Chapter 2

Recombineering Linear BACs

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Abstract

Recombineering is a powerful genetic engineering technique based on homologous recombination that can be used to accurately modify DNA independent of its sequence or size. One novel application of recombineering is the assembly of linear BACs in *E. coli* that can replicate autonomously as linear plasmids. A circular BAC is inserted with a short telomeric sequence from phage N15, which is subsequently cut and rejoined by the phage protelomerase enzyme to generate a linear BAC with terminal hairpin telomeres. Telomere-capped linear BACs are protected against exonuclease attack both in vitro and in vivo in *E. coli* cells and can replicate stably. Here we describe step-by-step protocols to linearize any BAC clone by recombineering, including inserting and screening for presence of the N15 telomeric sequence, linearizing BACs in vivo in *E. coli*, extracting linear BACs, and verifying the presence of hairpin telomere structures. Linear BACs may be useful for functional expression of genomic loci in cells, maintenance of linear viral genomes in their natural conformation, and for constructing innovative artificial chromosome structures for applications in mammalian and plant cells.

Key words Linear BAC, Recombineering, *E. coli*, Genomic DNA, Chromosome, Phage N15, Plasmid

1 Introduction

BACs are important resources for the functional study of mammalian genes because of their capacity to clone several hundred kilobases of DNA, which can accommodate large chromosomal segments containing complete genes and all their natural regulatory signals. However, all the currently available BAC clones are in circular form, while mammalian genes are naturally carried on linear chromosomes. The ability to assemble linear BACs may provide a useful model system for eukaryotic studies because they will more closely represent the structure of natural mammalian chromosomes. While linear artificial chromosomes constructs based on truncation of actual human chromosomes are already available, the techniques required to build such linear vectors are extremely tedious and not applicable to BAC clones isolated from existing libraries [1, 2].

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In the microbial world, although it is more common to find DNA maintained in circular form there are exceptions where linear chromosomes and plasmids can be found, e.g., *Borrelia*, *Streptomyces*, *Agrobacterium*, and a number of gram-negative bacteria [3–6]. In *E. coli*, the prophage of the lambda-like N15 phage replicates as a linear plasmid with telomeres. The N15 core telomeric sequence (*telRL*) is a 56 bp site that is contained within a larger 310 bp DNA sequence known as the telomerase occupancy site (*tos*) [6–8]. The *tos* site is the target for the viral protelomerase enzyme TelN to process into left (*tel*L) and right (*tel*R) telomeres, enabling its prophage to replicate as a linear plasmid. We recently adapted the phage N15 system to assemble linear BACs using recombineering technology in *E. coli* [9–12]. In this technique, a DNA fragment containing the phage N15 telomeric (*tos*) sequence is inserted into a BAC followed by its resolution in vivo in *E. coli* into separate left (*tel*L) and right (*tel*R) telomeres by the phage protelomerase enzyme TelN, resulting in a self-replicating linear BAC [9].

Specifically, a *tos* sequence is amplified along with a selectable zeocin resistance marker (Zeo) (Fig. 1a) as a PCR cassette and inserted into a target BAC via homologous recombination (Fig. 1b) [11]. By design, primers TosZeoF2 and TosZeoR2 that are used to amplify the *tos*-Zeo cassette (Fig. 1a) contain homology sequences that target this DNA into a site centered on the unique *Sfi*I restriction site located within the vector section of many BAC clones (Fig. 1b), leaving its genomic insert intact.

The resulting recombinant BAC (in this chapter any BAC clone containing the recombined *tos*-Zeo cassette is referred to as "BAC-*tos*") remains circular at this stage and carries the *tos* sequence as well as the zeocin marker within its backbone (Fig. 1b) [9]. After recombination, zeo^r colonies are screened by PCR to verify presence of the intact *tos* sequence (Fig. 1c). Primers Sfi1 and Sfi2, positioned outside the junction where the recombination occurs, amplify the *tos*-Zeo insertion, generating a 1.5 kb PCR product.

To linearize the BAC-*tos* in vivo, it is transferred into a $telN^+$ *E. coli* strain that constitutively expresses active TelN enzyme for resolution of the *tos* sequence by the protelomerase, generating the terminal *telL* and *tel*R telomeres (Fig. 1d) [9]. The linear BAC-tos can be column extracted for further downstream applications (Fig. 1e). The presence of telomeres on the linear BAC-*tos* can be verified by treatment with RecBCD (exonuclease V), which hydrolyzes DNA with open ends. Linear BAC-*tos* with terminal telomeres will be covalently closed and therefore resist RecBCD digestion (Fig. 1f) [9].

The linearization protocols in this chapter are described using a 100-kb BAC (BAC4396) that contains the entire human beta-globin locus [9, 13]. But the same principles can be applied to any BAC clone. The biggest advantage of linearizing BACs by recombineering is that it is not limited by size or sequence

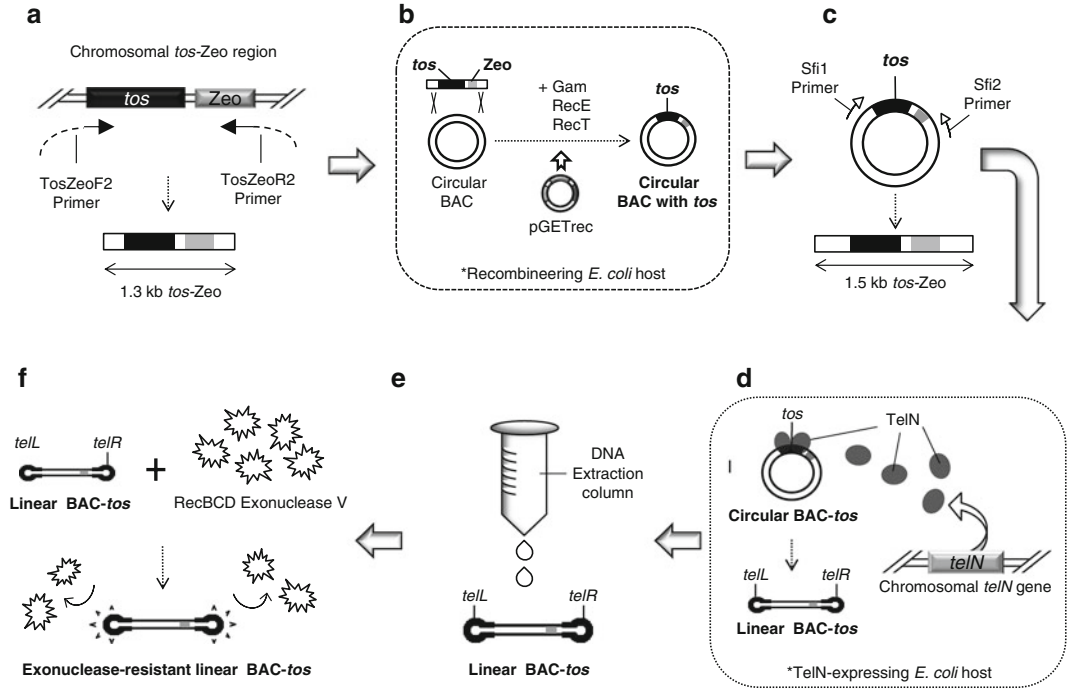

Fig. 1 Overview of BAC linearization. (**a**) Synthesizing *tos*-Zeo cassette for recombineering. A *tos*-Zeo cassette is amplified by PCR from a chromosomal *tos*-Zeo region, using primers TosZeoF2 and TosZeoR2. The resulting 1.3 kb *tos*-Zeo cassette contains the phage N15 telomeric sequence *tos* and a Zeocin resistance gene, with overhangs that complement DNA sequence at the target site on the circular BAC. (**b**) Recombineering the *tos-Zeo* sequence into BACs. First, the *tos*-Zeo cassette is transformed into an *E. coli* host carrying the target circular BAC. Expression of the recombineering enzymes (Gam, RecE, RecT) from plasmid pGETrec then induces insertion of the *tos*-Zeo cassette into the circular BAC by recombineering [11]. The precise insertion of *tos*-Zeo cassette into target BAC results in a circular BAC containing the *tos* sequence. The BAC remains circular at this stage. (**c**) PCR screening for recombinants. Zeo^R colonies that arise after recombineering are screened by PCR for the presence of the integrated *tos*-Zeo cassette. Primers Sfi1 and Sfi2 are located flanking the recombinant junction, and produce a 1.5 kb PCR product when the *tos*-Zeo cassette is correctly targeted into a BAC. (**d**) In vivo linearization of BAC-*tos*. The circular BAC-*tos* is transformed into a *telN*$^+$ *E. coli* constitutively expressing the TelN protein, resolving the *tos* sequence into individual left (*telL*) and right (*telR*) telomeres and generating a linear BAC-*tos* [9]. (**e**) Extraction of linear BAC-*tos*. Linearized BAC-*tos* DNA is then column extracted for downstream applications. (**f**) Detecting presence of telomeres. To test for the integrity of telomeric ends formed on the linearized BAC-*tos*, the DNA is incubated with RecBCD exonuclease V that degrades open-ended linear DNA. As the telomeric ends on TelN-linearized BAC-*tos* are covalently closed, they will be resistant to exonuclease attack. Diagram is not drawn to scale

composition. As a result, virtually any BAC may be linearized through the incorporation of a *tos* site, followed by telomere resolution in a TelN-expressing host [9]. Linear BACs assembled using this method may be useful for functional expression of genomic loci in cells, maintenance of linear viral genomes in their natural conformation, and for constructing innovative artificial chromosome structures for applications in mammalian and plant cells.

2 Materials

2.1 Bacterial Strains and Plasmids

1. Plasmid pGETrec [11]: This plasmid is ampicillin resistant and contains the *recE*, *recT*, and *gam* genes that are under the control of an arabinose inducible promoter, P_{BAD} [14].
2. Target BAC: BAC4396 [13]. This 100-kb BAC contains the entire human beta-globin gene cluster on a pBeloBAC11 backbone [13].
3. Bacterial host for recombineering: *Escherichia coli* DH10B. DH10B is F^- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) φ80dlacZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*Δ139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G [15]. DH10B is routinely used as the recombineering host because it is commonly used for cloning and functional studies of large BACs [16, 17]. Therefore by directly performing recombineering in this 'working' strain there will not be a need to isolate and transform the newly recombineered BAC into a different strain for downstream experiments.
4. The tos-*Zeo* carrying strain *asd*$^-$ *zeo*^{r} *tos*$^+$ *cm*^{r} DH10B [9]: This strain serves as the template to amplify the 1.3 kb *tos*-Zeo PCR cassette used for recombineering into BACs (Fig. 1a). It carries a 519 bp N15 genomic sequence that includes the 310 bp *tos* (N15 positions 24,471–24,989 bp; Gen-Bank Accession No. NC_001901) [9] (*see* **Note 1**).
5. TelN-expressing host, *telN*$^+$ DH10B (full genotype: *asd*$^-$ *zeo*^{r} *telN*$^+$ DH10B [9]): This strain is auxotrophic for diaminopimelic acid (DAP), and must be grown under zeocin selection and supplemented with 5 mM of DAP in nutrient-rich Brain Heart Infusion (BHI) broth or agar (*see* **Note 2**).

2.2 Enzymes

1. PCR enzyme pack, e.g., Long PCR Enzyme Mix from Thermo Scientific (*see* **Note 3**).
2. RecBCD (Exonuclease V): Epicentre RecBCD Enzyme Pack, including ATP.
3. Restriction enzymes and their respective buffers, e.g., *Dpn*I and SuRE/Cut buffer A from Roche.

2.3 DNA Isolation and Purification Kits

1. Plasmid extraction kit, e.g., QIAprep® Spin Miniprep Kit by Qiagen Inc.
2. DNA Gel Extraction Kit, e.g., QIAquick® Gel Extraction Kit by Qiagen Inc.
3. DNA extraction kit for linear DNA: NucleoBond® Xtra Midi/Maxi Kit (Macherey-Nagel Inc.) (*see* **Note 4**).

2.4 Primers

1. Primers for recombineering the tos-*Zeo* cassette into BAC4396:

   TosZeoF2: 5′-GAA ACC TGT CGT GCC AGC TGC ATT AAT GAA TCG GCC AAC GCG AAC CCC TTG CGG CCG CCC ttc ccc cgt ttc taa gtc tc-3′

   TosZeoR2: 5′-GCG GAT GAA TGG CAG AAA TTC GAT GAT AAG CTG TCA AAC ATG AGA ATT GGT CGA CGG CCC gca ata aac aag ttt cga gg-3′

   These primers have 60 nt sequence at the 5′ end (in upper case letters) that is homologous to a sequence centered around the *Sfi*I restriction site on the vector section of BAC4396, and a 20 nt sequence at the 3′ end (in lower case letters) that is complementary to the *tos*-Zeo region of the *asd*$^-$ *zeo*^{r} *tos*$^+$ *cm*^{r} DH10B strain (*see* Subheading 2.1) (*see* **Note 5**). Using the genomic DNA of *asd*$^-$ *zeo*^{r} *tos*$^+$ *cm*^{r} DH10B strain as the PCR template, the amplified *tos*-Zeo cassette will contain flanking sequences that are complementary to the BAC4396 (*see* **Note 6**).

2. Primers for screening BAC4396-tos clones that are generated after recombineering with *tos*-Zeo:

   Sfi1: 5′-aaa gtg taa agc ctg ggg tg-3′

   Sfi2: 5′-aac agt act gcg atg agt gg-3′

   These primers anneal just outside the recombineering target site on BAC4396, amplifying across the recombineering junctions. Recombinant BAC4396-tos clones will give a 1.5 kb product while non-recombinant BAC4396 clones without the 1.3 kb tos-*Zeo* cassette insertion will give a 300 bp product, enabling detection of the recombination event rapidly using PCR screening.

2.5 Antibiotics, Reagents, and Chemicals

1. Diaminopimelic acid (DAP), 100 mM: Dissolve 9.5 g DAP in 50 mL of distilled water in a clean beaker. Heat solution up to 50 °C while stirring. When powder has completely dissolved, cool to room temperature before filter-sterilizing.
2. Antibiotics:

No.	Selective antibiotic	Working concentration (μg/mL)	Diluent	Preparation
1	Ampicillin	100	Water	Filter-sterilized
2	Chloramphenicol	12.5	Ethanol	Not sterilized
3	Streptomycin	20	Water	Filter-sterilized
4	Zeocin	25	Water	Purchased from Invitrogen Inc. and used directly

3. Brain Heart Infusion (BHI) media, e.g., BHI agar or broth from BD Inc.
4. TE buffer for dissolving and eluting DNA: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
5. 10 % (w/v) L-(+)-arabinose for inducing pGETrec: Dissolve 5 g of dry L-(+)-arabinose in 50 mL of sterile water, filter with 0.2 μm syringe filter under sterile condition. Prepare freshly before recombineering. Keep at 4 °C until used.
6. Ice-cold 10 % (v/v) glycerol for preparing electrocompetent cells: Mix 100 mL of glycerol with 900 mL of water and autoclave. Cool at 4 °C overnight, then chill at −20 °C for 1–2 h before use (*see* **Note 7**).
7. 3 M Sodium acetate for DNA precipitation, pH 5.2: Dissolve 24.6 g of sodium acetate anhydrous in 80 mL of distilled water. Adjust pH to 5.2 with glacial acetic acid. Top up solution to 100 mL final volume and autoclave before use (*see* **Note 8**).
8. Absolute ethanol (100 % v/v) for DNA precipitation: Chill at −20 °C at least an hour before use.
9. 70 % ethanol (v/v) for DNA precipitation: Mix 700 mL of ethanol with 300 mL of distilled water.
10. Isopropanol for DNA extraction: Use at room temperature.

2.6 Specialized Equipment and Consumables

1. Electroporation unit: Gene Pulser Xcell Microbial System (Bio-Rad Inc.).
2. Gene Pulser® 0.2 cm electrode gap cuvette (Bio-Rad Inc.). Cool at −20 °C about 1 h before use (*see* **Note 9**).
3. Pulsed field gel electrophoresis (PFGE) system, e.g., CHEF-DR III System (Bio-Rad Inc.).

3 Methods

3.1 PCR Amplifying and Purifying the tos-Zeo Cassette

The main objective of this set of PCR is to amplify the *tos* sequence as part of a *tos*-Zeo cassette, and insert it into a chosen BAC clone (Fig. 1a). High fidelity DNA polymerase with proofreading activity should be used in order to ensure accurate amplification of the targeting cassette.

1. Grow strain *asd*$^-$ *zeo*^{r} *tos*$^+$ *cm*^{r} DH10B [9] in 5 mL of BHI broth (BHI is updated in Subheading 2.5) supplemented with 5 mM of DAP and zeocin at 30 °C with 150 rpm shaking for 30–48 h.
2. Use 1 μL aliquot of the *asd*$^-$ *zeo*^{r} *tos*$^+$ *cm*^{r} DH10B culture as template to obtain DNA. Prepare the following mixture in a PCR tube for each reaction (*see* **Note 10**).

No.	Component	Volume (μL)
1	10× Long PCR Buffer with 15 mM $MgCl_2$	5
2	10 mM dNTPs (Thermo Scientific)	1
3	TosZeoF2 Primer (0.5 μg/μL)	1
4	TosZeoR2 Primer (0.5 μg/μL)	1
5	*asd*$^-$ *zeo*^{r} *tos*$^+$ *cm*^{r} DH10B culture	1
6	Long PCR Enzyme Mix (5 U/μL)	0.5
7	Nuclease-free water	40.5
	Total	50

3. Subject the mixture to the following cycling condition:

Step	Temperature (°C)	Duration (s)	
1	95	120	
2	95	30	
3	45	30	35 cycles
4	68	120	
5	68	600	
6	4	Forever	

4. Electrophorese the PCR product in a 1 % (w/v) agarose gel (*see* **Note 11**).
5. Stain the gel with ethidium bromide or other nucleic acid stains, and visualize gel with a UV light box to confirm that a 1.3 kb band is present (*see* **Note 12**). Carefully excise the 1.3 kb band into clean microcentrifuge tubes with a clean, sharp scalpel.
6. Extract the DNA from the excised agarose using a gel extraction kit according to the manufacturer's instructions, e.g., QIAquick® Gel Extraction Kit by Qiagen Inc.
7. If using QIAquick® Gel Extraction Kit by Qiagen Inc., weigh the gel slices into a 2 mL microcentrifuge tube. By putting maximum 400 mg of gel into each tube, add 3 volumes of Buffer QG to the gel e.g., for every 100 mg of gel 300 μL of Buffer QG is added.
8. Incubate the gel and QG buffer mixture at 50 °C for 10 min or until gel has completely dissolved. Vortex the mixture every 2–3 min to help dissolve the gel.
9. Add 1 volume of isopropanol to the mixture, then mix by inverting the tube.
10. Apply the mixture from each tube into one QIAquick column, then centrifuge at 15,000 × *g* for 1 min at room temperature. Discard flow-through.

11. Add 500 μL of Buffer QG to column and repeat centrifuge at 15,000 × *g* for 1 min at room temperature. Discard flow-through.
12. Add 750 μL of Buffer PE to column. Incubate column for 2–5 min, then centrifuge at 15,000 × *g* for 1 min at room temperature.
13. Discard flow-through, then repeat centrifuge at 15,000 × *g* for 1 min at room temperature to remove residual Buffer PE.
14. To elute the PCR product, transfer column to a clean collection tube, then add 50 μL of distilled water (pH 8.5) into the center of the column. Let the column stand for 1 min before centrifuging at 15,000 × *g* for 1 min at room temperature.
15. To remove potential contaminating bacterial genomic DNA of the *asd*$^-$ *zeo*^{r} *tos*$^+$ *cm*^{r} DH10B strain, the purified PCR product is digested with *Dpn*I. *Dpn*I is able to specifically digest methylated bacterial DNA while leaving the newly synthesized PCR product intact, which is unmethylated. Set up the *Dpn*I digestion as follows:

No.	Component	Volume (μL)
1	10× SuRE/Cut buffer A	6
2	Purified PCR product in water	50
3	*Dpn*I (10 U/μL)	3
4	Nuclease-free water	1
	Total	60

16. Precipitate the PCR product by mixing the *DpnI-digested* DNA with 50 μL of distilled water (pH 8.5), 10 μL of 3 M sodium acetate (pH 5.2), and 250 μL of −20 °C absolute ethanol.
17. Shake the mixture vigorously and incubate at −80 °C for 30 min.
18. Centrifuge at 15,000 × *g* for 15 min at room temperature.
19. Wash the pellet with 250 μL of 70 % ethanol and centrifuge at 15,000 × *g* for 5 min at room temperature.
20. Remove the remaining supernatant with a micropipette tip and let the pellet air-dry for 1–3 min.
21. Resuspend the pellet in 5–10 μL of TE buffer.

3.2 Transferring the Recombineering Plasmid pGETrec into DH10B (BAC4396)

Transforming plasmid pGETrec into an *E. coli* strain introduces the recombineering machinery into this host [11]. Therefore, in order to introduce recombineering ability into DH10B (BAC4396), i.e., DH10B strain carrying the human beta-globin

BAC4396, pGETrec is transformed into this host. This step generates strain DH10B (pGETrec, BAC4396), where both plasmid pGETrec and BAC4396 coexist in a host that is proficient to carry out recombineering (Fig. 1b) (*see* **Note 13**).

Perform all procedures under sterile condition unless otherwise specified. Keep cells cool on ice at all times. Precool all plasticware (centrifuge bottles, microcentrifuge tubes, pipette tips, serological pipettes, etc.) and glassware at –20 °C for a few hours. These materials should be kept at –20 °C up until immediately before they come into contact with bacterial cells (*see* **Note 14**).

1. Grow a 5 mL overnight starter culture of DH10B (BAC4396) in LB at 220 rpm and 37 °C, supplemented with chloramphenicol.
2. Inoculate a fresh 250 mL of LB broth containing chloramphenicol with the DH10B (BAC4396) starter culture.
3. Grow the culture in a shaker at 220 rpm and 37 °C until it reaches exponential stage, specifically at OD_{600} of 0.55–0.65 (*see* **Note 15**).
4. Harvest the cells by aliquoting the culture into a precooled sterile centrifuge bottle.
5. Incubate the culture at –20 °C for 20 min, swirling the bottle gently at 3-min intervals.
6. Centrifuge the bottle at 6,800 × *g*, at –2 °C, for 12 min.
7. Sit the bottle on ice, and carefully pour off the broth, leaving the pellet intact at the bottom of the bottle.
8. Gently, but thoroughly, resuspend the cell pellet in 250 mL of ice-cold 10 % (v/v) glycerol using a precooled 10 mL serological pipette (*see* **Note 16**).
9. Centrifuge the resuspended culture at 6,800 × *g*, at –2 °C, for 12 min.
10. Repeat the glycerol washing steps with 250 mL of ice-cold 10 % (v/v) glycerol (*see* **steps 7–9**) twice.
11. Carefully pour off all supernatant, leaving the pellet at the bottom.
12. Leave the bottle upright on ice for 2–3 min so that any residual 10 % (v/v) glycerol from the sides is collected at the bottom.
13. Resuspend the pellet in the residual glycerol using a precooled 1 mL micropipette tip with a wide bore (*see* **Note 17**).
14. Collect the entire volume of resuspended cells into one chilled microcentrifuge tube before dispensing 45 μL of aliquots each into individual chilled microcentrifuge tubes (*see* **Note 18**).
15. Using a precooled pipette tip, add 1–2 μL (approximately 0.01–0.05 μg) of pGETrec plasmid into a tube containing

45 μL of the DH10B (BAC4396) electrocompetent cells. Transfer the cells and DNA mixture into a prechilled 0.2 cm electrode gap cuvette; keep cuvette on ice until it is ready for electroporation.

16. To estimate transformation efficiency, perform a control transformation reaction by adding a clean preparation of 10 pg of pUC19 control plasmid DNA (e.g., obtained from Invitrogen Inc.) into one tube of electrocompetent cells (*see* **Note 19**).
17. Transfer the cells and control plasmid mixture into a prechilled 0.2 cm electrode gap cuvette; keep cuvette on ice until it is ready for electroporation.
18. Just before electroporation, firmly tap the cuvette on a flat surface, e.g., the lab bench twice to bring all cells down to the bottom.
19. Wipe the cuvette dry before placing it into the electroporation cuvette chamber (*see* **Note 20**).
20. Set the electroporation unit to the following setting: 2.5 kV, 200 Ω, and 25 μF.
21. Press the pulse button located on the pulse controller unit. As soon as a beep sounds, release the pulse button (*see* **Note 21**).
22. Immediately remove the cuvette from chamber and add 1 mL of LB broth without any antibiotic (*see* **Note 22**).
23. Using a glass Pasteur pipette transfer the cells from the cuvette into a 14 mL round bottom snap-cap tube (*see* **Note 23**).
24. Grow the cells for 60 min at 220 rpm, 37 °C. This will help the cells recover from the electroporation treatment.
25. At the end of the recovery incubation, serially dilute transformed cells and plate them onto LB agar plates containing chloramphenicol and ampicillin.
26. Incubate plates at 37 °C for overnight.
27. Pick 15–20 *zeo*^{r} *amp*^{r} colonies [i.e., putative DH10B (pGETrec, BAC4396) colonies] onto a single LB agar plate containing chloramphenicol and ampicillin with a sterile tooth pick, and grow the plate at 37 °C overnight.
28. Pick and grow each colony from the plate in LB broth, supplemented with chloramphenicol and ampicillin, at 220 rpm, 37 °C, overnight (*see* **Note 24**).

3.3 Recombineering tos-Zeo Cassette into BAC4396

The main objective of this recombineering step is to introduce the *tos* sequence into the circular BAC4396 by insertion of the *tos*-Zeo cassette (Fig. 1b). The *tos*-Zeo cassette is electroporated into DH10B (pGETrec, BAC4396) (*see* Subheading 3.2) and, once plasmid pGETrec is induced to express the recombineering genes, will insert into the target site on BAC4396 (Fig. 1b).

All procedures listed here should be performed under sterile condition unless otherwise specified. Keep cells cool on ice at all times. Precool all plasticware (centrifuge bottles, microcentrifuge tubes, pipette tips, serological pipettes, etc.) and glassware at –20 °C for at least a few hours until immediately before they come into contact with bacterial cells (*see* **Note 14**).

1. Grow a 5 mL starter culture of DH10B (pGETrec, BAC4396) in LB at 150 rpm and 30 °C for 30–48 h, supplemented with ampicillin and chloramphenicol (*see* **Notes 1** and **25**).
2. Inoculate a fresh 250 mL of LB broth containing ampicillin and chloramphenicol with the starter culture of DH10B (pGETrec, BAC4396) culture.
3. Grow the culture in a shaker at 150 rpm and 30 °C until it reaches exponential stage, specifically at OD_{600} of 0.45–0.50, before the culture is induced in the next step (*see* **Note 26**).
4. To induce the expression of the recombineering genes *recE*, *recT*, and *gam* on plasmid pGETrec, supplement the culture with L-(+)-arabinose to a final concentration of 0.2 % (w/v) under sterile condition (*see* **Note 27**).
5. Return the culture to grow at 150 rpm and 30 °C, until OD_{600} reaches between 0.55 and 0.65 (*see* **Note 28**).
6. Harvest cells by aliquoting the culture into a precooled sterile centrifuge bottle.
7. Incubate the culture at –20 °C for 20 min, swirling the bottle gently at 3-min intervals.
8. Centrifuge the bottle at 6,800 × *g*, at –2 °C, for 12 min.
9. Sit the bottle on ice, and carefully pour off the broth, leaving the pellet intact at the bottom of the bottle.
10. Gently but thoroughly resuspend the cell pellet in 250 mL of ice-cold 10 % (v/v) glycerol using a precooled 10 mL serological pipette.
11. Centrifuge the resuspended culture at 6,800 × *g*, at –2 °C, for 12 min.
12. Repeat the glycerol washing steps with 250 mL of ice-cold 10 % (v/v) glycerol (*see* **steps 9–11**) twice.
13. Sit the bottle on ice, and carefully remove all supernatant with a precooled serological pipette.
14. Leave the bottle upright on ice for 2–3 min, so that residual 10 % (v/v) glycerol is collected at the bottom.
15. Resuspend the pellet in the residual glycerol using a precooled 1 mL micropipette tip with a wide bore.
16. Collect the entire volume of resuspended cells into one chilled microcentrifuge tube, before dispensing 45 μL of aliquots each into individual chilled microcentrifuge tubes.

17. Using a precooled pipette tip, add 1–2 μL of precooled DNA in TE buffer containing approximately 0.3–1.0 μg of purified *tos*-Zeo PCR cassette (*see* Subheading 3.1) into a tube of the DH10B (pGETrec, BAC4396) electrocompetent cells. Transfer the cells and DNA mixture into a prechilled 0.2 cm electrode gap cuvette; keep cuvette on ice until it is ready for electroporation.
18. To estimate transformation efficiency, perform a control transformation reaction by adding a clean preparation of 10 pg of pZeoSV2 (+) control plasmid DNA into one tube of electrocompetent cells (*see* **Notes 29** and **30**).
19. Transfer the cells and DNA mixture into a prechilled 0.2 cm electrode gap cuvette; keep cuvette on ice until it is ready for electroporation.
20. Just before electroporation, firmly tap the cuvette on a flat surface, e.g., the lab bench twice to bring all cells down to the bottom.
21. Wipe the cuvette dry before placing it into the electroporation cuvette chamber.
22. Set the electroporation unit to the following setting: 2.5 kV, 200 Ω, and 25 μF.
23. Press the pulse button located on the pulse controller unit. As soon as a beep sounds, release the pulse button (*see* **Note 21**).
24. Immediately remove the cuvette from chamber and add 1 mL of BHI broth without any antibiotic added.
25. Using a glass Pasteur pipette transfer cells from the cuvette into a 14 mL round bottom snap-cap tube, and grow them for 120 min at 150 rpm, 30 °C. This will help the cells recover from the electroporation treatment (*see* **Note 31**).
26. At the end of recovery incubation, serially dilute transformed cells and plate onto LB agar plates containing chloramphenicol and zeocin (*see* **Note 32**).
27. Incubate plates at 30 °C for at least 20 h.
28. Pick 15–20 *zeo*^{r} *cm*^{r} colonies [i.e., putative DH10B (BAC4396-tos) colonies], spot them onto a single LB agar plate containing chloramphenicol and zeocin with a sterile tooth pick and grow the plate at 30 °C for 24–48 h.
29. Pick and grow each colony from the plate in LB broth, supplemented with chloramphenicol and zeocin, at 150 rpm, 30 °C for 30–48 h (*see* **Note 24**).

3.4 PCR Screening of BAC-tos

Perform PCR screening using primers Sfi1 and Sfi2 (Fig. 1c) on the cultures grown from Subheading 3.3, **step 29** to identify correctly inserted *tos*-Zeo in individual *zeo*^{r} *cm*^{r} colonies, i.e.,

putative DH10B (BAC4396-tos) clones. Set up the PCR according to the instructions provided by the enzyme manufacturer; optimization may be necessary for a different enzyme (*see* **Note 33**).

1. Grow 5 mL each of the *zeo*^{r} *cm*^{r} colonies at 150 rpm at 30 °C for 30–48 h in LB broth supplemented with chloramphenicol and zeocin.
2. Mix the following components in a PCR tube for each reaction:

No.	Component	Volume (μL)
1	10× Long PCR Buffer with 15 mM $MgCl_2$	5
2	10 mM dNTPs (Thermo Scientific)	1
3	Sfi1 Primer (0.5 μg/μL)	1
4	Sfi2 Primer (0.5 μg/μL)	1
5	DH10B (BAC4396-tos) culture	1
6	Long PCR Enzyme Mix (5 U/μL)	0.5
7	Nuclease-free water	40.5
	Total	50

3. Subject the reaction to the following cycling condition:

Step	Temperature (°C)	Duration (s)	
1	95	120	30 cycles
2	95	30	
3	55	30	
4	68	120	
5	68	600	
6	4	Forever	

4. Electrophorese the resulting PCR product in a 1 % (w/v) agarose gel. Positive recombinants with the *tos*-Zeo cassette correctly integrated, i.e., BAC4396-tos, will produce a 1.5 kb band, while non-recombinants without *tos*-Zeo cassette integration (or with *tos*-Zeo integration at other unintended sites) will produce a 300 bp product (Fig. 1c) (*see* **Note 34**).

3.5 Separating Recombinant BAC-tos from pGETrec Plasmid

In this section, the newly recombineered BAC4396-tos, which is generated in Subheading 3.3 and validated in Subheading 3.4, is isolated from the recombineering host and re-transformed into freshly made DH10B competent cells (*see* **Note 32**).

1. Purify circular BAC4396-tos from DH10B (BAC4396-tos) clones (Subheading 3.4), which have been previously verified

by sequencing, using standard plasmid extraction kit according to manufacturer's instructions, e.g., QIAprep Spin Miniprep Kit by Qiagen Inc.

2. If using QIAprep Spin Miniprep Kit, pellet down DH10B (BAC4396-tos) which has been grown in 5 mL of LB broth for 20–24 h at 30 °C and 150 rpm by centrifuging at 6,800 × *g*, room temperature for 5 min. Remove all supernatant by pipetting.
3. Resuspend the pellet in 250 μL of Buffer P1 containing RNase A, then transfer cell suspension to a microcentrifuge tube.
4. Add 250 μL of Buffer P2, invert tube to mix suspension thoroughly.
5. Add 350 μL of Buffer N3, then immediately invert tube to mix.
6. Centrifuge sample for 10 min at 15,000 × *g*, room temperature for 10 min.
7. Carefully pipette out supernatant from tube without removing the white debris, then load into a QIAprep column.
8. Wash the column by adding 500 μL of Buffer PB and centrifuging at 15,000 × *g*, room temperature for 1 min. Discard flow-through.
9. Add 750 μL of Buffer PE into column and centrifuge at 15,000 × *g*, room temperature for 1 min. Discard flow-through.
10. Repeat centrifugation at 15,000 × *g*, room temperature for 1 min to remove residual Buffer PE.
11. To elute the DNA, transfer column to a clean collection tube, then add 50 μL of TE buffer to the column. Let column stand for 1 min, then centrifuge at 15,000 × *g*, room temperature for 1 min.
12. Digest the eluted DNA with *Dpn*I, followed by precipitation as described in Subheading 3.1, **steps 15–21**.
13. Quantify the precipitated BAC4396-tos DNA by spectrophotometer, and set up a restriction digestion reaction with a small DNA aliquot:

No.	Component	Volume (μL)
1	10× SuRE/Cut buffer A	2
2	~100 ng BAC4396-tos, diluted in water	17
3	*Nar*I (10 U/μL)	1
	Total	20

14. Visualize the digested BAC4396-tos by pulsed-field electrophoresis (PFGE) to verify the intactness of the isolated BAC4396-tos.
15. Grow a 5 mL starter culture of DH10B in LB at 150 rpm for 30–48 h at 30 °C, supplemented with streptomycin.
16. Inoculate a fresh 250 mL of LB broth containing streptomycin with the DH10B starter culture.
17. Grow the culture in a 30 °C shaker at 150 rpm until it reaches an OD_{600} of 0.55–0.65 (*see* **Note 15**).
18. Harvest cells by aliquoting the culture into a precooled sterile centrifuge bottle.
19. Incubate the culture at –20 °C for 20 min, swirling the bottle gently at 3-min intervals.
20. Centrifuge the bottle at 6,800 × *g*, –2 °C, for 12 min.
21. Sit the bottle on ice, and carefully remove the broth, leaving the pellet intact at the bottom of the bottle.
22. Gently but thoroughly resuspend the cell pellet in 250 mL of ice-cold 10 % (v/v) glycerol using a precooled 10 mL serological pipette.
23. Centrifuge the resuspended culture at 6,800 × *g*, at –2 °C, for 12 min.
24. Repeat the glycerol washing steps with 250 mL of ice-cold 10 % (v/v) glycerol (*see* **steps 21–23**) twice.
25. Sit the bottle on ice, and carefully remove all supernatant with a precooled serological pipette.
26. Leave the bottle upright on ice for 2–3 min, so that residual 10 % (v/v) glycerol collects at the bottom.
27. Resuspend the pellet in the residual glycerol using a precooled 1 mL micropipette tip with a wide bore.
28. Collect the entire volume of resuspended cells into one chilled microcentrifuge tube, before dispensing 45 μL of aliquots each into individual chilled microcentrifuge tubes.
29. Using a precooled wide bore pipette tip, add 1–2 μL of BAC4396-tos DNA (100–200 ng) in TE buffer into a tube containing a 45 μL aliquot of DH10B electrocompetent cells. Transfer the cells and DNA mixture into a prechilled 0.2 cm electrode gap cuvette; keep cuvette on ice until it is ready for electroporation.
30. Perform a control transformation reaction by adding a clean preparation of 10 pg of pZeoSV2 (+) control plasmid DNA into one tube of electrocompetent cells.
31. Transfer the cells and control DNA mixture into a prechilled 0.2 cm electrode gap cuvette; keep cuvette on ice until it is ready for electroporation.

32. Just before electroporation, firmly tap the cuvette on a flat surface, e.g., the lab bench twice to bring all cells down to the bottom.
33. Wipe the cuvette dry before placing it into the electroporation cuvette chamber.
34. Set the electroporation unit to the following setting: 2.5 kV, 200 Ω and 25 μF.
35. Press the pulse button located on the pulse controller unit. As soon as a beep sounds, release the pulse button (*see* **Note 21**).
36. Immediately remove the cuvette from chamber and add 1 mL of LB broth without adding any antibiotic.
37. Using a glass Pasteur pipette transfer cells from the cuvette into a 14 mL round bottom snap-cap tube, and grow them for 120 min at 150 rpm and 30 °C to help the cells recover from the electroporation treatment.
38. At the end of recovery incubation, serially dilute transformed cells and plate onto LB agar plates containing chloramphenicol and zeocin.
39. Incubate plates at 30 °C for 16–48 h.
40. Pick 15–20 *zeo*^{r} *cm*^{r} colonies [i.e., putative DH10B (BAC4396-tos) colonies] onto a single LB agar plate containing chloramphenicol and zeocin with a sterile tooth pick and grow the plate at 30 °C for 24–48 h.
41. Pick and grow each colony from the plate in LB broth, supplemented with chloramphenicol and zeocin, at 150 rpm, 30 °C for 36–48 h (*see* **Note 24**).

3.6 In Vivo Linearization of BAC-tos

In this section circular BAC4396-tos DNA is introduced into a TelN-expressing *E. coli* host cell (*telN*$^+$ *E. coli*) (Fig. 1d) to resolve the *tos* sequence into telomeres in vivo, linearizing the BAC. All steps prior to electroporation are performed at room temperature unless otherwise stated. During electroporation, care must be taken to keep the cells chilled as much as possible to maximize their viability and thence transformation efficiency.

1. Isolate circular BAC4396-tos from PCR verified DH10B (BAC4396-tos) clones with standard plasmid extraction kit, e.g., QIAprep Spin eluting the DNA in 50 μL of TE buffer (*see* Subheading 3.5, **steps 2–11**). Run a diluted sample by PFGE to confirm identity and integrity of isolated DNA (*see* **Note 35**).
2. Once verified, precipitate the DNA. Mix eluted DNA with 50 μL of distilled water (pH 8.5), 10 μL of 3 M sodium acetate (pH 5.2) and 250 μL of −20 °C absolute ethanol.

3. Shake the mixture vigorously and incubate at −80 °C for 30 min (*see* **Note 36**).
4. Centrifuge at 15,000 × *g* for 15 min at room temperature.
5. Wash the pellet with 250 μL of 70 % ethanol and centrifuge at 15,000 × *g* for 5 min at room temperature.
6. Remove the remaining supernatant with a micropipette tip and let the pellet air-dry for 5–10 min.
7. Resuspend the pellet in 5–10 μL of TE buffer.
8. Evaluate the quality of precipitated DNA by PFGE.
9. Grow the *telN*+ DH10B strain in 5 mL of LB broth supplemented with streptomycin, zeocin, and 5 mM of DAP at 30 °C with 150 rpm shaking for 30–48 h.
10. Dilute the 5 mL *telN*+ DH10B culture into 250 mL of LB broth containing streptomycin, zeocin and 5 mM of DAP.
11. Grow the diluted culture in a 30 °C shaker at 150 rpm until the culture reaches an OD_{600} of 0.55–0.65 (*see* **Note 15**).
12. Harvest the cells by aliquoting the culture into a precooled sterile centrifuge bottle.
13. Incubate the culture at −20 °C for 20 min, swirling the bottle gently at 3-min intervals.
14. Centrifuge the bottle at 6,800 × *g*, at −2 °C, for 12 min.
15. Sit the bottle on ice, and carefully remove the broth, leaving the pellet intact at the bottom of the bottle.
16. Gently but thoroughly resuspend the cell pellet in 250 mL of ice-cold 10 % (v/v) glycerol using a precooled 10 mL serological pipette.
17. Centrifuge the resuspended culture at 6,800 × *g*, at −2 °C, for 12 min.
18. Repeat the glycerol washing steps with 250 mL of ice-cold 10 % (v/v) glycerol (*see* **steps 15–17**) twice.
19. Sit the bottle on ice and carefully remove all supernatant with a precooled serological pipette.
20. Leave the bottle upright on ice for 2–3 min, so that residual 10 % (v/v) glycerol is collected at the bottom.
21. Resuspend the pellet in the residual glycerol using a precooled 1 mL micropipette tip with a wide bore.
22. Collect the entire volume of resuspended cells into one chilled microcentrifuge tube, before dispensing 45 μL aliquots each into individual chilled microcentrifuge tubes.
23. Electroporate the *telN*+ DH10B cells with 0.1–0.5 μg of the precipitated BAC4396-tos DNA. For control, electroporate

10 pg of pUC19 plasmid following the protocol described in Subheading 3.2 (*see* **steps 15–23**).

24. Grow the cells at 30 °C, 150 rpm for 120 min for recovery.
25. At the end of recovery growth, serially dilute cells and plate them onto BHI agar plates containing chloramphenicol, streptomycin, zeocin and 5 mM of DAP. Incubate plates at 30 °C for 24–48 h.
26. Pick individual transformed *telN*$^+$ DH10B (BAC4396-tos) colonies onto a fresh BHI agar plate containing chloramphenicol, zeocin and 5 mM of DAP with a sterile tooth pick and incubate at 30 °C until colonies arise (30–48 h).
27. Grow each clone in BHI broth supplemented with chloramphenicol, zeocin and 5 mM of DAP at 150 rpm, 30 °C, for 30–48 h until they reach late-log growth (*see* **Notes 1** and **37**).

3.7 Extraction of Linear BAC-tos

Linear BAC-*tos* is extracted from *telN*$^+$ DH10B (BAC4396-tos) to check for the identity of the newly recombineered BAC-*tos* and to obtain purified linear DNA for downstream applications (Fig. 1e). To obtain large quantity of intact linear BAC4369-tos DNA, we use the Macherey-Nagel's NucleoBond® Xtra Midi or Maxi Kit (*see* **Note 38**).

The procedures described below are based on the protocol provided by the manufacturer for purification of low-copy plasmid using NucleoBond® Xtra Midi Kit, with minor modifications. Perform all steps at room temperature unless otherwise specified.

1. Grow 5 mL of *telN*$^+$ DH10B (BAC4396-tos) starter culture in LB broth containing chloramphenicol, zeocin and 5 mM of DAP for 36–48 h at 30 °C with 150 rpm shaking.
2. Dilute the 5 mL starter culture into 200 mL of LB broth supplemented with chloramphenicol, zeocin and 5 mM of DAP, and grow for 36–48 h at 30 °C with 150 rpm shaking.
3. Warm the Elution Buffer ELU to 50 °C.
4. Harvest the cultures into a centrifuge bottle and centrifuge at 6,000 × *g*, 4 °C for 15 min.
5. Carefully pour off the supernatant and completely resuspend the pellet in 16 mL of Resuspension Buffer RES + RNase A with a serological pipette.
6. Add in 16 mL of Lysis Buffer LYS and invert the bottle gently five times (*see* **Note 39**). Incubate at room temperature for 5 min.
7. While the suspension is incubating, equilibrate a NucleoBond® Xtra Column and its inserted column filter by directly pipetting 12 mL of Equilibration Buffer EQU onto the rim of the column filter, making sure that the entire column is wet. Let the column empty by gravity flow (*see* **Note 40**).

8. Add 16 mL of Neutralization Buffer NEU to the suspension. Immediately invert the bottle 10–15 times (*see* **Note 41**).
9. Gently invert the suspension again three times, and without delay carefully pour the content into the equilibrated column. Continue to refill the column until all of the suspension has been loaded. Let the column empty by gravity flow.
10. Wash the column by directly pipetting 5 mL of Equilibration Buffer EQU onto the rim of the column filter. Let the column empty by gravity flow.
11. Remove and discard the column filter (*see* **Note 42**).
12. Wash the column by adding 8 mL of Wash Buffer WASH into the column. Let the column empty by gravity flow.
13. Remove the flow-through collection tray and place a sterile centrifuge tube of 10 mL or bigger capacity directly underneath the column (*see* **Note 43**).
14. Elute the BAC DNA by adding 5 mL of pre-warmed Elution Buffer ELU (*see* **step 3**), 1 mL at a time, into the column (*see* **Note 44**). The eluate should flow directly into the centrifuge tube.
15. Add 3.5 mL of room-temperature isopropanol to the eluted DNA. Secure the tube tightly and vortex vigorously for 10 s. Incubate the mixture at room temperature for 2 min.
16. Centrifuge the mixture at 17,000 × *g* for 45 min at room temperature (*see* **Note 45**).
17. Carefully remove the supernatant with a pipette tip while avoiding the pellet (*see* **Note 46**).
18. Wash the pellet with 2 mL of room-temperature 70 % (v/v) ethanol. Centrifuge at 17,000 × *g* for 5 min at room temperature.
19. Carefully remove the supernatant with a pipette tip and briefly centrifuge for a few seconds again to bring down the residual ethanol. Remove the last remaining residual ethanol with a micropipette tip.
20. Dry the pellet at room temperature for 5–10 min (*see* **Note 47**).
21. Gently resuspend the pellet in 100–150 μL of TE buffer, and incubate in a 37 °C water bath with constant agitation for 30–60 min to dissolve the pellet (*see* **Note 48**).
22. Determine the DNA yield using a spectrophotometer and check the quality by electrophoresing a small aliquot.
23. Subject an aliquot of the DNA to restriction digestion and analyze the restriction patterns by PFGE (Fig. 2). For single-site digestion, e.g., by *Nar*I (Fig. 2a), two bands are observed for linearized BAC4396-tos (lanes 5–7), while only one band will be present for circular BAC4396-tos (lane 8). For restriction

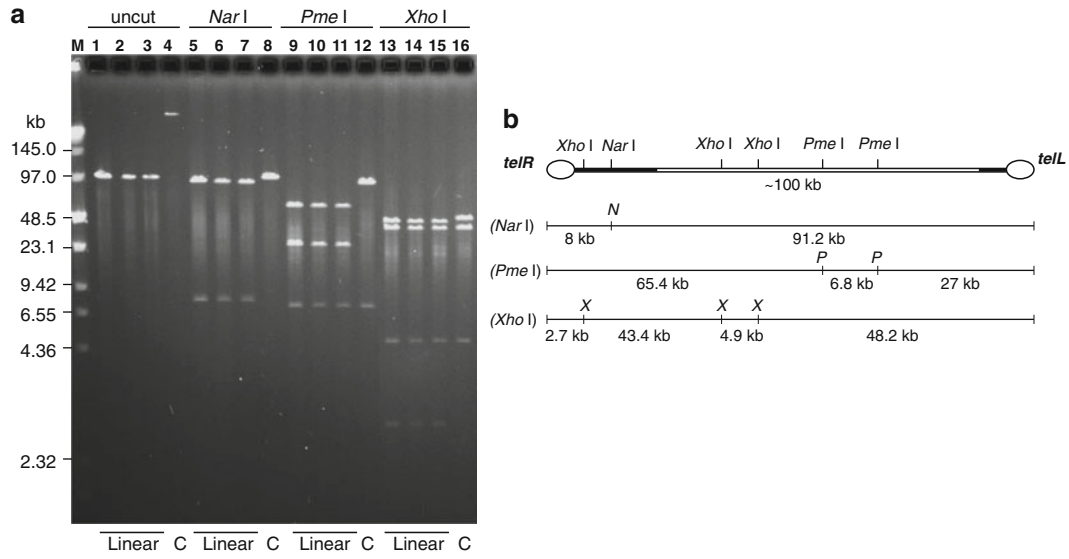

Fig. 2 In vivo linearization of BAC-*tos*. (**a**) Pulsed-field gel electrophoresis analysis of three independent clones of the 100 kb BAC4396-tos DNA, linearized in vivo, compared with the parent circular BAC4396-tos. *Lane M*, low range PFG marker (New England Biolabs Inc.); *lanes 1–3*, uncut TelN-linearized 100 kb BAC4396-tos; *lanes 5–7*, *9–11*, and *13–15* show TelN-linearized BAC4396-tos digested with *Nar*I, *Pme*I, and *Xho*I, respectively. *Lane 4*, uncut circular 100 kb BAC4396-tos; *lanes 8*, *12*, and *16*, circular 100 kb BAC4396-tos digested with *Nar*I, *Pme*I, and *Xho*I, respectively. The conformation of the DNA is indicated as either Linear or circular (labeled 'C'). (**b**) Predicted *Nar*I, *Pme*I, and *Xho*I restriction fragments for linear BAC4396-tos. Both **a** and **b** are reprinted from [9] with permission from Elsevier

digestions with more than one cut site, e.g., by *Pme*I and *Xho*I (Fig. 2b), an additional band is observed for linearized BAC4396-tos (lanes 9–11, and lanes 13–15) compared to its circular counterpart (lanes 12 and 16), hence verifying the linear conformation of BAC4396-tos.

3.8 Detecting the Presence of Telomeres on Linear BAC-tos

To detect the presence of *telL* and *telR* telomeres on the linear BAC4396-tos, a RecBCD assay is performed [9] (Fig. 1f). The presence of telomeres will enable BAC4396-tos to resist RecBCD enzyme digestion and remain intact (*see* **Note 49**). Perform all steps at room temperature unless otherwise stated.

1. Mix 300 ng of linear BAC4396-tos, diluted in 20.5 μL of distilled water, with 2.5 μL of 10× Reaction Buffer, 1 μL of 25 mM ATP and 1 μL of 10 U/μL RecBCD.
2. Incubate the mixture at 37 °C for 45 min with gentle mixing every 15–20 min.
3. Perform the same reaction on intact circular BAC4396-tos (positive control for RecBCD resistance) and restriction enzyme-linearized circular BAC4396-tos (positive control for RecBCD exonuclease activity).

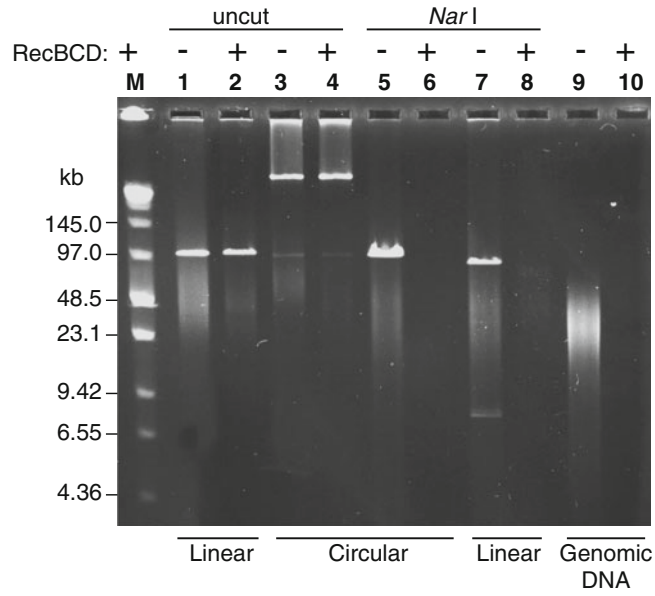

Fig. 3 Detection of hairpin telomeres on BAC-*tos* ends by RecBCD digestion. Linear BAC4396-tos with telomeres is resistant to RecBCD exonuclease digestion. Linear BAC4396-tos is treated in vitro with RecBCD and resolved using pulsed-field gel electrophoresis. *Lane M*, low range PFG marker (New England Biolabs Inc.); *lanes 1–2*, uncut TelN-linearized BAC4396-tos; *lanes 3–4*, uncut circular BAC4396-tos; *lanes 5–6*, circular BAC4396-tos digested with *Nar*I; *lanes 7–8*, TelN-linearized BAC4396-tos digested with *Nar*I; *lanes 9–10*, total genomic DNA from HT1080 cells. Lanes marked '+' denote DNA treated with RecBCD exonuclease, while lanes marked '–' denote control DNA not treated with RecBCD exonuclease prior to electrophoresis. Reprinted from [9] with permission from Elsevier

4. Examine the samples by PFGE (Fig. 3). BAC4396-tos linearized by TelN will be resistant to RecBCD attack and travel as an intact 100 kb band (lane 2), demonstrating presence of the protective telomeres. Circular BAC4396-tos will similarly be resistant due to the absence of open ends, and travel as a supercoiled band (lane 4). When the same circular and linear BAC4396-tos are first linearized by restriction enzyme *Nar*I to expose their DNA ends (lanes 5 and 7), both samples will be destroyed by subsequent RecBCD digestion (lanes 6 and 8).

4 Notes

1. This *asd*$^{-}$ *zeo*^{r} *tos*$^{+}$ *cm*^{r} DH10B strain as well as other strains containing *tos* DNA or that will be inserted with *tos* DNA, should be grown at less vigorous conditions in order to prevent rearrangement of the N15 telomeric sequence [9, 18].

All cultures on plates should be incubated at 30 °C for at least 20–24 h. The exact timeline can be judged by looking for the presence of well grown colonies on the plate. All liquid culture should be grown at 30 °C with shaking at 150 rpm for 30–48 h. For liquid culture, it is important to determine the exact time it needs to grow until it reaches late log phase. An OD_{600} reading should be taken at the end of the growth in order to ensure the culture density is at the late log phase. For the DH10B host, we always aim for OD_{600} of 1.0–1.2. This is the reading we commonly get at the late log phase for this strain. We recommend taking the OD_{600} reading when the strains are grown for the first time in the lab and ensuring they are given enough time to reach a value of 1.0–1.2. Once this is worked out, all subsequent growth of the strain can be based on this timeline.

2. The chromosomally integrated TelN gene is constitutively expressed in this *telN*$^+$ DH10B strain and it is used for in vivo resolution of *tos*-containing BACs into linear BACs (Fig. 1d) [9].
3. For amplification of the 1.3 kb *tos*-Zeo cassette, we strongly recommend using long-template PCR enzymes that possess proofreading activity because the sequences of the targeting cassette, especially the *tos* element, need to be faultless in order for TelN to process it during resolution. However, for routine screening of recombinants by PCR, where a size difference between recombinants and non-recombinant is used to detect a recombineering event, regular DNA Taq Polymerase without proofreading activity, e.g., DNA Taq Polymerase by Invitrogen Inc., may be used.
4. The Resuspension Buffer RES should be kept at 4 °C after addition of RNase.
5. When designing long primers for recombineering, we recommend ordering a high-quality HPLC-purification step in order to quality control for full-length products.
6. The targeted region around the *Sfi*I site can be found on the backbone of BAC clones from many BAC libraries. Therefore TosZeoF2 and TosZeoR2 may be used as universal primers for targeting the *tos*-Zeo cassette into BAC clones.
7. The 10 % (v/v) glycerol needs to be ice-cold to preserve viability of the electrocompetent cells in order to maximize recombineering efficiency.
8. When working with glacial acetic acid, work in a fume hood with safety goggles and protective gloves.
9. The cuvettes should be chilled before electroporation. This will help maximize transformation and/or recombineering efficiency.

10. We typically prepare eight or more reactions and pool them together to obtain enough PCR product for purification.
11. We like to make an elongated well on the gel by taping several wells on the well comb together during agarose gel preparation. After PCR the contents of all the tubes are loaded slowly into this long well. This way the DNA band will migrate in one long well and can be subsequently cut out and purified cleanly without having to deal with intervening agarose fragments between wells.
12. Always wear protective gear and goggles when working under UV light.
13. Plasmid pGETrec [11] is a 6.6 kb high-copy plasmid that carries the recombineering genes, *recE*, *recT*, and *gam*. Because of its small size calcium chloride transformation [19, 20] can also be used for transformation of this plasmid into the *E. coli* host.
14. Exposing cells to room temperature will severely reduce transformation efficiency and the resulting number of positive clones.
15. The time required to reach OD_{600} of 0.55–0.65 may vary between cultures and should be monitored closely. To keep track of the growth, start monitoring the culture early. About 2 h after inoculation, start taking the OD_{600} reading approximately every 30 min. As the OD_{600} begins to approach 0.4, take more frequent readings, e.g., every 10 min, or even 5 min, judging on the rate of growth (i.e., for faster growing cultures, take readings at shorter intervals). As soon as the reading is anywhere within this range, stop the growth by aliquoting the culture into a precooled sterile centrifuge bottle and place it on ice for 15–20 min, then proceed to make the competent cells. If, however, the culture overgrows past OD_{600} 0.65, it should be discarded and a fresh culture started.
16. Glycerol, placed at −20 °C before use, could partially freeze and form ice crystals. Do not pour crystallized glycerol into the bottle and resuspend the cells. If glycerol is found partially frozen, swirl it under running tap water for a few minutes to thaw the solution completely. It should still remain extremely cold after this quick thawing process. Pour 250 mL into the bottle containing the cell pellet and resuspend the pellet by gently pipetting it up and down. This resuspension step should be carried out fast, within 1–2 min, and the bottle returned to the centrifuge for the following step.
17. Typically 1 mL or less of cells is collected from this residual glycerol.
18. Approximately 10–20 tubes of 45 μL aliquots can be routinely obtained. For each electroporation one 45 μL tube is used. The remaining electrocompetent cells may be stored at −80 °C at this point for future use.

19. The choice of control plasmid DNA differs for each transformation. For this electroporation, pUC19 is used as the control plasmid. This is because pUC19 confers resistance to ampicillin, of which the parental cells DH10B (BAC4396) will be susceptible to. Therefore DH10B (BAC4396) transformed with pUC19 will be selected on LB plates containing both chloramphenicol and ampicillin, with BAC4396 giving resistance to chloramphenicol and pUC19 to ampicillin.
20. Drying the cuvette's surface reduces the occurrence of electrical arcing, which could significantly reduce the viability of the cells. Avoid touching the aluminum electrodes because this could raise the temperature of the mixture and reduce electroporation efficiency.
21. If a "pop" sound is heard, arcing has occurred. Discard this electroporation reaction and repeat using another batch of competent cells, DNA, and cuvette. Plating these "popped" cells is unlikely to give any recombinants.
22. Adding antibiotic at this recovery stage will dramatically reduce cell viability, effectively ruining the experiment.
23. It is not necessary to sterilize the Pasteur pipettes as long as they are clean. A bulb should be used at the end of the pipette to gently suck the cells up and down in the cuvette. After electroporation the cells will be very fragile and therefore harsh pipetting should be avoided as it will lyse the cells.
24. For storage purpose, thoroughly mix 750 μL of the grown culture with 250 μL of 50 % (v/v) glycerol/LB medium by vigorous shaking. Flash-freeze the tube in a −80 °C isopropanol bath and store the stock culture at −80 °C. To maintain a working stock, streak each colony onto a LB agar plate containing the appropriate antibiotics, and re-streak every fortnight to maintain stability of the strain.
25. In our example we use BAC4396. For a specific BAC clone that is to be used, it is necessary to first transform pGETrec into the DH10B strain containing this BAC clone to generate the recombineering strain, DH10B (pGETrec, BAC clone) (*see* Subheading 3.2).
26. It is important to not overgrow the culture past OD_{600} of 0.50, as overgrown cultures will result in limited recombinants. In order to accurately keep track of the growth, start monitoring the culture early.
27. Even after the culture is removed from the incubator it will continue growing. Therefore it is very important to work fast. Remove the culture from the incubator, take the OD_{600} reading, and when the growth target is reached, induce with arabinose and immediately return the culture into the incubator.

28. The time required to reach OD_{600} of 0.55–0.65 may vary between cultures and should be monitored closely. A reading may be taken 20–30 min after induction in order to judge how fast the culture is growing. It is common to find the growth rate slowing down a little after induction, an effect of the bacterial cells being forced to overproduce the recombineering proteins from plasmid pGETrec [12, 18]. The most important thing is to stop the growth of the culture as it moves into the window between OD_{600} of 0.55–0.65. As soon as the reading is anywhere within this range, stop the growth by aliquoting the culture into a precooled sterile centrifuge bottle and place it on ice for 15–20 min and proceed to make the competent cells. If, however, the culture overgrows past OD_{600} 0.65, it should be discarded and a fresh culture started. Overgrown cultures will suffer from diminished recombineering proficiency and the experiment is unlikely to work.
29. For this electroporation, we typically transform the zeocin-resistant plasmid pZeoSV2(+) (Invitrogen Inc.) and select on LB plates containing zeocin, ampicillin, and chloramphenicol. Since DH10B (pGETrec, BAC4396) will already be ampicillin (and chloramphenicol) resistant, and will naturally grow on LB plates containing this ampicillin, the control plasmid must be resistant to an antibiotic other than ampicillin and chloramphenicol—hence, zeocin is used.
30. In our hands, a transformation efficiency of at least 1×10^8 CFU/μg is achieved routinely and consistently. Efficiencies lower than this could result in very few recombinants, or none at all. Therefore we strongly recommend fine-tuning the competent cell preparation to reach an efficiency of at least 1×10^8 CFU/μg before proceeding. The key to achieving this is to (1) work quickly during preparation of the competent cells, and (2) ensure that all instructions for using precooled materials and reagents are followed closely.
31. For the recovery stage, the cells should be grown at the slower condition of 150 rpm and 30 °C (as opposed to the standard 220 rpm and 37 °C) in order to promote the stability of the repeat-containing *tos* sequence [9, 18]. Because of the slower growth condition, the cells are incubated for 120 min, much longer time than usually done for electroporation. This longer incubation helps the cells undergo recombination as efficient as cells grown at standard conditions.
32. We have often found pGETrec plasmid persisting in the transformed cells even if ampicillin selection is not added. But at this stage, elimination of pGETrec is not a priority. The first step is to identify the correct recombinant containing the *tos-Zeo* cassette by PCR screening, described in Subheading 3.4.

Only after determining the correct recombinants by PCR screening, the modified BAC will be moved away from pGETrec by a re-transformation step in Subheading 3.5 into freshly prepared DH10B competent cells [11].

33. We use the Long PCR Enzyme Mix from Thermo Scientific Inc., but any other long PCR kit should be suitable because the PCR is only performed for screening purpose.
34. It is strongly recommended to confirm that the *tos*-Zeo cassette is correctly recombined, i.e., by sequencing several PCR positive clones and by restriction digestion checking, before proceeding any further with these modified BACs.
35. We routinely digest the BAC4396-tos with a single cutting restriction enzyme, e.g., *Nar*I before electrophoresis [9]. However, for a chosen BAC any restriction enzyme that can verify its intactness can be used. Once you are satisfied that the BAC is intact and not rearranged it can be transferred into the telN$^+$ *E. coli* strain (Fig. 1d).
36. Vortexing large BAC DNA can damage it. Therefore shake the tube to mix.
37. For storage purpose, thoroughly mix 750 μL of the grown culture with 250 μL of 50 % (v/v) glycerol/BHI medium by vigorous shaking. Flash-freeze the tube in a −80 °C isopropanol bath and store this stock culture at −80 °C. To maintain a working stock, streak each colony onto a BHI agar plate containing chloramphenicol, streptomycin, zeocin, and 5 mM of DAP, and re-streak every fortnight to maintain viability of the DAP-auxotrophic clones.
38. We have used conventional plasmid extraction kit, e.g., QIAprep® Spin Miniprep Kit (Qiagen Inc.) to successfully isolate intact linear BAC-*tos* for routine plasmid checking. Nevertheless we find the NucleoBond® kits to produce the highest yield of linear DNA in excellent purity (260 nm:280 nm ratio > 1.80). Using the NucleoBond® Xtra Midi kit we typically achieve a yield of around 50 μg of linear DNA from 200 mL of culture.
39. Avoid vortexing to mix as this will generate contaminating chromosomal DNA.
40. Place a collection tray at the bottom to collect unwanted flow-through. The buffer will not completely run dry from the column; as long as the column is not dripping anymore it is ready for use.
41. Make sure the bottle is no more than two-third full so that the suspension can be mixed homogeneously.
42. Make sure the off-white precipitate in the column filter does not get into the column or it may clog the column and reduce the purification yield and/or quality.

43. Be sure to use high quality tubes that can withstand very high speed centrifugation of up to 17,000 × *g* for at least 45 min. We regularly use the Nalgene™ Oak Ridge High-Speed Polypropylene Copolymer (PPCO) 10 mL centrifuge tubes.
44. Adding 1 mL aliquots of the Elution Buffer ELU gives a higher yield compared to directly adding 5 mL in one go.
45. It will be a good idea to mark the edge of the tube that faces outward in the centrifuge rotor to indicate the position of the pellet, so that the translucent pellet will not be disturbed or lost during subsequent washing steps.
46. Be very careful when removing supernatant. The pellet is translucent to the naked eye and can easily dislodge from the bottom of the tube.
47. Do not over dry the pellet or it will take a long time to dissolve.
48. Dissolving the pellet could take up to 2–3 h depending on the size of pellet and how dry it is.
49. The protocol described here uses RecBCD Exonuclease V from Epicentre Inc. Different suppliers may have different composition of buffer and enzyme concentration, therefore perform the RecBCD digestion according to the manufacturer's instructions. We have also tested a RecBCD Exonuclease V pack from New England BioLabs Inc., which produces similar results.

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