(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
07 December 2017 (07.12.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/210148 A1

(51) International Patent Classification:
C12N 15/09 (2006.01) *C12N 15/81* (2006.01)
C12N 1/19 (2006.01)

(21) International Application Number:
PCT/US2017/034917

(22) International Filing Date:
30 May 2017 (30.05.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/343,338 31 May 2016 (31.05.2016) US

(71) Applicant: **IGC BIO, INC.** [US/US]; 55 Rawson Rd., Brookline, Massachusetts 02445 (US).

(72) Inventors: **ZIMMERMAN, Lior**; Moshe Sharet 3A, 6209207 Tel Aviv (IL). **BARAN, Dror**; Nahalat Yitzhak 18, 6744815 Tel Aviv (IL).

(74) Agent: **COHEN, Mark S.** et al.; PEARL COHEN ZEDEK LATZER BARATZ LLP, 1500 Broadway, 12TH Floor, New York, New York 10036 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

WO 2017/210148 A1

(54) Title: COMPOSITIONS AND METHODS FOR SELF-RECOMBINATION PLASMID TRANSFORMATION

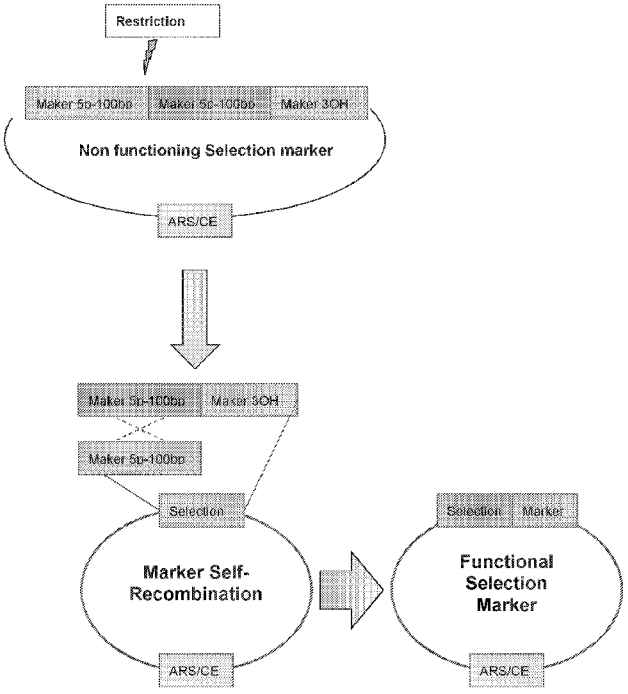


FIGURE 1

(57) Abstract: The invention relates to compositions and methods for improving a plasmid transformation. Specifically, the invention relates to a linear plasmid transformation and a self-homologous recombination. The invention further relates to a combination of a selectable marker having a start codon and its homologous selectable marker lacking a start codon, and uses thereof, for improving a plasmid transformation.

[Continued on next page]

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

COMPOSITIONS AND METHODS FOR SELF-RECOMBINATION PLASMID TRANSFORMATION

FIELD OF THE INVENTION

[0001] The invention relates to compositions and methods for improving plasmid transformation efficiency. Specifically, the invention relates to a linear plasmid transformation and a self-homologous recombination. The invention further relates to a combination of a selectable marker having a start codon and its homologous selectable marker lacking a start codon, in a plasmid, and uses thereof, for improving plasmid transformation efficiency.

BACKGROUND OF THE INVENTION

[0002] An expression/cloning vector, otherwise known as an expression/cloning construct, is usually a plasmid designed for protein expression, cloning or manipulation in cells. The vector is used to introduce a specific gene into a target cell, and can commandeer the cell's mechanism for protein synthesis to produce the protein encoded by the gene. Expression vectors are the basic tools in biotechnology for the production of proteins.

[0003] The plasmid is engineered to contain regulatory sequences that act as enhancer and promoter regions and lead to efficient transcription of the gene carried on the expression vector. The goal of a well-designed expression vector is the efficient production of protein.

[0004] Different organisms may be used to express a target protein. The expression vector used therefore will have elements specific for use in the particular organism.

[0005] Yeast cells offer many of the advantages of producing proteins in microbes (e.g., growth speed, easy genetic manipulation, low cost media) while offering some of the attributes of higher eukaryotic systems (e.g., post translational modifications, secretory expression). Several yeast protein expression systems exist in organisms from the genera *Saccharomyces*, *Pichia*, *Kluyveromyces*, *Hansenula* and *Yarrowia*.

[0006] Yeast plasmid transformation is a common molecular genetic manipulation by which an exogenous DNA is being delivered into the yeast cell.

[0007] Homologous recombination in yeast have been exploited to generate large DNA libraries (>10^8) by transforming a linearized plasmid backbone with library DNA inserts that are homologously recombined to form autonomous replicating circular plasmids. It has been shown that homologous recombination leads to DNA shuffling between DNA inserts sharing high homology generating chimeric inserts which may lead to alternate representation of DNA libraries upon transformation. While transformation of circular plasmids lowers DNA shuffling events, it suffers from lower transformation efficiency.

[0008] Accordingly, there exists a need for improved systems and method for plasmid transformation.

SUMMARY OF THE INVENTION

[0009] In one aspect, the invention provides a recombinant nucleic acid molecule comprising: a first nucleic acid sequence of a selectable marker operably linked to a second nucleic acid sequence of said selectable marker, wherein said second nucleic acid sequence lacks a start codon and is capable of combining with said first nucleic acid sequence by homologous recombination, wherein said first nucleic acid sequence has a restriction enzyme cleavage site at its 3' end and said second nucleic acid sequence has a restriction enzyme cleavage site at its 5' end. In one example, the invention provides a vector comprising the recombinant nucleic acid molecule. In an exemplary embodiment, the vector is a plasmid vector, for example, a yeast plasmid vector.

[00010] In another aspect, the invention provides a method for transforming a gene of interest to a host cell, the method comprising: providing a vector comprising a recombinant nucleic acid molecule, said molecule comprising a first nucleic acid sequence of a selectable marker operably linked to a second nucleic acid sequence of said selectable marker, wherein said second nucleic acid sequence lacks a start codon and is capable of recombining with said first nucleic acid sequence by homologous recombination, wherein said first nucleic acid sequence has a restriction enzyme cleavage site at its 3' end and said second nucleic acid sequence has a restriction enzyme cleavage site at its 5' end; providing one or more restriction enzymes capable of cleaving said restriction enzyme cleavage site of said first nucleic acid sequence and said restriction enzyme cleavage site of said second nucleic acid sequence; facilitating a transformation of the cleaved nucleic acid sequence into said host cell; and facilitating a

homologous recombination between said first and second nucleic acid sequences in order to form a circular plasmid in said cell with an operational selection marker .

[00011] In yet another aspect, the invention provides a method for enhancing a transformation efficiency, the method comprising: providing a vector comprising a recombinant nucleic acid molecule, said molecule comprising a first nucleic acid sequence of a selectable marker operably linked to a second nucleic acid sequence of said selectable marker, wherein said second nucleic acid sequence lacks a start codon and is capable of combining with said first nucleic acid sequence by homologous recombination, wherein said first nucleic acid sequence has a restriction enzyme cleavage site at its 3' end and said second nucleic acid sequence has a restriction enzyme cleavage site at its 5' end; providing one or more restriction enzymes capable of cleaving said restriction enzyme cleavage site of said first nucleic acid sequence and said restriction enzyme cleavage site of said second nucleic acid sequence; facilitating a transformation of the cleaved nucleic acid sequence into said host cell; and facilitating a homologous recombination between said first and second nucleic acid sequences in order to form a circular plasmid in said cell.

[00012] In a further aspect, the invention provides a kit comprising a recombinant nucleic acid molecule, said molecule comprising: a first nucleic acid sequence of a selectable marker operably linked to a second nucleic acid sequence of said selectable marker, wherein said second nucleic acid sequence lacks a start codon and is capable of combining with said first nucleic acid sequence by homologous recombination, wherein said first nucleic acid sequence has a restriction enzyme cleavage site at its 3' end and said second nucleic acid sequence has a restriction enzyme cleavage site at its 5' end.

[00013] Other features and advantages of the present invention will become apparent from the following detailed description examples and figures. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[00014] The invention will be better understood from a reading of the following detailed description taken in conjunction with the drawings:

[00015] Figure 1 shows a schematic detailing the self-recombination cloning strategy. The circular plasmid is digested in the selectable marker and forms a linear dsDNA plasmids. By homologous recombination, the plasmid is delivered into the yeast cell and re-circularized forming a plasmid that can be selected by it's full framed selectable marker.

DETAILED DESCRIPTION OF THE INVENTION

[00016] The invention provides compositions and methods for linear plasmid transformation by self-homologous recombination. Specifically, the invention provides a combination of a selectable marker having a start codon and its homologous selectable marker lacking a start codon in a plasmid, and uses thereof, for improving plasmid transformation efficiency.

[00017] The inventors of this application have developed a new plasmid transformation strategy in which a linearized plasmid with two homology regions is being transformed and re-circularized by self-homologous recombination, forming a full-framed selectable marker.

[00018] In one aspect, provided herein is a recombinant nucleic acid molecule comprising: a first nucleic acid sequence of a selectable marker operably linked to a second nucleic acid sequence of said selectable marker, wherein said second nucleic acid sequence lacks a start codon and is capable of combining with said first nucleic acid sequence by homologous recombination.

[00019] The first nucleic acid sequence may include a restriction enzyme cleavage site at its 3' end and the second nucleic acid sequence may include a restriction enzyme cleavage site at its 5' end.

[00020] Any suitable restriction enzyme cleavage site known to one of skilled in the art can be used. Examples of restriction enzyme for cleavage include, for example, but not limited to, type I, II, III, IV, V, and artificial restriction enzymes.

[00021] Type I enzymes are complex, multisubunit, combination restriction-and-modification enzymes that cut DNA at random far from their recognition sequences. Type II enzymes cut DNA at defined positions close to or within their recognition sequences. One of the most

common Type II enzymes, referred to as "Type IIS" are those that cleave outside of their recognition sequence to one side.

[00022] Type III enzymes cleave outside of their recognition sequences and require two sequences in opposite orientations within the same DNA molecule to accomplish cleavage. Type IV enzymes recognize modified, typically methylated DNA. Type V restriction enzymes utilize guide RNAs to target specific non-palindromic sequences found on invading organisms. Artificial restriction enzymes are well known in the art. Artificial restriction enzymes can be engineered to bind to desired DNA sequences.

[00023] In a particular embodiment, the restriction enzyme of the invention is a type II restriction enzyme, for example, a type IIs restriction endonuclease, such as BsaI, known to one of skilled in the art. Type IIs enzymes cut outside of their recognition site; the restriction sites in the plasmid are eliminated by the digestion and that way the marker recombination sites are 100% homologous.

[00024] Restriction sites and enzymes for yeast plasmid vectors are well known in the art. *See e.g.*, Ma *et al.*, 1987, *Gene*, vol. 58, pages 201-216. Examples of restriction enzymes include, for example, but are not limited to, *Aat*I, *Ava*I, *Bam*HI, *Bal*I, *Bst*EI, *Bss*HII, *Bsm*I, *BSP*MI, *Bst*XI, *Cla*I, *Dra*III, *Spe*I, *Sph*I, *Bgl*II, *Hind*III, *Nru*I, *Kpn*I, *Bcl*I, *Sma*I, *Mst*II, *Nco*I, *Nde*I, *Nhe*I, *Xho*I, *Pst*I, *Pvu*II, *Hpa*I, *Eco*RI, *Sal*I, *Sca*I, *Sna*BI, *Ssp*I, *Stu*I, *Sty*I, *Tth*I, *Pvu*I, *Bsp*MII, *Ava*III, *Xba*I, *Apa*I, and *Xma*III.

[00025] With respect to the selectable marker, any suitable selectable marker, known to one of skilled in the art can be used. The term "selectable marker," as used herein, may refer to a gene introduced into a cell that confers a trait suitable for artificial selection. In one example, the selectable marker is a type of reporter gene used in laboratory microbiology, molecular biology, and genetic engineering to indicate the success of a transfection or other procedure meant to introduce foreign nucleic acid into a cell.

[00026] In one embodiment, the selectable marker is a positive selectable marker. Positive selection marker may confer selective advantage to the host organism. In one example, the positive selectable marker is an antibiotic resistant gene, which allows the host organism to survive antibiotic selection. The host cells that have been subjected to a procedure to introduce foreign DNA are grown on a medium containing an antibiotic, and those colonies that can grow

have successfully taken up and expressed the introduced genetic material. In one embodiment, the positive selectable marker may confer resistance to antibiotics such as, for example, zeocin, geneticin, nourseothricin. In one embodiment, the selectable marker is a gene that encodes an enzyme that can complement an auxotrophy, for example a TRP marker In the lab i actually do this using TRP marker that can synthesize tryptophan and grow on media that lacks it.

[00027] In another embodiment, the selectable marker is a negative selectable marker. Negative or counterselectable markers are selectable markers that eliminate or inhibit growth of the host organism upon selection. An example of a negative selectable marker includes thymidine kinase, which makes a host sensitive to ganciclovir selection.

[00028] In yet another embodiment, the selectable marker is a combination of a positive and a negative selectable marker. Such a marker can serve as both a positive and a negative marker by conferring an advantage to the host under one condition, but inhibits growth under a different condition. In one example, the combination of positive and negative selectable marker includes an enzyme that can complement an auxotrophy (positive selection) and be able to convert a chemical to a toxic compound (negative selection).

[00029] Additional examples of selectable markers include, but are not limited to, β-lactamase which confers ampicillin resistance to bacterial hosts; neo gene from Tn5, which confers resistance to kanamycin and geneticin; and mutant FabI gene (mFabI), which confers triclosan resistance to the host.

[00030] In a particular embodiment, the selectable marker is a yeast marker, for example, *URA3*, an orotidine-5' phosphate decarboxylase from yeast, which is a positive and negative selectable marker.

[00031] Useful markers for other expression systems are well known to those of skill in the art. These and other selectable markers can be obtained from commercially available plasmids, using techniques well known in the art.

[00032] The nucleic acid sequence of a selectable marker may be of any suitable length. In a particular embodiment, the length of said selectable marker nucleic acid sequence ranges from approximately 20 bp to approximately 100 bp.

[00033] In one embodiment, the selectable marker comprises a start codon. In another embodiment, the selectable marker lacks a start codon. In yet another embodiment, the invention relates to a combination of a start codon containing selectable marker and its homologous start codon lacking selectable marker, wherein the start codon lacking selectable marker is capable of combiding with the start codon containing selectable marker by homologous recombination. In one example, the invention provides a vector comprising a start codon containing selectable marker fused or operably linked to its homologous start codon lacking selectable marker by a linker. The linker may comprise one or more cleavable restriction sites discussed herein.

[00034] In one example, the start codon is a standard AUG (or ATG) codon, found in both prokaryotes and eukaryotes. In another example, the start codon is a non-AUG (or non-ATG) codon. Alternate start codons (non AUG) are very rare in eukaryotic genomes. However, naturally occurring non-AUG start codons have been reported for some cellular mRNAs. *See* Ivanov *et al.*, 2011, *Nucleic Acids Research* vol. 39 (10), pages 4220–4234.

[00035] The selectable marker or any gene of interest in the invention may also comprise a stop codon. In the genetic code, a stop codon (or termination codon) may refer to a nucleotide triplet within messenger RNA that signals a termination of translation. Examples of stop codon include, for example, but not limited to UAG, UAA, and UGA.

[00036] The invention also includes a recombinant vector comprising any of the nucleic acid molecules described herein. Vector can be any suitable vector known to one of skilled in the art. In a particular embodiment, the vector is a plasmid vector. The term "plasmid," as used herein may refer to a small DNA molecule within a cell that is physically separated from a chromosomal DNA and can replicate independently. Plasmids are considered *replicons*, a unit of DNA capable of replicating autonomously within a suitable host.

[00037] In one example, the plasmid is a yeast plasmid. Yeast is organism that naturally harbour plasmids. Both circular and linear plasmids are encompassed within the scope of the invention. For example, the gene URA3, that codes an enzyme related to the biosynthesis of pyrimidine nucleotides (T, C).

[00038] In another embodiment, the plasmid is a Yeast Replicative Plasmid (YRp). In some embodiments, YRp transports a sequence of chromosomal DNA that includes an origin of replication.

[00039] Other suitable vecotors are also within the scope of the invention. Other exemplary vectors include, but are not limited to, phagemids, cosmids, and phage nucleic acids or other nucleic acid molecules that are capable of replication in a prokaryotic or eukaryotic host.

[00040] In one embodiment, the vector is an expression vector, wherein the nucleic acid encoding an antibody is operably linked to an expression control sequence. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the nucleic acid molecules of the invention. The vectors may also contain genetic expression cassettes containing an independent terminator sequence, sequences permitting replication of the vector, or other elements known to one of skill in the art. When the vector contains nucleic acids encoding both a heavy and light chain or portions thereof, the nucleic acid encoding the heavy chain may be under the same or a separate promoter. The separate promoters may be identical or may be different types of promoters.

[00041] Suitable promoters include constitutive promoters and inducible promoters. Representative expression control sequences/promoters include, for example, the glycolytic promoters of yeast, *e.g.*, the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, *e.g.*, *Pho5*, the promoters of the yeast alpha mating factors, the *lac* system, the *trp* system, the *tac* system, the *trc* system, major operator and promoter regions of phage lambda, the control region of *fd* coat protein, promoters derived from the human cytomegalovirus, metallothionine promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, *e.g.*, the early and late promoters of SV40.

[00042] In some embodiments, promoters useful in yeast expression systems include, for example, promoters from sequences encoding enzymes in the metabolic pathway such as alcohol dehydrogenase (ADH) (EPO Publication No. 284,044), enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase (GAP or GAPDH), hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate kinase (PyK) (EPO Publication No. 329,203) promoters. In some embodiments, the expression construct comprises a synthetic hybrid promoter. Examples of such hybrid promoters include the ADH regulatory sequence linked to the GAP transcription activation region (U.S. Pat. Nos. 4,876,197 and 4,880,734), as well as promoters which consist of the regulatory sequences of either the ADH2, GAL4, GAL10, or PHO5 genes, combined with the transcriptional activation region of a

glycolytic enzyme gene such as GAP or PyK (EPO Publication No. 164,556). These and other promoters can be obtained from commercially available plasmids, using techniques well known in the art.

[00043] In another embodiment, transcription termination and polyadenylation sequences are also present in the expression constructs. These sequences are located 3' to the translation stop codon for the coding sequence. Transcription terminator/polyadenylation signal sequences are well known in the art.

[00044] A host of the present invention may be eukaryotic or prokaryotic. Suitable eukaryotic cells include yeast and other fungi, insect cells, plant cells, human cells, and animal cells, including mammalian cells, such as hybridoma lines, COS cells, NS0 cells and CHO cells. Suitable prokaryotic hosts include, for example, *E. coli*, such as *E. coli* SG-936, *E. coli* HB 101, *E. coli* W3110, *E. coli* X1776, *E. coli* X2282, *E. coli* DHI, and *E. coli* MRC1, *Pseudomonas*, *Bacillus*, such as *Bacillus subtilis*, and *Streptomyces*.

[00045] The term “host cell”, as used herein, refers to a cell or population of cells into which a nucleic acid molecule or vector of the invention is introduced. “A population of host cells" refers to a group of cultured cells into which a nucleic acid molecule or vector of the present invention can be introduced and expressed. The host may contain a nucleic acid or vector encoding only one chain or portion thereof (*e.g.*, the heavy or light chain); or it may contain a nucleic acid or vector encoding both chains or portions thereof, either the same or separate nucleic acids and/or vectors.

[00046] Nucleic acid molecules comprising nucleotide sequences of interest can be stably integrated into a host cell genome or maintained on a stable episomal element in a suitable host cell using various gene delivery techniques well known in the art. *See, e.g.*, U.S. Pat. No. 5,399,346. A number of appropriate host cells for use with the above systems are also known. For example, yeast hosts useful in the present invention include, but not limited to, *Saccharomyces cerevisiae*, *Candida albicans, Candida maltosa, Hansenula polymorpha, Kluyveromyces fragilis, Kluyveromyces lactis, Pichia guillerimondii, Pichia pastoris, Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Similarly, bacterial hosts such as *E. coli*, *Bacillus subtilis*, and *Streptococcus spp.*, may find use with the present expression constructs. Insect cells for use with baculovirus expression vectors include, but not limited to, *Aedes*

aegypti, Autographa californica, Bombyx mori, Drosophila melanogaster, and *Spodoptera frugiperda.* Mammalian cell lines are known in the art and include immortalized cell lines available from the American Type Culture Collection (ATCC), such as, but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human embryonic kidney cells, human hepatocellular carcinoma cells (e.g., Hep G2), Madin-Darby bovine kidney ("MDBK") cells, as well as others.

[00047] In another aspect, the present invention can be used in expression constructs to express a wide variety of substances. AJ gene of interest can be expressed. In an exemplary embodiment, the the present invention is used to express an antibody, for example, a monoclonal antibody, a polyclonal antibody, a humanized recombinant, or a fragment thereof. In one embodiment, the invention is used to express the nucleic acid sequence that encodes a heavy or light chain immunoglobulin.

[00048] A vector of the invention can be constructed by methods or techniques well known in the art.

[00049] Construction of yeast strains are well known and fully disclosed, for example, in U.S. Patent 5,635,369 and U.S. Patent Application Publication 2002/0160380, which are incorporated by reference herein in their entirety. Plasmid construction by homologous recombination yeast is also known in the art. *See e.g.*, Ma *et al.*, 1987, *Gene*, vol. 58, pages 201-216, which is incorporated by reference herein in its entirety.

[00050] A wide variety of methods, known to one of skilled in the art, can be used to deliver the expression constructs to cells. Such methods include, for example, but are not limited to, lithium acetate transformation, DEAE dextran-mediated transfection, calcium phosphate precipitation, polylysine- or polyomithine-mediated transfection, electroporation, sonoporation, protoplast fusion, liposomes, peptoid delivery, or microinjection.

[00051] In another aspect, provided herein is a method for transforming a gene of interest to a host cell, the method comprising: providing a vector comprising a recombinant nucleic acid molecule, said molecule comprising a first nucleic acid sequence of a selectable marker operably linked to a second nucleic acid sequence of said selectable marker, wherein said second nucleic acid sequence lacks a start codon and is capable of combining with said first nucleic acid sequence by homologous recombination, wherein said first nucleic acid sequence has a

restriction enzyme cleavage site at its 3' end and said second nucleic acid sequence has a restriction enzyme cleavage site at its 5' end; providing one or more restriction enzymes capable of cleaving said restriction enzyme cleavage site of said first nucleic acid sequence and said restriction enzyme cleavage site of said second nucleic acid sequence; facilitating a transformation of the cleaved nucleic acid sequence into said host cell; facilitating a homologous recombination between said first and second nucleic acid sequences in order to form a circular plasmid in said cell. In yet another aspect, provided herein is a method for enhancing a transformation efficiency.

[00052] The invention also provides a kit comprising a recombinant nucleic acid molecule, said molecule comprising: a first nucleic acid sequence of a selectable marker operably linked to a second nucleic acid sequence of said selectable marker, wherein said second nucleic acid sequence lacks a start codon and is capable of combining with said first nucleic acid sequence by homologous recombination, wherein said first nucleic acid sequence has a restriction enzyme cleavage site at its 3' end and said second nucleic acid sequence has a restriction enzyme cleavage site at its 5' end.

[00053] Kits for contructing vectors of or performing assays are provided by the present invention. The kits can include a container that includes some or all of the reagents and methods for contructing vectors or carrying out the assays. The kits can also include labels or instructions for contructing vectors or carrying out the assays.

[00054] The term "nucleic acid," as used herein, may include both double- and single-stranded sequences and refers to, but not limited to, cDNA from yeast, procaryotic or eucaryotic mRNA, genomic DNA sequences, or procaryotic DNA, and especially synthetic DNA sequences. The term also captures sequences that include any of the known base analogs of DNA and RNA.

[00055] The term "operably linked," as used herein, refers to an arrangement of elements wherein the components so described are configured so as to perform their desired function.

[00056] The term "recombinant" as used herein to describe a nucleic acid molecule means a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation is not associated with all or a portion of the polynucleotide with which it is associated in nature. The term "recombinant" as used with respect to a protein or polypeptide means a polypeptide produced by expression of a recombinant polynucleotide. In general, the

gene of interest is cloned and then expressed in transformed organisms, as described further below. The host organism expresses the foreign gene to produce the protein under expression conditions.

[00057] The term "promoter," as used herein, refers to a DNA regulatory region capable of binding RNA polymerase in a host cell and initiating transcription of a downstream (3' direction) coding sequence operably linked thereto. For purposes of the present invention, a promoter sequence includes the minimum number of bases or elements necessary to initiate transcription of a gene of interest at levels detectable above background. Within the promoter sequence is a transcription initiation site, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

[00058] The term "host cell," as used herein, refers to a cell which has been transformed, or is capable of transformation, by an exogenous DNA sequence.

[00059] The term "expression construct," as used herein, refer to an assembly which is capable of directing the expression of the sequence(s) or gene(s) of interest. The expression construct includes control elements, as described above, such as a promoter or promoter/enhancer which is operably linked to (so as to direct transcription of) the sequence(s) or gene(s) of interest, and often includes a polyadenylation sequence as well. Within certain embodiments of the invention, the expression construct described herein may be contained within a plasmid construct. In addition to the components of the expression construct, the plasmid construct may also include, one or more selectable markers, a signal which allows the plasmid construct to exist as single-stranded DNA (e.g., an origin of replication).

[00060] All patents, patent applications, and scientific publications cited herein are hereby incorporated by reference in their entirety.

[00061] The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLES

EXAMPLE 1

Linear Plasmid Transformation and Self-homologous Recombination in Yeast

[00062] Our invention presents a new plasmid transformation strategy in which a linearized plasmid with two homology regions is being transformed and re-circularized by self-homologous recombination, forming a full-framed selectable marker.

[00063] Our invention utilizes yeast homologous recombinations machinery for the transformation of linear autonomous replicating plasmid and it's re-circularization by specific self-recombination site located within the plasmid selective marker open reading frame. The selective marker self-recombination cassette construct contain the initial 100 bp of the selective gene, following by a stop-codon and two inverted type IIs restriction site and the entire selective marker lacking an initiation ATG codon. Lacking the initiation codon, the selective marker fail to translate and to gain any auxotrophic activity or antibiotic resistance. By plasmid linearization using the cassette type IIs restriction, the linear plasmid can be self recombined utilizing the initial 100 bp of the selective marker and its homologues initiation-lacking marker. thus, circular plasmid transformation can be selected by the gain of functionality of the selection marker.

[00064] Figure 1 shows a schematic detailing the self-recombination cloning strategy. As shown in Figure 1, the circular plasmid is digested in the selectable marker and forms a linear dsDNA plasmids. The plasmid is delivered into the yeast cell and re-circulized forming a plasmid that can be selected by it's full framed selectable marker.

[00065] The invention offers an improved transformation efficiency than the conventional circular plasmid, while preventing notorious DNA shuffling between DNA constructs.

[00066] Having described preferred embodiments of the invention with reference to the accompanying drawings, it is to be understood that the invention is not limited to the precise embodiments, and that various changes and modifications may be effected therein by those skilled in the art without departing from the scope or spirit of the invention as defined in the appended claims.

WHAT IS CLAIMED IS:

1. A recombinant nucleic acid molecule comprising: a first nucleic acid sequence of a selectable marker operably linked to a second nucleic acid sequence of said selectable marker, wherein said second nucleic acid sequence lacks a start codon and is capable of combining with said first nucleic acid sequence by homologous recombination, wherein said first nucleic acid sequence has a restriction enzyme cleavage site at its 3' end and said second nucleic acid sequence has a restriction enzyme cleavage site at its 5' end.

2. The nucleic acid molecule of claim 1, wherein said restriction enzyme cleavage site of said first nucleic acid sequence is cleavable by a type IIs restriction enzyme.

3. The nucleic acid molecule of claim 2, wherein said restriction enzyme is a type IIs restriction enzyme.

4. The nucleic acid molecule of claim 1, wherein said restriction enzyme cleavage site of said second nucleic acid sequence is cleavable by a type IIs restriction enzyme.

5. The nucleic acid molecule of claim 4, wherein said restriction enzyme is a type IIs restriction enzyme.

6. The nucleic acid molecule of claim 1, wherein said selectable marker is a reporter gene that indicates the success of a transfection or transformation to introduce a foreign nucleic acid into a cell.

7. The nucleic acid molecule of claim 1, wherein the length of said selectable marker nucleic acid sequence ranges from approximately 20 bp to approximately 100 bp.

8. The nucleic acid molecule of claim 1, wherein said start codon is AUG or ATG.

9. The nucleic acid molecule of claim 1, wherein said molecule comprises a nucleic acid sequence encoding a gene of interest.

10. The nucleic acid molecule of claim 1, wherein said molecule comprises a nucleic acid sequence encoding a self-replication gene that facilitates the self-replication of a plasmid.

11. A vector comprising said recombinant nucleic acid molecule of claim 1.

12. The vector of claim 11, wherein said vector is a plasmid vector.

13. The vector of claim 12, wherein said plasmid is a self-replicating plasmid.

14. The vector of claim 13, wherein said plasmid is a yeast plasmid.

15. An expression library comprising a vector of claim 11, 12, 13, or 14.

16. A host cell comprising a plasmid of claim 12, 13, or 14.

17. The cell of claim 16, wherein said cell is a yeast cell.

18. A method for transforming a gene of interest to a host cell, the method comprising:

providing a vector comprising a recombinant nucleic acid molecule, said molecule comprising a first nucleic acid sequence of a selectable marker operably linked to a second nucleic acid sequence of said selectable marker, wherein said second nucleic acid sequence lacks a start codon and is capable of combining with said first nucleic acid sequence by homologous recombination, wherein said first nucleic acid sequence has a restriction enzyme cleavage site at its 3' end and said second nucleic acid sequence has a restriction enzyme cleavage site at its 5' end;

providing one or more restriction enzymes capable of cleaving said restriction enzyme cleavage site of said first nucleic acid sequence and said restriction enzyme cleavage site of said second nucleic acid sequence;

facilitating a transformation of the cleaved nucleic acid sequence into said host cell;

facilitating a homologous recombination between said first and second nucleic acid sequences in order to form a circular plasmid in said cell.

19. The method of claim 18, wherein said restriction enzyme cleavage site of said first nucleic acid sequence is cleavable by a type IIs restriction enzyme.

20. The method of claim 19, wherein said restriction enzyme is a type II s1 restriction enzyme.

21. The method of claim 18, wherein said restriction enzyme cleavage site of said second nucleic acid sequence is cleavable by a type II restriction enzyme.

22. The method of claim 21, wherein said restriction enzyme is a type II s1 restriction enzyme.

23. The method of claim 18, wherein said selectable marker is a reporter gene that indicates the success of a transfection or transformation to introduce a foreign nucleic acid into a cell.

24. The method of claim 18, wherein the length of said selectable marker nucleic acid sequence ranges from approximately 5 bp to approximately 100 bp.

25. The method of claim 18, wherein said start codon is AUG or ATG.

26. The method of claim 18, wherein said molecule comprises a nucleic acid sequence encoding a gene of interest.

27. The method of claim 18, wherein said molecule comprises a nucleic acid sequence encoding a self-replication gene that facilitates the self-replication of a plasmid.

28. The method of claim 18, wherein said vector is a plasmid vector.

29. The method of claim 18, wherein said vector is a self-replicating plasmid vector.

30. The method of claim 18, wherein said vector is a yeast plasmid vector.

31. The method of claim 18, wherein said cell is a yeast cell.

32. A method for enhancing a transformation efficiency, the method comprising:

providing a vector comprising a recombinant nucleic acid molecule, said molecule comprising a first nucleic acid sequence of a selectable marker operably linked to a second nucleic acid sequence of said selectable marker, wherein said second nucleic acid sequence lacks a start codon and is capable of combining with said first nucleic acid sequence by homologous recombination, wherein said first nucleic acid sequence has a restriction enzyme cleavage site at its 3' end and said second nucleic acid sequence has a restriction enzyme cleavage site at its 5' end;

providing one or more restriction enzymes capable of cleaving said restriction enzyme cleavage site of said first nucleic acid sequence and said restriction enzyme cleavage site of said second nucleic acid sequence;

facilitating a transformation of the cleaved nucleic acid sequence into said host cell;

facilitating a homologous recombination between said first and second nucleic acid sequences in order to form a circular plasmid in said cell.

33. A kit comprising a recombinant nucleic acid molecule, said molecule comprising: a first nucleic acid sequence of a selectable marker operably linked to a second nucleic acid sequence of said selectable marker, wherein said second nucleic acid sequence lacks a start codon and is capable of combining with said first nucleic acid sequence by homologous recombination, wherein said first nucleic acid sequence has a restriction enzyme cleavage site at its 3' end and said second nucleic acid sequence has a restriction enzyme cleavage site at its 5' end.

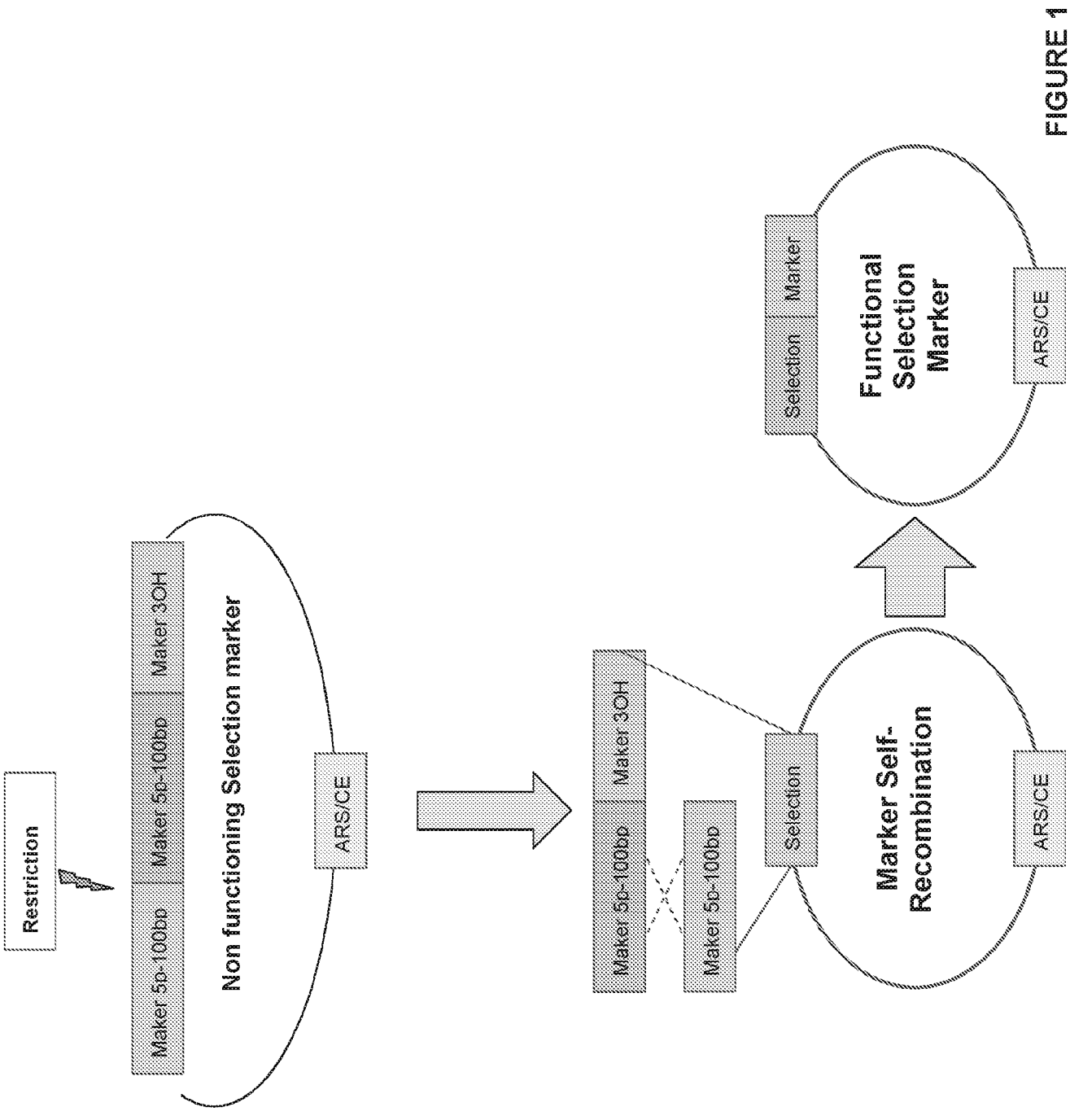

Restriction
Maker 5p-100bp
Maker 5p-100bp
Maker 3OH
Non functioning Selection marker
ARS/CE
Maker 5p-100bp
Maker 3OH
Maker 5p-100bp
Selection
Marker Self-Recombination
ARS/CE
Selection
Marker
Functional Selection Marker
ARS/CE

FIGURE 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/34917

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/09, C12N 1/19, C12N 15/81 (2017.01)
CPC - C12N 15/8213, C12N 15/905, C12N 1/19, C12N 15/81

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	ABASTADO et al., Intramolecular recombination between partially homologous sequences in Escherichia coli and Xenopus laevis oocytes. Proc Natl Acad Sci U S A., September 1987, Vol 84, No 18, pp 6496-6500. Especially p 6496 col 2 para 4; p 6497 col 1 para 2-5; p 6497 col 2 para 1; p 6497 col 2 para 4-5; p 6499 col 1 para 4-5	1, 6-13, 18, 23-29, 32-33 --- 2-5, 14-17, 19-22, 30-31
Y	US 2013/0267021 A1 (WEBER et al.) 10 October 2013 (10.10.2013) para [0007], [0233], [0315], [0335]	2-5, 14-17, 19-22, 30-31

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 July 2017 (30.07.2017)	23 AUG 2017

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774