

Assessing the reverse transcriptase and strand displacement activities of DNA polymerases via quantitative capillary electrophoresis

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INTRODUCTION

DNA polymerases (DNAPs) are essential enzymes, responsible for the accurate replication of genetic information and the maintenance, repair, and evolution of genomes (1,2). Beyond their biological roles, DNAPs are critical tools in biotechnology, enabling foundational methods such as the polymerase chain reaction (PCR), molecular diagnostics, and next-generation sequencing (3,4). Importantly, DNA polymerases display remarkable structural and functional diversity, and understanding this diversity is key to advancing both basic biological insight and technological innovation.

At their core, all DNAPs catalyze DNA synthesis through a 5'–3' nucleotidyl transferase activity. Many polymerases also encode specialized functions that support their diverse *in vivo* roles, including 3'–5' exonuclease proofreading, 5'–3' flap endonuclease activity, template-dependent uracil (dU) stalling, reverse transcriptase activity, and strand displacement capability (5–8). But when considering the right DNA polymerase for a DNA-dependent biotechnology workflow, understanding which specialized activities are present, and to what extent, is critical. While defined sequence motifs or domains can often predict activities such as exonuclease proofreading, flap endonuclease function, or uracil stalling, properties like strand displacement and reverse transcription are less straightforward and typically require experimental validation.

MATERIALS

- T4 DNA Polymerase (NEB #M0203)
- DNA Polymerase I (*E. coli*) (NEB #M0209)
- DNA Polymerase I, Large (Klenow) Fragment (NEB #M0210)
- Klenow Exo- (NEB #M0212)
- M-MuLV Reverse Transcriptase (NEB #M0253)
- Vent® DNA Polymerase (NEB #M0254)
- Vent (exo-) DNA Polymerase (NEB # M0257)
- Deep Vent® (NEB #M0258)
- Deep Vent (exo-) (NEB #M0259)
- ThermoTaq™ DNA Polymerase (NEB #M0261)
- Phi29 DNA Polymerase (NEB #M0269)
- Taq DNA polymerase (NEB #M0273)
- T7 DNA Polymerase (NEB #M0274)
- Bst DNA Polymerase, Large Fragment (NEB #M0275)
- AMV Reverse Transcriptase (NEB #M0277)
- LongAmp® Taq DNA Polymerase (NEB #M0323)
- *Sulfolobus* DNA Polymerase IV (NEB #M0327)
- Bst DNA Polymerase, Full Length (NEB #M0328)
- Bsu DNA Polymerase, Large Fragment (NEB #M0330)
- Hemo KlenTaq® (NEB #M0332)
- Protoscript® II Reverse Transcriptase (NEB #M0368)
- Bst 3.0® DNA Polymerase (NEB #M0374)
- Warmstart® RTx Reverse Transcriptase (NEB #M0380)
- OneTaq® DNA Polymerase (NEB #M0480)
- Epimark® Hot Start Taq DNA Polymerase (NEB #M0490)
- Q5® High-Fidelity DNA Polymerase (NEB #M0491)
- Q5U® High-Fidelity DNA Polymerase (NEB #M0515)
- Phusion™ High-Fidelity DNA Polymerase (NEB #M0530)
- Bst 2.0® DNA Polymerase (NEB #M0537)
- Induro® Reverse Transcriptase (NEB #M0681)
- Bst-XT DNA Polymerase (NEB #M9204)
- Deoxynucleotide (dNTP) Solution Mix (NEB #N0447)
- Phi29-XT Standalone Enzyme
- Template Switching RT Standalone Enzyme
- Luna® RT Standalone Enzyme

FIGURE 1: Schematics of reverse transcriptase and strand displacement activities

Reverse transcription (RT)

Using a DNA-dependent DNA polymerase



Strand displacement

Using a DNA-dependent DNA polymerase



Using a DNA-dependent DNA polymerase with RT activity or a reverse transcriptase



Strand displacement activity is the capacity of a DNA polymerase to extend a nascent DNA strand while displacing downstream DNA that is hybridized to the template (Figure 1) (9). This property varies widely among polymerases, with some capable of efficient displacement, without any hindrance on activity, while others are substantially blocked by the downstream strand. Many bacterial family A DNA polymerases, such as *E. coli* DNA polymerase I, contain 5'–3' flap endonuclease activity for its biological role in lagging-strand DNA synthesis that is fundamentally at odds with strand displacement, as this domain degrades downstream DNA. When the domain responsible for this nuclease activity is removed, creating the “Klenow Fragment”, or “Large Fragment” form, it enables strand displacement in some family members (e.g., *E. coli*, *G. stearothermophilus*) but does not in others (e.g., *T. aquaticus*), demonstrating our inability to predict strand displacement *de novo* (10). However, some structural insight has been gained from the study of strand-displacing polymerases, as certain DNAPs exhibit distinct structural features that enable exceptional strand displacement activity. For example, structural and biochemical studies of phi29 DNA polymerase revealed that the enzyme forms a nearly closed ring around the primer–template duplex (11). This configuration allows forward translocation during DNA synthesis to mechanically destabilize and unwind the downstream duplex, effectively functioning as an intrinsic helicase and supporting highly processive synthesis, up to tens of thousands of base pairs. More recently, analysis of yeast mitochondrial DNA polymerase identified a strand-displacement loop and a wedge-helix element that act cooperatively to destabilize the downstream duplex and promote strand displacement (12). For many other polymerases, the structural determinants of strand displacement remain unclear; therefore, quantitatively assessing this activity is essential for informed enzyme selection and optimization in DNA-dependent applications where different properties and behaviors are essential for achieving the intended outcome.

DNAPs can also exhibit RNA-dependent DNA polymerase activity, the defining property of reverse transcriptases (RTs), which preferentially use RNA rather than DNA as a template for DNA synthesis (Figure 1). Some RTs contain conserved structural motifs predictive of this

function, such as the “YMDD” sequence found in the active site of retroviral RTs and certain retrotransposon enzymes (13). However, a lack of this motif is not sufficient evidence that a given polymerase cannot use RNA templates, and bona fide DNA-dependent DNA polymerases display a wide range of “RT” activity; therefore, if RNA is present some DNA polymerases may copy RNA. As many workflows may contain mixed DNA and RNA samples and may require utilization of only one type of template, it is important to consider the capabilities of the desired enzyme for potential suitability. Quantitative evaluation of polymerase activities on known substrate types is necessary to fully understand the properties of the DNA polymerases.

Here, we demonstrate the use of capillary electrophoresis (CE) to quantitatively assess both the reverse transcriptase activity of our commercially available DNA-dependent DNAPs and the strand displacement activity of our DNAPs and RTs. Previous work at NEB has shown the utility of CE in quantitatively characterizing a number of different DNA-modifying enzymes, including DNA ligases, endonucleases, and polymerases. This method is routinely used internally to evaluate enzyme performance and product quality. Here, we observe substantial variation in both RT and strand displacement activities among various polymerases. For several hyperthermophilic DNAPs, increased temperature correlates with enhanced apparent strand displacement activity, likely due to thermal destabilization of the DNA duplex rather than true intrinsic enzymatic strand displacement. Further, the presence or absence of proofreading 3'–5' exonuclease activity can also affect the strand displacement capacity of certain DNAPs. The results presented here have been used to update NEB's DNA Polymerase Selection Chart and Reverse Transcriptase Selection Chart so that polymerase users can make better informed decisions during DNAP and RT selection.

METHODS

Enzymes, buffers, and oligonucleotides

All reagents, including DNA polymerases, reverse transcriptases, deoxynucleoside triphosphates (dNTPs), and buffers, are from New England Biolabs (NEB). All oligonucleotides used in this study were synthesized by Integrated DNA Technologies® (Coralville, IA).

Assessing the RT activity of DNA-dependent DNA polymerases

To assess the RT activity of our commercially available DNA polymerases, a capillary electrophoresis-based primer-extension assay was utilized. A 5'-FAM labeled 30 nt primer (5'-/56-FAM/AGT GAA TTC GAG CTC GGT ACC CGG GGA TCC-3') and 72 nt RNA template (5'-rUrCrG rArGrA rArGrC rArCrG rArArA rGrCrA rGrGrG rUrGrC rCrUrG rCrArG rGrUrC rGrArC rUrCrU rArGrA rGrGrA rUrCrC rCrCrG rGrGrU rArCrC rGrArG rCrUrC rGrAr ArUrU rCrArC rU-3') were annealed in a 1:1.25 ratio, respectively, in 1x annealing buffer (10 mM Tris-HCl, 20 mM NaCl, pH 7.4) to a final concentration of 1 μ M by heating to 90°C for 5 mins then slowly cooling to room temperature. DNAPs were diluted to 3 μ M in their preferred storage buffer. Each DNAP at 100 nM final was incubated with 10 nM final of RT substrate and 100 μ M final dNTPs in a 20 μ L reaction for 5 min in their preferred buffer and optimized reaction temperature (found on the product webpage, for hyperthermophilic DNA polymerases 65°C was used), followed by quenching with an equal volume of 10 mM EDTA. Reaction products were diluted to 1 nM with dH₂O and analyzed by capillary electrophoresis as previously described (14).

Assessing the strand displacement activity of DNA-dependent DNA polymerases and Reverse Transcriptases

To assess the strand displacement activity of our commercially available DNA polymerases, a capillary electrophoresis-based primer-extension assay was utilized. For DNA-dependent DNAPs a 5'-FAM labeled 50 nt primer (5'-/56-FAM/ TAT AAC AGT TGA TTC CCA ATT CTG CGA ACG AGT AGA TTT AGT TTG ACC AT-3'), a 90 nt 5'-monophosphate downstream oligonucleotide (5'-/5Phos/TCG CAA ATG GTC AAT AAC CTG TTT AGC TAT ATT TTC ATT TGG GGC GCG AGC TGA AAA GGT GGC ATC AAT TCT ACT AAT AGT AGT AGC ATT-3'), and 150 nt DNA template (5'- AAT GCT ACT ACT ATT AGT AGA ATT GAT GCC ACC TTT TCA GCT CGC GCC CCA AAT GAA AAT ATA GCT AAA CAG GTT ATT GAC CAT TTG CGA AAT GTA TCT AAT GGT CAA ACT AAA TCT ACT CGT TCG CAG AAT TGG GAA TCA ACT GTT ATA-3') were annealed in a 1:3:2 ratio, respectively, in annealing buffer as described above.

To assess the strand displacement activity of RTs, the 30 nt 5'-FAM DNA primer and 72 nt RNA template (described above in RT activity assays), along with a 22 nt 5' phosphate downstream DNA oligonucleotide (5'-/5Phos/CAG GCA CCC TGC TTT CGT GCT T-3') were annealed in a 1:2:3 ratio, respectively, in 1x annealing buffer as described above. DNAPs and RTs were diluted to 3 μ M in their preferred storage buffer. Each DNAP/RT at 100 nM final was incubated with 10 nM final of substrate and 100 μ M final dNTPs in a 20 μ L reaction for 5 min in their preferred buffer and optimized reaction temperature (found on the product webpage, for hyperthermophilic DNA polymerases 65°C was used), followed by quenching with an equal volume of 10 mM EDTA. Reaction products were diluted to 1 nM with dH₂O and analyzed by capillary electrophoresis as previously described (14). For DNAPs and RTs whose optimal reaction temperature was greater than 37°C, strand displacement assays were additionally performed at 15°C below the optimal temperature, i.e., if 65°C was the optimal temperature, an additional reaction was performed at 50°C. As a control, primer extension reactions were performed, as described above, except utilizing a construct that lacked the downstream oligonucleotide to ensure the activity of each DNAP & RT tested (data not shown).

RESULTS

Capillary Electrophoresis as a quantitative tool to characterize DNA and RNA modifying enzymes

Capillary electrophoresis (CE) is an analytical technique that separates charged molecules in a narrow capillary tube according to their size-to-charge ratio as they migrate under an applied electric field (Figure 2) (15,16). Smaller, more highly charged molecules migrate through the capillary more rapidly than larger molecules. CE is commonly used to separate biomolecules such as proteins, charged metabolites, and nucleic acids, including DNA and RNA polymers. These molecules are typically labeled with fluorescent dyes (fluorophores), allowing them to be detected by laser-induced fluorescence as they pass a detector within the capillary. The instrument records this fluorescence signal and generates electropherogram traces that report the migration time and relative abundance of each fluorescently labeled species (Figure 2). Although several

capillary electrophoresis platforms are available, at New England Biolabs we use the Agilent® 3730XL fragment analyzer—originally developed for Sanger sequencing—to perform quantitative CE analysis (Figure 2) (17). This instrument enables simultaneous five-dye detection and automated fragment analysis, with one dye channel dedicated to an internal size standard to accurately determine fragment length and enable alignment of migration times across samples.

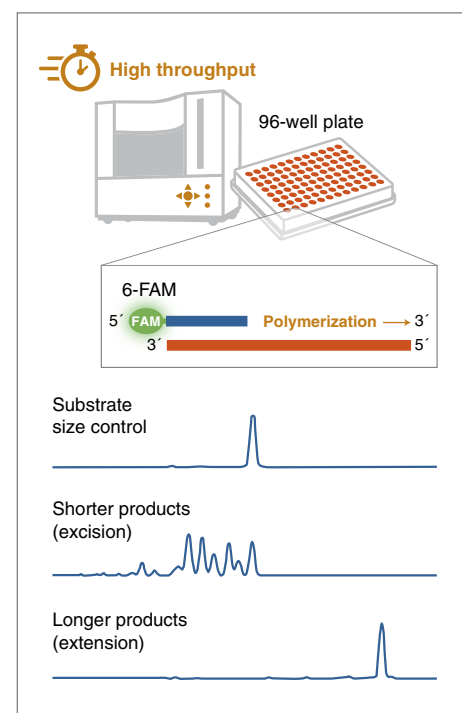
Over the years, we have used capillary electrophoresis (CE) to characterize the activity of a wide range of DNA- and RNA-modifying enzymes, including DNA and RNA ligases, endo- and exonucleases, DNA helicases, and DNA and RNA polymerases (14,18). Given the utility of this platform, CE is heavily relied upon by our Research department for enzyme discovery and characterization, our Product development teams for precise enzyme development, and by our Production and Quality departments to analyze our enzymes for activity and contaminants. Given the importance and reliance of this system, we have a dedicated DNA sequencing core that runs and maintains our suite of Agilent 3730XL instruments, and a bioinformatics team that has developed specialized software that enables efficient and rapid analysis of CE traces.

CE has proven particularly informative for studying DNA polymerases because of the diverse set of activities that can influence their performance in different applications. These activities, which include 5'–3' polymerase activity, 3'–5' proofreading exonuclease activity, 5'–3' flap endonuclease activity, dU stalling, strand displacement and reverse transcription can all be quantitatively assessed using CE.

Reverse transcriptase ability of DNA-dependent DNA polymerases

DNA polymerases are divided into eight families based on sequence and structural similarity: A, B, C, D, X, Y, archaeo-eukaryotic primases (AEPs), and reverse transcriptases (RTs) (19). All families except RTs are primarily DNA-dependent DNA polymerases that function in genome replication and repair. In contrast, reverse transcriptases are RNA-dependent DNA polymerases, typically encoded by retroviruses or retroelements, that synthesize DNA using an RNA template (20). This process generates cDNA intermediates that

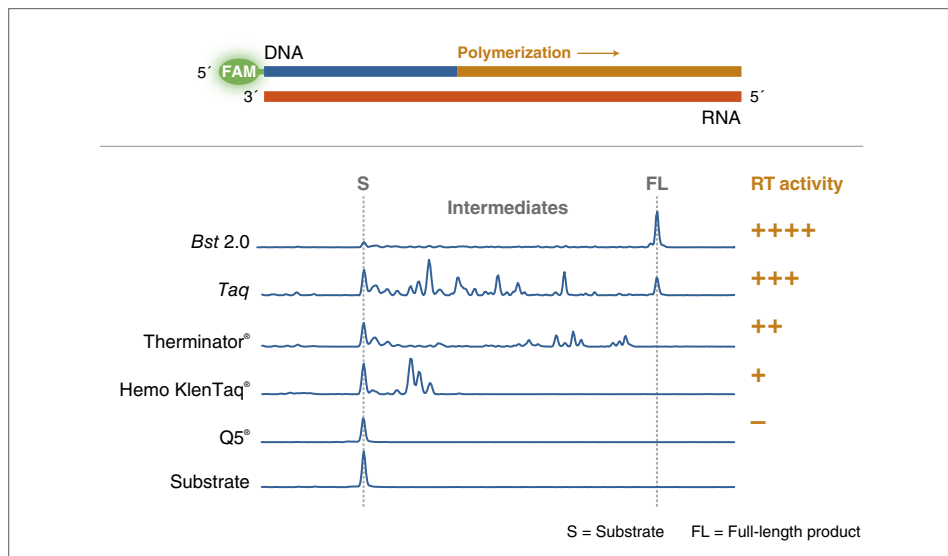
FIGURE 2: Capillary electrophoresis using the ABI 3730xl DNA Analyzer



DNA and RNA samples labeled with fluorescent dyes are separated by capillary electrophoresis on an ABI 3730xl DNA Analyzer. Up to 96 samples are loaded into individual capillaries and subjected to an electric field within a polymer matrix. Negatively charged nucleic acids migrate toward the positive electrode, with smaller fragments moving faster than larger ones, resulting in separation based on size and charge. As fragments pass a detection window, attached fluorophores are excited by a laser and emit fluorescence that is recorded by the instrument, allowing visualization and sizing of DNA and RNA fragments across the 96-capillary array. Examples are shown for CE traces from 5' 6-FAM labeled DNA alone, excised by a DNA polymerase exonuclease activity or extended by a DNA polymerase with dNTPs.

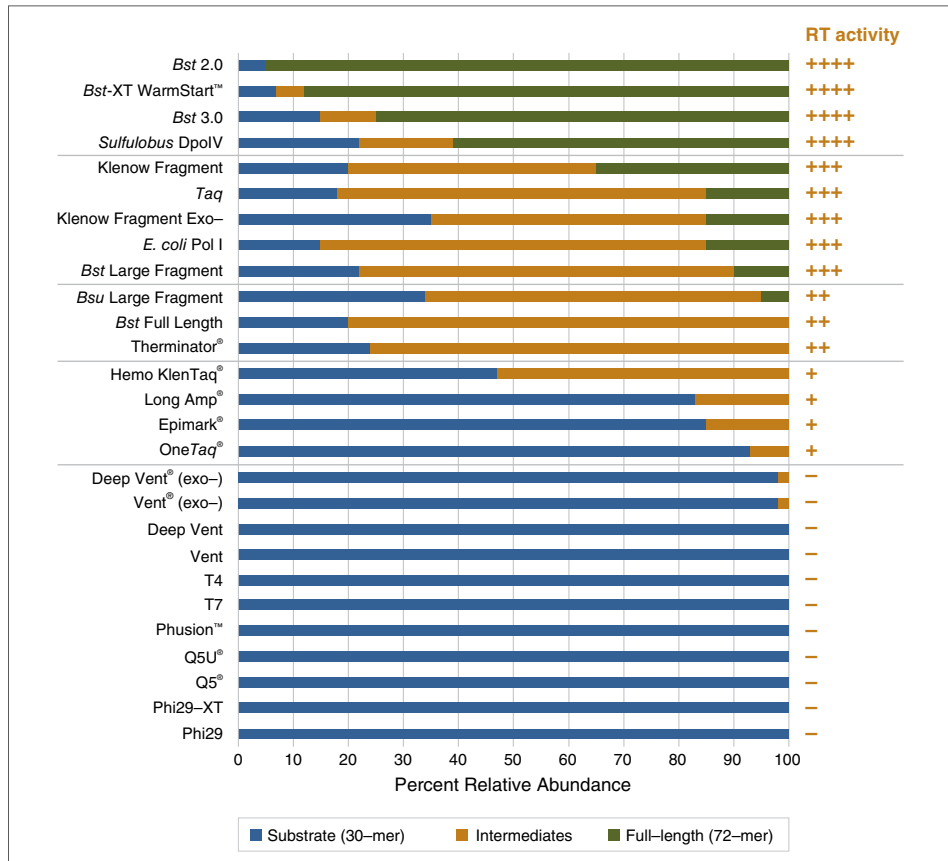
are used during viral replication and genome integration. Because RTs can convert RNA into cDNA, they enable RNA molecules to be amplified, sequenced, cloned, and quantitatively analyzed using DNA-based technologies, making them powerful tools in biotechnology. However, the distinction between RTs and other DNA polymerase families is not always clear-cut. Even bona fide DNA polymerases involved in DNA-templated replication and repair can exhibit reverse transcriptase activity, meaning they can utilize RNA as a template.

FIGURE 3: Quantitating the reverse transcriptase activity of NEB DNA-dependent DNAPs



A CE-based assay was used to quantitate the ability of DNAPs to perform RNA dependent DNA synthesis by utilizing a -FAM labeled DNA primer annealed to an RNA template. Following incubation with each DNAP and dNTPs CE product were analyzed. Resultant CE traces are shown from DNAP RT activity assays.

FIGURE 4: Quantitation of DNA polymerase reverse transcriptase activity



The reverse transcriptase activity of DNA-dependent DNA polymerases determined by the relative abundance of substrate, intermediates and full-length product.

To quantitatively assess the ability of our commercially available DNA polymerases (DNAPs) to synthesize DNA utilizing an RNA template, we designed a construct consisting of a 30-nt 5'-FAM-labeled DNA oligonucleotide annealed to a 72-nt RNA template (Figure 3). Each polymerase was incubated with the hybrid DNA-RNA construct and dNTPs in its recommended reaction buffer and at its optimal reaction temperature. Reactions were then quenched, analyzed by capillary electrophoresis (CE), and RT activity was quantified.

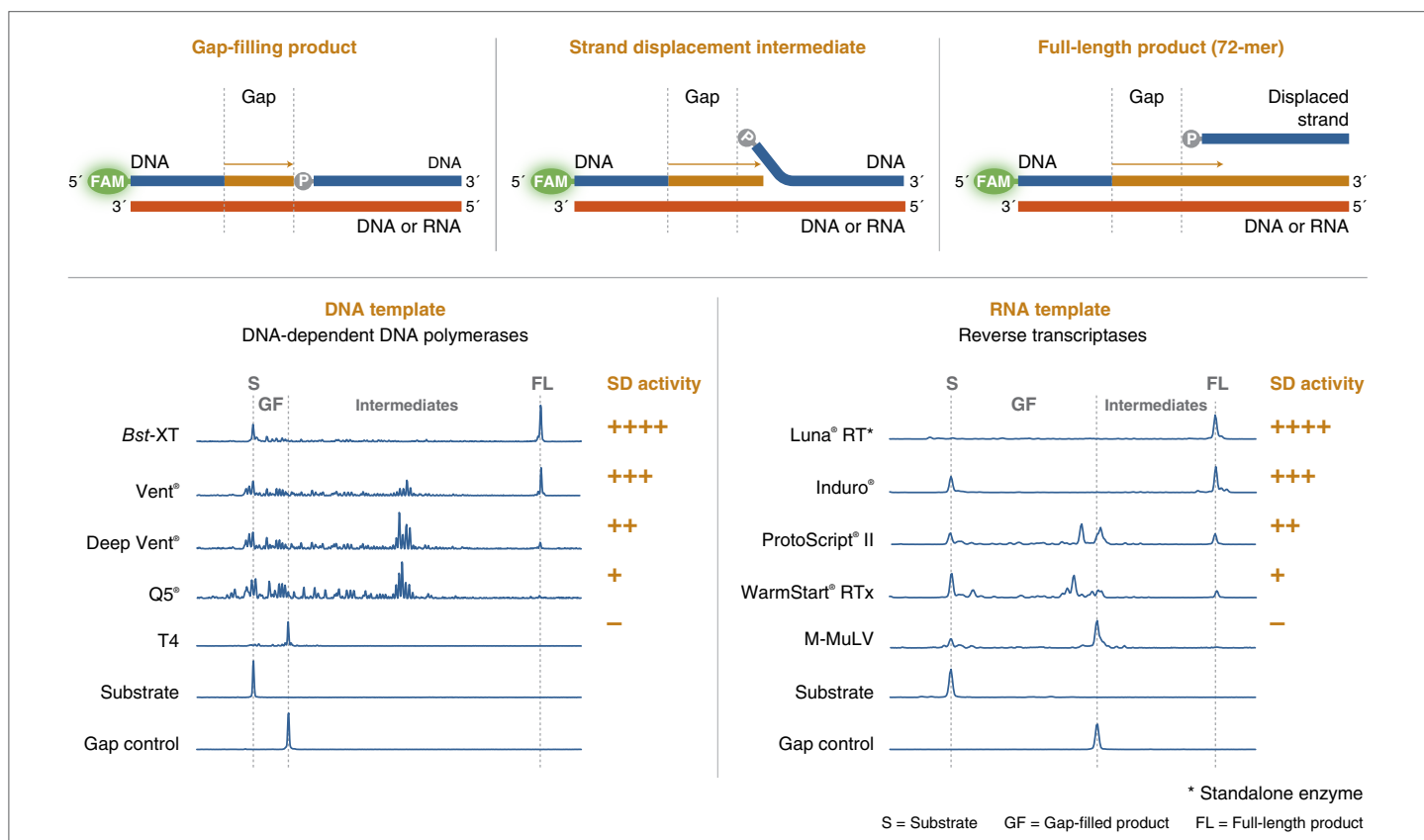
Using discrete quantitation bins, we categorized RT activity for each DNAP based on the relative amounts of DNA intermediates and full-length products generated (Figure 4). DNAPs with “++++” RT designation are those in which $\geq 50\%$ of product is full length, “+++” RT designation are those in which $\geq 10\%$ of product is full length, “++” designation are those in which $\geq 75\%$ of product is intermediates, “+” designation are those in which at least 5% of product is intermediates, and “-” indicates no observable RT activity. These experiments confirmed that many DNA polymerases exhibit varying levels of RT activity. As expected, we observed robust RT activity for previously reported RT capable DNA-dependent DNA polymerases such as *Bst*-XT and *Bst* 2.0 (Figure 3 & Figure 4). Our results also revealed strong RT activity for Klenow fragment and *Sulfolobus* DNA Polymerase IV. In addition, several other DNA-dependent DNA polymerases, including *Taq* and Hemo KlenTaq, exhibited detectable RT activity, although at lower levels. We observed no RT activity for the hyperthermophilic archaeal family B DNA polymerases, such as Q5, Phusion, Vent and Deep Vent, and for the viral T4, T7 and phi29 DNA polymerases.

Strand displacement ability of DNA-dependent DNA polymerases

During DNA repair and Okazaki fragment maturation, DNA polymerases often must displace or digest downstream DNA to allow joining of DNA through ligation. Most bacterial family A DNA polymerases possess an intrinsic 5'-3' flap endonuclease activity that can digest displaced downstream DNA (21). Examples include *Bst* full-length, *E. coli* Pol I, and *Taq* DNA polymerase. In contrast, other DNA polymerases lack this intrinsic activity and instead may displace downstream DNA, generating a 5' flap that must be processed by a separate, stand-alone flap endonuclease, like flap endonuclease 1 (FEN1) (22).



FIGURE 5: Quantitating the strand displacement activity of DNA-dependent DNA polymerases and RNA-dependent reverse transcriptases



A CE-based assay was used to quantitate the ability of DNAPs to facilitate strand displacement by utilizing a 5'-FAM labeled DNA primer annealed to either a DNA or RNA template with a downstream DNA oligonucleotide. Following incubation with each DNAP or RT and dNTPs CE product were analyzed. Resultant CE trace are shown from CE strand displacement assays using either a DNA or RNA template.

The extent to which individual DNA polymerases can displace downstream DNA varies widely. This property can be critical for certain applications, particularly those in which a probe must be cleaved or gaps in DNA need to be filled. This property is essential for most isothermal amplification techniques, where no thermal denaturation is used to separate dsDNA but rather the strand displacement activity of the polymerase itself. Further, while strand displacement activity of NEB DNA polymerases has been measured over the years using a variety of methodologies, a unified quantitative method has not been applied to systematically quantitate and compare these strand displacement abilities across all DNAPs.

To quantitatively assess the ability of our commercially available DNAPs that lack intrinsic 5'-3' flap endonuclease activity to perform strand displacement synthesis, we designed a construct consisting of a 50-nt 5'-FAM-labeled DNA oligonucleotide and a 90-nt 5'-phosphate DNA oligonucleotide annealed to a 150-nt DNA template, creating a 10-nt gap between the

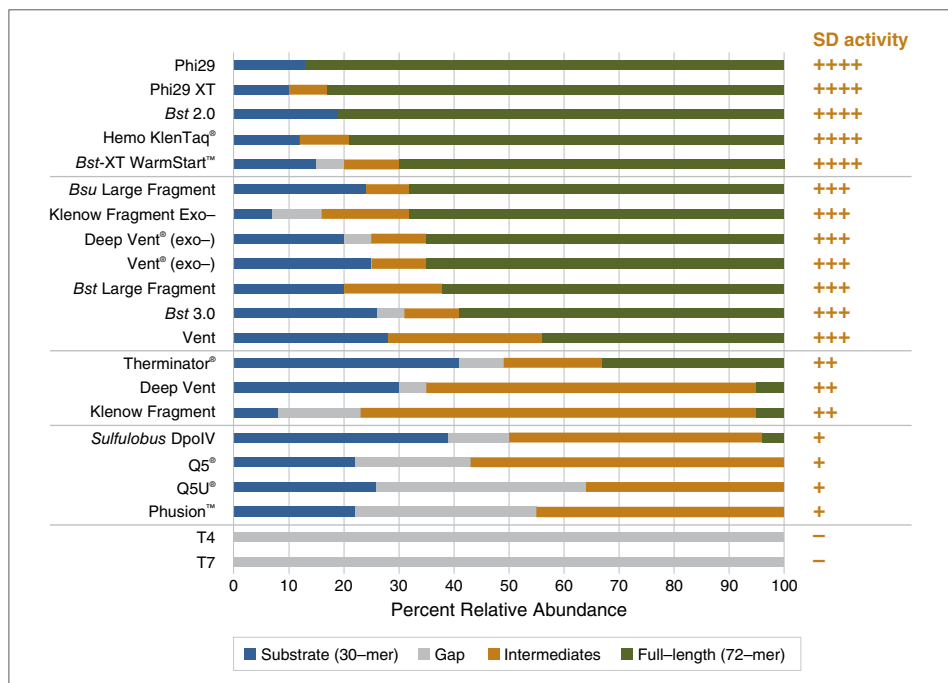
upstream FAM primer and downstream blocking oligonucleotide (Figure 5). Each polymerase was incubated with the strand displacement construct and dNTPs in its recommended reaction buffer and at its optimal reaction temperature. Reactions were then quenched, analyzed by CE, and strand displacement activity was quantified. Similar to RT reactions, we used discrete quantitation bins to categorize strand displacement activity for each DNAP based on the relative amounts of DNA intermediate products and full-length products generated (Figure 6). DNAPs with "++++" strand displacement designation are those in which $\geq 70\%$ of product is full length, "+++" strand displacement designation are those in which $\geq 40\%$ of product is full length, "++" designation are those in 5% of product is full length, "+" designation are those in which intermediate products are observed without full length products, and "-" are those in which 100% of product is gap product. As expected, we observed a wide variety of strand displacement activities across the various DNAPs with some polymerases readily able to displace the downstream 90-nt

oligonucleotide and synthesize full-length 150 nt DNA, such as *Bst*-XT 2.0 (Figure 5 & Figure 6). Other polymerases displayed varying levels of strand displacement activities as observed by accumulation of strand displacement intermediate products, such as DeepVent, Vent and *Sulfolobus* DNA Polymerase IV. Two DNA polymerases, T4 and T7, completely lacked any observable strand displacement activity and just gap-filled, creating the 60-nt product.

Exonuclease activity and temperature affect the strand displacement activity of DNA polymerases

While different DNA polymerases display varying degrees of strand displacement activity, likely due to unique structural architectures that enable their diverse biological roles, we also observed that 3'-5' exonuclease activity and temperature can influence the strand displacement ability of DNAPs. For several of our DNAPs, we have exonuclease-deficient variants, in which critical residues within the proofreading exonuclease active site have been mutated, including Klenow exo-, Vent exo-, and DeepVent exo-.

FIGURE 6: Quantitation of strand displacement activity of DNA-dependent DNA polymerases

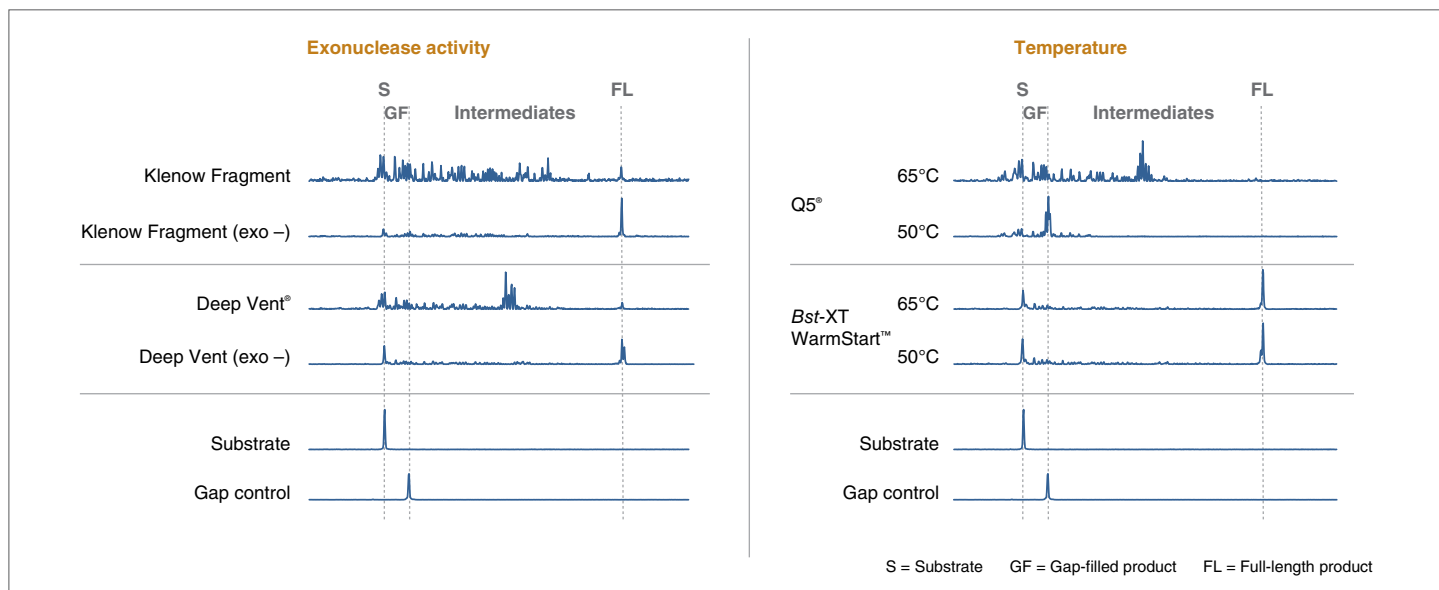


The strand displacement activity of DNA-dependent DNA polymerases determined by the relative abundance of substrate, gap, intermediates and full-length product.

In comparisons of strand displacement activities between *exo+* and *exo-* DNAPs, we observed that *exo-* variants often display more robust strand displacement activity than their *exo+* counterparts (Figure 6 and Figure 7). One likely explanation is that *exo+* polymerases can partition the primer terminus into the exonuclease site when synthesis is impeded, such as during strand displacement against downstream DNA. This proofreading transfer can lead to pauses and removal of nucleotides, limiting forward progression. In contrast, *exo-* variants lack exonucleolytic cleavage and therefore favor continued polymerization, promoting forward DNA synthesis and more efficient strand displacement. Of course, this enhancement in strand displacement synthesis comes at the cost of fidelity, as *exo-* DNA polymerases are more error prone.

In addition to exonuclease activity, we observed that temperature can influence the strand displacement activity of DNA polymerases. For enzymes with optimal reaction temperatures above 37°C, strand displacement assays were conducted at both the optimal temperature and at 15°C below this value. For hyperthermophilic archaeal family B DNA polymerases (e.g., Q5, Phusion, Vent, Deep Vent, and Terminator), while we observed some strand displacement at 65°C (i.e., “+” or “++”), strand displacement activity was minimal at the lower temperature (Figure 7), and in many instances the polymerases were unable to strand displace. This reduction likely results from decreased

FIGURE 7: The effect of exonuclease activity and temperature on the strand displacement activity of DNA-dependent DNA polymerases



CE traces of strand displacement assays showing the effect of mutating the exonuclease activity of Klenow fragment and Deep Vent, where *exo-* DNAPs show more robust strand displacement activity compared to *exo+*. Resultant CE traces are shown of strand displacement assays showing the effect of temperature on the strand displacement activity of Q5 and Bst-XT, where a decrease in temperature inhibit Q5 strand displacement activity, but not Bst-XT.

thermal destabilization of the downstream duplex DNA. At elevated temperatures, transient base-pair opening and fraying occur more frequently, thereby lowering the energetic barrier for polymerase progression through duplex regions. Consequently, the enzyme may partially rely on thermally induced strand separation to sustain strand displacement synthesis, leading to an apparent increase in strand displacement activity at higher temperatures. Therefore, careful consideration is needed when performing reactions with such DNAPs to ensure the desired properties are in play. In contrast, *Bst* family DNA polymerases retained robust strand displacement activity even at lower temperatures, suggesting the presence of intrinsic structural features that support efficient strand displacement independent of thermal assistance (Figure 7).

Strand displacement ability of RNA-dependent reverse transcriptases and DNAP-dependent DNA polymerases capable of robust RT activity

In addition to assessing the strand displacement activity of our DNA-dependent DNA polymerases, we also quantitated the strand displacement activity of our RNA-dependent RTs. Here, we used a similar construct that was used to assess the RT activity of our DNA-dependent DNA polymerases, except we included a 22-nt downstream blocking DNA oligonucleotide (Figure 5). As with all previous reactions, each RT was incubated with the RT strand displacement construct and dNTPs in its recommended reaction buffer and at its optimal reaction temperature.

Reactions were then quenched, analyzed by CE, and strand displacement activity was quantified. RT strand displacement activity was categorized using the same bin designation as above (Figure 9). Here we observed a wide degree of RT strand displacement activity, with very robust strand displacement activity for Luna and Induro RT, and decreasing degrees of strand displacement activity for Protoscript II, AMV, and Warmstart. Neither Template Switching nor M-MuLV RT was capable of strand displacement activity. Conventional wisdom has held that RTs are capable of strand displacement due to a need to get through inherent RNA structures, but this does not hold true for all RTs.

Utilizing the same strand displacement RT construct, we also measured the strand displacement activity of DNA-dependent DNAPs that were capable of robust RT and strand displacement activities (i.e., $\geq + + +$ for both activities) (Figure 8). Interestingly, for all DNAPs tested, the presence of the downstream oligonucleotide inhibited strand displacement activity to some degree (Figure 8). For example, for *Bst*-XT, which displayed very robust RT and DNA-dependent strand displacement activities, we observed the accumulation of intermediate products on the RT strand displacement construct. These results are even more pronounced for *Taq* and Klenow Fragment, in which the presence of the downstream DNA completely inhibits strand displacement activity (Figure 8).

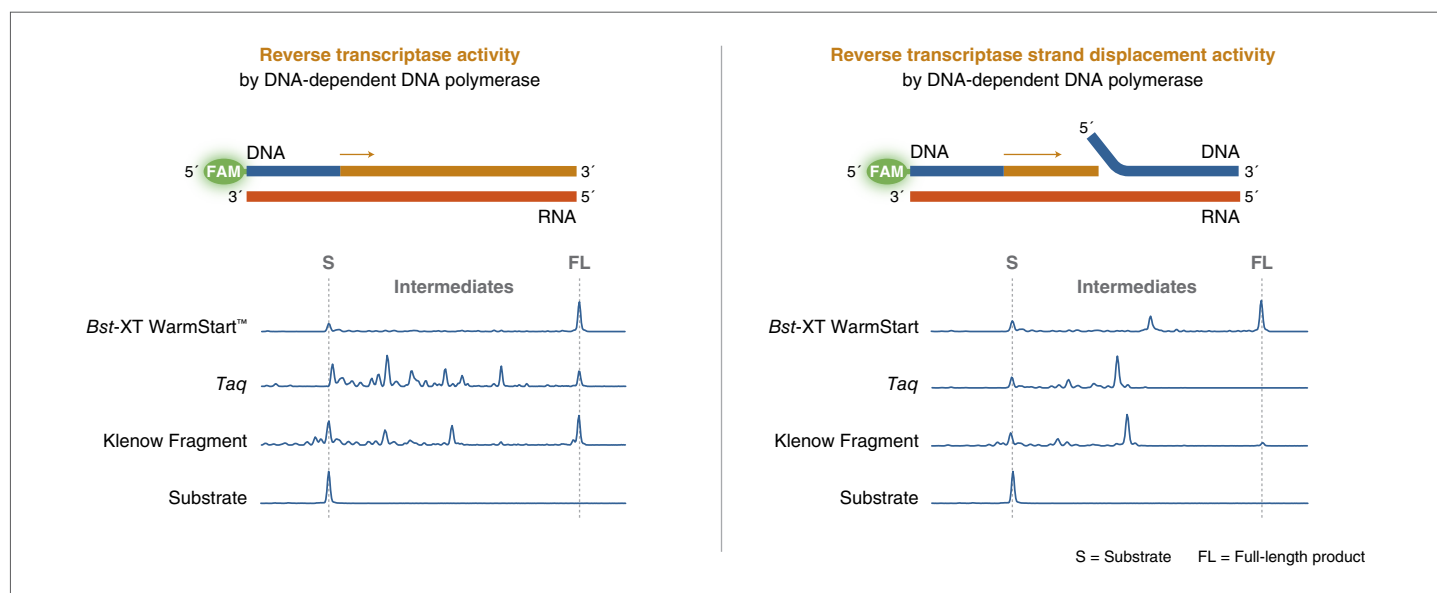
CONCLUSIONS

Due to the central role of DNA polymerases in biology and biotechnology, a detailed molecular understanding of their intrinsic properties, including proofreading, strand displacement, and reverse transcriptase activity, is essential for selecting the appropriate enzyme for a given application. In this study, we leveraged a highly sensitive capillary electrophoresis-based approach to quantitatively assess the RT activities of commercially available DNA-dependent DNA polymerases, as well as the strand displacement activities of both DNA polymerases and reverse transcriptases. These data provide a framework to guide enzyme selection based on functional performance. Notably, these properties are difficult to predict from sequence or structural information alone, underscoring the need for careful biochemical characterization.

While bona fide reverse transcriptases are specialized DNA polymerases that preferentially utilize RNA templates, some DNA-dependent DNA polymerases can also use RNA to varying extents. Here, we observed a wide range of RT activity across the enzymes tested. The highest activity was found among members of the *Bst* family, including full-length *Bst*, Large Fragment, and engineered variants such as 2.0, 3.0, and XT. These enzymes belong to the bacterial family A DNA polymerases, and more broadly, many bacterial family A polymerases examined here exhibited measurable RT activity, suggesting that RNA templating may be an intrinsic feature of this class.



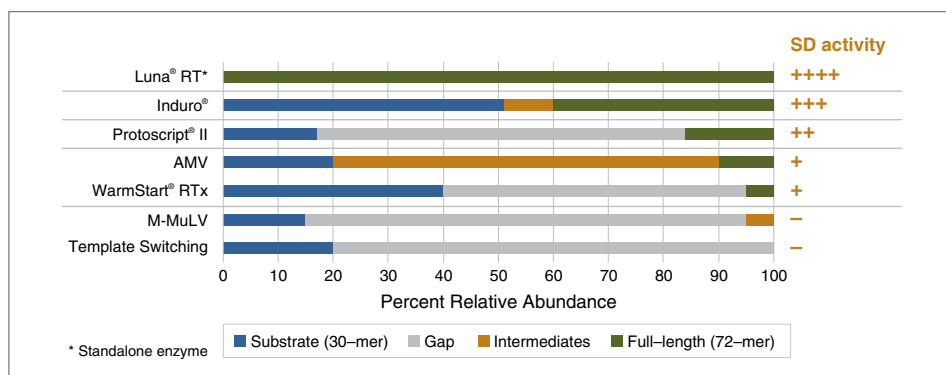
FIGURE 8: The effect of downstream DNA on the RT activity of DNA-dependent DNA polymerases



CE constructs used to assess the RT and RT SD activity of DNA-dependent DNA polymerases. Resultant CE traces are shown of representative DNA-dependent DNA polymerases and their RT activity, where the presence of downstream DNA impeded RT synthesis.



FIGURE 9: Quantitation of reverse transcriptase strand displacement activity



The strand displacement activity of reverse transcriptases determined by the relative abundance of substrate, gap, intermediates and full-length product.

In contrast, the viral T7 DNA polymerase, also a family A enzyme, showed no detectable RT activity. Archaeal family B polymerases, including Q5, Phusion, and Vent, likewise exhibited no RT activity, with the engineered Terminator representing a notable exception. This exception is likely attributable to engineered mutations that expand the active site and binding pocket, facilitating accommodation of RNA templates (23). Although *Bst*-like polymerases lack 3'–5' proofreading exonuclease activity, this does not appear to be sufficient for explaining the differential RT activity between the families. Family B enzymes possess a robust proofreading domain that could, in principle, interfere with RT, but exonuclease-deficient variants of family B polymerases still failed to exhibit meaningful RT activity. Additionally, one family A polymerase with 3'–5' exonuclease activity, *E. coli* Pol I, demonstrated measurable RT activity with or without its proofreading activity intact. This indicates that proofreading alone does not explain the absence of RT function. Instead, the high fidelity of archaeal family B polymerases, likely arising from a highly constrained binding pocket optimized for DNA, may limit their ability to accommodate RNA templates. These distinctions have clear practical implications: high-fidelity family B polymerases are well suited for applications such as sequencing, where minimizing unintended RT activity is desirable, whereas the intrinsic RT activity of *Bst*-like enzymes is advantageous in isothermal amplification methods such as loop-mediated isothermal amplification (LAMP), particularly for RNA virus detection.

In addition to robust RT activity, *Bst* family polymerases also exhibit strong strand displacement activity. Interestingly, wild-

type *Bst* DNA polymerase contains a 5'–3' exonuclease activity required *in vivo* for Okazaki fragment processing. Removal of this domain, as in *Bst* Large Fragment and related engineered variants (2.0, 3.0, and XT), results in markedly enhanced strand displacement activity. This suggests that strand displacement activity is not a primary physiological function of these enzymes, but rather an emergent property of their structural architecture. The persistence of strong strand displacement activity across a range of temperatures further supports the idea that it arises from inherent structural features, although the precise determinants remain unclear. Importantly, this robust strand displacement capability underpins the utility of *Bst* polymerases in LAMP, enabling continuous DNA synthesis with concurrent strand displacement and eliminating the need for thermal denaturation (24). By contrast, hyperthermophilic archaeal polymerases exhibited less strand displacement activity at 65°C compared to *Bst*, which was largely lost at lower temperatures. This observation suggests that the apparent strand displacement activity at elevated temperatures may be driven, at least in part, by thermal destabilization of the downstream duplex rather than an intrinsic property of the enzyme to actively displace strands. Consistent with this, these polymerases are typically employed in PCR workflows that rely on thermal cycling for strand separation. Two DNA polymerases, bacteriophage T7 and T4 DNA polymerases, both display no apparent strand displacement activity and are often recommended by NEB for gap-filling workflows in which strand displacement activity is not desired. It is important to note that both T7 and T4 polymerases possess robust 3'→5' exonuclease proofreading activity and, in the absence of, or under low concentrations

of, dNTPs, can engage in exonucleolytic “chewing back,” resulting in a gap larger than intended. Therefore, care should be taken when utilizing these DNA polymerases for gap-filling protocols, with particular attention to maintaining appropriate dNTP concentrations to suppress exonuclease activity, minimize template resection, and ensure accurate and complete gap filling.

Finally, although some polymerases displayed both robust RT and strand displacement activities, their strand displacement efficiency was reduced when acting on DNA–RNA hybrids. This is likely due to the increased stability and compact A-form geometry of DNA–RNA duplexes relative to B-form DNA–DNA duplexes, which may present a greater barrier to strand invasion and displacement (25). Taken together, these results highlight that RT and strand displacement activities are distinct, context-dependent properties that do not necessarily co-occur or translate equivalently across substrate types. Functional performance is shaped by a combination of structural features, substrate geometry, and reaction conditions, rather than enzyme family classification alone. It is important to note, however, that while the CE-based assay employed here provides a sensitive and quantitative window into these intrinsic biochemical properties, it relies on short, fluorescently labeled oligonucleotide substrates, whereas DNA polymerases are typically required to act on long genomic or plasmid DNA in most applied contexts. Substrate length, sequence context, and secondary structure may all modulate the magnitude of the activities reported here, and these measurements are therefore best interpreted as a comparative ranking of intrinsic enzyme properties rather than absolute predictors of performance in every workflow. With this consideration in mind, empirical characterization remains essential for selecting the optimal polymerase for defined workflows.

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