Generation of long tracts of disease-associated DNA repeats

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The generation of long uninterrupted DNA repeats is important for the study of repeat instability associated with several human genetic diseases, including myotonic dystrophy type 1. However, obtaining defined lengths of long repeats in vitro has been problematic. Strand slippage and/or DNA secondary structure formation may prevent efficient ligation. For example, a purified (CTG)₁₄₀•(CAG)₁₄₀ repeat fragment containing 4-bp AGCA/TGCT overhanging ends ligated poorly using T4 or Escherichia coli DNA ligase, although limited repeat ligation occurred using thermostable DNA ligase. Here we describe a general procedure for ligating multimers of DNA repeats. Multimers are efficiently ligated when slippage is prevented or when DNA repeats contain a single G/C overhang. A cloning vector is designed from which pure repeat fragments containing a G/C overhang can be generated for further ligation. (CAG)_n•(CTG)_n DNA molecules longer than 800 bp were generated using this approach. This approach also worked for (GAA)_n•(TTC)_n, (CCTG)_n•(CAGG)_n, and (ATTCT)_n•(AGAAT)_n tracts associated with Friedreich ataxia, DM2, and spinocerebellar ataxia type 10, respectively.

INTRODUCTION

DNA repeat expansions are associated with many genetic neurodegenerative diseases and three fragile sites (1–4). Long disease-relevant repeats often possess unique properties including the ability to form alternative DNA secondary structures that may contribute to their instability. Biophysical and biochemical studies have shown that the disease-associated DNA repeats form several secondary structures, including hairpins, parallel DNA, triplex DNA, quadruplex DNA, slipped-strand DNA, and unpaired DNA structures (5–10). The probabilities of secondary structure formation and genetic instability are generally proportional to repeat length (6,9–11).

For the identification of molecular mechanisms involved in trinucleotide repeat instability leading to disease, the generation of long pure trinucleotide repeats has been an essential but difficult step, even though different methods have been employed (12–18). The problem of generating proteins containing long tracts of Gln encoded by CAG repeats has been solved by cloning Gln-encoding tracts that are not pure

repeats (19,20). In *Escherichia coli*, the maximum number of (CTG)_n•(CAG)_n repeats that can be maintained ranges from 180 to 240, and these are typically stabilized by sequence interruptions within the repeat tract (12). Pure CTG tracts have been produced in vitro by thermal cycling protocols (21,22). A similar procedure has been used to generate long ATTCT repeats (23). In addition, a pure (CTG)₂₅₀ tract was made by the dimerized ligation of CTG repeats (17,18). Because cells from patients with these disorders maintain thousands of repeats, the generation of pure repeat tracts, longer than those stably maintained in bacteria, would be useful for mammalian cell studies, in which thousands of repeats are relatively stable. Here we discuss how technical difficulties encountered in the cloning of longer repeat DNAs may result from strand slippage of pure duplex repeats, which can change the nature of the overhang at the ends of the DNA molecules. We describe procedures for cloning pure repeats in *E. coli* as substrates for subsequent ligation in vitro to generate tracts of 800 repeats or more.

MATERIALS AND METHODS

Plasmids and *Escherichia coli* Growth Conditions

Plasmid pACT, kindly provided by Dr. Charles A. Thornton (Neuromuscular Disease Center Clinic, University of Rochester, Rochester, NY, USA), contains 130–140 (CAG)•(CTG) repeats inserted between the *Bbs*I and *Bsa*I sites in plasmid pACYC177. Transformed cells (*E. coli* DH5α or HB101) were grown in Luria-Bertani (LB) broth (10 g Bacto[®] tryptone, 5 g yeast extract, 10 g NaCl per liter of water) supplemented with 10 μg/mL kanamycin at 30°C and harvested before the cultures reached stationary phase (A_{650} of 0.7), conditions important for maintaining the stability of repeats in *E. coli*.

Plasmid Purification

We utilized an alkaline lysis method for purifying plasmid DNA (24), with slight modification. A 500-mL culture was grown in LB, supplemented with 10 μg/mL kanamycin for pACT and

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30 µg/mL ampicillin for pLCtg44 and pLCtg27, at 30°C until the cultures reached late log phase ($A_{650} = 0.7$). The culture flasks were then chilled as quickly as possible to 0°–4°C. After centrifugation at 4000× *g* for 5 min at 4°C, the pellet was resuspended in 25 mL ice-cold M9 buffer (for 1 L of M9 buffer, combine 1 g of NH_4Cl , 5.8 g of Na_2HPO_4 , 3 g of KH_2PO_4 , pH 7.0) and centrifuged at 4000× *g* for 5 min at 4°C. The pellet was resuspended in 18 mL alkaline lysis solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, and 10 mM EDTA), 2 mL 10 mg/mL lysozyme were added, and the cells were incubated for 5 min on ice. For lysis of the cells, 40 mL alkaline lysis solution II [0.2 M NaOH and 1% sodium dodecyl sulfate (SDS)] were added, the sample was mixed, and the solution was incubated for 10 min on ice. Twenty milliliters of ice-cold alkaline lysis solution III [3 M potassium acetate and 11.5% (v/v) glacial acetic acid] were added, and the solution was mixed well by swirling several times, followed by incubation for 10 min on ice. After centrifugation at 4000× *g* for 30 min at 4°C, the supernatant was filtered through 4 layers of cheesecloth, 0.6 volumes of isopropanol were added, and the solution was mixed well and incubated for 20 min on ice. The precipitated nucleic acids were recovered by centrifugation at 3000× *g* for 30 min at 4°C and dissolved in 5 mL TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). The DNA was deproteinized by extraction 2 to 3 times with 2 mL phenol and 2 to 3 times with 2 mL of chloroform:isoamyl alcohol (24:1). Five milliliters of ice-cold 5 M LiCl were added to precipitate RNA, and the solution was incubated for 10 min at 0°–4°C. After centrifugation at 10,000× *g* for 20 min at 4°C, the supernatant was collected, and 0.6 volumes of isopropanol were added. The sample was mixed well and then incubated for 2–12 h at 4°C. The precipitated plasmid DNA was recovered by centrifugation at 10,000× *g* for 20 min at 4°C, and the pellet was dissolved in 3.9 mL TE. Ethidium bromide (0.4 mL of 5 mg/mL) and 4.25 g CsCl were added, and the mixture was incubated for 10 min at room temperature and then centrifuged at 10,000× *g* for 10 min

at room temperature to pellet residual RNA. The supernatant was centrifuged for 12–16 h at 145,700× *g* in a VTi65.2 rotor (Beckman Coulter, Fullerton, CA, USA). The band of closed circular DNA (below the linear DNA band) was visualized by illumination with 360 nm ultraviolet (UV) light and collected through an 18-gauge hypodermic needle. The ethidium bromide was removed by repeated extractions with TEN-saturated *n*-butanol, and the DNA was dialyzed (Spectra/Por® 2 membrane; Spectrum Laboratories, Rancho Dominguez, CA, USA) for a

minimum of 16–24 h against three 1-L changes of TEN (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 50 mM NaCl).

Ligation and Cloning of Long (CAG)•(CTG) Repeats

Ligation of (CAG)_{130–140}•(CTG)_{130–140} fragments. The (CAG)_{130–140}•(CTG)_{130–140} *BbsI*-*BsaI* fragment from pACT was purified with a 1% agarose gel using an Ultra-Clean™ DNA Purification Kit (Mo Bio Laboratories, Carlsbad, CA, USA). One microgram of the gel-purified

Figure 1. Ligation of long trinucleotide repeats with 4-bp 5' overhanging ends using thermostable DNA Ligase. (A) An approximately 400-bp DNA fragment containing 130–140 (CTG)•(CAG) repeats was cut from plasmid pACT using *BbsI* and *BsaI*, and gel-purified and ligated using several protocols, as described in the text. (B) The gel-purified DNA fragments of (CTG)_{130–140}•(CAG)_{130–140} repeats were ligated by T4 (T4), *Escherichia coli* (E), or thermostable (Ts) DNA ligase at 4°, 16°, or 24°C. DNAs are separated on a 1.2% agarose gel. M, molecular weight marker consisting of a 100-bp ladder (New England Biolabs). (C) The (CTG)_{130–140}•(CAG)_{130–140} fragment was ligated using thermostable DNA ligase and a thermal cycling procedure. The units of enzyme are indicated above the lanes. The efficient ligation under this condition required at least 15 U of the thermostable ligase and produced a ladder of ligated products of 800, 1200, and 1600 bp.

(CAG)₁₃₀₋₁₄₀•(CTG)₁₃₀₋₁₄₀ fragment was incubated with 400 U T4 DNA ligase (New England Biolabs, Beverly, MA, USA or Roche Applied Science, Indianapolis, IN, USA) or 10 U *E. coli* DNA ligase (New England Biolabs) in the supplied buffer in 20 μ L for 16, 24, or 48 h at 4° or 16°C (Figure 1B). For high-temperature ligation, 1 μ g of the gel-purified (CAG)₁₃₀₋₁₄₀•(CTG)₁₃₀₋₁₄₀ fragment was incubated with 5, 10, 15, 20, or 25 U of thermostable DNA ligase from *Thermus scodoductus* (Roche Applied Science) at 37°, 45°, 55°, or 65°C with or without heating at 95°C for 5 min (Figure 1B). High-temperature ligation on a DNA thermal cycler (GeneAmp® PCR System 9700; PE Applied Biosystems, Foster City, CA, USA) utilized one microgram of (CTG)₁₃₀₋₁₄₀•(CAG)₁₃₀₋₁₄₀ fragment and 5, 10, 15, 20, or 25 U of the thermostable DNA ligase at 95°C for 20 s and 60°, 65°, or 77°C for 30 min. This cycle was repeated 15–30 times.

Cloning (CAG)_n•(CTG)_n tracts containing G/C overhanging ends.

pLC plasmids, derived from pUC8, contain 5'-AATTCGAGTCGCG CG(CTG)_nGCGCGACTCGG-3' between the *EcoRI* and *BamHI* sites. pLCctg7, pLCctg27, and pLCctg44, contain 7, 27, and 44 (CAG)•(CTG) repeats, respectively. These plasmids were constructed by the ligation of (CAG)₇•(CTG)₇ fragments flanked with G/C overhanging ends, formed by hybridization of (GCT)₇ and (CAG)₇. (The lengths of cloned repeat tracts are frequently a bit different from that expected, given the genetic instability of these sequences.) One to six micrograms of oligonucleotides (18–20 nucleotides) were phosphorylated using 1.5 mM ATP and 10 U T4 polynucleotide kinase in a 20- μ L reaction volume (New England Biolabs). The DNA was precipitated with ethanol and then resuspended in 20 μ L of TEN. Complementary pairs of oligonucleotides in TEN (2–12 μ g in 40 μ L) were hybridized by boiling for 3 min and then slowly cooled overnight to 4°C. The annealed DNA fragments (3 μ g)

were ligated to an *EcoRI*-*PleI* adaptor (0.3 μ g) formed by hybridization of 5'-GGAATTCGAGTCGCGC-3' with 5'-CGCGCGACTCGAATTCC-3' at a 1:10 (adaptor to repeat) weight ratio using 400 U of T4 DNA ligase in 20 μ L at 16°C for 1 h. This was followed by the addition of a *BamHI*-*PleI* adaptor (0.6 μ g) formed by the hybridization of 5'-GGCGCGACTCGGATCCG-3' and 5'-CGGATCCGAGTCGCGC-3' at twice the concentration of the first adaptor (1:10:2 weight ratio), and ligation was continued for 1 additional hour. After ligation, the DNA was digested with *EcoRI* and *BamHI* and ligated into pUC8 that had also been digested with *EcoRI* and *BamHI*. The length and purity of the repeat tracts were determined by dideoxy DNA sequencing.

For secondary cloning of longer repeats, the repeat sequences in pLC-based plasmids were amplified by PCR using two primers, 5'-GGAATTCGAGTCGCGC-3' and 5'-CGGATCCGAGTCGCGC-3' (two of the oligonucleotides used for linker construction). For PCR, REDTaq™ (Sigma, St. Louis, MO, USA) was used as directed with denaturation for 2 min at 94°C; 30 cycles of 94° for 30 s, 50° for 30 s, and 72°C for 2 min; followed by a final extension at 72°C for 5 min. The PCR products were digested with *PleI*, and then the DNA fragments containing (CAG)_n•(CTG)_n fragments with G/C overhanging ends were ligated and separated on a 5% polyacrylamide gel. The ligation products purified from the gel as previously described (25) were cloned into the *EcoNI* site of pLC-PNP. pLC-PNP contains the sequence 5'-GAATTCGAGTCCTGGGGGAGGACTCGGATCC-3' cloned between the *EcoRI* and *BamHI* sites of pUC8. This insert contains an *EcoNI* site flanked by two *PleI* sites. It is designed such that a pure repeat with a single G/C overhanging end can be ligated into the *EcoNI* site and then cut out using *PleI* to regenerate the G/C overhang.

Agarose and Polyacrylamide Gel Analysis

The ligation products of DNA repeat fragments were analyzed by separation on a 1.2% agarose gel by electropho-

Figure 2. Ligation of annealed oligonucleotides containing different overhanging ends. (A) Synthesized oligonucleotide pairs creating DNA fragments 1–12 were mixed, boiled, and annealed by slowly cooling to 4°C. The oligonucleotides were designed to contain one-base overhanging ends (1–4 and 7–12) or four-base overhanging ends (5 and 6). (B) Oligonucleotides 1–9 were ligated using T4 DNA ligase at 4°, 16°, or 24°C for 12 h and separated on a 10% polyacrylamide gel. (C) Oligonucleotides 10–12 were ligated at 16°C. M, molecular weight marker (100-bp ladder).

resis in TAE buffer (15 mM Tris, pH 7.8, 33 mM Na acetate, 2 mM EDTA) at 7 V/cm for 2 h at room temperature. The ligation products were analyzed by separation on a 10% native polyacrylamide gel by electrophoresis in TBE buffer (90 mM Tris, pH 8.3, 90 mM boric acid, 2.5 mM EDTA) at 8–10 V/cm for 2–3 h at room temperature. The gels were stained with ethidium bromide and visualized.

RESULTS

Ligation of the (CAG)_{130–140}•(CTG)_{130–140} Fragment

To obtain long pure (CAG)•(CTG) repeats in vitro, (CAG)_{130–140}•(CTG)_{130–140} fragments flanked with four-base overhanging ends (AGCA/TGCT) were purified with a 1% agarose gel after digestion of pACT with *Bbs*I and *Bsa*I (Figure 1A). Ligation was then performed as previously described (17). The gel-purified (CAG)_{130–140}•(CTG)_{130–140} fragments were ligated using T4, *E. coli*, or thermostable DNA ligases. However, following ligation by T4 and *E. coli* DNA ligases at 4° or 24°C, only about 5% of the fragment was ligated (Figure 1B). Ligation may be prevented by the formation

of secondary structures within the (CAG)_{130–140}•(CTG)_{130–140} fragments or slippage of the fragment leading to a single A/T overhang at the 5' ends of the top and bottom strands. To test if the block to ligation could be alleviated by a high-temperature ligation, which may melt potential secondary structures at the DNA ends or promote slippage, we performed ligation using a thermostable ligase at high temperature. The melting temperature (T_m) of (CAG)_{130–140}•(CTG)_{130–140} in 6 mM NaCl (diluted from the storage buffer containing 100 mM NaCl of *Tsc* thermostable ligase) is approximately 87°C. The T_m is given by $T_m = 81.5 + 0.41 (\%GC) - 500/L + 16.6 \log[M]$ where L refers to the length of oligonucleotide, GC is the G+C content, and $[M]$ is the concentration of monovalent cations, in this case Na⁺. Based on the high T_m of 87°C, the ligation using thermostable DNA ligase was performed by 15 cycles of incubation at 95° for 20 s and 65°C for 30 min. From densitometric analysis of the distribution of DNA in individual lanes using ImageQuant® software (Amersham Biosciences, Piscataway, NJ, USA), 40%–50% of the (CAG)_{130–140}•(CTG)_{130–140} fragments appeared to have been ligated into multimers under these conditions in the reaction using 25 U (Figure 1C). At least 15 U of the

thermostable ligase were required for optimal ligation (Figure 1C). When the denaturation time was increased from 20 s up to 5 min or the denaturation temperature was decreased from 95° to below 89°C, ligation did not occur (data not shown). Therefore, to obtain long trinucleotide repeats in vitro by ligation of (CAG)_{130–140}•(CTG)_{130–140} fragments, the optimal conditions were a short denaturation at 95° for 20 s and ligation at 65°C with thermostable DNA ligase. These results demonstrate that thermostable DNA ligase and a thermal cycling procedure can be used to improve the ligation of long pure (CAG)_n•(CTG)_n repeats in vitro.

Ligation of DNA Repeats Containing a One-Base G/C Overhang

It was possible that the initial four-base 5'-AGCA/5'-TGCT overhanging ends of the (CAG)_{130–140}•(CTG)_{130–140} fragment were fractious to ligation. To identify the nature of the overhanging ends that would support efficient ligation, we performed ligations with several short duplex DNAs containing (CAG)_n•(CTG)_n tracts with various overhanging ends (Figure 2A) using T4 DNA ligase at 4°, 16°, or 24°C (Figure 2B). The ligation of duplex DNA containing (CAG)₂•(CTG)₂

Figure 3. Secondary ligation of cloned trinucleotide repeats containing G/C overhanging ends. (A) Scheme for generation of pure repeat containing fragment from pLC-based plasmids. DNA fragments containing (CAG)_n•(CTG)_n repeats with the *Eco*RI-*Ple*I and *Bam*HI-*Ple*I adaptors were digested with *Eco*RI and *Bam*HI, and the fragments were inserted into pUC8. The repeat sequences were then amplified from the pLC plasmids by PCR. The PCR products were digested with *Ple*I, the pure (CAG)_n•(CTG)_n fragments were gel-purified and then ligated to form oligomers. (B) Purified oligomers were then inserted into plasmid pLC-PNP that had been cut with *Eco*NI to obtain plasmids containing longer pure repeats. (C) To demonstrate the method, DNA repeats were cut from PCR fragments made from plasmids pLCctg44 and pLCctg27, which contained (CAG)₄₄•(CTG)₄₄ and (CAG)₂₇•(CTG)₂₇ repeats, respectively. Shown on a 5% polyacrylamide gel are DNA repeat fragments that were purified [(CTG)₂₇ and (CTG)₄₄] and then ligated [(CTG)₂₇]_n and [(CTG)₄₄]_n. Lane M contains the 100-bp molecular weight marker.

tracts with one-base G/C overhanging ends at 4°, 16°, or 24°C produced a ladder of ligation products (Figure 2B, lane 1), but duplex DNAs containing (CAG)_{3, 6, or 12}•(CTG)_{3, 6, or 12} tracts with one-base A/T overhanging ends were poorly ligated at 4°C (Figure 2B, lanes 2–4). Moreover, duplex DNAs containing the (CAG)_{3 or 6}•(CTG)_{3 or 6} tracts and a 12-bp unique sequence spacer with one- or four-base (G/C or AGCA/TGCT) overhanging ends were ligated efficiently (Figure 2B, lanes 5, 6, and 8). The ligation of molecules containing the four-base overhang was clearly the most efficient. However, a duplex DNA containing the repeats and the 12-bp spacer with one-base A/T overhanging ends ligated poorly (Figure 2B, lane 7). A duplex DNA containing (CAG)₄•(CTG)₄ with a 6-bp mismatch spacer and one-base A/T overhanging ends was also ligated poorly (Figure 2B, lane 9). These results support the idea that potential slippage resulting in one-base A/T overhanging ends of (CAG)_{130–140}•(CTG)_{130–140} fragment may be responsible for the poor ligation of this DNA fragment.

To demonstrate the generality of ligating disease-related DNA repeats containing a single G/C overhang, we synthesized (GAA)_n•(TTC)_n, (CCTG)_n•(CAGG)_n, and (ATTCT)_n•(AGAAT)_n tracts with single-base G/C overhanging ends and performed ligation reactions. With these repeats, more than 15–20 short fragments were ligated to the adaptors (Figure 2C).

Generation of Long (CAG)•(CTG) Repeats

To clone long (CAG)•(CTG) repeats, multiple (CAG)₇•(CTG)₇ tracts with G/C overhanging ends were ligated to *EcoRI*-*PleI* and *BamHI*-*PleI* adaptors (shown in Figure 3 and described in Materials and Methods). The DNA was then cut with *EcoRI* and *BamHI*, and the *EcoRI*-*BamHI* DNA fragment was inserted into pUC8 cut with *EcoRI* and *BamHI*, and the plasmid was transformed into bacteria (Figure 3A). Plasmids pLCtg27 and pLCtg44 contain pure (CAG)₂₇•(CTG)₂₇ and (CAG)₄₄•(CTG)₄₄ repeats, respec-

tively, cloned into pUC8. *PleI* digestion generates the expected fragments of 81 and 132 bp, respectively (Figure 3C). Following this initial cloning, two options are available for generating a longer pure repeat DNA fragment. First, the fragment could be liberated by *PleI* digestion from plasmid purified from *E. coli*. Second, a region of plasmid DNA containing the (CAG)•(CTG) repeat could be amplified by PCR, and then the pure repeat fragment could be cut out by *PleI* digestion. Following either procedure, the fragment can be gel-purified for further ligation. Figure 3 shows the PCR procedure for plasmids pLCtg27 and pLCtg44. Following gel purification, the pure (CAG)₂₇•(CTG)₂₇ and (CAG)₄₄•(CTG)₄₄ fragments were ligated into ladders and separated on a 5% polyacrylamide gel. At least 6–10 fragments were ligated in this experiment.

An additional plasmid, pLC-PNP, was constructed to facilitate a second stage cloning of longer repeats. This plasmid was constructed by ligation of a duplex fragment formed by hybridization of 5'-AATTCGAGTCCTGGGGGAGGACTCG-3' with 5'-GATCCGAGTCCTCCCCAGGACTCG-3' between the *EcoRI* and *BamHI* sites in pUC8 (Figure 3B). Digestion with *EcoNI* generates a linear plasmid flanked with G/C overhanging ends that will accept multimers of the pure repeat fragments liberated by *PleI* digestion. Plasmids containing multimers of the original *PleI* fragments were obtained (data not shown), and these can be cut again with *PleI* to liberate fragments flanked with G/C overhanging ends for another round of ligation, if desired.

DISCUSSION

The generation of pure disease-associated DNA repeat tracts, longer than those stably maintained in bacteria, is important for biophysical studies of these repeats and for use in the introduction of these repeats into mammalian cells to ascertain the biochemical basis for the disease state. In contrast to bacteria where repeat tracts longer than 150–200 repeats are very unstable, cells from patients

with these repeat-expansion disorders maintain thousands of repeats in relatively stable fashion. We describe a simple approach for generating disease-associated DNA repeats that involves multiple rounds of cloning pure repeat fragments containing single-base G/C overhanging ends. This is advisable for several reasons. It is often difficult to obtain large amounts of long repeats, consisting of many ligated fragments, in an initial ligation reaction. Second, very long repeat tracts are difficult to clone, and resulting clones frequently contain repeat interruptions (mutations). By a stepwise cloning approach, the length and purity of the cloned repeat tract can be monitored more easily. Finally, with long pure cloned repeats near the limit of that maintained stably in bacteria (approximately 200 repeats), it is easier to generate pure repeats of very long length (>800 repeats) for use in biochemical studies or for introduction into mammalian cells.

Previous technical difficulties in ligating, cloning, and generating long repeat DNAs may result from strand slippage or the formation of secondary structures at the ends or within DNA repeat sequences. The inability to easily ligate the (CAG)_{130–140}•(CTG)_{130–140} fragment with the AGCA/TGCT overhanging end presumably results from facile strand slippage during fragment preparation, converting a four-base overhang to a one-base A/T overhang, which, under our conditions, ligates poorly. Slippage can occur by base unpairing or “fraying” at the DNA ends, followed by misalignment, and then transmission of a bubble down the helix. Strand slippage occurs easily in short repeat DNA fragments (26). Many approaches were attempted to prevent slippage and obtain ligation but without success. The limited ligation that occurs during the thermal cycling protocol presumably results from fraying at the ends and slippage, forming a population of ends, some of which are able to hybridize and ligate. Our results strongly suggest that for molecules with an overhang of one repeat length or more, slippage generating a single A/T overhang may reduce ligation efficiency. The potential problems to ligating long repeats can be overcome by the design of oligo-

nucleotides that hybridize to produce DNA fragments with single-base G/C overhanging ends.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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