# Enzymatic assembly of DNA molecules up to several hundred kilobases

Daniel G Gibson<sup>1</sup>, Lei Young<sup>1</sup>, Ray-Yuan Chuang<sup>1</sup>, J Craig Venter<sup>1,2</sup>, Clyde A Hutchison III<sup>2</sup> & Hamilton O Smith<sup>2</sup>

We describe an isothermal, single-reaction method for assembling multiple overlapping DNA molecules by the concerted action of a 5' exonuclease, a DNA polymerase and a DNA ligase. First we recessed DNA fragments, yielding single-stranded DNA overhangs that specifically annealed, and then covalently joined them. This assembly method can be used to seamlessly construct synthetic and natural genes, genetic pathways and entire genomes, and could be a useful molecular engineering tool.

For nearly 40 years, scientists have had the ability to join DNA sequences and produce combinations that are not present in nature. This 'recombinant DNA technology' was initiated soon after the discovery of DNA ligase<sup>1</sup> and restriction endonucleases<sup>2</sup>. Since then, multiple approaches for joining DNA molecules through the use of restriction enzymes<sup>3,4</sup> and PCR<sup>5-8</sup> have been adapted. Ligation-independent cloning strategies have also been developed<sup>9,10</sup>. We had recently described an *in vitro* recombination method that we had used to join 101 DNA cassettes into four quarter molecules of the Mycoplasma genitalium genome, each 136-166 kilobases (kb)<sup>11</sup> (Supplementary Figs. 1-3 and Supplementary Results online). Because we performed this recombination method in a thermocycler (to which we refer as 'thermocycled' here), individual reactions were carried out in only two steps. Here we improved this two-step thermocycled method by using exonuclease III and antibody-bound Taq DNA polymerase, which allow for one-step thermocycled in vitro recombination (Supplementary Fig. 4 and Supplementary Results online).

We now present an *in vitro* recombination system that differs from the ones above by its capacity to assemble and repair overlapping DNA molecules in a single isothermal step. This approach can be used to join DNA molecules that are as large as 583 kb and to clone joined products in *Escherichia coli* that are as large as 300 kb. All reagents and enzymes are commercially available, and all that is required for DNA assembly is for the reagent-enzyme mix (which can be stored at -20 °C until needed) to be combined with overlapping DNA molecules and then incubated at 50 °C for as few as 15 min (Online Methods). This approach dramatically simplifies the construction of large DNA molecules from constituent parts.

Exonucleases that recess double-stranded DNA from 5' ends will not compete with polymerase activity. Thus, all enzymes required for DNA assembly can be simultaneously active in a single isothermal reaction. Furthermore, circular products can be enriched as they are not processed by any of the three enzymes in the reaction. We optimized a 50 °C isothermal assembly system using the activities of the 5' T5 exonuclease (Epicentre), Phusion DNA polymerase (New England Biolabs (NEB)) and *Taq* DNA ligase (NEB) (**Fig. 1**). *Taq* DNA polymerase (NEB) can be used in place of Phusion DNA polymerase (data not shown), but the latter is preferable as it has inherent proofreading activity for removing noncomplementary sequences (for example, partial restriction sites) from assembled molecules.

To test this system, we cleaved two restriction fragments that overlapped by ~450 base pairs (bp) from the 6-kb pRS415 vector and then reassembled them into a circle (**Fig. 2a**). After 6–8 min at 50 °C, the linear substrate DNA was completely reacted, and the major product was the 6-kb circle, which migrated just below the 4-kb linear position on an agarose gel. T5 exonuclease actively degrades linear DNA molecules, but closed circular DNA molecules are not degraded<sup>12</sup>. We confirmed the circularity of this assembled product by treating it with additional T5 exonuclease (**Fig. 2a**). To demonstrate that this assembled product was the predicted 6-kb circle, we digested it with *Not* I (a single-cutter) and observed the



**Figure 1** | One-step isothermal *in vitro* recombination. Two adjacent DNA fragments (magenta and green) sharing terminal sequence overlaps (black) were joined into a covalently sealed molecule in a one-step isothermal reaction. T5 exonuclease removed nucleotides from the 5' ends of double-stranded DNA molecules, complementary single-stranded DNA overhangs annealed, Phusion DNA polymerase filled the gaps and *Taq* DNA ligase sealed the nicks. T5 exonuclease is heat-labile and is inactivated during the 50 °C incubation.

<sup>&</sup>lt;sup>1</sup>The J. Craig Venter Institute, Synthetic Biology Group, Rockville, Maryland, USA, and <sup>2</sup>San Diego, California, USA. Correspondence should be addressed to D.G.G. (dgibson@jcvi.org).

RECEIVED 5 JANUARY; ACCEPTED 16 MARCH; PUBLISHED ONLINE 12 APRIL 2009; DOI:10.1038/NMETH.1318

## **BRIEF COMMUNICATIONS**

Figure 2 | Examples of the one-step isothermal assembly method. (a) Two DNA molecules (4,024 bp, green (i) and 2,901 bp, magenta (ii)), overlapping by  $\sim$  450 bp at the termini (black, overlaps labeled A and B), were reacted for 0-16 min to form a 6-kb circle, pRS415 (iv). Linear assembly products (iii) were then removed by incubation with additional tenfold excess T5 exonuclease after the 16-min incubation (T5). (**b**) Not I digest of the assembled circles (pRS415) shown in a. (c) Fragments F1-F3 were assembled into an 8-kb PCR-amplified pCC1BAC (Epicentre) containing 40-bp overlaps to F1 and F3, using the indicated amounts of T5 exonuclease. The  $\sim$  23-kb circular assembly products are indicated by the arrow. (d) Not I digestion of BACs purified from ten *E. coli* clones after electroporation of the 4 U ml<sup>-1</sup> T5 exonuclease reaction shown in c. \*, correct 15-kb insert. (e) Assembly of guarter M. genitalium genomes C25-49 and C50-77 with BAC25-77 (ref. 11) to produce Mgen25-77. (f) Not I digestion of BACs purified from ten E. coli clones after electroporation of the assembly



reaction shown in **e**. The correct insert size (310 kb) is indicated by the arrow. (**g**) Assembly of quarter *M. genitalium* genomes *C1–24*, *C25–49*, *C50–77* and *C78–101* (ref. 11) to produce a complete *M. genitalium* genome. DNA products were analyzed by conventional gel electrophoresis (**a**,**b**) and by field-inversion gel electrophoresis (**c**–**g**). M, 1-kb DNA extension markers;  $\lambda$ , lambda markers.

6-kb linear fragment (**Fig. 2b**). We concluded that DNA molecules can be assembled and repaired in a single isothermal step using this method.

We next determined whether DNA molecules with overlaps of only 40 bp could be joined. We accomplished this when we reduced the concentration of T5 exonuclease (**Fig. 2c**). Three 5-kb DNA fragments, F1–F3, were efficiently assembled into an 8-kb bacterial artificial chromosome (BAC). Furthermore, when we transformed these assembled DNA molecules into *E. coli*, we obtained 4,500 colonies, and nine out of ten colonies tested had the predicted 15-kb insert (**Fig. 2d**).

During the construction of the synthetic M. genitalium genome, we could not use our two-step thermocycled in vitro recombination method to clone assembled DNA molecules larger than  $\sim$  150 kb in E. coli<sup>11</sup>. To determine whether the isothermal assembly method could be used to join and clone DNA fragments of larger size, we reacted two synthetic M. genitalium quarter DNA molecules, C25-49 (144 kb) and C50-77 (166 kb), with BAC25-77 (8 kb), a cloning vector specific for the assembly of these two DNA molecules. The 318-kb Mgen25-77 product was efficiently produced, so we conclude that DNA fragments this size can be joined by this method (Fig. 2e). To determine whether this method could be used to clone assembled DNA fragments this size, we transformed a fraction of this assembly reaction into E. coli. We obtained several hundred clones, and 5 out of 10 colonies screened had the correct insert size (310 kb; Fig. 2f). Thus, this DNA assembly system can be used to join and clone DNA molecules up to several hundred kilobases in length in E. coli, the approximate upper limit for transformation into this bacterium<sup>13</sup>. In a direct comparison of all our assembly methods, we found that only the one-step in vitro recombination methods could be used to clone assembled DNA fragments this size (Supplementary Fig. 5 online).

During *in vitro* recombination, errors may be introduced in the assembled DNA. However, sequencing of 30 cloned DNA molecules (210 repaired junctions) after two-step thermocycled assembly revealed only 4 errors (**Supplementary Table 1** online). This equates to only about 1 error per 50 DNA molecules joined. Therefore, if our hierarchical scheme to assemble the *M. genitalium* genome was used<sup>11</sup> without sequence verification at intermediate steps, 3–4 errors would likely be present. We expect that the number of mutations would be even lower with the isothermal assembly system because gaps are filled in by Phusion DNA polymerase, which has higher fidelity than *Taq* polymerase.

Our isothermal method can be used to assemble DNA molecules of unprecedented sizes, and we used it to assemble the complete synthetic 583-kb *M. genitalium* genome (**Fig. 2g**). The size limit for *in vitro* DNA assembly is not known, but products as large as 900 kb have been observed (**Supplementary Fig. 6** online). Of the three *in vitro* recombination methods, we prefer the one-step-isothermal system because of its simplicity. This approach could be very useful for cloning multiple inserts into a vector without relying on the availability of restriction sites and for rapidly constructing large DNA molecules. For example, regions of DNA too large to be amplified by PCR can be divided into multiple overlapping PCR amplicons and then assembled into one piece. The one-step thermocycled method could be used to generate linear assemblies as the exonuclease is inactivated during the reaction (**Supplementary Figs. 4** and **5**).

Synthetic biologists are engineering genetic pathways for the production of biofuels, pharmaceuticals and industrial compounds<sup>14,15</sup>. Here we provide efficient methods for constructing these pathways, from natural or synthetic DNA.

## METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

# **BRIEF COMMUNICATIONS**

#### ACKNOWLEDGMENTS

This work was supported by the Office of Science (Biological and Environmental Research) United States Department of Energy grant number DE-FG02-02ER63453, and Synthetic Genomics, Inc.

#### AUTHOR CONTRIBUTIONS

D.G.G., L.Y., R.-Y.C., J.C.V., C.A.H. and H.O.S. designed research; D.G.G., L.Y., R.-Y.C., C.A.H. and H.O.S. performed research; D.G.G., L.Y., R.-Y.C., J.C.V., C.A.H. and H.O.S. analyzed data; and D.G.G., C.A.H. and H.O.S. wrote the paper.

#### COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemethods/.

#### Published online at http://www.nature.com/naturemethods/ Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Gellert, M. Proc. Natl. Acad. Sci. USA 57, 148-155 (1967).

- 2. Smith, H.O. & Wilcox, K.W. J. Mol. Biol. 51, 379-391 (1970).
- 3. Shetty, R.P., Endy, D. & Knight, T.F. Jr. J. Biol. Eng. 2, 5 (2008).
- Yount, B., Denison, M.R., Weiss, S.R. & Baric, R.S. J. Virol. 76, 11065–11078 (2002).
- Horton, R.M., Cai, Z.L., Ho, S.N. & Pease, L.R. *Biotechniques* 8, 528–535 (1990).
- 6. Horton, R.M. Mol. Biotechnol. 3, 93-99 (1995).
- 7. Bang, D. & Church, G.M. Nat. Methods 5, 37-39 (2008).
- Geu-Flores, F., Nour-Eldin, H.H., Nielsen, M.T. & Halkier, B.A. Nucleic Acids Res. 35, e55 (2007).
- 9. Aslanidis, C. & de Jong, P.J. Nucleic Acids Res. 18, 6069-6074 (1990).
- 10. Li, M.Z. & Elledge, S.J. Nat. Methods 4, 251-256 (2007).
- 11. Gibson, D.G. et al. Science 319, 1215-1220 (2008).
- 12. Sayers, J.R. & Eckstein, F. J. Biol. Chem. 265, 18311-18317 (1990).
- Sheng, Y., Mancino, V. & Birren, B. Nucleic Acids Res. 23, 1990–1996 (1995).
- 14. Endy, D. Nature 438, 449-453 (2005).
- 15. Drubin, D.A., Way, J.C. & Silver, P.A. Genes Dev. 21, 242-254 (2007).



## **ONLINE METHODS**

Preparation of DNA molecules for in vitro recombination. The DNA molecules used in the assembly analyses were derived from several sources including (i) the assembly intermediates of the synthetic M. genitalium genome<sup>11</sup>, (ii) PCR products derived from plasmids (F6 and F8), Clostridium cellulolyticum genomic DNA (F1-F4) and Mycoplasma gallisepticum genomic DNA (F5 and F7) and (iii) pRS415 restriction fragments. E. coli strains carrying each of M. genitalium cassettes 66-69 (contained in pENTR223), each of M. genitalium cassettes 78-85 (contained in pBR322), C1-24, C25-49, C50-77 and C78-101 (each contained in pCC1BAC) or pRS415 were propagated in LB medium containing the appropriate antibiotic and incubated at 30 °C or 37 °C for 16 h. The cultures were collected and the DNA molecules were purified using Qiagen's HiSpeed Plasmid Maxi Kit according to the manufacturer's instructions, with the exception of C1-24, C25-49, C50-77 and C78-101, which were not column-purified. Instead, after neutralization of the lysed cells, these DNA molecules were centrifuged then precipitated with isopropanol. DNA pellets were dissolved in Tris-EDTA (TE) buffer (pH 8.0) then RNase treated, phenol-chloroform extracted and ethanol precipitated. DNA pellets were dissolved in TE buffer. Cassettes 66-69 and 78-85 were excised from the vectors by restriction digestion with either Fau I or Bsm BI and C1-24, C25-49, C50-77 and C78-101 were excised by digestion with Not I. To generate the 4,024-bp and 2,901-bp overlapping fragments of pRS415, DNA was digested with Pvu II and Sca I or Psi I, respectively. Restriction digestions were terminated by phenol-chloroform extraction and ethanol precipitation. DNA was dissolved in TE buffer, then quantified by gel electrophoresis with known DNA standards. Fragments F1-F8 were generated by PCR using the Phusion Hot Start High-Fidelity DNA polymerase with HF buffer (NEB) according to the manufacturer's instructions. PCR products were extracted from agarose gels after electrophoresis and purified using the QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's instructions, except DNA was eluted from the columns with TE buffer pH 8.0. Fragments F1-F4 were amplified from Clostridium cellulolyticum genomic DNA using primers F1-For and F1-Rev, F2-For and F2-Rev, F3-For and F3-Rev, and F4-For and F4-Rev, respectively. F5 and F7 were amplified from Mycoplasma gallisepticum genomic DNA using primers F5-For and F5-Rev, and F7-For and F7-Rev, respectively. F6 and F8 were amplified from pRST2 (ref. 16) using primers F6-For and F6-Rev, and F8-For and F8-Rev, respectively. Primer sequences are listed in Supplementary Table 2 online.

**Two-step thermocycled DNA assembly.** A 4× chew-back and anneal (CBA) reaction buffer (20% PEG-8000, 800 mM Tris-HCl pH 7.5, 40 mM MgCl<sub>2</sub>, 4 mM DTT) was used for thermocycled DNA assembly. DNA molecules were assembled in 20-µl reactions consisting of 5 µl 4× CBA buffer, 0.2 µl of 10 mg ml<sup>-1</sup> BSA (NEB) and 0.4 µl of 3 U µl<sup>-1</sup> T4 polymerase (NEB). T7 polymerase can be substituted for T4 polymerase (data not shown). Approximately 10–100 ng of each ~6 kb DNA segment was added in equimolar amounts. For larger DNA segments, proportional amounts of DNA were added (for example, 250 ng of each 150 kb DNA segment). Assembly reactions were prepared in 0.2 ml PCR tubes and cycled as follows: 37 °C from 0 to 18 min as indicated in the text, 75 °C for 20 min, 0.1 °C s<sup>-1</sup> to 60 °C, held at 60 °C for 30 min, then cooled to 4 °C at a rate of 0.1 °C s<sup>-1</sup>. In general, a

chew-back time of 5 min was used for overlaps less than 80 bp and 15 min for overlaps greater than 80 bp. Ten microliters of the CBA reactions were then added to 25.75  $\mu$ l of *Taq* repair buffer (TRB), which consisted of 5.83% PEG-8000, 11.7 mM MgCl<sub>2</sub>, 15.1 mM DTT, 311  $\mu$ M each of the four dNTPs and 1.55 mM NAD. Four microliters of 40 U  $\mu$ l<sup>-1</sup> *Taq* DNA ligase and 0.25  $\mu$ l of 5 U  $\mu$ l<sup>-1</sup> *Taq* polymerase were added and the reactions were incubated at 45 °C for 15 min. For the T4 polymerase fill-in assembly method, 10  $\mu$ l of the CBA reaction was mixed with 0.2  $\mu$ l of 10 mM dNTPs and 0.2  $\mu$ l of 3 U  $\mu$ l<sup>-1</sup> T4 polymerase. This reaction was carried out at 37 °C for 30 min.

**One-step thermocycled DNA assembly.** A  $4 \times$  chew-back, anneal and repair (CBAR) reaction buffer (20% PEG-8000, 600 mM Tris-HCl pH 7.5, 40 mM MgCl<sub>2</sub>, 40 mM DTT, 800 µM each of the four dNTPs and 4 mM NAD) was used for one-step thermocycled DNA assembly. DNA molecules (added in amounts described above for CBA reactions) were assembled in 40 µl reactions consisting of 10  $\mu$ l 4× CBAR buffer, 0.35  $\mu$ l of 4 U  $\mu$ l<sup>-1</sup> ExoIII (NEB), 4  $\mu$ l of 40 U  $\mu$ l<sup>-1</sup> Taq DNA ligase and 0.25  $\mu$ l of 5 U  $\mu$ l<sup>-1</sup> Ab-Taq polymerase (Applied Biosystems). ExoIII was diluted 1:25 from 100 U  $\mu$ l<sup>-1</sup> in its stored buffer (50% glycerol, 5 mM KPO<sub>4</sub>, 200 mM KCl, 5 mM 2-mercaptoethanol, 0.05 mM EDTA and 200 µg ml<sup>-1</sup> BSA, pH 6.5). DNA assembly reactions are prepared in 0.2 ml PCR tubes and cycled using the following conditions: 37 °C for 5 or 15 min as indicated in the text, 75 °C for 20 min, 0.1 °C s<sup>-1</sup> to 60 °C, then held at 60 °C for 1 h. In general, a chewback time of 5 min was used for overlaps less than 80 bp and 15 min for overlaps greater than 80 bp. ExoIII is less active on 3' protruding termini<sup>17</sup>, which can result from digestion with certain restriction enzymes. This can be overcome by removing the overhangs to form blunt ends with the addition of T4 polymerase and dNTPs, as described above, before assembly (data not shown).

**One-step isothermal DNA assembly.** A  $5 \times$  isothermal reaction buffer (25% PEG-8000, 500 mM Tris-HCl pH 7.5, 50 mM MgCl<sub>2</sub>, 50 mM DTT, 1 mM each of the four dNTPs and 5 mM NAD) was used for one-step DNA assembly at 50 °C. DNA molecules (added in amounts described above for CBA reactions) were assembled in 40  $\mu$ l reactions consisting of 8  $\mu$ l 5× isothermal buffer, 0.8  $\mu$ l of 0.2 U  $\mu$ l<sup>-1</sup> or 1.0 U  $\mu$ l<sup>-1</sup> T5 exonuclease, 4  $\mu$ l of 40 U  $\mu$ l<sup>-1</sup> Taq DNA ligase and 0.5  $\mu$ l of 2 U  $\mu$ l<sup>-1</sup> Phusion DNA polymerase. T5 exonuclease was diluted 1:50 or 1:10 from 10 U µl<sup>-1</sup> in its stored buffer (50% glycerol, 50 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 1 mM DTT, 0.1 M NaCl and 0.1% Triton X-100) depending on the overlap size. For overlaps shorter than 150 bp, 0.2 U  $\mu$ l<sup>-1</sup> T5 exonuclease is used. For overlaps larger than 150 bp, 1.0 U  $\mu$ l<sup>-1</sup> T5 exonuclease was used. All isothermal assembly components can be stored at -20 °C in a single mixture at 1.33× concentration for more than one year. The enzymes are still active after more than ten freeze-thaw cycles. To constitute a reaction, 5 µl DNA was added to 15 µl of this mixture. Incubations were carried out at 50 °C for 15 to 60 min, with 60 min being optimal.

**One-step isothermal DNA assembly protocol.** Six milliliters of  $5 \times$  isothermal reaction buffer were prepared by combining 3 ml of 1 M Tris-HCl pH 7.5, 150 µl of 2 M MgCl<sub>2</sub>, 60 µl of 100 mM dGTP, 60 µl of 100 mM dATP, 60 µl of 100 mM dTTP, 60 µl of 100 mM dCTP, 300 µl of 1 M DTT, 1.5 g PEG-8000 and

Bdr

300 µl of 100 mM NAD. This buffer can be aliquoted and stored at -20 °C. An assembly master mixture was prepared by combining 320  $\mu$ l 5× isothermal reaction buffer, 0.64  $\mu$ l of 10 U  $\mu$ l<sup>-1</sup> T5 exonuclease, 20 µl of 2 U µl<sup>-1</sup> Phusion DNA polymerase, 160 µl of 40 U  $\mu$ l<sup>-1</sup> Taq DNA ligase and water up to a final volume of 1.2 ml. Fifteen microliters of this reagent-enzyme mix were aliquoted and stored at -20 °C. This mixture can tolerate numerous freeze-thaw cycles and remains stable even after one year. The exonuclease amount is ideal for the assembly of DNA molecules with 20-150 bp overlaps. For DNA molecules overlapping by greater than 150 bp, 3.2  $\mu$ l of 10 U  $\mu$ l<sup>-1</sup> T5 exonuclease was used to prepare the assembly master mixture above. Frozen 15 µl assembly mixture aliquots were thawed and then kept on ice until ready to be used. Five microliters of the DNA to be assembled were added to the master mixture in equimolar amounts. Between 10 and 100 ng of each  $\sim 6$  kb DNA fragment was added. For larger DNA segments, proportional amounts of DNA were added (for example, 250 ng of each 150 kb DNA segment). Incubations were performed at 50 °C for 15 to 60 min (60 min was optimal).

**Rolling-circle amplification (RCA) of assembled products.** RCA was carried out as previously described<sup>18</sup>. One microliter of the repaired or unrepaired reaction was mixed with 1 µl of 100 mM NaOH and incubated at room temperature (18–22 °C) for 5 min to denature the double-stranded DNA. One microliter of this alkaline-treated mixture was then added to 19 µl of RCA components in a 0.2 ml PCR tube. The final reaction concentrations for RCA are as follows: 37 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM MgCl<sub>2</sub>, 5 mM (NH4)<sub>2</sub>SO<sub>4</sub>, 100 µg ml<sup>-1</sup> BSA, 1 mM DTT, 3.25 mM random hexamers (Fidelity System), 1 U ml<sup>-1</sup> yeast pyrophosphatase (United States Biochemical) and 250 U ml<sup>-1</sup> phi29 DNA polymerase (NEB). The reaction was incubated at 30 °C for 20 h then terminated by incubation at 65 °C for 10 min.

**Cloning the DNA assembly products.** To clone assembled products, reactions were carried out in the presence of PCR-amplified BACs containing 40 bp of overlapping sequence to the ends of the assembled product. *Not* I restriction sites were also included to allow release of the vector<sup>11</sup>. To produce BAC-F1/F3, primers

BACF1 For and BACF3 Rev were used in PCR. To produce BAC66-69, primers BAC66 For and BAC69 Rev were used in PCR. To produce BAC25-77, primers BAC25 For and BAC77 Rev were used in PCR. Primer sequences are listed in Supplementary Table 2. In general, pCC1BAC was used as DNA template. However, for cloning Mgen25-77, a version of pCC1BAC, named KanBAC, was constructed that contains the kanamycin resistance gene in place of the chloramphenicol resistance gene. Samples (up to 1 µl) of the assembly reactions were transformed into 30 µl TransforMax EPI300 (Epicentre) electrocompetent E. coli cells in a 1-mm cuvette (BioRad) at 1,200 V, 25  $\mu$ F and 200  $\Omega$  using a Gene Pulser Xcell Electroporation system (BioRad). Cells were allowed to recover at 30 °C or 37 °C for 2 h in 1 ml SOC medium then plated onto LB medium containing 12.5 µg ml<sup>-1</sup> chloramphenicol or LB medium containing 25 µg ml<sup>-1</sup> kanamycin. After incubation at 30 °C or 37 °C for 24-48 h, individual colonies were selected and grown in 3 ml LB medium with 12.5 µg ml<sup>-1</sup> chloramphenicol or 25 µg ml<sup>-1</sup> kanamycin overnight at 30 °C or 37 °C. DNA was prepared from these cells by alkaline lysis using the P1, P2 and P3 buffers (Qiagen) followed by isopropanol precipitation. DNA pellets were dissolved in TE buffer containing RNase and then digested with Not I to release the insert from the BAC.

Agarose gel analyses of assembled DNA molecules and cloned products. U-5 field-inversion gel electrophoresis analysis was performed on 0.8% E-gels (Invitrogen) and the parameters were forward 72 V, initial switch 0.1 s, final switch 0.6 s, with linear ramp and reverse 48 V, initial switch 0.1 s, final switch 0.6 s, with linear ramp. U-2 field-inversion gel electrophoresis analysis was performed on 1% agarose gels (BioRad) in 1× TAE buffer with 0.5  $\mu$ g ml<sup>-1</sup> ethidium bromide without circulation, and the parameters were forward 90 V, initial switch 5.0 s, final switch 30 s, with linear ramp, and reverse 60 V, initial switch 5.0 s, final switch 30 s, with linear ramp. DNA bands were visualized with a BioRad Gel Doc or an Amersham Typhoon 9410 Fluorescence Imager.

- 16. Lartigue, C., Duret, S., Garnier, M. & Renaudin, J. Plasmid 48, 149-159 (2002).
- 17. Henikoff, S. Gene 28, 351-359 (1984).
- Hutchison, C.A., III, Smith, H.O., Pfannkoch, C. & Venter, J.C. Proc. Natl. Acad. Sci. USA 102, 17332–17336 (2005).