Split Selectable Markers

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Abstract

Selectable markers, such as antibiotic resistance or fluorescent protein genes, are widely adopted in transgenesis and genome editing for selecting engineered cells with desired genotype but are limited in choices. We demonstrate here the concept of split selectable markers each allowing for selection of multiple "unlinked" transgenes with one selection scheme. Future development of split selectable markers may support enrichment or selection of "hyper-engineered" cells containing tens of transgenes or genetic modifications.

Main

Selectable markers are often used in genetic engineering to isolate cells with desired 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 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 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 marketrons. 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 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 *Npu*DnaE [4,5] and *Ssp*DnaB [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 cytometry. For hygromycin (Hygro) resistance gene, one "native" *Ssp*DnaB split point (G200:S201) with flanking residues "GS" and one "native" *Npu*DnaE 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 1c; Plasmid pairs 3,4 and 5,6). Cells transduced 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 *Npu*DnaE 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 *Npu*DnaE 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 1c). Similarly, for puromycin

(Puro) (Fig 1d), neomycin (Neo) (Fig 1e) and blasticidin (Blast) (Fig 1f) 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 (Fig 1c~f). To facilitate adoption of Intres markers, we created Gateway-compatible lentiviral vectors for convenient restriction-ligation-independent LR clonase recombination of transgenes [8] (Fig 2a). We tested the functionality of these vectors by recombining TagBPF2 and mCherry, respectively to the N- and C-Intres vectors and found robust selection of double transgenic cells (Fig 2b). 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 2c). 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 2c). Details of the split points of Intres genes and plasmids are presented in Supplementary fig 1~4 and Supplementary table 1.

To test whether split fluorescent markers can be used for transgene selection, we screened for *Npu*DnaE split points for mScarlet fluorescent protein (Fig 3a) 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 3b).

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 (Fig 4a and b). 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 4c). 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 *Npu*DnaE inteins, two using *Npu*DnaE for the first intein and *Ssp*DnaB for the second intein, as well as two using *Ssp*DnaB for the first intein and *Npu*DnaE for the second intein (Fig 4d). 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 5a). 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 5b). 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 5c). We further tested the feasibility of 4-markertron hygromycin Intres genes (Supplementary Fig 6a). Here, we used an enhanced variant of *Npu*DnaE intein known as *Npu*DnaGEP [10] fused with leucine zipper motifs [11] in combination with the *Ssp*DnaB 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 (Supplementary Fig 6b).

In this study, 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 to enable selection of cells with double transgenesis. 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 five or more transgenes.

Acknowledgements

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Figure Legends

Figure 1. Split selectable marker for antibiotic co-selection of two separate transgenic vectors. (a) The coding sequence of a selectable marker is split into an N-terminal fragment (Mar_N) and a C-terminal fragment (ker_C) and separately cloned upstream of an N-terminal fragment of a split intein (Int_N) and downstream of a C-terminal fragment of the split intein (Int_C), 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. (b) 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. (c) 2-markertron hygromycin (Hygro) intein-split resistance (Intres) genes. Top schematics 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 *Npu*DnaE intein while square lollipops represent those using *Ssp*DnaB 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. (d) 2-markertron puromycin (Puro) Intres genes. Top schematics show 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). (e) 2-markertron neomycin (Neo) resistance genes. Top schematics show 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). (f) 2-markertron blasticidin (Blast) Intres genes. Top schematics show split points tested for blasticidin resistance gene while bottom column plot shows percentages of double transgnic cells in the non-selected (white columns) and the selected (cyan colums) cultures.

Figure 2. Gateway-compatible lentiviral destination vectors with 2-markertron Intres markers. (a) Gateway-compatiable 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. (b) 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). (c) 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.

Figure 3. Split mScarlet for fluorescence-mediated co-selection of two separate transgenic vectors. (a) 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. (b) To screen for NpuDnaE 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. (c) 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).

Figure 4. Multi-split selectable markers for co-selection of three or more transgenic vectors. (a) 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. (b) 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. (c) 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. (d) 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 *Npu*DnaE and *Ssp*DnaB 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.

Figure 5. Gateway-compatible lentiviral destination vectors with 3-markertron hygromycin Intres genes. (a) Gateway-compatiable 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. (b) 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. (c) 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).

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Figure 1

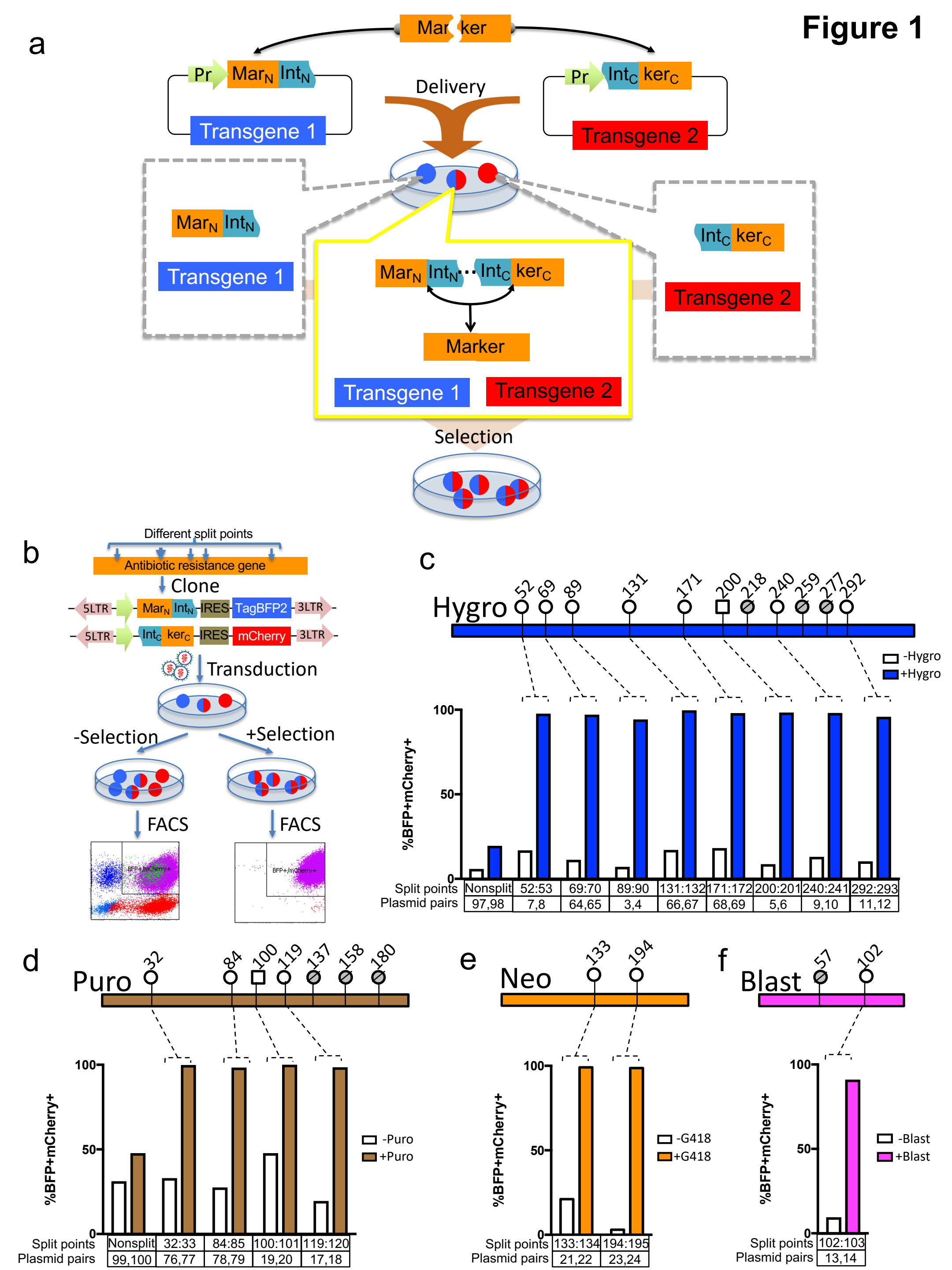
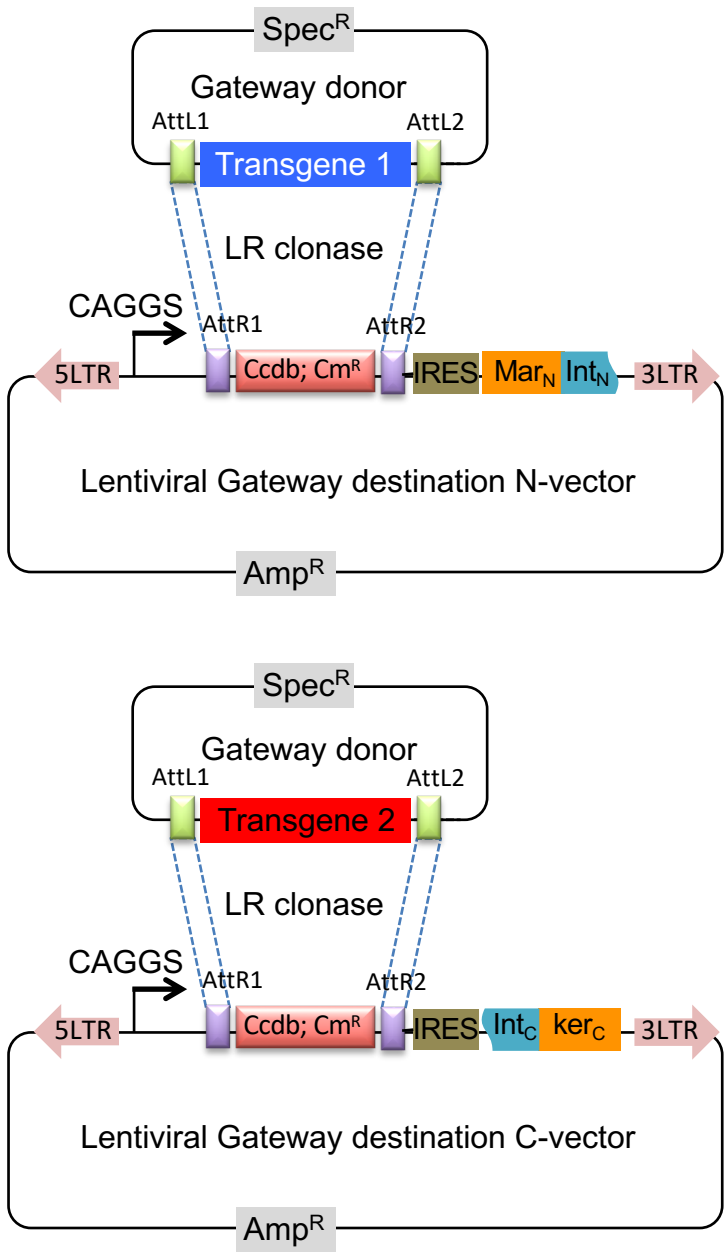

a
Mar ker
Pr
MarN IntN
Transgene 1
Delivery
Pr
IntC kerC
Transgene 2
MarN IntN
Transgene 1
MarN IntN ··· IntC kerC
Marker
Transgene 1
Transgene 2
IntC kerC
Transgene 2
Selection
b
Different split points
Antibiotic resistance gene
Clone
5LTR
MarN IntN
IRES
TagBFP2
3LTR
5LTR
IntC kerC
IRES
mCherry
3LTR
Transduction
-Selection
+Selection
FACS
FACS
BFP+/mCherry+
BFP+/mCherry+
c
Hygro
52 69 89 131 171 200 218 240 259 277 292
-Hygro
+Hygro
%BFP+mCherry+
100
50
0
Split points | Nonsplit | 52:53 | 69:70 | 89:90 | 131:132 | 171:172 | 200:201 | 240:241 | 292:293
Plasmid pairs | 97,98 | 7,8 | 64,65 | 3,4 | 66,67 | 68,69 | 5,6 | 9,10 | 11,12
d
Puro
32 84 100 119 137 158 180
%BFP+mCherry+
100
50
0
-Puro
+Puro
Split points | Nonsplit | 32:33 | 84:85 | 100:101 | 119:120
Plasmid pairs | 99,100 | 76,77 | 78,79 | 19,20 | 17,18
e
Neo
133 194
%BFP+mCherry+
100
50
0
-G418
+G418
Split points | 133:134 | 194:195
Plasmid pairs | 21,22 | 23,24
f
Blast
57 102
%BFP+mCherry+
100
50
0
-Blast
+Blast
Split points | 102:103
Plasmid pairs | 13,14

Figure 2

a



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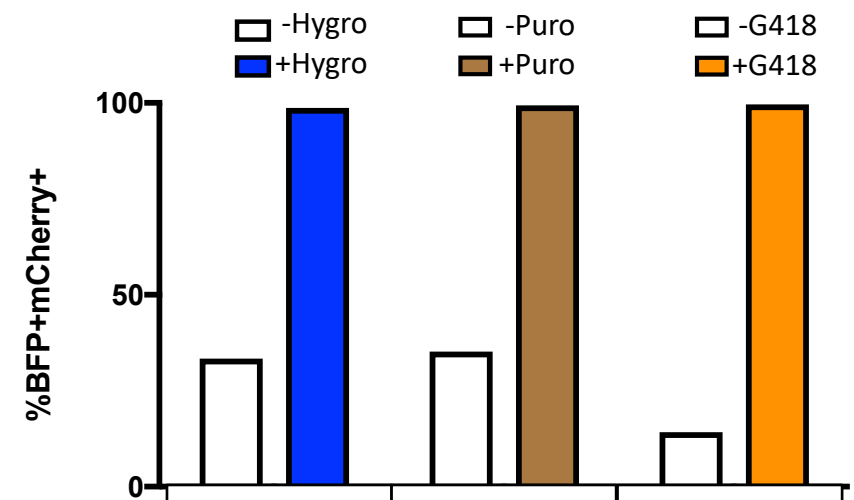


Resistance	Hygro	Puro	Neo
Dest. Plasmids	49,50	53,54	57,58
Final Plasmids	51,52	55,56	59,60

c

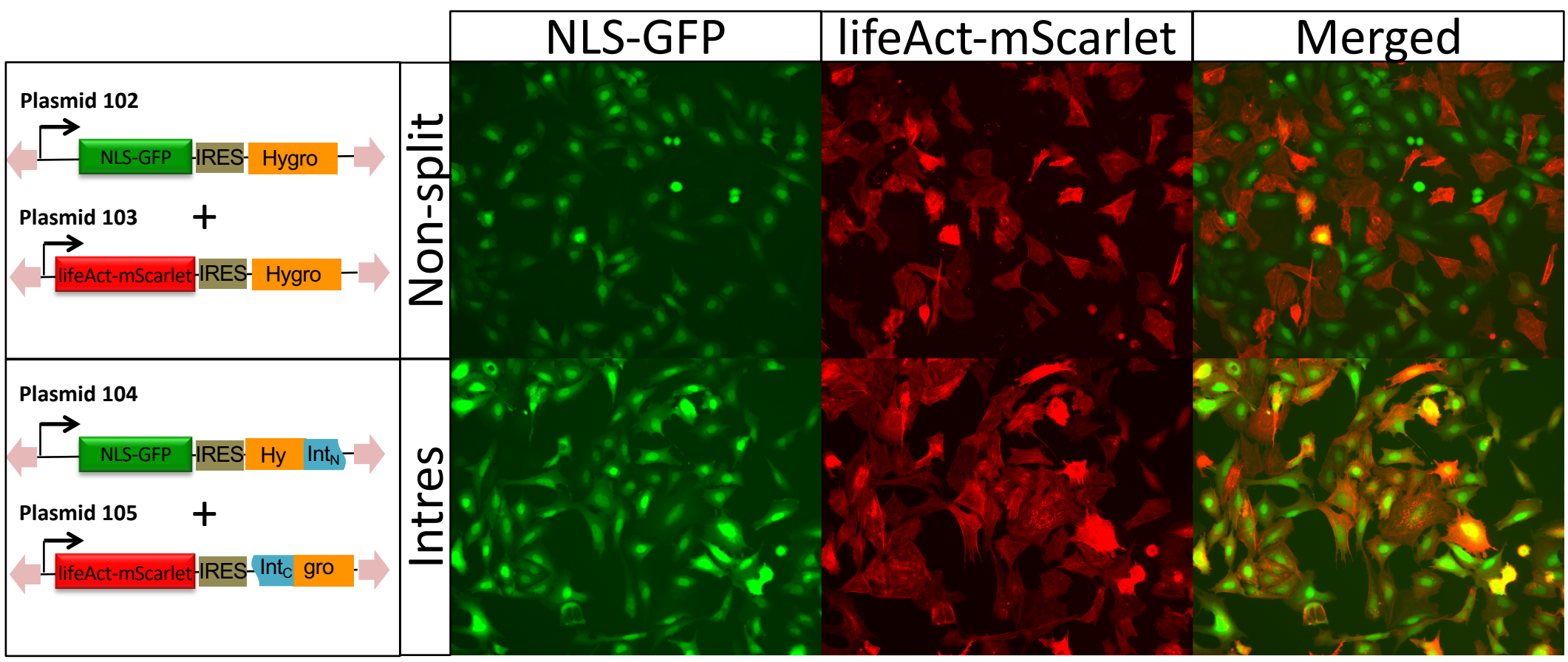


Figure 3

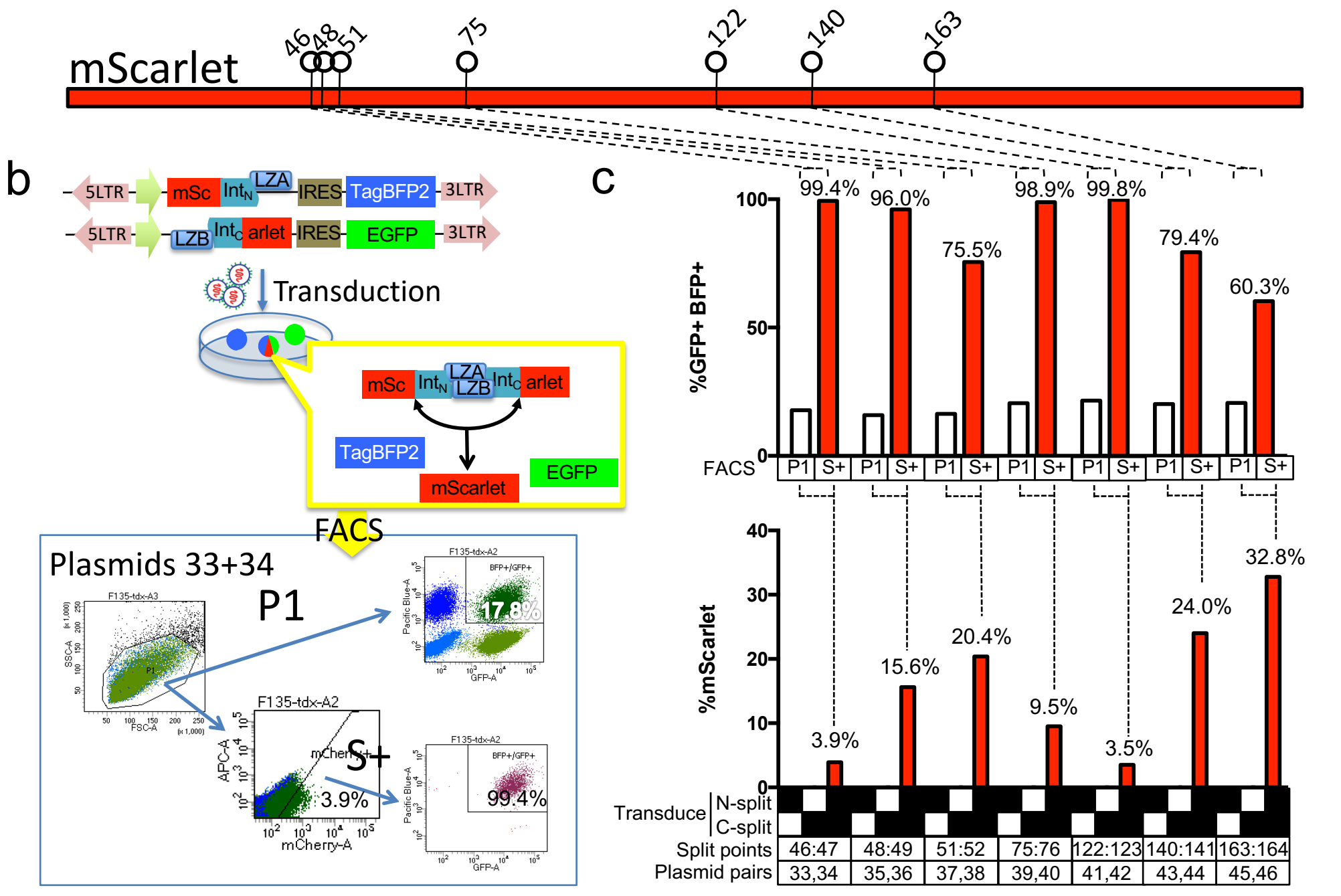

a
mScarlet
46
48
51
75
122
140
163
b
5LTR
mSc
Int$_N$
LZA
IRES
TagBFP2
3LTR
5LTR
LZB
Int$_C$
arlet
IRES
EGFP
3LTR
Transduction
mSc
Int$_N$
LZA
LZB
Int$_C$
arlet
TagBFP2
mScarlet
EGFP
FACS
Plasmids 33+34
P1
F135-tdx-A3
SSC-A
FSC-A
F135-tdx-A2
APC-A
mCherry-A
S+
3.9%
F135-tdx-A2
BFP+/GFP+
17.8%
Pacific Blue-A
GFP-A
F135-tdx-A2
BFP+/GFP+
99.4%
Pacific Blue-A
GFP-A
c
%GFP+ BFP+
100
50
0
99.4%
96.0%
75.5%
98.9%
99.8%
79.4%
60.3%
FACS
P1
S+
%mScarlet
40
30
20
10
0
3.9%
15.6%
20.4%
9.5%
3.5%
24.0%
32.8%
Transduce
N-split
C-split
Split points
46:47
48:49
51:52
75:76
122:123
140:141
163:164
Plasmid pairs
33,34
35,36
37,38
39,40
41,42
43,44
45,46

Figure 4

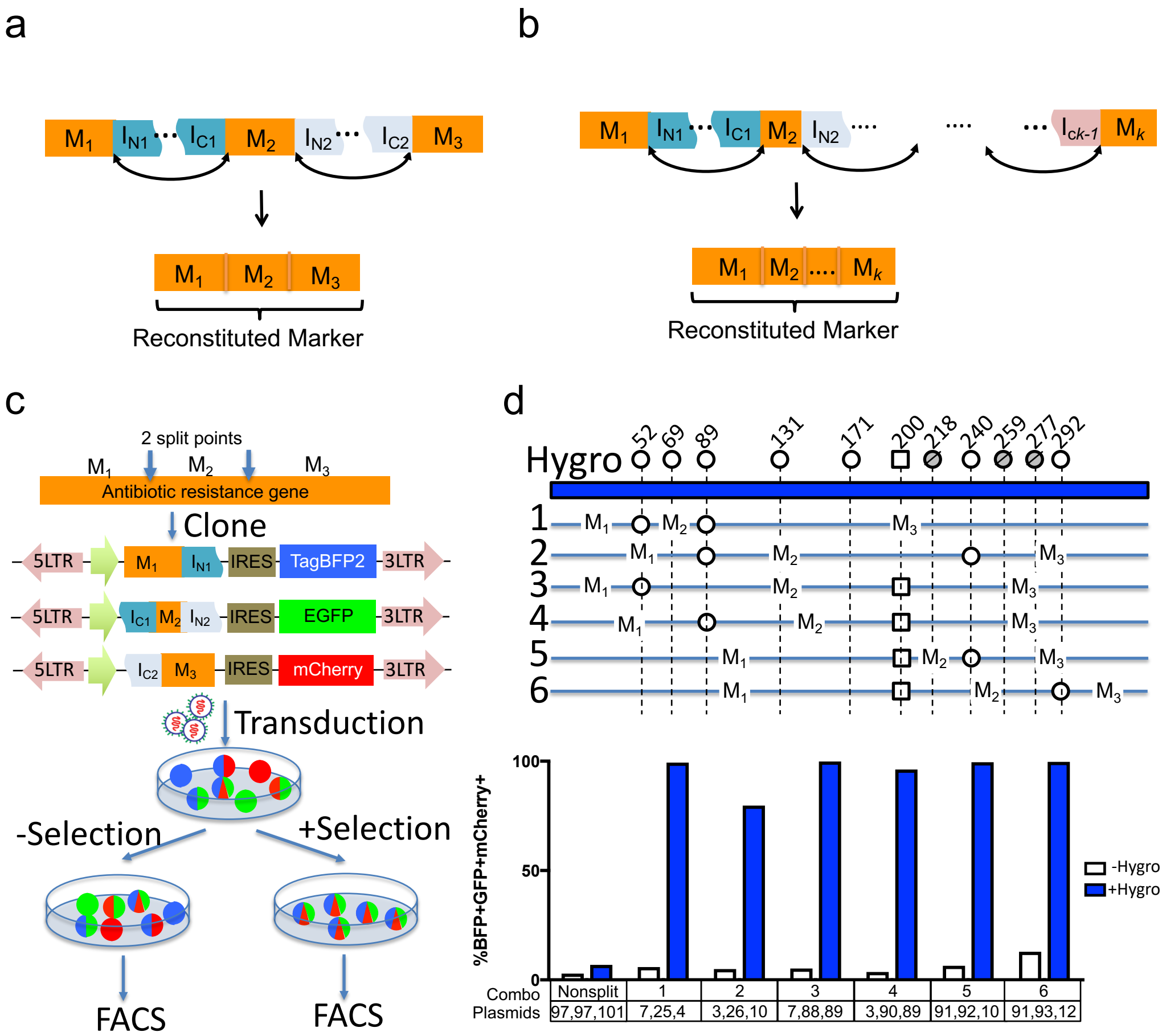
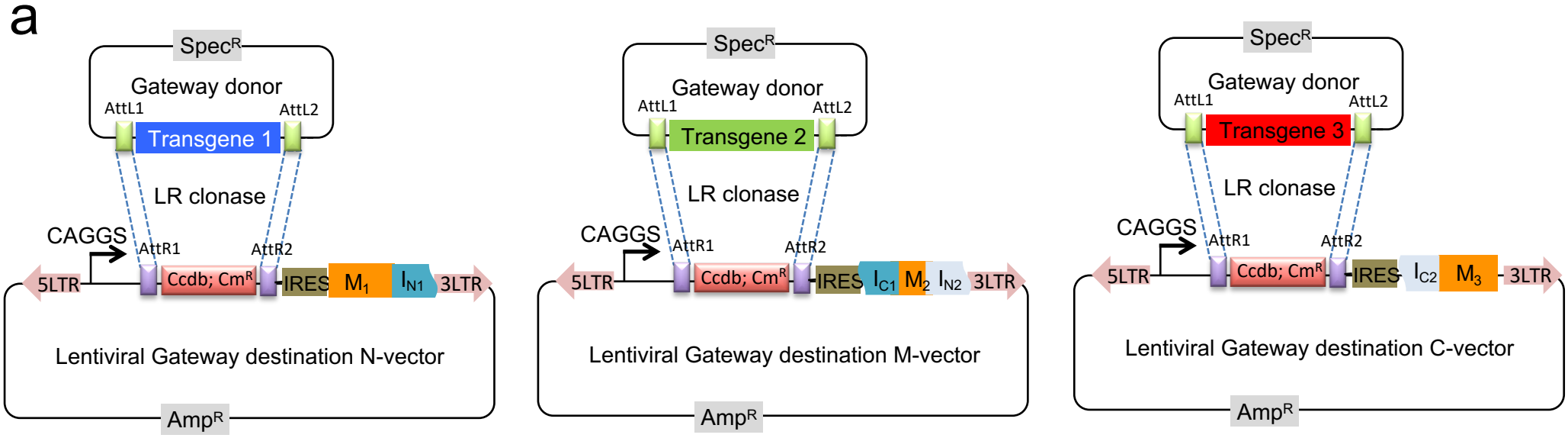
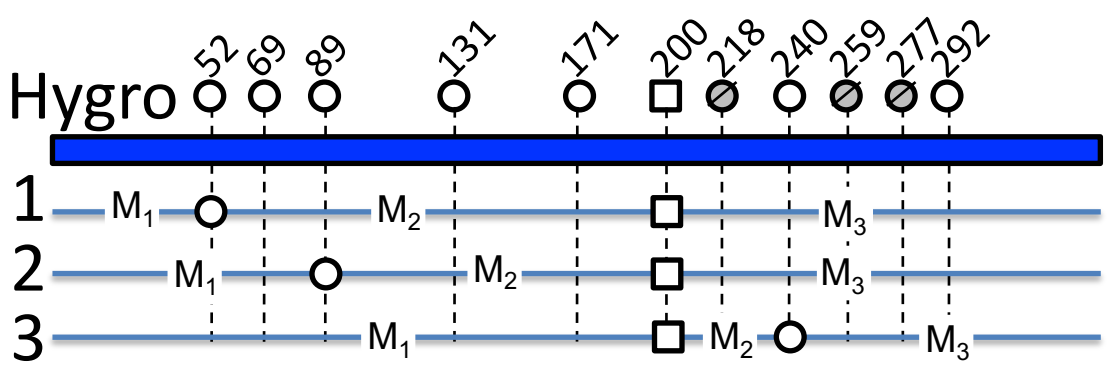

a
M1 IN1 IC1 M2 IN2 IC2 M3
M1 M2 M3
Reconstituted Marker
b
M1 IN1 IC1 M2 IN2 .... Ick-1 Mk
M1 M2 .... Mk
Reconstituted Marker
c
2 split points
M1 M2 M3
Antibiotic resistance gene
Clone
5LTR M1 IN1 IRES TagBFP2 3LTR
5LTR IC1 M2 IN2 IRES EGFP 3LTR
5LTR IC2 M3 IRES mCherry 3LTR
Transduction
-Selection
+Selection
FACS
FACS
d
Hygro
52 69 89 131 171 200 218 240 259 277 292
1 M1 M2 M3
2 M1 M2 M3
3 M1 M2 M3
4 M1 M2 M3
5 M1 M2 M3
6 M1 M2 M3
%BFP+GFP+mCherry+
100
50
0
-Hygro
+Hygro
Combo Nonsplit 1 2 3 4 5 6
Plasmids 97,97,101 7,25,4 3,26,10 7,88,89 3,90,89 91,92,10 91,93,12

Figure 5

a



b

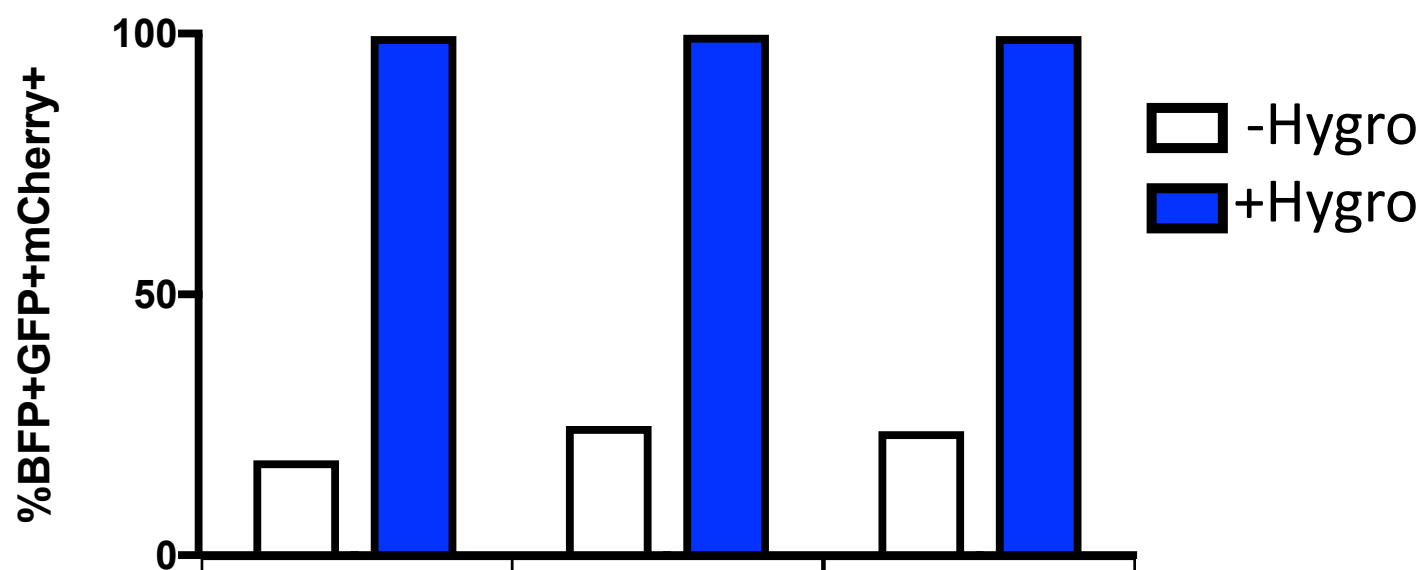


Transgene 1 = TagBFP
Transgene 2 = EGFP
Transgene 3 = mCherry

↓ LR clonase

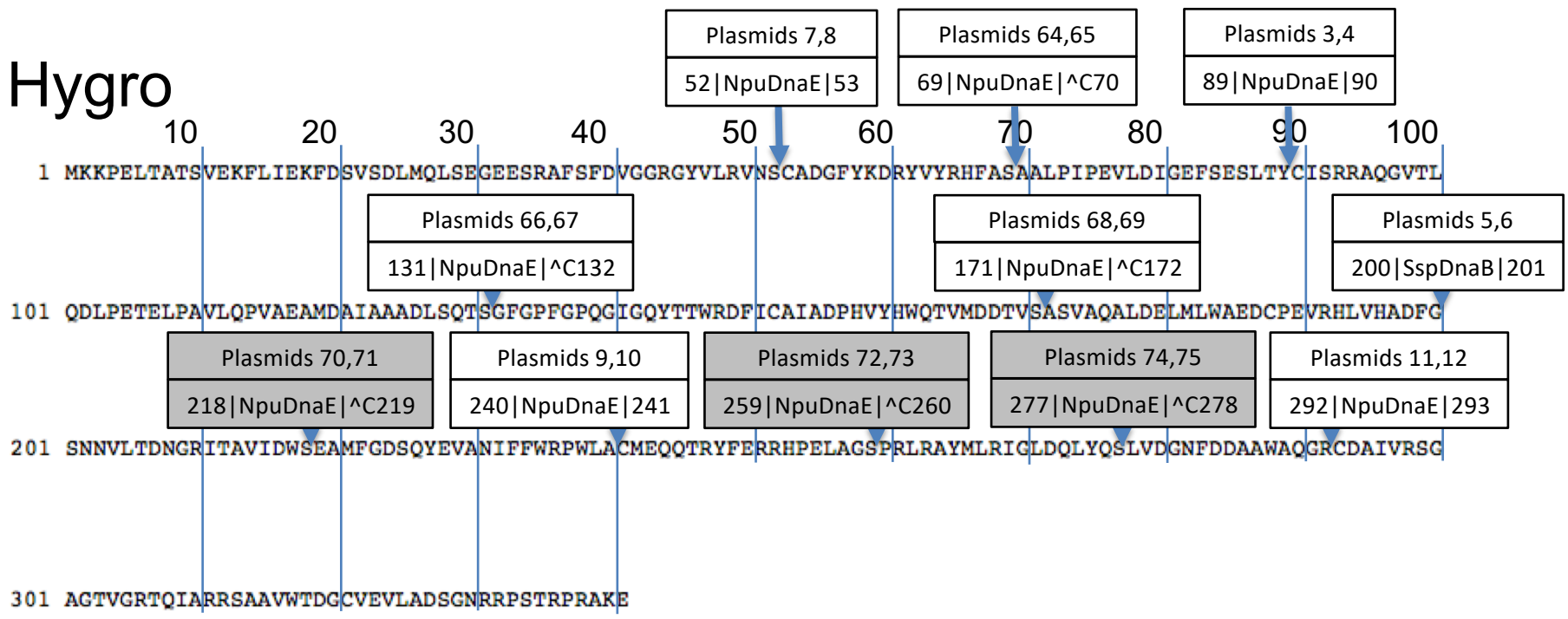
Dest. Plasmids	→	Final Plasmids
115,116,117		118,119,120
121,122,123		124,125,126
127,128,129		130,131,132

c

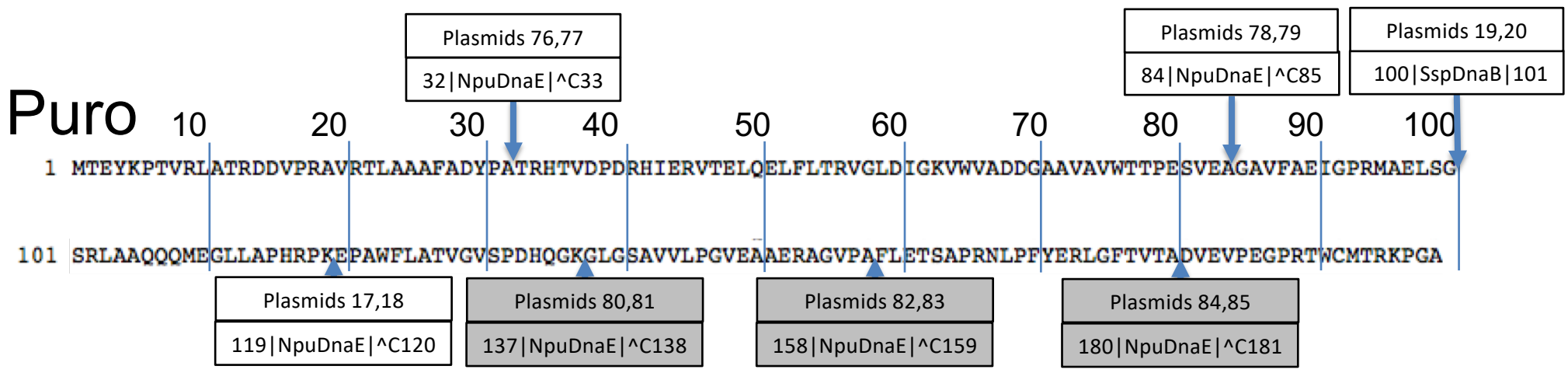


Combo	1	2	3
Dest. Plasmids	115,116,117	121,122,123	127,128,129
Final Plasmids	118,119,120	124,125,126	130,131,132

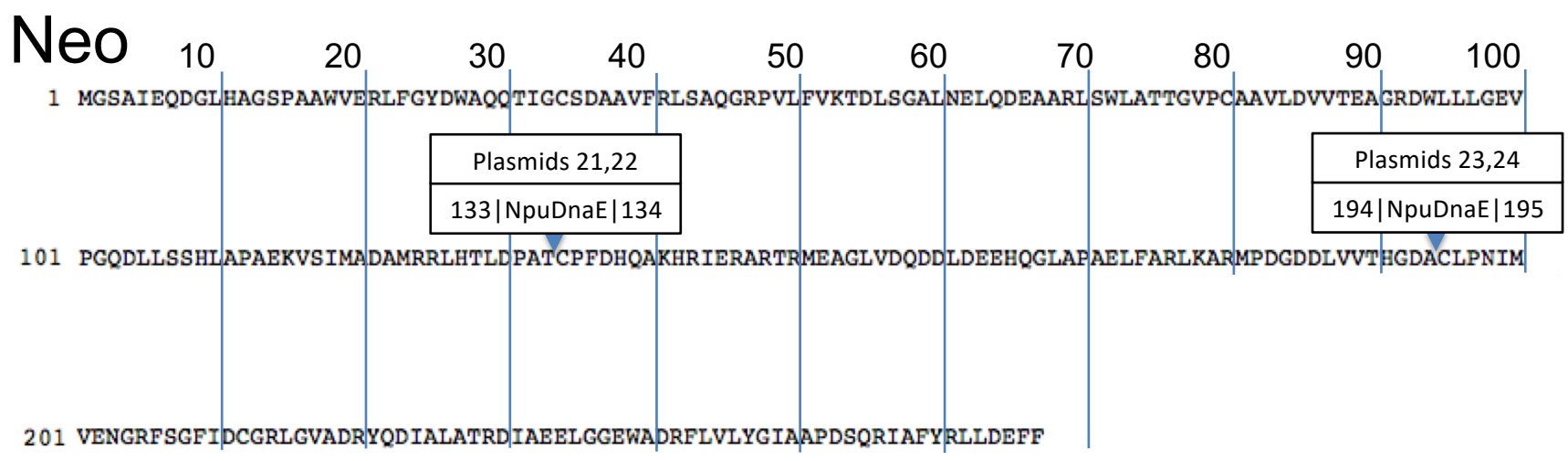
Supplementary Figure 1



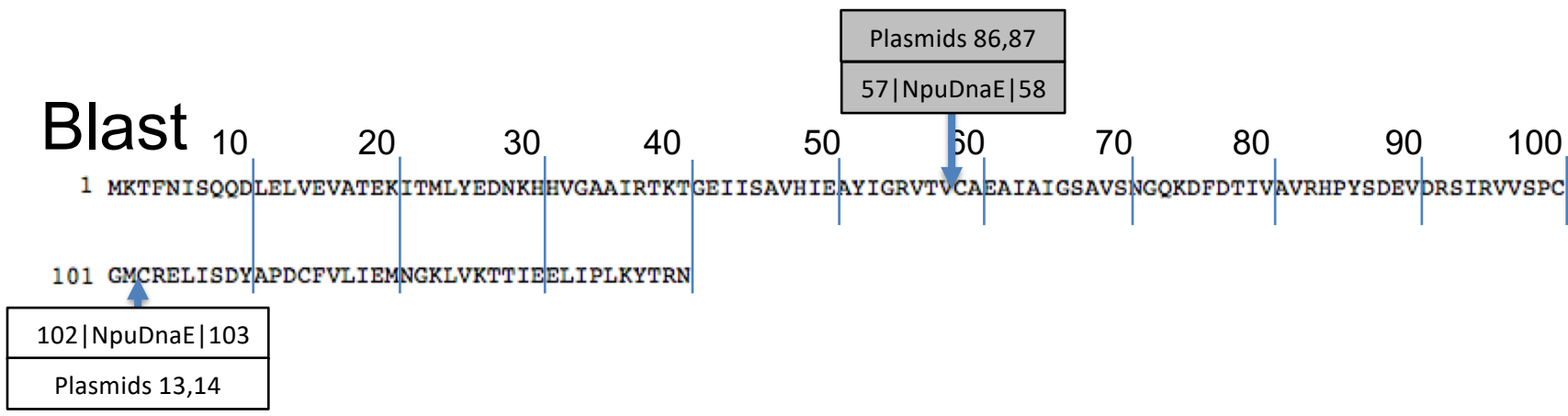
Supplementary Figure 2



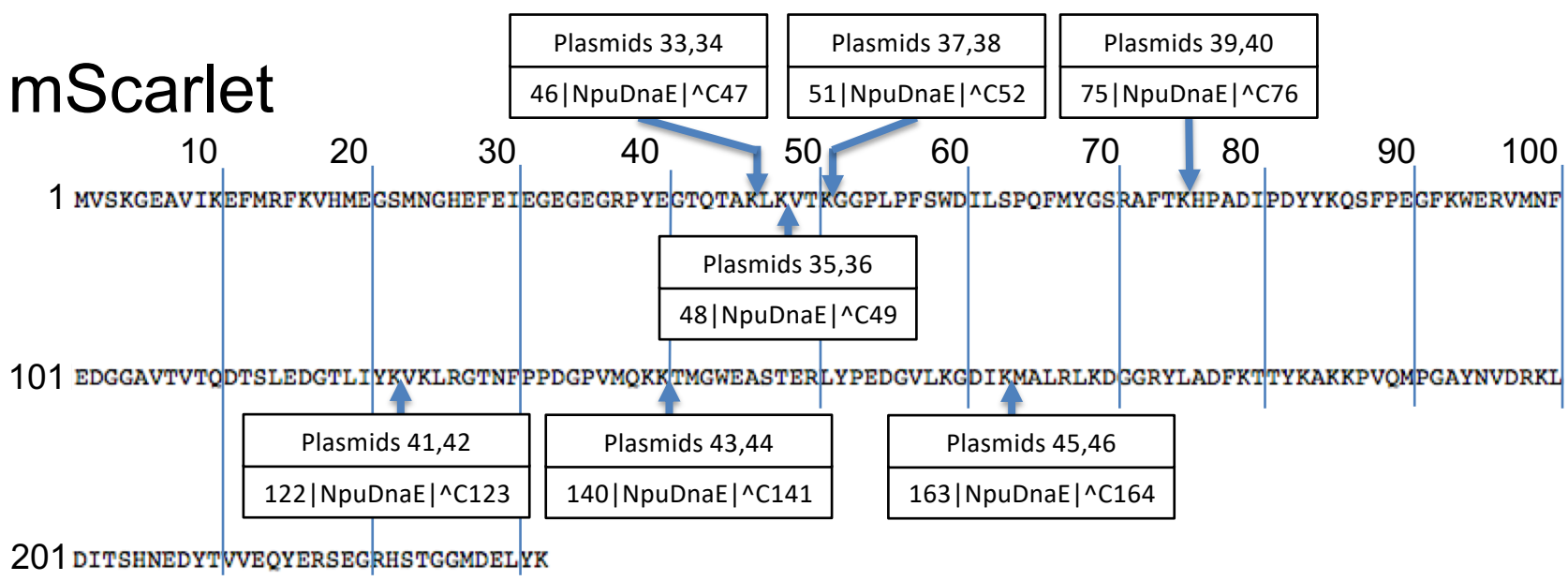
Supplementary Figure 3



Supplementary Figure 4

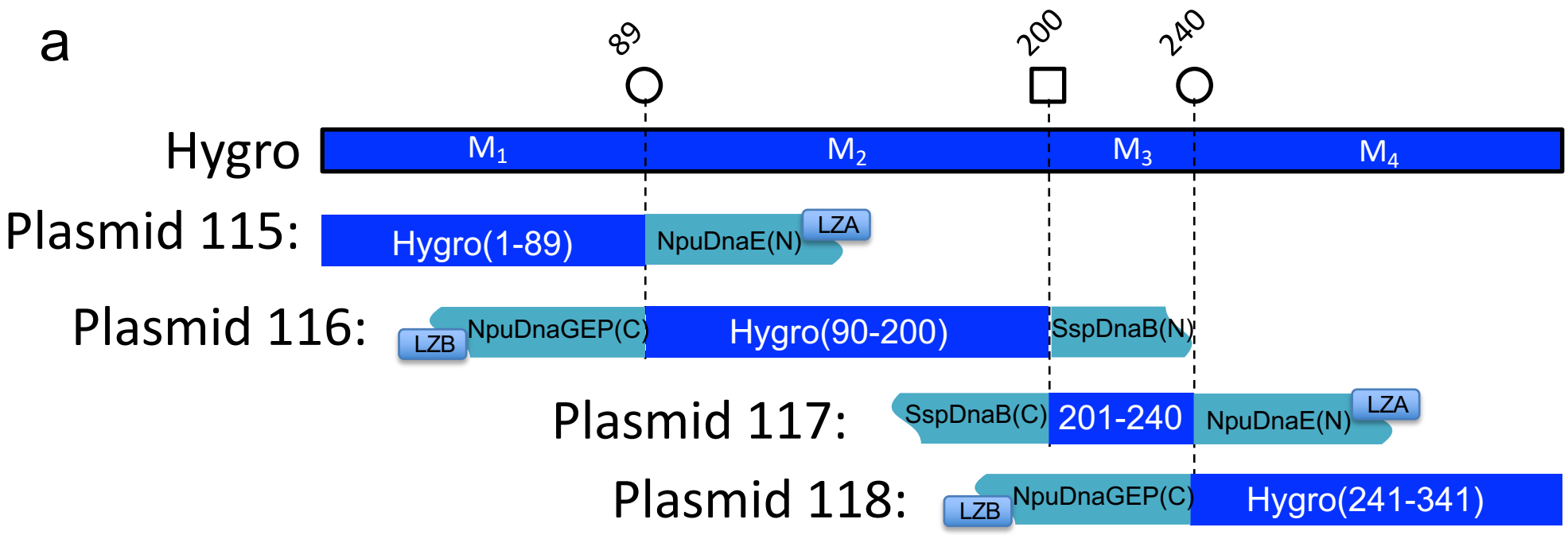


Supplementary Figure 5



Supplementary Figure 6

a



b

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

Split Selectable Markers

Online 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 (Gift from David Root; Addgene: http://www.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) [1], 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. Plasmids created in this study are listed in Supplementary Table 1. Plasmid maps will be available on http://intr.es Plasmid will be deposited to Addgene for distribution.

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 45 μM PES filters then stored at -80C until use.

Transduction

The day prior to transduction, U2OS cells were seeded into 12-well plates at a density of $1.5x10^5$ 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-transduction. Four days post-transduction, cells were split into duplicate plates. Five days post-transduction, media with antibiotics (130µg/mL Hygromycin, 2µg/mL Puromycin, 700µg/mL G418, or 6µg/mL Blastidicin) was added to each respective well of one replicate plate (the other remained under no selection). Antibiotics selection continued for 2 weeks before analysis on FACS.

Fluorescence-Activated Cell Sorting

Cells were trypisinized, suspended in media then analyzed on a LSRFortessa X-20 (BD Bioscience) flow cytometer using FACSDiVa software, version 8, on an HP Z230 workstation. Fifty thousand events were collected each run.

References

1. Li, M.Z. & Elledge, S.J. SLIC: a method for sequence-and ligation-independent cloning. *Gene Synthesis: Methods and Protocols*, 51-59 (2012).

Split Selectable Markers

Supplementary Table 1

Plasmid #*	Plasmid Name	Markertron	Plasmid Filename
3	pLX-Hygro(1-89)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-89)-NpuDnaE(N)	pLX-[HYGro-NpuDnaE(N)-25-65-85]-IRES-TagBFP2-25-79-47
4	pLX-NpuDnaE(C)-Hygro(90-341)-IRES-mCherry	NpuDnaE(C)-Hygro(90-341)	pLX-[NpuDnaE(C)_hygRO-25-65-86]-IRES-mCherry-25-79-57
5	pLX-Hygro(1-200)-SspDnaB(N)-IRES-TagBFP2	Hygro(1-200)-SspDnaB(N)	pLX-[HYGro(A)-SspDnaB(N)-25-65-72]-IRES-TagBFP2-25-79-42
6	pLX-SspDnaB(C)-Hygro(201-341)-IRES-mCherry	SspDnaB(C)-Hygro(201-341)	pLX-[SspDnaB(C)_hygRO(A)-25-81-23]-IRES-mCherry-25-91-56
7	pLX-Hygro(1-52)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-52)-NpuDnaE(N)	pLX-[HYGro(B)-NpuDnaE(N)-25-105-15]-IRES-TagBFP2-25-117-16
8	pLX-NpuDnaE(C)-Hygro(53-341)-IRES-mCherry	NpuDnaE(C)-Hygro(53-341)	pLX-[NpuDnaE(C)_hygRO(B)-25-105-16]-IRES-mCherry-25-117-22
9	pLX-Hygro(1-240)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-240)-NpuDnaE(N)	pLX-[HYGro(C)-NpuDnaE(N)-25-125-46]-IRES-TagBFP2-MD1-73-3
10	pLX-NpuDnaE(C)-Hygro(241-341)-IRES-mCherry	NpuDnaE(C)-Hygro(241-341)	pLX-[NpuDnaE(C)_hygRO(C)-25-105-18]-IRES-mCherry-25-117-23
11	pLX-Hygro(1-292)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-292)-NpuDnaE(N)	pLX-[HYGro(D)-NpuDnaE(N)[KozakDel3]-25-105-19]-IRES-TagBFP2-25-117-18
12	pLX-NpuDnaE(C)-Hygro(293-341)-IRES-mCherry	NpuDnaE(C)-Hygro(293-341)	pLX-[NpuDnaE(C)_hygRO(D)-25-105-20]-IRES-mCherry-25-117-24
13	pLX-Blast(1-102)-NpuDnaE(N)-IRES-TagBFP2	Blast(1-102)-NpuDnaE(N)	pLX-[Blast(B)-NpuDnaE(N)-25-119-7]-IRES-TagBFP2-MD1-63-6
14	pLX-NpuDnaE(C)-Blast(103-140)-IRES-mCherry	NpuDnaE(C)-Blast(103-140)	pLX-[NpuDnaE(C)_Blast(B)-25-119-8]-IRES-mCherry-MD1-63-9
17	pLX-Puro(1-119)-NpuDnaE(N)-IRES-TagBFP2	Puro(1-119)-NpuDnaE(N)	pLX-[PuroKC2(N)-NpuDnaE(N)-25-131-28"]-IRES-TagBFP2-25-133-5
18	pLX-NpuDnaE(C)-Puro(insCys;120-199)-IRES-mCherry	NpuDnaE(C)-Puro(insCys;120-199)	pLX-[NpuDnaE(C)_PuroKC2(C)-25-131-35"]-IRES-mCherry-25-133-13
19	pLX-Puro(1-100)-SspDnaB(N-S0)-IRES-TagBFP2	Puro(1-100)-SspDnaB(N-S0)	pLX-[Puro(A)_SspDnaB(N-S0)-26-3-37]-IRES-TagBFP2-26-9-14
20	pLX-SspDnaB(C-S0)-Puro(101-199)-IRES-mCherry	SspDnaB(C-S0)-Puro(101-199)	pLX-[SspDnaB(C-S0)_Puro(A)-26-1-19]-IRES-mCherry-26-7-26
21	pLX-Neo(1-133)-NpuDnaE(N)-IRES-TagBFP2	Neo(1-133)-NpuDnaE(N)	pLX-[Neo(A)-NpuDnaE(N)-25-125-1]-IRES-TagBFP2-MD1-73-1
22	pLX-NpuDnaE(C)-Neo(134-267)-IRES-mCherry	NpuDnaE(C)-Neo(134-267)	pLX-[NpuDnaE(C)_Neo(A)-25-119-2]-IRES-mCherry-F79-4

23	pLX-Neo(1-194)-NpuDnaE(N)-IRES-TagBFP2	Neo(1-194)-NpuDnaE(N)	pLX-[Neo(B)-NpuDnaE(N)-25-125-4]-IRES-TagBFP2-MD1-73-2
24	pLX-NpuDnaE(C)-Neo(195-267)-IRES-mCherry	NpuDnaE(C)-Neo(195-267)	pLX-[NpuDnaE(C)_Neo(B)-25-125-6]-IRES-mCherry-MD1-73-4
25	pLX-NpuDnaE(C)_Hygro(53-89)-NpuDnaE(N)-IRES-GFP	NpuDnaE(C)_Hygro(53-89)-NpuDnaE(N)	pLX-[NpuDnaE(C)_HygroBA_NpuDnaE(N)-25-155-79]-IRES-GFP-26-3-21
26	pLX-NpuDnaE(C)_Hygro(53-239)-NpuDnaE(N)-IRES-GFP	NpuDnaE(C)_Hygro(53-239)-NpuDnaE(N)	pLX-[NpuDnaE(C)_HygroAC_NpuDnaE(N)-25-155-83]-IRES-GFP-26-3-22
27			pCR8-BsaI->ccdbCam<-BsaI-NpuDnaE(N)-MD1-68-15
28			pCR8-NpuDnaE(C)_BsaI->ccdbCam<-BsaI-MD1-68-18
31			pCR8-BsaI->ccdbCam<-BsaI-SspDnaB(N-S0)-25-135-18
32			pCR8-SspDnaB(C-S0)_BsaI->ccdbCam<-BsaI-25-155-41
33	pLX-mScarlet(1-46)-NpuDnaE(N)_LZA-IRES-TagBFP2	mScarlet(1-46)-NpuDnaE(N)_LZA	pLX-[mScarlet(1-46)-NpuDnaE(N)_LZA-26-103-38]-IRES-TagBFP2-26-107-16
34	pLX-LZB_NpuDnaE(C)-mScarlet(insCys;47-232)-IRES-TagBFP2	LZB_NpuDnaE(C)-mScarlet(insCys;47-232)	pLX-[LZB_NpuDnaE(C)-mScarlet(insCys;47-232)-26-103-59]-IRES-GFP-26-107-33
35	pLX-mScarlet(1-48)-NpuDnaE(N)_LZA-IRES-TagBFP2	mScarlet(1-48)-NpuDnaE(N)_LZA	pLX-[mScarlet(1-48)-NpuDnaE(N)_LZA-26-103-39]-IRES-TagBFP2-26-107-17
36	pLX-LZB_NpuDnaE(C)-mScarlet(insCys;49-232)-IRES-GFP	LZB_NpuDnaE(C)-mScarlet(insCys;49-232)	pLX-[60-pCR8-LZB_NpuDnaE(C)-mScarlet(insCys;49-232)]-IRES-GFP-26-107-34
37	pLX-mScarlet(1-51)-NpuDnaE(N)_LZA -IRES-TagBFP2	mScarlet(1-51)-NpuDnaE(N)_LZA	pLX-[mScarlet(1-51)-NpuDnaE(N)_LZA-26-103-40]-IRES-TagBFP2-26-107-18
38	pLX-LZB_NpuDnaE(C)-mScarlet(insCys;52-232)-IRES-GFP	LZB_NpuDnaE(C)-mScarlet(insCys;52-232)	pLX-[LZB_NpuDnaE(C)-mScarlet(insCys;52-232)-26-103-61]-IRES-GFP-26-107-35
39	pLX-mScarlet(1-75)-NpuDnaE(N)_LZA-IRES-TagBFP2	mScarlet(1-75)-NpuDnaE(N)_LZA	pLX-[mScarlet(1-75)-NpuDnaE(N)_LZA-26-103-41]-IRES-TagBFP2-26-107-19
40	pLX-LZB_NpuDnaE(C)-mScarlet(insCys;76-232)-IRES-GFP	LZB_NpuDnaE(C)-mScarlet(insCys;76-232)	pLX-[LZB_NpuDnaE(C)-mScarlet(insCys;76-232)-26-103-62]-IRES-GFP-26-107-36
41	pLX-mScarlet(1-122)-NpuDnaE(N)_LZA-IRES-TagBFP2	mScarlet(1-122)-NpuDnaE(N)_LZA	pLX-[mScarlet(1-122)-NpuDnaE(N)_LZA-26-103-44]-IRES-TagBFP2-26-107-22
42	pLX-LZB_NpuDnaE(C)-mScarlet(insCys;123-232)-IRES-GFP	LZB_NpuDnaE(C)-mScarlet(insCys;123-232)	pLX-[LZB_NpuDnaE(C)-mScarlet(insCys;123-232)-26-103-65]-IRES-GFP-26-107-39
43	pLX-mScarlet(1-140)-NpuDnaE(N)_LZA-IRES-TagBFP2	mScarlet(1-140)-NpuDnaE(N)_LZA	pLX-[24-pCR8-mScarlet(1-140)-NpuDnaE(N)_LZA]-IRES-TagBFP2-26-107-24
44	pLX-LZB_NpuDnaE(C)-mScarlet(insCys;141-232)-IRES-GFP	LZB_NpuDnaE(C)-mScarlet(insCys;141-232)	pLX-[LZB_NpuDnaE(C)-mScarlet(insCys;141-232)-26-103-66]-IRES-GFP-26-107-40
45	pLX-mScarlet(1-163)-NpuDnaE(N)_LZA-IRES-TagBFP2	mScarlet(1-163)-NpuDnaE(N)_LZA	pLX-[48-pCR8-mScarlet(1-163)-NpuDnaE(N)_LZA]-IRES-TagBFP2-26-107-25
46	pLX-LZB_NpuDnaE(C)-mScarlet(insCys;164-232)-IRES-GFP	LZB_NpuDnaE(C)-mScarlet(insCys;164-232)	pLX-[LZB_NpuDnaE(C)-mScarlet(insCys;164-232)-26-103-68]-IRES-GFP-26-107-41
47	pCR8-TagBFP2	TagBFP2	pCR8-TagBFP2-25-119-29
48	pCR8-mCherry	mCherry	pCR8-mCherry-25-121-14
49	pLX-DEST-IRES-Hygro(1-89)-NpuDnaE(N)	Hygro(1-89)-NpuDnaE(N)	pLX-DEST-IRES-HYGro-NpuDnaE(N)-25-105-7

50	pLX-DEST-IRES-NpuDnaE(C)-Hygro(90-341)	NpuDnaE(C)-Hygro(90-341)	pLX-DEST-IRES-NpuDnaE(C)_hygRO-25-105-9
51	pLX-[TagBFP2]-IRES-Hygro(1-89)-NpuDnaE(N)	Hygro(1-89)-NpuDnaE(N)	pLX-[TagBFP2-25-119-29]-IRES-HYGro-NpuDnaE(N)-MD1-68-1
52	pLX-[mCherry]-IRES-NpuDnaE(C)-Hygro(90-341)	NpuDnaE(C)-Hygro(90-341)	pLX-[mCherry-25-121-14]-IRES-NpuDnaE(C)_hygRO-MD1-68-3
53	pLX-DEST-IRES-Puro(1-119)-NpuDnaE(N)	Puro(1-119)-NpuDnaE(N)	pLX-DEST-IRES-PuroKC2(N)-NpuDnaE(N)-26-113-44
54	pLX-DEST-IRES-NpuDnaE(C)-Puro(120-199)	NpuDnaE(C)-Puro(insCys;120-199)	pLX-DEST-IRES-NpuDnaE(C)_PuroKC2(C)-26-23-75
55	pLX-[TagBFP2]-IRES-Puro(1-119)-NpuDnaE(N)	Puro(1-119)-NpuDnaE(N)	pLX-[TagBFP2-25-119-29]-IRES-PuroKC2(N)-NpuDnaE(N)-26-121-18
56	pLX-[mCherry]-IRES-NpuDnaE(C)-Puro(120-199)	NpuDnaE(C)-Puro(insCys;120-199)	pLX-[mCherry-25-121-14]-IRES-NpuDnaE(C)_PuroKC2(C)-26-107-60
57	pLX-DEST-IRES-Neo(1-194)-NpuDnaE(N)	Neo(1-194)-NpuDnaE(N)	pLX-DEST-IRES-Neo(B)-NpuDnaE(N)-26-103-6
58	pLX-DEST-IRES-NpuDnaE(C)-Neo(195-267)	NpuDnaE(C)-Neo(195-267)	pLX-DEST-IRES-NpuDnaE(C)_Neo(B)-26-45-8
59	pLX-[TagBFP2]-IRES-Neo(1-194)-NpuDnaE(N)	Neo(1-194)-NpuDnaE(N)	pLX-[TagBFP2-25-119-29]-IRES-Neo(B)-NpuDnaE(N)-26-107-55
60	pLX-[mCherry]-IRES-NpuDnaE(C)-Neo(195-267)	NpuDnaE(C)-Neo(195-267)	pLX-[mCherry-25-121-14]-IRES-NpuDnaE(C)_Neo(B)-26-107-56
64	pLX-Hygro(1-69)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-69)-NpuDnaE(N)	pLX-[Hygro69-NpuDnaE(N)-26-61-17]-IRES-TagBFP2-26-69-5
65	pLX-NpuDnaE(C)-Hygro(^C;70-341)-IRES-mCherry	NpuDnaE(C)-Hygro(^C;70-341)	pLX-[NpuDnaE(C)_HygroSC69-26-61-30]-IRES-mCherry-26-69-21
66	pLX-Hygro(1-131)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-131)-NpuDnaE(N)	pLX-[Hygro131-NpuDnaE(N)-26-61-18]-IRES-TagBFP2-26-69-6
67	pLX-NpuDnaE(C)-Hygro(^C;132-341)-IRES-mCherry	NpuDnaE(C)-Hygro(^C;132-341)	pLX-[NpuDnaE(C)_HygroSC131-26-61-31]-IRES-mCherry-26-69-22
68	pLX-Hygro(1-171)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-171)-NpuDnaE(N)	pLX-[Hygro171-NpuDnaE(N)-26-61-19]-IRES-TagBFP2-26-69-7
69	pLX-NpuDnaE(C)-Hygro(^C;172-341)-IRES-mCherry	NpuDnaE(C)-Hygro(^C;172-341)	pLX-[NpuDnaE(C)_HygroSC171-26-61-32]-IRES-mCherry-26-69-23
70	pLX-Hygro(1-218)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-218)-NpuDnaE(N)	pLX-[Hygro218-NpuDnaE(N)-26-61-20]-IRES-TagBFP2-26-69-8
71	pLX-NpuDnaE(C)-Hygro(^C;219-341)-IRES-mCherry	NpuDnaE(C)-Hygro(^C;219-341)	pLX-[NpuDnaE(C)_HygroSC218-26-61-33]-IRES-mCherry-26-69-24
72	pLX-Hygro(1-259)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-259)-NpuDnaE(N)	pLX-[Hygro259-NpuDnaE(N)-26-65-45]-IRES-TagBFP2-26-69-9
73	pLX-NpuDnaE(C)-Hygro(^C;260-341)-IRES-mCherry	NpuDnaE(C)-Hygro(^C;260-341)	pLX-[NpuDnaE(C)_HygroSC259-26-61-34]-IRES-mCherry-26-69-25
74	pLX-Hygro(1-277)-NpuDnaE(N)-IRES-TagBFP2	Hygro(1-277)-NpuDnaE(N)	pLX-[Hygro277-NpuDnaE(N)-26-65-46]-IRES-TagBFP2-26-69-10
75	pLX-NpuDnaE(C)-Hygro(^C; 278-341)-IRES-mCherry	NpuDnaE(C)-Hygro(^C;278-341)	pLX-[NpuDnaE(C)_HygroSC277-26-61-35]-IRES-mCherry-26-69-26
76	pLX-Puro(1-32)-NpuDnaE(N)-IRES-TagBFP2	Puro(1-32)-NpuDnaE(N)	pLX-[PuroAC32-NpuDnaE(N)-26-61-24]-IRES-TagBFP2-26-69-12

77	pLX-NpuDnaE(C)-Puro(^C;33-199)-IRES-mCherry	NpuDnaE(C)-Puro(^C;33-199)	pLX-[NpuDnaE(C)_PuroAC32-26-61-37]-IRES-mCherry-26-69-28
78	pLX-Puro(1-84)-NpuDnaE(N)-IRES-TagBFP2	Puro(1-84)-NpuDnaE(N)	pLX-[PuroAC84-NpuDnaE(N)-26-61-25]-IRES-TagBFP2-26-69-13
79	pLX-NpuDnaE(C)-Puro(^C;85-199)-IRES-mCherry	NpuDnaE(C)-Puro(^C;85-199)	pLX-[NpuDnaE(C)_PuroAC84-26-61-38]-IRES-mCherry-26-69-29
80	pLX-Puro(1-137)-NpuDnaE(N)-IRES-TagBFP2	Puro(1-137)-NpuDnaE(N)	pLX-[PuroKC3(N)-NpuDnaE(N)-25-131-29"]-IRES-TagBFP2-25-133-6
81	pLX-NpuDnaE(C)-Puro(^C;138-199)-IRES-mCherry	NpuDnaE(C)-Puro(^C;138-199)	pLX-[NpuDnaE(C)_PuroKC3(C)-25-131-36"]-IRES-mCherry-25-133-14
82	pLX-Puro(1-158)-NpuDnaE(N)-IRES-TagBFP2	Puro(1-158)-NpuDnaE(N)	pLX-[PuroAC158-NpuDnaE(N)-26-61-26]-IRES-TagBFP2-26-69-14
83	pLX-NpuDnaE(C)-Puro(^C;159-199)-IRES-mCherry	NpuDnaE(C)-Puro(^C;159-199)	pLX-[NpuDnaE(C)_PuroAC158-26-61-39]-IRES-mCherry-26-69-30
84	pLX-Puro(1-180)-NpuDnaE(N)-IRES-TagBFP2	Puro(1-180)-NpuDnaE(N)	pLX-[PuroAC180-NpuDnaE(N)-26-61-27]-IRES-TagBFP2-26-69-15
85	pLX-NpuDnaE(C)-Puro(^C;181-199)-IRES-mCherry	NpuDnaE(C)-Puro(^C;181-199)	pLX-[NpuDnaE(C)_PuroAC180-26-61-40]-IRES-mCherry-26-69-31
86	pLX-Blast(1-58)-NpuDnaE(N)-IRES-TagBFP2	Blast(1-58)-NpuDnaE(N)	pLX-[Blast(A)-NpuDnaE(N)-25-119-5]-IRES-TagBFP2-MD1-63-5
87	pLX-NpuDnaE(C)-Blast(59-140)-IRES-mCherry	NpuDnaE(C)-Blast(59-140)	pLX-[NpuDnaE(C)_Blast(A)-25-119-6]-IRES-mCherry-MD1-63-8
88	pLX-NpuDnaE(C)-HygroBA-SspDnaB(N-S0)-IRES-EGFP	NpuDnaE(C)-Hygro(53-200)-SspDnaB(N-S0)	pLX-[NpuDnaE(C)_HygroBA_SspDnaB(N-S0)-26-37-59]-IRES-GFP-26-43-59
89	pLX-SspDnaB(C-S0)-Hygro(201-341)-IRES-mCherry	SspDnaB(C-S0)-Hygro(201-341)	pLX-[SspDnaB(C-S0)_Hygro(A)-26-1-17]-IRES-mCherry-26-7-24
90	pLX-NpuDnaE(C)-Hygro(90-200)-SspDnaB(N-S0)-IRES-EGFP	NpuDnaE(C)-Hygro(90-200)-SspDnaB(N-S0)	pLX-[NpuDnaE(C)_HygroAA_SspDnaB(N-S0)-26-37-60]-IRES-GFP-26-43-60
91	pLX-Hygro(1-200)-SspDnaB(N-S0)-IRES-TagBFP2	Hygro(1-200)-SspDnaB(N-S0)	pLX-[Hygro(A)_SspDnaB(N-S0)-26-1-10]-IRES-TagBFP2-26-7-15
92	pLX-SspDnaB(C-S0)-Hygro(201-240)-NpuDnaE(N)-IRES-EGFP	SspDnaB(C-S0)-Hygro(201-240)-NpuDnaE(N)	pLX-[SspDnaB(C-S0)_HygroAC_NpuDnaE(N)-26-37-61]-IRES-GFP-26-43-61
93	pLX-SspDnaB(C-S0)-Hygro(201-292)-NpuDnaE(N)-IRES-EGFP	SspDnaB(C-S0)-Hygro(201-292)-NpuDnaE(N)	pLX-[SspDnaB(C-S0)_HygroAD_NpuDnaE(N)-26-37-62]-IRES-GFP-26-43-62
94	pLX-DEST-IRES-TagBFP2		pLX-DEST-IRES-TagBFP2-25-65-63,25-131-25
95	pLX-DEST-IRES-EGFP		pLX-DEST-IRES-GFP-25-81-21
96	pLX-DEST-IRES-mCherry		pLX-DEST-IRES-mCherry-25-65-64,25-131-26
97	pLX-Hygro-IRES-TagBFP2	Non-split Hygro	pLX-[Hygro-24-127-20]-IRES-TagBFP2-25-91-23
98	pLX-Hygro-IRES-mCherry	Non-split Hygro	pLX-[Hygro-24-127-20]-IRES-mCherry-25-117-21
99	pLX-Puro-IRES-TagBFP2	Non-split Puro	pLX-[Puro-24-127-21]-IRES-TagBFP2-25-117-13
100	pLX-Puro-IRES-mCherry	Non-split Puro	pLX-[Puro-24-127-21]-IRES-mCherry-25-91-24
101	pLX-Hygro-IRES-EGFP	Non-split Hygro	pLX-[Hygro-24-127-20]-IRES-GFP-27-61-1
102	pLX-NLS_GFP-IRES-Hygro	Non-split Hygro	pLX-[NLSGFP-6-29-B1]-IRES-Hygro-28-73-13
103	pLX-LifeAct_mCherry-IRES-Hygro	Non-split Hygro	pLX-[LifeAct_mScarlet_N1-26-69-64]-IRES-Hygro-27-131-30

104	pLX-NLS_GFP-IRES-Hygro(1-89)-NpuDnaE(N)	Hygro(1-89)-NpuDnaE(N)	pLX-[NLSGFP-6-29-B1]-IRES-HYGro-NpuDnaE(N)-28-73-11
105	pLX-LifeAct_mScarlet-IRES- NpuDnaE(C)-Hygro(90-341)	NpuDnaE(C)-Hygro(90-341)	pLX-[LifeAct_mScarlet_N1-26-69-64]-IRES-NpuDnaE(C)_hygRO-28-73-12