博士論文

Development of elemental technologies for a simple and rapid nucleic acid amplification test

（簡便・迅速な核酸増幅検査のための要素技術開発）

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(1) Mutations to create thermostable reverse transcriptase with bacterial family A DNA polymerase from *Thermotoga petrophila* K4
Sotaro Sano, Yosuke Yamada, Tomoki Shinkawa, Satoru Kato, Takashi Okada, Hiroki Higashibata, and Shinsuke Fujiwara
Journal of Bioscience and Bioengineering, **113** (3), 315-321 (2012).

(2) Rapid multiplex nucleic acid amplification test developed using paper chromatography chip and azobenzene-modified oligonucleotides
Sotaro Sano, Shigehiko Miyamoto, Seiji Kawamoto
Journal of Bioscience and Bioengineering, **126** (3), 397-403 (2018).

３．参考論文

Method for colorimetric detection of double-stranded nucleic acid using leuco triphenylmethane dyes
Shigehiko Miyamoto, **Sotaro Sano**, Koji Takahashi, Takaaki Jikihara
Analytical Biochemistry, **473** (15), 28-33 (2015).

主論文

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General introduction

1. Introduction

Since the development of polymerase chain reaction (PCR) (1), technologies aimed at the analysis of nucleic acid have drastically improved. These are applied in the pathogen detection, food poisoning detection, and companion diagnostics (2-4), and they are referred to as nucleic acid amplification tests (NATs). NAT has become an indispensable diagnostics tools for medical care and food industries, as it can be used to detect targets from the trace amounts of DNA or RNA. As one example, to detect herpes simplex virus (HSV), which may cause severe diseases, such as HSV encephalitis and neonatal HSV infections, only 10 copies of HSV DNA are necessary (5). However, to perform NAT, complicated procedures and expensive instruments are usually required, which prevents a wider application of NATs in the developing countries and smaller institutions, such as clinics and quarantine stations, despite the large demand in these fields. Especially considering the recent pandemics of emerging and re-emerging infectious diseases, the point of care (POC)-testing, referring to the diagnostic

on site, has attracted attention due to its high infection control potential (6-8). It is important for the POC-testing tools to be able to operate easily, output results quickly and, perform with inexpensive instruments. However, generally NAT requires complex procedures, long time for outputting results and expensive devices.

2. Enzyme for nucleic acid amplification

Basically, NAT consists of amplification and detection procedures. Various amplification methods besides PCR have been developed. Some methods are characterized as isothermal amplification which proceeding amplification reaction without thermal cycling (9-12). These methods are suitable for POC-NAT, since just a heat regulator is required for the reaction. However, main method for nucleic acid amplification is still PCR, because it is easy to make multiplex detection forms (13).

For the amplification procedure, some enzymes are used as reagents. The most important enzyme for PCR is thermostable DNA-dependent DNA polymerase which catalyzes DNA synthesis using primer oligonucleotides and template DNA. In the previous studies, various thermostable DNA-

dependent DNA polymerases were developed and now we can select the enzymes with specialized performances (14-16).

DNA-dependent DNA polymerase for PCR is roughly divided to two groups, one is family A DNA polymerase derived from thermophilic bacteria and the other one is family B DNA polymerase derived from thermophilic archaea. There are Taq DNA polymerase and KOD DNA polymerase as representatives of each group (14, 16). As common features of these enzymes are heat residence property and reacting at high temperature (usually around 70°C). The enzyme possessing 3′-5′ exonuclease (proof-reading) activity, has higher accuracy in DNA synthesis, so application of these enzymes to diagnostics are important to high precision testing (15, 16).

As one of basic derivative methods of PCR, reverse transcript (RT)-PCR which detects RNA is frequently used (17). RT-PCR has additional reverse transcript step which synthesizing DNA from RNA templates before PCR step. The method is important, not only for the RNA virus detection, but also for the ultra-high sensitivity detection, because some RNA molecules (*e.g.* 16S rRNA in bacteria) are contained larger copies than genome DNA per one cell (18). In the RT step, reverse transcriptase (RNA-dependent DNA

polymerase) derived from retrovirus is mainly used (19). Since suitable reaction conditions of reverse transcriptase and PCR-use DNA polymerase are different, RT and PCR reactions are usually performed separately. Therefore, we need two steps complicated operations to perform RT-PCR, and it reduces the simplicity of this method.

3. Detection technologies for amplified products

The detection procedure of NAT is a step to detect amplified nucleic acid over million times in amplification procedure. In the molecular biology laboratory, electrophoresis and ethidium bromide staining are widely used to detect PCR products (20). However, these methods require technical skill, complicated procedures, some special instruments and carcinogenic reagents. Therefore, it is difficult to perform PCR products detection in the place without such skilled personnel and special instruments.

To make the detection procedure of NAT simply, some technologies have been developed. One is real-time PCR method which monitors amplified DNA using intercalating fluorescent dye or probe in real-time (21, 22). As this method integrates amplification and detection process, we need no additional

operation for detection. However, the machine for real-time PCR is expensive and requires regular maintenances, so that it is still difficult to install the machine to smaller institutions.

Paper-based chromatography chip systems are suitable for the development of POC-NATs, because they require simple procedures, the use of inexpensive instruments, and low manufacturing costs. Previously, Corstjens *et al.*, (23) reported the development of a chromatography chip comprising a streptavidin immobilized on a chip and anti-digoxigenin (DIG) antibody-immobilized phosphor particles. Using PCR and biotin and DIG-modified primers, the obtained amplified products can be detected on the chip as colored lines. However, according to this system, multiplex detection is limited by the antigen-antibody combination. Another group developed a chromatography chip using DNA-DNA hybridization, comprising streptavidin immobilized on a paper chip and single-strand DNA immobilized on colloidal gold nanoparticles (24), which enabled the development of multiplex detection systems, due to the different complementary sequence combinations. However, since PCR products are double-strand DNA, melting and temperature regulation are necessary, which reduces the usability of the

system. To make the technology more suitable for POC-testing, simplicity and rapidity in operation processes are required.

4. Research objective of this thesis

The objective of this thesis is to develop elemental technologies for a simple and rapid NAT which contribute to realize the point of care (POC)-NAT. Concretely, I focused on the amplification and the detection processes of NAT which consisting major procedures of this method, and conducted a study to accomplish objectives described below respectively.

In chapter 1, I tried to develop high fidelity RT-PCR-use enzyme possessing both DNA-dependent DNA polymerase activity and reverse transcriptase activity, to make RT-PCR procedures a simple format. In chapter 2, I tried to develop a simple and rapid detection system for multiplex-PCR products using a paper base DNA chromatography chip to make multiplex-PCR detection procedures simple and rapid format.

Chapter 1: Development of thermostable enzyme possessing both reverse transcriptase activity and DNA-dependent DNA polymerase activity

1-1. Introduction

Reverse transcriptase catalyzes DNA polymerization using RNA template (RNA-dependent DNA polymerase) (19). This enzyme is applied to reverse transcript (RT)-PCR which is useful for RNA virus detection and ultra-high sensitivity detection. In this application, reverse transcriptase has central roles, and retroviral enzymes, such as the Moloney murine leukemia virus (MMLV) or the avian myeloblastosis virus (AMV), are mainly utilized. However, retroviral reverse transcriptase has two major drawbacks for the application to RT-PCR.

One is low fidelity in reaction, because of the lack of 3′-5′ exonuclease (proof-reading) activity. Application of enzyme with high accuracy is preferable for the precise diagnostics. Attempts were made to create a retroviral reverse transcriptase with high fidelity. The accuracy of reverse transcriptase derived from human immunodeficiency virus type 1 was increased by an amino acid substitution at a unique position (25). The

addition of an *Escherichia coli* DNA polymerase III ε subunit (possessing 3′-5′ exonuclease activity) also increased the fidelity of MMLV reverse transcriptase (26).

The other drawback of retroviral reverse transcriptase is instability which requests not only delicate handlings, but also causes insufficient RT reactions. Single-strand template DNA or RNA forms unfavorable stem loop-like structure depending on the nucleotide sequence, especially at low temperatures. This unfavorable structure inhibits the DNA synthesis. To prevent the inhibition, incubation at temperatures above 50°C is preferred in enzymatic DNA synthesis. However, a retroviral enzyme is easily denatured in such high temperature.

To develop a reverse transcriptase with highly stability, several approaches have been tried (27–36), and some genetically modified reverse transcriptases are available as reagents. The stabilized MMLV reverse transcriptase was developed by site-directed mutagenesis following suppression of RNase H activity (29, 30). However, it has not been accomplished to improve the fidelity and stability of a retroviral enzyme as same as a PCR-use DNA polymerase. Also, since suitable reaction conditions

of reverse transcriptase and PCR-use DNA polymerase are different, RT and PCR reactions are usually performed separately. Therefore, we need two step complicated operations to perform RT-PCR with retroviral enzymes.

Another approach to create thermostable reverse transcriptase was attempted using thermostable DNA-dependent DNA polymerases from thermophiles (31-36). Thermophilic archaeal family B DNA polymerases, such as from *Pyrococcus furiosus* (15) or *Thermococcus kodakarensis* (16) are reported to possess a higher accuracy in PCR than enzymes derived from *T. aquaticus* and *T. thermophilus*. However, these family B DNA polymerases were considered unsuitable as a source to develop a thermostable reverse transcriptase, because their reaction stalls when an uracil-containing template was incorporated (37). On the other hand, some DNA polymerases I (PolI) derived from thermophilic bacteria are known to possess reverse transcriptase activity (31-34). It has been known that chimeric PolI from *Thermus s*pecies Z05 and *Thermotoga maritima* possesses reverse transcriptase activity while maintaining DNA-dependent DNA polymerase activity and thermal stability (36). However, it has been unclear the template DNA/RNA recognition system of PolI type DNA polymerase.

In this chapter, I have constructed various mutants of DNA polymerase I from a newly isolated hyperthermophilic bacterium, *Thermotoga petrophila* K4 and have attempted to identify the key positions of amino acid residue for template DNA/RNA recognition. Also, I have tried to create the thermostable reverse transcriptase with DNA-dependent DNA polymerase activity useful for simple one-step RT-PCR.

1-2. Material and methods

1-2-1. Materials

Thermotoga maritima MSB8 (DSM 3109) was obtained from the Biological Resource Center, National Institute of Technology and Evaluation (NITE), Japan. *Escherichia coli* DH5α [*F*$^-$, *mcrA,* Δ(*mrr*$^-$ *hsdRMS*$^-$ *mcrBC*), *Φ80dlacZ,* Δ*M15,* Δ*lacX74, deoR, recA1, araD139,* Δ(*ara leu*)7697*, galU, galK,* λ$^-$*, rpsL, endA1, nupG*] was utilized for plasmid construction. *E. coli* BL21-CodonPlus (DE3)-RP [*F*$^-$ *ompT hsdSB* (rB$^-$ mB$^-$) *dcm*$^+$ *Tet*^{r} *gal*λ (DE3) *endA Hte* [*argU proL Cam*^{r}]] (Stratagene, USA) and the expression vector pET-21a (Novagen, USA) were utilized for overexpression of the target genes. *E. coli* DH5α and *E. coli* BL21-CodonPlus (DE3)-RP were aerobically cultured in LB liquid medium (1% tryptone, 0.5% yeast extract, 1% NaCl; adjusted to pH 7.3 with NaOH) at 37°C. Plasmid pUC19 was utilized as a vector for cloning the DNA polymerase I gene. *E. coli* TH2 [*supE44, hsdS20* (rB$^-$ mB$^-$)*, recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1, thi-1, trpR624*] was used for fidelity analysis was obtained from Takara Bio (Japan). The final concentration of ampicillin, chloramphenicol and streptomycin, when added to LB liquid medium, were 100 μg/ml, 12 μg/ml and 50 μg/ml, respectively.

Restriction enzymes and other enzymes for cloning were obtained from Takara Bio (Japan), Toyobo (Japan) or Roche Diagnostics (Switzerland). A BigDye Terminator cycle-sequencing ready reaction kit, version 3.1, and a DNA sequencer 3130 Genetic Analyzer (Applied Biosystems, USA) were used for DNA sequencing analysis. All chemicals and the culture medium were obtained from Wako Pure Chemical Industries (Japan) and Nacalai Tesque (Japan).

1-2-2. Isolation of the environmental microorganism

Water samples collected from a hydrothermal vent (73°C) at Kodakara Island, Kagoshima, Japan were filtrated with 0.45 μm filter. The filters were added to 2× YT liquid medium (1.0% yeast extract, 1.6% tryptone, 1.0% NaCl) and were anaerobically incubated at 83°C for 12 h. After culturing, the grown microorganisms were placed in a 2× YT solid plate (2× YT medium containing 1% gelrite® instead of agar) and incubated at 83°C for 12 h. From a cultured plate, a single colorless colony was isolated. The isolated microorganism (*Thermotoga prtrophila* K4) on a 2× YT gelrite® plate was cultured on the K4 medium with 1.6% tryptone, 1.0% yeast extract, trace

minerals (1.5 g of nitrilotriacetic acid, 3 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $MnSO_4 \cdot 5H_2O$, 1 g of NaCl, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.18 g of $CoSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.18 g of $ZnSO_4 \cdot 7H_2O$, 0.01 g of $CuSO_4 \cdot 5H_2O$, 0.01 g of H_3BO_3, 0.01 g of $Na_2MoO_4 \cdot 2H_2O$, 0.014 g of $NiCl_2$, 0.53 mg of Na_2SeO_3, 0.93 mg of $Na_2WO_4 \cdot 2H_2O$, 0.92 mg of $Na_2WO_4 \cdot 2H_2O$, 4 mg of $KAl(SO_4)_2 \cdot 12H_2O$ per liter), and artificial sea water (5.54 g of NaCl, 1.4 g of $MgSO_4 \cdot 7H_2O$, 1.1 g of $MgCl_2 \cdot 6H_2O$, 0.45 g of $CaCl_2 \cdot 2H_2O$, 0.13 g of KCl, 20 mg of NaBr, 6 mg of H_2BO_3, 3 mg of $SrCl_2 \cdot 6H_2O$, 2 mg of citric acid, 10 μg of KI per liter) at 80°C under anaerobic conditions. The pH of K4 medium was adjusted to 6.8 with KOH. The antibiotics susceptibility analysis of the isolated microorganism was carried out with K4 medium containing the final concentration 100 μg/ml of rifampicin as described in the previous study (38).

1-2-3. Electron microscopic photography

Electron micrographs of the isolated microorganism were taken as same as the previous report (38). Cultured microorganisms were putted on a VECO Cu 400 mesh (Electron Microscopy Sciences, USA) pasted with a 2% solution of isoamyl acetate and negatively stained by 1% ammonium

molybdate. Electron micrographs of the isolated microorganism were taken with a JEM-1010 (JEOL, Japan) with accelerating voltage at 80 kV. An imaging plate (Fujifilm, Japan) was used as a detector.

1-2-4. Molecular phylogenetic analysis

DNA manipulations were carried out by standard techniques as described previously by Sambrook and Russell (20). Genomic DNA of the isolated microorganism was prepared by the Sarkosyl method and purified by CsCl equilibrium density gradient ultracentrifugation. Molecular phylogenetic analysis was performed by comparing nucleotide sequences of 16S rDNA region. To obtain the sequence of 16S rDNA region, PCR was carried out using primers 16SFFw and 16SFRv with 1 unit of KOD Plus polymerase (Toyobo, Japan) under the following condition (2 min at 94°C, followed by 30 cycles of 15 s at 94°C, 30 s at 60°C, 30 s at 68°C). Primer sequences used in this chapter are listed in Table 1.

1-2-5. Cloning and purification of K4 DNA polymerase

The PCR product with primer set (Pol-Fw and Pol-Rv) amplifying the

open reading frame of DNA polymerase I gene in newly isolated strain K4 (K4PolI) was digested with *Nde* I and *Sal* I and cloned into the pET-21a vector digested with the same restriction enzymes. *E. coli* BL21-CodonPlus (DE3)-RP was transformed with the constructed plasmid pET-K4PolI. Overexpression of K4PolI was induced by the addition of IPTG (final concentration, 1 mM). After 3 h of induction, cultured cells were harvested with centrifugation (8000× g, 10 min). The cells were disrupted by sonication and lysate sample was centrifuged (8000× g, 10 min). The obtained supernatant sample was incubated at 85°C for 30 min and centrifuged again (8000× g, 10 min). The supernatant of incubated sample was then applied to a heparin column (1.6 by 2.5 cm; HiTrap™ heparin; GE Healthcare, USA) equilibrated with the buffer A (10 mM Tris-HCl [pH 8.0], 0.1 M NaCl, 1 mM DTT, 10% glycerol). Trapped proteins were eluted by a linear gradient of 0.1 to 1.5 M NaCl in the buffer A. The recovered sample was dialyzed with buffer A and applied to an anion exchange column (Mono Q® HR 5/5; GE Healthcare, USA). Proteins were eluted by a linear gradient of 0.1 to 1.0 M NaCl in the buffer A. The recovered sample was dialyzed with buffer B (10 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 1 mM DTT, 10% glycerol) and applied to a gel filtration

column (Superdex™ 200; GE Healthcare, USA) equilibrated with buffer B. Fractions containing DNA-dependent DNA polymerase activity were collected and dialyzed with buffer A. DNA polymerase activity was confirmed by monitoring DNA-dependent DNA amplification of the TK0149 gene from *Thermococcus kodakarensis* using primer set, TK0149-Fw and TK0149-Rv, and plasmid pUD2-TK0149 DNA as a template (39).

1-2-6. 3′-5′ exonuclease activity evaluation

The 3′-5′ exonuclease activity was evaluated by calculating the released dNMP (nmol) from DNA on 1 μg of enzyme in 1 min. The reaction mixture (50 μl) contained 50 mM bicine KOH (pH 8.2), 115 mM CH_3COOK, 8% glycerol, 1 mM $Mn(CH_3COO)_2$, 0.2 μM 5′ fluorescently labeled synthetic oligo-deoxyribonucleotide (3′-5′ EXO), and 20 μg purified polymerase. The reaction was started by the addition of an enzyme to the mixture and quenched by 50 mM EDTA. The reaction temperature was set at 68°C. Reacted samples were then separated on a 10% (w/v) polyacrylamide gel (containing 7 M urea) electrophoresis. The fluorescent intensity of separated fragments were measured with an Odyssey infrared imaging system (LI-COR,

USA).

1-2-7. Accuracy evaluation in PCR

The accuracy of DNA polymerase was examined according to the previously reported procedure with slight modifications (40). The *rpsL* gene of *E. coli* encodes the small ribosomal protein S12 which is the target molecule of streptomycin (Sm). *E. coli* TH2 (*rpsL20*) shows Sm resistant phenotype (Sm^r) by lack of functional S12 protein. The PCR fidelity was analyzed as the mutation frequency in PCR products with the full-length of the plasmid pKF3 possessing *cat* gene for chloramphenicol (Cm) resistance and *rpsL* gene for streptomycin sensitive phenotype (Sm^s). An inverse PCR products using pKF3 as a template were self-ligated. *E. coli* TH2 cells were transformed with the amplified and self-ligated pKF3. TH2 cells possessing wild-type *rpsL* gene in pKF3 show Sm^s and Cm^r phenotype. On the other hand, the cells possessing mutated *rpsL* gene show Sm^r and Cm^r phenotype. The mutation frequency was calculated as the ratio of the number of TH2 cell colonies with Sm^r and Cm^r phenotype to the total number of the Sm^s and Cm^r phenotype. An inverse PCR was performed with two adjacent primers, Fw-m and Rv-m,

template pKF3 plasmid and DNA polymerase in a 50 µl of optimized reaction mixture containing 0.6 mM $(NH_4)_2SO_4$, 1 mM KCl, 12 mM Tris-HCl (pH 8.0), 0.01% Triton X-100, 0.0001% BSA, 1 mM $MgCl_2$, 200 µM dNTPs, 0.3 µM of primers, and 1 µg of DNA polymerase from isolated microorganism with PC707 thermal cycler (Aspec, Japan). The reaction was performed as follows: 2 min at 94°C followed by 40 cycles of 15 s at 94°C, 30 s at 60°C, and 1 min at 68°C. The PCR products were blunted with T4 DNA polymerase (Takara Bio, Japan) and then self-ligated with Ligation high (Toyobo, Japan) and used to transform *E. coli* TH2.

1-2-8. Modeling of tertiary structure and mutant construction

Protein tertiary structure modeling was carried out with a Swiss model, and Klenow fragment of *E. coli* was used as a template (41). All K4PolI mutants used in this chapter were constructed by an inverse PCR using plasmid pET21a-K4PolI as a template. For the mutant plasmids construction, the following primer sets were used: T326A, T326A-Fw, and T326A-Rv; L329A, L329A-Fw, and L329A-Rv; Q384A, Q384A-Fw, and Q384ARv; K387A, K387A-Fw, and K387A-Rv; F388A, F388A-Fw, and F388A-Rv; M408A,

M408AFw, and M408A-Rv; N422A, N422A-Fw, and N422A-Rv; Y438A, Y438A-Fw, and Y438A-Rv; F451A, F451A-Fw, and F451A-Rv (see nucleotide sequences, Table 1). 5′ terminuses of all primers were phosphorylated by T4 DNA kinase (Takara Bio, Japan). The 50 µl PCR mixture containing 0.6 mM $(NH_4)_2SO_4$, 1 mM KCl, 12 mM Tris-HCl (pH 8.0), 0.01% Triton X-100, 0.0001% BSA, 1 mM $MgCl_2$, 200 µM dNTPs, 0.3 µM each of corresponding Fw and Rv primer sets, 10 ng template DNA (pET-K4PolI) and 0.5 U of KOD Plus polymerase (Toyobo, Japan). The amplified fragments were self-ligated to obtain plasmids, pET21a-K4PolI-T326A, -L329A, -Q384A, -K387A, -F388A, M408A, -N422A, -Y438A, and -F451A.

1-2-9. Reverse transcriptase activity evaluation

RT-PCR was performed for assay of RNA-dependent and DNA-dependent DNA polymerase activity. Total RNA isolated from *T. kodakarensis* cells, which was collected at the logarithmic growth phase with the RNeasy Midi Kit (Qiagen, Germany), was used as the template for RT-PCR, and the 16S rRNA was selected as the target. In the case of K4PolI mutants, reverse transcript reaction and DNA amplification were carried out

in one tube. The 50 µl RT-PCR mixture contained 50 mM bicine KOH (pH 8.2), 115 mM CH_3COOK, 8% glycerol, 1 mM $Mn(CH_3COO)_2$, 0.3 µM 16S-Fw and 16S-Rv primer sets, *T. kodakarensis* total RNA (100 ng), and 1 µg of purified K4 DNA polymerase. RT-PCR was carried out as follows: 1 min at 55°C, 30 min at 68°C, and 2 min at 94°C followed by 40 cycles of 15 s at 94°C, 30 s at 60°C, and 1 min at 68°C. The amplified DNA was analyzed with agarose gel electrophoresis, and stained with ethidium bromide. In the case of reverse transcript with the MMLV reverse transcripatase, complementary DNA was synthesized from the total RNA (100 ng) of *T. kodakarensis* at 42°C for 30 min using primer 16S-Rv in a total volume of 20 µl containing 30 mM KCl, 50 mM Tris-HCl (pH 8.5), 8 mM $MgCl_2$, 0.3 µM 16S-Rv primer, 1 mM dNTP, and 1 µl of MMLV reverse transcriptase (20 unit of ReverTra Ace®, Toyobo, Japan). The solution was heated at 85°C for 10 min to inactivate MMLV reverse transcriptase. 1 µl of the solution was then added to a 50 µl PCR mixture containing 0.6 mM $(NH_4)_2SO_4$, 1 mM KCl, 12 mM Tris-HCl (pH 8.0), 0.01% Triton X-100, 0.0001% BSA, 1 mM $MgCl_2$, 200 µM dNTPs, 0.3 µM of 16S-Fw 16S-Rv primer sets, and 1 unit of KOD Plus DNA polymerase. PCR was performed as follows: 2 min at 94°C followed by 40 cycles of 15 s at 94°C,

30 s at 60°C, and 1 min at 68°C.

1-2-10. DNA- and RNA-dependent DNA polymerase activity assay

DNA- and RNA-dependent DNA polymerase activity were analyzed respectively with comparing the threshold cycle (Ct) values of DNA amplification. The Ct value, which is a threshold cycle number needed to reach the constant fluorescent intensity, was utilized as a parameter to analyze DNA-dependent DNA polymerase activity. ABI PRISM 7000 (Applied Biosystems, USA) was utilized as a thermal cycler for PCR and real-time PCR fluorescent detector. SYBR® Green I Nucleic Gel Stain (excitation wavelength, 494 nm; fluorescent wavelength, 521 nm) (Life Technologies, USA) was utilized as a double-strand DNA-specific fluorescent intercalator. For the Ct value measurement to evaluate DNA-dependent DNA polymerase, 16S rDNA of *T. kodakarensis* was selected as a target region. The 25 μl PCR mixture contained 50 mM bicine KOH (pH 8.2), 115 mM KCl, 8% glycerol, 1 mM $Mn(CH_3COO)_2$, template DNA (50 ng), 5×10^4 diluted SYBR® Green I solution, 0.3 μM primer sets (16S-Fw and 16S-Rv), and 1 μg of wild-type K4PolI or mutants. PCR was carried out with the thermal cycler as follows: 1 min at

94°C followed by 40 cycles of 15 s at 94°C, 30 s at 60°C, and 1 min at 68°C. The fluorescent intensity was analyzed and the Ct value was obtained with the supplied software. To investigate RNA-dependent DNA polymerase (reverse transcriptase activity), complementary DNA synthesis activity was analyzed. Complementary DNA of the 16S rRNA was synthesized using the 16S-Rv primer by 1 μg of K4PolI or MMLV reverse transcriptase (ReverTra Ace®, Toyobo, Japan) using total RNA (5 μg) extracted from *T. kodakarensis*. After the reverse transcription, the samples were reacted with RNase A following phenol chloroform treatment to inactivate the enzymes, and complementary DNA was collected by ethanol precipitation. Using the synthesized complementary DNA as a template, PCR was performed by primer sets 16S-Fw and 16S-Rv with 5 unit of Taq DNA polymerase (Roche Diagnostics) and 5×10^{4}-fold diluted SYBR® Green I solution. PCR condition was as follow; 40 cycles of 15 s at 94°C, 30 s at 60°C, and 1 min at 68°C. The fluorescent intensity was analyzed and the Ct value was obtained.

1-2-11. Accession number of nucleotide sequence

The nucleotide sequences of DNA polymerase and 16S rDNA derived

from *Thermotoga petrophila* K4 reported in this chapter have been listed to the DDBJ nucleotide sequence database under the accession numbers AB547905 and AB547906, respectively.

Table 1. Oligonucleotides used in chapter 1.

No.	Primer sequences	Primer sequences (5'-3')	Mer	Remarks
1	16SFFw	TATATGGAGGGTTTGATCCTGGC	23	
2	16SFRv	GAAAGGAGGTGATCCAGCCGC	21	
3	Pol-Fw	AAAAAAACATATGGCGAGACTATTTCTCTTTG	32	*Nde* I recognition sequence is underlined *Sal* I recognition sequence is underlined 5' end is labeled by Cy5.5
4	Pol-Rv	AAGTCGACTCACGACCATGTTTTGCC	26	
5	3′-5′ EXO	[Cy5.5] GGTCAGTGCTGCAACATTTTGCTGCCGGTC	30	
6	T326A-Fw	GCTGTAGATCTTGAAGCGTCTTCCCTCGAT	30	
7	T326A-Rv	GAACGAAGGGGATTCTTTCAGCTTCTCTAC	30	
8	L329A-Fw	TTGAAACGTCTTCCGCGGATCCCTTCGAC	29	
9	L329A-Rv	GATCTACAGCGAACGAAGGGGATTCTTTC	29	
10	Q384A-Fw	CGGAGCAAAGATCGTTGGTGCGAATCTG	28	
11	Q384A-Rv	GGGTCCTCCAGAATTTCTTTGAGCTTTTTC	30	
12	K387A-Fw	AGAATCTGGCGTTCGATTACAAGGTGTTG	29	
13	K387A-Rv	GACCAACGATCTTTGCTCCGGGGTCC	26	
14	F388A-Fw	AGAATCTGAAAGCGGATTACAAGGTGTTG	29	
15	F388A-Rv	GACCAACGATCTTTGCTCCGGGGTCC	26	
16	M408A-Fw	ACTTTGACACAGCGATAGCGGCTTACC	27	
17	M408A-Rv	GCGGAGGAACAGGTTCAACACCCTTCACC	29	
18	N422A-Fw	CGAAAAGAAGTTCGCGCTGGACGATCTCG	29	
19	N422A-Rv	TTCGGTTCAATAAGGTAAGCCGCTATC	27	
20	Y438A-Fw	AAATGACCTCTGCGCAGGAACTCATGTC	28	
21	Y438A-Rv	TGTAACCAAGAAATTTCAGCGCGAGATCG	29	
22	F451A-Fw	GTTGTTTGGTGCGAGTTTTGCCGATGTTCC	30	
23	F451A-Rv	GGTGAAGAGAAGGACATGAGTTCCTGGTAA	30	
24	16S-Fw	GGCAGGATGAAGGCCAGGCTGAAGGTCTTG	30	
25	16S-Rv	CGTATTCGCCGCGCGATGATGACACGCGGG	30	
26	PolI-up	GCCATAAACACTCCCATACAGGG	23	
27	PolI-down	AGAATCAGAGGAATGATTTCGTG	23	
28	PolI-Fw	TGAACGAGAAGGTGCTGTCCCGCGGAACTC	30	
29	PolI-Rv	ACAACTTTCAGTGCCGTCCCCGTCGAG	27	
30	Fw-m	AAAAAGCGCGCACAGCCCAGCTTGGAGCG	29	
31	Rv-m	AAAAAGCGCGCGAACCCCCCGTTCAGCCCG	30	

1-3. Results

1-3-1. Isolated hyperthermophile *Thermotoga petrophila* K4

A hyperthermophilic microorganism was isolated from a hot spring (73°C) at Kodakara Island, Kagoshima, Japan. Pure cultivation was performed by isolating a microorganism from a single colony of the 2× YT solid medium. Growth of the microorganism was optimized by K4 medium. The isolated thermophile was anaerobic heterotrophic rod-shaped microorganism with a "toga" which is a unique characteristic of Thermotogales (Fig. 1A). The size of the microorganism was about 2 μm× 6 μm; growth of the microorganism was confirmed at the range of 50-85°C, the optimum growth temperature was 80°C (Figs. 1A, B). Genome DNA was prepared from the liquid culture, and 16S rDNA region was amplified with PCR. Sequencing analysis of 16S rDNA showed that the isolated microorganism belonged to the genus *Thermotoga*, and this strain was most closely related to the hyperthermophilic bacterium *Thermotoga petrophila* RKU-1 (99% of 16S rDNA sequence identity) (Fig. 1C). *T. petrophila* RKU-1 and *T. maritima* MSB8 are distinguished on the basis of their antibiotic sensitivity against rifampicin (38). Growth of the microorganism was not

confirmed in a medium with rifampicin, as same as *T. petrophila* RKU-1. Based on the morphology, growth temperature, phylogenetic analysis, and rifampicin sensitivity, I designated the newly isolated strain as *Thermotoga petrophila* K4.

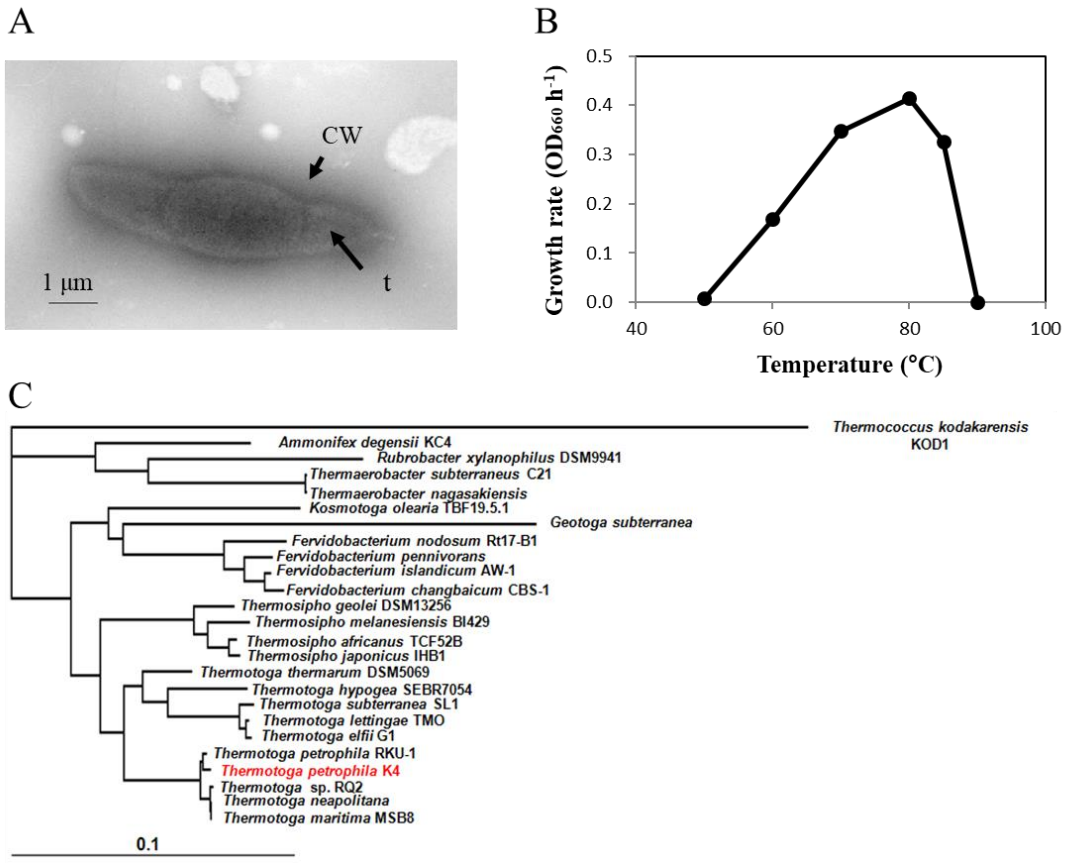


Fig. 1. Electron microscopy, growth profile, and phylogenetic tree of newly isolated T. petrophila K4. (A) Electron microscopy of *T. petrophila* K4. The arrows indicate the toga (t) and the cell wall (cw). Bar: 1 μm. (B) Effects of the temperature on the specific growth rates of *T. petrophila* K4. The growth rates were obtained from calculating a linear regression analysis along the logarithmic phase of the growth profile. (C) Phylogenetic neighbor-joining tree of the members of thermophilic bacteria including *T. petrophila* K4 based on the 16S rDNA. The sequences of 16S rDNA were got from the GenBank/EMBL/DDBJ database. The sequence of *Thermococcus kodakarensis* was used as an out group. Bar length shows rate of the base substitution: 0.1 means 1 substitution per 10 nucleotide lengths. The arrows show the position of *T. petrophila* K4.

1-3-2. Cloning of the DNA polymerase I gene from *T. petrophila* K4

To clone the DNA polymerase I (PolI) gene from *T. petrophila* K4, the PolI regions of genus *Thermotoga* were aligned and compared. The alignment analysis showed, the regions of the PolI start and stop codons were highly conserved in the three *Thermotoga* genera (*T. maritima* MSB8, *T. petrophila* RKU-1, and *Thermotoga* sp. RQ2). Two primers complementary binding to the conserved regions, Pol-Fw and Pol-Rv were designed and utilized for PCR using a genome DNA of *T. petrophila* K4 as a template. An about 2600 bp DNA fragment was successfully amplified. Sequencing analysis showed that it contained part of the PolI region. To obtain further upstream and downstream regions, gene walking was performed using a single primer method (42). Primer PolI-up that possesses a nucleotide sequence of the complementary strand of PolI and primer PolI-down that possesses the nucleotide sequence of the sense strand of PolI were utilized for PCR using a *T. petrophila* K4 genome DNA as a template. As the result of reactions, about 7000 bp and about 1500 bp DNA fragments were confirmed respectively. Sequencing analysis revealed amplified fragments contained the 5′ end and 3′ end regions of the PolI. Next, two kinds of primers (PolI-Fw and PolI-Rv),

which were designed to recognize the 5′ and 3′ regions, were ustlized for PCR. The 3207 bp DNA fragment that contained entire PolI open reading frame consists of 2679 bases coding for a protein with 893 amino acid residues, was amplified. The estimated molecular mass of the PolI was 101.7 kDa. Sequence comparison analysis showed that the DNA polymerase I from *T. petrophila* K4 (K4PolI) was most closely related to that derived from *Thermotoga* sp. RQ2 (97% identity in amino acid sequence). K4PolI consists of three preserved regions, the 5′-3′ exonuclease region, the 3′-5′ exonuclease region, and DNA polymerase region. The putative metal catiion-binding sites (Asp323, Glu325, Asp389, Asp468, and Tyr464) in the 3′-5′ exonuclease region were also conserved, suggesting that K4PolI possesses 3′-5′ exonuclease activity that contributes to high accuracy in PCR (15, 16).

1-3-3. Characteristics of K4PolI

The recombinant protein of K4PolI was obtained with overexpression in *E. coli* and purified (Fig. 2A), and its enzymatic characteristics were analyzed and compared with those of DNA polymerases from various sources. As a result of PCR with K4PolI, a specific DNA fragment from the DNA

template was obtained (Fig. 2B). The 3′-5′ exonuclease activity of K4PolI was investigated with a 5′ fluorescently labeled oligo-deoxyribonucleotide (3′-5′ EXO). The 3′-5′ exonuclease activity of K4PolI was confirmed and it was calculated as 0.44 nmol min^{-1} μg^{-1}. Mutation frequency was evaluated according to a reported method (40). In this assay, the error rate of K4PolI, Taq DNA polymerase, MSB8 DNA polymerase and KOD DNA polymerase were 0.35%, 0.50%, 0.46% and 0.26%, respectively (Fig. 2C).

To investigate whether K4PolI has the reverse transcriptase activity, the RNA-dependent DNA polymerase activity was examined with RT-PCR assay. Total RNA from *T. kodakarensis* was used as template of RT-PCR with K4PolI. The reacted sample was analyzed by agarose gel electrophoresis. However, no amplified DNA was confirmed (Fig. 2D). It shows that the K4PolI does not have the reverse transcriptase activity.

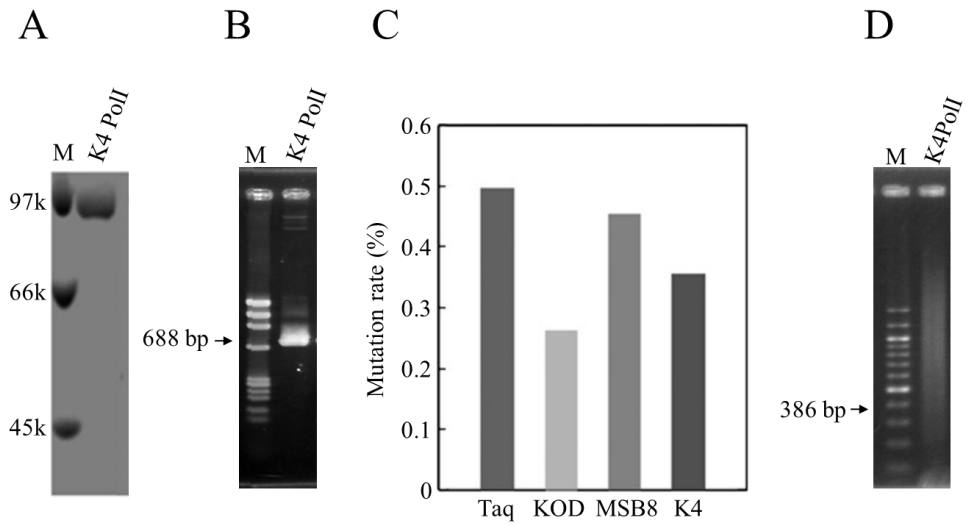


Fig. 2. Properties of wild-type K4PolI. (A) 0.1% SDS-10% PAGE of purified K4PolI. Lane M shows the molecular weight markers (phosphorylase b, 97,000; albumin, 66,000; ovalbumin, 45,000). (B) PCR using K4PolI. The TK0149 gene of *T. kodakarensis* was targeted for PCR as described in materials and methods, and 688 bp DNA was generated. Agarose gel (0.8%) was stained with ethidium bromide. M is a DNA molecular marker; lambda phage DNA digested with *Hae* III. The sizes from the top are 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp. (C) Mutation frequency in PCR. The mutation rate was obtained by calculating the percentage (%) of colonies with mutated plasmids to the number of total colonies harboring wild-type plasmid pKF3 as described in materials and methods. Taq, DNA polymerase I derived from *Thermus aquaticus*; KOD, family B DNA polymerase derived from *Thermococcus kodakarensis*; MSB8, DNA polymerase I derived from *Thermotoga maritima* MSB8; K4, DNA polymerase I derived from *Thermotoga petrophila* K4. (D) RT-PCR using K4PolI. The structured region of 16S rRNA from *T. kodakarensis* was targeted for reverse transcription. Reverse transcription and PCR were performed by two primers, 16S-Fw and 16S-Rv, using total RNA of *T. kodakarensis* as a template. The arrow shows the position of a predicted RT-PCR product (386 bp). M is the DNA molecular marker (100 bp DNA ladder; Toyobo, Japan).

1-3-4. Predicted structure of K4PolI

As mentioned above, wild-type of K4PolI possesses only DNA-dependent DNA polymerase activity but not reverse transcriptase activity. This characteristic means to be caused by specificity of the template recognition. DNA polymerase I derived from bacteria is classified as family A, which accepts an uracil containing DNA as a template in DNA synthesis. This property differentiates it family B DNA polymerase derived from archaea (37). I estimated that K4PolI also accepted an uracil-containing DNA and it distinguishes DNA from RNA by recognizing the difference in structure of the 2′ hydroxyl group of ribose. To get further precise information about template DNA binding region of K4PolI, protein crystallization was attempted under various conditions with changing the buffers and template oligonucleotides. However, successful crystallization was not obtained. The K4PolI structure was hence speculated *in silico*, using the *E. coli* Klenow fragment (1kfs) as a modeling template (41). The modeled structure showed that there was a single-strand DNA binding site in the 3′-5′ exonuclease domain of K4PolI. The amino acid residues, Thr326, Leu329, Gln384, Lys387, Phe388, Met408, Asn422, Tyr438, and Phe451, were estimated to locate around a single-strand

DNA template. It was hypothesized that some of these amino acid residues played a key role in template DNA/RNA distinction by steric interference with the 2′ hydroxyl group of ribose. Brautigam *et al.* reported the template-binding situation of Klenow polymerase using oligo-deoxyribonucleotides (oligo dA, dC, and dG) and suggested these amino acid residues contribute the binding between enzyme and DNA (41). In K4PolI, the amino acid residues Thr326, Leu329, Gln384, Lys387, Phe388, Met408, Asn422, Tyr438, and Phe451 were selected as the site introduce mutation, and each amino acid residue was changed to alanine with the expectation that the mutant would accept ribonucleotide by decreasing the side-chain bulkiness surrounding the ribonucleotide template (Fig. 3). These K4PolI mutants were designated as T326A, L329A, Q384A, K387A, F388A, M408A, N422A, Y438A, and F451A, respectively.

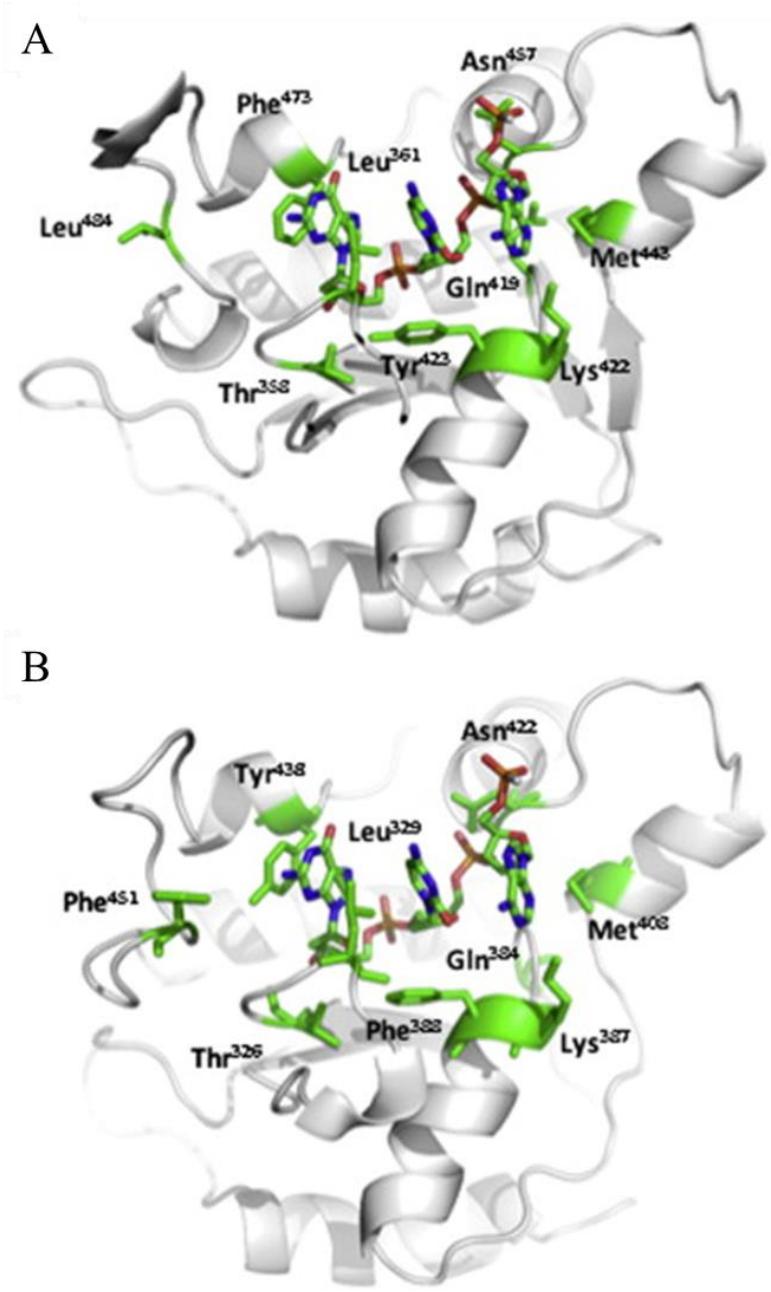


Fig. 3. Modeled structure of 3′-5′ exonuclease domain with single-strand DNA. (A) Structure of a Klenow fragment with single-strand DNA (1kfs). The template-binding situation using synthetic oligonucleotides (oligo dA, dC, and dG) is indicated. (B) Predicted structure of K4PolI based on the Klenow fragment crystal structure (1kfs).

1-3-5. Characteristics of the K4PolI mutants

All K4PolI mutants were overexpressed in *E. coli* and purified (Fig. 4A). The DNA-dependent DNA polymerase activities were confirmed by PCR. The PCR products synthesized with the purified mutants are indicated in Fig. 4B. As all mutated amino acid residues that I focused on are located within the 3′-5′ exonuclease domain, the activity was investigated (Fig. 4C). The 3′-5′ exonuclease values of T326A, L329A, Q384A, K387A, M408A, and Y438A were decreased to about 0.7 to 4.5% of those of the wild-type enzyme. No 3′-5′ exonuclease activity was confirmed from mutant F388A. Mutants N422A and F451A showed 48% and 117% of 3′-5′ exonuclease activity comparing to the wild-type enzyme, respectively.

Next, I examined whether mutants enable RT-PCR in a single-step format to amplify RNA. If DNA is directly amplified from RNA, it appears that mutants have both reverse transcriptase and DNA-dependent DNA polymerase activity. In addition, mutants were expected to amplify DNA from structured RNA as they work at higher temperature condition. To examine these two possibilities, the 16S rRNA of *T. kodakarensis*, which forms a stable structure with a free energy change (-ΔG) of -265.6 kcal/mol, was used as a

target template RNA. When RT-PCR was performed to generate complementary DNA using 16S rRNA-specific primers (16S-Fw and 16S-Rv), the mutants T326A, L329A, Q384A, F388A, M408A, and Y438A led to the synthesis of only expected size of DNA (Fig. 4D). The mutant F388A also generated the DNA but in less amount. In contrast, when MMLV reverse transcriptase was used for the complementary DNA synthesis, nonspecific DNAs were amplified with specific DNA after PCR with Taq DNA polymerase (Fig. 4D).

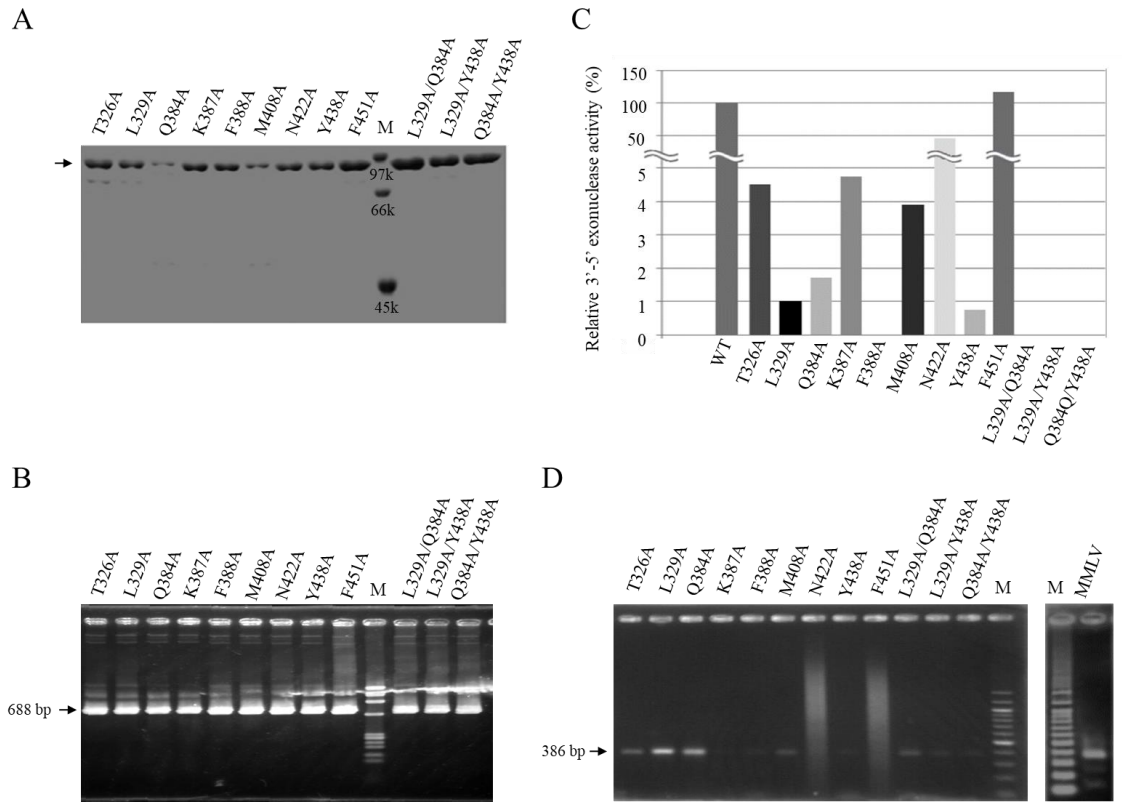


Fig. 4. Properties of K4PolI mutants. (A) 0.1% SDS-10% PAGE of purified K4PolI mutants. Lane M shows the molecular weight markers (phosphorylase b, 97,000; albumin, 66,000; ovalbumin, 45,000). (B) PCR using K4PolI mutants. The TK0149 gene of *T. kodakarensis* was targeted for PCR as described in materials and methods, and 688 bp DNA was generated. Agarose gel (0.8%) was stained with ethidium bromide. M is a DNA molecular marker; lambda phage DNA digested with *Hae* III. The sizes from the top are 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp. (C) Relative 3′-5′ exonuclease activity. The activity of the wild-type K4PolI (0.44 nmol/min/μg) is defined as 100%. (D) RT-PCR using various enzymes. The structured region of 16S rRNA from *T. kodakarensis* was targeted for reverse transcription. Reverse transcription and PCR were performed by two primers, 16S-Fw and 16S-Rv, using total RNA of *T. kodakarensis* as a template. The arrow shows the position of an RT-PCR product (386 bp). In the case of RT-PCR with MMLV reverse transcriptase, reverse transcription was performed

with primer 16S-Rv. Using the reverse transcripted DNA as a template, DNA was generated by two primers, 16S-Fw and 16S-Rv, with Taq DNA polymerase. M is the DNA molecular marker (100 bp DNA ladder; Toyobo, Japan).

Next, to examine the performance of mutant enzymes for DNA-dependent DNA polymerase, Ct values of PCR were analyzed. SYBR® Green I was used as a fluorescent intercalator for double-strand DNA. As indicated in Fig. 5A, no significant difference was observed in the Ct values among T326A, M408A, N422A, and the wild-type. These results showed that amino acid mutation to alanine in those positions does not affect their DNA-dependent DNA polymerase activity. On the other hand, L329A, Q384A, F388A, and Y438A showed lower Ct values than the wild-type, indicating that they got higher DNA-dependent DNA polymerase activity than the wild-type enzyme.

Next, reverse transcriptase activity was quantitatively investigated. First, complementary DNA of the 16S rRNA region of *T. kodakarensis* was generated using the 16S-Rv primer by various mutants. Total RNA purified from *T. kodakarensis* was used as RNA template. After the reverse transcription, the sample was treated with RNase A following phenol chloroform extraction to inactivate the enzymes, and generated DNA was prepared by ethanol precipitation. Then, the DNA was used as a template, and amplified by Taq DNA polymerase with a fluorescent intercalator SYBR®

Green I. Under this condition, an enzyme with higher reverse transcriptase activity would show a lower Ct value in the PCR. As indicated in Fig. 5B, L329A, Q384A, and M408A showed lower Ct values than T326A, F388A, and Y438A. The Ct values of K4PolI mutants were higher than those of MMLV, which means reverse transcriptase activity of MMLV is higher than those of K4PolI mutants. L329A, Q384A, and Y438A mutants showed lower Ct values for DNA-dependent DNA polymerase activity than those of other mutant enzymes (Fig. 5A). Leu329, Gln384, and Tyr438 seem to be favorable residues to modify the substrate recognition specificity without compromising DNA-dependent DNA polymerase activity. In order to analyze the effect of double mutation on DNA polymerase activity, three kinds of mutants were constructed. The double-substitution mutants (L329A and Q384A, L329A and Y438A, and Q384A and Y438A) were designated as L329A/Q384A, L329A/Y438A, and Q384A/Y438A, respectively. No detectable 3′-5′ exonuclease activity was observed from these double mutants (Fig. 4D). In the RT-PCR analysis, L329A/Q384A showed the lowest Ct value among the mutants, indicating the highest reverse transcriptase activity (Fig. 5B).

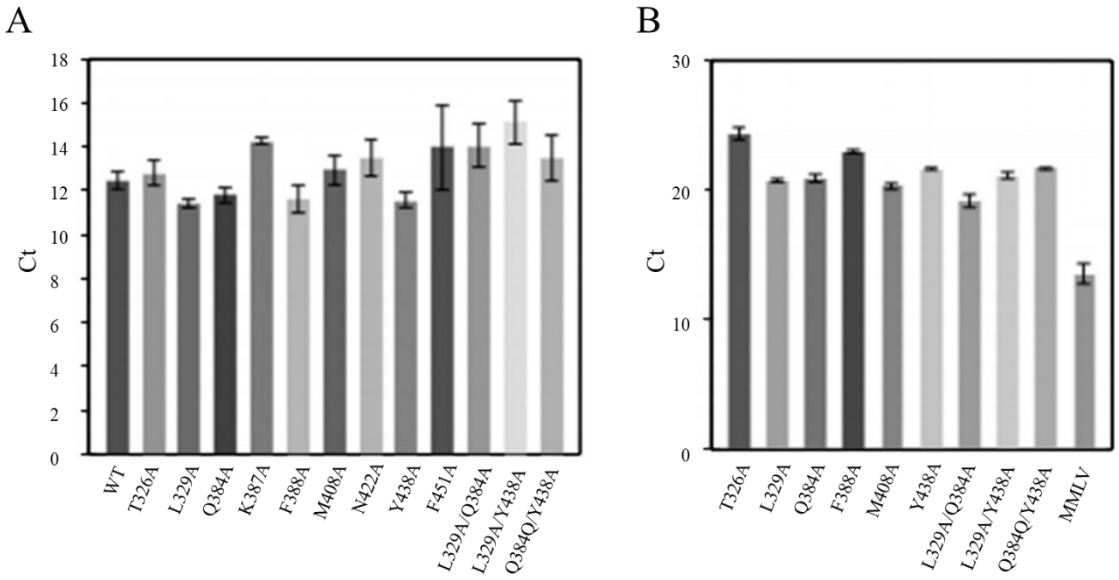


Fig. 5. Quantitative analysis of DNA- and RNA-dependent DNA polymerase activity. (A) Comparison of DNA-dependent DNA polymerase activity. For Ct value assays in PCR, genome DNA of *T. kodakarensis* (100 ng) was utilized as a template to generate the 16S rDNA region as a target, and the fluorescent intensity was investigated. (B) Comparison of RNA-dependent DNA polymerase activity. For Ct value assays in the RT reaction, complementary DNA of the 16S rRNA region was generated with K4PolI derived enzymes or MMLV reverse transcriptase using total RNA obtained from *T. kodakarensis* cells. Following the reverse transcript reaction, a sample was treated with phenol chloroform to irreversibly inactivate the enzyme. Using reverse transcripted DNA as a template, DNA was generated with Taq DNA polymerase. The fluorescent intensity was analyzed by ABI PRISM 7000, and the Ct value was calculated using the supplied software.

1-4. Discussion

In this chapter, a hyperthermophilic bacteria was isolated. This newly isolated strain was classified as *Thermotoga petrophila* and named K4 strain, on the basis of phylogenetic analysis as well as its growth profiles, typical toga-like shape and rifampicin sensitivity. The DNA polymerase I from *T. petrophila* K4 (K4PolI) possesses sufficient performance to perform PCR was obtained (Fig. 2B). K4PolI has 3′-5′ exonuclease activity, which contributes to accuracy in PCR (Figs. 2C). K4PolI is applicable for PCR as thermostable DNA polymerase with accuracy, as same as the other DNA polymerases with the 3′-5′ exonuclease activity.

The DNA-dependent DNA polymerase generally accepts DNA and dNTP and excludes RNA and rNTP. The precise mechanisms to distinguish suitable substrates from unsuitable ones are still unknown. Some approaches to clarify the recognition mechanisms have been carried out, and two kinds of mechanisms for substrate distinction have been proposed. One mechanism is for rNTP/dNTP distinction. The Klenow DNA polymerase accepts only dNTP and excludes rNTP by sterically blocking the 2′ hydroxyl group of rNTP interacting with Glu710 (43). The bulkiness of the 2′ hydroxyl group of ribose

appears to interfere with the substrate-binding site of Klenow DNA polymerase. Family B DNA polymerase derived from the hyperthermophilic archaea *Thermococcus litoralis* indicated a similar hindrance effect (44). Tyr412 of *T. litoralis* DNA polymerase acts as a steric gate for the 2′ hydroxyl group of ribose and excludes rNTP. Another mechanism has been proposed for template recognition. As bacterial DNA polymerase I does not recognize the absence of the 5-methyl group in uracil, it accepts an uracil-containing template. Also, the 2′ hydroxyl group of ribose is predicted as the factor to distinguish DNA from RNA for 3′-5′ exonuclease activity of bacterial DNA-dependent DNA polymerase (45). In Klenow DNA polymerase, Asn420 and Tyr423 in the 3′-5′ exonuclease domain interfered with the 2′ hydroxyl group of the template and played a key role for RNA exclusion (46). On the other hand, the archaeal family B DNA polymerase does not accept the template containing uracil, and DNA synthesis is prematurely ended at the uracil-containing position; this is a different characteristic from that of bacterial DNA polymerase (37). Family B DNA polymerase, therefore, strictly recognizes DNA and RNA than bacterial DNA polymerase I.

In this study, I focused on the 3′-5′ exonuclease domain of K4PolI and

introduced mutations to make K4PolI to accept a RNA as the template because the modeled structure of K4PolI shows that there is a template DNA-binding region at the 3′-5′ exonuclease domain (Fig. 3). Among the mutant constructs, T326A, L329A, Q384A, F388A, M408A, and Y438A showed reverse transcriptase activity with DNA-dependent DNA polymerase activity, and they are applicable to simple one-step RT-PCR (Fig. 4D).

All the mutants that gained reverse transcriptase activity showed reduced 3′-5′ exonuclease activity. In the RT-PCR experiment, no specific complementary DNA were generated from N422A and F451A (Fig. 4D), which have full 3′-5′ exonuclease activity (Fig. 4C) comparable to that of the wild-type enzyme. The results suggest that gain of the reverse transcriptase activity is correlated with loss of the 3′-5′ exonuclease activity. There are studies that mutations into 3′-5′ exonuclease domain enhanced reverse transcriptase activity (33, 35). On the other hand, RT-PCR product was not efficiently synthesized from F388A (Fig. 4D), even though F388A possessed no detectable 3′-5′ exonuclease activity (Fig. 4C). To optimize the reverse transcriptase activity, further structural study about the template recognition site is required.

Among the constructed mutants, L329A/Q384A possesses the highest reverse transcriptase activity (Fig. 5B). However, in RT-PCR experiments, L329A/Q384A generated DNA with less efficiency, as shown in Fig. 4C. This may be due to the low DNA-dependent DNA polymerase activity, as indicated by the higher Ct value shown in Fig. 5A. To improve the efficiency of RT-PCR, additional mutations might be required combining with L329A/Q384A.

In this chapter, I obtained K4PolI mutants which possess 3′-5′ exonuclease activity, reverse transcriptase activity and DNA-dependent DNA polymerase activity. Also, RT-PCR is able to be performed at simple one step format with these developed enzymes. The mutants will contribute to realize POC-NAT as simple RT-PCR-use enzymes.

Chapter 2: Rapid nucleic acid amplification test developed using paper chromatography chip and azobenzene-modified oligonucleotides

2-1. Introduction

As simple detection technologies of amplified nucleic acid, DNA chromatography methods using a chip made of paper have been improved. The detection chip for PCR products with single-strand DNA tags were developed as well (47, 48). Kaneka corporation designated this detection system as the Kaneka DNA chromatography chip (KDCC) (49), which uses special primers with the sequences complementary to the 3′ sequences of the target region, and single-strand DNA tag sequence at the 5′ end, containing a modification. During PCR, elongation by the DNA polymerases is stopped at the modification site and the amplified products with single-strand DNA tag are generated, so that they would specifically bind to the DNA probes on the chromatography chip and allow colorimetric particle labeling at room temperature. After the addition of PCR products on the chip, they can rapidly be confirmed as colored lines, without special operation such as electrophoresis or fluorescence staining, which simplifies the analysis.

For efficient KDCC-based detection, synthesizing PCR products with ssDNA tags at 5′ ends is required, and they are generated by blocking DNA polymerase-mediated product elongation, using modification sites in the primers. Insufficient elongation inhibition makes the worse performance and the detection limit of KDCC, because the tag is covered with complementary DNA and not recognized in room temperature. Some modification substances that inhibit DNA polymerase elongation have been identified, such as fluorescent dyes, methylated base, uracil base, phosphoramidite, azobenzene, and C3 (47, 48, 50). These substances can be utilized to introduce modification in the used primers, such that DNA polymerase activity is inhibited; therefore, they are useful for KDCC. However, they have not been investigated in detail.

Shortening time for the amplification is equally important for the development of POC-NATs, and several approaches have been performed to this end. A thermal cycler with high ramp-rate can be applied in order to adjust the arbitrary temperature with high speed (51), while another approach may be the application of DNA polymerase with a high extension speed, allowing shorter time for DNA synthesis (16). On the other hand, the binding efficiency of a primer and a template is strongly correlated with the

applicability of these approaches in specific tests.

As primer modifications, non-nucleotide type modifications are more suitable, because nucleotide modifications tend to be overcome by DNA polymerase (52), resulting in the synthesis of undetectable products with KDCC. However, the modifications that do not imitate nucleotides usually decline the efficiency of primer-template bindings, so that they interfere the proper formation of complementary double-strand DNA (53) and increase the time necessary for the efficient amplification in PCR. Therefore, to optimize the KDCC-based PCR products detection, the utilization of modifications that inhibit the elongation by DNA polymerase and do not affect primer-template binding is important. Here, I searched for conjugate substances for the optimum primer modification with high elongation efficiently and superior primer-template binding property. The candidate substances were applied to KDCC detection method as modified primers, and their detection performances were analyzed. Additionally, I further developed a rapid and sensitive pathogenic virus detection system using the modified primers and KDCC.

2-2. Material and methods

2-2-1. Materials

In the PCR, bacterial A and archaeal family B DNA polymerases can be used. Here, I used family A DNA polymerase derived from thermophilic bacteria, *Thermus aquaticus* (Taq; New England Biolabs, USA) and family B DNA polymerase derived from hyperthermophilic archaea, *Thermococcus kodakarensis* (KOD exo (-); Toyobo, Japan) in the assay (14, 16). All oligonucleotides (Table 2; (54)) were obtained from Kaneka Eurogentec (Belgium). Substances selected as modifications were azobenzene, trimethylene (C3), triethylene glycol (S9), and inverted nucleotides (INs) (Fig. 6). Azobenzene, under visible light, forms trans-isomer, while under ultra-violet light (UV), it changes a form to cis-isomer (55). Lambda phage DNA was obtained from Takara Bio (Japan). HSV-1/2 DNA was obtained from Bio-Rad (USA).

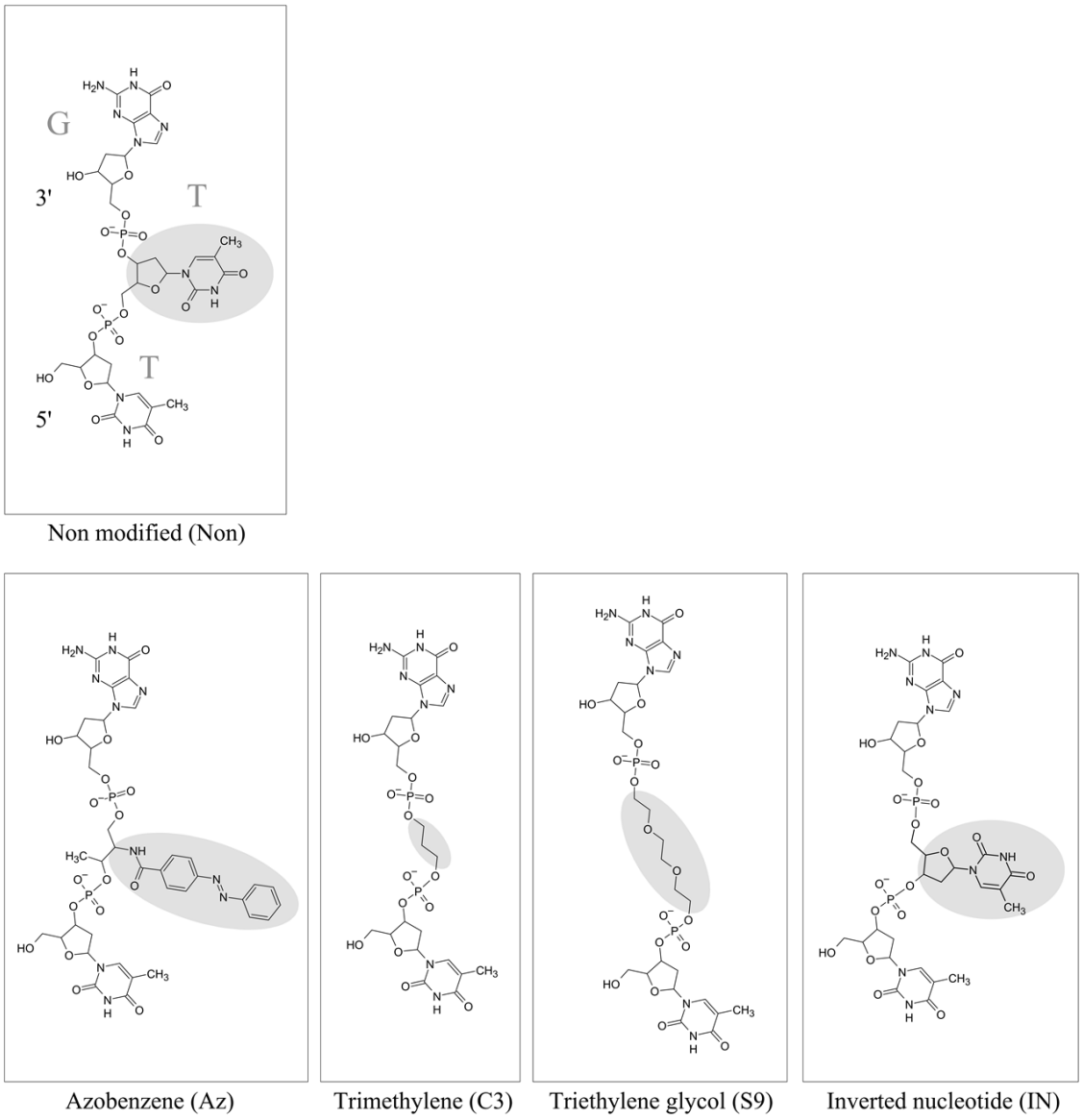


Fig. 6. Modification substances used in this chapter. Shadowed parts of the molecules show modification sites. For example, one of the modifications was added to between 5′ thymidine and 3′ guanidine.

2-2-2. DNA chromatography chip

Principle of KDCC is indicated in Fig. 7. Two procedures, PCR amplification and detection, are employed to detect target molecules. For the amplification, DNA-tag added modified primers are utilized. These primers have two domains, a primer domain, capable of hybridizing to the target sequence, and a tag domain, which can bind to the solid-phase DNA probe on chip or colloidal gold nanoparticles. These two domains are connected with the modification site that blocks the DNA polymerase-associated elongation. The PCR products amplified with these primers possesses a single-strand DNA tag at 5′ end, which specifically binds to the probe DNA on the chromatography chip or colloidal gold nanoparticles.

Following the PCR, one aliquot of amplicon is used for KDCC detection, with a development buffer, to develop the PCR products on the chromatography chip, through the capillary action. During development, the PCR products interact with colloidal gold nanoparticles that immobilizing the DNA probes, forming an PCR products-colloidal gold nanoparticle complex. As the complex moves on the chromatography chip, it hybridizes with the solid phase DNA probes on a chromatography chip, which consists of the

sequence complementary to the other tag sequence, and is captured by the probe, resulting in a red colored signal originating from the aggregation of colloidal gold nanoparticles, which allows the visual detection of the PCR products. By utilizing multiple sets of primer pairs with different tags, in combination with a multiplex PCR method, it is possible to simultaneously detect a number of targets.

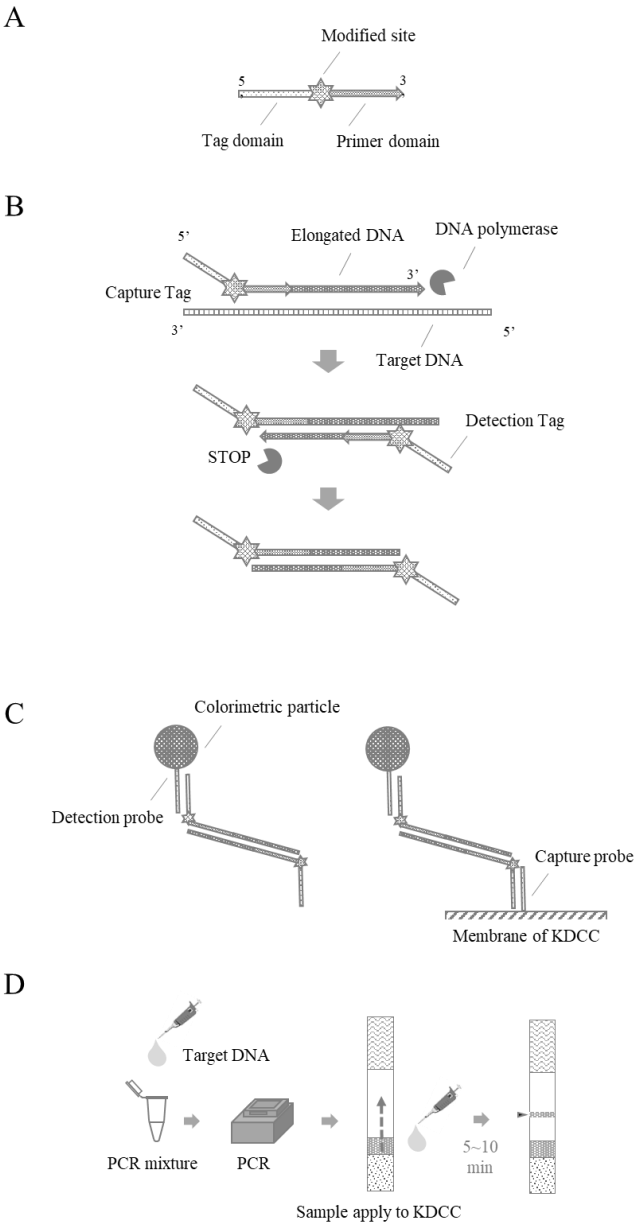


Fig. 7. Principles of KDCC. (A) Structure of the DNA-tagged primer, with a primer domain, hybridizing to the target sequence, and a tag domain, binding to the DNA probe on a chromatography chip or colloidal gold nanoparticles. These two domains are connected with the modification site. (B) The generated PCR products, with ssDNA tags at 5′ end. (C) Captured PCR product and colloidal gold nanoparticles on the chromatography chip. (D) KDCC detection procedures. PCR product with ssDNA tags can be detected as a colored line on the chromatography chip.

2-2-3. Elongation inhibition evaluation

The elongation inhibition assays were carried out by the previously reported method, with slight modifications (50), using Taq and KOD exo (-) DNA polymerases. The reaction mixtures of Taq DNA polymerase contained 0.0125 U/μL Taq DNA polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotides (dNTPs), 0.5 μM Cy3-probe, 1 μM of each modified oligonucleotide in the total volume of 20 μL. The reaction mixtures of KOD DNA polymerase contained 0.0125 U/μL KOD exo (-) DNA polymerase, 1× KOD exo (-) buffer, 0.2 mM dNTPs, 0.5 μM Cy3-probe, and 1 μM of each modified oligonucleotide in 20 μL. Elongation reaction conditions were as follows: 1 min at 94°C, followed by 30 cycles of 5 s at 95°C, 5 s at 60°C, and 5 s at 72°C. Following the incubation, 1 μL of 0.5 M EDTA was added as a reaction terminating reagent to the mixtures, 2.5 μL of each solution were mixed with sample buffer containing 10 M urea and 1x TBE buffer (0.089 M Tris borate, 0.089 M boric acid, 0.002 M EDTA), and the reacted samples were electrophoresed on a 20% denaturing polyacrylamide gel, 7 M urea in TBE buffer. Following the electrophoresis, polyacrylamide gels were analyzed with fluorescent gel imager, ImageQuant LAS 4000 (GE Healthcare, USA). In this

evaluation, 50-mer DNA templates and 5′ Cy3 labeled 17-mer primers were used. Each DNA template possessed a modified site at position 25. After incubation reaction, if the sequence elongation by DNA polymerase was completely inhibited, 25-mer products were generated, whereas, if it was not blocked efficiently, 50-mer products were identified (Fig. 8.).

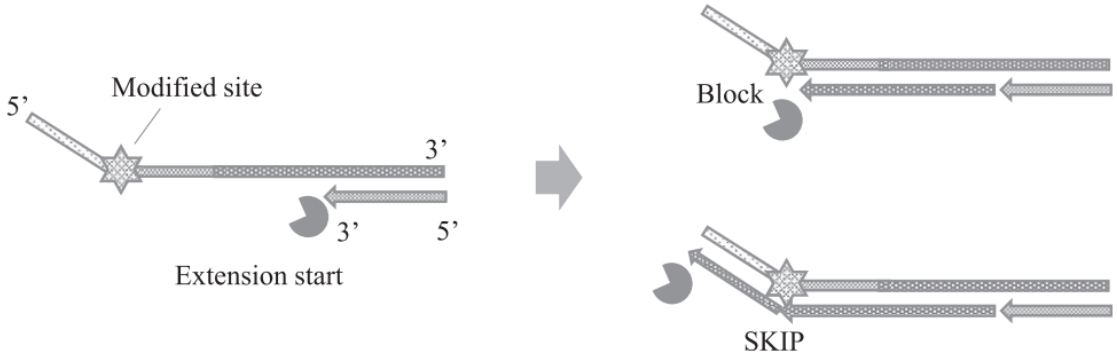


Fig. 8. DNA elongation with templates with modified site. DNA polymerase generates complementary sequences using the DNA templates until it reaches the modified site, where the elongation is blocked or it continues due to the translesion synthesis (skipping the modified site).

2-2-4. Real-time PCR

The reaction mixtures of Taq DNA polymerase contained 0.0125 U/μL Taq DNA polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 μM of forward and reverse primers each, and 1/20,000 SYBR® Green I (Takara Bio, Japan) in 20 μL. The reaction mixtures of KOD DNA polymerase contained 0.0125 U/μL KOD exo (-) DNA polymerase, 1× KOD exo (-) buffer, 0.2 mM dNTPs, 0.3 μM forward and reverse primers each, and 1/20,000 SYBR® Green I in 20 μL. 100 pg/test lambda phage DNA was used as a template DNA. Combinations of the primers were as follows: azobenzene (forward primer: T50 (Az); reverse primer: R (Az)), C3 (forward primer: T50 (C3); reverse primer: R (C3)), S9 (forward primer: T50 (S9); reverse primer: R (S9)), IN (forward primer: T50 (IN); reverse primer: R (IN)). Non-modified primers (forward primer: T25; reverse primer: R) were used as a control. Real-time PCR was carried out with LightCycler® 96 (Roche Diagnostics, Switzerland).

2-2-5. Melting temperature assay

Tm values of each modified oligonucleotide were evaluated with UV-

vis spectrophotometer TMSPC-8 (Shimadzu, Japan). Oligonucleotides containing modified site (T50 (Az), T50 (C3), T50 (S9) and T50 (In); 1 μM each) and a nonmodified oligonucleotide (T50) were hybridized with 1 μM complementary oligonucleotide (C25) in the hybridizing buffer (100 mM NaCl, 10 mM Na_2HPO_4, 1 mM Na_2EDTA, [pH 7.0]) (56). Absorbance at 260 nm of the hybridized samples was measured at temperatures between 25°C and 80°C with a ramp rate of 0.5°C /min. The Tm values were calculated with the preprogrammed fitting methods offered in the Tm analysis software provided with the TMSPC-8 UV-vis spectrophotometer.

2-2-6. PCR products detection with KDCC

Two types of KDCCs were designed. KDCC-1 was constructed with linearly immobilizing capture DNA probe (I1) onto the nitrocellulose membranes, whereas a detection probe (A1) was immobilized on the colloidal gold nanoparticles. KDCC-3 was constructed by linearly immobilizing the capture DNA probes (I1, I2, and I3) onto the nitrocellulose membrane and a detection probe (A1) on the colloidal gold nanoparticles. Chromatography chips were assembled by the previously described protocol (47).

PCR reactions were carried out with both Taq and KOD DNA polymerases. The reaction mixtures of Taq DNA polymerase contained 2.5 U/μL Taq DNA polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.3 μM forward and reverse primers each, in 20 μL. The reaction mixtures of KOD DNA polymerase contained 2.5 U/μL KOD exo (-) DNA polymerase and 1 KOD exo (-) buffer, 0.2 mM dNTPs, and 0.3 μM forward and reverse primers each in 20 μL. 100 pg/test lambda phage DNA, HSV-1, and HSV-2 DNA sequences were used as template DNAs. PCR analyses were carried out with LifeECO (Bioer, China). Following the amplification, 5 μL of PCR products were tested on KDCCs, and developed with 65 μL of development buffer at room temperature. After 5 min, color signals from the chromatography chip were analyzed with immuno-chromatography reader, C10066-10 (Hamamatsu Photonics, Japan) and inspected visually.

Table 2. Oligonucleotides used in chapter 2.

No.	Name	Primer sequence (5′ to 3′)	Mer	Remarks
1	Cy3-P	[Cy3]ACCTCTTCCAGCGAGAAC	18	5′ fluorescent dye (Cy3) modified
2	T25	GCTATAAGTTCTCGCTGGAAGAGGT	25	
3	T50	TCGAGTGACAGCTAATGTGTGATTGCTATAAGTTCTCGCTGGAAGAGGT	50	
4	T50 (Az)	TCGAGTGACAGCTAATGTGTGATT-[**Az]**-GCTATAAGTTCTCGCTGGAAGAGGT	50	
5	T50 (C3)	TCGAGTGACAGCTAATGTGTGATT-[**C3]**-GCTATAAGTTCTCGCTGGAAGAGGT	50	
6	T50 (S9)	TCGAGTGACAGCTAATGTGTGATT-[**S9]**-GCTATAAGTTCTCGCTGGAAGAGGT	50	
7	T50 (IN)	TCGAGTGACAGCTAATGTGTGATT-[**IN]**-GCTATAAGTTCTCGCTGGAAGAGGT	50	
8	R	GATAGGATTAGAAGGTCGAACCGT	24	
9	R (Az)	ATTTTTCACTGGGTTTATAGT-[**Az]**-GATAGGATTAGAAGGTCGAACCGT	45	
10	R (C3)	ATTTTTCACTGGGTTTATAGT-[**C3]**-GATAGGATTAGAAGGTCGAACCGT	45	
11	R (S9)	ATTTTTCACTGGGTTTATAGT-[**S9]**-GATAGGATTAGAAGGTCGAACCGT	45	
12	R (In)	ATTTTTCACTGGGTTTATAGT-[**IN]**-GATAGGATTAGAAGGTCGAACCGT	45	
13	I1	ATCACACATTAGCTGTCACTCGATGCA	27	Capture probe (immobilized on membrane)
14	I2	TTAGAGAGTTATCGTAGACCTCGCA	25	
15	I3	TGGCAACATTTTTCACTGGGTTTATAG	27	
16	A1	CTATAAACCCAGTGAAAAATGTTGCCA**[C6-SH]**	27	Detection probe (immobilized on Au nano-colloid), 3′ C6-Thiol modified
17	C25	ACCTCTTCCAGCGAGAACTTATAGC	25	Complementary sequence of T25
18	i1-hsv-1f	TGCATCGAGTGACAGCTAATGTGTGAT-**[Az]-**CTGTGGTGTTTTTGGCATCA	47	Primers were designed based on (54)
19	a1-hsv-1r	TGGCAACATTTTTCACTGGGTTTATAG-**[Az]-**GGTGGTGGAGGAGACGTTG	46	
20	a1-hsv-2f	TGGCAACATTTTTCACTGGGTTTATAG-**[Az]-**CATGGGGCGTTTGACCT	44	
21	i2-hsv-2r	TGCGAGGTCTACGATAACTCTCTAA-**[Az]-**TACACAGTGATCGGGATGCT	45	

Az: Azobenzene, C3: Trimethylene, S9: Triethylene glycol, IN: Inverted nucleotide (thymidine)

2-3. Results

2-3-1. Elongation inhibition efficiency

I examined the elongation inhibition efficiency of the modification substances; azobenzene, C3, S9, and IN. Following the electrophoresis, the inhibition efficiency was calculated from detected fluorescence intensity as follows: (25-mer product)/(25-mer product +>25-mer product) [%]. I confirmed that azobenzene, C3, S9, and INs inhibited the elongation of sequences when utilized both DNA polymerases. Azobenzene modification of the template oligonucleotide was shown to be the most efficient (Taq: 100%, KOD: 96.3 ±0.4%), followed by C3 modifications (Taq: 95.7 ±5.2%, KOD: 81.9 ±0.4%), S9 (Taq: 96.3 ±2.7%, KOD: 77.0 ±2.8%), and, finally, INs (Taq: 84.2 ±7.1%, KOD: 81.2 ±0.6%) (Fig. 9). While, products longer than 25 nucleotides were detected as well after all modifications, except when azobenzene-Taq DNA polymerase combination was utilized.

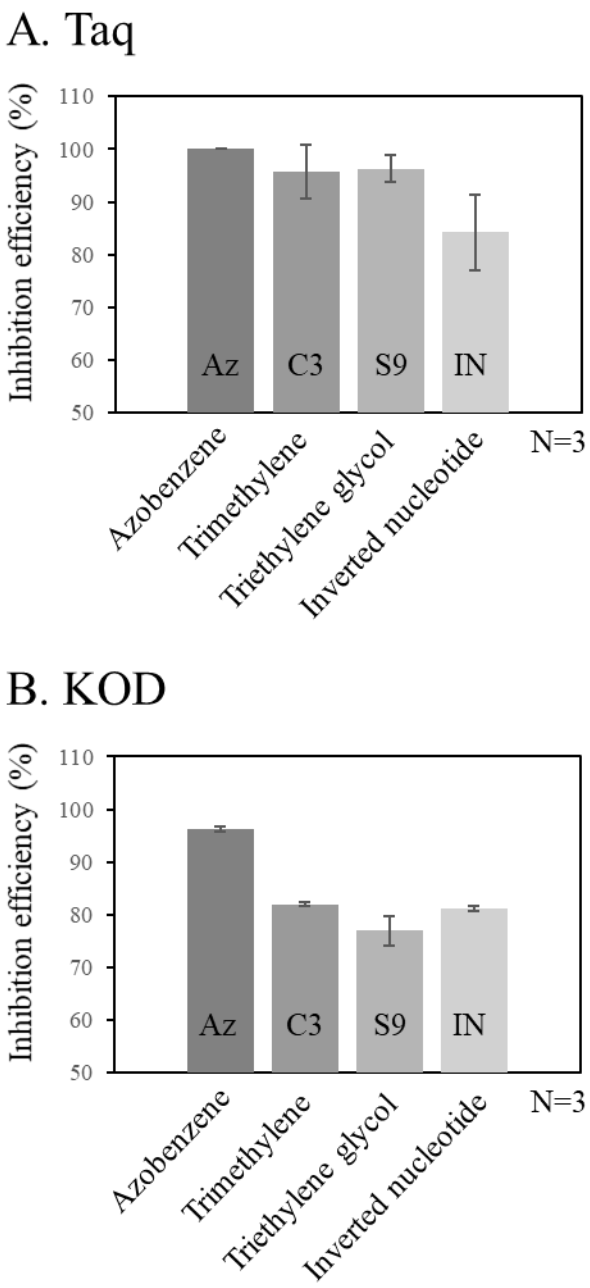


Fig. 9. Efficiency of DNA elongation inhibition. Inhibition efficiency was calculated based on the fluorescence intensity of (25-mer product)/(25-mer product) + > 25-mer product) [%]. (A) Taq DNA polymerase analysis. (B) KOD exo (-) DNA polymerase analysis (25-mer product, properly generated product; 50-mer, translesion elongated product).

2-3-2. Amplification efficiency

The amplification efficiencies of each modified oligonucleotide were examined with real-time PCR. Lambda phage DNA was utilized as the template DNA. The Cq value, which is a quantification cycle number needed to reach the constant fluorescent intensity, was utilized as a parameter to analyze amplification efficiency. Cq analysis indicated that the PCR with azobenzene-modified primers had the lowest Cq values (Taq: 15.1 ±0.09, KOD: 13.6 ±0.06), followed by C3-modified (Taq: 16.0 ±0.32, KOD: 15.2 ±0.38), S9 (Taq: 16.8 ±0.33, KOD: 15.9 ±0.54), and IN modified (Taq: 17.0 ±0.60, KOD: 16.7 ±0.38) primers. The Cq values obtained using azobenzene-modified primers were shown to be very similar to those Cq values obtained with non-modified primers (Taq: 15.3 ±0.09, KOD: 13.0 ±0.06) (Fig. 10).

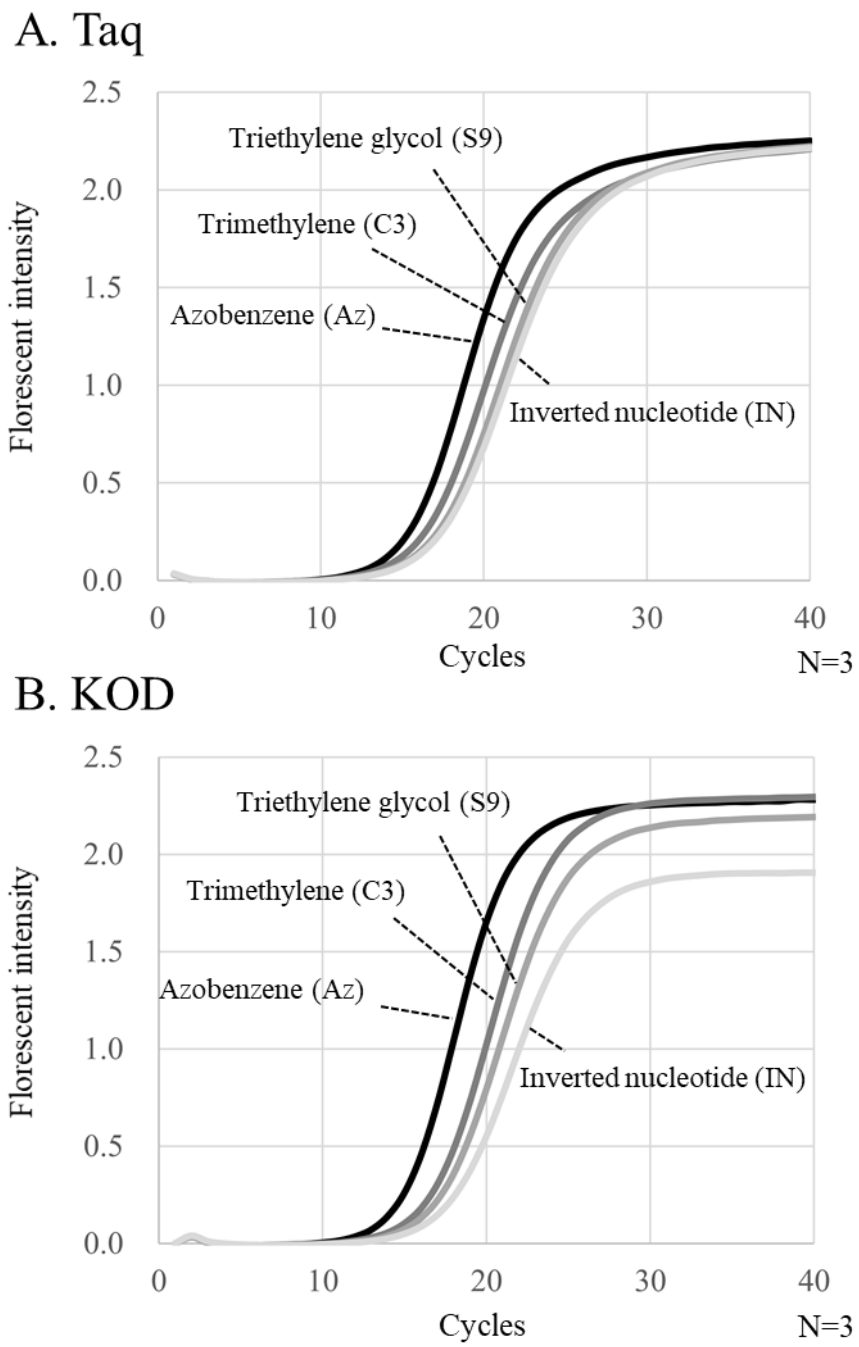


Fig. 10. Real-time PCR assay. Amplification efficiency of each modified primer was examined. Fluorescence intensity and Cq values were obtained. (A) Taq DNA polymerase analysis. (B) KOD exo (-) DNA polymerase analysis.

2-3-3. Melting temperature

Tm values of each modified oligonucleotide were measured by analyzing absorbance of the hybridized samples at 260 nm. The analyses indicated that azobenzene-modified primers showed the highest Tm values (65.24 ±0.43), followed by the non-modified primer (63.79 ±0.10), S9 (63.55 ±0.52), C3 (63.27 ±0.60), and IN (62.66 ±0.44) (Fig. 11).

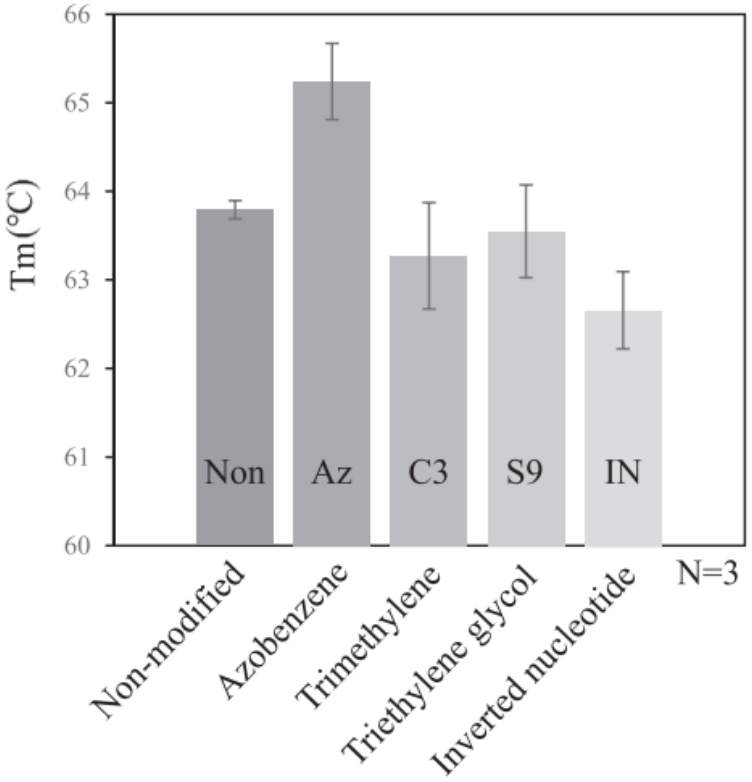
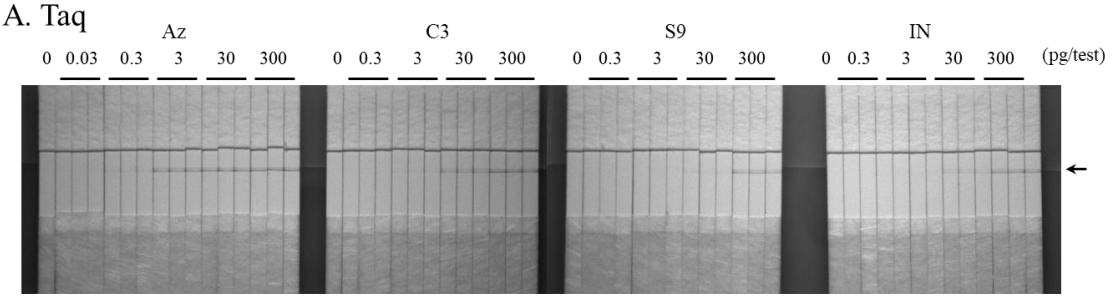


Fig. 11. Melting temperature (Tm) assays, using modified oligonucleotides. Tm values obtained using the modified primers were measured and compared.

2-3-4. Detection limit profiles of KDCC

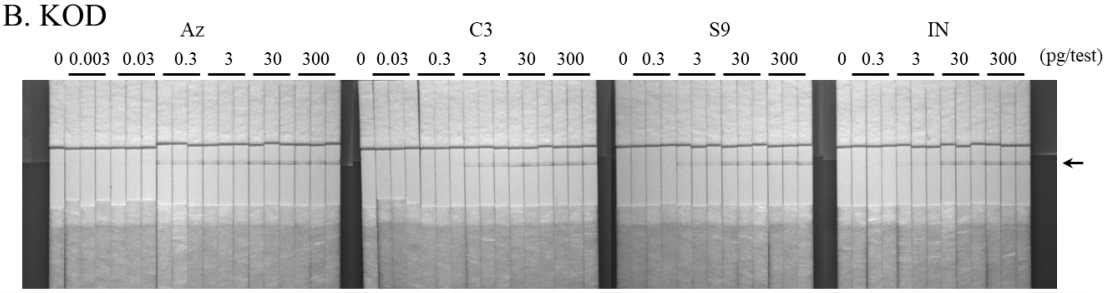
Using all modified oligonucleotides as primers, I have carried out the PCR analyses. Primer combinations and template DNA were the same as described for the assay of amplification efficiency. KDCC-1 was utilized to investigate all products, and PCR products obtained by using the azobenzene-modified primers indicated the lowest detection limit (Taq: 0.3 pg/test, KOD: 0.03 pg/test) with the same reaction time (Fig. 12). The detection limit was shown to be 10-fold lower than that obtained using C3-modfidied primers, and 100-fold lower than those obtained using S9- and IN-modified primers. Furthermore, by adding a larger amount of template DNA enhanced the signal intensity observed on the chromatography chip, showing the potential of KDCC for quantitative analyses.

A. Taq



	pg/test						
	0	0.003	0.03	0.3	3	30	300
Azobenzene (Az)	-	-	-	+	++	+++	+++
Trimethylene (C3)	-	n. d.	n. d.	-	+	++	+++
Triethylene glycol (S9)	-	n. d.	n. d.	-	-	+	+++
Inverted nucleotide (IN)	-	n. d.	n. d.	-	-	++	+++

B. KOD



	pg/test						
	0	0.003	0.03	0.3	3	30	300
Azobenzene (Az)	-	-	+	++	+++	+++	+++
Trimethylene (C3)	-	n. d.	-	+	++	+++	+++
Triethylene glycol (S9)	-	n. d.	n. d.	-	++	+++	+++
Inverted nucleotide (IN)	-	n. d.	n. d.	-	+	+++	+++

Fig. 12. KDCC detection of PCR products. Color signals obtained on chips were analyzed visually and with immuno-chromatography reader. (A) PCR products obtained using Taq DNA polymerase. (B) PCR products obtained using KOD exo (-) DNA polymerase. n. d., no data; minus, color signal was not visually detectable; plus sign, color signal was visually detectable; two plus signs, easily detectable; three plus signs, a stronger signal. Az, azobenzene-modified primers used; C3, C3-modified primers used; S9, S9-modified primers used; IN, IN modified primers used.

2-3-5. Detection of herpes simplex virus (HSV) with KDCC

Using KOD DNA polymerase and HSV-1/2 DNA as templates, multiplex-PCR assays were carried out. Primers for the virus detection were designed as previously reported (54) and azobenzene was used as a modifier for primers. KDCC-3 was utilized for the assays. HSV-1 and HSV-2 were shown to be properly detected as red colored lines on KDCCs (Fig. 13), while the required time for the detection was less than 5 min.

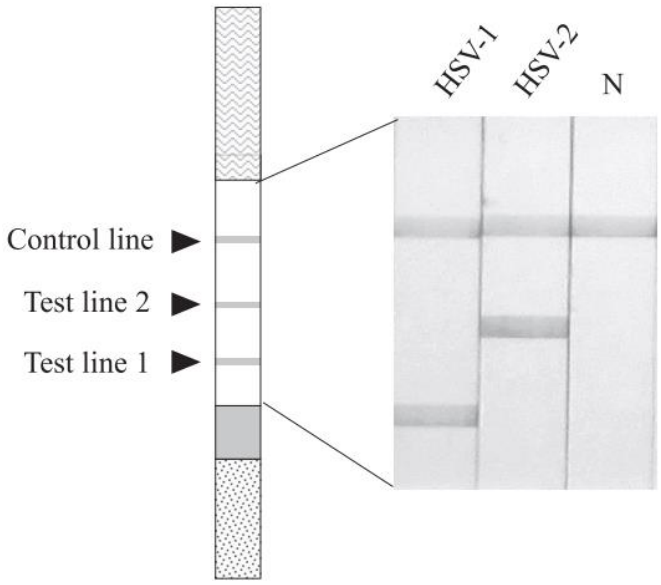


Fig. 13. HSV-1/2 detection with KDCC. HSV-1/2 DNA samples were amplified with PCR using azobenzene-modified primers. PCR products were assayed on KDCC-3, and the signals were visually detected within 5 min. N, non-template negative control.

2-4. Discussion

Here, I investigated that the inhibition efficiency depended on the type of modification substance utilized, and azobenzene modifications were shown to induce the highest levels of elongation inhibition. The elemental mechanisms of these processes remain unclear, however, azobenzene characteristics, such as bulkiness, hydrophobicity, and intercalation properties (55), may relate to this effect. Furthermore, a considerable level of translesion elongation products were confirmed to be dependent on the modification substance, with the maximum rate of 23% of the total reacted products observed for the combination of KOD exo (-) DNA polymerase and S9 modifications. Additionally, in KDCC detection analyses, two modified primers (forward and reverse primers) were utilized. Since undetectable PCR products for KDCC are generated when translesion synthesis occurs, this could involve either forward or reverse primers, or both. In case of the combination of KOD exo (-) DNA polymerase and S9 modifications, an estimated ratio of generating detectable PCR products which possessed ssDNA tags both 5′ ends, was calculated to 59.29% ($0.77^2 \times 100$). This presumed value shows translesion synthesis and the synthesis of

undetectable products leads to a decline in KDCC detection performance. Although the reduction in detection sensitivity can be recovered by increasing the number of thermal cycles, longer time for reaction is not suitable for POC-testing. Therefore, all assays in this chapter pointed to azobenzene modifications as superior for the application in KDCC, due to the higher inhibition efficiency and the generation of detectable PCR products.

The type of DNA polymerase is related to the inhibition efficiency by modifications in a template. For example, although some bacterial family A DNA polymerases can accept ribonucleotide and uracil base, archaeal family B DNA polymerases are inhibited by the presence of ribonucleotide and uracil bases (32, 37, 57). Additionally, thymine dimers and abasic sites allow the translesion synthesis (52, 58). In this chapter, I investigated the elongation efficiencies of Taq and KOD exo (-) DNA polymerases, and showed that KOD DNA polymerase was easy to generate translesion elongation. This was more prominent when using C3- and S9-modified primers and KOD exo (-) DNA polymerase, which reduced inhibition efficiency, whereas the use of azobenzene primer led to the inhibition efficiency of > 96%. KOD DNA polymerase was shown to possess a high elongation speed and high tolerance

against impurities in DNA synthesis, making it suitable for the application in the ultra-rapid PCR and PCR with no purified samples or with crude samples (16, 59). As these are the characteristics required for POC-NATs, the use of KOD exo (-) represents a suitable approach in the development of tools for POC-testing.

Speed of the diagnostics is one of the key factors in POC-NAT development. To examine the speed of amplification, real-time PCR analysis were carried out, and indicated that the reaction speed when using all modified primers, except for azobenzene-modified ones, was reduced (increased Cq values), compared with that obtained when utilizing no modified primers. This may be due to the decline of primer-template hybridizing efficiency after the modification, which stalls DNA polymerase and induces the formation of inert complexes. On the other hand, when using azobenzene modified primers, the speed of amplification remained the same rate as that for the non-modified samples when Taq DNA polymerase was used, or it was higher than those obtained for other modifications when KOD exo (-) DNA polymerase was applied. This high-speed amplification may be related to the property of azobenzene, which acts as an intercalator and

enhancing primer-template binding. Based on the Tm analysis in this chapter, I support this hypothesis as the azobenzene-modified oligonucleotides have higher Tm values than other examined modified oligonucleotides (the buffer used in this assay was not completely similar to the one used in PCR; therefore, the Tm value may be altered in PCR results). Furthermore, this counteracts the negative effect of primer modification, and contributes to improvement of the amplification speed. The superiority of azobenzene-modified primers for utilization in KDCC assay was indicated throughout this chapter. The lowest detection limit was observed when using azobenzene-modified primers, with both DNA polymerases used. Additionally, these results suggest that, by using azobenzene-modified primers with the KDCCs, trace amounts of DNA can be rapidly detected, due to the efficient inhibition of DNA polymerase-mediated DNA elongation during the amplification procedure, in combination with a higher amplification efficiency compared with those obtained when using other modified primers.

Finally, rapid multiplex detection system for HSV-1/2 using KDCC and azobenzene-modified primers was developed, allowing a rapid (within 5 min) detection of HSV-1/2 DNA. By combining this system with simple and

rapid sample pretreatment and rapid PCR technologies, multiplex POC-NATs can be developed and widely used in future.

General conclusion

In this thesis study, I tried to develop elemental technologies for a simple and rapid NAT to realize point of care-NAT, which was useful for the earlier detection of pathogens. To accomplish the objective, I focused on the amplification and the detection processes of NAT which consisting major procedures of this method. At first, I conducted study about the amplification step and a technology to simplify the RT-PCR was developed. Secondly, about the detection step, a technology for a simple and rapid multiplex-PCR product detection was developed.

In chapter 1, to simplify the RT-PCR, thermostable proof-reading DNA polymerase possessing both reverse transcriptase activity and DNA-dependent DNA polymerase activity was developed from DNA polymerase I derived from newly isolated hyperthermophilic bacterium *Thermotoga petrophila* K4. The firstly-obtained wild-type DNA polymerase I (K4PolI) did not have detectable reverse transcriptase activity. Structure modeling of the enzyme estimated the site of template recognition. I designed the amino acid substituted mutants in the prospected recognition region. Enzymatic analysis of the mutants revealed that some of enzymes showed reverse transcriptase

activity with enough stability, DNA-dependent DNA polymerase activity and proof-reading activity. These K4PoI mutants are capable of applying simple one-step RT-PCR.

In chapter 2, a rapid detection technology for multiplex-PCR products was developed using azobenzen-modified primers and a paper-based DNA chromatography chip named KDCC. This research firstly discovered that the combination of azobenzene-modified primers and KDCC showed excellent detection performance than other modifications such as trimethylene, triethylene glycol, and inverted nucleotides. Also, the study revealed this superior property of azobenzene-modified primers was derived from efficient ss DNA tag addition and high-speed amplification in PCR. Constructed HSV-1/2 detection method with the developed technology will contribute rapid detection of pathogens.

To realize on-site NAT, the industrial study about combining developed technologies and building up as a diagnostic system is still remained. Also, further improvements in rapidity and usability will be required for the wider application. The developed technologies in this study are useful for development of the next generation diagnostic device and

realize POC-NAT, which contribute to earlier diagnostics of infectious diseases in smaller institutions and developing countries.

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