Supplementary Information

Serine integrase-based recombination enables direct plasmid assembly *in vivo*

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I. Supplementary Tables

Supplementary Table S1. *E. coli* strains.

Strain name	Purpose	Genotype	Origin
DH5α	Molecular cloning, plasmid assembly, chromoprotein-based color exhibition	F^- φ80 *lac*ZΔM15 Δ(*lac*ZYA-*arg* F) U169 *end*A1 *rec*A1 *hsd*R17(r_K^-,m_K^+) *sup*E44λ- *thi* -1 *gyr*A96 *rel*A1 *pho*A	TransGen Biotech
MG1655	Insertion of integrases (Bxb1, phiC31) into its genome for endogenous expression	K12 F^- *lambda- ilvG- rfb-50 rph-1*	Shanghai Weidi Biotechnology
DH5α λpir	Cloning R6K-ori plasmids	F^- φ80 *lac*ZΔM15 Δ(*lac*ZYA-*arg* F) *LAMpir* U169 *end*A1 *rec*A1 *hsd*R17(r_K^-,m_K^+) *sup*E44λ- *thi* -1 *gyr*A96 *rel*A1 *pho*A	Shanghai Weidi Biotechnology

Supplementary Table S2. Genetic parts.

Part name	Type	DNA sequence	Reference
BioBrick prefix	BioBrick assembly site	gaattcgcggccgcttctagag	1
BioBrick suffix	BioBrick assembly site	tactagtagcggccgctgcag	1
J23100	promoter	ttgacggctagctcagtcctaggtacagtgctagc	1, BBa_J23100
paraBAD	promoter (with native RBS)	aaagccatgacaaaaacgcgtaacaaaagtgtctataatcacggcagaaaagtccacattgattatttgc acggcgtcacactttgctatgccatagcatttttatccataagattagcggatcctacctgacgctttttatcgc aactctctactgtttctccat	From *E. coli* MG1655 genome (GenBank: U00096.3)
prhaBAD	promoter (with native RBS)	actggcctcctgatgtcgtcaacacggcgaaatagtaatcacgaggtcaggttcttaccttaaattttcgacg gaaaaccacgtaaaaaacgtcgatttttcaagatacagcgtgaattttcaggaaatgcggtgagcatcac atcaccacaattcagcaaattgtgaacatcatcacgttcatctttccctggttgccaatggcccatttttcctgtc agtaacgagaaggtcgcgaattcaggcgctttttagactggtcgtaatgaaattcagcaggatcacatt	From *E. coli* MG1655 genome (GenBank: U00096.3)
B0034	RBS	aaagaggagaaa	1, BBa_B0034
T7 terminator	terminator	ctagcataaccccttggggcctctaaacgggtcttgaggggttttttg	From plasmid vector pET22b
B0015	terminator	ccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaa cgctctctactagagtcacactggctcaccttcgggtgggcctttctgcgtttata	1, BBa_B0015
attP-Bxb1	integrase recognition site	gtcgtggtttgtctggtcaaccaccgcggtctcagtggtgtacggtacaaaccccgac	2
attB-Bxb1	integrase recognition site	tcggccggcttgtcgacgacggcggtctccgtcgtcaggatcatccgggc	2
attP-phiC31	integrase recognition site	gtgccccaactggggtaacctttgagttctctcagttggggg	2
attB-phiC31	integrase recognition site	tgcgggtgccagggcgtgcccttgggctccccgggcgcgtactcc	2
attP-TP901-1	integrase recognition site	gcgagtttttatttcgtttatttcaattaaggtaactaaaaaactccttt	2
attB-TP901-1	integrase recognition site	atgccaacacaattaacatctcaatcaaggtaaatgctttttgctttttttgc	2
araC	CDS	atggctgaagcgcaaaatgatcccctgctgccgggatactcgtttaatgcccatctggtggcgggtttaacg ccgattgaggccaacggttatctcgatttttttatcgaccgaccgctgggaatgaaaggttatattctcaatctc accattcgcggtcaggggtggtgaaaaatcagggacgagaatttgtttgccgaccgggtgatattttgctg ttcccgccaggagagattcatcactacggtcgtcatccggaggctcgcgaatggtatcaccagtgggttta ctttcgtccgcgcgcctactggcatgaatggcttaactggccgtcaatatttgccaatacggggttctttcgcc cggatgaagcgcaccagccgcatttcagcgacctgtttgggcaaatcattaacgccgggcaaggggaa gggcgctattcggagctgctggcgataaatctgcttgagcaattgttactgcggcgcatggaagcgattaa cgagtcgctccatccaccgatggataatcgggtacgcgaggcttgtcagtacatcagcgatcacctggca gacagcaattttgatatcgccagcgtcgcacagcatgtttgcttgtcgccgtcgcgtctgtcacatcttttccgc cagcagttagggattagcgtcttaagctggcgcgaggaccaacgtatcagccaggcgaagctgcttttga gcaccacccggatgcctatcgccaccgtcggtcgcaatgttggttttgacgatcaactctatttctcgcgggt atttaaaaaatgcaccggggccagcccgagcgagttccgtgccggttgtgaagaaaaagtgaatgatgt agccgtcaagttgtcataa	From *E. coli* MG1655 genome (GenBank: U00096.3)
Bxb1	CDS	atgagagccctggtagtcatccgcctgtcccgcgtcaccgatgctacgacttcaccggagcgtcagctgg agtcttgccagcagctctgcgcccagcgcggctgggacgtcgtcggggtagcggaggatctggacgtct ccggggcggtcgatccgttcgaccggaagcgcagaccgaacctggcccggtggctagcgttcgagga gcaaccgttcgacgtgatcgtggcgtaccgggtagaccggttgacccgatcgatccggcatctgcaaca gctggtccactgggccgaggaccacaagaagctggtcgtctccgcgaccgaagcgcacttcgatacga cgacgccgtttgcggcggtcgtcatcgcgcttatgggaacggtggcgcagatggaattagaagcgatca aagagcggaaccgttcggctgcgcatttcaatatccgcgccgggaaataccgaggatccctgccgccgt ggggatacctgcctacgcgcgtggacggggagtggcggctggtgccggaccctgtgcagcgagagcg	2

		catcctcgaggtgtatcaccgcgtcgtcgacaaccacgagccgctgcacctggtggcccacgacctgaa ccggcgtggtgtcctgtcgccgaaggactacttcgcgcagctgcaaggccgcgagccgcagggccggg agtggtcggctaccgcgctgaagcgatcgatgatctccgaggcgatgctcgggtacgcgactctgaacg gtaagaccgtccgagacgacgacggagccccgctggtgcgggctgagccgatcctgacccgtgagca gctggaggcgctgcgcgccgagctcgtgaagacctcccgggcgaagcccgcggtgtctaccccgtcgc tgctgctgcgggtgttgttctgcgcggtgtgcggggagcccgcgtacaagttcgccgggggaggacgtaa gcacccgcgctaccgctgccgctcgatggggttcccgaagcactgcgggaacggcacggtggcgatgg ccgagtgggacgcgttctgcgaggagcaggtgctggatctgctcggggacgcggagcgtctggagaaaa gtctgggtagccggctcggactccgcggtcgaactcgcggaggtgaacgcggagctggtggacctgac gtcgctgatcggctccccggcctaccgggccggctctccgcagcgagaagcactggatgcccgtattgc ggcgctggccgcgcggcaagaggagctggagggcctagaggctcgcccgtctggctgggagtggcgc gagaccgggcagcggttcggggactggtggcgggagcaggacaccgcggcaaagaacacctggctt cggtcgatgaacgttcggctgacgttcgacgtccgcggcgggctgactcgcacgatcgacttcggggatc tgcaagagtacgagcagcatctcaggctcggcagcgtggtcgaacggctacacaccgggatgtcgtaa	
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mCherry	CDS	atggtgagcaagggcgaggaggataacatggccatcatcaaggagttcatgcgcttcaaggtgcacatg gagggctccgtgaacggccacgagttcgagatcgagggcgagggcgagggccgcccctacgagggc acccagaccgccaagctgaaggtgaccaagggtggcccccctgcccttcgcctgggacatcctgtcccct cagttcatgtacggctccaaggcctacgtgaagcaccccgccgacatccccgactacttgaagctgtcctt ccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggcggcgtggtgaccgtgacccagg	2

		actcctccctgcaggacggcgagttcatctacaaggtgaagctgcgcggcaccaacttcccctccgacg gccccgtaatgcagaagaagacgatgggctgggaggcctcctccgagcggatgtaccccgaggacgg cgccctgaagggcgagatcaagcagaggctgaagctgaaggacggcggccactacgacgctgaggt caagaccacctacaaggccaagaagcccgtgcagctgcccggcgcctacaacgtcaacatcaagttg gacatcacctcccacaacgaggactacaccatcgtggaacagtacgaacgcgccgagggccgccact ccaccggcggcatggacgagctgtacaagtaa	
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		atgtttttgagttagttaaaactcagttgggctcaattccgattaatgaactatcatatgataataaaaagaaa atcgtcaacaaccttgtatcaaaggttgatgttactgctgataatgtagatatcatatttaaattccaactcgctt aa	
eforRed	CDS	atgtcagtgattaagcaggtaatgaagaccaagttgcaccttgagggcactgtcaatggccatgattttacg atcgagggtaaaggtgaaggcaagccgtacgaagggttacagcacatgaaaatgacagtcaccaaag gcgcgcctctgccgttttccgttcatattcttacacctagccacatgtatggaagcaaaccgtttaataagtat ccagcggatatcccagactaccacaaacagtcttttcccgaaggtatgtcttgggagcggtcgatgatttttg aagatggtggcgtatgcaccgccagtaatcactccagcataaacttgcaagagaactgtttcatctatgat gttaaatttcatggtgtgaacctgcctccggatgggcccgtaatgcaaaaaaccattgctggatgggagcc gagcgtggaaacactgtacgtgcgtgacgggatgttaaaaagtgacactgcaatggtttttaaactgaaa ggaggcggtcatcatcgtgttgatttcaaaacgacgtataaagccaaaaaacctgtcaagctgccagaat ttcatttcgttgaacatcgcctggaactgaccaaacacgataaagatttcacaacttgggaccagcaggag gcagccgaaggccatttctcaccgctgccgaaggctctcccataa	2
CyOFP1	CDS	atggttagcaagggcgaagagctgatcaaggagaacatgcgcagcaagctgtatctggaaggcagcgt gaacggccaccagttcaagtgtacccacgagggcgaaggcaaaccatacgaaggcaagcagacca accgcatcaaggtggttgaaggcggtccgctgccgtttgccttcgatattctggccacccacttcatgtacgg cagcaaggtgttcattaagtatccggcggatctgccggattacttcaagcagagctttccagagggcttcac gtgggaacgcgtgatggtgtttgaagatggcggtgtgctgaccgcgacccaagataccagtctgcaagat ggcgagctgatctacaatgtgaaggtgcgcggcgtgaatttcccagccaatggcccggtgatgcagaaa aagacgctgggttgggaaccgagcacggaaaccatgtatccagcggatggcggtctggaaggccgttg tgataaagcgctgaaactggtgggtggcggccatctgcacgtgaacttcaagaccacctacaagagcaa gaagccggtgaaaatgccgggcgttcattacgtggatcgccgtctggaacgcattaaagaggccgaca acgagacctacgtggaacagtacgaacacgccgttgcccgctacagcaatctgggcggtggcatggac gaactgtataaataa	2
fwYellow	CDS	atgacggcactgactgaaggcgcaaaactgttcgagaaagaaatcccatatatcactgagctggaaggt gacgttgaaggtatgaagtttatcatcaagggtgaaggtaccggtgacgcgagcgtcggtaaagtggatg ctcagttcatttgtaccacgggcgacgttccggttccgtggagcacgctggtcaccacgctgacgtatggtg ctcagtgctttgccaagtatccgcgccacattgcggatttcttcaaaagctgcatgccggaaggttacgtcc aagagcgcaccatcacctttgagggtgatggcgtgttcaagacccgtgcggaagtcacctttgaaaatgg cagcgtgtacaaccgtgtaaaactgaacggccagggtttcaagaaggacggccacgtgctgggcaaa aatctggagtttaactttacccctcattgtttgtacatttggggtgaccaagcgaatcatggcctgaagagcg cgttcaaaatcatgcatgagatcaccggctccaaagaggatttcattgttgccgatcacacccaaatgaat accccgattggtggtggtccggtgcacgtgccggagtaccaccacattacgtatcatgttaccctgtctaaa gacgtcaccgatcaccgtgaccatttgaacattgttgaggtgatcaaggcagttgacctggagacgtaccg ttaa	2
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tsPurple	CDS	atggcgagcttggttaagaaagatatgtgtgttaagatgacgatggaaggtactgtgaacggttatcacttta	2

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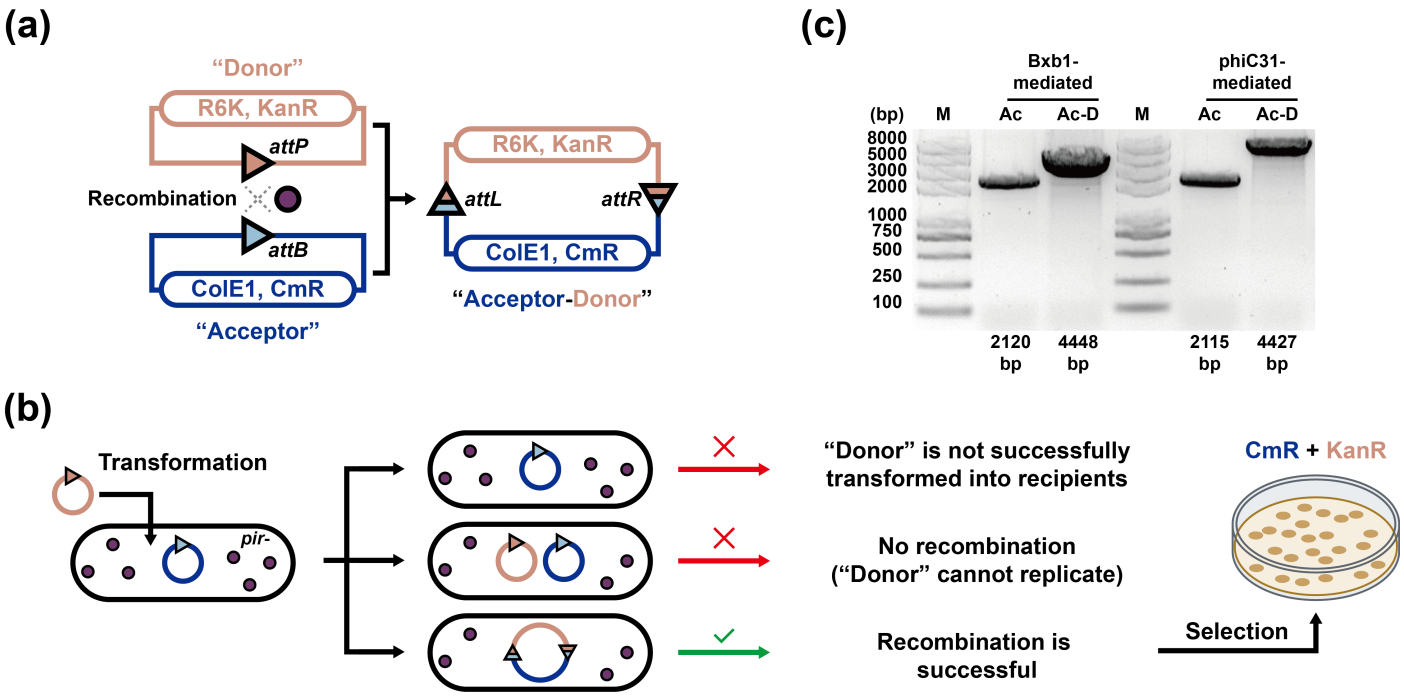
amajLime	CDS	atggcactgagcaacaagtttatcggtgatgatatgaaaatgacgtaccacatggacggctgcgtgaacg gccactactttacggtgaaaggtgaaggcaacggtaaaccgtatgaaggcacccagacgagcaccttta aagttacgatggcgaatggcggtccgctggcctttagtttcgatattctgtccaccgtctttaaatacggcaac cgttgcttcacggcgtatccgaccagcatgccggattactttaagcaagccttcccggacggcatgtcttatg aacgcacgtttacctacgaagatggcggtgtggcgaccgccagctgggaaatttctctgaaaggcaactg tttcgaacataagagtacctttcacggcgtgaatttcccggcagatggtccggttatggctaaaaagaccac gggttgggacccgtcatttgaaaaaatgacggtctgcgatggcatcctgaagggtgacgtgaccgcatttc tgatgctgcaaggcggcggcaattatcgttgtcaattccatacgtcctacaaaaccaaaaagccggttacg atgccgccgaaccatgtggttgaacaccgtattgcgcgcaccgatctggacaaaggtggcaattcagttc agctgacggaacatgcagtcgctcacatcacctcggtcgtgccgttctaa	2
rhaS	CDS	atgaccgtattacatagtgtggatttttttccgtctggtaacgcgtccgtggcgatagaaccccggctcccgc aggcggattttcctgaacatcatcatgattttcatgaaattgtgattgtcgaacatggcacgggtattcatgtgtt taatgggcagccctataccatcaccggtggcacggtctgtttcgtacgcgatcatgatcggcatctgtatga acataccgataatctgtgtctgaccaatgtgctgtatcgctcgccggatcgatttcagtttctcgccgggctga atcagttgctgccacaagagctggatgggcagtatccgtctcactggcgcgttaaccacagcgtattgcag caggtgcgacagctggttgcacagatggaacagcaggaaggggaaaatgatttaccctcgaccgcca gtcgcgagatcttgtttatgcaattactgctcttgctgcgtaaaagcagtttgcaggagaacctggaaaaca gcgcatcacgtctcaacttgcttctggcctggctggaggaccattttgccgatgaggtgaattgggatgccgt ggcggatcaattttctctttcactgcgtacgctacatcggcagcttaagcagcaaacgggactgacgcctc agcgatacctgaaccgcctgcgactgatgaaagcccgacatctgctacgccacagcgaggccagcgtt actgacatcgcctatcgctgtggattcagcgacagtaaccacttttcgacgcttttcgccgagagtttaactg gtcaccgcgtgatattcgccagggacgggatggctttctgcaataa	From *E. coli* MG1655 genome (GenBank: U00096.3)
rhaR	CDS	gtggcgcatcagttaaaacttctcaaagatgatttttttgccagcgaccagcaggcagtcgctgtggctgac cgttatccgcaagatgtctttgctgaacatacacatgatttttgtgagctggtgattgtctggcgcggtaatggc ctgcatgtactcaacgatcgccccttatcgcattacccgtggcgatctctttttacattcatgctgacgataaaca ctcctacgcttccgttaacgatctggttttgcagaatattatttattgcccggagcgtctgaagctgaatcttgac tggcaggggggcgattccgggatttaacgccagcgcagggcaaccacactggcgcttaggtagcatggg gatggcgcaggcgcggcaggttatcggtcagcttgagcatgaaagtagtcagcatgtgccgtttgctaac gaaatggctgagttgctgttcgggcagttggtgatgttgctgaatcgccatcgttacaccagtgattcgttgcc gccaacatccagcgaaacgttgctggataagctgattacccggctggcggctagcctgaaaagtccctttt gcgctggataaattttgtgatgaggcatcgtgcagtgagcgcgttttgcgtcagcaatttcgccagcagact ggaatgaccatcaatcaatatctgcgacaggtcagagtgtgtcatgcgcaatatcttctccagcatagccg cctgttaatcagtgatatttcgaccgaatgtggctttgaagatagtaactatttttcggtggtgtttacccgggaa accgggatgacgcccagccagtggcgtcatctcaattcgcagaaagattaa	From *E. coli* MG1655 genome (GenBank: U00096.3)

Supplementary Table S3. Vectors.

Vector name	Antibiotic resistance gene	Origin of replication	Reference
pvLW1	AmpR	pSC101	3, derived from pKD46
pvLW2	SmR	R6K	This work
pvLW3	KanR	R6K	This work
pvLW4	AmpR	R6K	This work
pvLW5	KanR (with *attP-TP901-1*)	R6K	This work
pSB1C3	CmR	ColE1	1, pSB1C3
pKD4	AmpR, KanR	R6K	3

Supplementary Table S4. Plasmids.

Plasmid name	Biobricks	Vector	Antibiotic resistance gene	Origin of replication
pLW1	araC - paraBAD - Bxb1 - (6xHis) - T7 terminator	pvLW1	AmpR	pSC101
pLW2	araC - paraBAD - phiC31 - (6xHis) - T7 terminator	pvLW1	AmpR	pSC101
pLW3	*attB-Bxb1*	pSB1C3	CmR	ColE1
pLW4	*attB-phiC31*	pSB1C3	CmR	ColE1
pLW5	*attP-Bxb1*	pvLW2	SmR	R6K
pLW6	*attP-phiC31*	pvLW2	SmR	R6K
pLW7	*attP-Bxb1*	pvLW3	KanR	R6K
pLW8	*attP-phiC31*	pvLW3	KanR	R6K
pLW9	J23100 - B0034 - mCherry - B0015 - *attP-Bxb1* - *attB-phiC31*	pvLW2	SmR	R6K
pLW10	J23100 - B0034 - sfGFP - B0015 - *attP-Bxb1* - *attB-phiC31*	pvLW2	SmR	R6K
pLW11	J23100 - B0034 - ECFP - B0015 - *attP-Bxb1* - *attB-phiC31*	pvLW2	SmR	R6K
pLW12	J23100 - B0034 - sfGFP - B0015 - *attP-phiC31* - *attB-Bxb1*	pvLW3	KanR	R6K
pLW13	J23100 - B0034 - ECFP - B0015 - *attP-Bxb1* - *attB-phiC31*	pvLW4	AmpR	R6K
pLW14	J23100 - B0034 - ECFP - B0015 - *attP-phiC31* - *attB-Bxb1*	pvLW3	KanR	R6K
pLW15	J23100 - B0034 - sfGFP - B0015 - *attP-Bxb1* - *attB-phiC31*	pvLW4	AmpR	R6K
pLW16	J23100 - B0034 - mCherry - B0015 - *attP-phiC31* - *attB-Bxb1*	pvLW2	SmR	R6K
pLW17	J23100 - B0034 - sfGFP - B0015 - *attP-Bxb1* - *attB-phiC31*	pvLW3	KanR	R6K
pLW18	J23100 - B0034 - ECFP - B0015 - *attP-phiC31* - *attB-Bxb1*	pvLW4	AmpR	R6K
pLW19	J23100 - B0034 - ECFP - B0015 - *attP-Bxb1* - *attB-phiC31*	pvLW3	KanR	R6K
pLW20	J23100 - B0034 - sfGFP - B0015 - *attP-phiC31* - *attB-Bxb1*	pvLW4	AmpR	R6K
pLW21	(derived from pKD4) homoL (csgA) - *attB-TP901-1* - *FRT* - KanR - *FRT* - homoR (csgA)	pKD4	AmpR, KanR	R6K
pLW22	araC - paraBAD - TP901-1 - (6xHis) - T7 terminator	pvLW1	AmpR	pSC101
pLW23	*attP-TP901-1* - araC - paraBAD - Bxb1 - (6xHis) - T7 terminator	pvLW5	KanR	R6K
pLW24	*attP-TP901-1* - araC - paraBAD - phiC31 - (6xHis) - T7 terminator	pvLW5	KanR	R6K
pLW25	J23100 - B0034 - eforRed - B0015 - *attB-Bxb1*	pSB1C3	CmR	ColE1
pLW26	J23100 - B0034 - CyOFP1 - B0015 - *attB-Bxb1*	pSB1C3	CmR	ColE1
pLW27	J23100 - B0034 - fwYellow - B0015 - *attB-Bxb1*	pSB1C3	CmR	ColE1
pLW28	J23100 - B0034 - amilCP - B0015 - *attB-Bxb1*	pSB1C3	CmR	ColE1
pLW29	J23100 - B0034 - tsPurple - B0015 - *attB-Bxb1*	pSB1C3	CmR	ColE1
pLW30	J23100 - B0034 - amajLime - B0015 - *attB-Bxb1*	pSB1C3	CmR	ColE1
pLW31	araC - paraBAD - B0034 - CyOFP1 - B0015 - *attP-Bxb1* - *attB-phiC31*	pvLW3	KanR	R6K
pLW32	rhaR - rhaS - prhaBAD - B0034 - tsPurple - B0015 - *attP-phiC31* - *attB-Bxb1*	pvLW2	SmR	R6K
pLW33	araC - paraBAD - B0034 - amilCP - B0015 - *attP-Bxb1* - *attB-phiC31*	pvLW3	KanR	R6K
pLW34	rhaR - rhaS - prhaBAD - B0034 - amajLime - B0015 - *attP-phiC31* - *attB-Bxb1*	pvLW2	SmR	R6K

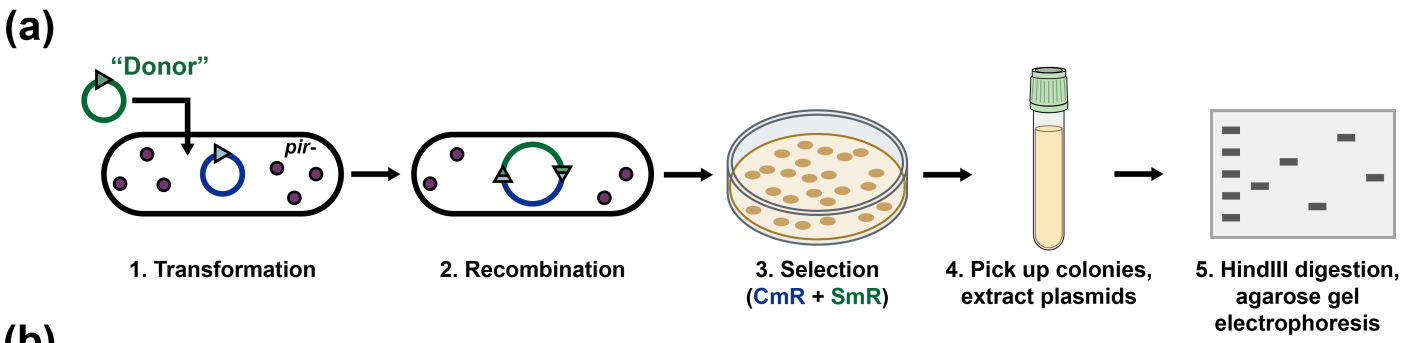


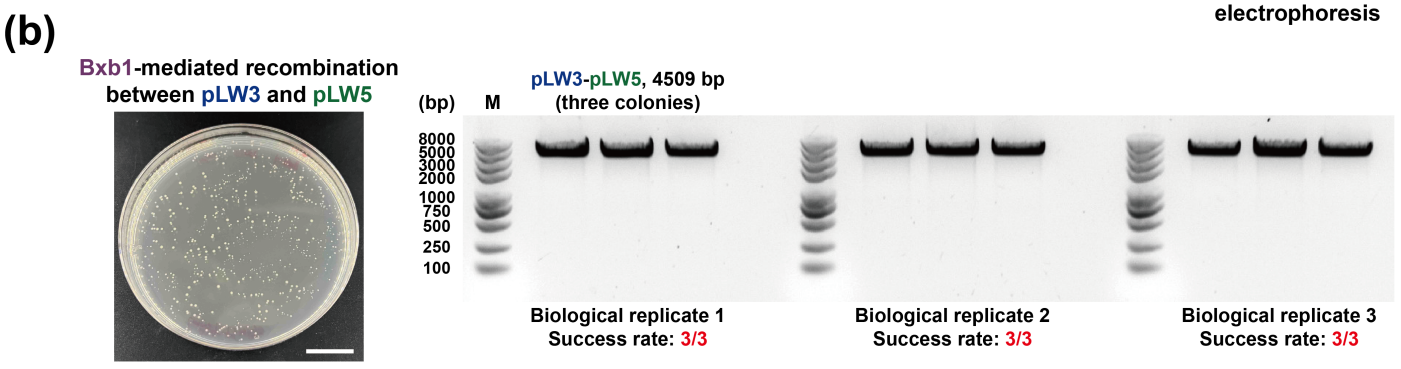
Supplementary Figure S1. Serine integrase enables *in vivo* plasmid assembly.

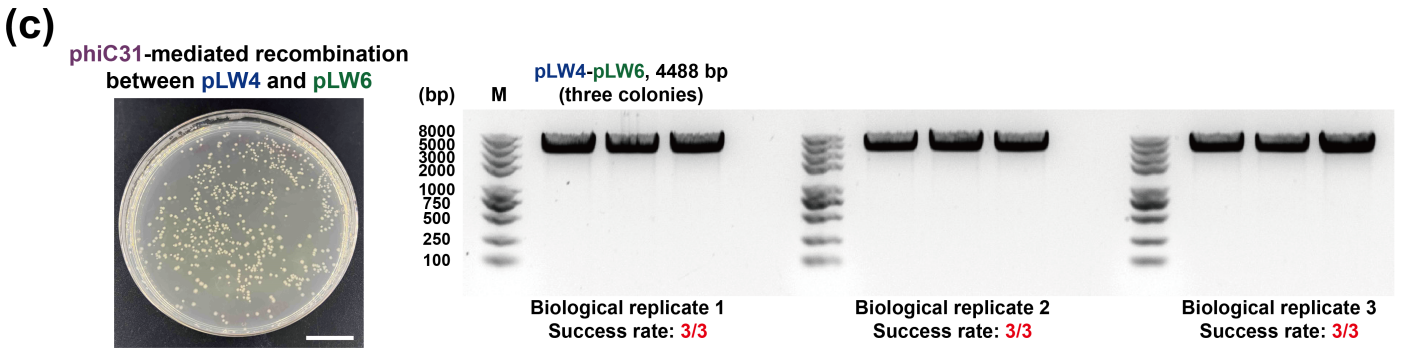
(**a**) Plasmid assembly between "Acceptor" and "Donor" (*attL*: attachment site in the left, *attR*: attachment site in the right). Note that the "Donor" plasmids here contain KanR rather than SmR.

(**b**) Schematic workflow of assembled plasmid selection.

(**c**) DNA fragment sizes of "Acceptor" (Ac, pLW3 for 2120 bp, and pLW4 for 2115 bp), "Acceptor-Donor" (Ac-D, pLW3-pLW7 for 4448 bp, and pLW4-pLW8 for 4427 bp) plasmids after linearization by restriction endonuclease (M: DNA marker).







Supplementary Figure S2. Success rate test of *in vivo* plasmid assembly.

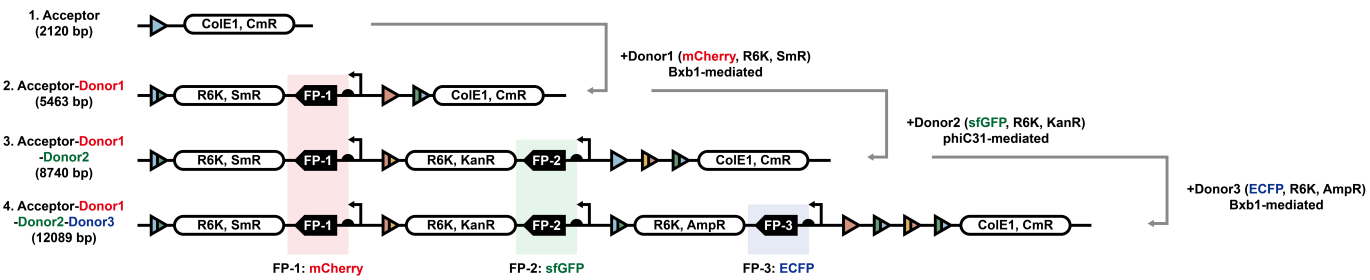
(**a**) Schematic workflow for testing the success rate of *in vivo* plasmid assembly.

(**b**) Results of Bxb1-mediated plasmid assembly between "Acceptor" (pLW3, 2120 bp) and "Donor" (pLW5, 2389 bp). The LB-agar plate (with chloramphenicol and streptomycin) was used for selecting *E. coli* strains that harboring assembled plasmids (pLW3-pLW5, 4509 bp).

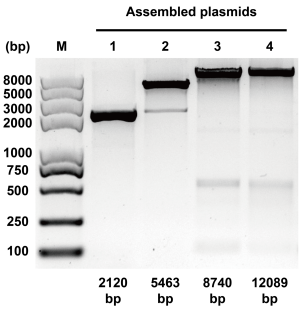
(**c**) Results of phiC31-mediated plasmid assembly between "Acceptor" (pLW4, 2115 bp) and "Donor" (pLW6, 2373 bp). The LB-agar plate (with chloramphenicol and streptomycin) was used for selecting *E. coli* strains that harboring assembled plasmids (pLW4-pLW6, 4488 bp).

Both of the experiments in (**b**) and (**c**) were performed with three biological replicates. In each replicate, three *E. coli* colonies were picked up from LB-agar plates. Scale bar: 2 cm.

(a)



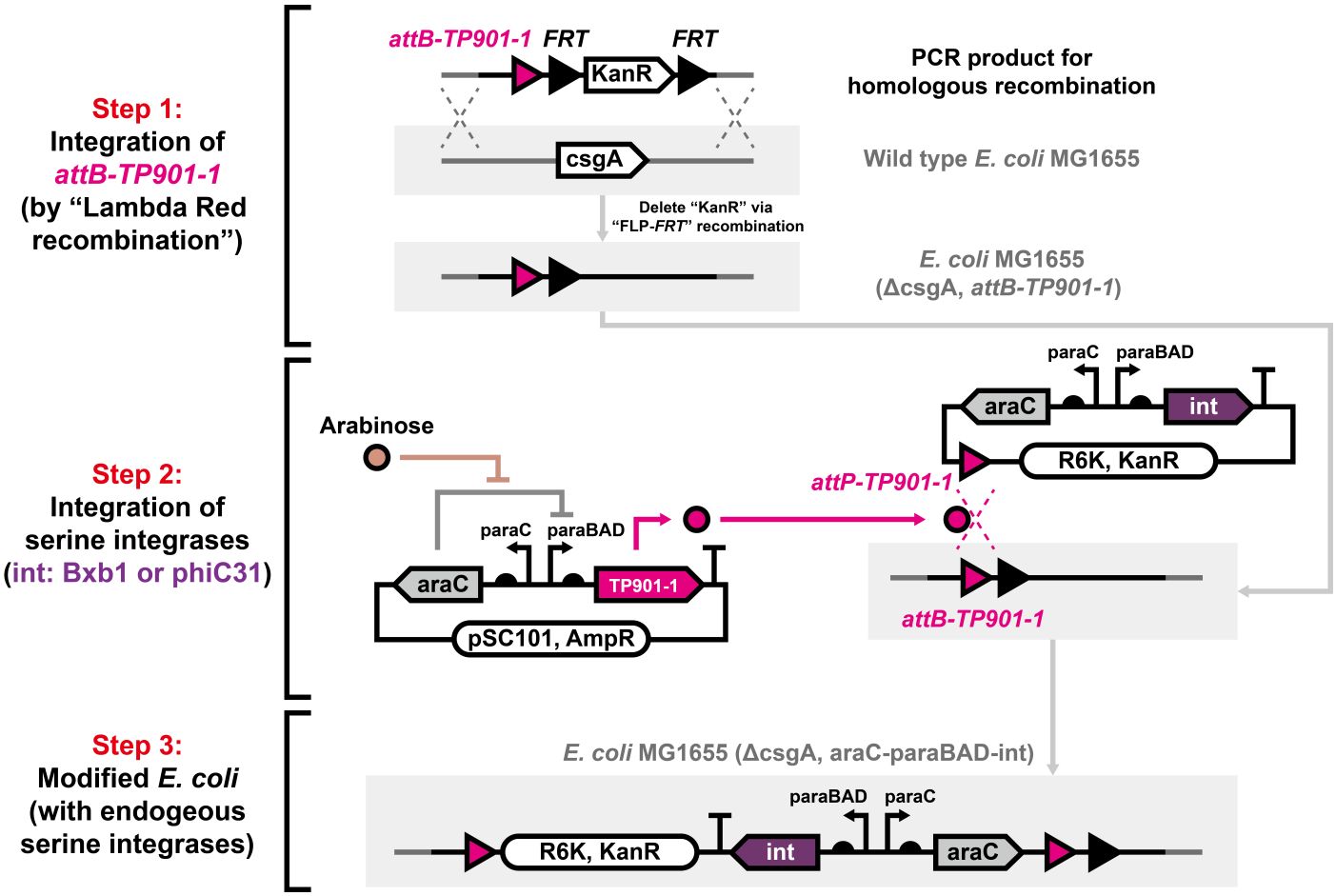
(b)



Supplementary Figure S3. Workflow and demonstration of *in vivo* plasmid assembly cascade.

(**a**) Schematic workflow of *in vivo* plasmid assembly cascade. This is an example of a three-round assembly cascade. “Acceptor”: pLW3, “Donor1”: pLW9 with mCherry, “Donor2”: pLW12 with sfGFP, “Donor3”: pLW13 with ECFP. FP: fluorescent protein.

(**b**) Agarose gel electrophoresis showed the DNA fragment sizes after linearization by restriction endonuclease (M: DNA marker). Sample 1: “Acceptor” pLW3 for 2120 bp, sample 2: “Acceptor-Donor1” for 5463 bp, sample 3: “Acceptor-Donor1-Donor2” for 8740 bp, sample 4: “Acceptor-Donor1-Donor2-Donor3” for 12089 bp.

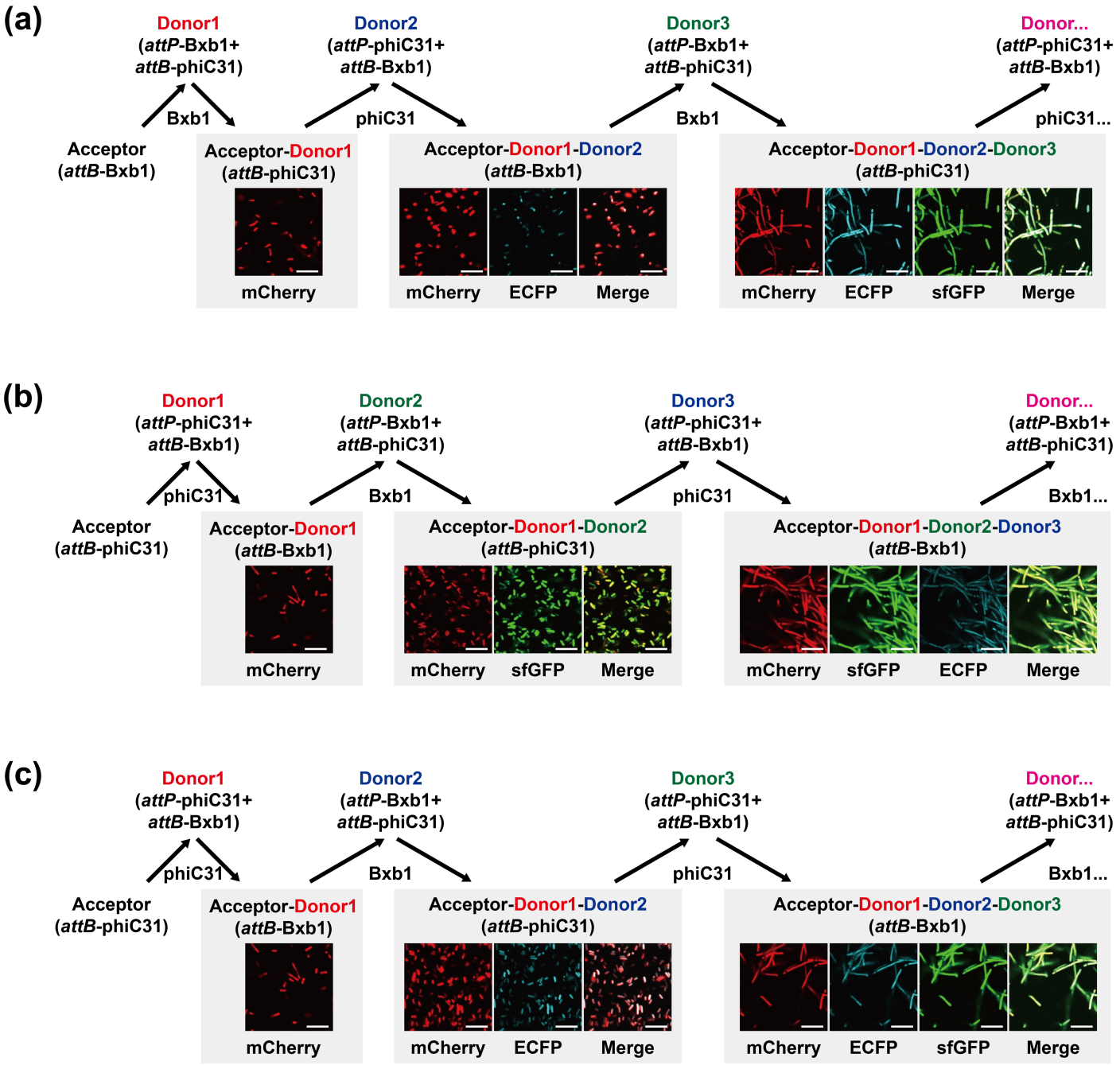


Supplementary Figure S4. Schematic workflow of *E. coli* MG1655 modification.

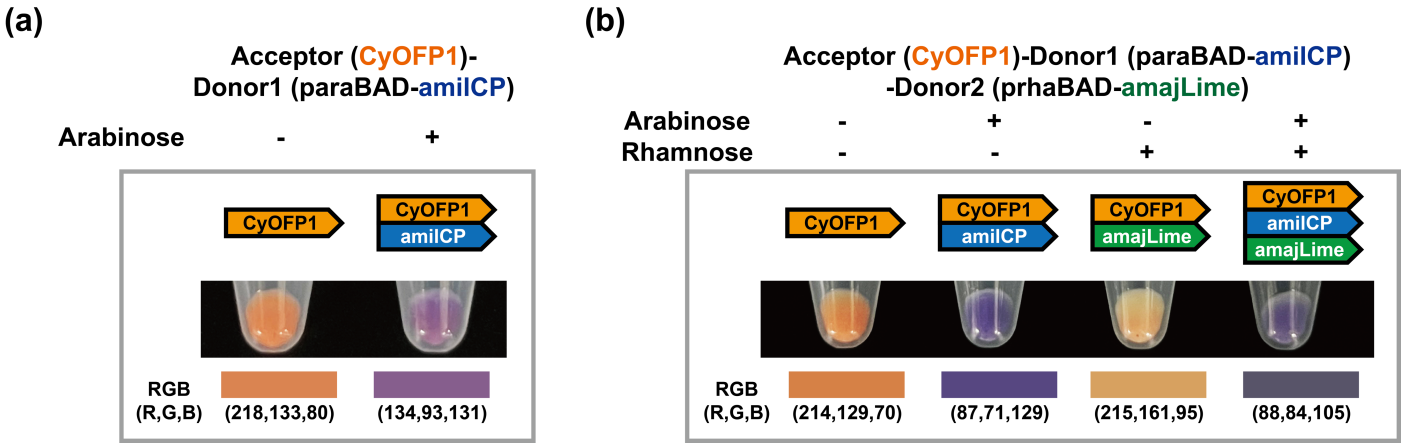
Step 1: the procedure of "Lambda Red recombination" was referred as previously reported [3]. Here we inserted the *attP-TP901-1* sequence into the original pKD4 [3] plasmid as PCR template (pLW21) for the next integration. After recombination, the DNA fragment between two *FRT* (including KanR) was deleted by pCP20 [3].

Step 2: After the integration of *attP-TP901-1* into wild type *E. coli* MG1655 genome, this modified *E. coli* strain was then transferred with a helper plasmid pLW22 for the expression of serine integrase TP901-1. Then this strain was induced by 1% (w/v) arabinose and prepared as recipient cell. Next, this recipient cell was electroporated with pLW23 (arabinose-induced Bxb1) or pLW24 (arabinose-induced phiC31) for genome integration. After that, by several days' continuous cultivation at 37°C for curing the helper plasmid pLW22 [Because this plasmid was constructed with pSC101, a high-temperature (over 30°C) sensitive replication ori. This helper plasmid could be cured at 37°C].

Step 3: Finally, this strain was integrated the integrases (Bxb1 or phiC31) into its genome and could be used for *in vivo* plasmid assembly.



Supplementary Figure S5. *In vivo* plasmid assembly cascade workflow and fluorescence visualization.
Note that all of the assembly workflow in (**a**), (**b**), and (**c**) were similar, and the differences were among the plasmid design and order of assembly. Scale bar: 10 μm.
(**a**) "Acceptor": pLW3, "Donor1": pLW9 with mCherry, "Donor2": pLW14 with ECFP, "Donor3": pLW15 with sfGFP.
(**b**) "Acceptor": pLW4, "Donor1": pLW16 with mCherry, "Donor2": pLW17 with sfGFP, "Donor3": pLW18 with ECFP.
(**c**) "Acceptor": pLW4, "Donor1": pLW16 with mCherry, "Donor2": pLW19 with ECFP, "Donor3": pLW20 with sfGFP.



Supplementary Figure S6. Generation of colored *E. coli* by *in vivo* plasmid assembly, another example.
(**a**) Two color phenotypes were generated from the "Acceptor-Donor1" plasmid (pLW26-pLW33). Arabinose: 1% (w/v).
(**b**) Four color phenotypes were generated from the "Acceptor-Donor1-Donor2" plasmid (pLW26-pLW33-pLW34). Arabinose: 1% (w/v), rhamnose: 1% (w/v).

III. Supplementary References

References

[1] *Registry of Standard Biological Parts*, <http://parts.igem.org/Main_Page?title=Main_Page> (2023).
[2] Ba, F.; Liu, Y.; Liu, W. Q.; Tian, X.; Li, J. SYMBIOSIS: synthetic manipulable biobricks via orthogonal serine integrase systems. *Nucleic Acids Res.* **2022**, *50*, 2973-2985.
[3] Datsenko, K. A.; Wanner, B. L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 6640-6645.