**De novo DNA synthesis using polymerase-nucleotide conjugates**

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Oligonucleotides are almost exclusively synthesized using the nucleoside phosphoramidite method, even though it is limited to the direct synthesis of ~200 mers and produces hazardous waste. Here, we describe an oligonucleotide synthesis strategy that uses the template-independent polymerase terminal deoxynucleotidyl transferase (TdT). Each TdT molecule is conjugated to a single deoxyribonucleoside triphosphate (dTTP) molecule that it can incorporate into a primer. After incorporation of the tethered dNTP, the 3′ end of the primer remains covalently bound to TdT and is inaccessible to other TdT–dTTP molecules. Cleaving the linkage between TdT and the incorporated nucleotide releases the primer and allows subsequent extension. We demonstrate that TdT–dTTP conjugates can quantitatively extend a primer by a single nucleotide in 10–20 s, and that the scheme can be iterated to write a defined sequence. This approach may form the basis of an enzymatic oligonucleotide synthesizer.

The overwhelming majority of biological research and bioengineering requires synthetic DNA, including oligonucleotides (oligos) and longer constructs such as synthetic genes and even entire chromosomes1,2. Massively parallel oligo synthesis3 has dramatically reduced the cost of high-throughput and genome-wide functional screens4 and target capture for next-generation sequencing (NGS). De novo DNA synthesis also enables other emerging applications such as DNA nanotechnology5 and DNA-based data archiving6.

Today, essentially all synthetic DNA is manufactured using the nucleoside phosphoramidite method pioneered by Marvin Caruthers and colleagues over 35 years ago7 — a development that marked an inflection point in biological research8. However, after decades of fine-tuning and improvements in liquid handling, the upper limit of phosphoramidite-based oligo synthesis is now about 200–300 nt, in practice9. As a result, longer molecules must be assembled from oligos in a process that is failure-prone and not amenable to all target sequences10, rendering some DNA sequences inaccessible to study.

Proposals for enzymatic de novo synthesis of oligonucleotides with a defined sequence date back to at least 1962 (refs. 11,12). Enzymatic oligo synthesis promises several potential advantages over chemical synthesis: 1) the exquisite specificity of enzymes and mild conditions in which they function may reduce the formation of side products and DNA damage such as depurination, thereby enabling the direct synthesis of longer oligos; 2) reactions take place in aqueous conditions and need not generate hazardous waste; 3) synthesis could be initiated from natural DNA (i.e., DNA without protecting groups on the nucleophilic positions of the bases); and 4) enzyme engineering techniques such as high-throughput screens and selections can be employed to optimize the system in ways that are not possible using organic chemistry alone.

Terminal deoxynucleotidyl transferase (TdT) is the only known polymerase whose predominant activity is to indiscriminately add deoxynucleotide triphosphates (dTTPs) to the 3′ end of single-stranded DNA13, making it the natural candidate for use in enzymatic oligo synthesis. However, thus far there have been no demonstrations of a practical oligo synthesis method based on TdT. Detailed proposals to employ TdT for stepwise DNA synthesis using ‘reversible terminator’ dNTPs (RTdNTPs) in a scheme analogous to sequencing by synthesis14 date back to 1986 (refs. 15–21). There is at least one obstacle to this approach: RTdNTPs with removable groups on the 3′ OH that, once incorporated into a primer, directly block further elongation22–26, which can be rationalized in light of the co-crystal structure of TdT with dCPT27 (Supplementary Fig. 1). An alternative scheme proposes using 3′ O-unblocked RTdNTPs with inhibitory groups attached to the base27, but thus far there are no reports of successful de novo DNA synthesis based on this approach, suggesting that it may be difficult to develop 3′ O-unblocked RTdNTPs that are rapidly incorporated, yet sufficiently terminate further elongation.

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We conceived of an approach for reversible termination wherein each polymerase molecule is site-specifically labeled with a tethered nucleoside triphosphate (Fig. 1a). When a polymerase incorporates its tethered dNTP into a primer, it remains covalently attached to the 3' end, blocking further elongation by other polymerase–dNTP conjugates. The linker can then be cleaved to deprotect the 3' end of the primer for subsequent extension. This simple two-step reaction cycle of extension and deprotection can be iterated to write a defined sequence. (b) Chemical structures of two types of TdT–linker–dNTP conjugates used in this study, based on the amine-to-thiol crosslinkers PEG₂–SPDP (upper, “TdT–PEG₂–dTTP”) and BP-23354 (lower, “TdT–dTTP”) and the dTTP analogs 5-aminoallyl-dUTP (aa-dUTP) and 5-propargylamino-dUTP (pa-dUTP), respectively. Upon cleavage of the linker, the atoms indicated in red remain attached to the nucleobase and are referred to as a scar. The cleavable bond is indicated with a black dotted line.

Figure 1 TdT–dNTP conjugates for reversible termination of primer elongation. (a) Scheme for two-step oligonucleotide extension using TdT–dNTP conjugates consisting of a TdT molecule site-specifically labeled with a dNTP via a cleavable linker. In the extension step, a DNA primer is exposed to an excess of TdT–dNTP conjugate. Upon incorporation of the tethered nucleotide into the 3' end of the primer, the conjugate becomes covalently attached and prevents further extensions by other TdT–dNTP molecules. In the deprotection step, the remaining TdT–dNTP conjugates are inactivated (or removed) and the linkage between the incorporated nucleotide and TdT is cleaved by addition of the cleavage reagent (e.g., DTT, 365 nm light, peptidase), thereby releasing the primer for subsequent extension. The cycle can be iterated to extend a primer by a defined sequence. (b) Chemical structures of two types of TdT–linker–dNTP conjugates used in this study, based on the amine-to-thiol crosslinkers PEG₂–SPDP (upper, “TdT–PEG₂–dTTP”) and BP-23354 (lower, “TdT–dTTP”) and the dTTP analogs 5-aminoallyl-dUTP (aa-dUTP) and 5-propargylamino-dUTP (pa-dUTP), respectively. Upon cleavage of the linker, the atoms indicated in red remain attached to the nucleobase and are referred to as a scar. The cleavable bond is indicated with a black dotted line.
of the primer; instead, those reactions produced a variety of primer extension products (Fig. 2b), consistent with TdT-catalyzed incorporation of free nucleotides.

To test the influence of the linker on the incorporation kinetics of the nucleoside, we coupled the photocleavable linker to the dTTP analog 5-propargylamino-dUTP and quenched the maleimide moiety with βMe (βMe-linker–dTTP, Supplementary Fig. 7a), and then tested its incorporation kinetics. When incorporated freely from solution, βMe-linker–dTTP was a less efficient substrate than dTTP (Supplementary Fig. 7b). However, once attached to TdT (Supplementary Fig. 8), the linker–nucleotide was incorporated much faster than from solution (Supplementary Fig. 7b), suggesting that the extremely high effective concentration of tethered nucleotide with respect to the catalytic site partially compensates for the decreased incorporation efficiency of the linker–nucleotide.

Next, we prepared a complete set of photocleavable TdT–dNTP conjugates using the dNTP analogs 5-propargylamino-dUTP and 5-propargylamino-dCTP as well as 7-propargylamino-7-deaza-dATP and 7-propargylamino-7-deaza-dGTP (Supplementary Fig. 5b). Each conjugate was able to convert a primer into its respective singly extended product (Fig. 2c). 16 µM TdT–dCTP, TdT–dGTP, and TdT–dTTP almost completely extended 25 nM primer in 8 s, and TdT–dATP did so in 15 s. The slightly slower extension of the primer by TdT–dATP is in agreement with the reduced incorporation rate of free ddATP compared to the other three ddNTPs under these conditions (Supplementary Fig. 9).

Besides the predominant singly-extended product, we observed the formation of a small amount (~4%) of doubly-extended primer visible after 2 min (Fig. 2c). We sought to investigate whether the double extension (i.e., ‘non-termination’) was dependent on the conjugate concentration, which would suggest an intermolecular process, (i.e., incorporation of the dNTP tethered to one polymerase molecule by another polymerase molecule), or concentration-independent, which would suggest an intramolecular process (i.e., incorporation of multiple dNTPs tethered to the same polymerase molecule). Dilution of the TdT reaction after an initial incubation sufficient to quantitatively form +1 product had little effect on the formation of the +2 product during 14 min of subsequent incubation, suggesting an intramolecular process. (Supplementary Fig. 10a). We hypothesized that the majority of double extensions resulted from conjugates labeled off-target with a second dNTP, so we produced another set of conjugates with reduced loading of linker–dNTP (maleimide) in the labeling reaction to improve labeling specificity (Supplementary Fig. 11). These new conjugates indeed displayed improved termination relative to the original conjugates, with a substantial reduction in +2 product formation measured at 5 min (Supplementary Fig. 10b).

Since DNA synthesized using the photocleavable conjugates would ultimately contain a propargylamino scar on each base (Fig. 1b), we investigated whether DNA containing only scarred bases could serve as a template for accurate synthesis of complementary DNA. We prepared a single-stranded DNA molecule with a defined sequence containing 146 sequential N-acetyl-propargylamino nucleotides (Supplementary Note 1) and used Taq polymerase to synthesize (natural) complementary DNA, which we then PCR-amplified and cloned. Sequencing of 69 clones revealed an error rate of 6 × 10−4/nt (95% CI: 0.2 – 1.4 × 10−3/nt), suggesting that the propargylamino DNA produced using conjugates with the presented attachment chemistry can be amplified without many errors (Supplementary Fig. 12), in accordance with reports that comparable modifications do not prevent normal base-pairing29,30.

Figure 2 TdT–dNTP conjugates can extend a DNA molecule by a single nucleotide in 10–20 s, enabling stepwise DNA synthesis. (a) Monitoring of reaction cycle intermediates by SDS-PAGE with two-color fluorescence imaging. Oligonucleotides are visualized using 5′ FAM fluorescence (red channel) and protein is visualized using the fluorescent stain Lumitein (green channel). Exposing the primer (i) to an excess of the TdT–dCTP conjugate results in a new band containing both protein and DNA (yellow in composite), indicating the formation of a primer-TdT complex. Irradiation of the complex with 365 nm light recovers the distinct migration of the protein and DNA bands, indicating dissociation of the primer-TdT complex (ii). When exposed to fresh TdT–dCTP conjugate, extension products of cycle 1 again form a primer-TdT complex, which again is dissociated by 365 nm irradiation (iii). In contrast, no primer-TdT complex formation is observed if the TdT–dCTP conjugates are irradiated before the incubation with the primer (iv). This experiment was repeated with conjugates of all four nucleotides with similar results. (b) Capillary electropherograms of reaction products from panel a. Each reaction cycle quantitatively extends the primer (i) by a single nucleotide (ii, iii). Exposure of the primer to pre-photoalyzed conjugates results in a distribution of extension products (iv). The ladder of product standards was generated by incorporating free dNTP analogs using TdT under conditions selected to give 0-4 extensions. The electropherogram time axis is normalized using the elution times of the ladder of product standards. The intensity axis is normalized to the height of the tallest peak. (c) Capillary electropherograms of reaction time courses for the extension of a 25 nM DNA primer by 16 µM TdT–dATP, TdT–dCTP, TdT–dGTP, and TdT–dTTP conjugates, followed by photolysis. The primer is completely converted into the singly extended product in 10–20 s depending on the type of conjugate. A small amount of double extension (i.e., ‘non-termination’) is detectable in the reactions quenched at 120 s as a peak co-eluting with the +2 peaks of the ladders. The ladder of product standards was generated as described above. This experiment was performed twice with similar results.
Finally, to demonstrate the feasibility of TdT–dNTP conjugates for stepwise de novo DNA synthesis, we subjected a double-stranded DNA molecule with a 3' overhang (Supplementary Fig. 13) to ten cycles of extension and deprotection using conjugates corresponding to the sequence 5'-CTAGTCAGCT-3'. (For deprotection, we used 1 min of irradiation with a 405 nm laser (Supplementary Fig. 14).) We then poly-A tailed the product using TdT with free dATP to create a (reverse) primer binding site, PCR-amplified the product (Fig. 3a), and analyzed the amplicon by (Fig. 3b) NGS. Of 4,861 reads, we found that 3,913 (80.5%) contained the complete oligonucleotide as intended. By aligning the reads against the target sequence (Supplementary Fig. 15a), we were able to estimate the yields of individual steps of the synthesis (Fig. 3c), which ranged from 99.5% (step 6) to 93.4% (step 10). The average yield of all steps was 97.7%, with deletions as the predominant source of errors (1.3%) and the remaining errors arising from insertions (1.0%). To demonstrate that the synthesis of repeats is possible, we also synthesized the sequence 5'-CCC-3' and analyzed the products by NGS. Of 474 reads, 418 (88.2%) contained the intended sequence, implying an average stepwise yield of 0.8821/3 = 96.1%, assuming three independent steps (Supplementary Fig. 15b).

Here, we developed polymerase–nucleotide conjugates for reversible termination of chain extension by a polymerase. We have shown that active polymerase–nucleotide conjugates can be prepared by tethering a nucleoside triphosphate to various points on the surface of a polymerase using two different linkers, so we expect the approach to be quite general. As long as the tethered nucleotide can reach the active site of the polymerase in a productive conformation, it will be incorporated into a primer; and as long as the tether is short enough that the nucleotide is hindered from accessing the active sites of other conjugates, termination will be achieved.

Using TdT–dNTP conjugates, we report enzymatic de novo synthesis of a 10-mer oligonucleotide. Recently, Mathews et al. demonstrated the addition of 4 nucleotides to a primer by TdT using 3' O-nitrobenzyl RTdNTPs with 60-min coupling times (stepwise yields not reported)21. Our demonstration used coupling times of 1.5 min (C, G, and T) and 3 min (A) and achieved an average stepwise yield of 97.7%, comparable to early demonstrations of phosphoramidite-based DNA synthesis7. We used 1 min of irradiation with a 405 nm laser for deprotection, though similar nitrobenzyl moieties can be cleaved in 10 s using a more powerful 365 nm light source31 without causing DNA damage32. Our demonstration included a 3 min acetylation step to neutralize the scar produced by each photolysis step, but we have not conclusively determined whether this improves yields or can be omitted. Scars could be reduced or eliminated in future conjugates by changing the attachment chemistry.

Compared to reversible termination strategies employing 3'-modified RTdNTPs, we propose that a key advantage of our approach is that it enables use of 3'-unblocked dNTPs. It is recognized that 3' O-modified RTdNTPs are typically poor substrates for natural polymerases33, and considerable effort has been expended to engineer polymerases for sequencing by synthesis that can better accept RTdNTPs34. In spite of these efforts, 3' O-modified RTdNTPs still lag behind natural dNTPs in incorporation kinetics35. By contrast, tethered 3'-unblocked dNTPs are identical to the native substrates in the region that contacts the highly conserved catalytic site, so we expect they can achieve native-like incorporation kinetics. While the presented conjugates do not match the incorporation speed of TdT on natural dNTPs, our results suggest that the bulky photocleavable linker is responsible for the inhibition (Supplementary Fig. 7). However, dNTPs with a similar photocleavable group attached to the base via a hydroxymethyl linkage can be rapidly incorporated by a
Several challenges remain in the implementation of a practical enzymatic oligonucleotide synthesizer based on TdT–dNTP conjugates. First, a suitable solid support for the growing DNA must be identified. Second, the extension yields must be increased to consistently exceed 99% to enable the accurate synthesis of longer molecules. We are currently investigating the origin of the remaining non-termination of ~1% per step in our demonstration synthesis. Though eight steps of our demonstration synthesis had yields exceeding 98%, the remaining two steps had yields of 93–94%, and the cause of this variability remains to be identified. Because TdT contacts the last four bases of the primer, it is possible that its extension kinetics depend to a certain extent on its terminal sequence, so extending different termini may require adjusted coupling times. Also, it may be necessary to reduce the formation of DNA secondary structures during synthesis in order to achieve consistent extension times, since TdT has reduced activity on blunt and recessed ends. Strategies for disrupting secondary structures include using base modifications that are removed after the synthesis is completed, and engineering TdT to function at elevated temperatures or in the presence of co-solvents. Finally, for commercialization, conjugate manufacturing costs must be brought down to practical levels. While each synthesis cycle consumes a stoichiometric quantity of TdT enzyme, we do not expect that reagent costs will hamper the implementation of the method (Supplementary Note 2). Therefore, we believe that the presented scheme offers a promising starting point for the development of a practical enzymatic DNA synthesis technology.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.P. and D.H.A. conceived the method, designed and performed experiments, analyzed data, and wrote the manuscript with input from all other authors. T.D.R., S.B., J.S.K., R.B., H.M.B., A.N.T., and P.W.K. performed experiments and analyzed data, and all authors discussed and interpreted results. A.K.S., N.J.H. and J.D.K. supervised the research. All authors read and corrected the manuscript.

COMPETING INTERESTS

S.P. and D.H.A. have filed international patent application WO2017223517A1 on polymerase–nucleotide conjugates and are cofounders of Ansa Biotechnologies, Inc.


ONLINE METHODS

The description of experiments that are shown in Supplementary Figures are described in Supplementary Note 4. This includes all experiments involving the conjugates based on the disulfide-forming amine-to-thiol crosslinker PE-G5-SPDP.

DNA construction. The TdT amino acid sequence used consists of the residues 132–510 from the short isoform (TdT510) of M. musculus TdT (NCBI Accession number: NP_001036693.1) and lacks the N-terminal BRCT domain27. For the initial demonstration of tethered dNTP incorporation employing the PE-G5-SPDP crosslinker, TdT was expressed fused to an N-terminal His-Tag (TdTwt). The gene encoding tethered dNTP incorporation was cloned into pET19b using isothermal assembly27. To generate the TdTΔ5cs mutant, surface-exposed cysteine residues were identified in the crystal structure of TdT (PDB ID: 4127) and the mutations Cys188Ala (PDB ID 4127 numbering), Cys216Ser, Cys302Ala, Cys378Ala, and Cys438Ser were introduced using site-directed mutagenesis28. Based on this surface-cysteine free TdT variant, mutants with a single surface-exposed cysteine for linker attachment were then constructed by the re-insertion of cysteines into four positions near the catalytic site using site-directed mutagenesis, generating the mutants TdTΔ180 (Glu180Cys), TdTΔc180 (Ala188Cys), TdTΔ253 (Ser253Cys), and TdTΔ302 (Ala302Cys) (Supplementary Fig. 4). To generate the TdT variant that was used with the photocleavable linker, maltose binding protein (MBP) was inserted between the His-Tag and the TdT domain of TdT302 to yield MBTD302. The sequence encoding MBP from E. coli was amplified from pMAL-c5X (NEB) and inserted into pET19b (Supplementary Note 3). In addition, sequences of the plasmids coding for all TdT mutants were likewise generated.

The amino acid sequences of all TdT mutants used can be found in Supplementary Note 3. In addition, sequences of the plasmids coding for all TdT variants can be downloaded from the JBEI Public registry (https://public-registry.jbei.org/folders/355). Respective cloning and expression strains harboring the plasmids were added to JBEI strain archive and are available upon request (Data availability and descriptions in Supplementary Note 4). This includes all experiments involving the conjugates described in the supplementary note 4.

Preparation of TdT-dNTP conjugates using MTdTc302 and the photocleavable maleimide-NHS carbonate crosslinker (BP-23534). The scheme for the preparation of TdT–dNTP conjugates is shown in Supplementary Figure 5. The photocleavable NHS carbonate-maleimide crosslinker (Supplementary Fig. 5a) was purchased from Broadpharm (catalog number BP-23534). The complete set of propargylamino-dNTPs (pa-dNTPs) was purchased from TriLink Biotechnologies. First, the pa-dNTP was coupled to BP-23534 to form a thiol-reactive “linker-dNTP” (maleimide) in a 30 µL reaction containing 1 µL of the respective 100 mM pa-dNTP (100 nmol), 1 µL of 10× TdT pH 7.4 Storage Buffer, 26 µL of D₂O, and 2 µL of 100 mM BP-23534 dissolved in anhydrous DMSO (200 nmol) added last. The reaction was incubated at RT for 1 h with shaking. Initially, the linker concentration is above the solubility limit, but after the reaction makes some progress, enough (soluble) linker-dNTP product is formed that the remaining (unreacted) linker fully dissolves. The crude products (and buffer salts) were triturated using ethyl acetate (~2 mL) and centrifuged at 15,000g for 10 min to pellet the linker-dNTPs. The supernatant was removed and the linker-dNTP-containing pellets were dried by speed-vac or lyophilization and stored at −80 °C.

MTdT302 labeling with linker-dNTPs and purification of TdT–dNTP conjugates. To site-specifically label TdT at surface cysteine residues with a linker–dNTP, a dried linker–dNTP pellet was resuspended in 1× TdT pH 6.5 Storage Buffer and added to MTdT302 (conc. 10–15 µg/mL by absorbance) in 1× TdT pH 6.5 Storage Buffer. The (nominal) nucleotide concentration in the labeling reactions ranged from 0.1 mM to 2.5 mM, depending on the experiment. (The nominal nucleotide concentration was calculated based on the assumption that all (linker-)dNTPs precipitate quantitatively during trituration.) Unless indicated otherwise, all conjugates employing linker BP-23534 were prepared with nominal nucleotide concentrations of 0.1 mM (dGTP, dCTP) and 0.2 mM (dATP, dTTP). The labeling reaction was then incubated for 1 h at RT, and TdT–dNTP conjugates were purified using amyllose affinity chromatography to remove free (i.e., untheter) dNTPs: A spin column purification was performed using 0.8 mL spin columns (Pierce) that were filled with 250 µL of amyllose resin (NEB), and all centrifugation steps were performed at 50 RCF. All reagents and buffers used throughout the procedure were precooled on ice. Prior to binding, the amyllose resin was washed twice with 500 µL of TdT pH 6.5 Storage Buffer. A typical 15 µL linker–dNTP labeling reaction containing ~200 µg of MTdT302 was diluted into 200 µL TdT pH 6.5 Storage Buffer and loaded onto the spin column containing the amyllose resin, which was then incubated in a shaker block at 800 r.p.m. for 10 min for binding. Next, the column was washed twice with TdT pH 6.5 Storage Buffer, and then twice with TP8 Buffer (50 mM potassium acetate, 20 mM Tris-acetate, pH 7.9). Each washing step involved 1) addition of 500 µL buffer to the column, 2) incubation of the column for 1 min while shaking at 800 r.p.m., 3) centrifugation at 50 g for 1 min, and 4) removal of the flow-through. Elution of TdT–dNTP conjugates was performed by 1) the addition of 150 µL TP8 Buffer + 10 mM maltose, 2) an incubation for 5 min while shaking at 800 r.p.m., and 3) centrifugation. The elution procedure was repeated twice, and the eluates were combined and concentrated using a 30 kDa MWCO column (Corning), diluted 1:10 with TP8 Buffer to reduce the maltose concentration, and concentrated to ~2.5 µg/µL. The conjugates can be frozen in liquid nitrogen and stored at −80 °C. Notably, we observed a substantial loss of activity when storing the conjugates in the presence of cobalt.

Capillary electrophoresis (CE). 20 µL samples containing 0.5–1.5 nM 5′-FAM labeled oligonucleotides and ~0.1 µL GeneScan 600 LIZ dye Size Standard in 75% Hi-Di formamide were submitted to the UC Berkeley Sequencing Facility for capillary electrophoresis (CE, also called fragment analysis). CE samples were run on an Applied Biosystems 3730xl DNA Analyzer with a 50 capillary array containing POP-7 Polymer, with 15 s of injection at 1.5 kV and a 41 min run at 15 kV, oven: 68 °C, buffer: 35 °C. Electrophrogram data files were processed using custom software written in R (r-project.org) with comparable functionality to the Peak Scanner software from Applied Biosystems. R scripts are available upon request from the authors. Further information on data analysis software and experimental design is available in the Life Sciences Reporting Summary.
High ionic strength in a CE sample causes poor injection and distorted peaks, so DNA samples from extension reactions were either diluted 50-fold with 75% formamide or desalted before CE.

It was observed that DNA containing multiple propargylamino groups had reduced injection yield and inconsistent migration in CE, likely due to the added positive charges. Therefore, all DNA samples containing propargylamino groups were derivitized using NHS-acetate before CE. Unless specified otherwise, acetylation reactions contained 20 mM NHS-acetate and 200 mM sodium bicarbonate.

**Generation of extension products of oligo P2** (5′-FAM-dT200) with pa-dNTPs for use as size standards (ladder) in CE assays. Oligo P2 (5′-FAM-dT200, Supplementary Table 2) extension products that were used as size standards (ladders) were generated by the incorporation of free pa-dNTPs using TdT. Reactions contained 100 nM oligo P2, 200 µM of one type of pa-dNTP, reaction buffer with cobalt (RBC: 50 mM potassium acetate, 20 mM tris-acetate, 10 mM magnesium acetate, 0.25 mM cobalt chloride, pH 7.9), and either 0.05 U/µL or 0.03 U/µL NEB TdT. Reactions were performed at 37 °C, and aliquots were quenched with EDTA to a final concentration of 3 mM after 2, 5, and 10 min. Quenched samples were then acetylated, desalted using the Oligo Clean and Concentrator Kit (OCC; Zymo Research), and analyzed by CE. Samples with detectable peaks for oligo P2 as well as the +1 and +2 pa-dNTP extension products were selected for use as ladders.

**Two cycle demonstration and pre-photolysis experiment using TdT-dCTP conjugates.** The conjugates used in this experiment were generated using 1 mM nucleotide in the MTdTC302-labeling reaction. All extension reactions contained 50 nM oligo P2, 0.25 mg/mL TdT-dCTP (or photolyzed TdT-dCTP, see below), and RBC. Reactions were performed at 37 °C and quenched after 2 min by the addition of an equal volume of 200 mM EDTA. Photolysis of the linker was performed using a Benchtop 2UV Transilluminator (UVP, LLC) on the 365 nm setting for 1 h on ice. The measured irradiance was ~5 mW/cm². Aliquots of all photolyzed samples were acetylated and desalted by OCC for CE. Samples for PAGE were combined with 2× SDS loading buffer (Novex) and run on an 8–16% PAGE gel (Bio-Rad). The gel was imaged on a Multilager III (Alpha Innotech) for green fluorescence (5′-FAM-labeled primer) and, after staining with Lumitein UV (Biotium), imaged for red fluorescence (total protein). Gel images were aligned and composited using Adobe Photoshop.

**Two cycle experiment:** a reaction containing TdT–dCTP conjugate and oligo P2 was performed and the reaction products were photolyzed. The DNA products were then purified by OCC and subjected to another extension reaction with TdT–dCTP, again followed by photolysis. Aliquots were taken after both extension reactions for PAGE and after both photocleavage reactions for PAGE and CE.

**Control (“pre-photolyzed conjugate”) experiment:** TdT–dCTP conjugate was irradiated with 365 nm light for 1 h on ice to generate a stoichiometric mixture of unlinked MTdTC302(linker) + pa-dCTP. The photocleavage products were then used in an extension reaction with oligo P2, and aliquots were taken for PAGE and CE.

**Fast primer extension time courses of all four TdT–dNTP conjugates.** The conjugates used in this experiment were generated using nucleotides at 1 mM in the maleimide-labeling reaction. Oligo P2 extension yield by 1.5 mg/mL (~16 µM) TdT–dNTP conjugates was measured at 8, 15, and 120 s. Reactions were performed in a 37 °C room by adding 4.5 µL of 2 mg/mL TdT–dNTP conjugate to 1.5 µL of 100 nM oligo P2 (final: 25 nM), both in RBC. After rapid mixing, 4.5 µL of the reaction were quenched in 18 µL Quenching Solution (94% H2O:Di Formamide with 10 mM EDTA) after 8 or 15 s. The remaining reaction volume was quenched with 6 µL Quenching Solution after 2 min. The samples were irradiated with 365 nm light on a Benchtop 2UV Transilluminator for 30 min. Photocleavage products were acetylated using 100 mM NHS-acetate in 400 mM bicarbonate buffer and then captured onto DynaBeads M-280 Streptavidin (Thermo Fisher Scientific) that were saturated with 5′-biotin-dA20 oligo. Beads were washed with 1× B&WBuffer (5 mM Tris HCl pH 7.5, 0.5 mM EDTA, 1 M NaCl), 0.1× B&WBuffer, 0.01× B&WBuffer, and then eluted with 75% formamide for analysis by CE.

**Extension of a DNA strand by the sequences 5′-CTAGCTAGCT-3′ and 5′-CCC-3′ using TdT–dNTP conjugates.** Generation of the double stranded DNA molecule with a 3′ overhang used as synthesis starter. The double-stranded DNA used as initial substrate for the synthesis (starter) was prepared from a 359 bp PCR product derived from the pET19b plasmid. The PCR was performed using Phusion (Thermo Fisher Scientific). For the following cycles, the manufacturer’s instructions and using primers C1 and C2 (see Supplementary Table 2) (PCR program: 98 °C for 1 min, then 35 cycles of two-step protocol: 98 °C for 10 s, 72 °C for 1 min). The PCR product was purified using the DNA Clean & Concentrator Kit (“DCC”, Zymo Research) and digested with PstI, cutting the restriction site inserted by C1, to generate a 3′ overhang. The digested product was purified (DCC) and tail-ddTTP to block the strand with the 3′ overhang from further incorporations (0.5 mM ddTTP, 1 U/µL NEB TdT in RBC at 37 °C for 30 min). After tailing, the DNA was purified (DCC) and digested with BstXI to generate a 5′ overhang (5′-ATT-3′) for extensions by TdT–dNTP conjugates. The digestion product was separated from undigested DNA by 2% TAE-agarose gel electrophoresis and gel-extracted using the Gel Recovery Kit (Zymo Research). A scheme for the preparation of the synthesis starter can be found in [Supplementary Figure 13](#).

**Synthesis overview.** Nucleotide additions were performed using TdT–dNTP conjugates at 1 mg/mL in RBC at 37 °C. Extension reactions with TdT–dCTP, TdT–dGTP and TdT–dTP were performed for 90 s, extensions with TdT–dATP for 180 s. Quenching of the reactions was performed by the addition of an equal volume of Quenching Buffer (100 mM NaHCO3, 300 mM NaCl, 0.1% Tween 20 (Sigma–Aldrich), 50 mM EDTA, 20 mM Sodium Azide, 40 mM NHS-acetate; NHS-acetate was added immediately before use). Photolysis was performed for 1 min using a 405 nm diode laser as described in “Supplementary Note 4”, section Linker photolysis time course using 405 nm light. After each cleavage step, the DNA products were purified using AMPure XP beads, and the recovered DNA was subjected to the next extension step. For the 10-mer synthesis, the following conjugates were used in the extension steps: 1) TdT–dCTP; 2) TdT–dGTP; 3) TdT–dATP; 4) TdT–dGTP; 5) TdT–dTP; 6) TdT–dCTP; 7) TdT–dATP; 8) TdT–dGTP; 9) TdT–dCTP; 10) TdT–dTP. To synthesize 5′-CCC-3′, three cycles with TdT–dCTP were performed.

**Detailed protocol of the extension cycles.** For the first step, 10 µL starter at ~30 nM was mixed with 2 µL of Cofactor Mix (300 mM potassium acetate, 120 mM Tris-acetate, 80 mM magnesium acetate and 2 mM cobalt chloride, pH 7.9). The 12 µL mixture was then added to 4 µL of TdT–dCTP at 4 µg/mL in TP8. The resulting 16 µL reaction in RBC was incubated for 90 s, before it was quenched by the addition of 16 µL Quenching Buffer. After quenching, the reaction was photolyzed for 1 min using a 405 nm laser as described in “as described in Supplementary Note 4”, section Linker photolysis time course using 405 nm light”. A 3 min incubation at RT was performed to allow the acetylation reaction to proceed (NHS-acetate is a component of the Quenching Buffer). Subsequently, 32 µL of FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific) at 0.32 U/µL in 40 mM Tris–HCl and 60 mM MgCl2 was added to the photolysis products to digest released dNTPs, and the reaction was incubated for 1 min at RT. The photophase treatment was performed because we found that there was a substantial amount of dNTP carry-over during the AMPure XP cleanup, and that the dNTPs could be incorporated in the next extension cycle, leading to insertions (see “Amplification and next-generation sequencing analysis of synthesis products”). Next, the DNA was purified using AMPure XP beads (Beckman Coulter): 115.2 µL AMPure XP beads were added to the 64 µL phophatase reaction, and a binding step of 5 min was performed. The solution was then transferred into a well of a 96-well plate on a magnetic rack and incubated for 2 min for sedimentation of the beads. The liquid was removed and the beads were washed first with 400 µL of 70% ethanol and then with 200 µL of 70% ethanol. Subsequently, the beads were dried for 90 s, and the DNA was eluted with 10 µL of Buffer EBT (1 mM Tris–HCl, 10 µM EDTA, 0.04% Tween–20, pH 8.5). For the following cycles, the 10 µL of purified synthesis product of the previous cycle were mixed with 2 µL of Cofactor Mix, and the 12 µL mixture was added to 4 µL of the respective TdT–dNTP at 4 µg/mL in TP8. The resulting 16 µL reaction was then incubated for either 90 or 180 s, depending on the type of TdT–dNTP. The reaction was quenched, photolyzed, acetylated, phosphatase-treated, and purified using AMPure beads in the same way as for the first synthesis step. The procedure was repeated until the complete sequence was synthesized.
Amplification and next-generation sequencing analysis of synthesis products. 10-cycle and 3-cycle synthesis products were A-tailed using 0.4 U/µL TdT (NEB) with 1 mM dATP in TdT Reaction Buffer (NEB) for 30 min at 37 °C. The tailing products were purified by DCC and PCR-amplified using HotStart Taq (NEB) with primers C3 and C4 (Supplementary Table 2) according to the manufacturer’s instructions. PCR program: 98 °C for 2 min, 49 °C for 20 s, 68 °C for 12 min, then 35 cycles of: 98 °C for 30 s, 49 °C for 20 s, 68 °C for 30 s. Amplicons were purified by DCC and submitted to the JBEI DIVA DNA Sequencing Service for barcoded Nextera (Illumina) library preparation as described previously40, multiplexed with other samples submitted by other users of the service. NGS was performed on a MiSeq (Illumina). Reads containing the “target region” sequence 5′-TCCAGATTT(N0–20)AAAAAA-3′ were identified using a BioPython script, and reads with a Q-score of at least 34 (error rate: ~1/2,500 nt) for all bases in the target region were retained for analysis. Singleton target regions accounted for 1.5% of the data set and were excluded from analysis to avoid artifactual errors such as read misassignment due to index switching.

As mentioned above, it was observed in independent experiments (data not shown) that AMPure XP beads can retain dNTPs in a manner that is resistant to washing. As a result, some of the dNTPs that are released during photolysis of a quenched extension reaction are carried over into the next extension step, causing a characteristic type of (non-double) insertion error, (e.g., the G insertion in “CTAGTCAGCG” observed in 0.27% of reads, Supplementary Fig. 15a). The effect was mitigated by a brief alkaline phosphatase treatment following the photolysis (see “Detailed protocol of the extension cycles”), but not completely eliminated. dNTP carryover-type insertions were definitively identified in 0.7% of all reads total and were manually removed before estimation of stepwise yields.

Life Sciences Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Cloning and expression strains with plasmids of all TdT variants used in this demonstration have been deposited in the JBEI strain archive (Supplementary Table 1) and are available upon request (https://public-registry.jbei.org/folders/355). The raw electropherogram data and custom analysis software that support the findings of this study are available from the corresponding authors upon request.