rendering the cell auxotrophic.

[00107] Host cell transformants comprising a chromosamlly integrated assembled or combined polynucleotide can also be identified by selecting host cell transformants exhibiting other traits encoded by individual DNA segments or by combinations of DNA segments, *e.g.*, expression of peptides that emit light, or by molecular analysis of individual host cell colonies, *e.g.*, by restriction enzyme mapping, PCR amplification, or sequence analysis of isolated assembled polynucleotides or chromosomal integration sites.

5.4 Combinatorial Methods of Polynucleotide Assembly and Host Cell Generation

[00108] In another aspect, the present invention provides rapid, robust, and high-throughput methods for the ordered assembly of multiple component polynucleotides into a plurality of assembled polynucleotides. The methods rely on the use of an assembly composition comprising assembly vectors that each comprise a DNA segment D, flanked by an annealable linker sequence LA or LB, a pair of annealable linker sequences LA and LB, or by an annealable linker sequence and a primer binding segment, *i.e.*, LA and PB or LB and PA, flanked by a pair of restriction sites RA and RB (FIG. 1B). However, to generate a diversity of assembled polynucleotides using the methods disclosed herein, annealable linker sequences and primer binding segments are chosen such that more than one combination of component polynucleotides can be assembled into an assembled polynucleotide in the reaction. Thus, in some embodimens, the assembly composition comprises at least two assembly vectors that have the same annealable linker sequence LA or LB or the same primer binding segment PA or PB, but differ with respect to the DNA segment. In other embodimens, the assembly composition comprises at least two assembly vectors that have the same pair of annealable linker sequences LA and LB, or the same annealable linker sequence and primer binding segment pair, *i.e.*, LA and PB or LB and PA but differ with respect to the DNA segment.

[00109] FIG. 10 presents an exemplary method of generating a plurality of assembled polynucleotides from seven (7) component polynucleotides in the same reaction. Assembly vectors comprising DNA segments to be assembled are pooled in a single tube and digested with SapI to release component polynucleotide fragments from the assembly vector backbones. Following heat inactivation of SapI, the component polynucleotides are subjected to denaturing conditions, followed by annealing conditions sufficient for hybridization of the complementary annealable linker pairs. Following primer extension in the presence of DNA polymerase and dNTPs, primers complementary to primer binding segments PA and PB are added to PCR amplify eight (8) different full-length assembled polynucleotides that comprise DNA segments $D_{01/02}$, $D_{1/2}$, D_3, and $D_{41/42}$ assembled in various possible combinations. Individual assembled polynucleotides can be isolated from the composition of mixed assembled polynucleotides, e.g., by another round of PCR amplification using primers complementary to regions of DNA segments D_{01}, D_{02}, D_{41}, and D_{42}. Alternatively, a set of assembled polynucleotides can be isolated by first and last assembly vectors comprising one of a group of primer binding segments PA and/or PB and using primers for PCR amplification that hybridize to only a select subgroup of primer binding segments PA and PB. The isolated assembled polynucleotides can be used, *e.g.*, to transform host cells to generate a plurality of host cells comprising assembled polynucleotides. Alternatively, host cells can be directly transformed with the composition of mixed assembled polynucleotides and host cell transformants comprising each assembled polynucleotide can be isolated, e.g., by molecular analysis of individual host cell colonies, or by selecting host cell transformants comprising selectable markers or exhibiting other traits encoded by individual DNA segments or by combinations of DNA segments.

[00110] In other embodiments, a plurality of host cells comprising a plurality of polynucleotides assembled by combinatorial methods are generated by transforming host cells with a composition comprising multiple assembled polynucleotides of which at least two assembled polynucleotides comprise non-functional segments of a selectable marker that upon homologous recombination generate a functional selectable marker, and by selecting host cells comprising a combined polynucleotide. FIG. 11 illustrates a combinatorial approach to generating a plurality of host cells comprising combined polynucleotides. In the example, assembled polynucleotides A1 and A2, each comprising the same upstream chromosomal targeting sequence and the same first portion of a selectable marker, and assembled polynucleotides B1 and B2, each comprising the same downstream chromosomal targeting sequence and the same second portion of a selectable marker, are combinatorially combined by host cell mediated homologous recombination to generate four different combined polynucleotides, A1/B1, A1/B2, A2/B1, and A2/B2, that can be inserted into a chromosome to generate four different host cells.

[00111] In yet other embodiments, a plurality of host cells comprising a plurality of polynucleotides assembled and combined by combinatorial methods are generated by transforming host cells with a component composition comprising multiple component polynucleotides of which at least two component polynucleotides comprise non-functional segments of a selectable marker that upon host cell mediated homologous recombination generate a functional selectable marker, and by selecting host cells comprising an assembled or combined polynucleotide.

5.5 Entry Vectors

[00112] In another aspect, provided herein is a vector, *i.e.*, an entry vector, that can be used to prepare an assembly vector. In some embodiments, an entry vector is a circular polynucleotide that comprises a selectable marker, an origin of replication, and a DNA segment immediately flanked by two restriction sites that facilitate the subcloning of different DNA segments to be assembled in the assembly methods provided herein. The entry vector further comprises one or two annealable linker sequences, or an annealable linker sequence and a primer binding segment, flanking the restriction sites. The entry vector further comprises an additional pair of restriction sites positioned at the outer flanks of the DNA segment, *e.g.*, that flank the one or two annealable linker sequences, or the annealable linker sequence and primer binding segment. Thus, in some embodiments, the entry vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a restriction site RY, a DNA segment D, a restriction site RZ, and a restriction site RB. In other embodiments, the entry vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a restriction site RY, a DNA segment D, a restriction site RZ, an annealable linker sequence LB, and a restriction site RB. In other embodiments, the entry vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a primer binding segment PA or an annealable linker sequence LA, a restriction site RY, a DNA segment D, a restriction site RZ, a primer binding segment PB or an annealable linker sequence LB, and a restriction site RB.

[00113] In some embodiments, the sequence of the DNA segment D of the entry vector is the lac Z reporter gene. The lac Z reporter gene is useful for facilitating blue/white selection of colonies transformed with vectors comprising DNA segments other than lac Z, *e.g.*, during the preparation of an assembly vector described herein.

[00114] In some embodiments, the entry vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a restriction site RY, a DNA segment D, a restriction site RZ, and a restriction site RB (*i.e.*, 5'-RA-LA-RY-D-RZ-RB-3'). In some embodiments, the entry vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a restriction site RY, a DNA segment D, a restriction site RZ, an annealable linker sequence LB, and a restriction site RB (*i.e.*, 5'-RA-RY-D-RZ-LB-RB-3'). In some embodiments, the entry vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a restriction site RY, a DNA segment D, a restriction site RZ, an annealable linker sequence LB, and a restriction site RB (*i.e.*, 5'-RA-LA-RY-D-RZ-LB-RB-3'). In some embodiments, the entry vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a primer binding segment PA, a restriction site RY, a DNA segment D, a restriction site RZ, an annealable linker sequence LB, and a restriction site RB (*i.e.*, 5'-RA-PA-RY-D-RZ-LB-RB-3'). In some embodiments, the entry vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a restriction site RY, a DNA segment D, a restriction site RZ, a primer binding segment PB, and a restriction site RB (*i.e.*, 5'-RA-LA-RY-D-RZ-PB-RB-3'). An exemplary entry vector is provided in FIG. 1A.

[00115] The primer binding segment can be any nucleotide sequence that is not complementary with any of the annealable linker sequences that are used to make an assembled polynucleotide. In some embodiments, the two primer binding segment includes a restriction endonuclease recognition and cleavage site. In some embodiments, the primer binding segment is simply one of the available linker sequences that are not being used in a particular assembly reaction. In some embodiments, the nucleic acid sequence of primer binding segment PA is selected from the group consisting of SEQ ID NOS: 24 and 25. In some embodiments, the nucleic acid sequence of primer binding segment PB is selected from the group consisting of SEQ ID NOS: 24 and 25. In some embodiments, the nucleic acid sequences of primer binding segment PA and primer binding segment PB are selected from the group consisting of SEQ ID NOS: 24 and 25. In preferable embodiments, PA and PB are not identical in sequence.

[00116] In some embodiments, the nucleic acid sequence of annealable linker sequence LA or LB is at least 24 nucleotides and has a T_m of at least 60 °C. In some embodiments, the nucleic acid sequence of annealable linker sequence LA is selected from the group consisting of SEQ ID NOS: 1 to 23. In some embodiments, the nucleic sequence

of annealable linker sequence LB is selected from the group consisting of SEQ ID NOS: 1 to 23. In some embodiments, the nucleic sequences of annealable linker sequence LA and annealable linker sequence LB are selected from the group consisting of SEQ ID NOS: 1 to 23.

[00117] The restriction sites RY and RZ can be utilized as cloning sites to introduce various DNA segments for the generation of an assembly vector. In some embodiments, RY and RZ are not identical in sequence. In some embodiments, RY and RZ are cleavable by the same restriction endonuclease. In some embodiments, RY and RZ are identical in sequence. In some embodiments, restriction sites RY and RZ are cleavable by a restriction endonuclease that generates staggered ends, *i.e.* termini having a 5' or 3' overhang. In other embodiments, restriction sites RY and RZ are cleavable by a restriction endonuclease that generates blunt ends.

[00118] Although restriction sites RY and RZ can be any restriction site known in the art, restriction sites recognized by the Type IIS restriction endonucleases are particularly useful. Type IIS restriction endonucleases have DNA binding domains that are distinct from their cleavage domains. Therefore, they recognize a specific sequence but cleave at a defined distance away. For example, the Type IIS restriction endonuclease SchI (which is also known as MlyI) binds to a recognition site containing the sequence GAGTC and cleaves four (4) base pairs away from the recognition site, creating a blunt ended DNA molecule. Type IIS restriction sites are particularly useful for the preparation of an assembly vector from an entry vector. For example, in a subcloning procedure wherein the DNA segment of an entry vector, for example lacZ, is replaced with a DNA segment of interest, excision of lacZ with a Type IIS restriction endonuclease can result in complete removal of the restriction site recognition sequence. As a result, upon ligation of the DNA segment of interest to the linearized entry vector, extraneous sequence between the annealable linker sequence or the primer binding segment and the newly introduced DNA segment is minimized.

[00119] Thus, in some embodiments, restriction sites RY and RZ are restriction sites recognizable and cleavable by any Type IIS restriction endonuclease known in the art. Suitable Type IIS restriction endonucleases include but are not limited to the following endonucleases and their isoschizomers, which are indicated in parentheses: Alw26I (BsmAI), AlwI (AclWI, BinI), AsuHPI (HphI), BbvI (Bst71I), BcefI, BstF5I (BseGI, FokI), FauI, HgaI, SapI (LguI), MboII, PleI, SapI, SchI (MlyI), SfaNI, and TspRI, AceIII, BbsI (BbvII, BpiI, BpuAI), Bce83I, BciVI, BfiI (BmrI), BpmI (GsuI), BsaI (Eco31I), BseRI, BsgI, BsmBI (Esp3I), BsmFI, BspMI, BsrDI (Bse3DI), Bsu6I (Eam1104I, EarI, Ksp632I), Eco57I, FauI, MmeI, RleAI, TaqII, and Tth111II. In particular embodiments, restriction sites RY and RZ are recognizable and cleavable by the SchI restriction endonuclease.

[00120] In some embodiments, RA and RB are not identical in sequence. In some embodiments, RA and RB are cleavable by the same restriction endonuclease. In some embodiments, RA and RB are identical in sequence. In some embodiments, restriction sites RA and RB are cleavable by a restriction endonuclease that generates staggered ends, *i.e.* termini having a 5' or 3' overhang. In other embodiments, restriction sites RA and RB are cleavable by a restriction endonuclease that generates blunt ends.

[00121] Although restriction sites RA and RB can be any restriction sites known in the art, restriction sites that are relatively infrequent in DNA (*e.g.*, cDNA) of one or more organisms (*i.e.*, an infrequent cutter) are particularly useful. In some embodiments, restriction sites RA and RB are recognizable and cleavable by a restriction endonuclease that has relatively infrequent restriction sites in human DNA. In some embodiments, restriction sites RA and RB are recognizable and cleavable by a restriction endonuclease that has relatively infrequent restriction sites in mouse DNA. In some embodiments, restriction sites RA and RB are recognizable and cleavable by a restriction endonuclease that has relatively infrequent restriction sites in yeast DNA, for example, in the DNA of *Saccharomyces cerevisiae*, *Pichia pastoris*, *Kluyveromyces lactis*, *Arxula adeninivorans*, or *Hansenula polymorpha*. In some embodiments, restriction sites RA and RB are recognizable and cleavable by a restriction endonuclease that has relatively few restriction sites in the DNA of bacteria, for example, in the DNA of *Escherichia coli* or *Bacillus subtilis*.

[00122] In some embodiments, restriction sites RA and RB are recognizable and cleavable by a Type IIS restriction endonuclease wherein the recognition site is distal to the polynucleotide sequence comprising, *e.g.*, PA/LA-D-PB/LB. In some embodiments, each restriction site RA and RB is independently recognizable and cleavable by a restriction endonuclease selected from the group consisting of MssI, NruI (Bsp68I, MluB2I, Sbo13I, SpoI), SnaBI (BstSNI, Eco105I), SrfI, and SwaI (BstRZ246I, BstSWI, MspSWI, SmiI), HpaI, HincII, PshAI, OliI, AluI, Alw26I, BalI, DraI, DpnI, EcoR47III, EcoRCRI, EcoRV, FokI, HaeIII, HincII, MboI, MspA1I, NaeI, RsaI, PvuII, ScaI, SmaI, SspI, StuI, XmnI, EcaBC3I, SciI, HincII, DraI, BsaBI, Cac8I, Hpy8I, MlyI, PshAI, SspD51, BfrBI, BsaAI, BsrBI, BtrI, CdiI, CviJI, CviRI, Eco47III, Eco78I, EcoICRI, FnuDII, FspAI, HaeI, LpnI, MlyI, MslI, MstI, NaeI, NlaIV, NruI, NspBII, OliI, PmaCI, PshAI, PsiI, SrfI, StuI, XcaI, XmnI, ZraI, and isoschizomers thereof. In a particular embodiment, restriction sites RA and

RB are recognizable and cleavable by the SapI or LguI restriction endonuclease. LguI is an isoschizomer of SapI having the same recognition and cleavage specificity.

[00123] In some embodiments, the entry vector provided herein also comprises one or more nucleic acid sequences that generally have some function in the replication, maintenance, or integrity of the vector (*e.g.*, origins of replication) as well as one or more selectable markers. Replication origins are unique polynucleotides that comprise multiple short repeated sequences that are recognized by multimeric origin-binding proteins and that play a key role in assembling DNA replication enzymes at the origin site. Suitable origins of replication for use in the entry and assembly vectors provided herein include but are not limited to *E. coli* oriC, colE1 plasmid origin, 2 μ and ARS (both useful in yeast systems), sfl, SV40 EBV oriP (useful in mammalian systems), or those found in pSC101. Selectable markers can be useful elements in vectors as they provide a means to select for or against growth of cells that have been successfully transformed with a vector containing the selectable marker and express the marker.

[00124] In some embodiments, any vector may be used to construct the entry vector as provided herein. In particular, vectors known in the art and those commercially available (and variants or derivatives thereof) may be engineered to include a restriction site RA, optionally a primer binding segment PA or an annealable linker sequence LA, a restriction site RY, a DNA segment D, a restriction site RZ, optionally a primer binding segment PB or an annealable linker sequence LB, and a restriction site RB, for use in the methods provided herein. Such vectors may be obtained from, for example, Vector Laboratories Inc., InVitrogen, Promega, Novagen, NEB, Clontech, Boehringer Mannheim, Pharmacia, EpiCenter, OriGenes Technologies Inc., Stratagene, Perkin Elmer, Pharmingen, Life Technologies, Inc., and Research Genetics. General classes of vectors of particular interest include prokaryotic and/or eukaryotic cloning vectors, expression vectors, fusion vectors, two-hybrid or reverse two-hybrid vectors, shuttle vectors for use in different hosts, mutagenesis vectors, transcription vectors, vectors for receiving large inserts, and the like. Other vectors of interest include viral origin vectors (M13 vectors, bacterial phage λ vectors, adenovirus vectors, and retrovirus vectors), high, low and adjustable copy number vectors, vectors that have compatible replicons for use in combination in a single host (PACYC184 and pBR322) and eukaryotic episomal replication vectors (pCDM8).

[00125] In particular embodiments, entry vectors for use in accordance with the methods provided herein are the pRYSE vectors, having the nucleotide sequences of SEQ ID

NO: 207 through 221. A schematic of the pRYSE vectors is provided in FIG. 4, and the preparation of the pRYSE vectors is described in Example 1 below.

5.6 Assembly Vectors

[00126] In another aspect, provided herein is a vector, *i.e.*, an assembly vector, that can be used in the assembly of a plurality of component polynucleotides into one or more assembled polynucleotides. In some embodiments, an assembly vector is a circular polynucleotide that comprises a selectable marker, an origin of replication, and a DNA segment flanked by an annealable linker sequence, an annealable linker sequence pair, or by an annealable linker sequence / primer binding segment pair, flanked by a pair of restriction sites. The restriction sites can serve to facilitate excision of the component polynucleotide from the assembly vector backbone during the assembly reaction. Thus, in some embodiments, the assembly vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a primer binding segment PA or an annealable linker sequence LA, a DNA segment D, and a restriction site RB. In some embodiments, the assembly vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a DNA segment D, a primer binding segment PB or an annealable linker sequence LB, and a restriction site RB. In certain embodiments, the assembly vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a primer binding segment PA or an annealable linker sequence LA, a DNA segment D, a primer binding segment PB or an annealable linker sequence LB, and a restriction site RB.

[00127] In some embodiments, the assembly vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a DNA segment D, and a restriction site RB (*i.e.*, 5'-RA-LA-D-RB-3'). In some embodiments, the assembly vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a DNA segment D, an annealable linker sequence LB, and a restriction site RB (*i.e.*, 5'-RA-D-LB-RB-3'). In some embodiments, the assembly vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a DNA segment D, an annealable linker sequence LB, and a restriction site RB (*i.e.*, 5'-RA-LA-D-LB-RB-3'). In some embodiments, the assembly vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a primer binding segment PA, a DNA segment D, an annealable linker sequence LB, and a restriction site RB (*i.e.*, 5'-RA-PA-D-LB-RB-3'). In some embodiments, the assembly vector is a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a DNA segment D, a primer binding segment PB, and a restriction site RB (*i.e.*, 5'-RA-LA-D-PB-RB-3'). Exemplary assembly vectors are provided in FIG. 1B and FIG. 2.

[00128] In some embodiments, the nucleic acid sequence of primer binding segment PA is selected from the group consisting of SEQ ID NOS: 24 and 25. In some embodiments, the nucleic acid sequence of primer binding segment PB is selected from the group consisting of SEQ ID NOS: 24 and 25. In some embodiments, the nucleic acid sequences of primer binding segment PA and primer binding segment PB are selected from the group consisting of SEQ ID NOS: 24 and 25. In preferable embodiments, the nucleic acid sequences of primer binding segment PA and primer binding segment PB are not identical.

[00129] In some embodiments, the nucleic acid sequence of annealable linker sequence LA or LB is at least 24 nucleotides and has a T_m of at least 60 °C. In some embodiments, the nucleic acid sequence of annealable linker sequence LA is selected from the group consisting of SEQ ID NOS: 1 to 23. In some embodiments, the nucleic acid sequence of annealable linker sequence LB is selected from the group consisting of SEQ ID NOS: 1 to 23. In some embodiments, the nucleic acid sequences of annealable linker sequence LA and annealable linker sequence LB are selected from the group consisting of SEQ ID NOS: 1 to 23.

[00130] In some embodiments, RA and RB are not identical in sequence. In some embodiments, RA and RB are cleavable by the same restriction endonuclease. In some embodiments, RA and RB are identical in sequence. In some embodiments, restriction sites RA and RB are cleavable by a restriction endonuclease that generates staggered ends, *i.e.* termini having a 5' or 3' overhang. In other embodiments, restriction sites RA and RB are cleavable by a restriction endonuclease that generates blunt ends.

[00131] Although restriction sites RA and RB can be any restriction sites known in the art, restriction sites that are relatively infrequent in DNA (*e.g.*, cDNA) of one or more organisms (*i.e.*, an infrequent cutter) are particularly useful. In some embodiments, restriction sites RA and RB are recognizable and cleavable by a restriction endonuclease that has relatively infrequent restriction sites in human DNA. In some embodiments, restriction sites RA and RB are recognizable and cleavable by a restriction endonuclease that has relatively infrequent restriction sites in mouse DNA. In some embodiments, restriction sites RA and RB are recognizable and cleavable by a restriction endonuclease that has relatively infrequent restriction sites in yeast DNA, for example, in the DNA of *Saccharomyces cerevisiae*, *Pichia pastoris*, *Kluyveromyces lactis*, *Arxula adeninivorans*, or *Hansenula polymorpha*. In some embodiments, restriction sites RA and RB are recognizable and cleavable by a restriction endonuclease that has relatively few restriction sites in the DNA of bacteria, for example, in the DNA of *Escherichia coli* or *Bacillus subtilis*.

[00132] In some embodiments, restriction sites RA and RB are recognizable and cleavable by a Type IIS restriction endonuclease. Illustrativce examples of suitable Type IIS restriction endonucleases include but are not limited to: MssI, NruI (Bsp68I, MluB2I, Sbo13I, SpoI), SnaBI (BstSNI, Eco105I), SrfI, and SwaI (BstRZ246I, BstSWI, MspSWI, SmiI), HpaI, HincII, PshAI, OliI, AluI, Alw26I, BalI, DraI, DpnI, EcoR47III, EcoRCRI, EcoRV, FokI, HaeIII, HincII, MboI, MspA1I, NaeI, RsaI, PvuII, ScaI, SmaI, SspI, StuI, XmnI, EcaBC3I, SciI, HincII, DraI, BsaBI, Cac8I, Hpy8I, MlyI, PshAI, SspD51, BfrBI, BsaAI, BsrBI, BtrI, CdiI, CviJI, CviRI, Eco47III, Eco78I, EcoICRI, FnuDII, FspAI, HaeI, LpnI, MlyI, MslI, MstI, NaeI, NlaIV, NruI, NspBII, OliI, PmaCI, PshAI, PsiI, SrfI, StuI, XcaI, XmnI, ZraI, or isoschizomers thereof. In a particular embodiment, restriction sites RA and RB are recognizable and cleavable by the SapI or LguI restriction endonuclease.

[00133] Preferably, the DNA segment of an assembly vector does not comprise a nucleic acid sequence that can be recognized and cleaved by a restriction endonuclease that can cleave any of restriction sites RA and RB within the assembly vector. This ensures that the DNA segment remains intact during the first stage of the assembly reaction, during which the component polynucleotide is excised from the assembly vector backbone. In particular embodiments, the DNA segment does not comprise a SapI/LguI site and RA and RB are cleavable by SapI or LguI. Site-directed mutagenesis (*see* Carter, *Bi°Chem. J.* 237:1-7 (1986); Zoller and Smith, *Methods Enzymol.* 154:329-50 (1987)), cassette mutagenesis, restriction selection mutagenesis (Wells *et al.*, *Gene* 34:315-323 (1985)), oligonucleotide-mediated (site-directed) mutagenesis, PCR mutagenesis, or other known techniques can be performed to modify any such sequence within the DNA segment either before or after ligation of the DNA segment to the entry vector.

[00134] In some embodiments, the assembly vector provided herein also comprises one or more nucleic acid sequences that generally have some function in the replication, maintenance, or integrity of the vector (*e.g.*, origins of replication) as well as one or more selectable markers. Replication origins are unique polynucleotides that comprise multiple short repeated sequences that are recognized by multimeric origin-binding proteins and that play a key role in assembling DNA replication enzymes at the origin site. Suitable origins of replication for use in the entry and assembly vectors provided herein include but are not limited to E. coli oriC, colE1 plasmid origin, 2 μ and ARS (both useful in yeast systems), sfl, SV40 EBV oriP (useful in mammalian systems), or those found in pSC101. Selectable markers can be useful elements in vectors as they provide a means to select for or against growth of cells that have been successfully transformed with a vector containing the selectable marker and express the marker.

[00135] In some embodiments, any vector may be used to construct the assembly vector as provided herein. In particular, vectors known in the art and those commercially available (and variants or derivatives thereof) may be engineered to include a restriction site RA, a primer binding segment PA or an annealable linker sequence LA, a DNA segment D, a primer binding segment PB or an annealable linker sequence LB, and a restriction site RB, for use in the methods provided herein. Such vectors may be obtained from, for example, Vector Laboratories Inc., InVitrogen, Promega, Novagen, NEB, Clontech, Boehringer Mannheim, Pharmacia, EpiCenter, OriGenes Technologies Inc., Stratagene, Perkin Elmer, Pharmingen, Life Technologies, Inc., and Research Genetics. General classes of vectors of particular interest include prokaryotic and/or eukaryotic cloning vectors, expression vectors, fusion vectors, two-hybrid or reverse two-hybrid vectors, shuttle vectors for use in different hosts, mutagenesis vectors, transcription vectors, vectors for receiving large inserts, and the like. Other vectors of interest include viral origin vectors (M13 vectors, bacterial phage λ vectors, adenovirus vectors, and retrovirus vectors), high, low and adjustable copy number vectors, vectors that have compatible replicons for use in combination in a single host (PACYC184 and pBR322) and eukaryotic episomal replication vectors (pCDM8).

[00136] An assembly vector can be prepared from an entry vector. To prepare an assembly vector from an entry vector, the entry vector can be digested with one or more restriction endonucleases capable of cleaving RY and RZ thereby linearizing the vector such that it can accept a DNA segment. The DNA segment can be ligated into RY and RZ sites using standard cloning techniques to generate an assembly vector of the invention. For example, the DNA segment may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell, or by PCR amplification and cloning. *See*, for example, Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, 3d. ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001); Glover, D.M. (ed.), *DNA Cloning: A Practical Approach*, 2d. ed., MRL Press, Ltd., Oxford, U.K. (1995).

[00137] An assembly vector can also be prepared from another vector that does not comprise an annealable linker sequence, an annealable linker sequence pair, or an annealable linker sequence / primer binding segment pair flanking the site of insertion of the DNA segment. To prepare an assembly vector from such a vector, the vector can be digested with one or more restriction endonucleases capable of cleaving the vector at a site suitable for insertion of a DNA fragment, *e.g.*, at a multiple cloning site, thereby linearizing the vector such that it can accept a DNA fragment. The DNA fragment to be inserted can be obtained by standard procedures known in the art such as, for example, cloning, chemical synthesis, or PCR amplification. The DNA fragment comprises a DNA segment flanked by an annealable linker sequence, an annealable linker sequence pair or an annealable linker sequence / primer binding segment pair. Thus, in some embodiments, the DNA fragment comprises, in a 5' to 3' orientation, an annealable linker sequence LA or a primer binding segment PA, a DNA segment D, and an annealable linker sequence LB or a primer binding segment PB (*i.e.*, 5'-LA-D-LB-3' or 5'-PA-D-LB-3' or 5'-LA-D-PB-3'). In some embodiments, the DNA fragment comprises, in a 5' to 3' orientation, a DNA segment D, and an annealable linker sequence LB or a primer binding segment PB (*i.e.*, 5'-D-LB-3' or 5'-D-PB-3'). In some embodiments, the DNA fragment comprises, in a 5' to 3' orientation, an annealable linker sequence LA or a primer binding segment PA, and a DNA segment D, (*i.e.*, 5'-LA-D-3' or 5'-PA-D-3'). The DNA fragment can further comprise a pair of restriction sites that flank the annealable linker sequence, the annealable linker sequence pair or the annealable linker sequence / primer binding segment pair and that upon cleavage by a restriction endonuclease produce termini that are compatible with termini produced by linearising the vector into which the DNA fragment is to be inserted. Alternatively, the DNA fragment can generated such that it contains such compatible termini and does not require additional digestion with a restriction endonuclease to produce the compatible termini. Upon ligation of the DNA fragment with the linearized vector to generate an assembly vector, the restriction sites used to generate the compatible termini may be preserved to serve as restriction sites RA and RB of the assembly vector. Alternatively, the ligation may remove the original restriction sites but additional restriction sites may be present in the linearised vector that can serve as restriction sites RA and RB of the assembly vector.

[00138] Exemplary methods for generating an assembly vector from an entry vector (*i.e.*, a pRYSE vector) or from another vector (*i.e.*, a pMULE vector) are provided in Example 6 below.

5.7 Annealable Linker Sequences

[00139] In another aspect, provided herein are annealable linker sequences that flank the DNA segment located within entry vectors and assembly vectors. Annealable linker sequences provide sequence overlap between adjacent component polynucleotides in an assembly reaction, and thus serve to prime a component polynucleotide for assembly into an assembled polynucleotide. Thus, in preferred embodiments, the annealable linker sequences LA and LB of the entry and assembly vectors are optimized to provide efficient and accurate priming to complementary annealable linker sequences during an assembly reaction.

[00140] In some embodiments, the length of an annealable linker sequence is long enough to provide adequate specificity with its complement annealable linker sequence, yet short enough to readily anneal to its complement annealable linker sequence at the annealing temperature of the assembly reaction. In some embodiments the length of an annealable linker sequence is long enough to allow for host cell mediated homologous recombination with its complement annealable linker sequence.

[00141] In some embodiments, the annealable linker sequence is about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, or 80 nucleotides in length. In some embodiments, the annealable linker sequence is at least 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, or 30 nucleotides in length. In some embodiments, the anealable linker sequence is greater than 30, 40, 50, 60, 70, 80, 90, 100, 500, 1000, 5000, or 10,000 nucleotides in length. In some embodiments, the annealable linker is at least 18 nucleotides in length and is a number divisible by three, so as to facilitate read-through transcription of the linker when ligated to an encoding DNA segment. In particular embodiments, the annealable linker is 18, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, 51, 54, 57, or 60 nucleotides in length.

[00142] In some embodiments, an annealable linker sequence has a relatively high melting temperature (T_m), *i.e.,* the temperature at which one half of an annealed annealable linker sequence duplex will dissociate to become single stranded. The T_m of an annealable linker can be calculated according to SantaLucia, PNAS, 95:-1460-1465 (1998) using a nearest neighbor algorithm. A relatively high T_m may provide for more specific priming during an assembly reaction. A relatively high T_m may also allow combination of the annealing and extension steps of PCR or reduce the amount of time needed to adjust temperatures between the annealing and extension steps of PCR and thus enable greater efficiency in using the assembly methods of the invention. Thus, in some embodiments, an annealable linker sequence duplex has a T_m of about 60 °C – 80 °C. In some embodiments, an annealable linker sequence duplex has a T_m of about 65 °C – 75 °C. In some embodiments, an annealable linker sequence duplex has a T_m of greater than 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C, 85 °C, or 90 °C.

[00143] In some embodiments, annealable linker sequences do not form appreciable secondary structures (*e.g.*, hairpins, self-dimers) produced via intramolecular (*i.e.*, within the same molecule) interactions under the conditions of the methods described herein, either at the DNA level or at the RNA level or at both the DNA and the RNA level. The presence of secondary structures in DNA can lead to poor or no assembled polynucleotide yield of the assembly reaction. The presence of secondary structures in RNA can lead to decreased translation efficiencies, which are of particular concern when the annealable linker sequence is used to assemble component polynucleotides comprising a promoter and a protein coding sequence inco a assembled polynucleotide in which the annealable linker sequence is positioned between the promoter and the protein coding sequence. Accordingly, annealable linker sequences useful in the assembly methods of the invention are designed to not form secondary RNA and/or DNA structures. The ability of an annealable linker sequence to form secondary RNA or DNA structures can be determined using software tools such as, for example, IDT Oligo Analyzer (Integrated DNA Technologies, Coralville, IA), mFold (Zuker 2003 *Nucleic Acids Res.* 31 (13), 3406-15), or RNAfold (Hofacker & Stadler (2006) *Bioinformatics* 22 (10): 1172–6). In general, these tools calculate the Gibbs free energy (ΔG) for transition of a sequence from the linear to the folded state. The larger ΔG, the less likely that the sequence will form a secondary structure. Accordingly, in some embodiments, annealable linker sequences are designed to have large ΔG values for the transition from linear to folded states. In some embodiments, annealable linker sequences are designed to have ΔG values for the transition from linear to folded states that are equal to or greater than the ΔG values for the transition from linear to folded states of the n-bases that lie immediately upstream of the coding sequences of highly expressed genes in the *Saccharomyces cerevisiae* genome, wherein n represents an integer that corresponds to the number of bases in the annealable linker sequence. In some embodiments, annealable linker sequences are 36 bases long and have a ΔG value for the transition from linear to folded states of -1 or greater.

[00144] In some embodiments, annealable linker sequences are also designed to avoid unintended intermolecular interactions (*i.e.*, between different molecules). Thus, in some embodiments, an annealable linker sequence does not anneal substantially with any other sequences within the assembly vector that contains the annealable linker sequence (*e.g.*, vector backbone sequences) and/or with any other sequences within other assembly vectors of the assembly compositions aside from the complementary annealable linker sequences required for polynucleotide assembly by the methods provided herein. In some embodiments, an annealable linker sequence does not anneal substantially with other

annealable linker sequences within assembly vectors of the assembly compositions provided herein.

[00145] In some embodiments, an annealable linker sequence has a high G-C content, *i.e.*, the number of guanine and cytosine nucleotides in the annealable linker sequence as a percentage of the total number of bases in the annealable linker sequence. Annealable linker sequences that have a high G-C content are generally useful in the methods of the invention because a high G-C content generally provides for a high T_m, which in turn may provide for more specific priming during an assembly reaction and for time and process savings by allowing combination of the annealing and extension steps of SOE/PCR. In some embodiments, the G-C content of the annealable linker sequence is between about 20-80%. In some embodiments, the G-C content of the annenalable linker sequence is between about 40-60%. In some embodiments, the G-C content of the annealable linker sequence is about 40, 45, 50, 55, 60, or 70%. In particular embodiments, an annealable linker sequence has a G-C content of greater than 70%. Illustrative examples of annealable linker sequences that have a high G-C content, do not form appreciable secondary DNA structures, and have a T_m of 70 °C or greater are SEQ ID NOS: 1 to 8.

[00146] In some embodiments, an annealable linker sequence has a high A-T content, *i.e.*, the number of adenine and thymine nucleotides in the annealable linker sequence as a percentage of the total number of bases in the annealable linker sequence. A high A-T content may provide for reduced propensity of the annealable linker sequence to form substantial secondary structures, which may be of particular concern when the annealable linker sequence is used to assemble component polynucleotides comprising a promoter and a protein coding sequence into a assembled polynucleotide in which the annealable linker sequence is positioned between the promoter and the protein coding sequence. In some embodiments, the A-T content of the annealable linker sequence is between about 20-80%. In some embodiments, the A-T content of the annealable linker sequence is between about 40-60%. In some embodiments, the A-T content of the annealable linker sequence is about 30, 35, 40, 45, 50, 55, or 60%. In some embodiments, the annealable linker sequence has an A-T content of greater than 30%. In some embodiments, the sequence of the 3'-most 26 bases of an annealable linker sequence fulfills the following consensus motif: 5'-ANNNNNNNNANNNAANTANNTTNANA-3', wherein A stands for adenine, N for any nucleotide, and T for thymine. This consensus motif is frequently found in the 26 bases that lie upstream of the start codons of highly expressed genes in the genome of *Saccharomyces cerevisiae*. Illustrative examples of annealable linker sequences that comprise this consensus motif, have a relatively high A-T content, do not form appreciable secondary RNA or DNA structures, and have a T_m of 65 °C or greater are SEQ ID NOS: 9 to 23.

[00147] In some embodiments, an annealable linker sequence comprises one or more restriction sites. Incorporation of restriction sites into an annealable linker sequence allows for the excision of a DNA segment from an entry or assembly vector while maintaining the restriction sites RA and RB within the entry vector or assembly vector. Restriction sites within the annealable linker sequence also facilitate directional subcloning of DNA segments into other entry or assembly vectors. This feature facilitates the efficient construction of assembly vectors comprising the same DNA segment but having different annealable linker sequence pairs or primer binding segment / annealable linker sequence pairs, for instance, to generate a library of assembly vectors comprising different annealable linker sequence pairs as described below. This feature can also obviate the need to re-amplify and sequence a DNA segment to create additional assembly vectors comprising the DNA segment. Thus, in some embodiments, the annealable linker sequence comprises a unique restriction site. In some embodiments, the restriction site is a 7-base pair restriction site, *i.e.*, is cleavable by a restriction endonuclease that recognizes a 7-base pair nucleotide sequence. In some embodiments, the restriction site is a 8-base pair restriction site. In particular embodiments, the restriction site within the annealable linker sequence is recognized and cleavable by MreI, FseI, SbfI, AsiSI, NotI, AscI, or BbvCI.

[00148] In some embodiments, the annealable linker sequence comprises a sequence that allows for read-through transcription once the linker is ligated to an encoding DNA segment. In some embodiments, an annealable linker sequence allows for read-through transcription in both the 5' to 3' and 3' to 5' orientation. In these embodiments, the length of the annealable linker sequence, preferably, is a number of nucleotides divisible by three (3).

[00149] In particular embodiments, an annealable linker sequence does not comprise codons that are rarely used in *Escherichia coli* (*E. coli*) or *Saccharomyces cerevisiae* (*S. cerevisiae*). Efficient expression of heterologous genes in *E. coli* or *S. cerevisiae* can be adversely affected by the presence of infrequently used codons, and expression levels of the heterologous protein often rise when rare codons are replaced by more common ones. *See, e.g.*, Williams *et al.*, *Nucleic Acids Res*. 16: 10453-10467, 1988 and Hoog *et al.*, *Gene* 43: 13-21, 1986. Accordingly, an annealable linker sequence that comprises a read-through sequence preferably does not comprise rare codons used in *E. coli* or *S. cerevisiae,* so as to enable efficient expression of proteins encoded by a assembled polynucleotide comprising the annealable linker sequence.

[00150] In some embodiments, the set of annealable linker sequences are unique sequences that are not found in an intended host organism. In some embodiments, the set of annealable linker sequences are unique sequences that are not found in E. coli. In other embodiments, the set of annealable linker sequences are unique sequences that are not found in S. cerevisiase.

[00151] In some embodiments, suitable annealable linker sequences are identified in a test assembled polynucleotide. A test assembled polynucleotide comprises the annealable linker sequence to be tested and additional elements that permit testing of the annealable linker sequence. For example, to test whether an annealable linker is suitable for assembling a first component polynucleotide comprising a promoter sequence and a second component polynucleotide comprising a protein coding sequence to be put under the control of the promoter in the assembled polynucleotide, a test assembled polynucleotide can be assembled from the first component polynucleotide comprising, in a 5' to 3' orientation, a primer binding segment or an annealable linker sequence, a DNA segment comprising the promoter, and the annealable linker sequence to be tested, and the second component polynucleotide comprising, in a 5' to 3' orientation, the annealable linker sequence to be tested, a DNA segment encoding a reporter gene (*e.g.*, green fluourescent protein (GFP)), and a primer binding segment or annealable linker sequence. The test assembled polynucleotide can be tested *in vivo* or *in vitro* for the efficiency of expression of the reporter gene. Similar test assembled polynucleotides can be assembled to test the suitability of annealable linker sequences for assembling component polynucleotides comprising DNA segments comprising other elements, such as an enhancer, terminator, poly-A tail, nuclear localization signal, mRNA stabilization signal, selectable marker, epitope tag coding sequence, degradation signal, and the like. The test assembled polynucleotide may comprise additional component polynucleotides that enable testing, such as for example, genomic targeting sequences and selectable markers that enable introduction of the test assembled polynucleotide into host cells and selection of positive transformants for *in vivo* testing.

[00152] Table 1 presents the T_m, restriction sites, and read-through amino acids of exemplary annealable linker sequences corresponding to SEQ ID NOS: 1-23.

Table 1 - Sequence and Characteristics of Annealable Linker Sequences								
Annealable Linker Sequence	Seq. Name	Length (bases)	% G-C	% A-T	Melt Temp. (T_m)	Restriction Enzyme Site	Read-Through Amino Acids	
							Fwd	Rev
SEQ ID NO: 1	RYSE 1	24	79.2	20.8	72.4			
SEQ ID NO: 2	RYSE 2	24	75.0	25.0	71.4	MreI		
SEQ ID NO: 3	RYSE 3	24	75.0	25.0	73.7	FseI		TAGQA RGD
SEQ ID NO: 4	RYSE 4	24	70.8	29.2	71.5	SbfI	NLQA ASAD	IGARG LQV
SEQ ID NO: 5	RYSE 5	24	70.8	29.2	71.2	AsiSI	NAIAD AAD	IGGVG DRV
SEQ ID NO: 6	RYSE 6	24	70.8	29.2	70.9	NotI	KAAA GEGD	ISLASG RL
SEQ ID NO: 7	RYSE 7	24	70.8	29.2	71.5	AscI	KARH GRRD	
SEQ ID NO: 8	RYSE 8	24	75.0	25.0	70.7	BbvCI		
SEQ ID NO: 9	RYSE 9	36	50.0	50.0	67.4			
SEQ ID NO: 10	RYSE 10	36	52.8	47.2	67.7			
SEQ ID NO: 11	RYSE 11	36	58.3	41.7	69.2			
SEQ ID NO: 12	RYSE 12	36	50.0	50.0	67.4			
SEQ ID NO: 13	RYSE 13	36	58.3	41.7	69.4			
SEQ ID NO: 14	RYSE 14	36	52.8	47.2	67.4			
SEQ ID NO: 15	RYSE 15	36	52.8	47.2	67.8			
SEQ ID NO: 16	RYSE 16	36	52.8	47.2	67.8			
SEQ ID NO: 17	RYSE 17	36	52.8	47.2	68.4			
SEQ ID NO: 18	RYSE 18	36	50.0	50.0	67.8			
SEQ ID NO: 19	RYSE 19	36	52.8	47.2	68.1			
SEQ ID NO: 20	RYSE 20	36	55.6	44.4	68.3			
SEQ ID NO: 21	RYSE 21	36	55.6	44.4	67.9			
SEQ ID NO: 22	RYSE 22	36	52.8	47.2	67.4			

SEQ ID NO: 23	RYSE 23	36	55.6	44.4	68.8			

5.8 Libraries

[00153] In another aspect, provided herein is a library comprising a plurality of assembly vectors. The library can serve to facilitate the efficient assembly of a plurality of component polynucleotides into one or more assembled polynucleotides that are functional in prokaryotes or eukaryotes, and thus facilitate the generation of unique organisms, *e.g.*, recombinant strains of bacteria or yeast, without the need for time-consuming restriction endonuclease and ligase enzyme based cloning techniques. The assembly methods and compositions provided herein can facilitate the efficient replacement or introduction of functional DNA units, *e.g.*, promoters, enhancers, origins of replication, *etc.*, within an expression construct, and thus can provide for efficient optimization of the replication of, and/or expression from, the expression construct within a host organism.

[00154] The library may comprise a plurality of assembly vectors assembled within a single composition or container, *e.g.*, a composition or container suitable for performing the assembly methods provided herein. Alternatively, the library may comprise a plurality of assembly vectors that are not assembled within the same composition or container. In some embodiments, the library comprises at least 3, at least 6, at least 10, at least 20, at least 50, or more than 50 assembly vectors, each comprising a DNA segment.

[00155] In some embodiments, the library comprises a plurality of assembly vectors wherein each of the assembly vectors comprises, in a 5' to 3' orientation, a first restriction site RA, a DNA segment D, an annealable linker sequence LB, and a second restriction site RB. In some embodiments, the library comprises a plurality of assembly vectors wherein each of the assembly vectors comprises, in a 5' to 3' orientation, a first restriction site RA, a primer binding segment PA or a first annealable linker sequence LA, a DNA segment D, and a second restriction site RB. In some embodiments, the library comprises a plurality of assembly vectors wherein each of the assembly vectors comprises, in a 5' to 3' orientation, a first restriction site RA, a first annealable linker sequence LA, a DNA segment D, an annealable linker sequence LB or a primer binding segment PB, and a second restriction site RB. In some embodiments, the annealable linker sequence pair or annealable linker sequence / primary binding segment pair within each assembly vector of the library does not comprise the same sequence. In some embodiments, the nucleic acid sequence of the annealable linker sequence LA and/or LB within each assembly vector is selected from the group consisting of SEQ ID NOS: 1 to 23. In some embodiments, the nucleic acid sequence of the primer binding segment PA or PB within each assembly vector is selected from the group consisting of SEQ ID NOS: 24 and 25.

[00156] In some embodiments, the library comprises at least one of each of the following vectors:

(a) a vector that consists of a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a DNA segment D, an annealable linker sequence LB, and a restriction site RB;

(b) a vector that consists of a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a DNA segment D, an annealable linker sequence LB, and a restriction site RB; and

(c) a vector that consists of a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a DNA segment D, and a restriction site RB_0.

[00157] In some embodiments, the library comprises at least one of each of the following vectors:

(a) a vector that consists of a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, a primer binding segment PA, a DNA segment D, an annealable linker sequence LB, and a restriction site RB;

(b) a vector that consists of a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a DNA segment D, an annealable linker sequence LB, and a restriction site RB; and

(c) a vector that consists of a circular polynucleotide that comprises, in a 5' to 3' orientation, a restriction site RA, an annealable linker sequence LA, a DNA segment D, a primer binding segment PB, and a restriction site RB_0.

[00158] In some embodiments, the nucleic acid sequence of primer binding segment PA is selected from the group consisting of SEQ ID NOS: 24 and 25. In some embodiments, the nucleic acid sequence of primer binding segment PB is selected from the group consisting of SEQ ID NOS: 24 and 25. In some embodiments, the nucleic acid sequences of primer binding segment PA and primer binding segment PB are selected from the group consisting of SEQ ID NOS: 24 and 25.

[00159] In some embodiments, the nucleic acid sequence of any of the annealable linker sequences LA and annealable linker sequneces LB in the library are selected from the group consisting of SEQ ID NOS: 1 to 23. In some embodiments, the nucleic acid sequences of at least one of the annealable linker sequences LA and at least one of the annealable linker sequences LB in the library are selected from the group consisting of SEQ ID NOS: 1 to 23. In some embodiments, the nucleic acid sequence of each of the annealable linker sequences LA and annealable linker sequences LB in the library is selected from the group consisting of SEQ ID NOS: 1 to 23.

[00160] In some embodiments, the DNA segment D comprises a nucleic sequence selected from the group consisting of a selectable marker, a promoter, a genomic targeting sequence, a nucleic acid sequence encoding an epitope tag, a nucleic acid sequence encoding a gene of interest, a nucleic acid sequence encoding a termination codon, and lacZ.

[00161] In some embodiments, the library comprises at least one of each of the following nucleic acid molecules:

(a) a first nucleic acid molecule wherein the first nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_0, any DNA segment selected from the group D_0, an annealable linker sequence LB_0, and a second restriction site RB_0;

(b) an intermediate nucleic acid molecule wherein the intermediate nucleic acid molecule n is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_n, a first annealable linker sequence LA_n, any DNA segment selected from the group D_n, a second annealable linker sequence LB_n, and a second restriction site RB_n, and wherein n represents an integer from one to the number of intermediate nucleic acid molecules; and

(c) a last nucleic acid molecule wherein the last nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_m, an annealable linker sequence LA_m, any DNA segment selected from the group D_m, a second restriction site RB_m wherein m represents an integer one greater than the number of intermediate nucleic acid molecules;

whereupon cleavage of restriction sites RA_0 through RB_m and denaturation of the resulting linear nucleic acid molecules, each annealable linker sequence $LB_{(p-1)}$ is capable of hybridizing to the complement of annealable linker sequence LA_p wherein p represents the integers from 1 to m, and wherein each group D_0,... D_n,...and D_m consists of one or more DNA segments. In some embodiments, a first nucleic acid molecule further comprises a primer binding segment PA positioned 5' to the DNA segment selected from the group D_0. In some embodiments, a last nucleic acid molecules further comprises a primer binding segment PB positioned 3' to the DNA segment selected from the group D_m.

[00162] In some embodiments, upon cleavage of restriction sites RA_0 through RB_m and denaturation of the resulting linear nucleic acid molecules, each annealable linker sequence $LB_{(p-1)}$ is capable of selectively hybridizing to the complement of annealable linker sequence LA_p compared to the other annealable linker seuences, or their complements, in the components composition. In some embodiments, each annealable linker sequence $LB_{(p-1)}$ is identical in sequence to annealable linker sequence LA_p.

[00163] In a particular embodiment, the restriction sites RA_0 through RB_m are cleavable by the same restriction endonuclease so as to facilitate excision of the component polynucleotides from the assembly vectors. In some embodiments, the restrictions sites RA_0 through RB_m are cleavable by SapI and LguI restriction endonucleases.

[00164] In some embodiments, the nucleic acid sequence of primer binding segment PA is selected from the group consisting of SEQ ID NOS: 24 and 25. In some embodiments, the nucleic acid sequence of primer binding segment PB is selected from the group consisting of SEQ ID NOS: 24 and 25. In some embodiments, the nucleic acid sequences of primer binding segment PA and primer binding segment PB are selected from the group consisting of SEQ ID NOS: 24 and 25. In preferable embodiments, the nucleic acid sequences of primer binding segment PA and primer binding segment PB are not identical.

[00165] In some embodiments, the nucleic acid sequence of any of the annealable linker sequences LA and annealable linker sequences LB in the library is selected from the group consisting of SEQ ID NOS: 1 to 23. In some embodiments, the nucleic acid sequences of at least one of the annealable linker sequences LA and at least one of the annealable linker sequences LB in the library are selected from the group consisting of SEQ ID NOS: 1 to 23. In some embodiments, the nucleic acid sequence of each of the annealable linker sequences LA and annealable linker sequences LB in the library is selected from the group consisting of SEQ ID NOS: 1 to 23. In some embodiments, the nucleic acid sequence of each of the annealable linker sequences LA in the composition are not identical to one another. In some embodiments, the nucleic acid sequence of each of the annealable linker sequences LB in the composition are not identical to one another.

[00166] In a particular embodiment, the library comprises the following nucleic acid molecules:

(a) two first nucleic acid molecules, wherein one first nucleic acid molecule comprises, in a 5' to 3' orientation, a first restriction site RA_0, a primer binding segment PA, a DNA segment D_{01}, an annealable linker sequence LB_0, and a second restriction site RB_0, wherein another first nucleic acid molecule comprises, in a 5' to 3' orientation, a first restriction site RA_0, a primer binding segment PA, a DNA segment D_{02}, an annealable linker sequence LB_0, and a second restriction site RB_0, wherein DNA segment D_{01} encodes a first genomic targeting sequence, wherein DNA segment D_{02} encodes a second genomic targeting sequence located downstream of the first genomic targeting sequence in a target genome, and wherein DNA segment D_{02} is positioned in opposite orientation as DNA segment D_{01} relative to primer binding segment PA and annealable linker sequence LB_0;

(b) at least one intermediate nucleic acid molecule comprising, in a 5' to 3' orientation, a first restriction site RA_n, a first annealable linker sequence LA_n, a DNA segment D_n, a second annealable linker sequence LB_n, and a second restriction site RB_n, wherein n represents an integer from one to the number of intermediate nucleic acid molecules; and

(c) two last nucleic acid molecules, wherein one last nucleic acid molecule comprises, in a 5' to 3' orientation, a first restriction site RA_m, an annealable linker sequence LA_m, a DNA segment D_{m1}, a primer binding segment PB, and a second restriction site RB_m, wherein another last nucleic acid molecule comprises, in a 5' to 3' orientation, a first restriction site RA_m, an annealable linker sequence LA_m, a DNA segment D_{m2}, a primer binding segment PB, and a second restriction site RB_m, wherein m represents an integer one greater than the number of intermediate nucleic acid molecules, wherein DNA segment D_{m1} encodes a first segment of a selectable marker, wherein DNA segment D_{m2} encodes a second segment of the selectable marker, wherein DNA segment D_{m2} is positioned in opposite orientation as DNA segment D_{m1} relative to annealable linker sequence LA_m and primer binding segment PB, wherein neither DNA segment D_{m1} nor DNA segment D_{m2} produces a functional selectable marker but whereupon homologous recombination of DNA segments D_{m1} and D_{m2} a functional selectable marker is generated;

wherein each annealable linker sequence $LB_{(p-1)}$ is identical to annealable linker sequence LA_p, wherein p represents the integers from 1 to m.

[00167] In some embodiments, the library comprises a plurality of assembly vectors wherein each assembly vector comprises the same annealable linker sequence, annealable linker sequence pair or annealable linker sequence / primary binding segment pair but differs in the sequence of their respective DNA fragment D.

[00168] In other embodiments, the library comprises a plurality of assembly vectors wherein each assembly vector comprises the same DNA segment D flanked by a unique annealable linker sequence, annealable linker sequence pair or annealable linker sequence / primer binding segment pair. Such a library may serve to facilitate the rapid assembly of DNA segment D into a particular position or orientation relative to the other DNA segments being assembled into the assembled polynucleotide.

[00169] In some embodiments, the members of the library comprise DNA segments that have shared structural or functional characteristics. For example, a library can comprise a plurality of assembly vectors comprising the same functional DNA unit. Exemplary functional DNA units include but are not limited to protein-coding sequences, reporter genes, fluorescent markers, promoters, enhancers, terminators, introns, exons, poly-A tails, multiple cloning sites, nuclear localization signals, nuclear export signals, mRNA stabilization signals, selectable markers, integration loci, epitope tags, and degradation signals. In some embodiments, the library comprises a plurality of assembly vectors wherein each assembly vector comprises the same promoter. The assembly vectors can comprise any prokaryotic or eukaryotic promoter sequence known in the art. Exemplary eukaryotic promoters include but are not limited to a metallothionein promoter, a constitutive adenovirus major late promoter, a dexamethasone-inducible MMTV promoter, a SV40 promoter, a MRP po*l III* promoter, a constitutive MPSV promoter, an RSV *prom*oter, a tetracycline-inducible CMV promoter (such as the human immediate-early CMV promoter), and a constitutive CMV promoter. In particular embodiments, the assembly vectors comprise a yeast promoter sequence. Exemplary yeast promoters include *b*ut are not limited to PGAL3, PGAL7, PCTR3, PMET3, PPGK1, PTDH1, PTDH3, PFBA1, PTEF1, PENO1, PENO2, PCYC1, PTDH2, PCUP1, PGAL80, PGAL2, PBNA6, PTMA29, PSBP1, PPUP3, PACS2, PTPO1, PRPT1, PAAT2, PAHP1, PSSE1, PTEF2, PNPL3, PPET9, PTUB2, POLE1, PCPR1, PIPPP1, and PSOD1.

[00170] In some embodiments, the library comprises a plurality of assembly vectors wherein each assembly vector comprises the same terminator sequence. The assembly vectors can comprise any prokaryotic or eukaryotic terminator sequence known in the art. In particular embodiments, the assembly vectors comprise a yeast terminator sequence. Exemplary yeast terminators include but are not limited to TADH1, TENO1, TENO2, TCYC1, TNDT80, TTDH3, TTDH1, and TPGK1.

[00171] In some embodiments, the library comprises a plurality of assembly vectors wherein each assembly vector comprises the same selectable marker. The assembly vectors can comprise any prokaryotic or eukaryotic selectable marker known in the art. Examples of selectable markers include but are not limited to antibiotic resistance markers (*e.g.*, genes encoding resistance to kanamycin, ampicillin, chloramphenicol, gentamycin, or trimethoprim) and metabolic markers (*e.g.*, amino acid synthesis genes or transfer RNA genes).

5.9 Kits

[00172] In another aspect, provided herein is a kit for the assembly of a polynucleotide, said kit comprising two or more of the following: (a) one or more entry vectors described herein; (b) one or more restriction endonucleases capable of cleaving the restriction sites RA and RB of said one or more entry vectors; (c) one or more restriction endonucleases capable of cleaving the restriction sites RY and RZ of said entry vectors; and (d) oligonucleotide primers capable of annealing to primer binding segments PA and PB of said one or more entry vectors.

[00173] In some embodiments, restriction sites RA and RB of each entry vector of the kit are recognizable and cleavable by SapI restriction endonuclease, and the kit comprises SapI restriction endonuclease. In some embodiments, restriction sites RY and RZ of each entry vector of the kit are recognizable and cleavable by SchI (or MlyI) restriction endonuclease, and the kit comprises SchI (or MlyI) restriction endonuclease.

[00174] In some embodiments, the nucleic acid sequence of primer binding segment PA of one or more entry vectors in the kit is selected from the group consisting of SEQ ID NOS: 24 and 25. In some embodiments, the nucleic acid sequence of primer binding segment PB one or more entry vectors in the kit is selected from the group consisting of SEQ ID NOS: 24 and 25. In preferable embodiments, the nucleic acid sequences of primer binding segment PA and primer binding segment PB are not identical.

[00175] In some embodiments, the nucleic sequence of annealable linker sequence LA of one or more entry vectors in the kit is selected from the group consisting of SEQ ID NOS: 1 to 23. In some embodiments, the nucleic sequence of annealable linker sequence LB one or more entry vectors in the kit is selected from the group consisting of SEQ ID NOS: 1 to 23. In some embodiments, the nucleic sequences of annealable linker sequence LA and annealable linker sequence LB of all the entry vectors in the kit are selected from the group consisting of SEQ ID NOS: 1 to 23.

[00176] In some embodiments, the kit comprises pRYSE vector #1, the sequence of which is provided herein as SEQ ID NO: 221. In some embodiments, the kit comprises pRYSE vector #2, the sequence of which is provided herein as SEQ ID NO: 207. In some embodiments, the kit comprises pRYSE vector #3, the sequence of which is provided herein as SEQ ID NO: 208. In some embodiments, the kit comprises pRYSE vector #4, the sequence of which is provided herein as SEQ ID NO: 209. In some embodiments, the kit comprises pRYSE vector #5, the sequence of which is provided herein as SEQ ID NO: 210. In some embodiments, the kit comprises pRYSE vector #6, the sequence of which is provided herein as SEQ ID NO: 211. In some embodiments, the kit comprises pRYSE vector #7, the sequence of which is provided herein as SEQ ID NO:212. In some embodiments, the kit comprises pRYSE vector #8, the sequence of which is provided herein as SEQ ID NO: 213. In some embodiments, the kit comprises pRYSE vector #9, the sequence of which is provided herein as SEQ ID NO: 214. In some embodiments, the kit comprises pRYSE vector #10, the sequence of which is provided herein as SEQ ID NO: 215. In some embodiments, the kit comprises pRYSE vector #11, the sequence of which is provided herein as SEQ ID NO: 216. In some embodiments, the kit comprises pRYSE vector #12, the sequence of which is provided herein as SEQ ID NO: 217. In some embodiments, the kit comprises pRYSE vector #13, the sequence of which is provided herein as SEQ ID NO: 218. In some embodiments, the kit comprises pRYSE vector #14, the sequence of which is provided herein as SEQ ID NO:219. In some embodiments, the kit comprises pRYSE vector #15, the sequence of which is provided herein as SEQ ID NO: 220.

[00177] In some embodiments, the kit further comprises instructions for use that describe the polynucleotide assembly method disclosed herein. In some embodiments, a polynucleotide polymerase, such as a thermostable DNA polymerase (*e.g.*, Pfu DNA polymerase), and deoxyribonucleoside triphosphates (dNTPs) are also present in the kit. In some embodiments, two or more assembly vectors each comprising a component polynucleotide to be assembled into an assembled polynucleotide may be provided in the kit. For example, assembly vectors may be provided that comprise a component polynucleotide useful for calibration and/or for use as a positive control to verify correct performance of the kit. Other examples include but are not limited to assembly vectors comprising as a component polynucleotide a protein-coding sequence, reporter gene, fluorescent marker coding sequence, promoter, enhancer, terminator, intron, exon, poly-A tail, multiple cloning site, nuclear localization signal, mRNA stabilization signal, selectable marker, integration loci, epitope tag coding sequence, and degradation signal.

6. EXAMPLES

[00178] The invention is illustrated by the following examples, which are not intended to be limiting in any way. The *Saccharyomices cerevisiae* constructs described in the Examples were derived from *Saccharyomices cerevisiae* strain CEN.PK2. Unlike *Saccharyomices cerevisiae* strain S288c, the genomic sequence of strain CEN.PK2 is not publically available. Some of the constructs described were sequence-verified, and so the sequences provided are those of the actual CEN.PK2-derived constructs. For constructs that were not sequence-verified, the sequences provided are based on the published genomic sequence of strain S288c, and thus may include polymorphic differences to the sequences of the actual CEN.PK2-derived constructs.

Example 1

[00179] This example describes methods for making pRYSE vectors. pRYSE vectors comprise, in a 5' to 3' orientation, a first SapI restriction enzyme recognition site, a first annealable linker sequence or primer binding segment, a first SchI restriction enzyme recognition site, a green fluourescent protein (GFP) or lacZ marker gene, a second SchI restriction enzyme recognition site, a second annealable linker sequence or primer binding segment, and a second SapI restriction enzyme recognition site.

[00180] A DNA fragment encoding β-lactamase was PCR amplified from the pUC19 vector (GenBank accession L09137) using primers JCB158-17C (SEQ ID NO: 229) and JCB158-17D (SEQ ID NO: 230) after the SchI restriction enzyme recognition site in the bla gene of pUC19 had been removed by site-directed mutagenesis of pUC19 using PCR primers JCB158-17A (SEQ ID NO: 227) and JCB158-17B (SEQ ID NO: 228). The PCR product was gel purified, and then ligated into the TOPO vector (Invitrogen, Carlsbad, CA), from which it was liberated again by digesting the construct to completion using SphI and MfeI restriction enzymes, yielding the "bla DNA fragment".

[00181] DNA fragments 1040 (SEQ ID NO: 224), 1041 (SEQ ID NO: 225), and 1042 (SEQ ID NO: 226) were generated synthetically (Biosearch Technologies, Novato, CA). DNA fragments 1040 and 1041 were digested to completion using *BstXI* restriction enzyme, and each digested fragment was ligated with the 2.65 kb vector backbone that was generated by cutting to completion pAM1466 (SEQ ID NO: 223; generated synthetically by Biosearch Technologies, Novato, CA) using restriction enzymes *SacI* and *KpnI*. The 1040_pAM1466 DNA construct was digested to completion using *BsmBI* and *BstXI* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, and an approximately 3.5 kb DNA fragment comprising the 1040 DNA fragment was gel purified. The 1041_pAM1466 DNA construct was digested to completion using *BsaI* and *BstXI* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, and an approximately 0.9 kb 1041 DNA fragment comprising the 1041 DNA fragment was gel purified. The purified DNA fragments were ligated, yielding DNA construct 1040_1041_pAM1466. DNA fragment 1042 was joined to DNA construct 1040_1041 by a PCR "stitching" reaction using primers JO36 (SEQ ID NO: 69) and JO37 (SEQ ID NO: 70) to generate the 1040_1041 DNA fragment, primers JO38 (SEQ ID NO: 71) and JO39 (SEQ ID NO: 72) to generate the 1042 DNA fragment with a terminal sequence that overlapped a terminal sequence of the 1040_1041 DNA fragment, and primers JO39 (containing a *SphI* restriction enzyme recognition site) (SEQ ID NO: 72) and JO36 (containing a *MfeI* restriction enzyme recognition site) (SEQ ID NO: 69) to join the two PCR products. The 1040_1041_1042 PCR product was digested to completion using *SphI* and *MfeI* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 2.4 kb 1040_1041_1042 DNA fragment was gel purified, and the purified DNA fragment was ligated to the gel purified *bla* fragment, yielding the "1040_1041_1042_bla" DNA construct.

[00182] The segment of the 1040_1041_1042_bla DNA construct encoding the GFP gene was PCR amplified using PCR primers 1 and 2 (see Table 2). To the amplified GFP fragment terminal *SacI* and *XhoI* restriction enzymes recognition sites were added by PCR amplification using as templates the gel-extracted GFP fragments generated in the first round of PCR reactions, and PCR primers 3 and 4 (see Table 2). The amplified PCR products were gel extracted, then digested to completion using *XhoI* and *SacI* restriction enzymes, the restriction enzymes were heat inactivated for 20 minutes at 65°C, and the digested PCR products were column purified and then ligated with the gel purified approximately 2.2 kb DNA fragment that resulted from digesting the 1040_1041_1042_bla DNA construct to completion using *XhoI* and *SacI* restriction enzymes. The resulting vectors were PCR amplified using PCR primers 5 and 6 (see Table 3), the reaction mixtures were resolved by gel electrophoresis, and the approximately 2.2 kb "pRYSE vector backbones" were gel purified.

Table 2 - PCR Primers used to Generate GFP Inserts Flanked by Annealable Linker Pairs or Annealable Linker / Primer Binding Segment Pairs and SacI and XhoI Restriction Enzyme Sites

GFP Fragment	Annealable Linker or Primer Binding Segment 1	Annealable Linker or Primer Binding Segment 2	Primer 1	Primer 2	Primer 3	Primer 4
1	Pme1-5'	RYSE 1	J018 (SEQ ID NO: 73)	J073 (SEQ ID NO: 106)	J055 (SEQ ID NO: 88)	J064 (SEQ ID NO: 97)
2	RYSE 1	RYSE 2	J019 (SEQ ID NO: 74)	J074 (SEQ ID NO: 107)	J056 (SEQ ID NO: 89)	J065 (SEQ ID NO: 98)
3	RYSE 2	RYSE 3	J020 (SEQ ID NO: 75)	J029 (SEQ ID NO: 82)	J057 (SEQ ID NO: 90)	J066 (SEQ ID NO: 99)
4	RYSE 3	RYSE 4	J021 (SEQ ID NO: 76)	J030 (SEQ ID NO: 83)	J058 (SEQ ID NO: 91)	J067 (SEQ ID NO: 100)
5	RYSE 4	RYSE 5	J022 (SEQ ID NO: 77)	J031 (SEQ ID NO: 84)	J059 (SEQ ID NO: 92)	J068 (SEQ ID NO: 101)
6	RYSE 5	RYSE 6	J023 (SEQ ID NO: 78)	J032 (SEQ ID NO: 85)	J060 (SEQ ID NO: 93)	J069 (SEQ ID NO: 102)
7	RYSE 6	RYSE 7	J024 (SEQ ID NO: 79)	J033 (SEQ ID NO: 86)	J061 (SEQ ID NO: 94)	J070 (SEQ ID NO: 103)
8	RYSE 7	RYSE 8	J025 (SEQ ID NO: 80)	J034 (SEQ ID NO: 87)	J062 (SEQ ID NO: 95)	J071 (SEQ ID NO: 104)
9	RYSE 2	Pme1-3'	J020 (SEQ ID NO: 75)	J075 (SEQ ID NO: 108)	J057 (SEQ ID NO: 90)	J072 (SEQ ID NO: 105)
10	RYSE 3	Pme1-3'	J021 (SEQ ID NO: 76)	J075 (SEQ ID NO: 108)	J058 (SEQ ID NO: 91)	J072 (SEQ ID NO: 105)
11	RYSE 4	Pme1-3'	J022 (SEQ ID NO: 77)	J075 (SEQ ID NO: 108)	J059 (SEQ ID NO: 92)	J072 (SEQ ID NO: 105)
12	RYSE 5	Pme1-3'	J023 (SEQ ID NO: 78)	J075 (SEQ ID NO: 108)	J060 (SEQ ID NO: 93)	J072 (SEQ ID NO: 105)
13	RYSE 6	Pme1-3'	J024 (SEQ ID NO: 79)	J075 (SEQ ID NO: 108)	J061 (SEQ ID NO: 94)	J072 (SEQ ID NO: 105)
14	RYSE 7	Pme1-3'	J025 (SEQ ID NO: 80)	J075 (SEQ ID NO: 108)	J062 (SEQ ID NO: 95)	J072 (SEQ ID NO: 105)

15	RYSE 8	Pme1-3'	J026 (SEQ ID NO: 81)	J075 (SEQ ID NO: 108)	J063 (SEQ ID NO: 96)	J072 (SEQ ID NO: 105)

Table 3 – Annealable Linker Sequence Pairs or Annealable Linker Sequnece / Primer Binding Segment Pairs Present in pRYSE Vectors, and PCR Primers Used to Generate pRYSE Vector Backbones

pRYSE vector	Annealable Linker or Primer Binding Segment 1 (see Table 1)	Annealable Linker or Primer Binding Segment 2 (see Table 1)	Primer 5	Primer 6
1	Pme1-5'	RYSE 1	S001 (SEQ ID NO: 46)	S002 (SEQ ID NO: 47)
2	RYSE 1	RYSE 2	S003 (SEQ ID NO: 48)	S004 (SEQ ID NO: 49)
3	RYSE 2	RYSE 3	S005 (SEQ ID NO: 50)	S006 (SEQ ID NO: 51)
4	RYSE 3	RYSE 4	S007 (SEQ ID NO: 52)	S008 (SEQ ID NO: 53)
5	RYSE 4	RYSE 5	S009 (SEQ ID NO: 54)	S010 (SEQ ID NO: 55)
6	RYSE 5	RYSE 6	S011 (SEQ ID NO: 56)	S012 (SEQ ID NO: 57)
7	RYSE 6	RYSE 7	S013 (SEQ ID NO: 58)	S014 (SEQ ID NO: 59)
8	RYSE 7	RYSE 8	S015 (SEQ ID NO: 60)	S016 (SEQ ID NO: 61)
9	RYSE 2	Pme1-3'	S005 (SEQ ID NO: 50)	S018 (SEQ ID NO: 63)
10	RYSE 3	Pme1-3'	S007 (SEQ ID NO: 52)	S018 (SEQ ID NO: 63)
11	RYSE 4	Pme1-3'	S009 (SEQ ID NO: 54)	S018 (SEQ ID NO: 63)
12	RYSE 5	Pme1-3'	S011 (SEQ ID NO: 56)	S018 (SEQ ID NO: 63)
13	RYSE 6	Pme1-3'	S013 (SEQ ID NO: 58)	S018 (SEQ ID NO: 63)
14	RYSE 7	Pme1-3'	S015 (SEQ ID NO: 60)	S018 (SEQ ID NO: 63)
15	RYSE 8	Pme1-3'	S017 (SEQ ID NO: 62)	S018 (SEQ ID NO: 63)

[00183] The *lacZ* gene was PCR amplified from the pUC19 vector using primers S027 (SEQ ID NO: 65) and S028 (SEQ ID NO: 66), which each comprise a SchI restriction enzyme recognition site. The reaction mixture was resolved by gel electrophoresis, the approximately 0.5 kb PCR product was gel purified, and the purified PCR product was ligated with each of the pRYSE vector backbones. Site-directed mutagenesis was performed on the resulting vectors using PCR primers L012 (SEQ ID NO: 231) and L013 (SEQ ID NO: 232) to remove a SchI restriction enzyme recognition site from the origin of replication. Finally, a second site-directed mutagenesis was performend using PCR primers S036 (SEQ ID NO: 67) and S037 (SEQ ID NO: 68) to remove the SchI restriction enzyme recognition site from the *lacZ* fragment, thus yielding pRYSE vectors 1 through 15 (see FIG. 4 for a plasmid map of the pRYSE vectors, and SEQ ID NOS: 207 through 221 for the nucleotide sequence of pRYSE vectors 1 through 15).

Example 2

[00184] This example describes alternative methods for making pRYSE vectors.

[00185] pRYSE vectors 1 through 15 can be generated synthetically using as template SEQ ID NOS: 207 through 221 (*e.g.*, by Biosearch Technologies, Novato, CA). Additional pRYSE vectors comprising different annealable linker sequences can be generated synthetically using as template SEQ ID NO: 221 in which the Pme1-5' primer binding segment and/or the RYSE 1 annealable linker sequence are changed to another suitable annealable linker sequence or primer binding segment (see Table 1).

Example 3

[00186] This example describes methods for making a pMULE vector, comprising, in a 5' to 3' orientation, a first SapI restriction enzyme recognition site, a first SchI restriction enzyme recognition site, a *lacZ* marker gene, a second SchI restriction enzyme recognition site, and a second SapI restriction enzyme recognition site. The pMULE vector can be used to clone Mules.

[00187] The backbone of pRYSE vector 8 was PCR amplified using primers K162 (SEQ ID NO: 109) and K163 (SEQ ID NO: 110). The reaction mixture was resolved by gel electrophoresis, and the approximately 2.2 kb vector backbone was gel purified. A DNA fragment comprising the *lacZ* gene was generated by digesting to completion pRYSE vector 8 using SchI restriction enzyme, heat inactivating the enzyme at 65°C for 20 minutes, resolving the reaction mixture by gel electrophoresis, and gel purifying the approximately 0.5 kb DNA fragment. The purified DNA fragment comprising the *lacZ* gene was ligated with the purified vector backbone, yielding the pMULE vector (see FIG. 7 for a plasmid map).

Example 4

[00188] This example describes methods for making "Bits". Bits are DNA fragments that can be inserted into pRYSE vectors to generate assembly vectors comprising component polynucleotides that can be assembled into assembled polynucleotides using methods disclosed herein. Bits may encode genes or genetic elements of interest (*e.g.*, promoters, terminators, selectable markers, integration loci, epitope tags, localization signals, degradation signals, fluorescent markers, multiple cloning sites). Bits were PCR amplified from a template using primers as described in Table 4.

Table 4 - Amplified Bits

Bit	Type *	Primers	Size (bp)	Template
atoB	Gs	L229 (SEQ ID NO: 40) L230 (SEQ ID NO: 41)	1185	plasmid DNA comprising the *atoB* gene from *Escherichia coli* (GenBank accession number NC_000913 REGION: 2324131..2325315)
mvaS	Gs	L235 (SEQ ID NO: 42) L236 (SEQ ID NO: 43)	1152	synthetic DNA fragment comprising *mvaS* gene from *Enter°C°Ccus faecalis* (GenBank accession number AF290092 REGION: 142..1293) codon-optimized for expression in *Saccharomyces cerevisiae* and comprising at position 110 an alanine to glycine modification to increase enzyme activity (see Steussy *et al.* (2006) *Bi°Chemistry* 45(48):14407-14414)
ERG13-1	GsT	L109 (SEQ ID NO: 235) L110 (SEQ ID NO: 26)	1726	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
3' NDT80	D	L221 (SEQ ID NO: 34) L222 (SEQ ID NO: 35)	516	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
5' NDT80	U	L219 (SEQ ID NO: 32) L220 (SEQ ID NO: 33)	495	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
tP_{FBA1}	P	L225 (SEQ ID NO: 37) L057 (SEQ ID NO: 234)	526	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
tP_{TDH3}	P	L224 (SEQ ID NO: 36) L054 (SEQ ID NO: 233)	559	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
ERG10-1	Gs	L226 (SEQ ID NO: 38) L227 (SEQ ID NO: 39)	1182	synthesized fragment encoding the acetyl-CoA acetyltransferase of *Ralstonia eutropha* (GenBank accession NC_008313 REGION: 183291..184469) codon-optimized for expression in *Saccharomyces cerevisiae* and followed by an additional stop codon
tENO1	T	L248 (SEQ ID NO: 44) L176 (SEQ ID NO: 27)	265	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
tTDH3	T	L185 (SEQ ID NO: 28) L186 (SEQ ID NO: 29)	260	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
HphA	M	TRIX_L_193 (SEQ ID NO: 184) TRIX_L_194 (SEQ ID NO: 185)	1912	plasmid DNA comprising the TEF1 promoter and terminator of *Kluyveromyces lactis* (GenBank accession CR382122 REGIONS:788874..789380 and 787141..787496, respectively) and the hph gene of *Klebsiella pneumonia*
tHMG1	GsT	TRIX_L_232 (SEQ ID NO: 186)	1742	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA

		TRIX_L_233 (SEQ ID NO: 187)		
$tP_{GAL1,10}$	P	TRIX_L_266 (SEQ ID NO: 190) TRIX_L_267 (SEQ ID NO: 191)	620	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
ERG10-2	GsT	TRIX_L_106 (SEQ ID NO: 170) TRIX_L_107 (SEQ ID NO: 171)	1467	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
ERG13-2	GsT	TRIX_L_109 (SEQ ID NO: 172) TRIX_L_110 (SEQ ID NO: 173)	1726	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
GAL80US	U	JU-218-168-130-GAL80US-F (SEQ ID NO: 134) JU-219-168-130-GAL80US-R (SEQ ID NO: 135)	500	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
GAL80DS	D	JU-220-168-130-GAL80DS-F (SEQ ID NO: 136) JU-221-168-130-GAL80DS-R (SEQ ID NO: 137)	500	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
P_{TDH3}	P	L224 (SEQ ID NO: 36) TRIX_L_053 (SEQ ID NO: 169)	583	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA

Table 4 - Amplified Bits (continued)				
Bit	**Type ***	**Primers**	**Size (bp)**	**Template**
NatA	M	TRIX_L_193 (SEQ ID NO: 184) TRIX_L_194 (SEQ ID NO: 185)	1456	plasmid DNA comprising the TEF1 promoter and terminator of *Kluyveromyces lactis* (GenBank accession CR382122 REGIONS:788874..789380 and 787141..787496, respectively) and the nat1 gene of *S. noursei*
ERG12	GsT	TRIX_L_112 (SEQ ID NO: 174) TRIX_L_113 (SEQ ID NO: 175)	1582	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
ERG8	GsT	TRIX_L_118 (SEQ ID NO: 178) TRIX_L_119 (SEQ ID NO: 179)	1616	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
P_{GAL4oc}	P	TRIX_K_131 (SEQ ID NO: 165) PW-91-093-CPK422-G (SEQ ID NO: 162)	270	plasmid DNA comprising an "operative constitutive" version of the promoter of the GAL4 gene of *Saccharomyces cerevisiae* strain CEN.PK2 (Griggs & Johnston (1991) *PNAS* **88**(19):8597-8601)
GAL4-1	G	JU-286-275-31-GAL4-F (SEQ ID NO: 140) JU-285-275-31-GAL4-FIX-R2 (SEQ ID NO: 139)	526	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
GAL4-2	G	JU-284-275-31-GAL4-FIX-F2 (SEQ ID NO: 138) JU-287-275-31-GAL4-R (SEQ ID NO: 141)	2414	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
KanA	M	TRIX_L_193 (SEQ ID NO: 184) TRIX_L_194 (SEQ ID NO: 185)	1696	plasmid DNA comprising the TEF1 promoter and terminator of *Kluyveromyces lactis* (GenBank accession CR382122 REGIONS:788874..789380 and 787141..787496, respectively) and the kanR gene of Tn903 transposon
ERG19	GsT	TRIX_L_115 (SEQ ID NO: 176) TRIX_L_116 (SEQ ID NO: 177)	1441	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
ERG20	GsT	TRIX_L_124 (SEQ ID NO: 182) TRIX_L_125 (SEQ ID NO: 183)	1319	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
P_{GAL7}	P	TRIX_L_34 (SEQ ID NO:	500	*Saccharomyces cerevisiae* strain

		166) TRIX_L_35 (SEQ ID NO: 167)		CEN.PK2 genomic DNA
tP_{GAL7}	P	TRIX_L_34 (SEQ ID NO: 166) TRIX_L_36 (SEQ ID NO: 168)	476	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
IDI1	GsT	TRIX_L_121 (SEQ ID NO: 180) TRIX_L_122 (SEQ ID NO: 181)	1127	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
tP_{CTR3}	P	TRIX_K_0142 (SEQ ID NO: 163) TRIX_K_0143 (SEQ ID NO: 164)	710	plasmid DNA comprising promoter of the CTR3 gene of *Saccharomyces cerevisiae* strain CEN.PK2
LEU2US	U	JU-164-168-110-LEU2 US-f (SEQ ID NO: 129) JU-165-168-110-LEU2 US-r (SEQ ID NO: 130)	500	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
LEU2DS	D	JU-162-168-110-LEU2 DS-f (SEQ ID NO: 127) JU-163-168-110-LEU2 DS-r (SEQ ID NO: 128)	500	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
ERG9US	U	JU-108-168-110-ERG9 US-f (SEQ ID NO: 126) JU-172-168-110-ERG9 US-r1 (SEQ ID NO: 133)	499	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
ERG9CD S	G	JU-106-168-110-ERG9 CDS-f (SEQ ID NO: 124) JU-107-168-110-ERG9 CDS-r (SEQ ID NO: 125)	501	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
STE5US	U	TRIX_RN017 (SEQ ID NO: 192) TRIX_RN018 (SEQ ID NO: 193)	600	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
STE5DS	D	TRIX_RN019 (SEQ ID NO: 194) TRIX_RN020 (SEQ ID NO: 195)	600	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA

Table 4 - Amplified Bits (continued)				
Bit	**Type ***	**Primers**	**Size (bp)**	**Template**
URA3	M	JU-169-168-110-URA3-f (SEQ ID NO: 131) JU-170-168-110-URA3-r (SEQ ID NO: 132)	1554	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
* G=gene; s=stop codon; T=terminator; M=marker; D=downstream integration region; U=upstream integration region; P=promoter.				

[00189] PCR amplifications were done using the Phusion DNA polymerase (New England Biolabs, Ipswich, MA) as per manufacturer's suggested protocol. The PCR reactions were resolved by gel electrophoresis, the bits were gel purified, and the purified bits were treated with T4 polynucleotide kinase (PNK) (New England Biolabs, Ipswich, MA) as per manufacturer's suggested protocol. The PNK was heat inactivated at 65°C for 20 minutes, and the samples were stored at -20°C.

Example 5

[00190] This example describes methods for making "MULEs." MULEs are DNA fragments that can be inserted into pMULE vectors to generate assembly vectors comprising components polynucleotides that can be assembled into assembled polynucleotides using methods disclosed herein. MULEs may encode genes or genetic elements of interest (*e.g.*, promoters, terminators, selectable markers, integration loci, epitope tags, localization signals, degradation signals, fluorescent markers, multiple cloning sites) flanked by annealable linker sequence pairs or annealable linker sequence / primer binding segment pairs. MULEs were PCR amplified from a template using primers of which the 3' end anneals to the target sequence and the 5' end comprises an annealable linker sequence or a primer binding segment (see Table 1 for suitable annealable linker sequences), as described in Table 5.

Table 5 - Amplified MULEs				
MULE	**Type ***	**Primers**	**Size (bp)**	**Template**
tHMG1 -a	G	KMH8-276-1-linker4.tHMG1.fwd (SEQ ID NO: 157) KMH9-276-1-linker9.tHMG1.rev (SEQ ID NO: 160)	1794	RABit 254 plasmid DNA
ERG12	G	KMH46-276-43-ERG12linker4.fwd (SEQ ID NO: 151) KMH14-276-4-linker9.ERG12.rev (SEQ ID NO: 145)	1634	RABit 250 plasmid DNA
ERG19	G	KMH47-276-43-ERG19linker4.fwd (SEQ ID NO: 152) KMH15-276-4-linker9.ERG19.rev (SEQ ID NO: 146)	1493	RABit 241 plasmid DNA
P_{TDH3}-a	P	KMH81-276-116-TDH3.rev.tHMG1 (SEQ ID NO: 155) S004 (SEQ ID NO: 49)	626	RABit 54 plasmid DNA
P_{TDH3}-b	P	KMH91-276-116-TDH3.rev.FS (SEQ ID NO: 158) S004 (SEQ ID NO: 49)	546	RABit 54 plasmid DNA
tHMG1 -b	G	KMH82-276-116-tHMG1.fwd.TDH3 (SEQ ID NO: 156) S009 (SEQ ID NO: 54)	1801	RABit 20 plasmid DNA
IME1US	U	KB454-266-53 (SEQ ID NO: 142) KB455-266-53 (SEQ ID NO: 143)	578	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
IME1DS	D	KMH93-276-130-3'IME.linker4.fwd (SEQ ID NO: 161) KB457-266-53 (SEQ ID NO: 144)	554	*Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA
LEU2	M	VH296-235-55-Leu2 12-1 F (SEQ ID NO: 30) VH296-235-55-Leu2 12-1 R (SEQ ID NO: 31)	1795	plasmid DNA comprising LEU2 locus of *Saccharomyces cerevisiae* strain CEN.PK2 (Sikorski RS, Hieter (1989) Genetics 122(1):19-27)

FS-a	G	KMH5-276-1-linker3.FS(Kozak).fwd (SEQ ID NO: 153) KMH7-276-1-linker4.TCYC1.rev (SEQ ID NO: 154)	198 1	plasmid DNA comprising coding sequence of farnesene synthase of *Artemisia annua* (GenBank accession number AY835398) codon-optimized for expression in *Saccharomyces cerevisiae* and terminator of CYC1 gene of *Saccharomyces cerevisiae* strain CEN.PK2
FS-b	G	KMH92-276-116-FS.fwd.TDH3 (SEQ ID NO: 159) KMH7-276-1-linker4.TCYC1.rev (SEQ ID NO: 154)	197 6	plasmid DNA comprising coding sequence of farnesene synthase of *Artemisia annua* (GenBank accession number AY835398) codon-optimized for expression in *Saccharomyces cerevisiae* and terminator of CYC1 gene of *Saccharomyces cerevisiae* strain CEN.PK2

Table 5 - Amplified MULEs (continued)				
MULE	**Type ***	**Primers**	**Size (bp)**	**Template**
URA3b laster	M	VH228-235-7-URA3LOF3RYSE12-1F (SEQ ID NO: 204) VH229-235-7-URA3LOF3RYSE12-1R (SEQ ID NO: 205)	1565	URA-3 blaster template **
* G=gene; s=stop codon; T=terminator; M=marker; D=downstream integration region; U=upstream integration region; P=promoter. ** The URA-3 blaster template was made by first generating DNA fragments flanking sequence A (generated from a synthetic DNA fragment comprising SEQ ID NO: 206 using PCR primers TRIX_Z025 (SEQ ID NO: 196) and TRIX_Z026 (SEQ ID NO: 197)), flanking sequence B (generated from a synthetic DNA fragment comprising SEQ ID NO: 206 using PCR primers TRIX_Z027 (SEQ ID NO: 198) and TRIX_Z028 (SEQ ID NO: 199)), URA3-c (generated from *Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA using PCR primers TRIX_Z033 (SEQ ID NO: 200) and TRIX_Z036 (SEQ ID NO: 203)), and URA3-d (generated from *Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA using PCR primers TRIX_Z034 (SEQ ID NO: 201) and TRIX_Z035 (SEQ ID NO: 202)). DNA fragments flanking sequence A, URA3-c, and URA-3-d were then stitched together into DNA fragment A using PCR primers TRIX_Z025 and TRIX_Z034, and DNA fragments URA3-c, URA3-d, and flanking sequence B were stitched together into DNA fragment B using PCR primers TRIX_Z028 and TRIX_Z033. Finally, DNA fragments A and B were stitched together using PCR primers TRIX_Z025 and TRIX_Z028, yielding the URA-3 blaster template.				

[00191] PCR amplifications were done using the Phusion DNA polymerase (New England Biolabs, Ipswich, MA) as per manufacturer's suggested protocol. The PCR reactions were resolved by gel electrophoresis, the MULEs were gel purified, and the purified MULEs were treated with T4 polynucleotide kinase (PNK) (New England Biolabs, Ipswich, MA) as per manufacturer's suggested protocol. The PNK was heat inactivated at 65°C for 20 minutes, and the samples were stored at -20°C.

Example 6

[00192] This example describes methods for inserting Bits into pRYSE vectors or MULEs into the pMULE vector to generate assembly vectors.

[00193] pRYSE vectors 1 through 8 and pRYSE vector 15 were digested to completion using SchI restriction enzyme, and the digested DNA fragments were treated with Antarctic Phosphatase (New England Biolabs, Ipswich, MA). The phosphatase was heat inactivated at 65°C for 20 minutes, the reaction mixtures were resolved by gel electrophoresis, and the approximately 2.2 kb pRYSE vector backbones (lacking lacZ) were gel purified. Purified pRYSE vector backbones were ligated with Bits as detailed in Table 6, thus yielding assembly vectors.

[00194] The pMULE vector is digested to completion using SchI restriction enzyme, the reaction mixture is resolved by gel electrophoresis, and the approximately 2.2 kb pMULE vector backbone (lacking *lacZ*) is gel purified. The purified pMULE vector backbone is treated with a phosphatase (*e.g.*, Antarctic Phosphatase (New England Biolabs, Ipswich, MA), CIAP (New England Biolabs, Ipswich, MA), SAP (New England Biolabs, Ipswich, MA; Fermentas, Glen Burnie, MD), or FastAP (Fermentas, Glen Burnie, MD)), the phosphatase is heat inactivated (*e.g.*, 20 min at 65°C), and the pMULE vector backbone is ligated with MULEs, thus yielding assembly vectors.

Table 6 - Assembly Vectors Generated

Bit (see Table 4)	**pRYSE Vector** (see Table 3)	**Assembly Vector**
atoB	4	2
mvaS	7	5
ERG13-1	7	12
3' NDT80	15	29
	10	24
5' NDT80	1	30
	1	97
tP_{FBA1}	6	35
tP_{TDH3}	3	53
ERG10-1	4	60
tENO1	8	62
tTDH3	5	64
GAL80US	1	270
HphA	2	22
tHMG1	3	254
$tP_{GAL1,10}$	4	229
ERG10-2	5	244
ERG13-2	6	253
$tP_{GAL1,10}$	7	228
tHMG1	8	255
GAL80DS	15	271
LEU2US	1	187
NatA	2	262
ERG12	3	250
ERG8	5	252
P_{GAL4oc}	6	268
GAL4 *	7	265
LEU2DS	14	263
ERG9US	1	186
KanA	2	261
ERG19	3	241
ERG20	5	251
tP_{GAL7}	6	249
IDI1	7	237
tP_{CTR3}	8	269
ERG9CDS	15	185
P_{GAL7}	3	44
STE5US	1	567
URA3	2	556 (orientation 1)
		555 (orientation 2)
P_{TDH3}	3	54
tHMG1	4	20
STE5DS	11	563

Ligations were performed using 50 ng vector backbone, 3 molar excess Bit, and a ligase (*e.g.*, Quick Ligase (New England Biolabs, Ipswich, MA), T4 DNA ligase (regular and high concentration; vendor, Fermentas, Glen Burnie, MD), Fast Ligase (Fermentas, Glen Burnie, MD)) as per manufacturer's suggested protocol. * Bit GAL4 was generated by stitching together Bits GAL4-1 and GAL4-2 (see Table 4) using primers JU-286-275-31-GAL4-F (SEQ ID NO: 140) and JU-287-275-31-GAL4-R (SEQ ID NO: 141).

[00195] Assembly vectors were transformed into chemically competent TOP10 *Escherichia coli* parent cells (Invitrogen, Carlsbad, CA). Host cell transformants were selected on Luria Bertoni (LB) agar containing 100 ug/mL carbenicillin and 40 ug/mL X-gal. Single white colonies were transferred from LB agar to culture tubes containing 5 mL of LB liquid medium and carbenicillin, and the cultures were incubated overnight at 37°C on a rotary shaker at 250 rpm. Plasmid DNAs were extracted and sequenced to identify clones containing the correct sequence in the correct orientation. The cells were stored at -80°C in cryo-vials in 1 mL stock aliquots made up of 400 uL sterile 50% glycerol and 600 uL liquid culture.

<u>Example 7</u>

[00196] This example describes methods for assembling component polynucleotides into a assembled polynucleotide using assembly vectors and/or MULEs.

[00197] Assembly vectors (see Table 7) were placed together in one tube (333 fmole of each RABit) and digested using LguI restriction enzyme (Fermentas, Glen Burnie, MD). The restriction enzyme was removed by column centrifugation or heat inactivated for 20 minutes at 65°C. For assembly reactions involving MULEs or assembled polynucleotides, 333 fmole of each MULE or assembled polynucleotide (see Table 7) were placed together in one tube or were added to the digested assembly vectors. The samples were split into three 30 uL reactions; water, buffer, dNTPs, and DNA polymerase were added to each reaction mixture, and a first round of PCR amplification was initiated. Samples were placed on ice, 0.5 uM of each terminal primer (Table 7) were added to the reaction mixtures, and a second round of PCR amplification was performed. The three PCR reaction mixtures were combined in one tube, the reaction mixtures were resolved by gel electrophoresis, and the PCR products were gel purified.

Table 7 - Terminal Primers for Assembly of Assembled polynucleotides				
Assembly	**Assembly Vectors** (see Table 6) **or MULEs** (see Table 5) **To Be Combined** *	**Assembled polynucleotide Size (kb) (Sequence)**	**Terminal Primer 1**	**Terminal Primer 2**
1	30_22_53_60	4.3	S000 (SEQ ID NO: 45)	S009 (SEQ ID NO: 54)
2	30_22_53	3.1	S000 (SEQ ID NO: 45)	S007 (SEQ ID NO: 52)
3	22_53_60	3.7	S002 (SEQ ID NO: 47)	S009 (SEQ ID NO: 54)
4	30_22	2.5	S000 (SEQ ID NO: 45)	S005 (SEQ ID NO: 50)
5	22_53	2.5	S002 (SEQ ID NO: 47)	S007 (SEQ ID NO: 52)
6	53_60	1.8	S004 (SEQ ID NO: 49)	S009 (SEQ ID NO: 54)
7	30_22_53_60_64_35_12_62_29	7.7 (SEQ ID NO: 222)	S000 (SEQ ID NO: 45)	S019 (SEQ ID NO: 64)
8	30_22_53_60_64_35_5_62_29	7.1	S000 (SEQ ID NO: 45)	S019 (SEQ ID NO: 64)
9	30_22_53_2_64_35_5_62_29	7.1	S000 (SEQ ID NO: 45)	S019 (SEQ ID NO: 64)
10	60_64_35_5_62_29	4.1	S006 (SEQ ID NO: 51)	S019 (SEQ ID NO: 64)
11	2_64_35_5_62_29	4.1	S006 (SEQ ID NO: 51)	S019 (SEQ ID NO: 64)
Phase I-A	270_22_254_229_244_253	8.1 (SEQ ID NO: 111)	S000 (SEQ ID NO: 45)	S013 (SEQ ID NO: 58)
Phase I-B	228_255_271	3.0 (SEQ ID NO: 112)	S013 (SEQ ID NO: 58)	S019 (SEQ ID NO: 64)
Phase II complete	187_262_250_229_252_268_265_263	9.7 (SEQ ID NO:113)	S000 (SEQ ID NO: 45)	S019 (SEQ ID NO: 64)

Phase III-A	186_261_241_229	4.4 (SEQ ID NO: 114)	S000 (SEQ ID NO: 45)	S008 (SEQ ID NO: 53)
Phase III-B	251_249_237_269_185	4.3 (SEQ ID NO: 115)	S009 (SEQ ID NO: 54)	S018 (SEQ ID NO: 63)
Phase I marker recycling	270_URA3blaster_44_FS-a_tHMG1-a	6.3 (SEQ ID NO: 116)	S000 (SEQ ID NO: 45)	S019 (SEQ ID NO: 64)
Phase II marker recycling	187_ URA3blaster_44_FS-a_ERG12	6.2 (SEQ ID NO: 117)	S000 (SEQ ID NO: 45)	S019 (SEQ ID NO: 64)
Phase III marker recycling	186_URA3blaster_44_FS-a_ERG19	6.0 (SEQ ID NO: 118)	S000 (SEQ ID NO: 45)	S019 (SEQ ID NO: 64)
STE5 knockout	567_556_P_{TDH3}-a_tHMG1-b_563	5.2 (SEQ ID NO: 119)	S000 (SEQ ID NO: 45)	S019 (SEQ ID NO: 64)
IME1 knockout	IME1US_LEU2_P_{TDH3}-b_FS-b_IME1DS	5.4 (SEQ ID NO: 120)	S000 (SEQ ID NO: 45)	S019 (SEQ ID NO: 64)

The first round of PCR amplification was performed as follows: one cycle of denature at 98°C for 2 minutes; 5 cycles of denature at 98°C for 30 seconds and anneal/extend at 72°C for 30 seconds per kilobase PCR product. The second round of PCR amplification was performed as follows: one cycle of denature at 98°C for 2 minutes; 35 rounds of denature at 98°C for 12 seconds and anneal/extend at 72°C for 20-25 seconds per kilobase PCR product; one cycle of final extend at 72°C for 7 minutes; and a final hold at 4°C. When the annealing temperature was not 72°C (*i.e.*, when it was either 54°C or 65°C), in the first round of PCR amplification a 1 minute annealing step followed by a 30 seconds per kilobase PCR product extension step at 72°C was used, and for the second round of PCR amplification a 15 seconds annealing step followed by a 20 seconds per kilobase PCR product extension step at 72°C was used.

* Assembly vectors are designated with numbers, and MULEs with names.

[00198] As shown in FIGS. 5 and 6, 2 to 9 component polynucleotides were correctly assembled into up to 7.7 kb long assembled polynucleotides.

Example 8

[00199] This example describes methods for generating genetically altered host microorganisms using assembled polynucleotides assembled by the methods disclosed herein.

[00200] Phase I-A and Phase I-B assembled polynucleotides (see Table 7) were cloned into the TOPO Zero Blunt II cloning vector (Invitrogen, Carlsbad, CA), yielding plasmids TOPO-Phase I-A and TOPO-Phase I-B, respectively. The constructs were propagated in TOP10 cells (Invitrogen, Carlsbad, CA) grown on LB agar containing 50 μg/ml kanamycin. Each plasmid was digested to completion using NotI restriction endonuclease, the Phase I-A and Phase I-B inserts were gel extracted using a gel purification kit (Qiagen, Valencia, CA), and equal molar ratios of the purified DNA fragments were ligated using T4 DNA ligase (New England Biolabs, Ipswich, MA), yielding the Phase I complete assembled polynucleotide. The Phase I complete assembled polynucleotide was cloned into the TOPO Zero Blunt II cloning vector (Invitrogen, Carlsbad, CA), yielding plasmid TOPO-Phase I. The construct was propagated in TOP10 cells (Invitrogen, Carlsbad, CA) grown on LB agar containing 50 μg/ml kanamycin.

[00201] The Phase II complete assembled polynucleotide (see Table 7) was cloned into the TOPO Zero Blunt II cloning vector (Invitrogen, Carlsbad, CA), yielding plasmid TOPO-Phase II. The construct was propagated in TOP10 cells (Invitrogen, Carlsbad, CA) grown on LB agar containing 50 μg/ml kanamycin.

[00202] The Phase III-A and Phase III-B assembled polynucleotides (see Table 7) were cloned into the TOPO Zero Blunt II cloning vector (Invitrogen, Carlsbad, CA), yielding plasmids TOPO-Phase III-A and TOPO-Phase III-B, respectively. The constructs were propagated in TOP10 cells (Invitrogen, Carlsbad, CA) grown on LB agar containing 50 μg/ml kanamycin. Each plasmid was digested to completion using BamHI and SbfI restriction endonuclease, the Phase III-A and Phase III-B inserts were gel extracted using a gel purification kit (Qiagen, Valencia, CA), and equal molar ratios of the purified DNA fragments were ligated using T4 DNA ligase (New England Biolabs, Ipswich, MA), yielding the Phase III complete assembled polynucleotide. The Phase III complete assembled polynucleotide was cloned into the TOPO Zero Blunt II cloning vector (Invitrogen, Carlsbad, CA), yielding plasmid TOPO-Phase III. The construct was propagated in TOP10 cells (Invitrogen, Carlsbad, CA) grown on LB agar containing 50 μg/ml kanamycin.

[00203] For yeast cell transformations, 25 ml of Yeast Extract Peptone Dextrose (YPD) medium was inoculated with a single colony of a starting host strain. The culture was grown overnight at 30°C on a rotary shaker at 200 rpm. The OD600 of the culture was measured, and the culture was then used to inoculate 50 ml of YPD medium to an OD600 of 0.15. The newly inoculated culture was grown at 30°C on a rotary shaker at 200 rpm up to an OD600 of 0.7 to 0.9, at which point the cells were transformed with 1 μg of DNA. The cells

were allowed to recover in YPD medium for 4 hours before they were plated on agar containing a selective agent to identify the host cell transformants.

[00204] Starter host strain Y1198 was generated by resuspending active dry PE-2 yeast (isolated in 1994 at Santelisa Vale, Sertãozinho, Brazil) in 5 mL of YPD medium containing 100 ug/mL carbamicillin and 50 ug/mL kanamycin. The culture was incubated overnight at 30°C on a rotary shaker at 200 rpm. An aliquot of 10 uL of the culture was then plated on a YPD plate and allowed to dry. The cells were serially streaked for single colonies, and incubated for 2 days at 30°C. Twelve single colonies were picked, patched out on a new YPD plate, and allowed to grow overnight at 30°C. The strain identities of the colonies were verified by analyzing their chromosomal sizes on a Bio-Rad CHEF DR II system (Bio-Rad, Hercules, CA) using the Bio-Rad CHEF Genomic DNA Plug Kit (Bio-Rad, Hercules, CA) according to the manufacturer's specifications. One colony was picked and stocked as strain Y1198.

[00205] Strains Y1661, Y1662, Y1663, and Y1664 were generated from strain Y1198 by rendering the strain haploid. Strain Y1198 was grown overnight in 5 mL of YPD medium at 30°C in a glass tube in a roller drum. The OD600 was measured, and the cells were diluted to an OD600 of 0.2 in 5 mL of YP medium containing 2% potassium acetate. The culture was grown overnight at 30°C in a glass tube in a roller drum. The OD600 was measured again, and 4 OD600*mL of cells was collected by centrifugation at 5,000g for 2 minutes. The cell pellet was washed once with sterile water, and then resuspended in 3 mL of 2% potassium acetate containing 0.02% raffinose. The cells were grown for 3 days at 30°C in a glass tube in a roller drum. Sporulation was confirmed by microscopy. An aliquot of 33 uL of the culture was transferred to a 1.5 mL microfuge tube and was centrifuged at 14,000rpm for 2 minutes. The cell pellet was resuspended in 50 uL of sterile water containing 2 uL of 10 mg/mL Zymolyase 100T (MP Biomedicals, Solon, OH), and the cells were incubated for 10 minutes in a 30°C waterbath. The tube was transferred to ice, and 150 uL of ice cold water was added. An aliquot of 10 uL of this mixture was added to a 12 mL YPD plate, and tetrads were dissected on a Singer MSM 300 dissection microscope (Singer, Somerset, UK). The YPD plate was incubated at 30°C for 3 days, after which spores were patched out onto a fresh YPD plate and grown overnight at 30°C. The mating types of each spore from 8 four-spore tetrads were analyzed by colony PCR. A single 4 spore tetrad with 2 MATA and 2 MATalpha spores was picked and stocked as strains Y1661 (MATA), Y1662 (MATA), Y1663 (MATalpha), and Y1664 (MATalpha).

[00206] Host strain 1515 was generated by transforming strain Y1664 with plasmid TOPO-Phase I digested to completion using PmeI restriction endonuclease. Host cell transformants were selected on YPD medium containing 300 ug/mL hygromycin B.

[00207] Host strain 1762 was generated by transforming strain Y1515 with plasmid TOPO-Phase II digested to completion using PmeI restriction endonuclease. Host cell transformants were selected on YPD medium containing 100 ug/mL nourseothricin.

[00208] Host strain 1770 was generated by transforming strain Y1762 in two steps with expression plasmid pAM404 and plasmid TOPO-Phase III digested to completion using PmeI restriction endonuclease. Expression plasmid pAM404 was derived from plasmid pAM353, which was generated by inserting a nucleotide sequence encoding a β-farnesene synthase into the pRS425-Gal1 vector (Mumberg et. al. (1994) *Nucl. Acids. Res.* 22(25): 5767-5768). The nucleotide sequence insert was generated synthetically, using as a template the coding sequence of the β-farnesene synthase gene of *Artemisia annua* (GenBank accession number AY835398) codon-optimized for expression in *Saccharomyces cerevisiae* (SEQ ID NO: 121). The synthetically generated nucleotide sequence was flanked by 5' *BamHI* and 3' *XhoI* restriction sites, and could thus be cloned into compatible restriction sites of a cloning vector such as a standard pUC or pACYC origin vector. The synthetically generated nucleotide sequence was isolated by digesting to completion the DNA synthesis construct using *BamHI* and *XhoI* restriction enzymes. The reaction mixture was resolved by gel electrophoresis, the approximately 1.7 kb DNA fragment comprising the β-farnesene synthase coding sequence was gel extracted, and the isolated DNA fragment was ligated into the *BamHI XhoI* restriction site of the pRS425-Gal1 vector, yielding expression plasmid pAM353. The nucleotide sequence encoding the β-farnesene synthase was PCR amplified from pAM353 using primers GW-52-84 pAM326 BamHI (SEQ ID NO: 188) and GW-52-84 pAM326 NheI (SEQ ID NO: 189). The resulting PCR product was digested to completion using *BamHI* and *NheI* restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 1.7 kb DNA fragment comprising the β-farnesene synthase coding sequence was gel extracted, and the isolated DNA fragment was ligated into the *BamHI NheI* restriction site of vector pAM178 (SEQ ID NO: 122), yielding expression plasmid pAM404. Host cell transformants with pAM404 were selected on Complete Synthetic Medium (CSM) lacking methionine and leucine. Host cell transformants with pAM404 and Phase III complete assembled polynucleotide were selected on CSM lacking methionine and leucine and containing 200 ug/mL G418.

[00209] Host strain 1793 was generated by transforming strain Y1770 with a URA3 knockout construct (SEQ ID NO: 123). The knockout construct was generated by first generating DNA fragments URA3US (generated from *Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA using PCR primers KMH33-276-21-URA3 5'.fwd (SEQ ID NO: 147) and KMH34-276-21-URA3 5'.rev (SEQ ID NO: 148)) and URA3DS (generated from *Saccharomyces cerevisiae* strain CEN.PK2 genomic DNA using PCR primers KMH35-276-21-URA3 3'.fwd (SEQ ID NO: 149) and KMH36-276-21-URA3 3'.rev (SEQ ID NO: 150); followed by stitching the two DNA fragments together using PCR primers KMH33-276-21-URA3 5'.fwd and KMH36-276-21-URA3 3'.rev. Host cell transformants were selected on YPD medium containing 5-FOA.

[00210] Host strain YAAA was generated by transforming strain Y1793 with the Phase I marker recycling assembled polynucleotide (see Table 7). Host cell transformants were selected on CSM lacking methionine and uracil. The URA3 marker was excised by growing the cells overnight in YPD medium at 30°C on a rotary shaker at 200 rpm, and then plating the cells onto agar containing 5-FOA. Marker excision was confirmed by colony PCR.

[00211] Host strain YBBB was generated by transforming strain YAAA with the Phase II marker recycling assembled polynucleotide (see Table 7). Host cell transformants were selected on CSM lacking methionine and uracil. The URA3 marker was excised by growing the cells overnight in YPD medium at 30°C on a rotary shaker at 200 rpm, and then plating the cells onto agar containing 5-FOA. Marker excision was confirmed by colony PCR.

[00212] Host strain Y1912 was generated by transforming strain YBBB with the Phase III marker recycling assembled polynucleotide (see Table 7). Host cell transformants were selected on CSM lacking methionine and uracil. The URA3 marker was excised by growing the cells overnight in YPD medium at 30°C on a rotary shaker at 200 rpm, and then plating the cells onto agar containing 5-FOA. Marker excision was confirmed by colony PCR.

[00213] Host strain Y1913 was generated by transforming strain Y1912 with the STE5 knockout assembled polynucleotide (see Table 7). Host cell transformants were selected on CSM lacking methionine and uracil.

[00214] Host strain Y1915 was generated from strain Y1913 by curing the strain from pAM404 and transforming the resulting strain with the IME1 knockout assembled polynucleotide (see Table 7). Strain Y1913 was propagated in non-selective YPD medium at 30°C on a rotary shaker at 200 rpm. Approximately 100 cells were plated onto YPD solid

media and allowed to grow for 3 days at 30°C before they were replica-plated no CSM plates lacking methionine and leucine where they were grown for another 3 days at 30°C. Cured cells were identified by their ability to grow on minimal medium containing leucine and their inability to grow on medium lacking leucine. A single such colony was picked and transformed with the IME1 knockout assembled polynucleotide. Host cell transformants were selected on CSM lacking methionine and uracil.

Example 9

This example describes methods for selecting annealable linker sequences to be used to assemble component polynucleotides encoding a promoter and a protein coding sequence into a assembled polynucleotide by the inventive methods disclosed herein.

MULEs encoding promoters followed by two different candidate annealable linker sequences, annealable linker sequence RYSE 15 (R15; SEQ ID NO: 15) and annealable linker sequence RYSE 7 (R7; SEQ ID NO: 7), as well as MULEs encoding GFP preceded by the two annealable linker sequences, were PCR amplified as described in Table 8.

Table 8 - Amplified MULEs Encoding Promoters and GFP with Annealable Linker Sequences RYSE 15 (R15) or Annealable Linker Sequence RYSE 7 (R7)

MULE	Type *	Primers	Size (bp)	Template
pGAL1-R15	P	Plan X19 (SEQ ID NO: 236) Plan X20 (SEQ ID NO: 237)	698	*S. cerevisiae* strain CEN.PK2 genomic DNA
pTDH3-R15	P	Plan X47(SEQ ID NO: 238) Plan X48(SEQ ID NO: 239)	613	*S. cerevisiae* strain CEN.PK2 genomic DNA
pCYC1-R15	P	Plan X11(SEQ ID NO: 240) Plan X12(SEQ ID NO: 241)	645	*S. cerevisiae* strain CEN.PK2 genomic DNA
pGAL1-R7	P	Plan X19 (SEQ ID NO: 236) Plan X64 (SEQ ID NO: 242)	692	*S. cerevisiae* strain CEN.PK2 genomic DNA
pTDH3-R7	P	Plan X47(SEQ ID NO: 238) Plan X71(SEQ ID NO: 243)	607	*S. cerevisiae* strain CEN.PK2 genomic DNA
pCYC1-R7	P	Plan X11(SEQ ID NO: 240) Plan X78(SEQ ID NO: 244)	639	*S.cerevisiae* strain CEN.PK2 genomic DNA
R7-GFP	GsT	Plan X96(SEQ ID NO: 247) Plan X88(SEQ ID NO: 245)	1378	RABit 634 plasmid DNA **
A-GFP	GsT	Plan X89(SEQ ID NO: 246) Plan X88(SEQ ID NO: 245)	1385	RABit 634 plasmid DNA **

PCR reactions contained: 67 uL ddH2O, 20 uL 5x HF Buffer, 2 uL of each Primer (10uM), 1 uL dNTP mix (200 uM), 1 uL Phusion DNA Polymerase (New England Biolabs, Ipswich, MA), and 9 uL Y002 genomic DNA or RABit 634 plasmid DNA.

PCR amplification was performed as follows: 1 cycle of denature at 98°C for 2 minutes; 9 cycles of denature at 98°C for 15 seconds, anneal at 61°C for 30 seconds decreasing by 1°C each cycle, and extend at 72°C for 1 minute; 26 rounds of denature at 98°C for 15 seconds, anneal at 52°C for 30 seconds, and extend at 72°C for 1 minute; 1 cycle of final extend at 72°C for 7 minutes; and a final hold at 4°C.

* G=gene; s=stop codon; T=terminator; P=promoter.

** RABit 634 comprises the coding sequence of the green fluourescent protein (GFP) followed by the terminator of the ADH1 gene of *Saccharomyces cerevisiae.*

The PCR reactions were resolved by gel electrophoresis, the MULEs were gel purified, and the purified MULEs were used to assemble test assembled polynucleotides. To this end, MULEs and assembly vectors (see Table 6) to be assembled (see Table 9) were placed together in a tube (333 fmole of each assembly vector, 667 fmole for each MULE) and digested using LguI restriction enzyme (Fermentas, Glen Burnie, MD). The restriction enzyme was heat inactivated for 20 minutes at 65°C. The samples were split into three 30 uL reactions; water, buffer, dNTPs, and DNA polymerase were added to each reaction mixture, and a first round of PCR amplification was initiated. Terminal primers were then added to the reaction mixtures, and a second round of PCR amplification was performed (see Table 9).

The three PCR reaction mixtures were combined in one tube, the reaction mixtures were resolved by gel electrophoresis, and the PCR products were gel purified.

Table 9 - Terminal Primers for Assembly of Test Assembled polynucleotides

Assembly	**MULEs** (see Table 8) **and Assembly Vectors** (see Table 6) **To Be Combined** *	**Assembled polynucleotide Size (kb)**	**Terminal Primer 1**	**Terminal Primer 2**
1	97_555_pGAL1-A_A-GFP_24	4.7	S000 (SEQ ID NO: 45)	S019 (SEQ ID NO: 64)
2	97_555_pTDH3-A_A-GFP_24	4.6		
3	97_555_pCYC1-A_A-GFP_24	4.7		
7	97_555_ pGAL1-R7_R7-GFP _24	4.7		
8	97_555_pTDH3-R7_R7-GFP _24	4.6		
9	97_555_pCYC1-R7_R7-GFP _24	4.6		

PCR reactions contained: 41 uL ddH2O, 20 uL 5x HF Buffer, 5 uL of each terminal primer (1 uM), 2 uL dNTP mix (200 uM), 1.8 uL Phusion DNA Polymerase, and 30 uL MULE or LguI digested assembly vector.

The first round of PCR amplification was performed as follows: 1 cycle of denature at 98°C for 2 minutes; 5 cycles of denature at 98°C for 30 seconds, anneal at 60°C for 30 seconds, and extend at 72°C for 2.5 minutes; followed by a hold at 4°C for addition of the two terminal primers. The second round of PCR amplification was performed as follows: 1 cycle of denature at 98°C for 2 minutes; 35 rounds of denature at 98°C for 12 seconds, anneal at 60°C for 30 seconds, and extend at 72°C for 2.5 minutes; 1 cycle of final extend at 72°C for 7 minutes; and a final hold at 4°C.

* Assembly vectors are designated with numbers, and MULEs with names.

The test assembled polynucleotides were used to transform a *Saccharomyces cerevisiae* host strain that was URA3 deficient and had a deletion of the GAL80 locus. Host cell transformants were selected on CSM lacking uracil, and correct genomic integration of the assembled polynucleotide was confirmed by colony PCR. Two verified colonies from each transformation were picked into 360 uL Bird Seed Medium (BSM) containing 2% sucrose, and the cultures were incubated for 48 hours at 30°C on a rotary shaker at 999 rpm. An aliquot of 14.4 uL was taken from each well and transferred to 1.1 mL BSM containing 4% sucrose on a 96-well block plate, and cultured for another 6 hours at 30°C on a rotary shaker at 999 rpm, at which point 100 uL of each culture was transferred to a well of a clear bottom 96-well plate for analysis of GFP expression. GFP expression in each well was analyzed by measuring 515 nm emission after 485 nm excitation on an M5 Plate reader spectrophotometer (Molecular Devices, Sunnyvale, CA). Measured GFP concentrations were normalized for cell culture growth by dividing by the OD600 reading for each culture.

As shown in Table 10, annealable linker sequence RYSE 15 enabled increased GAL1, TDH3, and CYC1 promoter driven expression of the GFP reporter gene in the test assembled polynucleotides compared to annealable linker sequence RYSE 7.

Table 10 – GFP Expression in Host Cells Harboring Test Assembled polynucleotides Comprising Either Annealable Linker Sequence RYSE 15 (R15) or Annealable Linker Sequence RYSE 7 (R7) Between Promoter and GFP Reporter

Annealable linker sequence positioned between promoter and GFP reporter gene in test assembled polynucleotide	Average %GFP expression (compared to average %GFP expression obtained with host cells harboring one of 3 seamless control constructs*; average for 2 independent host cell isolates)			
	GAL1 promoter	TDH3 promoter	CYC1 promoter	Average across all three promoters
R15	79.34	91.42	81.92	84.22
R7	27.43	54.68	46.31	42.81

* The seamless control constructs had an identical structure as the test assembled polynucleotides except that the promoter sequences were seamlessly linked to the GFP reporter gene (*i.e.*, without an intervening annealable linker sequence).

Example 10

[00215] This example describes methods for the high-throughput combinatorial assembly of polynucleotides, and methods for the high-throughput generation of host cells comprising combinatorially combined polynucleotides.

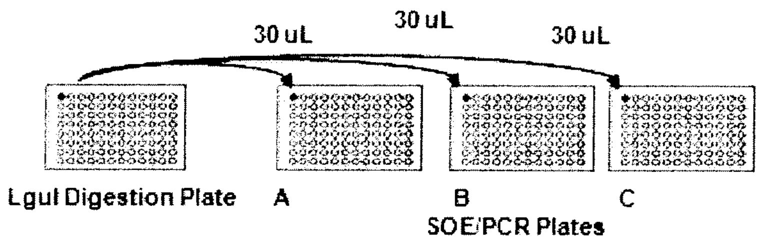
[00216] The component polynucleotides used in this example, and the expected assembled and combined polynucleotides generated from these component polynucleotides, are schematically illustrated in FIG. 12A. The component polynucleotides comprised DNA segments encoding an upstream and a downstream chromosomal targeting sequence (US and DS), 6 different promoters (P), 35 different proteins (G), and a 5' and a 3' segment of the URA3 selectable marker (URA and RA3, respectively), flanked by annealable linker sequences pairs or primer binding segment / annealable linker sequence pairs.

[00217] Component polynucleotides were released from assembly vectors by digesting RABits or MULES using LguI restriction endonuclease. To this end, 96-well plates ("LguI Digestion Plates") were set up as shown in the table below, and the plates were incubated at

37°C for 75 min, after which the LguI restriction endonuclease was heat inactivated at 65°C for 20 min in a PCR machine.

LguI **Digestion Plates**	
Component (per well)	**Volume (uL)**
667 fMoles RABit or MULE	Variable
10x Tango Buffer (Fermentas, Glen Burnie, MD)	10
LguI (Fermentas, Glen Burnie, MD)	2.5
ddH2O	to 100

[00218] Component polynucleotides were assembled by SOE. For each LguI Digestion Plate, triplicate 96-well plates ("SOE/PCR Plates") were set up and thermocycled in a PCR machine as shown in the table below.

SOE/PCR Plates	
Component (per well)	**Volume (uL)**
ddH2O	41
5x Phusion HF Buffer (New England Biolabs, Ipswich, MA)	20
10 mM dNTP mix	2
Phusion DNA polymerase (New England Biolabs, Ipswich, MA)	1.8
LguI-digested RABits or MULEs to be assembled	30 
	Total: 95

Thermocycling conditions			
Initial Denature		98°C	2 min
7 cycles	Denature Anneal Extend	98°C 67°C 72°C	30 sec 30 sec 5 min
Hold		4°C	∞

[00219] Assembled polynucleotides were PCR amplified. Each SOE/PCR Plate received additional reagent and was thermocycled in a PCR machine as shown in the table below. Corresponding wells on SOE/PCR plates were pooled into 96-deep well blocks, and assembled polynucleotides were purified using the Omega Biotek E-Z 96® Cycle-Pure Kit (Omega Bio-Tek Inc., Norcross, GA) as per manufacturer's suggested protocol (approximate end-volumes of 45 uL).

SOE/PCR Plates			
Additional Component (per well)	**Volume (uL)**		
10 mM stock of terminal primers S000 (SEQ ID NO: 45) and S019 (SEQ ID NO: 64)	10		
Thermocycling conditions			
Initial Denature		98°C	2 min
35 cycles	Denature Anneal Extend	98°C 67°C 72°C	12 sec 30 sec 4.5 min
Final Extend		72°C	7 min
Hold		4°C	∞

[00220] FIG. 12B shows exemplary assembled polynucleotides (boxed) resolved on a 1% agarose gel.

[00221] Purified assembled polynucleotides were digested with LguI restriction endonuclease to generate sticky ends for cloning. To this end, 96-well plates ("LguI Assembled Polynucleotide Digestion Plates") were set up as shown in the table below, and the plates were incubated at 37°C for 60 min, after which the LguI restriction endonuclease was heat inactivated at 65°C for 20 min in a PCR machine. LguI digested assembled polynucleotides were gel purified using the ZR-96 Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corporation, Orange, CA) as per manufacturer's recommended protocol.

LguI Assembled Polynucleotide LguI Digestion Plates	
Component (per well)	**Volume (uL)**
Purified assembled polynucleotide	43
10x Tango Buffer	5
LguI	2

[00222] Assembled polynucleotides were ligated into a pUC-19 based vector backbone. When no insert is ligated into this vector, a pTRC promoter (i.e., promoter of the

TRC gene of *Saccharomyces cerevisiae*) drives expression of GluRS and kills the host cell. 96-well plates ("Ligation Plates") were set up as shown in the table below, and the plates were incubated at 24°C for 15 min, and then at 16°C overnight. Ligation products were purified using the ZR-96 DNA Clean & Concentrator™-5 (Zymo Research Corporation, Orange, CA) as per manufacturer's suggested protocol.

Ligation Plates	
Component (per well)	**Volume (uL)**
ddH2O	5
10x T4 DNA Ligase Buffer	2
Vector backbone	2
Purified assembled polynucleotide	10
T4 DNA ligase (NEB, Ipswich, MA)	1

[00223] Ligation products were electroporated into E. coli competent cells. Pre-chilled 96-well electroporation plates were set up and electroporations were carried out as shown in the table below.

Electroporation Plates		
Component (per well)		**Volume (uL)**
Purified ligation products		10
Lucigen 10G competent cells (Lucigen Corporation, Middleton, WI)		25
Electroporation settings		
2400V	750 Ω	25 uF

[00224] 1.1 mL 96-well culture plates ("Culture Plates") containing 250 uL of pre-warmed SOC were set up, and 100 uL SOC was taken from each well and added to the electroporated cells immediately after electroporation. The SOC and cells were mixed, and 100 uL of each mixture was transferred back to the Culture Plates. The Culture Plates were incubated at 37°C for 1 hour in a Multitron II Incubator Shaker (ATR Biotech, Laurel, MD). Two dilutions of cells (3 ul and 240 ul) were plated on LB agar comprising 50 ug/mL kanamycin, and incubated overnight at 37°C. Colonies were picked and grown in 96 deep well plates comprising 1 mL LB medium with kanamycin per well, and DNA was extracted for restriction analysis using LguI restriction endonuclease. Results of such restriction analysis for 22 of 24 exemplary colonies comprising an approximately 8 kb combined polynucleotide are shown in FIG. 12C.

[00225] Yeast cells comprising chromosomally integrated combined polynucleotides were generated by host cell mediated homologous recombination between terminal chromosomal targeting sequences and selectable marker segments of the assembled polynucleotides. To this end, 96-well PCR plates ("Yeast Transformation Plates") were set up and heat shock transformations were carried out in a PCR machine as shown in the table below.

Yeast Transformation Plates	
Component (per well)	**Volume (uL)**
Miniprep DNA (20 ng/uL)	10
Competent yeast cells *	40
PEG/SS/LiAc master mix **	200
Heat shock	
30°C	30 min
42°C	45 min
24 °C (optional)	30 min
*Prepared by growing cells in 100 mL YPD overnight, diluting the culture and growing to an OD600 of about 0.8 overnight, spinning the cultures at 3,000g for 5 min, washing the cell pellet with 1 L ddH2O, washing the cell pellet with 1 L 100mM lithium acetate (LiAc), and resuspending the cell pellet to a total volume of 18 mL in 100 mM LiAc. ** Master mix sufficient for 4 PCR plates contains 100 mL 50% PEG, 4 mL boiled (95°C for at least 10 min) single-stranded DNA, 15 mL 1M LiAc.	

[00226] The Yeast Transformation Plates were spun at 2,000g for 2 min, supernatants were removed, and cell pellets were washed three times with 200 uL ddH2O. Cell pellets were resuspended with 100 uL cold Bird Seed Media (BSM) taken from previously prepared pre-chilled 96-well culture plates ("Seed Plates") containing 360 uL cold BSM per well. The suspended cells were transferred to the Seed Plates, and were grown overnight at 30°C in a Multitron II Incubator Shaker. The Seed Plates were spun at 3,000g for 5 min, all but 60 uL of the liquid was removed, and covered Seed Plates were shaken at 1,000rpm to resuspend the cell pellets.

Example 11

[00227] This example describes methods for generating yeast cells comprising assembled polynucleotides generated by host cell mediated homologous recombination.

[00228] The assembled polynucleotide and component polynucleotides used in this example, and the expected chromosomal locus obtained upon assembly and chromosomal integration, are schematically illustrated in FIG. 13A.

[00229] Yeast cell transformations were carried out as described in the table below. Following heat shock, the cells were spun down, supernatant was removed, cells were resuspended in 400 uL ddH2O, and host cell transformants were selected for by plating 100-200 uL of the cell suspension on agar lacking uracil.

Yeast Transformation	
Component	**Volume (uL)**
Component and assembled polynucleotides (300-500 ng each)	20
Competent yeast cells *	cell pellet *
50% PEG solution	240
1 M LiAc pH 8.4-8.9	36
Boiled (95°C for 5 min) single-stranded DNA (10 mg/mL) (Invitrogen, Carlsbad, CA)	10
ddH2O	54
Heat shock	
42°C	40 min
*Prepared by growing cells from a colony in 25 mL YPD overnight at 30°C to an OD600 of 0.7-0.9, spinning down the cells, washing the cell pellet with 5-10 mL ddH2O, washing the cell pellet with 1 mL ddH2O, washing the cell pellet with 1 mL 100 mM lithium acetate (LiAc), spinning in microcentrifuge for 30 sec to pellet the cells, and discarding the supernatant.	

[00230] Successful integration of assembled polynucleotides was determined by cPCR using cPCR primers A, B, E, and F (5' junction of chromosomal integration site) or cPCR primers C, D, G, and H (3' junction of chromosomal integration site) (FIG. 13A). As shown in FIG. 13B, all 8 colonies analyzed produced the 700 bp PCR band indicative of a positive chromosomal integration event of the expected assembled polynucleotide and lacked the 950 bp PCR band that the native locus would have produced.

Example 12

[00231] This example describes methods for the high-throughput generation of yeast cells comprising combinatorially assembled and combinatorially combined polynucleotides generated by host cell mediated homologous recombination.

[00232] The component polynucleotides used in this example, and the expected combined polynucleotides obtained upon assembly and combination by host cell mediated homologous recombination, are schematically illustrated in FIG. 14A. The component polynucleotides comprised DNA segments encoding an upstream and a downstream chromosomal targeting sequence (US and DS), 6 different promoters (P), 35 different proteins (G), and a 5' and a 3' segment of the URA3 selectable marker (URA and RA3, respectively), flanked by annealable linker sequences pairs or primer binding segment / annealable linker sequence pairs.

[00233] Component polynucleotides were released from assembly vectors by digesting RABits or MULES using LguI restriction endonuclease. To this end, 96-well plates ("LguI Digestion Plates") were set up as shown in the table below, and the plates were incubated at 37°C for 75 min, after which the LguI restriction endonuclease was heat inactivated at 65°C for 20 min in a PCR machine.

LguI **Digestion Plates**	
Component (per well)	**Volume (uL)**
667 fMoles RABit or MULE	Variable
10x Tango Buffer (Fermentas, Glen Burnie, MD)	5
LguI (Fermentas, Glen Burnie, MD)	2.5
ddH2O	to 50

[00234] To generate yeast cells comprising chromosomally integrated combinatorially assembled and combinatorially combined polynucleotides 96-well PCR plates ("Yeast Transformation Plates") were set up and heat shock transformations were carried out in a PCR machine as shown in the table below.

Yeast Transformation Plates	
Component (per well)	**Volume (uL)**
Component polynucleotides	10
Competent yeast cells *	40
PEG/SS/LiAc master mix **	200
Heat shock	
30°C	30 min
42°C	45 min

24 °C (optional)	30 min
*Prepared by growing cells in 100 mL YPD overnight, diluting the culture and growing to an OD600 of about 0.8 overnight, spinning the cultures at 3,000g for 5 min, washing the cell pellet with 1 L ddH2O, washing the cell pellet with 1 L 100mM lithium acetate (LiAc), and resuspending the cell pellet to a total volume of 18 mL in 100 mM LiAc. ** Master mix sufficient for 4 PCR plates contains 100 mL 50% PEG, 4 mL boiled (95°C for at least 10 min) single-stranded DNA, 15 mL 1M LiAc.	

[00235] The Yeast Transformation Plates were spun at 2,000g for 2 min, supernatants were removed, and cell pellets were washed three times with 200 uL ddH2O. Cell pellets were resuspended with 100 uL cold Bird Seed Media (BSM) taken from previously prepared pre-chilled 96-well culture plates ("Seed Plates") containing 360 uL cold BSM per well. The suspended cells were transferred to the Seed Plates, and were grown overnight at 30°C in a Multitron II Incubator Shaker. The Seed Plates were spun at 3,000g for 5 min, all but 60 uL of the liquid was removed, and covered Seed Plates were shaken at 1,000rpm to resuspend the cell pellets. Various dilutions of cells were plated on agar lacking uracil, and incubated overnight at 37°C. Colonies of yeast cell transformants harboring a functional URA3 selectable marker were picked and analyzed.

[00236] The scope of the claims should not be limited by the preferred embodiments set forth in the examples, but should be given the broadest interpretation consistent with the description as a whole.

The embodiments of the present invention for which an exclusive property or privilege is claimed are defined as follows:

1. A composition comprising:

(a) one or more first nucleic acid molecules, wherein each first nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_0, any DNA segment selected from the group D_0, an annealable linker sequence LB_0, and a second restriction site RB_0;

(b) one or more intermediate nucleic acid molecules, wherein each intermediate nucleic acid molecule n is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_n, a first annealable linker sequence LA_n, any DNA segment selected from the group D_n, a second annealable linker sequence LB_n, and a second restriction site RB_n, and wherein n represents an integer from one to the number of intermediate nucleic acid molecules; and

(c) one or more last nucleic acid molecules, wherein each last nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_m, an annealable linker sequence LA_m, any DNA segment selected from the group D_m, a second restriction site RB_m, wherein m represents an integer one greater than the number of intermediate nucleic acid molecules;

whereupon cleavage of restriction sites RA_0 through RB_m and denaturation of the resulting linear nucleic acid molecules, each annealable linker sequence $LB_{(p-1)}$ is capable of hybridizing to the complement of annealable linker sequence LA_p and serving as a point of initiation for synthesis of a complementary polynucleotide, wherein n is an integer that varies from 1 to (m-1), wherein p represents an integer from 1 to m, and wherein each group $D_0, \ldots D_n, \ldots$ and D_m independently consists of one or more DNA segments.

2. The composition of claim 1, wherein each of said one or more first nucleic acid molecules further comprises a primer binding segment PA positioned 5' to the DNA segment selected from the group D_0, and wherein each of said one or more last nucleic acid molecules further comprises a primer binding segment PB positioned 3' to the DNA segment selected from the group D_m.

3. The composition of claim 1 or 2, wherein upon cleavage of restriction sites RA_0 through RB_m and denaturation of the resulting linear nucleic acid molecules, each annealable linker sequence $LB_{(p-1)}$ is capable of selectively hybridizing to the complement of annealable linker sequence LA_p compared to the other annealable linker sequences, or their complements, in the composition.

4. The composition of claim 1 or 2, wherein each annealable linker $LB_{(p-1)}$ is identical in sequence to annealable linker sequence LA_p, or the complements thereof.

5. The composition of claim 1 or 2, comprising one first nucleic acid molecule and one last nucleic acid molecule.

6. The composition of claim 1 or 2, wherein each of restriction sites RA_0 through RB_m are cleavable by a Type IIS restriction endonuclease.

7. The composition of claim 1 or 2, wherein the restriction sites RA_0 through RB_m are cleavable by the same Type IIS restriction endonuclease.

8. The composition of claim 1 or 2, wherein the restrictions sites RA_0 through RB_m are cleavable by SapI or LguI restriction endonuclease.

9. The composition of claim 1, wherein each of two or more annealable linker sequences is independently at least 24 nucleotides in length and has a melting temperature of at least 60°C.

10. The composition of claim 1 or 2, wherein each of two or more annealable linker sequences independently has a G-C content of at least 70% and a melting temperature of at least 70°C.

11. The composition of claim 1 or 2, wherein each of two or more annealable linker sequences independently has an A-T content of at least 30% and a melting temperature of at least 65°C, and comprise a sequence motif

5'ANNNNNNNNANNNAANTANNTTNANA-3', wherein A stands for adenine, N for any nucleotide, and T for thymine.

12. The composition of claim 1 or 2, wherein two or more annealable linker sequences have sequences selected from the group consisting of SEQ ID NOS: 1 to 8, and the complements thereof.

13. The composition of claim 1 or 2, wherein each of the annealable linker sequences has a sequence selected from the group consisting of SEQ ID NOS: 1 to 8, and the complements thereof.

14. The composition of claim 2, wherein each of the primer binding segments has a sequence selected from the group consisting of SEQ ID NOS: 9 to 10, and the complements thereof.

15. The composition of claim 1 or 2, further comprising one or more restriction endonucleases capable of cleaving the restriction sites RB_0 through RB_m.

16. The composition of claim 8, further comprising SapI or LguI restriction endonuclease.

17. A composition comprising a plurality of linear nucleic acid molecules formed by digesting the composition of claim 1 or claim 2 with one or more restriction endonucleases that cleave the restriction sites RA_0 through RB_m.

18. The composition of claim 17, wherein each linear nucleic acid molecule comprises sticky ends.

19. The composition of claim 2, further comprising a first primer that is complimentary to primer binding segment PA, or the complement thereof, and a second primer that is complimentary to primer binding segment PB, or the complement thereof.

20. The composition of claim 1 or 2, further comprising a DNA polymerase.

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21. A composition comprising a plurality of linear nucleic acid molecules formed by digesting the composition of claim 8 with SapI or LguI restriction endonuclease.

22. A library of nucleic acid molecules comprising at least one of each of the following nucleic acid molecules:

(a) a first nucleic acid molecule wherein the first nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_0, any DNA segment selected from the group D_0, an annealable linker sequence LB_0, and a second restriction site RB_0;

(b) an intermediate nucleic acid molecule wherein the intermediate nucleic acid molecule n is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_n, a first annealable linker sequence LA_n, any DNA segment selected from the group D_n, a second annealable linker sequence LB_n, and a second restriction site RB_n, and wherein n represents an integer from one to the number of intermediate nucleic acid molecules; and

(c) a last nucleic acid molecule wherein the last nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_m, an annealable linker sequence LA_m, any DNA segment selected from the group D_m, a second restriction site RB_m wherein m represents an integer one greater than the number of intermediate nucleic acid molecules;

whereupon cleavage of restriction sites RA_0 through RB_m and denaturation of the resulting linear nucleic acid molecules, each annealable linker sequence $LB_{(p-1)}$ is capable of hybridizing to the complement of annealable linker sequence LA_p and serving as a point of initiation for synthesis of a complementary polynucleotide.wherein n is an integer that varies from 1 to (m-1), wherein p represents an integer from 1 to m, and wherein each group D_0,... D_n,...and D_m independently consists of one or more DNA segments.

23. The library of claim 22, wherein said first nucleic acid molecule further comprises a primer binding segment PA positioned 5' to the DNA segment selected from the group D_0, and said last nucleic acid molecules further comprises a primer binding segment PB positioned 3' to the DNA segment selected from the group D_m.

24. The library of claim 22 or 23, wherein each of two or more annealable linker sequences is independently at least 24 nucleotides in length and has a melting temperature of at least 60°C.

25. The library of claim 22 or 23, wherein each of two or more annealable linker sequences independently has a G-C content of at least 70% and a melting temperature of at least 70°C.

26. The library of claim 22 or 23, wherein each of two or more annealable linker sequences independently has an A-T content of at least 30% and a melting temperature of at least 65°C, and comprise a sequence motif 5'ANNNNNNNNANNNAANTANNTTNANA-3', wherein A stands for adenine, N for any nucleotide, and T for thymine.

27. The library of claim 22 or 23, wherein two or more of the annealable linker sequences are independently selected from the group consisting of SEQ ID NOS: 1 to 8, and complements thereof.

28. The library of claim 22 or 23, wherein each of the annealable linker sequences are independently selected from the group consisting of SEQ ID NOS: 1 to 8, and complements thereof.

29. The library of claim 23, wherein each of the primer binding segments are independently selected from the group consisting of SEQ ID NOS: 9 and 10, and complements thereof.

30. The library of claim 22 or 23, comprising two first nucleic acid molecules and two last nucleic acid molecules,

wherein one of the first nucleic acid molecules comprises a DNA segment D_{01} that comprises a first segment of a genomic targeting sequence, and the other first nucleic acid molecule comprises a DNA segment D_{02} that comprises a second segment of the genomic targeting sequence, wherein DNA segments D_{01} and D_{02} are oriented in the same orientation relative to restriction sites RA_0 and RB_0 of the two first nucleic acid molecules;

wherein one of the last nucleic acid molecules comprises a DNA segment D_{m1} that encodes a first non-functional segment of a selectable marker, and the other last nucleic acid molecule comprises a DNA segment D_{m2} that encodes a second non-functional segment of the selectable marker, wherein DNA segments D_{m1} and D_{m2} are oriented in opposite orientations relative to restriction sites RA_m and RB_m of the two last nucleic acid molecules; and

wherein recombination by gap repair between the first non-functional segment of the selectable marker and the second non-functional segment of the selectable marker results in the creation of a functional selectable marker.

31. A method of generating an assembled polynucleotide from a plurality of component polynucleotides comprising the steps of:

(a) digesting an assembly composition with one or more restriction endonucleases to generate a components composition, the assembly composition comprising:

(i) one or more first nucleic acid molecules, wherein each first nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_0, any primer binding segment selected from the group PA, any DNA segment selected from the group D_0, an annealable linker sequence LB_0, and a second restriction site RB_0;

(ii) one or more intermediate nucleic acid molecules wherein each intermediate nucleic acid molecule n is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_n, a first annealable linker sequence LA_n, any DNA segment selected from the group D_n, a second annealable linker sequence LB_n, and a second restriction site RB_n, and wherein n represents an integer from one to the number of intermediate nucleic acid molecules; and

(iii) one or more last nucleic acid molecules, wherein each last nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_m, an annealable linker sequence LA_m, a DNA segment selected from the group D_m, any primer binding segment selected from the group PB, a second restriction site RB_m wherein m represents an integer one greater than the number of intermediate nucleic acid molecules; whereupon cleavage of restriction sites RA_0

through RB_m and denaturation of the resulting linear nucleic acid molecules, each annealable linker sequence $LB_{(p-1)}$ is capable of hybridizing to the complement of annealable linker sequence LA_p and serving as a point of initiation for synthesis of a complementary polynucleotide, wherein n is an integer that varies from 1 to (m-1), wherein p represents an integer from 1 to m, and wherein each group D_0,... D_n,...and D_m consists of one or more DNA segments; and

(b) contacting the components composition with DNA polymerase, deoxyribonucleoside triphosphates and one or more first primers and one or more second primers, under conditions suitable for denaturation of the nucleic acid molecules, annealing of annealable linker sequence $LB_{(p-1)}$ to annealable linker sequence LA_p, and extension therefrom; wherein each said first primer is capable of hybridizing to one of said primer binding segments selected from the group PA and each said second primer is capable of hybridizing to one of said primer binding segments selected from the group PB; and subjecting the components composition to polymerase chain reaction,

wherein a polynucleotide is assembled which comprises, in a 5' to 3' orientation, one DNA segment selected from each of the groups D_0,... D_n,...and D_m.

32. The method of claim 31, wherein the restriction sites RA_0 through RB_m are cleaved by a Type IIS restriction endonuclease.

33. The method of claim 31, wherein the assembly composition comprises one first nucleic acid molecule and one last nucleic acid molecule.

34. The method of claim 31, wherein each of two or more annealable linker sequences is independently at least 24 nucleotides in length and has a melting temperature of at least 60°C.

35. The method of claim 31, wherein each of two or more annealable linker sequences independently has a G-C content of at least 70% and a melting temperature of at least 70°C.

36. The method of claim 31, wherein each of two or more annealable linker sequences independently has an A-T content of at least 30% and a melting temperature of at least 65°C, and comprise a sequence motif 5'ANNNNNNNNANNNAANTANNTTNANA-3', wherein A stands for adenine, N for any nucleotide, and T for thymine.

37. The method of claim 31, wherein each annealable linker sequence $LB_{(p-1)}$ is identical in sequence to annealable linker sequence LA_p.

38. The method of claim 31, wherein the restriction sites RA_0 through RB_m are cleaved by the same Type IIS restriction endonuclease.

39. The method of claim 31, wherein the restrictions sites RA_0 through RB_m are cleaved by SapI or LguI restriction endonuclease, and the restriction endonuclease of step (a) is SapI or LguI.

40. The method of claim 31, wherein each of two or more annealable linker sequences are independently selected from the group consisting of SEQ ID NOS: 1 to 8, and complements thereof.

41. The method of claim 31, wherein each of the annealable linker sequences are independently selected from the group consisting of SEQ ID NOS: 1 to 8, and complements thereof.

42. The method of claim 31, wherein each of the primer binding segments are selected from the group consisting of SEQ ID NOS: 9 and 10, and complements thereof.

43. The method of claim 31, wherein the DNA polymerase is Pfu.

44. A method of generating a host cell comprising a polynucleotide comprising the steps of:

(a) transforming a host cell with one or more polynucleotides assembled according to claim 31; and

(b) selecting the host cell comprising the one or more assembled polynucleotides.

45. The method of claim 44, wherein the assembled polynucleotide comprises a selectable marker and step (b) comprises propagating the transformed host cell on selectable media.

46. The method of claim 44, further comprising transforming the host cell with a linearized plasmid comprising:

(i) a first region of homology with primer binding segment PA; and

(ii) a second region of homology with primer binding segment PB, wherein said first and second regions of homology are of sufficient length to initiate host cell mediated homologous recombination between said polynucleotide and said plasmid to form a circularized plasmid in the host cell.

47. A method of generating a host cell comprising a polynucleotide, the method comprising the steps of:

(a) transforming a host cell with a composition comprising:

(i) one or more first linear nucleic acid molecules, wherein each molecule comprises, in a 5' to 3' orientation, any DNA segment selected from the group D_0 and an annealable linker sequence LB_0;

(ii) one or more intermediate linear nucleic acid molecules, wherein each intermediate linear nucleic acid molecule comprises, in a 5' to 3' orientation, a first annealable linker sequence LA_n, any DNA segment selected from the group D_n, and a second annealable linker sequence LB_n, wherein n represents an integer from one to the number of intermediate nucleic acid molecules; and

(iii) one or more last linear nucleic acid molecules, wherein each last linear nucleic acid molecule comprises, in a 5' to 3' orientation, an annealable linker sequence LA_m, and any DNA segment selected from the group D_m, wherein m represents an integer one greater than the number of intermediate nucleic acid molecules;

wherein n is an integer that varies from 1 to (m-1),

wherein each group $D_0,\ldots D_n,\ldots$ and D_m consists of one or more DNA segments,

wherein each annealable linker sequence $LB_{(p-1)}$ comprises a region of homology with annealable linker sequence LA_p of sufficient length to initiate host cell mediated homologous recombination between $LB_{(p-1)}$ and LA_p wherein p represents an integer from 1 to m, wherein said homologous recombination results in the assembly of a polynucleotide; and

(b) selecting a host cell comprising an assembled polynucleotide, wherein the assembled polynucleotide comprises in a 5' to 3' orientation, one DNA segment selected from each of the groups $D_0, \ldots D_n, \ldots$ and D_m.

48. The method of claim 47, wherein

(a) each of the one or more first linear nucleic acid molecules further comprises a first region of homology with a first integration site of the host cell genome; and

(b) each of the one or more last linear nucleic acid molecules further comprises a second region of homology with a second integration site of the host cell genome,

wherein said first and second regions of homology are of sufficient length to initiate host cell mediated homologous recombination with said first and second integration sites, respectively, wherein said homologous recombination results in integration of the assembled polynucleotide into the host cell genome.

49. The method of claim 47, wherein at least one homologous recombination of an annealable linker sequence $LB_{(p-1)}$ and LA_p forms a nucleic acid sequence that encodes a selectable marker gene.

50. The method of claim 47, wherein step (a) further comprises transforming the host cell with a linearized plasmid, wherein the linearized plasmid comprises:

(i) a first region of homology with the one or more first linear nucleic acid molecules; and

(ii) a second region of homology with the one or more last linear nucleic acid molecules, wherein said first and second regions of homology are of sufficient length to initiate host cell mediated homologous recombination between the assembled polynucleotide and said plasmid to form a circularized plasmid in the host cell.

51. The method of claim 47, wherein the composition comprises one first nucleic acid molecule and one last nucleic acid molecule.

52. The method of claim 47, wherein two or more annealable linker sequences have a G-C content of at least 70% and a melting temperature of at least 70°C.

53. The method of claim 47, wherein two or more annealable linker sequences have an A-T content of at least 30% and a melting temperature of at least 65°C, and comprise a sequence motif 5'ANNNNNNNNANNNAANTANNTTNANA-3', wherein A stands for adenine, N for any nucleotide, and T for thymine.

54. The method of claim 47, wherein each annealable linker sequence $LB_{(p-1)}$ is identical in sequence to annealable linker sequence LA_p.

55. A method of generating a host cell comprising a polynucleotide, the method comprising the steps of:

(a) digesting with one or more restriction endonucleases a composition comprising:

(i) one or more first nucleic acid molecules, wherein each first nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_0, a primer binding segment PA, any DNA segment selected from the group D_0, an annealable linker sequence LB_0, and a second restriction site RB_0;

(ii) one or more intermediate nucleic acid molecules wherein each intermediate nucleic acid molecule n is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_n, a first annealable linker sequence LA_n, any DNA segment selected from group D_n, a second annealable linker sequence LB_n, and a second restriction site RB_n, and wherein n represents an integer from one to the number of intermediate nucleic acid molecules; and

(iii) one or more last nucleic acid molecules, wherein each last nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_m, an annealable linker sequence LA_m, any DNA segment selected from the group D_m, a primer binding segment PB, a second restriction site RB_m, wherein m represents an integer one greater than the number of intermediate nucleic acid molecules; whereupon cleavage of restriction sites RA_0 through RB_m and denaturation of the resulting linear nucleic acid molecules, each annealable linker sequence $LB_{(p-1)}$ is capable of hybridizing to the complement of annealable linker sequence LA_p, wherein p represents an integer from 1 to m, wherein n is an integer that varies from 1 to (m-1), wherein each group $D_0, \ldots D_n, \ldots$ and D_m consists of one or more DNA segments;

wherein the one or more restriction endonucleases are capable of cleaving the restriction sites RA_0 through RB_m; and

(b) transforming a host cell with the digested composition resulting from step (a), wherein each annealable linker sequence $LB_{(p-1)}$ comprises a region of homology with annealable linker sequence LA_p of sufficient length to initiate host cell mediated homologous recombination between $LB_{(p-1)}$ and LA_p, wherein said homologous recombination results in assembly of said polynucleotide, wherein p represents an integer from 1 to m; and

(c) selecting a host cell comprising the assembled polynucleotide, wherein the assembled polynucleotide comprises in a 5' to 3' orientation, one DNA segment selected from each of groups $D_0, \ldots D_n, \ldots$ and D_m.

56. The method of claim 55, wherein

(a) each of the one or more first linear nucleic acid molecules further comprises a first region of homology with a first integration site of the host cell genome; and

(b) each of the one or more last linear nucleic acid molecules further comprises a second region of homology with a second integration site of the host cell genome,

wherein said first and second regions of homology are of sufficient length to initiate host cell mediated homologous recombination with said first and second integration sites, respectively, wherein said homologous recombination results in integration of the assembled polynucleotide into the host cell genome.

57. The method of claim 55, wherein at least one homologous recombination of an annealable linker sequence $LB_{(p-1)}$ and LA_p forms a nucleic acid sequence that encodes a selectable marker gene.

58. The method of claim 55, wherein step (b) further comprises transforming the host cell with a linearized plasmid, wherein the linearized plasmid comprises:

(i) a first region of homology with the one or more first linear nucleic acid molecules; and

(ii) a second region of homology with the one or more last linear nucleic acid molecules, wherein said first and second regions of homology are of sufficient length to initiate host cell mediated homologous recombination between an assembled polynucleotide and said plasmid to form a circularized plasmid in the host cell.

59. The method of claim 55, wherein each of two or more annealable linker sequences is independently at least 24 nucleotides in length and have a melting temperature of at least 60°C.

60. The method of claim 55, wherein each of two or more annealable linker sequences independently has a G-C content of at least 70% and a melting temperature of at least 70°C.

61. The method of claim 55, wherein each of two or more annealable linker sequences independently has an A-T content of at least 30% and a melting temperature of at least 65°C, and comprise a sequence motif 5'ANNNNNNNNANNNAANTANNTTNANA-3', wherein A stands for adenine, N for any nucleotide, and T for thymine.

62. The method of claim 55, wherein each annealable linker sequence $LB_{(p-1)}$ is identical in sequence to annealable linker sequence LA_p, or a complement thereof.

63. The method of claim 55, wherein the restriction sites RA_0 through RB_m are cleaved by a Type IIS restriction endonuclease.

64. The method of claim 55, wherein the restriction sites RA_0 through RB_m are cleaved by the same Type IIS restriction endonuclease.

65. The method of claim 55, wherein the restrictions sites RA_0 through RB_m are cleaved by SapI or LguI restriction endonuclease, and the restriction endonuclease of step (a) is SapI or LguI.

66. A method of generating a host cell comprising a polynucleotide, the method comprising the steps of:

(a) digesting with one or more restriction endonucleases a composition comprising:

(i) one or more first nucleic acid molecules, wherein each first nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_0, a primer binding segment PA, any DNA segment selected from the group D_0, an annealable linker sequence LB_0, and a second restriction site RB_0; and

(ii) one or more last nucleic acid molecules, wherein each last nucleic acid molecule is circular and comprises, in a 5' to 3' orientation, a first restriction site RA_1, an annealable linker sequence LA_1, any DNA segment selected from the group D_1, a primer binding segment PB, a second restriction site RB_1, whereupon cleavage of restriction sites RA_0 through RB_1 and denaturation of the resulting linear nucleic acid molecules, each annealable linker sequence LB_0 is capable of hybridizing to the complement of annealable linker sequence LA_1, wherein each group D_0 and D_1 consists of one or more DNA segments;

wherein the one or more restriction endonucleases are capable of cleaving the restriction sites RA_0 through RB_1;

(b) transforming a host cell with the digested composition resulting from step (a), wherein each annealable linker sequence LB_0 comprises a region of homology with annealable linker sequence LA_1 of sufficient length to initiate host cell mediated homologous recombination between LB_0 and LA_1, wherein said homologous recombination results in assembly of said polynucleotide; and

(c) selecting a host cell comprising the assembled polynucleotide, wherein the assembled polynucleotide comprises in a 5' to 3' orientation, one DNA segment selected from each of groups D_0 and D_1.

67. The method of claim 66, wherein

(a) each of the one or more first linear nucleic acid molecules further comprises a first region of homology with a first integration site of the host cell genome; and

(b) each of the one or more last linear nucleic acid molecules further comprises a second region of homology with a second integration site of the host cell genome,

wherein said first and second regions of homology are of sufficient length to initiate host cell mediated homologous recombination with said first and second integration sites, respectively, wherein said homologous recombination results in integration of the assembled polynucleotide into the host cell genome.

68. The method of claim 66, wherein at least one homologous recombination of an annealable linker sequence LB_0 and LA_1 forms a nucleic acid sequence that encodes a selectable marker gene.

69. The method of claim 66, wherein step (b) further comprises transforming the host cell with a linearized plasmid, wherein the linearized plasmid comprises:

(i) a first region of homology with the one or more first linear nucleic acid molecules; and

(ii) a second region of homology with the one or more last linear nucleic acid molecules, wherein said first and second regions of homology are of sufficient length to initiate host cell mediated homologous recombination between an assembled polynucleotide and said plasmid to form a circularized plasmid in the host cell.

70. The method of claim 66, wherein each of two or more annealable linker sequences is independently at least 24 nucleotides in length and have a melting temperature of at least 60°C.

71. The method of claim 66, wherein each of two or more annealable linker sequences independently has a G-C content of at least 70% and a melting temperature of at least 70°C.

72. The method of claim 66, wherein each of two or more annealable linker sequences independently has an A-T content of at least 30% and a melting temperature of at least 65°C, and comprise a sequence motif 5'ANNNNNNNNANNNAANTANNTTNANA-3', wherein A stands for adenine, N for any nucleotide, and T for thymine.

73. The method of claim 66, wherein each annealable linker sequence LB_0 is identical in sequence to annealable linker sequence LA_0, or a complement thereof.

74. The method of claim 66, wherein the restriction sites RA_0 through RB_1 are cleaved by a Type IIS restriction endonuclease.

75. The method of claim 66, wherein the restriction sites RA_0 through RB_1 are cleaved by the same Type IIS restriction endonuclease.

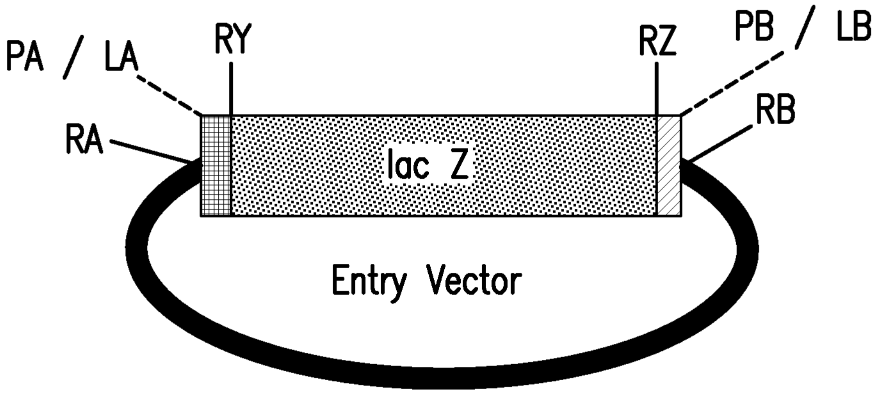

PA / LA
RY
RZ
PB / LB
RA
RB
lac Z
Entry Vector

FIG.1A

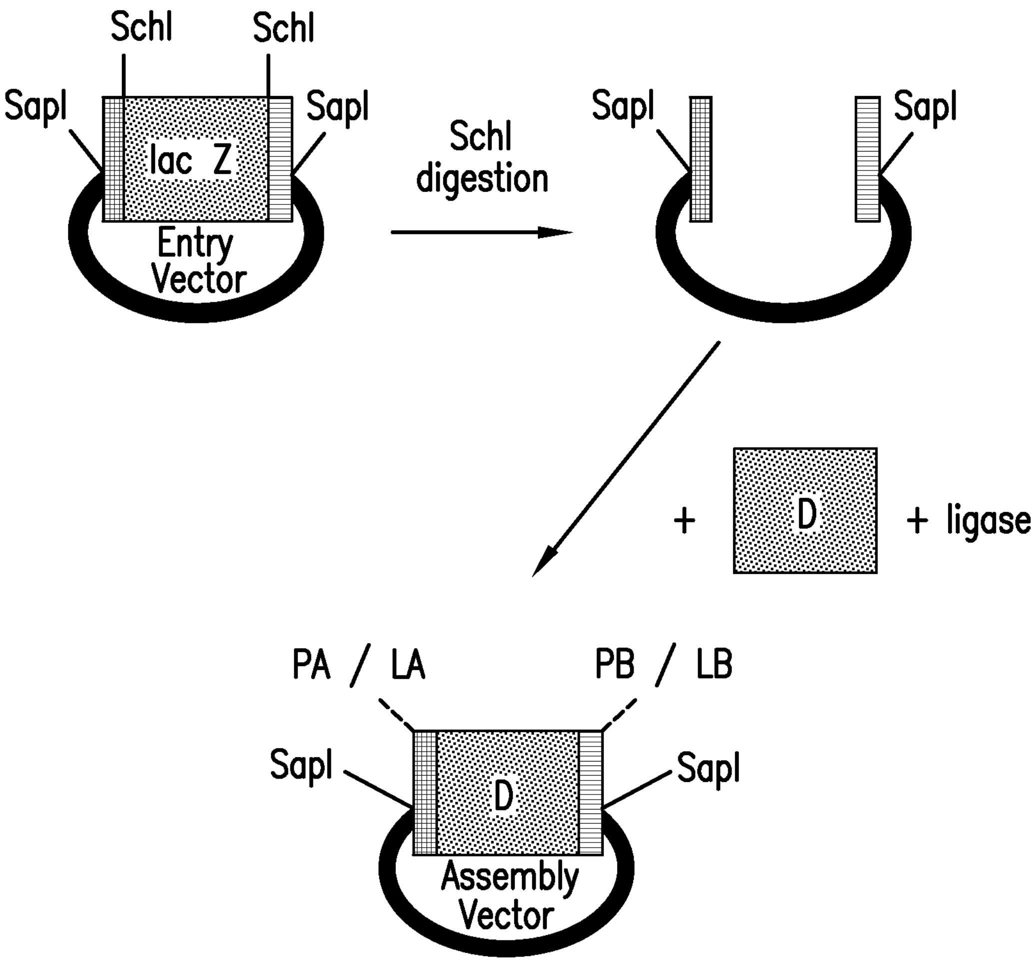

SchI
SchI
SapI
SapI
lac Z
Entry Vector
SchI digestion
SapI
SapI
+
D
+ ligase
PA / LA
PB / LB
SapI
SapI
D
Assembly Vector

FIG.1B

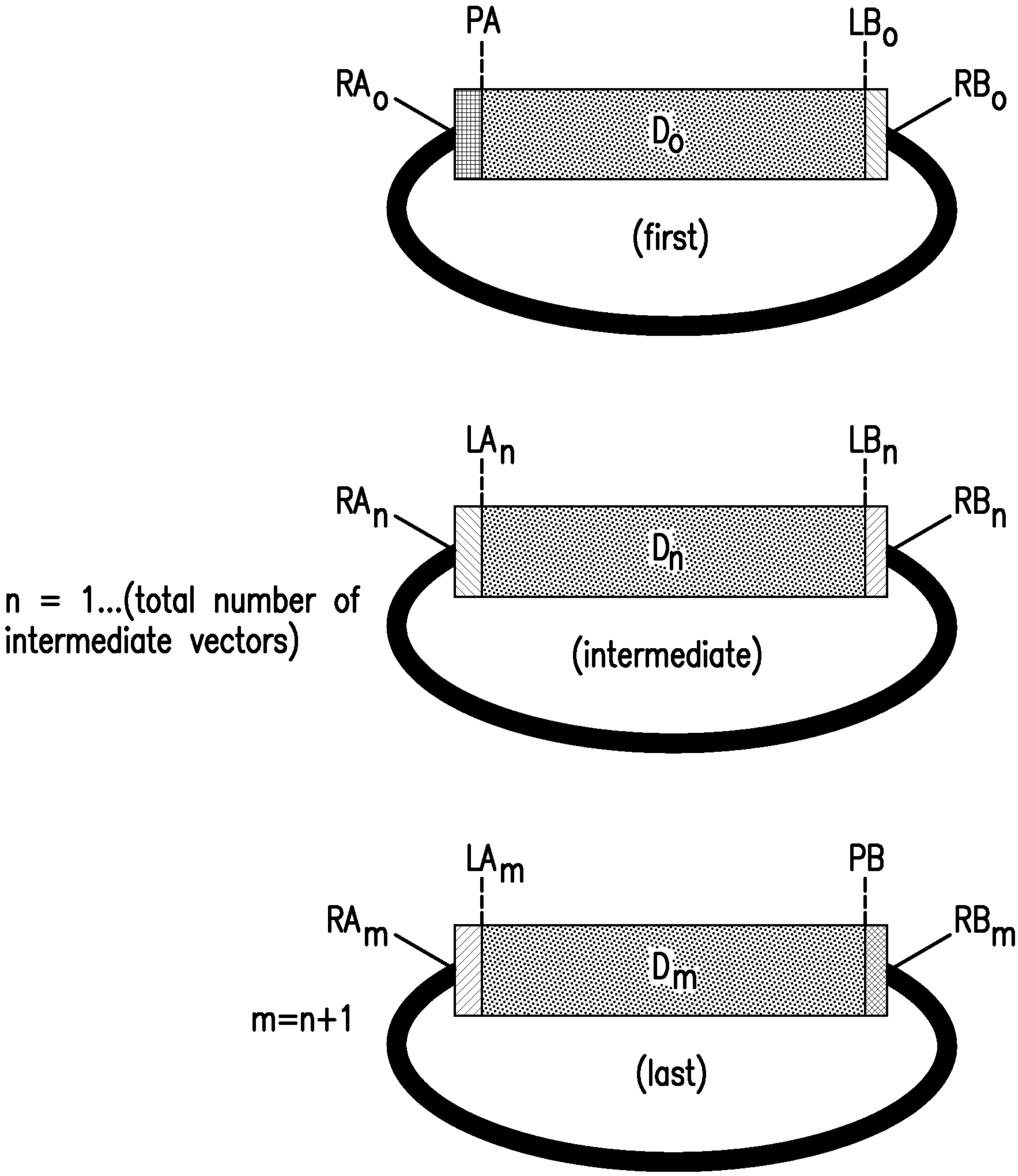

PA
LB_o
RA_o
RB_o
D_o
(first)
LA_n
LB_n
RA_n
RB_n
D_n
n = 1...(total number of intermediate vectors)
(intermediate)
LA_m
PB
RA_m
RB_m
D_m
m=n+1
(last)

FIG.2

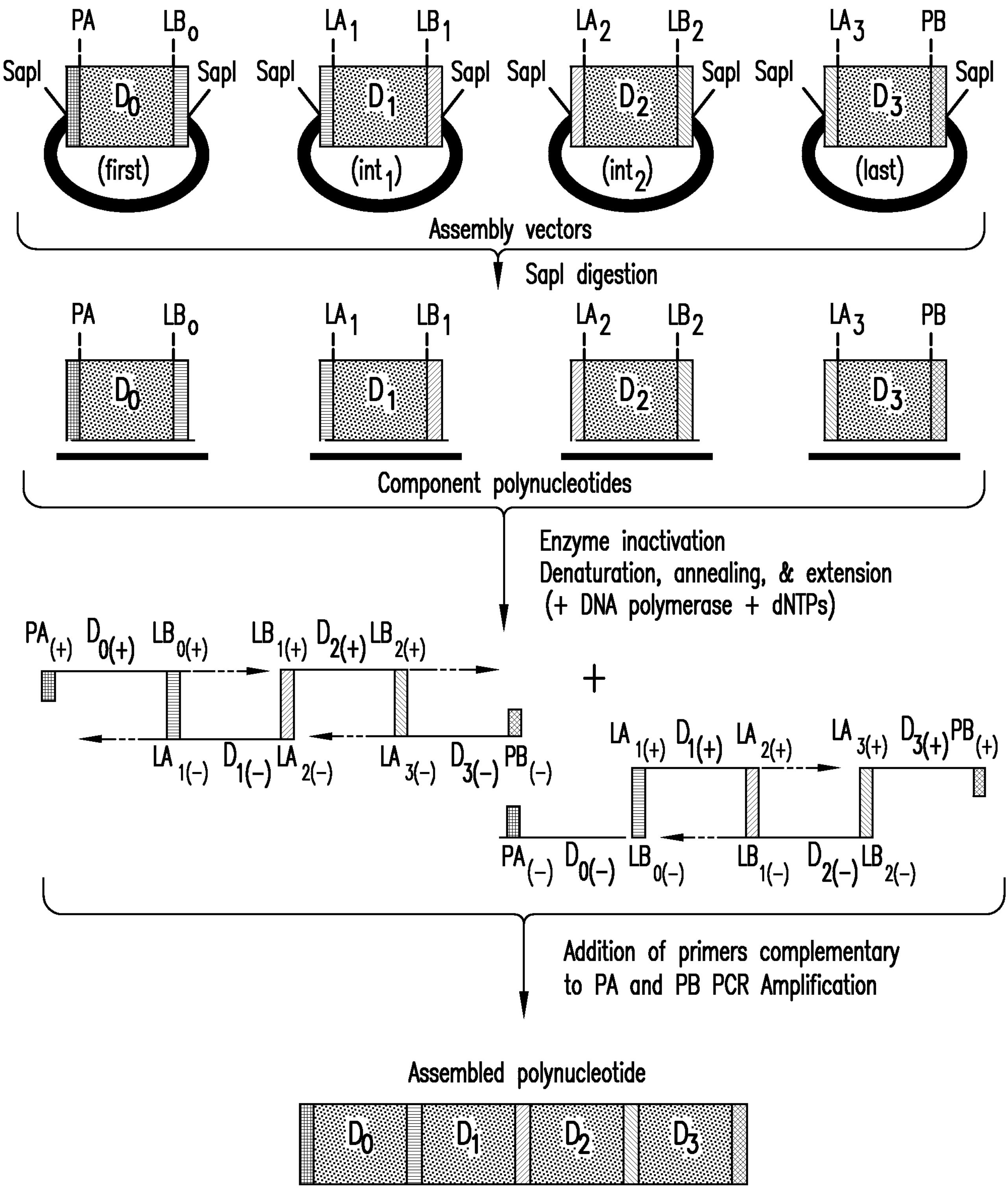

PA
LB0
LA1
LB1
LA2
LB2
LA3
PB
SapI
D0
D1
D2
D3
(first)
(int1)
(int2)
(last)
Assembly vectors
SapI digestion
Component polynucleotides
Enzyme inactivation
Denaturation, annealing, & extension
(+ DNA polymerase + dNTPs)
PA(+) D0(+) LB0(+) LB1(+) D2(+) LB2(+)
LA1(-) D1(-) LA2(-) LA3(-) D3(-) PB(-)
+
LA1(+) D1(+) LA2(+) LA3(+) D3(+) PB(+)
PA(-) D0(-) LB0(-) LB1(-) D2(-) LB2(-)
Addition of primers complementary
to PA and PB PCR Amplification
Assembled polynucleotide

FIG.3

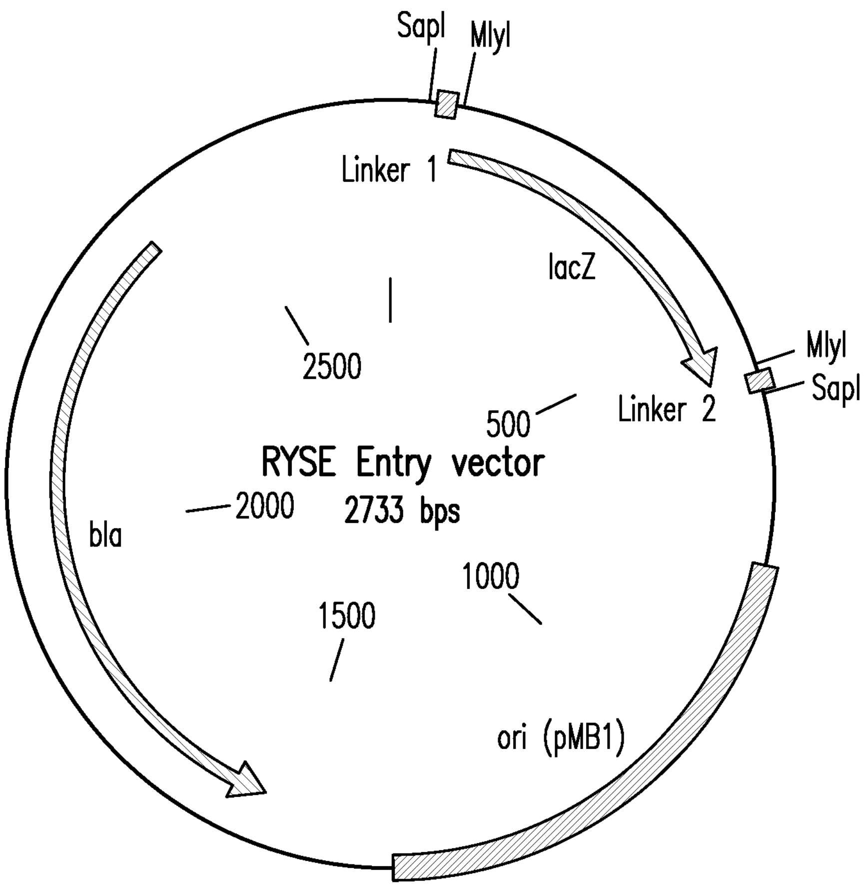

SapI
MlyI
Linker 1
lacZ
MlyI
SapI
Linker 2
2500
500
RYSE Entry vector
2733 bps
2000
bla
1000
1500
ori (pMB1)

FIG.4

Column Purification

Heat Inactivation

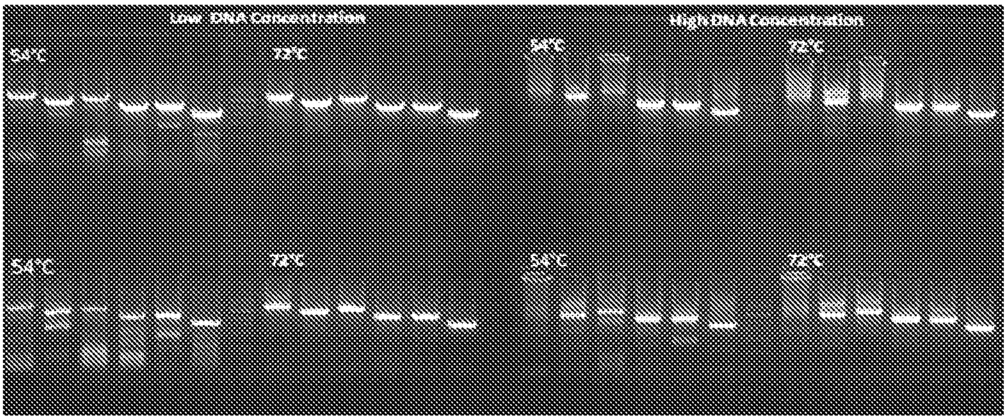


FIG.5

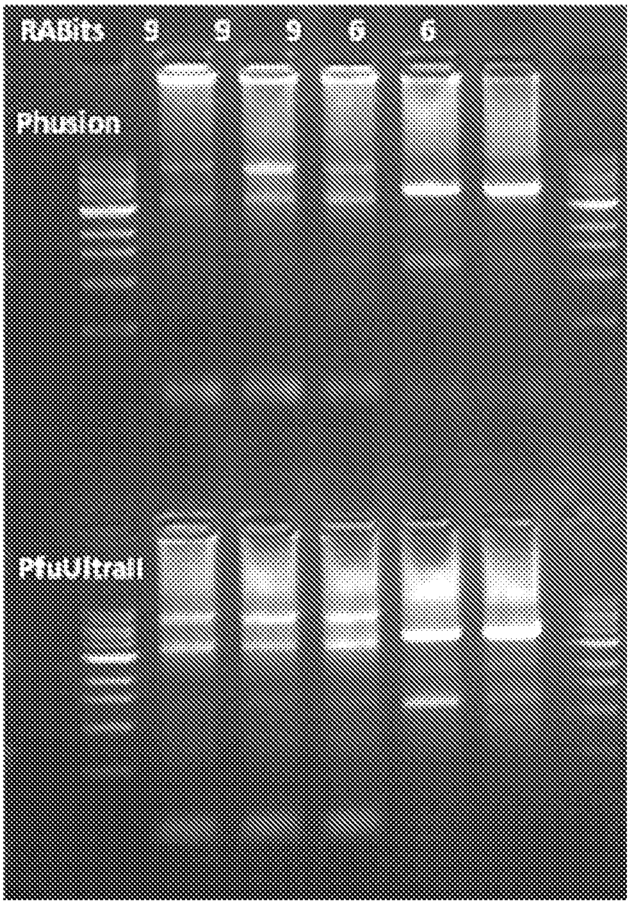


FIG.6

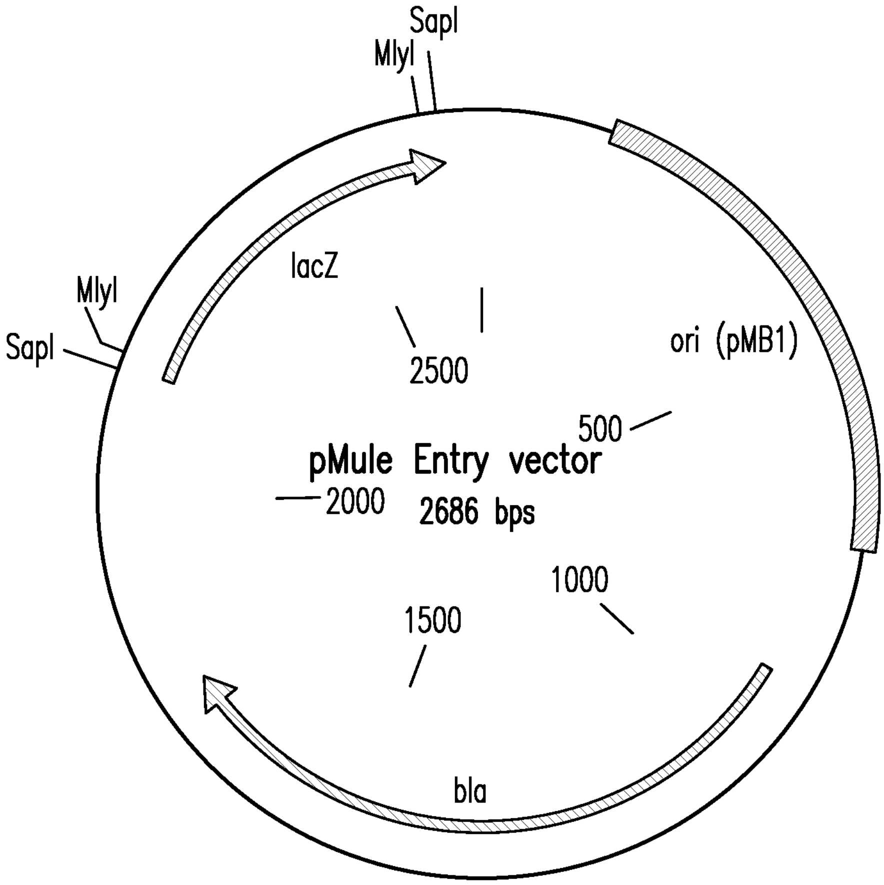

SapI
MlyI
MlyI
SapI
lacZ
2500
ori (pMB1)
500
pMule Entry vector
2000
2686 bps
1000
1500
bla

FIG.7

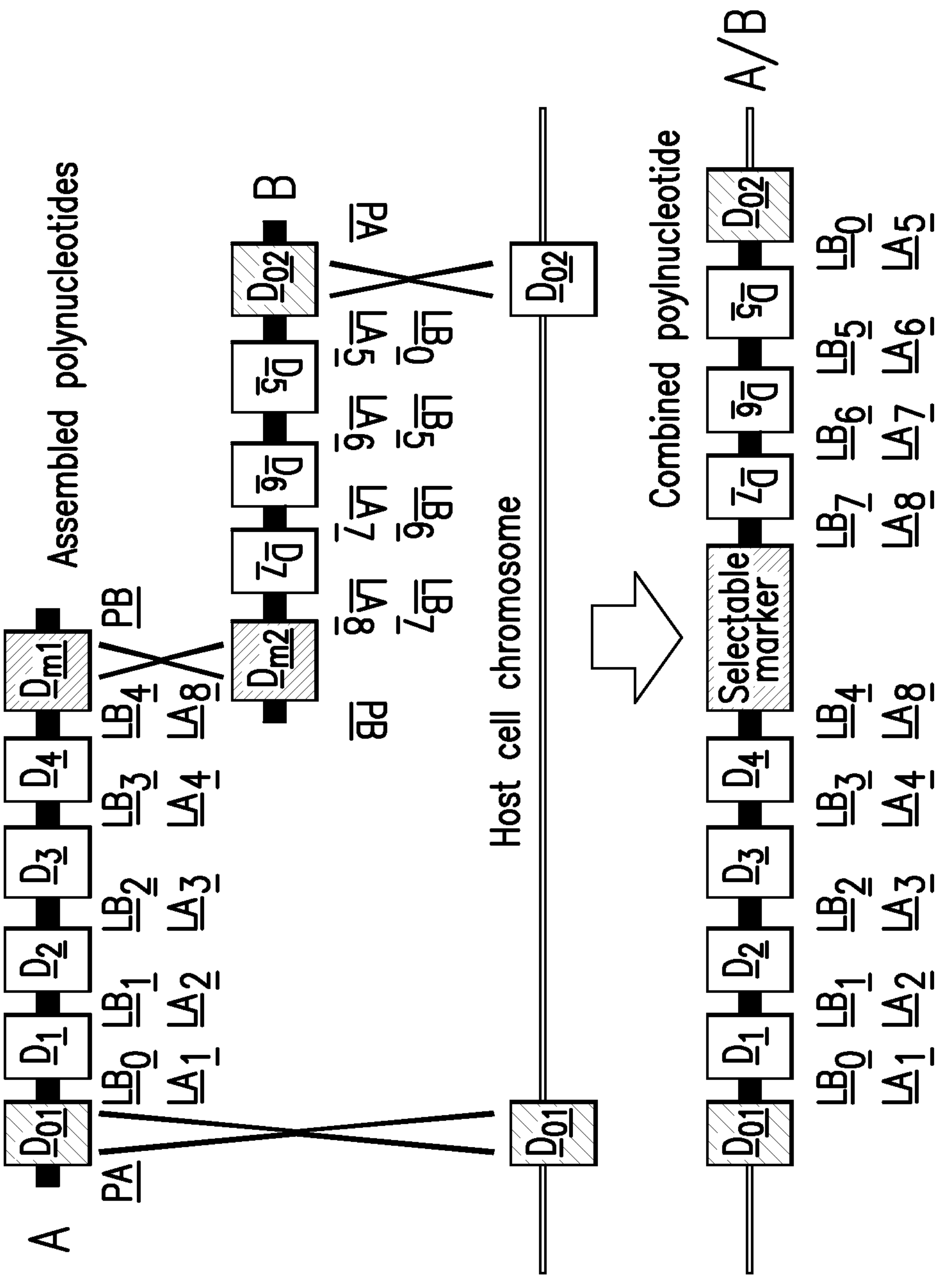

Assembled polynucleotides
A
B
PA
PB
D01
D1
D2
D3
D4
Dm1
Dm2
D7
D6
D5
D02
LB0
LB1
LB2
LB3
LB4
LB5
LB6
LB7
LA1
LA2
LA3
LA4
LA5
LA6
LA7
LA8
Host cell chromosome
Combined poylnucleotide
Selectable marker
A/B

FIG.8

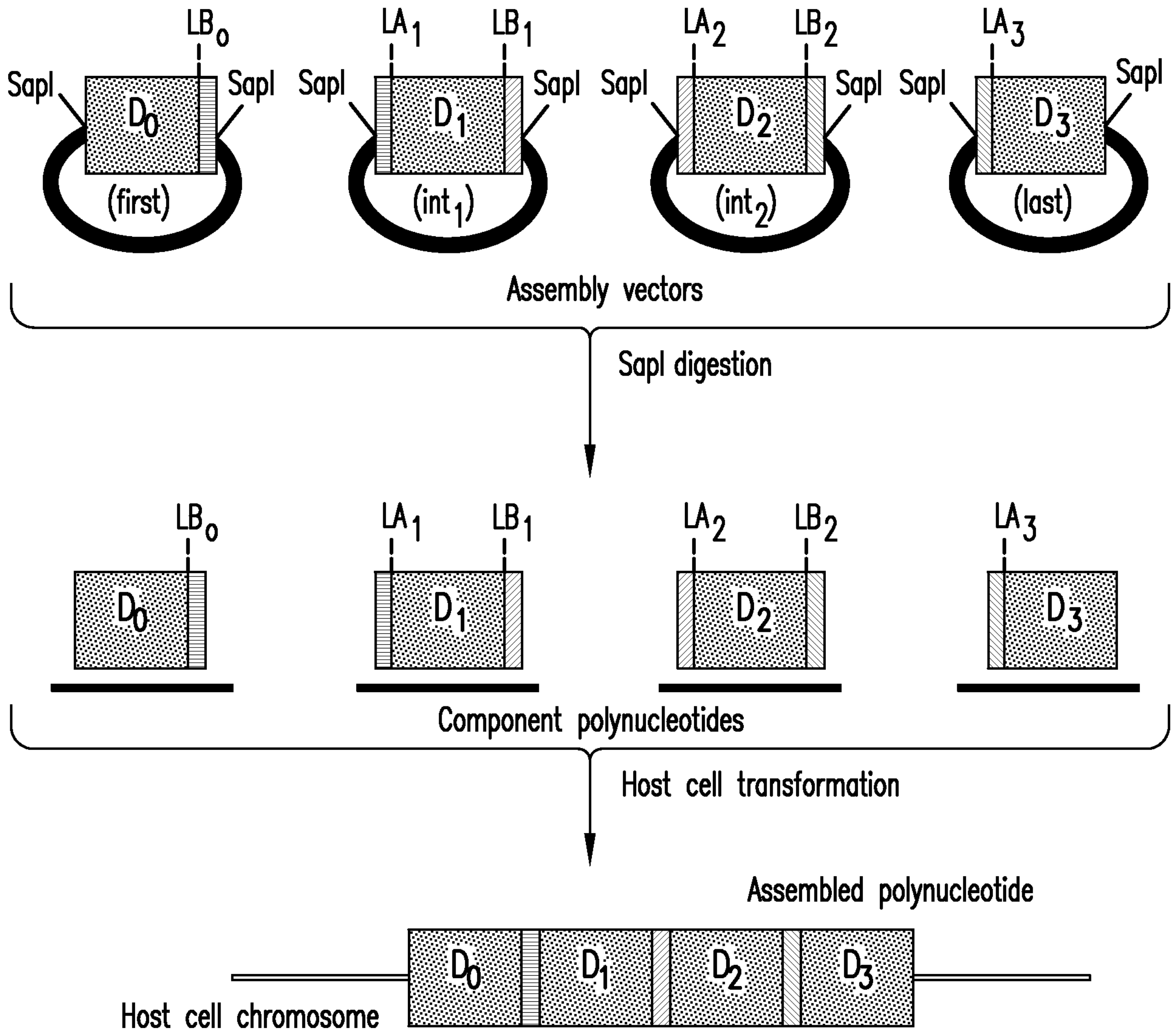

LB0
LA1
LB1
LA2
LB2
LA3
SapI
SapI
SapI
SapI
SapI
SapI
SapI
SapI
D0
D1
D2
D3
(first)
(int1)
(int2)
(last)
Assembly vectors
SapI digestion
LB0
LA1
LB1
LA2
LB2
LA3
D0
D1
D2
D3
Component polynucleotides
Host cell transformation
Assembled polynucleotide
D0
D1
D2
D3
Host cell chromosome

FIG.9

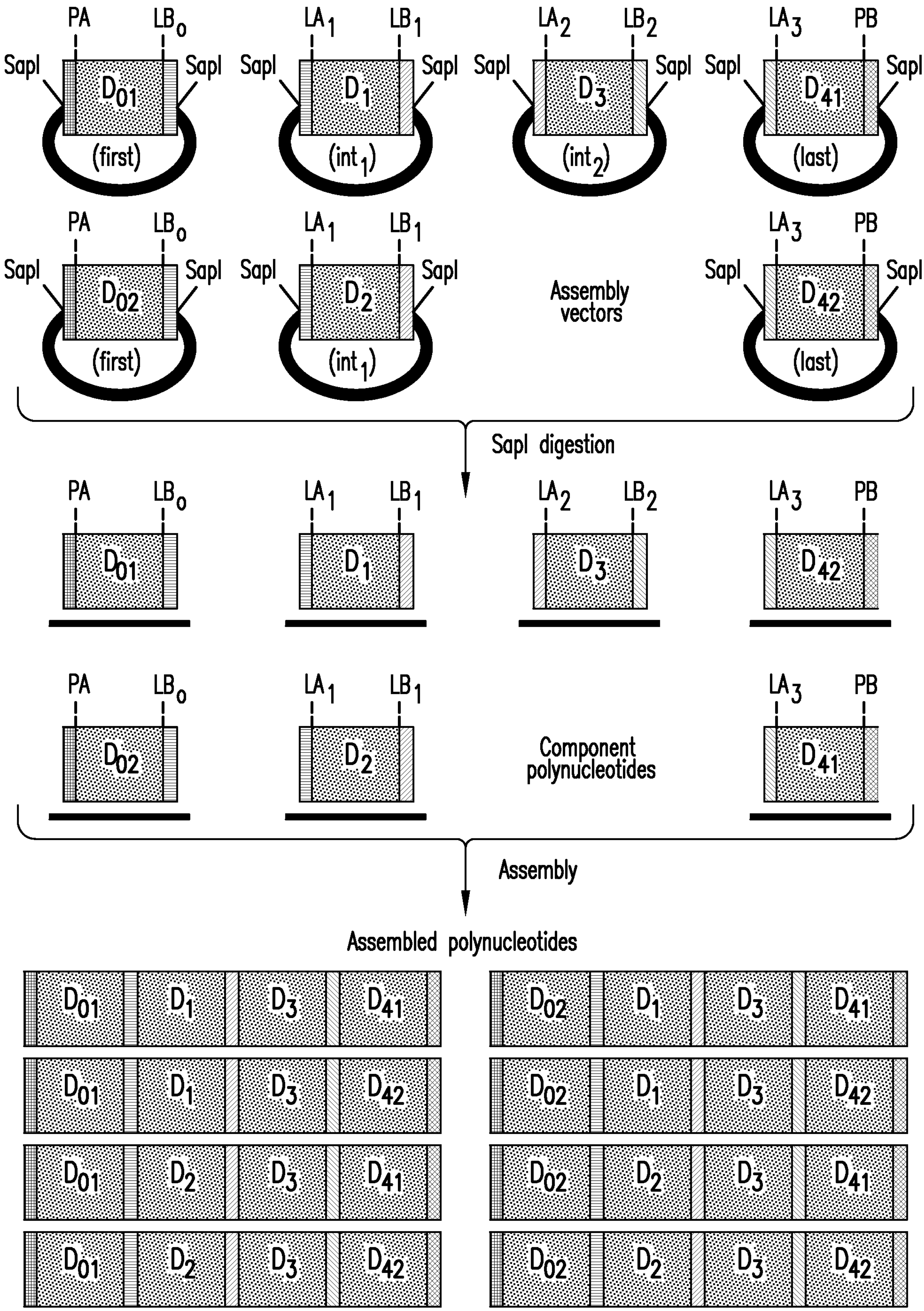

PA
LB0
SapI
D01
(first)
LA1
LB1
D1
(int1)
LA2
LB2
D3
(int2)
LA3
PB
D41
(last)
D02
D2
Assembly vectors
D42
SapI digestion
Component polynucleotides
Assembly
Assembled polynucleotides

FIG.10

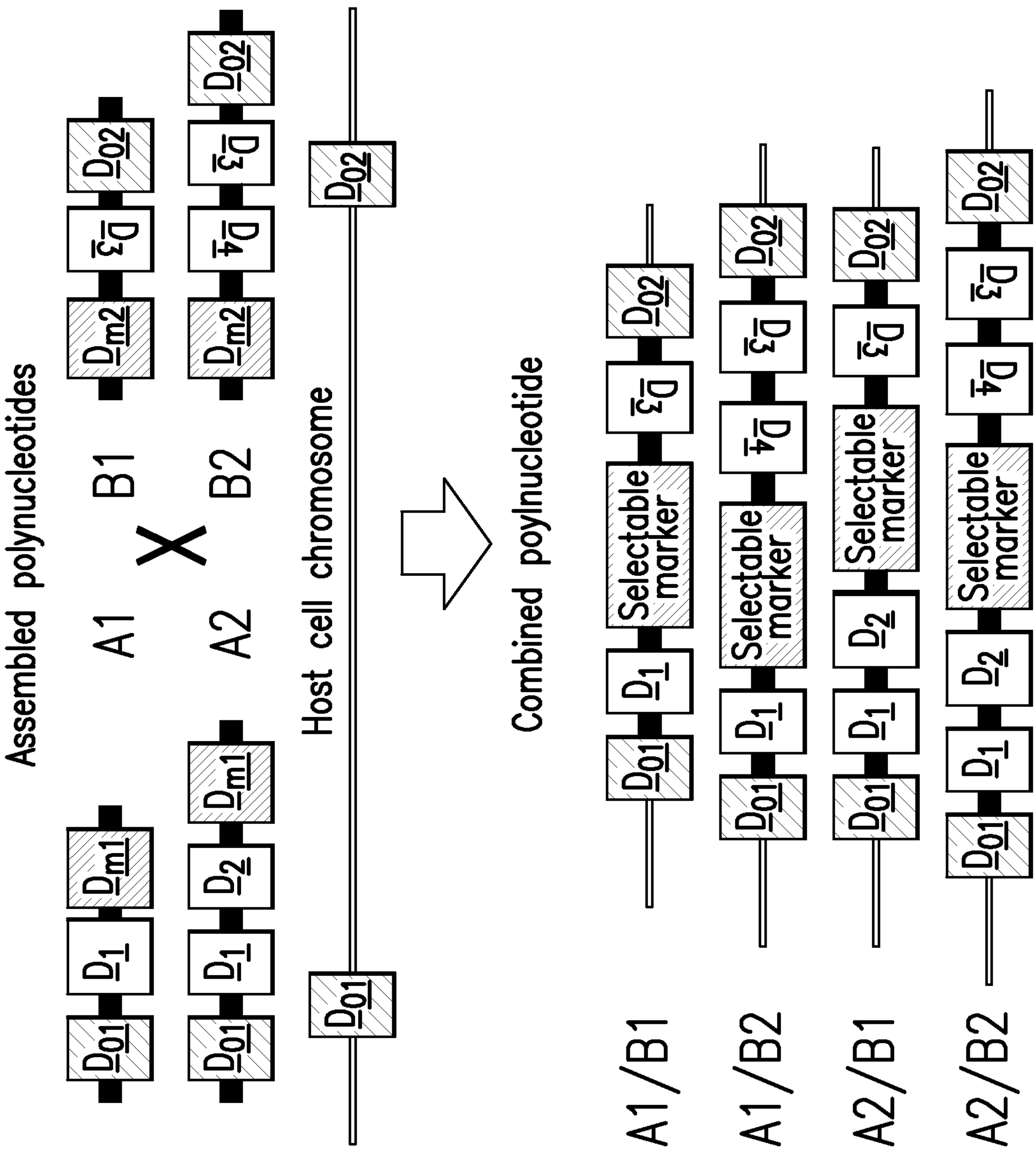

Assembled polynucleotides
A1
B1
A2
B2
X
D01
D1
Dm1
D2
Dm2
D3
D4
D02
Host cell chromosome
Combined poylnucleotide
Selectable marker
A1/B1
A1/B2
A2/B1
A2/B2

FIG.11

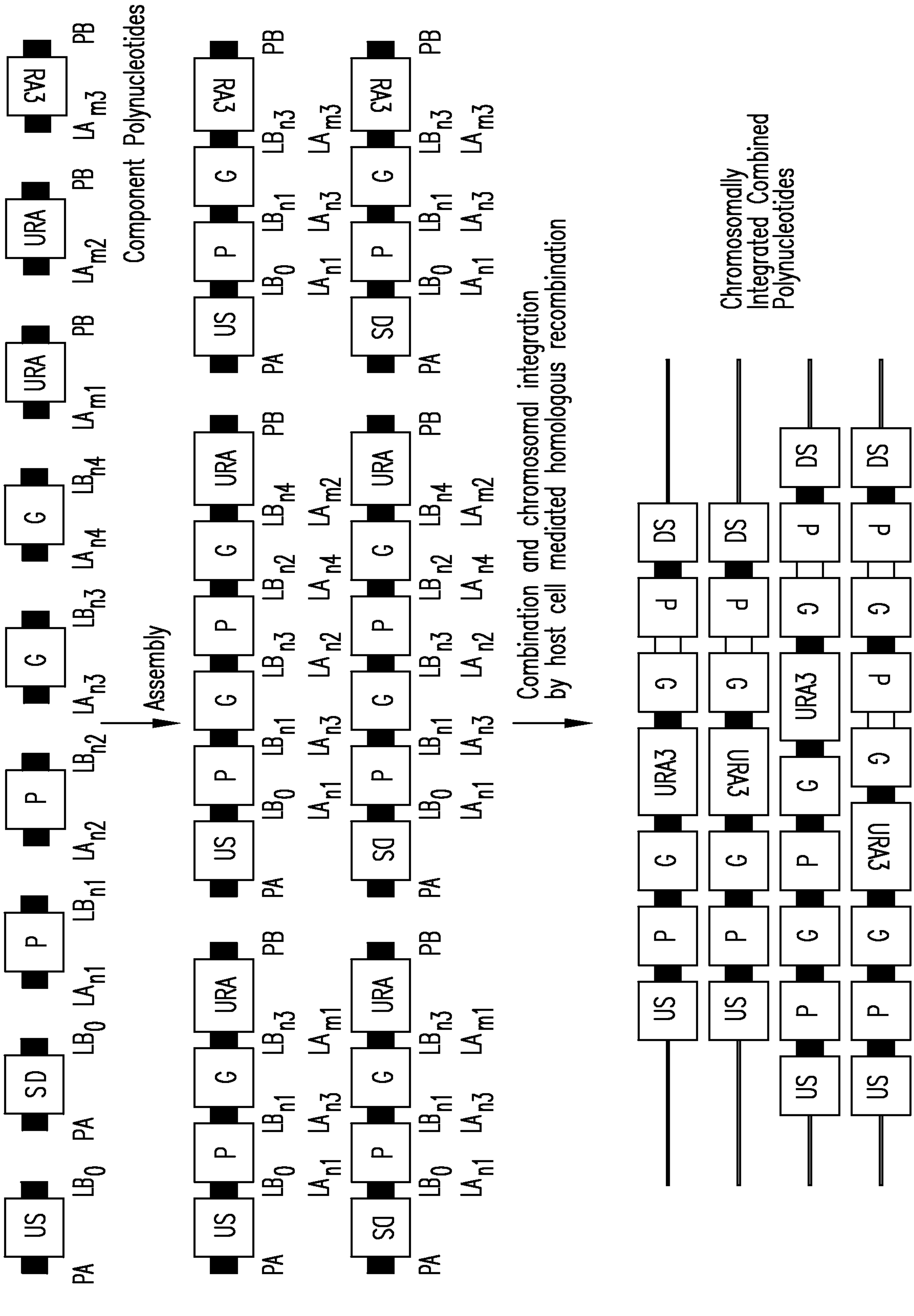
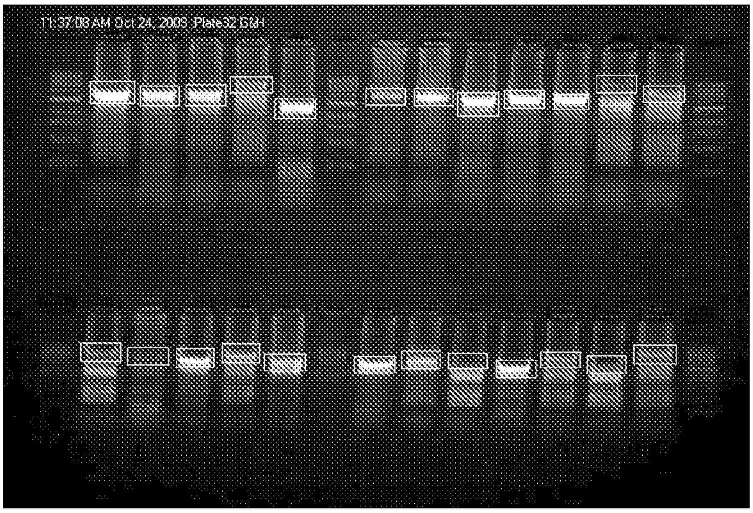

Component Polynucleotides
US
SD
P
G
URA
RA3
PA
PB
LB0
LAn1
LBn1
LAn2
LBn2
LAn3
LBn3
LAn4
LBn4
LAm1
LAm2
LAm3
Assembly
DS
Combination and chromosomal integration
by host cell mediated homologous recombination
URA3
Chromosomally
Integrated Combined
Polynucleotides

FIG.12A

Top panel

	Ladder						Ladder								Ladder
Assembled Polynucleotide Size (bp)		3909	3896	4027	6425	2709		5310	5415	3518	3874	3933	8459	5370	
Number of Assembled Component Polynucleotides		4	4	4	6	4		6	6	4	4	4	6	6	
Assembly Number		1621	1622	1623	1624	1625		1626	1627	1628	1629	1630	1631	1632	



	Ladder						Ladder								Ladder
Assembly Number		1633	1634	1635	1636	1637		1638	1639	1640	1641	1642	1643	1644	
Number of Assembled Component Polynucleotides		6	4	4	6	4		4	6	6	4	6	6	6	
Assembled Polynucleotide Size (bp)		5877	3848	4035	5135	3603		3516	6457	5492	3458	5453	4758	6818	

Bottom panel

FIG.12B

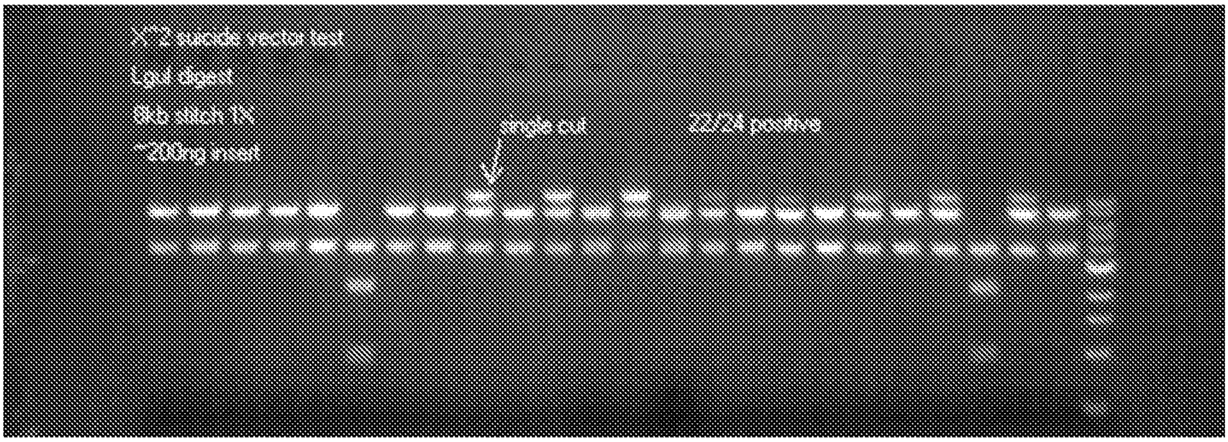

suicide vector test
LguI digest
8kb stitch 1X
~200ng insert
single cut
22/24 positive

FIG.12C

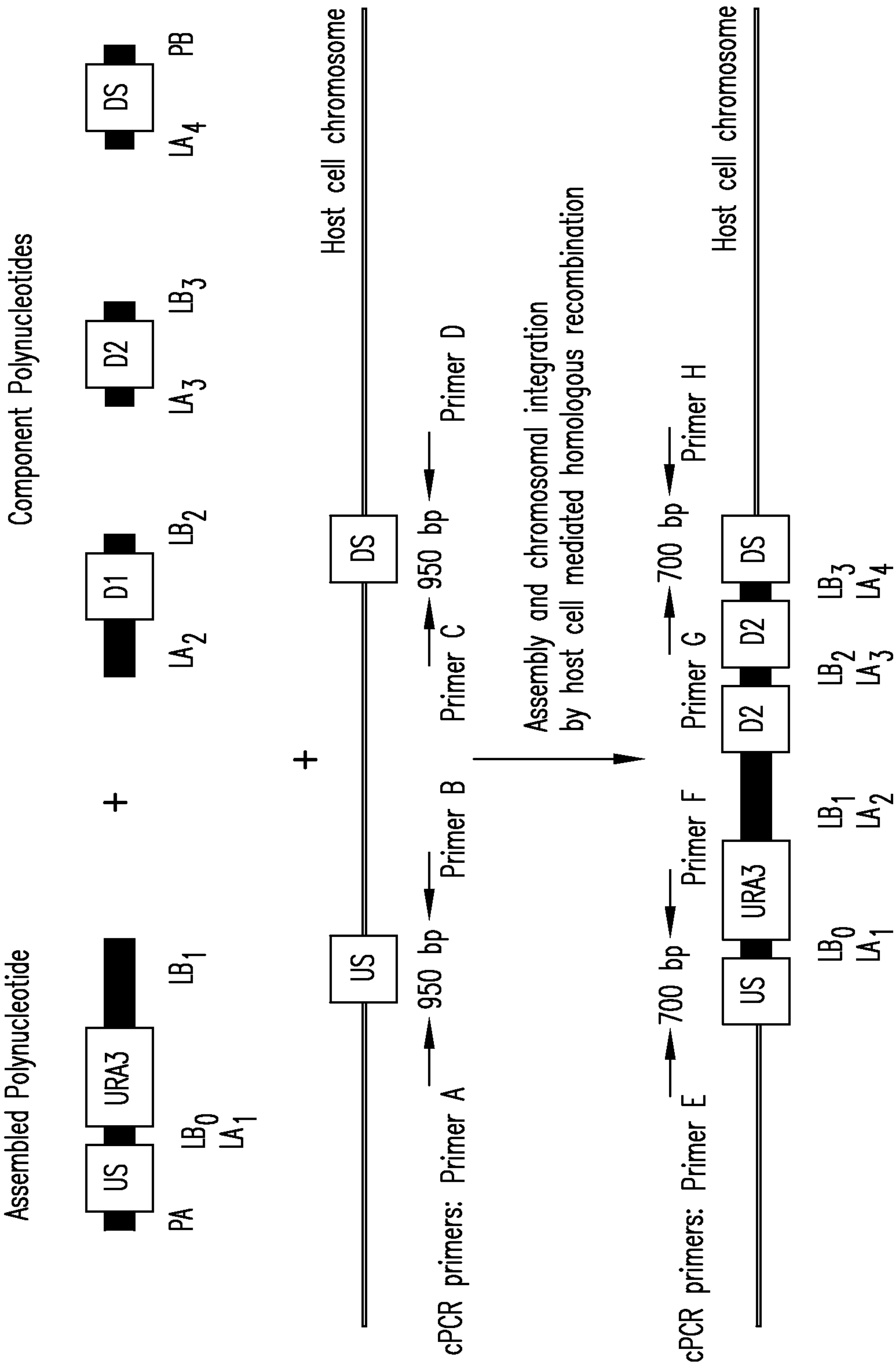

Assembled Polynucleotide
US
URA3
PA
LB_0
LA_1
LB_1
+
Component Polynucleotides
D1
LA_2
LB_2
D2
LA_3
LB_3
DS
LA_4
PB
+
US
DS
Host cell chromosome
cPCR primers: Primer A
950 bp
Primer B
Primer C
950 bp
Primer D
Assembly and chromosomal integration
by host cell mediated homologous recombination
cPCR primers: Primer E
700 bp
Primer F
Primer G
700 bp
Primer H
US
URA3
D2
D2
DS
LB_0
LA_1
LB_1
LA_2
LB_2
LA_3
LB_3
LA_4
Host cell chromosome

FIG.13A

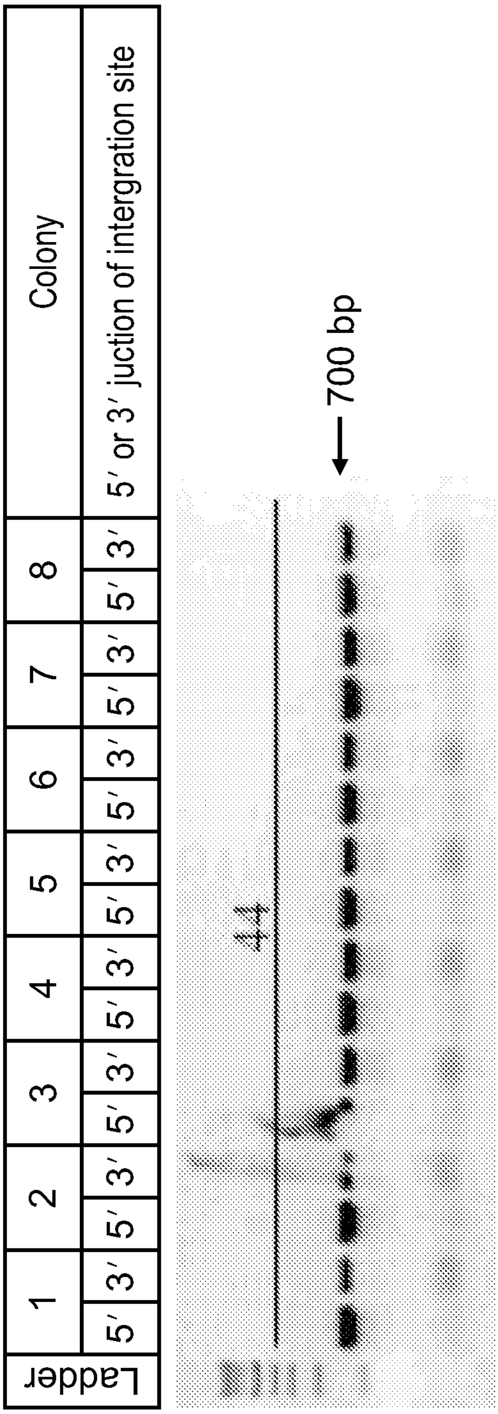

Ladder
Colony
1
2
3
4
5
6
7
8
5′ or 3′ juction of intergration site
5′
3′
5′
3′
5′
3′
5′
3′
5′
3′
5′
3′
5′
3′
5′
3′
700 bp

FIG.13B

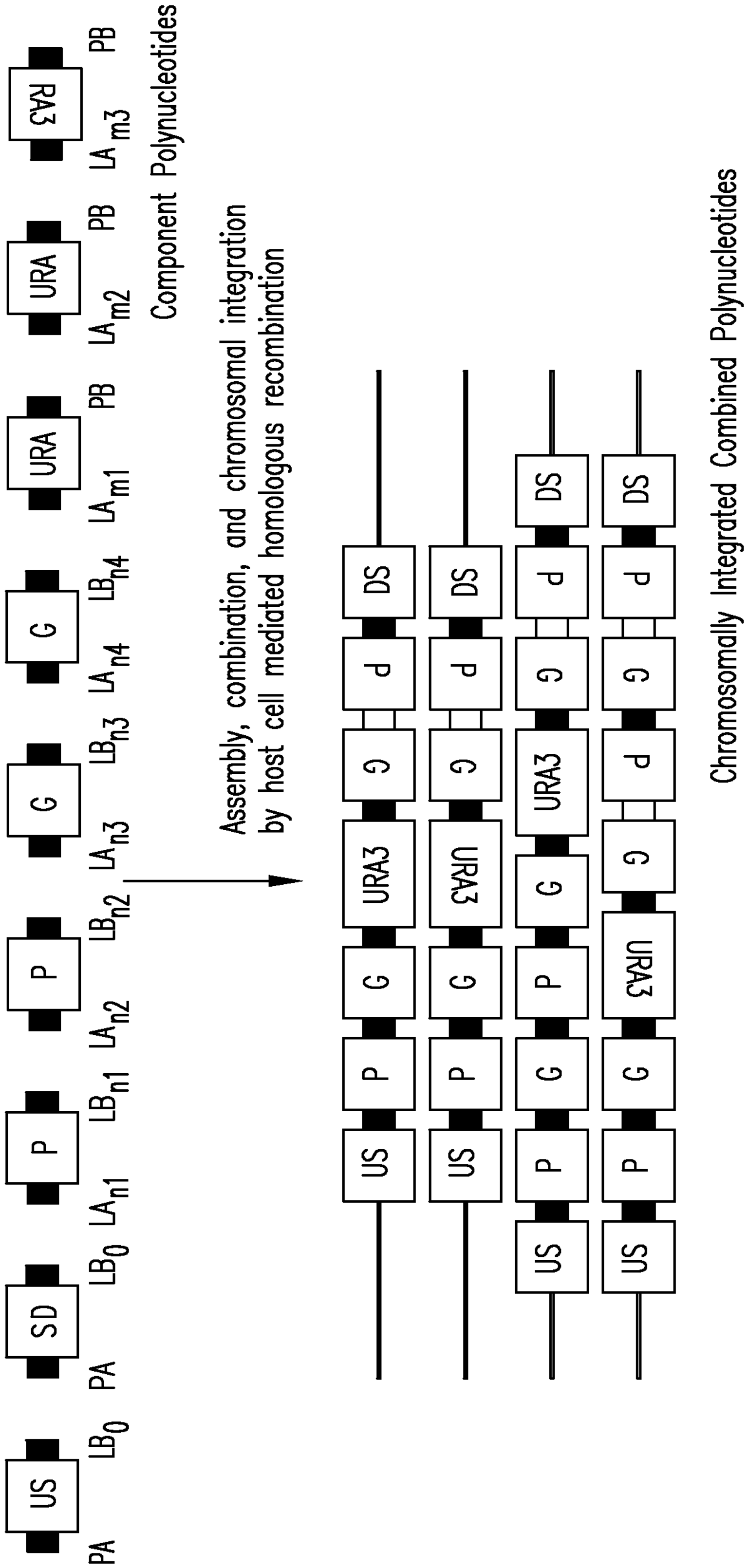
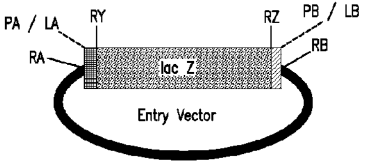
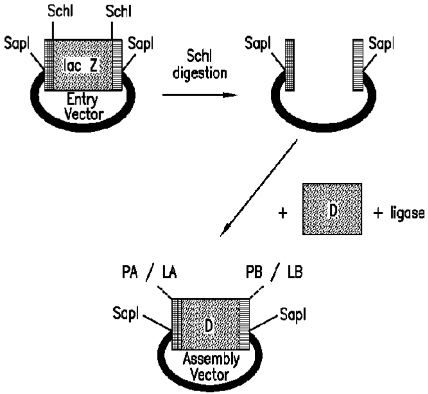

US
SD
P
P
G
G
URA
URA
RA3
PA
LB_0
PA
LB_0
LA_{n1}
LB_{n1}
LA_{n2}
LB_{n2}
LA_{n3}
LB_{n3}
LA_{n4}
LB_{n4}
LA_{m1}
PB
LA_{m2}
PB
LA_{m3}
PB
Component Polynucleotides
Assembly, combination, and chromosomal integration
by host cell mediated homologous recombination
US
P
G
URA3
G
P
DS
Chromosomally Integrated Combined Polynucleotides

FIG.14


PA / LA
RY
RZ
PB / LB
RA
RB
lac Z
Entry Vector

A


SchI
SchI
SapI
SapI
lac Z
Entry Vector
SchI digestion
SapI
SapI
+
D
+ ligase
PA / LA
PB / LB
SapI
SapI
D
Assembly Vector

B