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SCIENCE DIRECT
Bioorganic & Medicinal Chemistry
Bioorganic & Medicinal Chemistry 14 (2006) 2518–2526

Direct PCR amplification of various modified DNAs having amino acids: Convenient preparation of DNA libraries with high-potential activities for in vitro selection

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Received 9 October 2005; revised 12 November 2005; accepted 15 November 2005
Available online 15 December 2005

Abstract—We synthesized modified 2′-deoxyuridine triphosphates bearing amino acids at the C5 position and investigated their substrate properties for *KOD Dash* DNA polymerase during polymerase chain reaction (PCR). PCR using C5-modified dUTP having an amino acyl group (arginyl, histidyl, lysyl, phenylalanyl, tryptophanyl, leucyl, prolyl, glutaminyl, seryl, *O*-benzyl seryl or threonyl group) gave the corresponding full-length PCR products in good yield. Although dUTP analogues bearing aspartyl, glutamyl or cysteinyl were found to be poor substrates for PCR catalyzed by *KOD Dash* DNA polymerase, optimization of the reaction conditions resulted in substantial generation of full-length product. In the case of reaction using dUTP analogue having a cysteinyl group, addition of a reducing agent improved the reaction yield. Thus, PCRs using *KOD Dash* DNA polymerase together with amino acyl dUTP provide convenient and efficient preparation of various modified DNA libraries with potential protein-like activities.

1. Introduction

In vitro selection techniques have been used to select DNA catalysts and DNA aptamers that have activities similar to those of protein–enzymes and antibodies.[1–6] To date, various functionally modified nucleic acids have been developed to enhance activity or to diversify function.[7–15] In particular, Perrin et al.[13,14] and Sidorov et al.[15] invented modified DNAzymes with two functionalities, imidazolyl and a primary amino group, that can catalyze RNA hydrolysis in the absence of divalent metal ions. These examples demonstrated that a DNA molecule modified with plural functionalities found in the active sites of protein–enzymes can exhibit protein-like activity. However, preparation of modified DNAs having plural functionalities is difficult using polymerase reactions. These modified DNA libraries were prepared by primer extension reaction using C8- or C7-modified dATP and C5-modified dUTP together with a genetically engineered variant of T7 DNA polymerase (*Sequenase*) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (*SuperScript* II), respectively. Thus, primer extension[16–18] or one-primer polymerase chain reaction (PCR) is used widely instead of symmetric PCR, which can amplify DNA exponentially, to prepare modified DNA for in vitro selection. It is vital in symmetric PCR that the modified nucleoside triphosphates act as good substrates for thermostable DNA polymerase and that the modified DNAs work as templates during the amplification process. Therefore, the combinations of modified nucleoside triphosphates and thermostable DNA polymerases that result in efficient amplification by PCR are limited.

Among commercially available thermostable DNA polymerases, it is known that *KOD Dash* DNA polymerase from *Pyrococcus kodakaraensis* shows high polymerase activity and excellent fidelity.[19–22] For instance, polymerization reaction rate with *KOD Dash* DNA polymerase is twofold faster than that with *Taq* DNA polymerase and about sixfold faster than that with *Pfu* DNA polymerase. Moreover, the fidelity of *KOD Dash* DNA polymerase is about 3.4-fold higher than that of *Taq* DNA polymerase. Thus, *KOD Dash* DNA

Keywords: Modified DNA; Modified thymidine triphosphate; Polymerase chain reaction; Amino acid.
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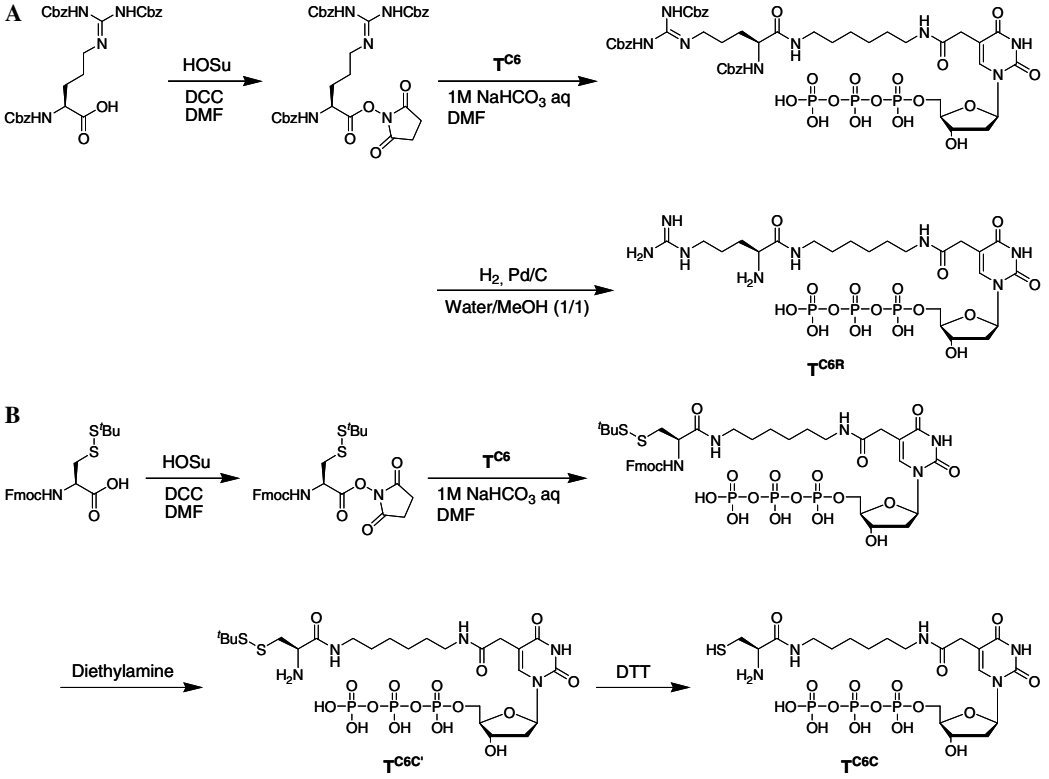
doi:10.1016/j.bmc.2005.11.030

polymerase is the one of the best catalysts for PCR. In a previous study, *KOD Dash* DNA polymerase was found to accept 5-(2-(6-aminohexylamino)-2-oxoethyl)-dUTP ($\mathbf{T^{C6}}$) as an alternative to TTP during PCR, efficiently yielding the corresponding modified DNA.[23] We report here the convenient and efficient preparation of modified DNAs having two different functionalities (primary amino group and a group such as imidazolyl, guanidinyl, hydroxyl, etc.) by PCR using a series of C5-modified dUTPs carrying amino acids.

2. Results

All aminoacyl dUTPs, except for sulfur-containing analogues, such as $\mathbf{T^{C6C}}$ and $\mathbf{T^{C6C'}}$, were synthesized from amino acids with hydrogenation-sensitive protecting groups such as benzyloxycarbonyl and benzyl groups (Scheme 1). Reaction of the amino acid with *N*-hydroxysuccinimide in the presence of *N*,*N*′-dicyclohexylcarbodiimide gave the corresponding *N*-succinimide ester. Then, coupling of $\mathbf{T^{C6}}$ with the ester followed by hydrogenation to remove the protecting groups gave $\mathbf{T^{C6H}}$, $\mathbf{T^{C6K}}$, $\mathbf{T^{C6R}}$, $\mathbf{T^{C6F}}$, $\mathbf{T^{C6W}}$, $\mathbf{T^{C6L}}$, $\mathbf{T^{C6P}}$, $\mathbf{T^{C6Q}}$, $\mathbf{T^{C6S}}$, $\mathbf{T^{C6S'}}$, $\mathbf{T^{C6T}}$, $\mathbf{T^{C6D}}$, and $\mathbf{T^{C6E}}$. The sulfur-containing analogues ($\mathbf{T^{C6C}}$ and $\mathbf{T^{C6C'}}$) were synthesized from *N*-α-(9-fluorenylmethoxycarbonyl)-*S*-*tert*-butylthio-L-cysteine because sulfur inhibits catalysis of the Pd/C used for the hydrogenation reaction. After the coupling reaction, the Fmoc and *S*-*t*-butyl groups were successfully removed by treatment with diethylamine and dithiothreitol (DTT), respectively.

We first investigated the effects of linker structure on amplification efficiency of PCR. Under condition 3, the analogue $\mathbf{T^{C6}}$ with a 2-(6-aminohexylamino)-2-oxoethyl linker was found to be the best substrate for PCR among several known C5-modified dUTPs used in this study (Fig. 1, Table 1). The formation of the corresponding natural-type DNA from the natural substrate TTP was 1.4 times larger compared with that from $\mathbf{T^{C6}}$ under optimized conditions for the formation of natural-type DNA, where the enzyme quantity was reduced to suppress the non-specific amplification. With regard to relative yields of PCR using *KOD Dash* DNA polymerase, that of $\mathbf{T^{C2}}$ with 2-(2-aminoethylamino)-2-oxoethyl linker four methylene units shorter than that in $\mathbf{T^{C6}}$ decreased to 0.48. While use of $\mathbf{T^{AL}}$ provided a product in a relative yield of 0.77, use of $\mathbf{T^{PR}}$, which is known to be a substrate for PCR using *Taq* DNA polymerase,[32] did not provide any product. Moreover, use of $\mathbf{T^{DH}}$ was found to decrease the relative yield to 0.34, which was lower than when $\mathbf{T^{C2}}$ or $\mathbf{T^{AL}}$ was used. These results indicated that the distance between amino groups with positive charge and nucleobase, the chemical structure of the linker as well as a type of DNA polymerase affects generation of the PCR product. Interestingly however, these five modified dUTPs were found to be good substrates for template-directed primer extension reactions (see Supplementary material). Therefore, when preparing modified DNAs by PCR, template activities of the PCR product as well as substrate properties of the modified nucleoside triphosphates for thermostable DNA polymerases are important factors in amplifying modified DNAs.



Scheme 1. Synthetic routes of modified dUTPs bearing (A) argininyl group and (B) cysteinyl group via 2-(6-aminohexylamino)-2-oxoethyl linker at the C5 position of uracil, respectively.

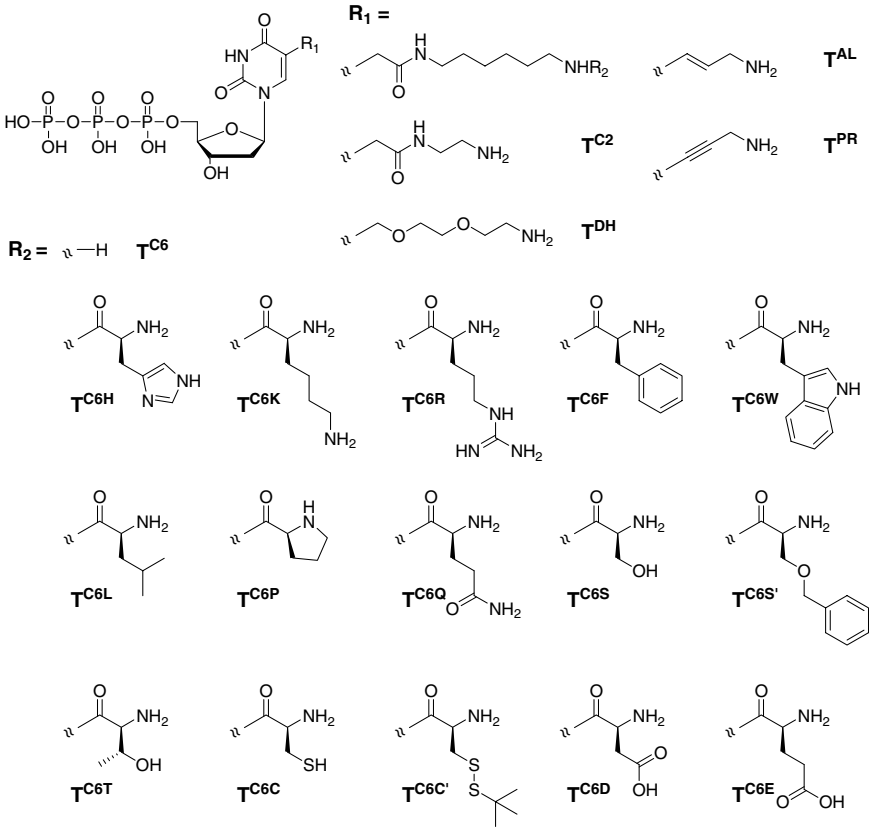


Figure 1. Chemical structures and abbreviations of C5-modified dUTP analogs used in this study.

Table 1. Relative yield of the modified DNAs from dUTP analogs by PCR

dUTP analog	Relative yield
$\mathbf{T^{C6}}$	1
$\mathbf{T^{C2}}$	0.48
$\mathbf{T^{DH}}$	0.34
$\mathbf{T^{AL}}$	0.77
$\mathbf{T^{PR}}$	0

Relative yield is expressed by the ratio of the resulting modified DNA from each dUTP analogue to that from $\mathbf{T^{C6}}$ under the PCR condition 3 (20 cycles, 0.5 min at 94 °C for denaturing, 0.5 min at 52 °C for annealing, and 1 min at 74 °C for extension). All experiments were run five times and the averaged data are shown.

We then investigated the effects of tethered amino acids on amplification efficiency of PCR. Fifteen amino acyl dUTPs were synthesized and their substrate properties were evaluated by PCR assays (Figs. 2A–C, Fig. 3, and Table 2). In all cases, relative yields of PCR were similar or somewhat improved by changing the reaction conditions. However, it is still necessary to optimize PCR parameters, such as temperature and time for each process as well as number of cycles. For example, with $\mathbf{T^{C6D}}$ or $\mathbf{T^{C6E}}$, a small amount of the full-length product of modified DNA was provided under condition 2, while no product was yielded under condition 1. The results of PCR assays indicated that the chemical structure of the side chain residue on the tethered amino acid considerably affects the amplification efficiency of PCR, and relative yields were found to be as follows: anionic residue < cationic residue <aromatic residue ⩽ aliphatic residue ≈ neutral hydrophilic residue, except for $\mathbf{T^{C6C}}$ or $\mathbf{T^{C6C'}}$. For $\mathbf{T^{C6C}}$, PCR under conditions 1 and 2 provided no product, presumably due to dimerization of $\mathbf{T^{C6C}}$ by disulfide bond formation,[24–26] because addition of the reducing agent dithiothreitol (DTT) to the reaction mixture improved generation of the PCR product (Fig. 4). Actually, in our previous study, a dUTP analogue with a thiol group forms a disulfide bond, which was found to be reduced by DTT.[26] For $\mathbf{T^{C6C'}}$, the PCR product obtained in the absence of DTT formed smears (Fig. 3, lane 15). Addition of DTT to the reaction mixture led to a sharp band of gel mobility equal to that derived from $\mathbf{T^{C6C}}$ in the presence of DTT, although long-chain DNA which could be formed by non-specific amplification was present in a substantial amount (Fig. 4). The sharp band was identified by sequencing involving a cloning method as described in experimental section.

To confirm the accurate incorporation of amino acyl dUTPs instead of natural TTP during PCR, modified DNAs derived from $\mathbf{T^{C6H}}$, $\mathbf{T^{C6R}}$, $\mathbf{T^{C6F}}$, $\mathbf{T^{C6W}}$, $\mathbf{T^{C6L}}$, $\mathbf{T^{C6C}}$ (with 4 mM DTT) or $\mathbf{T^{C6E}}$ were sequenced (Figs. 2C–F). Five samples were chosen at random from among single colonies derived from each modified DNA, and their sequences were confirmed. Misincorporation was not observed in any of the samples analyzed, indicating that the modified dUTPs were accurately incorporated into the corresponding modified DNAs, in which the original sequence was conserved (Fig. 5).

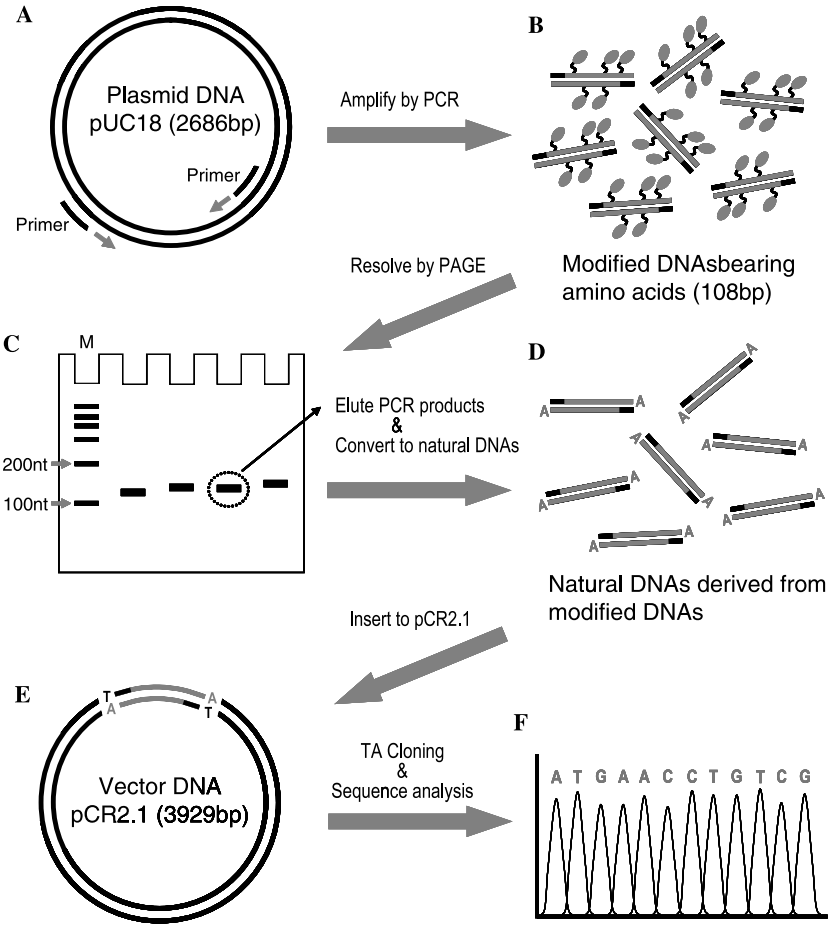


Figure 2. Schematic illustration of PCR assays (A–C) and sequence analyses of PCR products (C–F).

3. Discussion

We synthesized C5-modified dUTPs bearing amino acids by coupling of $\mathbf{T^{C6}}$ with commercially available protected amino acids and subsequent removal of the protecting groups by conventional hydrogenation, which is applicable to $\mathbf{T^{C6}}$ derivatives because the linker arm is not sensitive to catalytic reduction. This synthetic method would also be useful for tethering a variety of short peptides to the amino terminus of $\mathbf{T^{C6}}$. Preparations of modified DNA with 2–4 functionalities by symmetric PCR simultaneously using 2–4 different modified nucleoside triphosphates have been reported[27,28]; however, multiple functionalities could be conveniently incorporated into DNA by using only a single type of dUTP analogue bearing amino acids or peptides.

Using a series of amino acyl dUTPs instead of natural TTP, a variety of modified DNAs with amino acids were prepared by PCR catalyzed with *KOD Dash* DNA polymerase. The relative yields of PCR products were found to depend on the structure of substituents in the analogue, as reported in previous studies.[23,25–33] The substrate specificity of the modified nucleotides is likely varied with their chemical structures, amino acid sequences, and ternary structure of DNA polymerase; however, direct experimental evidence to explain this phenomenon has not yet been reported.

Sequence analyses of the PCR products showed that the original sequences were conserved in the products derived from modified dUTPs, indicating that the modified DNAs obtained in this method are applicable to in vitro selection. Modification of DNA with residues found in the catalytic centers of enzymes (histidyl, lysyl, seryl, cysteinyl, aspartyl or glutamyl) would enhance the potential of libraries for catalytic DNA. Modification with residues such as phenylalanyl, tryptophanyl, leucyl or threonyl may cause local distortion of conformation that induces formation of unique hydrophobic cavities for catalytic- or binding sites via hydrogen bonding or π-π stacking interactions between strands of DNA. In addition, highly active DNA aptamers against anionic target molecules, which cannot be obtained from natural DNA, may emerge from modified DNA libraries having cationic guanidinyl (Arg) or amino groups (Lys). A combinatorial modified DNA library containing arginyl uracil bases was prepared using $\mathbf{T^{C6R}}$, and screening for glutamic acid-binding aptamers from this library is currently underway. Moreover, apart from applications to in vitro selection, enzymatic preparation of modified DNAs could be applied to conferring new abilities to DNA. For instance, serial sequences of uracil with guanidinyl groups, which were enzymatically attached to the 3′-end of oligodeoxynucleotide (ODN) using a $\mathbf{T^{C6}}$ derivative, performed as a carrier for cellular uptake of ODN[34]. Thus, expansion of modified DNA repertories with unique functionalities,

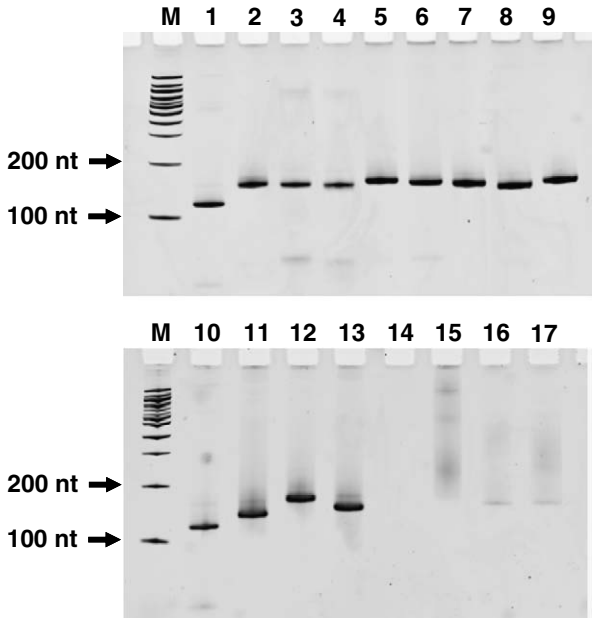


Figure 3. PAGE gel image of the ethidium bromide stained and UV visualized 108 nt PCR product derived from pUC18 and the C5-modified dUTP analogs under condition 2. The full experimental conditions are described in Materials and methods. The reaction mixtures containing dATP, dGTP, dCTP and $\mathbf{T^{C6}}$ (lane 1, 10), $\mathbf{T^{C6H}}$ (lane 2), $\mathbf{T^{C6K}}$ (lane 3), $\mathbf{T^{C6R}}$ (lane 4), $\mathbf{T^{C6F}}$ (lane 5), $\mathbf{T^{C6W}}$ (lane 6), $\mathbf{T^{C6L}}$ (lane 7), $\mathbf{T^{C6P}}$ (lane 8), $\mathbf{T^{C6Q}}$ (lane 9), $\mathbf{T^{C6S}}$ (lane 11), $\mathbf{T^{C6S'}}$ (lane 12), $\mathbf{T^{C6T}}$ (lane 13), $\mathbf{T^{C6C}}$ (lane 14), $\mathbf{T^{C6C'}}$ (lane 15), $\mathbf{T^{C6D}}$ (lane 16) or $\mathbf{T^{C6E}}$ (lane 17) were electrophoresed on denaturing PAGE, respectively. All reaction mixtures, except for positive control, do not contain natural TTP. A reaction mixture containing all four natural dNTPs for positive control provided the PCR product, and a reaction mixture containing dATP, dGTP, and dCTP for negative control did not result in formation of any product (data omitted).

such as amino acyl or peptidyl groups, would enhance the potential of DNA as a functional molecule.

4. Experimental

4.1. General methods

Mass spectral analysis for nucleoside analogues was performed on an *ABI* MDS-Sciex API-100 spectrometer

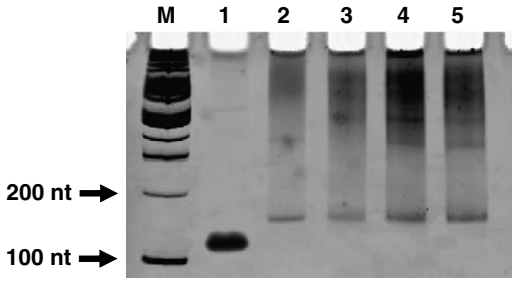


Figure 4. PAGE gel image of the ethidium bromide stained and UV visualized 108 nt PCR product derived from pUC18 and the C5-modified dUTP analogs ($\mathbf{T^{C6}}$, $\mathbf{T^{C6C}}$ or $\mathbf{T^{C6C'}}$) with or without dithiothreitol (DTT) under condition 2. The reaction mixtures containing dATP, dGTP, dCTP, and $\mathbf{T^{C6}}$ (lane 1), $\mathbf{T^{C6C'}}$ with 2 mM DTT (lane 2), $\mathbf{T^{C6C}}$ with 2 mM DTT (lane 3), $\mathbf{T^{C6C}}$ with 4 mM DTT (lane 4) or $\mathbf{T^{C6C}}$ with 10 mM DTT (lane 5) were electrophoresed on denaturing PAGE, respectively. All reaction mixtures, except for positive control, do not contain natural TTP. A reaction mixture containing all four natural dNTPs with 2 mM DTT for positive control provided the PCR product, and a reaction mixture containing dATP, dGTP, and dCTP with 2 mM DTT for negative control did not result in formation of any product (data omitted).

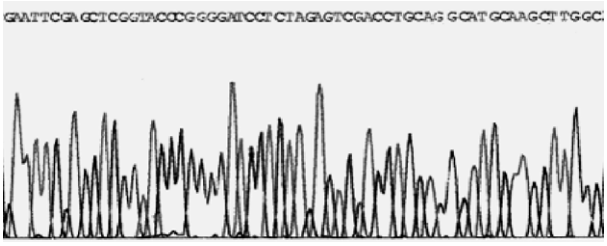


Figure 5. Sequence analysis of the PCR product derived from pUC18 and phenylalanyl dUTP ($\mathbf{T^{C6F}}$) instead of natural TTP. The figure shows DNA sequences (25–108) excluding the primer region.

under atmospheric pressure ionization conditions. UV analyses were performed on a Shimadzu UV-1200 spectrometer. ^{1}H and ^{31}P NMR spectra were recorded on a JEOL JNM-AL300 or JNM-LA500 FT-NMR spectrometer. Tetramethylsilane (TMS) and 85% phosphoric acid were used as the internal standards for ^{1}H and ^{31}P NMR, respectively. Sodium salt of the dUTP analogue, into which the corresponding triethylammonium salt

Table 2. Relative yield of the modified DNAs with amino acids by PCR

dUTP analog	Relative yield		dUTP analog	Relative yield	
	Condition 1	Condition 2		Condition 1	Condition 2
$\mathbf{T^{C6}}$	1	1	$\mathbf{T^{C6Q}}$	1.22	1.29
$\mathbf{T^{C6H}}$	0.70	0.79	$\mathbf{T^{C6S}}$	1.14	1.29
$\mathbf{T^{C6K}}$	0.63	0.61	$\mathbf{T^{C6S'}}$	0.96	1.19
$\mathbf{T^{C6R}}$	0.56	0.59	$\mathbf{T^{C6T}}$	1.21	1.28
$\mathbf{T^{C6F}}$	1.08	1.24	$\mathbf{T^{C6C}}$	0	0
$\mathbf{T^{C6W}}$	0.82	0.94	$\mathbf{T^{C6C'}}$	ND	ND
$\mathbf{T^{C6L}}$	1.18	1.28	$\mathbf{T^{C6D}}$	0	0.08
$\mathbf{T^{C6P}}$	1.16	1.13	$\mathbf{T^{C6E}}$	0	0.10

Relative yield is expressed by the ratio of the resulting modified DNA from each dUTP analogue to that from $\mathbf{T^{C6}}$ under the PCR condition 1 or 2; condition 1 (30 cycles, 0.5 min at 94 °C for denaturing, 0.5 min at 52 °C for annealing, and 1 min at 74 °C for extension) and condition 2 (30 cycles, 0.5 min at 94 °C for denaturing, 1 min at 50 °C for annealing, and 2 min at 74 °C for extension). All experiments were run five times and the averaged data are shown.

ND, not determined because smear band appeared.

was converted with Dowex 50WX8 (Na^+ form), was used for NMR measurement. Reversed-phase HPLC was performed using a JASCO Gulliver system with UV detection at 260 nm and a packed column (C18, 4.6 × 250 mm).

4.2. Materials

All amino acids with protecting groups were purchased from WATANABE Chemical Industries. The following dUTP analogues were used as reference samples: 5-(2-(6-aminohexylamino)-2-oxoethyl)-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6}}$); 5-(2-(2-aminoethylamino)-2-oxoethyl)-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C2}}$); 5-((2-(2-aminoethoxy)ethoxy)methyl)-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{DH}}$); 5-((*E*)-3-aminoprop-1-enyl)-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{AL}}$); and 5-(3-aminoprop-1-ynyl)-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{PR}}$). Among these, the analogues $\mathbf{T^{C6}}$, $\mathbf{T^{C2}}$, and $\mathbf{T^{DH}}$were synthesized according to procedures reported previously,[23,30] while the analogues $\mathbf{T^{AL}}$ and $\mathbf{T^{PR}}$ were purchased from TriLink BioTechnologies and from JENA Bioscience, respectively. Natural 2′-deoxynucleoside-5′-triphosphates (dATP, dGTP, dCTP, and TTP) were obtained from Roche Diagnostics. *KOD Dash* DNA polymerase was obtained from Toyobo. Primer oligonucleotides (#1: 5′-GGAA ACAGCTATGACCATGATTAC-3′, #1F: 5′-FAM-labeled-GGAAACAGCTATGACCATGATTAC-3′, #2: 5′-CGACGTTGTAAAACGACGGCCAGT-3′, and #3: 5′-AAGTTGGGTAACGCCAGGGTTTTC-3′) were purchased from *JBioS* and pUC18 template DNA was obtained from TaKaRa Biomedicals.

4.3. Synthesis of aminoacyl dUTP analogues

All aminoacyl dUTPs, except for sulfur-containing analogs, such as $\mathbf{T^{C6C}}$ and $\mathbf{T^{C6C'}}$, were prepared using protocol similar to that for arginyl dUTP ($\mathbf{T^{C6R}}$), as follows. Aminoacyl dUTPs were synthesized by coupling $\mathbf{T^{C6}}$ with commercially available protected amino acids followed by hydrogenation to remove the protecting groups (Scheme 1). For synthesis of $\mathbf{T^{C6C}}$ and $\mathbf{T^{C6C'}}$, cysteine protected with an Fmoc group was used. The yields were calculated from the estimation that the molar absorption coefficient of the product is the sum of that of 5-(2-(6-aminohexylamino)-2-oxoethyl)-2′-deoxyuridine and a tethered amino acid.

4.3.1. *N*-α, *N*-ω$_1$,*N*-ω$_2$-tricarbobenzoxy-arginine *N*-hydroxysuccinimide ester. *N*,*N*′-Dicyclohexylcarbodiimide (64 mg, 1.5 equiv) in dry DMF (1.0 mL) was added dropwise to a stirred solution of *N*-α,ω$_1$,ω$_2$-tricarbobenzoxy-arginine (120 mg), and hydroxysuccinimide (29 mg, 1.2 equiv) in dry DMF (1.0 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and at room temperature for 18 h, and was then filtered to remove DCUrea. The filtrate was evaporated and the resulting residue was dried well and used for the next reaction without further purification. Assuming that the succinimide ester was obtained in quantitative yield, the crude ester was dissolved in dry DMF such that the final concentration would be 200 mM.

4.3.2. 5-((2-(6-(Arginamido)hexylamino)-2-oxoethyl))-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6R}}$). The analogue $\mathbf{T^{C6}}$ (20 OD_{260nm}, 2.14 μmol) was dissolved in distilled water (100 μL), and a mixture of sodium bicarbonate buffer (1.0 M, 80 μL) and DMF (215 μL) was then added. To the above mixture, *N*-α,*N*-ω$_1$,*N*-ω$_2$-tricarbobenzoxy-L-arginine*N*-succinimidyl ester in dry DMF (200 mM, 215 μL, 20 equiv) was added and the reaction mixture was stirred at room temperature for 3 h. After evaporation, the residue was suspended with water and centrifuged to remove insoluble matter, and the supernatant was evaporated to dryness. The residue was purified by reversed-phase HPLC with a 3.5–70% gradient of acetonitrile in 50 mM triethylammonium acetate buffer (pH 7.2) over 50 min at a flow rate of 1.0 mL/min to give 5-((2-(6-(*N*-α,*N*-ω$_1$,*N*-ω$_2$-tricarbobenzoxy-arginamido)hexylamino)-2-oxoethyl))-dUTP ($\mathbf{T^{C6R'}}$, 1.26 μmol) in 59% yield; ESI-MS (negative ion mode) *m*/*z*: found 1181.5; calcd for [(M−H)$^-$] 1181.3. A suspension containing 5% Pd/C (ca. 10 mg) in 1 mL water/methanol (1:1) was stirred under an H_2 atmosphere. After activation of Pd/C, 10 drops of the suspension were added to the analogue $\mathbf{T^{C6R'}}$ (5.78 μmol) in 1 mL water/methanol (1:1), and this was stirred under an H_2 atmosphere at room temperature for 3 h. The reaction mixture was filtered with a membrane filter (Millex-LH, Millipore) to remove the catalyst and then the filtrate was evaporated to dryness. The residue was purified by reversed-phase HPLC with a 0–56% gradient of acetonitrile in triethylammonium acetate buffer (50 mM, pH 7.2) over 50 min at a flow rate of 1.0 mL/min to give the analogue $\mathbf{T^{C6R}}$ (2.08 μmol) in 36% yield. ^{1}H NMR (300 MHz, D_2O): δ = 7.77 (s, 1H), 6.21 (t, 1H), 4.07 (m, 3H), 3.86 (t, 1H), 3.23 (s, 2H), 3.20 (m, 1H), 3.06 (m, 6H), 2.28 (m, 2H), 1.78 (m, 2H), 1.53 (m, 2H), 1.37 (m, 4H), 1.17 (m, 4H); ^{31}P NMR (500 MHz, D_2O): δ = −7.79 (d), −10.94 (d), −22.04 (t); ESI-MS (negative ion mode) *m*/*z*: found 779.5; calcd for [(M−H)$^-$] 779.2.

4.3.3. 5-((2-(6-(Asparatamido)hexylamino)-2-oxoethyl))-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6D}}$). This product was prepared by coupling of $\mathbf{T^{C6}}$ (0.35 μmol) with *N*-α-carbobenzoxy-L-aspartic acid α-*N*-succinimidyl β-benzyl diester followed by hydrogenation to give the analogue $\mathbf{T^{C6D}}$ (0.14 μmol) in 40% yield from $\mathbf{T^{C6}}$. ESI-MS (negative ion mode) *m*/*z*: found 738.1; calcd for [(M−H)$^-$] 738.1.

4.3.4. 5-((2-(6-(*S*-*tert*-Butylthio-cysteinamido)hexylamino)-2-oxoethyl))-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6C'}}$). The analogue $\mathbf{T^{C6}}$ (10 OD_{260nm}, 1.07 μmol) was dissolved in distilled water (120 μL) and sodium bicarbonate buffer (1.0 M, 20 μL) was then added. To the above mixture, *N*-α-(9-fluorenylmethoxycarbonyl)-*S*-*tert*-butylthio-L-cysteine *N*-succinimidyl ester in dry DMF (100 mM, 260 μL, 25 equiv) was added and the reaction mixture was stirred at room temperature for 3 h. After evaporation, the residue was suspended in water and centrifuged to remove insoluble materials, and the supernatant was evaporated to dryness. The residue was purified by reversed-phase HPLC with a 3.5–56% gradient of acetonitrile in 50 mM triethylammonium acetate buffer (pH 7.2) over 35 min at a flow rate of

1.0 mL/min to give 5-((2-(6-(*N*-α-(9-fluorenylmethoxycarbonyl)-*S*-*tert*-butylthio-cysteinamido)hexylamino)-2-oxoethyl))-dUTP ($\mathbf{T^{C6C'}}$-Fmoc, 0.50 μmol) in 47% yield from $\mathbf{T^{C6}}$; ESI-MS (negative ion mode) *m*/*z*: found 1036.2; calcd for [(M−H)$^-$] 1036.2. The analogue $\mathbf{T^{C6C'}}$-Fmoc (0.50 μmol) was dissolved with water (1 mL) and a drop of diethylamine was added at 0 °C. The reaction mixture was stirred for 2 h at 0 °C and was then evaporated to dryness. The residue was purified by reversed-phase HPLC with a 3.5–56% gradient of acetonitrile in triethylammonium acetate buffer (50 mM, pH 7.2) over 35 min at a flow rate of 1.0 mL/min to give the analogue $\mathbf{T^{C6C'}}$ (0.34 μmol) in 68% yield from $\mathbf{T^{C6C'}}$-Fmoc. ESI-MS (negative ion mode) *m*/*z*: found 814.1; calcd for [(M−H)$^-$] 814.1.

4.3.5. 5-((2-(6-(Cysteinamido)hexylamino)-2-oxoethyl))-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6C}}$). The *tert*-butylthio-protecting group was removed by addition of Tris–HCl buffer (10 mM, pH 8.0) and DTT (10 mM) to the analogue $\mathbf{T^{C6C'}}$ (0.66 μmol). The reaction mixture was stirred for 10 min at room temperature and then evaporated to dryness. The residue was purified by reversed-phase HPLC with a 3.5–56% gradient of acetonitrile in triethylammonium acetate buffer (50 mM, pH 7.2) over 35 min at a flow rate of 1.0 mL/min to give the analogue $\mathbf{T^{C6C}}$ (0.053 μmol) in 8% yield from $\mathbf{T^{C6C'}}$. ESI-MS (negative ion mode) *m*/*z*: found 726.1; calcd for [(M−H)$^-$] 726.1.

4.3.6. 5-((2-(6-(Glutaminamido)hexylamino)-2-oxoethyl))-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6Q}}$). This product was prepared by coupling of $\mathbf{T^{C6}}$ (0.66 μmol) with *N*-α-carbobenzoxy-L-glutamine α-*N*-succinimidyl ester followed by hydrogenation to give the analogue $\mathbf{T^{C6Q}}$ (0.51 μmol) in 77% yield from $\mathbf{T^{C6}}$. ESI-MS (negative ion mode) *m*/*z*: found 751.4; calcd for [(M−H)$^-$] 751.2.

4.3.7. 5-((2-(6-(Glutaminamido)hexylamino)-2-oxoethyl))-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6E}}$). This product was prepared by coupling of $\mathbf{T^{C6}}$ (0.89 μmol) with *N*-α-carbobenzoxy-L-glutamic acid α-*N*-succinimidyl γ-benzyl diester followed by hydrogenation to give the analogue $\mathbf{T^{C6E}}$ (0.73 μmol) in 82% yield from $\mathbf{T^{C6}}$; ESI-MS (negative ion mode) *m*/*z*: found 752.2; calcd for [(M−H)$^-$] 752.1.

4.3.8. 5-((2-(6-(Histidinamido)hexylamino)-2-oxoethyl))-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6H}}$). This product was prepared by coupling of $\mathbf{T^{C6}}$ (0.65 μmol) with *N*-α, *N*-π-dicarbobenzoxy-L-histidine α-*N*-succinimidyl ester followed by hydrogenation to give the analogue $\mathbf{T^{C6H}}$ (0.15 μmol) in 23% yield from $\mathbf{T^{C6}}$. ESI-MS (negative ion mode) *m*/*z*: found 760.1; calcd for [(M−H)$^-$] 760.2.

4.3.9. 5-((2-(6-(Leucinamido)hexylamino)-2-oxoethyl))-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6L}}$). This product was prepared by coupling of $\mathbf{T^{C6}}$ (0.35 μmol) with *N*-α-carbobenzoxy-L-leucine α-*N*-succinimidyl ester followed by hydrogenation to give the analogue $\mathbf{T^{C6L}}$ (0.15 μmol) in 43% yield from $\mathbf{T^{C6}}$. ESI-MS (negative ion mode) *m*/*z*: found 736.0; calcd for [(M−H)$^-$] 736.2.

4.3.10. 5-((2-(6-(Lysinamido)hexylamino)-2-oxoethyl))-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6K}}$). This product was prepared by coupling of $\mathbf{T^{C6}}$ (0.86 μmol) with *N*-α-*N*-ε-di-carbobenzoxy-L-lysine α-*N*-succinimidyl ester followed by hydrogenation to give the analogue $\mathbf{T^{C6K}}$ (0.72 μmol) in 84% yield from $\mathbf{T^{C6}}$. ESI-MS (negative ion mode) *m*/*z*: found 753.3; calcd for [(M−H)$^-$] 753.2.

4.3.11. 5-((2-(6-(Tryptophanamido)hexylamino)-2-oxoethyl))-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6W}}$). This product was prepared by coupling of $\mathbf{T^{C6}}$ (0.63 μmol) with *N*-α-carbobenzoxy-L-tryptophan α-*N*-succinimidyl ester followed by hydrogenation to give the analogue $\mathbf{T^{C6W}}$ (0.24 μmol) in 38% yield from $\mathbf{T^{C6}}$. ESI-MS (negative ion mode) *m*/*z*: found 809.0; calcd for [(M−H)$^-$] 809.2.

4.3.12. 5-((2-(6-(Phenylalaninamido)hexylamino)-2-oxoethyl))-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6F}}$). This product was prepared by coupling of $\mathbf{T^{C6}}$ (0.66 μmol) with *N*-α-carbobenzoxy-L-phenylalanine α-*N*-succinimidyl ester followed by hydrogenation to give the analogue $\mathbf{T^{C6F}}$ (0.18 μmol) in 27% yield from $\mathbf{T^{C6}}$. ESI-MS (negative ion mode) *m*/*z*: found 770.2; calcd for [(M−H)$^-$] 770.2.

4.3.13. 5-((2-(6-(Prolinamido)hexylamino)-2-oxoethyl))-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6P}}$). This product was prepared by coupling of $\mathbf{T^{C6}}$ (0.41 μmol) with *N*-α-carbobenzoxy-L-proline α-*N*-succinimidyl ester followed by hydrogenation to give the analogue $\mathbf{T^{C6P}}$ (0.12 μmol) in 29% yield from $\mathbf{T^{C6}}$. ESI-MS (negative ion mode) *m*/*z*: found 720.1; calcd for [(M−H)$^-$] 720.2.

4.3.14. 5-((2-(6-(*O*-Benzyl-serinamido)hexylamino)-2-oxoethyl))-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6S'}}$). Coupling of the analogue $\mathbf{T^{C6}}$ with *N*-α-carbobenzoxy-*O*-benzyl-L-serine α-*N*-succinimidyl ester was performed similarly to the procedure described for $\mathbf{T^{C6R}}$. The analogue $\mathbf{T^{C6S''}}$, *5-((2-(6-(N*-α-carbobenzoxy-*O-benzyl-serinamido)hexylamino)-2-oxoethyl))*-2′-dUTP (0.49 μmol) was obtained in 54% yield from $\mathbf{T^{C6}}$ (0.90 μmol). A suspension containing 5% Pd/C (ca. 10 mg) in 1 mL of water/methanol (1:1) was stirred under an H_2 atmosphere. After activation of Pd/C, ten drops of the suspension were added to $\mathbf{T^{C6S''}}$ (0.35 μmol) in 1 mL of water/methanol (1:1), and this was stirred under an H_2 atmosphere at room temperature for 3 h. The reaction mixture was filtered with a membrane filter (Millex-LH, Millipore) to remove the catalyst, and the filtrate was evaporated to dryness. The residue was purified by reversed-phase HPLC with a 3.5-70% gradient of acetonitrile in triethylammonium acetate buffer (50 mM, pH 7.2) over 35 min at a flow rate of 1.0 mL/min to give the analogue $\mathbf{T^{C6S'}}$ (0.077 μmol) in 22% yield from $\mathbf{T^{C6S''}}$. ESI-MS (negative ion mode) *m*/*z*: found 800.2; calcd for [(M−H)$^-$] 800.2.

4.3.15. 5-((2-(6-(Serinamido)hexylamino)-2-oxoethyl))-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6S}}$). A suspension containing 5% Pd/C (ca. 10 mg) in 1 mL water/methanol (1:1) was stirred under H_2 atmosphere. After activation of Pd/C, ten drops of the suspension were added to $\mathbf{T^{C6S''}}$ (0.25 μmol) in 1 mL water/methanol (1:1), and this was

then stirred under an H_2 atmosphere at room temperature overnight. The reaction mixture was filtered with a membrane filter (Millex-LH, Millipore) to remove the catalyst, and the filtrate was evaporated to dryness. The residue was purified by reversed-phase HPLC with a 3.5–70% gradient of acetonitrile in triethylammonium acetate buffer (50 mM, pH 7.2) over 35 min at a flow rate of 1.0 mL/min to give the analogue $\mathbf{T^{C6S}}$ (0.038 μmol) in 15% yield from $\mathbf{T^{C6S''}}$. ESI-MS (negative ion mode) *m*/*z*: found 710.0; calcd for [(M−H)$^-$] 710.1.

4.3.16. 5-((2-(6-(Threoninamido)hexylamino)-2-oxoethyl))-2′-deoxyuridine-5′-triphosphate ($\mathbf{T^{C6T}}$). This product was prepared by coupling of $\mathbf{T^{C6}}$ (0.58 μmol) with *N*-α-carbobenzoxy-*O*-benzyl-L-threonine α-*N*-succinimidyl ester followed by hydrogenation to give the analogue $\mathbf{T^{C6T}}$ (0.070 μmol) in 12% yield from $\mathbf{T^{C6}}$. ESI-MS (negative ion mode) *m*/*z*: found 724.0; calcd for [(M−H)$^-$] 724.1.

4.4. PCR amplification

PCR experiments were performed with a 20 μL reaction volume containing 10 ng pUC 18 template DNA, primers #1 and #2 at 0.4 μM each, 1.0 U *KOD Dash* DNA polymerase, reaction buffer supplied with enzyme (at 1times concentration), and triphosphates at 200 μM each; PCR with natural triphosphates dATP, dGTP, dCTP, and TTP was used as a positive control; PCR with one of the dUTP analogues ($\mathbf{T^{C6}}$, $\mathbf{T^{C2}}$, $\mathbf{T^{DH}}$, $\mathbf{T^{AL}}$, $\mathbf{T^{PR}}$, $\mathbf{T^{C6H}}$, $\mathbf{T^{C6K}}$, $\mathbf{T^{C6R}}$, $\mathbf{T^{C6F}}$, $\mathbf{T^{C6W}}$, $\mathbf{T^{C6L}}$, $\mathbf{T^{C6P}}$, $\mathbf{T^{C6Q}}$, $\mathbf{T^{C6S}}$, $\mathbf{T^{C6S'}}$, $\mathbf{T^{C6T}}$, $\mathbf{T^{C6C}}$, $\mathbf{T^{C6C'}}$, $\mathbf{T^{C6D}}$ or $\mathbf{T^{C6E}}$) instead of TTP was used for the actual assays (Fig. 1); PCR with dATP, dGTP, and dCTP (water was added instead of TTP) was used as a negative control. For all amplifications, a hot start (1 min at 94 °C) was used, followed by amplification and final incubation (5 min at 74 °C). Conditions for the amplification process (denaturing, annealing, and extension) were as follows; condition 1 (30 cycles, 0.5 min at 94 °C, 0.5 min at 52 °C, and 1 min at 74 °C); condition 2 (30 cycles, 0.5 min at 94 °C, 1 min at 50 °C, and 2 min at 74 °C); condition 3 (20 cycles, 0.5 min at 94 °C, 0.5 min at 52 °C, and 1 min at 74 °C). PCR products were resolved by denaturing PAGE, visualized by ethidium bromide staining, and gel images were recorded on a Molecular Imager® FX (Bio-Rad). The intensity of each band corresponding to full-length DNA product was quantified using Quantity One® software. The amount of full-length PCR product formed during reaction with $\mathbf{T^{C6}}$ was set at 1. Here, calculated relative yields are averages of five independent experiments.

4.5. Cloning and sequencing of PCR products

Modified DNA was purified by agarose gel electrophoresis and eluted from the gel into TBE buffer (45 mM Tris-borate, 1 mM EDTA). The eluted sample was converted into natural DNA by a second PCR using *KOD Dash* DNA polymerase under condition 1. After purification by agarose gel electrophoresis and elution, the dsDNA product was re-amplified by a third PCR using *Taq* DNA polymerase under condition 3 and was subjected to the TA Cloning® method: *Taq* DNA polymerase has a non-template-dependent activity that adds a single 2′-deoxyadenosine at the 3′-end of PCR product. Cloning was performed using a TA Cloning® kit (Invitrogen) according to the instruction manual and five samples were chosen at random from among single colonies derived from modified DNA for sequencing. Plasmids extracted from the single colonies were sequenced with a Genetic Analyzer 310 (ABI) according to standard protocol using a BigDye Terminator® v3.0 Sequencing Kit together with primer #3.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (B) and (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and PRESTO from the Japan Science and Technology Agency.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.11.030.

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