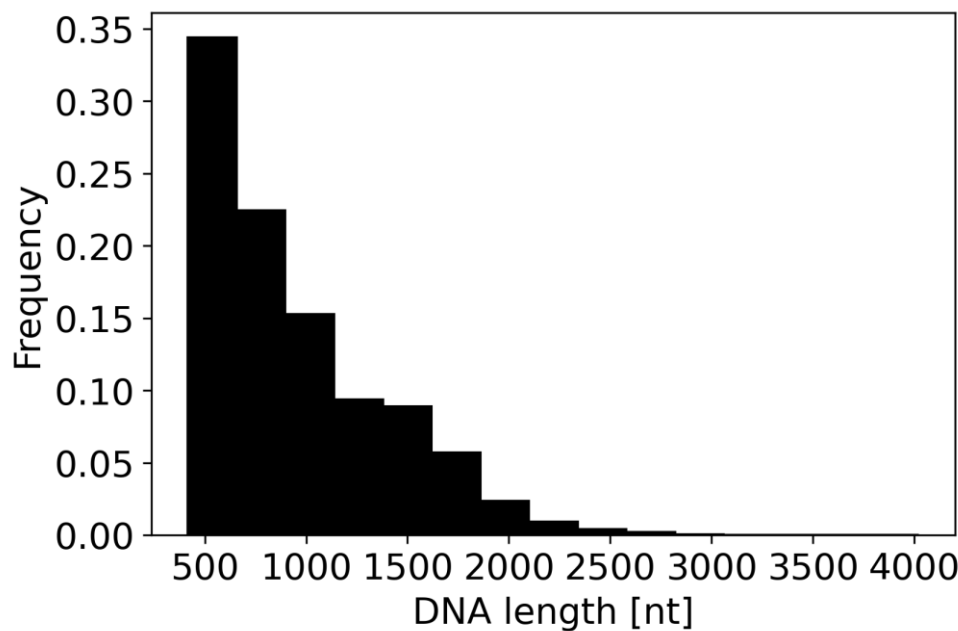
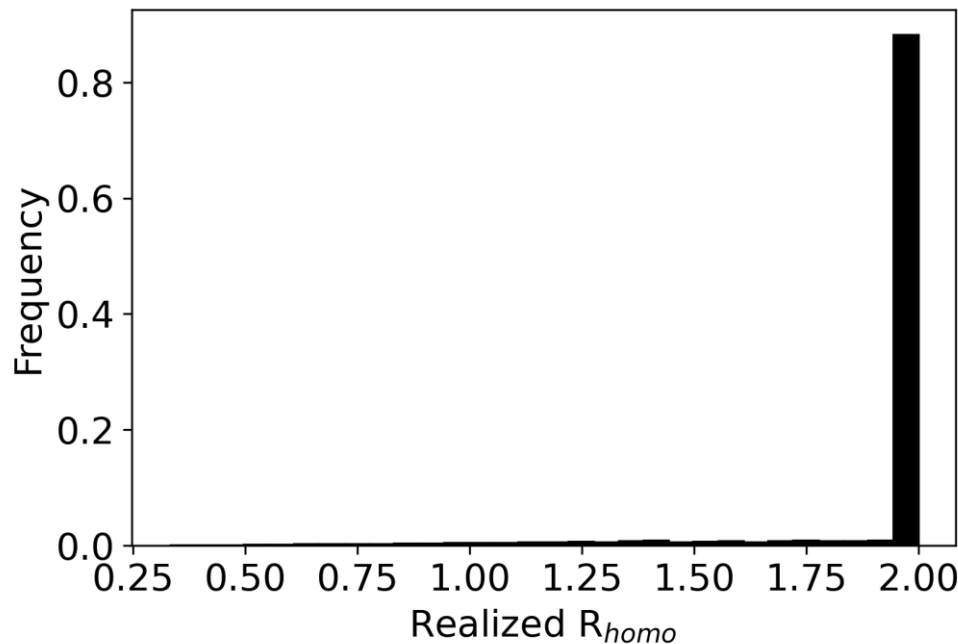


# Supplementary information

## Supplementary Figures



Supplementary Figure 1. Distribution of lengths of DNA inserts for tagging or deleting S288C genes designed by PIPOLine with default parameters. For tagging, the length of the insert does not include the tag nor the linker, which the user needs to choose (and ensure compatibility with restriction sites in the plasmid).



Supplementary Figure 2. Distribution of realized  $R_{homo}$  parameters for tagging or deleting all S288C genes when using the default input value of 2 for  $R_{homo}$ . The realized  $R_{homo}$  can be smaller than the desired  $R_{homo}$  if the rules for PIPOLine cannot be satisfied otherwise. The average  $R_{homo}$  is  $(1.93 \pm 0.22 \text{ STD})$ .

## Supplementary Note 1: List of PIPOLine input parameters

Argument	Description	Default Value
--backbone_path	Plasmid backbone used for PIPO	'./Test_examples/pETUL_backbone.fsa'
--MCS_start_ind	Starting nucleotide of the multiple cloning site sequence (1-based counting)	1
--MCS_end_ind	Ending nucleotide of the multiple cloning site sequence (1-based counting)	108
--min_homology	Minimal length of DNA sequence used for homologous recombination during pop-in	70
--R	Ratio between DNA sequence length used for pop-out vs. pop-in	2
--Gene_path	ORF of the gene with 1000 bp upstream and downstream (in FASTA format)	'./Test_examples/CLB2_3p_labeling/CLB2_pm_1000.fsa'
--linker_path	Linker	'./Test_examples/long_linker.fsa'
--modality	Modality of the program that can assume one of the following values: <ul style="list-style-type: none"> <li>– 0 for gene deletion</li> <li>– 5 for gene tagging at the 5' terminus</li> <li>– 3 for gene tagging at the 3' terminus</li> </ul>	0
--enzyme_path	List of enzymes that can be used for cloning and plasmid linearization prior to transformation	'./Test_examples/raw_enzyme_list.txt'
--FPG_paths	List of fluorescent protein coding genes that should be considered during plasmid design	['./Test_examples/CLB2_3p_labeling/mCherry_FPG.fsa', './Test_examples/CLB2_3

		p_labeling/ymNeonGreen_FPG.fsa']
--popular_enzyme_path	List of enzymes to be considered for adding around linker and FPG	-
--assembled_plasmid_name	Name of the output FASTA file	-

## Supplementary Note 2: Example of PIPOLine calls for gene deletion and gene tagging

Call used for gene deletion:

```
python.exe ./main.py
--backbone_path './Test_examples/pETUL_backbone.fsa'
--MCS_start_ind 1
--MCS_end_ind 108
--min_homology 70
--R 2
--Gene_path './Test_examples/GAL4_deletion/GAL4_pm_1000_gene.fsa'
--modality 0 --enzyme_path './Test_examples/raw_enzyme_list.txt'
```

Call used for gene tagging:

```
python.exe ./main.py
--backbone_path ./Test_examples/pETUL_backbone.fsa
--MCS_start_ind 1
--MCS_end_ind 108
--min_homology 70
--R 2
--Gene_path ./Test_examples/CLB2_3p_labeling/CLB2_pm_1000.fsa
--linker_path ./Test_examples/long_linker.fsa
--modality 5 --enzyme_path ./Test_examples/raw_enzyme_list.txt
--popular_enzyme_path ./Test_examples/Popular_enzymes.txt
--FPG_paths ./Test_examples/CLB2_3p_labeling/mCherry_FPG.fsa
./Test_examples/CLB2_3p_labeling/ymNeonGreen_FPG.fsa
./Test_examples/CLB2_3p_labeling/ymTq2_FPG.fsa
```

### Supplementary Note 3: Sequence of pETURALEU backbone

The following sequence is also supplied with annotations in gbk format in the **Supplementary Files**:

```
GGTACCGGGCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGATCCACTAGT
TCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTCCCTTTAGTGAGGGTTAATTGCGCGCTTGCGGTAATC
ATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATAcGAGCCGGAAGCATAAAGTGT
AAAGCCTGGGGTGCCTAATGAGTGAGcTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCAGTCGGGAAAC
CTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTTCGTATTGGGCGCTCTCCGCTTCC
TCGCTCACTGACTCGCTGCGCTCGGTCGTTTCGGCTGCGGCGAGCGGTATCAGCTCAAAAGGCGGTAATACGGTTA
TCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAG
GCCGCGTTGCTGGCGTTTTTTCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGG
CGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGC
CGTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAG
TTCGGTGTAGGTCGTTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTTCAGCCCGACCGCTGCGCCTTATCCGG
TAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAG
AGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTG
GTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAAACAAACCACCGCTG
GTAGCGGTGGTTTTTTTGTTCGCAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTT
CTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCA
CCTAGATCCTTTTAAATTAATAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCA
ATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGA
TAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAG
ATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCTGCAACTTTATCCGCTCCATCCAGT
CTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTTCGCCAGTTAATAGTTTTCGCAACGTTGTTGCCATTGCTACAG
GCATCGTGGTGTACGCTCGTCGTTTGGTATGGCTTCATTTCAGCTCCGTTCCCAACGATCAAGGCGAGTTACATGATC
CCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCACTGTTATC
ACTCATGGTTATGGCAGCACTGCATAATTCTTACTGTATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACT
CAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGC
CACATAGCAGAACTTTAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGT
TGAGATCCAGTTCGATGTAACCCACTCGTGACCCAACTGATCTTCAGCATCTTTTACTTTACCAGCGTTTCTGGGTGA
GCAAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCT
TTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAAC
AAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACTTA
TAAAAATAGGCGTATCACGAGGCCCTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTTGACACATGCAGCT
CCcGGACTCTAATTTGTGAGTTTAGTATACATGCATTTACTTATAATACAGTTTTTTAGTTTTGCTGGCCGCATCTTCTCA
AATATGCTTCCAGCCTGCTTTTCTGTAACGTTACCCCTCTACCTTAGCATCCCTTCCCTTTGCAATAGTCCTCTTCCAA
CAATAATAATGTCAGATCCTGTAGAGACCACATCATCCACGGTTCTATACTGTTGACCCAATGCGTCTCCCTTGTCATCT
AAACCCACACCGGGTGTGATAATCAACCAATCGTAACCTTCATCTCTTCCACCCATGTCTCTTTGAGCAATAAAGCCGAT
AACAAAATCTTTGTGCTCTTCGCAATGTCAACAGTACCCTTAGTATATTCTCCAGTAGATAGGGAGCCCTTGATGAC
AATTCTGCTAACATCAAAAGGCCTCTAGGTTCTTTGTTACTTCTTCTGCCGCTGCTTCAAAACGCTAACAACTCTGG
GCCACCACACCGTGTGCATTGTAATGTCTGCCATTCTGCTATTCTGTATACACCCGAGAGTACTGCAATTTGACT
GTATTACCAATGTCAGCAAATTTTCTGTCTTCGAAGAGTAAAAAATTGTACTTGCGGATAATGCCTTAGCGGCTTAA
CTGTGCCCTCCATGGAAAAATCAGTCAAGATATCCACATGTGTTTTAGTAAACAAATTTTGGGACCTAATGCTTCAAC
TAACTCCAGTAATTCCTTGGTGGTACGAACATCCAATGAAGCACACAAGTTTGTTCGTTTTCGTGCATGATATTAAT
AGCTTGGCAGCAACAGGACTAGGATGAGTAGCAGCACGTTCTTATATGTAGCTTTCGACATGATTTATCTTCGTTTCC
TGCAGGTTTTTGTCTGTGCAAGTTGGGTTAAGAATACTGGGCAATTCATGTTTCTTCAACACTACATATGCGTATATAT
ACCAATCTAAGTCTGTGCTCCTTCTTCGTTCTTCTTCTGTTTCGGAGATTACCGAATCAAAAAAATTTCAAGGAAACCG
AAATCAAAAAAAGAATAAAAAAATGATGAATTGAAAAGTGGTATGGTGAAGTCTCAGTACTCCgGGAGACGG
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GGGCTGGCTTAATATGCGGCATCAGAGCAGATTGTAAGTGTGAGAGTGCACCATATCGACTACGTCGTTAAGGCCGTTTC
TGACAGAGTAAAATTCTTGAGGGAACTTTCACCATATGGGAAATGGTTCAAGAAGGTATTGACTTAACTCCATCAA
ATGGTCAGGTCATTGAGTGTTTTTATTTGTTGATTTTTTTTTTTTAGAGAAAATCCTCCAATATATAAATTAGGAATC
ATAGTTTCATGATTTTCTGTTACACCTAACTTTTTGTGTGGTGCCCTCCTCCTGTCAATATTAATGTTAAAGTGAATTC
```

TTTTTCCTTATCACGTTGAGCCATTAGTATCAATTTGCTTACCTGTATTCTTTACATCCTCCTTTTTCTCCTTCTTGATAA  
 ATGTATGTAGATTGCGTATATAGTTTTCGTCTACCCTATGAACATATTCCATTTTGTAAATTCGTGTCGTTTCTATTATGAA  
 TTTCATTTATAAAGTTTATGTACAAATATCATAAAAAAGAGAATCTTTTTAAGCAAGGATTTTCTTAACCTCTTCGGCG  
 ACAGCATCACCGACTTCGGTGGTACTGTTGGAACCACCTAAATCACCAGTTCTGATACCTGCATCCAAAACCTTTTTAA  
 CTGCATCTTCAATGGCCTTACCTTCTTCAGGCAAGTTCAATGACAATTTCAACATCATTGCAGCAGACAAGATAGTGGC  
 GATAGGGTCAACCTTATTCTTTGGCAAATCTGGAGCAGAACCGTGGCATGGTTCGTACAAACCAAATGCGGTGTTCTT  
 GTCTGGCAAAGAGGCCAAGGACGCAGATGGCAACAAACCAAGGAACCTGGGATAACGGAGGCTTCATCGGAGATG  
 ATATCACCAAACATGTTGCTGGTGATTATAATACCATTTAGGTGGGTTGGGTTCTTAAGTAGGATCATGGCGGCAGAA  
 TCAATCAATTGATGTTGAACCTTCAATGTAGGGAATTCGTTCTTGATGGTTTTCTCCACAGTTTTTCTCCATAATCTTGA  
 AGAGGCCAAAAGATTAGCTTTATCCAAGGACCAAATAGGCAATGGTGGCTCATGTTGTAGGGCCATGAAAGCGGCCA  
 TTCTTGATGATTCTTGCATTCTGGAACGGTGTATTGTTCACTATCCCAAGCGACACCATCACCATCGTCTTCTTTCTCT  
 TACCAAAGTAAATACCTCCACTAATTCTCTGACAACAACGAAGTCAGTACCTTTAGCAAATTGTGGCTTGATTGGAGA  
 TAAGTCTAAAAGAGAGTCGGATGCAAAGTTACATGGTCTTAAGTTGGCGTACAATTGAAGTTCTTTACGGATTTTTAG  
 TAAACCTTGTTCAAGTCTAACACTACCGGTACCCATTTAGGACCACCCACAGCACCTAACAAAACGGCATCAGCCTTC  
 TTGGAGGCTTCCAGCGCTCATCTGGAAGTGGAACACCTGTAGCATCGATAGCAGCACCACCAATTAATGATTTTCG  
 AAATCGAATTGACATTGGAACGAACATCAGAAATAGCTTTAAGAACCTTAATGGCTTCGGCTGTGATTTCTTGACCAA  
 CGTGGTCACCTGGCAAAACGACGATCTTCTTAGGGGCGAGACATTAGAATGGTATATCCTTGAAATATATATATATAT  
 TGCTGAAATGTAAAAGGTAAGAAAAGTTAGAAAAGTAAGACGATTGCTAACCACCTATTGGAACCAATAGGTCCT  
 TAAATAATATTGTCAACTTCAAGTATTGTGATGCAAGCATTTAGTCATGAACGCTTCTCTATTCTATATGAAAAGCCGG  
 TTCCGGCGCTCTCACCTTCTTTTTCTCCCAATTTTTAGTTGAAAAAGGTATATGCGTCAGGCGACCTCTGAAATTAA  
 CAAAAAATTTCCAGTCATCGAATTTGATTCTGTGCGATAGCGCCCTGTGTGTTCTCGTTATGTTGAGGAAAAAATAA  
 TGTTGCTAAGAGATTGCAACTCTTGATCTTACGATACCTGAGTATCCACAGTTAACTGCGGTCAAGATATTTCTT  
 GAATCAGGCGCCTTAGACCGCTCGGCCAAACAACCAATTACTTGTGAGAAATAGAGTATAATTATCCTATAAATATA  
 ACGTTTTTGAACACACATGAACAAGGAAGTACAGGACAATTGATTTTGAAGAGAATGTGGATTTTGATGTAATTGTTG  
 GGATTCCATTTTAAATAAGGCAATAATATTAGGTATGTAGATATACTAGAAGTTCTCCTCGACCGGTCGATATGCGGTG  
 TGAAATACCGCACAGATGCGTAAGGAGAAAAATACCGCATCAGGAAATTGTAAgCGTTAATATTTTGTAAAAATTCGCG  
 TTAATTTTTTGTAAATCAGCTCATTTTTTAAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAGAATAGA  
 CCGAGATAGGGTTGAGTGTTGTTCCAGTTTGAACAAGAGTCCACTATTAAGAACGTGGACTCCAACGTCAAAGGG  
 CGAAAAACCGTCTATCAGGGCGATGGCCACTACGTGAACCATCACCTAATCAAGTTTTTTGGGGTCGAGGTGCCGT  
 AAAGCACTAAATCGGAACCTTAAAGGGAGCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAA  
 AGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCA  
 CACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGAT  
 CGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAAGTTGGGTAACGCCA  
 GGGTTTTCCAGTCACGACGTTGTAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTG

## Supplementary Note 4: Restriction enzymes and their cut sites

The following default list of restriction enzymes and cut sites is included in the PIPOLine package in file raw\_enzyme\_list.txt:

AatII	Bme1580I	BstEII-HF®	HindIII	NlaIII	SbfI
GACGTC	GKGCMC	GGTNACC	AAGCTT	CATG	CCTGCAGG
AccI	BmgBI	BstNI	HindIII-HF®	NlaIV	SbfI-HF®
GTMKAC	CACGTC	CCWGG	AAGCTT	GGNNCC	CCTGCAGG
Acc65I	Bmrl	BstUI	Hinfl	NmeAIII	Scal-HF®
GGTACC	ACTGGG	CGCG	GANTC	GCCGAG	AGTACT
Acil	BmtI	BstXI	HinP1I	NotI	ScrFI
CCGC	GCTAGC	CCANNNNN	GCGC	GCGGCCGC	CCNGG
AcII	BmtI-HF®	BstYI	HpaI	NotI-HF®	SexAI
AACGTT	GCTAGC	RGATCY	GTTAAC	GCGGCCGC	ACCWGGT
AcuI	BpmI	BstZ17I	HpaII	NruI	SfaNI
CTGAAG	CTGGAG	GTATAC	CCGG	TCGCGA	GCATC

AfeI	Bpu10I	BstZ17I-HF®	HphI	NruI-HF®	SfiI
AGCGCT	CCTNAGC	GTATAC	GGTGA	TCGCGA	CTRYAG
AflII	BpuEI	Bsu36I	Hpy99I	NsiI	SfiI
					GGCCNNNNNG
CTTAAG	CTTGAG	CCTNAGG	CGWCG	ATGCAT	GCC
AflIII	BsaI	BtgI	Hpy166II	NsiI-HF®	SfoI
ACRYGT	GGTCTC	CCRYGG	GTNNAC	ATGCAT	GGCGCC
AgeI	BsaI-HF®	BtgZI	Hpy188I	NspI	SgrAI
ACCGGT	GGTCTC	GCGATG	TCNGA	RCATGY	CRCCGGYG
AgeI-HF®	BsaI-HF®v2	BtsCI	Hpy188III	Nt.AlwI	SmaI
ACCGGT	GGTCTC	GGATG	TCNNGA	GGATC	CCCGGG
AhdI	BsaAI	BtsIMutI	HpyAV	Nt.BbvCI	SmlI
GACNNNNN					
GTC	YACGTR	CAGTG	CCTTC	CCTCAGC	CTYRAG
AleI	BsaBI	BtsaI	HpyCH4III	Nt.BsmAI	SnaBI
CACNNNNG	GATNNNN				
TG	ATC	GCA GTG	ACNGT	GTCTC	TACGTA
AluI	BsaHI	Cac8I	HpyCH4IV	Nt.BspQI	SpeI
AGCT	GRCGYC	GCNNGC	ACGT	GCTCTTC	ACTAGT
AlwI	BsaJI	Clal	HpyCH4V	Nt.BstNBI	SpeI-HF®
GGATC	CCNNGG	ATCGAT	TGCA	GAGTC	ACTAGT
AlwNI	BsaWI	CspCI	I-CeuI	PacI	SphI
CAGNNNCT		CAANNNNN	TA ACTATAACGGTCCTAA		
G	WCCGGW	GTGG	GGTAGCGA	TTAATTAA	GCATGC
Apal	BsaXI	CviAII	I-SceI	PaeR7I	SphI-HF®
	ACNNNNN		TAGGGATAACAGGGTAA		
GGGCCC	CTCC	CATG	T	CTCGAG	GCATGC
ApaLI	BseRI	CviKI-1	KasI	PaqCI	SrfI
GTGCAC	GAGGAG	RGCY	GGCGCC	CACCTGC	GGCCGGGC
ApeKI	BseYI	CviQI	KpnI	PciI	SspI
GCWGC	CCCAGC	GTAC	GGTACC	ACATGT	AATATT
ApoI	BsgI	DdeI	KpnI-HF®	PfiFI	SspI-HF®
RAATTY	GTGCAG	CTNAG	GGTACC	GACNNNGTC	AATATT
ApoI-HF®	BsiEI	DpnI	MboI	PfiMI	StuI
RAATTY	CGRYCG	GATC	GATC	CCANNNNNTGG	AGGCCT
AscI	BsiHKAI	DpnII	MboII	PhoI	StyI
GGCGCGCC	GWGCWC	GATC	GAAGA	GGCC	CCWWGG
Asel	BsiWI	DraI	MfeI	PI-PspI	StyI-HF®
				TGGCAAACAGCTATTATGGGTATTAT	
ATTAAT	CGTACG	TTTAAA	CAATTG	GGGT	CCWWGG
AsiSI	BsiWI-HF®	DraIII-HF®	MfeI-HF®	PI-SceI	StyD4I
		CACNNNGT		ATCTATGTCGGGTGCGGAGAAAGAG	
GCGATCGC	CGTACG	G	CAATTG	GTAATGAAATGG	CCNGG
AvaI	BsII	DrdI	MluI	PleI	Swal
	CCNNNNN	GACNNNNN			
CYCGRG	NNGG	NGTC	ACGCGT	GAGTC	ATTTAAAT
AvaII	BsmI	EaeI	MluI-HF®	PluTI	TaqαI
GGWCC	GAATGC	YGGCCR	ACGCGT	GGCGCC	TCGA
AvrII	BsmAI	EagI	MluCI	PmeI	TfiI
CCTAGG	GTCTC	CGGCCG	AATT	GTTTAAAC	GAWTC
BaeI	BsmBI	EagI-HF®	MlyI	PmlI	TseI
ACNNNNGT					
AYC	CGTCTC	CGGCCG	GAGTC	CACGTG	GCWGC

BaeGI	BsmBI-v2	EarI	MmeI	PpuMI	Tsp45I
GKGC MC	CGTCTC	CTCTTC	TCCRAC	RGGWCCY	GTSAC
BamHI	BsmFI	EciI	MnII	PshAI	Tsp509I
GGATCC	GGGAC	GGCGGA	CCTC	GACNNNGTC	AATT
BamHI-HF®	BsoBI	Eco53kI	MscI	PsiI	TspMI
GGATCC	CYCGRG	GAGCTC	TGGCCA	TTATAA	CCCGGG
BanI	Bsp1286I	EcoNI CCTNNNNN	MseI	PspGI	TspRI
GGYRCC	GDGCHC	AGG	TTAA	CCWGG	CASTG
BanII	BspCNI	EcoO109I	MslI	PspOMI	Tth111I
GRG CYC	CTCAG	RGGNCCY	CAYNNNNRTG	GGGCCC	GACNNNGTC
BbsI	BspDI	EcoP15I	MspI	PspXI	XbaI
GAAGAC	ATCGAT	CAGCAG	CCGG	VCTCGAGB	TCTAGA
BbsI-HF®	BspEI	EcoRI	MspA1I	PstI	XcmI CCANNNNNNN NNTGG
GAAGAC	TCCGGA	GAATTC	CMGCKG	CTGCAG	XhoI
BbvI	BspHI	EcoRI-HF®	MwoI	PstI-HF®	CTCGAG
GCAGC	TCATGA	GAATTC	GCNNNNNNNGC	CTGCAG	XmaI
BbvCI	BspMI	EcoRV	NaeI	PvuI	CCCGGG
CCTCAGC	ACCTGC	GATATC	GCCGGC	CGATCG	XmnI
BccI	BspQI	EcoRV-HF®	NarI	PvuI-HF®	GAANNNTTC
CCATC	GCTCTTC	GATATC	GGCGCC	CGATCG	ZraI
BceAI	BsrI	Esp3I	Nb.BbvCI	PvuII	GACGTC
ACGGC	ACTGG	CGTCTC	CCTCAGC	CAGCTG	
BcgI CGANNNNN NTGC	BsrBI	FatI	Nb.BsmI	PvuII-HF®	
	CCGCTC	CATG	GAATGC	CAGCTG	
BciVI	BsrDI	FauI	Nb.BsrDI	RsaI	
GTATCC	GCAATG	CCCGC	GCAATG	GTAC	
BclI	BsrFI	Fnu4HI	Nb.BssSI	RsrII	
TGATCA	RCCGGY	GCNGC	CACGAG	CGGWCCG	
BclI-HF®	BsrGI	FokI	Nb.BtsI	SacI	
TGATCA	TGTACA	GGATG	GCAGTG	GAGCTC	
BcoDI	BsrGI-HF®	FseI	NciI	SacI-HF®	
GTCTC	TGTACA	GGCCGGCC	CCSGG	GAGCTC	
BfaI	BssHII	FspI	NcoI	SacII	
CTAG	GCGCGC	TGCGCA	CCATGG	CCGCGG	
BfuAI	BssKI	HaeII	NcoI-HF®	SalI	
ACCTGC	CCNGG	RGCGCY	CCATGG	GTCGAC	
BfuCI	BssSαI	HaeIII	NdeI	SalI-HF®	
GATC	CACGAG	GGCC	CATATG	GTCGAC	
BglI GCCNNNNN GGC	BstAPI GCANNNN NTGC	HgaI	NgoMIV	SapI	
		GACGC	GCCGGC	GCTCTTC	
BglII	BstBI	HhaI	NheI	Sau3AI	
AGATCT	TTCGAA	GCGC	GCTAGC	GATC	
BlpI	BstEII	HincII	NheI-HF®	Sau96I	
GCTNAGC	GGTNACC	GTyrAC	GCTAGC	GGNCC	

### Supplementary Note 5: Default linker used for tagging

CCGCGGGGTGCTTCTGTTGGTGCTTCTGTTTCTGTTGGTCCGCGGTGGAGCTCAATG

### Supplementary Note 6: Sequence of yeast-optimized mScarletI (ymScarletI) from ref. (Botman et al., 2019) with restriction sites for frequently-used restriction enzymes removed

The following sequence is also supplied with annotations in gbk format in the **Supplementary Files**:

ATGGTTAGTAAAGGTGAAGCTGTTATAAAAGAATTTATGAGGTTTAAAGTTCATATGGAAGGTTCAATGAATGGTCAT  
GAATTTGAAATTGAAGGTGAAGGTGAAGGTAGACCATATGAAGGTACACAAACTGCTAAATTGAAAGTTACTAAAGG  
TGGTCCATTACCATTTTCTTGGGATATTTGTCTCCACAATTCATGTATGGTTCAAGAGCTTTTATTAAGCATCCTGCTG  
ATATTCCAGATTATTATAAAACAATCTTTTCTGAAGGTTTTAAATGGGAAAGAGTTATGAATTTTGAAGATGGTGGTGC  
TGTTACTGTTACACAAGATACTTCTTTGGAAGATGGTACTTTAATCTATAAAAGTTAAATTGAGAGGTACTAATTTTCCAC  
CAGACGGTCCAGTTATGCAAAAGAAAACCTATGGGTTGGGAAGCATCTACTGAAAGATTGTATCCTGAAGATGGTGT  
TTGAAAGGAGACATTAAGATGGCTTTGAGATTGAAAGATGGTGGTAGATACCTGGCCGACTTCAAGACCACCTATAA  
AGCTAAAAAACAGTTCAAATGCCAGGTGCATATAATGTTGATAGAAAGTTAGATATAACGTCGCATAACGAGGACTA  
TACTGTAGTTGAACAATATGAACGTAGTGAAGGTAGACATAGTACCGGAGGAATGGATGAATTGTATAAATAA

### Supplementary Table 1: Strains used in the study

Strain name	Genotype	Source
yVG2873	<i>W303 MATa WHI5::pVG264::URA3, LEU2</i>	This study
yVG2874	<i>W303 MATa WHI5::pVG265::URA3, LEU2</i>	This study
yVG2875	<i>W303 MATa WHI5::pVG266::URA3, LEU2</i>	This study
yVG2876	<i>W303 MATa WHI5::pVG267::URA3, LEU2</i>	This study
yVG2877	<i>W303 MATa WHI5::pVG268::URA3, LEU2</i>	This study
yVG2878	<i>W303 MATa HTB2::pVG288::URA3, LEU2</i>	This study
yVG2879	<i>W303 MATa HTB2::pVG289::URA3, LEU2</i>	This study
yVG2880	<i>W303 MATa HTB2::pVG290::URA3, LEU2</i>	This study
yVG2881	<i>W303 MATa HTB2::pVG291::URA3, LEU2</i>	This study

### Supplementary Table 2: Plasmids used in the study

The annotated sequences for the plasmids below are included as gbk files in the **Supplementary Files**:

Plasmid name	Description	Yeast selection marker	Bacterial selection marker	Source of the plasmid
pETURALEU	Empty backbone containing both <i>URA3</i> and <i>LEU2</i> selectable markers	<i>URA3, LEU2</i>	<i>Amp</i>	This study
pVG254	Whi5 marking plasmid using ymScarletI and $R_{\text{homo}} = 0.5$	<i>URA3, LEU2</i>	<i>Amp</i>	This study
pVG265	Whi5 marking plasmid using ymScarletI and $R_{\text{homo}} = 1$	<i>URA3, LEU2</i>	<i>Amp</i>	This study
pVG266	Whi5 marking plasmid using ymScarletI and $R_{\text{homo}} = 1.5$	<i>URA3, LEU2</i>	<i>Amp</i>	This study
pVG267	Whi5 marking plasmid using ymScarletI and $R_{\text{homo}} = 2$	<i>URA3, LEU2</i>	<i>Amp</i>	This study
pVG268	Whi5 marking plasmid using ymScarletI with $R_{\text{homo}} = 4$	<i>URA3, LEU2</i>	<i>Amp</i>	This study



pVG288	Htb2 marking plasmid using ymScarletI and $R_{\text{homo}} = 0.5$	<i>URA3, LEU2</i>	<i>Amp</i>	This study
pVG289	Htb2 marking plasmid using ymScarletI and $R_{\text{homo}} = 1$	<i>URA3, LEU2</i>	<i>Amp</i>	This study
pVG290	Htb2 marking plasmid using ymScarletI and $R_{\text{homo}} = 1.5$	<i>URA3, LEU2</i>	<i>Amp</i>	This study
pVG291	Htb2 marking plasmid using ymScarletI and $R_{\text{homo}} = 2$	<i>URA3, LEU2</i>	<i>Amp</i>	This study

Supplementary Table 3: Number of analyzed colonies for Fig. 4

Gene	$R_{\text{homo}}$	Number of colonies analyzed in desired versus undesired pop-out	Method for analysis
<i>HTB2</i>	0.5	100	Fluorescence microscopy
<i>HTB2</i>	1	95	Fluorescence microscopy
<i>HTB2</i>	1.5	105	Fluorescence microscopy
<i>HTB2</i>	2	100	Fluorescence microscopy
<i>WHI5</i>	0.5	44	PCR
<i>WHI5</i>	1	18	PCR
<i>WHI5</i>	1.5	38	PCR
<i>WHI5</i>	2	28	PCR
<i>WHI5</i>	4	42	PCR