Table S1. Synthesized Fragments of Different Lengths of Homologous Arms for Upstream and Downstream of the *LacZ* Gene Without Selection Markers*

Homology arm length	**Sequence (5' -3')**
10bp	GGAAACAGCTTAATAACCGG
20bp	ATTTCACACAGGAAACAGCTTAATAACCGGGCAGGCCATG
30bp	GCGGATAACAATTTCACACAGGAAACAGCTTAATAACCGGGCAGGCCATGTCTG CCCGTA
40bp	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTTAATAACCGGGCAG GCCATGTCTGCCCGTATTTCGCGTAA
50bp	TATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTTAATA ACCGGGCAGGCCATGTCTGCCCGTATTTCGCGTAAGGAAATCCAT
60bp	TTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAA ACAGCTTAATAACCGGGCAGGCCATGTCTGCCCGTATTTCGCGTAAGGAAATCC ATTATGTACTAT
70bp	CACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTC ACACAGGAAACAGCTTAATAACCGGGCAGGCCATGTCTGCCCGTATTTCGCGTA AGGAAATCCATTATGTACTATTTAAAAAACA
80bp	CCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGA TAACAATTTCACACAGGAAACAGCTTAATAACCGGGCAGGCCATGTCTGCCCGT ATTTCGCGTAAGGAAATCCATTATGTACTATTTAAAAAACACAAACTTTTG
90bp	ATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATT GTGAGCGGATAACAATTTCACACAGGAAACAGCTTAATAACCGGGCAGGCCATG TCTGCCCGTATTTCGCGTAAGGAAATCCATTATGTACTATTTAAAAAACACAAAC TTTTGGATGTTCGGT
100bp	TAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTT GTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTTAATAACCGG GCAGGCCATGTCTGCCCGTATTTCGCGTAAGGAAATCCATTATGTACTATTTAAAA AACACAAACTTTTGGATGTTCGGTTTATTCTTTT

*The green and blue regions represent the upstream and downstream regions, respectively.

CGGGATCCTTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTCAATCCGCCGTTTGTTC
CCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAA
AAGTGGCACCGAGTCGGTGCttttttccgaaaaaaaaaccccgcccctgacagggcggggttttttttaattaacagaaaatt
attttaaatttcctcTTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTCGCCTGGCAGCAGTGG
CGTCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAA
AAAGTGGCACCGAGTCGGTGCTTTTTTTGAATTCCG

Figure S1. Synthesized Fragment for Constructing the pTargetF-*lacZ*-2 Plasmid

The fragment is 364 bp in length, with underlined regions indicating the BamHI and EcoRI restriction enzyme cutting sites. The yellow region represents the N_{20} region, while lowercase letters denote the connecting regions between sgRNAs.

CGGGATCCTTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTGGCCAGATTGAGAAAC
AATTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAA
AAAGTGGCACCGAGTCGGTGCttttttccgaaaaaaaaaccccgcccctgacagggcggggttttttttaattaacagaaa
attattttaaatttcctcTTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTCGACATCCGTCGTAT
CGGCGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGA
AAAAGTGGCACCGAGTCGGTGCttttttccgaaaaaaaaaccccgcccctgacagggcggggttttttttaattaacagaa
aattattttaaatttcctcTTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTCCCACGCCGATGAT
CCTCTGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTG
AAAAAGTGGCACCGAGTCGGTGCttttttccgaaaaaaaaaccccgcccctgacagggcggggttttttttaattaacag
aaaattattttaaatttcctcTTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTTACTGAGCGGCGC
AGTGGTCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTT
GAAAAAGTGGCACCGAGTCGGTGCttttttccgaaaaaaaaaccccgcccctgacagggcggggttttttttaattaac
agaaaattattttaaatttcctcTTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTGAAGTACGCTA
CATGCCAAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
TTGAAAAAGTGGCACCGAGTCGGTGCttttttccgaaaaaaaaaccccgcccctgacagggcggggttttttttaatta
acagaaaattattttaaatttcctcTTGACAGCTAGCTCAGTCCTAGGTATAATACTAGTCAGCCTCAGC
AGCCGCAGGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCA
ACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTGAATTCCG

Figure S2. Synthesized Fragment for Constructing the pTargetF-3 Plasmid

The fragment is 1204 bp in length, with underlined regions indicating the BamHI and EcoRI restriction enzyme cutting sites. The green region represents the N_{20} region, while lowercase letters denote the connecting regions between sgRNAs.

AATTCTCATGTTTGACAGCTTATCATCGATAAGCTTTAATGCGGTAGTTTATCACAGTTA
AATTGCTAACGCAGTCAGGCACCGTGTATGAAATCTAACAATGCGCTCATCGTCATCCT
CGGCACCGTCACCCTGGATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCCGGGCC
TCTTGCGGGATATCGTCCATTCCGACAGCATCGCCAGTCACTATGGCGTGCTGCTAGCG
CTATATGCGTTGATGCAATTTCTATGCGCACCCGTTCTCGGAGCACTGTCCGACCGCTTT
GGCCGCCGCCCAGTCCTGCTCGCTTCGCTACTTGGAGCCACTATCGACTACGCGATCAT
GGCGACCACACCCGTCCTGTGGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACC
GGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATGGGGAAGATC
GGGCTCGCCACTTCGGGCTCATGAGCGCTTGTTTCGGCGTGGGTATGGTGGCAGGCCC
CGTGGCCGGGGGACTGTTGGGCGCCATCTCCTTGCATGCACCATTCCTTGCGGCGGCG
GTGCTCAACGGCCTCAACCTACTACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGG
GAGAGCGTCGACCGATGCCCTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGGTGGGC
GCGGGGCATGACTATCGTCGCCGCACTTATGACTGTCTTCTTTATCATGCAACTCGTAG
GACAGGTGCCGGCAGCGCTCTGGGTCATTTTCGGCGAGGACCGCTTTCGCTGGAGCGC
GACGATGATCGGCCTGTCGCTTGCGGTATTCGGAATCTTGCACGCCCTCGCTCAAGCCT
TCGTCACTGGTCCCGCCACCAAACGTTTCGGCGAGAAGCAGGCCATTATCGCCGGCAT
GGCGGCCGACGCGCTGGGCTACGTCTTGCTGGCGTTCGCGACGCGAGGCTGGATGGC
CTTCCCCATTATGATTCTTCTCGCTTCCGGCGGCATCGGGATGCCCGCGTTGCAGGCCAT
GCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAGCTTCAAGGATCGCTCGCGGCT
CTTACCAGCCTAACTTCGATCATTGGACCGCTGATCGTCACGGCGATTTATGCCGCCTC
GGCGAGCACATGGAACGGGTTGGCATGGATTGTAGGCGCCGCCCTATACCTTGTCTGC
CTCCCCGCGTTGCGTCGCGGTGCATGGAGCCGGGCCACCTCGACCTGAATGGAAGCCG
GCGGCACCTCGCTAACGGATTCACCACTCCAAGAATTGGAGCCAATCAATTCTTGCGG
AGAACTGTGAATGCGCAAACCAACCCTTGGCAGAACATATCCATCGCGTCCGCCATCT
CCAGCAGCCGCACGCGGCGCATCTCGATATAGATA

Figure S3. Expression Cassette for the Tetracycline Resistance Gene

Each upstream and downstream homologous arm for the *RecA* gene is 100 bp.

CAATTTCTACAAAACACTTGATACTGTATGAGCATACAGTATAATTGCTTCAACAGAAC
ATATTGACTATCCGGTATTACCCGGCATGACAGGAGTAAAATCGTCTTGTTTGATACACA
AGGGTCGCATCTGCGGCCCTTTTGCTTTTTTAAGTTGTAAGGATATGCCATGACAGAAT
CAACATCCCGTCGCCCGGCATA

Each upstream and downstream homologous arm for the *RecBCD* gene is 100 bp.

AAGAACATCCGCAACAAGGGATTTACACAACCCGACCCAACGCCGGGTTGATTGCCCT
GATGGATGAGATGTTTGCCGGTATGACCCTGGAGGAGGCGTAACGTAATTGCCGGATG
CGACGTACGAGTGTTACGCATGTCGCATCCGACGATTTTCATCGCTTACCCTAAATCCG
CCATCAACACTTTGGATTTACGCTG

Each upstream and downstream homologous arm for the *SSB* gene is 100 bp.

TTCCCGGAACCGAGGTCACAACATAGTAAAAGCGCTATTGGTAATGGTACAATCGCGC
GTTTACACTTATTCAGAACGATTTTTTTCAGGAGACACGAACTTTGTCATTAAAACAAT
AGGTTATATTGTTTTAAGGTGGATGATTAAAGCATCTGCCAGCCATAAAAAAGAAGCCT
CCGTTATGGAGGCTTCTACGTATC

Figure S4. Donor DNA Fragments for Three Genes Without Selection Markers

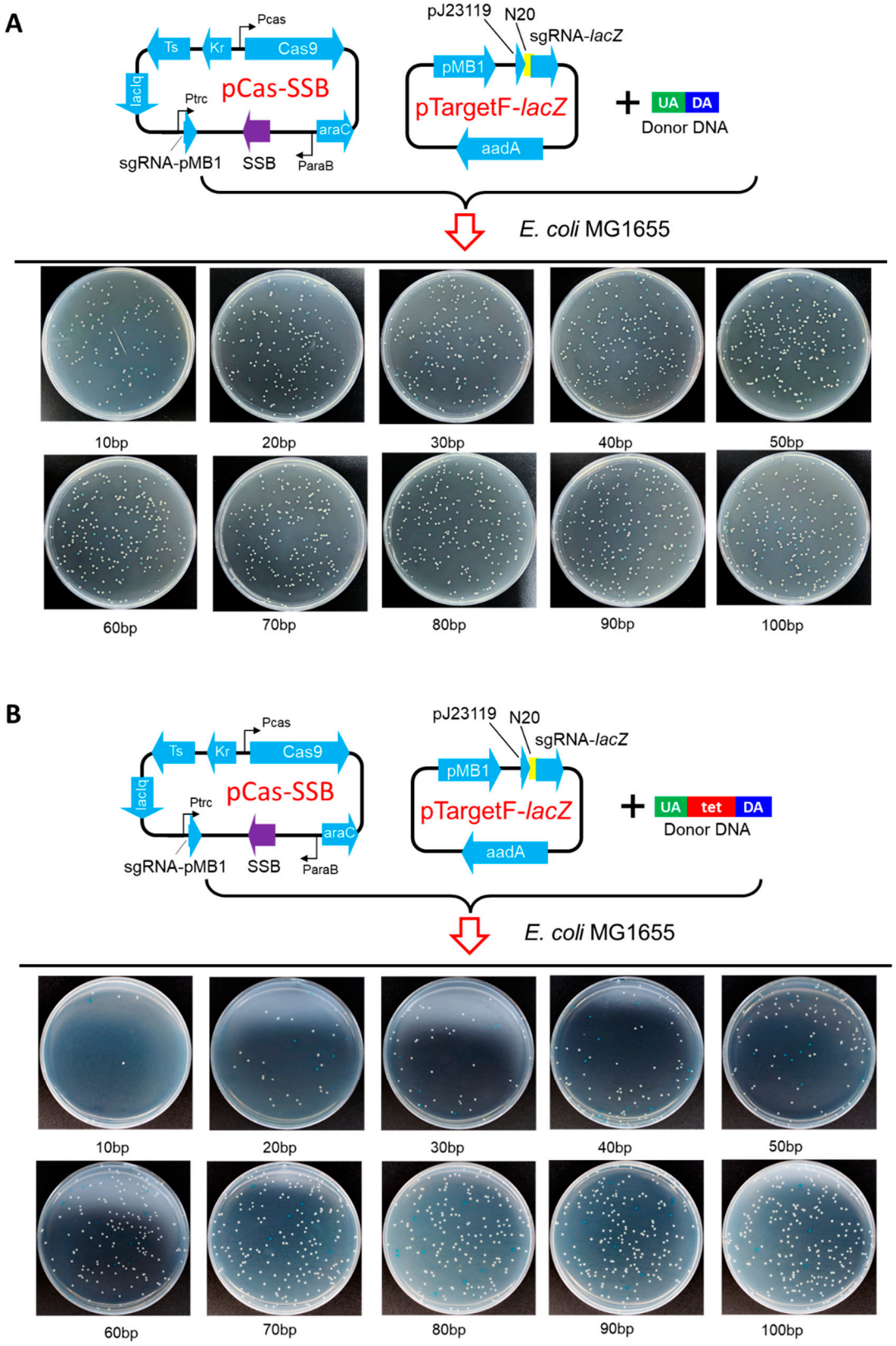


Figure S5. Transformation of MG1655 with Homologous Arms of Different Lengths, with and without Selection Markers, Mediated by SSB/CRISPR-Cas9

(A) Transformation results of homologous arm fragments of different lengths without selection markers under SSB/CRISPR-Cas9 mediation. (B) Transformation results of homologous arm

fragments of different lengths with selection markers under SSB/CRISPR-Cas9 mediation. The pCas-SSB plasmid contains the Streptococcus pyogenes Cas9 protein gene, driven by its native promoter P_{cas}. The *Escherichia coli* SSB protein gene possesses an arabinose-inducible promoter P_{araB} and the arabinose-inducible transcription factor *araC*. The sgRNA-PMB1 is under the control of an IPTG-inducible promoter P_{trc} and guides the pMB1 replication, elimination of pTargetF-*lacZ*, lac repressor (lacIq), temperature-sensitive replication origin repA101 (Ts), and kanamycin resistance gene (Kr). Plasmid pTargetF-3 harbors two sgRNAs each targeting *lacZ* gene.

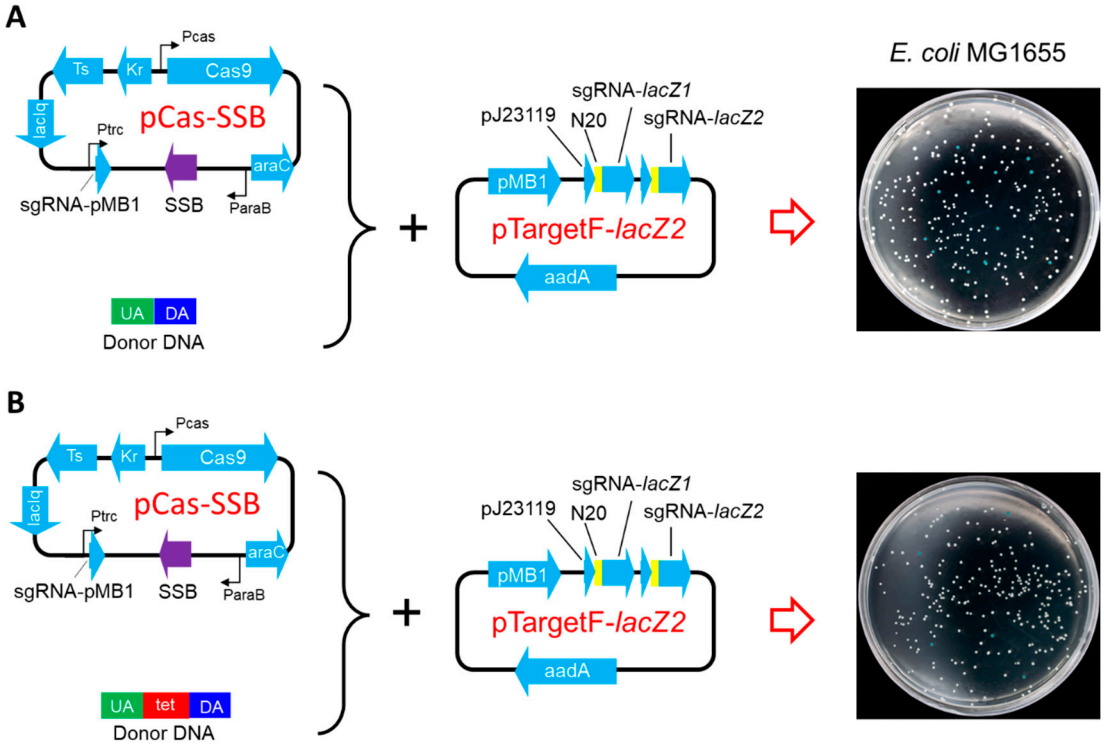


Figure S6. Dual Target Site Editing of Target Genes with Two Different Homologous Fragments under SSB/CRISPR-Cas9 Mediation

(A) Dual editing of the *lacZ* gene using homologous fragments without selection markers. (B) Dual editing of the *lacZ* gene using homologous fragments with selection markers. Plasmid pTargetF-*lacZ*-2 harbors two sgRNAs each targeting *lacZ* gene, directing two cleavage sites for *lacZ* gene.