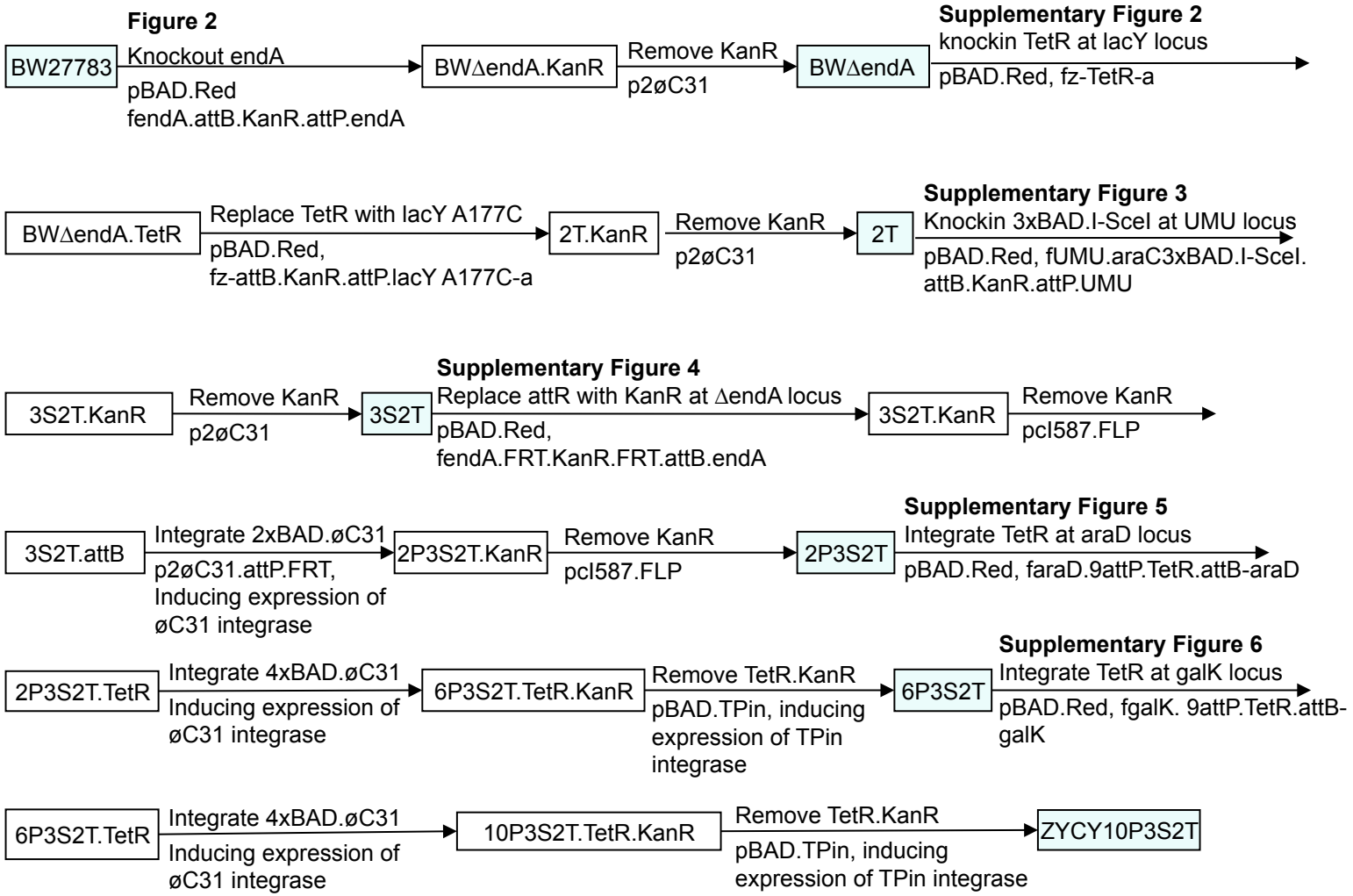
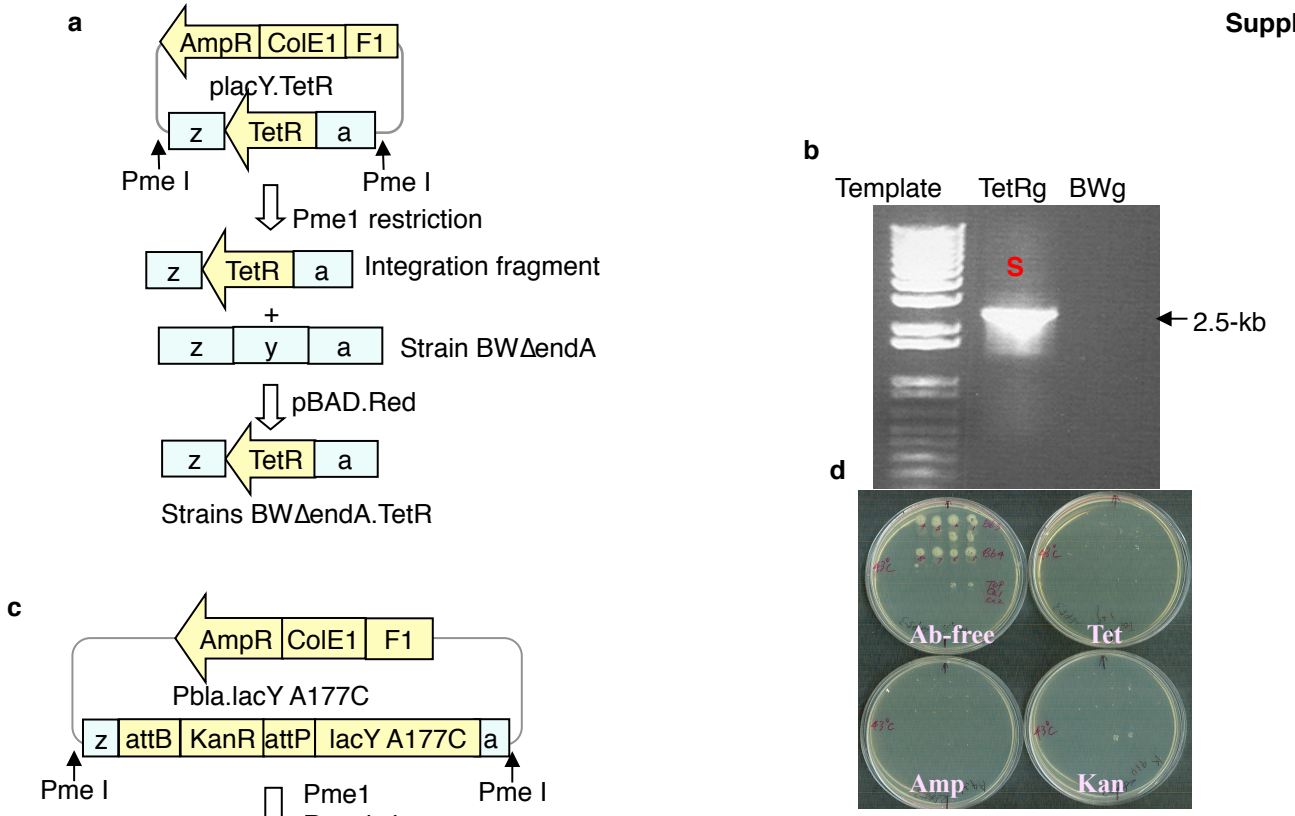
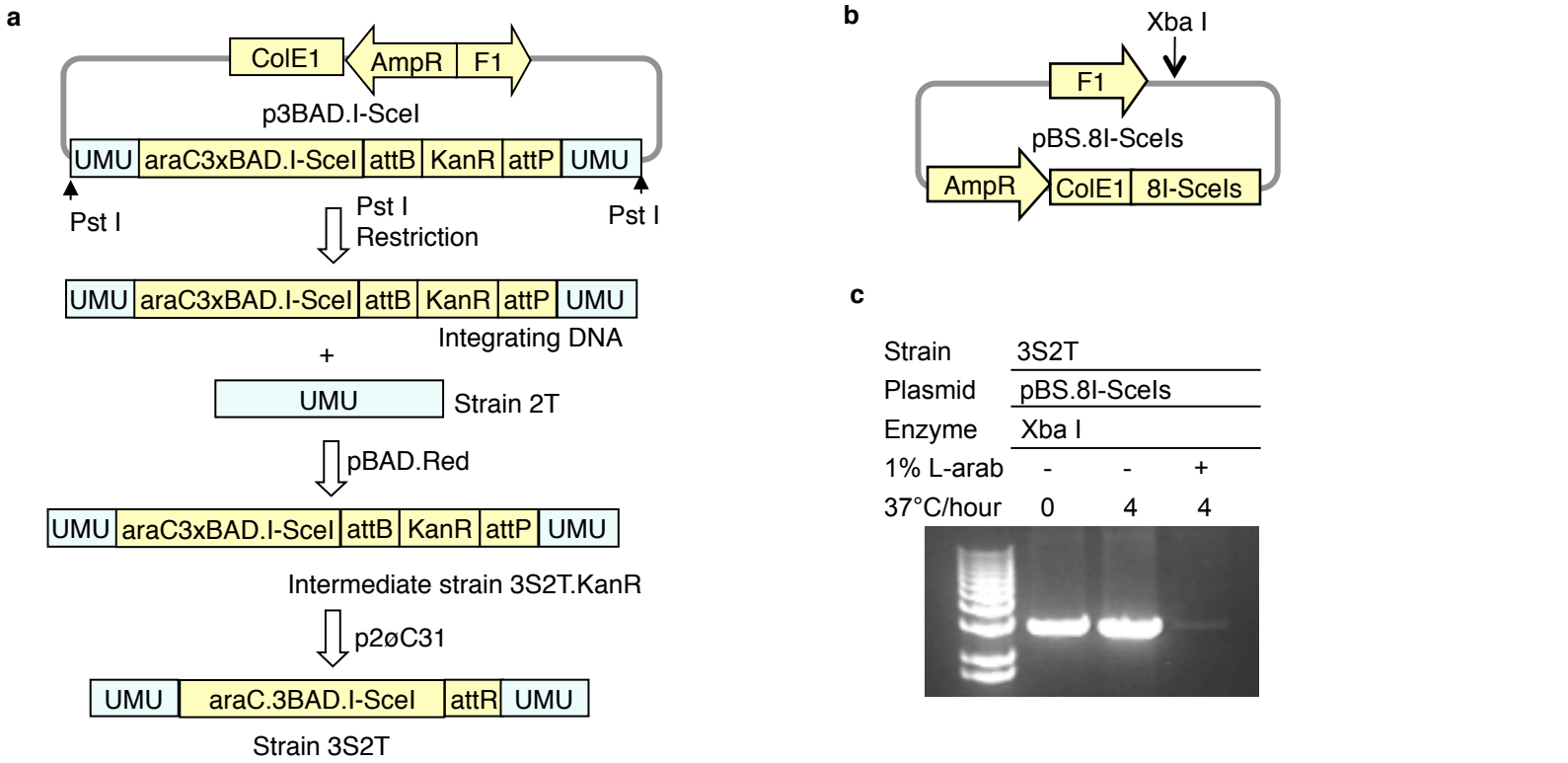


Supplementary Figure 1. A flowchart summarizing the stepwise genetic manipulation of the bacterial genome. The bacterial strains are boxed.

Supplementary Figure 2

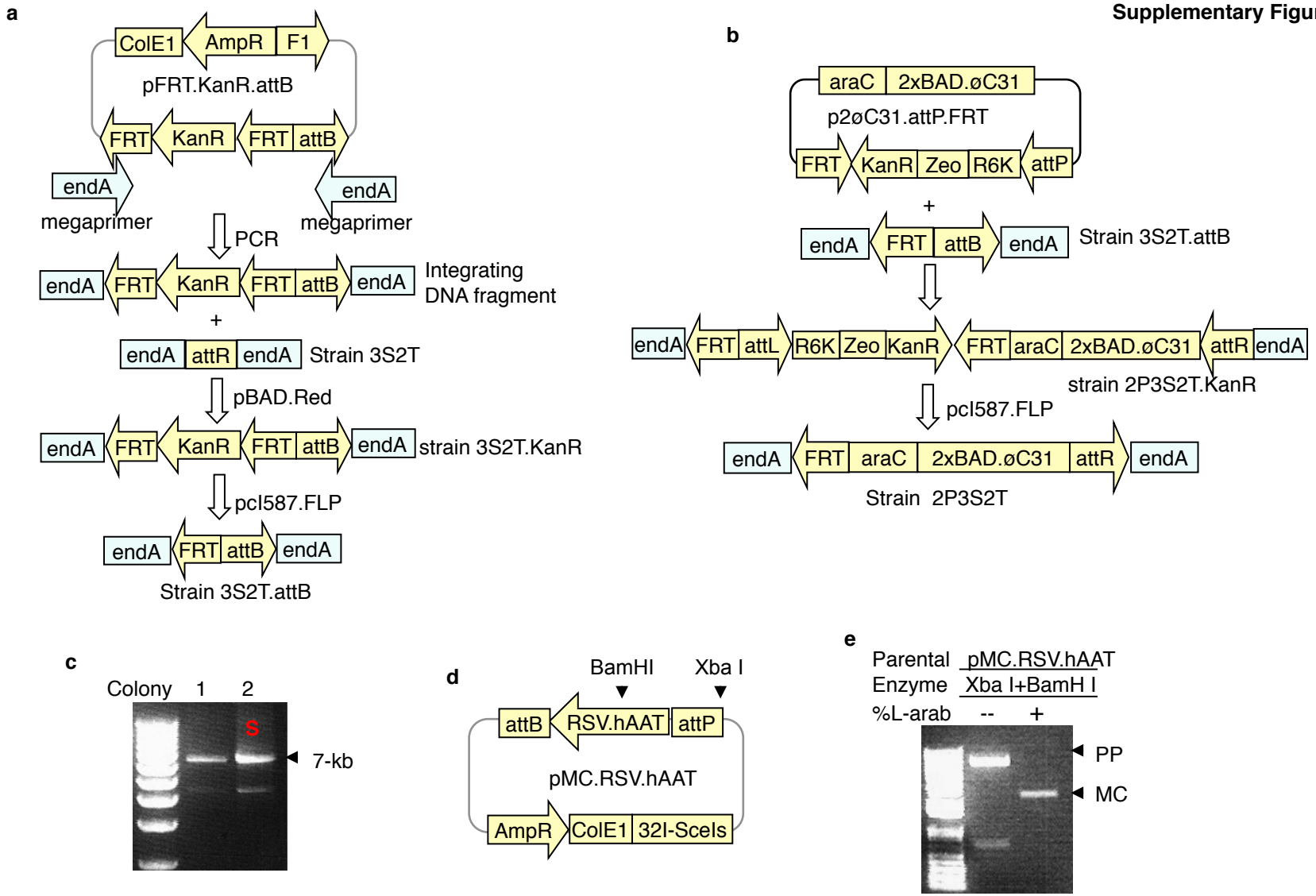


Supplementary Figure 2. Integration of 2nd L-arabinose transporter mutant lacY A177C. (a) Flow chart illustrating procedure for preparing target site. placY.TetR, the plasmid used to prepare the Pme1-restricted integrating fragment; z, y and a, the structural genes of lactose operon; the boxed z and a, PCR-generated 425- and 227-bp of z- and a-specific sequences. (b) PCR illustration of integrant. 2.5-kb, integrant-specific PCR product; TetRg, genome of strain BWΔendA.TetR; BWg, genome of BW27783. (c) Mutant lacY A177C knock-in strategy. pbla.lacY A177C, the plasmid for producing the Pme1-restricted integrated DNA fragment; bla, the beta-galactosidase gene promoter. (d) Selection of the strain 2T. Colonies that lost all three antibiotic resistance phenotypes were selected using the 4 agar plates containing different antibiotics. Ab-free, antibiotic-free; Tet, tetracycline (12μg/ml); Amp, ampicillin (25 μg/ml); Kan, kanamycin (25 μg/ml).



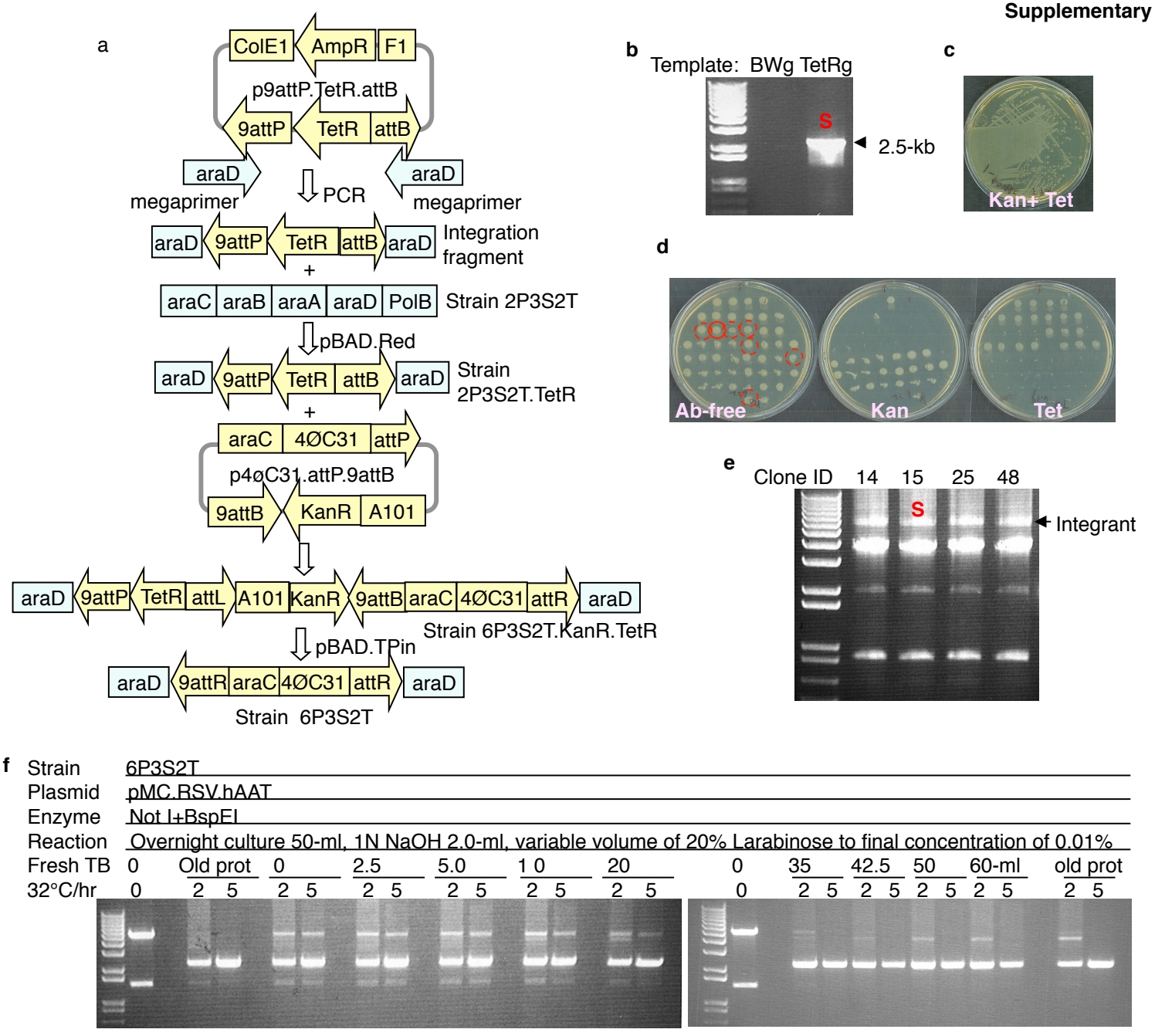
Supplementary Figure 3. Integration of BAD.I-SceI gene. (a) Flow chart showing the BAD.I-SceI gene knock-in procedure. p3BAD.I-SceI, the plasmid encoding 3 tandem copies of the I-SceI gene under the control of BAD (BAD.I-SceI); UMU, the UMU D gene; the boxed UMU, PCR-generated 737- and 647-bp UMU-specific product, respetcively; araC, the repressor gene of araC.BDA system; integrating DNA, the DNA fragment derived from p3BAD.I-SceI via Pst 1 restriction; strain 2T, the E. coli expressing 2 constitutive L-arabinose transporters cp8.araE and bla.lacY A177C (Supplementary Figure 2); strain 3S2T, generated by Red-mediated integration of the above integrating fragment to the UMU gene of strain 2T. (b) pBS.8I-SceIs, a pBlueScript base plasmid encoding 8 tandem copies of the I-SceI site. (c) Demonstration of function of the integrated I-SceI gene in strain 3S2T. An overnight culture of the 3S2Tstrain transformed with pBS.8I-SceIs was re-suspended in LB with or without 1% L-arabinose and incubated at 37° for 4 hours; Xba I, the restriction enzyme used to cut the plasmid before electrophoresis.

Supplementary Figure 4



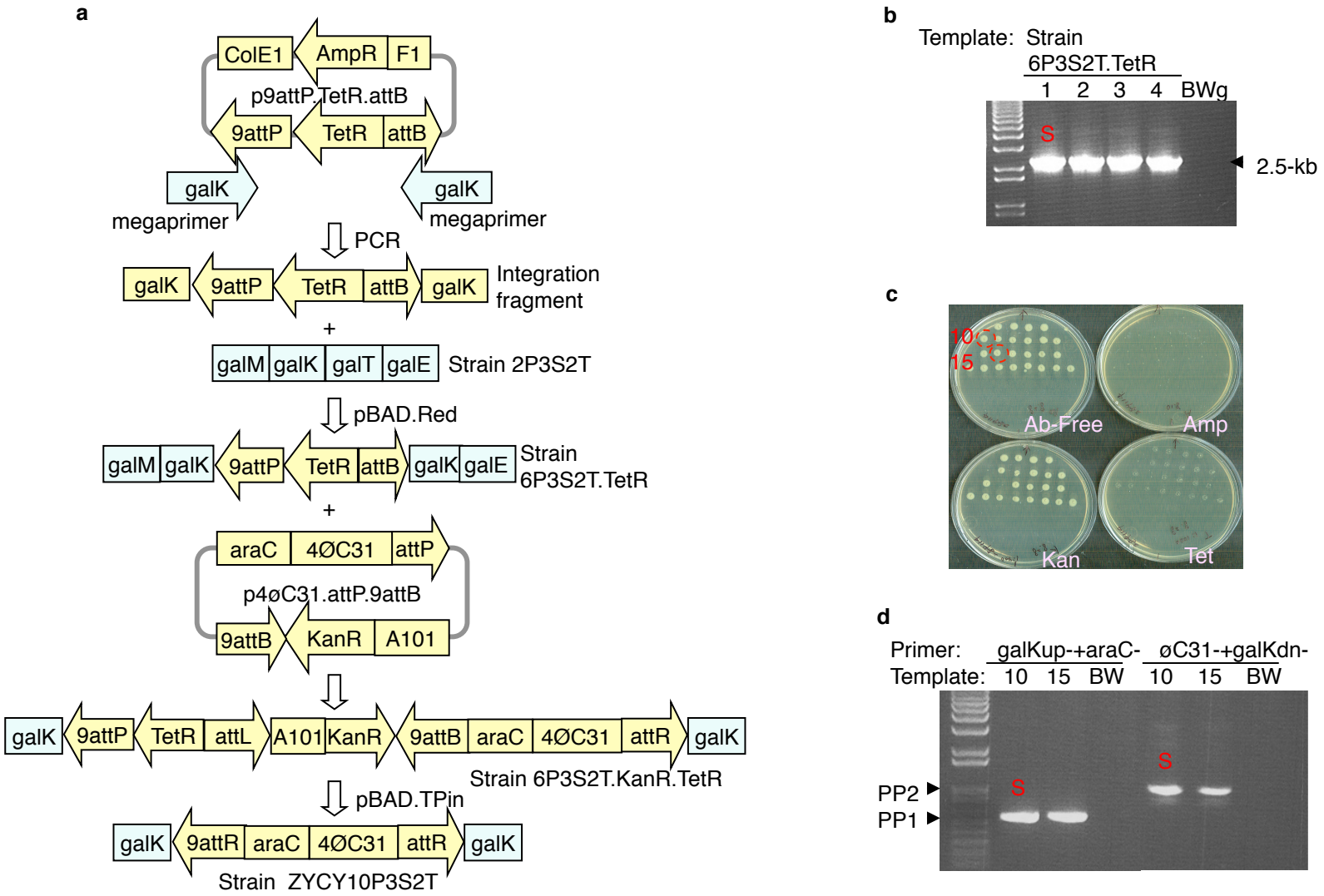
Supplementary Figure 4. Integration of 2 copies of BAD.øC31 gene. (a) Preparation of genomic target site. pFRT.KanR.attB, the plasmid serving as PCR template; FRT, the binding site of flipase (FLP); megaprimers, 329- and 754-bp of endA sequences, generated by PCR, to serve as PCR primers for generating the integrating DNA fragment; the PCR product was integrated into the modified endA site of strain 3S2T via Red-mediated reaction; subsequently, the KanR was eliminated via FLP-mediated reaction between the two flanking FRTs, resulting in the strain 3S2T.attB; cI587, the bacteriophage Lambda temperature sensitive promoter. (b) Integration of BAD.øC31. p2øC31.attP.FRT, a conditional replicating plasmid carrying R6K origin capable of supporting plasmid replication only in the cell expressing the pir protein; Zeo, zeocin. (c) PCR illustration of the integrant. 7-kb, the size of the PCR product generated by a pair of endA-specific primers. S, partial DNA sequencing of the DNA in the band. (d) The minicircle producer plasmid pMC.RSV.HAAT. R, the Rous Sarcoma virus long terminal repeat (promoter); H, human alpha-1 antitrypsin cDNA; B, bovine growth hormone gene polyadenylation signal; 32I-SceIs, 32 consecutive I-SceI cutting sites. (e) Illustration of genomic øC31 integrase function. The minicircle was made according to the protocol described previously [6]. Xba I+BamHI, 2 restriction enzymes used to cut the DNA before electrophoresis.

Supplementary Figure 5



Supplementary Figure 5. Integration of 4 copies of BAD.øC31. (**a**) Flow chart illustrating the integration procedure. p9attP.TetR.attB, the plasmid served as template for PCR-generation of the integrating DNA fragment; 9attB and 9attP, the bacterial and phage attachment sites of the bacteriophage TP901-1 integrase (TPin); the boxed araD, PCR-generated 275- and 310-bp araD-specific product, respectively; p4øC31.attP.9attB and pBAD.TPin, two complementing plasmids carrying the temperature-sensitive A101 origin of replication; 9attR, the recombination product between 9attB and 9attP. (**b**) PCR illustration of the altered araD locus. 2.5-kb, size of the PCR product generated by a TetR- and an araD-specific primers. (**c**) Selection of strain 6P3S2T.KanR.TetR. Kan+Tet, the plate containing both Kan and Tet. (**d**) Elimination of KanR and TetR. , marked colonies that lost both KanR and TetR are circled. (**e**) PCR illustration of integrant. Integrant, PCR product generated by a pair of araD-specific primers. (**f**) Optimizing minicircle production procedure. Reaction, minicircle formation reaction each comprised a 50-ml overnight culture, 2-ml 1N sodium hydroxide, indicated volume of fresh TB and L-arabinose to a final concentration of 0.01%; old prot, previous protocol for minicircle production [6].

Supplementary Figure 6



Supplementary Figure 6. Integration of the 7th to 10th copies of BAD.øC31. (**a**) The integration procedure was the same as that for integration of the 3rd to 6th copies of the BAD.øC31 cassette (**Supplementary Figure 5a**); galK, galactokinase gene. (**b**) PCR reaction to confirm the prepared target. 2.5-kb, the expected size of the PCR product generated by a TetR- and a galK-specific primers. (**c**) Screening of strain ZYCY10P3S2T; the the AmpR^-/KanR^-/TetR^- colonies are circled. (**d**) PCR illustration of the integrant elements; galKup, the up-stream galK-specific primer; galKdn, the down-stream galK-specific primer.

Supplementary Figure 7. Plasmid glossary

1. P2øC31-a plasmid expresses øC31 integrase under the control of the L-arabinose-araC.BAD system; carrying a BAD.I-SceI cassette and an I-SceI site, the plasmid can be eliminated through overnight culture with 1% L-arabinose.
2. p2øC31.attP.FRT- a plasmid for integrating 2 copies of BAD.øC31 cassette through two FRT sites in the plasmid and in the genomic target, respectively; carrying the conditional R6K DNA origin and capable of replicating only in the cell expressing the pir protein, this plasmid will be cured in BW27783 and other common laboratory strain lacking the pir gene.
3. p2øC31.hFIX, an old version of minicircle producer plasmid encoding the ApoE.hFIX expression cassette, as described previously [2] . The makeup of the plasmid is shown as p2øC31.Transgene when the expression cassette is the ApoE.hFIX (ApoE HCR enhancer, hAAT promoter, hFIX minigene, and bovine poly A signal).
4. P2øC31.Transgene - the previous minicircle producer plasmid.
5. p3BAD.I-SceI -a plasmid for generating fragment DNA encoding 3 copies of BAD.I-SceI cassette to be integrated through homologous recombination.
6. p4øC31.attP.9attP - a plasmid encoding 4 copies of BAD.øC31 cassette capable of integrating through the øC31 integrase-mediated recombination between the attP site in the plasmid and attB site in the genomic target.
7. p9attP.TetR.attB -a plasmid for integrating the TetR flanked with the attB and 9attP sites into the genomic target site; 9attP, the plasmid attachment site of the bacteriophage TP901-1 integrase (TPin).
8. pBAD.RED -a plasmid expressing the bacteriophage Lambda RED homologous recombinase system under the control of the L-arabinose-araC.BAD system; control under the heat sensitive DNA replication origin A101, it can be eliminated by growing the cells at 43°C overnight.
9. pBAD.TPin - a plasmid expressing the TPin to mediate the elimination of unwanted or harmful DNA elements through recombination between 9attB and 9attP, under the control of the L-arabinose-araC.BAD system; similar to that of p2øC31, the plasmid carrying the BAD.I-SceI cassette and an I-SceI site, allowing its own degradation when inducing expression of I-SceI by L-arabinose.

10. pbla.LacY A177C -a plasmid for integrating the bla.LacY.A177C cassette at the disrupted LacY region. Bla, promoter of beta-lactosidase gene; A177C, an Alanine to Cysteine mutation at codon 177.
11. pBS.8I-SceIs - a pBlueScript base plasmid carrying 8 consecutive I-SceI sites.
12. pcI587.FLP - a plasmid expressing flipase, which mediates recombination between 2 FRT sites, under the control of bacteriophage Lambda pR promoter/cI587 repressor system.
13. pFRT.KanR.attB **-**a plasmid for generating PCR product encoding an attB site and the KanR flanked with 2 FRT sites up- and down-stream, respectively.
14. pKanR.endA **-** a plasmid for generating pme1-fragment to interrupt the endA gene.
15. pLacY.TetR **-** a plasmid for generating DNA fragment to disrupt the LacY locus with the selection marker, TetR.
16. pMC.ApoE.hFIX **-**a new version of minicircle producer plasmid for producing minicircle encoding the ApoE.hFIX expression cassette.
17. pMC.CMV.LGNSO **-** a new version of minicircle producing plasmid for production of the minicircle encoding the reporter *gfp* and a set of 4 transcription factors, including the *LIN28, NANOG, SOX2 and OCT4* , for converting somatic cells to iPS cells through reprogramming [1].
18. pMC.RSV.hAAT **-** a new version of minicircle producer plasmid for production of minicircle encoding the RSV.hAAT expression cassette (RSV-LTR promoter, hAAT cDNA, and bovine poly A signal).

Supplementary Figure 8. Minicircle production protocol

Materials:

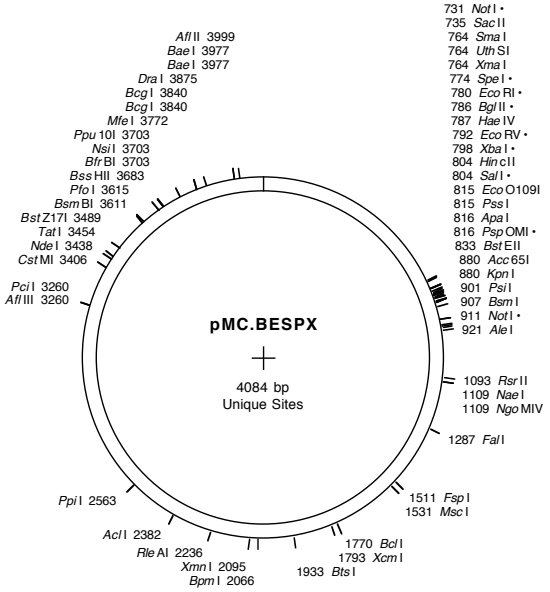
1. E. coli strain: ZYCY10P3S2T
2. Empty minicircle producing plasmid pMC.BESPX
3. Terrific Broth (TB) Powder (Invitrogen, Carlsbad, CA);
4. Luria-Bertani broth (LB-medium) powder (MP Biomedicals, Solon, OH);
5. Plasmid purification kits (Qiagen Volancia, CA).

Procedure:

1. Preparation of competent cell of ZYCY10P3S2T following standard protocol;
2. Transformation of the ZYCY10P3S2T competent cell with minicircle producing plasmid carrying the transgene of interest following standard protocol;
3. Preparation of minicircle:
    - On Day 1 morning, inoculate bacteria from 2 colonies in one each of 2-ml of LB containing kanamycin (50 µg/ml) at 37°C with shaking at 250 rpm;
    - On Day 1 evening, prepare plasmid DNA and confirm the ID of the minicircle producing plasmid through restriction mapping;
    - One Day 1 evening, cast an overnight culture by combining 100-µl of the culture from above step with 400-ml TB containing kanamycin (50 µg/ml) in 2 liter flask and incubate with shaking as in above step; if prepare smaller volume, keep the ratio of flask size to the TB volume at 5:1 (vol:vol) to ensure appropriate aeration;
    - On Day 2 morning (culture for 16-18 hours), the OD600 reading of the overnight culture will be between 4 to 5, pH 6.5; otherwise, adjust to pH 6.5 using 1N Sodium Hydroxide;
    - For a 400-ml overnight culture, prepare a Minicircle Induction Mix comprising 400-ml LB, 16-ml 1N NaOH and 400-µl 20% L-arabinose (final concentration in the Minicircle Induction Reaction will be 0.01%);
    - Cast the Minicircle Induction Reaction by combine the Minicircle Induction Mix with the overnight culture and incubate at 32°C with shaking at 250 rpm for 5-hours; the incubation can be extended for additional 1 to 3 hours if desired;
    - After pelleting the bacteria, use Qiagen kit to isolate minicircle according to manufacturer's protocol with modifications: double the volume of P1, P2 and P3 buffers. For example, for 400-ml overnight culture, use 100-ml each of P1, P2, P3 buffers and one 2500 column;
    - Note: to have the best yield, ensure that the bacteria are completely resuspended in P1 buffer; to minimize contamination of bacterial genomic DNA, avoid excessive shaking of the bacteria suspension after addition of P2 buffer.

Supplementary Figure 9. Empty Minicircle producer plasmid pMC.BESPX

(1) DNA Strider map of ZYCY10P3S2T



Notes

- The sequence 1-730 encodes 32 consecutive cutting sites of homing endocnuclease I-SceI;
- The attB sequence “GGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCG” locates between 740-774 bp;
- The attP sequence “CCCAACTGGGGTAACCTTTGAGTTCTCTAGTTGGGG” locates between 825-860 bp;
- The following unique sites can be used to insert the transgene expression cassette of your interest: BglII, EcoRI, EcoRV, SalI, PspOMI and XbaI; pMC.BESPX carries neither promoter nor polyadenylation signal;
- It carries a Kanamycin resistance gene.

(2) DNA sequence of pMC.BESPX

ACATTACCCTGTTATCCCTAGATGACATTACCCTGTTATCCCAGAtGACA
TTACCCTGTTATCCCTAGATGACATTACCCTGTTATCCCTAGATGACATT
TACCCTGTTATCCCTAGATGACATTACCCTGTTATCCCAGATGACATTAC
CCTGTTATCCCTAGATACATTACCCTGTTATCCCAGATGACATACCCTGT
TATCCCTAGATGACATTACCCTGTTATCCCAGATGACATTACCCTGTTAT
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Supplementary Figure 9. Minicircle producer plasmid pMC.BESPX. DNA sequence and a DNA strider map are provided.