# CasHRA (Cas9-facilitated Homologous Recombination Assembly) to construct megabase-sized DNA

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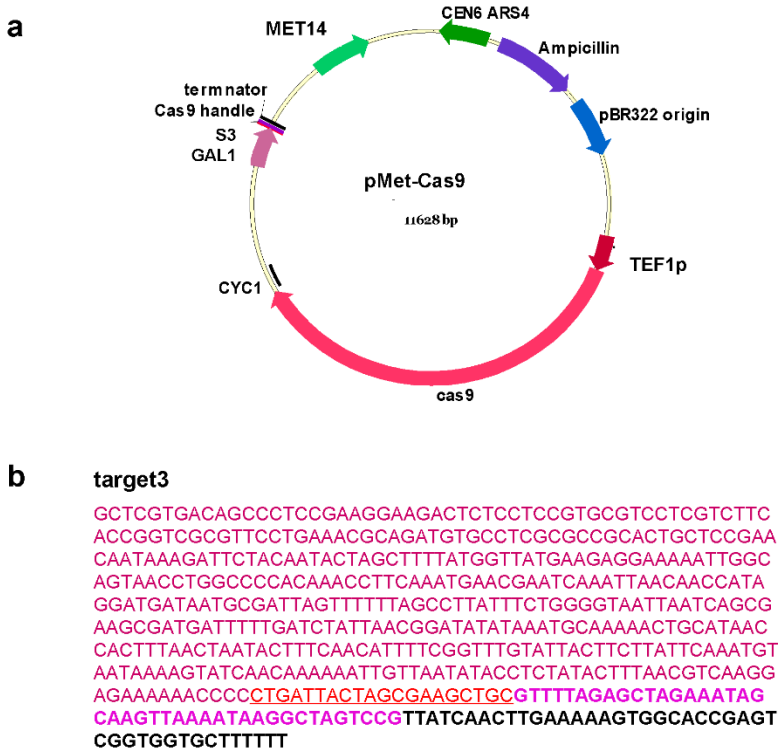
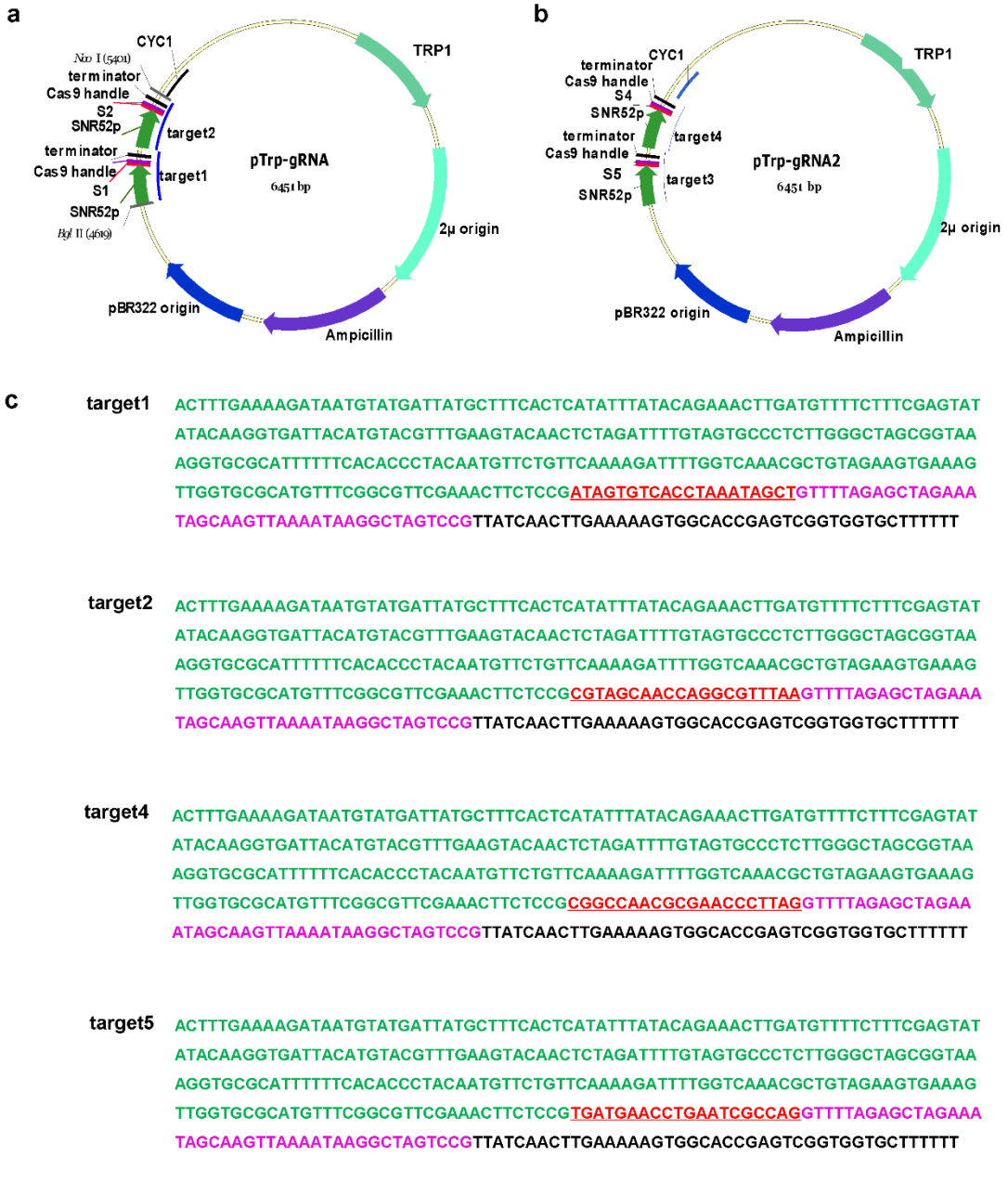
**Supplementary Figure 1:**




**Supplementary Figure 1: The Cas9 expression plasmid pMet-Cas9.** (a) The schematic map of pMet-Cas9. The yeast replication element (*CEN6 ARS4*) and selection marker (*MET14*, methionine auxotrophic selection) were indicated by green and light green arrowheads separately. The expression of *cas9* (marked by pink) was driven by the strong promoter TEF1p (red color). The *E. coli* replication element (pBR322) and selection marker (ampicillin resistance gene) were shown by blue and violet arrowheads separately. The plasmid pMet-Cas9 contained a galactose inducible guide RNA targeting pTrp replication origin at S3 site. The sequence of target 3 detail was shown in (b). Target 3 consisted GAL1 promoter (marked in rose), base-pairing region for S3 (underlined and marked in red), Cas9 handle (marked in purple), and terminator (black color).

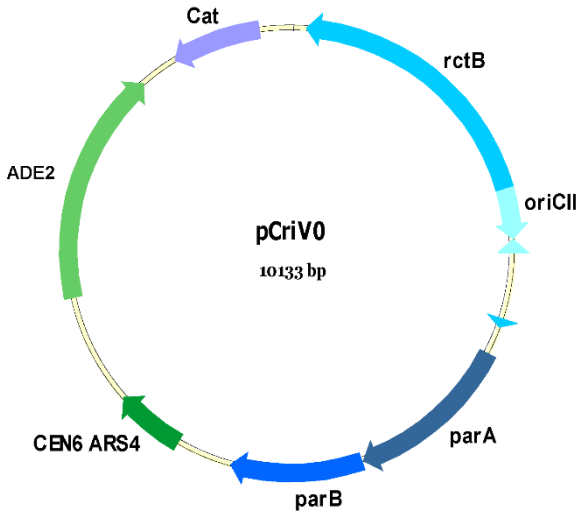
**Supplementary Figure 2:**




**Supplementary Figure 2: Targeting guide RNA expression plasmid pTrp-gRNA and pTrp-gRNA2.** The vector backbone of pTrp-gRNA and pTrp-gRNA2 were from pTrp, which contained yeast replication element (2 micron origin, allowed high copy number replication in yeast) and selection marker (*TRP1*) were indicated by green and light green arrowheads separately. The pBR322 replication origin and the ampicillin resistance gene were shown by blue and violet

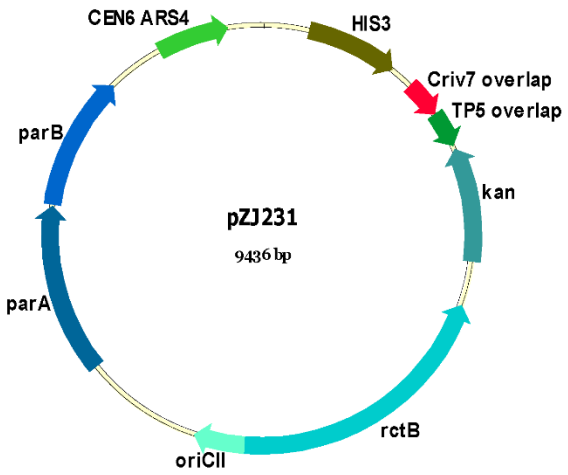
arrowheads separately. The guide RNA expression sequences target 1, 2 and target 4, 5, were synthesized by company and cloned into pTrp to construct pTrp-gRNA (a) and pTrp-gRNA2 (b), respectively. The sequence details of target 1, 2, 4, 5 were shown in (c), which consisted SNR52 promoter (marked in green), base-pairing region for corresponding targeting sites S1, S2, S4, S5 (underlined and marked in red), Cas9 handle (marked in purple), and terminator (black color).

**Supplementary Figure 3:**



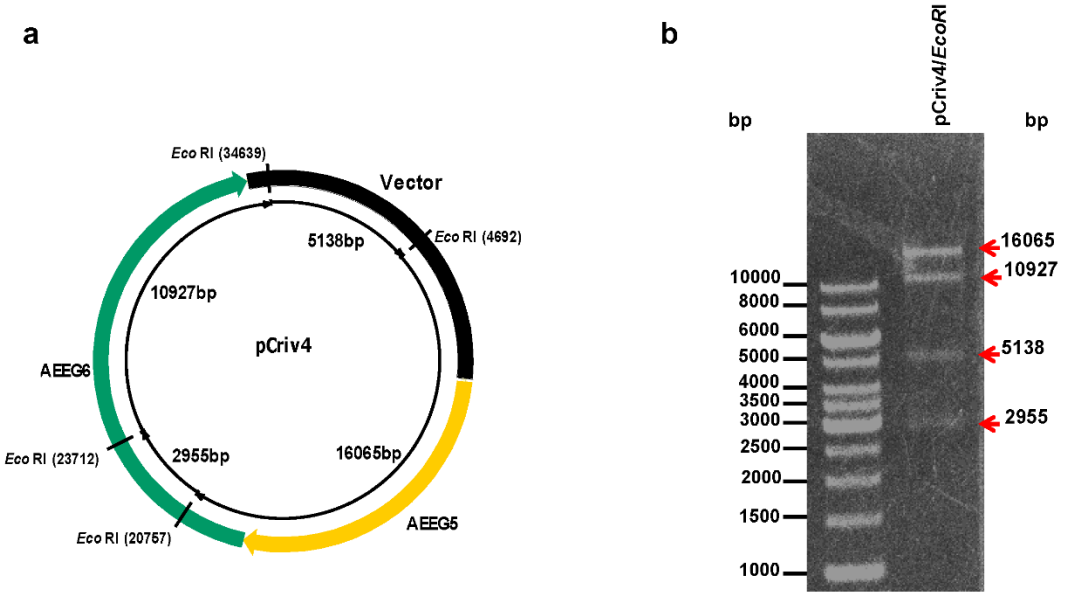

**Supplementary Figure 3: The schematic map of the assembly vector pCriv0.** The assembly vector pCriv0 contained the replication element (*CEN6/ARS4*), allowing single copy maintenance in yeast, and the selection marker *ADE2*. The origin of *Vibrio cholerae* chromosome II (oriCII) and replication protein coding gene (*rctB*), as well as the partition protein coding genes *parA* and *parB* allowed single copy maintenance in *E. coli*.

**Supplementary Figure 4:**



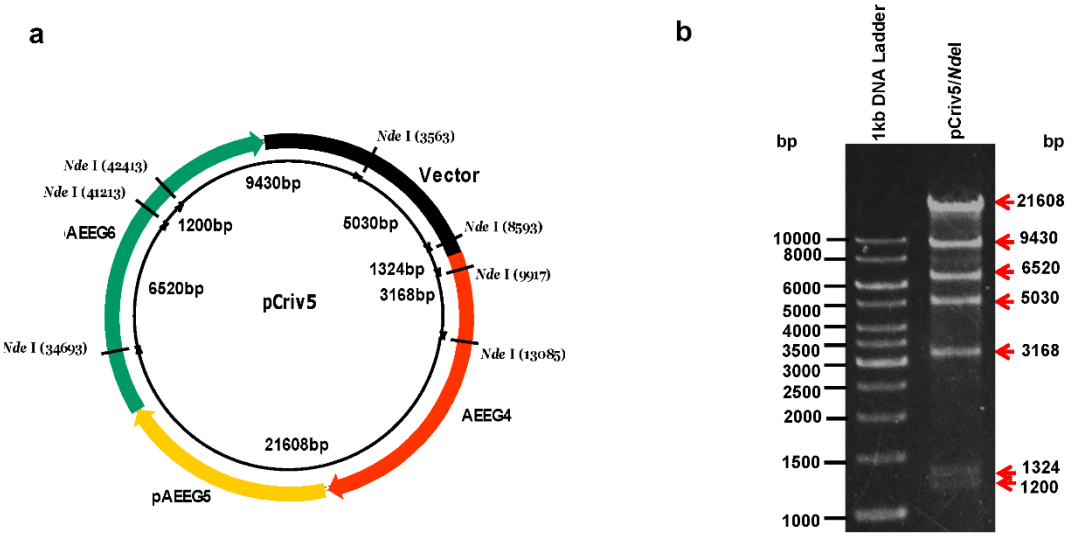

**Supplementary Figure 4: The schematic map of the MGE-syn1.0 assembly vector pZJ231.** The assembly vector pZJ231 contained the yeast replication element (*CEN6/ARS4*) for single copy maintenance in yeast. The selection marker in yeast is *HIS3*. The origin of *Vibrio cholerae* chromosome II (oriCII) and replication protein coding gene (*rctB*), as well as the partition protein coding genes *parA* and *parB* allowed single copy number maintenance in *E. coli*. The selection marker in *E. coli* is kanamycin resistance gene. The about 500 bp overlaps to Criv7 and TP5 were PCR amplified from *E. coli* MDS42 and cloned in pZJ231.

**Supplementary Figure 5:**



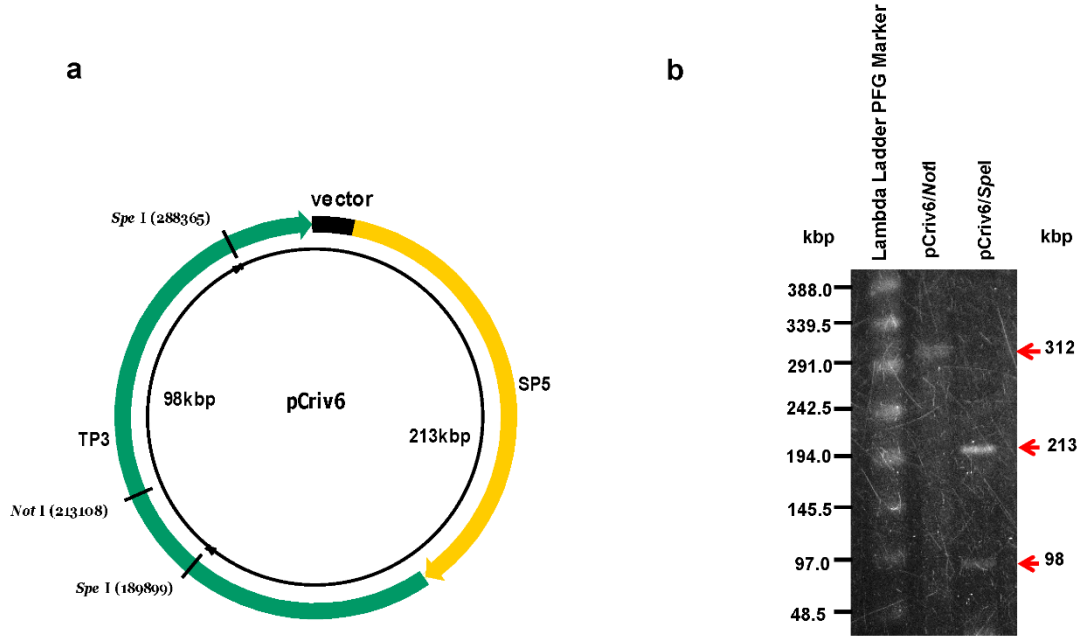

**Supplementary Figure 5: Analysis of pCriv4.** (a) Map of pCriv4 assembled from two small circular DNAs, which contained AEEG5 and AEEG6. The total size of Criv4 (25 kbp) and the vector (10 kbp) was 35 kbp. (b) The gel electrophoresis analysis of *EcoR*I digested pCriv4, which released five fragments with size of 16065, 10927, 5138, and 2955 bp.

**Supplementary Figure 6:**



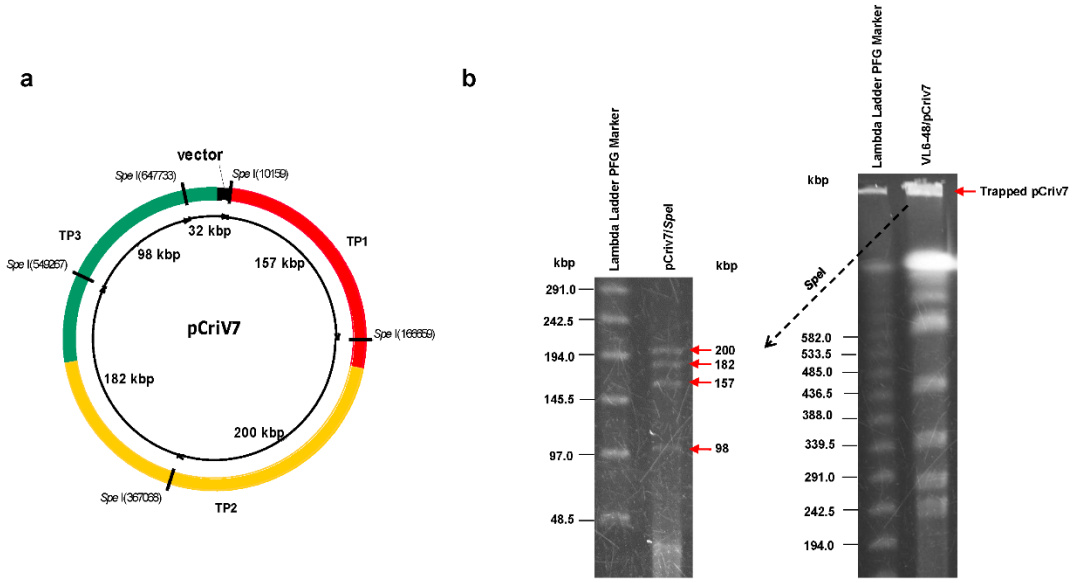

**Supplementary Figure 6: Analysis of pCriv5.** (a) Map of pCriv5 assembled from three small circular DNAs, which contained AEEG4, AEEG5 and AEEG6. The total size of Criv5 (38 kbp) and the vector (10 kbp) was 48 kbp. (b) The gel electrophoresis analysis of *Nde*I digested pCriv5, which released seven fragments with size of 21608, 9430, 6520, 5030, 3168, 1324, and 1200 bp.

**Supplementary Figure 7:**




**Supplementary Figure 7: Analysis of pCriv6.** (a) Map of pCriv6 assembled from two large circular DNAs, which contained SP5 and TP6. The total size of Criv4 (302 kbp) and the vector (10 kbp) was 312 kbp. (b) The pulsed-field gel electrophoresis (PFGE) confirmation of pCriv6. *Not*I digestion of pCriv6 resulted in one 312 kbp linear fragment. *Spe*I digestion of pCriv6 released two fragments with size of 213 and 98 kbp.

**Supplementary Figure 8:**




**Supplementary Figure 8:** Analysis of the assembled pCriv7. (a) Map of pCriv7, assembled from three large circular DNAs, containing TP1 (177 kbp, marked in red), TP2 (298 kbp, marked in yellow), and TP3 (185 kbp, marked in green). The total size of Criv7 (660 kbp) and the assembly vector (10 kbp) was 670 kbp. (b) PFGE analysis of pCriv7. To separate out the linear yeast chromosomal DNA, the yeast agarose plug was subjected to PFGE at 6 V/cm, switch time 10–60 second, 14 ℃ for 20 hours. The circular pCriv7 that trapped inside the plug was subjected to *Spe*I digestion, following by another round of PFGE carried out at 6 V/cm, switch time 1–25 second, 14 ℃ for 20 hours. The *Spe*I digestion of pCriv7 released five fragments with size of 200, 182, 157, 98, and 32 kbp. The smallest band (32 kbp) could not be clearly separated from the short fragments of yeast chromosome under the experiment condition.

**Supplementary Table 1: Plasmids used in this study.**

| Plasmid name | Description | Resource |
|---|---|---|
| p415-GalL-hCAS9 | p415-GAlL-cas9-SUP4 3'flanking region | Purchased from AddGene |
| p426-crRNA | p426-SNR52-CAN.Y gRNA-SUP4 3'flanking region | Purchased from AddGene |
| pAG36 | natMax4 *CEN URA3* Tef1p | Goldstein and McCusker 1999 |
| pZQ233 | VoriII-Cm-ARS4/CEN6-HIS3 | Constructed by our lab |
| pET28 | kanamycin resistance, His-tagged protein expression vector | Preserved in our lab |
| pMetcas9 | p415-GalL-hCAS9：Met14-Tef1p-Cas9-CYC1t | This study |
| pMet-Cas9 | pMetcas9：GAL1p-gRNA-S0-CYC1t | This study |
| pTrp | p426-crRNA：Trp1-SNR52-CYC1t | This study |
| pTrp-gRNA | pTRP：sgRNA.target1&2 | This study |
| pTrp-gRNA2 | pTRP：sgRNA.target3&4 | This study |
| pCriv0 | Ade2-the replication origin of *Vibrio cholerae* chromosome II | This study |
| pZJ231 | VoriII-Kan-ARS4/CEN6-HIS3 | This study |
| pAEEG4 | Containing 13700 bp of *Escherichia coli* essential genes | Constructed by our lab |
| pAEEG5 | Containing 9969 bp of *E. coli* essential genes | Constructed by our lab |
| pAEEG6 | Containing 15212 bp of *E. coli* essential genes | Constructed by our lab |
| pTP1 | Containing 177147 bp of *E. coli* essential genes | Constructed by our lab |
| pTP2 | Containing 297952 bp of *E. coli* essential genes | Constructed by our lab |
| pTP3-L | Containing 184475 bp of *E. coli* essential genes | Constructed by our lab |
| pTP3-U | Containing 184475 bp of *E. coli* essential genes | Constructed by our lab |
| pSP5 | Containing 116643 bp of *E. coli* essential genes | Constructed by our lab |
| pTP4 | Containing 184794 bp of *E. coli* essential genes | Constructed by our lab |
| pTP5 | Containing 184593 bp of *E. coli* essential genes | Constructed by our lab |
| pCriv7 | Containing 659554 bp of *E. coli* essential genes | This study |

**Supplementary Table 2: Primers used in this study.**

| Primer name | Sequence (5′ – 3′) | Description |
|---|---|---|
| **For construction of pMet-Cas9** | | |
| Met14-F | AGTAATTGGTTGTTTGGCCGAGCGGTCTAAGGCGCCTGATTCAAGAAATATCTTGACCGCAAGATTACGTTTAAAGGAGCATTAACAG | Forward primer for MET14 |
| Met14-R | TCGTTTCTATTATGAATTTCATTTATAAAGTTTATGTACAAATATCATAAAAAAAGAGAATCTCAAATTACAAATGCTTACGGATGAT | Reverse primer for MET14 |
| Tef1p-F | CTCTATACTTTAACGTCAAGGAGAAAAAACCCCGGATTCTAGCTTGCCTTGTCCCCGCCGGGGTCA | Forward primer for Tef1 promotor |
| Tef1p-R | TTGTGCCGATATCGAGCCCAATGGAGTACTTCTTGTCCATGGTTGTTTATGTTCGGATGTGATG | Reverse primer for Tef1 promotor |
| GalL-F | GCAGCTTCGCTAGTAATCAGGGGGTTTTTTCTCCTTGACGTTAAAG | Forward primer for GAlL promotor |
| GalL-R | TGATTCTAGAGCTCGTGACAGCCCTCCGAAGGA | Reverse primer for GAlL promotor |
| Cas-S3-F | ACTTCTAGAAAAGCCTTCGAGCGTCCCAAAACCT | Forward primer for S3 handle Cas9 |
| Cas-S3-R | CTGATTACTAGCGAAGCTGCGTTTTAGAGCTAGAAATAGCAAGTT | Reverse primer for S3 handle Cas9 |
| **For construction of pTrp** | | |
| Trp1-F | GTTTAGTATACATGCATTTACTTATAATACAGTTTTTCGCGCGTTTCGGTGATGACGGT | Forward primer for TRP1 with f1 origin overlap |
| Trp1-R | ATAACTTCGTATAATGTATGCTATACGAAGTTATGATCGGCAAGTGCACAAACAATAC | Reverse primer for TRP1 with 5'loxp overlap |
| 2μ ori-F | ATAACTTCGTATAGCATACATTATACGAAGTTATCAGGAGGTACTAGACTACCTTTCAT | Forward primer for 2u origin with 5'loxp overlap |
| 2μ ori-R | ATAACTTCGTATAATGTATGCTATACGAAGTTATAAGTGCCACCTGAACGAAGCATCTG | Reverse primer for 2u origin with 3'loxp overlap |
| pBR322-F | CTTATAACTTCGTATAGCATACATTATACGAAGTTATTTCGGGGAAATGTGCGCGGAAC | Forward primer for pBR322 with 3'loxp overlap |
| pBR322-R | AATTACATGACTCGACCATGGCCTAGGAGATCTAGCTCCAGCTTTTGTTCCCTTTAGTG | Reverse primer for pBR322 with f1 origin overlap |
| f1ori-F | GCTGGAGCTAGATCTCCTAGGCCATGGTCGAGTCATGTAATTAGTTATGTCACGCTTAC | Forward primer for f1 origin with pBR322 overlap |
| f1ori-R | GAGGTTTTCACCGTCATCACCGAAACGCGCGAAAAACTGTATTATAAGTAAATGCATGT | Reverse primer for f1 origin with TRP1 overlap |
| **For construction of pCriv0** | | |

| | | |
|---|---|---|
| pCriv0-VF | CCAACGCGAACCGCGGCCGCGGCGTTTAAG GGCACCAATAACTGC | Forward primer for the replication origin of *V. cholerae* chromosome II with ADE2 overlap |
| pCriv0-VR | GATGATATACTGGTAGTGCGCTGTGGTATG GTGCACTCTCAGTAC | Reverse primer for the replication origin of *V. cholerae* chromosome II with ADE2 overlap |
| ade2-F | GAGAGTGCACCATACCACAGCGCACTACCA GTATATCATCTCATTTC | Forward primer for ADE2 with the replication origin of V. cholerae chromosome II overlap |
| ade2-R | CCCTTAAACGCCGCGGCCGCGGTTCGCGTT GGCCGATTCATTAAT | Reverse primer for ADE2 with the replication origin of V. cholerae chromosome II overlap |
| **For construction of pZJ231** | | |
| ZJ231-F | GCAAGACGTTTCCCGTTGAATATGGCTCATT TTAGCTTCCTTAGCTCCTGAAAATCTCG | Forward primer for oriCII and CEN6 ARS4 |
| ZJ231-R | CCACTAAGCCAAGCTATTTAGGTGACACTA TCTTCAGAATGACACGTATAGAATGATGC | Reverse primer for oriCII and CEN6 ARS4 |
| Criv7-ov-F | CATTCTGAAGATAGTGTCACCTAAATAGCTT GGCTTAGTGGGAAGAGGTAGGGGGATG | Forward primer for Criv7 overlap |
| Criv7-ov-R | CAATTTCATGTCTGTGACGCGACTCACTCAC TGCGGTTGACTACT | Reverse primer for Criv7 overlap |
| TP5-ov-F | TCAACCGCAGTGAGTGAGTCGCGTCACAGA CATGAAATTGGTAAG | Forward primer for TP5 overlap |
| TP5-ov-R | AGAATTAACCCTTAAACGCCTGGTTGCTAC GCGACGAAGCAACAAGCCCTTC | Reverse primer for TP5 overlap |
| kan-F | CGCGTAGCAACCAGGCGTTTAAGGGTTAAT TCTTAGAAAAACTCATCGAGCATC | Forward primer for kanamycin resistant gene |
| kan-R | GTTATCGAGATTTTCAGGAGCTAAGGAAGC TAAAATGAGCCATATTCAACGGGAAACG | Reverse primer for kanamycin resistant gene |
| **For generation of unique linear assembly vector** | | |
| pCriv4-VF | ACGCATAACCATAGCGAAAATAGTGGCGCA GTGTAAGGTTGTTGTGAATATTGAGTTGCAG GCGTTTAAGGGCACCAATAACTGC | Forward primer for linearized pCriv4 vector with EEG6 overlap |
| pCriv4-VR | GCAATCGGCGAGCTACGCCAACCTGTCTGG CCTGCGGAGGTATACGGCAGTATTAGCCAC GGTTCGCGTTGGCCGATTCATTAAT | Reverse primer for linearized pCriv4 vector with EEG5 overlap |
| pCriv5-VF | CTTACGCATAACCATAGCGAAAATAGTGGC GCAGTGTAAGGTTGTTGTGAATATTGAGTTG CAGGCGTTTAAGGGCACCAATAACTGC | Forward primer for linearized pCriv5 vector with EEG6 overlap |

| | | |
|---|---|---|
| pCriv5-VR | GGAGATAAAATCCCCCCTTTTTGGTTAACTA ATTGTATGGGAATGGTTAATTATTCACCAGA TGGTTCGCGTTGGCCGATTCATTAAT | Reverse primer for linearized pCriv5 vector with EEG4 overlap |
| pCriv6-VF | CCCGCCACGAAATTGAGGATGCTAAGGCTG TTGTAGACCGGAAACGGTGTTCACGCCGCA TCCGGCGTTTAAGGGCACCAATAACTGC | Forward primer for linearized pCriv6 vector with TP3 overlap |
| pCriv6-VR | ATCCAGGTACTATGAGCCCAATCCAACACG GGGAAGTGTTCGTTACTGAAGACGGCGCTG AAAGGTTCGCGTTGGCCGATTCATTAAT | Reverse primer for linearized pCriv6 vector with SP5 overlap |
| pCriv7-VF | GCCACGAAATTGAGGATGCTAAGGCTGTTG TAGACCGGAAACGGTGTTCACGCCGCATCC GGCGTTTAAGGGCACCAATAACTGC | Forward primer for linearized pCriv7 vector with TP3 overlap |
| pCriv7-VR | ATCTTCTCTTTATCAGCAGTAAACTAGTGGG TATTCATCCCCCTACCTCTTCCCACTAAGGG TTCGCGTTGGCCGATTCATTAAT | Reverse primer for linearized pCriv7 vector with TP1 overlap |
| pCriv8-VF | TGTGATTGAGCAGCAACTGGAG | Forward primer for linearized assembly vector for MGE-syn1.0 |
| pCriv8-VR | GCGATATGGCAGGACAAGACGT | Reverse primer for linearized assembly vector for MGE-syn1.0 |
| **For verification of assemblies** | | |
| EEG5-T1 | GCTACAAATGTAGCGTTGAGGTG | Forward primer for verifying pCriv4 and pCriv5 |
| EEG6-T1 | CATCATCACGCTGGGTAAAGG | Reverse primer for verifying pCriv4 and pCriv5 |
| EEG4-T3 | AGTCGCTCGTGTTCCGCT | Forward primer for verifying pCriv5 |
| EEG5-T2 | CTGTGGATAGCCGCCAGAG | Reverse primer for verifying pCriv5 |
| pCriv6-T2 | CTCAAACAGATCATCGTC | Forward primer for verifying pCriv6 and pCriv7 |
| pCriv6-T3 | AAGAGCAAGATAAAAGGTA | Reverse primer for verifying pCriv6 and pCriv7 |
| pCriv7-T2 | CGCTGGATTTGTCACCTTC | Forward primer for verifying pCriv7 |
| pCriv7-T3 | CGATTTGCCAGTTGTTCCAG | Reverse primer for verifying pCriv7 |
| pCriv8-T1 | CGCATTGCAGTATTCTGACTACG | Forward primer for verifying MGE-syn1.0 |
| pCriv8-T2 | CAATGTTGAACGCGGATCG | Reverse primer for verifying MGE-syn1.0 |
| pCriv8-T3 | GAGTCAGAGAGTTAAAGCGAACTGC | Forward primer for verifying MGE-syn1.0 |

| | | |
|---|---|---|
| pCriv8-T4 | CAACACCCTGACGCGTAAG | Reverse primer for verifying MGE-syn1.0 |

**Supplementary Table 3: Sequencing results of assemblies overlaps.**

| Name of assembly | Overlaps | colony 1 | colony 2 | colony 3 | colony 4 | colony 5 | colony 6 | colony 7 | colony 8 |
|---|---|---|---|---|---|---|---|---|---|
| pCriv4 | Overlap 1 (vector-AEEG5) | one 13-bp insertion | correct | one 13-bp insertion | correct | correct | | | |
| | Overlap 2 (AEEG5-AEEG6) | correct | correct | correct | correct | correct | | | |
| | Overlap 3 (AEEG6-vector) | three 1-bp deletions | correct | correct | correct | correct | | | |
| pCriv5 | Overlap 1 (vector-AEEG4) | correct | correct | correct | correct | two 1-bp deletion | | | |
| | Overlap 2 (AEEG4-AEEG5) | correct | correct | correct | correct | correct | | | |
| | Overlap 2 (AEEG5-AEEG6) | correct | correct | correct | correct | correct | | | |
| | Overlap 3 (AEEG6-vector) | correct | correct | correct | correct | one 1-bp deletion | | | |
| pCriv6 | Overlap 1 (vector-SP5) | correct | correct | one 1-bp deletion | correct | correct | | | |
| | Overlap 2 (SP5-TP3) | correct | correct | correct | correct | correct | | | |
| | Overlap 3 (TP3-vector) | correct | correct | one 1-bp deletion | correct | correct | | | |
| pCriv7 | Overlap 1 (vector-TP1) | correct | correct | correct | correct | correct | one 16-bp insertion | one 16-bp insertion | correct |
| | Overlap 2 (TP1-TP2) | correct | correct | correct | correct | correct | correct | correct | correct |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Overlap 2 (TP2-TP3) | correct | correct | correct | correct | correct | correct | correct | correct |
| | Overlap 3 (TP3-vector) | correct | correct | correct | correct | correct | one 1-bp mutation | correct | correct |
| MGE-syn1.0 | Overlap 1 (vector-Criv7) | correct | correct | correct | correct | correct | | | |
| | Overlap 2 (Criv7-TP4) | correct | correct | correct | correct | correct | | | |
| | Overlap 2 (TP4-TP5) | correct | correct | correct | correct | correct | | | |
| | Overlap 3 (TP5-vector) | correct | correct | correct | correct | correct | | | |