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An *in vitro* screening technique for DNA polymerases that can incorporate modified nucleotides. Pseudo-thymidine as a substrate for thermostable polymerases

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ABSTRACT

DNA polymerases are desired that incorporate modified nucleotides into DNA with diminished pausing, premature termination and infidelity. Reported here is a simple *in vitro* assay to screen for DNA polymerases that accept modified nucleotides based on a set of primer extension reactions. In combination with the scintillation proximity assay (SPA™), this allows rapid and simple screening of enzymes for their ability to elongate oligonucleotides in the presence of unnatural nucleotides. A proof of the concept is obtained using pseudo-thymidine (ψT), the C-nucleoside analog of thymidine, as the unnatural substrate. The conformational properties of ψT arising from the carbon–carbon bond between the sugar and the base make it an interesting probe for the importance of conformational restraints in the active site of polymerases during primer elongation. From a pool of commercially available thermostable polymerases, the assay identified *Taq* DNA polymerase as the most suitable enzyme for the PCR amplification of oligonucleotides containing ψT. Subsequent experiments analyzing PCR performance and fidelity of *Taq* DNA polymerase acting on ψT are presented. This is the first time that PCR has been performed with a C-nucleoside.

INTRODUCTION

The expansion of the nucleic acid-based chemistry and biology by novel, functionalized nucleotides and non-standard base pairs is driven by the possibility of identifying RNA and DNA molecules with new catalytic and binding properties (1–3) and of better understanding the physical organic chemistry underlying strand–strand interaction in nucleic acids (4). Additional nucleotide building blocks in a DNA strand could be used to introduce structural motifs and functional groups, including thiols (5) and imidazoles (6), not found in standard nucleic acids. Sophisticated techniques such as *in vitro* selection (7) could be applied to functionalized oligonucleotides to generate more powerful ligands, receptors and catalysts than can be obtained from *in vitro* selection experiments with standard oligonucleotide libraries.

To implement this vision requires molecular biological tools for manipulating an expanded genetic alphabet. Enzymes, in particular DNA polymerases that utilize unnatural substrates with the same efficiency as the natural polymerases, would be especially valuable. While many enzymes accept components of an expanded genetic alphabet (8), natural polymerases have clearly been optimized for the structures of the natural nucleotides (9). When they encounter an unnatural base either in the template or as a triphosphate, they frequently pause or abort copying. Even when the pausing is modest, it can have consequences in a PCR amplification, possibly increasing the rate of misincorporation and resulting in a loss of sequence information.

For these reasons, many efforts have been made to find empirically polymerases that have an improved ability to accept elements of an expanded genetic alphabet. To date, polymerase variants have been screened using gel-based assays. These experiments, which have examined approximately 10^2 polymerases, have detected variants with improved ability to accept non-standard nucleotides (9–11). Based on the results, a crude statistical estimate suggests that satisfactory polymerases will be attained, not after millions, but after thousands of variants are inspected. The goal of a polymerase-based molecular biology of an expanded genetic alphabet is therefore accessible, but only if a higher throughput screen is developed. In view of the present interest of many laboratories in improving polymerases for a variety of nucleic acid analogs (12–14), it is appropriate to describe such a screening method at this time.

MATERIALS AND METHODS

Chemical synthesis of pseudo-thymidine ψT (1) and ψTTP (3)

The phosphoramidite of pseudo-thymidine (ψT, **1**) was prepared from pseudo-uridine (15). The triphosphate **3** (ψTTP) of **1** was synthesized from 3'-acetyl-pseudo-thymidine, using the procedure of Ludwig and Eckstein (16). The 5' position of pseudo-thymidine was protected with 4,4'-dimethoxytrityl chloride (in pyridine, 16 h, room temperature), followed by acetylation of the 3'-hydroxyl group with acetic anhydride (4 equiv. in pyridine, 4 h, room temperature). After evaporation of

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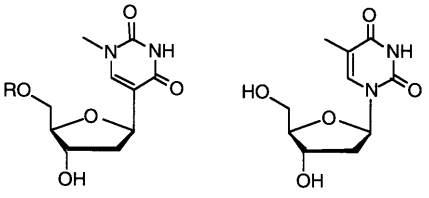
the solvents, the residue was detritylated with trifluoroacetic acid in dichloromethane, yielding 3'-acetyl-pseudo-thymidine (8.5 mg, 30 μmol). This intermediate was dissolved in a mixture of pyridine and dioxane (30 μl/89 μl) and reacted with a solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (36 μl, 188 mg in 0.93 ml dioxane). After 10 min, tributylammonium pyrophosphate (17 mg, 37 μmol) in DMF (91 μl) and tributylamine (31 μl) were added and the reaction mixture shaken for another 10 min. The reaction was quenched by addition of iodine solution (0.6 ml, 1% in pyridine/water 98:2). After shaking the mixture at room temperature for 15 min, the excess iodine was destroyed with a few drops of Na_2SO_3 solution (5%). The solution was evaporated and dried completely under high vacuum. The residue was reacted with water (0.8 ml) at room temperature for 30 min. The solution was lyophilized and the residue incubated in concentrated ammonia (1 ml) at 55°C overnight. After degassing and lyophilizing the solution, the crude product was purified by reverse phase HPLC (Waters Preparative Nova Pak HR C18, 7.8 × 300 mm, flow rate 4 ml/min, 10 mM triethylammonium acetate, pH 7.0, 10 mM triethylammonium acetate pH 7.0 + 20% acetonitrile, 0–25% B in 30 min). The product fractions were pooled and lyophilized three times to remove excess triethylammonium acetate, yielding **3** (2 mg, 14%) as a white/yellow foam.

^{1}H-NMR (300 MHz, D_2O) δ: 7.85 (s, 1H, H-6), 5.18 (q, 1H, H-1', J = 6.04, 9.61 Hz), 4.62 (hept, 1H, H-3', J = 2.5, 5.3 Hz), 4.20 (m, 1H, H-4'), 3.71 (dd, 1H, H-5'$_A$, J = 4.4, 11.7 Hz), 3.62 (dd, 1H, H-5'$_B$, J = 6.3, 11.7 Hz), 3.47 (s, 3H, H-(NCH_3), 3.27 (q, triethylammonium), 2.34 (ddd, 1H, H-2'$_A$, J = 2.47, 6.05, 13.5 Hz), 2.18 (ddd, 1H, H-2'$_B$, J = 5.7, 9.61, 13.5 Hz), 1.35 (t, triethylammonium), p.p.m. ^{31}P-NMR (300 MHz, D_2O) δ: 3.05 (d, γP, J = 18.5 Hz), 2.23 (d, αP, J = 24.4 Hz), –9.73 (t, βP, J = 18.5, 24.4 Hz), p.p.m. MS: (ESI): 481 (M-H), 503 (M-H, Na). UV: (Tris–HCl, 100 mM, pH 7.0): λ_{max} = 271 nm (ε = 8800 M^{-1} cm^{-1}).



1 R = H
3 R = O(P_3O_9)$^{3-}$

2

DNA synthesis, digestion and sequencing

Oligodeoxyribonucleotides containing **1** were prepared on an Applied Biosystems DNA synthesizer. The coupling times for **1** were doubled. No further adjustments of the protocol were necessary. The oligonucleotides were deprotected under standard conditions (ammonia solution, 55°C, overnight) and PAGE purified (17).

Successful incorporation of **1** by chemical synthesis or enzymatic primer extension was shown through enzymatic digestion of the oligonucleotides with phosphodiesterase (*Crotalus durissus terrificus*; Boehringer Mannheim) and alkaline phosphatase (bovine calf intestine; Boehringer Mannheim). Subsequent reverse phase HPLC analysis (5% acetonitrile in 10 mM triethylammonium acetate, pH 7, over 15 min) was used to quantify the composition of the oligonucleotides (**1**; λ_{max} = 271 nm, ε = 6000 M^{-1} cm^{-1}).

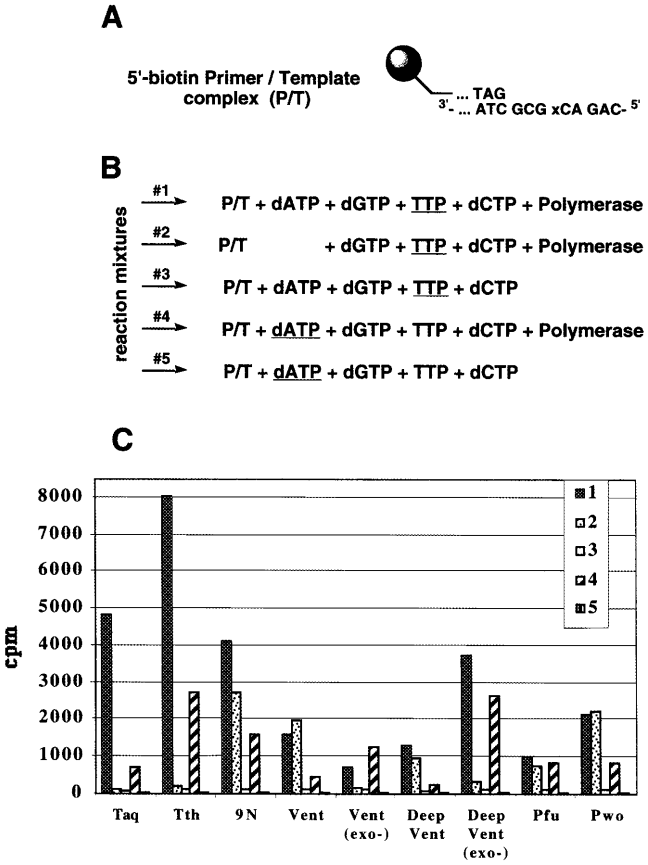


Figure 1. (**A**) Primer/template complex (P/T 21/30) with 5'-biotinylated primer. The position of **1** is marked as X. (**B**) Each polymerase is tested in five separate reactions. The underlined dNTP is tritium-labeled and functions as a reporter for successful incorporation. The first set (reactions 1–3) uses TTP as a reporter. A signal in reaction 1 indicates successful incorporation of a nucleotide opposite the unnatural base in the template and continuation of primer elongation. Reaction 2, lacking the correct hydrogen bonding partner for **1**, measures the tendency towards misincorporation of dGTP, dCTP or TTP for polymerases lacking exonuclease activity (see text). Reactions 3 and 5 provides information concerning background, reaction 4 measures the incorporation of dATP opposite the unnatural nucleotide. (**C**) Graphical presentation of primer extension by various thermostable DNA polymerases. Vent (but not Vent exo$^-$), Deep Vent (but not Deep Vent exo$^-$), *Pfu* and *Pwo* DNA polymerases all have high exonuclease activity. For each enzyme, all five experiments were performed and the amount of tritium incorporation was measured by liquid scintillation counting.

The accuracy of the PCR amplification was further analyzed by Sanger dideoxy sequencing with *Taq* DNA polymerase (18) and chemical sequencing using the protocol of Maxam and Gilbert (17) (see Standing start experiments and PCR amplification below).

Polymerase screening, primer extension and scintillation counting

A series of commercially available thermostable polymerases was tested with the assay: *Taq* DNA polymerase and *Tth* DNA polymerase (Promega), *Pfu* DNA polymerase (cloned; Stratagene), *Pwo* DNA polymerase (Boehringer Mannheim), Vent™, Vent (exo$^-$)™, Deep Vent™, Deep Vent (exo$^-$)™ and 9°N DNA polymerase (all from New England Biolabs). Each enzyme

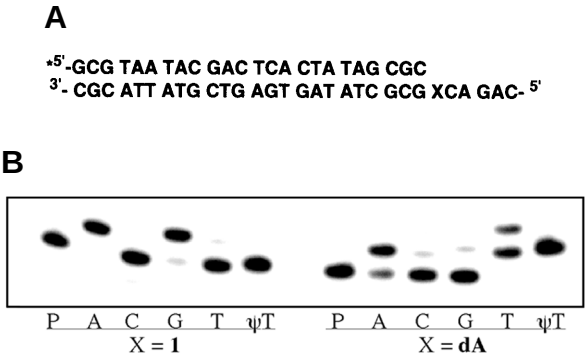


Figure 2. Standing start experiments with *Taq* DNA polymerase. (**A**) The 5'-^{32}P-labeled primer/template complex (P/T 24/30) is incubated with a single nucleoside triphosphate. X marks the position of **1** or dA. The reaction conditions were chosen to allow multiple hits by polymerases to indicate possible mismatches. (**B**) The reaction mixtures were analyzed by PAGE (10%, 7 M urea) against the original primer P.

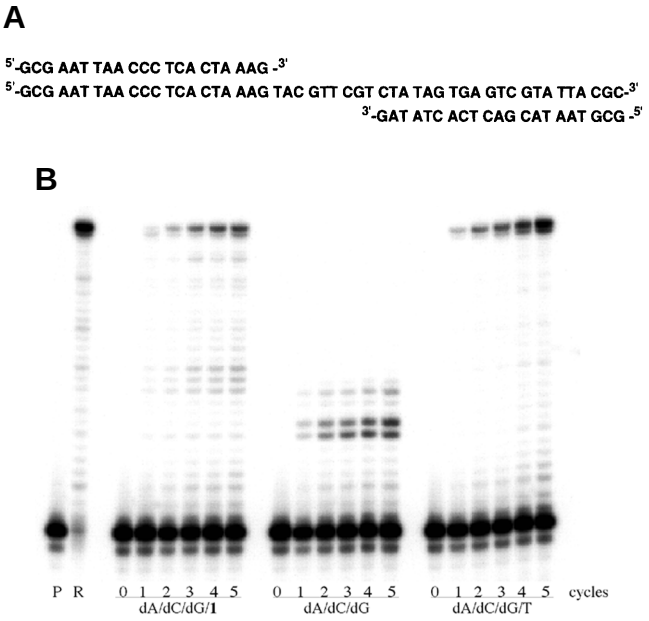


Figure 3. PCR-like amplification over five cycles. (**A**) The template strand (51mer) is complemented by the two primers (T3 and T7). Amplification during each cycle required incorporation of 13 thymidines or thymidine analogs to obtain full-length product. (**B**) Three separate PCR experiments with 5'-^{32}P-labeled primer in the presence of the triphosphates of dA, dC, dG and **1**, TTP or no thymidine analog were performed. Positive control R (^{32}P-labeled template) and primer P are shown.

was incubated in five separate reactions (Fig. 1B) for 30 min at 72°C with a mixture containing the supplier's reaction buffer, 1 μM each dNTP (Promega) as well as either [^{3}H]TTP (90–130 Ci/mmol; Amersham) or [^{3}H]dATP (23 Ci/mmol; ICN) and 0.2 pmol of the primer/template complex (Fig. 1A) (total volume 50 μl). The reaction was quenched with EDTA (20 μl, 0.5 M, pH 8.0) and the mixture was mixed with SPA™ beads (Amersham) or loaded into a Flashplate™ (NEN). After incubation at room temperature for 15–30 min, the samples were counted in a Topcount NXT Scintillation Microtiterplate Reader (Packard Instruments, Meriden, CT).

Standing start experiments and PCR amplification

The qualitative fidelity of *Taq* DNA polymerase was investigated by standing start experiments and PCR amplification over 10 cycles. Primers with a 5'-^{32}P-label were obtained by enzymatic phosphorylation with polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (>5000 Ci/mmol; Amersham) and were ethanol precipitated twice. After mixing primer (0.5 μM), template (0.625 μM) (Fig. 2A), the corresponding dNTP (200 μM) and reaction buffer (supplied with the polymerase), the enzyme (2.5 U per 8 μl reaction mixture) was added on ice and the sample cycled once (1 min, 94°C; 30 s, 55°C; 10 min, 72°C). The reaction was quenched with EDTA (0.5 M, pH 8) and ethanol precipitated before loading on a 10% PAGE gel (7 M urea). The gel was analyzed with a Molecular Imager® (Bio-Rad, Hercules, CA) (Fig. 2).

For the PCR amplification experiments, primer (1 μM each, forward primer 5'-^{32}P labeled) and template (20 nM) (Fig. 3A), as well as the appropriate dNTPs (200 μM each), were mixed with reaction buffer and *Taq* DNA polymerase (25 U) and adjusted to a final volume of 100 μl with water. The experiments were cycled (initial 5 min, 94°C; 30 s, 94°C; 30 s, 55°C; 10 min, 72°C; polishing for 5 min, 72°C) 10 times. During each annealing phase, an aliquot (8 μl) was taken, quenched with EDTA and precipitated with ethanol. The samples were separated on a 10% PAGE gel (7 M urea) and analyzed with the Molecular Imager® (see Supplementary Material).

The single-stranded oligonucleotides for DNA sequencing were produced by replacing the forward primer with a 5'-biotinylated analog. After 10 cycles, the quenched PCR reaction mixture was loaded onto a pre-equilibrated streptavidin–agarose column (Fluka Chemicals), washed with 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA (pH 8.0) and the unbiotinylated strand was eluted with 0.2 M NaOH. After ethanol precipitation and PAGE purification, the single-stranded material was used for Sanger dideoxy and Maxam–Gilbert sequencing. Sequence analysis by the dideoxy method required some adjustment of the experimental conditions. When the temperature of the primer extension reaction was set at 70°C, the following dNTP/ddNTP/Mg concentrations were found suitable to sequence short oligonucleotides with *Taq* DNA polymerase: dA/ddA/Mg, 15 μM/2 mM/2.24 mM; dT/ddT/Mg, 15 μM/3 mM/3.24 mM; dG/ddG/Mg, 15 μM/0.5 mM/0.74 mM; dC/ddC/Mg, 15 μM/1 mM/1.24 mM. After annealing equimolar amounts of ^{32}P-labeled forward primer (T7) with the purified template, all reaction mixtures were incubated with primer/template complex (0.25 μM) and 10 U of *Taq* DNA polymerase for 10 min. For the chemical sequence analysis, the protocol of Maxam–Gilbert for purine and pyrimidine analysis was employed. After 5'-^{32}P-labeling of the amplified oligonucleotides with [γ-^{32}P]ATP (>5000 Ci/mmol) and T4 polynucleotide kinase (NEB), ~100 000 c.p.m. (2 μl) were used per derivatization. Purine bases (A+G) were depurinated in 2.3% formic acid (20–30 min at 37°C), followed by strand cleavage with piperidine (150 μl, 1 M, 90°C, 30 min). Pyrimidine bases were derivatized by hydrazine treatment (23 μl, 20°C, 4–10 min) and quenched with hydrazine stop solution (200 μl) (17) and ethanol (750 μl); following lyophilization, the pyrimidine bases were cleaved with piperidine (100 μl, 1 M,

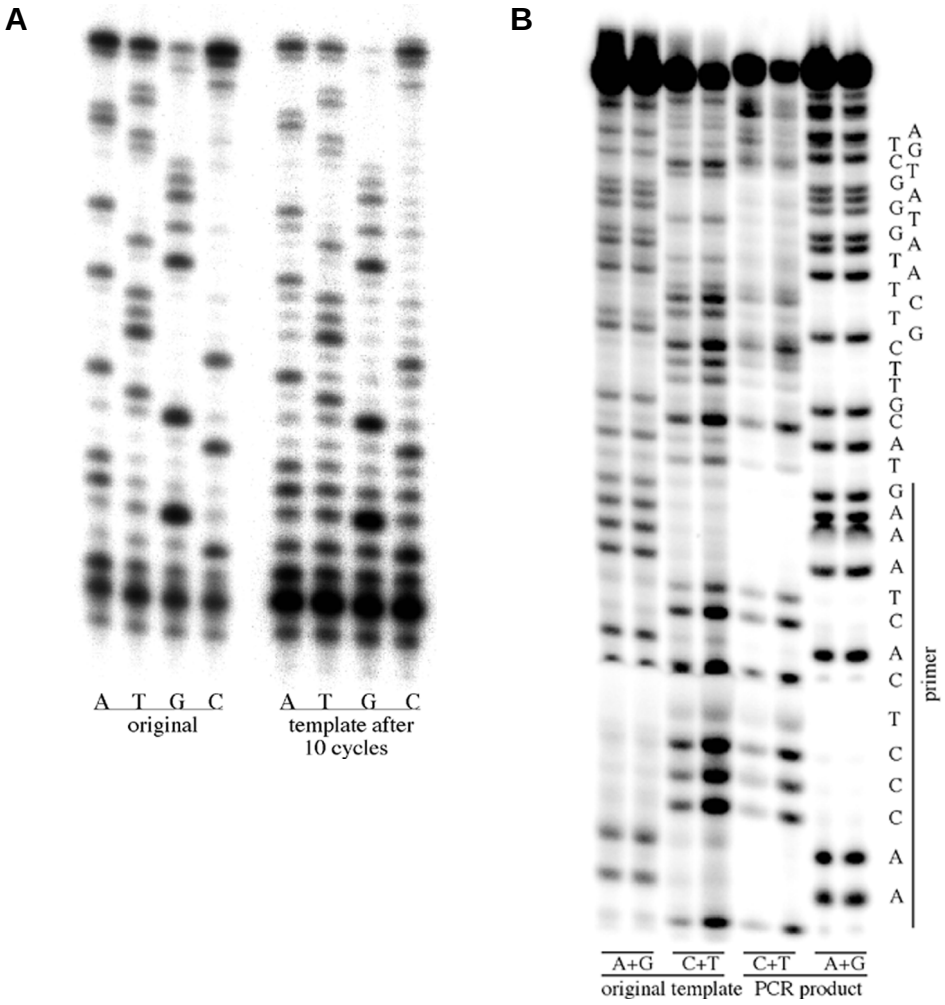


Figure 4. Sequencing of PCR-generated oligonucleotides amplified using ψTTP instead of TTP. (**A**) Sanger dideoxynucleotide sequencing. The sequencing patterns from the original template and the PCR product are indistinguishable. The additional weak bands are attributed to background termination of *Taq* DNA polymerase and extended exposure time. (**B**) Chemical sequencing by the Maxam–Gilbert method. Each sequence was incubated for two different time periods (A+G, 21 and 30 min; C+T, 4 and 10 min). The depurination pattern (A+G) for the original template and the PCR product are identical. The cleavage reaction for C+T is not as efficient. The single nucleotide primer extension experiments showed that ψT is most likely to be replaced by a purine (Fig. 2). The sequencing pattern showed no evidence for substantial replacement of ψT by purines.

90°C, 30 min). All reaction mixtures from the dideoxy sequencing, as well as from the Maxam–Gilbert derivatization, were electrophoretically separated in acrylamide (10%, 7 M urea) and analyzed by autoradiology (Fig. 4A and B).

Gel fidelity assay for 1

Once a polymerase that accepted **1** was identified by the high throughput screen, kinetic data for the incorporation of **1** by the polymerase were determined by single nucleotide incorporation (19). The same primer/template complex as in the previous qualitative analysis was used (Fig. 2A). In the absence of dNTP, primer (^{32}P-labeled, 20 pmol), template (25 pmol) and *Taq* DNA polymerase (0.1 U) were mixed in reaction buffer (final volume 8 µl), denatured at 94°C (1 min) and annealed at 55°C (60 min). The experiment was initiated upon addition of the appropriate dNTP (2 µl; Table 1) at 55°C and the reaction was quenched after the previously determined time to ensure that a maximum of 20% of the primer was extended. After separation by PAGE, the products were quantified using the Molecular Imager® and MultiAnalyst® software (Bio-Rad). Kinetic parameters (Table 1) were determined by plotting [dNTP] versus [dNTP]/*v*, where *v* is the primer extension velocity [ratio $(n + 1)/\{n + (n + 1)\}$ per second, where *n* is the pixel counts per band; Hanes–Woolf plot].

RESULTS AND DISCUSSION

In vitro screening assay/SPA

The scintillation proximity assay (SPA™; Amersham/Pharmacia Biotech, Arlington Heights, IL) was recently introduced as an alternative to polyacrylamide gel electrophoresis and filter binding assays for measuring polymerase activity (20). In the assay, tritium-labeled dNTP is added to the reaction mixture together with 5'-biotinylated primer. Following an elongation period, streptavidin-coated plastic beads containing scintillant

Table 1. Kinetic parameters for ψT incorporation by *Taq* DNA polymerase

Template	dNTP	V_{max} (% min^{-1})	K_m (μM)	Efficiency (V_{max}/K_m)	Accuracy
ψT	dATP	3.25 (± 0.35)	17.7 (± 2.56)	1.8×10^5	1
	dGTP	0.19 (± 0.01)	51.3 (± 2.10)	3.8×10^3	0.021
dA	TTP	9.65 (± 0.15)	8.98 (± 0.24)	1.1×10^6	1
	ψTTP	6.14 (± 0.10)	13.6 (± 1.69)	4.5×10^5	0.41
	dATP	0.68 (± 0.01)	183 (± 0.19)	3.7×10^3	0.003

Steady-state parameters for a single nucleotide incorporation into P/T 24/30 (Fig. 2A). The optimal reaction times were determined in a time course experiment (dA template: TTP = 180 s, ψTTP = 240 s, dATP = 20 min; ψT template: dATP = 300 s, dGTP = 20 min). To determine the kinetic data, the dNTP concentration was varied from 3.12 to 200 μM (a total of seven concentrations). All experiments were run three times and the averaged data are shown. Error limits are given in parentheses.

are added to the reaction. The 5'-biotinylated primer–template complex binds with high affinity, placing the incorporated tritium-labeled nucleotides in close proximity to the plastic bead. Because of the relatively low energy of tritium decay particles, only isotopic sources in the vicinity of the plastic bead result in a detectable signal, which therefore represents the amount of labeled dNMP incorporated. Unincorporated [^{3}H]dNTP, still present in solution, accounts only for background counts. An analogous strategy can be implemented using Flashplates (NEN™; Life Science Products, Boston, MA), a microtiter plate where the streptavidin is on the surface of the plastic wells and scintillant is embedded in the plastic. Setting up the primer extension reactions requires ~15 min while incubation with polymerase usually runs for 30–60 min, making the plate, screening 96 variants, available for counting (typically 5 min per well) in <2 h.

The combination of primer extension experiments with SPA was tested to screen for DNA polymerases able to incorporate dATP opposite pseudo-thymidine (**1**), the C-nucleoside analog of thymidine (**2**), in a template. Pseudo-thymidine was prepared from the commercially available pseudo-uridine (15; Materials and Methods) and converted to its phosphoramidite. Oligonucleotides containing **1** were prepared by phosphoramidite-based DNA synthesis.

The experiments are illustrated schematically in Figure 1. The template (30mer; Fig. 1A) was designed to contain no 2'-deoxyadenosine between the 3'-end of the primer (position 21) and **1** (X, position 25). After annealing with a 5'-biotinylated primer (P/T 21/30), the complex was incubated with the polymerase and the standard dNTPs in the presence of [^{3}H]TTP. Successful incorporation of dATP opposite **1** permits further elongation of the primer beyond position 25 and consequently incorporation of tritium-labeled TTP at position 27.

To learn whether the polymerase extended past **1** in the template via misincorporation, analogous experiments were run in the absence of dATP (Fig. 1B). Thus, experiment 1 tests for primer extension beyond the unnatural nucleotide, experiment 2 tests the specificity of the incorporation in the absence of dATP (the appropriate hydrogen bonding partner for **1**), while experiment 3 measures the background radioactivity of the reaction mixture.

Incorporation of the correct nucleoside triphosphate opposite **1** can also be measured using tritium-labeled dATP in a second, independent set of reactions (Fig. 1B, reactions 4 and 5). Given the specific activity of the [^{3}H]dTTP and [^{3}H]dATP used, a polymerase that successfully completes elongation with no mismatching should generate approximately seven times more radioactivity in the products in experiments 1–3 than in experiments 4 and 5. A lower ratio indicates failure to complete incorporation past the initial dA to yield full-length product when [^{3}H]dTTP is incorporated.

A set of commercially available thermostable polymerases suitable for PCR were screened for their ability to incorporate **1** using the P/T 21/30 complex. The set of five reactions was run for each polymerase. After quenching with EDTA, the amount of tritium incorporation was determined by scintillation counting on SPA beads (Fig. 1C). Results were shown to be linear as a function of time and the concentration of polymerase, with results reproducible to within 10%.

In these experiments, the amount of label introduced in the parallel experiments with [^{3}H]dTTP and [^{3}H]dATP approached the ratio of 7:1 for *Taq* and Deep Vent DNA polymerases only. The ratio was lower for the other polymerases examined, suggesting that they aborted elongation after [^{3}H]dATP was incorporated but before [^{3}H]dTMP could be incorporated.

Interestingly, the data from the screen also showed a direct correlation between the amount of products containing [^{3}H]dTMP synthesized in the absence of dATP (experiment 2) and the exonuclease activity of the polymerase. Thus, the Vent (but not Vent exo$^-$), Deep Vent (but not Deep Vent exo$^-$), *Pfu*, and *Pwo* DNA polymerases, all having high exonuclease activity, all displayed a high ratio of signal in experiment 2 relative to that in experiment 1. This could, of course, arise by primer extension involving misincorporation opposite **1** followed by full-length extension, by skipping over **1** followed by full-length extension or perhaps by incorporation of [^{3}H]dTMP opposite **1**. More likely, however, once exo$^+$ polymerases stalled at the unnatural nucleotide in the template, the exonuclease activity operated to digest the primer to position 19, exposing an A in the template, which then directed the incorporation of [^{3}H]dTMP. This phenomenon is well known in polymerase biochemistry (21), especially when triphosphate concentrations are as low as those used here.

Standing start experiments

Based on the screening using the scintillation proximity assay, the DNA polymerases from *Thermus aquaticus* and *Thermus thermophilus* were chosen for standing start experiments (19),

the first because of the high ratio of [^{3}H]dTMP to [^{3}H]dAMP incorporation in the parallel experiments 1 and 4, the second because of an evidently high fidelity. Two primer/template complexes (Fig. 2A, P/T 24/30) carrying either **1** or 2'-deoxyadenosine at position 25 were prepared. An extended reaction time of 30 min allowed the polymerase to undergo several cycles of polymerization (multiple hit conditions). The resulting gel, while not suitable for quantitative analysis, identified nucleoside triphosphates that might be misincorporated opposite **1** in the template, as well as the competitors of ψTTP for incorporation opposite dA in the template.

Tth DNA polymerase performed rather poorly, showing significant misincorporation of purines opposite **1** under these conditions (data not shown). *Taq* DNA polymerase (Fig. 2B), however, extended the primer by placing a purine nucleotide, preferentially 2'-deoxyadenosine, opposite **1** in the template. *Taq* DNA polymerase also incorporated ψTTP efficiently opposite dA in the template. The only nucleoside triphosphate misincorporated to a significant extent was dATP.

Steady-state kinetics of ψT incorporation by gel fidelity assay

A quantitative comparison of the efficiency of incorporation of ψT and its natural competitors was made by measuring single nucleotide insertion by varying the dNTP concentration under single turnover conditions. Applying the same primer–template complex as above (P/T 24/30), a series of kinetic studies was carried out using steady-state methods (22,23). Based on the above qualitative findings, kinetic data were determined only for the possible competitors and **1** itself. The insertion of dATP and dGTP opposite ψT in the template and the incorporation of TTP, ψTTP and dATP opposite dA were studied. Kinetic parameters derived from the data are presented in Table 1. The insertion of dATP opposite ψT in the template is 50-fold more efficient than insertion of dGTP. In the opposite direction, incorporation of ψTTP vis-à-vis dA in the template is only 2-fold less efficient than TTP, the natural hydrogen bonding partner of dA. The insertion of dATP, on the other hand, is 3×10^2-fold lower and probably the result of non-template-specific polymerization.

PCR amplification

Once performance with good fidelity and efficiency under single nucleotide incorporation conditions was achieved, primer elongation by *Taq* DNA polymerase under PCR-like conditions was tested. A reaction mixture containing a standard oligonucleotide (51mer; Fig. 3A) flanked by the T3 and the T7 primer-binding sequence was prepared and PCR amplified in the presence of dATP, dCTP, dGTP and ψTTP. Two control reactions, one substituting ψTTP with standard TTP, the other performed in the absence of any thymidine analog, were run simultaneously. A sample was taken after each cycle and analyzed by PAGE (Fig. 3B). The reaction mixture lacking thymidine or its analog generated no full-length product; the primer extension reaction was terminated completely at the position of the first adenosine in the template. In the presence of ψTTP, the desired full-length oligonucleotide represented the primary product. Although up to three ψT residues in succession need to be incorporated to produce full-length product, only a small fraction of the extended primer appeared as an early termination product. Further PCR amplification up to 10 cycles gave similar results (see Supplementary Material) and suggested that *Taq* DNA polymerase could be used for PCR-like amplification of sequences containing ψT.

Amplification fidelity/sequencing

In addition to the evidence of ψT incorporation by *Taq* DNA polymerase under conditions of PCR amplification, further proof for sequence-specific amplification of an oligonucleotide in the presence of ψTTP was sought. Modifying the original PCR protocol, the T7 primer was replaced by its 5'-biotinylated analog. After the regular PCR cycles, the amplified material was loaded onto a streptavidin column and washed. Single-stranded oligonucleotides were isolated by elution with sodium hydroxide solution and purified from the excess primer by PAGE. A complete digestion of the PCR product to give nucleosides by phosphodiesterase and alkaline phosphatase, followed by quantitation by reverse phase HPLC analysis, gave the predicted ratio of nucleosides.

The material from PCR amplification with ψT was then used for Sanger dideoxy (Fig. 4A) and Maxam–Gilbert (Fig. 4B) sequencing. The positions of ψT could be identified in the Maxam–Gilbert experiment by the missing bands in the C+T pattern, as the C-nucleoside does not undergo strand cleavage upon hydrazine/piperidine treatment and therefore does not appear as a distinct band in the sequencing gel. In both experiments, the sequence pattern of the products amplified with ψTTP was indistinguishable from the pattern of the original template after 10 cycles of PCR amplification. While the sensitivity of the experiment cannot exclude the generation of some products having sequences different from the starting sequence (something that happens even with natural templates), these results show a high level of fidelity at the individual steps in the PCR reaction. This is the first time that PCR has been performed with a C-nucleoside.

Conclusions

We have tested a new *in vitro* screening assay for thermostable DNA polymerases. The combination of a set of primer extension reactions and the SPA technology for its rapid and simple quantification represents a novel method to search for polymerases that can utilize functionalized or structurally modified nucleotides. The method is applicable to any nucleoside analog, whether integrated in an oligonucleotide or present as the triphosphate. To further simplify and speed up the procedure, the entire assay has been transferred into 96-well microtiter plates (Flashplates), making it suitable for high throughput screening of protein libraries.

For pseudo-thymidine, the best polymerase obtained from the screen was *Taq* DNA polymerase. Kinetic studies were then performed to demonstrate that the polymerase does perform with **1** as expected from the results of the screen. The fidelity of synthesis of the A-ΨT base pair is lower than with the conventional base pairs. It is sufficient, however, for the amplification of typical oligonucleotides for *in vitro* selection (up to 150 nt over 10 cycles).

The results from the kinetic experiments can be put into perspective with previously published data on related nucleoside analogs such as difluorotoluene deoxynucleoside (12,24). While the structural distortion of the nucleoside analog as seen for **1** reduces the accuracy of replication ~2-fold as measured by kinetic experiments (Table 1, dA template versus TTP and ψTTP), additional removal of the Watson–Crick hydrogen

bonding pattern in the difluoro compound causes the accuracy for incorporation of dFTP compared to TTP opposite dA drop by a factor of 40 (12). Although quantitative comparison would require the experiments to be measured in parallel, these preliminary data may help address the question of the individual contribution of hydrogen bonding and geometry to the overall fidelity of DNA polymerases.

SUPPLEMENTARY MATERIAL

Gel picture of PCR amplification in the presence of ψTTP, dATP, dGTP and dCTP. A total of 10 cycles were run and an aliquot was removed during each annealing phase.

See supplementary material in NAR Online.

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