Electronic Supplementary Material (ESI) for Analyst.


# POSITIVE FEEDBACK DRIVES A SECONDARY NONLINEAR PRODUCT BURST DURING A BIPHASIC DNA AMPLIFICATION REACTION - ELECTRONIC SUPPLEMENTARY INFORMATION

BURCU ÖZAY,‡ SHANNON D. MURPHY,‡ ESTHER E. STOPPS, TOMÁŠ GEDEON, AND STEPHANIE E. MCCALLA*
‡AUTHORS CONTRIBUTED EQUALLY TO THIS WORK

| Oligo Name | Sequence |
|---|---|
| LS2 template | 5'-TCCGGA GAAT TAATGACTCT TCCGGA GAAT-3' $NH_2$ |
| LS2 trigger | 5'$PO_3$-ATTC TCCGGA-3' |
| LS2 dimer | 5'$PO_3$-ATTC TCCGGA GAAT-3' |
| LS2 top strand | 5'$PO_3$-ATTCTCCGGAAGAGTCATTAATTCTCCGGA-3' |
| LS2 20nt top strand | 5'$PO_3$-ATTCTCCGGAAGAGTCATTA-3' |
| LS2 trigger A-tailed | 5'$PO_3$-ATTC TCCGGA A-3' |
| LS3 lowpG3 template | 5'-TCCGGA GTTTGG TAATGACTCT TCCGGA GTTTGG-3' $NH_2$ |
| LS3 lowpG3 trigger | 5'$PO_3$-CCAAAC TCCGGA-3' |
| LS3 lowpG3 dimer | 5'$PO_3$-CCAAAC TCCGGA GTTTGG-3' |
| LS3 lowpG3 top strand | 5'$PO_3$-CCAAACTCCGGAAGAGTCATTACCAAACTCCGGA-3' |
| LS3 lowpG3 22nt top strand | 5'$PO_3$-CCAAACTCCGGAAGAGTCATTA-3' |
| LS3 lowpG3 trigger A-tailed | 5'$PO_3$-CCAAAC TCCGGA A-3' |
| LS3 sp template | 5'-CGCG GTTTGG TAATGACTCT CGCG GTTTGG-3' $NH_2$ |
| LS3 sp trigger | 5'$PO_3$-CCAAAC CGCG-3' |
| LS3 sp dimer | 5'$PO_3$-CCAAAC CGCG GTTTGG-3' |
| LS3 sp top strand | 5'$PO_3$-CCAAACCGCGAGAGTCATTACCAAACCGCG-3' |
| LS3 sp 20nt top strand | 5'$PO_3$-CCAAACCGCGAGAGTCATTA-3' |
| LS3 sp trigger A-tailed | 5'$PO_3$-CCAAAC CGCG A-3' |
| EXPAR1 template | 5'-CTCACGCTAC GGACGACTCT CTCACGCTAC-3' $PO_3$ |
| EXPAR1 trigger | 5'-GTAGCGTGAG-3' |

**Table SI 1:** Oligonucleotide sequences.

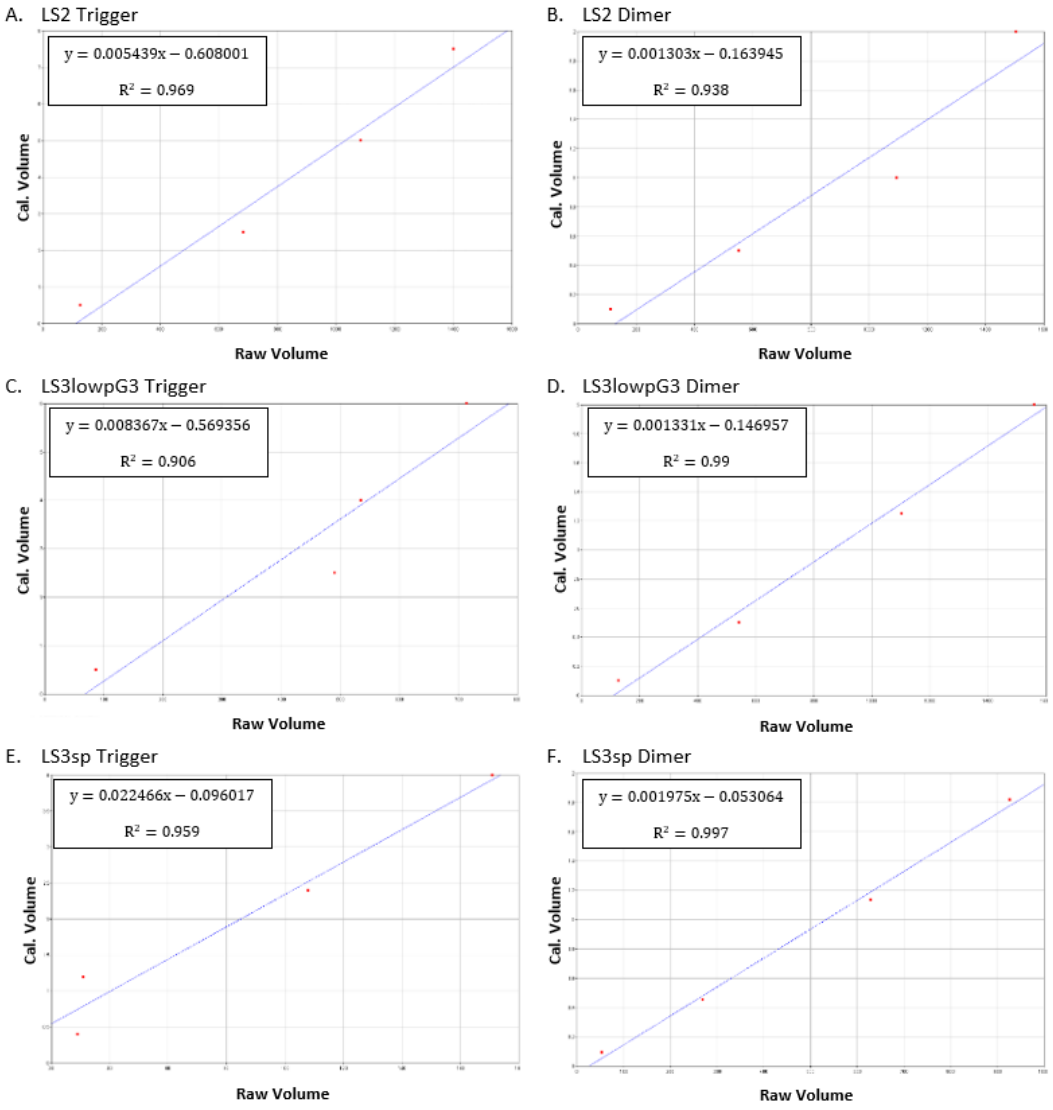


**Figure SI 1:** Example calibration curves of trigger and dimer standards for PAGE gel analysis, as calculated by GelAnalyzer 19.1 software (www.gelanalyzer.com).

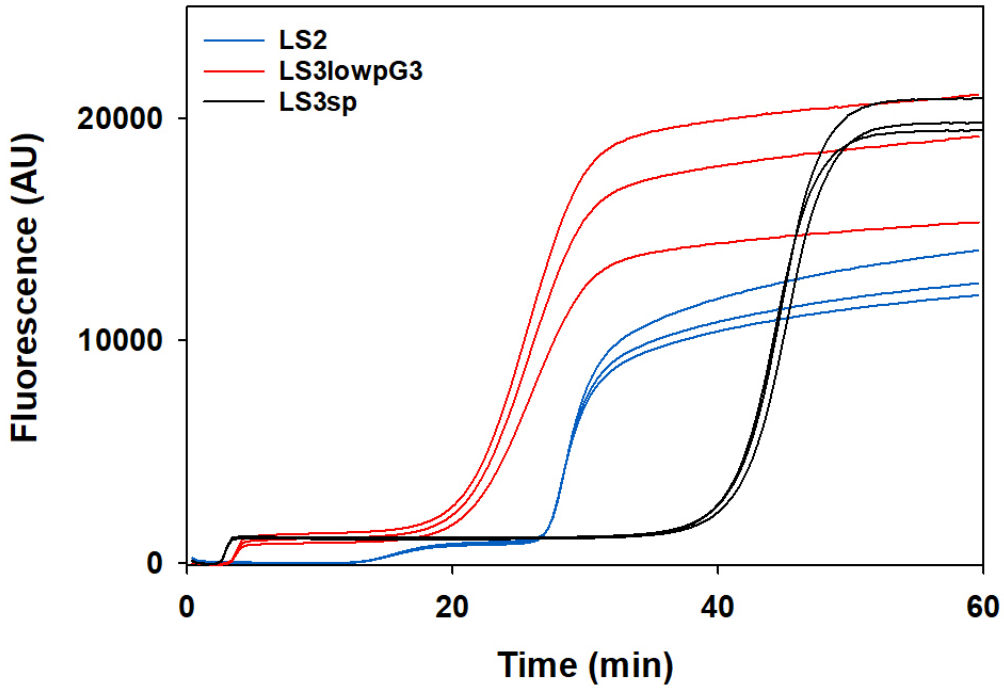


**Figure SI 2:** Fluorescence traces for three different templates shows the distinct biphasic response and large fluorescent output of UDAR. Three technical replicates from the same master mix are shown for each template.

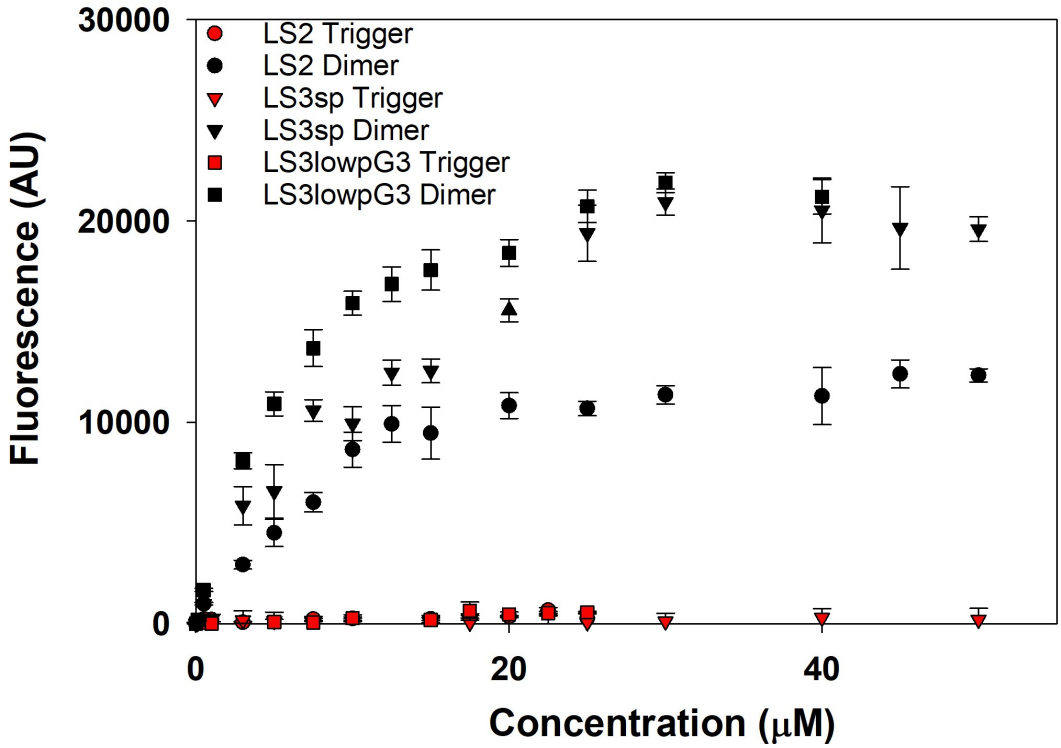


**Figure SI 3:** Staining performance of SYBR Green II for triggers and dimers of three different UDAR templates. Samples were prepared in UDAR buffer and fluorescence measurements were conducted by a qPCR machine at 55°C. While triggers provide minimal signal, extended trigger dimers and monomers $(S + D)$ create a significant fluorescent signal.

| | $\kappa_1$ | $\kappa_2$ | $k_1$ | $k_2$ | $\kappa_3$ |
|---|---|---|---|---|---|
| Second Phase Fit, LS2 and LS3lowpG | 1,$10^5$ | 0.01,1 | N/A | N/A | N/A |
| Second Phase Fit, LS3sp | 1,$10^{10}$ | 0.01,2 | N/A | N/A | N/A |
| Full Reaction, Initial Fit | N/A | N/A | $10^{-5}$,100 | $0.01, 10^4$ | 0.01,$\kappa_2$ |
| Full Reaction, Final Fit | N/A | N/A | $10^{-5}$,100 | N/A | 0.01,$\kappa_2$ |
| Full Reaction, Final Fit, LS3sp | N/A | N/A | $10^{-5}$,200 | N/A | 0.01,$\kappa_2$ |

**Table SI 2:** Constraints used during optimization with the MATLAB function fmincon.

[h]

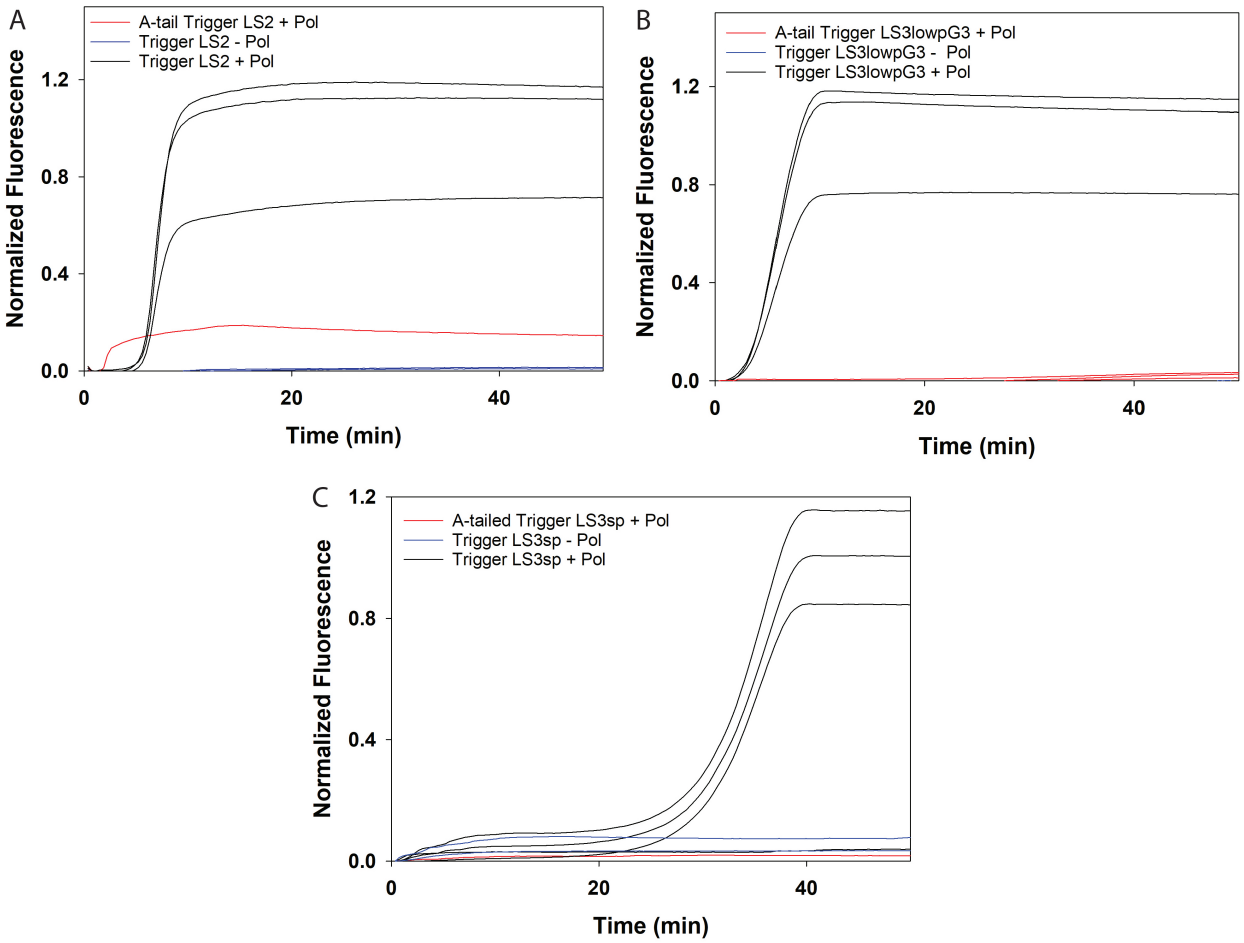


**Figure SI 4:** UDAR second phase reaction fluorescence traces for three different triggers and their A-tailed versions, with three technical replicates each from the same master mix. Samples with no polymerase added were included as background fluorescence controls. A-tailed triggers did not reproduce second phase production of highly fluorescent products. A-tailing of products by the polymerase was therefore not included in the mathematical model.

**Computationally investigating polymerase association with double stranded DNA:** It is possible that polymerase bound to double stranded DNA, which could decrease the unbound polymerase concentration in the reaction. We investigated this possibility by both including polymerase in the competition term for long double stranded DNA ($\kappa_3 = \kappa_4$), including polymerase in the competition term for all double stranded DNA ($\kappa_3 = \kappa_4 = \kappa_5$), or excluding polymerase from the competition term ($\kappa_4$ and $\kappa_5$ excluded). The model fits were optimal when using $\kappa_4$, although the model reproduces the reaction output without including sequestration of polymerase by double stranded DNA (Fig. SI 5 and Table SI 3). There were modest changes in the final fit parameters when these different competitive $\kappa$ values were used (Table SI 4). It is possible that sequestration of polymerase by double stranded DNA did not need to be included in the final model, as the effect on the fit was modest.

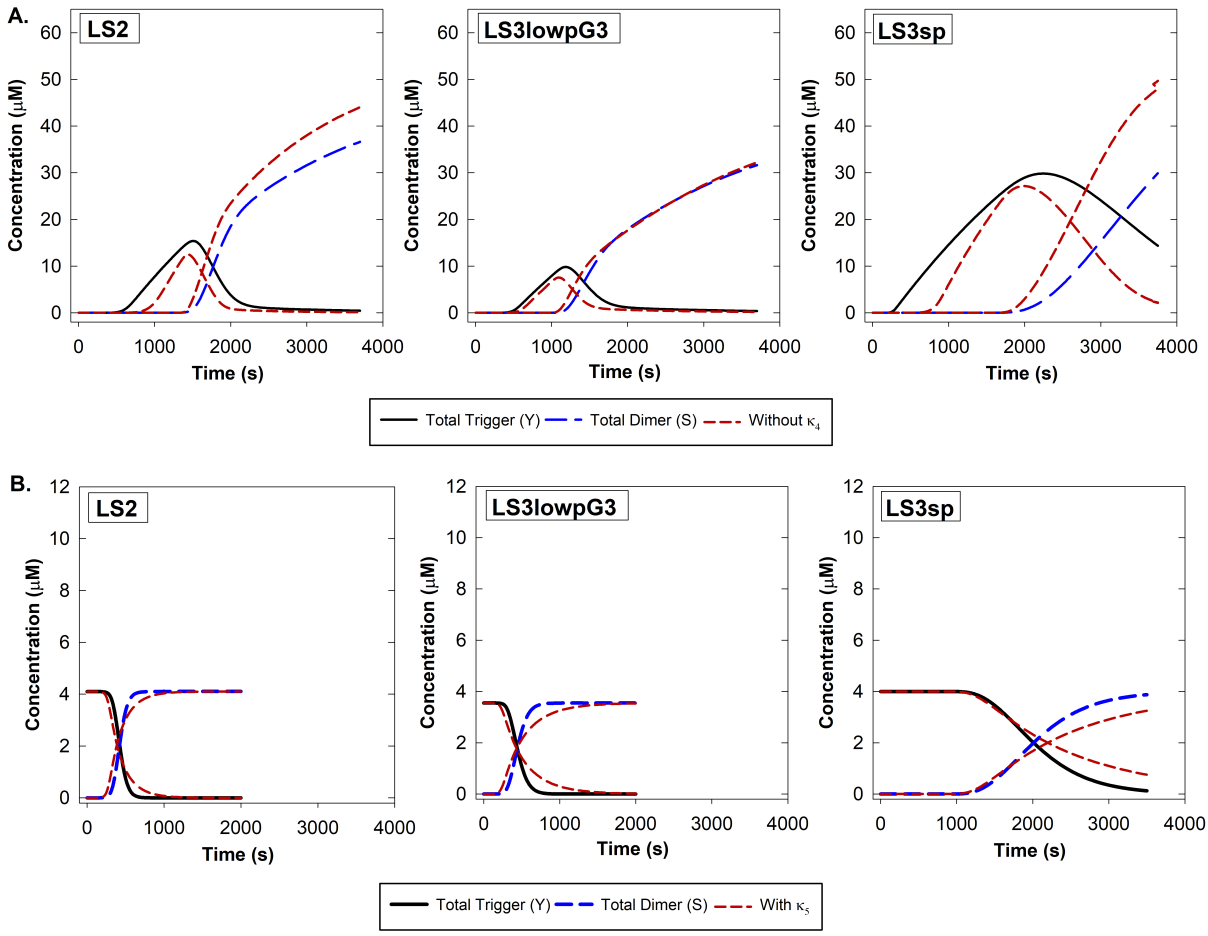


**Figure SI 5:** The effect of excluding polymerase rebinding to $W$ and $V$ ($\kappa_4$) in the full fit and including competition from double stranded dimer $D$ ($\kappa_5$) in the second phase fit. A) Excluding $\kappa_4$, which acknowledges the possibility of Bst polymerase rebinding to fully dsDNA, results in a full fit that sill retains the rise and fall of $Y$ and the steep second phase rise; however, the fit error increases. Therefore, $\kappa_4$ was included in the final model. B) Including competition from the double stranded dimer $D$ ($\kappa_5$) also increases the model error and causes a decreased rate of second phase growth. Therefore, competition due to polymerase rebinding to $D$ was not included in the final model.

| | **Final Full Fit** | **Full fit without $\kappa_4$ (no dsDNA inhibition)** | **Final second phase fit** | **Second phase fit with $\kappa_5$ (ds Dimer inhibition)** |
|---|---|---|---|---|
| LS2 | 2.4E+04 | 4.4E+04 | 8.1 | 11.1 |
| LS3lowpG3 | 5.9E+03 | 8.8E+03 | 11.6 | 14.0 |
| LS3sp | 4.0E+04 | 7.7E+04 | 105.0 | 122.5 |

**Table SI 3:** Comparison of least errors for fits using different assumptions for $\kappa_4$ and $\kappa_5$, which relate to polymerase rebinding to fully double stranded DNA.

| Parameter | LS2 | LS3lowpG3 | LS3sp | |
|---|---|---|---|---|
| Full fit with $\kappa_4$ | | | | |
| $k_1$ | 7.6E-01 | 5.2E-02 | 2.6E-01 | $s^{-1}$ |
| $k_2$ | 2.6E+01 | 2.6E+01 | 2.6E+01 | $s^{-1}$ |
| $\kappa_3$ | 2.6E-02 | 5.8E-02 | 3.6E-02 | $\mu$M |
| $\kappa_4$ | 2.6E-02 | 5.8E-02 | 3.6E-02 | $\mu$M |
| Full fit without $\kappa_4$ | | | | |
| $k_1$ | 3.0E-01 | 4.8E-02 | 2.7E-02 | $s^{-1}$ |
| $k_2$ | 2.7E+01 | 2.7E+01 | 2.7E+01 | $s^{-1}$ |
| $\kappa_3$ | 1.0E-02 | 9.3E-02 | 1.0E-02 | $\mu$M |
| $\kappa_4$ | N/A | N/A | N/A | $\mu$M |
| Second phase fit without $\kappa_5$ | | | | |
| $\kappa_1$ | 1.0+04 | 1.0E+04 | 1.0E+06 | $\mu$M |
| $\kappa_2$ | 3.5E-01 | 3.5E-01 | 8.9E-01 | $\mu$M |
| Second phase fit with $\kappa_5$ | | | | |
| $\kappa_1$ | 5.0+03 | 1.0E+03 | 1.0E+06 | $\mu$M |
| $\kappa_2$ | 3.0E-01 | 3.2E-01 | 8.1E-01 | $\mu$M |
| $\kappa_5$ | 3.0E-01 | 3.2E-01 | 8.1E-01 | $\mu$M |

**Table SI 4:** Comparison of selected model parameters with and without simulated polymerase binding to fully double stranded DNA. The full fit with $\kappa_4$, as seen in the main text, is shown with the full fit without $\kappa_4$ for comparison. The inclusion of $\kappa_4$ assumes that the polymerase can rebind the fully double stranded template $W$. The second phase fit of $\kappa_1$ and $\kappa_2$, as seen in the main text, is compared with a fit that additionally includes $\kappa_5$. The inclusion of $\kappa_5$ assumes that the polymerase can rebind fully double stranded dimer $D$.

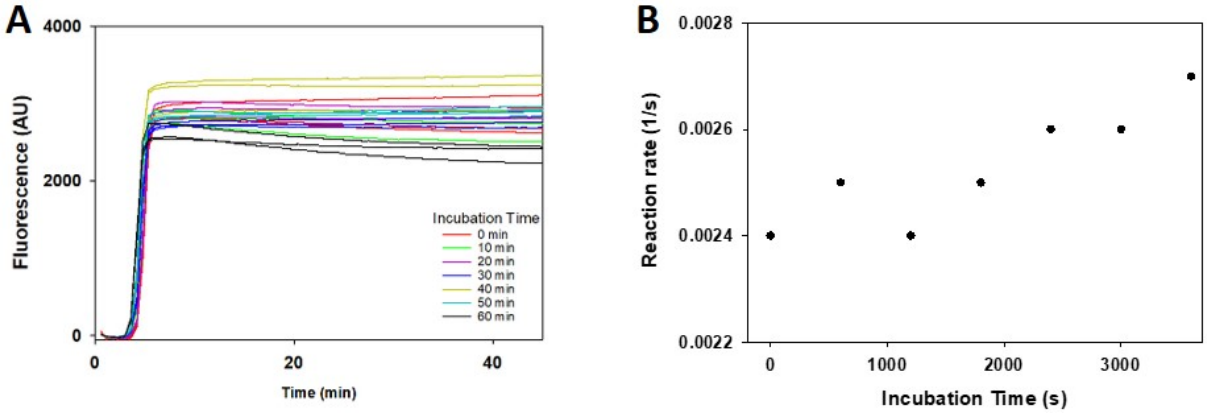


**Figure SI 6:** Polymerase activity did not notably change after 55°C incubation. A) Fluorescence-time traces of EXPAR samples, prepared with polymerase that was pre-incubated at 55°C. Six EXPAR reaction mixes were prepared with polymerase and without nicking endonuclease and template, and incubated for 0, 10, 20, 30, 50 and 60 min, respectively. Once the nicking endonuclease and an EXPAR template are added, 55°C incubation is continued and the fluorescence readings were performed every 24 seconds in a qPCR machine. B) The reaction rate, assumed to be a saturating exponential conversion between single stranded and double stranded template, minimally changes over time.

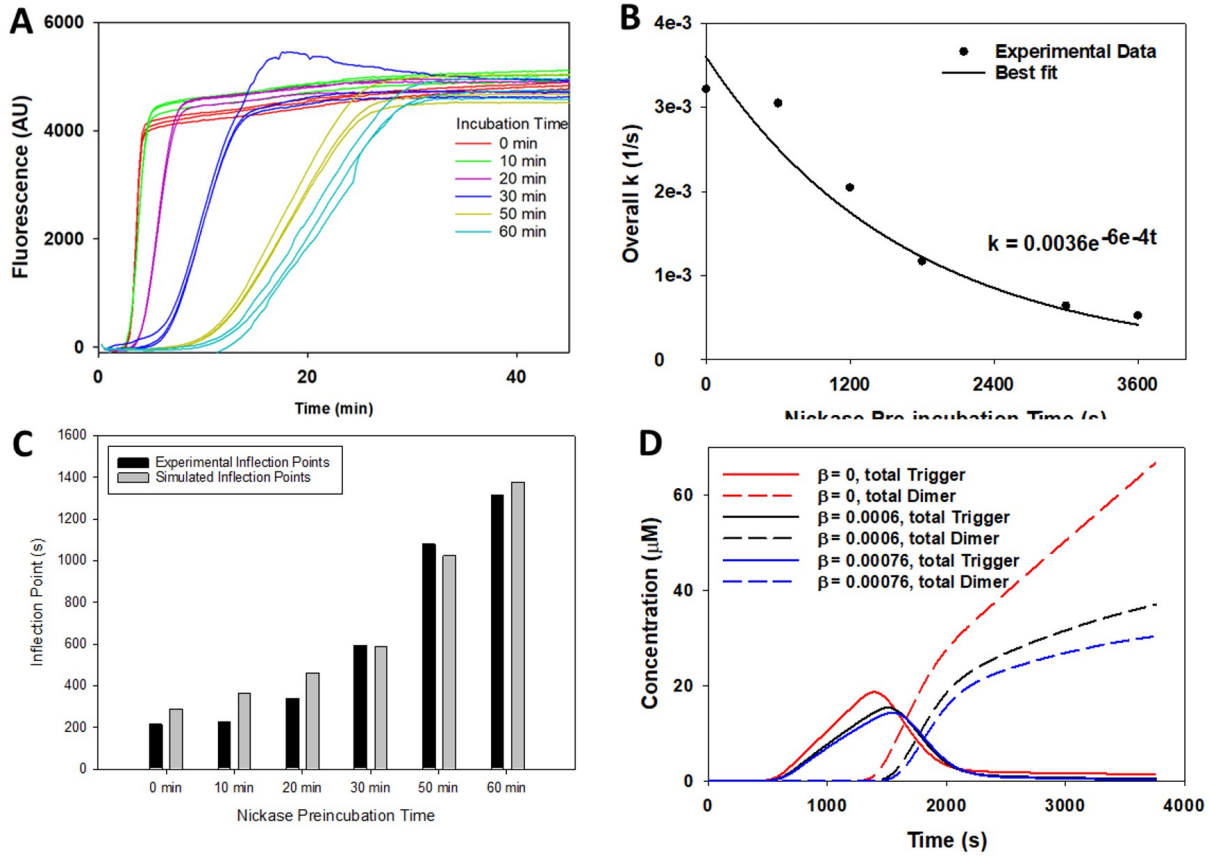


**Figure SI 7:** Nicking endonuclease activity changed over time with 55°C incubation. A) Fluorescence-time traces of EXPAR samples, prepared with nicking endonuclease that was pre-incubated at 55°C. Six EXPAR reaction mixes were prepared with nicking endonuclease and without polymerase and template, and incubated for 0, 10, 20, 30, 50 and 60 min, respectively. Once the polymerase and an EXPAR template are added, 55°C incubation is continued and the fluorescence readings were performed every 18 seconds in a qPCR machine. B) The reaction rate, assumed to be a saturating exponential conversion between single stranded and double stranded template, exponentially decreased over nickase incubation time.

**Verifying nicking endonuclease deactivation:** Nicking endonuclease slowly deactivates over time during EXPAR and UDAR reactions. To quantify this, nicking endonuclease was pre-incubated at 55°C for varying times in reaction buffer and then used in a subsequent EXPAR reaction (Fig. SI 7A). This reaction was originally assumed to fit the saturating exponential

$$F = F_{max}(1 - exp(-kt)),$$

where $F_{max}$ is the maximum fluorescence, $F$ is the fluorescence, and $k$ is the overall reaction rate of the EXPAR reaction. The overall reaction rate is therefore $k = \frac{ln(2)}{IF}$, where the inflection point $IF$ is assumed to approximate the point at which half of the maximum fluorescence occurs. The calculated overall reaction rate $k$ exponentially decayed with pre-incubation time (Fig. SI 7B). The decay rate was taken as the model parameter $\beta$, which assumes that the overall reaction rate was proportional to the nicking rate $k_2[N]$. As this calculation carries several assumptions, we aimed to verify the validity of this calculation by fitting the inflection points found in a mathematical model of EXPAR with nicking endonuclease deactivation with our experimental data to extract $\beta$, and compare this value to the originally calculated $\beta$. The following

equations describe an EXPAR reaction:

$$\begin{aligned}
[\dot{Y}] &= -k_1(K_a[T][Y]) + k_2[N][W] \\
[\dot{W}] &= k_3[V] - k_2[N][W] + k_1(K_a[T][Y]) \\
[\dot{V}] &= -k_3[V] + k_2[N][W] \\
[\dot{N}] &= -\beta[N] \\
T &= T_0 - W - V
\end{aligned}$$

Here, $T$ is the linear EXPAR template, $T_0$ is the initial concentration of template, $W$ is fully double stranded template, $V$ is nicked template, and $[N]$ is the nicking endonuclease. The parameter $K_a = 0.0694$ is the association constant of the EXPAR trigger calculated as described in the main text. The parameters $k_1 = 0.5s^{-1}$ and $k_2 = 25.75s^{-1}\mu M^{-1}$ were found in this study, and were the average value of the extension of a 10nt trigger bound to template and average overall nicking rate, respectively. The parameter $k_3$ describes extension of $V$ to $W$. As with the original mathematical model introduced with EXPAR [1], we did not account for polymerase competition in this model. The observed increase in fluorescence during an EXPAR reaction is due to the single stranded template $T$ becoming double stranded template $W$ or $V$. We therefore define the species $T_d = W + V$, and find the ODE system:

$$\begin{aligned}
[\dot{Y}] &= -k_1(K_a[T][Y]) + k_2[N][W] \\
[\dot{T_d}] &= k_1(K_a[T][Y]) \\
[\dot{N}] &= -\beta[N] \\
T &= T_0 - T_d
\end{aligned}$$

We assume that $W \approx T_d$, as the two species should be similar in concentration; the species $W$ is seen as the main template-derived product during the first phase of UDAR. The initial conditions were

$$N_0 = 0.026exp(-\beta t_p)$$

and $T_0 = 0.1$, where $t_p$ is the pre-incubation time in seconds and $T_0$ is the initial concentration of template expressed in $\mu M$. We fit parameter $\beta$ by minimizing the function

$$\sum_{i=1}^{n}(IF_i - \widehat{IF_i})^2$$

. Here, $n$ is the number of pre-incubation data sets, $IF_i$ is the experimentally determined inflection point found by numerically calculating the point where the second derivative of fluorescence is zero, and $\widehat{IF_i}$ is the inflection point in the rise of species $T_d$ found in the second ODE model above. We constrained $\beta$ to be between $0.0001 - 0.0008$. The resulting best fit value, $\beta = 0.000757$, differed from the original calculation by 26% and reproduced the inflection points across all pre-incubation times, including the EXPAR reaction that was not pre-incubated and therefore completed in less than 10 minutes (Fig. SI 7C). This difference minimally changes the output of the mathematical model (Fig. SI 7D), but the exclusion of $\beta$ produces a large final product rise that is not seen in our reaction. We therefore maintained the original calculated value of $\beta = 0.0006$.

## References

[1] J. Van Ness, L. K. Van Ness, and D. J. Galas, "Isothermal reactions for the amplification of oligonucleotides," *Proceedings of the National Academy of Sciences*, vol. 100, no. 8, pp. 4504–4509, 2003, ISSN: 0027-8424. DOI: 10.1073/pnas.0730811100. eprint: https://www.pnas.org/content/100/8/4504.full.pdf. [Online]. Available: https://www.pnas.org/content/100/8/4504.