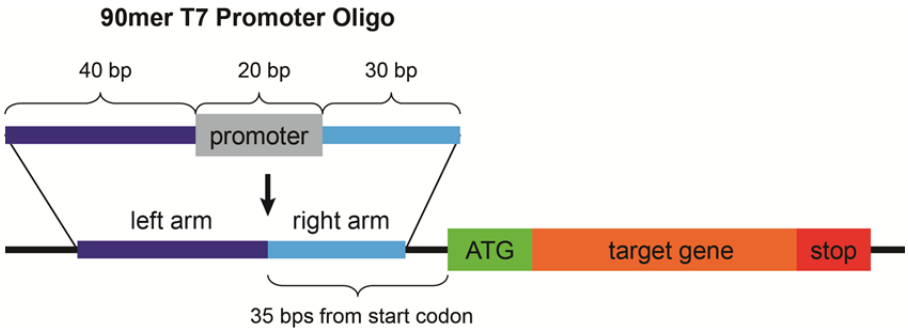
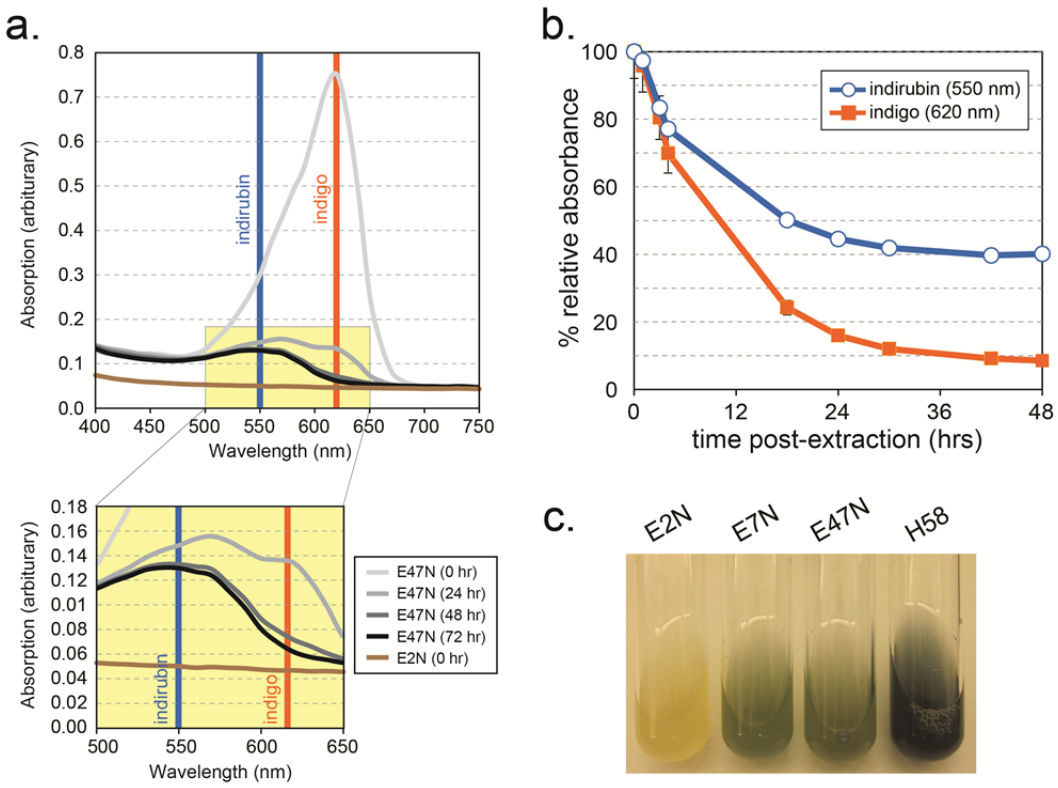


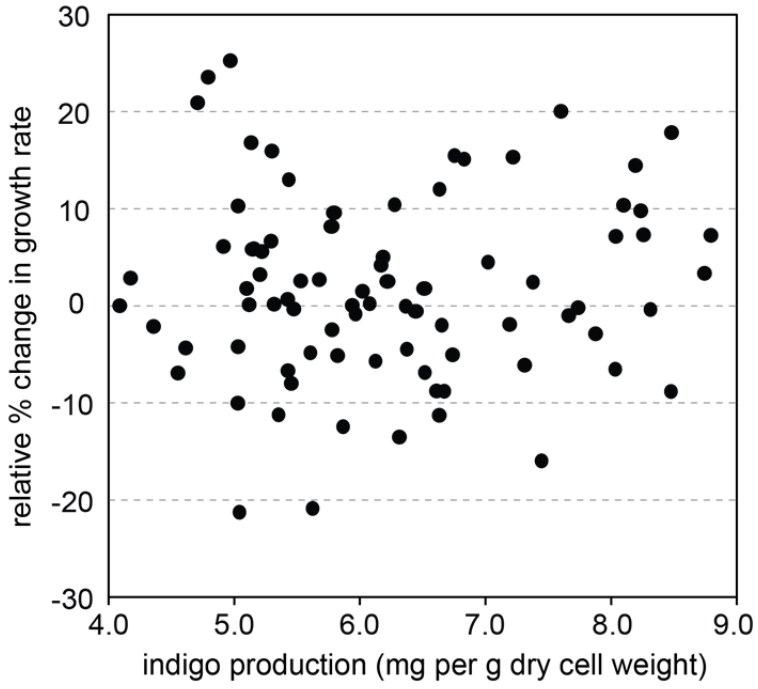
Supplementary Fig. 1

Insertion design used to introduce T7 promoters upstream of genes/operons. The T7 promoter was inserted 35 bp upstream of the open reading frame to avoid disrupting the ribosomal binding site.



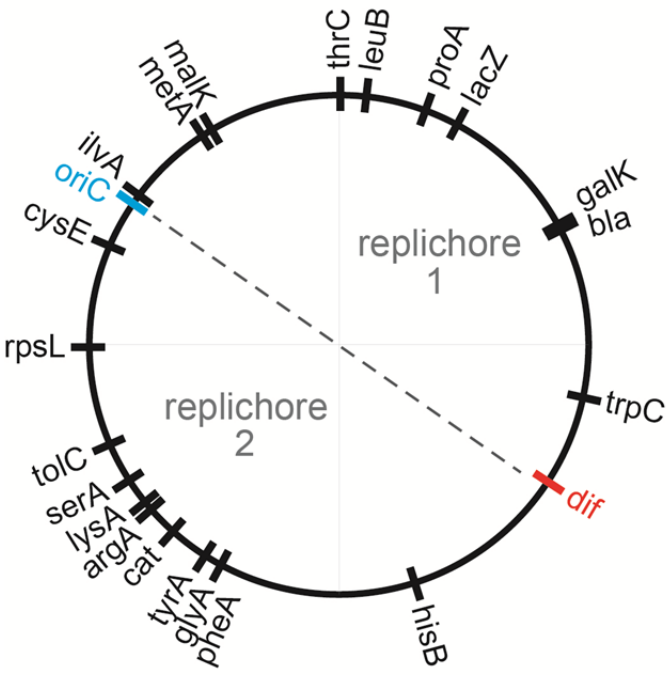
Supplementary Fig. 2

a, Absorbance spectrum of extracted pigment from E47N measured at 400 to 750 nm in DMSO after incubation at room temperature for 0, 24, 48 and 72 hours (gray to black lines). The insert highlights the relevant wavelengths 550 nm and 620 nm corresponding to max absorbance peaks for indirubin and indigo respectively. Indirubin levels stabilized after 48 hrs. **b,** Calculation of relative absorbance change over time for 550 nm peak (indirubin) and 620 nm peak (indigo). Absorbance at 620 nm dropped substantially after 2 days likely due to indigo instability. Absorbance at 550 nm stabilized to ~40% of initial levels, likely after interference of from indigo at 620 nm has disappeared. Indirubin levels (Ab_{550}) was thus used to quantify pigment production. **c,** Picture of 3 mL cultures of E2N ancestral control, E7N and E47N controls and high pigment producer clone H58 grown in LB-min media for 24 hrs. The blue pigmented cultures were the result of indigo and indirubin production.



Supplementary Fig. 3

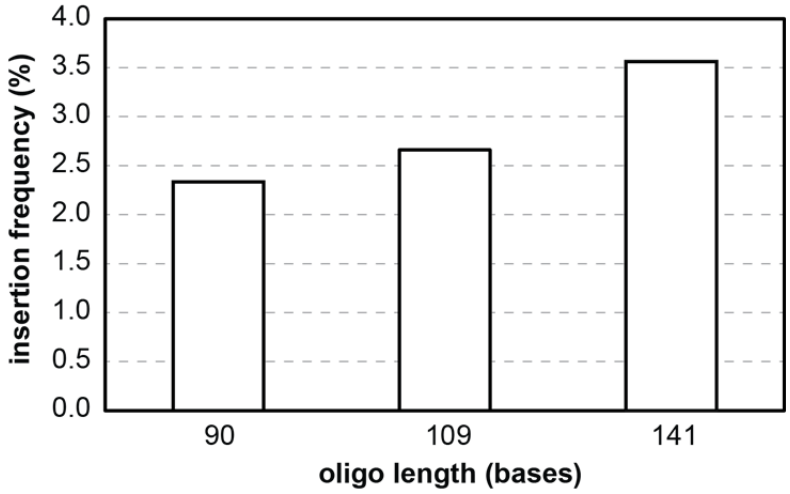
Indigo production level and relative % change in growth rate does not appear to have any notable correlation for strains H1-H80, E2N, E7N, and E47N.



REPLIC	MARKER	LOCATION
1	thrC	3783
1	leuB	81795
1	proA	260812
1	lacZ	365478
1	galK	788768
1	ΔbioA::bla	808383
	dif	1588788
1	trpC	1317764
2	hisB	2091541
2	pheA	2735846
2	glyA	2683484
2	tyrA	2738026
2	ΔmutS::cat	2855406
2	argA	2947499
2	lysA	2976834
2	serA	3056383
2	tolC	3176216
2	rpsL	3472446
2	cysE	3780470
	oriC	3923883
1	ilvA	3953577
1	metA	4212352
1	malK	4245059

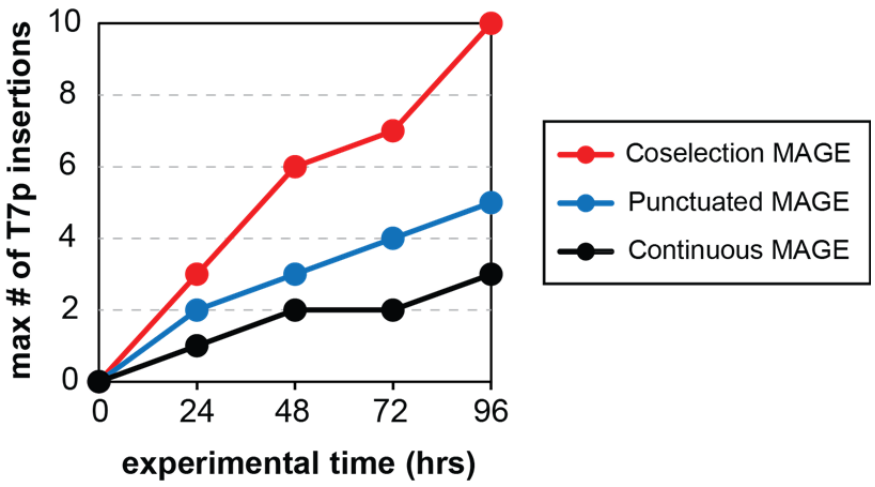
Supplementary Fig. 4

Genomic map and list of 21 available Co-Selection markers that can be used to enhance MAGE efficiency. The genomic coordinate of each CoS marker is given. CoS markers most efficiently enhance nearby target sites within ~500 kb on the same replichore due to the nature of the bidirectional θ-replication of the *E. coli* genome. CoS oligos that inactivate or restore each marker are provided in Supplementary Table 2.



Supplementary Fig. 5

Characterization of the insertion efficiency of a 19 bp sequence (ATGATGATGATGATGATGA) versus length (and homology arm) of the oligo. Insertion of the 19 bp sequence inactivated the *lacZ* gene by generating a premature stop codon. The 90-base oligo (GATTCACTGGCCGTCGTTTTACAACGTCGTGACTGATGATGATGATGATGATGAGGAAAAC CCTGGCGTTACCCAACTTAATCGCCTTGC) has 35-36 bases of homology to the target sequence on each arm. The 109-base oligo (CATGATTACGGATTCACTGGCCGTCGTTTTACAACGTCGTGACTGATGATGATGATGATGA TGAGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCC) has 45 bases of homology to the target sequence on each arm. The 141-base oligo (AGGAAACAGCTATGACCATGATTACGGATTCACTGGCCGTCGTTTTACAACGTCGTGACT GATGATGATGATGATGATGAGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCAC ATCCCCCTTTCGCCAGCTGG) has 61 bases of homology to the target sequence on each arm. Oligos were obtained from Integrated DNA Technology's Ultramer Custom Oligo service. Increased insertion efficiency was observed with increased homology in the absence of inhibitory oligo secondary structures. All oligos had minimal secondary structures (ΔG of -7.7 to -11.4 kcal/mol).



Supplementary Fig. 6

Comparison of the length of time in experimental hours needed for different MAGE strategies to generate clones with 20bp T7 promoter insertions. Continuous MAGE is the standard MAGE process ~10 cycles/day with no clone isolation until the end of the cycling process. Punctuated MAGE is the MAGE process with ~4 cycles/day with plating, clone isolation, and enrichment for the most mutated clone every 24 hrs. Coselection MAGE is our enhanced MAGE process using CoS markers to generate clones with up to 3 insertions per day.

Supplementary Table 1

Summary of Stages of CoS-MAGE to generate the H77 strain.

Stage	Best Strains	CoS marker	Genes with T7 insertions	Distance from CoS marker (bp)
0	E47N			
1	H46	cat	aroH	1068923
			arof	117280
			aroC	410972
2	H63	galK	ppsA	1003911
			ydiB	992966
			aroL	382425
3	H65	malK	aroE	816765
4	H77	bla	aroG	32881
			aroA	149505
			trpE	510878

*Distance values are calculated from start codon of CoS marker genes to start codon of target genes.

Supplementary Table 3

Side-by-side comparison of various genome engineering strategies to make a strain that contains 20bp T7 insertion mutation across 10 genomic loci.

	dsDNA Recombineering	continuous MAGE	punctuated MAGE	coselection MAGE
Multiplexibility	no	yes	yes	yes
Insertion efficiency	n/a	0.02	0.02	>0.2 near CoS
# of loci	10	10	10	10
Per locus insertion eff	n/a	0.002	0.002	>0.02 near CoS
# of cycles needed	n/a	610	40	16
Days of experiments	20	61	10	4
Max # of mutations made per day	0.5	0.1	1.0	2.5

Supplementary Note

Co-Selection Mechanism and Enhancement Analysis

Here, we speculate on some mechanistic rationale for the observed improvements in insertion efficiency by CoS MAGE. Our findings that nearby sites are found to be incorporated at higher frequency in these enriched cells suggest a strong temporal component of this process, which can be explained by the replicative process. We believe that these consecutive oligo integration events are in fact happening along the same lagging strand of the replicating DNA. Additionally, this mechanism seems to fall in line with the observation that CoS markers do not confer much benefit to other far-off sites or cross-replichore sites, thus further pointing to a local replication fork accessibility mechanism. For any loci, it is accessible to lagging strand oligo integration at max once every 25 min with each 1-3 kb stretch of DNA existing in the open state for only ~1 second every cell doubling (assuming 25 min doublings). The CoS marker may perhaps be viewed as artificially synchronizing DNA replication in the cell population by selecting for replication forks that are open and have undergone an oligo-mediated integration event at a given site.

The crucial feature of CoS-MAGE is the increased capability to enrich for cells with higher number of mutations and longer base insertions, thus extending the multiplexing capabilities of MAGE. With CoS-MAGE, ~5% of cells had 3 insertions. With standard MAGE, no 3-insert cells were found when the same numbers of colonies were screened. Standard MAGE generates mutations based on a binomial distribution. Assuming that the efficiency of each insertion is 2% for a set of 12 targets, then the frequency of generating 1 or more inserts is 21.5% within the population as calculated by $\sum_{n=1}^{12}\binom{12}{n}(0.02)^{n}(1-0.02)^{12-n}$. Isolation of a 1-mutation clone at a 95% likelihood would need to satisfy the condition $(1\text{-}0.215)^{x} < 0.05$, which gives an *x* of 12; meaning that 12 clones need to be screened to find one that has at least 1 mutation at 95% confidence. To find a clone with 3 or more mutations in this population

(frequency of 0.2%), one would need to screen 1948 colonies. Thus, there is a 2 order of magnitude difference in the screening requirements for finding a 1 vs 3 mutation clone. Given our CoS-MAGE experimental results of ~5% abundance of cells with 3-inserts or more, we can back calculate to find that our individual insertion efficiency would be 8% (vs 2% with standard MAGE). Thus with co-selection, the effect on multiplexing is exponential such that we are able to reduce the screening requirement for finding highly modified cells by substantial enrichment (>2 log). Further comparisons of CoS MAGE with other modalities of MAGE and dsDNA recombineering techniques are outlined in **Supplementary Table 3**.