NUCLEIC ACIDS, COMPOSITIONS AND METHODS FOR THE EXCISION OF TARGET NUCLEIC ACIDS

[0001] This application claims benefit of U.S. Provisional Application No. 61/378,350, filed August 30, 2010~~, which is hereby incorporated by reference in its entirety~~.

1. FIELD OF THE INVENTION

[0002] The nucleic acids, compositions, and methods provided herein generally relate to the fields of molecular biology and genetic engineering.

2. BACKGROUND

[0003] Genetic engineering techniques to excise a target nucleic acid from a host cell genome or an episome are needed in a variety of fields including metabolic engineering, industrial microbiology, synthetic biology, and basic molecular genetics research. Previous methods for removal of target nucleic acids, however, have been restricted and limited in application. Site specific recombinase methods of removal, for example, leave behind deleterious specific recombinase binding sites that create potential genomic instabilities within the host cells. Other methods can produce excision events at low frequency, thus necessitating methods for growth-selection of rare host cells that undergo the excision event. There exists a need for nucleic acids, compositions, and methods that can allow for high frequency and high fidelity excision of a target nucleic acid from a host cell genome or an episome without creating potential genomic instabilities.

3. SUMMARY

[0004] Provided herein are nucleic acids, compositions, and methods that allow for the excision of one or more loci from the genome of a host cell. In a first aspect, provided herein is an excisable nucleic acid construct comprising, in a 5' to 3' orientation: a) a first tandem repeat nucleic acid, b) a first homing endonuclease recognition site, c) a target nucleic acid, d) a second homing endonuclease recognition site and e) a second tandem repeat nucleic acid. In some embodiments, the excisable nucleic acid construct is integrated into the host cell genome. In some embodiments, the second homing endonuclease recognition site is optional.

[0005] The first and second homing endonuclease recognition sites allow for a homing endonuclease to cleave the excisable nucleic acid construct. A homing endonuclease bound to a homing endonuclease recognition site can cleave the excisable nucleic acid construct at or adjacent to the homing endonuclease recognition site. In some embodiments, each of the first and second homing endonuclease recognition sites independently comprises 14-40 nucleotide

endonuclease is expressed within a host cell using a homing endonuclease expression vector. Any homing endonuclease expression vector may be used including the expression vectors described above. The homing endonuclease expression vector may comprise a selectable marker, *e.g.,* a counter-selectable marker, that allows for selection of host cells that do not contain the expression vector subsequent to excision of the target DNA segment. The expression vector used may also be a transient vector that has no selectable marker, or is one that is not selected for. In particular embodiments, the progeny of a host cell comprising a transient vector loses the vector over time. In other embodiments, the excisable nucleic acid construct can be contacted with a purified form of the homing endonuclease.

[0099] In some embodiments, cleavage of each of ES1 and ES2 advantageously creates three nucleic acid fragments (Fig. 3A and 3B): (1) a left arm of a genomic nucleic acid; (2) a nucleic acid fragment comprising the target DNA segment; and (3) a right arm of the genomic nucleic acid. After cleavage, endogenous 5' to 3' exonucleases found in the host cell rapidly degrade one strand of each nucleic acid fragment, destroying the nucleic acid fragment comprising the target DNA segment and leaving long 3' tails on the left (4) and right (5) arms of the genomic nucleic acid (Fig. 3C) comprising DR1 and DR2 as complementary regions (Fig.4A). The complementary regions form a heteroduplex (Fig. 4B, 6) and undergo recombination facilitated by host cell proteins. In some embodiments, the complementary regions advantageously undergo recombination via the single strand annealing pathway. The extreme 3' ends of the tails on the right (7) and left (8) arms are not complementary and thus hang out of the heteroduplex formed by the complementary portions. These extreme non-complementary 3' ends are advantageously cleaved by a flap nuclease. Finally, repair DNA synthesis and DNA ligase fill in the heteroduplex and seal nicks, creating an intact genomic nucleic acid with a precise excision of the target DNA segment (Fig. 4C). In embodiments in which DR1 and DR2 share 100% nucleotide sequence identity with one another, DR1 and DR1 advantageously recombine to create a genomic nucleic acid comprising a third tandem repeat that shares 100% nucleotide sequence identity with DR1 and DR2.

~~[00100]~~ [0100]In another aspect, provided herein is a method of simultaneously excising at least two target nucleic acids from the genome of a host cell in which at least two excisable nucleic acid constructs have been genomically integrated. In some embodiments, the method comprises contacting the at least two excisable nucleic acid constructs with one or more homing endonucleases in the host cell such that one or more homing endonucleases cleave at or adjacent

to at least one of the first or second homing endonuclease recognition sites of each excisable nucleic acid construct.

~~[00101]~~ [0101] In some embodiments, the host cell comprises two or more excisable nucleic acid constructs wherein each excisable nucleic acid construct comprises unique ES sites, *i.e.,* ES sites not shared among other excisable nucleic acid constructs within the host cell. In these embodiments, one or more homing endonucleases are provided in the host cell to effect the simultaneous excision of the more than one target DNA segments. In some embodiments, more than one homing endonuclease is provided in the host cell to effect the simultaneous excision of the more than one target DNA segments.

~~[00102]~~ [0102] In other embodiments, each of the genomically integrated excisable nucleic acid constructs share at least one identical ES region, such that contact of each of the ES region of each excisable nucleic acid construct with a single homing endonuclease capable of cleaving the ES results in the simultaneous excision of the target DNA segment of each excisable nucleic acid construct. In some embodiments, the methods provided herein allow for the simulataneuous excision of at least 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 target nucleic acids from the genome of a host cell. In some embodiments, the simultaneous multiple excision is effected with a single homing endonuclease having specificity for a shared ES site within each of the integrated excisable nucleic acid constructs. In other embodiments, the simultaneous multiple excision is effected with a plurality of homing endonucleases, each having specificity for at least one ES site within an integrated excisable nucleic acid construct.

~~[00103]~~ [0103] An advantage of the methods presented is that DR1 and DR2 of a particular excisable nucleic acid construct may comprise any tandem repeat that can mediate the recombination of the excisable nucleic acid construct upon cleavage. Therefore, multiple excisable nucleic acid constructs, each with unique tandem repeats, can be used within the same cell without concerns of genomic instabilities due to recombination of tandem repeats between different excisable nucleic acid constructs. Further, the methods can advantageously be used for removing selectable markers to enable their reuse in the same host cell or its progeny.

~~[00104]~~ [0104] In other embodiments, the excision event can be used to promote, suppress, or alter the expression of an endogenous gene of interest in the host cell. For example, in some embodiments, the first genomic integration sequence comprises a nucleotide sequence that is homologous to a nucleotide sequence positioned 5' of the coding sequence of the endogenous gene of interest, and the second genomic integration sequence comprises a nucleotide sequence

nucleotide sequence that is homologous to a nucleotide sequence positioned within the coding sequence of the endogenous gene of interest, and the target DNA segment comprises a nucleotide sequence encoding a promoter that can be induced or repressed, for example, by addition of an inducer or repressor, respectively, to the culture medium in which the host cell is cultivated. Upon integration of the integrating sequence at the target locus, the native promoter of the target gene is replaced with the inducible or repressible promoter from the target DNA segment, rendering production of the gene product of the gene of interest dependent on the presence of the inducing or repressing agent in the culture medium. Similarly, the target DNA segment of the excisable nucleic acid construct may comprise a nucleotide sequence encoding a repressor that can be induced or repressed by addition of an inducer or repressor, respectively. Such exogenous regulation of the expression of the gene of interest can be removed as desired by inducing an excision event as described herein, such that the regulatable promoter or repressor is excised from the host cell genome.

~~[00105]~~ [0105]In other embodiments, integration of the excisable nucleic acid construct into the host cell genome can be used to disrupt the expression of an endogenous gene of interest, for example, by interrupting the operable linkage between the coding sequence of the endogenous gene of interest and its native promoter element. Where restoration of the expression of the endogenous gene is desired, an excision event in accordance with the methods described herein can be induced to operably re-link the native promoter element with the coding sequence of the endogenous gene of interest, that is, to bring the native promoter element back within operable proximity of the coding sequence for the endogenous gene of interest.

5.6 Kits

~~[00106]~~ [0106] In another aspect, provided herein is a kit for the excision of a target DNA segment from the genome of a host cell. In some embodiments, the kit comprises: (a) an excisable nucleic acid construct comprising, in a 5' to 3' orientation: (i) a first tandem repeat (DR1), (ii) a first homing endonuclease recognition site (ES1), (iii) a target DNA segment (D), and (iv) a second tandem repeat (DR2); and (b) a vector comprising a homing endonuclease nucleic acid encoding a homing endonuclease capable of binding to and cleaving at or adjacent to at least one of the first or second homing endonuclease recognition sites. In some embodiments, the kit comprises: (a) an excisable nucleic acid construct comprising, in a 5' to 3' orientation: (i) a first tandem repeat (DR1), (ii) a target DNA segment (D), (iii) a first homing endonuclease recognition site (ES1), and (iv) a second tandem repeat (DR2); and (b) a vector comprising a homing endonuclease nucleic acid encoding a homing endonuclease capable of

binding to and cleaving at or adjacent to at least one of the first or second homing endonuclease recognition sites. In some embodiments, the kit comprises: (a) an excisable nucleic acid construct comprising, in a 5' to 3' orientation: (i) a first tandem repeat nucleic acid (DR1), (ii) a first homing endonuclease recognition site (ES1), (iii) a target nucleic acid, (iv) a second homing endonuclease recognition site (ES2), and (v) a second tandem repeat nucleic acid (DR2); and (b) a vector comprising a homing endonuclease nucleic acid encoding a homing endonuclease capable of binding to and cleaving at or adjacent to at least one of the first or second homing endonuclease recognition sites.

~~[00107]~~ [0107]In some embodiments, each of the first and second tandem repeat nucleic acids independently comprises at least 18 nucleotide base pairs. In some embodiments, each of DR1 and DR2 independently consists of 18 to 500 nucleotide base pairs. In some embodiments, each of the first and second tandem repeat nucleic acids independently consists of 18 to 500, 18 to 495, 18 to 490, 18 to 485, 18 to 480, 18 to 475, 18 to 470, 18 to 465, 18 to 460, 18 to 455, 18 to 450, 18 to 445, 18 to 440, 18 to 435, 18 to 430, 18 to 425, 18 to 420, 18 to 415, 18 to 410, 18 to 405, 18 to 400, 18 to 395, 18 to 390, 18 to 385, 18 to 380, 18 to 375, 18 to 370, 18 to 365, 18 to 360, 18 to 355, 18 to 350, 18 to 345, 18 to 340, 18 to 335, 18 to 330, 18 to 325, 18 to 320, 18 to 315, 18 to 310, 18 to 305, 18 to 300, 18 to 295, 18 to 290, 18 to 285, 18 to 280, 18 to 275, 18 to 270, 18 to 265, 18 to 260, 18 to 255, 18 to 250, 18 to 245, 18 to 240, 18 to 235, 18 to 230, 18 to 225, 18 to 220, 18 to 215, 18 to 210, 18 to 205, 18 to 200, 18 to 195, 18 to 190, 18 to 185, 18 to 180, 18 to 175, 18 to 170, 18 to 165, 18 to 160, 18 to 155, 18 to 150, 18 to 145, 18 to 140, 18 to 135, 18 to 130, 18 to 125, 18 to 120, 18 to 115, 18 to 110, 18 to 105, 18 to 100, 18 to 95, 18 to 90, 18 to 85, 18 to 80, 18 to 75, 18 to 70, 18 to 65, 18 to 60, 18 to 55, 18 to 50, 18 to 45, 18 to 40, 18 to 35, 18 to 30, 18 to 25, or 18 to 20 nucleotide base pairs. In some embodiments, each of DR1 and DR2 consists of 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94 , 95 96, 97 ,98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194 , 195, 196, 197, 198, 199 or 200 nucleotide base pairs.

~~[00108]~~ [0108]In some embodiments, the excisable nucleic acid construct further comprises a first genomic integration sequence linked 5' of the first tandem repeat nucleic acid and a second genomic integration sequence linked 3' of the second tandem repeat nucleic acid. In some embodiments, the kit provides first and second genomic integration sequences that are specific to the host cell for which integration of the excisable nucleic acide construct and subsequent excision of the target nucleic acid is desired. For example, for use in yeast, the kit provides, in some embodiments, a nucleic acid construct comprising first and second yeast-specific genomic integration sequences that share suffificent length and homology to initiate homologous recombination with a selected yeast genomic locus. In some embodiments, the kit provides a plurality of nucleic acid constructs, each comprising a unique pair of first and second genomic integration sequences targeted to a specific yeast genomic locus.

~~[00109]~~ [0109] In a particular embodiment, the kit provided herein comprises:(a) an excisable nucleic acid construct comprising, in a 5' to 3' orientation: (i) a first tandem repeat nucleic acid of at least 18 nucleotide base pairs, (ii) a first I-SceI site, (iii) a target nucleic acid, (iv) a second I-SceI site, and (v) a second tandem repeat nucleic acid of at least 18 nucleotide base pairs; and (b) a vector comprising a nucleic acid encoding I-SceI. In some embodiments, the excisable nucleic acid construct further comprises a first integration site linked 5' of the first homing endonuclease recognition site and a second integration site linked 3' of the second tandem repeat nucleic acid.

~~[00110]~~ [0110] In another particular embodiment, the kit provided herein comprises:(a) an excisable nucleic acid construct comprising, in a 5' to 3' orientation: (i) a first tandem repeat nucleic acid, (ii) a first F-CphI site, (iii) a target nucleic acid, (iv) a second F-CphI site, and (v) a second tandem repeat nucleic acid; and (b) a vector comprising a nucleic acid encoding F-CphI. In some embodiments, the excisable nucleic acid construct further comprises a first integration site linked 5' of the first homing endonuclease recognition site and a second integration site linked 3' of the second tandem repeat nucleic acid.

~~[00111]~~ [0111] In certain embodiments, the kits provided herein further comprise one or more host cells suitable for transformation with the excisable nucleic acid constructs and/or the vectors described above.

~~[00112]~~ [0112] In some embodiments, the kit further comprises instructions for use that describe the methods of excising a target DNA segment from the genome of a host cell disclosed herein. In some embodiments, the kit comprises an excisable nucleic acid construct comprising

a target DNA segment, wherein the target DNA segment is selected from, *e.g.*, 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, or degradation signal.

6. EXAMPLES

6.1 Example 1: Construction of xMarker Constructs

~~[00113]~~ [0113]The compositions and methods described herein were implemented to prepare and characterize a series of excisable selectable markers for use in *S. cerevisiae*, described herein as "xMarkers." The first generation of markers utilized the I-SceI endonuclease and tested the parameters of the DNA construct shown in **FIG. 1**, which parameters include: (1) different lengths of the direct repeats flanking the endonuclease sites and (2) one vs. two endonuclease sites. The second generation of markers applied the results of the first generation tests toward broader application of the I-SceI endonuclease. The third generation xMarkers demonstrated the usefulness and extensibility to other endonucleases listed in Table 1 below.

Table 1: Recognition and cleavage sites for the endonucleases.

~~Meganuclease~~ Endonucleases	minimal site size (base pairs)	site size used (base pairs)	sequence of site used
I-SceI	18	18	TAGGGATAACAGGGTAAT (SEQ ID NO:3)
VDE (PI-SceI)	31	31	TATGTCGGGTGCGGAGAAAG AGGTAATGAAA (SEQ ID NO:4)
F-Cph	20	24	GATGCACGAGCGCAACGCTC ACAA (SEQ ID NO:5)
PI-MgaI (*pps1*)	22	24	GCGTAGCTGCCCAGTATGAGT CAG (SEQ ID NO:6)
PI-MtuII (*pps1*)	unknown	40	ACGTGCACTACGTAGAGGGTC GCACCGCACCGATCTACAA (SEQ ID NO:7)

~~[00114]~~ [0114] With respect to the reagents used in the experiments described below, restriction enzymes were obtained from New England Biolabs and Fermentas. Phusion, a high-fidelity thermostable polymerase from Finnzymes, was used for construction of DNA used in cloning of plasmids or in yeast transformations for chromosomal integrations. A low-fidelity thermostable polymerase kit was used for PCR of genomic DNA from yeast colonies (Qiagen Taq PCR kit). Oligonucleotides were obtained from Integrated DNA Technologies (IDT). Other chemicals were obtained from Sigma, Fisher, and Zymo Research (e.g., standard molecular biology buffer components like Tris and EDTA and standard yeast reagents like lithium acetate and yeast nitrogen base). Competent E. coli cells used for DNA cloning were purchased from Invitrogen. DNA minipreps were performed with the miniprep kit from Qiagen. Molecular biology, yeast molecular genetics, and yeast cell culture techniques were performed according to standard protocols.

6.1.1. Construction of First Generation xMarkers

~~[00109]~~ [0115] The initial series of xMarkers used *URA3* as the selectable marker, and each member of the series differed in the number of I-SceI cleavage sites (1 or 2) and in the length of direct repeats (20, 40, 60, or 80 bp). *URA3* is a counterselectable marker whose presence can be selected by growth on medium lacking uracil and whose absence can be selected by growth on medium containing 5-fluoroorotic acid. Direct repeat sequences were designed to consist of a 112 bp stretch of DNA, wherein each segment of 20 bp had a GC content of ~50%, and that stretches of 20, 40, 60, and 80 bp had little predicted secondary structure at temperatures above 30°C. The I-SceI cleavage sites all shared the same 18 bp sequence: 5'-TAGGGATAACAGGGTAAT-3' (SEQ ID NO:3). Table 2 lists all tested first generation xMarkers with the number of cleavage sites and their orientation relative to each other, the length and sequence of the direct repeats (DR), and the sequence of the I-SceI cleavage site(s). For the xMarkers with two I-SceI sites, the sequence of elements was DR→/ I-SceI site→/ URA3/ I-SceI site→/ DR→. For the xMarker with one I-SceI site, the sequence of elements was DR→/ I-SceI site→/ URA3/ DR→.

Table 2: List of characteristics of individual elements in first generation I-SceI xMarkers, with order and orientation of the elements as shown in FIG. 1

name of URA3 xMarker	length of DR (bp)	I-SceI sites (#)	orientation of I-SceI sites to each other	DR (scar) sequence
20mer-direct	20	2	direct	AAGATCCGATCGACCGAGAA (SEQ ID NO: 8)
40mer-direct	40	2	direct	AAGATCCGATCGACCGAGAACTGAGA ACGGTGCAATGATC (SEQ ID NO:9)
60mer-direct (aka s0x-URA3)	60	2	direct	AAGATCCGATCGACCGAGAACTGAGA ACGGTGCAATGATC-AACATGATCTGCGACGAGCT (SEQ ID NO:10)
80mer-direct	80	2	direct	AAGATCCGATCGACCGAGAACTGAGA ACGGTGCAATGATC-AACATGATCTGCGACGAGCTTGAGGA TGCAAATGGCTGAC (SEQ ID NO:11)
60mer-single	60	1	solo	AAGATCCGATCGACCGAGAACTGAGA ACGGTGCAATGATCAACATGATCTGC GACGAGCT (SEQ ID NO:12)

~~[00116]~~ [0116] Due to the repeated sites in the xMarkers, construction required that the left half and the right half be constructed separately before joining the two halves. The first generation xMarkers were created in three steps.

~~[00117]~~ [0117] First, the sequences flanking the selectable marker on the left (5') and on the right (3') were constructed separately by annealing of phosphorylated oligonucleotides. The left and right double-stranded flanking sequences created by annealing were designed to have complementary, non-palindromic, 3' single-stranded overhangs of five bases (TAGAC on top and GTCTA on bottom). Table 3 lists the oligos used for each xMarker. For each annealing reaction, an equimolar mixture of oligonucleotides in DNA ligase buffer was heated to 95°C in a heat block for 5 minutes and then the heat block was turned off and allowed to cool slowly to room temperature over the course of 1-2 hours.

Table 3: List of oligonucleotides mixed for annealing to make left segments and right segments flanking the marker in first generation I-SceI xMarkers.

name of URA3 xMarker	oligos annealed for left segment	oligos annealed for right segment
20mer-direct	KB411, KB412	KB464, KB429
40mer-direct	KB415, KB416	KB419, KB420
60mer-direct (aka s0x-URA3)	KB423, KB425, KB426, KB427, KB428	KB419, KB423, KB424, KB429
80mer-direct	KB423, KB425, KBB427, KB431, KB432, KB433	KB419, KB423, KB429, KB432, KB434
60mer-single	KB423, KB425, KB426, KB427, KB428	KB421, KB423, KB424, KB430

~~[00118]~~ [0118] Second, the corresponding left and right flanking DNAs from the annealing mixtures were mixed with the RYSE 12 entry vector for a 3-way ligation. The right end of the left repeat sequence was joined to the left end of the right repeat sequence by a sticky end ligation, and on the opposite ends the left and right repeat sequences were joined to the plasmid by blunt end ligations. The spacer between the I-SceI cleavage sites contained divergent SchI restriction sites, separated by an XbaI site. These plasmids were called xMarker entry vectors, because they did not yet have a marker, and they had only the flanking sequences to direct cleavage and repair.

~~[00119]~~ [0119] 3Third, the URA3 selectable marker was ligated into the xMarker entry vectors. The URA3 marker was amplified by PCR from a plasmid (RaBit 12-0-M-555) to give a blunt-ended marker. For the directly repeated dual I-SceI site xMarkers, the URA3 PCR used oligonucleotides KB439 and KB440; for the single I-SceI site xMarker, the oligonucleotides were KB439 and KB441. The xMarker entry vectors were digested with SchI to give a blunt-ended linear plasmid. Then the two fragments were ligated together using ligase standard conditions. This generated the xMarker structure shown in **FIG. 1**. Individual isolates of

ligated plasmids were recovered and the desired plasmids were identified by DNA sequencing of the insert in the plasmid.

6.1.2. Construction of Second Generation xMarkers

~~[00120]~~ [0120]The second generation of xMarkers were all created with 60 bp direct repeats to aid DNA repair and with two I-SceI cleavage sites in direct repeat. Each xMarker had a unique 60 bp sequence, which was chosen to be a semi-random sequence, as indicated below in Table 4.

Table 4: List of 2nd generation I-SceI xMarkers and their 60 bp repeat sequences.

name of xMarker	selection	sequence of 60 bp repeat
s1x_hphA	hygromcycin B resistance (hph)	CGTTACGAAGCACACACTAGTTAGCGTCGAGACA CATAGCGACGCTAGAACTTGCGACTT (SEQ ID NO:13)
s2x_hphA	hygromcycin B resistance (hph)	GTACTGCCTAGTAGAAACGGATCTCCACGTACTA GAGTCCACCTGGTATCTATTAGCCCG (SEQ ID NO:14)
s3x_kanA	kanamycin/G418 resistance (kan)	CGAGGATTAACGTGTAAGGCCCTAAGCTATGTAC CGCATCTCCTAAGAGAGTGTGACCCA (SEQ ID NO:15)
s4x_kanA	kanamycin/G418 resistance (kan)	TTAATCAGCGCCCAGAGACTAGCACTGAATGATC AACGGGTAGTTCACACACTGCCAGAC (SEQ ID NO:16)
s5x_natA	nourseothricin resistance (nat)	ATGGAATCACGGGGCTATTCCACTTGCTAATAAC GAGGCGCTTATCAACGGCGAGCACAT (SEQ ID NO:17)
s6x_natA	nourseothricin resistance (nat)	AGTCAAAGCGCGATTCGCTAGGAATGAGAGCGA GAACGAACCGGAGTATATCACAATCGC (SEQ ID NO:18)
s7x_URA3	uracil prototropy	ACTAGAGCGAAATGGAGAGGTACGTGATCCTACT AGAGCCCACGCTATCATACAGTTGGC (SEQ ID

	(URA3)	NO:19)
s8x_URA3	uracil prototropy (URA3)	GTACGTCCGTACTTATGCTGAGCGCTCCTACACG AAAAACTCACCGTGACTAGCATAACG (SEQ ID NO:20)

~~[00121]~~ [0121] The 60 bp sequences were chosen to satisfy two criteria: (1) the first, second, and third 20 bp windows had a melting temperature of 60°±2°C and (2) no sequence window 13 bp or longer that appeared in the 60 bp sequence was identical to any native sequence in the yeast genome. This set included four different selectable markers: URA3; hygromycin B phosphotransferase or *hph*; nourseothricin acetyltransferase or *nat*; and aminoglycoside phosphotransferase or *kan*. These three drug resistance genes from bacteria were all controlled by adjacent sequences corresponding to the promoter and terminator of the *TEF1* gene from *Kluyveromyces lactis*, and the suffix "A" was appended to indicate this *TEF1*-derived regulatory control; the cassettes were then called *hphA*, *natA*, and *kanA*.

~~[00122]~~ [0122] Due to the repeated sites in the xMarkers, construction required that the left half and the right half be constructed separately before joining the two halves. The second generation xMarkers were created in two steps, using a strategy different from the first generation. The 60 bp sequence and a 20 bp I-SceI cleavage site were introduced to the left and right of the marker by priming PCR amplification of the marker with long-tailed oligonucleotides listed in Table 5 below.

Table 5: List of primers used for PCR amplification of left and right portions of 2nd generation I-SceI xMarkers.

name of xMarker	**left portion primers**	**right portion primers**	**restriction enzyme**	**alternative left portion primers**	**alternative right portion primers**	**alternative restriction enzyme**
s1x_hphA	KB469, KB467	KB470, KB468	NdeI	KB469, KB492	KB470, KB491	RsrII or BanI
s2x_hphA	KB471, KB	KB472,	NdeI	KB471,	KB472,	RsrII or

	467	KB468		KB492	KB491	BanI
s3x_kanA	KB475, KB473	KB476, KB474	PvuI	KB475, KB494	KB476, KB493	NciI or BstXI
s4x_kanA	KB477, KB473	KB478, KB474	PvuI	KB477, KB494	KB478, KB493	NciI or BstXI
s5x_natA	KB481, KB479	KB482, KB480	StyI	none	none	none
s6x_natA	KB483, KB479	KB484, KB480	StyI	none	none	none
s7x_URA3	KB487, KB485	KB488, KB486	NcoI	KB487, KB496	KB488, KB495	BsiHKAI or AlwNI
s8x_URA3	KB489, KB485	KB490, KB486	NcoI	KB489, KB496	KB490, KB495	BsiHKAI or AlwNI

~~[00123]~~ [0123] The oligonucleotides contained a priming region of 20-22 bp at the 3' end and a 5' tail of 80 bp containing the unique 60 bp sequence and the I-SceI site. Two slightly different I-SceI sites were used on the left and the right of the marker: on the left, version 1 (v1) was 5'-GCTAGGGATAACAGGGTAAT-3'(SEQ ID NO:21) and on the right, version 2 (v2) was 5'-ACTAGGGATAACAGGTTTAT-3' (SEQ ID NO:22). The second generation xMarkers all had a structure of elements summarized by DR (60 bp)→/ I-SceI site (v1)→/ marker/ I-SceI site(v2)→/ DR (60 bp)→.

~~[00124]~~ [0124] The xMarker construction overview is as follows. First, the left portion of the marker and the right portion of the marker were amplified separately by PCR. The primers were designed so that a middle segment of the marker was included in both the left and right PCR products, and so that this overlap segment included a unique restriction site that left single-stranded complementary overhangs. Second, the two PCR products were digested separately with the chosen restriction enzyme, gel-purified, and added to a three-piece ligation mixture with the linearized RYSE 12 entry vector. Similar to the first generation ligation, the left and right portions annealed and ligated using sticky ends, while the outermost ends of the marker construct participated in a blunt-ended ligation with the recipient plasmid. A difference from the first generation method is that after the ligation, the product already contains the marker gene and construction is completed. Individual isolates of ligated plasmids were recovered and the

desired plasmids were identified by DNA sequencing of the insert in the plasmid. These plasmids were new xMarker 12 RaBits that could be used for RYSE-based stitching of compound DNA constructs, just as previous 12 RaBits were used.

~~[00125]~~ [0125]The xMarker details are described below. The unique restriction sites internal to the markers that were used initially for each marker are as follows: hphA, NdeI (RsrII or BanI); kanA, PvuI (NciI or BstXI); natA, StyI; and URA3, NcoI (BsiHKAI or AlwNI). The enzymes chosen later were used if the initial enzymes did not yield positive clones; their benefit is that the single-stranded overhangs are not palindromic, so that it is not likely to get a ligated plasmid with two left or two right portions. Amplification of the left portion of a marker was performed with a ~20 bp reverse primer that annealed to the top strand on the right of the indicated restriction site and with a primer to the left edge of the marker that included a 5' tail, such that the 5' oligonucleotide had a structure as follows: 60 bp sequence→/ I-SceI site (v1)→/ ~20 bp forward primer. Amplification of the right portion of a marker was performed with a ~20 bp forward primer that annealed to the bottom strand on the left of the indicated restriction site and with a primer to the right edge of the marker that included a 5' tail, such that the 3' oligonucleotide had a structure like this: 60 bp sequence (reverse complement)→/ I-SceI site (v2, reverse complement)→/ ~20 bp reverse primer. Table 4 contains a list of the primers used for amplification of the left and right portions of each xMarker. The templates used for PCR amplification of the markers were RaBit plasmids: 12-0-M-21 for hphA, 12-0-M-261 for kanA, 12-0-M-262 for natA, and 12-0-M-555 for URA3. The finished xMarker second generation plasmids were themselves 12 RaBits that could be used for RYSE-based stitching of compound DNA constructs, just as previous 12 RaBits were used.

6.1.3. Construction of I-SceI Expression Plasmids

~~[00126]~~ [0126] The I-SceI gene was placed under control of the *S. cerevisiae* promoter for *GAL1* and cloned into a set of CEN.ARS plasmids with various markers. The yeast-*E.coli* shuttle vectors with CEN.ARS sequences and *LEU2* (pRS415 aka pAM63) and *URA3* (pRS416 aka pAM63) markers were previously described (*see, e.g.*, *Gene* 1992;110:119-22; and *Genetics* 1989;122:19-27). Derivatives of pRS416 were made by replacing the auxotrophic markers with the drug resistance markers kanA (pAM1110), natA (pAM1111), and hphA (pAM1112). Each of the vectors was digested at a unique blunt-ended restriction site within the polylinker/multiple cloning site, either EcoRV (drug resistance markers) or SmaI (URA3 and LEU2), and then treated with phosphatase. The linearized vectors were ligated to a tripartite stitched PCR

product that had been treated with polynucleotide kinase (PNK) to phosphorylate the 5' ends. The PCR product was made by stitching together three pieces of DNA having overlapping ends using the oligonucleotide primers called RYSE4 and RYSE11: (1) the promoter from *S. cerevisiae GAL1* with RYSE linkers 2 and 3, provided by a SapI-liberated insert from RaBit 23-0-P-39, (2) the I-SceI coding sequence with RYSE linkers 3 and 4, provided by a PCR product from a custom-synthesized gene used as template and primers 00177-JD-75AN and 00177-JD-75AO, and (3) the terminator from *S. cerevisiae TDH* with RYSE linkers 4 and 5, provided by a SapI-liberated insert from RaBit 45-0-T-64. Individual isolates of ligated plasmids were recovered and the desired plasmids were identified by DNA sequencing of the P_{GAL1}-I-SceI-T_{TDH3} insert in the plasmid. The expression plasmids were called pAM1592 (URA3), pAM1593 (kanA), pAM1594 (natA), and pAM1595 (hphA). The P_{GAL1} promoter is highly expressed when cells are grown in galactose and, in wild-type cells (GAL80+ GAL4), is not expressed when cells are grown in glucose. However, in mutants lacking the GAL80 repressor (gal80Δ), P_{GAL1} is expressed even in the absence of galactose; glucose repression of P_{GAL1} is further reduced by addition of a GAL4 mutant with a promoter mutation, called $GAL4_{OC}$ (for Operator Constitutive).

~~[00127]~~ [0127]As described above, expression of the endonuclease was placed under the control of a strong promoter that was inducibly expressed in some host strain genetic backgrounds (GAL80+) and constitutively expressed in others (gal80Δ +/- $GAL4_{OC}$). Many other inducible promoters or constitutive promoters are expected to work well. Even if the promoter is constitutive, the expression of endonuclease can readily be eliminated after a desired time period by losing the plasmid; about half of the cells lose these plasmids after 10 generations in non-selective media (*see, e.g.*, *Gene* 1992;110:119-22; and *Genetics* 1989;122:19-27).

6.1.4. Construction of Third Generation xMarkers: Additional Endonucleases

~~[00128]~~ [0128]The initial series of xMarkers for other endonucleases (other than I-SceI) used *URA3* as the selectable marker. Each member of the series of xMarkers contained a recognition/cleavage site for a different endonuclease [VDE, F-CphI, PI-MgaI (*pps1*), PI-MtuII (*pps1*)]. *See* Table 1 above for a description of the cleavage sites. All *xURA3* markers contained two copies of the same 50 bp sequence in directly repeated orientation flanking the cleavage sites; this unique 50 bp sequence destined to be the scar after excision was called xM0. Table 6 below provides a description of the scar sequences.

xM8	F-CphI	TBD	GTACGTCCGTACTTATGCTGAGCGCTCCT ACACGAAAAACTCACCGTGAC (SEQ ID NO:31)
xM9	F-CphI	TBD	GCATTAAGTCGTAGCTAGCGGATTCTCTC TTCGTGCATCCTAGCAAATGG (SEQ ID NO:32)

~~[00129]~~ [0129] The scar sequences were chosen to satisfy the following criteria: (1) GC content of ~50%, (2) the first and second sequence of 20 bp (within the 50 bp total) had a melting temperature of 60°±2°C, (3) minimal predicted secondary structure at temperatures above 30°C, and (4) no sequence window 13 bp or longer that appeared in the 50 bp sequence was identical to any native sequence in the yeast genome. The general sequence and orientation of elements in the xMarkers was as shown in Figure 1: DR (50bp)→ , cleavage site→ , URA3 , cleavage site→ , DR(50bp)→ .

~~[00130]~~ [0130] Due to the repeated sites in the xMarkers, creation of the DNA molecules required that the left half and the right half be constructed separately before joining the two halves. Construction of the markers described here used the "2nd Generation" strategy described above in section 6.2.

~~[00131]~~ [0131] First, the left portion of the marker and the right portion of the marker were amplified separately by PCR. Four oligonucleotides were designed for PCR amplification of each xMarker: two "outer" oligonucleotides for PCR annealed to the ends of the marker and two "inner" oligonucleotides annealed inside the marker gene. The outer oligonucleotides contained 20-22 bp of sequence at the 3' end that was complementary to the template and 74-90 bp of sequence at the 5' end that did not anneal to the template and served to introduce the cleavage and scar sequences. The outer oligonucleotides were phosphorylated with PNK prior to inclusion in a PCR reaction, to facilitate ligation in later steps. The inner oligonucleotides were designed so that a middle segment of the marker was included in both the left and right PCR products, and so that this overlap segment included a unique restriction site that could be used to generate single-stranded, complementary overhangs. When possible, it was advantageous to choose restriction enzymes that generated non-palindromic overhangs, to reduce the likelihood of ligation of two left portions (or two right portions) together.

~~[00132]~~ [0132]Second, the two PCR products were digested separately with the chosen restriction enzyme and gel-purified. Third, the left and right segments, each having a sticky end and a blunt end, were added the linearized RYSE 12 entry vector plasmid for a three-piece ligation. The left and right portions annealed and ligated using sticky ends, while the outermost ends of the marker construct participated in a blunt-ended ligation with the recipient plasmid. Individual clones of ligated plasmids were recovered and the desired plasmids were identified by DNA sequencing. These plasmids were new xMarker 12 RaBits that could be used for RYSE-based stitching of compound DNA constructs, just as previous 12 RaBits were used.

~~[00133]~~ [0133]After the first set of xURA3 markers was tested, the F-CphI endonuclease was chosen for further work. Additional xMarkers with different selectable markers were made for use with F-CphI. This set included six different selectable markers: URA3; hygromycin B phosphotransferase or *hph*; nourseothricin acetyltransferase or *nat*; aminoglycoside phosphotransferase or *kan*; zeocin resistance gene or *ble*; and phosphinothricin N-acetyltransferase or *pat*. These drug resistance genes from bacteria were all controlled by adjacent sequences corresponding to the promoter and terminator of the *TEF1* gene from *Kluyveromyces lactis*, and the suffix "A" was appended to indicate this *TEF1*-derived regulatory control; the cassettes were then called *hphA*, *natA*, *kanA*, *zeoA*, and *patA*. Table 7 lists the restriction sites and the inner oligonucleotides for each marker; and Table 8 lists the outer oligonucleotides for each marker.

Table 7: Templates, restriction sites, and "inner" oligonucleotides used for construction of 3rd generation xMarkers

Marker	Template for PCR	Internal restriction enzymes for 3-way ligations	reverse inner oligonucleotide (RIO)	forward inner oligonucleotide (FIO)
URA3	RaBit 12-0-M-555	BsiHKAI, PpuMI, BslI, or AlwNI	KB496-266-100	KB495-266-100
hphA	RaBit 12-0-M-21	RsrII or BanI	KB492-266-100	KB491-266-100
kanA	RaBit 12-0-M-261	BstXI	KB494-266-100 or TD_187	KB493-266-100 or TD_186

~~[00134]~~ [0134]Table 9 below describes plasmids containing the endonuclease genes I-SceI, F-CphI, PI-MtuII (pps1), PI-MgaI (pps1) and VDE. The endonuclease genes were either chemically synthesized (I-SceI, F-CphI, PI-MtuII (pps1), PI-MgaI (pps1)) or amplified by PCR from *S. cerevisiae* genomic DNA (VDE).

Table 9: Plasmids containing endonuclease genes

Plasmid	**backbone**	**yeast marker**	**endonuclease**	**promoter**	**terminator**
pAM1592	pRS416	URA3	I-SceI	GAL1	TDH3
pAM1593	pAM1110	kanA	I-SceI	GAL1	TDH3
pAM1594	pAM1111	natA	I-SceI	GAL1	TDH3
pAM1595	pAM1112	hphA	I-SceI	GAL1	TDH3
pAM1677	pAM1112	hphA	VDE with N-terminal SV40 NLS	ACS2	ADE6
pAM1678	pUC19	none	PI-MgaI (pps1) with N-terminal SV40 NLS	none	none
pAM1679	pUC19	none	PI-MtuII (pps1) with N-terminal SV40 NLS	none	none
pAM1680	pUC19	none	F-CphI	none	none
pAM1749	pAM1112	hphA	F-CphI	ACS2	ADE6
pAM1750	pAM1112	hphA	PI-MgaI (pps1) with N-terminal SV40 NLS	ACS2	ADE6
pAM1751	pAM1112	hphA	PI-MtuII (pps1) with N-terminal SV40 NLS	ACS2	ADE6
pAM1799	pAM1112	hphA	F-CphI	GAL1	TDH3

pAM1800	pAM1801/ pAM1799	zeoA	F-CphI	GAL1	TDH3
pAM1862	pAM64 = pRS416	URA3	F-CphI	GAL1	TDH3
pAM1863	pAM1110	kanA	F-CphI	GAL1	TDH3
pAM1864	pAM1111	natA	F-CphI	GAL1	TDH3
pAM1865	pAM1112	hphA	PI-MtuII (pps1) with N-terminal SV40 NLS	GAL1	TDH3
pAM1866	pAM1112	hphA	PI-MgaI (pps1) with N-terminal SV40 NLS	GAL1	TDH3
pAM1867	pAM1112	hphA	VDE with N-terminal SV40 NLS	GAL1	TDH3

~~[00135]~~ [0135]Since three of the endonucleases (all but F-CphI and I-SceI) were too large (>25 kD; 353-456 amino acids) to freely travel through the nuclear pores from the intracellular site of synthesis (cytoplasm) to the site of action (nucleus), a DNA sequence was added to the 5' end of the coding sequence to append the SV40 nuclear localization sequence (NLS) to the amino-terminus of the protein. This NLS was described to be essential for the activity of VDE during mitotic (normal proliferative) growth in *S. cerevisiae*, since this native yeast enzyme naturally enters the nucleus only during meiosis. *See, e.g., Mol Cell Biol* 2003;23:1726-36. The NLS was added to VDE using oligonucleotides with tails containing the coding sequence for the SV40 NLS; the NLS was added to PI-MtuII and PI-MgaI as part of the chemical syntheses of the entire genes. PCR-based "stitching" or "overlap extension" was used to fuse the endonuclease coding sequences, with or without NLS, with a promoter and a terminator. A first set of constructs was made with the *S. cerevisiae* promoter from *ACS2* and the terminator from *ADE6*, without using the RYSE linkers. For this first set, the promoter-gene-terminator PCR stitching products were digested with SacI and XhoI, whose unique restriction sites had been introduced by tails on the oligonucleotides used for priming the PCR reactions of the individual

pieces; then the constructs were ligated into the recipient plasmid that had been digested with SacI and XhoI and treated with phosphatase. A second set used the *S. cerevisiae* promoter from *GAL1* and the terminator from *TDH3*; this set used the RYSE linkers and RYSE RaBits for the promoter (23-0-P-39) and terminator (45-0-T-64). For this second set of plasmids, the promoter-gene-terminator constructs were stitched with the RYSE primers RYSE4 and RYSE11 that had been previously phosphorylated using polynucleotide kinase (PNK), so that the blunt-ended stitched product could be efficiently ligated into a CEN.ARS plasmid with a yeast marker that had been linearized with a restriction enzyme that created blunt ended double-strand breaks. Finally, individual isolates of ligated plasmids were recovered and the desired plasmids were identified by DNA sequencing of the "promoter-gene-terminator" insert in the plasmid. The details of the plasmid construction are described below.

~~[00136]~~ [0136]The recipient plasmids were all based on the "pRS" series of yeast-*E.coli* shuttle vectors with CEN.ARS sequences and yeast selectable markers, e.g., the *URA3* marked version (pRS416 aka pAM64) was previously described (*see, e.g.*, *Gene* 1992;110:119-22; and *Genetics* 1989;122:19-27). Derivatives of pRS416 were made by replacing the auxotrophic markers with the drug resistance markers kanA (pAM1110), natA (pAM1111), and hphA (pAM1112). For the second set of plasmids, pAM1112 was digested at a unique blunt-ended restriction site (EcoRV) within the polylinker/multiple cloning site, and then treated with phosphatase. The linearized vector was ligated to tripartite stitched PCR products that had been treated with polynucleotide kinase (PNK) to phosphorylate the 5' ends. For the first set of plasmids, the tripartite stitched PCR products and the pAM1112 recipient plasmid were both digested with XhoI and SacI and then mixed for ligation.

~~[00137]~~ [0137]The "promoter-gene-terminator" PCR products for endonuclease gene expression were made by stitching together three pieces of DNA having overlapping ends. The oligonucleotides and templates used to create the three pieces, and the oligonucleotides used for stitching together the three pieces are shown in Table 10.

Table 10: Pieces used PCR stitches of promoter-endonuclease-terminator

identity	promoter piece: oligos and template OR RaBit	endonuclease piece: oligos and template	terminator piece: oligos and template OR RaBit	priming oligos for stitching	treatment of stitched product prior to

P_{GAL1}-PI-MgaI-T_{TDH3}	23-0-P-39	KB589, KB593, pAM1678	45-0-T-64	PNK-treated RYSE4, RYSE11	none
P_{GAL1}-PI-MtuII-T_{TDH3}	23-0-P-39	KB589, KB592, pAM1679	45-0-T-64	PNK-treated RYSE4, RYSE11	none

6.1.6. Construction of I-SceI Expression Plasmids

~~[00138]~~ [0138]The I-SceI gene was placed under control of the *S. cerevisiae* promoter for *GAL1* and cloned into a set of CEN.ARS plasmids with various markers. The yeast-*E.coli* shuttle vectors with CEN.ARS sequences and *LEU2* (pRS415 aka pAM63) and *URA3* (pRS416 aka pAM63) markers were previously described (*see, e.g.*, *Gene* 1992;110:119-22; and *Genetics* 1989;122:19-27). Derivatives of pRS416 were made by replacing the auxotrophic markers with the drug resistance markers kanA (pAM1110), natA (pAM1111), and hphA (pAM1112). Each of the vectors was digested at a unique blunt-ended restriction site within the polylinker/multiple cloning site, either EcoRV (drug resistance markers) or SmaI (URA3 and LEU2), and then treated with phosphatase. The linearized vectors were ligated to a tripartite stitched PCR product that had been treated with polynucleotide kinase (PNK) to phosphorylate the 5' ends. The PCR product was made by stitching together three pieces of DNA having overlapping ends using the oligonucleotide primers called RYSE4 and RYSE11: (1) the promoter from *S. cerevisiae GAL1* with RYSE linkers 2 and 3, provided by a Sap1-liberated insert from RaBit 23-0-P-39, (2) the I-SceI coding sequence with RYSE linkers 3 and 4, provided by a PCR product from a custom-synthesized gene used as template and primers 00177-JD-75AN and 00177-JD-75AO, and (3) the terminator from *S. cerevisiae TDH* with RYSE linkers 4 and 5, provided by a Sap1-liberated insert from RaBit 45-0-T-64. Individual isolates of ligated plasmids were recovered and the desired plasmids were identified by DNA sequencing of the P_{GAL1}-I-SceI-T_{TDH3} insert in the plasmid. The expression plasmids were called pAM1592 (URA3), pAM1593 (kanA), pAM1594 (natA), and pAM1595 (hphA). The P_{GAL1} promoter is highly expressed when cells are grown in galactose and, in wild-type cells (GAL80+ GAL4), is not expressed when cells are grown in glucose. However, in mutants lacking the GAL80 repressor (gal80Δ), P_{GAL1}

is expressed even in the absence of galactose; glucose repression of P_{GAL1} is further reduced by addition of a GAL4 mutant with a promoter mutation, called $GAL4_{OC}$ (for Operator Constitutive).

~~[00139]~~ [0139]Expression of the endonuclease was placed under the control of a strong promoter that was inducibly expressed in some host strain genetic backgrounds (GAL80+) and constitutively expressed in others (gal80Δ +/- $GAL4_{OC}$). Many other inducible promoters or constitutive promoters are expected to work well. Even if the promoter is constitutive, the expression of endonuclease can readily be eliminated after a desired time period by losing the plasmid; about half of the cells lose these plasmids after 10 generations in non-selective media (*see, e.g., Gene* 1992;110:119-22; and *Genetics* 1989;122:19-27).

6.1.7. Construction of F-CphI Expression Plasmids

~~[00140]~~ [0140]After the F-CphI expression plasmid with the hphA marker (pAM1799) was made and tested, the P_{GAL1}-F-CphI-T_{TDH3} cassette was subcloned into other CEN.ARS plasmids with different markers (pAM1110, pAM1111, pAM64) using the restriction enzymes XhoI and XbaI, each of which cleaved a unique site in the recipient plasmids and pAM1799. The plasmids were all cut with XhoI and XbaI, the plasmid vectors were treated with phosphatase, and the P_{GAL1}-F-CphI-T_{TDH3} cassette was ligated with the other backbones. After ligation, the correct plasmid isolates were identified by restriction digestion.

~~[00141]~~ [0141]The zeocin-resistance plasmid (pAM1800) was created by a method different from the others. Rather than stitch together the "promoter-gene-terminator" PCR product for P_{GAL1}-F-CphI-T_{TDH3} and then ligate into a recipient plasmid with the zeocin resistance marker, the construction started with the F-CphI expression plasmid with the hphA marker (pAM1799) and exchanged the marker by exploiting yeast homologous recombination in vivo to substitute the zeocin resistance gene in place of the hygromycinB resistance gene. First, pAM1799 was linearized with NdeI, a restriction enzyme that cut a unique site in the hygromycin B resistance coding sequence. Second, the zeocin resistance gene was PCR-amplified from pAM1500 (or any Topo plasmid) using oligonucleotides with long tails (JU183 and JU184) that introduced sequence from the P_{TEF} promoter and T_{TEF} terminator that was homologous to the sequence in pAM1799 that controlled expression of the hph marker. Third, the two pieces of DNA were gel-purified and transformed into yeast for "gap repair" recombination that precisely replaced the hygromycin B resistance gene with the zeocin resistance gene. The correct plasmid was verified by DNA sequencing.

[00142] [0142]The P_{GAL1} promoter is highly expressed when cells are grown in galactose and is not expressed when wild-type cells (with genotype GAL80 GAL4) are grown in glucose. However, in mutants lacking the GAL80 repressor (gal80Δ), P_{GAL1} is expressed even in the absence of galactose; glucose repression of P_{GAL1} is further reduced by addition of a GAL4 mutant with a promoter mutation, called $GAL4_{OC}$ (for Operator Constitutive). The P_{ACS2} promoter is moderately expressed in all carbon sources.

6.2 Example 2: Excision of Selectable markers from Chromosomal DNA

[00143] [0143]This example demonstrates the utility of the xMarker constructs described in Example 1 in mediating excision of a selectable marker from the chromosomal DNA of a host cell. As described below, the construct is transformed into cells, cells are plated on media selective for the xMarker, and the correct integration is confirmed by colony PCR; such strains perform just like any other strain made with a standard marker and the xMarker is stably maintained. Third, when the excision of the xMarker is desired, the strain is transformed with a single-copy (CEN.ARS) plasmid containing its own marker and an expression construct for the homing endonuclease gene, as described in the sections below. After growing under conditions that select for the presence of the plasmid for several generations and induce expression of the homing endonuclease gene, the strain is tested for loss of the xMarker. Finally, the strain is grown under conditions that permit loss of the homing endonuclease expression plasmid and isolates are tested for loss of the plasmid. At the end of this process, the strain is ready for re-use of the xMarkers.

[00144] [0144]For this strategy, it is advantageous that the xMarker is stable for a period of time sufficient for selection and verification of the cells that correctly integrated the desired DNA construct. High stability is ensured by using cells that lack the endonuclease gene until it is introduced by a second transformation. However, an alternative approach utilizes a host strain with a stably integrated endonuclease gene under the control of an inducible promoter. In this alternative scheme, the xMarker would be stable until the strain was cultured under conditions that induced expression of the endonuclease from the regulated promoter. If promoters with the desired characteristics are available, this approach would save time and effort required for transformation of the endonuclease plasmid.

6.2.1. Initial Testing of xMarkers using I-SceI:

[00145] [0145]To test this approach for precise excision of target DNA, the efficiency and fidelity of excision of selectable markers from chromosomal DNA were measured in *S.*

cerevisiae. First, the xMarkers were placed between two stretches of >500 bp sequence corresponding to the regions upstream and downstream of a chosen gene (generically, GOI=gene of interest) to generate a knock-out construct. Second, yeast cells were transformed with this DNA construct to delete the gene of interest and replace its coding sequence with the xMarker, via homologous recombination involving a double crossover event. Third, mutants were transformed with an I-SceI expression plasmid; plasmid-bearing isolates were allowed time to express the endonuclease and undergo DNA cleavage and repair. Fourth, colonies that were unable to grow under marker-selective conditions were identified and counted. Fifth, the chromosomal DNA sequence of the deleted gene of interest (goiΔ) locus was determined to assess if the marker was excised and repaired cleanly as expected. The details of this series of steps are presented below for the first generation markers.

~~[00146]~~ [0146] First, a knock-out construct with the structure GOI US/ xMarker/ GOI DS (GOI=gene of interest, US=upstream, DS=downstream) was stitched together using overlap extension PCR with three elements. I-SceI xMarkers were initially tested in the context of a ndt80Δ construct; NDT80 is a meiosis-specific gene that was chosen because its deletion in mitotically growing cells has no discernable phenotype. Each PCR reaction used as templates three RaBits liberated from their plasmid backbones by digestion with SapI: (1) NDT80 upstream (01-0-U-30), (2) an xMarker RaBit, and (3) NDT80 DS (29-0-D-23). The primers for PCR were RYSE0 and RYSE19. After PCR, the reaction mixture was loaded on an agarose gel and the desired full-length product was purified from the gel.

~~[00147]~~ [0147] Second, yeast cells were transformed with gel-purified PCR products and plated on selective plates. Selective plates for the first generation xMarkers were CSM-Uracil; selective plates for the drug resistance markers were YPD+drug (hygromycin B, nourseothricin, or G418). All xMarker tests were performed in strains derived from the CEN.PK2 or S288c strain backgrounds. The initial test of the first generation xMarkers involved transformation of the PCR products into a strain (Y1625) whose relevant genotype was GAL80^{+} GAL4 gal1Δ; this genotype conferred wild-type inducible expression from P_{GAL1}, but inability to eat galactose so that this sugar was a "gratuitous inducer." Desired tranformants had the mutant genotype ndt80Δ::xURA3, with several versions of the xURA3 marker as listed in Table 2. Colonies that arose on the CSM-U plates were tested for the presence of the desired chromosomal locus by PCR amplification of genomic DNA from cells lysed by boiling ("colony PCR") using primer pairs that amplified across the novel DNA junctions created by recombination. Amplification of the new 5' junction with primers RYSE3 and AET0065-186-63-F-pNDT80 gave a product of

1024 bp; amplification of the new 3' junction with primers RYSE4 and AET0072-186-63-R-tNDT80 gave a product of 1024 bp. Two isolates for each xMarker variant were chosen for further analysis.

~~[00148]~~ [0148] Third, isolates with the genotype ndt80Δ::xMarker were transformed with an I-SceI expression plasmid. For the first generation ndt80Δ::xURA3 strains, the natA-marked plasmid (pAM1594) was transformed using standard transformation conditions, the cells were allowed time to express the new marker gene during outgrowth in liquid YPD for 3-6 hrs, then plated on YPD+nourseothricin plates. Half of each transformation was plated on regular YPD (2% dextrose) and half was plated on YPDG (1% dextrose + 1% galactose). Since the host strain genotype was GAL80^{+} GAL4 gal1Δ, the GAL1 promoter of the I-SceI gene was not induced on dextrose; on the mixed dextrose and galactose, the GAL1 promoter was expected to remain off while dextrose was high, but then to turn on as the dextrose was consumed and the galactose in the plates took effect.

~~[00149]~~ [0149]Fourth, isolates that had excised the marker were identified. The xURA3 markers have the advantage of counterselection on 5-FOA plates, which permit growth only of cells lacking URA3. In contrast, isolates that have excised the other xMarkers must be identified by screening, e.g., replica plating from non-selective plates on which all isolates grow to selective plates on which the desired isolates fail to grow. The first generation xMarkers used URA3 to take advantage of counterselection. In accordance with expectation, when the plasmid-harboring transformants described above were streaked from the YPD plate onto 5-FOA, no colonies grew because I-SceI was not expressed on YPD. When plasmid-harboring transformants were streaked from the YPDG plate onto 5-FOA, many colonies grew. The YPDG plate includes galactose and induces exptession of I-SceI. The 5-FOA plate selectes against cells harboring intact URA3. Based on the number of 5-FOA colonies growing from restreaks of the I-SceI transformants relative to a control ura3Δ strain, it is estimated that ≥30% of the cells had lost URA3 from between dual I-SceI sites; the frequency appeared lower for the xURA3 with a single I-SceI site. This suggested that without I-SceI expression the URA3 xMarker was rarely excised ($<10^{-6}$ of cells) and after I-SceI expression was induced the URA3 was frequently excised. Excision frequency was subsequently tested more directly.

~~[00150]~~ [0150] Fifth, the chromosomal locus where the xMarker had been integrated was sequenced to assess the scar remaining after I-SceI cleavage and subsequent DNA double-strand break repair. Colonies demonstrated to have lost marker function were used for colony PCR

with a primer pair that annealed upstream and downstream of the junction between the xMarker and the GOI targeting sequences, and then the PCR product was sequenced. In the case of ndt80Δ::xMarkers, the primers annealed 208 bp upstream (JU-197-168-125-NDT80US-F) and 234 bp downstream (JU-198-168-125-NDT80DS-R) of the junctions between the xMarker and the NDT80 sequences. The expected sizes of PCR products was 462-522 bp if URA3 was cleanly excised and 2072 bp if URA3 was completely intact. All 27 of the 5-FOA resistant colonies used for colony PCR gave a ~500 bp product expected for excision. Sequencing of these 27 PCR products showed in all cases the expected perfect scar depicted in Figure 1 (see Table 11). As provided in Table 11, all 27 colonies tested showed xMarker integration followed by I-SceI expression and xMarker excision.

Table 11: Results showing fidelity of excision by sequencing of 1st generation I-SceI xMarker scars after marker excision

name of URA3 xMarker	length of DR (bp)	I-SceI sites (#)	scars sent for sequencing (#)	perfect scars (%)
20mer-direct	20	2	8	100
40mer-direct	40	2	4	100
60mer-direct (aka s0x-URA3)	60	2	4	100
80mer-direct	80	2	8	100
60mer-single	60	1	3	100

~~[00151]~~ [0151]The first and second generation xMarkers (Tables 2 and 4) were also tested in different *S. cerevisiae* strains in which the GAL1 promoter was constitutive (gal80Δ $GAL4_{OC}$). The ndt80Δ::xMarker constructs were stitched together and transformed into yeast as above. Transformation mixtures with xURA3 were directly plated onto CSM-U plates; those with drug resistance markers were first grown for 3-6 hrs in liquid YPD in a shaking incubator before plating on YPD+drug plates. Two transformants of each xMarker variant that were verified by colony PCR to have the ndt80Δ::xMarker integration were independently transformed with one of the I-SceI expression plasmids and plated on selective plates as described above.

~~[00152]~~ [0152]Since P_{GAL1}-I-SceI expression was constitutive, the colonies on the plasmid transformation selective plates were expected to contain a mixed population of cells that maintained or excised the xMarker. Several colonies from each plate were restreaked on non-selective YPD plates that permitted growth of cells that had lost the plasmid or the marker or both. Colonies from these restreaks were patched on YPD plates, grown overnight, then replica plated to YPD+drug, CSM-uracil, and 5-FOA plates. Many patches grew on both 5-FOA and CSM-uracil, revealing a mixed population of cells in the patch and suggesting that further outgrowth under non-selective conditions prior to isolation of colonies was required to obtain a homogeneous population for analysis. Although the frequency of marker excision was generally underestimated in this experiment due to the mixed patches, generally at least 18% and more often 35-50% of the patches had at least some cells that had excised the marker (Table 12). Most of the patches (53-88%) had completely lost the I-SceI expression plasmid before replica plating. In parallel with the patching and replica plating, 90 colonies on the YPD restreak plates from two independent ndt80Δ::s1x-hphA strains were tested for marker excision by colony PCR; 48% (43/90) of the colonies yielded a band of the size expected for a precise excision of the hphA marker.

Table 12: Results showing frequency of excision of 2nd generation I-SceI xMarkers and frequency of loss of the endonuclease expression plasmid

		% of patches with at least some cells with			
xMarker	**plasmid marker**	**intact xMarker**	**excised xMarker**	**retained plasmid**	**lost plasmid**
s1x-hphA	URA3	65	n/a	28	100
s5x-natA	URA3	98	n/a	48	100
s7x-URA3	hphA	58	46	13	n/a
s8x-URA3	hphA	79	40	19	n/a
20mer-URA3	hphA	92	19	15	n/a
60mer-URA3 (aka s0x-URA3)	hphA	35	75	15	n/a

80mer-direct	hphA	48	58	33	n/a

~~[00109]~~ [0153] Additional xMarkers have been constructed for investigations using experiments similar to those described above. In addition, tests of simultaneous excision of multiple xMarkers integrated at different loci in the same strain have been successful and have shown no signs of genomic instability (*e.g.*, chromosomal translocations) (*see* Example 6.2.4 below).

6.2.2. Testing of xMarkers using other endonucleases:

~~[00154]~~ [0154] Four additional endonucleases were tested using a set of xURA3 markers shown in Table 6. Each xMarker contained the URA3 selectable marker gene flanked by two endonuclease cleavage sites, which in turn were flanked by a directly repeated sequence of 50 base pairs. For each, a knock-out construct with the structure GOI US/ xMarker/ GOI DS (GOI=gene of interest, US=upstream, DS=downstream) was stitched together using overlap extension PCR with three elements. The new-endonuclease URA3 xMarkers were initially tested with HXT3 as the gene of interest (GOI). Each PCR reaction used as templates three RaBits liberated from their plasmid backbones by digestion with SapI: (1) HXT3 upstream (01-0-U-407), (2) an xMarker RaBit (x0.URA.VDE, x0.URA.F-CphI, 12-0-x0.URA.PI-MtuII, 12-0-x0.URA.PI-MgaI), and (3) HXT3 DS (29-0-D-408). The primers used for PCR stitching were RYSE0 and RYSE19. After PCR, the reaction mixture was loaded on an agarose gel and the desired full-length product was purified from the gel. Yeast cells were transformed with gel-purified PCR products and plated on selective plates for URA3(CSM-Uracil). Desired transformants had the mutant genotype hxt3Δ::xURA3, with several versions of the xURA3 marker as listed in Table 6. Colonies that arose on the CSM-Uracil plates were tested for the presence of the desired chromosomal locus by PCR amplification of genomic DNA from cells lysed by boiling ("colony PCR") using primer pairs that amplified across the novel DNA junctions created by recombination. The primers were KB502, KB503, and CPK904; the latter two produced a 738 bp fragment for hxt3Δ::xURA3 and the former two produced a 538 bp fragment for intact HXT3. Two isolates for each xMarker variant were chosen for further analysis.

~~[00155]~~ [0155] The xURA3 markers have the advantage of counterselection on 5-FOA plates, which permit growth only of cells lacking URA3. In contrast, isolates that have excised other xMarkers must be identified by screening, *e.g.*, replica plating from non-selective plates on

which all isolates grow to selective plates on which the desired isolates fail to grow. The first generation xMarkers used URA3 to take advantage of counterselection, which permitted quantification of rare excision events. In accordance with expectation, when the strains containing xURA3 markers and lacking the endonuclease expression plasmids were plated or streaked onto 5-FOA, no colonies grew. This indicated that spontaneous excision of the xMarker was very rare in the absence of endonuclease-catalyzed cleavage. This suggested that without endonuclease expression the URA3 xMarker was rarely excised ($<10^{-6}$ of cells).

~~[00156]~~ [0156]Isolates with the confirmed genotype hxt3Δ::xURA3 were transformed with the cognate expression plasmids for endonucleases that were marked with the marker gene hphA (pAM1799, pAM1865, pAM1866). Cells from transformation mixtures were allowed time to express the new marker gene (hphA) during outgrowth in liquid YPD for 3-6 hrs, then plated on YPD+hygromycinB plates. Since the host strain genotype was *gal80Δ GAL4oc*, the *GAL1* promoter driving endonuclease gene expression was constitutively expressed and did not need an inducer. After three days growth, the transformant colonies were restreaked on YPD+hygromycinB plates and grown another three days. Colonies from the restreaks (four colonies per endonuclease) were resuspended in 3 ml of YPD and grown overnight in nonselective conditions to permit loss of the plasmids. Cell density was determined, cultures were diluted, and cells were plated at an estimated density of 150, 15,000, or 150,000 cells per plate on three different solid media: YPD, YPD+hygromycinB, and 5-FOA. All cells were expected to form colonies on YPD, only cells that had maintained the endonuclease expression plasmid were expected to form colonies on hygromycin B, and only cells that had excised the xURA3 marker were expected for form colonies on 5-FOA.

~~[00157]~~ [0157]The results shown in Table 13 indicate that F-CphI mediated high efficiency xMarker excision, PI-MtuII mediated low efficiency xMarker excision, and PI-MgaI mediated undetectable levels of xMarker excision. The loss of the CEN.ARS endonuclease expression plasmids was a high frequency event, suggesting that it would be easy to isolate cells that had lost the endonuclease prior to another round of transformations with new xMarkers.

Table 13: Results comparing xMarker excision efficiency of different endonucleases and frequency of loss of expression plasmid after several generations of non-selective growth

Endonuclease	% of cells that lost xURA3 xMarker	% of cells that lost hphA-marked endonuclease expression plasmid

F-CphI	90.8%	31.9%
PI-MgaI	0.058%	76.5%
PI-MtuII	<0.0007%	ND
VDE	ND	ND

~~[00158]~~ [0158] To determine if the F-CphI mediated excision of the marker left behind a "perfect" scar, the 5-FOA resistant (functionally ura3$^-$) colonies were subjected to colony PCR using oligonucleotide primers that flanked the integration site of the xURA3 marker (oligonucleotides KB503 and KB604); the 533 bp PCR product was sent for DNA sequencing with oligonucleotide primer KB503. Of 16 colonies tested, all had a "perfect" scar in which the only DNA sequence remaining from the xMarker was a single copy of the 50 bp sequence.

6.2.3. Further tests of F-Cph xMarker excision frequency and fidelity

~~[00159]~~ [0159] Additional xMarkers with F-CphI cleavage sites and various selectable markers were created and tested (natA, kanA, hphA, zeoA). The excision frequencies and fidelities of excision of these markers were tested in haploid and diploid *S. cerevisiae* strains, both singly and in combinations. The frequency of excision was often 100% of the colonies tested and always >80%. The fidelity of excision was nearly 100%. Almost all of the excision events in many independent cultures gave the expected scar; excision left behind only one copy of the 50 bp unique sequence that was introduced as a direct repeat in the xMarker and the marker itself was absent (Table 14).

Table 14 - xMarker excision frequencies for different endonucleases and DRs of at least 50 bp length

Endo-nuclease	Length of DR (bp)	Num. of ES's	xMarker Targeting Construct	Endo-nuclease Expression Plasmid	xMarker Excision			Excision Precision	
					Num. of Cells Tested [a]	Num. of Cells with Excised *xMarker*	Excision Frequency	Num. of Cells Tested	Num. of Cells with Perfect Scar [b]
F-CphI	50	2	HXT3-US_ xM0.URA.F-CphI _ HXT3-DS	pAM1799	402	365	0.91	ND	ND
	50	2	GAL80-US_xM0.URA.F-	pAM1800	8	8	1	8	8

			CphI_GAL80-DS						
	50	2	GAL80-US_xM1.nat.F-CphI_GAL80-DS	pAM1800	16	16	1	8	8
	50	2	GAL80-US_xM3.kan.F-CphI_GAL80-DS	pAM1800	8	8	1	8	8
	50	2	GAL80-US_xM4.nat.F-CphI_GAL80-DS	pAM1800	8	8	1	8	8
	50	2	GAL80-US_xM6.zeo.F-CphI_GAL80-DS	pAM1862/ pAM1864	16	16	1	16	16
PI-MgaI	50	2	HXT3-US_ xM0.URA.PI-MgaI _ HXT3-DS	pAM1866	~6.6 x 10^5	0	<1.5 x 10^{-6}	ND	ND
PI-MtuII	50	2	HXT3-US_ xM0.URA.PI-MtuII _ HXT3-DS	pAM1865	~6.6 x 10^5	419	6.3 x 10^{-4}	ND	ND
VDE	50	2	HXT3-US_ xM0.URA.VDE _ HXT3-DS	pAM1677	535	9	1.7 x 10^{-2}	ND	ND
I-SceI	60	2	NDT80-US_ s60M.URA.I-SceI_NDT80-DS	pAM1595	288	155	0.54	4	4
	80	2	NDT80-US_ s80M.URA.I-SceI_NDT80-DS	pAM1595	237	76	0.32	8	8

[)] Number of colonies on YPD plates, adjusted by dilution factor.

[)] Perfect scar = after excision only DNA sequence remaining from the xMarker is a single copy of the DR sequence.

ND = not determined.

~~[00160]~~ [0160] Strains with xMarkers at the NDT80 locus (deleting the NDT80 gene) were made as follows. The stitched PCR product used to transform cells was made by stitching the xMarker (as a 12 RaBit) with the 01-0-U-97 and 29-0-U-23 RaBits with the oligonucleotides called RYSE0 and RYSE19. After transformation, the identity of the correct isolates was verified by colony PCR using a pair of oligonucleotide primers in which one was outside of the transformed DNA (CPK650) and the other was inside the marker (e.g., KB561 and KB562 for URA3; KB563 and KB564 for natA, kanA, and hygA), which gave a PCR product of ~1.1 kb. The removal of native NDT80 sequence was verified by the absence of a PCR product from colony PCR with oligonucleotides AET83 and AET84, which gave a PCR product of 442 bp from the parental strain with an intact NDT80 locus. After transformation with a F-CphI expression plasmid (pAM1800), individual colonies were tested for the intended excision by colony PCR with JU197 and JU198, which gave a band of 492 bp for a perfect excision that left

behind only one copy of the 50 bp scar sequence; this band was run on a gel to visualize and separate from any other DNA, then extracted from the gel and sent for DNA sequencing using the same oligonucleotides used to prime the PCR reaction.

~~[00161]~~ [0161]Strains with xMarkers at the GAL80 locus (deleting the GAL80 gene) were made as follows. The stitched PCR product used to transform cells was made by stitching the xMarker (as a 12 RaBit) with the 01-0-U-270 and 29-0-U-95 RaBits with the oligonucleotides called RYSE0 and RYSE19. After transformation, the identity of the correct isolates was verified by colony PCR. Oligonucleotides JU436 and RYSE3 amplified the vicinity around the 5' junction where the transformed DNA integrated within the GAL80 upstream sequence to give a PCR product of 572 bp (alternatively, JU210 and RYSE3 were used to give a PCR product of 182 bp); JU221 and RYSE4 gave a 386 bp product from amplification of the 3' junction of the marker with the GAL80 downstream sequence bp (alternatively, JU439 and RYSE4 were used to give a PCR product of 531 bp); a negative control was colony PCR with primers JU212 and JU210, which gave a 290 bp product for an intact GAL80 locus and no product for the desired locus. After transformation with a F-CphI expression plasmid (pAM1800 or pAM1799), individual colonies were tested for the intended excision by colony PCR with JU210 and JU211, which gave a band of 277 bp for a perfect excision that left behind only one copy of the 50 bp scar sequence; this band was run on a gel to visualize and separate from any other DNA, then extracted and sequenced using the same oligonucleotides used to prime the PCR reaction.

~~[00162]~~ [0162]After transformation with an expression plasmid for F-CphI, eight of the colonies that arose on selective plates were randomly selected for colony PCR to diagnose excision. For the following xMarkers all eight colonies gave the expected PCR product and the DNA sequencing verified perfect scars: x0URA3, x1nat, x3kan, x4nat, and x6zeo. The x2hph marker gave anomalous results; sequencing of the scars and resequencing xMarker (the 12 RaBit) revealed that the direct repeats were not as intended, and instead only 19 bp were directly repeated flanking the marker with a scrambling of the adjacent sequences. Despite the small region provided for repair of the double-stranded break the excision frequency and fidelity was good; in the first trial, six out of eight colonies had clearly excised the marker and left behind scars of variable lengths; in the second trial, eight of eight colonies had excised the marker and left behind scars of 19 bp. This suggests that 17-18 bp would be sufficient for the direct repeat length, and 50 bp is more than enough to guide repair of the chromosome after cleavage by F-CphI.

[00163] [0163]High frequency and precision of excision of the xMarkers was observed in several circumstances. The simplest circumstance was a single xMarker in each strain. A more complicated circumstance was excision of an xMarker in a heterozygous diploid strain that had one intact GAL80 allele and one allele that had been deleted by an xMarker; in this case, the intact GAL80 locus remained intact after the excision of the xMarker at the disrupted allele. This is important, because it might have happened that the cleaved chromosome ends near the xMarker, after action of the F-CphI, could have used the intact second copy of the chromosome as a template for repair, thereby leading to a gene conversion event that would restore the intact GAL80 locus to the chromosome from which it had been deleted. This gene conversion event was not seen, and instead the cell apparently preferentially repaired the broken chromosome by an intrachromosomal single-stranded annealing mechanism.

6.2.4. Simultaneous multiple xMarker excision

[00164] [0164]This example demonstrates the utility of the xMarker constructs in mediating simultaneous excision of multiple selectable markers from the chromosomal DNA of a host cell, where each xMarker was used to mark an integration into a unique locus.

[00165] [0165]Strains with xMarkers at both the GAL80 and GAL4 loci were made as follows. Hygromycin sensitive cells were transformed with the xMarker Targeting construct GAL80-US_xM4.Hph.FCphI_GAL80-DS. Transformation was outgrown in 3mL liquid YPD medium for 5 hrs and then plated on YPD plus hygromycin. Transformants were verified by colony PCR. Oligonucleotides HJ53 and HJ848 amplified the vicinity around the 5' junction where the transformed DNA integrated within the GAL80 upstream sequence to give a PCR product of 761 bp (alternatively, HJ53 and HJ253 were used to give a PCR product of 1 kb); H727 and HJ258 gave a 1033 bp product from amplification of the 3' junction of the marker with the GAL80 downstream sequence bp (alternatively, HJ727 and HJ54 were used to give a PCR product of 627 bp). A chosen isolate was then transformed with the xMarker Targeting construct GAL4-US_xM0.Kan.FCphI_GAL4-DS. Transformation was outgrown in 3mL liquid YPD medium for 5 hrs and then plated on YPD plus G418. Transformants were verified by colony PCR. Oligonucleotides HJ270 and HJ253 amplified the vicinity around the 5' junction where the transformed DNA integrated within the GAL4 upstream sequence to give a PCR product of 1.1 kb (alternatively, HJ270 and HJ54 were used to give a PCR product of 774 bp); H239 and HJ706 gave a 793 bp product from amplification of the 3' junction of the marker with the GAL4 downstream sequence bp (alternatively, HJ239 and HJ241 were used to give a PCR product of 1038 bp).

[00166] [0166] Isolates with the genotype gal80Δ::xHph gal4Δ::xKan were transformed with a natA-marked F-CphI expression plasmid (pAM1864). Transformation was outgrown in 3mL liquid YPD medium for 5 hrs and then plated on YPD plus nourseothricin. After 3 days of incubation at 30 °C, 4 transformants were streaked on fresh YPD to lose pAM1864. To test xMarker excision and loss of pAM1864, 50 colonies (about 12-13 colonies from each streak) were patched on a YPD plate and then replica plated on YPD, YPD plus hygromycin, G418, or nourseothricin 24 hrs later. Plates were examined 48 hrs after replica plating.

[00167] [0167] The frequency of simultaneous multiple integratrion was 100%, that is, 50/50 colonies lost resistance to both hygromycin and G418, indicating that all the colonies tested had lost both xMarkers. About 20% (10/50) colonies also lost resistance to nourseothricin, indicating that 20% of the colonies lost the F-CphI plasmid. Similar frequencies were observed for two additional strains in which two xMarker constructs were genomically integrated. In a second gal80Δ::xHph gal4Δ::xKan strain in which F-CphI was expressed, 50/50 colonies lost resistance to both hygromycin and G418, and 10/50 colonies also lost resistance to nourseothricin. In a third gal80Δ::xHph gal4Δ::xKan strain in which F-CphI was expressed, 48/48 colonies lost resistance to both hygromycin and G418, and 5/50 colonies also lost resistance to nourseothricin. Colonies that lost resistance to all three drugs were also colony PCR verified. As expected, all of them had both xMarkers excised by F-CphI.

[00168] [0168] This example demonstrates a high frequency of perfect excision of two markers from a single haploid strain. Further, this example demonstrates that there was no evidence for chromosomal translocation or genomic instability. These results indicate that xMarker constructs facilitate highly efficient simultaneous multiple excision of target DNA from a host cell genome.

6.2.5. Genomic Stability of xMarker constructs comprising DRs of varying length

[00169] [0169] This example demonstrates the stability of xMarker constructs comprising direct repeats (DRs) of varying lengths when integrated in a yeast genome, in the absence of endonuclease expression.

[00170] [0170] It is advantageous that a conditional gene deletion system be tightly controlled so that no significant excision occurs prior to induction and under non-inducing conditions. In the context of genome engineering, high rates of spontaneous homologous recombination events can lead to genomic instability, undesired loss of selectable markers, and consequently, a loss in the ability to select for desired genotypes.

~~[00171]~~ [0171] Native, endogenous, spontaneous homologous recombination events can catalyze a rare recombinational crossover between directly repeated sequences (generally, >300 bp) that deletes the intervening DNA. Published reports have shown that a sequence can be excised from a yeast host cell genome via spontaneous recombination crossover between DRs flanking the sequence ("loop-out"). The rate at which such loop-out occurs between DRs of 300 bp to 1.1 kb length have been reported to lie between 1 in 1,000 cells (1×10^{-3}) to 1 in 10,000 cells (1×10^{-4}) (Alani *et al.* (1987) Genetics 116(4):541-5 (10^{-4} for direct repeat lengths of 1100 bp); Wach *et al.* (1994) Yeast 10(13):1793-808 (10^{-3} to 10^{-4} for direct repeat length of 430 bp); Erdeniz *et al.* (1997) Genome Res 7(12):1174-83) (10^{-3} to 10^{-4} for direct repeat lengths of ~300 to 3000 bp). To determine the genomic stability of integrated xMarker constructs having varying lengths of direct repeats, the rates of spontaneous recombination crossover between DRs of various lengths were assessed in the absence of endonuclease expression.

~~[00172]~~ [0172] Strains with xMarkers integrated at the NDT80 locus (deleting the NDT80 gene), having direct repeat lengths of 50, 60, 80 and 198 bp, were made as follows. The integration vectors were made by stitching the following URA3 xMarker variants with the 01-0-U-30 and 29-0-D-23 RaBits, using the RYSE0 and RYSE19 oligonucleotides as primers:

Table 15: List of characteristics of individual elements of xMarkers used to assess the effect of tandem repeat length on genomic stability

name of URA3 xMarker	length of DR (bp)	ES sites (#)	orientation of ES sites to each other	DR (scar) sequence
50mer-direct (aka xM0_URA3)	50	F-CphI (2)	direct	AAGATCCGATCGACCGAGAACTGAG AACGGTGCAATGATCAACATGATCT (SEQ ID NO:23)
60mer-direct (aka s0x-URA3)	60	I-SceI (2)	direct	AAGATCCGATCGACCGAGAACTGAG AACGGTGCAATGATCAACATGATCTG CGACGAGCT (SEQ ID NO:10)

80mer-direct	80	I-SceI (2)	direct	AAGATCCGATCGACCGAGAACTGAG AACGGTGCAATGATCAACATGATCTG CGACGAGCTTGAGGATGCAAATGGCT GAC (SEQ ID NO:85)
198mer-direct (aka Rabit 606)	198	0	direct	TCACTATTATTCCATAAGATGATCATT AGCATTACGTTCAAAACGAGTACAAA TAACTTAAGTAATAACACGAGCCATA TGACCATTAGCAAGATGACAAGCAAG TTAAGACCAATCAGCTTCCATCATAG CATCAGCTTAACGTTCACCATTAATA AGAGTAGAAATTTCACCTTCAAGACA ATAACGATTTTCGTG (SEQ ID NO:145)

~~[00173]~~ [0173] After transformation into a ura3- strain, cells were plated on ura3- plates, and colonies were verified for correct integration by colony PCR. Oligonucleotides AET0065-186-63-F-pNDT80 and RYSE3 amplified the vicinity around the 5' junction where the transformed DNA integrated within the NDT80 upstream sequence to give a PCR product of 1024 bp; AET0072-186-63-R-tNDT80 and RYSE4 gave a 1024 bp product from amplification of the 3' junction of the marker with the NDT80 downstream sequence. These results indicate integration of the xMarker and disruption of NDT80. Two confirmed isolates for each xMarker variant were chosen for further analysis.

~~[00174]~~ [0174] Colonies were restreaked on CSM lacking uracil, and then, to permit spontaneous excision of the URA3 marker between the DRs, the cells were grown in liquid YPD for 2 days, being diluted every 12-24 hours (resulting in approximately 25 doublings). Since both URA3+ and ura3- colonies grow well in this culture medium, there is no selection for gene excision. In addition, cells comprising native URA3, *i.e.,* URA3 not flanked by any direct repeats, were grown as a control for assessing the rate of spontaneous mutation, which can lead to point mutation(s) that reduce or inactivate the activity of URA3 (leading to survival of host cells on 5-FOA), without loop-out of the URA3 coding sequence.

~~[00175]~~ [0175] Cultures were finally plated on solid medium containing 5-FOA and grown for another 2.5 days before colonies were counted. 16 individual ura3- (5-FOA resistant) colonies for each genotype were confirmed by colony PCR for spontaneous loop-out of the URA3 gene,

or alternatively, for the presence of an intact URA3 gene. Primers were used that annealed 208 bp upstream (JU-197-168-125-NDT80US-F) and 234 bp downstream (JU-198-168-125-NDT80DS-R) of the junctions between the xMarker and the NDT80 sequences. The expected sizes of PCR products were 462-522 bp if URA3 was cleanly excised and 2072 bp if URA3 was intact.

~~[00176]~~ [0176] As shown in Table 16, the average frequency of 5-FOA resistance in cells comprising URA3 not flanked by direct repeats was approximately 1.7 x 10^{-6} cells, which indicates the intrinsic rate of spontaneous mutation leading to inactivation of URA3. The average frequency of 5-FOA resistance in cells comprising URA3 flanked by direct repeats of 50-80 bp length was approximately 4.5 x 10^{-6} to 1.14 x 10^{-5} cells, and the average frequency of 5-FOA resistance in cells comprising URA3 flanked by direct repeats of 198 bp length was approximately 4.7 x 10^{-5} cells.

~~[00177]~~ [0177] Colony PCR? (cPCR) results confirmed that some amount of the 5-FOA resistant xMarker colonies arose as a result of spontaneous mutation leading to inactivation of URA3, as opposed to loop-out of the URA3 marker. In particular, for colonies comprising integrated xMarker constructs having 50 bp and 80 bp DR lengths, four out of twelve (4/12) 5-FOA resistant colonies screened by cPCR had intact URA3 genes, indicating that 5-FOA resistance in these colonies arose from inactivating spontaneous mutations in URA3 rather than URA3 loop-out. Thus, the frequency of DR-mediated spontaneous loop-out in the xMarker strains is expected to be even lower than the observed frequency of 5-FOA resistance. Even with the understanding of this limitation, using 5-FOA resistance as a proxy, these results indicate that the frequency of spontaneous loop-out associated with DR lengths of about 50 to 200 bp is 1 to 3 orders of magnitude lower than the rates of spontaneous loop-out reported for DRs of 300 bp to 1.1 kb (10^{-6} to 10^{-5} versus 10^{-4} to 10^{-3}, as discussed above).

~~[00178]~~ [0178] These results demonstrate that a DR length of about 200 bp provides a 1-2 log reduction in the number of spontaneous loop-out events compared to a DR length of at least 300 bp. In particular, the frequency of spontaneous loop-out events for a DR length of 198 was 1.7 x 10^{-6} cells compated to the frequency of approximately 4.7 x 10^{-5} cells for no direct repeats.

~~[00179]~~ [0179] The results for shorter DR lengths are even more dramatic. As described above, the results demonstrate that a DR length in the range of 50 to 80 bp provides a 2-3 log reduction in the number of spontaneous loop-out events compared to a DR length of at least 300 bp (10^{-6} to 10^{-5} versus 10^{-4} to 10^{-3}).

~~[00180]~~ [0180] Furthermore, these results indicate that the rates of spontaneous URA3 loop-out approach the rate of spontaneous mutation of URA3 as the lengths of the DRs decrease below 200. DR lengths of 50, 60 and 80 resulted in only a roughly 2.7 to 7-fold increase in loss of URA3 due to loop-out compared to spontaneous inactivating mutation.

~~[00181]~~ [0181]These results suggest that DR lengths of about 200 bp or shorter provide substantial advantages with respect to genomic stability when compared to DR lengths of 300 bp or higher. The excision of target DNA is controlled tightly and no significant excision prior to induction with a homing endonuclease. Accordingly, the xMarker methods and compositions described herein provide improved retention of target DNA, *e.g.,* selectable markers, under non-inducing conditions, which can result in greater genomic stability and more efficient selection of desired genotypes during strain engineering.

Table 16 – Spontaneous recombination events mediated by direct repeats (DRs) of various lengths

DR length	isolate	avg. # of colonies	Std. dev.	# of cells plated	frequency of 5-FOA resistance	avg. 5-FOA resistance	# of colonies tested by cPCR	# of colonies lacking URA3 gene	# of colonies with URA3 gene
0	a	32.17	16.69	2.68E+07	1.20E-06		16	0	16
0	b	57.67	34.14	2.72E+07	2.12E-06	1.66E-06			
50	a	240.83	157.67	2.76E+07	8.74E-06		16	12	4
50	b	164.67	68.95	2.74E+07	6.02E-06	7.38E-06			
60	a	260.83	142.14	2.74E+07	9.52E-06		16	15	1
60	b	361.00	214.56	2.72E+07	1.33E-05	1.14E-05			
80	a	113.83	53.15	2.58E+07	4.41E-06		16	12	4
80	b	116.33	22.91	2.49E+07	4.67E-06	4.54E-06			
198	a	1212.00	343.09	2.52E+07	4.81E-05		16	16	0
198	b	1118.67	132.32	2.44E+07	4.58E-05	4.69E-05			

~~[00182]~~ [0182] These results demonstrate that the compositions and methods described herein, for creating and excising specialized variants of selectable markers, work with high frequency,

efficiency, fidelity and stability. The I-SceI and F-CphI endonucleases work well for this approach, with F-CphI showing unexpectedly exceptional excision frequency and fidelity in *S. cerevisiae* cells. The excision events themselves do not cause genomic instability even in diploid cells and even in cells with more than one xMarker. Major advantages of this approach include the ability to simultaneously excise many xMarkers at once and the ability to choose a large variety of unique scar sequences, such that even repeated use and recycling of xMarkers in the same strain can be designed such that each scar is unique. This is an advantage over Flp/FRT or Cre/lox systems that necessarily leave behind multiple copies of the binding and cleavage site for the site-specific recombinase, littered throughout the genome, waiting to be cleaved again upon re-introduction of the recombinase, which potentially causes translocations and excisions of chromosome segments. Another advantage is that the excision frequency is often higher than 50%, and thus the approach can be used to excise any desired target DNA, using a screening approach to identify the desired product strains, even when there is no selection method available to permit growth only of the successfully excised product isolates.

~~[00183]~~ [0183]~~All publications, patents and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.~~ Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED:

1. An excisable nucleic acid construct comprising, in a 5' to 3' orientation:

(a) a first tandem repeat nucleic acid;

(b) a first F-CphI endonuclease recognition site;

(c) a target nucleic acid;

(d) a second F-CphI endonuclease recognition site; and

(e) a second tandem repeat nucleic acid.

2. The excisable nucleic acid construct of claim 1, wherein each of the first and second tandem repeat nucleic acids independently consists of 18 to 200 nucleotide base pairs.

3. The excisable nucleic acid construct of claim 1, wherein each of the first and second tandem repeat nucleic acids independently consists of 18 to 150 nucleotide base pairs.

4. The excisable nucleic acid construct of claim 1, wherein each of the first and second tandem repeat nucleic acids independently consists of 18 to 100 nucleotide base pairs.

5. The excisable nucleic acid construct of claim 1, wherein each of the first and second tandem repeat nucleic acids independently comprises 18-80 nucleotide base pairs.

6. The excisable nucleic acid construct of any one of claims 1 to 5, wherein the target nucleic acid encodes a selectable marker.

7. The excisable nucleic acid construct of claim 6, wherein the selectable marker is selected from the group consisting of: URA3, hygromycin B phosphotransferase, aminoglycoside phosphotransferase, zeocin resistance gene and phosphinothricin N-acetyltransferase.

8. The excisable nucleic acid construct of any one of claims 1 to 7, further comprising a first genomic integration site linked 5' of the first tandem repeat nucleic acid, and a second integration site linked 3' of the second tandem repeat nucleic acid.

9. The excisable nucleic acid construct of any one of claims 1 to 8, wherein the target nucleic acid comprises a promoter element operably linked to a nucleic acid encoding F-CphI endonuclease.

10. A host cell comprising the excisable nucleic acid construct of any one of claims 1 to 7.

11. The host cell of claim 10, further comprising a vector comprising a nucleic acid encoding F-CphI endonuclease.

12. The host cell of claim 11, wherein the vector comprises a promoter element that controls the expression of the nucleic acid encoding F-CphI endonuclease.

13. The host cell of claim 12, wherein the promoter element is an inducible promoter.

14. The host cell of of any one of claims 1 to 13 that is a yeast cell.

15. The host cell of claim 14 that is a haploid yeast cell.

16. The host cell of claim 14 that is a diploid yeast cell.

17. The host cell of claim 14 that is a *Saccharomyces cerevisiae* cell.

18. The host cell of any one of claims 1 to 17, wherein the excisable nucleic acid construct is integrated into the host cell genome.

19. A method of excising at least one target nucleic acid from the genome of a host cell comprising the excisable nucleic acid construct of claim 1, wherein the method comprises: contacting the excisable nucleic acid construct with F-CphI in the host cell.

20. The method of claim 19, wherein said excising operably links a promoter element to a gene of interest.

21. The method of claim 19, wherein the target nucleic acid encodes a selectable marker.

22. The method of claim 21, wherein the selectable marker is selected from the group consisting of: URA3, hygromycin B phosphotransferase, aminoglycoside phosphotransferase, zeocin resistance gene and phosphinothricin N-acetyltransferase.

23. The method of any one of claims 1 to 22, wherein the host cell is a yeast cell.

24. The method of claim 23, wherein the host cell is a haploid yeast cell.

25. The method of claim 23, wherein the host cell is a diploid yeast cell.

26. The method of claim 23, wherein the host cell is a *Saccharomyces cerevisiae* cell.

27. An excisable nucleic acid construct comprising, in a 5' to 3' orientation:

(a) a first genomic integration sequence;

(b) a first tandem repeat nucleic acid consisting of 18 to 200 nucleotide base pairs;

(c) a first homing endonuclease recognition site;

(d) a target nucleic acid;

(e) a second homing endonuclease recognition site;

(f) a second tandem repeat nucleic acid consisting of 18 to 200 nucleotide base pairs; and

(g) a second genomic integration sequence,

wherein each of the first and second genomic integration sequences is of sufficient length and identity to initiate homologous recombination with a yeast genomic locus.

28. The excisable nucleic acid construct of claim 27, wherein each of the first and second tandem repeat nucleic acids independently consists of 18 to 150 nucleotide base pairs.

29. The excisable nucleic acid construct of claim 27, wherein each of the first and second tandem repeat nucleic acids independently consists of 18 to 100 nucleotide base pairs.

30. The excisable nucleic acid construct of claim 27, wherein each of the first and second homing endonuclease recognition sites independently consists of 18 to 80 nucleotide base pairs.

31. The excisable nucleic acid construct of any one of claims 27 to 30, wherein at least one of the first or second homing endonuclease recognition sites is a recognition site for a homing endonuclease selected from the group consisting of: an LAGLIDADG homing endonuclease, an HNH homing endonuclease, a His-Cys box homing endonuclease, a GIY-YIG homing endonuclease, and a cyanobacterial homing endonuclease.

32. The excisable nucleic acid construct of any one of claims 27 to 30, wherein each of the first and second homing endonuclease recognition sites is independently a recognition site for a homing endonuclease selected from the group consisting of: an LAGLIDADG homing endonuclease, a HNH homing endonuclease, an His-Cys box homing endonuclease, a GIY-YIG homing endonuclease, and a cyanobacterial homing endonuclease.

33. The excisable nucleic acid construct of any one of claims 27 to 30, wherein at least one of the first or second homing endonuclease recognition sites is a recognition site for a homing endonuclease selected from the group consisting of: I-CreI , I-MsoI, I-SceI, I-SceIV, H-

DreI, I-HmuI, I-PpoI, I-DirI, I-NjaI, I-NanI, I-NitI, I-TevI, I-TevII, I-TevIII, F-TevI, F-TevII, F-CphI, PI-MgaI, I-CsmI, I-CeuI, and PI-SceI.

34. The excisable nucleic acid construct of any one of claims 27 to 30, wherein each of the first and second homing endonuclease recognition sites is independently a recognition site for a homing endonuclease selected from the group consisting of: I-CreI , I-MsoI, I-SceI, I-SceIV, H-DreI, I-HmuI, I-PpoI, I-DirI, I-NjaI, I-NanI, I-NitI, I-TevI, I-TevII, I-TevIII, F-TevI, F-TevII, F-CphI, PI-MgaI, I-CsmI, I-CeuI, and PI-SceI.

35. The excisable nucleic acid construct of any one of claims 27 to 30, wherein at least one of the first or second homing endonuclease recognition sites is a recognition site for I-SceI.

36. The excisable nucleic acid construct of any one of claims 27 to 30, wherein at least one of the first or second homing endonuclease recognition sites is a recognition site for F-CphI.

37. The excisable nucleic acid construct of any one of claims 27 to 36, wherein the target nucleic acid encodes a selectable marker.

38. The excisable nucleic acid construct of claim 37, wherein the selectable marker is selected from the group consisting of: URA3, hygromycin B phosphotransferase, aminoglycoside phosphotransferase, zeocin resistance gene and phosphinothricin N-acetyltransferase.

39. The excisable nucleic acid construct of any one of claims 27 to 38, wherein each of the first and second genomic integration sequences independently consists of about 50 to 5000 nucleotide base pairs.

40. The excisable nucleic acid construct of any one of claims 27 to 38, wherein each of the first and second genomic integration sequences independently consists of about 500 nucleotide base pairs.

41. A yeast cell comprising the excisable nucleic acid construct of any one of claims 27 to 40.

42. The yeast cell of claim 41 that is a haploid yeast cell.

43. The yeast cell of claim 41 that is a diploid yeast cell.

44. The yeast cell of claim 41 that is a *Saccharomyces cerevisiae* cell.

45. A yeast cell comprising:

(a) an excisable nucleic acid construct comprising, in a 5' to 3' orientation:

(i) a first tandem repeat nucleic acid consisting of 18 to 200 nucleotide base pairs;

(ii) a first homing endonuclease recognition site;

(iii) a target nucleic acid;

(iv) a second homing endonuclease recognition site; and

(v) a second tandem repeat nucleic acid consisting of 18 to 200 nucleotide base pairs; and

(b) a vector comprising a homing endonuclease nucleic acid encoding a homing endonuclease capable of binding to and cleaving at or adjacent to at least one of the first or second homing endonuclease recognition sites.

46. The yeast cell of claim 45, wherein the homing endonuclease nucleic acid encodes a homing endonuclease capable of binding to and cleaving at or adjacent to each of the first and second homing endonuclease recognition sites.

47. The yeast cell of claim 45 or 46, wherein the homing endonuclease nucleic acid encodes a homing endonuclease selected from the group consisting of: an LAGLIDADG homing endonuclease, a HNH homing endonuclease, an His-Cys box homing endonuclease, a GIY-YIG homing endonuclease, and a cyanobacterial homing endonuclease.

48. The yeast cell of claim 45or 46, wherein the homing endonuclease nucleic acid encodes a homing endonuclease selected from the group consisting of: I-CreI , I-MsoI, I-SceI, I-SceIV, H-DreI, I-HmuI, I-PpoI, I-DirI, I-NjaI, I-NanI, I-NitI, I-TevI, I-TevII, I-TevIII, F-TevI, F-TevII, F-CphI, PI-MgaI, I-CsmI, I-CeuI, and PI-SceI.

49. The yeast cell of claim 45 or 46, wherein the homing endonuclease nucleic acid encodes I-SceI.

50. The yeast cell of claim 45 or 46, wherein the homing endonuclease nucleic acid encodes F-CphI.

51. The yeast cell of any one of claims 45 to 50, wherein the vector comprises a promoter element that controls the expression of the homing endonuclease nucleic acid.

52. The yeast cell of claim 51, wherein the promoter element is an inducible promoter.

53. The yeast cell of claim 51, wherein the promoter element is a constitutive promoter.

54. The yeast cell of any one of claims 45 to 53, wherein each of the first and second tandem repeat nucleic acids independently consists of 18 to 150 nucleotide base pairs.

55. The yeast cell of any one of claims 45 to 53, wherein each of the first and second tandem repeat nucleic acids independently consists of 18 to 100 nucleotide base pairs.

56. The yeast cell of any one of claims 45 to 53, wherein each of the first and second homing endonuclease recognition sites independently consists of 18 to 80 nucleotide base pairs.

57. The yeast cell of any one of claims 45 to 56 that is a *Saccharomyces cerevisiae* cell.

58. The yeast cell of any one of claims 45 to 57, wherein the excisable nucleic acid construct is integrated into the yeast cell genome.

59. A kit comprising:

(a) the excisable nucleic acid construct of claim 27; and

(b) a vector comprising a homing endonuclease nucleic acid encoding a homing endonuclease capable of binding to and cleaving at or adjacent to at least one of the first or second homing endonuclease recognition sites.

60. The kit of claim 59, wherein the homing endonuclease nucleic acid encodes a homing endonuclease capable of binding to and cleaving at or adjacent to each of the first and second homing endonuclease recognition sites.

61. The kit of claim 59 or 60, wherein the homing endonuclease nucleic acid encodes a homing endonuclease selected from the group consisting of: an LAGLIDADG homing endonuclease, a HNH homing endonuclease, an His-Cys box homing endonuclease, a GIY-YIG homing endonuclease, and a cyanobacterial homing endonuclease.

62. The kit of claim 59 or 60, wherein the homing endonuclease nucleic acid encodes a homing endonuclease selected from the group consisting of: I-CreI , I-MsoI, I-SceI, I-SceIV, H-DreI, I-HmuI, I-PpoI, I-DirI, I-NjaI, I-NanI, I-NitI, I-TevI, I-TevII, I-TevIII, F-TevI, F-TevII, F-CphI, PI-MgaI, I-CsmI, I-CeuI, and PI-SceI.

63. The kit of claim 59 or 60, wherein the homing endonuclease nucleic acid encodes I-SceI.

64. The kit of claim 59 or 60, wherein the homing endonuclease nucleic acid encodes F-CphI.

65. The kit of any one of claims 59 to 64, wherein the vector comprises a promoter element that controls the expression of the homing endonuclease nucleic acid.

66. The kit of claim 65, wherein the promoter element is an inducible promoter.

67. The kit of claim 65, wherein the promoter element is a constitutive promoter.

68. The kit of any one of claims 59 to 67, wherein each of the first and second tandem repeat nucleic acids independently consists of 18 to 150 nucleotide base pairs.

69. The kit of any one of claims 59 to 67, wherein each of the first and second tandem repeat nucleic acids independently consists of 18 to 100 nucleotide base pairs.

70. The kit of any one of claims 59 to 67, wherein each of the first and second homing endonuclease recognition sites independently consists of 18 to 80 nucleotide base pairs.

71. A method of excising at least one target nucleic acid from the genome of a yeast cell comprising an excisable nucleic acid construct, wherein the excisable nucleic acid construct comprises, in a 5' to 3' orientation:

(a) a first tandem repeat nucleic acid consisting of 18 to 200 nucleotide base pairs;

(b) a first homing endonuclease recognition site;

(c) a target nucleic acid;

(d) a second homing endonuclease recognition site; and

(e) a second tandem repeat nucleic acid consisting of 18 to 200 nucleotide base pairs,

wherein the method comprises contacting the excisable nucleic acid construct with a homing endonuclease in the yeast cell such that the homing endonuclease cleaves at or adjacent to at least one of the first or second homing endonuclease recognition sites.

72. The method of claim 71, wherein each of the first and second tandem repeat nucleic acids independently consists of 18 to150 nucleotide base pairs.

73. The method of claim 71, wherein each of the first and second tandem repeat nucleic acids independently consists of 18 to 100 nucleotide base pairs.

74. The method of claim 71, wherein each of the first and second homing endonuclease recognition sites independently consists of 18 to 80 nucleotide base pairs.

75. The method of any one of claims 71 to 74, wherein the homing endonuclease cleaves at or adjacent to each of the first and second homing endonuclease recognition sites.

76. The method of any one of claims 71 to 75, wherein at least one of the first or second homing endonuclease recognition sites is a recognition site for a homing endonuclease selected from the group consisting of: an LAGLIDADG homing endonuclease, an HNH homing endonuclease, a His-Cys box homing endonuclease, a GIY-YIG homing endonuclease, and a cyanobacterial homing endonuclease.

77. The method of any one of claims 71 to 75, wherein each of the first and second homing endonuclease recognition sites is independently a recognition site for a homing endonuclease selected from the group consisting of: an LAGLIDADG homing endonuclease, a HNH homing endonuclease, an His-Cys box homing endonuclease, a GIY-YIG homing endonuclease, and a cyanobacterial homing endonuclease.

78. The method of any one of claims 71 to 75, wherein at least one of the first or second homing endonuclease recognition sites is a recognition site for a homing endonuclease selected from the group consisting of: I-CreI , I-MsoI, I-SceI, I-SceIV, H-DreI, I-HmuI, I-PpoI, I-DirI, I-NjaI, I-NanI, I-NitI, I-TevI, I-TevII, I-TevIII, F-TevI, F-TevII, F-CphI, PI-MgaI, I-CsmI, I-CeuI, and PI-SceI.

79. The method of any one of claims 71 to 75, wherein each of the first and second homing endonuclease recognition sites is independently a recognition site for a homing endonuclease selected from the group consisting of: I-CreI , I-MsoI, I-SceI, I-SceIV, H-DreI, I-HmuI, I-PpoI, I-DirI, I-NjaI, I-NanI, I-NitI, I-TevI, I-TevII, I-TevIII, F-TevI, F-TevII, F-CphI, PI-MgaI, I-CsmI, I-CeuI, and PI-SceI.

80. The method of any one of claims 71 to 75, wherein at least one of the first or second homing endonuclease recognition sites is a recognition site for I-SceI.

81. The method of any one of claims 71 to 75, wherein at least one of the first or second homing endonuclease recognition sites is a recognition site for F-CphI.

82. The method of any one of claims 71 to 81, wherein the target nucleic acid encodes a selectable marker.

83. The method of claim 82, wherein the selectable marker is selected from the group consisting of: URA3, hygromycin B phosphotransferase, aminoglycoside phosphotransferase, zeocin resistance gene and phosphinothricin N-acetyltransferase.

84. The method of any one of claims 71 to 83, wherein the yeast cell is a haploid yeast cell.

85. The method of any one of claims 71 to 83, wherein the yeast cell is a diploid yeast cell.

86. The method of any one of claims 71 to 83, wherein the yeast cell is a *Saccharomyces cerevisiae* cell.

87. The method of any one of claims 71 to 86, wherein said excising the at least one target nucleic acid from the genome of the yeast cell results in the activation of expression of a gene of interest.

88. The method of claim 45, wherein said excising the at least one target nucleic acid from the genome of the yeast cell results in the suppression of expression of a gene of interest.

89. A method of simultaneously excising at least two target nucleic acids from the genome of a host cell comprising at least two excisable nucleic acid constructs, wherein each excisable nucleic acid construct independently comprises, in a 5' to 3' orientation:

(a) a first tandem repeat nucleic acid;

(b) a first homing endonuclease recognition site;

(c) a target nucleic acid;

(d) a second homing endonuclease recognition site; and

(e) a second tandem repeat nucleic acid;

wherein the method comprises: contacting the at least two excisable nucleic acid constructs with one or more homing endonucleases in the host cell such that at least one homing endonuclease cleaves at or adjacent to at least one of the first or second homing endonuclease recognition sites of each excisable nucleic acid construct.

90. The method of claim 89, wherein each of the first and second tandem repeat nucleic acids of each excisable nucleic acid construct independently consists of 18 to200 nucleotide base pairs.

91. The method of claim 89, wherein each of the first and second tandem repeat nucleic acids of each excisable nucleic acid construct independently consists of 18 to150 nucleotide base pairs.

92. The method of claim 89, wherein each of the first and second tandem repeat nucleic acids of each excisable nucleic acid construct independently consists of 18 to 100 nucleotide base pairs.

93. The method of claim 89, wherein each of the first and second homing endonuclease recognition sites of each excisable nucleic acid construct independently consists of 18 to 80 nucleotide base pairs.

94. The method of any one of claims 89 to 93, wherein the homing endonuclease cleaves at or adjacent to each of the first and second homing endonuclease recognition sites of each excisable nucleic acid construct.

95. The method of any one of claims 89 to 94, wherein at least one of the first or second homing endonuclease recognition sites of each excisable nucleic acid construct is a recognition site for a homing endonuclease selected from the group consisting of: an LAGLIDADG homing endonuclease, an HNH homing endonuclease, a His-Cys box homing endonuclease, a GIY-YIG homing endonuclease, and a cyanobacterial homing endonuclease.

96. The method of any one of claims 89 to 94, wherein each of the first and second homing endonuclease recognition sites of each excisable nucleic acid construct is independently a recognition site for a homing endonuclease selected from the group consisting of: an LAGLIDADG homing endonuclease, a HNH homing endonuclease, an His-Cys box homing endonuclease, a GIY-YIG homing endonuclease, and a cyanobacterial homing endonuclease.

97. The method of any one of claims 89 to 94, wherein at least one of the first or second homing endonuclease recognition sites of each excisable nucleic acid construct is a recognition site for a homing endonuclease selected from the group consisting of: I-CreI , I-MsoI, I-SceI, I-SceIV, H-DreI, I-HmuI, I-PpoI, I-DirI, I-NjaI, I-NanI, I-NitI, I-TevI, I-TevII, I-TevIII, F-TevI, F-TevII, F-CphI, PI-MgaI, I-CsmI, I-CeuI, and PI-SceI.

98. The method of any one of claims 89 to 94, wherein each of the first and second homing endonuclease recognition sites of each excisable nucleic acid construct is independently a recognition site for a homing endonuclease selected from the group consisting of: I-CreI , I-MsoI, I-SceI, I-SceIV, H-DreI, I-HmuI, I-PpoI, I-DirI, I-NjaI, I-NanI, I-NitI, I-TevI, I-TevII, I-TevIII, F-TevI, F-TevII, F-CphI, PI-MgaI, I-CsmI, I-CeuI, and PI-SceI.

99. The method of any one of claims 89 to 94, wherein at least one of the first or second homing endonuclease recognition sites of at least one excisable nucleic acid construct is a recognition site for I-SceI.

100. The method of any one of claims 89 to 94, wherein at least one of the first or second homing endonuclease recognition sites of at least one excisable nucleic acid construct is a recognition site for F-CphI.

101. The method of any one of claims 89 to 94, wherein at least one of the first or second homing endonuclease recognition sites of each excisable nucleic acid construct is a recognition site for I-SceI.

102. The method of any one of claims 89 to 94, wherein at least one of the first or second homing endonuclease recognition sites of each excisable nucleic acid construct is a recognition site for F-CphI.

103. The method of any one of claims 89 to 102, wherein the target nucleic acid of each excisable nucleic acid construct encodes a selectable marker.

104. The method of claim 103, wherein the selectable marker is selected from the group consisting of: URA3, hygromycin B phosphotransferase, aminoglycoside phosphotransferase, zeocin resistance gene and phosphinothricin N-acetyltransferase.

105. The method of any one of claims 89 to 104, wherein the host cell is a yeast cell.

106. The method of claim 105, wherein the yeast cell is a haploid yeast cell.

107. The method of claim 105, wherein the yeast cell is a diploid yeast cell.

108. The method of claim 105, wherein the yeast cell is a *Saccharomyces cerevisiae* cell.

WHAT IS CLAIMED:

1. An excisable nucleic acid construct comprising, in a 5' to 3' orientation:

(a) a first tandem repeat nucleic acid;

(b) a first F-CphI endonuclease recognition site;

(c) a target nucleic acid;

(d) a second F-CphI endonuclease recognition site; and

(e) a second tandem repeat nucleic acid.

2. The excisable nucleic acid construct of claim 1, wherein each of the first and second tandem repeat nucleic acids independently consists of 18 to 200 nucleotide base pairs.

3. The excisable nucleic acid construct of claim 1, wherein each of the first and second tandem repeat nucleic acids independently consists of 18 to 150 nucleotide base pairs.

4. The excisable nucleic acid construct of claim 1, wherein each of the first and second tandem repeat nucleic acids independently consists of 18 to 100 nucleotide base pairs.

5. The excisable nucleic acid construct of claim 1, wherein each of the first and second tandem repeat nucleic acids independently comprises 18-80 nucleotide base pairs.

6. The excisable nucleic acid construct of any one of claims 1 to 5, wherein the target nucleic acid encodes a selectable marker.

7. The excisable nucleic acid construct of claim 6, wherein the selectable marker is selected from the group consisting of: URA3, hygromycin B phosphotransferase, aminoglycoside phosphotransferase, zeocin resistance gene and phosphinothricin N-acetyltransferase.

8. The excisable nucleic acid construct of any one of claims 1 to 7, further comprising a first genomic integration site linked 5' of the first tandem repeat nucleic acid, and a second integration site linked 3' of the second tandem repeat nucleic acid.

9. The excisable nucleic acid construct of any one of claims 1 to 8, wherein the target nucleic acid comprises a promoter element operably linked to a nucleic acid encoding F-CphI endonuclease.

10. A host cell comprising the excisable nucleic acid construct of any one of claims 1 to 7.

11. The host cell of claim 10, further comprising a vector comprising a nucleic acid encoding F-CphI endonuclease.

12. The host cell of claim 11, wherein the vector comprises a promoter element that controls the expression of the nucleic acid encoding F-CphI endonuclease.

13. The host cell of claim 12, wherein the promoter element is an inducible promoter.

14. The host cell of of any one of claims 1 to 13 that is a yeast cell.

15. The host cell of claim 14 that is a haploid yeast cell.

16. The host cell of claim 14 that is a diploid yeast cell.

17. The host cell of claim 14 that is a *Saccharomyces cerevisiae* cell.

18. The host cell of any one of claims 1 to 17, wherein the excisable nucleic acid construct is integrated into the host cell genome.

19. A method of excising at least one target nucleic acid from the genome of a host cell comprising the excisable nucleic acid construct of claim 1, wherein the method comprises: contacting the excisable nucleic acid construct with F-CphI in the host cell.

20. The method of claim 19, wherein said excising operably links a promoter element to a gene of interest.

21. The method of claim 19, wherein the target nucleic acid encodes a selectable marker.

22. The method of claim 21, wherein the selectable marker is selected from the group consisting of: URA3, hygromycin B phosphotransferase, aminoglycoside phosphotransferase, zeocin resistance gene and phosphinothricin N-acetyltransferase.

23. The method of any one of claims 1 to 22, wherein the host cell is a yeast cell.

24. The method of claim 23, wherein the host cell is a haploid yeast cell.

25. The method of claim 23, wherein the host cell is a diploid yeast cell.

26. The method of claim 23, wherein the host cell is a *Saccharomyces cerevisiae* cell.

NUCLEIC ACIDS, COMPOSITIONS AND METHODS FOR THE EXCISION OF TARGET NUCLEIC ACIDS

ABSTRACT

Nucleic acids, compositions, and methods that allow for the excision of one or more loci from the genome of a host cell are provided herein. In particular, provided herein is an excisable nucleic acid construct comprising, in a 5' to 3' orientation: a first tandem repeat nucleic acid, a first homing endonuclease recognition site, a target nucleic acid, a second homing endonuclease recognition site, and a second tandem repeat nucleic acid. In some embodiments, the excisable nucleic acid construct is integrated into the host cell genome, and the target nucleic acid can be excised from the host cell genome by contacting the homing endonuclease recognition sites with one or more appropriate homing endonucleases.