SUBSTRATE SPECIFICITY STUDY OF AI-GENERATED BOS TAURUS TERMINAL DEOXYNUCLEOTIDE TRANSFERASE VARIANTS

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Terminal deoxynucleotidyl transferase (TdT) is a unique DNA polymerase that catalyses the addition of nucleotides to the 3' terminus of a DNA molecule without the need for a template. The ability of TdT to incorporate modified nucleotides has potential applications in creating functionalized DNA for diagnostic tools, therapeutics, and advanced materials for DNA origami and biosensors [1]. Due to the mesophilic nature of wild-type TdT, its efficiency is limited in vitro, particularly when working with modified nucleotides or structured DNA templates, as the enzyme's activity decreases at elevated temperatures needed for substrate accessibility and reduced secondary structure formation [2].

This study aims to evaluate the efficiency of novel AI-generated TdT variants with enhanced thermostability and the ability to incorporate various modified nucleotides and ribonucleotides into oligonucleotide chains, comparing their performance to that of the wild-type enzyme.

AI-designed TdT variants and N-terminal truncated protein variants were produced in *Escherichia coli* KRX strain. The recombinant proteins were purified using a two-step chromatography approach and used for enzymatic primer extension reactions by incorporation of various unnatural nucleotides such as alkyne-modified nucleotides (5-propargylamino-dUTP, C8-alkyne-dUTP, 5-ethynyl-UTP/dUTP) and thio-modified nucleotides (4-thio-UTP, 6-thio-GTP/dGTP) at various temperatures.

The AI-generated TdT variants demonstrated similar or better activity in extending ssDNAs with all used nucleoside triphosphates by incorporating one to several nucleotides onto the 3′-end of the primer compared to the wild-type enzyme. Among the tested variants, TdT-P15 showed no reduction in enzymatic activity even at 70 °C, which is 25 degrees above the thermostability limit of the wild-type enzyme. However, TdT-P15 variant possessed diminished thermostability after N-terminus truncation. This suggests that N-terminus may be also responsible for protein thermostability.

The successful development of TdT variants with enhanced thermostability and ability to incorporate modified nucleotides opens new paths for enzymatic DNA synthesis and modification applications.

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