

Bacterial exonuclease III expands its enzymatic activities on single-stranded DNA

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Abstract

Bacterial exonuclease III (ExoIII), widely acknowledged for specifically targeting double-stranded DNA (dsDNA), has been documented as a DNA repair-associated nuclease with apurinic/apyrimidinic (AP)-endonuclease and 3' → 5' exonuclease activities. Due to these enzymatic properties, ExoIII has been broadly applied in molecular biosensors. Here, we demonstrate that ExoIII (*Escherichia coli*) possesses highly active enzymatic activities on ssDNA. By using a range of ssDNA fluorescence-quenching reporters and fluorophore-labelled probes coupled with mass spectrometry analysis, we found ExoIII cleaved the ssDNA at 5'-bond of phosphodiester from 3' to 5' end by both exonuclease and endonuclease activities. Additional point mutation analysis identified the critical residues for the ssDNase action of ExoIII and suggested the activity shared the same active center with the dsDNA-targeted activities of ExoIII. Notably, ExoIII could also digest the dsDNA structures containing 3'-end ssDNA. Considering most ExoIII-assisted molecular biosensors require the involvement of single-stranded DNA (ssDNA) or nucleic acid aptamer containing ssDNA, the activity will lead to low efficiency or false positive outcome. Our study revealed the multi-enzymatic activity and the underlying molecular mechanism of ExoIII on ssDNA, illuminating novel insights for understanding its biological roles in DNA repair and the rational design of ExoIII-ssDNA involved diagnostics.

eLife assessment

This manuscript highlights single-stranded DNA exo- and endo-nuclease activities of ExoIII as a potential caveat and an underestimated source of decreased efficiency in its use in biosensor assays. The data present **solid** evidence for the ssDNA nuclease activity of ExoIII and identifies residues that contribute to it. The findings are **useful**, but some aspects in the study remain **incomplete**.

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Introduction

Endogenous and exogenous stresses frequently result in DNA damage. Base damage is one of the most common DNA lesions caused by alkylation, oxidation, deamination, and depurination/depyrimidination¹. Such structural changes in double-stranded DNA (dsDNA) immediately recruit a range of downstream enzymes to the lesion locus for repair, a process known as base excision repair (BER)². Exonuclease III (ExoIII) has been widely recognized as one of the most critical players in BER^{3–5}. Based on the enzymatic activities involved, BER has been conceptually divided into the following five steps⁶: 1) glycosylase excision on the damaged base forming the apurinic/apyrimidinic (AP) site; 2) AP endonuclease (ExoIII) incision on the phosphodiester backbone to create and extend a ssDNA gap; 3) 5'-deoxyribose phosphate (dRp) removal; 4) polymerase-directed gap filling; 5) ligase-aided nick sealing. During the BER process, ExoIII performs the dual roles of AP endonuclease and exonuclease in *E. coli*⁷. Once the damaged bases are removed by glycosylases leaving an AP site, ExoIII first acts as an endonuclease to hydrolyze the phosphodiester bond at the 5'-end of the AP site, then as an exonuclease to degrade the downstream single-stranded DNA (ssDNA) of the duplex region from 3' to 5' end. These two ExoIII-dominated enzymatic activities create a proper length of ssDNA gap on dsDNA, which is required for polymerase binding at the locus to re-synthesize nucleotides⁸. Structural analysis revealed that the α_M helix of ExoIII stabilized the substrate by stretching into the major groove of dsDNA in the catalytic process⁵. Interestingly, different from the typical multifunctional enzymes, i.e., DNA polymerase⁹, with separate active sites, the multiple activities of ExoIII on dsDNA lie in the same active site^{4, 5}.

During the 1960s–1990s, Kornberg's laboratory demonstrated that ExoIII specifically recognizes dsDNA but is inactive to ssDNA based on an ssDNA oligo of five consecutive Ts (5' pTpTpTpTpT 3')^{10, 11}, and other researchers showed ExoIII had limited enzymatic activity on dsDNA containing 3'-protruding ssDNA (≥ 4 nt)^{12, 13}. Hence, ExoIII was previously proposed to produce ssDNA from dsDNA¹⁴. Combined with novel materials, chemicals, or mechanical platforms, ExoIII has been widely employed in detecting nucleic acids, metals, toxins, and other types of small molecules¹⁵. Based on the specific enzymatic activities of ExoIII on dsDNA in detections, the ExoIII-assisted biosensors can be divided into two categories: AP-endonuclease and exonuclease-based methods. As a promising replacement for PCR technique¹⁶, TwistAmp® Liquid exo kit (<https://www.twistdx.co.uk/product/twistamp-liquid-exo/>) represents one of the most successful AP-endonuclease-based nucleic acid detection, which combines recombinase polymerase amplification (RPA)¹⁷ and ExoIII. In the application of the commercial kit, a 46~52 nt ssDNA probe is required to base-pair with the target sequence and the probe should also contain an AP site (usually simulated by a tetrahydrofuran [THF] residue) labelled with a fluorophore and a quencher, respectively, at or near the 5'- and 3'-end of AP. Once the ssDNA probe binds to the complementary sequence with the aid of recombinase and forms a dsDNA region containing an AP, ExoIII recognizes and cleaves the AP site, which separates the fluorophore and

quencher, leading to a fluorescence release. In the exonuclease-based methods, ssDNA or nucleic acid aptamer contained ssDNA is often configured to form dsDNA substrate for the exonuclease activity of ExoIII¹⁵. However, the digesting of ExoIII on ssDNA probe has been inadvertently observed in three ExoIII-developed detection methods^{18–20} and a biochemical study²¹. As the studies displayed limitations in proving the ssDNase activity of ExoIII: a) no additional procedure safeguards the ssDNA substrate from forming a secondary structure containing dsDNA duplex in or between ssDNA, which can easily happen, and b) only stays on the phenomenon without further in-depth characterization and mechanism exploring, ExoIII has therefore still been publicly described (<https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/exonuclease-III>), studied^{3,4,8,22}, and commercialized as a dsDNA-specific enzyme that is inactive or has limited activity on ssDNA (<https://www.thermofisher.cn/order/catalog/product/EN0191> and <https://international.neb.com/products/m0206-exonuclease-iii-e-coli#Product%20Information>) for over a half-century. The definition of the enzymatic activity of ExoIII on ssDNA remains ambiguous.

To address this long-standing controversy, we used several sensitive fluorescence-quenching (FQ) ssDNA reporters to detect the enzymatic activity of ExoIII on ssDNA. FQ is distance-dependent, where increasing the distance between the fluorophore and the quencher increases the fluorescence intensity. As a sensitive and visible physical phenomenon, it has been transformed into one of the most influential techniques for monitoring dynamic alterations of macromolecule conformation in complex interactions^{23–25}. The DNA or RNA-constructed FQ reporter has been successfully employed in CRISPR-based diagnostics^{26,27} as an efficient sensor of collateral trans-cleavage activity of Cas12a on ssDNA²⁸ and Cas13a on RNA²⁹. Once the nuclease cuts the ssDNA or RNA connector and disconnects the fluorophore and quencher, fluorescence will be significantly released as a positive readout for the detection. Combining with mass spectrometry and structure analysis, our finding demonstrated the ssDNase activity of ExoIII and its potential biological role in DNA repair. Furthermore, it also alerted a risk of false positive or low efficiency within the widely adopted ExoIII-ssDNA combined diagnostic approach.

Results

ExoIII rapidly cleaved the ssDNA FQ reporters

As an ultrasensitive biosensor of the ssDNase activity, the ssDNA FQ (Figure 1A) (5 nt) of CRISPR-based detection systems²⁶ were utilized for assaying the ssDNase activity of ExoIII and its digesting rate. To examine if the substrates form the dsDNA region internally or between ssDNA strands, the dsDNA-specific nuclease T7 exo³⁰ was used as the negative control, while LbCas12a²⁸ was designated as the positive control. Once the ssDNA FQ reporter is cleaved by nuclease, the fluorescent signal will be released. In contrast to T7 exo and LbCas12a, ExoIII (5 U/μl) triggered a substantial fluorescent signal promptly upon adding to FQ reporter (5 μM) (a total of 50 pmol), and most of the ssDNA substrate (over 90%) was cleaved in the first minute (Figure 1B), which suggested that the commercial ExoIII (5 U/μl) degraded over 2.7×10^{13} (45 pmol) phosphodiester bonds per minute in the reaction, significantly faster than 0.7 pmol phosphodiester bonds per minute of LbCas12a and that of other nucleases (Figure 1C). Strikingly, we found even at room temperature (22°C), the fluorescence release initiated immediately when 0.5 μl ExoIII was mixed with FQ reporter (5 μM) under LED blue light (Supplementary video S1). In summary, T7 exo showed no significance with D-T7 exo; APE1 exhibited a significantly detectable fluorescence signal compared to its deactivated counterpart (D-APE1), albeit much weaker than that of ExoIII and LbCas12a (Figure 1C).

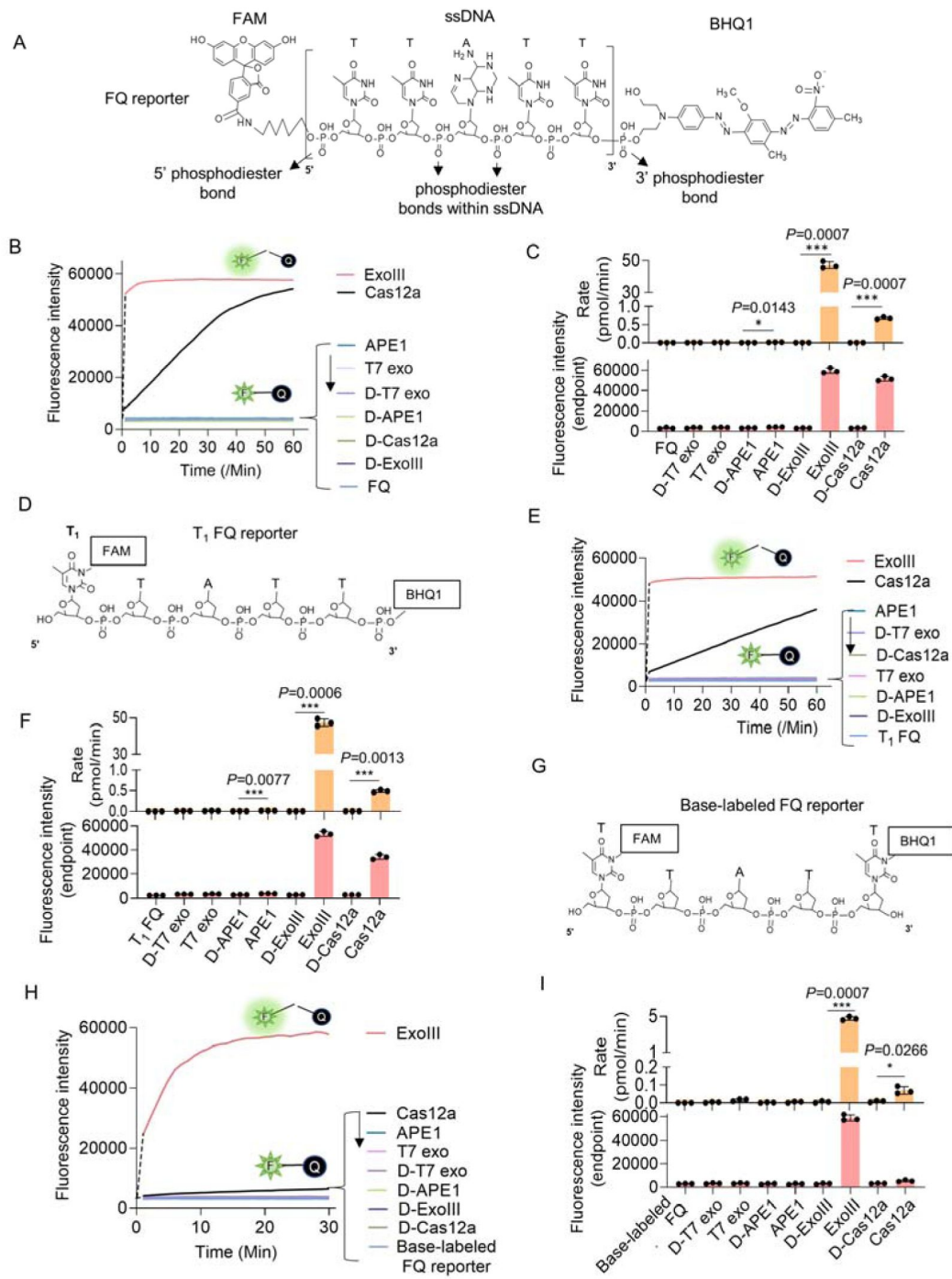


Figure 1.

ExoIII (5 U/μl) rapidly cleaved the ssDNA FQ reporter (5 μM) of the CRISPR/Cas12a system with an average rate of 4.5~45 pmol ($2.7\sim 27\times 10^{12}$) phosphodiester bonds per minute. **(A)** The typical structure of the ssDNA FQ is presented, in which three types of putative susceptible sites for ExoIII digestion are outlined, including 5' phosphodiester bond, phosphodiester bonds within ssDNA, and 3' phosphodiester bond. **(B)** The fluorescence intensity of FQ reporter (5 μM) treated with four active nucleases (T7 exonuclease (T7 exo) (5 U/μl), APE1 (5 U/μl), ExoIII (5 U/μl), and Cas12a/crRNA (0.1 μM)) or their heat-deactivated counterparts (D-T7 exo, D-APE1, D-ExoIII, D-Cas12a) was monitored every minute for 60 min. The dsDNA nuclease T7 exo was a negative control, also used to examine if the ssDNA substrates in the reaction formed dsDNA regions internally and between ssDNA strands, while LbCas12a with a trans-cleavage activity on ssDNA was a positive control. The D-nuclease served as the internal control of each active nuclease. The curve was plotted with an average value of three repeats. **(C)** The average rate of ExoIII digestion on FQ reporter was calculated by the formula: (the fluorescence produced/total fluorescence× 50 pmol)/reaction time. The total fluorescence means the fluorescence intensity generated when the input FQ reporter (50 pmol) was all cleaved. P (APE1 vs. D-APE1) = 0.0143; P (ExoIII vs. D-ExoIII) = 0.0007; P (Cas12a vs. D-Cas12a) = 0.0007. $*P<0.05$, and $***P<0.001$. The fluorescence intensity at the endpoint of the reaction was plotted based on three repeats, after the FQ reporter respectively treated with four active nucleases and their heat-deactivated counterpart (D-nuclease). **(D)** The 5' T₁ FAM-labelled FQ reporter structure is designed and presented, in which the 5' phosphodiester bond is removed and 3' phosphodiester bond retained. **(E)** Real-time monitoring on the fluorescence generated upon 5' T₁ FAM-labelled FQ reporter (5 μM) treated with four nucleases (T7 exonuclease (T7 exo) (5 U/μl), APE1 (5 U/μl), ExoIII (5 U/μl), and Cas12a-crRNA (0.1 μM)) or deactivated nucleases (D-T7 exo, D-APE1, D-ExoIII, D-Cas12a) was performed for 60 min. The average value of four repeats was calculated and curved. **(F)** The average rate of ExoIII digestion on T₁-labelled FQ reporter was calculated by the formula: (the fluorescence produced/total fluorescence× 50 pmol)/reaction time. After the FQ reporter was treated with the four nucleases or their deactivated ones, the fluorescence intensities at the reaction endpoint were measured and plotted. P (APE1 vs. D-APE1) = 0.0077; P (ExoIII vs. D-ExoIII) = 0.0007; P (Cas12a vs. D-Cas12a) = 0.0013. **(G)** The structure of the base-labelled FQ reporter for ExoIII is diagrammed, in which both 5' and 3' phosphodiester bonds are removed. **(H)** The digestion of the four nucleases and their deactivated counterparts on the base-labelled FQ reporter was monitored for 30 min. The average value of three repeats was calculated and curved. **(I)** The average rate of ExoIII digestion on the base-labelled FQ reporter was calculated by the formula: (the fluorescence produced/total fluorescence× 50 pmol)/reaction time. After the cleavage reactions on the FQ reporter, the fluorescence intensities at the reaction endpoint were measured and plotted. P (ExoIII vs. D-ExoIII) = 0.000006; P (Cas12a vs. D-Cas12a) = 0.0185. The statistical analysis was performed using a two-tailed *t*-test. $*P<0.05$, $***P<0.001$. The dashed line in the figure does not indicate the real-time fluorescence generated in the reaction but only represents a trend in the period for monitor machine to initiate (~2 minutes).

Given that ExoIII has long been recognized as a dsDNA-specific nuclease, we initially speculated that the ssDNA activity on FQ reporters might be ascribed to its cleavage at the 5' or 3' end phosphodiester bonds of ssDNA reporter rather than the typical phosphodiester bonds within ssDNA. To narrow down the possibility, we first removed the 5' end phosphodiester by designing the FQ reporter with FAM labelled at its 5' first thymine (T₁) base instead of the 5' end phosphate (**Figure 1D**). Logically, the 5' FAM T₁-labelled reporter should not produce fluorescence if ExoIII cuts at the 5' end phosphodiester bond. But fluorescence monitoring assays showed that ExoIII (5 U/μl) digested over 90% of 5' FAM T₁-labelled FQ reporter (5 μM) in the first minute (**Figure 1E**), indicating a speed of 2.7×10^{13} (45 pmol) phosphodiester bonds per minute (**Figure 1F**); APE1 and LbCas12a also exhibited a stronger fluorescence intensity compared to their deactivated counterparts (D-APE1 and D-LbCas12a) (**Figure 1F**). Treated with ExoIII, the fluorescence of the FQ reporter was generated rapidly even before incubating at 37°C (**Supplementary video S2**). The evidence implied that ExoIII cleaved the FQ reporter not at 5' end phosphodiester bonds.

Subsequently, we removed the 3' end phosphodiester bonds by labelling the BHQ1 at the reporter's 3' end thymine (**Figure 1G**). The fluorescence monitoring assay showed that the ExoIII-treated FQ reporter (5 μ M) released a significant fluorescence signal compared to other nucleases. Slower than the previous two FQ reporters, it cleaved over 90% of the base-labelled FQ reporter in 10 min (**Figure 1H**), demonstrating that the commercial ExoIII (5 U/ μ l) digested about 2.7×10^{12} (4.5 pmol) phosphodiester bonds or nucleotides of ssDNA in the FQ reporter (**Figure 1I**). Compared with the massive fluorescence induced by ExoIII, the modifications on thymine may impede the binding and cleaving of the LbCas12a-crRNA complex on the ssDNA substrate, leading to less fluorescence generated (**Figure 1I**). Combining all the results of three types of FQ reporters, we demonstrated ExoIII might harbor a powerful ssDNase activity.

ExoIII is an efficient 3' $\rightarrow$ 5' ssDNase

To exclude the possibility of sequence-specific driven catalysis of ExoIII, we tested three ssDNA oligos with fluorophore labelled at the 5' end (5 μ M) (**Figure 2A**). After 40 min of incubation with ExoIII (5 U/ μ l) at 37°C, the reaction mixture of three probes was separated by gel electrophoresis. The result indicated that the ssDNA was degraded by ExoIII and LbCas12a compared with the undigested product of APE1 and T7 exo (**Figure 2B** and **2C**). The reaction products gradually decreased in length as time prolonged (**Figure 2D**), suggesting that ExoIII shortened Probe 1 into smaller fragments gradually and rapidly from 3' to 5' end. The time course analysis on the digestion demonstrated the digesting rate of ExoIII on the ssDNA stood at 20~400 pmol nucleotides per minute in the 15 min (**Figure 2E**). Similarly, Probe 2 was processed into shorter fragments by ExoIII and LbCas12a compared to other nucleases (**Figure 2F** and **2G**) and were digested gradually and promptly over time (**Figure 2H** and **2I**). Probe 3 also resembled the digestion over time (**Figure 2J**, **2K**, **2L**, and **2M**). The gradual and successive degradation on the three ssDNA substrates in the 40 min indicated that ExoIII (5 U/ μ l) digested the ssDNA (5 μ M) in the 3' $\rightarrow$ 5' direction with an estimated average speed of approximately 1.5×10^{13} (25 pmol) phosphodiester bonds or nucleotides per minute, and the digestion rate varied with the encountered nucleotides of ssDNA substrate. We also compared the digestion rates of ExoIII on ssDNA and dsDNA. The result showed that ExoIII required 10 minutes to digest the 30-nt single-stranded DNA (ssDNA) (**Supplementary Figure S1A**), whereas it could digest the same sequence on double-stranded DNA (dsDNA) within 1 minute (**Supplementary Figure S1B**). This indicated that ExoIII digested the dsDNA at a rate at least ten times faster than ssDNA. Even not as rapid as the dsDNA, the ssDNase activity of ExoIII surpasses that of the conventional ssDNA-specific nuclease ExoI²⁰, suggesting a potential biological significance of ExoIII in bacteria related to ssDNA.

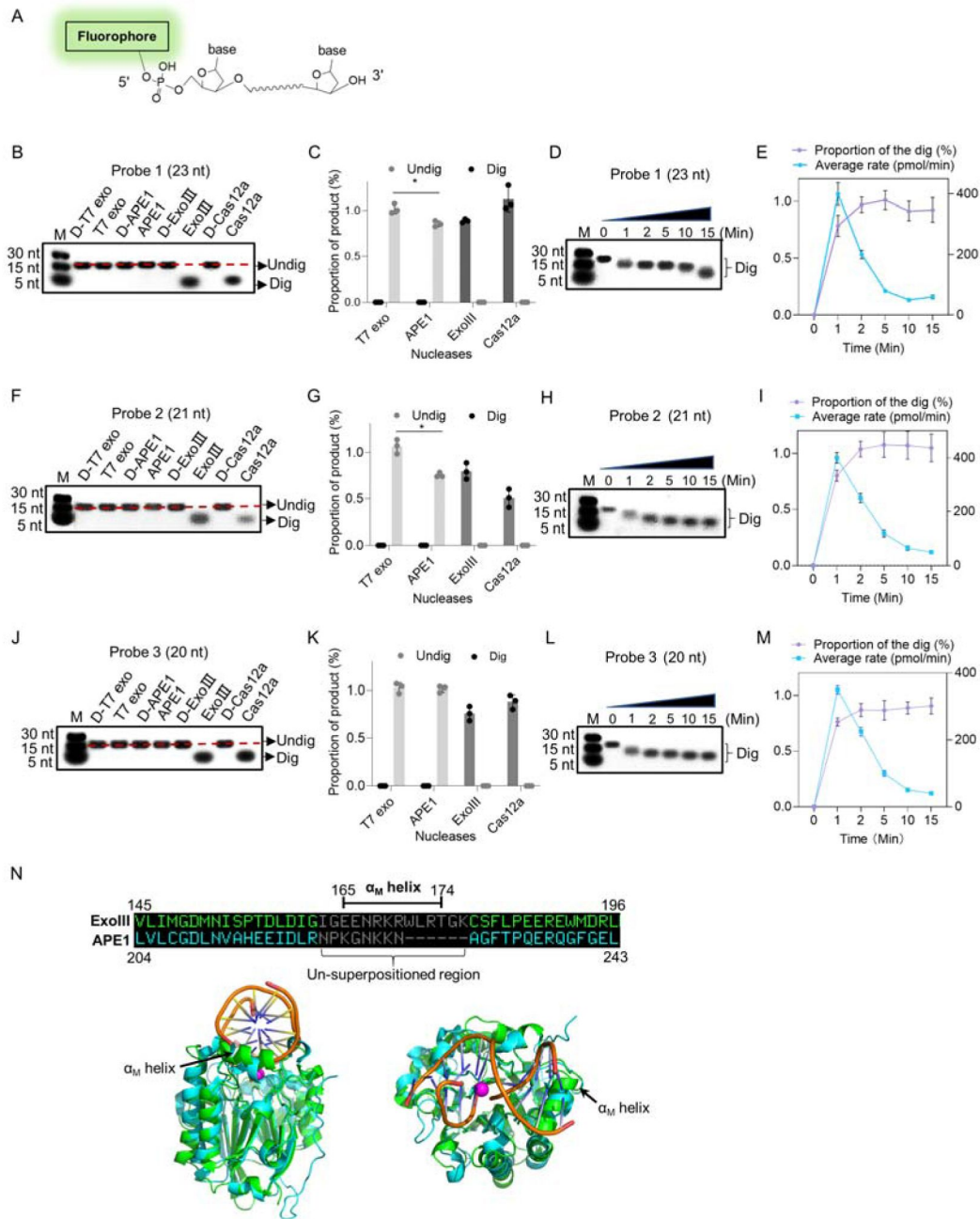


Figure 2.

ExoIII (5 U/μl) digested the 5' end fluorophore-labelled ssDNA substrates (5 μM) in a 3' to 5'-end direction with a estimated rate of $\sim 10^{13}$ nucleotides per minute. **(A)** The 5' fluorophore-labelled ssDNA structure for the following ExoIII digestion is shown. The squiggle line represents the ~ 20 nucleotides of the ssDNA oligo. **(B, F, and J)** The ssDNA oligo of Probe 1, Probe 2, or Probe 3 (5 μM) was treated with the four nucleases (T7 exo (5 U/μl), APE1 (5 U/μl), ExoIII (5 U/μl), and Cas12a-crRNA (0.1 μM)) and their deactivate counterpart (D-T7 exo, D-APE1, D-ExoIII, D-Cas12a) for 30 min. The reaction products were analyzed by gel electrophoresis. Undig, undigested; dig, digested; M, maker. **(C, G, and K)** The gray intensity of the digested or undigested bands produced by the three probes was measured by ImageJ. The undigested or digested proportions were calculated with the formula: Proportion = (Intensity of the undigested or digested band produced in the active nuclease treatment)/(Intensity of the undigested band produced in the corresponding deactivated nuclease treatment). P (APE1 vs. T7 exo on Probe 1) = 0.04; P (APE1 vs. T7 exo on Probe 2) = 0.02; $*P < 0.05$. The statistical analysis was performed using a two-tailed t -test. **(D, H, and L)** The time course of ExoIII digestion on the three probes was analyzed by gel electrophoresis, and the gray intensity of the generated bands was measured by ImageJ. **(E, I, and M)** The digesting rate of ExoIII on the ssDNA was calculated by: the digested proportion $\times$ 50 pmol $\times$ digested nucleotides/reaction time. The digested proportion was calculated by the formula: (gray intensity of the digested band produced)/(gray intensity of the band at 0 s). Detailed information on these oligos is described in **Supplementary Table 4**. All experiments were repeated three times, and the representative ones were shown. **(N)** The structure alignment between ExoIII (PDB ID:1AKO) and APE1-dsDNA (PDB ID: 1DE8 and NDB ID: SWN5) was performed by Pymol, and the un-superpositioned region (Residues 165-174, α_M helix) in the enzyme-substrate binding surface between ExoIII and APE1 was displayed. The aligned structures were shown from different angles by Pymol.

To investigate the massive disparity of ssDNase activity between the two highly similar homologs, ExoIII and APE1, we performed structure alignment and found the structure of the α_M helix of ExoIII deteriorated in APE1 (**Figure 2N**). As α_M lies in the binding interface of ExoIII and substrate ⁵, it might play a critical role in the digestion of ssDNA. The degenerated ssDNA-cleavage activity of APE1 may evolve to minimize the risk of genomic instability in human. Whether it is associated with related human cancers remains to be further studied.

Mass spectrometry analysis revealed the endonuclease activity of ExoIII on ssDNA

To provide high-resolution information regarding the ExoIII actions on the ssDNA substrates (used in **Figure 2**), we parsed the products of ExoIII by ESI-mass spectrometry. The major products of the three ssDNA probes are identified and listed (**Supplementary Table S1**) and all the reaction products are included in **Supplementary File S1**. After being treated with ExoIII, Probe 1 was digested to the three major products (Product 1 of Probe 1, termed as P1P1, Mass peak = 2914.9 Da; P1P2, Mass peak = 2601.5 Da; P1P3, Mass peak = 5395.5 Da) (**Figure 3A**), which respectively matched to an 8-nt (Theoretical mass = 2915.6 Da), 7-nt (Theoretical mass = 2602.4 Da) and 16-nt (Theoretical mass = 5397.2 Da) ssDNA fragments of Probe 1, and these undigested nucleotides accounted for $\sim 15\%$ of the input (**Figure 3B**). Probe 2 was digested to two major products (P2P1, Mass peak = 4639.6 Da; P2P2, Mass peak = 957.7 Da) (**Figure 3C**), which respectively matched to a 13-nt (Theoretical mass = 4641.6 Da) and 3-nt (Theoretical mass = 957.6 Da) ssDNA fragments of Probe 2, and these remaining oligos accounted for $\sim 0.6\%$ of the input (**Figure 3D**). Probe 3 was digested into four products (P3P1, Mass peak = 2936.9; P3P2, Mass peak = 3240.9; P3P3, Mass peak = 2632.7; P3P4, Mass peak = 2328.7) (**Figure 3E**) respectively matched to an 8-nt (Theoretical mass = 2937.6 Da), 9-nt (Theoretical mass = 3241.8 Da), 7-nt (Theoretical mass = 2633.4 Da) and 6-nt (Theoretical mass = 2329.2 Da) fragments of Probe 3, and these leftover accounted for $\sim 28\%$ of the input (**Figure 3F**). According to the mass peak intensities of reaction products (**Supplementary Table S2**), the majority of ssDNA substrates (5 μM) were degraded into mononucleotides ($\sim 85\%$

for Probe 1, ~99% for Probe 2 and ~72% for Probe 3) by ExoIII (5 U/ μ l). As the control, the mass spectrometry analysis of the three probes without ExoIII digestion is provided (**Supplementary Figure S2A, S2B, and S2C**).

Furthermore, we found these undigested fragments above (P1P1, P1P2, P2P1, P3P1, P3P2, P3P3, and P3P4) retained an intact 5' end fluorophore and a free 3'-OH, which indicated the ExoIII catalyzed the cleavage of ssDNA at the 5'-side bond of phosphodiester. And 17 ssDNA fragments (4 fragments from Probe 1 and 13 fragments from Probe 2 included in **Supplementary File S1**) were identified from the middle part of the ssDNA, implying ExoIII possessed an endonuclease activity on ssDNA, which might be activated by specific sequences. Combined with the results of **Figure 2**, we confirmed that ExoIII is an efficient ssDNase, and its digestion rate varied based on the sequences of ssDNA.

Reaction conditions for the ssDNase activity of ExoIII

To explore if this enzymatic action of ExoIII requires Mg^{2+} , we added EDTA to the enzymatic reaction. The endpoint fluorescence intensity of the FQ reporter showed that the addition of EDTA completely inhibited the fluorescence generation compared with the reaction without EDTA (**Figure 4A**). The gel analysis on the cleavage product of three ssDNA probes showed that adding EDTA stopped the catalytic digestion of ExoIII on ssDNA (**Figure 4B**). These EDTA-added experiments demonstrated that Mg^{2+} was required for the ssDNase activity of ExoIII. Subsequently, we added different concentrations of ExoIII (0, 0.025, 0.125, 0.250, 0.500, and 1.250 μ M) to the ssDNA probe (0.5 μ M). The result showed the amount of ExoIII (0.025 μ M) degraded ~10 nucleotides of ssDNA substrate (0.5 μ M) in 10 min (**Figure 4C and 4D**), suggesting each molecular of ExoIII was capable of cleaving off one nucleotide by 3 seconds. To explore the suitable temperature for the ssDNase activity, ExoIII was incubated with the ssDNA probe at 0, 16, 25, 37, 42, and 50°C, respectively. The gel results indicated the ssDNase activity of ExoIII was active at 16, 25, and 37°C but receded when the temperature was raised to 42°C and 50°C (**Figure 4E and 4F**), suggesting the suitable temperature for the ssDNase activity of ExoIII was probably at a range of 16~42°C.

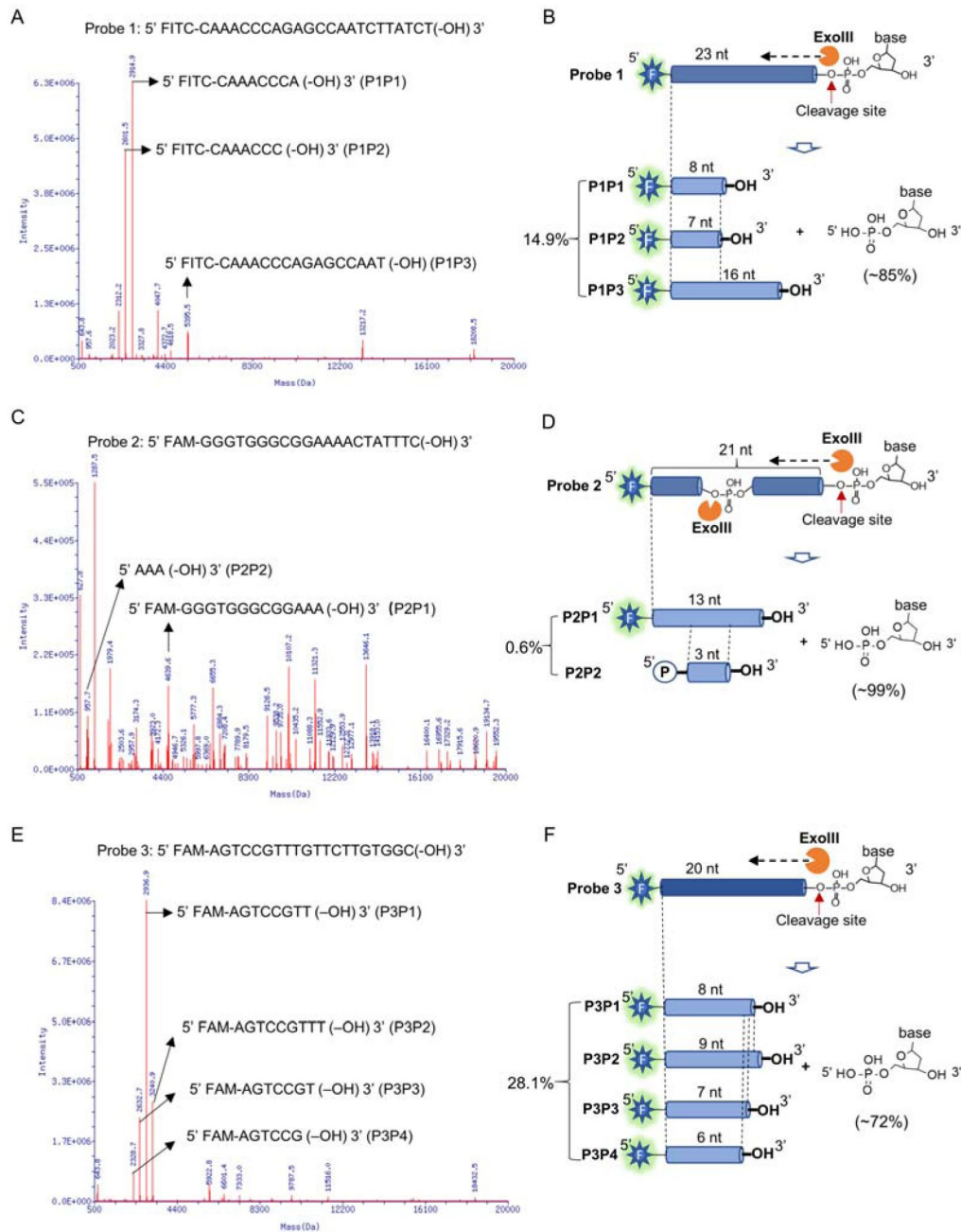


Figure 3.

Mass spectrometry analysis revealed the exonuclease and endonuclease activities of ExoIII on ssDNA. After the ssDNA probe (5 μ M) was incubated with ExoIII (5 U/ μ l) at 37°C for 30 min, the reaction product was analyzed by mass spectrometry. The detection range of mass spectrometry is 500~20000 Da. **(A)** The mass peaks of ssDNA Probe 1 digested by ExoIII are present. The three major peaks of reaction products of Probe 1 (P1P1, P1P2 and P1P3) are indicated by arrows. The information related to major peaks is provided in Supplementary Table S1. **(B)** The digestion process, including the cleavage site, product and relevant proportion of Probe 1 is illuminated based on the mass spectrometry analysis. The three major mass peaks match three fragments of Probe 1 (8 nt, 7 nt and 16 nt), respectively. **(C)** The mass peaks of ssDNA Probe 2 digested by ExoIII are displayed. The two major peaks (P2P1 and P2P2) are labelled by arrows. **(D)** The digestion process of Probe 2 is illuminated based on the mass spectra analysis. The two major mass peaks of reaction products match two fragments of Probe 2 (13 nt and 3 nt). **(E)** The mass peaks of Probe 3 digested by ExoIII are displayed. The major peaks (P3P1, P3P2, P3P3, and P3P4) are labelled by arrows. **(F)** The reaction process of Probe 3 is illuminated based on the mass spectra analysis and gel result ([Figure 3L](#)). The four major mass peaks of reaction products match the fragments of Probe 3 (8 nt, 9 nt, 7 nt, and 6 nt). As the control, the mass peaks of undigested ssDNA probes are displayed in **Supplementary Figure S1**.

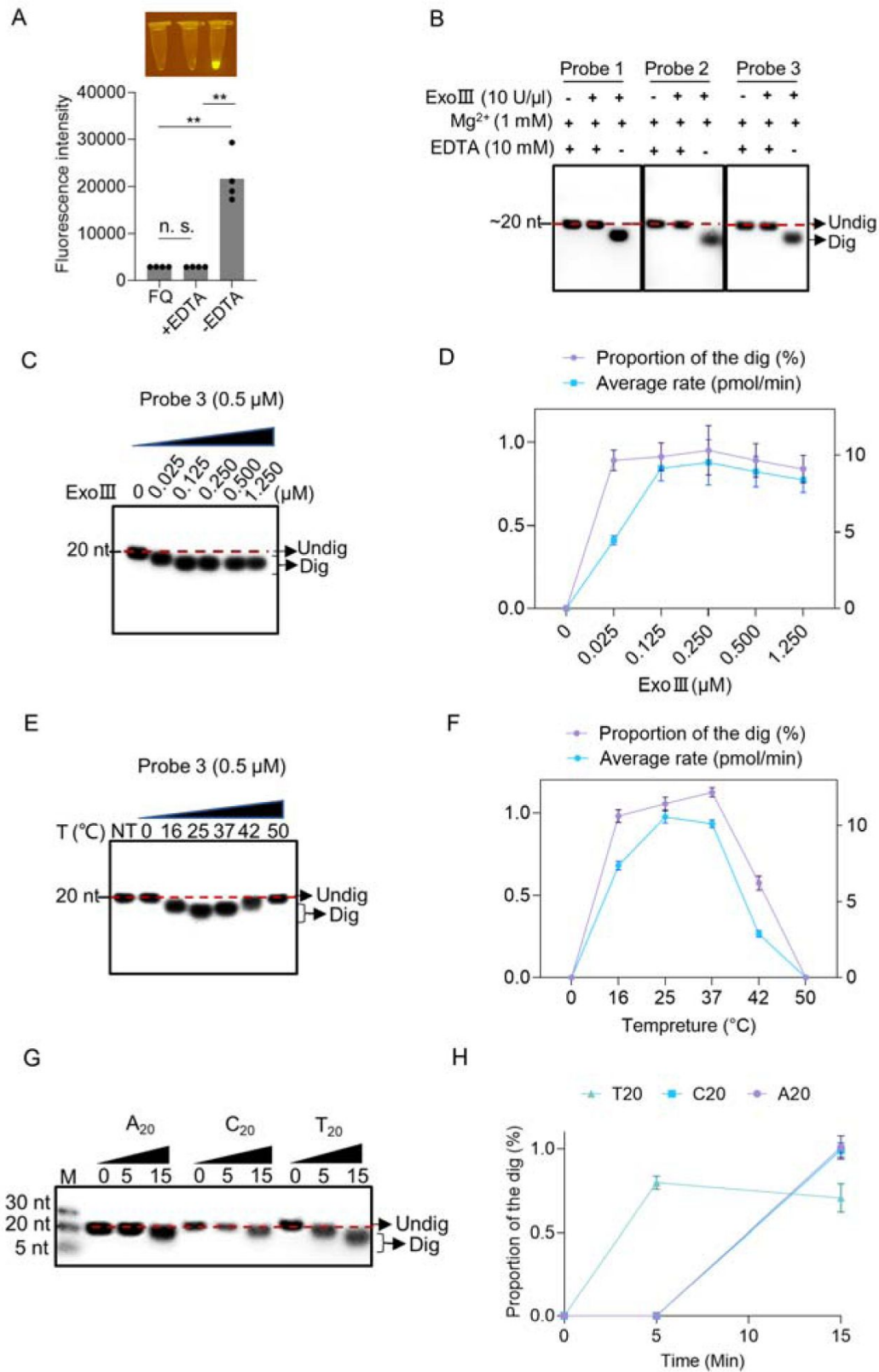


Figure 4.

Enzymatic reaction conditions for the ssDNase activity of ExoIII were investigated. **(A)** EDTA (10 mM) was added with the mixture of FQ reporter (2.5 μ M) and $MgCl_2$ (1 mM) before incubating with commercial ExoIII (5 U/ μ l) for 10 min. The fluorescence intensity generated in 10 min was measured and plotted on four repeats. The corresponding fluorescent tubes at the reaction endpoint were captured under LED UV light. The reaction without adding EDTA was regarded as a control. $**P < 0.01$. The n.s. indicates no significance. Statistical significance was determined by a two-tailed *t*-test. **(B)** Three ssDNA probes (5 μ M) with or without adding EDTA were digested by commercial ExoIII (5 U/ μ l) for 10 min, and the reaction products were analyzed by gel. **(C)** Different amounts of purified ExoIII (0, 0.025, 0.125, 0.250, 0.500, and 1.250 μ M) were incubated with ssDNA Probe 1 (0.5 μ M) for 10 min, and the reaction products were analyzed by gel electrophoresis. **(D)** The digesting rate of ExoIII on the ssDNA was calculated by: the digested proportion $\times$ 50 pmol $\times$ digested nucleotides/reaction time. The digested proportion of Probe 3 was calculated by: (Gray intensity of the digested band)/(Gray intensity of the undigested band with 0 μ M ExoIII). **(E)** The ssDNA substrate (0.5 μ M) was incubated with ExoIII (0.5 μ M) at different temperatures (0, 16, 25, 37, 42, and 50°C) for 10 min, and the products were analyzed by gel electrophoresis. NT, no treatment. **(F)** The digested proportion of Probe 3 was calculated by: (Gray intensity of the digested band)/(Gray intensity of the undigested band (NT)). **(G)** The 5' FAM-labelled ssDNA probes constructed by 20 consecutive identical bases (A_{20} , C_{20} , T_{20}) were digested by ExoIII over 15 min, and the reaction products were separated by gel electrophoresis. The fluorescence intensity difference between the three oligos is caused by the bases covalently linked with a fluorophore. G_{20} was not tested here as it easily forms a G-quadruplex structure. **(H)** The gray intensity was determined by ImageJ. A digested proportion curve over 15 min was plotted from three repeats. The digested proportion was calculated by the formula: (Intensity of the digested band produced)/(Intensity of the band at 0 s). Data represents the average value of three repeats and is expressed as mean $\pm$ SD.

Intriguingly, focusing on the major products of the three ssDNA probes in [Figure 3](#), we found digestion of ExoIII seemingly always stalled in or ahead of a zone of serial identical bases ([Supplementary Table S1](#)), suggesting this type of sequence feature might affect the digestion of ExoIII. To explore if this phenomenon always occurs upon serial identical bases, we performed enzymatic reactions on three 5' FAM-labelled ssDNA probes consisting of 20 nt identical nucleotides. The reaction product was analyzed by gel after these ssDNA substrates (5 μ M) were incubated with purified ExoIII (2.5 μ M) for 15 min. The result of the time course analysis indicated that A_{20} , C_{20} , and T_{20} displayed an obviously slower degradation than the ssDNA substrates in [Figure 2](#) ($A_{20} \approx C_{20} > T_{20}$) ([Figure 4G](#) and [4H](#)), suggesting the consecutive identical nucleotides in ssDNA might slow down the digestion of ExoIII.

ExoIII in the commercial isothermal amplification diagnostic kits digested the ssDNA FQ reporter and probes

Due to the well-acknowledged dsDNA activities of ExoIII, most ExoIII-assisted diagnostic methods require ssDNA to form an appropriate dsDNA intermediate ([Supplementary Table S2](#)). As a promising replacement for PCR technique [16](#), RPA [17](#), [31](#), and MIRA [32](#), [33](#) kits, as well as their derivative products, the MIRA- and RPA-ExoIII kits [34](#), [35](#), have been widely used for nucleic acid detection. To explore if ExoIII contained in the commercial detection kits digests the ssDNA probe or primer, we used an FQ reporter and 5' FAM-labelled ssDNA probes to test the commercial kits of MIRA-ExoIII and RPA-ExoIII, while the RPA and MIRA detection kits (without ExoIII) served as their controls. Our result showed that the RPA-ExoIII and MIRA-ExoIII detection kits exhibited significant fluorescence signals over time compared with those of the RPA and MIRA kits ([Figure 5A](#)). The distinct difference in fluorescence brightness between the ExoIII-presence group (MIRA-ExoIII and RPA-ExoIII) and ExoIII-absence group (MIRA and RPA) can be directly visualized under LED UV blue light ([Figure 5B](#)). These results suggested that the ssDNA FQ reporter was cleaved by ExoIII contained in the commercial detection kit, and this type of FQ reporter was unsuitable for developing the ExoIII-based detection.

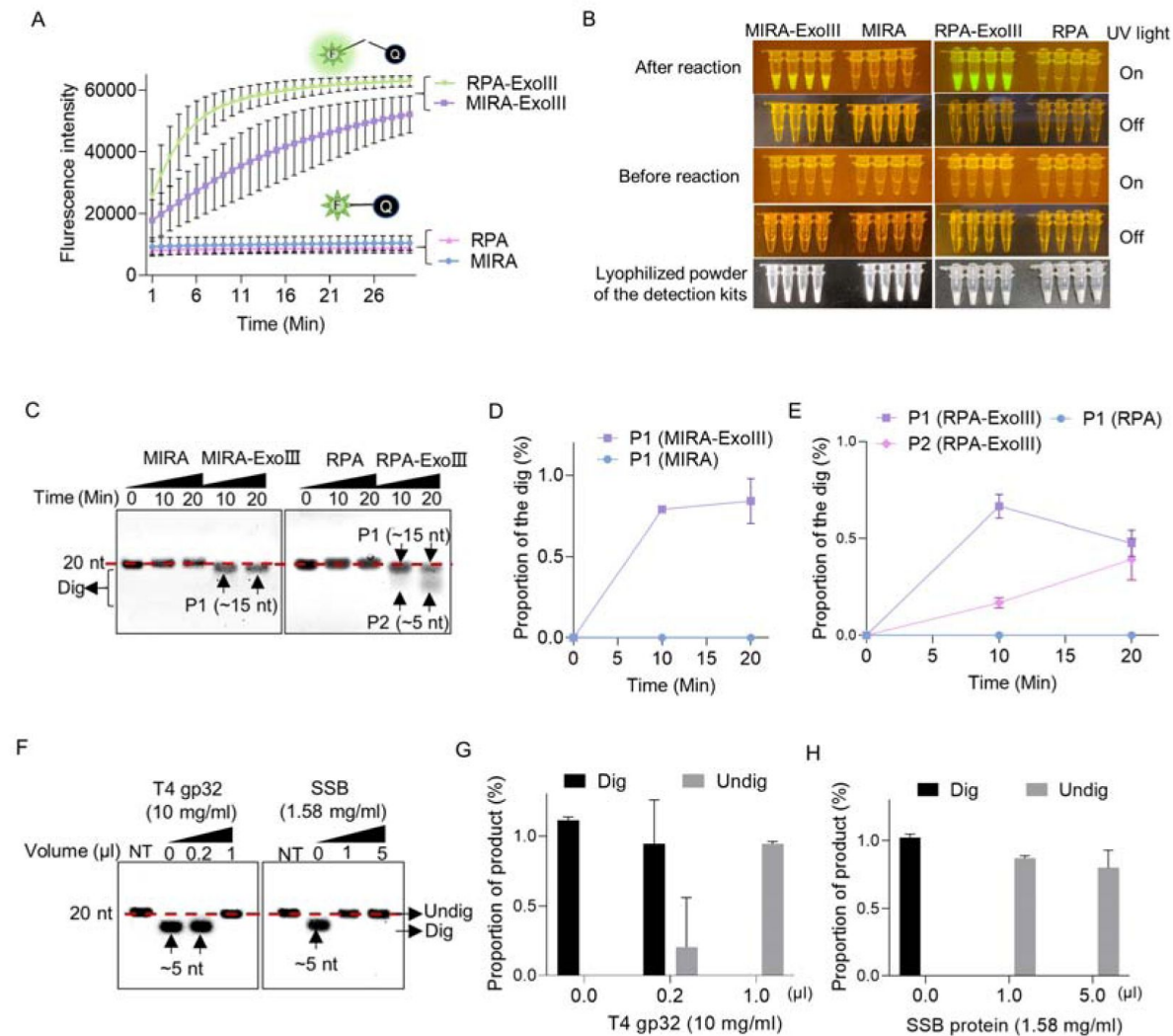


Figure 5.

ExoIII in the isothermal amplification kits digested the ssDNA FQ reporter and 5' FAM-labelled ssDNA probes, while the single-stranded DNA binding protein protected the ssDNA probe from ExoIII digestion. **(A)** The fluorescence of the FQ reporter generated in four detection kits (MIRA, MIRA-ExoIII (containing ExoIII), RPA, and RPA-ExoIII (containing ExoIII)) was monitored for 30 min. MIRA and RPA were the control of their corresponding counterparts, respectively. The average value of three repeats was calculated and plotted. **(B)** Before and after these four commercial kits were incubated with the FQ reporter for 30 min, the reaction tubes with fluorescence generated were visualized under LED blue light by the naked eye. MIRA-ExoIII indicates the ExoIII-containing MIRA kit; MIRA indicates the MIRA kit without ExoIII. **(C)** The 5' FAM-labelled ssDNA probe (Probe 1) was incubated with the four detection kits for 20 min, and the reaction products were analyzed by gel electrophoresis. Undig, undigested; dig, digested. **(D, E)** The analysis on the gray intensities of the band was performed by ImageJ. The digested proportion was calculated by: (Intensity of the digested band)/(Intensity of the undigested band at 0 min). **(F)** Different amounts of single-stranded DNA binding protein (T4 gp32 or SSB (*E. coli*)) were incubated with the ssDNA oligo (Probe 1) for 10 min before being treated with ExoIII for 10 min. The reaction products of ExoIII digestion were separated by gel electrophoresis. The ssDNA oligo treated with deactivated ExoIII served as control. **(G, H)** The gray intensity of bands was measured by ImageJ, and the digested or undigested proportions were calculated by: (Intensity of the digested or undigested band)/(Intensity of the band produced in the treatment of deactivated ExoIII).

Furthermore, to investigate the degradation degree of ssDNA caused by ExoIII in the detection kits, we tested a 5' FAM-labelled ssDNA probe in these detection reactions. We found that 80% of the ssDNA probe added to the MIRA-ExoIII kits was digested to a 15-nt product (P1) (**Figure 5C** and **5D**), while in RPA-ExoIII kit, 50% and 40% of the ssDNA probe were degraded into a ~15-nt (P1) and ~5-nt (P2) in 20 min, respectively, presenting a more intensified degradation than MIRA-ExoIII (**Figure 5C** and **5E**). It suggested ExoIII in the detection kits was capable of digesting the ssDNA probe or primer, leading to a low detection efficiency.

The different degrees of degradation between these two kinds of commercial kits might be caused by different amounts of T4 gp32 added, as T4 gp32 is a ssDNA-binding protein protecting the ssDNA from nuclease degradation³⁶. To investigate whether the ssDNA-binding protein prevents ssDNA from ExoIII digestion, we incubated the ssDNA with two types of ssDNA-binding proteins (T4 gp32 or SSB) before ExoIII digestion. The ssDNA probe pre-incubated with 1 µl T4 gp32 (10 mg/ml), 1 µl or 5 µl SSB (1.58 mg/ml) showed no digested band compared to the control (0 µl) (**Figure 5F**, **5G** and **5H**), which suggested the single-stranded DNA-binding protein was capable of protecting ssDNA from ExoIII digestion. These results indicated that adding more T4 gp32 could offset or minimize the effects of ExoIII' ssDNase activity in the commercial kits. The findings also suggested that ssDNA-binding proteins may regulate *in vivo* the degradation of ssDNA mediated by ExoIII in DNA repair or metabolism processes.

Key amino acid residues for ssDNase activity in ExoIII

To identify the key residues that determine the ssDNase activity of ExoIII, we predicted the structure of ExoIII-ssDNA by molecular docking. And the predicted residues (S217, R216, D214, W212, K176, R170, N153, K121, and Q112) (**Supplementary Figure S3A**) were expressed and purified for mutation analysis (**Figure 6A**, **Supplementary Figure S4A**). Residues (W212, F213, K121, Y109, and D151) reported to play essential roles in the exonuclease and AP-endonuclease activities of ExoIII^{4,8} were also mutated for analysis. The result of the fluorescence monitoring assay on the FQ reporter indicated the mutants of S217A, R216A, K176A, or R170A displayed equal cleavage efficiency with wild type ExoIII on the FQ reporter; D214A, W212A, F213A, Y109A, N153A, and D151N produced no fluorescence signal; K121A or Q112A showed much weaker cleavage than the wild type (**Figure 6B**). Meanwhile, gel electrophoresis on the digested products showed a consistent result with the fluorescence monitoring assay, but K121A exhibited undetectable digestion on the ssDNA probe (**Figure 6C** and **6D**). These results indicated the residues (D214, W212, F213, N153, D151, K121, and Y109) were crucial in the catalyzing of ExoIII on ssDNA. As six of these critical residues (W212, F213, N153, Y109, K121, and D151) have also been found to play critical roles in the dsDNA exonuclease and AP-endonuclease activities of ExoIII (4), the ssDNase activity of ExoIII most likely shares the same active center with the other two enzymatic activities on dsDNA.

Among these mutants, K121A of ExoIII has been reported to have an AP-endonuclease activity on dsDNA⁴. With the weakened ssDNase activity demonstrated in our study, K121A can replace the wild type to be applied in the AP-endonuclease-based commercial kits with minimized effects of ssDNase activity. By comparing the digested products generated by S217A, R216A, K176A, R170A, Q112A, and wild type, we found that R170A produced a larger fragment than other mutants and wild type (**Figure 6E**). It suggested that mutation of R170A affected the efficiency of ExoIII digestion on ssDNA. Based on the conservation indicator of all residues of ExoIII illustrated (<https://consurfdb.tau.ac.il/index.php>), these identified residues (W212, F213, D214, N153, Y109, K121, D151 and R170) were highly conserved among 300 homolog proteins (**Figure 6F** and **6G**). The structure of ExoIII is constructed as a four-layered sandwich, and its active center on dsDNA is located at the valley between the two inner layers^{4,5} (**Figure 6G**).

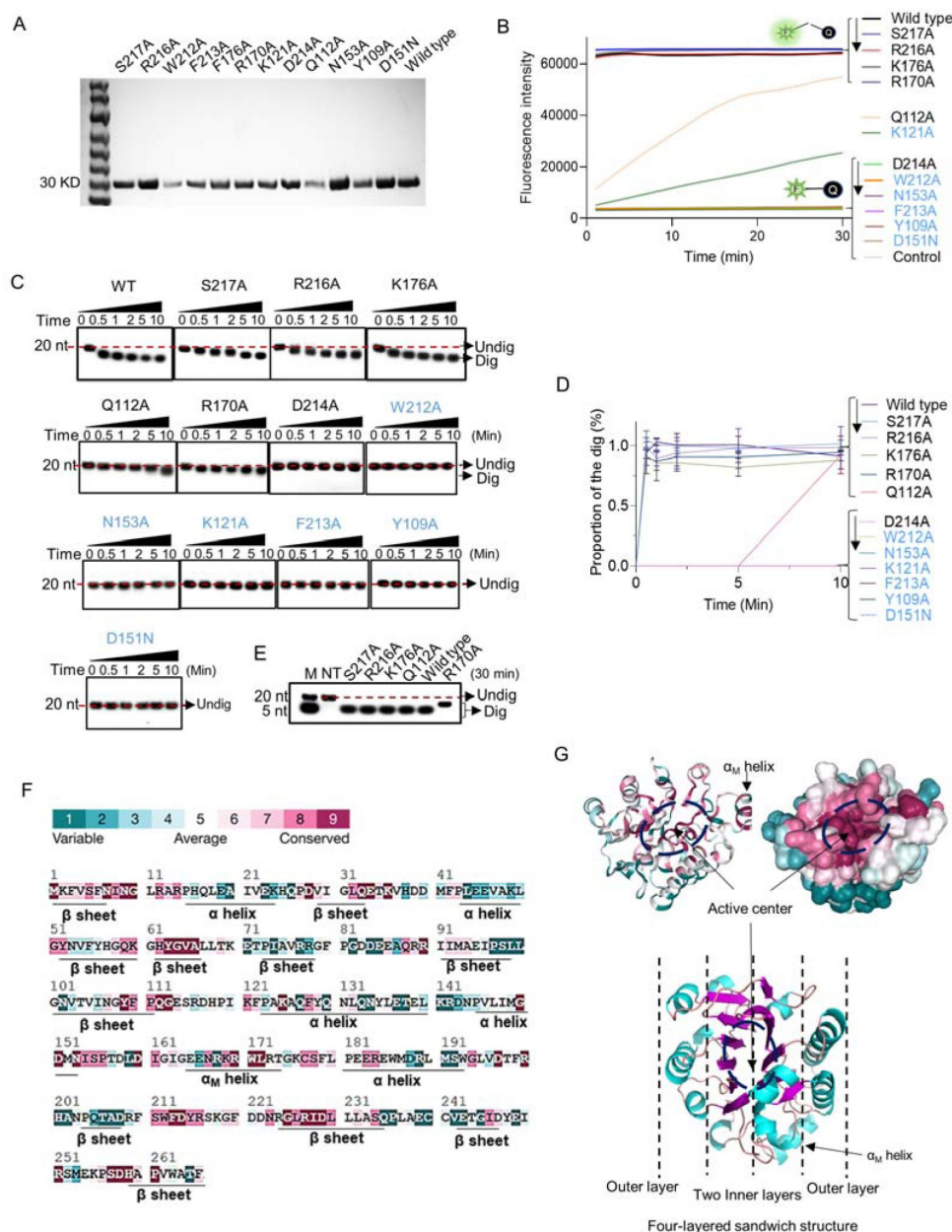


Figure 6.

Point mutation of ExoIII identified the critical amino acid residues that determine the ssDNase activity. **(A)** All purified mutants and wildtype proteins used in the study were loaded into the 8% PAGE electrophoresis and stained with Coomassie Brilliant Blue. **(B)** The FQ reporter (5 μ M) was incubated with ExoIII mutants (2.5 μ M) (S217A, R216A, D214A, F213A, W212A, K176A, D151N, R170A, N153A, K121A, Q112A, Y109A) and wild type (WT) and the generated fluorescence was monitored for 30 min. FQ reporter treated with deactivated wildtype ExoIII was regarded as a control. The mutations in blue indicated they are previously-reported key residues for dsDNA-targeted activities of ExoIII. **(C)** The ssDNA oligo of Probe 3 (20 nt) was incubated with the ExoIII mutants, and the reaction products were analyzed by gel electrophoresis. **(D)** The gray intensity of the bands was determined by ImageJ. The average intensity value of three repeats was used to calculate the proportion of the digested band with: (Intensity of the digested band)/(Intensity of the undigested band at 0 min). **(E)** The digested products of S217A, R216A, K176A, R170A, Q112A, and wild type, after 30 min of incubation with ssDNA substrate, were compared with each other by gel electrophoresis. **(F)** The residue conservation of ExoIII (PDB ID:1AKO) was investigated using the ConSurf database (<https://consurfdb.tau.ac.il/index.php>). The conservation degree of ExoIII residues is calculated among 300 homologs, which the color scale indicates. **(G)** The cartoon and surface style of the ExoIII structure (PDB ID:1AKO) with conservation-indicated color is displayed (upper part). The four-layered sandwich structure of ExoIII is displayed by Pymol (lower part): the pink indicates β -sheet; the blue represents α -helices; the red indicates the random coils or turns.

R170A has an attenuated AP-endonuclease activity

The residue of R170 is located with the protruding structure α_M helix of ExoIII, which helps ExoIII bind to dsDNA by stretching into the major groove [5](#). To investigate if the mutation of R170A also affects the AP-endonuclease and exonuclease activities of ExoIII on dsDNA, we designed specific dsDNA substrates for the two types of activities. On the AP-endonuclease substrate, the mutation of R170 took a much longer time (10 min) to digest compared to the wild type (1 min)

(**Supplementary Figure S5A and S5B**), suggesting the mutation seriously impaired the efficiency of AP-endonuclease activity. The digestion process is described in **Supplementary Figure S5C**. On the exonuclease substrate, the mutant of R170A produced a much larger product than the wild type (**Supplementary Figure S5D and S5E**). It might be because R170A retained the intact exonuclease activity on dsDNA but not on ssDNA, leading to a larger ssDNA intermediate compared to the wild type. The exonuclease activities of ExoIII on dsDNA and ssDNA involved in the digestion are described in **Supplementary Figure S5F**. Altogether, we deemed that R170 in the α_M helix structure may help recognize or stabilize the AP site on dsDNA or ssDNA substrate for further docking into the active site, and the mutation of R170 affects the AP-endonuclease and ssDNase activities of ExoIII but still retains its dsDNA exonuclease activity.

The proposed enzymatic action model of ExoIII on ssDNA

Since the ssDNase activity probably shares the same active center with its dsDNA-targeted activity, an appropriate ssDNA substrate for ExoIII should possess a 'V'-shaped micro-structure, similar to the ssDNA conformation in the B-formed dsDNA (**Figure 7A**). According to the ExoIII-ssDNA structure and the previously reported functions of the key residues [4](#), four primary functional units for enzymatic actions of ExoIII are defined: capturing unit (R170, at 3' side of the cleavage site and three nucleotides away from it), phosphate-stabilizing unit (Y109, K121, and N153, at 5' side of cleavage site and three nucleotides away from it), sugar ring-stacking unit (W212, F213, and D214) and phosphodiester-cleaving unit (D151), all of which also represent four sequential steps in the digestion process (**Figure 7B**). Unit 1 (R170), located at the surface of ExoIII and functioning at the 3' side of the cleavage site, is most likely the first contact with the substrate, the involvement of which naturally drives the endonuclease action on ssDNA. In the endonuclease model, interactions of unit 1 and unit 2 with ssDNA substrates can help convert the ~6 nucleotides at 3' end of ssDNA to form a 'V' shape intermediate, suitable for the following actions of unit 3 and unit 4. Certain ssDNA substrates, such as the consecutive identical bases (**Figure 4**), might have difficulty in converting to the necessary conformation, resulting in the inhibition of ExoIII digestion; Meanwhile, the substrates with "V" conformation internally possibly attract units 2 and 3 of ExoIII binding to the site and triggering the endonucleolytic digestion, which explains the products of endonuclease activity in the mass spectrometry analysis (**Figure 3**). In the exonuclease model, some types of ssDNA (possibly short ssDNA or others with suitable micro-structure) might directly enter the active center without contacting unit 1 and sequentially be processed by units 2, 3, and 4 (**Figure 7C**).

Combining with the structure analysis and the digestion results of **Figure 2** and **3**, we proposed the enzymatic actions of ExoIII digestion on ssDNA: the ssDNA substrate tends to be captured by the outer-layered unit 1, then stabilized by the inter-layered units 2 and 3, finally cleaved by unit 4 of ExoIII, producing short fragments of ssDNA (endonuclease activity). The short ssDNA (~3 nt) or others with suitable micro-structure might directly enter into the active center and be processed by unit 2, unit 3, and unit 4 into nucleotides (exonuclease activity) (**Figure 7D**). The model reflects a distributive catalyzation of ExoIII on ssDNA. In summary, ssDNA substrate is likely to undergo fragmentation by the endonuclease activity of ExoIII, and the resulting small fragments are subsequently digested by the exonuclease activity of ExoIII into mononucleotides. Without the base-pairing of complementary strands, the conformation of ssDNA is easily affected by ion concentration, interacted protein, temperature, and other factors in the solution. All these elements together forge the specific catalytic pattern of ExoIII on ssDNA.

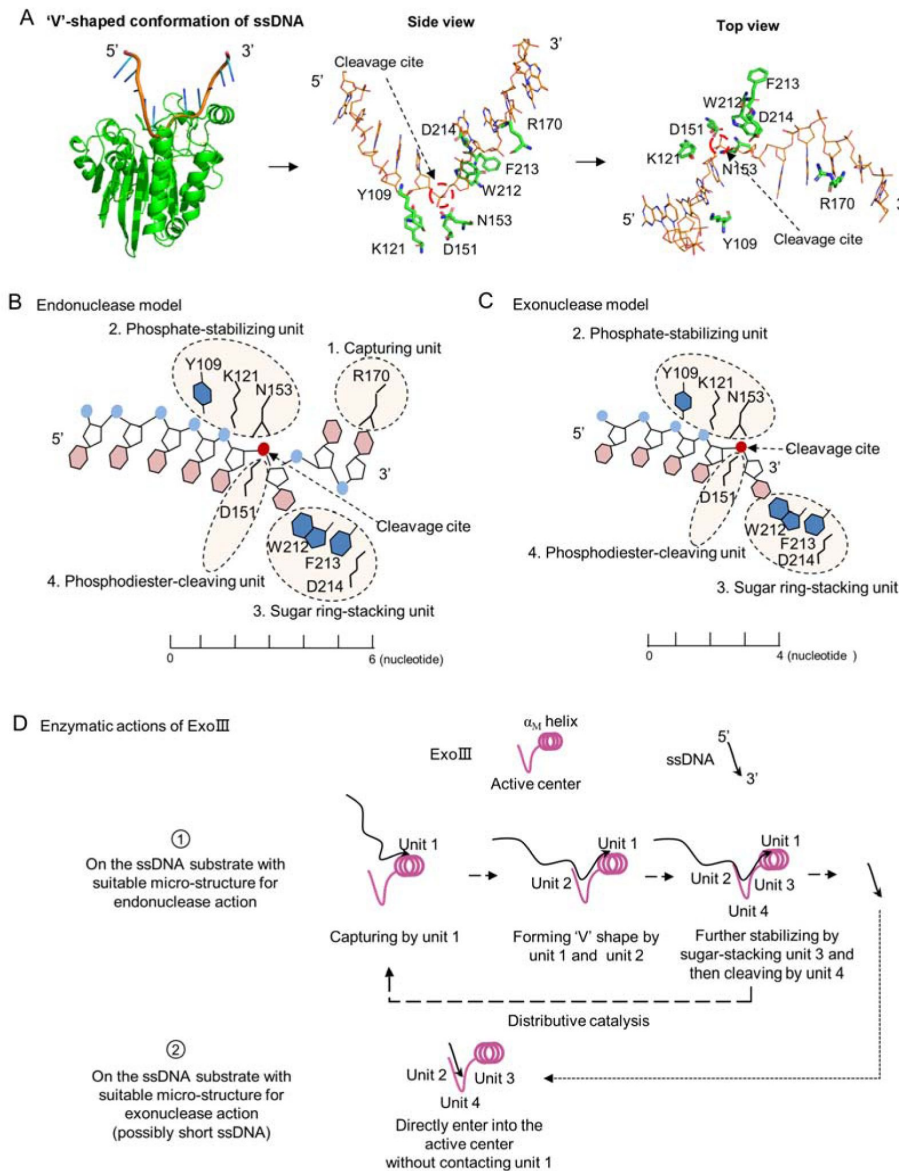


Figure 7.

A theoretical model for the enzymatic actions of ExoIII on ssDNA is proposed. **(A)** The ExoIII-ssDNA structure was obtained by structure alignment between ExoIII (PDB:1AKO) and APE1-dsDNA (PDB: 5WN5) by Pymol. The key residues and the cleavage site of ExoIII are labelled in situ and presented by side and top view. **(B)** According to the ExoIII-ssDNA structure and the previously reported functions of the key residues (4), four functional units of ExoIII on ssDNA are primarily defined: capturing unit (R170, at 3' side of the cleavage site and three nucleotides away from it), phosphate-stabilizing unit (Y109, K121, and N153, at 5' side of cleavage site and three nucleotides away from it) and sugar ring-stacking unit (W212, F213, and D214) and phosphodiester-cleaving unit (D151). In the model of endonuclease, Unit 1(R170), located at the surface of ExoIII, is most likely the first contact with the substrate, and binding at the 3' side of the cleavage site naturally drives the endonucleolytic cleavage on ssDNA. Interactions of unit 1 and unit 2 with substrates may help convert the ~6 nucleotides at 3' end of ssDNA to form a 'V' shape intermediate for docking into the active center and being processed by unit 3 and unit 4. **(C)** In the exonuclease model, some types of ssDNA (possibly short ssDNA or others with suitable micro-structure) directly enter into the active center without contacting unit 1, and sequentially processed by unit 2, 3 and 4. **(D)** The ssDNA substrate tends to be captured by the unit 1, then stabilized by the unit 2 and 3, and finally cleaved by unit 4 of ExoIII, producing short fragments of ssDNA (endonuclease activity), which may reflect the distributive catalysis of ExoIII on ssDNA. The short ssDNA (~3 nt) or others with suitable microstructure would directly enter into the active center and be processed by units 2, 3, and 4 into mononucleotides (exonuclease activity). Most ssDNA substrates might be digested by both endonuclease and exonuclease activity of ExoIII.

ExoIII digested the dsDNA structures containing 3' end ssDNA

To explore the biological significance of the ssDNase activity of ExoIII, we examined several dsDNA substrates containing ssDNA (dsDNA with 3' ssDNA flap, dsDNA with 3' protruding ssDNA tail, bubble and fork-shaped dsDNA) that might occur in the biological process. After being treated with ExoIII, our result demonstrated that ExoIII was capable of digesting the dsDNA with a 3' flap (**Figure 8A**). The 3' flap-removing capability of ExoIII may originate from its ssDNase activity (**Figure 8B**). The analysis of the gray intensity indicated the dsDNA with 3' flap was digested with time (**Figure 8C**). Based on our results and the gap creation ability of ExoIII previously reported⁸, we proposed the novel biological role of ExoIII in DNA repair: when the 3' flap occurs, ExoIII recognize and remove the ssDNA flap by the ssDNase activity; then it continues to digest the ssDNA on dsDNA and create a gap; the resulting intermediate recruits the DNA polymerase to re-synthesize the complementary strand and fill the ssDNA gap; finally, the nick left is sealed by DNA ligase (**Figure 8D**).

We next tested its ability to cleave the dsDNA substrates with a protruding ssDNA (≥ 4 nt) at the 3' end, previously reported as resistant to ExoIII digestion¹². Our results on the dsDNA with a 10-base protruding structure indicated that ExoIII could rapidly digest the dsDNA with a 3' end 10-base ssDNA tail (**Supplementary Figure S6A and S6B**). But for the dsDNA with 4-base protruding ssDNA at the 3' end, the digestion occurred at 10 min, suggesting the efficiency of digestion significantly decreased to about one-tenth of the 10-base protruding structure (**Supplementary Figure S6C and S6D**). Unlike the previous study¹², our results demonstrated that ExoIII could catalyze dsDNA structure with 3' end protruding bases (≥ 4 nt). Furthermore, we found when the 3' end 4-base protruding ssDNA was replaced with four adenylates, the dsDNA showed no digestion over 10 min (**Supplementary Figure S6E and S6F**), suggesting the 3' A₄-protruding ssDNA was resistant to the ssDNase activity of ExoIII and protected the dsDNA duplex from ExoIII digestion. Therefore, dsDNA with 3' A₄-protruding ssDNA can be used for related enzymatic research of ExoIII or as the aptamer of biosensors, as the previous typical substrate may not protect itself from being cleaved by the ssDNase activity and the following exonuclease of ExoIII. Based on the structural analysis that indicated the α_M helix was exactly 4~5 nt apart from the AP site (located in the active center) (**Supplementary Figure S6G and S6H**), we speculated that the lower efficiency of digestion on the 4-base protruding dsDNA might be because the topology of dsDNA-ssDNA junction site was not well recognized by α_M helix, which was unfavourable for the ssDNA further docking into the active center.

The fork structure was slightly digested by ExoIII in the first minute and completely degraded over 10 min (**Supplementary Figure S7A**). To test if the ssDNA binding protein affects the digestion of ExoIII, the fork structure was incubated with T4 gp32 for 10 min before adding ExoIII. The result indicated that 1 μ L T4 gp32 (10 mg/mL) protected over 40% of the substrate from digestion, significantly higher than 0.2 μ L (~10%) (**Supplementary Figure S7A**). This protection effect suggested that ExoIII started the digestion of the structure from the 3' end ssDNA, and the binding of T4 gp32 to the 3' end ssDNA prevented the dsDNA from being digested (**Supplementary Figure S7B**). The analysis on gray intensity showed ExoIII was able to digest the fork dsDNA structure and the single binding protein delayed the process by binding to the ssDNA part (**Supplementary Figure S7C and S7D**). The bubble structure showed no apparent digestion over time (**Supplementary Figure S7E and 7F**). Collectively, these results indicated ExoIII may function in more types of dsDNA structures with 3' ssDNA in DNA-repair or -metabolism processes.

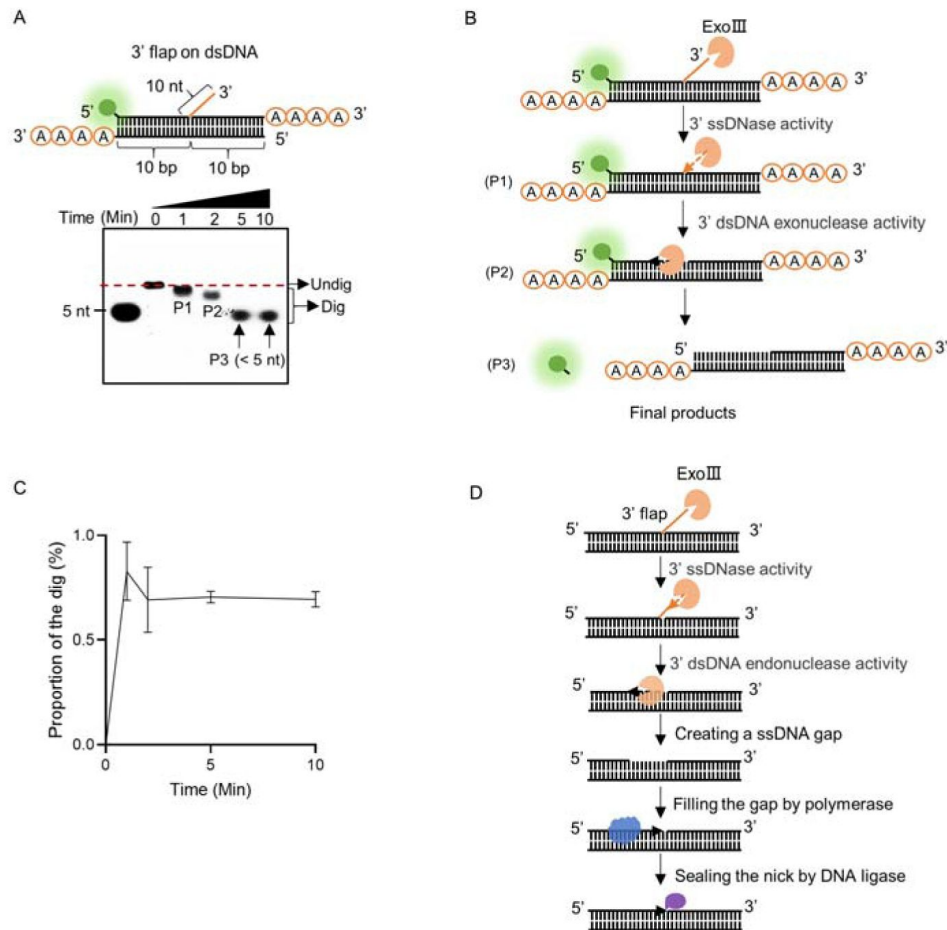


Figure 8.

ExoIII efficiently digested the 3' flap ssDNA on dsDNA. **(A)** The constitution of the dsDNA structure with 3' flap is displayed. The time course analysis of ExoIII (2.5 μ M) digestion on the structure (5 μ M) was performed, and the products were analyzed by gel electrophoresis. **(B)** According to the activities of ExoIII on ssDNA and dsDNA, the process of enzymatic reaction on the dsDNA structure was outlined. **(C)** The digested proportions of ssDNA substrates were calculated by: (gray intensity of the digested band)/(gray intensity of the undigested band at 0 min) and plotted based on an average value of three repeats. **(D)** Based on our result and the gap-creation ability of ExoIII [8](#), a novel biological role of ExoIII in DNA repair was proposed: when the 3' ssDNA flap occurs on dsDNA during biological processes such as BER or DNA replication, ExoIII recognizes and removes the ssDNA flap by its ssDNase activity; then it continues to digest and create a ssDNA gap on the dsDNA by exonuclease activity; the resulted intermediate recruits the DNA polymerase to re-synthesize the complementary strand; finally, the nick left is sealed by DNA ligase.

Discussion

Defining the ssDNase activity of ExoIII is crucial for understanding its biological roles and guiding the current diagnostic applications. In this study, we adopted the sensitive ssDNase indicator FQ reporters, a range of short ssDNA substrates (~20 nt), and mass spectrometry analysis for prudent confirmation and systematic characterization of the ssDNase activity of ExoIII. Our results uncovered that ExoIII possessed highly efficient 3' → 5' exonuclease and endonuclease activities on ssDNA, with the cleavage site at the 5' side bond of the phosphodiester. As the discovery may remould the roles of ExoIII in its industrialized application and biological understanding in the DNA repair process, we assessed its effects by testing the popular commercialized kits containing ExoIII and investigated its possible biological functions through the dsDNA structures containing ssDNA that might occur *in vivo*. Our results showed that ExoIII digested ssDNA probes or primer in the isothermal amplification kit and dsDNA structures containing 3'-end ssDNA. To minimize the effects of ExoIII' ssDNase activity, we advise: a) adding more single-strand DNA binding protein T4 gp32 protects the primer or ssDNA probe from degradation of ExoIII in the isothermal amplification kits; b) adding four or more consecutive adenylates at the 3'-end of the dsDNA structure or ssDNA helps inhibit the degradation of ExoIII; c) with limited ssDNase activity, K121A and R170A mutants can replace the wild type and be applied in the AP-endonuclease and exonuclease based diagnostics, respectively; d) raise the reaction temperature to ≥ 42°, as the ssDNase activity of ExoIII was found highly suppressed in the temperature range. Considering that the ssDNase activity of ExoIII shares the same active center as its dsDNA-targeted activity, examining its biological roles *in vivo* by individual point mutation seems challenging. But digesting the dsDNA with 3' flap ssDNA or 3' protruding ssDNA tail *in vitro* indicated the ssDNase activity of ExoIII may confer a 3' flap-cleaving ability, suggesting it might be competent to deal with more types of DNA lesions. During this, the ssDNA-binding proteins may play a regulatory role in ExoIII-related ssDNA degradation *in vivo*. As a multi-functional nuclease, the enzymatic activities of ExoIII identified so far are summarized in **Supplementary Table S3**.

The endonuclease activity of ExoIII on ssDNA was confirmed for the first time by mass spectrometry analysis on Probe 1 and Probe 2. It might explain why digested products of ExoIII and LbCas12a only accounted for 50%~80% of their deactivated counterparts (**Figure 3C**, **3G** [↗](#), and **3K** [↗](#)). The lost 20~50% fluorescence is possibly caused by ExoIII or LbCas12a cleaving the substrates and forming products with different lengths, which travelled in the gel at different speeds, finally leading to being dispersed or invisible under UV light. Combining these results with the analysis of the constructed crystal structure of ExoIII-ssDNA, we proposed a theoretical model for understanding the underlying mechanism of endonuclease and exonuclease actions of ExoIII on ssDNA. Steven A. Benner and his coworkers observed that ExoIII could bypass two consecutive nucleotides with phosphorothioate modification and continued deleting the ssDNA ²¹[↗](#), which might be well-explained by endonuclease activity in the model. In addition, its human homolog APE1 has been reported to have a strong binding affinity to the G-rich oligo (5'-AGGGCGGTGTGGGAAGAGGGAAGAGGGGAGG-3', a 32-nt sequence of the oncogene KRAS promoter) ³⁷[↗](#). Thus, ExoIII also possibly bound to the G-rich region of Probe 2 and activated its endonuclease activity, producing more middle fragments than the other two ssDNA probes, as indicated in mass spectrometry analysis.

Another interesting finding is that the consecutive identical bases of ssDNA stalled the digestion of ExoIII. In the early 1960s, the inactivity of ExoIII on ssDNA was coined by Kornberg's laboratory based on an ssDNA oligo of five consecutive Ts (5' pTpTpTpTpT 3') ¹¹[↗](#) (which might not be sufficiently prudent to conclude that ExoIII is inactive on all ssDNA). In 2007, Steven A. Benner and his coworkers pointed out for the first time that ExoIII could degrade ssDNA by incubating with two long ssDNA oligos (51 nt) ²¹[↗](#). An approximate 43-mer band occurred as the superior product when the concentration of ExoIII decreased, but the reason remains unclear. Here, based on our

conclusion, the 43-mer intermediate exactly corresponds to the product generated when ExoIII stalled its digestion ahead of the consecutive GGG (Position 40-42) of the ssDNA substrate in 3' → 5' direction (5'-GCGTAATACGACTCACTATAGACGAGCGTACTTTAGTGAGGGTTAATT CGC-3'). In addition, adding 10 T bases to an ssDNA at the 3' end has been indicated to suppress ExoIII digestion¹⁸; a *Saccharomyces cerevisiae* homolog of ExoIII, CCR4 (an ssDNA exonuclease), has also been reported to have a slower degradation on the substrate with 3' end five consecutive As and was unable to cleave when the ssDNA contained ten consecutive As within³⁸. Based on our proposed model, the sequence feature of consecutive identical bases in ssDNA may create an unfavorable local microstructure that can impede ExoIII digestion.

Our work uncovers the long-underestimated ssDNase activity of ExoIII, dissects the underlying enzymatic behaviors of ExoIII on ssDNA and advances the mechanism understanding of its biological roles in DNA repair. Future investigations might be conducted: 1) crystal structure of the ExoIII-ssDNA complex (endonuclease and exonuclease model); 2) potential biological roles of the ssDNase activity *in vivo*. A better understanding of the underlying enzymatic actions of this multifunctional enzyme will facilitate efficient and reliable applications of ExoIII-based nucleic acid diagnostics.

Materials and methods

Material and reagents

To avoid any potential secondary structure formed, we used short ssDNA oligos (5~23 nt) (Sunya Biotechnology Co., Hangzhou, Zhejiang Province, China) as the substrate for the nuclease treatment. The FQ reporter was a synthesized ssDNA oligo labelled with a fluorophore (FAM or FITC) at its 5' end and a quencher (BHQ1) at its 3' end. The FQ ssDNA reporter is a sensitive sensor of ssDNA-cleaving activity, enabling a fluorescence signal's visible emission upon ssDNase-like activity-mediated cleavage²⁶. The other reporter (termed the FB reporter) was an ssDNA oligo labelled with FAM at the 5' end and biotin at the 3' end, which is tailored for use in lateral flow strips and yields a positive result upon cleavage by ssDNase activity, as indicated by the appearance of two lines on the strip²⁶. All the sequences and related modifications are detailed in **Supplementary Table S4**. ExoIII (NEB#M0206V), APE1 (NEB#M0282S), T7 exonuclease (T7 exo) (NEB#M0263L) and LbCas12a (NEB#M0653S) were bought from New England Biolabs (Ipswich, MA, USA). The solutions of MgCl₂ and EDTA-2Na (referred to as EDTA) (Sinopharm Chemical Reagent Co., Ltd. Shanghai, China) were respectively prepared with deionized water to concentrations of 10 mM (pH = 7) and 100 mM (pH = 8). The 5' FAM-labelled ssDNA constructed with 5 nt, 15 nt, 20 nt, and 30 nt adenylates were synthesized and used as the ssDNA marker in the gel electrophoresis.

Protein expression and purification

The protein expression and purification followed the method in the previous study⁴. The wild type of ExoIII gene (*E. coli*) (GeneID:946254) and the DNA fragments of mutants (S217A, R216A, D214A, F213A, W212A, K176A, D151N, R170A, N153A, K121A, Q112A, Y109A) were cloned into pET-30a (6×His–ExoIII) by using ClonExpress II One Step Cloning Kit (Vazyme Biotech, Nanjing, China). These vectors were transformed into Rosetta (DE3) *E. coli* (LGC Biosearch Technologies, Hoddlesdon, UK). After the plasmids were confirmed by sequencing, cells were placed in a 37°C incubator (adding 100 µg/mL kanamycin) and shaken until the OD₆₀₀ = 0.6. Then, the culture was added with 1 mM IPTG (final concentration) and incubated at 16°C shaking incubator for 16 hours. Cells were collected by centrifugation at 7500 × g for 10 min and resuspended in 20 mL PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH=7.8). The suspensions were sonicated with 4 s pulse-on and 6 s pulse-off (P = 150W) for 15~30 min. Products were centrifuged at 15000 × g for 15 min, and then the supernatants were retained and filtered by micro syringe filters (0.45 µm). The protein was purified with HisSep Ni-NTA 6FF Chromatography Column

(Yeasen Biotechnology Co., Ltd., Shanghai, China). After dialysis in PBS buffer, these proteins were aliquoted with 20% glycerol and stored at -80°C . The tag does not affect the enzymatic activity of ExoIII ⁴ (Supplementary Figure S3).

Fluorescence monitoring assay on FQ reporter

In the assay, 5 μM ssDNA FQ reporter was incubated with ExoIII (5 U/ μl), T7 exo (5 U/ μl), APE1 (5 U/ μl) or LbCas12a-crRNA-activator complex (0.1 μM). To form the LbCas12a-crRNA-activator complex, LbCas12a crRNA and the activator dsDNA are pre-incubated at 37°C for 10 min before incubating with FQ reporter. The corresponding buffer for each nuclease was added based on the manufacturer's document, and the total reaction volume was supplemented to 10 μl by the nuclease-free water. During the incubation, the fluorescence value was recorded every minute for over 30~60 min at 37°C by Mx3500P (Agilent, CA, USA). The endpoint detection here was defined as detecting the accumulated fluorescence once at the end of the reaction. The image of fluorescence excited by LED blue light was captured in an Ultra Slim LED illuminator (Miulab, Hangzhou, Zhejiang Province, China).

Enzymatic reaction on ssDNA

The enzymatic reaction contained 5 μM fluorophore-labelled ssDNA oligos, 5 U/ μl commercial ExoIII or 2.5 μM purified wildtype and mutant ExoIII, 1 μl 10x NEbuffer #1. The total reaction volume was supplemented to 10 μl by nuclease-free water. Before being treated with T7 exonuclease (T7 exo) (5 U/ μl), APE1 (5 U/ μl), ExoIII or LbCas12a-crRNA-activator complex (0.1 μM), the ssDNA oligos were incubated at 95°C for 5 min, then cooled down in the ice. The nucleases were deactivated by heat at 95°C for 5 min and used as controls (D-T7 exo, D-APE1, D-ExoIII, and D-LbCas12a). After incubating in a 37°C water bath for 40 min, enzymatic reaction products were subjected to gel electrophoresis.

Different dsDNA substrates were prepared to investigate the roles of ExoIII in DNA repair. An equivalent amount of complementary ssDNAs (10 μM) was mixed and annealed to each other by heating at 95°C for 5 min and cooling down at room temperature for 30 min. The generated dsDNA (5 μM) was incubated with ExoIII (2.5 μM), and the reaction product was then analyzed by gel electrophoresis.

The trans-cleavage reaction of LbCas12a on ssDNA

For the reaction of LbCas12a on ssDNA, crRNA and dsDNA activators for the trans-cleavage activity of LbCas12a were prepared. The crRNA was synthesized using the T7 *in vitro* transcription kit (Vazyme Biotech, Nanjing, China). The dsDNA template for transcription was designed by annealing a single-stranded DNA (ssDNA) comprising T7 promoter and crRNA sequence (SUNYA Biotechnology Co., Ltd, Zhejiang, China) to its complementary oligonucleotides. A total volume of 20 μl was prepared by mixing template dsDNA (~100 ng), 2 μl of T7 RNA polymerase, 8 μl of NTP buffer, 2 μl of reaction buffer, and nuclease-free water. The reaction mix was incubated at 37°C water bath for 16 h. The transcription product was treated with TURBO™ DNase (Thermo Fisher Scientific, Massachusetts, USA) at 37°C for 30 min and purified by Monarch RNA Cleanup Kit (New England Biolabs, Massachusetts, USA). The concentration of crRNA was determined by Nanodrop 2000 (Thermo Fisher Scientific, Massachusetts, USA). The dsDNA activator for the trans-cleavage activity of LbCas12a was created by annealing complementary ssDNAs containing PAM and the target sequence against crRNA. To form the LbCas12a-crRNA-activator complex, LbCas12a crRNA, and the activator dsDNA are pre-incubated at 37°C for 10 min before incubating with an ssDNA probe or FQ reporter. The sequences are presented (Supplementary Table S4).

The fluorescence-based gel electrophoresis assay

The gel was prepared with a concentration of 6% (wt/vol) agarose (HydraGene Co., Ltd, Xiamen, Fujian Province, China). After incubating with ExoIII, the reaction mixtures mixed with a 6×loading buffer (Takara, Beijing, China) were loaded into the gel. The electrophoresis (EPS300, Tanon, Shanghai, China) was carried out at 130 V for ~40 min in Tris-acetate-EDTA (TAE) running buffer (400 mM Tris, 25 mM EDTA, adding acetic acid to pH =7.8). A dark environment was required during electrophoresis to slow down fluorescence extinction. The abundance of reaction products was indicated by fluorescence bands under UV light, which was visualized and imaged by a multifunctional ultra-sensitive imaging system (Shenhua Science Technology Co. Ltd., Hangzhou, Zhejiang Province, China).

Mass spectrometry analysis

After 5 μM fluorophore-labelled ssDNA oligo was incubated with 5 U/μl ExoIII at 37°C for 0.5 h, the enzymatic reaction was stopped by heating at 95°C for 5min. The reaction product was then analyzed by mass spectrometry. The reaction containing only ssDNA oligo or ExoIII was prepared as the control. Before incubation at 37°C, the ssDNA oligos were pre-incubated at 95°C for 5 min and cooled in the ice. Hippo Biotechnology Co., Ltd. performed the mass spectrometry (Thermo LTQ-XL, Massachusetts, USA) analysis on the samples (Huzhou, Zhejiang, China). The detection range of molecular weight in mass spectrometry is 500~20000 Da. Electron Spray Ionization (ESI) was used to ionize the molecules.

Molecular docking

Molecular docking of ExoIII (PDB code: 1AKO) and ssDNA (NDB ID: 1S40) was performed in a webserver (<https://wenmr.science.uu.nl/haddock2.4/>)^{39,41}. Before the docking, we performed structure alignment or superimposing on ExoIII and APE1-dsDNA (PDB ID: 1DE8 and NDB ID: 5WN5) complex through TM-align (Version 20190822) (<https://zhanggroup.org/TM-align/>)⁴². We extracted the structure of the ExoIII-ssDNA complex by PyMOL⁴³ (**Supplementary Figure S7**). Residues of ExoIII located within 5 Å of the ssDNA and the last four nucleotides at the 3' end of the ssDNA were selected to input in the HADDOCK website for molecular docking. Docking parameters were set by default. The output structure of ExoIII-ssDNA with the highest score was visualized by PyMOL for further analysis.

Evaluation of the ExoIII' ssDNase activity in the commercial diagnostic kits

To examine the effects of ExoIII' ssDNase activity on the commercial isothermal amplification detection kit containing ExoIII, we tested four kits including RPA (recombinase polymerase amplification) with ExoIII (TwistAmp® exo) or without ExoIII (TwistAmp® Basic) (Abbott, Illinois, US), MIRA (Multienzyme Isothermal Rapid Amplification) with (Fluorescence type) or without ExoIII (Basic type) (Amplification Future company, Weifang, Shandong Province, China) by FQ reporter and 5'-FAM-labelled ssDNA probe. All the reagents were added to the lyophilized powder following the usage instructions. Then 5 μl ssDNA FQ reporter or probe (10 μM) was added to the reaction mix. The tubes containing the reaction mixture were incubated in Mx3500P (Agilent, Santa Clara, CA, USA) at 37°C for 30 min to monitor the fluorescence generated. The endpoint fluorescence was visualized under an LED blue light transilluminator (THBC-470) (TUOHE ELECTROMECHANICAL TECHNOLOGY CO., LTD, Shanghai, China).

Data availability

The data underlying this article are available from the corresponding author upon request.

Acknowledgements

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Contributions

H.W. performed the majority of experiments and wrote the manuscript; C.Y., Q.L., Z.J. and N.L. helped with electrophoresis experiments, protein purification and figure preparation; C.J., C.Z., C.Z., and G.Z. helped with the data analysis and offered technical support; M.Y. and Y.L. acquired funding and supervised the research; all authors discussed the results and commented on the manuscript.

Conflict of Interest

The authors declare no competing interests.

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Reviewer #2 (Public Review):

Summary:

This paper describes some experiments addressing 3' exonuclease and 3' trimming activity of bacterial exonuclease III. The quantitative activity is in fact very low, despite claims to the contrary. The work is of low interest with regard to biology, but possibly of use for methods development. Thus the paper seems better suited to a methods forum.

Strengths:

Technical approaches.

Comments on revised version:

All concerns have been addressed.

<https://doi.org/10.7554/eLife.95648.2.sa1>

Reviewer #3 (Public Review):

Overall:

ExoIII has been described and commercialized as a dsDNA specific nuclease. Several lines of evidence, albeit incomplete, have indicated this may not be entirely true. Therefore, Wang et al comprehensively characterize the endonuclease and exonuclease enzymatic activities of ExoIII on ssDNA. A strength of the manuscript is the testing of popular kits that utilize ExoIII and coming up with and testing practical solutions (e.g., addition of SSB proteins ExoIII variants such as K121A and varied assay conditions).

Comments:

- (1) The footprint of ExoIII on DNA is expected to be quite a bit larger than 5-nt, see structure in manuscript reference #5. Therefore, the substrate design in Figure 1A seems inappropriate for studying the enzymatic activity and it seems likely that ExoIII would be interacting with the FAM and/or BHQ1 ends as well as the DNA. Could this cause quenching? Would this represent real ssDNA activity? Is this figure/data necessary for the manuscript?
- (2) Based on the descriptions in the text, it seems there is activity with some of the other

nucleases in 1C, 1F, and 1I other than ExoIII and Cas12a. Can this be plotted on a scale that allows the reader to see these relative to one other?

(3) The sequence alignment in Figure 2N and corresponding text indicate a region of ExoIII lacking in APE1 that may be responsible for their differences in substrate specificity in regards to ssDNA. Does the mutational analysis support this hypothesis?

<https://doi.org/10.7554/eLife.95648.2.sa0>

Author response:

The following is the authors' response to the original reviews.

eLife assessment

This manuscript highlights single-stranded DNA exo- and endo-nuclease activities of ExoIII as a potential caveat and an underestimated source of decreased efficiency in its use in biosensor assays. The data present convincing evidence for the ssDNA nuclease activity of ExoIII and identifies residues that contribute to it. The findings are useful, but the study remains incomplete as the effect on biosensor assays was not established.

Reviewer #1 (Public Review):

Summary:

In this manuscript, the authors show compelling data indicating that ExoIII has significant ssDNA nuclease activity that is posited to interfere with biosensor assays. This does not come as a surprise as other published works have indeed shown the same, but in this work, the authors provide a deeper analysis of this underestimated activity.

Response: Thank you so much for reviewing and summarizing our work.

Strengths:

The authors used a variety of assays to examine the ssDNA nuclease activity of ExoIII and its origin. Fluorescence-based assays and native gel electrophoresis, combined with MS analysis clearly indicate that both commercial and laboratory purified ExoIII contain ssDNA nuclease activity. Mutational analysis identifies the residues responsible for this activity. Of note is the observation in this submitted work that the sites of ssDNA and dsDNA exonuclease activity overlap, suggesting that it may be difficult to identify mutations that affect one activity but not the other. In this regard, it is of interest the observation by the authors that the ssDNA nuclease activity depends on the sequence composition of the ssDNA, and this may be used as a strategy to suppress this activity when necessary. For example, the authors point out that a 3' A4-protruding ssDNA could be employed in ExoIII-based assays due to its resistance to digestion. However, this remains an interesting suggestion that the authors do not test, but that would have strengthened their conclusion.

Response: Thank you so much for the positive evaluation and insightful comments on our manuscript. In the revised version, we have modified the manuscript to address the reviewer's concerns by providing point-to-point responses to all the comments.

Weaknesses:

The authors provide a wealth of experimental data showing that E. coli ExoIII has ssDNA nuclease activities, both exo- and endo-, however this work falls short in showing that

indeed this activity practically interferes with ExoIII-driven biosensor assays, as suggested by the authors. Furthermore, it is not clear what new information is gained compared to the one already gathered in previously published works (e.g. references 20 and 21). Also, the authors show that ssDNA nuclease activity has sequence dependence, but in the context of the observation that this activity is driven by the same site as dsDNA Exo, how does this differ from similar sequence effects observed for the dsDNA Exo? (e.g. see Linxweiler, W. and Horz, W. (1982). Nucl. Acids Res. 10, 4845-4859).

Response: We agree with the reviewer regarding the limitations in showing the practical influence of the ssDNase activity in the commercial detection kit. Different from the biosensor in reference 20, our results showed a potential impact of ExoIII on another frequently used detection system, as the primer and probe required for the detection kit could be digested by ExoIII, leading to a lower detection efficiency. Since the activities of ExoIII on ssDNA and dsDNA share a same active center, we reason that the difference in sequence specificity of ExoIII on these two types of substrates might be caused in two aspects: on the nuclease, some unidentified residues of ExoIII that play an auxiliary role in digesting ssDNA but not in dsDNA, might exist, which contribute to the difference we observed; on the substrate structure, without the base-pairing of complementary sequence, the structure of ssDNA is more flexible (changeable with environmental factors such as ions and temperature) than that of dsDNA. The two aspects may collectively result in the difference in sequence specificity of ExoIII on ssDNA and dsDNA. We believe that cryo-electronic microscopy-based structure analysis of the ExoIII-ssDNA complex would provide more comprehensive and direct evidence.

Because of the claim that the underestimated ssDNA nuclease activity can interfere with commercially available assays, it would have been appropriate to test this. The authors only show that ssDNA activity can be identified in commercial ExoIII-based kits, but they do not assess how this affects the efficiency of a full reaction of the kit. This could have been achieved by exploiting the observed ssDNA sequence dependence of the nuclease activity. In this regard, the work cited in Ref. 20 showed that indeed ExoIII has ssDNA nuclease activity at concentrations as low as 50-fold less than what test in this work. Ref 20 also tested the effect of the ssDNA nuclease activity in Targeted Recycle Assays, rather than just testing for its presence in a kit.

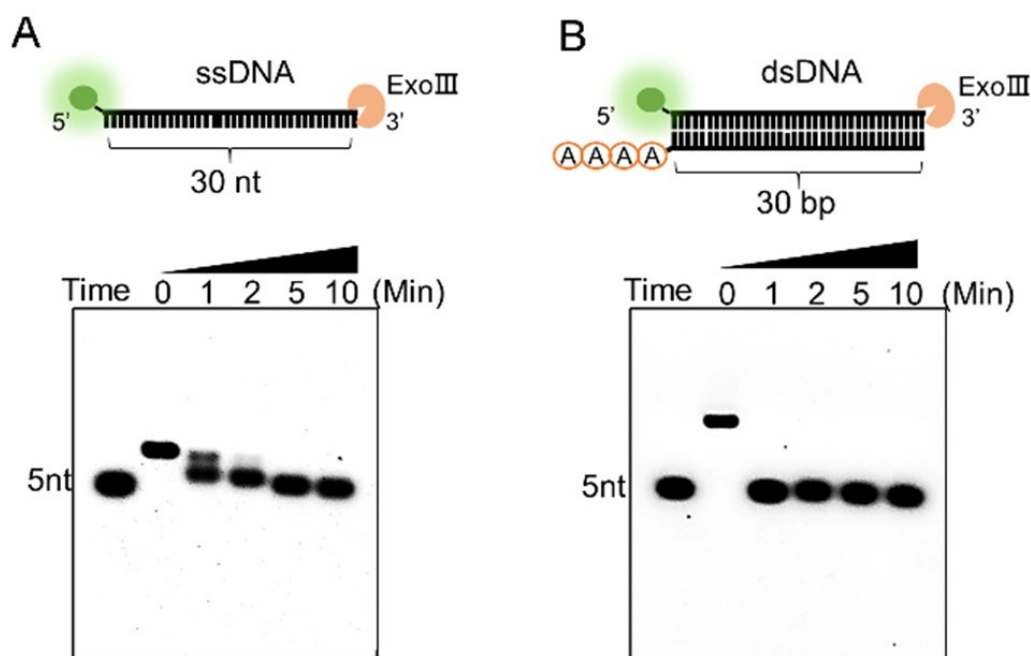
Response: Thanks so much for your comments. Logically, to evaluate the practical influence, we need to compare the current and improved detection kits. Our result suggested that raising the temperature or using the mutant may minimize the ssDNase activity of ExoIII. But the RAA or RPA-ExoIII detection kit is multiple-component system consisting of recombinase T4 UvsX, loading factor T4 UvsY, ssDNA binding protein T4 gp32 polymerase Bsu and ExoIII (Analyst. 2018 Dec 17;144(1):31-67. doi: 10.1039/c8an01621f), which collectively decide the performance of the kit. By increasing the temperature, the activities or functions of other proteins contained in the detection kit would also be affected, and the resultant change in detection efficiency would not reflect the real practical influence of the ssDNase activity of ExoIII; By replacing the wild type with the mutant, the other four proteins need to be prepared and combined with an optimized ratio for rebuilding the detection system, which is challenging. The targeted recycle assays in Ref 20 is a simple system composed of ExoIII and corresponding nucleic acid adapters, which could be easily simulated by the researchers for evaluation. Being a much more complex system, the RAA or RPA-ExoIII detection kit is difficult to manipulate for displaying the practical influence. Thank you again for your insightful suggestions; and we may conduct a systematic investigation improve the detection kit in future studies.

Because of the implication that the presence of ssDNA exonuclease activity may have in reactions that are supposed to only use ExoIII dsDNA exonuclease, it is surprising that in

this submitted work no direct comparison of these two activities is done. Please provide an experimental determination of how different the specific activities for ssDNA and dsDNA are.

Response: As for your suggestion, we have compared the digesting rate of two activities by using an equal amount of the commercial ExoIII (10 U/μL) on the two types of substrates (10 μM). The results below revealed that ExoIII required 10 minutes to digest the 30-nt single-stranded DNA (ssDNA) (A), whereas it could digest the same sequence on double-stranded DNA (dsDNA) within 1 minute (B) (in a newly produced Supplementary Figure S1). This indicated that ExoIII digested the dsDNA at a rate at least ten times faster than ssDNA. In conjunction with these results, a recent study has shown that the ssDNase activity of ExoIII surpasses that of the conventional ssDNA-specific nuclease ExoI (Biosensors (Basel), 2023, May 26; 13(6):581, doi: 10.3390/bios13060581), suggesting a potential biological significance of ExoIII in bacteria related to ssDNA, even though the digesting rate is not as rapid as the dsDNA. The corresponding text has been added to the result (Lines 200-207).

Author response image 1.



Reviewer #2 (Public Review):

Summary:

This paper describes some experiments addressing 3' exonuclease and 3' trimming activity of bacterial exonuclease III. The quantitative activity is in fact very low, despite claims to the contrary. The work is of low interest with regard to biology, but possibly of use for methods development. Thus the paper seems better suited to a methods forum.

Response: We thank you for your time and effort in improving our work. In the following, we have revised the manuscript by providing point-to-point responses to your comments.

Strengths:

Technical approaches.

Response: Thanks for your evaluation.

Weaknesses:

The purity of the recombinant proteins is critical, but no information on that is provided. The minimum would be silver-stained SDS-PAGE gels, with some samples overloaded in order to detect contaminants.

Response: As suggested, we have performed the silver-stained SDS-PAGE on the purified proteins. The result below indicated that no significant contaminant was found, except for a minor contaminant in S217A (in a newly produced Supplementary Figure S4).

Author response image 2.



Lines 74-76: What is the evidence that BER in E. coli generates multinucleotide repair patches in vivo? In principle, there is no need for the nick to be widened to a gap, as DNA Pol I acts efficiently from a nick. And what would control the extent of the 3' excision?

Response: Thank you for the insightful questions. The team of Gwangrog Lee lab has found that ExoIII is capable of creating a single-stranded DNA (ssDNA) gap on dsDNA during base excision repair, followed by the repair of DNA polymerase I. The gap size is decided by the rigidity of the generated ssDNA loop and the duplex stability of the dsDNA (Sci Adv. 2021 Jul 14;7(29):eabg0076. doi: 10.1126/sciadv.abg0076).

Figure 1: The substrates all report only the first phosphodiester cleavage near the 3' end, which is quite a limitation. Do the reported values reflect only the single phosphodiester cleavage? Including the several other nucleotides likely inflates that activity value. And how much is a unit of activity in terms of actual protein concentration? Without that, it's hard to compare the observed activities to the many published studies. As best I know, Exo III was already known to remove a single-nucleotide 3'-overhang, albeit more slowly than the digestion of a duplex, but not zero! We need to be able to calculate an actual specific activity: pmol/min per µg of protein.

Response: Yes, once the FQ reporter is digested off even one nucleotide or phosphodiester, fluorescence will be generated, and the value reflects how many phosphodiesters at least have been cleaved during the period, based on which the digesting rate or efficiency of the nuclease on ssDNA could be calculated. The following Figure 2 and 3 showed ExoIII could digest the ssDNA from the 3' end, not just a single nucleotide. Since the "unit" has been widely used in numerous studies (Nature. 2015 Sep 10;525(7568):274-7; Cell. 2021 Aug 19;184(17):4392-4400.e4; Nat Nanotechnol. 2018 Jan;13(1):34-40.), its inclusion here aids in facilitating comparisons and evaluations of the activity in these studies. And the actual activity of ExoIII had been calculated in Figure 4D.

Figures 2 & 3: These address the possible issue of 1-nt excision noted above. However, the question of efficiency is still not addressed in the absence of a more quantitative approach, not just "units" from the supplier's label. Moreover, it is quite common that commercial enzyme preparations contain a lot of inactive material.

Response: Thanks for your comments. In fact, numerous studies have used the commercial ExoIII (Nature. 2015 Sep 10;525(7568):274-7; Cell. 2021 Aug 19;184(17):4392-4400.e4; Nat Nanotechnol. 2018 Jan;13(1):34-40.). Using this universal label of “units” helps researchers easily compare or evaluate the activity and its influence. The commercial ExoIII is developed by New England Biolabs Co., Ltd., and its quality has been widely examined in a wide range of scientific investigations.

Figure 4D: This gets to the quantitative point. In this panel, we see that around 0.5 pmol/min of product is produced by 0.025 μ mol = 25,000 pmol of the enzyme. That is certainly not very efficient, compared to the digestion of dsDNA or cleavage of an abasic site. It's hard to see that as significant.

Response: Thanks for your comments; the possible confusion could have arisen due to the arrangement of the figure. Please note that based on Figure 4D, the digestion rate of 0.025 μ M ExoIII on the substrate is approximately 5 pmol/min (as shown on the right vertical axis), rather than 0.5 pmol/min. Given that the reaction contained ExoIII with a concentration of 0.025 μ M in a total volume of 10 μ L, the quantity of ExoIII was determined to be 0.25 pmol (0.025 μ mol/L $\times$ 10 μ L, rather than 25,000 pmol), resulting in a digestion rate of 5 pmol/min. It suggested each molecule of ExoIII could digest one nucleotide in 3 seconds (5 pmol nucleotides / 0.25 pmol ExoIII / 60second = 0.33 nucleotides/molecular/second). While it may not be as rapid as the digestion of ExoIII on dsDNA, a recent study has shown that the ssDNase activity of ExoIII surpasses that of the conventional ssDNA-specific nuclease ExoI (Biosensors (Basel), 2023, May 26; 13(6):581, doi: 10.3390/bios13060581), suggesting a potential biological significance of ExoIII in bacteria related to ssDNA.

Line 459 and elsewhere: as noted above, the activity is not "highly efficient". I would say that it is not efficient at all.

Response: We respectfully disagree with this point. Supported by the outcomes from fluorescence monitoring of FQ reporters, gel analysis of the ssDNA probe, and mass spectrometry findings, the conclusion is convincing, and more importantly, our findings align with a recent study (Biosensors 2023, 13(6), 581; <https://doi.org/10.3390/bios13060581>).

Reviewer #3 (Public Review):

Overall:

ExoIII has been described and commercialized as a dsDNA-specific nuclease. Several lines of evidence, albeit incomplete, have indicated this may not be entirely true. Therefore, Wang et al comprehensively characterize the endonuclease and exonuclease enzymatic activities of ExoIII on ssDNA. A strength of the manuscript is the testing of popular kits that utilize ExoIII and coming up with and testing practical solutions (e.g. the addition of SSB proteins ExoIII variants such as K121A and varied assay conditions).

Response: We really appreciate the reviewer for pointing out the significance and strength of our work. Additionally, we have responded point-by-point to the comments and suggestions.

Comments:

(1) The footprint of ExoIII on DNA is expected to be quite a bit larger than 5-nt, see structure in manuscript reference #5. Therefore, the substrate design in Figure 1A seems inappropriate for studying the enzymatic activity and it seems likely that ExoIII would be interacting with the FAM and/or BHQ1 ends as well as the DNA. Could this cause

quenching? Would this represent real ssDNA activity? Is this figure/data necessary for the manuscript?

Response: Thanks so much for your questions. The footprint of ExoIII on the dsDNA appears to exceed 5 nucleotides based on the structural analysis in reference #5. However, the footprint may vary when targeting ssDNA. Mass spectrometry analysis in our study demonstrated that ExoIII degraded a ~20-nucleotide single-stranded DNA substrate to mononucleotides (Figure 3), suggesting its capability to digest a 5-nt single-stranded DNA into mononucleotides as well. Otherwise, the reaction product left would only be 5-nt ssDNA fragment. Thus, the 5-nt FQ reporter is also a substrate for ExoIII. ExoIII possibly interacts with BHQ1 and affects its quenching efficiency on FAM to trigger the fluorescence release, as shown in Figure 1A, but this possibility has already been ruled out by the development of the RPA-ExoIII detection kit. As pointed out in the introduction part, the kit requires a probe labeled with fluorophore and quencher. If ExoIII could affect the fluorophore and quencher causing fluorescence release, the detection kit would yield a false-positive result regardless of the presence of the target, rendering the detection system ineffective. Thus, ExoIII does not interfere with the fluorophore and quencher. The digestion of ExoIII on the ssDNA within the FQ reporter was the sole cause of fluorescence release, and the emitted fluorescence represented the ssDNA activity. The result suggested that the FQ reporter might offer an effective approach to sensitively detect or quantitatively study the ssDNase activity of a protein that has not been characterized.

(2) Based on the descriptions in the text, it seems there is activity with some of the other nucleases in 1C, 1F, and 1I other than ExoIII and Cas12a. Can this be plotted on a scale that allows the reader to see them relative to one other?

Response: Thanks so much for your suggestions. We attempted to adjust the figure, but due to most of the values being less than or around 0.005, it was challenging to re-arrange for presentation.

(3) The sequence alignment in Figure 2N and the corresponding text indicates a region of ExoIII lacking in APE1 that may be responsible for their differences in substrate specificity in regards to ssDNA. Does the mutational analysis support this hypothesis?

Response: Our result indicated that the mutation of R170 located in the region (α M helix) resulted in lower digesting efficiency on ssDNA than the wild type, which showed that R170 was an important residue for the ssDNase activity, partially supported the hypothesis. Further investigation is needed to determine whether the structure of the α M helix accounts for the distinctions observed between ExoIII and APE1. Future research may require more residue mutations in this area for validation.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

- *A significant fraction of amplitude is missing in the presented fluorescence time courses reporting on ssDNA nuclease activity (Figs 1 B, E, and H). Please indicate the dead time of mixing in these experiments, and if necessary include additional points in this time scale. It is unacceptable for the authors to simply connect the zero-time point and the first experimental point with a dashed line.*

Response: We thank the reviewer for pointing out the critical detail. We agree that simply connecting with a dashed line is an inappropriate way for indicating the real fluorescence generated in the initial stage. The fluorescence monitor machine needs about two minutes to

initiate from the moment we place the reaction tube into the machine. But ExoIII can induce significant fluorescence immediately, reaching the peak within ~40 seconds, as shown in the video data. Therefore, it is difficult to record the initial real-time fluorescence generated. To avoid misleading, we have added a description in the legend as follows: “The dashed line used in the figure does not indicate the real-time fluorescence generated in the reaction but only represents a trend in the period for the monitor machine to initiate (~2 minutes).” The text was added in Lines 836-838.

- *The authors chose to utilize a 6% agarose electrophoresis to analyze digestion products. However, while this approach clearly shows that the substrates are being digested, it does not allow us to clearly estimate the extent. It would be appropriate to include control denaturing PAGE assays to test the extent of reaction, especially for dsDNA that contains a ssDNA extension, as in Figure 8, or for selected mutants to test whether exo activity may be limited to just a few nts, that may not be resolved with the lower resolution agarose gels.*

Response: We agree with the reviewer that denaturing PAGE assays usually is the choice for high-resolution analysis. And we performed this experiment on the short ssDNA, but observed that the bands of digestion products frequently shifted more or less in the gel. Of note, the other independent study also showed a similar phenomenon (Nucleic Acids Res. 2007;35(9):3118-27. doi: 10.1093/nar/gkm168). Even slight band shifting would significantly interfere with our analysis of the results, especially on the short ssDNA utilized in the study. After numerous attempts, we discovered that 6% agarose gel electrophoresis could detect the digested ssDNA bands with lower resolution than PAGE, but less shifting was observed. Considering all the factors, the 6% agarose gel was finally selected to analyze the digestion process.

Reviewer #2 (Recommendations For The Authors):

Line 158: tipycal should be typical

Response: Thanks so much, and as the reviewer pointed, we have corrected the typo.

Lines 299-300: "ssD-NA" should not be hyphenated, i.e., it should be ssDNA. .

Response: Thank you for pointing this out. We have rectified the error and thoroughly reviewed the entire paper for any necessary corrections.

Reviewer #3 (Recommendations For The Authors):

Figure 2A should indicate the length of the substate. The legend says omitted nucleotides - I assume they were present in the substrate and just not in the figure? The authors should be very clear about this. Moreover, the text and figure do not well describe the design differences between the three probes. Are they the same except just 23, 21, and 20 nt in length? Are the sequences selected at random?

Response: Thank you for your questions. The lengths of probes were described in the figure (23, 21, and 20 nt). The legend has been reworded in Line 843 as “The squiggle line represents the ~20 nucleotides of the ssDNA oligo.” And the sequences of three ssDNA substrates were randomly selected, and all the detailed information was provided in Supplementary Table S4.

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