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Chemical modification and blocking ratio assessment of Taq DNA polymerases using a 2,4,6-trinitrobenzenesulfonic acid (TNBS)-based assay

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ABSTRACT: Mainstreaming modification approaches of commercial hot-start Taq DNA polymerases include antibody modification, nucleic acid adaptor modification, and chemical modification, among which the chemical modification possesses merits of lower cost and higher efficiency excluding animal-derived matters compared with the former two. In chemical modification, the overall modification ratio of lysine residues is a key parameter to indicate the blocking ratio of modified enzyme without compromising its stability. In this study, 2,4,6-trinitrobenzenesulfonic acid (TNBS)-based assay was applied for chemically modified Taq DNA polymerase activity blocking ratio for the first time. The results showed that at the modification ratio of about 50%, the enzyme activity was completely blocked. This detection method's features were easy-operation and time-saving for the screening of optimized anhydride modified Taq DNA polymerase in multiple incubation conditions. Notably, this new protocol provided a practical reference for identification of novel chemical compounds that can modify DNA polymerases, thereby facilitating the development of advanced enzymes.

KEYWORDS: Taq DNA polymerase, TNBS-based assay, activity blocking ratio, chemical modification

INTRODUCTION

Taq DNA polymerase is the most widely used bio-enzyme that represents a central player in multiple PCR applications including qPCR, Taqman assay, digital PCR, and relevant nucleic acid detection contexts [1, 2]. Undesirable amplification occurs when the PCR system is prepared under ambient temperature. However, due to the fact that Taq DNA polymerase retains partial enzymatic activities, it leads to the non-specific binding of primers and the template [3]. With the increasing demand for specificity and accuracy in the nucleic acid assay field, scientists have developed the hot start PCR technology to abolish non-specific amplifications, which is said to be one of the most effective solutions. The principle of hot start PCR technology lies on a modified DNA polymerase that is inactive at low temperatures but initiates the reaction at high temperatures (i.e. above the primer annealing temperature) in conjunction or dissociation with certain modifying blocker, respectively [4].

Thus far, mainstreaming modification approaches of commercial hot start Taq DNA polymerases include antibody modification, nucleic acid adaptor modification, and chemical modification, among which the last one possesses merits of lower cost and higher efficiency excluding animal-derived matters compared with the former two. Anhydride serves as one of the most used chemical ligands to Taq DNA polymerases. In chemical modification, the inhibition of enzymatic activity at ambient temperatures is achieved by the conjunction of an optimal chemical ligand at lysine (Lys) residues of the DNA polymerase [5–7]. The chemical modification principle of Taq DNA polymerase is to use small molecule chemical groups to chemically react with the free amino group (positively charged) of the Lys residues in the enzyme, thereby blocking the polymerase activity of the enzyme [8]. The overall modification ratio of Lys free amino is a key parameter to indicate the blocking ratio of modified enzyme without compromise of its stability. However, existing methods for evaluating the effects of chemical modifications on DNA polymerases relied on the identification of PCR amplification product contents through agarose gel electrophoresis or fluorescence quantitative PCR. Consequently, the ratio of DNA polymerase activity blocked by compounds was indirectly inferred [9]. These methods were relatively cumbersome to operate and failed to reflect the proportion of Lys residues on the DNA polymerase that had been modified, which hindered the rapid screening of novel modifiers. Still, we lack a practical way to quantitatively analyze the binding rate of modifiers and the Taq DNA polymerase.

A concise but effective strategy to identify low-cost chemical modifiers of Taq DNA polymerase with excellent blocking efficiency other than compromising its polymerase amplification capacity has been urgently demanded in the bio-enzymatic research and

development. In this sense, we aimed to establish a rapid quantitative assay of Taq DNA polymerase chemical modification ratio, using TNBS, Under alkaline conditions (negative charge), a reaction occurs between the free amino group with positive charge on the amino acid, forming an intermediate complex. Taking advantage of the property that TNBS reacts with free amino groups, quantitative measurement of the corresponding amino acid can be performed at 420 nm (in alkaline solution) [10]. We found in the literature that the polymerase activity region of Taq polymerase only has free amino groups with positive charge on lys, and the exposed free amino groups can be used as targets for chemical modification of the enzyme [11]. Unmodified lys still has free amino groups, which can react chemically with TNBS and increase the absorbance at 420 nm.

This property enables us to use this reagent in the detection of chemical blocking ratio of Taq DNA polymerase. At first, a simple linear regression was plotted after the measurement of individual absorbance. Then, the chemical modification ratio was deduced by calculating the values of slope ratio between modified and unmodified proteins using the following equation: Modification ratio $= \left(1 - \frac{\text{slope}_{\text{modified Taq pol}}}{\text{slope}_{\text{unmodified Taq pol}}}\right) \times 100\%$. Given that the molecular weight of Taq DNA polymerase changes after tetrahydrobenzene anhydride (THPA) modification and the difference value could be recorded using a MALDI-TOF-MS assay [12], we designated another chemical modification ratio value as the ratio value of the above difference value and numbers of unmodified lys. This value was adopted as a reference to verify the efficiency and accuracy of the TNBS-based method as well as a testing factor associating with the blocking efficiency and amplification capacity of variant chemical modified Taq DNA polymerases.

MATERIALS AND METHODS

Preparation of hot-start Taq DNA polymerase

Hot-start Taq DNA Polymerase modified with THPA, Citraconic Anhydride (CTA, Sigma, St. Louis, MO, USA), and Maleic Anhydride (MAn, Sigma) was prepared. Firstly, the amount of acid anhydride to be added was calculated based on the mixing molar ratios of Taq DNA polymerase and the acid anhydride, which were 100:1, 200:1, 300:1, 400:1, 500:1, 600:1 and 700:1. The formula for calculating the amount of the acid anhydride to be added was as follows: V = mixing molar ratio × protein concentration × protein volume/94000 × 103/molar concentration of the acid anhydride solution. Then, a 0.5 M acid anhydride stock solution was prepared using dimethyl sulfoxide, and the acid anhydride was added to the protein solution. The mixture was incubated overnight at 4 °C with rotation at 100 rpm using a shaker. The incubation solution was centrifuged at 18,000*g* for 10 min at 4 °C, and the chemically modified protein was obtained by filtration through a 0.22 μM filter membrane.

Feasibility and accuracy of TNBS-based method measured chemical modification

In detail, 0.1% TNBS solution (Sigma) and 0.1 M CB solution containing sodium carbonate and sodium bicarbonate (Sinopharm, Beijing, China; pH 9.0) were prepared in advance as described in a previous report [13]. Lys reacts with TNBS through dehydrating reaction of the free amino group upstream of the lys molecule. They combine in a 1:1 ratio of free amino group to TNBS molecule. One molecule of Taq DNA polymerase has 42 free amino groups, and some of them will be blocked by the reaction with TNBS. By fixing the amount of TNBS added to the reaction system at 0.2 μM and changing the amount of Taq DNA polymerase added, the molar ratio of TNBS:Taq DNA polymerase was set to a range of 10:1–180:1, which corresponded to a range of 1×10^{-3} μM to 15×10^{-3} μM of added Taq DNA polymerase protein, with 1×10^{-3} μM as the starting point and increasing in increments of 1×10^{-3} μM for a total of 15 increments. In this experiment, we investigated the linear detection range of Taq DNA polymerase using TNBS. The enzyme-labeled instrument (Thermo Fisher Scientific, Waltham, MA, USA) was used according to the manufacturer's instructions, with a detection wavelength of 420 nm and a reaction temperature of 40 °C. A total of 60 μl of CB and 60 μl of 0.1% TNBS were added to a 180 μl reaction system. Taq DNA polymerase was added in an appropriate amount, and the reaction mixture was brought up to 180 μl with water. The mixed reaction system was added to a 96-well plate, and the appropriate wells were selected for testing. After a 30 min incubation period, the absorbance was read and the program was started to export the reaction results. The absorbance changes with increasing amounts of Taq DNA polymerase were analyzed linearly.

In a 180 μl reaction system, Taq DNA polymerase was modified with different molar ratios of THPA of 0.2, 0.4, 0.6, 0.8, and 1.0×10^{-3} μM. (The self-made Taq DNA polymerase was modified with an acid anhydride as aforementioned.) Taq-HS Polymerase (BestEnzymes Biotech, Lianyungang, China), a commercial chemical modified hot start Taq DNA polymerase, was included as the positive control. The experimental procedure was conducted as described above, and the linear equation for the relationship between the amount added and the change in absorbance was plotted. The degree of chemical modification was calculated using the formula. Based on this method, the linear equation for Taq DNA polymerase modified with tetrahydrofuran was plotted (Fig. S1a-b), and the degree of chemical modification was calculated

Table 1 Chemical modification ratio values obtained by TNBS-based and MALDI-TOF-MS-based methods.

Molar ratio of THPA to Taq DNA polymerase	Regression equation[a]	r[b]	TNBS-based method detected ratio[c]	MAIDI-TOF-MS detected ratio[d]
0	$y = 0.0279x + 0.0730$[f]	0.9944	0.000	0.000
100:1	$y = 0.0191x + 0.0790$[g]	0.9949	31.54	31.74 ± 0.21
200:1	$y = 0.0180x + 0.0083$[g]	0.9983	35.48	38.32 ± 0.72
300:1	$y = 0.0164x + 0.0913$[g]	0.9991	41.22	43.22 ± 1.15
400:1	$y = 0.0159x + 0.0905$[g]	0.999	43.01	46.99 ± 0.64
500:1	$y = 0.0137x + 0.1003$[g]	0.9972	50.90	51.95 ± 1.10
600:1	$y = 0.0139x + 0.0094$[g]	0.9994	50.18	51.66 ± 1.31
700:1	$y = 0.0136x + 0.0971$[g]	0.9993	51.25	52.18 ± 1.11
PC[e]	$y = 0.0135x - 0.0993$[g]	0.9973	51.61	53.39 ± 0.52

Quantifications are means of three replicates ± SE. [a] y = absorbance, x = sample concentration (1×10^{-3} µmol); [b]coefficient of determination; [c]Modification ratio $= \left(1 - \frac{\text{slope}_{\text{modified Taq pol}}}{\text{slope}_{\text{unmodified Taq pol}}}\right)\times 100\%$; [d]Modification ratio $= \frac{\text{MW}_{\text{modification Taq pol}} - \text{MW}_{\text{unmodification Taq pol}}}{42\times \text{MW}_{\text{THPA}}} \times 100\%$; [e] Taq HS polymerase; [f] slope unmodified Taq pol; and [g] slope modified Taq pol.

(Table 1).

Subsequently, the MALDI-TOF-MS-based determination were included to bolster the TNBS-based method. The molecular weights of Taq DNA polymerase modified with different molar ratios of THPA using MALDI-TOF-MS was detected consistent with the samples detected by TNBS method. At first, we prepared 10 mg/ml 2,5-dihydroxybenzoic acid (DHB) by dissolving 10 mg DHB (Thermo Fisher Scientific) in eppendorf tubes containing 1 ml 50% acetonitrile (Thermo Fisher Scientific). 1.0 µl/µg sampling solution and 1.0 µl erucic acid (SA, Thermo Fisher Scientific) solution were added and mixed. The mixtures were then evaporated to dry at room temperature. The MS (Shimadzu, Kyodo, Japan) setting parameters were cation – reflection model, laser intensity at 5500, detector voltage at 0.75 V, 10000–200000 m/z.

The relationship between the chemical modification ratio of Taq DNA polymerase measured by TNBS method and the activity

Fluorescence quantitative method was used to detect the blocking rate of Taq DNA polymerase with chemical modification degree >50%. A 20 µl reaction mixture consisted of 2.5 µl 10 µM T2-5′ TAGCGAAGGATGTGAACCTAATCCCTGCTCCCGCGGC CGATCTGCCGGCCGCGGGAGCA-3′ (General Bio, Chuzhou, China) as the template [14], 5 µg modified Taq DNA polymerase, 2 µl 10 × Taq reaction buffer (BestEnzyme Biotech, Lianyungang, China), and 0.5 µl 10 mM dNTP (Vazyme, Nanjing, China), and 1 µl 200 × Picogreen. The commercial Taq-HS DNA polymerase (BestEnzymes Biotech) and unmodified Taq DNA polymerase were used as positive and negative controls, respectively, while NTC was a no template control. The reaction was run using CFX Connect Real-Time System (BioRad, California, USA) at 55 °C for 2 min in 5 rounds of cycles before reading the RFU value, which was regarded as the block rate [14].

Functional detection of the chemical modification ratio of Taq DNA polymerase

According to the method provided in the instruction manual of Taq-HS DNA polymerase (BestEnzymes Biotech), whether the Taq DNA polymerase modified by three anhydrides could be effectively used in the TaqMan method was investigated. The sequences of primer pairs were 5′-CGAAATCGGTAGACGCTACG-3′ (forward) and 5′-TCCACGTCTCTGACTTSCCTT-3′ (reverse), while the probe sequence is 5′-GCAATCCT GAGCCAAATCC-3′ (General Bio). The 20 µl qPCR reaction system consisted of 1 µl tomato genomic DNA of different amounts (3, 30, and 300 pg), 2 µl 10 × Taq reaction buffer, 0.4 µl 10 mM dNTP, 1 µl modified Taq DNA polymerase of each type (5 U/µl), 1 µl of each primer (4 µM), and 1 µl probe (5 µM). The running procedure was 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s and 60 °C for 60 s. Taq-HS DNA polymerase was included as the positive control, and Taq DNA polymerase was the negative control. The efficiency values of amplification were calculated using ABI Step I software.

RESULTS AND DISCUSSION

Detection range and feasibility analysis of TNBS method

Fig. 1a illustrates that as the concentration of Taq DNA polymerase reacting with TNBS increased, the absorbance exhibited a unidirectional upward trend. However, a good linear relationship between absorbance values and the concentration of Taq DNA polymerase was observed only within the range from 2×10^{-3} µM to 10×10^{-3} µM. Within this linear range, the addition of 0.2, 0.4, 0.6, 0.8, and 1.0×10^{-3} µM of Taq DNA polymerase still showed a good linear relationship with the change in absorbance, $R^2 = 0.9908$.

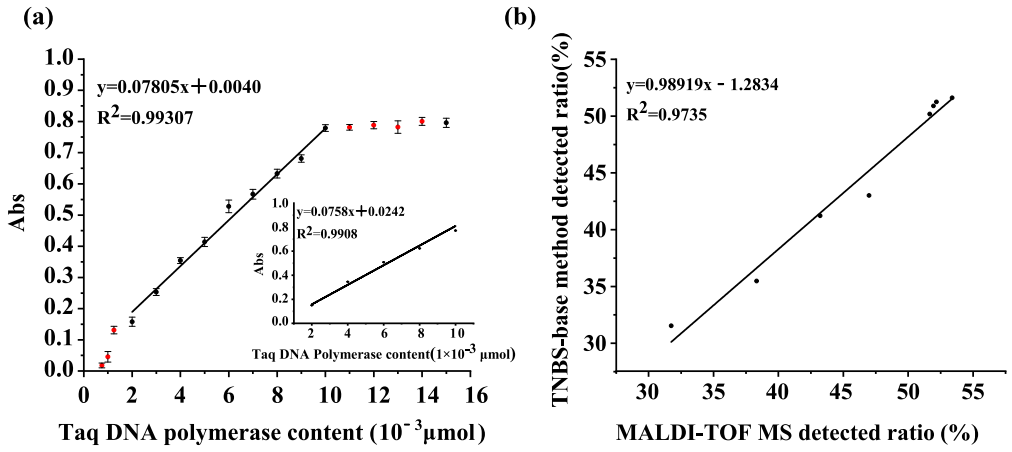

Fig. 1 Detection range and feasibility analysis of TNBS method. (a), Linear regression analysis of the addition of Taq DNA polymerase and the change of absorbance; (b), Linear regression analysis of chemically modified ratio based on TNBS and MALDI-TOF-MS method. x-axis represents TNBS-based method detected ratio; y-axis represents MALDI-TOF-MS detected ratio, $y = 0.9880x + 2.317$, $R^2 = 0.9773$.

Literature review showed that the TNBS method could detect amino acids in the range of 0.05–0.4 µM [15]. Since Taq DNA polymerase contains 42 Lys residues, $2 \sim 10 \times 10^{-3}$ µM of Taq DNA polymerase corresponds to 0.084∼0.42 µM of Lys, which matched the range provided in the literature [16]. Therefore, the range of TNBS detection for Taq DNA polymerase was determined to be from $2{\times}10^{-3}$ µM to $10{\times}10^{-3}$ µM.

By using the linear equation of the change in absorbance of Taq DNA polymerase protein amount with TNBS-modification at the five selected points, the modification ratio was calculated using the ratio of the amount of the product of the reaction between the free amino group of Lys and TNBS for the modified Taq DNA polymerase to that for the unmodified Taq DNA polymerase. The formula for the modification ratio was Modification ratio $= \left(1 - \frac{\text{slope}_{\text{modified Taq pol}}}{\text{slope}_{\text{unmodified Taq pol}}}\right) \times 100\%$. The chemical modification degree was then calculated based on this formula.

MALDI-TOF-MS is the most classic methods used to determine the molecular mass of Taq DNA polymerase [17–19]. In this study, MALDI-TOF-MS was used to measure the molecular weight of Taq DNA polymerase before and after THPA modification. The theoretical chemical modification degree was calculated by directly comparing the difference in molecular weight of the DNA polymerase before and after chemical modification to the ratio of the number of free Lys amino groups (Table 1). Linear regression analysis was performed to compare and verify the accuracy and feasibility of using TNBS method to measure the chemical modification degree of Taq DNA polymerase, and the results were compared with those obtained from the traditional MALDI-TOF-MS method. According to Fig. 1b, the two methods showed a good linear relationship, $R^2 = 0.97$, demonstrating that the TNBS method had a good linear fit with the traditional method.

The correlation between chemical modification degree and blocking ratio measured by TNBS method

Fig. 2a shows that when the mixed molar ratio of THPA to Taq DNA polymerase was greater than 500:1, the chemical modification degree detected by TNBS method did not change and remained at 50%. This indicated that all the exposed free amines of Taq DNA polymerase had bound to THPA. The blocking rate of Taq DNA polymerase was detected using a fluorescence quantitative method (Fig. 2b). Since the polymerization activity of Taq DNA Polymerase was not closed in the positive control system, T2 was amplified, emitting fluorescence and producing a higher RFU value. In the negative control system, there was no polymerase, and T2 could not be amplified. We used the RFU value of the negative control as the blank background. If the fluorescence curve RFU value is close to the negative control, it indicates that the polymerase activity is closed, and there is no amplification. The reaction fluorescence curve RFU value of Taq DNA polymerase modified with THPA (chemical modification degree >50%) was very close to the negative control, indicating that T2 did not amplify in the system, and the polymerase activity was completely closed with a blocking rate >99%.

We found that when the chemical modification degree was >50%, the polymerization activity of Taq

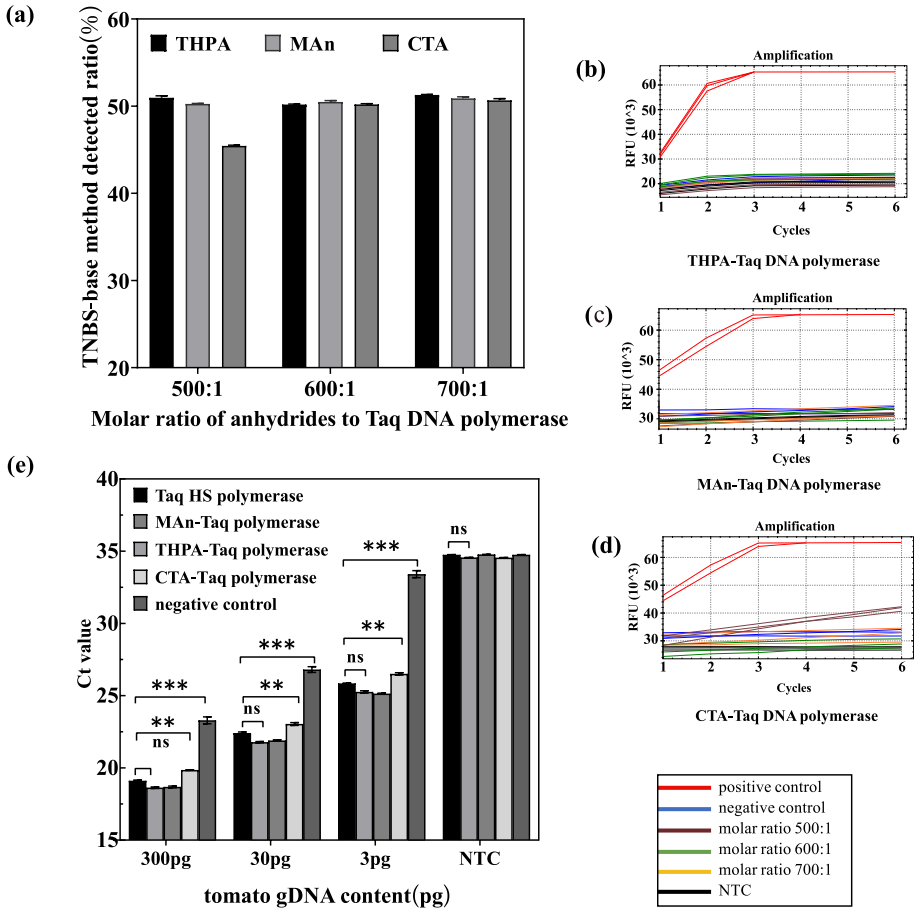

Fig. 2 Activity and function assays of Taq DNA polymerase. (a), Histogram showing chemical modification ratios; x-axis represents different reaction systems at a variety of molar ratios of anhydrides and Taq DNA polymerases; y-axis represents chemical modification ratio values. (b, c, and d), qPCR assays of different modified Taq DNA polymerases using three types of anhydrides. In panels 2(b-d), x-axis represents reaction cycles, and y-axis represents Fluorescence value (RFU). (e), three types of anhydride modified Taq DNA polymerase remain capable in the TaqMan assay application to distinct extents; x-axis represents tomato gDNA contents; and y-axis represents Ct values. ** $p < 0.01$, significant difference; *** $p < 0.001$, highly significant difference; ns, no significant difference.

DNA polymerase was completely closed with a blocking rate >99%. To verify this idea, we used two other anhydrides to modify Taq DNA polymerase with mixed molar ratios of 500:1, 600:1, and 700:1. TNBS and fluorescence quantitative methods were used to detect the chemical modification degree (Fig. S1b) and activity, respectively. Thorough analyses of the association between the chemical modification ratios and the blocking rates were conducted, and the results were shown in Fig. 2a-d. When the mixed molar ratio of Taq DNA polymerase and MAn or THPA was ⩾500:1, the chemical modification degree was ⩾50%, and the blocking rate of the enzyme was >99%. When Taq DNA polymerase was mixed with CTA at a molar ratio of ⩾600:1, the chemical modification degree was >50%, and the blocking rate of the enzyme was >99%.

By analyzing the relationship between the degree of chemical modification and the blocking rate of Taq DNA polymerase modified with three types of anhydride, it was ultimately determined that when the degree of chemical modification was greater than 50%, the enzyme's blocking rate was >99%.

Functional testing was performed on Taq DNA polymerase modified with three types of acid anhydride (chemical modification degree ⩾50%) (Fig. 2e). We also investigated whether the function of the Taq DNA polymerase unblocking after acid anhydride modification with a chemical modification degree of ⩾50% was affected by the acid anhydride. Taq DNA polymerase modified with MAn and THPA showed amplification function for different concentrations of tomato gDNA that was consistent with commercially available chemically modified hot-start Taq DNA polymerase. However, the CTA of Taq DNA polymerase modified

with citraconic anhydride was weaker than that of the commercially available chemically modified hot-start Taq DNA polymerase. Hence, it was speculated that a high molar ratio of acid anhydride and Taq DNA polymerase could reduce the enzyme's activity [8, 20].

CONCLUSION

In summary, the results suggested that the TNBS-based and MALDI-TOF-MS-based were comparable methods in quantitative determination of modification ratios of chemical modified Taq DNA polymerases. The TNBS-based method could be used to detect the activity blocking ratio of Taq DNA polymerase.

When the modification ratio was about 50%, the enzyme activity was completely blocked. The method was cost savings and easier to operate than the qPCR assay, and the modified Taq DNA polymerase activity could be detected rapidly. Particularly, it was recommended to use TNBS-based method to quickly screen the most optimized anhydride modified Taq DNA polymerase in multiple incubation conditions, providing a practical reference for identification of novel chemical compounds that can modify DNA polymerases, thereby facilitating the development of advanced enzymes.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2024.015.

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Appendix A. Supplementary data

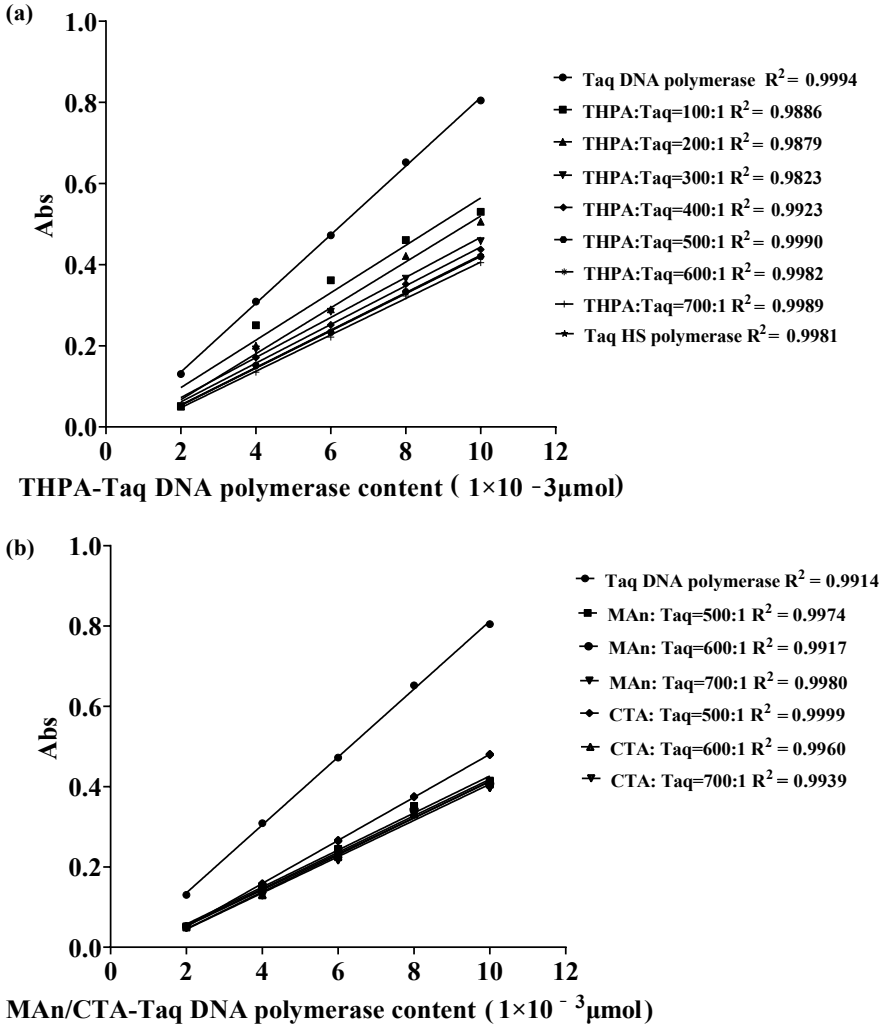

Fig. S1 TNBS-based method detected the chemical modification of Taq DNA polymerase. (a), Linear fitting graph of THPATaq DNA polymerase; (b), Linear fitting graph of CTA/MAnTaq DNA polymerase.