

NUCLEIC ACID ENZYME SUBSTRATES

INCORPORATION BY CROSS-REFERENCE

This application claims priority from Australian Provisional Patent Application No. 2011903686 filed on 9 September 2011, the entire content of which is incorporated herein by cross-reference.

TECHNICAL FIELD

The invention relates generally to the field of nucleic acid enzymes. More specifically, the invention relates to substrates for nucleic acid enzymes and methods utilising the substrates.

BACKGROUND OF THE INVENTION

A wide variety of nucleic acid molecules with enzymatic or catalytic activity have been discovered in the last 20 years. RNA enzymes ("ribozymes") occur in nature but can be engineered to specifically recognize and modify a target RNA substrate. *In vitro* evolution techniques have facilitated the discovery and development of many more catalytic nucleic acids, including deoxyribonucleic acids often referred to as "deoxyribozymes", "DNA enzymes" or "DNAzymes". *In vitro* evolved DNAzymes and/or ribozymes have been discovered which have the capacity to catalyse a broad range of reactions including cleavage of nucleic acids, ligation of nucleic acids, porphyrin metallation, and formation of carbon-carbon bonds, ester bonds or amide bonds.

In particular, DNAzymes and ribozymes have been characterized which specifically cleave distinct nucleic acid sequences after hybridizing via Watson Crick base pairing. DNAzymes are capable of cleaving either RNA or DNA molecules. Ribozymes are also able to cleave both RNA and DNA target sequences. The "10-23" and "8-17" DNAzymes are capable of cleaving nucleic acid substrates at specific RNA phosphodiester bonds to create reaction products which have 2', 3'-cyclic phosphate and 5'-hydroxyl groups. Examples of deoxyribozymes (DNAzymes), which can ligate 2', 3'-cyclic phosphate and 5'-hydroxyl products include the "7Z81" and "7Z48" ligases.

More recently, Multi-component Nucleic Acid enzymes (MNAzymes) have been described which have the capacity to self-assemble from two or more oligonucleotide components (also referred to herein as "partzymes") in the presence of a MNAzyme assembly facilitator (e.g. a target molecule to be detected).

The versatile nature of catalytic nucleic acids has facilitated their use in many different applications. A key element to the successful use of catalytic nucleic acids is their capacity to modify an appropriate substrate. In general, the substrate is substantially complementary to the hybridizing arms of the catalytic nucleic acid and contains a specific sequence or sequence motif at the site of catalytic action. The nature of the interaction between a given catalytic nucleic acid and its substrate is determinative of how efficiently the enzyme engages and/or catalytically modifies its substrate, and is thus a fundamental consideration in designing any system that utilises catalytic nucleic acids.

Catalytic nucleic acids have *in vitro* diagnostic applications in the detection of nucleic acids, proteins and small molecules. These applications often involve amplification of either the target or the signal to generate sufficient signal for robust detection of the analyte of interest.

Methods that employ catalytic nucleic acids require substrates that are modified with a sufficient rate of catalytic activity to allow effective discrimination over background noise. Different methods may require the use of different reaction temperatures and so there is a necessity for substrates that are efficiently modified (e.g. cleaved) at the required temperatures. Methods such as those utilizing MNazymes and DNazymes permit multiplexed analysis of many targets simultaneously in a single reaction, but the ability to multiplex and distinguish between the multiple targets is dependent on the existence of a suitable range of substrates, usually at least one per target. The number of substrates known in the art that are modified (e.g. cleaved) with high efficiency is currently insufficient for mass multiplexing.

The DNzyme and MNzyme substrates previously known in the art were derived by screening multiple possible substrates to empirically determine those that were cleaved most efficiently. Often this screening was performed using large numbers of DNzymes targeted to cleave theoretically possible cleavage sites within full length mRNA. This screening was usually performed under physiological conditions (temperature and ionic strength, composition and pH of buffers). This bias towards finding efficiently cleaved sequences of mRNA at physiological conditions exists because such studies were focused on therapeutic uses of DNzymes as inhibitors of RNA expression *in vivo*. Such studies provide a range of laborious protocols for empirical measurement of a large number of putative substrates to find the few that are cleaved efficiently (see for example Cairns *et al.*, 1999 Nat Biotech 17:480-486). These studies resulted in a limited set of design guidelines for the selection of efficiently cleaved substrates, and in many cases the guidelines focused on the design of the DNzyme rather than the substrate as the

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: $GU \geq AU > GC \ggg AC$.

5 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*.

10 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
15 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
20 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

25 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 provides a series of principles which has facilitated the development of new efficiently cleaved substrates. The present invention thus provides catalytic nucleic acid enzyme substrates with
30 properties that enhance catalytic nucleic acid function thereby addressing a need existing in the art.

In a first aspect, the present invention 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:

35 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.

5 In one embodiment of the 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 first aspect, seven or more, or eight or more of N₅-N₁₃ are cytosine nucleotides.

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

In one embodiment of the first aspect, seven or more, or eight or more of N₄-N₁₃ are cytosine nucleotides.

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

In one embodiment of the 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 first aspect, six or more, seven or more, or eight or more
25 of N₄-N₁₂ are cytosine nucleotides.

In one embodiment of the first aspect, 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 first aspect, eight of N₄-N₁₂ are cytosine nucleotides and
30 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 first aspect, six or more, seven or more, or eight or more
of N₅-N₁₂ are cytosine nucleotides.

In one embodiment of the 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.

5 In one embodiment of the 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 first aspect, any one or more of N₁, N₂, N₈ and/or N₉ is a cytosine nucleotide.

10 In one embodiment of the 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 first aspect, two, one or none of N₉-N₁₅ are guanine nucleotides.

15 In one embodiment of the 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 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.

20 In one embodiment of the 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 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.

25 In one embodiment of the 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 first aspect, more than eight, more than nine, more than ten, or eleven of N₁-N₁₄ are cytosine nucleotides.

30 In one embodiment of the 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 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.

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In one embodiment of the first aspect, the polynucleotide substrate further comprises a detectable label for detecting the polynucleotide substrate.

In one embodiment of the first aspect, the polynucleotide substrate further comprising a detectable portion and a quencher portion, wherein a detectable effect
5 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 first aspect, the purine ribonucleotide comprises guanine.

In one embodiment of the first aspect, the pyrimidine ribonucleotide comprises uracil.

10 In one embodiment of the 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,
15 68°C, 70°C, 72°C, or 76°C.

In one embodiment of the 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.

20 In one embodiment of the first aspect, the polynucleotide substrate is capable of catalytic modification by an MNAzyme.

In one embodiment of the first aspect, the polynucleotide substrate is capable of catalytic modification by a DNAzyme.

In one embodiment of the first aspect, the polynucleotide substrate comprises a
25 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.

30 In one embodiment of the first aspect, the polynucleotide substrate comprises a detectable label for detection by fluorescence spectroscopy.

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

In a second aspect, the present invention 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 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),
5 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,
10 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

15 (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.

20 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
25 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.

30 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 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
35 multi-component nucleic acid enzyme (MNzyme),

said MNzyme 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 MNzyme assembly facilitator to form a catalytically active multi-component nucleic acid enzyme (MNzyme), wherein each of said at least 5 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 MNzyme, the substrate arm portion of the first and second oligonucleotide partzymes act as substrate arms of the MNzyme, 10 and the catalytic core portion of the first and second oligonucleotide partzymes act as a catalytic core of the MNzyme;

and wherein the sensor arms of the MNzyme interact with said MNzyme 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 15 core of the MNzyme, said catalytic core capable of modifying said polynucleotide substrate, and wherein said substrate arms of said MNzyme engage said polynucleotide substrate so that said catalytic core of said MNzyme can modify said polynucleotide substrate.

In one embodiment of the third or fourth aspect, the catalytic core portion of each 20 said oligonucleotide partzyme comprises DNA or an analogue thereof.

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

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

25 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 30 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 35 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
5 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
10 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.

15 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.

20 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,
25 72°C, or 76°C.

In a fifth aspect, the present invention 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.

30 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 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
5 Table 6, 8, 10, 13, 16, 20, 22 and/or 24.

In a seventh aspect, the present invention 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.

10 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
15 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.

20 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
25 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
30 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.

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

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

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

In one embodiment of the first, second, fifth, and sixth aspect, the polynucleotide substrate the polynucleotide substrate is capable of hybridising to said MNzyme 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 MNzyme.

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 (MNzyme).

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 and sixth aspect, the modifying is cleavage of the polynucleotide substrate by the MNzyme.

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 (MNzyme). By way of exemplary disclosure, a MNzyme 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 MNzyme 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

directs the assembly of partzyme components in a highly specific fashion which is amenable to modulation. In some embodiments, the assembly facilitator may be, for example, a target nucleic acid sequence present in a test sample. In other embodiments, the assembly facilitator may be, for example a synthetic oligonucleotide included in the milieu to direct the self-assembly of the partzyme components in the presence of a detectable entity or event. Modification of the substrate by the assembled MNzyme can provide a “detectable effect” which may be detected and/or quantified. For example, when the substrate is dual labelled with a fluorophore (F) and a quencher (Q), cleavage of this “reporter substrate” by an active MNzyme separates the fluorophore and the quencher resulting in a concomitant increase in fluorescence.

Figure 2 provides a flow chart showing exemplary applications of methods for target detection using MNzymes. MNzymes can be used for (1) direct detection; (2) detecting amplicons generated, for example, by PCR, SDA, LAMP, RCA, TMA, 3SR or NASBA either during, or following, target amplification; and (3) initiating a signal amplification cascade.

Figure 3 provides a depiction of exemplary MNzymes and a method for target detection using MNzymes that cleave substrates tethered to a support. In this embodiment, the MNzyme forms only in the presence of an assembly facilitator (target). When the MNzyme cleaves the tethered substrate between a fluorophore and quencher, a signal is generated. As shown here, upon cleavage between fluorophore F and quencher Q, there is a resultant increase in fluorescence. In general, the method may be designed such that either fluorophore F or quencher Q may stay attached to the support once cleavage occurs. Panel (i): The support shown has only one substrate type (Substrate 1) tethered to it. Panel (ii): There may be multiple substrate types tethered in different positions. Each substrate can be cleaved only by a MNzyme formed in the presence of a specific MNzyme assembly facilitator molecule – here, Targets 1 and 2 facilitate the self-assembly of MNzymes 1 and 2 respectively. Thus, in this example MNzyme 1 only self-assembles in the presence of Target 1 and only cleaves Substrate 1. Similarly, MNzyme 2 only self-assembles in the presence of Target 2 and only cleaves Substrate 2. The signal can be localised by positioning of the substrate on the surface, thus allowing specific detection of different assembly facilitators. The exemplary assay in Panel (ii) requires two distinct substrate sequences.

Figure 4 shows an exemplary assay using catalytically modified substrate products as MNzyme assembly facilitator components. In this strategy an initiating MNzyme (Mt) is formed in the presence of a target (T). The initiating MNzyme (Mt) cleaves a

first substrate (S1) to create a first assembly facilitator component (S1f), which directs formation of a first cascading MNzyme (cascading MNzyme Mc1). In this example the first cascading MNzyme (Mc1) comprises two partzymes and three assembly facilitator components designated F1, F2 and S1f. Mc1 can cleave an additional substrate (S2) thus liberating an additional assembly facilitator component (S2f), which directs formation of a second cascading MNzyme (cascading MNzyme Mc2). In this example the second cascading MNzyme (Mc2) comprises two partzymes and three assembly facilitator components designated F3, F4 and S2f. Mc2 can then cleave more of the first substrate (S1) thus creating more of the first assembly facilitator component (S1f). This leads to the formation of further first cascading MNzyme (Mc1) thereby forming an amplification cascade. This exemplary assay requires two distinct substrate sequences.

Figure 5 provides a graph illustrating Ct values generated by MNzyme qPCR using a range of different universal substrates for detection of the human TFRC gene. The identity of the universal substrates used in reactions are indicated on the x-axis (where "2" refers to Sub2, "3" refers to Sub3 etc.). The same primer set and genomic DNA were used for all reactions, and all partzymes had the same target-sensor regions and catalytic domains. The only differences between reactions were the substrate-sensor arms of the partzymes and the fluorescently labelled universal substrates. Thus the difference in Ct values between reactions is correlated with efficiency of cleavage of the universal substrates. Lower Ct values indicate a faster number of cycles to achieve a threshold level of fluorescence, and therefore indicate more efficiently cleaved universal substrates.

Figure 6 provides graphs illustrating the signal to noise ratios resulting from MNzyme mediated cleavage of a range of universal substrates in an isothermal format. Target was a synthetic oligonucleotide. The signal to noise ratio was calculated from normalized fluorescence data collected during cleavage reactions at a range of reaction temperatures. The identity of the universal substrates used in reactions are indicated on the x-axis (where "2" refers to Sub2, "3" refers to Sub3 etc.). In **Figure 6 (i)** the different columns of data refer to results for different reaction temperatures (as indicated in the legend 52°C, 54°C, 56°C and 58°C). **Figure 6 (i)** illustrates the signal to noise ratio on the y-axis. **Figure 6 (ii)** illustrates the standard deviation of the average of the signal to noise ratio from all four temperatures tested and the different series of substrates are indicated by different shading of columns.

Figure 7 provides graphs illustrating linear amplification plots generated by MNzyme qPCR using a range of different universal substrates for detection of a range

of human genes. Each combination of substrates and targets was run at an annealing temperature of a) 52°C or b) 58°C. The universal substrates tested with each gene are indicated by symbols located at the top left of each plot and were (i) Sub3 and Sub61 with CYP2C9, (ii) Sub6, Sub72, Sub74 and Sub79, with TP53, (iii) Sub60, Sub61 and Sub79 with B2M, (iv) Sub49 and Sub75 with HMBS, (v) Sub2, Sub72 and Sub80 with TFRC and (iv) Sub55, Sub80 and Sub88 with RPL13a. The same primer set and genomic DNA were used for all reactions with each different gene, and all partzymes had the same catalytic domains and target-sensor regions matched to the particular gene. The only differences between reactions were the substrate-sensor arms of the partzymes and the fluorescently labelled universal substrates. Thus the difference in the shape of the amplification plot and the Ct values between reactions for the same gene is correlated with efficiency of cleavage of the universal substrates. The steeper the curves and the earlier Ct values indicate a faster number of cycles to achieve a threshold fluorescence, and therefore indicate more efficiently cleaved universal substrates.

Figure 8 provides graphs illustrating the signal to noise ratio resulting from DNazyme mediated cleavage of a range of universal substrates in an isothermal format. The signal to noise ratio was calculated from normalized fluorescence data collected during cleavage reactions at a range of reaction temperatures. The identity of the universal substrates used in reactions is indicated on the x-axis (where "2" refers to Sub2, "3" refers to Sub3 etc.). In **Figure 8 (i)** the different columns of data refer to results for different reaction temperatures (as indicated in the legend - 50°C to 60°C) with the signal to noise ratio on the y-axis. In **Figure 8 (ii)** the columns of data refer to the standard deviation of the average of the signal to noise ratio from all six temperatures tested and the different series of substrates are indicated by different shading of columns.

Figure 9 provides graphs illustrating linear amplification plots from MNazyme qPCR performed with a range of different universal substrates (Sub44, Sub55, Sub61, Sub65, Sub72 and Sub74) to investigate non-specific cleavage activity with a subset of partzymes (designed with substrate sensor arms to be complementary to Sub44, Sub55, Sub72 and Sub74) for detection of the human RPL13a gene. Each universal substrate was tested individually with partzyme pairs designed to bind with full complementarity to the other substrates to determine if a signal could be detected. The partzymes complementary to (i) Sub72, (ii) Sub74, (iii) Sub55 and (iv) Sub44, were tested with all substrates. In Panel (a) the bottom line of the table indicates the substrate to which the partzymes in the experiment exhibit full complementarity. The other rows of the table show an alignment of the sequences of the other substrates tested in each experiment

with differences between the bottom substrate and other substrates sequences indicated by letters that are grey and underlined. In Panel (b) linear amplification plots are illustrated. The normalised fluorescence (y-axis) is plotted against the cycle number (x-axis). Individual amplification curves are labelled on the right of the plot to indicate which amplification curve relates to each substrate. The threshold fluorescence is indicated by a solid horizontal line above the x-axis. An increasing signal above the threshold fluorescence indicates cleavage of the universal substrate.

Figure 10 provides graphs illustrating the signal to noise ratio resulting from DNAzyme mediated cleavage of each universal substrate individually with DNAzymes designed to bind with full complementarity to the other substrates, in an isothermal format. The normalized signal to noise ratio was calculated from normalized fluorescence data collected at reaction temperatures of (i) 52°C or (ii) 58°C. The identity of the universal substrates used in the reactions is indicated on the x-axis. The different columns of data refer to the cleavage results for each DNAzyme as indicated in the legend, with the signal to noise ratio on the y-axis.

Figure 11 illustrates exponential amplification plots generated with amplification at either 52°C (Panel (i)) or 58°C (Panel (ii)) for two multiplex MNAzyme qPCR reactions Multiplex 1 using series 1 substrates (Sub2, Sub3, Sub4, Sub6 and Sub7) and Multiplex 2 using series 2 and 3 substrates (Sub55, Sub61, Sub74, Sub79 and Sub80). Both multiplexes measured the human genes TFRC, HPRT, TP53, RPL13a and CYP2C9 in a single reaction vessel. For each different gene measured in the two multiplex formats the same primer sets and genomic DNA were used at both temperatures and for all universal substrates, and all partzymes had the same catalytic domains and target-sensor regions matched to the particular gene. The only difference between reactions was the substrate-sensor arms of the partzymes and the fluorescently labelled universal substrates. The universal substrates tested with each gene are indicated at the top left of each plot. The amplification plots for Multiplex 1 (crosses) and Multiplex 2 (circles) were overlaid to allow for a direct comparison between the two multiplexes at two different DNA concentrations, 100 ng (cross and circle plot on the left) and 391 pg (cross and circle plot on the right). The difference in the shape of the amplification plots correlated with efficiency of cleavage of the universal substrates. The steeper the curves and the earlier Ct values indicate a faster number of cycles to achieve a threshold fluorescence, and therefore indicate more efficiently cleaved universal substrates.

DEFINITIONS

Certain terms are used herein which shall have the meanings set forth as follows.

As used herein, the singular form “a”, “an” and “the” include plural references unless the context clearly dictates otherwise. For example, the term “a polynucleotide substrate” also includes a plurality of polynucleotide substrates.

The term “comprising” means “including principally, but not necessarily solely”. Furthermore, variations of the word “comprising”, such as “comprise” and “comprises”, have correspondingly varied meanings.

Use of the term “about” herein in reference to a recited numerical value includes the recited numerical value and numerical values within plus or minus ten percent of the recited value.

Use of the term “between” herein when referring to a range of numerical values encompasses the numerical values at each endpoint of the range. For example, a polynucleotide of between 10 nucleotides and 20 nucleotides in length is inclusive of a polynucleotide of 10 nucleotides in length and a polynucleotide of 20 nucleotides in length.

The terms “polynucleotide” and “nucleic acid” are used interchangeably herein and refer to a single- or double-stranded polymer of deoxyribonucleotide and/or ribonucleotide bases, and/or analogues, derivatives, variants, fragments or combinations thereof, including but not limited to DNA, methylated DNA, alkylated DNA, RNA, methylated RNA, microRNA, siRNA, shRNA, mRNA, tRNA, snoRNA, stRNA, smRNA, pre- and pri-microRNA, other non-coding RNAs, ribosomal RNA, derivatives thereof, amplicons thereof or any combination thereof. By way of non-limiting example, the source of a nucleic acid may be selected from the group consisting of synthetic, mammalian, human, animal, plant, fungal, bacterial, viral, archael sources or any combination thereof.

The term “oligonucleotide” typically denotes a segment of DNA or a DNA-containing nucleic acid molecule, or RNA or RNA-containing molecule, or a combination thereof. An oligonucleotide may thus comprise or consist of deoxyribonucleotide and/or ribonucleotide bases, and/or analogues, derivatives, variants, fragments or combinations thereof, including but not limited to DNA, methylated DNA, alkylated DNA, RNA, methylated RNA, microRNA, siRNA, shRNA, mRNA, tRNA, snoRNA, stRNA, smRNA, pre- and pri-microRNA, other non-coding RNAs, ribosomal RNA, derivatives thereof, amplicons thereof or any combination thereof. Examples of oligonucleotides include nucleic acid targets; substrates, for example, those which can be modified by a DNAzyme

or an MNzyme with cleavage, ligase or other enzymatic activity; primers such as those used for *in vitro* target amplification by methods such as PCR; and components of MNzymes including, but not limited to partzymes, and assembly facilitators.

5 The term "pyrimidine nucleotide" encompasses any nucleotide comprising a pyrimidine base including, but not limited to, cytosine, thymine and uracil. A pyrimidine nucleotide may comprise a ribose sugar molecule (ie. a "pyrimidine ribonucleotide") or a deoxyribose sugar molecule (ie. a "pyrimidine deoxyribonucleotide").

The term "purine nucleotide" encompasses any nucleotide comprising a purine base including, but not limited to, adenine and guanine. A purine nucleotide may comprise a
10 ribose sugar molecule (ie. a "purine ribonucleotide") or a deoxyribose sugar molecule (ie. a "purine deoxyribonucleotide").

The terms "nucleic acid enzyme", "catalytic nucleic acid", "nucleic acid with catalytic activity", and "catalytic nucleic acid enzyme" are used herein interchangeably and shall mean a DNA or DNA-containing molecule or complex or an RNA or RNA-
15 containing molecule or complex, or a combination thereof being a DNA-RNA hybrid molecule or complex, which may bind at least one substrate and catalyse a modification (such as ligation or cleavage) of the at least one substrate. The nucleotide residues in the catalytic nucleic acids may include the bases A, C, G, T, and U, as well as derivatives and analogues thereof. The terms above include uni-molecular nucleic acid enzymes which
20 may comprise a single DNA or DNA-containing molecule (also known in the art as a "DNA enzyme", "deoxyribozyme" or "DNzyme") or an RNA or RNA-containing molecule (also known in the art as a "RNA enzyme" or "ribozyme") or a combination thereof, being a DNA-RNA hybrid molecule which may recognize at least one substrate and catalyse a modification (such as ligation or cleavage) of the at least one substrate. The
25 terms above include nucleic acid enzymes which comprise a DNA or DNA-containing complex or an RNA or RNA-containing complex or a combination thereof, being a DNA-RNA hybrid complex which may recognize at least one substrate and catalyse a modification (such as ligation or cleavage) of the at least one substrate. The terms "nucleic acid enzyme", "catalytic nucleic acid", "nucleic acid with catalytic activity", and
30 "catalytic nucleic acid enzyme" include within their meaning MNzymes.

The terms "MNzyme" and "multi-component nucleic acid enzyme" as used herein have the same meaning and refer to two or more oligonucleotide sequences (e.g. partzymes) which, only in the presence of an MNzyme assembly facilitator (for example, a target), form an active nucleic acid enzyme that is capable of catalytically
35 modifying a substrate. MNzymes can catalyse a range of reactions including cleavage

of a substrate, ligation of substrates and other enzymatic modifications of a substrate or substrates. An exemplary MNzyme comprising partzyme A and partzyme B which has cleavage activity is depicted in **Figure 1**. MNzymes with endonuclease or cleavage activity are also known as “MNzyme cleavers”. With reference to **Figure 1**, partzymes A and B each bind to an assembly facilitator (e.g. a target DNA or RNA sequence) through Watson-Crick base pairing. The MNzyme only forms when the sensor arms of partzymes A and B hybridize adjacent to each other on the assembly facilitator. The substrate arms of the MNzyme engage the substrate, the modification (e.g. cleavage) of which is catalyzed by the catalytic core of the MNzyme, formed by the interaction of the catalytic domains of partzymes A and B. Cleavage of a DNA/RNA chimeric reporter substrate is exemplified in the drawing. The MNzyme cleaves the substrate between a fluorophore and a quencher dye pair, thus generating signal. The terms “multi-component nucleic acid enzyme” and “MNzyme” comprise bipartite structures, composed of two molecules, or tripartite structures, composed of three nucleic acid molecules, or other multipartite structures, for example those formed by four or more nucleic acid molecules.

It will be understood that the terms “MNzyme” and “multi-component nucleic acid enzyme” as used herein encompass all known MNzymes and modified MNzymes including those disclosed in any one or more of PCT patent publication numbers WO/2007/041774, WO/2008/040095, WO2008/122084, and related US patent publication numbers 2007-0231810, 2010-0136536, and 2011-0143338 (the contents of each of these documents are incorporated herein by reference in their entirety). Non-limiting examples of MNzymes and modified MNzymes encompassed by the terms “MNzyme” and “multi-component nucleic acid enzyme” include MNzymes with cleavage catalytic activity (as exemplified herein), disassembled or partially assembled MNzymes comprising one or more assembly inhibitors, MNzymes comprising one or more aptamers (“apta-MNzymes”), MNzymes comprising one or more truncated sensor arms and optionally one or more stabilizing oligonucleotides, MNzymes comprising one or more activity inhibitors, multi-component nucleic acid inactive proenzymes (MNAi), and MNzymes with ligase catalytic activity (“MNzyme ligases”), each of which is described in detail in one or more of WO/2007/041774, WO/2008/040095, WO2008/122084, US 2007-0231810, US 2010-0136536, and/or US 2011-0143338.

As used herein, the terms “partzyme”, “component partzyme”, “partzyme component”, “component oligonucleotide”, “oligonucleotide component” and

“oligonucleotide partzyme” refer to a DNA-containing or RNA-containing or DNA-RNA-containing oligonucleotide, two or more of which, only in the presence of an MNzyme assembly facilitator as herein defined, can together form an “MNzyme.” In certain preferred embodiments, one or more component partzymes, and preferably at least two, may comprise three regions or domains: a “catalytic” domain, which forms part of the catalytic core that catalyzes a modification; a “sensor arm” domain, which may associate with and/or bind to an assembly facilitator; and a “substrate arm” domain, which may associate with and/or bind to a substrate. Illustrations of these regions or domains are shown in **Figure 1**. Partzymes may comprise at least one additional component including but not limited to an aptamer, referred to herein as an “apta-partzyme.” A partzyme may comprise multiple components, including but not limited to, a partzyme component with a truncated sensor arm and a stabilizing arm component which stabilises the MNzyme structure by interacting with either an assembly facilitator or a substrate.

The terms “assembly facilitator molecule”, “assembly facilitator”, “MNzyme assembly facilitator molecule”, and “MNzyme assembly facilitator” as used herein refer to entities that can facilitate the self-assembly of component partzymes to form a catalytically active MNzyme by interaction with the sensor arms of the MNzyme. As used herein, assembly facilitators may facilitate the assembly of MNzymes which have cleavage, ligase or other enzymatic activities. In preferred embodiments an assembly facilitator is required for the self-assembly of an MNzyme. An assembly facilitator may be comprised of one molecule, or may be comprised of two or more “assembly facilitator components” that may pair with, or bind to, the sensor arms of one or more oligonucleotide “partzymes”. The assembly facilitator may be a target. The target may be a nucleic acid 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. The nucleic acid may be amplified. The amplification may comprise one or more of: polymerase chain reaction (PCR), strand displacement amplification, loop-mediated isothermal amplification, rolling circle amplification, transcription-mediated amplification, self-sustained sequence replication, ligase chain reaction, nucleic acid sequence based amplification, or reverse transcription polymerase chain reaction (RT-PCR).

An “assembly facilitator component” is a molecule which can be used to control the assembly of active MNAzymes or facilitate the transition from inactive MNAzyme components to active MNAzymes.

The term “target” as used herein includes any natural or synthetic entity, constituent or analyte which is sought to be detected, identified or quantitated by a method which uses a particular nucleic acid enzyme such as an MNAzyme(s), with or without an additional amplification step and/or cascade. Targets therefore encompass the broadest range of detectable entities, constituents or analytes for which methods of sensitive detection, identification and/or quantification are desirable. Some exemplary targets include, but are not limited to, nucleic acid, protein, polypeptide, peptide, glycoproteins, lipids, lipoproteins, entire organisms, cells, viruses, bacteria, archaea, yeast, fungi, antibodies, metabolites, pathogens, toxins, contaminants, poisons, small molecules, polymers, metal ions, metal salts, prions or any derivatives, portions or combinations thereof. Other targets are also contemplated for use herein. It will be understood that the target may also be an assembly facilitator or assembly facilitator component.

A “detectable effect” is an effect that can be detected or quantified as an indication that modification of substrate/s has occurred. The magnitude of the effect may be indicative of the quantity of an input such as an assembly facilitator (e.g. a target). The detectable effect may be detected by a variety of methods, including fluorescence spectroscopy, surface plasmon resonance, mass spectroscopy, NMR, electron spin resonance, polarization fluorescence spectroscopy, circular dichroism, immunoassay, chromatography, radiometry, photometry, scintigraphy, electronic methods, UV, visible light or infra red spectroscopy, enzymatic methods or any combination thereof.

The terms “polynucleotide substrate” and “substrate” as used herein include any single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases, or analogues, derivatives, variants, fragments or combinations thereof, including but not limited to DNA, methylated DNA, alkylated DNA, RNA, methylated RNA, microRNA, siRNA, shRNA, mRNA, tRNA, snoRNA, stRNA, smRNA, pre- and pri-microRNA, other non-coding RNAs, ribosomal RNA, derivatives thereof, amplicons thereof or any combination thereof (including mixed polymers of deoxyribonucleotide and ribonucleotide bases), which is capable of being recognized, acted upon or modified by an enzyme including a catalytic nucleic acid enzyme. A “polynucleotide substrate” or “substrate” may be modified by various enzymatic activities including but not limited to cleavage or ligation. Modification of a “polynucleotide substrate” or “substrate” may provide a “detectable effect” for monitoring the catalytic activity of an enzyme.

A “reporter substrate” as used herein is a substrate that is particularly adapted to facilitate measurement of either the disappearance of a substrate or the appearance of a product in connection with a catalyzed reaction. Reporter substrates can be free in solution or bound (or “tethered”), for example, to a surface, or to another molecule. A reporter substrate can be labelled by any of a large variety of means including, for example, fluorophores (with or without one or more additional components, such as quenchers), radioactive labels, biotin (e.g. biotinylation) or chemiluminescent labels.

As used herein, a “generic substrate” or a “universal substrate” is a substrate, for example, a reporter substrate, that is recognized by and acted on catalytically by a plurality of MNazymes, each of which can recognize a different assembly facilitator. The use of such substrates facilitates development of separate assays for detection, identification or quantification of a wide variety of assembly facilitators using structurally related MNazymes all of which recognize a universal substrate. These universal substrates can each be independently labelled with one or more labels. In preferred embodiments, independently detectable labels are used to label one or more universal substrates to allow the creation of a convenient system for independently or simultaneously detecting a variety of assembly facilitators using MNazymes. In some embodiments, substrates cleaved by MNazymes could be reconstituted, and hence recycled, using an MNzyme or DNAzyme ligase. In some embodiments, substrate(s) cleaved or ligated by MNazymes can be further used as components or modulators of additional MNzyme(s) or DNAzyme(s).

In some embodiments, “universal substrates” may be tethered to a solid support in different positions to provide a substrate array. In such embodiments, the tethered universal substrates may all be labelled with the same fluorophore. In certain cases, each universal substrate can be cleaved only by an MNzyme formed in the presence of a specific MNzyme assembly facilitator molecule and signal can be localised by positioning of the substrate on the surface, thus allowing specific detection of different assembly facilitators.

The term “product” refers to the new molecule or molecules that are produced as a result of enzymatic modification of a substrate. As used herein the term “cleavage product” refers to a new molecule produced as a result of cleavage or endonuclease activity by an enzyme. The term “ligation product” refers to a new molecule produced as a result of the ligation of substrates by an enzyme.

As used herein, use of the terms “melting temperature” and “T_m” in the context of a polynucleotide substrate of the present invention will be understood to be a reference to

the melting temperature (T_m) as calculated using the Wallace rule, whereby $T_m = 2^\circ\text{C}(A+T) + 4^\circ\text{C}(G+C)$ (see Wallace *et al.*, (1979) Nucl. Acids Res. 6(11):3543-3558), unless specifically indicated otherwise.

As used herein, the term "base" will be understood to encompass the entire
 5 ribonucleotide or deoxyribonucleotide to which the base is attached.

ABBREVIATIONS

The following abbreviations are used herein and throughout the specification:

- 10 *MNAzyme*: multi-component nucleic acid enzyme, or multipartite nucleic acid enzyme;
- DNAzyme*: deoxyribonucleic acid enzyme;
- Ribozyme*: ribonucleic acid enzyme;
- Partzyme*: Partial enzyme containing oligonucleotide
- PCR*: polymerase chain reaction;
- 15 *qPCR*: Real-time quantitative PCR;
- NF-H₂O*: nuclease-free water;
- LNA*: locked nucleic acid;
- F*: fluorophore;
- Q*: quencher;
- 20 *N*= A, C, T/U, G, or any analogue thereof;
- N'*= any nucleotide complementary to *N*, or able to base pair with *N*;
- (N)_x*: any number of *N*;
- (N')_x*: any number of *N'*;
- W*: A or T;
- 25 *R*: A, G, or AA;
- rN*: any ribonucleotide;
- (rN)_x*: any number of *rN*;
- rR*: A or G ribonucleotide;
- rY*: C or U; ribonucleotide
- 30 *M*: A or C;
- H*: A, C, or T/U;
- D*: G, A, or T/U;
- JOE* or *6-JOE*: 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescein;
- FAM* or *6-FAM*: 6-Carboxyfluorescein.
- 35 *BHQ1*: Black Hole Quencher® 1

BHQ2: Black Hole Quencher® 2

IB: Iowa Black® FQ

IBR: Iowa Black® RQ

shRNA: short hairpin RNA

5 *siRNA*: short interfering RNA

mRNA: messenger RNA

tRNA: transfer RNA

snoRNA: small nucleolar RNA

stRNA: small temporal RNA

10 *smRNA*: small modulatory RNA

pre-microRNA: precursor microRNA

pri-microRNA: primary microRNA

UV: ultra violet

15 **DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS**

It is to be understood at the outset, that the figures and examples provided herein are to exemplify rather than limit the present invention and its various embodiments.

A need exists for catalytic nucleic acid substrates with properties that facilitate improved catalytic nucleic acid function. In particular, many applications involving
20 MNazymes and DNazymes will benefit significantly from the provision of new universal substrate families having increased capacity for catalytic modification by different MNazymes with the same or distinct target specificities. For example, these substrate families would be advantageous in increasing the efficiency and/or accuracy of multiplex assays involving MNazymes. Additional universal substrates are particularly
25 useful in applications where substrate arrays are created by tethering substrates to solid supports.

The present invention provides a set of guidelines for producing universal oligonucleotide substrates with a higher probability of being catalytically modified (e.g. cleaved) efficiently over a broad temperature range with improved performance at
30 elevated temperatures. These guidelines include, but are not limited to, any one or more of: (i) seven or more cytosine nucleotides in the ten bases surrounding the two central ribonucleotides; (ii) bases immediately adjacent to the two central ribonucleotides are cytosines (N_8 and N_9); (iii) total pyrimidine content of the oligonucleotide substrate is greater than 64%; (iv) total T_m of the oligonucleotide substrate is 66°C or greater,
35 applicable if the reaction temperature for catalytic modification (e.g. cleavage) of the

oligonucleotide substrate by the nucleic acid enzyme is above 50°C; and/or (v) a low number of guanine nucleotides (e.g. three, two, one or none) in the 10 bases surrounding the two central ribonucleotides.

The development of these guidelines has facilitated the development of catalytic nucleic acid enzyme substrates with features that augment catalytic nucleic acid function. It has been identified that the catalytic modification of nucleotide/s within a substrate targeted by a given nucleic acid enzyme can be enhanced by the presence of certain specific nucleotides proximate to those which are catalytically modified.

Accordingly, certain aspects of the present invention relate to polynucleotide substrates for catalytic nucleic acid enzymes. The polynucleotide substrates may comprise a series of pyrimidine nucleotides 5' (ie. upstream) and/or 3' (ie. downstream) of nucleotide/s that are catalytically modified by a nucleic acid enzyme that targets the substrate. The pyrimidine nucleotides may be cytosine nucleotides.

Other aspects of the present invention relate to the use of polynucleotide substrates described herein as substrates for nucleic acid enzymes (e.g. a DNAzyme, ribozyme or an MNAzyme). In certain embodiments, the substrates are used as substrates for MNAzymes. In certain embodiments, the substrates are used as substrates for DNAzymes.

Additional aspects of the present invention relate to methods for detecting a target molecule. The methods comprise modifying a polynucleotide substrate described herein to provide a detectable effect. In certain embodiments, the methods comprise modifying the polynucleotide substrate using an MNAzyme that is capable of detecting a target.

Further aspects of the present invention relate to kits comprising one or more polynucleotide substrate/s described herein. The kits may comprise a nucleic acid enzyme capable of catalytically modifying the substrate/s. In certain embodiments, the nucleic acid enzyme may be an MNAzyme.

1. Catalytic nucleic acid enzyme substrate/s

The present invention provides polynucleotide substrates for catalytic nucleic acid enzymes. The present invention also provides substrate families the members of which have increased capacity for catalytic modification by different nucleic acids (e.g. MNAzymes) with the same or distinct target specificities.

The polynucleotide substrates comprise at least one sequence motif that can be modified by a catalytic nucleic acid enzyme. No limitation exists regarding the particular type of catalytic nucleic acid enzyme that may modify a polynucleotide substrate of the

present invention. The sequence motif may comprise any one or more of at least one DNA nucleotide, at least one RNA nucleotide, at least one analogue of a DNA nucleotide, and at least one analogue of a RNA nucleotide.

Non-limiting examples of suitable sequence motifs include those recognised and modified by DNazymes (e.g. 10-23 DNazymes; 8-17 DNazymes; “7Z81”, “7Z48” and “7Q10” DNzyme ligases; “UV1C” thymine dimer photoreversion DNazymes, “DAB22” carbon-carbon bond forming DNazymes; and derivations thereof), ribozymes (e.g. hammerhead ribozymes; homodimeric ribozymes, heterodimeric ribozymes; and derivations thereof), and MNazymes (see, for example, MNazymes described in PCT patent publication numbers WO/2007/041774, WO/2008/040095 and WO2008/122084, and related US patent publication numbers 2007-0231810, 2010-0136536, and 2011-0143338; each of which is incorporated herein by reference in its entirety).

Non-limiting examples of suitable sequence motifs include those set out in **Table 1** below.

Table 1: Exemplary catalytic motifs

Catalytic Enzyme	Substrate Catalytic Motif
8-17 DNzyme	(N') _x (rN) _x G (N') _x
10-23 DNzyme	(N') _x rR rY (N') _x

N = A, C, T, G or any analogue; *N'* = any nucleotide complementary to *N*; (*N*)_x or (*N'*)_x = any number of nucleotides; *W* = A or T; *R* = A, G or AA; *rN* = any ribonucleotide base; (*rN*)_x = any number of ribonucleotides; *rR* = A or G ribonucleotides; *rY* = C or U ribonucleotides; *M* = A or C; *H* = A, C or T; *D* = G, A or T

Catalytic nucleic acids have been shown to tolerate only certain modifications in the area that forms the catalytic core (Perreault *et al.*, 1990 Nature 344(6266): 565-7.; Perreault *et al.*, 1991 Biochemistry 30(16): 4020-5; Zaborowska *et al.*, 2002 J Biol Chem. 277(43): 240617-22; Cruz *et al.*, 2004 Chem Biol. Jan;11(1): 57-6; Silverman, 2004 Chem Biol. Jan;11(1): 7-8). Examples of sequences responsible for catalytic activity of DNazymes are listed in **Table 2**.

Table 2: Exemplary sequences for some active DNazymes and their substrates

DNzyme type	DNzyme sequence	Substrate sequence
8-17	(N) _x TNNNAGCNNNWCGR(N) _x SEQ ID NO: 189	(N') _x (rN) _x G (N') _x
10-23	(N) _x GGMTMGHNDNNNMGD(N) _x SEQ ID NO: 190	(N') _x rR rY (N') _x

For the DNAzyme sequence N is generally A, C, T or G or any analogue, in some instances N can be U; for the substrate sequence N = A, C, T/U, G or any analogue; ; N' = any nucleotide complementary to N; (N)_x or (N')_x = any number of nucleotides; W = A or T, in some instances W can be U; R = A, G or AA; rN = any ribonucleotide base; (rN)_x = any number of ribonucleotides; rR = A or G ribonucleotide; rY = C or U ribonucleotide; M = A or C; H = A, C or T, in some instances H can be U; D = G, A or T, in some instances D can be U.

The polynucleotide substrates may comprise multiple sequence motifs. The motifs may be recognised and modified by one type of catalytic nucleic acid enzyme. Alternatively, different sequence motifs within the substrate may be recognised and modified by different types of catalytic nucleic acid enzymes.

As noted above, polynucleotide substrates of the present invention comprise at least one sequence motif capable of modification by a catalytic nucleic acid enzyme. In some embodiments, nucleotides in the proximity of the sequence motif are pyrimidine nucleotides. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleotides preceding and/or succeeding (i.e. following) the sequence motif may be pyrimidine nucleotides. Any one or more of the pyrimidine nucleotides may be cytosine nucleotides.

In other embodiments, the sequence motif is preceded and/or succeeded (i.e. followed) directly (i.e. in continuous sequence) by one or more pyrimidine nucleotides. For example, the sequence motif may be directly preceded and/or directly succeeded by a sequence of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides. Any one or more of the pyrimidine nucleotides may be cytosine nucleotides.

In further embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more nucleotides within ten nucleotides 5' (i.e. upstream) and/or within ten nucleotides 3' (i.e. downstream) of the sequence motif are pyrimidine nucleotides. Any one or more of the pyrimidine nucleotides may be cytosine nucleotides.

In still further embodiments, more than 9, more than 10, or more than 11 nucleotides of the polynucleotide substrate may be cytosine nucleotides. For example, the substrate may comprise or consist of 10, 11, 12, 13, 14, 15, or more than 15 nucleotides, and 9, 10, 11 or more than 11 of these nucleotides may be cytosine nucleotides.

Additionally or alternatively, less than 5, less than 4, less than 3, or less than 2 nucleotides of the polynucleotide substrate may be guanine nucleotides. For example, the substrate may comprise or consist of 10, 11, 12, 13, 14, 15, or more than 15 nucleotides, and 4, 3, 2, 1 or none of these nucleotides may be guanine nucleotides.

No particular limitation exists regarding the length of a polynucleotide substrate of the present invention. For example, the substrate may be less than 100, 75, 50, 40, 30, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, or 5 nucleotides in

length. For example, the substrate may be between 5 and 30, 10 and 15, 10 and 20, 10 and 25, 10 and 30, 16 and 23, 16 and 21, 16 and 18, 18 and 21, 18 and 23, or 21 and 23 nucleotides in length.

A polynucleotide substrate of the present invention may be designed to possess a specific melting temperature (T_m) as calculated using the Wallace rule, whereby $T_m = 2^\circ\text{C}(\text{A}+\text{T}) + 4^\circ\text{C}(\text{G}+\text{C})$ (see Wallace *et al.*, (1979) Nucl. Acids Res. 6(11):3543-3558). In certain embodiments, the substrate may be recognised and catalytically modified by an MNzyme, and the T_m of the bases that are bound by the partzyme substrate arm/s of the MNzyme may be between about 52°C and about 76°C , between about 55°C and about 75°C , between about 60°C and about 70°C , between about 65°C and about 70°C , between about 64°C and 68°C , or between 64°C and 70°C (as calculated using the Wallace rule).

In other embodiments, the substrate may be recognised and catalytically modified by an MNzyme or a DNzyme, and the T_m of the bases that are bound by the partzyme substrate arm/s of the MNzyme may be between 68°C and 90°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 .

By way of non-limiting example only, a polynucleotide substrate of the present invention may comprise a sequence defined in any one or more of SEQ ID NOs: 25-27, 29-30, 72-90, or 172-175. In certain embodiments, the polynucleotide substrate may consist of a sequence defined in any one or more of SEQ ID NOs: 25-27, 29-30, 72-90, or 172-175.

In some embodiments, a polynucleotide substrate of the present invention may comprise a sequence defined by SEQ ID NO: 28. In other embodiments, the polynucleotide substrate may consist of a sequence defined by SEQ ID NO: 28.

In some embodiments, polynucleotide substrates of the present invention are capable of catalytic modification by an MNzyme comprising two oligonucleotide partzymes. The sequences of the polynucleotide substrate and the oligonucleotide partzymes may be any specific combination of three sequences (as depicted by SEQ ID NOs) that is shown in Table 6, 8, 10, 13, 16, 20, 22 and/or 24.

In other embodiments, polynucleotide substrates of the present invention are capable of catalytic modification by a DNzyme. The sequences of the polynucleotide substrate and DNzyme may be any specific pair of sequences (as depicted by SEQ ID NOs) that is shown in Table 15.

Polynucleotide substrates of the present invention may contain one or more substitutions such as analogues, derivatives, modified or altered bases, ribonucleotides, alterations of the sugar or phosphate backbone, various deletions, insertions, substitutions, duplications or other modifications, or any combination of these, well known to those skilled in the art.

Non-limiting examples of additions or substitutions include LNA phosphoramidite, 4-acetylcytidine, 5-(carboxyhydroxymethyl)uridine, 2'-O-methylcytidine, 5-carboxymethylaminomethyl thiouridine, dihydrouridine, 2'-O-methylpseudouridine, beta D-galactosylqueosine, 2'-O-methylguanosine, inosine, N6-isopentenyladenosine, 1-methyladenosine, 1-methylpseudouridine, 1-methylguanosine, 1-methylinosine, 2,2-dimethylguanosine, 2-methyladenosine, 2-methylguanosine, 3-methylcytidine, 5-methylcytidine, N6-methyladenosine, 7-methylguanosine, 5-methylaminomethyluridine, 5-methoxyaminomethyl-2-thiouridine, beta D-mannosylmethyluridine, 5-methoxycarbonylmethyluridine, 5-methoxyuridine, 2-methylthio-N6-isopentenyladenosine, N-((9-beta-ribofuranosyl-2-methylthiopurine-6-yl)carbamoyl)threonine, N-((9-beta-ribofuranosylpurine-6-yl)N-methylcarbamoyl)threonine, uridine-5-oxyacetic acid methylester, uridine-5-oxyacetic acid (v), wybutoxosine, pseudouridine, queosine, 2-thiocytidine, 5-methyl-2-thiouridine, 2-thiouridine, 4-thiouridine, 5-methyluridine, N-((9-beta-D-ribofuranosylpurine-6-yl)carbamoyl)threonine, 2'-O-methyl-5-methyluridine, 2'-O-methyluridine, wybutosine, 3-(3-amino-3-carboxypropyl)uridine, beta D-arabinosyl uridine, and beta D-arabinosyl thymidine.

Non-limiting examples of derivatives include functionally equivalent nucleic acids or nucleotides, including any fusion molecules produced integrally (e.g. by recombinant means) or added post-synthesis (e.g. by chemical means). Such fusions may comprise oligonucleotides of the invention with RNA or DNA added thereto or conjugated to a polypeptide (e.g. puromycin or other polypeptide), a small molecule (e.g. psoralen), a microcarrier or nanocarrier, or an antibody.

Non-limiting examples of analogues include compounds having a physical structure that is related to a DNA or RNA molecule or residue, and may be capable of forming a hydrogen bond with a DNA or RNA residue or an analogue thereof (i.e. it is able to anneal with a DNA or RNA residue or an analogue thereof to form a base-pair), but such bonding is not so required for said compound to be encompassed within the term "analogue". Such analogues may possess different chemical and biological properties to the ribonucleotide or deoxyribonucleotide residue to which they are structurally related.

Methylated, iodinated, brominated or biotinylated residues are examples of analogues. Active DNazymes have been described which contain nucleotide analogues, including deoxyinosine, C-5-immidazole deoxyuridine, 3-(aminopropynyl)-7-deaza-dATP, 2'-O-methyl RNA, 2' O-methyl cap. Other analogues could also be compatible with catalytic activity of DNazymes and MNazymes. Alteration of a nucleic acid with catalytic activity, for example by substitution of one base for another, by substitution of an analogue for a base, or alteration of the sugar component or phosphodiester backbone, can be straight forward for the skilled artisan. For example, alterations can be made during synthesis, or by modification of specific bases after synthesis. Empirical testing of catalytic nucleic acids incorporating alterations such as base changes or base analogues allows for assessment of the impact of the altered sequences, or specific analogues, on catalytic activity. Analogues of the bases A, C, G, T and U are known in the art, and a subset is listed in **Table 3**.

Table 3: Exemplary nucleotide analogues

Abbreviation	Name
ac4c	4-acetylcytidine
chm5u	5-(carboxyhydroxymethyl)uridine
Cm	2'-O-methylcytidine
Cmm5s2u	5-carboxymethylaminomethyl thiouridine
D	Dihydrouridine
Fm	2'-O-methylpseudouridine
Galq	beta, D-galactosylqueosine
Gm	2'-O-methylguanosine
I	Inosine
i6a	N6-isopentenyladenosine
m1a	1-methyladenosine
m1f	1-methylpseudouridine
m1g	1-methylguanosine
M1l	1-methylinosine
m22g	2,2-dimethylguanosine
m2a	2-methyladenosine
m2g	2-methylguanosine
m3c	3-methylcytidine
m5c	5-methylcytidine
m6a	N6-methyladenosine
m7g	7-methylguanosine
mam5u	5-methylaminomethyluridine
mam5s2u	5-methoxyaminomethyl-2-thiouridine
Manq	beta, D-mannosylmethyluridine
mcm5s2u	5-methoxycarbonylmethyluridine
Mo5u	5-methoxyuridine

Abbreviation	Name
Ms2i6a	2-methylthio-N6-isopentenyladenosine
Ms2t6a	N-((9-beta-ribofuranosyl-2-methylthiopurine-6-yl)carbamoyl)threonine
Mt6a	N-((9-beta-ribofuranosylpurine-6-yl)N-methyl-carbamoyl)threonine
Mv	Uridine-5-oxyacetic acid methylester
o5u	Uridine-5-oxyacetic acid (v)
Osyw	Wybutoxosine
P	Pseudouridine
Q	Queosine
s2c	2-thiocytidine
s2t	5-methyl-2-thiouridine
s2u	2-thiouridine
s4u	4-thiouridine
T	5-methyluridine
t6a	N-((9-beta-D-ribofuranosylpurine-6-yl)carbamoyl)threonine
Tm	2'-O-methyl-5-methyluridine
Um	2'-O-methyluridine
Yw	Wybutosine
X	3-(3-amino-3-carboxypropyl)uridine, (acp3)u
AraU	beta D-arabinosyluridine
AraT	beta D-arabinosylthymidine

Polynucleotide substrates of the present invention may incorporate additional entities such as labelled nucleic acids, nanoparticles, microparticles, proteins, antibodies, RNA, DNA, nucleic acid analogues, proteins, glycoproteins, lipoproteins, peptide nucleic acids, locked nucleic acids, peptide-nucleic acid chimeras, or any combination thereof. The nanoparticles may be gold nanoparticles.

Polynucleotide substrates of the present invention may be catalytically modified by a catalytic nucleic acid enzyme. Non-limiting examples of potential catalytic modifications include cleavage of nucleic acids, ligation of nucleic acids, phosphorylation of nucleic acids, nucleic acid capping, amino acid adenylation, cofactor synthesis, RNA polymerization, template-directed polymerization, RNA-protein conjugation, aldol reaction, alcohol oxidation, aldehyde reduction, purine and pyrimidine nucleotide synthesis, alkylation, amide synthesis, urea synthesis, formation of peptide bonds, peptidyl-RNA synthesis, acyl transfer, aminoacylation, carbonate hydrolysis, phosphorothioate alkylation, porphyrin metallation, formation of carbon-carbon bonds, Pd nanoparticle formation, biphenyl isomerization, formation of ester bonds, formation of amide bonds, DNA deglycosylation, thymine dimer photoreversion and phosphoramidate cleavage.

In certain applications, it may be desirable to detect product/s arising from catalytic modification of polynucleotide substrates of the present invention. This can be achieved using any number of standard techniques known in the art.

For example, the substrate may comprise a detectable portion and a quencher portion, wherein upon modification of said substrate by a catalytic nucleic acid, a detectable effect provided by said detectable portion is increased or decreased. The detectable effect may be detected 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.

Additionally or alternatively, product/s arising from catalytic modification of polynucleotide substrates of the present invention may be detected on the basis of size (e.g. by standard electrophoresis), nucleic acid sequencing, fluorescence resonance energy transfer, chemiluminescence, potentiometry, mass spectrometry, plasmon resonance, colorimetry, polarimetry, flow cytometry, scanometry, and DNA sequencing or any combination thereof.

Nucleic acid product/s arising from catalytic modification of polynucleotide substrates of the present invention may be amplified in order to assist detection using techniques such as, for example the polymerase chain reaction (PCR).

Polynucleotide substrates of the present invention may be recognized and modified by catalytic nucleic acid enzymes (e.g. MNAzymes) designed to detect a target that differs from the substrate to be modified by the enzyme. Accordingly, polynucleotide substrates of the present invention may be "generic" or "universal" substrates that are recognized by and acted on catalytically by a plurality of catalytic nucleic acid enzymes (e.g. a plurality of MNAzymes), each of which can recognize a different target. The use of such substrates may facilitate the development of separate assays for detection, identification or quantification of a wide variety of targets using catalytic nucleic enzymes which recognize a universal substrate. The universal substrates may each be independently labelled with one or more labels. In certain embodiments, independently detectable labels may be used to label one or more universal substrates to allow for the independent or simultaneous detection of a variety of targets using MNAzymes. For example, a series of universal substrates may be used in a multiplex reaction allowing simultaneous detection of multiple targets.

Polynucleotide substrates of the present invention may be provided bound, attached or tethered to an insoluble or solid support for use in various applications (e.g. enzymatic cascades or any other signal transduction cascades). The support may be an insoluble material, or a matrix which retains the substrate and excludes it from freely moving in the bulk of the reaction mixture. Such supports are known in the art for immobilizing or localizing substrates, including nucleic acid targets. The skilled addressee will appreciate that the support can be selected from a wide variety of matrices, polymers, and the like in a variety of forms including beads convenient for use in microassays, as well as other materials compatible with the reaction conditions. In certain preferred embodiments, the support can be a plastic material, such as plastic beads or wafers, or that of the well or tube in which a particular assay is conducted. In certain embodiments, the support may be a microcarrier or a nanocarrier. The attachment of the substrate to the support may be designed so that upon modification (e.g. cleavage) of the substrate by the catalytic nucleic acid (e.g. MNAzyme), a portion of the modified substrate remains attached to the support, while the other is freed to move into the bulk of the reaction mixture, away from the portion remaining attached.

2. Exemplary Methods

Polynucleotide substrates of the present invention may be used in any number of potential applications utilising catalytic nucleic acids which recognise/modify the substrates.

For example, the substrates may be used in applications involving DNAzymes (e.g. 10-23 DNAzymes; 8-17 DNAzymes; "7Z81", "7Z48" and "7Q10" DNAzyme ligases; "UV1C" thymine dimer photoreversion DNAzymes, "DAB22" carbon-carbon bond forming DNAzymes; and derivations thereof), ribozymes (e.g. hammerhead ribozymes; homodimeric ribozymes, heterodimeric ribozymes; and derivations thereof), and/or MNAzymes.

In certain embodiments of the invention, the substrates may be used as substrates for MNAzymes. The features of MNAzymes and various applications using MNAzymes are described in detail in PCT patent publication numbers WO/2007/041774, WO/2008/040095 and WO2008/122084, and related US patent publication numbers 2007-0231810, 2010-0136536, and 2011-0143338 (the contents of each of these documents are incorporated herein by reference in their entirety).

MNAzymes are capable of self-assembling from two or more oligonucleotide components, also referred to herein as partzymes. The partzyme oligonucleotides self-

assemble in the presence of an MNzyme self assembly facilitator to form an MNzyme. MNzymes are therefore catalytically active nucleic acid enzymes. In some embodiments, the presence of an MNzyme can be detected, and is indicative of the presence of a target, because the MNzyme forms only in the presence of the target, wherein the target comprises the assembly facilitator.

In preferred embodiments, the MNzyme structures are based on one or more DNzymes and/or ribozymes. More preferred are those MNzyme structures which are based on a particular DNzyme structure. Presently preferred structures are based on DNzymes including the 10-23 and 8-17 DNzymes. In various embodiments the MNzymes comprise either or both ribonucleotide bases and deoxyribonucleotide bases. In more preferred embodiments, an MNzyme structure is based at least in part on the structure of a DNzyme. In other preferred embodiments, MNzymes comprise at least some deoxyribonucleotide bases or analogues thereof. In more preferred embodiments, the catalytic core of an MNzyme comprises one or more deoxyribonucleotide bases or analogues thereof. In still more preferred embodiments, one or more deoxyribonucleotide bases or analogues thereof are involved in the catalysis of a substrate. In other embodiments, at least one deoxyribonucleotide base, or its analogue, in the catalytic core improves catalytic activity. In yet other embodiments, there is a strict requirement for at least one deoxyribonucleotide base, or its analogue, in the catalytic core of the MNzyme for catalysis to occur at a measurable rate, relative to that of a comparable MNzyme without the deoxyribonucleotide base present.

The MNzymes may contain one or more substitutions such as analogues, derivatives, modified or altered bases, ribonucleotides, alterations of the sugar or phosphate backbone, various deletions, insertions, substitutions, duplications or other modifications, or any combination of these, well known to those skilled in the art. Such modifications, substitutions, deletions, insertions, etc may be made in the sensor and/or substrate arms and/or in the catalytic core portions, such that the molecule retains catalytic activity. Substitutions and modifications to arms that bind the substrate or assembly facilitator may be well tolerated and in fact are the basis of allowing tailoring of the molecules to different substrates/assembly facilitators. For example, modification of the sensor arms will allow tailoring to different assembly facilitators, while modification of the substrate arms will allow tailoring to different substrates.

The MNzyme may comprise either deoxyribonucleotides or ribonucleotides, or even both. MNzymes comprising at least one and more preferably, all, deoxyribonucleotide component oligonucleotides are preferred. Also preferred are

MNAzymes comprising at least one deoxyribonucleotide base, or its analogue, within the catalytic core of the MNAzyme. Even more preferred are those embodiments where such a base is required for catalytic activity.

MNAzyme assembly and disassembly may also be controlled by changing the microenvironment. Examples of such changes include, but are not limited to, temperature, divalent cation type and concentration, salt concentration, pH, additives, and the presence or absence of critical components essential for assembly and/or activity of an active MNAzyme. Accordingly, disassembled or partially assembled MNAzymes may be prevented from assembling into a catalytically active MNAzyme in the presence of an assembly facilitator by modulating the microenvironment, thus providing a “molecular switch”.

A basic example of a MNAzyme structure is depicted in **Figure 1**. The structure shown comprises partzyme A and partzyme B, the sensor arms (i) of which have base-paired with an MNAzyme assembly facilitator molecule, for example a target DNA or RNA. Partzymes A and B by interacting with the assembly facilitator have allowed the partial catalytic cores (iii) to come into close proximity and thereby form a single catalytic core. The substrate arms (ii) of the MNAzyme have interacted with and base-paired with a substrate, depicted here as a Reporter Substrate. Thus the MNAzyme has self-assembled and this process is facilitated through the presence of the MNAzyme assembly facilitator molecule. In the absence of assembly facilitator, no MNAzyme will form. Modification (in this case, cleavage) of a polynucleotide substrate of the present invention is catalyzed by the catalytic core of the MNAzyme at the MNAzyme Modification Site (e.g. Cleavage Site within the substrate denoted by a cross (X)). The polynucleotide substrate in this particular embodiment comprises a detectable portion having a detectable signal, for example fluorophore F, and a quencher portion Q having a quenching effect on the detectable signal F through the action of quencher Q. Upon cleavage at the MNAzyme Cleavage Site, there is a substantial increase in detectable signal, here fluorescence, which can readily be detected and quantified if so desired.

Figure 1 can further be understood to depict an example of a basic method of using MNAzymes to detect a target, which in some embodiments comprises an assembly facilitator. More specifically, partzyme A and partzyme B are shown in **Figure 1**, each comprising a substrate arm portion (ii), catalytic core portion (iii), and a sensor arm portion (i). In the presence of a target, the sensor arm portions of partzyme A and partzyme B can begin to hybridize to, and base pair with complementary portions, of the target, for example a DNA or RNA sequence. Upon contacting the target in this fashion,

the MNAzyme self-assembles forming a catalytic core which can modify a substrate which is bound by the substrate arms. Preferably the presence of the MNAzyme is detected through the detection or measurement of its catalytic activity. The substrate arms of the thus assembled MNAzyme can engage a polynucleotide substrate of the present invention through the interaction of the complementary sequences on the substrate arms and the substrate. Once the substrate is so engaged with the substrate arms, the catalytic core can promote the modification (e.g. cleavage) of the substrate, which can in turn be measured or detected, directly or indirectly.

The skilled artisan will readily appreciate that the methods described herein may involve amplification of a target before, during or after MNAzyme catalytic activity. Such target amplification finds particular application in embodiments of the present invention where the amount of target being sought to be detected, identified or quantified is of such quantum so as to provide a signal that may otherwise not be detectable. Such amplification may comprise 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).

Figure 2 provides exemplary applications of methods for target detection using MNAzymes. Strategy 1 exemplifies MNAzymes adapted for detection of targets including DNA, RNA and proteins. As described above (see description of **Figure 1**) an MNAzyme composed of two separate oligonucleotides with recognition sequences for both a target and a substrate forms when the oligonucleotides recognize and bind a target. The substrate, e.g. reporter substrate, is modified by the catalytic action of the MNAzyme and causes generation of a detectable signal, either directly (Strategy 1), during or after target amplification (Strategy 2) or via a signal cascade (Strategy 3). In some embodiments, both target and signal amplification occur simultaneously or sequentially.

Strategy 2 of **Figure 2** exemplifies the use of an MNAzyme adapted to monitor the accumulation of amplicons during, or following, *in vitro* amplification of nucleic acid targets. Techniques for *in vitro* amplification of nucleic acid sequences are known in the art. These include techniques mediated by a DNA polymerase, such as the polymerase chain reaction ("PCR") (see, for example, U.S. Patent No. 4,683,202; U.S. Patent No. 4,683,195; U.S. Patent No. 4,800,159; U.S. Patent No. 4,965,188; U.S. Patent No. 5,176,995), strand displacement amplification ("SDA"), rolling circle amplification ("RCA"), reverse transcription polymerase chain reaction (RT-PCR) and loop-mediated

isothermal amplification ("LAMP"). Other target amplification techniques are mediated by an RNA polymerase, for example, transcription-mediated amplification ("TMA"), self-sustained sequence replication ("3SR") and nucleic acid sequence replication based amplification ("NASBA"). The amplification products ("amplicons") produced by PCR, RT-PCR, SDA, RCA and LAMP are composed of DNA, whereas RNA amplicons are produced by TMA, 3SR and NASBA.

With further reference to strategy 2 of **Figure 2** an MNzyme that recognises and modifies a polynucleotide substrate of the present invention can be used in conjunction with target amplification methods which include, for example, the aforementioned PCR, RT-PCR, SDA, RCA, LAMP, TMA, 3SR and NASBA. The accumulation of amplicons produced by PCR using either asymmetric or symmetric primer ratios can be monitored using MNzymes. Examples 1, 2, 4, 6, 8 and 9 of the present specification demonstrate the detection of PCR amplicons in real time utilising MNzymes that catalytically modify various polynucleotide substrates of the present invention.

Again referring to strategy 2 of **Figure 2**, a target nucleic acid may be amplified in accordance with a procedure for amplifying that nucleic acid (ie. DNA or RNA). Preferably, standard methods of *in vitro* amplification are used. The amplicons generated during the amplification may serve as target assembly facilitators for an MNzyme. The MNzyme activity, which is made detectable by modification of a polynucleotide substrate of the present invention by the MNzyme, is indicative of the presence of the target. The skilled artisan will appreciate that assays of this nature can be conducted in a single vessel under conditions that permit both the target nucleic acid amplification and the MNzyme assembly and catalytic activity. Additionally or alternatively, they can be conducted subsequent to, or at time points throughout, the target nucleic acid amplification, by removing samples at the end or during the course of the amplification reactions.

Strategy 3 of **Figure 2** shows an overview of a method of using a MNzyme to initiate amplification of a signal through the use of a signal cascade. Example 3 of the present specification demonstrates the isothermal direct detection of a target utilising MNzymes that catalytically modify various polynucleotide substrates of the present invention, which could be used to initiate a signal cascade.

The skilled addressee will appreciate that methods or protocols that combine target amplification with catalytic nucleic acid activity may require specific reaction conditions (e.g. those described in Examples 1, 2, 4, 6, 8 and 9 of the present specification). Preferably, reaction conditions are compatible with both polymerase activity (for

amplification), and catalytic nucleic acid modification of a substrate (for detection). Protocols for determining conditions for concurrent catalytic activity and polymerase activity at high temperature, such as during PCR, have been described for DNAzymes. The influence of factors including DNAzyme arm length, buffer, temperature, divalent ion concentration and effects of additives are known in the art. DNA enzymes are suited for use in combination with *in vitro* amplification strategies. For example, they are not irreversibly denatured by exposure to high temperatures during amplification.

In certain embodiments, a polynucleotide substrate of the present invention capable of recognition and modification by an MNAzyme may be bound, attached or tethered to an insoluble or solid support. For example, with reference to **Figure 3, Panel (i)**, an exemplary method for detecting targets using an MNAzyme and a polynucleotide substrate of the present invention anchored to a support is depicted. In this embodiment, the substrate is preferably a substrate with a detectable portion comprising a detectable signal, for example a fluorophore, and a quencher portion which diminishes or eliminates the detectable signal while the detectable portion and the quencher portion of the substrate remain in close proximity, for example, until the substrate is modified (e.g. by cleavage). The substrate is attached to a support. Preferably the support is an insoluble material, or a matrix which retains the substrate and excludes it from freely moving in the bulk of the reaction mixture. The attachment of the substrate to the support may be designed such that upon modification (e.g. by cleavage) of the substrate by the MNAzyme, either the detectable portion or the quencher portion, but not both, remains attached to the support, while the other is freed to move into the bulk of the reaction mixture, away from the portion remaining attached. Thus, in a cleavage example, the detectable signal vastly increases as the quencher portion and the detectable portion are separated upon cleavage. In the embodiment shown in **Figure 3, Panel (i)**, the fluorophore-containing detectable portion remains attached after cleavage. This has the benefit of allowing localization of the signal on the support but in certain instances, the fluorophore/s may be released into solution. In a further embodiment where, for example, ligation occurs, the quencher may be ligated to a fluorophore thus decreasing the detectable signal.

In certain embodiments, multiple universal substrates may be tethered to a solid support in different positions to provide a substrate array. With reference to **Figure 3 Panel (ii)**, two substrates may be attached at defined positions on a solid surface. Each universal substrate can be cleaved only by an MNAzyme formed in the presence of a specific MNAzyme assembly facilitator molecule (e.g. target 1 or target 2) and signal can be localised by positioning of the substrate on the surface (position 1 or position 2), thus

allowing specific detection of different assembly facilitators. In such embodiments tethered universal substrates may all be labelled with the same fluorophore. In other embodiments tethered universal substrates may be labelled with different fluorophores. The strategy depicted in **Figure 3 (ii)** can be extended to create universal substrate arrays with many different universal substrates attached in defined positions on a solid surface. In such embodiments increasing the number of universal substrates that are available for use in arrays may provide an advantage by allowing the creation of more complex arrays. Such universal arrays of universal substrates may have utility for use in highly multiplexed analysis of target analytes. The present invention provides additional universal substrates with features that may augment catalytic activity function which may be useful in improving the capacity to perform increasingly more complex analysis using MNazymes.

In certain embodiments, a polynucleotide substrate of the present invention may be recognised and modified by a MNzyme to provide an assembly facilitator, assembly facilitator component, or partzyme for a second different MNzyme.

With reference to **Figure 4**, an initiating MNzyme (Mt) may be formed in the presence of a target (T). The initiating MNzyme (Mt) cleaves a (first) polynucleotide substrate of the present invention (S1) to create a first assembly facilitator component (S1f), which directs formation of a first cascading MNzyme (cascading MNzyme Mc1). In this example the first cascading MNzyme (Mc1) comprises two partzymes and three assembly facilitator components designated F1, F2 and S1f. Mc1 can cleave an additional substrate (S2) thus liberating an additional assembly facilitator component (S2f), which directs formation of a second cascading MNzyme (cascading MNzyme Mc2). In this example the second cascading MNzyme (Mc2) comprises two partzymes and three assembly facilitator components designated F3, F4 and S2f. Mc2 can then cleave more of the first substrate (S1) thus creating more of the first assembly facilitator component (S1f). This leads to the formation of further first cascading MNzyme (Mc1) thereby forming an amplification cascade. The skilled addressee will recognise that **Figure 4** shows three assembly facilitator components are required to facilitate active MNzyme assembly. More or less assembly facilitator components could be utilised in a similar schema.

The skilled artisan will readily understand that the methods described herein may be optimized using a variety of experimental parameters in order to optimize the detection, identification and/or quantification of a target, and/or the recognition and catalytic modification of a polynucleotide substrate of the present invention by a catalytic nucleic

acid (e.g. an MNzyme or a DNzyme). The particular experimental parameters that are optimized, and the level of such optimization, will depend upon the particular method being employed and the particular target and/or substrate involved. Such parameters include, but are not limited to, time, temperature, concentration of salts, detergents, cations and other reagents including but not limited to dimethylsulfoxide (DMSO), and length, complementarity, GC content and melting point (T_m) of nucleic acids.

In some embodiments, for example those methods involving detection of sequence variation and/or detection of methylated DNA, the experimental parameters, and preferably including the temperature at which the method is performed, may be optimized so as to discriminate between binding of an MNzyme component nucleic acid to a target nucleic acid that does or does not comprise a sequence variation or a methylated nucleotide, respectively. The temperature at which such methods may be performed may be in the range of about 20°C to about 96°C, about 20°C to about 75°C, 20°C to about 60°C or about 20 to about 55°C.

In some embodiments, optimized reactions for practicing the methods of using MNzymes and DNzymes are provided herein. In such optimized reactions, catalytic activity is increased by up to 10, 20, or 30% above unoptimized reactions. More preferred reaction conditions improve catalytic activity by at least 35%, or 40%, and preferably up to 50% or more. In still more preferred embodiments, optimized reactions have an increase of catalytic activity of more than 50%, and up to 66%, 75% or even 100%. In yet more preferred embodiments, a fully optimized reaction method will offer 100, 200 or even 300% or more increase in catalytic activity. Other preferred reaction conditions can improve the catalytic activity by up to 1,000% or more over methods practiced with unoptimized reaction conditions. A highly preferred reaction condition for optimizing the methods provided herein is the inclusion of certain divalent cations. The catalytic activity of most nucleic acid enzymes may be influenced in a concentration-dependent fashion by the concentration of divalent cations. Preferred optimized reactions are optimized for one or more of Ba^{2+} , Sr^{2+} , Mg^{2+} , Ca^{2+} , Ni^{2+} , Co^{2+} , Mn^{2+} , Zn^{2+} , and Pb^{2+} .

In some embodiments, the use of polynucleotide substrates of the present invention in assays with nucleic acid enzymes (e.g. MNzymes or DNzymes) may increase a detectable effect (e.g. an increase or decrease in fluorescent signal) arising from catalytic modification of the substrate by the enzyme above the detectable effect gained using a known substrate in the same assay under the same conditions. For example, the detectable effect may be increased by more than 2%, more than 3%, more than 4%, more than 5%, more than 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 20%, 25%, 30%, 40%, or

more than 50% compared to the known substrate. In certain embodiments, the detectable effect is a fluorescent signal.

In some embodiments, the methods of the invention involve using a polynucleotide substrate for an MNAzyme in combination with an MNAzyme comprising two
5 oligonucleotide partzymes. The sequences of the polynucleotide substrate and the oligonucleotide partzymes may be any specific combination of three sequences (as depicted by SEQ ID NOs) that is shown in Table 6, 8, 10, 13, 16, 20, 22 and/or 24.

In some embodiments, the methods of the invention involve using a polynucleotide substrate in combination with a DNAzyme. The sequences of the polynucleotide substrate
10 and DNAzyme may be any specific pair of sequences (as depicted by SEQ ID NOs) that is shown in Table 15.

3. Kits

Also provided herein are kits comprising one or more polynucleotide substrates of
15 the present invention.

The kits may comprise additional reagents for practising the methods disclosed herein. For example, the kits may comprise one or more catalytic nucleic acids capable of recognising and modifying the substrate. Non-limiting examples of suitable catalytic nucleic acids include DNAzymes (e.g. 10-23 DNAzymes; 8-17 DNAzymes; "7Z81",
20 "7Z48" and "7Q10" DNAzyme ligases; "UV1C" thymine dimer photoreversion DNAzymes, "DAB22" carbon-carbon bond forming DNAzymes; and derivations thereof), ribozymes (e.g. hammerhead ribozymes; homodimeric ribozymes, heterodimeric ribozymes; and derivations thereof), and MNAzymes.

Kits of the present invention may be "compartmentalised" kits. A
25 compartmentalised kit encompasses any kit in which reagents are provided in separate containers such as, for example, small glass containers, plastic containers or strips of plastic or paper. Such containers may allow the efficient transfer of reagents from one compartment to another compartment whilst avoiding cross-contamination of samples and reagents, and/or allow the addition of agents or solutions of each container from one
30 compartment to another in a quantitative fashion. Such kits may also include a container which will accept a sample to be tested, a container which contains reagents to be used in the assay, containers which contain wash reagents, and containers which contain a detection reagent.

In certain embodiments, the kits comprise one or more polynucleotide substrates of
35 the present invention and a plurality of oligonucleotide partzymes designed to assemble

an MNzyme capable of recognising and catalytically modifying the polynucleotide substrate in the presence of target. The target may act as an assembly facilitator causing assembly of the oligonucleotide partzymes into a catalytically active MNzyme capable of recognising and modifying the polynucleotide substrate.

5 In some embodiments, the kits comprise a polynucleotide substrate of the present invention and an MNzyme comprising two oligonucleotide partzymes. The sequences of the polynucleotide substrate and the oligonucleotide partzymes may be any specific combination of three sequences (as depicted by SEQ ID NOs) that is shown in Table 6, 8, 10, 13, 16, 20, 22 and/or 24.

10 In other embodiments, the kits comprise a polynucleotide substrate of the present invention and a DNzyme. The sequences of the polynucleotide substrate and DNzyme may be any specific pair of sequences (as depicted by SEQ ID NOs) that is shown in Table 15.

Individual oligonucleotide partzymes may be present in the same container.
15 Alternatively, individual oligonucleotide partzyme/s may be present in separate containers. It will be understood that not all components for all MNzymes intended to be used in a given method need necessarily to be provided in a kit as such component/s may be generated as part of a cascade reaction.

In other embodiments, components for additional catalytic nucleic acids with, for
20 example, either cleavage or ligase activity may also form part of the kits of the present invention. In yet other embodiments the kits of the present invention may include DNzymes or components thereof.

Kits of the present invention may include instructions for using the kit components in order to conduct desired methods.

25 Kits and methods of the invention may be used in conjunction with automated analysis equipment and systems including, but not limited to, real time PCR machines.

Kits of the present invention may include additional reagents for conducting target amplification reactions (e.g. PCR) including, for example, oligonucleotide primers, buffers, magnesium ions, polymerase enzymes and the like.

30 Kits of the present invention may comprise one or more assemblies comprising one or more solid supports and one or more polynucleotide substrates of the present invention. One or more of the solid supports may be bound to one or more of the polynucleotide substrates. In certain embodiments, the kits may comprise one or more assemblies comprising a plurality of different solid supports. The plurality of different solid supports

may be bound to a plurality of different polynucleotide substrates of the present invention.

EXAMPLES

In the following examples, the ability of MNazymes based on the 10-23 DNAzyme, and 10-23 DNAzymes, to efficiently cleave universal substrates was tested. The universal substrates tested included some of those previously known in the art (Table 4) and the novel substrates designed according to either all or a subset of the design guidelines of the present invention (Table 5). These examples demonstrate the robustness, as indicated by efficient cleavage in a range of conditions, of universal substrates designed according to all or a subset of the design guidelines of the present invention.

Table 4: Previously known universal substrates used in Examples

Name	Sequence*
Sub2 (SEQ ID NO: 21)	AAGGTTTCCTCguCCCTGGGCA
Sub3 (SEQ ID NO: 22)	CAGCACAACCguCACCAACCG
Sub6 (SEQ ID NO: 23)	ATCACGCCTCguTCCTCCCAG
Sub7 (SEQ ID NO: 24)	TTAACATGGCACguTGGCTGTGATA
Sub4 (SEQ ID NO: 171)	CATGGCGCACguTGGGAGAAGTA

*Uppercase letters indicate DNA, lower case letters indicate RNA.

Table 5: Improved universal substrates from the current invention used in Examples

Name	Sequence*
Sub44 (SEQ ID NO: 25)	CAGGTCTCCTCguCCCTATAGTGA
Sub45 (SEQ ID NO: 26)	ACGGGTCCCguCTCCTTTGGAA
Sub46 (SEQ ID NO: 27)	ACCGCACCTguCCCCAGCTC
Sub49 (SEQ ID NO: 28)	TAAACTTGGCTCguTGGCTGTGATA
Sub55 (SEQ ID NO: 29)	ACCGCACCTCguCCCCAGCTC
Sub59 (SEQ ID NO: 33)	CCTCCTCCCTguCCCTCCTCCT
Sub60 (SEQ ID NO: 30)	GCCAACCACguCCAACACGAC
Sub60T (SEQ ID NO: 72)	TGCCAACCACguCCAACACGAC
Sub61 (SEQ ID NO: 73)	CTCGACCCCguCTCCACGCCA
Sub65 (SEQ ID NO: 74)	TCTCGACCTCguCTCCACGCCA
Sub72 (SEQ ID NO: 75)	ATCACGCCTCguCTCCTCCCAG
Sub73 (SEQ ID NO: 76)	TGGCGTCCCCguCCCCTCGTG

Sub74 (SEQ ID NO: 77)	ATCACTCCCCguCCCCTCCCAG
Sub75 (SEQ ID NO: 78)	TGACCCTCCTCguCTCCCCACTA
Sub77 (SEQ ID NO: 79)	CTCCTCCCTCguCCCTCCTCCT
Sub79 (SEQ ID NO: 80)	TCCTCTCCCCguCCCCTTCAACC
Sub80 (SEQ ID NO: 81)	AACCGCCCTCguCCCGTGAACC
Sub82 (SEQ ID NO: 82)	CTCCTCCCTCguCCCTCGTCCA
Sub83 (SEQ ID NO: 83)	TCCGCTCCCCguCCCCTGCAAC
Sub84 (SEQ ID NO: 84)	ACCGCACCTCguCTCCTCCCAG
Sub85 (SEQ ID NO: 85)	ACCGCACCTCguCCCCTCCCAG
Sub86 (SEQ ID NO: 86)	ATCACGCCTCguCCCCAGCTC
Sub87 (SEQ ID NO: 87)	ATCACTCCCCguCCCCAGCTC
Sub88 (SEQ ID NO: 88)	CTCCTCCCTCguCCCCAGCTC
Sub89 (SEQ ID NO: 89)	ACCGCACCTCguCCCTCCTCCT
Sub90 (SEQ ID NO: 90)	CTCGACCCTCguCCCTCGTCCA
Sub55(16) (SEQ ID NO: 172)	GCACCTCguCCCCAGC
Sub55(18) (SEQ ID NO: 173)	CGCACCTCguCCCCAGCT
Sub55(23A) (SEQ ID NO: 174)	AACCGCACCTCguCCCCAGCTCA
Sub55(23C) (SEQ ID NO: 175)	CACCGCACCTCguCCCCAGCTCC

*Uppercase letters indicate DNA, lower case letters indicate RNA.

Example 1: Use of universal substrates with MNazymes in real-time quantitative PCR (qPCR), at an annealing temperature of 52°C.

5 MNazymes can be used to monitor amplification of target nucleic acids in real-time using *in vitro* target amplification methods such as PCR, referred to as MNzyme qPCR. Further, real-time monitoring during qPCR using MNzyme substrates labelled with fluorophore and quencher pairs generates a curve on which a threshold line, of an arbitrary level of fluorescence, can be placed over the exponential phase of the reactions, producing a value which can be known as a Ct (cycle threshold). Reactions that produce a lower Ct value are indicative of more efficient cleavage of a specific substrate since such reactions reach the threshold cycle faster. In this example amplification and detection are performed in a one-step process, wherein PCR amplification and MNzyme-mediated detection occur simultaneously in a single tube. The amount of time taken to reach the threshold fluorescence, measured by the Ct value generated, can be influenced by the sequence of the universal substrate.

In this example, previously known universal substrates from series 1 (Sub2, Sub3, Sub6 and Sub7, see Table 4) are compared to new improved universal substrates, series 2 (Sub44, Sub45, Sub46, Sub49, Sub55 and Sub60, see Table 5) that are the subject of the

present invention to determine if the series 2 substrates have the same, higher or lower level of activity in real-time PCR as series 1 substrates. The level of activity was determined by the Ct obtained for each reaction containing individual substrates during real-time PCR.

1.1. Partzyme Oligonucleotides

In the experiments conducted to measure the efficiency of cleavage of universal substrates previously known (Table 4) and novel universal substrates (Table 5) in real-time, all the partzyme oligonucleotides A and B were designed with sensor arms complementary to the same sequence of the human RPLPO gene. The sequences of the A and B partzymes are listed below from 5' to 3', where the bases underlined hybridize to their matched substrate. The "-P" indicates 3' phosphorylation of the oligonucleotide.

SEQ ID NO: 1 partzyme A RPLPOA/2-P:

CAAACGAGTCCTGGCCTTGTCTACAACGAGAGGAAACCTT

SEQ ID NO: 2 partzyme B RPLPOB/2-P:

TGCCCAGGGAGGCTAGCTGTGGAGACGGATTACACCTTC

SEQ ID NO: 3 partzyme A RPLPOA/3-P:

CAAACGAGTCCTGGCCTTGTCTACAACGAGGTTGTGCTG

SEQ ID NO: 4 partzyme B RPLPOB/3-P:

CGGTTGGTGAGGCTAGCTGTGGAGACGGATTACACCTTC

SEQ ID NO: 5 partzyme A RPLPOA/6-P:

CAAACGAGTCCTGGCCTTGTCTACAACGAGAGGCGTGAT

SEQ ID NO: 6 partzyme B RPLPOB/6-P:

CTGGGAGGAAGGCTAGCTGTGGAGACGGATTACACCTTC

SEQ ID NO: 7 partzyme A RPLPOA/7-P:

CAAACGAGTCCTGGCCTTGTCTACAACGAGTGCCATGTTAA

SEQ ID NO: 8 partzyme B RPLPOB/7-P:

TATCACAGCCAAGGCTAGCTGTGGAGACGGATTACACCTTC

SEQ ID NO: 9 partzyme A RPLPOA/44-P:

CAAACGAGTCCTGGCCTTGTCTACAACGAGAGGAGACCTG

SEQ ID NO: 10 partzyme B RPLPOB/44-P:

TCACTATAGGGAGGCTAGCTGTGGAGACGGATTACACCTTC

SEQ ID NO: 11 partzyme A RPLPOA/45-P:

CAAACGAGTCCTGGCCTTGTCTACAACGAGGGACCCGT

SEQ ID NO: 12 partzyme B RPLPOB/45-P:

TTCCAAAGGAGAGGCTAGCTGTGGAGACGGATTACACCTTC

SEQ ID NO: 13 partzyme A RPLPOA/46-P:

CAAACGAGTCCTGGCCTTGTCTACAACGAAGGTGCGGT

SEQ ID NO: 14 partzyme B RPLPOB/46-P:

5 GAGCTGGGGAGGCTAGCTGTGGAGACGGATTACACCTTC

SEQ ID NO: 15 partzyme A RPLPOA/49-P:

CAAACGAGTCCTGGCCTTGTCTACAACGAGAGCCAAGTTTA

SEQ ID NO: 16 partzyme A RPLPOA/55-P:

CAAACGAGTCCTGGCCTTGTCTACAACGAGAGGTGCGGT

10 SEQ ID NO: 17 partzyme A RPLPOA/60-P:

CAAACGAGTCCTGGCCTTGTCTACAACGAGTGGTTGGC

SEQ ID NO: 18 partzyme B RPLPOB/60-P:

GTCGTGTTGGAGGCTAGCTGTGGAGACGGATTACACCTTC

15 1.2. Reporter Substrates

The reporter substrates tested in this example are shown below with the sequence, 5' to 3'. The lower case bases represent RNA and the upper case bases represent DNA. In the current example the substrates, other than Sub60, were end labelled with a 6-FAM moiety at the 5' end and a quencher moiety at the 3' end. The quencher molecule was
 20 either Black Hole Quencher 1 (indicated by a "B" in the name of the substrate below) or Iowa Black® FQ (indicated by an "IB" in the name of the substrates below). Sub60 was end labelled with a quencher moiety at the 5' end and a FAM moiety at the 3' end (due to the 5' terminal base being a "G" which is known to quench FAM fluorescence). Cleavage of the substrates was monitored between 510-530 nm (FAM emission wavelength range
 25 on CFX96 (BioRad)) with excitation between 450-490 nm (FAM excitation wavelength range on CFX96 (BioRad)).

SEQ ID NO: 21 Sub2-FIB:

AAGGTTTCCTCguCCCTGGGCA

SEQ ID NO: 22 Sub3-FB:

30 CAGCACAACCGuACCAACCG

SEQ ID NO: 23 Sub6-FIB:

ATCACGCCTCguTCCTCCCAG

SEQ ID NO: 24 Sub7-FB:

TTAACATGGCACguTGGCTGTGATA

35 SEQ ID NO: 25 Sub44-FIB:

CAGGTCTCCTCguCCCTATAGTGA

SEQ ID NO: 26 Sub45-FIB:

ACGGGTCCCguCTCCTTTGGAA

SEQ ID NO: 27 Sub46-FIB:

5 ACCGCACCTguCCCCAGCTC

SEQ ID NO: 28 Sub49-FB:

TAAACTTGGCTCguTGGCTGTGATA

SEQ ID NO: 29 Sub55-FIB:

ACCGCACCTCguCCCCAGCTC

10 SEQ ID NO: 30 Sub60-IBF:

GCCAACCACguCCAACACGAC

1.3. PCR primers for amplification of RPLPO

The target PCR amplicon for this example was generated by *in vitro* PCR
15 amplification of human genomic DNA using the oligonucleotide PCR primers listed
below. Primer sequences are written 5' to 3'.

SEQ ID NO: 31 Forward primer 5RPLPO:

CCCATTCTATCATCAACGGGTA

SEQ ID NO: 32 Reverse primer 3RPLPO:

20 GCCCACTGTGGTCCTGGTG

1.4. Target sequence

The target sequence for this example was a PCR amplicon of the RPLPO gene
generated by *in vitro* PCR amplification of human genomic DNA extracted from K562
25 cells.

1.5. Reaction Components: Amplification and detection of a target sequence

Real-time PCR amplification and detection of the target sequence was performed in
a total reaction volume of 25 μ L. All reactions were conducted in a CFX96 Real-Time
30 PCR Detection System (Bio-Rad). The cycling parameters were 95°C for 10 minutes, 10
cycles of 95°C for 15 seconds and 60°C for 30 seconds (-1°C per cycle for the latter
temperature), 40 cycles of 95°C for 15 seconds and 52°C for 60 seconds (data collected at
the 52°C step). Reactions were set up with substrates and their associated partzymes as in
Table 6. Each set of reaction conditions were run in duplicate and contained 80 nM
35 5RPLPO and 400 nM of 3RPLPO, 200 nM each of partzyme A and partzyme B, 200 nM

of substrate, 8 mM MgCl₂, 200 μM of each dNTP, 10 units RiboSafe RNase inhibitor (Bioline), 1 x Immobuffer (Bioline), 2 units of Immolase (Bioline) and either genomic DNA template (50 ng) or no-DNA target (nuclease free H₂O (NF-H₂O)). Separate reactions were set up to test each substrate with its matched partzymes. The same PCR primers were used for all reactions and all partzymes had the same target-sensing portions. Any differences in efficiency of reactions will therefore be attributable to differences in the efficiency of cleavage of the substrates.

Table 6: Partzyme combinations used for each universal substrate

Substrate	Partzyme A	Partzyme B
Sub2 SEQ ID NO: 21	RPLPOA/2-P SEQ ID NO: 1	RPLPOB/2-P SEQ ID NO: 2
Sub3 SEQ ID NO: 22	RPLPOA/3-P SEQ ID NO: 3	RPLPOB/3-P SEQ ID NO: 4
Sub6 SEQ ID NO: 23	RPLPOA/6-P SEQ ID NO: 5	RPLPOB/6-P SEQ ID NO: 6
Sub7 SEQ ID NO: 24	RPLPOA/7-P SEQ ID NO: 7	RPLPOB/7-P SEQ ID NO: 8
Sub44 SEQ ID NO: 25	RPLPOA/44 SEQ ID NO: 9	RPLPOB/44-P SEQ ID NO: 10
Sub45 SEQ ID NO: 26	RPLPOA/45-P SEQ ID NO: 11	RPLPOB/45-P SEQ ID NO: 12
Sub46 SEQ ID NO: 27	RPLPOA/46-P SEQ ID NO: 13	RPLPOB/46-P SEQ ID NO: 14
Sub49 SEQ ID NO: 28	RPLPOA/49-P SEQ ID NO: 15	RPLPOB/7-P SEQ ID NO: 8
Sub55 SEQ ID NO: 29	RPLPOA/55-P SEQ ID NO: 16	RPLPOB/46-P SEQ ID NO: 14
Sub60 SEQ ID NO: 30	RPLPOA/60-P SEQ ID NO: 17	RPLPOB/60-P SEQ ID NO: 18

1.6. Results: Amplification of target and cleavage of reporter substrate

Each MNzyme qPCR reaction containing human genomic DNA, with each different substrate, showed an increase in fluorescence over time for the real-time detection of RPLPO from human genomic DNA. For all substrates, the fluorescence of the no-DNA target control was lower than that in the DNA target-containing reactions. This demonstrates that the increase in fluorescence produced in target-containing reactions is due to target dependent assembly of catalytically active MNzymes that then cleaved one of the universal substrates.

The series 1 and 2 substrates all crossed the threshold producing a Ct value, as seen in **Table 7**. The series 1 substrates had Ct values in the range from 16.9 (Sub6) to 18.4

(Sub3 and Sub7) and the series 2 substrates had Ct values in the range of 17.1 (Sub55) to 19.2 (Sub45). This indicates that the series 2 substrates are highly active and very comparable to series 1 substrates under the reaction conditions tested. These results demonstrate that, on average, the substrates that were cleaved with the greatest efficiency (i.e. lowest Ct) were those with a higher number of pyrimidines in the eight bases surrounding the ribonucleotides in the substrate (underlined in Table 7).

Table 7: Efficiency of cleavage of universal substrates (listed in order of cleavage efficiency based on Ct)

Name	Sequence [^]	# pyrimidines in 8 bases surrounding ribonucleotides	Tm*	Ct
Sub6	ATCAGCCTC <u>gu</u> TCCTCCCAG SEQ ID NO: 23	8	64	16.9
Sub2	AAGGTTT <u>TCCTCgu</u> CCCTGGGCA SEQ ID NO: 21	8	66	17.0
Sub55	ACCGCACCTC <u>gu</u> CCCCAGCTC SEQ ID NO: 29	8	68	17.1
Sub44	CAGGTCTCCTC <u>gu</u> CCCTATAGTGA SEQ ID NO: 25	8	70	18.0
Sub46	ACCGCACCT <u>gu</u> CCCCAGCTC SEQ ID NO: 27	7	64	18.1
Sub60	GCCAACCAC <u>gu</u> CCAACACGAC SEQ ID NO: 30	5	64	18.3
Sub3	CAGCACAACC <u>gu</u> CACCAACCG SEQ ID NO: 22	5	64	18.4
Sub7	TTAACATGGCAC <u>gu</u> TGGCTGTGATA SEQ ID NO: 24	4	68	18.4
Sub49	TAAACTTGGCTC <u>gu</u> TGGCTGTGATA SEQ ID NO: 28	5	68	18.5
Sub45	ACGGGTCCC <u>gu</u> CTCCTTTGGAA SEQ ID NO: 26	8	66	19.2~

[^] Uppercase represent DNA and lowercase represent RNA

* Tm given here equates to the melting temperature of the bases bound to the two partzymes calculated using the Wallace rule. When the substrate is bound to the MNAzyme based on the 10-23 DNAzyme the "g" ribonucleotide remains unbound therefore does not contribute to the overall bound Tm.

~ Only one replicate due to experimental error.

Example 2: Use of universal substrates with MNAzymes qPCR at an annealing temperature of 58°C.

MNAzymes can be used to monitor amplification of target nucleic acids in real-time using *in vitro* target amplification methods such as PCR. Furthermore, real-time monitoring during qPCR using MNAzyme substrates labelled with fluorophore and

quencher pairs generates a curve on which a threshold line, of an arbitrary level of fluorescence, can be placed over the exponential phase of the reactions, producing a value which can be known as a Ct (cycle threshold). Reactions that produce a lower Ct value are indicative of more efficient cleavage of a specific substrate since such reactions reach the threshold cycle faster. In this example, amplification and detection are performed in a one-step process, wherein PCR amplification and MNazyme-mediated detection occur simultaneously in a single tube. Where all other reaction conditions are the same the Ct value can be influenced by the sequence of the universal substrate. The annealing/detection temperature for MNazyme qPCR used in the art is between 50 and 54°C. This temperature was dictated by the fact that the universal substrates known in the art had a limitation on the temperature at which they were efficiently cleaved with 54°C being the upper limit for the series 1 universal substrates. There is a need for universal substrates that cleave at higher temperatures to allow greater flexibility in design of primers and partzymes that anneal at higher temperatures. This design flexibility for primers and partzymes could be of great benefit for many applications such as genetic targets of interest that have high percentages of G and C bases in their sequence, requiring higher reaction temperatures and hence partzymes and primers with higher Tms for specific detection.

Investigation into efficiency of cleavage of substrates based on the performance of the series 1 and 2 substrates, lead to the development of guidelines to aid in a third round of substrate designs, resulting in the series 3 substrates. These guidelines included but were not limited to (i) seven or more cytosine nucleotides in the ten bases surrounding the ribonucleotides (N₄-N₁₃), (ii) bases immediately adjacent to the ribonucleotides are cytosines (N₈ and N₉) (iii) total content of substrate has >64% pyrimidines and (iv) total Tm of the oligonucleotide is 66°C or greater (where this latter guideline is only applicable if the reaction temperature for substrate cleavage is above 50°C).

In this example, the series 1 universal substrates (Sub2, Sub3 and Sub6) are compared to the series 2 universal substrates (Sub44, Sub 45, Sub46, Sub60T and Sub55), and the series 3 substrates (Sub61, Sub65, Sub72, Sub73, Sub74, Sub75, Sub77, Sub79, Sub80, Sub82, Sub83, Sub84, Sub85, Sub86, Sub87, Sub88, Sub89 and Sub90) to compare the cleavage efficiency of all substrates in real-time PCR at 58°C to ensure that the design guidelines produce universal substrates with a high probability of applicability to MNazyme qPCR at an elevated temperature. The level of cleavage efficiency was determined by measuring the Ct value for reactions containing different universal substrates.

2.1. Partzyme Oligonucleotides

In the experiments conducted to measure the efficiency of cleavage of the series 1, 2 and 3 universal substrates in real-time PCR, all the partzyme oligonucleotides A and B were designed with sensor arms complementary to the same sequence of the human TFRC gene. The sequences of the A and B partzymes are listed below from 5' to 3', where the bases underlined hybridize to their matched universal substrate. The "-P" indicates 3' phosphorylation of the oligonucleotide.

SEQ ID NO: 34 partzyme A TFRCA/2-P:
 10 GGAATATGGAAGGAGACTGTCACAACGAGAGGAAACCTT
 SEQ ID NO: 35 partzyme B TFRCB/2-P:
 TGCCCAGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
 SEQ ID NO: 36 partzyme A TFRCA/3-P:
 GGAATATGGAAGGAGACTGTCACAACGAGGTTGTGCTG
 15 SEQ ID NO: 37 partzyme B TFRCB/3-P:
 CGGTTGGTGAGGCTAGCTCCTCTGACTGGAAAACAGACT
 SEQ ID NO: 38 partzyme A TFRCA/6-P:
 GGAATATGGAAGGAGACTGTCACAACGAGAGGCGTGAT
 SEQ ID NO: 39 partzyme B TFRCB/6-P:
 20 CTGGGAGGAAGGCTAGCTCCTCTGACTGGAAAACAGACT
 SEQ ID NO: 40 partzyme A TFRCA/44-P:
 GGAATATGGAAGGAGACTGTCACAACGAGAGGAGACCTG
 SEQ ID NO: 41 partzyme B TFRCB/44-P:
 TCACTATAGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
 25 SEQ ID NO: 42 partzyme A TFRCA/45-P:
 GGAATATGGAAGGAGACTGTCACAACGAGGGACCCGT
 SEQ ID NO: 43 partzyme B TFRCB/45-P:
 TTCCAAAGGAGAGGCTAGCTCCTCTGACTGGAAAACAGACT
 SEQ ID NO: 44 partzyme A TFRCA/46-P:
 30 GGAATATGGAAGGAGACTGTCACAACGAAGGTGCGGT
 SEQ ID NO: 45 partzyme B TFRCB/46-P:
 GAGCTGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
 SEQ ID NO: 46 partzyme A TFRCA/55-P:
 GGAATATGGAAGGAGACTGTCACAACGAGAGGTGCGGT
 35 SEQ ID NO: 48 partzyme A TFRCA/60-P:

GGAATATGGAAGGAGACTGTCACAACGAGTGGTTGGC
SEQ ID NO: 49 partzyme B TFRCB/60-P:
GTCGTGTTGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 50 partzyme A TFRCA/61-P:
5 GGAATATGGAAGGAGACTGTCACAACGAGGGGTTCGAG
SEQ ID NO: 51 partzyme B TFRCB/61-P:
TGGCGTGGAGAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 52 partzyme A TFRCA/65-P:
GGAATATGGAAGGAGACTGTCACAACGAGAGGTTCGAGA
10 SEQ ID NO: 55 partzyme B TFRCB/72-P:
CTGGGAGGAGAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 56 partzyme A TFRCA/73-P:
GGAATATGGAAGGAGACTGTCACAACGAGGGGACGCCA
SEQ ID NO: 57 partzyme B TFRCB/73-P:
15 CACGAGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 58 partzyme A TFRCA/74-P:
GGAATATGGAAGGAGACTGTCACAACGAGGGGAGTGAT
SEQ ID NO: 59 partzyme B TFRCB/74-P:
CTGGGAGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
20 SEQ ID NO: 60 partzyme A TFRCA/75-P:
GGAATATGGAAGGAGACTGTCACAACGAGAGGAGGGTCA
SEQ ID NO: 61 partzyme B TFRCB/75-P:
TAGTGGGGAGAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 62 partzyme A TFRCA/77-P:
25 GGAATATGGAAGGAGACTGTCACAACGAGAGGGAGGAG
SEQ ID NO: 63 partzyme B TFRCB/77-P:
AGGAGGAGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 64 partzyme A TFRCA/79-P:
GGAATATGGAAGGAGACTGTCACAACGAGGGGAGAGGA
30 SEQ ID NO: 65 partzyme B TFRCB/79-P:
GGTTGAAGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 66 partzyme A TFRCA/80-P:
GGAATATGGAAGGAGACTGTCACAACGAGAGGGCGGTT
SEQ ID NO: 67 partzyme B TFRCB/80-P:
35 GGTTCACGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT

SEQ ID NO: 68 partzyme B TFRCB/82-P:

TGGACGAGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT

SEQ ID NO: 69 partzyme A TFRCA/83-P:

GGAATATGGAAGGAGACTGTCACAACGAGGGGAGCGGA

5 SEQ ID NO: 70 partzyme B TFRCB/83-P:

GTTGCAGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT

SEQ ID NO: 71 partzyme A TFRCA/90-P:

GGAATATGGAAGGAGACTGTCACAACGAGAGGGTCGAG

10 2.2. Reporter Substrates

The reporter substrates for this example are shown below with the sequence, 5' to 3'. The lower case bases represent RNA and the upper case bases represent DNA. In the current example, the substrates were end labelled with a 6-FAM moiety at the 5' end (indicated by a "F" in the name of the substrates below) and an Iowa Black® FQ quencher moiety at the 3' end (indicated by a "IB" in the name of the substrates below). The sequence of Sub60 was been modified to include a "T" at the 5' end, this enabled it to be 5' end-labelled with 6-FAM. The partzyme A substrate binding sequence has not changed and therefore cleavage efficiency is comparable to the Sub60 sequence in Example 1 which lacks the extra "T" at the 5' end. Cleavage of the substrates was monitored between 510-530 nm (FAM emission wavelength range on CFX96 (BioRad)) with 15 excitation between 450-490 nm (FAM excitation wavelength range on CFX96 (BioRad)).

SEQ ID NO: 21 Sub2-FIB:

AAGGTTTCCTCguCCCTGGGCA

SEQ ID NO: 22 Sub3-FIB:

25 CAGCACAACCGuACCAACCG

SEQ ID NO: 23 Sub6-FIB:

ATCACGCCTCguTCCTCCCAG

SEQ ID NO: 25 Sub44-FIB:

CAGGTCTCCTCguCCCTATAGTGA

30 SEQ ID NO: 26 Sub45-FIB:

ACGGGTCCCguCTCCTTTGGAA

SEQ ID NO: 27 Sub46-FIB:

ACCGCACCTguCCCCAGCTC

SEQ ID NO: 29 Sub55-FIB:

35 ACCGCACCTCguCCCCAGCTC

SEQ ID NO: 72 Sub60T-FIB:
TGCCAACCACguCCAACACGAC
SEQ ID NO: 73 Sub61-FIB:
CTCGACCCCguCTCCACGCCA
5 SEQ ID NO: 74 Sub65-FIB:
TCTCGACCTCguCTCCACGCCA
SEQ ID NO: 75 Sub72-FIB:
ATCACGCCTCguCTCCTCCCAG
SEQ ID NO: 76 Sub73-FIB:
10 TGGCGTCCCCguCCCCTCGTG
SEQ ID NO: 77 Sub74-FIB:
ATCACTCCCCguCCCCTCCCAG
SEQ ID NO: 78 Sub75-FIB:
TGACCCTCCTCguCTCCCCACTA
15 SEQ ID NO: 79 Sub77-FIB:
CTCCTCCCTCguCCCTCCTCCT
SEQ ID NO: 80 Sub79-FIB:
TCCTCTCCCCguCCCCTTCAACC
SEQ ID NO: 81 Sub80-FIB:
20 AACCGCCCTCguCCCGTGAACC
SEQ ID NO: 82 Sub82-FIB:
CTCCTCCCTCguCCCTCGTCCA
SEQ ID NO: 83 Sub83-FIB:
TCCGCTCCCCguCCCCTGCAAC
25 SEQ ID NO: 84 Sub84-FIB:
ACCGCACCTCguCTCCTCCCAG
SEQ ID NO: 85 Sub85-FIB:
ACCGCACCTCguCCCCTCCCAG
SEQ ID NO: 86 Sub86-FIB:
30 ATCACGCCTCguCCCCAGCTC
SEQ ID NO: 87 Sub87-FIB:
ATCACTCCCCguCCCCAGCTC
SEQ ID NO: 88 Sub88-FIB:
CTCCTCCCTCguCCCCAGCTC
35 SEQ ID NO: 89 Sub89-FIB:

ACCGCACCTCguCCCTCCTCCT

SEQ ID NO: 90 Sub90-FIB:

CTCGACCCTCguCCCTCGTCCA

2.3. Target sequence and PCR primers for amplification of TFRC

The target sequence for this example was a PCR amplicon from the TFRC gene generated by *in vitro* amplification of human genomic DNA, extracted from the IM9 cell line (Promega), using the oligonucleotide PCR primers listed below. The sequence in bold in the primer sequences corresponds to a universal tag (U1 or U2) that increases the T_m of the primer without affecting the specificity of the primer to the gene target. This tag improves amplification efficiency in PCR reactions. Primer sequences are listed 5' to 3'.

SEQ ID NO: 91 Forward primer 5TFRC_U1:

GCTAAAACAATAACTCAGAACTTACG

SEQ ID NO: 92 Reverse primer 3TFRC_U2:

CAGCTTTCTGAGGTTACCATCCTA

2.4. Reaction Components: Amplification and quantification of target sequence

Real-time PCR amplification and detection of the target sequence was performed in a total reaction volume of 25 μ L. All reactions were conducted in a CFX96 Real-Time PCR Detection System (Bio-Rad). Reactions were set up with substrates and their associated partzymes as in Table 8. The cycling parameters were, 95°C for 2 minutes, 50 cycles of 95°C for 15 seconds and 58°C for 60 seconds (data collected at the 58°C step). Each set of reaction conditions were run in duplicate and contained 40 nM 5TFRC_U1, 200 nM of 3TFRC_U2, 200 nM each of partzyme A and partzyme B, 200 nM substrate, 8 mM $MgCl_2$, 200 μ M of each dNTP, 10 units RiboSafe RNase inhibitor (Bioline), 1 x Immobuffer (Bioline), 2 units of MyTaqHS™ DNA polymerase (Bioline) and either genomic DNA template (50 ng) or no target (NF-H₂O).

Table 8: Partzyme combinations used for each universal substrate

Substrate	Partzyme A	Partzyme B
Sub2 SEQ ID NO: 21	TFRCA/2-P SEQ ID NO: 34	TFRCB/2-P SEQ ID NO: 35
Sub3 SEQ ID NO: 22	TFRCA/3-P SEQ ID NO: 36	TFRCB/3-P SEQ ID NO: 37
Sub6	TFRCA/6-P	TFRCB/6-P

SEQ ID NO: 23	SEQ ID NO: 38	SEQ ID NO: 39
Sub44	TFRCA/44	TFRCB/44-P
SEQ ID NO: 25	SEQ ID NO: 40	SEQ ID NO: 41
Sub45	TFRCA/45-P	TFRCB/45-P
SEQ ID NO: 26	SEQ ID NO: 42	SEQ ID NO: 43
Sub46	TFRCA/46-P	TFRCB/46-P
SEQ ID NO: 27	SEQ ID NO: 44	SEQ ID NO: 45
Sub55	TFRCA/55-P	TFRCB/46-P
SEQ ID NO: 29	SEQ ID NO: 46	SEQ ID NO: 45
Sub60T	TFRCA/60-P	TFRCB/60-P
SEQ ID NO: 72	SEQ ID NO: 48	SEQ ID NO: 49
Sub61	TFRCA/61-P	TFRCB/61-P
SEQ ID NO: 73	SEQ ID NO: 50	SEQ ID NO: 51
Sub65	TFRCA/65-P	TFRCB/61-P
SEQ ID NO: 74	SEQ ID NO: 52	SEQ ID NO: 51
Sub72	TFRCA/6-P	TFRCB/72-P
SEQ ID NO: 75	SEQ ID NO: 38	SEQ ID NO: 55
Sub73	TFRCA/73-P	TFRCB/73-P
SEQ ID NO: 76	SEQ ID NO: 56	SEQ ID NO: 57
Sub74	TFRCA/74-P	TFRCB/74-P
SEQ ID NO: 77	SEQ ID NO: 58	SEQ ID NO: 59
Sub75	TFRCA/75-P	TFRCB/75-P
SEQ ID NO: 78	SEQ ID NO: 60	SEQ ID NO: 61
Sub77	TFRCA/77-P	TFRCB/77-P
SEQ ID NO: 79	SEQ ID NO: 62	SEQ ID NO: 63
Sub79	TFRCA/79-P	TFRCB/79-P
SEQ ID NO: 80	SEQ ID NO: 64	SEQ ID NO: 65
Sub80	TFRCA/80-P	TFRCB/80-P
SEQ ID NO: 81	SEQ ID NO: 66	SEQ ID NO: 67
Sub82	TFRCA/77-P	TFRCB/82-P
SEQ ID NO: 82	SEQ ID NO: 62	SEQ ID NO: 68
Sub83	TFRCA/83-P	TFRCB/83-P
SEQ ID NO: 83	SEQ ID NO: 69	SEQ ID NO: 70
Sub84	TFRCA/55-P	TFRCB/72-P
SEQ ID NO: 84	SEQ ID NO: 46	SEQ ID NO: 55
Sub85	TFRCA/55-P	TFRCB/74-P
SEQ ID NO: 85	SEQ ID NO: 46	SEQ ID NO: 59
Sub86	TFRCA/6-P	TFRCB/46-P
SEQ ID NO: 86	SEQ ID NO: 38	SEQ ID NO: 45
Sub87	TFRCA/74-P	TFRCB/46-P
SEQ ID NO: 87	SEQ ID NO: 58	SEQ ID NO: 45
Sub88	TFRCA/77-P	TFRCB/46-P
SEQ ID NO: 88	SEQ ID NO: 62	SEQ ID NO: 45
Sub89	TFRCA/55-P	TFRCB/77-P
SEQ ID NO: 89	SEQ ID NO: 46	SEQ ID NO: 63
Sub90	TFRCA/90-P	TFRCB/82-P
SEQ ID NO: 90	SEQ ID NO: 71	SEQ ID NO: 68

2.5. Results: Amplification of target and cleavage of reporter substrate

Each MNzyme qPCR reaction containing human genomic DNA showed an increase in fluorescence over time for the real-time detection of TFRC from human genomic DNA. For all reactions the fluorescence of the no-DNA target control was lower than that in the DNA target-containing reactions. This demonstrates that the increase in fluorescence produced in target-containing reactions is due to target dependent assembly of catalytically active MNzymes that then cleaved one of the universal reporter substrates.

Comparison of the Ct values for each universal substrate (**Figure 5** and **Table 9**) show the series 1 substrates (Sub2, Sub3 and Sub6) and series 2 substrates (Sub44, Sub45, Sub46 and Sub60T) all have Ct values > 27, whereas the other series 2 and all series 3 substrates tested (Sub55, Sub61, Sub65, Sub72, Sub73, Sub74, Sub75, Sub77, Sub79, Sub80, Sub82, Sub83, Sub84, Sub85, Sub86, Sub87, Sub88, Sub89 and Sub90) had Ct values less than 27. This indicates that the latter series 2 universal substrate and all series 3 universal substrates tested showed increased efficiency of the MNzyme cleavage reaction at a higher annealing/detection temperature than that previously possible for MNzyme qPCR. This improved efficiency of cleavage now permits efficient and robust detection of target using MNzyme qPCR at a higher temperature than previously possible. This may also prove beneficial when using DNA polymerase formulations that require a higher temperature for amplification.

Of note is the importance of the nature of the nucleotide sequence of these efficiently cleaved substrates and the proximity of specific nucleotides to the ribonucleotides of the substrates. These features form the basis of a set of guidelines that result in universal substrates with a higher probability of being cleaved efficiently at elevated temperatures. These design guidelines include but are not limited to (not all may be necessary): (i) seven or more cytosine nucleotides in the ten bases surrounding the ribonucleotides (N₄-N₁₃); (ii) the bases immediately adjacent to the ribonucleotides are cytosines (N₈ and N₉); (iii) total content of substrate has >64% pyrimidine's; (iv) total T_m of the oligonucleotide is 66°C or greater (where this latter guideline is only applicable if the reaction temperature for substrate cleavage is above 50°C) (**Table 9**). In addition, it was observed that a low number of guanine nucleotides (e.g. three, two, one or none) in the 10 bases surrounding the ribonucleotides is also beneficial.

All universal substrates in **Figure 5** that had at Ct < 27 at an annealing temperature of 58°C obeyed three or more of these design guidelines (**Table 9**).

Table 9: Efficiency of cleavage of universal substrates (listed in order of cleavage efficiency based on Ct)

Name	Sequence [^]	# C's N ₄ -N ₁₃	CguC	%C/T ⁺	Tm [*]	Ct	# (i – iv) [~]
Sub80	AACCGCCCTCguCCCGTGAACC SEQ ID NO: 81	7	Yes	65	70	25.2	4
Sub55	ACCGCACCTCguCCCCAGCTC SEQ ID NO: 29	7	Yes	74	68	25.4	4
Sub85	ACCGCACCTCguCCCCCTCCCAG SEQ ID NO: 85	7	Yes	75	72	25.4	4
Sub79	TCCTCTCCCCguCCCCCTCCTACC SEQ ID NO: 80	8	Yes	90	72	25.5	4
Sub61	CTCGACCCCGuCTCCACGCCA SEQ ID NO: 73	7	Yes	74	68	25.5	4
Sub65	TCTCGACCTCguCTCCACGCCA SEQ ID NO: 74	6	Yes	75	68	25.5	3
Sub89	ACCGCACCTCguCCCTCCTCCT SEQ ID NO: 89	7	Yes	85	70	25.7	4
Sub90	CTCGACCCCTCguCCCTCGTCCA SEQ ID NO: 90	8	Yes	80	70	25.7	4
Sub87	ATCACTCCCCguCCCCAGCTC SEQ ID NO: 87	8	Yes	79	66	25.7	4
Sub83	TCCGCTCCCCguCCCCCTGCAAC SEQ ID NO: 83	8	Yes	80	72	25.8	4
Sub86	ATCACGCCTCguCCCCAGCTC SEQ ID NO: 86	7	Yes	74	66	25.9	4
Sub88	CTCCTCCCTCguCCCCAGCTC SEQ ID NO: 88	8	Yes	89	68	26.2	4
Sub82	CTCCTCCCTCguCCCTCGTCCA SEQ ID NO: 82	8	Yes	90	70	26.2	4
Sub74	ATCACTCCCCguCCCCCTCCCAG SEQ ID NO: 77	8	Yes	80	70	26.2	4
Sub84	ACCGCACCTCguCTCCTCCCAG SEQ ID NO: 84	6	Yes	75	70	26.3	3
Sub75	TGACCCCTCCTCguCTCCCCACTA SEQ ID NO: 78	7	Yes	81	70	26.3	4
Sub73	TGGCGTCCCCguCCCCCTCGTG SEQ ID NO: 76	8	Yes	74	70	26.3	4
Sub77	CTCCTCCCTCguCCCTCCTCCT SEQ ID NO: 79	8	Yes	100	70	26.3	4
Sub72	ATCACGCCTCguCTCCTCCCAG SEQ ID NO: 75	6	Yes	75	68	26.9	3
Sub2	AAGGTTTCCTCguCCCTGGGCA SEQ ID NO: 21	6	Yes	60	66	27.2	2
Sub60T [§]	TGCCAACACguCCAACACGAC SEQ ID NO: 72	6	Yes	53	64	27.3	2
Sub46	ACCGCACCTguCCCCAGCTC SEQ ID NO: 27	7	No	72	64	27.5	2
Sub45	ACGGGTCCCguCTCCTTTGGAA SEQ ID NO: 26	6	Yes	60	66	27.6	2
Sub3	CAGCACAACCguCACCAACCG SEQ ID NO: 22	6	Yes	53	64	28.2	1
Sub44	CAGTCTCCTCguCCCTATAGTGA SEQ ID NO: 25	6	Yes	64	70	28.4	2
Sub6	ATCACGCCTCguTCCTCCCAG SEQ ID NO: 23	6	No	74	64	29.0	1

^ uppercase bases represent DNA and lowercase bases represent RNA and position of base in a substrate is represented by (N_x)-N₁-N₂-N₃-N₄-N₅-N₆-N₇-N₈-rR-rY-N₉-N₁₀-N₁₁-N₁₂-N₁₃-N₁₄-N₁₅-(N_x)

+ % C/T (pyrimidines) of sequence length shown above for each substrate, does not include ribonucleotides

* T_m given here equates to the melting temperature of the bound bases calculated using the Wallace rule – only calculated for bases that hybridize to their complement. When the substrate is bound to the MNAzyme based on the 10-23 DNAzyme the “g” ribonucleotide remains unbound therefore does not contribute to the overall bound T_m.

~ The number of the design guidelines (i), (ii), (iii) and/or (iv) that have been met by the substrate sequence.

§ The additional “T” in Sub60T is not bound by a partzyme arm and is therefore not included in the calculations of % C/T and T_m.

Example 3: Use of universal substrates with MNAzymes in a format for direct detection of a nucleic acid target.

Investigation into efficiency of cleavage of substrates based on the performance of the series 1 and 2 substrates, lead to the development of guidelines to aid in designing a third round of substrates, series 3. These guidelines included but were not limited to (i) seven or more cytosine nucleotides in the ten bases surrounding the ribonucleotides (N₄-N₁₃), (ii) bases immediately adjacent to the ribonucleotides are cytosines (N₈ and N₉) (iii) total content of substrate has >64% pyrimidines and (iv) total T_m of the oligonucleotide is 66°C or greater (where this latter guideline is only applicable if the reaction temperature for substrate cleavage is above 50°C).

MNAzymes can be used to directly detect target nucleic acids in an isothermal reaction without any target amplification. This method of direct target detection can be used to assess the efficiency of cleavage of substrates. Partzymes were designed to test the efficiency of cleavage of a range of universal substrates when coupled with direct detection of the gene TFRC at a range of temperatures. In this example, the previously known series 1 universal substrates (Sub2, Sub3, and Sub6) were compared to the series 2 universal substrates (Sub44, Sub45, Sub46, Sub49, Sub55 and Sub60T) and series 3 substrates (Sub61, Sub65, Sub72, Sub73, Sub74, Sub75, Sub77, Sub79, Sub80, Sub82, Sub83, Sub84, Sub85, Sub86, Sub87, Sub88, Sub89 and Sub90) to determine if the design guidelines derived from analyses of series 1 and 2 substrates would be useful in the development of series 3 substrates that are cleaved with the same or a higher level of activity as substrates from series 1 and 2. The level of cleavage efficiency was determined by calculating the signal to noise ratio (from results of a “test” reaction containing template and a no template control reaction) after 10 minutes over a temperature range of 52, 54, 56 and 58°C. The standard deviation of the signal to noise ratios over this

temperature range was also calculated as a measure of robustness of the substrates with regards to temperature.

3.1. Partzyme Oligonucleotides

In the experiments conducted to measure the cleavage efficiency of series 1, 2 and 3 universal substrates described in **Tables 4 and 5** using direct target detection, all the partzyme oligonucleotides A and B were designed with sensor arms complementary to the same sequence of the human TFRCA gene. The sequences of the A and B partzymes are listed below from 5' to 3', where the bases underlined hybridize to the substrate. The "P" indicates 3' phosphorylation of the oligonucleotide.

SEQ ID NO: 34 partzyme A TFRCA/2-P:

GGAATATGGAAGGAGACTGTCACAACGAGAGGAAACCTT

SEQ ID NO: 35 partzyme B TFRCA/2-P:

TGCCCAGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT

SEQ ID NO: 36 partzyme A TFRCA/3-P:

GGAATATGGAAGGAGACTGTCACAACGAGGTTGTGCTG

SEQ ID NO: 37 partzyme B TFRCA/3-P:

CGGTTGGTGAGGCTAGCTCCTCTGACTGGAAAACAGACT

SEQ ID NO: 38 partzyme A TFRCA/6-P:

GGAATATGGAAGGAGACTGTCACAACGAGAGGCGTGAT

SEQ ID NO: 39 partzyme B TFRCA/6-P:

CTGGGAGGAAGGCTAGCTCCTCTGACTGGAAAACAGACT

SEQ ID NO: 40 partzyme A TFRCA/44-P:

GGAATATGGAAGGAGACTGTCACAACGAGAGGAGACCTG

SEQ ID NO: 41 partzyme B TFRCA/44-P:

TCACTATAGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT

SEQ ID NO: 42 partzyme A TFRCA/45-P:

GGAATATGGAAGGAGACTGTCACAACGAGGGACCCGT

SEQ ID NO: 43 partzyme B TFRCA/45-P:

TTCCAAAGGAGAGGCTAGCTCCTCTGACTGGAAAACAGACT

SEQ ID NO: 44 partzyme A TFRCA/46-P:

GGAATATGGAAGGAGACTGTCACAACGAAGGTGCGGT

SEQ ID NO: 45 partzyme B TFRCA/46-P:

GAGCTGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT

SEQ ID NO: 93 partzyme A TFRCA/49-P:

GGAATATGGAAGGAGACTGTCACAACGAGAGCCAAGTTTA
SEQ ID NO: 94 partzyme B TFRCB/49-P:
TATCACAGCCAAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 46 partzyme A TFRCA/55-P:
5 GGAATATGGAAGGAGACTGTCACAACGAGAGGTGCGGT
SEQ ID NO: 48 partzyme A TFRCA/60-P:
GGAATATGGAAGGAGACTGTCACAACGAGTGGTTGGC
SEQ ID NO: 49 partzyme B TFRCB/60-P:
GTCGTGTTGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
10 SEQ ID NO: 50 partzyme A TFRCA/61-P:
GGAATATGGAAGGAGACTGTCACAACGAGGGGTTCGAG
SEQ ID NO: 51 partzyme B TFRCB/61-P:
TGGCGTGGAGAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 52 partzyme A TFRCA/65-P:
15 GGAATATGGAAGGAGACTGTCACAACGAGAGGTTCGAGA
SEQ ID NO: 55 partzyme B TFRCB/72-P:
CTGGGAGGAGAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 56 partzyme A TFRCA/73-P:
GGAATATGGAAGGAGACTGTCACAACGAGGGGACGCCA
20 SEQ ID NO: 57 partzyme B TFRCB/73-P:
CACGAGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 58 partzyme A TFRCA/74-P:
GGAATATGGAAGGAGACTGTCACAACGAGGGGAGTGAT
SEQ ID NO: 59 partzyme B TFRCB/74-P:
25 CTGGGAGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 60 partzyme A TFRCA/75-P:
GGAATATGGAAGGAGACTGTCACAACGAGAGGAGGGTCA
SEQ ID NO: 61 partzyme B TFRCB/75-P:
TAGTGGGAGAGGCTAGCTCCTCTGACTGGAAAACAGACT
30 SEQ ID NO: 62 partzyme A TFRCA/77-P:
GGAATATGGAAGGAGACTGTCACAACGAGAGGGAGGAG
SEQ ID NO: 63 partzyme B TFRCB/77-P:
AGGAGGAGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 64 partzyme A TFRCA/79-P:
35 GGAATATGGAAGGAGACTGTCACAACGAGGGGAGAGGA

SEQ ID NO: 65 partzyme B TFRCB/79-P:
GGTTGAAGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
 SEQ ID NO: 66 partzyme A TFRCA/80-P:
 GGAATATGGAAGGAGACTGTCACAACGAGAGGGCGGTT
 5 SEQ ID NO: 67 partzyme B TFRCB/80-P:
GGTTCACGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
 SEQ ID NO: 68 partzyme B TFRCB/82-P:
TGGACGAGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
 SEQ ID NO: 69 partzyme A TFRCA/83-P:
 10 GGAATATGGAAGGAGACTGTCACAACGAGGGGAGCGGA
 SEQ ID NO: 70 partzyme B TFRCB/83-P:
GTTGCAGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
 SEQ ID NO: 71 partzyme A TFRCA/90-P:
 GGAATATGGAAGGAGACTGTCACAACGAGAGGGTCGAG

15

3.2. Reporter Substrates

The reporter substrates for this example are shown below with the sequence, 5' to 3'. The lower case bases represent RNA and the upper case bases represent DNA. In the current example, the substrates were end labelled with a 6-FAM moiety at the 5' end (indicated by a "F" in the name of the substrates below) and an Iowa Black® FQ quencher moiety at the 3' end (indicated by an "IB" in the name of the substrates below). Cleavage of the substrates was monitored between 510-530 nm (FAM emission wavelength range on CFX96 (BioRad)) with excitation between 450-490 nm (FAM excitation wavelength range on CFX96 (BioRad)).

25 SEQ ID NO: 21 Sub2-FIB:
 AAGGTTTCCTCguCCCTGGGCA
 SEQ ID NO: 22 Sub3-FIB:
 CAGCACAACCGuACCAACCG
 SEQ ID NO: 23 Sub6-FIB:
 30 ATCACGCCTCguTCCTCCCAG
 SEQ ID NO: 25 Sub44-FIB:
 CAGGTCTCCTCguCCCTATAGTGA
 SEQ ID NO: 26 Sub45-FIB:
 ACGGGTCCCguCTCCTTTGGAA
 35 SEQ ID NO: 27 Sub46-FIB:

ACCGCACCTGuCCCCAGCTC
SEQ ID NO: 28 Sub49-FB:
TAAACTTGGCTCguTGGCTGTGATA
SEQ ID NO: 29 Sub55-FIB:
5 ACCGCACCTCguCCCCAGCTC
SEQ ID NO: 72 Sub60T-FIB:
TGCCAACCACguCCAACACGAC
SEQ ID NO: 73 Sub61-FIB:
CTCGACCCCguCTCCACGCCA
10 SEQ ID NO: 74 Sub65-FIB:
TCTCGACCTCguCTCCACGCCA
SEQ ID NO: 75 Sub72-FIB:
ATCACGCCTCguCTCCTCCCAG
SEQ ID NO: 76 Sub73-FIB:
15 TGGCGTCCCCguCCCCTCGTG
SEQ ID NO: 77 Sub74-FIB:
ATCACTCCCCguCCCCTCCCAG
SEQ ID NO: 78 Sub75-FIB:
TGACCCTCCTCguCTCCCCACTA
20 SEQ ID NO: 79 Sub77-FIB:
CTCCTCCCTCguCCCTCCTCCT
SEQ ID NO: 80 Sub79-FIB:
TCCTCTCCCCguCCCCTTCAACC
SEQ ID NO: 81 Sub80-FIB:
25 AACCGCCCTCguCCCGTGAACC
SEQ ID NO: 82 Sub82-FIB:
CTCCTCCCTCguCCCTCGTCCA
SEQ ID NO: 83 Sub83-FIB:
TCCGCTCCCCguCCCCTGCAAC
30 SEQ ID NO: 84 Sub84-FIB:
ACCGCACCTCguCTCCTCCCAG
SEQ ID NO: 85 Sub85-FIB:
ACCGCACCTCguCCCCTCCCAG
SEQ ID NO: 86 Sub86-FIB:
35 ATCACGCCTCguCCCCAGCTC

SEQ ID NO: 87 Sub87-FIB:

ATCACTCCCCguCCCCAGCTC

SEQ ID NO: 88 Sub88-FIB:

CTCCTCCCTCguCCCCAGCTC

5 SEQ ID NO: 89 Sub89-FIB:

ACCGCACCTCguCCCTCCTCCT

SEQ ID NO: 90 Sub90-FIB:

CTCGACCCTCguCCCTCGTCCA

10 3.3. Target sequence

The target sequence for this example was a synthetic oligonucleotide AF-TFRC with the sequence, 5' to 3' below. This target sequence has the same sequence as a section of the TFRC gene.

SEQ ID NO: 95 Assembly Facilitator AF-TFRC:

15 AGTCTGTTTTCCAGTCAGAGGGACAGTCTCCTTCCATATTCC

3.4. Reaction Components: Direct isothermal detection of target sequence

Detection of the target sequence was measured by an increase in fluorescent signal caused by cleavage of the reporter substrate by the catalytically active MNAzyme. The total volume of all reactions was 25 μ L and all reactions were conducted on the CFX96™
20 Real-Time PCR Detection Systems (BioRad), with each combination of partzymes and substrates (**Table 10**) being tested at 52°C, 54°C, 56°C, 58°C and 60°C. Fluorescence for each reaction was programmed to be read after 1 second for the first 50 cycles and then programmed to be read after 25 seconds for the next 50 cycles. All reactions contained 1
25 x PCR Buffer II (Applied Biosystems), 10 mM MgCl₂, and 0.2 μ M of Partzymes A and B and 0.2 μ M substrate (tested in combinations as in **Table 10**). Each reaction was performed in duplicate as either a "test" with 10 nM target sequence (AF-TFRC) or no-template control (NF- H₂O).

30 **Table 10: Partzyme combinations used for each substrate**

Substrate	Partzyme A	Partzyme B
Sub2 SEQ ID NO: 21	TFRCA/2-P SEQ ID NO: 34	TFRCB/2-P SEQ ID NO: 35
Sub3 SEQ ID NO: 22	TFRCA/3-P SEQ ID NO: 36	TFRCB/3-P SEQ ID NO: 37
Sub6	TFRCA/6-P	TFRCB/6-P

SEQ ID NO: 23	SEQ ID NO: 38	SEQ ID NO: 39
Sub44	TFRCA/44	TFRCB/44-P
SEQ ID NO: 25	SEQ ID NO: 40	SEQ ID NO: 41
Sub45	TFRCA/45-P	TFRCB/45-P
SEQ ID NO: 26	SEQ ID NO: 42	SEQ ID NO: 43
Sub46	TFRCA/46-P	TFRCB/46-P
SEQ ID NO: 27	SEQ ID NO: 44	SEQ ID NO: 45
Sub49	TFRCA/49-P	TFRCB/49-P
SEQ ID NO: 28	SEQ ID NO: 93	SEQ ID NO: 94
Sub55	TFRCA/55-P	TFRCB/46-P
SEQ ID NO: 29	SEQ ID NO: 46	SEQ ID NO: 45
Sub60T	TFRCA/60-P	TFRCB/60-P
SEQ ID NO: 72	SEQ ID NO: 48	SEQ ID NO: 49
Sub61	TFRCA/61-P	TFRCB/61-P
SEQ ID NO: 73	SEQ ID NO: 50	SEQ ID NO: 51
Sub65	TFRCA/65-P	TFRCB/61-P
SEQ ID NO: 74	SEQ ID NO: 52	SEQ ID NO: 51
Sub72	TFRCA/6-P	TFRCB/72-P
SEQ ID NO: 75	SEQ ID NO: 38	SEQ ID NO: 55
Sub73	TFRCA/73-P	TFRCB/73-P
SEQ ID NO: 76	SEQ ID NO: 56	SEQ ID NO: 57
Sub74	TFRCA/74-P	TFRCB/74-P
SEQ ID NO: 77	SEQ ID NO: 58	SEQ ID NO: 59
Sub75	TFRCA/75-P	TFRCB/75-P
SEQ ID NO: 78	SEQ ID NO: 60	SEQ ID NO: 61
Sub77	TFRCA/77-P	TFRCB/77-P
SEQ ID NO: 79	SEQ ID NO: 62	SEQ ID NO: 63
Sub79	TFRCA/79-P	TFRCB/79-P
SEQ ID NO: 80	SEQ ID NO: 64	SEQ ID NO: 65
Sub80	TFRCA/80-P	TFRCB/80-P
SEQ ID NO: 81	SEQ ID NO: 66	SEQ ID NO: 67
Sub82	TFRCA/77-P	TFRCB/82-P
SEQ ID NO: 82	SEQ ID NO: 62	SEQ ID NO: 68
Sub83	TFRCA/83-P	TFRCB/83-P
SEQ ID NO: 83	SEQ ID NO: 69	SEQ ID NO: 70
Sub84	TFRCA/55-P	TFRCB/72-P
SEQ ID NO: 84	SEQ ID NO: 46	SEQ ID NO: 55
Sub85	TFRCA/55-P	TFRCB/74-P
SEQ ID NO: 85	SEQ ID NO: 46	SEQ ID NO: 59
Sub86	TFRCA/6-P	TFRCB/46-P
SEQ ID NO: 86	SEQ ID NO: 38	SEQ ID NO: 45
Sub87	TFRCA/74-P	TFRCB/46-P
SEQ ID NO: 87	SEQ ID NO: 58	SEQ ID NO: 45
Sub88	TFRCA/77-P	TFRCB/46-P
SEQ ID NO: 88	SEQ ID NO: 62	SEQ ID NO: 45
Sub89	TFRCA/55-P	TFRCB/77-P
SEQ ID NO: 89	SEQ ID NO: 46	SEQ ID NO: 63
Sub90	TFRCA/90-P	TFRCB/82-P
SEQ ID NO: 90	SEQ ID NO: 71	SEQ ID NO: 68

3.5. Results: Direct isothermal detection of target sequence

Each reaction with each universal substrate showed an increase in fluorescence over time for reactions containing the synthetic template AF-TFRC (target sequence corresponding to a portion of the TFRC gene). For all substrates, the fluorescence of the no-template control was lower than that in the target sequence-containing reactions. This demonstrates that the increase in fluorescence produced in target-containing reactions is due to target dependent assembly of catalytically active MNazymes that then cleaved the universal reporter substrate.

For each reaction, the raw fluorescent data points obtained from the CFX96 were normalised by dividing each data point by the value obtained for the paired no-template reaction at the first reading. This normalised data was then used to calculate the signal to noise value at approximately the 10 minute mark by dividing test data points by the no template data points. This calculation was performed for each substrate at each reaction temperature. The signal to noise value provides a measurement of the efficiency of the cleavage of substrates (**Figure 6, (i)**), with a high signal to noise indicating efficient cleavage. The standard deviation of the signal to noise ratio for each substrate over the temperature range was also calculated and plotted to determine substrates with consistently high activity over the tested range of temperatures (**Figure 6, (ii)**). A low value for this standard deviation indicates minimal change of signal to noise levels between temperatures. This suggests that these substrates are robust with respect to temperature.

Analysis of the signal to noise ratio for each substrate at the range of temperatures (**Figure 6, (i)**) shows that the series 1 substrates (Sub2, Sub3 and Sub6) had a higher signal to noise at the lower temperatures measured. However the cleavage efficiency of these substrates dropped dramatically as the reaction temperature was increased to 58°C. A similar pattern was seen for a subset of the series 2 substrates (Sub44, Sub45, Sub46, and Sub60T). The series 2 substrate, Sub49, performed poorly at the lower temperatures and slightly better at the higher but overall had lower signal to noise than other substrates. The series 2 substrate Sub60T showed a decrease in signal to noise with increasing temperature, and overall lower signal to noise at the lower temperatures than the majority of other series 1 and 2 substrates. The other series 2 substrate (Sub55), and a subset of the series 3 substrates (Sub61, Sub65, Sub74, Sub79, Sub80, Sub82, Sub83, Sub84, Sub85, Sub86, Sub87, Sub88, Sub89 and Sub90) displayed high fluorescence levels and hence were efficiently cleaved across all the temperatures tested. The series 3 substrates Sub73, Sub75 and Sub77 displayed roughly the same signal to noise value across the

temperatures tested, however the overall signal to noise level was low. These three substrates all gave good results with relatively low Ct values when tested with MNAzyme qPCR (see Example 2). Comparison of the data for these substrates for Examples 2 and 3 may indicate that when a constant temperature in the range of 52 to 58°C is used the turnover of these substrates is lower thus affecting the cleavage efficiency. This decreased substrate turnover could be related to the “off” rate of cleaved products. In Example 2, the cleaved products would dissociate from the partzyme substrate arms at least once a cycle when the temperature was increased to above 90°C as part of the thermocycling profile of PCR.

Overall, the substrates that conform to the design guidelines (**Table 9**) showed a greater signal to noise ratio across the tested temperature range than the substrates that fell outside these guidelines (**Figure 6, (i)**). More specifically reactions with Sub55, Sub61, Sub65, Sub72, Sub74, Sub79, Sub80, Sub82, Sub83, Sub84, Sub85, Sub86, Sub87, Sub88, Sub89 and Sub90 displayed high signal to noise values at every temperature tested demonstrating that these are robust substrates over a range of temperatures. This improvement was further evident when the standard deviation was calculated from the signal to noise ratio across the temperatures for each substrate (**Figure 6, (ii)**). This measure of variability paired with the absolute values of signal to noise indicated that, over the temperature range tested, the series 2 substrate Sub55, and the series 3 substrates Sub61, Sub65, Sub74, Sub79, Sub80, Sub82, Sub83, Sub85, Sub86, Sub87, Sub88, Sub89 and Sub90, had a high signal to noise ratio with little variability across a broad temperature range. There were three series 3 substrates, Sub65, Sub72 and Sub84, that only match three of the four design guidelines and two of these, Sub72 and Sub84, had a slightly greater standard deviation of the signal to noise than the series 3 substrates that match all four of the design guidelines as specified in **Table 9**.

Substrates that had signal to noise values less than 1.6 at 3 or more temperatures (Sub60, Sub73, Sub75 and Sub77) were not considered robust with respect to the range of temperatures tested.

These data suggest that compliance with all four of these design guidelines (**Table 9**) will, in general, produce substrates that are cleaved efficiently and robustly over a range of temperatures.

A study of the sequence of the most successful substrates from series 2 and 3 shows that these substrates share common features. Substrates that have little variation between signal to noise ratios over the temperatures tested (**Figure 6, (i)** and **(ii)**) generally contained seven or more cytosine nucleotides within the bases N₄ to N₁₃. This indicates

that the core region, i.e. the 10 bases surrounding the ribonucleotides, is highly influential on substrate activity. In addition, it was observed that a low number of guanine nucleotides (e.g. three, two, one or none) in the 10 bases surrounding the ribonucleotides is also beneficial.

5

Example 4: Use of universal substrates with MNAzyme qPCR, at annealing temperatures of 52°C and 58°C.

MNAzymes can be used to monitor amplification of target nucleic acids in real-time using *in vitro* target amplification methods such as PCR. Further, real-time monitoring during qPCR using MNAzyme substrates labelled with fluorophores and quencher pairs generates a curve that can indicate the efficiency of a reaction by its Ct value and steepness (reaction rate). In this example amplification and detection are performed in a one-step process, wherein PCR amplification and MNAzyme-mediated detection occur simultaneously in a single tube. The rate of production of signal (measured by Ct and steepness of reaction curves) at different annealing temperatures such as 52°C and 58°C (the temperature which data was collected), can be influenced by the sequence of the universal substrate.

The annealing/detection temperature for MNAzyme qPCR used in the art is between 50 and 54°C. This temperature was dictated by the fact that the universal substrates known in the art had a limitation on the temperature at which they were efficiently cleaved with 54°C being the upper limit for the series 1 universal substrates. There is a need for universal substrates that cleave at higher temperatures to allow greater flexibility in design of primers and partzymes that anneal at higher temperatures. This design flexibility for primers and partzymes would be of great benefit for many applications such as genetic targets of interest that have high percentages of G and C bases in their sequence, requiring partzymes and primers with higher Tms for specific detection. Utility of universal substrates would be greatly increased if substrates existed that were efficiently cleaved at a range of temperatures between 52 and 58°C.

In this example, partzymes corresponding to series 1, 2 and 3 universal substrates, were designed to target a range of genes, as outlined in **Table 11**. One skilled in the art would appreciate that any gene sequence or gene transcript or any other nucleic acid amplification product could be used as a target as described here. Each combination of partzymes and their associated universal substrates were tested at annealing temperatures of 52°C and 58°C in qPCR. The results from this comparison will determine if series 1, 2 and 3 substrates targeted to different genes, and at different annealing temperatures allow

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the same, higher or lower level of cleavage efficiency in real-time PCR. The level of cleavage efficiency was determined by measuring the Ct value and looking at the steepness of reaction curves for reactions containing different universal substrates.

5 **Table 11. Substrates used to detect different genes by MNzyme qPCR**

Gene	Series 1 substrate	Series 2 substrate	Series 3 substrate
CYP2C9	Sub3	-	Sub61
TP53	Sub6	-	Sub72, Sub74, and Sub79
B2M	-	Sub60	Sub61 and Sub79
HMBS	-	Sub49	Sub75
TFRC	Sub2	-	Sub72 and Sub80
RPL13a	-	Sub55	Sub80 and Sub88

4.1. Partzyme Oligonucleotides

In the experiments conducted to measure the efficiency of cleavage of the universal substrates in real-time PCR, the partzyme oligonucleotides A and B were designed with
 10 sensor arms complementary to the human CYP2C9, TP53, B2M, HMBS, RPL13a or TFRC genes. The sequences of the A and B partzymes are listed below from 5' to 3', where the bases underlined hybridize to the substrate. The “-P” indicates 3' phosphorylation of the oligonucleotide.

SEQ ID NO: 96 partzyme A CYP2C9A/3-P:
 15 GGAAGAGGAGCATTTGAGGAACAACGAGGTTGTGCTG
 SEQ ID NO: 97 partzyme B CYP2C9B/3-P:
 CGGTTGGTGAGGCTAGCTCCGTGTTCAAGAGGAAGC
 SEQ ID NO: 98 partzyme A CYP2C9A/61-P:
 GGAAGAGGAGCATTTGAGGAACAACGAGGGGTCGAG
 20 SEQ ID NO: 99 partzyme B CYP2C9B/61-P:
 TGGCGTGAGAGGCTAGCTCCGTGTTCAAGAGGAAGC
 SEQ ID NO: 100 partzyme A TP53A/6-P:
 GACGGAACAGCTTTGAGGTGACAACGAGAGGCGTGAT
 SEQ ID NO: 101 partzyme B TP53B/6-P:
 25 CTGGGAGGAAGGCTAGCTCGTGTTTGTGCCTGTCCTGG
 SEQ ID NO: 103 partzyme B TP53B/72-P:

CTGGGAGGAGAGGCTAGCTCGTGTTTGTGCCTGTCCTGG
SEQ ID NO: 104 partzyme A TP53A/74-P:
GACGGAACAGCTTTGAGGTGACAACGAGGGGAGTGAT
SEQ ID NO: 105 partzyme B TP53B/74-P:
5 CTGGGAGGGGAGGCTAGCTCGTGTTTGTGCCTGTCCTGG
SEQ ID NO: 106 partzyme A TP53A/79-P:
GACGGAACAGCTTTGAGGTGACAACGAGGGGAGAGGA
SEQ ID NO: 107 partzyme B TP53B/79-P:
GGTTGAAGGGGAGGCTAGCTCGTGTTTGTGCCTGTCCTGG
10 SEQ ID NO: 108 partzyme A B2MA/60-P:
ATTCAGGTTTACTCACGTCATCACAACGAGTGGTTGGC
SEQ ID NO: 109 partzyme B B2MB/60-P:
GTCGTGTTGGAGGCTAGCTCAGCAGAGAATGGAAAGTCAAA
SEQ ID NO: 110 partzyme A B2MA/61-P
15 ATTCAGGTTTACTCACGTCATCACAACGAGGGGTCGAG
SEQ ID NO: 111 partzyme B B2MB/61-P
TGGCGTGGAGAGGCTAGCTCAGCAGAGAATGGAAAGTCAAA
SEQ ID NO: 112 partzyme A B2MA/79-P
ATTCAGGTTTACTCACGTCATCACAACGAGGGGAGAGGA
20 SEQ ID NO: 113 partzyme B B2MB/79-P
GGTTGAAGGGGAGGCTAGCTCAGCAGAGAATGGAAAGTCAAA
SEQ ID NO: 114 partzyme A HMBSA/49-P:
GCCATGTCTGGTAACGGCAAACAACGAGAGCCAAGTTTA
SEQ ID NO: 115 partzyme B HMBSB/49-P:
25 TATCACAGCCAAGGCTAGCTTGCGGCTGCAACGGCGGTG
SEQ ID NO: 116 partzyme A HMBSA/75-P:
GCCATGTCTGGTAACGGCAAACAACGAGAGGAGGGTCA
SEQ ID NO: 117 partzyme B HMBSB/75-P:
TAGTGGGGAGAGGCTAGCTTGCGGCTGCAACGGCGGTG
30 SEQ ID NO: 34 partzyme A TFRCA/2-P:
GGAATATGGAAGGAGACTGTCACAACGAGAGGAAACCTT
SEQ ID NO: 35 partzyme B TFRCB/2-P:
TGCCCAGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 38 partzyme A TFRCA/72-P:
35 GGAATATGGAAGGAGACTGTCACAACGAGAGGCGTGAT

SEQ ID NO: 55 partzyme B TFRCB/72-P:
CTGGGAGGAGAGGCTAGCTCCTCTGACTGGAAAACAGACT
 SEQ ID NO: 66 partzyme A TFRCA/80-P:
GGAATATGGAAGGAGACTGTCACAACGAGAGGGCGGTT
 5 SEQ ID NO: 67 partzyme B TFRCB/80-P:
GGTTCACGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
 SEQ ID NO: 118 partzyme A RPL13aA/55-P
TTGACAAATACACAGAGGTCACAACGAGAGGTGCGGT
 SEQ ID NO: 119 partzyme B RPL13aB/55-P
 10 GAGCTGGGGAGGCTAGCTCTCAAGACCCACGGACTCCT
 SEQ ID NO: 120 partzyme A RPL13aA/80-P
AATTGACAAATACACAGAGGTCACAACGAGAGGGCGGTT
 SEQ ID NO: 121 partzyme B RPL13aB/80-P
GGTTCACGGGAGGCTAGCTCTCAAGACCCACGGACTCCT
 15 SEQ ID NO: 122 partzyme A RPL13aA/88-P
AATTGACAAATACACAGAGGTCACAACGAGAGGGAGGAG

4.2. Reporter Substrates

In the current example, the substrates were 5' end labelled with a fluorophore and
 20 3' end labelled with a quencher moiety. **Table 12** depicts the Substrate –
 fluorophore/quencher combinations. Some substrates were tested with more than one
 particular fluorophore/quencher combination. Cleavage of the substrates was monitored at
 various emission and excitation wavelengths (**Table 12**).

25 **Table 12. Substrates and their fluorescent labelling**

Substrate	Name	Fluorophore	Quencher^	Excitation*	Emission*
Sub49	Sub49-FB	6-FAM	BHQ1	450-490	510-530
Sub3	Sub3-FIB		IB		
Sub61	Sub61-FIB				
Sub75	Sub75-FIB				
Sub55	Sub55-HIB	HEX	IB	515-535	560-580
Sub80	Sub80-HIB				
Sub88	Sub88-HIB				
Sub6	Sub6-TRB2	Texas Red	BHQ2	560-590	610-650
Sub72	Sub72-TRIBR		IBR		
Sub74	Sub74-TRIBR				
Sub79	Sub79-TRIBR				
Sub2	Sub2-Q670B2	Quasar 670	BHQ2	620-650	675-690
Sub72	Sub72-Q670B2				

Sub80	Sub80-Q670B2				
Sub60	Sub60-Q705B2				
Sub61	Sub61-Q705B2	Quasar 705	BHQ2	672-684	705-730
Sub79	Sub79-Q705B2				

^ BHQ1; black hole quencher 1, BHQ2; black hole quencher 2, IB; Iowa black® FQ, IBR; Iowa black® RQ

*CFX96 Real-Time PCR Detection System (Biorad) excites, and measures emission of, each fluorophore over a range of wavelengths for each channel.

5 The reporter substrates tested in this example are shown below with the sequence, 5' to 3'. The lower case bases represent RNA and the upper case bases represent DNA.

SEQ ID NO: 21 Sub2:

AAGGTTTCCTCguCCCTGGGCA

SEQ ID NO: 22 Sub3:

10 CAGCACAACCguACCAACCG

SEQ ID NO: 23 Sub6:

ATCACGCCTCguTCCTCCCAG

SEQ ID NO: 28 Sub49:

TAAACTTGGCTCguTGGCTGTGATA

15 SEQ ID NO: 29 Sub55:

ACCGCACCTCguCCCCAGCTC

SEQ ID NO: 30 Sub60:

GCCAACCACguCCAACACGAC

SEQ ID NO: 73 Sub61:

20 CTCGACCCCguCTCCACGCCA

SEQ ID NO: 75 Sub72:

ATCACGCCTCguCTCCTCCCAG

SEQ ID NO: 77 Sub74:

ATCACTCCCCguCCCCTCCCAG

25 SEQ ID NO: 78 Sub75:

TGACCCTCCTCguCTCCCCACTA

SEQ ID NO: 80 Sub79:

TCCTCTCCCCguCCCCTTCAACC

SEQ ID NO: 81 Sub80:

30 AACCGCCCTCguCCCGTGAACC

SEQ ID NO: 88 Sub88:

CTCCTCCCTCguCCCCAGCTC

4.3. Target sequence and PCR primers for amplification of the CYP2C9, TP53, B2M, HMBS, TFRC and RPL13a genes

Human genomic DNA extracted from IM9 cell line (Promega) was used as template for *in vitro* amplification of the target genes. The amplicons were generated by qPCR using the oligonucleotide PCR primers listed below. Primer sequences are listed from 5' to 3'. The sequence in bold in the primer sequences corresponds to a universal tag (U1, U2 or U3) that increases the T_m of the primer without affecting the specificity of the primer to the gene target. This tag improves amplification efficiency in PCR reactions.

10 SEQ ID NO: 91 Forward primer 5TFRC_U1
GCTAAAACAATAACTCAGAACTTACG

SEQ ID NO: 92 Reverse primer 3TFRC_U2
CAGCTTTCTGAGGTTACCATCCTA

SEQ ID NO: 123 Forward primer 5B2M_U1
15 **GCTAATCTTTTCCCGATATTCCTCAG**

SEQ ID NO: 124 Reverse primer 3B2M_U2
CAGCCCAGACACATAGCAATTCAG

SEQ ID NO: 125 Forward primer 5TP53_U3
CTAACTTACTGCCTCTTGCTTCTC

20 SEQ ID NO: 126 Reverse primer 3TP53_U2
CAGCTCTGTGCGCCGGTCTCTC

SEQ ID NO: 127 Forward primer 5RPL13a_U3
CTAAACCGGAAGAAGAAACAGCTCA

SEQ ID NO: 128 Reverse primer 3RPL13a_U2
25 **CAGGAGGAATTAACAGTCTTTATTGG**

SEQ ID NO: 129 Forward primer 5CYP2C9_U3
CTAACCTCATGACGCTGCGGAA

SEQ ID NO: 130 Reverse primer 3CYP2C9_U2
CAGATATGGAGTAGGGTCACCCA

30 SEQ ID NO: 131 Forward primer 5HMBS_U3
CTAAACCCACACACAGCCTACTTTC

SEQ ID NO: 132 Reverse primer 3HMBS_U2
CAGAGCCCAAAGTGTGCTGGTCA

4.4. Reaction Components: Amplification and quantification of target sequence

Real-time PCR amplification and detection of the target sequence was performed in a total reaction volume of 25 μ L. All reactions were conducted in a CFX96 Real-Time PCR Detection System (Bio-Rad). Reactions were set up with substrates and their associated partzymes as in **Table 13**. The cycling parameters were either;

1) 95°C for 2 minutes, 50 cycles of 95°C for 15 seconds and 52°C for 60 seconds (data collected at the 52°C step) or

2) 95°C for 2 minutes, 50 cycles of 95°C for 15 seconds and 58°C for 60 seconds (data collected at the 58°C step).

Each set of reaction conditions was run in duplicate and contained 40 nM forward primer and 200 nM of reverse primer, 200 nM each of partzyme A and partzyme B, 200 nM substrate, 8 mM MgCl₂, 200 μ M of each dNTP, 10 units RiboSafe RNase inhibitor (Bioline), 1 x Immobuffer (Bioline), 2 units of MyTaqHS™ DNA polymerase (Bioline) and either genomic DNA template (100 ng) or no target (NF-H₂O).

Table 13: Oligonucleotide combinations used for each universal substrate

Substrate	Partzyme A	Partzyme B	Primers
Sub3 SEQ ID NO: 22	CYP2C9A/3-P SEQ ID NO: 96	CYP2C9B/3-P SEQ ID NO: 97	5CYP2C9_U1 and 3CYP2C9_U2
Sub61 SEQ ID NO: 73	CYP2C9A/61-P SEQ ID NO: 98	CYP2C9B/61-P SEQ ID NO: 99	SEQ ID NO: 129 and SEQ ID NO: 130 respectively
Sub6 SEQ ID NO: 23	TP53A/6-P SEQ ID NO: 100	TP53B/6-P SEQ ID NO: 101	5TP53_U1 and 3TP53_U2 SEQ ID NO: 125 and SEQ ID NO: 126 respectively
Sub72 SEQ ID NO: 75	TP53A/6-P SEQ ID NO: 100	TP53B/72-P SEQ ID NO: 103	
Sub74 SEQ ID NO: 77	TP53A/74-P SEQ ID NO: 104	TP53B/74-P SEQ ID NO: 105	
Sub79 SEQ ID NO: 80	TP53A/79-P SEQ ID NO: 106	TP53B/79-P SEQ ID NO: 107	
Sub60 SEQ ID NO: 30	B2MA/60-P SEQ ID NO: 108	B2MB/60-P SEQ ID NO: 109	5B2M_U1 and 3B2M_U2 SEQ ID NO: 123 and SEQ ID NO: 124 respectively
Sub61 SEQ ID NO: 73	B2MA/61-P SEQ ID NO: 110	B2MB/61-P SEQ ID NO: 111	
Sub79 SEQ ID NO: 80	B2MA/79-P SEQ ID NO: 112	B2MB/79-P SEQ ID NO: 113	
Sub49 SEQ ID NO: 28	HMBSA/49-P SEQ ID NO: 114	HMBSB/49-P SEQ ID NO: 115	5HMBS_U1 and 3HMBS_U2
Sub75 SEQ ID NO: 72	HMBSA/75-P SEQ ID NO: 116	HMBSB/75-P SEQ ID NO: 117	SEQ ID NO: 131 and SEQ ID NO: 132 respectively
Sub2 SEQ ID NO: 21	TFRCA/2-P SEQ ID NO: 34	TFRCB/2-P SEQ ID NO: 35	5TFRC_U1 and 3TFRC_U2
Sub72 SEQ ID NO: 75	TFRCA/72-P SEQ ID NO: 38	TFRCB/72-P SEQ ID NO: 55	SEQ ID NO: 91 and SEQ ID NO: 92 respectively

SEQ ID NO: 81	SEQ ID NO: 66	SEQ ID NO: 67	
Sub55 SEQ ID NO: 29	RPL13aA/55-P SEQ ID NO: 118	RPL13aB/55-P SEQ ID NO: 119	5RPL13a_U1 and 3RPL13a_U2 SEQ ID NO: 127 and SEQ ID NO: 128 respectively
Sub80 SEQ ID NO: 81	RPL13aA/80-P SEQ ID NO: 120	RPL13aB/80-P SEQ ID NO: 121	
Sub88 SEQ ID NO: 88	RPL13aA/88-P SEQ ID NO: 122	RPL13aB/55-P SEQ ID NO: 119	

4.5. Results: Amplification of target and cleavage of reporter substrate

Each MNazyme qPCR reaction containing human genomic DNA showed an increase in fluorescence over time for the real-time detection of the genes CYP2C9, TP53, B2M, HMBS, RPL13a and TFRC, at annealing temperatures of both 52°C and 58°C (**Figure 7**). For all universal substrates, the fluorescence of the no-DNA target control was lower than that in the DNA target-containing reactions. This demonstrates that the increase in fluorescence produced in target-containing reactions is due to target dependent assembly of catalytically active MNazymes that then cleaved one of the universal reporter substrates.

Results from MNazyme qPCR detection of the CYP2C9 and TP53 genes showed that all universal substrates tested performed equivalently at 52°C with less than 0.5 Ct difference between the substrates and similar slopes of the amplification curves (**Table 14** and **Figure 7, (i)a** and **(ii)a** respectively). The series 1 substrates tested (Sub3 and Sub6) performed worse at the higher temperature of 58°C than the series 3 substrates tested (Sub61, Sub72, Sub74 and Sub79) with a difference of more than 1 Ct between the substrates and a much shallower slope for the amplification curves for Sub3 and Sub6 indicating a less efficient reaction (**Table 14** and **Figure 7, (i)b** and **(ii)b** respectively). These data show that the improved design of these series 3 substrates (Sub61, Sub72, Sub74 and Sub79) leads to more efficient cleavage at 58°C, and that this improved performance at elevated temperatures does not prevent these substrates from being efficiently cleaved at a lower temperature.

Results from MNazyme qPCR detection of the B2M and HMBS genes showed that all the universal substrates tested performed equivalently at 52°C with only approximately 0.5 Ct difference between the substrates and similar slopes of the amplification curves (**Table 14** and **Figure 7, (iii)a** and **(iv)a** respectively). The series 2 substrates tested (Sub60 and Sub49) performed worse at the higher temperature of 58°C than the series 3 substrates tested (Sub61 and Sub75, and Sub79) with a difference of more than 1 Ct between the substrates and a shallower slope for the amplification curves for Sub60 and

Sub49 indicating a less efficient reaction (**Table 14** and **Figure 7, (iii)b** and **(iv)b** respectively). These data show that the improved design of these series 3 substrates (Sub61, Sub75 and Sub79) leads to more efficient cleavage at 58°C, but that this improved performance at elevated temperatures does not prevent the substrates from being efficiently cleaved at a lower temperature. The better performance of the series 3 substrates versus the series 2 substrates at 58°C is attributable to the fact that the series 3 substrates follow all the design guidelines for highly active substrates and the series 2 substrates do not (see **Table 9**).

Results from MNAzyme qPCR detection of the TFRC gene showed that all universal substrates tested performed equivalently at 52°C with only approximately 0.5 Ct difference between the substrates and similar slopes of the amplification curves (**Table 14** and **Figure 7, (v)a**). The series 3 substrates tested (Sub72 and Sub80) both performed better at 58°C than the series 1 substrate tested (Sub2) with a difference of more than 1 Ct between the substrates and a shallower slope for the amplification curve for Sub2 indicating a less efficient reaction (**Table 14** and **Figure 7, (v)b**). The series 3 substrate, Sub80, performed better at 58°C than the series 3 substrate, Sub72, with a greater than 1 Ct difference between the substrates. Sub80 follows all of the design guidelines for highly active substrates and Sub72 does not (see **Table 9**).

Results from MNAzyme qPCR detection of the RPL13a gene showed that at 52°C the series 2 substrate Sub55 and the series 3 substrate Sub88 were better than the series 3 substrate Sub80 (**Table 14** and **Figure 7, (vi)a**). At 58°C the series 3 substrate Sub80 and the series 2 Sub55 performed the same, and both these substrates performed better than the series 3 substrate Sub88 (**Table 14** and **Figure 7, (vi)b**). The series 2 substrate Sub55 meets all the all of the design guidelines for highly active substrates (see **Table 9**) and would therefore be expected to perform well. The series 3 Sub88 also meets all of the design guidelines for highly active substrates (**Table 9**) but has not performed as well as Sub55. Overall, the design guidelines show a high probability of producing substrates that are efficiently cleaved under MNAzyme qPCR conditions.

Overall, with a range of different target sequences, the series 1, 2 and 3 substrates performed comparably in MNAzyme qPCR performed at 52°C. At 58°C, the series 3 substrates out-performed the series 1 and 2 substrates, with the exception of the series 2 substrate Sub55 which, as explained above, falls within the all the design guidelines for highly active substrates. These data show that the design guidelines do, in general, produce substrates that are robust and efficiently cleaved at a range of temperatures in the context of thermocycling protocols used for qPCR.

Of note is that some of the series 3 substrates, indicated by (^) in **Table 14**, have very high T_m's, and therefore at lower temperatures they may have poorer turnover over of the cleaved substrate and hence even though the activity is comparable to the other substrates, the final fluorescence value is lower.

5

Table 14. Substrate cleaved by MNazymes targeted to different genes.

Gene	Substrate	52°C			58°C		
		Amplification curve*	Ct	Diff in Ct	Amplification curve*	Ct	Diff in Ct
CYP2C9	Sub3	Sub3 = Sub61	22.9	< 0.5	Sub61 > Sub3	26.7	> 1.0
	Sub61		22.5			25.2	
TP53	Sub6	All equal (^Sub79)	23.0	< 0.5	Sub79 > Sub74 > Sub72 >> Sub6	27.4	(Sub79 & Sub6) > 1.5
	Sub72		22.7			24.2	
	Sub74		22.7			25.1	
	Sub79		22.6			25.8	
B2M	Sub60	Sub60 = Sub61 > Sub79 (^Sub79)	24.0	(Sub60 & Sub61) 0.1	Sub61 = Sub79 >> Sub60	28.5	> 2.0
	Sub61		23.9			25.5	
	Sub79		24.6			25.7	
HMBS	Sub49	Sub49 = Sub75 (^Sub75)	25.1	0.1	Sub75 > Sub49	28.1	> 1.0
	Sub75		25.0			27.0	
TFRC	Sub2	Sub72 > Sub2 = Sub80 (^Sub80)	23.8	0.6	Sub80 > Sub72 >> Sub2	27.1	(Sub80 & Sub2) > 2.0
	Sub72		23.2			25.7	
	Sub80		23.4			24.8	
RPL13a	Sub55	Sub55 = Sub88 > Sub80 (^Sub80)	20.3	(Sub55 & Sub88) < 0.5	Sub80 > Sub55 > Sub88	22.6	(Sub55 & Sub88) > 2.0
	Sub80		21.0			22.3	
	Sub88		20.3			24.3	

*The shape of the amplification curve (steepness and time taken to reach plateau) demonstrates the reaction efficiency.

^ Tm of these substrates is too high for lower reaction temperatures and this can result in poor turnover over of the cleaved portions of the substrate and thus lower final fluorescence values.

Example 5: Use of universal substrates with DNazymes

5 The 10-23 DNzyme is a unimolecular structure that can directly bind to, and modify, a substrate sequence. The 10-23 DNzyme has utility in *in vitro* diagnostic applications. Due to similarity in the catalytic region, the 10-23 DNzyme can bind and cleave substrates that are cleavable by the MNzyme based on the 10-23 DNzyme. Unlike the MNzyme, the DNzyme does not need a target sequence to form the active
10 core, and therefore the binding and subsequent cleavage of the substrate by the 10-23 DNzyme is not influenced by the target sequence and does not utilize a split catalytic core. The ability of matched 10-23 DNzymes to cleave the series 1 universal substrates (Sub2, Sub3, and Sub6), the series 2 universal substrates (Sub44, Sub45, Sub49, Sub55 and Sub60T) and the series 3 substrates (Sub61, Sub72, Sub73, Sub74, Sub75, Sub77,
15 Sub79, Sub80, Sub84, Sub85, Sub86, Sub87, Sub88, and Sub89) was measured to determine if the design guidelines of the present invention lead to the development of substrates that can be cleaved by the 10-23 DNzyme with high activity and robustly over a range of temperatures.

20 5.1. 10-23 DNzyme Oligonucleotides

A series of 10-23 DNzymes were designed with sensor arms complementary to the substrates described above and listed in **Tables 4 and 5**. The sequences of the DNzymes are listed below from 5' to 3', where the bases underlined hybridize to the substrate and the bases in *italics* form the catalytic core. Some DNzyme sequences
25 below contain an extra G at the very 5' and 3' ends (e.g. Dz55). These added bases do not hybridize with the substrates and do not impact on the efficiency at which the DNzyme cleaved the substrate.

SEQ ID NO: 133 DNzyme Dz2

TGCCCAGGGAGGCTAGCTACAACGAGAGGAAACCTT

30 SEQ ID NO: 134 DNzyme Dz3

CGGTTGGTGAGGCTAGCTACAACGAGGTTGTGCTG

SEQ ID NO: 135 DNzyme Dz6

CTGGGAGGAAGGCTAGCTACAACGAGAGGCGTGAT

SEQ ID NO: 136 DNzyme Dz44

35 TCACTATAGGGAGGCTAGCTACAACGAGAGGAGACCTG

SEQ ID NO: 137 DNAzyme Dz45
TTCCAAAGGAGAGGCTAGCTACAACGAGGGACCCGT

SEQ ID NO: 138 DNAzyme Dz49
TATCACAGCCAAGGCTAGCTACAACGAGAGCCAAGTTTA

5 SEQ ID NO: 139 DNAzyme Dz55
GGAGCTGGGGAGGCTAGCTACAACGAGAGGTGCGGTG

SEQ ID NO: 140 DNAzyme Dz60
GTCGTGTTGGAGGCTAGCTACAACGAGTGGTTGGC

SEQ ID NO: 141 DNAzyme Dz61
10 GTGGCGTGGAGAGGCTAGCTACAACGAGGGGTCGAGG

SEQ ID NO: 142 DNAzyme Dz72
GCTGGGAGGAGAGGCTAGCTACAACGAGAGGCGTGATG

SEQ ID NO: 143 DNAzyme Dz73
GCACGAGGGGAGGCTAGCTACAACGAGGGGACGCCAG

15 SEQ ID NO: 144 DNAzyme Dz74
GCTGGGAGGGGAGGCTAGCTACAACGAGGGGAGTGATG

SEQ ID NO: 145 DNAzyme Dz75
GTAGTGGGGAGAGGCTAGCTACAACGAGAGGAGGGTCAG

SEQ ID NO: 146 DNAzyme Dz77
20 GAGGAGGAGGGAGGCTAGCTACAACGAGAGGGAGGAGG

SEQ ID NO: 147 DNAzyme Dz79
GGGTTGAAGGGGAGGCTAGCTACAACGAGGGGAGAGGAG

SEQ ID NO: 148 DNAzyme Dz80
GGGTTACGGGAGGCTAGCTACAACGAGAGGGCGGTTGG

25 SEQ ID NO: 149 DNAzyme Dz84
GCTGGGAGGAGAGGCTAGCTACAACGAGAGGTGCGGTG

SEQ ID NO: 150 DNAzyme Dz85
GCTGGGAGGGGAGGCTAGCTACAACGAGAGGTGCGGTG

SEQ ID NO: 151 DNAzyme Dz86
30 GGAGCTGGGGAGGCTAGCTACAACGAGAGGCGTGATG

SEQ ID NO: 152 DNAzyme Dz87
GGAGCTGGGGAGGCTAGCTACAACGAGGGGAGTGATG

SEQ ID NO: 153 DNAzyme Dz88
GGAGCTGGGGAGGCTAGCTACAACGAGAGGGAGGAGG

35 SEQ ID NO: 154 DNAzyme Dz89

GAGGAGGAGGGAGGCTAGCTACAACGAGAGGTGCGGTG

5.2. Reporter Substrates

In the current example, the substrates were end labelled with a 6-FAM moiety at the 5' end (indicated by a "F" in the name of the substrates below) and an Iowa Black® FQ quencher moiety at the 3' end (indicated by a "IB" in the name of the substrates below). Cleavage of the substrates was monitored between 510-530 nm (FAM emission wavelength range on CFX96 (BioRad)) with excitation between 450-490 nm (FAM excitation wavelength range on CFX96 (BioRad)). The reporter substrates for this example are shown below with the sequence, 5' to 3'. The lower case bases represent RNA and the upper case bases represent DNA.

SEQ ID NO: 21 Sub2-FIB:

AAGGTTTCCTCguCCCTGGGCA

SEQ ID NO: 22 Sub3-FIB:

CAGCACAACCguACCAACCG

SEQ ID NO: 23 Sub6-FIB:

ATCACGCCTCguTCCTCCCAG

SEQ ID NO: 25 Sub44-FIB:

CAGGTCTCCTCguCCCTATAGTGA

SEQ ID NO: 26 Sub45-FIB:

ACGGGTCCCguCTCCTTTGGAA

SEQ ID NO: 28 Sub49-FB:

TAAACTTGGCTCguTGGCTGTGATA

SEQ ID NO: 29 Sub55-FIB:

ACCGCACCTCguCCCCAGCTC

SEQ ID NO: 72 Sub60T-FIB:

TGCCAACCACguCCAACACGAC

SEQ ID NO: 73 Sub61-FIB:

CTCGACCCCguCTCCACGCCA

SEQ ID NO: 75 Sub72-FIB:

ATCACGCCTCguCTCCTCCCAG

SEQ ID NO: 76 Sub73-FB:

TGGCGTCCCCguCCCCTCGTG

SEQ ID NO: 77 Sub74-FIB:

ATCACTCCCCguCCCCTCCCAG

SEQ ID NO: 78 Sub75-FIB:
 TGACCCTCCTCguCTCCCCACTA
 SEQ ID NO: 79 Sub77-FB:
 CTCCTCCCTCguCCCTCCTCCT
 5 SEQ ID NO: 80 Sub79-FIB:
 TCCTCTCCCCguCCCCTTCAACC
 SEQ ID NO: 81 Sub80-FIB:
 AACCGCCCTCguCCCGTGAACC
 SEQ ID NO: 84 Sub84-FIB:
 10 ACCGCACCTCguCTCCTCCCAG
 SEQ ID NO: 85 Sub85-FIB:
 ACCGCACCTCguCCCCTCCCAG
 SEQ ID NO: 86 Sub86-FIB:
 ATCACGCCTCguCCCCAGCTC
 15 SEQ ID NO: 87 Sub87-FIB:
 ATCACTCCCCguCCCCAGCTC
 SEQ ID NO: 88 Sub88-FIB:
 CTCCTCCCTCguCCCCAGCTC
 SEQ ID NO: 89 Sub89-FIB:
 20 ACCGCACCTCguCCCTCCTCCT

5.3. Reaction Components: Cleavage of a substrate by a DNAzyme at temperatures between 50°C and 60°C

Cleavage of a substrate was measured by an increase in fluorescent signal caused by
 25 the binding and subsequent cleavage by a matched DNAzyme. Separate reactions were
 set up to measure the cleavage of each substrate with its matched DNAzyme
 (oligonucleotides as in **Table 15**). Reactions contained 1 x PCR Buffer II (Applied
 Biosystems), 10 mM MgCl₂, 200 nM Substrate and NF-H₂O in total volume of 25 µL.
 Each reaction was run in duplicate as either a “test” (addition of 1 nM DNAzyme) or
 30 “control” (addition of NF-H₂O) reaction. Reactions were performed on a CFX96™ Real-
 Time PCR Detection System (BioRad) at 50, 52, 54, 56, 58 and 60°C. Fluorescence for
 each reaction was programmed to be read after 1 second for the first 50 cycles and then
 programmed to be read after 25 seconds for the next 50 cycles.

Table 15: DNAzymes tested with matching Substrates

Substrate	DNAzyme
Sub2 SEQ ID NO: 21	Dz2 SEQ ID NO: 133
Sub3 SEQ ID NO: 22	Dz3 SEQ ID NO: 134
Sub6 SEQ ID NO: 23	Dz6 SEQ ID NO: 135
Sub44 SEQ ID NO: 25	Dz44 SEQ ID NO: 136
Sub45 SEQ ID NO: 26	Dz45 SEQ ID NO: 137
Sub49 SEQ ID NO: 28	Dz49 SEQ ID NO: 138
Sub55 SEQ ID NO: 29	Dz55 SEQ ID NO: 139
Sub60T SEQ ID NO: 72	Dz60 SEQ ID NO: 140
Sub61 SEQ ID NO: 73	Dz61 SEQ ID NO: 141
Sub72 SEQ ID NO: 75	Dz72 SEQ ID NO: 142
Sub73 SEQ ID NO: 76	Dz73 SEQ ID NO: 143
Sub74 SEQ ID NO: 77	Dz74 SEQ ID NO: 144
Sub75 SEQ ID NO: 78	Dz75 SEQ ID NO: 145
Sub77 SEQ ID NO: 79	Dz77 SEQ ID NO: 146
Sub79 SEQ ID NO: 80	Dz79 SEQ ID NO: 147
Sub80 SEQ ID NO: 81	Dz80 SEQ ID NO: 148
Sub84 SEQ ID NO: 84	Dz84 SEQ ID NO: 149
Sub85 SEQ ID NO: 85	Dz85 SEQ ID NO: 150
Sub86 SEQ ID NO: 86	Dz86 SEQ ID NO: 151
Sub87 SEQ ID NO: 87	Dz87 SEQ ID NO: 152
Sub88 SEQ ID NO: 88	Dz88 SEQ ID NO: 153
Sub89 SEQ ID NO: 89	Dz89 SEQ ID NO: 154

5.5. Results: Cleavage of a substrate by a DNAzyme at various temperatures

Each test reaction containing DNAzymes with matched substrates showed an increase in fluorescence over time. There was no increase in fluorescence of water only control reactions (no DNAzyme added). This demonstrates that the increase in fluorescence produced in the DNAzyme-containing reactions was due to the binding and subsequent catalytic cleavage of the reporter substrate by the DNAzyme.

For each substrate data set (test and control reactions), the raw fluorescence data points were exported into Excel (Microsoft), duplicate values were averaged and then normalised. Normalisation was performed by dividing each averaged data point by the averaged value of the no-DNAzyme reaction at the first reading of reactions containing the same substrate (e.g. the averaged data for the test reactions for Sub61 was divided by the averaged fluorescence at cycle 1 for the Sub61 no-DNAzyme control reaction; the averaged data for the no-DNAzyme control reactions for Sub61 was divided by the averaged fluorescence at cycle 1 for the Sub61 no-DNAzyme control reaction.) These normalised data were then used to calculate the signal to noise ratio at approximately 10 minutes after the start of the reaction, by dividing the normalized fluorescence of the test

reaction at 10 minutes by the normalized fluorescence of the no-DNAzyme reaction at 10 minutes. This calculation of signal to noise was performed for each combination of DNAzyme and substrate and at each temperature tested. The signal to noise value was then plotted on a bar graph to compare the efficiency of cleavage of each substrate by its matched DNAzyme at the various temperatures (**Figure 8, (i)**). The standard deviation of the signal to noise ratio for each substrate over the temperature range was also calculated and plotted to determine substrates with consistent signal to noise over the tested range of temperatures (**Figure 8, (ii)**). This suggests that these substrates are robust with respect to temperature.

Due to experimental error there are no data for Sub2 at 54°C or for Sub79 at 58°C, however this has minimal impact on the overall interpretation of the data.

Analysis of the signal to noise ratio for each substrate (**Figure 8, (i)**) shows that the series 1 substrates (Sub2, Sub3 and Sub6) had high signal to noise at the lower temperatures measured. However the cleavage efficiency of these substrates dropped dramatically as the reaction temperature was increased. A similar pattern was seen for a subset of the series 2 substrates (Sub44, Sub45, Sub49 and Sub60T). The other series 2 substrate (Sub55), and the majority of the series 3 substrates tested (Sub61, Sub72, Sub74, Sub75, Sub79, Sub80, Sub84, Sub85, Sub86, Sub87, Sub88 and Sub89) displayed high signal to noise across all the temperatures tested. The series 3 substrates Sub73 and Sub77 had uniformly low signal to noise at all temperatures tested indicating that although the new design guidelines provide a good probability of design of robust substrates (83% success rate for this application), there will still be some sequences that meet these guidelines but that are unsuitable for a subset of MNAzyme and/or DNAzyme diagnostic applications. The results for Sub73 and Sub77 also prove that methods utilizing DNAzymes for mass screening of potential substrate sequences to find those that are suitable for MNAzyme diagnostic applications may be missing out on detection of substrates that would be robust and efficiently cleaved by MNAzymes in qPCR applications and vice versa (see Example 2 for the successful utilization of Sub73 and Sub77 in MNAzyme qPCR, **Figure 5**).

Overall, the majority of the substrates that conform to all of the design guidelines (**Table 9**) showed a greater signal to noise ratio across the tested temperature range than the substrates that fell outside one or more of these guidelines (**Figure 8, (i)**). More specifically reactions with Sub55, Sub61, Sub72, Sub74, Sub75, Sub79, Sub80, Sub84, Sub85, Sub86, Sub87, Sub88 and Sub89 displayed high signal to noise values at every temperature tested, demonstrating that these are robust substrates over a range of

temperatures. This improvement was further evident when the standard deviation was calculated from the signal to noise ratio across the temperatures for each substrate (Figure 8, (ii)). This measure of variability indicated that, over the temperature range tested, the series 2 substrate, Sub55, and the series 3 substrates Sub61, Sub72 Sub74, Sub75, Sub79, Sub80, Sub82, Sub83, Sub85, Sub86, Sub87, Sub88 and Sub89, had similar signal to noise across the temperatures tested demonstrating that they are robust substrates across a broad temperature range. Note that although Sub60T, Sub73 and Sub77 do demonstrate a low standard deviation in signal to noise over the temperature range, the absolute signal to noise is very low at all temperatures excluding these substrates from being a robust substrate in this application.

Example 6: Testing for non-specific cleavage of universal substrates with MNAzyme qPCR.

MNAzymes can be used to monitor amplification of target nucleic acids in real-time using *in vitro* target amplification methods such as PCR. Amplification and detection are performed in a one-step process, wherein PCR amplification and MNAzyme-mediated detection occur simultaneously in a single tube. Multiple targets can be amplified and detected in a single reaction vessel using partzymes with target sensor arms specific to the individual targets. Partzymes for the detection of a first target will bind to and cleave a first substrate, partzymes for the detection of a second target will bind to and cleave a second substrate and so on. For the detection of targets to be specific, there can be no non-specific cleavage of a substrate by partzymes designed to cleave any other substrate in the reaction mix.

The degree of complementarity of the substrate sensor arms of MNAzyme partzymes with other substrates present in the reaction impacts on the specificity of binding. Full complementarity of bases closest to the ribonucleotides are more crucial to specific cleavage. The design guidelines for creation of efficiently cleaved universal substrates include constraints around the sequence composition of universal substrates i.e. seven or more cytosine nucleotides in the ten bases surrounding the ribonucleotides (N₄-N₁₃); the bases immediately adjacent to the ribonucleotides are cytosines (N₈ and N₉); total content of substrate has >64% pyrimidines. These constraints may lead to similarities in the sequence of the substrate close to the ribonucleotides, and possibly result in non-specific cleavage of a universal substrate by partially matched partzymes, especially in a multiplex format where a range of universal substrates are present with their associated partzymes in a single reaction mix. If a substrate is cleaved in a non-

specific manner by partially matched partzymes designed to specifically cleave a second substrate, then that particular combination of substrates may not be suitable for use in multiplex format.

In this example, the universal substrates Sub44, Sub55, Sub61, Sub65, Sub72 and Sub74 were tested for non-specific cleavage activity by the partzymes associated with Sub44, Sub55, Sub72 and Sub74. This involved testing each universal substrate individually with partzyme pairs designed to bind with full complementarity to the other substrates to see if a signal was detected in a MNAzyme qPCR format. The partzymes of Sub72 and Sub74 were chosen for this test as their respective substrates differ by only 3 bases (**Figure 9, (i)a and (ii)a**). The partzymes of Sub55 were chosen to be tested with Sub61 and Sub65 as they are very similar in the central region around the ribonucleotides (N₄-N₁₃) (**Figure 9, (iii)a**). The partzymes of Sub44 were chosen as a control as they are less similar to the other substrates (**Figure 9, (iv)a**).

6.1. Partzyme Oligonucleotides

In the experiments conducted to test for non-specific cleavage by non-complementary partzymes, partzyme oligonucleotides A and B were designed with target sensor arms complementary to the human RPL13a gene and substrate sensor arms complementary to each of the universal substrates as discussed above. The sequences of the A and B partzymes are listed below from 5' to 3', where the bases underlined hybridize to the substrate. The "-P" indicates 3' phosphorylation of the oligonucleotide.

SEQ ID NO: 155 partzyme A RPL13aA/44-P:
AATTGACAAATACACAGAGGTCACAACGAGAGGAGACCTG

SEQ ID NO: 156 partzyme B RPL13aB/44-P:
TCACTATAGGGAGGCTAGCTCTCAAGACCCACGGACTCCT

SEQ ID NO: 118 partzyme A RPL13aA/55-P
TTGACAAATACACAGAGGTCACAACGAGAGGTGCGGT

SEQ ID NO: 119 partzyme B RPL13aB/55-P
GAGCTGGGGAGGCTAGCTCTCAAGACCCACGGACTCCT

SEQ ID NO: 157 partzyme A RPL13aA/72-P:
AATTGACAAATACACAGAGGTCACAACGAGAGGCGTGAT

SEQ ID NO: 158 partzyme B RPL13aB/72-P:
CTGGGAGGAGAGGCTAGCTCTCAAGACCCACGGACTCCT

SEQ ID NO: 159 partzyme A RPL13aA/74-P:
AATTGACAAATACACAGAGGTCACAACGAGGGGAGTGAT

SEQ ID NO: 160. partzyme B RPL13aB/74-P:

CTGGGAGGGGAGGCTAGCTCTCAAGACCCACGGACTCCT

6.2. Reporter Substrates

5 In the current example, the substrates were end labelled with a 6-FAM moiety at the 5' end (indicated by a "F" in the name of the substrates below) and an Iowa Black® FQ quencher moiety at the 3' end (indicated by a "IB" in the name of the substrates below). Cleavage of the substrates was monitored at 530 nm (FAM emission wavelength) with excitation at 485 nm (FAM excitation wavelength). The reporter substrates for this
10 example are shown below with the sequence, 5' to 3'. The lower case bases represent RNA and the upper case bases represent DNA.

SEQ ID NO: 25 Sub44-FB:

CAGGTCTCCTCguCCCTATAGTGA

SEQ ID NO: 29 Sub55-FB:

15 ACCGCACCTCguCCCCAGCTC

SEQ ID NO: 73 Sub61-FB:

CTCGACCCCGuCTCCACGCCA

SEQ ID NO: 74 Sub65-FB:

TCTCGACCTCguCTCCACGCCA

20 SEQ ID NO: 75 Sub72-FB:

ATCACGCCTCguCTCCTCCCAG

SEQ ID NO: 77 Sub74-FB:

ATCACTCCCCguCCCCTCCCAG

25 6.3. Target sequence and PCR primers for amplification of RPL13a

The target sequence for this example was a PCR amplicon from the RPL13a gene generated by *in vitro* PCR amplification of human genomic DNA extracted from the IM9 cell line (Promega) using the oligonucleotide PCR primers listed below. The reporter substrates for this example are shown below with the sequence, 5' to 3'.

30 SEQ ID NO: 161 Forward primer 5RPL13a:

ACCGGAAGAAGAAACAGCTCA

SEQ ID NO: 162 Reverse primer 3RPL13a:

GAGGAATTAACAGTCTTTATTGG

6.4. *Reaction Components: Amplification and measurement of specific and non-specific cleavage of universal substrates*

Real-time PCR amplification and detection of the target sequence was performed in a total reaction volume of 25 μ L. All reactions were conducted in an Mx3005P QPCR system (Stratagene). The cycling parameters were, 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds and 52°C for 60 seconds (data collected at 52°C). Reactions were set up with substrates and partzymes as in **Table 16**. Each set of reaction conditions was tested in duplicate and contained 40 nM 5RPL13a and 200 nM of 3RPL13a, 200 nM of partzyme A, 200 nM partzyme B, 200 nM substrate, 8 mM MgCl₂, 200 μ M of each dNTP, 10 units RNasin (Promega), 1 x Immobuffer (Bioline), 2 units of MyTaqHS™ DNA polymerase (Bioline) and either genomic DNA template (50 ng) or no target (NF-H₂O).

Table 16: Partzyme combinations used for each universal substrate

Partzymes	Substrate	Testing for:
RPL13aA/44-P SEQ ID NO: 155 and RPL13aB/44-P SEQ ID NO: 156	Sub44 SEQ ID NO: 25	Specific cleavage
	Sub55 SEQ ID NO: 29	Non-specific cleavage
	Sub61 SEQ ID NO: 73	Non-specific cleavage
	Sub65 SEQ ID NO: 74	Non-specific cleavage
	Sub72 SEQ ID NO: 75	Non-specific cleavage
	Sub74 SEQ ID NO: 77	Non-specific cleavage
RPL13aA/55-P SEQ ID NO: 118 and RPL13aB/55-P SEQ ID NO: 119	Sub44 SEQ ID NO: 25	Non-specific cleavage
	Sub55 SEQ ID NO: 29	Specific cleavage
	Sub61 SEQ ID NO: 73	Non-specific cleavage
	Sub65 SEQ ID NO: 74	Non-specific cleavage

	Sub72 SEQ ID NO: 75	Non-specific cleavage
	Sub74 SEQ ID NO: 77	Non-specific cleavage
RPL13aA/72-P SEQ ID NO: 157 and RPL13aB/72-P SEQ ID NO: 158	Sub44 SEQ ID NO: 25	Non-specific cleavage
	Sub55 SEQ ID NO: 29	Non-specific cleavage
	Sub61 SEQ ID NO: 73	Non-specific cleavage
	Sub65 SEQ ID NO: 74	Non-specific cleavage
	Sub72 SEQ ID NO: 75	Specific cleavage
	Sub74 SEQ ID NO: 77	Non-specific cleavage
RPL13aA/74-P SEQ ID NO: 159 and RPL13aB/74-P SEQ ID NO: 160	Sub44 SEQ ID NO: 25	Non-specific cleavage
	Sub55 SEQ ID NO: 29	Non-specific cleavage
	Sub61 SEQ ID NO: 73	Non-specific cleavage
	Sub65 SEQ ID NO: 74	Non-specific cleavage
	Sub72 SEQ ID NO: 75	Non-specific cleavage
	Sub74 SEQ ID NO: 77	Specific cleavage

6.5. *Results: Measurement of specific and potential non-specific cleavage by a MNzyme*

There was an increase in fluorescence in all reactions that contained genomic DNA
 5 and a universal substrate with partzymes with substrate sensor arms that were fully
 complementary to the universal substrate (i.e. reactions testing for specific cleavage). The
 fluorescence of the no-DNA target controls was lower than the fluorescence in the

reactions testing for specific cleavage, demonstrating that the increase in fluorescence in reactions testing for specific cleavage was due to target dependent assembly of catalytically active MNazymes that then cleaved the fully complementary universal reporter substrates.

5 There was no increase in fluorescence in any reaction testing cross-reactivity (Figure 9, (i)b - (iv)b). This demonstrates that partzymes designed to cleave these closely related universal substrates only cleaved substrates with which they had full complementarity. These data show that these universal substrates are compatible in multiplex MNzyme qPCR assays.

10 **Example 7: Testing for non-specific cleavage of universal substrates with DNazymes**

The 10-23 DNzyme is a unimolecular structure that can directly bind to and modify a substrate sequence. Unlike the MNzyme, the 10-23 DNzyme does not need a target sequence to form the active core therefore the binding and subsequent cleavage of
15 the substrate by the 10-23 DNzyme is not influenced by the target sequence or having a split catalytic core.

The degree of complementarity of the sensor arms of DNazymes with the substrate impacts on the specificity of binding. Full complementarity of bases closest to the ribonucleotides are more crucial to specific cleavage. The design guidelines for creation
20 of efficiently cleaved universal substrates include constraints around the sequence composition of universal substrates i.e. seven or more cytosine nucleotides in the ten bases surrounding the ribonucleotides (N₄-N₁₃); the bases immediately adjacent to the ribonucleotides are cytosines (N₈ and N₉); total content of substrate has >64% pyrimidines. These constraints may lead to similarities in the sequence of the substrate
25 close to the ribonucleotides, and possibly result in non-specific cleavage of a universal substrate by partially matched DNazymes, especially in a multiplex format where a range of universal substrates are present with their associated DNazymes in a single reaction mix. If a substrate is cleaved in a non-specific manner by partially matched DNazymes designed to specifically cleave a second substrate, then that particular combination of
30 substrates may not be suitable for use in multiplex format.

In this example, the universal substrates Sub55, Sub61, Sub72, Sub74, Sub75, Sub79, Sub80 and Sub85 and their associated 10-23 DNazymes were tested for non-specific cleavage activity. This involved testing every universal substrate individually with the DNazymes designed to bind with full complementarity to all the other substrates

to see if a signal could be detected in an isothermal detection format. These substrates were chosen to be tested as they have similar sequences in different areas of the substrate.

7.1. 10-23 DNase Oligonucleotides

5 The 10-23 DNases used in the experiments conducted to test for non-specific cleavage by non-complementary DNases are listed below from 5' to 3', where the bases underlined hybridize to the substrate and the bases in italics form the catalytic core. Some DNase sequences below contain an extra G at the very 5' and 3' ends. These added bases do not hybridize with the substrate sequence and do not impact on the efficiency at which the DNase cleaved the substrate.

SEQ ID NO: 139 DNase Dz55
 GGAGCTGGGGAGGCTAGCTACAACGAGAGGTGCGGTG
 SEQ ID NO: 141 DNase Dz61
 GTGGCGTGGAGAGGCTAGCTACAACGAGGGGTCGAGG
 15 SEQ ID NO: 142 DNase Dz72
 GCTGGGAGGAGAGGCTAGCTACAACGAGAGGCGTGATG
 SEQ ID NO: 144 DNase Dz74
 GCTGGGAGGGAGGCTAGCTACAACGAGGGGAGTGATG
 SEQ ID NO: 145 DNase Dz75
 20 GTAGTGGGGAGAGGCTAGCTACAACGAGAGGAGGGTCAG
 SEQ ID NO: 147 DNase Dz79
 GGGTTGAAGGGGAGGCTAGCTACAACGAGGGGAGAGGAG
 SEQ ID NO: 148 DNase Dz80
 GGGTTCACGGGAGGCTAGCTACAACGAGAGGCGGTTGG
 25 SEQ ID NO: 150 DNase Dz85
 GCTGGGAGGGGAGGCTAGCTACAACGAGAGGTGCGGTG

7.2. Reporter Substrates

In the current example, the substrates were end labelled with a 6-FAM moiety at the 5' end (indicated by a "F" in the name of the substrates below) and an Iowa Black® FQ quencher moiety at the 3' end (indicated by a "IB" in the name of the substrates below). Cleavage of the substrates was monitored between 510-530 nm (FAM emission wavelength range on CFX96 (BioRad)) with excitation between 450-490 nm (FAM excitation wavelength range on CFX96 (BioRad)). The reporter substrates for this

example are shown below with the sequence, 5' to 3'. The lower case bases represent RNA and the upper case bases represent DNA.

SEQ ID NO: 29 Sub55-FIB:

ACCGCACCTCguCCCCAGCTC

5 SEQ ID NO: 73 Sub61-FIB:

CTCGACCCCGuCTCCACGCCA

SEQ ID NO: 75 Sub72-FIB:

ATCACGCCTCguCTCCTCCCAG

SEQ ID NO: 77 Sub74-FIB:

10 ATCACTCCCCguCCCCTCCCAG

SEQ ID NO: 78 Sub75-FIB:

TGACCCTCCTCguCTCCCCACTA

SEQ ID NO: 80 Sub79-FIB:

TCCTCTCCCCguCCCCTTCAACC

15 SEQ ID NO: 81 Sub80-FIB:

AACCGCCCTCguCCCGTGAACC

SEQ ID NO: 85 Sub85-FIB:

ACCGCACCTCguCCCCTCCCAG

20 7.3. Reaction Components: Measurement of specific and potential non-specific cleavage of universal substrates by a DNAzyme at 52°C and 58°C

Cleavage of universal substrates was measured by monitoring fluorescent signal caused by the binding and subsequent modification of a substrate by a DNAzyme. Cleavage of a universal substrate by a DNAzyme will result in the separation of the fluorophore and quencher producing in an increase in fluorescence. All reactions, as outlined in **Table 17**, contained 1x PCR Buffer II (Applied Biosystems), 10 mM MgCl₂, 200 nM Substrate and NF-H₂O in total volume of 25 µL. Each reaction was run in duplicate as either a "test" (addition of 10 nM DNAzyme) or "control" (addition of NF-H₂O) reaction. Reactions were performed on a CFX96™ Real-Time PCR Detection System (BioRad) at 52 and 58°C. Fluorescence for each reaction was programmed to be read after 1 second for the first 50 cycles and then programmed to be read after 25 seconds for the next 50 cycles.

Table 17: DNAzyme-Substrate combinations tested for specific and non-specific cleavage

	Dz55	Dz61	Dz72	Dz74	Dz75	Dz79	Dz780	Dz85
Sub55	test	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Sub61	-ve	test	-ve	-ve	-ve	-ve	-ve	-ve
Sub72	-ve	-ve	test	-ve	-ve	-ve	-ve	-ve
Sub74	-ve	-ve	-ve	test	-ve	-ve	-ve	-ve
Sub75	-ve	-ve	-ve	-ve	test	-ve	-ve	-ve
Sub79	-ve	-ve	-ve	-ve	-ve	test	-ve	-ve
Sub80	-ve	-ve	-ve	-ve	-ve	-ve	test	-ve
Sub85	-ve	-ve	-ve	-ve	-ve	-ve	-ve	test

test; substrate and DNAzyme arms fully complementary (testing for specific cleavage)

-ve; control reactions, substrate and DNAzyme arms not fully complementary (testing for non-specific cleavage)

7.5. Results: Measurement of specific and potential non-specific cleavage of universal substrates by a DNAzyme

There was an increase in fluorescence in each 'test' reaction containing DNAzymes and their fully complementary substrates. There was no increase in fluorescence in any reaction that did not contain DNAzyme (No DNAzyme added). This demonstrates that the increase in fluorescence produced in the DNAzyme-containing 'test' reactions was due to the binding and subsequent catalytic cleavage of the reporter substrate by the DNAzyme.

For each substrate data set (test and control reactions), the raw fluorescence data points were exported into Excel (Microsoft), duplicate values were averaged and then normalised. Normalisation was performed by dividing each averaged data point by the averaged value of the no-DNAzyme reaction after the first reading of reactions containing the same substrate (e.g. the averaged fluorescence for the test reaction for Sub61 was divided by the averaged cycle 1 fluorescence (after the first 8 seconds) for the Sub61 no-DNAzyme reaction; the averaged fluorescence for the no-DNAzyme control reaction for Sub61 was divided by the averaged cycle 1 fluorescence for the no-DNAzyme control reaction.) These normalised data were then used to calculate the signal to noise ratio at 10 minutes, by dividing the test normalized fluorescence at 10 minutes by the no-DNAzyme normalized fluorescence at 10 minutes. This calculation of signal to noise was performed for each temperature. The signal to noise ratio was then plotted on a bar graph to compare the efficiency of cleavage of each universal substrate with each DNAzyme at the various temperatures tested (**Figure 10, (i) and (ii)**).

Some reactions with various combinations of substrates and non-complementary DNazymes showed a slightly raised fluorescence level compared to the paired no-DNAzyme control reaction. This signal did not increase over time and is therefore not indicative of cleavage of the universal substrate by the non-complementary DNAzyme. Detection plots showing this horizontal background fluorescence all had a signal to noise of less than 1.2. Any reaction showing a signal to noise above 1.2 was therefore deemed to either (i) indicate cleavage of a universal substrate (either specific or non-specific), or (ii) indicate that a particular combination of substrate and non-complementary DNAzyme produce a level of background noise that would not be distinguishable from specific cleavage when in a multiplex format.

All combinations of universal substrates with their fully matched DNazymes showed high signal to noise ratios (above the threshold of 1.2) at both temperatures tested (**Figure 10, (i) and (ii)**).

There was no non-specific cleavage of the universal substrates Sub61, Sub74, Sub75, Sub79 and Sub80 by any non-complementary DNAzyme at either temperature (**Figure 10, (i) and (ii)**).

At 52°C some universal substrates were cleaved by DNazymes that were not fully matched to the substrate (where cleavage is defined as a signal to noise ratio above the threshold of 1.2 as described above). The combinations showing non-specific cleavage were: Sub85 non-specifically cleaved by Dz55, Sub72 non-specifically cleaved by Dz74 and Dz85, and Sub55 non-specifically cleaved by Dz85 (**Figure 10, (i)**).

The non-specific cleavage of Sub55 by Dz85 could be expected as Sub55 and Dz85 differ by only four bases. Alignment of Sub55 with Dz85 shows the four base mismatch is at the distal end of the 3' substrate arm away from the critical region adjacent to the ribonucleotides (**Table 18**). Further, one of the 4 bases is a G/T mismatch which is known in the art to bind with some affinity, albeit weaker than for A/T and G/C matches. The non-specific cleavage of Sub85 by Dz55 may also be expected as Sub85 and Dz55 differ by only five bases and this five base mismatch is found at the distal end of the 3' substrate arm away from the critical region adjacent to the ribonucleotides (**Table 18**). However unlike the reverse scenario above, there are no G/T mismatches present and therefore it appears the Sub85 is not cleaved as efficiently as the reverse combination of Sub55 and Dz85. The non-specific cleavage of Sub72 by Dz74 could be expected as Sub72 and Sub74 differ by only 3 bases. Alignment of Sub72 with Dz74 shows the two mismatches closest to the ribonucleotides are G/T mismatches (**Table 18**). This also explains why Sub74 is not cleaved by Dz72 as the two mismatches are C/A and occur very close to the

ribonucleotides and this is enough to disable cleavage of the substrate by the DNAzyme (Table 18). The non-specific cleavage of Sub72 by Dz85 can be explained by the alignment of the sequences in Table 18, which shows that the mismatch bases are primarily G/T and this would therefore lead Dz85 to bind and cleave Sub72. Again, the reverse situation of Dz72 non-specifically cleaving Sub85 would not be expected as the relevant mismatches become the more destabilising C/A mismatches which are unlikely to lead to strong enough binding between the DNAzyme and the substrate to lead to cleavage.

Table 18. Alignment of Substrates and DNAzymes where non-specific cleavage was observed.

	Name of oligonucleotide	Sequence [^]
Substrate	Sub55 (Seq ID: 29)	ACCGCACCTCguCCCC <u>AGCTC</u>
DNAzyme	Dz85 (Seq ID: 150)	GTGGCGTGGAG AGGGG <u>AGGGT</u> CG GA GG AC CT ACATCGA
Substrate	Sub85 (Seq ID: 85)	ACCGCACCTCguCCCC <u>TCCAG</u>
DNAzyme	Dz55 (Seq ID: 139)	GTGGCGTGGAG AGGGG <u>TCCAGG</u> GA GG AC CT ACATCGA
Substrate	Sub72 (Seq ID: 75)	ATCAC <u>G</u> CCTCguCTCCTCCCA
DNAzyme	Dz74 (Seq ID: 144)	GTAGTG <u>AGGGG</u> AGGGGAGGGTCG GA GG AC CT ACATCGA
Substrate	Sub72 (Seq ID: 75)	ATCA <u>C</u> GCCTCguCTCCTCCAG
DNAzyme	Dz85 (Seq ID: 150)	GTGG <u>C</u> TGGAG AGGGGAGGGTCG GA GG AC CT ACATCGA
Substrate	Sub74 (Seq ID: 77)	ATCACT <u>TCCC</u> CguCCCTCCAG
DNAzyme	Dz72 (Seq ID: 142)	GTAGTG <u>CGGAG</u> AGAGGAGGGTCG GA GG AC CT ACATCGA

^ Substrate sequence written 5' to 3' and DNAzyme sequence written 3' to 5'. Mismatched bases are in bold and underlined and mismatched bases that are G/T are only in bold. The DNAzyme core sequences are in italics.

5 Increasing the reaction temperature to 58°C created more stringent conditions for hybridization of oligonucleotides and resulted in the loss of almost all the non-specific cleavage seen at 52°C (**Figure 10, (ii)**). The only combination of universal substrate and DNAzyme that showed non-specific cleavage at 58°C was Sub55 being cleaved non-specifically by Dz85. This non-specificity is to be expected as Sub55 and Dz85 differ by
10 only four bases and this four base mismatch is found at the distal end of the substrate arm away from the critical region adjacent to the ribonucleotides and one of the mismatches is a G/T (**Table 18**). Even though there are only three bases different between Sub72 and Sub74, this three base mismatch occurs in the critical region close to the ribonucleotides and are therefore more critical for specificity and will be more destabilising at higher
15 temperatures that create more stringent conditions for binding of oligonucleotides.

 These results demonstrate that the design guidelines produce universal substrates that can be effectively multiplexed at a range of temperatures for applications involving 10-23 DNAzymes. There is a need for some optimization of reaction temperature to provide sufficient stringency of binding for some combinations of substrates, but overall
20 the guidelines produce sets of substrates that can be multiplexed. One skilled in the art can appreciate that the length of substrate and DNAzyme binding arms can be adjusted to create more stringent binding at lower and higher temperatures.

Example 8: Use of universal substrates with multiplex MNAzyme qPCR for the
25 **quantification of five different nucleic acid targets in a multiplex reaction with annealing temperatures of 52°C or 58°C.**

 Multiple targets can be simultaneously amplified and detected in real time using *in vitro* target amplification methods such as qPCR. Further, the amplification of the targets can be simultaneously monitored in real-time in one multiplexed reaction that comprises
30 multiple unique MNAzymes. Each MNAzyme can be designed with sensor arms specific for one target and substrate arms specific for a unique member of a series of universal substrates. Each target can be individually detected if each of the series of universal substrates is labelled with a different fluorophore. The amplification and detection of the multiple targets are performed in a one-step process, wherein PCR
35 amplification and MNAzyme-mediated detection occur simultaneously in a single tube.

Real-time monitoring generates an amplification curve that can indicate the efficiency of a reaction by the shape of the curve (steepness and speed to reach plateau).

The annealing/detection temperature for MNzyme qPCR used in the art is between 50 and 54°C. This temperature was dictated by the fact that the universal substrates known in the art had a limitation on the temperature at which they were efficiently cleaved with 54°C being the upper limit for efficient cleavage of the series 1 universal substrates. There is a need for a panel of universal substrates that can be combined in a multiplex reaction and efficiently cleaved at higher temperatures. Not only does this allow greater flexibility in design of primers and partzymes that anneal at higher temperatures and the targeting of G/C rich templates, but it would also enable MNzyme qPCR detection to be multiplexed with other real-time chemistries well known in the art such as TaqMan® in which the standard annealing/detection temperature ranges from 60 - 65°C. Utility of universal substrates would be greatly increased if substrates existed that worked well together at a greater range of temperatures.

In this example, two multiplex reactions were performed both comprising MNzymes designed to detect five different targets, namely human TFRC, HPRT, TP53, RPL13a and CYP2C9 genes. In Multiplex 1, each target MNzyme was designed to cleave one of the series 1 universal substrates, Sub2, Sub3, Sub4, Sub6 and Sub7 and in Multiplex 2 each target MNzyme was designed to cleave one of the improved series 2 or 3 universal substrates, Sub55, Sub61, Sub74, Sub79 and Sub80. It will be appreciated that any number of targets can be used in accordance with the method and that those skilled in the art can design appropriate partzymes to detect any target.

The two multiplex reactions were compared to determine the cleavage efficiency of each set of universal substrates by looking at the shape of the curve (steepness and speed to reach plateau). In this example, amplification and detection are compared at a temperature favourable to all substrates, 52°C, and a temperature outside of the efficient range for series 1 substrates, 58°C.

8.1. Partzyme Oligonucleotides

The sequences of the partzymes A and B for each target are listed below from 5' to 3'. For each target, the partzymes were designed to be used with one of the original series 1 universal substrates and one of the novel improved substrates from series 3. In the following sequences, the bases underlined hybridize to the substrate. The "-P" indicates 3' phosphorylation of the oligonucleotide.

SEQ ID NO: 157 partzyme A RPL13aA/6-P
AATTGACAAATACACAGAGGTCACAACGAGAGGCGTGAT
SEQ ID NO: 163 partzyme B RPL13aB/6-P
CTGGGAGGAAGGCTAGCTCTCAAGACCCACGGACTCCT
5 SEQ ID NO: 120 partzyme A RPL13aA/80-P
AATTGACAAATACACAGAGGTCACAACGAGAGGGCGGTT
SEQ ID NO: 121 partzyme B RPL13aB/80-P
GTTTCACGGGAGGCTAGCTCTCAAGACCCACGGACTCCT
SEQ ID NO: 96 partzyme A CYP2C9A/3-P
10 GGGAAGAGGAGCATTTGAGGAACAACGAGGTTGTGCTG
SEQ ID NO: 97 partzyme B CYP2C9B/3-P
CGGTTGGTGAGGCTAGCTCCGTGTTCAAGAGGAAGC
SEQ ID NO: 98 partzyme A CYP2C9A/61-P
GGGAAGAGGAGCATTTGAGGAACAACGAGGGGTCGAG
15 SEQ ID NO: 99 partzyme B CYP2C9B/61-P
TGGCGTGAGAGGCTAGCTCCGTGTTCAAGAGGAAGC
SEQ ID NO: 164 partzyme A TP53A/4-P
GACGGAACAGCTTTGAGGTGACAACGAGTGCGCCATG
SEQ ID NO: 165 partzyme B TP53B/4-P
20 TACTTCTCCCAAGGCTAGCTCGTGTTTGTGCCTGTCCTGG
SEQ ID NO: 106 partzyme A TP53A/79-P:
GACGGAACAGCTTTGAGGTGACAACGAGGGGAGAGGA
SEQ ID NO: 107 partzyme B TP53B/79-P:
GGTTGAAGGGGAGGCTAGCTCGTGTTTGTGCCTGTCCTGG
25 SEQ ID NO: 34 partzyme A TFRCA/2-P:
GGAATATGGAAGGAGACTGTCACAACGAGAGGAAACCTT
SEQ ID NO: 35 partzyme B TFRCB/2-P:
TGCCCAGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 58 partzyme A TFRCA/74-P:
30 GGAATATGGAAGGAGACTGTCACAACGAGGGGAGTGAT
SEQ ID NO: 59 partzyme B TFRCB/74-P:
CTGGGAGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT
SEQ ID NO: 166 partzyme A HPRTA/7-P
CTGAATAGAAATAGTGATAGATCACAACGAGTGCCATGTTAA
35 SEQ ID NO: 167 partzyme B HPRTB/7-P

TATCACAGCCAAGGCTAGCTCATTCCTATGACTGTAGATTTTA

SEQ ID NO: 168 partzyme A HPRTA/55-P

CTGAATAGAAATAGTGATAGATCACAACGAGAGGTGCGGT

SEQ ID NO: 169 partzyme B HPRTB/55-P

GAGCTGGGGAGGCTAGCTCATTCCTATGACTGTAGATTTTA

8.2. Reporter Substrates

For this example, in each multiplex five different universal substrates were used together in the one reaction chamber. Each universal substrate in each multiplex was labelled with one of five different fluorophores. In the current example, the substrates were 5' end labelled with a fluorophore and 3' end labelled with a quencher moiety (Table 19). Cleavage of the substrates was monitored at various emission and excitation wavelengths (Table 19).

Table 19. Substrates and their fluorescent labelling

Substrate	Name	Fluorophore	Quencher [^]	Excitation*	Emission*
Multiplex 1					
Sub2	Sub2-Q705B2	Quasar 705	BHQ2	672-684	705-730
Sub6	Sub6-Q670B2	Quasar 670	BHQ2	620-650	675-690
Sub4	Sub4-TRB2	Texas Red	BHQ2	560-590	610-650
Sub7	Sub7-JB	JOE	BHQ1	515-535	560-580
Sub3	Sub3-FIB	6-FAM	IB	450-490	510-530
Multiplex 2					
Sub74	Sub74-Q705B2	Quasar 705	BHQ2	672-684	705-730
Sub80	Sub80-Q670B2	Quasar 670	BHQ2	620-650	675-690
Sub79	Sub79-TRIBR	Texas Red	IBR	560-590	610-650
Sub55	Sub55-HIB	HEX	IB	515-535	560-580
Sub61	Sub61-FIB	6-FAM	IB	450-490	510-530

[^] BHQ1; black hole quencher 1, BHQ2; black hole quencher 2, IB; Iowa black® FQ, IBR; Iowa black® RQ

*CFX96 Real-Time PCR Detection System (Biorad) excites, and measures emission of, each fluorophore over a range of wavelengths for each channel.

The reporter substrates tested in this example are shown below with the sequence, 5' to 3'. The lower case bases represent RNA and the upper case bases represent DNA.

SEQ ID NO: 21 Sub2-Q705B2:

AAGGTTTCCTCguCCCTGGGCA

SEQ ID NO: 22 Sub3-FIB:

CAGCACAACCguCACCAACCG

SEQ ID NO: 23 Sub6-Q670B2:

ATCACGCCTCguTCCTCCCAG
 SEQ ID NO: 24 Sub7-JB:
 TTAACATGGCACguTGGCTGTGATA
 SEQ ID NO: 171 Sub4-TRB2:
 5 CATGGCGCACguTGGGAGAAGTA
 SEQ ID NO: 73 Sub61-FIB:
 CTCGACCCCguCTCCACGCCA
 SEQ ID NO: 77 Sub74-Q705B2:
 ATCACTCCCCguCCCCTCCCAG
 10 SEQ ID NO: 80 Sub79-TRIBR:
 TCCTCTCCCCguCCCCTTCAACC
 SEQ ID NO: 81 Sub80-Q670B2:
 AACCGCCCTCguCCCGTGAACC
 SEQ ID NO: 29 Sub55-HIB:
 15 ACCGCACCTCguCCCCAGCTC

8.3 Target sequences and PCR primers for amplification of the *CYP2C9*, *TP53*, *HPRT*, *TFRC* and *RPL13a* genes

The target PCR amplicons for all five genes were generated by *in vitro*
 20 amplification of Human DNA extracted from IM9 cell line (Promega). The amplicons
 were generated using the oligonucleotide PCR primers listed 5' to 3' below. The
 sequence in bold corresponds to a universal tag (U1, U2 or U3) that increases the T_m of
 the primer without affecting the specificity of the primer to the gene target. This tag
 improves amplification efficiency in PCR reactions.

25 SEQ ID NO: 91 Forward primer 5TFRC_U1
GCTAAACAATAACTCAGAACTTACG
 SEQ ID NO: 92 Reverse primer 3TFRC_U2
CAGCTTTCTGAGGTACCATCCTA
 SEQ ID NO: 125 Forward primer 5TP53_U3
 30 **CTAACTTACTGCCTCTTGCTTCTC**
 SEQ ID NO: 126 Reverse primer 3TP53_U2
CAGCTCTGTGCGCCGGTCTCTC
 SEQ ID NO: 127 Forward primer 5RPL13a_U3
CTAAACCGGAAGAAGAAACAGCTCA
 35 SEQ ID NO: 128 Reverse primer 3RPL13a_U2

CAGGAGGAATTAACAGTCTTTATTGG

SEQ ID NO: 129 Forward primer 5CYP2C9_U3

CTAACCTCATGACGCTGCGGAA

SEQ ID NO: 130 Reverse primer 3CYP2C9_U2

CAGATATGGAGTAGGGTCACCCA

SEQ ID NO: 176 Forward primer 5HPRT_U3

CTAACTTTGCTGACCTGCTGGATTA

SEQ ID NO: 177 Reverse primer 3HPRT_U2

CAGCAATAGCTCTTCAGTCTGATAA

8.4. Reaction Components: Amplification and detection of target sequences in a multiplex MNazyme qPCR format

Real-time amplification and detection of the target sequences was performed in total reaction volume of 25 μ L. All reactions were conducted in a CFX96 Real-Time PCR Detection System (Bio-Rad). The cycling parameters were either, 1) 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds and 52°C for 60 seconds or 2) 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds and 58°C for 60 seconds. Fluorescent data were collected at either the 52°C or 58°C step. Each multiplex reaction was run in duplicate and contained 10 mM MgCl₂, 200 μ M of each dNTP, 10 units Ribosafe RNase inhibitor (Bioline), 1 x Immobuffer (Bioline), 2 units MyTaqHS (Bioline). The identity of the partzymes, primers and substrates and their respective concentrations were as listed in **Table 20**. Reactions contained either DNA template (100 ng or 391 pg) or no target control (NF-H₂O).

Multiplex reactions were set up with primers, substrates and their associated partzymes as in **Table 20** (Multiplex 1 or Multiplex 2). The same PCR primers were used for both multiplex reactions and all partzymes had the same target-sensing portions. Any differences in efficiency of reactions detecting the same target will therefore be attributable to differences in the efficiency of cleavage of the substrates.

Table 20. Oligonucleotide combinations used for each multiplex reaction

Target	5' primer (20 nM except * = 80 nM)	3'Primer (400 nM except * = 200 nM)	PzA & PzB (200 nM each)		Substrate (200 nM each)
Multiplex 1					
TFRC	5TFRC_U3	3TFRC_U2	TFRCA/2-P SEQ ID NO: 34	TFRCB5/2-P SEQ ID NO: 35	Sub2-Q705B2 SEQ ID NO: 21
HPRT	5HPRT_U3*	3HPRT_U2	HPRTA/7-P SEQ ID NO: 166	HPRTB5/7-P SEQ ID NO: 167	Sub7-JB SEQ ID NO: 24
TP53	5TP53_U1	3TP53_U2*	TP53A/4-P SEQ ID NO: 164	TP53B5/4-P SEQ ID NO: 165	Sub4-TRB2 SEQ ID NO: 171
RPL13a	5RPL13a_U1	3RPL13a_U2	RPL13aA/6-P SEQ ID NO: 157	RPL13aB/6-P SEQ ID NO: 163	Sub6Q670B2 SEQ ID NO: 23
CYP2C9	5CYP2C9_U1	3CYP2C9_U2	CYP2C9A/3-P SEQ ID NO: 96	CYP2C9B/3-P SEQ ID NO: 97	Sub3-FIB SEQ ID NO: 22
Multiplex 2					
TFRC	5TFRC_U3	3TFRC_U2	TFRCA/74-P SEQ ID NO: 58	TFRCB/74-P SEQ ID NO: 59	Sub74-Q705B2 SEQ ID NO: 77
HPRT	5HPRT_U3*	3HPRT_U2	HPRTA/55-P SEQ ID NO: 168	HPRTB/55-P SEQ ID NO: 169	Sub55-HIB SEQ ID NO: 29
TP53	5TP53_U1	3TP53_U2*	TP53A/79-P SEQ ID NO: 106	TP53B/79-P SEQ ID NO: 107	Sub79-TRIBR SEQ ID NO: 80
RPL13a	5RPL13a_U1	3RPL13a_U2	RPL13aA/80-P SEQ ID NO: 120	RPL13aB/80-P SEQ ID NO: 121	Sub80-Q670B2 SEQ ID NO: 81
CYP2C9	5CYP2C9_U1	3CYP2C9_U2	CYP2C9A/61-P SEQ ID NO: 98	CYP2C9B/61-P SEQ ID NO: 99	Sub61-FIB SEQ ID NO: 73

8.5. Results: Amplification of target and cleavage of reporter substrate in multiplex MNazyme qPCR format

Each multiplex reaction containing human genomic DNA showed an increase in fluorescence over time for the real-time detection of the genes CYP2C9, TP53, HPRT, RPL13a and TFRC. For all reactions, the fluorescence of the no-DNA target control was lower than that in the DNA target-containing reactions. This demonstrates that the increase in fluorescence produced in target-containing reactions is due to target dependent assembly of catalytically active MNazymes that then cleaved one of the universal substrates.

The amplification plots of the CYP2C9, TP53, HPRT, RPL13a and TFRC genes at 52°C demonstrated that Multiplex 2 (Figure 11, (i)), which used the new improved series 2 and 3 universal substrates, had steeper curves that reached plateau faster than those observed for Multiplex 1 (Figure 11, (i)), which used series 1 universal substrates. There was only a small difference in the amplification plots for detection of TFRC with Sub2 versus Sub74 at 52°C. One skilled in the art would appreciate that all of the amplification

plots of Multiplex 1, using series 1 vs series 2 and 3 substrates, are of a sufficiently good quality to be readily acceptable in the field for detection of these targets.

The amplification plots of the CYP2C9, TP53, HPRT, RPL13a and TFRC genes at 58°C demonstrated that Multiplex 2 (**Figure 11, (ii)**), which used the new improved series 2 and 3 universal substrates, had considerably steeper curves and reached plateau substantially faster than Multiplex 1 (**Figure 11, (ii)**), which used series 1 universal substrates. Further, one skilled in the art would acknowledge that the amplification curves produced by use of Sub2, Sub3, Sub6 and Sub7 in Multiplex 1 may not be of sufficient quality for robust detection of target and thus may not be generally acceptable in the field.

Overall, the new series 2 and 3 substrates show improved quality, and therefore robustness, of multiplex data at temperatures currently in use with MNAzyme qPCR reactions and enable multiplex detection at a temperature much higher than previously capable. The new design guidelines increase the probability of designing universal substrates that extend the ability to multiplex at temperatures currently used for MNAzyme qPCR and that produce robust data at higher reaction temperatures.

Example 9: Use of universal substrates, designed to be functional at various temperatures, with MNAzymes in real-time PCR.

A new series of highly active substrates has been invented, using a novel set of design guidelines, for use with the 10-23 DNAzyme or MNAzyme based on the 10-23 DNAzyme.

MNAzymes can be tailored to produce a detectable effect, via cleavage or ligation of a substrate, at various temperatures. The efficiency and stringency of catalytic activity of an MNAzyme or DNAzyme can be manipulated by changing the reaction temperature. Another way to optimise efficiency and stringency of catalytic activity is to modify the T_m and/or length of the substrate(s) and matching partzymes or matching DNAzyme. The T_m of a substrate can be increased by adding nucleotides on to the 5' and/or 3' ends of the substrate sequence. These changes can be made within the design guidelines. The nucleotides chosen for this extension can also have an effect on the T_m . Addition of extra G or C bases to the 3' or 5' end will have a greater impact on the T_m than the addition of extra A or T bases. Similarly, the T_m of substrates can be reduced by removing nucleotides from the 3' or 5' end. MNAzyme partzymes and DNAzymes can be truncated or extended to match the adjusted substrate sequence.

In this example, the length and base composition of the series 2 substrate, Sub55, was modified to produce a range of derivative substrates with a variety of T_m 's (**Table**

21). Modifications included truncating the substrate by removal of one to three nucleotides from each of the 5' and 3' ends; extending the substrate by the addition of nucleotides to both of the 5' and 3' ends. The effect of adding different nucleotides to produce this extension was also tested by designing substrates with either additional A or C nucleotides at the 5' and 3' ends. The resulting substrates were then tested in a MNazyme qPCR reaction to assess the flexibility of design of derivatives of substrates and their utility at a range of temperatures. The PCR amplification and MNazyme-mediated detection were performed in a one-step process, wherein PCR amplification and MNazyme-mediated detection occurred simultaneously in a single tube. The efficiency of cleavage of substrates can be measured by the Ct value generated at several annealing temperatures. Reactions that produce a lower Ct value are indicative of more efficient cleavage of a specific substrate since such reactions reach the threshold cycle faster.

Table 21: Sub55 and derivatives

Name	Sequence [^]	Length (bp)	Tm*
Sub55(16)	GCACCTCguCCCCAGC SEQ ID NO: 172	16	52
Sub55(18)	CGCACCTCguCCCCAGCT SEQ ID NO: 173	18	58
Sub55	ACCGCACCTCguCCCCAGCTC SEQ ID NO: 29	21	68
Sub55(23A)	AACCGCACCTCguCCCCAGCTCA SEQ ID NO: 174	23	72
Sub55(23C)	CACCGCACCTCguCCCCAGCTCC SEQ ID NO: 175	23	76

[^] All sequences written 5' to 3'; uppercase represents DNA and lowercase represents RNA

* Tm given here equates to the melting temperature of the bases bound to the two partzymes calculated using the Wallace rule. When the substrate is bound to the MNazyme based on the 10-23 DNAzyme, or the 10-23 DNAzyme itself, the "g" ribonucleotide remains unbound therefore does not contribute to the overall bound Tm.

9.1. Partzyme Oligonucleotides

In the experiments conducted to measure the rate of catalytic activity of Sub55 and its' derivatives described in Table 21, the partzyme oligonucleotides A and B were designed with target sensor arms complementary to the human TFRC gene. The sequences of the A and B partzymes are listed below from 5' to 3', where the bases underlined hybridize to the substrate. The "-P" indicates 3' phosphorylation of the oligonucleotide.

SEQ ID NO: 179 partzyme A TFRCA/55(18)-P:
 GGAATATGGAAGGAGACTGTCACAACGAGAGGTGCG

SEQ ID NO: 180 partzyme B TFRCB/55(18)-P:
 AGCTGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT

5 SEQ ID NO: 181 partzyme A TFRCA/55(16)-P:
 GGAATATGGAAGGAGACTGTCACAACGAGAGGTGC

SEQ ID NO: 182 partzyme B TFRCB/55(16)-P:
 GCTGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT

SEQ ID NO: 183 partzyme A TFRCA/55(23A)-P:
 10 GGAATATGGAAGGAGACTGTCACAACGAGAGGTGCGGTT

SEQ ID NO: 184 partzyme B TFRCB/55(23A)-P:
 TGAGCTGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT

SEQ ID NO: 185 partzyme B TFRCA/55(23C)-P:
 GGAATATGGAAGGAGACTGTCACAACGAGAGGTGCGGTG

15 SEQ ID NO: 186 partzyme B TFRCB/55(23C)-P:
 GGAGCTGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT

SEQ ID NO: 46 partzyme A TFRCA/55-P:
 GGAATATGGAAGGAGACTGTCACAACGAGAGGTGCGGT

SEQ ID NO: 45 partzyme B TFRCB/55-P:
 20 GAGCTGGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT

9.2. Reporter Substrates

In the current example, the substrates were end labelled with a Quasar 670 moiety at the 5' end and a BHQ2 moiety at the 3' end. Cleavage of the substrates was monitored at 665 nm (Quasar 670 emission wavelength) with excitation at 635 nm (Quasar 670
 25 excitation wavelength). The reporter substrates tested in this example are shown below with the sequence, 5' to 3'. The lower case bases represent RNA and the upper case bases represent DNA.

SEQ ID NO: 29 Sub55-Q670B2:
 30 ACCGCACCTCguCCCCAGCTC

SEQ ID NO: 172 Sub55(16)-Q670B2:
 GCACCTCguCCCCAGC

SEQ ID NO: 173 Sub55(18)-Q670B2:
 CGCACCTCguCCCCAGCT

35 SEQ ID NO: 174 Sub55(23A)-Q670B2:

AACCGCACCTCguCCCCAGCTCA

SEQ ID NO: 175 Sub55(23C)-Q670B2:

CACCGCACCTCguCCCCAGCTCC

5 9.3. Target sequence and PCR primers for amplification of TFRC

The target sequence for this example was a PCR amplicon generated by *in vitro* amplification of human genomic DNA, extracted from the IM9 cell line (Promega), using the oligonucleotide PCR primers listed below. Primer sequences are listed 5' to 3'.

10 SEQ ID NO: 187 Forward primer 5TFRC:

AACAATAACTCAGAACTTACG

SEQ ID NO: 188 Reverse primer 3TFRC:

CTTTCTGAGGTTACCATCCTA

15 9.4. Reaction Components: Amplification and detection of target sequence

Real-time amplification and detection of the target sequence was performed in a total reaction volume of 25 μ L. All reactions were conducted in a Mx3005P QPCR system (Stratagene/Agilent). The cycling parameters were varied by the annealing temperature, (underlined as follows), and were either,

- 20 1) 95°C for 10 minutes, 5 cycles of 95°C for 15 seconds and 55°C for 30 seconds, 50 cycles of 95°C for 15 seconds and 50°C for 60 seconds, or
- 2) 95°C for 10 minutes, 5 cycles of 95°C for 15 seconds and 55°C for 30 seconds, 50 cycles of 95°C for 15 seconds and 52°C for 60 seconds, or
- 3) 95°C for 10 minutes, 5 cycles of 95°C for 15 seconds and 55°C for 30
- 25 seconds, 50 cycles of 95°C for 15 seconds and 55°C for 60 seconds, or
- 4) 95°C for 10 minutes, 5 cycles of 95°C for 15 seconds and 55°C for 30 seconds, 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

All fluorescent data were collected at the annealing temperature. Reactions were set up with substrates and their associated partzymes as in **Table 22**. Each set of reaction

30 conditions were run in duplicate and contained 40 nM 5TFRC, 200 nM of 3TFRC, 200 nM each of partzyme A and partzyme B, 200 nM of substrate, 8 mM MgCl₂, 200 μ M of each dNTP, 10 units Rnasin (Promega), 1 x Immobuffer (Bioline), 1 unit of Immolase (Bioline) and either genomic DNA template (100 ng) or no target (NF-H₂O). Separate reactions were set up to test each substrate with its matched partzymes. The same PCR

35 primers were used for all reactions and all partzymes had the same target-sensing

portions. Any differences in efficiency of reactions will therefore be attributable to differences in the efficiency of cleavage of the substrates at various temperatures.

Table 22: Partzyme combinations used for each universal substrate

Substrate	Partzyme A	Partzyme B
Sub55 SEQ ID NO: 29	TFRCA/55-P SEQ ID NO: 46	TFRCB/55-P SEQ ID NO: 45
Sub55(16) SEQ ID NO: 172	TFRCA/55(16)-P SEQ ID NO: 181	TFRCB/55(16)-P SEQ ID NO: 182
Sub55(18) SEQ ID NO: 173	TFRCA/55(18)-P SEQ ID NO: 179	TFRCB/55(18)-P SEQ ID NO: 180
Sub55(23A) SEQ ID NO: 174	TFRCA/55(23A)-P SEQ ID NO: 183	TFRCB/55(23A)-P SEQ ID NO: 184
Sub55(23C) SEQ ID NO: 175	TFRCA/55(23C)-P SEQ ID NO: 185	TFRCB/55(23C)-P SEQ ID NO: 186

5

9.5. Results: Amplification of target and cleavage of reporter substrate

Each reaction containing human genomic DNA showed an increase in fluorescence over time for real time detection of the TFRC gene using Sub55 and various derivatives. The fluorescence of the no-DNA target control was lower than that in the DNA target-
10 containing reactions. This demonstrates that the increase in fluorescence produced in target-containing reactions is due to target dependent assembly of catalytically active MNazymes that then cleaved one of the universal substrates.

The efficiency of the reactions, measured by the Ct, was dependent on the compatibility of the reaction temperature (annealing/cleavage temperature) and the Tm of
15 the substrate used in the reaction. The various Sub55 derivatives have different lengths and nucleotide compositions and thus different melting temperatures (Tm) and are therefore expected to perform differently at the various annealing temperatures tested (50, 52, 55, and 60°C). The results, in **Table 23**, show the Ct for each substrate at the different reaction temperatures. The Ct values in bold indicate the substrates(s) which performed
20 most efficiently at the temperatures indicated.

At lower temperatures the shorter substrate (Sub55(18)) performed better (had the lowest Ct value). As the annealing temperature increased, substrates with increased length and therefore Tm, performed optimally. One skilled in the art could produce derivatives of substrates that could be efficiently cleaved at a chosen reaction temperature by
25 lengthening or shortening the substrate arms from the 5' and/or 3' ends, and/or changing the nucleotide composition at the 5' and 3' ends of the substrate.

Table 23: Ct values for MNAzyme qPCR performed using Sub55 and derivatives at various annealing temperatures

Name	50°C	52°C	55°C	60°C
Sub55(16)	26.1	26.7	31.2	n/a
Sub55(18)	23.9	23	24.8	n/a
Sub55	24.4	22.8	23.0	26.8
Sub55(23A)	25.3	23.9	23.0	25.8
Sub55(23C)	25.8	24.1	23.9	26.2

Example 10: Use of universal substrates with MNAzymes qPCR at an annealing temperature of 58°C.

MNAzymes can be used to monitor amplification of target nucleic acids in real-time using *in vitro* target amplification methods such as PCR. Furthermore, real-time monitoring during qPCR using MNAzyme substrates labelled with fluorophore and quencher pairs generates a curve on which a threshold line, of an arbitrary level of fluorescence, can be placed over the exponential phase of the reactions, producing a value which can be known as a Ct (cycle threshold). Reactions that produce a lower Ct value are indicative of more efficient cleavage of a specific substrate since such reactions reach the threshold cycle faster. In this example, amplification and detection are performed in a one-step process, wherein PCR amplification and MNAzyme-mediated detection occur simultaneously in a single tube. Where all other reaction conditions are the same the Ct value can be influenced by the sequence of the universal substrate. The annealing/detection temperature for MNAzyme qPCR used in the art is between 50 and 54°C. This temperature was dictated by the fact that the universal substrates known in the art had a limitation on the temperature at which they were efficiently cleaved with 54°C being the upper limit for the series 1 universal substrates. There is a need for universal substrates that cleave at higher temperatures to allow greater flexibility in design of primers and partzymes that anneal at higher temperatures. This design flexibility for primers and partzymes could be of great benefit for many applications such as genetic targets of interest that have high percentages of G and C bases in their sequence, requiring higher reaction temperatures and hence partzymes and primers with higher Tms for specific detection.

Investigation into efficiency of cleavage of substrates based on the performance of the series 1 and 2 substrates, lead to the development of guidelines to aid in a third round of substrate designs, resulting in the series 3 substrates. These guidelines included but were not limited to (i) seven or more cytosine nucleotides in the ten bases surrounding the ribonucleotides (N₄-N₁₃), (ii) bases immediately adjacent to the ribonucleotides are

cytosines (N₈ and N₉) (iii) total content of substrate has >64% pyrimidines and (iv) total T_m of the oligonucleotide is 66°C or greater (where this latter guideline is only applicable if the reaction temperature for substrate cleavage is above 50°C).

In this example, the series 2 universal substrate, Sub59 is compared to the series 3 substrate, Sub77 to compare the cleavage efficiency in real-time PCR at 58°C to ensure that the design guidelines produce universal substrates with a high probability of applicability to MNazyme qPCR at an elevated temperature. The level of cleavage efficiency was determined by measuring the C_t value for reactions containing different universal substrates.

10.1. Partzyme Oligonucleotides

In the experiments conducted to measure the efficiency of cleavage of the series 2 and series 3 universal substrate in real-time PCR, all the partzyme oligonucleotides A and B were designed with sensor arms complementary to the same sequence of the human TFRC gene. The sequences of the A and B partzymes are listed below from 5' to 3', where the bases underlined hybridize to their matched universal substrate. The “-P” indicates 3' phosphorylation of the oligonucleotide.

SEQ ID NO: 47 partzyme A TFRCA/59-P:

GGAATATGGAAGGAGACTGTCACAACGAAGGGAGGAGG

SEQ ID NO: 62 partzyme A TFRCA/77-P:

GGAATATGGAAGGAGACTGTCACAACGAGAGGGAGGAG

SEQ ID NO: 63 partzyme B TFRCB/77-P:

AGGAGGAGGGAGGCTAGCTCCTCTGACTGGAAAACAGACT

10.2. Reporter Substrates

The reporter substrates for this example are shown below with the sequence, 5' to 3'. The lower case bases represent RNA and the upper case bases represent DNA. In the current example, the substrates were end labelled with a 6-FAM moiety at the 5' end (indicated by a “F” in the name of the substrates below) and an Iowa Black[®] FQ quencher moiety at the 3' end (indicated by a “IB” in the name of the substrates below). Cleavage of the substrates was monitored at 530 nm (FAM emission wavelength on Mx3005P (Stratagene)) with excitation at 485 nm (FAM excitation wavelength on Mx3005P (Stratagene)).

SEQ ID NO: 33 Sub59-FIB:

CCTCCTCCCTguCCCTCCTCCT

SEQ ID NO: 79 Sub77-FIB:

CTCCTCCCTCguCCCTCCTCCT

10.3. Target sequence and PCR primers for amplification of TFRC

The target sequence for this example was a PCR amplicon from the TFRC gene generated by *in vitro* amplification of human genomic DNA, extracted from the IM9 cell line (Promega), using the oligonucleotide PCR primers listed below. Primer sequences are listed 5' to 3'.

SEQ ID NO: 187 Forward primer 5TFRC:

AACAATAACTCAGAACTTACG

SEQ ID NO: 188 Reverse primer 3TFRC:

CTTTCTGAGGTTACCATCCTA

10.4. Reaction Components: Amplification and quantification of target sequence

Real-time PCR amplification and detection of the target sequence was performed in a total reaction volume of 25 μ L. All reactions were conducted in an Mx3005P QPCR system (Stratagene). Reactions were set up with substrates and their associated partzymes as in **Table 24**. The cycling parameters were, 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds and 58°C for 60 seconds (data collected at the 58°C step). Each set of reaction conditions were run in duplicate and contained 40 nM 5TFRC, 200 nM of 3TFRC, 200 nM each of partzyme A and partzyme B, 200 nM substrate, 8 mM MgCl₂, 200 μ M of each dNTP, 10 units RNasin (Promega), 1 x Immobuffer (Bioline), 2 units of MyTaqHS™ DNA polymerase (Bioline) and either genomic DNA template (50 ng) or no target (NF-H₂O).

Table 24: Partzyme combinations used for each universal substrate

Substrate	Partzyme A	Partzyme B
Sub59 SEQ ID NO: 33	TFRCA/59-P SEQ ID NO: 47	TFRCB/77-P SEQ ID NO: 63
Sub77 SEQ ID NO: 79	TFRCA/77-P SEQ ID NO: 62	TFRCB/77-P SEQ ID NO: 63

10.5. Results: Amplification of target and cleavage of reporter substrate

Each MNAzyme qPCR reaction containing human genomic DNA showed an increase in fluorescence over time for the real-time detection of TFRC from human genomic DNA. For all reactions the fluorescence of the no-DNA target control was lower

than that in the DNA target-containing reactions. This demonstrates that the increase in fluorescence produced in target-containing reactions is due to target dependent assembly of catalytically active MNazymes that then cleaved one of the universal reporter substrates.

The reactions with the series 2, substrate Sub59, showed an averaged Ct value of 28.5 while the reactions with the series 3 substrate Sub77 showed an averaged Ct value of 26.5.

Of note is that Sub59 and Sub77 have the same T_m and %C/T but differ in the number of cytosines in the central region N₄-N₁₃ and the composition of N₈. In the central region surrounding the ribonucleotides Sub77 has a cytosine in position N₈ which appears to have led to improved cleavage efficiency over Sub59 indicated by a lower Ct value (26.5). Sub59 contains a thymine at position N₈ and an added cytosine at the distal end of the 5' arm which has led to a reduced cleavage reaction and hence a higher Ct value (28.5).

Of note is the importance of the nature of the nucleotide sequence of the efficiently cleaved substrate and the proximity of specific nucleotides to the ribonucleotides of the substrates. These features form the basis of a set of guidelines that result in universal substrates with a higher probability of being cleaved efficiently at elevated temperatures. These design guidelines include but are not limited to (not all may be necessary): (i) seven or more cytosine nucleotides in the ten bases surrounding the ribonucleotides (N₄-N₁₃); (ii) the bases immediately adjacent to the ribonucleotides are cytosines (N₈ and N₉); (iii) total content of substrate has >64% pyrimidine's; (iv) total T_m of the oligonucleotide is 66°C or greater (where this latter guideline is only applicable if the reaction temperature for substrate cleavage is above 50°C) (Table 25). The earlier Ct value for the series 3 substrate, Sub77, is expected as this substrate complies with all of these design guidelines, while Sub59 complies with only two of these design guidelines (Table 25).

Table 25: Efficiency of cleavage of universal substrates (listed in order of cleavage efficiency based on Ct)

Name	Sequence [^]	# C's N ₄ -N ₁₃	CguC	%C/T ⁺	T _m [*]	Ct	# (i-iv) [~]
Sub77	CTCCTCCCTCguCCCTCCTCCT SEQ ID NO: 79	8	Yes	100	70	26.5	4
Sub59	CCTCCTCCCTguCCCTCCTCCT SEQ ID NO: 33	6	No	100	70	28.5	2

[^] uppercase bases represent DNA and lowercase bases represent RNA and position of base in a substrate is represented by (N_x)-N₁-N₂-N₃-N₄-N₅-N₆-N₇-N₈-rR-rY-N₉-N₁₀-N₁₁-N₁₂-N₁₃-N₁₄-N₁₅-(N_x)

* % C/T (pyrimidines) of sequence length shown above for each substrate, does not include ribonucleotides

* T_m given here equates to the melting temperature of the bound bases calculated using the Wallace rule – only calculated for bases that hybridize to their complement. When the substrate is bound to the MNAzyme based on the 10-23 DNAzyme the “g” ribonucleotide remains unbound therefore does not contribute to the overall bound T_m.

~ The number of the design guidelines (i), (ii), (iii) and/or (iv) that have been met by the substrate sequence.

REFERENCES

- PCT International Publication No. WO/2007/041774
- 10 PCT International Publication No. WO/2008/040095
- PCT International Publication No. WO/2008/122084
- U.S. Patent No. 4,683,202
- U.S. Patent No. 4,683,195
- U.S. Patent No. 4,800,159
- 15 U.S. Patent No. 4,965,188
- U.S. Patent No. 5,176,995
- US publication number 2007-0231810
- US publication number 2010-0136536
- US publication number 2011-0143338
- 20 Cairns, M.J, Hopkins, T.M., Witherington, G., Wang, L. and Sun, L (1999) Target site selection for an RNA-cleaving catalytic DNA. *Nat. Biotech.* 17: 480-486.
- Cruz, R.P., Withers, J.B. and Li, Y. (2004) Dinucleotide junction cleavage versatility of 8-17 deoxyribozyme. *Chem Biol. Jan*;11(1): 57-67.
- Perreault, J., Labuda, D., Usman, N., Yang, J. and Cedergren, R. (1991)
- 25 Relationship between 2'-hydroxyls and magnesium binding in the hammerhead RNA domain: a model for ribozyme catalysis. *Biochemistry* 30(16): 4020-5.
- Perreault, J., Wu, T., Cousineau, B., Ogilvie, K. and Cedergren, R. (1990) Mixed deoxyribo- and ribo-oligonucleotides with catalytic activity. *Nature* 344(6266): 565-7.
- Silverman, S. (2004) Breaking up is easy to do (if you're a DNA enzyme that
- 30 cleaves RNA). *Chem Biol. Jan*;11(1): 7-8. Wallace, R.B., Shaffer, J., Murphy, R.F., Bonner, J., Hirose, T. and Itakura K. (1979) Hybridization of synthetic oligodeoxyribonucleotides to ϕ_{x174} DNA: the effect of single base pair mismatch. *Nucl. Acids Res.* 6(11): 3543- 3558.
- Zaborowska, Z., Furste, J., Erdmann, V. and Kurreck, J. (2002) Sequence
- 35 requirements in the catalytic core of the "10-23" DNA enzyme. *J Biol Chem.* 277(43): 240617-22.

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;

5 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

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

10 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, 72-90, or 172-175.

3. The isolated polynucleotide substrate according to claim 1 or claim 2, wherein
15 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, 85-90,
20 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.
25

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
30 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, 85-90, 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.

5 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 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, 85-88, 90 or 172-175.

15 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.

20 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, 85-88 or 172-175.

25 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.

30 15. The isolated polynucleotide substrate according to any one of claims 1 to 14, wherein any one or more of N_1 , N_2 , 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, 72-90, 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 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.

19. The isolated polynucleotide substrate according to claim 17, wherein 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, 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-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, 33, 73-90 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, 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.

5 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
10 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.

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

30. The isolated polynucleotide substrate of any one of claims 1 to 29, wherein a
20 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.

25 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

30 (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

presence of said target to form at least a first catalytically active multi-component nucleic acid enzyme (MNAzyme);

(b) providing the isolated polynucleotide substrate of any one of claims 1 to 31, wherein said polynucleotide substrate is capable of being modified by said first MNAzyme, wherein said modification of said polynucleotide substrate by said MNAzyme 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 MNAzyme, and

(2) the catalytic activity of said at least first MNAzyme; and

(d) detecting said detectable effect.

33. The method according to claim 32, wherein said target is a nucleic acid.

34. The method according to claim 33, wherein said 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 primicroRNA, other non-coding RNAs, ribosomal RNA, derivatives thereof, amplicons, or any combination thereof.

35. The method according to claim 33 or claim 34, wherein the nucleic acid is amplified.

36. The method according to claim 35, wherein said 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).

37. The method according to any one of claims 32 to 36, wherein 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: 170 and SEQ ID NO: 176; 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.

20

38. The method according to any one of claims 32 to 37, wherein the polynucleotide substrate hybridises with substrate arms of said MNAzyme 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.

25

39. The method according to any one of claims 32 to 38, said method further comprising providing:

30 two or more additional oligonucleotide partzymes capable of self-assembling in the presence of a different target to form a second catalytically active MNAzyme; and

at least one additional polynucleotide substrate;

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

35

40. The method according to claim 39, wherein said additional polynucleotide substrate is not capable of being modified by said first MNAzyme.

41. The method of any one of claims 32 to 40, wherein said 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.

42. The method of any one of claims 32 to 41, wherein said detecting in part (d) comprises use of fluorescence spectroscopy.

43. The method of any one of claims 32 to 42, wherein said detecting in part (d) comprises detection of a FRET detectable effect.

44. Use of the isolated polynucleotide substrate of any one of claims 1 to 31 as a substrate for a catalytic nucleic acid enzyme.

45. The use of claim 44, wherein the catalytic nucleic acid enzyme is a multi-component nucleic acid enzyme (MNAzyme),

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 MNzyme, said catalytic core capable of modifying said polynucleotide substrate, and wherein said substrate arms of said MNzyme engage said polynucleotide substrate so that said catalytic core of said MNzyme can modify said polynucleotide substrate.

5

46. The use of claim 45, wherein the catalytic core portion of each said oligonucleotide partzyme comprises DNA or an analogue thereof.

47. The use of claim 45 or claim 46, wherein said assembly facilitator is a target
10 to be identified, detected or quantitated.

48. The use of any one of claims 44 to 47, wherein 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
15 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.

49. A kit comprising the isolated polynucleotide substrate of any one of claims 1
20 to 31.

50. The kit according to claim 49, further comprising a catalytic nucleic acid enzyme capable of catalytically modifying said polynucleotide substrate.

51. A kit comprising the isolated polynucleotide substrate of any one of claims 1
25 to 31 and a plurality of oligonucleotide partzymes designed to assemble a multi-component nucleic acid enzyme (MNzyme) capable of detecting at least one target, wherein said MNzyme is capable of catalytically modifying the polynucleotide substrate.

30

52. An assembly comprising a solid support bound to a polynucleotide substrate
of any one of claims 1 to 31.

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