

Appendix for:

Precision design of stable genetic circuits carried in highly-insulated *E. coli* genomic landing pads

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Figure 1. Terminator characterization assay.

Figure 2. Comparison of expression at different integration sites between *E. coli* strains.

Figure 3. Comparison of expression levels between landing pads.

Figure 4. Comparison between plasmid RPU and genome RPU_G fluorescence levels.

Figure 5. Characterization of the sensor array at Landing Pad #1.

Figure 6. Genetic schematics for the genome-encoded NOT gates.

Figure 7. Optimization of NOT gates.

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Figure 9. Example of a circuit designed using “tandem” NOR gates.

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Figure 12. Conversion of x-axis of response functions from [IPTG] to RPU_G

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Figure 14. Plasmid and genome constructs used in this study.

Note 1. Detailed protocol for inserting payloads into genome landing pads

Table 1. Double terminator sequences and strengths

Table 2. Transposon library results for *E. coli* DH10β

Table 3. ON/OFF levels for genome-encoded sensors

Table 4. Sequence of landing pads used in this study

Table 5. Sequence of genetic parts used in this study

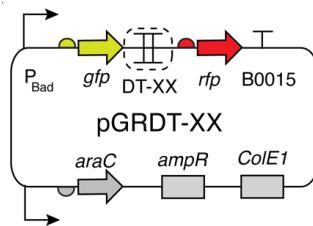
Table 6. Sequence of plasmids used in this study

Table 7. Sequence of genome integrated constructs used In this study

Table 8. Strains used in this study

References

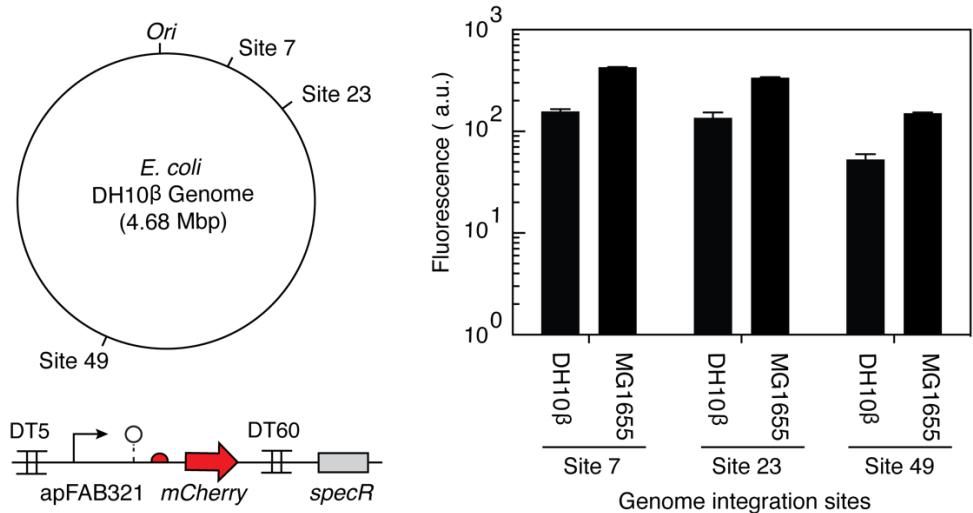
a



b

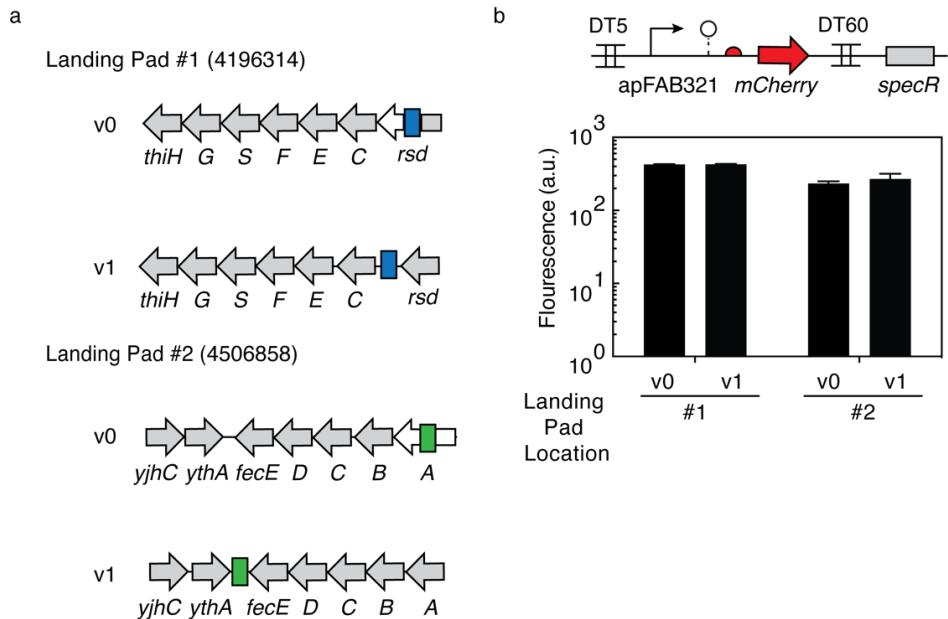
Name	Forward Terminator Strength					Reverse Terminator Strength						
	Day 1		Day 2		Day 3	Day 1		Day 2		Day 3		
	GFP	RFP	GFP	RFP	GFP	RFP	GFP	RFP	GFP	RFP		
DT3	4100	0.4	3200	0.8	3100	0.8	3200	8.4	2900	10	3600	14
DT5	3100	0.9	4600	0.4	3500	0.5	3600	34	4700	51	3300	27
DT19	3400	2.0	2700	1.8	2200	1.5	2900	1300	3200	1500	4100	1400
DT34	4400	3.6	3300	2.4	3200	3.2	2600	950	3200	900	3800	1400
DT36	3400	2.5	3800	2.2	4500	3.8	3000	390	2900	470	3300	510
DT42	3200	0.4	3900	1.4	3900	1.0	6700	130	700	160	800	200
DT54	3100	0.6	3700	1.0	7700	2.7	3200	55	3500	60	4200	52
DT56	2700	5.4	3400	6.5	3300	7.1	540	20	560	26	660	41
DT60	3400	16	3200	14	4200	21	2900	48	3600	53	3800	79
DT65	2400	2.6	1500	1.4	3000	5.4	2200	1200	3000	1400	3100	1900
DT82	2200	6.3	1800	3.9	3000	11	2900	500	3300	570	3500	720
DT83	1800	1.7	4200	5.9	9100	2.9	2100	210	2700	310	3000	400
DT86	4800	11	2500	4.4	4000	12	3000	3100	4000	4700	4100	5200
DT100	5400	0.7	4500	0.5	9500	1.7	2700	120	3200	100	4100	180
DT101	4100	0.4	4600	0.7	3200	0.5	2900	10	5000	13	5200	20
DT103	3500	2.2	3000	1.6	3500	3.0	2400	260	3400	410	3200	500
DT104	3200	3.1	4400	4.9	3000	2.1	2400	11	3700	17	3300	19

Appendix Figure 1: Terminator characterization assay. (a) The plasmid to measure terminator strength is shown, where XX refers to the terminator name. The terminator to be measured is inserted between *gfp* and *rfp* genes in an operon and the measured fluorescences are used to calculate terminator strength T_s (Methods). (b) The raw fluorescence measurements for each terminator are shown.

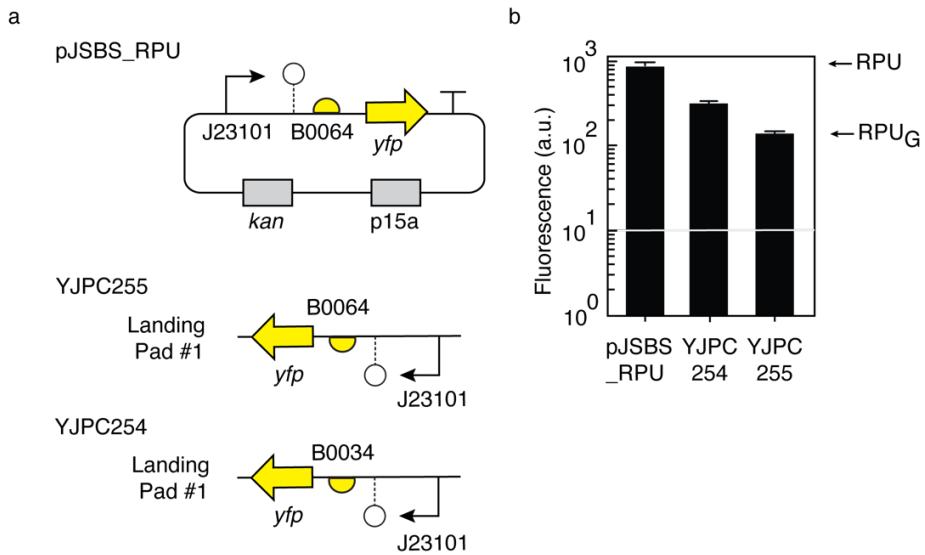


Appendix Figure 2: Comparison of expression at different integration sites between *E. coli* strains.

Three sites are shown, selected from the Tn5 library (Appendix Table 2) (corresponding to nt positions #4294255, #4614289, #1983293 in the genome sequence NCBI CP000948.1). The construct integrated at these sites is shown. The means of three experiments performed on different days are shown and the error bars are the standard deviations of these measurements.

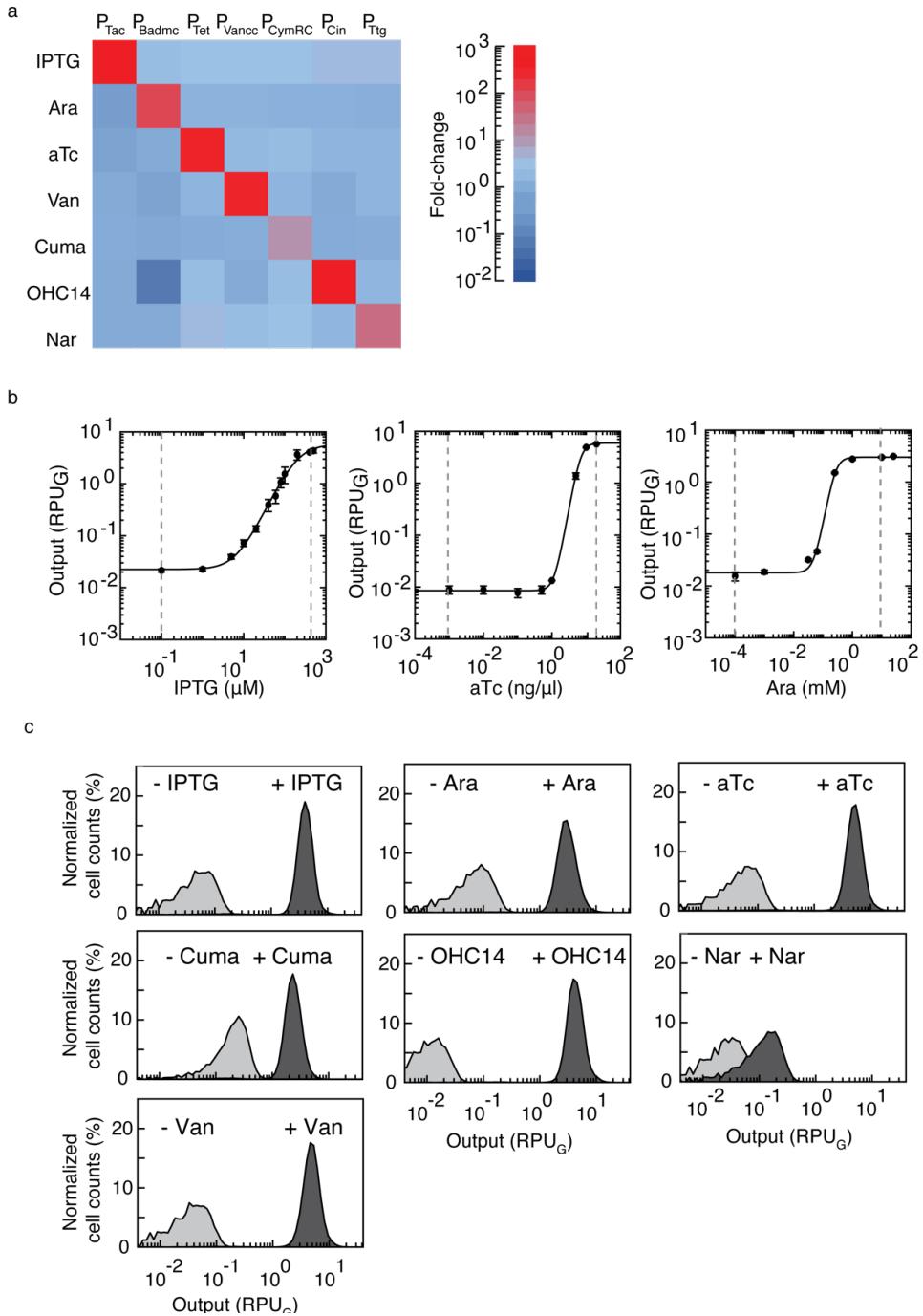


Appendix Figure 3: Comparison of expression levels between landing pads. **(a)** The locations of the landing pads in the genome are shown. **(b)** Experiments were performed where a constitutive expression cassette was evaluated at these positions (but not containing the context of the double terminators and integration sites) (Methods). The means are shown for three experiments performed on different days and the error bars are the standard deviations of these measurements.



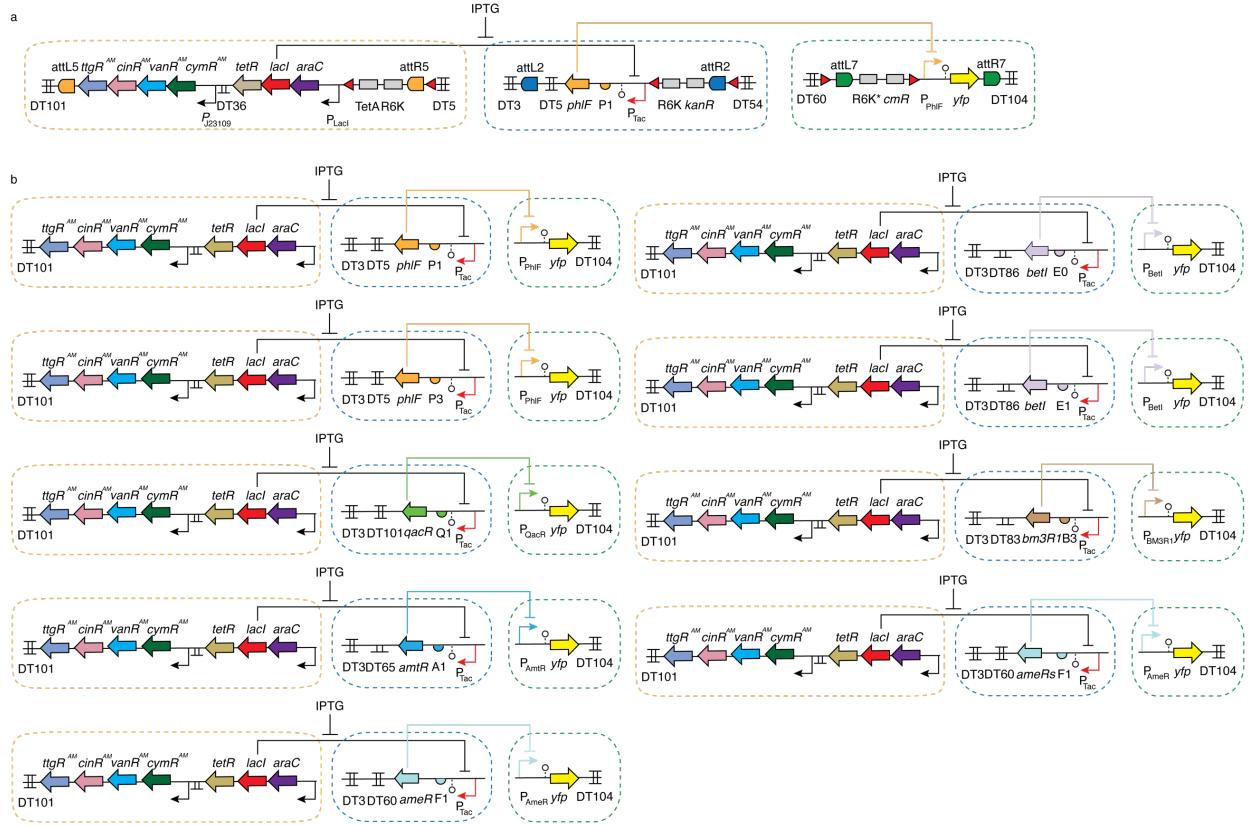
Appendix Figure 4: Comparison between plasmid RPU and genome RPU_G fluorescence levels. (a)

The constructs used for evaluating the reference promoter are shown, with the plasmid names on the left¹. (b) Fluorescence measurements of cells carrying the constructs are shown (Methods). The horizontal line shows the background fluorescence of cells. By comparing expression levels between the YJPC255 and pJSBS_RPU plasmids, the conversion factor between RPU and RPU_G was calculated (1 RPU = 6.3 RPU_G). Part sequences are provided in Supplementary Table 5. The means are shown for three experiments performed on different days and the error bars are the standard deviations of these measurements.

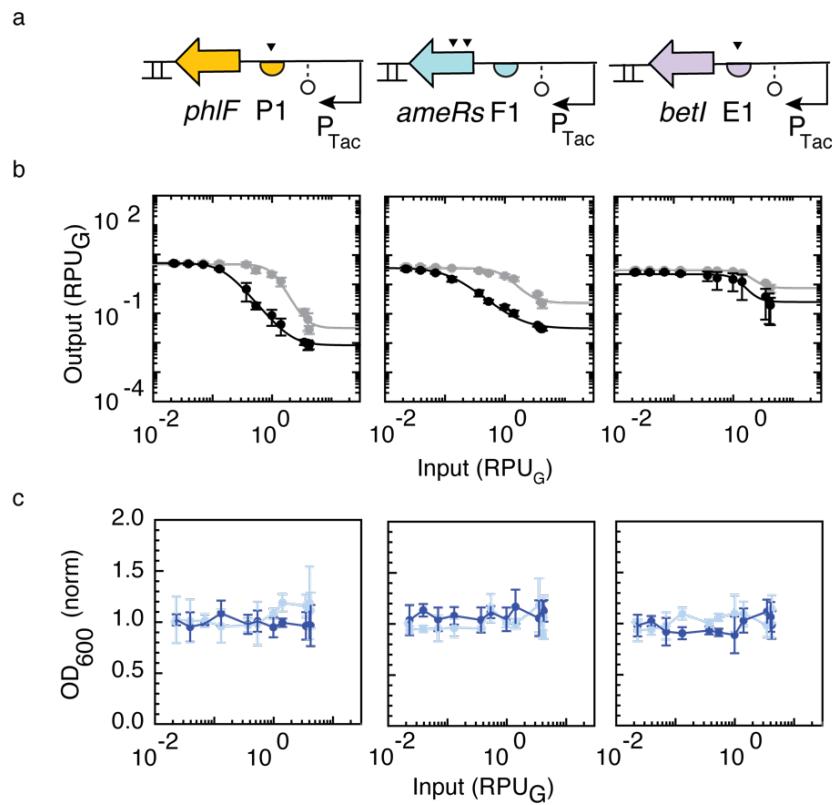


Appendix Figure 5: Characterization of the sensor array at Landing Pad #1. The genetic construct showing the sensors is shown in Figure 2c. **(a)** Sensor orthogonality. For these experiments, the output promoter of each sensor is transcriptionally fused to *yfp* and inserted into Landing pad #1 (Appendix Figure S18). The cross reactivity of the sensors is shown when the following inducer concentrations were used: 12.5 mM Ara, 1 mM IPTG, 20 ng/μl aTc, 500 μM Cuma, 200 μM Van, 10 μM OHC14, and 1 mM Nar.

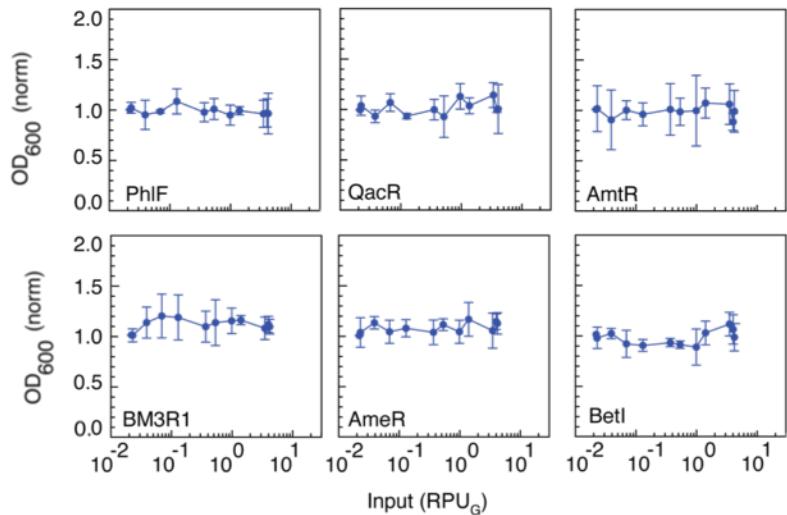
The fold-change is calculated from the median fluorescence in the presence of inducer divided by that measured in its absence. Growth and measurement conditions are provided in the Methods. The data represents the mean from three replicates measured on different days. **(b)** Full response functions for the three sensors used in the circuit designs. The P_{Tac} response function shown was used to convert the x-axis of the NOT gate response functions from concentration to RPU_G (Appendix Figure S12). The means are shown for three experiments performed on different days and the error bars are the standard deviation of these measurements. Two gray dotted lines indicate inducer concentrations used for Cello prediction and circuit design. **(c)** The fluorescence distribution measured from flow cytometry and was converted into RPU_G (Methods). For each output promoter, both the OFF (without inducer, light gray) and ON (with inducer, darker gray) states are shown in the same plot. The experiments were repeated three times on different days with similar results. The mean of three replicates are shown in Figure 2C.



Appendix Figure 6: Genetic schematics for the genome-encoded NOT gates. (a) Schematic representation of the P1-PhIF-NOT gate on the genome. This construct is shown with the additional parts in the landing pad (e.g., antibiotic resistance genes). Genetic parts and the integration sequences are provided in Appendix Tables 4, 5 and 6. **(b)** Design of 9 NOT gates implemented on the genome (genomic parts not shown for clarity). The constructs inserted into each landing pad are: landing pad #1 (blue), landing pad #2 (green) and landing pad #3 (orange).

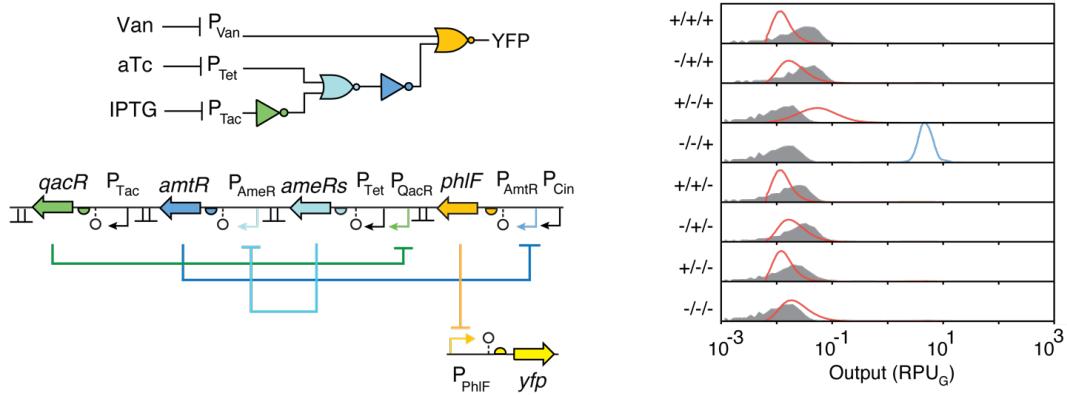


Appendix Figure 7: Optimization of the NOT gates. (a) Schematics of each gate; the changes made during optimization are indicated by the triangles. For the PhlF gate, the RBS was changed from P3 (CTTTACGAGGGCGATCCT) to P1 (CTATGGACTATGTTGAAAGGGAGAAATACTAG). Two mutations were made in *ameR* (R25H and R43S) to create *ameRs*. For the Betl gate, the RBS was changed from E0 (CCCCCCGAGGAGTAGCAC) to E1 (CCTTCCGAGGAGGAGCACA). (b) The response functions before (gray) and after (black) optimization. (c) The impact on the growth of cells is shown before (light blue) and after (dark blue) optimization. The means are shown for three experiments performed on different days and the error bars are the standard deviations of these measurements.

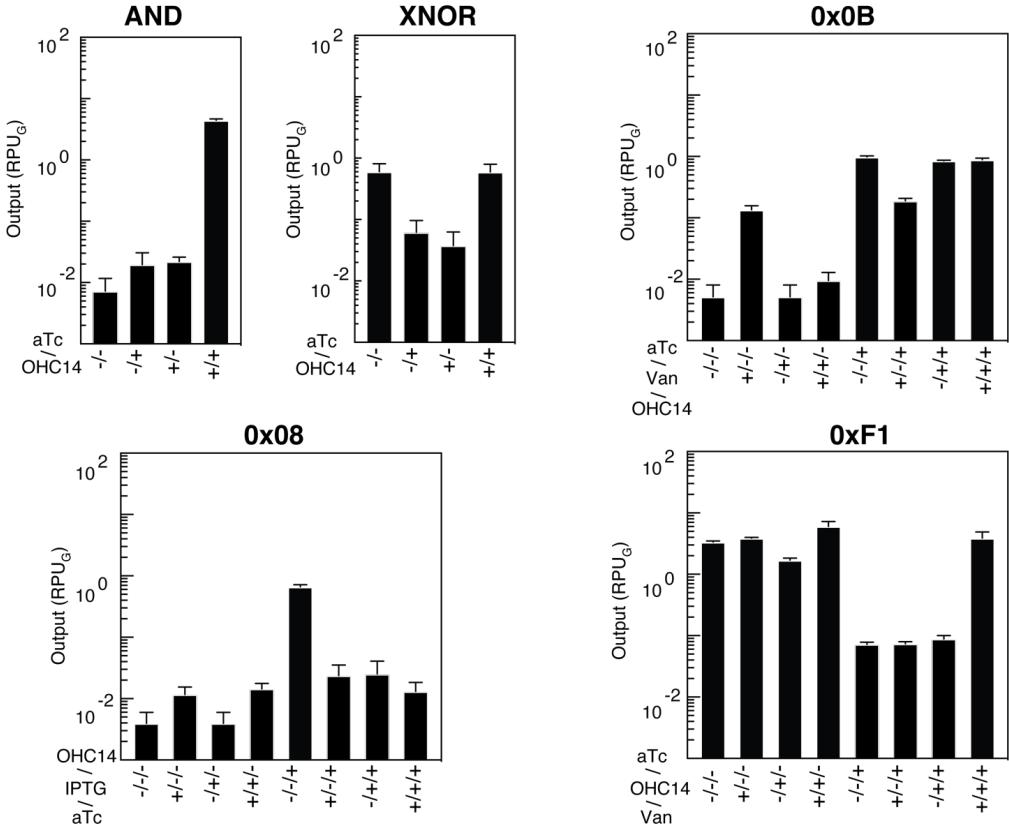


Appendix Figure 8: **Growth impact of repressor expression from Landing Pad #1.** Changes in OD_{600} upon the expression of repressors are shown (Methods). These data were used to create the blue lines shown in Figure 2D. The means are shown for three experiments performed on different days and the error bars are the standard deviations of these measurements. Note that the data used to plot the growth impact of PhiF, Betl, AmeRs gates were also used in Appendix Figure S7.

0x08

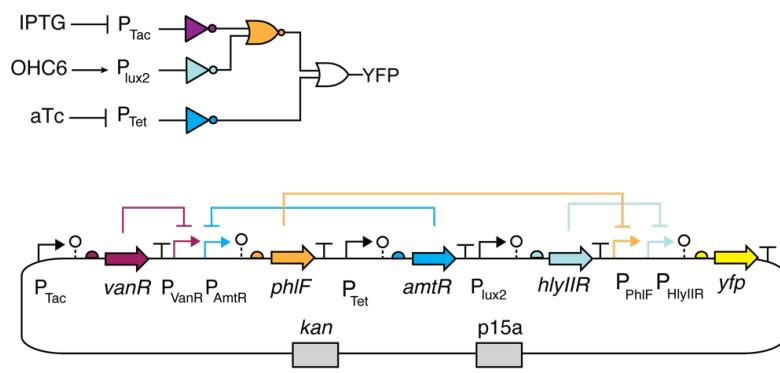


Appendix Figure 9: Example of a circuit designed using “tandem” NOR gates. The gates are based on two promoters in series driving the expression of the repressor (Figure 3F). The wiring diagram, repressor assignment, and genetic construct designed by Cello is shown. The predicted outputs are shown as blue (on) or red (off) distributions and the experimental data as gray distributions (Methods). The concentrations of inducers used are: 200 mM Van, 20 ng/ μ l aTc, and 1mM IPTG. The experiments were repeated three times on different days with similar results.

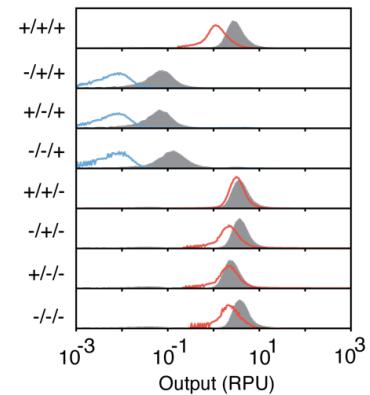


Appendix Figure 10: Replicate measurements are shown for the genetic circuits. This figure corresponds to the cytometry distributions in Figure 4. The concentrations of inducers used are: 1mM IPTG, 20 ng/ μ l aTc, 200 mM Van, and 10 μ M of OHC14. These experiments were performed three times on different days and the means and standard deviations of the measurements are shown.

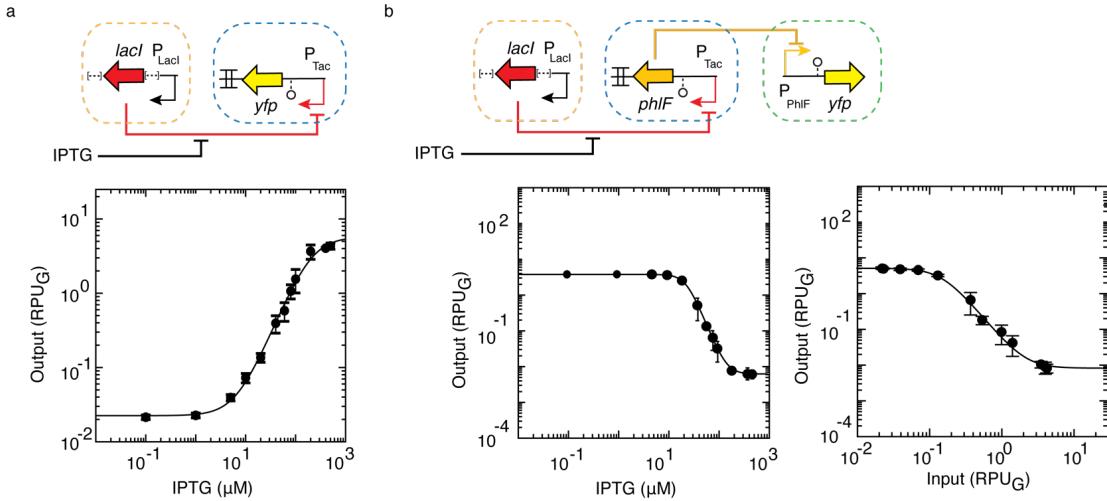
OxF1- Plasmid Encoded



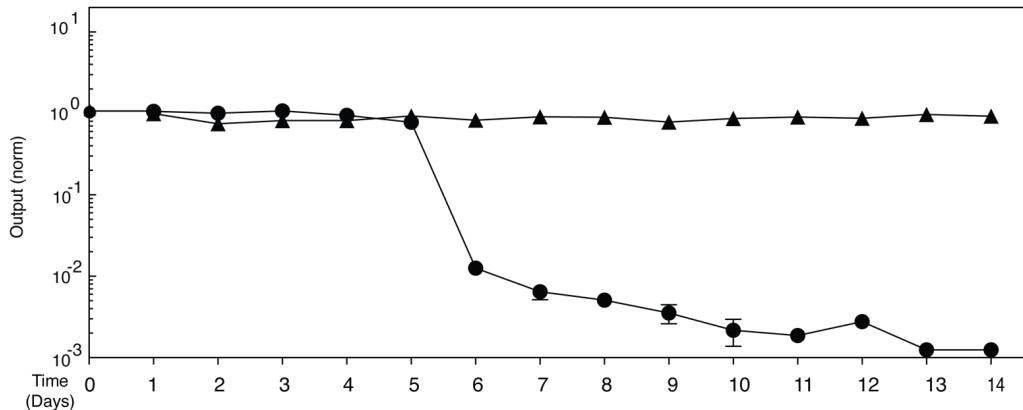
aTc / IPTG / OHC6



Appendix Figure 11: Cello-designed plasmid circuit for the OxF1 logic operation. This logic operation corresponds to the genome-encoded circuit shown in Figure 4 and used for the evolutionary stability experiments in Figure 5. The following inducer concentrations are used: 2 ng/ μ l aTc, 0.2 mM IPTG and 10^{-4} ng/ μ l OHC6. The distributions predicted by Cello are shown in blue (ON states) or red (OFF states) and the grey distributions are the experimental measurements. The experiments were repeated three times on different days with similar results.

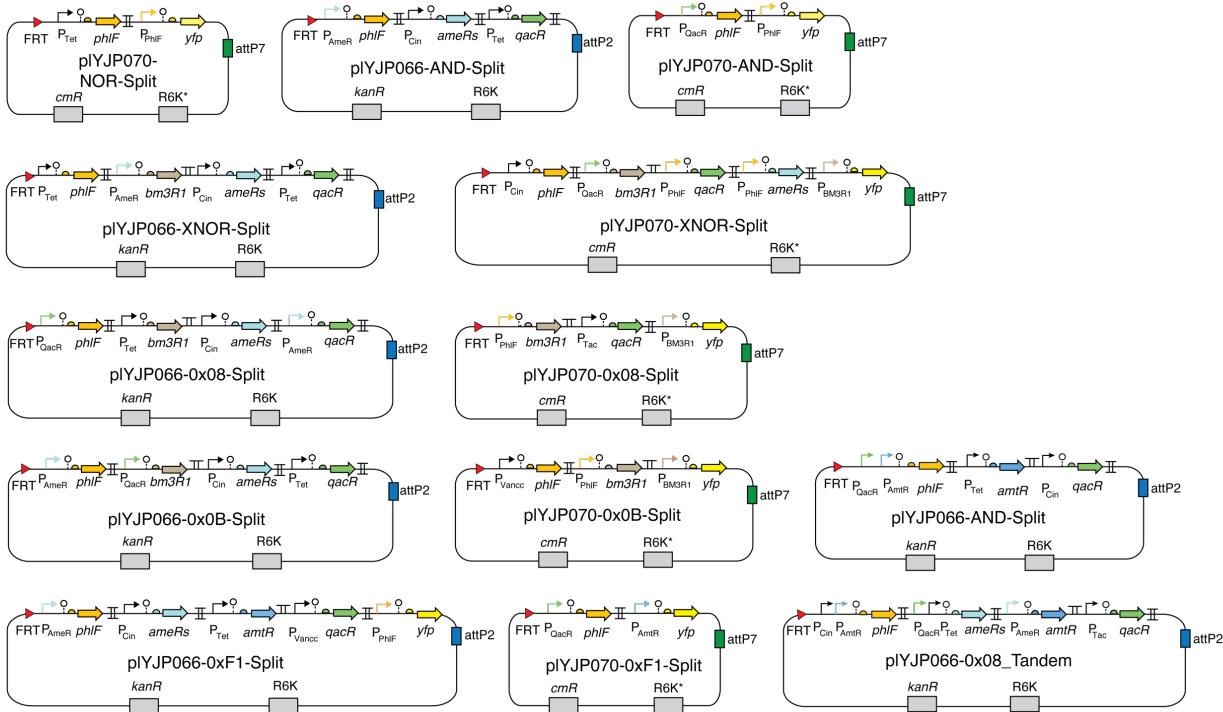


Appendix Figure 12: Conversion of x-axis of response functions from [IPTG] to RPU_G . The constructs at each landing pad are shown: blue (LP #1), green (LP #2) and orange (LP #3). In all subfigures, the concentrations of IPTG used were 0.1, 1, 5, 10, 20, 40, 60, 80, 100, 200, 400, 500 μM . **(a)** The construct and response function of the IPTG sensor were first used to obtain the activity of P_{Tac} . (The [...] indicates the remainder of the sensor array, removed from the schematic for clarity). **(b)** The response function of the PhIF gate was first obtained as a function of IPTG (top). Then, the P_{Tac} activity at each IPTG concentration (part a) is used to convert the x-axis (bottom), which is used to fit to the response function to parameterize the gate. The data represent the means of three experiments performed on different days and the error bars are the standard deviations.

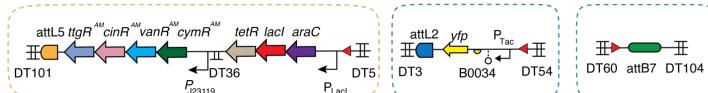


Appendix Figure 13: Performance of genome- and plasmid- encoded circuits when passaged for two weeks. The constructs tested are: plasmid (OxF1, YJP_DHC54, circle) and genome (YJP_MKC267, triangle). YJP_MKC267 was streaked on LB agar (2%) plates with Kan (50 µg/ml) and Cm (35 µg/ml) antibiotics and grown overnight. YJP_DHC54 was streaked on LB agar (2%) plates containing Kan (50 µg/ml) and grown overnight. Three individual colonies were inoculated in the M9 media without antibiotics. The circuits were tested under the presence of + 200 µM vanillic acid (genome) or + 2 ng/µl aTc (plasmid) (the other inducers are not present) in M9 media without antibiotics. Each day, cells were diluted 10,000-fold into 500 µl of fresh M9 media containing the same inducer noted above and were grown for 8 hours at 37 °C. After the incubation at 37 °C and 900 rpm in a Multitron Pro incubator shaker (In Vitro Technologies, VIC, Australia), 30 µl of cells were added to 200 µl 1x PBS with 2 mg/ml Kan for flow cytometry analysis and 100 µl of cells were mixed with 80% autoclaved glycerol (VWR chemical BDH1172-1LP) and stored at -80 °C. Another aliquot was diluted 100-fold into fresh media with the same inducer and incubated overnight. The cycle continued for 14 days by repeating this protocol. The means of three replicates are shown and the error bars are the standard deviations of these measurements. The error bars are often smaller than the data points. The output of the OxF1 circuit was measured and normalized by the output at t = 0 days. Wild type E. coli MG1655 autofluorescence was measured as the output value of 0.005 a.u. (dotted line).

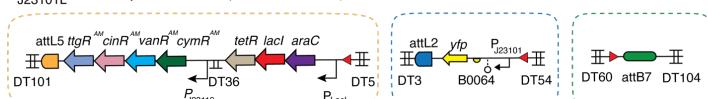




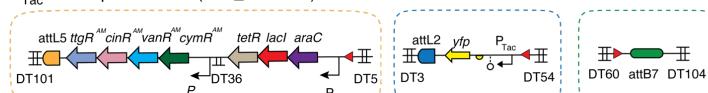
P_{J23101H}-YFP reporter strain (YJP_MKC254)



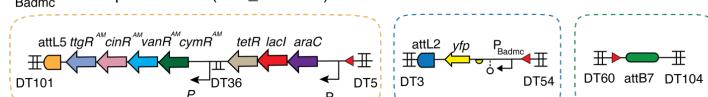
P_{J23101L}-YFP reporter strain (YJP_MKC255)



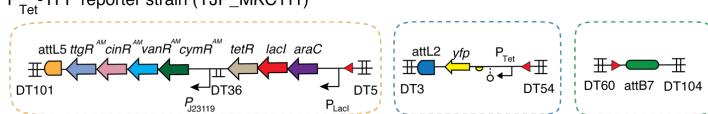
P_{Tac}-YFP reporter strain (YJP_MKC108)



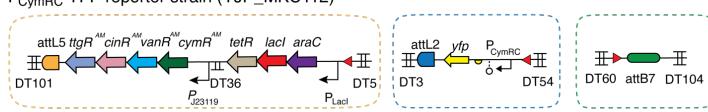
P_{Badmc}-YFP reporter strain (YJP_MKC110)



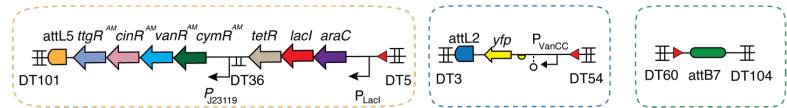
P_{Tet}-YFP reporter strain (YJP_MKC111)



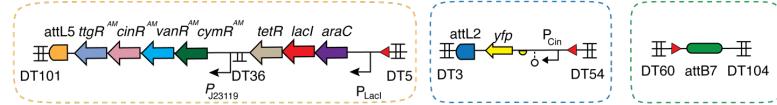
P_{CymRC}-YFP reporter strain (YJP_MKC112)



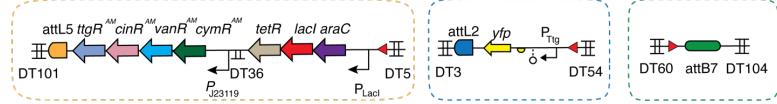
P_{VanCC} -YFP reporter strain (YJP_MKC113)



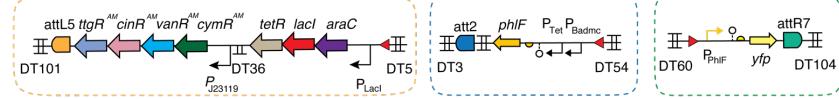
P_{Cin} -YFP reporter strain (YJP_MKC114)



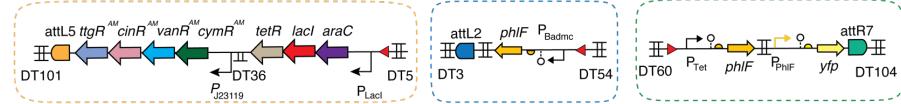
P_{Ttg} -YFP reporter strain (YJP_MKC115)



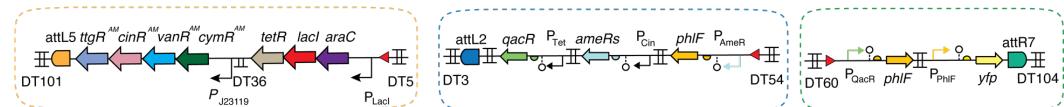
PhIF-NOR gate- Tandem design (YJP_MKC258)



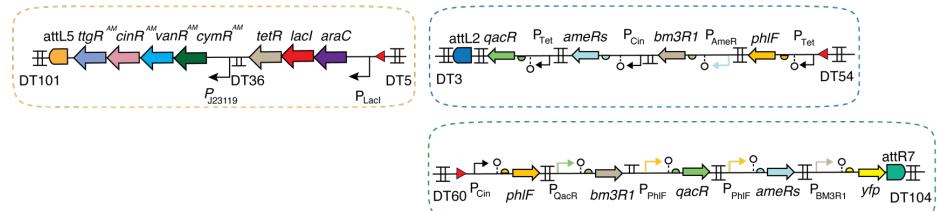
PhIF-NOR gate- Split design (YJP_MKC259)



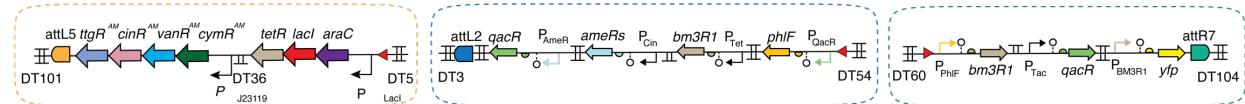
AND circuit- Split- (YJP_MKC262)



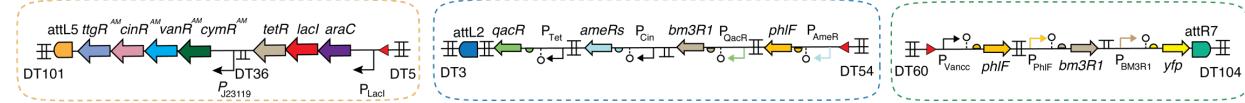
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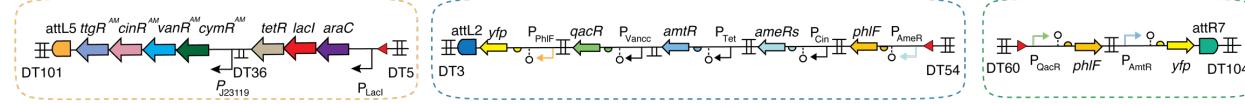
0x08 circuit (YJP_MKC264)

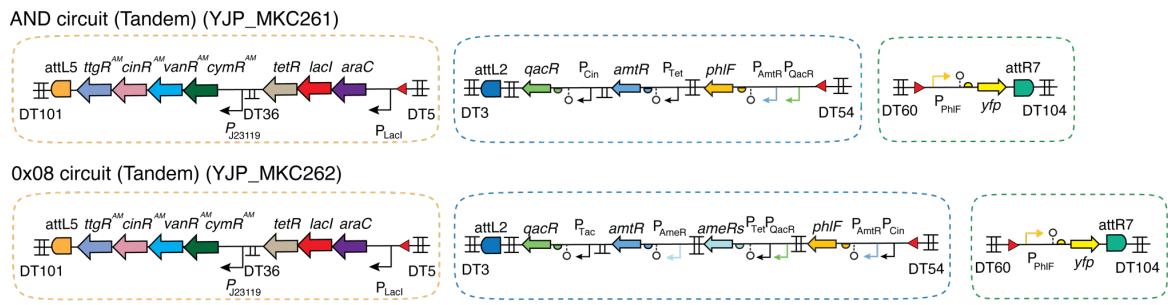


0x0B circuit (YJP_MKC265)



0xF1 circuit (YJP_MKC266)





Appendix Figure 14: Plasmid and genome constructs used in this study. All genetic parts are provided in Appendix Table 5. Plasmid sequences are provided in Appendix Table 6.

Appendix Note 1: Detailed protocol for inserting payloads into genome landing pads

PREPARATION OF LANDING PAD CONSTRUCTS

Construct plasmids containing the payloads to be inserted in landing pads 1, 2, 3.

1. Clone payloads to one of pYJP066-KanR, pYJP070-CmR or pYJP064-TetR plasmids to target landing pad 1, 2, and 3, respectively.
2. Transform the cloned product into *E. coli* EC100D pir+ competent cells and sequence verify the construct
3. Miniprep plasmids (~250 ng/μl).

PREPARATION OF ELECTROCOMPETANT CELLS CONTAINING LANDING PADS

1. Streak cells harboring empty landing pads (*E. coli* MG1655 YJPC173) on a 2% LB-agar plate without antibiotics and incubate overnight at 37 °C.
2. Pick a single colony and inoculate into 2 ml LB no salt media (water + 10 g/L tryptone + 5 g/L yeast extract, autoclaved) without antibiotics and incubate overnight at 37 °C, shaking at 250 rpm.
3. Dilute cells 50-fold 1 ml into 50 ml LB no salt medium in a 250-ml Erlenmeyer flask.
4. Grow 1-2 hours until reaching OD₆₀₀ = 0.55-0.65.
5. Pre-chill 50-ml Falcon tubes, electroporation cuvettes and 10% glycerol solution while cells are growing.
6. Transfer cells to pre-chilled 50-ml Falcon tubes.
7. Centrifuge cells at 4000g and 4°C for 10 minutes.
8. Remove the supernatant.
9. Resuspend cell pellet in 4 ml of ice-chilled 10% glycerol.
10. Centrifuge cells at 4000g and 4°C for 10 minutes.
11. Remove the supernatant.
12. Repeat Steps 9-11 twice more for a total of three glycerol washes.
13. Resuspend cell pellet in 175 μl of ice-chilled 10% glycerol, yielding electrocompetent cells.
14. If making frozen aliquots, which we have found decreases transformation efficiency, aliquot 100 μl cells into pre-chilled 200-μl PCR strip tubes. Each 100 μl aliquot will yield a single transformation.

TRANSFORMATION WITH PAYLOAD DNA

15. Aliquot 100 μl cells into pre-chilled electroporation cuvettes.
16. Add 1500 ng of the integrase plasmid (pYJP053) and 500-1000 ng of the DNA containing the plasmid with payload.
 1. In order to ensure that the electroporation does not arc, it is important to ensure minimal salts. Salts in prepped plasmids can be removed using plasmid dialysis
 2. For plasmid dialysis:
 - a. Fill appropriate number of wells in a 24-well plate with autoclaved Milli-Q purified water (one per transformation).
 - b. Place DNA dialysis disc (Millipore Sigma, USA, VSWP01300) floating on top of water.
 - c. Spot plasmids to be transformed on disc and leave for 30 minutes.
 - d. Carefully retrieve plasmids from discs using pipette without submerging disc.
 3. For higher throughput dialysis method, we have also used Pierce micro-dialysis plates (ThermoFisher Scientific, 88260).
17. Electroporate the mixture (Using the Eppendorf Electroporator 2510, we found that 2500 mA and a time constant after the transformation >4.0 worked best).
18. Immediately after electroporation, add 500 μl of SOC recovery media to the cells.

19. Incubate the cells at 30 °C for 1-2 hours and then spread on a 2% LB agar plate with appropriate antibiotic (Insertion in landing pad #1, #2 and #3 was selected with 50 µg/ml Kan, 35 µg/ml Cm or 5 ng/ml Tet, respectively).
20. Incubate overnight at 37 °C.
21. Pick colony and inoculate in 200 µl LB with appropriate antibiotic for 3 hours. Typically, we pick three colonies for a higher likelihood of identifying a successful integration.

PCR SCREENING FOR SUCCESSFUL INTEGRATION

22. Primers that can amplify the junction between integrated constructs and the adjacent genomic DNA were used to validate the integration of the payload. The following primers are used at concentrations of 10 µM.
 - a. LP #1: oYJP2164 (AATAAACAAATAGGCATGGTCTAAGAAACCATT)
oYJP3436 (CCTGATCAGGTTCCCGCGGATCCCGAATAAACGGTC)
 - b. LP #2: oYJP2164 (AATAAACAAATAGGCATGGTCTAAGAAACCATT)
oYJP3526 (TGCAAAGGCGATAGGTGAAATAATGTCGGCGACAGCGG)
 - c. LP #3: oYJP2166 (CACAAAACGGTTACAAGCATAAAATCTCTG)
oYJP2826(AGAGATGACAGAAAAATTTCATCTGTGACAGAGAAAAAGTAGCCGAAG ATG)

23. Prepare an aliquot of cells for the PCR reaction. Take 30 µl of the cultures from Step 21 and incubate them at 98 °C for 10 minutes.
24. Perform the PCR reaction. Our preference is NEB Hi-Fi Phusion Master Mix (NEB M0531) with the following volumes: 12.5 µl Phusion Master Mix 2X, 8.5 µl water, 1.25 µl, 1.25 µl Primer #1, 1.25 µl Primer #2, 1 µl template (the cell mixture from Step 23) and 0.5 µl DMSO. The thermocycler parameters are: 90s initial denaturation (98 °C), 20s denaturation (98 °C), 30 s anneal (60 °C), 1 min/kb extension (72 °C), repeat denaturation/anneal/extension steps 29 times, 1 kb/min final extension (72 °C).
25. Confirm the amplicon size using gel electrophoresis by adding 5 µl of PCR product into 1% agarose gel. NEB 1kb ladder was run together to confirm the size.

REMOVAL OF ANTIBIOTIC RESISTANCE MARKERS

26. Inoculate the 170 µl culture from Step 21 to 4 ml SOB without antibiotics for 3 hours at 37 °C.
27. Once cells reach OD₆₀₀=0.4, Centrifuge cells at 4700g and 4 °C for 10 minutes.
28. Remove the supernatant.
29. Add 1 ml of ice-chilled 10% glycerol to the cell pellet.
30. Transfer the resuspended cells into a 1.5 ml Eppendorf tube and centrifuge at 21000g and 4 °C for 30 seconds.
31. Remove the supernatant.
32. Repeat steps 30 to 31.
33. Add 20 ng of the FLP encoding plasmid (pE-FLP)
34. Electroporate the mixture using the Eppendorf Electroporator 2510 at 2500 mA with time constant after the transformation >4.0.
35. Immediately after electroporation, add 1 ml of SOC recovery media to the cells.
36. Incubate the cells at 30 °C for 1 hour and then spread on a 2% LB agar plate with 100 µg/ml.
37. Incubate the cells overnight at 30 °C
38. Pick colony and streak the colony on 2% LB agar plate with no antibiotics.
39. Pick 5-6 colonies from the streak after 14 hours of incubation and resuspend each to 30 µl LB without antibiotics.

40. Inoculate the resuspended cells into 5 different media by adding 5 μ l of resuspended cells. The five media compositions are: LB without antibiotics, with Amp 100 μ g/ml, Kan 50 μ g/ml, Cm 35 μ g/ml, Tet 5 ng/ml. Incubate at 37 °C overnight.
41. Find colonies that only grew on LB without antibiotics.
42. Take 30 μ l of the cultures from Step 41 and incubate them at 98 °C for 10 minutes.
43. PCR amplify using following oligos to confirm the removal of antibiotic markers from landing pads.
44. Primers that can amplify the junction between integrated constructs and the adjacent genomic DNA were used to validate the integration of the payload. The following primers are used at concentrations of 10 μ M.
 - a. LP #1: oYJP3436 (CCTGATCAGGTTCCGGATCCGAATAACGGTC)
oYJP3437 (AGGCCTGGAAAGCGCGCTTGTGCTGGAAGATAAG)
 - b. LP #2: oYJP3525 (ACCAATTGGCGCGCTTCGCAATAAAATCTCCCTCG)
oYJP3526 (TGCAAAGGCGATAGGTGAAATAATGTCGGCGACAGCGG)
 - c. LP #3: oYJP2826
(AGAGATGACAGAAAAATTTCATTCTGTGACAGAGAAAAAGTAGCCGAAGATG)
oYJP2827 (CCGCGTAACCTGGCAAAATCGGTTACGGTTGAGTAA)
45. Perform the PCR reaction. Our preference is NEB Hi-Fi Phusion Master Mix (NEB M0531) with the following volumes: 12.5 μ l Phusion Master Mix 2X, 8.5 μ l water, 1.25 μ l, 1.25 μ l Primer #1, 1.25 μ l Primer #2, 1 μ l template (the cell mixture from Step 42) and 0.5 μ l DMSO. The thermocycler parameters are: 90s initial denaturation (98 °C), 20s denaturation (98 °C), 30 s anneal (66 °C), 1 min/kb extension (72 °C), repeat denaturation/anneal/extension steps 29 times, 1 kb/min final extension (72 °C).
46. Confirm the amplicon size using gel electrophoresis by adding 5 μ l of PCR product into 1% agarose gel. NEB 1kb ladder was run together to confirm the size.
47. Once the size is confirmed, add 25% autoclaved glycerol (v/v) to create glycerol stock.
48. Store glycerol stock at -80 °C.

Appendix Table 1: Double terminator sequences and strengths

Name	Sequence	T _S (forward) ^a	T _S (reverse) ^a
DT3	CCGGCTTATCGGTAGTTACCTGATTACGAAAAACCGCCTTCGGGGGGTTTGCTTTGGAGGGGCAGAAAGATGAATGACT GTCCACGACGTATAACCAAAAGAAAAAAAAAAAACCCGCCCTGACAGGGGGGGTTTTTTT	3000	120
DT5	TCGGCAATTAAAAAGGGCTAACACGCCGCTTTTACGTCTGCACTCGTACCAAATTCAGAAAAGGGCTCCGAAAG GGGGCTTTTTGGTCTC	4700	50
DT19	TTACGCCAAAAACTTAAAGCCGGCTTGTGCACTACCTTGCAGTAATGCGGTGACAGGATCGCGGTTTCTCTTCTC AACTCGTACCAAAGACGAAACAATAGCGCTGAAAGCGTCTTTCGTTGGTCC	770	1.2
DT34	GCTGATGCCAGAAAAGGGCTCTGAATTTCAGGCCCTTTTACATGGATTGCTGGTACCAAATTCAGAAAAGAGCGCTTCGA GCGTCTTTCTGTTGGTCC	570	1.4
DT36	GATCTAACTAAAAAGGCCCTGGGCCCTTTCTTCACTGTAAACAACGGAAACGCCATTGCGCCGTTTTGGCCT	680	3.2
DT42	AGTTAACCAAAAGGGGGATTITATCCCTTAAATTTCCTCGCAGATAGCAAAAGGCCCTTGGCGCTTTTACATTG GTG	2500	2.2
DT54	GGAAACACAGAAAAAGCCGCACCTGACAGTGCAGGCTTTTCGACCAAAGGCTGGTACCAAATTCAGAAAAGACACCC GAAAGGTGTTTCTGTTGGTCC	1800	30
DT56	TACCAACGTCAAAAAAACGGCCTTTAGCGCGTTTATTTCAACCTTCCAGGCATCAAATAAACGAAAGGCTCAGTGA AAGACTGGCCTTCGTTTATCTGTGTTGCTGGTAAACGCTCTC	240	11
DT60	ACATTTAATAAAAAGGGCGTCGAAGATGCCCTTTTACGTATGACACAGTGA AAAATGGCGCCATGGCGCCATT ATG	110	29
DT65	TGCTCGTACCAAGGCCCTGCAATTCAACAGGGGCTTTTATCCAATTCCATGGCTCGAATTTCGGACCTTTCTCCGC	400	1.0
DT82	CTTATCCATAACAAAGCGGGTAATCCGGCTTGTGATCTGAACAATAATGGATGCCCTCGTAAGCGGGGCAATT CT	170	2.8
DT83	AGCGTCAAAAGGCCGGATTCCGCCCTTTTATTAGGCAGCATGCTGCCAGGTATCCCCCTGGCACCTCTT	600	4.4
DT86	TAATCTTCTAGCGTAGCCGGGAAGTCGGTCACGCTACCTCTTGAAGAAACAGCAACATCCAAAAGCGCGCTCAGCGC GTTTTCTGTTTCT	210	0.4
DT100	GTGAAGTGA AAAATGGCGCACATTGCGCATT TTTGTCTGCCATTACCGCTCTGAAAATCAACGGCAGGTACTGAC TTGCCCTGTTTTATCCCTTCCACACCG	4700	12
DT101	TCTTTAAAAGAACCTCCGATTGGCGCTTGTGCTGCCTTGTGATACTCTGCTGAAGTAATTCTGCCAGTGAAAATGGGCC CATCGGGCCATTTTATGCTTCATTAGAAAGCAAAAGCGCTGAGAAAGCAGGCTTGTGAAATGGCTCTGAC	2800	160
DT103	AAAGTTCTGAAAAGGGTCACTCGGTGCCCTTTTATGCCACGGTTGAGCAGTGACTGCTTAAATCCGCCAGCGCC GATTTTTATTGTCGGTTAAAGCA	790	4.0
DT104	GCAGACAAAAAAATGGCGACAAATGGCCCATTTCTACTCACAGGTACTATTGTTGAAATTGAAAAGGGCTGGGCC TTTTGCATTGTTGACGGCATATAATTGTTATGCAAGGGCCCTGATGGGGCTTTTTATTAAATCGATAACCGAGA	580	101

a. The means of three replicates performed on different days are shown. The conditions and calculation of T_S are provided in the Methods and Appendix Figure 1.

Appendix Table 2: Transposon library results for *E. coli* DH10 β

Number	Position ^a	Strand	Expression ^{b,c}	Relative OD ₆₀₀ ^{b,d}
1	3986119	-	160	1.5
2	239205	+	140	1.3
3	1703536	+	50	1.1
4	4403940	-	110	0.9
5	2354832	-	90	1.1
6	3821587	+	120	1.3
7 (LP#1)	4294255	-	160	1.1
8	3636005	-	130	1.1
9	216090	+	110	1.2
10	130791	+	80	1.0
11	1974358	-	60	1.2
12	953620	+	80	1.4
13	3942414	+	180	1.1
14	4577260	+	110	1.0
15	4185554	-	130	1.2
16	3635939	+	160	1.0
17	3293003	-	130	1.2
18	970929	-	80	1.2
19	2968336	-	110	1.0
20	3280886	-	60	1.2
21	3839522	-	120	1.1
22	3007855	+	90	1.4
23 (LP#3)	4000978	-	150	1.2
24	4160155	+	140	1.0
25	311535	+	80	1.3
26	4158775	+	130	1.1
27	2612824	-	80	1.1
28	3119521	-	110	1.2
29	268509	+	90	0.9
30	3286750	+	100	1.0
31	4575120	-	110	0.9
32	4648089	+	90	1.4
33	4525992	+	60	1.1
34	3149195	-	70	0.8
35	3851188	-	140	1.1
36	3792231	+	150	1.0
37	2691678	-	100	1.2
38	4078450	-	190	1.0
39	3008076	-	90	0.9
40	3357859	-	90	1.3
41	124347	-	90	0.8
42	4161116	+	100	0.9
43	6673	-	140	0.8
44	1434023	-	60	1.0
45	3268733	+	110	0.9
46	2763676	-	100	1.0
47	391229	+	120	1.5
48 (LP#2)	4614289	+	140	1.3
49	1983293	+	50	0.7
50	4519171	-	80	1.0

a. The number is based on the nucleotide numbering of the *E. coli* DH10 β genome (NCBI CP000948.1)

b. The means of three experiments performed on different days are shown.

c. The expression is measured as mCherry fluorescence (Methods). The construct is shown in Figure 1b

d. The relative OD₆₀₀ is calculated by dividing the OD₆₀₀ by that of wild-type *E. coli* DH10 β (Methods)

Appendix Table 3: ON/OFF levels for genome-encoded sensors

Inducer	Output Promoter	Strain	Y_{\min} (RPUs _G)	Y_{\max} (RPUs _G)
Ara	P _{Badmc}	YJP_MKC110	0.04	3.33
IPTG	P _{Tac}	YJP_MKC108	0.02	4.20
aTc	P _{Tet}	YJP_MKC111	0.02	5.41
Cuma	P _{CymRC}	YJP_MKC112	0.19	2.39
Van	P _{VanCC}	YJP_MKC113	0.02	3.79
OHC14	P _{Cin}	YJP_MKC114	0.01	4.38
Nar	P _{Ttg}	YJP_MKC115	0.01	0.22

Appendix Table 4: Sequence of landing pads used in this study

- a. All landing pad sequences contain the antibiotic resistance marker.
 - b. The nucleotide numbering is based on *E. coli* MG1655 K-12 (NCBI U00096.3)
 - c. The following colors are used for annotations. Blue: Promoters; Purple: Ribozymes; Green: Repressors, YFP and Sensor ORFs; Red: Terminators; Orange: FRT sites and att sites; yellow highlight: RBSs; Gray highlight: antibiotic resistance gene, and origin of rep; Green highlight: Transposase (*Tn5*, *Tn7*) recognition sites

Appendix Table 5: Sequence of genetic parts used in this study

Part Name	Part Class	Part Sequence
P _{Badmc}	Promoter	AACGATCGTTGGCTGTAGCATTATTCATAAGATTAGCGGATCTACCTGACGTTTATCGCAACTCTCTATATTTCTCCATACCG
P _{Tet}	Promoter	AACGATCGTTGGCTGCCTCATCGTGATAGAGATTGACATCCCTACAGTGATAGATAATGAGCAC
P _{Cin}	Promoter	TGGTAGCACAAGCTCCCTTGTGCGTCCAACCGGACGCCGGCTCTAAAGCGGGTGGCATTTTCAGATTGCGCTCTCGCGCTTCAGTCTTGT TTGCGCATGTCGTTACCGAACCGCTGACACTTTGCGGACATGCTGATCCCCCATCTGGGGGGCTATCTGAGGGAAATTCCGATCC GGCTGGCTGAACCATTCGCT
P _{CymRC}	Promoter	TTCCGATGTTAGGAGTAACAAACAGACAACTGGCTGTTGATTATGAAAATTTCGTTAGATAATAGATT
P _{T_{tg}}	Promoter	TACGCTGCCACGTGCAACCAGCAGTATTACAAACAACCATGAAATGAAAGTATTCCTTAGCAA
P _{Tac}	Promoter	AACGATCGTTGGCTGTGTTGACAATTAAATCATCGGCTGTTAATGTTGGAATTGAGCGCTCACATT
P _{VanCC}	Promoter	GAGCCTACCTGCCTATTGGATCCAATTGACAGCTAGCTCAGTCTAGGACATTGGATCCAATT
P _{AmrR}	Promoter	CTTGCCAACCAAATGATTGTTACCCCTTGACAGTTCTATGCACTAGATAATGCTAG
P _{AmeR}	Promoter	TCGTCACTAGAGGGCGTAGTGACAAACTTGACAACCTCATCCTCTAGTAGGCTGCTAGC
P _{PhIF}	Promoter	CGACGTACGGTGGAACTGATTGTTACCAATTGACATGACGAAACGTACCGTATCGTTAGGT
P _{QacR}	Promoter	GGTATGGAAGCTACGTTACCAATTGACAGCTAGCTCAGTCTACTTTAGTATATAGACCGTGGATCGTATA
P _{BetI}	Promoter	AGCGGGGTGAGAGGGATTGTTACCAATTGACAGTTGACGTTACATATAATGCTAG
P _{BM3R1}	Promoter	AACGATGCGCATGTCGTTACCAATTGACGGAACTGCTCGATATGCTAG
P _{HlyIIR}	Promoter	ACCAAGAACTGCGATTGCTTACCAATTGCGATATTAAATTCGTTAAATGCTAGC
P _{LacI}	Promoter	TGGTCCAAAACCTTCGCGGTATGGCATGATAGCGCCGGAAGAGAGTCATTGAG
P _{Lux2}	Promoter	ATAGCTCTTACCGGACCTGTTAGGATGTCAGGTTACCGAACGAAATGGTTATTTCGAATAAA
BBa_J23105	Promoter	GAAGACGGGAGGGAGACCGGCGCCTTACGGCTAGCTCAGTCTAGGACTATGCTAGCAAGGT
BBa_J23104	Promoter	GCTTCAAATGCGGATTGACAGCTAGCTCAGTCTAGGTTAGGTTAGCTAG
BBa_J23119	Promoter	GAATCGCACCAGACAGTTGTCATTGACAGCTAGCTCAGTCTAGGATAATGCTAGC
BBa_J23101	Promoter	GATAAGCTCTAACTTTACAGCTAGCTCAGTCTAGGTTAGGTTAGCTAG
Tn7L	att sites	AACAGATAACTGAAATCTAGTCCAAACTATTTGTCATTTAATTTCTATTAGCTTACGACGCTACACCCAGTTCCATCTATTGTCACTCT TCCCTAAATACTCTAAAAACTCCATTCCACCCCTCCAGTCCAACTATTGTCGCCACA
Tn7R	att sites	TGTGGCCGACAATAAAGCTTAAACTGAAACAAATAGACTCTAAACTATGCAAAATAGCTTAAACTAGACAGAAATGTTGAAACTGAAATCAGTCC AGTTATGCTGTGAAAAGCATACTGGACATTGTTATGGCTAAAGCAAACCTCTGAAGTGCATAATTGCCGCGTGTAAAGGGCGTG
attR2	att sites	GGACGGCCAGAAGGGAGTAGCTCTCGCCGAGAACCTCTGCAAGGACTGCTTGGCT
attR5	att sites	GCACCGACCGCAGCACAGCGTAGGGCTCCAGGCCGTCACCTGATCCGGCCTC
attR7	att sites	GTGTTATAAACCTGTGAGAGTTAAGTTACATGGCAAAGTTGATGACCCGGTGTGCGTT
attL2	att sites	GCTCATGTTGTCAGCGAGATTCTCGCCGGACCGTCACACTGCTCAGTC
attL5	att sites	GCACCGACCGCAGCACAGCGTAGGGCTCCAGGCCGTCACCTGACTGCGTATTAGGG
attL7	att sites	AGACGAGAACGTTCCGCTGGTCAAGTCTACCGAGGACTCTGCGAGGTTAGCT
attB2	att sites	GACGAGCTGAGCAGTATGTCAGCGGTCGGCAGAGACTCTCCCTCTGCGCGTCC
attP2	att sites	GCTCATGTTGTCAGCGAGATTCTCGCCGGAGAACTCTGCAAGGACTGCTCTGGCT
attB5	att sites	GAGCCCGGATCAGGGAGTGGACGGCTGGGAGCGCTACAGCTGTGGCTGGTGC
attP5	att sites	CCCTAAACGCAAGTCGATAACTCTCTGGAGCGTTGACAACCTGCGCACCTGATCTG
attB7	att sites	AGACGAGAACGTTCCGCTGGTCAAGTGGCAAAGTTGATGACCCGGTGTGCGTT
attP7	att sites	GTGTTATAAACCTGTGAGAGTTAAGTTACATGGCTAACTTACGAGGTTAGCT
FRT	FRT	GAAGTCCTATTCCTAGAAAGTATAGAACTTC
A1	RBS	AATGTCCTAATAATCAGCAAAGAGTTACTAG
F2	RBS	ACGCTATGGACTATGTTCAACAGGAGCTAATAG
B3	RBS	CCAAACGAGGCCGGAGG
E0	RBS	CCCCCCGAGGAGTAGCAC
E1	RBS	CCTTCGGAGGAGGAGACA
P1	RBS	CTATGGACTATGTTGAAAGGGAGAAATCTAG
P2	RBS	GGAGCTATGGACTATGTTGAAAGGCTGAAATCTAG
P3	RBS	CTTACGAGGGCGATCT
H1	RBS	ACCCCCGAG
V1	RBS	AAGACCAATTAAAGGTTGAACT
BBa_B0032	RBS	TCACACAGGAAAGTACTAG
BBa_B0064	RBS	TACTAGAGAAAGAGGGAAATCTAG
BBa_B0034	RBS	TACTAGAGAAAGAGGGAAATCTAG
AraJ	Ribozyme	AGTGGCTGATCTGAAACTCGATCACCTGATGAGCTCAAGGCAGAGCGAAACCACCTACAAATAATTGTTAA
SarJ	Ribozyme	AGACTGTCGCCGGATGTTGATCCGACCTGACGATGGCCAAAAGGGCGAACAGTCCTCTACAAATAATTGTTAA
RiboJ00	Ribozyme	AGCTGTCACCGGATGTTGCTTCCGGTCTGATGAGTCGGTGGAGCACAAAGCCTCTACAAATAATTGTTAA

<i>oriT</i>	Conjugation element	CTTTCCGCTGCATAACCCCTGCTCGGGGTCAATTATAGCGATTTTCGGTATATCCATCCTTTCGCACGATAACAGGATTTGCCAAGGGTTCG TGTAGACTTCCTTGGTGTATCCAACGGCTCAGCCGGCAGGATAGGTGAAGTAGGCCACCCCGAGCGGGTGTCCCTTCACTGTCCCTTATTC GCACCTGGCGGTGCTAACGGGAATCCTGCTCT
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Appendix Table 6: Sequence of plasmids used in this study

		TCAAGTGAGAAATACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAGCTTATGCATTCTTCCAGACTTGTCAACAGGCCAG CCATTACGCTCGTCATAAAATCACTCGCATCAACCAAACCTTATTCAATTGTGATTGCCCTGAGCGAGCAGAAATACGCGATCGCTG TTAAAAGGACAATTACAAAAGGAATCGAATGCAACCGCCGAGGAACACTGCCAGCCATCAACAAATTTTCACCTGAATCAGGATAT TCTTCTAAATACCTGGAATGCTGTTTCCCGGGGATCGAGTGGTAGTAACCATGTCATCATCAGGAGTACGGATAAAATGCTTGATGGTC GGAAGAGGCATAAATTCCGTCAAGCTTATGCTGACCATCTCATCTGTAACATCATTGCCAACGCTACCTTGGCATTTTCAGAAAC AACTCTGGCGCATGGGCCTTCCCATAAAATCGATAGATTGTCGCACCTGATTGGCCGACATTATGGCAGGCCATTATACCCATATAAA TCAGCATCCATGTTGGAATTAAATCGGGCTCGAGCAAGACGTTCCCGTTGAATATGGCTCATAAACACCCCTTGATTAATCTGTTATG TAAGCACACAGTTTATTTGTCATGATGATATTTTATCTTGTGCAATGTAACATCAGAGATTGAGACACAAGTGCTTGTG ATAAAATCGAACTTTGCTGAGTTGAAGGATCAGATCACCGCATCTCCGACAACCGCAGACCGTTCCGTGGCAAAGCAAAGCTCAAATC ACCAACTGGTCCACCTACAACAAAGCTCTCATCAACCGTGGCTCCCTCACTTCTGGCTGGATGATGGGGCGATTAGGCCCTGGTATGAG TCAGCAACACCTTCTTCAAGAGGCCAGACCTCAGCGC
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- a. *Annotation colors. Blue: Promoters; Purple: Ribozymes; Green: Repressors, YFP and Sensor ORFs; Red: Terminators; Orange: FRT sites and att sites; yellow highlight: RBSs; Gray highlight: antibiotic resistance gene and origin of rep; Green highlight: Transposase (Tn5, Tn7) recognition sites*

Appendix Table 7: Sequence of genome integrated constructs used In this study

- a. Annotation colors. Blue: Promoters; Purple: Ribozymes; Green: Repressors, YFP and Sensor ORFs; Red: Terminators; Orange: FRT sites and att sites; yellow highlight: RBSS; Gray highlight: antibiotic resistance gene and origin of rep; Green highlight: Transposase (*Tn5*, *Tn7*) recognition sites

Appendix Table 8: Strains used in this study

Strain Name	Plasmid Integrated	Related figure	Marker removal
YJP_DHC404	pIYJP019	Fig 1b	N
YJP_MKC172	Landing pad DNA, pIYJP072	Fig 1c	N
YJP_MKC173	Landing pad DNA, pIYJP072	Fig 1c, Fig 5	Y
YJP_MKC174	pIYJP064-Sensor	Fig 2c, Appendix Fig 5	N
YJP_MKC108	pIYJP064-Sensor, pIYJP067-PTac	Fig 2c, Appendix Fig 5	N
YJP_MKC110	pIYJP064-Sensor, pIYJP067-PBadmc	Fig 2c, Appendix Fig 5	N
YJP_MKC111	pIYJP064-Sensor, pIYJP067-PTet	Fig 2c, Appendix Fig 5	N
YJP_MKC112	pIYJP064-Sensor, pIYJP067-PCymRC	Fig 2c, Appendix Fig 5	N
YJP_MKC113	pIYJP064-Sensor, pIYJP067-PVanCC	Fig 2c, Appendix Fig 5	N
YJP_MKC114	pIYJP064-Sensor, pIYJP067-PCinR	Fig 2c, Appendix Fig 5	N
YJP_MKC115	pIYJP064-Sensor, pIYJP067-PTtg	Fig 2c, Appendix Fig 5	N
YJP_MKC254	pIYJP067-PJ23101H	Fig 2a, 2b	Y
YJP_MKC255	pIYJP067-PJ23101L	Appendix Fig 4	Y
YJP_MKC140	pIYJP064-Sensor, pIYJP066-P3-PhlF, pIYJP070-PPhlF	Appendix Fig 7	N
YJP_MKC141	pIYJP064-Sensor, pIYJP066-Q1-QacR, pIYJP070-PQacR	Fig 2d	N
YJP_MKC142	pIYJP064-Sensor, pIYJP066-A1-AmtR, pIYJP070-PAmtR	Fig 2d	N
YJP_MKC146	pIYJP064-Sensor, pIYJP066-F1-AmeR, pIYJP070-PAmeR	Appendix Fig 7	N
YJP_MKC149	pIYJP064-Sensor, pIYJP066-B3-BM3R1, pIYJP070-PBM3R1	Fig 2d	N
YJP_MKC154	pIYJP064-Sensor, pIYJP066-F1-AmeRs, pIYJP070-PAmeR	Fig 2d	N
YJP_MKC155	pIYJP064-Sensor, pIYJP066-E0-Betl, pIYJP070-PBetl	Appendix Fig 7	N
YJP_MKC156	pIYJP064-Sensor, pIYJP066-P1-PhlF, pIYJP070-PPhlF	Fig 2d	N
YJP_MKC158	pIYJP064-Sensor, pIYJP066-E1-Betl, pIYJP070-PBetl	Fig 2d	N
YJP_MKC258	pIYJP064-Sensor, pIYJP066-NOR-Tandem, pIYJP070-PPhlF	Fig 3a, 3c	N
YJP_MKC259	pIYJP064-Sensor, pIYJP066-NOR-Split, pIYJP070-NOR-Split	Fig 3b, 3c	N
YJP_MKC260	pIYJP064-Sensor, pIYJP066-AND-Tandem, pIYJP070-PPhlF	Fig 3d, 3f	Y
YJP_MKC261	pIYJP064-Sensor, pIYJP066-0x08-Tandem, pIYJP070-PPhlF	Fig 3f, Appendix Fig 9	Y
YJP_MKC262	pIYJP064-Sensor, pIYJP066-AND-Split, pIYJP070-AND-Split	Fig 3e, 4	Y
YJP_MKC263	pIYJP064-Sensor, pIYJP066-XNOR-Split, pIYJP070-XNOR-Split	Fig 4	Y
YJP_MKC264	pIYJP064-Sensor, pIYJP066-0x08-Split, pIYJP070-0x08-Split	Fig 4	Y
YJP_MKC265	pIYJP064-Sensor, pIYJP066-0x0B-Split, pIYJP070-0x0B-Split	Fig 4	Y
YJP_MKC266	pIYJP064-Sensor, pIYJP066-0xF1-Split, pIYJP070-0xF1-Split	Fig 4	Y
YJP_MKC267	pIYJP064-Sensor, pIYJP066-0xF1-Split, pIYJP070-0xF1-Split	Fig 5	N

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