Published in final edited form as:
Anal Biochem. 2009 April 1; 387(1): 139–141. doi:10.1016/j.ab.2009.01.015.

Recombination between linear double-stranded DNA substrates *in vivo*

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Abstract

Recombineering technology in *E. coli* enables targeting of linear donor DNA to circular recipient DNA using short shared homology sequences. In this work, we demonstrate that recombineering is also able to support recombination between a pair of linear DNA substrates (linear/linear recombineering) *in vivo* in *E. coli*. Linear DNA up to 100 kb is accurately modified and remains intact without undergoing rearrangements after recombination. This system will be valuable for direct *in vivo* manipulation of large linear DNA including the N15 and PY54 prophages and linear animal viruses, and for assembly of linear constructs as artificial chromosome vectors.

Keywords

BAC; linear; recombineering; recombination; phage; *E. coli*; chromosome; telomere

Recombineering technology enables facile modification of large circular DNA using homologous recombination in *E. coli* without dependence on suitably placed restriction sites or *in vitro* enzymatic manipulations [1–3]. In the presence of short 40–60 nucleotide homology sequences, expression of the *E. coli recE* and *recT* genes and/or the bacteriophage λ *red* genes facilitates targeting of linear donor DNA (*e.g.*, linear PCR products) to a specific site on circular recipient DNA *in vivo* [1,2].

To date, recombineering has been demonstrated for the following substrates: 1) linear DNA and a circular replicon (including plasmids [2,4], PACs and BACs [1,2,5], and the *E. coli* chromosome [6,7]), and 2) linear DNA and a linearized plasmid vector backbone that becomes recircularized after gap-repair following *in vivo* recombination [8]. However, so far, recombineering between a pair of linear DNA substrates has not been tested *in vivo* because of the lack of a system that could serve as linear recipient plasmids in *E. coli*.

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We recently developed a system to assemble BACs up to 100 kb as linear plasmids capped with telomeres derived from the bacteriophage N15 [9]. This linear BAC was resistant to RecBCD, which degrades linear DNA in *E. coli*, and was functional after transfer into human cells and produced correctly spliced β-globin transcript [9]. In this work, using this linear BAC DNA system, we demonstrate recombineering between a pair of linear DNA substrates (linear/linear recombineering) *in vivo* in *E. coli* and discuss its application.

To investigate recombineering between a pair of linear DNA substrates, a linear PCR product (donor DNA) was electroporated into electrocompetent *E. coli* DH10B containing a resident linear 100 kb human β-globin BAC and plasmid pGETrec [strain *telN*$^+$ DH10B (pGETrec, linear BAC)] [9] (Figure 1a). The linear BAC serves as the recipient DNA (Figure 1a), while plasmid pGETrec provides the recombineering enzymes, Gam, RecE, and RecT. To initiate linear/linear recombineering the linear donor DNA (Kan60) is electroporated into these cells as described in Figure 1a.

First, to provide transient expression of the recombineering enzymes in this strain, the cellswere grown at 150 rpm at 30°C and plasmid pGETrec was induced for 10 mins with arabinose when the OD_{600} reached 0.55 [3] (Figure 1a). The linear donor DNA (Kan60) contains the kanamycin resistance gene (Km^r) flanked by 60 bp of homology that directs this 1162 bp DNA to recombine to a sequence centered around the *Bst* 1107I site on the vector backbone of the linear BAC recipient DNA (Figure 1a). Kan60 was PCR amplified using primers kan60F (5′-TTC CGG TCA CAC CAC ATA CGT TCC GCC ATT CTT ATG CGA TGC ACA TGC TGT ATG CCG GTA caa gaa atc aca gcc gaa gc-3′) and kan60R (5′-AGA CTT CCG TTG AAC TGA TGG ACT TAT GTC CCA TCA GGC TTT GCA GAA CTT TCA GCG gta gcg tga tct gat cct tca act-3′) from pCyPAC7 (a gift from the late Dr. Panos Ioannou) according to our standard protocol [1] and electroporated into the host cells (Figure 1a).

After recombineering between the linear DNA substrate pair (linear/linear substrates), 58 Km^r recombinants were obtained (Figure 1b). In comparison, a parallel set of linear/circular substrates were recombined by electroporation of the same linear Kan60 donor fragment into a strain containing a circular β-globin BAC recipient [DH10B (pGETrec, circular BAC)] [10]. This recombination event produced 211 Km^r recombinants (Figure 1b). Although linear/circular recombinantion produced about 3.6 times more colonies than linear/linear recombination, it is statistically not significant to suggest that recombination favors linear/circular substrates (Student's t-test: $t = 2.39$, $df = 3$, $p > 0.10$). While linear/circular recombineering generated more clones, it is important to note that the linear/linear substrates produced an average of more than 50 recombinants per electroporation, far more colonies than needed for analysis. Thus, the linear/linear technique works as efficiently as standard linear/circular recombination and can be applied using existing recombineering protocols.

To verify that linear/linear recombineering precisely targeted the Kan60 fragment to the *Bst*1107I site on the vector backbone of the linear BAC, a PCR screening assay was employed with primers KF and KR [6] (black arrows in Figure 1a) to amplify across this site. In total, at least 20 independent Km^r colonies were screened using this assay and all clones were positive for a 1.4 kb product that is expected when the Kan60 PCR fragment recombined correctly into the *Bst*1107I site of the linear BAC (Figure 1a). Figure 2a shows five of the Km^r recombinants (lanes 1–5) analyzed that produced this 1.4 kb PCR product. As control for this assay, the non-recombinant parent linear BAC produced a 288 bp fragment (Figure 2a, lane 6), indicating absence of Kan60 insertion. This PCR assay confirmed that the linear/linear recombination between Kan60 was precise to the targeted *Bst*1107I site on the linear BAC DNA to produce the linear BAC::Kan60 (Figure 1a).

Next, to examine the integrity of the modified linear BAC::Kan60 from the *telN*$^+$ DH10B (pGETrec, linear BAC::Kan60) strain after recombineering, this DNA was analyzed by pulsed field gel electrophoresis (PFGE). In order to purify the linear BAC::Kan60 for analysis without plasmid pGETrec contamination, plasmid pGETrec was first eliminated from the *telN*$^+$ DH10B (pGETrec, linear BAC::Kan60) strain by addition of arabinose in the medium and omitting ampicillin selection for pGETrec [6].

Arabinose induction triggers elimination of pGETrec due to the toxicity of the recombineering proteins when over-expressed from this plasmid [3], leaving only the linear BAC::Kan60 DNA in the host cells. Pure linear BAC::Kan60 DNA was then purified from the *telN*$^+$ DH10B (linear BAC::kan60) cells using the NucleoBond Plasmid Midi Kit (BD Biosciences, Clontech) and analyzed by PFGE after *Nar* I restriction digestion.

In the control digestion (Figure 2b, lane P) *Nar* I cuts the unmodified linear BAC DNA once to produce an 8024 bp vector fragment (see schematic below Figure 2b) and an intact ~ 95 kb fragment containing the β-globin insert. The recombinant linear BAC::Kan60 was also cut once with *Nar* I but produced a larger 9.2 kb vector band (Figure 2b, lanes 1–3), in addition to the 95 kb β-globin insert fragment, indicating insertion of the 1162 bp Kan60 PCR fragment into the vector backbone as intended (refer to schematic below Figure 2b). The *Nar* I PFGE (Figure 2b) demonstrated: i) that linear/linear recombineering between the linear Kan60 fragment and the 100 kb linear BAC DNA was precise, and ii) the stability of the linear BAC::Kan60 DNA was retained after linear/linear recombineering without undergoing gross rearrangements.

Recombineering technology has emerged as an important tool for manipulation of genomic loci for functional studies in *in vitro* and *in vivo* models [11–13]. The linear/linear recombineering mechanism described in this work functions as effectively as standard recombineering and should specifically appeal to researchers who are interested in applying this technique to manipulate large linear substrates such as the phages N15 and PY54 [14], animal viruses such as vaccinia virus [15], and for assembly of large linear constructs for use as artificial chromosome vectors [16] in mammalian cells.

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a **b**

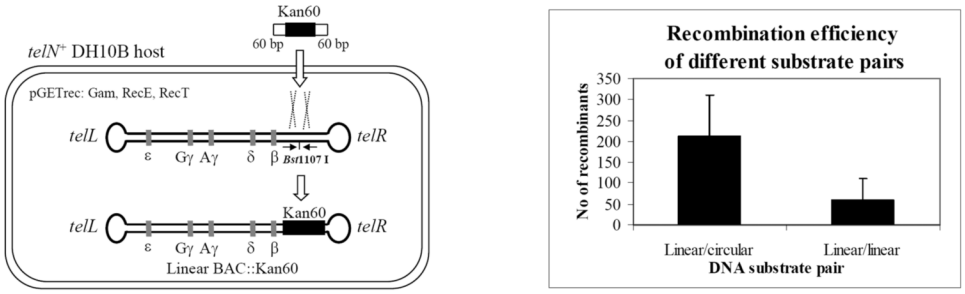

Figure 1. Recombineering linear double stranded DNA substrates *in vivo*.

a) A linear Kan60 PCR product (donor) was recombined into the *Bst* 1107 I site of a resident linear 100 kb β-globin BAC (recipient) in a *telN*$^+$ DH10 strain to generate a linear BAC::Kan60 recombinant. The coexisting plasmid pGETrec provided the recombineering enzymes, Gam, RecE, and RecT. Black arrows flanking the *Bst* 1107 I site on the linear BAC represent primers KF and KR. b) Comparison of *in vivo* recombineering efficiencies between linear/linear and linear/circular substrate pairs. Recombination between linear/circular substrates: linear Kan60 PCR product and circular β-globin BAC. Recombination between linear/linear substrates: linear Kan60 PCR product and linear BAC. Km^r colonies were scored as recombination events. The average number of 3 independent experiments is provided for each recombination substrate pair.

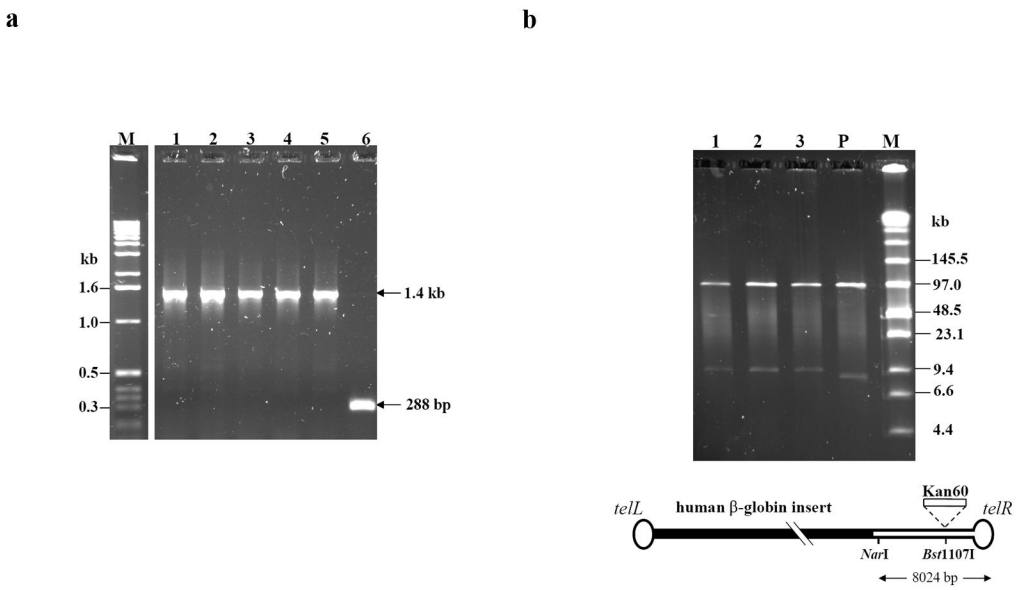

Figure 2. Analyses of recombinant linear BACs after recombineering with Kan60 DNA

a) PCR analysis across the *Bst* 1107 I recombination junction using primers KF and KR. M, 1 kb plus DNA marker (Invitrogen Corp.); lanes 1–5, Km^r colonies containing recombinant linear BAC::Kan60 with Kan60 insertion; lane 6, unmodified parent linear BAC. b) PFGE of retrofitted linear BACs after *Nar* I digestion. Lanes 1–3, DNA from recombinant linear BAC::Kan60 containing Kan60 insertion; lane P, unmodified parent linear BAC; lane M, low-range PFGE marker (New England Biolabs). Schematic below the PFGE shows predicted *Nar* I fragments for the recombinant linear BAC::Kan60 and unmodified linear BAC. *Nar* I cuts the unmodified linear BAC DNA to produce an 8024 bp vector fragment. In the recombinant linear BAC::Kan60 containing the 1162 bp Kan60 insertion, *Nar* I digestion released a larger 9.2 kb vector band.