Synergistic and non-specific nucleic acid production by T7 RNA polymerase and *Bsu* DNA polymerase catalyzed by single-stranded polynucleotides

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ABSTRACT

Point-of-care molecular diagnostic tests show great promise for providing accurate, timely results in low-infrastructure healthcare settings and at home. The design space for these tests is limited by a variety of possible background reactions, which often originate from relatively weak promiscuous activities of the enzymes used for nucleic acid amplification. When this background signal is amplified alongside the signal of the intended biomarker, the dynamic range of the test can be severely compromised. Therefore, a detailed knowledge of potential side reactions arising from enzyme promiscuity can improve rational design of point-of-care molecular diagnostic tests. Towards this end, we report a previously unknown synergistic reaction between T7 RNA polymerase and *Bsu* DNA polymerase that produces nucleic acid in the presence of single-stranded DNA or RNA. This reaction occurs in the absence of any previously reported substrates for either polymerases and compromises a theoretical microRNA amplification scheme utilizing these polymerases.

1. Introduction

Point-of-care diagnostic tests based on the detection of RNA or DNA biomarkers have the potential to reduce equipment and skilled-labor costs, decrease turnaround times, and provide diagnostic results in settings where infrastructure is insufficient for conventional diagnostic methods [1]. Recent research efforts to realize these benefits have led to the development of a wide variety of novel isothermal nucleic acid amplification systems [2]. A subset of these systems operate at temperatures close to that of commercial cell-free protein synthesis (CFPS) systems, particularly those using mesophilic enzymes such as T7 RNA polymerase (T7 RNAP), *Bsu* DNA polymerase (*Bsu* DNAP), T4 DNA Ligase, SplintR® Ligase, and φ29 DNA polymerase. This temperature compatibility

may enable the design of new systems which utilize isothermal nucleic acid amplification schemes in conjunction with reactions taking place in a CFPS system. A two-pot system based on this hybrid approach has recently been demonstrated for the detection of Zika virus [3].

The potential design space for such systems is large; therefore, it is helpful to understand the constraints due to factors such as enzyme compatibility to more quickly identify ineffective reaction schemes. Towards this end, we document a previously unknown synergistic nucleic acid polymerization reaction by two mesophilic polymerases, T7 RNAP and *Bsu* DNAP, which are commonly used in isothermal nucleic acid amplification reactions [4–7]. This interaction was first observed while testing a theoretical microRNA detection system (Fig. 1a). The reaction system was designed to use a ssDNA template (Supplementary Table 1) containing a binding site for microRNA 141 (miR-141) as well as the complement of a promoter sequence for T7 RNAP and two regions complementary to a defined trigger sequence. A second version of this template was also created in which the 3′ end has been blocked by phosphorylation (3′-P) to prevent any background reactions involving extension of the 3′ end of the template, a common concern in many

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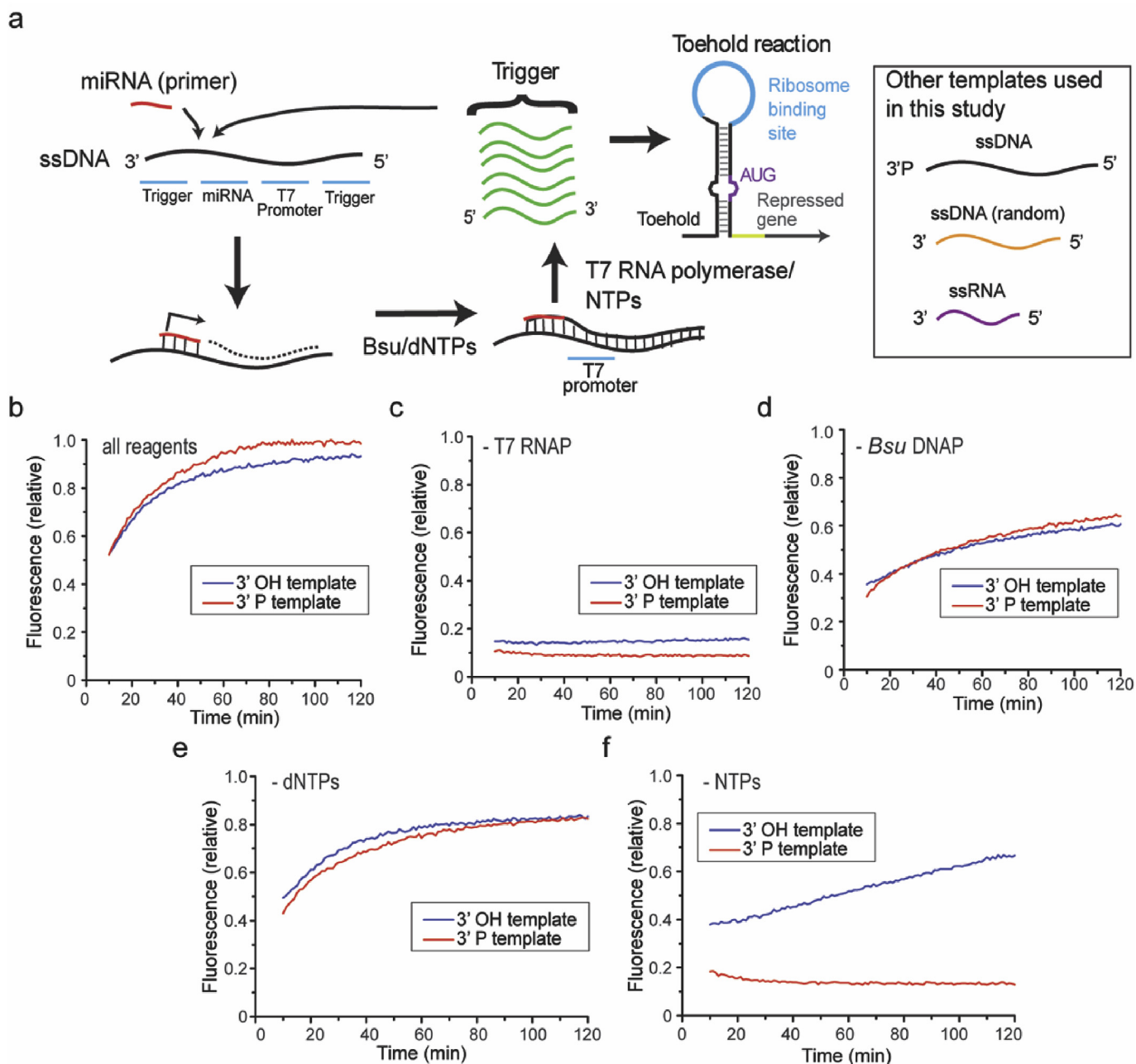
Fig. 1. a) Envisioned reaction scheme for the detection of miR-141 using T7 RNAP, *Bsu* DNAP using a toehold switch. Subfigures b through f compare background nucleic acid synthesis over time by T7 RNAP and *Bsu* DNAP in the absence of miR-141 for 3′-OH and 3′-P templates using Quant-iT RNA dye when the following reagents were omitted: b) none (all reagents added), c) T7 RNAP, d) *Bsu* DNAP, e) dNTPs, f) NTPs. Background subtraction for all experiments was performed by comparison to a reaction in which both T7 RNAP and *Bsu* DNAP were omitted. All experiments shown were repeated twice independently, producing results consistent with the presented data (additional data is presented in Fig. S1).

isothermal amplification systems. The 30 nt trigger sequence encoded by the ssDNA template is designed to activate a downstream toehold switch [8]. Upon addition of miR-141, which acts as a primer for *Bsu* DNAP, the region of the template encoding the T7 promoter and one copy of the trigger sequence becomes double-stranded. Once a double-stranded promoter is present, T7 RNAP is expected to begin transcribing copies of the 30 nt trigger sequence. The transcribed trigger RNA can then bind additional ssDNA template molecules, acting as a primer similar to the original miR-141 target. This scheme bears similarities to exponential amplification reaction (EXPAR) [9] but uses T7 RNAP in place of a nickase. In a one-pot format, a fraction of the 30 nt trigger RNA

molecules would concurrently activate a toehold switch controlling the translation of a colorimetric or fluorescent reporter protein. This toehold switch would be constitutively transcribed from a separate template plasmid within the reaction. Alternatively, a two-pot format could be used in which the amplification reaction is allowed to run for a fixed time before adding the product to a CFPS reaction containing the template for the toehold switch.

2. Results and discussion

The production of trigger RNA from the miR-141-primed amplification reaction was attempted under a variety of

conditions using both 3′-OH and 3′-P templates, and reactions were monitored by real-time fluorescence measurement with an intercalating dye to observe nucleic acid products. Surprisingly, similar rates of nucleic acid synthesis were consistently observed regardless of the presence or concentration of miR-141 (data not shown). This background nucleic acid synthesis reaction in the absence of miR-141 can be seen in Fig. 1b, which shows real-time fluorescence data for both 3′-OH and 3′-P templates. These data show a sharp increase in fluorescence demonstrating a nucleic acid synthesis reaction despite a lack of any primed ssDNA template or double-stranded T7 promoter. A wide variety of possible substrates have been previously reported for T7 RNAP [10–14] and, to a lesser extent, *Bsu* DNAP [15]; however, to the best of the authors' knowledge, neither T7 RNAP nor *Bsu* DNAP are known to act on unprimed ssDNA with a blocked 3′ end. This reaction occurred at a similar rate for the 3′-OH and 3′-P templates, suggesting that extension of the 3′ end of the ssDNA template is not the main mechanism of this reaction (Fig. 1b). In order to identify which reaction components are necessary for this behavior, reactions omitting T7 RNAP (Fig. 1c), *Bsu* DNAP (Fig. 1d), dNTPs (Fig. 1e), and NTPs (Fig. 1f) were carried out concurrently and normalized to the same maximum fluorescence value.

Comparison between the conditions in which T7 RNAP was included (Fig. 1b) and was omitted (Fig. 1c) clearly demonstrates the necessity of T7 RNAP in the mechanism driving the background reaction. Even when *Bsu* DNAP was omitted entirely (Fig. 1d), T7 RNAP appeared to be capable of unprimed nucleic acid synthesis under our reaction conditions; however, the removal of *Bsu* DNAP did noticeably slow the reaction rate. This suggests that, although *Bsu* DNAP is incapable of carrying out this unprimed nucleic acid synthesis reaction alone, it catalyzes the reaction carried out by T7 RNAP, possibly by stabilizing interactions between T7 RNAP and the ssDNA substrate or (more likely) by slow, unprimed synthesis of short dsDNA regions along the ssDNA which can then act as primers to be extended by T7 RNAP. The hypothesis that *Bsu* DNAP may exhibit such slow, unprimed synthesis activity on a ssDNA template is supported by the discovery of a similar behavior in thermophilic DNA polymerases at 55 °C [16]. Interestingly, removal of dNTPs (Fig. 1e) produced results which were intermediate between the condition in which *Bsu* DNAP was omitted and the condition in which nothing was omitted. This lends support to the hypothesis that *Bsu* DNAP may also be increasing T7 RNAP activity through direct interactions with T7 RNAP and/or the ssDNA template rather than solely through DNA synthesis. Interestingly, the omission of NTPs (Fig. 1f) completely inhibited the reaction for the 3′-P template but not the 3′-OH template. The reaction observed in this case was relatively slow and linear, whereas the reactions observed under other conditions increased sharply at early time points before leveling off. These observations suggest that the reaction in the absence of NTPs may proceed via a unique mechanism. A possible mechanism involves extension of the 3′ end of the 3′-OH template by *Bsu* DNAP, with T7 RNAP playing a supporting role (removal of T7 greatly inhibited the reaction as shown in Fig. 1c). Because this 3′ extension reaction is relatively slow, it likely does not contribute substantially to product formation under other conditions where NTPs are present.

In order to determine approximate molecular weights of the products produced by this background reaction, all aforementioned experiments were repeated and, after a 2-hour incubation at 37 °C, run on a 4% agarose gel stained with SYBR Gold Nucleic Acid Gel Stain (see discussion about SYBR Gold vs. Quant-iT dyes in supplemental method). Agarose gel electrophoresis data for both 3′-OH (Fig. 2a) and 3′-P (Fig. 2b) templates were in general agreement with the data obtained by real-time reaction monitoring. Under all conditions, the original template band migrated the same distance

and either maintained or gained intensity relative to the control condition (lane 1) in which both enzymes were omitted. This confirms that 3′ end extension of the original template, which would be expected to increase molecular weight, is not the main mechanism of nucleic acid synthesis in these reactions. A band corresponding to the expected trigger size of 30 nt can be seen for both the 3′-OH (Fig. 2a, lane 6) and 3′-P (Fig. 2b, lane 6) templates in lanes corresponding to reactions where no components have been omitted; note that this does not imply proper functioning of the envisioned reaction system because the trigger was transcribed in the absence of miR-141. For both 3′-OH (Fig. 2a, lanes 3, 5, and 6) and 3′-P (Fig. 2b, lanes 3, 5, and 6) templates, a prominent high molecular weight smear was observed under all conditions containing both T7 RNAP and NTPs. The small amount of product formation by T7 RNAP in the absence of *Bsu* DNAP suggests a weak but previously unobserved ability of T7 RNAP to transcribe RNA from a ssDNA template with no free 3′ end. When both *Bsu* DNAP and dNTPs were added as well, the intensity of this smear increased (Fig. 2a, lane 6 and Fig. 2b, lane 6). This increase provides additional evidence for the existence of a synergistic reaction between T7 RNAP and *Bsu* DNAP which leads to non-specific nucleic acid synthesis. The production of nucleic acids longer than the original template implies non-template-directed extension of the 3′ ends of products after template-directed synthesis and/or repeated cycles of template-directed synthesis, melting, and annealing.

To investigate the dependence of this reaction on the sequence and length of the ssDNA template, analogous experiments were performed in which the ssDNA template is replaced with a 51 nt single-stranded DNA polynucleotide with an unrelated sequence (Fig. 2c), which is not predicted to form secondary structures and does not contain a T7 promoter sequence, miR-141 binding site, or trigger binding site. Additionally, experiments were performed in which the ssDNA template was replaced by a 30 nt single-stranded RNA polynucleotide corresponding to the trigger sequence. The reactions observed in the presence of the 51 nt ssDNA and 30 nt ssRNA polynucleotides appear qualitatively similar to that observed using the original ssDNA template. Importantly, this is not the result of template-independent *ab initio* synthesis, which was not found to occur under the conditions tested (data not shown). A high molecular weight smear was observed when T7 RNAP, *Bsu* DNAP, NTPs, and dNTPs were all present (Fig. 2c, lane 6 and Fig. 2d, lane 6). In reactions containing the 51 nt ssDNA polynucleotide, a weaker smear could also be observed when either *Bsu* DNAP or dNTPs were removed but T7 RNAP and NTPs were included (Fig. 2c, lanes 3 and 5). Interestingly, no similar smear was apparent in analogous reactions containing the 30 nt ssRNA polynucleotide (Fig. 2d, lanes 3 and 5); the reason for this difference is unclear and warrants further investigation. All agarose gel experiments were repeated independently, and comparable results were obtained (Fig. S3). To further understand the observed reactions, all agarose gel experiments were repeated with DNase (Fig. S4) or RNase (Fig. S5) added post-reaction. Additionally, an experiment was performed for all four polynucleotide templates with all other reagents added, followed by a combined RNase and DNase digest (Fig. 2e). Double digestions with both RNase and DNase led to a greater loss in product than either digestion alone, in general agreement with our previous experiments suggesting that both T7 and *Bsu* contributed to the observed nucleic acid products. Interestingly, lane 4 of Fig. S3a-d showed a very noticeable smear not present in the undigested reactions. This result represents a potential avenue of further mechanistic investigation. A possible mechanism may include increased dsDNA formation by *Bsu* polymerase in the absence of T7 activity due to the removal of NTPs, leading to inefficient digestion by DNase I and a smear of various DNA-DNase complexes.
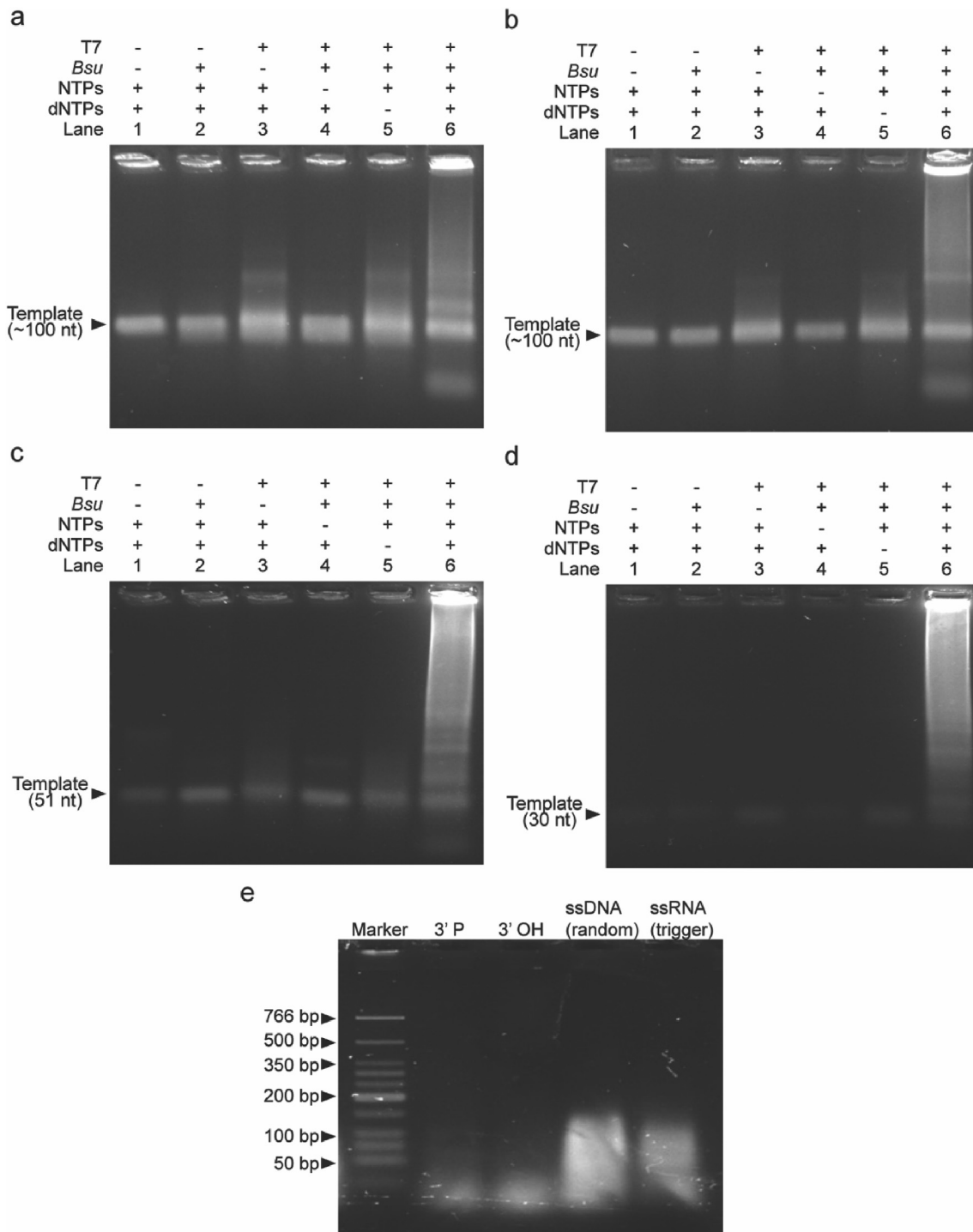
Fig. 2. Nucleic acid products for varying reaction conditions visualized by agarose gel electrophoresis using SYBR Gold after a 2-h incubation in the absence of miR-141. In addition to the components denoted above each lane, reactions contained the following polynucleotides: a) 3′-OH ssDNA template, b) 3′-P ssDNA template, c) random 51 nt ssDNA, and d) 30 nt ssRNA. a) and b) were imaged at 1.5 s exposure while c) and d) were imaged at 0.5 s exposure to display a similar intensity of the high molecular weight smear. 1.5 s exposure images of c) and d) are provided in Fig. S2. e) Nucleic acid products with T7, *Bsu*, NTPs, dNTPs added for various different templates listed for 2 h followed by DNase I (2 units) and RNase A (100 μg/ml) treatment for 30 min at 37 °C.

Although the presence of multiple enzymes each capable of acting on a variety of substrate types makes detailed mechanistic understanding of these results challenging, several commonalities observed across experiments shed light on the nature of the synergistic reaction between T7 RNAP and *Bsu* DNAP. The primary discovery of this work is that T7 RNAP and *Bsu* DNAP, when added together in the presence of dNTPs, NTPs and a single-stranded polynucleotide template, were capable of producing a smear of high molecular weight nucleic acid products regardless of template length, sequence characteristics, or even whether the template is composed of RNA or DNA. This reaction did not require a primer, and was not dependent on extension of the 3′ end of the original

polynucleotide template. Although T7 RNAP was able to produce a faint smear of high molecular weight nucleic acid products from a ssDNA template in the absence of *Bsu* DNAP, the addition of *Bsu* DNAP increased the reaction rate and led to a larger amount of product formed. This interaction limits the use of these two enzymes together in isothermal nucleic acid amplification and detection schemes, where even low levels of background nucleic acid production can be amplified and lead to compromised dynamic range. Interestingly, this reaction occurred in the absence of any previously known substrate for either T7 RNAP or *Bsu* DNAP. Preliminary experiments showed that this may be generalizable to T7 RNAP with other DNAPs such as *Bst*, φ29, and Klenow (exo-). This work contributes towards a greater understanding of potentially problematic interactions between enzymes relevant to the development of novel isothermal amplification and detection schemes, and may prove generalizable to related DNA and RNA polymerases.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.synbio.2018.02.005.

References

[1] Zarei M. Advances in point-of-care technologies for molecular diagnostics. Biosens Bioelectron 2017;98:494–506.
[2] Yan L, Zhou J, Zheng Y, Gamson AS, Roembke BT, Nakayama S, Sintim HO. Isothermal amplified detection of DNA and RNA. Mol Biosyst 2014;10(5):970–1003.
[3] Pardee K, Green AA, Takahashi MK, Braff D, Lambert G, Lee JW, Ferrante T, Ma D, Donghia N, Fan M, Daringer NM, Bosch I, Dudley DM, O'Connor DH, Gehrke L, Collins JJ. Rapid, low-cost Detection of Zika virus using programmable biomolecular components. Cell 2016;165(5):1255–66.
[4] Zhang Y, Tanner NA. Isothermal amplification of long, discrete DNA fragments facilitated by single-stranded binding protein. Sci Rep 2017;7(1):8497.
[5] Hoser MJ, Mansukoski HK, Morrical SW, Eboigbodin KE. Strand Invasion Based Amplification (SIBA(R)): a novel isothermal DNA amplification technology demonstrating high specificity and sensitivity for a single molecule of target analyte. PLoS One 2014;9(11):e112656.
[6] Kievits T, van Gemen B, van Strijp D, Schukkink R, Dircks M, Adriaanse H, Malek L, Sooknanan R, Lens P. NASBA isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection. J Virol Methods 1991;35(3):273–86.
[7] Wharam SD, Marsh P, Lloyd JS, Ray TD, Mock GA, Assenberg R, McPhee JE, Brown P, Weston A, Cardy DL. Specific detection of DNA and RNA targets using a novel isothermal nucleic acid amplification assay based on the formation of a three-way junction structure. Nucleic Acids Res 2001;29(11). E54-4.
[8] Green AA, Silver PA, Collins JJ, Yin P. Toehold switches: de-novo-designed regulators of gene expression. Cell 2014;159(4):925–39.
[9] Van Ness J, Van Ness LK, Galas DJ. Isothermal reactions for the amplification of oligonucleotides. Proc Natl Acad Sci U S A 2003;100(8):4504–9.
[10] Zhou W, Reines D, Doetsch PW. T7 RNA polymerase bypass of large gaps on the template strand reveals a critical role of the nontemplate strand in elongation. Cell 1995;82(4):577–85.
[11] Rong M, He B, McAllister WT, Durbin RK. Promoter specificity determinants of T7 RNA polymerase. Proc Natl Acad Sci U S A 1998;95(2):515–9.
[12] Cazenave C, Uhlenbeck OC. RNA template-directed RNA synthesis by T7 RNA polymerase. Proc Natl Acad Sci U S A 1994;91(15):6972–6.
[13] McGinness KE, Joyce GF. Substitution of ribonucleotides in the T7 RNA polymerase promoter element. J Biol Chem 2002;277(4):2987–91.
[14] Arnaud-Barbe N, Cheynet-Sauvion V, Oriol G, Mandrand B, Mallet F. Transcription of RNA templates by T7 RNA polymerase. Nucleic Acids Res 1998;26(15):3550–4.
[15] Okazaki T, Kornberg A. Enzymatic synthesis of deoxyribonucleic acid. Xv. purification and properties of a polymerase from Bacillus subtilis. J Biol Chem 1964;239:259–68.
[16] Tan E, Erwin B, Dames S, Ferguson T, Buechel M, Irvine B, Voelkerding K, Niemz A. Specific versus nonspecific isothermal DNA amplification through thermophilic polymerase and nicking enzyme activities. Biochemistry 2008;47(38):9987–99.