

(12) STANDARD PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. **AU 2018347421 B2**

(54) Title
Transgenic selection methods and compositions

(51) International Patent Classification(s)
C12N 9/14 (2006.01) **C12N 15/09** (2006.01)
C07K 14/435 (2006.01) **C12N 15/62** (2006.01)
C07K 19/00 (2006.01)

(21) Application No: **2018347421** (22) Date of Filing: **2018.10.11**

(87) WIPO No: **WO19/075200**

(30) Priority Data

(31) Number	(32) Date	(33) Country
62/616,281	2018.01.11	US
62/624,629	2018.01.31	US
62/608,478	2017.12.20	US
62/571,672	2017.10.12	US

(43) Publication Date: **2019.04.18**

(44) Accepted Journal Date: **2024.08.22**

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(56) Related Art
US 6858775 B1



(51) International Patent Classification:

C07K 14/435 (2006.01) C12N 15/09 (2006.01)

C07K 19/00 (2006.01) C12N 15/62 (2006.01)

C12N 9/14 (2006.01)

(21) International Application Number:

PCT/US2018/055412

(22) International Filing Date:

11 October 2018 (11.10.2018)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/571,672 12 October 2017 (12.10.2017) US

62/608,478 20 December 2017 (20.12.2017) US

62/616,281 11 January 2018 (11.01.2018) US

62/624,629 31 January 2018 (31.01.2018) US

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(81) Designated States (unless otherwise indicated, for every

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

(84) Designated States (unless otherwise indicated, for every

kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: TRANSGENIC SELECTION METHODS AND COMPOSITIONS

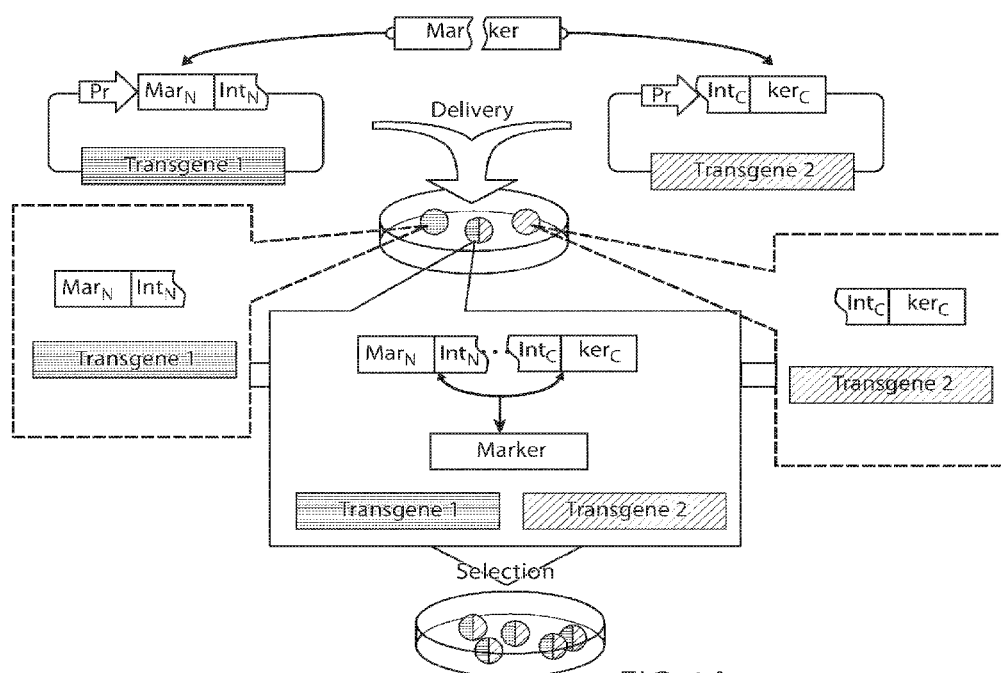


FIG. 1A

(57) **Abstract:** The present disclosure provides a split intein selectable marker system for the production and selection of transgenic cells.



Published:

- *with international search report (Art. 21(3))*
- *with sequence listing part of description (Rule 5.2(a))*

TRANSGENIC SELECTION METHODS AND COMPOSITIONS**RELATED APPLICATIONS**

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional application number 62/616,281, filed January 11, 2018, U.S. provisional application number 62/608,478, filed December 20, 2017, U.S. provisional application number 62/624,629, filed January 31, 2018, U.S. provisional application number 62/571,672, filed October 12, 2017, which is incorporated by reference herein in its entirety.

SEQUENCE LISTING

This application contains a Sequence Listing in computer readable form (filename: J022770007WO00-SEQ-HJD; 1.50 MB – ASCII text file; created October 3, 2018), which is incorporated herein by reference in its entirety and forms part of the disclosure.

BACKGROUND

Selectable markers are widely adopted in transgenesis and genome editing for selecting engineered cells with a desired genotype. Antibiotic resistance genes (encoding antibiotic resistance proteins) provide resistance to specific antibiotics so that only cells expressing these resistance genes survive and multiply. Antibiotic resistance genes/antibiotics available for use in eukaryotic cells include *hygB*/Hygromycin, *neo*/ Geneticin[®]/G418, *pac*/Puromycin, *Sh bla*/Phleomycin D1 (Zeocin[™]), and *bsd*/Blasticidin. Fluorescent proteins, such as green fluorescent protein (GFP) provide another means of cell selection, for example, via fluorescent-activated cell sorting (FACS) techniques or fluorescent microscopy.

SUMMARY

There is a limited number of antibiotic resistance genes/antibiotics available for use in eukaryotic (e.g., mammalian) cells, thus selection schemes for identifying cells containing multiple transgenes are limited. Not only is there a limited number of distinct genes that confer antibiotic resistance in eukaryotic cells, but simultaneous use of as few as three different antibiotic resistance genes can adversely affect the health of transgenic cells. While antibiotic selection can be performed serially, this process is time-consuming. These limitations on selections schemes for identifying transgenic cells are problematic when there is a need to identify cells into which multiple transgenes have been introduced (e.g., to generate a transgenic organism, e.g., animal model, such as a mouse model).

Provided herein are methods, compositions and kits useful for the production and/or identification of, for example, cells and/or organisms harboring two or more transgenes (e.g., double-transgenics, triple-transgenics, etc.). For example, the compositions and kits may be used for the production and/or identification of cells and/or organisms harboring two, three, or four transgenes. This technology is based, at least in part, on a protein splicing mechanism initiated by an intein auto-processing domain, which facilitates the joining (conjugation) specifically in multi-transgenic cells of multiple (e.g., two, three, or four) separate selectable marker protein fragments (double-transgenic cells, triple-transgenic cells, or quadruple-transgenic cells). Joining of the two, three, four, or more separate selectable marker protein fragments in the multi-transgenic cells produces a full-length selectable marker protein that confers, for example, antibiotic resistance (an antibiotic resistance protein) or is capable of fluorescence under an appropriate wavelength of light (fluorescent protein). Cells expressing a full-length antibiotic resistance gene survive in the presence of the corresponding antibiotic and thus are selected as multi-transgenic (e.g., double-transgenic, triple-transgenic, or quadruple-transgenic) cells. Likewise, cells expressing a full-length functioning fluorescent protein fluoresce under the appropriate wavelength of light and thus are selected as multi-transgenic (e.g., double-transgenic, triple-transgenic, or quadruple-transgenic) cells.

Thus, the present disclosure provides, in some embodiments, methods comprising delivering to a composition comprising eukaryotic cells two or more vectors, wherein each vector comprises (i) a nucleotide sequence encoding a selectable marker protein fragment linked to an N-terminal intein protein fragment and/or a C-terminal intein protein fragment and (ii) a nucleotide sequence encoding a molecule of interest, wherein the intein protein fragments, when joined in frame to form full-length function proteins, catalyze joining of the selectable marker protein fragments to produce a full-length selectable marker protein. For example, when two vectors are delivered to a population of cells (e.g., under transfection conditions), some cells will take up the first vector (the vector is introduced in the cells), some cells will take up the second vector, and some cells will take up both vectors. Only those cells that take up both vectors are capable of expressing a full-length functioning selectable marker protein, thus only those cells are selected as double-transgenic cells.

In some embodiments, methods herein comprising delivering to a composition comprising eukaryotic cells (a) a first vector comprising (i) a nucleotide sequence encoding a first selectable marker protein fragment (e.g., antibiotic resistance protein fragment or fluorescent protein fragment) upstream from a nucleotide sequence encoding an N-terminal intein protein fragment and (ii) a nucleotide sequence encoding a first molecule, and (b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal intein protein fragment upstream from a second selectable marker protein fragment (e.g., antibiotic resistance protein fragment or fluorescent

protein fragment) and (ii) a nucleotide sequence encoding a second molecule, wherein the N-terminal intein protein fragment and the C-terminal intein protein fragment catalyze joining of the first selectable marker protein fragment to the second selectable marker protein fragment to produce a full-length selectable marker protein. When the two vectors are delivered to a population of cells (e.g., under transfection conditions), some cells will take up the first vector (the vector is introduced in the cells), some cells will take up the second vector, and some cells will take up both vectors. Only those cells that take up both vectors are capable of expressing a full-length functioning selectable marker protein, thus only those cells are selected as double-transgenic cells.

In other embodiments, methods comprise delivering to eukaryotic cells (a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker protein (e.g., antibiotic resistance protein or fluorescent protein), which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest, (b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, and (c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a third molecule of interest, wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the selectable marker protein to the central fragment of the selectable markerprotein, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of central fragment of the selectable markerprotein to the C-terminal fragment of the selectable markerprotein, to produce a full-length selectable markerprotein. When the three vectors are delivered to a population of cells (e.g., under transfection conditions), some cells will take up the first vector (the vector is introduced in the cells), some cells will take up the second vector, some cells will take up the third vector, some cells will take up two different vectors, and some cells will take up all three vectors. Only those cells that take up all three vectors are capable of expressing a full-length functional selectable marker protein, thus only those cells are selected as triple-transgenic cells.

In still other embodiments, methods comprise delivering to eukaryotic cells (a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker protein (e.g., antibiotic resistance protein or fluorescent protein), which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest, (b) a second vector comprising (i) a nucleotide sequence

encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a first central fragment of the selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, (c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a second central fragment of the selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a third intein and (ii) a nucleotide sequence encoding a third molecule of interest, and (d) a fourth vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the third intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a third molecule of interest, wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the selectable marker protein to the first central fragment of the selectable marker protein, the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of first central fragment of the selectable marker protein to the second central fragment of the selectable marker protein, the N-terminal fragment and the C-terminal fragment of the third intein catalyze joining of second central fragment of the selectable marker protein to the C-terminal fragment of the selectable marker protein to produce a full-length selectable marker protein. When the four vectors are delivered to a population of cells (e.g., under transfection conditions), some cells will take up the first vector (the vector is introduced in the cells), some cells will take up the second vector, some cells will take up the third vector, some will take up the fourth vector, some cells will take up two different vectors, some cells will take up three different vectors, and some will take up all four vectors. Only those cells that take up all four vectors are capable of expressing a full-length functional selectable marker protein, thus only those cells are selected as quadruple-transgenic cells.

It should be understood that any one embodiment described herein, including those only disclosed in the examples or one section of the specification, is intended to be able to combine with any one or more other embodiments unless explicitly disclaimed.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1B. Split selectable marker for antibiotic co-selection of two separate transgenic vectors. **(FIG. 1A)** The coding sequence of a selectable marker is split into an N-terminal fragment (MarN) and a C-terminal fragment (kerC) and separately cloned upstream of an N-terminal fragment of a split intein (IntN) and downstream of a C-terminal fragment of the split intein (IntC), respectively, on two different vectors each carrying a different transgene. These vectors are delivered to cells yielding sub-populations of cells containing either one of the vectors or both of

the vectors. Only in cells with both vectors expressing the two intein-split selectable marker fragments (“markertrons”) undergo protein trans-splicing to reconstitute a full-length selectable marker, allowing specific selection and enrichment of the double transgenic cells. **(FIG. 1B)** To screen for split points compatible for inteins for an antibiotic resistance gene, we identified potential split points according to the junctional requirement for the type of intein tested, then cloned the corresponding N-terminal and C-terminal fragments to the split intein scaffolds on lentiviral vectors equipped with TagBFP2 or mCherry fluorescent proteins, which serve as our test transgenes to evaluate selection efficiency. These are delivered into cells via lentiviral transduction. The cells were then split into replicate plates, one subjected to antibiotic selection while the other maintained in non-selective media. Following antibiotic selection, the replicate cultures were analyzed by flow cytometry.

FIGS. 2A-2F. Details of the split points of Intein-split resistance (Intres) genes (also referred to as selectable marker genes) and plasmids. **(FIG. 2A)** Split points for hygromycin resistance protein (SEQ ID NO: 1). Amino acid sequence of hygromycin resistance protein is presented with clouds labeling the split points characterized in this study. Within the label, the top row indicates the plasmid numbers corresponding to **Table 1**. The bottom row indicates the residue number of the last amino acid in the N-terminal fragment, the species of the intein used, and the residue number of the first amino acid in the C-terminal fragment. “^C” indicates an insertion of a Cysteine. **(FIG. 2B)** Split points for puromycin resistance protein (SEQ ID NO: 2). Amino acid sequence of puromycin resistance protein is presented with clouds labeling the split points characterized in this study. Within the label, the top row indicates the plasmid numbers corresponding to **Table 1**. The bottom row indicates the residue number of the last amino acid in the N-terminal fragment, the species of the intein used, and the residue number of the first amino acid in the C-terminal fragment. “^C” indicates an insertion of a Cysteine. **(FIG. 2C)** Split points for neomycin resistance protein (SEQ ID NO: 3). Amino acid sequence of neomycin resistance gene is presented with clouds labeling the split points characterized in this study. Within the label, the top row indicates the plasmid numbers corresponding to **Table 1**. The bottom row indicates the residue number of the last amino acid in the N-terminal fragment, the species of the intein used, and the residue number of the first amino acid in the C-terminal fragment. **(FIG. 2D)** Split points for blasticidin resistance protein (SEQ ID NO: 4). Amino acid sequence of blasticidin resistance gene is presented with clouds labeling the split points characterized in this study. Within the label, the top row indicates the plasmid numbers corresponding to **Table 1**. The bottom row indicates the residue number of the last amino acid in the N-terminal fragment, the species of the intein used, and the residue number of the first amino acid in the C-terminal fragment. **(FIG. 2E)** Split points for green fluorescent protein (SEQ ID NO: 5). **(FIG. 2F)** Split points for mScarlet fluorescent protein (SEQ

ID NO: 6). Amino acid sequence of mScarlet gene is presented with clouds labeling the split points characterized in this study. Within the label, the top row indicates the plasmid numbers corresponding to **Table 1**. The bottom row indicates the residue number of the last amino acid in the N-terminal fragment, the species of the intein used, and the residue number of the first amino acid in the C-terminal fragment. “^C” indicates an insertion of a Cysteine.

FIG. 3. 2-markertron hygromycin (Hygro) intein-split resistance (Intres) genes. Top schematic shows the split points tested for hygromycin resistance gene. The last residue of the N-terminal fragment is indicated on top of the lollipops. Circle lollipops represent split points using NpuDnaE intein while square lollipops represent those using SspDnaB intein. Crossed-out and shaded lollipops indicate split pairs that failed to endow cells with hygromycin resistance. The column plot below shows the percentage of double-transgenic cells (BFP+ mCherry+) in the non-selected (white columns) and the selected cultures (blue columns) analyzed by flow cytometry.

FIG. 4. 2-markertron puromycin (Puro) Intres genes. Top schematic shows split points tested for puromycin resistance genes while bottom column plots show percentage of double transgenic cells in the non-selected (white columns) and the selected cultures (brown columns).

FIG. 5. 2-markertron neomycin (Neo) resistance genes. Top schematic shows split points tested for neomycin resistance genes while bottom column plots show percentage of double transgenic cells in the non-selected (white columns) and the selected cultures (orange columns).

FIG. 6. 2-markertron blasticidin (Blast) Intres genes. Top schematic shows split points tested for blasticidin resistance gene while bottom column plot shows percentages of double transgenic cells in the non-selected (white columns) and the selected (cyan columns) cultures.

FIGS. 7A-7C. Gateway-compatible lentiviral destination vectors with 2-markertron Intres markers. (**FIG. 7A**) Gateway-compatible lentiviral destination vector kits for each split Intres marker consists of an N-vector and C-vector. N-vector contains viral LTRs, CAGGS promoter, gateway destination cassette AttL, ccdB gene, chloramphenicol resistance gene that allows LR clonase-mediated recombination of Gateway donor vector carrying transgenes, followed by internal ribosomal entry site (IRES) that allows polycistronic expression of the N-markertron. Similarly, C-vector contains the C-markertron and allows recombination of another transgene. (**FIG. 7B**) TagBFP2 (as transgene 1) and mCherry (as transgene 2) were cloned into the 2-markertron Intres plasmids by Gateway recombination and delivered to cells by lentiviral transduction, followed by antibiotic selection and flow cytometry analysis. Column plot shows the percentage of BFP+mCherry+ double-positive cell in the selected culture from the 2-markertron hygromycin (Hygro, blue column), puromycin (Puro, brown column), and neomycin (Neo, orange column) experiments versus their corresponding non-selective cultures (white columns). (**FIG. 7C**) NLS-GFP (as transgene 1) that labels nucleus with GFP fluorescence and lifeAct-mScarlet (as transgene

2) that labels F-actin with mScarlet fluorescence were recombined into lentiviral vectors expressing full non-split hygromycin resistance gene or lentiviral vectors with 2-markertron hygromycin Intres genes and used to transduce U2OS cells to make dual-label cells. Representative fluorescence microscopic images show GFP, mScarlet and merged channels of cells after hygromycin selection for two weeks.

FIGS. 8A-8C. Split mScarlet for fluorescence-mediated co-selection of two separate transgenic vectors. **(FIG. 8A)** 2-markertron mScarlet proteins. Top schematics shows the split points tested for mScarlet. The last residue of the N-terminal fragment is indicated on top of the lollipops. **(FIG. 8B)** To screen for *Npu*DnaE intein-compatible split points for mScarlet, we identified potential split points according to the junctional requirement for *Npu*DnaE intein, then cloned the corresponding N-terminal and C-terminal fragments to the split inteins scaffolds on lentiviral vectors equipped with TagBFP2 or EGFP fluorescent proteins, which serve as our test transgenes to evaluate the selection efficiency. These are delivered into cells via lentiviral transduction. Cells with both lentiviruses contain the necessary protein splicing machinery and mScarlet fragments to reconstitute the full-length mScarlet fluorescent protein, as well as express both TagBFP2 and EGFP transgenes. Cells were subjected to FACS analysis. Boxed schematic shows an example of FACS analysis of the plasmid pair 33+34. P1 population was gated for forward scatter and side scatter for live singlet cells. From those, 17.8% of cells are double positive for TagBFP and EGFP transgenes. When the P1 cells were further gated for mScarlet-positive (mCherry channel), 99.4% of cells are double positive for TagBFP and EGFP. **(FIG. 8C)** The column plot below shows the percentage of mScarlet-positive cells of each of the indicated split points. The column plot above shows the percentage of TagBFP+ EGFP+ cells among the P1 cells (white columns) and the mScarlet-positive subset of P1 cells (red columns).

FIGS. 9A-9D. Multi-split selectable markers for co-selection of three or more transgenic vectors. **(FIG. 9A)** A selectable marker is partitioned into three fragments (M_1 , M_2 and M_3). The first marker fragment (M_1) is fused upstream of the N-terminal fragment of the first split intein (I_{N1}). The second marker fragment (M_2) is fused downstream of the C-terminal fragment of the first split intein (I_{C1}) and upstream of the N-terminal fragment of the second split intein (I_{N2}). The third marker fragment (M_3) is fused downstream of the C-terminal fragment of the second split intein (I_{C2}). The first split intein catalyzes the joining of M_1 to M_2 while the second split intein catalyzes the joining of M_2 to M_3 , effectively reconstituting the full selectable marker. **(FIG. 9B)** A design of a k -split selectable marker via an “intein chain” mechanism. Similar to the 3-split scenario, the selectable marker is partitioned into k fragments, and are reconstituted through protein trans-splicing mediated by intervening split inteins. **(FIG. 9C)** Split points identified from 2-split selectable markers were used in combination to produce 3-split selectable markers. The

corresponding fragments were cloned into lentiviral vectors to result in the 3-split selectable marker structure and a reporter fluorescent transgene per vector. Cells were then transduced with viruses prepared from these vectors, split into selective or non-selective media. After appropriate selection period, the cultures were analyzed by flow cytometry. **(FIG. 9D)** 3-markertron hygromycin (Hygro) Intres. Top schematics shows the split points tested for hygromycin resistance gene, with residue numbers of the last amino acid of the N-terminal fragments indicated above circle or square lollipops, representing *NpuDnaE* and *SspDnaB* inteins, respectively. Six 3-markertron hygromycin Intreses were tested, each indicated with a numbered line with circle or square indicating the two split points used for each case. Column plot below shows the percentage of triple transgenic (BFP+ GFP+ mCherry+) cells from the non-selective (white columns) and selective (blue columns) cultures for the 3-markertron hygromycin Intres indicated by the numbers below.

FIGS. 10A-10C. Gateway-compatible lentiviral destination vectors with 3-markertron hygromycin Intres genes. **(FIG. 10A)** Gateway-compatible lentiviral destination vector with viral LTRs, CAGGS promoter, gateway destination cassette *AttL*, *ccdB* gene, chloramphenicol resistance gene that allows LR clonase-mediated recombination of Gateway donor vector carrying transgenes, followed by internal ribosomal entry site (IRES) that allows polycistronic expression of the each of the three 3-split hygromycin markertrons. **(FIG. 10B)** TagBFP2 (as transgene 1) and EGFP (as transgene 2) and mCherry (as transgene 3) were cloned into the 3-split Intres plasmids by Gateway recombination and delivered to cells by lentiviral transduction, followed by antibiotic selection and flow cytometry analysis. **(FIG. 10C)** Column plot shows the percentage of BFP+GFP+mCherry+ triple-positive cells in the hygromycin selected (blue columns) versus their corresponding non-selective cultures (white columns).

FIG. 11. Four-split Hygro intres. (a) Markertrons of 4-split hygro intres expressed by four different plasmids. Plasmid 115 expresses a markertron created by fusing amino acid 1~89 of Hygromycin resistance gene [Hygro(1-89)] to *NpuDnaE*(N) and a leucine Zipper A motif (LZA). Plasmid 116 expresses a markertron created by fusing, from N- to C-termini, Leucine Zipper B motif (LZB)-*NpuDnaGEP*(C), Hygro(90-200) and *SspDnaB*(N). Plasmid 117 expresses a markertron created by fusing, from N- to C-termini, *SspDnaB*(C), Hygro(201-240), *NpuDnaE*(N)-LZA. Plasmid 118 expresses a markertron created by fusing LZB-*NpuDnaGEP*(C) to Hygro(241-341).

FIGS. 12A-12E. Intres markers allow enrichment of biallelic targeted cells from CRISPR/Cas-mediated knock-in experiments. Targeting construct pairs containing homology arms for AAVS1 safe harbor locus were designed to contain full length (FL) non-split or split Intres markers and tested for ability to enrich for biallelic targeted cells via antibiotic selection. **(FIG. 12A)** Plasmids 107 and 108 contains FL Neomycin (Neo) resistance gene driven by endogenous

PPP1R12C promoter at the AAVS1 locus, FL Hygromycin (Hygro) gene and rtTA Dox-responsive transactivator driven by an EF1a promoter, as well as FL Blasticidin (Blast) expressed as well as EGFP (plasmid 107) and mScarlet (plasmid 108) from a dox-inducible TetO promoter. Plasmid 106 contains Cas9 and an sgRNA targeting the AAVS locus. 2A: self-cleaving 2A peptides. Plasmids 106, 107 and 108 were co-transfected into HEK293T cells, split, and passaged in dox-containing hygromycin, blasticidin or non-selective media for two weeks, and analyzed by flow cytometry to assay efficiency of biallelic targeting. **(FIG. 12B)** Plasmids 109 and 110 contain similar structure as Plasmids 107 and 108, but having split Blast Intres in place of the FL Blast. **(FIG. 12C)** Plasmids 111 and 112 contain an EF1a-driven FL Blast and TetO-driven FL Hygro, nitroreductase (NTR), fluorescent protein (EGFP or mCherry) separated by 2A peptides. **(FIG. 12D)** Plasmids 113 and 114 are similar to Plasmids 111 and 112 but with Hygro Intres in place of FL Hygro. **(FIG. 12E)** Flow cytometry analysis of cells transfected with Plasmid 106 (Cas9+AAVS-sgRNA) and the indicated targeting construct pairs, two weeks after culturing in dox-containing non-selective media (Selection: None), blasticidin selection media (Blast) and hygromycin selection media (Hygro).

DETAILED DESCRIPTION

Provided herein, in some aspects, are methods of producing transgenic (e.g., multi-transgenic, such as double transgenic or triple transgenic) organisms, into which more than one transgene (or other genetic element) is introduced. As shown in FIG. 1A, an exemplary method of the present disclosure comprises delivering to a population of cells (a) a vector encoding a first selectable marker protein fragment upstream from an N-terminal intein protein fragment and a first transgene of interest, and (b) another vector encoding a C-terminal intein protein fragment upstream from a second selectable marker protein fragment and a second (e.g., a different) transgene of interest. Some cells of the population will take up a single vector (carrying only a fragment of the intein, a fragment of the selectable marker protein, and a single transgene), while other cells of the population will take up both vectors (and thus both intein fragments, both selectable marker protein fragments, and both transgenes of interest). In cells that take up both vectors, following translation, the intein protein fragments spontaneously and non-covalently assemble (cooperatively fold) into an intein structure to catalyze joining of the first selectable marker protein fragment to the second selectable marker protein fragment to produce a full-length selectable marker protein, which enables specific selection of those double transgenic cells. For example, if the selectable marker protein is an antibiotic resistance protein, only double-transgenic cells expressing the full-length (functional) antibiotic resistance protein will survive selection in the presence of the particular antibiotic. If the selectable marker protein is a fluorescent protein, as another example, only double-transgenic cells expressing the full-length (functional) fluorescent protein will emit a detectable signal such that

only those signal-emitting cells are selected.

Another exemplary method of the present disclosure comprises delivering to a population of cells (a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of an antibiotic resistance protein, which is upstream from a nucleotide sequence encoding an N-terminal
5 fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest, (b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the antibiotic resistance protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, and (c) a
10 third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the antibiotic resistance protein and (ii) a nucleotide sequence encoding a third molecule of interest. Some cells of the population will take up a single vector (carrying only a fragment of the intein, a fragment of the selectable marker protein, and a single transgene), while other cells of the
15 population will take up two vectors or all three vectors (and thus all intein fragments, all selectable marker protein fragments, and all transgenes of interest). In cells that take up all three vectors, following translation, the intein protein fragments spontaneously and non-covalently assemble (cooperatively fold) into an intein structure to catalyze joining of the N-terminal fragment of the selectable marker protein to the central fragment, and the central fragment to the C-terminal
20 fragment of the selectable marker protein to produce a full-length selectable marker protein, which enables specific selection of those triple-transgenic cells. For example, if the selectable marker protein is an antibiotic resistance protein, only triple-transgenic cells expressing the full-length (functional) antibiotic resistance protein will survive selection in the presence of the particular antibiotic. If the selectable marker protein is a fluorescent protein, as another example, only triple-
25 transgenic cells expressing the full-length (functional) fluorescent protein will emit a detectable signal such that only those signal-emitting cells are selected.

Inteins

An intein (*intervening protein*) carries out a unique auto-processing event known as protein
30 splicing in which it excises itself out from a larger precursor polypeptide through the cleavage of two peptide bonds and, in the process, ligates the flanking extein (*external protein*) sequences through the formation of a new peptide bond. This rearrangement occurs post-translationally (or possibly co-translationally), as intein genes are found embedded in frame within other protein-coding genes. Furthermore, intein-mediated protein splicing is spontaneous; it requires no external
35 factor or energy source, only the folding of the intein domain. In nature, the precursor protein

contains three segments—an N-extein (N-terminal portion of the protein) followed by the intein followed by a C-extein (C-terminal portion of the protein). Following splicing, the resulting protein contains the N-extein linked to the C-extein.

There are two types of inteins: *cis*-splicing inteins are single polypeptides that are embedded in a host protein, whereas *trans*-splicing inteins (referred to as split inteins) are separate polypeptides that mediate protein splicing after the intein pieces and their protein cargo associate (see, e.g., Paulus, H *Annu Rev Biochem* 69:447–496 (2000); and Saleh L, Perler FB *Chem Rec* 6:183–193 (2006)). Split inteins catalyze a series of chemical rearrangements that require the intein to be properly assembled and folded. The first step in splicing involves an N-S acyl shift in which the N-extein polypeptide is transferred to the side chain of the first residue of the intein. This is then followed by a *trans*-(thio)esterification reaction in which this acyl unit is transferred to the first residue of the C-extein (which is either serine, threonine, or cysteine) to form a branched intermediate. In the penultimate step of the process, this branched intermediate is cleaved from the intein by a transamidation reaction involving the C-terminal asparagine residue of the intein. This then sets up the final step of the process involving an S-N acyl transfer to create a normal peptide bond between the two exteins (Lockless, SW, Muir, TW *PNAS* 106(27): 10999–11004 (2009)).

To date, there are at least 70 different intein alleles, distinguished not only by the type of host gene in which the inteins are embedded, but also the integration point within that host gene (Perler, FB *Nucleic Acids Res.* 30: 383–384 (2002); Pietrokovski, S *Trends Genet.* 17: 465–472 (2001)). A small fraction (less than 5%) of the identified intein genes encode split inteins. Unlike the more common contiguous inteins, split inteins are transcribed and translated as two separate polypeptides, the N-intein and C-intein, each fused to one extein. Upon translation, the intein fragments spontaneously and non-covalently assemble (cooperatively fold) into the canonical intein structure to carry out protein splicing *in trans*. The first two split inteins to be characterized, from the cyanobacteria *Synechocystis* species PCC6803 (*Ssp*) and *Nostoc punctiforme* PCC73102 (*Npu*), are orthologs naturally found inserted in the α subunit of DNA Polymerase III (DnaE). *Npu* is especially notable due its remarkably fast rate of protein *trans*-splicing ($t_{1/2} = 50$ s at 30 °C). This half-life is significantly shorter than that of *Ssp* ($t_{1/2} = 80$ min at 30 °C) (Shah, NH et al. *J. Am. Chem. Soc.* 135: 5839 (2013)).

Herein, split inteins are used to catalyze the joining of two fragments (e.g., an N-terminal fragment and a C-terminal fragment) of a selectable marker protein, such as an antibiotic resistance protein or a fluorescent protein to produce a functional, full-length protein (e.g., FIGS. 1A and 1B).

A split intein may be a natural split intein or an engineered split intein. Natural split inteins naturally occur in a variety of different organisms. The largest known family of split inteins is found within the DnaE genes of at least 20 cyanobacterial species (Caspi J, et al. *Mol. Microbiol.* 50:

1569–1577 (2003)). Thus, in some embodiments of the present disclosure, a natural split intein is selected from DnaE inteins. Non-limiting examples of DnaE inteins include *Synechocystis sp.* DnaE (*SspDnaE*) inteins and *Nostoc punctiforme* (*NpuDnaE*) inteins.

In some embodiments, a split intein is an engineered split intein. Engineered split inteins may be produced from contiguous inteins (where a contiguous intein is artificially split) or may be modified natural split inteins that, for example, promote efficient protein purification, ligation, modification and cyclization (e.g., *NpuGEP* and *CfAGEP*, as described by Stevens, AJ *PNAS* 114(32): 8538–8543 (2017)). Methods for engineering split inteins are described, for example, by Aranko, AS et al. *Protein Eng Des Sel.* 27(8): 263–271 (2014), incorporated herein by reference. In some embodiments, the engineered split intein is engineered from DnaB inteins (Wu, H, et al. *Biochim Biophys Acta* 1387(1–2): 422–432 (1998)). For example, the engineered split intein may be a *SspDnaB* S1 intein. In some embodiments, the engineered split intein is engineered from GyrB inteins. For example, the engineered split intein may be a *SspGyrB* S11 intein.

In some embodiments, wherein triple-transgenics are produced, for example, the first intein may be the same as the second intein (e.g., both DnaE inteins). In other embodiments, two different inteins may be used (e.g., a DnaE intein and a DnaB intein). In some embodiments, the first intein is a *NpuDnaE* intein and the second intein is a *NpuDnaE* intein.

Selectable Marker Proteins

Transgenic (e.g., double and/or triple transgenic) cells of the present disclosure are selected based on their expression of a full-length selectable marker protein. A selectable marker protein, generally, confers a trait suitable for artificial selection. Examples of selectable marker proteins include antibiotic resistance proteins and fluorescent proteins.

An antibiotic resistance gene is a gene encoding a protein that confers resistance to a particular antibiotic or class of antibiotics. Non-limiting examples of antibiotic resistance genes for use in eukaryotic cells include those encoding proteins that confer resistance to hygromycin, G418, puromycin, phleomycin D1 or blasticidin. Non-limiting examples of antibiotic resistance genes for use in prokaryotic cells include those encoding proteins that confer resistance to hygromycin, G418, puromycin, phleomycin D1, blasticidin, kanamycin, spectinomycin, streptomycin, ampicillin, carbenicillin, bleomycin, erythromycin, polymyxin D, tetracycline and chloramphenicol.

Hygromycin B is an antibiotic produced by the bacterium *Streptomyces hygroscopicus*. It is an aminoglycoside that kills bacteria, fungi and higher eukaryotic cells by inhibiting protein synthesis. Hygromycin phosphotransferase (HPT), encoded by the *hpt* gene (also referred to as the *hph* or *aphIV* gene) originally derived from *Escherichia coli*, detoxifies the aminocyclitol antibiotic hygromycin B. Thus, in some embodiments, the selectable marker gene of the present disclosure is

the *hpt* gene.

G418 (GENETICIN[®]) is an aminoglycoside antibiotic similar in structure to gentamicin B1. It is produced by *Micromonospora rhodorangea*. G418 blocks polypeptide synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells. Resistance to G418 is conferred by the
5 *neo* gene from Tn5 encoding an aminoglycoside 3'-phosphotransferase, APT 3' II. G418 is an analog of neomycin sulfate, and has similar mechanism as neomycin. Thus, in some embodiments, the selectable marker gene of the present disclosure is the *neo* gene.

Puromycin is an aminonucleoside antibiotic, derived from *Streptomyces alboniger*, that causes premature chain termination during translation taking place in the ribosome. Puromycin is
10 selective for either prokaryotes or eukaryotes. Resistance to puromycin is conferred through expression of the puromycin N-acetyl-transferase (*pac*) gene. Thus, in some embodiments, the selectable marker gene of the present disclosure is the *pac* gene.

Phleomycin D1 (e.g., ZEOCIN[®]) is a glycopeptide antibiotic and one of the phleomycins from *Streptomyces verticillus* belonging to the bleomycin family of antibiotics. It is a broad-
15 spectrum antibiotic that is effective against most bacteria, filamentous fungi, yeast, plant, and animal cells. It causes cell death by intercalating into DNA and induces double strand breaks of the DNA. Resistance to phleomycin D1 is conferred by the product of the *Sh ble* gene first isolated from *Streptoalloteichus hindustanus*. Thus, in some embodiments, the selectable marker gene of the present disclosure is the *Sh ble* gene.

Blasticidin S is an antibiotic that is produced by *Streptomyces griseochromogenes*. Blasticidin prevents the growth of both eukaryotic and prokaryotic cells by inhibiting termination
20 step of translation and peptide bond formation (to lesser extent) by the ribosome. Resistance to blasticidin is conferred by at least three different genes: *bls* (an acetyltransferase) from *Streptoverticillum* spp.; *bsr* (a blasticidin-S deaminase) from *Bacillus cereus* (other *bsr* genes are known as well); and *bsd* (another deaminase) from *Aspergillus terreus*. Thus, in some
25 embodiments, the selectable marker gene of the present disclosure is the *bls* gene, the *bsr* gene, or the *bsd* gene.

Non-limiting examples of fluorescent proteins that may be used as provided herein include TagCFP, mTagCFP2, Czurite, ECFP2, mKalama1, Sirius, Sapphire, T-Sapphire, ECFP, Cerulean,
30 SCFP3C, mTurquoise, mTurquoise2, monomeric Midoriishi-Cyan, TagCFP, mTFP1, EGFP, Emerald, Superfolder GFP, Monomeric Czami Green, TagGFP2, mUKG, mWasabi, Clover, mNeonGreen, EYFP, Citrine, Venus, SYFP2, TagYFP, Monomeric Kusabira-Orange, mKOκ, mKO2, mOrange, mOrange2, mRaspberry, mCherry, mStrawberry, mScarlet, mTangerine, tdTomato, TagRFP, TagRFP-T, mCppl, mRuby, mRuby2, mPlum, HcRed-Tandem, mKate2,
35 mNeptune, NirFP, TagRFP657, IFP1.4 and iRFP.

Full-length selectable marker genes, in some embodiments, are produced by joining in the same cell two selectable marker gene fragments. In some embodiments, with reference to any full-length protein, one of the fragments is an N-terminal fragment (N-extein), while the other fragment is a C-terminal fragment (C-extein). Thus, in some embodiments, a first antibiotic resistance protein fragment is an N-terminal antibiotic resistance protein fragment, and a second antibiotic resistance protein fragment is a C-terminal antibiotic resistance protein fragment. In other embodiments, a first fluorescent protein fragment is an N-terminal fluorescent protein fragment, and a second fluorescent protein fragment is a C-terminal fluorescent protein fragment.

In other embodiments, full-length selectable marker genes are produced by joining in the same cell three or more selectable marker gene fragments. In some embodiments, with reference to any full-length protein, one of the fragments is an N-terminal fragment, one or more (e.g., 1, 2, or 3) of the fragments is a central fragment, and one of the fragments is a C-terminal fragment.

An N-terminal fragment may be any protein fragment that includes the free amine group (-NH₂) of the full-length protein. A C-terminal fragment may be any protein fragment that includes the free carboxyl group (-COOH). A central fragment may be any protein fragment that is located between the N-terminal fragment and the C-terminal fragment of the full-length protein.

For example, amino acids 1-89 of the gene encoding hygromycin (a 341-amino acid protein) may be referred to as the N-terminal protein fragment, while amino acids 90-341 may be referred to as the C-terminal fragment. Similarly, with reference to FIG. 5, amino acids 1-200 of the gene encoding hygromycin may be referred to as the N-terminal protein fragment, while amino acids 201-341 may be referred to as the C-terminal fragment. FIG. 6 shows additional examples where amino acids 1-53, 1-240, or 1-292 are considered the N-terminal protein fragments of full length hygromycin containing amino acids 54-341, 241-341, or 293-341 as the respective C-terminal fragments.

As another example, amino acids 1-52 of the gene encoding hygromycin (a 341-amino acid protein) may be referred to as the N-terminal protein fragment, amino acids 53-89 may be referred to as the central protein fragment, and amino acids 90-341 may be referred to as the C-terminal fragment. Similarly, amino acids 1-89 of the gene encoding hygromycin may be referred to as the N-terminal protein fragment, amino acids 90-240 may be referred to as the central fragment, and amino acids 241-341 may be referred to as the C-terminal fragment.

Transgenes and Other Molecules of Interest

The methods and compositions of the present disclosure are used, in some embodiments, to produce multi-transgenic (e.g., double and/or triple transgenic) cells and/or organisms. Thus, in some embodiments, the methods use one vector that encodes a first molecule (a first molecule of

interest) and another vector that encodes a second molecule (a second molecule of interest). In some embodiments, the methods use yet another vector that encodes a third molecule of interest.

Additional vectors (e.g., encoding additional central fragments of a selectable marker protein) may encode additional molecules of interest. Molecules of interest may be, for example, polypeptides

5 (e.g., proteins and peptides) or polynucleotides (e.g., nucleic acids, such as DNA or RNA).

In some embodiments, the first molecule (e.g., located on the first vector) is a protein. In some embodiments, the second molecule (e.g., located on the second vector) is a protein. In some embodiments, the third molecule (e.g., located on the third vector) is a protein. Examples of proteins of interest include, but are not limited to, enzymes, cytokines, transcription factors, hormones, growth factors, blood factors, antigens and antibodies.

10 In some embodiments, the first molecule is a peptide. In some embodiments, the second molecule is a peptide. In some embodiments, the third molecule is a peptide.

In some embodiments, the first molecule is a messenger RNA (mRNA). In some embodiments, the second molecule is a mRNA. In some embodiments, the third molecule is a mRNA. The mRNA, in some embodiments, encodes a vaccine or other antigenic molecule.

15 In some embodiments, the first molecule is a non-coding RNA (a RNA that does not encode a protein). In some embodiments, the second molecule is a non-coding RNA. In some embodiments, the third molecule is a non-coding RNA. Examples of non-coding RNA include, but are not limited to, RNA interference molecules, such as microRNA (miRNA), antisense RNA, short-interfering RNA (siRNA) or short-hairpin RNA (shRNA).

Vectors

Methods of the present disclosure include the use of at least two or at least three different vectors. A vector is any nucleic acid that may be used as a vehicle to carry exogenous (foreign) genetic material into a cell. A vector, in some embodiments, is a DNA sequence that includes an insert (e.g., transgene) and a larger sequence that serves as the backbone of the vector. Non-limiting examples of vectors include plasmids, viruses/viral vectors, cosmids, and artificial chromosomes, any of which may be used as provided herein. In some embodiments, the vector is a viral vector, such as a viral particle. In some embodiments, the vector is an RNA-based vector, such as a self-replicating RNA vector. In some embodiments, the first vector is a plasmid, the second vector is a plasmid, and/or the third vector is a plasmid. A vector, as provided herein, includes a promoter operably linked to a nucleic acid encoding a fragment of an intein and a fragment of selectable marker protein. In some embodiments, a vector also comprises a promoter operably linked to a nucleic acid, such as a transgene, encoding a molecule of interest.

35 In some embodiments, one vector (e.g., a first vector) comprises a nucleotide sequence

encoding a first selectable marker protein fragment upstream from a nucleotide sequence encoding an N-terminal intein protein fragment, while the other vector (e.g., a second vector) comprises a nucleotide sequence encoding a C-terminal intein protein fragment upstream from a second antibiotic resistance protein fragment (see, e.g., FIG. 1A). This configuration is equivalent to one vector (e.g., a first vector) comprising a nucleotide sequence encoding an N-terminal intein protein fragment downstream from a nucleotide sequence encoding a first selectable marker protein fragment, and the other vector (e.g., a second vector) comprising a second antibiotic resistance protein fragment downstream from a nucleotide sequence encoding a C-terminal intein protein fragment. The terms “upstream” and “downstream” refer to relative positions in a nucleic acid. Each nucleic acid has a 5' end and a 3' end, so named for the carbon position on the deoxyribose (or ribose) ring. When considering double-stranded DNA, for example, upstream is toward the 5' end of the coding strand and downstream is toward the 3' end.

In some embodiments, (a) a first vector comprises a nucleotide sequence encoding an N-terminal fragment of an antibiotic resistance protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein, (b) a second vector comprises a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the antibiotic resistance protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein, and (c) a third vector comprises a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the antibiotic resistance protein. This configuration is equivalent to a (a) a first vector comprising a nucleotide sequence encoding an N-terminal fragment of a first intein, which is downstream from a nucleotide sequence encoding an N-terminal fragment of an antibiotic resistance protein, (b) a second vector comprising a nucleotide sequence encoding an N-terminal fragment of a second intein, which is downstream from a nucleotide sequence encoding a central fragment of the antibiotic resistance protein, which is downstream from a nucleotide sequence encoding a C-terminal fragment of the first intein, and (c) a third vector comprising a C-terminal fragment of the antibiotic resistance protein, which is downstream from a nucleotide sequence encoding a C-terminal fragment of the second intein.

Cells

Methods of the present disclosure may be used for the production of transgenic cells and organisms by introducing into host cells the vectors (e.g., first and second vectors) described herein. The cells into which the vectors are introduced may be eukaryotic or prokaryotic. In some embodiments, the cells are eukaryotic. Examples of eukaryotic cells for use as provided herein include mammalian cells, plant cells (e.g., crop cells), insect cells (e.g., *Drosophila*) and fungal cells

(e.g., *Saccharomyces*). Mammalian cells may be, for example, human cells (stem cells or cells from an established cell line), primate cells, equine cells, bovine cells, porcine cells, canine cells, feline cells, or rodent cells (e.g., mouse or rat). Examples of mammalian cells for use as provided herein include, but are not limited to, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) 293 cells, HeLa cells, and NS0 cells. In some embodiments, the cells are prokaryotic. Examples of prokaryotic cells for use as provided herein include bacterial cells. Bacterial cells may be, for example, *Escherichia* spp. (e.g., *Escherichia coli*), *Streptococcus* spp. (e.g., *Streptococcus pyogenes*, *Streptococcus viridans*, *Streptococcus pneumoniae*), *Neisseria* spp. (e.g., *Neisseria giberria*, *Neisseria meningitidis*), *Corynebacterium* spp. (e.g., *Corynebacterium diphtheriae*), *Bacillus* spp. (e.g., *Bacillus anthracis*, *Bacillus subtilis*), *Lactobacillus* spp., *Clostridium* spp. (e.g., *Clostridium tetani*, *Clostridium perfringens*, *Clostridium novyi*), *Mycobacterium* spp. (e.g., *Mycobacterium tuberculosis*), *Shigella* spp. (e.g., *Shigella flexneri*, *Shigella dysenteriae*), *Salmonella* spp. (e.g., *Salmonella typhi*, *Salmonella enteritidis*), *Klebsiella* spp. (e.g., *Klebsiella pneumoniae*), *Yersinia* spp. (e.g., *Yersinia pestis*), *Serratia* spp. (e.g., *Serratia marcescens*), *Pseudomonas* spp. (e.g., *Pseudomonas aeruginosa*, *Pseudomonas mallei*), *Eikenella* spp. (e.g., *Eikenella corrodens*), *Haemophilus* spp. (e.g., *Haemophilus influenza*, *Haemophilus ducreyi*, *Haemophilus aegyptius*), *Vibrio* spp. (e.g., *Vibrio cholera*, *Vibrio natriegens*), *Legionella* spp. (e.g., *Legionella micdadei*, *Legionella bozemani*), *Brucella* spp. (e.g., *Brucella abortus*), *Mycoplasma* spp. (e.g., *Mycoplasma pneumoniae*) or *Streptomyces* spp. (e.g., *Streptomyces coelicolor*, *Streptomyces lividans*, *Streptomyces albus*).

Delivery and Selection Methods

Methods of the present disclosure, in some embodiments, include delivering vectors to a composition comprising cells and maintaining the composition under conditions that permit introduction of nucleic acid (e.g., first, second, and third vector) into the cells and permit nucleic acid expression in the cells to produce eukaryotic cells. Conditions required for the introduction of nucleic acid (e.g., vectors) into cells are well known. These conditions include, for example, transformation (of prokaryotic cells) conditions, transfection (of eukaryotic cells) conditions, transduction (via virus/viral vector) conditions, and electroporation conditions, any of which may be used as provided herein. Thus, in some embodiments, methods of the present disclosure include transfecting eukaryotic (e.g., mammalian) cells, while in other embodiments, the methods include transforming prokaryotic (e.g., bacterial) cells.

The selection of transgenic, e.g., multi-transgenic cells, such as double, triple, and/or quadruple transgenic cells depends on the type of selectable marker used. For example, if the selectable marker protein is an antibiotic resistance protein, the selection step may include exposing

the cells to a specific antibiotic and selecting only those cells that survive. If the selectable marker protein is a fluorescent protein, the selection step may include simply viewing the cells under a microscope and selecting cells that fluoresce, or the selection step may include other fluorescent selection methods, such as fluorescence-activated cell sorting (FACS) sorting.

5 In some embodiments, cells are transduced with viral vectors (e.g., viruses) carrying the nucleic acids as described herein. In some embodiments, prior to transduction (or other transfection method), cells are seeded, for example, on well plates (e.g., 12-well plates) at a density of 1×10^4 to 1×10^6 per well. In some embodiments 100 μ L to 500 μ L, e.g., 100, 150, 200, 250, 300, 350, 400, 450, or 500 μ L of each viral vector is added to each well.

Kits

The present disclosure also provides kits that may be used, for example, to produce and screen for transgenic cells and/or organisms. The kits may include any two or more components as described herein. For example, a kit may comprise (a) a first vector comprising a nucleotide
15 sequence encoding a first selectable marker protein fragment upstream from a nucleotide sequence encoding an N-terminal intein protein fragment; and (b) a second vector comprising a nucleotide sequence encoding a C-terminal intein protein fragment upstream from a second selectable marker protein fragment, wherein the N-terminal intein protein fragment and the C-terminal intein protein fragment catalyze joining of the first selectable marker protein fragment to the second selectable
20 marker protein fragment to produce a full-length antibiotic resistance protein.

In some embodiments, the kits include any two or more components as described herein. For example, a kit may comprise (a) a first vector comprising a nucleotide sequence encoding an N-terminal fragment of an antibiotic resistance protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein, (b) a second vector comprising a nucleotide
25 sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the antibiotic resistance protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein, and (c) a third vector comprising a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the antibiotic resistance protein, wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze
30 joining of N-terminal fragment of the antibiotic resistance protein to the central fragment of the antibiotic resistance protein, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of central fragment of the antibiotic resistance protein to the C-terminal fragment of the antibiotic resistance protein, to produce a full-length antibiotic resistance protein.

35 In some embodiments, the kits further comprise any one or more of the following

components: buffers, salts, cloning enzymes (e.g., LR clonase), competent cells (e.g., competent bacterial cells), transfection reagents, antibiotics, and/or instructions for performing the methods described herein.

5 Additional Embodiments

Additional embodiments of the present disclosure are encompassed by the following numbered paragraphs:

1. A method comprising delivering to eukaryotic cells
 - (a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment
10 of an antibiotic resistance protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a nucleotide sequence encoding a first molecule of interest; and
 - (b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal
15 fragment of the intein, which is upstream from a C-terminal fragment of the antibiotic resistance protein and (ii) a nucleotide sequence encoding a second molecule of interest,
wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of the N-terminal fragment and the C-terminal fragment of the antibiotic resistance protein to produce a full-length antibiotic resistance protein.
2. The method of paragraph 1 further comprising maintaining the eukaryotic cells under
20 conditions that permit introduction of the first and second vectors into the eukaryotic cells to produce transgenic eukaryotic cells.
3. The method of paragraph 2 further comprising selecting the transgenic eukaryotic cells that comprise the full-length antibiotic resistance protein.
4. The method of any one of paragraphs 1-3, wherein the eukaryotic cells are mammalian cells.
- 25 5. The method of any one of paragraphs 1-4, wherein the antibiotic resistance protein confers resistance to hygromycin, G418, puromycin, phleomycin D1 or blasticidin.
6. The method of any one of paragraphs 1-5, wherein the intein is a split intein.
7. The method of paragraph 6, wherein the split intein is a natural split intein.
8. The method of paragraph 7, wherein the natural split intein is selected from DnaE inteins.
- 30 9. The method of paragraph 8, wherein the DnaE inteins are selected from *Synechocystis sp.* DnaE (*SspDnaE*) inteins and *Nostoc punctiforme* (*NpuDnaE*) inteins.
10. The method of paragraph 6, wherein the split intein is an engineered split intein.
11. The method of paragraph 10, wherein the engineered split intein is engineered from DnaB inteins.
- 35 12. The method of paragraph 11, wherein the engineered split intein is a *SspDnaB* S1 intein.

13. The method of paragraph 12, wherein the engineered split intein is engineered from GyrB inteins.

14. The method of paragraph 13, wherein the engineered split intein is a *SspGyrB* S11 intein.

15. The method of any one of paragraphs 1-14, wherein the first and/or second molecule is a protein.

16. The method of any one of paragraphs 1-15, wherein the first and/or second molecule is a non-coding ribonucleic acid (RNA).

17. The method of paragraph 16, wherein the non-coding RNA is a microRNA (miRNA), antisense RNA, short-interfering RNA (siRNA) or short-hairpin RNA (shRNA).

18. The method of any one of paragraphs 1-17, wherein the first and/or second vector is a plasmid vector or a viral vector.

19. A method comprising delivering to eukaryotic cells

(a) a first vector comprising (i) an N-terminal fragment of a *hygB* gene, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a first molecule of interest; and

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the *hygB* gene and (ii) a second molecule of interest,

wherein the N-terminal fragment and C-terminal fragment of the intein catalyze joining of a protein fragment encoded by the N-terminal fragment of the *hygB* gene to a protein fragment encoded by the C-terminal fragment of the *hygB* gene to produce full-length hygromycin B phosphotransferase.

20. The method of paragraph 19, wherein the first amino acid of the protein fragment encoded by the second *hygB* gene fragment is cysteine.

21. The method of paragraph 23, wherein

the protein fragment encoded by the N-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 1-89 of SEQ ID NO: 1, and the protein fragment encoded by the C-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 90-341 of SEQ ID NO: 1;

the protein fragment encoded by the N-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 1-200 of SEQ ID NO: 1, and the protein fragment encoded by the C-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 201-341 of SEQ ID NO: 1;

the protein fragment encoded by the N-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 1-53 of SEQ ID NO: 1, and the protein fragment

encoded by the C-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 54-341 of SEQ ID NO: 1;

the protein fragment encoded by the N-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 1-240 of SEQ ID NO: 1, and the protein fragment
5 encoded by the C-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 241-341 of SEQ ID NO: 1;

the protein fragment encoded by the N-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 1-292 of SEQ ID NO: 1, and the protein fragment
10 encoded by the C-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 293-341 of SEQ ID NO: 1.

22. The method of any one of paragraphs 23-21, wherein

the N-terminal fragment of the intein is identified by SEQ ID NO:16, and the C-terminal fragment of the intein is identified by SEQ ID NO:17;

the N-terminal fragment of the intein is identified by SEQ ID NO:7, and the C-terminal
15 fragment of the intein is identified by SEQ ID NO:8; or

the N-terminal fragment of the intein is identified by SEQ ID NO:18 or SEQ ID NO:9, and the C-terminal fragment of the intein is identified by SEQ ID NO:19 or SEQ ID NO:10.

23. A method comprising delivering to eukaryotic cells

(a) a first vector comprising (i) a N-terminal fragment of a *bsr* gene, which is upstream
20 from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a first molecule of interest; and

(b) a second vector comprising (ii) a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the *bsr* gene and (ii) a second molecule of interest,

25 wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of a protein fragment encoded by the N-terminal fragment of the *bsr* gene to a protein fragment encoded by the C-terminal fragment of the *bsr* gene to produce full-length blasticidin-S deaminase.

24. The method of paragraph 23, wherein the protein fragment encoded by the N-terminal fragment of the *bsr* gene comprises an amino acid sequence identified by amino acids 1-102 of SEQ
30 ID NO: 4, and the protein fragment encoded by the C-terminal fragment of the *bsr* gene comprises an amino acid sequence identified by amino acids 103-140 of SEQ ID NO: 4.

25. The method of paragraph 22 or 23, wherein

the N-terminal fragment of the intein is identified by SEQ ID NO:16, and the C-terminal fragment of the intein is identified by SEQ ID NO:17;

35 the N-terminal fragment of the intein is identified by SEQ ID NO:7, and the C-terminal

fragment of the intein is identified by SEQ ID NO:8; or

the N-terminal fragment of the intein is identified by SEQ ID NO:18 or SEQ ID NO:9, and the C-terminal fragment of the intein is identified by SEQ ID NO:19 or SEQ ID NO:10.

26. A method comprising delivering to eukaryotic cells

(a) a first vector comprising (i) a N-terminal fragment of a *pac* gene, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a first molecule of interest; and

(b) a second vector comprising (ii) a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the *pac* gene and (ii) a second molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of a protein fragment encoded by the N-terminal fragment of the *pac* gene to a protein fragment encoded by the C-terminal fragment of the *pac* gene to produce full-length puromycin N-acetyl-transferase.

27. The method of paragraph 26, wherein

the protein fragment encoded by the N-terminal fragment of the *pac* gene comprises an amino acid sequence identified by amino acids 1-63 of SEQ ID NO: 2, and the protein fragment encoded by the C-terminal fragment of the *pac* gene comprises an amino acid sequence identified by amino acids 64-199 of SEQ ID NO: 2;

the protein fragment encoded by the N-terminal fragment of the *pac* gene comprises an amino acid sequence identified by amino acids 1-119 of SEQ ID NO: 2, and the protein fragment encoded by the C-terminal fragment of the *pac* gene comprises an amino acid sequence identified by amino acids 120-199 of SEQ ID NO: 2;

the protein fragment encoded by the N-terminal fragment of the *pac* gene comprises an amino acid sequence identified by amino acids 1-100 of SEQ ID NO: 2, and the protein fragment encoded by the C-terminal fragment of the *pac* gene comprises an amino acid sequence identified by amino acids 101-199 of SEQ ID NO: 2.

28. The method of paragraph 26 or 27, wherein

the N-terminal fragment of the intein is identified by SEQ ID NO:16, and the C-terminal fragment of the intein is identified by SEQ ID NO:17;

the N-terminal fragment of the intein is identified by SEQ ID NO:7, and the C-terminal fragment of the intein is identified by SEQ ID NO:8; or

the N-terminal fragment of the intein is identified by SEQ ID NO:18 or SEQ ID NO:9, and the C-terminal fragment of the intein is identified by SEQ ID NO:19 or SEQ ID NO:10.

29. A method comprising delivering to eukaryotic cells

(a) a first vector comprising (i) a N-terminal fragment of a *neo* gene, which is upstream

from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a first molecule of interest; and

(b) a second vector comprising (ii) a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the *neo* gene and (ii) a second molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of a protein fragment encoded by the N-terminal fragment of the *neo* gene to a protein fragment encoded by the C-terminal fragment of the *neo* gene to produce full-length aminoglycoside 3'-phosphotransferase.

30. The method of paragraph 29, wherein

the protein fragment encoded by the N-terminal fragment of the *neo* gene comprises an amino acid sequence identified by amino acids 1-133 of SEQ ID NO: 3 and the protein fragment encoded by the C-terminal fragment of the *neo* gene comprises an amino acid sequence identified by amino acids 134-267 of SEQ ID NO: 3; or

the protein fragment encoded by the N-terminal fragment of the *neo* gene comprises an amino acid sequence identified by amino acids 1-194 of SEQ ID NO: 3 and the protein fragment encoded by the C-terminal fragment of the *neo* gene comprises an amino acid sequence identified by amino acids 195-267 of SEQ ID NO: 3.

31. The method of paragraph 29 or 30, wherein

the N-terminal fragment of the intein is identified by SEQ ID NO:16, and the C-terminal fragment of the intein is identified by SEQ ID NO:17;

the N-terminal fragment of the intein is identified by SEQ ID NO:7, and the C-terminal fragment of the intein is identified by SEQ ID NO:8; or

the N-terminal fragment of the intein is identified by SEQ ID NO:18 or SEQ ID NO:9, and the C-terminal fragment of the intein is identified by SEQ ID NO:19 or SEQ ID NO:10.

32. A method comprising delivering to a composition comprising eukaryotic cells

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a fluorescent protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a nucleotide sequence encoding a first molecule of interest; and

(b) a second vector comprising (ii) a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the fluorescent protein and (ii) a nucleotide sequence encoding a second molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of the N-terminal fragment of the fluorescent protein to the C-terminal fragment of the fluorescent protein to produce a full-length fluorescent protein.

33. The method of paragraph 51 further comprising maintaining the eukaryotic cells under conditions that permit introduction of the first and second vectors into the eukaryotic cells to produce transgenic eukaryotic cells.
34. The method of paragraph 33 further comprising selecting the transgenic eukaryotic cells that
5 comprise the full-length fluorescent protein.
35. The method of any one of paragraphs 32-34, wherein the eukaryotic cells are mammalian cells.
36. The method of any one of paragraphs 32-35, wherein the fluorescent protein is selected from TagCFP, mTagCFP2, Czurite, ECFP2, mKalama1, Sirius, Sapphire, T-Sapphire, ECFP, Cerulean,
10 SCFP3C, mTurquoise, mTurquoise2, monomeric Midoriishi-Cyan, TagCFP, mTFP1, EGFP, Emerald, Superfolder GFP, Monomeric Czami Green, TagGFP2, mUKG, mWasabi, Clover, mNeonGreen, EYFP, Citrine, Venus, SYFP2, TagYFP, Monomeric Kusabira-Orange, mKOκ, mKO2, mOrange, mOrange2, mRaspberry, mCherry, mStrawberry, mTangerine, tdTomato, TagRFP, TagRFP-T, mCppl, mRuby, mRuby2, mPlum, HcRed-Tandem, mKate2, mNeptune,
15 NirFP, TagRFP657, IFP1.4 and iRFP.
37. The method of any one of paragraphs 32-36, wherein the intein is a split intein.
38. The method of paragraph 37, wherein the split intein is a natural split intein.
39. The method of paragraph 38, wherein the natural split intein is selected from DnaE inteins.
40. The method of paragraph 39, wherein the DnaE inteins are selected from *Synechocystis sp.*
20 DnaE (*SspDnaE*) inteins and *Nostoc punctiforme* (*NpuDnaE*) inteins.
41. The method of paragraph 40, wherein the split intein is an engineered split intein.
42. The method of paragraph 41, wherein the engineered split intein is engineered from DnaB inteins.
43. The method of paragraph 42, wherein the engineered split intein is a *SspDnaB* S1 intein.
- 25 44. The method of paragraph 42, wherein the engineered split intein is engineered from GyrB inteins.
45. The method of paragraph 44, wherein the engineered split intein is a *SspGyrB* S11 intein.
46. The method of any one of paragraphs 32-45, wherein the first and/or second molecule is a protein.
- 30 47. The method of any one of paragraphs 32-46, wherein the first and/or second molecule is a non-coding ribonucleic acid (RNA).
48. The method of paragraph 47, wherein the non-coding RNA is a microRNA (miRNA), antisense RNA, short-interfering RNA (siRNA) or short-hairpin RNA (shRNA).
49. The method of any one of paragraphs 32-48, wherein the first and/or second vector is a
35 plasmid vector or a viral vector.

50. A method comprising delivering to eukaryotic cells

(a) a first vector comprising (i) an N-terminal fragment of an *egfp* gene, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a first molecule of interest; and

5 (b) a second vector comprising (ii) a nucleotide sequence encoding a C-terminal fragment of an intein, which is upstream from a C-terminal fragment of an *egfp* gene and (ii) a second molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of a protein fragment encoded by the N-terminal fragment of the *egfp* gene to a protein fragment
10 encoded by the C-terminal fragment of the *egfp* gene to produce full-length EGFP protein.

51. The method of paragraph 50, wherein the protein fragment encoded by the N-terminal fragment of the *egfp* gene comprises an amino acid sequence identified by amino acids 1-175 of SEQ ID NO: 5, and the protein fragment encoded by the C-terminal fragment of the *egfp* gene comprises an amino acid sequence identified by amino acids 175-239 of SEQ ID NO: 5.

15 52. The method of paragraph 50 or 51, wherein

the N-terminal fragment of the intein is identified by SEQ ID NO:16, and the C-terminal fragment of the intein is identified by SEQ ID NO:17;

the N-terminal fragment of the intein is identified by SEQ ID NO:7, and the C-terminal fragment of the intein is identified by SEQ ID NO:8; or

20 the N-terminal fragment of the intein is identified by SEQ ID NO:18 or SEQ ID NO:9, and the C-terminal fragment of the intein is identified by SEQ ID NO:19 or SEQ ID NO:10.

53. A method comprising delivering to eukaryotic cells

(a) a first vector comprising (i) an N-terminal fragment of an *mScarlet* gene, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a first
25 molecule of interest; and

(b) a second vector comprising (ii) a nucleotide sequence encoding a C-terminal fragment of an intein, which is upstream from a C-terminal fragment of an *mScarlet* gene and (ii) a second molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining
30 of a protein fragment encoded by the N-terminal fragment of the *mScarlet* gene to a protein fragment encoded by the C-terminal fragment of the *mScarlet* gene to produce full-length mScarlet protein.

54. The method of paragraph 53, wherein

the protein fragment encoded by the N-terminal fragment of the *mScarlet* gene comprises an
35 amino acid sequence identified by amino acids 1-46 of SEQ ID NO: 6, and the protein fragment

encoded by the C-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 47-232 of SEQ ID NO: 6;

the protein fragment encoded by the N-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 1-48 of SEQ ID NO: 6, and the protein fragment
5 encoded by the C-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 49-232 of SEQ ID NO: 6;

the protein fragment encoded by the N-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 1-51 of SEQ ID NO: 6, and the protein fragment
10 encoded by the C-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 52-232 of SEQ ID NO: 6;

the protein fragment encoded by the N-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 1-75 of SEQ ID NO: 6, and the protein fragment
15 encoded by the C-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 76-232 of SEQ ID NO: 6;

the protein fragment encoded by the N-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 1-122 of SEQ ID NO: 6, and the protein fragment
20 encoded by the C-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 123-232 of SEQ ID NO: 6;

the protein fragment encoded by the N-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 1-140 of SEQ ID NO: 6, and the protein fragment
25 encoded by the C-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 141-232 of SEQ ID NO: 6;

the protein fragment encoded by the N-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 1-163 of SEQ ID NO: 6, and the protein fragment
30 encoded by the C-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 164-232 of SEQ ID NO: 6.

55. The method of paragraph 53 or 54, wherein

the N-terminal fragment of the intein is identified by SEQ ID NO:16, and the C-terminal fragment of the intein is identified by SEQ ID NO:17;

30 the N-terminal fragment of the intein is identified by SEQ ID NO:7, and the C-terminal fragment of the intein is identified by SEQ ID NO:8; or

the N-terminal fragment of the intein is identified by SEQ ID NO:18 or SEQ ID NO:9, and the C-terminal fragment of the intein is identified by SEQ ID NO:19 or SEQ ID NO:10.

56. A eukaryotic cell, comprising

35 (a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment

of an antibiotic resistance protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a nucleotide sequence encoding a first molecule of interest; and

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the antibiotic resistance protein and (ii) a nucleotide sequence encoding a second molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of the N-terminal fragment and the C-terminal fragment of the antibiotic resistance protein to produce a full-length antibiotic resistance protein.

57. The cell of paragraph 56, wherein the eukaryotic cells are mammalian cells.

58. The cell of paragraph 56 or 57, wherein the antibiotic resistance protein confers resistance to hygromycin, G418, puromycin, phleomycin D1 or blasticidin.

59. The cell of any one of paragraphs 56-58, wherein the intein is a split intein.

60. The cell of paragraph 59, wherein the split intein is a natural split intein.

61. The cell of paragraph 60, wherein the natural split intein is selected from DnaE inteins.

62. The cell of paragraph 61, wherein the DnaE inteins are selected from *Synechocystis sp.* DnaE (*SspDnaE*) inteins and *Nostoc punctiforme* (*NpuDnaE*) inteins.

63. The cell of paragraph 59, wherein the split intein is an engineered split intein.

64. The cell of paragraph 63, wherein the engineered split intein is engineered from DnaB inteins.

65. The cell of paragraph 64, wherein the engineered split intein is a *SspDnaB* S1 intein.

66. The cell of paragraph 65, wherein the engineered split intein is engineered from GyrB inteins.

67. The cell of paragraph 66, wherein the engineered split intein is a *SspGyrB* S11 intein.

68. The cell of any one of paragraphs 56-67, wherein the first and/or second molecule is a protein.

69. The cell of any one of paragraphs 56-68, wherein the first and/or second molecule is a non-coding ribonucleic acid (RNA).

70. The cell of paragraph 69, wherein the non-coding RNA is a microRNA (miRNA), antisense RNA, short-interfering RNA (siRNA) or short-hairpin RNA (shRNA).

71. The cell of any one of paragraphs 56-70, wherein the first and/or second vector is a plasmid vector or a viral vector.

72. A cell comprising

(a) a first vector comprising (i) an N-terminal fragment of a *hygB* gene, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a first

molecule of interest; and

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the *hygB* gene and (ii) a second molecule of interest,

wherein the N-terminal fragment and C-terminal fragment of the intein catalyze joining of a protein fragment encoded by the N-terminal fragment of the *hygB* gene to a protein fragment encoded by the C-terminal fragment of the *hygB* gene to produce full-length hygromycin B phosphotransferase.

73. The cell of paragraph 72, wherein the first amino acid of the protein fragment encoded by the second *hygB* gene fragment is cysteine.

74. The cell of paragraph 73, wherein

the protein fragment encoded by the N-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 1-89 of SEQ ID NO: 1, and the protein fragment encoded by the C-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 90-341 of SEQ ID NO: 1;

the protein fragment encoded by the N-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 1-200 of SEQ ID NO: 1, and the protein fragment encoded by the C-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 201-341 of SEQ ID NO: 1;

the protein fragment encoded by the N-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 1-53 of SEQ ID NO: 1, and the protein fragment encoded by the C-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 54-341 of SEQ ID NO: 1;

the protein fragment encoded by the N-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 1-240 of SEQ ID NO: 1, and the protein fragment encoded by the C-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 241-341 of SEQ ID NO: 1;

the protein fragment encoded by the N-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 1-292 of SEQ ID NO: 1, and the protein fragment encoded by the C-terminal fragment of the *hygB* gene comprises an amino acid sequence identified by amino acids 293-341 of SEQ ID NO: 1.

75. The cell of any one of paragraphs 72-74, wherein

the N-terminal fragment of the intein is identified by SEQ ID NO:16, and the C-terminal fragment of the intein is identified by SEQ ID NO:17;

the N-terminal fragment of the intein is identified by SEQ ID NO:7, and the C-terminal

fragment of the intein is identified by SEQ ID NO:8; or

the N-terminal fragment of the intein is identified by SEQ ID NO:18 or SEQ ID NO:9, and the C-terminal fragment of the intein is identified by SEQ ID NO:19 or SEQ ID NO:10.

76. A eukaryotic cell, comprising

(a) a first vector comprising (i) a N-terminal fragment of a *bsr* gene, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a first molecule of interest; and

(b) a second vector comprising (ii) a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the *bsr* gene and (ii) a second molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of a protein fragment encoded by the N-terminal fragment of the *bsr* gene to a protein fragment encoded by the C-terminal fragment of the *bsr* gene to produce full-length blasticidin-S deaminase.

77. The cell of paragraph 76, wherein the protein fragment encoded by the N-terminal fragment of the *bsr* gene comprises an amino acid sequence identified by amino acids 1-102 of SEQ ID NO: 4, and the protein fragment encoded by the C-terminal fragment of the *bsr* gene comprises an amino acid sequence identified by amino acids 103-140 of SEQ ID NO: 4.

78. The cell of paragraph 76 or 77, wherein

the N-terminal fragment of the intein is identified by SEQ ID NO:16, and the C-terminal fragment of the intein is identified by SEQ ID NO:17;

the N-terminal fragment of the intein is identified by SEQ ID NO:7, and the C-terminal fragment of the intein is identified by SEQ ID NO:8; or

the N-terminal fragment of the intein is identified by SEQ ID NO:18 or SEQ ID NO:9, and the C-terminal fragment of the intein is identified by SEQ ID NO:19 or SEQ ID NO:10.

79. A eukaryotic cell, comprising

(a) a first vector comprising (i) a N-terminal fragment of a *pac* gene, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a first molecule of interest; and

(b) a second vector comprising (ii) a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the *pac* gene and (ii) a second molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of a protein fragment encoded by the N-terminal fragment of the *pac* gene to a protein fragment encoded by the C-terminal fragment of the *pac* gene to produce full-length puromycin N-acetyl-transferase.

80. The cell of paragraph 79, wherein

the protein fragment encoded by the N-terminal fragment of the *pac* gene comprises an amino acid sequence identified by amino acids 1-63 of SEQ ID NO: 2, and the protein fragment encoded by the C-terminal fragment of the *pac* gene comprises an amino acid sequence identified by amino acids 64-199 of SEQ ID NO: 2;

5 the protein fragment encoded by the N-terminal fragment of the *pac* gene comprises an amino acid sequence identified by amino acids 1-119 of SEQ ID NO: 2, and the protein fragment encoded by the C-terminal fragment of the *pac* gene comprises an amino acid sequence identified by amino acids 120-199 of SEQ ID NO: 2;

10 the protein fragment encoded by the N-terminal fragment of the *pac* gene comprises an amino acid sequence identified by amino acids 1-100 of SEQ ID NO: 2, and the protein fragment encoded by the C-terminal fragment of the *pac* gene comprises an amino acid sequence identified by amino acids 101-199 of SEQ ID NO: 2.

81. The cell of paragraph 79 or 80, wherein

15 the N-terminal fragment of the intein is identified by SEQ ID NO:16, and the C-terminal fragment of the intein is identified by SEQ ID NO:17;

the N-terminal fragment of the intein is identified by SEQ ID NO:7, and the C-terminal fragment of the intein is identified by SEQ ID NO:8; or

the N-terminal fragment of the intein is identified by SEQ ID NO:18 or SEQ ID NO:9, and the C-terminal fragment of the intein is identified by SEQ ID NO:19 or SEQ ID NO:10.

20 82. A eukaryotic cell, comprising

(a) a first vector comprising (i) a N-terminal fragment of a *neo* gene, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a first molecule of interest; and

25 (b) a second vector comprising (ii) a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the *neo* gene and (ii) a second molecule of interest,

30 wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of a protein fragment encoded by the N-terminal fragment of the *neo* gene to a protein fragment encoded by the C-terminal fragment of the *neo* gene to produce full-length aminoglycoside 3'-phosphotransferase.

83. The cell of paragraph 82, wherein

35 the protein fragment encoded by the N-terminal fragment of the *neo* gene comprises an amino acid sequence identified by amino acids 1-133 of SEQ ID NO: 3 and the protein fragment encoded by the C-terminal fragment of the *neo* gene comprises an amino acid sequence identified by amino acids 134-267 of SEQ ID NO: 3; or

the protein fragment encoded by the N-terminal fragment of the *neo* gene comprises an amino acid sequence identified by amino acids 1-194 of SEQ ID NO: 3 and the protein fragment encoded by the C-terminal fragment of the *neo* gene comprises an amino acid sequence identified by amino acids 195-267 of SEQ ID NO: 3.

5 84. The cell of paragraph 82 or 83, wherein

the N-terminal fragment of the intein is identified by SEQ ID NO:16, and the C-terminal fragment of the intein is identified by SEQ ID NO:17;

the N-terminal fragment of the intein is identified by SEQ ID NO:7, and the C-terminal fragment of the intein is identified by SEQ ID NO:8; or

10 the N-terminal fragment of the intein is identified by SEQ ID NO:18 or SEQ ID NO:9, and the C-terminal fragment of the intein is identified by SEQ ID NO:19 or SEQ ID NO:10.

85. A eukaryotic cell, comprising

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a fluorescent protein, which is upstream from a nucleotide sequence encoding an N-terminal
15 fragment of an intein and (ii) a nucleotide sequence encoding a first molecule of interest; and

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the fluorescent protein and (ii) a nucleotide sequence encoding a second molecule of interest,

20 wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of the N-terminal fragment of the fluorescent protein to the C-terminal fragment of the fluorescent protein to produce a full-length fluorescent protein.

86. The cell of paragraph 85 further comprising maintaining the eukaryotic cells under conditions that permit introduction of the first and second vectors into the eukaryotic cells to produce transgenic eukaryotic cells.

25 87. The cell of paragraph 86 further comprising selecting the transgenic eukaryotic cells that comprise the full-length fluorescent protein.

88. The cell of any one of paragraphs 85-87, wherein the eukaryotic cells are mammalian cells.

89. The cell of any one of paragraphs 85-88, wherein the fluorescent protein is selected from TagCFP, mTagCFP2, Czurite, ECFP2, mKalama1, Sirius, Sapphire, T-Sapphire, ECFP, Cerulean,
30 SCFP3C, mTurquoise, mTurquoise2, monomeric Midoriishi-Cyan, TagCFP, mTFP1, EGFP, Emerald, Superfolder GFP, Monomeric Czami Green, TagGFP2, mUKG, mWasabi, Clover, mNeonGreen, EYFP, Citrine, Venus, SYFP2, TagYFP, Monomeric Kusabira-Orange, mKOκ, mKO2, mOrange, mOrange2, mRaspberry, mCherry, mStrawberry, mTangerine, tdTomato, TagRFP, TagRFP-T, mCppl, mRuby, mRuby2, mPlum, HcRed-Tandem, mKate2, mNeptune,
35 NirFP, TagRFP657, IFP1.4 and iRFP.

90. The cell of any one of paragraphs 85-89, wherein the intein is a split intein.
91. The cell of paragraph 90, wherein the split intein is a natural split intein.
92. The cell of paragraph 91, wherein the natural split intein is selected from DnaE inteins.
93. The cell of paragraph 92, wherein the DnaE inteins are selected from *Synechocystis sp.* DnaE (*SspDnaE*) inteins and *Nostoc punctiforme* (*NpuDnaE*) inteins.
94. The cell of paragraph 93, wherein the split intein is an engineered split intein.
95. The cell of paragraph 94, wherein the engineered split intein is engineered from DnaB inteins.
96. The cell of paragraph 95, wherein the engineered split intein is a *SspDnaB* S1 intein.
97. The cell of paragraph 95, wherein the engineered split intein is engineered from GyrB inteins.
98. The cell of paragraph 97, wherein the engineered split intein is a *SspGyrB* S11 intein.
99. The cell of any one of paragraphs 85-98, wherein the first and/or second molecule is a protein.
100. The cell of any one of paragraphs 85-99, wherein the first and/or second molecule is a non-coding ribonucleic acid (RNA).
101. The cell of paragraph 100, wherein the non-coding RNA is a microRNA (miRNA), antisense RNA, short-interfering RNA (siRNA) or short-hairpin RNA (shRNA).
102. The cell of any one of paragraphs 85-101, wherein the first and/or second vector is a plasmid vector or a viral vector.
103. A eukaryotic cell, comprising
- (a) a first vector comprising (i) an N-terminal fragment of an *egfp* gene, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a first molecule of interest; and
- (b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of an intein, which is upstream from a C-terminal fragment of an *egfp* gene and (ii) a second molecule of interest,
- wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of a protein fragment encoded by the N-terminal fragment of the *egfp* gene to a protein fragment encoded by the C-terminal fragment of the *egfp* gene to produce full-length EGFP protein.
104. The cell of paragraph 103, wherein the protein fragment encoded by the N-terminal fragment of the *egfp* gene comprises an amino acid sequence identified by amino acids 1-175 of SEQ ID NO: 5, and the protein fragment encoded by the C-terminal fragment of the *egfp* gene comprises an amino acid sequence identified by amino acids 175-239 of SEQ ID NO: 5.
105. The cell of paragraph 103 or 104, wherein

the N-terminal fragment of the intein is identified by SEQ ID NO:16, and the C-terminal fragment of the intein is identified by SEQ ID NO:17;

the N-terminal fragment of the intein is identified by SEQ ID NO:7, and the C-terminal fragment of the intein is identified by SEQ ID NO:8; or

5 the N-terminal fragment of the intein is identified by SEQ ID NO:18 or SEQ ID NO:9, and the C-terminal fragment of the intein is identified by SEQ ID NO:19 or SEQ ID NO:10.

106. A eukaryotic cell, comprising

(a) a first vector comprising (i) an N-terminal fragment of an *mScarlet* gene, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a first molecule of interest; and

(b) a second vector comprising (ii) a nucleotide sequence encoding a C-terminal fragment of an intein, which is upstream from a C-terminal fragment of an *mScarlet* gene and (ii) a second molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of a protein fragment encoded by the N-terminal fragment of the *mScarlet* gene to a protein fragment encoded by the C-terminal fragment of the *mScarlet* gene to produce full-length mScarlet protein.

107. The cell of paragraph 106, wherein

the protein fragment encoded by the N-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 1-46 of SEQ ID NO: 6, and the protein fragment encoded by the C-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 47-232 of SEQ ID NO: 6;

the protein fragment encoded by the N-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 1-48 of SEQ ID NO: 6, and the protein fragment encoded by the C-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 49-232 of SEQ ID NO: 6;

the protein fragment encoded by the N-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 1-51 of SEQ ID NO: 6, and the protein fragment encoded by the C-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 52-232 of SEQ ID NO: 6;

the protein fragment encoded by the N-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 1-75 of SEQ ID NO: 6, and the protein fragment encoded by the C-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 76-232 of SEQ ID NO: 6;

the protein fragment encoded by the N-terminal fragment of the *mScarlet* gene comprises an

amino acid sequence identified by amino acids 1-122 of SEQ ID NO: 6, and the protein fragment encoded by the C-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 123-232 of SEQ ID NO: 6;

the protein fragment encoded by the N-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 1-140 of SEQ ID NO: 6, and the protein fragment encoded by the C-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 141-232 of SEQ ID NO: 6;

the protein fragment encoded by the N-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 1-163 of SEQ ID NO: 6, and the protein fragment encoded by the C-terminal fragment of the *mScarlet* gene comprises an amino acid sequence identified by amino acids 164-232 of SEQ ID NO: 6.

108. The cell of paragraph 106 or 107, wherein

the N-terminal fragment of the intein is identified by SEQ ID NO:16, and the C-terminal fragment of the intein is identified by SEQ ID NO:17;

the N-terminal fragment of the intein is identified by SEQ ID NO:7, and the C-terminal fragment of the intein is identified by SEQ ID NO:8; or

the N-terminal fragment of the intein is identified by SEQ ID NO:18 or SEQ ID NO:9, and the C-terminal fragment of the intein is identified by SEQ ID NO:19 or SEQ ID NO:10.

109. A composition comprising the cell of any one of paragraph 85-108.

110. A kit, comprising

(a) a first vector comprising a nucleotide sequence encoding an N-terminal fragment of an antibiotic resistance protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein; and

(b) a second vector comprising a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the antibiotic resistance protein,

wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of the N-terminal fragment and the C-terminal fragment of the antibiotic resistance protein to produce a full-length antibiotic resistance protein.

111. The kit of paragraph 110, wherein the antibiotic resistance protein confers resistance to hygromycin, G418, puromycin, phleomycin D1 or blasticidin.

112. A kit, comprising

(a) a first vector comprising a nucleotide sequence encoding an N-terminal fragment of a fluorescent protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein; and

(b) a second vector comprising a nucleotide sequence encoding a C-terminal fragment of

the intein, which is upstream from a C-terminal fragment of the fluorescent protein,

wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of the N-terminal fragment of the fluorescent protein to the C-terminal fragment of the fluorescent protein to produce a full-length fluorescent protein.

5 113. The kit of paragraph 112, wherein the fluorescent protein is selected from TagCFP, mTagCFP2, Czurite, ECFP2, mKalama1, Sirius, Sapphire, T-Sapphire, ECFP, Cerulean, SCFP3C, mTurquoise, mTurquoise2, monomeric Midoriishi-Cyan, TagCFP, mTFP1, EGFP, Emerald, Superfolder GFP, Monomeric Czami Green, TagGFP2, mUKG, mWasabi, Clover, mNeonGreen, EYFP, Citrine, Venus, SYFP2, TagYFP, Monomeric Kusabira-Orange, mKOκ, mKO2, mOrange, 10 mOrange2, mRaspberry, mCherry, mStrawberry, mTangerine, tdTomato, TagRFP, TagRFP-T, mCppl, mRuby, mRuby2, mPlum, HcRed-Tandem, mKate2, mNeptune, NirFP, TagRFP657, IFP1.4 and iRFP.

114. The kit of any one of paragraphs 110-113, wherein the intein is a split intein.

115. The kit of paragraph 114, wherein the split intein is a natural split intein or an engineered 15 split intein.

116. The kit of paragraph 115, wherein the natural split intein is selected from DnaE inteins.

117. The kit of paragraph 116, wherein the DnaE inteins are selected from *Synechocystis sp.* DnaE (*SspDnaE*) inteins and *Nostoc punctiforme* (*NpuDnaE*) inteins.

118. The kit of paragraph 115, wherein the engineered split intein is engineered from DnaB 20 inteins or GyrB inteins.

119. The kit of paragraph 118, wherein the engineered split intein is a *SspDnaB* S1 intein.

120. The kit of paragraph 118, wherein the engineered split intein is a *SspGyrB* S11 intein.

121. The kit of any one of paragraphs 112-120, further comprising any one or more of the following components: buffers, salts, cloning enzymes, competent cells, transfection reagents, 25 antibiotics, and/or instructions for performing the methods described herein.

122. A method comprising delivering to eukaryotic cells

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of an antibiotic resistance protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of 30 interest,

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the antibiotic resistance protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest,

35 and

(c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the antibiotic resistance protein and (ii) a nucleotide sequence encoding a third molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the antibiotic resistance protein to the central fragment of the antibiotic resistance protein, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of central fragment of the antibiotic resistance protein to the C-terminal fragment of the antibiotic resistance protein, to produce a full-length antibiotic resistance protein.

123. The method of paragraph 112 further comprising maintaining the eukaryotic cells under conditions that permit introduction of the first, second, and third vectors into the eukaryotic cells to produce transgenic eukaryotic cells.

124. The method of paragraph 123 further comprising selecting the transgenic eukaryotic cells that comprise the full-length antibiotic resistance protein.

125. The method of any one of paragraphs 112-124, wherein the eukaryotic cells are mammalian cells.

126. The method of any one of paragraphs 112-125, wherein the antibiotic resistance protein confers resistance to hygromycin, G418, puromycin, phleomycin D1 or blasticidin.

127. The method of paragraph 126, wherein the antibiotic resistance protein confers resistance to hygromycin.

128. The method of any one of paragraphs 112-127, wherein the first intein is a split intein.

129. The method of any one of paragraphs 112-128, wherein the second intein is a split intein.

130. The method of paragraph 128 or 129, wherein the split intein is a natural split intein.

131. The method of paragraph 130, wherein the natural split intein is selected from DnaE inteins.

132. The method of paragraph 131, wherein the DnaE inteins are selected from *Synechocystis* sp.

DnaE (*Ssp*DnaE) inteins and *Nostoc punctiforme* (*Npu*DnaE) inteins.

133. The method of paragraph 132, wherein the first intein is an *Npu*DnaE intein and the second intein is an *Npu*DnaE intein.

134. The method of any one of paragraphs 112-133, wherein the first molecule of interest, second molecule of interest, third molecule of interest, or any combination thereof is a protein.

135. The method of any one of paragraphs 112-133, wherein the first molecule of interest, second molecule of interest, third molecule of interest, or any combination thereof is a non-coding ribonucleic acid (RNA).

136. The method of paragraph 135, wherein the non-coding RNA is a microRNA (miRNA), antisense RNA, short-interfering RNA (siRNA) or short-hairpin RNA (shRNA).

137. The method of any one of paragraphs 112-136, wherein the first vector, second vector, third

vector, or any combination thereof, is a plasmid vector or a viral vector.

138. A method comprising delivering to eukaryotic cells

(a) a first vector comprising (i) an N-terminal fragment of a *hygB* gene, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest,

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a central fragment of the *hygB* gene, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, and

(c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a C-terminal fragment of the *hygB* gene and (ii) a nucleotide sequence encoding a third molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of the protein fragment encoded by N-terminal fragment of the *hygB* gene to a protein fragment encoded by the central fragment of the *hygB* gene, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of the protein fragment encoded by the central fragment of the *hygB* gene to the protein fragment encoded by the C-terminal fragment of the *hygB* gene, to produce a full-length hygromycin B phosphotransferase.

139. The method of paragraph 138, wherein the first vector encodes the sequence identified by SEQ ID NO: 29, the second vector encodes the sequence identified by SEQ ID NO: 61, and the third vector encodes the sequence identified by SEQ ID NO: 23.

140. The method of paragraph 138, wherein the first vector encodes the sequence identified by SEQ ID NO: 21, the second vector encodes the sequence identified by SEQ ID NO: 61, and the third vector encodes the sequence identified by SEQ ID NO: 35.

141. A eukaryotic cell comprising:

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of an antibiotic resistance protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest,

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the antibiotic resistance protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, and

(c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of

the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the antibiotic resistance protein and (ii) a nucleotide sequence encoding a third molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the antibiotic resistance protein to the central fragment of the antibiotic resistance protein, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of central fragment of the antibiotic resistance protein to the C-terminal fragment of the antibiotic resistance protein, to produce a full-length antibiotic resistance protein.

142. The eukaryotic cell of paragraph 112, wherein the eukaryotic cells are mammalian cells.

143. The eukaryotic cell of paragraph 141 or 142, wherein the antibiotic resistance protein confers resistance to hygromycin, G418, puromycin, phleomycin D1 or blasticidin.

144. The eukaryotic cell of paragraph 143, wherein the antibiotic resistance protein confers resistance to hygromycin.

145. The eukaryotic cell of any one of paragraphs 141-144, wherein the first intein is a split intein.

146. The eukaryotic cell of any one of paragraphs 142-145, wherein the second intein is a split intein.

147. The eukaryotic cell of paragraph 145 or 146, wherein the split intein is a natural split intein.

148. The eukaryotic cell of paragraph 147, wherein the natural split intein is selected from DnaE inteins.

149. The eukaryotic cell of paragraph 148, wherein the DnaE inteins are selected from *Synechocystis* sp. DnaE (*SspDnaE*) inteins and *Nostoc punctiforme* (*NpuDnaE*) inteins.

150. The eukaryotic cell of paragraph 149, wherein the first intein is an *NpuDnaE* intein and the second intein is an *NpuDnaE* intein.

151. The eukaryotic cell of any one of paragraphs 142-150, wherein the first molecule of interest, second molecule of interest, third molecule of interest, or any combination thereof is a protein.

152. The eukaryotic cell of any one of paragraphs 142-150, wherein the first molecule of interest, second molecule of interest, third molecule of interest, or any combination thereof is a non-coding ribonucleic acid (RNA).

153. The eukaryotic cell of paragraph 152, wherein the non-coding RNA is a microRNA (miRNA), antisense RNA, short-interfering RNA (siRNA) or short-hairpin RNA (shRNA).

154. The eukaryotic cell of any one of paragraphs 142-153, wherein the first vector, second vector, third vector, or any combination thereof, is a plasmid vector or a viral vector.

155. A composition comprising the eukaryotic cell of any one of paragraph 142-154.

156. A kit comprising:

(a) a first vector comprising a nucleotide sequence encoding an N-terminal fragment of an

antibiotic resistance protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein,

(b) a second vector comprising a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the antibiotic resistance protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein, and

(c) a third vector comprising a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the antibiotic resistance protein,

wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the antibiotic resistance protein to the central fragment of the antibiotic resistance protein, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of central fragment of the antibiotic resistance protein to the C-terminal fragment of the antibiotic resistance protein, to produce a full-length antibiotic resistance protein.

157. The kit of paragraph 156, wherein the antibiotic resistance protein confers resistance to hygromycin, G418, puromycin, phleomycin D1 or blasticidin.

158. The kit of paragraph 157, wherein the antibiotic resistance protein confers resistance to hygromycin.

159. The kit of any one of paragraphs 156-158, wherein the first intein is a split intein.

160. The kit of any one of paragraphs 156-159, wherein the second intein is a split intein.

161. The kit of paragraph 159 or 160, wherein the split intein is a natural split intein.

162. The kit of paragraph 161, wherein the natural split intein is selected from DnaE inteins.

163. The kit of paragraph 162, wherein the DnaE inteins are selected from *Synechocystis sp.* DnaE (*SspDnaE*) inteins and *Nostoc punctiforme* (*NpuDnaE*) inteins.

164. The kit of paragraph 163, wherein the first intein is an *NpuDnaE* intein and the second intein is an *NpuDnaE* intein.

165. The kit of any one of paragraphs 156-164, wherein the first molecule of interest, second molecule of interest, third molecule of interest, or any combination thereof is a protein.

166. The kit of any one of paragraphs 156-164, wherein the first molecule of interest, second molecule of interest, third molecule of interest, or any combination thereof is a non-coding ribonucleic acid (RNA).

167. The kit of paragraph 166, wherein the non-coding RNA is a microRNA (miRNA), antisense RNA, short-interfering RNA (siRNA) or short-hairpin RNA (shRNA).

168. The kit of any one of paragraphs 156-167, wherein the first vector, second vector, third vector, or any combination thereof, is a plasmid vector or a viral vector.

169. A method comprising delivering to eukaryotic cells

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a fluorescent protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest,

5 (b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the fluorescent protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, and

(c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of
10 the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the fluorescent protein and (ii) a nucleotide sequence encoding a third molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the fluorescent protein to the central fragment of the fluorescent protein, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze
15 joining of central fragment of the fluorescent protein to the C-terminal fragment of the fluorescent protein, to produce a full-length fluorescent protein.

170. The method of paragraph 169 further comprising maintaining the eukaryotic cells under conditions that permit introduction of the first, second, and third vectors into the eukaryotic cells to produce transgenic eukaryotic cells.

20 171. The method of paragraph 170 further comprising selecting the transgenic eukaryotic cells that comprise the full-length fluorescent protein.

172. The method of any one of paragraphs 169-171, wherein the eukaryotic cells are mammalian cells.

173. The method of any one of paragraphs 169-172, wherein the fluorescent protein is selected
25 from TagCFP, mTagCFP2, Czurite, ECFP2, mKalama1, Sirius, Sapphire, T-Sapphire, ECFP, Cerulean, SCFP3C, mTurquoise, mTurquoise2, monomeric Midoriishi-Cyan, TagCFP, mTFP1, EGFP, Emerald, Superfolder GFP, Monomeric Czami Green, TagGFP2, mUKG, mWasabi, Clover, mNeonGreen, EYFP, Citrine, Venus, SYFP2, TagYFP, Monomeric Kusabira-Orange, mKOκ, mScarlet, mKO2, mOrange, mOrange2, mRaspberry, mCherry, mStrawberry, mTangerine,
30 tdTomato, TagRFP, TagRFP-T, mCppl, mRuby, mRuby2, mPlum, HcRed-Tandem, mKate2, mNeptune, NirFP, TagRFP657, IFP1.4 and iRFP.

174. The method of paragraph 173, wherein the fluorescent protein is mScarlet.

175. The method of any one of paragraphs 169-174, wherein the first intein is a split intein.

176. The method of any one of paragraphs 169-175, wherein the second intein is a split intein.

35 177. The method of paragraph 175 or 176, wherein the split intein is a natural split intein.

178. The method of paragraph 177, wherein the natural split intein is selected from DnaE inteins.

179. The method of paragraph 178, wherein the DnaE inteins are selected from *Synechocystis sp.* DnaE (*Ssp*DnaE) inteins and *Nostoc punctiforme* (*Npu*DnaE) inteins.

180. The method of paragraph 179, wherein the first intein is an *Npu*DnaE intein and the second
5 intein is an *Npu*DnaE intein.

181. The method of any one of paragraphs 169-170, wherein the first molecule of interest, second molecule of interest, third molecule of interest, or any combination thereof is a protein.

182. The method of any one of paragraphs 169-180, wherein the first molecule of interest, second molecule of interest, third molecule of interest, or any combination thereof is a non-coding
10 ribonucleic acid (RNA).

183. The method of paragraph 182, wherein the non-coding RNA is a microRNA (miRNA), antisense RNA, short-interfering RNA (siRNA) or short-hairpin RNA (shRNA).

184. The method of any one of paragraphs 169-183, wherein the first vector, second vector, third vector, or any combination thereof, is a plasmid vector or a viral vector.

15 185. A method comprising delivering to eukaryotic cells

(a) a first vector comprising (i) an N-terminal fragment of a mScarlet gene, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest,

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of
20 the first intein, which is upstream from a central fragment of the mScarlet gene, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, and

(c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a C-terminal fragment of the mScarlet gene and (ii) a
25 nucleotide sequence encoding a third molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of the protein fragment encoded by N-terminal fragment of the mScarlet gene to a protein fragment encoded by the central fragment of the mScarlet gene, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of the protein fragment encoded by the
30 central fragment of the mScarlet gene to the protein fragment encoded by the C-terminal fragment of the mScarlet gene, to produce a full-length mScarlet protein.

186. The method of paragraph 185, wherein the first vector encodes the sequence identified by SEQ ID NO: 121, the second vector encodes the sequence identified by SEQ ID NO: 123, and the third vector encodes the sequence identified by SEQ ID NO: 125.

35 187. A eukaryotic cell comprising:

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a fluorescent protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest,

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the fluorescent protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, and

(c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the fluorescent protein and (ii) a nucleotide sequence encoding a third molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the fluorescent protein to the central fragment of the fluorescent protein, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of central fragment of the fluorescent protein to the C-terminal fragment of the fluorescent protein, to produce a full-length fluorescent protein.

188. The eukaryotic cell of paragraph 187 wherein the eukaryotic cells are mammalian cells.

189. The eukaryotic cell of paragraph 187 or 188, wherein the fluorescent protein is selected from TagCFP, mTagCFP2, Czurite, ECFP2, mKalama1, Sirius, Sapphire, T-Sapphire, ECFP, Cerulean, SCFP3C, mTurquoise, mTurquoise2, monomeric Midoriishi-Cyan, TagCFP, mTFP1, EGFP, Emerald, Superfolder GFP, Monomeric Czami Green, TagGFP2, mUKG, mWasabi, Clover, mNeonGreen, EYFP, Citrine, Venus, SYFP2, TagYFP, Monomeric Kusabira-Orange, mKOκ, mScarlet, mKO2, mOrange, mOrange2, mRaspberry, mCherry, mStrawberry, mTangerine, tdTomato, TagRFP, TagRFP-T, mCppl, mRuby, mRuby2, mPlum, HcRed-Tandem, mKate2, mNeptune, NirFP, TagRFP657, IFP1.4 and iRFP.

190. The eukaryotic cell of paragraph 189, wherein the fluorescent protein is mScarlet.

191. The eukaryotic cell of any one of paragraphs 187-190, wherein the first intein is a split intein.

192. The eukaryotic cell of any one of paragraphs 185-191, wherein the second intein is a split intein.

193. The eukaryotic cell of paragraph 191 or 192, wherein the split intein is a natural split intein.

194. The eukaryotic cell of paragraph 193, wherein the natural split intein is selected from DnaE inteins.

195. The eukaryotic cell of paragraph 194, wherein the DnaE inteins are selected from *Synechocystis sp.* DnaE (*SspDnaE*) inteins and *Nostoc punctiforme* (*NpuDnaE*) inteins.

196. The eukaryotic cell of paragraph 195, wherein the first intein is an *NpuDnaE* intein and the

second intein is an *Npu*DnaE intein.

197. The eukaryotic cell of any one of paragraphs 185-196, wherein the first molecule of interest, second molecule of interest, third molecule of interest, or any combination thereof is a protein.

198. The eukaryotic cell of any one of paragraphs 185-196, wherein the first molecule of interest, second molecule of interest, third molecule of interest, or any combination thereof is a non-coding ribonucleic acid (RNA).

199. The eukaryotic cell of paragraph 198, wherein the non-coding RNA is a microRNA (miRNA), antisense RNA, short-interfering RNA (siRNA) or short-hairpin RNA (shRNA).

200. The eukaryotic cell of any one of paragraphs 185-199, wherein the first vector, second vector, third vector, or any combination thereof, is a plasmid vector or a viral vector.

201. A composition comprising the eukaryotic cell of any one of paragraph 185-200.

202. A kit comprising:

(a) a first vector comprising a nucleotide sequence encoding an N-terminal fragment of a fluorescent protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein,

(b) a second vector comprising a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the fluorescent protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein, and

(c) a third vector comprising a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the fluorescent protein,

wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the fluorescent protein to the central fragment of the fluorescent protein, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of central fragment of the fluorescent protein to the C-terminal fragment of the fluorescent protein, to produce a full-length fluorescent protein.

203. The kit of paragraph 202, wherein the fluorescent protein is selected from TagCFP, mTagCFP2, Czurite, ECFP2, mKalama1, Sirius, Sapphire, T-Sapphire, ECFP, Cerulean, SCFP3C, mTurquoise, mTurquoise2, monomeric Midoriishi-Cyan, TagCFP, mTFP1, EGFP, Emerald, Superfolder GFP, Monomeric Czami Green, TagGFP2, mUKG, mWasabi, Clover, mNeonGreen, EYFP, Citrine, Venus, SYFP2, TagYFP, Monomeric Kusabira-Orange, mKOκ, mScarlet, mKO2, mOrange, mOrange2, mRaspberry, mCherry, mStrawberry, mTangerine, tdTomato, TagRFP, TagRFP-T, mCapple, mRuby, mRuby2, mPlum, HcRed-Tandem, mKate2, mNeptune, NirFP, TagRFP657, IFP1.4 and iRFP.

204. The kit of paragraph 203, wherein the fluorescent protein is mScarlet.
205. The kit of any one of paragraphs 202-204, wherein the first intein is a split intein.
206. The kit of any one of paragraphs 202-205, wherein the second intein is a split intein.
207. The kit of paragraph 206, wherein the split intein is a natural split intein.
- 5 208. The kit of paragraph 207, wherein the natural split intein is selected from DnaE inteins.
209. The kit of paragraph 208, wherein the DnaE inteins are selected from *Synechocystis sp.* DnaE (*Ssp*DnaE) inteins and *Nostoc punctiforme* (*Npu*DnaE) inteins.
210. The kit of paragraph 209, wherein the first intein is an *Npu*DnaE intein and the second intein is an *Npu*DnaE intein.
- 10 211. The kit of any one of paragraphs 202-210, wherein the first molecule of interest, second molecule of interest, third molecule of interest, or any combination thereof is a protein.
212. The kit of any one of paragraphs 202-210, wherein the first molecule of interest, second molecule of interest, third molecule of interest, or any combination thereof is a non-coding ribonucleic acid (RNA).
- 15 213. The kit of paragraph 212, wherein the non-coding RNA is a microRNA (miRNA), antisense RNA, short-interfering RNA (siRNA) or short-hairpin RNA (shRNA).
214. The kit of any one of paragraphs 202-213, wherein the first vector, second vector, third vector, or any combination thereof, is a plasmid vector or a viral vector.
215. The kit of any one of paragraphs 202-214, further comprising any one or more of the
- 20 following components: buffers, salts, cloning enzymes, competent cells, transfection reagents, antibiotics, and/or instructions for performing the methods described herein.
216. A transgenic selection method comprising delivering to a composition comprising eukaryotic cells (a) a first vector comprising (i) a nucleotide sequence encoding a first selectable marker protein fragment (e.g., antibiotic resistance protein fragment or fluorescent protein
- 25 fragment) upstream from a nucleotide sequence encoding an N-terminal intein protein fragment and (ii) a nucleotide sequence encoding a first molecule, and (b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal intein protein fragment upstream from a second selectable marker protein fragment (e.g., antibiotic resistance protein fragment or fluorescent protein fragment) and (ii) a nucleotide sequence encoding a second molecule, wherein the N-
- 30 terminal intein protein fragment and the C-terminal intein protein fragment catalyze joining of the first selectable marker protein fragment to the second selectable marker protein fragment to produce a full-length selectable marker protein.
217. A transgenic selection method comprising delivering to eukaryotic cells (a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker
- 35 protein (e.g., antibiotic resistance protein or fluorescent protein), which is upstream from a

nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest, (b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, and (c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a third molecule of interest, wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the selectable marker protein to the central fragment of the selectable markerprotein, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of central fragment of the selectable markerprotein to the C-terminal fragment of the selectable markerprotein, to produce a full-length selectable markerprotein.

218. A transgenic selection method comprising delivering to eukaryotic cells (a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker protein (e.g., antibiotic resistance protein or fluorescent protein), which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest, (b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a first central fragment of the selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, (c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a second central fragment of the selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a third intein and (ii) a nucleotide sequence encoding a third molecule of interest, and (d) a fourth vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the third intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a third molecule of interest, wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the selectable marker protein to the first central fragment of the selectable marker protein, the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of first central fragment of the selectable markerprotein to the second central fragment of the selectable marker protein, the N-terminal fragment and the C-terminal fragment of the third intein catalyze joining of second central fragment

of the selectable marker protein to the C-terminal fragment of the selectable marker protein to produce a full-length selectable marker protein.

219. The method of any one of paragraphs 216-218 further comprising maintaining the eukaryotic cells under conditions that permit introduction of the vectors into the eukaryotic cells to produce transgenic eukaryotic cells.

220. The method of paragraph 219 further comprising selecting the transgenic eukaryotic cells that comprise the full-length selectable marker protein.

221. The method of any one of paragraphs 216-220, wherein the eukaryotic cells are mammalian cells.

222. The method of any one of paragraphs 216-221, wherein the antibiotic resistance protein confers resistance to hygromycin, G418, puromycin, phleomycin D1 or blasticidin.

223. The method of any one of paragraphs 216-222, wherein the intein is a split intein.

224. The method of paragraph 223, wherein the split intein is a natural split intein.

225. The method of paragraph 224, wherein the natural split intein is selected from DnaE inteins.

226. The method of paragraph 225, wherein the DnaE inteins are selected from *Synechocystis sp.* DnaE (*SspDnaE*) inteins and *Nostoc punctiforme* (*NpuDnaE*) inteins.

227. The method of paragraph 223, wherein the split intein is an engineered split intein.

228. The method of paragraph 227, wherein the engineered split intein is engineered from DnaB inteins.

229. The method of paragraph 228, wherein the engineered split intein is a *SspDnaB* S1 intein.

230. The method of paragraph 229, wherein the engineered split intein is engineered from GyrB inteins.

231. The method of paragraph 230, wherein the engineered split intein is a *SspGyrB* S11 intein.

232. The method of any one of paragraphs 216-231, wherein the molecules are selected from proteins.

233. The method of any one of paragraphs 216-231, wherein the molecules are selected from non-coding ribonucleic acids (RNAs).

234. The method of paragraph 233, wherein the non-coding RNAs are selected from microRNAs (miRNAs), antisense RNAs, short-interfering RNAs (siRNAs), and short-hairpin RNAs (shRNAs).

235. The method of any one of paragraphs 216-234, wherein the vectors are selected from plasmid vectors and viral vectors.

EXAMPLES

The present disclosure is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the disclosure, and should not be construed as a limitation

thereof.

Example 1. Antibiotic Resistance Markers

Selectable markers are often used in genetic engineering to isolate cells with desired
5 genotypes [1]. However, there are a limited number of well-characterized antibiotic resistance genes
for use in eukaryotic cells and a limited number of fluorescent proteins whose spectra can be
unambiguously differentiated by equipment in ordinary laboratories. Researchers often run into the
problem of not having enough choices of selectable markers if they are to incorporate multiple
transgenes into a cell. On the other hand, selection with multiple antibiotics at the same time is often
10 harsh to cells. “Selectable marker recycling” may provide a work-around, however, requiring
multiple rounds of transgenesis, selection and removal of selection markers [2]. To allow multiple
transgenes to be selected by one selection scheme at the same time, we have created split antibiotics
resistance and fluorescent protein genes wherein a gene encoding an antibiotic resistance or
fluorescent protein is split into two or more segments fused to inteins (“markertrons”) that can be
15 rejoined by protein trans-splicing [3] (**FIG. 1A**). Each markertron is inserted onto a transgenic
vector carrying a specific transgene. Delivery of transgenic vectors containing a set of markertrons
yield cells harboring a subset or a complete set of the markertrons. Only cells containing a complete
set of markertrons produce a fully reconstituted marker protein via protein splicing and thus passes
through selection while cells with partial sets of markertrons are eliminated, achieving co-selection
20 of cells containing all intended transgenes.

We started out with engineering 2-markertron intein-split resistance (Intres) genes for
double transgenesis. Since flanking residues and local protein folding can affect efficiency of intein-
mediated trans-splicing, we set out to identify split points in each of the four commonly used
antibiotic resistance genes compatible with two well-characterized split inteins derived from
25 NpuDnaE [4, 5] and SspDnaB [6]. To facilitate assessment of the effectiveness of double transgenic
selection, we cloned markertrons onto lentiviral vectors expressing TagBFP2 or mCherry
fluorescent proteins as test transgenes (**FIG. 1B**). Viral preparations were transduced into U2OS
cells which were then split into replicate plates with non-selective or selective media. Following
appropriate passages for antibiotics selection, the two cell cultures were analyzed by flow
30 cytometry. For hygromycin (Hygro) resistance gene, one “native” SspDnaB split point (G200:S201)
with flanking residues “GS” and one “native” NpuDnaE split point (Y89:C90) with “YC” residues
were tested. Both enabled successful selection when both N- and C-markertrons were transduced
yielding >99% BFP+ mCherry+ double transgenic cells in selected cultures compared to <10%
double-positive cells in non-selected culture (**FIG. 3**; Plasmid pairs 3,4 and 5,6). Cells transduced
35 with either of the two markertrons did not survive hygromycin selection. In contrast, double

transgenesis with conventional full-length non-split hygromycin vectors only allow for ~20% enrichment of BFP+ mCherry+ cells (Plasmid pairs 97,98). We screened three additional potential split points (52S:53C), (240A:241C), and (292R:293C) for NpuDnaE with the obligatory cysteine residue on the C-extein junction and a residue on the N-extein junction that supported substantial trans-splicing activities in a previous report 7. We also incorporated six additional NpuDnaE split points by inserting an “artificial” cysteine on the C-extein junction to support splicing at ectopic sites yielding additional split points. In total, eight out of eleven split points tested supported hygromycin selection (**FIG. 3**). Similarly, for puromycin (Puro) (**FIG. 4**), neomycin (Neo) (**FIG. 5**) and blasticidin (Blast) (**FIG. 6**) resistance genes, we identified four, two, and one functional Intres pair(s), respectively. In all of these cases, cells transduced with either markertrons did not survive selection, while cells transduced with both yielded >95% double transgenic cells in selective cultures compared to <50% in non-selective cultures with the exception of Blasticidin(102) Intres, achieving lower but still significant enrichment of 91% double transgenic cells (**FIGS. 3-6**). Details of the split points of Intres genes and plasmids are presented in **FIGS. 2A-2D** and **Table 1**.

Table 1. Plasmids

Plasmid #	Plasmid Name	Markertron
3	pLX-Hygro(1-89)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-89)-NpuDnaE(N)
4	pLX-NpuDnaE(C)-Hygro(90-341)-IRES-mCherry	NpuDnaE(C)-Hygro(90-341)
5	pLX-Hygro(1-200)-SspDnaB(N)-IRES-TagBFP2	Hygro(1-200)-SspDnaB(N)
6	pLX-SspDnaB(C)-Hygro(201-341)-IRES-mCherry	SspDnaB(C)-Hygro(201-341)
7	pLX-Hygro(1-52)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-52)-NpuDnaE(N)
8	pLX-NpuDnaE(C)-Hygro(53-341)-IRES-mCherry	NpuDnaE(C)-Hygro(53-341)
9	pLX-Hygro(1-240)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-240)-NpuDnaE(N)
10	pLX-NpuDnaE(C)-Hygro(241-341)-IRES-mCherry	NpuDnaE(C)-Hygro(241-341)
11	pLX-Hygro(1-292)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-292)-NpuDnaE(N)
12	pLX-NpuDnaE(C)-Hygro(293-341)-IRES-mCherry	NpuDnaE(C)-Hygro(293-341)
13	pLX-Blast(1-102)-NpuDnaE(N)-IRES-TagBFP2	Blast(1-102)-NpuDnaE(N)
14	pLX-NpuDnaE(C)-Blast(103-140)-IRES-mCherry	NpuDnaE(C)-Blast(103-140)
17	pLX-Puro(1-119)-NpuDnaE(N)-IRES-TagBFP2	Puro(1-119)-NpuDnaE(N)
18	pLX-NpuDnaE(C)-Puro(insCys; 120-199)-IRES-mCherry	NpuDnaE(C)-Puro(insCys; 120-199)
19	pLX-Puro(1-100)-SspDnaB(N-S0)-IRES-TagBFP2	Puro(1-100)-SspDnaB(N-S0)
20	pLX-SspDnaB(C-S0)-Puro(101-199)-IRES-mCherry	SspDnaB(C-S0)-Puro(101-199)
21	pLX-Neo(1-133)-NpuDnaE(N)-IRES-TagBFP2	Neo(1-133)-NpuDnaE(N)
22	pLX-NpuDnaE(C)-Neo(134-267)-IRES-mCherry	NpuDnaE(C)-Neo(134-267)
23	pLX-Neo(1-194)-NpuDnaE(N)-IRES-TagBFP2	Neo(1-194)-NpuDnaE(N)
24	pLX-NpuDnaE(C)-Neo(195-267)-IRES-mCherry	NpuDnaE(C)-Neo(195-267)
25	pLX-NpuDnaE(C)_Hygro(53-89)-NpuDnaE(N)-IRES-GFP	NpuDnaE(C)_Hygro(53-89)-NpuDnaE(N)
26	pLX-NpuDnaE(C)_Hygro(53-239)-NpuDnaE(N)-IRES-GFP	NpuDnaE(C)_Hygro(53-239)-NpuDnaE(N)
27	pCR8-Bsal->ccdbCam<-Bsal-NpuDnaE(N)-MD1-68-15	
28	pCR8-NpuDnaE(C)_Bsal->ccdbCam<-Bsal-MD1-68-18	
29	pCR8-Bsal->ccdbCam<-Bsal-SspDnaE(N)-MD1-68-12	
30	pCR8-SspDnaE(C)_Bsal->ccdbCam<-Bsal-MD1-68-13	
31	pCR8-Bsal->ccdbCam<-Bsal-SspDnaB(N-S0)-25-135-18	
32	pCR8-SspDnaB(C-S0)_Bsal->ccdbCam<-Bsal-25-155-41	

Plasmid #	Plasmid Name	Markertron
33	pLX-mScarlet(1-46)-NpuDnaE(N)_LZA-IRES-TagBFP2	mScarlet(1-46)-NpuDnaE(N)_LZA
34	pLX-LZB_NpuDnaE(C)-mScarlet(insCys;47-232)-IRES-TagBFP2	LZB_NpuDnaE(C)-mScarlet(insCys;47-232)
35	pLX-mScarlet(1-48)-NpuDnaE(N)_LZA-IRES-TagBFP2	mScarlet(1-48)-NpuDnaE(N)_LZA
36	pLX-LZB_NpuDnaE(C)-mScarlet(insCys;49-232)-IRES-GFP	LZB_NpuDnaE(C)-mScarlet(insCys;49-232)
37	pLX-mScarlet(1-51)-NpuDnaE(N)_LZA-IRES-TagBFP2	mScarlet(1-51)-NpuDnaE(N)_LZA
38	pLX-LZB_NpuDnaE(C)-mScarlet(insCys;52-232)-IRES-GFP	LZB_NpuDnaE(C)-mScarlet(insCys;52-232)
39	pLX-mScarlet(1-75)-NpuDnaE(N)_LZA-IRES-TagBFP2	mScarlet(1-75)-NpuDnaE(N)_LZA
40	pLX-LZB_NpuDnaE(C)-mScarlet(insCys;76-232)-IRES-GFP	LZB_NpuDnaE(C)-mScarlet(insCys;76-232)
41	pLX-mScarlet(1-122)-NpuDnaE(N)_LZA-IRES-TagBFP2	mScarlet(1-122)-NpuDnaE(N)_LZA
42	pLX-LZB_NpuDnaE(C)-mScarlet(insCys;123-232)-IRES-GFP	LZB_NpuDnaE(C)-mScarlet(insCys;123-232)
43	pLX-mScarlet(1-140)-NpuDnaE(N)_LZA-IRES-TagBFP2	mScarlet(1-140)-NpuDnaE(N)_LZA
44	pLX-LZB_NpuDnaE(C)-mScarlet(insCys;141-232)-IRES-GFP	LZB_NpuDnaE(C)-mScarlet(insCys;141-232)
45	pLX-mScarlet(1-163)-NpuDnaE(N)_LZA-IRES-TagBFP2	mScarlet(1-163)-NpuDnaE(N)_LZA
46	pLX-LZB_NpuDnaE(C)-mScarlet(insCys;164-232)-IRES-GFP	LZB_NpuDnaE(C)-mScarlet(insCys;164-232)
47	pCR8-TagBFP2	TagBFP2
48	pCR8-mCherry	mCherry
49	pLX-DEST-IRES-Hygro(1-89)-NpuDnaE(N)	Hygro(1-89)-NpuDnaE(N)
50	pLX-DEST-IRES-NpuDnaE(C)-Hygro(90-341)	NpuDnaE(C)-Hygro(90-341)
51	pLX-[TagBFP2]-IRES-Hygro(1-89)-NpuDnaE(N)	Hygro(1-89)-NpuDnaE(N)
52	pLX-[mCherry]-IRES-NpuDnaE(C)-Hygro(90-341)	NpuDnaE(C)-Hygro(90-341)
53	pLX-DEST-IRES-Puro(1-119)-NpuDnaE(N)	Puro(1-119)-NpuDnaE(N)
54	pLX-DEST-IRES-NpuDnaE(C)-Puro(120-199)	NpuDnaE(C)-Puro(insCys;120-199)
55	pLX-[TagBFP2]-IRES-Puro(1-119)-NpuDnaE(N)	Puro(1-119)-NpuDnaE(N)
56	pLX-[mCherry]-IRES-NpuDnaE(C)-Puro(120-199)	NpuDnaE(C)-Puro(insCys;120-199)
57	pLX-DEST-IRES-Neo(1-194)-NpuDnaE(N)	Neo(1-194)-NpuDnaE(N)
58	pLX-DEST-IRES-NpuDnaE(C)-Neo(195-267)	NpuDnaE(C)-Neo(195-267)
59	pLX-[TagBFP2]-IRES-Neo(1-194)-NpuDnaE(N)	Neo(1-194)-NpuDnaE(N)
60	pLX-[mCherry]-IRES-NpuDnaE(C)-Neo(195-267)	NpuDnaE(C)-Neo(195-267)
64	pLX-Hygro(1-69)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-69)-NpuDnaE(N)
65	pLX-NpuDnaE(C)-Hygro(^C;70-341)-IRES-mCherry	NpuDnaE(C)-Hygro(^C;70-341)
66	pLX-Hygro(1-131)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-131)-NpuDnaE(N)
67	pLX-NpuDnaE(C)-Hygro(^C;132-341)-IRES-mCherry	NpuDnaE(C)-Hygro(^C;132-341)
68	pLX-Hygro(1-171)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-171)-NpuDnaE(N)
69	pLX-NpuDnaE(C)-Hygro(^C;172-341)-IRES-mCherry	NpuDnaE(C)-Hygro(^C;172-341)
70	pLX-Hygro(1-218)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-218)-NpuDnaE(N)
71	pLX-NpuDnaE(C)-Hygro(^C;219-341)-IRES-mCherry	NpuDnaE(C)-Hygro(^C;219-341)
72	pLX-Hygro(1-259)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-259)-NpuDnaE(N)
73	pLX-NpuDnaE(C)-Hygro(^C;260-341)-IRES-mCherry	NpuDnaE(C)-Hygro(^C;260-341)
74	pLX-Hygro(1-277)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-277)-NpuDnaE(N)
75	pLX-NpuDnaE(C)-Hygro(^C;278-341)-IRES-mCherry	NpuDnaE(C)-Hygro(^C;278-341)
76	pLX-Puro(1-32)-NpuDnaE(N)-IRES-TagBFP2	Puro(1-32)-NpuDnaE(N)
77	pLX-NpuDnaE(C)-Puro(^C;33-199)-IRES-mCherry	NpuDnaE(C)-Puro(^C;33-199)
78	pLX-Puro(1-84)-NpuDnaE(N)-IRES-TagBFP2	Puro(1-84)-NpuDnaE(N)
79	pLX-NpuDnaE(C)-Puro(^C;85-199)-IRES-mCherry	NpuDnaE(C)-Puro(^C;85-199)
80	pLX-Puro(1-137)-NpuDnaE(N)-IRES-TagBFP2	Puro(1-137)-NpuDnaE(N)
81	pLX-NpuDnaE(C)-Puro(^C;138-199)-IRES-mCherry	NpuDnaE(C)-Puro(^C;138-199)
82	pLX-Puro(1-158)-NpuDnaE(N)-IRES-TagBFP2	Puro(1-158)-NpuDnaE(N)

Plasmid #	Plasmid Name	Marker
83	pLX-NpuDnaE(C)-Puro(^C;159-199)-IRES-mCherry	NpuDnaE(C)-Puro(^C;159-199)
84	pLX-Puro(1-180)-NpuDnaE(N)-IRES-TagBFP2	Puro(1-180)-NpuDnaE(N)
85	pLX-NpuDnaE(C)-Puro(^C;181-199)-IRES-mCherry	NpuDnaE(C)-Puro(^C;181-199)
86	pLX-Blast(1-58)-NpuDnaE(N)-IRES-TagBFP2	Blast(1-58)-NpuDnaE(N)
87	pLX-NpuDnaE(C)-Blast(59-140)-IRES-mCherry	NpuDnaE(C)-Blast(59-140)
88	pLX-NpuDnaE(C)-HygroBA-SspDnaB(N-S0)-IRES-EGFP	NpuDnaE(C)-Hygro(53-200)-SspDnaB(N-S0)
89	pLX-SspDnaB(C-S0)-Hygro(201-341)-IRES-mCherry	SspDnaB(C-S0)-Hygro(201-341)
90	pLX-NpuDnaE(C)-Hygro(90-200)-SspDnaB(N-S0)-IRES-EGFP	NpuDnaE(C)-Hygro(90-200)-SspDnaB(N-S0)
91	pLX-Hygro(1-200)-SspDnaB(N-S0)-IRES-TagBFP2	Hygro(1-200)-SspDnaB(N-S0)
92	pLX-SspDnaB(C-S0)-Hygro(201-240)-NpuDnaE(N)-IRES-EGFP	SspDnaB(C-S0)-Hygro(201-240)-NpuDnaE(N)
93	pLX-SspDnaB(C-S0)-Hygro(201-292)-NpuDnaE(N)-IRES-EGFP	SspDnaB(C-S0)-Hygro(201-292)-NpuDnaE(N)
94	pLX-DEST-IRES-TagBFP2	
95	pLX-DEST-IRES-EGFP	
96	pLX-DEST-IRES-mCherry	
97	pLX-Hygro-IRES-TagBFP2	Non-split Hygro
98	pLX-Hygro-IRES-mCherry	Non-split Hygro
99	pLX-Puro-IRES-TagBFP2	Non-split Puro
100	pLX-Puro-IRES-mCherry	Non-split Puro
101	pLX-Hygro-IRES-EGFP	Non-split Hygro
102	pLX-NLS_GFP-IRES-Hygro	Non-split Hygro
103	pLX-LifeAct_mCherry-IRES-Hygro	Non-split Hygro
104	pLX-NLS_GFP-IRES-Hygro(1-89)-NpuDnaE(N)	Hygro(1-89)-NpuDnaE(N)
105	pLX-LifeAct_mScarlet-IRES- NpuDnaE(C)-Hygro(90-341)	NpuDnaE(C)-Hygro(90-341)
106	pX330-AAVS1	
107	pAAVS1-Nst-EF1aHygro2ArtTA3(-)_TetO-Blast-P2A-EGFP	Non-split Blast
108	pAAVS1-Nst-EF1aHygro2ArtTA3(-)_TetO-Blast-P2A-mScarlet	Non-split Blast
109	pAAVS1-Nst-EF1aHygro2ArtTA3(-)_TetO-Blast(1-102)_NpuDnaE(N)-P2A-EGFP	Blast(1-102)-NpuDnaE(N)
110	pAAVS1-Nst-EF1aHygro2ArtTA3(-)_TetO-NpuDnaE(C)_Blast(103-140)-P2A-mScarlet	NpuDnaE(C)-Blast(103-140)
111	pAAVS1-Nst-EF1aBlast2ArtTA3(-)_TetO-Hygro-P2A-NTR-E2A-EGFP	Non-split Hygro
112	pAAVS1-Nst-EF1aBlast2ArtTA3(-)_TetO-Hygro-P2A-NTR-E2A-mCherry	Non-split Hygro
113	pAAVS1-Nst-EF1aBlast2ArtTA3(-)_TetO- Hygro(1-89)-NpuDnaE(N)-P2A-NTR-E2A-EGFP	Hygro(1-89)-NpuDnaE(N)
114	pAAVS1-Nst-EF1aBlast2ArtTA3(-)_TetO- NpuDnaE(C)-Hygro(90-341)-P2A-NTR-E2A-mCherry	NpuDnaE(C)-Hygro(90-341)
115	pLX-Hygro(1-89)_NpuDnaE(N)_LZA-IRES-TagBFP2	Hygro(1-89)-NpuDnaE(N)-LZA
116	pLX-LZB_NpuDnaGEP(C)_Hygro(90-200)_SspDnaB(N-S0)-IRES-GFP	LZB-NpuDnaGEP(C)-Hygro(90-200)-SspDnaB(N-S0)
117	pLX-SspDnaB(C-S0)_Hygro(201-240)_NpuDnaE(N)_LZA-IRES-GFP	SspDnaB(C-S0)-Hygro(201-240)-NpuDnaE(N)-LZA
118	pLX-LZB_NpuDnaGEP(C)_Hygro(241-341)-IRES-mCherry	LZB-NpuDnaGEP(C)-Hygro(241-341)

Example 2. Gateway-Compatible Lentiviral Vectors

To facilitate adoption of Intres markers, we created Gateway-compatible lentiviral vectors for convenient restriction-ligation-independent LR clonase recombination of transgenes 8 (FIG.

7A). We tested the functionality of these vectors by recombining TagBFP2 and mCherry, respectively to the N- and C-Intres vectors and found robust selection of double transgenic cells (FIG. 7B). One potential utility of Intres vectors is to install different fluorescent markers in cells to label different cellular compartments. To explore such utility, we cloned in NLS-GFP and LifeAct-mScarlet 9, which label nucleus and F-actin, respectively, by Gateway recombination to conventional full length (FL) non-split hygromycin selectable vectors or 2-markertron hygromycin Intres vectors and transduced cells with either sets of plasmids, followed by antibiotic selection (FIG. 7C). The sample transduced with non-split selectable plasmids contained both singly and doubly labelled cells, while cell transduced with Intres plasmids were all doubly labelled (FIG. 7C).

Example 3. Fluorescent Markers

To test whether split fluorescent markers can be used for transgene selection, we screened for NpuDnaE split points for mScarlet fluorescent protein (FIG. 8A) and identified four split points allowing for >96% enrichment of double transgenic cells and three other split points enabling >60% enrichment of double transgenic cells in mScarlet-gated population, compared to <20% double transgenic cells in non-gated population (FIG. 8B).

Example 4. Higher Degree Split Markers

With the split points identified for 2-markertron Intres genes, we set out to engineer higher degree split markers. We tested combinations of splits points to partition a marker gene into three or more markertrons to allow for co-selection of more than two “unlinked” transgenes with one antibiotics (FIGS. 9A-9B). To identify pairs of split points that would allow such “Intres chain”, we cloned 3-split markertrons into three lentiviral vectors each carrying one of three fluorescent transgenes TagBFP2, EGFP, or mCherry, that will allow us to assess effectiveness of selection by flow cytometry (FIG. 9C). Since hygromycin resistance gene is the longest and provides the most split points for testing, we focused on engineering 3-markertron hygromycin Intres. We tested two 3-markertron hygromycin Intres using two intervening NpuDnaE inteins, two using NpuDnaE for the first intein and SspDnaB for the second intein, as well as two using SspDnaB for the first intein and NpuDnaE for the second intein (FIG. 9D). Five of these six 3-markertron hygromycin Intres enabled >97% and with the remaining one enabling 80% triple transgenic selection in hygromycin-selected cultures compared to <15% triple transgenic cells in non-selected cultures. Samples with leave-one-out transduction did not yield any viable cells after hygromycin selection while cells transduced with non-split hygromycin vectors yielded only 7% triple transgenic cells after selection.

To facilitate the use of 3-markertron Intres, we created Gateway compatible lentiviral vectors with these markers (FIG. 10A). Three sets of these vectors were each tested by recombining

TagBFP (as transgene 1), EGFP (as transgene 2) and mCherry (as transgene 3) into the N-, M-, and C-Intres Gateway destination vectors and used to transduce U2OS cells, which were then split and cultured in hygromycin selection or non-selective media (**FIG. 10B**). Two weeks after selection, cells were analyzed by flow cytometry. All three sets of 3-markertron hygromycin Intres plasmids support triple transgenic cell selection of >99% compared to <25% in the non-selected cultures (**FIG. 10C**).

We further tested the feasibility of 4-markertron hygromycin Intres genes (**FIG. 11**). Here, we used an enhanced variant of NpuDnaE intein known as NpuDnaGEP 10 fused with leucine zipper motifs 11 in combination with the SspDnaB intein. While transduction of all four plasmids containing constituent markertrons produced cells that survived hygromycin selection, leave-one-out transduction did not yield any survival (**Table 2**).

Table 2. Survival of cells transduced with (“+”) or without (“-“) lentiviruses prepared from the indicated plasmids.

	Plasmid 115	Plasmid 116	Plasmid 117	Plasmid 118	Survival
Sample 1	+	+	+	+	Yes
Sample 2	-	+	+	+	No
Sample 3	+	-	+	+	No
Sample 4	+	+	-	+	No
Sample 5	+	+	+	-	No

Example 5. Biallelic Knock-In at the AAVS1 Locus

CRISPR/Cas has recently emerged as a powerful technology for genome engineering and editing. Although gene knockout based on NHEJ-mediated insertions/deletions (indels) occur at high frequency, precise editing and knock-in based on homology directed repair (HDR) using exogenous repair templates (a.k.a targeting constructs) are inefficient. We tested whether split selectable markers can be used to enrich for cells with biallelic knock-in at the AAVS1 locus. We constructed targeting constructs with homology arms flanking the target site, and splice acceptor-2A peptide to trap the markertrons within intron one of the host gene PPP1R12C. However, we did not obtain any live cells after CRISPR/Cas knock-in experiments using these targeting constructs and two weeks of antibiotic selection (data not shown). We suspected that the endogenous promoter of the host gene PPP1R12C might not drive sufficient expression of markertrons to reconstitute enough antibiotic resistance protein to counteract actions of the antibiotics. We thus tested an alternative strategy to express Intres markertrons by TetO promoter whose activity can be titrated by doxycycline (dox) concentration. To allow comparison of Intres-mediated biallelic selection versus full-length (FL) non-split selectable markers, we implemented several different targeting

construct designs. First, we drive expression of a full-length (FL) resistance gene (e.g., Hygro) together with rtTA under a constitutive EF1a promoter and a separate test Intres (e.g., Blast Intres) under a dox-inducible TetO promoter (**FIG. 12B**, Plasmids 109 and 110). This allow comparison of full length and split selectable markers within the same constructs. To allow fair comparison of full length versus split markers driven by the same TetO promoter, we constructed two similar plasmids 107 and 108 (cf. Plasmids 109 and 110), wherein full-length antibiotic resistance gene (Blast) is placed downstream of the TetO promoter (**FIG. 12A**). To enable single-cell quantification of biallelic targeting and to demonstrate the feasibility of incorporating two transgenes into two AAVS1 alleles, we appended EGFP and mScarlet fluorescent genes downstream of the test split or non-split markers via self-cleaving 2A peptide. Similarly, to test Hygro Intres, we swapped the EF1a and TetO-driven markers so that FL Hygro or Hygro Intres were placed downstream of TetO and FL Blast downstream of EF1a (**FIGS. 12C-12D**; Plasmids 111-114). We co-transfected pX330-AAVS1 (Plasmid 106) containing Cas9 and sgRNA targeting AAVS1, and the different pairs of targeting constructs to HEK293T cells, split into triplicate doxycycline-containing media without antibiotics, with blasticidin, or with hygromycin at the subsequent passages. Two weeks after selection, we analyzed the cultures for biallelic targeting by flow cytometric measurement of GFP and RFP fluorescence (**FIG. 12E**). As expected, non-selected cultures harbored small fraction (<1%) of biallelic knock-in GFP+/RFP+ cells (**FIG. 12E**; Selection = None). Selection of antibiotics where corresponding FL antibiotic resistance genes were present on targeting constructs yielded < 30% biallelic knock-in cells (**FIG. 12E**; Blast: TC a,c,d; Hygro: TC a,b,c). In contrast, selection of antibiotics where corresponding Intres are present on the targeting constructs yielded 75% (Fig 6e; Blast Intres: TC b) and 88% (Fig 6e; Hygro Intres: TC d) biallelic knock-in cells.

In the Examples above, we have engineered split antibiotic resistance and fluorescent protein genes that can allow selection for two or more “unlinked” transgenes. By inserting unnatural residues at selectable markers, we showed that novel high-efficiency split points can be utilized, expanding the positions available for engineering. We demonstrated that split selectable markers can be incorporated into lentiviral vectors or gene targeting constructs in CRISPR/Cas9 genome editing experiments to enable enrichment of cells with double transgenesis or biallelic knock-ins. By combining two or more splits points, we showed that 3- and 4-split markers can be generated to allow higher degree transgenic selection. Future development of even higher-degree split selectable markers may enable “hyper-engineering” of cells containing tens of transgenes or targeted knock-ins.

Materials and Methods

Cloning

To generate a test plasmid for each markertron, we first generated a Gateway donor plasmid containing its ORF and then recombine into lentiviral destination vector with TagBFP2 (Plasmid 94: pLX-DEST-IRES-TagBFP2), EGFP (Plasmid 95: pLX-DEST-IRES-EGFP), or mCherry (Plasmid 96: pLX-DEST-IRES-mCherry) reporters, which were derived from pLX302 (addgene.org/25896/) by removing Puromycin resistance gene and inserting IRES-fluorescent genes downstream of the Gateway cassette. The markertron-ORF Gateway donor plasmids were generated either by a nested fusion PCR procedure to combine intein with the coding sequence of fragments of the selectable marker followed by insertion into the pCR8-GW-TOPO plasmid by sequence- and ligation-independent cloning (SLIC) (Li, M.Z. & Elledge, S.J. SLIC: a method for sequence-and ligation-independent cloning. *Gene Synthesis: Methods and Protocols*, 51-59 (2012)), or PCR-amplifying the relevant fragment of the selectable marker followed by insertion into “scaffold” plasmids (Plasmids 27~32) containing the intein sequences by SLIC. DNA sequences encoding inteins were codon optimized for Homo sapiens, and synthesized as GBlock (IDT), with AC1947GB encoding NpuDnaE intein, AC1949GB encoding SspDnaB intein. Selectable marker fragments were amplified from plasmids containing these markers. See **Table 1** for plasmids.

Cell Culture

All cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Sigma) with 10% fetal bovine serum (FBS)(Lonza), 4% Glutamax (Gibco), 1% Sodium Pyruvate (Gibco) and penicillin-streptomycin (Gibco). Incubator conditions were 37°C and 5% CO₂.

Virus Production

A viral packaging mix of pLP1, pLP2, and VSV-G were co-transfected with each lentiviral vector into Lenti-X 293T cells (ClonTech), seeded the day before in 6-well plates at a concentration of 1.2×10^6 cells per well, using Lipofectamine 3000. Media was changed 6h after transfection then incubated overnight. 28 hour post transfection, the media supernatant containing virus was filtered using 45uM PES filters then stored at -80°C until use.

Transduction

The day prior to transduction, target cells (HEK293T, MCF7, U2-OS) were seeded into 12-well plates at a density of 1.5×10^5 cells per well. Prior to transduction, media was changed to media containing 10 µg/mL polybrene, 1 mL per well. 250 µL of each respective virus (500 µL total for experimental samples with two viruses added) was added to each well and incubated overnight. Media was changed 24 hour post infection. 4 day post infection cells were split into duplicate plates. 5 day post infection media with antibiotic (hygromycin) was added to each respective well of one replicate plate (the other remained under no selection). Antibiotic selection continued for 2

weeks before analysis on FACS.

Fluorescent-Activated Cell Sorting

Cells were trypsinized, suspended in media then analyzed on a LSRFortessa X-20 (BD Bioscience) flow cytometer using FACSDiVa software, version 8, on an HP Z230 workstation.

5 Fifty thousand events were collected each run.

CONSTRUCTS AND SEQUENCES

NpuDnaE(N)

CLSYETEILTVEYGLLPKIVEKRIECTVYSVDNNGNIYTQPVAQWHDRGEQEVFEYCLEDGSLIRATKDHKF
10 MTVDGQMLPIDEIFERELDLMRVDNLPN (SEQ ID NO: 7)

NpuDnaE(C)

IKIATRKYLGKQNVYDIGVERDHNFALKNGFIASN (SEQ ID NO: 8)

SspDnaB(N-S0)

CISGDSLISLASTGKRVSIDLLDEKDFEIWAINETMKLESASVSRVFCTGKKLVYILKTRLGRTIKATANHRF
15 LTIDGWRKRLDELSLKEHIALPRKLESSSLQL (SEQ ID NO: 9)

SspDnaB(C-S0)

20 SPEIEKLSQSDIYWDSIVSITETGVVEEVDLTVPGPHNFVANDIIVHN (SEQ ID NO: 10)

NpuDnaE(N)-LZA

CLSYETEILTVEYGLLPKIVEKRIECTVYSVDNNGNIYTQPVAQWHDRGEQEVFEYCLEDGSLIRATKDHKF
MTVDGQMLPIDEIFERELDLMRVDNLPNGGGGSGSAQLEKELQALEKKLAQLEWENQALEKELAQ (SEQ ID
25 NO: 11)

LZB-NpuDnaGFP(C)

AQLKKKLQANKKELAQKWKQLQALKKKLAQGGGGSGSMIKIATRKYLGKQNVYDIGVGEPHNFALKNGFIA
30 SN (SEQ ID NO: 12)

NpuDnaGFP(C)

IKIATRKYLGKQNVYDIGVGEPHNFALKNGFIASN (SEQ ID NO: 13)

LZA

35 AQLEKELQALEKKLAQLEWENQALEKELAQ (SEQ ID NO: 14)

LZB

AQLKKKLQANKKELAQKWKQLQALKKKLAQ (SEQ ID NO: 15)

40

SspDnaE(N)

CLSFGTEILTVEYGPLPIGKIVSEEINCSVYSVDPEGRVYTQAIAQWHDRGEQEVLEYELEDGSVIRATSDHRFL
TTDYQLLAIEEIFARQLDLLTLENIKQTTEEALDNHRLPFPLLDAGTIK (SEQ ID NO:16)

5 **SspDnaE(C)**

VKVIGRRSLGVQRIFDIGLPQDHNFLLANGAIAAN (SEQ ID NO:17)

SspDnaB(N)

10 CISGDSLISLA (SEQ ID NO:18)

SspDnaB(C)

STGKRVS IKDLLDEKDFEIWAIN EQTMKLESAKVS RVFCTGKKLVYILKTRLGRTIKATANHRFLTIDGWKRLD
ELSLKEHIALPRKLESSSLQLSPEIEKLSQSDIYWDSIVSITETGVVEEVFDLTVPGPHNFVANDIIVHN (SEQ ID
15 NO:19)

Plasmid 3: pLX-Hygro(1-89)-NpuDnaE(N)-IRES-TagBFP2

Protein = Hygro(1-89)-NpuDnaE(N)

Vector sequence (SEQ ID NO:20)

20 Amino acid sequence (SEQ ID NO:21)

Plasmid 4: pLX-NpuDnaE(C)-Hygro(90-341)-IRES-mCherry

Protein = NpuDnaE(C)-Hygro(90-341)

Vector sequence (SEQ ID NO:22)

25 Amino acid sequence (SEQ ID NO:23)

Plasmid 5: pLX-Hygro(1-200)-SspDnaB(N)-IRES-TagBFP2

Protein = Hygro(1-200)-SspDnaB(N)

Vector sequence (SEQ ID NO:24)

30 Amino acid sequence (SEQ ID NO:25)

Plasmid 6: pLX-SspDnaB(C)-Hygro(201-341)-IRES-mCherry

Protein = SspDnaB(C)-Hygro(201-341)

Vector sequence (SEQ ID NO:26)

35 Amino acid sequence (SEQ ID NO:27)

Plasmid 7: pLX-Hygro(1-52)-NpuDnaE(N)-IRES-TagBFP2

Protein = Hygro(1-52)-NpuDnaE(N)

Vector sequence (SEQ ID NO:28)

40 Amino acid sequence (SEQ ID NO:29)

Plasmid 8: pLX-NpuDnaE(C)-Hygro(53-341)-IRES-mCherry

Protein = NpuDnaE(C)-Hygro(53-341)

Vector sequence (SEQ ID NO:30)

Amino acid sequence (SEQ ID NO:31)

5

Plasmid 9: pLX-Hygro(1-240)-NpuDnaE(N)-IRES-TagBFP2

Protein = Hygro(1-240)-NpuDnaE(N)

Vector sequence (SEQ ID NO:32)

Amino acid sequence (SEQ ID NO:33)

10

Plasmid 10: pLX-NpuDnaE(C)-Hygro(241-341)-IRES-mCherry

Protein = NpuDnaE(C)-Hygro(241-341)

Vector sequence (SEQ ID NO:34)

Amino acid sequence (SEQ ID NO:35)

15

Plasmid 11: pLX-Hygro(1-292)-NpuDnaE(N)-IRES-TagBFP2

Protein = Hygro(1-292)-NpuDnaE(N)

Vector sequence (SEQ ID NO:36)

20

Amino acid sequence (SEQ ID NO:37)

Plasmid 12: pLX-NpuDnaE(C)-Hygro(293-341)-IRES-mCherry

Protein = NpuDnaE(C)-Hygro(293-341)

25

Vector sequence (SEQ ID NO:38)

Amino acid sequence (SEQ ID NO:39)

Plasmid 13: pLX-Blast(1-102)-NpuDnaE(N)-IRES-TagBFP2

Protein = Blast(1-102)-NpuDnaE(N)

30

Vector sequence (SEQ ID NO:40)

Amino acid sequence (SEQ ID NO:41)

Plasmid 14: pLX-NpuDnaE(C)-Blast(103-140)-IRES-mCherry

35

Protein = NpuDnaE(C)-Blast(103-140)

Vector sequence (SEQ ID NO:42)

Amino acid sequence (SEQ ID NO:43)

40

Plasmid 17: pLX-Puro(1-119)-NpuDnaE(N)-IRES-TagBFP2

Protein = Puro(1-119)-NpuDnaE(N)

Vector sequence (SEQ ID NO:44)

Amino acid sequence (SEQ ID NO:45)

45

Plasmid 18: pLX-NpuDnaE(C)-Puro(120-199)-IRES-mCherry

Protein = NpuDnaE(C)-Puro(insCys;120-199)

Vector sequence (SEQ ID NO:46)

Amino acid sequence (SEQ ID NO:47)

5 **Plasmid 19:** pLX-Puro(1-100)-SspDnaB(N-S0)-IRES-TagBFP2

Protein = Puro(1-100)-SspDnaB(N-S0)

Vector sequence (SEQ ID NO:48)

Amino acid sequence (SEQ ID NO:49)

10 **Plasmid 20:** pLX-SspDnaB(C-S0)-Puro(101-199)-IRES-mCherry

Protein = SspDnaB(C-S0)-Puro(101-199)

Vector sequence (SEQ ID NO:50)

Amino acid sequence (SEQ ID NO:51)

15 **Plasmid 21:** pLX-Neo(1-133)-NpuDnaE(N)-IRES-TagBFP2

Protein = Neo(1-133)-NpuDnaE(N)

Vector sequence (SEQ ID NO:52)

Amino acid sequence (SEQ ID NO:53)

20 **Plasmid 22:** pLX-NpuDnaE(C)-Neo(134-267)-IRES-mCherry

Protein = NpuDnaE(C)-Neo(134-267)

Vector sequence (SEQ ID NO:54)

Amino acid sequence (SEQ ID NO:55)

25 **Plasmid 23:** pLX-Neo(1-194)-NpuDnaE(N)-IRES-TagBFP2

Protein = Neo(1-194)-NpuDnaE(N)

Vector sequence (SEQ ID NO:56)

Amino acid sequence (SEQ ID NO:57)

30 **Plasmid 24:** pLX-NpuDnaE(C)-Neo(195-267)-IRES-mCherry

Protein = NpuDnaE(C)-Neo(195-267)

Vector sequence (SEQ ID NO:58)

Amino acid sequence (SEQ ID NO:59)

35 **Plasmid 25:** pLX-NpuDnaE(C)_Hygro(53-89)-NpuDnaE(N)-IRES-GFP

Protein = NpuDnaE(C)_Hygro(53-89)-NpuDnaE(N)

Vector sequence (SEQ ID NO:60)

Amino acid sequence (SEQ ID NO:61)

40 **Plasmid 26:** pLX-NpuDnaE(C)_Hygro(53-239)-NpuDnaE(N)-IRES-GFP

Protein = NpuDnaE(C)_Hygro(53-239)-NpuDnaE(N)

Vector sequence (SEQ ID NO:62)

Amino acid sequence (SEQ ID NO:63)

Plasmid 27: pCR8-BsaI->ccdbCam<-BsaI-NpuDnaE(N)-MD1-68-15 (SEQ ID NO:64)

Plasmid 28: pCR8-NpuDnaE(C)_BsaI->ccdbCam<-BsaI-MD1-68-18 (SEQ ID NO:65)

Plasmid 29: pCR8-BsaI->ccdbCam<-BsaI-SspDnaE(N)-MD1-68-12 (SEQ ID NO:66)

Plasmid 30: pCR8-SspDnaE(C)_BsaI->ccdbCam<-BsaI-MD1-68-13 (SEQ ID NO:67)

Plasmid 31: pCR8-BsaI->ccdbCam<-BsaI-SspDnaB(N-S0)-25-135-18 (SEQ ID NO:68)

Plasmid 32: pCR8-SspDnaB(C-S0)_BsaI->ccdbCam<-BsaI-25-155-41 (SEQ ID NO:69)

Plasmid 33: pLX-mScarlet(1-46)-NpuDnaE(N)_LZA-IRES-TagBFP2

Protein = mScarlet(1-46)-NpuDnaE(N)_LZA

Vector sequence (SEQ ID NO:70)

Amino acid sequence (SEQ ID NO:71)

Plasmid 34: pLX-LZB_NpuDnaE(C)-mScarlet(insCys;47-232)-IRES-TagBFP2

Protein = LZB_NpuDnaE(C)-mScarlet(insCys;47-232)

Vector sequence (SEQ ID NO:72)

Amino acid sequence (SEQ ID NO:73)

Plasmid 35: pLX-mScarlet(1-48)-NpuDnaE(N)_LZA-IRES-TagBFP2

Protein = mScarlet(1-48)-NpuDnaE(N)_LZA

Vector sequence (SEQ ID NO:74)

Amino acid sequence (SEQ ID NO:75)

Plasmid 36: pLX-LZB_NpuDnaE(C)-mScarlet(insCys;49-232)-IRES-GFP

Protein = LZB_NpuDnaE(C)-mScarlet(insCys;49-232)

Vector sequence (SEQ ID NO:76)

Amino acid sequence (SEQ ID NO:77)

Plasmid 37: pLX-mScarlet(1-51)-NpuDnaE(N)_LZA -IRES-TagBFP2

Protein = mScarlet(1-51)-NpuDnaE(N)_LZA

Vector sequence (SEQ ID NO:78)

Amino acid sequence (SEQ ID NO:79)

Plasmid 38: pLX-LZB_NpuDnaE(C)-mScarlet(insCys;52-232)-IRES-GFP

Protein = LZB_NpuDnaE(C)-mScarlet(insCys;52-232)

Vector sequence (SEQ ID NO:80)

Amino acid sequence (SEQ ID NO:81)

5 Plasmid 39: pLX-mScarlet(1-75)-NpuDnaE(N)_LZA-IRES-TagBFP2

Protein = mScarlet(1-75)-NpuDnaE(N)_LZA

Vector sequence (SEQ ID NO:82)

Amino acid sequence (SEQ ID NO:83)

10 Plasmid 40: pLX-LZB_NpuDnaE(C)-mScarlet(insCys;76-232)-IRES-GFP

Protein = LZB_NpuDnaE(C)-mScarlet(insCys;76-232)

Vector sequence (SEQ ID NO:84)

Amino acid sequence (SEQ ID NO:85)

15 Plasmid 41: pLX-mScarlet(1-122)-NpuDnaE(N)_LZA-IRES-TagBFP2

Protein = mScarlet(1-122)-NpuDnaE(N)_LZA

Vector sequence (SEQ ID NO:86)

Amino acid sequence (SEQ ID NO:87)

20 Plasmid 42: pLX-LZB_NpuDnaE(C)-mScarlet(insCys;123-232)-IRES-GFP

Protein = LZB_NpuDnaE(C)-mScarlet(insCys;123-232)

Vector sequence (SEQ ID NO:88)

Amino acid sequence (SEQ ID NO:89)

25

Plasmid 43: pLX-mScarlet(1-140)-NpuDnaE(N)_LZA-IRES-TagBFP2

Protein = mScarlet(1-140)-NpuDnaE(N)_LZA

Vector sequence (SEQ ID NO:90)

Amino acid sequence (SEQ ID NO:91)

30

Plasmid 44: pLX-LZB_NpuDnaE(C)-mScarlet(insCys;141-232)-IRES-GFP

Protein = LZB_NpuDnaE(C)-mScarlet(insCys;141-232)

Vector sequence (SEQ ID NO:92)

Amino acid sequence (SEQ ID NO:93)

35

Plasmid 45: pLX-mScarlet(1-163)-NpuDnaE(N)_LZA-IRES-TagBFP2

Protein = mScarlet(1-163)-NpuDnaE(N)_LZA

Vector sequence (SEQ ID NO:94)

Amino acid sequence (SEQ ID NO:95)

40

Plasmid 46: pLX-LZB_NpuDnaE(C)-mScarlet(insCys;164-232)-IRES-GFP

Protein = LZB_NpuDnaE(C)-mScarlet(insCys;164-232)

Vector sequence (SEQ ID NO:96)

Amino acid sequence (SEQ ID NO:97)

Plasmid 47: pCR8-TagBFP2

5 Protein = TagBFP2

Vector sequence (SEQ ID NO:98)

Amino acid sequence (SEQ ID NO:99)

Plasmid 48: pCR8-mCherry

10 Protein = mCherry

Vector sequence (SEQ ID NO:100)

Amino acid sequence (SEQ ID NO:101)

Plasmid 49: pLX-DEST-IRES-Hygro(1-89)-NpuDnaE(N)

15 Protein = Hygro(1-89)-NpuDnaE(N)

Vector sequence (SEQ ID NO:102)

Amino acid sequence (SEQ ID NO:103)

Plasmid 50: pLX-DEST-IRES-NpuDnaE(C)-Hygro(90-341)

20 Protein = NpuDnaE(C)-Hygro(90-341)

Vector sequence (SEQ ID NO: 104)

Amino acid sequence (SEQ ID NO: 105)

Plasmid 51: pLX-[TagBFP2]-IRES-Hygro(1-89)-NpuDnaE(N)

25 Vector sequence (SEQ ID NO: 106)

Plasmid 52: pLX-[mCherry]-IRES-NpuDnaE(C)-Hygro(90-341)

Vector sequence (SEQ ID NO: 107)

30 **Plasmid 53:** pLX-DEST-IRES-Puro(1-119)-NpuDnaE(N)

Protein = Puro(1-119)-NpuDnaE(N)

Vector sequence (SEQ ID NO: 108)

Amino acid sequence (SEQ ID NO:109)

35 **Plasmid 54:** pLX-DEST-IRES-NpuDnaE(C)-Puro(120-199)

Protein = NpuDnaE(C)-Puro(120-199)

Vector sequence (SEQ ID NO:110)

Amino acid sequence (SEQ ID NO:111)

40 **Plasmid 55:** pLX-[TagBFP2]-IRES-Puro(1-119)-NpuDnaE(N)

Vector sequence (SEQ ID NO:112)

Plasmid 56: pLX-[mCherry]-IRES-NpuDnaE(C)-Puro(120-199)

Vector sequence (SEQ ID NO:113)

Plasmid 57: pLX-DEST-IRES-Neo(1-194)-NpuDnaE(N)

Protein = Neo(1-194)-NpuDnaE(N)

5 Vector sequence (SEQ ID NO:114)

Amino acid sequence (SEQ ID NO:115)

Plasmid 58: pLX-DEST-IRES-NpuDnaE(C)-Neo(195-267)

Protein = NpuDnaE(C)-Neo(195-267)

10 Vector sequence (SEQ ID NO:116)

Amino acid sequence (SEQ ID NO:117)

Plasmid 59: pLX-[TagBFP2]-IRES-Neo(1-194)-NpuDnaE(N)

Vector sequence (SEQ ID NO:118)

15

Plasmid 60: pLX-[mCherry]-IRES-NpuDnaE(C)-Neo(195-267)

Vector sequence (SEQ ID NO:119)

Plasmid 61: pLX-mScarlet(1-51)-NpuDnaE(N)-LZA-IRES-TagBFP2

20 Protein = mScarlet(1-51)-NpuDnaE(N)-LZA

Vector sequence (SEQ ID NO:120)

Amino acid sequence (SEQ ID NO:121)

Plasmid 62: pLX-LZB-NpuDnaE(C)-mScarlet(^C;52-163)-NpuDnaE(N)_LZA-IRES-EGFP

25 Protein = LZB-NpuDnaE(C)-mScarlet(^C;52-163)-NpuDnaE(N)_LZA

Vector sequence (SEQ ID NO:122)

Amino acid sequence (SEQ ID NO:123)

Plasmid 63: pLX-LZB-NpuDnaE(C)-mScarlet(^C;164-232)-IRES-EGFP

30 Protein = LZB-NpuDnaE(C)-mScarlet(^C;164-232)

Vector sequence (SEQ ID NO:124)

Amino acid sequence (SEQ ID NO:125)

Plasmid 64: pLX-Hygro(1-69)-NpuDnaE(N)-IRES-TagBFP2

35 Protein = Hygro(1-69)-NpuDnaE(N)

Vector sequence (SEQ ID NO:126)

Amino acid sequence (SEQ ID NO:127)

Plasmid 65: pLX-NpuDnaE(C)-Hygro(^C;70-341)-IRES-mCherry

40 Protein = NpuDnaE(C)-Hygro(^C;70-341)

Vector sequence (SEQ ID NO:128)

Amino acid sequence (SEQ ID NO:129)

Plasmid 66: pLX-Hygro(1-131)-NpuDnaE(N)-IRES-TagBFP2

Protein = Hygro(1-131)-NpuDnaE(N)

5 Vector sequence (SEQ ID NO:130)

Amino acid sequence (SEQ ID NO:131)

Plasmid 67: pLX-NpuDnaE(C)-Hygro(^C;132-341)-IRES-mCherry

10 Protein = NpuDnaE(C)-Hygro(^C;132-341)

Vector sequence (SEQ ID NO:132)

Amino acid sequence (SEQ ID NO:133)

Plasmid 68: pLX-Hygro(1-171)-NpuDnaE(N)-IRES-TagBFP2

15 Protein = Hygro(1-171)-NpuDnaE(N)

Vector sequence (SEQ ID NO:134)

Amino acid sequence (SEQ ID NO:135)

Plasmid 69: pLX-NpuDnaE(C)-Hygro(^C;172-341)-IRES-mCherry

20 Protein = NpuDnaE(C)-Hygro(^C;172-341)

Vector sequence (SEQ ID NO:136)

Amino acid sequence (SEQ ID NO:137)

Plasmid 70: pLX-Hygro(1-218)-NpuDnaE(N)-IRES-TagBFP2

25 Protein = Hygro(1-218)-NpuDnaE(N)

Vector sequence (SEQ ID NO:138)

Amino acid sequence (SEQ ID NO:139)

Plasmid 71: pLX-NpuDnaE(C)-Hygro(^C;219-341)-IRES-mCherry

30 Protein = NpuDnaE(C)-Hygro(^C;219-341)

Vector sequence (SEQ ID NO:140)

Amino acid sequence (SEQ ID NO:141)

Plasmid 72: pLX-Hygro(1-259)-NpuDnaE(N)-IRES-TagBFP2

35 Protein = Hygro(1-259)-NpuDnaE(N)

Vector sequence (SEQ ID NO:142)

Amino acid sequence (SEQ ID NO:143)

Plasmid 73: pLX-NpuDnaE(C)-Hygro(^C;260-341)-IRES-mCherry

40 Protein = NpuDnaE(C)-Hygro(^C;260-341)

Vector sequence (SEQ ID NO:144)

Amino acid sequence (SEQ ID NO:145)

Plasmid 74: pLX-Hygro(1-277)-NpuDnaE(N)-IRES-TagBFP2

Protein = Hygro(1-277)-NpuDnaE(N)

5 Vector sequence (SEQ ID NO:146)

Amino acid sequence (SEQ ID NO:147)

Plasmid 75: pLX-NpuDnaE(C)-Hygro(^C; 278-341)-IRES-mCherry

Protein = NpuDnaE(C)-Hygro(^C;278-341)

10 Vector sequence (SEQ ID NO:148)

Amino acid sequence (SEQ ID NO:149)

Plasmid 76: pLX-Puro(1-32)-NpuDnaE(N)-IRES-TagBFP2

Protein = Puro(1-32)-NpuDnaE(N)

15 Vector sequence (SEQ ID NO:150)

Amino acid sequence (SEQ ID NO:151)

Plasmid 77: pLX-NpuDnaE(C)-Puro(^C;33-199)-IRES-mCherry

Protein = NpuDnaE(C)-Puro(^C;33-199)

20 Vector sequence (SEQ ID NO:152)

Amino acid sequence (SEQ ID NO:153)

Plasmid 78: pLX-Puro(1-84)-NpuDnaE(N)-IRES-TagBFP2

Protein = Puro(1-84)-NpuDnaE(N)

25 Vector sequence (SEQ ID NO:154)

Amino acid sequence (SEQ ID NO:155)

Plasmid 79: pLX-NpuDnaE(C)-Puro(^C;85-199)-IRES-mCherry

Protein = NpuDnaE(C)-Puro(^C;85-199)

30 Vector sequence (SEQ ID NO:156)

Amino acid sequence (SEQ ID NO:157)

Plasmid 80: pLX-Puro(1-137)-NpuDnaE(N)-IRES-TagBFP2

Protein = Puro(1-137)-NpuDnaE(N)

35 File = pLX-[PuroKC3(N)-NpuDnaE(N)-25-131-29"]-IRES-TagBFP2-25-133-6

Vector sequence (SEQ ID NO:158)

Amino acid sequence (SEQ ID NO:159)

Plasmid 81: pLX-NpuDnaE(C)-Puro(^C;138-199)-IRES-mCherry

40 Protein = NpuDnaE(C)-Puro(^C;138-199)

Vector Sequence (SEQ ID NO:160)

Amino acid sequence (SEQ ID NO:161)

Plasmid 82: pLX-Puro(1-158)-NpuDnaE(N)-IRES-TagBFP2

Protein = Puro(1-158)-NpuDnaE(N)

5 Vector Sequence (SEQ ID NO:162)

Amino acid sequence (SEQ ID NO:163)

Plasmid 83: pLX-NpuDnaE(C)-Puro(^C;159-199)-IRES-mCherry

Protein = NpuDnaE(C)-Puro(^C;159-199)

10 Vector Sequence (SEQ ID NO:164)

Amino acid sequence (SEQ ID NO:165)

Plasmid 84: pLX-Puro(1-180)-NpuDnaE(N)-IRES-TagBFP2

Protein = Puro(1-180)-NpuDnaE(N)

15 Vector Sequence (SEQ ID NO:166)

Amino acid sequence (SEQ ID NO:167)

Plasmid 85: pLX-NpuDnaE(C)-Puro(^C;181-199)-IRES-mCherry

Protein = NpuDnaE(C)-Puro(^C;181-199)

20 Vector Sequence (SEQ ID NO:168)

Amino acid sequence (SEQ ID NO:169)

Plasmid 86: pLX-Blast(1-58)-NpuDnaE(N)-IRES-TagBFP2

Protein = Blast(1-58)-NpuDnaE(N)

25 Vector Sequence (SEQ ID NO:170)

Amino acid sequence (SEQ ID NO:171)

Plasmid 87: pLX-NpuDnaE(C)-Blast(59-140)-IRES-mCherry

Protein = NpuDnaE(C)-Blast(59-140)

30 Vector Sequence (SEQ ID NO:172)

Amino acid sequence (SEQ ID NO:173)

Plasmid 88: pLX-NpuDnaE(C)-HygroBA-SspDnaB(N-S0)-IRES-EGFP

Protein = NpuDnaE(C)-Hygro(53-200)-SspDnaB(N-S0)

35 Vector Sequence (SEQ ID NO:174)

Amino acid sequence (SEQ ID NO:175)

Plasmid 89: pLX-SspDnaB(C-S0)-Hygro(201-341)-IRES-mCherry

Protein = SspDnaB(C-S0)-Hygro(201-341)

40 Vector Sequence (SEQ ID NO:176)

Amino acid sequence (SEQ ID NO:177)

Plasmid 90: pLX-NpuDnaE(C)-Hygro(90-200)-SspDnaB(N-S0)-IRES-EGFP

Protein = NpuDnaE(C)-Hygro(90-200)-SspDnaB(N-S0)

Vector Sequence (SEQ ID NO:178)

Amino acid sequence (SEQ ID NO:179)

5

Plasmid 91: pLX-Hygro(1-200)-SspDnaB(N-S0)-IRES-TagBFP2

Protein = Hygro(1-200)-SspDnaB(N-S0)

Vector sequence (SEQ ID NO:180)

Amino acid sequence (SEQ ID NO:181)

10

Plasmid 92: pLX-SspDnaB(C-S0)-Hygro(201-240)-NpuDnaE(N)-IRES-EGFP

Protein = SspDnaB(C-S0)-Hygro(201-240)-NpuDnaE(N)

Vector sequence (SEQ ID NO:182)

Amino acid sequence (SEQ ID NO:183)

15

Plasmid 93: pLX-SspDnaB(C-S0)-Hygro(201-292)-NpuDnaE(N)-IRES-EGFP

Protein = SspDnaB(C-S0)-Hygro(201-292)-NpuDnaE(N)

Vector sequence (SEQ ID NO:184)

Amino acid sequence (SEQ ID NO:185)

20

Plasmid 94: pLX-DEST-IRES-TagBFP2 (SEQ ID NO:186)

Plasmid 95: pLX-DEST-IRES-EGFP (SEQ ID NO:187)

25 **Plasmid 96:** pLX-DEST-IRES-mCherry (SEQ ID NO:188)

Plasmid 97: pLX-Hygro-IRES-TagBFP2

Vector sequence (SEQ ID NO:189)

30 **Plasmid 98:** pLX-Hygro-IRES-mCherry

Vector sequence (SEQ ID NO:190)

Plasmid 99: pLX-Puro-IRES-TagBFP2

Vector sequence (SEQ ID NO:191)

35

Plasmid 100: pLX-Puro-IRES-mCherry

Vector sequence (SEQ ID NO:192)

Plasmid 101: pLX-Hygro-IRES-EGFP

40 Vector sequence (SEQ ID NO:193)

Plasmid 102: pLX-NLS_GFP-IRES-Hygro

Vector sequence (SEQ ID NO:194)

Plasmid 103: pLX-LifeAct_mCherry-IRES-Hygro

Vector sequence (SEQ ID NO:195)

5

Plasmid 104: pLX-NLS_GFP-IRES-Hygro(1-89)-NpuDnaE(N)

Vector sequence (SEQ ID NO:196)

Plasmid 105: pLX-LifeAct_mScarlet-IRES- NpuDnaE(C)-Hygro(90-341)

10 Vector sequence (SEQ ID NO:197)

Plasmid 106: pX330-AAVS1

sgRNA spacer sequence: gACCCACAGTGGGGCCACTA (First g does not match genome) (SEQ ID NO:198)

Vector sequence (SEQ ID NO:199)

15

Plasmid 107: pAAVS1-Nst-EF1aHygro2ArtTA3(-)_TetO-Blast-P2A-EGFP

Vector sequence (SEQ ID NO:200)

Plasmid 108: pAAVS1-Nst-EF1aHygro2ArtTA3(-)_TetO-Blast-P2A-mScarlet

20 Vector sequence (SEQ ID NO:201)

Plasmid 109: pAAVS1-Nst-EF1aHygro2ArtTA3(-)_TetO-Blast(1-102)_NpuDnaE(N)-P2A-EGFP

Vector sequence (SEQ ID NO:202)

25 **Plasmid 110:** pAAVS1-Nst-EF1aHygro2ArtTA3(-)_TetO-NpuDnaE(C)_Blast(103-140)-P2A-mScarlet

Vector sequence (SEQ ID NO:203)

Plasmid 111: pAAVS1-Nst-EF1aBlast2ArtTA3(-)_TetO-Hygro-P2A-NTR-E2A-EGFP

Vector sequence (SEQ ID NO:204)

30

Plasmid 112: pAAVS1-Nst-EF1aBlast2ArtTA3(-)_TetO-Hygro-P2A-NTR-E2A-mCherry

Vector sequence (SEQ ID NO:205)

Plasmid 113: pAAVS1-Nst-EF1aBlast2ArtTA3(-)_TetO- Hygro(1-89)-NpuDnaE(N)-P2A-NTR-E2A-EGFP

35 Vector sequence (SEQ ID NO:206)

Plasmid 114: pAAVS1-Nst-EF1aBlast2ArtTA3(-)_TetO- NpuDnaE(C)-Hygro(90-341)-P2A-NTR-E2A-mCherry

Vector sequence (SEQ ID NO:207)

40 **Plasmid 115:** pLX-Hygro(1-89)_NpuDnaE(N)_LZA-IRES-TagBFP2

Protein = Hygro(1-89)-NpuDnaE(N)-LZA

Vector sequence (SEQ ID NO:208)

Amino acid sequence (SEQ ID NO:209)

Plasmid 116: pLX-LZB_NpuDnaGEP(C)_Hygro(90-200)_SspDnaB(N-S0)-IRES-GFP

Protein = LZB-NpuDnaGEP(C)-Hygro(90-200)-SspDnaB(N-S0)

5 Vector sequence (SEQ ID NO:210)

Amino acid sequence (SEQ ID NO:211)

Plasmid 117: pLX-SspDnaB(C-S0)_Hygro(201-240)_NpuDnaE(N)_LZA-IRES-GFP

Protein = SspDnaB(C-S0)-Hygro(201-240)-NpuDnaE(N)-LZA

10 Vector sequence (SEQ ID NO:212)

Amino acid sequence (SEQ ID NO:213)

Plasmid 118: pLX-LZB_NpuDnaGEP(C)_Hygro(241-341)-IRES-mCherry

Protein = LZB-NpuDnaGEP(C)-Hygro(241-341)

15 Vector sequence (SEQ ID NO:214)

Amino acid sequence (SEQ ID NO:215)

AC1947GB (SEQ ID NO:216)

AC1949GB (SEQ ID NO:217)

20 pCR8-ccdbCam (SEQ ID NO:218)

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22 Jul 2024
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All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

5 The indefinite articles “a” and “an,” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least one.”

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

0 In the claims, as well as in the specification above, all transitional phrases such as “comprising,” “including,” “carrying,” “having,” “containing,” “involving,” “holding,” “composed of,” and the like are to be understood to be open-ended, *i.e.*, to mean including but not limited to. Only the transitional phrases “consisting of” and “consisting essentially of” shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

5 The terms “about” and “substantially” preceding a numerical value mean $\pm 10\%$ of the recited numerical value.

Where a range of values is provided, each value between the upper and lower ends of the range are specifically contemplated and described herein.

0 In the first aspect, the present invention provides a method comprising delivering to eukaryotic cells

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a nucleotide sequence encoding a first molecule of interest; and

25 (b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a second molecule of interest,

30 wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of the N-terminal fragment and the C-terminal fragment of the selectable marker protein to produce a full-length selectable marker protein.

In the second aspect, the present invention provides a eukaryotic cell, comprising

35 (a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a nucleotide sequence encoding a first molecule of interest; and

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a second molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of the N-terminal fragment and the C-terminal fragment of the selectable marker protein to produce a full-length antibiotic resistance protein.

In a third aspect, the present invention provides a kit, comprising

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a nucleotide sequence encoding a first molecule of interest; and

(b) a second vector comprising a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a second molecule of interest,

wherein when the kit is used, the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of the N-terminal fragment and the C-terminal fragment of the selectable marker protein to produce a full-length selectable marker protein.

In a fourth aspect, the present invention provides a method comprising delivering to eukaryotic cells

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest,

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, and

(c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a third molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the selectable marker protein to the central fragment of the selectable marker protein, and the N-terminal fragment and the C-terminal fragment of the second

intein catalyze joining of central fragment of the selectable marker protein to the C-terminal fragment of the selectable marker protein, to produce a full-length selectable marker protein.

In a fifth aspect, the present invention provides a eukaryotic cell comprising:

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest,

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, and

(c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a third molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the selectable marker protein to the central fragment of the selectable marker protein, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of central fragment of the selectable marker protein to the C-terminal fragment of the selectable marker protein, to produce a full-length selectable marker protein.

In another aspect, the present invention provides a kit comprising:

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest,

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, and

(c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a third molecule of interest,

wherein when the kit is used, the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the selectable marker protein to the central

fragment of the selectable marker protein, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of central fragment of the selectable marker protein to the C-terminal fragment of the selectable marker protein, to produce a full-length selectable marker protein.

What is claimed is:

CLAIMS

1. A method comprising delivering to eukaryotic cells
 - (a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a nucleotide sequence encoding a first molecule of interest; and
 - (b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a second molecule of interest, wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of the N-terminal fragment and the C-terminal fragment of the selectable marker protein to produce a full-length selectable marker protein.
2. The method of claim 1 further comprising maintaining the eukaryotic cells under conditions that permit introduction of the first and second vectors into the eukaryotic cells to produce transgenic eukaryotic cells.
3. The method of claim 2 further comprising selecting the transgenic eukaryotic cells that comprise the full-length selectable marker protein.
4. The method of any one of claims 1-3, wherein the eukaryotic cells are mammalian cells.
5. The method of any one of claims 1-4, wherein the selectable marker protein is an antibiotic resistance protein.
6. The method of claim 5, wherein the antibiotic resistance protein confers resistance to hygromycin, G418, puromycin, phleomycin D1 or blasticidin.
7. The method of claim 5 or 6, wherein the antibiotic resistance protein is encoded by a *hygB* gene, a *bsr* gene, a *pac* gene, or a *neo* gene.
8. The method of any one of claims 1-4, wherein the selectable marker protein is a fluorescent protein.

9. The method of claim 8, wherein the fluorescent protein is selected from TagCFP, mTagCFP2, Czurite, ECFP2, mKalama1, Sirius, Sapphire, T-Sapphire, ECFP, Cerulean, SCFP3C, mTurquoise, mTurquoise2, monomeric Midoriishi-Cyan, TagCFP, mTFP1, EGFP, Emerald, Superfolder GFP, Monomeric Czami Green, TagGFP2, mUKG, mWasabi, Clover, mNeonGreen, EYFP, Citrine, Venus, SYFP2, TagYFP, Monomeric Kusabira-Orange, mKOκ, mKO2, mOrange, mOrange2, mRaspberry, mCherry, mStrawberry, mScarlet, mTangerine, tdTomato, TagRFP, TagRFP-T, mCapple, mRuby, mRuby2, mPlum, HcRed-Tandem, mKate2, mNeptune, NirFP, TagRFP657, IFP1.4 and iRFP.

10. The method of any one of claims 1-9, wherein the intein is a split intein.

11. The method of claim 10, wherein the split intein is a natural split, optionally wherein the natural split intein is selected from DnaE inteins, and optionally wherein the DnaE inteins are selected from *Synechocystis sp.* DnaE (*SspDnaE*) inteins and *Nostoc punctiforme* (*NpuDnaE*) inteins.

12. The method of claim 10, wherein the split intein is an engineered split intein, optionally wherein the engineered split intein is engineered from DnaB inteins or GyrB inteins, and optionally wherein the engineered split intein is a *SspDnaB* S1 intein or a *SspGyrB* S11 intein.

13. The method of any one of claims 1-12, wherein the first and/or second molecule is a protein or a non-coding ribonucleic acid (RNA), optionally wherein the non-coding RNA is a microRNA (miRNA), antisense RNA, short-interfering RNA (siRNA) or short-hairpin RNA (shRNA).

14. The method of any one of claims 1-13, wherein the first and/or second vector is a plasmid vector or a viral vector.

15. A eukaryotic cell, comprising

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a nucleotide sequence encoding a first molecule of interest; and

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a second molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the intein catalyze joining

of the N-terminal fragment and the C-terminal fragment of the selectable marker protein to produce a full-length antibiotic resistance protein.

16. A kit, comprising

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of an intein and (ii) a nucleotide sequence encoding a first molecule of interest; and

(b) a second vector comprising a nucleotide sequence encoding a C-terminal fragment of the intein, which is upstream from a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a second molecule of interest,

wherein when the kit is used, the N-terminal fragment and the C-terminal fragment of the intein catalyze joining of the N-terminal fragment and the C-terminal fragment of the selectable marker protein to produce a full-length selectable marker protein.

17. A method comprising delivering to eukaryotic cells

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest,

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, and

(c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a third molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the selectable marker protein to the central fragment of the selectable marker protein, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of central fragment of the selectable marker protein to the C-terminal fragment of the selectable marker protein, to produce a full-length selectable marker protein.

18. The method of claim 17 further comprising maintaining the eukaryotic cells under conditions that permit introduction of the first, second, and third vectors into the eukaryotic cells to produce transgenic eukaryotic cells.

19. The method of claim 18 further comprising selecting the transgenic eukaryotic cells that comprise the full-length selectable marker protein.

20. A eukaryotic cell comprising:

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest,

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, and

(c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a third molecule of interest,

wherein the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the selectable marker protein to the central fragment of the selectable marker protein, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of central fragment of the selectable marker protein to the C-terminal fragment of the selectable marker protein, to produce a full-length selectable marker protein.

21. A kit comprising:

(a) a first vector comprising (i) a nucleotide sequence encoding an N-terminal fragment of a selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a first intein and (ii) a nucleotide sequence encoding a first molecule of interest,

(b) a second vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the first intein, which is upstream from a nucleotide sequence encoding a central fragment of the selectable marker protein, which is upstream from a nucleotide sequence encoding an N-terminal fragment of a second intein and (ii) a nucleotide sequence encoding a second molecule of interest, and

(c) a third vector comprising (i) a nucleotide sequence encoding a C-terminal fragment of the second intein, which is upstream from a nucleotide sequence encoding a C-terminal fragment of the selectable marker protein and (ii) a nucleotide sequence encoding a third molecule of interest,

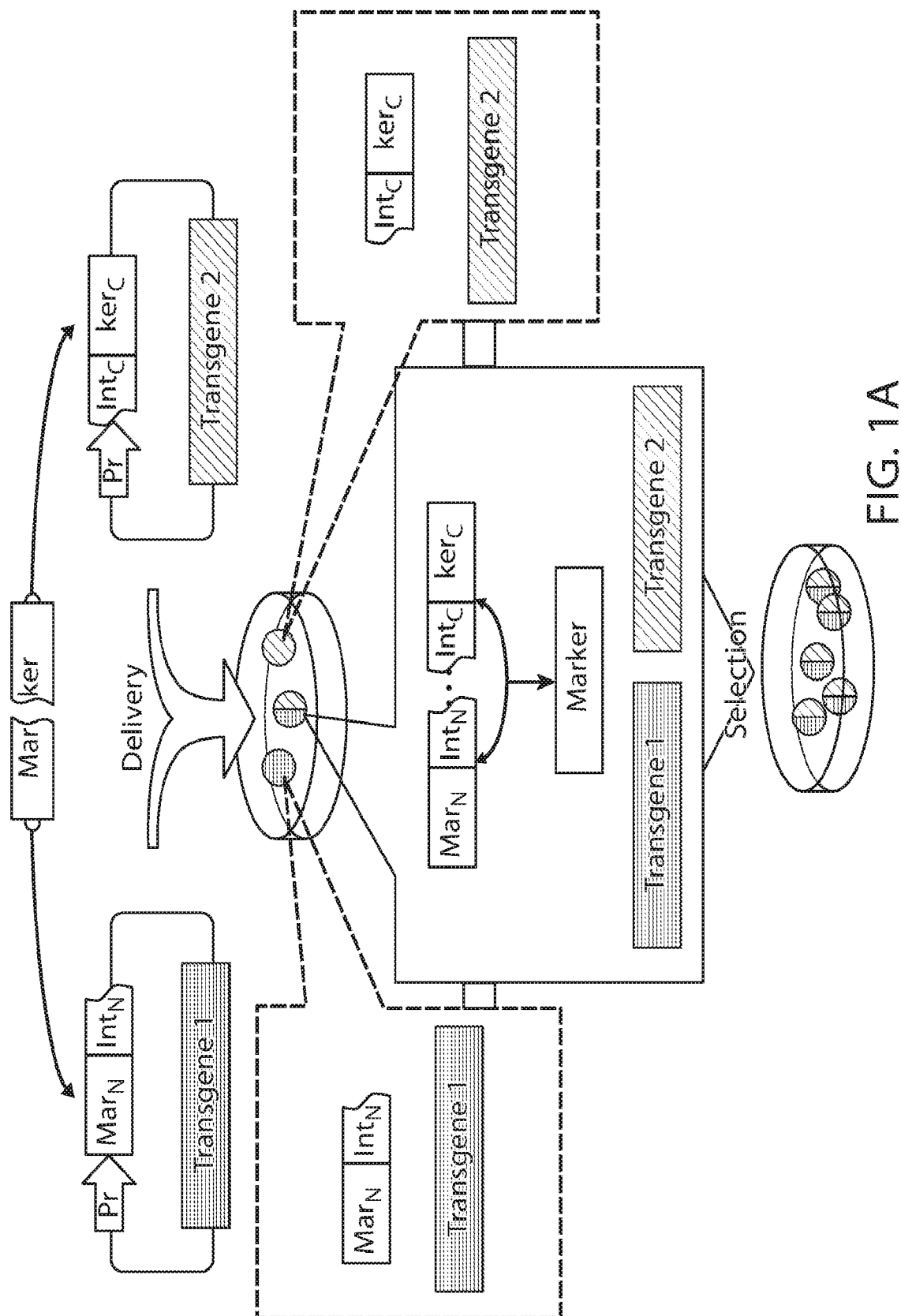
wherein when the kit is used, the N-terminal fragment and the C-terminal fragment of the first intein catalyze joining of N-terminal fragment of the selectable marker protein to the central

fragment of the selectable marker protein, and the N-terminal fragment and the C-terminal fragment of the second intein catalyze joining of central fragment of the selectable marker protein to the C-terminal fragment of the selectable marker protein, to produce a full-length selectable marker protein.

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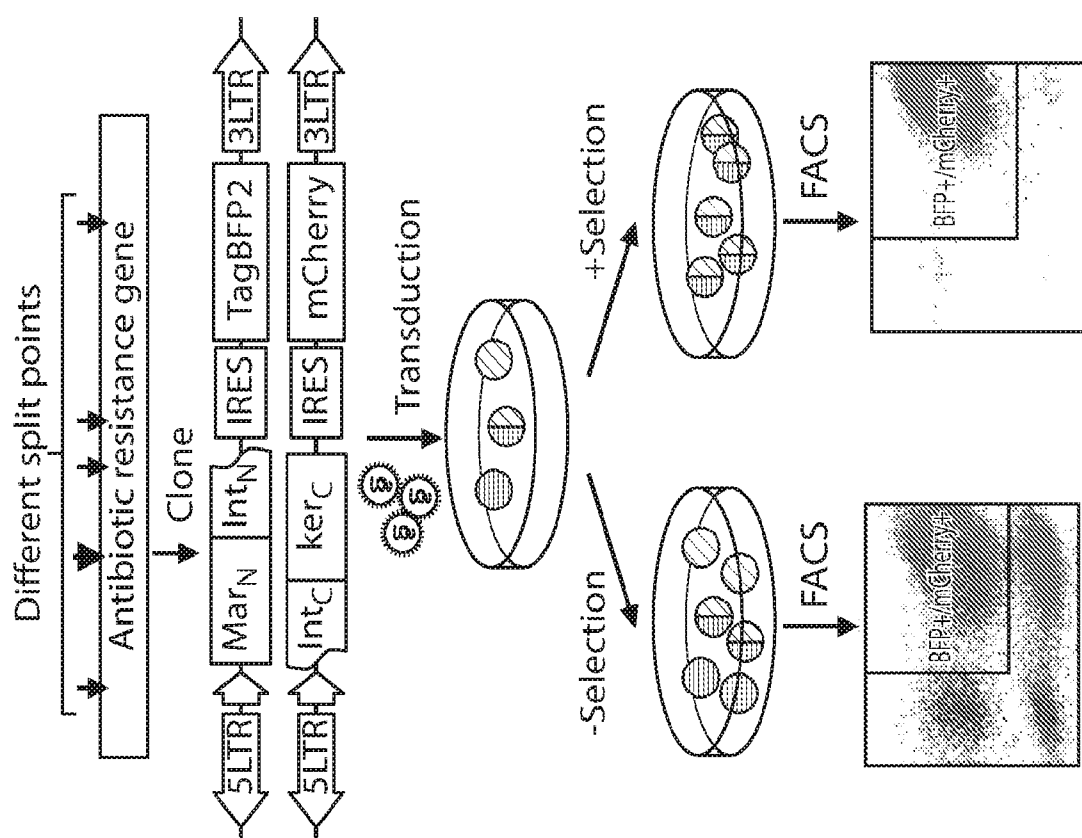


FIG. 1B

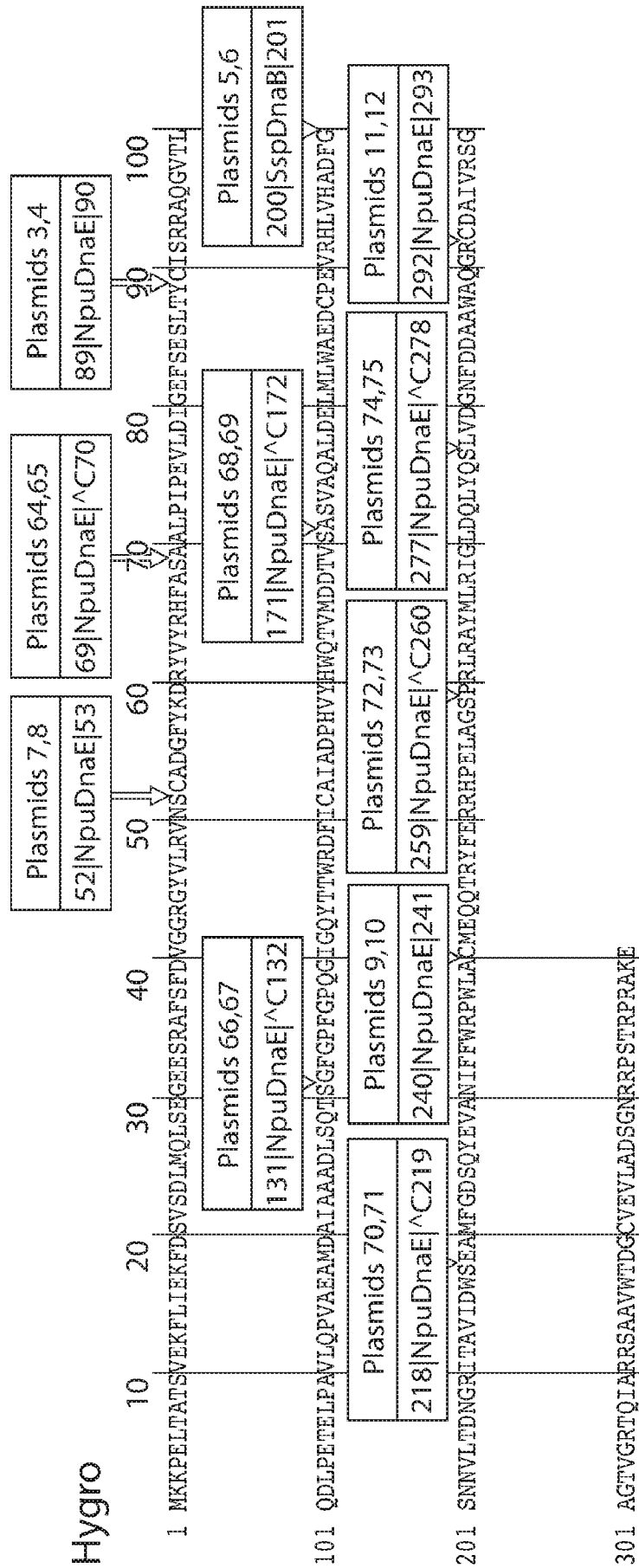


FIG. 2A

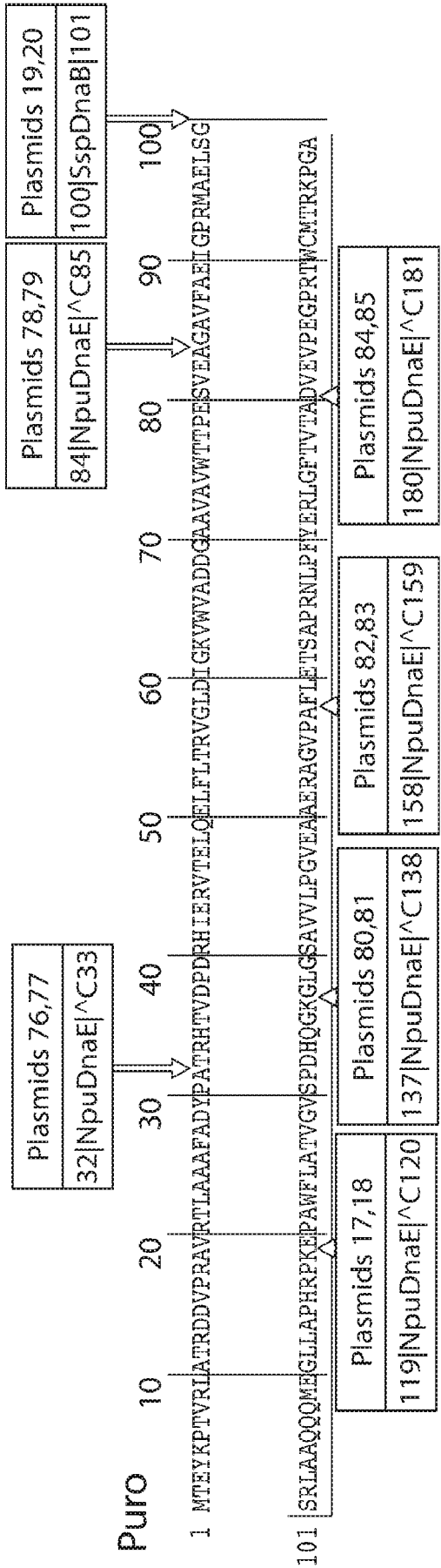


FIG. 2B

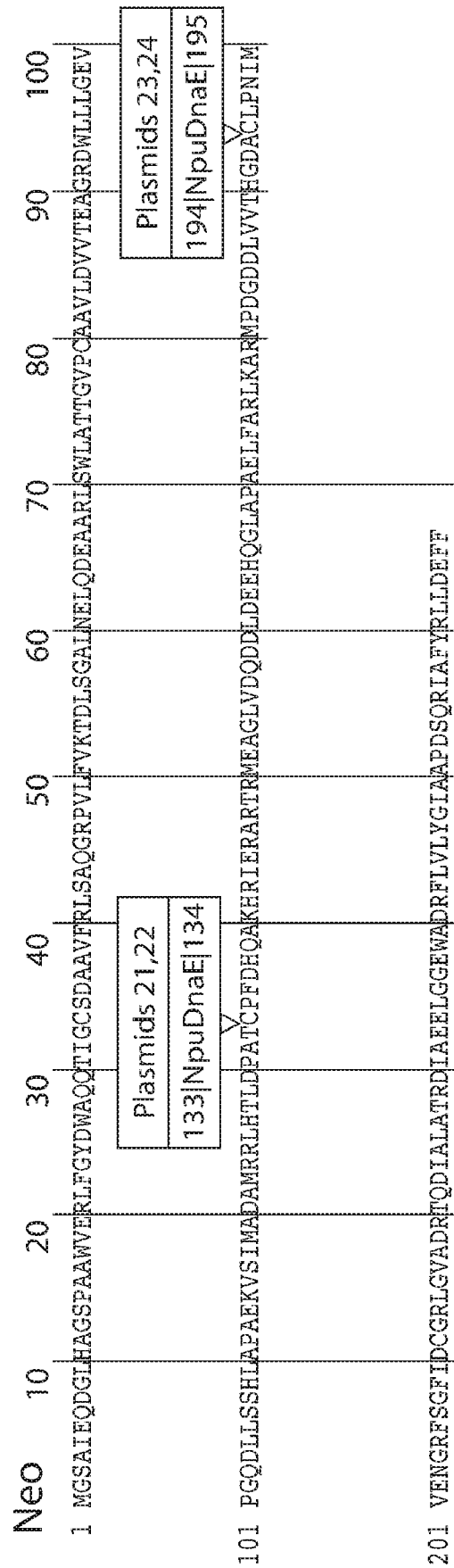


FIG. 2C

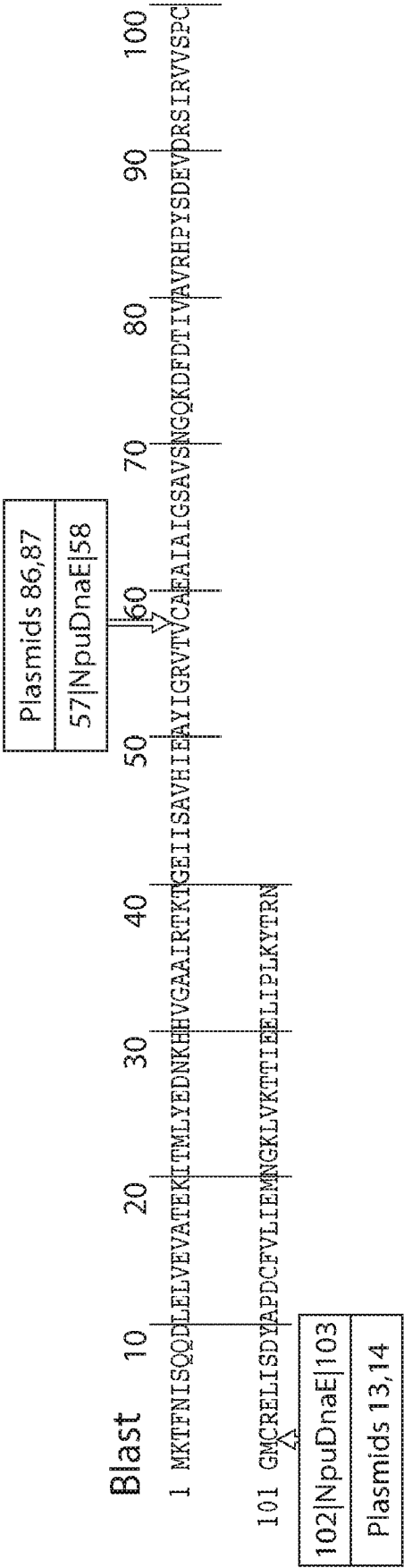


FIG. 2D

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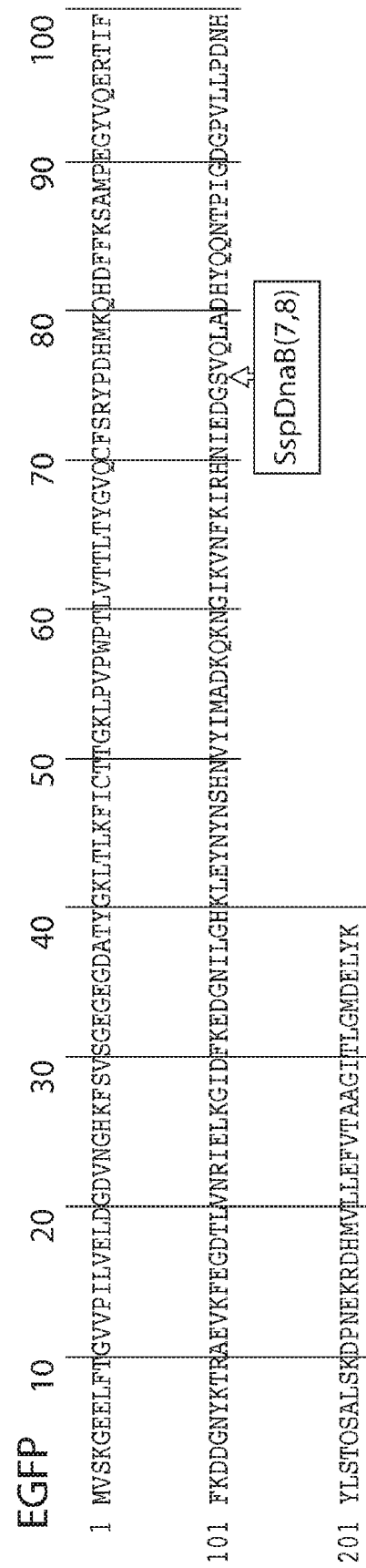


FIG. 2E

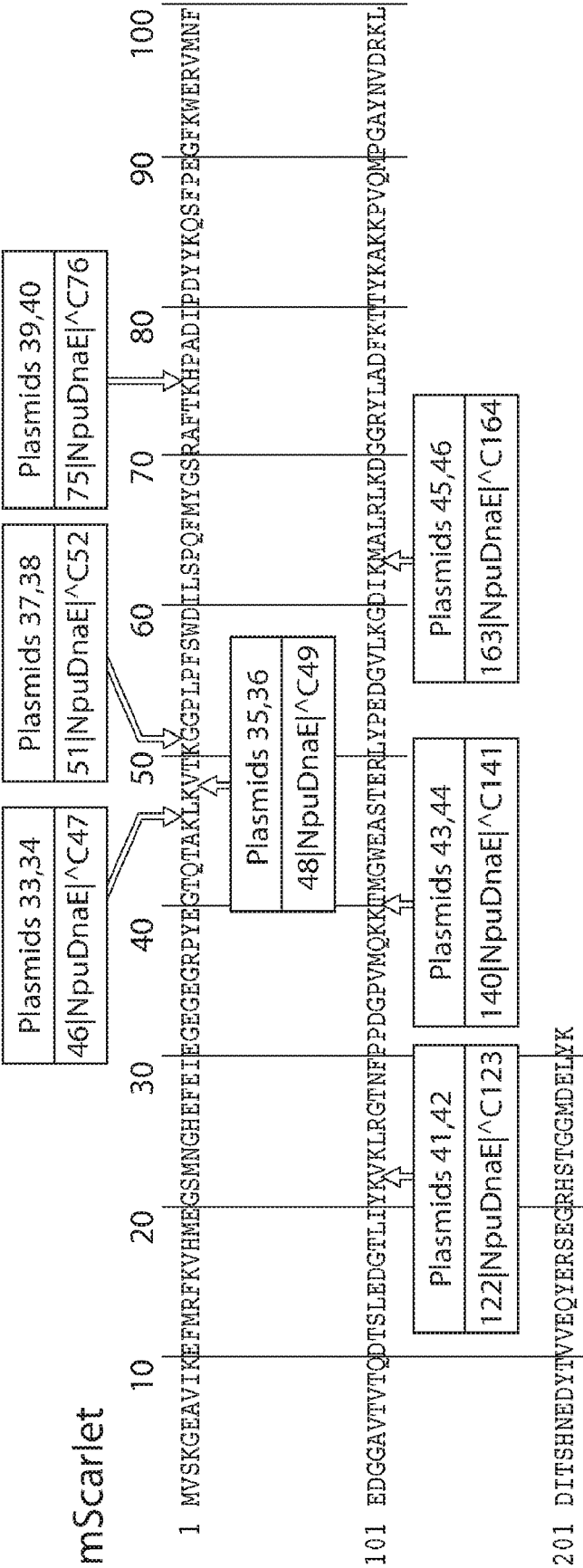


FIG. 2F

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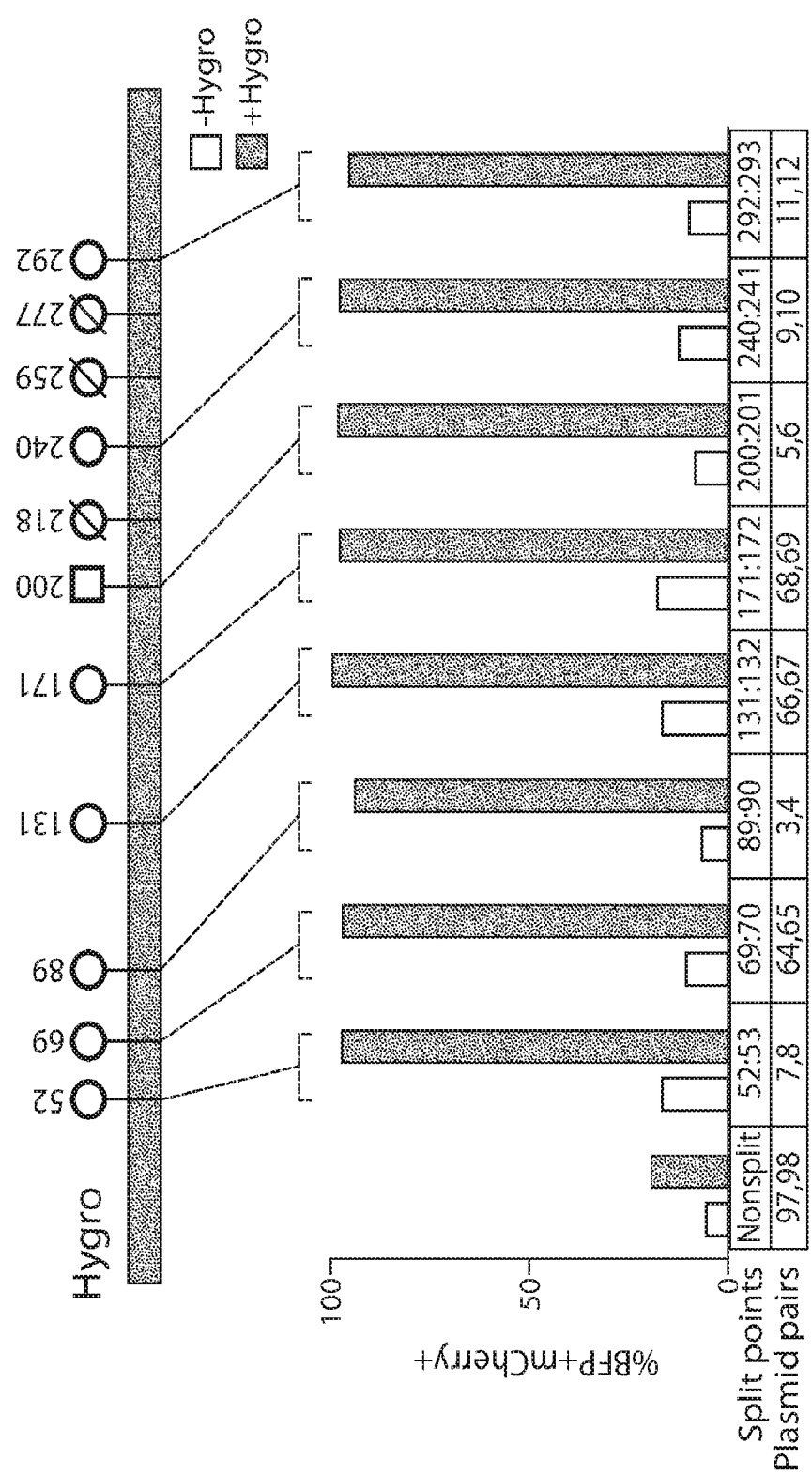


FIG. 3

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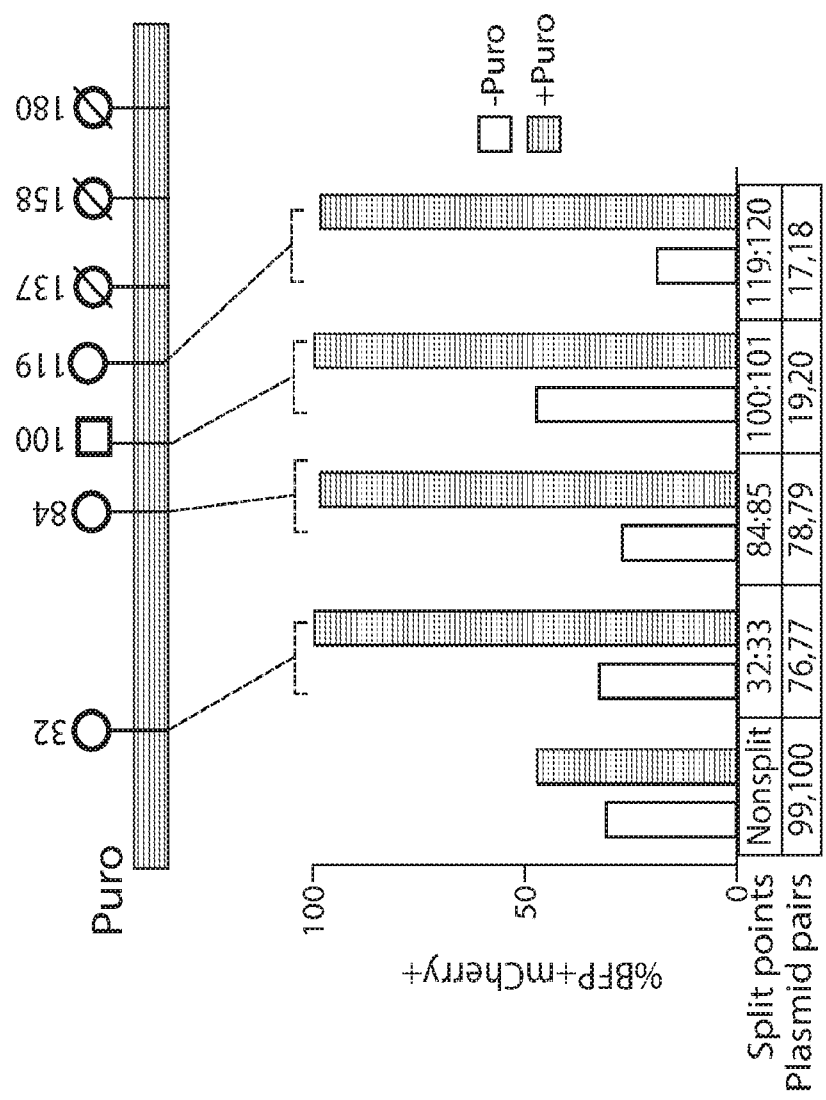


FIG. 4

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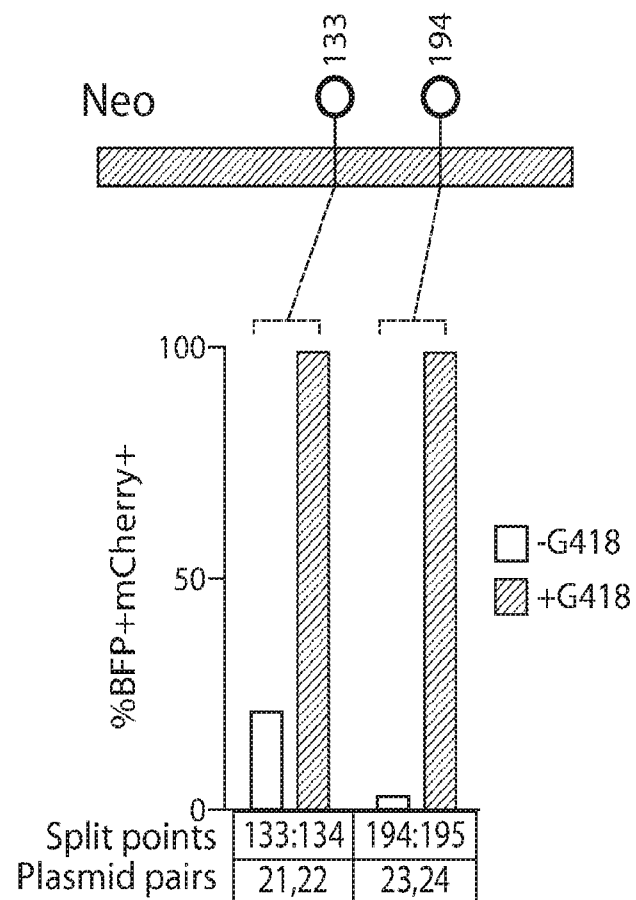


FIG. 5

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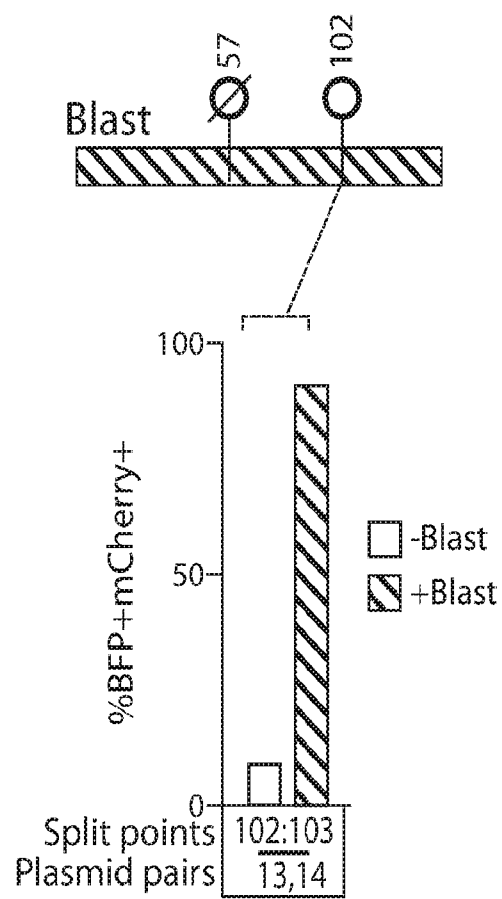


FIG. 6

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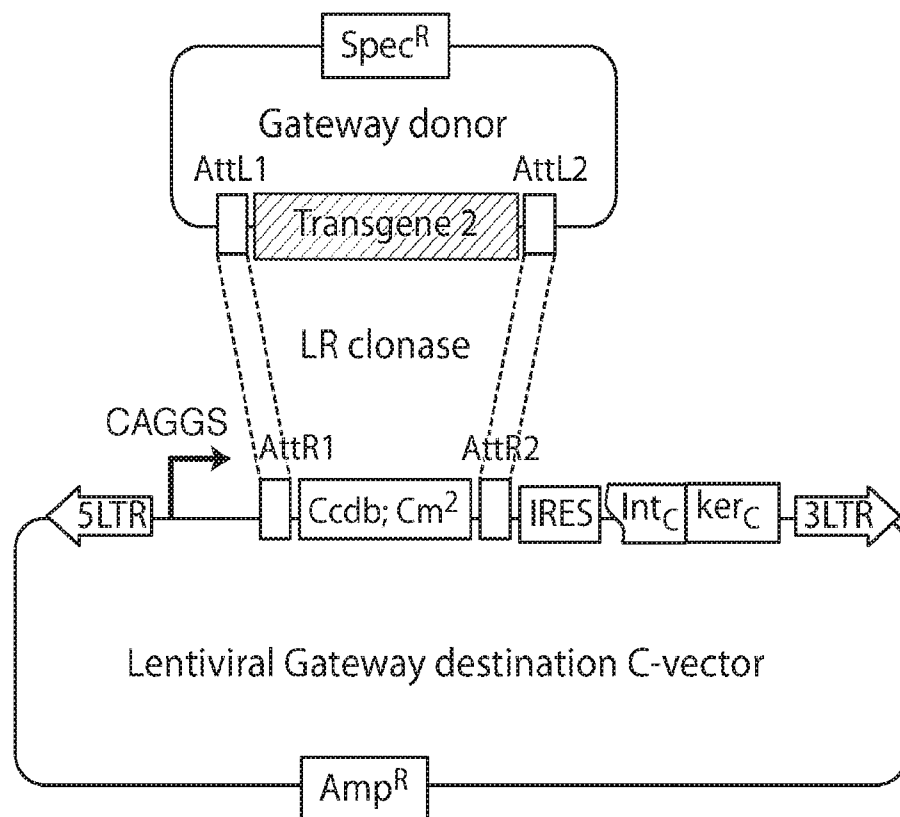
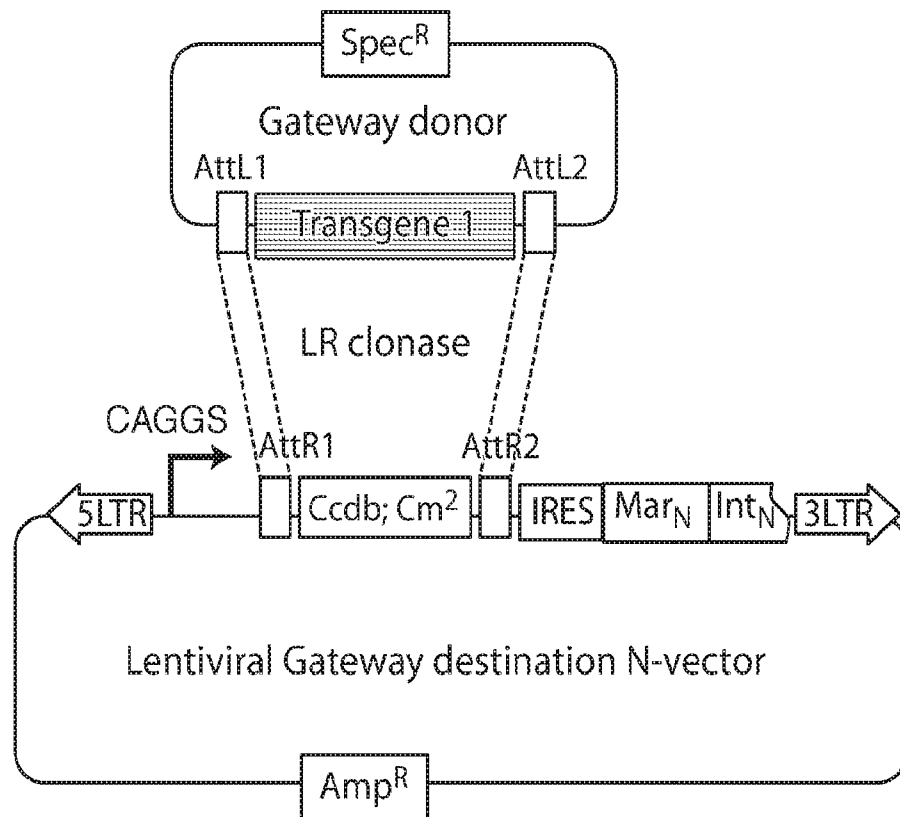


FIG. 7A

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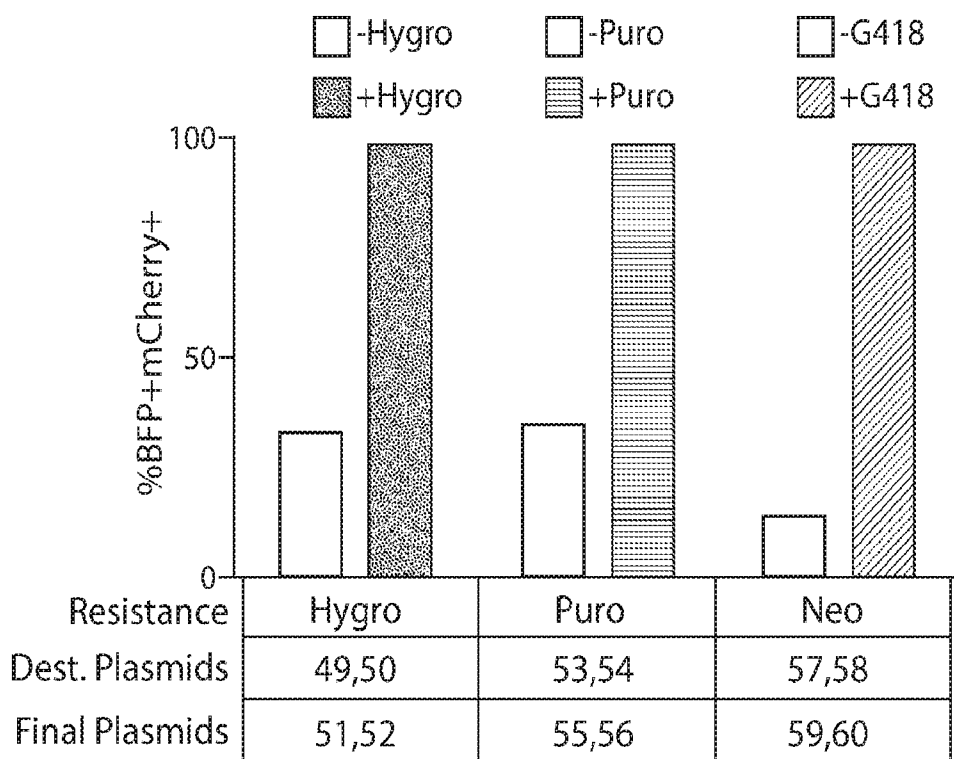


FIG. 7B

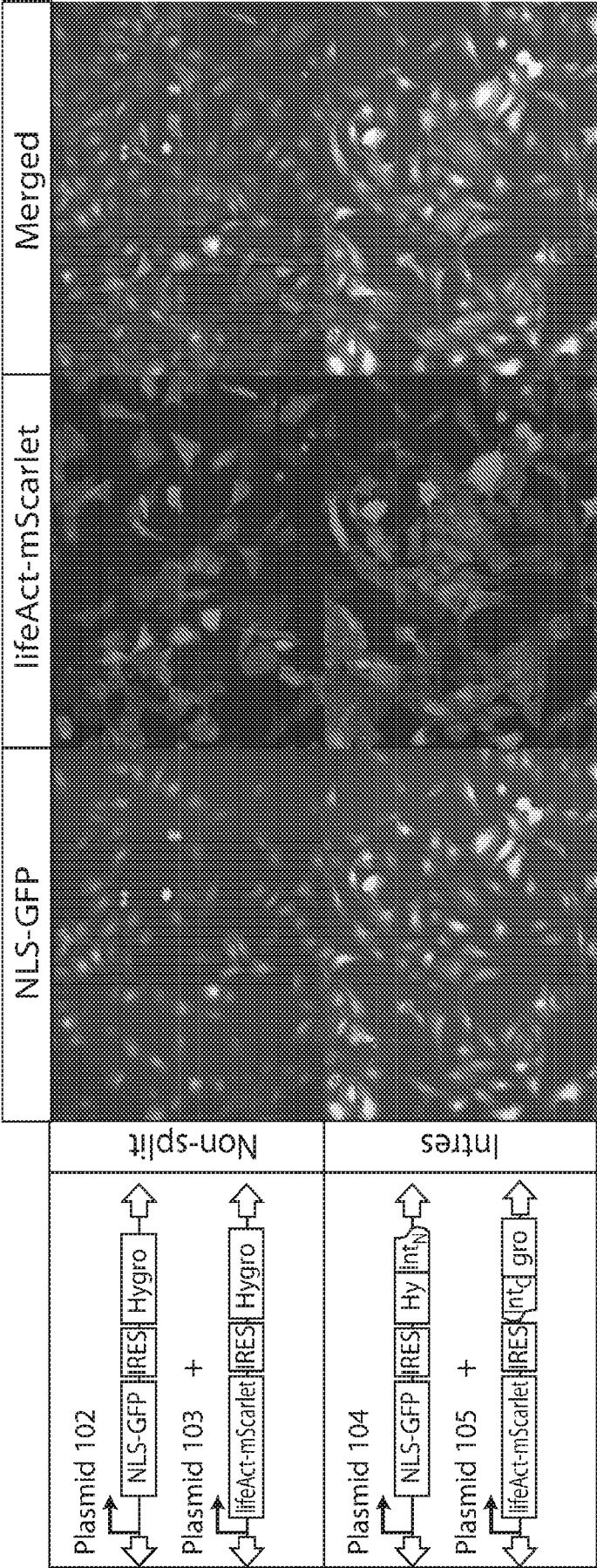


FIG. 7C

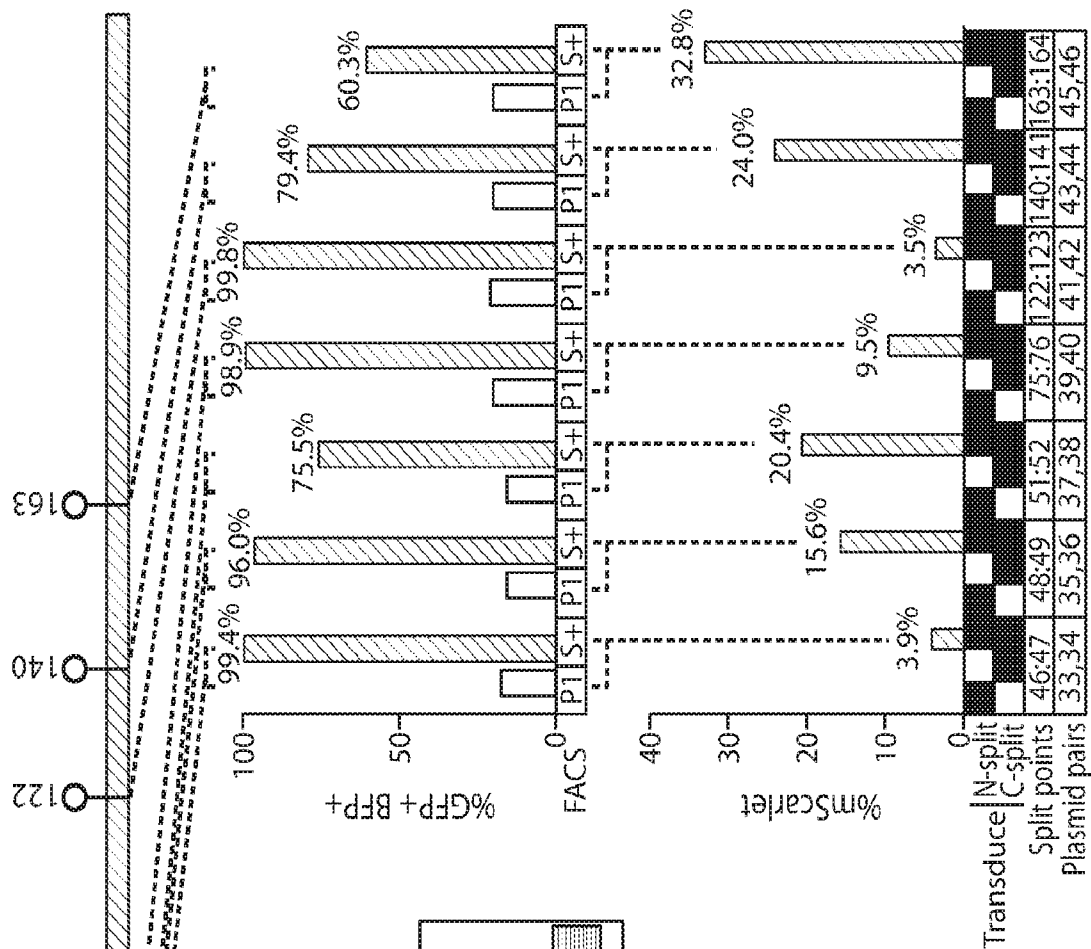


FIG. 8C

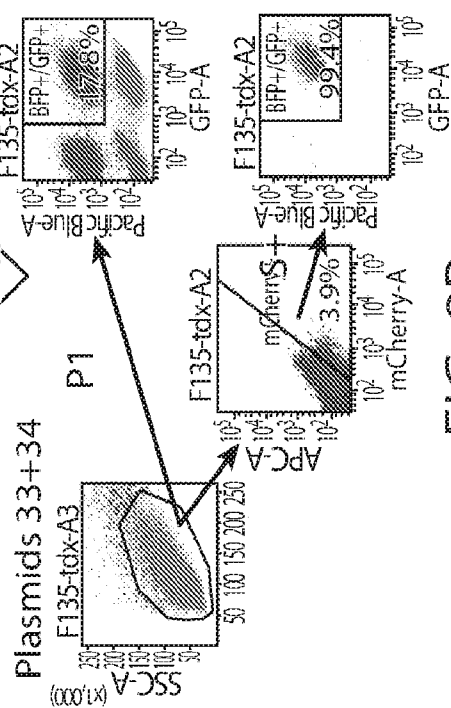


FIG. 8B

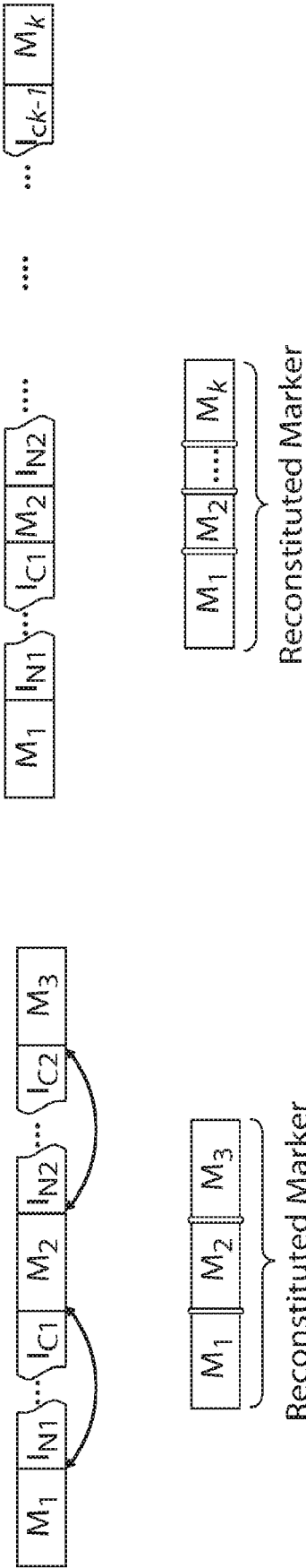


FIG. 9B

FIG. 9A

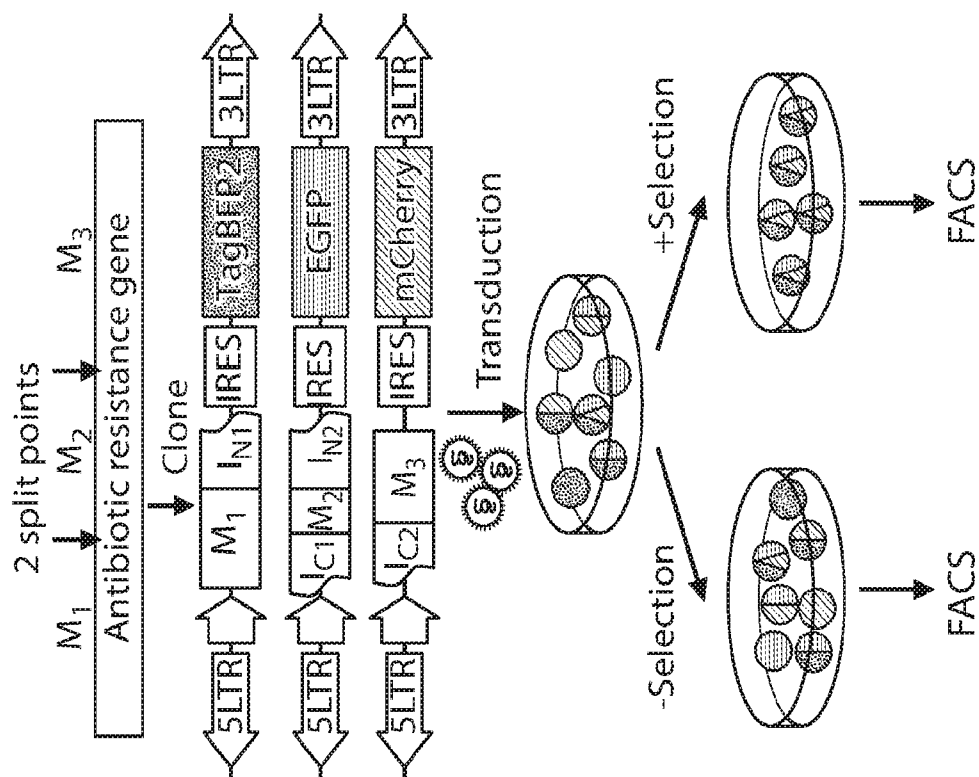


FIG. 9C

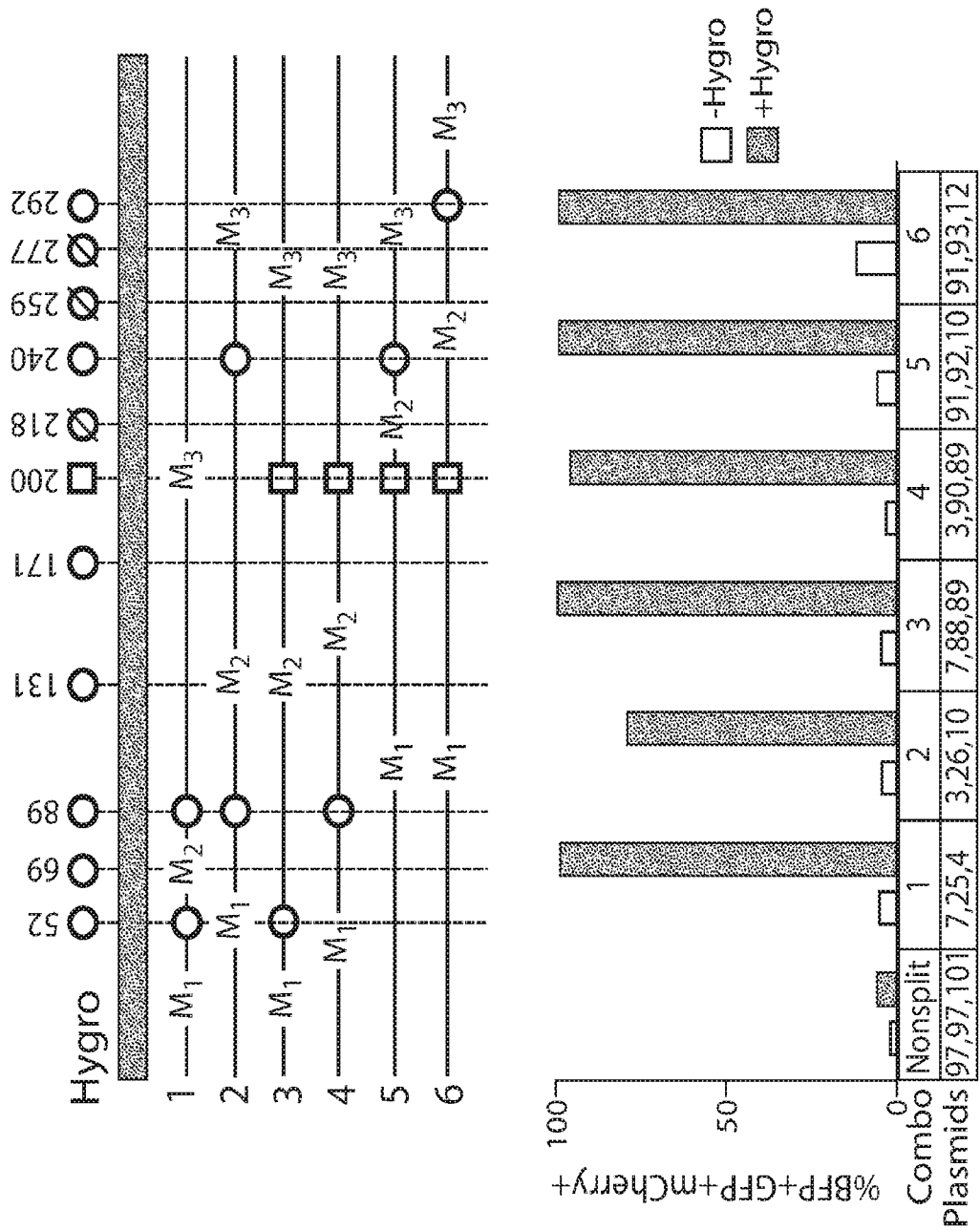


FIG. 9D

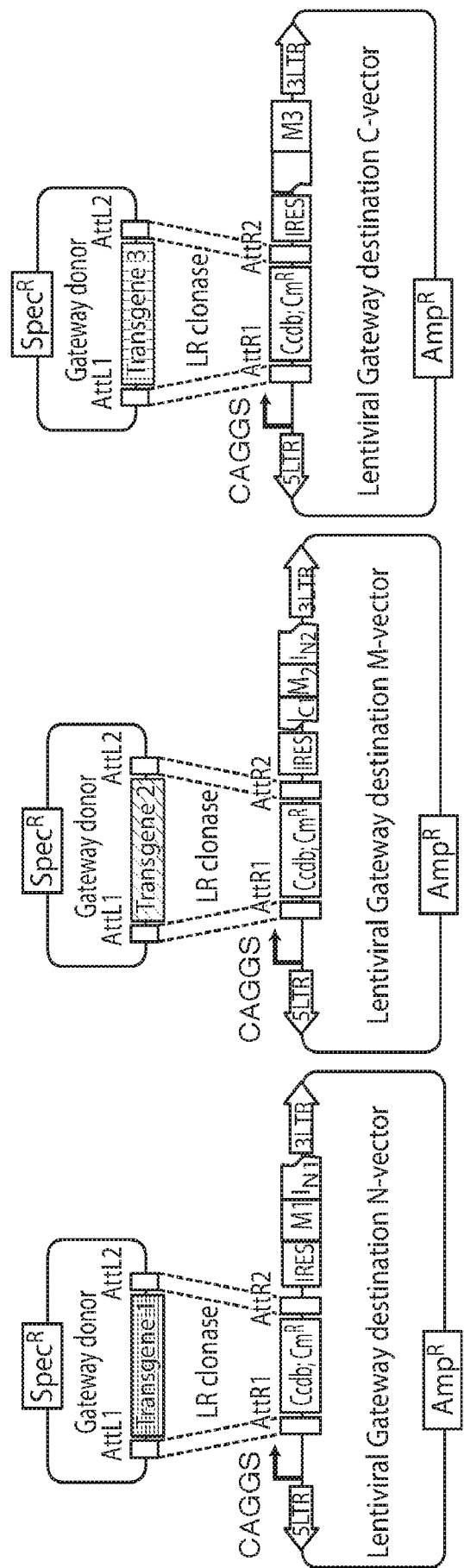


FIG. 10A

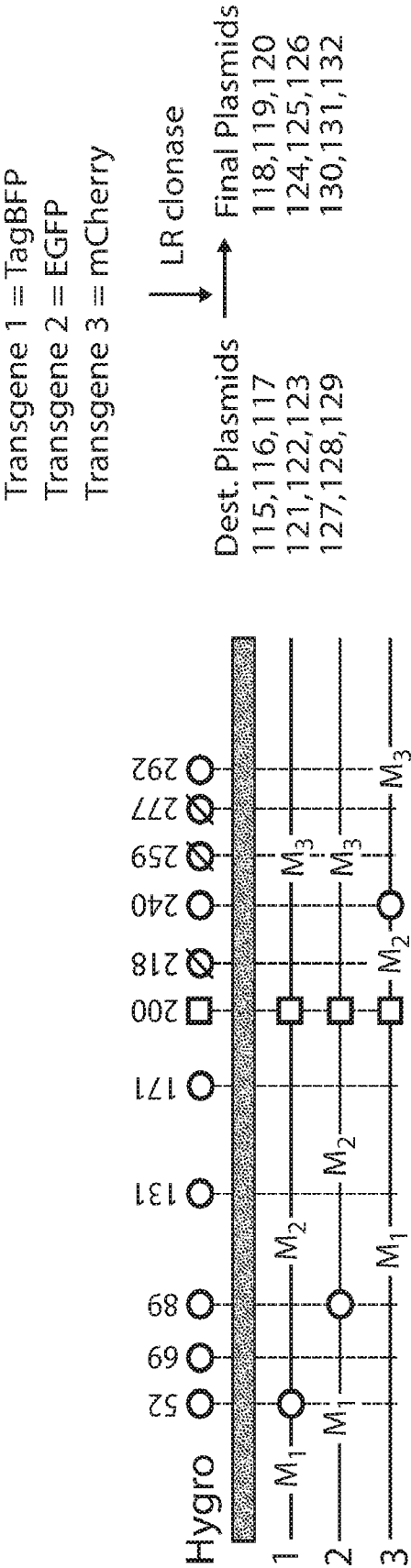


FIG. 10B

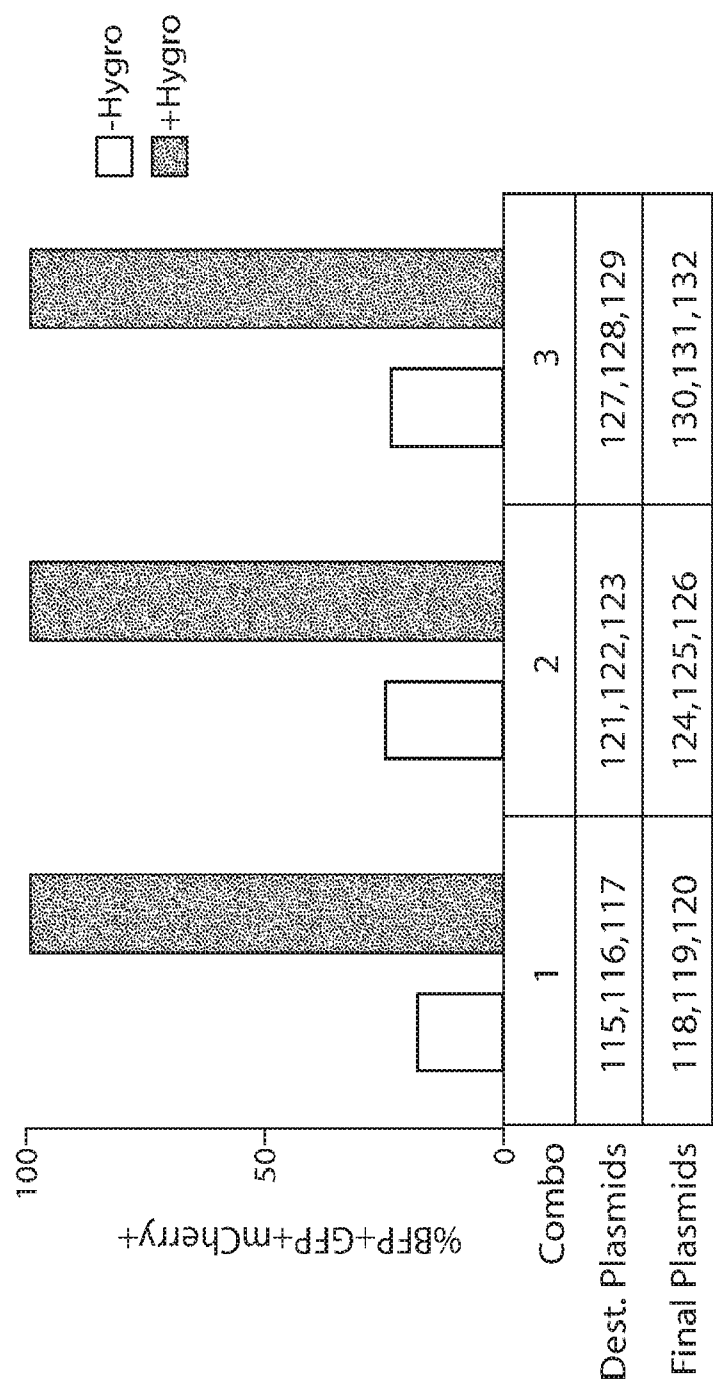


FIG. 10C

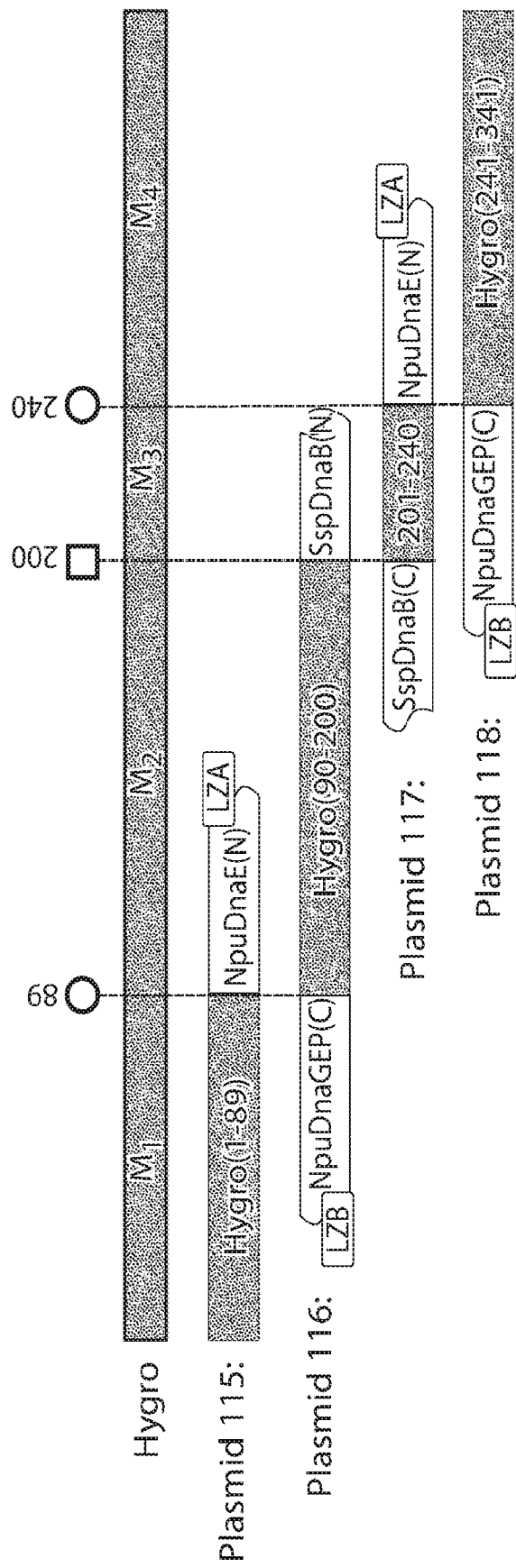


FIG. 11

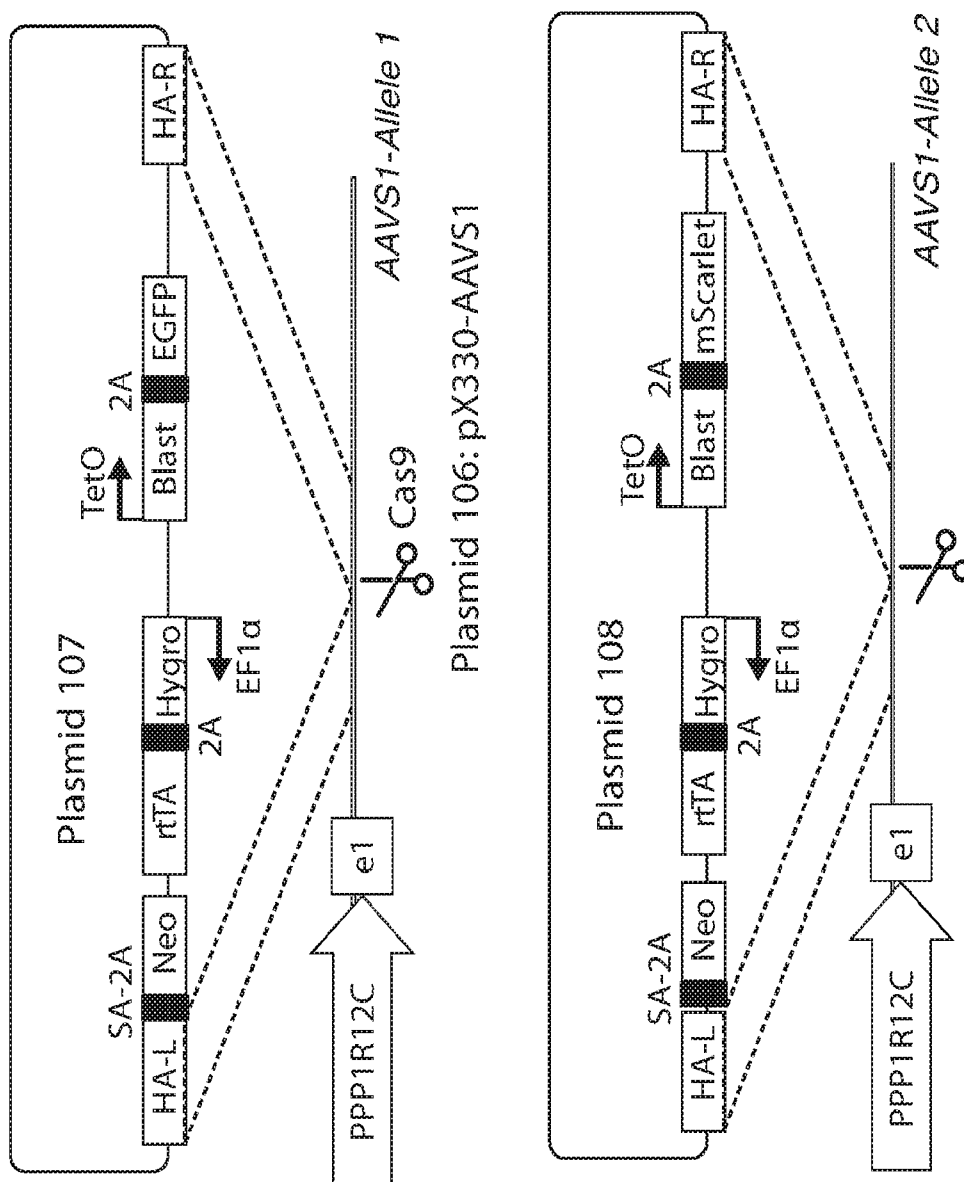


FIG. 12A

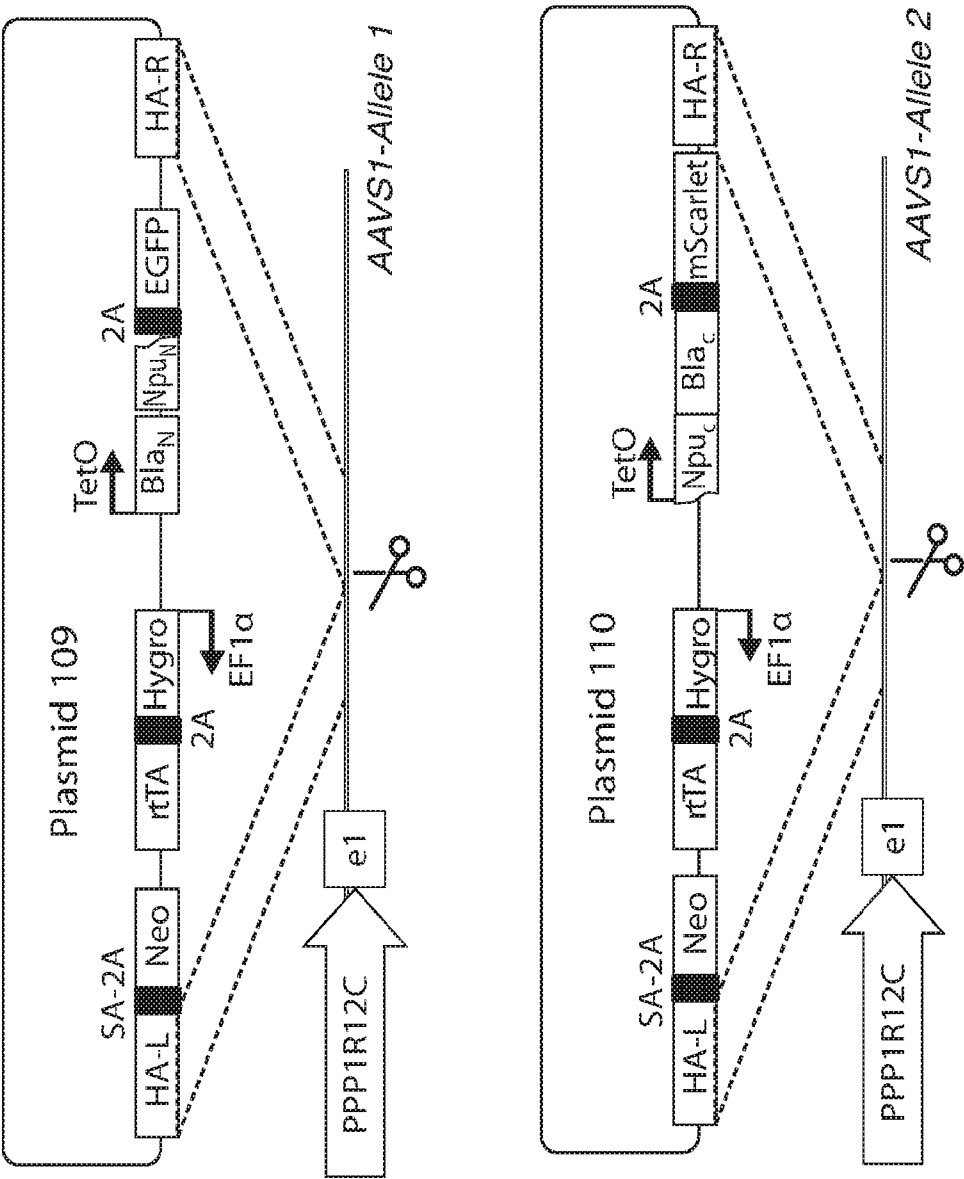


FIG. 12B

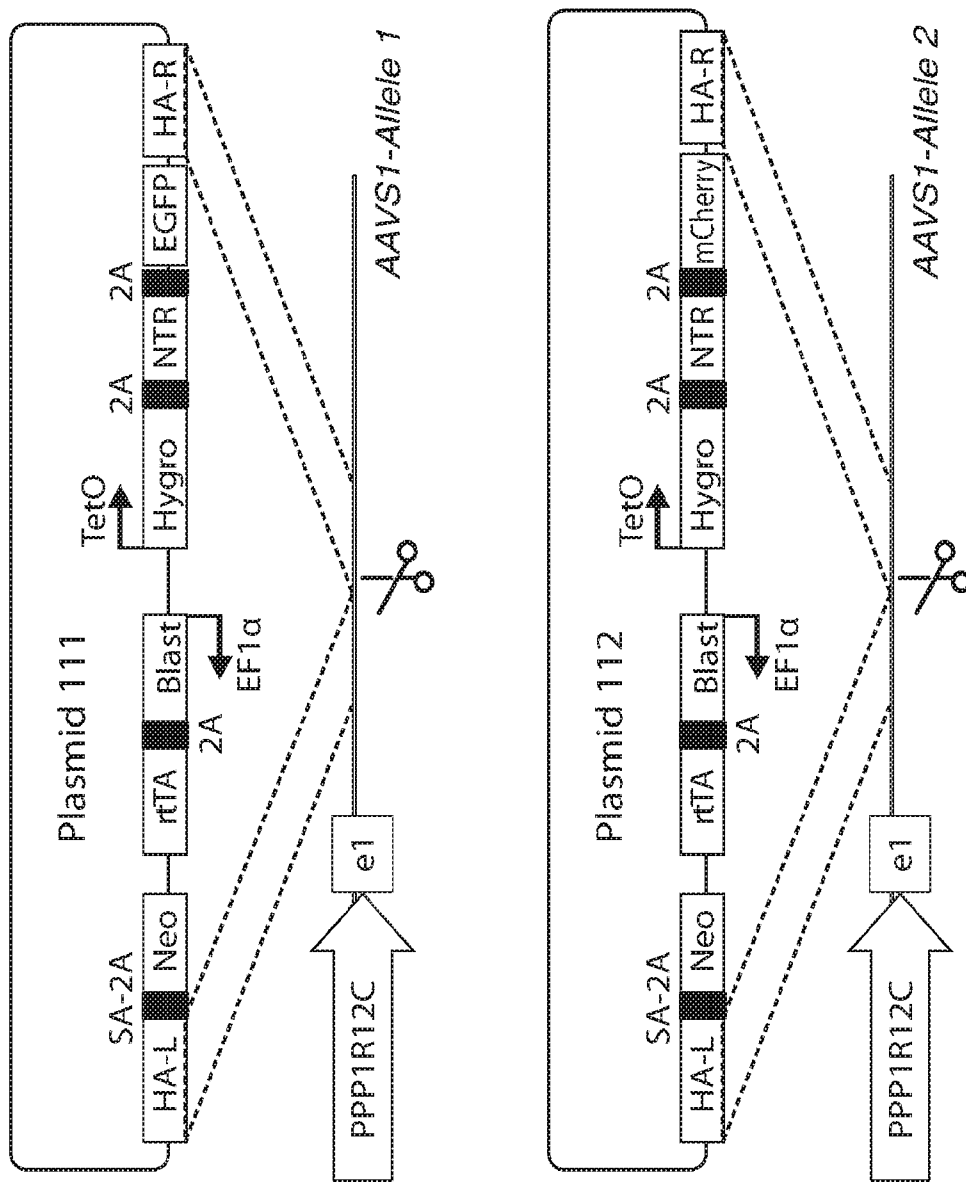


FIG. 12C

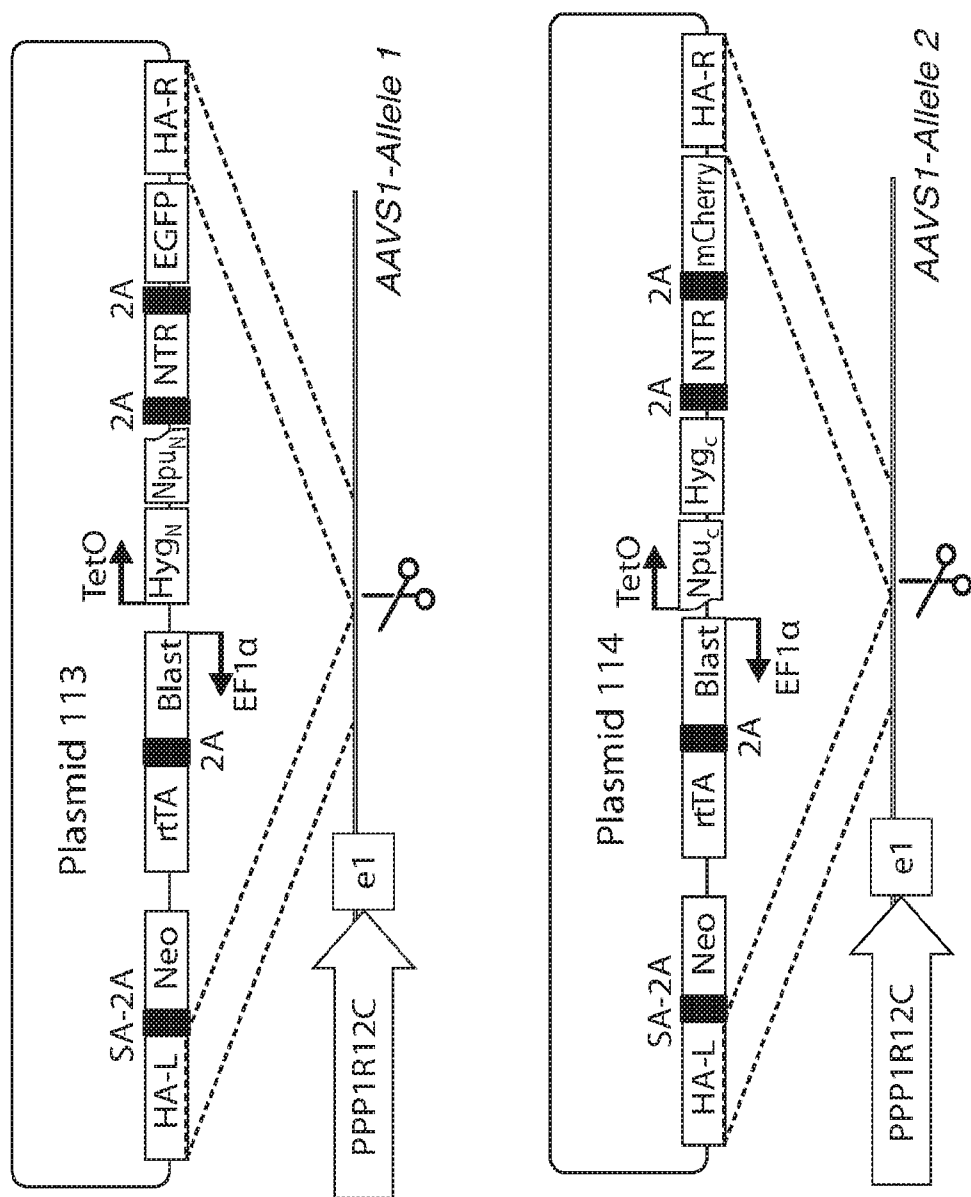


FIG. 12D

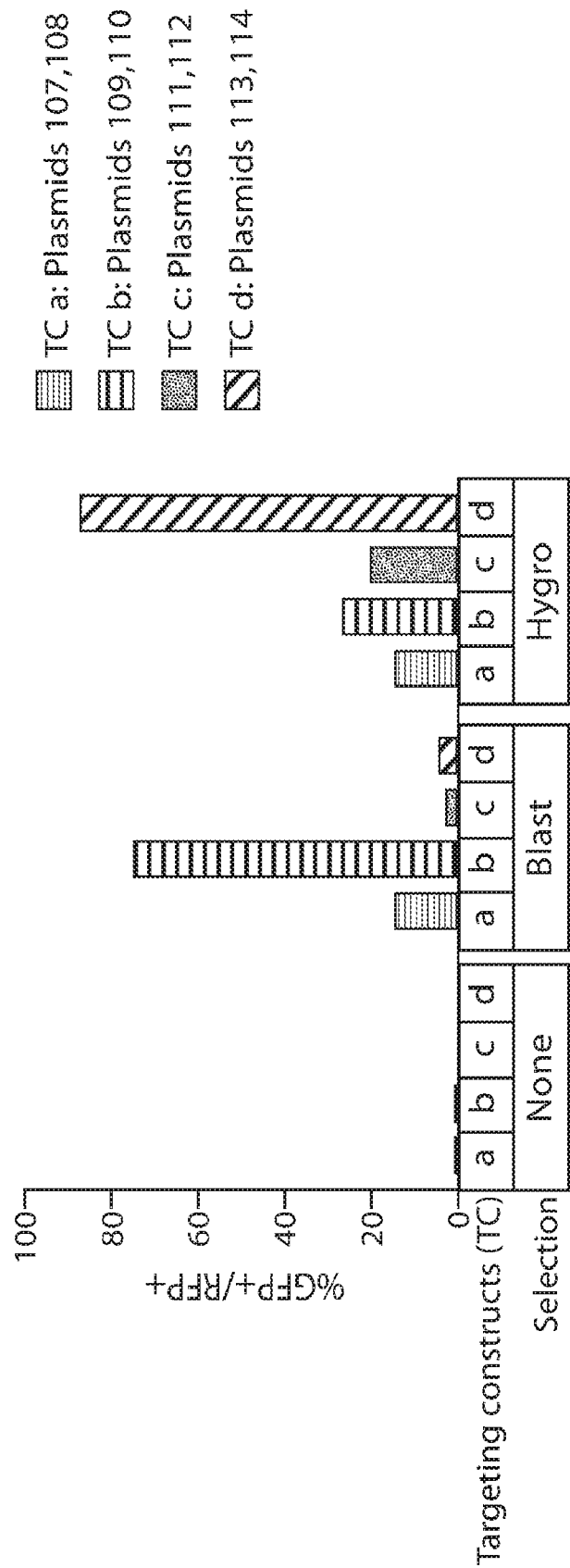


FIG. 12E

SEQUENCE LISTING

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<120> TRANSGENIC SELECTION METHODS AND COMPOSITIONS

<130> J0227.70007W000

<140> Not Yet Assigned

<141> Concurrently Herewith

<150> US 62/624,629

<151> 2018-01-31

<150> US 62/616,281

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<150> US 62/608,478

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<160> 218

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<210> 1

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20 25 30

Glu Ser Arg Ala Phe Ser Phe Asp Val Gly Gly Arg Gly Tyr Val Leu
 35 40 45

Arg Val Asn Ser Cys Ala Asp Gly Phe Tyr Lys Asp Arg Tyr Val Tyr
 50 55 60

Arg His Phe Ala Ser Ala Ala Leu Pro Ile Pro Glu Val Leu Asp Ile
 65 70 75 80

Gly Glu Phe Ser Glu Ser Leu Thr Tyr Cys Ile Ser Arg Arg Ala Gln
 85 90 95

Gly Val Thr Leu Gln Asp Leu Pro Glu Thr Glu Leu Pro Ala Val Leu
 100 105 110

Gln Pro Val Ala Glu Ala Met Asp Ala Ile Ala Ala Ala Asp Leu Ser
 115 120 125

Gln Thr Ser Gly Phe Gly Pro Phe Gly Pro Gln Gly Ile Gly Gln Tyr
 130 135 140

Thr Thr Trp Arg Asp Phe Ile Cys Ala Ile Ala Asp Pro His Val Tyr
 145 150 155 160

His Trp Gln Thr Val Met Asp Asp Thr Val Ser Ala Ser Val Ala Gln
 165 170 175

Ala Leu Asp Glu Leu Met Leu Trp Ala Glu Asp Cys Pro Glu Val Arg
 180 185 190

His Leu Val His Ala Asp Phe Gly Ser Asn Asn Val Leu Thr Asp Asn
 195 200 205

Gly Arg Ile Thr Ala Val Ile Asp Trp Ser Glu Ala Met Phe Gly Asp

210

215

220

Ser Gln Tyr Glu Val Ala Asn Ile Phe Phe Trp Arg Pro Trp Leu Ala
 225 230 235 240

Cys Met Glu Gln Gln Thr Arg Tyr Phe Glu Arg Arg His Pro Glu Leu
 245 250 255

Ala Gly Ser Pro Arg Leu Arg Ala Tyr Met Leu Arg Ile Gly Leu Asp
 260 265 270

Gln Leu Tyr Gln Ser Leu Val Asp Gly Asn Phe Asp Asp Ala Ala Trp
 275 280 285

Ala Gln Gly Arg Cys Asp Ala Ile Val Arg Ser Gly Ala Gly Thr Val
 290 295 300

Gly Arg Thr Gln Ile Ala Arg Arg Ser Ala Ala Val Trp Thr Asp Gly
 305 310 315 320

Cys Val Glu Val Leu Ala Asp Ser Gly Asn Arg Arg Pro Ser Thr Arg
 325 330 335

Pro Arg Ala Lys Glu
 340

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<211> 199

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Pro	Arg	Ala	Val	Arg	Thr	Leu	Ala	Ala	Ala	Phe	Ala	Asp	Tyr	Pro	Ala	20	25	30	
Thr	Arg	His	Thr	Val	Asp	Pro	Asp	Arg	His	Ile	Glu	Arg	Val	Thr	Glu	35	40	45	
Leu	Gln	Glu	Leu	Phe	Leu	Thr	Arg	Val	Gly	Leu	Asp	Ile	Gly	Lys	Val	50	55	60	
Trp	Val	Ala	Asp	Asp	Gly	Ala	Ala	Val	Ala	Val	Trp	Thr	Thr	Pro	Glu	65	70	75	80
Ser	Val	Glu	Ala	Gly	Ala	Val	Phe	Ala	Glu	Ile	Gly	Pro	Arg	Met	Ala	85	90	95	
Glu	Leu	Ser	Gly	Ser	Arg	Leu	Ala	Ala	Gln	Gln	Gln	Met	Glu	Gly	Leu	100	105	110	
Leu	Ala	Pro	His	Arg	Pro	Lys	Glu	Pro	Ala	Trp	Phe	Leu	Ala	Thr	Val	115	120	125	
Gly	Val	Ser	Pro	Asp	His	Gln	Gly	Lys	Gly	Leu	Gly	Ser	Ala	Val	Val	130	135	140	
Leu	Pro	Gly	Val	Glu	Ala	Ala	Glu	Arg	Ala	Gly	Val	Pro	Ala	Phe	Leu	145	150	155	160
Glu	Thr	Ser	Ala	Pro	Arg	Asn	Leu	Pro	Phe	Tyr	Glu	Arg	Leu	Gly	Phe	165	170	175	
Thr	Val	Thr	Ala	Asp	Val	Glu	Val	Pro	Glu	Gly	Pro	Arg	Thr	Trp	Cys				

180

185

190

Met Thr Arg Lys Pro Gly Ala
195

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<211> 264

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Met Ile Glu Gln Asp Gly Leu His Ala Gly Ser Pro Ala Ala Trp Val
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Glu Arg Leu Phe Gly Tyr Asp Trp Ala Gln Gln Thr Ile Gly Cys Ser
20 25 30

Asp Ala Ala Val Phe Arg Leu Ser Ala Gln Gly Arg Pro Val Leu Phe
35 40 45

Val Lys Thr Asp Leu Ser Gly Ala Leu Asn Glu Leu Gln Asp Glu Ala
50 55 60

Ala Arg Leu Ser Trp Leu Ala Thr Thr Gly Val Pro Cys Ala Ala Val
65 70 75 80

Leu Asp Val Val Thr Glu Ala Gly Arg Asp Trp Leu Leu Leu Gly Glu
85 90 95

Val Pro Gly Gln Asp Leu Leu Ser Ser His Leu Ala Pro Ala Glu Lys
100 105 110

Val Ser Ile Met Ala Asp Ala Met Arg Arg Leu His Thr Leu Asp Pro
115 120 125

Ala Thr Cys Pro Phe Asp His Gln Ala Lys His Arg Ile Glu Arg Ala
130 135 140

Arg Thr Arg Met Glu Ala Gly Leu Val Asp Gln Asp Asp Leu Asp Glu
145 150 155 160

Glu His Gln Gly Leu Ala Pro Ala Glu Leu Phe Ala Arg Leu Lys Ala
165 170 175

Arg Met Pro Asp Gly Glu Asp Leu Val Val Thr His Gly Asp Ala Cys
180 185 190

Leu Pro Asn Ile Met Val Glu Asn Gly Arg Phe Ser Gly Phe Ile Asp
195 200 205

Cys Gly Arg Leu Gly Val Ala Asp Arg Tyr Gln Asp Ile Ala Leu Ala
210 215 220

Thr Arg Asp Ile Ala Glu Glu Leu Gly Gly Glu Trp Ala Asp Arg Phe
225 230 235 240

Leu Val Leu Tyr Gly Ile Ala Ala Pro Asp Ser Gln Arg Ile Ala Phe
245 250 255

Tyr Arg Leu Leu Asp Glu Phe Phe
260

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<212> PRT

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1 5 10 15

Ala Thr Glu Lys Ile Thr Met Leu Tyr Glu Asp Asn Lys His His Val
20 25 30

Gly Ala Ala Ile Arg Thr Lys Thr Gly Glu Ile Ile Ser Ala Val His
35 40 45

Ile Glu Ala Tyr Ile Gly Arg Val Thr Val Cys Ala Glu Ala Ile Ala
50 55 60

Ile Gly Ser Ala Val Ser Asn Gly Gln Lys Asp Phe Asp Thr Ile Val
65 70 75 80

Ala Val Arg His Pro Tyr Ser Asp Glu Val Asp Arg Ser Ile Arg Val
85 90 95

Val Ser Pro Cys Gly Met Cys Arg Glu Leu Ile Ser Asp Tyr Ala Pro
100 105 110

Asp Cys Phe Val Leu Ile Glu Met Asn Gly Lys Leu Val Lys Thr Thr
115 120 125

Ile Glu Glu Leu Ile Pro Leu Lys Tyr Thr Arg Asn
130 135 140

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Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
1 5 10 15

Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
20 25 30

Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
35 40 45

Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
50 55 60

Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65 70 75 80

Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
85 90 95

Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
100 105 110

Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
115 120 125

Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
130 135 140

Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145 150 155 160

Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
165 170 175

Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
180 185 190

Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
195 200 205

Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
210 215 220

Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
225 230 235

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<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic Polypeptide

<400> 6

Met Val Ser Lys Gly Glu Ala Val Ile Lys Glu Phe Met Arg Phe Lys
1 5 10 15

Val His Met Glu Gly Ser Met Asn Gly His Glu Phe Glu Ile Glu Gly
20 25 30

Glu Gly Glu Gly Arg Pro Tyr Glu Gly Thr Gln Thr Ala Lys Leu Lys
35 40 45

Val Thr Lys Gly Gly Pro Leu Pro Phe Ser Trp Asp Ile Leu Ser Pro
50 55 60

Gln Phe Met Tyr Gly Ser Arg Ala Phe Thr Lys His Pro Ala Asp Ile
65 70 75 80

Pro Asp Tyr Tyr Lys Gln Ser Phe Pro Glu Gly Phe Lys Trp Glu Arg
85 90 95

Val Met Asn Phe Glu Asp Gly Gly Ala Val Thr Val Thr Gln Asp Thr
100 105 110

Ser Leu Glu Asp Gly Thr Leu Ile Tyr Lys Val Lys Leu Arg Gly Thr
115 120 125

Asn Phe Pro Pro Asp Gly Pro Val Met Gln Lys Lys Thr Met Gly Trp
130 135 140

Glu Ala Ser Thr Glu Arg Leu Tyr Pro Glu Asp Gly Val Leu Lys Gly
145 150 155 160

Asp Ile Lys Met Ala Leu Arg Leu Lys Asp Gly Gly Arg Tyr Leu Ala
165 170 175

Asp Phe Lys Thr Thr Tyr Lys Ala Lys Lys Pro Val Gln Met Pro Gly
180 185 190

Ala Tyr Asn Val Asp Arg Lys Leu Asp Ile Thr Ser His Asn Glu Asp
195 200 205

Tyr Thr Val Val Glu Gln Tyr Glu Arg Ser Glu Gly Arg His Ser Thr
210 215 220

Gly Gly Met Asp Glu Leu Tyr Lys
225 230

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<223> Synthetic Polypeptide

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Cys Leu Ser Tyr Glu Thr Glu Ile Leu Thr Val Glu Tyr Gly Leu Leu
1 5 10 15

Pro Ile Gly Lys Ile Val Glu Lys Arg Ile Glu Cys Thr Val Tyr Ser
20 25 30

Val Asp Asn Asn Gly Asn Ile Tyr Thr Gln Pro Val Ala Gln Trp His
35 40 45

Asp Arg Gly Glu Gln Glu Val Phe Glu Tyr Cys Leu Glu Asp Gly Ser
50 55 60

Leu Ile Arg Ala Thr Lys Asp His Lys Phe Met Thr Val Asp Gly Gln
65 70 75 80

Met Leu Pro Ile Asp Glu Ile Phe Glu Arg Glu Leu Asp Leu Met Arg
85 90 95

Val Asp Asn Leu Pro Asn
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<400> 8

Ile Lys Ile Ala Thr Arg Lys Tyr Leu Gly Lys Gln Asn Val Tyr Asp
1 5 10 15

Ile Gly Val Glu Arg Asp His Asn Phe Ala Leu Lys Asn Gly Phe Ile
20 25 30

Ala Ser Asn
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<210> 9

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<212> PRT

<213> Artificial Sequence

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<223> Synthetic Polypeptide

<400> 9

Cys Ile Ser Gly Asp Ser Leu Ile Ser Leu Ala Ser Thr Gly Lys Arg
1 5 10 15

Val Ser Ile Lys Asp Leu Leu Asp Glu Lys Asp Phe Glu Ile Trp Ala
20 25 30

Ile Asn Glu Gln Thr Met Lys Leu Glu Ser Ala Lys Val Ser Arg Val
35 40 45

Phe Cys Thr Gly Lys Lys Leu Val Tyr Ile Leu Lys Thr Arg Leu Gly
50 55 60

Arg Thr Ile Lys Ala Thr Ala Asn His Arg Phe Leu Thr Ile Asp Gly
65 70 75 80

Trp Lys Arg Leu Asp Glu Leu Ser Leu Lys Glu His Ile Ala Leu Pro
85 90 95

Arg Lys Leu Glu Ser Ser Ser Leu Gln Leu
100 105

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<400> 10

Ser Pro Glu Ile Glu Lys Leu Ser Gln Ser Asp Ile Tyr Trp Asp Ser
1 5 10 15

Ile Val Ser Ile Thr Glu Thr Gly Val Glu Glu Val Phe Asp Leu Thr
20 25 30

Val Pro Gly Pro His Asn Phe Val Ala Asn Asp Ile Ile Val His Asn
35 40 45

<210> 11

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Cys Leu Ser Tyr Glu Thr Glu Ile Leu Thr Val Glu Tyr Gly Leu Leu
1 5 10 15

Pro Ile Gly Lys Ile Val Glu Lys Arg Ile Glu Cys Thr Val Tyr Ser
20 25 30

Val Asp Asn Asn Gly Asn Ile Tyr Thr Gln Pro Val Ala Gln Trp His
35 40 45

Asp Arg Gly Glu Gln Glu Val Phe Glu Tyr Cys Leu Glu Asp Gly Ser
50 55 60

Leu Ile Arg Ala Thr Lys Asp His Lys Phe Met Thr Val Asp Gly Gln
65 70 75 80

Met Leu Pro Ile Asp Glu Ile Phe Glu Arg Glu Leu Asp Leu Met Arg
85 90 95

Val Asp Asn Leu Pro Asn Gly Gly Gly Gly Ser Gly Ser Ala Gln Leu
100 105 110

Glu Lys Glu Leu Gln Ala Leu Glu Lys Lys Leu Ala Gln Leu Glu Trp
115 120 125

Glu Asn Gln Ala Leu Glu Lys Glu Leu Ala Gln
130 135

<210> 12

<211> 68

<212> PRT

<213> Artificial Sequence

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<223> Synthetic Polypeptide

<400> 12

Ala Gln Leu Lys Lys Lys Leu Gln Ala Asn Lys Lys Glu Leu Ala Gln
1 5 10 15

Leu Lys Trp Lys Leu Gln Ala Leu Lys Lys Lys Leu Ala Gln Gly Gly
20 25 30

Gly Gly Ser Gly Ser Met Ile Lys Ile Ala Thr Arg Lys Tyr Leu Gly
35 40 45

Lys Gln Asn Val Tyr Asp Ile Gly Val Gly Glu Pro His Asn Phe Ala
50 55 60

Leu Lys Asn Gly
65

<210> 13
<211> 35
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic Polypeptide

<400> 13

Ile Lys Ile Ala Thr Arg Lys Tyr Leu Gly Lys Gln Asn Val Tyr Asp
1 5 10 15

Ile Gly Val Gly Glu Pro His Asn Phe Ala Leu Lys Asn Gly Phe Ile
20 25 30

Ala Ser Asn
35

<210> 14
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<400> 14

Ala Gln Leu Glu Lys Glu Leu Gln Ala Leu Glu Lys Lys Leu Ala Gln
1 5 10 15

Leu Glu Trp Glu Asn Gln Ala Leu Glu Lys Glu Leu Ala Gln
20 25 30

<210> 15

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<220>

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<400> 15

Ala Gln Leu Lys Lys Lys Leu Gln Ala Asn Lys Lys Glu Leu Ala Gln
1 5 10 15

Leu Lys Trp Lys Leu Gln Ala Leu Lys Lys Lys Leu Ala
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<210> 16

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<223> Synthetic Polypeptide

<400> 16

Cys Leu Ser Phe Gly Thr Glu Ile Leu Thr Val Glu Tyr Gly Pro Leu
1 5 10 15

Pro Ile Gly Lys Ile Val Ser Glu Glu Ile Asn Cys Ser Val Tyr Ser

20

25

30

Val Asp Pro Glu Gly Arg Val Tyr Thr Gln Ala Ile Ala Gln Trp His
 35 40 45

Asp Arg Gly Glu Gln Glu Val Leu Glu Tyr Glu Leu Glu Asp Gly Ser
 50 55 60

Val Ile Arg Ala Thr Ser Asp His Arg Phe Leu Thr Thr Asp Tyr Gln
 65 70 75 80

Leu Leu Ala Ile Glu Glu Ile Phe Ala Arg Gln Leu Asp Leu Leu Thr
 85 90 95

Leu Glu Asn Ile Lys Gln Thr Glu Glu Ala Leu Asp Asn His Arg Leu
 100 105 110

Pro Phe Pro Leu Leu Asp Ala Gly Thr Ile Lys
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Lys Thr Arg Leu Gly Arg Thr Ile Lys Ala Thr Ala Asn His Arg Phe
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Leu Thr Ile Asp Gly Trp Lys Arg Leu Asp Glu Leu Ser Leu Lys Glu

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His Ile Ala Leu Pro Arg Lys Leu Glu Ser Ser Ser Leu Gln Leu Ser
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4304