1. Materials and Methods

1.1 Construction of DNA Template on the Streptavidin Magnetic Beads

The overall scheme for constructing DNA template immobilized on streptavidin magnetic beads is shown above. (i) Resuspend the magnetic beads in the vial (or vortex for 20 s), transfer 100 μL of streptavidin magnetic beads into a 1.5 mL tube. Place the tube into a magnetic stand to collect the beads against the side of the tube. Remove and discard the supernatant. (ii) Add 1 mL Buffer I (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 M NaCl, 0.01%-0.1% Tween-20) to the beads, invert the tube several times or vortex gently for 15 s to mix. Remove and discard the supernatant from magnetic separation. Repeat this step for 2 times. (iii) Add 500 μL of biotinylated DNA template diluted with Buffer I, makes the beads at a final concentration of 2 mg/ml. Rotate the tube for 30 min at room temperature or 2 h at 4ºC. (iv) Separate the biotinylated DNA template coated beads with a magnetic stand. (v) Add 1 mL Buffer I to the beads, invert the tube several times or vortex gently for 15 s to mix. Remove and discard the supernatant from magnetic separation. Repeat this step for 2 times. (vi) Binding is now complete. Resuspend the beads in a buffer at a desired concentration with a low salt concentration, suitable for downstream applications. Use the beads immediately, or store at 4ºC for late use.

1.2 Detection of the reaction rates of fluorophore-labeled nucleotides on a chip

The primer (SP) was hybridized with the DNA template (T1) immobilized on the chip, and a 10 μL solution containing 200 μM dGTP-Cy5 or dGTP-N_3-Cy5, 1 unit 9°N DNA polymerase, and 20 nM $MnCl_2$ was added. The extension reaction was carried out at 60℃ for varying durations of 10 s, 20 s, 40 s, 60 s, and 100 s. Subsequently, the chip was washed with 2×SSC,1% SDS for 5 min, followed by washing with 0.2×SSC, 0.1% SDS for 5 min. After rinsing with dH_2O, the chip was scanned using a fluorescence scanner.

1.3 Polymerase extension with fluorophore-labeled nucleotides on a chip

The primer (SP) was hybridized with the DNA template (T1) immobilized on the chip, and a 10 μL solution containing 200 μM dGTP-Cy5 or dGTP-N_3-Cy5, 1 unit 9°N DNA polymerase, and 20 mM $MnCl_2$ was spotted on the DNA chip and incubated for 20 s at

60℃ (2). The chip was then washed with 2×SSC,1% SDS for 5 min, followed by washing with 0.2×SSC, 0.1% SDS for 5 min. After rinsing with dH_2O, the chip was scanned using a fluorescence scanner.

1.4 Decoding algorithm

For a single sequencing run with dual-mononucleotide addition, a set of two-digit strings ($N^1M^1, N^2M^2, N^3M^3, ..., N^kM^k$) is obtained sequentially. Assuming that conjugated mixes X'Y*' and W'Z*' are alternately introduced to react with template in each sequencing cycle, and a two-digit string N^iM^i is obtained in i cycle. The decoding algorithm, converting the two-digit strings into base-encoding, is as follow:

(1) if $N^i > 0, M^i = 0, i = 1,2,...,k-1$, there are $N^i - 1$ base(s) X and one base Y.

(2) if $N^i > 0, M^i = 0, i = k$, there are $N^i - 1$ base(s) X and an encoding (XY).

(3) if $N^i \geq 0, M^i > 0, i = 1,2,...,k$, there are N^i base(s) X, $M^i - 1$ base(s) W and an encoding (WZ).

(4) if $N^i \geq 0, M^i > 0, N^{i+1} = 0, M^{i+1} > 0, i = 1,2,...,k-1$, there are N^i base(s) X, $M^i - 1$ base(s) W and one base Z.

2. Results and Discussion

2.1 DNA polymerase screening

To identify DNA polymerases compatible with 3'-O-azidomethyl reversible terminators, we tested four types of DNA polymerases (Therminator DNA polymerase, T7 DNA polymerase, Phusion DNA polymerase, and Klenow), along with an additional 9°N DNA polymerase provided by Huang Lab. The polymerase extension reactions were carried out using the 3'-O-azidomethyl reversible terminators. Results showed minimal signal at position S when using T7 DNA polymerase, Phusion DNA polymerase, and Klenow, indicating no effective polymerization of the reversible terminators. Therminator DNA polymerase showed a slight signal at position S, and subsequent sequencing reactions confirmed that the first base matched the template

base A, indicating partial incorporation of the reversible terminators. In contrast, using 9°N DNA polymerase for single nucleotide incorporation showed a strong signal at position S, with subsequent sequencing reactions being terminated, demonstrating effective incorporation of reversible terminators into the synthetic chain and termination of further nucleotide extension. Therefore, we selected 9°N DNA polymerase for our sequencing experiments.

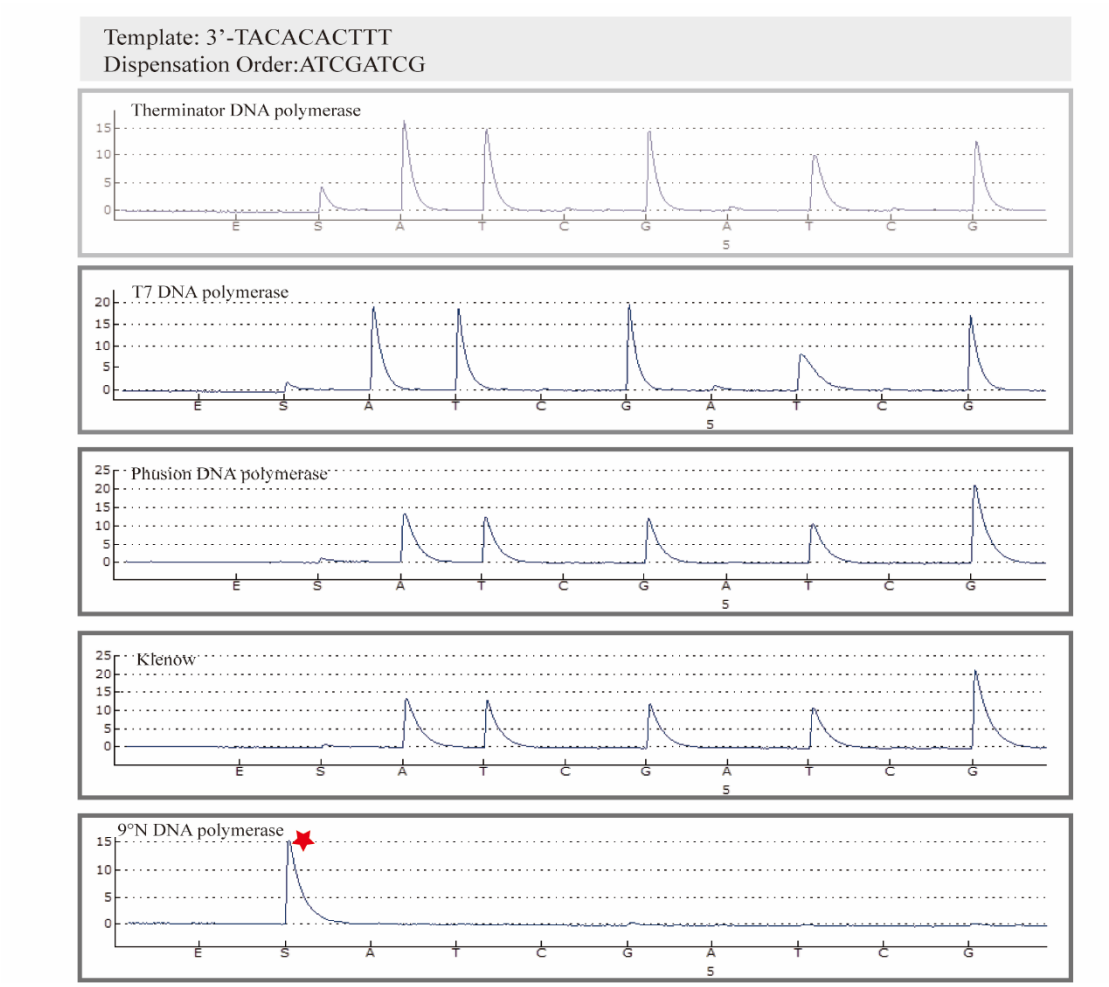

Fig.S1 Efficiency comparison of different DNA polymerases. 9°N DNA polymerase effectively incorporates reversible terminators into the synthetic chain and subsequent nucleotide extension reactions is terminated.

2.2 The optimization of enzymatic reaction

Through exploration of reaction temperature and enzyme concentration, we ultimately selected the enzymatic reaction conditions as follows: 1 unit of 9°N DNA polymerase reacting at 60°C for 20 s.

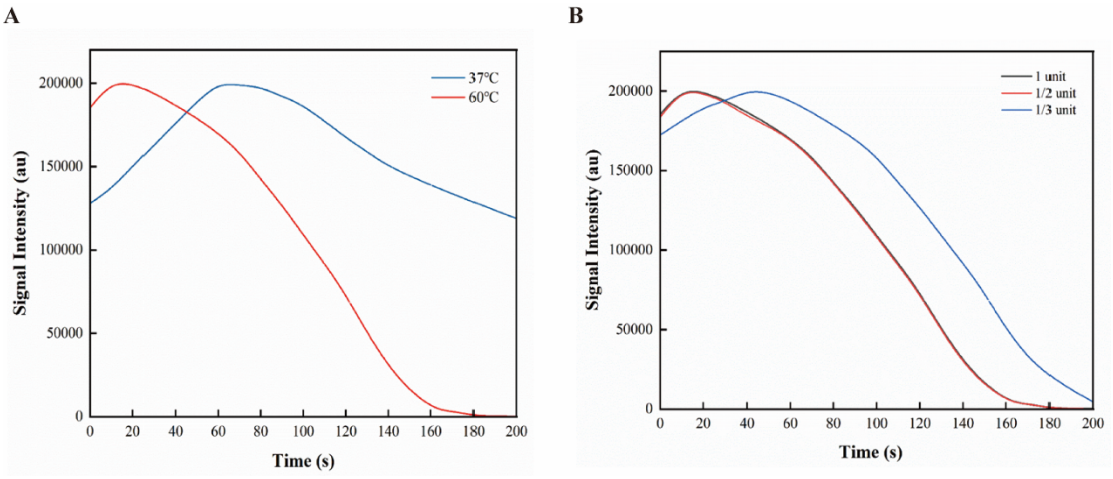

Fig.S2 The optimization of enzymatic reaction. (A) The enzymatic activity of DNA polymerase at different temperature. **(B)** The enzymatic activity of DNA polymerase at different concentration.

2.3 Reaction rates of the four different types of nucleotides

The results presented in Fig. S3 demonstrate that natural nucleotides achieved complete reaction in approximately 2 s, while unlabeled 3’ blocked nucleotides required about 15 s to reach complete reaction. This indicates that both natural nucleotides and unlabeled 3’ blocked nucleotides can completely react under 9°N DNA polymerase. When these two types of nucleotides are present at equivalent concentrations, the reaction rate of natural nucleotides is slightly faster than that of unlabeled 3’ blocked nucleotides, with both types completing within 20 s.

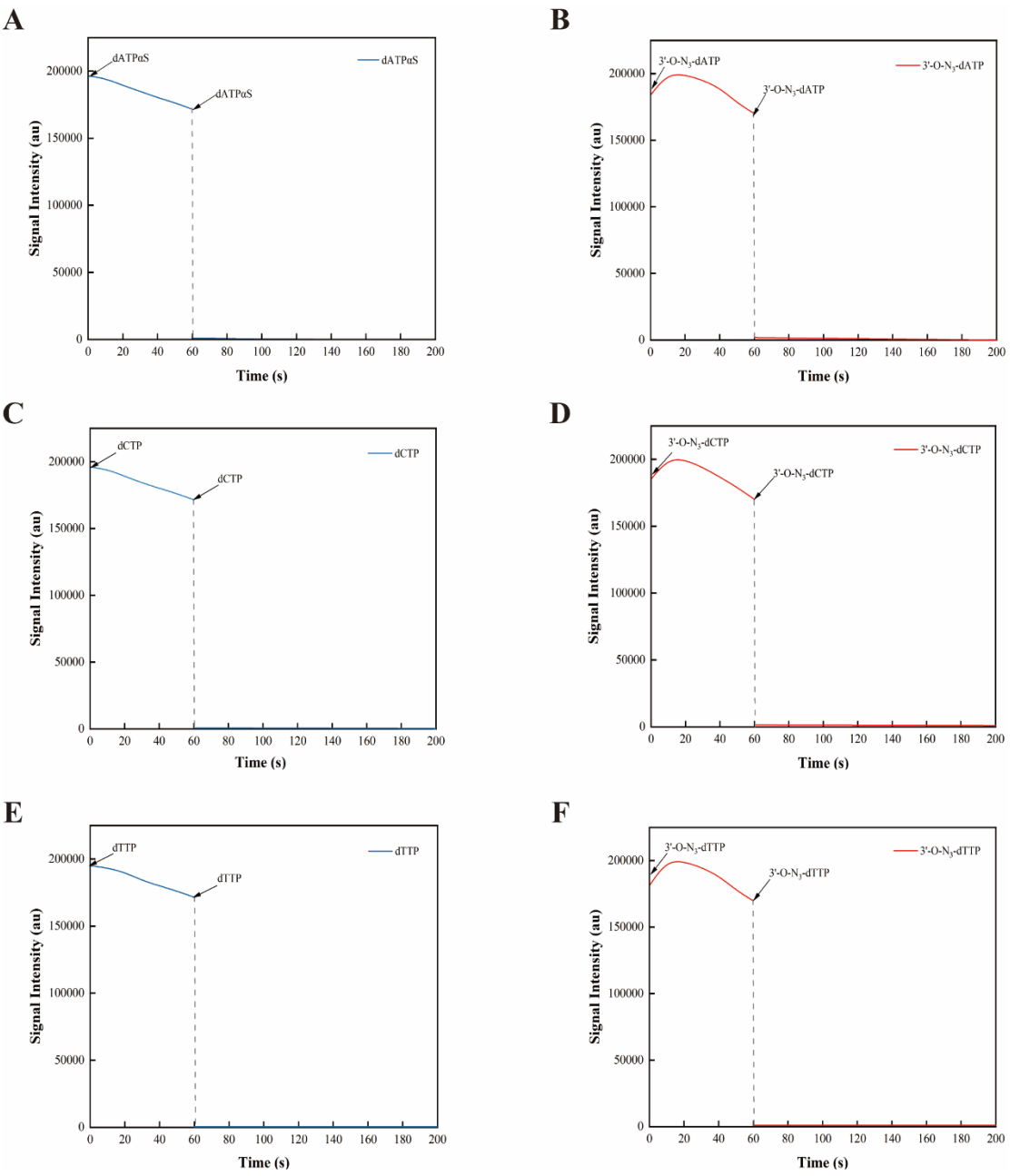

Fig.S3 Reaction rates of natural nucleotides and labeled 3' blocked nucleotides. The instrument recording time lags behind the actual reaction time by 2~3 s. **(A)** dATPαS. **(B)** 3'-O-N_3-dATP. **(C)** dCTP. **(D)** 3'-O-N_3-dCTP. **(E)** dTTP. **(F)** 3'-O-N_3-dTTP.

Fig. S4 illustrates the reaction rates of fluorophore-labeled unblocked nucleotides and fluorophore-labeled 3' blocked nucleotides under 9°N DNA polymerase. Despite the labeled nucleotide being slower by approximately 10 s compared to the corresponding unlabeled one, both types of labeled nucleotides can almost completely react within a short time (approximately 20 s).

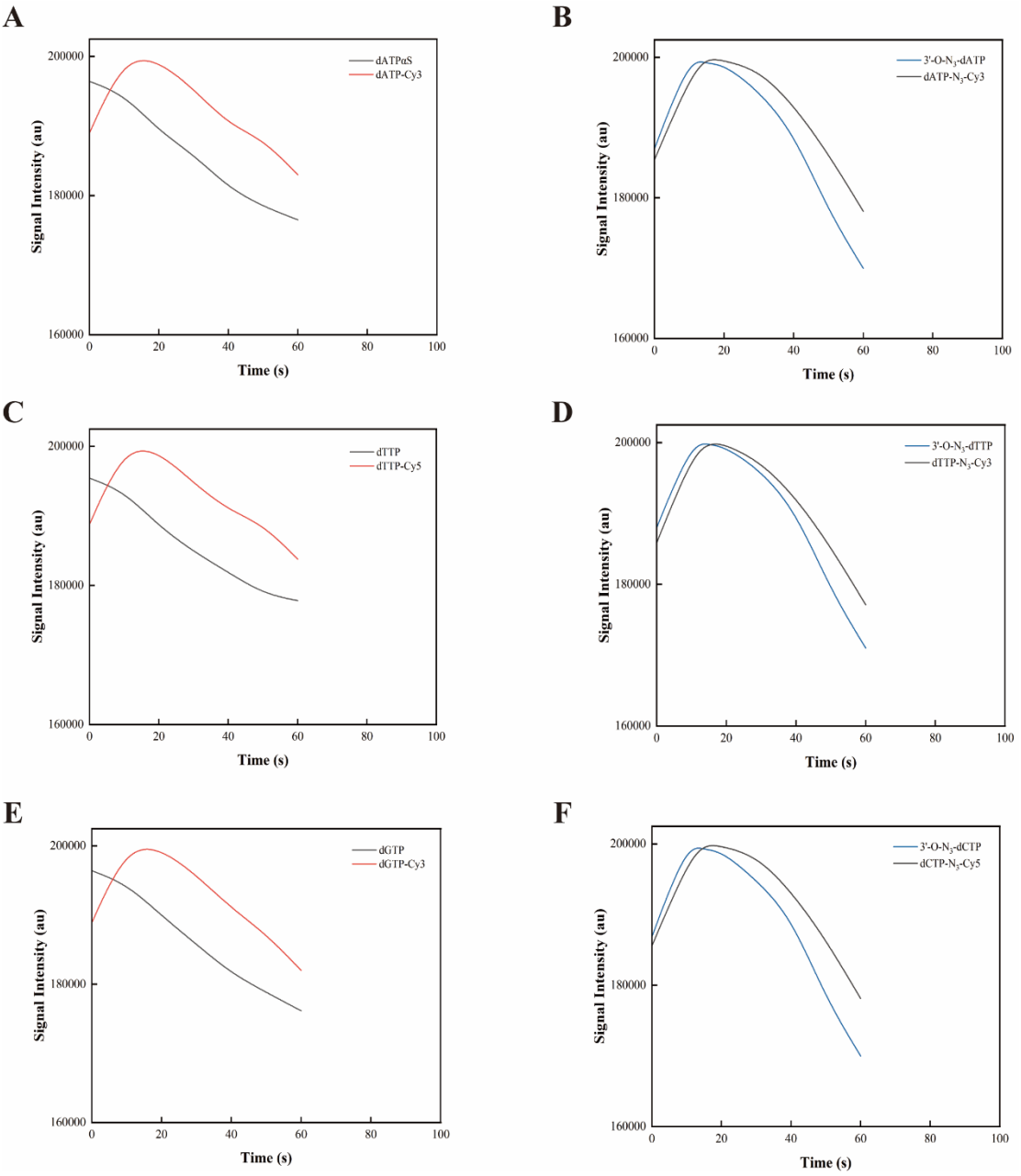

Fig.S4 Reaction rates of labeled unblocked nucleotides and labeled 3' blocked nucleotides. The instrument recording time lags behind the actual reaction time by 2~3 s. **(A)** dATP-Cy3. **(B)** dATP -N_3- Cy3. **(C)** dTTP-Cy5. **(D)** dTTP -N_3- Cy3. **(E)** dGTP-Cy3. **(F)** dCTP -N_3- Cy5.

2.4 Reaction rates of labeled nucleotides on DNA chip

We investigated the efficiency of nucleotide extension by 9°N DNA polymerase using two types of fluorophore-labeled nucleotides on a DNA chip. As shown in Fig. S5, the fluorescence signal generated by the nucleotide extension reaction steadily increased with reaction time. However, once reaching its peak intensity, the signal plateaued, indicating completion of the extension reaction at this point. Comparison of the time

required for complete extension between the two types of labeled nucleotides reveals that the reaction rate of labeled 3' unblocked nucleotides under 9°N DNA polymerase is slightly faster than that of labeled 3' blocked nucleotides, with both rates being very similar. Both types of nucleotides reached their signal peaks at approximately 25 s, signifying complete extension. Therefore, for the correctable two-color fluorogenic DNA decoding sequencing, we ultimately selected the polymerase extension conditions as follows: reaction at 60°C for 20 s.

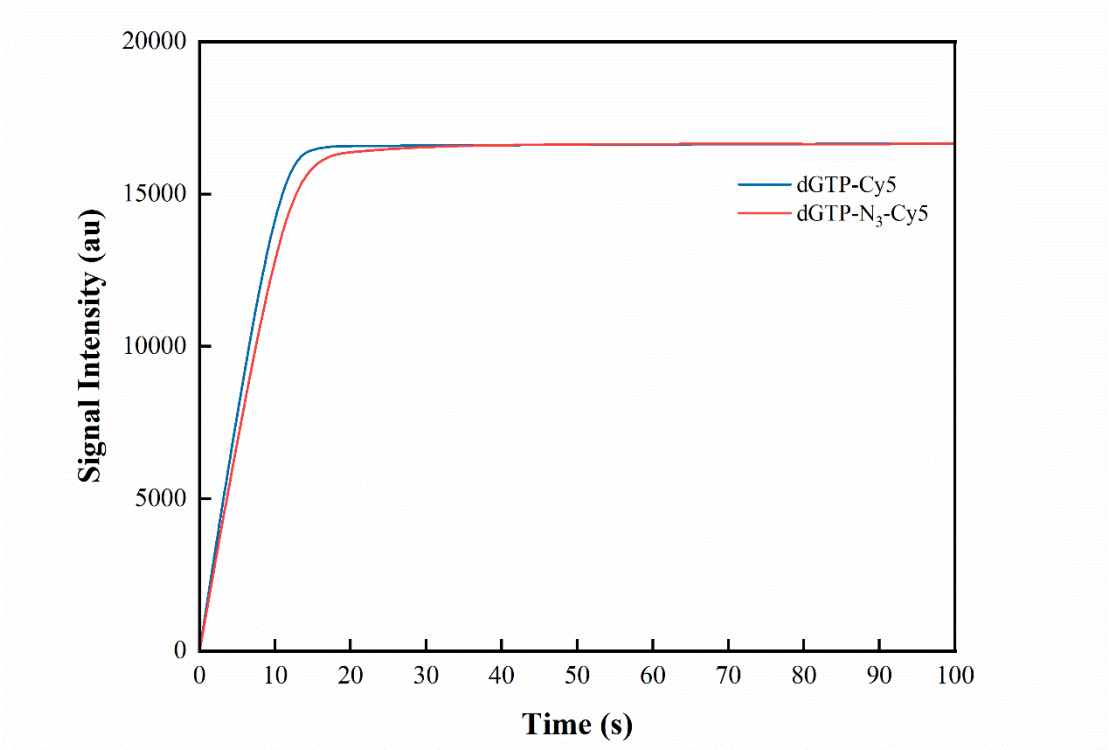

Fig. S5 The reaction rates of two different types of labeled nucleotides. dGTP-Cy5 and dGTP-N3-Cy5 were added for nucleotide extension reaction on the DNA chip, and the reaction temperature was 60 ℃.

2.5 Polymerase extension with fluorophore-labeled nucleotides on a chip

We investigated the polymerase extension reaction under 9°N DNA polymerase using two types of fluorophore-labeled nucleotides on a DNA chip. For a DNA template with the starting sequence CGTACGCT, in the first experiment, dGTP-Cy5 was added, and DNA polymerase incorporated dGTP-Cy5 into the synthetic chain, pairing with the first base C of the template and generating a fluorescence signal. In the second experiment, dGTP-N_3-Cy5 was added, and similarly, under DNA polymerase, dGTP-N_3-Cy5 was

incorporated into the synthetic chain, producing a fluorescence signal. This demonstrates that both types of labeled nucleotides can be accurately incorporated in a base-specific manner in the polymerase reaction. By comparing the fluorescence signals generated from extending a single nucleotide with these two types of labeled nucleotides, we found that the fluorescence intensity of the labeled unblocked nucleotides is slightly higher than that of the labeled 3' blocked nucleotides. This suggests that labeled unblocked nucleotides have slightly higher reaction efficiency than labeled 3' blocked nucleotides, although their produced fluorescence signals are very close (Fig. S6). Therefore, the method of simultaneously incorporating these two types of labeled nucleotides to record sequencing information is feasible.

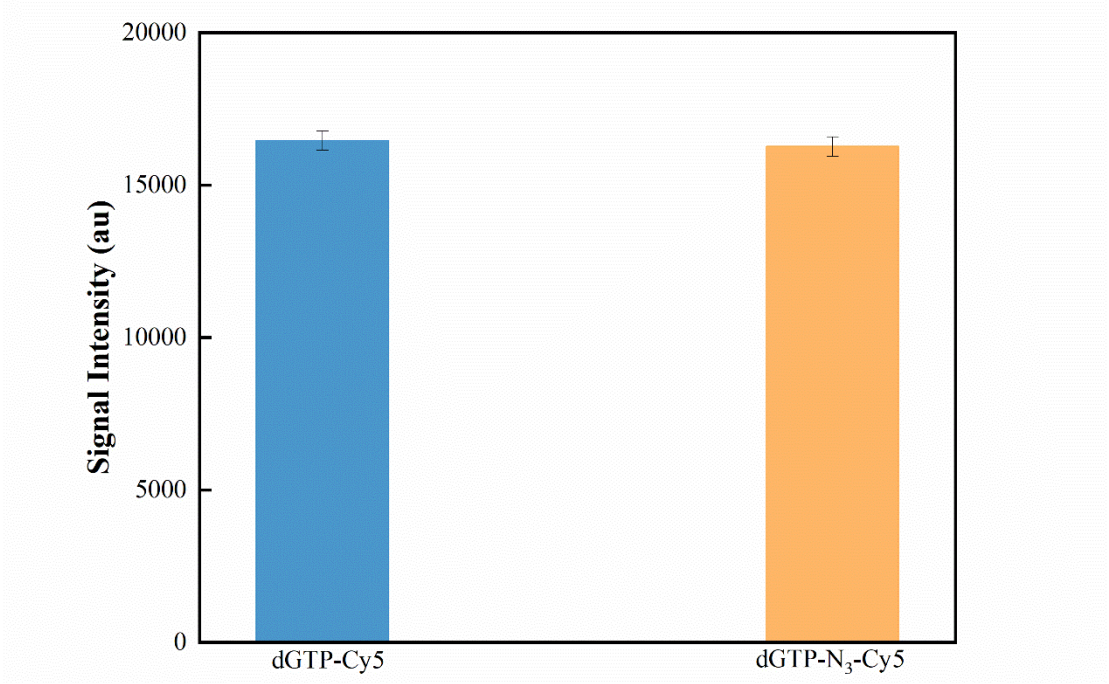

Fig. S6 Polymerase extension with fluorophore-labeled nucleotides on a chip. dGTP-Cy5 and dGTP-N3-Cy5 were added for nucleotide extension reaction on the DNA chip, and then the fluorescence signal was scanned.

2.6 The optimization of cleavage reaction

In order to optimize the cleavage reaction conditions, we investigated the optimal parameters using fluorophore-labeled 3' blocked nucleotides. The completeness of the cleavage reaction was assessed by monitoring fluorescence intensity along the extension chain. Fig. S7 shows our study on optimal cleavage reaction conditions using template T1 for sequencing. We treated the fluorophore-labeled 3' blocked nucleotides

with TCEP at 37°C, 50°C, and 60°C to remove their protective groups. As the reaction time increased, the detected fluorescence signal gradually weakened, and when the signal intensity decreased to background levels, it indicated completion of the cleavage reaction. Comparing the rates of fluorescence signal decay at 37°C, 50°C, and 60°C revealed a more rapid decrease in fluorescence signal at 60°C, indicating faster cleavage at this temperature. Therefore, we determined that the optimal reaction condition was at 60°C for 5 min.

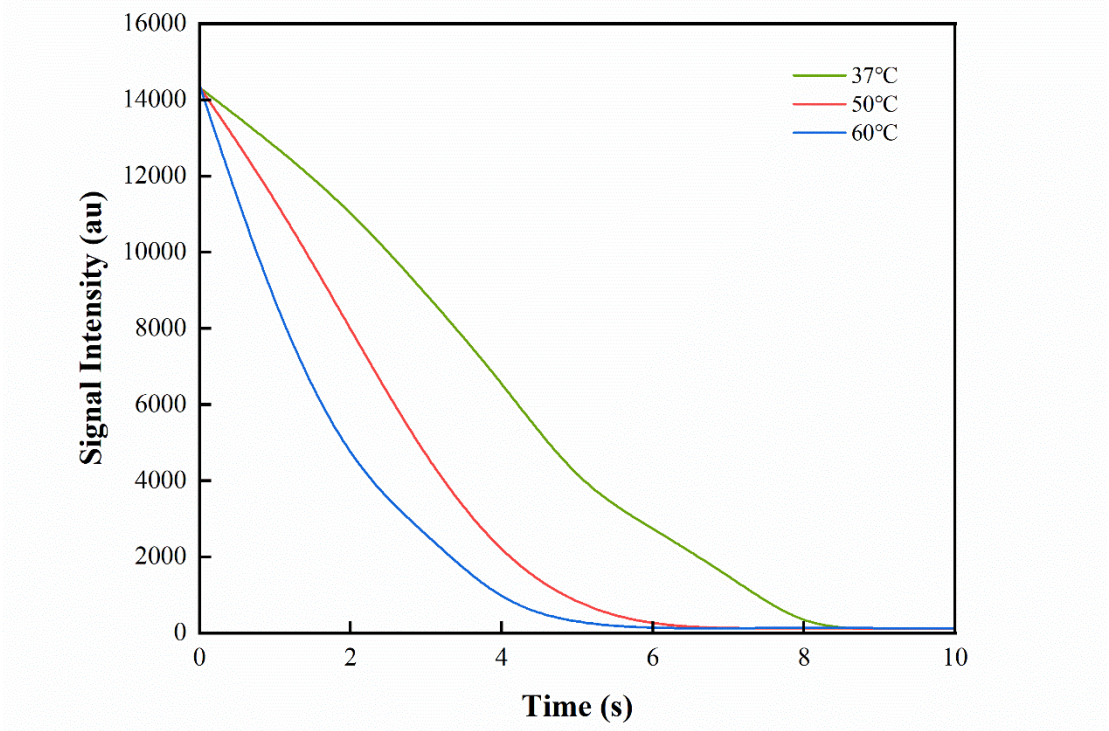

Fig. S7 The optimization of cleavage reaction.

2.7 Sequencing of homopolymer

For any homopolymer regions, they can be extended exclusively in at least one of the two cycles. So, there is always a sequencing cycle that the homopolymer is encoded in such a way that only one base information can be measured per reaction cycle, so every homopolymer can be extend exclusively in at least one of the two cycles. Moreover, in the correctable two-color fluorogenic DNA decoding sequencing, as long as a single sequencing cycle can distinguish between 1 and multiple nucleotides (2 or more), the true sequence can be identified by single-base sequencing of the 'homopolymer region' using the second set of sequencing data. To determine the number of nucleotides

extended in ambiguous reactions, we generally follow the principle that the relationship between the number of extended nucleotides and intensity tends to introduce deletions rather than insertions (meaning we prefer to read one less base rather than one more: deleted bases can be supplemented by comparing the results of two parallel sequencing runs; in a reaction where n nucleotides are extended, there must be n single-nucleotide extensions or (n-1) single-nucleotide extensions and one double-nucleotide extension at the same position in the other sequencing run. Insertion errors, however, are difficult to eliminate).

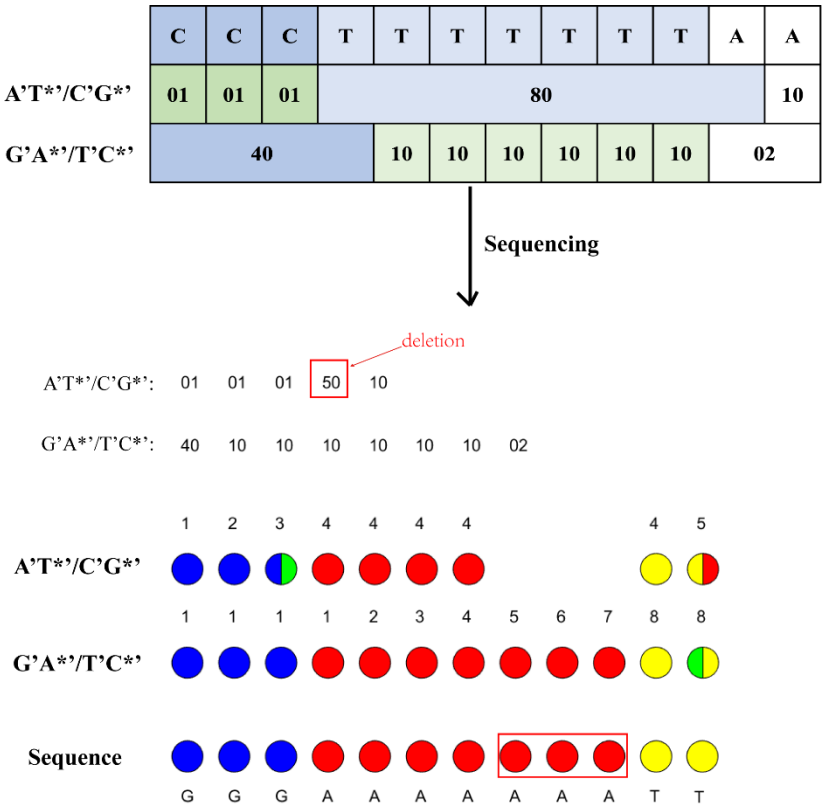

Fig. S8 Sequencing of homopolymer with two different sequencing runs.

Table S1. Primer and templates used in used in this study

Template	Sequence (5'-3')
T1	5'-NH2-TCAGTGACCGACGACTGTTATCTCGCATGCAGATGATCCAAATTGCAC
T2	5'-NH2-TCAGTGACCGACGACTGTTATCTCGCATGTAGATGATCCAAATTGCAC
T3	5'-NH2-TCAGTGACCGACGACTGTTATCTCATGCCCAGATGATCCAAATTGCAC
T4	5'-NH2-TCAGTGACCGACGACTGTTATCTAGCCCCCAGATGATCCAAATTGCAC
T5	5'-NH2-TCAGTGACCGACGACTGTTATTGCCCCCCCAGATGATCCAAATTGCAC

SP	CAATTTGGATCATCT
F-P[5301]	CAATTACCCACATAGGATGAA
R-P[5301]	Biotin-AGGCGTAGGTAGAAGTAGAGGTT
S-P	CAAATGGGCCATTATCGAAGAATTCACAAAAAAC

References

1. J. Wu, S. Zhang, Q. Meng, H. Cao, Z. Li, X. Li, S. Shi, D.H. Kim, L. Bi, N. J. Turro, J. Ju. 3'-O-modified nucleotides as reversible terminators for pyrosequencing, P. Natl. Acad. Sci. USA, 2007, **104,** 16462-16467.