

DNAzyme can be easily adjusted and the mRNA cannot. One common guideline generated from these studies is that the exact sequence of the R-Y ribonucleotide motif at the cleavage site of the substrate is important with cleavage efficiency being in the following order: $GU \geq AU > GC \gg AC$.

The efficiency of cleavage of a full length mRNA under *in vitro* conditions is not an absolute measure of the cleavage efficiency in a cellular environment as the latter includes ribonuclear proteins and other confounding factors that cannot be easily mimicked *in vitro*.

The design guidelines generated in the past have some use in selection of sites within a long mRNA molecule that may be efficiently cleaved by DNAzymes and MNAzymes under physiological conditions, but have limited ability to predict which substrates will be cleaved with sufficient efficiency for utility in *in vitro* diagnostic applications. *In vitro* diagnostic applications may require conditions very different from the physiological conditions generally screened and used to establish the limited substrate design guidelines that exist in the art.

There is a need for a set of guidelines, or sequence motifs, for substrate sequences that predict with greater certainty if a substrate will be efficiently cleaved by a MNAzyme or DNAzyme in conditions suitable for *in vitro* diagnostic applications. There is also a need for catalytic nucleic acid substrates with properties that facilitate improved catalytic nucleic acid function. These properties may include, for example, an ability to facilitate improved catalytic nucleic acid function over a range of conditions and/or a capacity to extend the number of targets that can be simultaneously detected in a multiplex reaction.

SUMMARY OF THE INVENTION

While many have attempted to establish a sequence motif or set of design guidelines which consistently produces efficient substrate sequences, to date no effective sequence or set of guidelines has been identified. The present ~~invention~~-disclosure provides a series of principles which has facilitated the development of new efficiently cleaved substrates. The present ~~invention~~-disclosure thus provides catalytic nucleic acid enzyme substrates with properties that enhance catalytic nucleic acid function thereby addressing a need existing in the art.

In a first aspect, the present ~~invention~~-disclosure provides an isolated polynucleotide substrate for a catalytic nucleic acid enzyme, said polynucleotide substrate comprising a sequence $N_1-N_2-N_3-N_4-N_5-N_6-N_7-N_8-rR-rY-N_9-N_{10}-N_{11}-N_{12}-N_{13}-N_{14}-N_{15}$ wherein:

rR is a purine ribonucleotide;

In a second aspect, the present ~~invention~~-disclosure provides an isolated polynucleotide substrate for a catalytic nucleic acid enzyme, said polynucleotide substrate comprising or consisting of a sequence defined by SEQ ID NO: 28.

In one embodiment of the second aspect, the catalytic nucleic acid enzyme is an MNzyme comprising a pair of oligonucleotide partzymes, said pair comprising or consisting of SEQ ID NOs: 15 and 8, SEQ ID NOs: 93 and 94, or SEQ ID NOs: 114 and 115.

In one embodiment of the second aspect, the catalytic nucleic acid enzyme is a DNzyme comprising or consisting of a sequence defined by SEQ ID NO: 138.

In a third aspect, the present ~~invention~~-disclosure provides a method for detecting the presence of at least one target comprising:

(a) providing two or more oligonucleotide partzymes, wherein at least a first oligonucleotide partzyme and a second oligonucleotide partzyme self-assemble in the presence of said target to form at least a first catalytically active multi-component nucleic acid enzyme (MNzyme);

(b) providing the isolated polynucleotide substrate of the first or second aspect, wherein said polynucleotide substrate is capable of being modified by said first MNzyme, wherein said modification of said polynucleotide substrate by said MNzyme provides a detectable effect;

(c) contacting said two or more oligonucleotide partzymes with a sample putatively containing said target under conditions permitting:

- (1) the self-assembly of said at least first MNzyme, and
- (2) the catalytic activity of said at least first MNzyme; and

(d) detecting said detectable effect.

In one embodiment of the third aspect, the target is an MNzyme assembly facilitator.

In one embodiment of the third aspect, the target is a nucleic acid.

In one embodiment of the third aspect, the target is a nucleic acid that hybridizes to one or more sensor arms of said MNzyme by base pair complementarity.

In one embodiment of the third aspect, the nucleic acid is selected from the group consisting of DNA, methylated DNA, alkylated DNA, RNA, methylated RNA, microRNA, siRNA, shRNA, tRNA, mRNA, snoRNA, stRNA, smRNA, pre- and pri-microRNA, other non-coding RNAs, ribosomal RNA, derivatives thereof, amplicons, or any combination thereof.

In one embodiment of the third aspect, the nucleic acid is amplified.

In one embodiment of the third aspect, the amplification comprises one or more of: polymerase chain reaction (PCR), strand displacement amplification (SDA), loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), transcription-mediated amplification (TMA), self-sustained sequence replication (3SR), nucleic acid sequence based amplification (NASBA), or reverse transcription polymerase chain reaction (RT-PCR).

In one embodiment of the third aspect, the polynucleotide substrate hybridises with substrate arms of said MNzyme at a temperature of between 50°C and 90°C, between 50°C and 65°C, between 50°C and 60°C, between 52°C and 58°C, between 66°C and 76°C, between 68°C and 76°C, between 64°C and 70°C, between 70°C and 76°C, between 70°C and 75°C, between 72°C and 76°C, 52°C, 58°C, 64°C, 66°C, 68°C, 70°C, 72°C, or 76°C.

In one embodiment of the third aspect, the method further comprises providing:

- (a) two or more additional oligonucleotide partzymes capable of self-assembling in the presence of a different target to form a second catalytically active MNzyme; and
- (b) at least one additional polynucleotide substrate;

wherein said additional polynucleotide substrate is capable of being modified by said second MNzyme in the presence of said different target.

In one embodiment of the third aspect, the additional polynucleotide substrate is not capable of being modified by said first MNzyme.

In one embodiment of the third aspect, the detecting in part (d) comprises use of fluorescence spectroscopy, surface plasmon resonance, mass spectroscopy, NMR, electron spin resonance, polarization fluorescence spectroscopy, circular dichroism, immunoassay, chromatography, radiometry, electrochemical, photometry, scintigraphy, electronic methods, UV, visible light or infra-red spectroscopy, enzymatic methods, or any combination thereof.

In one embodiment of the third aspect, the detecting in part (d) comprises use of fluorescence spectroscopy.

In one embodiment of the third aspect, the detecting in part (d) comprises detection of a FRET detectable effect.

In one embodiment of the third aspect, the catalytic core of said MNzyme comprises DNA or an analogue thereof.

In a fourth aspect, the present ~~invention~~-disclosure provides use of the isolated polynucleotide substrate of the first or second aspect as a substrate for a catalytic nucleic acid enzyme.

In one embodiment of the fourth aspect, the catalytic nucleic acid enzyme is a multi-component nucleic acid enzyme (MNzyme),

SEQ ID NO: 45; SEQ ID NO: 62 and SEQ ID NO: 45; SEQ ID NO: 46 and SEQ ID NO: 63; SEQ ID NO: 71 and SEQ ID NO: 68; SEQ ID NO: 98 and SEQ ID NO: 99; SEQ ID NO: 100 and SEQ ID NO: 103; SEQ ID NO: 104 and SEQ ID NO: 105; SEQ ID NO: 106 and SEQ ID NO: 107; SEQ ID NO: 108 and SEQ ID NO: 109; SEQ ID NO: 110 and SEQ ID NO: 111; SEQ ID NO: 112 and SEQ ID NO: 113; SEQ ID NO: 116 and SEQ ID NO: 117; SEQ ID NO: 118 and SEQ ID NO: 119; SEQ ID NO: 120 and SEQ ID NO: 121; SEQ ID NO: 122 and SEQ ID NO: 119; SEQ ID NO: 155 and SEQ ID NO: 156; SEQ ID NO: 157 and SEQ ID NO: 158; SEQ ID NO: 159 and SEQ ID NO: 160; SEQ ID NO: 168 and SEQ ID NO: 169; SEQ ID NO: 179 and SEQ ID NO: 180; SEQ ID NO: 181 and SEQ ID NO: 182; SEQ ID NO: 183 and SEQ ID NO: 184 or SEQ ID NO: 185 and SEQ ID NO: 186.

In one embodiment of the third or fourth aspect, said oligonucleotide substrate and said first and second oligonucleotide partzymes are defined by a combination of sequences as set forth in Table 6, 8, 10, 13, 16, 20, 22 and/or 24.

In one embodiment of the fourth aspect, the catalytic nucleic acid enzyme is a DNAzyme, and the DNAzyme and oligonucleotide substrate are defined by a combination of sequences as set forth in Table 15.

In one embodiment of the fourth aspect, the target is a nucleic acid that hybridizes to one or more sensor arms of said MNAzyme by base pair complementarity.

In one embodiment of the fourth aspect, the polynucleotide substrate hybridises with said catalytic nucleic acid enzyme at a temperature of between 50°C and 90°C, between 50°C and 65°C, between 50°C and 60°C, between 52°C and 58°C, between 66°C and 76°C, between 68°C and 76°C, between 64°C and 70°C, between 70°C and 76°C, between 70°C and 75°C, between 72°C and 76°C, 52°C, 58°C, 64°C, 66°C, 68°C, 70°C, 72°C, or 76°C.

In a fifth aspect, the present ~~invention~~-disclosure provides a kit comprising the isolated polynucleotide substrate of the first or second aspect.

In one embodiment of the fifth aspect, the kit further comprises a catalytic nucleic acid enzyme capable of catalytically modifying said polynucleotide substrate.

In one embodiment of the fifth aspect, the catalytic nucleic acid enzyme is a multi-component nucleic acid enzyme (MNAzyme).

In a sixth aspect, the present ~~invention~~-disclosure provides a kit comprising the isolated polynucleotide substrate of the first or second aspect and a plurality of oligonucleotide partzymes designed to assemble a multi-component nucleic acid enzyme (MNAzyme)

capable of detecting at least one target, wherein said MNAzyme is capable of catalytically modifying the polynucleotide substrate.

In one embodiment of sixth aspect, said oligonucleotide substrate and said plurality of oligonucleotide partzymes are defined by a combination of sequences as set forth in Table 6, 8, 10, 13, 16, 20, 22 and/or 24.

In a seventh aspect, the present ~~invention~~ disclosure provides an assembly comprising a solid support bound to a polynucleotide substrate of the first or second aspect.

In one embodiment of the first or third to seventh aspects, any one or more of N_1 - N_{15} are deoxyribonucleotides.

In one embodiment of the first or third to seventh aspects, any one or more of N_1 - N_{15} are ribonucleotides.

In one embodiment of the first or third to seventh aspects, all of N_1 - N_{15} are deoxyribonucleotides.

In one embodiment of the first or third to seventh aspects, all of N_1 - N_{15} are ribonucleotides.

In one embodiment of the first or third to seventh aspects, N_1 - N_{15} comprises a mixture of deoxyribonucleotides and ribonucleotides.

In one embodiment of the seventh aspect, the assembly comprises a plurality of different solid supports bound to a plurality of different polynucleotide substrates.

In one embodiment of the first, second, fourth, or fifth aspect, the catalytic nucleic acid enzyme is a DNAzyme.

In one embodiment of the first, second, fourth, or fifth aspect, the catalytic nucleic acid enzyme is a ribozyme.

In one embodiment of the first, second, fourth, or fifth aspect, the catalytic nucleic acid enzyme is a multi-component nucleic acid enzyme (MNAzyme).

In one embodiment of the above aspects, the catalytic nucleic acid enzyme is capable of modifying the polynucleotide substrate by cleavage.

In one embodiment of the first, second, or fifth aspect, the catalytic nucleic acid enzyme is an MNAzyme comprising first and second oligonucleotide partzymes, and said oligonucleotide substrate and said first and second oligonucleotide partzymes are defined by a combination of sequences as set forth in Table 6, 8, 10, 13, 16, 20, 22 and/or 24.

In one embodiment of the first, second, or fifth aspect, the catalytic nucleic acid enzyme is a DNAzyme, and the DNAzyme and oligonucleotide substrate are defined by a combination of sequences as set forth in Table 15.