# Electronic Supplementary Information

## MNAzymes provide a universal mechanism for triggering DNAzyme synthesis cascades.

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### Materials and methods

**1. Chemicals.** All synthetic oligonucleotides were purchased from either Integrated DNA Technologies (Coralville, IA, USA) or Biosearch Technologies (Petaluma, CA, USA). The oligonucleotide sequences are listed in Table S1. The strand-displacing polymerase '*Bst* 2.0 warm start', Restriction Enzyme 'Nt.AlwI' and T4 Polynucleotide Kinase (PNK) enzymes as well as the NEB Buffer 2 were purchased from New England Biolabs Inc. (Ipswich, MA, USA). The deoxynucleotide tri-phosphates (dNTPs) were purchased from Bioline Australia (Sydney, NSW, Australia).

**2. Isothermal fluorescence assays.** Reactions contained a master mix consisting of 1x NEB buffer 2 and 200 µM of deoxynucleotide tri-phosphates (dNTPs) at a final volume of 25 µL. All reactions were set-up on ice to initially inhibit enzyme activity until the reactions were raised to the reaction temperature and fluorescently monitored. Reactions were incubated at a constant temperature (specified for each experiment) in a CFX96™ Real-Time PCR detection system (Bio-Rad) with fluorescence measured every 30 seconds. All reactions were run in duplicate and results in figures are the averages of the duplicate reactions, plotted using Microsoft Excel (Version 14).

**2.1. Hairpinned primer assay.** Isothermal fluorescence assays were performed at a temperature of 53˚C. In addition to the master mix, reactions included 0.7 units of *Bst* 2.0 warm start polymerase and 2.5 units of Nt.AlwI. All reactions contained 50 nM of each of Partzyme A1 and Partzyme B1 as well as 100 nM of Dz-hp-template (Temp D4) and 200 nM of fluorescent substrate (Dz Sub1). The following controls and reactions were tested and results are presented in Figure 1.

- 'No primer control' (black trace), containing all oligonucleotides listed above only.
- 'Linear primer (positive control)' (red trace), containing all oligonucleotides listed above, with 20 nM of control 'linear primer' (Linear PR1).
- 'hp-primer (- target)' (blue trace), containing all oligonucleotides listed above, with 30 nM of hairpinned primer (HpPR), but no MNAzyme target.
- 'hp-primer (+ target)' (green trace), containing all oligonucleotides listed above, with 30 nM of hairpinned primer (HpPR) and 20 nM of MNAzyme target (Target 1).

**2.2. PNK assay.** Isothermal fluorescence assays were performed at a temperature of 51˚C. In addition to the master mix, reactions contained 0.6 units of *Bst* 2.0 warm start polymerase and 2.2 units of Nt.AlwI. All reactions also contained the following oligonucleotide components: 25 nM of

Dz-hp-template (Temp D5), 200 nM of DNAzyme substrate (Dz Sub2), 50 nM each of Partzyme A2 and Partzyme B2 required for MNAzyme assembly and 100 nM of MNAzyme substrate (Mz Sub). The following controls and reactions were tested and results are presented in Figure 2 with the signal from the FAM channel shown on the left and signal from the Q670 channel on the right:

- 'No PNK (- Target)' (black trace), containing all oligonucleotides listed above only.
- 'No PNK (+ Target)' (red trace), containing all oligonucleotide components listed above, along with 10 nM of MNAzyme target (Target 2).
- 'PNK present (-Target)' (blue trace), containing all oligonucleotide components listed above, along with 4 units of T4 PNK.
- 'PNK present (+ Target)' (green trace), containing all oligonucleotide components listed above, along with 10 nM of MNAzyme target (Target 2) and 4 units of T4 PNK.

**2.3. Hairpinned primer limit of detection assay.** Isothermal fluorescence assays were performed at a temperature of 53˚C. In addition to the master mix, reactions included 0.7 units of *Bst* 2.0 warm start polymerase and 2.5 units of Nt.AlwI. All reactions contained 50 nM of each of Partzyme A1 and Partzyme B1 as well as 100 nM of Dz-hp-template (Temp D4) and 200 nM of fluorescent substrate (Dz Sub1). The following controls and reactions were tested and results are presented in Figure S2.

- 'No primer control' (black trace), containing all oligonucleotides listed above only.
- 'No target' (grey trace), containing all oligonucleotides listed above, with 30 nM of hairpinned primer (HpPR), but no MNAzyme target.
- '20 nM target' (red trace), containing all oligonucleotides listed above, with 30 nM of hairpinned primer (HpPR) and 20 nM of MNAzyme target (Target 1).
- '5 nM target' (dark blue trace), containing all oligonucleotides listed above, with 30 nM of hairpinned primer (HpPR) and 5 nM of MNAzyme target (Target 1).
- '2.5 nM target' (green trace), containing all oligonucleotides listed above, with 30 nM of hairpinned primer (HpPR) and 2.5 nM of MNAzyme target (Target 1).
- '1 nM target' (purple trace), containing all oligonucleotides listed above, with 30 nM of hairpinned primer (HpPR) and 1 nM of MNAzyme target (Target 1).
- '500 pM target' (orange trace), containing all oligonucleotides listed above, with 30 nM of hairpinned primer (HpPR) and 500 pM of MNAzyme target (Target 1).
- '100 pM target' (light blue trace), containing all oligonucleotides listed above, with 30 nM of hairpinned primer (HpPR) and 100 pM of MNAzyme target (Target 1).

**2.4. PNK limit of detection assay.** Isothermal fluorescence assays were performed at a temperature of 51˚C. In addition to the master mix, reactions contained 0.6 units of *Bst* 2.0 warm start polymerase, 2.2 units of Nt.AlwI and 4 units of T4 PNK. All reactions also contained the following oligonucleotide components: 25 nM of Dz-hp-template (Temp D5), 200 nM of DNAzyme substrate (Dz Sub2), 50 nM each of Partzyme A2 and Partzyme B2 required for MNAzyme assembly and 100 nM of MNAzyme substrate (Mz Sub). The following controls and reactions were tested and results

are presented in Figure S3 with the signal from the FAM channel shown on the top and signal from the Q670 channel underneath:

- 'No Target' (black trace), containing all oligonucleotides listed above only.
- '10 nM Target' (red trace), containing all oligonucleotide components listed above, along with 10 nM of MNAzyme target (Target 2).
- '1 nM Target' (dark blue trace), containing all oligonucleotide components listed above, along with 1 nM of MNAzyme target (Target 2).
- '500 pM Target' (green trace), containing all oligonucleotide components listed above, along with 500 pM of MNAzyme target (Target 2).
- '100 pM Target' (purple trace), containing all oligonucleotide components listed above, along with 100 pM of MNAzyme target (Target 2).
- '75 pM Target' (orange trace), containing all oligonucleotide components listed above, along with 75 pM of MNAzyme target (Target 2).
- '50 pM Target' (light blue trace), containing all oligonucleotide components listed above, along with 50 pM of MNAzyme target (Target 2).

**2.3. Template variation assay.** Isothermal fluorescence assays were performed at a temperature of 54˚C. In addition to the master mix, reactions included 0.8 units of *Bst* 2.0 warm start polymerase and 2.5 units of Nt.AlwI. All reactions contained 200 nM of fluorescent substrate (Dz Sub1). The following controls and reactions were tested and results are presented in Figure S1.

- 'Design 1 (Primer absent)' (dashed green trace), containing 100 nM of Design 1 template (Temp D1) only.
- 'Design 1 (Primer present)' (solid green trace), containing 100 nM of Design 1 template (Temp D1) with 20 nM of primer (Linear PR1).
- 'Design 2 (Primer absent)' (dashed purple trace), containing 100 nM of Design 2 template (Temp D2) only.
- 'Design 2 (Primer present)' (solid purple trace), containing 100 nM of Design 2 template (Temp D2) with 20 nM of primer (Linear PR2).
- 'Design 3 (Primer absent)' (dashed red trace), containing 100 nM of Design 3 template (Temp D3) only.
- 'Design 3 (Primer present)' (solid red trace), containing 100 nM of Design 3 template (Temp D3) with 20 nM of primer (Linear PR1).
- 'Design 4 (Primer absent)' (dashed blue trace), containing 100 nM of Design 4 template (Temp D4) only.
- 'Design 4 (Primer present)' (solid blue trace), containing 100 nM of Design 4 template (Temp D4) with 20 nM of primer (Linear PR1).

**Table S1.** Oligonucleotide sequences

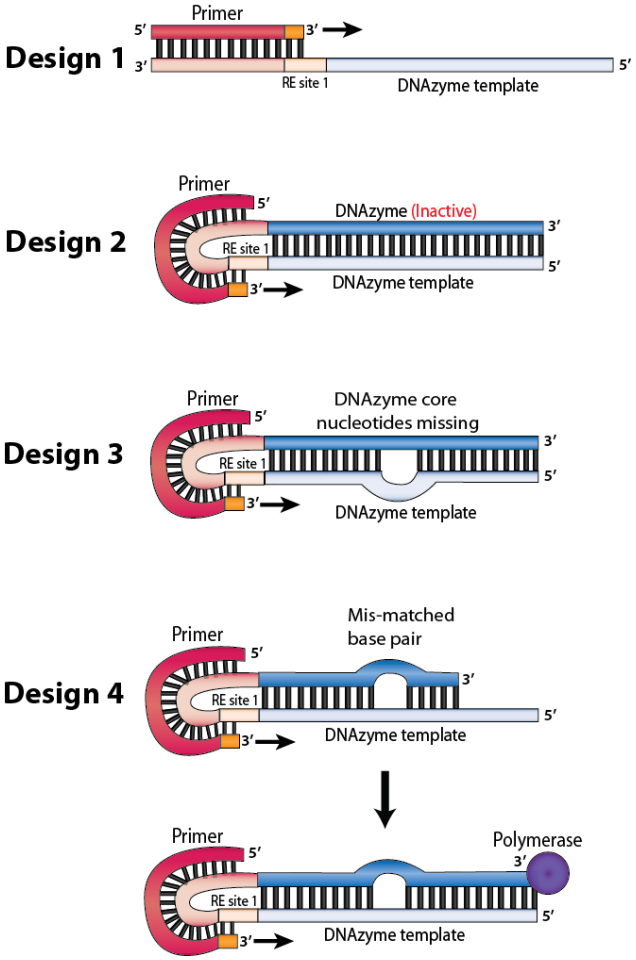
| Fig# | Oligo name | Oligo type | Sequence 5'-3' |
|---|---|---|---|
| 1 | Partzyme A1 | Partzyme A | **TAAACTTGTGGTAGTTGGAG**ACAACGA*GAGGAAACCTT*/3Phos/ |
| 1 | Partzyme B1 | Partzyme B | *TGCCCAGGGA*GGCTAGCT**CTGGTGGCGTAGGCAAGAGTGCC**/3Phos/ |
| 2 | Partzyme A2 | Partzyme A | **GGAATATGGAAGGAGACTGTC**ACAACGA*GGGACCCGT*/3Phos/ |
| 2 | Partzyme B2 | Partzyme B | *TTCCAAAGGAGA*GGCTAGCT**CCTCTGACTGGAAAACAGACT**/3Phos/ |
| 1 | Target 1 | Target | CAAGGCACTCTTGCCTACGCCACCAGCTCCAACTACCACAAGTTTATA TTCA |
| 2 | Target 2 | Target | AGTCTGTTTTCCAGTCAGAGGGACAGTCTCCTTCCATATTCC |
| 1, S1 | Dz Sub1 | Substrate | ACGGGTCCCguCTCCTTTGGAA |
| 2 | Dz Sub2 | Substrate | ACGGGTCCCguCTCCTTTGGAA |
| 2 | Mz Sub | Substrate | CGAAGGTTTCCTCguCCCTGGGCACG |
| 1, S1 | Temp D4 | Template | ACGGGTCCCTCGTTGTAGCTAGCCTCTCCTTTGGTCGCTGATCCTGTAC TTGACCAAAGGAGATGCTAGC |
| 2 | Temp D5 | Template | GATCCGACTAGGACGGGTCCCTCGTTGTAGCTAGCCTCTCCTTTGGGCT GATCCGAGGAAACCTTCCAAAGGAGAAAGCTA |
| S1 | Temp D1 | Template | ACGGGTCCCTCGTTGTAGCTAGCCTCTCCTTTGGAATCGCTGATCCTGT ACTT |
| S1 | Temp D2 | Template | ACGGGTCCCTCGTTGTAGCTAGCCTCTCCTTTGGTCGTCGATCCTGAGA C**CAAAGGAGAGGCTAGCTACAACGAGGGACCCGT** |
| S1 | Temp D3 | Template | ACGGGTCCCTCGTTGTAGCTAGCCTCTCCTTTGGTCGCTGATCCTGTAC TTGACCAAAGGAGAGGCTACAACGAGGGACCCGT |
| 1, S1 | Linear PR1 | Linear primer | AAGTACAGGATCAG |
| S1 | Linear PR2 | Linear primer | TCAGGATCGA |
| 1 | HpPR | Hairpin primer | CTGATCCTGTACTTCCGAGCATCCTTTTTGGATGCTC*AGGTTTCCTCguC CCTGGGC***AAGTAC** |

**Partzymes**. Partzyme sequences used in these experiments are displayed such that the boxed bases represent the partial catalytic core, bases in **bold** hybridize to the target and bases in *italics* hybridize to the substrate. /3Phos/ indicates 3' phosphorylation.

**Fluorescent substrates.** MNAzyme and DNAzyme activity were monitored by cleavage of dual-labelled nucleic acid substrates composed of DNA (upper case) and RNA (lower case) bases. Underlined bases indicate the position of the fluorophore (either 6-FAM (F), or Quasar 670 (Q670)), located 5' of the ribonucleotide bases and the quencher moiety (Iowa Black (IB), Black Hole Quencher 1 (B) or Black Hole Quencher 2 (B2)) located 3' of the ribonucleotide bases.

**Templates.** Template sequences used in these experiments are displayed such that the Boxed bases indicate bases of complete or partial DNAzyme catalytic cores. Underlined bases represent the DNAzyme template region which corresponds to the antisense of an active DNAzyme. Bases shaded in grey represent one strand of a double-stranded RE recognition sequence.

**Hairpinned primer.** The sequence of the hairpinned primer is displayed such that bases in **bold** indicate regions of the primer portion which are complementary to the inhibitory portion. Underlined bases represent regions of the inhibitory portion which are complementary to the primer portion. Bases in *italics* represent a substrate sequence within the hairpinned oligonucleotides. Uppercase bases represent DNA and lower case bases represent RNA.




**Scheme S1. Template variations for DNAzyme synthesis.** For each of the variations, the DNAzyme template portion is shown in pale blue, partial RE recognition site (RE site 1) in pale orange and primer-binding site in pale red. Any DNAzyme or partial DNAzyme portion is shown in blue. In each case, the primer fully binds to the primer-binding site and partially binds to the RE site 1, thus is drawn as both red and orange according to the regions of the template which it hybridizes to.
Design 1 - A single-stranded template that does not contain any sense DNAzyme sequence or portion thereof.
Design 2 - A complete hairpinned DNAzyme containing a complete DNAzyme hybridized to its complement, which functions to both block the DNAzyme portion and provide a template for new DNAzyme synthesis.
Design 3 - A hairpinned structure with DNAzyme portion lacking essential core nucleotides.
Design 4 - A hairpinned structure with a partial DNAzyme portion comprising a mutated base with respect to the consensus sequence for an active catalytic core, which is thus mis-matched with the template. The template contains the anti-sense of the active DNAzyme sequence. Extension by the polymerase enzyme (purple) of the partial mutated sequence is designed to further close the hairpin but should not produce an active DNAzyme. This was the design chosen for further studies.

The purpose of creating Designs 2-4 as hairpinned molecules were because it was hypothesized that in these designs the DNAzyme template should always remain blocked within a double-stranded conformation. Further as new DNAzymes are synthesized, they may be less likely to bind to ‘vacant’ templates which could function to block and temporarily inactivate these newly synthesized DNAzymes. Design 2 was initially designed for this purpose; however Design 3 then aimed to improve the specificity of the reaction by removing the initial presence of a DNAzyme, such that active DNAzymes would only be present following the extension of a primer. Design 4 was the final adaptation and makes use of the polymerase to complete the remainder of the molecule at the 3’ end rather than require it to be present initially.

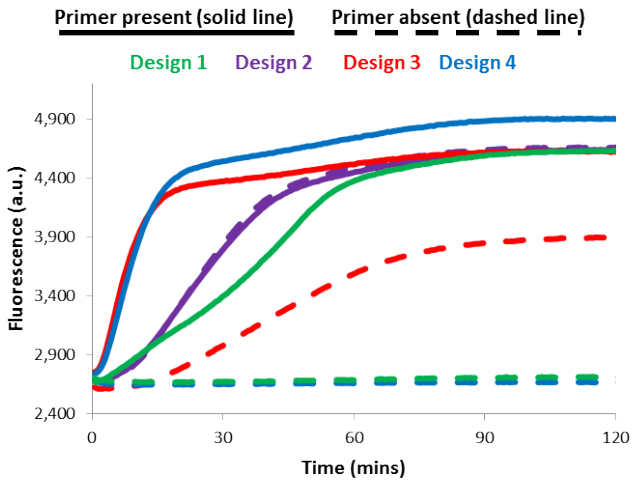



**Figure S1. Raw fluorescent data obtained from an experiment with each of Scheme S1 Designs 1 to 4.**
An increase in the 'Primer absent' signal (dashed lines) indicates non-specific background signal via either the DNAzyme not being completely blocked within the hairpin (Design 2 only), residual activity of the partial DNAzyme (Design 3) or via primer-independent DNAzyme synthesis (Designs 2 and 3).
An increase in the 'Primer present' signal (solid lines) above that of their respective signals in the absence of primer, indicates catalytic activity from either the bound DNAzyme released from within the hairpin (Design 2 only) or from new DNAzymes synthesized via primer-initiated extension (Designs 1 to 4).

Design 4 produced the greatest signal-to-noise difference and consequently was selected for experiments with the hairpinned primer strategy and used as a basis for the template in the PNK strategy in this manuscript. This result agrees with the rationale for the creation of this design, in that its advantage comes from the presence of the following features. Firstly, a complete DNAzyme was not present initially; therefore it only became present following the extension of the primer, helping to improve specificity. As the anti-sense template always remained within a double-stranded formation, this improved the speed of the signal, as synthesized DNAzymes were less able to re-hybridize and be blocked by vacant templates. This was also observed in the previous study outlining synthesis of nucleic acid modifying DNAzymes [1] and found that the addition of complementary 'blocking' sequences to their templates helped to improve the speed of their DNAzyme synthesis reactions. Their blocking sequences were however complete DNAzyme sequences potentially capable of catalysis, which is in contrast to the catalytically inactive, mutant DNAzymes used to block templates in this manuscript.

The final feature employed to improve specificity was the presence of recessed 3' termini. Since this inactive DNAzyme is initially truncated, the polymerase can extend at this 3' end as well as from the primer. This alternate polymerase activity may be providing an initial "distraction" for the polymerase which in turn may be preventing it from initiating aberrant DNA synthesis via a little-understood mechanism that is commonly reported in protocols that combine a RE and a polymerase[2-8]. Yan et al [9] have also observed and exploited this idea in their strategy which involved synthesis of peroxidase-mimicking DNAzymes. This group also reported a lower background signal using a similar 3' recessed structure. Further improvements in specificity could be achieved by providing 'background templates' into the reaction, such as those demonstrated in a recent study which used a combination of Exonuclease I and DNAzymes in a method for protein detection [10]. In this study, the addition of

random, unrelated DNA to reactions helped lower background signal from non-specific Exonuclease I activity.

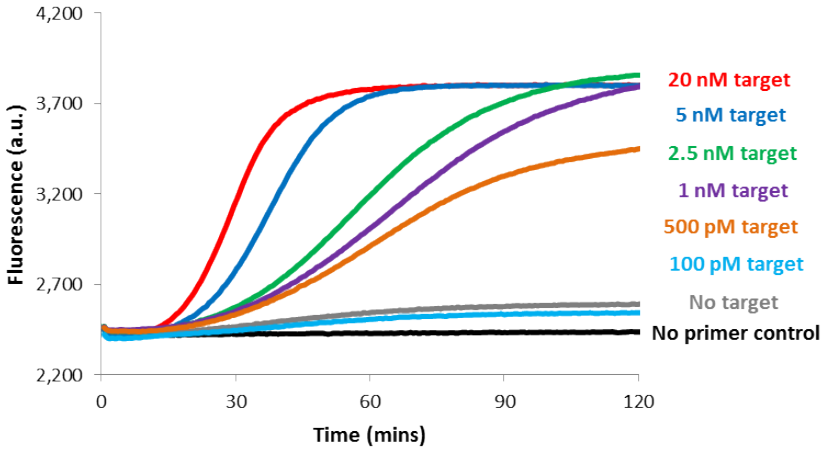



**Figure S2. Raw fluorescence data obtained from an experiment determining the limit of target detection with the hairpinned primer strategy.**

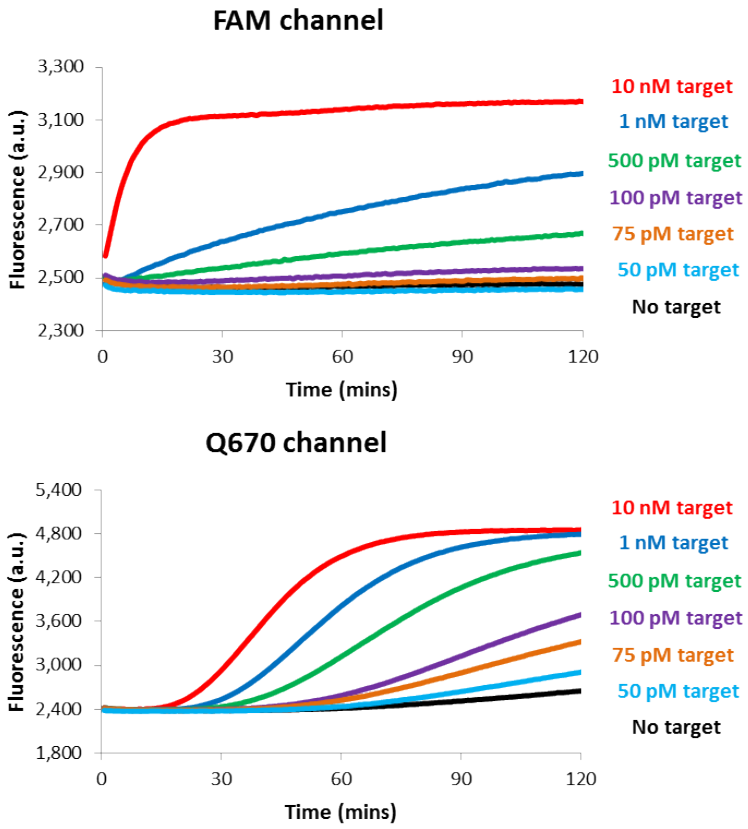



**Figure S3. Raw fluorescence data obtained from an experiment determining the limit of target detection with the PNK strategy.** Fluorescence signal from the FAM channel (top) corresponds to cleavage of the MNAzyme substrate and fluorescence signal from the Q670 channel (bottom) corresponds to cleavage of the DNAzyme substrate.

## References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