

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 >>> 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

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 disclosure provides a series of principles which has facilitated the development of new efficiently cleaved substrates. The present 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 disclosure provides an isolated polynucleotide substrate for a catalytic nucleic acid enzyme, said polynucleotide substrate comprising a sequence N₁-N₂-N₃-N₄-N₅-N₆-N₇-N₈-rR-rY-N₉-N₁₀-N₁₁-N₁₂-N₁₃-N₁₄-N₁₅ wherein:

rR is a purine ribonucleotide;

rY is a pyrimidine ribonucleotide;
 each of N₁-N₁₅ are nucleotides;
 six or more of N₅-N₁₃ are cytosine nucleotides; and
 less than three of N₉-N₁₅ are guanine nucleotides.

In a first aspect of the present disclosure, there is provided an isolated polynucleotide substrate for a catalytic nucleic acid enzyme, said polynucleotide substrate comprising a sequence N₁-N₂-N₃-N₄-N₅-N₆-N₇-N₈-rR-rY-N₉-N₁₀-N₁₁-N₁₂-N₁₃-N₁₄-N₁₅, wherein:

rR is a purine ribonucleotide;
rY is a pyrimidine ribonucleotide;
each of N₁-N₁₅ are nucleotides;
six or more of N₅-N₁₃ are cytosine nucleotides;
N₉ is a cytosine nucleotide; and
less than three of N₉-N₁₅ are guanine nucleotides;

and wherein said polynucleotide substrate:

comprises at least one DNA nucleotide; and
does not consist of a sequence defined in SEQ ID NO: 75 or SEQ ID NO: 86.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 25-27, 29-30, 33, 72-90, or 172-175.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, seven or more, or eight or more of N₅-N₁₃ are cytosine nucleotides.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, seven or more of N₅-N₁₃ are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 29, 73, 76-80, 82-83, 85-90, or 172-175.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, eight of N₅-N₁₃ are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 76, 77, 80, 83 or 87.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, seven or more, or eight or more of N₄-N₁₃ are cytosine nucleotides.

In one embodiment of the isolated polynucleotide substrates disclosed herein~~first aspect~~, seven or more of N₄-N₁₃ are cytosine nucleotides and the polynucleotide substrate comprises of a sequence defined by any one of SEQ ID NOs: 27, 29, 73, 76-83, 85-90, or 172-175.

In one embodiment of the isolated polynucleotide substrates disclosed herein~~first aspect~~, eight of N₄-N₁₃ are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 76, 77, 79-80, 82-83, 87, 88 or 90.

In one embodiment of the isolated polynucleotide substrates disclosed herein~~first aspect~~, six or more, seven or more, or eight or more of N₄-N₁₂ are cytosine nucleotides.

In one embodiment of the isolated polynucleotide substrates disclosed herein, seven or more of N₄-N₁₂ are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 27, 29, 73, 76, 77, 79-83, 85-88, 90 or 172-175.

In one embodiment of the isolated polynucleotide substrates disclosed herein~~first aspect~~, eight of N₄-N₁₂ are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 76, 77, 80, 83, 87, or 88.

In one embodiment of the isolated polynucleotide substrates disclosed herein~~first aspect~~, six or more, seven or more, or eight or more of N₅-N₁₂ are cytosine nucleotides.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, seven or more of N₅-N₁₂ are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 29, 73, 76, 77, 80, 83, 85-88 or 172-175.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, eight of N₅-N₁₂ are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 76, 77, 80, 83, or 87.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, any one or more of N₁, N₂, N₈ and/or N₉ is a cytosine nucleotide.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, N₈ and N₉ are cytosine nucleotides, and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 25-26, 29-30, 72-90, or 172-175.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, two, one or none of N₉-N₁₅ are guanine nucleotides.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, one or none of N₉-N₁₅ are guanine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 25-27, 29-30, 33, 72-80, 82-90, or 172-175.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, none of N₉-N₁₅ are guanine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 25, 26, 30, 33, 72, 75, 77-80, 84-85, or 89.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, more than ten, more than eleven, more than twelve, or more than thirteen of N₁-N₁₅ are pyrimidine nucleotides.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, eleven, twelve, or more than twelve of N₁-N₁₅ are pyrimidine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one SEQ ID NOs: 25-27, 29, 33, 73-90 or 173-175.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, thirteen or fourteen of N₁-N₁₅ are pyrimidine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one SEQ ID NOs: 75, 77-80, 82-85 or 88-89.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, more than eight, more than nine, more than ten, or eleven of N₁-N₁₄ are cytosine nucleotides.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, ten or eleven of N₁-N₁₄ are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one SEQ ID NOs: 33, 76-80, 82-83, 85, 87, 88, or 89.

In one embodiment of the isolated polynucleotide substrates disclosed herein^{first aspect}, eleven of N₁-N₁₄ are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one SEQ ID NOs: 77-79.

In one embodiment of the isolated polynucleotide substrates disclosed herein~~first aspect~~, the polynucleotide substrate further comprises a detectable label for detecting the polynucleotide substrate.

In one embodiment of the isolated polynucleotide substrates disclosed herein~~first aspect~~, the polynucleotide substrate further comprising a detectable portion and a quencher portion, wherein a detectable effect provided by the detectable portion is increased or decreased upon modification of the polynucleotide substrate by said catalytic nucleic acid enzyme.

In one embodiment of the isolated polynucleotide substrates disclosed herein~~first aspect~~, the purine ribonucleotide comprises guanine.

In one embodiment of the isolated polynucleotide substrates disclosed herein~~first aspect~~, the pyrimidine ribonucleotide comprises uracil.

In one embodiment of the isolated polynucleotide substrates disclosed herein~~first aspect~~, a portion of the polynucleotide substrate that binds to said catalytic nucleic acid enzyme has a melting temperature (T_m) 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 isolated polynucleotide substrates disclosed herein~~first aspect~~, the catalytic nucleic acid enzyme is:

(i) a multi-component nucleic acid enzyme (MNAzyme) and said portion binds to at least one substrate arm of said MNAzyme; or

(ii) a DNAzyme.

In one embodiment of the isolated polynucleotide substrates disclosed herein~~first aspect~~, the polynucleotide substrate is capable of catalytic modification by an MNAzyme.

In one embodiment of the isolated polynucleotide substrates disclosed herein, the polynucleotide substrate is capable of catalytic modification by a DNAzyme.

In one embodiment of the isolated polynucleotide substrates disclosed herein~~first aspect~~, the polynucleotide substrate comprises a detectable label for detection by 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 isolated polynucleotide substrates disclosed herein~~first aspect~~, the polynucleotide substrate comprises a detectable label for detection by fluorescence spectroscopy.

In one embodiment of the isolated polynucleotide substrates disclosed herein~~first aspect~~, the polynucleotide substrate comprises a detectable label for detection by Fluorescence Resonance Energy Transfer (FRET) spectroscopy.

~~In a second aspect,~~ The present disclosure also 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 second aspect,~~ the present 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 second aspect,~~ the target is an MNzyme assembly facilitator.

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

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

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

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

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

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

In a ~~fourth-third~~ aspect, the present 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-third~~ aspect, the catalytic nucleic acid enzyme is a multi-component nucleic acid enzyme (MNzyme),

said MNAzyme comprising at least two or more oligonucleotide partzymes wherein at least a first oligonucleotide partzyme and a second oligonucleotide partzyme self-assemble in the presence of an MNAzyme assembly facilitator to form a catalytically active multi-component nucleic acid enzyme (MNAzyme), wherein each of said at least first and said second oligonucleotide partzymes comprise a substrate arm portion, a catalytic core portion, and a sensor arm portion;

wherein upon self-assembly, the sensor arm portion of said first and second oligonucleotide partzymes act as sensor arms of the MNAzyme, the substrate arm portion of the first and second oligonucleotide partzymes act as substrate arms of the MNAzyme, and the catalytic core portion of the first and second oligonucleotide partzymes act as a catalytic core of the MNAzyme;

and wherein the sensor arms of the MNAzyme interact with said MNAzyme assembly facilitator so as to maintain the first and second oligonucleotide partzymes in proximity for association of their respective catalytic core portions to form the catalytic core of the MNAzyme, said catalytic core capable of modifying said polynucleotide substrate, and wherein said substrate arms of said MNAzyme engage said polynucleotide substrate so that said catalytic core of said MNAzyme can modify said polynucleotide substrate.

In one embodiment of the ~~third-second~~ or ~~fourth-third~~ aspect, the catalytic core portion of each said oligonucleotide partzyme comprises DNA or an analogue thereof.

In one embodiment of the ~~fourth-third~~ aspect, the assembly facilitator is a target to be identified, detected or quantitated.

In one embodiment of the ~~third-second~~ or ~~fourth-third~~ aspect, the first and second oligonucleotide partzymes comprise respective sequences defined by:

SEQ ID NO: 9 and SEQ ID NO: 10; SEQ ID NO: 11 and SEQ ID NO: 12; SEQ ID NO: 13 and SEQ ID NO: 14; SEQ ID NO: 16 and SEQ ID NO: 14; SEQ ID NO: 17 and SEQ ID NO: 18; SEQ ID NO: 40 and SEQ ID NO: 41; SEQ ID NO: 42 and SEQ ID NO: 43; SEQ ID NO: 44 and SEQ ID NO: 45; SEQ ID NO: 46 and SEQ ID NO: 45; SEQ ID NO: 47 and SEQ ID NO: 63; SEQ ID NO: 48 and SEQ ID NO: 49; SEQ ID NO: 50 and SEQ ID NO: 51; SEQ ID NO: 52 and SEQ ID NO: 51; SEQ ID NO: 38 and SEQ ID NO: 55; SEQ ID NO: 56 and SEQ ID NO: 57; SEQ ID NO: 58 and SEQ ID NO: 59; SEQ ID NO: 60 and SEQ ID NO: 61; SEQ ID NO: 62 and SEQ ID NO: 63; SEQ ID NO: 64 and SEQ ID NO: 65; SEQ ID NO: 66 and SEQ ID NO: 67; SEQ ID NO: 62 and SEQ ID NO: 68; SEQ ID NO: 69 and SEQ ID NO: 70; SEQ ID NO: 46 and SEQ ID NO: 55; SEQ ID NO: 46 and SEQ ID NO: 59; SEQ ID NO: 38 and SEQ ID NO: 45; SEQ ID NO: 58 and

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~~-second or ~~fourth~~-third 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~~-third 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~~-third 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~~-third 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~~-fourth aspect, the present disclosure provides a kit comprising the isolated polynucleotide substrate of the first ~~or second~~ aspect.

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

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

In a ~~sixth~~-fifth aspect, the present 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-fifth~~ 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-sixth~~ aspect, the present 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-sixth~~ aspects, any one or more of N₁-N₁₅ are deoxyribonucleotides.

In one embodiment of the first ~~or third~~ to ~~seventh-sixth~~ aspects, any one or more of N₁-N₁₅ are ribonucleotides.

In one embodiment of the first ~~or third~~ to ~~seventh-sixth~~ aspects, all of N₁-N₁₅ are deoxyribonucleotides.

In one embodiment of the first ~~or third~~ to ~~seventh-sixth~~ aspects, all of N₁-N₁₅ are ribonucleotides.

In one embodiment of the first ~~or third~~ to ~~seventh-sixth~~ aspects, N₁-N₁₅ comprises a mixture of deoxyribonucleotides and ribonucleotides.

In one embodiment of the ~~seventh-sixth~~ 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~~third, or ~~fifth-fourth~~ aspect, the catalytic nucleic acid enzyme is a DNAzyme.

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

In one embodiment of the first, ~~second, fourth~~third, or ~~fifth-fourth~~ 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-fourth~~ 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-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 ~~first, second, or fifth~~ fourth aspect, the polynucleotide substrate is capable of hybridising to said catalytic nucleic acid enzyme by complementary base pairing.

In one embodiment of the ~~fourth~~ third aspect, the polynucleotide substrate hybridises to said catalytic nucleic acid enzyme by complementary base pairing.

In one embodiment of the ~~third, second and fourth~~ third aspect, the polynucleotide substrate hybridises to said MNAzyme by complementary base pairing.

In one embodiment of the ~~first, second, fifth~~ fourth, and ~~sixth~~ fifth aspect, the polynucleotide substrate the polynucleotide substrate is capable of hybridising to said MNAzyme by complementary base pairing.

In one embodiment of the above aspects, a portion of the isolated polynucleotide substrate binds to at least one substrate arm of said MNAzyme.

In one embodiment of the above aspects, the polynucleotide substrate is a universal substrate capable of being bound and catalytically modified by more than one different type of catalytic nucleic acid enzyme.

In one embodiment of the above aspects, the polynucleotide substrate is a universal substrate capable of being bound and catalytically modified by more than one different type of multi-component nucleic acid enzyme (MNAzyme).

In one embodiment of the above aspects, at least one of said oligonucleotide partzymes, assembly facilitator or substrate comprises DNA or an analogue thereof.

In one embodiment of the ~~third, second and sixth~~ fifth aspect, the modifying is cleavage of the polynucleotide substrate by the MNAzyme.

BRIEF DESCRIPTION OF THE FIGURES

Preferred embodiments of the present invention will now be described, by way of example only, with reference to the accompanying figures wherein:

Figure 1 is a diagram depicting an exemplary design of a Multi-component nucleic acid (MNAzyme). By way of exemplary disclosure, a MNAzyme is comprised of two oligonucleotide components (partzyme A and partzyme B), which self assemble in the presence of an assembly facilitator. When the two partzymes assemble in the presence of the assembly facilitator, a catalytically active MNAzyme forms which is capable of modifying (e.g. cleaving or ligating) a substrate. The two component partzymes have (i) sensor arms, which bind to the assembly facilitator, (ii) substrate arms, which bind the substrate, and (iii) partial catalytic core sequences. The presence of an assembly facilitator molecule (e.g. a target nucleic acid sequence) provides the “input” signal which

CLAIMS:

1. 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;

rY is a pyrimidine ribonucleotide;

each of N_1-N_{15} are nucleotides;

six or more of N_5-N_{13} are cytosine nucleotides; and

N_9 is a cytosine nucleotide; and

less than three of N_9-N_{15} are guanine nucleotides;

and wherein said polynucleotide substrate:

comprises at least one DNA nucleotide; and

does not consist of a sequence defined in SEQ ID NO: 75 or SEQ ID NO: 86.

2. The isolated polynucleotide substrate according to claim 1, wherein the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 25-27, 29-30, 33, 72-74, 76-85, 87-90, or 172-175.

3. The isolated polynucleotide substrate according to claim 1 or claim 2, wherein seven or more, or eight or more of N_5-N_{13} are cytosine nucleotides.

4. The isolated polynucleotide substrate according to claim 3, wherein seven or more of N_5-N_{13} are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 29, 73, 76-80, 82-83, 85, 87-90, or 172-175.

5. The isolated polynucleotide substrate according to claim 3, wherein eight of N_5-N_{13} are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 76, 77, 80, 83 or 87.

6. The isolated polynucleotide substrate according to claim 1 or claim 2, wherein seven or more, or eight or more of N_4-N_{13} are cytosine nucleotides.

7. The isolated polynucleotide substrate according to claim 6, wherein seven or more of N_4-N_{13} are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 27, 29, 73, 76-83, 85, 87-90, or 172-175.

8. The isolated polynucleotide substrate according to claim 6, wherein eight of N_4 - N_{13} are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 76, 77, 79-80, 82-83, 87, 88 or 90.

9. The isolated polynucleotide substrate according to claim 1 or claim 2, wherein six or more, seven or more, or eight or more of N_4 - N_{12} are cytosine nucleotides.

10. The isolated polynucleotide substrate according to claim 9, wherein seven or more of N_4 - N_{12} are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 27, 29, 73, 76, 77, 79-83, 85, 87, -88, 90 or 172-175.

11. The isolated polynucleotide substrate according to claim 9, wherein eight of N_4 - N_{12} are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 76, 77, 80, 83, 87, or 88.

12. The isolated polynucleotide substrate according to claim 1 or claim 2, wherein six or more, seven or more, or eight or more of N_5 - N_{12} are cytosine nucleotides.

13. The isolated polynucleotide substrate according to claim 12, wherein seven or more of N_5 - N_{12} are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 29, 73, 76, 77, 80, 83, 85, 87, -88 or 172-175.

14. The isolated polynucleotide substrate according to claim 12, wherein eight of N_5 - N_{12} are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 76, 77, 80, 83, or 87.

15. The isolated polynucleotide substrate according to any one of claims 1 to 14, wherein any one or more of N_1 , N_2 , and/or N_8 ~~and/or~~ N_9 is a cytosine nucleotide.

16. The isolated polynucleotide substrate according to claim 15, wherein N_8 and N_9 are cytosine nucleotides, and the polynucleotide substrate comprises or consists of a sequence defined by any one of SEQ ID NOs: 25-26, 29-30, 72-74, 76-85, 87-90, or 172-175.

17. The isolated polynucleotide substrate according to any one of claims 1 to 16, wherein two, one or none of N₉-N₁₅ are guanine nucleotides.

18. The isolated polynucleotide substrate according to claim 17, wherein one or
5 none of N₉-N₁₅ are guanine nucleotides and the polynucleotide substrate comprises or
consists of a sequence defined by any one of SEQ ID NOs: 25-27, 29-30, 33, 72-74, 76-
80, 82-85, 87-90, or 172-175.

19. The isolated polynucleotide substrate according to claim 17, wherein none of
10 N₉-N₁₅ are guanine nucleotides and the polynucleotide substrate comprises or consists of
a sequence defined by any one of SEQ ID NOs: 25, 26, 30, 72, ~~75~~, 77-80, 84-85, or 89.

20. The isolated polynucleotide substrate according to any one of claims 1 to 19,
wherein more than ten, more than eleven, more than twelve, or more than thirteen of N₁-
15 N₁₅ are pyrimidine nucleotides.

21. The isolated polynucleotide substrate according to claim 20, wherein eleven,
twelve, or more than twelve of N₁-N₁₅ are pyrimidine nucleotides and the polynucleotide
substrate comprises or consists of a sequence defined by any one SEQ ID NOs: 25-27, 29,
20 33, 73, 74, 76-85, 87-90 or 173-175.

22. The isolated polynucleotide substrate according to claim 20 or claim 21
wherein thirteen or fourteen of N₁-N₁₅ are pyrimidine nucleotides and the polynucleotide
substrate comprises or consists of a sequence defined by any one SEQ ID NOs: ~~75~~, 77-80,
25 82-85 or 88-89.

23. The isolated polynucleotide substrate according to any one of claims 1 to 22,
wherein more than eight, more than nine, more than ten, or eleven of N₁-N₁₄ are cytosine
nucleotides.

24. The isolated polynucleotide substrate according to claim 23, wherein ten or
eleven of N₁-N₁₄ are cytosine nucleotides and the polynucleotide substrate comprises or
consists of a sequence defined by any one SEQ ID NOs: 33, 76-80, 82-83, 85, 87, 88, or
89.

25. The isolated polynucleotide substrate according to claim 23 or claim 24 wherein eleven of N₁-N₁₄ are cytosine nucleotides and the polynucleotide substrate comprises or consists of a sequence defined by any one SEQ ID NOs: 77-79.

26. The isolated polynucleotide substrate of any one of claims 1 to 25, further comprising a detectable label for detecting the polynucleotide substrate.

27. The isolated polynucleotide substrate of any one of claims 1 to 25, further comprising a detectable portion and a quencher portion, wherein a detectable effect provided by the detectable portion is increased or decreased upon modification of the polynucleotide substrate by said catalytic nucleic acid enzyme.

28. The isolated polynucleotide substrate of any one of claims 1 to 27, wherein the purine ribonucleotide comprises guanine.

29. The isolated polynucleotide substrate of any one of claims 1 to 28, wherein the purine ribonucleotide comprises guanine and the pyrimidine ribonucleotide comprises uracil.

30. The isolated polynucleotide substrate of any one of claims 1 to 29, wherein a portion of the substrate that binds to said catalytic nucleic acid enzyme has a melting temperature (T_m) 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.

31. The isolated polynucleotide substrate of any one of claims 1 to 30, wherein said catalytic nucleic acid enzyme is:

- (a) a multi-component nucleic acid enzyme (MNAzyme) and said portion binds to at least one substrate arm of said MNAzyme; or
- (b) a DNAzyme.

32. 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